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(54) Title: RNA COMPOSITIONS COMPRISING LIPID NANOPARTICLES OR LIPID RECONSTRUCTED NATURAL MESSENGER PACKS

(57) Abstract: Disclosed herein are RNA compositions including one or more polynucleotides encoding one or more gene editing systems, formulated within a lipid reconstructed natural messenger pack (LNMP) comprising natural lipids and an ionizable lipid. The disclosure also includes a method for making an RNA composition, comprising reconstituting a film comprising purified NMP lipids in the presence of an ionizable lipid to produce a LNMP comprising the ionizable lipid, and loading into the LNMPs with one or more polynucleotides encoding one or more gene editing systems. The disclosure also includes an RNA composition that is repeat dosable.

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RNA COMPOSITIONS COMPRISING LIPID NANOPARTICLES OR LIPID RECONSTRUCTED NATURAL MESSENGER PACKS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of priority to U.S. Provisional Application No. 63/424,208, filed November 10, 2022; U.S. Provisional Application No. 63/447,494, filed February 22, 2023; and U.S. Provisional Application No. 63/527,780, filed July 19, 2023, all of which are herein incorporated by reference in their entirety.

BACKGROUND

[0002] Commercial or developing mRNA therapeutics are typically based on whole microorganisms, protein antigens, peptides, polysaccharides or deoxyribonucleic acid (DNA) vaccines and their combinations. The use of RNA polynucleotides as therapeutics is a new and emerging field.

[0003] A need therefore exists for developing an enhanced RNA delivery system for a more effective, easily scalable, and stable delivery of RNA therapeutics.

SUMMARY OF THE INVENTION

[0004] In one aspect, provided herein is a RNA composition for gene editing, comprising one or more polynucleotides encoding one or more components of a gene editing system or one or more gene editing systems. The one or more polynucleotides are formulated within a complex lipid particle (CLP), such as a lipid reconstructed natural messenger packs (LNMPs) comprising natural lipids and an ionizable lipid. The ionizable lipid has two or more of the characteristics listed below:

(i) at least 2 ionizable amines;

(ii) at least 3 lipid tails, wherein each of the lipid tails is at least 6 carbon atoms in length;

(iii) a pKa of about 4.5 to about 7.5;

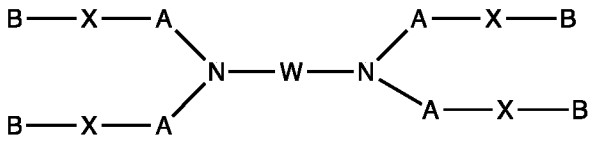
(iv) an ionizable amine and a heteroorganic group separated by a chain of at least two atoms; and

(v) an N:P ratio of at least 3 (or at least 4).

[0005] In another aspect, provided herein is a method for making a RNA composition. The method comprises reconstituting a film comprising purified NMP lipids in the presence of an ionizable lipid to produce a lipid reconstructed natural messenger packs (LNMP) comprising the ionizable lipid described herein. The method further comprises loading into the LNMPs with one or more polynucleotides encoding one or more components of a gene editing system or one or more gene editing systems.

[0006] In another aspect, provided herein is an RNA composition for gene editing, comprising: one or more polynucleotides encoding one or more components of a gene editing system or one or more gene editing systems, formulated within a plurality of lipid nanoparticles (LNPs) comprising synthetic structural lipids and an ionizable lipid.

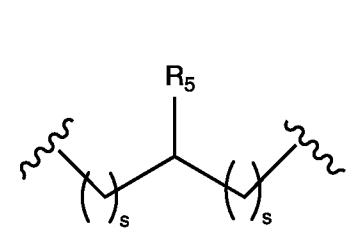
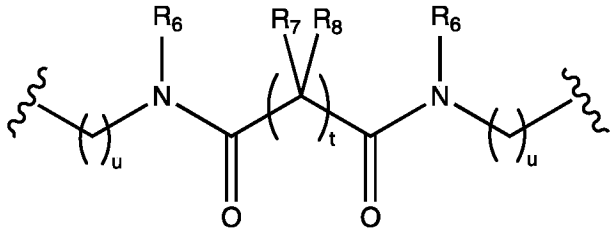
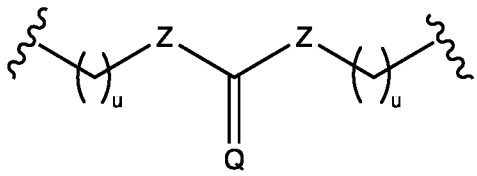
[0007] In all these aspects of the invention, the ionizable lipid may be selected from one the following groups of compounds:

i) a compound of formula  (I), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing, wherein:

each **A** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally substituted with heteroatom or substituted with OH, SH, or halogen;

each **B** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally substituted with heteroatom or substituted with OH, SH, or halogen;

each **X** is independently a biodegradable moiety; and

W is  ,  ; or  , wherein:

$\mathbf{R_5}$ is OH, SH, $N\mathbf{R_{10}}\mathbf{R_{11}}$;

each $\mathbf{R_6}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each $\mathbf{R_7}$ and each $\mathbf{R_8}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $N\mathbf{R_{10}}\mathbf{R_{11}}$, wherein each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 alkyl, or $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring; or $\mathbf{R_7}$ and $\mathbf{R_8}$ are taken together to form a ring;

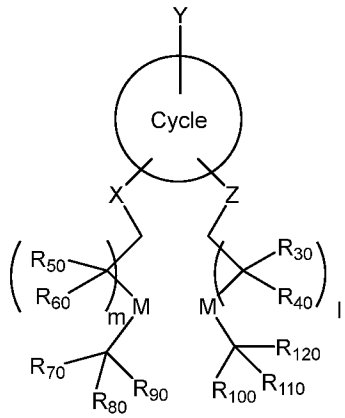
each **s** is independently 1, 2, 3, 4, or 5;

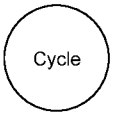
each **u** is independently 1, 2, 3, 4, or 5;

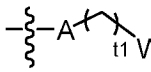
t is 1, 2, 3, 4 or 5;

each **Z** is independently absent, O, S, or $N\mathbf{R_{12}}$, wherein $\mathbf{R_{12}}$ is H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl, provided that when Z is not absent, the adjacent $\mathbf{R_1}$ and $\mathbf{R_2}$ cannot be OH, $N\mathbf{R_{10}}\mathbf{R_{11}}$, or SH; and

Q is O, S, or $N\mathbf{R_{13}}$, wherein each $\mathbf{R_{13}}$ is H, C_1-C_5 alkyl;

ii) a compound of formula  (II), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing, wherein:

 is cyclic or heterocyclic moiety;

Y is alkyl, hydroxy, hydroxyalkyl or  ;

A is absent, -O-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -N(R^7)C(O)N(R^7)-, -S-, -S-S-, or a bivalent heterocycle;

each of X and Z is independently absent, -O-, -CO-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, or -S-;

each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxy, hydroxyalkyl, or aminoalkyl;

each M is independently a biodegradable moiety;

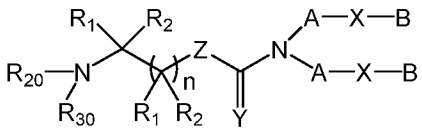
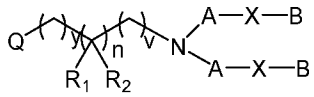
each of R_{30}, R_{40}, R_{50}, R_{60}, R_{70}, R_{80}, R_{90}, R_{100}, R_{110}, and R_{120} is independently H, C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with heteroatom or substituted with OH, SH, or halogen, or cycloalkyl or substituted cycloalkyl;

each of l and m is an integer from 1 to 10;

t1 is an integer from 0 to 10; and

W is hydroxyl, substituted or unsubstituted hydroxyalkyl, substituted or unsubstituted amino, substituted or unsubstituted aminocarbonyl, or substituted or unsubstituted heterocylyl or heteroaryl; and

iii) a compound of formula

 (III) or  (V), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein:

R_{20} and **R_{30}** are each independently H, C_1-C_5 branched or unbranched alkyl, or C_2-C_5 branched or unbranched alkenyl, or **R_{20}** and **R_{30}** together with the adjacent N atom form a 3 to 7 membered cyclic ring, optionally substituted with **R^a**;

R^a is H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, or SH;

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, OH, halogen, SH, or $N\mathbf{R_{10}R_{11}}$, or

$\mathbf{R_1}$ and $\mathbf{R_2}$ are taken together to form a cyclic ring;

each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring;

n is 0, 1, 2, 3 or 4;

Y is O or S;

Z is absent, O, S, or $N(\mathbf{R_{12}})(\mathbf{R_{12}})$, wherein each $\mathbf{R_{12}}$ is independently H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl, provided that when Z is not absent, the adjacent $\mathbf{R_1}$ and $\mathbf{R_2}$ cannot be OH, $N\mathbf{R_{10}R_{11}}$, or SH;

u is 0, 1, 2, 3, 4, 5, 6, 7, or 8;

v is 0, 1, 2, 3, or 4;

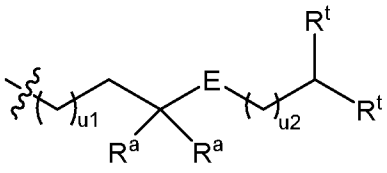
y is 0, 1, 2, 3, or 4;

each **A** is each independently C_1-C_{16} branched or unbranched alkyl, or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen;

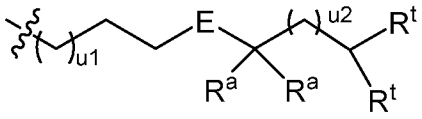
each **B** is each independently C_1-C_{16} branched or unbranched alkyl, or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen; and

each **X** is independently a biodegradable moiety; and

iv) a lipid comprising at least one head group and at least one tail group of formula (TI) or (TI'):



(TI) or



(TI'), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing,

wherein:

E is each independently -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -C(O-R_{13})-O-, -C(O)O(CH_2)$_r$-, -C(O)N(R^7)(CH_2)$_r$-, -S-S-, or -C(O-R_{13})-O-(CH_2)$_r$-, wherein each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxyalkyl, or aminoalkyl;

R_{13} is branched or unbranched C_3-C_{10} alkyl;

r is 1, 2, 3, 4, or 5;

R^a is each independently C_1-C_5 alkyl, C_2-C_5 alkenyl, or C_2-C_5 alkynyl;

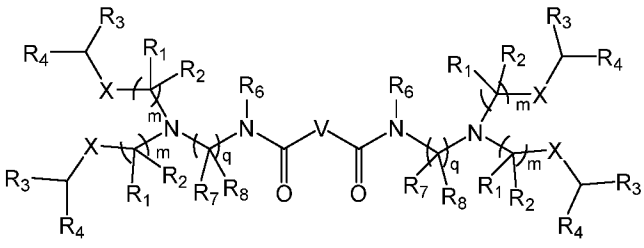
u1 and u2 are each independently 0, 1, 2, 3, 4, 5, 6, or 7;

R^t is each independently H, C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with heteroatom or substituted with OH, SH, or halogen, or cycloalkyl or substituted cycloalkyl;

 represents the bond connecting the tail group to the head group; and

wherein the lipid has a pKa from about 4 to about 8.

[0008] In some embodiments, the ionizable lipid is a compound of group i), represented by a formula



of (IX), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein:

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ is independently H, C_1-C_3 branched or unbranched alkyl, OH, halogen, SH, or $N\mathbf{R_{10}R_{11}}$, or

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ are independently taken together with the carbon atom(s) to which they are attached to form a cyclic ring;

each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 branched or unbranched alkyl, or $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring;

each $\mathbf{R_3}$ and each $\mathbf{R_4}$ is independently H, C_2-C_{14} branched or unbranched alkyl (e.g., C_3-C_{10} branched or unbranched alkyl), or C_3-C_{10} branched or unbranched alkenyl, provided that at least one of $\mathbf{R_3}$ and $\mathbf{R_4}$ is not H;

each **X** is independently a biodegradable moiety;

each **q** is independently 2, 3, 4,or 5;

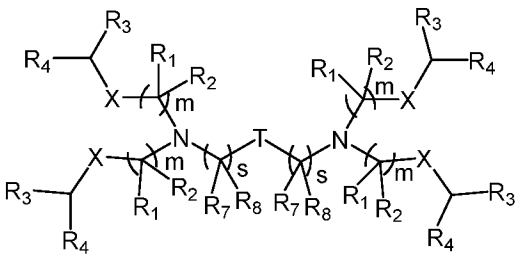
V is branched or unbrachned C_2-C_{10} alkylene, C_2-C_{10} alkenylene, C_2-C_{10} alkynylene, or C_2-C_{10} heteroalkylene, optionally substituted with one or more OH, SH, and/or halogen groups;

each $\mathbf{R_6}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each $\mathbf{R_7}$ and each $\mathbf{R_8}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $(CH_2)_v\mathbf{R_{17}}$, or $N\mathbf{R_{10}R_{11}}$, wherein each **v** is independently 0, 1, 2, 3, 4, or 5, and $\mathbf{R_{17}}$ is OH, SH, or $N(CH_3)_2$; and

each **m** is independently 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. In some embodiments, **V** is a branched or unbrachned C_2-C_3 alkylene, and each $\mathbf{R_6}$ is independently H or methyl.

[0009] In some embodiments, the ionizable lipid is a compound of group i), represented by a formula



of (XI), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein:

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ is independently H, C_1-C_3 branched or unbranched alkyl, OH, halogen, SH, or $N\mathbf{R_{10}R_{11}}$, or

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ are independently taken together with the carbon atom(s) to which they are attached to form a cyclic ring;

each R_{10} and R_{11} is independently H, C_1-C_3 branched or unbranched alkyl, or R_{10} and R_{11} are taken together to form a heterocyclic ring;

each R_3 and each R_4 is independently H, C_2-C_{14} branched or unbranched alkyl (e.g., C_3-C_{10} branched or unbranched alkyl), or C_3-C_{10} branched or unbranched alkenyl, provided that at least one of R_3 and R_4 is not H;

each **X** is independently a biodegradable moiety;

each **s** is independently 1, 2, 3, 4, or 5;

T is –NHC(O)O-, –OC(O)NH-, or a divalent heterocyclic optionally substituted with one or more -$(CH_2)_vOH$, -$(CH_2)_vSH$, -$(CH_2)_v$-halogen groups,

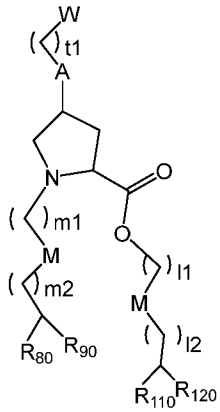
each R_7 and each R_8 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $(CH_2)_vR_{17}$, or $NR_{10}R_{11}$, wherein R_{17} is OH, SH, or $N(CH_3)_2$;

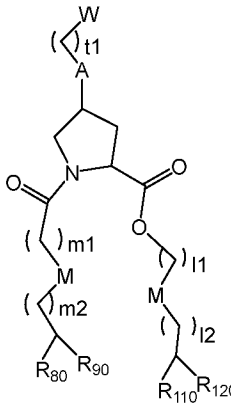
each **v** is independently 0, 1, 2, 3, 4, or 5; and

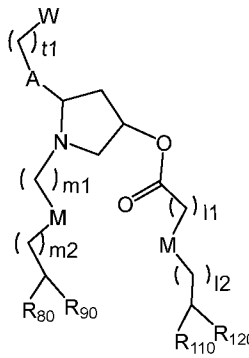
each **m** is independently 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. In some embodiments, T is a divalent piperazine or a divalent dioxopiperazine.

[0010] In some embodiments, in the above formulas for group i), **X** is -OCO-, -COO-, -NHCO-, or -CONH-.

[0011] In some embodiments, the ionizable lipid is a compound of group ii), represented by one of the following formulas:


(IVA-1),


(IVA-2), or


(IVA-3), wherein:

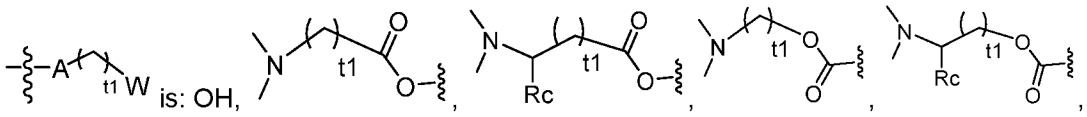
each m1 is independently an integer from 3 to 6,

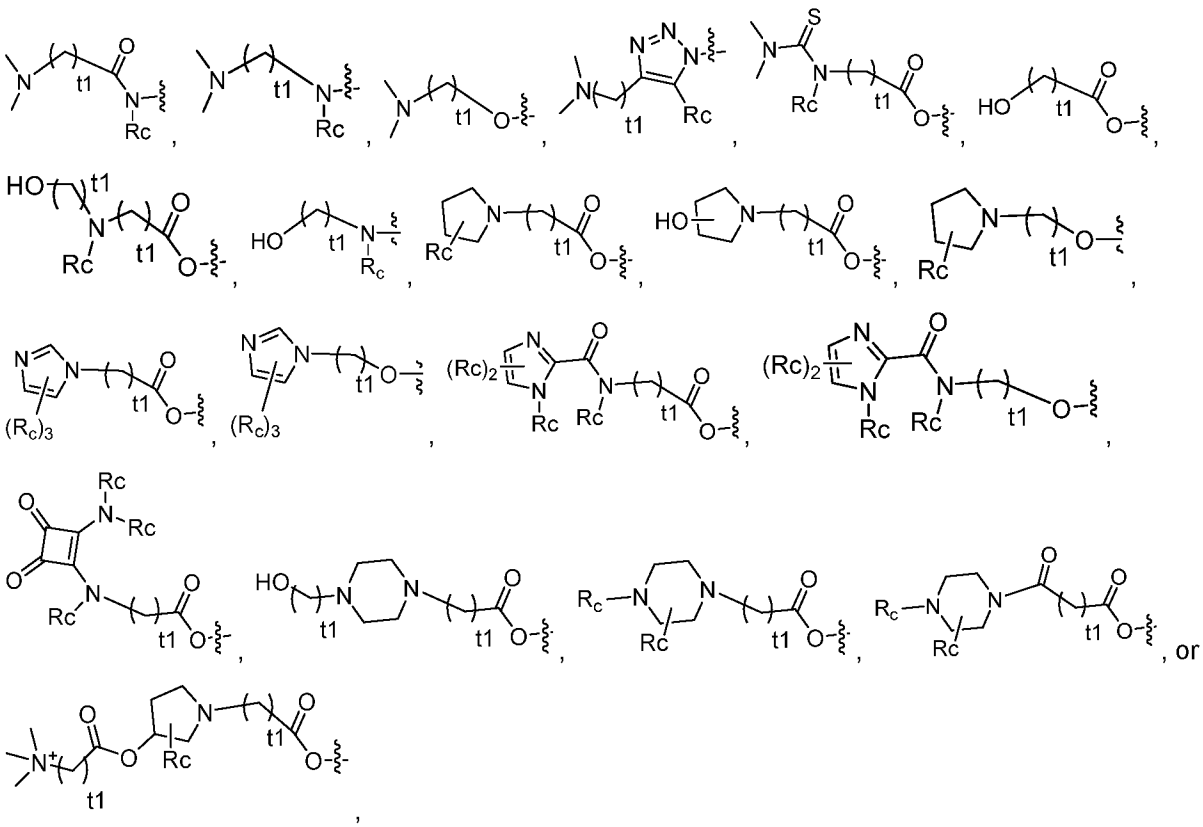
each l1 is independently an integer from 4 to 8,

m2 and l2 are each independently an integer from 0 to 3,

R_{80} and R_{90} are each independently unsubstituted C_5-C_8 alkyl or alkenyl; or R_{80} is H or unsubstituted C_1-C_4 alkyl or alkenyl, and R_{90} is unsubstituted C_5-C_{11} alkyl or alkenyl; and

R_{110} and R_{120} are each independently unsubstituted C_5-C_8 alkyl or alkenyl; or R_{110} is H or unsubstituted C_1-C_4 alkyl or alkenyl, and R_{120} is unsubstituted C_5-C_{11} alkyl or alkenyl. In some embodiments, M is -OC(O)- or -C(O)O-;


is: OH,



each R^c is independently H or C_1-C_3 alkyl;

each t1 is independently 1, 2, 3, or 4;

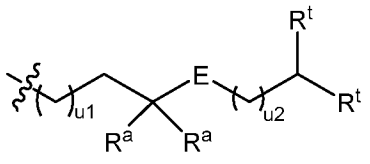
each of R_{80} and R_{90} is independently H or C_1-C_{12} branched or unbranched alkyl; and

each of R_{110} and R_{120} is independently H or C_1-C_{12} branched or unbranched alkyl, provided that at least one of R_{80} and R_{90} is not H, and at least one of R_{110} and R_{120} is not H.

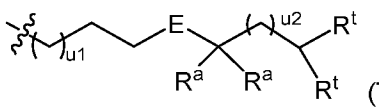
[0012] In some embodiments, the ionizable lipid is a compound of group iii), wherein $\mathbf{R_1}$ and $\mathbf{R_2}$ are each H, or each $\mathbf{R_1}$ is H, and one of the $\mathbf{R_2}$ variables is OH; and X is –OC(O)- or –C(O)O-. In some embodiments, the ionizable lipid is a compound of group iii), represented by formula III), wherein $\mathbf{R_{20}}$ and $\mathbf{R_{30}}$ are each independently H or C_1-C_3 branched or unbranched alkyl; or $\mathbf{R_{20}}$ and $\mathbf{R_{30}}$ together with the adjacent N atom form a 3 to 7 membered cyclic ring, optionally substituted with $\mathbf{R^a}$; $\mathbf{R^a}$ is H or OH; **Z** is absent, S, O, or NH; and n is 0, 1, or 2. In some embodiments, the ionizable lipid is a compound of group iii), represented by formula V),

[0013] In some embodiments, the ionizable lipid is a compound of group iv), wherein the lipid comprises at least one head group and at least one tail group, wherein:

the tail group has a structure of formula (TI) or formula TI'

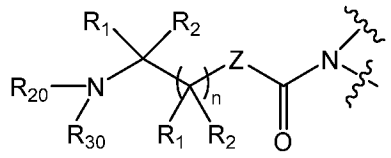


(TI) or



(TI'); and

the head group has a structure of one of the following formulas:

i)  (HA-I),

wherein:

R_{20} and R_{30} are each independently H, C_1-C_5 branched or unbranched alkyl, or C_2-C_5 branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or substituted with OH, SH, halogen, or cycloalkyl groups; or

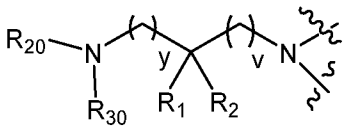
R_{20} and R_{30}, together with the adjacent N atom, form a 3 to 7 membered heterocylic or heteroaromatic ring containing one or more heteroatoms, optionally substituted with one or more OH, SH, halogen, alkyl, or cycloalkyl groups;

each of R_1 and R_2 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, OH, halogen, SH, or $NR_{10}R_{11}$; or R_1 and R_2 together form a cyclic ring;

each of R_{10} and R_{11} is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl; or R_{10} and R_{11} together form a heterocyclic ring;

n is 0, 1, 2, 3 or 4; and

Z is absent, O, S, or NR_{12}, wherein R_{12} is H or C_1-C_7 branched or unbranched alkyl; provided that when Z is not absent, the adjacent R_1 and R_2 cannot be OH, $NR_{10}R_{11}$, SH;

ii)  (HA-V).

wherein:

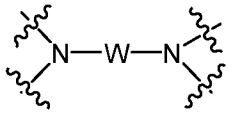
R_1 is H, C_1-C_3 alkyl, OH, halogen, SH, or $NR_{10}R_{11}$;

R_2 is OH, halogen, SH, or $NR_{10}R_{11}$; or R_1 and R_2 can be taken together to form a cyclic ring;

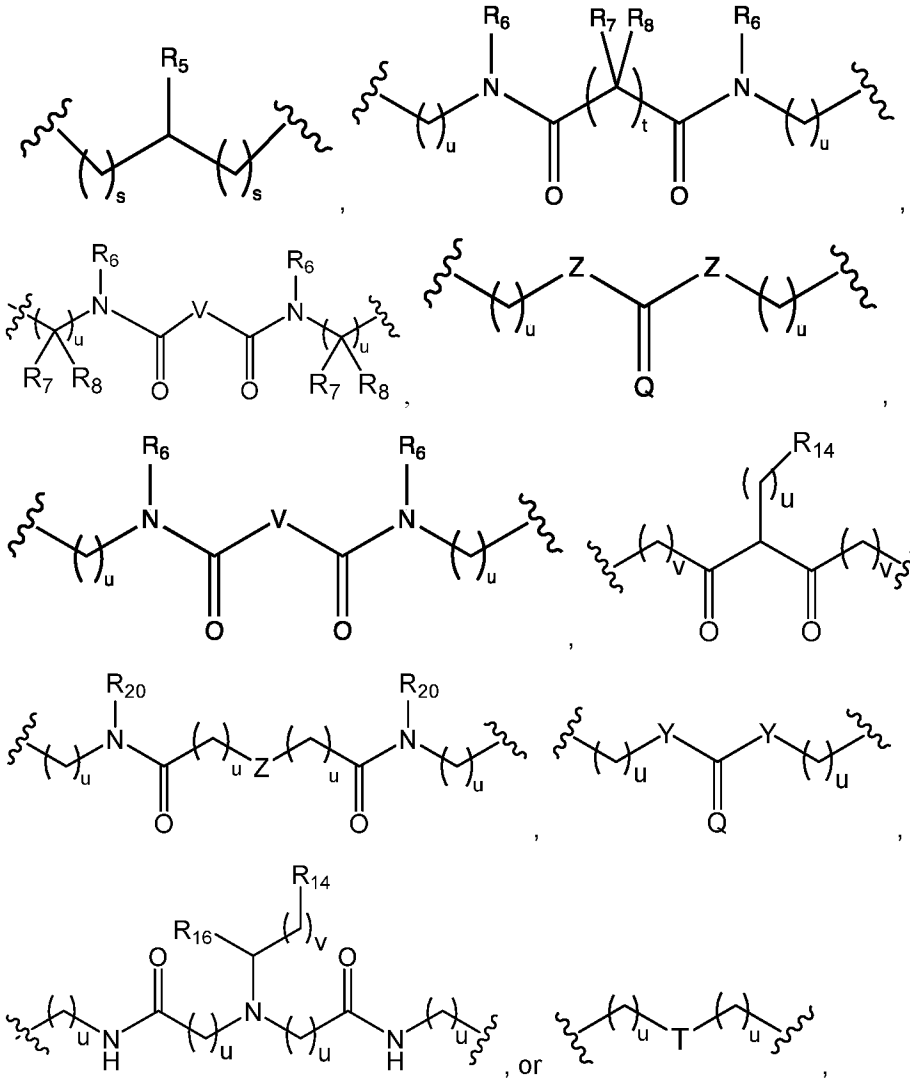
R_{10} and R_{11} are each independently H or C_1-C_3 alkyl; or R_{10} and R_{11} can be taken together to form a heterocyclic ring;

R_{20} and R_{30} are each independently H, C_1-C_5 branched or unbranched alkyl, C_2-C_5 branched or unbranched alkenyl; or R_{20} and R_{30} can be taken together to form a cyclic ring; and

each of v and y is independently 1, 2, 3, or 4;

iii)  (HB-I),

wherein W is



wherein

R_5 is OH, SH, $(CH_2)_sOH$, or $NR_{10}R_{11}$;

each R_6 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each R_7 and R_8 are independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, $(CH_2)_vOH$, $(CH_2)_vSH$, $(CH_2)_sN(CH_3)_2$, or $NR_{10}R_{11}$, wherein each R_{10} and R_{11} is independently H or C_1-C_3 alkyl, or R_{10} and R_{11} are taken together to form a heterocyclic ring; or R_7 and R_8 are taken together to form a ring;

each R_{20} is independently H, or C_1-C_3 branched or unbranched alkyl;

R_{14} is a heterocyclic, $NR_{10}R_{11}$, $C(O)NR_{10}R_{11}$, $NR_{10}C(O)NR_{10}R_{11}$, or $NR_{10}C(S)NR_{10}R_{11}$, wherein each R_{10} and R_{11} is independently H, C_1-C_3 alkyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloalkenyl, optionally substituted with one or more NH and/or oxo groups, or R_{10} and R_{11} are taken together to form a heterocyclic ring;

R_{16} is H, =O, =S, or CN;

each of s, u, and t is independently 1, 2, 3, 4, or 5;

each v is independently 0, 1, 2, 3, 4, or 5;

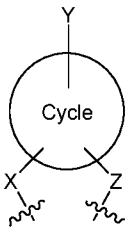
each Y is a divalent heterocyclic;

each Z is independently absent, O, S, or NR_{12}, wherein R_{12} is H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl;

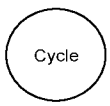
Q is O, S, CH_2, or NR_{13}, wherein each R_{13} is H, C_1-C_5 alkyl;

V is branched or unbrachned C_2-C_{10} alkylene, C_2-C_{10} alkenylene, C_2-C_{10} alkynylene, or C_2-C_{10} heteroalkylene, optionally substituted with one or more OH, SH, and/or halogen groups; and

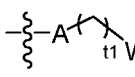
T is –NHC(O)O-, –OC(O)NH-, or a divalent heterocyclic; and

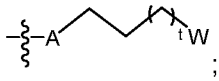
iv) 

(HC-I), wherein:



is cyclic or heterocyclic moiety;

Y is alkyl, hydroxy, hydroxyalkyl, 

, or 

;

A is absent, -O-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -N(R^7)C(O)N(R^7)-, -S-, or -S-S-;

each of X and Z is independently absent, -O-, -C(O)-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, or -S-;

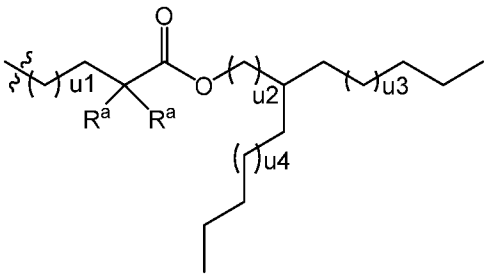
each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxy, alkoxy, hydroxyalkyl, alkylamino, alkylaminoalkyl, or aminoalkyl;

t1 is an integer from 0 to 10; and

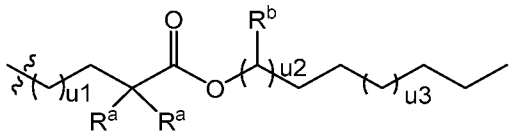
W is hydroxyl, substituted or unsubstituted hydroxyalkyl, substituted or unsubstituted amino, substituted or unsubstituted aminocarbonyl, or substituted or unsubstituted heterocyclyl or heteroaryl; and

wherein the lipid has a pKa from about 4 to about 8.

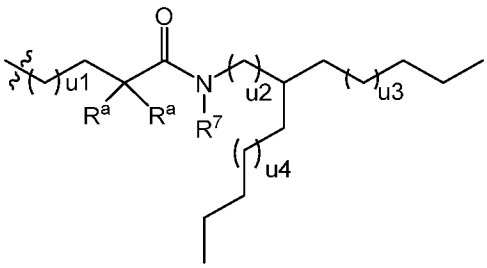
[0014] In some embodiments, the ionizable lipid is a compound of group iv), and wherein at least one tail group of the lipid has one of the following formulas:



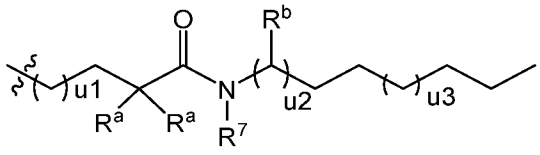
(TII),



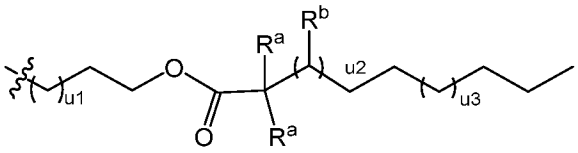
(TIII),



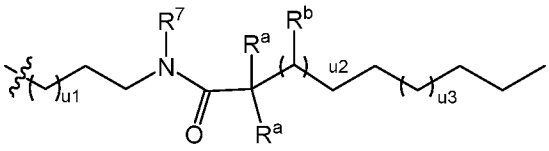
(TIV),



(TV),



(TII'), and



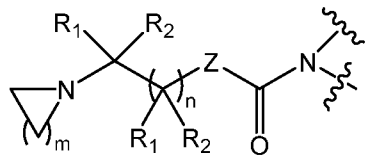
(TIII'), wherein:

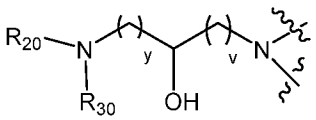
R^7 is each independently H or methyl;

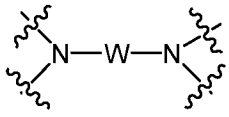
R^b is in each occasion independently H or C_1-C_4 alkyl; and

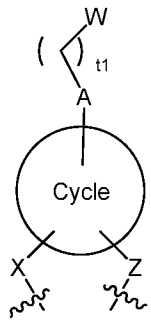
u3 and u4 are each independently 0, 1, 2, 3, 4, 5, 6, or 7; and

the head group has a structure of one of the following formulas:

i)  (HA-IA), wherein m is 1, 2, 3, 4, 5, 6, 7 or 8;

ii)  (HA-VI).

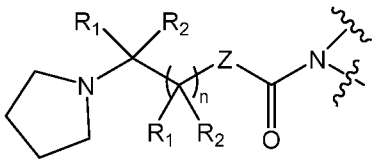
iii)  (HB-I), and

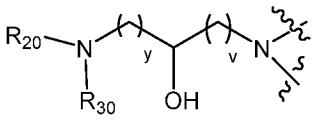
iv)  (HC-IB).

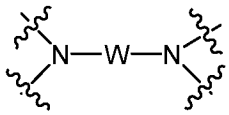
[0015] In some embodiments, at least one tail group has the structure of formula (TII), (TIII), (TIV), (TV), (TII'), and/or (TIII'), wherein u1 is 3-5, u2 is 0-3, u3 and u4 are each independently 1-7, and R^a is each independently methyl.

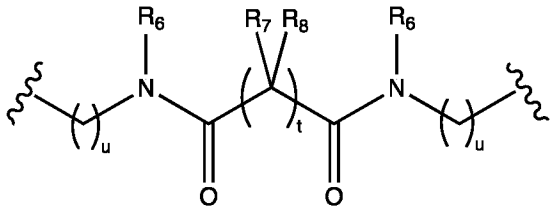
[0016] In some embodiments, the tail group has the structure of formula (TII) or formula (TIII), wherein each R^a is methyl; u1 is 3-5, u2 is 0-3; and u3 and u4 are each independently 1-4.

[0017] In some embodiments, the head group has the structure of one of the following formulas

i)  (HA-III);

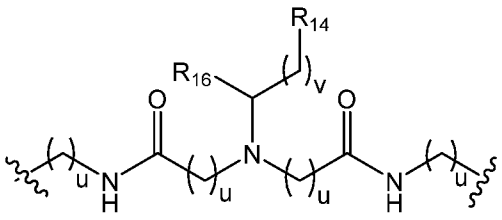
ii)  (HA-VI), wherein each R_{20} and R_{30} are independently C_1-C_3 alkyl.

iii)  (HB-I), wherein:

W is  , wherein:

each R_6, R_7, and R_8 are independently H or methyl; and

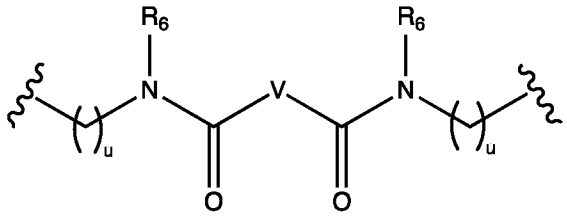
each of u and t is independently 1, 2, or 3; or

W is  , wherein:

R_{16} is H or =O;

R_{14} is a nitrogen-containing 5- or 6- membered heterocyclic, $NR_{10}R_{11}$, $C(O)NR_{10}R_{11}$, $NR_{10}C(O)NR_{10}R_{11}$, or $NR_{10}C(S)NR_{10}R_{11}$, wherein each R_{10} and R_{11} is independently H or C_1-C_3 alkyl; and

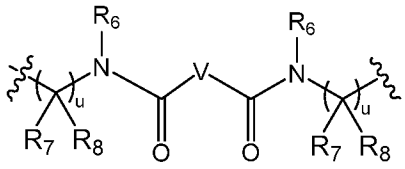
each of u and v is independently 1, 2, or 3; or

W is  , wherein:

each R_6 is independently H or methyl;

each u is independently 1, 2, or 3; and

V is C_2-C_6 alkylene or C_2-C_6 alkenylene; or

W is  , wherein:

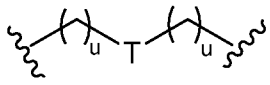
each R_6 is independently H or methyl;

each R_7 is independently H;

each R_8 is methyl;

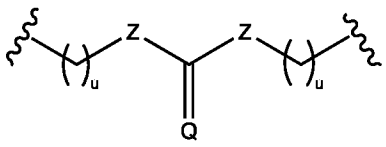
each u is independently 1, 2, or 3; and

V is C_2-C_6 alkylene or C_2-C_6 alkenylene; or

W is  , wherein:

each u is independently 1, 2, or 3; and

T is a divalent nitrogen-containing 5- or 6- membered heterocyclic; or

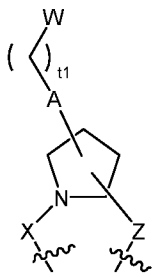
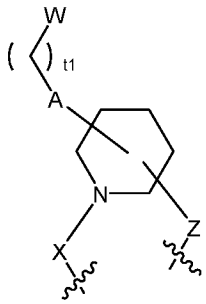
W is  , wherein:

each u is independently 1, 2, or 3;

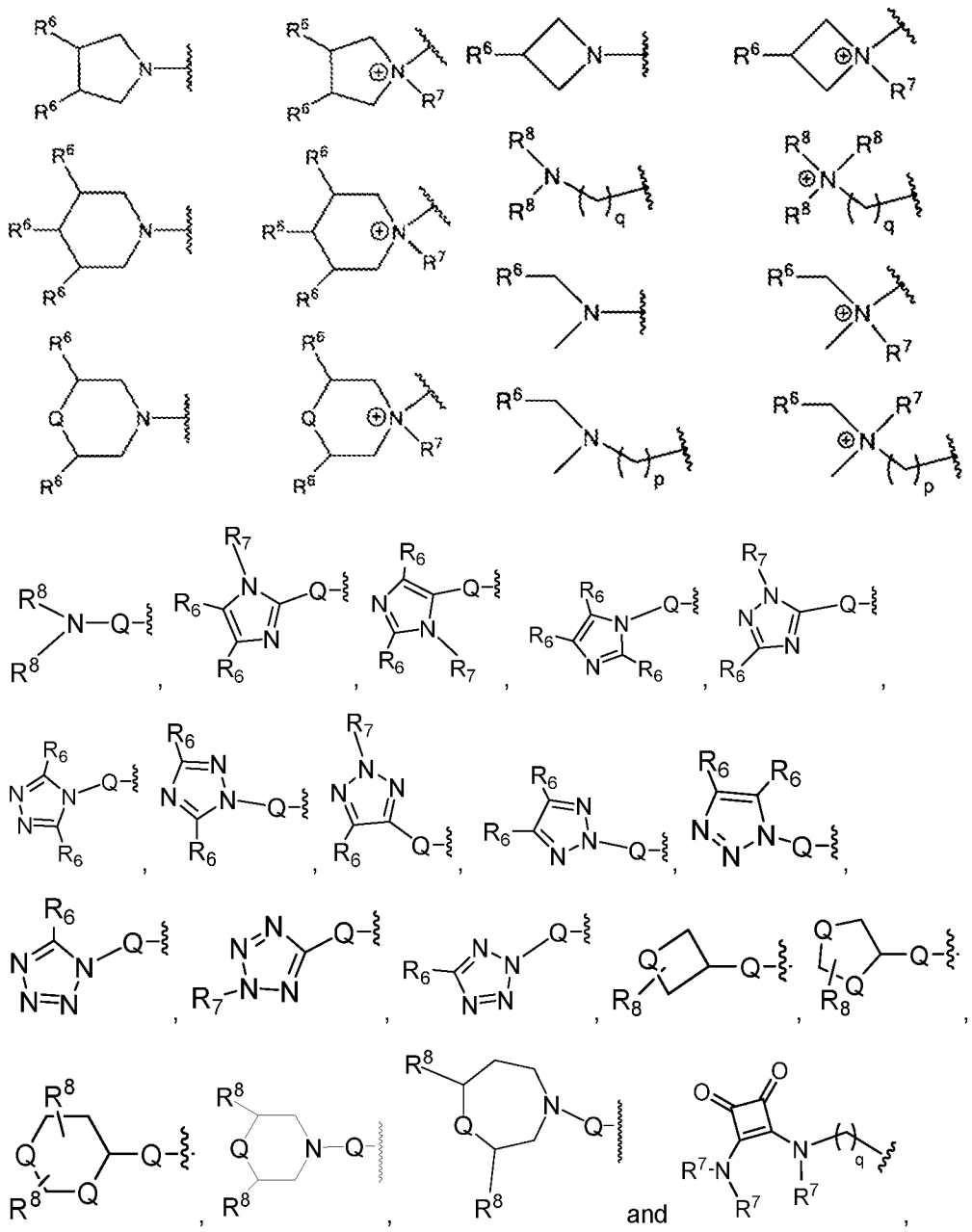
Q is O;

each Z is independently $NR_{12;}$ and

R_{12} is H or C_1-C_3 alkyl; and

iv)  (HC-IIA) or  (HC-IIC), wherein:

W is hydroxyl, substituted or unsubstituted hydroxyalkyl, one of the following moieties:



wherein

each Q is independently absent, -O-, -C(O)-, -C(S)-, -C(O)O-, -$(CH_2)_qC(R^7)_2$-, -C(O)N(R^7)-, -C(S)N(R^7)-, or -N(R^7);

R^6 is independently H, alkyl, hydroxyl, hydroxyalkyl, alkoxy, -O-alkylene-O-alkyl, -O-alkylene-N(R^7)$_2$, amino, alkylamino, aminoalkyl, thiol, thiolalkyl, or $N^+(R^7)_3$–alkylene-Q-;

each R^8 is independently H, alkyl, hydroxyalkyl, amino, aminoalkyl, alkylamino, thiol, thiolalkyl, heterocyclyl, heteroaryl; or two R^8 together with the nitrogen atom form a ring, optionally substituted with one or more alkyl, hydroxy, hydroxyalkyl, alkoxy, alkylaminoalkyl, alkylamino, or aminoalkyl;

q is 0, 1, 2, 3, 4, or 5; and

p is 0, 1, 2, 3, 4, or 5.

[0018] In some embodiments, the ionizable lipid is a compound in Table I, Table II, Table III, or Table IV.

[0019] In some embodiments, the ionizable lipid is Lipid No. 2252, 2272, 2320, 2439, 2356, 2243, 2431, 2455, 2454, 2424, 2425, 2433, 2275, 2220, 2335, or 2282.

[0020] In the CLP (or LNMP) formulation or LNP formulations, more than one ionizable lipid can be used for the ionizable lipid component: one or more of the ionizable lipids from the compounds of formulas in groups i)-iv) can be used alone or in combination with a different ionizable lipid from the compounds of formulas in groups i)-iv).

[0021] In all these aspects of the invention, in some embodiments, the CLP is a LNMP, and the CLP formulation encapsulating one or more polynucleotides is a LNMP. Thus, all the embodiments below describing the features relating to LNMP and LNMP formulation are applicable to CLP and CLP formulation.

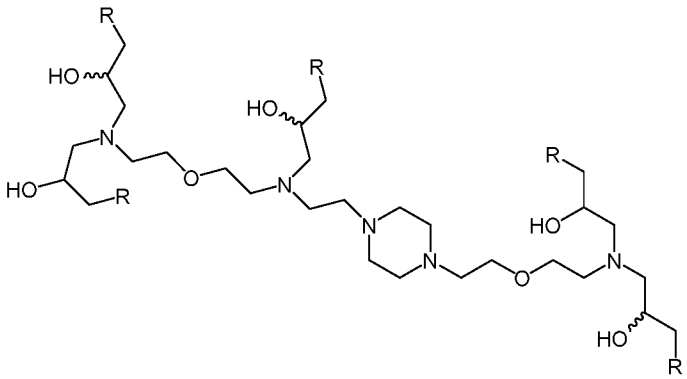
[0022] In some embodiments, the polynucleotide is encapsulated by the lipid reconstructed natural messenger packs (LNMPs). In some embodiments, the polynucleotide is encapsulated by the lipid reconstructed plant messenger packs (LPMPs). In some embodiments, the polynucleotide is embedded on the surface of the LNMPs. In some embodiments, the polynucleotide is conjugated to the surface of the LNMPs.

[0023] In some embodiments, the LNMP is produced by a method comprising lipid extrusion. In some embodiments, the LNMP is produced by a method comprising processing a solution comprising a lipid extract of the NMPs in a microfluidics device comprising an aqueous phase, thereby producing the LNMPs. In some embodiments, the aqueous phase comprises the polynucleotides.

[0024] In some embodiments, the natural lipids of the LPMPs are extracted from a plant source, such as lemon or algae.

[0025] In the CLP formulations (e.g., LNMP) or LNP formulations, for the ionizable lipid component, the ionizable lipids from the compounds of formulas in groups i)-iv) can be used in combination with one or more other ionizable lipids.

[0026] In some embodiments, the ionizable lipid of the LNMPs is selected from the group consisting of 1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl) (2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-200), MD1 (cKK-E12), OF2, EPC, ZA3-Ep10, TT3, LP01, 5A2-SC8, Lipid 5, SM-102 (Lipid H), and ALC-315. In one embodiment, the ionizable lipid is C12-200. In some embodiments, the ionizable lipid is



(III), wherein R is C8-C14 alkyl group.

[0027] In some embodiments, the reconstitution is performed in the presence of a sterol, thereby

producing a LNMP that comprises natural lipids, an ionizable lipid, and a sterol.

[0028] In some embodiments, the reconstitution is performed in the presence of a PEGylated lipid (or a PEG-lipid conjugate), thereby producing a LNMP that comprises natural lipids, a ionizable lipid, and a PEG-lipid conjugate.

[0029] In some embodiments, the LNMPs further comprise a sterol and a polyethylene glycol (PEG)-lipid conjugate. In some embodiments, the LNPs further comprise a sterol and a polyethylene glycol (PEG)-lipid conjugate.

[0030] In some embodiments, the sterol is cholesterol or sitosterol.

[0031] In some embodiments, PEG lipid conjugate comprises a PEG-2k. In some embodiments, the PEG-lipid conjugate is C14-PEG2k, C18-PEG2k, or DMPE-PEG2k. In some embodiments, the PEG-lipid conjugate is PEG-DMG or PEG-PE. In some embodiments, the PEG lipid conjugate is PEG2000-DMG or PEG2000-PE.

[0032] In some embodiments, the amount of the PEG lipid conjugate is about 1.5-2.5 mol%.

[0033] In some embodiments, the amount of the ionizable lipid is about 30-50 mol%, about 30-40 mol%, or about 45-55%. In one embodiment, the amount of the ionizable lipid is about 50 or 35 mol%.

[0034] In some embodiments, the LNMP comprises:

about 20 mol% to about 50 mol% of the ionizable lipid,

about 20 mol% to about 60 mol% of the natural lipids,

about 7 mol% to about 50 mol% of the sterol, and

about 0.5 mol% to about 3 mol% of the polyethylene glycol (PEG)-lipid conjugate.

[0035] In one embodiment, the LNMPs comprise the ionizable lipid:natural lipids:sterol:PEG-lipid at a molar ratio of about 35:50:12.5:2.5. In one embodiment, the LNMPs comprise the ionizable lipid:natural lipids:sterol:PEG-lipid at a molar ratio of about 35:20:42.5:2.5. In one embodiment, the LNMPs comprise the ionizable lipid:natural lipids:sterol:PEG-lipid at a molar ratio of about 35:16:46.5:2.5. In one embodiment, the LNMPs comprise the ionizable lipid:natural lipids:sterol:PEG-lipid at a molar ratio of about 50:10:38.5:1.5. In one embodiment, the LNMPs comprise the ionizable lipid:natural lipids:sterol:PEG-lipid at a molar ratio of about 50:20:28.5:1.5.

[0036] In some embodiments, the LNMPs comprise:

natural lipids extracted from lemon or algae,

the ionizable lipid (e.g., from Table I, Table II, Table III, or Table IV),

cholesterol, and

DMPE-PEG2k.

[0037] In one embodiment, the LNMPs comprise:

natural lipids extracted from lemon,

the ionizable lipid from Table I, Table II, Table III, or Table IV,

cholesterol, and

DMPE-PEG2k. The LNMPs may comprise the ionizable lipid:lemon lipids:cholesterol: DMPE-PEG2k at a molar ratio of about 35:50:12.5:2.5, about 35:20:42.5:2.5, about 35:16:46.5:2.5, about

50:10:38.5:1.5, or about 50:20:28.5:1.5.

[0038] In one embodiment, the LNMPs comprise:

natural lipids extracted from algae,

the ionizable lipid (e.g., from Table I, Table II, Table III, or Table IV),

cholesterol, and

DMPE-PEG2k. The LNMPs may comprise the ionizable lipid:algae lipids:cholesterol: DMPE-PEG2k at a molar ratio of about 35:20:42.5:2.5, about 35:50:12.5:2.5, about 35:16:46.5:2.5, about 50:10:38.5:1.5, or about 50:20:28.5:1.5.

[0039] In some embodiments, the LNP comprises:

about 20 mol% to about 50 mol% of the ionizable lipid,

about 5 mol% to about 40 mol% of the synthetic structural lipids,

about 20 mol% to about 50 mol% of the sterol, and

about 0.5 mol% to about 3 mol% of the polyethylene glycol (PEG)-lipid conjugate.

[0040] In one embodiment, the LNP comprises ionizable lipid: synthetic structural lipid:sterol:PEG-lipid at a molar ratio of about 35:50:12.5:2.5, about 35:20:42.5:2.5, about 35:16:46.5:2.5, about 50:10:38.5:1.5, or about 50:20:28.5:1.5.

[0041] In some embodiments, the LNPs comprise:

synthetic structural lipids,

an ionizable lipid (e.g., from Table I, Table II, Table III, or Table IV),

cholesterol, and

a PEG-lipid.

[0042] In some embodiments, the (e.g. LNPs) comprise:

synthetic structural lipids,

an ionizable lipid (e.g., 2252, 2272, 2320, 2439, 2356, 2243, 2431, 2455, 2454, 2424, 2425, 2433, 2275, 2220, 2335, or 2282),

cholesterol, and

DMG-PEG2000.

[0043] In some embodiments, the LNMP is a lipophilic moiety selected from the group consisting of a lipoplex, a liposome, a lipid nanoparticle, a polymer-based carrier, an exosome, a lamellar body, a micelle, and an emulsion. In one embodiment, the LNMP is a liposome selected from the group consisting of a cationic liposome, a nanoliposome, a proteoliposome, a unilamellar liposome, a multilamellar liposome, a ceramide-containing nanoliposome, and a multivesicular liposome. In one embodiment, the LNMP is a lipid nanoparticle.

[0044] In some embodiments, the LNMP has a size of less than about 200 nm. In one embodiment, the LNMP has a size of less than about 150 nm. In one embodiment, the LNMP has a size of less than about 100 nm. In one embodiment, the LNMP has a size of about 55 nm to about 80 nm. In one embodiment, the LNMP has a size of about 80 nm to about 100 nm.

[0045] In some embodiments, the LNMP or LNP has an N:P ratio of at least 3, for instance, an N:P ratio of 3 to 100, 3 to 50, 3 to 30, 3 to 20, 3 to 15, 3 to 12, 6 to 30, 6 to 20, 6 to 15, or 6 to 12. In one embodiment, the N/P ratio is 6 ± 1. In one embodiment, the N/P ratio is 3 ± 1. In one embodiment,

the N/P ratio is 15 ± 1.

[0046] In one embodiment, the polypeptide is erythropoietin or Epogen, or a fragment or subunit thereof.

[0047] In some embodiments, one or more polynucleotides encode one or more components of a gene editing system. In some embodiments, one or more polynucleotides encoded one or more gene editing systems. In some embodiments, the gene editing system comprises an RNA-guided DNA-binding agent.

[0048] In some embodiments, the polynucleotide may be a mRNA, an siRNA or siRNA precursor, a microRNA (miRNA) or miRNA precursor, a plasmid, a Dicer substrate small interfering RNA (dsiRNA), a short hairpin RNA (shRNA), an asymmetric interfering RNA (aiRNA), a peptide nucleic acid (PNA), a morpholino, a locked nucleic acid (LNA), a piwi-interacting RNA (piRNA), a ribozyme, a deoxyribozyme (DNAzyme), an aptamer, a circular RNA (circRNA), a guide RNA (gRNA), a single-guide RNA (sgRNA), or a DNA molecule encoding any of these RNAs.

[0049] In one embodiment, the one or more polynucleotides comprise an mRNA or modified mRNA.

[0050] In some embodiments, the mRNA is derived from (a) a DNA molecule, or (b) an RNA molecule. In the mRNA, T is optionally substituted with U.

[0051] In some embodiments, the mRNA is derived from a DNA molecule. The DNA molecule can further comprise a promoter. In some embodiments, the promoter is a T7 promoter, a T3 promoter, or an SP6 promoter. In some embodiments, the promoter is located at the 5' UTR.

[0052] In some embodiments, the mRNA is derived from an RNA molecule. The RNA molecule may be a self-replicating RNA molecule.

[0053] In some embodiments, the mRNA is an RNA molecule. The RNA molecule may further comprise a 5' cap. The 5' cap can have a Cap 1 structure, a Cap 1 (m6A) structure, a Cap 2 structure, a Cap 3 structure, a Cap 0 structure, or any combination thereof.

[0054] In some embodiments, the mRNA comprises a 5' untranslated region (UTR) and/or a 3' UTR.

[0055] In some embodiments, the mRNA comprises a 5' UTR. The 5' UTR may comprise a Kozak sequence.

[0056] In some embodiments, the mRNA comprises a 3' UTR. In some embodiments, the 3' UTR comprises one or more sequences derived from an amino-terminal enhancer of split (AES). In some embodiments, the 3' UTR comprises a sequence derived from mitochondrially encoded 12S rRNA (mtRNRl).

[0057] In some embodiments, the mRNA comprises a poly(A) sequence. In one embodiment, the poly(A) sequence is a 110-nucleotide sequence consisting of a sequence of 30 adenosine residues, a 10-nucleotide linker sequence, and a sequence of 70 adenosine residues.

[0058] In some embodiments, the RNA-guided DNA-binding agent of the gene editing system is a Cas nuclease mRNA. In some embodiments, the Cas nuclease mRNA is a Class II Cas nuclease mRNA. In one embodiment, the Class II Cas nuclease is a Cas9 nuclease mRNA.

[0059] In some embodiments, the one or more polynucleotides comprise a gRNA or modified gRNA. In one embodiment, the gRNA is a dual-guide RNA (dgRNA) or an sgRNA. In some embodiments, the gRNA is a modified gRNA comprising a modification selected from the group consisting of 2'-O-

methyl (2'-O-Me) modified nucleotide, a phosphorothioate (PS) bond between nucleotides, and a 2'-fiuoro (2'-F) modified nucleotide. In some embodiments, the gRNA is a modified gRNA comprising a modification at one or more of the first five nucleotides at the 5' end or the 3' end. In some embodiments, the gRNA is a modified gRNA comprising PS bonds between the first four nucleotides or the last four nucleotides. In some embodiments, the modified gRNA further comprises 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end or the 3' end.

[0060] In some embodiments, the one or more polynucleotides comprise a gRNA and a Class II Cas nuclease mRNA. In some embodiments, the gRNA and Class 2 Cas nuclease mRNA are present in a ratio ranging from about 10:1 to about 1:10 by weight. In some embodiments, the gRNA and Class 2 Cas nuclease mRNA are present in a ratio ranging from about 5:1 to about 1:5 by weight. In some embodiments, the gRNA and Class 2 Cas nuclease mRNA are present in a ratio ranging from about 2:1 to about 1:2 by weight. In some embodiments, the gRNA and Class 2 Cas nuclease mRNA are present in a ratio of about 2:1 by weight or about 1:1 by weight.

[0061] In some embodiments, the one or more polynucleotides comprise an RNA comprising an open reading frame encoding an RNA-guided DNA-binding agent, wherein the open reading frame has a uridine content ranging from its minimum uridine content to 150% of the minimum uridine content. In some embodiments, the one or more polynucleotides comprise an mRNA comprising an open reading frame encoding an RNA-guided DNA-binding agent, wherein the open reading frame has a uridine dinucleotide content ranging from its minimum uridine dinucleotide content to 150% of the minimum uridine dinucleotide content.

[0062] In some embodiments, the RNA composition further comprises at least one template nucleic acid.

[0063] In some embodiments, the RNA composition has a total lipid:polynucleotide weight ratio of about 50:1 to about 10:1. In one embodiment, the RNA composition has a total lipid:polynucleotide weight ratio of about 44: 1 to about 24: 1. In one embodiment, the RNA composition has a total lipid:polynucleotide weight ratio of about 40: 1 to about 28: 1. In one embodiment, the RNA composition has a total lipid:polynucleotide weight ratio of about 38: 1 to about 30:1. In one embodiment, the RNA composition has a total lipid:polynucleotide weight ratio of about 37: 1 to about 33:1.

[0064] In some embodiments, the RNA composition, e.g., the aqueous phase, further comprises a HEPES or TRIS buffer. The HEPES or TRIS buffer may have a pH of about 7.0 to about 8.5. The HEPES or TRIS buffer can be at a concentration of about 7 mg/mL to about 15 mg/mL. The aqueous phase may further comprise about 2.0 mg/mL to about 4.0 mg/mL of NaCl.

[0065] In some embodiments, the RNA composition, e.g., the aqueous phase comprises water, PBS, or a citrate buffer. In one embodiment, the aqueous phase comprises a citrate buffer having a pH of about 3.2.

[0066] In some embodiments, the aqueous phase and the lipid solution are mixed at a 3:1 volumetric ratio.

[0067] In some embodiments, the RNA composition further comprises one or more cryoprotectants. The one or more cryoprotectants may be sucrose, glycerol, or a combination thereof. In one

embodiment, the RNA composition comprises a combination of sucrose at a concentration of about 70 mg/mL to about 110 mg/mL and glycerol at a concentration of about 50 mg/mL to about 70 mg/mL.

[0068] In some embodiments, the RNA composition is a lyophilized composition. The lyophilized RNA composition may comprise one or more lyoprotectants. The lyophilized RNA composition may comprise a poloxamer, potassium sorbate, sucrose, or any combination thereof. In one embodiment, the lyophilized RNA composition comprises a poloxamer, e.g., poloxamer 188.

[0069] In some embodiments, the RNA composition is a lyophilized composition. In one embodiment, the lyophilized RNA composition comprises about 0.01 to about 1.0 % w/w of the polynucleotides. In one embodiment, the lyophilized RNA composition comprises about 1.0 to about 5.0 % w/w lipids. In one embodiment, the lyophilized RNA composition comprises about 0.5 to about 2.5 % w/w of TRIS buffer. In one embodiment, the lyophilized RNA composition comprises about 0.75 to about 2.75 % w/w of NaCl. In one embodiment, the lyophilized RNA composition comprises about 85 to about 95 % w/w of a sugar, e.g., sucrose. In one embodiment, the lyophilized RNA composition comprises about 0.01 to about 1.0 % w/w of a poloxamer, e.g., poloxamer 188. In one embodiment, the lyophilized RNA composition comprises about 1.0 to about 5.0 % w/w of potassium sorbate.

[0070] In another aspect, provided herein is a method of delivering a gene editing system to a subject in need thereof, the method comprising administering to the subject a RNA composition comprising:

one or more polynucleotides encoding one or more components of a gene editing system or one or more gene editing systems, formulated within

(a) a plurality of lipid nanoparticles (LNPs) comprising synthetic structural lipids and an ionizable lipid; or

(b) a lipid reconstructed natural messenger packs (LNMPs) comprising natural lipids and an ionizable lipid,

wherein the ionizable lipid has two or more of the characteristics listed below:

(i) at least 2 ionizable amines;

(ii) at least 3 lipid tails; wherein each of the lipid tails is at least 6 carbon atoms in length;

(iii) a pKa of about 4.5 to about 7.5;

(iv) an ionizable amine and a heteroorganic group separated by a chain of at least two atoms; and

(v) an N:P ratio of at least 3.

[0071] In another aspect, provided herein is a method of gene editing in a cell or a subject, comprising contacting the cell with or administering to the subject:

one or more polynucleotides encoding one or more components of a gene editing system or one or more gene editing systems, formulated within

(a) a plurality of lipid nanoparticles (LNPs) comprising synthetic structural lipids and an ionizable lipid; or

(b) a lipid reconstructed natural messenger packs (LNMPs) comprising natural lipids and an ionizable lipid,

wherein the ionizable lipid has two or more of the characteristics listed below:

(i) at least 2 ionizable amines;

(ii) at least 3 lipid tails; wherein each of the lipid tails is at least 6 carbon atoms in length;

(iii) a pKa of about 4.5 to about 7.5;

(iv) an ionizable amine and a heteroorganic group separated by a chain of at least two atoms; and

(v) an N:P ratio of at least 3,

wherein the one or more components of a gene editing system or one or more gene editing systems are delivered to the cell or subject to modify the genome of the cell or the subject.

[0072] In some embodiments, the RNA composition is administered at least one time.

[0073] In some embodiments, the RNA composition is administered at least twice, at least three times, at least four times, at least five times, at least six times, at least seven times, at least eight times, at least nine times, at least ten times, at least fifteen times, at least twenty times, or more. In some embodiments, the RNA composition is administered 2-8 times. In some embodiments, the delivery of the gene editing system, or the result of gene editing improves upon multiple administrations.

[0074] In some embodiments, the RNA composition is administered to the subject once, twice, three times, four times, five times, six times, seven times, eight times, nine times, ten times or more. In some embodiments, the RNA composition is administered to the subject eight times. In some embodiments, the RNA composition is administered to the subject five times a week apart. In some embodiments, the RNA composition is administered to the subject twice, three times, four times, five times, six times, seven times, eight times, ten times or more, wherein the RNA composition is administered to the subject one week apart, two weeks apart, three weeks apart, four weeks apart, five weeks apart, or more.

[0075] In some embodiments, the RNA composition further comprises at least one template nucleic acid.

[0076] In some embodiments, at least two RNA compositions are administered into the subject or contacted with the cell: a first RNA composition comprising a mRNA and the second RNA composition comprising a guide RNA nucleic acid. In some embodiments, the first and second RNA compositions are administered simultaneously. In some embodiments, the first and second RNA compositions are administered sequentially.

[0077] In some embodiments, a single RNA composition is contacted with the cell or administered into the subject, wherein the single RNA composition comprises an mRNA and a guide RNA nucleic acid.

[0078] In these aspects of the invention, the RNA composition may be administered by oral, intravenous, intradermal, intramuscular, intranasal, intraocular, or rectal, and/or subcutaneous administration. In certain embodiments, the RNA composition is administered by oral, intravenous, intramuscular, and/or subcutaneous administration.

[0079] In some embodiments, the RNA composition is administered at a dosage level sufficient to deliver about 0.01 mg/kg to about 4 mg/kg of the mRNA to the subject. In some embodiments, the

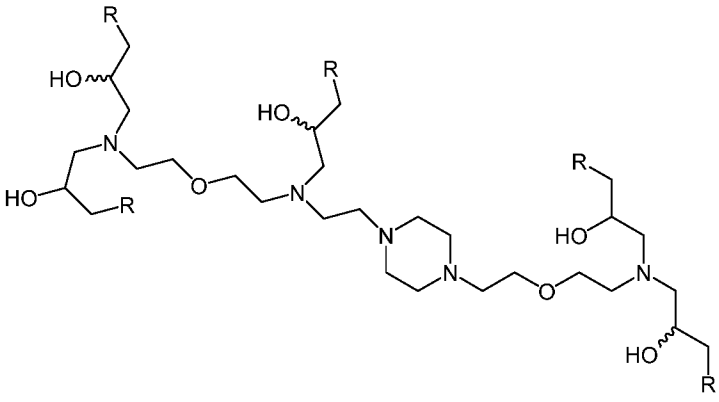
RNA composition is administered at a dosage level sufficient to deliver 0.125 mg/kg, 0.3 mg/kg, 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, or 3 mg/kg of the mRNA to the subject.

[0080] In some embodiments, the method further comprises administering an additional therapeutic agent to the subject.

[0081] In some embodiments, the additional therapeutic agent is administered prior to, concurrent with, or after the administration of the RNA composition.

[0082] In these aspects of the invention relating to a method for delivering a gene editing system or method of gene editing, the RNA composition may be formulated with b) CLPs such as LNMPs, as described herein.

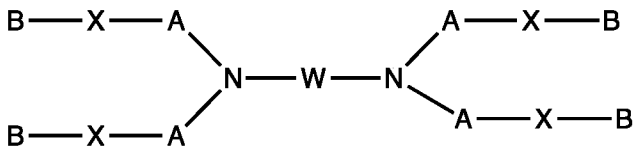
[0083] In some embodiments, the ionizable lipid of the LNMPs is selected from the group consisting of 1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl) (2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-200), MD1 (cKK-E12), OF2, EPC, ZA3-Ep10, TT3, LP01, 5A2-SC8, Lipid 5, SM-102 (Lipid H), and ALC-315. In one embodiment, the ionizable lipid is C12-200. In some embodiments, the ionizable lipid is



(III), wherein R is C8-C14 alkyl group.

[0084] In these aspects of the invention relating to a method for delivering a gene editing system or method of gene editing, the RNA composition may be formulated with a) LNPs, as described herein.

[0085] In these aspects of the invention relating to a method for delivering a gene editing system or method of gene editing, the ionizable lipid in CLP (such as LNMP) or LNP may be selected from one of the following groups of compounds:

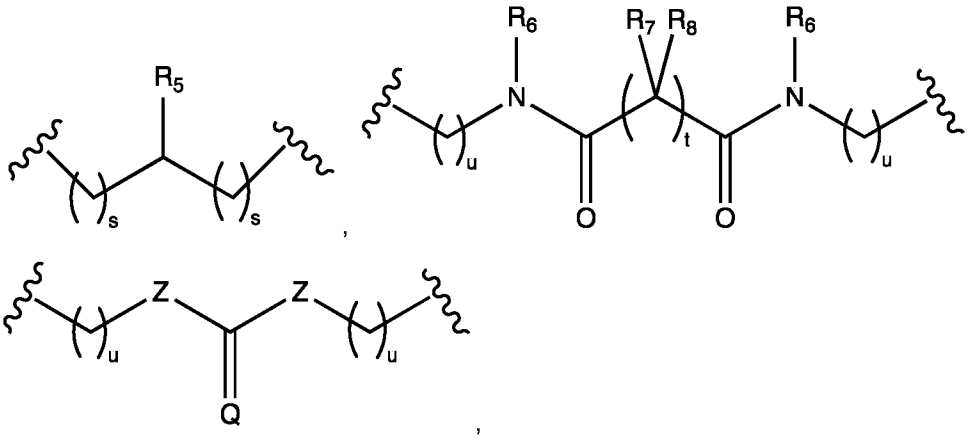
i) a compound of formula  (I), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing, wherein:

each **A** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally substituted with heteroatom or substituted with OH, SH, or halogen;

each **B** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally substituted with heteroatom or substituted with OH, SH, or halogen;

each **X** is independently a biodegradable moiety; and

W is



; or

wherein

R_5 is OH, SH, $NR_{10}R_{11}$;

each R_6 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each R_7 and each R_8 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $NR_{10}R_{11}$, wherein each R_{10} and R_{11} is independently H, C_1-C_3 alkyl, or R_{10} and R_{11} are taken together to form a heterocyclic ring;

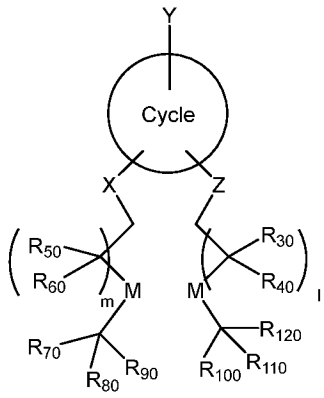
each **s** is independently 1, 2, 3, 4, or 5;

each **u** is independently 1, 2, 3, 4, or 5;

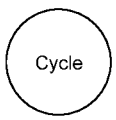
t is 1, 2, 3, 4 or 5;

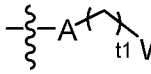
each **Z** is independently absent, O, S, or NR_{12}, wherein R_{12} is H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl; and

Q is O, S, or NR_{13}, wherein each R_{13} is H, C_1-C_5 alkyl;

ii) a compound of formula  (II), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing,

wherein:

 is cyclic or heterocyclic moiety;

Y is alkyl, hydroxy, hydroxyalkyl or  ;

A is absent, -O-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-,

-N(R^7)C(O)-, -C(O)N(R^7)-, -N(R^7)C(O)N(R^7)-, -S-, -S-S-, or a bivalent heterocycle;

each of X and Z is independently absent, -O-, -CO-, -N(R^7)-, -O-alkylene-; -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, or -S-;

each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxy, hydroxyalkyl, or aminoalkyl;

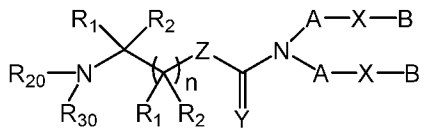
each M is independently a biodegradable moiety;

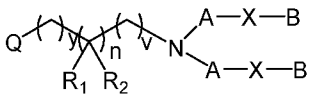
each of R_{30}, R_{40}, R_{50}, R_{60}, R_{70}, R_{80}, R_{90}, R_{100}, R_{110}, and R_{120} is independently H, C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with heteroatom or substituted with OH, SH, or halogen, or cycloalkyl or substituted cycloalkyl;

each of l and m is an integer from 1 to 10;

t1 is an integer from 0 to 10; and

W is hydroxyl, substituted or unsubstituted hydroxyalkyl, substituted or unsubstituted amino, substituted or unsubstituted aminocarbonyl, or substituted or unsubstituted heterocylyl or heteroaryl; and

iii) a compound of formula  (III) or

 (V), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein:

R_{20} and **R_{30}** are each independently H, C_1-C_5 branched or unbranched alkyl, or C_2-C_5 branched or unbranched alkenyl, or **R_{20}** and **R_{30}** together with the adjacent N atom form a 3 to 7 membered cyclic ring, optionally substituted with **R^a**;

R^a is H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, or SH;

each **R_1** and each **R_2** is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, OH, halogen, SH, or N**R_{10}****R_{11}**, or

R_1 and **R_2** are taken together to form a cyclic ring;

each **R_{10}** and **R_{11}** is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or **R_{10}** and **R_{11}** are taken together to form a heterocyclic ring;

n is 0, 1, 2, 3 or 4;

Y is O or S;

Z is absent, O, S, or N(**R_{12}**)(**R_{12}**), wherein each **R_{12}** is independently H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl, provided that when Z is not absent, the adjacent **R_1** and **R_2** cannot be OH, N**R_{10}****R_{11}**, or SH;

u is 0, 1, 2, 3, 4, 5, 6, 7, or 8;

v is 0, 1, 2, 3, or 4;

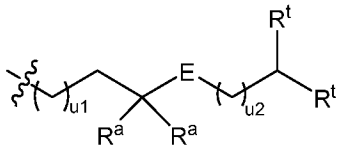
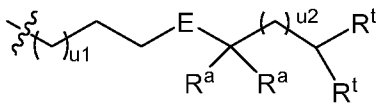
y is 0, 1, 2, 3, or 4;

each **A** is each independently C_1-C_{16} branched or unbranched alkyl, or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen;

each **B** is each independently C_1-C_{16} branched or unbranched alkyl, or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen; and

each **X** is independently a biodegradable moiety; and

iv) a lipid comprising at least one head group and at least one tail group of formula (TI) or (TI')


(TI) or

(TI'), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing,

wherein:

E is each independently -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -C(O-R_{13})-O-, -C(O)O(CH_2)$_r$-, -C(O)N(R^7) (CH_2)$_r$-, -S-S-, or -C(O-R_{13})-O-(CH_2)$_r$-, wherein each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxyalkyl, or aminoalkyl;

R_{13} is branched or unbranched C_3-C_{10} alkyl;

r is 1, 2, 3, 4, or 5;

R^a is each independently C_1-C_5 alkyl, C_2-C_5 alkenyl, or C_2-C_5 alkynyl;

u1 and u2 are each independently 0, 1, 2, 3, 4, 5, 6, or 7;

R^t is each independently H, C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with heteroatom or substituted with OH, SH, or halogen, or cycloalkyl or substituted cycloalkyl;


represents the bond connecting the tail group to the head group; and

wherein the lipid has a pKa from about 4 to about 8.

[0086] In some embodiments, the ionizable lipid is a compound in Table I, Table II, Table III, or Table IV. In some embodiments, the ionizable lipid is 2272, 2320, 2439, 2356, 2243, 2431, 2455, 2454, 2424, 2433, 2425, 2275, 2220, or 2335.

[0087] In another aspect, provided herein is a method of repeatable dosing to a subject in need thereof, the method comprising administering to the subject a RNA composition comprising:

one or more polynucleotides (e.g., those encoding one or more components of a gene editing system or one or more gene editing systems), formulated within

(a) a plurality of lipid nanoparticles (LNPs) comprising synthetic structural lipids and an ionizable lipid; or

(b) a lipid reconstructed natural messenger packs (LNMPs) comprising natural lipids and an ionizable lipid,

wherein the ionizable lipid has two or more of the characteristics listed below:

(i) at least 2 ionizable amines;

(ii) at least 3 lipid tails; wherein each of the lipid tails is at least 6 carbon atoms in length;

(iii) a pKa of about 4.5 to about 7.5;

(iv) an ionizable amine and a heteroorganic group separated by a chain of at least two atoms;

and

(v) an N:P ratio of at least 3.

[0088] In some embodiments, the RNA composition is administered at least twice, at least three times, at least four times, at least five times, at least six times, at least seven times, at least eight times, at least nine times, at least ten times, at least fifteen times, at least twenty times, or more. In some embodiments, the RNA composition is administered 2-8 times. In some embodiments, the delivery of the RNA composition or the result of dosing improves upon multiple administrations.

[0089] In some embodiments, the RNA composition is administered to the subject once, twice, three times, four times, five times, six times, seven times, eight times, nine times, ten times or more. In some embodiments, the RNA composition is administered to the subject eight times. In some embodiments, the RNA composition is administered to the subject five times a week apart. In some embodiments, the RNA composition is administered to the subject twice, three times, four times, five times, six times, seven times, eight times, ten times or more, wherein the RNA composition is administered to the subject one week apart, two weeks apart, three weeks apart, four weeks apart, five weeks apart, or more.

DEFINITIONS

[0090] As used herein, the term "effective amount," "effective concentration," or "concentration effective to" refers to an amount of a LNMP, or nucleic acid composition, sufficient to effect the recited result or to reach a target level (e.g., a predetermined or threshold level) in or on a target organism.

[0091] As used herein, the term "therapeutic agent" refers to an agent that can act on an animal, e.g., a mammal (e.g., a human), an animal pathogen, or a pathogen vector, such as an antifungal agent, an antibacterial agent, a virucidal agent, an anti-viral agent, an insecticidal agent, a nematicidal agent, an antiparasitic agent, or an insect repellent.

[0092] As defined herein, the term "nucleic acid" and "polynucleotide" are interchangeable and refer to RNA or DNA that is linear or branched, single or double stranded, or a hybrid thereof, regardless of length (e.g., at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 100, 150, 200, 250, 500, 1000, or more nucleic acids). The term also encompasses RNA/DNA hybrids. Nucleotides are typically linked in a nucleic acid by phosphodiester bonds, although the term "nucleic acid" also encompasses nucleic acid analogs having other types of linkages or backbones (e.g., phosphoramide, phosphorothioate, phosphorodithioate, O-methylphosphoroamidate, morpholino, locked nucleic acid (LNA), glycerol nucleic acid (GNA), threose nucleic acid (TNA), and peptide nucleic acid (PNA) linkages or backbones, among others). The nucleic acids may be single-stranded, double-stranded, or contain portions of both single-stranded and double-stranded sequence. A nucleic acid can contain any combination of deoxyribonucleotides and ribonucleotides, as well as any combination of bases, including, for example, adenine, thymine, cytosine, guanine, uracil, and modified or non-canonical bases (including, e.g., hypoxanthine, xanthine, 7-methylguanine, 5,6-dihydrouracil, 5-methylcytosine, and 5 hydroxymethylcytosine).

[0093] As used herein, the term "peptide," "protein," or "polypeptide" encompasses any chain of naturally or non-naturally occurring amino acids (either D- or L-amino acids), regardless of length (e.g., at least 2, 3, 4, 5, 6, 7, 10, 12, 14, 16, 18, 20, 25, 30, 40, 50, 100, 150, 200, 250, 300, 350, 400,

450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, or more than 1000 amino acids), the presence or absence of post-translational modifications (e.g., glycosylation or phosphorylation), or the presence of, e.g., one or more non-amino acyl groups (for example, sugar, lipid, etc.) covalently linked to the peptide, and includes, for example, natural proteins, synthetic, or recombinant polypeptides and peptides, hybrid molecules, peptoids, or peptidomimetics. The polypeptide may be, e.g., at least 0.1, at least 1, at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, or more than 50 kD in size. The polypeptide may be a full-length protein. Alternatively, the polypeptide may comprise one or more domains of a protein.

[0094] As used herein, the term “animal” refers to humans and non-human animals (including for example, dogs, cats, horses, rabbits, zoo animals, cows, pigs, sheep, chickens, and non-human primates).

[0095] As used herein the term "pathogen" refers to an organism, such as a microorganism or an invertebrate, which causes disease or disease symptoms in an animal by, e.g., (i) directly infecting the animal, (ii) producing agents that causes disease or disease symptoms in an animal (e.g., bacteria that produce pathogenic toxins and the like), and/or (iii) by eliciting an immune (e.g., inflammatory response) in animals (e.g., biting insects, e.g., bedbugs). As used herein, pathogens include, but are not limited to, bacteria, protozoa, parasites, fungi, nematodes, insects, viroids and viruses, or any combination thereof, wherein each pathogen is capable, either by itself or in concert with another pathogen, of eliciting disease or symptoms in humans.

[0096] As used herein, the term “heterologous” refers to an agent (e.g., a polypeptide) that is either (1) exogenous to the plant (e.g., originating from a source that is not the plant or plant part from which the PMP is produced) (e.g., an agent which is added to the PMP using loading approaches described herein) or (2) endogenous to the plant cell or tissue from which the PMP is produced, but present in the PMP (e.g., added to the PMP using loading approaches described herein, genetic engineering, as well as *in vitro* or *in vivo* approaches) at a concentration that is higher than that found in nature (e.g., higher than a concentration found in a naturally-occurring plant extracellular vesicle).

[0097] As used herein, “percent identity” between two sequences is determined by the BLAST 2.0 algorithm, which is described in Altschul et al., (1990) *J. Mol. Biol.* 215:403-410. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information.

[0098] As used herein, the term “modified NMPs” or “modified LNMPs” refers to a composition including a plurality of NMPs or LNMPs that include one or more heterologous agents (e.g., one or more exogenous lipids, such as a ionizable lipids, e.g., a NMP or LNMP comprising an ionizable lipid and a sterol and/or a PEGylated lipid) capable of increasing cell uptake (e.g., animal cell uptake, plant cell uptake, bacterial cell uptake, or fungal cell uptake) of the NMP or LNMP, or a portion or component thereof, relative to an unmodified NMP or LNMP; capable of enabling or increasing delivery of a heterologous functional agent (e.g., an agricultural or therapeutic agent) by the NMP or LNMP to a cell, and/or capable of enabling or increasing loading (e.g., loading efficiency or loading capacity) of a heterologous functional agent (e.g., an agricultural or therapeutic agent). The NMPs or LNMPs may be modified *in vitro* or *in vivo*.

[0099] As used herein, the term “unmodified NMPs” or “unmodified LNMPs” refers to a composition including a plurality of NMPs or LNMPs that lack a heterologous cell uptake agent capable of increasing cell uptake (e.g., animal cell uptake, plant cell uptake, bacterial cell uptake, or fungal cell uptake) of the NMP.

[0100] As used herein, the term “modified PMPs” or “modified LPMPs” refers to a composition including a plurality of PMPs or LPMPs that include one or more heterologous agents (e.g., one or more exogenous lipids, such as a ionizable lipids, e.g., a PMP or LPMP comprising an ionizable lipid and a sterol and/or a PEGylated lipid) capable of increasing cell uptake (e.g., animal cell uptake, plant cell uptake, bacterial cell uptake, or fungal cell uptake) of the PMP or LPMP, or a portion or component thereof, relative to an unmodified PMP or LPMP; capable of enabling or increasing delivery of a heterologous functional agent (e.g., an agricultural or therapeutic agent) by the PMP or LPMP to a cell, and/or capable of enabling or increasing loading (e.g., loading efficiency or loading capacity) of a heterologous functional agent (e.g., an agricultural or therapeutic agent). The PMPs or LPMPs may be modified *in vitro* or *in vivo*.

[0101] As used herein, the term “unmodified PMPs” or “unmodified LPMPs” refers to a composition including a plurality of PMPs or LPMPs that lack a heterologous cell uptake agent capable of increasing cell uptake (e.g., animal cell uptake, plant cell uptake, bacterial cell uptake, or fungal cell uptake) of the PMP.

[0102] As used herein, the term “cell uptake” refers to uptake of a NMP or LNMP or a portion or component thereof (e.g., a polynucleotide carried by the NMP or LNMP) by a cell, such as an animal cell, a plant cell, bacterial cell, or fungal cell. For example, uptake can involve transfer of the NMP (e.g., LNMP) or a portion of component thereof from the extracellular environment into or across the cell membrane, the cell wall, the extracellular matrix, or into the intracellular environment of the cell). Cell uptake of NMPs (e.g., LNMPs) may occur via active or passive cellular mechanisms. Cell uptake includes aspects in which the entire NMP (e.g., LNMP) is taken up by a cell, e.g., taken up by endocytosis. In some embodiments, one or more polynucleotides are exposed to the cytoplasm of the target cell following endocytosis and endosomal escape. In some embodiments, a modified LNMP (e.g., a LNMP comprising an ionizable lipid, e.g., a LNMP comprising an ionizable lipid and a sterol and/or a PEGylated lipid) has an increased rate of endosomal escape relative to an unmodified LNMP. Cell uptake also includes aspects in which the NMP (e.g., LNMP) fuses with the membrane of the target cell. In some embodiments, one or more polynucleotides are exposed to the cytoplasm of the target cell following membrane fusion. In some embodiments, a LNMPs has an increased rate of fusion with the membrane of the target cell (e.g., is more fusogenic) relative to an unmodified LNMP.

[0103] As used herein, the term “cell-penetrating agent” refers to agents that alter properties (e.g., permeability) of the cell wall, extracellular matrix, or cell membrane of a cell (e.g., an animal cell, a plant cell, a bacterial cell, or a fungal cell) in a manner that promotes increased cell uptake relative to a cell that has not been contacted with the agent.

[0104] As used herein, the term "plant" refers to whole plants, plant organs, plant tissues, seeds, plant cells, seeds, and progeny of the same. Plant cells include, without limitation, cells from seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots,

gametophytes, sporophytes, pollen, and microspores. Plant parts include differentiated and undifferentiated tissues including, but not limited to the following: roots, stems, shoots, leaves, pollen, seeds, fruit, harvested produce, tumor tissue, and various forms of cells and culture (e.g., single cells, protoplasts, embryos, and callus tissue). The plant tissue may be in a plant or in a plant organ, tissue, or cell culture. In addition, a plant may be genetically engineered to produce a heterologous protein or RNA.

[0105] As used herein, the term "Bacteria" refers to whole bacteria or parts of bacteria. Further divisions of bacteria can be classified as coccals, bacillus, spirillum, or vibrio, and varying phylums include but are not limited to Proteobacteria, Firmicutes, Bacteroids, sphingobacteria, Flavobacteria, Fusobacteria, Spirochaetes, Chlorobia, Cyanobacteria, Thermomicrobia, Xenobacteria, or Aquificae. Example of specific bacteria species include Staphylococcus aureus, Escherichia coli, Streptococcus pneumoniae, and Pseudomonas aeruginosa. Parts of bacteria include cellular components such as peptidoglycan, outer membranes, inner membranes, cell walls, RNA polymerase, metabolic products, polypeptides, proteins. Flagella, pili, ribosomes, mesosome, cytoplasm, or chromosome. A bacteria may be genetically engineered to produce a heterologous protein or RNA, or may be genetically engineered to not produce an endogenous protein or RNA.

[0106] As used herein, the term "Arthropod" refers to any animal within the phylum Arthropoda, or any animal section, part, organ, tissue, egg, cell, or progeny of the same. Example animals include insects, spiders, and crustaceans. Arthropod cells include, without limitation, cells from eggs, suspension cultures, embryos, tissue, organs, exoskeletion, segments, and appendages. Arthropod parts include body segments, appendages, exoskeleton, eggs, organs, embryos, and various forms of cells and culture. Arthropod tissue may be in an arthropod or in an organ, tissue, or cell culture. An arthropod may be genetically engineered to produce a heterologous protein or RNA. An arthropod may be genetically engineered to not produce an endogenous protein or RNA.

[0107] As used herein, the term "Fungi" refers to whole fungi, fungi organs, fungi tissue, spores, fungi cells, and progeny of the same. Example fungi include yeasts, mushrooms, molds, and mildews. Fungi cells include without limitation cells from spores, suspension cultures, mycelium, hyphae, thallus, cell walls, tissue, gametophytes, sporophytes, and organs. Fungal tissue may be in a fungus or in an organ, tissue, or cell culture. A fungus may be genetically engineered to produce a heterologous protein or RNA. A fungus may be genetically engineered to not produce an endogenous protein or RNA.

[0108] As used herein, the term "Archaea" refers to whole archaea or parts of archaea. Example archaea include euryarchaeota, crenarchaeota, and koraarchaeota. Parts of archaea include cellular components such as RNA polymerases, glycerol-ether lipids, membranes, cell walls, polypeptides, proteins, and metabolic products. An archaea may be genetically engineered to produce a heterologous protein or RNA, or may be genetically engineered to not produce an endogenous protein or RNA.

[0109] As used herein, the term "extracellular vesicle" or "EV" refers to an enclosed lipid-bilayer structure naturally occurring in an organism or cell. Optionally, the EV includes one or more EV markers. As used herein, the term "EV marker" refers to a component that is naturally associated with

a specific organism, such as a protein, a nucleic acid, a small molecule, a lipid, or a combination thereof. In some instances, the EV marker is an identifying marker of an EV but is not a pesticidal agent. In some instances, the EV marker is an identifying marker of an EV and also a pesticidal agent (e.g., either associated with or encapsulated by the plurality of NMPs, or not directly associated with or encapsulated by the plurality of NMPs).

[0110] As used herein, the term "natural messenger pack" or "NMP" refers to a lipid structure (e.g., a lipid bilayer, unilamellar, multilamellar structure; e.g., a vesicular lipid structure), that is about 5-2000 nm (e.g., at least 5-1000 nm, at least 5-500 nm, at least 400-500 nm, at least 25-250 nm, at least 50-150 nm, or at least 70-120 nm) in diameter that is derived from (e.g., enriched, isolated or purified from) a natural source or segment, portion, or extract thereof, including lipid or non-lipid components (e.g., peptides, nucleic acids, or small molecules) associated therewith and that has been enriched, isolated or purified from a natural source, such as a Plant, Arthropod, Fungi, Archaea, or Bacteria ; an Arthropod, Plant, Fungi, Archaea, or Bacteria part; or an Arthropod, Plant, Fungi, Archaea, or Bacteria cell, the enrichment or isolation removing one or more contaminants or undesired components from the source. NMPs are also able to be isolated from other natural sources, such as algae or animal organs. NMPs may be highly purified preparations of naturally occurring EVs. Preferably, at least 1% of contaminants or undesired components from the source are removed (e.g., at least 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 45%, 50%, 55%, 60%, 70%, 80%, 90%, 95%, 96%, 98%, 99%, or 100%) of one or more contaminants or undesired components from the source, e.g., cell wall components; membrane components; chitin; organelles (e.g., mitochondria; nucleolus; golgi complex; ribosomes, endoplasmic reticulum and nuclei); chromatin; or molecular aggregates (e.g., protein aggregates, protein-nucleic acid aggregates, lipoprotein aggregates, or lipido-proteic structures). Preferably, a NMP is at least 30% pure (e.g., at least 40% pure, at least 50% pure, at least 60% pure, at least 70% pure, at least 80% pure, at least 90% pure, at least 99% pure, or 100% pure) relative to the one or more contaminants or undesired components from the source as measured by weight (w/w), spectral imaging (% transmittance), or conductivity (S/m).

[0111] As used herein, the term "plant extracellular vesicle", "plant EV", or "EV" refers to an enclosed lipid-bilayer structure naturally occurring in a plant. Optionally, the plant EV includes one or more plant EV markers. As used herein, the term "plant EV marker" refers to a component that is naturally associated with a plant, such as a plant protein, a plant nucleic acid, a plant small molecule, a plant lipid, or a combination thereof, including but not limited to any of the plant EV markers listed in the Appendix. In some instances, the plant EV marker is an identifying marker of a plant EV but is not a pesticidal agent. In some instances, the plant EV marker is an identifying marker of a plant EV and also a pesticidal agent (e.g., either associated with or encapsulated by the plurality of PMPs or LPMPs, or not directly associated with or encapsulated by the plurality of PMPs or LPMPs).

[0112] As used herein, the term "plant messenger pack" or "PMP" refers to a lipid structure (e.g., a lipid bilayer, unilamellar, multilamellar structure; e.g., a vesicular lipid structure), that is about 5-2000 nm (e.g., at least 5-1000 nm, at least 5-500 nm, at least 400-500 nm, at least 25-250 nm, at least 50-150 nm, or at least 70-120 nm) in diameter that is derived from (e.g., enriched, isolated or purified from) a plant source or segment, portion, or extract thereof, including lipid or non-lipid components

(e.g., peptides, nucleic acids, or small molecules) associated therewith and that has been enriched, isolated or purified from a plant, a plant part, or a plant cell, the enrichment or isolation removing one or more contaminants or undesired components from the source plant. PMPs may be highly purified preparations of naturally occurring EVs. Preferably, at least 1% of contaminants or undesired components from the source plant are removed (e.g., at least 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 45%, 50%, 55%, 60%, 70%, 80%, 90%, 95%, 96%, 98%, 99%, or 100%) of one or more contaminants or undesired components from the source plant, e.g., plant cell wall components; pectin; plant organelles (e.g., mitochondria; plastids such as chloroplasts, leucoplasts or amyloplasts; and nuclei); plant chromatin (e.g., a plant chromosome); or plant molecular aggregates (e.g., protein aggregates, protein-nucleic acid aggregates, lipoprotein aggregates, or lipido-proteic structures). Preferably, a PMP is at least 30% pure (e.g., at least 40% pure, at least 50% pure, at least 60% pure, at least 70% pure, at least 80% pure, at least 90% pure, at least 99% pure, or 100% pure) relative to the one or more contaminants or undesired components from the source plant as measured by weight (w/w), spectral imaging (% transmittance), or conductivity (S/m).

[0113] A lipid reconstructed NMP (LNMP) is used herein. For instance, a lipid reconstructed PMP (LPMP) is used herein. The terms “lipid reconstructed NMP” and “LNMP” refer to a NMP that has been derived from a lipid structure (e.g., a lipid bilayer, unilamellar, multilamellar structure; e.g., a vesicular lipid structure) derived from (e.g., enriched, isolated or purified from) an Arthropod, Plant, Fungi, Archaea, or Bacteria source, wherein the lipid structure is disrupted (e.g., disrupted by lipid extraction) and reassembled or reconstituted in a liquid phase (e.g., a liquid phase containing a cargo) using standard methods, e.g., reconstituted by a method comprising lipid film hydration, multifluidics, and/or solvent injection, to produce the LNMP, as is described herein. The method may, if desired, further comprise sonication, freeze/thaw treatment, and/or lipid extrusion, e.g., to reduce the size of the reconstituted NMPs. Alternatively, LNMPs may be produced using a microfluidic device (such as a NanoAssemblr® IGNITE™ microfluidic instrument (Precision NanoSystems)).

[0114] A lipid reconstructed PMP (LPMP) is used herein as a subcategory of lipid reconstructed NMPs. The terms “lipid reconstructed PMP” and “LPMP” refer to a PMP that has been derived from a lipid structure (e.g., a lipid bilayer, unilamellar, multilamellar structure; e.g., a vesicular lipid structure) derived from (e.g., enriched, isolated or purified from) a plant source, wherein the lipid structure is disrupted (e.g., disrupted by lipid extraction) and reassembled or reconstituted in a liquid phase (e.g., a liquid phase containing a cargo) using standard methods, e.g., reconstituted by a method comprising lipid film hydration and/or solvent injection, to produce the LPMP, as is described herein. The method may, if desired, further comprise sonication, freeze/thaw treatment, and/or lipid extrusion, e.g., to reduce the size of the reconstituted LPMPs. Alternatively, LPMPs may be produced using a microfluidic device (such as a NanoAssemblr® IGNITE™ microfluidic instrument (Precision NanoSystems)).

[0115] As used herein, the term “pure” refers to a PMP preparation in which at least a portion (e.g., at least 20%, 25%, 30%, 40%, 45%, 50%, 55%, 60%, 70%, 80%, 90%, 95%, 96%, 98%, 99%, or 100%) of plant cell wall components, plant organelles (e.g., mitochondria, chloroplasts, and nuclei), or plant molecule aggregates (protein aggregates, protein-nucleic acid aggregates, lipoprotein

aggregates, or lipido-proteic structures) have been removed relative to the initial sample isolated from a plant, or part thereof.

[0116] As used herein, the term "complex lipid particle" refers to a lipid particle that has a complexity characterized by comprising a wide variety of lipids, including structural lipids extracted from one or more natural sources (such as plants or bacteria), and optionally at least one exogenous ionizable lipid. The complex lipid particle may comprise between 10% w/w and 99% w/w structural lipids derived from a lipid structure from one or more natural sources, e.g., it may contain at least 10% w/w, at least 20% w/w, at least 30% w/w, at least 40% w/w, at least 50% w/w, at least 60% w/w, at least 70% w/w, at least 80% w/w, at least 90% w/w, at least 95% w/w, or about 99% w/w lipids derived from a lipid structure from one or more natural sources. In some instances, a complex lipid particle incorporating natural lipid extracts may also be referred to as a natural messenger pack (NMP). For instance, a complex lipid particle incorporating plant lipid extracts may also be referred to as a plant messenger pack (PMP). In some instances, a complex lipid particle incorporating natural lipid extracts and at least one exogenous ionizable lipid may also be referred to as a lipid reconstructed natural messenger pack (LNMP). For instance, a complex lipid particle incorporating plant lipid extracts and at least one exogenous ionizable lipid may also be referred to as a lipid reconstructed plant messenger pack (LPMP). Thus, any disclosure herein describing the features relating to LNMP and LNMP formulation are applicable to CLP and CLP formulation.

[0117] The complex lipid particle may contain 3-1000 lipids extracted from one or more natural (e.g., plant, bacteria) sources. The complex lipid particle may contain natural (e.g., plant, bacteria) lipids from at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 different classes or sub-classes of lipids from the natural (e.g., plant, bacteria) source. The complex lipid particle may comprise all or a fraction of the lipid species present in the lipid structure from the natural (e.g., plant, bacteria) source, e.g., it may contain at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or virtually 100% of the lipid species present in the lipid structure from the natural source. The complex lipid particle may comprise all or a fraction of the lipid species present in the lipid structure from a particular natural source. For instance, it may contain at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or virtually 100% of the lipid species present in the lipid structure from a plant source or from a bacteria source.

[0118] The complex lipid particle may comprise reduced or minimized protein matter endogenous to the one or more natural (i.e. plant, bacteria) sources, e.g., it may contain 0% w/w, less than 1% w/w, less than 5% w/w, less than 10% w/w, less than 15% w/w, less than 20% w/w, less than 30% w/w, less than 40% w/w, or less than 50% w/w of the protein matter endogenous to the one or more natural (e.g., plant, bacteria) sources. In some instances, the lipid bilayer of the complex lipid particle does not contain proteins.

[0119] The complex lipid particle may also include synthetic structural lipids such as neutral lipids as the structural lipid component. The structural lipid component of the complex lipid particle may comprise between 10% w/w and 99% w/w structural lipids derived from a synthetic lipid structure (as opposed to the lipids extracted from a natural source), e.g., it may contain at least 10% w/w, at least

20% w/w, at least 30% w/w, at least 40% w/w, at least 50% w/w, at least 60% w/w, at least 70% w/w, at least 80% w/w, at least 90% w/w, at least 95% w/w, or about 99% w/w lipids derived from a synthetic lipid structure.

[0120] The complex lipid particle may further comprise at least two exogenous lipids. The complex lipid particle may include at least 1% w/w, at least 2% w/w, at least 5% w/w, at least 10% w/w, at least 15% w/w, at least 20% w/w, at least 25% w/w, at least 30% w/w, at least 40% w/w, at least 50% w/w, at least 60% w/w, at least 70% w/w, at least 80% w/w, or about 90% w/w exogenous lipids. Exemplary exogenous lipids include sterols and PEG-lipid conjugate. The complex lipid particle may be used to encapsulate one or more exogenous nucleic acids or polynucleotides encoding one or more peptides, polypeptides, or proteins, to enable delivery of the exogenous nucleic acids or polynucleotides to a target cell or tissue.

[0121] As used herein, the term “exogenous lipid” refers to a lipid that is exogenous to the natural source (e.g., plant, bacteria), i.e., a lipid originates from a source that is not the natural source from which the lipids are extracted (e.g., a lipid that is added to the complex lipid particle formulation using method described herein). The term “exogenous lipid” does not exclude a natural-derived lipid (such as a plant-derived sterol). That is to say, an exogenous lipid can be a natural-derived lipid (such as a plant-derived sterol that is exogenous to the plant source from which the lipids are extracted, e.g., an exogenous lipid can be a plant derived sterol that is added to the complex lipid particle formulation). As another example, an exogenous lipid can be a natural-derived lipid that is exogenous to the particular natural source from which the lipids are extracted (e.g., a bacteria-derived lipid that is exogenous to the plant source from which the lipids are extracted, or vice versa). An exogenous lipid may be a cell-penetrating agent, may be capable of increasing delivery of one or more polynucleotides by the complex lipid formulation to a cell, and/or may be capable of increasing loading (e.g., loading efficiency or loading capacity) of a polynucleotide. In some embodiments, the exogenous lipid may be a stabilizing lipid. In some embodiments, the exogenous lipid may be a structural lipid (e.g., a synthetic structural lipid). Exemplary exogenous lipids include ionizable lipids, synthetic structural lipids, sterols, and PEGylated lipids.

[0122] As used herein, the term “cationic lipid” refers to an amphiphilic molecule (e.g., a lipid or a lipidoid) that is positively charged, containing a cationic group (e.g., a cationic head group).

[0123] As used herein, the term “ionizable lipid” refers to an amphiphilic molecule (e.g., a lipid or a lipidoid, e.g., a synthetic lipid or lipidoid) containing a group (e.g., a head group) that can be ionized, e.g., dissociated to produce one or more electrically charged species, under a given condition (e.g., pH). It has been surprisingly found that ionizable lipids comprising alkyl chains with multiple sites of unsaturation, e.g., at least two or three sites of unsaturation, are particularly useful for forming lipid particles with increased membrane fluidity. A number of ionizable lipids and related analogs, suitable for use herein, have been described in U.S. Patent Publication Nos. 20060083780 and 20060240554; U.S. Pat. Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390, the disclosures of which are herein incorporated by reference in their entirety for all purposes. In some embodiments, ionizable lipids are ionizable such that they can dissociate to exist in a positively charged form depending on pH. The ionization of an ionizable lipid

affects the surface charge of a lipid nanoparticle comprising the ionizable lipid under different pH conditions. The surface charge of the lipid nanoparticle in turn can influence its plasma protein absorption, blood clearance, and tissue distribution (Semple, S.C., et al., Adv. Drug Deliv Rev 32:3-17 (1998)) as well as its ability to form endosomolytic non-bilayer structures (Hafez, I.M., et al., Gene Ther 8: 1188-1196 (2001)) that can influence the intracellular delivery of nucleic acids.

[0124] In some embodiments, ionizable lipids are those that are generally neutral, e.g., at physiological pH (e.g., pH about 7), but can carry net charge(s) at an acidic pH or basic pH. In one embodiment, ionizable lipids are those that are generally neutral at pH about 7, but can carry net charge(s) at an acidic pH. In one embodiment, ionizable lipids are those that are generally neutral at pH about 7, but can carry net charge(s) at a basic pH. In some embodiments, ionizable lipids do not include those cationic lipids or anionic lipids that generally carry net charge(s) at physiological pH (e.g., pH about 7).

[0125] As used herein, the term “lipidoid” refers to a molecule having one or more characteristics of a lipid.

[0126] As used herein, the term “stable LNMP formulation” or “stable CLP formulation” refers to a CLP formulation or a LNMP composition that over a period of time (e.g., at least 24 hours, at least 48 hours, at least 1 week, at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 30 days, at least 60 days, or at least 90 days) retains at least 5% (e.g., at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) of the initial number of CLPs or LNMPs (e.g., CLPs or LNMPs per mL of solution) relative to the number of CLPs or LNMPs in the CLP formulation or LNMP formulation (e.g., at the time of production or formulation) optionally at a defined temperature range (e.g., a temperature of at least 24°C (e.g., at least 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, or 30°C), at least 20°C (e.g., at least 20°C, 21°C, 22°C, or 23°C), at least 4°C (e.g., at least 5°C, 10°C, or 15°C), at least -20°C (e.g., at least -20°C, -15°C, -10°C, -5°C, or 0°C), or -80°C (e.g., at least -80°C, -70°C, -60°C, -50°C, -40°C, or -30°C)); or retains at least 5% (e.g., at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) of its activity (e.g., cell wall penetrating activity and/or activity of the mRNA formulated within the CLP or LNMP) relative to the initial activity of the CLP or LNMP (e.g., at the time of production or formulation) optionally at a defined temperature range (e.g., a temperature of at least 24°C (e.g., at least 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, or 30°C), at least 20°C (e.g., at least 20°C, 21°C, 22°C, or 23°C), at least 4°C (e.g., at least 5°C, 10°C, or 15°C), at least -20°C (e.g., at least -20°C, -15°C, -10°C, -5°C, or 0°C), or -80°C (e.g., at least -80°C, -70°C, -60°C, -50°C, -40°C, or -30°C)).

[0127] Alternatively, the expression refers to a CLP formulation or LNMP composition that over a period of time (e.g., at least 24 hours, at least 48 hours, at least 1 week, at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 30 days, at least 60 days, or at least 90 days) retains at least 5% (e.g., at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) of its activity relative to the initial activity of the CLP formulation or LNMP formulation (e.g., at the time of production or formulation) optionally at a defined temperature range (e.g., a temperature of at least 24°C (e.g., at least 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, or

30°C), at least 20°C (e.g., at least 20°C, 21°C, 22°C, or 23°C), at least 4°C (e.g., at least 5°C, 10°C, or 15°C), at least -20°C (e.g., at least -20°C, -15°C, -10°C, -5°C, or 0°C), or -80°C (e.g., at least -80°C, -70°C, -60°C, -50°C, -40°C, or -30°C)).

[0128] Alternatively, the expression refers to a CLP formulation or a LNMP formulation that over a period of time (e.g., at least 24 hours, at least 48 hours, at least 1 week, at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 30 days, at least 60 days, or at least 90 days) retains their particle size, i.e., the particle size does not increase, or has an increase of no more than 5% (e.g., no more than 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 150%, 2-fold, 2.5-fold, or 3-fold) relative to the initial size of the CLPs or LNMPs (e.g., at the time of production or formulation) optionally at a defined temperature range (e.g., a temperature of at least 24°C (e.g., at least 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, or 30°C), at least 20°C (e.g., at least 20°C, 21°C, 22°C, or 23°C), at least 4°C (e.g., at least 5°C, 10°C, or 15°C), at least -20°C (e.g., at least -20°C, -15°C, -10°C, -5°C, or 0°C), or -80°C (e.g., at least -80°C, -70°C, -60°C, -50°C, -40°C, or -30°C)).

[0129] In some embodiments, the stable CLP or LNMP formulation continues to encapsulate or remains associated with an exogenous peptide, polypeptide, or protein with which the CLP or LNMP formulation has been loaded, e.g., continues to encapsulate or remains associated with an exogenous peptide, polypeptide, or protein for at least 24 hours, at least 48 hours, at least 1 week, at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 30 days, at least 60 days, at least 90 days, or 90 or more days.

[0130] As used herein, the term “treatment” refers to administering a pharmaceutical composition to an animal for prophylactic and/or therapeutic purposes.

[0131] As used herein, the term “gene editing system” or “genome editing system” refers to any genome editing technology including the CRISPR-Cas nuclease system, transcription activator-like effector nucleases (TALENS), zinc-finger nucleases (ZFNs), or other known means of inducing gene editing.

[0132] As used herein, an "RNA-guided DNA binding agent" means a polypeptide or complex of polypeptides having RNA and DNA binding activity, or a DNA-binding subunit of such a complex, wherein the DNA binding activity is sequence-specific and depends on the sequence of the RNA. Exemplary RNA-guided DNA binding agents include Cas cleavases/nickases and inactivated forms thereof ("dCas DNA binding agents"). "Cas nuclease", as used herein, encompasses Cas cleavases, Cas nickases, and dCas DNA binding agents. Cas cleavases/nickases and dCas DNA binding agents include a Csm or Cmr complex of a type III CRISPR system, the Cas 10, Csm1, or Cmr2 subunit thereof, a Cascade complex of a type I CRISPR system, the Cas3 subunit thereof, and Class II Cas nucleases. As used herein, a "Class II Cas nuclease" is a single-chain polypeptide with RNA-guided DNA binding activity. Class II Cas nucleases include Class II Cas cleavases/nickases (e.g., H840A, D 10A, or N863A variants), which further have RNA-guided DNA cleavase or nickase activity, and Class II dCas DNA binding agents, in which cleavase/nickase activity is inactivated. Class II Cas nucleases include, for example, Cas9, Cpf1, C2c1, C2c2, C2e3, HF Cas9 (e.g., N497A, R661A, Q695A, Q926A variants), HypaCas9 (e.g., N692A, M694A, Q695A, H69SA variants), eSPCas9(1.0) (e.g, K810A,

K1003A, R1060A variants), and eSPCas9(1.1) (e.g., K848A, K1003A, R1060A variants) proteins and modifications thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0133] Figure 1A is a graph depicting the TTR concentration (ug/mL) in the blood of the mice at D0, D2, and D7, dosed intravenously with either 3 mg/kg recLemon LPMP (1:1 Cas9 mRNA:sgRNA mTTR_G211) or an equivalent volume of PBS. N=5/group. Figure 1B shows the amount of gene editing (%) of the target gene (TTR) in the bone marrow, spleen, and the targeted organ, the liver, with an intravenous dose of either an LPMP delivering CRISPR/Cas gene editing components (3 mg/kg recLemon LPMP; 1:1 Cas9 mRNA:sgRNA mTTR_G211) or an equivalent volume of PBS. N=5/group.

[0134] Figure 2A depicts the TTR concentration (ug/mL) in the blood of the mice dosed intravenously with various exemplary recLemon LPMPs (1 mg/kg recLemon LPMP; 1:1 Cas9 mRNA:sgRNA mTTR_G211) or an equivalent volume of PBS at D0, D3, D7, D14, D21, D28, and D35 post-dose. N=3/group. Figure 2B depicts the TTR concentration (ug/mL) in the blood of mice dosed intravenously with various exemplary LNPs (1 mg/kg LNP; 1:1 Cas9 mRNA:sgRNA mTTR_G211) or an equivalent volume of PBS at D0, D3, D7, D14, D21, D28, and D35 post-dose. N=3/group.

[0135] Figure 3A shows the percentage of the TTR protein from baseline in the liver 7 days after an intravenous dose of various exemplary recLemon LPMP formulations (Figure 5) delivering CRISPR/Cas gene editing components (1 mg/kg recLemon LPMP; 1:1 Cas9 mRNA:sgRNA mTTR_G211) or an equivalent volume of PBS. N=3/group. Figure 3B shows the percent of TTR protein from baseline in the liver 7 days after an intravenous dose of various LNP formulations (Figure 5) delivering CRISPR/Cas gene editing components (1 mg/kg LNP; 1:1 Cas9 mRNA:sgRNA mTTR_G211) or an equivalent volume of PBS. N=3/group.

[0136] Figure 4A shows the percentage of the TTR protein from baseline in the liver 21 days after an intravenous dose of various exemplary recLemon LPMP formulations delivering CRISPR/Cas gene editing components (1 mg/kg recLemon LPMP; 1:1 Cas9 mRNA:sgRNA mTTR_G211) or an equivalent volume of PBS. N=3/group. Figure 4B shows the percentage of the TTR protein from baseline in the liver 21 days after an intravenous dose of various LNP formulations delivering CRISPR/Cas gene editing components (1 mg/kg LNP; 1:1 Cas9 mRNA:sgRNA mTTR_G211) or an equivalent volume of PBS. N=3/group.

[0137] Figure 5A shows the percentage of the TTR protein from baseline in the liver 28 days after an intravenous dose of various exemplary recLemon LPMP formulations delivering CRISPR/Cas gene editing components (1 mg/kg recLemon LPMP; 1:1 Cas9 mRNA:sgRNA mTTR_G211) or an equivalent volume of PBS. N=3/group. Figure 5B shows the percentage of the TTR protein from baseline in the liver 28 days after an intravenous dose of various LNP formulations delivering CRISPR/Cas gene editing components (1 mg/kg LNP; 1:1 Cas9 mRNA:sgRNA mTTR_G211) or an equivalent volume of PBS. N=3/group.

[0138] Figure 6A shows the percentage of the TTR protein from baseline in the liver 35 days after an intravenous dose of various exemplary recLemon LPMP formulations delivering CRISPR/Cas

gene editing components (1 mg/kg recLemon LPMP; 1:1 Cas9 mRNA:sgRNA mTTR_G211) or an equivalent volume of PBS. N=3/group. Figure 6B shows the percentage of the TTR protein from baseline in the liver 35 days after an intravenous dose of various LNP formulations delivering CRISPR/Cas gene editing components (1 mg/kg LNP; 1:1 Cas9 mRNA:sgRNA mTTR_G211) or an equivalent volume of PBS. N=3/group.

[0139] Figure 7A depicts the TTR concentration (ug/mL) in the blood of the mice dosed intravenously with various exemplary recLemon LPMPs at dosage of 1.5 mg/kg or 0.3 mg/kg recLemon LPMP (1:1 Cas9 mRNA:sgRNA mTTR_G211) or an equivalent volume of PBS, at D0, D3, and D7 post-dose. N=6-7/group. Figure 7B shows the percentage of the TTR protein from baseline in the liver 7 days after an intravenous dose of various exemplary recLemon LPMP formulations delivering CRISPR/Cas gene editing components at dosage of 1.5 mg/kg or 0.3 mg/kg recLemon LPMP (1:1 Cas9 mRNA:sgRNA mTTR_G211) or an equivalent volume of PBS. N=6-7/group.

[0140] Figure 8A shows the whole body radiance (average radiance p/s/cm^2/sr) at 4 hours post-dose of intravenous administration of various exemplary recLemon LPMPs and LNP formulations (LNP 2272, recLemon LPMP 2272, LNP Lipid 5, LNP C12, recLemon LPMP C12_EE, or recLemon LPMP_CM) encapsulating 1:1 mRNA FLuc:hEPO, dosed at 0.5 mg/kg, in mice given 8 doses (each dose was one to two weeks apart). LNP Lipid 5 was measured starting from Dose 3. N=5/group. Figure 8B shows the systemic hEPO concentration (pg/mL) at pre-dose (0h), 4h post-dose, and 24h post-dose of intravenous administration of various exemplary recLemon LPMPs and LNP formulations (LNP 2272, recLemon LPMP 2272, LNP Lipid 5, LNP C12, recLemon LPMP C12_EE, or recLemon LPMP_CM) encapsulating 1:1 mRNA FLuc:hEPO, dosed at 0.5 mg/kg, in mice given 5 doses (each dose was one week apart. Pre-dose measures were not taken prior to Dose 4 and 5. LNP Lipid 5 was measured starting from Dose 3. N=5/group.

[0141] Figure 9A shows the whole body radiance (average radiance p/s/cm^2/sr) at 4 hours post-dose of intravenous administration of various exemplary recLemon LPMPs and LNP formulations (LNP 2272, recLemon LPMP 2272, LNP Lipid 5, LNP C12, recLemon LPMP C12_EE, or recLemon LPMP_CM) (0.125 mg/kg per dose; encapsulating 1:1 mRNA FLuc:hEPO) in mice given 5 doses (each dose was one week apart). N=5/group. Figure 9B shows the systemic hEPO concentration (pg/mL) at pre-dose (0h), 4h post-dose, and 24h post-dose of intravenous administration of various exemplary recLemon LPMPs and LNP formulations (LNP 2272, recLemon LPMP 2272, LNP Lipid 5, LNP C12, recLemon LPMP C12_EE, or recLemon LPMP_CM) (0.125 mg/kg per dose; encapsulating 1:1 mRNA FLuc:hEPO) in mice given 5 doses (each dose was one week apart). N=5/group.

[0142] Figure 10A shows the anti-PEG IgM antibody titers at 0h pre-dose of intravenous administration of various exemplary recLemon LPMPs and LNP formulations (LNP 2272, recLemon LPMP 2272, LNP Lipid 5, LNP C12, recLemon LPMP C12_EE, or recLemon LPMP_CM) (0.5 mg/kg per dose; encapsulating 1:1 mRNA FLuc:hEPO) in mice given 5 doses (each dose was one week apart). N=5/group. Figure 10B shows the anti-PEG IgG antibody titers at 0h pre-dose of intravenous administration of various exemplary recLemon LPMPs and LNP formulations (LNP 2272, recLemon LPMP 2272, LNP Lipid 5, LNP C12, recLemon LPMP C12_EE, or recLemon LPMP_CM) (0.5 mg/kg per dose; encapsulating 1:1 mRNA FLuc:hEPO) in mice given 5 doses (each dose was one week

apart). N=5/group.

[0143] Figure 11A shows the systemic hEPO concentration (pg/mL) post-dose of intravenous administration of various exemplary recLemon LPMPs and LNP formulations (recLemon LPMP 2272, recLemon LPMP 2320, or 2320 LNP) (1 mg/kg or 0.5 mg/kg per dose; encapsulating 3:1 mRNA FLuc:hEPO) in mice given 5 doses (each dose was one week apart). N=5/group. Figure 11B shows the whole body radiance (average radiance p/s/cm^2/sr) at 4 hours post-dose of intravenous administration of various exemplary recLemon LPMPs and LNP formulations (recLemon LPMP 2272, recLemon LPMP 2320, or 2320 LNP) (1 mg/kg or 0.5 mg/kg per dose; encapsulating 3:1 mRNA FLuc:hEPO) in mice given 5 doses (each dose was one week apart). N=5/group. Figures 11C and 11D show the anti-PEG IgG (Figure 11C) and anti-PEG IgM (Figure 11D) antibody titers at 0h pre-dose of various exemplary recLemon LPMPs and LNP formulations (recLemon LPMP 2272, recLemon LPMP 2320, or 2320 LNP) (1 mg/kg or 0.5 mg/kg per dose; encapsulating 3:1 mRNA Fluc:hEPO) in mice given 5 doses (each dose was one week apart). N=5/group.

[0144] Figure 12A shows the whole body radiance (average radiance p/s/cm^2/sr) at 4 hours post-dose of subcutaneous administration of various exemplary recLemon LPMPs and LNP formulations (recLemon LPMP 2272, recLemon LPMP 2320, recLemon LPMP 2439, 2272 LNP, 2320 LNP, or 2439 LNP) (0.2 mg/kg per dose; encapsulating 1:1 mRNA FLuc:hEPO) in mice given 5 doses (each dose was one week apart). N=5/group. Figures 12B and 12C show the anti-PEG IgG (12B) and anti-PEG IgM (12C) antibody titers at 0h pre-dose of various exemplary recLemon LPMPs and LNP formulations (recLemon LPMP 2272, recLemon LPMP 2320, recLemon LPMP 2439, 2272 LNP, 2320 LNP, or 2439 LNP) (0.2 mg/kg per dose; encapsulating 1:1 mRNA FLuc:hEPO) in mice given 5 doses (each dose was one week apart). N=5/group.

[0145] Figure 13A shows the whole body radiance (average radiance p/s/cm^2/sr) at 4 hours post-dose of either subcutaneous (SQ) or intravenous (IV) administration of recLemon LPMP 2272 or 2272 LNP (0.5 mg/kg per dose; encapsulating 1:1 mRNA FLuc:hEPO) in mice given 5 doses (each dose was two weeks apart). N=5/group. Figure 13B shows systemic hEPO concentration (pg/mL) post-dose of subcutaneous or intravenous administration of recLemon LPMP 2272 or 2272 LNP (0.5 mg/kg per dose; encapsulating 1:1 mRNA FLuc:hEPO) in mice given 5 doses (each dose was two weeks apart). N=5/group. Figures 13C and 13D show the anti-PEG IgG (Figure 13C) and anti-PEG IgM (Figure 13D) antibody titers at 0h pre-dose of recLemon LPMP 2272 or 2272 LNP (0.5 mg/kg per dose; encapsulating 1:1 mRNA FLuc:hEPO) in mice given 5 doses (each dose was two weeks apart). N=5/group. 2272 LNP and PBS were controls.

DETAILED DESCRIPTION

[0146] Featured herein are RNA compositions (e.g., mRNA/gRNA encoding gene editing systems), that can safely direct the body's cellular machinery to edit the genome, and methods of using these RNA compositions for delivering a gene editing system or for gene editing. The RNA compositions may be dosed repeatedly (e.g., 5 times, 6 times, 7 times, 8 times). These RNA compositions include one or more polynucleotides (e.g., RNA such as messenger RNA (mRNA or gRNA)) encoding one or more components of a gene editing system, or one or more gene editing systems, formulated within a

lipid nanoparticle (LNP) or a complex lipid particle (CLP). In some embodiments, the CLP is a lipid reconstructed natural messenger packs (LNMPs) comprising lipids extracted from one or more natural sources (i.e., natural lipids) and an ionizable lipid. NMPs are lipid assemblies produced wholly or in part from natural extracellular vesicles (EVs), or segments, portions, or extracts thereof. PMPs are lipid assemblies produced wholly or in part from plant extracellular vesicles (EVs), or segments, portions, or extracts thereof. LNMPs are NMPs derived from a lipid structure wherein the lipid structure is disrupted and reassembled or reconstituted in a liquid phase. For instance, LPMPs are PMPs derived from a lipid structure wherein the lipid structure is disrupted and reassembled or reconstituted in a liquid phase.

[0147] In another aspect, this disclosure also includes a method of repeatable dosing to a subject in need thereof, the method comprising administering to the subject the RNA composition comprising one or more polynucleotides, formulated within an LNP or LNMP, as described herein.

[0148] The disclosure also includes a method for making an RNA composition, comprising reconstituting a film comprising purified NMP lipids in the presence of an ionizable lipid to produce a LNMP comprising the ionizable lipid, and loading into the LNMPs with one or more polynucleotides encoding one or more components of a gene editing system or one or more gene editing systems.

Complex Lipid Particles and Lipid Reconstructed Natural Messenger Packs (LNMPs)

Complex Lipid Particles

[0149] Complex lipid particles (CLPs) described herein comprise a wide variety of lipids, including structural lipids extracted from one or more natural sources (such as plants or bacteria). In some embodiments, a complex lipid particle is a natural messenger pack (NMP) incorporating natural lipid extracts. In some embodiments, a complex lipid particle is a lipid reconstructed natural messenger pack (LNMP) incorporating natural lipid extracts and at least one exogenous ionizable lipid.

[0150] The complex lipid particles may also comprise at least exogenous ionizable lipid. The ionizable lipid has two or more of the characteristics listed below:

(i) at least 2 ionizable amines;

(ii) at least 3 lipid tails; wherein each of the lipid tails is at least 6 carbon atoms in length;

(iii) a pKa of about 4.5 to about 7.5;

(iv) an ionizable amine and a heteroorganic group separated by a chain of at least two atoms; and

(v) an N:P ratio of at least 3.

[0151] The complex lipid particle may comprise between 10% w/w and 99% w/w structural lipids derived from a lipid structure from one or more natural sources, e.g., it may contain at least 10% w/w, at least 20% w/w, at least 30% w/w, at least 40% w/w, at least 50% w/w, at least 60% w/w, at least 70% w/w, at least 80% w/w, at least 90% w/w, at least 95% w/w, or about 99% w/w lipids derived from a lipid structure from one or more natural sources.

[0152] In some embodiments, the complex lipid particle comprises about 10-95% w/w of the natural (e.g., plant, bacteria) lipids. For instance, the complex lipid particle comprises about 25-95% w/w, about 30-95% w/w, about 35-95% w/w, about 40-95% w/w, about 45-95% w/w, about 50-95% w/w,

about 55-95% w/w, about 60-95% w/w, about 65-95% w/w, about 70-95% w/w, about 75-95% w/w, about 80-95% w/w, or about 85-95% w/w of the natural (e.g., plant, bacteria) lipids based on the amounts of total lipids in the complex lipid formulation.

[0153] The complex lipid particle may contain 3-1000 lipids extracted from one or more natural (e.g., plant, bacteria) sources. In some embodiments, the natural source is a plant, plant extract, or fragment or part of a plant. In some embodiments, the natural source is a bacteria, bacteria fragment or part of a bacteria. In some embodiments, the natural source is lemon. In some embodiments the natural source is soy. In other embodiments, the natural source is E. coli.

[0154] In some embodiments, the complex lipid particle contains at least 10 natural lipids belonging to one or more of the classes selected from the group consisting of fatty acyls (FA), fatty acyl conjugates, phospholipids, glycerolipids, glycolipids, glycerophospholipids, sphingolipids, waxes, and sterol. For instance, the complex lipid particle contains at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 150, at least 200, at least 250, at least 300, at least 400, at least 500, at least 600, at least 700, or at least 800 natural lipids belonging to one or more of the classes selected from the group consisting of fatty acyls (FA), fatty acyl conjugates, phospholipids, glycerolipids, glycolipids, glycerophospholipids, sphingolipids, waxes, and sterol. In some embodiments, the complex lipid particle contains lipids from at least two or at least three of these different classes.

[0155] In some embodiments, the complex lipid particle contains at least 10 natural lipids belonging to one or more of the classes selected from the group consisting of glycerolipid, sphingolipid, and sterol. For instance, the complex lipid particle contains at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 150, at least 200, at least 250, at least 300, at least 400, at least 500, at least 600, at least 700, or at least 800 natural lipids belonging to one or more of the classes selected from the group consisting of glycerolipid, sphingolipid, and sterol. In some embodiments, the complex lipid particle contains lipids from at least two or at least three of these different classes.

[0156] In some embodiments, the complex lipid particle may contain one or more glycerolipids (GL) or glycerophospholipids (GP), which may also include glycolipids.

[0157] In some embodiments, the complex lipid particle may contain one or more glycerolipids selected from the group consisting of phospholipids (PL), galactolipids, triacylglycerols (TG), and sulfolipids (SL). In some embodiments, the CLPs contains one or more glycerophospholipids (GP) selected from the group consisting of phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), and phosphatidylinositols (PI). In some embodiments, the complex lipid particle contains one or more sphingolipids (SP) selected from the group consisting of sulfolipids (SL), glycosyl inositol phosphoryl ceramides (GIPC), glucosylceramides (GCer), ceramides (Cer), and free long-chain bases (LCB). In some embodiments, the complex lipid particle contains one or more phytosterols selected from the group consisting of campesterol, stigmasterol, β-sitosterol, Δ5-avenasterol, brassicasterol, avenasterol, 4-desmethyl sterol, 4α-monomethyl sterol, Δ5-sterol, Δ7-sterol, α-spinasterol, Δ5,Δ7-sterol, phytostanol, and sitosterol.

[0158] The CLP may contain one or more natural lipids belonging to one or more classes or sub-

classes selected from the group consisting of fatty acids, fatty esters, fatty aldehydes, fatty amides, acyclic oxylipins, cyclic oxylipins, glycerolipids, monoradylglycerols, diradylglycerols, triradylglycerols, estolides, glycosylmonoacylglycerols, sulfoquinovosylmonoacylglycerols, monogalactosylmonoacylglycerol, digalactosylmonoacylglycerol, sulfoquinovosyldiacylglycerols, monogalactosyldiacylglycerol, digalactosyldiacylglycerol, glycosyldiacylglycerols, glycerophospholipids, phospholipids, lysophospholipids, phosphatidylinositol phosphates, n-modified phospholipids, oxygenated/oxidized phospholipids, shingolipids, sphingoid bases, ceramides, phosphocereamides, glycophingolipids, sterols, cholesterol, cholesteryl ester, steryl esters, bile acids, sterylglycosides, and acylsterylglycosides. The complex lipid particle may contain one or more natural lipids belonging to one or more of the classes or sub-classes selected from the group consisting of the classes or sub-classes listed above. For instance, the complex lipid particle contains at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 150, at least 200, at least 250, at least 300, at least 400, at least 500, at least 600, at least 700, or at least 800 natural lipids belonging to one or more of the classes or sub-classes selected from the group consisting of the classes or sub-classes listed above.

[0159] In some embodiments, the CLP contains one or more natural lipids belonging to one or more of the sub-classes selected from the group consisting of acyl diacylglyceryl glucuronides, acylhexosylceramides, acylsterylglycosides, bile acids, acyl carnitines, cholesteryl esters, ceramides, cardiolipins, coenzyme Qs, diacylglycerols, digalactosyldiacylglycerols, diacylglyceryl glucuronides, dilysocardiolipins, fatty acids, fatty acid esters of hydroxyl fatty acids, hemibismonoacylglycerophosphates, hexosylceramides, lysophosphatidic acids, lysophophatidylcholines, lysophosphatidylethanolamines, N-acyl-lysophosphatidylethanolamines, lysophosphatidylglycerols, lysophosphatidylinositols, lysophosphatidylserines, monogalactosyldiacylglycerols, lysocardiolipins, N-acyl ethanolaminess, N-acyl glycines, N-acyl glycyl serines, phosphatidic acids, phosphatidylcholines, phosphatidylethanolamines, phosphatidylethanols, phosphatidylglycerols, phosphatidylinositols, ceramide phosphoinositols, phosphatidylmethanols, phosphatidylserines, steryl esters, stigmasterols, sulfatides, sulfonolipids, sphingomyelins, sulfoquinovosyl diacylglycerosl, sterols, and triacylglycerols. In some embodiments, the complex lipid particle contains at least 10 natural (e.g., plant, bacteria) lipids belonging to one or more of the sub-classes selected from the group consisting of the sub-classes listed above. For instance, the complex lipid particle contains at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 150, at least 200, at least 250, at least 300, at least 400, at least 500, at least 600, at least 700, or at least 800 natural lipids belonging to one or more of the sub-classes selected from the group consisting of the sub-classes listed above.

[0160] The complex lipid particle may contain 10 or more natural lipids belonging to one or more of the sub-classes selected from the group consisting of acylsterylglycosides, ceramides, digalactosyldiacylglycerols, diacylglyceryl glucuronides, hemibismonoacylglycerophosphates, hexosylceramides, lysophophatidylcholines, lysophosphatidylethanolamines,

monogalactosyldiacylglycerols, phosphatidylcholines, phosphatidylethanolamines, phosphatidylethanols, phosphatidylglycerols, phosphatidylinositols, sulfoquinovosyl diacylglycerosl, and sterols. For instance, the complex lipid particle contains at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 150, at least 200, at least 250, at least 300, at least 400, at least 400, at least 500, at least 600, at least 700, or at least 800 natural lipids belonging to one or more of the sub-classes selected from the group consisting of the sub-classes listed above.

[0161] The complex lipid particle may contain natural lipids from at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 different classes or sub-classes of lipids from the natural sources. In some embodiments, the complex lipid particle contains natural lipids from at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 different classes or sub-classes of lipids from a single natural source (e.g., from only the plant source, or from only the bacteria source). In some embodiments, the CLP may contain natural lipids from only one class or only one sub-class of lipids from the natural sources.

[0162] The identity (and class and subclass) and the amounts of the lipids extracted from the natural source(s) can be analyzed by lipidomics analysis by solubilizing the lipid extracts or complex lipid particles in compatible solvents and analyzing by a mass spectrometry (e.g., MS/MS). Other known methods, such as charged aerosol detection (CAD) (e.g., HPLC-CAD, normal-phase high-performance liquid chromatography (NP-HPLC-CAD), or reversed-phase high-performance liquid chromatography (RP-HPLC-CAD)), may also be used.

[0163] The complex lipid particle may comprise all or a fraction of the lipid species present in the lipid structure from the particular natural source(s), e.g., it may contain at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or virtually 100% of the lipid species present in the lipid structure from the particular natural source(s).

[0164] The complex lipid particle may comprise reduced or minimized protein matter endogenous to the one or more natural sources. For instance, the complex lipid particle may contain less than 50% w/w, less than 45% w/w, less than 40% w/w, less than 35% w/w, less than 30% w/w, less than 25% w/w, less than 20% w/w, less than 15% w/w, less than 10% w/w, less than 9% w/w, less than 8% w/w, less than 7% w/w, less than 6% w/w, less than 5% w/w, less than 4% w/w, less than 3% w/w, less than 2% w/w, less than 1% w/w, less than 0.5% w/w, less than 0.1% w/w, or essentially free of protein matter endogenous to the one or more natural sources. In some instances, the lipid bilayer of the complex lipid particle does not contain proteins. To calculate %w/w of residual protein matter endogenous to the one or more natural sources, protein concentration is divided by the concentration of the natural lipid extract and then multiplied by 100. Alternatively, %w/w is calculated as the percent of the mass of total protein endogenous to the one or more natural sources based on the mass of the total lipid extract.

[0165] The complex lipid particle may comprise reduced or minimized residual dsDNA matter endogenous to the one or more natural sources. For instance, the complex lipid particle may contain less than 15% w/w, less than 10% w/w, less than 5% w/w, less than 1% w/w, less than 0.5% w/w, less than 0.1% w/w, less than 0.05% w/w, less than 0.01% w/w, less than 0.005% w/w, less than

0.001% w/w, or essentially free of residual dsDNA matter endogenous to the one or more natural sources. In some instances, the lipid bilayer of the complex lipid particle does not contain residual dsDNA. To calculate %w/w of residual dsDNA matter endogenous to the one or more natural sources, total adjusted dsDNA concentration is divided by the concentration of the natural lipid extract and then multiplied by 100. Alternatively, %w/w is calculated as the percent of the mass of total residual dsDNA endogenous to the one or more natural sources based on the mass of the total lipid extract.

[0166] In some embodiments, the complex lipid particle further incorporates a synthetic structural lipid such as a neutral lipid. In some embodiments, the structural lipid component of the complex lipid particle may comprise between 10% w/w and 99% w/w structural lipids derived from a synthetic lipid structure, e.g., it may contain at least 10% w/w, at least 20% w/w, at least 30% w/w, at least 40% w/w, at least 50% w/w, at least 60% w/w, at least 70% w/w, at least 80% w/w, at least 90% w/w, at least 95% w/w, or about 99% w/w lipids derived from a synthetic lipid structure.

[0167] In addition to the exogenous ionizable lipid, the complex lipid particle may further comprise at least two other exogenous lipids. The complex lipid particle may include at least 1% w/w, at least 2% w/w, at least 5% w/w, at least 10% w/w, at least 15% w/w, at least 20% w/w, at least 25% w/w, at least 30% w/w, at least 40% w/w, at least 50% w/w, at least 60% w/w, at least 70% w/w, at least 80% w/w, at least 90% w/w, or about 95% w/w exogenous lipids. Exemplary exogenous lipids include ionizable lipids, synthetic structural lipids, sterols, and PEG-lipid conjugate. The complex lipid particle may further comprise at least two exogenous lipids. In some embodiments, the complex lipid particle contains an ionizable lipid, a sterol, and PEG-lipid conjugate. Additional exogenous lipids suitable for being included in the complex lipid particle are described herein below.

[0168] In some embodiments, the CLPs contain natural lipids comprising fatty acid-derived tails, said fatty acid-derived tails of the natural lipids being:

about 5 to 20% of fatty acid 16:0
about 0 to 10% of fatty acid 18:1 (C9)
about 0 to 10% of fatty acid 18:1 (C7)
about 5 to 30% of fatty acid 18:2
about 2 to 20% of fatty acid 18:3.

[0169] In some embodiments, the CLPs contain phosphatidylcholine (PC) lipids comprising fatty acid-derived tails, said fatty acid-derived tails of the PC lipids being:

about 10 to 20% of fatty acid 16:0
about 2 to 5% of fatty acid 18:0
about 7 to 15% of fatty acid 18:1
about 50 to 75% of fatty acid 18:2
about 2 to 10% of fatty acid 18:3.

[0170] In some embodiments, the CLPs contain phosphatidylethanolamines (PE) lipids comprising fatty acid-derived tails, said fatty acid-derived tails of the PE lipids being:

about 0.25 to 5% of fatty acid 14:0
about 25 to 45% of fatty acid 16:0

about 5 to 15% of fatty acid 16:1

about 10 to 25% of fatty acid 17:0

about 25 to 45% of fatty acid 18:1

about 2 to 7% of fatty acid 19:0.

[0171] In some embodiments, the CLPs contain natural lipids belonging to the sub-classes of phosphatidylethanolamines, phosphatidylglycerol, and cardiolipin, and comprising:

about 50 to 75 wt/wt% of phosphatidylethanolamines (PE)

about 15 to 30 wt/wt% of phosphatidylglycerol (PG)

about 5 to 15 wt/wt% of cardiolipin (CL).

[0172] In some embodiments, the CLPs contain natural lipids comprising :

about 10 to 50 wt/wt% of phosphatidylcholines (PC)

about 5 to 50 wt/wt% of phosphatidylethanolamines (PE)

about 0 to 15 wt/wt% of triacylglycerol (TG)

about 5 to 35 wt/wt% of hexosylceramides (HexCer)

about 0 to 5 wt/wt% of phosphatidylglycerol (PG)

about 0 to 7 wt/wt% of phosphatidylserines (PS)

about 0 to 10 wt/wt% of phosphatidylinositols (PI)

about 0 to 5 wt/wt% of cardiolipin (CL).

[0173] In some embodiments, the complex lipid particle contains less than 12% w/w of chloroplast endogenous to the one or more natural sources. In some embodiments, the complex lipid particle contains less than 20% w/w, less than 15% w/w, less than 10% w/w, less than 5% w/w, less than 1% w/w, less than 0.5% w/w, or less than 0.1% w/w of chloroplast endogenous to the one or more natural sources.

[0174] In some embodiments, the complex lipid particle contains less than 5% w/w of exogenous antioxidant.

[0175] In some embodiments, the CLPs contain natural lipids comprising about 0 to 20 wt/wt% of cardiolipin (CL).

Natural messenger pack (NMP)

[0176] A plurality of NMPs in a modified NMP formulation may be loaded with the exogenous peptide, polypeptide, or protein such that at least 5%, at least 10%, at least 15%, at least 25%, at least 50%, at least 75%, at least 90%, or at least 95% of NMPs in the plurality of NMPs encapsulate the exogenous peptide, polypeptide, or protein. In some embodiments the NMP is derived from an arthropod, fungi, archaea, or bacteria.

[0177] NMPs can include Arthropod, Plant, Fungi, Archaea, or Bacteria EVs, or segments, portions, or extracts, thereof, in which the EVs are about 5-2000 nm in diameter. For example, the NMP can include an EV, or segment, portion, or extract thereof, that has a mean diameter of about 5-50 nm, about 50-100 nm, about 100-150 nm, about 150-200 nm, about 200-250 nm, about 250-300 nm, about 300-350 nm, about 350-400 nm, about 400-450 nm, about 450-500 nm, about 500-550 nm, about 550-600 nm, about 600-650 nm, about 650-700 nm, about 700-750 nm, about 750-800 nm,

about 800-850 nm, about 850-900 nm, about 900-950 nm, about 950-1000nm, about 1000-1250nm, about 1250-1500nm, about 1500-1750nm, or about 1750-2000nm. In some instances, the NMP includes a Arthropod, Plant, Fungi, Archaea, or Bacteria EV, or segment, portion, or extract thereof, that has a mean diameter of about 5-1400 nm, 5-950 nm, about 5-900 nm, about 5-850 nm, about 5-800 nm, about 5-750 nm, about 5-700 nm, about 5-650 nm, about 5-600 nm, about 5-550 nm, about 5-500 nm, about 5-450 nm, about 5-400 nm, about 5-350 nm, about 5-300 nm, about 5-250 nm, about 5-200 nm, about 5-150 nm, about 5-100 nm, about 5-50 nm, or about 5-25 nm. In certain instances, the Arthropod, Plant, Fungi, Archaea, or Bacteria EV, or segment, portion, or extract thereof, has a mean diameter of about 50-200 nm. In certain instances, the EV, or segment, portion, or extract thereof, has a mean diameter of about 50-300 nm. In certain instances, the EV, or segment, portion, or extract thereof, has a mean diameter of about 200-500 nm. In certain instances, the EV, or segment, portion, or extract thereof, has a mean diameter of about 30-150 nm.

[0178] In some instances, the NMP may include a Arthropod, Plant, Fungi, Archaea, or Bacteria EV, or segment, portion, or extract thereof, that has a mean diameter of at least 5 nm, at least 50 nm, at least 100 nm, at least 150 nm, at least 200 nm, at least 250 nm, at least 300 nm, at least 350 nm, at least 400 nm, at least 450 nm, at least 500 nm, at least 550 nm, at least 600 nm, at least 650 nm, at least 700 nm, at least 750 nm, at least 800 nm, at least 850 nm, at least 900 nm, at least 950 nm, at least 1000 nm, or at least 1300. In some instances, the NMP includes a Arthropod, Fungi, Archaea, or Bacteria EV, or segment, portion, or extract thereof, that has a mean diameter less than 1400 nm, less than 1000 nm, less than 950 nm, less than 900 nm, less than 850 nm, less than 800 nm, less than 750 nm, less than 700 nm, less than 650 nm, less than 600 nm, less than 550 nm, less than 500 nm, less than 450 nm, less than 400 nm, less than 350 nm, less than 300 nm, less than 250 nm, less than 200 nm, less than 150 nm, less than 100 nm, or less than 50 nm. A variety of methods (e.g., a dynamic light scattering method) standard in the art can be used to measure the particle diameter of the EVs, or segment, portion, or extract thereof.

[0179] In some instances, the NMP may include an Arthropod, Plant, Fungi, Archaea, or Bacteria EV, or segment, portion, or extract thereof, that has a mean surface area of 77 nm^2 to 3.2 $x10^6$ nm^2 (e.g., 77-100 nm^2, 100-1000 nm^2, 1000-$1x10^4$ nm^2, $1x10^4$ - $1x10^5$ nm^2, $1x10^5$ -$1x10^6$ nm^2, or $1x10^6$-$3.2x10^6$ nm^2). In some instances, the NMP may include a Arthropod, Fungi, Archaea, or Bacteria EV, or segment, portion, or extract thereof, that has a mean volume of 65 nm^3 to $5.3x10^8$ nm^3 (e.g., 65-100 nm^3, 100-1000 nm^3, 1000-$1x10^4$ nm^3, $1x10^4$ - $1x10^5$ nm^3, $1x10^5$ -$1x10^6$ nm^3, $1x10^6$ -$1x10^7$ nm^3, $1x10^7$ -$1x10^8$ nm^3, $1x10^8$-$5.3x10^8$ nm^3). In some instances, the NMP may include a Arthropod, Plant, Fungi, Archaea, or Bacteria EV, or segment, portion, or extract thereof, that has a mean surface area of at least 77 nm^2, (e.g., at least 77 nm^2, at least 100 nm^2, at least 1000 nm^2, at least $1x10^4$ nm^2, at least $1x10^5$ nm^2, at least $1x10^6$ nm^2, or at least $2x10^6$ nm^2). In some instances, the NMP may include a Arthropod, Fungi, Archaea, or Bacteria EV, or segment, portion, or extract thereof, that has a mean volume of at least 65 nm^3 (e.g., at least 65 nm^3, at least 100 nm^3, at least 1000 nm^3, at least $1x10^4$ nm^3, at least $1x10^5$ nm^3, at least $1x10^6$ nm^3, at least $1x10^7$ nm^3, at least $1x10^8$ nm^3, at least $2x10^8$ nm^3, at least $3x10^8$ nm^3, at least $4x10^8$ nm^3, or at least $5x10^8$ nm^3.

[0180] In some instances, the NMP can have the same size as the Arthropod, Plant, Fungi,

Archaea, or Bacteria EV or segment, extract, or portion thereof. Alternatively, the NMP may have a different size than the initial EV from which the NMP is produced. For example, the NMP may have a diameter of about 5-2000 nm in diameter. For example, the NMP can have a mean diameter of about 5-50 nm, about 50-100 nm, about 100-150 nm, about 150-200 nm, about 200-250 nm, about 250-300 nm, about 300-350 nm, about 350-400 nm, about 400-450 nm, about 450-500 nm, about 500-550 nm, about 550-600 nm, about 600-650 nm, about 650-700 nm, about 700-750 nm, about 750-800 nm, about 800-850 nm, about 850-900 nm, about 900-950 nm, about 950-1000nm, about 1000-1200 nm, about 1200-1400 nm, about 1400-1600 nm, about 1600 – 1800 nm, or about 1800 – 2000 nm. In some instances, the NMP may have a mean diameter of at least 5 nm, at least 50 nm, at least 100 nm, at least 150 nm, at least 200 nm, at least 250 nm, at least 300 nm, at least 350 nm, at least 400 nm, at least 450 nm, at least 500 nm, at least 550 nm, at least 600 nm, at least 650 nm, at least 700 nm, at least 750 nm, at least 800 nm, at least 850 nm, at least 900 nm, at least 950 nm, at least 1000 nm, at least 1200 nm, at least 1400 nm, at least 1600 nm, at least 1800 nm, or about 2000 nm. A variety of methods (e.g., a dynamic light scattering method) standard in the art can be used to measure the particle diameter of the NMPs. In some instances, the size of the NMP is determined following loading of heterologous functional agents, or following other modifications to the NMPs.

[0181] In some instances, the NMP may have a mean surface area of 77 nm^2 to 1.3 $x10^7$ nm^2 (e.g., 77-100 nm^2, 100-1000 nm^2, 1000-$1x10^4$ nm^2, $1x10^4$ - $1x10^5$ nm^2, $1x10^5$ -$1x10^6$ nm^2, or $1x10^6$-$1.3x10^7$ nm^2). In some instances, the NMP may have a mean volume of 65 nm^3 to 4.2 $x10^9$ nm^3 (e.g., 65-100 nm^3, 100-1000 nm^3, 1000-$1x10^4$ nm^3, $1x10^4$ - $1x10^5$ nm^3, $1x10^5$ -$1x10^6$ nm^3, $1x10^6$ -$1x10^7$ nm^3, $1x10^7$ - $1x10^8$ nm^3, $1x10^8$-$1x10^9$ nm^3, or $1x10^9$ - 4.2 $x10^9$ nm^3). In some instances, the NMP has a mean surface area of at least 77 nm^2, (e.g., at least 77 nm^2, at least 100 nm^2, at least 1000 nm^2, at least $1x10^4$ nm^2, at least $1x10^5$ nm^2, at least $1x10^6$ nm^2, or at least $1x10^7$ nm^2). In some instances, the NMP has a mean volume of at least 65 nm^3 (e.g., at least 65 nm^3, at least 100 nm^3, at least 1000 nm^3, at least $1x10^4$ nm^3, at least $1x10^5$ nm^3, at least $1x10^6$ nm^3, at least $1x10^7$ nm^3, at least $1x10^8$ nm^3, at least $1x10^9$ nm^3, at least $2x10^9$ nm^3, at least $3x10^9$ nm^3, or at least $4x10^9$ nm^3).

[0182] In some instances, the NMP may include an intact Arthropod, Plant, Fungi, Archaea, or Bacteria EV. In some embodiments, the NMP may include a non-plant natural source such as algae or animal-derived organs EV, or segment, portion, or extract thereof. Alternatively, the NMP may include a segment, portion, or extract of the full surface area of the vesicle (e.g., a segment, portion, or extract including less than 100% (e.g., less than 90%, less than 80%, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, less than 10%, less than 5%, or less than 1%) of the full surface area of the vesicle) of a EV. The segment, portion, or extract may be any shape, such as a circumferential segment, spherical segment (e.g., hemisphere), curvilinear segment, linear segment, or flat segment. In instances where the segment is a spherical segment of the vesicle, the spherical segment may represent one that arises from the splitting of a spherical vesicle along a pair of parallel lines, or one that arises from the splitting of a spherical vesicle along a pair of non-parallel lines. Accordingly, the plurality of NMPs can include a plurality of intact EVs, a plurality of EV segments, portions, or extracts, or a mixture of intact and segments of EVs. One skilled in the art will appreciate that the ratio of intact to segmented EVs will

depend on the particular isolation method used. For example, grinding or blending a Arthropod, Fungi, Plant, Archaea, or Bacteria, or part thereof, may produce NMPs that contain a higher percentage of EV segments, portions, or extracts than a non-destructive extraction method, such as vacuum-infiltration.

[0183] In instances where, the NMP includes a segment, portion, or extract of a Arthropod, Fungi, Archaea, or Bacteria EV, the EV segment, portion, or extract may have a mean surface area less than that of an intact vesicle, e.g., a mean surface area less than 77 nm^2, 100 nm^2, 1000 nm^2, $1x10^4$ nm^2, $1x10^5$ nm^2, $1x10^6$ nm^2, or $3.2x10^6$ nm^2). In some instances, the EV segment, portion, or extract has a surface area of less than 70 nm^2, 60 nm^2, 50 nm^2, 40 nm^2, 30 nm^2, 20 nm^2, or 10 nm^2). In some instances, the NMP may include a Arthropod, Fungi, Archaea, or Bacteria EV, or segment, portion, or extract thereof, that has a mean volume less than that of an intact vesicle, e.g., a mean volume of less than 65 nm^3, 100 nm^3, 1000 nm^3, $1x10^4$ nm^3, $1x10^5$ nm^3, $1x10^6$ nm^3, $1x10^7$ nm^3, $1x10^8$ nm^3, or $5.3x10^8$ nm^3).

[0184] In instances where the NMP includes an extract of a Arthropod, Plant, Fungi, Archaea, or Bacteria EV, e.g., in instances where the NMP includes lipids extracted (e.g., with chloroform or ethanol) from a EV, the NMP may include at least 1%, 2%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or more than 99% of lipids extracted (e.g., with chloroform or with ethanol) from a Arthropod, Fungi, Archaea, or Bacteria EV. The NMPs in the plurality may include Arthropod, Plant, Fungi, Archaea, or Bacteria EV segments and/or EV-extracted lipids or a mixture thereof.

Plant Messenger Pack (PMPs)

[0185] A PMP is a lipid (e.g., lipid bilayer, unilamellar, or multilamellar structure) structure that includes a plant EV, or segment, portion, or extract (e.g., lipid extract) thereof. Plant EVs refer to an enclosed lipid-bilayer structure that naturally occurs in a plant and that is about 5-2000 nm in diameter. Plant EVs can originate from a variety of plant biogenesis pathways. In nature, plant EVs can be found in the intracellular and extracellular compartments of plants, such as the plant apoplast, the compartment located outside the plasma membrane and formed by a continuum of cell walls and the extracellular space. Alternatively, PMPs can be enriched plant EVs found in cell culture media upon secretion from plant cells. Plant EVs can be separated from plants, thereby providing PMPs, by a variety of methods further described herein. Further, the PMPs can optionally include a therapeutic agent, which can be introduced *in vivo* or *in vitro.*

[0186] PMPs can include plant EVs, or segments, portions, or extracts, thereof. Optionally, PMPs can also include exogenous lipids (e.g., sterols (e.g., cholesterol or sitosterol), ionizable lipids, and/or PEGylated lipids) in addition to lipids derived from plant EVs. In some embodiments, the plant EVs are about 5-1000 nm in diameter. For example, the PMP can include a plant EV, or segment, portion, or extract thereof, that has a mean diameter of about 5-50 nm, about 50-100 nm, about 100-150 nm, about 150-200 nm, about 200-250 nm, about 250-300 nm, about 300-350 nm, about 350-400 nm, about 400-450 nm, about 450-500 nm, about 500-550 nm, about 550-600 nm, about 600-650 nm, about 650-700 nm, about 700-750 nm, about 750-800 nm, about 800-850 nm, about 850-900 nm, about 900-950 nm, about 950-1000nm, about 1000-1250nm, about 1250-1500nm, about 1500-

1750nm, or about 1750-2000nm. In some instances, the PMP includes a plant EV, or segment, portion, or extract thereof, that has a mean diameter of about 5-950 nm, about 5-900 nm, about 5-850 nm, about 5-800 nm, about 5-750 nm, about 5-700 nm, about 5-650 nm, about 5-600 nm, about 5-550 nm, about 5-500 nm, about 5-450 nm, about 5-400 nm, about 5-350 nm, about 5-300 nm, about 5-250 nm, about 5-200 nm, about 5-150 nm, about 5-100 nm, about 5-50 nm, or about 5-25 nm. In certain instances, the plant EV, or segment, portion, or extract thereof, has a mean diameter of about 50-200 nm. In certain instances, the plant EV, or segment, portion, or extract thereof, has a mean diameter of about 50-300 nm. In certain instances, the plant EV, or segment, portion, or extract thereof, has a mean diameter of about 200-500 nm. In certain instances, the plant EV, or segment, portion, or extract thereof, has a mean diameter of about 30-150 nm.

[0187] In some instances, the PMP may include a plant EV, or segment, portion, or extract thereof, that has a mean diameter of at least 5 nm, at least 50 nm, at least 100 nm, at least 150 nm, at least 200 nm, at least 250 nm, at least 300 nm, at least 350 nm, at least 400 nm, at least 450 nm, at least 500 nm, at least 550 nm, at least 600 nm, at least 650 nm, at least 700 nm, at least 750 nm, at least 800 nm, at least 850 nm, at least 900 nm, at least 950 nm, or at least 1000 nm. In some instances, the PMP includes a plant EV, or segment, portion, or extract thereof, that has a mean diameter less than 1000 nm, less than 950 nm, less than 900 nm, less than 850 nm, less than 800 nm, less than 750 nm, less than 700 nm, less than 650 nm, less than 600 nm, less than 550 nm, less than 500 nm, less than 450 nm, less than 400 nm, less than 350 nm, less than 300 nm, less than 250 nm, less than 200 nm, less than 150 nm, less than 100 nm, or less than 50 nm. A variety of methods (e.g., a dynamic light scattering method) standard in the art can be used to measure the particle diameter of the plant EV, or segment, portion, or extract thereof.

[0188] In some instances, the PMP may include a plant EV, or segment, portion, or extract thereof, that has a mean surface area of 77 nm^2 to 3.2 $x10^6$ nm^2 (e.g., 77-100 nm^2, 100-1000 nm^2, 1000-$1x10^4$ nm^2, $1x10^4$ - $1x10^5$ nm^2, $1x10^5$ -$1x10^6$ nm^2, or $1x10^6$-$3.2x10^6$ nm^2). In some instances, the PMP may include a plant EV, or segment, portion, or extract thereof, that has a mean volume of 65 nm^3 to $5.3x10^8$ nm^3 (e.g., 65-100 nm^3, 100-1000 nm^3, 1000-$1x10^4$ nm^3, $1x10^4$ - $1x10^5$ nm^3, $1x10^5$ -$1x10^6$ nm^3, $1x10^6$ -$1x10^7$ nm^3, $1x10^7$ -$1x10^8$ nm^3, $1x10^8$-$5.3x10^8$ nm^3). In some instances, the PMP may include a plant EV, or segment, portion, or extract thereof, that has a mean surface area of at least 77 nm^2, (e.g., at least 77 nm^2, at least 100 nm^2, at least 1000 nm^2, at least $1x10^4$ nm^2, at least $1x10^5$ nm^2, at least $1x10^6$ nm^2, or at least $2x10^6$ nm^2). In some instances, the PMP may include a plant EV, or segment, portion, or extract thereof, that has a mean volume of at least 65 nm^3 (e.g., at least 65 nm^3, at least 100 nm^3, at least 1000 nm^3, at least $1x10^4$ nm^3, at least $1x10^5$ nm^3, at least $1x10^6$ nm^3, at least $1x10^7$ nm^3, at least $1x10^8$ nm^3, at least $2x10^8$ nm^3, at least $3x10^8$ nm^3, at least $4x10^8$ nm^3, or at least $5x10^8$ nm^3.

[0189] In some instances, the PMP can have the same size as the plant EV or segment, extract, or portion thereof. Alternatively, the PMP may have a different size than the initial plant EV from which the PMP is produced. For example, the PMP may have a diameter of about 5-2000 nm in diameter. For example, the PMP can have a mean diameter of about 5-50 nm, about 50-100 nm, about 100-150 nm, about 150-200 nm, about 200-250 nm, about 250-300 nm, about 300-350 nm, about 350-400 nm,

about 400-450 nm, about 450-500 nm, about 500-550 nm, about 550-600 nm, about 600-650 nm, about 650-700 nm, about 700-750 nm, about 750-800 nm, about 800-850 nm, about 850-900 nm, about 900-950 nm, about 950-1000nm, about 1000-1200 nm, about 1200-1400 nm, about 1400-1600 nm, about 1600 – 1800 nm, or about 1800 – 2000 nm. In some instances, the PMP may have a mean diameter of at least 5 nm, at least 50 nm, at least 100 nm, at least 150 nm, at least 200 nm, at least 250 nm, at least 300 nm, at least 350 nm, at least 400 nm, at least 450 nm, at least 500 nm, at least 550 nm, at least 600 nm, at least 650 nm, at least 700 nm, at least 750 nm, at least 800 nm, at least 850 nm, at least 900 nm, at least 950 nm, at least 1000 nm, at least 1200 nm, at least 1400 nm, at least 1600 nm, at least 1800 nm, or about 2000 nm. A variety of methods (e.g., a dynamic light scattering method) standard in the art can be used to measure the particle diameter of the PMPs. In some instances, the size of the PMP is determined following loading of a therapeutic agent, or following other modifications to the PMPs.

[0190] In some instances, the PMP may have a mean surface area of 77 nm^2 to 1.3 $x10^7$ nm^2 (e.g., 77-100 nm^2, 100-1000 nm^2, 1000-$1x10^4$ nm^2, $1x10^4$ - $1x10^5$ nm^2, $1x10^5$ -$1x10^6$ nm^2, or $1x10^6$-$1.3x10^7$ nm^2). In some instances, the PMP may have a mean volume of 65 nm^3 to 4.2 $x10^9$ nm^3 (e.g., 65-100 nm^3, 100-1000 nm^3, 1000-$1x10^4$ nm^3, $1x10^4$ - $1x10^5$ nm^3, $1x10^5$ -$1x10^6$ nm^3, $1x10^6$ -$1x10^7$ nm^3, $1x10^7$ - $1x10^8$ nm^3, $1x10^8$-$1x10^9$ nm^3, or $1x10^9$ - 4.2 $x10^9$ nm^3). In some instances, the PMP has a mean surface area of at least 77 nm^2, (e.g., at least 77 nm^2, at least 100 nm^2, at least 1000 nm^2, at least $1x10^4$ nm^2, at least $1x10^5$ nm^2, at least $1x10^6$ nm^2, or at least $1x10^7$ nm^2). In some instances, the PMP has a mean volume of at least 65 nm^3 (e.g., at least 65 nm^3, at least 100 nm^3, at least 1000 nm^3, at least $1x10^4$ nm^3, at least $1x10^5$ nm^3, at least $1x10^6$ nm^3, at least $1x10^7$ nm^3, at least $1x10^8$ nm^3, at least $1x10^9$ nm^3, at least $2x10^9$ nm^3, at least $3x10^9$ nm^3, or at least $4x10^9$ nm^3).

[0191] In some instances, the PMP may include an intact plant EV. Alternatively, the PMP may include a segment, portion, or extract of the full surface area of the vesicle (e.g., a segment, portion, or extract including less than 100% (e.g., less than 90%, less than 80%, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, less than 10%, less than 5%, or less than 1%) of the full surface area of the vesicle) of a plant EV. The segment, portion, or extract may be any shape, such as a circumferential segment, spherical segment (e.g., hemisphere), curvilinear segment, linear segment, or flat segment. In instances where the segment is a spherical segment of the vesicle, the spherical segment may represent one that arises from the splitting of a spherical vesicle along a pair of parallel lines, or one that arises from the splitting of a spherical vesicle along a pair of non-parallel lines. Accordingly, the plurality of PMPs can include a plurality of intact plant EVs, a plurality of plant EV segments, portions, or extracts, or a mixture of intact and segments of plant EVs. One skilled in the art will appreciate that the ratio of intact to segmented plant EVs will depend on the particular isolation method used. For example, grinding or blending a plant, or part thereof, may produce PMPs that contain a higher percentage of plant EV segments, portions, or extracts than a non-destructive extraction method, such as vacuum-infiltration.

[0192] In instances where, the PMP includes a segment, portion, or extract of a plant EV, the EV segment, portion, or extract may have a mean surface area less than that of an intact vesicle, (e.g., a mean surface area less than 77 nm^2, 100 nm^2, 1000 nm^2, $1x10^4$ nm^2, $1x10^5$ nm^2, $1x10^6$ nm^2, or

$3.2x10^6$ nm^2). In some instances, the EV segment, portion, or extract has a surface area of less than 70 nm^2, 60 nm^2, 50 nm^2, 40 nm^2, 30 nm^2, 20 nm^2, or 10 nm^2. In some instances, the PMP may include a plant EV, or segment, portion, or extract thereof, that has a mean volume less than that of an intact vesicle, (e.g., a mean volume of less than 65 nm^3, 100 nm^3, 1000 nm^3, $1x10^4$ nm^3, $1x10^5$ nm^3, $1x10^6$ nm^3, $1x10^7$ nm^3, $1x10^8$ nm^3, or $5.3x10^8$ nm^3).

[0193] In instances where the PMP includes an extract of a plant EV, e.g., in instances where the PMP includes lipids extracted (e.g., with chloroform or ethanol) from a plant EV, the PMP may include at least 1%, 2%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or more than 99%, of lipids extracted (e.g., with chloroform or ethanol) from a plant EV. The PMPs in the plurality may include plant EV segments and/or plant EV-extracted lipids or a mixture thereof.

Production of NMPs

[0194] NMPs may be produced from Arthropod, Fungi, Archaea, or Bacteria EVs, or a segment, portion or extract (e.g., lipid extract) thereof, that occur naturally in Arthropod, Fungi, Archaea, or Bacteria, or parts thereof, including tissues or cells. In some embodiments, the NMP may include a non-plant natural source such as algae or animal-derived organs EV, or segment, portion, or extract thereof. An exemplary method for producing NMPs includes (a) providing an initial sample from a source, or a part thereof, wherein the source or part thereof comprises EVs; and (b) isolating a crude NMP fraction from the initial sample, wherein the crude NMP fraction has a decreased level of at least one contaminant or undesired component from the source or part thereof relative to the level in the initial sample. The method can further include an additional step (c) comprising purifying the crude NMP fraction, thereby producing a plurality of pure NMPs, wherein the plurality of pure NMPs have a decreased level of at least one contaminant or undesired component from the Arthropod, Fungi, Archaea, or Bacteria or part thereof relative to the level in the crude EV fraction. Each production step is discussed in further detail, below. Exemplary methods regarding the isolation and purification of NMPs is found, for example, in Rutter and Innes, *Plant Physiol.* 173(1): 728-741, 2017; Rutter et al, *Bio. Protoc.* 7(17): e2533, 2017; Regente et al, *J of Exp. Biol.* 68(20): 5485-5496, 2017; Mu et al, Mol. Nutr. Food Res., 58, 1561–1573, 2014, and Regente et al, *FEBS Letters*. 583: 3363-3366, 2009, each of which is herein incorporated by reference.

[0195] For example, a plurality of NMPs may be isolated from a Arthropod, Fungi, Archaea, or Bacteria by a process which includes the steps of: (a) providing an initial sample from a source, or a part thereof, wherein the source or part thereof comprises EVs; (b) isolating a crude NMP fraction from the initial sample, wherein the crude NMP fraction has a decreased level of at least one contaminant or undesired component from the Arthropod, Fungi, Archaea, or Bacteria or part thereof relative to the level in the initial sample (e.g., a level that is decreased by at least 1%, 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 45%, 50%, 55%, 60%, 70%, 80%, 90%, 95%, 96%, 98%, 99%, or 100%); and (c) purifying the crude NMP fraction, thereby producing a plurality of pure NMPs, wherein the plurality of pure NMPs have a decreased level of at least one contaminant or undesired component from the source or part thereof relative to the level in the crude EV fraction (e.g., a level that is decreased by at least 1%, 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 45%, 50%, 55%, 60%,

70%, 80%, 90%, 95%, 96%, 98%, 99%, or 100%).

[0196] The NMPs provided herein can include a Arthropod, Fungi, Archaea, or Bacteria EV, or segment, portion, or extract thereof, isolated from a variety of sources. NMPs may be isolated from any genera of Arthropod, Fungi, Archaea, or Bacteria , including but not limited to crabs, crawfish, shrimp, spiders, scorpions, crickets, grasshoppers, beetles, millipedes, ticks, mites, centipedes, ants, wasps, dragonflies, flies, gnats, other insects and crustaceans, yeast, mushrooms, puffballs, stinkhorns, boletes, smuts, bunts, bracket fungi, jelly fungi, toadstools, molds, rusts, earth stars, chanterelles, ergot, pyrolobus, picrophilus, methanogens, crenarchaeota, nanoarchaeota, ignicoccus, cenarchaeum, halophiles, Escherichia, Acinetobacter, Agrobacterium, Anabaena, Anaplasma, Aquifex, Azoarcus, Azospirillum, Azotobacter, Bartonella, Bordetella, Bradyrhizobium, Brucella, Buchnera, Burkholderia, Candidatus, Chromobacterium, Coxiella, Crocosphaera, Dechloromonas, Desulfitobacterium, Desulfotalea, Erwinia, Francisella, Fusobacterium, Gloeobacter, Gluconobacter, Helicobacter, Legionella, Magnetospirillum, Mesorhizobium, Methylobacterium, Methylococcus, Neisseria, Nitrosomonas, Nostoc, Photobacterium, Photorhabdus, Phyllobacterium, Polaromonas, Prochlorococcus, Pseudomonas, Psychrobacter, Ralstonia, Rubrivivax, Salmonella, Shewanella, Shigella, Sinorhizobium, Synechococcus, Synechocystis, Thermosynechococcus, Thermotoga, Thermus, Thiobacillus, Trichodesmium, Vibrio, Wigglesworthia, Wolinella, Xanthomonas, Xylella, Yersinia, Bacillus, Bifidobacterium, Clostridium, Corynebacterium, Deinococcus, Enterococcus, Exiguobacterium, Geobacillus, Lactobacillus, Listeria, Leuconostoc, Moorella, Oceanobacillus, Rhizobium, Rickettsia, Staphylococcus, Streptococcus, Symbiobacterium, or Thermoanaerobacter.

[0197] NMPs may be produced from a whole Arthropod, Fungi, Archaea, or Bacteria (e.g., a whole insect, spider, crustacean, fungi, or single-cell of archaea or bacteria) or alternatively from one or more source parts (e.g., segments, organs, eggs, spores, mycelium, tissue, membrane or cell wall). For example, NMPs can be produced from organs/structures/tissues/cell cultures (e.g., body segments, appendages, organs, eggs, exoskeleton, embryos, spores, mycelium, hyphae, thallus, suspension cultures, cell walls, inner or outer membranes, gametophytes, sporophytes, polymerases, glycerol-ether lipids, metabolic products, flagella, pili, ribosomes or organelles) or progeny of same. The source may be at any stage of development. In some embodiments, the NMP is produced from an insect or fungi, (e.g. cricket, yeast, or mushroom). In some embodiments, the NMP is produced from a bacteria or archaea (e.g. E. coli). In some embodiments, the NMP is produced from an algae (e.g. kelp or chlorella). In some embodiments, the NMP is produced from an animal organ (e.g brain or blood).

[0198] NMPs can be produced from a Arthropod, Fungi, Archaea, or Bacteria, or part thereof, by a variety of methods. Any method that allows release of the EV-containing fraction of a source, or an otherwise extracellular fraction that contains NMPs comprising secreted EVs (e.g., cell culture media) is suitable in the present methods. EVs can be separated from the source or source part by either destructive (e.g., grinding or blending) or non-destructive (washing or vacuum infiltration) methods. For instance, the Arthropod, Fungi, Archaea, or Bacteria, or part thereof, can be vacuum-infiltrated, ground, blended, or a combination thereof to isolate EVs from the source or source part, thereby producing NMPs. For instance, the isolating step may involve (b) isolating a crude NMP fraction from

the initial sample (e.g., a Arthropod, Fungi, Archaea, or Bacteria or part, or a sample derived from a Arthropod, Fungi, Archaea, or Bacteria or part), wherein the crude NMP fraction has a decreased level of at least one contaminant or undesired component from the source or part thereof relative to the level in the initial sample;, wherein the isolating step involves vacuum infiltrating the Arthropod, Fungi, Archaea, or Bacteria (e.g., with a vesicle isolation buffer) to release and collect the desired fraction. Alternatively, the isolating step may involve (b) grinding or blending the source to release the EVs, thereby producing NMPs.

[0199] Upon isolating the Arthropod, Fungi, Archaea, or Bacteria EVs, thereby producing NMPs, the NMPs can be separated or collected into a crude NMP fraction. For instance, the separating step may involve separating the plurality of NMPs into a crude NMP fraction using centrifugation (e.g., differential centrifugation or ultracentrifugation) and/or filtration to separate the NMP-containing fraction from large contaminants, including tissue debris, cells, or cell organelles. As such, the crude NMP fraction will have a decreased number of large contaminants, including, for example, tissue debris, cells, or cell organelles (e.g., nuclei, mitochondria, etc), as compared to the initial sample from the source or source part.

[0200] The crude NMP fraction can be further purified by additional purification methods to produce a plurality of pure NMPs. For example, the crude NMP fraction can be separated from other source components by ultracentrifugation, e.g., using a density gradient (iodixanol or sucrose), size-exclusion, and/or use of other approaches to remove aggregated components (e.g., precipitation or size-exclusion chromatography). The resulting pure NMPs may have a decreased level of contaminants or undesired components from the source (e.g., one or more non-NMP components, such as protein aggregates, nucleic acid aggregates, protein-nucleic acid aggregates, free lipoproteins, lipido-proteic structures), nuclei, cell wall components, cell organelles, or a combination thereof) relative to one or more fractions generated during the earlier separation steps, or relative to a pre-established threshold level, e.g., a commercial release specification. For example, the pure NMPs may have a decreased level (e.g., by about 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or more than 100%; or by about 2x fold, 4x fold, 5x fold, 10x fold, 20x fold, 25x fold, 50x fold, 75x fold, 100x fold, or more than 100x fold) of source organelles or cell wall components relative to the level in the initial sample. In some instances, the pure NMPs are substantially free (e.g., have undetectable levels) of one or more non-NMP components, such as protein aggregates, nucleic acid aggregates, protein-nucleic acid aggregates, free lipoproteins, lipido-proteic structures), nuclei, cell wall components, cell organelles, or a combination thereof. Further examples of the releasing and separation steps can be found in WO 2021/041301. The NMPs may be at a concentration of, e.g., $1x10^9$, $5x10^9$, $1x10^{10}$, $5x10^{10}$, $5x10^{10}$, $1x10^{11}$, $2x10^{11}$, $3x10^{11}$, $4x10^{11}$, $5x10^{11}$, $6x10^{11}$, $7x10^{11}$, $8x10^{11}$, $9x10^{11}$, $1x10^{12}$, $2x10^{12}$, $3x10^{12}$, $4x10^{12}$, $5x10^{12}$, $6x10^{12}$, $7x10^{12}$, $8x10^{12}$, $9x10^{12}$, $1x10^{13}$, or more than $1x10^{13}$ NMPs/mL.

[0201] For example, protein aggregates may be removed from isolated NMPs. For example, the isolated NMP solution can be taken through a range of pHs (e.g., as measured using a pH probe) to precipitate out protein aggregates in solution. The pH can be adjusted to, e.g., pH 3, pH 5, pH 7, pH 9, or pH 11 with the addition of, e.g., sodium hydroxide or hydrochloric acid. Once the solution is at

the specified pH, it can be filtered to remove particulates. Alternatively, the isolated NMP solution can be flocculated using the addition of charged polymers, such as Polymin-P or Praestol 2640. Briefly, Polymin-P or Praestol 2640 is added to the solution and mixed with an impeller. The solution can then be filtered to remove particulates. Alternatively, aggregates can be solubilized by increasing salt concentration. For example, NaCl can be added to the isolated NMP solution until it is at, e.g., 1 mol/L. The solution can then be filtered to isolate the NMPs. Alternatively, aggregates are solubilized by increasing the temperature. For example, the isolated NMPs can be heated under mixing until the solution has reached a uniform temperature of, e.g., 50°C for 5 minutes. The NMP mixture can then be filtered to isolate the NMPs. Alternatively, soluble contaminants from NMP solutions can be separated by size-exclusion chromatography column according to standard procedures, where NMPs elute in the first fractions, whereas proteins and ribonucleoproteins and some lipoproteins are eluted later. The efficiency of protein aggregate removal can be determined by measuring and comparing the protein concentration before and after removal of protein aggregates via BCA/Bradford protein quantification. In some embodiments, protein aggregates are removed before the exogenous peptide, polypeptide, or protein is encapsulated by the NMP. In other embodiments, protein aggregates are removed after the exogenous peptide, polypeptide, or protein is encapsulated by the NMP.

[0202] In some embodiments, the preparation of NMPs from natural sources is through an ethanol extraction method. In some aspects, a 3:2 ethyl acetate:ethanol solvent aids in extraction. In some embodiments, the preparation of NMPs from natural sources is through a modified Matyash extraction method. In some aspects, a 1:2 MeOH:MTBE solvent aids in extraction.

[0203] Any of the production methods described herein can be supplemented with any quantitative or qualitative methods known in the art to characterize or identify the NMPs at any step of the production process. NMPs may be characterized by a variety of analysis methods to estimate NMP yield, NMP concentration, NMP purity, NMP composition, or NMP sizes. NMPs can be evaluated by a number of methods known in the art that enable visualization, quantitation, or qualitative characterization (e.g., identification of the composition) of the NMPs, such as microscopy (e.g., transmission electron microscopy), dynamic light scattering, nanoparticle tracking, spectroscopy (e.g., Fourier transform infrared analysis), or mass spectrometry (protein and lipid analysis). In certain instances, methods (e.g., mass spectroscopy) may be used to identify EV markers present on the NMP. To aid in analysis and characterization, of the NMP fraction, the NMPs can additionally be labelled or stained. For example, the NMPs can be stained with 3,3'-dihexyloxacarbocyanine iodide ($DIOC_6$), a fluorescent lipophilic dye, PKH67 (Sigma Aldrich); Alexa Fluor® 488 (Thermo Fisher Scientific), or DyLight™ 800 (Thermo Fisher). In the absence of sophisticated forms of nanoparticle tracking, this relatively simple approach quantifies the total membrane content and can be used to indirectly measure the concentration of NMPs (Rutter and Innes, *Plant Physiol.* 173(1): 728-741, 2017; Rutter et al, *Bio. Protoc.* 7(17): e2533, 2017). For more precise measurements, and to assess the size distributions of NMPs, nanoparticle tracking, nano flow cytometry, or Tunable Resistive Pulse Sensing can be used.

[0204] During the production process, the NMPs can optionally be prepared such that the NMPs are

at an increased concentration (e.g., by about 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or more than 100%; or by about 2x fold, 4x fold, 5x fold, 10x fold, 20x fold, 25x fold, 50x fold, 75x fold, 100x fold, or more than 100x fold) relative to the EV level in a control or initial sample. The isolated NMPs may make up about 0.1% to about 100% of the NMP composition, such as any one of about 0.01% to about 100%, about 1% to about 99.9%, about 0.1% to about 10%, about 1% to about 25%, about 10% to about 50%, about 50% to about 99%. In some instances, the composition described herein includes at least any of 0.1%, 0.5%, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more NMPs, e.g., as measured by wt/vol, percent NMP protein composition, and/or percent lipid composition (e.g., by measuring fluorescently labelled lipids)). In some instances, the concentrated agents are used as commercial products, e.g., the final user may use diluted agents, which have a substantially lower concentration of active ingredient. In some embodiments, the composition described herein is formulated as a NMP concentrate formulation, e.g., an ultra-low-volume concentrate formulation. In some embodiments, the NMPs in the composition are at a concentration effective to increase the fitness of an organism, e.g., a plant, an animal, an insect, a bacterium, or a fungus. In other aspects, the NMPs in the composition are at a concentration effective to decrease the fitness of an organism, e.g., a plant, an animal, an insect, a bacterium, or a fungus.

[0205] NMPs can be produced from a variety of Arthropod, Fungi, Archaea, or Bacteria, or one or more parts thereof (e.g., segments, organs, eggs, spores, mycelium, tissue, membrane or cell wall). For example, NMPs can be produced from organs/structures/tissues/cell cultures (e.g., body segments, appendages, organs, eggs, exoskeleton, embryos, spores, mycelium, hyphae, thallus, suspension cultures, cell walls, inner or outer membranes, gametophytes, sporophytes, polymerases, glycerol-ether lipids, metabolic products, flagella, pili, ribosomes or organelles) or progeny of same. The source may be at any stage of development. In some embodiments, the NMP is produced from an insect or fungi, (e.g. cricket, yeast, or mushroom). In some embodiments, the NMP is produced from a bacteria or archaea (e.g. E. coli). In some embodiments, the NMP is produced from an algae (e.g. kelp or chlorella). In some embodiments, the NMP is produced from an animal organ (e.g brain or blood).

[0206] NMPs can be produced and purified by a variety of methods, for example, by using a density gradient (iodixanol or sucrose) in conjunction with ultracentrifugation and/or methods to remove aggregated contaminants, e.g., precipitation or size-exclusion chromatography.

[0207] In some instances, the NMPs of the present compositions and methods can be isolated from a Arthropod, Fungi, Archaea, or Bacteria, or part thereof, and used without further modification to the NMP. In other instances, the NMP can be modified prior to use, as outlined further herein.

Production of PMPs

[0208] PMPs may be produced from plant EVs, or a segment, portion or extract (e.g., lipid extract) thereof, that occur naturally in plants, or parts thereof, including plant tissues or plant cells. An exemplary method for producing PMPs includes (a) providing an initial sample from a plant, or a part thereof, wherein the plant or part thereof comprises EVs; and (b) isolating a crude PMP fraction from the initial sample, wherein the crude PMP fraction has a decreased level of at least one contaminant

or undesired component from the plant or part thereof relative to the level in the initial sample. The method can further include an additional step (c) comprising purifying the crude PMP fraction, thereby producing a plurality of pure PMPs, wherein the plurality of pure PMPs have a decreased level of at least one contaminant or undesired component from the plant or part thereof relative to the level in the crude EV fraction. Each production step is discussed in further detail, below. Exemplary methods regarding the isolation and purification of PMPs is found, for example, in Rutter and Innes, *Plant Physiol.* 173(1): 728-741, 2017; Rutter et al, *Bio. Protoc.* 7(17): e2533, 2017; Regente et al, *J of Exp. Biol.* 68(20): 5485-5496, 2017; Mu et al, Mol. Nutr. Food Res., 58, 1561–1573, 2014, and Regente et al, *FEBS Letters*. 583: 3363-3366, 2009, each of which is herein incorporated by reference.

[0209] In some instances, a plurality of PMPs may be isolated from a plant by a process which includes the steps of: (a) providing an initial sample from a plant, or a part thereof, wherein the plant or part thereof comprises EVs; (b) isolating a crude PMP fraction from the initial sample, wherein the crude PMP fraction has a decreased level of at least one contaminant or undesired component from the plant or part thereof relative to the level in the initial sample (e.g., a level that is decreased by at least 1%, 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 45%, 50%, 55%, 60%, 70%, 80%, 90%, 95%, 96%, 98%, 99%, or 100%); and (c) purifying the crude PMP fraction, thereby producing a plurality of pure PMPs, wherein the plurality of pure PMPs have a decreased level of at least one contaminant or undesired component from the plant or part thereof relative to the level in the crude EV fraction (e.g., a level that is decreased by at least 1%, 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 45%, 50%, 55%, 60%, 70%, 80%, 90%, 95%, 96%, 98%, 99%, or 100%).

[0210] The PMPs can include a plant EV, or segment, portion, or extract thereof, produced from a variety of plants. PMPs may be produced from any genera of plants (vascular or nonvascular), including but not limited to angiosperms (monocotyledonous and dicotyledonous plants), gymnosperms, ferns, selaginellas, horsetails, psilophytes, lycophytes, algae (e.g., unicellular or multicellular, e.g., archaeplastida), or bryophytes. In certain instances, PMPs can be produced using a vascular plant, for example monocotyledons or dicotyledons or gymnosperms. For example, PMPs can be produced using alfalfa, apple, Arabidopsis, banana, barley, a Brassica species (e.g., *Arabidopsis thaliana* or *Brassica napus*), canola, castor bean, chicory, chrysanthemum, clover, cocoa, coffee, cotton, cottonseed, corn, crambe, cranberry, cucumber, dendrobium, dioscorea, eucalyptus, fescue, flax, gladiolus, liliacea, linseed, millet, muskmelon, mustard, oat, oil palm, oilseed rape, papaya, peanut, pineapple, ornamental plants, Phaseolus, potato, rapeseed, rice, rye, ryegrass, safflower, sesame, sorghum, soybean, sugarbeet, sugarcane, sunflower, strawberry, tobacco, tomato, turfgrass, wheat or vegetable crops such as lettuce, celery, broccoli, cauliflower, cucurbits; fruit and nut trees, such as apple, pear, peach, orange, grapefruit, lemon, lime, almond, pecan, walnut, hazel; vines, such as grapes, kiwi, hops; fruit shrubs and brambles, such as raspberry, blackberry, gooseberry; forest trees, such as ash, pine, fir, maple, oak, chestnut, popular; with alfalfa, canola, castor bean, corn, cotton, crambe, flax, linseed, mustard, oil palm, oilseed rape, peanut, potato, rice, safflower, sesame, soybean, sugarbeet, sunflower, tobacco, tomato, or wheat.

[0211] PMPs may be produced using a whole plant (e.g., a whole rosettes or seedlings) or alternatively from one or more plant parts (e.g., leaf, seed, root, fruit, vegetable, pollen, phloem sap,

or xylem sap). For example, PMPs can be produced using shoot vegetative organs/structures (e.g., leaves, stems, or tubers), roots, flowers and floral organs/structures (e.g., pollen, bracts, sepals, petals, stamens, carpels, anthers, or ovules), seed (including embryo, endosperm, or seed coat), fruit (the mature ovary), sap (e.g., phloem or xylem sap), plant tissue (e.g., vascular tissue, ground tissue, tumor tissue, or the like), and cells (e.g., single cells, protoplasts, embryos, callus tissue, guard cells, egg cells, or the like), or progeny of same. For instance, the isolation step may involve (a) providing a plant, or a part thereof. In some examples, the plant part is an Arabidopsis leaf. The plant may be at any stage of development. For example, the PMPs can be produced using seedlings, e.g., 1 week, 2 week, 3 week, 4 week, 5 week, 6 week, 7 week, or 8 week old seedlings (e.g., Arabidopsis seedlings). Other exemplary PMPs can include PMPs produced using roots (e.g., ginger roots), fruit juice (e.g., grapefruit juice), vegetables (e.g., broccoli), pollen (e.g., olive pollen), phloem sap (e.g., Arabidopsis phloem sap), or xylem sap (e.g., tomato plant xylem sap).

[0212] In some embodiments, the PMPs are produced from algae or lemon.

[0213] PMPs can be produced using a plant, or part thereof, by a variety of methods. Any method that allows release of the EV-containing apoplastic fraction of a plant, or an otherwise extracellular fraction that contains PMPs comprising secreted EVs (e.g., cell culture media) is suitable in the present methods. EVs can be separated from the plant or plant part by either destructive (e.g., grinding or blending of a plant, or any plant part) or non-destructive (washing or vacuum infiltration of a plant or any plant part) methods. For instance, the plant, or part thereof, can be vacuum-infiltrated, ground, blended, or a combination thereof to isolate EVs from the plant or plant part, thereby producing PMPs. For instance, the isolating step may involve vacuum infiltrating the plant (e.g., with a vesicle isolation buffer) to release and collect the apoplastic fraction. Alternatively, the isolating step may involve grinding or blending the plant to release the EVs, thereby producing PMPs.

[0214] Upon isolating the plant EVs, thereby producing PMPs, the PMPs can be separated or collected into a crude PMP fraction (e.g., an apoplastic fraction). For instance, the separating step may involve separating the plurality of PMPs into a crude PMP fraction using centrifugation (e.g., differential centrifugation or ultracentrifugation) and/or filtration to separate the plant PMP-containing fraction from large contaminants, including plant tissue debris or plant cells. As such, the crude PMP fraction will have a decreased number of large contaminants, including plant tissue debris or plant cells, as compared to the initial sample from the plant or plant part. Depending on the method used, the crude PMP fraction may additionally comprise a decreased level of plant cell organelles (e.g., nuclei, mitochondria or chloroplasts), as compared to the initial sample from the plant or plant part.

[0215] In some instances, the isolating step may involve separating the plurality of PMPs into a crude PMP fraction using centrifugation (e.g., differential centrifugation or ultracentrifugation) and/or filtration to separate the PMP-containing fraction from plant cells or cellular debris. In such instances, the crude PMP fraction will have a decreased number of plant cells or cellular debris, as compared to the initial sample from the source plant or plant part.

[0216] The crude PMP fraction can be further purified by additional purification methods to produce a plurality of pure PMPs. For example, the crude PMP fraction can be separated from other plant components by ultracentrifugation, e.g., using a density gradient (iodixanol or sucrose) and/or use of

other approaches to remove aggregated components (e.g., precipitation or size-exclusion chromatography). The resulting pure PMPs may have a decreased level of contaminants or other undesired components from the source plant (e.g., one or more non-PMP components, such as protein aggregates, nucleic acid aggregates, protein-nucleic acid aggregates, free lipoproteins, lipido-proteic structures), nuclei, cell wall components, cell organelles, or a combination thereof) relative to one or more fractions generated during the earlier separation steps, or relative to a pre-established threshold level, e.g., a commercial release specification. For example, the pure PMPs may have a decreased level (e.g., by about 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or more than 100%; or by about 2x fold, 4x fold, 5x fold, 10x fold, 20x fold, 25x fold, 50x fold, 75x fold, 100x fold, or more than 100x fold) of plant organelles or cell wall components relative to the level in the initial sample. In some instances, the pure PMPs are substantially free (e.g., have undetectable levels) of one or more non-PMP components, such as protein aggregates, nucleic acid aggregates, protein-nucleic acid aggregates, free lipoproteins, lipido-proteic structures), nuclei, cell wall components, cell organelles, or a combination thereof. The PMPs may be at a concentration of, e.g., $1x10^9$, $5x10^9$, $1x10^{10}$, $5x10^{10}$, $5x10^{10}$, $1x10^{11}$, $2x10^{11}$, $3x10^{11}$, $4x10^{11}$, $5x10^{11}$, $6x10^{11}$, $7x10^{11}$, $8x10^{11}$, $9x10^{11}$, $1x10^{12}$, $2x10^{12}$, $3x10^{12}$, $4x10^{12}$, $5x10^{12}$, $6x10^{12}$, $7x10^{12}$, $8x10^{12}$, $9x10^{12}$, $1x10^{13}$, or more than $1x10^{13}$ PMPs/mL.

[0217] For example, protein aggregates may be removed from PMPs. For example, the PMPs can be taken through a range of pHs (e.g., as measured using a pH probe) to precipitate out protein aggregates in solution. The pH can be adjusted to, e.g., pH 3, pH 5, pH 7, pH 9, or pH 11 with the addition of, e.g., sodium hydroxide or hydrochloric acid. Once the solution is at the specified pH, it can be filtered to remove particulates. Alternatively, the PMPs can be flocculated using the addition of charged polymers, such as Polymin-P or Praestol 2640. Briefly, Polymin-P or Praestol 2640 is added to the solution and mixed with an impeller. The solution can then be filtered to remove particulates. Alternatively, aggregates can be solubilized by increasing salt concentration. For example, NaCl can be added to the PMPs until it is at, e.g., 1 mol/L. The solution can then be filtered to isolate the PMPs. Alternatively, aggregates are solubilized by increasing the temperature. For example, the PMPs can be heated under mixing until the solution has reached a uniform temperature of, e.g., 50°C for 5 minutes. The PMP mixture can then be filtered to isolate the PMPs. Alternatively, soluble contaminants from PMP solutions can be separated by size-exclusion chromatography column according to standard procedures, where PMPs elute in the first fractions, whereas proteins and ribonucleoproteins and some lipoproteins are eluted later. The efficiency of protein aggregate removal can be determined by measuring and comparing the protein concentration before and after removal of protein aggregates via BCA/Bradford protein quantification.

[0218] Any of the production methods described herein can be supplemented with any quantitative or qualitative methods known in the art to characterize or identify the PMPs at any step of the production process. PMPs may be characterized by a variety of analysis methods to estimate PMP yield, PMP concentration, PMP purity, PMP composition, or PMP sizes. PMPs can be evaluated by a number of methods known in the art that enable visualization, quantitation, or qualitative characterization (e.g., identification of the composition) of the PMPs, such as microscopy (e.g.,

transmission electron microscopy), dynamic light scattering, nanoparticle tracking, spectroscopy (e.g., Fourier transform infrared analysis), or mass spectrometry (protein and lipid analysis). In certain instances, methods (e.g., mass spectroscopy) may be used to identify plant EV markers present on the PMP, such as markers disclosed in the Appendix. To aid in analysis and characterization, of the PMP fraction, the PMPs can additionally be labelled or stained. For example, the PMPs can be stained with 3,3'-dihexyloxacarbocyanine iodide ($DIOC_6$), a fluorescent lipophilic dye, PKH67 (Sigma Aldrich); Alexa Fluor® 488 (Thermo Fisher Scientific), or DyLight™ 800 (Thermo Fisher). In the absence of sophisticated forms of nanoparticle tracking, this relatively simple approach quantifies the total membrane content and can be used to indirectly measure the concentration of PMPs (Rutter and Innes, *Plant Physiol.* 173(1): 728-741, 2017; Rutter et al, *Bio. Protoc.* 7(17): e2533, 2017). For more precise measurements, and to assess the size distributions of PMPs, nanoparticle tracking can be used.

[0219] During the production process, the PMPs can optionally be prepared such that the PMPs are at an increased concentration (e.g., by about 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or more than 100%; or by about 2x fold, 4x fold, 5x fold, 10x fold, 20x fold, 25x fold, 50x fold, 75x fold, 100x fold, or more than 100x fold) relative to the EV level in a control or initial sample. The PMPs may make up about 0.1% to about 100% of the PMP composition, such as any one of about 0.01% to about 100%, about 1% to about 99.9%, about 0.1% to about 10%, about 1% to about 25%, about 10% to about 50%, about 50% to about 99%, or about 75% to about 100%. In some instances, the composition described herein includes at least any of 0.1%, 0.5%, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more PMPs, e.g., as measured by wt/vol, percent PMP protein composition, and/or percent lipid composition (e.g., by measuring fluorescently labelled lipids). In some instances, the concentrated agents are used as commercial products, e.g., the final user may use diluted agents, which have a substantially lower concentration of active ingredient. In some embodiments, the composition described herein is formulated as an agricultural concentrate formulation, e.g., an ultra-low-volume concentrate formulation.

Lipid Reconstructed Natural Messenger Packs (LNMPs)

[0220] A lipid reconstructed NMP (LNMP) is used herein. LNMP refers to a NMP that has been derived from a lipid structure (e.g., a lipid bilayer, unilamellar, multilamellar structure; e.g., a vesicular lipid structure) derived from (e.g., enriched, isolated or purified from) a natural source, wherein the lipid structure is disrupted (e.g., disrupted by lipid extraction) and reassembled or reconstituted in a liquid phase (e.g., a liquid phase containing a cargo) using standard methods, e.g., reconstituted by a method comprising lipid film hydration and/or solvent injection, to produce the LNMP, as is described herein. The method may, if desired, further comprise sonication, freeze/thaw treatment, and/or lipid extrusion, e.g., to reduce the size of the reconstituted LNMPs. Alternatively, LNMPs may be produced using a microfluidic device (such as a NanoAssemblr® IGNITE™ microfluidic instrument (Precision NanoSystems)).

[0221] In some embodiments, the LNMPs are produced by a process which comprises the steps of (a) providing a plurality of purified NMPs (e.g., NMPs purified as described in Section IA herein); (b) processing the plurality of NMPs to produce a lipid film; (c) reconstituting the lipid film in an organic

solvent or solvent combination, thereby producing a lipid solution; and (d) processing the lipid solution of step (c) in a microfluidics device comprising an aqueous phase, thereby producing the LNMPs.

[0222] In some instances, processing the plurality of NMPs to produce a lipid film includes extracting lipids from the plurality of NMPs, e.g., extracting lipids using the Bligh-Dyer method (Bligh and Dyer, *J Biolchem Physiol*, 37: 911-917, 1959). The extracted lipids may be provided as a stock solution, e.g., a solution in chloroform:methanol. Producing the lipid film may comprise, e.g., evaporation of the solvent with a stream of inert gas (e.g., nitrogen).

Lipid Reconstructed Plant Messenger Packs (LPMPs)

[0223] A lipid reconstructed PMP (LPMP) is used herein. LPMP refers to a PMP that has been derived from a lipid structure (e.g., a lipid bilayer, unilamellar, multilamellar structure; e.g., a vesicular lipid structure) derived from (e.g., enriched, isolated or purified from) a plant source, wherein the lipid structure is disrupted (e.g., disrupted by lipid extraction) and reassembled or reconstituted in a liquid phase (e.g., a liquid phase containing a cargo) using standard methods, e.g., reconstituted by a method comprising lipid film hydration and/or solvent injection, to produce the LPMP, as is described herein. The method may, if desired, further comprise sonication, freeze/thaw treatment, and/or lipid extrusion, e.g., to reduce the size of the reconstituted LPMPs. Alternatively, LPMPs may be produced using a microfluidic device (such as a NanoAssemblr® IGNITE™ microfluidic instrument (Precision NanoSystems)).

[0224] In some embodiments, the LPMPs are produced by a process which comprises the steps of (a) providing a plurality of purified PMPs (e.g., PMPs purified as described in Section IA herein); (b) processing the plurality of PMPs to produce a lipid film; (c) reconstituting the lipid film in an organic solvent or solvent combination, thereby producing a lipid solution; and (d) processing the lipid solution of step (c) in a microfluidics device comprising an aqueous phase, thereby producing the LPMPs.

[0225] In some instances, processing the plurality of PMPs to produce a lipid film includes extracting lipids from the plurality of PMPs, e.g., extracting lipids using the Bligh-Dyer method (Bligh and Dyer, *J Biolchem Physiol*, 37: 911-917, 1959). The extracted lipids may be provided as a stock solution, e.g., a solution in chloroform:methanol. Producing the lipid film may comprise, e.g., evaporation of the solvent with a stream of inert gas (e.g., nitrogen).

Natural lipids

[0226] A LNMP may comprise between 10% and 100% lipids derived from the lipid structure from the natural source (e.g., lemon or algae), e.g., may contain at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or 100% lipids derived from the lipid structure from the natural source. A LNMP may comprise all or a fraction of the lipid species present in the lipid structure from the source (e.g., lemon or algae), e.g., it may contain at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or 100% of the lipid species present in the lipid structure from the source. A LNMP may comprise none, a fraction, or all of the protein species present in the lipid structure from the source (e.g., lemon or algae), e.g., may contain 0%, less than 1%, less than 5%, less than 10%, less than 15%, less than 20%, less than 30%, less than 40%, less than 50%, less than

60%, less than 70%, less than 80%, less than 90%, less than 100%, or 100% of the protein species present in the lipid structure from the natural source (e.g., lemon or algae). In some instances, the lipid bilayer of the LNMP does not contain proteins. In some instances, the lipid structure of the LNMP contains a reduced amount of proteins relative to the lipid structure from the natural source.

[0227] In some embodiments, the natural lipids of the LNMPs are extracted from a plant source, such as lemon or algae. In some embodiments, the natural lipids are replaced with synthetic structural lipids to form a LNP formulation.

Exogenous lipids

[0228] The LNMPs may be modified to contain a heterologous agent (e.g., a cell-penetrating agent) that is capable of increasing cell uptake (e.g., animal cell uptake (e.g., mammalian cell uptake, e.g., human cell uptake), plant cell uptake, bacterial cell uptake, or fungal cell uptake) relative to an unmodified LNMP. For example, the modified LNMPs may include (e.g., be loaded with, e.g., encapsulate or be conjugated to) or be formulated with (e.g., be suspended or resuspended in a solution comprising) a plant cell-penetrating agent, such as an ionizable lipid. Each of the modified LNMPs may comprise at least 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more than 90% ionizable lipid.

[0229] LNMPs may include one or more exogenous lipids, e.g., lipids that are exogenous to the plant (e.g., originating from a source that is not the plant or plant part from which the LNMP is produced). The lipid composition of the LNMP may include 0%, less than 1%, or at least 1%, 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more than 95% exogenous lipid. In some examples, the exogenous lipid (e.g., ionizable lipid) is added to amount to 25% or 40% (w/w) of total lipids in the preparation. In some examples, the exogenous lipid is added to the preparation prior to step (b), e.g., mixed with extracted NMP lipids prior to step (b).

[0230] Exemplary exogenous lipids include ionizable lipids. The ionizable lipids in the LNMP compositions herein include one or more from the compounds of groups i)-iv) as described herein.

[0231] Exogenous lipids may also include cationic lipids.

[0232] In some instances, the exogenous lipid may also include an ionizable lipid or cationic lipid chosen from 1,1‘-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl) (2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-200), DLin-MC3-DMA (MC3), dioleoyl-3-trimethylammonium propane (DODAP), DC-cholesterol, DOTAP, Ethyl PC, GL67, DLin-KC2-DMA (KC2), MD1 (cKK-E12), OF2, EPC, ZA3-Ep10, TT3, LP01, 5A2-SC8, Lipid 5 (Moderna), a cationic sulfonamide amino lipid, an amphiphilic zwitterionic amino lipid, DODAC, DOBAQ, YSK05, DOBAT, DOBAQ, DOPAT, DOMPAQ, DOAAQ, DMAP-BLP, DLinDMA, DODMA, DOTMA, DSDMA, DOSPA, DODAC, DOBAQ, DMRIE, DOTAP-cholesterol, GL67A, and 98N12-5 or a combination thereof.

[0233] In some embodiments, the exogenous lipid may also include an ionizable lipid or cationic lipid chosen from C12-200, MC3, DODAP, DC-cholesterol, DOTAP, Ethyl PC, GL67, KC2, MD1, OF2, EPC, ZA3-Ep10, TT3, LP01, 5A2-SC8, Lipid 5 (Moderna), a cationic sulfonamide amino lipid, and an amphiphilic zwitterionic amino lipid or a combination thereof. In some embodiments, the ionizable

lipid is chosen from C12-200, MC3, DODAP, and DC-cholesterol or combinations thereof. In some instances, the ionizable lipid is an ionizable lipid. In some embodiments, the ionizable lipid is 1,1‘-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl) (2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-200) or (6Z,9Z,28Z,31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate, DLin-MC3-DMA (MC3). In some instances, the exogenous lipid is a cationic lipid. In some embodiments, the cationic lipid is DC-cholesterol or dioleoyl-3-trimethylammonium propane (DOTAP).

[0234] In some instances, the LNMPs comprise at least 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more than 90% ionizable lipid.

[0235] In some instances, the LNMPs comprise a molar ratio of least 0.1%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% 80%, 85%, 90%, or more than 90% ionizable lipid, e.g., 1%-10%, 10%-20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% ionizable lipid, e.g., about 30%-75% ionizable lipid (e.g., about 30%-75% ionizable lipid). In some embodiments, the LNMP comprises 25% ionizable lipid. In some embodiments, the LNMP comprises a molar ratio of 35% ionizable lipid. In some embodiments, the LNMP comprises a molar ratio of 50% ionizable lipid. In some embodiments, the LNMP comprises 40% MC3. In some embodiments, the LNMP comprises a molar ratio of 50% ionizable lipid. In some embodiments, the LNMP comprises 20% or 40% DC-cholesterol. In some embodiments, the LNMP comprises 25% or 40% DOTAP.

[0236] The agent may increase uptake of the LNMP as a whole or may increase uptake of a portion or component of the LNMP (e.g., the mRNA therapeutic) carried by the LNMP. The degree to which cell uptake is increased may vary depending on the plant or plant part to which the composition described herein is delivered, the LNMP formulation, and other modifications made to the LNMP, For example, the modified LNMPs may have an increased cell uptake (e.g., animal cell uptake, plant cell uptake, bacterial cell uptake, or fungal cell uptake) of at least 1%, 2%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% relative to an unmodified LNMP. In some instances, the increased cell uptake is an increased cell uptake of at least 2x-fold, 4x-fold, 5x-fold, 10x-fold, 100x-fold, or 1000x-fold relative to an unmodified LNMP.

[0237] In some embodiments, a LNMP that has been modified with a ionizable lipid more efficiently encapsulates a negatively charged a polynucleotide than a LNMP that has not been modified with an ionizable lipid. In some aspects, a LNMP that has been modified with an ionizable lipid has altered biodistribution relative to a LNMP that has not been modified with an ionizable lipid. In some aspects, a LNMP that has been modified with an ionizable lipid has altered (e.g., increased) fusion with an endosomal membrane of a target cell relative to a LNMP that has not been modified with an ionizable lipid.

Ionizable lipids

[0238] In some embodiments, the ionizable lipid has at least one (e.g., one, two, three, four or all five) of the characteristics listed below:

(i) at least 2 ionizable amines (e.g., at least 2, at least 3, at least 4, at least 5, at least 6, or more than 6 ionizable amines, e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more than 12 ionizable amines);

(ii) at least 3 lipid tails (e.g., at least 3, at least 4, at least 5, at least 6, or more than 6 lipid tails, e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more than 12 lipid tails), wherein each of the lipid tails is independently at least 6 carbon atoms in length (e.g., at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, or more than 18 carbon atoms in length, e.g., 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more than 25 carbon atoms in length);

(iii) an acid dissociation constant (pKa) of from about 4.5 to about 7.5 (e.g., a pKa of about 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, or 7.5 (e.g., a pKa of from about 6.5 and about 7.5 (e.g., a pKa of about 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, or 7.5));

(iv) an ionizable amine and a heteroorganic group; and

(v) an N:P (amines of ionizable lipid: phosphates of mRNA) ratio of at least 3 (or at least 4);

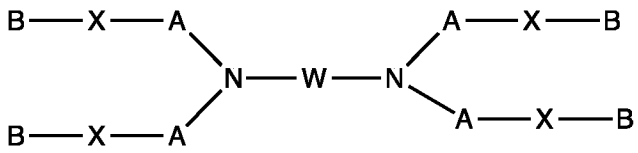
[0239] In some embodiments, the ionizable lipid is an ionizable amine and a heteroorganic group. In some embodiments, the heteroorganic group is hydroxyl. In some embodiments, the heteroorganic group comprises a hydrogen bond donor. In some embodiments, the heteroorganic group comprises a hydrogen bond acceptor. In some embodiments, the heteroorganic group is -OH, -SH, -(CO)H, -CO_2H, -NH_2, -$CONH_2$, optionally substituted C_1-C_6 alkoxy, or fluorine.

[0240] In some embodiments, the ionizable lipid is an ionizable amine and a heteroorganic group separated by a chain of at least two atoms

[0241] The ionizable lipid in the LNMP compositions included one of the compounds from group i) to group iv) as discussed below.

Ionizable lipid compounds i)

[0242] In some embodiments, the ionizable lipid is represented by the following formula I:



(I)

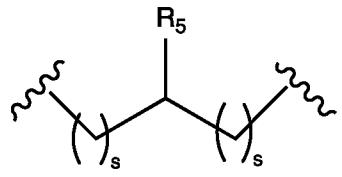
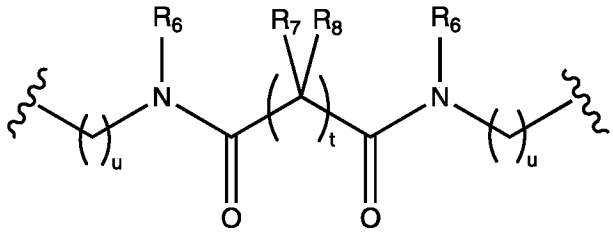
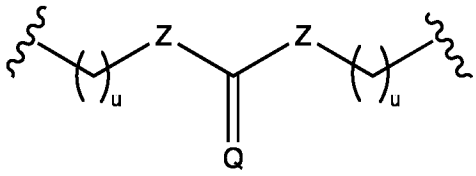
a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing,

wherein

each **A** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally substituted with heteroatom or substituted with OH, SH, or halogen;

each **B** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally substituted with heteroatom or substituted with OH, SH, or halogen;

each **X** is independently a biodegradable moiety; and

W is  ,  , or  , wherein

R_5 is OH, SH, $NR_{10}R_{11}$;

each **R_6** is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each **R_7** and each **R_8** is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $NR_{10}R_{11}$, wherein each **R_{10}** and **R_{11}** is independently H, C_1-C_3 alkyl, or **R_{10}** and **R_{11}** are taken together to form a heterocyclic ring;

each **s** is independently 1, 2, 3, 4, or 5;

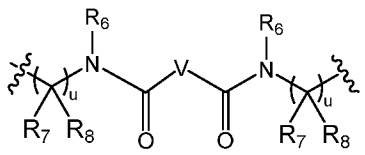
each **u** is independently 1, 2, 3, 4, or 5;

t is 1, 2, 3, 4 or 5;

each **Z** is independently absent, O, S, or NR_{12}, wherein **R_{12}** is H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl, provided that when Z is not absent, the adjacent **R_1** and **R_2** cannot be OH, $NR_{10}R_{11}$, or SH; and

Q is O, S, or NR_{13}, wherein each **R_{13}** is H, C_1-C_5 alkyl.

[0243] In some embodiments, **B** is C_3-C_{20} alkyl.

[0244] In some embodiments, **W** in formula (I) may alternatively be  , wherein:

V is branched or unbrachned C_2-C_{10} alkylene, C_2-C_{10} alkenylene, C_2-C_{10} alkynylene, or C_2-C_{10} heteroalkylene, optionally substituted with one or more OH, SH, and/or halogen groups;

each **R_6** is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

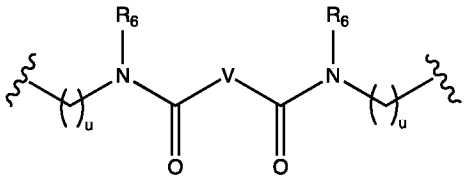
each **R_7** and each **R_8** is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $(CH_2)_vR_{17}$, or $NR_{10}R_{11}$, wherein each **R_{10}** and **R_{11}** is independently H, C_1-C_3 alkyl, or **R_{10}** and **R_{11}** are taken together to form a heterocyclic ring;

each **v** is independently 0, 1, 2, 3, 4, or 5;

R_{17} is OH, SH, or $N(CH_3)_2$; and

each **u** is independently 1, 2, 3, 4, or 5.

[0245] In some embodiments, **W** in formula (I) may alternatively be

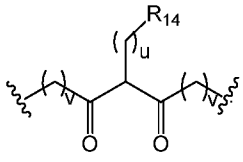


, wherein:

V is C_2-C_{10} alkenylene, C_2-C_{10} alkynylene, or C_2-C_{10} heteroalkylene;

each $\mathbf{R_6}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl; and

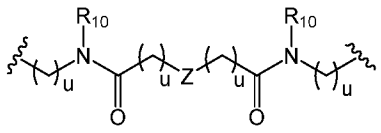
each **u** is independently 1, 2, 3, 4, or 5.

[0246] In some embodiments, W in formula (I) may alternatively be  , wherein:

$\mathbf{R_{14}}$ is a heterocyclic;

each **v** is independently 0, 1, 2, 3, 4, or 5; and

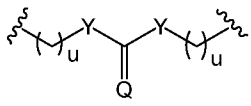
each **u** is independently 1, 2, 3, 4, or 5.

[0247] In some embodiments, W in formula (I) may alternatively be  , wherein:

Z is O, S, -C((CH$_2$)$_v$N($\mathbf{R_{15}}$)$_2$)-, or N($\mathbf{R_{15}}$), wherein $\mathbf{R_{15}}$ is H, C_1-C_4 branched or unbranched alkyl, and **v** is 0, 1, 2, 3, 4, or 5;

each $\mathbf{R_{10}}$ is independently H, or C_1-C_3 alkyl; and

each **u** is independently 0, 1, 2, 3, 4, or 5.

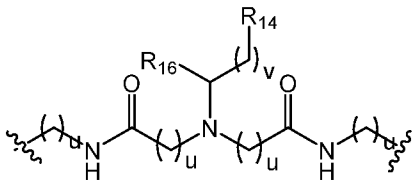
[0248] In some embodiments, **W** in formula (I) may alternatively be  , wherein:

each **Y** is a divalent heterocyclic;

Q is O, S, or NH; and

each **u** is independently 1, 2, 3, 4, or 5.

[0249] In some embodiments, **W** in formula (I) may alternatively be



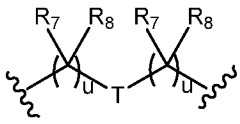
, wherein:

$\mathbf{R_{14}}$ is a heterocyclic, N$\mathbf{R_{10}R_{11}}$, C(O)N$\mathbf{R_{10}R_{11}}$, or C(S)N$\mathbf{R_{10}R_{11}}$, wherein each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 alkyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloalkenyl, optionally substituted with one or more NH and/or oxo groups, or $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring;

$\mathbf{R_{16}}$ is H, =O, =S, or CN;

each **v** is independently 0, 1, 2, 3, 4, or 5; and

each **u** is independently 1, 2, 3, 4, or 5.

[0250] In some embodiments, **W** in formula (I) may alternatively be  , wherein:

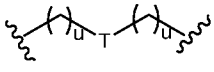
T is –NHC(O)O-, –OC(O)NH-, or a divalent heterocyclic optionally substituted with one or more -$(CH_2)_vOH$, -$(CH_2)_vSH$, and/or -$(CH_2)_v$-halogen groups;

each R_7 and each R_8 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $(CH_2)_vR_{17}$, or $NR_{10}R_{11}$, wherein each R_{10} and R_{11} is independently H, C_1-C_3 alkyl, or R_{10} and R_{11} are taken together to form a heterocyclic ring;

R_{17} is OH, SH, or $N(CH_3)_2$;

each **v** is independently 0, 1, 2, 3, 4, or 5; and

each **u** is independently 1, 2, 3, 4, or 5.

[0251] In some embodiments, **W** in formula (I) may alternatively be  , wherein:

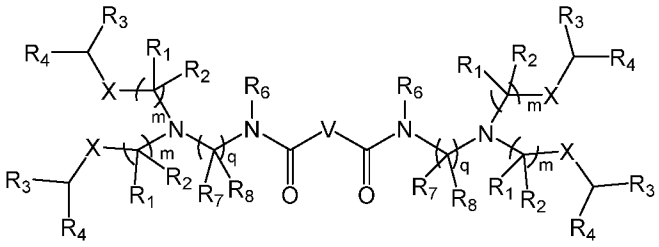
T is –NHC(O)O-, –OC(O)NH-, or a divalent heterocyclic; and

each **u** is independently 1, 2, 3, 4, or 5.

[0252] In some embodiments, when Z is not absent, the adjacent R_1 and R_2 cannot be OH, $NR_{10}R_{11}$, or SH.

[0253] In some embodiments, the heterocyclic is a piperazine, piperazine dione, piperazine-2,5-dione, piperidine, pyrrolidine, piperidinol, dioxopiperazine, bis-piperazine, aromatic or heteroaromatic.

[0254] In some embodiments, the ionizable lipid is represented by formula (IX):



(IX), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein

each R_1 and each R_2 is independently H, C_1-C_3 branched or unbranched alkyl, OH, halogen, SH, or $NR_{10}R_{11}$, or

each R_1 and each R_2 are independently taken together with the carbon atom(s) to which they are attached to form a cyclic ring;

each R_{10} and R_{11} is independently H, C_1-C_3 branched or unbranched alkyl, or R_{10} and R_{11} are taken together to form a heterocyclic ring;

each R_3 and each R_4 is independently H, C_2-C_{14} branched or unbranched alkyl (e.g., C_3-C_{10} branched or unbranched alkyl), or C_3-C_{10} branched or unbranched alkenyl, provided that at least one of R_3 and R_4 is not H;

each **X** is independently a biodegradable moiety;

each **q** is independently 2, 3, 4,or 5;

V is branched or unbrachned C_2-C_{10} alkylene, C_2-C_{10} alkenylene, C_2-C_{10} alkynylene, or C_2-C_{10} heteroalkylene, optionally substituted with one or more OH, SH, and/or halogen groups;

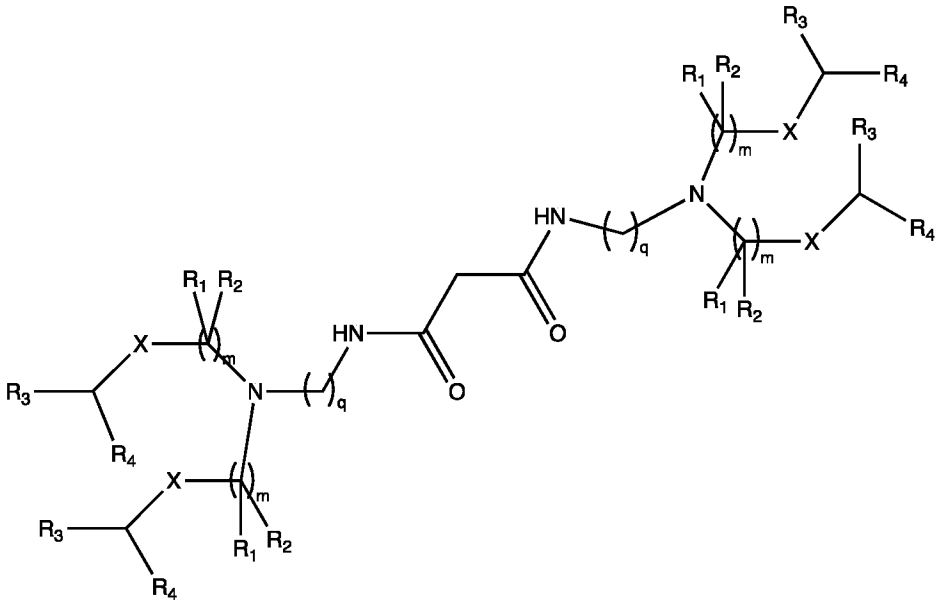
each $\mathbf{R_6}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each $\mathbf{R_7}$ and each $\mathbf{R_8}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $(CH_2)_v\mathbf{R_{17}}$, or $N\mathbf{R_{10}R_{11}}$, wherein each **v** is independently 0, 1, 2, 3, 4, or 5, and $\mathbf{R_{17}}$ is OH, SH, or $N(CH_3)_2$; and

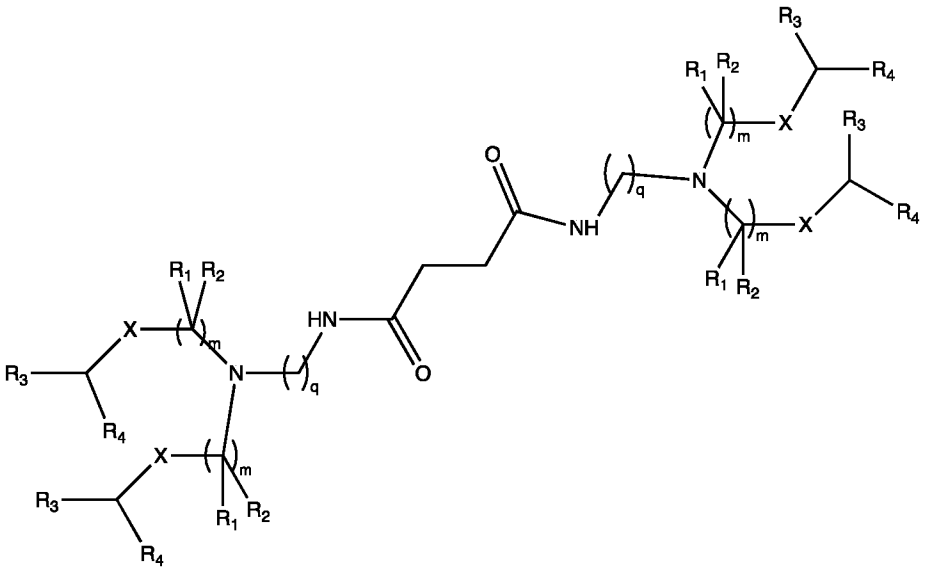
each **m** is independently 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

[0255] In some embodiments, **V** is a branched or unbrachned C_2-C_3 alkylene. In some embodiments, **V** is a C_2-C_3 alkylene substituted with OH. In some embodiments, **V** is a branched or unbrachned C_2-C_3 alkenylene. In some embodiments, each $\mathbf{R_6}$ is independently H or methyl.

[0256] In some embodiments, the ionizable lipid is represented by one of the following formulas

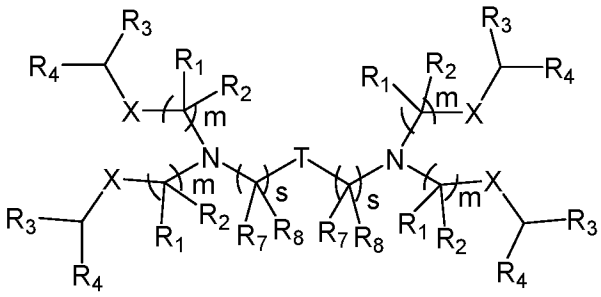


or



, wherein the definition for the variables are the same as those in formula (X).

[0257] In some embodiments, the disclosure relates to ionizable lipids of Formula (XI):



(XI), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ is independently H, C_1-C_3 branched or unbranched alkyl, OH, halogen, SH, or $N\mathbf{R_{10}R_{11}}$, or

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ are independently taken together with the carbon atom(s) to which they are attached to form a cyclic ring;

each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 branched or unbranched alkyl, or $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring;

each $\mathbf{R_3}$ and each $\mathbf{R_4}$ is independently H, C_2-C_{14} branched or unbranched alkyl (e.g., C_3-C_{10} branched or unbranched alkyl), or C_3-C_{10} branched or unbranched alkenyl, provided that at least one of $\mathbf{R_3}$ and $\mathbf{R_4}$ is not H;

each **X** is independently a biodegradable moiety;

each **s** is independently 1, 2, 3, 4, or 5;

T is –NHC(O)O-, –OC(O)NH-, or a divalent heterocyclic optionally substituted with one or more $-(CH_2)_vOH$, $-(CH_2)_vSH$, $-(CH_2)_v$-halogen groups,

each $\mathbf{R_7}$ and each $\mathbf{R_8}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $(CH_2)_v\mathbf{R_{17}}$, or $N\mathbf{R_{10}R_{11}}$, wherein $\mathbf{R_{17}}$ is OH, SH, or $N(CH_3)_2$;

each **v** is independently 0, 1, 2, 3, 4, or 5; and

each **m** is independently 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

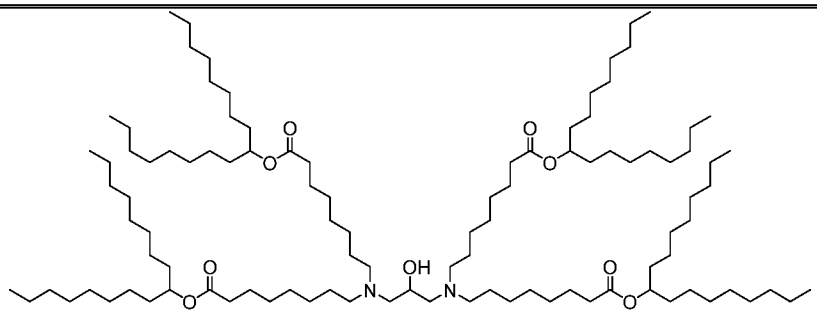
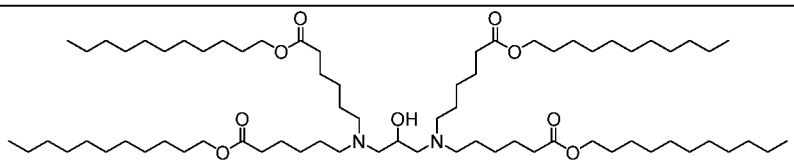
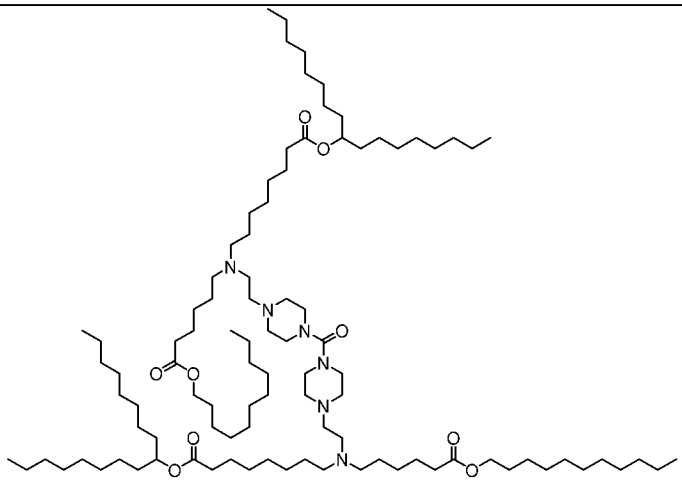
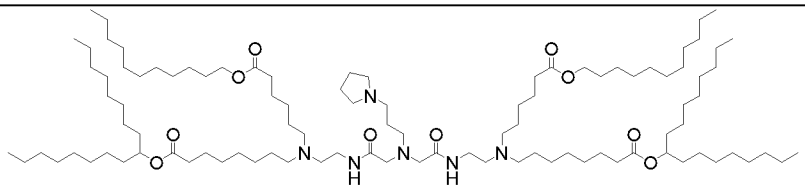
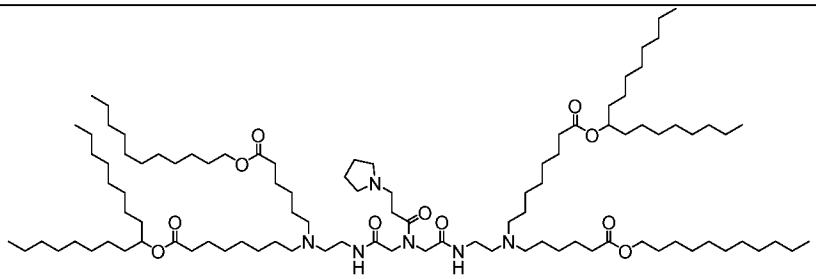
[0258] In some embodiments, T is a divalent heterocyclic (e.g., a divalent piperazine, or a divalent dioxopiperazine) optionally substituted with $-(CH_2)_vOH$, wherein **v** is independently 0, 1, or 2.

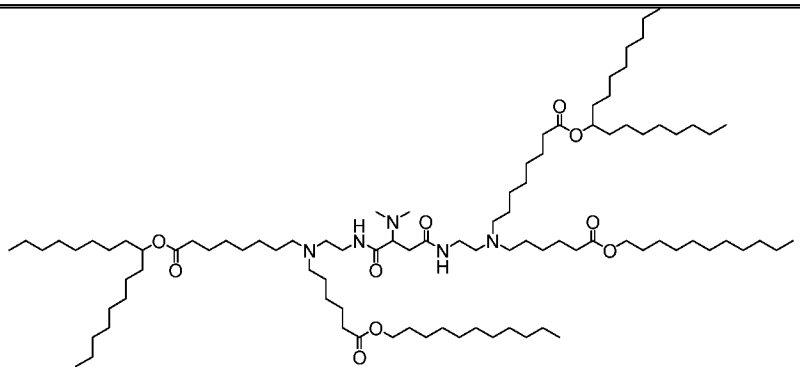
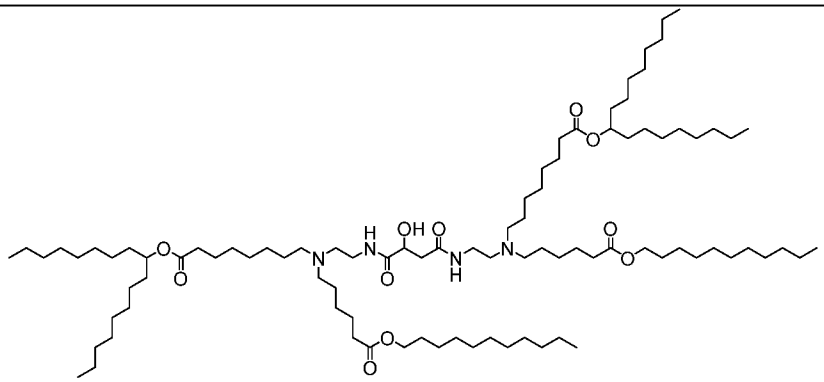
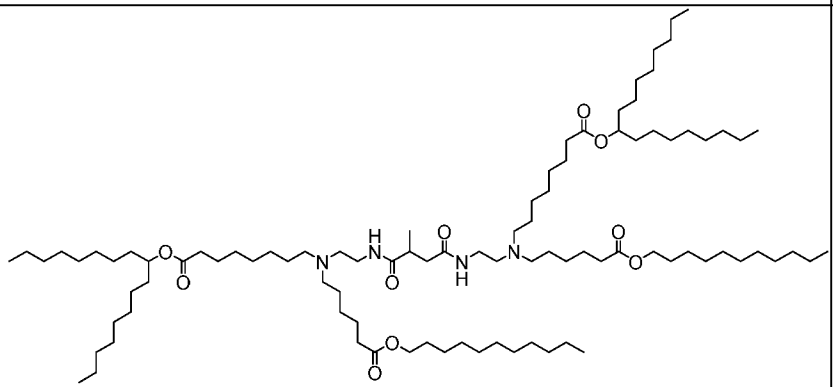
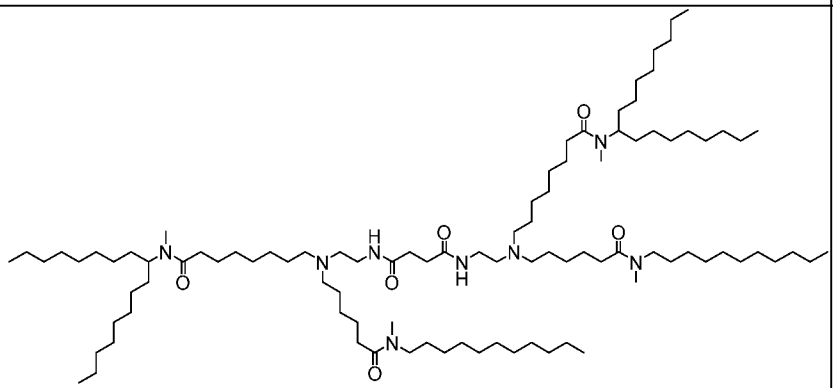
[0259] In some embodiments, in each of the above formulas, X is –OC(O)-, -C(O)O-, -SS-, $-N(\mathbf{R^{18}})C(O)-$, $-C(O)N(\mathbf{R^{18}})-$, $-C(O\text{-}\mathbf{R_{13}})\text{-}O-$, $-C(O)O(CH_2)_a-$, $-OC(O)(CH_2)_a-$, $-C(O)N(\mathbf{R^{18}})(CH_2)_a-$, $-N(\mathbf{R^{18}})C(O)(CH_2)_a-$, $-C(O\text{-}\mathbf{R_{13}})\text{-}O\text{-}(CH_2)_a-$, wherein each $\mathbf{R^{18}}$ is independently H, alkyl, alkenyl, cycloalkyl, hydroxyalkyl, or aminoalkyl, each $\mathbf{R_{13}}$ is independently C_3-C_{10} alkyl, and each **a** is independently 0-16. In one embodiment, each **X** is independently -OCO-, -COO-, -NHCO-, or -CONH-. In one embodiment, at least one X is -SS-.

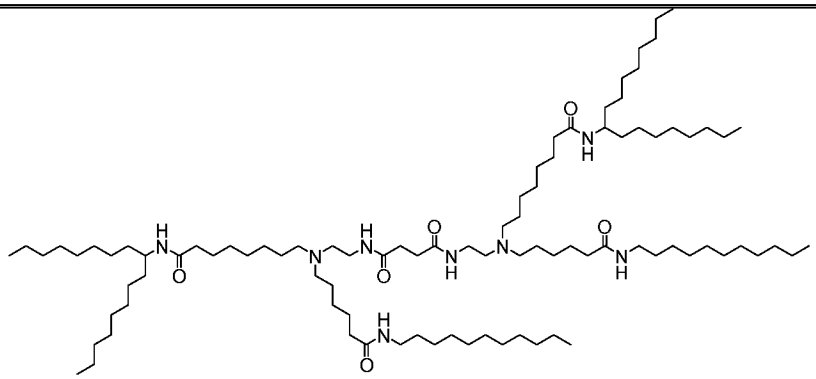
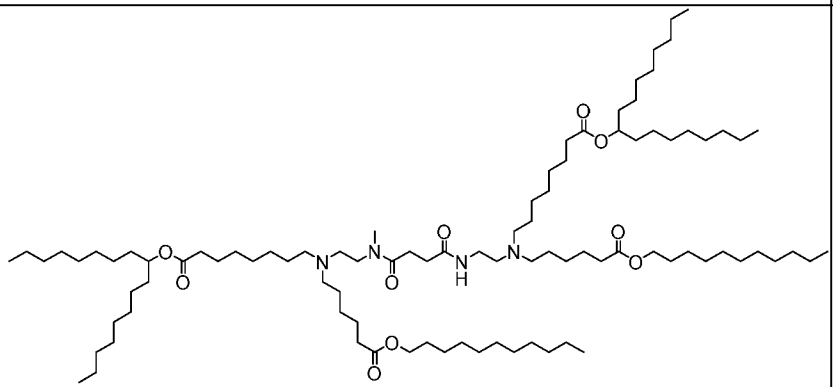
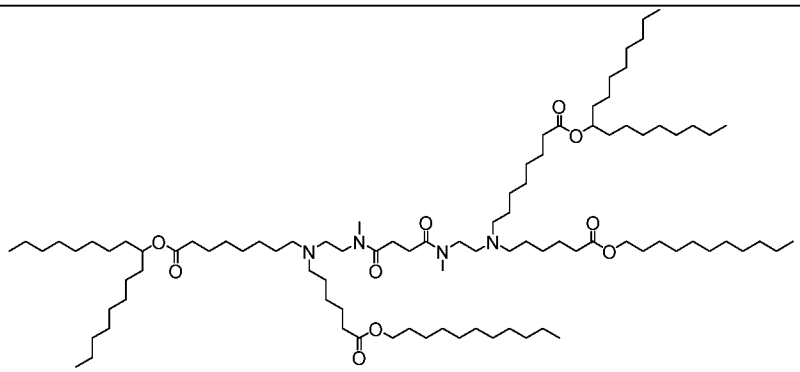
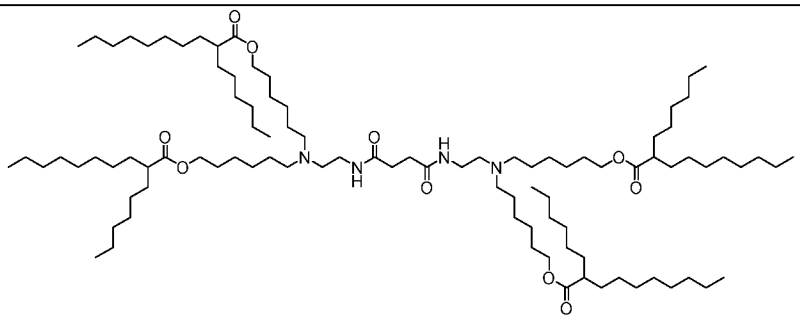
[0260] More embodiments of the ionizable lipid of formula (I), in the Ionizable lipid compounds group i), may be found in PCT Application No. PCT/US22/50725, filed on November 22, 2022, the content of which is incorporated herein by reference in its entirety. In particular, all the ionizable lipids of formulas (I)-(XII) of PCT Application No. PCT/US22/50725 are suitable for use as the ionizable lipids in this disclosure, and are incorporated herein by reference in its entirety.

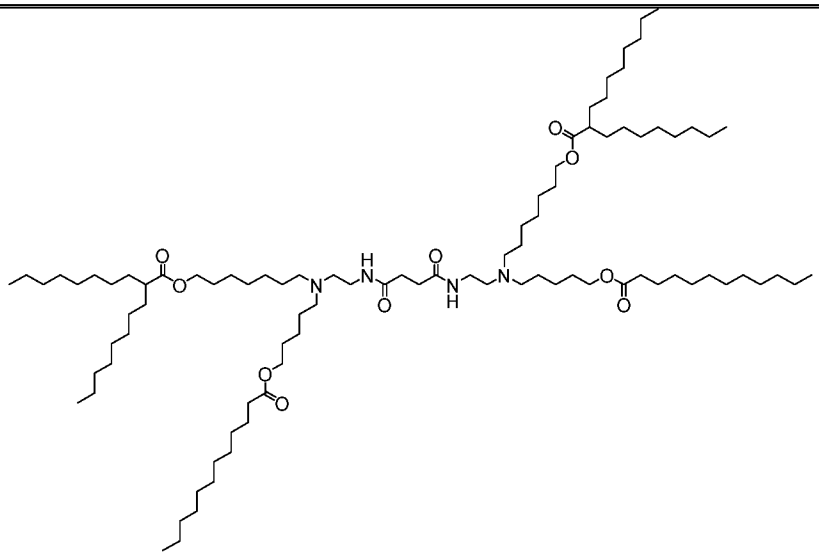
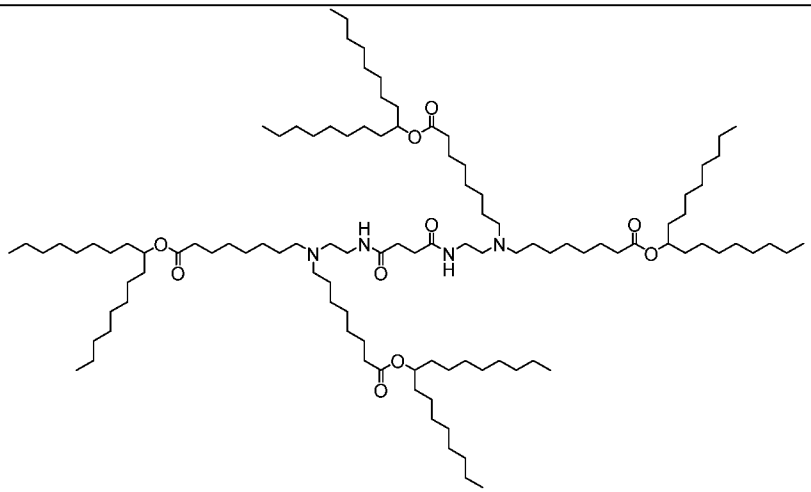
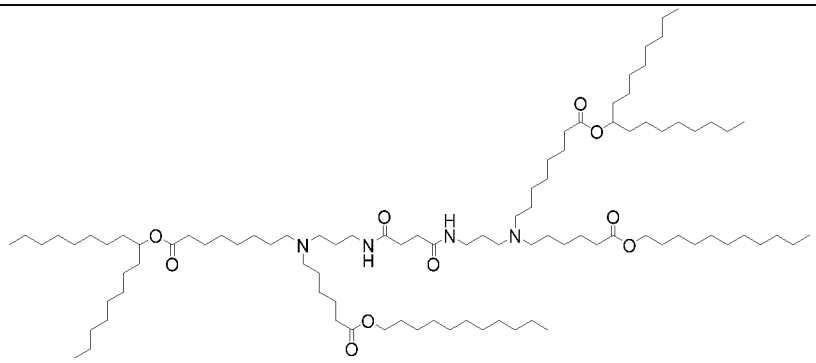
[0261] Certain exemplary ionizable lipid compounds disclosed herein are set forth in Table I below.

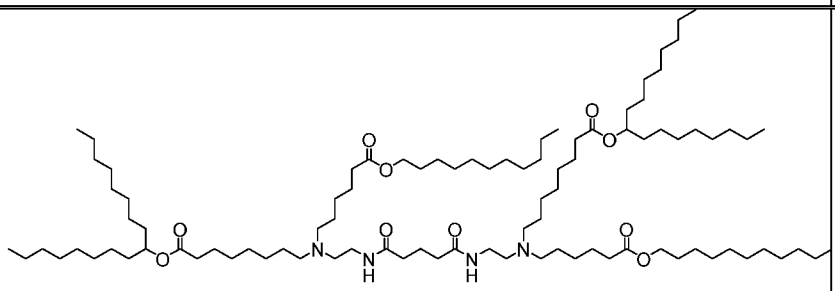
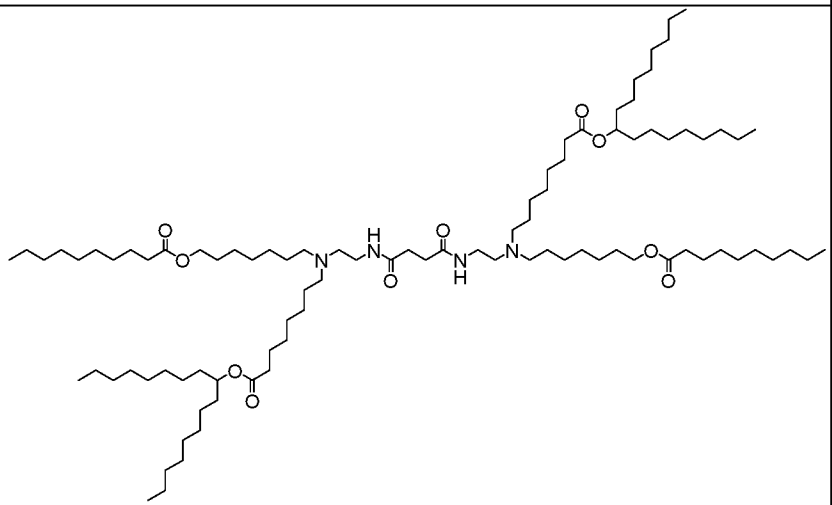
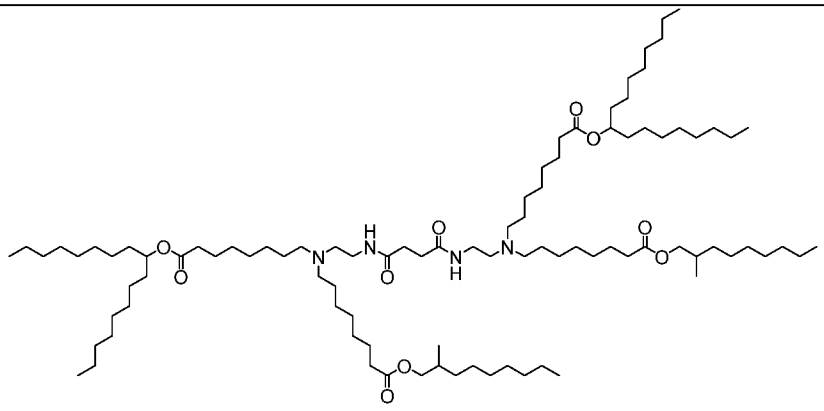
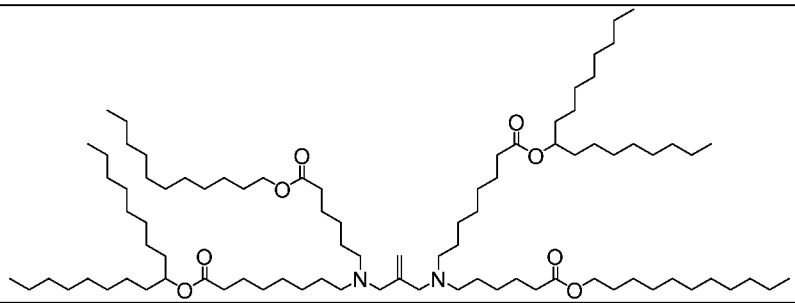
Table I. Exemplary ionizable lipid compounds.

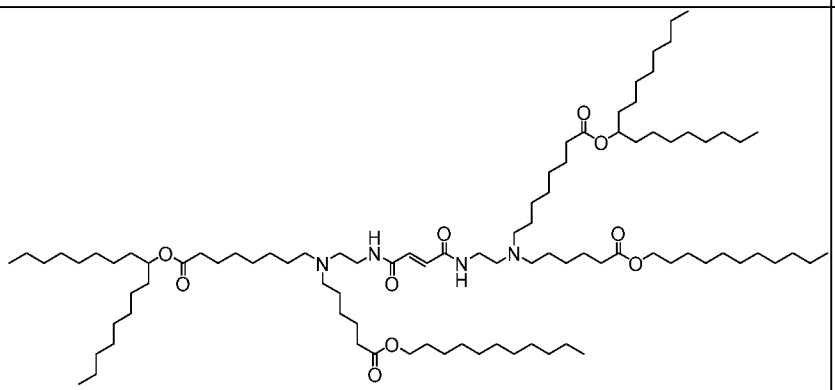
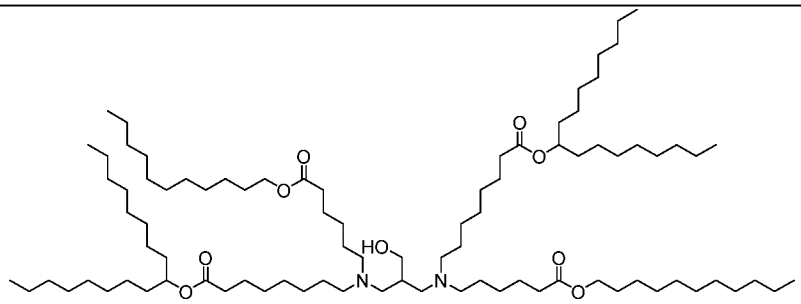
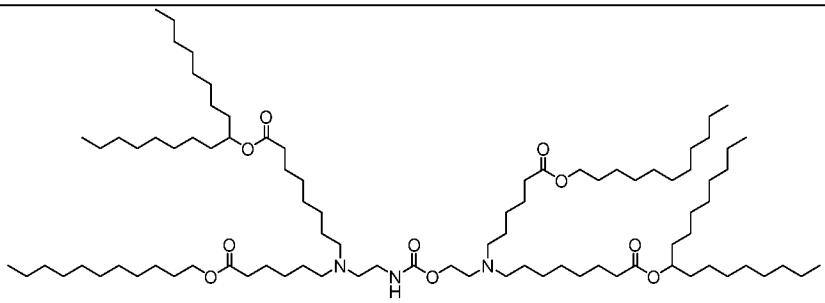
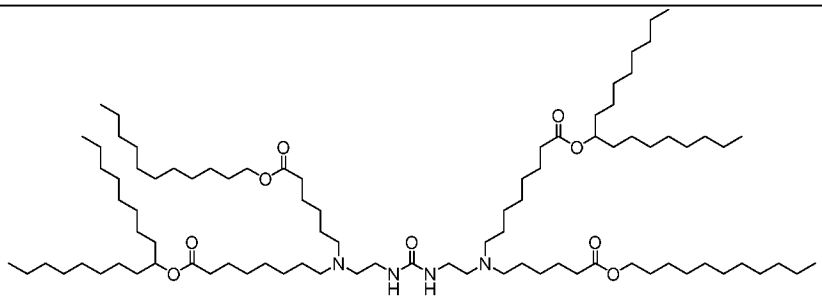
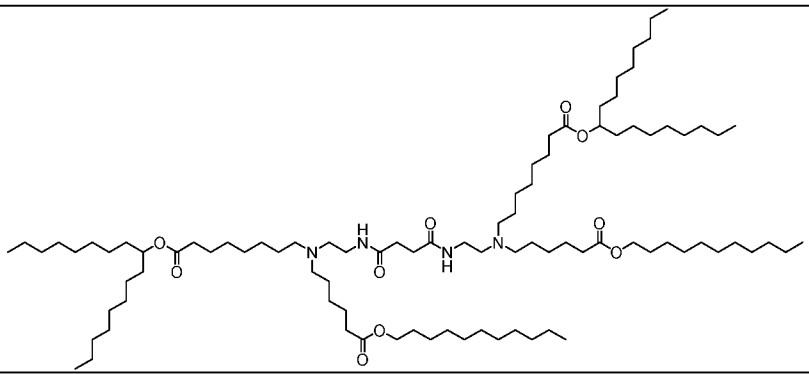
Lipid No.	Structure	IUPAC name
2303		heptadecan-9-yl 8-[(3-{bis[8-(heptadecan-9-yloxy)-8-oxooctyl]amino}-2-hydroxypropyl)[8-(heptadecan-9-yloxy)-8-oxooctyl]amino]octanoate
2302		undecyl 6-[(3-{bis[6-oxo-6-(undecyloxy)hexyl]amino}-2-hydroxypropyl)[6-oxo-6-(undecyloxy)hexyl]amino]hexanoate
2299		heptadecan-9-yl 8-[(2-{4-[4-(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)piperazine-1-carbonyl]piperazin-1-yl}ethyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate
2289		heptadecan-9-yl 8-({2-[2-({[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)carbamoyl]methyl}[3-(pyrrolidin-1-yl)propyl]amino)acetamido]ethyl}[6-oxo-6-
2288		heptadecan-9-yl 8-({2-[2-(N-{[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)carbamoyl]methyl}-3-(pyrrolidin-1-yl)propanamido)acetamido]ethyl}[6-oxo-6-

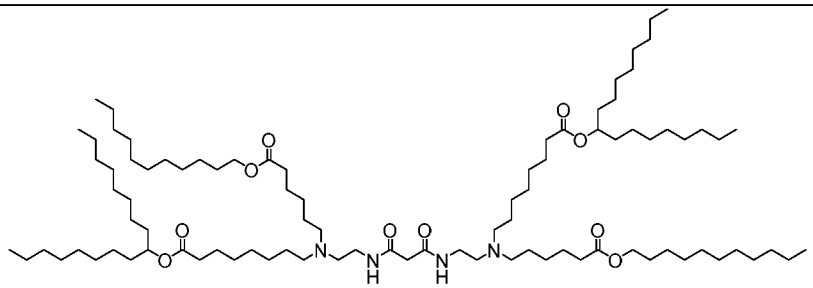
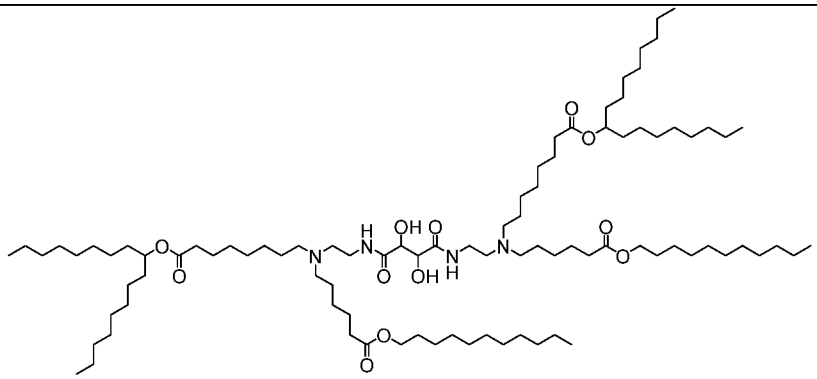
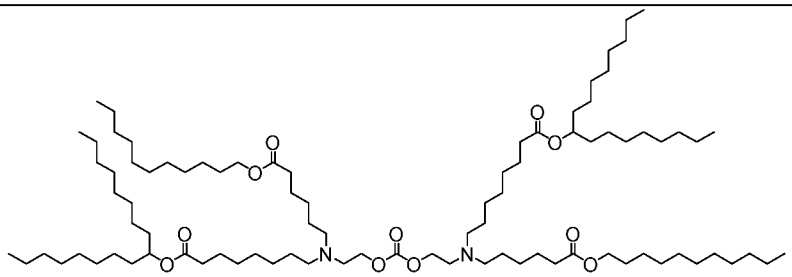
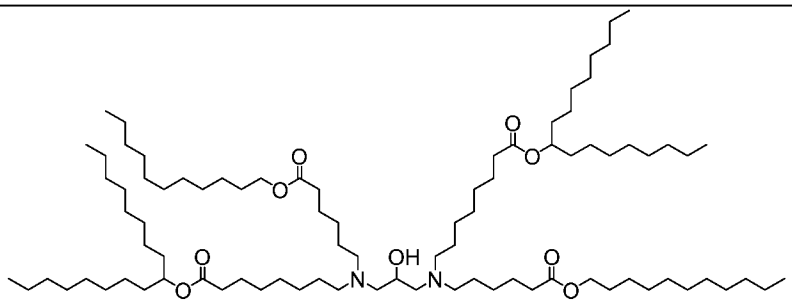
Lipid No.	Structure	IUPAC name
2286		heptadecan-9-yl 8-({2-[2-(dimethylamino)-3-[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)carbamoyl]propanamido]ethyl}[6-oxo-6-(undecyloxy)hexyl]amino)octanoate
2285		heptadecan-9-yl 8-[(2-{3-[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)carbamoyl]-2-hydroxypropanamido}ethyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate
2284		heptadecan-9-yl 8-[(2-{3-[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)carbamoyl]-2-methylpropanamido}ethyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate
2283		N,N'-bis[2-({7-[(heptadecan-9-yl)(methyl)carbamoyl]heptyl}({5-[methyl(undecyl)carbamoyl]pentyl})amino)ethyl]butanediamide

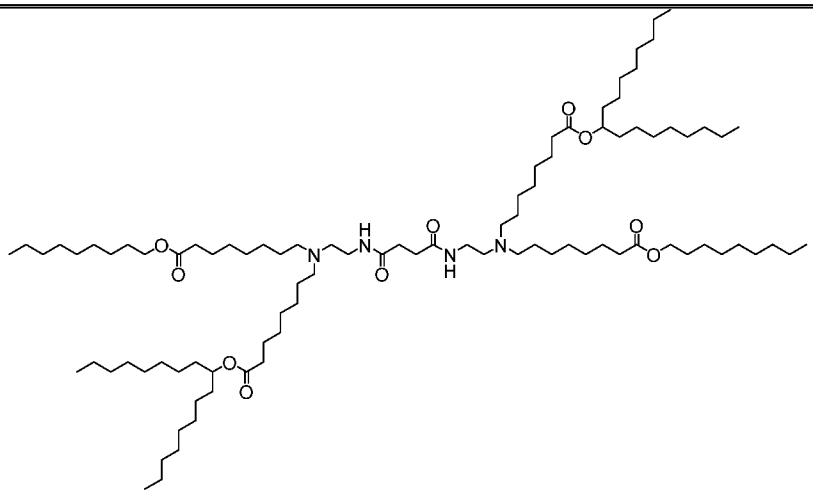
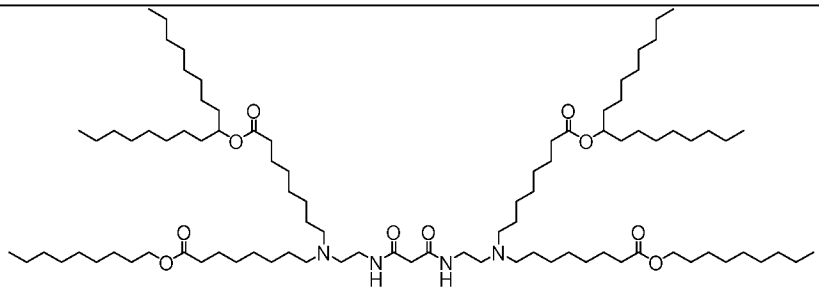
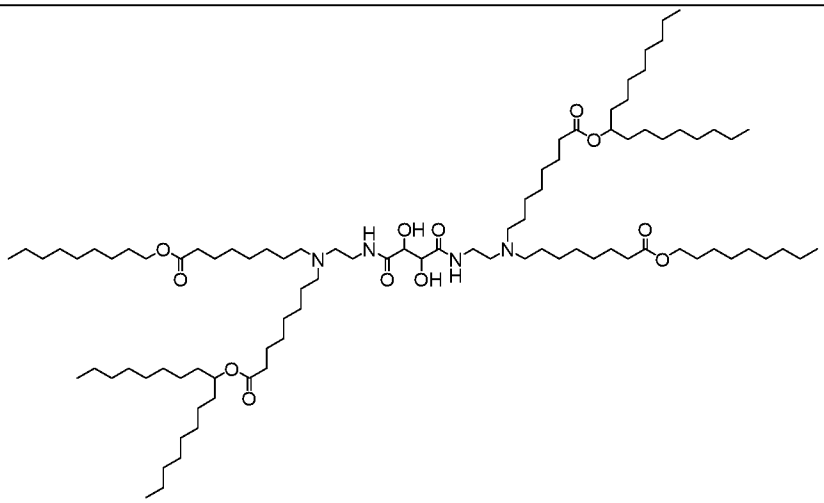
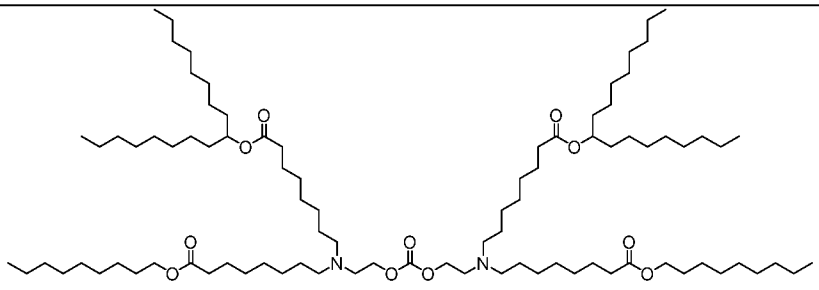
Lipid No.	Structure	IUPAC name
2282		N,N'-bis[2-({7-[(heptadecan-9-yl)carbamoyl]heptyl}[5-(undecylcarbamoyl)pentyl]amino)ethyl]butanediamide
2281		heptadecan-9-yl 8-[(2-{3-[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)carbamoyl]-N-methylpropanamido}ethyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate
2280		heptadecan-9-yl 8-[(2-{3-[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)(methyl)carbamoyl]-N-methylpropanamido}ethyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate
2279		6-({2-[3-({2-[bis({6-[(2-hexyldecanoyl)oxy]hexyl})amino]ethyl}carbamoyl)propanamido]ethyl}({6-[(2-hexyldecanoyl)oxy]hexyl})amino)hexyl 2-hexyldecanoate

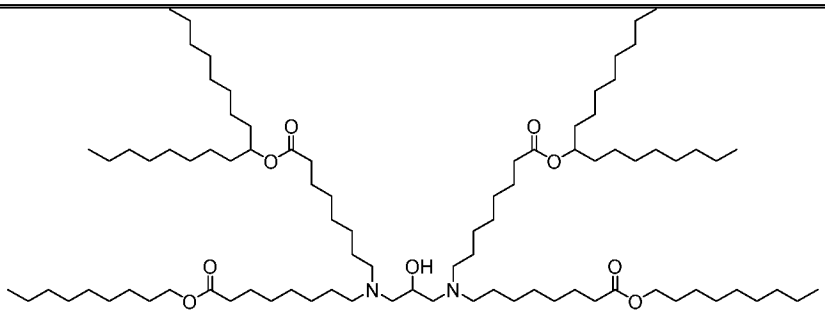
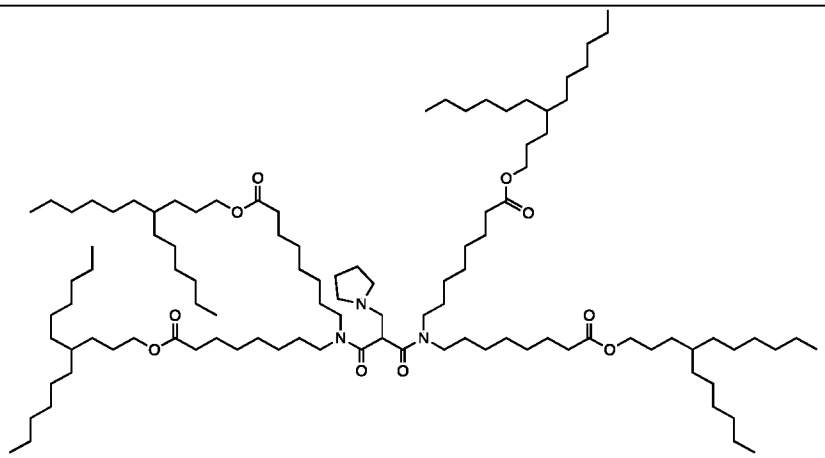
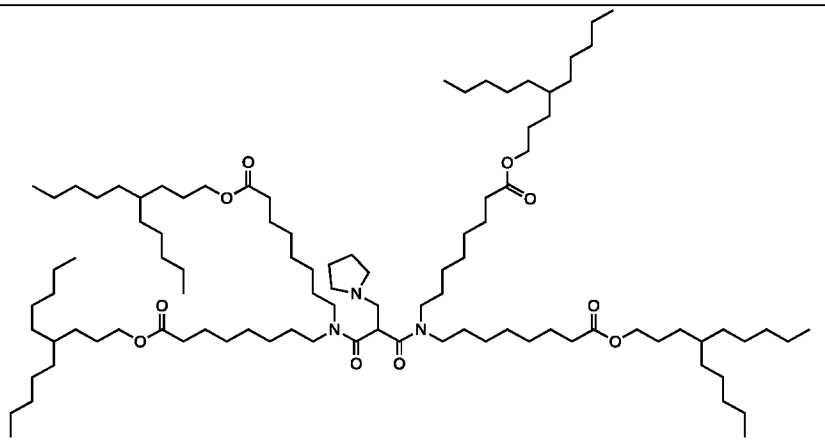
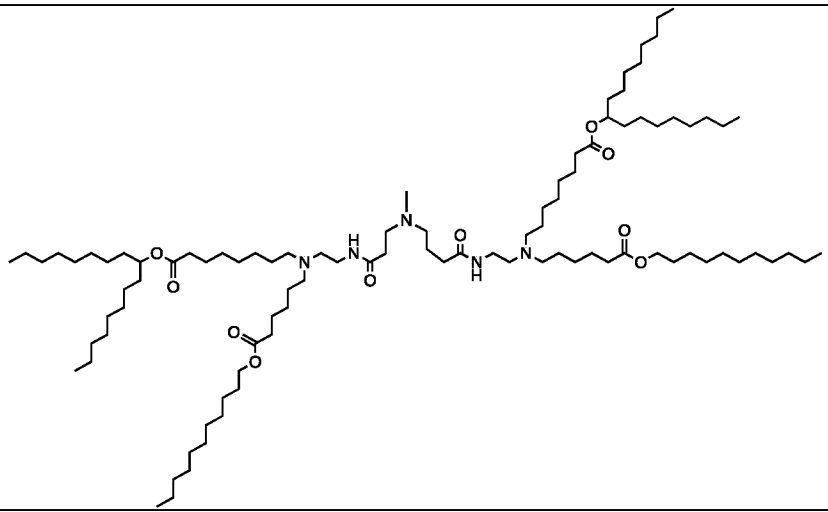
Lipid No.	Structure	IUPAC name
2278		5-[(2-{3-[(2-{[5-(dodecanoyloxy)pentyl]({7-[(2-octyldecanoyl)oxy]heptyl})amino}ethyl)carbamoyl]propanamido}ethyl)({7-[(2-octyldecanoyl)oxy]heptyl})amino]pentyl dodecanoate
2275		heptadecan-9-yl 8-[(2-{3-[(2-{bis[8-(heptadecan-9-yloxy)-8-oxooctyl]amino}ethyl)carbamoyl]propanamido}ethyl)[8-(heptadecan-9-yloxy)-8-oxooctyl]amino]octanoate
2274		heptadecan-9-yl 8-[(3-{3-[(3-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}propyl)carbamoyl]propanamido}propyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate

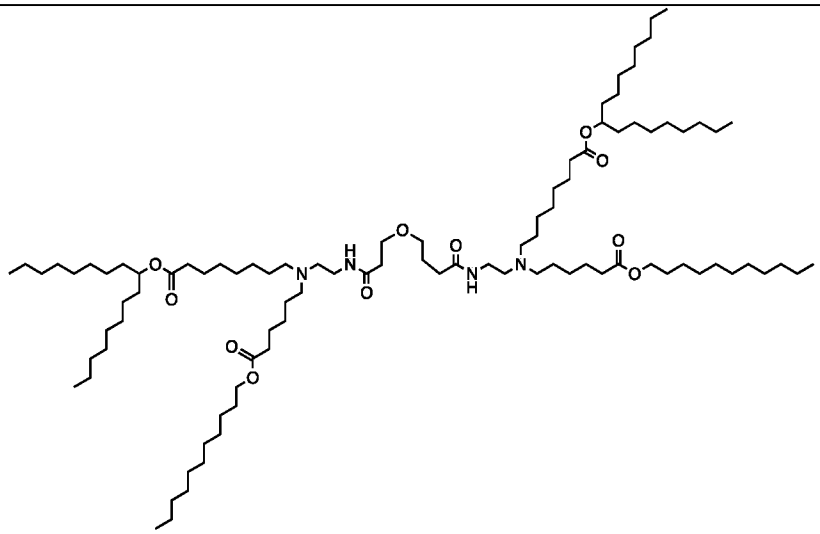
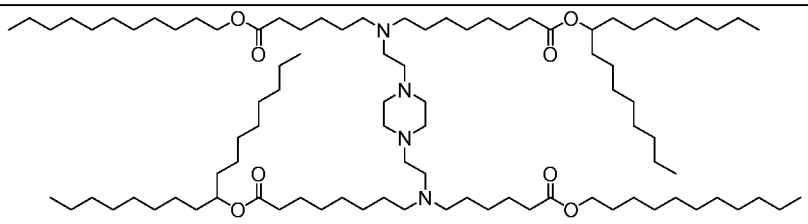
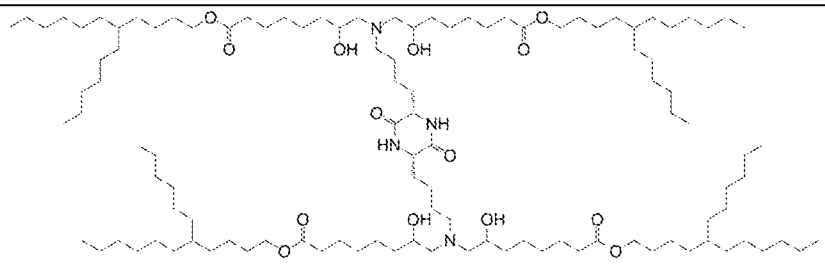
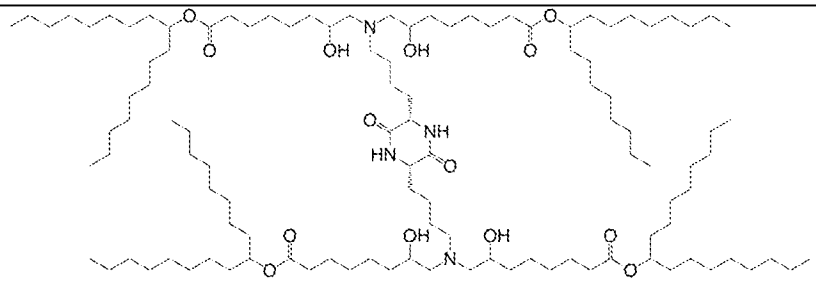
Lipid No.	Structure	IUPAC name
2273		heptadecan-9-yl 8-[(2-{4-[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)carbamoyl]butanamido}ethyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate
2272		7-[(2-{3-[(2-{[7-(decanoyloxy)heptyl][8-(heptadecan-9-yloxy)-8-oxooctyl]amino}ethyl)carbamoyl]propanamido}ethyl)[8-(heptadecan-9-yloxy)-8-oxooctyl]amino]heptyl decanoate
2271		heptadecan-9-yl 8-[(2-{3-[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl]({8-[(2-methylnonyl)oxy]-8-oxooctyl})amino}ethyl)carbamoyl]propanamido}ethyl)({8-[(2-methylnonyl)oxy]-8-oxooctyl})amino]octanoate
2253		heptadecan-9-yl 8-{[2-({[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}methyl)prop-2-en-1-yl][6-oxo-6-(undecyloxy)hexyl]amino}octanoate

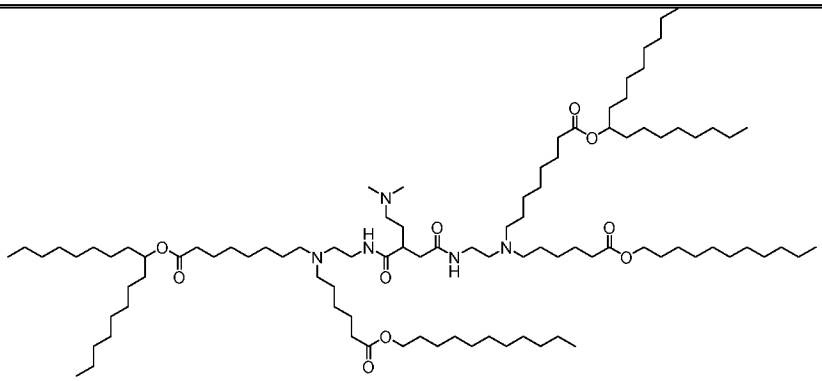
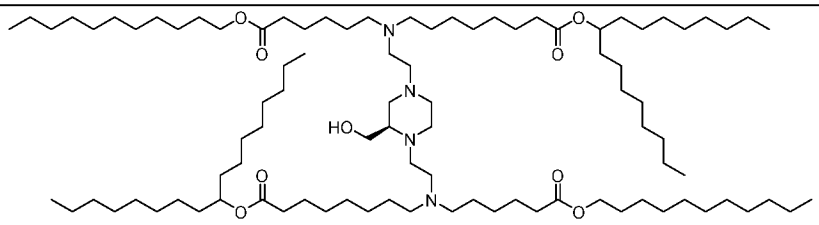
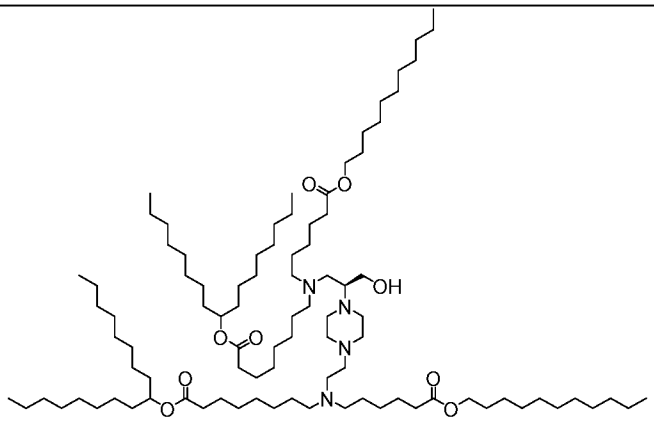
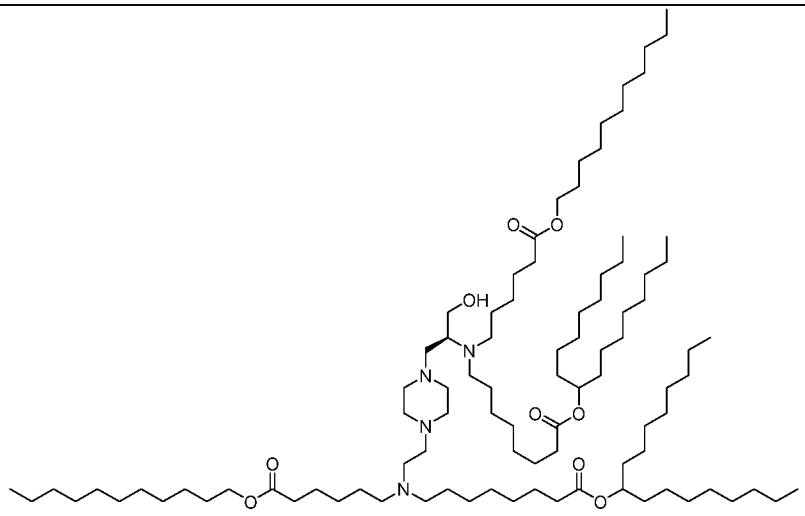
Lipid No.	Structure	IUPAC name
2252		heptadecan-9-yl 8-({2-[(2E)-3-[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)carbamoyl]prop-2-enamido]ethyl}[6-oxo-6-(undecyloxy)hexyl]amino)octanoate
2251		heptadecan-9-yl 8-{[2-({[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}methyl)-3-hydroxypropyl][6-oxo-6-(undecyloxy)hexyl]amino}octanoate
2247		heptadecan-9-yl 8-[(2-{[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethoxy)carbonyl]amino}ethyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate
2246		heptadecan-9-yl 8-[(2-{[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)carbamoyl]amino}ethyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate
2221		heptadecan-9-yl 8-[(2-{3-[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)carbamoyl]propanamido}ethyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate

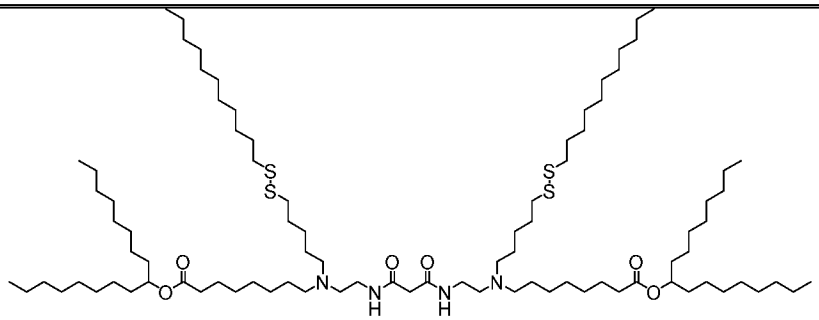
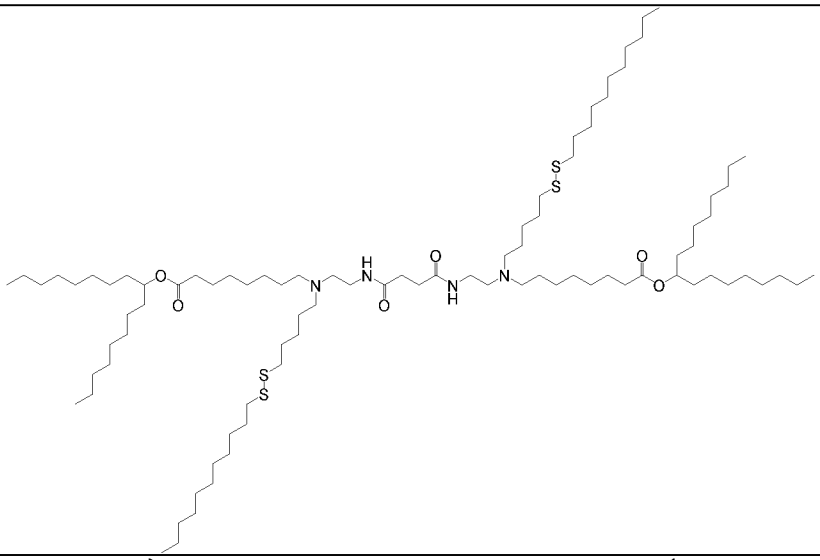
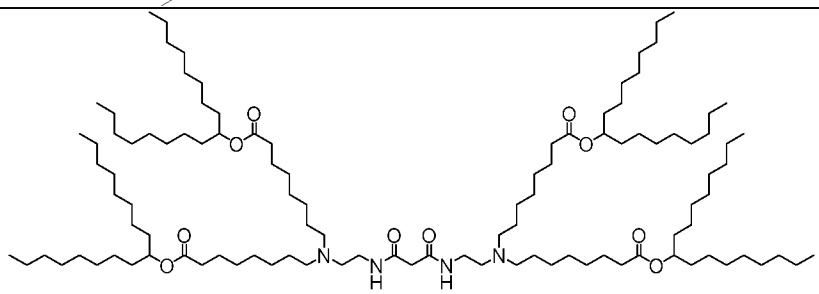
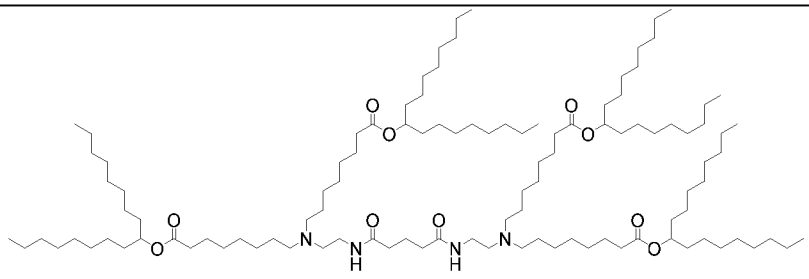
Lipid No.	Structure	IUPAC name
2220		heptadecan-9-yl 8-[(2-{2-[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)carbamoyl]acetamido}ethyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate
2219		heptadecan-9-yl 8-[(2-{3-[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)carbamoyl]-2,3-dihydroxypropanamido}ethyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate
2218		heptadecan-9-yl 8-[(2-{[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethoxy)carbonyl]oxy}ethyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate
2217		heptadecan-9-yl 8-[(3-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}-2-hydroxypropyl)[6-oxo-6-(undecyloxy)hexyl]amino]octanoate

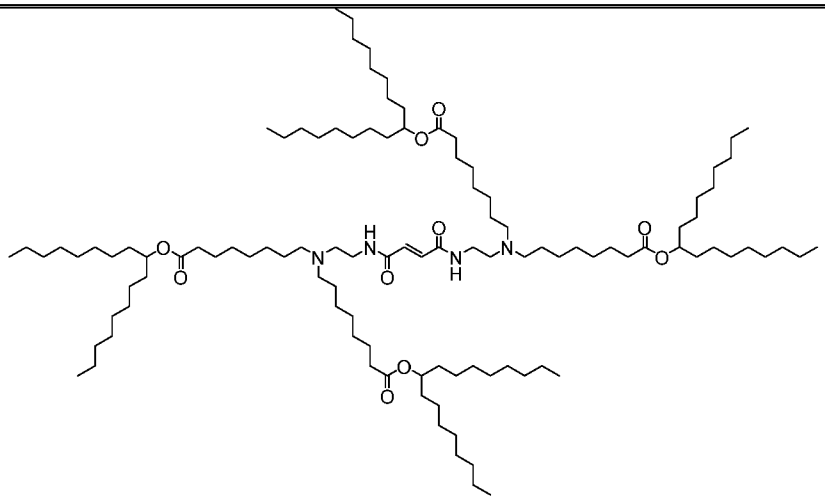
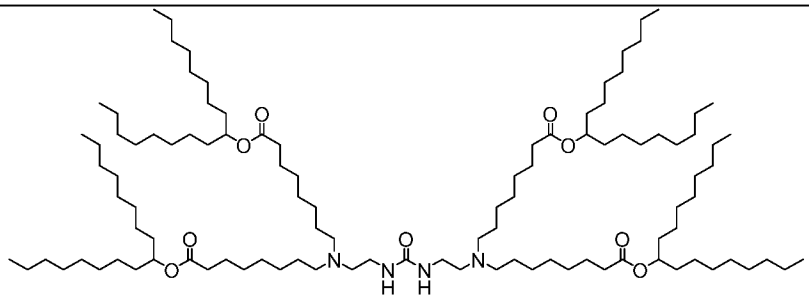
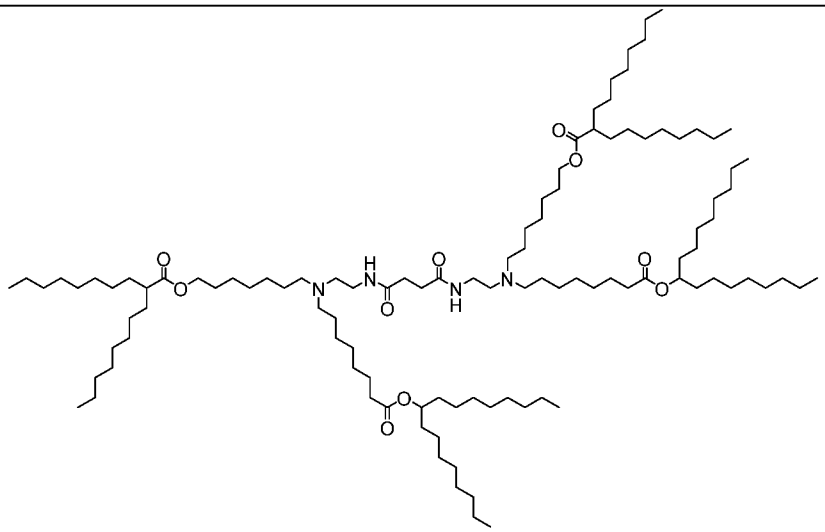
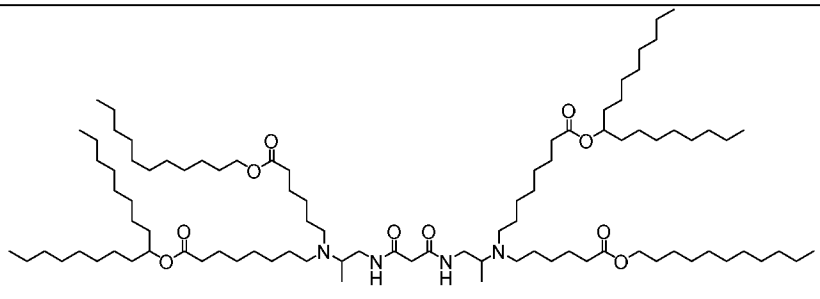
Lipid No.	Structure	IUPAC name
2213		heptadecan-9-yl 8-[(2-{3-[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][8-(nonyloxy)-8-oxooctyl]amino}ethyl)carbamoyl]propanamido}ethyl)[8-(nonyloxy)-8-oxooctyl]amino]octanoate
2212		heptadecan-9-yl 8-[(2-{2-[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][8-(nonyloxy)-8-oxooctyl]amino}ethyl)carbamoyl]acetamido}ethyl)[8-(nonyloxy)-8-oxooctyl]amino]octanoate
2211		heptadecan-9-yl 8-[(2-{3-[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][8-(nonyloxy)-8-oxooctyl]amino}ethyl)carbamoyl]-2,3-dihydroxypropanamido}ethyl)[8-(nonyloxy)-8-oxooctyl]amino]octanoate
2210		heptadecan-9-yl 8-[(2-{[(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][8-(nonyloxy)-8-oxooctyl]amino}ethoxy)carbonyl]oxy}ethyl)[8-(nonyloxy)-8-oxooctyl]amino]octanoate

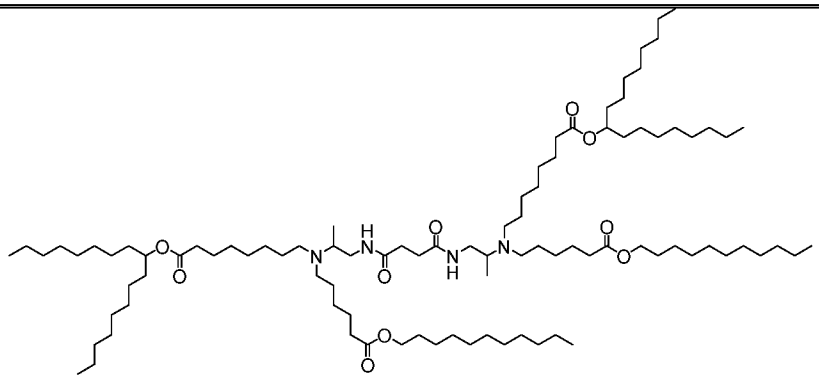
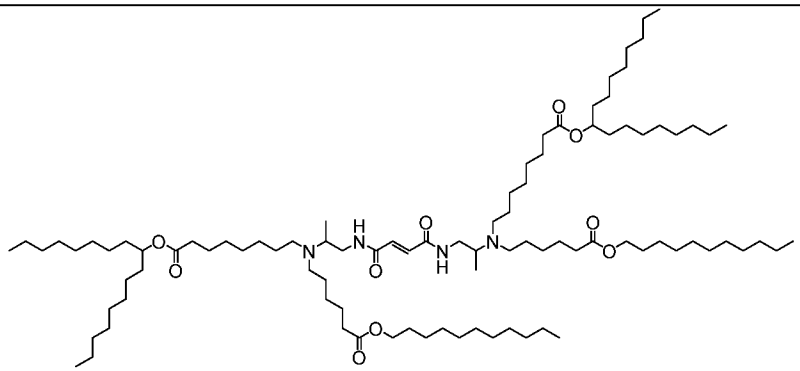
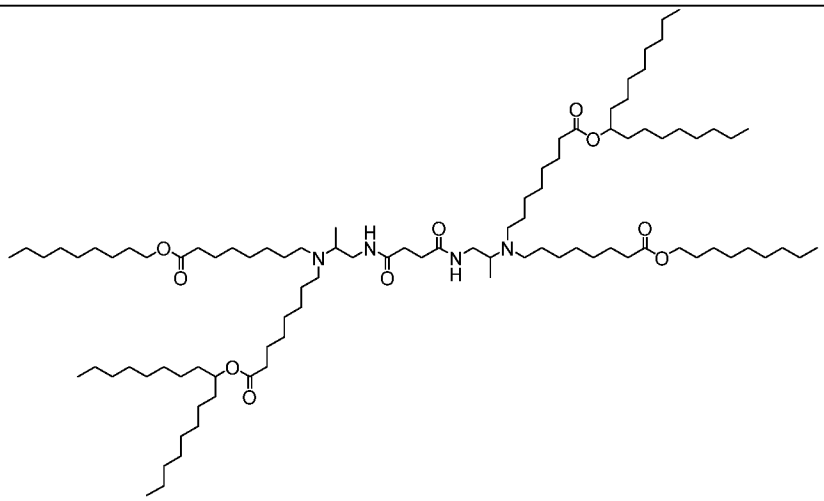
Lipid No.	Structure	IUPAC name
2209		heptadecan-9-yl 8-[(3-{[8-(heptadecan-9-yloxy)-8-oxooctyl][8-(nonyloxy)-8-oxooctyl]amino}-2-hydroxypropyl)[8-(nonyloxy)-8-oxooctyl]amino]octanoate
		
		
		

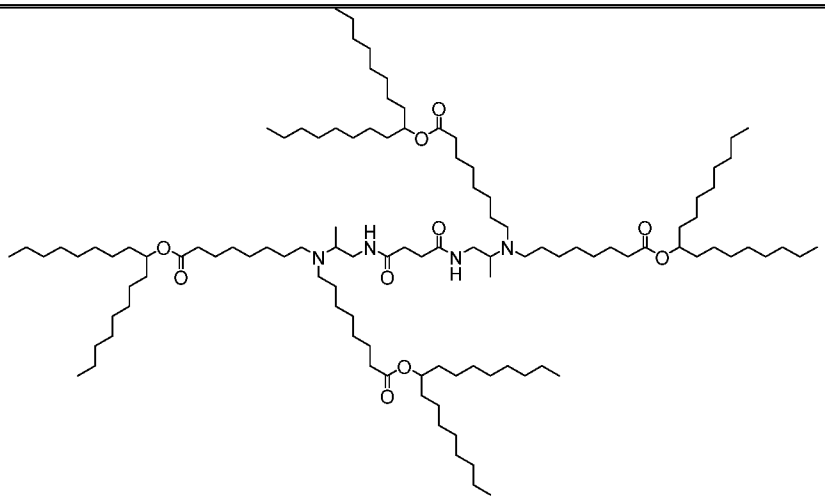
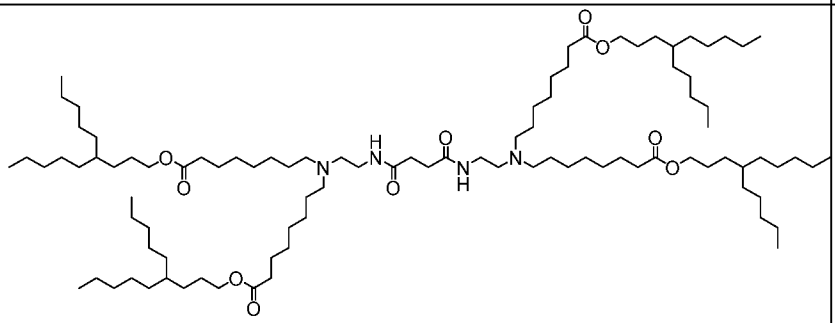
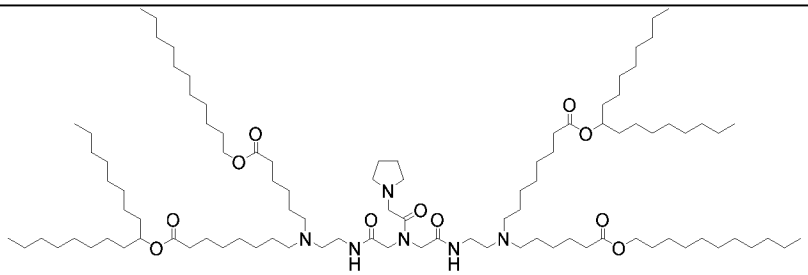
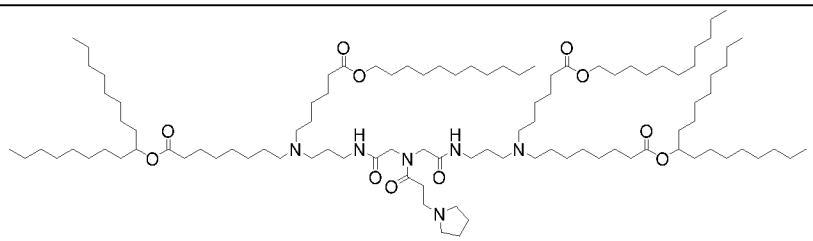
Lipid No.	Structure	IUPAC name
		
2248		heptadecan-9-yl 8-({2-[4-(2-{[8-(heptadecan-9-yloxy)-8-oxooctyl][6-oxo-6-(undecyloxy)hexyl]amino}ethyl)piperazin-1-yl]ethyl}[6-oxo-6-(undecyloxy)hexyl]amino)octanoate
2190		5-hexylundecyl 8-({4-[(2S,5S)-5-{4-[bis({8-[(5-hexylundecyl)oxy]-2-hydroxy-8-oxooctyl})amino]butyl}-3,6-dioxopiperazin-2-yl]butyl}({8-[(5-hexylundecyl)oxy]-2-hydroxy-8-oxooctyl})amino)-7-hydroxyoctanoate
2189		heptadecan-9-yl 8-({4-[(2S,5S)-5-(4-{bis[8-(heptadecan-9-yloxy)-2-hydroxy-8-oxooctyl]amino}butyl)-3,6-dioxopiperazin-2-yl]butyl}[8-(heptadecan-9-yloxy)-2-hydroxy-8-oxooctyl]amino)-7-hydroxyoctanoate

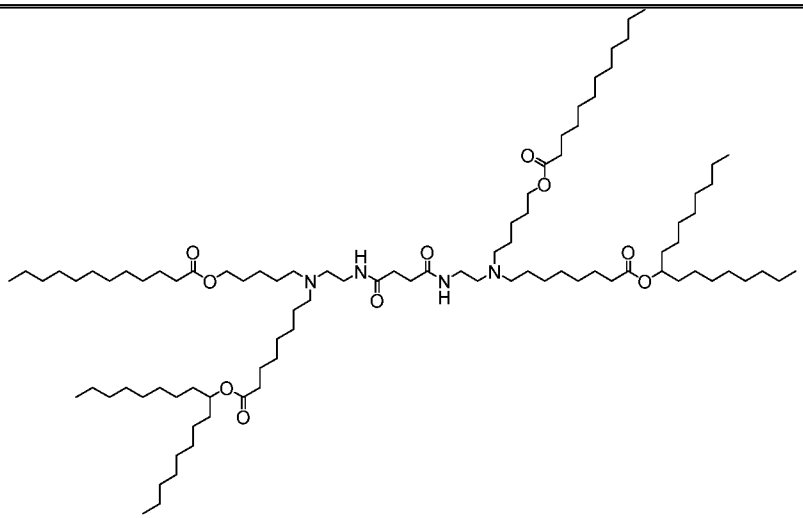
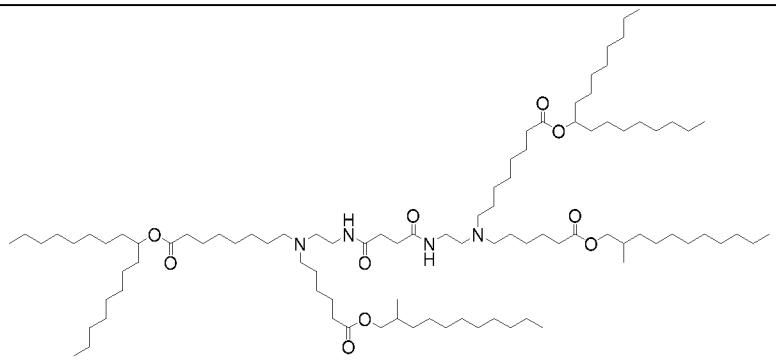
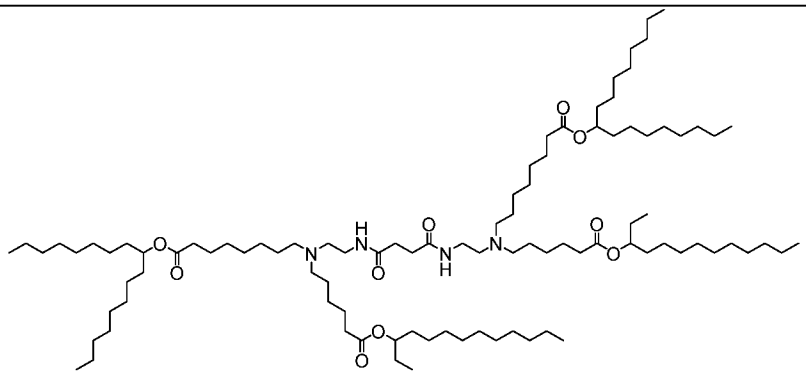
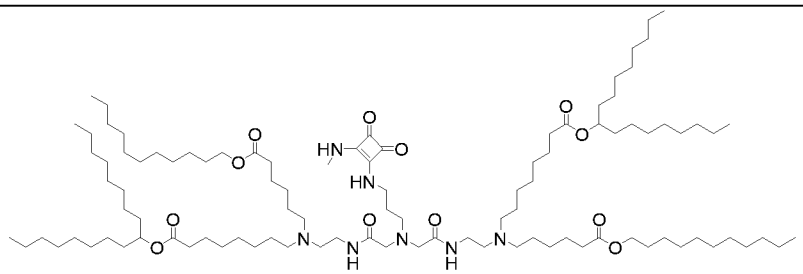
Lipid No.	Structure	IUPAC name
2287		di(heptadecan-9-yl) 14-(2-(dimethylamino)ethyl)-13,16-dioxo-9,20-bis(6-oxo-6-(undecyloxy)hexyl)-9,12,17,20-tetraazaoctacosanedioate
2315		di(heptadecan-9-yl) 8,8'-(((2-(hydroxymethyl)piperazine-1,4-diyl)bis(ethane-2,1-diyl))bis((6-oxo-6-(undecyloxy)hexyl)azanediyl))(R)-dioctanoate
2316		heptadecan-9-yl (S)-8-((2-(4-(1-((8-(heptadecan-9-yloxy)-8-oxooctyl)(6-oxo-6-(undecyloxy)hexyl)amino)-3-hydroxypropan-2-yl)piperazin-1-yl)ethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate
2317		heptadecan-9-yl (S)-8-((2-(4-(2-((8-(heptadecan-9-yloxy)-8-oxooctyl)(6-oxo-6-(undecyloxy)hexyl)amino)-3-hydroxypropyl)piperazin-1-yl)ethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate

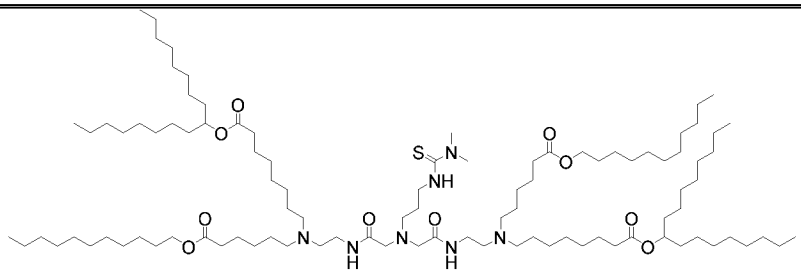
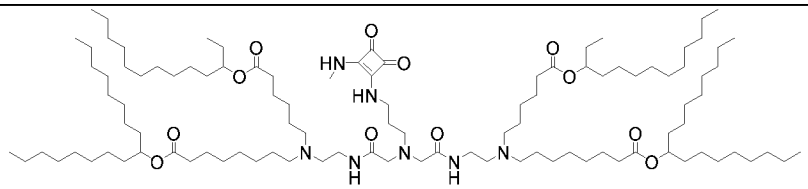
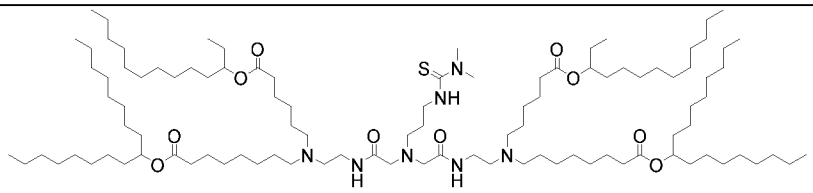
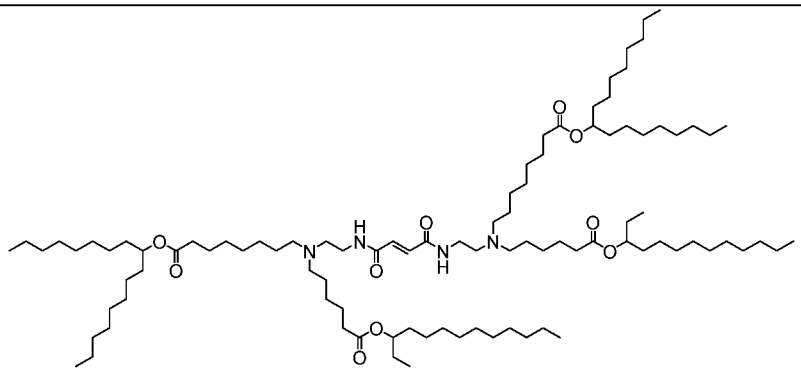
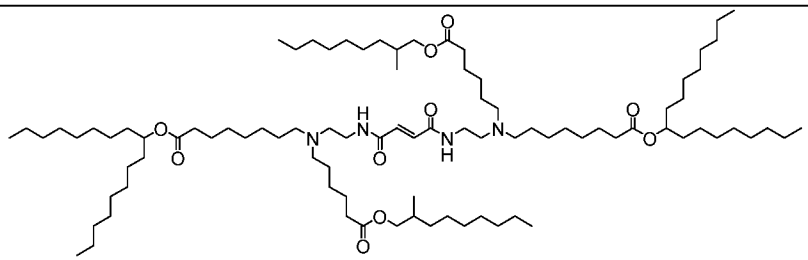
Lipid No.	Structure	IUPAC name
2318		di(heptadecan-9-yl) 13,15-dioxo-9,19-bis(5-(undecyldisulfaneyl)pentyl)-9,12,16,19-tetraazaheptacosanedioate
2439		
2319		di(heptadecan-9-yl) 9,19-bis(8-(heptadecan-9-yloxy)-8-oxooctyl)-13,15-dioxo-9,12,16,19-tetraazaheptacosanedioate
2320		di(heptadecan-9-yl) 9,21-bis(8-(heptadecan-9-yloxy)-8-oxooctyl)-13,17-dioxo-9,12,18,21-tetraazanonacosanedioate

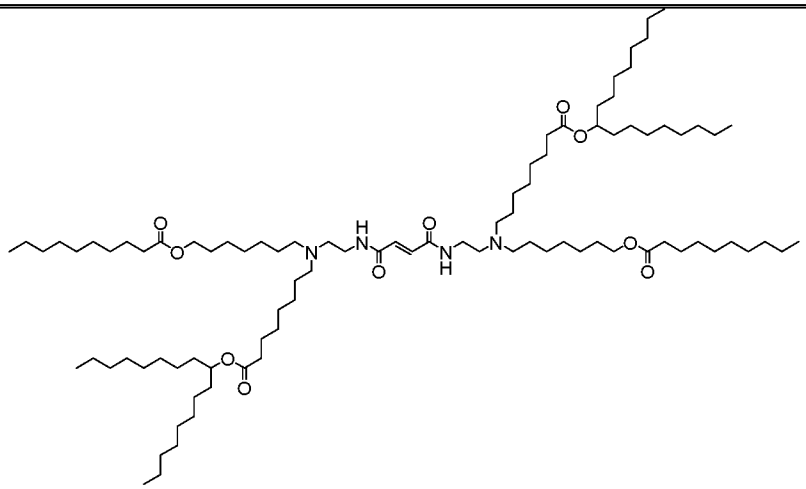
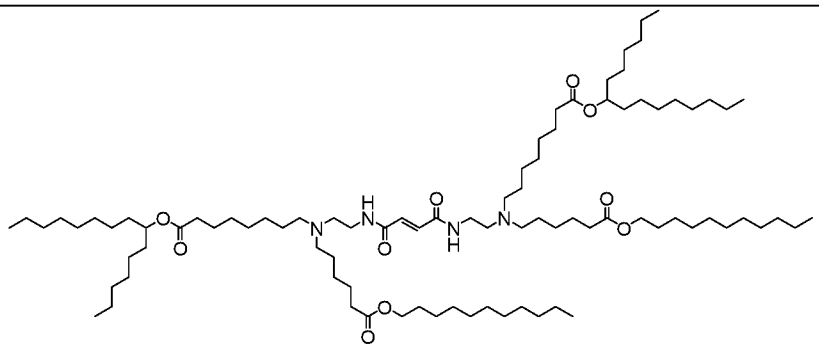
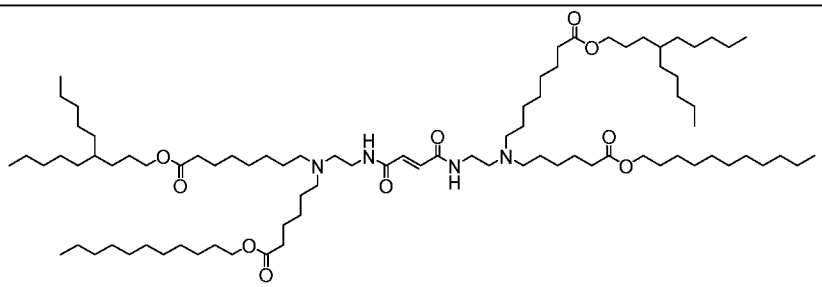
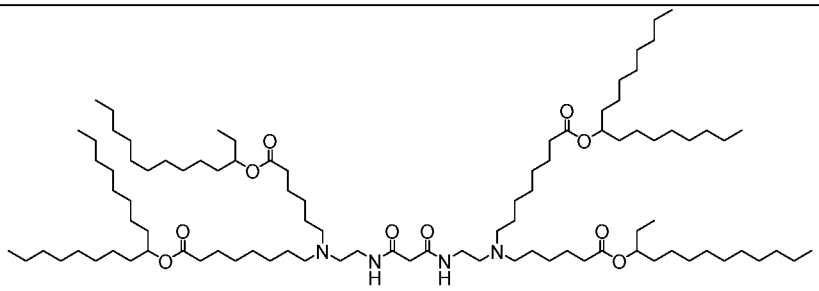
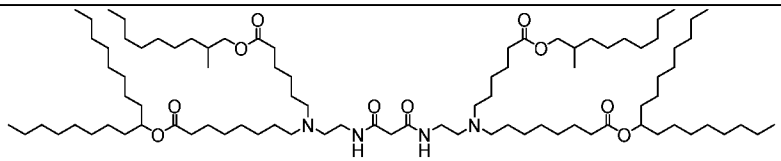
Lipid No.	Structure	IUPAC name
2321		di(heptadecan-9-yl) (E)-9,20-bis(8-(heptadecan-9-yloxy)-8-oxooctyl)-13,16-dioxo-9,12,17,20-tetraazaoctacos-14-enedioate
2322		di(heptadecan-9-yl) 9,17-bis(8-(heptadecan-9-yloxy)-8-oxooctyl)-13-oxo-9,12,14,17-tetraazapentacosanedioate
2323		di(heptadecan-9-yl) 9,20-bis(7-((2-octyldecanoyl)oxy)heptyl)-13,16-dioxo-9,12,17,20-tetraazaoctacosanedioate
2324		di(heptadecan-9-yl) 10,18-dimethyl-13,15-dioxo-9,19-bis(6-oxo-6-(undecyloxy)hexyl)-9,12,16,19-tetraazaheptacosanedioate

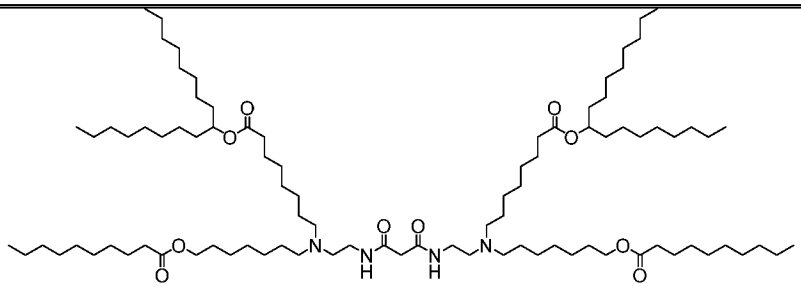
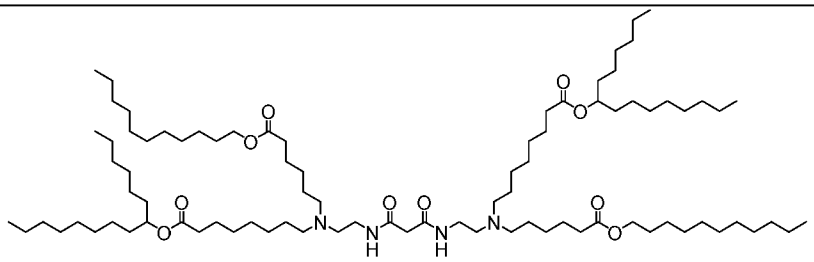
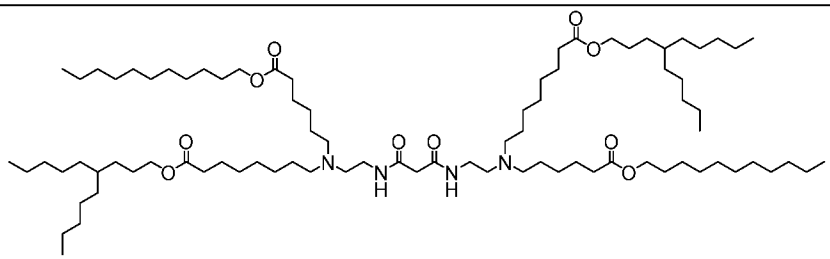
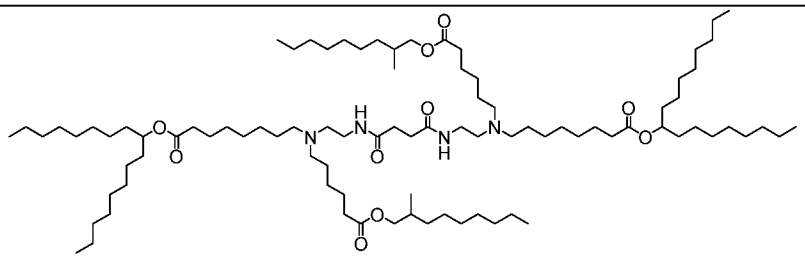
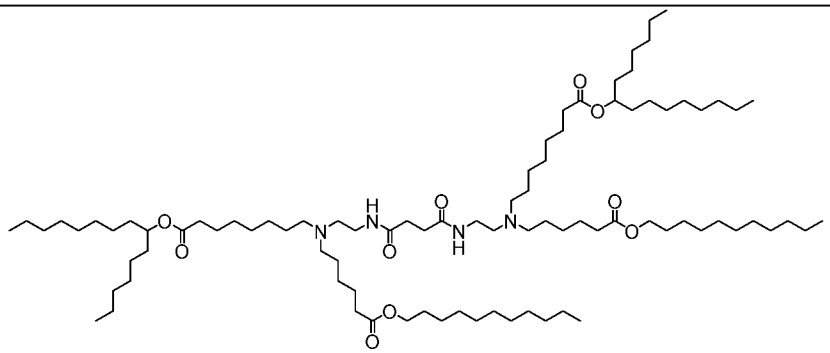
Lipid No.	Structure	IUPAC name
2325		di(heptadecan-9-yl) 10,19-dimethyl-13,16-dioxo-9,20-bis(6-oxo-6-(undecyloxy)hexyl)-9,12,17,20-tetraazaoctacosanedioate
2326		di(heptadecan-9-yl) (E)-10,19-dimethyl-13,16-dioxo-9,20-bis(6-oxo-6-(undecyloxy)hexyl)-9,12,17,20-tetraazaoctacos-14-enedioate
2327		di(heptadecan-9-yl) 10,19-dimethyl-9,20-bis(8-(nonyloxy)-8-oxooctyl)-13,16-dioxo-9,12,17,20-tetraazaoctacosanedioate

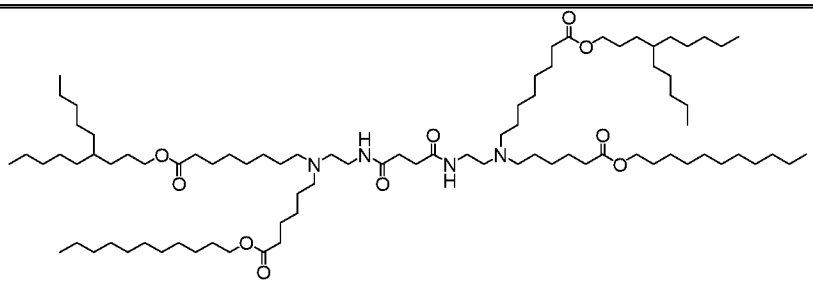
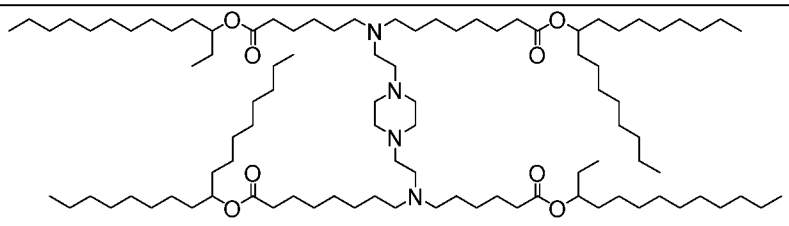
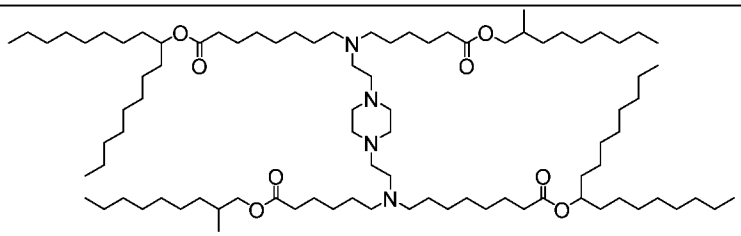
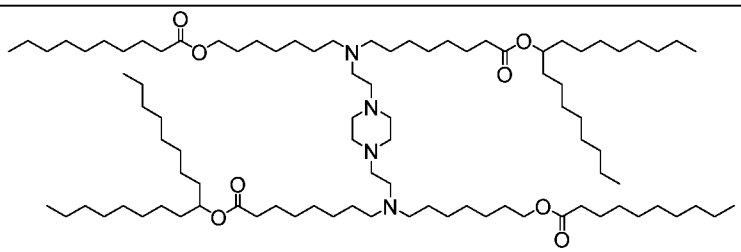
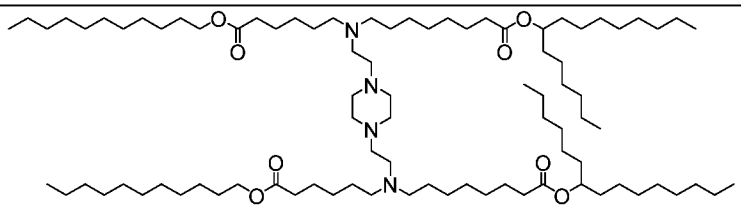
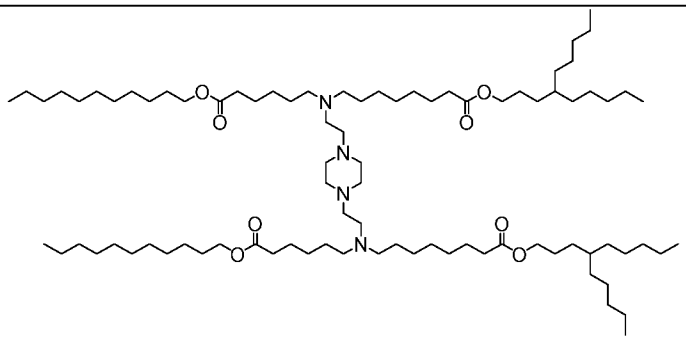
Lipid No.	Structure	IUPAC name
2328		di(heptadecan-9-yl) 9,20-bis(8-(heptadecan-9-yloxy)-8-oxooctyl)-10,19-dimethyl-13,16-dioxo-9,12,17,20-tetraazaoctacosanedioate
2332		bis(4-pentylnonyl) 13,16-dioxo-9,20-bis(8-oxo-8-((4-pentylnonyl)oxy)octyl)-9,12,17,20-tetraazaoctacosanedioate
2334		di(heptadecan-9-yl) 13,17-dioxo-9,21-bis(6-oxo-6-(undecyloxy)hexyl)-15-(2-(pyrrolidin-1-yl)acetyl)-9,12,15,18,21-pentaazanonacosanedioate
2353		di(heptadecan-9-yl) 14,18-dioxo-9,23-bis(6-oxo-6-(undecyloxy)hexyl)-16-(3-(pyrrolidin-1-yl)propanoyl)-9,13,16,19,23-pentaazahentriacontanedioate

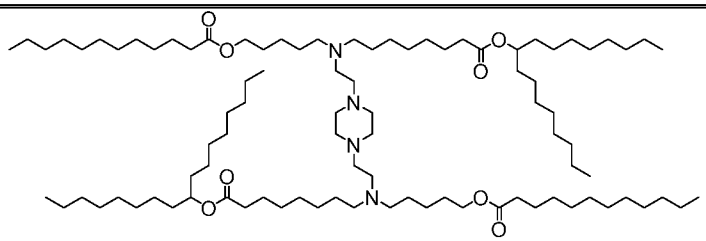
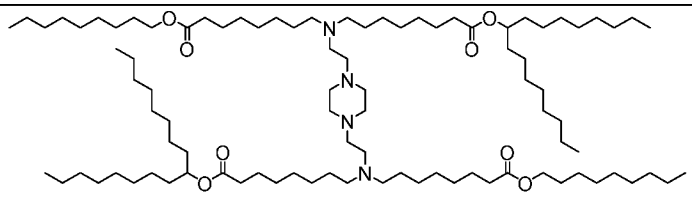
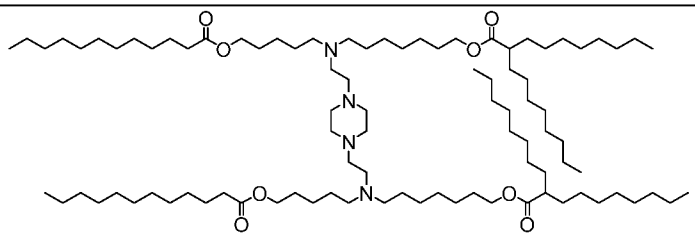
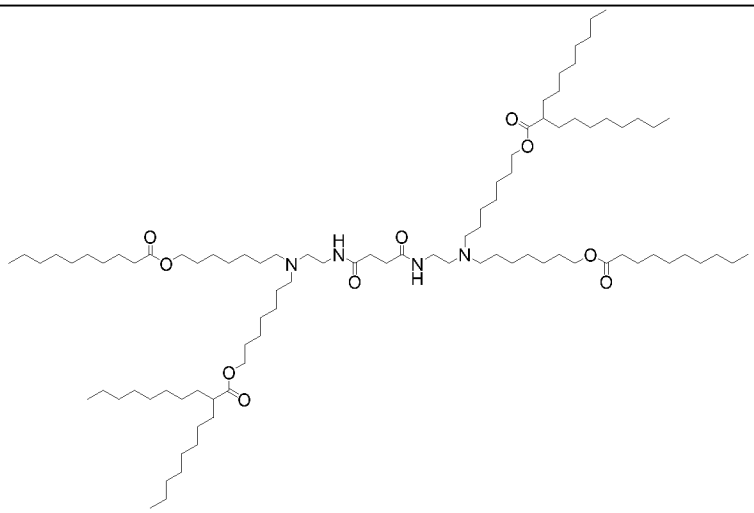
Lipid No.	Structure	IUPAC name
2354		di(heptadecan-9-yl) 9,20-bis(5-(dodecanoyloxy)pentyl)-13,16-dioxo-9,12,17,20-tetraazaoctacosanedioate
2355		di(heptadecan-9-yl) 9,20-bis(6-((2-methylundecyl)oxy)-6-oxohexyl)-13,16-dioxo-9,12,17,20-tetraazaoctacosanedioate
2356		di(heptadecan-9-yl) 13,16-dioxo-9,20-bis(6-oxo-6-(tridecan-3-yloxy)hexyl)-9,12,17,20-tetraazaoctacosanedioate
2357		di(heptadecan-9-yl) 15-(3-((2-(methylamino)-3,4-dioxocyclobut-1-en-1-yl)amino)propyl)-13,17-dioxo-9,21-bis(6-oxo-6-(undecyloxy)hexyl)-9,12,15,18,21-pentaazanonacosanedioate

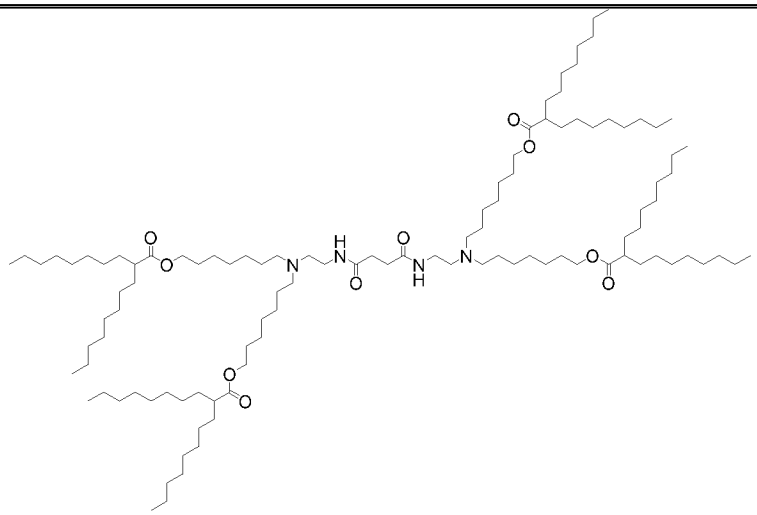
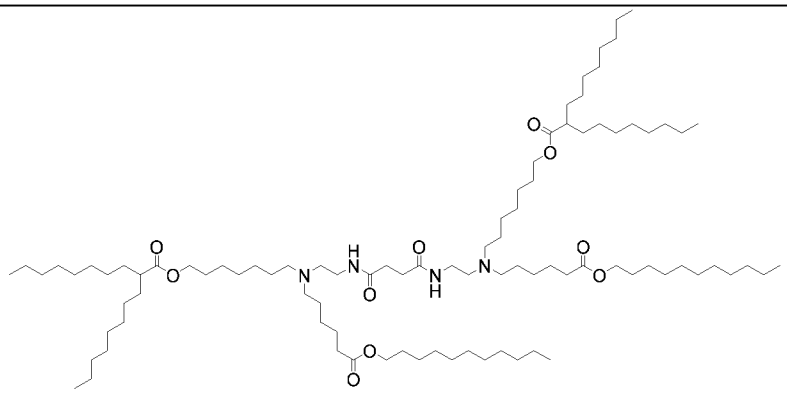
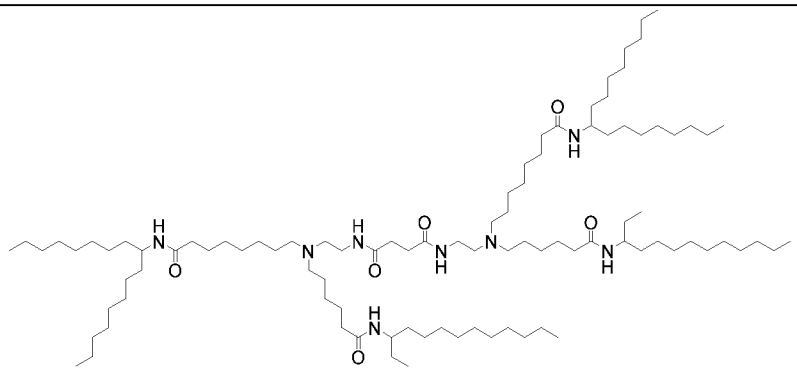
Lipid No.	Structure	IUPAC name
2358		di(heptadecan-9-yl) 15-(3-(3,3-dimethylthioureido)propyl)-13,17-dioxo-9,21-bis(6-oxo-6-(undecyloxy)hexyl)-9,12,15,18,21-pentaazanonacosanedioate
2361		di(heptadecan-9-yl) 15-(3-((2-(methylamino)-3,4-dioxocyclobut-1-en-1-yl)amino)propyl)-13,17-dioxo-9,21-bis(6-oxo-6-(tridecan-3-yloxy)hexyl)-9,12,15,18,21-pentaazanonacosanedioate
2362		di(heptadecan-9-yl) 15-(3-(3,3-dimethylthioureido)propyl)-13,17-dioxo-9,21-bis(6-oxo-6-(tridecan-3-yloxy)hexyl)-9,12,15,18,21-pentaazanonacosanedioate
2378		di(heptadecan-9-yl) (E)-13,16-dioxo-9,20-bis(6-oxo-6-(tridecan-3-yloxy)hexyl)-9,12,17,20-tetraazaoctacos-14-enedioate
2379		di(heptadecan-9-yl) (E)-9,20-bis(6-((2-methylnonyl)oxy)-6-oxohexyl)-13,16-dioxo-9,12,17,20-tetraazaoctacos-14-enedioate

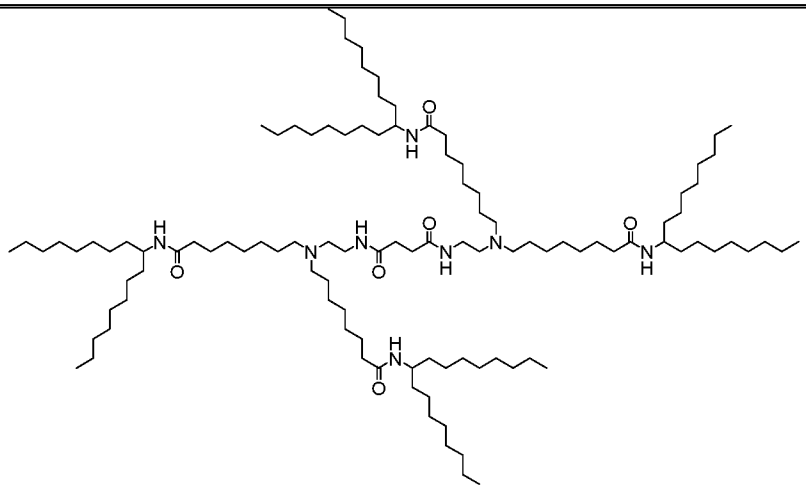
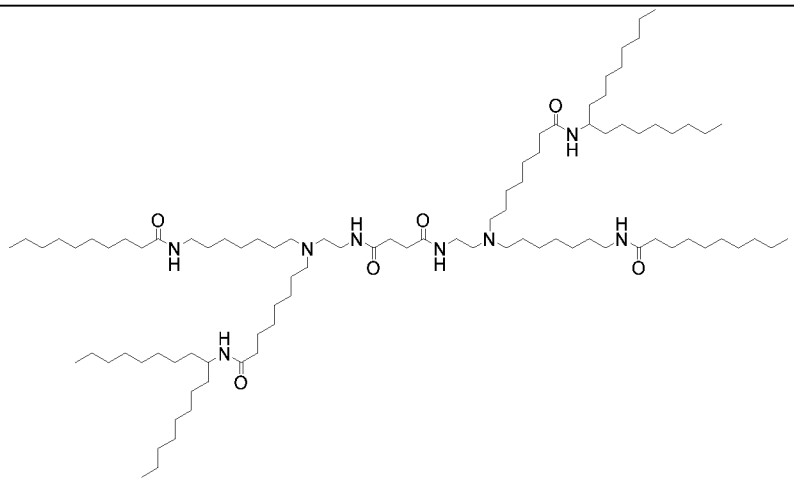
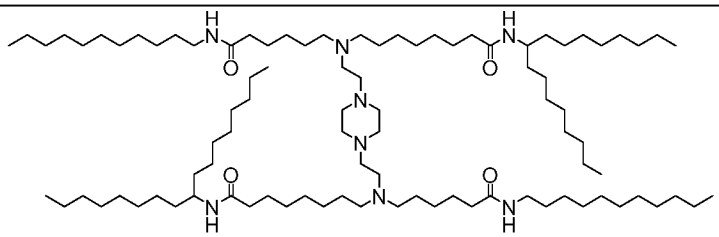
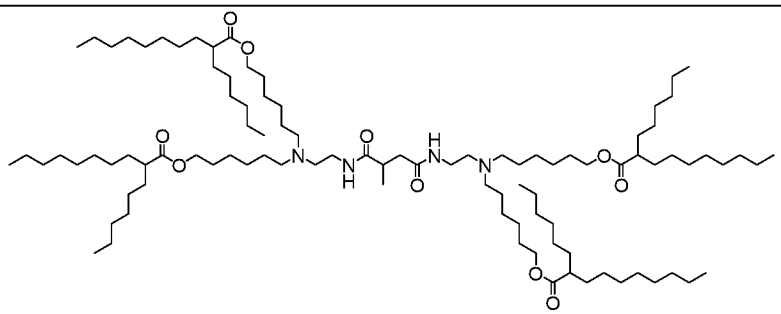
Lipid No.	Structure	IUPAC name
2380		di(heptadecan-9-yl) (E)-9,20-bis(7-(decanoyloxy)heptyl)-13,16-dioxo-9,12,17,20-tetraazaoctacos-14-enedioate
2381		di(pentadecan-7-yl) (E)-13,16-dioxo-9,20-bis(6-oxo-6-(undecyloxy)hexyl)-9,12,17,20-tetraazaoctacos-14-enedioate
2382		bis(4-pentylnonyl) (E)-13,16-dioxo-9,20-bis(6-oxo-6-(undecyloxy)hexyl)-9,12,17,20-tetraazaoctacos-14-enedioate
2384		di(heptadecan-9-yl) 13,15-dioxo-9,19-bis(6-oxo-6-(tridecan-3-yloxy)hexyl)-9,12,16,19-tetraazaheptacosanedioate
2385		di(heptadecan-9-yl) 9,19-bis(6-((2-methylnonyl)oxy)-6-oxohexyl)-13,15-dioxo-9,12,16,19-tetraazaheptacosanedioate

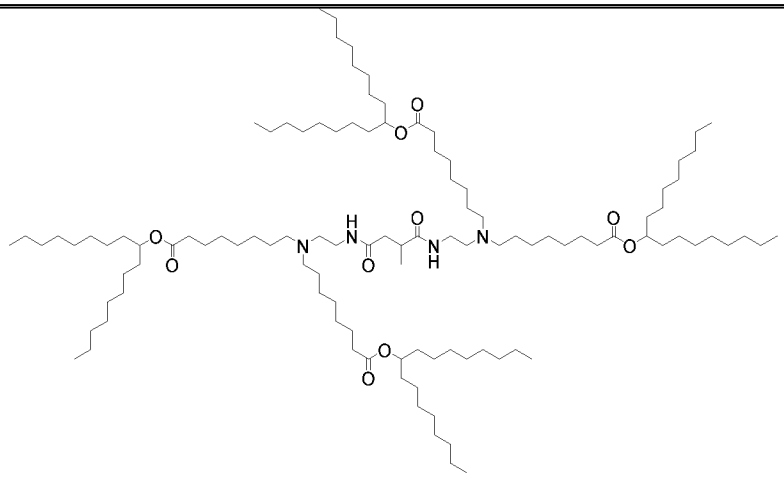
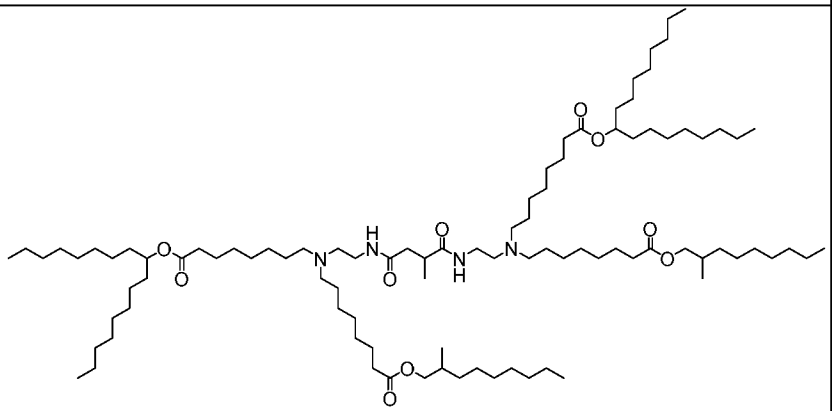
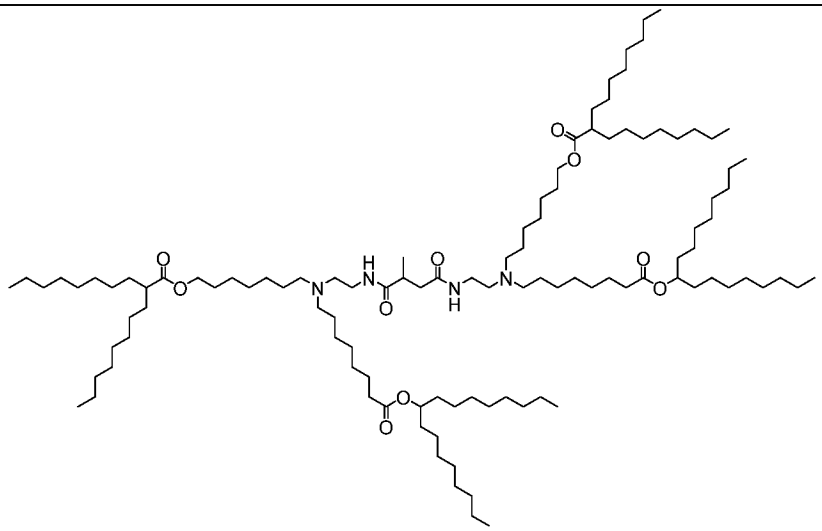
Lipid No.	Structure	IUPAC name
2386		di(heptadecan-9-yl) 9,19-bis(7-(decanoyloxy)heptyl)-13,15-dioxo-9,12,16,19-tetraazaheptacosanedioate
2387		di(pentadecan-7-yl) 13,15-dioxo-9,19-bis(6-oxo-6-(undecyloxy)hexyl)-9,12,16,19-tetraazaheptacosanedioate
2388		bis(4-pentylnonyl) 13,15-dioxo-9,19-bis(6-oxo-6-(undecyloxy)hexyl)-9,12,16,19-tetraazaheptacosanedioate
2389		di(heptadecan-9-yl) 9,20-bis(6-((2-methylnonyl)oxy)-6-oxohexyl)-13,16-dioxo-9,12,17,20-tetraazaoctacosanedioate
2390		di(pentadecan-7-yl) 13,16-dioxo-9,20-bis(6-oxo-6-(undecyloxy)hexyl)-9,12,17,20-tetraazaoctacosanedioate

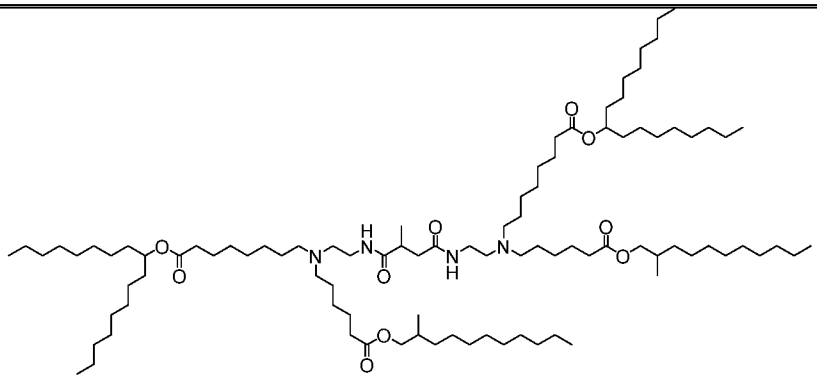
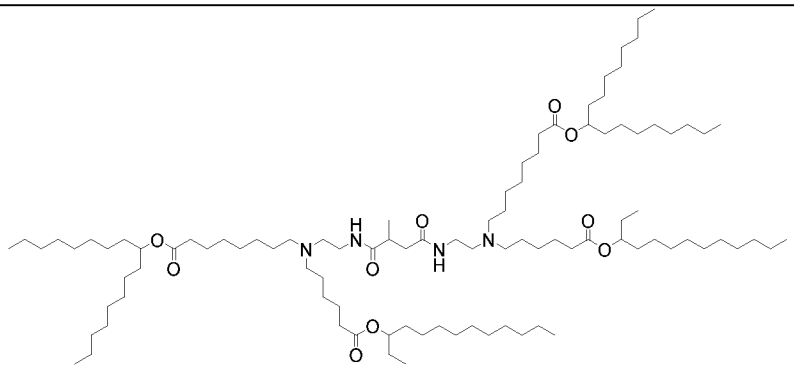
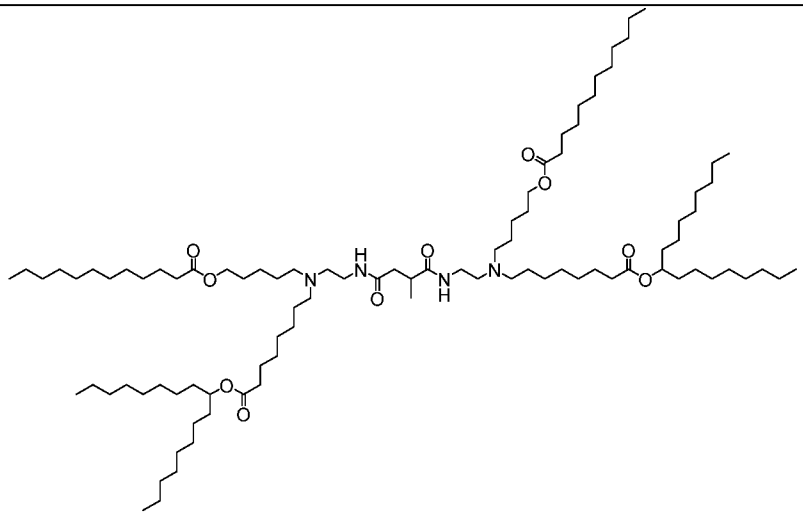
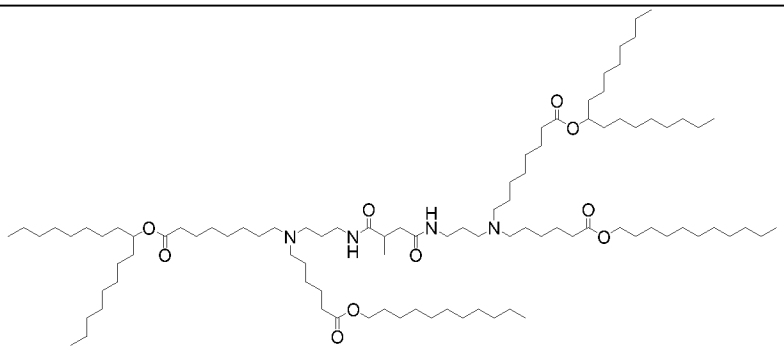
Lipid No.	Structure	IUPAC name
2391		bis(4-pentylnonyl) 13,16-dioxo-9,20-bis(6-oxo-6-(undecyloxy)hexyl)-9,12,17,20-tetraazaoctacosanedioate
2393		di(heptadecan-9-yl) 8,8'-((piperazine-1,4-diylbis(ethane-2,1-diyl))bis((6-oxo-6-(tridecan-3-yloxy)hexyl)azanediyl))dioctanoate
2394		di(heptadecan-9-yl) 8,8'-((piperazine-1,4-diylbis(ethane-2,1-diyl))bis((6-((2-methylnonyl)oxy)-6-oxohexyl)azanediyl))dioctanoate
2395		((piperazine-1,4-diylbis(ethane-2,1-diyl))bis((8-(heptadecan-9-yloxy)-8-oxooctyl)azanediyl))bis(heptane-7,1-diyl) bis(decanoate)
2396		di(pentadecan-7-yl) 8,8'-((piperazine-1,4-diylbis(ethane-2,1-diyl))bis((6-oxo-6-(undecyloxy)hexyl)azanediyl))dioctanoate
2397		bis(4-pentylnonyl) 8,8'-((piperazine-1,4-diylbis(ethane-2,1-diyl))bis((6-oxo-6-(undecyloxy)hexyl)azanediyl))dioctanoate

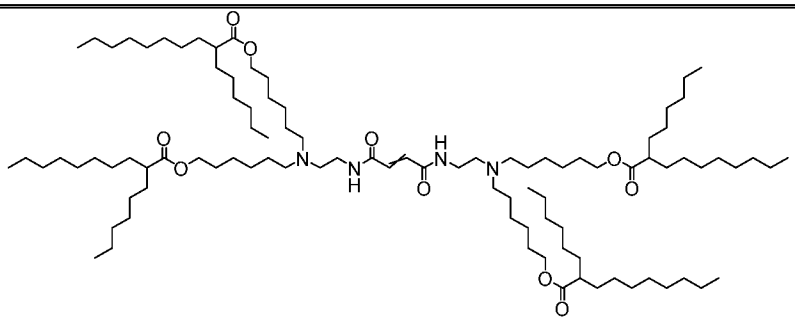
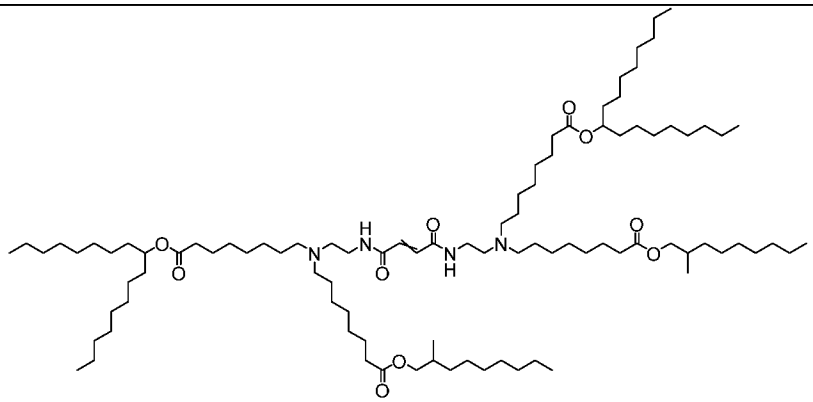
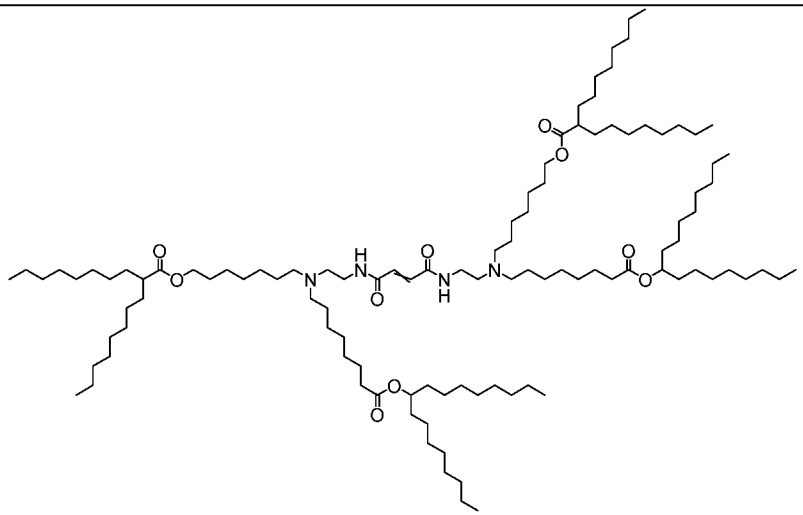
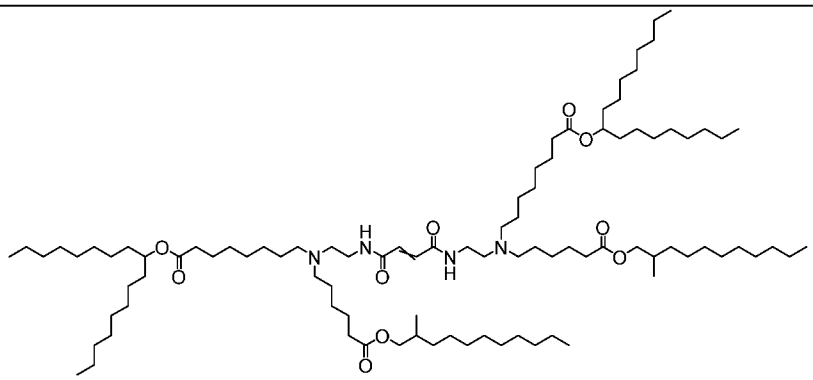
Lipid No.	Structure	IUPAC name
2399		((piperazine-1,4-diylbis(ethane-2,1-diyl))bis((8-(heptadecan-9-yloxy)-8-oxooctyl)azanediyl))bis(pentane-5,1-diyl) didodecanoate
2400		di(heptadecan-9-yl) 8,8'-((piperazine-1,4-diylbis(ethane-2,1-diyl))bis((8-(nonyloxy)-8-oxooctyl)azanediyl))dioctanoate
2401		((piperazine-1,4-diylbis(ethane-2,1-diyl))bis((7-((2-octyldecanoyl)oxy)heptyl)azanediyl))bis(pentane-5,1-diyl) didodecanoate
2403		8,19-bis(7-(decanoyloxy)heptyl)-12,15-dioxo-8,11,16,19-tetraazahexacosane-1,26-diyl bis(2-octyldecanoate)

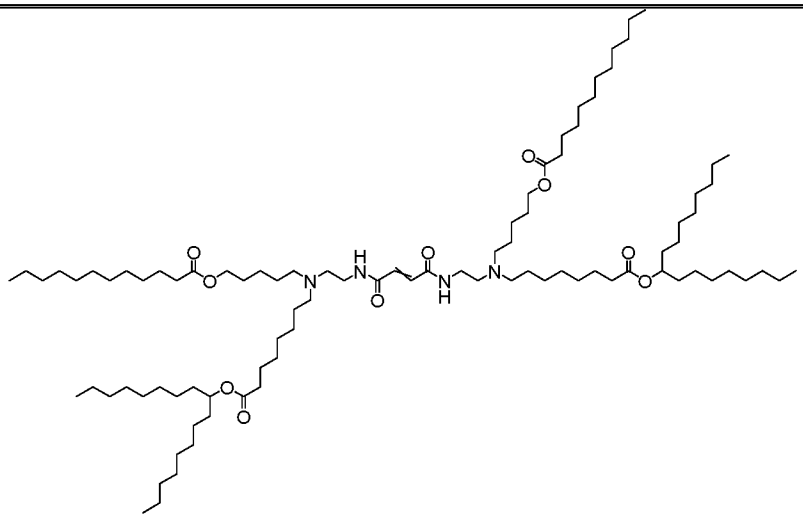
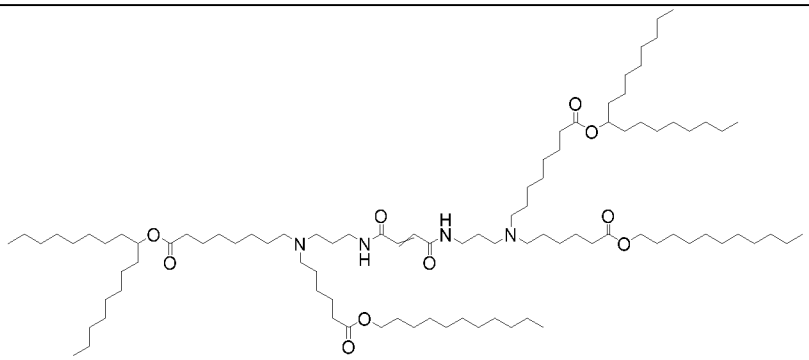
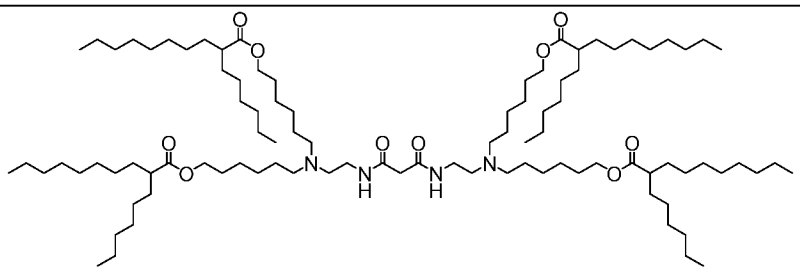
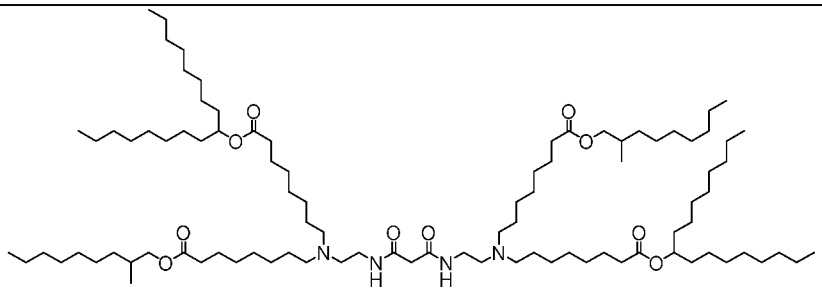
Lipid No.	Structure	IUPAC name
2404		7-[2-[[4-[2-[bis[7-(2-octyldecanoyloxy)heptyl]amino]ethylamino]-4-oxo-butanoyl]amino]ethyl-[7-(2-octyldecanoyloxy)heptyl]amino]heptyl 2-octyldecanoate
2405		diundecyl 7,18-bis(7-((2-octyldecanoyl)oxy)heptyl)-11,14-dioxo-7,10,15,18-tetraazatetracosanedioate
2421		N1,N4-bis(2-((8-(heptadecan-9-ylamino)-8-oxooctyl)(6-oxo-6-(tridecan-3-ylamino)hexyl)amino)ethyl)succinamide

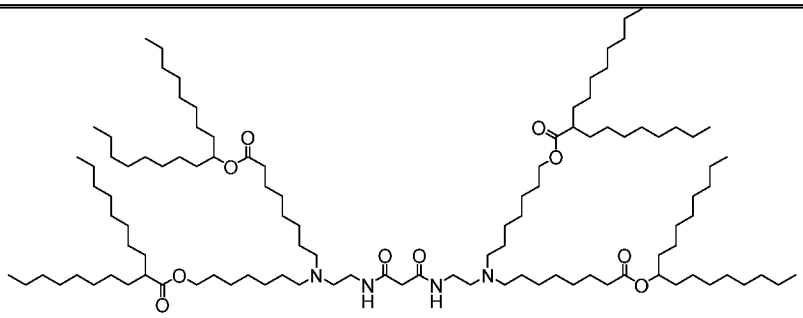
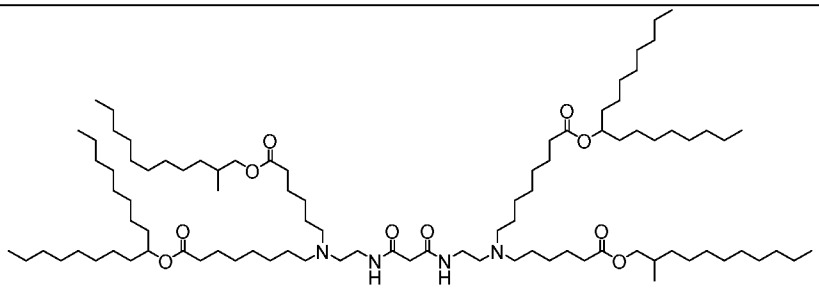
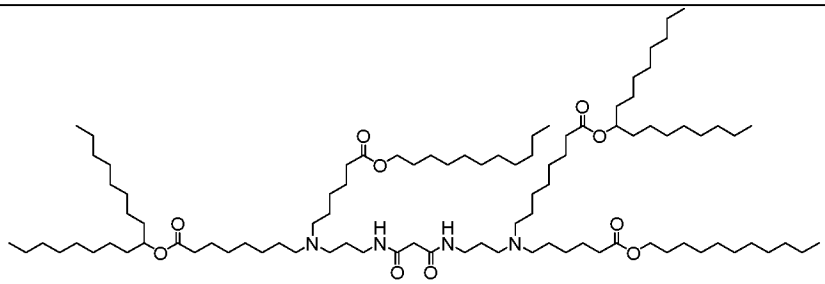
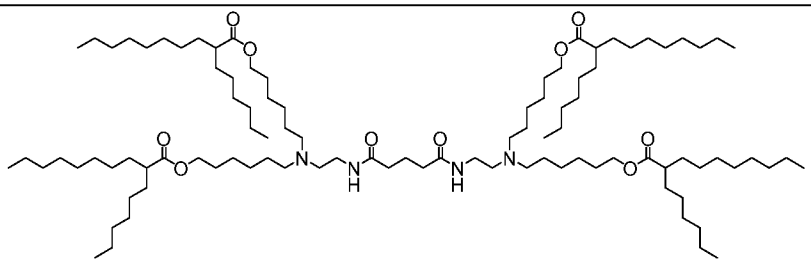
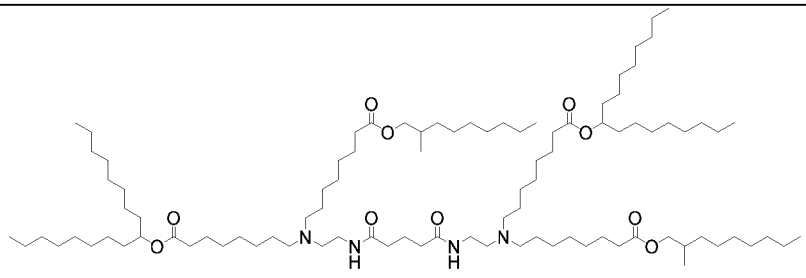
Lipid No.	Structure	IUPAC name
2426		N1,N4-bis(2-(bis(8-(heptadecan-9-ylamino)-8-oxooctyl)amino)ethyl)succinamide
2427		N1,N4-bis(2-((7-decanamidoheptyl)(8-(heptadecan-9-ylamino)-8-oxooctyl)amino)ethyl)succinamide
2428		8,8'-((piperazine-1,4-diylbis(ethane-2,1-diyl))bis((6-oxo-6-(undecylamino)hexyl)azanediyl))bis(N-(heptadecan-9-yl)octanamide)
		6-[2-[[4-[2-[bis[6-(2-hexyldecanoyloxy)hexyl]amino]ethylamino]-3-methyl-4-oxo-butanoyl]amino]ethyl-[6-(2-hexyldecanoyloxy)hexyl]amino]hexyl 2-hexyldecanoate

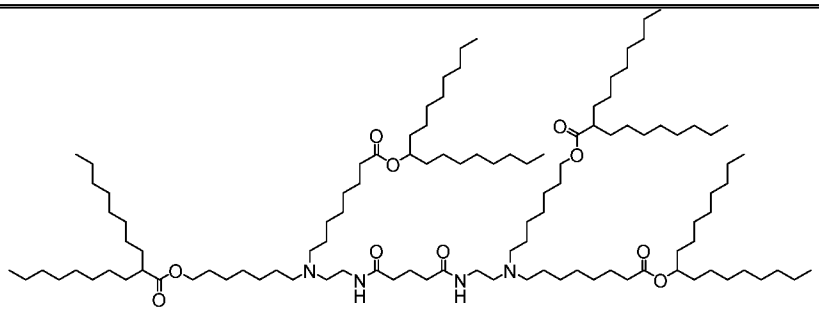
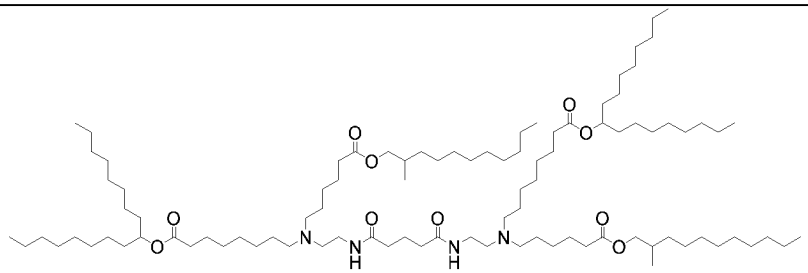
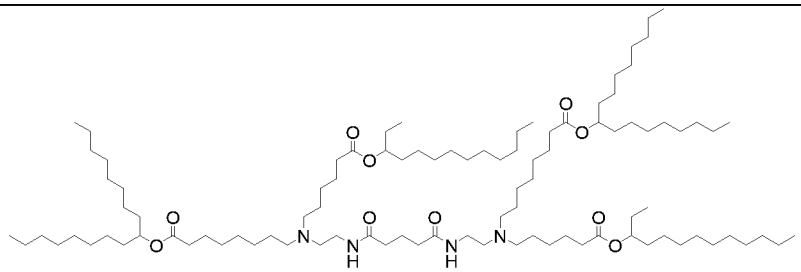
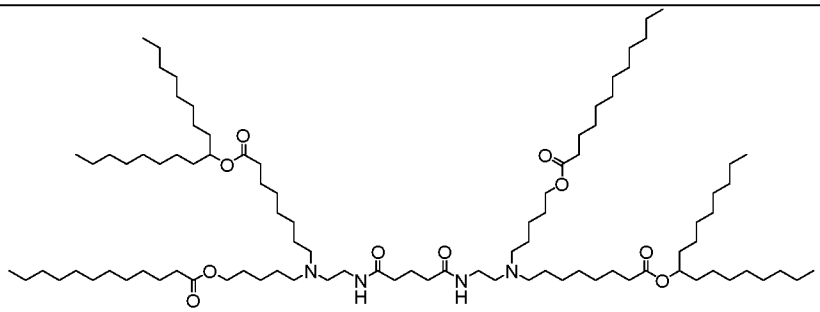
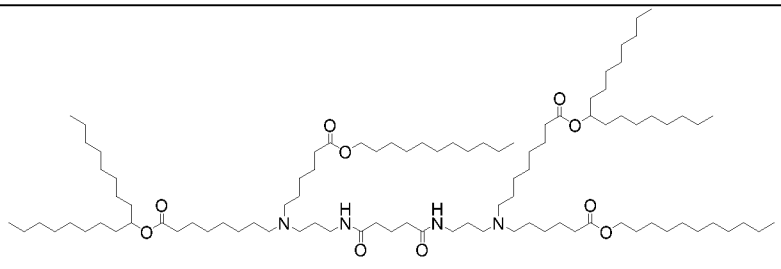
Lipid No.	Structure	IUPAC name
		di(heptadecan-9-yl) 9,20-bis(8-(heptadecan-9-yloxy)-8-oxooctyl)-14-methyl-13,16-dioxo-9,12,17,20-tetraazaoctacosanedioate
		di(heptadecan-9-yl) 14-methyl-9,20-bis(8-((2-methylnonyl)oxy)-8-oxooctyl)-13,16-dioxo-9,12,17,20-tetraazaoctacosanedioate
		di(heptadecan-9-yl) 14-methyl-9,20-bis(7-((2-octyldecanoyl)oxy)heptyl)-13,16-dioxo-9,12,17,20-tetraazaoctacosanedioate

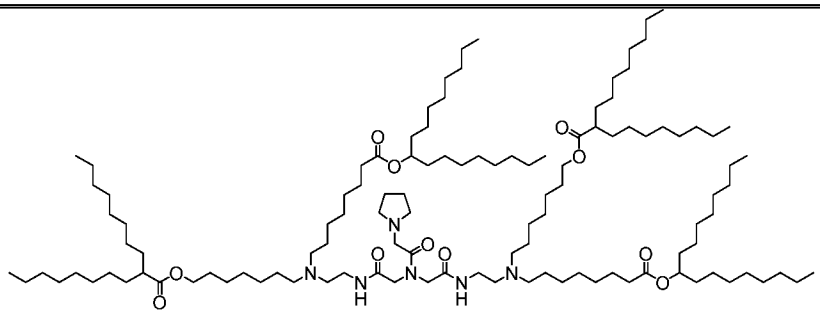
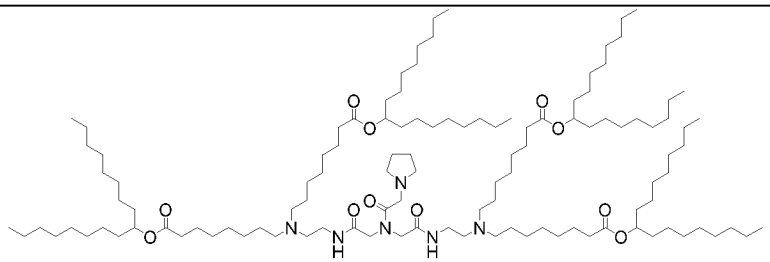
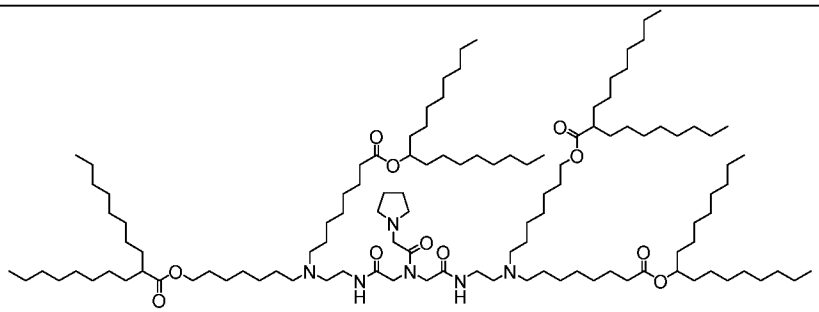
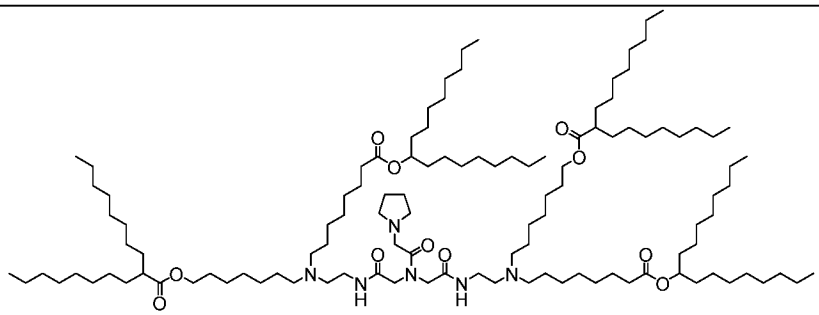
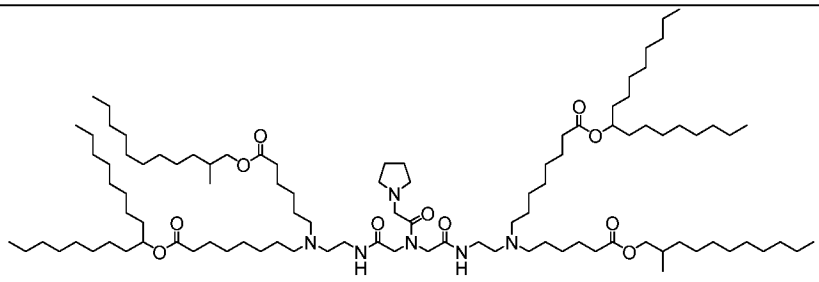
Lipid No.	Structure	IUPAC name
		di(heptadecan-9-yl) 14-methyl-9,20-bis(6-((2-methylundecyl)oxy)-6-oxohexyl)-13,16-dioxo-9,12,17,20-tetraazaoctacosanedioate
		di(heptadecan-9-yl) 14-methyl-13,16-dioxo-9,20-bis(6-oxo-6-(tridecan-3-yloxy)hexyl)-9,12,17,20-tetraazaoctacosanedioate
		di(heptadecan-9-yl) 9,20-bis(5-(dodecanoyloxy)pentyl)-14-methyl-13,16-dioxo-9,12,17,20-tetraazaoctacosanedioate
		di(heptadecan-9-yl) 15-methyl-14,17-dioxo-9,22-bis(6-oxo-6-(undecyloxy)hexyl)-9,13,18,22-tetraazatriacontanedioate

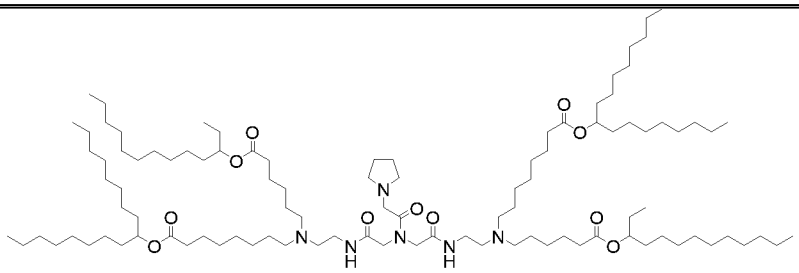
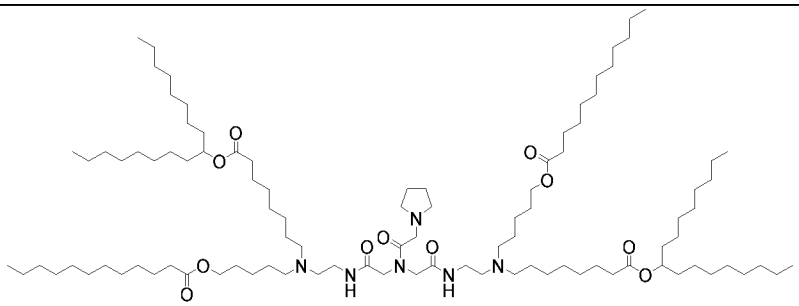
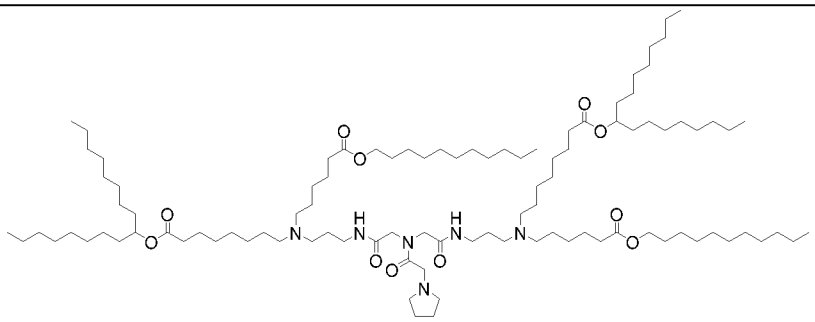
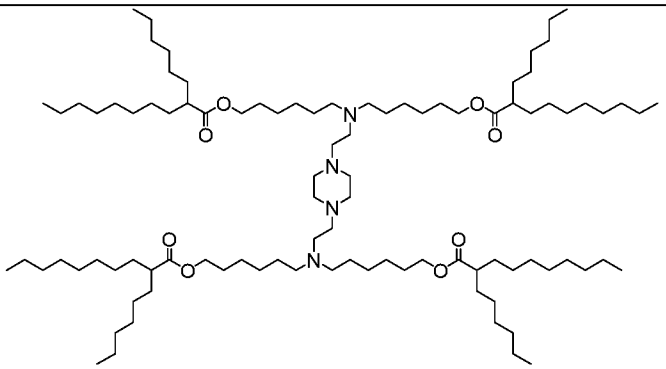
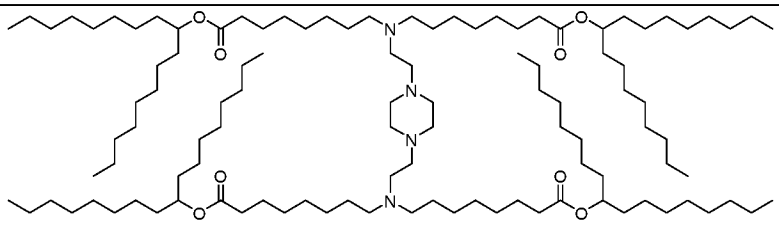
Lipid No.	Structure	IUPAC name
		6-[2-[[4-[2-[bis[6-(2-hexyldecanoyloxy)hexyl]amino]ethylamino]-4-oxo-but-2-enoyl]amino]ethyl-[6-(2-hexyldecanoyloxy)hexyl]amino]hexyl 2-hexyldecanoate
		di(heptadecan-9-yl) 9,20-bis(8-((2-methylnonyl)oxy)-8-oxooctyl)-13,16-dioxo-9,12,17,20-tetraazaoctacos-14-enedioate
		di(heptadecan-9-yl) 9,20-bis(7-((2-octyldecanoyl)oxy)heptyl)-13,16-dioxo-9,12,17,20-tetraazaoctacos-14-enedioate
		di(heptadecan-9-yl) 9,20-bis(6-((2-methylundecyl)oxy)-6-oxohexyl)-13,16-dioxo-9,12,17,20-tetraazaoctacos-14-enedioate

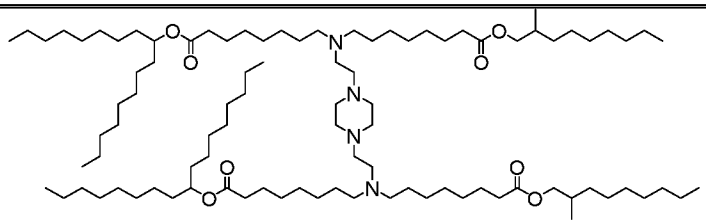
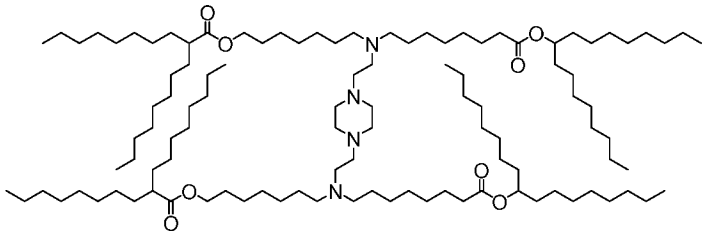
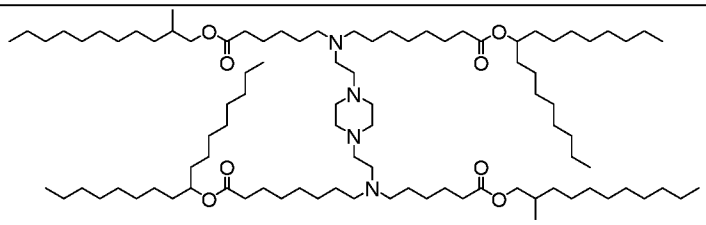
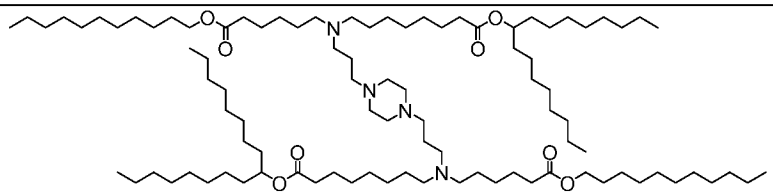
Lipid No.	Structure	IUPAC name
		di(heptadecan-9-yl) 9,20-bis(5-(dodecanoyloxy)pentyl)-13,16-dioxo-9,12,17,20-tetraazaoctacos-14-enedioate
		di(heptadecan-9-yl) 14,17-dioxo-9,22-bis(6-oxo-6-(undecyloxy)hexyl)-9,13,18,22-tetraazatriacont-15-enedioate
		6-[2-[[4-[2-[bis[6-(2-hexyldecanoyloxy)hexyl]amino]ethylamino]-4-oxo-but-2-enoyl]amino]ethyl-[6-(2-hexyldecanoyloxy)hexyl]amino]hexyl 2-hexyldecanoate
		di(heptadecan-9-yl) 9,19-bis(8-((2-methylnonyl)oxy)-8-oxooctyl)-13,15-dioxo-9,12,16,19-tetraazaheptacosanedioate

Lipid No.	Structure	IUPAC name
		di(heptadecan-9-yl) 9,19-bis(7-((2-octyldecanoyl)oxy)heptyl)-13,15-dioxo-9,12,16,19-tetraazaheptacosanedioate
		di(heptadecan-9-yl) 9,19-bis(6-((2-methylundecyl)oxy)-6-oxohexyl)-13,15-dioxo-9,12,16,19-tetraazaheptacosanedioate
		di(heptadecan-9-yl) 14,16-dioxo-9,21-bis(6-oxo-6-(undecyloxy)hexyl)-9,13,17,21-tetraazanonacosanedioate
		6-[2-[[5-[2-[bis[6-(2-hexyldecanoyloxy)hexyl]amino]ethylamino]-5-oxo-pentanoyl]amino]ethyl-[6-(2-hexyldecanoyloxy)hexyl]amino]hexyl 2-hexyldecanoate
		di(heptadecan-9-yl) 9,21-bis(8-((2-methylnonyl)oxy)-8-oxooctyl)-13,17-dioxo-9,12,18,21-tetraazanonacosanedioate

Lipid No.	Structure	IUPAC name
		di(heptadecan-9-yl) 9,21-bis(7-((2-octyldecanoyl)oxy)heptyl)-13,17-dioxo-9,12,18,21-tetraazanonacosanedioate
		di(heptadecan-9-yl) 9,21-bis(6-((2-methylundecyl)oxy)-6-oxohexyl)-13,17-dioxo-9,12,18,21-tetraazanonacosanedioate
		di(heptadecan-9-yl) 13,17-dioxo-9,21-bis(6-oxo-6-(tridecan-3-yloxy)hexyl)-9,12,18,21-tetraazanonacosanedioate
		di(heptadecan-9-yl) 9,21-bis(5-(dodecanoyloxy)pentyl)-13,17-dioxo-9,12,18,21-tetraazanonacosanedioate
		di(heptadecan-9-yl) 14,18-dioxo-9,23-bis(6-oxo-6-(undecyloxy)hexyl)-9,13,19,23-tetraazahentriacontanedioate

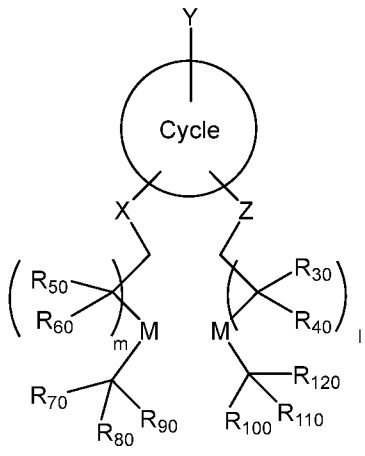
Lipid No.	Structure	IUPAC name
		6-[2-[[2-[[2-[2-[bis[6-(2-hexyldecanoyloxy)hexyl]amino]ethylamino]-2-oxo-ethyl]-(2-pyrrolidin-1-ylacetyl)amino]acetyl]amino]ethyl-[6-(2-hexyldecanoyloxy)hexyl]amino]hexyl 2-hexyldecanoate
		di(heptadecan-9-yl) 9,21-bis(8-(heptadecan-9-yloxy)-8-oxooctyl)-13,17-dioxo-15-(2-(pyrrolidin-1-yl)acetyl)-9,12,15,18,21-pentaazanonacosanedioate
		di(heptadecan-9-yl) 9,21-bis(8-((2-methylnonyl)oxy)-8-oxooctyl)-13,17-dioxo-15-(2-(pyrrolidin-1-yl)acetyl)-9,12,15,18,21-pentaazanonacosanedioate
		di(heptadecan-9-yl) 9,21-bis(7-((2-octyldecanoyl)oxy)heptyl)-13,17-dioxo-15-(2-(pyrrolidin-1-yl)acetyl)-9,12,15,18,21-pentaazanonacosanedioate
		di(heptadecan-9-yl) 9,21-bis(6-((2-methylundecyl)oxy)-6-oxohexyl)-13,17-dioxo-15-(2-(pyrrolidin-1-yl)acetyl)-9,12,15,18,21-pentaazanonacosanedioate

Lipid No.	Structure	IUPAC name
		di(heptadecan-9-yl) 13,17-dioxo-9,21-bis(6-oxo-6-(tridecan-3-yloxy)hexyl)-15-(2-(pyrrolidin-1-yl)acetyl)-9,12,15,18,21-pentaazanonacosanedioate
		di(heptadecan-9-yl) 9,21-bis(5-(dodecanoyloxy)pentyl)-13,17-dioxo-15-(2-(pyrrolidin-1-yl)acetyl)-9,12,15,18,21-pentaazanonacosanedioate
		di(heptadecan-9-yl) 14,18-dioxo-9,23-bis(6-oxo-6-(undecyloxy)hexyl)-16-(2-(pyrrolidin-1-yl)acetyl)-9,13,16,19,23-pentaazahentriacontanedioate
		((piperazine-1,4-diylbis(ethane-2,1-diyl))bis(azanetriyl))tetrakis(hexane-6,1-diyl) tetrakis(2-hexyldecanoate)
		tetra(heptadecan-9-yl) 8,8',8'',8'''-((piperazine-1,4-diylbis(ethane-2,1-diyl))bis(azanetriyl))tetraoctanoate

Lipid No.	Structure	IUPAC name
		di(heptadecan-9-yl) 8,8'-((piperazine-1,4-diylbis(ethane-2,1-diyl))bis((8-((2-methylnonyl)oxy)-8-oxooctyl)azanediyl))dioctanoate
		((piperazine-1,4-diylbis(ethane-2,1-diyl))bis((8-(heptadecan-9-yloxy)-8-oxooctyl)azanediyl))bis(heptane-7,1-diyl) bis(2-octyldecanoate)
		di(heptadecan-9-yl) 8,8'-((piperazine-1,4-diylbis(ethane-2,1-diyl))bis((6-((2-methylundecyl)oxy)-6-oxohexyl)azanediyl))dioctanoate
		di(heptadecan-9-yl) 8,8'-((piperazine-1,4-diylbis(propane-3,1-diyl))bis((6-oxo-6-(undecyloxy)hexyl)azanediyl))dioctanoate

Ionizable lipid compounds ii)

[0262] In some embodiments, the ionizable lipid is represented by the following formula II:

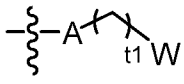


(II),

a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing,

wherein:

is cyclic or heterocyclic moiety;

Y is alkyl, hydroxy, hydroxyalkyl or  ;

A is absent, -O-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -N(R^7)C(O)N(R^7)-, -S-, -S-S-, or a bivalent heterocycle;

each of X and Z is independently absent, -O-, -CO-, -N(R^7)-, -O-alkylene-; -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, or -S-;

each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxy, hydroxyalkyl, or aminoalkyl;

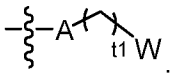
each M is independently a biodegradable moiety;

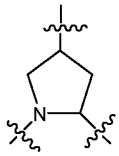
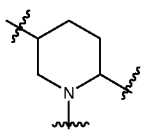
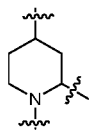
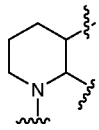
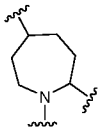
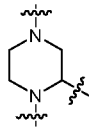
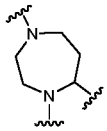
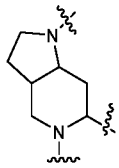
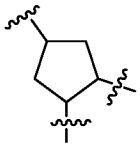
each of R_{30}, R_{40}, R_{50}, R_{60}, R_{70}, R_{80}, R_{90}, R_{100}, R_{110}, and R_{120} is independently H, C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with heteroatom or substituted with OH, SH, or halogen, or cycloalkyl or substituted cycloalkyl;

each of l and m is an integer from 1 to 10;

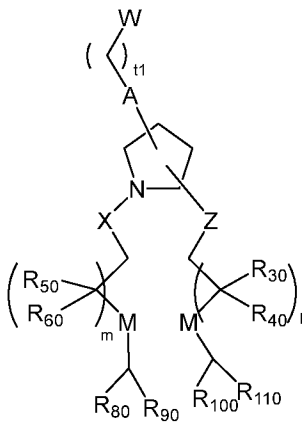
t1 is an integer from 0 to 10; and

W is hydroxyl, substituted or unsubstituted hydroxyalkyl, substituted or unsubstituted amino, substituted or unsubstituted aminocarbonyl, or substituted or unsubstituted heterocylyl or heteroaryl.

[0263] In some embodiments, Y is hydroxyl or  .

[0264] In some embodiments,  is selected from pyrrolidine, piperidine, piperazine, cyclohexane, cyclopentane, tetrahydrofuran, tetrahydropyran, morpholine, and dioxane. In some embodiments,  is selected from the group consisting of  .,  ,  ,  ,  ,  ,  ,  , and  .

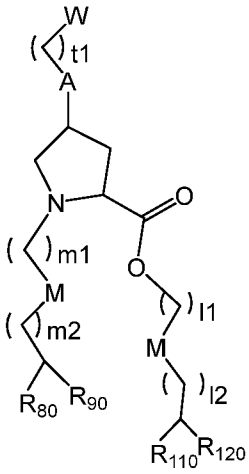
[0265] In some embodiments, the ionizable lipid is represented by formula:

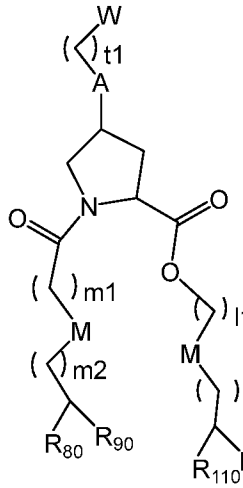


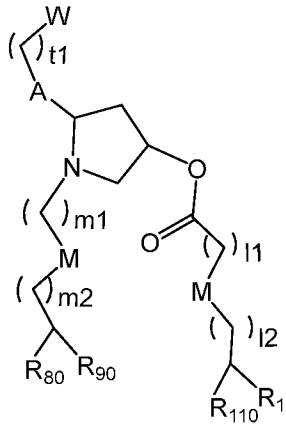
(IIA-1). All the variables in this formula have been defined and exemplified

as those described in the above embodiments.

[0266] In some embodiments, the ionizable lipid is represented by formula:


(IVA-1),


(IVA-2), or


(IVA-3),

wherein:

each m1 is independently an integer from 3 to 6,

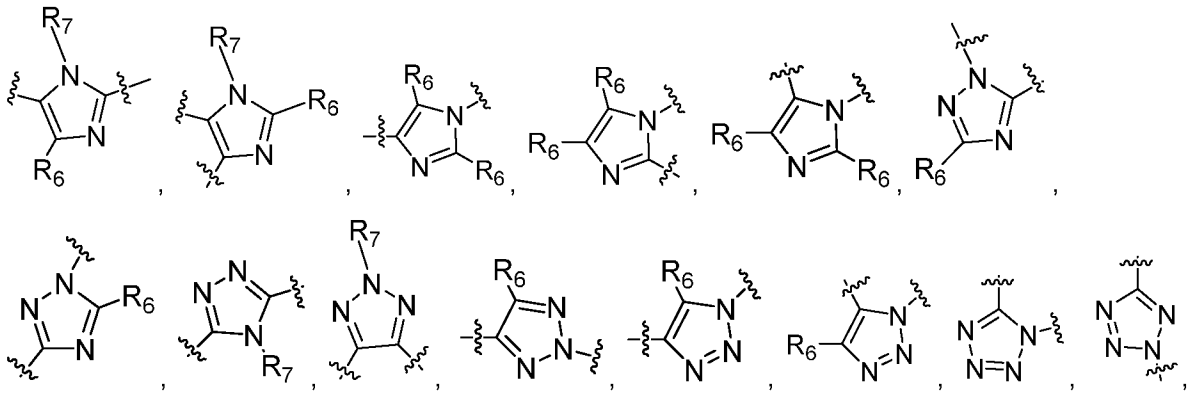
each l1 is independently an integer from 4 to 8,

m2 and l2 are each independently an integer from 0 to 3,

R_{80} and R_{90} are each independently unsubstituted C_5-C_8 alkyl; or R_{80} is H or unsubstituted C_1-C_4 alkyl, and R_{90} is unsubstituted C_5-C_{11} alkyl; and

R_{110} and R_{120} are each independently unsubstituted C_5-C_8 alkyl; or R_{110} is H or unsubstituted C_1-C_4 alkyl, and R_{120} is unsubstituted C_5-C_{11} alkyl. All the other variables in these formulas have been defined and exemplified as those described in the above embodiments. In some embodiments, in these formulas, R_{80} is H or unsubstituted C_1-C_2 alkyl, and R_{90} is unsubstituted C_6-C_{10} alkyl; and R_{110} and R_{120} are each independently unsubstituted C_5-C_8 alkyl. In some embodiments, R_{80}, R_{90}, R_{110}, and R_{120} are each independently unsubstituted C_5-C_8 alkyl.

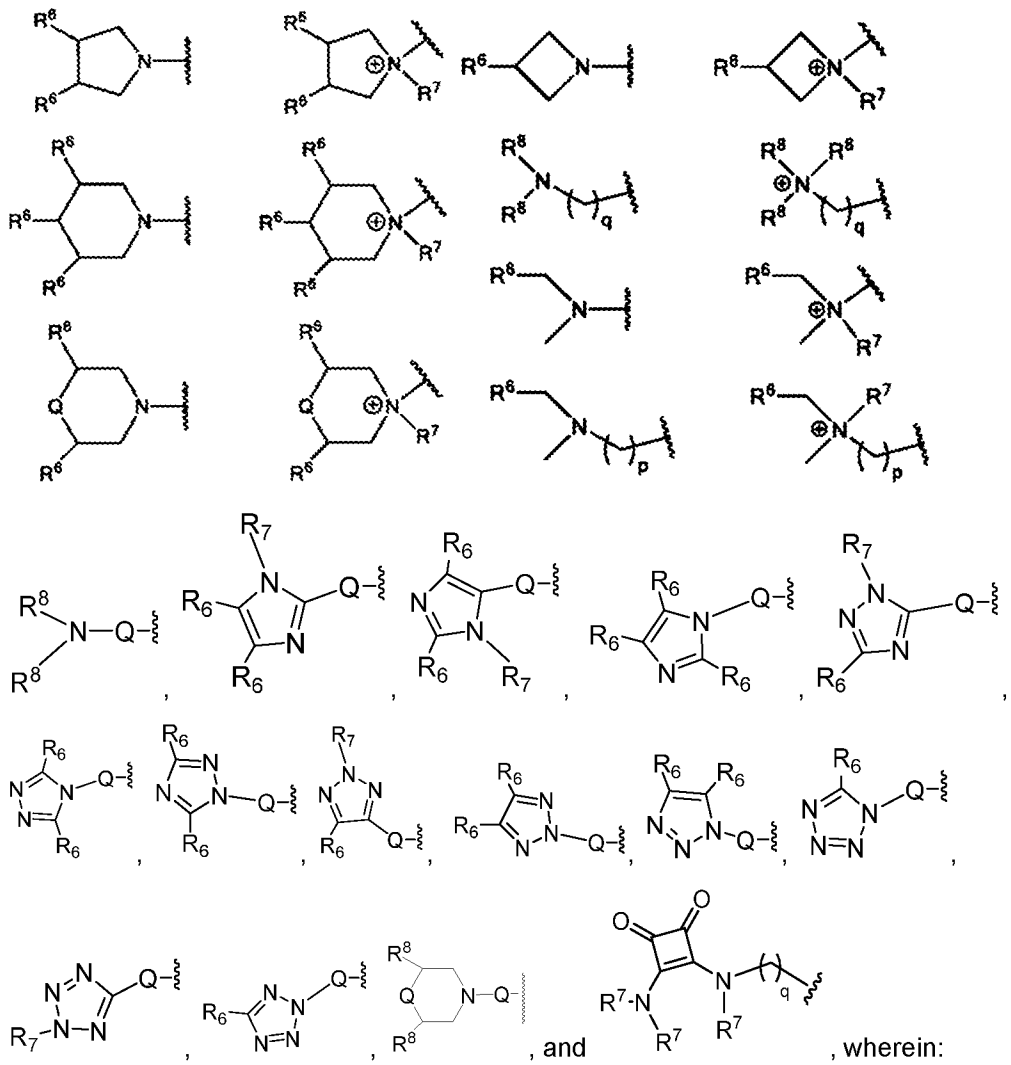
[0267] In some embodiments, in the above formulas, A is absent, -O-, -N(R^7)-, N(R^7)C(O)-,



-OC(O)-, or -C(O)O-, wherein R^6 is independently H, alkyl, hydroxyl, hydroxyalkyl, amino, aminoalkyl, thiol, thiolalkyl, or $N^+(R^7)_3$–alkylene-Q-; and R^7 is H or C_1-C_3 alkyl.

[0268] In some embodiments, in the above formulas, t1 is 0, 1, 2, 3 or 4; and t is 0, 1, or 2.

[0269] In some embodiments, in the above formulas, W is hydroxyl, hydroxyalkyl, or one of the following:



, wherein:

each Q is independently absent, -O-, -C(O)-, -C(S)-, -C(O)O-, -$C(R^7)_2$-, -C(O)N(R^7)-, -C(S)N(R^7)-, or -N(R^7)-;

each R^6 is independently H, alkyl, hydroxyl, hydroxyalkyl, alkoxy, amino, aminoalkyl, alkylamino,thiol, thiolalkyl, or $N^+(R^7)_3$–alkylene-Q-;

each R^8 is independently H, alkyl, hydroxyalkyl, amino, aminoalkyl, thiol, or thiolalkyl, or two R^8 together with the nitrogen atom may form a ring;

each q is independently 0, 1, 2, 3, 4, or 5; and

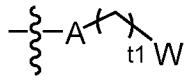
each p is independently 0, 1, 2, 3, 4, or 5.

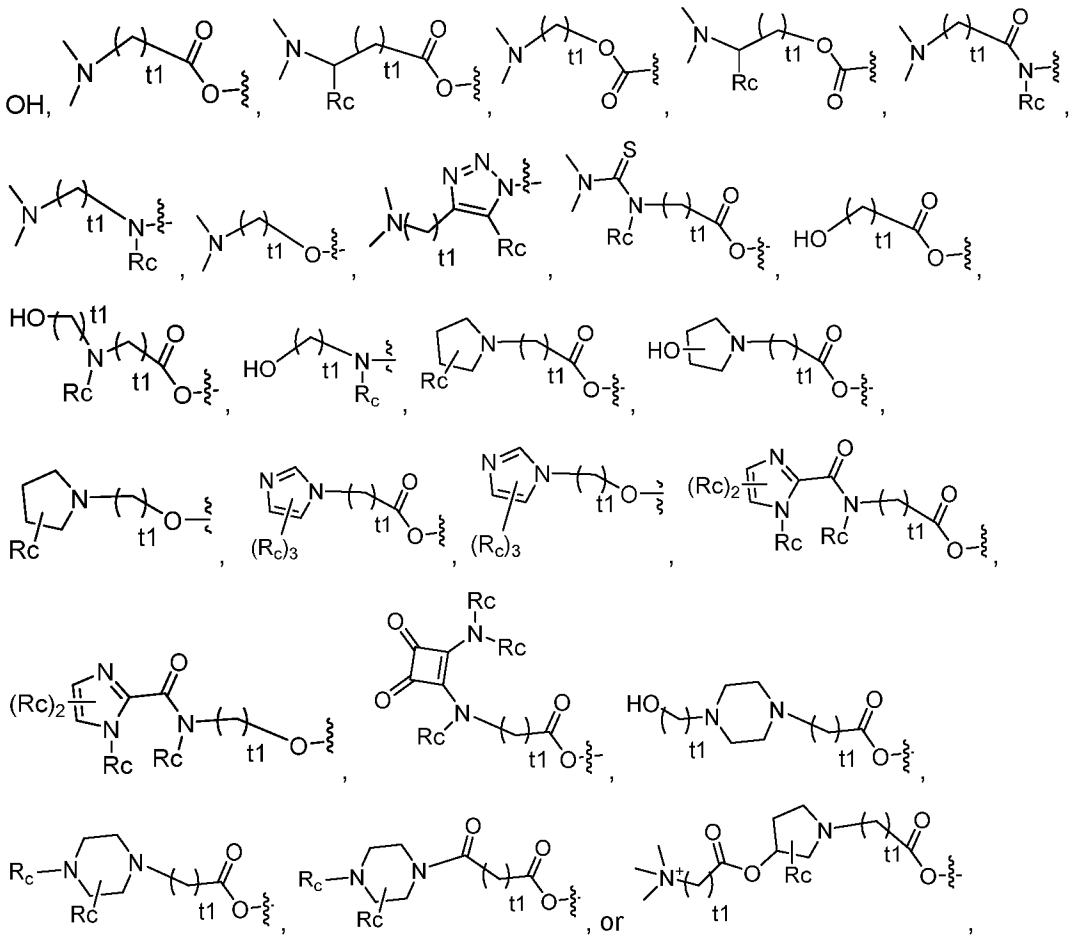
[0270] In some embodiments, in the above formulas,

X is absent, -O-, or –C(O)-;

Z is –O-, –C(O)O-, or –OC(O)-;

M is -OC(O)- or -C(O)O-;

Y or  is:



each R^c is independently H or C_1-C_3 alkyl;

each t1 is independently 1, 2, 3, or 4;

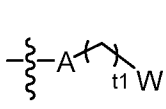
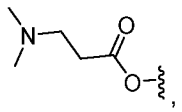
each of R_{30}, R_{40}, R_{50}, and R_{60} is H or C_1-C_4 branched or unbranched alkyl;

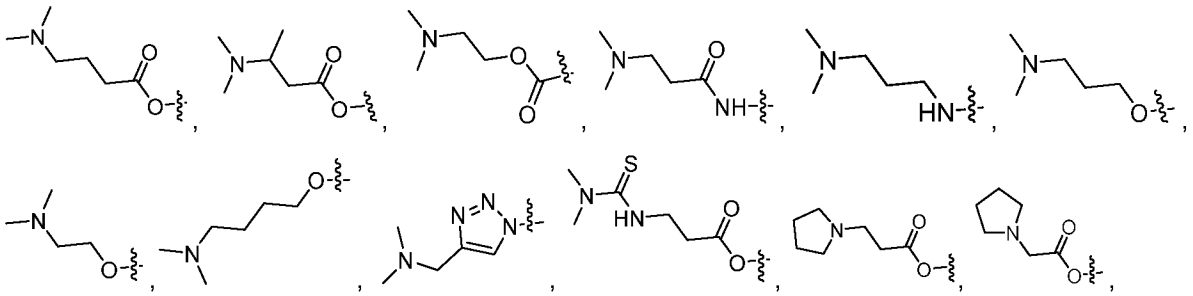
R_{70} is H; and each of R_{80} and R_{90} is independently H or C_1-C_{12} branched or unbranched alkyl;

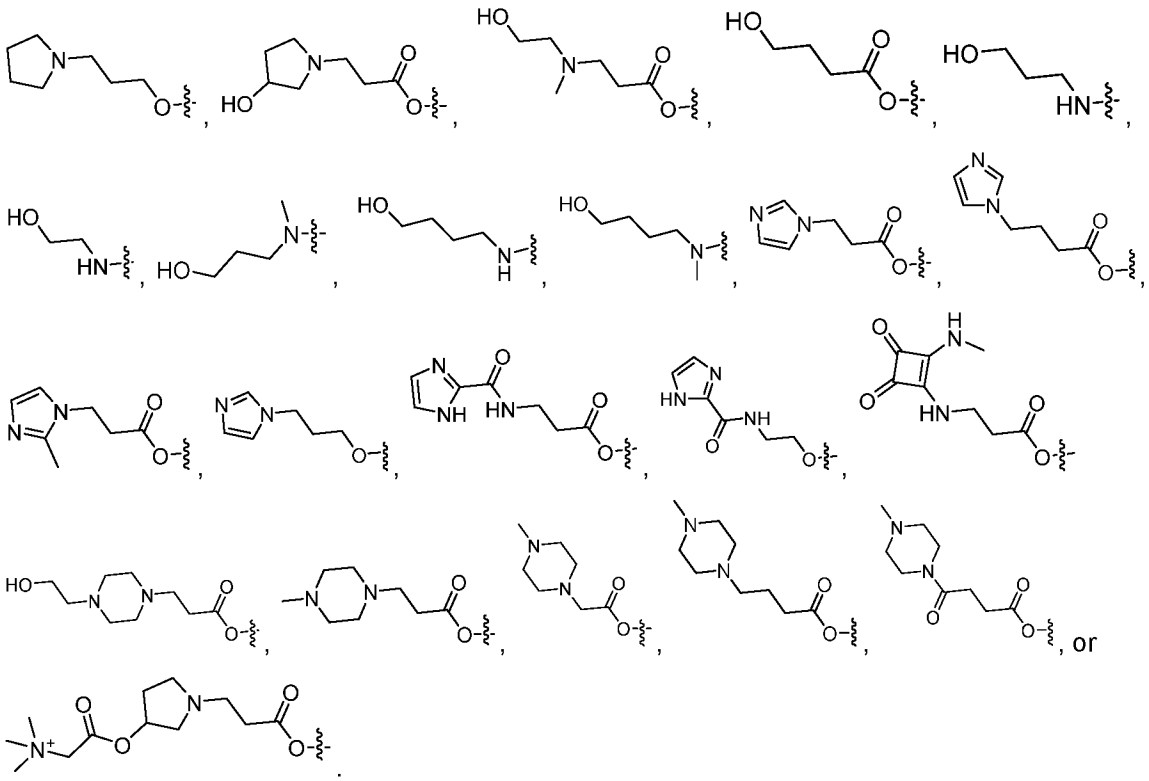
R_{100} is H; and each of R_{110} and R_{120} is independently H or C_1-C_{12} branched or unbranched alkyl, provided that at least one of R_{80} and R_{90} is not H, and at least one of R_{110} and R_{120} is not H;

l is from 3 to 7; and

m is from 1 to 5.

[0271] In some embodiments, in the above formulas, Y or  is: OH,  ,

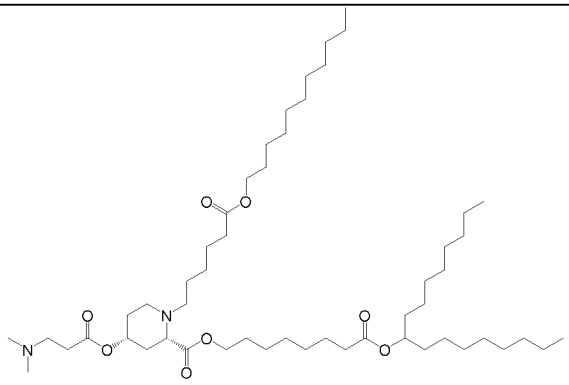


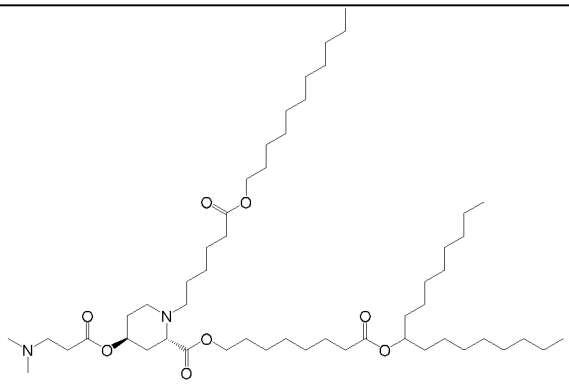
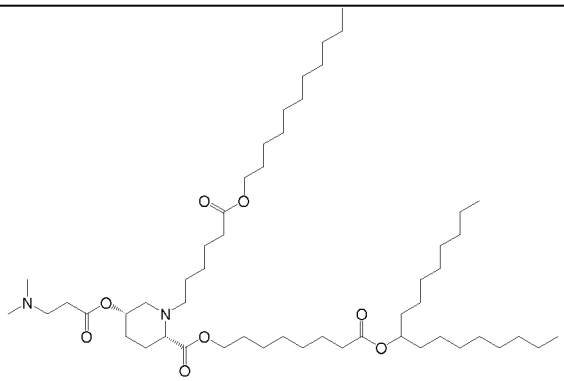
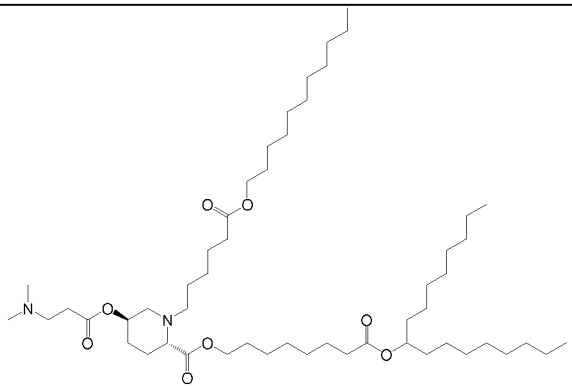
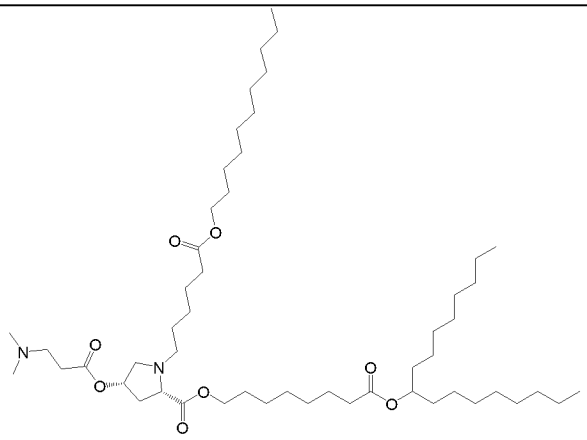


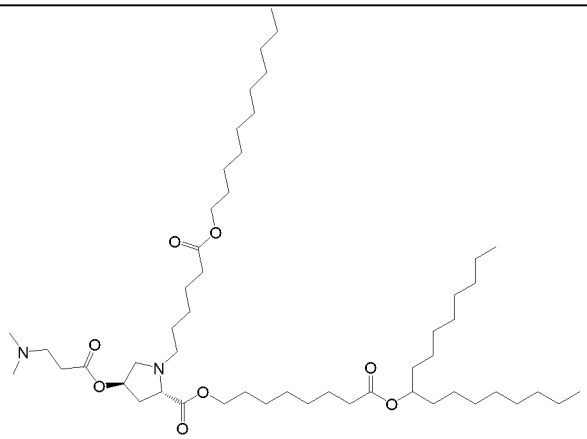
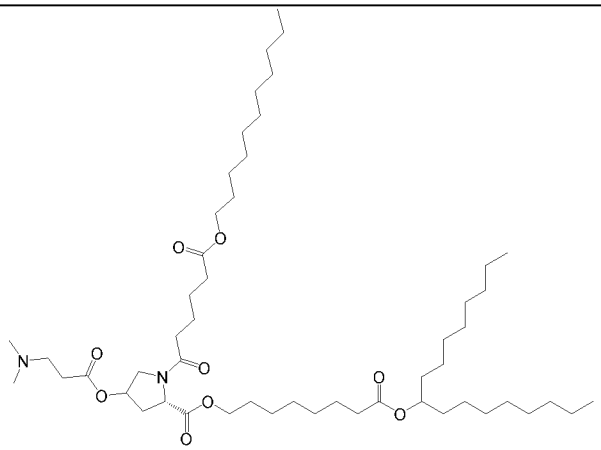
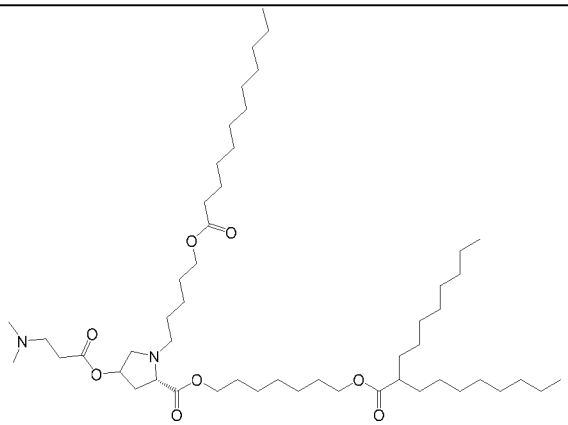
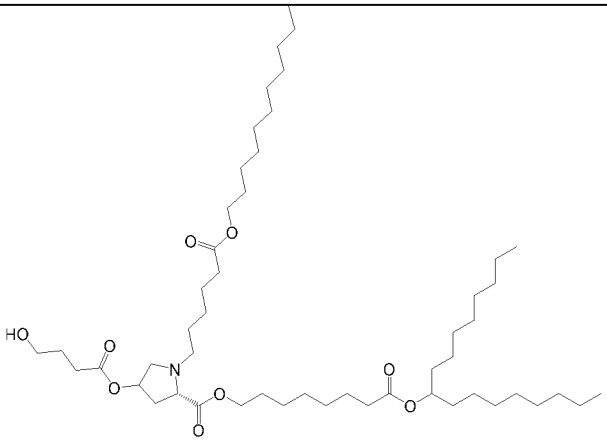
[0272] More embodiments of the ionizable lipid of formula (II), in the Ionizable lipid compounds group ii), may be found in PCT Application No. PCT/US23/16300, filed on March 24, 2023, the content of which is incorporated herein by reference in its entirety. In particular, all the ionizable lipids of formulas (I), (IA-1), (IA-2), (IIA)-(IIC), (IIA-1), (IIIA)-(IIIE), (IIIC-1), (IVA-1)-(IVA-3), (IVC-1)-(IVC-2), (VA-1)-(VA-9), (VC-1)-(VC-6) of PCT Application No. PCT/US23/16300 are suitable for use as the ionizable lipids in this disclosure, and are incorporated herein by reference in its entirety.

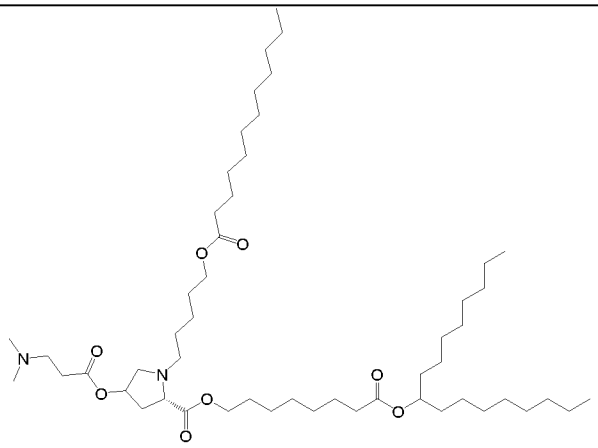
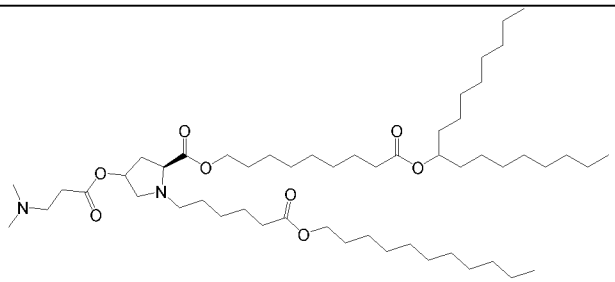
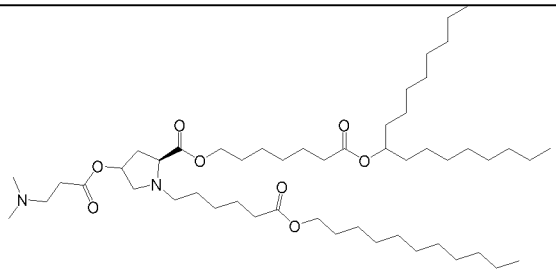
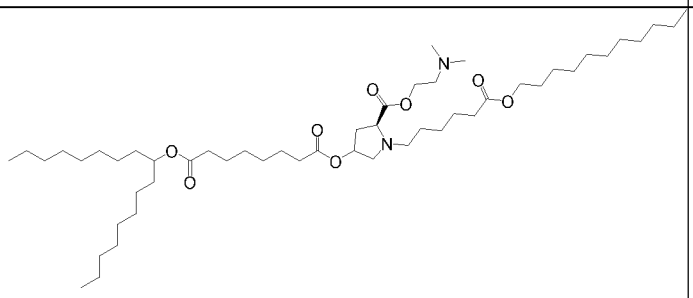
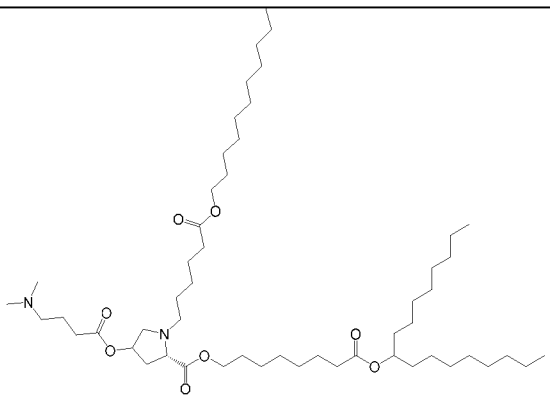
[0273] Certain exemplary ionizable lipid compounds disclosed herein are set forth in Table II below.

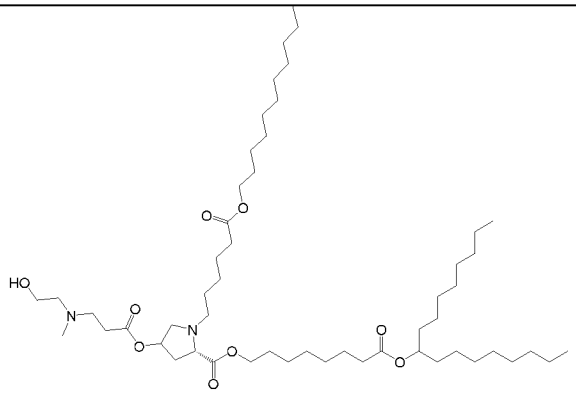
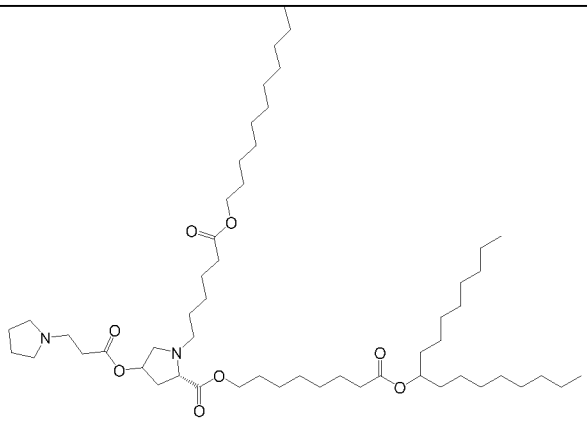
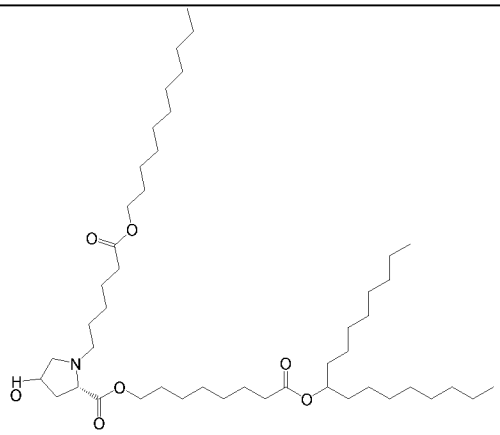
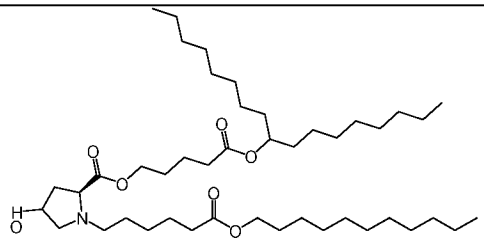
Table II. Exemplary ionizable lipid compounds.

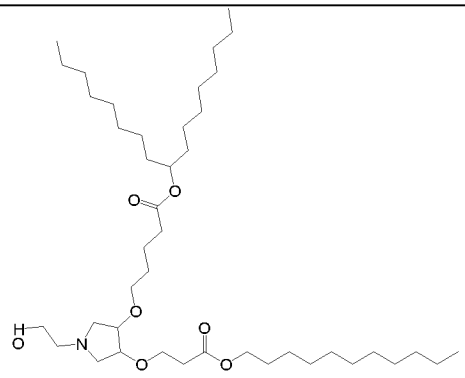
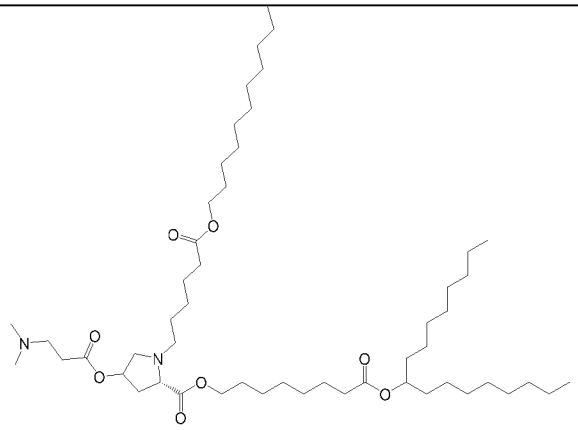
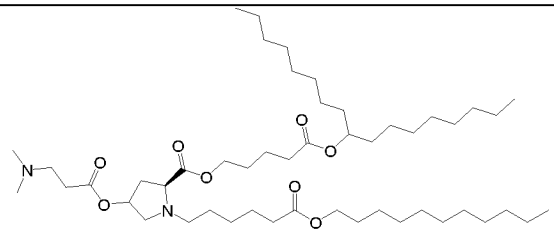
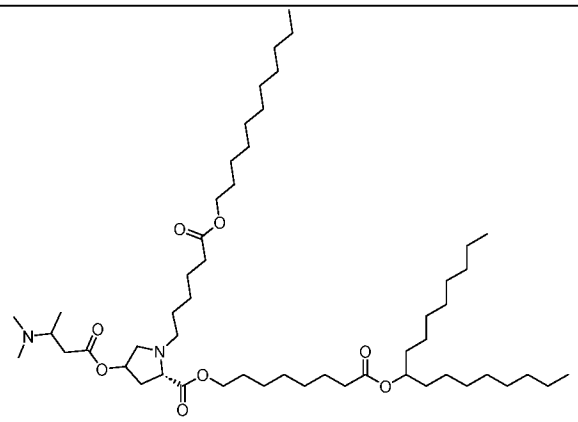
Lipid No.	Structure	IUPAC name
2310		heptadecan-9-yl 8-[(2S,4R)-4-{[3-(dimethylamino)propanoyl]oxy}-1-[6-oxo-6-(undecyloxy)hexyl]piperidine-2-carbonyloxy]octanoate

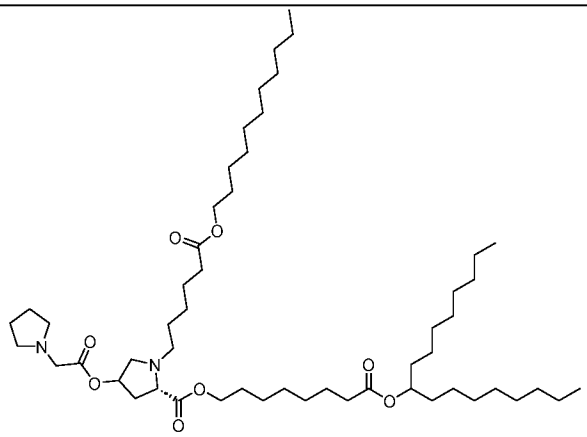
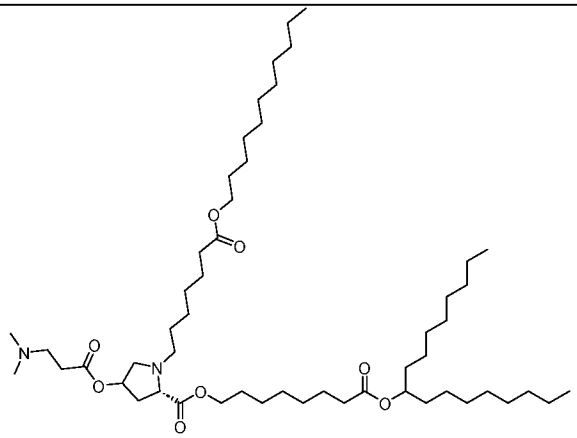
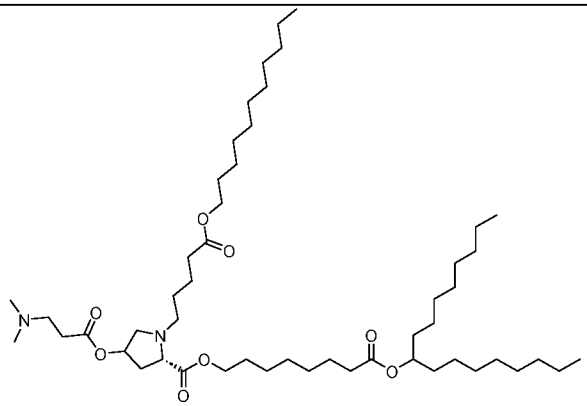
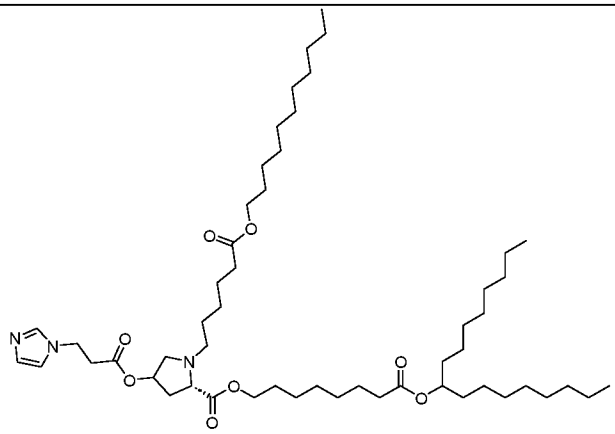
2309		heptadecan-9-yl 8-[(2S,4S)-4-{[3-(dimethylamino)propanoyl]oxy}-1-[6-oxo-6-(undecyloxy)hexyl]piperidine-2-carbonyloxy]octanoate
2308		heptadecan-9-yl 8-[(2S,5S)-5-{[3-(dimethylamino)propanoyl]oxy}-1-[6-oxo-6-(undecyloxy)hexyl]piperidine-2-carbonyloxy]octanoate
2307		heptadecan-9-yl 8-[(2S,5R)-5-{[3-(dimethylamino)propanoyl]oxy}-1-[6-oxo-6-(undecyloxy)hexyl]piperidine-2-carbonyloxy]octanoate
2306		heptadecan-9-yl 8-[(2S,4S)-4-{[3-(dimethylamino)propanoyl]oxy}-1-[6-oxo-6-(undecyloxy)hexyl]pyrrolidine-2-carbonyloxy]octanoate

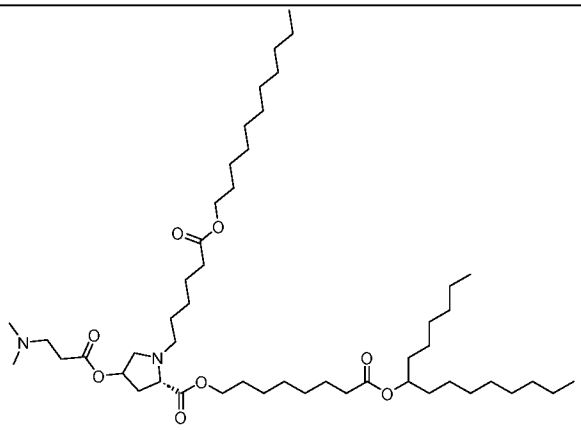
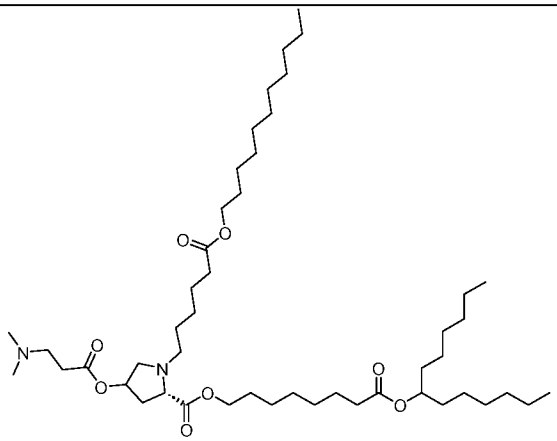
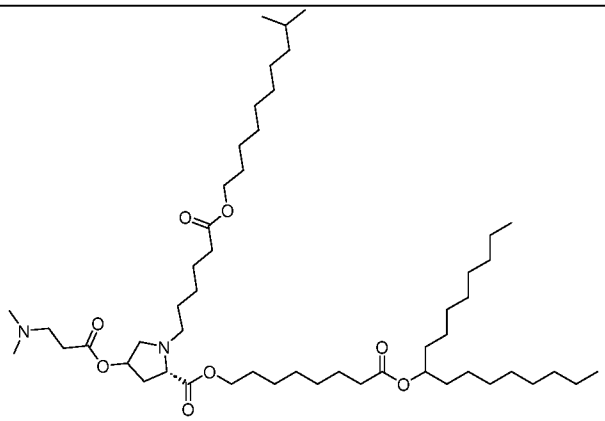
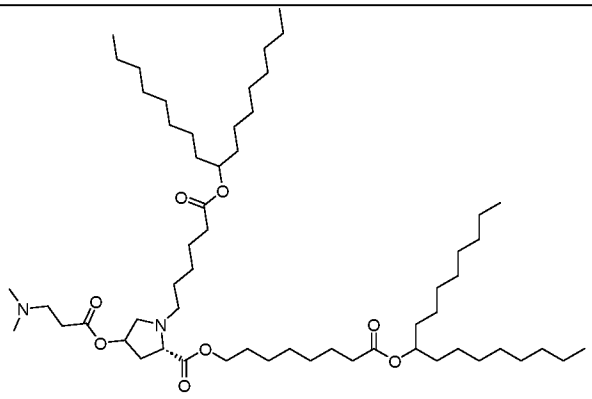
2305		heptadecan-9-yl 8-[(2S,4R)-4-{[3-(dimethylamino)propanoyl]oxy}-1-[6-oxo-6-(undecyloxy)hexyl]pyrrolidine-2-carbonyloxy]octanoate
2304		heptadecan-9-yl 8-[(2S)-4-{[3-(dimethylamino)propanoyl]oxy}-1-[6-oxo-6-(undecyloxy)hexanoyl]pyrrolidine-2-carbonyloxy]octanoate
2298		5-[(2S)-4-{[3-(dimethylamino)propanoyl]oxy}-2-[({7-[(2-octyldecanoyl)oxy]heptyl}oxy)carbonyl]pyrrolidin-1-yl]pentyl dodecanoate
2297		heptadecan-9-yl 8-[(2S)-4-[(4-hydroxybutanoyl)oxy]-1-[6-oxo-6-(undecyloxy)hexyl]pyrrolidine-2-carbonyloxy]octanoate

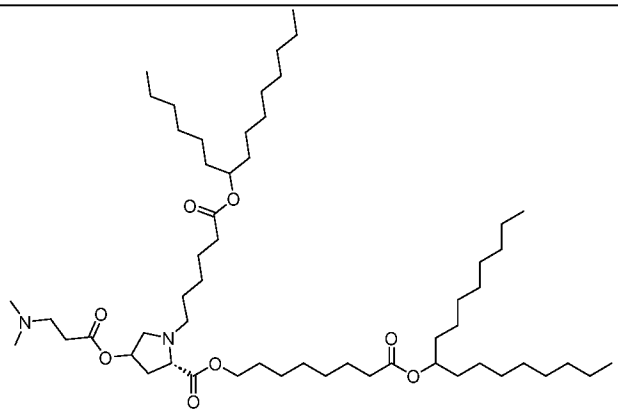
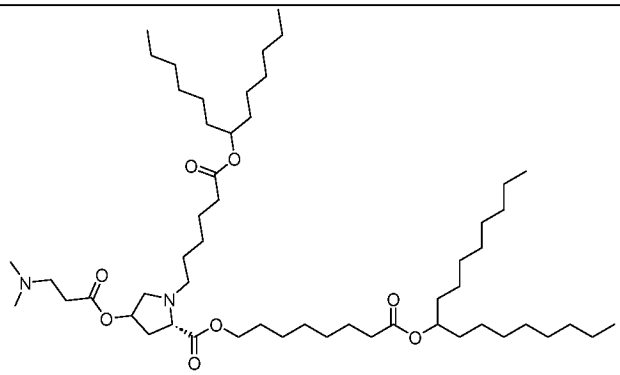
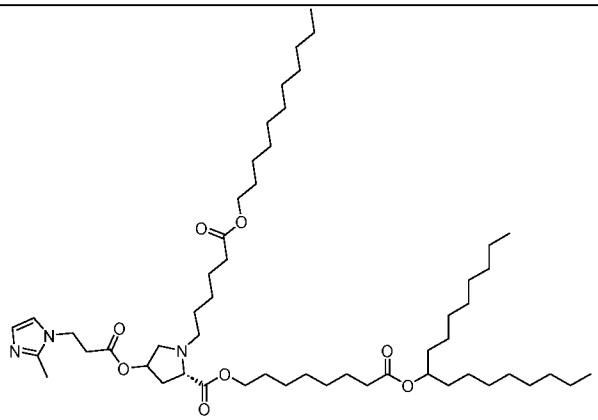
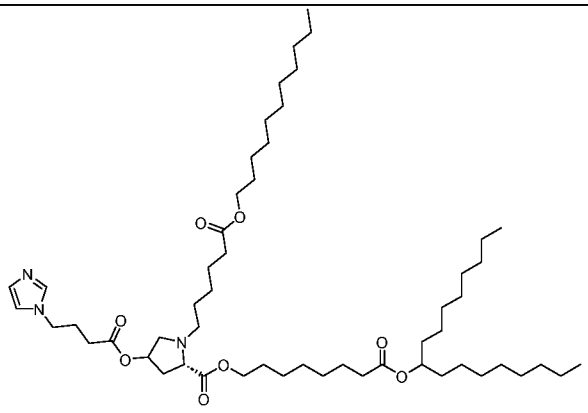
2296		5-[(2S)-4-{[3-(dimethylamino)propanoyl]oxy}-2-({[8-(heptadecan-9-yloxy)-8-oxooctyl]oxy}carbonyl)pyrrolidin-1-yl]pentyl dodecanoate
2295		heptadecan-9-yl 9-[(2S)-4-{[3-(dimethylamino)propanoyl]oxy}-1-[6-oxo-6-(undecyloxy)hexyl]pyrrolidine-2-carbonyloxy]nonanoate
2294		heptadecan-9-yl 7-[(2S)-4-{[3-(dimethylamino)propanoyl]oxy}-1-[6-oxo-6-(undecyloxy)hexyl]pyrrolidine-2-carbonyloxy]heptanoate
2293		(5S)-5-{[2-(dimethylamino)ethoxy]carbonyl}-1-[6-oxo-6-(undecyloxy)hexyl]pyrrolidin-3-yl 1-heptadecan-9-yl octanedioate
2292		heptadecan-9-yl 8-[(2S)-4-{[4-(dimethylamino)butanoyl]oxy}-1-[6-oxo-6-(undecyloxy)hexyl]pyrrolidine-2-carbonyloxy]octanoate

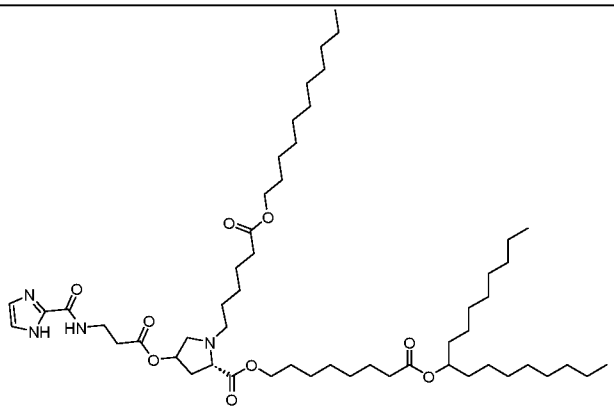
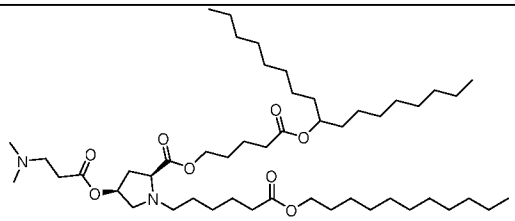
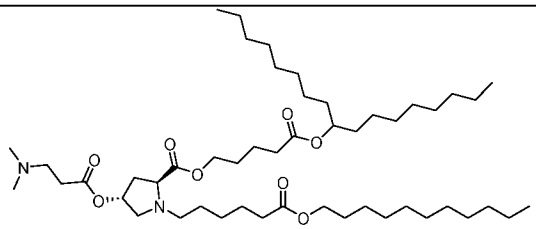
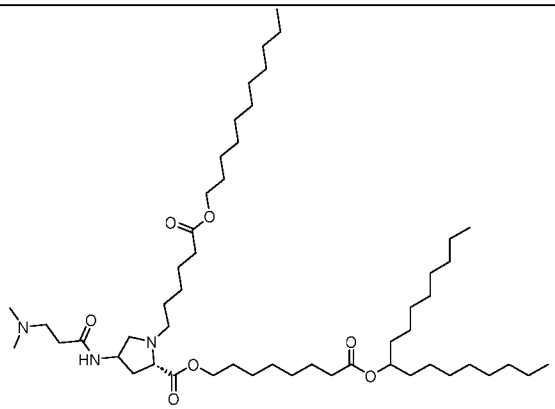
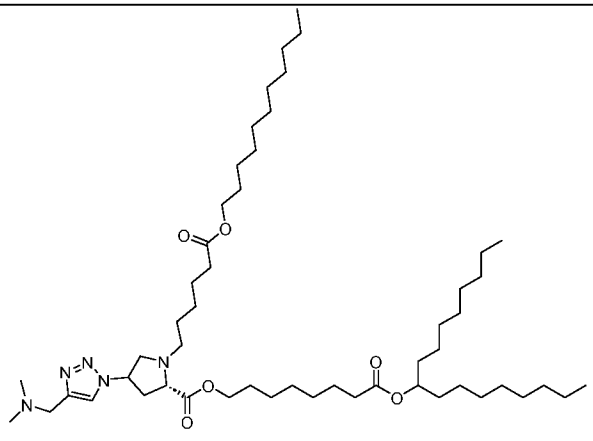
2291		heptadecan-9-yl 8-[(2S)-4-({3-[(2-hydroxyethyl)(methyl)amino]propanoyl}oxy)-1-[6-oxo-6-(undecyloxy)hexyl]pyrrolidine-2-carbonyloxy]octanoate
2290		heptadecan-9-yl 8-[(2S)-1-[6-oxo-6-(undecyloxy)hexyl]-4-{[3-(pyrrolidin-1-yl)propanoyl]oxy}pyrrolidine-2-carbonyloxy]octanoate
2270		heptadecan-9-yl 8-[(2S)-4-hydroxy-1-[6-oxo-6-(undecyloxy)hexyl]pyrrolidine-2-carbonyloxy]octanoate
2260		undecyl 6-[(2S)-2-({[5-(heptadecan-9-yloxy)-5-oxopentyl]oxy}carbonyl)-4-hydroxypyrrolidin-1-yl]hexanoate

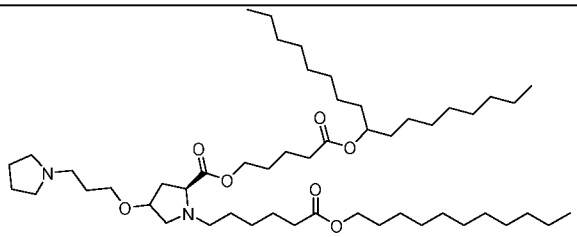
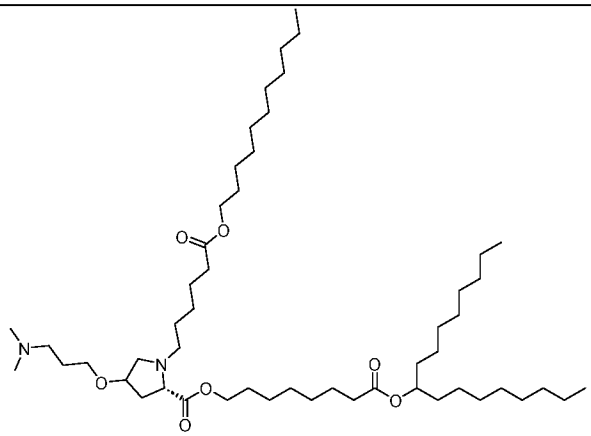
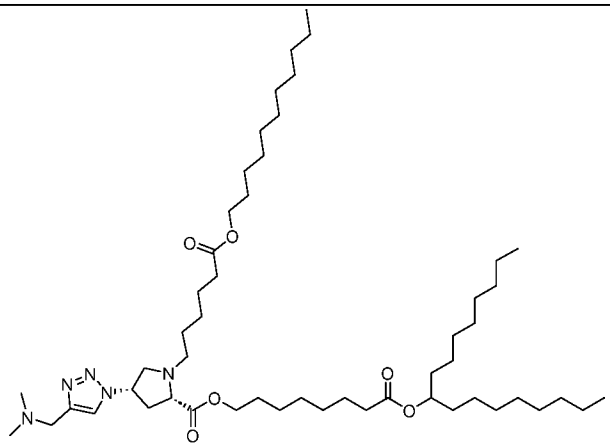
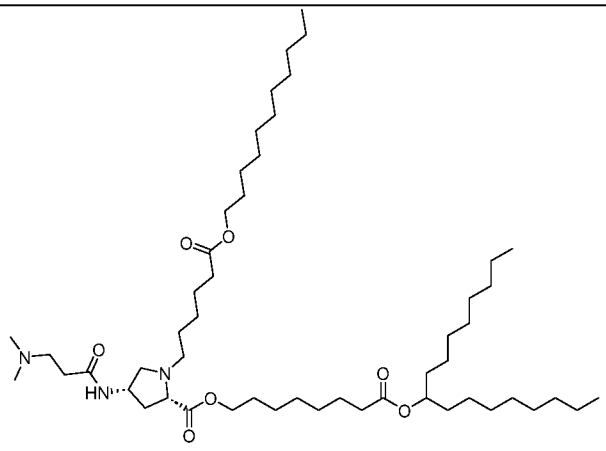
2240		heptadecan-9-yl 5-{[1-(2-hydroxyethyl)-4-[3-oxo-3-(undecyloxy)propoxy]pyrrolidin-3-yl]oxy}pentanoate
2231		heptadecan-9-yl 8-[(2S)-4-{[3-(dimethylamino)propanoyl]oxy}-1-[6-oxo-6-(undecyloxy)hexyl]pyrrolidine-2-carbonyloxy]octanoate
2230		undecyl 6-[(2S)-4-{[3-(dimethylamino)propanoyl]oxy}-2-({[5-(heptadecan-9-yloxy)-5-oxopentyl]oxy}carbonyl)pyrrolidin-1-yl]hexanoate
2329		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(dimethylamino)butanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate

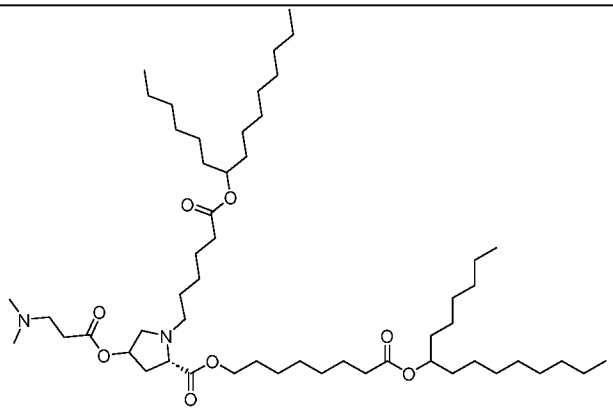
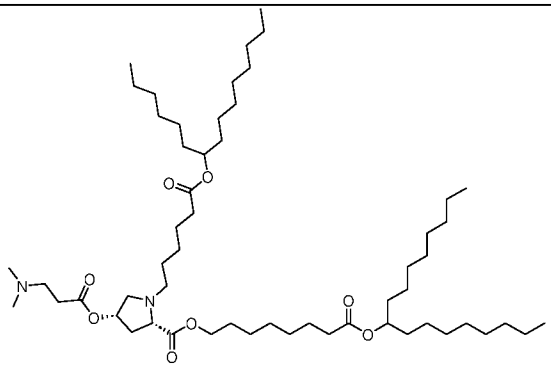
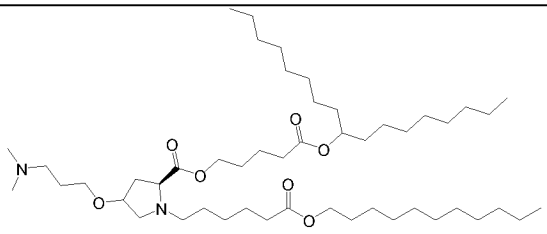
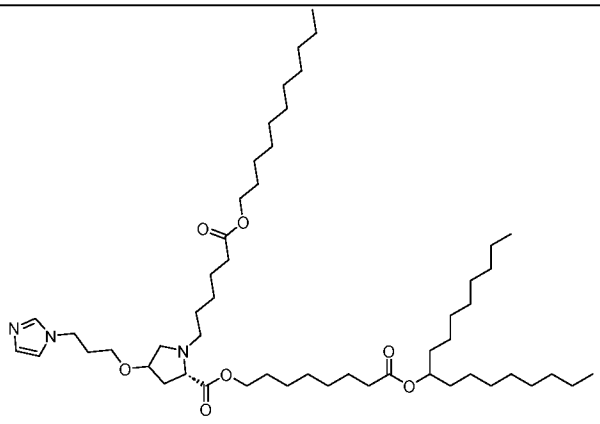
2336		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-1-(6-oxo-6-(undecyloxy)hexyl)-4-(2-(pyrrolidin-1-yl)acetoxy)pyrrolidine-2-carboxylate
2337		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(7-oxo-7-(undecyloxy)heptyl)pyrrolidine-2-carboxylate
2338		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(5-oxo-5-(undecyloxy)pentyl)pyrrolidine-2-carboxylate
2339		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(1H-imidazol-1-yl)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate

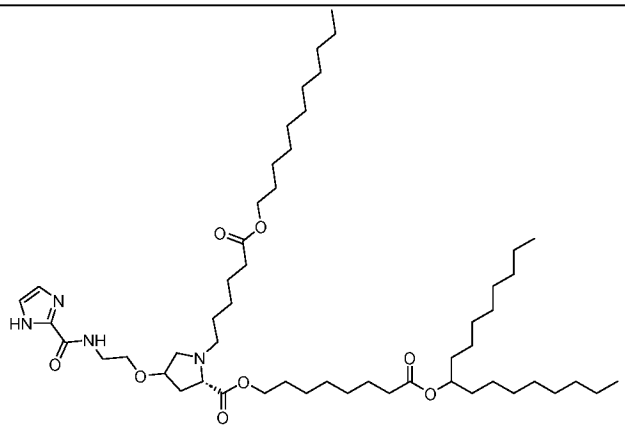
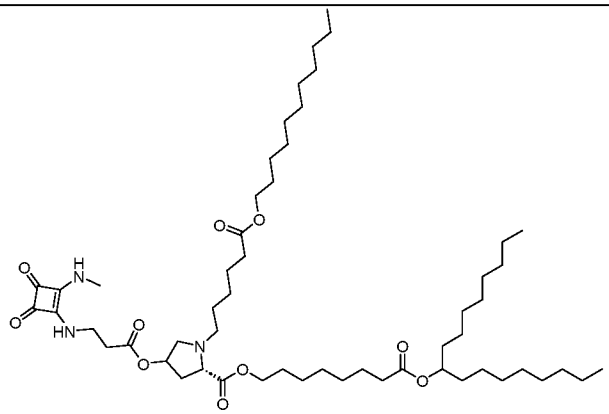
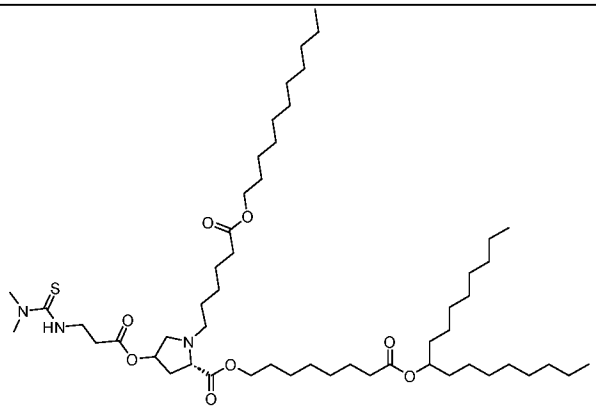
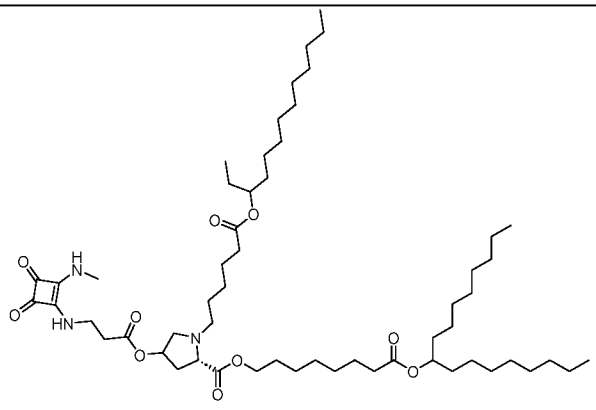
2340		8-oxo-8-(pentadecan-7-yloxy)octyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2341		8-oxo-8-(tridecan-7-yloxy)octyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2342		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-((9-methyldecyl)oxy)-6-oxohexyl)pyrrolidine-2-carboxylate
2343		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-(heptadecan-9-yloxy)-6-oxohexyl)pyrrolidine-2-carboxylate

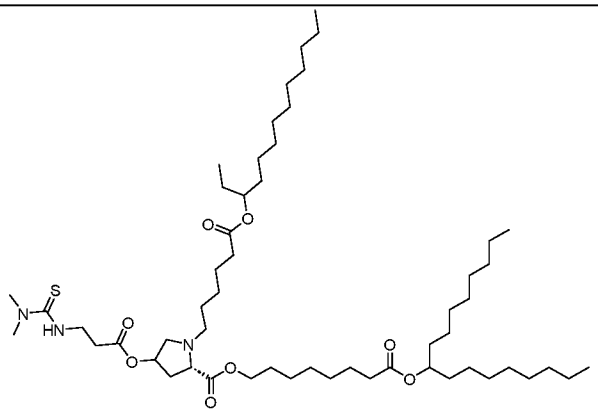
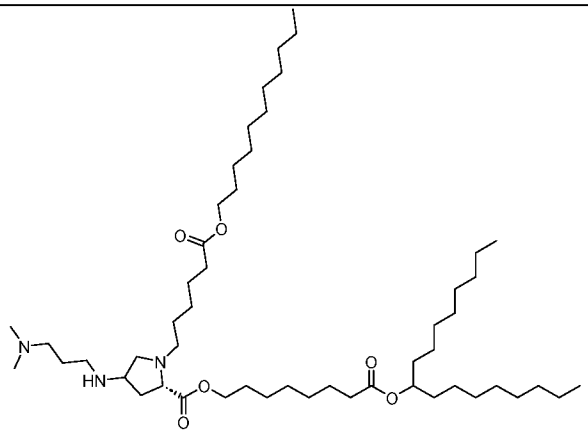
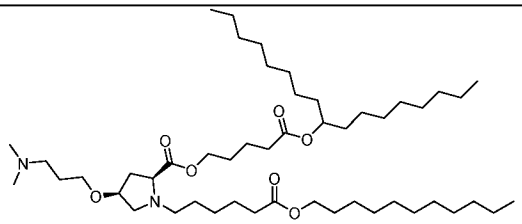
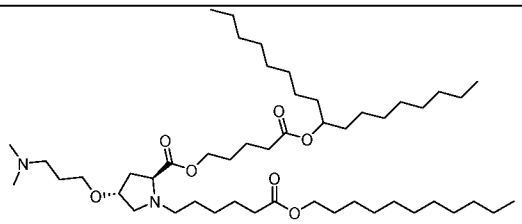
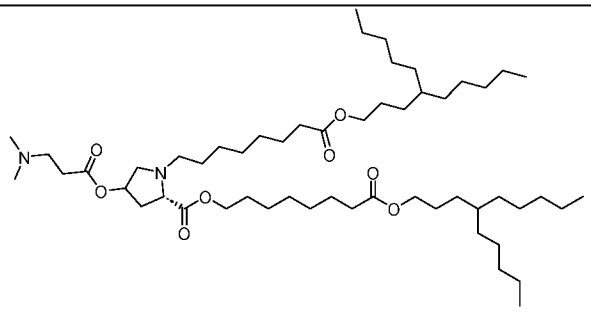
2344		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(pentadecan-7-yloxy)hexyl)pyrrolidine-2-carboxylate
2345		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(tridecan-7-yloxy)hexyl)pyrrolidine-2-carboxylate
2348		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(2-methyl-1H-imidazol-1-yl)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2349		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((4-(1H-imidazol-1-yl)butanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate

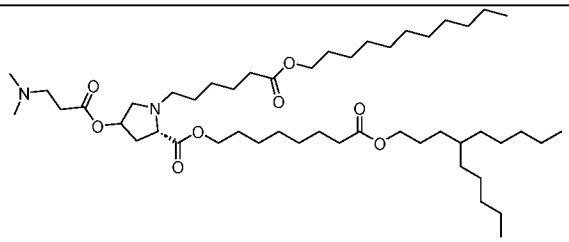
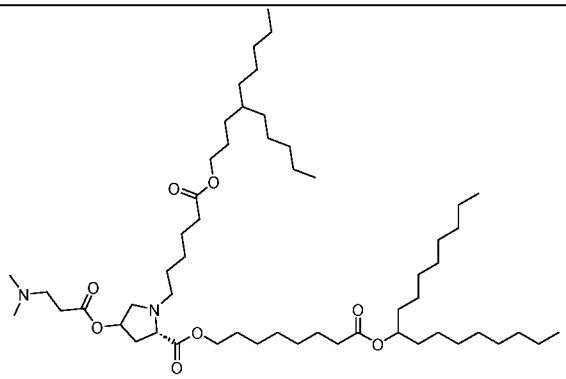
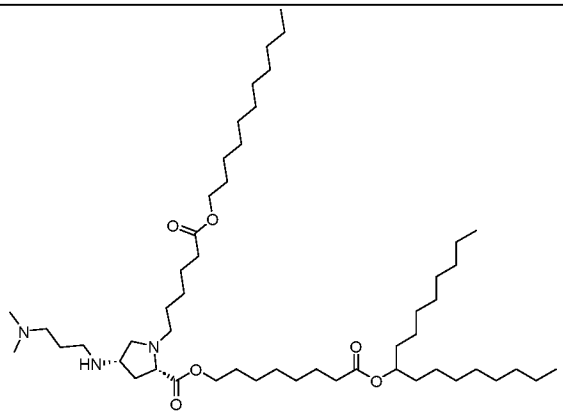
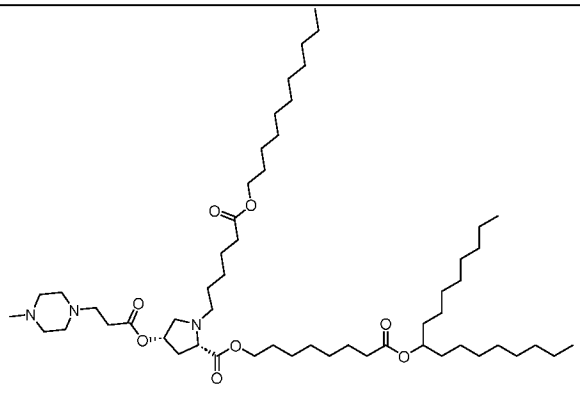
2352		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(1H-imidazole-2-carboxamido)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2371		5-(heptadecan-9-yloxy)-5-oxopentyl (2S,4S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2372		5-(heptadecan-9-yloxy)-5-oxopentyl (2S,4R)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2373		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-(3-(dimethylamino)propanamido)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2375		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-(4-((dimethylamino)methyl)-1H-1,2,3-triazol-1-yl)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate

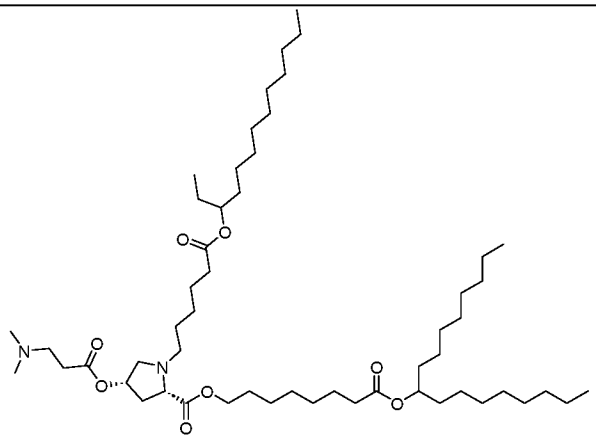
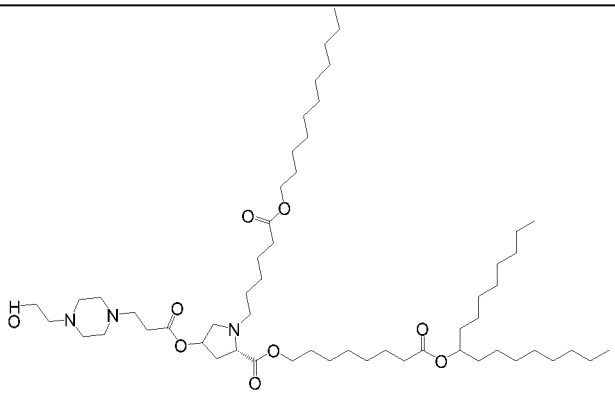
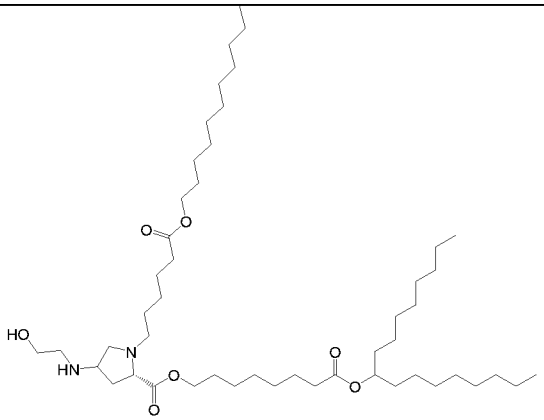
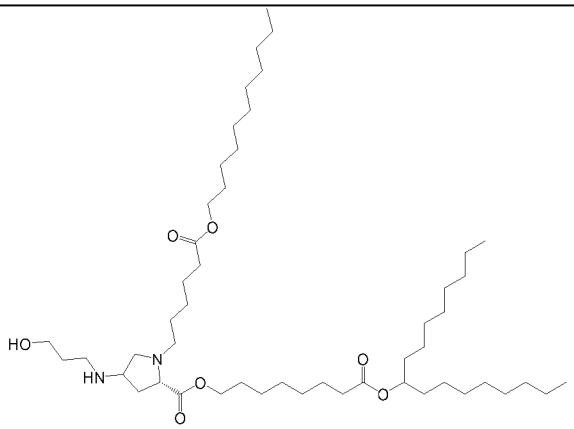
2376		5-(heptadecan-9-yloxy)-5-oxopentyl (2S)-1-(6-oxo-6-(undecyloxy)hexyl)-4-(3-(pyrrolidin-1-yl)propoxy)pyrrolidine-2-carboxylate
2377		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-(3-(dimethylamino)propoxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2436		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-(4-((dimethylamino)methyl)-1H-1,2,3-triazol-1-yl)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2437		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-(3-(dimethylamino)propanamido)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate

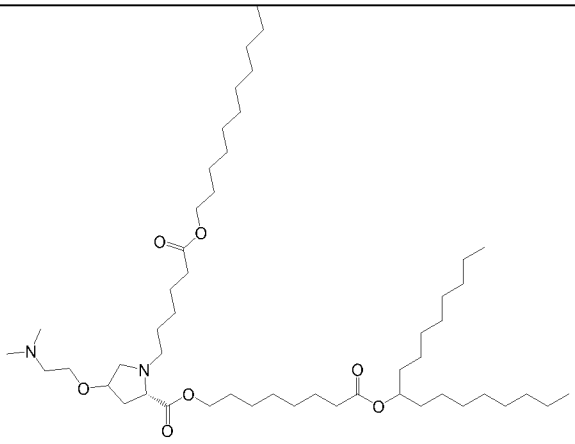
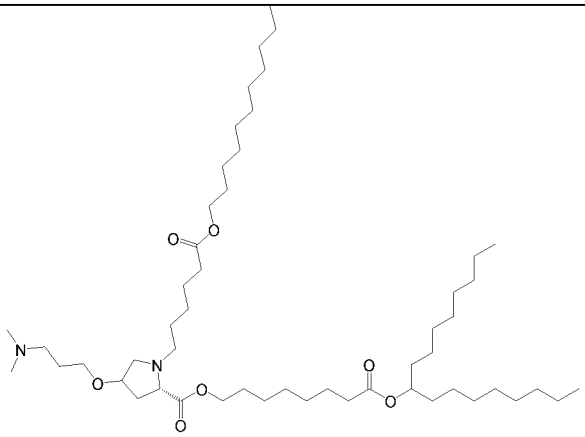
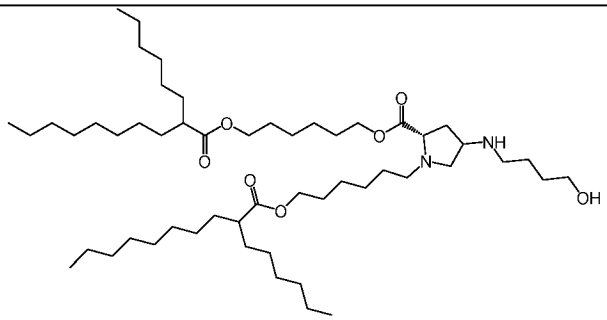
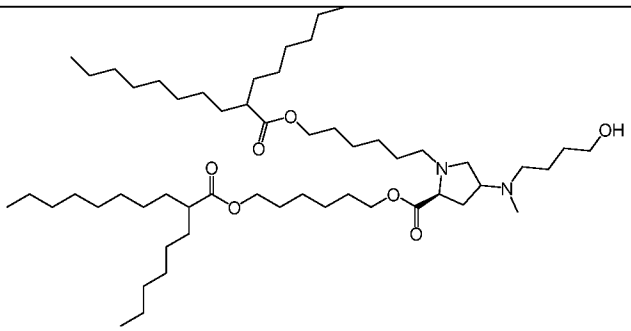
2440		8-oxo-8-(pentadecan-7-yloxy)octyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(pentadecan-7-yloxy)hexyl)pyrrolidine-2-carboxylate
2452		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(pentadecan-7-yloxy)hexyl)pyrrolidine-2-carboxylate
2232		undecyl 6-[(2S)-4-[3-(dimethylamino)propoxy]-2-({[5-(heptadecan-9-yloxy)-5-oxopentyl]oxy}carbonyl)pyrrolidin-1-yl]hexanoate
2350		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-(3-(1H-imidazol-1-yl)propoxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate

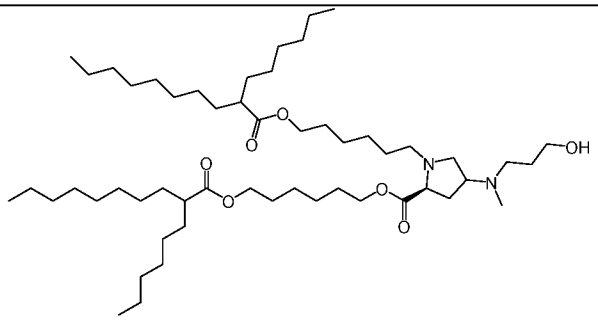
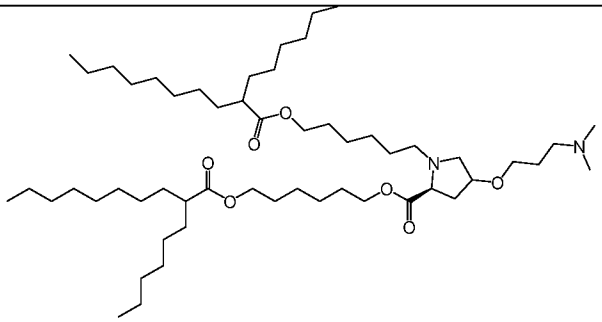
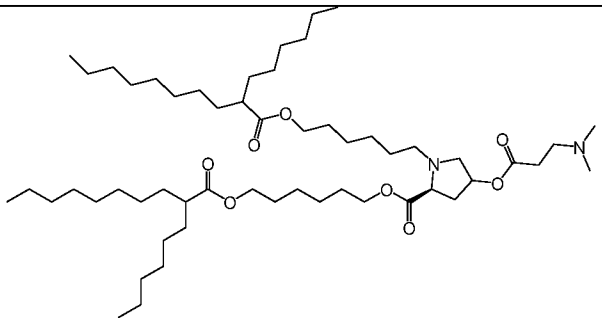
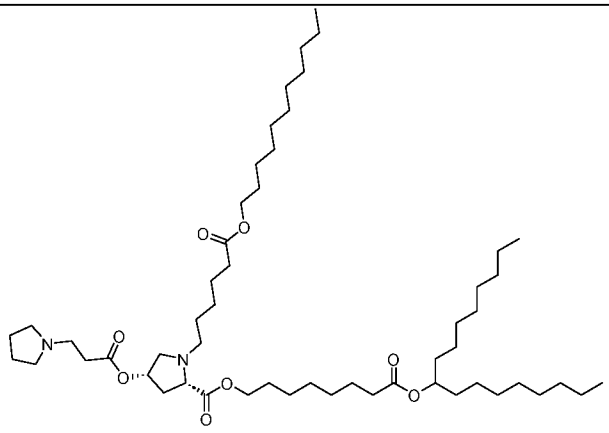
2351		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-(2-(1H-imidazole-2-carboxamido)ethoxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2359		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-((2-(methylamino)-3,4-dioxocyclobut-1-en-1-yl)amino)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2360		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(3,3-dimethylthioureido)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2363		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-((2-(methylamino)-3,4-dioxocyclobut-1-en-1-yl)amino)propanoyl)oxy)-1-(6-oxo-6-(tridecan-3-yloxy)hexyl)pyrrolidine-2-carboxylate

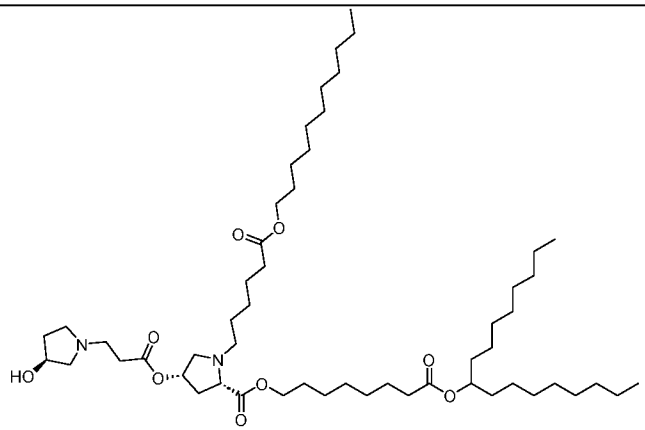
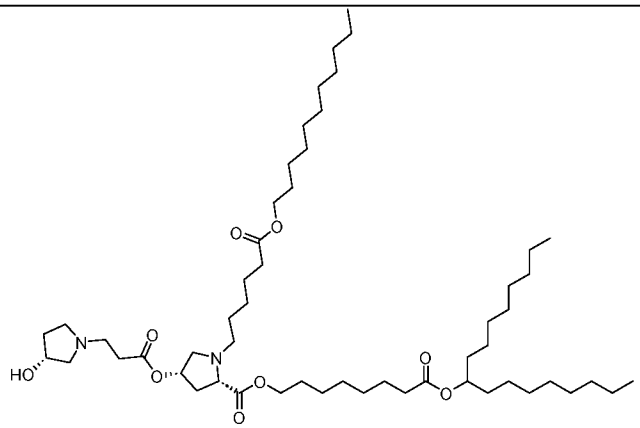
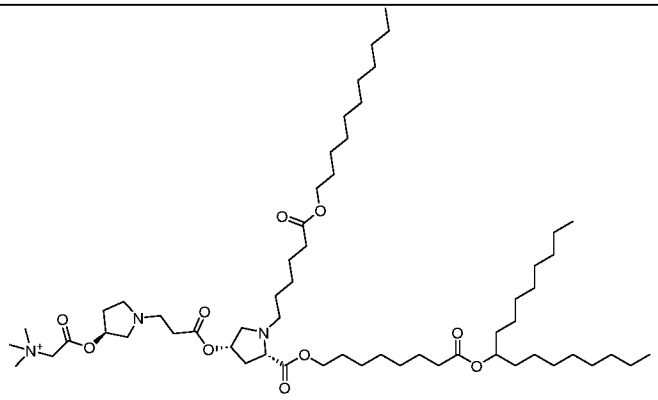
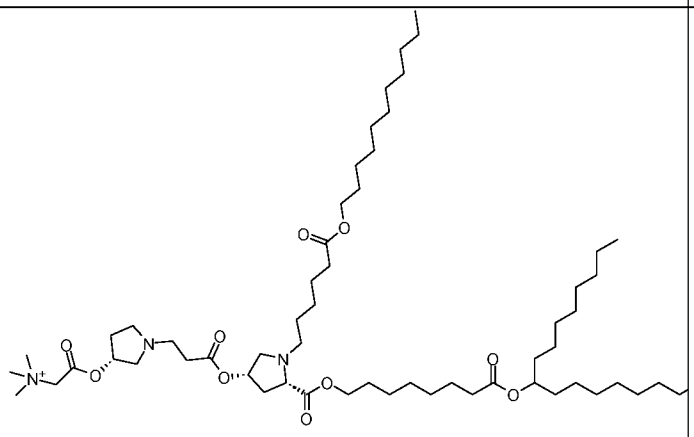
2364		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(3,3-dimethylthioureido)propanoyl)oxy)-1-(6-oxo-6-(tridecan-3-yloxy)hexyl)pyrrolidine-2-carboxylate
2374		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(dimethylamino)propyl)amino)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2422		5-(heptadecan-9-yloxy)-5-oxopentyl (2S,4S)-4-(3-(dimethylamino)propoxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2423		5-(heptadecan-9-yloxy)-5-oxopentyl (2S,4R)-4-(3-(dimethylamino)propoxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2429		8-oxo-8-((4-pentylnonyl)oxy)octyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(8-oxo-8-((4-pentylnonyl)oxy)octyl)pyrrolidine-2-carboxylate

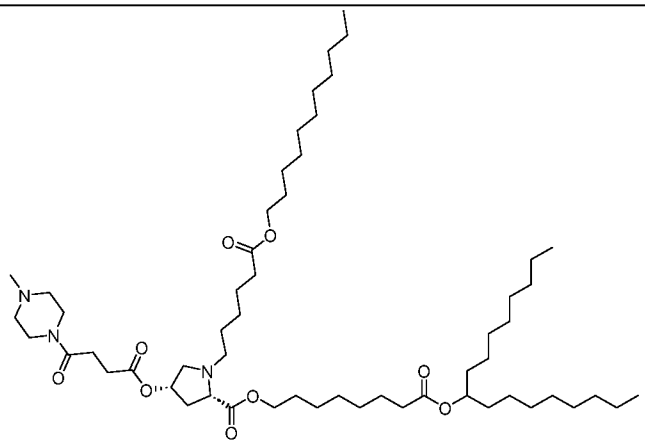
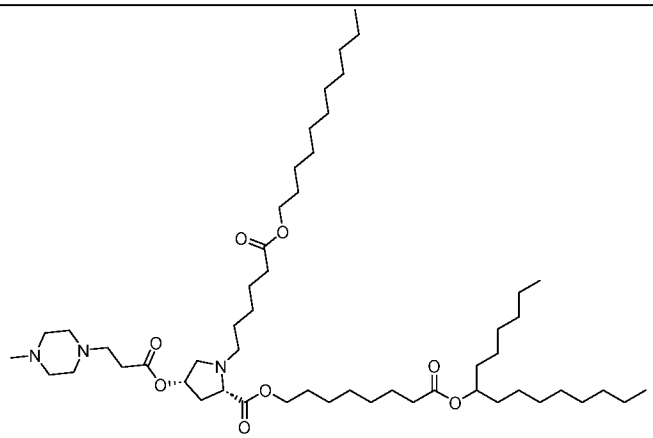
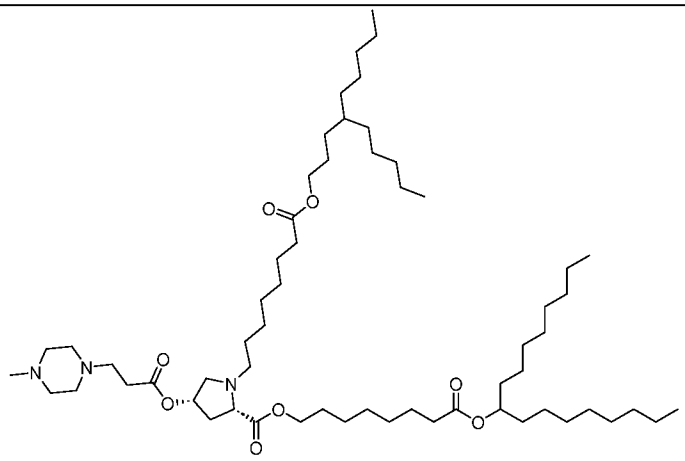
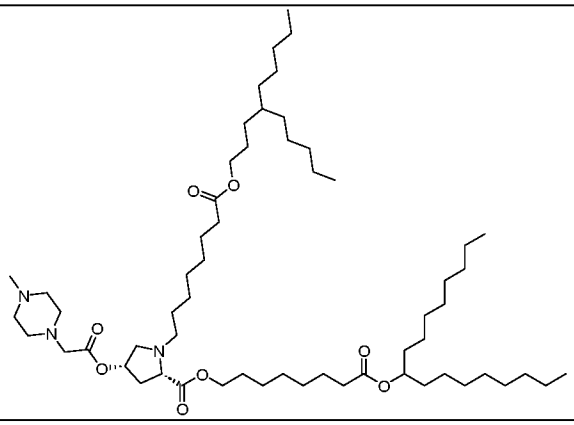
2430		8-oxo-8-((4-pentylnonyl)oxy)octyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2431		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-((4-pentylnonyl)oxy)hexyl)pyrrolidine-2-carboxylate
2438		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-((3-(dimethylamino)propyl)amino)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2451		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-((3-(4-methylpiperazin-1-yl)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate

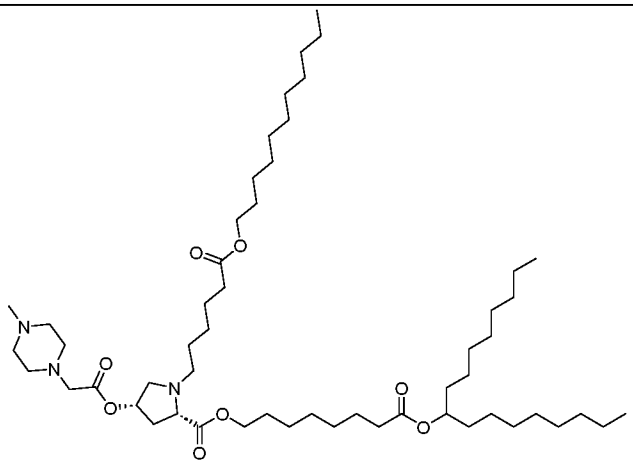
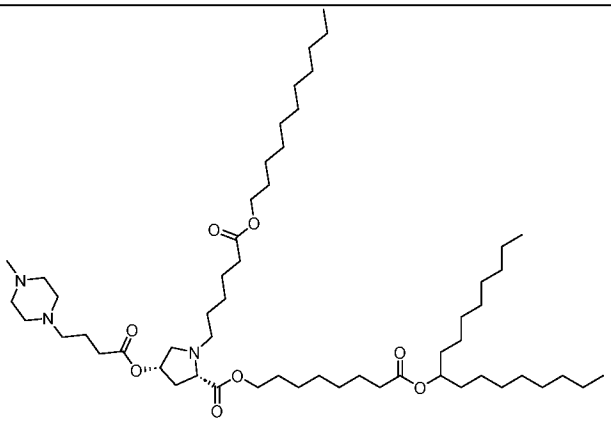
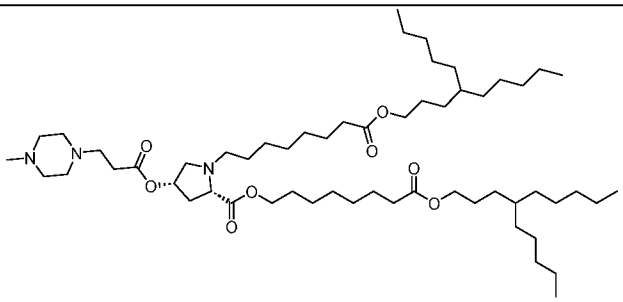
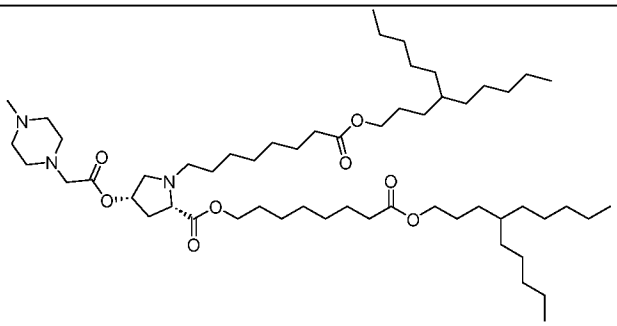
2455		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(tridecan-3-yloxy)hexyl)pyrrolidine-2-carboxylate
1		8-(heptadecan-9-yloxy)-8-oxooctyl (2*S*)-4-((3-(4-(2-hydroxyethyl)piperazin-1-yl)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2		8-(heptadecan-9-yloxy)-8-oxooctyl (2*S*)-4-((2-hydroxyethyl)amino)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
3		8-(heptadecan-9-yloxy)-8-oxooctyl (2*S*)-4-((3-hydroxypropyl)amino)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate

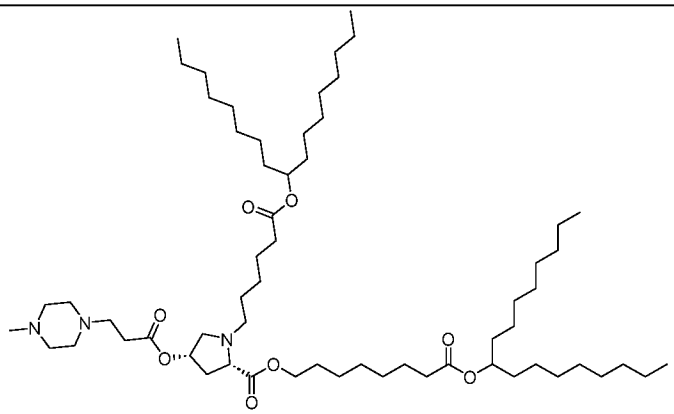
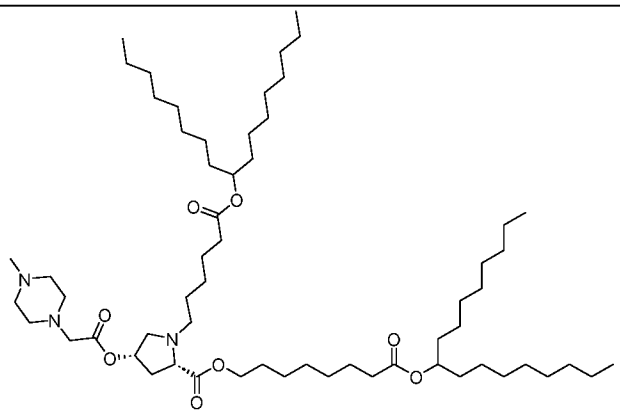
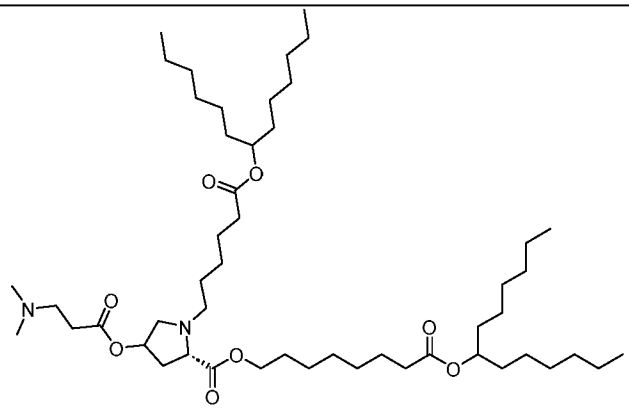
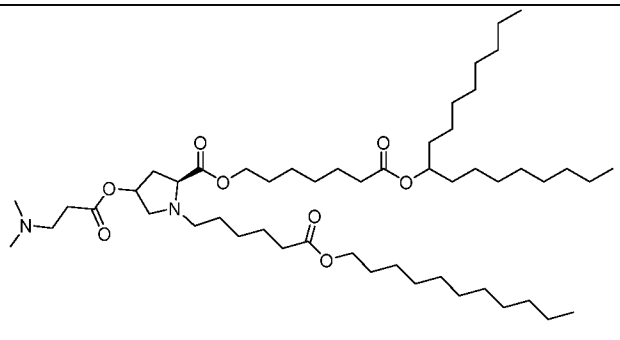
4		8-(heptadecan-9-yloxy)-8-oxooctyl (2*S*)-4-(2-(dimethylamino)ethoxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
5		8-(heptadecan-9-yloxy)-8-oxooctyl (2*S*)-4-(3-(dimethylamino)propoxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
6		6-((2-hexyldecanoyl)oxy)hexyl (2*S*)-1-(6-((2-hexyldecanoyl)oxy)hexyl)-4-((4-hydroxybutyl)amino)pyrrolidine-2-carboxylate
7		6-((2-hexyldecanoyl)oxy)hexyl (2*S*)-1-(6-((2-hexyldecanoyl)oxy)hexyl)-4-((4-hydroxybutyl)(methyl)amino)pyrrolidine-2-carboxylate

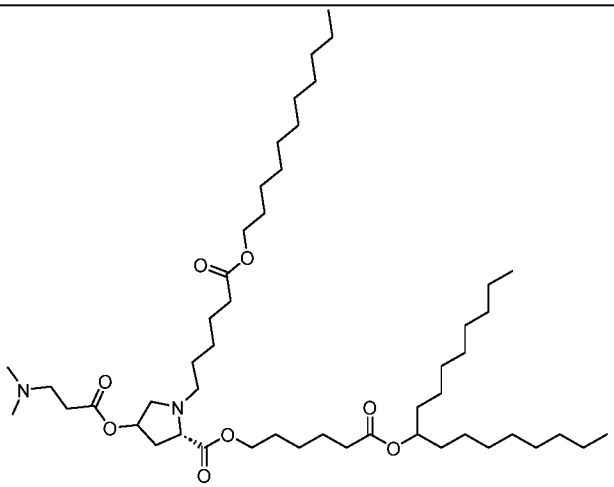
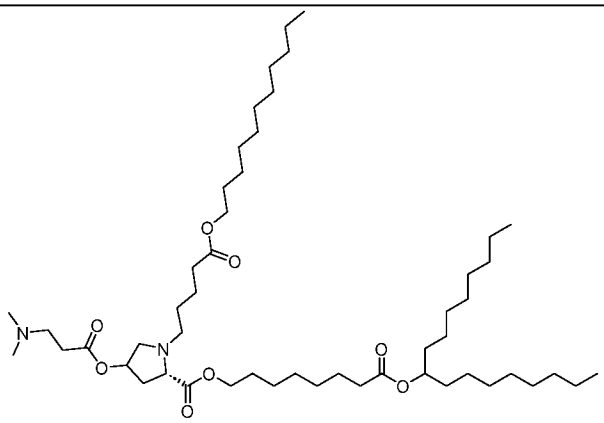
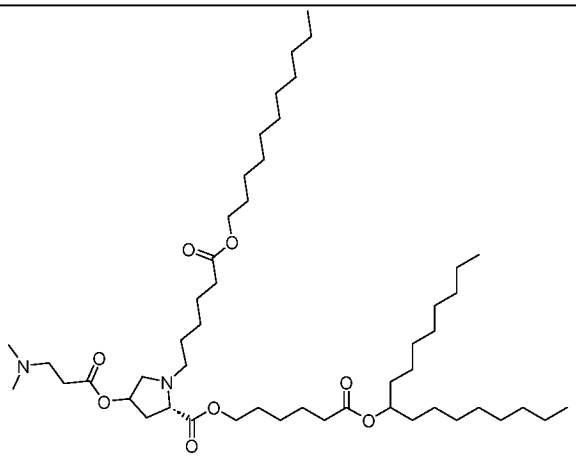
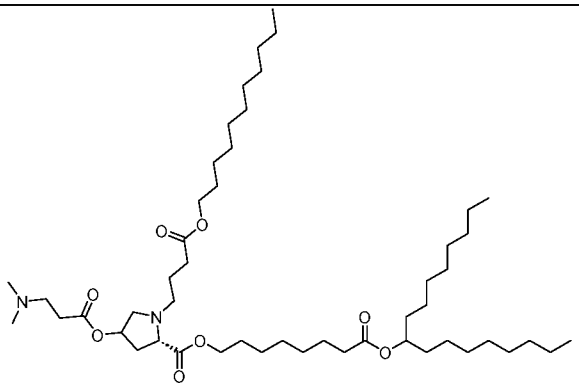
8		6-((2-hexyldecanoyl)oxy)hexyl (2*S*)-1-(6-((2-hexyldecanoyl)oxy)hexyl)-4-((3-hydroxypropyl)(methyl)amino)pyrrolidine-2-carboxylate
9		6-((2-hexyldecanoyl)oxy)hexyl (2*S*)-4-(3-(dimethylamino)propoxy)-1-(6-((2-hexyldecanoyl)oxy)hexyl)pyrrolidine-2-carboxylate
10		6-((2-hexyldecanoyl)oxy)hexyl (2*S*)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-((2-hexyldecanoyl)oxy)hexyl)pyrrolidine-2-carboxylate
11		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-1-(6-oxo-6-(undecyloxy)hexyl)-4-((3-(pyrrolidin-1-yl)propanoyl)oxy)pyrrolidine-2-carboxylate

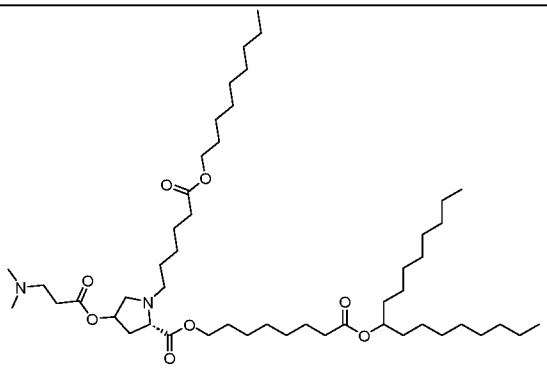
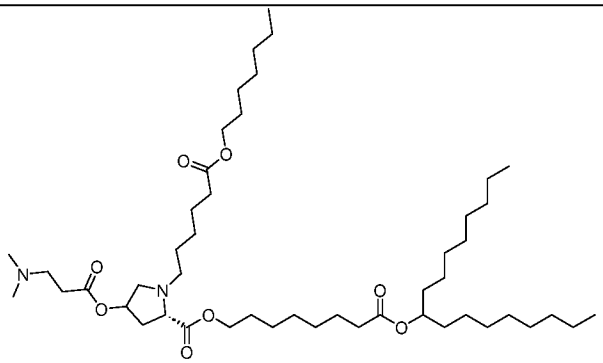
12.		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-((3-((S)-3-hydroxypyrrolidin-1-yl)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
13.		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-((3-((R)-3-hydroxypyrrolidin-1-yl)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
14.		2-(((S)-1-(3-(((3S,5S)-5-(((8-(heptadecan-9-yloxy)-8-oxooctyl)oxy)carbonyl)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidin-3-yl)oxy)-3-oxopropyl)pyrrolidin-3-yl)oxy)-N,N,N-trimethyl-2-oxoethan-1-aminium
15.		2-(((R)-1-(3-(((3S,5S)-5-(((8-(heptadecan-9-yloxy)-8-oxooctyl)oxy)carbonyl)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidin-3-yl)oxy)-3-oxopropyl)pyrrolidin-3-yl)oxy)-N,N,N-trimethyl-2-oxoethan-1-aminium

16.		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-((4-(4-methylpiperazin-1-yl)-4-oxobutanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
17.		8-oxo-8-(pentadecan-7-yloxy)octyl (2S,4S)-4-((3-(4-methylpiperazin-1-yl)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
18.		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-((3-(4-methylpiperazin-1-yl)propanoyl)oxy)-1-(8-oxo-8-((4-pentylnonyl)oxy)octyl)pyrrolidine-2-carboxylate
19.		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-(2-(4-methylpiperazin-1-yl)acetoxy)-1-(8-oxo-8-((4-pentylnonyl)oxy)octyl)pyrrolidine-2-carboxylate

20.		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-(2-(4-methylpiperazin-1-yl)acetoxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
21.		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-((4-(4-methylpiperazin-1-yl)butanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
22.		8-oxo-8-((4-pentylnonyl)oxy)octyl (2S,4S)-4-((3-(4-methylpiperazin-1-yl)propanoyl)oxy)-1-(8-oxo-8-((4-pentylnonyl)oxy)octyl)pyrrolidine-2-carboxylate
23.		8-oxo-8-((4-pentylnonyl)oxy)octyl (2S,4S)-4-(2-(4-methylpiperazin-1-yl)acetoxy)-1-(8-oxo-8-((4-pentylnonyl)oxy)octyl)pyrrolidine-2-carboxylate

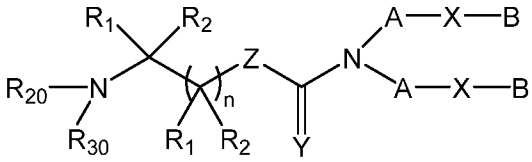
24.		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-1-(6-(heptadecan-9-yloxy)-6-oxohexyl)-4-((3-(4-methylpiperazin-1-yl)propanoyl)oxy)pyrrolidine-2-carboxylate
25.		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-1-(6-(heptadecan-9-yloxy)-6-oxohexyl)-4-(2-(4-methylpiperazin-1-yl)acetoxy)pyrrolidine-2-carboxylate
26.		8-oxo-8-(tridecan-7-yloxy)octyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(tridecan-7-yloxy)hexyl)pyrrolidine-2-carboxylate
27.		7-(heptadecan-9-yloxy)-7-oxoheptyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate

28.		6-(heptadecan-9-yloxy)-6-oxohexyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
29.		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(5-oxo-5-(undecyloxy)pentyl)pyrrolidine-2-carboxylate
30.		6-(heptadecan-9-yloxy)-6-oxohexyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
31.		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(4-oxo-4-(undecyloxy)butyl)pyrrolidine-2-carboxylate

32.		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-(nonyloxy)-6-oxohexyl)pyrrolidine-2-carboxylate
33.		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-(heptyloxy)-6-oxohexyl)pyrrolidine-2-carboxylate

Ionizable lipid compounds iii)

[0274] In some embodiments, the ionizable lipid is represented by formula



(III), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein:

$\mathbf{R_{20}}$ and $\mathbf{R_{30}}$ are each independently H, C_1-C_5 branched or unbranched alkyl, or C_2-C_5 branched or unbranched alkenyl, or

$\mathbf{R_{20}}$ and $\mathbf{R_{30}}$ together with the adjacent N atom form a 3 to 7 membered cyclic ring, optionally substituted with $\mathbf{R^a}$;

$\mathbf{R^a}$ is H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, or SH;

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, OH, halogen, SH, or $N\mathbf{R_{10}R_{11}}$, or

$\mathbf{R_1}$ and $\mathbf{R_2}$ are taken together to form a cyclic ring;

each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or

$\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring;

n is 0, 1, 2, 3 or 4;

Y is O or S;

Z is absent, O, S, or N(**R12**)(**R12**), wherein each **R12** is independently H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl, provided that when Z is not absent, the adjacent **R1** and **R2** cannot be OH, N**R10**R11, or SH;

each **A** is each independently C_1-C_{16} branched or unbranched alkyl, or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen;

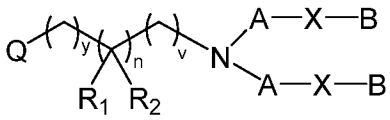
each **B** is each independently C_1-C_{16} branched or unbranched alkyl, or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen;

each **X** is independently a biodegradable moiety.

[0275] In some embodiments, **R20** and **R30** are each independently H or C_1-C_3 branched or unbranched alkyl. In some embodiments, **R20** and **R30** together with the adjacent N atom form a 3 to 7 membered cyclic ring, optionally substituted with **Ra**. In some embodiments, **Ra** is H, C_1-C_3 branched or unbranched alkyl or OH. In one embodiment, **Ra** is H or OH.

[0276] In some embodiments, **Z** is absent, S, O, or NH. In some embodiments, n is 0, 1, or 2.

[0277] In some embodiments, the ionizable lipid is represented by formula (V):



(V), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein

R1 is H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, OH, halogen, SH, or N**R10**R11, and

R2 is H, OH, halogen, SH, or N**R10**R11, or

R1 and **R2** are taken together to form a cyclic ring;

R10 and **R11** are each independently H or C_1-C_3 alkyl, or **R10** and **R11** are taken together to form a heterocyclic ring;

Q is OH or -$(OCH_2CH_2)_u$N**R20R30**,

R20 and **R30** are each independently H, C_1-C_5 branched or unbranched alkyl, or C_2-C_5 branched or unbranched alkenyl, or

R20 and **R30** together with the adjacent N atom form a 3 to 7 membered cyclic ring optionally substituted with **Ra**;

Ra is H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, or SH;

u is 0, 1, 2, 3, 4, 5, 6, 7, or 8;

v is 0, 1, 2, 3, or 4;

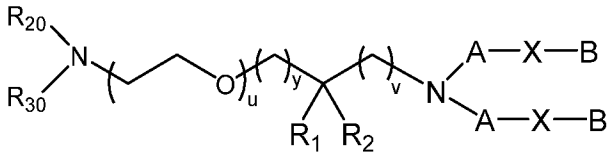
y is 0, 1, 2, 3, or 4;

each **A** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen;

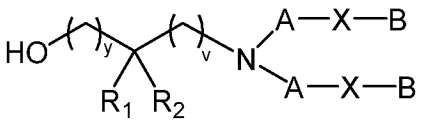
each **B** is each independently C_1-C_{16} branched or unbranched alkyl or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen; and

each **X** is independently a biodegradable moiety.

[0278] In some embodiments, the disclosure relates to ionizable lipids of one of the following formulas:



(VA),



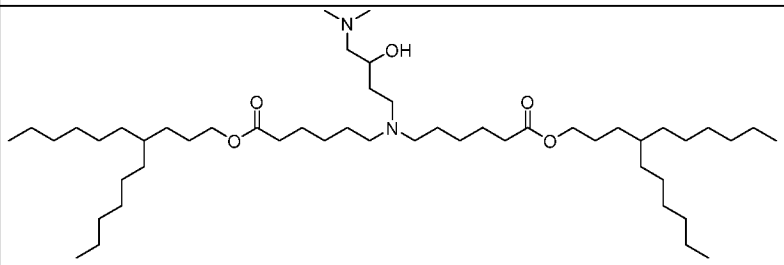
(VB), wherein: **u** is 0, 1, 2, 3, 4, 5, 6, 7, or 8; **v** is 0, 1, 2, 3, or 4; and **y** is 0, 1, 2, 3, or 4. Other variables are defined as in formulas III) and V) above.

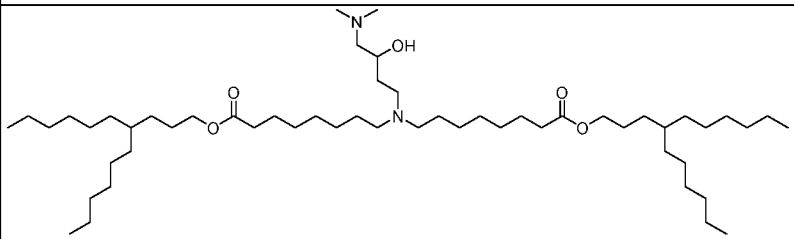
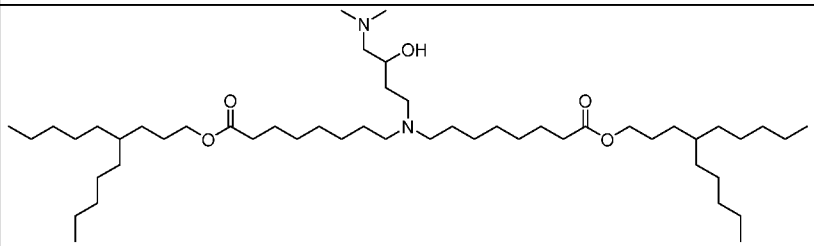
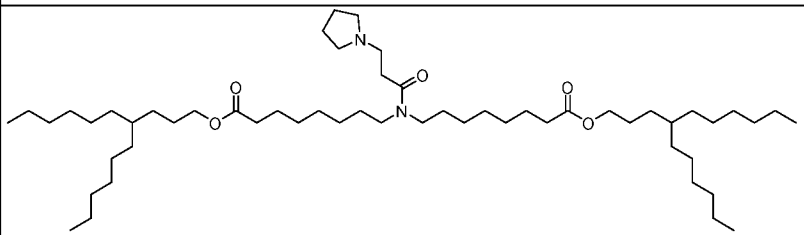
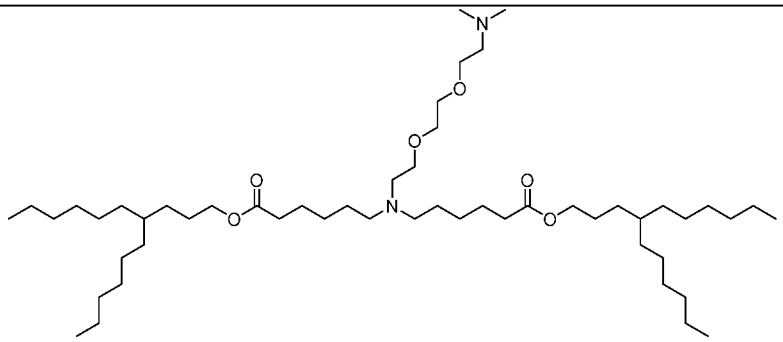
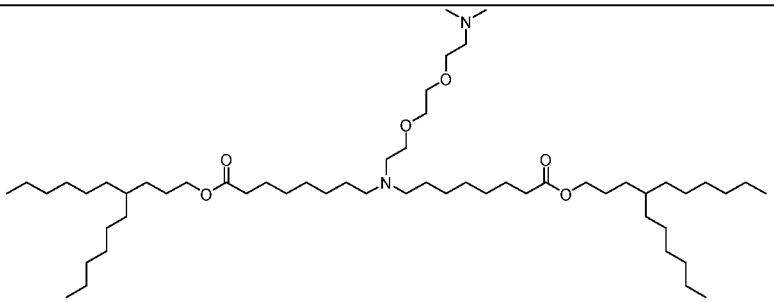
[0279] In some embodiments, in the above formulas, X is –OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -C(O-**R_{13}**)-O-, -C(O)O(CH_2)$_s$-, -OC(O)(CH_2)$_s$-, -C(O)N(R^7)(CH_2)$_s$-, -N(R^7)C(O)(CH_2)$_s$-, -C(O-**R_{13}**)-O-(CH_2)$_s$-, wherein each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxyalkyl, or aminoalkyl, each **R_{13}** is independently C_3-C_{10} alkyl, and each s is independently 0-16. In some embodiments, X is –OC(O)-, –C(O)O-, -C(O)O(CH_2)$_s$-, or -OC(O)(CH_2)$_s$-. In some embodiments, s is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

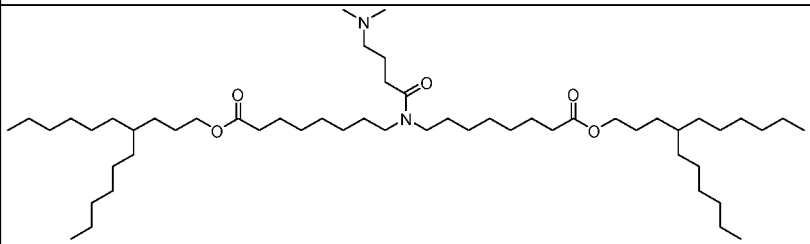
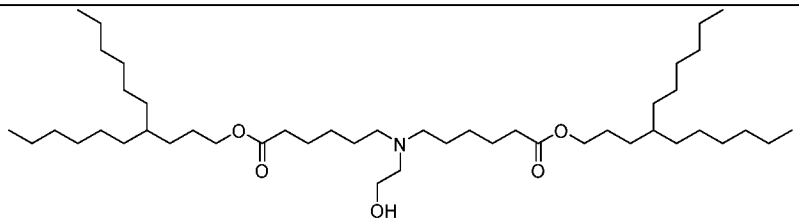
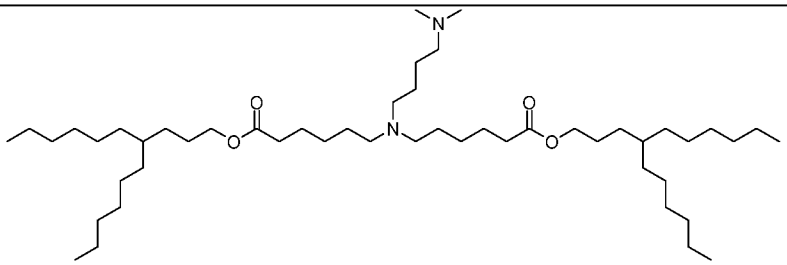
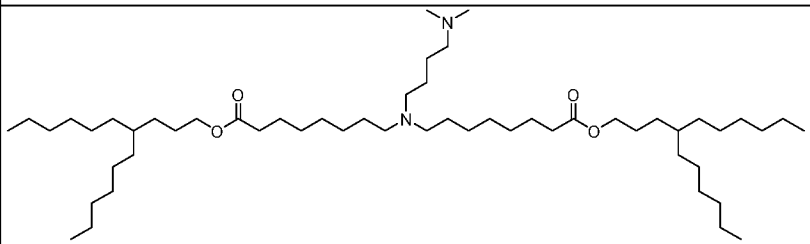
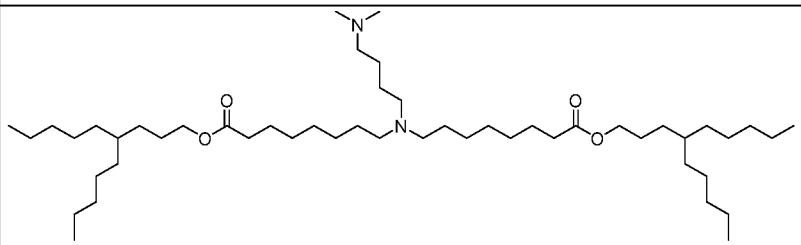
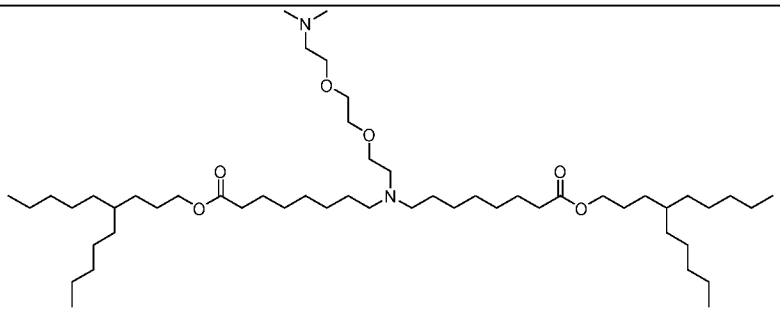
[0280] More embodiments of the ionizable lipid of formula (III) or (V), in the Ionizable lipid compounds group iii), may be found in PCT Application No. PCT/US22/50111, filed on November 16, 2022, the content of which is incorporated herein by reference in its entirety. In particular, all the ionizable lipids of formulas (IO)-(VIIO) and formulas (I)-(VIID) of PCT Application No. PCT/US22/50111 are suitable for use as the ionizable lipids in this disclosure, and are incorporated herein by reference in its entirety.

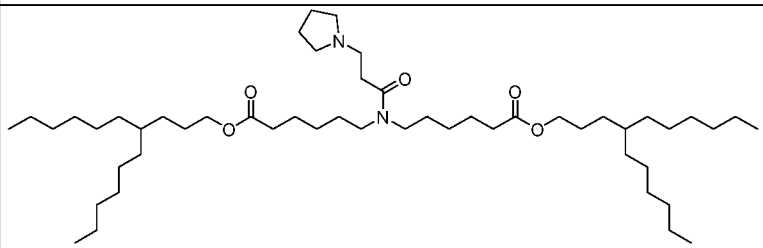
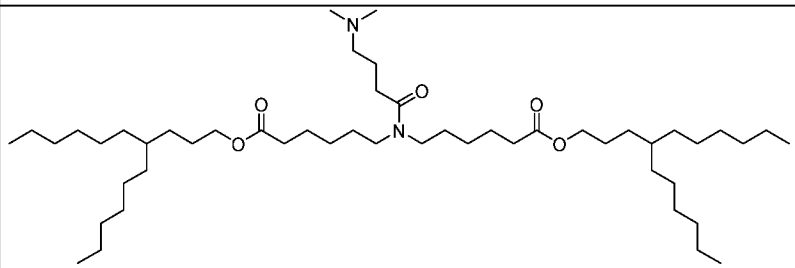
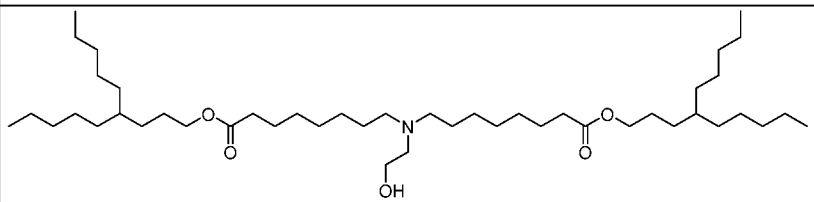
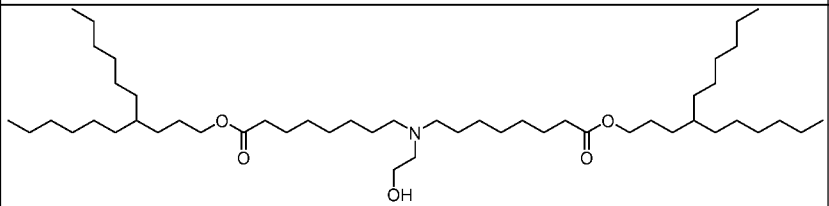
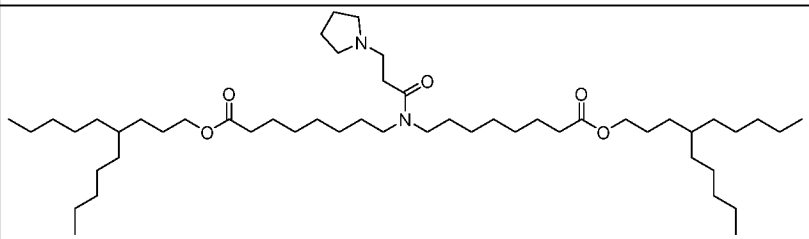
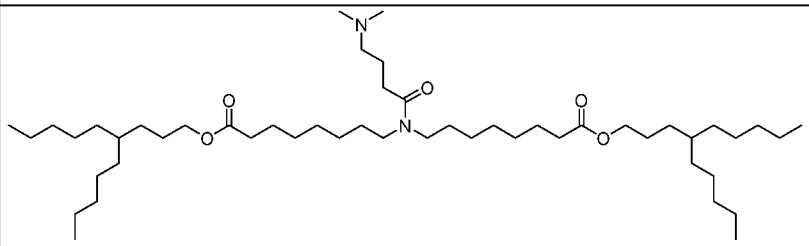
[0281] Certain exemplary ionizable lipid compounds disclosed herein are set forth in Table III below.

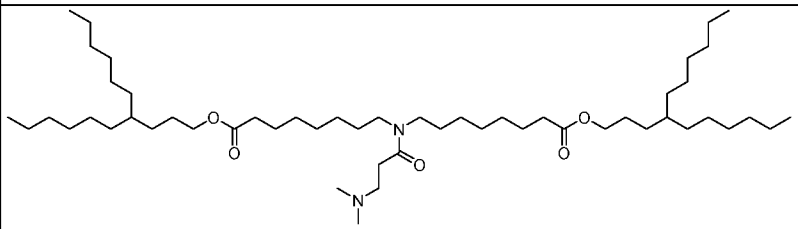
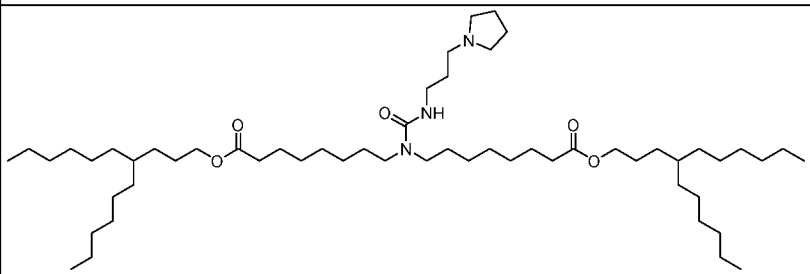
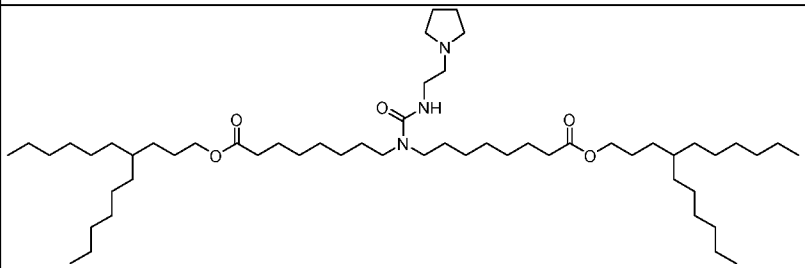
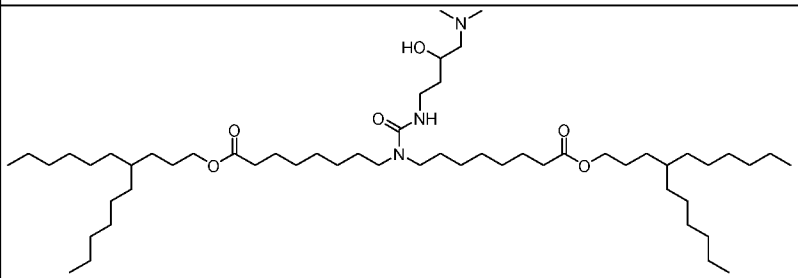
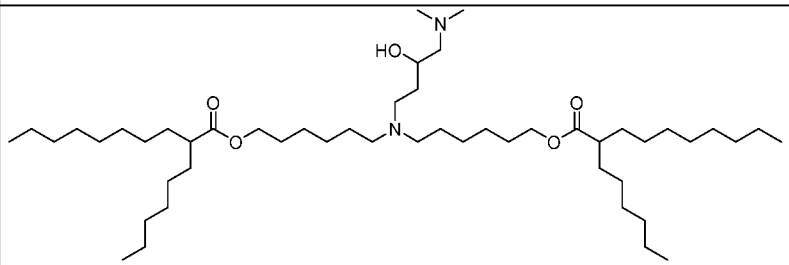
Table III. Exemplary ionizable lipid compounds.

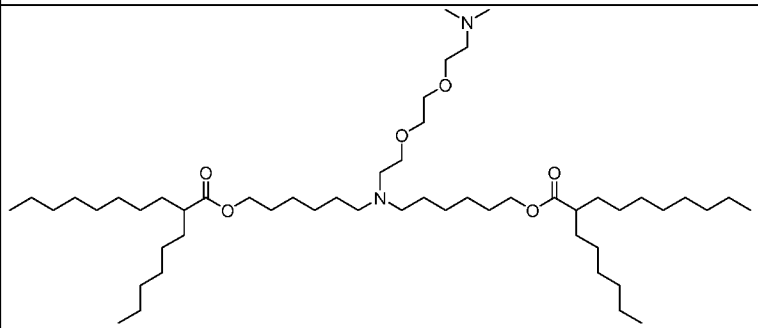
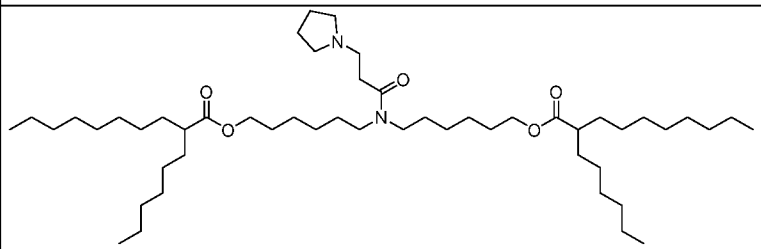
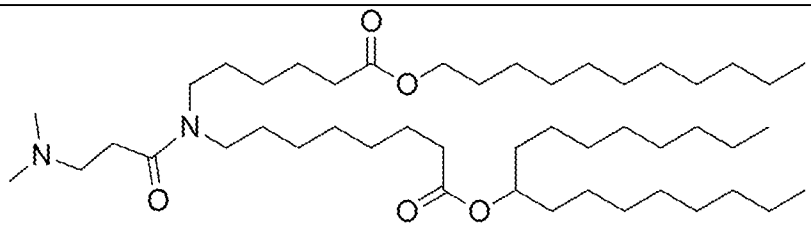
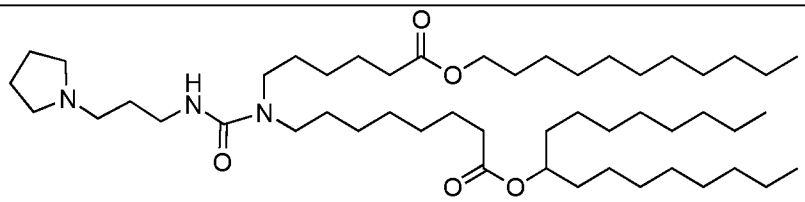
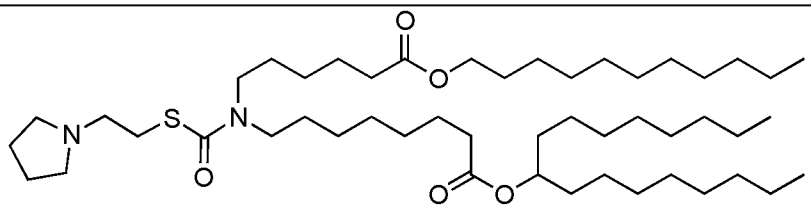
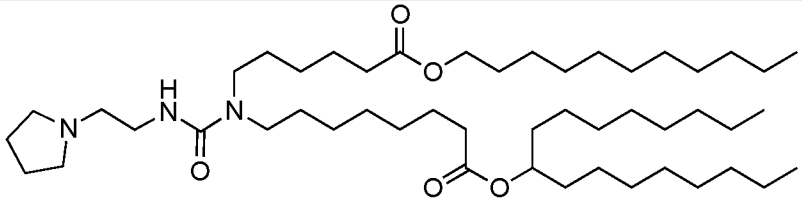
Lipid No.	Structure	IUPAC Name
2140		bis(4-hexyldecyl) 6,6'-((4-(dimethylamino)-3-hydroxybutyl)azanediyl)dihexanoate

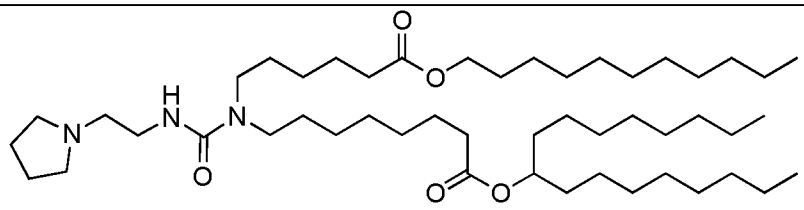
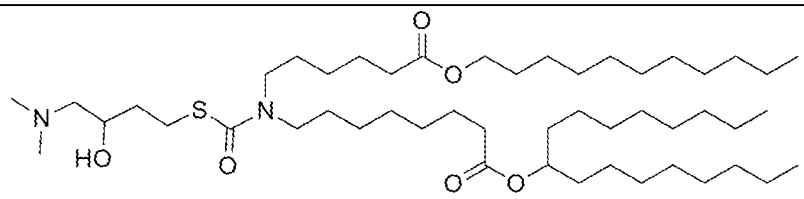
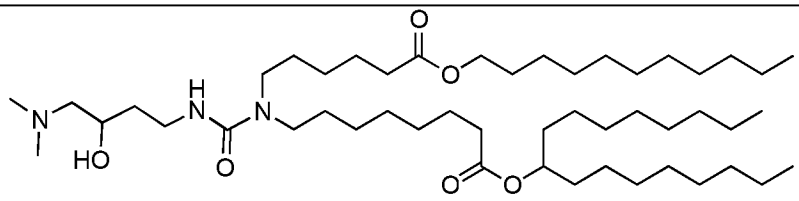
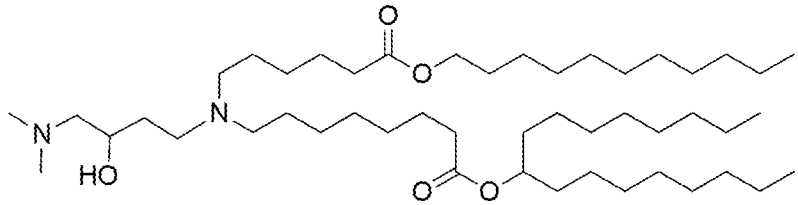
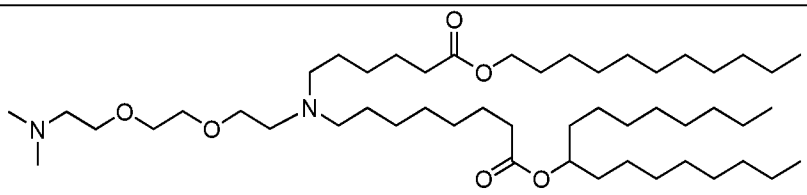
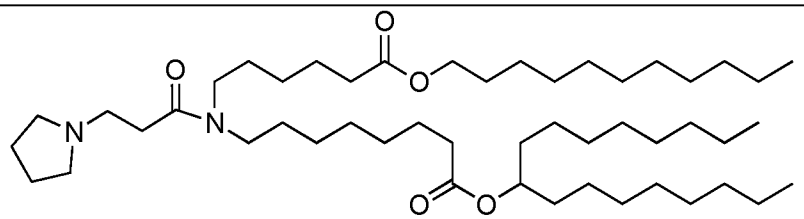
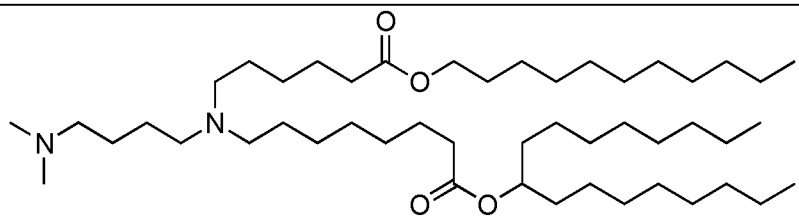
Lipid No.	Structure	IUPAC Name
2139		bis(4-hexyldecyl) 8,8'-((4-(dimethylamino)-3-hydroxybutyl)azanediyl)dioctanoate
2138		bis(4-pentylnonyl) 8,8'-((4-(dimethylamino)-3-hydroxybutyl)azanediyl)dioctanoate
2142		bis(4-hexyldecyl) 8,8'-((3-(pyrrolidin-1-yl)propanoyl)azanediyl)dioctanoate
2146		4-hexyldecyl 11-(6-((4-hexyldecyl)oxy)-6-oxohexyl)-2-methyl-5,8-dioxa-2,11-diazaheptadecan-17-oate
2145		4-hexyldecyl 11-(8-((4-hexyldecyl)oxy)-8-oxooctyl)-2-methyl-5,8-dioxa-2,11-diazanonadecan-19-oate

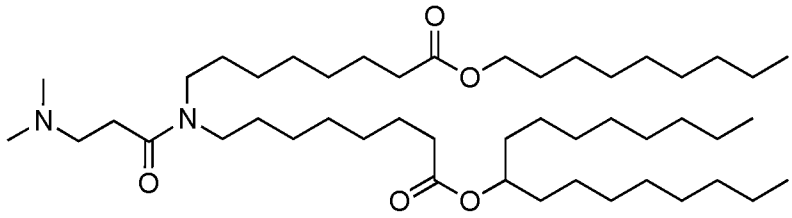
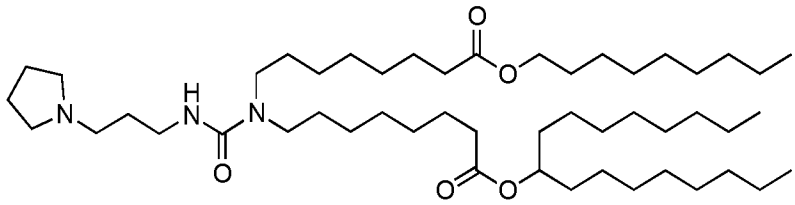
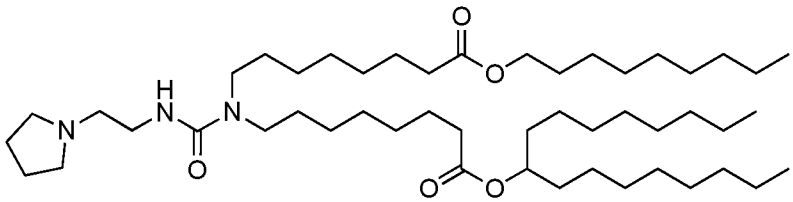
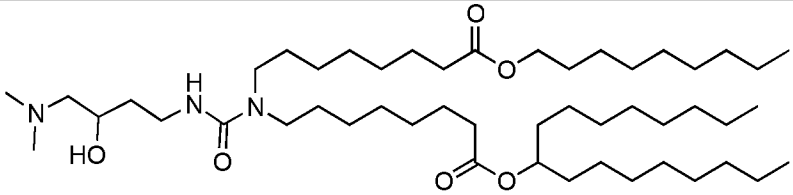
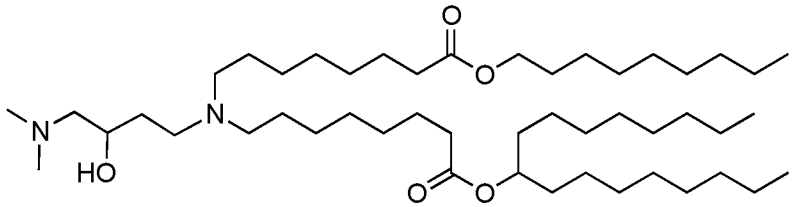
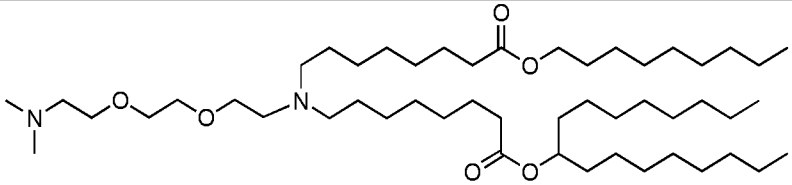
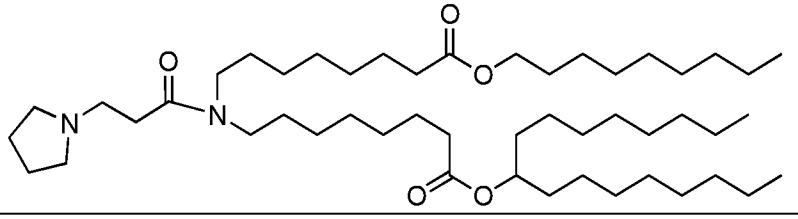
Lipid No.	Structure	IUPAC Name
2133		bis(4-hexyldecyl) 8,8'-((4-(dimethylamino)butanoyl)azanediyl)dioctanoate
2137		bis(4-hexyldecyl) 6,6'-((2-hydroxyethyl)azanediyl)dihexanoate
2131		bis(4-hexyldecyl) 6,6'-((4-(dimethylamino)butyl)azanediyl)dihexanoate
2130		bis(4-hexyldecyl) 8,8'-((4-(dimethylamino)butyl)azanediyl)dioctanoate
2129		bis(4-pentylnonyl) 8,8'-((4-(dimethylamino)butyl)azanediyl)dioctanoate
2144		4-pentylnonyl 2-methyl-11-(8-oxo-8-((4-pentylnonyl)oxy)octyl)-5,8-dioxa-2,11-diazanonadecan-19-oate

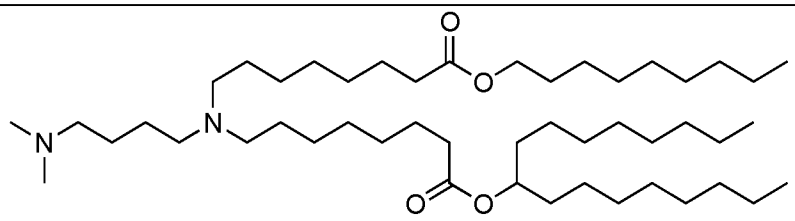
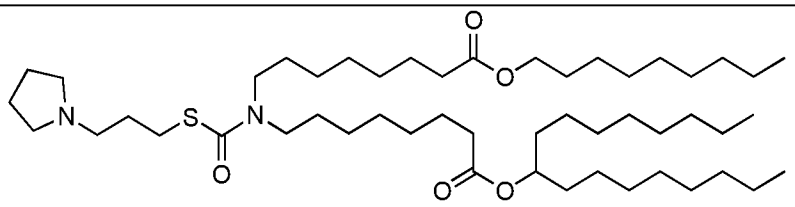
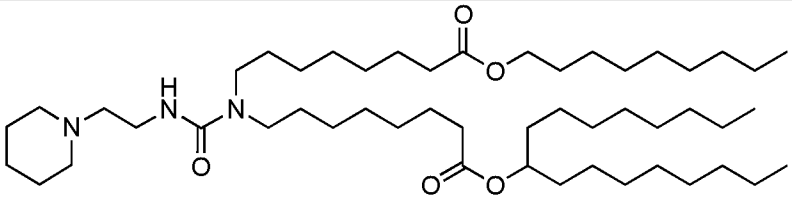
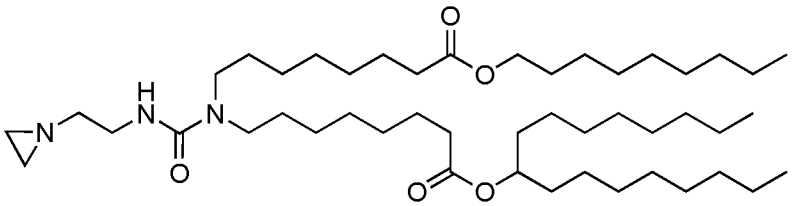
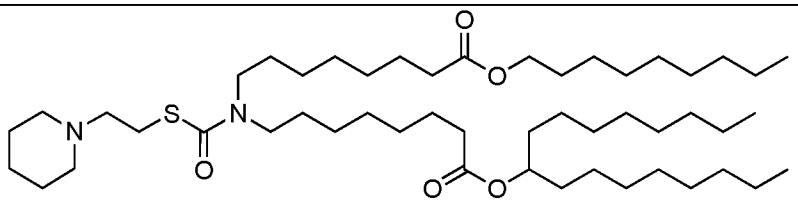
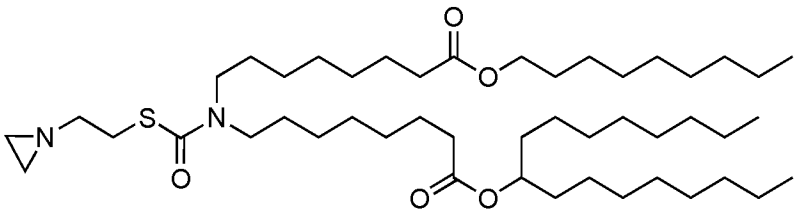
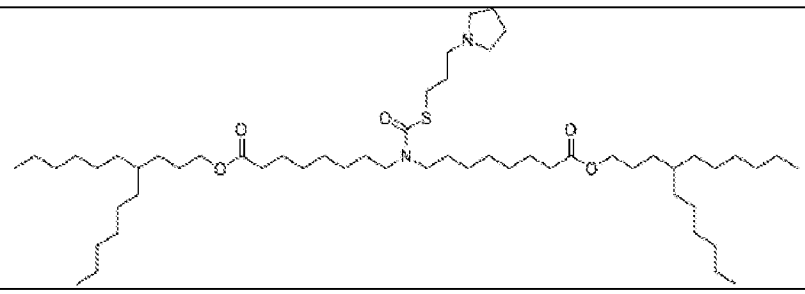
Lipid No.	Structure	IUPAC Name
2143		bis(4-hexyldecyl) 6,6'-((3-(pyrrolidin-1-yl)propanoyl)azanediyl)dihexanoate
2134		bis(4-hexyldecyl) 6,6'-((4-(dimethylamino)butanoyl)azanediyl)dihexanoate
2135		bis(4-pentylnonyl) 8,8'-((2-hydroxyethyl)azanediyl)dioctanoate
2136		bis(4-hexyldecyl) 8,8'-((2-hydroxyethyl)azanediyl)dioctanoate
2141		bis(4-pentylnonyl) 8,8'-((3-(pyrrolidin-1-yl)propanoyl)azanediyl)dioctanoate
2132		bis(4-pentylnonyl) 8,8'-((4-(dimethylamino)butanoyl)azanediyl)dioctanoate

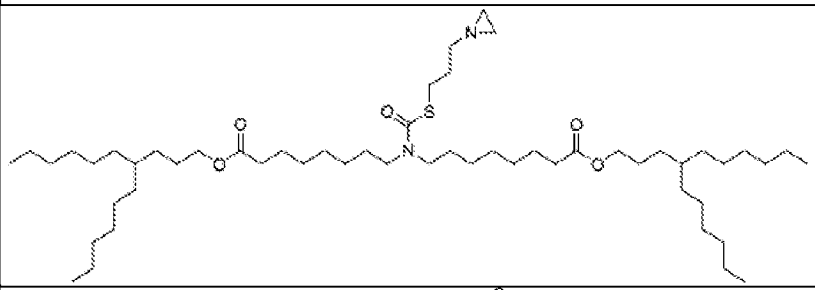
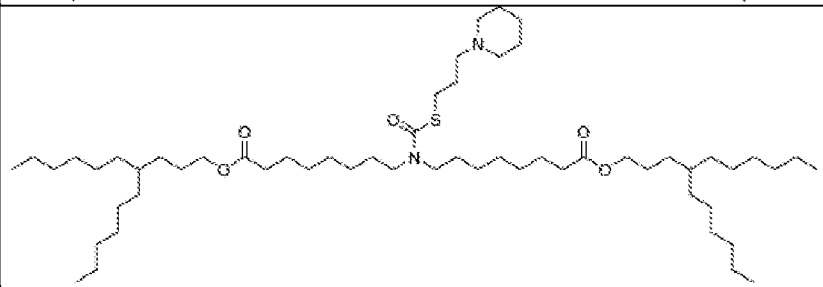
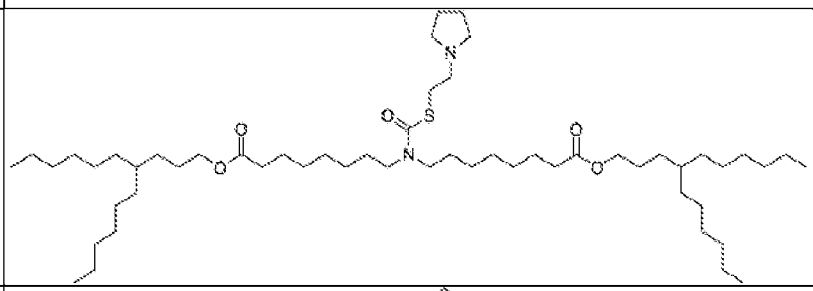
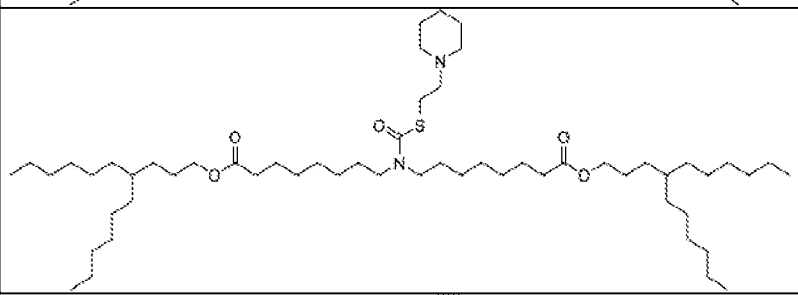
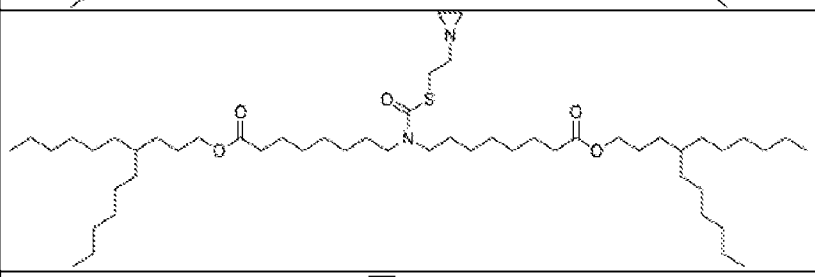
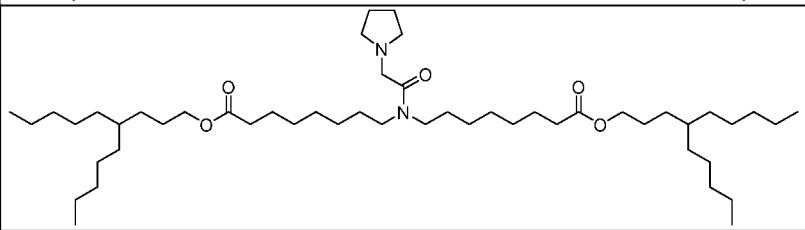
Lipid No.	Structure	IUPAC Name
2229		bis(4-hexyldecyl) 8,8'-((3-(dimethylamino)propanoyl)azanediyl)dioctanoate
2228		bis(4-hexyldecyl) 8,8'-(((3-(pyrrolidin-1-yl)propyl)carbamoyl)azanediyl)dioctanoate
2227		bis(4-hexyldecyl) 8,8'-(((2-(pyrrolidin-1-yl)ethyl)carbamoyl)azanediyl)dioctanoate
2226		bis(4-hexyldecyl) 8,8'-(((4-(dimethylamino)-3-hydroxybutyl)carbamoyl)azanediyl)dioctanoate
2225		((4-(dimethylamino)-3-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl) bis(2-hexyldecanoate)

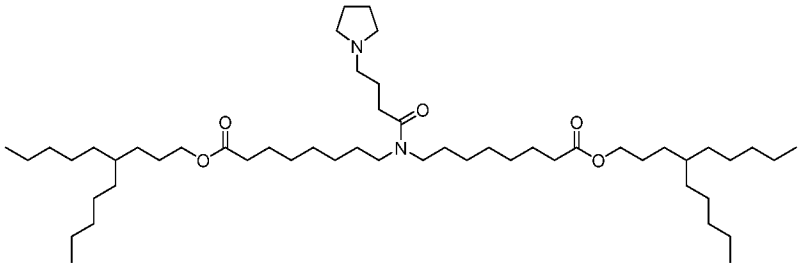
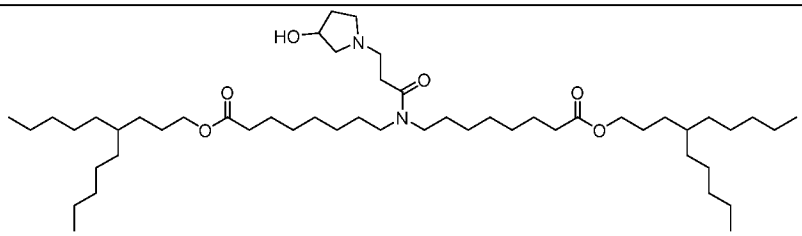
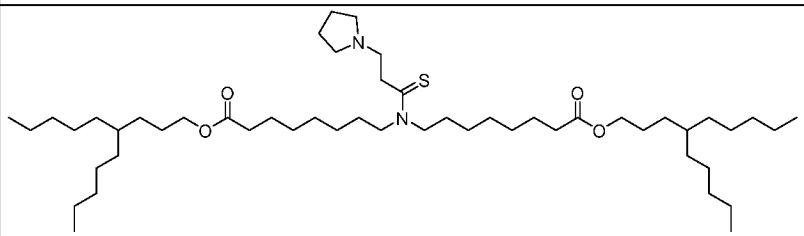
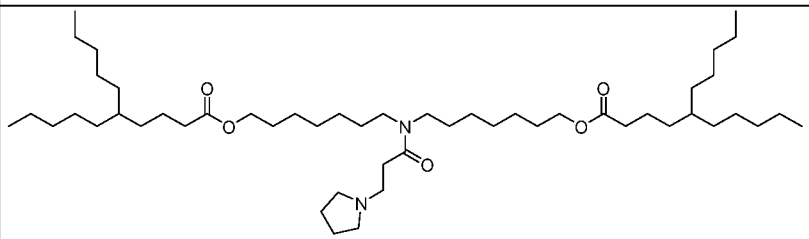
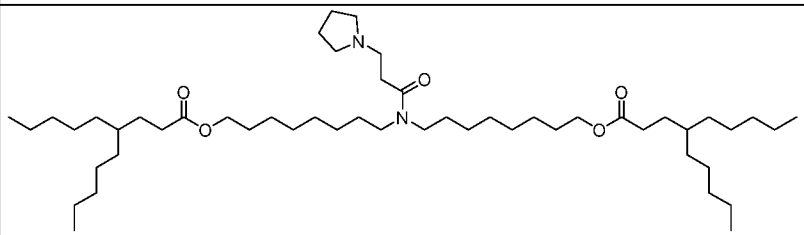
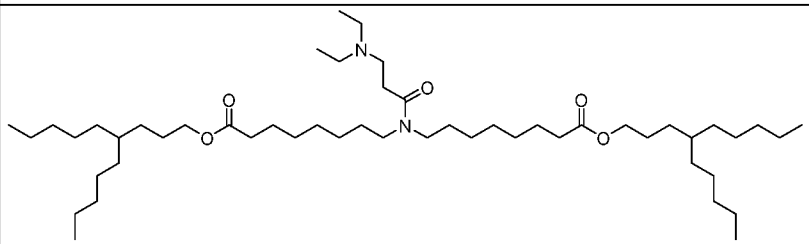
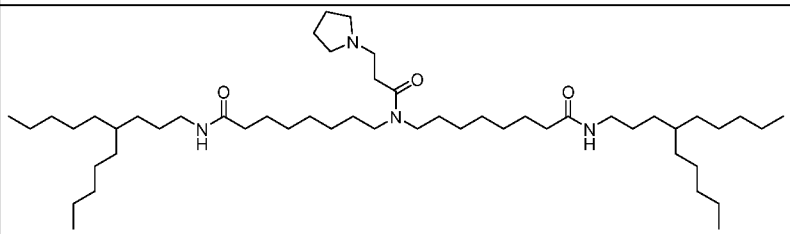
Lipid No.	Structure	IUPAC Name
2216		11-(6-((2-hexyldecanoyl)oxy)hexyl)-2-methyl-5,8-dioxa-2,11-diazaheptadecan-17-yl 2-hexyldecanoate
2215		((3-(pyrrolidin-1-yl)propanoyl)azanediyl)bis(hexane-6,1-diyl) bis(2-hexyldecanoate)
		
		
		
		

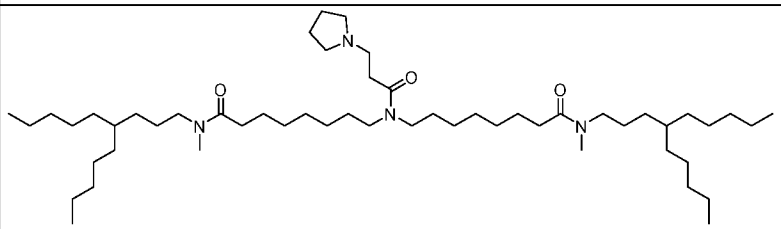
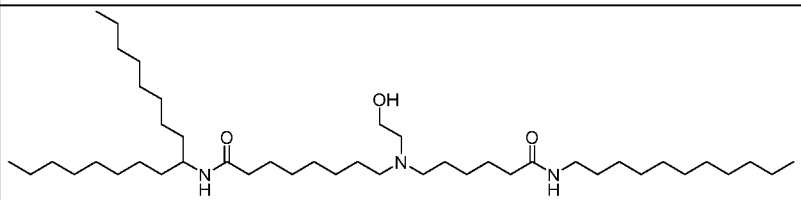
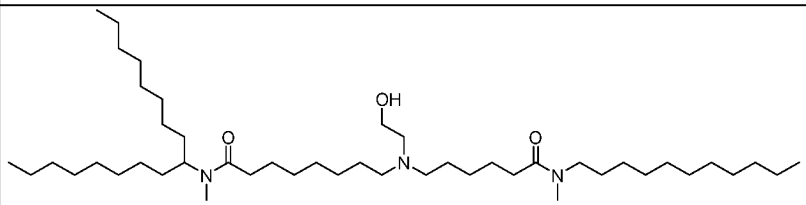
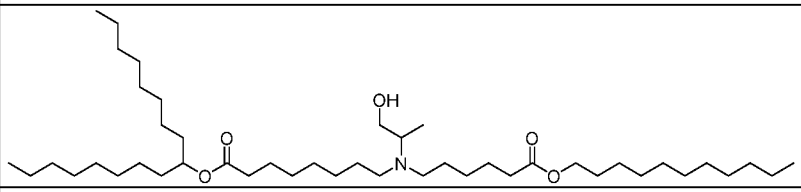
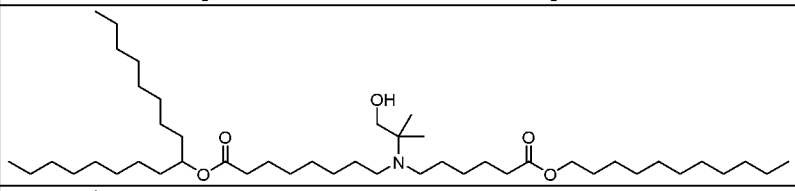
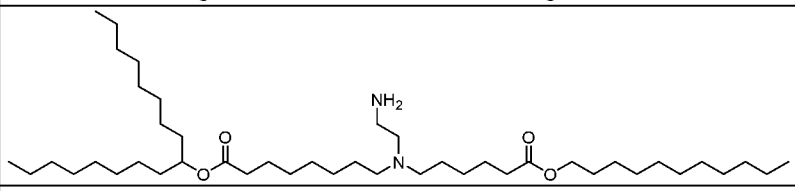
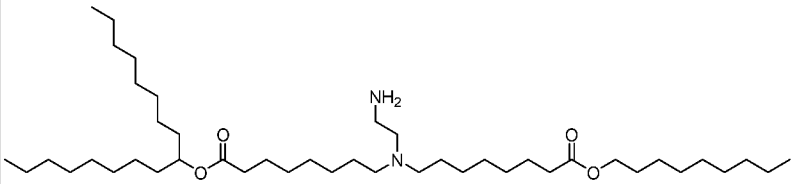
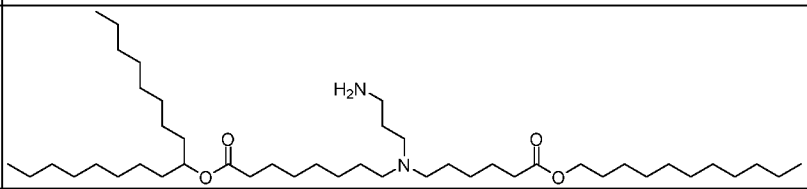
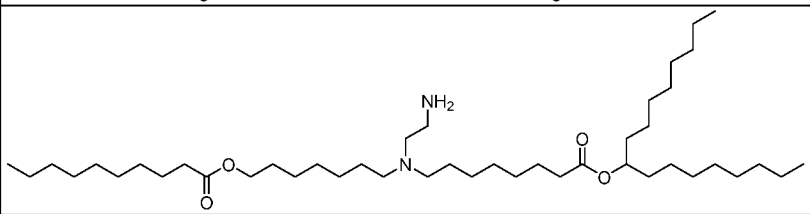
Lipid No.	Structure	IUPAC Name
		
		
		
		
		
		
		

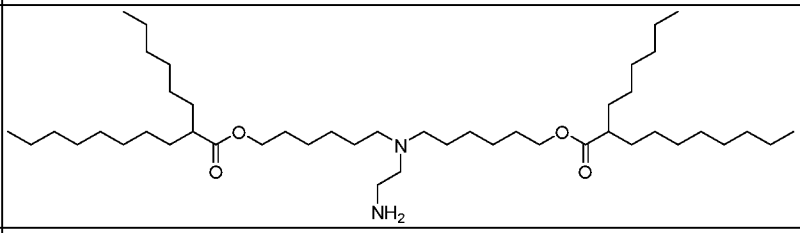
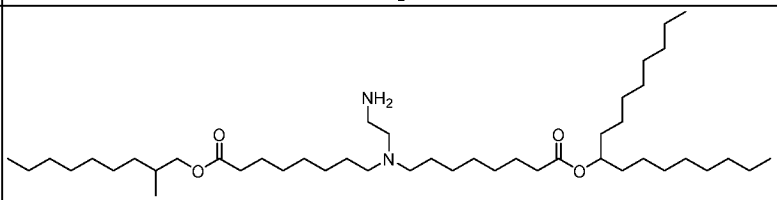
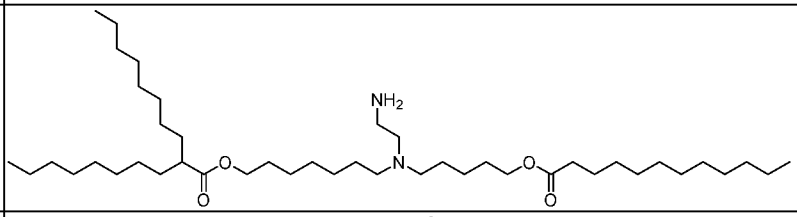
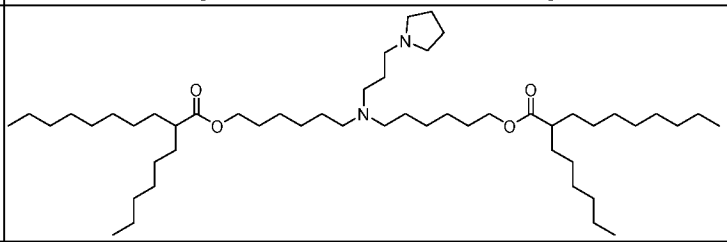
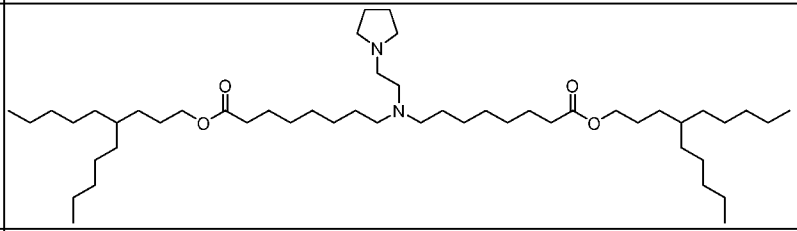
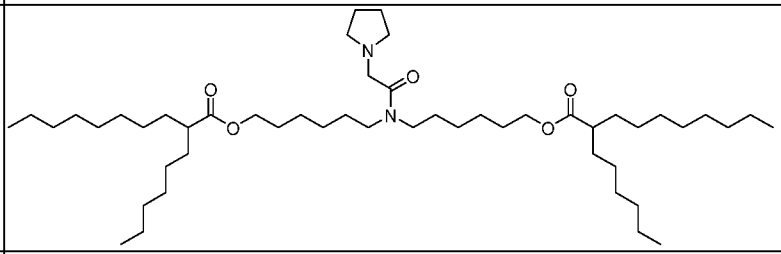
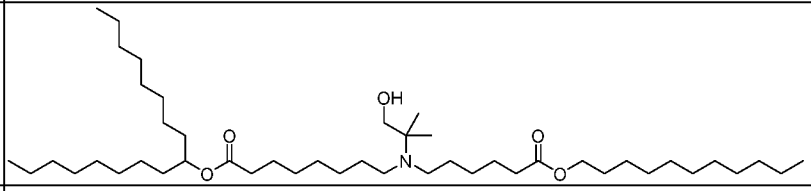
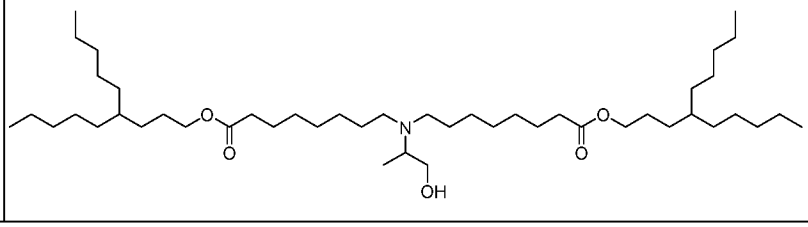
Lipid No.	Structure	IUPAC Name
		
		
		
		
		
		
		

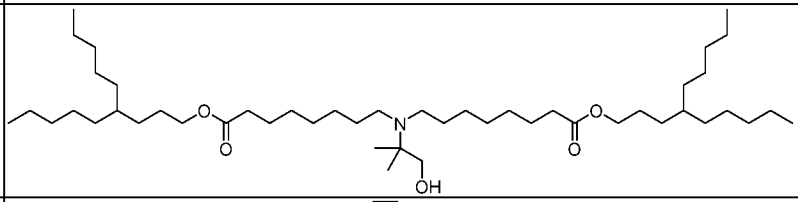
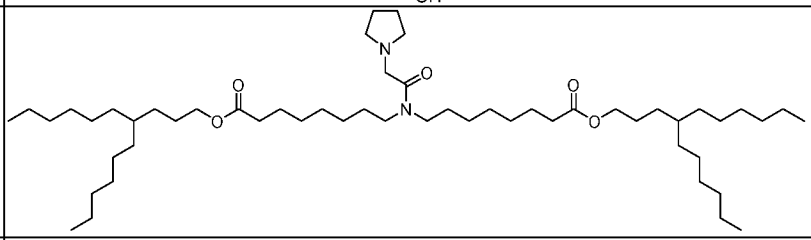
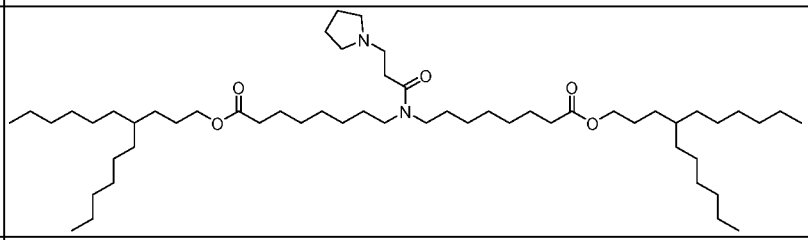
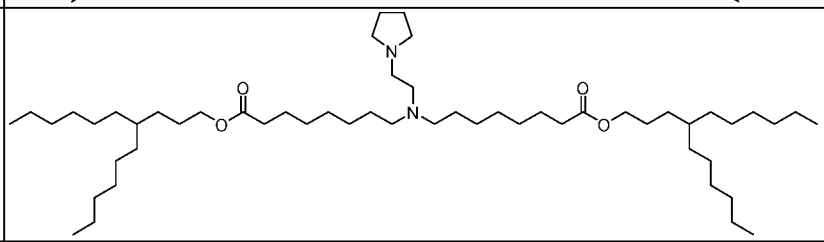
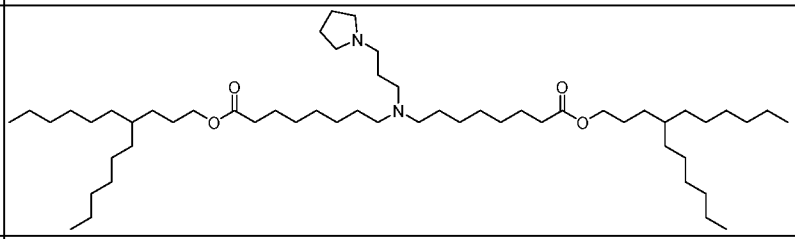
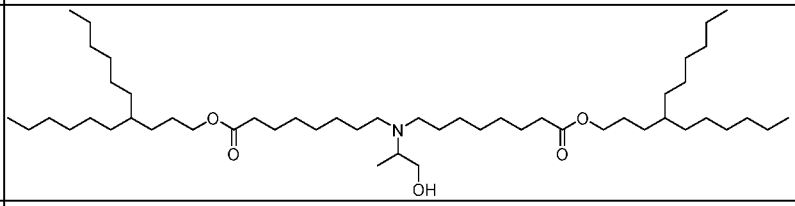
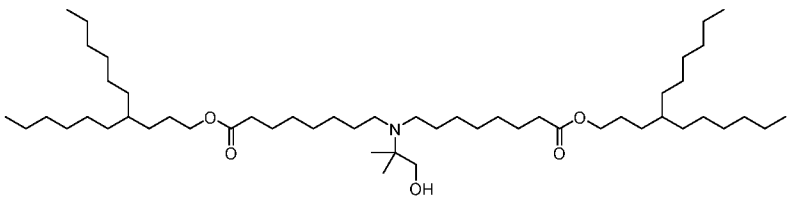
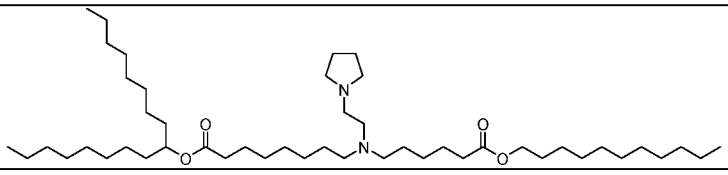
Lipid No.	Structure	IUPAC Name
		
		
		
		
		
		
		

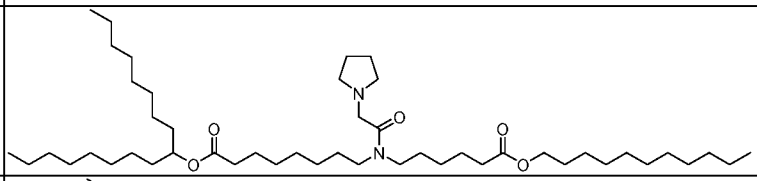
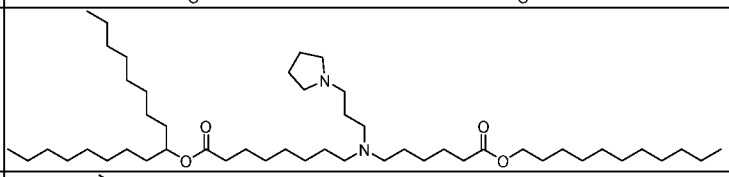
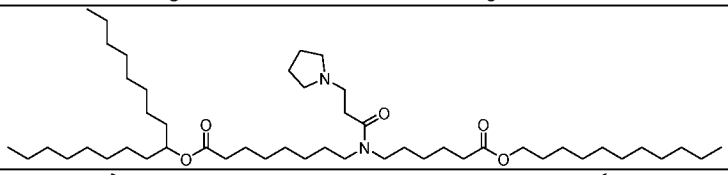
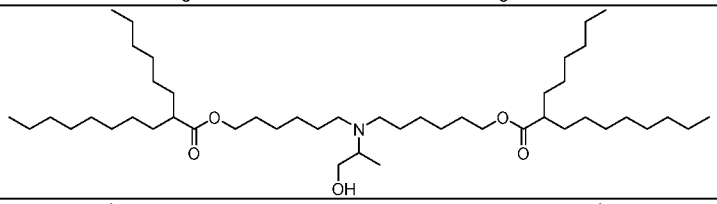
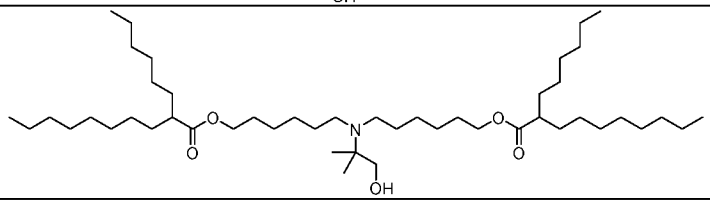
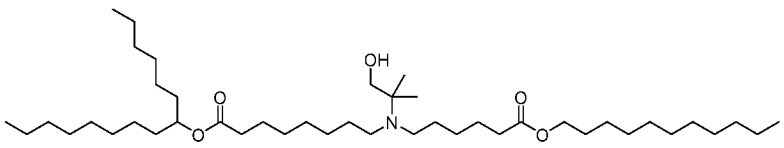
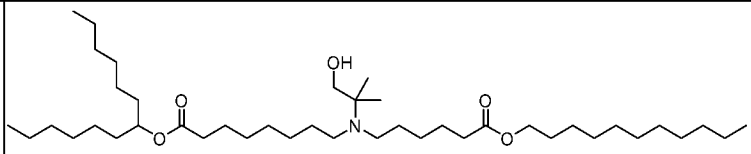
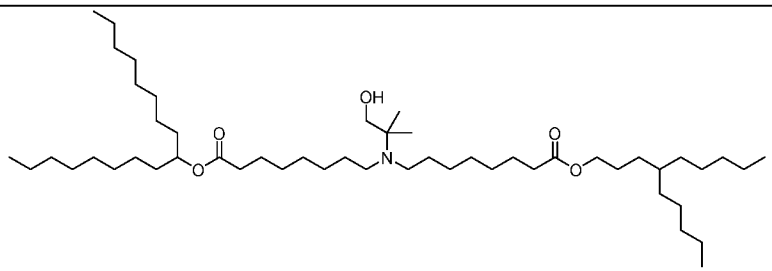
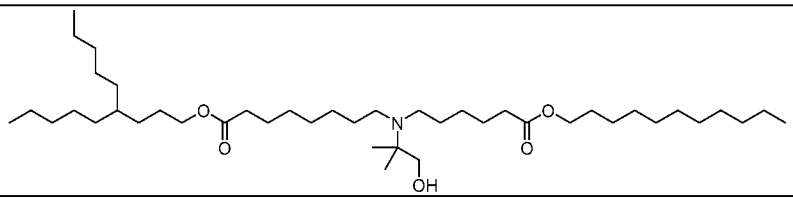
Lipid No.	Structure	IUPAC Name
		
		
		
		
		
2233		bis(4-pentylnonyl) 8,8'-((2-(pyrrolidin-1-yl)acetyl)azanediyl)dioctanoate

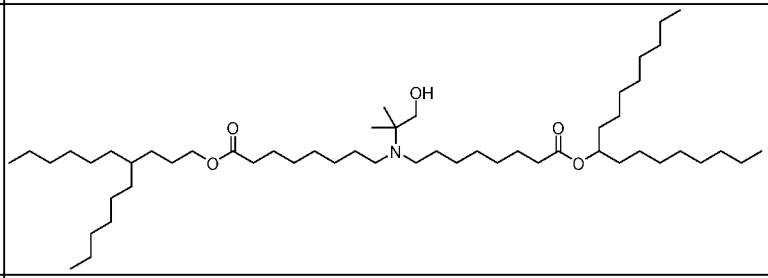
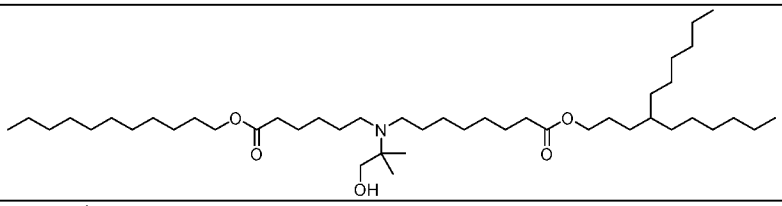
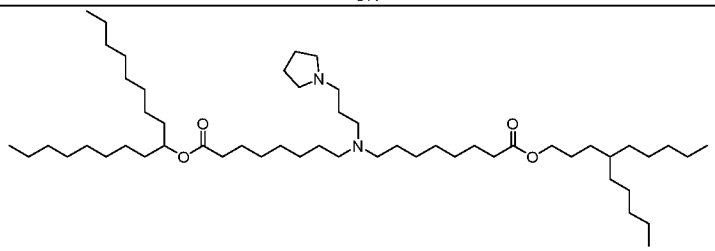
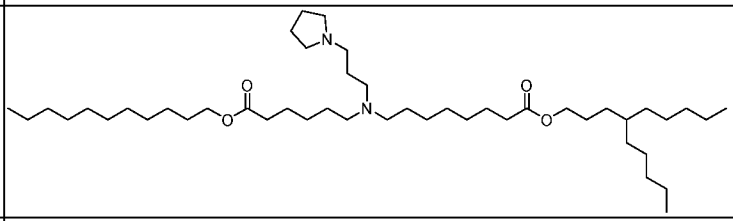
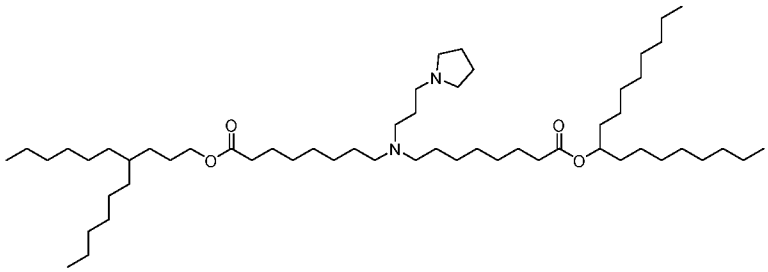
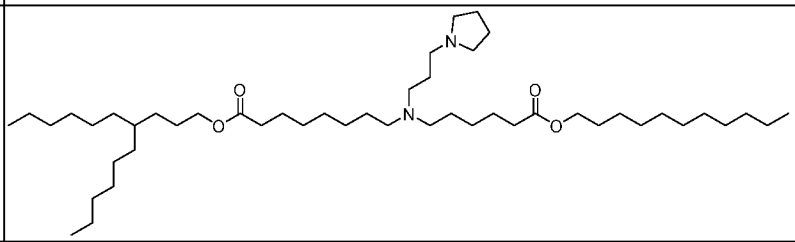
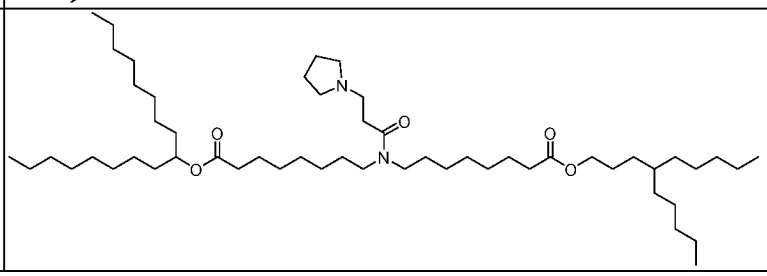
Lipid No.	Structure	IUPAC Name
2234		bis(4-pentylnonyl) 8,8'-((4-(pyrrolidin-1-yl)butanoyl)azanediyl)dioctanoate
2235		bis(4-pentylnonyl) 8,8'-((3-(3-hydroxypyrrolidin-1-yl)propanoyl)azanediyl)dioctanoate
2236		bis(4-pentylnonyl) 8,8'-((3-(pyrrolidin-1-yl)propanethioyl)azanediyl) dioctanoate
2237		((3-(pyrrolidin-1-yl)propanoyl)azanediyl)bis(heptane-7,1-diyl) bis(5-pentyldecanoate)
2238		((3-(pyrrolidin-1-yl)propanoyl)azanediyl)bis(octane-8,1-diyl) bis(4-pentylnonanoate)
2239		bis(4-pentylnonyl) 8,8'-((3-(diethylamino)propanoyl)azanediyl)dioctanoate
2241		8-(N-(8-oxo-8-((4-pentylnonyl)amino)octyl)-3-(pyrrolidin-1-yl)propanamido)-N-(4-pentylnonyl)octanamide

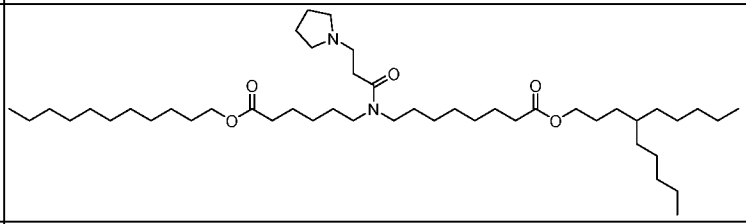
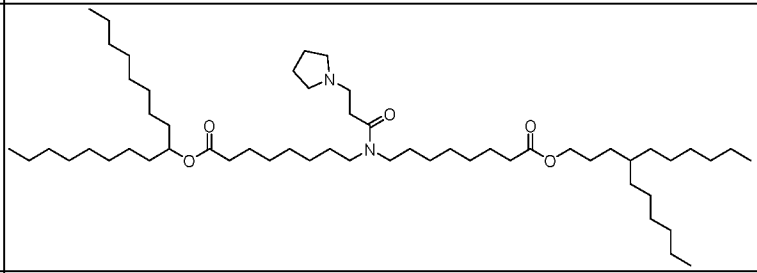
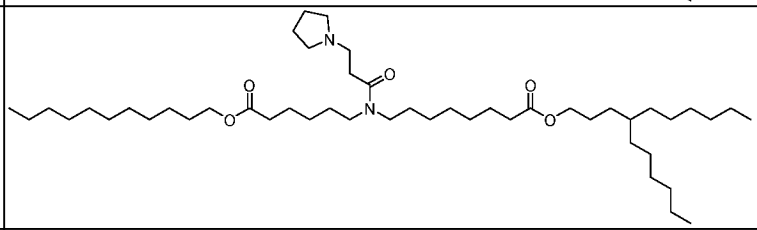
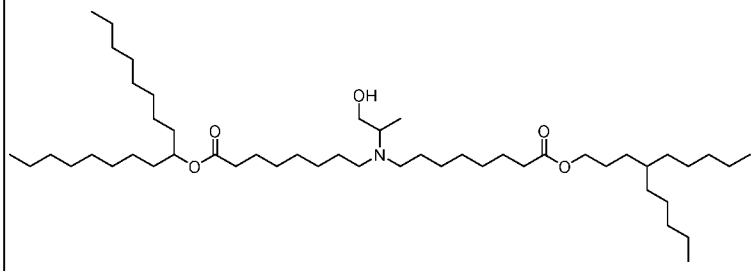
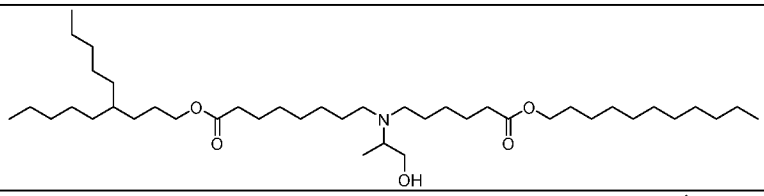
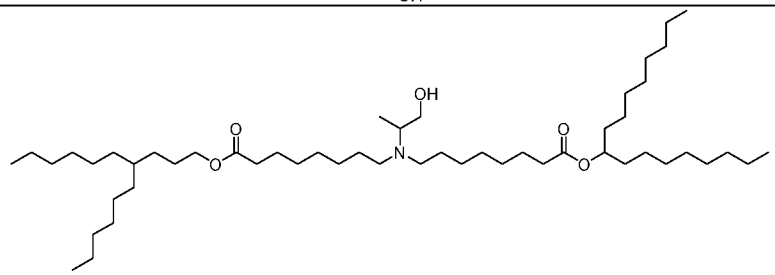
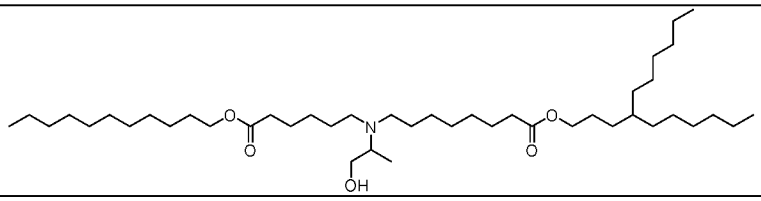
Lipid No.	Structure	IUPAC Name
2242		N-methyl-8-(N-(8-(methyl(4-pentylnonyl)amino)-8-oxooctyl)-3-(pyrrolidin-1-yl)propanamido)-N-(4-pentylnonyl)octanamide
2244		N-(heptadecan-9-yl)-8-((2-hydroxyethyl)(6-oxo-6-(undecylamino)hexyl)amino)octanamide
2245		N-(heptadecan-9-yl)-8-((2-hydroxyethyl)(6-(methyl(undecyl)amino)-6-oxohexyl)amino)-N-methyloctanamide
2249		heptadecan-9-yl 8-((1-hydroxypropan-2-yl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate
2250		heptadecan-9-yl 8-((1-hydroxy-2-methylpropan-2-yl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate
2276		heptadecan-9-yl 8-((2-aminoethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate
2277		heptadecan-9-yl 8-((2-aminoethyl)(8-(nonyloxy)-8-oxooctyl)amino)octanoate
2300		heptadecan-9-yl 8-((3-aminopropyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate
2301		7-((2-aminoethyl)(8-(heptadecan-9-yloxy)-8-oxooctyl)amino)heptyl decanoate

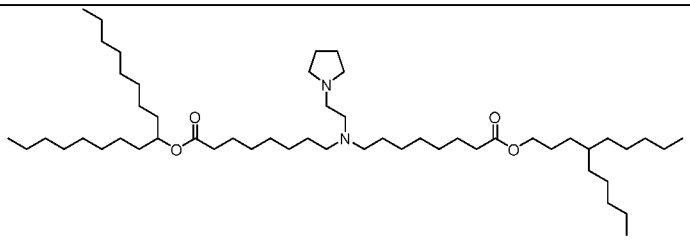
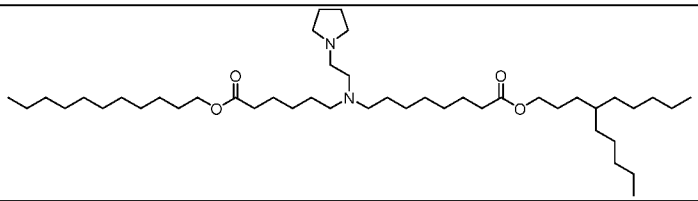
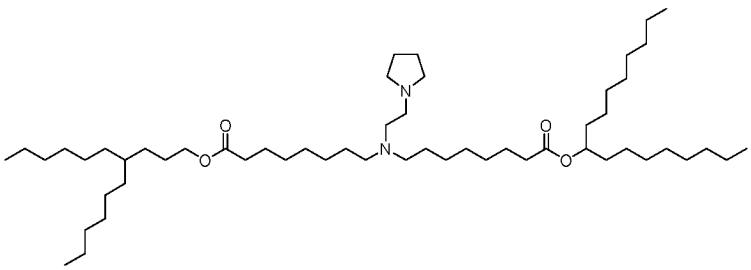
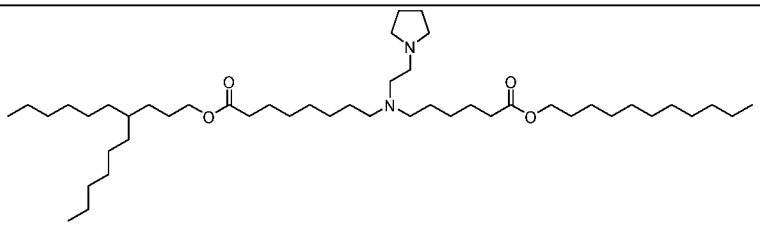
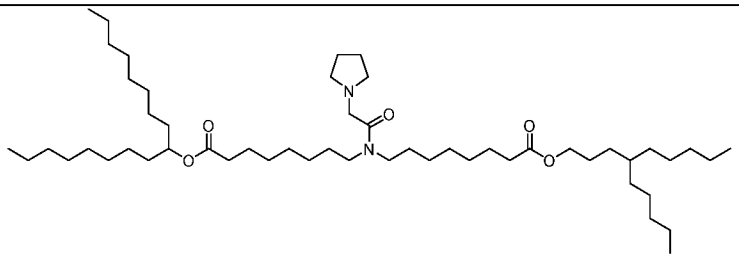
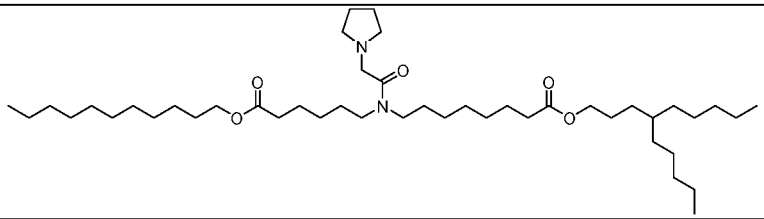
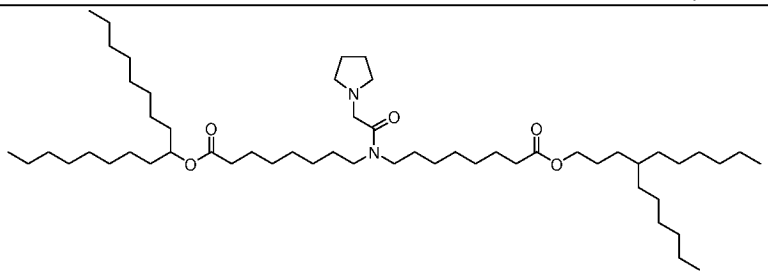
Lipid No.	Structure	IUPAC Name
2312		((2-aminoethyl)azanediyl)bis(hexane-6,1-diyl) bis(2-hexyldecanoate)
2313		heptadecan-9-yl 8-((2-aminoethyl)(8-((2-methylnonyl)oxy)-8-oxooctyl)amino)octanoate
2314		5-((2-aminoethyl)(7-((2-octyldecanoyl)oxy)heptyl)amino)pentyl dodecanoate
		((3-(pyrrolidin-1-yl)propyl)azanediyl)bis(hexane-6,1-diyl) bis(2-hexyldecanoate)
		bis(4-pentylnonyl) 8,8'-((2-(pyrrolidin-1-yl)ethyl)azanediyl)dioctanoate
		((2-(pyrrolidin-1-yl)acetyl)azanediyl)bis(hexane-6,1-diyl) bis(2-hexyldecanoate)
		heptadecan-9-yl 8-((1-hydroxy-2-methylpropan-2-yl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate
		bis(4-pentylnonyl) 8,8'-((1-hydroxypropan-2-yl)azanediyl)dioctanoate

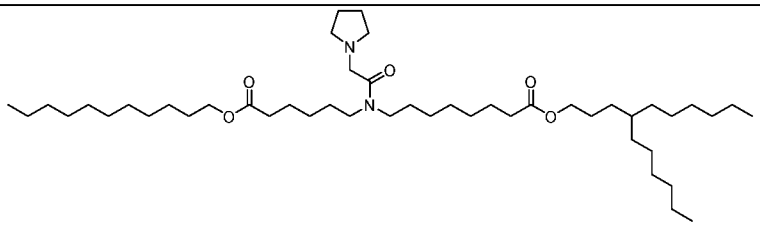
Lipid No.	Structure	IUPAC Name
		bis(4-pentylnonyl) 8,8'-((1-hydroxy-2-methylpropan-2-yl)azanediyl)dioctanoate
		bis(4-hexyldecyl) 8,8'-((2-(pyrrolidin-1-yl)acetyl)azanediyl)dioctanoate
		bis(4-hexyldecyl) 8,8'-((3-(pyrrolidin-1-yl)propanoyl)azanediyl)dioctanoate
		bis(4-hexyldecyl) 8,8'-((2-(pyrrolidin-1-yl)ethyl)azanediyl)dioctanoate
		bis(4-hexyldecyl) 8,8'-((3-(pyrrolidin-1-yl)propyl)azanediyl)dioctanoate
		bis(4-hexyldecyl) 8,8'-((1-hydroxypropan-2-yl)azanediyl)dioctanoate
		bis(4-hexyldecyl) 8,8'-((1-hydroxy-2-methylpropan-2-yl)azanediyl)dioctanoate
		heptadecan-9-yl 8-((6-oxo-6-(undecyloxy)hexyl)(2-(pyrrolidin-1-yl)ethyl)amino)octanoate

Lipid No.	Structure	IUPAC Name
		heptadecan-9-yl 8-(N-(6-oxo-6-(undecyloxy)hexyl)-2-(pyrrolidin-1-yl)acetamido)octanoate
		heptadecan-9-yl 8-((6-oxo-6-(undecyloxy)hexyl)(3-(pyrrolidin-1-yl)propyl)amino)octanoate
		heptadecan-9-yl 8-(N-(6-oxo-6-(undecyloxy)hexyl)-3-(pyrrolidin-1-yl)propanamido)octanoate
		((1-hydroxypropan-2-yl)azanediyl)bis(hexane-6,1-diyl) bis(2-hexyldecanoate)
		((1-hydroxy-2-methylpropan-2-yl)azanediyl)bis(hexane-6,1-diyl) bis(2-hexyldecanoate)
		pentadecan-7-yl 8-((1-hydroxy-2-methylpropan-2-yl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate
		tridecan-7-yl 8-((1-hydroxy-2-methylpropan-2-yl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate
		heptadecan-9-yl 8-((1-hydroxy-2-methylpropan-2-yl)(8-oxo-8-((4-pentylnonyl)oxy)octyl)amino)octanoate
		4-pentylnonyl 8-((1-hydroxy-2-methylpropan-2-yl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate

Lipid No.	Structure	IUPAC Name
		heptadecan-9-yl 8-((8-((4-hexyldecyl)oxy)-8-oxooctyl)(1-hydroxy-2-methylpropan-2-yl)amino)octanoate
		4-hexyldecyl 8-((1-hydroxy-2-methylpropan-2-yl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate
		heptadecan-9-yl 8-((8-oxo-8-((4-pentylnonyl)oxy)octyl)(3-(pyrrolidin-1-yl)propyl)amino)octanoate
		4-pentylnonyl 8-((6-oxo-6-(undecyloxy)hexyl)(3-(pyrrolidin-1-yl)propyl)amino)octanoate
		heptadecan-9-yl 8-((8-((4-hexyldecyl)oxy)-8-oxooctyl)(3-(pyrrolidin-1-yl)propyl)amino)octanoate
		4-hexyldecyl 8-((6-oxo-6-(undecyloxy)hexyl)(3-(pyrrolidin-1-yl)propyl)amino)octanoate
		heptadecan-9-yl 8-(N-(8-oxo-8-((4-pentylnonyl)oxy)octyl)-3-(pyrrolidin-1-yl)propanamido)octanoate

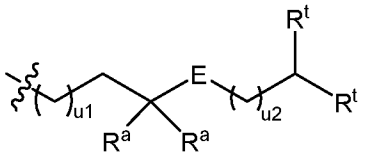
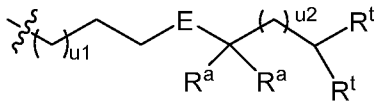
Lipid No.	Structure	IUPAC Name
		4-pentylnonyl 8-(N-(6-oxo-6-(undecyloxy)hexyl)-3-(pyrrolidin-1-yl)propanamido)octanoate
		heptadecan-9-yl 8-(N-(8-((4-hexyldecyl)oxy)-8-oxooctyl)-3-(pyrrolidin-1-yl)propanamido)octanoate
		4-hexyldecyl 8-(N-(6-oxo-6-(undecyloxy)hexyl)-3-(pyrrolidin-1-yl)propanamido)octanoate
		heptadecan-9-yl 8-((1-hydroxypropan-2-yl)(8-oxo-8-((4-pentylnonyl)oxy)octyl)amino)octanoate
		4-pentylnonyl 8-((1-hydroxypropan-2-yl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate
		heptadecan-9-yl 8-((8-((4-hexyldecyl)oxy)-8-oxooctyl)(1-hydroxypropan-2-yl)amino)octanoate
		4-hexyldecyl 8-((1-hydroxypropan-2-yl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate

Lipid No.	Structure	IUPAC Name
		heptadecan-9-yl 8-((8-oxo-8-((4-pentylnonyl)oxy)octyl)(2-(pyrrolidin-1-yl)ethyl)amino)octanoate
		4-pentylnonyl 8-((6-oxo-6-(undecyloxy)hexyl)(2-(pyrrolidin-1-yl)ethyl)amino)octanoate
		heptadecan-9-yl 8-((8-((4-hexyldecyl)oxy)-8-oxooctyl)(2-(pyrrolidin-1-yl)ethyl)amino)octanoate
		4-hexyldecyl 8-((6-oxo-6-(undecyloxy)hexyl)(2-(pyrrolidin-1-yl)ethyl)amino)octanoate
		heptadecan-9-yl 8-(N-(8-oxo-8-((4-pentylnonyl)oxy)octyl)-2-(pyrrolidin-1-yl)acetamido)octanoate
		4-pentylnonyl 8-(N-(6-oxo-6-(undecyloxy)hexyl)-2-(pyrrolidin-1-yl)acetamido)octanoate
		heptadecan-9-yl 8-(N-(8-((4-hexyldecyl)oxy)-8-oxooctyl)-2-(pyrrolidin-1-yl)acetamido)octanoate

Lipid No.	Structure	IUPAC Name
		4-hexyldecyl 8-(N-(6-oxo-6-(undecyloxy)hexyl)-2-(pyrrolidin-1-yl)acetamido)octanoate

Ionizable lipid compounds iv)

[0282] In some embodiments, the ionizable lipid is a lipid comprising at least one head group and at least one tail group of formula (TI) or (TI'):

 (TI) or  (TI'), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing,

wherein:

E is each independently a biodegradable group;

R^a is each independently C_1-C_5 alkyl, C_2-C_5 alkenyl, or C_2-C_5 alkynyl;

u1 and u2 are each independently 0, 1, 2, 3, 4, 5, 6, or 7;

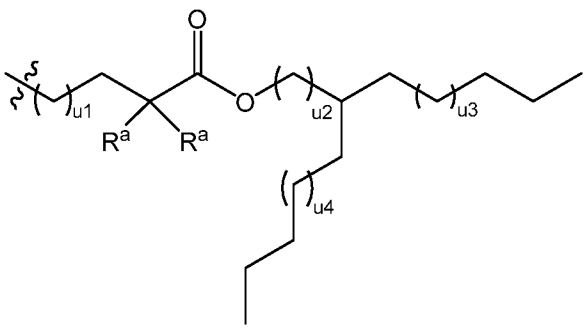
R^t is each independently H, C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with heteroatom or substituted with OH, SH, or halogen, or cycloalkyl or substituted cycloalkyl;

 represents the bond connecting the tail group to the head group; and

wherein the lipid has a pKa from about 4 to about 8.

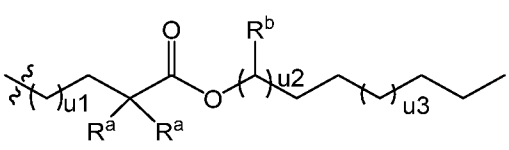
[0283] In some embodiments, E is each independently -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -C(O-R_{13})-O-, -C(O)O(CH_2)$_r$-, -C(O)N(R^7) (CH_2)$_r$-, -S-S-, or -C(O-R_{13})-O-(CH_2)$_r$-, wherein each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxyalkyl, or aminoalkyl; R_{13} is branched or unbranched C_3-C_{10} alkyl; and r is 1, 2, 3, 4, or 5. In some embodiments, E is each independently -OC(O)-, -C(O)O-, -N(R^7)C(O)-, or -C(O)N(R^7)-, wherein R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxyalkyl, or aminoalkyl.

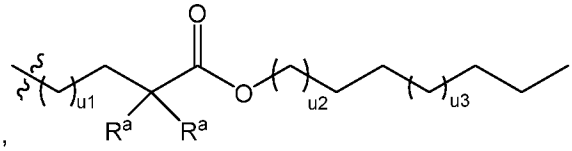
[0284] In some embodiments, the lipid comprises at least one head group and at least one tail group of formula (TII):

 (TII), wherein u3 and u4 are each independently 0,

1, 2, 3, or 4. The definitions of other variables in (TII) are the same as those defined above in (TI).

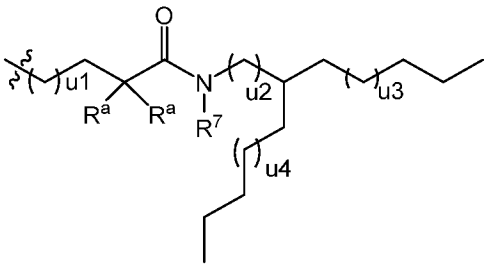
[0285] In some embodiments, the lipid comprises at least one head group and at least one tail group of formula (TIII):



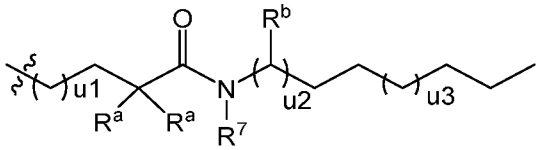
(TIII) (e.g., 

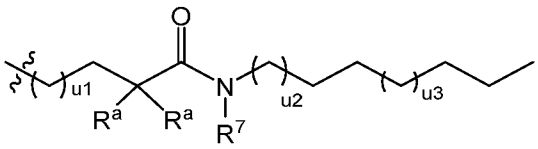
(TIIIa), wherein u3 is 0, 1, 2, 3, 4, 5, 6, or 7; and R^b is in each occasion independently H or C_1-C_4 alkyl. The definitions of other variables in (TIII) are the same as those defined above in (TI).

[0286] In some embodiments, the lipid comprises at least one head group and at least one tail group of formula (TIV):

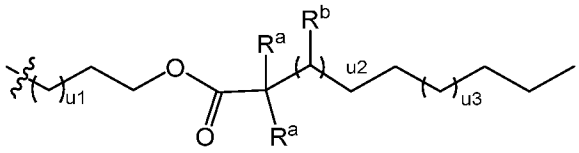


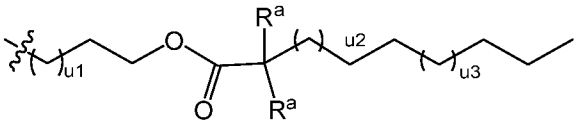
(TIV), wherein u3 and u4 are each independently 0, 1, 2, 3, or 4. The definitions of other variables in (TIV) are the same as those defined above in (TI).

[0287] In some embodiments, the lipid comprises at least one head group and at least one tail group of formula (TV) 

(TV) (e.g., 

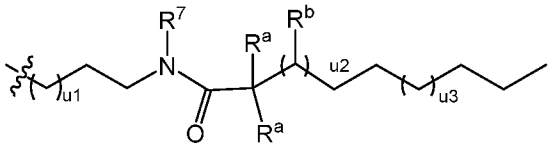
(TVa)), wherein u3 is 0, 1, 2, 3, 4, 5, 6, or 7; R^7 is each independently H or methyl; and R^b is in each occasion independently H or C_1-C_4 alkyl. The definitions of other variables in (TV) are the same as those defined above in (TI).

[0288] In some embodiments, the lipid comprises at least one head group and at least one tail group of formula (TII') 

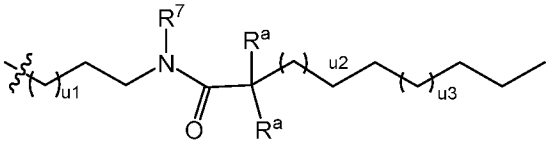
(TII') (e.g., 

(TII'a)), wherein u3 is 0, 1, 2, 3, 4, 5, 6, or 7; and R^b is in each occasion independently H or C_1-C_4 alkyl. The definitions of other variables in (TII') are the same as those defined above in (TI').

[0289] In some embodiments, the lipid comprises at least one head group and at least one tail group



of formula (TII') (TIII') (e.g.,



(TIII'a)), wherein u3 is 0, 1, 2, 3, 4, 5, 6, or 7; R^7 is each independently H or methyl; and R^b is in each occasion independently H or C_1-C_4 alkyl. The definitions of other variables in (TIII') are the same as those defined above in (TI').

[0290] In some embodiments, the lipid comprises at least one tail group of the formulas (TII), (TIII), (TIV), (TV), (TII'), and (TIII'), wherein

R^7 is each independently H or methyl;

R^b is in each occasion independently H or C_1-C_4 alkyl;

u3 and u4 are each independently 0, 1, 2, 3, 4, 5, 6, or 7; and

wherein the lipid has a pKa from about 4 to about 8.

[0291] In some embodiments, the lipid comprises tw, three, four, o or more tail groups that have a formula of (T), (TI), (TII), (TIII), (TIV), (TV), (TII'), and/or (TIII'), and each tail group may be the same or different.

[0292] In some embodiments, in any of the above formulas (T), (TI), (TII), and (TIII), (TIV), (TV), (TI'), (TII'), and/or (TIII'), R^a is each independently C_1-C_5 branched or unbranched alkyl, C_2-C_5 branched or unbranched alkenyl, or C_2-C_5 branched or unbranched alkynyl. In some embodiments, R^a is each independently C_1-C_3 branched or unbranched alkyl. In one embodiment, each R^a is methyl.

[0293] In some embodiments, in any of the above formulas (T), (TI), (TII), and (TIII), (TIV), (TV), (TI'), (TII'), and/or (TIII'), u1 is 3, 4, or 5. In some embodiments, in any of the above formulas (T), (TI), (TII), and (TIII), (TIV), (TV), (TI'), (TII'), and/or (TIII'), u2 is 0, 1, 2, or 3. In some embodiments, in any of the above formulas (T), (TI), (TII), and (TIII), (TIV), (TV), (TI'), (TII'), and/or (TIII'), u3 and u4 are each independently 1-7, for instance, u3 and u4 are each independently 1, 2, 3, or 4.

[0294] In some embodiments, the lipid comprises at least one tail of formula (TIII), wherein each R^a is methyl; R^b is in each occasion independently H, ethyl, or butyl; u1 is 3-5, u2 is 0-3, and u3 is 1-7 (e.g., 1-4). In some embodiments, the lipid comprises at least one two tails of formula (TIII), wherein the two tails of formula (TIII) are the same or different. In some embodiments, the lipid comprises at least three tails of formula (TIII), wherein each tail may be the same or different. In some embodiments, the lipid has four tails of formula (TIII), wherein each tail may be the same or different. In some embodiments, in each tail of formula (TIII), each R^a is methyl, and u1 is 3, u2 is 2, and u3 is 4.

[0295] In some embodiments, the lipid comprises at least one tail of formula (TII), wherein each R^a is methyl, u1 is 3-5, u2 is 0-3, u3 is 1-4, and u4 is 1-4. In some embodiments, the lipid has at least two tails of formula (TII), wherein the two tails of formula (TII) are the same. In some embodiments, the lipid has at least two tails of formula (TII), wherein the two tails of formula (TII) are or different. In some embodiments, the lipid comprises at least three tails of formula (TII), wherein each tail may be

the same or different. In some embodiments, the lipid has four tails of formula (TII), wherein each tail may be the same or different.

[0296] In some embodiments, the lipid comprises at least one tail of formula (TIV), wherein each R^a is methyl, u1 is 3-5, u2 is 0-3, u3 is 1-4, and u4 is 1-4. In some embodiments, the lipid comprises at least two tails of formula (TIV), wherein each tail may be the same or different. In some embodiments, the lipid comprises at least three tails of formula (TIV), wherein each tail may be the same or different. In some embodiments, the lipid comprises at least four tails of formula (TIV), wherein each tail may be the same or different.

[0297] In some embodiments, the lipid comprises at least two tails of formula (TV), wherein each tail may be the same or different. In some embodiments, the lipid comprises at least three tails of formula (TV), wherein each tail may be the same or different. In some embodiments, the lipid comprises at least four tails of formula (TV), wherein each tail may be the same or different.

[0298] In some embodiments, the lipid has at least two tails of formula (TII'), wherein each tail may be the same or different. In some embodiments, the lipid has at least three tails of formula (TII'), wherein each tail may be the same or different. In some embodiments, the lipid has at least four tails of formula (TII'), wherein each tail may be the same or different.

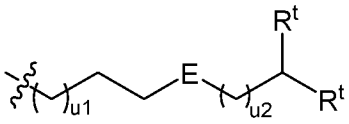
[0299] In some embodiments, the lipid has at least two tails of formula (TIII'), wherein each tail may be the same or different. In some embodiments, the lipid has at least three tails of formula (TIII'), wherein each tail may be the same or different. In some embodiments, the lipid has at least four tails of formula (TIII'), wherein each tail may be the same or different.

In some embodiments, the lipid has at least one tail of formula (TII) and/or at least one tail of formula (TIII); the lipid further comprises at least one tail that does not have a formula (T), (TI), (TII), (TIII), (TIV), (TV), (TII'), and/or (TIII'). That is to say, the lipid further comprises at least one tail that does not contain a gem-di functional groups bonded to the same carbon next to E (e.g., -C(O)O-).

[0300] In some embodiments, the lipid further comprises at least one tail that does not have a formula (T), (TI), (TII), (TIII), (TIV), (TV), (TI'), (TII'), and/or (TIII'). That is to say, the lipid further comprises at least one tail that does not contain a gem-di functional groups bonded to the same carbon next to E.

[0301] In some embodiments, the lipid further comprises at least one tail that does not have a formula (T), (TI), (TII), (TIII), (TIV), (TV), (TI'), (TII'), and/or (TIII'). That is to say, the lipid further comprises at least one tail that does not contain a gem-di functional groups bonded to the same carbon next to E.

[0302] In some embodiments, the lipid further comprises at least one tail of formula (TNG-I):



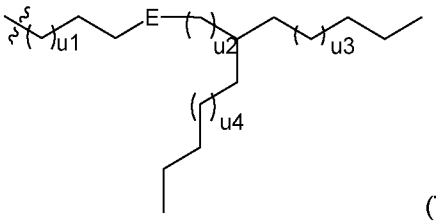
(TNG-I), wherein

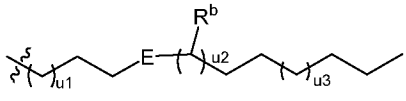
E is each independently a biodegradable group as described herein (e.g., -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -S-S-, or -C(O)N(R^7)-);

u1 and u2 are each independently 0, 1, 2, 3, 4, 5, 6, or 7; and

R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxyalkyl, or aminoalkyl.

[0303] In some embodiments, the at least one tail of formula (TNG-I) can be represented by



(TNG-II) or 

(TNG-III), wherein

u3 and u4 are each independently 0, 1, 2, 3, 4, 5, 6, or 7; and

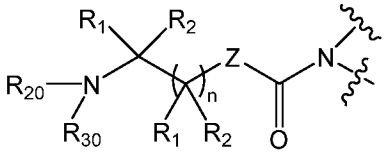
R^b is in each occasion independently H or C_1-C_4 alkyl.

[0304] All the embodiments above regarding the definitions of E, R^b, R^t, u1, u2, u3 and u4, as described above relating to the tail group containing a gem-di functional group bonded to the same carbon next to E, having a formula (T), (TI), (TII), (TIII), (TIV), (TV), (TII'), or (TIII'), are also applicable to the tail group that does not contain a gem-di functional groups bonded to the same carbon next to E, having a formula (TNG-I), (TNG-II), or (TNG-III).

[0305] In some embodiments, the lipid further comprises at least two tails that do not have a formula (T), (TI), (TII), (TIII), (TIV), (TV), (TI'), (TII'), and/or (TIII'). In some embodiments, the lipid comprises two tail groups of formula (TNG-II) or (TNG-III), and wherein each tail group may be the same or different,

[0306] In some embodiments, the lipid further comprises at least three tails that do not have a formula (T), (TI), (TII), (TIII), (TIV), (TV), (TI'), (TII'), and/or (TIII'). In some embodiments, the lipid comprises three tail groups of formula (TNG-II) or (TNG-III), and wherein each tail group may be the same or different,

[0307] In some embodiments, the head group of the lipid has a structure of formula (HA-I):



(HA-I),

wherein:

R_{20} and R_{30} are each independently H, C_1-C_5 branched or unbranched alkyl, or C_2-C_5 branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or substituted with OH, SH, halogen, or cycloalkyl groups; or

R_{20} and R_{30}, together with the adjacent N atom, form a 3 to 7 membered heterocylic or heteroaromatic ring containing one or more heteroatoms, optionally substituted with one or more OH, SH, halogen, alkyl, or cycloalkyl groups;

each of R_1 and R_2 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, OH, halogen, SH, or $NR_{10}R_{11}$; or R_1 and R_2 together form a cyclic ring;

each of R_{10} and R_{11} is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl; or R_{10} and R_{11} together form a heterocyclic ring;

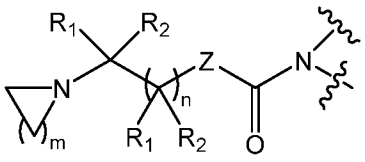
n is 0, 1, 2, 3 or 4;

Z is absent, O, S, or NR_{12}, wherein R_{12} is H or C_1-C_7 branched or unbranched alkyl; provided that when Z is not absent, the adjacent R_1 and R_2 cannot be OH, $NR_{10}R_{11}$, SH; and

 represents the bond connecting the head group to the tail group.

[0308] In some embodiments, R_{20} and R_{30} together with the adjacent N atom form a 3 to 7 membered heterocylic or heteroaromatic ring containing one or more heteroatoms, optionally substituted with one or more OH, SH, halogen, alkyl, or cycloalkyl groups.

[0309] In some embodiments, the head group of the ionizable lipid has a structure of formula (HA-I):

 (HA-IA),

wherein:

each of R_1 and R_2 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, OH, halogen, SH, or $NR_{10}R_{11}$; or R_1 and R_2 are taken together to form a cyclic ring;

each of R_{10} and R_{11} is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl; or R_{10} and R_{11} are taken together to form a heterocyclic ring;

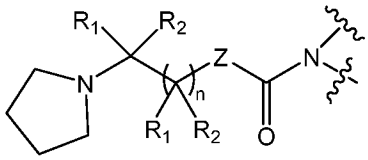
m is 1, 2, 3, 4, 5, 6, 7 or 8;

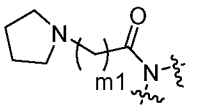
n is 0, 1, 2, 3 or 4;

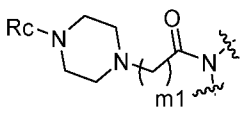
Z is absent, O, S, or NR_{12}, wherein R_{12} is H or C_1-C_7 branched or unbranched alkyl; provided that when Z is not absent, the adjacent R_1 and R_2 cannot be OH, $NR_{10}R_{11}$, SH; and

 represents the bond connecting the head group to the tail group.

[0310] In some embodiments, the head group of the ionizable lipid has a structure of formula (HA-III):

 (HA-III), wherein Z is absent, O, S, or NR_{12}; and R_{12} is H or C_1-C_7 branched or unbranched alkyl. The definitions of other variables in (HA-III) are the same as those defined above in (HA-IA).

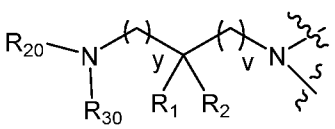
[0311] In some embodiments, the head group has a structure of:  or

 , wherein:

Rc is H or alkyl, optionally substituted with OH; and

m1 is 1, 2, or 3.

[0312] In some embodiments, the head group of the ionizable lipid has a structure of formula (HA-V):

 (HA-V),

wherein:

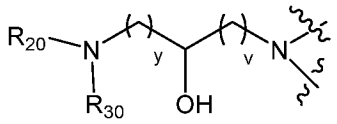
R_1 is H, C_1-C_3 alkyl, OH, halogen, SH, or $NR_{10}R_{11}$;

R_2 is OH, halogen, SH, or $NR_{10}R_{11}$; or R_1 and R_2 can be taken together to form a cyclic ring;

R_{10} and R_{11} are each independently H or C_1-C_3 alkyl; or R_{10} and R_{11} can be taken together to form a heterocyclic ring;

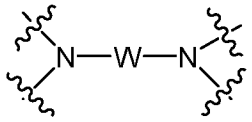
R_{20} and R_{30} are each independently H, C_1-C_5 branched or unbranched alkyl, C_2-C_5 branched or unbranched alkenyl; or R_{20} and R_{30} can be taken together to form a cyclic ring; and

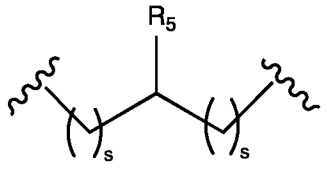
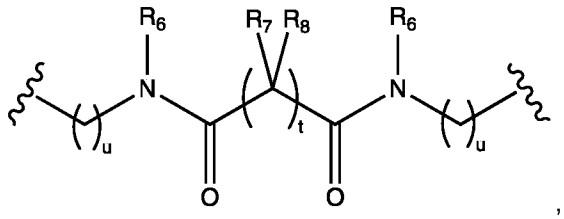
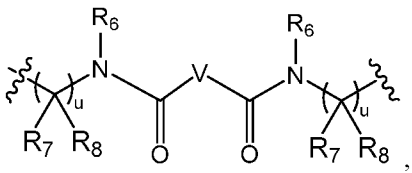
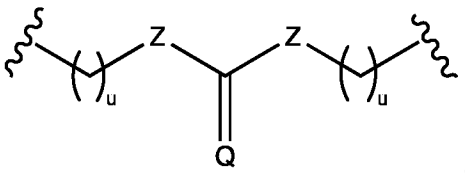
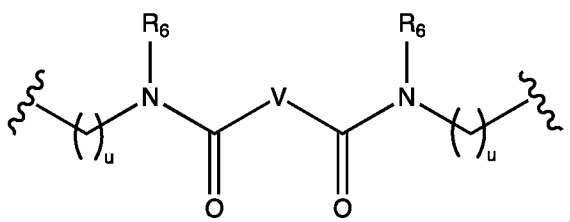
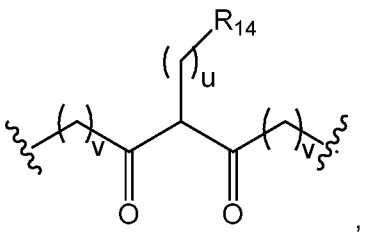
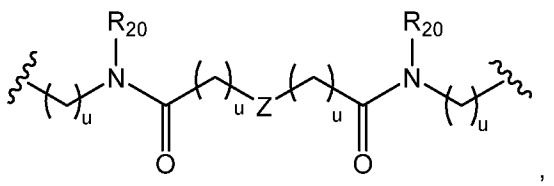
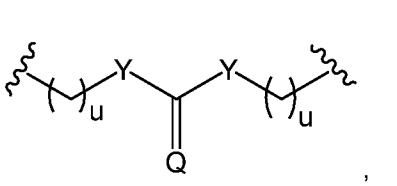
each of v and y is independently 1, 2, 3, or 4.

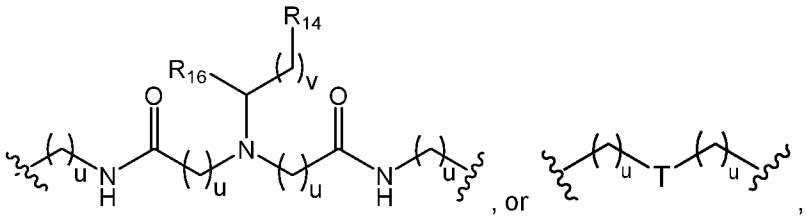
[0313] In some embodiments, the head group of the ionizable lipid has a structure of formula (HA-VI):  (HA-VI). The definitions of all variables in (HA-VI) are the same as those defined above in (HA-V).

[0314] In some embodiments, in any of the above formulas (HA-V) or (HA-VI), each R_{20} and R_{30} are independently C_1-C_3 alkyl. In one embodiment, each R_{20} and R_{30} are independently methyl.

[0315] In some embodiments, the head group of the ionizable lipid has a structure of formula (HB-I):

 (HB-I),

wherein W is  ,  ,  ,  ,  ,  ,  ,  .



wherein

R_5 is OH, SH, $(CH_2)_sOH$, or $NR_{10}R_{11}$;

each R_6 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each R_7 and R_8 are independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, $(CH_2)_vOH$, $(CH_2)_vSH$, $(CH_2)_sN(CH_3)_2$, or $NR_{10}R_{11}$, wherein each R_{10} and R_{11} is independently H or C_1-C_3 alkyl, or R_{10} and R_{11} are taken together to form a heterocyclic ring; or R_7 and R_8 are taken together to form a ring;

each R_{20} is independently H, or C_1-C_3 branched or unbranched alkyl;

R_{14} is a heterocyclic, $NR_{10}R_{11}$, $C(O)NR_{10}R_{11}$, $NR_{10}C(O)NR_{10}R_{11}$, or $NR_{10}C(S)NR_{10}R_{11}$, wherein each R_{10} and R_{11} is independently H, C_1-C_3 alkyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloalkenyl, optionally substituted with one or more NH and/or oxo groups, or R_{10} and R_{11} are taken together to form a heterocyclic ring;

R_{16} is H, =O, =S, or CN;

each of s, u, and t is independently 1, 2, 3, 4, or 5;

each v is independently 0, 1, 2, 3, 4, or 5;

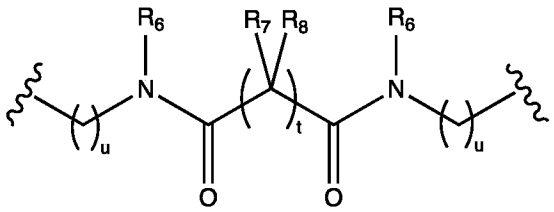
each Y is a divalent heterocyclic;

each Z is independently absent, O, S, or NR_{12}, wherein R_{12} is H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl;

Q is O, S, CH_2, or NR_{13}, wherein each R_{13} is H, C_1-C_5 alkyl;

V is branched or unbrachned C_2-C_{10} alkylene, C_2-C_{10} alkenylene, C_2-C_{10} alkynylene, or C_2-C_{10} heteroalkylene, optionally substituted with one or more OH, SH, and/or halogen groups; and

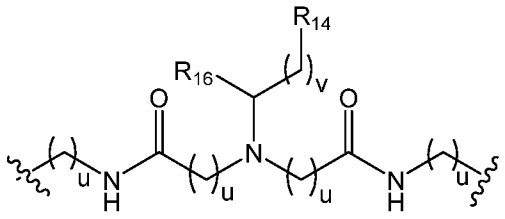
T is –NHC(O)O-, –OC(O)NH-, or a divalent heterocyclic.



[0316] In some embodiments, in formula (HB-I), W is , wherein:

each R_6, R_7, and R_8 are independently H or methyl; and

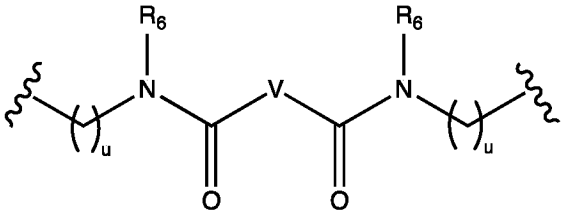
each of u and t is independently 1, 2, or 3.

[0317] In some embodiments, in formula (HB-I), W is  , wherein:

R_{16} is H or =O;

R_{14} is a nitrogen-containing 5- or 6- membered heterocyclic, $NR_{10}R_{11}$, $C(O)NR_{10}R_{11}$, $NR_{10}C(O)NR_{10}R_{11}$, or $NR_{10}C(S)NR_{10}R_{11}$, wherein each R_{10} and R_{11} is independently H or C_1-C_3 alkyl; and

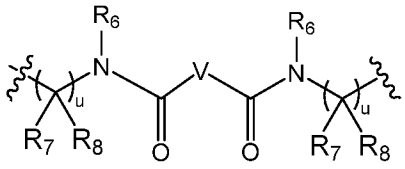
each of u and v is independently 1, 2, or 3.

[0318] In some embodiments, in formula (HB-I), W is  , wherein:

each R_6 is independently H or methyl;

each u is independently 1, 2, or 3; and

V is C_2-C_6 alkylene or C_2-C_6 alkenylene.

[0319] In some embodiments, in formula (HB-I), W is  , wherein:

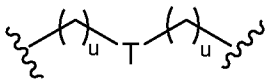
each R_6 is independently H or methyl;

each R_7 is independently H;

each R_8 is methyl;

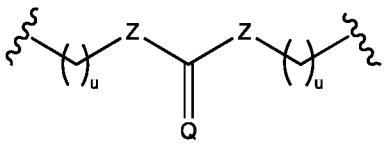
each u is independently 1, 2, or 3; and

V is C_2-C_6 alkylene or C_2-C_6 alkenylene.

[0320] In some embodiments, in formula (HB-I), W is  , wherein:

each u is independently 1, 2, or 3; and

T is a divalent nitrogen-containing 5- or 6- membered heterocyclic.

[0321] In some embodiments, in formula (HB-I), W is 

wherein:

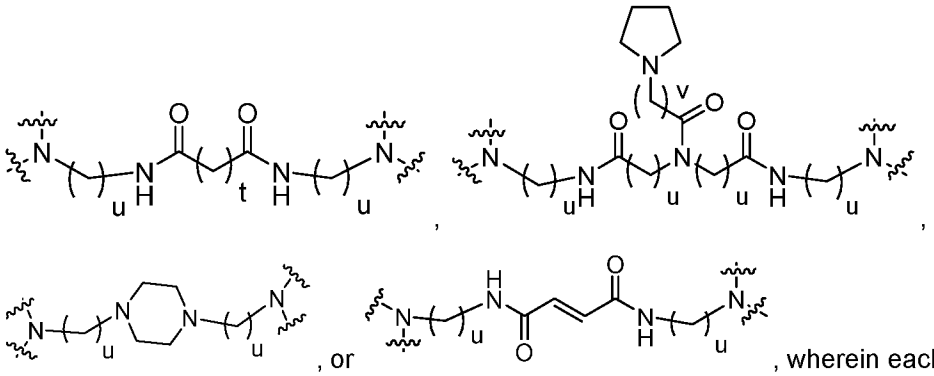
each u is independently 1, 2, or 3;

Q is O;

each Z is independently $NR_{12;}$ and

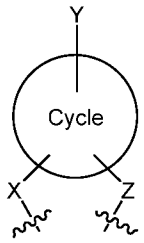
R_{12} is H or C_1-C_3 alkyl.

[0322] In some embodiments, the head group has the structure of:

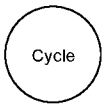


, wherein each of u and t is independently 1 or 2.

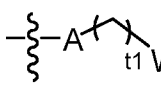
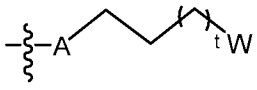
[0323] In some embodiments, the head group of the ionizable lipid has a structure of formula (HC-I):



(HC-I), wherein



is cyclic or heterocyclic moiety;

Y is alkyl, hydroxy, hydroxyalkyl,  , or  ;

A is absent, -O-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -N(R^7)C(O)N(R^7)-, -S-, or -S-S-;

each of X and Z is independently absent, -O-, -C(O)-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, or -S-;

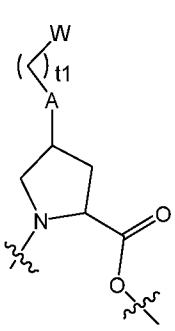
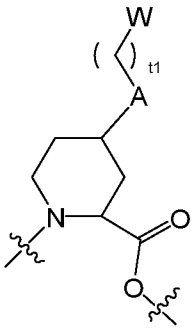
each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxy, alkoxy, hydroxyalkyl, alkylamino, alkylaminoalkyl, or aminoalkyl;

t is 0, 1, 2, or 3;

t1 is an integer from 0 to 10; and

W is hydroxyl, substituted or unsubstituted hydroxyalkyl, substituted or unsubstituted amino, substituted or unsubstituted aminocarbonyl, or substituted or unsubstituted heterocyclyl or heteroaryl.

[0324] In some embodiments, the head group has a structure of formula


(HC-IIIA) or 
(HC-IIIC).

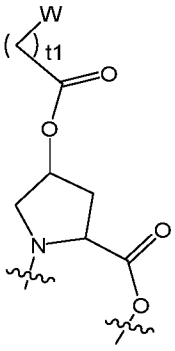
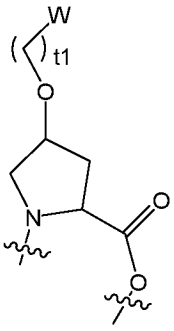
[0325] In some embodiments, in the above formulas,

A is absent, -O-, -N(R^7)-, -OC(O)-, or -C(O)O-;

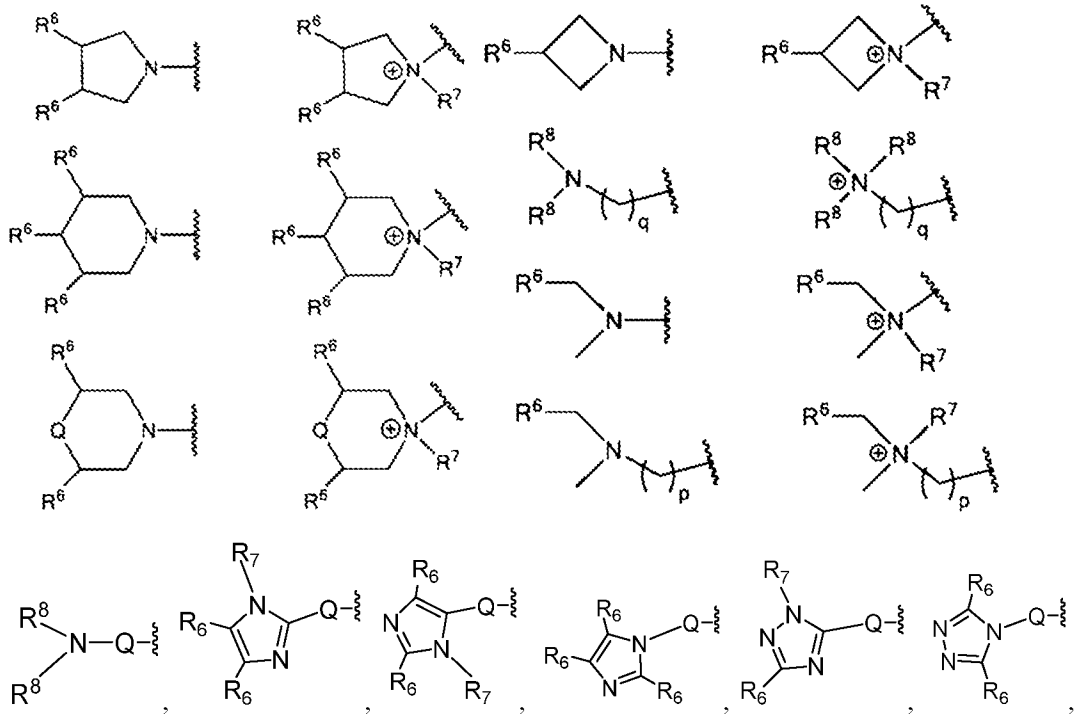
X is absent, -O-, or –C(O)-; and

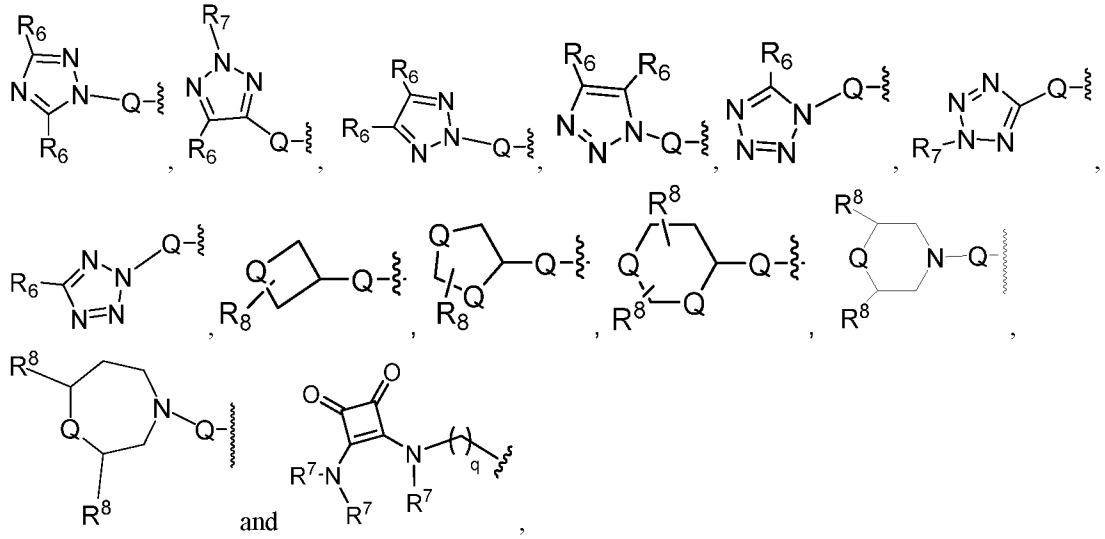
Z is –O-, –C(O)O-, or –OC(O)-.

[0326] In some embodiments, the head group has a structure of formula


(HC-IIID) or 
(HC-IIIE), wherein t1 is 0, 1, 2, or 3.

[0327] In some embodiments, W is hydroxyl, substituted or unsubstituted hydroxyalkyl, or one of the following moieties:





wherein

each Q is independently absent, -O-, -C(O)-, -C(S)-, -C(O)O-, -$(CH_2)_qC(R^7)_2$-, -C(O)N(R^7)-, -C(S)N(R^7)-, or -N(R^7);

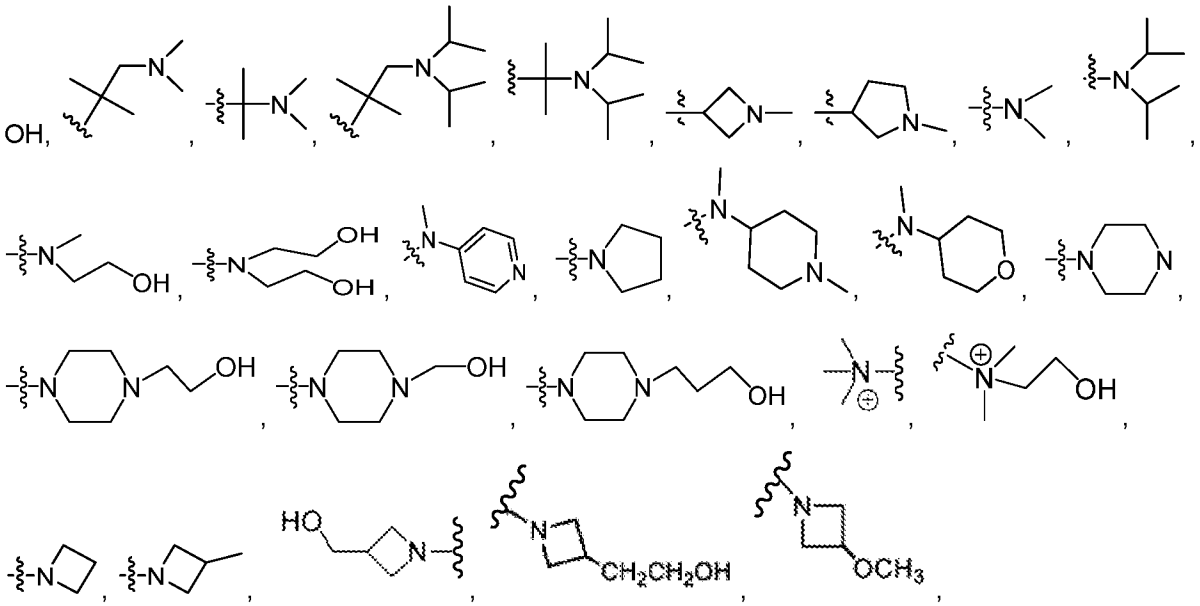
R^6 is independently H, alkyl, hydroxyl, hydroxyalkyl, alkoxy, -O-alkylene-O-alkyl, -O-alkylene-N(R^7)$_2$, amino, alkylamino, aminoalkyl, thiol, thiolalkyl, or $N^+(R^7)_3$–alkylene-Q-;

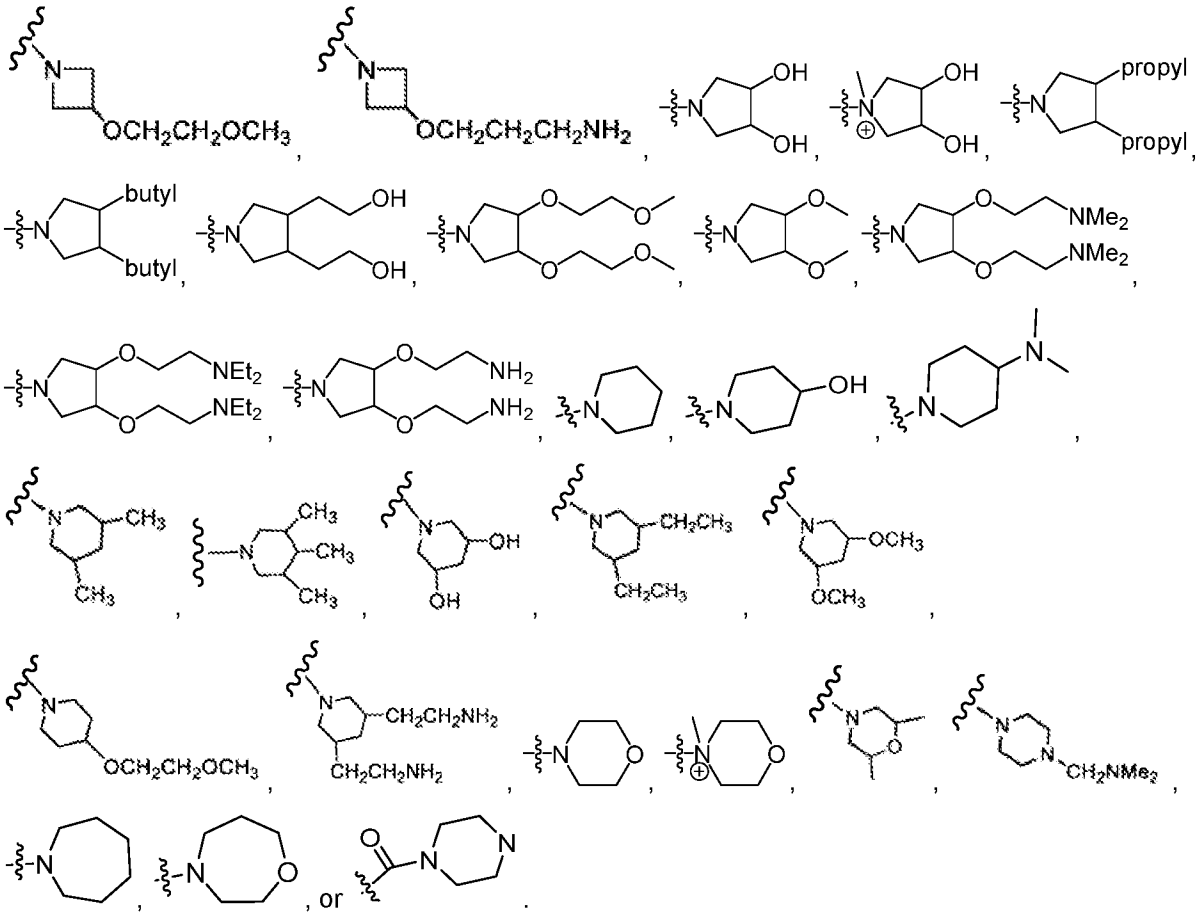
each R^8 is independently H, alkyl, hydroxyalkyl, amino, aminoalkyl, alkylamino, thiol, or thiolalkyl, heterocyclyl, heteroaryl, or two R^8 together with the nitrogen atom may form a ring, optionally substituted with one or more alkyl, hydroxy, hydroxyalkyl, alkoxy, alkylaminoalkyl, alkylamino, aminoalkyl;

q is 0, 1, 2, 3, 4, or 5; and

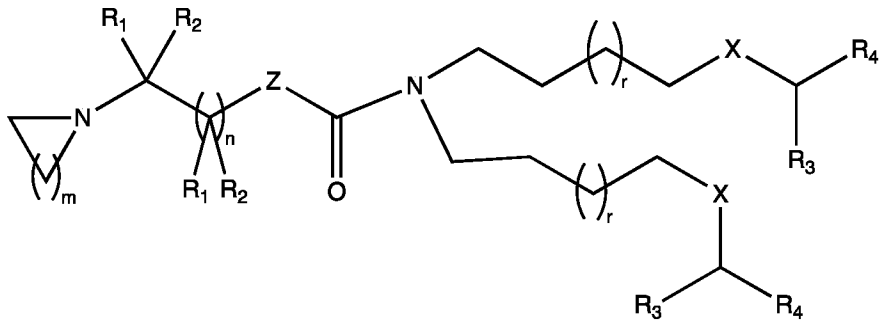
p is 0, 1, 2, 3, 4, or 5.

[0328] In some embodiments, W is one of the following:





[0329] In some embodiments, the ionizable lipid is represented by formula of



(LA-II), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein

R_1 is each independently H, C_1-C_3 alkyl, OH, halogen, SH, or $NR_{10}R_{11}$; R_1 and R_2 can be taken together to form a cyclic ring; R_{10} and R_{11} are each independently H, C_1-C_3 alkyl, and R_{10} and R_{11} can be taken together to form a heterocyclic ring;

R_2 is each independently H, C_1-C_3 alkyl, OH, halogen, SH, or $NR_{10}R_{11}$; R_1 and R_2 can be taken together to form a cyclic ring; R_{10} and R_{11} are each independently H, C_1-C_3 alkyl, and R_{10} and R_{11} can be taken together to form a heterocyclic ring;

m is 1, 2, 3, 4, 5, 6, 7 or 8;

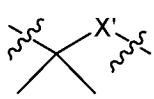
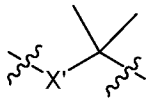
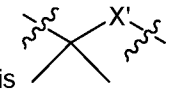
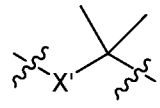
n is 0, 1, 2, 3 or 4;

r is each independently 0, 1, 2, 3, 4, 5, 6, 7 or 8;

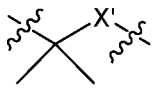
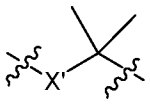
R_3 is each independently H, or C_3-C_{10} alkyl;

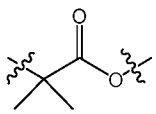
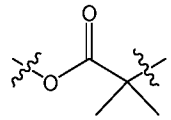
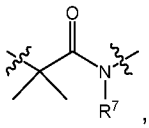
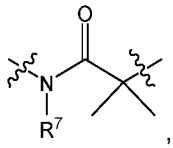
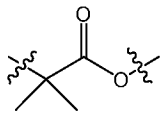
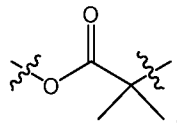
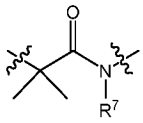
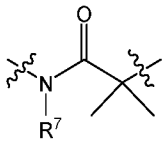
R_4 is each independently H, or C_3-C_{10} alkyl; provided that at least one of R_3 and R_4 is not H;

Z is absent, O, S, or NR_{12}; wherein R_{12} is C_1-C_7 alkyl;

each X is independently X',  , or  , provided that at least one X in the formula is  or  ; and

X' is a biodegradable moiety.

[0330] In some embodiments, each X is  or  . In some embodiments, X' is -OCO-, -COO-, -NR^7CO-, -CONR^7-, -C(O-R_{13})-O-(acetal), -COO$(CH_2)_s$-, -CONH$(CH_2)_s$-, or -C(O-R_{13})-O-$(CH_2)_s$-; wherein R^7 is H or C_1-C_3 alkyl; and R_{13} is C_3-C_{10} alkyl.

[0331] In some embodiments, at least one X in the formula is  ,  ,  , or  , wherein R^7 is H or methyl. In one embodiment, each X is  or  . In one embodiment, each X is  or  , wherein R^7 is H or methyl.

[0332] In some embodiments, m =3. In some embodiments, n = 0 or 1. In some embodiments, each R R_1 and R_2 is H. In some embodiments, Z is absent.

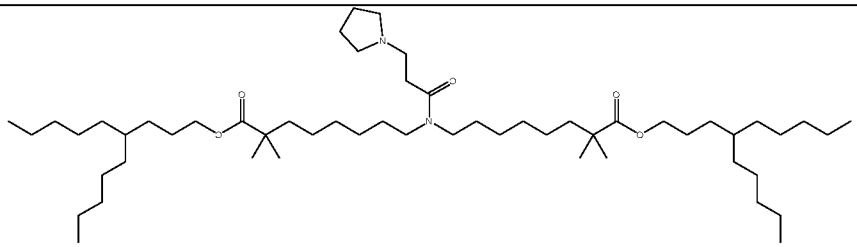
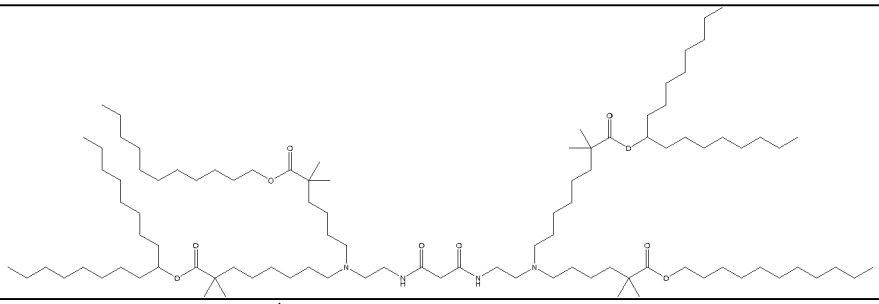
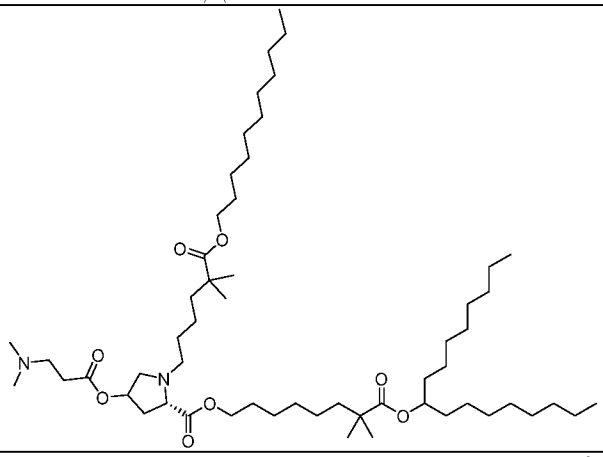
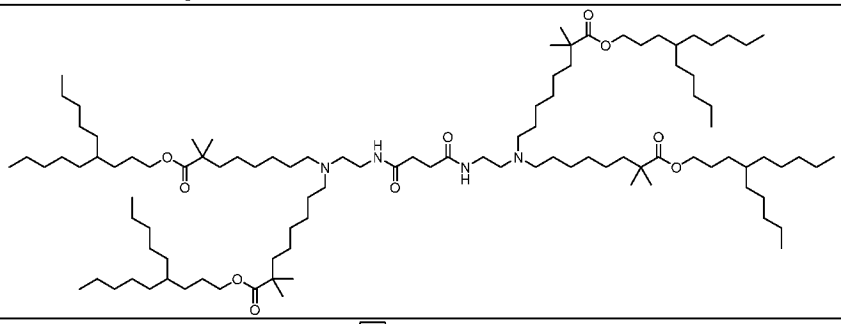
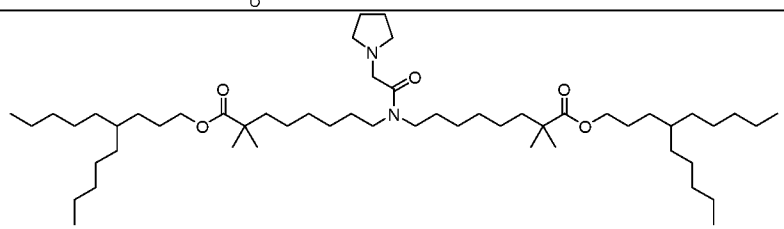
[0333] In some embodiments, Z is S. In some embodiments, Z is O. In some embodiments, Z is NH.

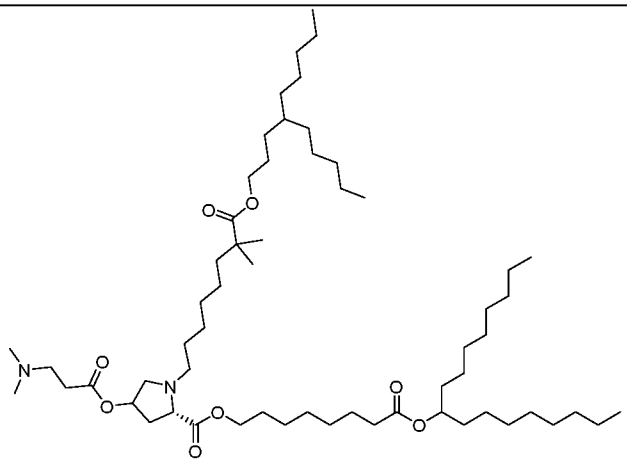
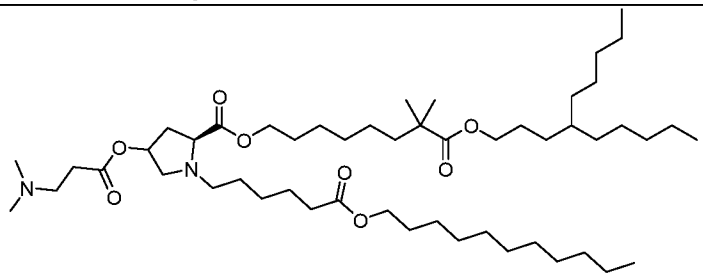
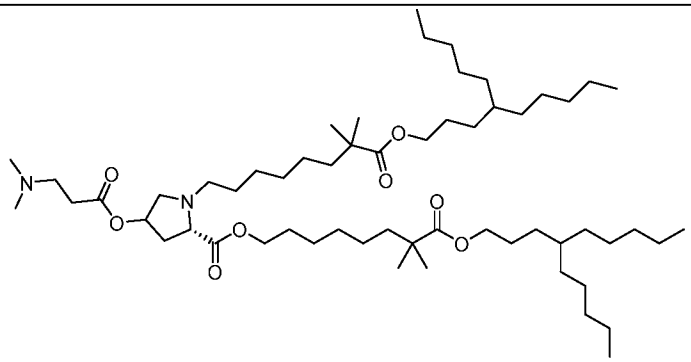
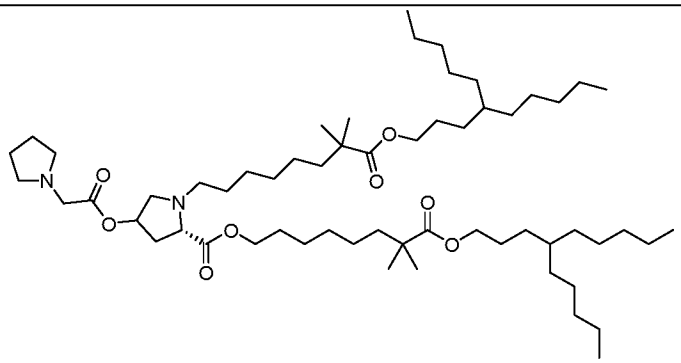
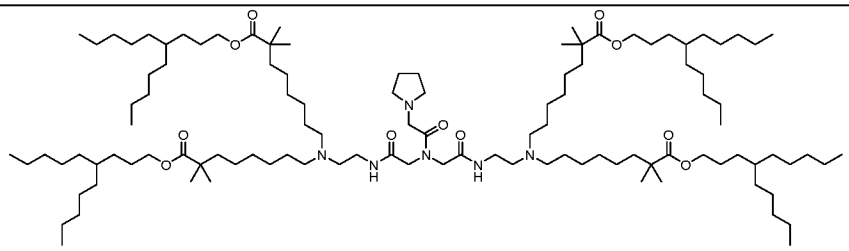
[0334] In some embodiments, r is 3. In some embodiments, r is 4.

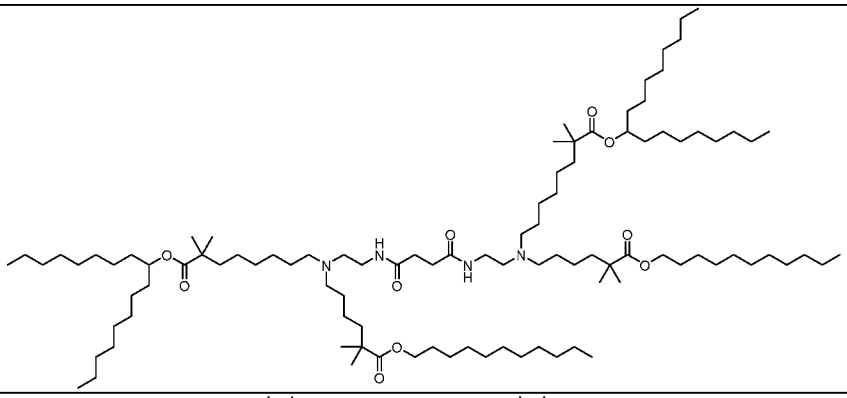
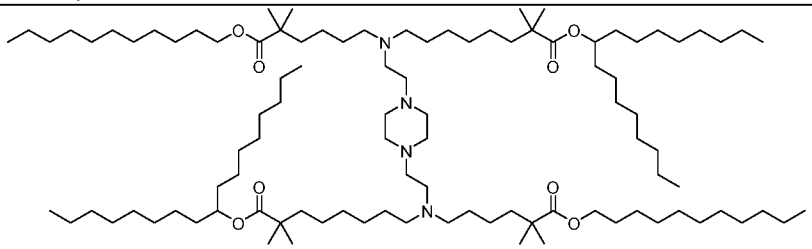
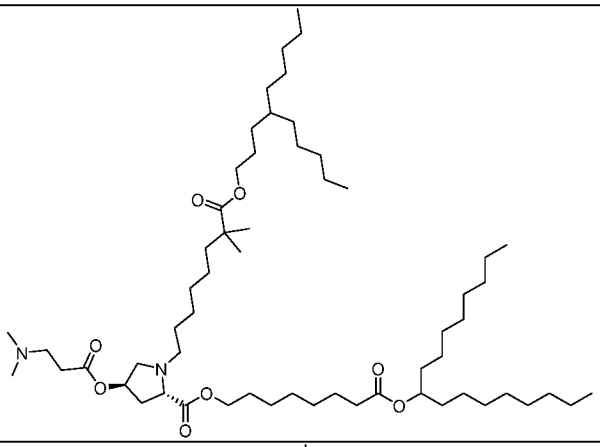
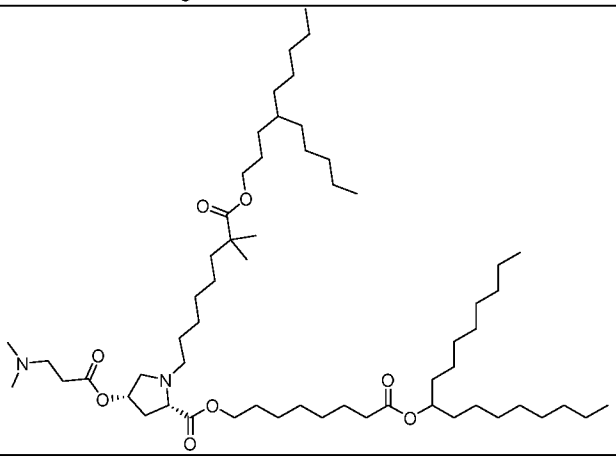
[0335] More embodiments of the above ionizable lipids comprising at least one head group (e.g., head group of formulas (HA-I) to (HA-VII), (HB-I), and (HC-I) to (HC-IIIE)), and at least one tail group of formula (TI) or (T1') (e.g., tail group of formula (TII), (TIII), TIV, TV, TII', or TIII'), in the Ionizable lipid compounds group iv), may be found in PCT Application No. PCT/US23/31669, filed on August 31, 2023, the content of which is incorporated herein by reference in their entirety. Moreover, all the ionizable lipids of formulas (LA-I)-(LA-VII), (LB-1)-(LB-VII), (LC-IA)-(LC-IC), (LC-IIA)-(LC-IIC), and (LC-IIIA)-(LC-IIIE) of PCT Application No. PCT/US23/31669, filed on August 31, 2023 are suitable for use as the ionizable lipids in this disclosure, and are incorporated herein by reference in its entirety.

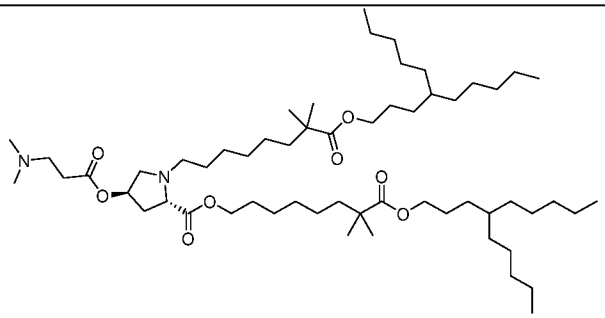
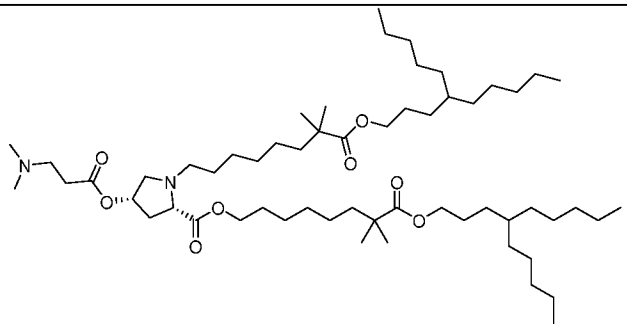
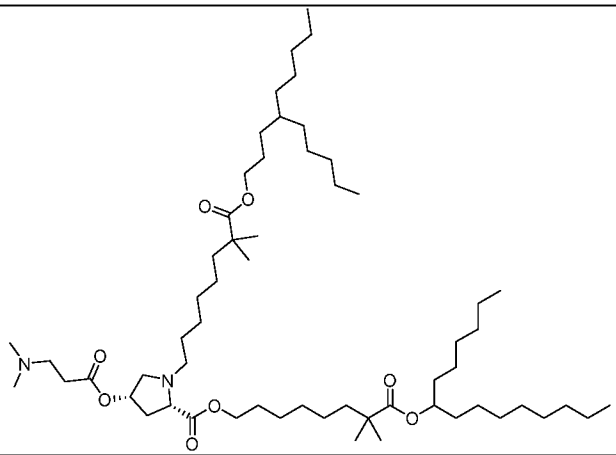
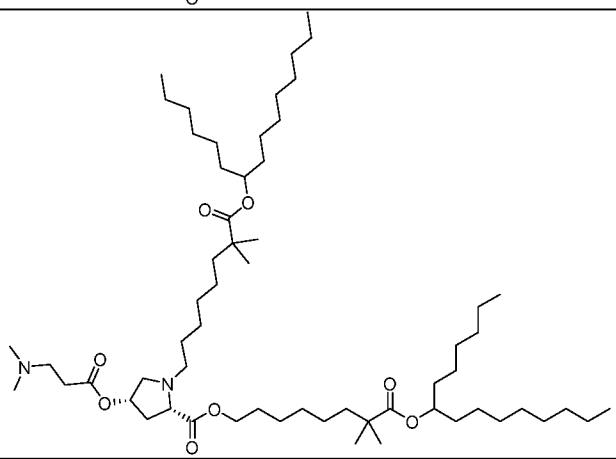
[0336] Certain exemplary ionizable lipid compounds disclosed herein are set forth in Table IV below.

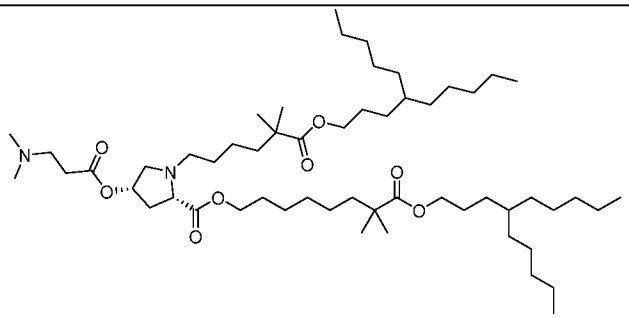
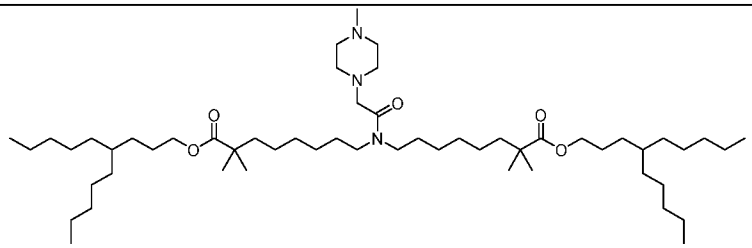
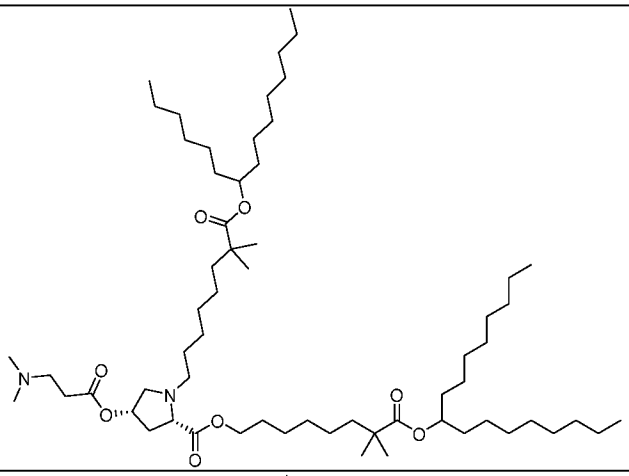
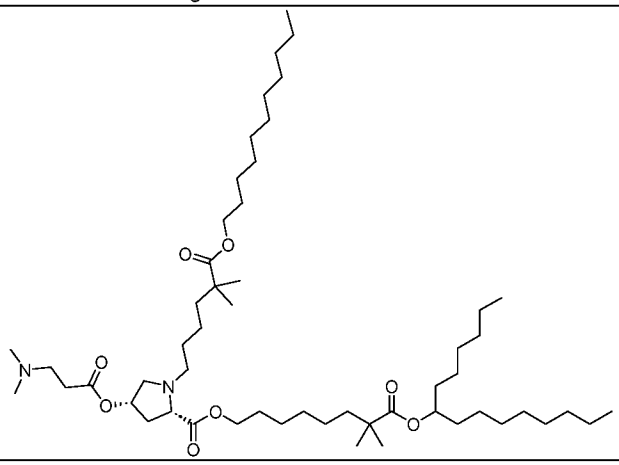
Table IV. Exemplary ionizable lipid compounds.

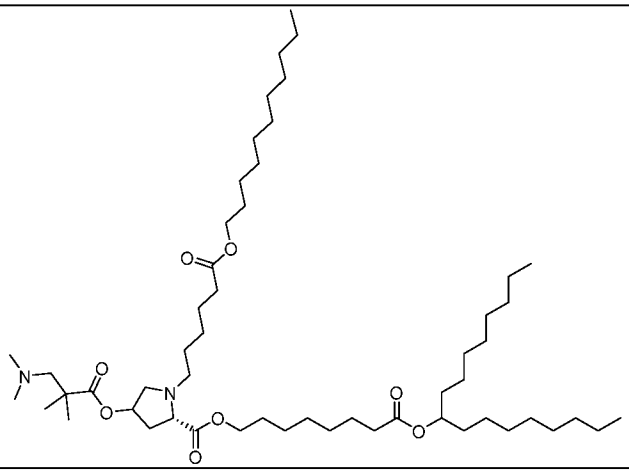
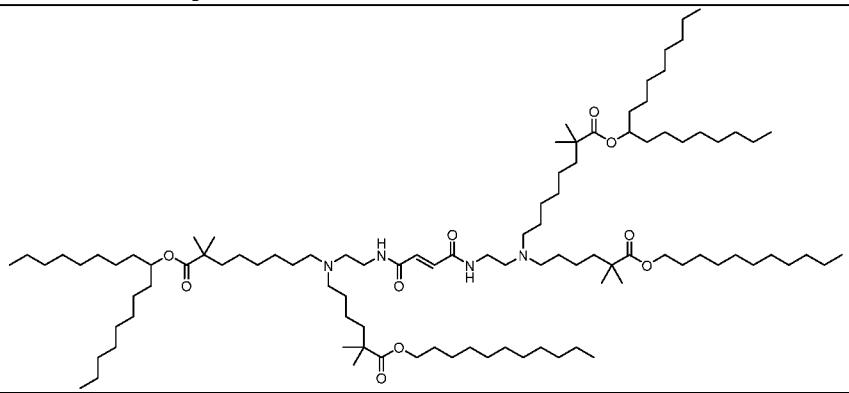
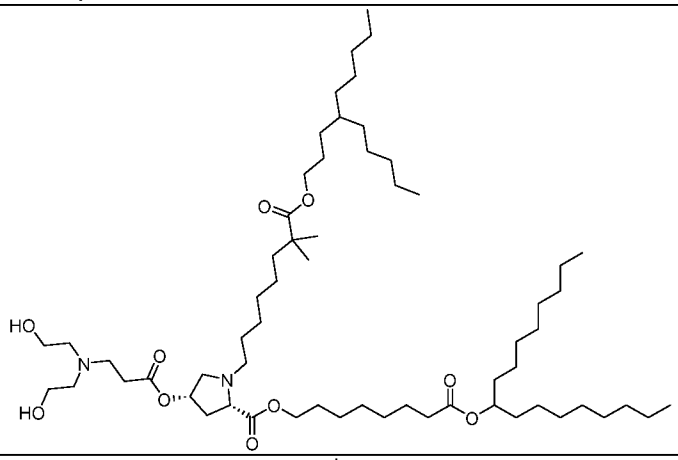
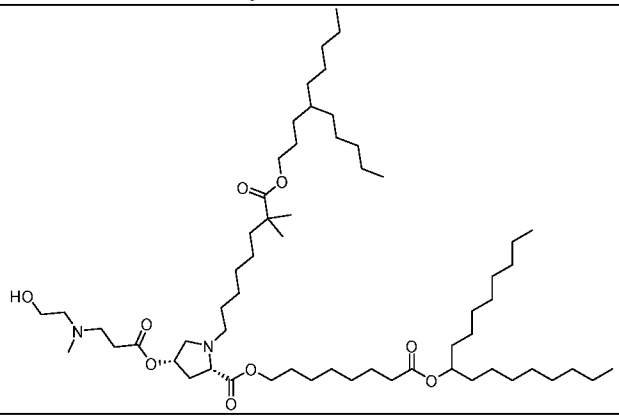
Lipid No.	Structure	IUPAC Name
2243		bis(4-pentylnonyl) 8,8'-((3-(pyrrolidin-1-yl)propanoyl)azanediyl)bis(2,2-dimethyloctanoate)
2330		di(heptadecan-9-yl) 9,19-bis(5,5-dimethyl-6-oxo-6-(undecyloxy)hexyl)-2,2,26,26-tetramethyl-13,15-dioxo-9,12,16,19-tetraazaheptacosanedioate
2331		8-(heptadecan-9-yloxy)-7,7-dimethyl-8-oxooctyl (2S)-1-(5,5-dimethyl-6-oxo-6-(undecyloxy)hexyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate
2333		bis(4-pentylnonyl) 9,20-bis(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-2,2,27,27-tetramethyl-13,16-dioxo-9,12,17,20-tetraazaoctacosanedioate
2335		bis(4-pentylnonyl) 8,8'-((2-(pyrrolidin-1-yl)acetyl)azanediyl)bis(2,2-dimethyloctanoate)

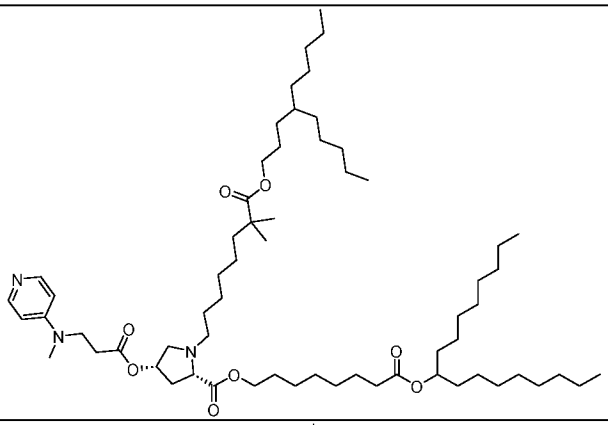
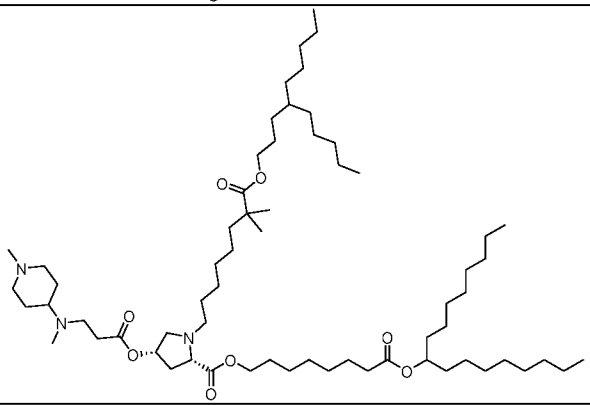
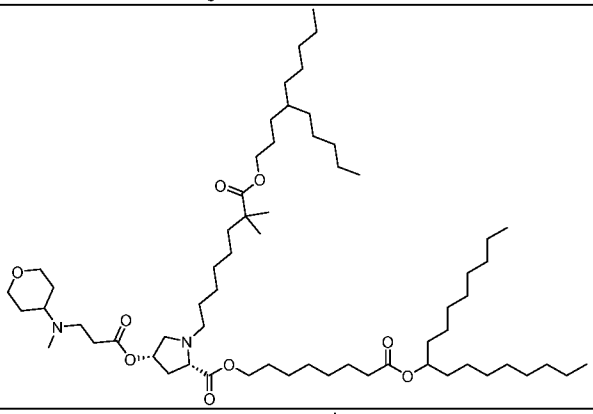
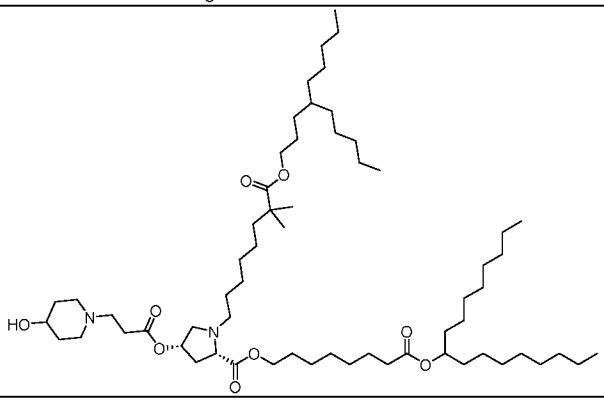
2365		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate
2366		7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl (2S)-4-((3-(dimethylamino)propanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2367		7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl (2S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate
2368		7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl (2S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-(2-(pyrrolidin-1-yl)acetoxy)pyrrolidine-2-carboxylate
2369		bis(4-pentylnonyl) 9,21-bis(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-2,2,28,28-tetramethyl-13,17-dioxo-15-(2-(pyrrolidin-1-yl)acetyl)-9,12,15,18,21-pentaazanonacosa

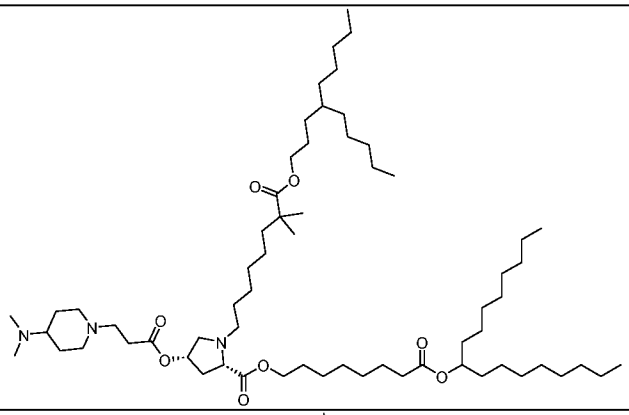
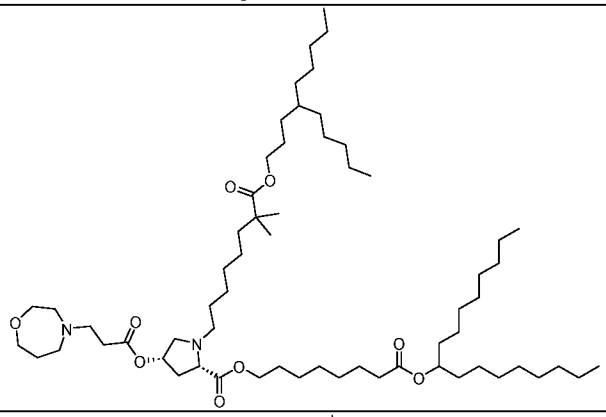
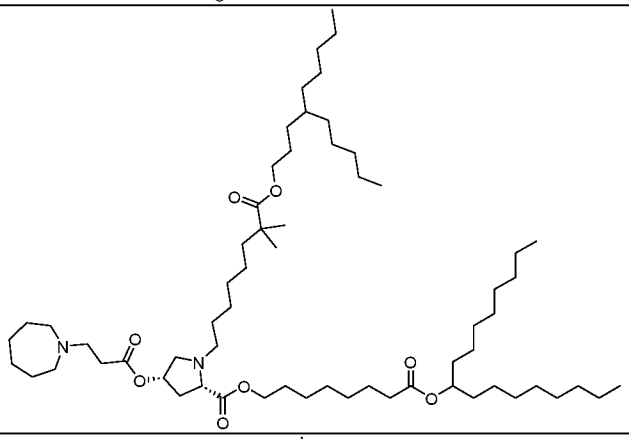
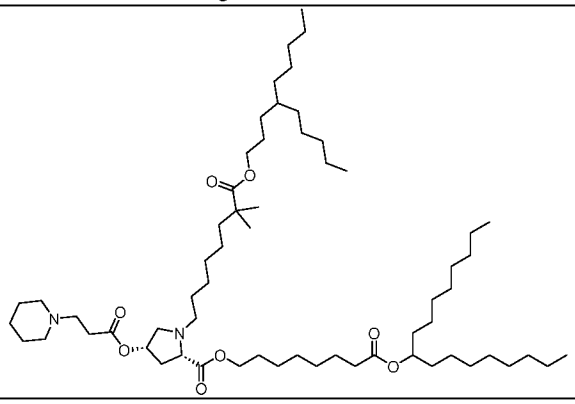
		nedioate
2392		di(heptadecan-9-yl) 9,20-bis(5,5-dimethyl-6-oxo-6-(undecyloxy)hexyl)-2,2,27,27-tetramethyl-13,16-dioxo-9,12,17,20-tetraazaoctacosanedioate
2398		di(heptadecan-9-yl) 8,8'-((piperazine-1,4-diylbis(ethane-2,1-diyl))bis((5,5-dimethyl-6-oxo-6-(undecyloxy)hexyl) azanediyl))bis(2,2-dimethyloctanoate)
2424		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4R)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate
2425		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate

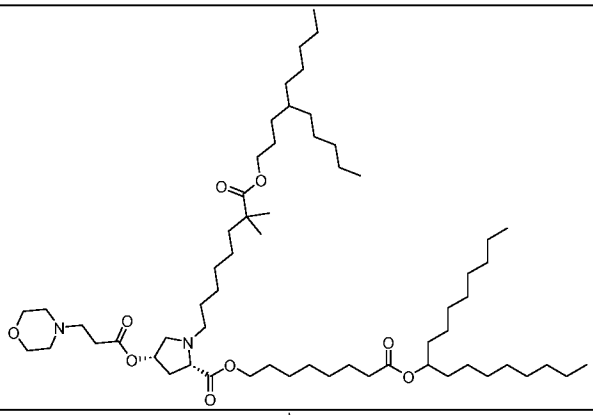
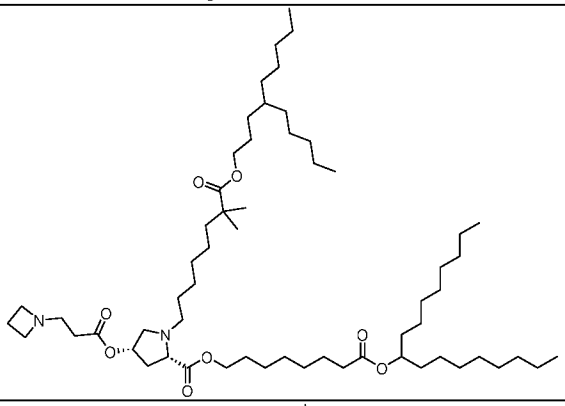
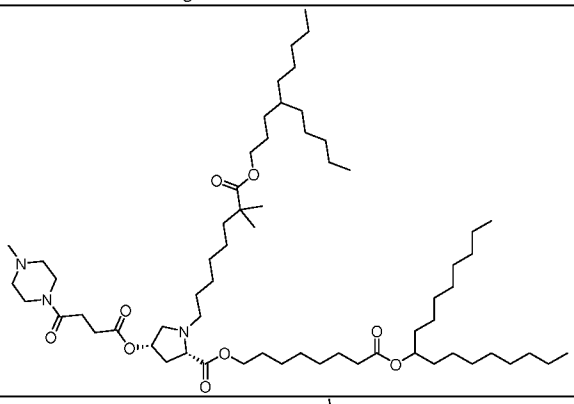
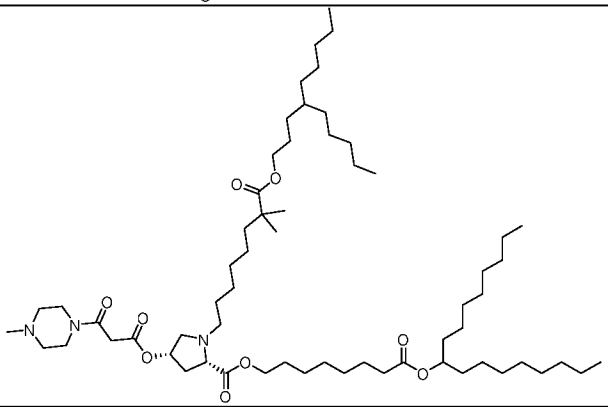
2432		7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl (2S,4R)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate
2433		7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate
2441		7,7-dimethyl-8-oxo-8-(pentadecan-7-yloxy)octyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate
2442		7,7-dimethyl-8-oxo-8-(pentadecan-7-yloxy)octyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-(pentadecan-7-yloxy)octyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate

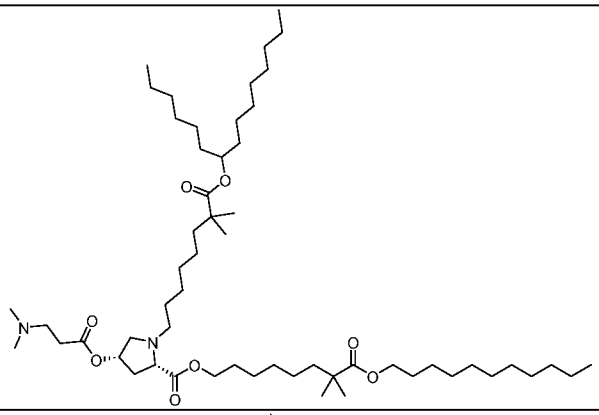
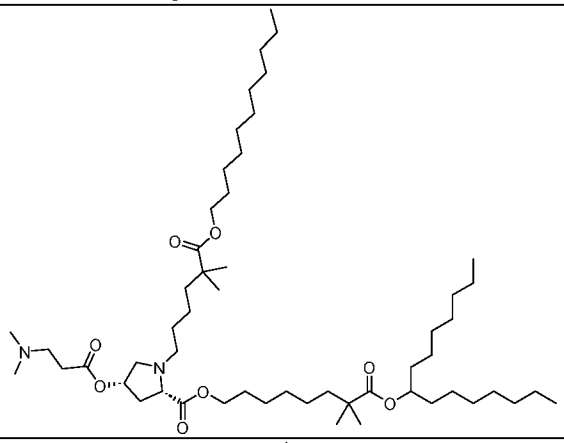
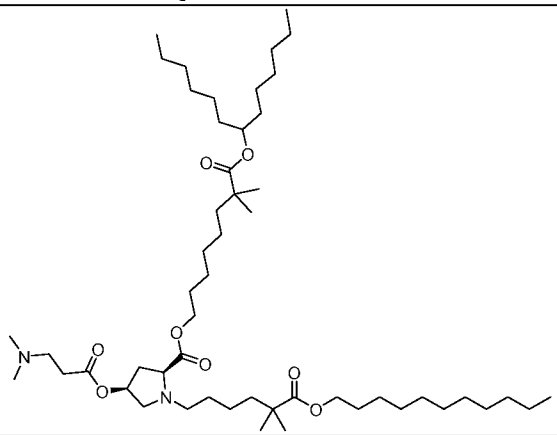
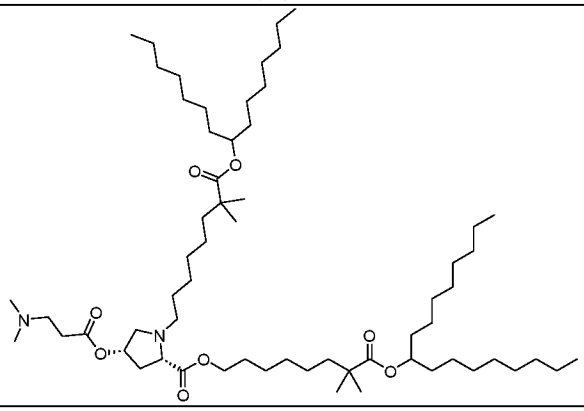
2443		7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl (2S,4S)-1-(5,5-dimethyl-6-oxo-6-((4-pentylnonyl)oxy)hexyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate
2454		bis(4-pentylnonyl) 8,8'-((2-(4-methylpiperazin-1-yl)acetyl)azanediyl)bis(2,2-dimethyloctanoate)
2481		8-(heptadecan-9-yloxy)-7,7-dimethyl-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-(pentadecan-7-yloxy)octyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate
2483		7,7-dimethyl-8-oxo-8-(pentadecan-7-yloxy)octyl (2S,4S)-1-(5,5-dimethyl-6-oxo-6-(undecyloxy)hexyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate

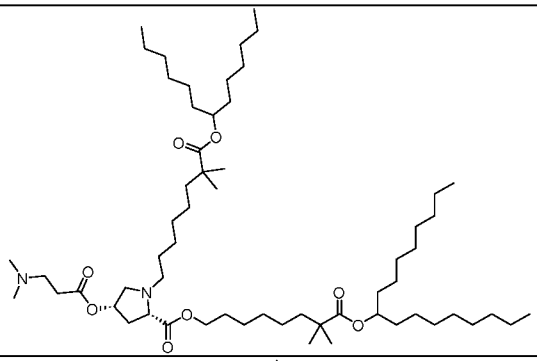
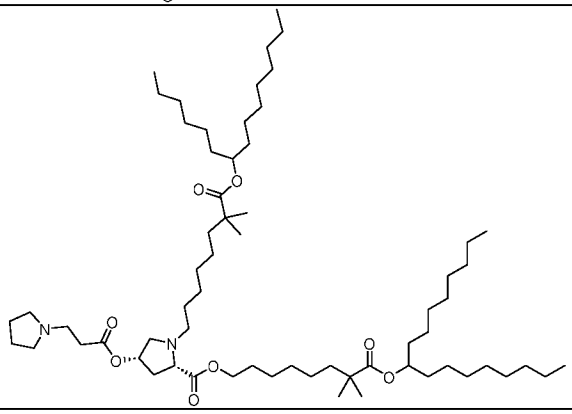
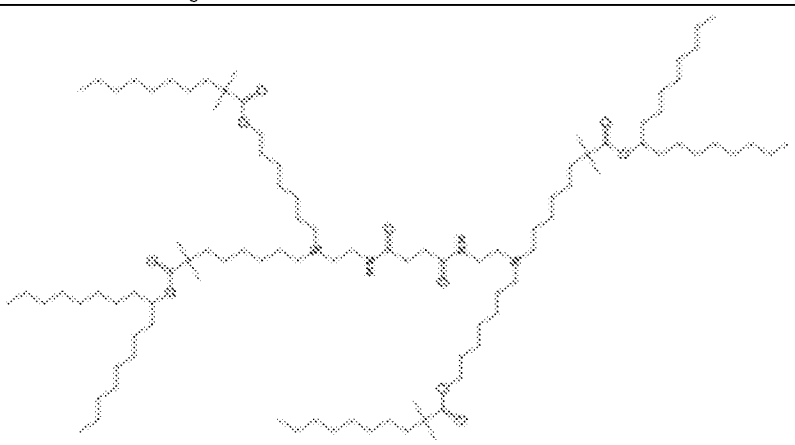
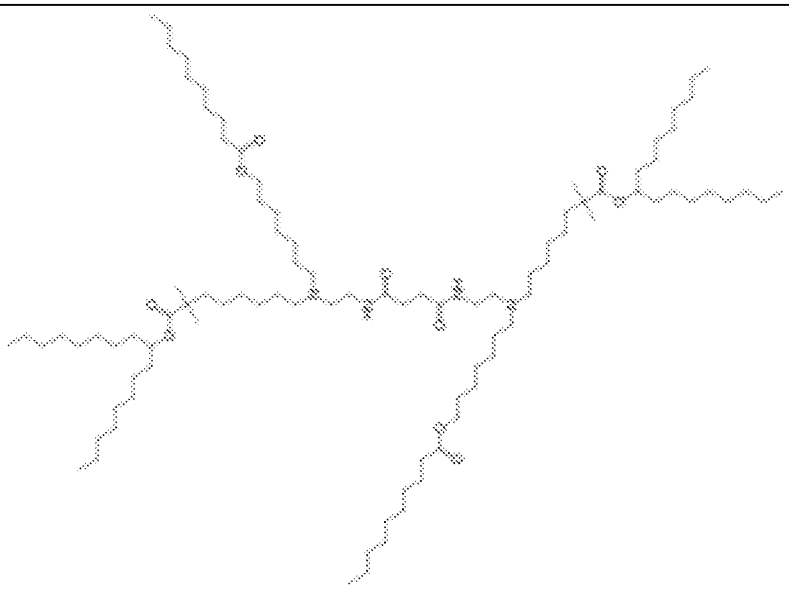
2370		8-(heptadecan-9-yloxy)-8-oxooctyl (2S)-4-((3-(dimethylamino)-2,2-dimethylpropanoyl)oxy)-1-(6-oxo-6-(undecyloxy)hexyl)pyrrolidine-2-carboxylate
2383		di(heptadecan-9-yl) (E)-9,20-bis(5,5-dimethyl-6-oxo-6-(undecyloxy)hexyl)-2,2,27,27-tetramethyl-13,16-dioxo-9,12,17,20-tetraazaoctacos-14-enedioate
2511		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-((3-(bis(2-hydroxyethyl)amino)propanoyl)oxy)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)pyrrolidine-2-carboxylate
2510		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-((2-hydroxyethyl)(methyl)amino)propanoyl)oxy)pyrrolidine-2-carboxylate

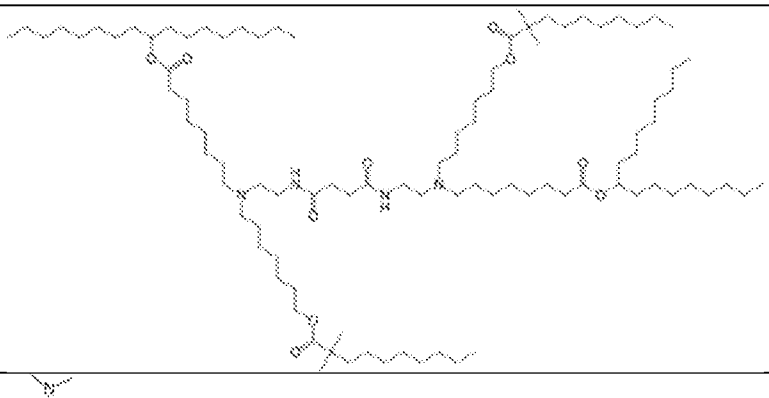
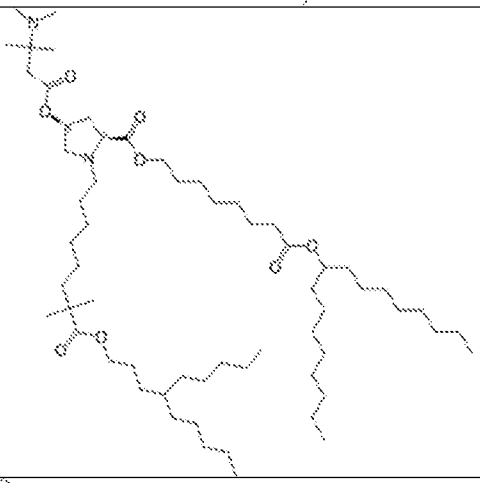
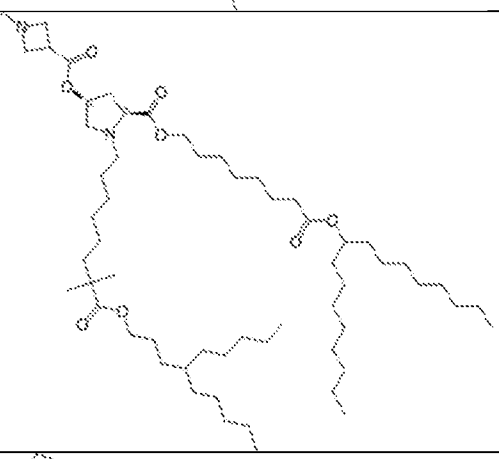
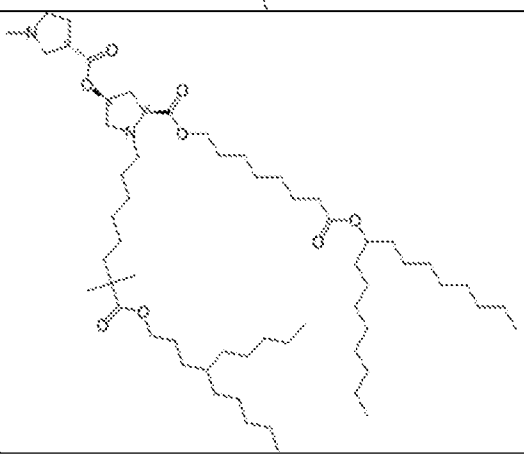
2509		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-(methyl(pyridin-4-yl)amino)propanoyl)oxy)pyrrolidine-2-carboxylate
2508		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-(methyl(1-methylpiperidin-4-yl)amino)propanoyl)oxy)pyrrolidine-2-carboxylate
2507		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-(methyl(tetrahydro-2H-pyran-4-yl)amino)propanoyl)oxy)pyrrolidine-2-carboxylate
2506		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-(4-hydroxypiperidin-1-yl)propanoyl)oxy)pyrrolidine-2-carboxylate

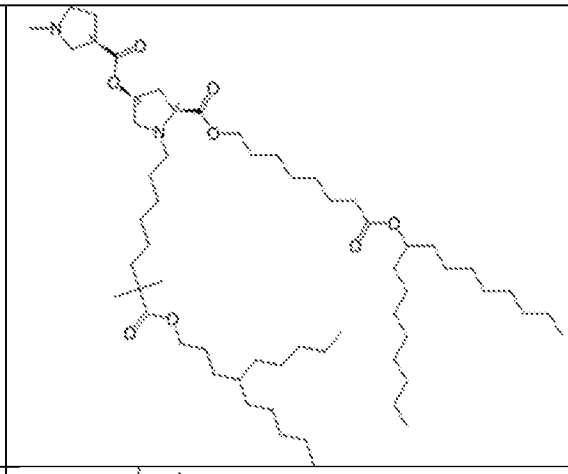
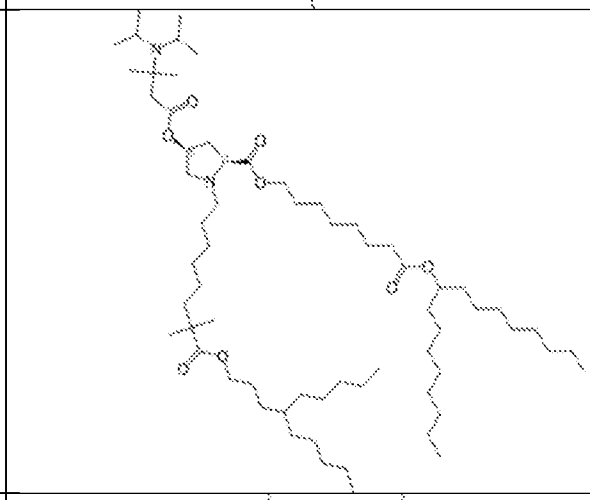
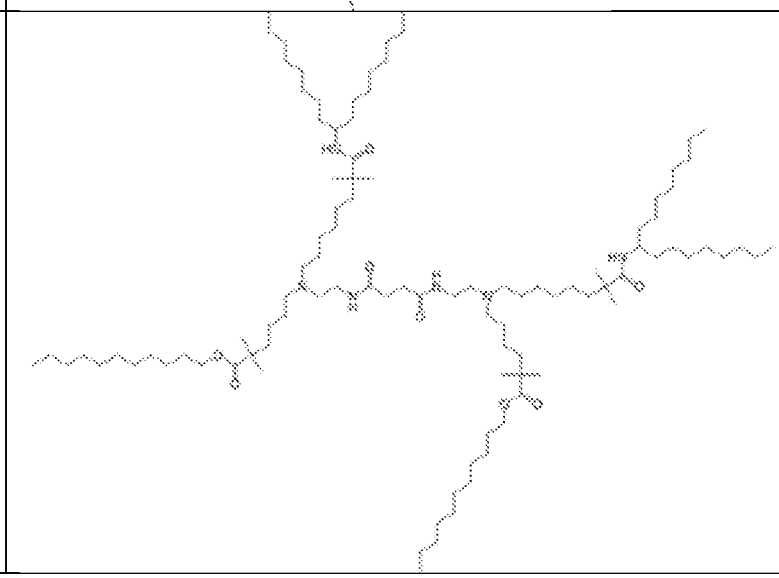
2505		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-(4-(dimethylamino)piperidin-1-yl)propanoyl)oxy)pyrrolidine-2-carboxylate
2504		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-((3-(1,4-oxazepan-4-yl)propanoyl)oxy)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)pyrrolidine-2-carboxylate
2503		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-((3-(azepan-1-yl)propanoyl)oxy)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)pyrrolidine-2-carboxylate
2502		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-(piperidin-1-yl)propanoyl)oxy)pyrrolidine-2-carboxylate

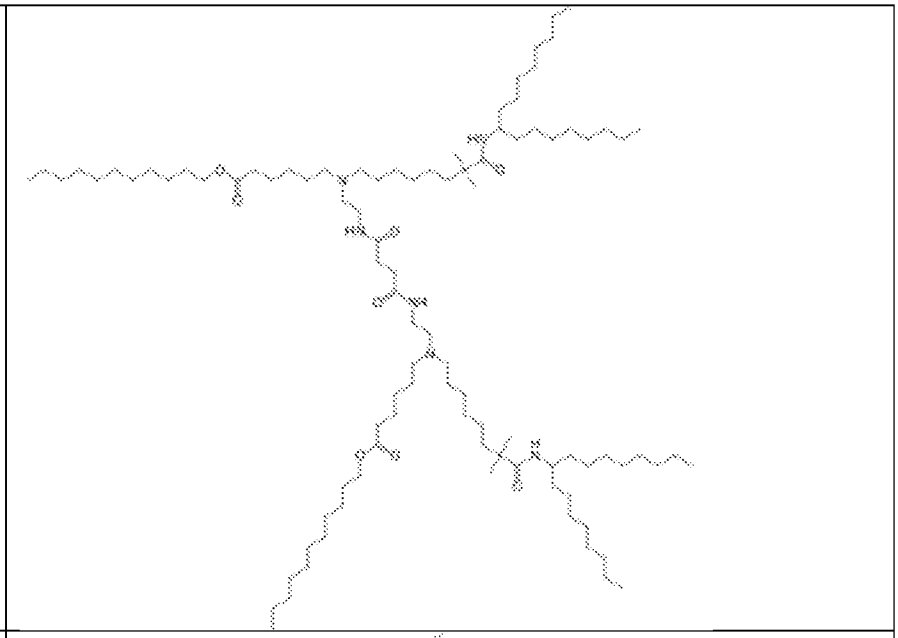
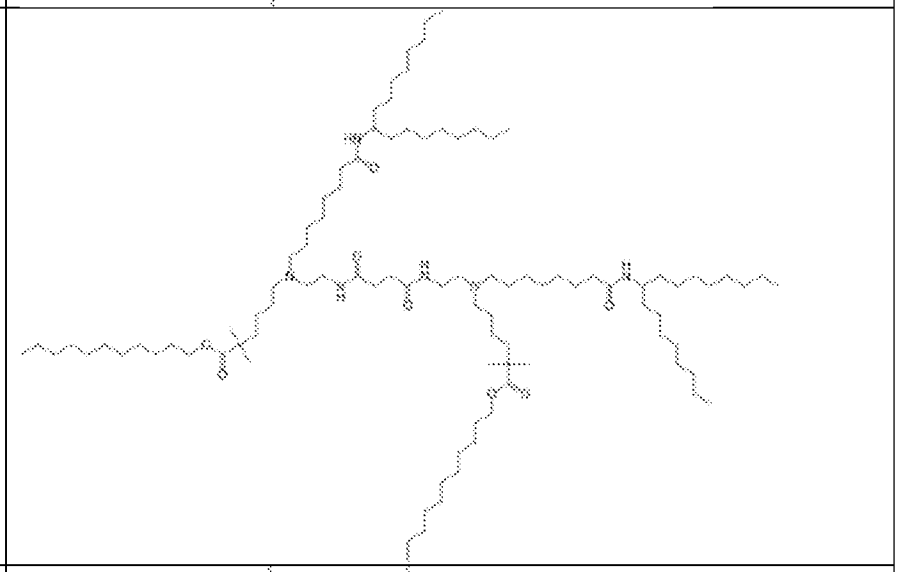
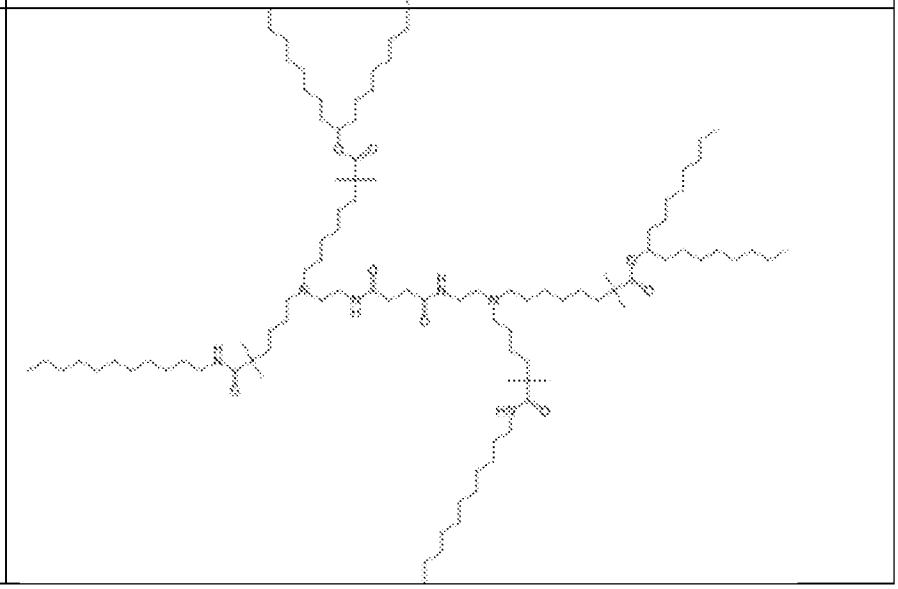
2501		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-morpholinopropanoyl)oxy)pyrrolidine-2-carboxylate
2500		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-4-((3-(azetidin-1-yl)propanoyl)oxy)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)pyrrolidine-2-carboxylate
2499		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((4-(4-methylpiperazin-1-yl)-4-oxobutanoyl)oxy)pyrrolidine-2-carboxylate
2498		8-(heptadecan-9-yloxy)-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-((4-pentylnonyl)oxy)octyl)-4-((3-(4-methylpiperazin-1-yl)-3-oxopropanoyl)oxy)pyrrolidine-2-carboxylate

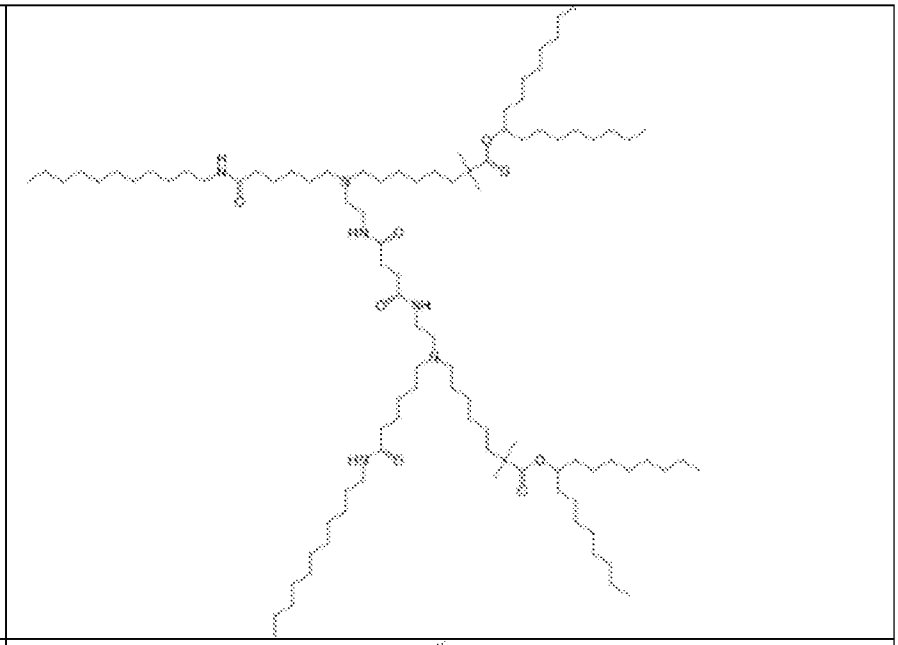
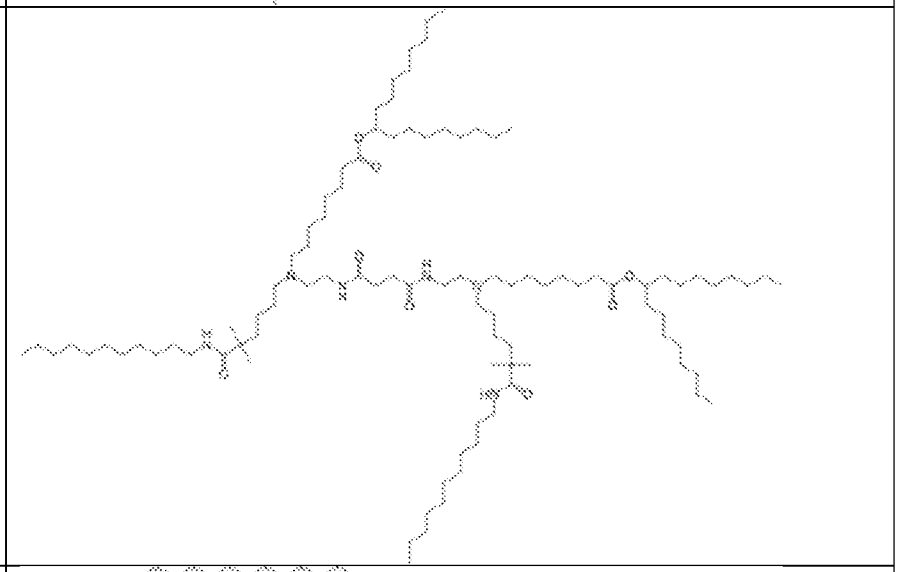
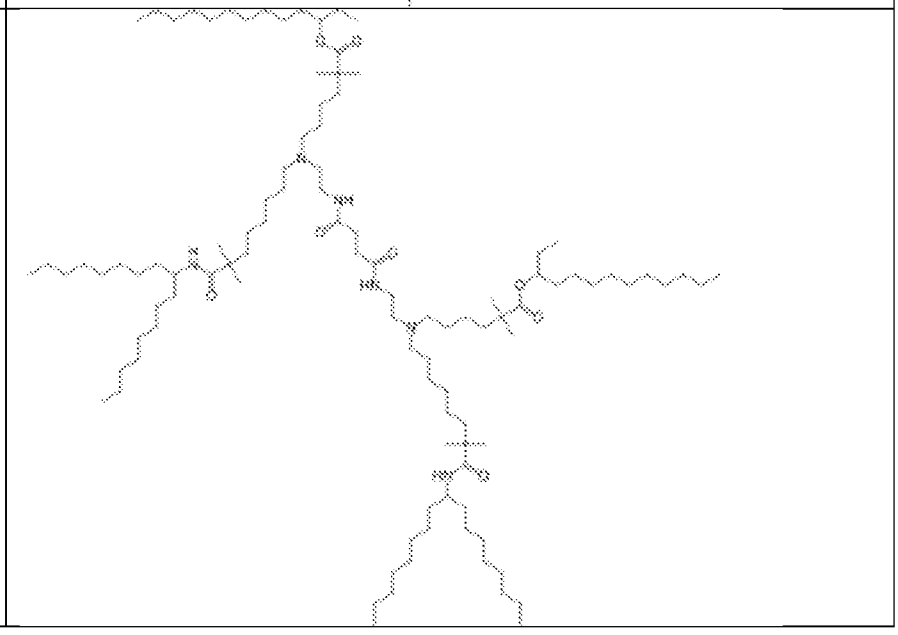
2488		7,7-dimethyl-8-oxo-8-(undecyloxy)octyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-(pentadecan-7-yloxy)octyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate
2487		7,7-dimethyl-8-oxo-8-(pentadecan-8-yloxy)octyl (2S,4S)-1-(5,5-dimethyl-6-oxo-6-(undecyloxy)hexyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate
2486		7,7-dimethyl-8-oxo-8-(tridecan-7-yloxy)octyl (2S,4S)-1-(5,5-dimethyl-6-oxo-6-(undecyloxy)hexyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate
2485		8-(heptadecan-9-yloxy)-7,7-dimethyl-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-(pentadecan-8-yloxy)octyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate

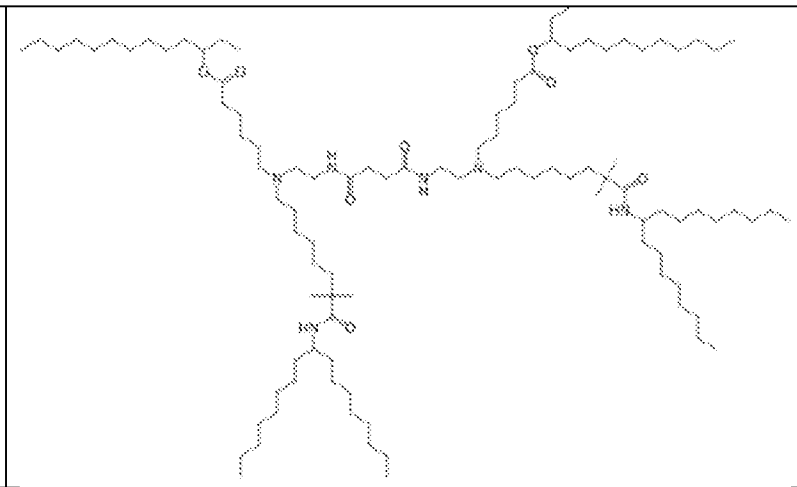
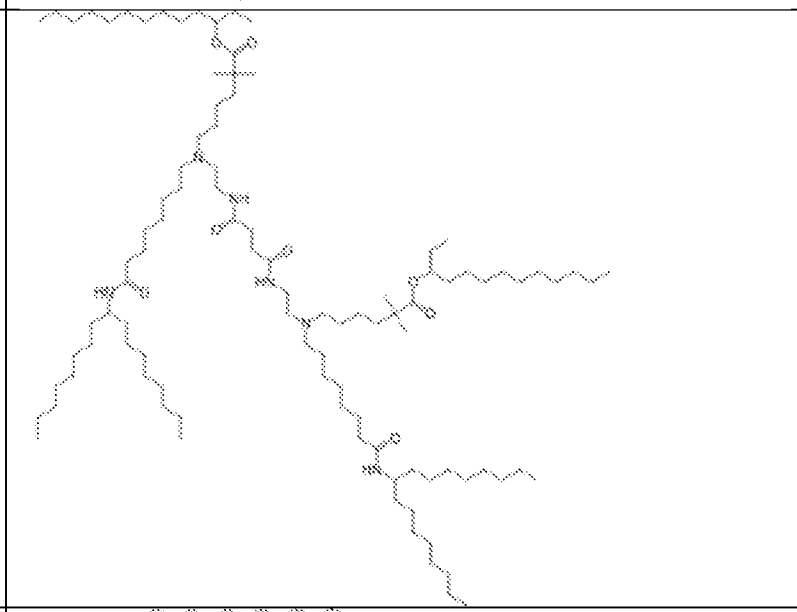
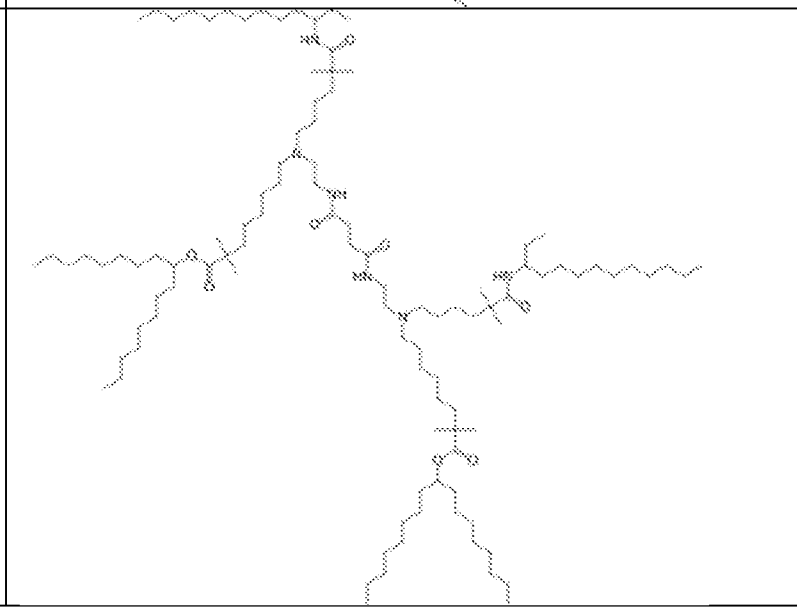
2484		8-(heptadecan-9-yloxy)-7,7-dimethyl-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-(tridecan-7-yloxy)octyl)-4-((3-(dimethylamino)propanoyl)oxy)pyrrolidine-2-carboxylate
2482		8-(heptadecan-9-yloxy)-7,7-dimethyl-8-oxooctyl (2S,4S)-1-(7,7-dimethyl-8-oxo-8-(pentadecan-7-yloxy)octyl)-4-((3-(pyrrolidin-1-yl)propanoyl)oxy)pyrrolidine-2-carboxylate
2535		
2536		

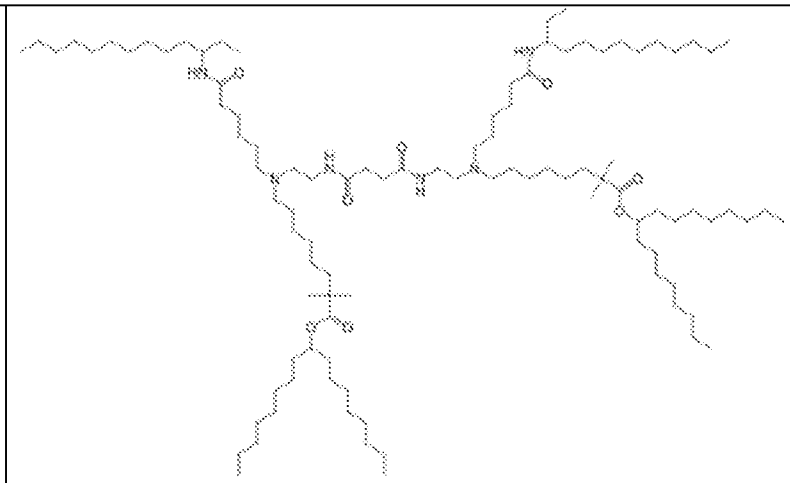
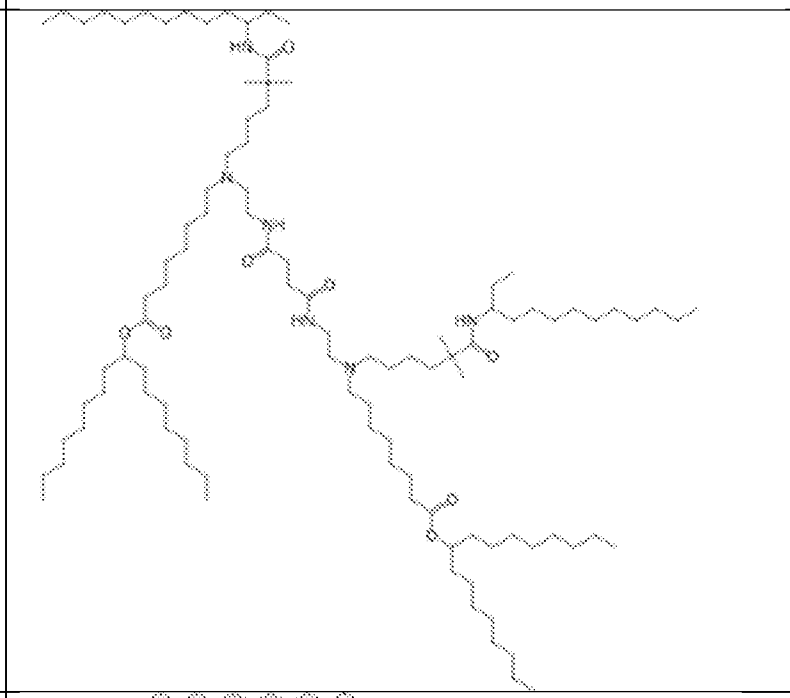
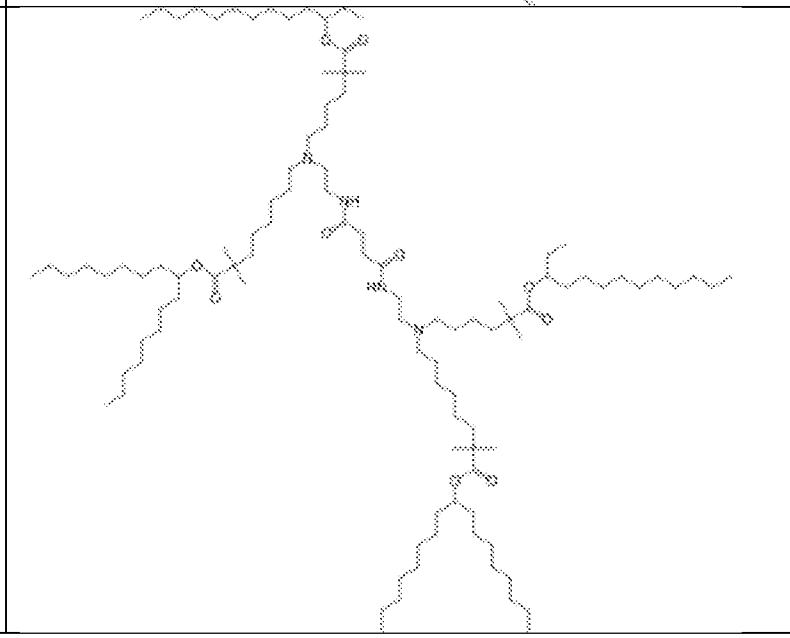
2537	
2544	
2545	
2546	

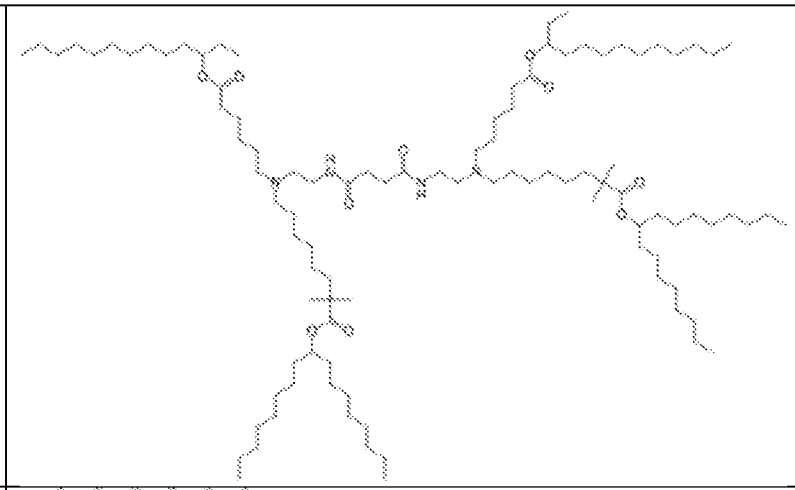
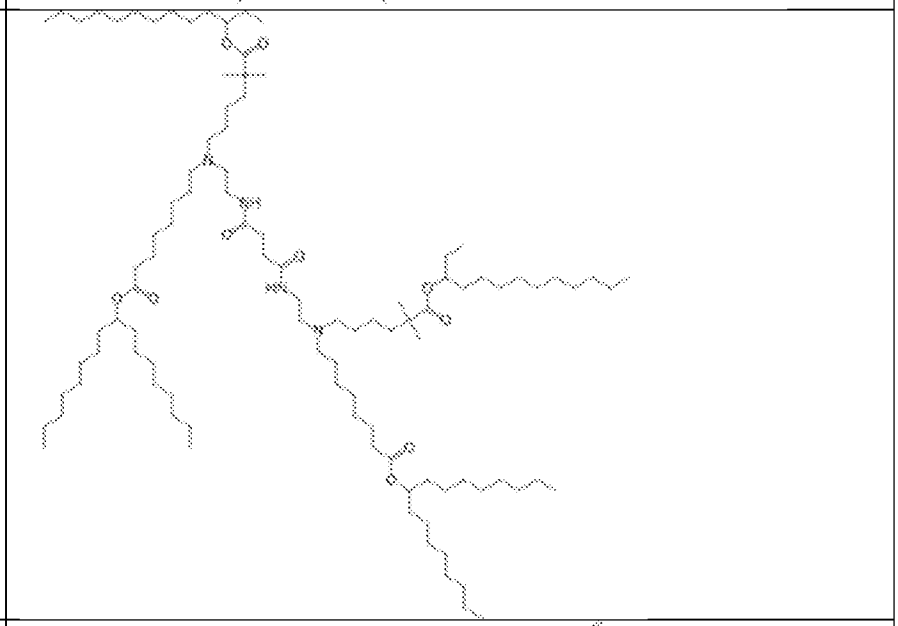
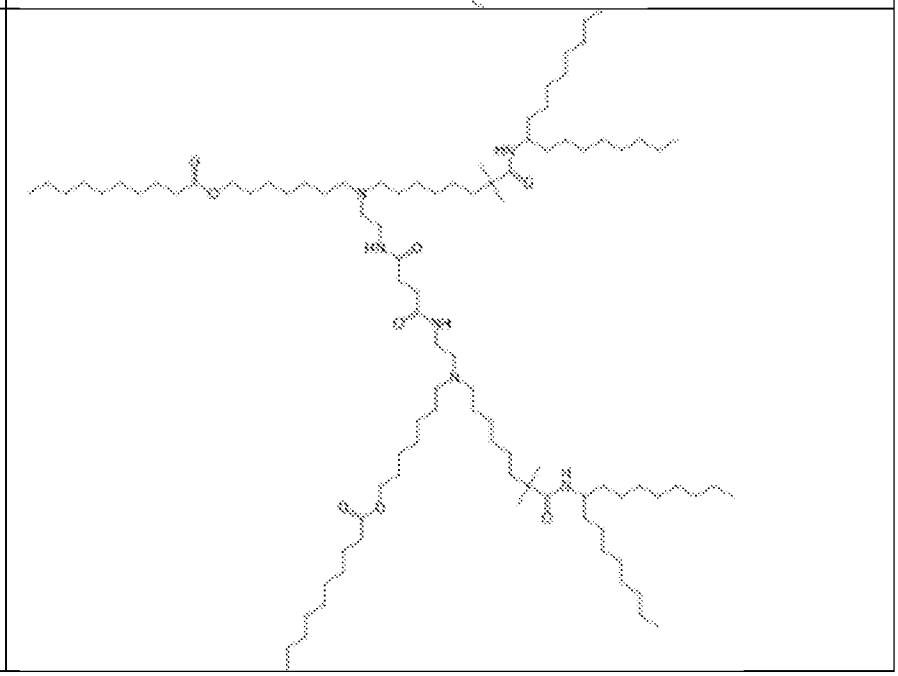
2547	
2551	
2552	

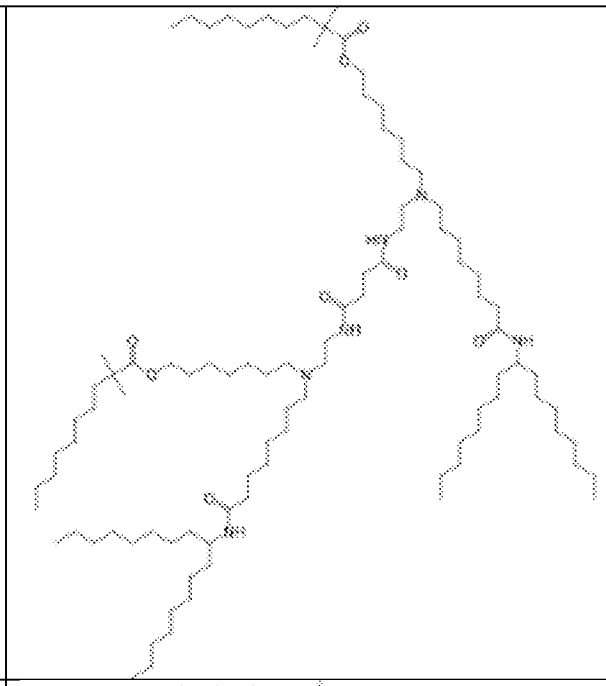
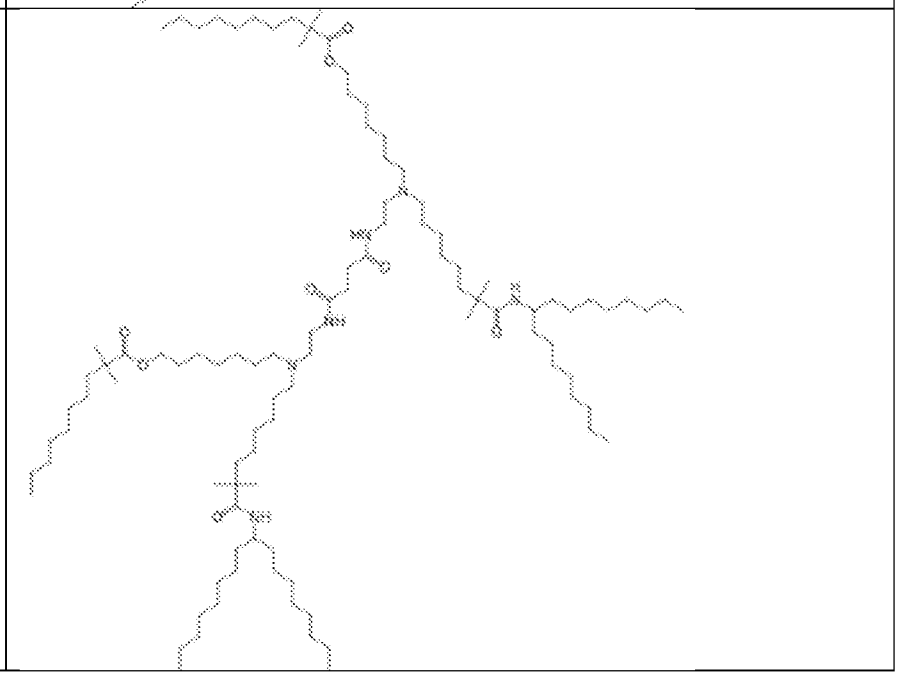
2553	
2554	
2555	

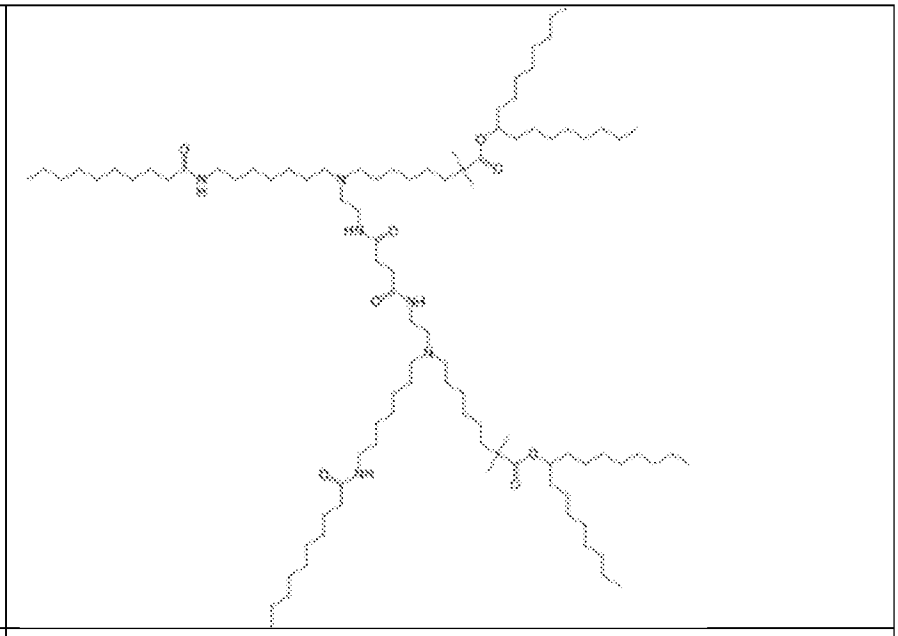
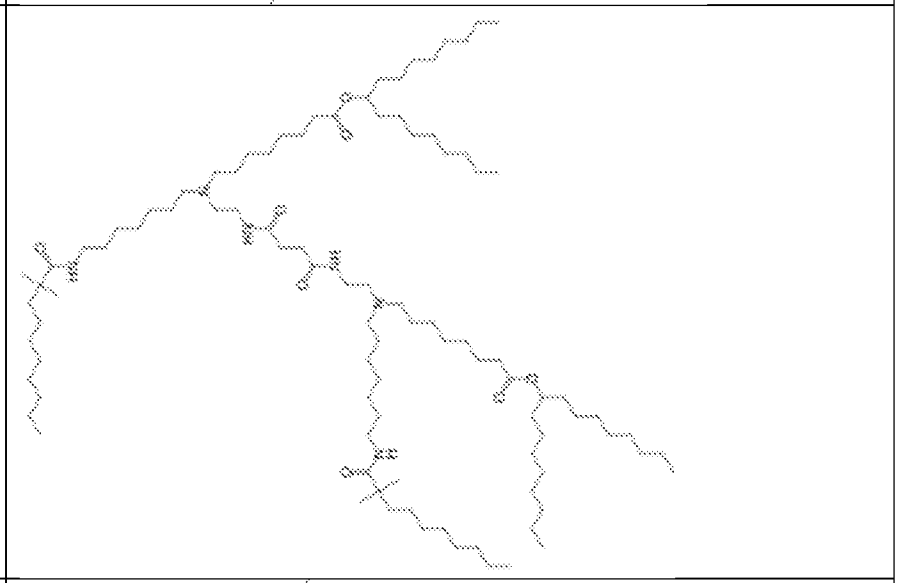
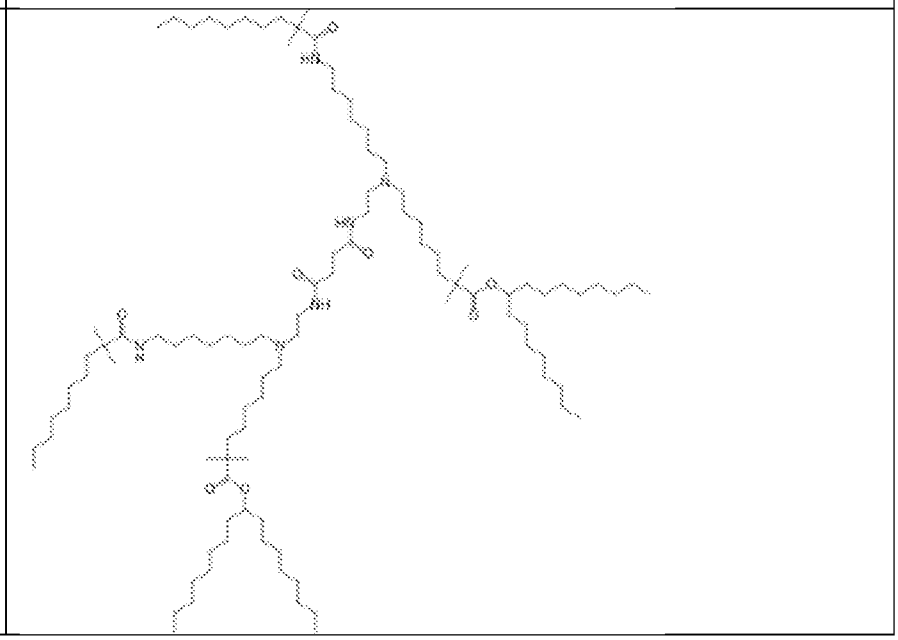
2556	
2557	
2559	

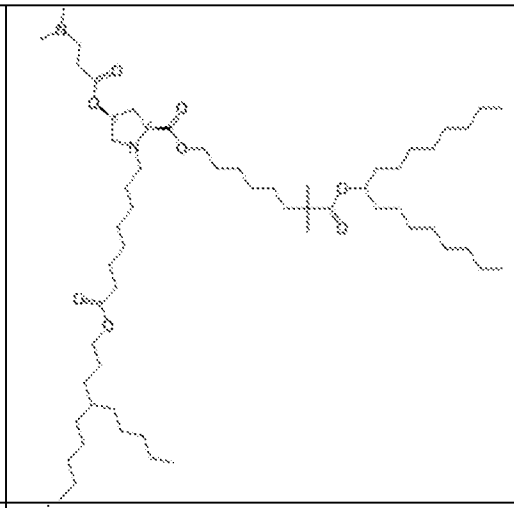
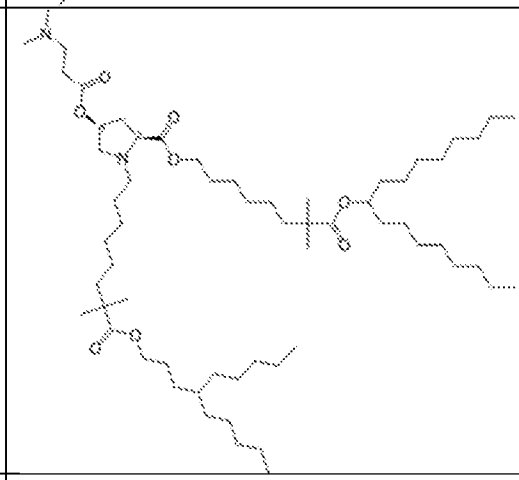
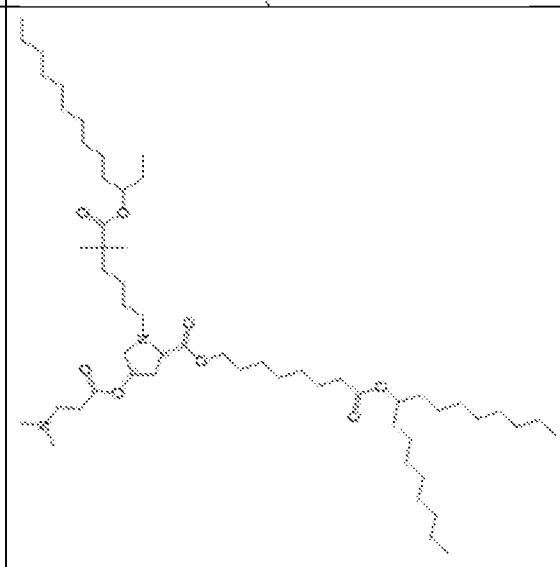
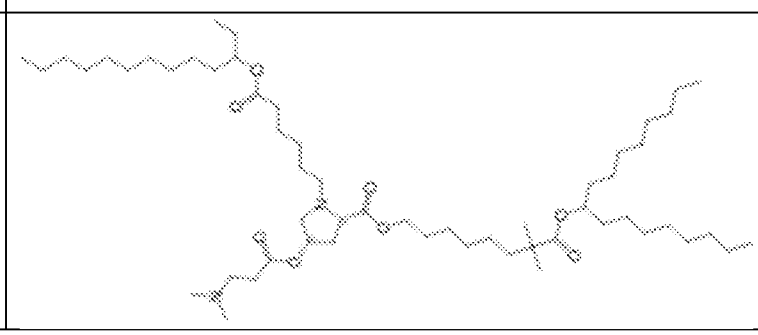
2560	
2561	
2563	

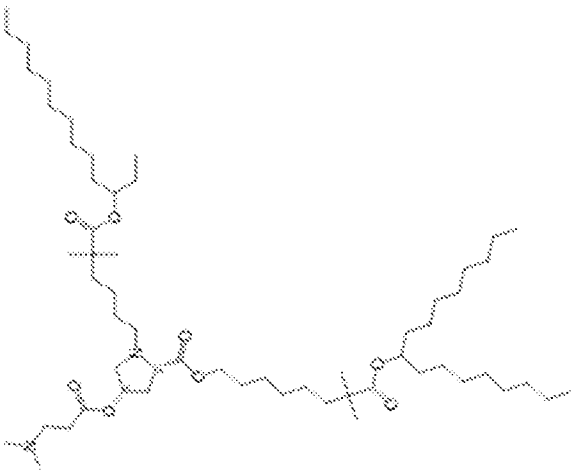
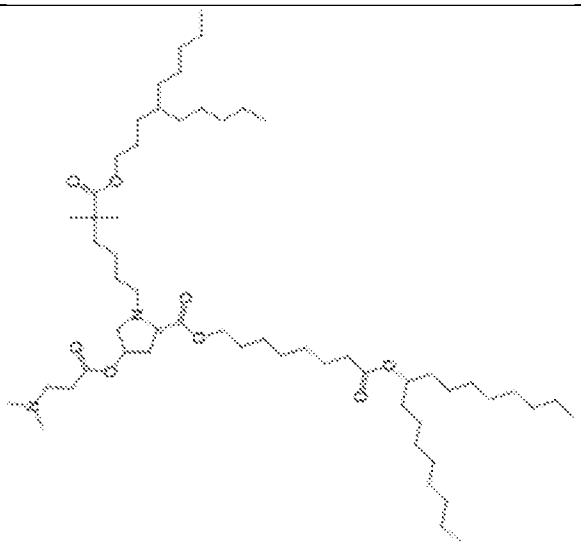
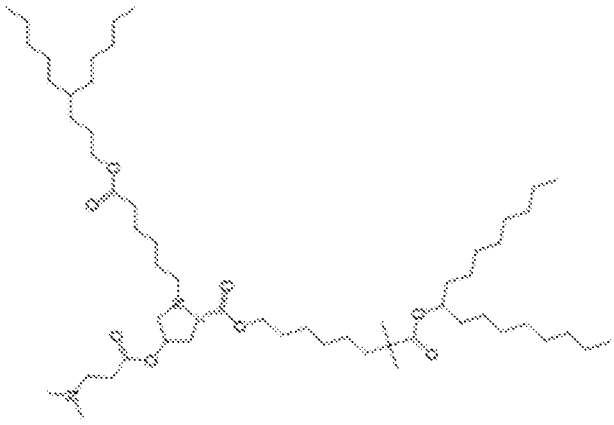
2564	
2565	
2566	

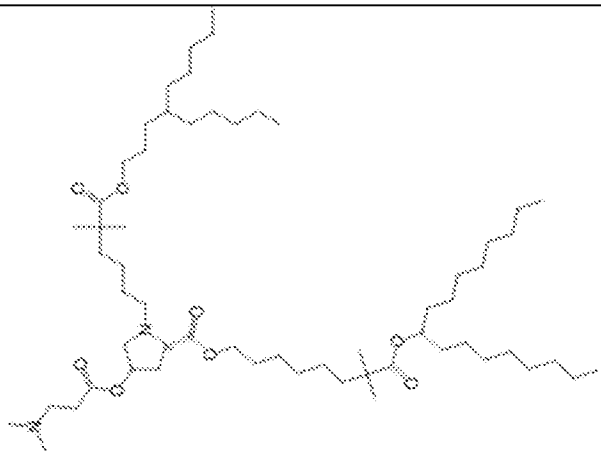
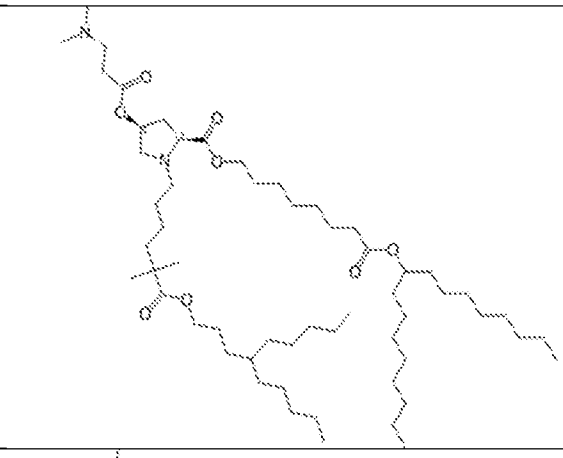
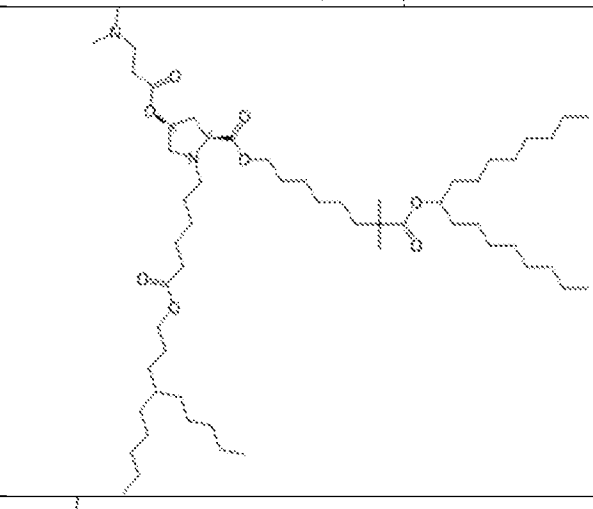
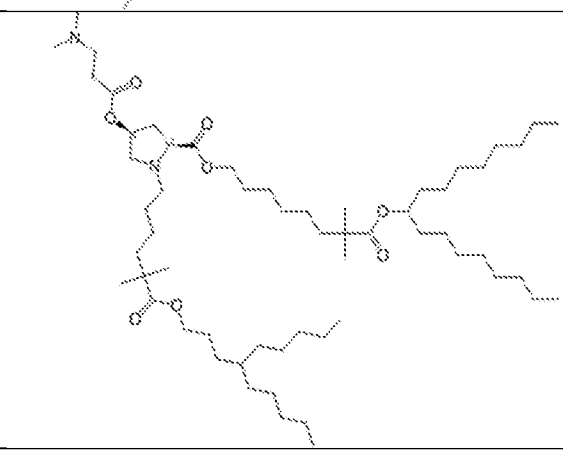
2567	
2568	
2569	

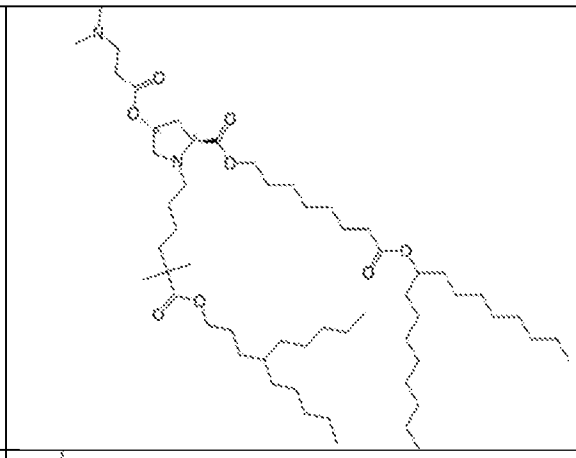
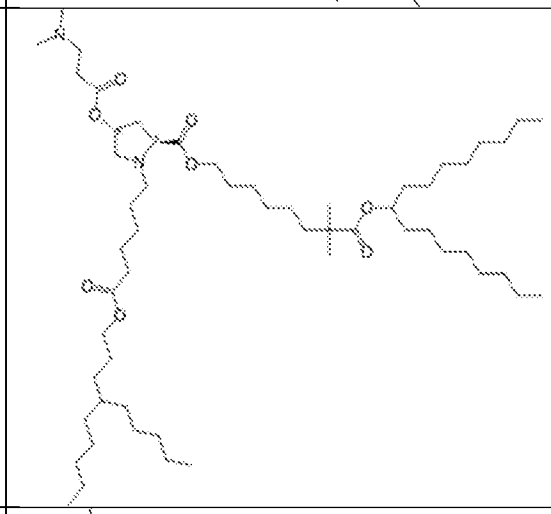
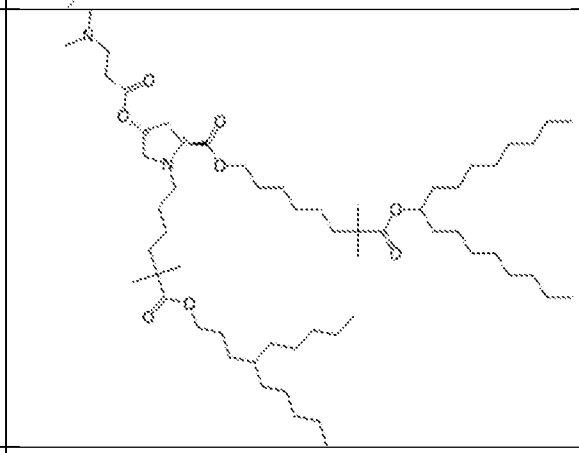
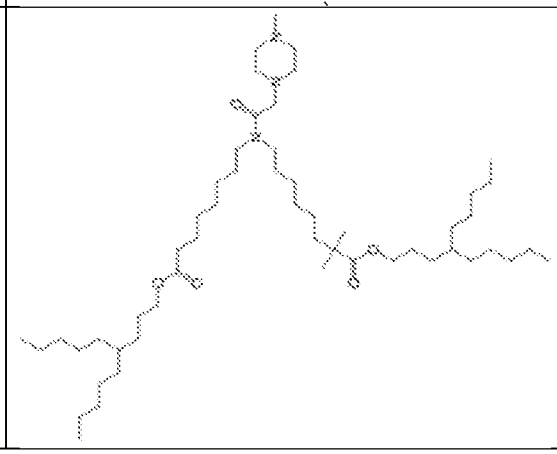
2570	
2571	

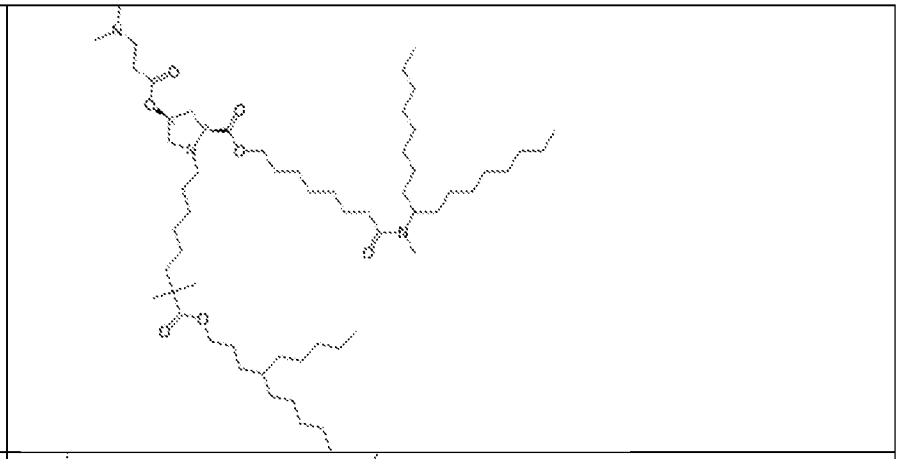
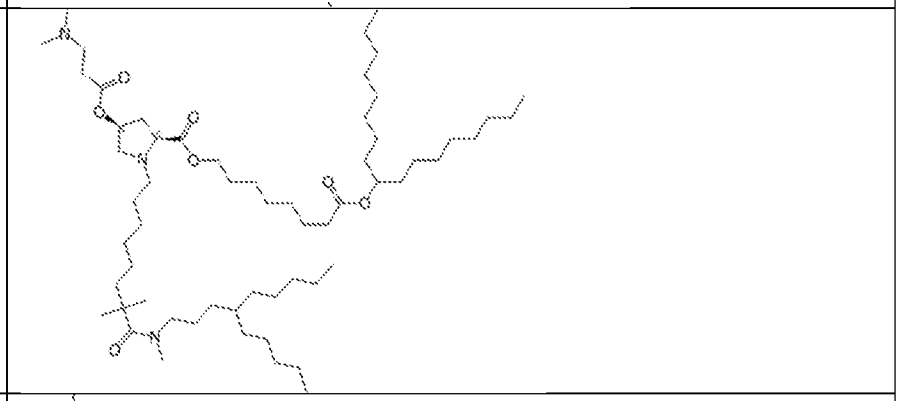
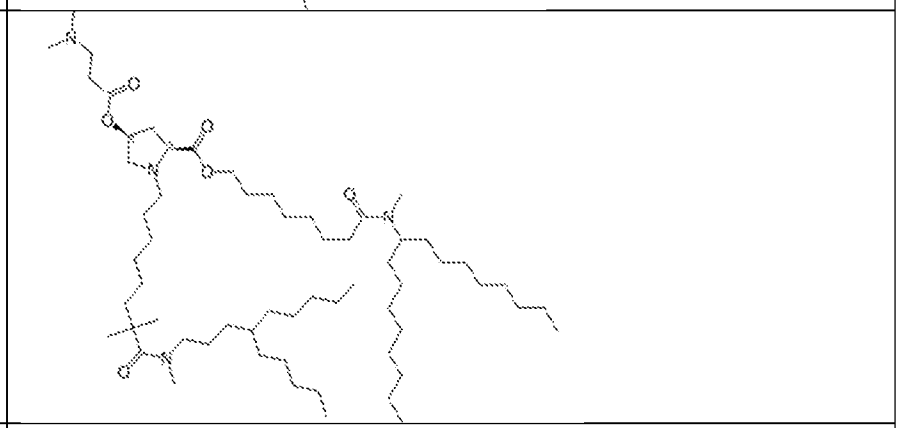
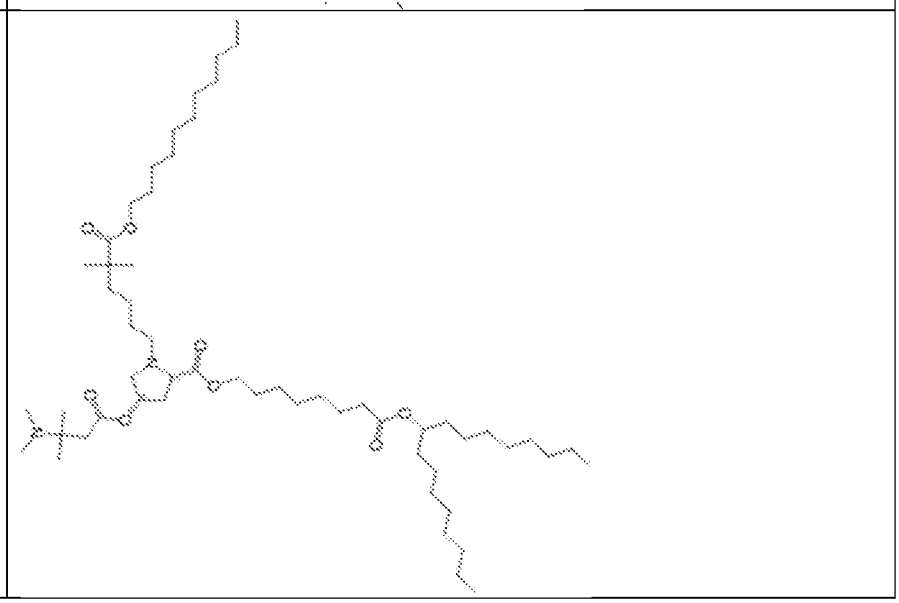
2573	
2574	
2575	

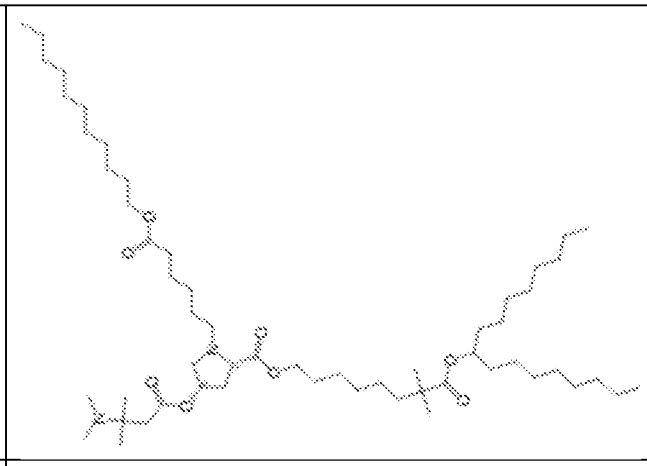
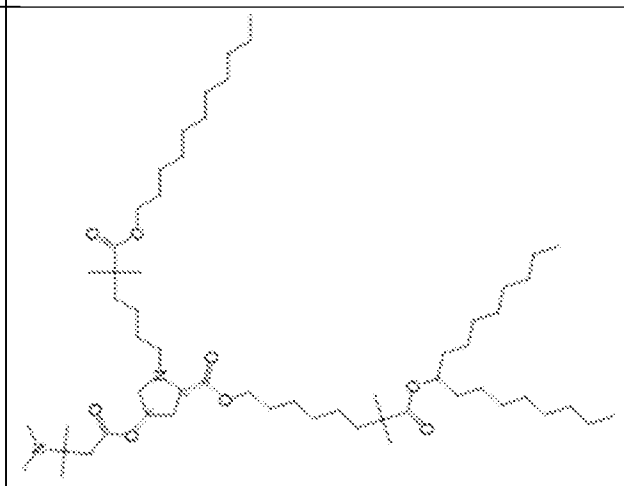
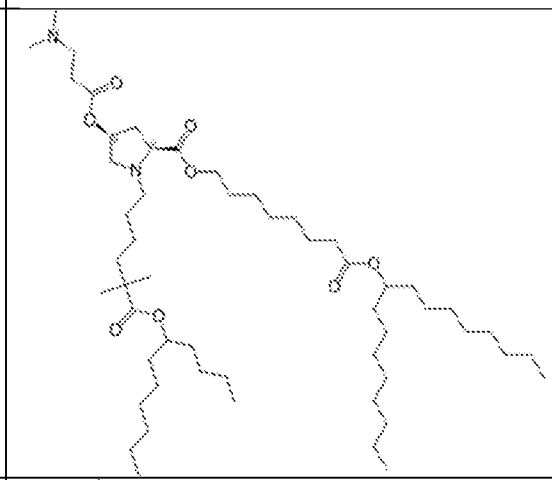
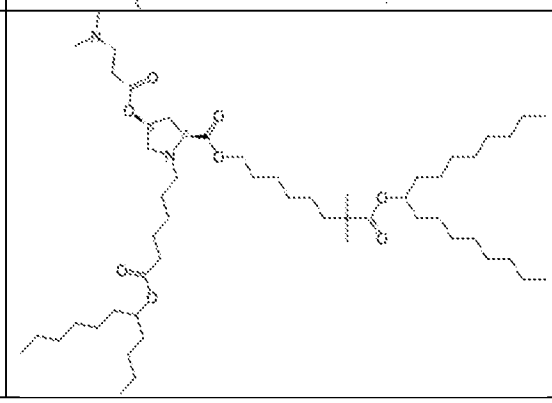
2577	
2578	
2580	
2581	

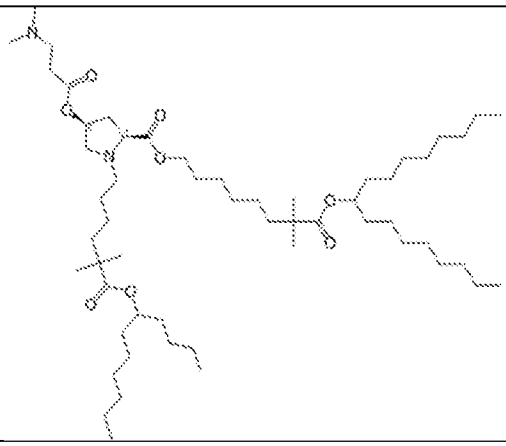
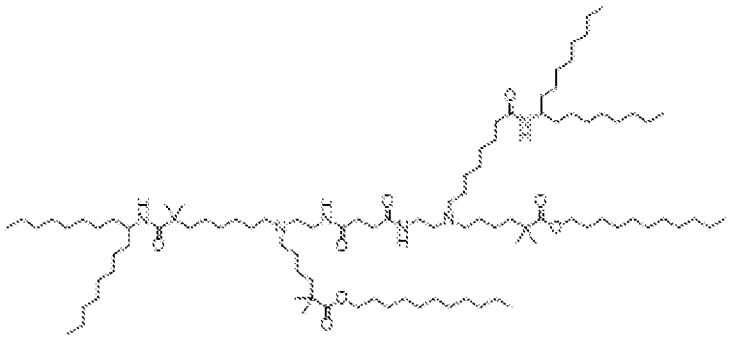
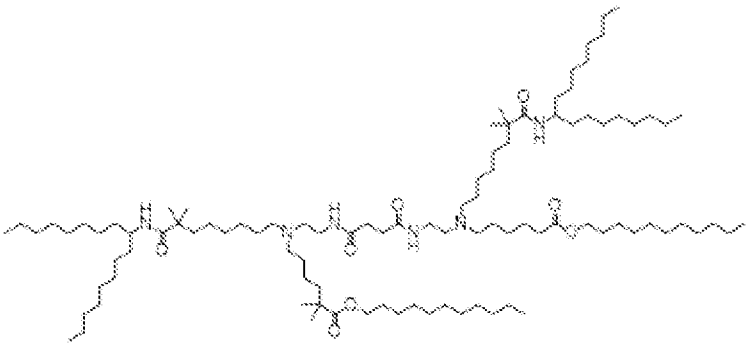
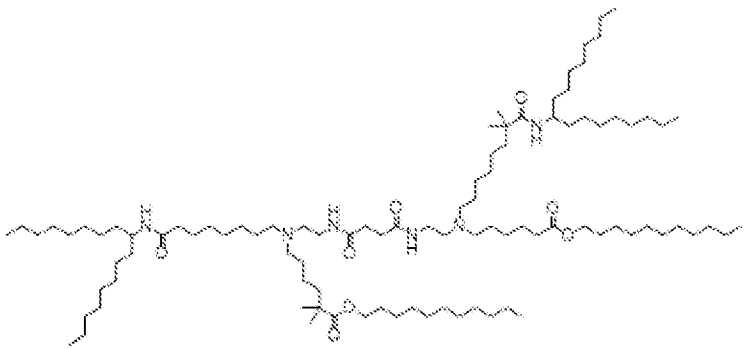
2582	
2583	
2584	

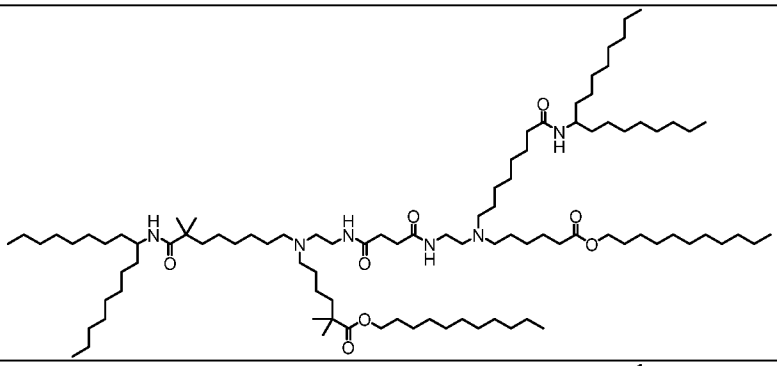
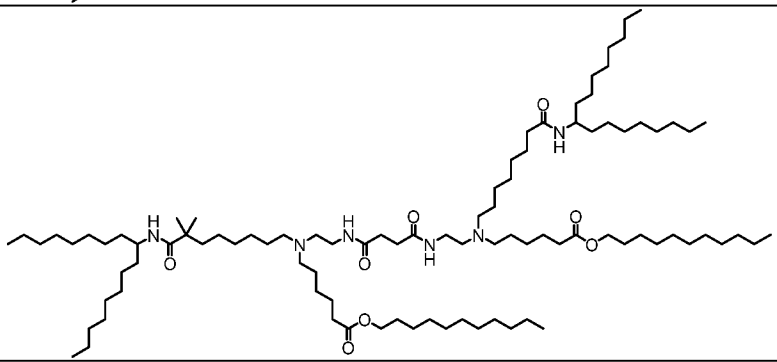
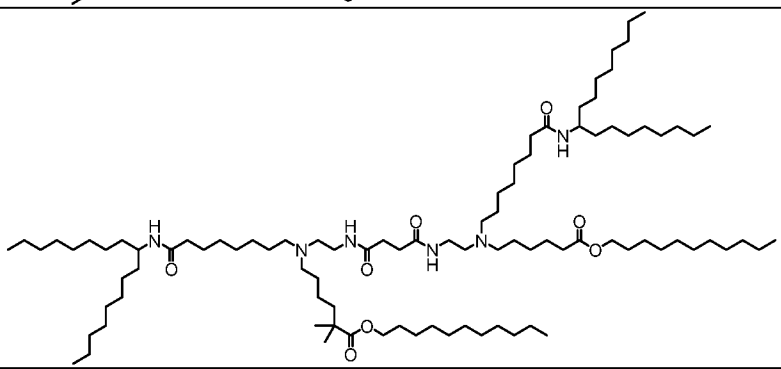
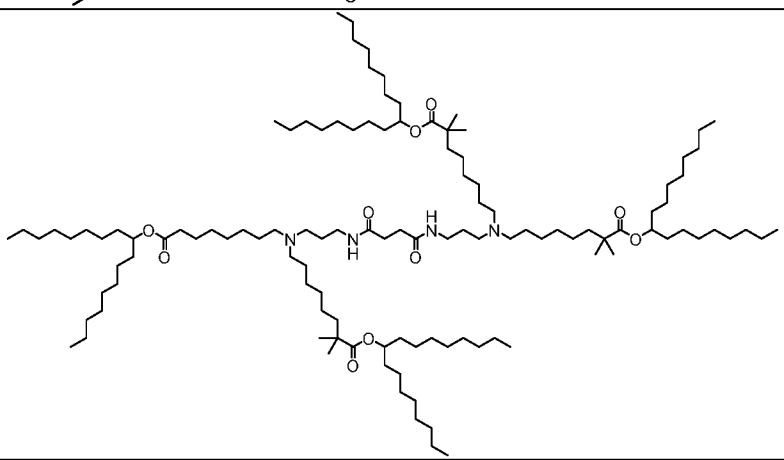
2585	
2586	
2587	
2588	

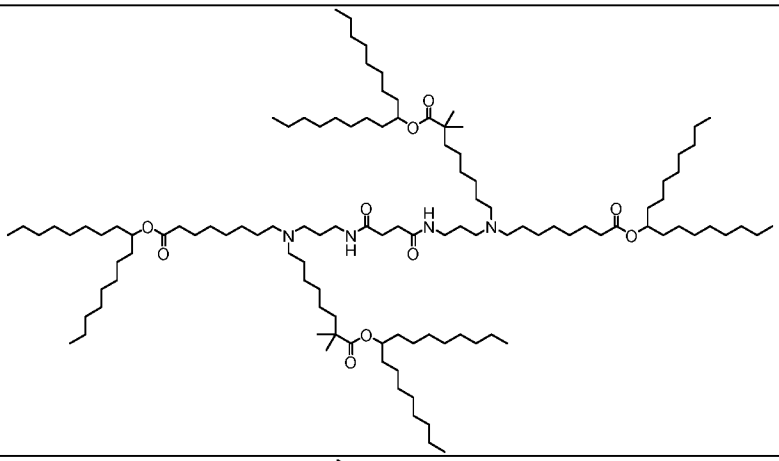
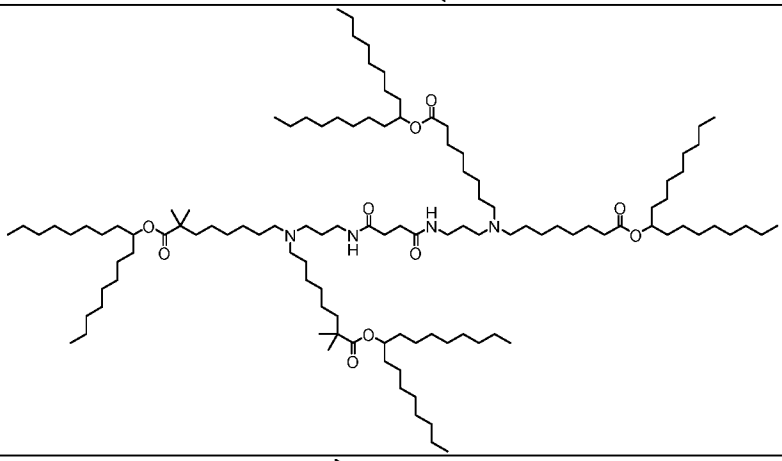
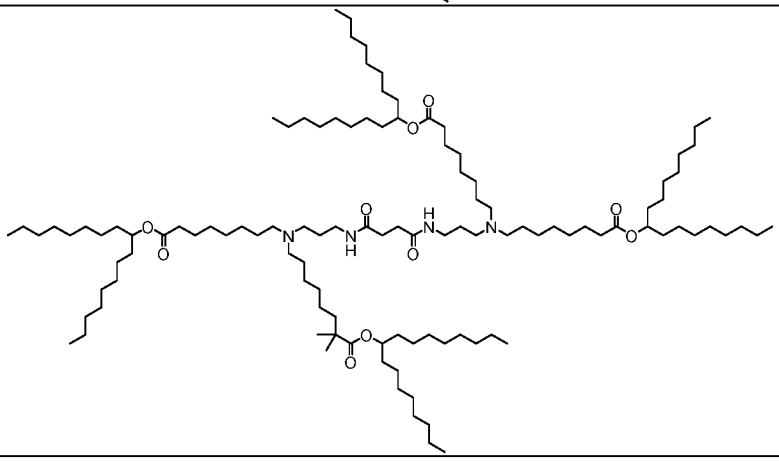
2589	
2590	
2591	
2592	

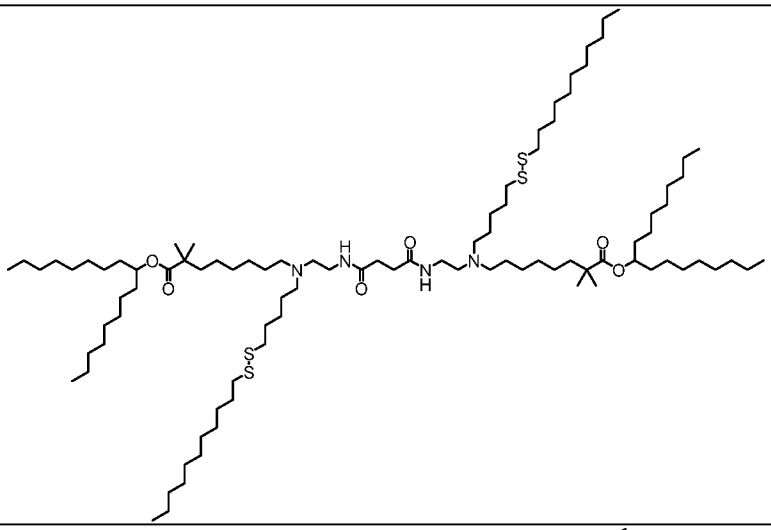
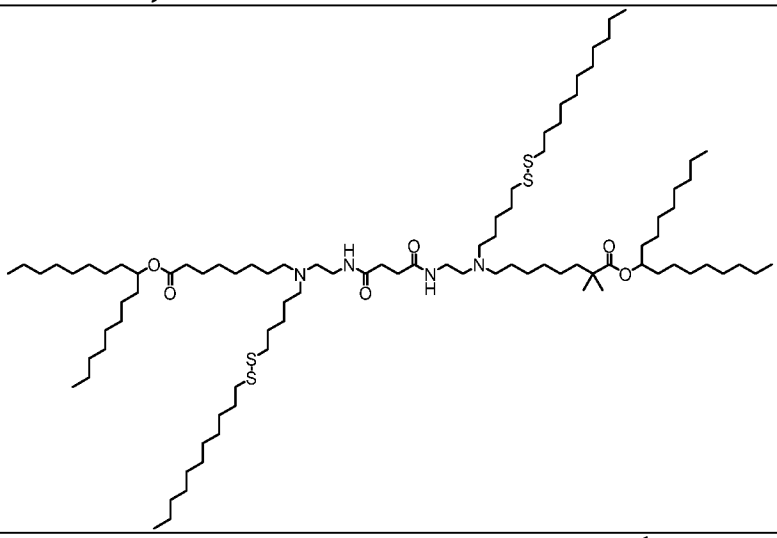
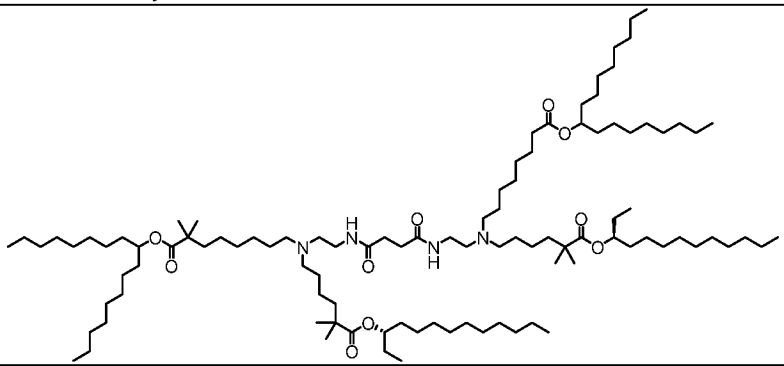
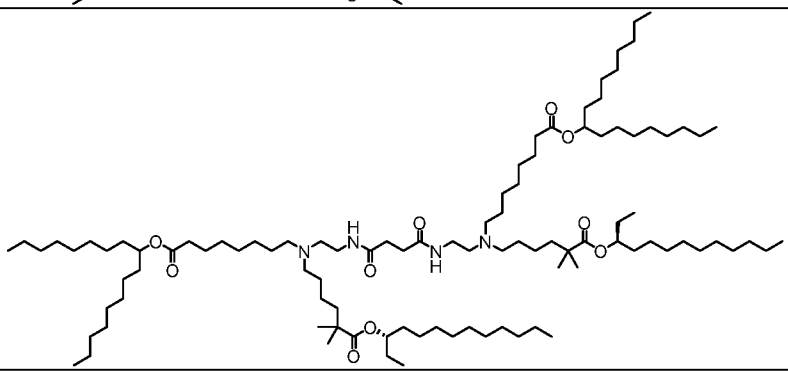
2599	
2600	
2601	
2608	

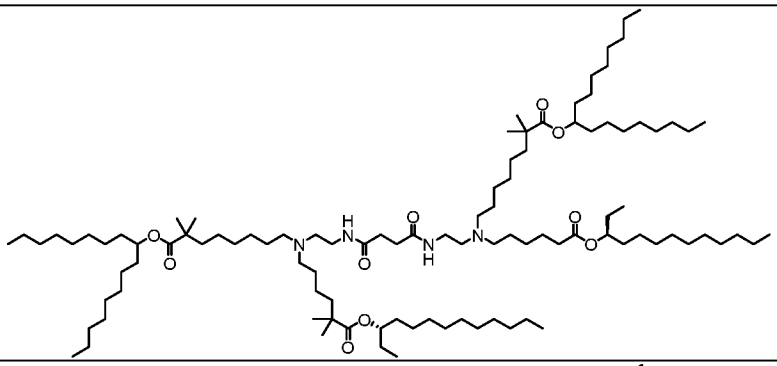
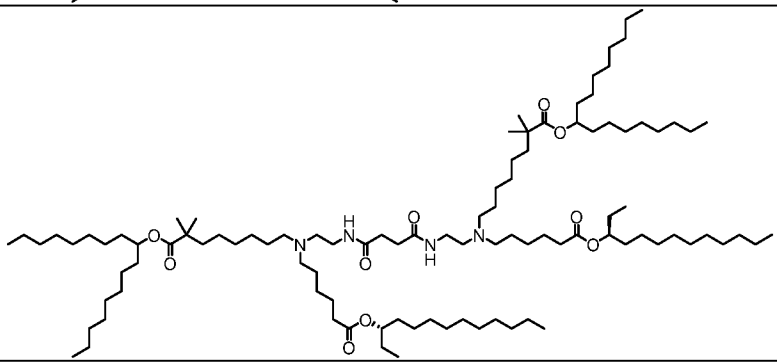
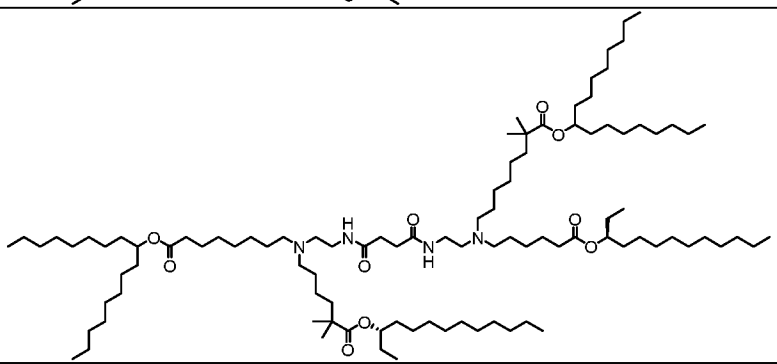
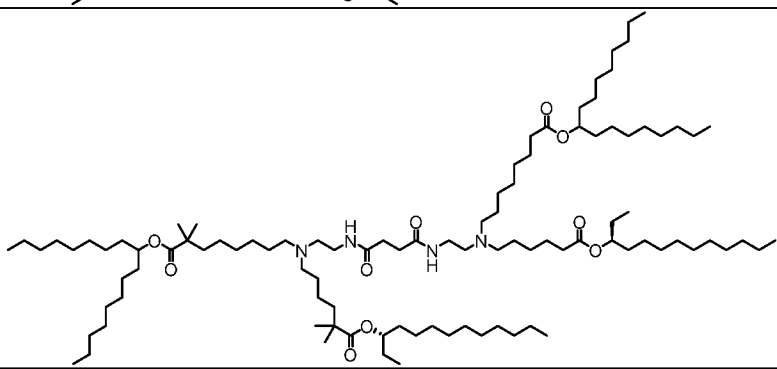
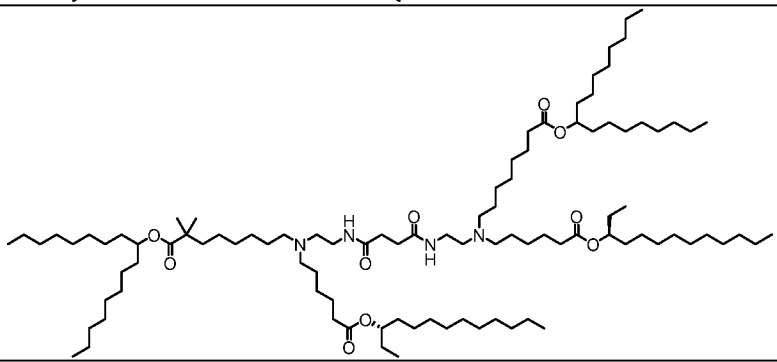
2609	
2610	
2611	
2612	

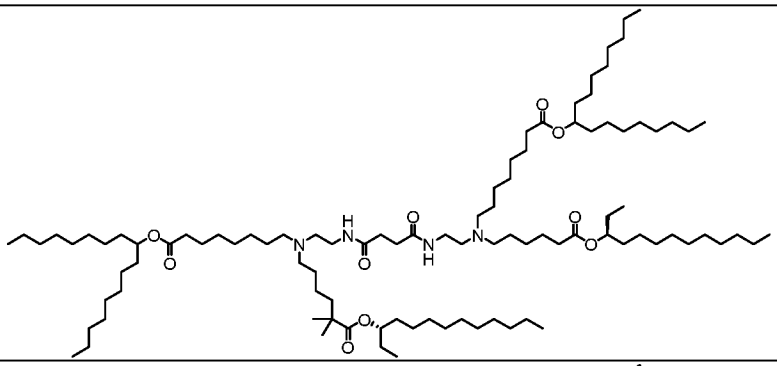
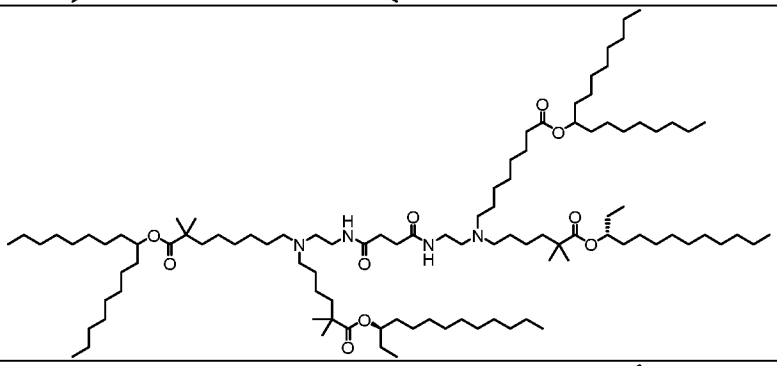
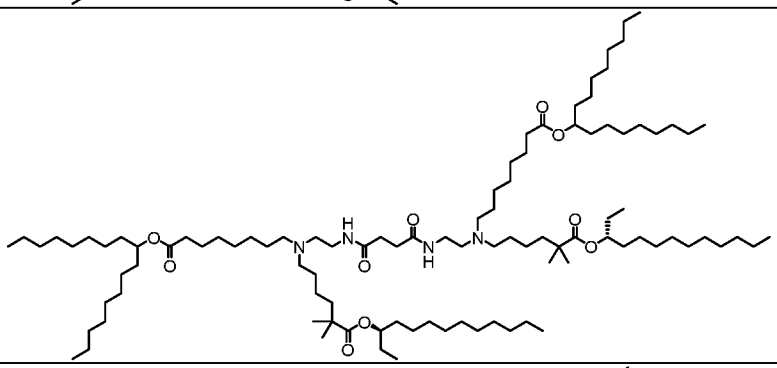
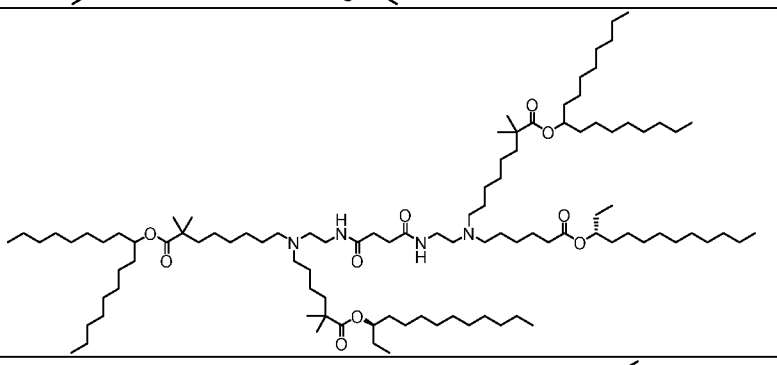
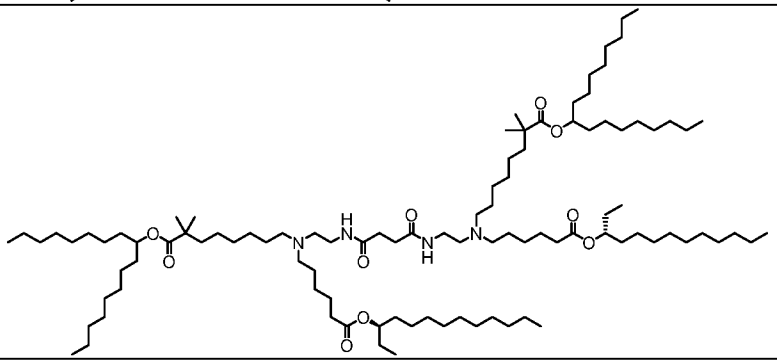
2613	
A1	
A2	
A3	

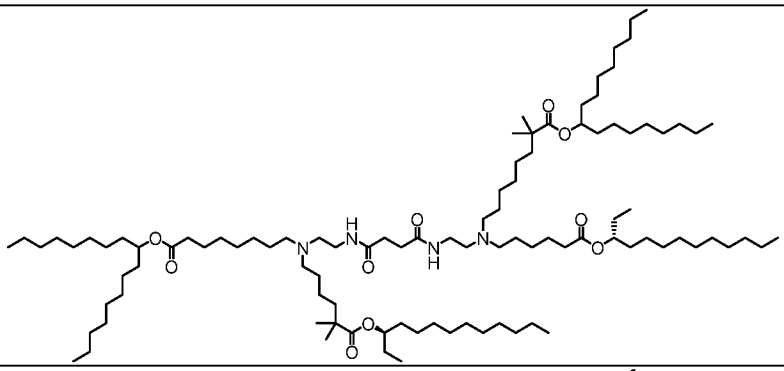
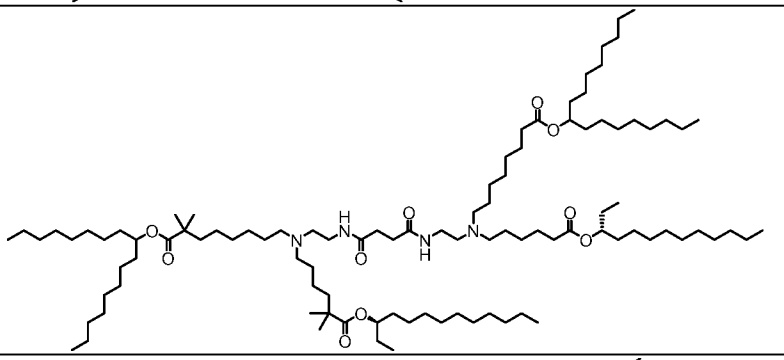
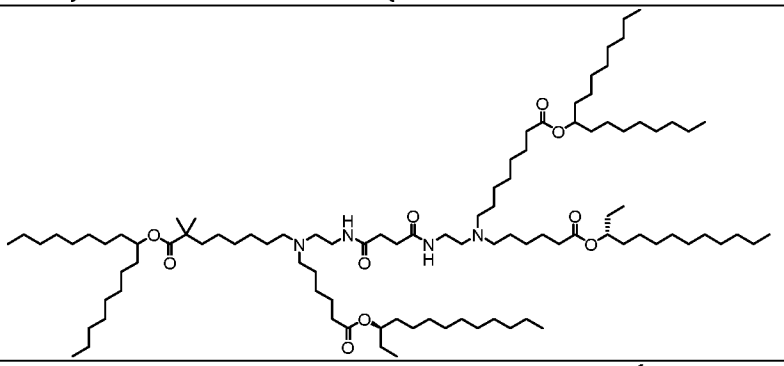
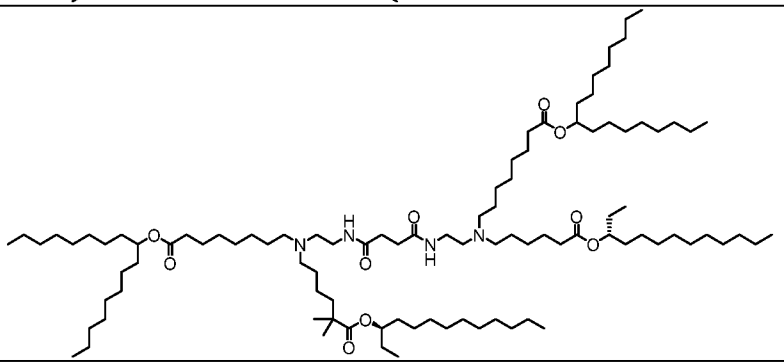
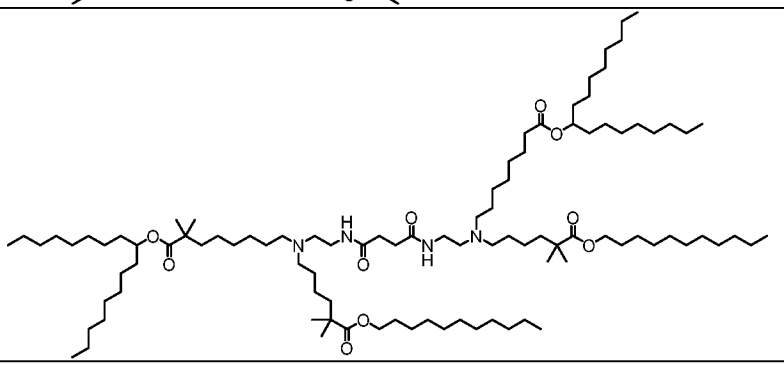
A4	
A5	
A6	
A7	

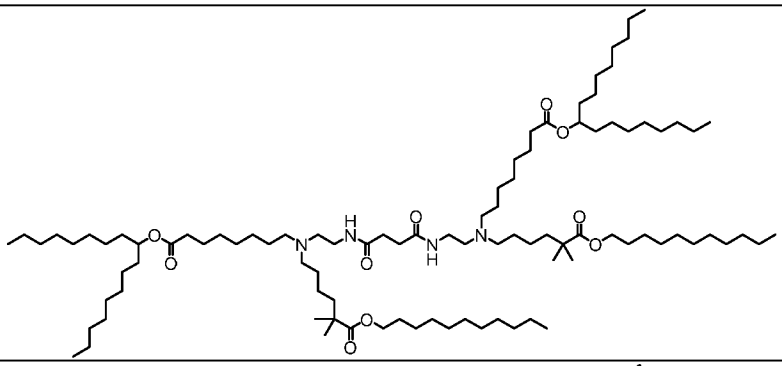
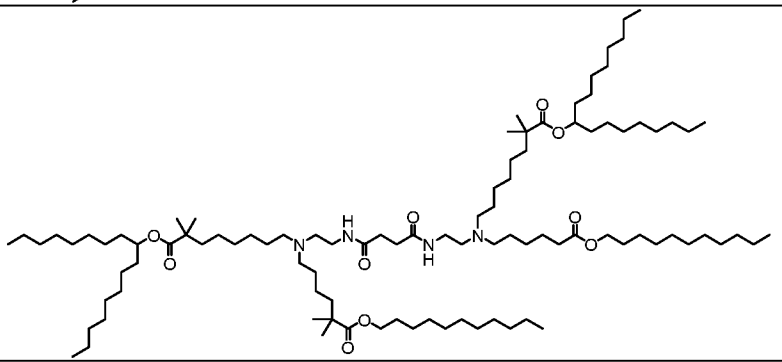
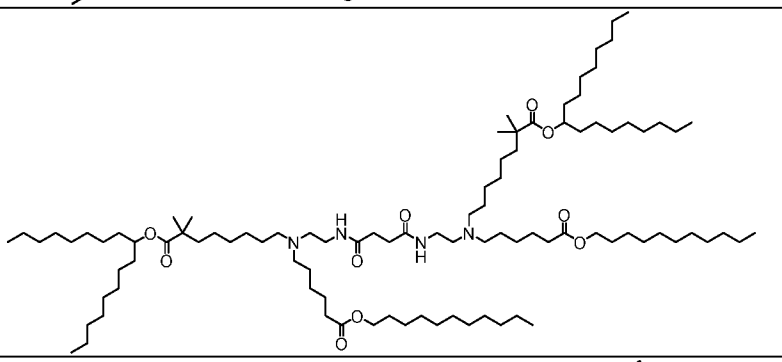
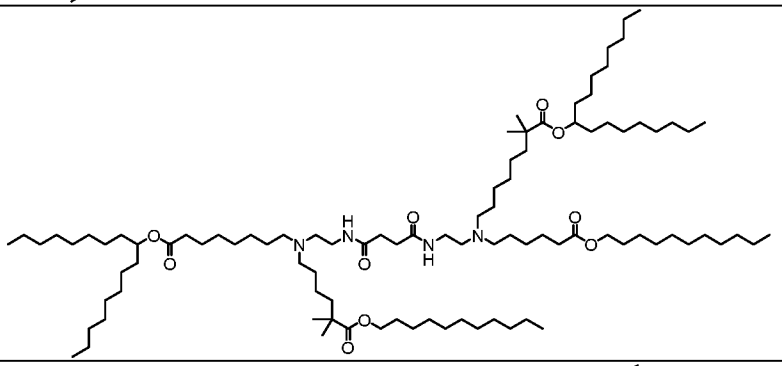
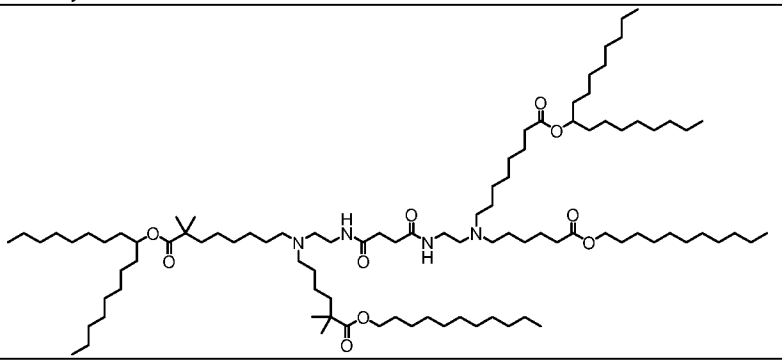
A8	
A9	
A10	

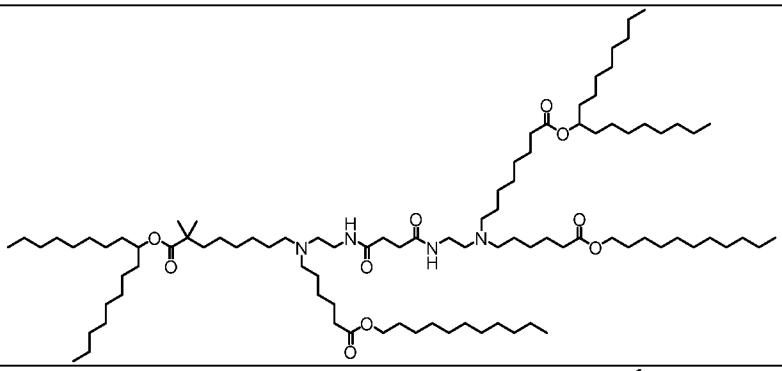
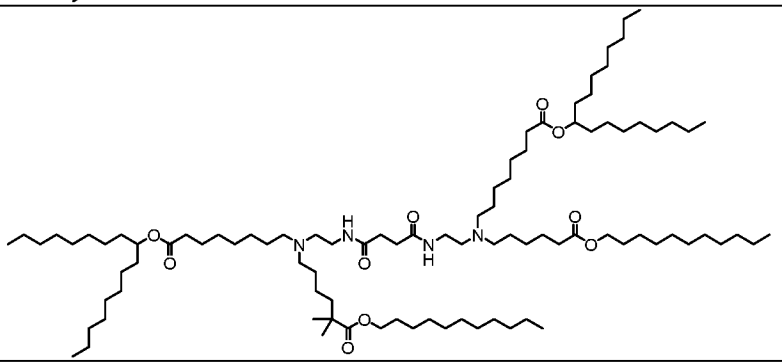
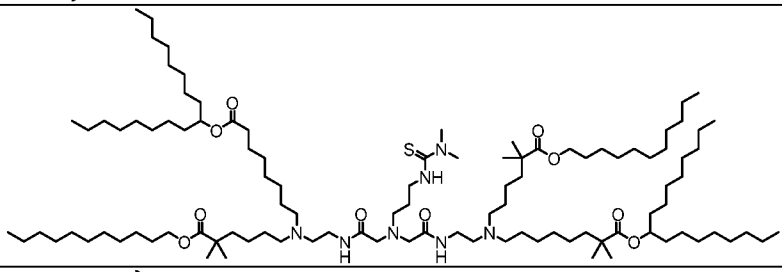
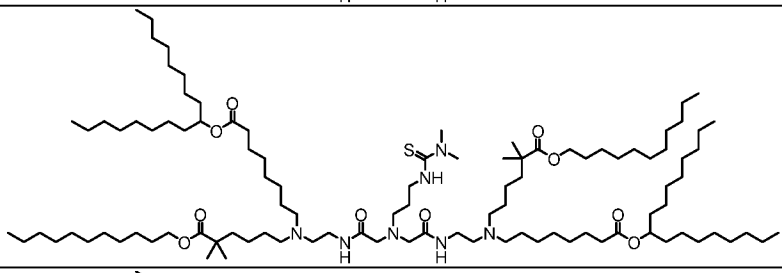
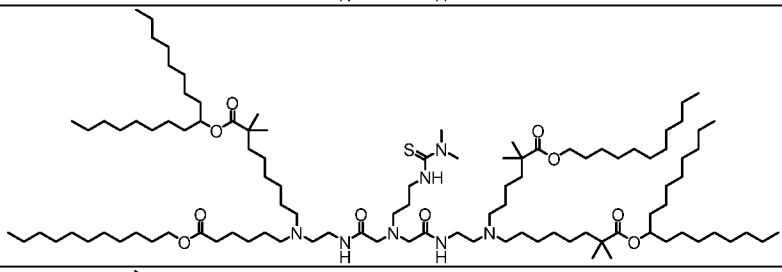
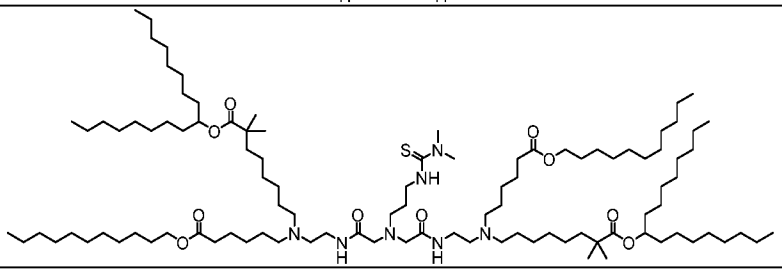
A11	
A12	
A13	
A14	

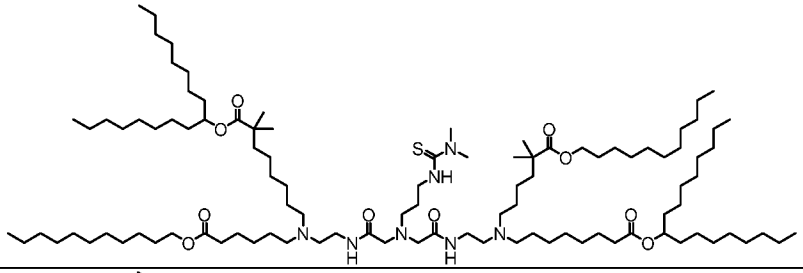
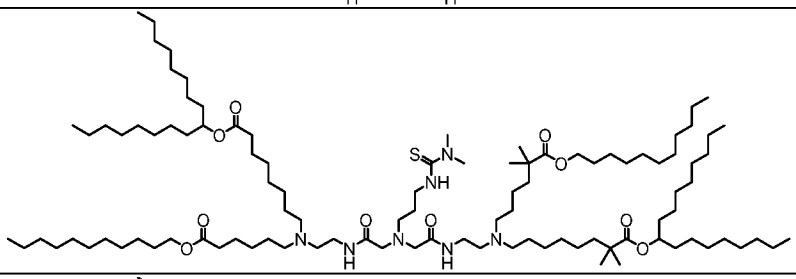
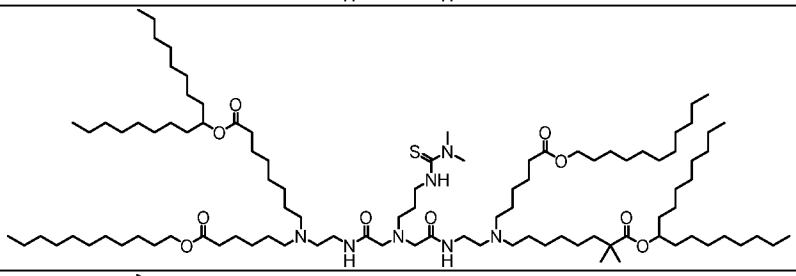
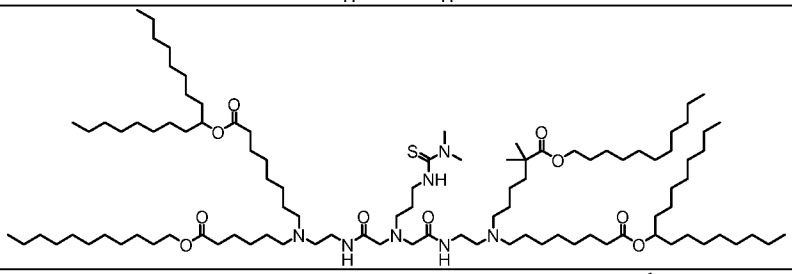
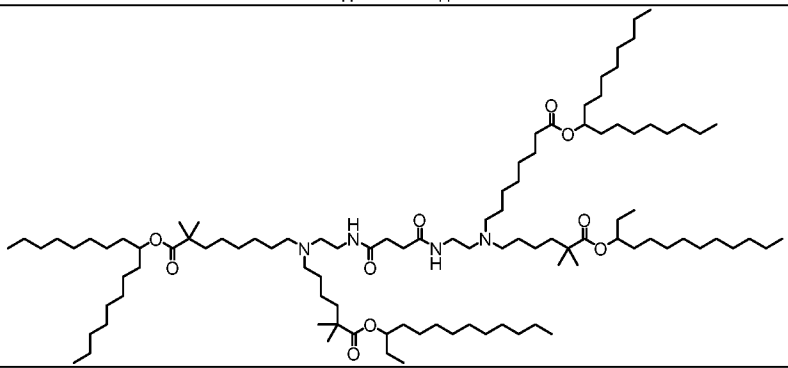
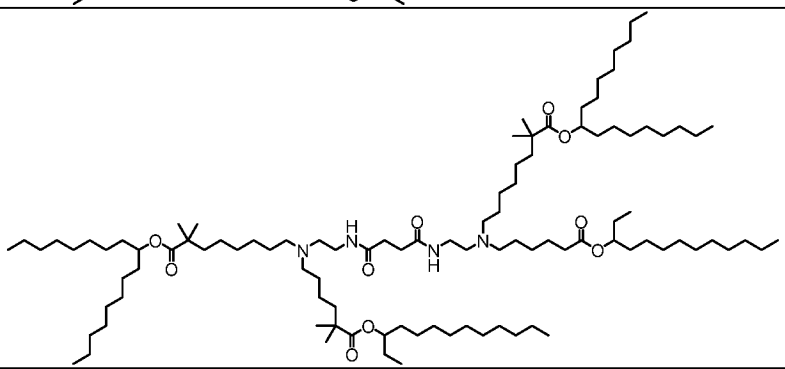
A15	
A16	
A17	
A18	
A19	

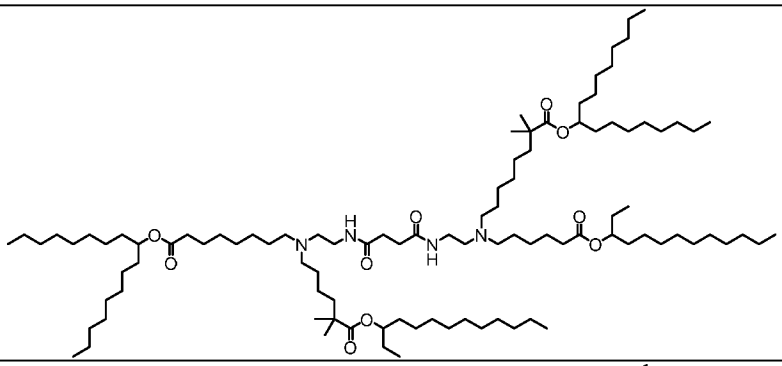
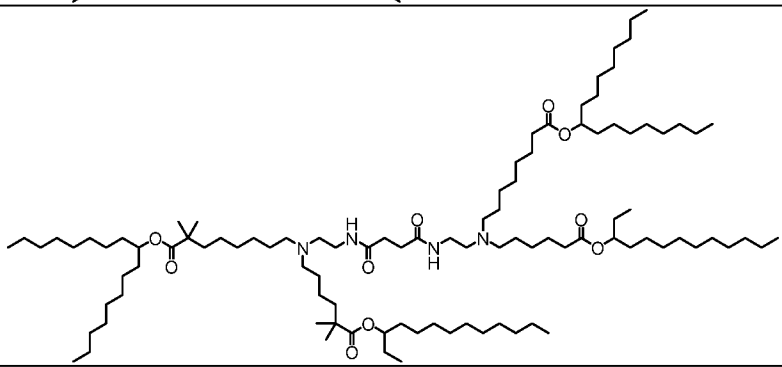
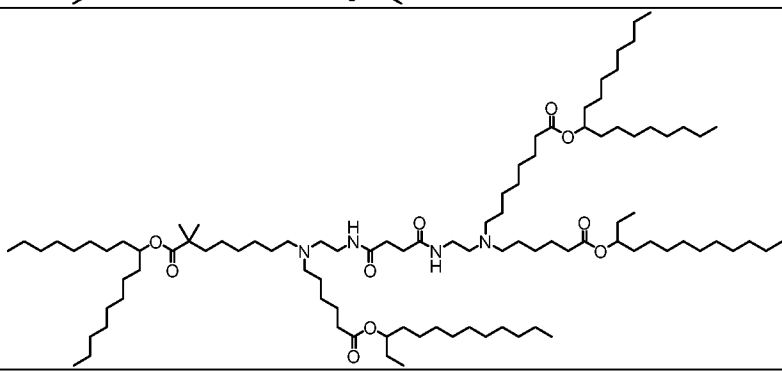
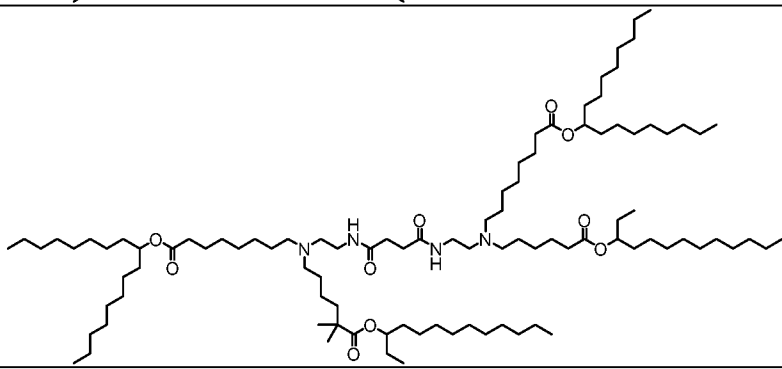
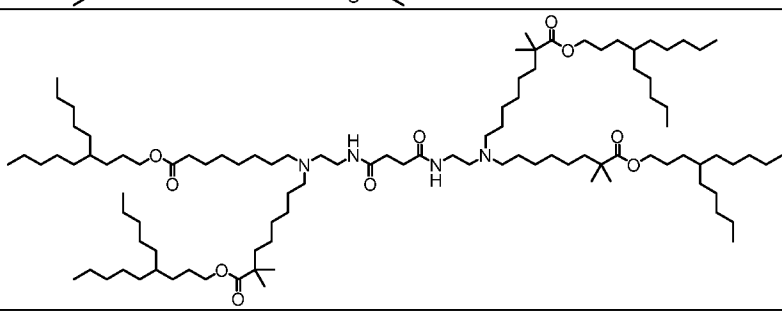
A20	
A21	
A22	
A23	
A24	

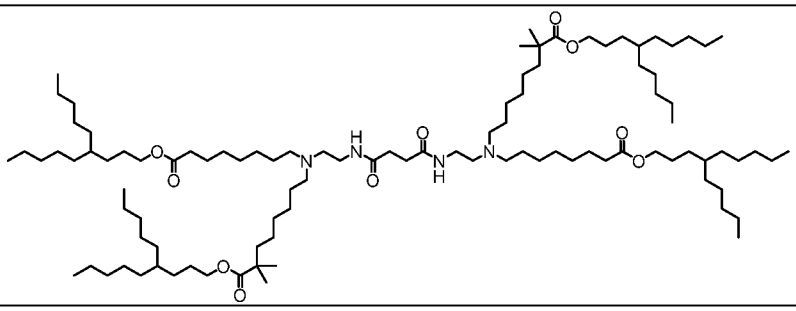
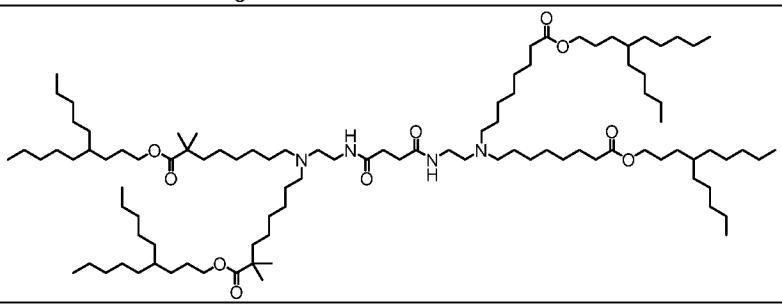
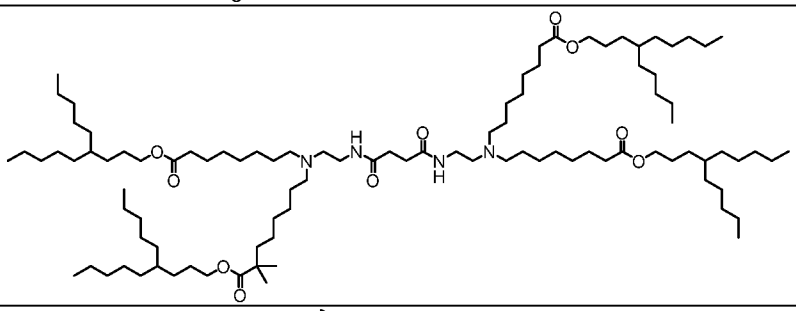
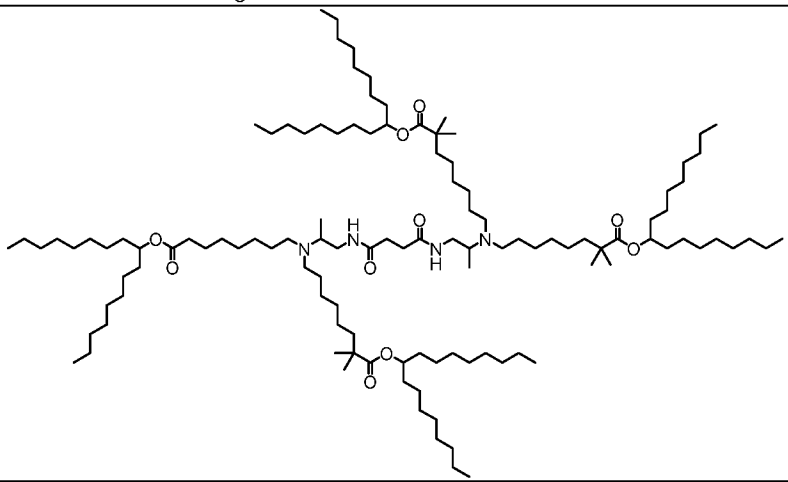
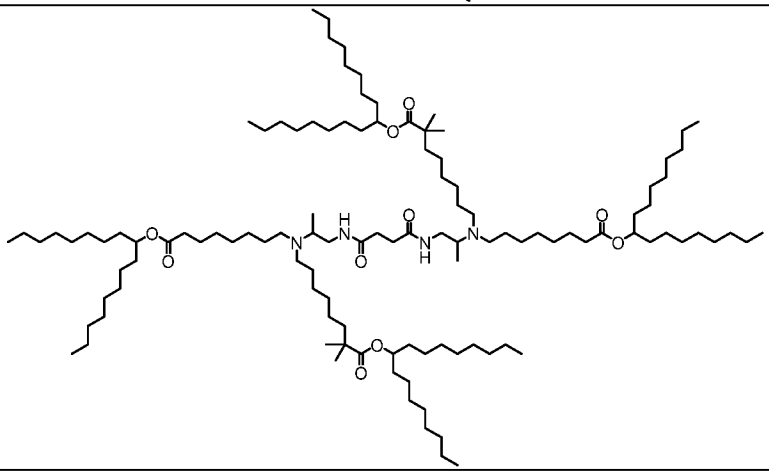
A25	
A26	
A27	
A28	
A29	

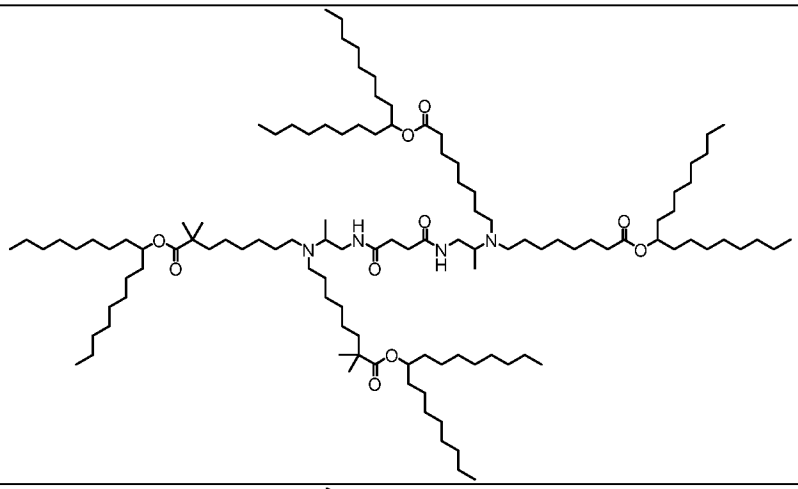
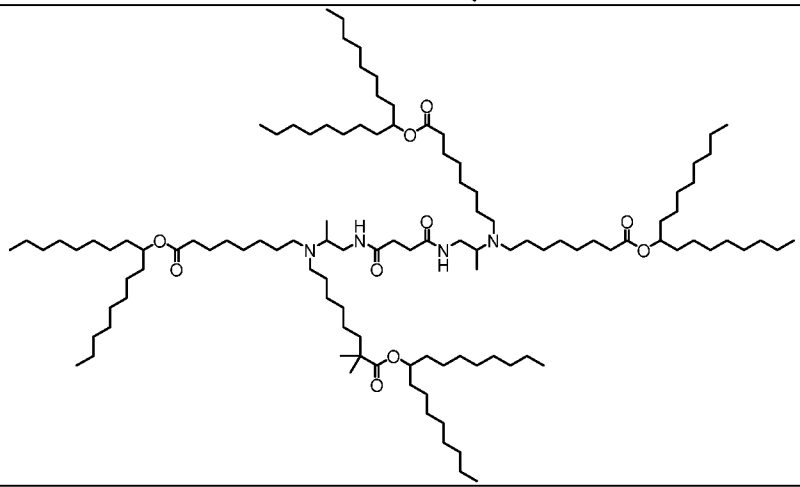
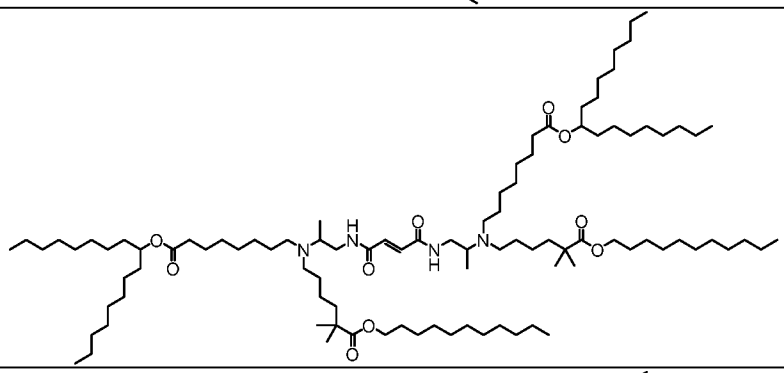
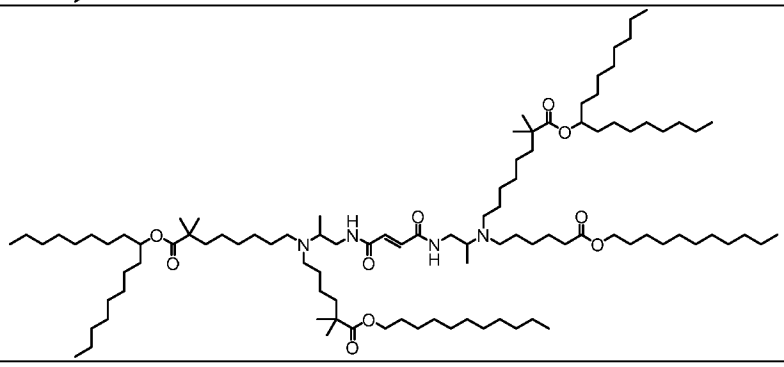
A30	
A31	
A32	
A33	
A34	

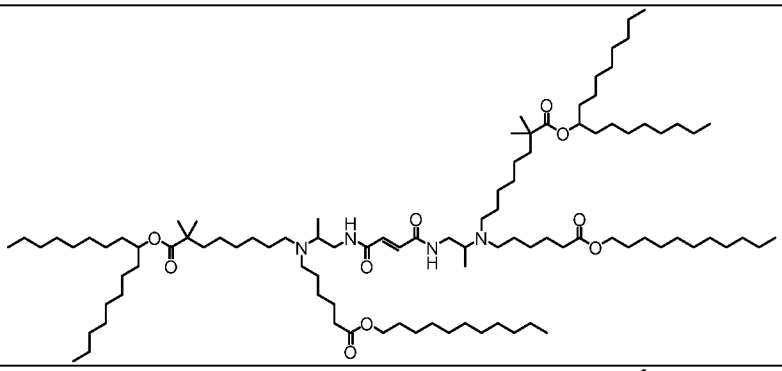
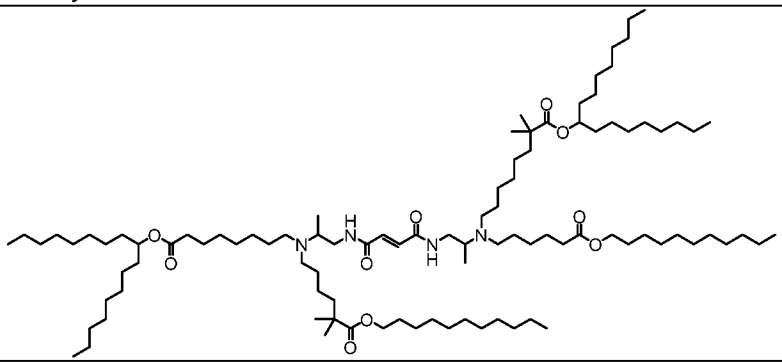
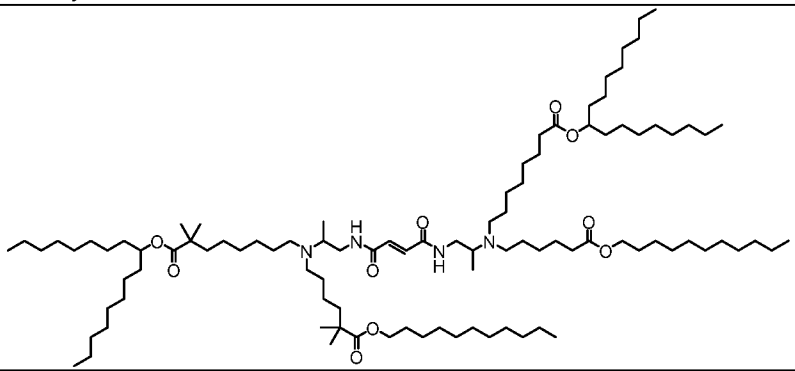
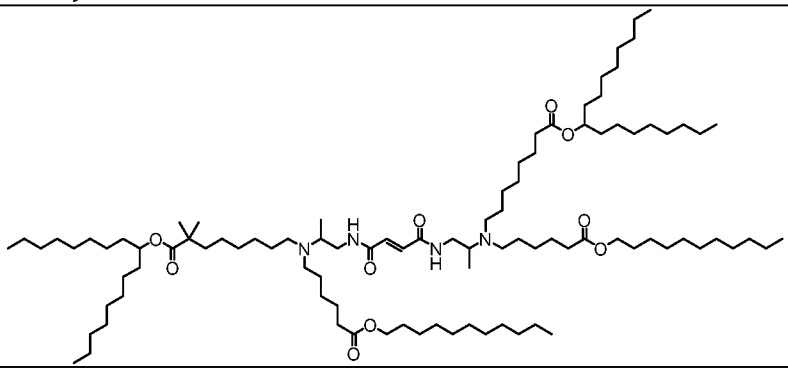
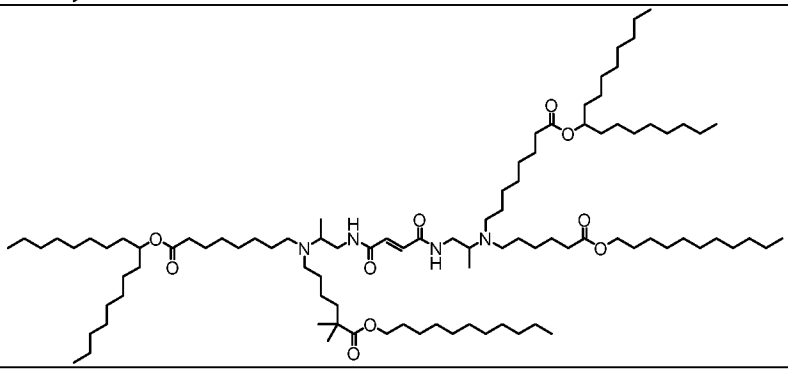
A35	
A36	
A37	
A38	
A39	
A40	

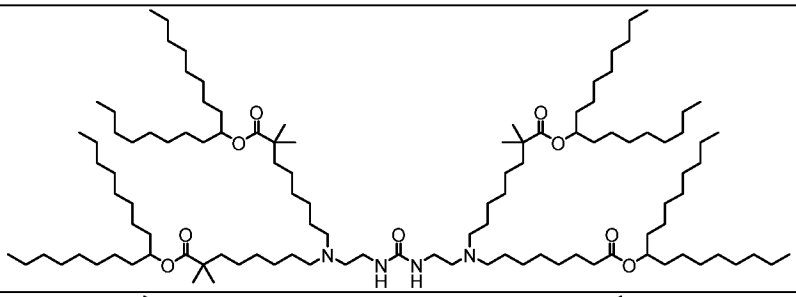
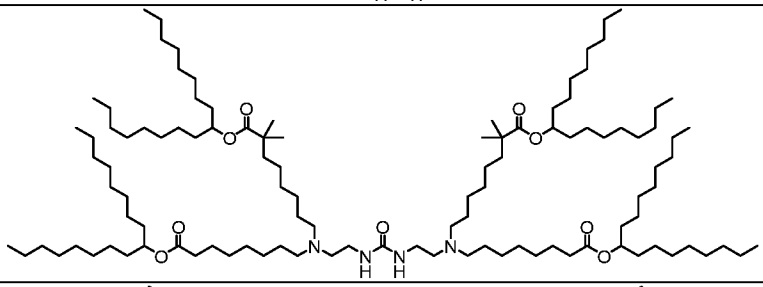
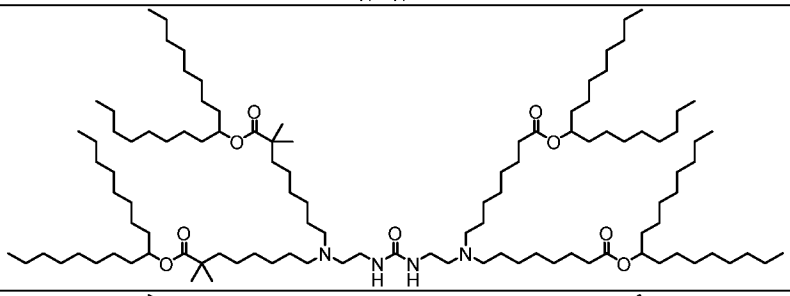
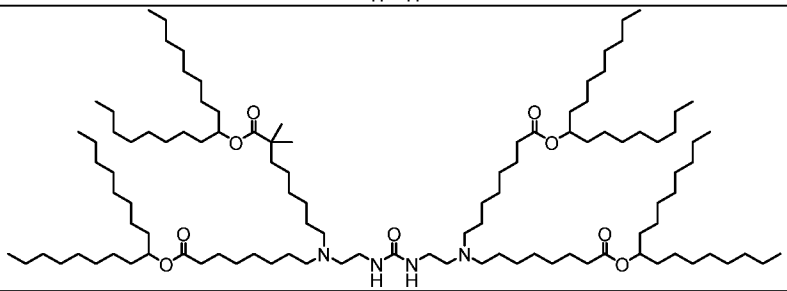
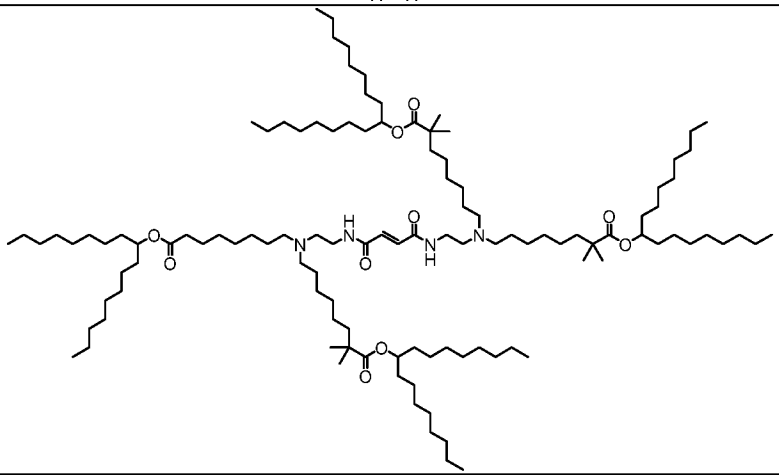
A41	
A42	
A43	
A44	
A45	
A46	

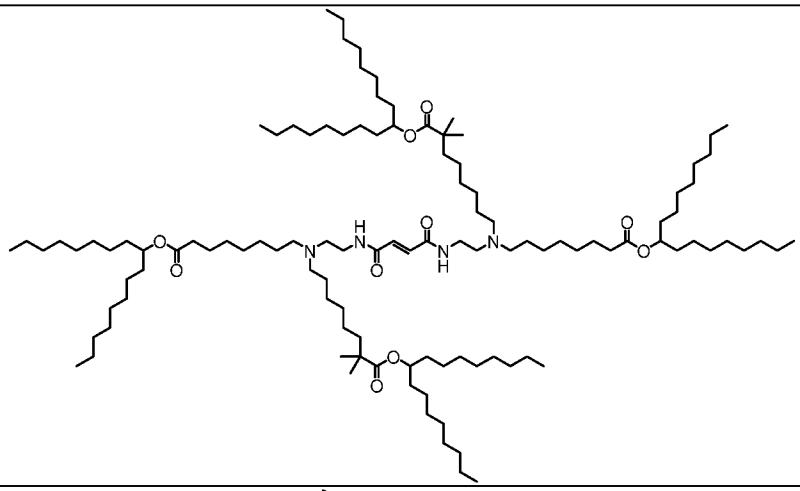
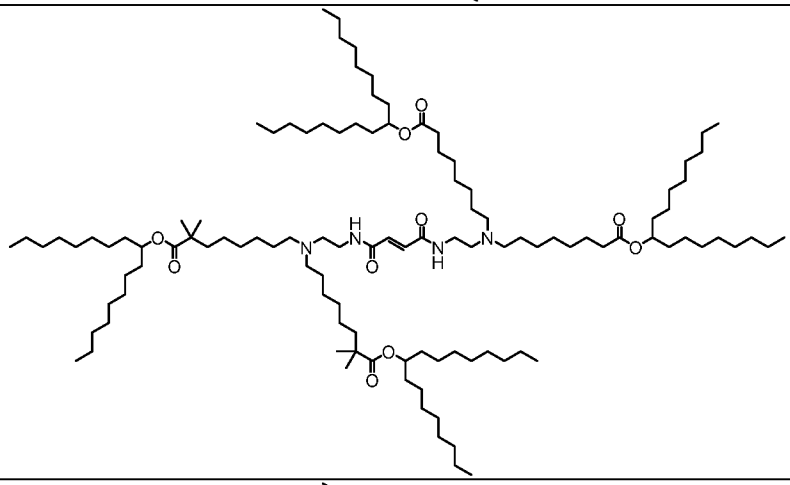
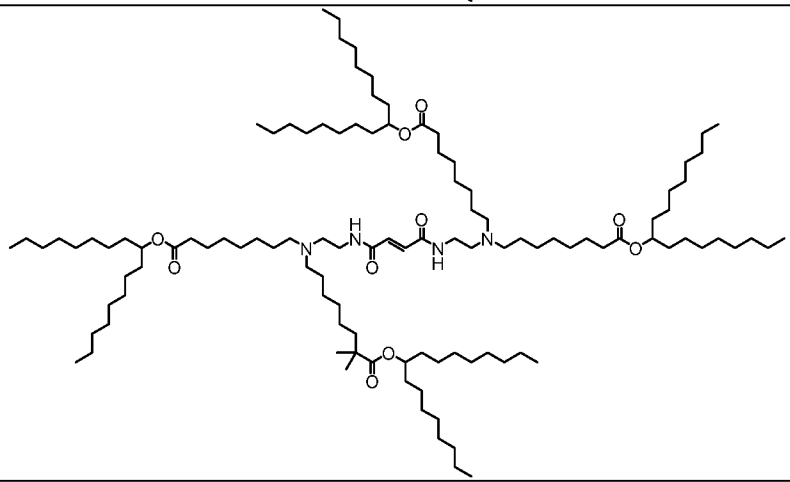
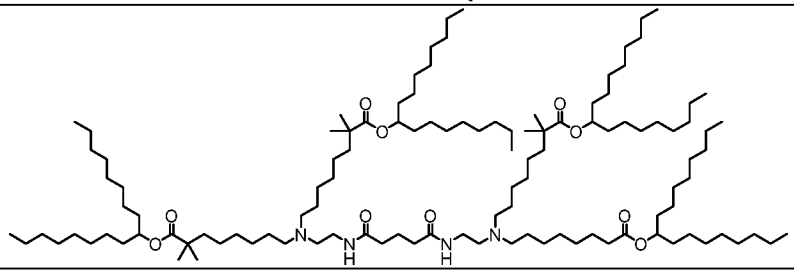
A47	
A48	
A49	
A50	
A51	

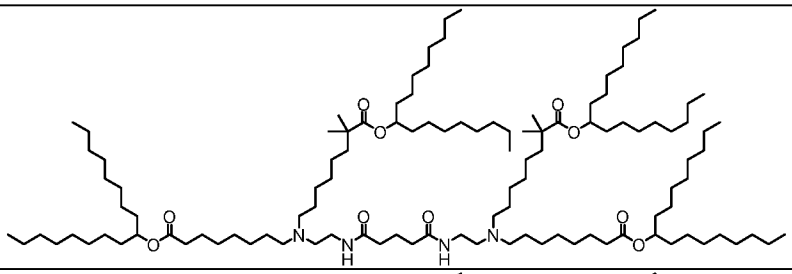
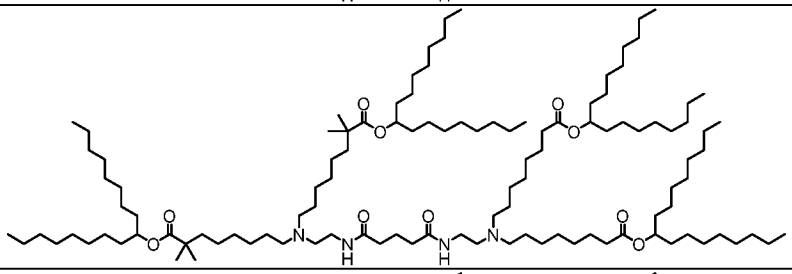
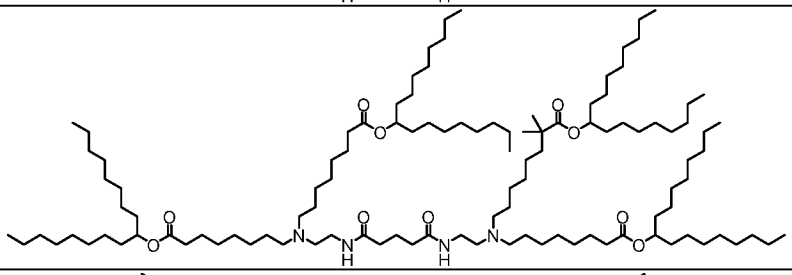
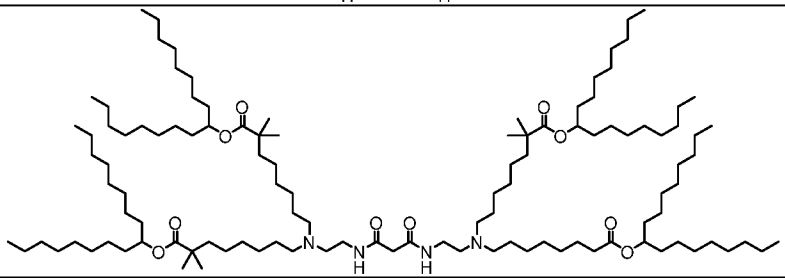
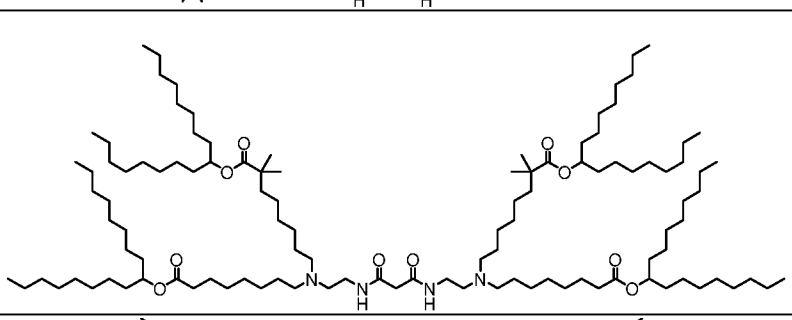
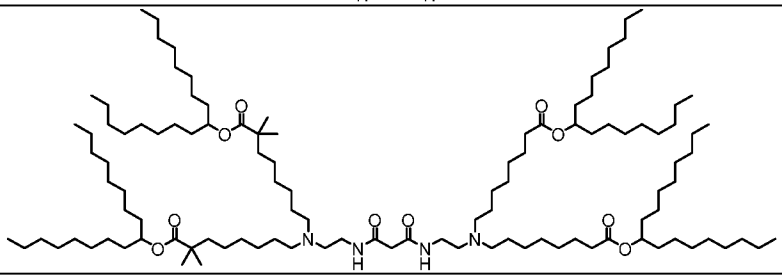
A52	
A53	
A54	
A55	
A56	

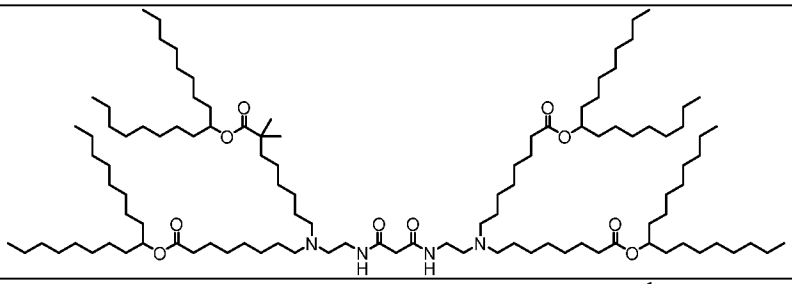
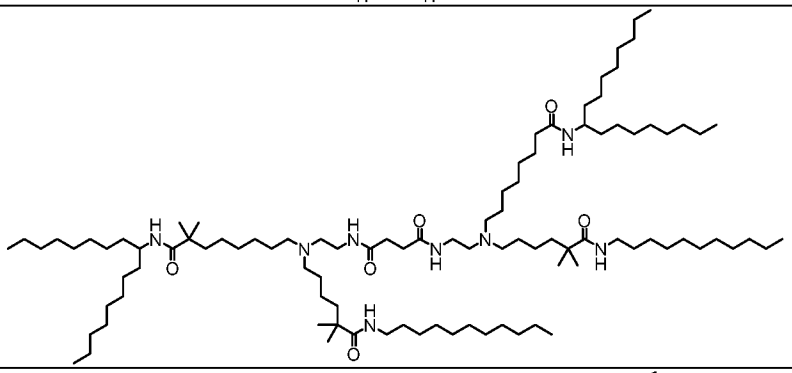
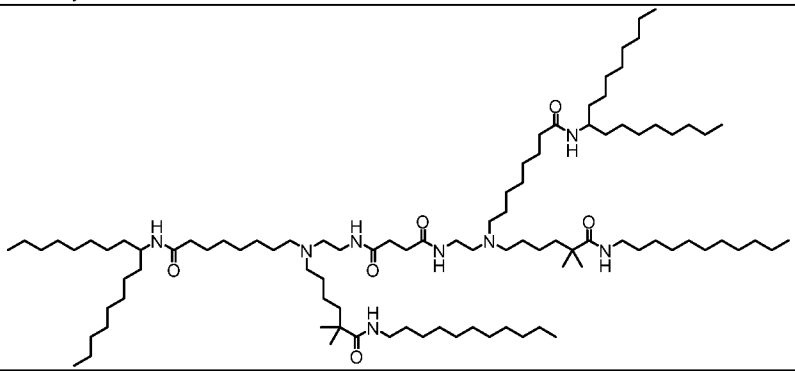
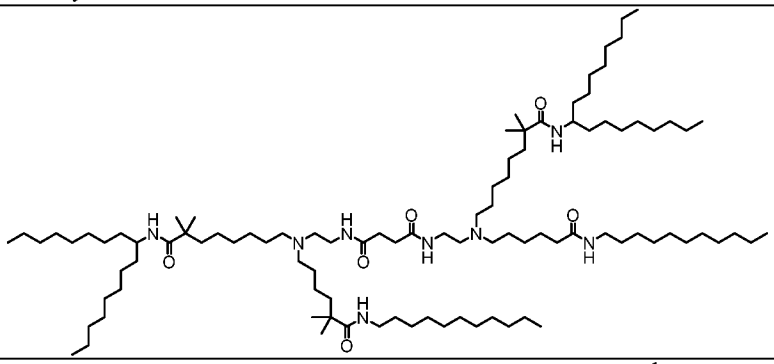
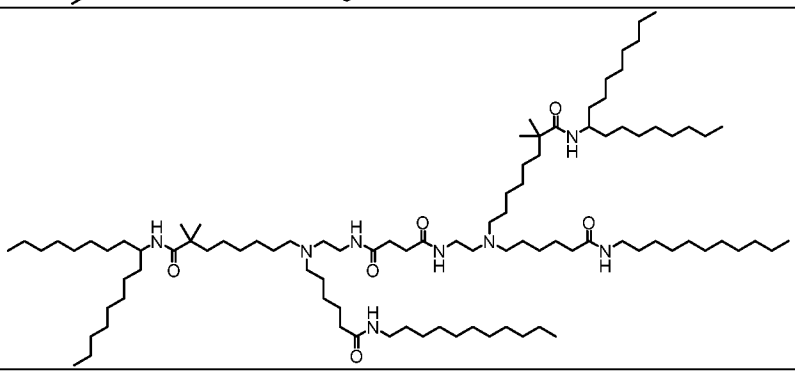
A57	
A58	
A59	
A60	

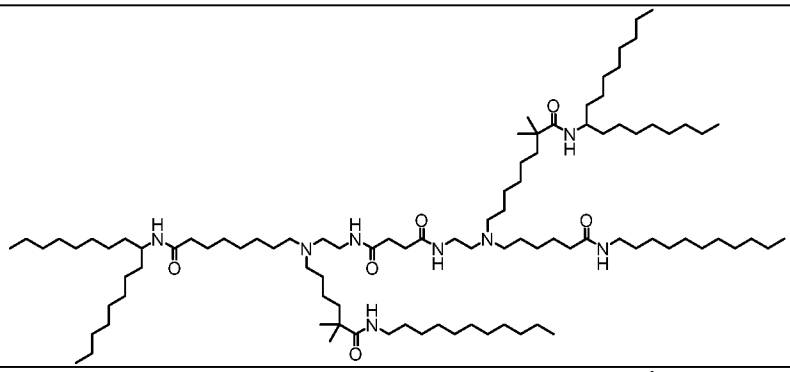
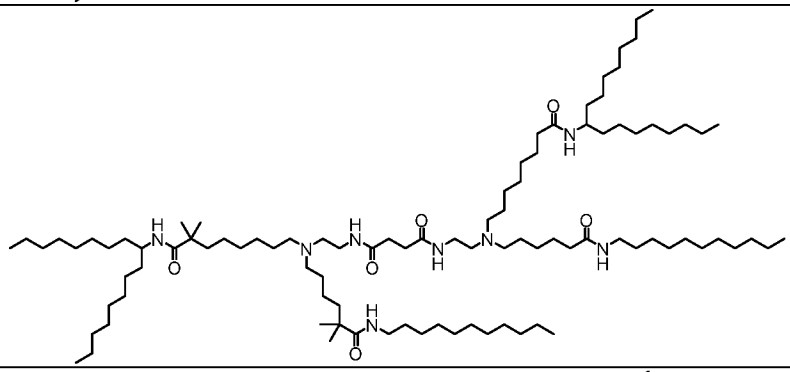
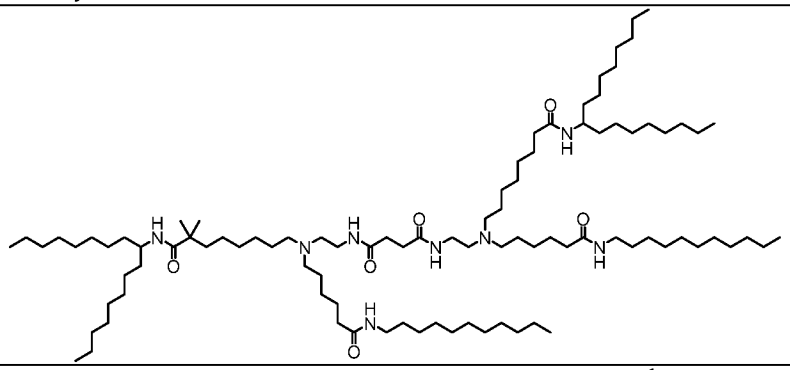
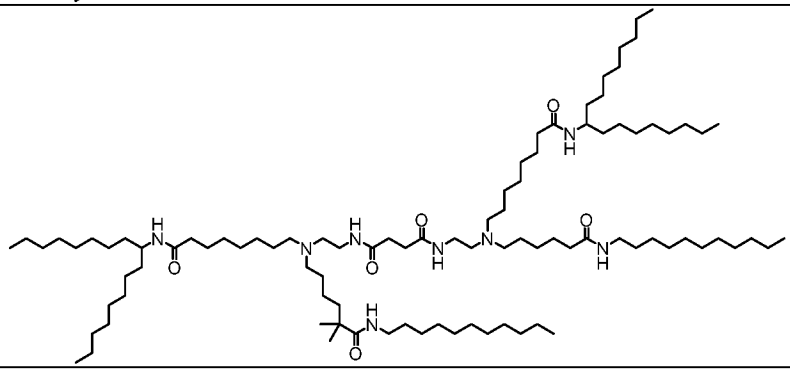
A61	
A62	
A63	
A64	
A65	

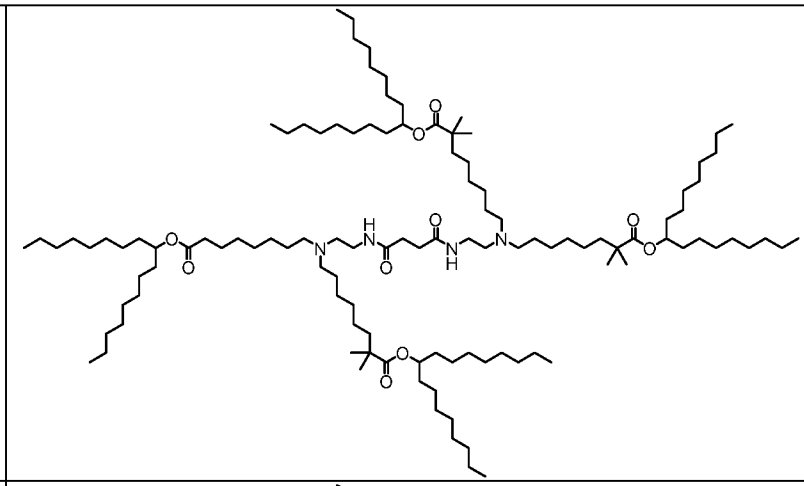
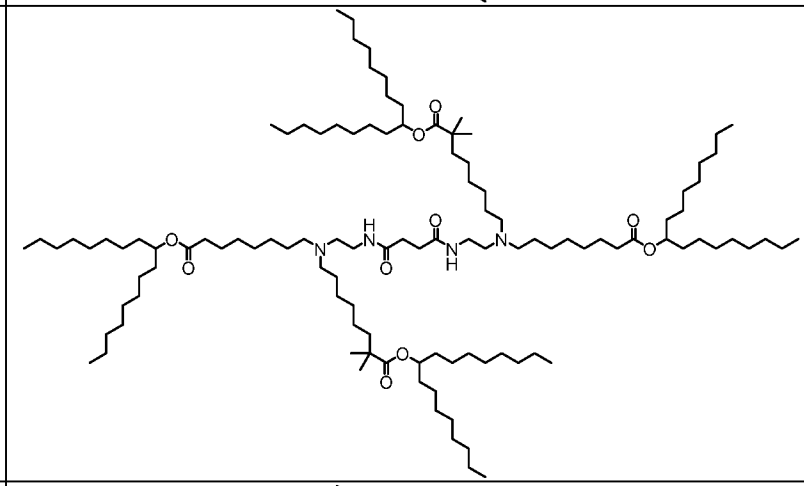
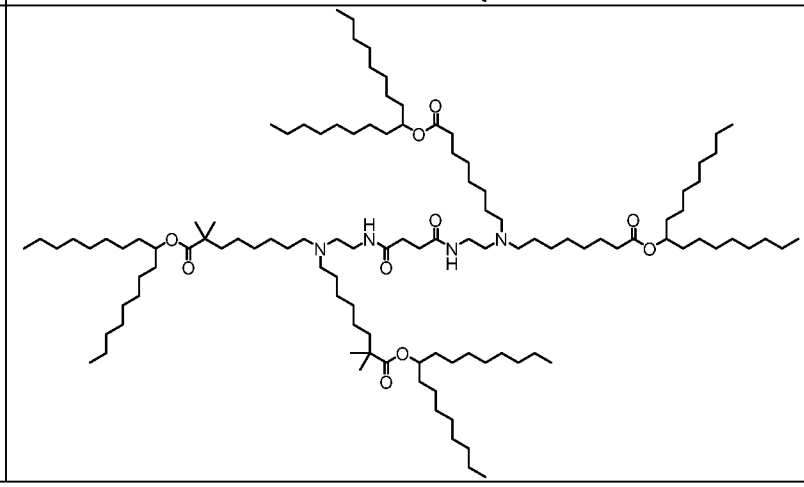
A66	
A67	
A68	
A69	
A70	

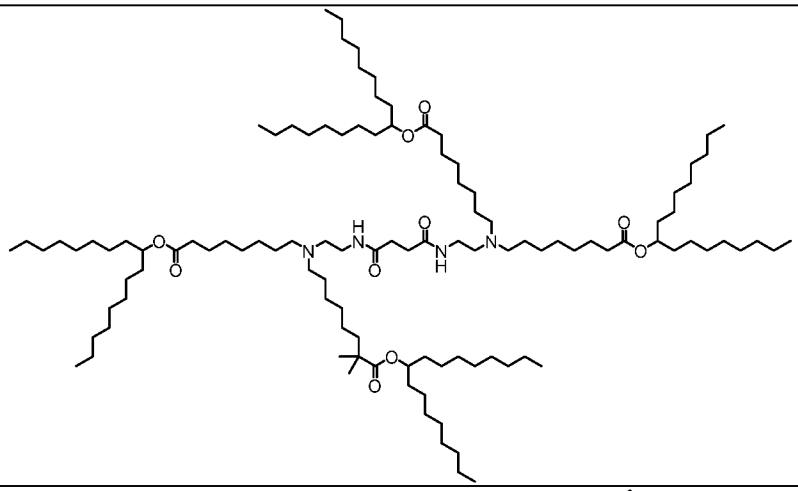
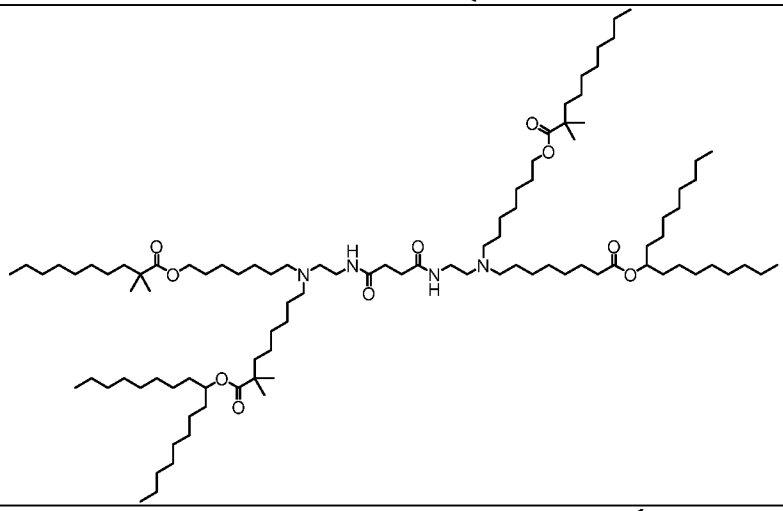
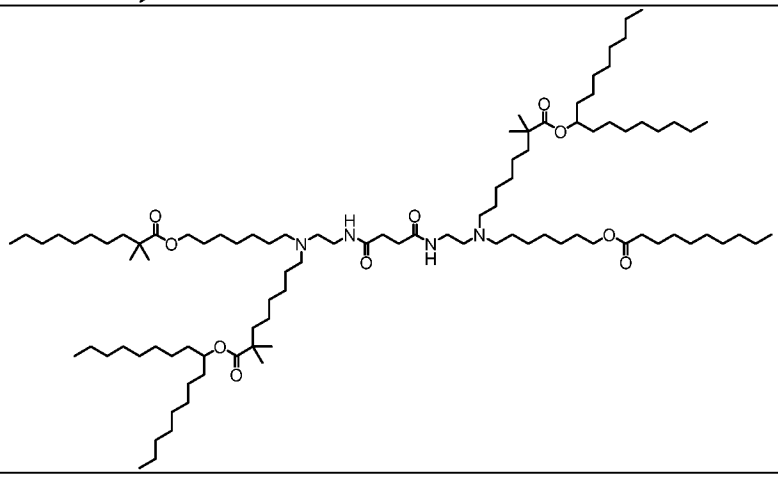
A71	
A72	
A73	
A74	

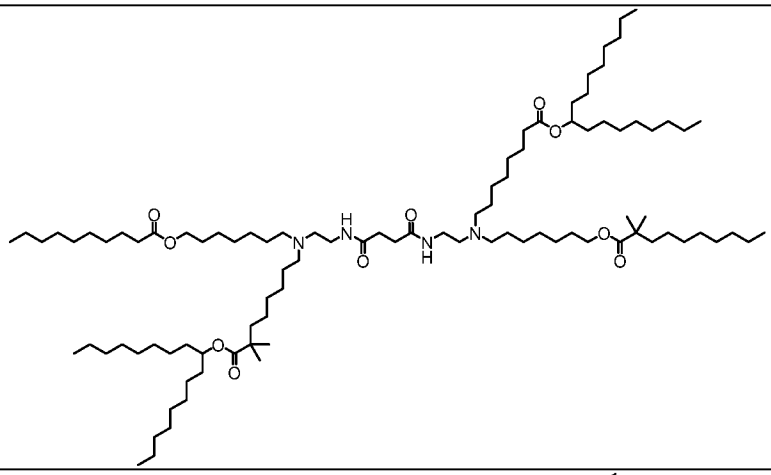
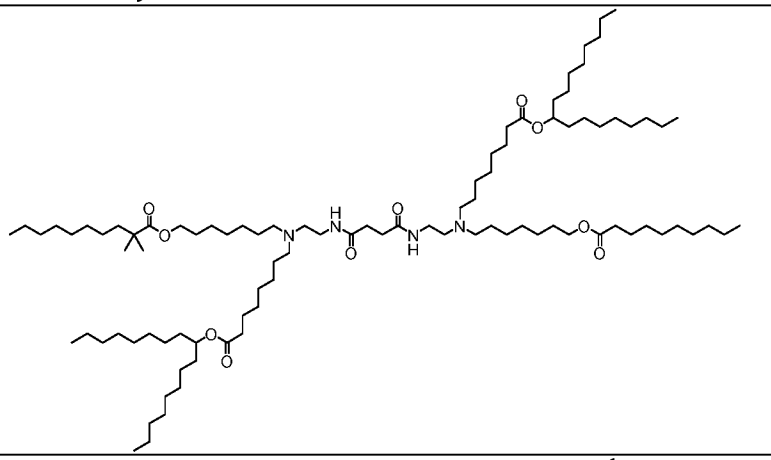
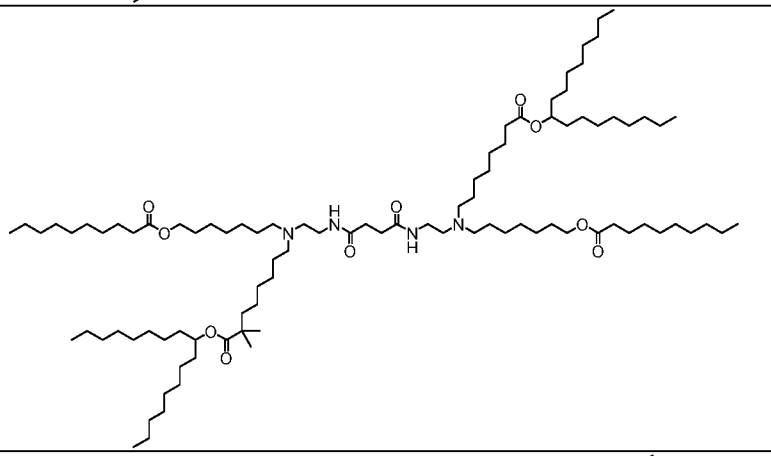
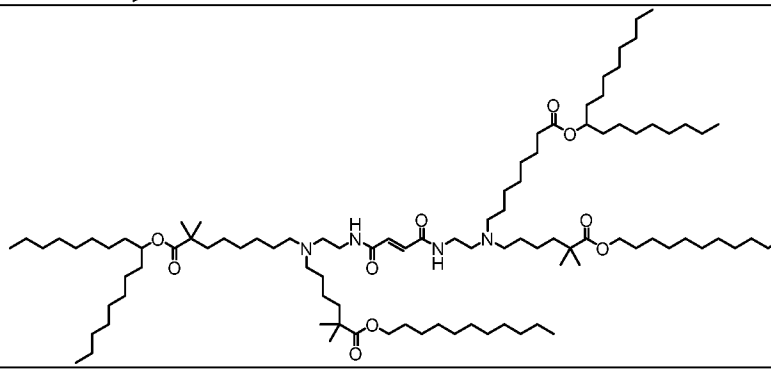
A75	
A76	
A77	
A78	
A79	
A80	

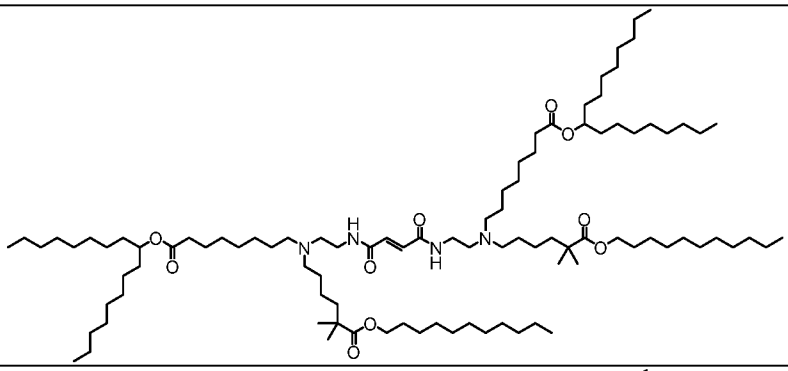
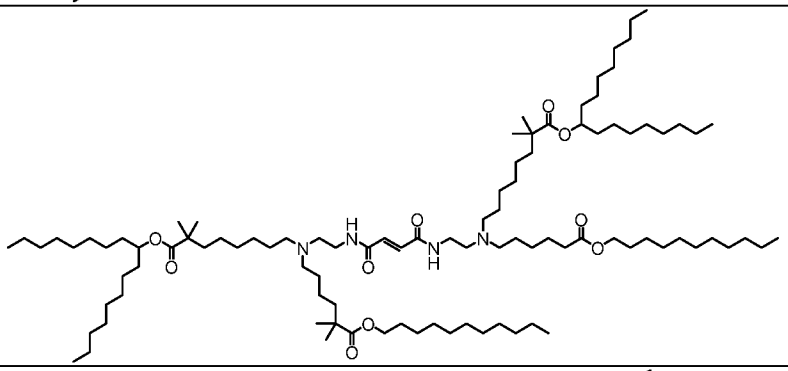
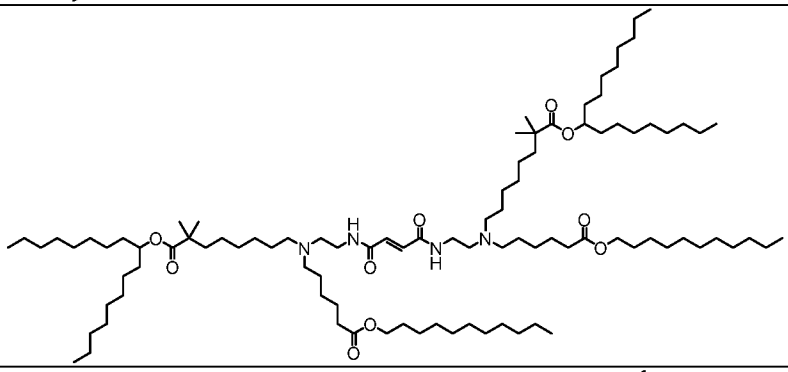
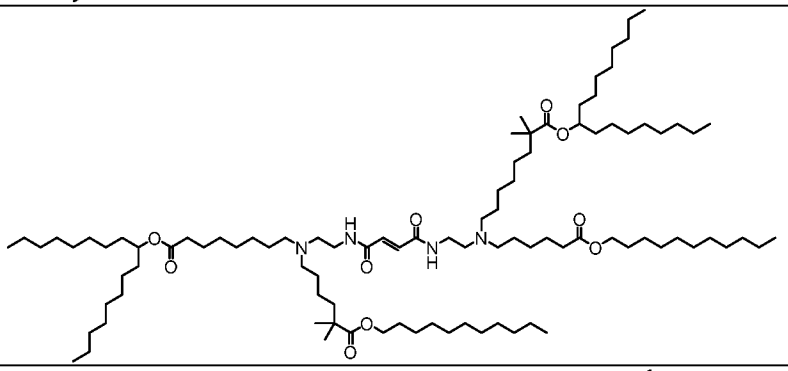
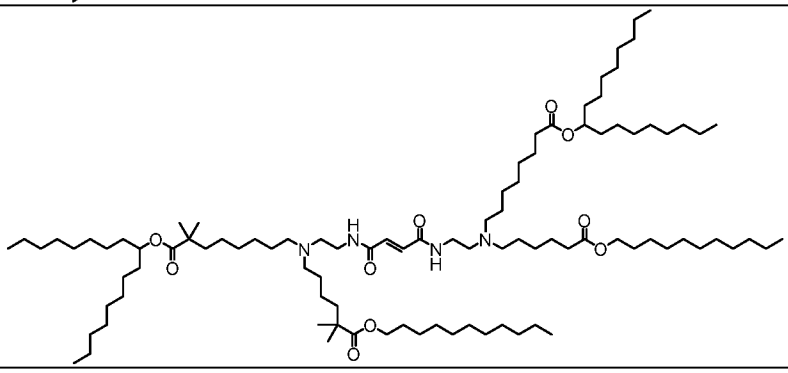
A81	
A82	
A83	
A84	
A85	

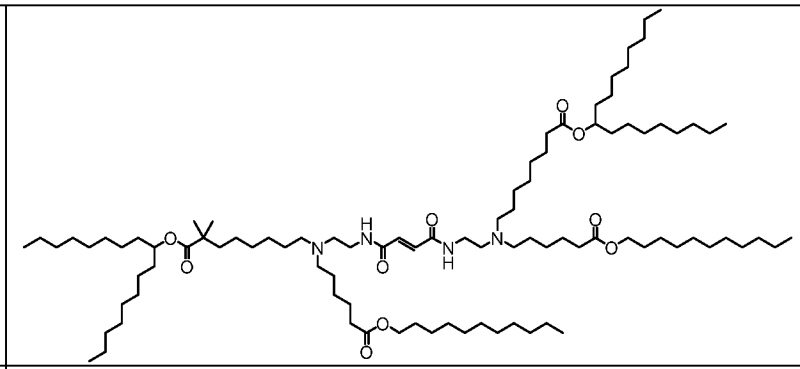
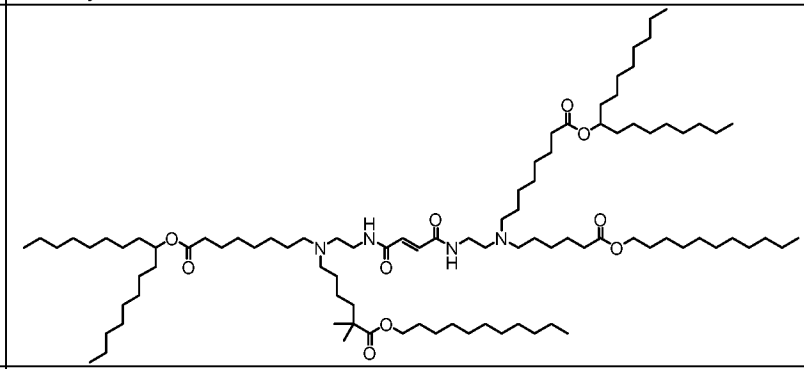
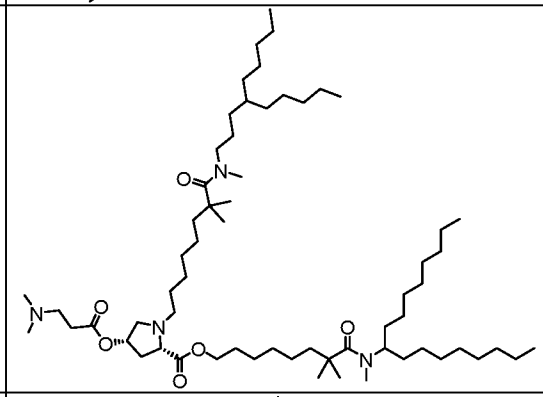
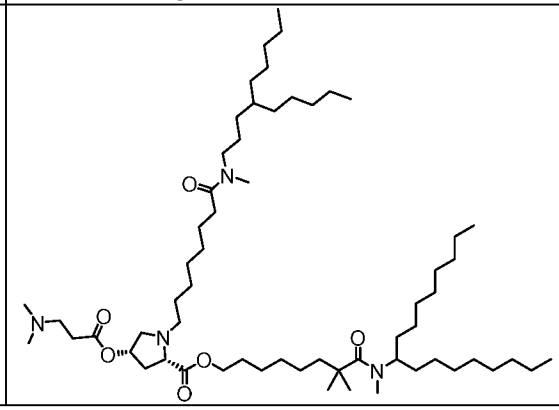
A86	
A87	
A88	
A89	

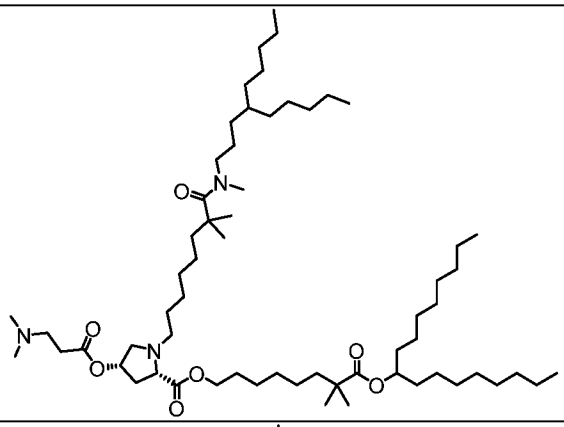
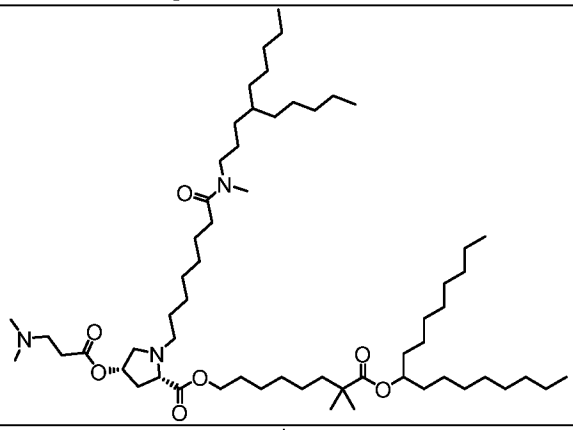
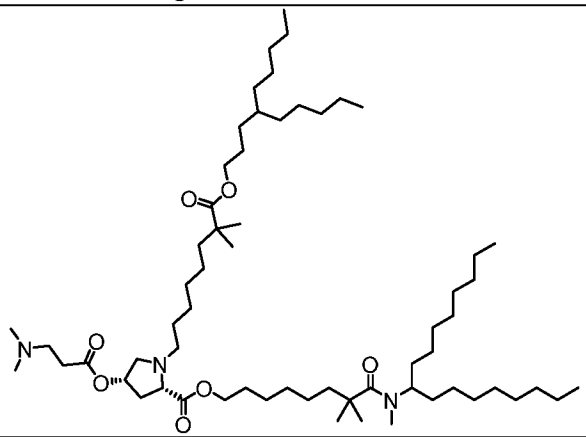
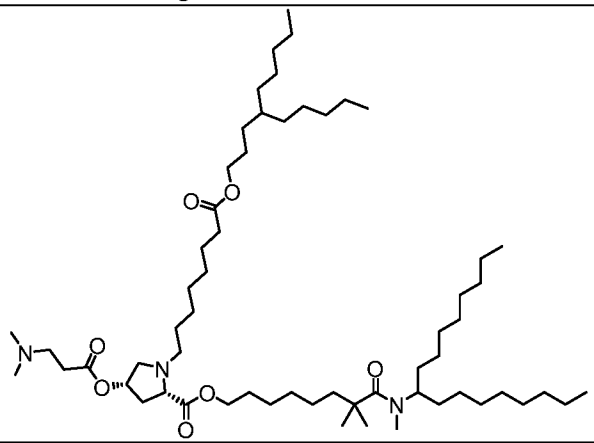
A90	
A91	
A92	

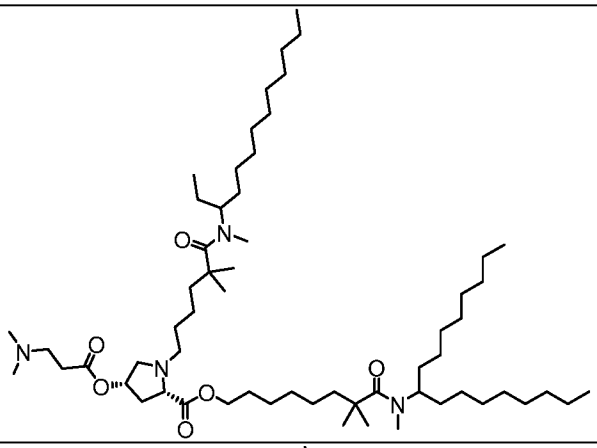
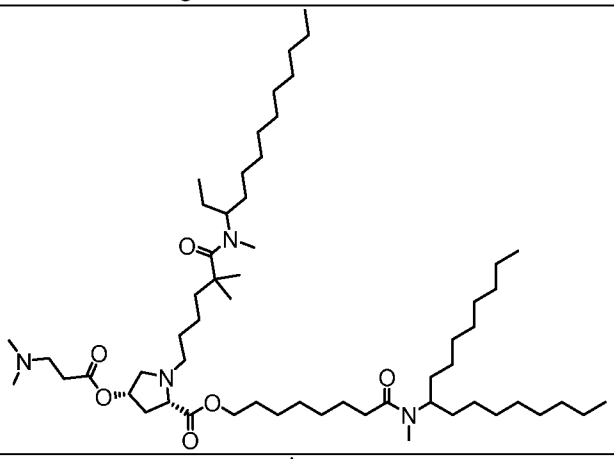
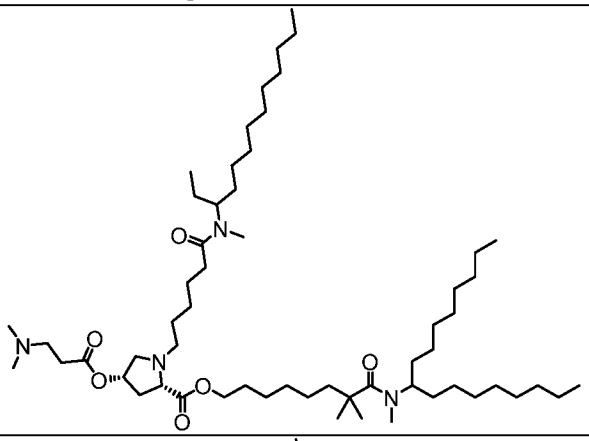
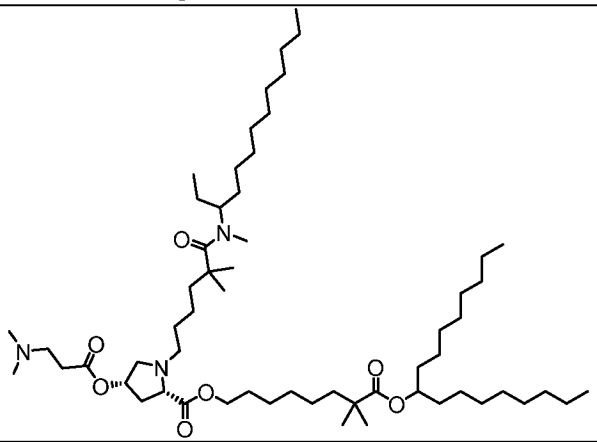
A93	
A94	
A95	

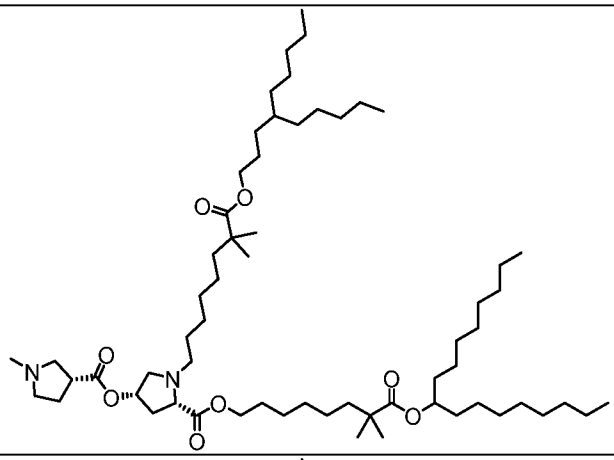
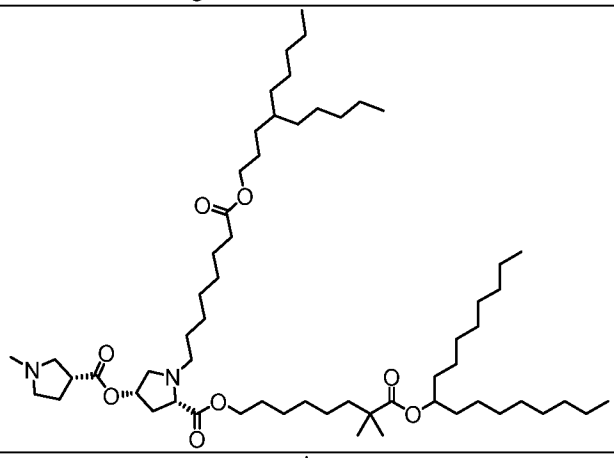
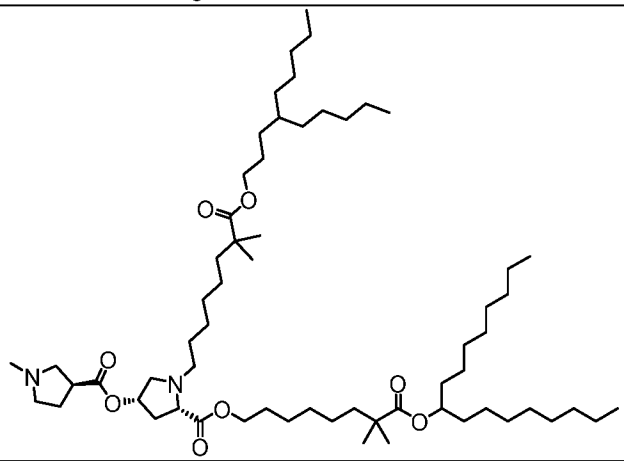
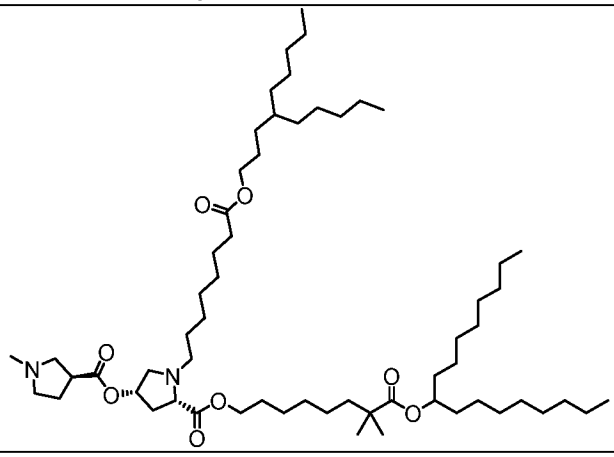
A96	
A97	
A98	
A99	

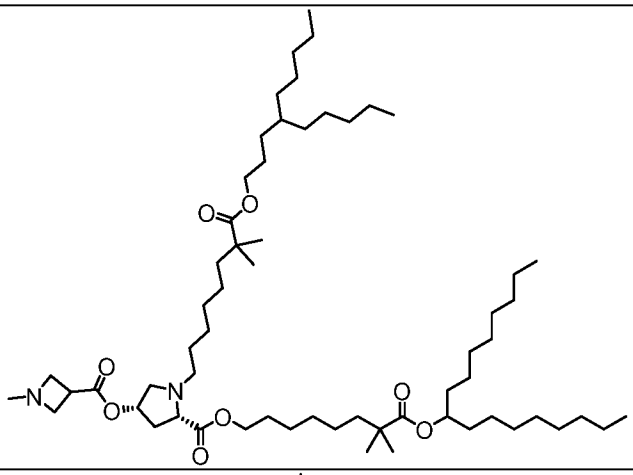
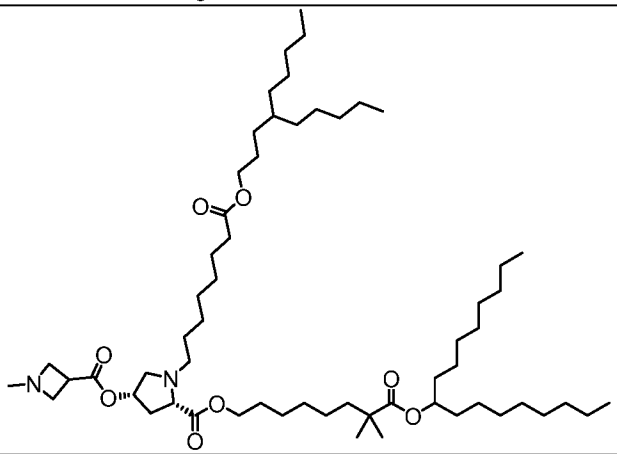
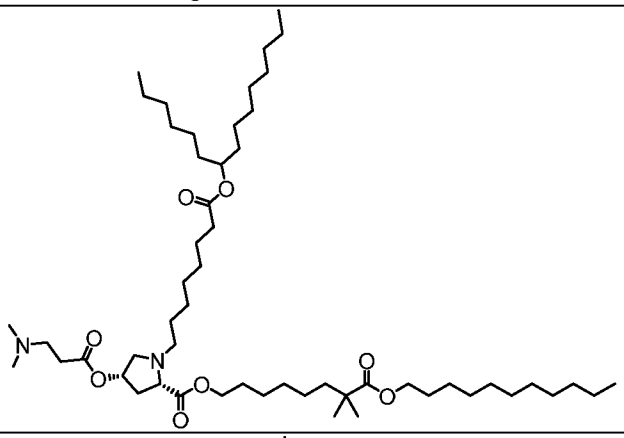
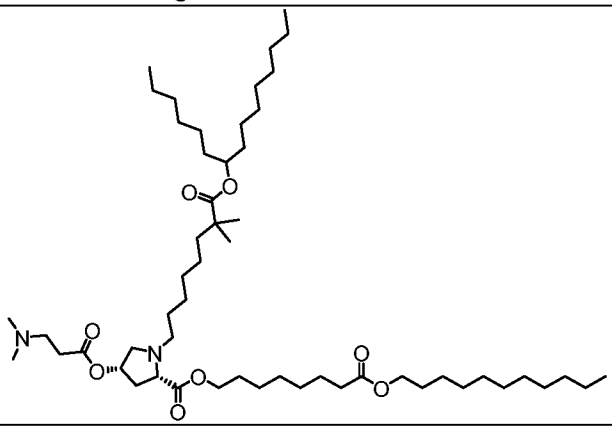
A100	
A101	
A102	
A103	
A104	

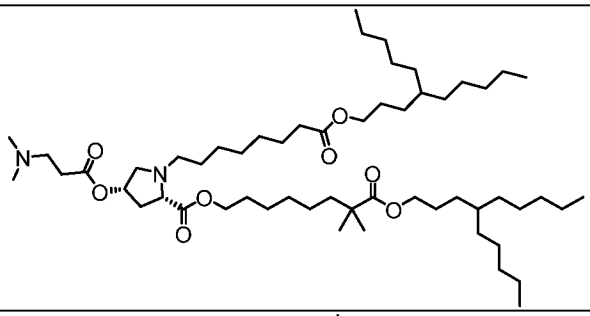
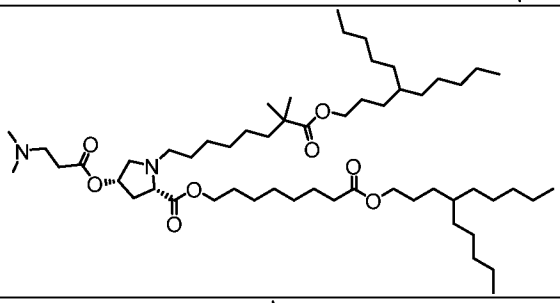
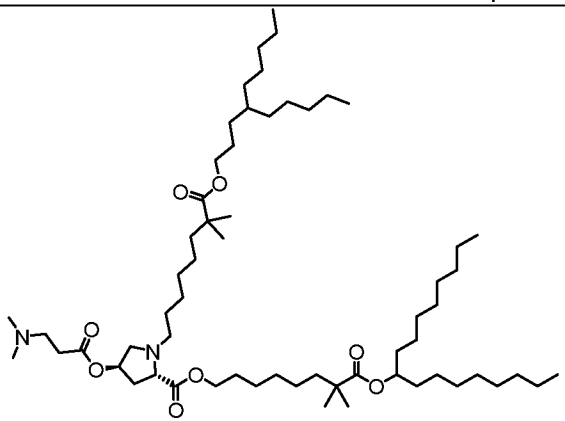
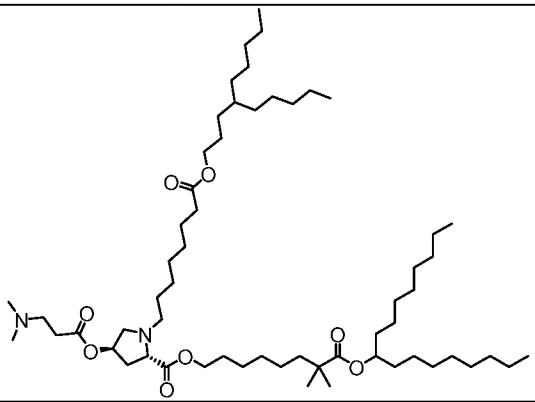
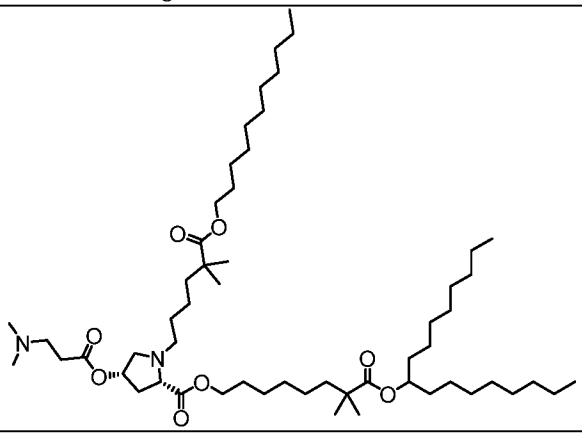
A105	
A106	
B1	
B2	

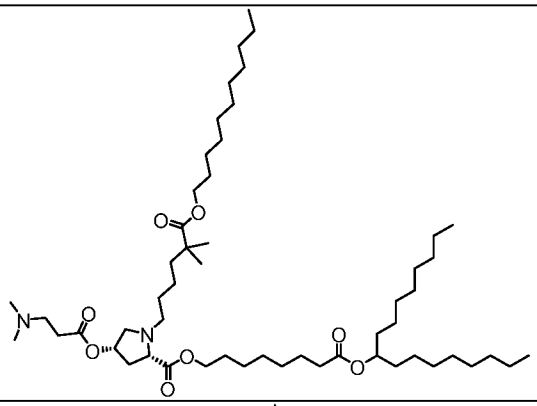
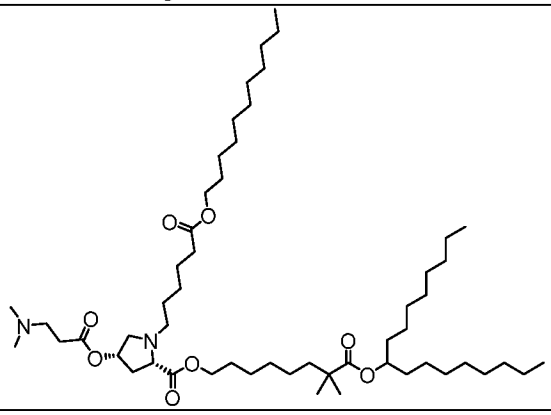
B3	
B4	
B5	
B6	

B7	
B8	
B9	
B10	

B11	
B12	
B13	
B14	

B15	
B16	
B17	
B18	

B19	
B20	
B21	
B22	
B23	

B24	
B25	

[0337] In some embodiments, a lipid membrane of the LNMPs (or LNPs) comprises at least 35% of the lipid compound from group i), e.g., at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% 80%, 85%, 90%, or more than 90% of the lipid compound from group i), e.g., 35%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% of the lipid compound from group i).

[0338] In some embodiments, a lipid membrane of the LNMPs (or LNPs) comprises at least 35% of the lipid compound from group ii), e.g., at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% 80%, 85%, 90%, or more than 90% of the lipid compound from group ii), e.g., 35%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% of the lipid compound from group ii).

[0339] In some embodiments, a lipid membrane of the LNMPs (or LNPs) comprises at least 35% of the lipid compound from group iii), e.g., at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% 80%, 85%, 90%, or more than 90% of the lipid compound from group iii), e.g., 35%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% of the lipid compound from group iii).

[0340] In some embodiments, a lipid membrane of the LNMPs (or LNPs) comprises at least 35% of the lipid compound from group iii), e.g., at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% 80%, 85%, 90%, or more than 90% of the lipid compound from group iii), e.g., 35%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% of the lipid compound from group iv).

[0341] In some instances, the LNMPs (or LNPs) comprise at least 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more than 90% ionizable lipid.

[0342] In some instances, the LNMPs (or LNPs) comprise a molar ratio of at least 0.1%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% 80%, 85%, 90%, or more than 90% ionizable lipid, e.g., 1%-10%, 10%-20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-80%, or 80%-90% ionizable lipid, e.g., about 25%-75% ionizable lipid (e.g., about

25%-75% ionizable lipid).

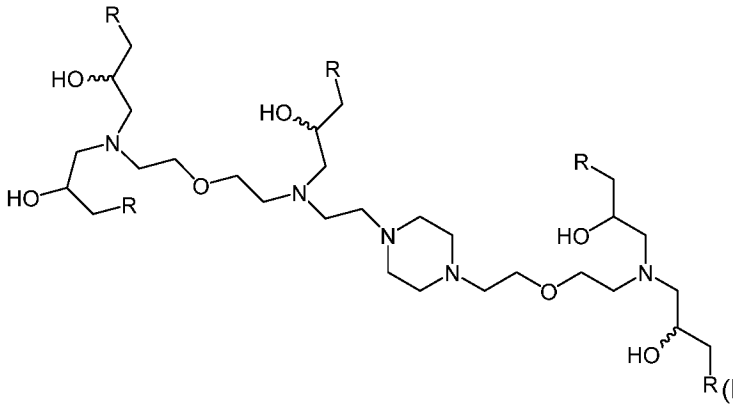
Other Ionizable lipids

[0343] In the LNMP or LNP formulations, more than one ionizable lipid can be used for the ionizable lipid component: one or more of the ionizable lipids from the compounds of formulas in groups i)-iv) can be used alone or in combination with a different ionizable lipid from the compounds of formulas in groups i)-iv).

[0344] In some embodiments, the ionizable lipid is not selected from 1‘-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl) (2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-200), MD1 (cKK-E12), OF2, EPC, ZA3-Ep10, TT3, LP01, 5A2-SC8, Lipid 5 (Moderna), and 98N12-5.

[0345] In some embodiments, the additional ionizable lipid is selected from the group consisting of 1,1’-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl) (2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-200), MD1 (cKK-E12), OF2, EPC, ZA3-Ep10, TT3, LP01, 5A2-SC8, Lipid 5, SM-102 (Lipid H), and ALC-315.

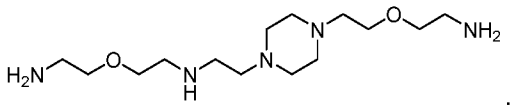
[0346] In some embodiments, the additional ionizable lipid is represented by the following formula III:



(III), wherein R is C_8-C_{14} alkyl group.

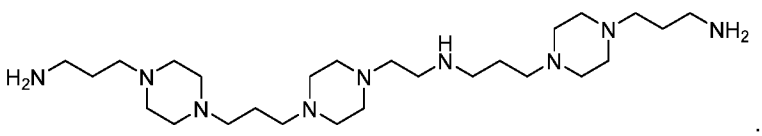
[0347] The ionizable lipid described herein may include an amine core described herein substituted with one or more (e.g., 1, 2, 3, 4, 5, or 6) lipid tails. In some embodiments, the ionizable lipid described herein include at least 3 lipid tails. A lipid tail may be a C_8-C_{18} hydrocarbon (e.g., C_6-C_{18} alkyl or C_6-C_{18} alkanoyl). An amine core may be substituted with one or more lipid tails at a nitrogen atom (e.g., one hydrogen atom attached to the nitrogen atom may be replaced with a lipid tail).

[0348] In some embodiments, the amine core has a structure of:



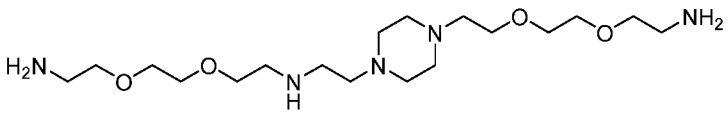
.

[0349] In some embodiments, the amine core has a structure of:

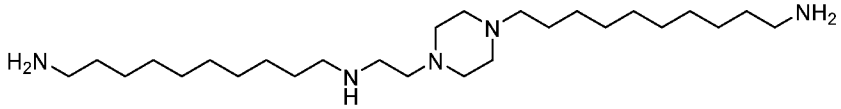


.

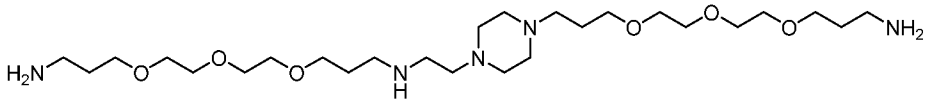
[0350] In some embodiments, the amine core has a structure of:

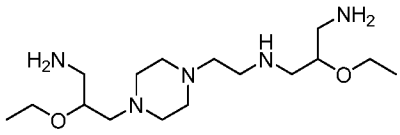


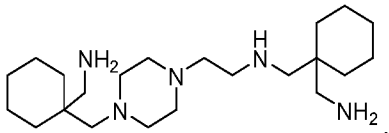
[0351] In some embodiments, the amine core has a structure of:

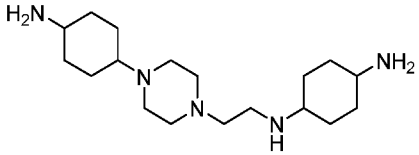


[0352] In some embodiments, the amine core has a structure of:



[0353] In some embodiments, the amine core has a structure of:  .

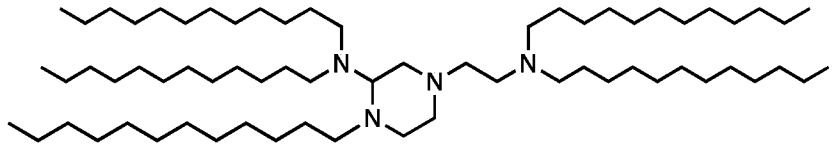
[0354] In some embodiments, the amine core has a structure of:  .

[0355] In some embodiments, the amine core has a structure of: 

.

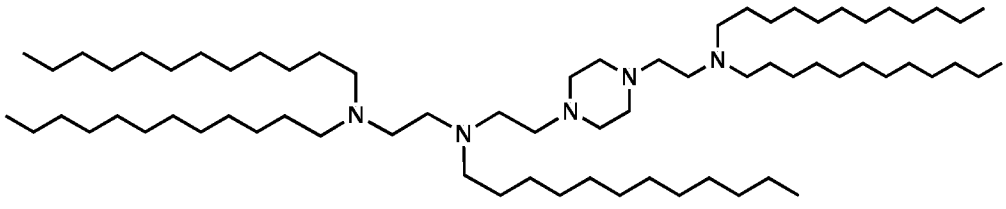
[0356] Other suitable lipids for use in the RNA composition and methods for making and using thereof include the lipids as described in International Patent Publication WO 2016/118725, which is incorporated herein by reference in its entirety.

[0357] In certain embodiments, the RNA composition and methods for making and using thereof include a lipid having a compound structure of:

 , and pharmaceutically acceptable salts thereof.

[0358] Other suitable lipids for use in the RNA composition and methods for making and using thereof include the lipids as described in International Patent Publication WO 2016/118724, which is incorporated herein by reference in its entirety.

[0359] In certain embodiments, the RNA composition and methods for making and using thereof include a lipid having a compound structure of:

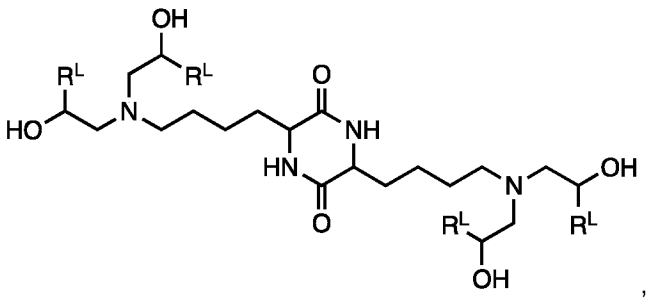


, and pharmaceutically acceptable salts thereof.

[0360] Other suitable lipids for use in the RNA composition and methods for making and using thereof include a lipid having the formula of 14,25-ditridecyl 15,18,21,24-tetraaza-octatriacontane, and pharmaceutically acceptable salts thereof.

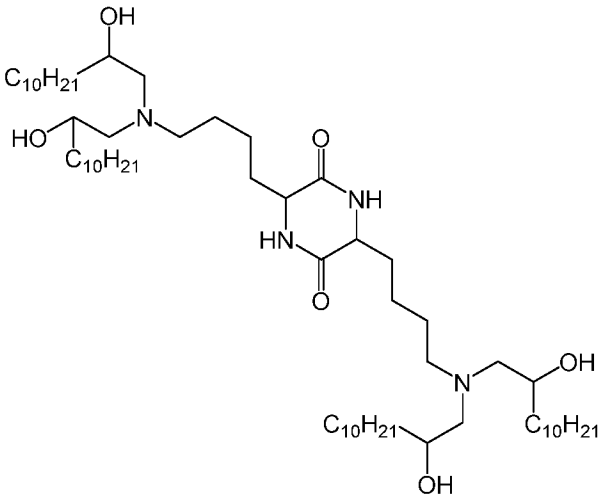
[0361] Other suitable lipids for use in the RNA composition and methods for making and using thereof include the lipids as described in International Patent Publications WO 2013/063468 and WO 2016/205691, each of which is incorporated herein by reference in its entirety.

[0362] In some embodiments, the RNA composition and methods for making and using thereof include a lipid of the following formula:



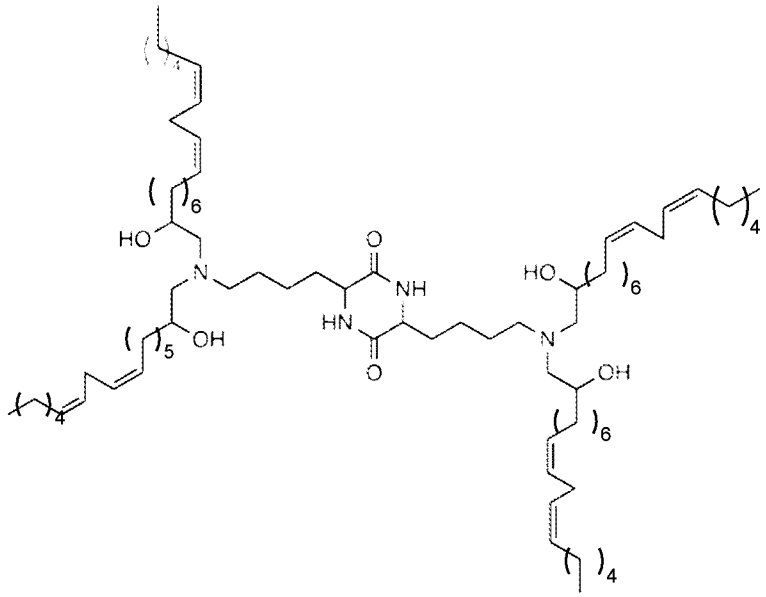
, or pharmaceutically acceptable salts thereof, wherein each instance of R^L is independently optionally substituted C6-C40 alkenyl.

[0363] In certain embodiments, the RNA composition and methods for making and using thereof include a lipid having a compound structure of:



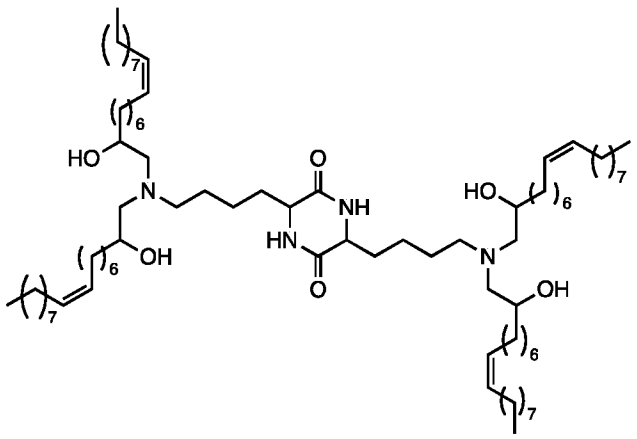
, and pharmaceutically acceptable salts thereof.

[0364] In certain embodiments, the RNA composition and methods for making and using thereof include a lipid having a compound structure of:



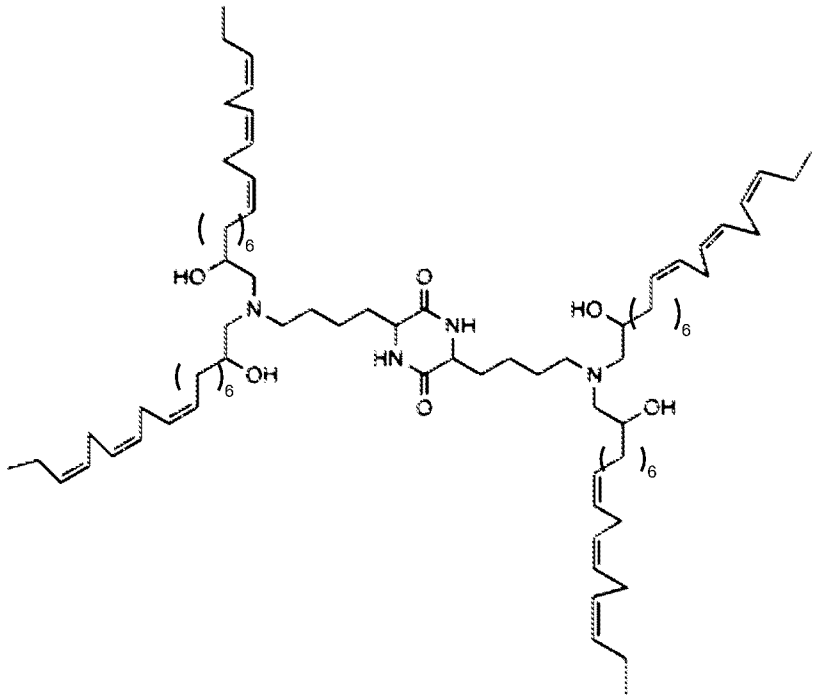
, and pharmaceutically acceptable salts thereof.

[0365] In certain embodiments, the RNA composition and methods for making and using thereof include a lipid having a compound structure of:



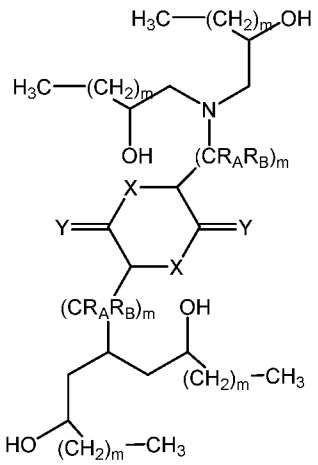
, and pharmaceutically acceptable salts thereof.

[0366] In certain embodiments, the RNA composition and methods for making and using thereof include a lipid having a compound structure of:



, and pharmaceutically acceptable salts thereof.

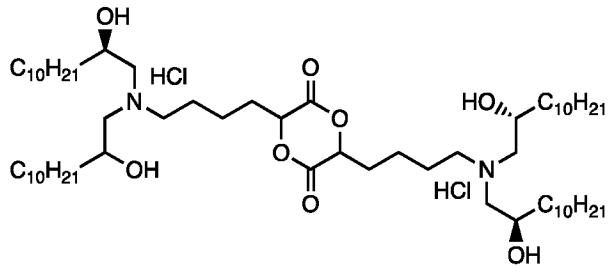
[0367] Other suitable lipids for use in the RNA composition and methods for making and using thereof include the lipids as described in International Patent Publication WO 2015/184256, which is incorporated herein by reference in its entirety. In some embodiments, the RNA composition and methods for making and using thereof include a lipid of the following formula:



, or a pharmaceutically acceptable salt thereof, wherein each X independently is O or S; each Y independently is O or S; each m independently is 0 to 20; each n independently is 1 to 6; each R_A is independently hydrogen, optionally substituted C1-50 alkyl, optionally substituted C2-50 alkenyl, optionally substituted C2-50 alkynyl, optionally substituted C3-10 carbocyclyl, optionally substituted 3-14 membered heterocyclyl, optionally substituted C6-14 aryl, optionally substituted 5-14 membered heteroaryl or halogen; and each RB is independently hydrogen, optionally substituted C1-50 alkyl, optionally substituted C2-50 alkenyl, optionally substituted C2-50 alkynyl, optionally substituted C3-10 carbocyclyl, optionally substituted 3-14 membered heterocyclyl, optionally substituted C6-14 aryl, optionally substituted 5-14 membered heteroaryl or halogen.

[0368] In certain embodiments, the RNA composition and methods for making and using thereof

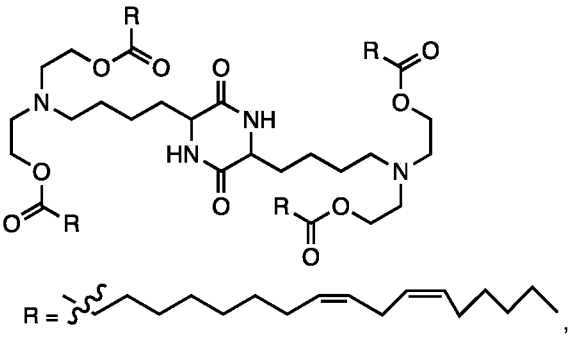
include a lipid,“Target 23”, having a compound structure of:



, (Target 23) and pharmaceutically acceptable salts thereof.

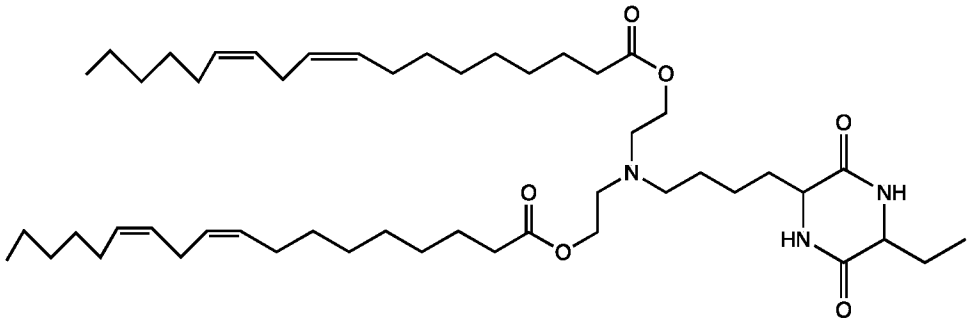
[0369] Other suitable lipids for use in the RNA composition and methods for making and using thereof include the lipids as described in International Patent Publication WO 2016/004202, which is incorporated herein by reference in its entirety.

[0370] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



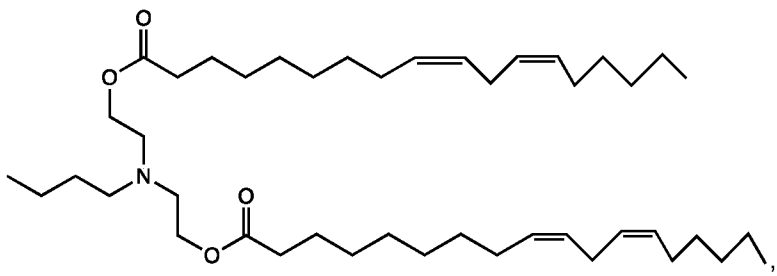
, or a pharmaceutically acceptable salt thereof.

[0371] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



, or a pharmaceutically acceptable salt thereof.

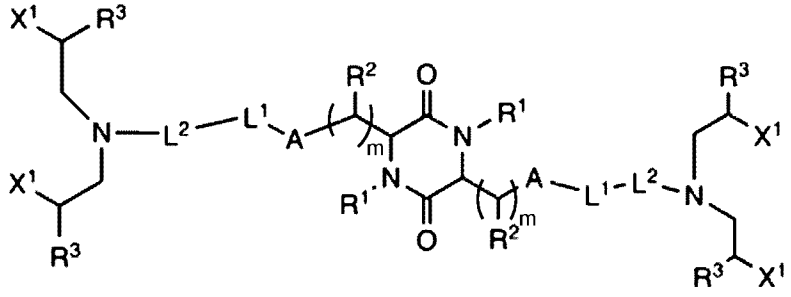
[0372] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



, or a pharmaceutically acceptable salt thereof.

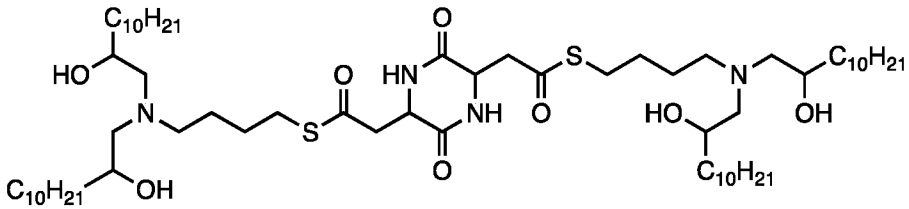
[0373] Other suitable lipids for use in the RNA composition and methods for making and using thereof include lipids as described in United States Provisional Patent Application Serial Number 62/758,179, which is incorporated herein by reference in its entirety.

[0374] In some embodiments, the RNA composition and methods for making and using thereof include a lipid of the following formula:



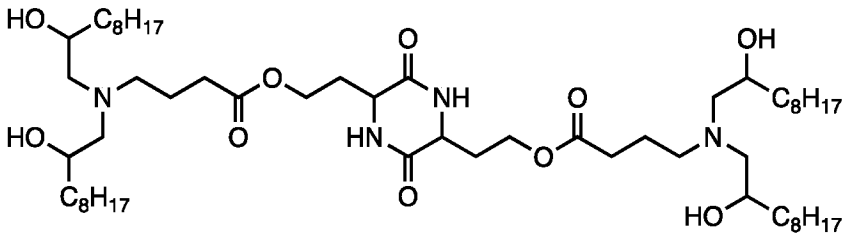
, or a pharmaceutically acceptable salt thereof, wherein each R^1 and R^2 is independently H or C1-C6 aliphatic; each m is independently an integer having a value of 1 to 4; each A is independently a covalent bond or arylene; each L^1 is independently an ester, thioester, disulfide, or anhydride group; each L^2 is independently C2-C10 aliphatic; each X^1 is independently H or OH; and each R^3 is independently C6-C20 aliphatic.

[0375] In some embodiments, the RNA composition and methods for making and using thereof include a lipid of the following formula:



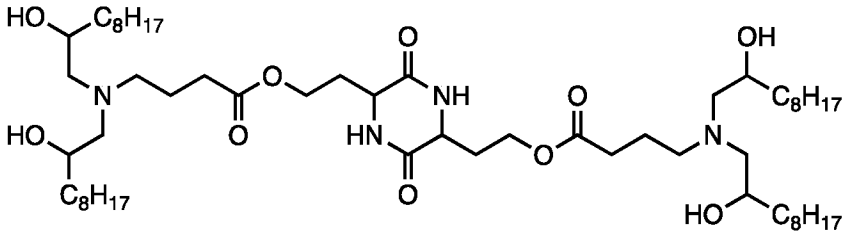
(Compound 1), or a pharmaceutically acceptable salt thereof.

[0376] In some embodiments, the RNA composition and methods for making and using thereof include a lipid of the following formula:



(Compound 2), or a pharmaceutically acceptable salt thereof.

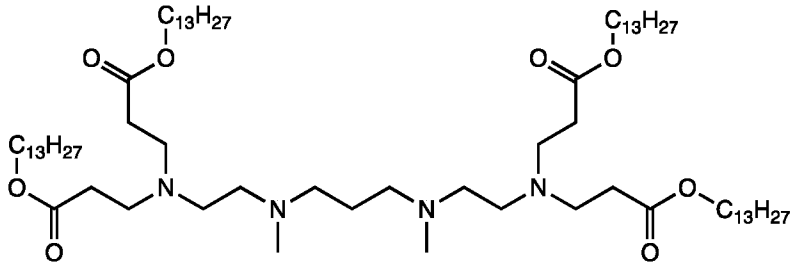
[0377] In some embodiments, the RNA composition and methods for making and using thereof include a lipid of the following formula:



(Compound 3), or a pharmaceutically acceptable salt thereof.

[0378] Other suitable lipids for use in the RNA composition and methods for making and using thereof include the lipids as described in J. McClellan, M. C. King, Cell 2010, 141, 210-217 and in Whitehead et al., Nature Communications (2014) 5:4277, which is incorporated herein by reference in its entirety.

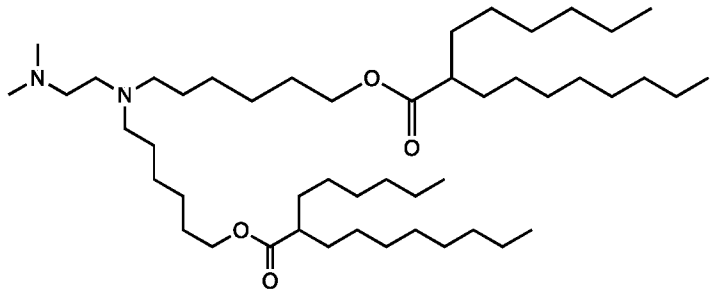
[0379] In certain embodiments, the lipids of the RNA composition and methods for making and using thereof include a lipid having a compound structure of:



, and pharmaceutically acceptable salts thereof.

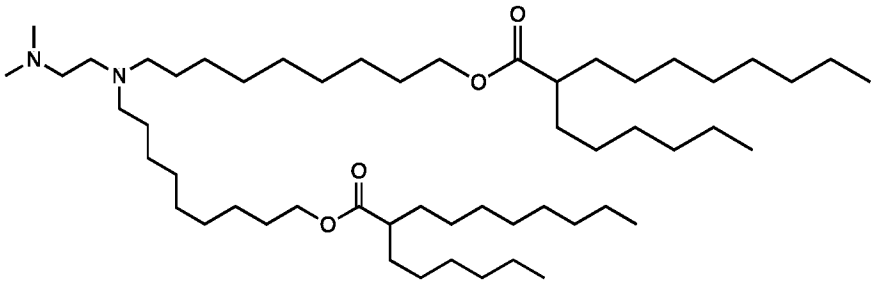
[0380] Other suitable lipids for use in the RNA composition and methods for making and using thereof include the lipids as described in International Patent Publication WO 2015/199952, which is incorporated herein by reference in its entirety.

[0381] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



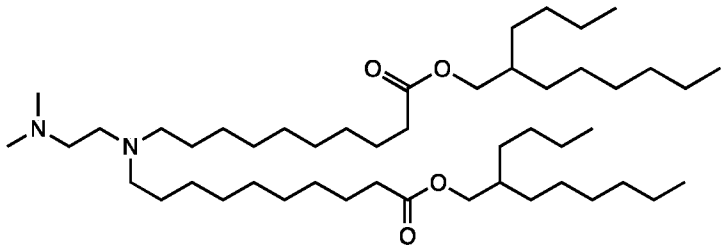
, and pharmaceutically acceptable salts thereof.

[0382] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



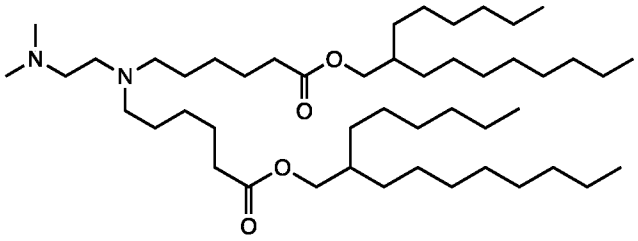
, and pharmaceutically acceptable salts thereof.

[0383] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



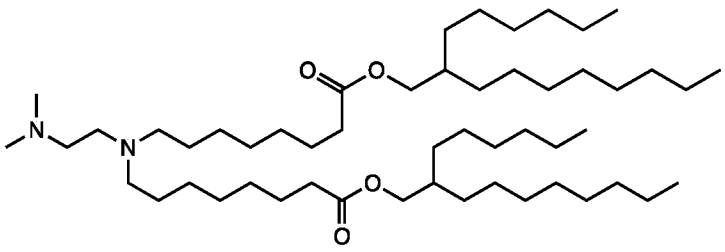
, and pharmaceutically acceptable salts thereof.

[0384] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



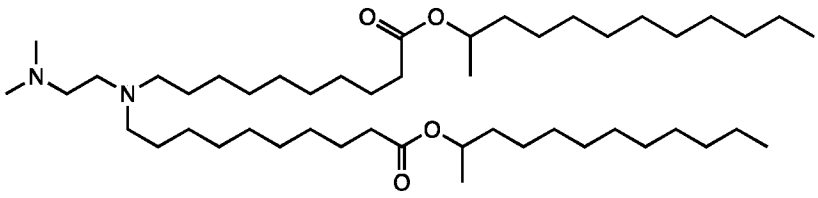
, and pharmaceutically acceptable salts thereof.

[0385] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



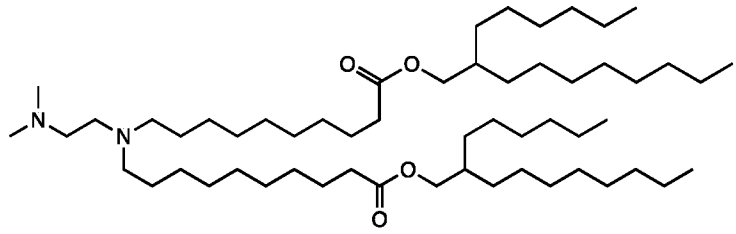
, and pharmaceutically acceptable salts thereof.

[0386] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



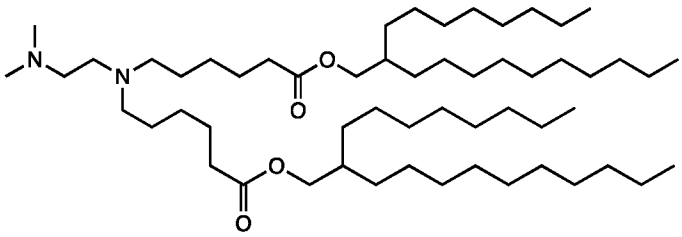
, and pharmaceutically acceptable salts thereof.

[0387] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



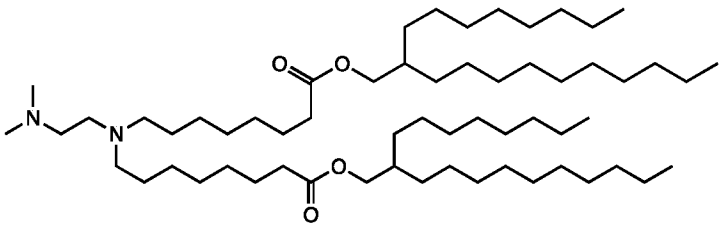
, and pharmaceutically acceptable salts thereof.

[0388] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



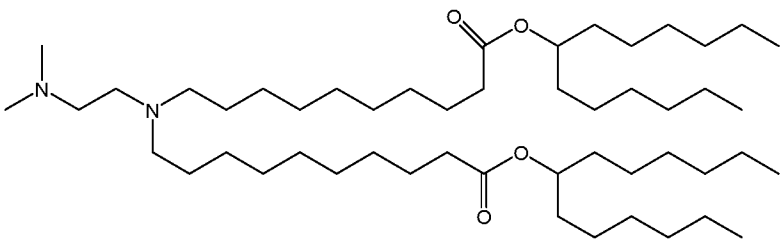
and pharmaceutically acceptable salts thereof.

[0389] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



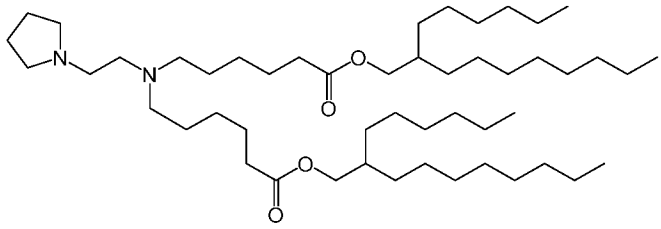
, and pharmaceutically acceptable salts thereof.

[0390] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



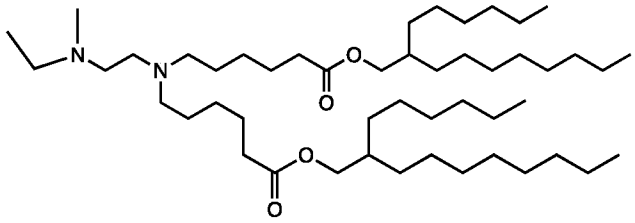
, and pharmaceutically acceptable salts thereof.

[0391] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



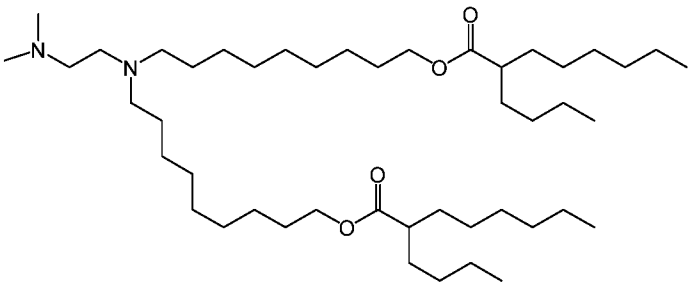
, and pharmaceutically acceptable salts thereof.

[0392] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



, and pharmaceutically acceptable salts thereof.

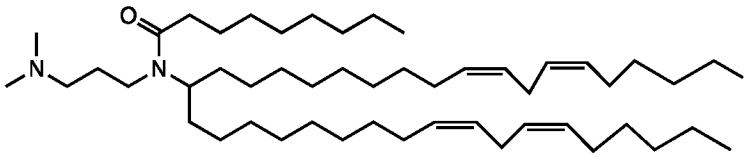
[0393] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



, and pharmaceutically acceptable salts thereof.

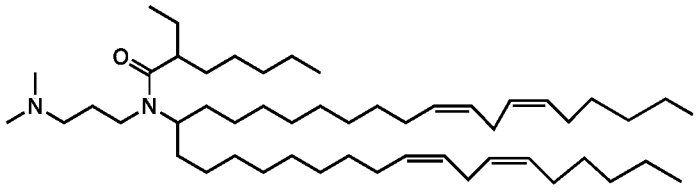
[0394] Other suitable lipids for use in the RNA composition and methods for making and using thereof include the lipids as described in International Patent Publication WO 2017/004143, which is incorporated herein by reference in its entirety.

[0395] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



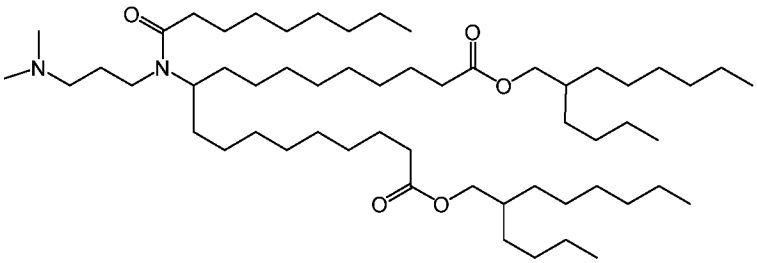
, and pharmaceutically acceptable salts thereof.

[0396] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



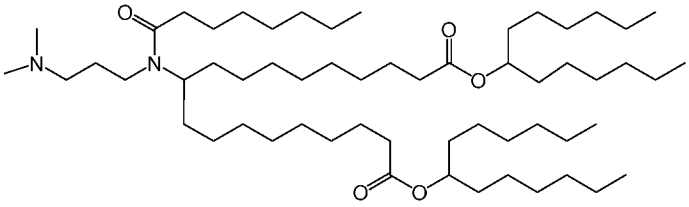
, and pharmaceutically acceptable salts thereof.

[0397] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



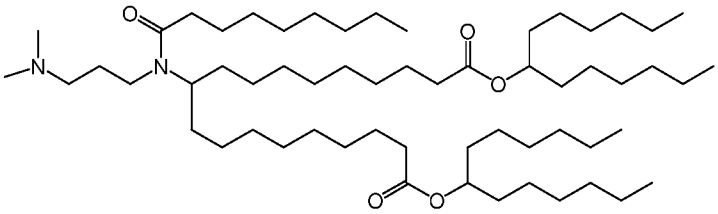
, and pharmaceutically acceptable salts thereof.

[0398] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



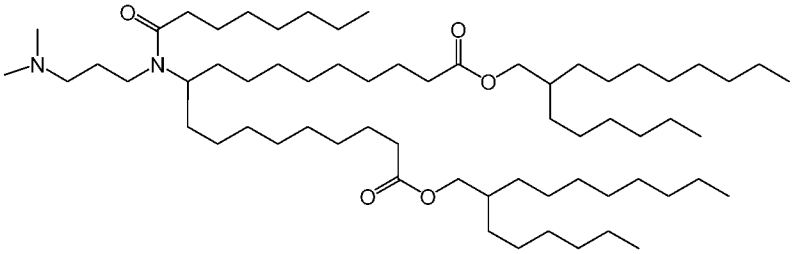
, and pharmaceutically acceptable salts thereof.

[0399] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



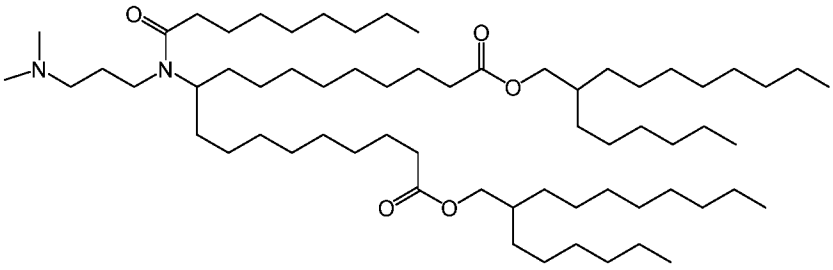
, and pharmaceutically acceptable salts thereof.

[0400] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



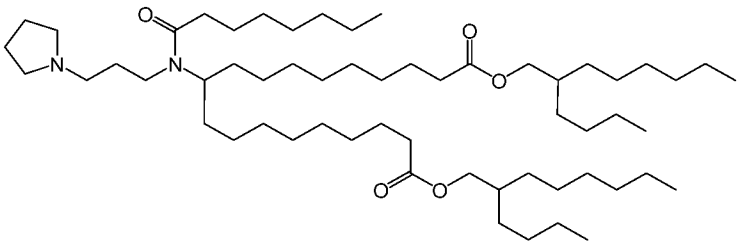
, and pharmaceutically acceptable salts thereof.

[0401] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



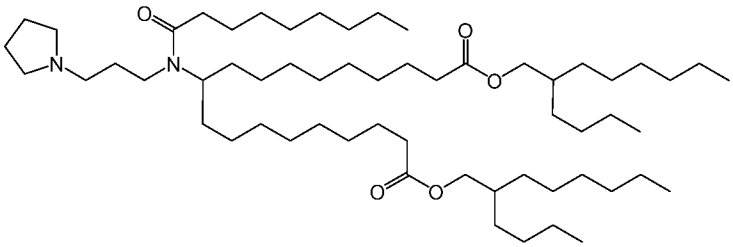
, and pharmaceutically acceptable salts thereof.

[0402] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



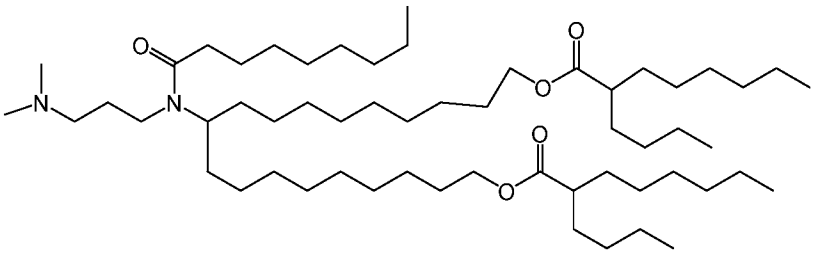
, and pharmaceutically acceptable salts thereof.

[0403] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



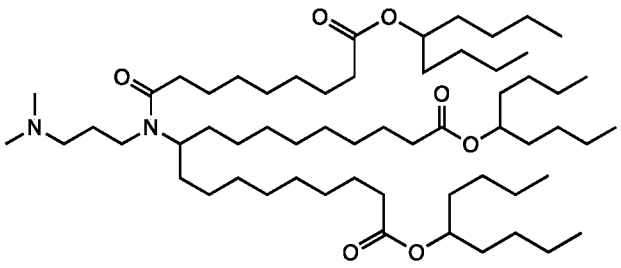
, and pharmaceutically acceptable salts thereof.

[0404] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



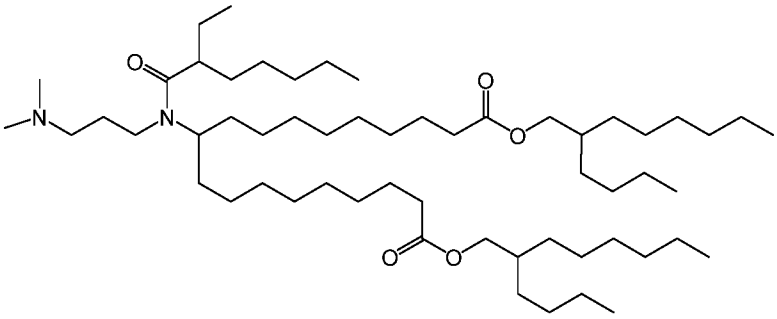
, and pharmaceutically acceptable salts thereof.

[0405] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



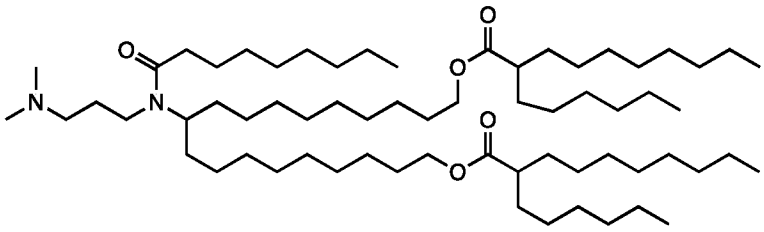
, and pharmaceutically acceptable salts thereof.

[0406] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



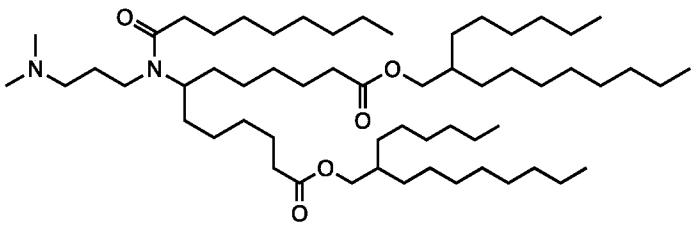
, and pharmaceutically acceptable salts thereof.

[0407] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



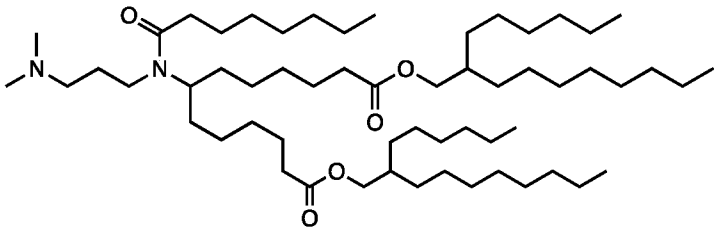
, and pharmaceutically acceptable salts thereof.

[0408] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



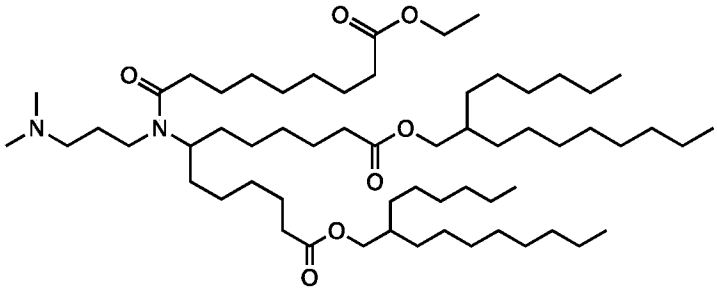
, and pharmaceutically acceptable salts thereof.

[0409] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



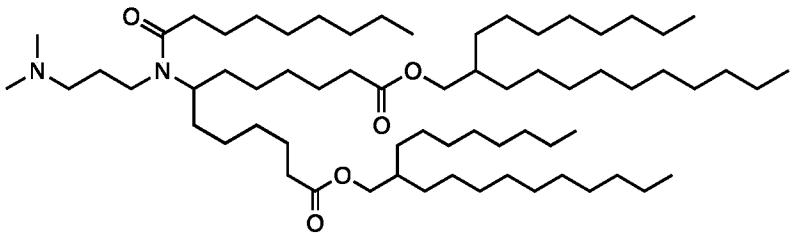
, and pharmaceutically acceptable salts thereof.

[0410] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



, and pharmaceutically acceptable salts thereof.

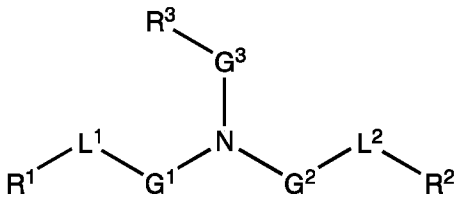
[0411] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



, and pharmaceutically acceptable salts thereof.

[0412] Other suitable lipids for use in the RNA composition and methods for making and using thereof include the lipids as described in International Patent Publication WO 2017/075531, which is incorporated herein by reference in its entirety.

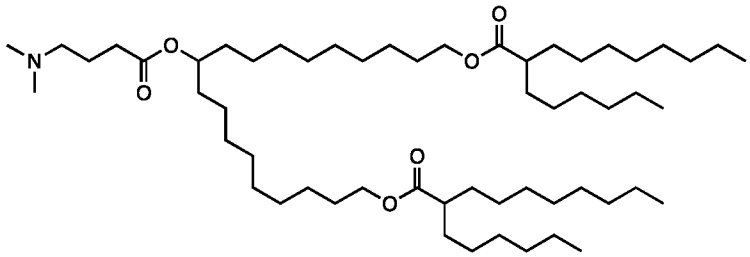
[0413] In some embodiments, the RNA composition and methods for making and using thereof include a lipid of the following formula:



, or a pharmaceutically acceptable salt thereof, wherein one of L^1 or L^2 is -O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -S(O)x, -S-S-, -C(=O)S-, -SC(=O)-, -$NR^aC(=O)$-, -$C(=O)NR^a$-, $NR^aC(=O)NR^a$-, -$OC(=O)NR^a$-, or -$NR^aC(=O)O$-; and the other of L^1 or L^2 is -O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -S(O) $_x$, -S-S-, -C(=O)S-, SC(=O)-, -$NR^aC(=O)$-, -$C(=O)NR^a$-, ,$NR^aC(=O)NR^a$-, -$OC(=O)NR^a$- or -$NR^aC(=O)O$- or a direct bond; G^1 and G^2 are each independently unsubstituted C_1-C_{12} alkylene or C_1-C_{12} alkenylene; G^3 is C_1-C_{24} alkylene, C_1-C_{24} alkenylene, C_3-C_8 cycloalkylene, C_3-C_8 cycloalkenylene; R^a is H or C_1-C_{12} alkyl; R^1 and R^2 are each independently C_6-C_{24} alkyl or C_6-

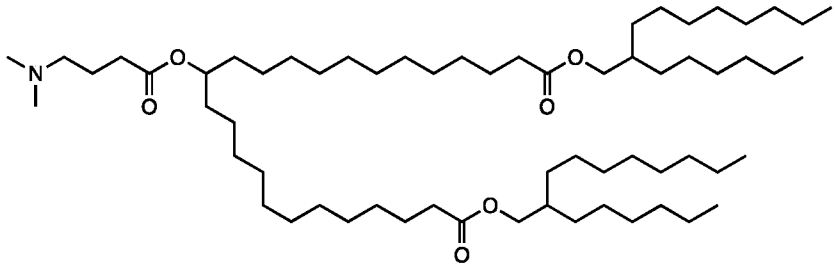
C_{24} alkenyl; R^3 is H, OR^5, CN, -C(=O)OR^4, -OC(=O)R^4 or -NR^5 C(=O)R^4; R^4 is C_1-C_{12} alkyl; R^5 is H or C_1-C_6 alkyl; and x is 0, 1 or 2.

[0414] Other suitable lipids for use in the RNA composition and methods for making and using thereof include the lipids as described in International Patent Publication WO 2017/117528, which is incorporated herein by reference in its entirety. In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



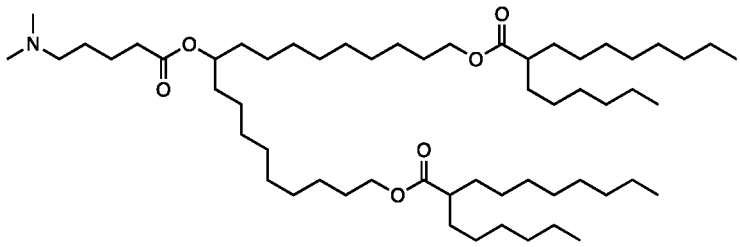
, and pharmaceutically acceptable salts thereof.

[0415] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



, and pharmaceutically acceptable salts thereof.

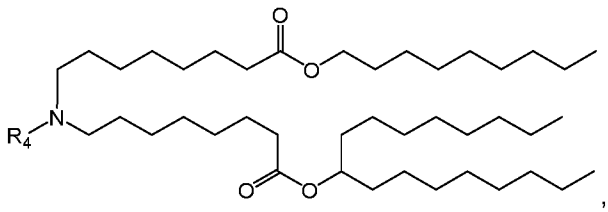
[0416] In some embodiments, the RNA composition and methods for making and using thereof include a lipid having the compound structure:



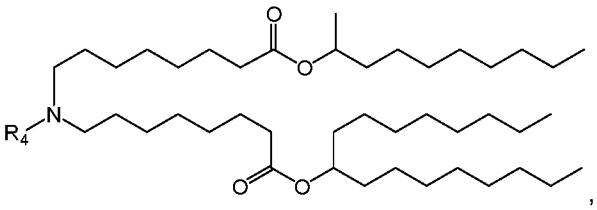
, and pharmaceutically acceptable salts thereof.

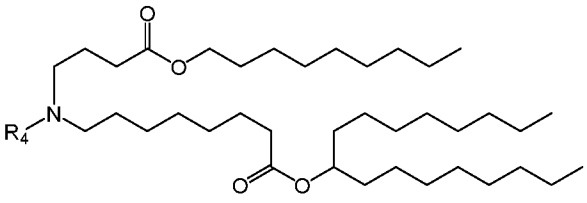
[0417] Other suitable lipids for use in the RNA composition and methods for making and using thereof include the lipids as described in International Patent Publication WO 2017/049245, which is incorporated herein by reference in its entirety.

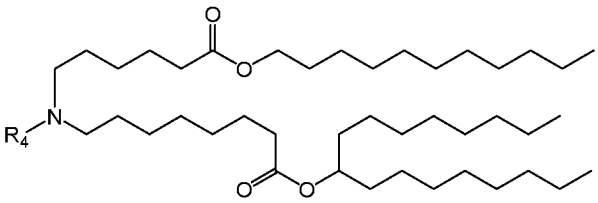
[0418] In some embodiments, the lipids of the RNA composition and methods for making and using thereof include a compound of one of the following formulas:



,

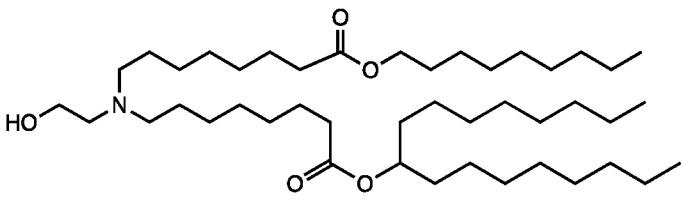

,


, and

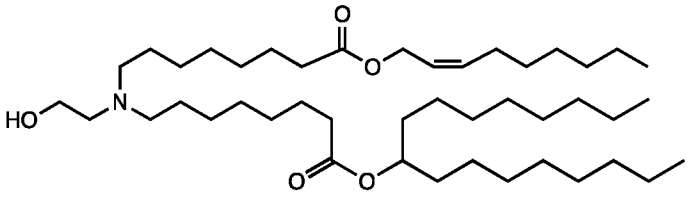

, and pharmaceutically acceptable salts thereof.

For any one of these four formulas, R_4 is independently selected from $-(CH_2)_nQ$ and $-(CH_2)_nCHQR$; Q is selected from the group consisting of -OR, -OH, $-O(CH_2)_nN(R)_2$, -OC(O)R, $-CX_3$, -CN, -N(R)C(O)R, -N(H)C(O)R, $-N(R)S(O)_2R$, $-N(H)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(H)C(O)N(R)_2$, -N(H)C(O)N(H)(R), $-N(R)C(S)N(R)_2$, $-N(H)C(S)N(R)_2$, -N(H)C(S)N(H)(R), and a heterocycle; and n is 1, 2, or 3.

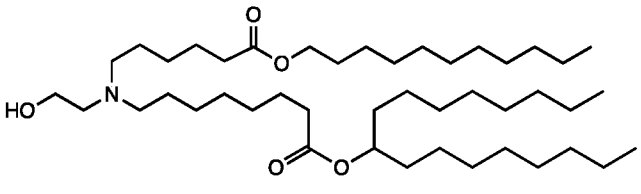
[0419] In certain embodiments, the RNA composition and methods for making and using thereof include a lipid having a compound structure of:


, and pharmaceutically acceptable salts thereof.

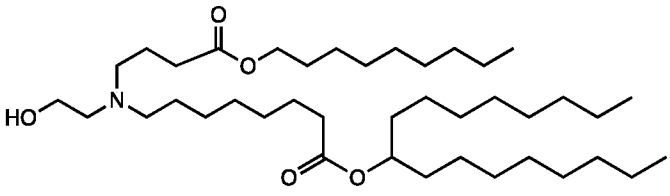
[0420] In certain embodiments, the RNA composition and methods for making and using thereof include a lipid having a compound structure of:


, and pharmaceutically acceptable salts thereof.

[0421] In certain embodiments, the RNA composition and methods for making and using thereof include a lipid having a compound structure of:

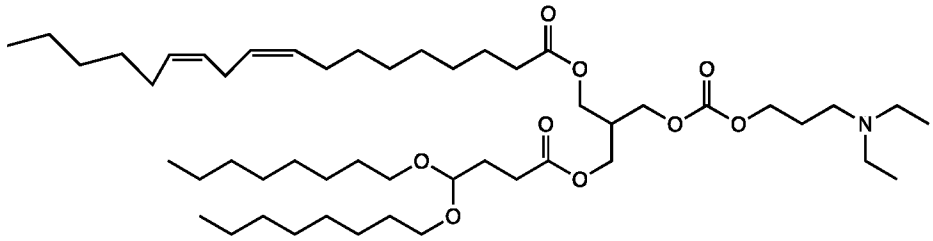

, and pharmaceutically acceptable salts thereof.

[0422] In certain embodiments, the RNA composition and methods for making and using thereof include a lipid having a compound structure of:



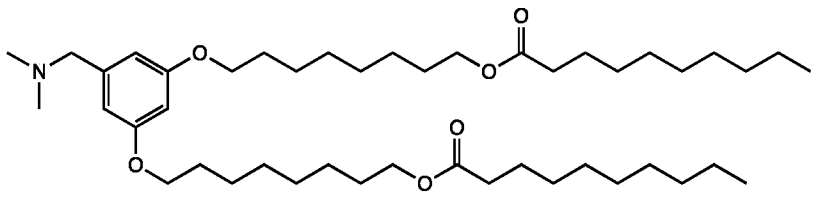
, and pharmaceutically acceptable salts thereof.

[0423] Other suitable lipids for use in the RNA composition and methods for making and using thereof include the lipids as described in International Patent Publication WO 2017/173054 and WO 2015/095340, each of which is incorporated herein by reference in its entirety. In certain embodiments, the RNA composition and methods for making and using thereof include a lipid having a compound structure of:



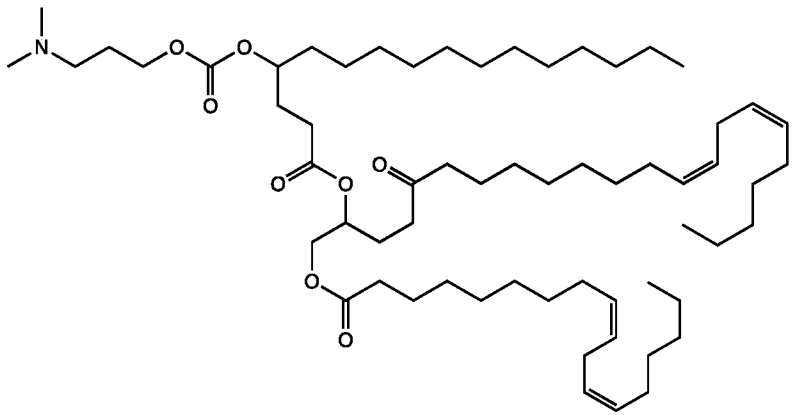
, and pharmaceutically acceptable salts thereof.

[0424] In certain embodiments, the RNA composition and methods for making and using thereof include a lipid having a compound structure of:



, and pharmaceutically acceptable salts thereof.

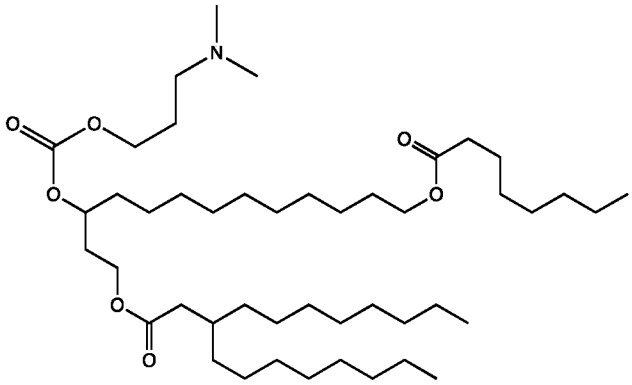
[0425] In certain embodiments, the RNA composition and methods for making and using thereof include a lipid having a compound structure of:



, and pharmaceutically acceptable salts thereof.

[0426] In certain embodiments, the RNA composition and methods for making and using thereof

include a lipid having a compound structure of:



, and pharmaceutically acceptable salts thereof.

[0427] In some embodiments, the LNMPs described herein may include a ionizable lipid as described in, may be formulated as described in, or may comprise or be comprised by a composition as described in WO2016118724, WO2016118725, WO2016187531, WO2017176974, WO2018078053, WO2019027999, WO2019036030, WO2019089828, WO2019099501, WO2020072605, WO2020081938, WO2020118041, WO2020146805, or WO2020219876, each of which is incorporated by reference herein in its entirety.

[0428] The ionizable lipids disclosed herein may be used to form the LNMP composition together with one or more natural lipids disclosed herein. In some embodiments, the LNMP composition is formulated to further comprise one or more therapeutic agents. In some embodiments, the LNMP composition is a lipid nanoparticle that encapsulates or is associated with the one or more therapeutic agents. In some embodiments, the therapeutic agents are one or more polynucleotides encoding one or more components of a gene editing system or one or more gene editing systems.

[0429] In some embodiments, the RNA composition disclosed herein has an N/P ratio of at least 3, for instance, an N/P ratio of 3 to 100, 3 to 50, 3 to 30, 3 to 20, 3 to 15, 3 to 12, about 3 to about 10, , 6 to 30, 6 to 20, 6 to 15, or 6 to 12. For example, the N/P ratio may be about 6 ± 1, or the N/P ratio may be about 6 ± 0.5. In some embodiments, the N/P ratio is about 6. . In some embodiments, the N/P ratio is about 3 (e.g., 3 ± 1 or 3 ± 0.5). In some embodiments, the RNA composition has an N/P ratio of about 12 to about 17, for example the N/P ratio is about 15 ± 1, or the N/P ratio is about 15 ± 0.5. In some embodiments, the N/P ratio is about 15. In some embodiments, the N/P ratio is about 12 (e.g., 12 ± 1 or 12 ± 0.5).

[0430] In some embodiments, the disclosure relates to a composition comprising (i) one or more compounds chosen from the ionizable lipids of Formula (I)-(III), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing and (ii) a lipid component. In some embodiments, the composition comprises 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the one or more compounds.

[0431] In some embodiments, the disclosure relates to a composition comprising (i) one or more lipid nanoparticles and (ii) one or more lipid components.

[0432] In some embodiments, one or more lipid components comprise one or more helper lipids and one or more PEG lipids. In some embodiments, the lipid component(s) comprise(s) one or more helper lipids, one or more PEG lipids, and one or more neutral lipids.

[0433] Non-limiting examples of neutral lipids include phospholipids such as lecithin,

phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane- 1 - carboxylate (DOPE-mal), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl- phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl- phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof. Other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids can also be used. The acyl groups in these lipids may be acyl groups derived from fatty acids having C_{10}-C_{24} carbon chains, e.g. , lauroyl, myristoyl, palmitoyl, stearoyl, or oleoyl.

[0434] In some embodiments, the RNA composition comprises a phytosterol or a combination of a phytosterol and cholesterol. In some embodiments, the phytosterol is selected from the group consisting of b-sitosterol, stigmasterol, b-sitostanol, campesterol, brassicasterol, and combinations thereof. In some embodiments, the phytosterol is selected from the group consisting of b-sitosterol, b-sitostanol, campesterol, brassicasterol, Compound S-140, Compound S-151, Compound S-156, Compound S-157, Compound S-159, Compound S-160, Compound S-164, Compound S-165, Compound S-170, Compound S-173, Compound S-175 and combinations thereof. In some embodiments, the phytosterol is selected from the group consisting of Compound S-140, Compound S-151, Compound S-156, Compound S-157, Compound S-159, Compound S-160, Compound S-164, Compound S-165, Compound S-170, Compound S-173, Compound S-175, and combinations thereof. In some embodiments, the phytosterol is a combination of Compound S-141, Compound S-140, Compound S-143 and Compound S-148. In some embodiments, the phytosterol comprises a sitosterol or a salt or an ester thereof. In some embodiments, the phytosterol comprises a stigmasterol or a salt or an ester thereof.

Other lipids and other agents

[0435] The exogenous lipid may be a cell-penetrating agent, may be capable of increasing delivery of a polypeptide by the LNMP to a cell, and/or may be capable of increasing loading (e.g., loading efficiency or loading capacity) of a polypeptide. Further exemplary exogenous lipids include sterols and PEGylated lipids.

[0436] The LNMPs can be modified with other components (e.g., lipids, e.g., sterols, e.g., cholesterol; or small molecules) to further alter the functional and structural characteristics of the LNMP. For example, the LNMPs can be further modified with stabilizing molecules that increase the stability of the LNMPs (e.g., for at least one day at room temperature, and/or stable for at least one week at 4°C).

[0437] In some embodiments, the LNMP is modified with a sterol, e.g., sitosterol, sitostanol, ß-sitosterol, 7α-hydroxycholesterol, pregnenolone, cholesterol (e.g., ovine cholesterol or cholesterol isolated from plants), stigmasterol, campesterol, fucosterol, or an analog (e.g., a glycoside, ester, or peptide) of any sterol. In some examples, the exogenous sterol is added to the preparation prior to step (b), e.g., mixed with extracted NMP lipids prior to step (b). The exogenous sterol may be added to amount to, e.g., 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more than 90% (w/w) of total lipids and sterols in the preparation.

[0438] In some embodiments, the sterol is cholesterol or sitosterol. In some instances, the LNMPs comprise a molar ratio of least 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, or more than 60% sterol (e.g., cholesterol or sitosterol), e.g., 1%-10%, 10%-20%, 20%-30%, 30%-40%, 40%-50%, or 50%-60% sterol. In some embodiments, the LNMP comprises a molar ratio of about 35%-50% sterol (e.g., cholesterol or sitosterol), e.g., about 36%, 38.5%, 42.5%, or 46.5% sterol. In some embodiments, the LNMP comprises a molar ratio of about 20%-40% sterol.

[0439] In some embodiments, a LNMP that has been modified with a sterol has altered stability (e.g., increased stability) relative to a LNMP that has not been modified with a sterol. In some aspects, a LNMP that has been modified with a sterol has a greater rate of fusion with a membrane of a target cell relative to a LNMP that has not been modified with a sterol.

[0440] In some instances, the LNMPs comprise an exogenous lipid and an exogenous sterol.

[0441] In some embodiments, the LNMP is modified with a PEGylated lipid. Polyethylene glycol (PEG) length can vary from 1kDa to 10kDa; in some aspects, PEG having a length of 2kDa is used. In some embodiments, the PEGylated lipid is C14-PEG2k, C18-PEG2k, or DMPE-PEG2k. In some instances, the LNMPs comprise a molar ratio of at least 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, 2%, 2.1%, 2.2%, 2.3%, 2.4%, 2.5%, 2.6%, 2.7%, 2.8%, 2.9%, 3%, 3.5%, 4%, 4.5%, 5%, 10%, 20%, 30%, 40%, 50%, or more than 50% PEGylated lipid (e.g., C14-PEG2k, C18-PEG2k, or DMPE-PEG2k), e.g., 0.1%-0.5%, 0.5%-1%, 1%-1.5%, 1.5%-2.5%, 2.5%-3.5%, 3.5%-5%, 5%-10%, 10%-20%, 20%-30%, 30%-40%, or 30%-50% PEGylated lipid. In some embodiments, the LNMP comprises a molar ratio of about about 0.1%-10% PEGylated lipid (e.g., C14-PEG2k, C18-PEG2k, or DMPE-PEG2k), e.g., about 1%-3% PEGylated lipid, e.g., about 1.5% or about 2.5% PEGylated lipid. In some embodiments, a LNMP that has been modified with a PEGylated lipid has altered stability (e.g., increased stability) relative to a LNMP that has not been modified with a PEGylated lipid. In some embodiments, a LNMP that has been modified with a PEGylated lipid has altered particle size relative to a LNMP that has not been modified with a PEGylated lipid. In some embodiments, a LNMP that has been modified with a PEGylated lipid is less likely to be phagocytosed than a LNMP that has not been modified with a PEGylated lipid. The addition of PEGylated lipids can also affect stability in GI tract and enhance particle migration through mucus. PEG may be used as a method to attach targeting moieties.

[0442] In some embodiments, the LNMPs are modified with an ionizable lipid and one or both of a sterol (e.g., cholesterol or sitosterol) and a PEGylated lipid (e.g., C14-PEG2k, C18-PEG2k, or DMPE-PEG2k).

[0443] In some embodiments, the LNMPs comprise a molar ratio of about 5%-50% natural lipids

(e.g., about 10%-20% natural lipids, e.g., about 10%, 12.5%, 16%, or 20% natural lipids); about 30%-75% ionizable lipids (e.g., about 35% or about 50% ionizable lipids); about 35%-50% sterol (e.g., about 36%, 38.5%, 42.5%, or 46.5% sterol); and about 0.1%-10% PEGylated lipid (e.g., about 1%-3% PEGylated lipid, e.g., about 1.5% or about 2.5% PEGylated lipid).

[0444] In some embodiments, the modified LNMPs comprise a molar ratio of about 5%-60% natural lipids (e.g., about 10%-20%, 20%-30%, 30%-40%, 40%-50%, or 50%-60% natural lipids, e.g., about 10%, 12.5%, 16%, 20%, 30%, 40%, 50%, or 60% natural lipids); about 25%-75% ionizable lipids (e.g., about 35% or about 50% ionizable lipids); about 10%-50% sterol (e.g., about 10%, 12.5%, 14%, 16%, 18%, 20%, 36%, 38.5%, 42.5%, or 46.5% sterol); and about 0.1%-10% PEGylated lipid (e.g., about 0.5%-5% PEGylated lipid, e.g., about 1%-3% PEGylated lipid, or about 1.5% or about 2.5% PEGylated lipid).

[0445] In some embodiments, the ionizable lipids, natural lipids, sterol, and PEGylated lipid comprise about 25%-75%, about 20%-60%, about 10%-45%, and about 0.5%-5%, respectively, of the lipids in the modified NMP.

[0446] In some embodiments, the ionizable lipids, natural lipids, sterol, and PEGylated lipid comprise about 30%-75%, about 20%-50%, about 10%-45%, and about 1%-5%, respectively, of the lipids in the modified NMP.

[0447] In some embodiments, the ionizable lipids, natural lipids, sterol, and PEGylated lipid comprise about 35%-75%, about 20%-50%, about 10%-45%, and about 1%-5%, respectively, of the lipids in the modified NMP.

[0448] In some embodiments, the ionizable lipids, natural lipids, sterol, and PEGylated lipid are formulated at a molar ratio of about 35:50:12.5:2.5.

[0449] In some embodiments, the ionizable lipids, natural lipids, sterol, and PEGylated lipid are formulated at a molar ratio of about 35:50:11.5:3.5.

[0450] In some embodiments, the ionizable lipids, natural lipids, sterol, and PEGylated lipid are formulated at a molar ratio of about 35:20:42.5:2.5.

[0451] In some embodiments, a LNMP that has been modified with a cationic lipid and a sterol and/or a PEGylated lipid more efficiently encapsulates a negatively charged cargo (e.g., a nucleic acid) than a LNMP that has not been modified with a cationic lipid and a sterol and/or a PEGylated lipid. The modified LNMP may have an encapsulation efficiency for the cargo (e.g., nucleic acid, e.g., RNA or DNA) that is at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99%, or more than 99%, e.g., may have an encapsulation efficiency of 5%-30%, 30%-50%, 50%-70%, 70%-80%, 80%-90%, 90%-95%, or 95%-100%.

[0452] Cell uptake of the modified LNMPs can be measured by a variety of methods known in the art. For example, the LNMPs, or a component thereof, can be labelled with a marker (e.g., a fluorescent marker) that can be detected in isolated cells to confirm uptake.

[0453] In some embodiments, a LNMP formulation provided herein comprises two or more different modified LNMPs, e.g., comprises modified LNMPs derived from different unmodified LNMPs (e.g., unmodified LNMPs from two or more different natural sources) and/or comprises modified LNMPs comprising different species and/or different ratios of ionizable lipids, sterols, and/or PEGylated lipids.

[0454] In some instances, the organic solvent in which the lipid film is dissolved is dimethylformamide:methanol (DMF:MeOH). Alternatively, the organic solvent or solvent combination may be, e.g., acetonitrile, acetone, ethanol, methanol, dimethylformamide, tetrahydrofuran, 1-buthanol, dimethyl sulfoxide, acetonitrile:ethanol, acetonitrile:methanol, acetone:methanol, methyl tert-butyl ether:propanol, tetrahydrofuran:methanol, dimethyl sulfoxide:methanol, or dimethylformamide:methanol.

[0455] The aqueous phase may be any suitable solution, e.g., a citrate buffer (e.g., a citrate buffer having a pH of about 3.2), water, or phosphate-buffered saline (PBS). The aqueous phase may further comprise a nucleic acid (e.g., an siRNA or siRNA precursor (e.g., dsRNA), miRNA or miRNA precursor, mRNA, or plasmid (pDNA)) or a small molecule.

[0456] The lipid solution and the aqueous phase may be mixed in the microfluidics device at any suitable ratio. In some examples, aqueous phase and the lipid solution are mixed at a 3:1 volumetric ratio.

[0457] LNMPs may optionally include additional agents, e.g., cell-penetrating agents, therapeutic agents, polynucleotides, polypeptides, or small molecules. The LNMPs can carry or associate with additional agents in a variety of ways to enable delivery of the agent to a target plant, e.g., by encapsulating the agent, incorporation of the agent in the lipid bilayer structure, or association of the agent (e.g., by conjugation) with the surface of the lipid bilayer structure. Nucleic acid molecules can be incorporated into the LNMPs either in vivo (e.g., in planta) or in vitro (e.g., in tissue culture, in cell culture, or synthetically incorporated).

Zeta Potential

[0458] The LNMPs comprising an ionizable lipid, and optionally a cationic lipid (e.g., DC-cholesterol or DOTAP) may have, e.g., a zeta potential of greater than -30 mV when in the absence of cargo, greater than -20 mV, greater than -5mV, greater than 0 mV, or about 30 mv when in the absence of cargo. In some examples, the LNMP has a negative zeta potential, e.g., a zeta potential of less than 0 mV, less than -10 mV, less than -20 mV, less than -30 mV, less than -40 mV, or less than -50 mV when in the absence of cargo. In some examples, the LNMP has a positive zeta potential, e.g., a zeta potential of greater than 0 mV, greater than 10 mV, greater than 20 mV, greater than 30 mV, greater than 40 mV, or greater than 50 mV when in the absence of cargo. In some examples, the LNMP has a zeta potential of about 0.

[0459] The zeta potential of the LNMP may be measured using any method known in the art. Zeta potentials are generally measured indirectly, e.g., calculated using theoretical models from the data obtained using methods and techniques known in the art, e.g., electrophoretic mobility or dynamic electrophoretic mobility. Electrophoretic mobility is typically measured using microelectrophoresis, electrophoretic light scattering, or tunable resistive pulse sensing. Electrophoretic light scattering is based on dynamic light scattering. Typically, zeta potentials are accessible from dynamic light scattering (DLS) measurements, also known as photon correlation spectroscopy or quasi-elastic light scattering.

Plant EV-Markers

[0460] The LPMPs in the RNA composition and methods of making and using thereof may have a

range of markers that identify the LPMPs as being produced using a plant EV, and/or including a segment, portion, or extract thereof. As used herein, the term "plant EV-marker" refers to a component that is naturally associated with a plant and incorporated into or onto the plant EV *in planta*, such as a plant protein, a plant nucleic acid, a plant small molecule, a plant lipid, or a combination thereof. Examples of plant EV-markers can be found, for example, in Rutter and Innes, *Plant Physiol.* 173(1): 728-741, 2017; Raimondo et al., *Oncotarget*. 6(23): 19514, 2015; Ju et al., *Mol. Therapy*. 21(7):1345-1357, 2013; Wang *et al., Molecular Therapy*. 22(3): 522-534, 2014; and Regente et al, *J of Exp. Biol.* 68(20): 5485-5496, 2017; each of which is incorporated herein by reference.

[0461] Additional examples of the suitable plant EV-markers include those described and listed in International Patent Application Publication No. WO 2021/041301, which is incorporated herein by reference in its entirety.

Bacterial EV-Markers

[0462] The bacterial components (e.g., bacterial lipids) in the bacteria-derived lipid composition and methods of making and using thereof may have a range of markers that identify the bacterial component as being produced. As used herein, the term "bacterial EV-marker" refers to a component that is naturally associated with a bacterium and incorporated into or onto the bacterial EV, such as a bacterial protein, a bacterial nucleic acid, a bacterial small molecule, a bacterial lipid, or a combination thereof.

Natural Source EV-Markers

[0463] The NMPs may have a range of markers that identify the NMP as being produced from a specific source EV, and/or including a segment, portion, or extract thereof. As used herein, the term "EV-marker" refers to a component that is naturally associated with a specific source and incorporated into or onto the EV *in vivo*, such as a protein, a nucleic acid, a small molecule, a lipid, or a combination thereof. Examples of source EV-markers include but are not limited to peptidoglycan, lipopolysaccharide, ester-linked lipids, ether-linked lipids, circular DNA, chitin, beta-glucan, pekilo, mycoprotein, cerato-platanins, exotoxins, diacylglycerol, triglycerides, phosphatidylcholine, phosphatidylinositol, ornithine lipids, glycolipids, sphingolipids, hopanoids, or ergosterol.

[0464] The source EV marker can include a lipid. Examples of lipid markers that may be found in the NMP include lipid A, lipopolysaccharide, ergosterol, ornithine lipids (OLs), sulfolipids, diacylglyceryl-N,N,N-trimethylhomoserine (DGTS), glycolipids (GLs), diacylglycerol (DAG), hopanoids (HOPs), glucosyleramide, sterylglycosides, ether-linked lipids, or a combination thereof.

[0465] Other EV markers may include lipids that accumulate in sources in response to abiotic or biotic stressors.

[0466] Alternatively, the source EV marker may include a protein. In some instances, the protein EV marker may be an antimicrobial or antiviral protein naturally produced by the source, including proteins that are secreted in response to abiotic or biotic stressors. Some examples of protein EV markers include but are not limited to cecropins, moricins, defensins, proline- and glycine-rich peptides, fungal immunomodulatory proteins, flagellin, encapsulin, streptavidin, internalin, pilin, halocin, or archaeocins. In some instances, the EV marker can include a protein involved in lipid metabolism,. In some instances, the protein EV marker is a cellular trafficking protein in the source.

In certain instances where the EV marker is a protein, the protein marker may lack a signal peptide that is typically associated with secreted proteins. Unconventional secretory proteins seem to share several common features like (i) lack of a leader sequence, (ii) absence of PTMs specific for ER or Golgi apparatus, and/or (iii) secretion not affected by brefeldin A which blocks the classical ER/Golgi-dependent secretion pathway. One skilled in the art can use a variety of tools freely accessible to the public to evaluate a protein for a signal sequence, or lack thereof.

[0467] In instances where the EV marker is a protein, the protein may have an amino acid sequence having at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, or 100% sequence identity to a known EV marker.

[0468] In some instances, the EV marker includes a nucleic acid encoded in the source, e.g., a Arthropod, Plant, Fungi, Archaea, or Bacteria RNA, DNA, or PNA. For example, the NMP may include dsRNA, mRNA, a viral RNA, a microRNA (miRNA), or a small interfering RNA (siRNA) encoded by the source. In some instances, the nucleic acid may be one that is associated with a protein that facilitates the long-distance transport of RNA. In some instances, the nucleic acid EV marker may be one involved in host-induced gene silencing (HIGS), which is the process by which a source silences foreign transcripts of pathogens. In some instances, the nucleic acid may be a microRNA.

[0469] In instances where the EV marker is a nucleic acid, the nucleic acid may have a nucleotide sequence having at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, or 100% sequence identity to a known EV marker.

[0470] In some instances, the EV marker includes a compound produced by the source. For example, the compound may a component of the cell wall (e.g. lipopolysaccharide). For example, the compound may be a defense compound produced in response to abiotic or biotic stressors, such as pathogens or extreme environmental stress.

[0471] In some instances, the NMP may also be identified as being produced from a source EV based on the lack of certain markers (e.g., lipids, polypeptides, or polynucleotides) that are not typically produced by these sources, but are generally associated with other organisms (e.g., markers of animal EVs or plant EVs). For example, in some instances, the NMP lacks lipids typically found in animal EVs or plant EVs.

[0472] EV markers can be identified using any approaches known in the art that enable identification of small molecules (e.g., mass spectroscopy, mass spectrometry), lipds (e.g., mass spectroscopy, mass spectrometry), proteins (e.g., mass spectroscopy, immunoblotting), or nucleic acids (e.g., PCR analysis). In some instances, a NMP composition described herein includes a detectable amount, e.g., a pre-determined threshold amount, of an EV marker described herein.

Loading of Agents (e.g., nucleic acids)

[0473] The LNMPs are modified to include a RNA agent (e.g., gene editing system; a nucleic acid molecule like mRNA or gRNA) to form the RNA composition. The LNMPs can carry or associate with such agents by a variety of means to enable delivery of the agent to a target organism (e.g., a target animal), e.g., by encapsulating the agent, incorporation of the component in the lipid bilayer structure, or association of the component (e.g., by conjugation) with the surface of the lipid bilayer structure of

the LNMP. In some instances, the agent is included in the LNMP formulation, as described herein.

[0474] The agent can be incorporated or loaded into or onto the LNMPs by any methods known in the art that allow association, directly or indirectly, between the LNMPs and agent. The agents can be incorporated into the LNMPs by an in vivo method (e.g., in planta, e.g., through production of LNMPs from a transgenic plant or source that comprises the agent), or in vitro (e.g., in tissue culture, or in cell culture), or both in vivo and in vitro methods.

[0475] In some instances, the LNMPs are loaded *in vitro*. The substance may be loaded onto or into (e.g., may be encapsulated by) the LNMPs using, but not limited to, physical, chemical, and/or biological methods (e.g., in tissue culture or in cell culture). For example, the agent may be introduced into LNMPs by one or more of electroporation, sonication, passive diffusion, stirring, lipid extraction, or extrusion. In some instances, the agent is incorporated into the LNMP using a microfluidic device, e.g., using a method in which natural lipids are provided in an organic phase, the heterologous functional agent is provided in an aqueous phase, and the organic and aqueous phases are combined in the microfluidics device to produce a LNMP comprising the heterologous functional agent. Loaded LNMPs can be assessed to confirm the presence or level of the loaded agent using a variety of methods, such as HPLC (e.g., to assess small molecules), immunoblotting (e.g., to assess proteins); and/or quantitative PCR (e.g., to assess nucleotides). However, it should be appreciated by those skilled in the art that the loading of a substance of interest into LNMPs is not limited to the above-illustrated methods.

[0476] In some instances, the agent can be conjugated to the LNMP, in which the agent is connected or joined, indirectly or directly, to the LNMP. For instance, one or more agents can be chemically linked to a LNMP, such that the one or more agents are joined (e.g., by covalent or ionic bonds) directly to the lipid bilayer of the LNMP. In some instances, the conjugation of various agents to the LNMPs can be achieved by first mixing the one or more agents with an appropriate cross-linking agent (e.g., N-ethylcarbo- diimide ("EDC"), which is generally utilized as a carboxyl activating agent for amide bonding with primary amines and also reacts with phosphate groups) in a suitable solvent. After a period of incubation sufficient to allow the agent to attach to the cross-linking agent, the cross-linking agent/ agent mixture can then be combined with the LNMPs and, after another period of incubation, subjected to a sucrose gradient (e.g., and 8, 30, 45, and 60% sucrose gradient) to separate the free agent and free LNMPs from the agent conjugated to the LNMPs. As part of combining the mixture with a sucrose gradient, and an accompanying centrifugation step, the LNMPs conjugated to the agent are then seen as a band in the sucrose gradient, such that the conjugated LNMPs can then be collected, washed, and dissolved in a suitable solution for use as described herein.

[0477] In some instances, the LNMPs are stably associated with the agent prior to and following delivery of the LNMP, e.g., to a plant or animal. In other instances, the LNMPs are associated with the agent such that the agent becomes dissociated from the LNMPs following delivery of the LNMP, e.g., to a plant or animal.

[0478] The LNMPs can be loaded or the LNMP can be formulated with various concentrations of the agent, depending on the particular agent or use. For example, in some instances, the LNMPs are

loaded or the LNMP is formulated such that the LNMP formulation disclosed herein includes about 0.001, 0.01, 0.1, 1.0, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 95 (or any range between about 0.001 and 95) or more wt% of an agent. In some instances, the LNMPs are loaded or the LNMP is formulated such that the LNMP formulation includes about 95, 90, 80, 70, 60, 50, 40, 30, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1.0, 0.1, 0.01, 0.001 (or any range between about 95 and 0.001) or less wt% of an agent. For example, the LNMP formulation can include about 0.001 to about 0.01 wt%, about 0.01 to about 0.1 wt%, about 0.1 to about 1 wt%, about 1 to about 5 wt%, or about 5 to about 10 wt%, about 10 to about 20 wt% of the agent. In some instances, the LNMP can be loaded or the LNMP is formulated with about 1, 5, 10, 50, 100, 200, or 500, 1,000, 2,000 (or any range between about 1 and 2,000) or more µg/ml of an agent. A LNMP of the invention can be loaded or a LNMP can be formulated with about 2,000, 1,000, 500, 200, 100, 50, 10, 5, 1 (or any range between about 2,000 and 1) or less µg/ml of an agent.

[0479] In some instances, the LNMPs are loaded or the LNMP is formulated such that the LNMP formulation disclosed herein includes at least 0.001 wt%, at least 0.01 wt%, at least 0.1 wt%, at least 1.0 wt%, at least 2 wt%, at least 3 wt%, at least 4 wt%, at least 5 wt%, at least 6 wt%, at least 7 wt%, at least 8 wt%, at least 9 wt%, at least 10 wt%, at least 15 wt%, at least 20 wt%, at least 30 wt%, at least 40 wt%, at least 50 wt%, at least 60 wt%, at least 70 wt%, at least 80 wt%, at least 90 wt%, or at least 95 wt% of an agent. In some instances, the LNMP can be loaded or the LNMP can be formulated with at least 1 µg/ml, at least 5 µg/ml, at least 10 µg/ml, at least 50 µg/ml, at least 100 µg/ml, at least 200 µg/ml, at least 500 µg/ml, at least 1,000 µg/ml, at least 2,000 µg/ml of an agent.

[0480] In some instances, the LNMP is formulated with the agent by suspending the LNMPs in a solution comprising or consisting of the agent, e.g., suspending or resuspending the LNMPs by vigorous mixing. The agent (e.g., cell-penetrating agent, e.g., nucleic acids, enzyme, detergent, ionic, fluorous, or zwitterionic liquid, or ionizable lipid may comprise, e.g., less than 1% or at least 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of the solution.

Pharmaceutical Formulations

[0481] The modified LNMPs are formulated into pharmaceutical compositions (i.e., a RNA composition), e.g., for administration to an animal (e.g., a human). The pharmaceutical composition may be administered to an animal (e.g., human) with a pharmaceutically acceptable diluent, carrier, and/or excipient. Depending on the mode of administration and the dosage, the pharmaceutical composition of the methods described herein will be formulated into suitable pharmaceutical compositions to permit facile delivery. The single dose may be in a unit dose form as needed.

[0482] The LNMP / RNA composition may be formulated for e.g., oral administration, intravenous administration (e.g., injection or infusion), intramuscular, or subcutaneous administration to an animal. For injectable formulations, various effective pharmaceutical carriers are known in the art (See, e.g., Remington: The Science and Practice of Pharmacy, 22nd ed., (2012) and ASHP Handbook on Injectable Drugs, 18th ed., (2014)).

[0483] Suitable pharmaceutically acceptable carriers and excipients are nontoxic to recipients at the dosages and concentrations employed. Acceptable carriers and excipients may include buffers such as phosphate, citrate, HEPES, and TAE, antioxidants such as ascorbic acid and methionine,

preservatives such as hexamethonium chloride, octadecyldimethylbenzyl ammonium chloride, resorcinol, and benzalkonium chloride, proteins such as human serum albumin, gelatin, dextran, and immunoglobulins, hydrophilic polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, histidine, and lysine, and carbohydrates such as glucose, mannose, sucrose, and sorbitol.

[0484] The LNMP / RNA composition may be formulated according to conventional pharmaceutical practice. The concentration of the compound in the formulation will vary depending upon a number of factors, including the dosage of the active agent (e.g., LNMPs and nucleic acids) to be administered, and the route of administration.

[0485] For oral administration to an animal, the LNMP / RNA composition can be prepared in the form of an oral formulation. Formulations for oral use can include tablets, caplets, capsules, syrups, or oral liquid dosage forms containing the active ingredient(s) in a mixture with non-toxic pharmaceutically acceptable excipients. These excipients may be, for example, inert diluents or fillers (e.g., sucrose, sorbitol, sugar, mannitol, microcrystalline cellulose, starches including potato starch, calcium carbonate, sodium chloride, lactose, calcium phosphate, calcium sulfate, or sodium phosphate); granulating and disintegrating agents (e.g., cellulose derivatives including microcrystalline cellulose, starches including potato starch, croscarmellose sodium, alginates, or alginic acid); binding agents (e.g., sucrose, glucose, sorbitol, acacia, alginic acid, sodium alginate, gelatin, starch, pregelatinized starch, microcrystalline cellulose, magnesium aluminum silicate, carboxymethylcellulose sodium, methylcellulose, hydroxypropyl methylcellulose, ethylcellulose, polyvinylpyrrolidone, or polyethylene glycol); and lubricating agents, glidants, and antiadhesives (e.g., magnesium stearate, zinc stearate, stearic acid, silicas, hydrogenated vegetable oils, or talc). Other pharmaceutically acceptable excipients can be colorants, flavoring agents, plasticizers, humectants, buffering agents, and the like. Formulations for oral use may also be provided in unit dosage form as chewable tablets, non-chewable tablets, caplets, capsules (e.g., as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium). The compositions disclosed herein may also further include an immediate-release, extended release or delayed-release formulation.

[0486] For parenteral administration to an animal, the LNMP / RNA compositions may be formulated in the form of liquid solutions or suspensions and administered by a parenteral route (e.g., subcutaneous, intravenous, or intramuscular). The pharmaceutical composition can be formulated for injection or infusion. Pharmaceutical compositions for parenteral administration can be formulated using a sterile solution or any pharmaceutically acceptable liquid as a vehicle. Pharmaceutically acceptable vehicles include, but are not limited to, sterile water, physiological saline, or cell culture media (e.g., Dulbecco's Modified Eagle Medium (DMEM), α-Modified Eagles Medium (α-MEM), and F-12 medium). Formulation methods are known in the art, see e.g., Gibson (ed.) Pharmaceutical Preformulation and Formulation (2nd ed.) Taylor & Francis Group, CRC Press (2009).

Polynucleotides

[0487] The LNMP / RNA composition includes one or more nucleic acid molecules, e.g., polynucleotides, which encode one or more wild type or engineered proteins, peptides, or

polypeptides. Exemplary polynucleotides, e.g., polynucleotide constructs, include gene editing system - encoding RNA polynucleotides, e.g., mRNAs and gRNAs.

[0488] Examples of polypeptides that can be used herein can include an enzyme (e.g., a metabolic recombinase, a helicase, an integrase, a RNAse, a DNAse, or an ubiquitination protein), a pore-forming protein, a signaling ligand, a cell penetrating peptide, a transcription factor, a receptor, an antibody, a nanobody, a gene editing protein (e.g., CRISPR-Cas system, TALEN, or zinc finger), riboprotein, a protein aptamer, or a chaperone.

[0489] Polypeptides included herein may include naturally occurring polypeptides or recombinantly produced variants. In some instances, the polypeptide may be a functional fragments or variants thereof (e.g., an enzymatically active fragment or variant thereof). For example, the polypeptide may be a functionally active variant of any of the polypeptides described herein with at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity, e.g., over a specified region or over the entire sequence, to a sequence of a polypeptide described herein or a naturally occurring polypeptide. In some instances, the polypeptide may have at least 50% (e.g., at least 50%, 60%, 70%, 80%, 90%, 95%, 97%, 99%, or greater) identity to a protein of interest.

[0490] The LNMP / RNA composition may include any number or type (e.g., classes) of polypeptides, such as at least about any one of 1 polypeptide, 2, 3, 4, 5, 10, 15, 20, or more polypeptides. A suitable concentration of each polypeptide in the LNMP / RNA composition depends on factors such as efficacy, stability of the polypeptide, number of distinct polypeptides in the formulation, and methods of application of the formulation. In some instances, each polypeptide in a liquid formulation is from about 0.1 ng/mL to about 100 mg/mL. In some instances, each polypeptide in a solid formulation is from about 0.1 ng/g to about 100 mg/g.

Nucleic Acids Encoding Peptides

[0491] In some instances, the LNMP / RNA composition include a heterologous nucleic acid encoding a polypeptide. Nucleic acids encoding a polypeptide may have a length from about 10 to about 50,000 nucleotides (nts), about 25 to about 100 nts, about 50 to about 150 nts, about 100 to about 200 nts, about 150 to about 250 nts, about 200 to about 300 nts, about 250 to about 350 nts, about 300 to about 500 nts, about 10 to about 1000 nts, about 50 to about 1000 nts, about 100 to about 1000 nts, about 1000 to about 2000 nts, about 2000 to about 3000 nts, about 3000 to about 4000 nts, about 4000 to about 5000 nts, about 5000 to about 6000 nts, about 6000 to about 7000 nts, about 7000 to about 8000 nts, about 8000 to about 9000 nts, about 9000 to about 10,000 nts, about 10,000 to about 15,000 nts, about 10,000 to about 20,000 nts, about 10,000 to about 25,000 nts, about 10,000 to about 30,000 nts, about 10,000 to about 40,000 nts, about 10,000 to about 45,000 nts, about 10,000 to about 50,000 nts, or any range therebetween.

[0492] The LNMP / RNA composition may also include active variants of a nucleic acid sequence of interest. In some instances, the variant of the nucleic acids has at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity, e.g., over a specified region or over the entire sequence, to a sequence of a nucleic acid of interest. In some instances, the invention

includes an active polypeptide encoded by a nucleic acid variant as described herein. In some instances, the active polypeptide encoded by the nucleic acid variant has at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity, e.g., over a specified region or over the entire amino acid sequence, to a sequence of a polypeptide of interest or the naturally derived polypeptide sequence.

[0493] Certain methods for expressing a nucleic acid encoding a protein may involve expression in cells, including insect, yeast, plant, bacteria, or other cells under the control of appropriate promoters. Expression vectors may include nontranscribed elements, such as an origin of replication, a suitable promoter and enhancer, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and termination sequences. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a heterologous DNA sequence. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described in Green et al., Molecular Cloning: A Laboratory Manual, Fourth Edition, Cold Spring Harbor Laboratory Press, 2012.

[0494] Genetic modification using recombinant methods is generally known in the art. A nucleic acid sequence coding for a desired gene can be obtained using recombinant methods known in the art, such as, for example by screening libraries from cells expressing the gene, by deriving the gene from a vector known to include the same, or by isolating directly from cells and tissues containing the same, using standard techniques. Alternatively, a gene of interest can be produced synthetically, rather than cloned.

[0495] Expression of natural or synthetic nucleic acids is typically achieved by operably linking a nucleic acid encoding the gene of interest to a promoter, and incorporating the construct into an expression vector. Expression vectors can be suitable for replication and expression in bacteria. Expression vectors can also be suitable for replication and integration in eukaryotes. Typical cloning vectors contain transcription and translation terminators, initiation sequences, and promoters useful for expression of the desired nucleic acid sequence.

[0496] Additional promoter elements, e.g., enhancers, regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 basepairs (bp) upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription.

[0497] One example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. Another example

of a suitable promoter is Elongation Growth Factor-1α (EF-1α). However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the creatine kinase promoter.

[0498] Alternatively, the promoter may be an inducible promoter. The use of an inducible promoter provides a molecular switch capable of turning on expression of the polynucleotide sequence to which it is operatively linked when such expression is desired, or turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a metallothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter.

[0499] The expression vector to be introduced can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other aspects, the selectable marker may be carried on a separate piece of DNA and used in a co-transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers include, for example, antibiotic-resistance genes, such as neo and the like.

[0500] Reporter genes may be used for identifying potentially transformed cells and for evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient source and that encodes a polypeptide whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells. Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (e.g., Ui-Tei et al., FEBS Letters 479:79-82, 2000). Suitable expression systems are well known and may be prepared using known techniques or obtained commercially. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter-driven transcription.

[0501] In some instances, an organism may be genetically modified to alter expression of one or more proteins. Expression of the one or more proteins may be modified for a specific time, e.g., development or differentiation state of the organism. In one instance, provided is a composition to alter expression of one or more proteins, e.g., proteins that affect activity, structure, or function. Expression of the one or more proteins may be restricted to a specific location(s) or widespread throughout the organism.

mRNA

[0502] The LNMP / RNA composition may include a mRNA molecule, e.g., a mRNA molecule

encoding a polypeptide. The mRNA molecule can be synthetic and modified (e.g., chemically). The mRNA molecule can be chemically synthesized or transcribed in vitro. The mRNA molecule can be disposed on a plasmid, e.g., a viral vector, bacterial vector, or eukaryotic expression vector. In some examples, the mRNA molecule can be delivered to cells by transfection, electroporation, or transduction (e.g., adenoviral or lentiviral transduction).

[0503] In some instances, the modified RNA agent of interest described herein has modified nucleosides or nucleotides. Such modifications are known and are described, e.g., in WO 2012/019168. Additional modifications are described, e.g., in WO 2015/038892; WO 2015/038892; WO 2015/089511; WO 2015/196130; WO 2015/196118 and WO 2015/196128 A2, which are herein incorporated by reference in their entirety.

[0504] In some instances, the modified RNA encoding a polypeptide of interest has one or more terminal modification, e.g., a 5' cap structure and/or a poly-A tail (e.g., of between 100-200 nucleotides in length). The 5' cap structure may be selected from the group consisting of CapO, Capl, ARCA, inosine, Nl-methyl-guanosine, 2'fluoro- guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido- guanosine. In some cases, the modified RNAs also contain a 5' UTR including at least one Kozak sequence, and a 3' UTR. Such modifications are known and are described, e.g., in WO 2012/135805 and WO 2013/052523, which are incorporated herein by reference in their entirety. Additional terminal modifications are described, e.g., in WO 2014/164253 and WO 2016/011306, WO 2012/045075, and WO 2014/093924, which are incorporated herein by reference in their entirety. Chimeric enzymes for synthesizing capped RNA molecules (e.g., modified mRNA) which may include at least one chemical modification are described in WO 2014/028429, which is incorporated herein by reference in its entirety.

[0505] In some instances, a modified mRNA (mmRNA) may be cyclized, or concatemerized, to generate a translation competent molecule to assist interactions between poly-A binding proteins and 5 '-end binding proteins. The mechanism of cyclization or concatemerization may occur through at least 3 different routes: 1) chemical, 2) enzymatic, and 3) ribozyme catalyzed. The newly formed 5'-/3'- linkage may be intramolecular or intermolecular. Such modifications are described, e.g., in WO 2013/151736.

[0506] Methods of making and purifying modified RNAs are known and disclosed in the art. For example, modified RNAs are made using only in vitro transcription (IVT) enzymatic synthesis. Methods of making IVT polynucleotides are known in the art and are described in WO 2013/151666, WO 2013/151668, WO 2013/151663, WO 2013/151669, WO 2013/151670, WO 2013/151664, WO 2013/151665, WO 2013/151671, WO 2013/151672, WO 2013/151667 and WO 2013/151736, which are incorporated herein by reference in their entirety. Methods of purification include purifying an RNA transcript including a polyA tail by contacting the sample with a surface linked to a plurality of thymidines or derivatives thereof and/or a plurality of uracils or derivatives thereof (polyT/U) under conditions such that the RNA transcript binds to the surface and eluting the purified RNA transcript from the surface (WO 2014/152031); using ion (e.g., anion) exchange chromatography that allows for separation of longer RNAs up to 10,000 nucleotides in length via a scalable method (WO 2014/144767); and subjecting a modified mRNA sample to DNAse treatment (WO 2014/152030).

[0507] Formulations of modified RNAs are known and are described, e.g., in WO 2013/090648. For example, the formulation may be, but is not limited to, nanoparticles, poly(lactic-co-glycolic acid)(PLGA) microspheres, lipidoids, lipoplex, liposome, polymers, carbohydrates (including simple sugars), cationic lipids, fibrin gel, fibrin hydrogel, fibrin glue, fibrin sealant, fibrinogen, thrombin, rapidly eliminated lipid nanoparticles (reLNPs) and combinations thereof.

[0508] Modified RNAs encoding polypeptides in the fields of human disease, antibodies, viruses, and a variety of in vivo settings are known and are disclosed in for example, Table 6 of International Publication Nos. WO 2013/151666, WO 2013/151668, WO 2013/151663, WO 2013/151669, WO 2013/151670, WO 2013/151664, WO 2013/151665, WO 2013/151736; Tables 6 and 7 International Publication No. WO 2013/151672; Tables 6, 178 and 179 of International Publication No. WO 2013/151671; Tables 6, 185 and 186 of International Publication No WO 2013/151667; which are incorporated herein by reference in their entirety. Any of the foregoing may be synthesized as an IVT polynucleotide, chimeric polynucleotide or a circular polynucleotide, and each may include one or more modified nucleotides or terminal modifications.

Inhibitory RNA

[0509] In some instances, the LNMP / RNA composition includes an inhibitory RNA molecule, e.g., that acts via the RNA interference (RNAi) pathway. In some instances, the inhibitory RNA molecule decreases the level of gene expression in a plant and/or decreases the level of a protein in the plant. In some instances, the inhibitory RNA molecule inhibits expression of a plant gene. For example, an inhibitory RNA molecule may include a short interfering RNA or its precursor, short hairpin RNA, and/or a microRNA or its precursor that targets a gene in the plant. Certain RNA molecules can inhibit gene expression through the biological process of RNA interference (RNAi). RNAi molecules include RNA or RNA-like structures typically containing 15-50 base pairs (such as about 18-25 base pairs) and having a nucleobase sequence identical (or complementary) or nearly identical (or substantially complementary) to a coding sequence in an expressed target gene within the cell. RNAi molecules include, but are not limited to short interfering RNAs (siRNAs), double-strand RNAs (dsRNA), short hairpin RNAs (shRNA), meroduplexes, dicer substrates, and multivalent RNA interference (U.S. Pat. Nos. 8,084,599 8,349,809, 8,513,207 and 9,200,276, which are incorporated herein by reference in their entirety). The inhibitory RNA molecule can be chemically synthesized or transcribed in vitro.

[0510] Additional examples of the inhibitory RNA molecules include those described in details in International Patent Application Publication No. WO 2021/041301, which is incorporated herein by reference in its entirety.

Gene Editing

[0511] In some instances, the LNP / RNA compositions or LNMP / RNA compositions may include a component of a gene editing system. For example, the agent may introduce an alteration (e.g., insertion, deletion (e.g., knockout), translocation, inversion, single point mutation, or other mutation) in a gene in the target cell. Exemplary gene editing systems include the zinc finger nucleases (ZFNs), Transcription Activator-Like Effector-based Nucleases (TALEN), and the clustered regulatory interspaced short palindromic repeat (CRISPR) system. ZFNs, TALENs, and CRISPR-based

methods are described, e.g., in Gaj et al., Trends Biotechnol. 31(7):397-405, 2013.

[0512] Additional descriptions about the component and process of the gene editing system may be found in International Patent Application Publication No. WO 2021/041301, which is incorporated herein by reference in its entirety. In regards to a CRISPR/Cas system, an endonuclease is directed to a target nucleotide sequence (e.g., a site in the genome that is to be sequence-edited) by sequence-specific, non-coding guide RNAs (eg sgRNA) that target single- or double-stranded DNA sequences. Three classes (I-III) of CRISPR systems are known, and class II CRISPR systems use a single Cas endonuclease (rather than multiple Cas proteins). One class II CRISPR system includes a type II Cas endonuclease such as Cas9, a CRISPR RNA (crRNA), and a trans-activating crRNA (tracrRNA). The crRNA contains a guide RNA, i.e., typically about 20-nucleotide RNA sequence that corresponds to a target DNA sequence. The crRNA also contains a region that binds to the tracrRNA to form a partially double-stranded structure which is cleaved by RNase III, resulting in a crRNA/tracrRNA hybrid. The RNAs serve as guides to direct Cas proteins to silence specific DNA/RNA sequences, depending on the spacer sequence. See, e.g., Horvath et al., Science 327:167-170, 2010; Makarova et al., Biology Direct 1 :7, 2006; Pennisi, Science 341 :833-836, 2013. The target DNA sequence must generally be adjacent to a protospacer adjacent motif (PAM) that is specific for a given Cas endonuclease; however, PAM sequences appear throughout a given genome. CRISPR endonucleases identified from various prokaryotic species have unique PAM sequence requirements; examples of PAM sequences include 5'-NGG (Streptococcus pyogenes), 5'-NNAGAA (Streptococcus thermophilus CRISPR1), 5'-NGGNG (Streptococcus thermophilus CRISPR3), and 5'-NNNGATT (Neisseria meningiditis).

[0513] Some endonucleases, such as Cas9 endonucleases, are associated with G-rich PAM sites, e.g., 5'-NGG, and perform blunt-end cleaving of the target DNA at a location 3 nucleotides upstream from (5' from) the PAM site. Another class II CRISPR system includes the type V endonuclease Cpfl (e.g. AsCpfl (from Acidaminococcus sp.) and LbCpfl (from Lachnospiraceae sp.)), which is smaller than Cas9. Cpfl -associated CRISPR arrays are processed into mature crRNAs without the requirement of a tracrRNA. Cpfl endonucleases, are associated with T-rich PAM sites, e.g., 5'-TTN, but can also recognize a 5'-CTA PAM motif. Cpfl cleaves the target DNA by introducing an offset or staggered double-strand break with a 4- or 5-nucleotide 5' overhang, for example, cleaving a target DNA with a 5-nucleotide offset or staggered cut located 18 nucleotides downstream from (3' from) from the PAM site on the coding strand and 23 nucleotides downstream from the PAM site on the complimentary strand; the 5-nucleotide overhang that results from such offset cleavage allows more precise genome editing by DNA insertion by homologous recombination than by insertion at blunt-end cleaved DNA. See, e.g., Zetsche et al., Cell 163:759-771 , 2015.,

[0514] For the purposes of gene editing, CRISPR arrays can be designed to contain one or multiple guide RNA sequences corresponding to a desired target DNA sequence; see, for example, Cong et al., Science 339:819-823, 2013; Ran et al., Nature Protocols 8:2281-2308, 2013. At least about 16 or 17 nucleotides of gRNA sequence are required by Cas9 for DNA cleavage to occur; for Cpfl at least about 16 nucleotides of gRNA sequence is needed to achieve detectable DNA cleavage. In practice, guide RNA sequences are generally designed to have a length of between 17-24 nucleotides (e.g.,

19, 20, or 21 nucleotides) and complementarity to the targeted gene or nucleic acid sequence. Custom gRNA generators and algorithms are available commercially for use in the design of effective guide RNAs.

[0515] Gene editing has also been achieved using a chimeric single guide RNA (sgRNA), an engineered (synthetic) single RNA molecule that mimics a naturally occurring crRNA-tracrRNA complex and contains both a tracrRNA (for binding the nuclease) and at least one crRNA (to guide the nuclease to the sequence targeted for editing). Chemically modified sgRNAs have also been demonstrated to be effective in genome editing; see, for example, Hendel et al., Nature Biotechnol. 985-991 , 2015.

[0516] Whereas wild-type Cas9 generates double-strand breaks (DSBs) at specific DNA sequences targeted by a gRNA, a number of CRISPR endonucleases having modified functionalities are available, for example: a nickase version of Cas9 generates only a single-strand break; a catalytically inactive Cas9 (dCas9) does not cut the target DNA but interferes with transcription by steric hindrance. dCas9 can further be fused with an effector to repress (CRISPRi) or activate (CRISPRa) expression of a target gene. For example, Cas9 can be fused to a transcriptional repressor (e.g., a KRAB domain) or a transcriptional activator (e.g., a dCas9-VP64 fusion). A catalytically inactive Cas9 (dCas9) fused to FokI nuclease (dCas9-FokI) can be used to generate DSBs at target sequences homologous to two gRNAs. See, e.g., the numerous CRISPR/Cas9 plasmids disclosed in and publicly available from the Addgene repository (Addgene, 75 Sidney St., Suite 550A, Cambridge, MA 02139; addgene.org/crispr/). A double nickase Cas9 that introduces two separate double-strand breaks, each directed by a separate guide RNA, is described as achieving more accurate genome editing by Ran et al., Cell 154:1380-1389, 2013.

[0517] CRISPR technology for editing the genes of eukaryotes is disclosed in US Patent Application Publications US 2016/0138008 A1 and US 2015/0344912 A1 , and in US Patents 8,697,359, 8,771 ,945, 8,945,839, 8,999,641 , 8,993,233, 8,895,308, 8,865,406, 8,889,418, 8,871 ,445, 8,889,356, 8,932,814, 8,795,965, and 8,906,616. Cpfl endonuclease and corresponding guide RNAs and PAM sites are disclosed in US Patent Application Publication 2016/0208243 A1 .

[0518] In some instances, the desired genome modification involves homologous recombination, wherein one or more double-stranded DNA breaks in the target nucleotide sequence is generated by the RNA-guided nuclease and guide RNA(s), followed by repair of the break(s) using a homologous recombination mechanism (homology-directed repair). In such instances, a donor template that encodes the desired nucleotide sequence to be inserted or knocked-in at the double-stranded break is provided to the cell or subject; suitable templates include single-stranded DNA templates and double-stranded DNA templates. In general, a donor template encoding a nucleotide change over a region of less than about 50 nucleotides is provided in the form of single-stranded DNA; larger donor templates (e.g., more than 100 nucleotides) are often provided as double-stranded DNA plasmids. In some instances, the donor template is provided to the cell or subject in a quantity that is sufficient to achieve the desired homology-directed repair but that does not persist in the cell or subject after a given period of time (e.g., after one or more cell division cycles). In some instances, a donor template has a core nucleotide sequence that differs from the target nucleotide sequence (e.g., a homologous

endogenous genomic region) by at least 1, at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, or more nucleotides. This core sequence is flanked by homology arms or regions of high sequence identity with the targeted nucleotide sequence; in some instances, the regions of high identity include at least 10, at least 50, at least 100, at least 150, at least 200, at least 300, at least 400, at least 500, at least 600, at least 750, or at least 1000 nucleotides on each side of the core sequence. In some instances where the donor template is in the form of a single-stranded DNA, the core sequence is flanked by homology arms including at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 100 nucleotides on each side of the core sequence. In instances, where the donor template is in the form of a double-stranded DNA, the core sequence is flanked by homology arms including at least 500, at least 600, at least 700, at least 800, at least 900, or at least 1000 nucleotides on each side of the core sequence. In one instance, two separate doublestrand breaks are introduced into the cell or subject's target nucleotide sequence with a double nickase Cas9 (see Ran et al., Cell 154:1380-1389, 2013), followed by delivery of the donor template.

[0519] In some instances, the RNA composition includes a gRNA and a targeted nuclease, e.g., a Cas9, e.g., a wild type Cas9, a nickase Cas9 (e.g., Cas9 D10A), a dead Cas9 (dCas9), eSpCas9, Cpfl , C2C1 , or C2C3, or a nucleic acid encoding such a nuclease. The choice of nuclease and gRNA(s) is determined by whether the targeted mutation is a deletion, substitution, or addition of nucleotides, e.g., a deletion, substitution, or addition of nucleotides to a targeted sequence. Fusions of a catalytically inactive endonuclease e.g., a dead Cas9 (dCas9, e.g., D10A; H840A) tethered with all or a portion of (e.g., biologically active portion of) an (one or more) effector domain create chimeric proteins that can be linked to the polypeptide to guide the RNA composition to specific DNA sites by one or more RNA sequences (sgRNA) to modulate activity and/or expression of one or more target nucleic acids sequences.

[0520] In instances, the agent includes a guide RNA (gRNA) for use in a CRISPR system for gene editing. In some instances, the agent includes a zinc finger nuclease (ZFN), or a mRNA encoding a ZFN, that targets (e.g., cleaves) a nucleic acid sequence (e.g., DNA sequence) of a gene. In some instances, the agent includes a TALEN, or an mRNA encoding a TALEN, that targets (e.g., cleaves) a nucleic acid sequence (e.g., DNA sequence) in a gene in the target organism.

[0521] For example, the gRNA can be used in a CRISPR system to engineer an alteration in a gene in the target organism. In other examples, the ZFN and/or TALEN can be used to engineer an alteration in a gene. Exemplary alterations include insertions, deletions (e.g., knockouts), translocations, inversions, single point mutations, or other mutations. The alteration can be introduced in the gene in a cell, e.g., in vitro, ex vivo, or in vivo. In some examples, the alteration increases the level and/or activity of a gene in the target organism. In other examples, the alteration decreases the level and/or activity of (e.g., knocks down or knocks out) a gene in the target organism. In yet another example, the alteration corrects a defect (e.g., a mutation causing a defect), in a gene in the target organism.

[0522] In some instances, the CRISPR system is used to edit (e.g., to add or delete a base pair) a target gene in the target organism. In other instances, the CRISPR system is used to introduce a

premature stop codon, e.g., thereby decreasing the expression of a target gene. In yet other instances, the CRISPR system is used to turn off a target gene in a reversible manner, e.g., similarly to RNA interference. In some instances, the CRISPR system is used to direct Cas to a promoter of a gene, thereby blocking an RNA polymerase sterically.

[0523] In some instances, a CRISPR system can be generated to edit a gene in the target organism, using technology described in, e.g., U.S. Publication No. 20140068797, Cong, Science 339: 819-823, 2013; Tsai, Nature Biotechnol. 32:6569-576, 2014; U.S. Patent Nos.: 8,871,445; 8,865,406; 8,795,965; 8,771,945; and 8,697,359.

[0524] In some instances, the CRISPR interference (CRISPRi) technique can be used for transcriptional repression of specific genes in the target organism. In CRISPRi, an engineered Cas9 protein (e.g., nuclease-null dCas9, or dCas9 fusion protein, e.g., dCas9-KRAB ordCas9-SID4X fusion) can pair with a sequence specific guide RNA (sgRNA). The Cas9-gRNA complex can block RNA polymerase, thereby interfering with transcription elongation. The complex can also block transcription initiation by interfering with transcription factor binding. The CRISPRi method is specific with minimal off-target effects and is multiplexable, e.g., can simultaneously repress more than one gene (e.g., using multiple gRNAs). Also, the CRISPRi method permits reversible gene repression.

[0525] In some instances, CRISPR-mediated gene activation (CRISPRa) can be used for transcriptional activation of a gene in the target organism. In the CRISPRa technique, dCas9 fusion proteins recruit transcriptional activators. For example, dCas9 can be fused to polypeptides (e.g., activation domains) such as VP64 or the p65 activation domain (p65D) and used with sgRNA (e.g., a single sgRNA or multiple sgRNAs), to activate a gene or genes in the target organism. Multiple activators can be recruited by using multiple sgRNAs - this can increase activation efficiency. A variety of activation domains and single or multiple activation domains can be used. In addition to engineering dCas9 to recruit activators, sgRNAs can also be engineered to recruit activators. For example, RNA aptamers can be incorporated into a sgRNA to recruit proteins (e.g., activation domains) such as VP64. In some examples, the synergistic activation mediator (SAM) system can be used for transcriptional activation. In SAM, MS2 aptamers are added to the sgRNA. MS2 recruits the MS2 coat protein (MCP) fused to p65AD and heat shock factor 1 (HSF1).

[0526] The CRISPRi and CRISPRa techniques are described in greater detail, e.g., in Dominguez et al., Nat. Rev. Mol. Cell Biol. 17:5-15, 2016, incorporated herein by reference. In addition, dCas9-mediated epigenetic modifications and simultaneous activation and repression using CRISPR systems, as described in Dominguez et al., can be used to modulate a gene in the target organism.

[0527] In some embodiments, the RNA-guided DNA-binding agent is a Class 2 Cas nuclease. In some embodiments, the RNA-guided DNA-binding agent has cleavase activity, which can also be referred to as double-strand endonuclease activity. In some embodiments, the RNA-guided DNA-binding agent comprises a Cas nuclease, such as a Class 2 Cas nuclease (which may be, e.g., a Cas nuclease of Type II V, or VI). Class 2 Cas nucleases include, for example, Cas9, Cpf1, C2c1, C2c2, and C2c3 proteins and modifications thereof. Examples of Cas9 nucleases include those of the type II CRISPR systems of S. pyogenes, S. aureus, and other prokaryotes, and modified (e.g., engineered or mutant) versions thereof.. In some embodiments, the Cas nuclease may be from a Type-IIA, Type-IIB,

or Type-IIC system.

[0528] Non-limiting exemplary species that the Cas nuclease can be derived from include Streptococcus pyogenes, Streptococcus thermophilus, Streptococcus sp., Staphylococcus aureus, Listeria innocua, Lactobacillus gasseri, Francisella novicida, Wolinella succinogenes, Sutterella wadsworthensis, Gammaproteobacterium, Neisseria meningitidis. Campylobacter jejuni, Pasteurella multocida, Fibrobacter succinogene, Rhodospirillum rubrum, Nocardiopsis dassonvillei, Streptomyces pristinaespiralis, Streptomyces viridochromogenes, Streptomyces viridochromogenes, Streptosporangium roseum, Streptosporangium roseum, Alicyclobacillus acidocaldarius. Bacillus pseudomycoides, Bacillus selenitireducens, Exiguobacterium sibiricum, Lactobacillus delbrueckii, Lactobacillus salivarius, Lactobacillus buchneri, Treponema denticola, Microscilla marina, Burkholderiales bacterium, Polaromonas naphthalenivorans, Polaromonas sp., Crocosphaera watsonii, Cyanothece sp., Microcystis aeruginosa, Synechococcus sp., Acetohalobium arabaticum, Ammonifex degensii, Caldicelulosiruptor becscii, Candidatus Desulforudis, Clostridium botulinum, Clostridium difficile, Finegoldia magna, Natranaerobius thermophilus, Pelotomaculum thermopropionicum, Ac idithio bacillus caldus, Acidithiobacillus ferrooxidans, Allochromatium vinosum, Marinobacter sp., Nitrosococcus halophilus, Nitrosococcus watsoni, Pseudoalteromonas haloplanktis, Ktedonobacier racemifer, Methanohalobium evestigatum, Anabaena variabilis, Nodularia spumigena, Nosioc sp., Arthrospira maxima, Aithrospira platensis, Arthrospira sp., Lyngbya sp., Microcoleus chthonoplastes, Oscillatoria sp., Peirotoga mobilis, Thermosipho africanus. Streptococcus pasteurianus, Neisseria cinerea, Campylobacter lari, Parvibaculum lavamentivorans, Corynebacterium diphtheria, Acidaminococcus sp., Lachnospiraceae bacterium ND2006, and Acaryochloris marina.

[0529] In some embodiments, the Cas nuclease is the Cas9 nuclease from Streptococcus pyogenes. In some embodiments, the Cas nuclease is the Cas9 nuclease from Streptococcus thermophilus. In some embodiments, the Cas nuclease is the Cas9 nuclease from Neisseria meningitidis. In some embodiments, the Cas nuclease is the Cas9 nuclease is from Staphylococcus aureus. In some embodiments, the Cas nuclease is the Cpf1 nuclease from Francisella novicida. In some embodiments, the Cas nuclease is the Cpf1 nuclease from Acidaminococcus sp. In some embodiments, the Cas nuclease is the Cpf1 nuclease from Lachnospiraceae bacterium ND2006. In further embodiments, the Cas nuclease is the Cpfl nuclease from Francisella tularensis, Lachnospiraceae bacterium, Butyrivibrio proteoclasticus, Peregrinibacteria bacterium, Parcubacferia bacterium, Smithella, Acidaminococcus, Candidatus Methanoplasma termitum, Eubacterium eligens, Moraxella bovoculi, Leptospira inadai, Porphyromonas crevioricanis, Prevotella disiens, or Porphyromonas macacae. In certain embodiments, the Cas nuclease is a Cpf1 nuclease from an Acidaminococcus or Lachnospiraceae.

[0530] In some embodiments, the Cas9 nuclease comprises more than one RuvC domain and/or more than one HNH domain. In some embodiments, the Cas9 nuclease is a wild type Cas9. In some embodiments, the Cas9 is capable of inducing a double strand break in target DNA. In certain embodiments, the Cas nuclease may cleave dsDNA, it may cleave one strand of dsDNA, or it may not have DNA cleavase or nickase activity. In some embodiments, chimeric Cas nucleases are used,

where one domain or region of the protein is replaced by a portion of a different protein. In some embodiments, a Cas nuclease domain may be replaced with a domain from a different nuclease such as Fok1. In some embodiments, a Cas nuclease may be a modified nuclease. In other embodiments, the Cas nuclease may be from a Type-1 CRISPR/Cas system. In some embodiments, the Cas nuclease may be a component of the Cascade complex of a Type-I CRISPR/Cas system. In some embodiments, the Cas nuclease may have an RNA cleavage activity.

[0531] In some embodiments, the RNA-guided DNA-binding agent has single-strand nickase activity, i.e., can cut one DNA strand to produce a single-strand break, also known as a "nick." In some embodiments, the RNA-guided DNA-bmding agent comprises a Cas nickase. A nickase is an enzyme that creates a stick in dsDNA, i.e., cuts one strand but not the other of the DNA double helix. In some embodiments, a Cas nickase is a version of a Cas nuclease (e.g., a Cas nuclease discussed above) in which an endonucleolytic active site is inactivated, e.g., by one or more alterations (e.g., point mutations) in a catalytic domain. See, e.g., U.S. Pat. No. 8,889,356 for discussion of Cas nickases and exemplary catalytic domain alterations. In some embodiments, a Cas nickase such as a Cas9 nickase has an inactivated RuvC or HNH domain. In some embodiments, the RNA-guided DNA-binding agent lacks cleavase and nickase activity. In some embodiments, the RNA-guided DNA-binding agent comprises a dCas DNA-binding polypeptide. A dCas polypeptide has DNA-binding activity while essentially lacking catalytic (cleavase/nickase) activity. In some embodiments, the dCas polypeptide is a dCas9 polypeptide. In some embodiments, the RNA-guided DNA-binding agent lacking cleavase and nickase activity or the dCas DNA-binding polypeptide is a version of a Cas nuclease (e.g., a Cas nuclease discussed above) in which its endonucleolytic active sites are inactivated, e.g., by one or more alterations (e.g., point mutations) in its catalytic domains. See, e.g., U.S. 2014/0186958 A1; U.S. 2015/0166980 A1.

[0532] In some embodiments, the RNA-guided DNA-binding agent comprises one or more heterologous functional domains (e.g., is or comprises a fusion polypeptide). In further embodiments, the heterologous functional domain may be an effector domain. When the RNA-guided DNA-binding agent is directed to its target sequence, e.g., when a Cas nuclease is directed to a target sequence by a gRNA, the effector domain may modify or affect the target sequence, In some embodiments, the effector domain may be chosen from a nucleic acid binding domain, a nuclease domain (e.g., a non-Cas nuclease domain), an epigenetic modification domain, a transcriptional activation domain, or a transcriptional repressor domain. In some embodiments, the heterologous functional domain is a nuclease, such as a FokI nuclease. See, e.g., U.S. Pat. No. 9,023,649. In some embodiments, the heterologous functional domain is a transcriptional activator or repressor. See, e.g., Qi et al., "Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression," Cell 152:1173-83 (2013); Perez-Pinera et al., "RNA-guided gene activation by CRISPR-Cas9-based transcription factors," Nat. Methods 10:973-6 (2013); Mali et al., "CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering," Nat. Biotechnol. 31:833-8 (2013); Gilbert et al., "CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes," Cell 154:442-51 (2013). As such, the RNA-guided DNA-binding agent essentially becomes a transcription factor that can be directed to bind a desired target sequence

using a guide RNA. In certain embodiments, the DNA modification domain is a methylation domain, such as a demethylation or methyltransferase domain. In certain embodiments, the effector domain is a DNA modification domain, such as a base-editing domain. In particular embodiments, the DNA modification domain is a nucleic acid editing domain that introduces a specific modification into the DNA, such as a deaminase domain. See, e.g., WO 2015/089406; U.S.

[0533] The nuclease may comprise at least one domain that interacts with a guide RNA ("gRNA"). Additionally, the nuclease may be directed to a target sequence by a gRNA. In Class 2 Cas nuclease systems, the gRNA interacts with the nuclease as well as the target sequence, such that it directs binding to the target sequence. In some embodiments, the gRNA provides the specificity for the targeted cleavage, and the nuclease may be universal and paired with different gRNAs to cleave different target sequences. In some embodiments of the present disclosure, the cargo for the LNMP formulation includes at least one gRNA. The gRNA may guide the Cas nuclease or Class 2 Cas nuclease to a target sequence on a target nucleic acid molecule. In some embodiments, a gRNA binds with and provides specificity of cleavage by a Class 2 Cas nuclease. In some embodiments, the gRNA and the Cas nuclease may form a ribonucleoprotein (RNP), e.g., a CRISPR/Cas complex such as a CRISPR/Cas9 complex which may be delivered by the LNMP composition. In some embodiments, the CRISPR/Cas complex may be a Type- II CRISPR/Cas9 complex. In some embodiments, the CRISPR/Cas complex may be a Type-V CRISPR/Cas complex, such as a Cpf1/guide RNA complex. Cas nucleases and cognate gRNAs may be paired. The gRNA scaffold structures that pair with each Class 2 Cas nuclease vary with the specific CRISPR/Cas system.

[0534] "Guide RNA", "gRNA", and simply "guide" are used herein interchangeably to refer to either a crRNA (also known as CRISPR RNA), or the combination of a CTRNA and a trRNA (also known as tracrRNA). The crRNA and trRNA may be associated as a single RNA molecule (single guide RNA, sgRNA) or in two separate RNA molecules (dual guide RNA, dgRNA). "Guide RNA" or "gRNA" refers to each type. The trRNA may be a naturally-occurring sequence, or a CrRNA sequence with modifications or variations compared to naturally-occurring sequences. As used herein, a "guide sequence" refers to a sequence within a guide RNA that is complementary to a target sequence and functions to direct a guide RNA to a target sequence for binding or modification (e.g., cleavage) by an RNA-guided DNA binding agent. A "guide sequence" may also be referred to as a "targeting sequence," or a "spacer sequence." A guide sequence can be 20 base pairs in length, e.g., in the case of Streptococcus pyogenes (i.e., Spy Cas9) and related Cas9 homologs/orthologs. Shorter or longer sequences can also be used as guides, e.g., 15-, 16-, 17-, 18-, 19-, 21-, 22-, 23-, 24-, or 25-nucleotides in length. In some embodiments, the target sequence is in a gene or on a chromosome, for example, and is complementary to the guide sequence. In some embodiments, the degree of complementarity or identity between a guide sequence and its corresponding target sequence may be about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%. In some embodiments, the guide sequence and the target region may be 100% complementary or identical. In other embodiments, the guide sequence and the target region may contain at least one mismatch. For example, the guide sequence and the target sequence may contain 1, 2, 3, or 4 mismatches, where the total length of the target sequence is at least 17, 18, 19, 20 or more base pairs. In some embodiments, the guide

sequence and the target region may contain 1-4 mismatches where the guide sequence comprises at least 17, 18, 19, 20 or more nucleotides. In some embodiments, the guide sequence and the target region may contain 1, 2, 3, or 4 mismatches where the guide sequence comprises 20 nucleotides.

[0535] Target sequences for Cas proteins include both the positive and negative strands of genomic DNA (i.e., the sequence given and the sequence's reverse compliment), as a nucleic acid substrate for a Cas protein is a double stranded nucleic acid. Accordingly, where a guide sequence is said to be "complementary to a target sequence", it is to be understood that the guide sequence may direct a guide RNA to bind to the reverse complement of a target sequence. Thus, in some embodiments, where the guide sequence binds the reverse complement of a target sequence, the guide sequence is identical to certain nucleotides of the target sequence (e.g., the target sequence not including the PAM) except for the substitution of U for T in the guide sequence. The length of the targeting sequence may depend on the CRISPR/Cas system and components used. For example, different Class 2 Cas nucleases from different bacterial species have varying optimal targeting sequence lengths. Accordingly, the targeting sequence may comprise 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21,22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, or more than 50 nucleotides in length. In some embodiments, the targeting sequence length is 0, 1, 2, 3, 4, or 5 nucleotides longer or shorter than the guide sequence of a naturally-occurring CRISPR/Cas system. In certain embodiments, the Cas nuclease and gRNA scaffold will be derived from the same CRISPR/Cas system. In some embodiments, the targeting sequence may comprise or consist of 18-24 nucleotides. In some embodiments, the targeting sequence may comprise or consist of 19-21 nucleotides. In some embodiments, the targeting sequence may comprise or consist of 20 nucleotides.

[0536] In some embodiments, the sgRNA is a "Cas9 sgRNA" capable of mediating RNA-guided DNA cleavage by a Cas9 protein. In certain embodiments, the gRNA comprises a crRNA and tracr RNA sufficient for forming an active complex with a Cas9 protein and mediating RNA-guided DNA cleavage. In certain embodiments, the gRNA comprises a crRNA sufficient for forming an active complex with a Cpf1 protein and mediating RNA-guided DNA cleavage. See Zetsche 2015. Certain embodiments of the invention also provide nucleic acids, e.g., expression cassettes, encoding the gRNA described herein. A "guide RNA nucleic acid" is used herein to refer to a guide UNA (e.g. an sgRNA or a dgRNA) and a guide RNA expression cassette, which is a nucleic acid that encodes one or more guide RNAs.

[0537] In some embodiments, the nucleic acid may comprise a nucleotide sequence encoding a crRNA. In some embodiments, the nucleotide sequence encoding the crRNA comprises a targeting sequence flanked by all or a portion of a repeat sequence from a naturally-occurring CRISPR/Cas system. In some embodiments, the nucleic acid may comprise a nucleotide sequence encoding a tracr RNA. In some embodiments, the crRNA and the tracr RNA may be encoded by two separate nucleic acids. In other embodiments, the crRNA and the tracr RNA may be encoded by a single nucleic acid. In some embodiments, the crRNA and the tracr RNA may be encoded by opposite strands of a single nucleic acid. In other embodiments, the crRNA and the tracr RNA may be encoded by the same strand of a single nucleic acid. In some embodiments, the gRNA nucleic acid encodes an sgRNA. In some embodiments, the gRNA nucleic acid encodes a Cas9 nuclease sgRNA. In come

embodiments, the gRNA nucleic acid encodes a Cpf1 nuclease sgRNA.

[0538] The nucleotide sequence encoding the guide RNA may be operabiy linked to at least one transcriptional or regulatory control sequence, such as a promoter, a 3' UTR, or a 5' UTR. In one example, the promoter may be a tRNA promoter, e.g., $tRNA^{Lys3}$, or a tRNA chimera. See Mefferd et al., RNA. 201521:1683-9; Scherer et al., Nucleic Acids Res. 2007 35: 2620-2628. In certain embodiments, the promoter may be recognized by RNA polymerase III (Pol III). Non-limiting examples of Pol III promoters also include U6 and H1 promoters. In some embodiments, the nucleotide sequence encoding the guide RNA may be operably linked to a mouse or human U6 promoter. In some embodiments, the gRNA nucleic acid is a modified nucleic acid. In certain embodiments, the gRNA nucleic acid includes a modified nucleoside or nucleotide. In some embodiments, the gRNA nucleic acid includes a 5' end modification, for example a modified nucleoside or nucleotide io stabilize and prevent integration of the nucleic acid. In some embodiments, the gRNA nucleic acid comprises a double-stranded DNA having a 5' end modification on each strand. In certain embodiments, the gRNA nucleic acid includes an inverted dideoxy-T or an inverted abasic nucleoside or nucleotide as the 5' end modification. In some embodiments, gRNA nucleic acid includes a label such as biotin, desthiobioten-TEG, digoxigenin, and fluorescent markers, including, for example, FAM, ROX, TAMRA, and AlexaFluor. In certain embodiments, more than one gRNA nucleic acid, such as a gRNA, can be used with a CRISPR/Cas nuclease system. Each gRNA nucleic acid may contain a different targeting sequence, such that the CRISPR/Cas system cleaves more than one target sequence. In some embodiments, one or more gRNAs may have the same or differing properties such as activity or stability within a CRISPR/Cas complex. Where more than one gRNA is used, each gRNA can be encoded on the same or on different gRNA nucleic acid. The promoters used to drive expression of the more than one gRNA may be the same or different.

[0539] In some embodiments, the RNA composition or formulation disclosed herein comprises an mRNA comprising an open reading frame (ORF) encoding an RNA-guided DNA binding agent, such as a Cas nuclease, or Class 2 Cas nuclease as described herein. In some embodiments, an mRNA comprising an ORF encoding an RNA-guided DNA binding agent, such as a Cas nuclease or Class 2 Cas nuclease, is provided, used, or administered. In some embodiments, the ORF encoding an RNA-guided DNA binding agent is a "modified RNA-guided DNA binding agent ORF" or simply a "modified ORF," which is used as shorthand to indicate that the ORF is modified in one or more of the following ways: (1) the modified ORF has a uridine content ranging from its minimum uridine content to 150% of the minimum uridine content; (2) the modified ORF has a uridine dinucleotide content ranging from its minimum uridine dinucleotide content to 150% of the minimum uridine dinucleotide content; (3) the modified ORF consists of a set of codons of which at least 75% of the codons are minimal uridine codon(s) for a given amino acid, e.g. the codon(s) with the fewest uridines (usually 0 or 1 except for a codon for phenylalanine, where the minimal uridine codon has 2 uridines); or (4) the modified ORF comprises at least one modified uridine. In some embodiments, the modified ORF is modified in at least two, three, or four of the foregoing ways. In some embodiments, the modified ORF comprises at least one modified uridine and is modified in at least one, two, three, or all of (1)-(3) above. "Modified uridine" is used herein to refer to a nucleoside other than thymidine with the same hydrogen bond

acceptors as uridine and one or more structural differences from uridine. In some embodiments, a modified uridine is a substituted uridine, i.e., a uridine in which one or more non-proton substituents (e.g., alkoxy, such as methoxy) takes the place of a proton. In some embodiments, a modified uridine is pseudouridine. In some embodiments, a modified uridine is a substituted pseudouridine, i.e., a pseudouridine in which one or more non-proton substituents (e.g., alkyl, such as methyl) takes the place of a proton. In some embodiments, a modified uridine is any of a substituted uridine, pseudouridine, or a substituted pseudouridine.

[0540] "Uridine position" as used herein refers to a position in a polynucleotide occupied by a uridine or a modified uridine. Thus, for example, a polynucleotide in which "100% of the uridine positions are modified uridines" contains a modified uridine at every position that would be a uridine in a conventional RNA (where all bases are standard A, U, C, or G bases) of the same sequence. Unless otherwise indicated, a U in a polynucleotide sequence of a sequence listing in, or accompanying, this disclosure can be a uridine or a modified uridine.

[0541] A cap can be included co-transcriptionally. For example, ARCA (anti-reverse cap analog; Thermo Fisher Scientific Cat. No. AM8045) is a cap analog comprising a 7- methylguanine 3'-methoxy-5'-triphosphate linked to the 5' position of a guanine ribonucleotide which can be incorporated in vitro into a transcript at initiation. ARCA results in a Cap0 cap in which the 2' position of the first cap-proximal nucleotide is hydroxy). See, e.g., Stepinski et al., (2001) "Synthesis and properties of mRNAs containing the novel 'anti- reverse' cap analogs 7-methyl(3'-O-methyl)GpppG and 7-methyl(3'deoxy)GpppG," RNA 7: 1486-1495.

[0542] CleanCap™ AG (m7G(5')ppp(5')(2'OMeA)pG; TriLink Biotechnologies Cat. No. N-7113) or CleanCap™ GG (m7G(5')ppp(5')(2'OMeG)pG; TriLink Biotechnologies Cat. No. N-7133) can be used to provide a Cap1 structure co-transcriptionally. 3'-O-methylated versions of CleanCap™ AG and CleanCap™ GG are also available from TriLink Biotechnologies as Cat. Nos. N-7413 and N-7433, respectively.

[0543] In some embodiments, the LNMPs described herein may include gene editing composition sequences as described in WO2019067992, WO2019090173, WO2019090175, WO2022159741, WO2022150608, WO2016201155, WO2022086846, WO2015153789, WO2017165826, WO2016154596, WO2018209158, WO2021050512, WO2021113494, WO2022229851, WO2020264254, each of which is incorporated by reference herein in its entirety.

RNA composition

[0544] The LNP / RNA composition or LNMP / RNA composition comprises one or more polynucleotides (e.g., mRNA, gRNA) encoding one or more gene editing systems for multiple purposes (e.g. therapeutic). The one or more polynucleotides (e.g., mRNA, gRNA) encode one or more gene editing systems.

[0545] The term “identity” refers to a relationship between the sequences of two or more polypeptides (e.g. antigens, anti-cancer proteins or signaling proteins) or polynucleotides (nucleic acids), as determined by comparing the sequences. Identity also refers to the degree of sequence relatedness between or among sequences as determined by the number of matches between strings

of two or more amino acid residues or nucleic acid residues. Identity measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (e.g., “algorithms”). Identity of related antigens, proteins, or nucleic acids can be readily calculated by known methods. “Percent (%) identity” as it applies to polypeptide or polynucleotide sequences is defined as the percentage of residues (amino acid residues or nucleic acid residues) in the candidate amino acid or nucleic acid sequence that are identical with the residues in the amino acid sequence or nucleic acid sequence of a second sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent identity. Methods and computer programs for the alignment are well known in the art. It is understood that identity depends on a calculation of percent identity but may differ in value due to gaps and penalties introduced in the calculation. Generally, variants of a particular polynucleotide or polypeptide (e.g., antigen, anti-cancer protein, or signaling protein) have at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% but less than 100% sequence identity to that particular reference polynucleotide or polypeptide as determined by sequence alignment programs and parameters described herein and known to those skilled in the art. Such tools for alignment include those of the BLAST suite (Stephen F. Altschul, et al (1997), “Gapped BLAST and PSI-BLAST: a new generation of protein database search programs”, Nucleic Acids Res. 25:3389-3402). Another popular local alignment technique is based on the Smith-Waterman algorithm (Smith, T.F. & Waterman, M.S. (1981) “Identification of common molecular subsequences.” J. Mol. Biol. 147:195-197). A general global alignment technique based on dynamic programming is the Needleman-Wunsch algorithm (Needleman, S.B. & Wunsch, C.D. (1970) “A general method applicable to the search for similarities in the amino acid sequences of two proteins.” J. Mol. Biol. 48:443-453). More recently, a Fast Optimal Global Sequence Alignment Algorithm (FOGSAA) has been developed that purportedly produces global alignment of nucleotide and protein sequences faster than other optimal global alignment methods, including the Needleman-Wunsch algorithm.

[0546] As such, polynucleotides encoding peptides or polypeptides containing substitutions, insertions and/or additions, deletions, and covalent modifications with respect to reference sequences, in particular the polypeptide sequences disclosed herein, are included within the scope of this disclosure. For example, sequence tags or amino acids, such as one or more lysines, can be added to peptide sequences (e.g., at the N-terminal or C-terminal ends). Sequence tags can be used for peptide detection, purification, or localization. Lysines can be used to increase peptide solubility or to allow for biotinylation. Alternatively, amino acid residues located at the carboxy and amino terminal regions of the amino acid sequence of a peptide or protein may optionally be deleted providing for truncated sequences. Certain amino acids (e.g., C-terminal or N-terminal residues) may alternatively be deleted depending on the use of the sequence, as for example, expression of the sequence as part of a larger sequence that is soluble or linked to a solid support. In some embodiments, sequences for (or encoding) signal sequences, termination sequences, transmembrane domains, linkers, multimerization domains (such as, e.g., foldon regions) and the like may be substituted with alternative sequences that achieve the same or a similar function. In some embodiments, cavities in

the core of proteins can be filled to improve stability, e.g., by introducing larger amino acids. In other embodiments, buried hydrogen bond networks may be replaced with hydrophobic resides to improve stability. In yet other embodiments, glycosylation sites may be removed and replaced with appropriate residues. Such sequences are readily identifiable to one of skill in the art. It should also be understood that some of the sequences provided herein contain sequence tags or terminal peptide sequences (e.g., at the N-terminal or C-terminal ends) that may be deleted, for example, prior to use in the preparation of an RNA (e.g., mRNA) vaccine.

[0547] In some embodiments, the polynucleotide is an mRNA. Messenger RNA (mRNA) is any RNA that encodes a (at least one) protein (a naturally- occurring, non-naturally-occurring, or modified polymer of amino acids) and can be translated to produce the encoded protein in vitro, in vivo, in situ, or ex vivo. The skilled artisan will appreciate that, except where otherwise noted, nucleic acid sequences set forth in the instant application may recite “T”s in a representative DNA sequence but where the sequence represents RNA (e.g., mRNA), the “T”s would be substituted for “U”s. Thus, any of the DNAs disclosed and identified by a particular sequence identification number herein also disclose the corresponding RNA (e.g., mRNA) sequence complementary to the DNA, where each “T” of the DNA sequence is substituted with “U.”

[0548] In some embodiments, the polynucleotide (e.g., mRNA) has an open reading frame (ORF) encoding one or more gene editing system. In some embodiments, the polynucleotide (e.g., mRNA) has an open reading frame (ORF) encoding an one or more polypeptide. An open reading frame (ORF) is a continuous stretch of DNA or RNA beginning with a start codon (e.g., methionine (ATG or AUG)) and ending with a stop codon (e.g., TAA, TAG or TGA, or UAA, UAG or UGA). An ORF typically encodes a protein. The sequences may further comprise additional elements, e.g., 5' and 3' UTRs.

[0549] In some embodiments, the RNA (e.g., mRNA) further comprises a 5' UTR, 3' UTR, a poly(A) tail and/or a 5' cap analog.

[0550] In some embodiments, the mRNA comprises a 5' untranslated region (UTR) and/or a 3' UTR.

[0551] In some embodiments, the mRNA is derived from (a) a DNA molecule; or (b) an RNA molecule. In the mRNA, T is optionally substituted with U.

[0552] In some embodiments, the mRNA is derived from a DNA molecule. The DNA molecule can further comprise a promoter. In some embodiments, the promoter is a T7 promoter, a T3 promoter, or an SP6 promoter. In some embodiments, the promoter is located at the 5’ UTR.

[0553] In some embodiments, the mRNA is an RNA molecule. The RNA molecule may be a self-replicating RNA molecule.

[0554] In some embodiments, the mRNA is an RNA molecule. The RNA molecule may further comprise a 5’ cap. The 5’ cap can have a Cap 1 structure, a Cap 1 (m6A) structure, a Cap 2 structure, a Cap 3 structure, a Cap 0 structure, or any combination thereof.

[0555] In some embodiments, the polynucleotide is an mRNA which encodes an IL-2 molecule. In one embodiment, the IL-2 molecule comprises a naturally occurring IL-2 molecule, a fragment of a naturally occurring IL-2 molecule, or a variant thereof. In one embodiment, the IL-2 molecule comprises a variant of a naturally occurring IL-2 molecule (e.g., an IL-2 variant, e.g., as described

herein), or a fragment thereof.

[0556] In one embodiment, the polynucleotide is an mRNA which encodes an IL-2 molecule comprising an amino acid sequence having at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identity to the amino acid sequence of an IL-2 molecule provided in any one of Tables I-III.

[0557] In some embodiments, the mRNA comprises a 5' untranslated region (UTR) and/or a 3' UTR.

[0558] In some embodiments, the mRNA comprises a 5' UTR. The 5' UTR may comprise a Kozak sequence.

[0559] In some embodiments, the mRNA comprises a 3' UTR. In some embodiments, the 3' UTR comprises one or more sequences derived from an amino-terminal enhancer of split (AES). In some embodiments, the 3' UTR comprises a sequence derived from mitochondrially encoded 12S rRNA (mtRNRl).

[0560] In some embodiments, the mRNA comprises a poly(A) sequence. In one embodiment, the poly(A) sequence is a 110-nucleotide sequence consisting of a sequence of 30 adenosine residues, a 10-nucleotide linker sequence, and a sequence of 70 adenosine residues.

Stabilizing Elements

[0561] Naturally occurring eukaryotic mRNA molecules can contain stabilizing elements, including, but not limited to untranslated regions (UTR) at their 5 '-end (5' UTR) and/or at their 3 '-end (3' UTR), in addition to other structural features, such as a 5 '-cap structure or a 3'-poly(A) tail. Both the 5' UTR and the 3' UTR are typically transcribed from the genomic DNA and are elements of the premature mRNA. Characteristic structural features of mature mRNA, such as the 5 '-cap and the 3'-poly(A) tail are usually added to the transcribed (premature) mRNA during mRNA processing.

[0562] In some embodiments, the polynucleotide has an open reading frame encoding at least one gene editing system having at least one modification, at least one 5' terminal cap, and is formulated within a lipid nanoparticle. 5 '-capping of polynucleotides may be completed concomitantly during the in vitro-transcription reaction using the following chemical RNA cap analogs to generate the 5'-guanosine cap structure according to manufacturer protocols: 3'-O-Me-m7G(5')ppp(5') G [the ARCA cap];G(5')ppp(5')A; G(5')ppp(5')G; m7G(5')ppp(5')A; m7G(5')ppp(5')G (New England BioLabs, Ipswich, MA). 5'- capping of modified RNA may be completed post-transcriptionally using a Vaccinia Vims Capping Enzyme to generate the “Cap 0” structure: m7G(5')ppp(5')G (New England BioLabs, Ipswich, MA). Cap 1 structure may be generated using both Vaccinia Vims Capping Enzyme and a 2'-0 methyl-transferase to generate m7G(5')ppp(5')G-2 '-O-methyl. Cap 2 structure may be generated from the Cap 1 stmcture followed by the 2'-0-methylation of the 5'- antepenultimate nucleotide using a 2'-O methyl-transferase. Cap 3 structure may be generated from the Cap 2 stmcture followed by the 2'-O-methylation of the 5'-preantepenultimate nucleotide using a 2'-O methyl-transferase. Enzymes may be derived from a recombinant source.

[0563] The 3 '-poly(A) tail is typically a stretch of adenine nucleotides added to the 3 '-end of the transcribed mRNA. It can, in some instances, comprise up to about 400 adenine nucleotides. In some embodiments, the length of the 3'-poly(A) tail may be an essential element with respect to the stability of the individual mRNA.

[0564] In some embodiments, the polynucleotide includes a stabilizing element. Stabilizing elements may include for instance a histone stem-loop. A stem-loop binding protein (SLBP), a 32 kDa protein has been identified. It is associated with the histone stem- loop at the 3 '-end of the histone messages in both the nucleus and the cytoplasm. Its expression level is regulated by the cell cycle; it peaks during the S-phase, when histone mRNA levels are also elevated. The protein has been shown to be essential for efficient 3 '-end processing of histone pre-mRNA by the U7 snRNP. SLBP continues to be associated with the stem-loop after processing, and then stimulates the translation of mature histone mRNAs into histone proteins in the cytoplasm. The RNA binding domain of SLBP is conserved through metazoa and protozoa; its binding to the histone stem-loop depends on the structure of the loop. The minimum binding site includes at least three nucleotides 5' and two nucleotides 3' relative to the stem- loop.

[0565] In some embodiments, the polynucleotide (e.g., mRNA) includes a coding region, at least one histone stem-loop, and optionally, a poly(A) sequence or polyadenylation signal. The poly(A) sequence or polyadenylation signal generally should enhance the expression level of the encoded protein. The encoded protein, in some embodiments, is not a histone protein, a reporter protein (e.g. Luciferase, GFP, EGFP, b-Galactosidase, EGFP), or a marker or selection protein (e.g. alpha-Globin, Galactokinase and Xanthine:guanine phosphoribosyl transferase (GPT)).

[0566] In some embodiments, the polynucleotide (e.g., mRNA) includes the combination of a poly(A) sequence or polyadenylation signal and at least one histone stem-loop, even though both represent alternative mechanisms in nature, acts synergistically to increase the protein expression beyond the level observed with either of the individual elements. The synergistic effect of the combination of poly(A) and at least one histone stem-loop does not depend on the order of the elements or the length of the poly(A) sequence. In some embodiments, an RNA (e.g., mRNA) does not include a histone downstream element (HDE). “Histone downstream element” (HDE) includes a purine-rich polynucleotide stretch of approximately 15 to 20 nucleotides 3' of naturally occurring stem-loops, representing the binding site for the U7 snRNA, which is involved in processing of histone pre-mRNA into mature histone mRNA. In some embodiments, the nucleic acid does not include an intron.

[0567] The polynucleotide (e.g., mRNA) may or may not contain an enhancer and/or promoter sequence, which may be modified or unmodified or which may be activated or inactivated. In some embodiments, the histone stem-loop is generally derived from histone genes and includes an intramolecular base pairing of two neighbored partially or entirely reverse complementary sequences separated by a spacer, consisting of a short sequence, which forms the loop of the structure. The unpaired loop region is typically unable to base pair with either of the stem loop elements. It occurs more often in RNA, as is a key component of many RNA secondary structures but may be present in single- stranded DNA as well. Stability of the stem-loop structure generally depends on the length, number of mismatches or bulges, and base composition of the paired region. In some embodiments, wobble base pairing (non-Watson-Crick base pairing) may result. In some embodiments, the at least one histone stem-loop sequence comprises a length of 15 to 45 nucleotides.

[0568] In some embodiments, the polynucleotide (e.g., mRNA) has one or more AU-rich sequences removed. These sequences, sometimes referred to as AURES are destabilizing sequences found in

the 3'UTR. The AURES may be removed from the RNA vaccines. Alternatively, the AURES may remain in the RNA vaccine.

Signal Peptides

[0569] In some embodiments, the polynucleotide (e.g., mRNA) has an ORF that encodes a signal peptide fused to the antigen. Signal peptides, comprising the N- terminal 15-60 amino acids of proteins, are typically needed for the translocation across the membrane on the secretory pathway and, thus, universally control the entry of most proteins both in eukaryotes and in prokaryotes to the secretory pathway. In eukaryotes, the signal peptide of a nascent precursor protein (pre-protein) directs the ribosome to the rough endoplasmic reticulum (ER) membrane and initiates the transport of the growing peptide chain across it for processing. ER processing produces mature proteins, wherein the signal peptide is cleaved from precursor proteins, typically by an ER-resident signal peptidase of the host cell, or they remain uncleaved and function as a membrane anchor. A signal peptide may also facilitate the targeting of the protein to the cell membrane. A signal peptide may have a length of 15-60 amino acids. For example, a signal peptide may have a length of 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 amino acids. In some embodiments, a signal peptide has a length of 20-60, 25-60, 30-60, 35- 60, 40-60, 45- 60, 50-60, 55-60, 15-55, 20-55, 25-55, 30-55, 35-55, 40-55, 45-55, 50-55, 15-50, 20-50, 25-50, 30-50, 35-50, 40-50, 45-50, 15-45, 20-45, 25-45, 30-45, 35-45, 40-45, 15-40, 20- 40, 25-40, 30-40, 35-40, 15-35, 20-35, 25-35, 30-35, 15-30, 20-30, 25-30, 15-25, 20-25, or 15-20 amino acids.

[0570] Signal peptides from heterologous genes (which regulate expression of genes other than coronavirus antigens in nature) are known in the art and can be tested for desired properties and then incorporated into a nucleic acid of the disclosure. In some embodiments, the signal peptide may comprise those described in WO 2021/154763, which is incorporated by reference in its entirety.

Fusion Proteins

[0571] In some embodiments, the polynucleotide (e.g., mRNA) encodes an antigenic fusion protein. Thus, the encoded antigen or antigens may include two or more proteins (e.g., protein and/or protein fragment) joined together. Alternatively, the protein to which a protein antigen is fused does not promote a strong immune response to itself, but rather to the coronavirus antigen. Antigenic fusion proteins, in some embodiments, retain the functional property from each original protein.

Scaffold Moieties

[0572] The polynucleotide (e.g., mRNA), in some embodiments, encodes fusion proteins that comprise coronavirus antigens linked to scaffold moieties. In some embodiments, such scaffold moieties impart desired properties to an antigen encoded by a nucleic acid of the disclosure. For example, scaffold proteins may improve the immunogenicity of an antigen, e.g., by altering the structure of the antigen, altering the uptake and processing of the antigen, and/or causing the antigen to bind to a binding partner.

[0573] In some embodiments, the scaffold moiety is protein that can self-assemble into protein nanoparticles that are highly symmetric, stable, and structurally organized, with diameters of 10- 150 nm, a highly suitable size range for optimal interactions with various cells of the immune system.

[0574] In some embodiments, bacterial protein platforms may be used. Non-limiting examples of these self-assembling proteins include ferritin, lumazine and encapsulin.

[0575] Ferritin is a protein whose main function is intracellular iron storage. Ferritin is made of 24 subunits, each composed of a four-alpha-helix bundle, that self-assemble in a quaternary structure with octahedral symmetry (Cho K.J. et al. J Mol Biol. 2009; 390:83-98). Several high- resolution structures of ferritin have been determined, confirming that Helicobacter pylori ferritin is made of 24 identical protomers, whereas in animals, there are ferritin light and heavy chains that can assemble alone or combine with different ratios into particles of 24 subunits (Granier T. et al. J Biol Inorg Chem. 2003; 8:105-111; Fawson D.M. et al. Nature. 1991; 349:541-544). Ferritin self-assembles into nanoparticles with robust thermal and chemical stability. Thus, the ferritin nanoparticle is well suited to carry and expose antigens.

[0576] Fumazine synthase (FS) is also well suited as a nanoparticle platform for antigen display. FS, which is responsible for the penultimate catalytic step in the biosynthesis of riboflavin, is an enzyme present in a broad variety of organisms, including archaea, bacteria, fungi, plants, and eubacteria (Weber S.E. Flavins and Flavoproteins. Methods and Protocols, Series: Methods in Molecular Biology. 2014). The FS monomer is 150 amino acids long, and consists of beta- sheets along with tandem alpha-helices flanking its sides. A number of different quaternary structures have been reported for FS, illustrating its morphological versatility: from homopentamers up to symmetrical assemblies of 12 pentamers forming capsids of 150 A diameter. Even FS cages of more than 100 subunits have been described (Zhang X. et al. J Mol Biol. 2006; 362:753-770).

[0577] Encapsulin, a novel protein cage nanoparticle isolated from thermophile Thermotoga maritima, may also be used as a platform to present antigens on the surface of self-assembling nanoparticles. Encapsulin is assembled from 60 copies of identical 31 kDa monomers having a thin and icosahedral T = 1 symmetric cage structure with interior and exterior diameters of 20 and 24 nm, respectively (Sutter M. et al. Nat Struct Mol Biol. 2008, 15: 939-947). Although the exact function of encapsulin in T. maritima is not clearly understood yet, its crystal structure has been recently solved and its function was postulated as a cellular compartment that encapsulates proteins such as DyP (Dye decolorizing peroxidase) and Flp (Ferritin like protein), which are involved in oxidative stress responses (Rahmanpour R. et al. FEBS J. 2013, 280: 2097-2104).

[0578] In some embodiments, the polynucleotide encodes a coronavirus antigen (e.g., SARS-CoV-2 S protein) fused to a foldon domain. The foldon domain may be, for example, obtained from bacteriophage T4 fibritin (see, e.g., Tao Y, et al. Structure. 1997 Jun 15; 5(6):789-98).

Linkers and Cleavable Peptides

[0579] In some embodiments, the polynucleotide (e.g., mRNA) encodes more than one polypeptide, referred to herein as fusion proteins. In some embodiments, the mRNA further encodes a linker located between at least one or each domain of the fusion protein. The linker can be, for example, a

cleavable linker or protease-sensitive linker. In some embodiments, the linker is selected from the group consisting of F2A linker, P2A linker, T2A linker, E2A linker, and combinations thereof. This family of self-cleaving peptide linkers, referred to as 2 A peptides, has been described in the art (see for example, Kim, J.H. et al. (2011) PLoS ONE 6:el8556). In some embodiments, the linker is an F2A linker. In some embodiments, the fusion protein contains three domains with intervening linkers, having the structure: domain-linker-domain-linker-domain.

[0580] Cleavable linkers known in the art may be used in connection with the disclosure. Exemplary such linkers include F2A linkers, T2A linkers, P2A linkers, E2A linkers (See, e.g., WO2017/127750, which is incorporated herein by reference in its entirety). The skilled artisan will appreciate that other art-recognized linkers may be suitable for use in the constructs of the disclosure (e.g., encoded by the nucleic acids of the disclosure). The skilled artisan will likewise appreciate that other polycistronic constructs (mRNA encoding more than one antigen/polypeptide separately within the same molecule) may be suitable for use as provided herein.

Sequence Optimization

[0581] In some embodiments, an ORF encoding one or more polypeptides of the disclosure is codon optimized. Codon optimization methods are known in the art. For example, an ORF of any one or more of the sequences provided herein may be codon optimized. Codon optimization, in some embodiments, may be used to match codon frequencies in target and host organisms to ensure proper folding; bias GC content to increase mRNA stability or reduce secondary structures; minimize tandem repeat codons or base runs that may impair gene construction or expression; customize transcriptional and translational control regions; insert or remove protein trafficking sequences; remove/add post translation modification sites in encoded protein (e.g., glycosylation sites); add, remove or shuffle protein domains; insert or delete restriction sites; modify ribosome binding sites and mRNA degradation sites; adjust translational rates to allow the various domains of the protein to fold properly; or reduce or eliminate problem secondary structures within the polynucleotide. Codon optimization tools, algorithms and services are known in the art - non-limiting examples include services from GeneArt (Life Technologies), DNA2.0 (Menlo Park CA) and/or proprietary methods. In some embodiments, the open reading frame (ORF) sequence is optimized using optimization algorithms.

[0582] In some embodiments, a codon optimized sequence shares less than 95% sequence identity to a naturally-occurring or wild-type sequence ORF (e.g., a naturally-occurring or wild- type mRNA sequence encoding a coronavirus antigen). In some embodiments, a codon optimized sequence shares less than 90% sequence identity to a naturally occurring or wild-type sequence (e.g., a naturally occurring or wild-type mRNA sequence encoding a coronavirus antigen). In some embodiments, a codon optimized sequence shares less than 85% sequence identity to a naturally occurring or wild-type sequence (e.g., a naturally occurring or wild-type mRNA sequence encoding a coronavirus antigen). In some embodiments, a codon optimized sequence shares less than 80% sequence identity to a naturally occurring or wild-type sequence (e.g., a naturally occurring or wild-type mRNA sequence encoding a coronavirus antigen). In some embodiments, a codon optimized

sequence shares less than 75% sequence identity to a naturally occurring or wild-type sequence (e.g., a naturally occurring or wild-type mRNA sequence encoding a coronavirus antigen).

[0583] In some embodiments, a codon optimized sequence shares between 65% and 85% (e.g., between about 67% and about 85% or between about 67% and about 80%) sequence identity to a naturally occurring or wild-type sequence (e.g., a naturally occurring or wild-type mRNA sequence encoding a coronavirus antigen). In some embodiments, a codon optimized sequence shares between 65% and 75% or about 80% sequence identity to a naturally occurring or wild- type sequence (e.g., a naturally occurring or wild-type mRNA sequence encoding a coronavirus antigen).

[0584] In some embodiments, a codon-optimized sequence encodes an antigen that is as immunogenic as, or more immunogenic than (e.g., at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 100%, or at least 200% more), a coronavirus antigen encoded by a non-codon-optimized sequence. When transfected into mammalian host cells, the modified mRNAs have a stability of between 12-18 hours, or greater than 18 hours, e.g., 24, 36, 48, 60, 72, or greater than 72 hours and are capable of being expressed by the mammalian host cells.

[0585] In some embodiments, a codon optimized RNA may be one in which the levels of G/C are enhanced. The G/C-content of nucleic acid molecules (e.g., mRNA) may influence the stability of the RNA. RNA having an increased amount of guanine (G) and/or cytosine (C) residues may be functionally more stable than RNA containing a large amount of adenine (A) and thymine (T) or uracil (U) nucleotides. As an example, WO02/098443 discloses a pharmaceutical composition containing an mRNA stabilized by sequence modifications in the translated region. Due to the degeneracy of the genetic code, the modifications work by substituting existing codons for those that promote greater RNA stability without changing the resulting amino acid. The approach is limited to coding regions of the RNA.

Chemically Unmodified Nucleotides

[0586] In some embodiments, the polynucleotide (e.g., mRNA) is not chemically modified and comprises the standard ribonucleotides consisting of adenosine, guanosine, cytosine and uridine. In some embodiments, nucleotides and nucleosides of the polynucleotide (e.g., mRNA) comprise standard nucleoside residues such as those present in transcribed RNA (e.g. A, G, C, or U). In some embodiments, nucleotides and nucleosides of the polynucleotide (e.g., mRNA) comprise standard deoxyribonucleosides such as those present in DNA (e.g. dA, dG, dC, or dT).

Chemical Modifications

[0587] The polynucleotide (e.g., mRNA) comprises, in some embodiments, an RNA having an open reading frame encoding a coronavirus antigen, wherein the nucleic acid comprises nucleotides and/or nucleosides that can be standard (unmodified) or modified as is known in the art. In some embodiments, nucleotides and nucleosides of the polynucleotide (e.g., mRNA) comprise modified nucleotides or nucleosides. Such modified nucleotides and nucleosides can be naturally occurring modified nucleotides and nucleosides or non-naturally occurring modified nucleotides and nucleosides. Such modifications can include those at the sugar, backbone, or nucleobase portion of the nucleotide and/or nucleoside as are recognized in the art.

[0588] The nucleic acids of the polynucleotide (e.g., mRNA) can comprise standard nucleotides and nucleosides, naturally- occurring nucleotides and nucleosides, non-naturally-occurring nucleotides and nucleosides, or any combination thereof.

[0589] Nucleic acids of the polynucleotide (e.g., DNA nucleic acids and RNA nucleic acids, such as mRNA nucleic acids), in some embodiments, comprise various (more than one) different types of standard and/or modified nucleotides and nucleosides. In some embodiments, a particular region of a nucleic acid contains one, two or more (optionally different) types of standard and/or modified nucleotides and nucleosides.

[0590] In some embodiments, a modified RNA nucleic acid (e.g., a modified mRNA nucleic acid), introduced to a cell or organism, exhibits reduced degradation in the cell or organism, respectively, relative to an unmodified nucleic acid comprising standard nucleotides and nucleosides.

[0591] In some embodiments, a modified RNA nucleic acid (e.g., a modified mRNA nucleic acid), introduced into a cell or organism, may exhibit reduced immunogenicity in the cell or organism, respectively (e.g., a reduced innate response) relative to an unmodified nucleic acid comprising standard nucleotides and nucleosides.

[0592] Nucleic acids (e.g., RNA nucleic acids, such as mRNA nucleic acids), in some embodiments, comprise non-natural modified nucleotides that are introduced during synthesis or post-synthesis of the nucleic acids to achieve desired functions or properties. The modifications may be present on internucleotide linkages, purine or pyrimidine bases, or sugars. The modification may be introduced with chemical synthesis or with a polymerase enzyme at the terminal of a chain or anywhere else in the chain. Any of the regions of a nucleic acid may be chemically modified.

[0593] The present disclosure provides for modified nucleosides and nucleotides of a nucleic acid (e.g., RNA nucleic acids, such as mRNA nucleic acids). A “nucleoside” refers to a compound containing a sugar molecule (e.g., a pentose or ribose) or a derivative thereof in combination with an organic base (e.g., a purine or pyrimidine) or a derivative thereof (also referred to herein as “nucleobase”). A “nucleotide” refers to a nucleoside, including a phosphate group. Modified nucleotides may by synthesized by any useful method, such as, for example, chemically, enzymatically, or recombinantly, to include one or more modified or non-natural nucleosides. Nucleic acids can comprise a region or regions of linked nucleosides. Such regions may have variable backbone linkages. The linkages can be standard phosphodiester linkages, in which case the nucleic acids would comprise regions of nucleotides.

[0594] Modified nucleotide base pairing encompasses not only the standard adenosine-thymine, adenosine-uracil, or guanosine-cytosine base pairs, but also base pairs formed between nucleotides and/or modified nucleotides comprising non-standard or modified bases, wherein the arrangement of hydrogen bond donors and hydrogen bond acceptors permits hydrogen bonding between a non-standard base and a standard base or between two complementary non-standard base structures, such as, for example, in those nucleic acids having at least one chemical modification. One example of such non-standard base pairing is the base pairing between the modified nucleotide inosine and adenine, cytosine or uracil. Any combination of base/sugar or linker may be incorporated into nucleic acids of the present disclosure.

[0595] In some embodiments, the polynucleotide (e.g., mRNA) comprises uridine at one or more or all uridine positions of the nucleic acid. In some embodiments, mRNAs are uniformly modified (e.g., fully modified, modified throughout the entire sequence) for a particular modification. For example, a nucleic acid can be uniformly modified with 1 -methyl-pseudouridine, meaning that all uridine residues in the mRNA sequence are replaced with 1 -methyl-pseudouridine. Similarly, a nucleic acid can be uniformly modified for any type of nucleoside residue present in the sequence by replacement with a modified residue such as those set forth above.

[0596] The nucleic acids of the polynucleotide (e.g., mRNA) may be partially or fully modified along the entire length of the molecule. For example, one or more or all or a given type of nucleotide (e.g., purine or pyrimidine, or any one or more or all of A, G, U, C) may be uniformly modified in a nucleic acid of the disclosure, or in a predetermined sequence region thereof (e.g., in the mRNA including or excluding the poly(A) tail). In some embodiments, all nucleotides X in a nucleic acid of the present disclosure (or in a sequence region thereof) are modified nucleotides, wherein X may be any one of nucleotides A, G, U, C, or any one of the combinations A+G, A+U, A+C, G+U, G+C, U+C, A+G+U, A+G+C, G+U+C or A+G+C.

[0597] The nucleic acid may contain from about 1% to about 100% modified nucleotides (either in relation to overall nucleotide content, or in relation to one or more types of nucleotide, i.e., any one or more of A, G, U or C) or any intervening percentage (e.g., from 1% to 20%, from 1% to 25%, from 1% to 50%, from 1% to 60%, from 1% to 70%, from 1% to 80%, from 1% to 90%, from 1% to 95%, from 10% to 20%, from 10% to 25%, from 10% to 50%, from 10% to 60%, from 10% to 70%, from 10% to 80%, from 10% to 90%, from 10% to 95%, from 10% to 100%, from 20% to 25%, from 20% to 50%, from 20% to 60%, from 20% to 70%, from 20% to 80%, from 20% to 90%, from 20% to 95%, from 20% to 100%, from 50% to 60%, from 50% to 70%, from 50% to 80%, from 50% to 90%, from 50% to 95%, from 50% to 100%, from 70% to 80%, from 70% to 90%, from 70% to 95%, from 70% to 100%, from 80% to 90%, from 80% to 95%, from 80% to 100%, from 90% to 95%, from 90% to 100%, and from 95% to 100%). It will be understood that any remaining percentage is accounted for by the presence of unmodified A, G, U, or C.

[0598] The mRNAs may contain at a minimum 1% and at maximum 100% modified nucleotides, or any intervening percentage, such as at least 5% modified nucleotides, at least 10% modified nucleotides, at least 25% modified nucleotides, at least 50% modified nucleotides, at least 80% modified nucleotides, or at least 90% modified nucleotides. For example, the nucleic acids may contain a modified pyrimidine such as a modified uracil or cytosine. In some embodiments, at least 5%, at least 10%, at least 25%, at least 50%, at least 80%, at least 90% or 100% of the uracil in the nucleic acid is replaced with a modified uracil (e.g., a 5-substituted uracil). The modified uracil can be replaced by a compound having a single unique structure, or can be replaced by a plurality of compounds having different structures (e.g., 2, 3, 4 or more unique structures). In some embodiments, at least 5%, at least 10%, at least 25%, at least 50%, at least 80%, at least 90% or 100% of the cytosine in the nucleic acid is replaced with a modified cytosine (e.g., a 5-substituted cytosine). The modified cytosine can be replaced by a compound having a single unique structure, or can be replaced by a plurality of compounds having different structures (e.g., 2, 3, 4 or more unique

structures).

Untranslated Regions (UTRs)

[0599] The polynucleotide (e.g., mRNA) may comprise one or more regions or parts that act or function as an untranslated region. Where mRNAs are designed to encode at least one protein of interest, the polynucleotide may comprise one or more of these untranslated regions (UTRs). Wild-type untranslated regions of a nucleic acid sequence are transcribed but not translated. In mRNA, the 5 ' UTR starts at the transcription start site and continues to the start codon but does not include the start codon; whereas, the 3' UTR starts immediately following the stop codon and continues until the transcriptional termination signal. There is growing body of evidence about the regulatory roles played by the UTRs in terms of stability of the nucleic acid molecule and translation. The regulatory features of a UTR can be incorporated into the polynucleotides of the present disclosure to, among other things, enhance the stability of the molecule. The specific features can also be incorporated to ensure controlled down-regulation of the transcript in case they are misdirected to undesired organs sites. A variety of 5' UTR and 3' UTR sequences are known and available in the art.

[0600] A 5' UTR is region of an mRNA that is directly upstream (5') from the start codon (the first codon of an mRNA transcript translated by a ribosome). A 5' UTR does not encode a protein (is non-coding). Natural 5' UTRs have features that play roles in translation initiation. They harbor signatures like Kozak sequences, which are commonly known to be involved in the process by which the ribosome initiates translation of many genes. Kozak sequences have the consensus CCR(A/G)CCAUGG, where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG), which is followed by another 'G'. 5' UTR also have been known to form secondary structures which are involved in elongation factor binding.

[0601] In some embodiments, the 5' UTR is a heterologous UTR, i.e., is a UTR found in nature associated with a different ORF. In another embodiment, a 5' UTR is a synthetic UTR, i.e., does not occur in nature. Synthetic UTRs include UTRs that have been mutated to improve their properties, e.g., which increase gene expression as well as those which are completely synthetic. Exemplary 5' UTRs include Xenopus or human derived a-globin or b- globin (8278063; 9012219), human cytochrome b-245 a polypeptide, and hydroxysteroid (17b) dehydrogenase, and Tobacco etch virus (U.S.8278063, 9012219, which are incorporated herein by reference in their entirety). CMV immediate-early 1 (IE1) gene (US2014/0206753, WO2013/185069, which are incorporated herein by reference in their entirety), the sequence GGGAUCCUACC (WO2014/144196) may also be used. In another embodiment, 5' UTR of a TOP gene is a 5' UTR of a TOP gene lacking the 5' TOP motif (the oligopyrimidine tract) (e.g., WO/2015/101414, W02015/101415, WO/2015/062738, WO2015/024667, WO2015/024667; 5' UTR element derived from ribosomal protein Large 32 (L32) gene (WO/2015/101414, W02015/101415, WO/2015/062738), 5' UTR element derived from the 5' UTR of an hydroxysteroid (17-b) dehydrogenase 4 gene (HSD17B4) (WO2015/024667), or a 5' UTR element derived from the 5' UTR of ATP5A1 (WO2015/024667) can be used. In some embodiments, an internal ribosome entry site (IRES) is used instead of a 5' UTR.

[0602] A 3' UTR is region of an mRNA that is directly downstream (3') from the stop codon (the

codon of an mRNA transcript that signals a termination of translation). A 3' UTR does not encode a protein (is non-coding). Natural or wild type 3' UTRs are known to have stretches of adenosines and uridines embedded in them. These AU rich signatures are particularly prevalent in genes with high rates of turnover. Based on their sequence features and functional properties, the AU rich elements (AREs) can be separated into three classes (Chen et al, 1995): Class I AREs contain several dispersed copies of an AUUUA motif within U-rich regions. C-Myc and MyoD contain class I AREs. Class II AREs possess two or more overlapping UUAUUUA(U/A)(U/A) nonamers. Molecules containing this type of AREs include GM-CSF and TNF-α. Class III ARES are less well defined. These U rich regions do not contain an AUUUA motif. c-Jun and Myogenin are two well-studied examples of this class.

[0603] Most proteins binding to the AREs are known to destabilize the messenger, whereas members of the ELAV family, most notably HuR, have been documented to increase the stability of mRNA. HuR binds to AREs of all the three classes. Engineering the HuR specific binding sites into the 3' UTR of nucleic acid molecules will lead to HuR binding and thus, stabilization of the message in vivo.

[0604] Introduction, removal or modification of 3 ' UTR AU rich elements (AREs) can be used to modulate the stability of the polynucleotide (e.g., mRNA). When engineering specific nucleic acids, one or more copies of an ARE can be introduced to make nucleic acids of the disclosure less stable and thereby curtail translation and decrease production of the resultant protein. Likewise, AREs can be identified and removed or mutated to increase the intracellular stability and thus increase translation and production of the resultant protein. Transfection experiments can be conducted in relevant cell lines, using nucleic acids of the disclosure and protein production can be assayed at various time points post-transfection. For example, cells can be transfected with different ARE-engineering molecules and by using an ELISA kit to the relevant protein and assaying protein produced at 6 hour, 12 hour, 24 hour, 48 hour, and 7 days post-transfection.

[0605] 3' UTRs may be heterologous or synthetic.

[0606] Those of ordinary skill in the art will understand that 5' UTRs that are heterologous or synthetic may be used with any desired 3' UTR sequence. For example, a heterologous 5' UTR may be used with a synthetic 3' UTR with a heterologous 3' UTR.

[0607] Non-UTR sequences may also be used as regions or subregions within a nucleic acid. For example, introns or portions of introns sequences may be incorporated into regions of nucleic acid sequences of the disclosure. Incorporation of intronic sequences may increase protein production as well as nucleic acid levels.

[0608] Combinations of features may be included in flanking regions and may be contained within other features. For example, the ORF may be flanked by a 5' UTR which may contain a strong Kozak translational initiation signal and/or a 3' UTR which may include an oligo(dT) sequence for templated addition of a poly- A tail. 5' UTR may comprise a first polynucleotide fragment and a second polynucleotide fragment from the same and/or different genes such as the 5' UTRs described in US Patent Application Publication No.2010/0293625 and PCT/US2014/069155, which are herein incorporated by reference in their entirety. It should be understood that any UTR from any gene may

be incorporated into the regions of a nucleic acid sequence. Furthermore, multiple wild-type UTRs of any known gene may be utilized. It is also within the scope of the present disclosure to provide artificial UTRs that are not variants of wild type regions. These UTRs or portions thereof may be placed in the same orientation as in the transcript from which they were selected or may be altered in orientation or location. Hence, a 5' or 3' UTR may be inverted, shortened, lengthened, made with one or more other 5' UTRs or 3' UTRs. As used herein, the term “altered” as it relates to a UTR sequence, means that the UTR has been changed in some way in relation to a reference sequence. For example, a 3' UTR or 5' UTR may be altered relative to a wild-type or native UTR by the change in orientation or location as taught above or may be altered by the inclusion of additional nucleotides, deletion of nucleotides, swapping or transposition of nucleotides. Any of these changes producing an “altered” UTR (whether 3' or 5') comprise a variant UTR.

[0609] In some embodiments, a double, triple or quadruple UTR such as a 5' UTR or 3' UTR may be used. As used herein, a “double” UTR is one in which two copies of the same UTR are encoded either in series or substantially in series. For example, a double beta-globin 3' UTR may be used as described in US Patent publication 2010/0129877, the contents of which are incorporated herein by reference in its entirety.

[0610] It is also within the scope of the present disclosure to have patterned UTRs. As used herein “patterned UTRs” are those UTRs which reflect a repeating or alternating pattern, such as ABABAB or AABBAABBAABB or ABCABCABC or variants thereof repeated once, twice, or more than 3 times. In these patterns, each letter, A, B, or C represent a different UTR at the nucleotide level.

[0611] In some embodiments, flanking regions are selected from a family of transcripts whose proteins share a common function, structure, feature or property. For example, polypeptides of interest may belong to a family of proteins that are expressed in a particular cell, tissue or at some time during development. The UTRs from any of these genes may be swapped for any other UTR of the same or different family of proteins to create a new polynucleotide. As used herein, a “family of proteins” is used in the broadest sense to refer to a group of two or more polypeptides of interest that share at least one function, structure, feature, localization, origin, or expression pattern.

[0612] The untranslated region may also include translation enhancer elements (TEE). As a non-limiting example, the TEE may include those described in US Application No. 2009/0226470, herein incorporated by reference in its entirety, and those known in the art. In vitro Transcription of RNA cDNA encoding the polynucleotides described herein may be transcribed using an in vitro transcription (IVT) system. In vitro transcription of RNA is known in the art and is described in International Publication WO 2014/152027, which is incorporated by reference herein in its entirety. In some embodiments, the RNA of the present disclosure is prepared in accordance with any one or more of the methods described in WO 2018/053209 and WO 2019/036682, each of which is incorporated by reference herein.

[0613] In some embodiments, the RNA transcript is generated using a non-amplified, linearized DNA template in an in vitro transcription reaction to generate the RNA transcript. In some embodiments, the template DNA is isolated DNA. In some embodiments, the template DNA is cDNA. In some embodiments, the cDNA is formed by reverse transcription of a RNA polynucleotide, for example, but

not limited to coronavirus mRNA. In some embodiments, cells, e.g., bacterial cells, e.g., E. coli, e.g., DH-1 cells are transfected with the plasmid DNA template. In some embodiments, the transfected cells are cultured to replicate the plasmid DNA, which is then isolated and purified. In some embodiments, the DNA template includes a RNA polymerase promoter, e.g., a T7 promoter located 5' to and operably linked to the gene of interest.

[0614] In some embodiments, an in vitro transcription template encodes a 5' untranslated (UTR) region, contains an open reading frame, and encodes a 3' UTR and a poly(A) tail. The particular nucleic acid sequence composition and length of an in vitro transcription template will depend on the mRNA encoded by the template.

[0615] A "5' untranslated region" (UTR) refers to a region of an mRNA that is directly upstream (i.e., 5') from the start codon (i.e., the first codon of an mRNA transcript translated by a ribosome) that does not encode a polypeptide. When RNA transcripts are being generated, the 5' UTR may comprise a promoter sequence. Such promoter sequences are known in the art. It should be understood that such promoter sequences will not be present in a vaccine of the disclosure.

[0616] A "3' untranslated region" (UTR) refers to a region of an mRNA that is directly downstream (i.e., 3') from the stop codon (i.e., the codon of an mRNA transcript that signals a termination of translation) that does not encode a polypeptide.

[0617] An "open reading frame" is a continuous stretch of DNA beginning with a start codon (e.g., methionine (ATG)), and ending with a stop codon (e.g., TAA, TAG or TGA) and encodes a polypeptide.

[0618] A "poly(A) tail" is a region of mRNA that is downstream, e.g., directly downstream (i.e., 3'), from the 3' UTR that contains multiple, consecutive adenosine monophosphates. A poly(A) tail may contain 10 to 300 adenosine monophosphates. For example, a poly(A) tail may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290 or 300 adenosine monophosphates. In some embodiments, a poly(A) tail contains 50 to 250 adenosine monophosphates. In a relevant biological setting (e.g., in cells, in vivo) the poly(A) tail functions to protect mRNA from enzymatic degradation, e.g., in the cytoplasm, and aids in transcription termination, and/or export of the mRNA from the nucleus and translation.

[0619] In some embodiments, a nucleic acid includes 200 to 3,000 nucleotides. For example, a nucleic acid may include 200 to 500, 200 to 1000, 200 to 1500, 200 to 3000, 500 to 1000, 500 to 1500, 500 to 2000, 500 to 3000, 1000 to 1500, 1000 to 2000, 1000 to 3000, 1500 to 3000, or 2000 to 3000 nucleotides).

[0620] An in vitro transcription system typically comprises a transcription buffer, nucleotide triphosphates (NTPs), an RNase inhibitor and a polymerase.

[0621] The NTPs may be manufactured in house, may be selected from a supplier, or may be synthesized as described herein. The NTPs may be selected from, but are not limited to, those described herein including natural and unnatural (modified) NTPs.

[0622] Any number of RNA polymerases or variants may be used in the method of the present disclosure. The polymerase may be selected from, but is not limited to, a phage RNA polymerase, e.g., a T7 RNA polymerase, a T3 RNA polymerase, a SP6 RNA polymerase, and/or mutant

polymerases such as, but not limited to, polymerases able to incorporate modified nucleic acids and/or modified nucleotides, including chemically modified nucleic acids and/or nucleotides. Some embodiments exclude the use of DNase.

[0623] In some embodiments, the RNA transcript is capped via enzymatic capping. In some embodiments, the polynucleotide (e.g., mRNA) comprises 5' terminal cap, for example, 7mG(5')ppp(5')NlmpNp.

Chemical Synthesis

[0624] Solid-phase chemical synthesis. The polynucleotide (e.g., mRNA) may be manufactured in whole or in part using solid phase techniques. Solid-phase chemical synthesis of nucleic acids is an automated method wherein molecules are immobilized on a solid support and synthesized step by step in a reactant solution. Solid-phase synthesis is useful in site-specific introduction of chemical modifications in the nucleic acid sequences.

[0625] Liquid Phase Chemical Synthesis. The synthesis of the polynucleotide (e.g., mRNA) by the sequential addition of monomer building blocks may be carried out in a liquid phase.

[0626] Combination of Synthetic Methods. The synthetic methods discussed above each has its own advantages and limitations. Attempts have been conducted to combine these methods to overcome the limitations. Such combinations of methods are within the scope of the present disclosure. The use of solid-phase or liquid-phase chemical synthesis in combination with enzymatic ligation provides an efficient way to generate long chain nucleic acids that cannot be obtained by chemical synthesis alone.

Ligation of Nucleic Acid Regions or Subregions

[0627] Assembling nucleic acids by a ligase may also be used. DNA or RNA ligases promote intermolecular ligation of the 5' and 3' ends of polynucleotide chains through the formation of a phosphodiester bond. Nucleic acids such as chimeric polynucleotides and/or circular nucleic acids may be prepared by ligation of one or more regions or subregions. DNA fragments can be joined by a ligase-catalyzed reaction to create recombinant DNA with different functions. Two oligodeoxynucleotides, one with a 5' phosphoryl group and another with a free 3' hydroxyl group, serve as substrates for a DNA ligase.

Purification

[0628] Purification of the nucleic acids described herein may include, but is not limited to, nucleic acid clean-up, quality assurance and quality control. Clean-up may be performed by methods known in the arts such as, but not limited to, AGENCOURT® beads (Beckman Coulter Genomics, Danvers, MA), poly-T beads, LNATM oligo-T capture probes (EXIQON® Inc, Vedbaek, Denmark) or HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC-HPLC). The term “purified” when used in relation to a nucleic acid such as a “purified nucleic acid” refers to one that is separated from at least one contaminant. A “contaminant” is any substance that makes another unfit, impure or inferior. Thus, a purified nucleic acid (e.g., DNA and RNA) is present in a form

or setting different from that in which it is found in nature, or a form or setting different from that which existed prior to subjecting it to a treatment or purification method.

[0629] A quality assurance and/or quality control check may be conducted using methods such as, but not limited to, gel electrophoresis, UV absorbance, or analytical HPLC.

[0630] In some embodiments, the nucleic acids may be sequenced by methods including, but not limited to reverse-transcriptase-PCR.

Quantification

[0631] In some embodiments, the polynucleotide (e.g., mRNA) may be quantified in exosomes or when derived from one or more bodily fluid. Bodily fluids include peripheral blood, serum, plasma, ascites, urine, cerebrospinal fluid (CSF), sputum, saliva, bone marrow, synovial fluid, aqueous humor, amniotic fluid, cerumen, breast milk, broncheo alveolar lavage fluid, semen, prostatic fluid, cowper's fluid or pre-ejaculatory fluid, sweat, fecal matter, hair, tears, cyst fluid, pleural and peritoneal fluid, pericardial fluid, lymph, chyme, chyle, bile, interstitial fluid, menses, pus, sebum, vomit, vaginal secretions, mucosal secretion, stool water, pancreatic juice, lavage fluids from sinus cavities, bronchopulmonary aspirates, blastocyl cavity fluid, and umbilical cord blood. Alternatively, exosomes may be retrieved from an organ selected from the group consisting of lung, heart, pancreas, stomach, intestine, bladder, kidney, ovary, testis, skin, colon, breast, prostate, brain, esophagus, liver, and placenta.

[0632] Assays may be performed using construct specific probes, cytometry, qRT-PCR, real time PCR, PCR, flow cytometry, electrophoresis, mass spectrometry, or combinations thereof while the exosomes may be isolated using immunohistochemical methods such as enzyme linked immunosorbent assay (ELISA) methods. Exosomes may also be isolated by size exclusion chromatography, density gradient centrifugation, differential centrifugation, nanomembrane ultrafiltration, immunoabsorbent capture, affinity purification, microfluidic separation, or combinations thereof.

[0633] These methods afford the investigator the ability to monitor, in real time, the level of nucleic acids remaining or delivered. This is possible because the nucleic acids of the present disclosure, in some embodiments, differ from the endogenous forms due to the structural or chemical modifications.

[0634] In some embodiments, the nucleic acid may be quantified using methods such as, but not limited to, ultraviolet visible spectroscopy (UV/Vis). A non-limiting example of a UV/Vis spectrometer is a NANODROP® spectrometer (ThermoFisher, Waltham, MA). The quantified nucleic acid may be analyzed in order to determine if the nucleic acid may be of proper size, check that no degradation of the nucleic acid has occurred. Degradation of the nucleic acid may be checked by methods such as, but not limited to, agarose gel electrophoresis, HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC- HPLC), liquid chromatography-mass spectrometry (LCMS), capillary electrophoresis (CE) and capillary gel electrophoresis (CGE).

Therapeutic and Prophylactic Compositions

[0635] Provided herein are compositions (e.g., pharmaceutical compositions), methods, kits and

reagents for use in humans and other mammals.

[0636] In some embodiments, LNMP / RNA composition (as vaccines) can be used in the priming of immune effector cells, for example, to activate peripheral blood mononuclear cells (PBMCs) ex vivo, which are then infused (re-infused) into a subject.

[0637] In exemplary embodiments, the LNMP / RNA composition can be administered to a subject (e.g., a mammalian subject, such as a human subject), and the RNA polynucleotides are translated in vivo.

[0638] The LNMP / RNA composition may be induced for translation of a polypeptide {e.g., gene editing system) in a cell, tissue or organism. In exemplary embodiments, such translation occurs in vivo, although there can be envisioned embodiments where such translation occurs ex vivo, in culture or in vitro. In exemplary embodiments, the cell, tissue or organism is contacted with an effective amount of a composition containing a LNMP / RNA composition that contains a polynucleotide that has at least one a translatable region encoding a gene editing system.

[0639] An "effective amount" of a LNMP / RNA composition is provided based, at least in part, on the target tissue, target cell type, means of administration, physical characteristics of the polynucleotide (e.g., size, and extent of modified nucleosides) and other components of LNMP / RNA composition, and other determinants.

[0640] In some embodiments, the LNMP / RNA composition may be used for treatment of cancer.

[0641] The LNMP / RNA composition may be administered prophylactically or therapeutically as part of an active immunization scheme to healthy individuals. In some embodiments, the amount of the LNMP / RNA composition provided to a cell, a tissue or a subject may be an amount effective for immune prophylaxis.

[0642] The LNMP / RNA composition may be administered with other prophylactic or therapeutic compounds. As a non-limiting example, a prophylactic or therapeutic compound may be an immune potentiator, adjuvant, or booster. As used herein, when referring to a composition, such as a vaccine, the term "booster" refers to an extra administration of the prophylactic (vaccine) composition. A booster (or booster vaccine) may be given after an earlier administration of the prophylactic composition. The time of administration between the initial administration of the prophylactic composition and the booster may be, but is not limited to, 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 15 minutes, 20 minutes 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 1 day, 36 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 10 days, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 1 year, 18 months, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years, 10 years, 11 years, 12 years, 13 years, 14 years, 15 years, 16 years, 17 years, 18 years, 19 years, 20 years, 25 years, 30 years, 35 years, 40 years, 45 years, 50 years, 55 years, 60 years, 65 years, 70 years, 75 years, 80 years, 85 years, 90 years, 95 years or more than 99 years. In exemplary embodiments, the time of administration between the initial administration of the prophylactic composition and the booster may

be, but is not limited to, 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 6 months or 1 year.

[0643] In one embodiment, the LNMP / RNA composition may be administered intramuscularly or intradermally similarly to the administration of vaccines known in the art.

[0644] The LNMP / RNA composition may be utilized in various settings depending on the severity or the degree or level of unmet medical need

[0645] The LNMP / RNA composition may be formulated or administered in combination with one or more pharmaceutically-acceptable excipients. In some embodiments, the LNMP / RNA composition comprises at least one additional active substances, such as, for example, a therapeutically- active substance, a prophylactically-active substance, or a combination of both. The LNMP / RNA composition may be sterile, pyrogen-free or both sterile and pyrogen-free. General considerations in the formulation and/or manufacture of pharmaceutical agents, such as vaccine compositions, may be found, for example, in Remington: The Science and Practice of Pharmacy 21st ed., Lippincott Williams & Wilkins, 2005 (incorporated herein by reference in its entirety).

[0646] In some embodiments, the LNMP / RNA compositions are administered to humans, human patients, or subjects. The phrase "active ingredient" generally refers to the LNMP / RNA composition or the polynucleotides contained therein, for example, RNA polynucleotides (e.g., mRNA polynucleotides) encoding gene editing or therapeutic polypeptides.

[0647] Formulations of the LNMP / RNA composition described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient (e.g., mRNA polynucleotide) into association with an excipient and/or one or more other accessory ingredients, and then, if necessary and/or desirable, dividing, shaping and/or packaging the product into a desired single- or multi-dose unit.

[0648] The LNMP / RNA composition can be formulated using one or more excipients to: (1) increase stability; (2) increase cell transfection; (3) permit the sustained or delayed release (e.g., from a depot formulation); (4) alter the biodistribution (e.g., target to specific tissues or cell types); (5) increase the translation of encoded protein in vivo; and/or (6) alter the release profile of encoded protein (antigen) in vivo. In addition to traditional excipients such as any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, excipients can include, without limitation, lipidoids, liposomes, lipid nanoparticles, polymers, lipoplexes, core-shell nanoparticles, peptides, proteins, cells transfected with cancer RNA vaccines (e.g., for transplantation into a subject), hyaluronidase, nanoparticle mimics and combinations thereof.

Kits

[0649] The present invention also provides a kit including a container having a RNA composition described herein. The kit may further include instructional material for applying or delivering the RNA composition to a subject in accordance with a method of the present invention. The skilled artisan will appreciate that the instructions for applying the RNA composition in the methods of the present invention can be any form of instruction. Such instructions include, but are not limited to, written

instruction material (such as, a label, a booklet, a pamphlet), oral instructional material (such as on an audio cassette or CD) or video instructions (such as on a video tape or DVD).

Other Embodiments:

Embodiment 1. A method for delivering a gene editing system to a subject in need thereof, the method comprising administering to the subject a RNA composition comprising:

one or more polynucleotides encoding one or more components of a gene editing system or one or more gene editing systems, formulated within

(a) a plurality of lipid nanoparticles (LNPs) comprising synthetic structural lipids and an ionizable lipid; or

(b) a lipid reconstructed natural messenger packs (LNMPs) comprising natural lipids and an ionizable lipid,

wherein the ionizable lipid has two or more of the characteristics listed below:

(i) at least 2 ionizable amines;

(ii) at least 3 lipid tails; wherein each of the lipid tails is at least 6 carbon atoms in length;

(iii) a pKa of about 4.5 to about 7.5;

(iv) an ionizable amine and a heteroorganic group separated by a chain of at least two atoms; and

(v) an N:P ratio of at least 3.

Embodiment 2. A method of gene editing, comprising:

contacting a cell with or administering into a subject a RNA composition comprising:

one or more polynucleotides encoding one or more components of a gene editing system or one or more gene editing systems, formulated within

(a) a plurality of lipid nanoparticles (LNPs) comprising synthetic structural lipids and an ionizable lipid; or

(b) a lipid reconstructed natural messenger packs (LNMPs) comprising natural lipids and an ionizable lipid,

wherein the ionizable lipid has two or more of the characteristics listed below:

(i) at least 2 ionizable amines;

(ii) at least 3 lipid tails; wherein each of the lipid tails is at least 6 carbon atoms in length;

(iii) a pKa of about 4.5 to about 7.5;

(iv) an ionizable amine and a heteroorganic group separated by a chain of at least two atoms; and

(v) an N:P ratio of at least 3,

wherein one or more components of the gene editing systems are delivered to the cell or subject to modify the genome of the cell or the subject.

Embodiment 3. The method of embodiment 1 or 2, wherein the gene editing system is CRISPR-Cas gene editing system.

Embodiment 4. The method of any one of embodiments 1-3, wherein the RNA composition further comprises at least one template nucleic acid.

Embodiment 5. The method of embodiment 1 or 2, wherein the RNA composition is administered at least one time.

Embodiment 6. The method of embodiment 1 or 2, wherein the RNA composition is administered at least two times, at least three times, at least four times, at least five times, at least six times, at least seven times, at least eight times, at least nine times, at least ten times, at least fifteen times, at least twenty times, or more.

Embodiment 7. The method of embodiment 1 or 2, wherein the RNA composition is administered 2-8 times.

Embodiment 8. The method of embodiment 6 or 7, wherein the delivery of the gene editing system, or the result of gene editing improves upon multiple administrations.

Embodiment 9. The method of embodiment 1 or 2, wherein at least two RNA compositions are administered into the subject or contacted with the cell: a first RNA composition comprising a mRNA, and the second RNA composition comprising a guide RNA nucleic acid.

Embodiment 10. The method of embodiment 9, wherein the first and second RNA compositions are administered simultaneously.

Embodiment 11. The method of embodiment 9, wherein the first and second RNA compositions are administered sequentially.

Embodiment 12. The method of embodiment 1 or 2, wherein a single RNA composition is contacted with the cell or administered into the subject, wherein the single RNA composition comprises an mRNA and a guide RNA nucleic acid.

Embodiment 13. The method of embodiment 1 or 2, wherein the RNA composition is administered via oral, intravenous, intramuscular, intranasal, or subcutaneous route.

Embodiment 14. The method of embodiment 1 or 2, wherein the RNA composition is administered more than once, within at least one week, at least two weeks, at least three weeks, or at least four weeks between administrations.

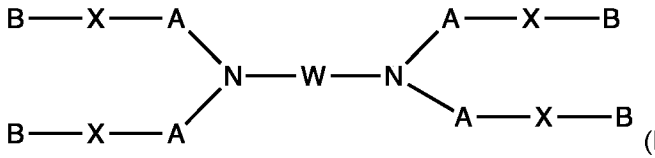
Embodiment 15. The method of embodiment 1 or 2, wherein the RNA composition is formulated with

b) LNMPs.

Embodiment 16. The method of embodiment 15, wherein the ionizable lipid is C12-200.

Embodiment 17. The method of embodiment 1 or 2, wherein the RNA composition is formulated with a) LNPs.

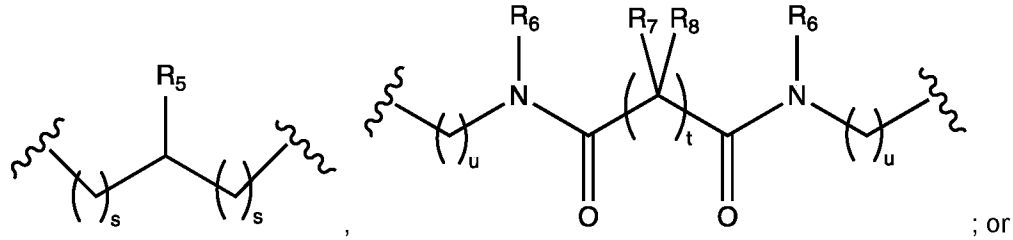
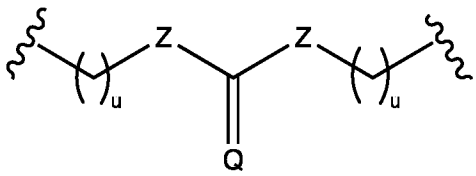
Embodiment 18. The method of embodiment 15 or 17, wherein the ionizable lipid is selected from one of the following groups of compounds:

i) a compound of formula  (I), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing, wherein:

each **A** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally substituted with heteroatom or substituted with OH, SH, or halogen;

each **B** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally substituted with heteroatom or substituted with OH, SH, or halogen;

each **X** is independently a biodegradable moiety; and

W is  ; or  , wherein

$\mathbf{R_5}$ is OH, SH, $N\mathbf{R_{10}}\mathbf{R_{11}}$;

each $\mathbf{R_6}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each $\mathbf{R_7}$ and each $\mathbf{R_8}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $N\mathbf{R_{10}}\mathbf{R_{11}}$, wherein each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 alkyl, or $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring;

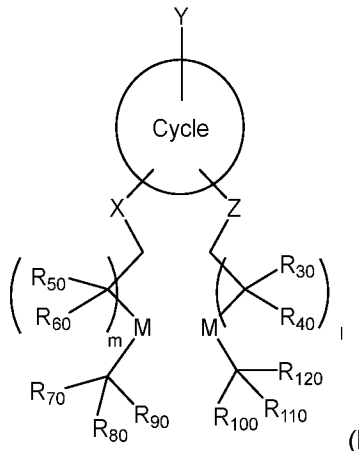
each **s** is independently 1, 2, 3, 4, or 5;

each **u** is independently 1, 2, 3, 4, or 5;

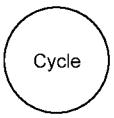
t is 1, 2, 3, 4 or 5;

each **Z** is independently absent, O, S, or $N\mathbf{R_{12}}$, wherein $\mathbf{R_{12}}$ is H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl, and

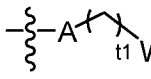
Q is O, S, or N**R**$_{13}$, wherein each **R**$_{13}$ is H, C_1-C_5 alkyl;

ii) a compound of formula 

(II), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing, wherein:



is cyclic or heterocyclic moiety;

Y is alkyl, hydroxy, hydroxyalkyl or 

;

A is absent, -O-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -N(R^7)C(O)N(R^7)-, -S-, -S-S-, or a bivalent heterocycle;

each of X and Z is independently absent, -O-, -CO-, -N(R^7)-, -O-alkylene-; -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, or -S-;

each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxy, hydroxyalkyl, or aminoalkyl;

each M is independently a biodegradable moiety;

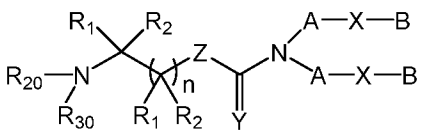
each of R_{30}, R_{40}, R_{50}, R_{60}, R_{70}, R_{80}, R_{90}, R_{100}, R_{110}, and R_{120} is independently H, C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with heteroatom or substituted with OH, SH, or halogen, or cycloalkyl or substituted cycloalkyl;

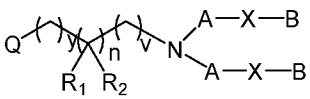
each of l and m is an integer from 1 to 10;

t1 is an integer from 0 to 10; and

W is hydroxyl, substituted or unsubstituted hydroxyalkyl, substituted or unsubstituted amino, substituted or unsubstituted aminocarbonyl, or substituted or unsubstituted heterocylyl or heteroaryl; and

iii) a compound of formula



(III) or 

(V), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein:

R$_{20}$ and **R**$_{30}$ are each independently H, C_1-C_5 branched or unbranched alkyl, or C_2-C_5 branched or unbranched alkenyl, or **R**$_{20}$ and **R**$_{30}$ together with the adjacent N atom form a 3 to 7 membered cyclic ring, optionally substituted with **R**^{a};

$\mathbf{R^a}$ is H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, or SH;

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, OH, halogen, SH, or $N\mathbf{R_{10}R_{11}}$, or

$\mathbf{R_1}$ and $\mathbf{R_2}$ are taken together to form a cyclic ring;

each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring;

n is 0, 1, 2, 3 or 4;

Y is O or S;

Z is absent, O, S, or $N(\mathbf{R_{12}})(\mathbf{R_{12}})$, wherein each $\mathbf{R_{12}}$ is independently H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl, provided that when Z is not absent, the adjacent $\mathbf{R_1}$ and $\mathbf{R_2}$ cannot be OH, $N\mathbf{R_{10}R_{11}}$, or SH;

u is 0, 1, 2, 3, 4, 5, 6, 7, or 8;

v is 0, 1, 2, 3, or 4;

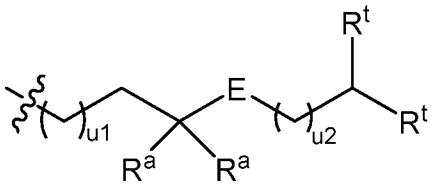
y is 0, 1, 2, 3, or 4;

each **A** is each independently C_1-C_{16} branched or unbranched alkyl, or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen;

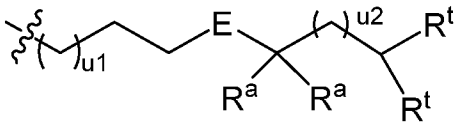
each **B** is each independently C_1-C_{16} branched or unbranched alkyl, or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen; and

each **X** is independently a biodegradable moiety; and

iv) a lipid comprising at least one head group and at least one tail group of formula (TI) or (TI')



(TI) or



(TI'), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing,

wherein:

E is each independently -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -C(O-R_{13})-O-, -C(O)O$(CH_2)_r$-, -C(O)N(R^7) $(CH_2)_r$-, -S-S-, or -C(O-R_{13})-O-$(CH_2)_r$-, wherein each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxyalkyl, or aminoalkyl;

R_{13} is branched or unbranched C_3-C_{10} alkyl;

r is 1, 2, 3, 4, or 5;

R^a is each independently C_1-C_5 alkyl, C_2-C_5 alkenyl, or C_2-C_5 alkynyl;

u1 and u2 are each independently 0, 1, 2, 3, 4, 5, 6, or 7;

R^t is each independently H, C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with heteroatom or substituted with OH, SH, or halogen, or cycloalkyl or substituted cycloalkyl;

 represents the bond connecting the tail group to the head group; and

wherein the lipid has a pKa from about 4 to about 8.

Embodiment 19. The method of embodiment 18, wherein the ionizable lipid is a compound in Table I, Table II, Table III, or Table IV.

Embodiment 20. The method of embodiment 19, wherein the ionizable lipid is 2272, 2320, 2439, 2356, 2243, 2431, 2455, 2454, 2424, 2433, 2425, 2275, 2220, or 2335.

Embodiment 21. An RNA composition for gene editing, comprising:

one or more polynucleotides encoding one or more components of a gene editing system or one or more gene editing systems,

formulated within a lipid reconstructed natural messenger packs (LNMPs) comprising natural lipids and an ionizable lipid, wherein the ionizable lipid has two or more of the characteristics listed below:

(i) at least 2 ionizable amines;

(ii) at least 3 lipid tails; wherein each of the lipid tails is at least 6 carbon atoms in length;

(iii) a pKa of about 4.5 to about 7.5;

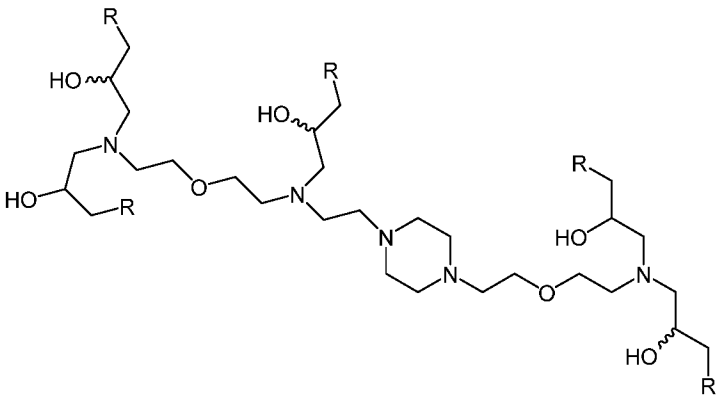
(iv) an ionizable amine and a heteroorganic group separated by a chain of at least two atoms; and

(v) an N:P ratio of at least 3.

Embodiment 22. The RNA composition of embodiment 21, wherein the ionizable lipid is selected from the group consisting of 1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl) (2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-200), MD1 (cKK-E12), OF2, EPC, ZA3-Ep10, TT3, LP01, 5A2-SC8, Lipid 5, SM-102 (Lipid H), and ALC-315.

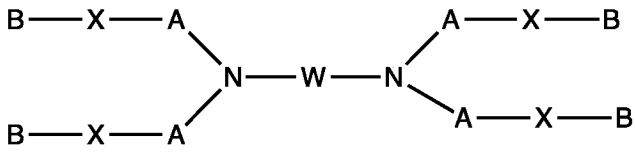
Embodiment 23. The RNA composition of embodiment 21, wherein the ionizable lipid is C12-200.

Embodiment 24. The RNA composition of embodiment 21, wherein the ionizable lipid is



(III), wherein R is C8-C14 alkyl group.

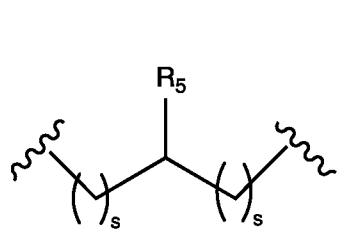
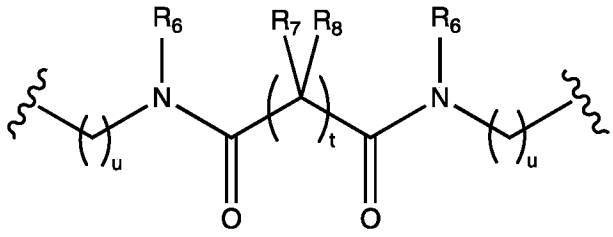
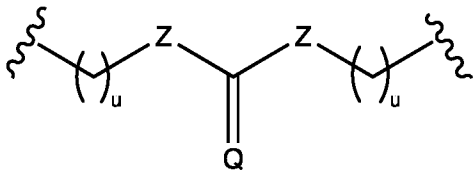
Embodiment 25. The RNA composition of embodiment 21, wherein the ionizable lipid is selected from one of the following groups of compounds:

i) a compound of formula  (I), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing, wherein:

each **A** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally substituted with heteroatom or substituted with OH, SH, or halogen;

each **B** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally substituted with heteroatom or substituted with OH, SH, or halogen;

each **X** is independently a biodegradable moiety; and

W is  ,  ; or  , wherein

$\mathbf{R_5}$ is OH, SH, $N\mathbf{R_{10}R_{11}}$;

each $\mathbf{R_6}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each $\mathbf{R_7}$ and each $\mathbf{R_8}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $N\mathbf{R_{10}R_{11}}$, wherein each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 alkyl, or $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring;

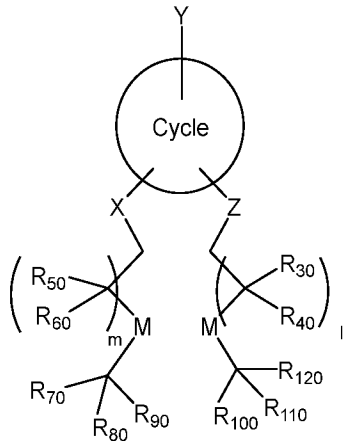
each **s** is independently 1, 2, 3, 4, or 5;

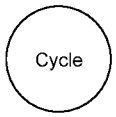
each **u** is independently 1, 2, 3, 4, or 5;

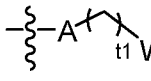
t is 1, 2, 3, 4 or 5;

each **Z** is independently absent, O, S, or $N\mathbf{R_{12}}$, wherein $\mathbf{R_{12}}$ is H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl, and

Q is O, S, or $N\mathbf{R_{13}}$, wherein each $\mathbf{R_{13}}$ is H, C_1-C_5 alkyl;

ii) a compound of formula  (II), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing, wherein:

 is cyclic or heterocyclic moiety;

Y is alkyl, hydroxy, hydroxyalkyl or  ;

A is absent, -O-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -N(R^7)C(O)N(R^7)-, -S-, -S-S-, or a bivalent heterocycle;

each of X and Z is independently absent, -O-, -CO-, -N(R^7)-, -O-alkylene-; -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, or -S-;

each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxy, hydroxyalkyl, or aminoalkyl;

each M is independently a biodegradable moiety;

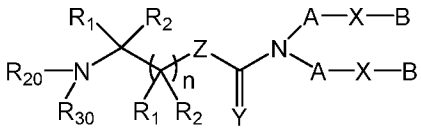
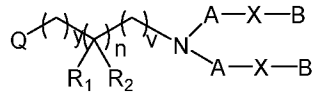
each of R_{30}, R_{40}, R_{50}, R_{60}, R_{70}, R_{80}, R_{90}, R_{100}, R_{110}, and R_{120} is independently H, C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with heteroatom or substituted with OH, SH, or halogen, or cycloalkyl or substituted cycloalkyl;

each of l and m is an integer from 1 to 10;

t1 is an integer from 0 to 10; and

W is hydroxyl, substituted or unsubstituted hydroxyalkyl, substituted or unsubstituted amino, substituted or unsubstituted aminocarbonyl, or substituted or unsubstituted heterocylyl or heteroaryl; and

iii) a compound of formula

 (III) or  (V), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein:

R_{20} and **R_{30}** are each independently H, C_1-C_5 branched or unbranched alkyl, or C_2-C_5 branched or unbranched alkenyl, or **R_{20}** and **R_{30}** together with the adjacent N atom form a 3 to 7 membered cyclic ring, optionally substituted with **R^a**;

R^a is H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, or SH;

each R_1 and each R_2 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, OH, halogen, SH, or $NR_{10}R_{11}$, or

R_1 and R_2 are taken together to form a cyclic ring;

each R_{10} and R_{11} is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or R_{10} and R_{11} are taken together to form a heterocyclic ring;

n is 0, 1, 2, 3 or 4;

Y is O or S;

Z is absent, O, S, or $N(R_{12})(R_{12})$, wherein each R_{12} is independently H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl, provided that when Z is not absent, the adjacent R_1 and R_2 cannot be OH, $NR_{10}R_{11}$, or SH;

u is 0, 1, 2, 3, 4, 5, 6, 7, or 8;

v is 0, 1, 2, 3, or 4;

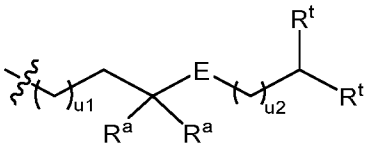
y is 0, 1, 2, 3, or 4;

each **A** is each independently C_1-C_{16} branched or unbranched alkyl, or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen;

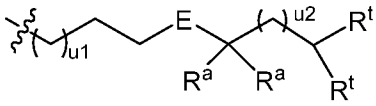
each **B** is each independently C_1-C_{16} branched or unbranched alkyl, or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen; and

each **X** is independently a biodegradable moiety; and

iv) a lipid comprising at least one head group and at least one tail group of formula (TI) or (TI')



(TI) or



(TI'), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing,

wherein:

E is each independently -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -C(O-R_{13})-O-, -C(O)O$(CH_2)_r$-, -C(O)N(R^7) $(CH_2)_r$-, -S-S-, or -C(O-R_{13})-O-$(CH_2)_r$-, wherein each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxyalkyl, or aminoalkyl;

R_{13} is branched or unbranched C_3-C_{10} alkyl;

r is 1, 2, 3, 4, or 5;

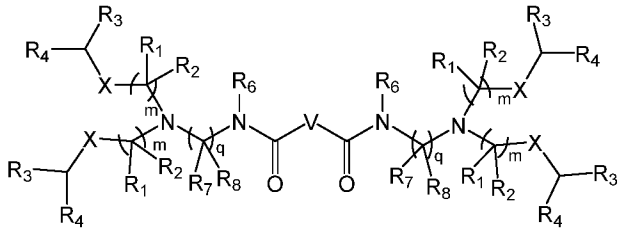
R^a is each independently C_1-C_5 alkyl, C_2-C_5 alkenyl, or C_2-C_5 alkynyl;

u1 and u2 are each independently 0, 1, 2, 3, 4, 5, 6, or 7;

R^t is each independently H, C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with heteroatom or substituted with OH, SH, or halogen, or cycloalkyl or substituted cycloalkyl;

 represents the bond connecting the tail group to the head group; and

wherein the lipid has a pKa from about 4 to about 8.

Embodiment 26. The RNA composition of embodiment 25, wherein the ionizable lipid is a compound of group i), represented by a formula of  (IX), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein:

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ is independently H, C_1-C_3 branched or unbranched alkyl, OH, halogen, SH, or $N\mathbf{R_{10}R_{11}}$, or

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ are independently taken together with the carbon atom(s) to which they are attached to form a cyclic ring;

each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 branched or unbranched alkyl, or $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring;

each $\mathbf{R_3}$ and each $\mathbf{R_4}$ is independently H, C_2-C_{14} branched or unbranched alkyl (e.g., C_3-C_{10} branched or unbranched alkyl), or C_3-C_{10} branched or unbranched alkenyl, provided that at least one of $\mathbf{R_3}$ and $\mathbf{R_4}$ is not H;

each **X** is independently a biodegradable moiety;

each **q** is independently 2, 3, 4,or 5;

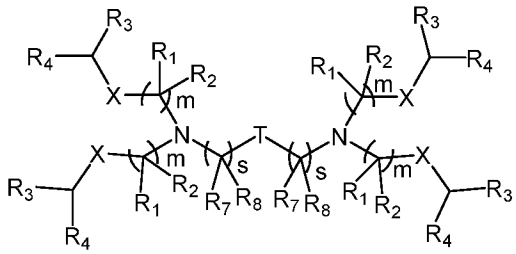
V is branched or unbranched C_2-C_{10} alkylene, C_2-C_{10} alkenylene, C_2-C_{10} alkynylene, or C_2-C_{10} heteroalkylene, optionally substituted with one or more OH, SH, and/or halogen groups;

each $\mathbf{R_6}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each $\mathbf{R_7}$ and each $\mathbf{R_8}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $(CH_2)_v\mathbf{R_{17}}$, or $N\mathbf{R_{10}R_{11}}$, wherein each **v** is independently 0, 1, 2, 3, 4, or 5, and $\mathbf{R_{17}}$ is OH, SH, or $N(CH_3)_2$; and

each **m** is independently 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

Embodiment 27. The RNA composition of embodiment 26, wherein **V** is a branched or unbranched C_2-C_3 alkylene, and each $\mathbf{R_6}$ is independently H or methyl.

Embodiment 28. The RNA composition of embodiment 25, wherein the ionizable lipid is a compound of group i), represented by a formula of  (XI), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein:

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ is independently H, C_1-C_3 branched or unbranched alkyl, OH, halogen, SH, or $N\mathbf{R_{10}R_{11}}$, or

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ are independently taken together with the carbon atom(s) to which they are attached to form a cyclic ring;

each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 branched or unbranched alkyl, or $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring;

each $\mathbf{R_3}$ and each $\mathbf{R_4}$ is independently H, C_2-C_{14} branched or unbranched alkyl (e.g., C_3-C_{10} branched or unbranched alkyl), or C_3-C_{10} branched or unbranched alkenyl, provided that at least one of $\mathbf{R_3}$ and $\mathbf{R_4}$ is not H;

each **X** is independently a biodegradable moiety;

each **s** is independently 1, 2, 3, 4, or 5;

T is –NHC(O)O-, –OC(O)NH-, or a divalent heterocyclic optionally substituted with one or more -$(CH_2)_v$OH, -$(CH_2)_v$SH, -$(CH_2)_v$-halogen groups,

each $\mathbf{R_7}$ and each $\mathbf{R_8}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $(CH_2)_v\mathbf{R_{17}}$, or $N\mathbf{R_{10}R_{11}}$, wherein $\mathbf{R_{17}}$ is OH, SH, or $N(CH_3)_2$;

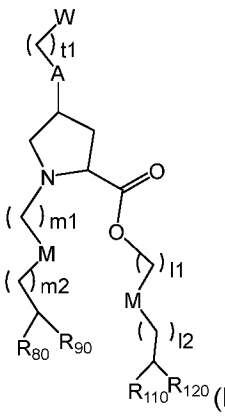
each **v** is independently 0, 1, 2, 3, 4, or 5; and

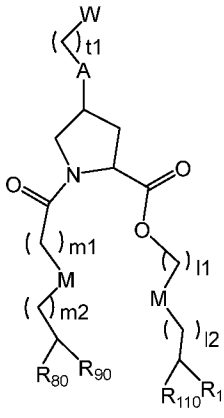
each **m** is independently 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

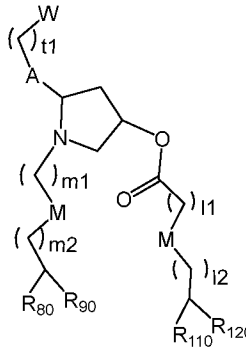
Embodiment 29. The RNA composition of embodiment 28, wherein T is a divalent piperazine or a divalent dioxopiperazine.

Embodiment 30. The RNA composition of any one of embodiments 26-29, wherein **X** is -OCO-, -COO-, -CONH-, or -NHCO-.

Embodiment 31. The RNA composition of embodiment 25, wherein the ionizable lipid is a compound of group ii), represented by one of the following formulas:


(IVA-1),


(IVA-2), or


(IVA-3),

wherein:

each m1 is independently an integer from 3 to 6,

each l1 is independently an integer from 4 to 8,

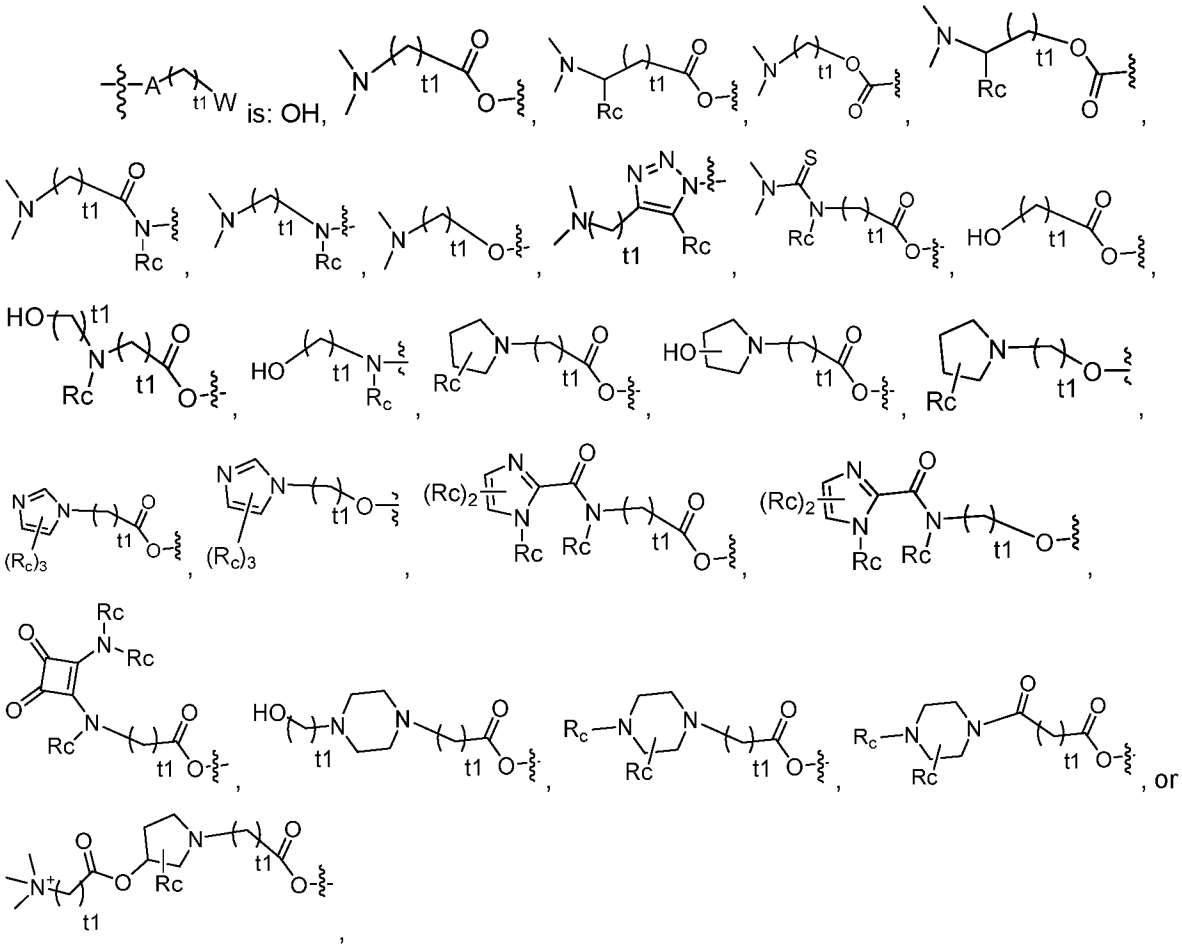
m2 and l2 are each independently an integer from 0 to 3,

R_{80} and R_{90} are each independently unsubstituted C_5-C_8 alkyl or alkenyl; or R_{80} is H or unsubstituted C_1-C_4 alkyl or alkenyl, and R_{90} is unsubstituted C_5-C_{11} alkyl or alkenyl; and

R_{110} and R_{120} are each independently unsubstituted C_5-C_8 alkyl or alkenyl; or R_{110} is H or unsubstituted C_1-C_4 alkyl or alkenyl, and R_{120} is unsubstituted C_5-C_{11} alkyl or alkenyl.

Embodiment 32. The RNA composition of embodiment 31, wherein:

M is -OC(O)- or -C(O)O-;



each R^c is independently H or C_1-C_3 alkyl;

each t1 is independently 1, 2, 3, or 4;

each of R_{80} and R_{90} is independently H or C_1-C_{12} branched or unbranched alkyl; and

each of R_{110} and R_{120} is independently H or C_1-C_{12} branched or unbranched alkyl, provided that at least one of R_{80} and R_{90} is not H, and at least one of R_{110} and R_{120} is not H.

Embodiment 33. The RNA composition of embodiment 25, wherein the ionizable lipid is a compound of group iii), wherein $\mathbf{R_1}$ and $\mathbf{R_2}$ are each H, or each $\mathbf{R_1}$ is H, and one of the $\mathbf{R_2}$ variables is OH; and X is –OC(O)- or –C(O)O-.

Embodiment 34. The RNA composition of embodiment 33, wherein the ionizable lipid is a compound of group iii), represented by formula III), wherein:

R_{20} and **R_{30}** are each independently H or C_1-C_3 branched or unbranched alkyl; or **R_{20}** and **R_{30}** together with the adjacent N atom form a 3 to 7 membered cyclic ring, optionally substituted with **R^a**;

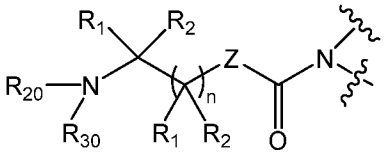
R^a is H or OH;

Z is absent, S, O, or NH; and

n is 0, 1, or 2.

Embodiment 35. The RNA composition of embodiment 33, wherein the ionizable lipid is a compound of group iii), represented by formula V),

Embodiment 36. The RNA composition of embodiment 25, wherein the ionizable lipid is a compound of group iv), comprising at least one head group and at least one tail group, wherein:
the tail group has a structure of formula (TI) (or TI'); and
the head group has a structure of one of the following formulas:

i)  (HA-I),

wherein:

R_{20} and R_{30} are each independently H, C_1-C_5 branched or unbranched alkyl, or C_2-C_5 branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or substituted with OH, SH, halogen, or cycloalkyl groups; or

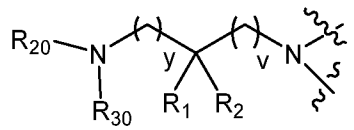
R_{20} and R_{30}, together with the adjacent N atom, form a 3 to 7 membered heterocylic or heteroaromatic ring containing one or more heteroatoms, optionally substituted with one or more OH, SH, halogen, alkyl, or cycloalkyl groups;

each of R_1 and R_2 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, OH, halogen, SH, or $NR_{10}R_{11}$; or R_1 and R_2 together form a cyclic ring;

each of R_{10} and R_{11} is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl; or R_{10} and R_{11} together form a heterocyclic ring;

n is 0, 1, 2, 3 or 4; and

Z is absent, O, S, or NR_{12}, wherein R_{12} is H or C_1-C_7 branched or unbranched alkyl; provided that when Z is not absent, the adjacent R_1 and R_2 cannot be OH, $NR_{10}R_{11}$, SH;

ii)  (HA-V).

wherein:

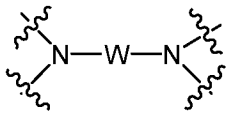
R_1 is H, C_1-C_3 alkyl, OH, halogen, SH, or $NR_{10}R_{11}$;

R_2 is OH, halogen, SH, or $NR_{10}R_{11}$; or R_1 and R_2 can be taken together to form a cyclic ring;

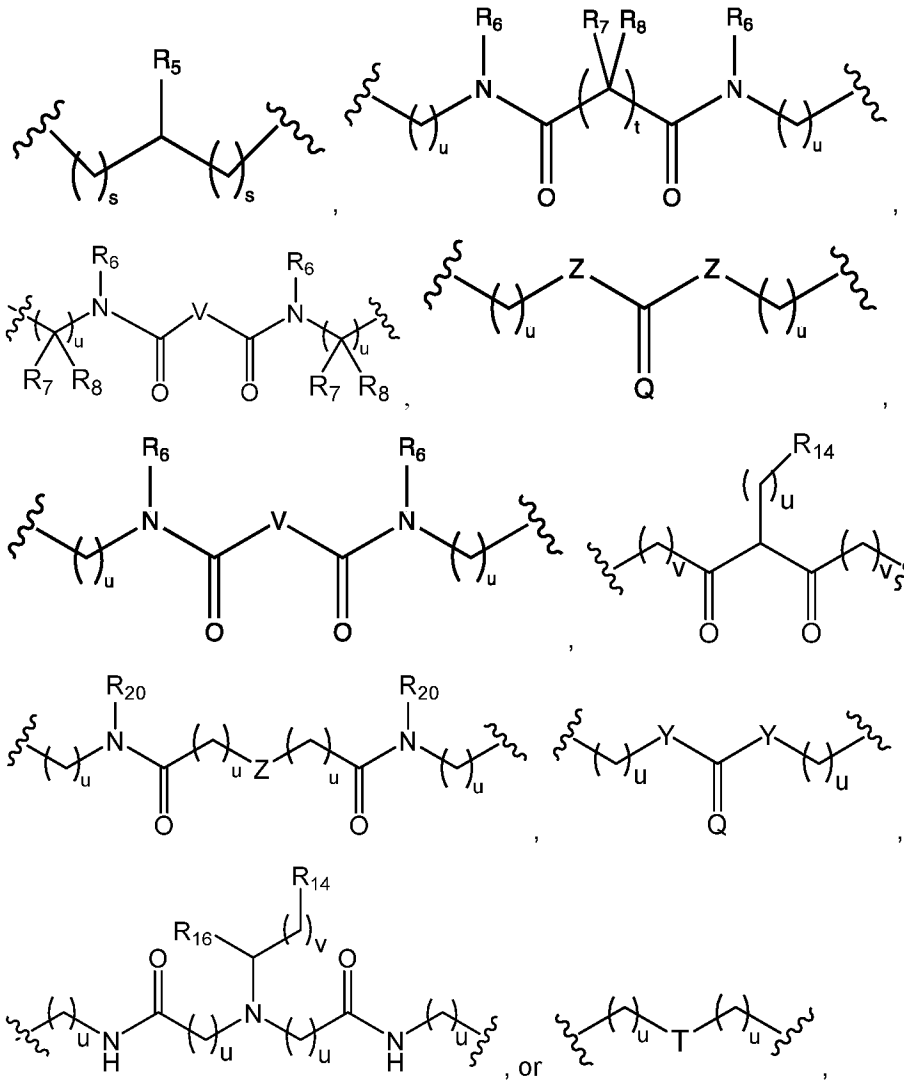
R_{10} and R_{11} are each independently H or C_1-C_3 alkyl; or R_{10} and R_{11} can be taken together to form a heterocyclic ring;

R_{20} and R_{30} are each independently H, C_1-C_5 branched or unbranched alkyl, C_2-C_5 branched or unbranched alkenyl; or R_{20} and R_{30} can be taken together to form a cyclic ring; and

each of v and y is independently 1, 2, 3, or 4;

iii)  (HB-I),

wherein W is



wherein

R_5 is OH, SH, $(CH_2)_sOH$, or $NR_{10}R_{11}$;

each R_6 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each R_7 and R_8 are independently H, C_1-C_3 branched or unbranched alkyl,

C_2-C_3 branched or unbranched alkenyl, halogen, $CH_2)_vOH$, $(CH_2)_vSH$, $(CH_2)_sN(CH_3)_2$, or $NR_{10}R_{11}$, wherein each R_{10} and R_{11} is independently H or C_1-C_3 alkyl, or R_{10} and R_{11} are taken together to form a heterocyclic ring; or R_7 and R_8 are taken together to form a ring;

each R_{20} is independently H, or C_1-C_3 branched or unbranched alkyl;

R_{14} is a heterocyclic, $NR_{10}R_{11}$, $C(O)NR_{10}R_{11}$, $NR_{10}C(O)NR_{10}R_{11}$, or $NR_{10}C(S)NR_{10}R_{11}$, wherein each R_{10} and R_{11} is independently H, C_1-C_3 alkyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloalkenyl, optionally substituted with one or more NH and/or oxo groups, or R_{10} and R_{11} are taken together to form a heterocyclic ring;

R_{16} is H, =O, =S, or CN;

each of s, u, and t is independently 1, 2, 3, 4, or 5;

each v is independently 0, 1, 2, 3, 4, or 5;

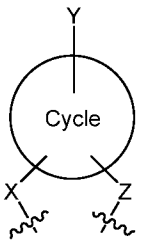
each Y is a divalent heterocyclic;

each Z is independently absent, O, S, or NR_{12}, wherein R_{12} is H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl;

Q is O, S, CH_2, or NR_{13}, wherein each R_{13} is H, C_1-C_5 alkyl;

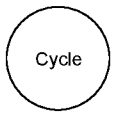
V is branched or unbranched C_2-C_{10} alkylene, C_2-C_{10} alkenylene, C_2-C_{10} alkynylene, or C_2-C_{10} heteroalkylene, optionally substituted with one or more OH, SH, and/or halogen groups; and

T is –NHC(O)O-, –OC(O)NH-, or a divalent heterocyclic; and

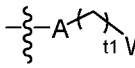
iv) 

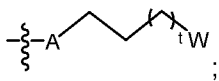
(HC-I),

wherein:



is cyclic or heterocyclic moiety;

Y is alkyl, hydroxy, hydroxyalkyl, 

, or 

;

A is absent, -O-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -N(R^7)C(O)N(R^7)-, -S-, or -S-S-;

each of X and Z is independently absent, -O-, -C(O)-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, or -S-;

each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxy, alkoxy, hydroxyalkyl, alkylamino, alkylaminoalkyl, or aminoalkyl;

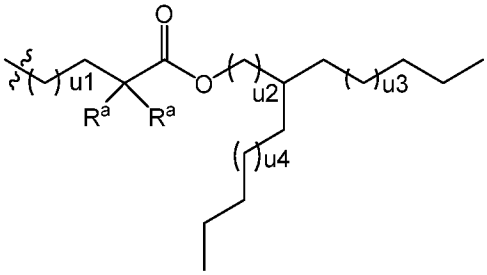
t1 is an integer from 0 to 10; and

W is hydroxyl, substituted or unsubstituted hydroxyalkyl, substituted or unsubstituted amino, substituted or unsubstituted aminocarbonyl, or substituted or

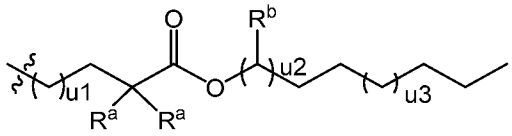
unsubstituted heterocyclyl or heteroaryl; and

wherein the lipid has a pKa from about 4 to about 8.

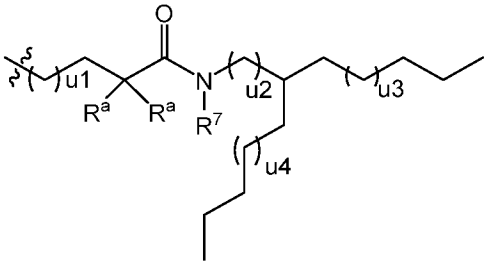
Embodiment 37. The RNA composition of embodiment 36, wherein the ionizable lipid is a compound of group iv), and wherein at least one tail group of the lipid has one of the following formulas:



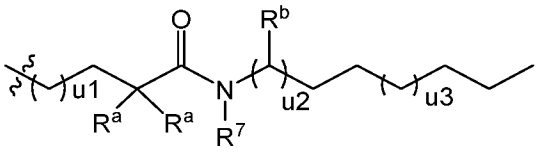
(TII),



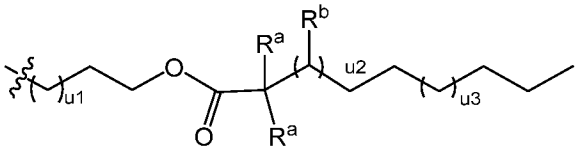
(TIII),



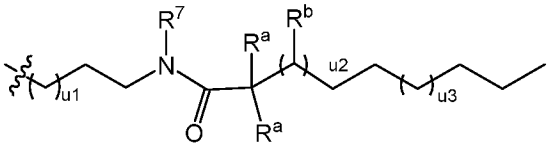
(TIV),



(TV),



(TII'), and



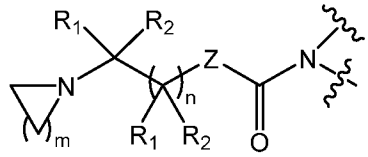
(TIII'), wherein:

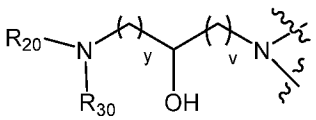
R^7 is each independently H or methyl;

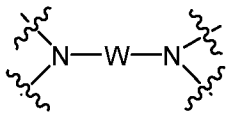
R^b is in each occasion independently H or C_1-C_4 alkyl; and

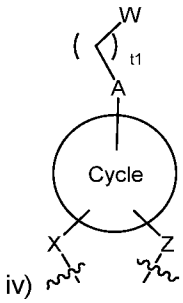
u3 and u4 are each independently 0, 1, 2, 3, 4, 5, 6, or 7; and

the head group has a structure of one of the following formulas:

i)  (HA-IA), wherein m is 1, 2, 3, 4, 5, 6, 7 or 8;

ii)  (HA-VI).

iii)  (HB-I), and

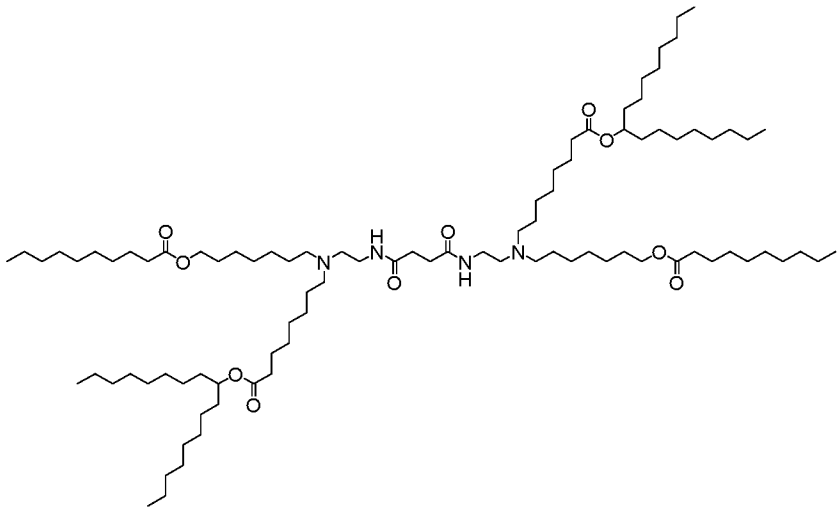


iv) (HC-IB).

Embodiment 38. The RNA composition of embodiment 37, wherein at least one tail group has the structure of formula (TII), (TIII) TIV), (TV), (TII'), and/or (TIII'), wherein u1 is 3-5, u2 is 0-3, wherein u3 and u4 are each independently 1-7, and R^a is each independently methyl.

Embodiment 39. The RNA composition of embodiment 25, wherein the ionizable lipid is a compound in Table I, Table II, Table III, or Table IV.

Embodiment 40. The RNA composition of embodiment 39, wherein the ionizable lipid is



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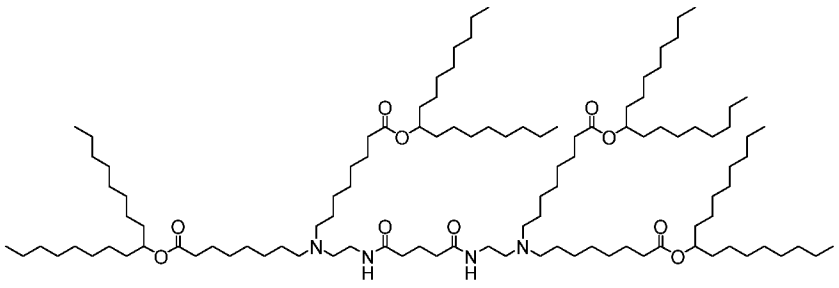

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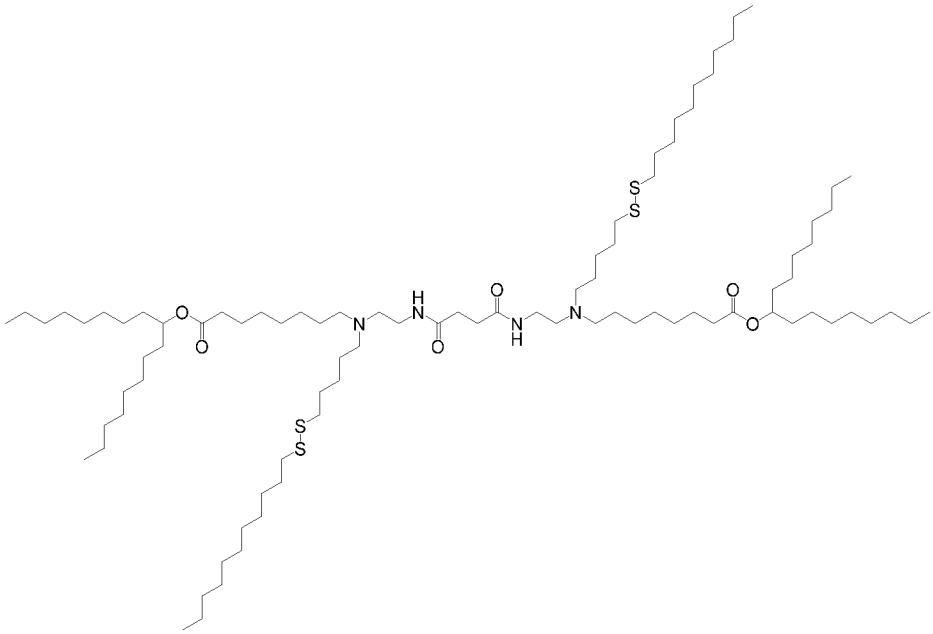

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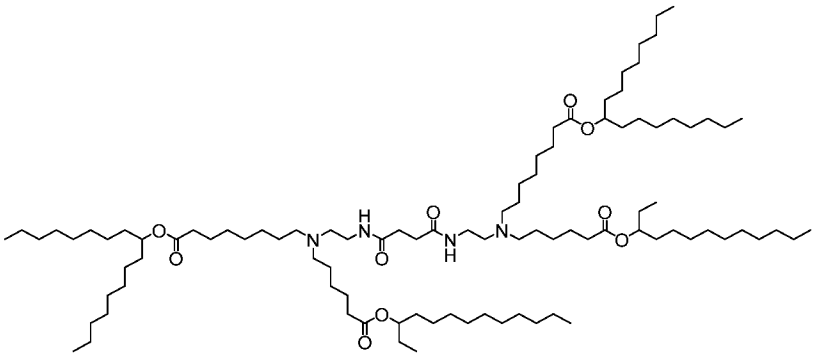

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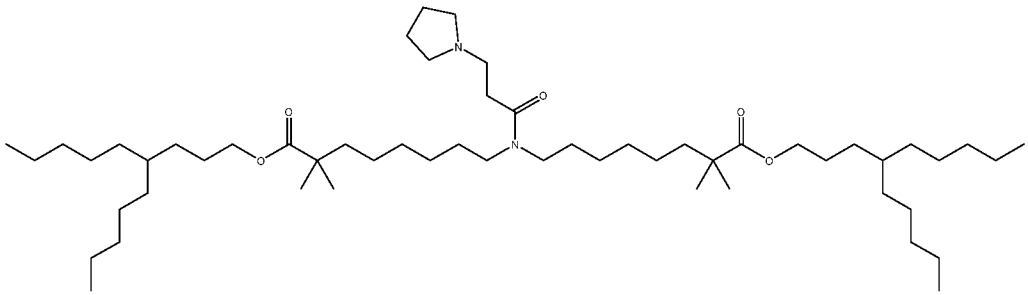

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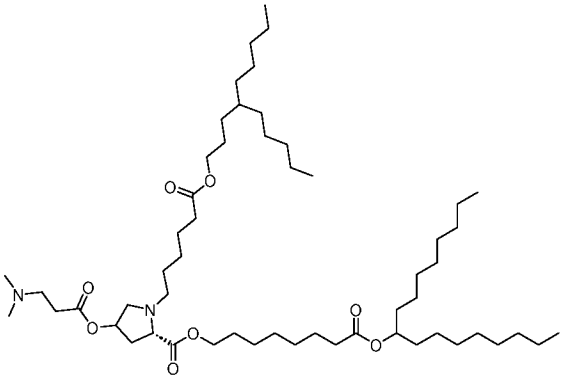

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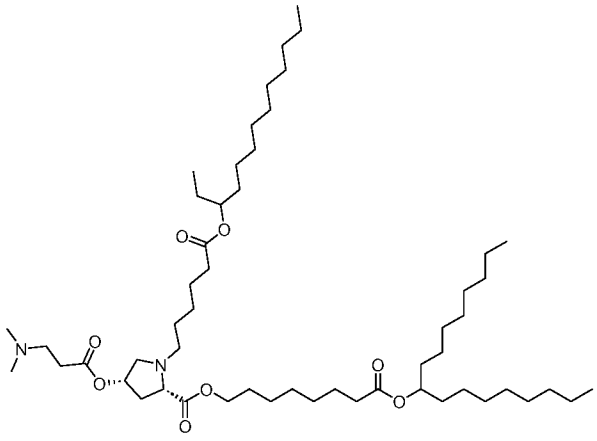

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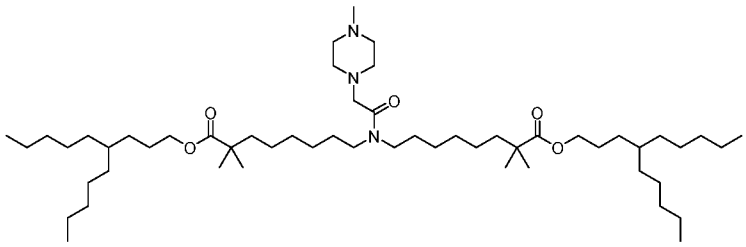

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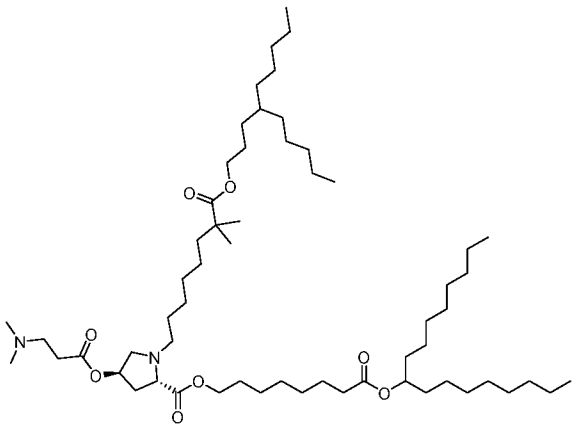

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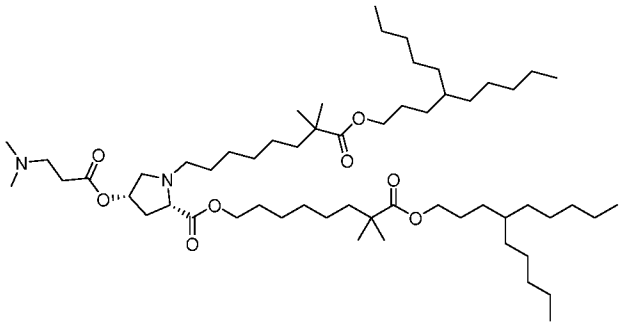

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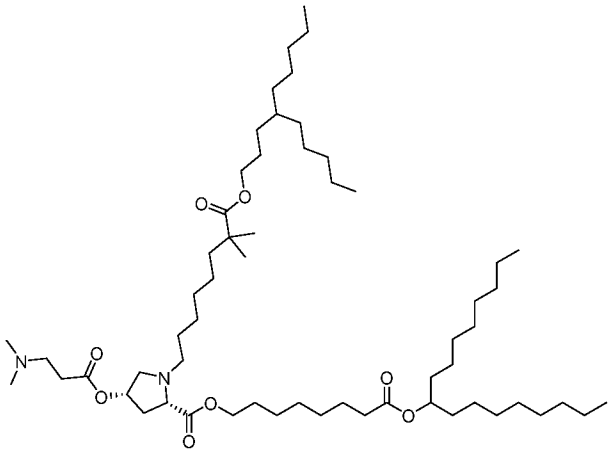

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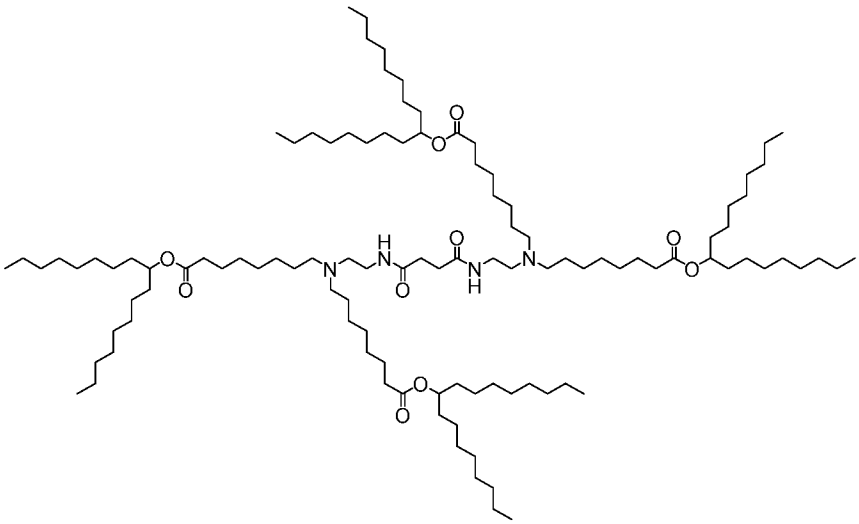

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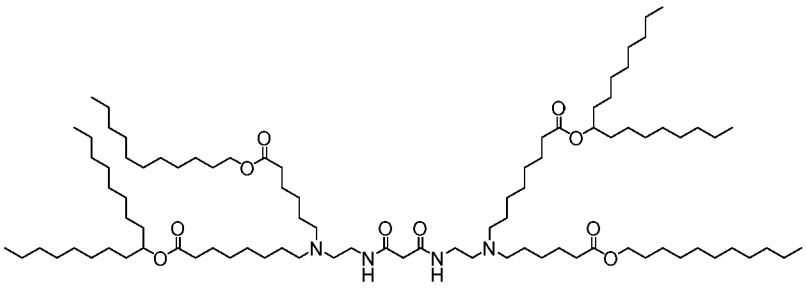
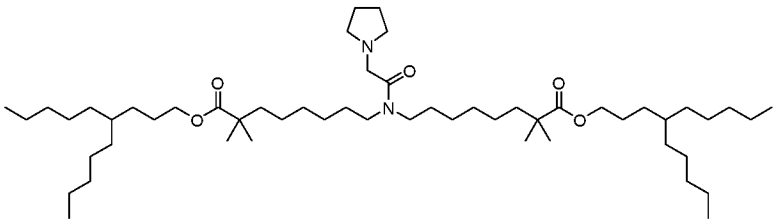


(2220), or



(2335).

Embodiment 41. The RNA composition of embodiment 21, wherein the natural lipids are extracted from lemon or algae.

Embodiment 42. The RNA composition of embodiment 21, wherein the LNMPs further comprise a sterol and a polyethylene glycol (PEG)-lipid conjugate.

Embodiment 43. The RNA composition of embodiment 42, wherein the sterol is cholesterol.

Embodiment 44. The RNA composition of embodiment 42, wherein the PEG lipid conjugate comprises a PEG-2k.

Embodiment 45. The RNA composition of embodiment 42, wherein the PEG lipid conjugate is a PEG-DMG or PEG-PE.

Embodiment 46. The RNA composition of embodiment 42, wherein the PEG lipid conjugate is a PEG2k-DMG or PEG-2k-PE.

Embodiment 47. The RNA composition of embodiment 21, wherein the LNMP comprises:

about 20 mol% to about 50 mol% of the ionizable lipid,
about 20 mol% to about 60 mol% of the natural lipids,
about 7 mol% to about 50 mol% of the sterol, and
about 0.5 mol% to about 3 mol% of the polyethylene glycol (PEG)-lipid conjugate.

Embodiment 48. The RNA composition of embodiment 47, wherein the LNMPs comprise ionizable lipid:natural lipids:sterol:PEG-lipid at a molar ratio of about 35:50:12.5:2.5, about 35:20:42.5:2.5, about 35:16:46.5:2.5, about 50:10:38.5:1.5, or about 50:20:28.5:1.5.

Embodiment 49. The RNA composition of embodiment 47, wherein the amount of the PEG lipid conjugate is about 1.5-2.5 mol%.

Embodiment 50. The RNA composition of embodiment 47, wherein the amount of the ionizable lipid is about 30-50 mol%.

Embodiment 51. The RNA composition of embodiment 50, wherein the amount of the ionizable lipid is about 50 or 35 mol%.

Embodiment 52. The RNA composition of embodiment 47, wherein the N/P ratio is 6 ± 1.

Embodiment 53. The RNA composition of embodiment 47, wherein the N/P ratio is 3 ± 1.

Embodiment 54. The RNA composition of embodiment 47, wherein the N/P ratio is 15 ± 1.

Embodiment 55. The RNA composition of any one of the preceding embodiments, wherein the gene editing system comprises an RNA-guided DNA-binding agent.

Embodiment 56. The RNA composition of embodiment 55, wherein the gene editing system is CRISPR-Cas gene editing system.

Embodiment 57. The RNA composition of embodiment 55, wherein the one or more polynucleotides comprise an mRNA or modified mRNA.

Embodiment 58. The RNA composition of embodiment 55, wherein the RNA-guided DNA-binding agent is a Cas nuclease mRNA.

Embodiment 59. The RNA composition of embodiment 58, wherein the Cas nuclease mRNA is a Class II Cas nuclease mRNA.

Embodiment 60. The RNA composition of embodiment 59, wherein the Class II Cas nuclease is a Cas9 nuclease mRNA.

Embodiment 61. The RNA composition of embodiment 55, wherein the one or more polynucleotides comprise a gRNA or modified gRNA.

Embodiment 62. The RNA composition of embodiment 55, wherein the one or more polynucleotides comprise a gRNA and a Class II Cas nuclease mRNA.

Embodiment 63. The RNA composition of embodiment 55, wherein the one or more polynucleotides comprise an RNA comprising an open reading frame encoding an RNA-guided DNA-binding agent, wherein the open reading frame has a uridine content ranging from its minimum uridine content to 150% of the minimum uridine content.

Embodiment 64. The RNA composition of embodiment 63, wherein the one or more polynucleotides comprise an mRNA comprising an open reading frame encoding an RNA-guided DNA-binding agent, wherein the open reading frame has a uridine dinucleotide content ranging from its minimum uridine dinucleotide content to 150% of the minimum uridine dinucleotide content.

Embodiment 65. The RNA composition of embodiment 61 or 62, wherein the gRNA is a dual-guide RNA (dgRNA) or an sgRNA.

Embodiment 66. The RNA composition of embodiment 61, wherein the gRNA is a modified gRNA comprising a modification selected from the group consisting of 2'-O-methyl (2'-O-Me) modified nucleotide, a phosphorothioate (PS) bond between nucleotides, and a 2'-fiuoro (2'-F) modified nucleotide.

Embodiment 67. The RNA composition of embodiment 61, wherein the gRNA is a modified gRNA comprising a modification at one or more of the first five nucleotides at the 5' end or the 3' end.

Embodiment 68. The RNA composition of embodiment 61, wherein the gRNA is a modified gRNA comprising PS bonds between the first four nucleotides or the last four nucleotides.

Embodiment 69. The RNA composition of any of embodiments 66-68, wherein the modified gRNA further comprises 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end or the 3' end.

Embodiment 70. The RNA composition of embodiment 62, wherein the gRNA and Class 2 Cas nuclease mRNA are present in a ratio ranging from about 10:1 to about 1:10 by weight.

Embodiment 71. The RNA composition of embodiment 62, wherein the gRNA and Class 2 Cas nuclease mRNA are present in a ratio ranging from about 5:1 to about 1:5 by weight.

Embodiment 72. The RNA composition of embodiment 62, wherein the gRNA and Class 2 Cas nuclease mRNA are present in a ratio ranging from about 2:1 to about 1:2 by weight.

Embodiment 73. The RNA composition of embodiment 62, wherein the gRNA and Class 2 Cas nuclease mRNA are present in a ratio of about 2:1 by weight or about 1:1 by weight.

Embodiment 74. The RNA composition of embodiment 55, further comprising at least one template nucleic acid.

EXAMPLES

[0650] The invention now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1: Preparation and modification of LPMP, and formulation LPMP with mRNAs

[0651] The preparation of plant messenger packs (PMP), modification of PMP to prepare lipid reconstructed plant messenger packs (LPMP) and formulation of PMP and LPMP with mRNAs may be accomplished utilizing the methods disclosed in International Patent Application Publication No. WO 2021/041301, which is incorporated herein by reference in its entirety.

[0652] In particular, all the experimental protocols disclosed in Examples 1-17 of International Patent Application Publication No. WO 2021/041301, are incorporated herein by reference in their entirety, including: Example 1. Isolation of Plant Messenger Packs from plants; Example 2. Production of purified Plant Messenger Packs (PMPs); Example 3. Plant Messenger Pack characterization; Example 4. Characterization of Plant Messenger Pack stability; Example 5. Loading PMPs with cargo; Example 6. Increasing PMP cellular uptake by formulation of PMPs with ionic liquids; Example 7. Modification of PMPs using ionizable lipids; Example 8. Formulation of LPMPs with microfluidics; Example 9. mRNA loading and delivery into lipid-reconstructed PMPs using ionizable lipids; Example

10. Cellular uptake of natural and reconstructed PMPs, with and without ionizable lipid modifications; Example 11. Increasing PMP cellular uptake by formulation of PMPs with cationic lipids; Example 12. Modification of PMPs using cationic lipids; Example 13. mRNA loading and delivery into lipid-reconstructed PMPs using cationic lipids; Example 14. Cellular uptake of natural and reconstructed PMPs, with and without cationic lipid modifications; Example 15. Improved loading using the cationic lipids GL67 and Ethyl PC; Example 16. Optimization of lipid ratios for mRNA loading; and Example 17. Optimization of lipid ratios for plasmid loading.

General procedure to prepare natural lipid extract component

[0653] The general preparation of a natural lipid extract component can begin with isolation of lipid extract from a natural source. Briefly, the method of extraction for plant lipids is as follows:

1) Plant matter (e.g. juice, pulp, or a blended part of the plant) is collected from the natural plant source.
2) The plant matter is filtered and concentrated.
3) The plant concentrate is diafiltered with a citrate-sodium chloride buffer to generate Diafiltered Intermediate (DFI), the starting material for extraction.
4) Lipids are extracted by adding an extracting solvent (e.g. dichloromethane (DCM) and methanol (MeOH)). Alternative extracting solvents in this process can include chloroform : methanol and ethyl acetate : ethanol.
5) Water is added to induce phase separation.
6) The resulting organic phase is collected and dried using a rotary evaporator (rotovap).
7) The dried lipids are resuspended in an aqueous solution (e.g. 90% DCM and 10% MeOH solution) and are transferred to vials to be dried (e.g. via Genevac or similar drying equipment).
8) The resulting lipid extract is stored dry at -20^{0}C.

Example 2: Preparation of an RNA composition

Manufacture and characterization of polynucleotides

[0654] The manufacture of polynucleotides and/or parts or regions thereof may be accomplished utilizing the methods taught in International Patent Application Publication No. WO 2014/152027, which is incorporated herein by reference in its entirety. Purification methods may include those taught in International Patent Application Publication Nos. WO2014/152030 and WO2014/152031, which are incorporated herein by reference in their entirety. Detection and characterization methods of the polynucleotides may be performed as taught in International Patent Application Publication No. WO2014/144039, which is incorporated herein by reference in its entirety. Characterization of the polynucleotides may be accomplished using polynucleotide mapping, reverse transcriptase sequencing, charge distribution analysis, detection of RNA impurities, or any combination of two or more of the foregoing. “Characterizing” comprises determining the RNA transcript sequence, determining the purity of the RNA transcript, or determining the charge heterogeneity of the RNA transcript, for example. Such methods are taught in, for example, International Patent Application Publication Nos. WO2014/144711 and WO2014/144767, which are incorporated herein by reference

in their entirety.

[0655] Additional details of the mRNA design and modifications may be found in WO 2021/154763 and WO 2021/188969, which are incorporated herein by reference in their entirety.

Formulation of LPMP with an mRNA

General protocols and experimental designs

[0656] The protocols and detailed experimental procedures of the preparation of plant messenger packs (PMP), and modification of PMP to prepare lipid reconstructed plant messenger packs (LPMP), and formulations of PMP and LPMP with mRNAs have been discussed in Example 1, which lists all the experimental protocols disclosed in Examples 1-17 of International Patent Application Publication No. WO 2021/041301, all of which are incorporated herein by reference in their entirety. These protocols and detailed experimental procedures were followed when preparing PMP, LPMP, and PMP or LPMP formulated with mRNAs in this example.

[0657] Briefly, the isolation and purification of crude plant messenger packs (PMPs) from lemon and algae, and characterization of these PMPs followed the experimental designs and protocols for plants sources described in Example 1: Isolation of Plant Messenger Packs from plants; Example 2: Production of purified Plant Messenger Packs (PMPs); Example 3: Plant Messenger Pack characterization; and Example 4: Characterization of Plant Messenger Pack stability, all of International Patent Application Publication No. WO 2021/041301, which is incorporated herein by reference in its entirety.

[0658] The modifications of natural PMP or reconstructed lemon LPMPs with cholesterol and PEG-lipid followed the experimental designs and protocols for plants sources described in Example 6: Increasing PMP cellular uptake by formulation of PMPs with ionic liquids; Example 7: Modification of PMPs using ionizable lipids; Example 10: Cellular uptake of natural and reconstructed PMPs, with and without ionizable lipid modifications; Example 11: Increasing PMP cellular uptake by formulation of PMPs with cationic lipids; Example 12: Modification of PMPs using cationic lipids; and Example 14: Cellular uptake of natural and reconstructed PMPs, with and without cationic lipid modifications, all of International Patent Application Publication No. WO 2021/041301, which is incorporated herein by reference in its entirety.

[0659] Formulations of the PMPs and lemon-lipid reconstructed LPMPs with mRNAs followed the experimental designs and protocols for nucleic acids loading described in Example 5: Loading PMPs with cargo; Example 8: Formulation of LPMPs with microfluidics; Example 9: mRNA loading and delivery into lipid-reconstructed PMPs using ionizable lipids; Example 13: mRNA loading and delivery into lipid-reconstructed PMPs using cationic lipids; Example 15: Improved loading using the cationic lipids GL67 and Ethyl PC; Example 16: Optimization of lipid ratios for mRNA loading; and Example 17: Optimization of lipid ratios for plasmid loading, all of International Patent Application Publication No. WO 2021/041301, which is incorporated herein by reference in its entirety.

Formulations for LPMP / mRNA with C12-200 as ionizable lipid

[0660] This example describes the formulation of lemon lipid reconstructed LPMP formulated with ionizable lipids, sterols, and PEG lipids, to encapsulate an mRNA for LPMP / mRNA formulation.

[0661] In this example, lemon PMP lipids were used as the PMP natural lipids; C12-200 [1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol)] was used as the ionizable lipids; cholesterol (14:0) was used as the sterols; DMPE-PEG2k was used as model PEGylated lipids.

[0662] **LNP formulations.** A LNP (lipid nanoparticle) formulation, as control, was prepared to result in ionizable lipid:structural lipid:sterol:PEG-lipid (C12-200:DOPE:cholesterol (14:0): DMPE-PEG2k) at a molar ratio of 35:16:46.5:2.5, respectively. To prepare this formulation, the above lipids were solubilized in ethanol, mixed at the above molar ratios, and diluted in ethanol (organic phase) to obtain total lipid concentration of 5.5 mM. An mRNA solution (aqueous phase) was prepared with RNAse-free water and 100 mM citrate buffer pH 3 for a final concentration of 50 mM citrate buffer. The formulations were maintained at an ionizable lipid to mRNA at an ionizable lipid nitrogen:mRNA phosphate (N:P) ratio of 15:1.

[0663] **LPMP formulations.** Reconstructed lemon (recLemon) LPMP formulation was prepared to result in ionizable lipid:natural lipids:sterol:PEG-lipid (C12-200:lemon lipid:cholesterol (14:0): DMPE-PEG2k) at a molar ratio of 35:50:12.5:2.5. To prepare this formulation, the above lipids were solubilized in ethanol, except for lemon lipid, which was solubilized in 4:1 DMF:methanol. The lipids were then mixed at the above molar ratios, and diluted to obtain total lipid concentration of 5.5 mM. An mRNA solution (aqueous phase) was prepared with RNAse-free water and 100 mM citrate buffer pH 3 for a final concentration of 50 mM citrate buffer.

[0664] The lipid mixture and mRNA solution were mixed at a 1:3 ratio by volume, respectively, on the NanoAssemblr Ignite (Precision Nanosystems) at a total flow rate of 9 mL/min. The resulting formulations were then loaded into Slide-A-Lyzer G2 dialysis cassettes (10k MWCO) and dialyzed in 200 times sample volume of 1x PBS for 4 hours at room temperature with gentle stirring. The PBS solution was refreshed, and the formulations were further dialyzed for at least 14 hours at 4 ºC with gentle stirring. The dialyzed formulations were then collected and concentrated by centrifugation at 3000xg (Amicon Ultra centrifugation filters, 100k MWCO).

[0665] The concentrated particles were characterized for size, polydispersity, and particle concentration using Zetasizer Ultra (Malvern Panalytical). The mRNA encapsulation efficiency was characterized by Quant-iT RiboGreen RNA Assay Kit (ThermoFisher Scientific). The particles were diluted to the desired mRNA concentration to get a final 10% sucrose solution in PBS. The formulations were then flash frozen in liquid nitrogen.

[0666] The resulting formulations with mRNA are shown in Table 2.

Table 2. The characterizations of a typical LPMP formulation with mRNA

Formulation	Ionizable: Structural: Sterol: PEG-lipid Molar ratio	Ionizable lipid	Structural lipid	Sterol	PEG-lipid	Cargo	N:P	Size (nm)	PDI	Encapsulation efficiency (%)
LNP / mRNA	*35:16:46.5:2.5*	C12-200	DOPE	Chol	DMPE-PEG2k	an mRNA	15:1	78.47	0.126	94.4

recLemon **LPMP / mRNA**	*35:50:12.5:2.5*	C12-200	Naturally derived lemon lipid	Chol	DMPE-PEG2k	an mRNA		96.49	0.300	94.8

Example 3. LPMP / mRNA and LNP / mRNA formulations for gene editing in mice

[0667] If not specified, the LNP / mRNA or LPMP / mRNA formulation were prepared according to those described in Example 2. The coding portion of the delivered cargo Cas9 mRNA (TriLink BioTechnologies, CleanCap Cas9 mRNA (modified)) is as follows:

AUGGCCCCCAAGAAGAAGCGGAAGGUGGGCAUCCACGGCGUGCCCGCCGCCGACAAGAAG
UACAGCAUCGGCCUGGACAUCGGCACCAACAGCGUGGGCUGGGCCGUGAUCACCGACGAG
UACAAGGUGCCCAGCAAGAAGUUCAAGGUGCUGGGCAACACCGACCGGCACAGCAUCAAG
AAGAACCUGAUCGGCGCCCUGCUGUUCGACAGCGGCGAGACCGCCGAGGCCACCCGGCU
GAAGCGGACCGCCCGGCGGCGGUACACCCGGCGGAAGAACCGGAUCUGCUACCUGCAGG
AGAUCUUCAGCAACGAGAUGGCCAAGGUGGACGACAGCUUCUUCCACCGGCUGGAGGAGA
GCUUCCUGGUGGAGGAGGACAAGAAGCACGAGCGGCACCCCAUCUUCGGCAACAUCGUGG
ACGAGGUGGCCUACCACGAGAAGUACCCCACCAUCUACCACCUGCGGAAGAAGCUGGUGG
ACAGCACCGACAAGGCCGACCUGCGGCUGAUCUACCUGGCCCUGGCCCACAUGAUCAAGU
UCCGGGGCCACUUCCUGAUCGAGGGCGACCUGAACCCCGACAACAGCGACGUGGACAAGC
UGUUCAUCCAGCUGGUGCAGACCUACAACCAGCUGUUCGAGGAGAACCCCAUCAACGCCA
GCGGCGUGGACGCCAAGGCCAUCCUGAGCGCCCGGCUGAGCAAGAGCCGGCGGCUGGAG
AACCUGAUCGCCCAGCUGCCCGGCGAGAAGAAGAACGGCCUGUUCGGCAACCUGAUCGCC
CUGAGCCUGGGCCUGACCCCCAACUUCAAGAGCAACUUCGACCUGGCCGAGGACGCCAAG
CUGCAGCUGAGCAAGGACACCUACGACGACGACCUGGACAACCUGCUGGCCCAGAUCGGC
GACCAGUACGCCGACCUGUUCCUGGCCGCCAAGAACCUGAGCGACGCCAUCCUGCUGAGC
GACAUCCUGCGGGUGAACACCGAGAUCACCAAGGCCCCCCUGAGCGCCAGCAUGAUCAAG
CGGUACGACGAGCACCACCAGGACCUGACCCUGCUGAAGGCCCUGGUGCGGCAGCAGCU
GCCCGAGAAGUACAAGGAGAUCUUCUUCGACCAGAGCAAGAACGGCUACGCCGGCUACAU
CGACGGCGGCGCCAGCCAGGAGGAGUUCUACAAGUUCAUCAAGCCCAUCCUGGAGAAGAU
GGACGGCACCGAGGAGCUGCUGGUGAAGCUGAACCGGGAGGACCUGCUGCGGAAGCAGC
GGACCUUCGACAACGGCAGCAUCCCCCACCAGAUCCACCUGGGCGAGCUGCACGCCAUCC
UGCGGCGGCAGGAGGACUUCUACCCCUUCCUGAAGGACAACCGGGAGAAGAUCGAGAAGA
UCCUGACCUUCCGGAUCCCCUACUACGUGGGCCCCCUGGCCCGGGGCAACAGCCGGUUC
GCCUGGAUGACCCGGAAGAGCGAGGAGACCAUCACCCCCUGGAACUUCGAGGAGGUGGU
GGACAAGGGCGCCAGCGCCCAGAGCUUCAUCGAGCGGAUGACCAACUUCGACAAGAACCU
GCCCAACGAGAAGGUGCUGCCCAAGCACAGCCUGCUGUACGAGUACUUCACCGUGUACAA
CGAGCUGACCAAGGUGAAGUACGUGACCGAGGGCAUGCGGAAGCCCGCCUUCCUGAGCG
GCGAGCAGAAGAAGGCCAUCGUGGACCUGCUGUUCAAGACCAACCGGAAGGUGACCGUGA
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AGGACAAGGACUUCCUGGACAACGAGGAGAACGAGGACAUCCUGGAGGACAUCGUGCUGA
CCCUGACCCUGUUCGAGGACCGGGAGAUGAUCGAGGAGCGGCUGAAGACCUACGCCCACC

UGUUCGACGACAAGGUGAUGAAGCAGCUGAAGCGGCGGCGGUACACCGGCUGGGGCCGG
CUGAGCCGGAAGCUGAUCAACGGCAUCCGGGACAAGCAGAGCGGCAAGACCAUCCUGGAC
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CUGACCUUCAAGGAGGACAUCCAGAAGGCCCAGGUGAGCGGCCAGGGCGACAGCCUGCAC
GAGCACAUCGCCAACCUGGCCGGCAGCCCCGCCAUCAAGAAGGGCAUCCUGCAGACCGUG
AAGGUGGUGGACGAGCUGGUGAAGGUGAUGGGCCGGCACAAGCCCGAGAACAUCGUGAU
CGAGAUGGCCCGGGAGAACCAGACCACCCAGAAGGGCCAGAAGAACAGCCGGGAGCGGAU
GAAGCGGAUCGAGGAGGGCAUCAAGGAGCUGGGCAGCCAGAUCCUGAAGGAGCACCCCGU
GGAGAACACCCAGCUGCAGAACGAGAAGCUGUACCUGUACUACCUGCAGAACGGCCGGGA
CAUGUACGUGGACCAGGAGCUGGACAUCAACCGGCUGAGCGACUACGACGUGGACCACAU
CGUGCCCCAGAGCUUCCUGAAGGACGACAGCAUCGACAACAAGGUGCUGACCCGGAGCGA
CAAGAACCGGGGCAAGAGCGACAACGUGCCCAGCGAGGAGGUGGUGAAGAAGAUGAAGAA
CUACUGGCGGCAGCUGCUGAACGCCAAGCUGAUCACCCAGCGGAAGUUCGACAACCUGAC
CAAGGCCGAGCGGGGCGGCCUGAGCGAGCUGGACAAGGCCGGCUUCAUCAAGCGGCAGC
UGGUGGAGACCCGGCAGAUCACCAAGCACGUGGCCCAGAUCCUGGACAGCCGGAUGAACA
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AGCUGGUGAGCGACUUCCGGAAGGACUUCCAGUUCUACAAGGUGCGGGAGAUCAACAACU
ACCACCACGCCCACGACGCCUACCUGAACGCCGUGGUGGGCACCGCCCUGAUCAAGAAGU
ACCCCAAGCUGGAGAGCGAGUUCGUGUACGGCGACUACAAGGUGUACGACGUGCGGAAGA
UGAUCGCCAAGAGCGAGCAGGAGAUCGGCAAGGCCACCGCCAAGUACUUCUUCUACAGCA
ACAUCAUGAACUUCUUCAAGACCGAGAUCACCCUGGCCAACGGCGAGAUCCGGAAGCGGC
CCCUGAUCGAGACCAACGGCGAGACCGGCGAGAUCGUGUGGGACAAGGGCCGGGACUUC
GCCACCGUGCGGAAGGUGCUGAGCAUGCCCCAGGUGAACAUCGUGAAGAAGACCGAGGUG
CAGACCGGCGGCUUCAGCAAGGAGAGCAUCCUGCCCAAGCGGAACAGCGACAAGCUGAUC
GCCCGGAAGAAGGACUGGGACCCCAAGAAGUACGGCGGCUUCGACAGCCCCACCGUGGCC
UACAGCGUGCUGGUGGUGGCCAAGGUGGAGAAGGGCAAGAGCAAGAAGCUGAAGAGCGU
GAAGGAGCUGCUGGGCAUCACCAUCAUGGAGCGGAGCAGCUUCGAGAAGAACCCCAUCGA
CUUCCUGGAGGCCAAGGGCUACAAGGAGGUGAAGAAGGACCUGAUCAUCAAGCUGCCCAA
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UGCAGAAGGGCAACGAGCUGGCCCUGCCCAGCAAGUACGUGAACUUCCUGUACCUGGCCA
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UGAUCCUGGCCGACGCCAACCUGGACAAGGUGCUGAGCGCCUACAACAAGCACCGGGACA
AGCCCAUCCGGGAGCAGGCCGAGAACAUCAUCCACCUGUUCACCCUGACCAACCUGGGCG
CCCCCGCCGCCUUCAAGUACUUCGACACCACCAUCGACCGGAAGCGGUACACCAGCACCA
AGGAGGUGCUGGACGCCACCCUGAUCCACCAGAGCAUCACCGGCCUGUACGAGACCCGGA
UCGACCUGAGCCAGCUGGGCGGCGACAGCGGCGGCAAGCGGCCCGCCGCCACCAAGAAG
GCCGGCCAGGCCAAGAAGAAGAAGGGCAGCUACCCCUACGACGUGCCCGACUACGCCUGA

[0668] The coding portion of the delivered cargo sgRNA mTTR_G211 (Synthego) is as follows:

UUACAGCCACGUCUACAGCA

and had an added Synthego modified EZ scaffold. The sgRNA was provided as a 50nmol powder aliquot, along with nuclease free water to resuspend in; sgRNA stock was dissolved at 1mg/mL in water prior to use.

Formulation and characterization

[0669] This example further describes the formulation of exemplary lemon LPMPs with ionizable lipids, sterols, and PEG lipids, to encapsulate an exemplary mRNA having gene-editing capabilities (e.g., 1:1 Cas9:mTTR_G211) for LPMP / mRNA formulation and LNPs formulated with ionizable lipids, sterols, and PEG lipids, to encapsulate mRNA (e.g., 1:1 Cas9:mTTR_G211).

[0670] **LNP formulation** was prepared according to Example 2, composed of ionizable lipid:structural lipid:sterol:PEG-lipid at given molar ratios outlined in Table 3. Lipids were solubilized in ethanol. These lipids were mixed at the indicated molar ratios and diluted in ethanol (organic phase) to 5.5 mM total lipid concentration and the mRNA solution (aqueous phase) was prepared with RNAse-free water and 100 mM citrate buffer pH 3 for a final concentration of 50 mM citrate buffer. Formulations were maintained at ionizable lipid to mRNA N:P ratio of 6:1 for each ionizable lipid as listed in Table 3.

[0671] Exemplary lemon **LPMP compositions** (recLemon LPMP) were prepared according to Example 2, composed of ionizable lipid:natural lipids:sterol:PEG-lipid at given molar ratios outlined in Table 3. Lipids were solubilized in ethanol. Formulations were then handled as above. Formulations were maintained at ionizable lipid to mRNA N:P ratio of 6:1 for each ionizable lipid as listed in Table 3.

[0672] The lipid mix and mRNA solution were mixed at a 1:3 ratio by volume, respectively, on the NANOASSEMBLR® IGNITE™ (Precision Nanosystems) at a total flow rate of 14 mL/minute. The resulting formulations were then loaded into Slide-A-Lyzer G2 dialysis cassettes (10k MWCO) and dialyzed against 1x PBS for 2 hours at room temperature. The PBS was refreshed, and the formulations were further dialyzed for at least 14 hours at 4 ^{0}C with gentle stirring. The dialyzed formulations were then collected and concentrated by tangential flow filtration (TFF) using Sartorius VIVAFLOW® 50R cassettes (100k MWCO) at fixed feed flowrate of 150ml/minute. The TFF-concentrated formulations were then further concentrated by centrifugation at 3000xg using AMICON® Ultra centrifugation filters (100k MWCO). The concentrated formulations were characterized for size, polydispersity, and particle concentration using a Zetasizer Ultra (Malvern Panalytical) and for mRNA encapsulation efficiency using QUANT-IT™ RIBOGREEN® RNA Assay Kit (ThermoFisher Scientific).

Table 3. Characterizations of LPMP/LNP formulations with gene editing capabilities

Formulation	Ionizable: Structural: Sterol: PEG-lipid Molar ratio	Ionizable lipid	Structural lipid	Sterol	PEG-lipid	Cargo	Size (nm)	Encapsulation efficiency (%)
recLemon LPMP	*35:20:42.5:2.5*	C12-200	Naturally derived lemon lipid	Chol	DMG-PEG	1:1 Cas9:sgG211	50-100	95.4
recLemon LPMP 2220	*35:20:42.5:2.5*	2220	Naturally derived lemon lipid	Chol	DMG-PEG	1:1 Cas9:sgG211	50-100	89.1

recLemon LPMP 2275	*35:20:42.5:2.5*	2275	Naturally derived lemon lipid	Chol	DMG-PEG	1:1 Cas9:sgG211	50-100	88.8
recLemon LPMP 2272	*35:20:42.5:2.5*	2272	Naturally derived lemon lipid	Chol	DMG-PEG	1:1 Cas9:sgG211	50-100	88.0
recLemon LPMP 2335	*50:20:28.5:1.5*	2335	Naturally derived lemon lipid	Chol	DMG-PEG	1:1 Cas9:sgG211	50-100	87.3
LNP 2220	*35:16:46.5:2.5*	2220	DOPE	Chol	DMG-PEG	1:1 Cas9:sgG211	50-100	88.2
LNP 2275	*35:16:46.5:2.5*	2275	DOPE	Chol	DMG-PEG	1:1 Cas9:sgG211	50-100	87.3
LNP 2272	*35:16:46.5:2.5*	2272	DOPE	Chol	DMG-PEG	1:1 Cas9:sgG211	50-100	87.6
LNP 2335	*50:10:38.5:1.5*	2335	DSPC	Chol	DMG-PEG	1:1 Cas9:sgG211	50-100	86.4

Editing of TTR gene in mice

[0673] 8-9 week old Bl6 mice were utilized for genetic editing screening efforts of the formulations provided in Table 7, with PBS acting as a negative control. Mice were obtained from Jackson Laboratories and allowed to acclimate for one week prior to manipulations. Animals were placed under a heat lamp for a few minutes before introducing them to a restraining chamber. The tail was wiped with alcohol pads (Fisher Scientific) and, for each LPMP or LNP composition described in Table 3, mice were bled prior to a 100 µL of a composition containing mRNA (1:1 Cas9:sgmTTR_G211) or PBS being injected intravenously using a 29G insulin syringe (Covidien). Blood collection was performed using a 25G insulin syringe (BD). Once all blood samples were collected, tubes are spun at 2000G for 10 minutes using a tabletop centrifuge and plasma was aliquoted into individual Eppendorf tubes (Fisher Scientific) and stored at -80 °C for subsequent TTR quantification. TTR levels in plasma were determined using TTR ELISA kit (Aviva Systems Biology), and instructions provided by the kit were followed accordingly, with plasma diluted 1:10,000. Mice were placed into a CO_2 chamber for euthanasia at the appropriate day post-dose. Tissue and marrow samples were taken, and gRNA was extracted. Briefly, primers were used to amplify the gene of interest (TTR) in the given samples, and the amplicons were sequenced. Sequences were then aligned to the mouse reference genome. The percentage of editing was calculated as the total number of sequences with insertions or deletions over the total number of sequence reads.

[0674] In an initial experiment, recLemon LPMP formulation (given in Table 3) was administered according to the doses and route in Table 4. Systemic concentrations of TTR protein in plasma were measured using an ELISA kit from mouse bleeds taken prior to the dose, two days post-dose (D2, 48h), and seven days post-dose (D7, 168h). The results are shown in Figures 1A and 1B. Figure 1A depicts a decrease in TTR concentration (ug/mL) in mice given recLemon LPMP (3 mg/kg, 1:1 Cas9:sgG211) as compared to the control mice given PBS, indicating a successful gene editing. The percentage of gene editing in the liver (the target organ as the major source of TTR), bone marrow, and spleen after 7 days post-dose was measured (Figure 1B). As shown in Figure 1B, the results of

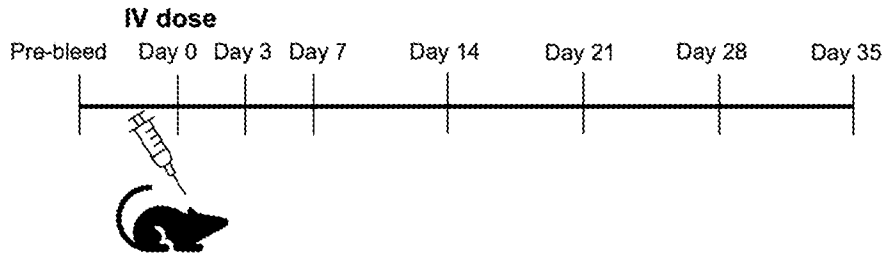
gene editing in the liver of the mice given recLemon LPMP exceeded the 10% lower limit of editing.

Table 4

Treatment	Number of Animals	Dose Route	Payload	Dose
PBS	5	IV; 100uL	-	-
recLemon LPMP	5	IV; 100uL	1:1 Cas9: sgG211	3 mg/kg

[0675] A variety of recLemon LPMPs and LNPs (formulations provided in Table 3) were further tested for gene editing capabilities. Doses were administered according to Table 5 and Scheme 1, which shows the experimental design with bleeds taken on the given days. Systemic concentrations of the TTR protein in plasma were measured from mouse bleeds taken at Day 0, 3 (72h), 7 (168h), 14 (336h), 21 (504h), 28 (672h), and 35 (840h). Figure 2A and 2B show that the majority of recLemon LPMP and LNP formulations performed well as evidenced through the decreased TTR protein, and the performances of the recLemon LPMP formulations were at least comparable to the performances of the LNP formulations.

[0676] The percentage of TTR protein from the baseline in the blood of mice 7, 21, 28, and 35 days after the intravenous dose was measured and the results are shown in Figures 3-6. The percentage of TTR protein from the baseline is calculated as the measured TTR post-dose over the measured TTR pre-dose times 100. As shown in Figures 3-6, the majority of recLemon LPMP and LNP formulations showed a decrease in TTR protein, with many around or below 10% of baseline; the performances of some recLemon LPMP formulations were comparable to the performances of the LNP formulations.



Scheme 1

Table 5

Treatment	Number of Animals	Dose Route	Payload	Dose
PBS	3	IV; 100uL	-	-
recLemon LPMP 2220	3	IV; 100uL	1:1 Cas9: sgG211	1 mg/kg
recLemon LPMP 2275	3	IV; 100uL	1:1 Cas9: sgG211	1 mg/kg
recLemon LPMP 2272	3	IV; 100uL	1:1 Cas9: sgG211	1 mg/kg
recLemon LPMP 2335	3	IV; 100uL	1:1 Cas9: sgG211	1 mg/kg
LNP 2220	3	IV; 100uL	1:1 Cas9: sgG211	1 mg/kg
LNP 2275	3	IV; 100uL	1:1 Cas9: sgG211	1 mg/kg
LNP 2272	3	IV; 100uL	1:1 Cas9: sgG211	1 mg/kg
LNP 2335	3	IV; 100uL	1:1 Cas9: sgG211	1 mg/kg

[0677] Several doses of various recLemon LPMP formulations (as provided in Table 3) were further tested for gene editing capabilities. Doses were administered according to Table 6 and Scheme 1, which shows the experimental design with bleeds taken on Days 0, 3, and 7. Systemic concentrations of the TTR protein in plasma were measured from mouse bleeds taken at Day 0, 3, and 7 (Figure 7A), and the percentage of the TTR protein from the baseline in the blood of mice 7 days after the intravenous dose was measured (Figure 7B). Figures 7A-7B show that both doses of the recLemon LPMP formulations across all recLemon LPMP formulations indicated a decrease in the TTR protein on Day 7, when compared to the results measured from the control (PBS) group.

Table 6

Treatment	Number of Animals	Dose Route	Payload	Dose
PBS	7	IV; 100uL	-	-
recLemon LPMP 2272	7	IV; 100uL	1:1 Cas9:sgG211	1.5 mg/kg
recLemon LPMP 2272	7	IV; 100uL	1:1 Cas9:sgG211	0.3 mg/kg
recLemon LPMP 2275	7	IV; 100uL	1:1 Cas9:sgG211	1.5 mg/kg
recLemon LPMP 2275	7	IV; 100uL	1:1 Cas9:sgG211	0.3 mg/kg
recLemon LPMP 2335	7	IV; 100uL	1:1 Cas9:sgG211	1.5 mg/kg
recLemon LPMP 2335	7	IV; 100uL	1:1 Cas9:sgG211	0.3 mg/kg

Example 4. LPMP/mRNA formulations for repeated dosing in mice

[0678] If not specified, the LNP / mRNA or LPMP / mRNA formulation were prepared according to those described in Example 2. The coding portion of the EPO mRNA used in this example is as follows:

AUGGGCGUGCACGAGUGCCCCGCCUGGCUGUGGCUGCUGCUGAGCCUGCUGAGCCUGC
CCCUGGGCCUGCCCGUGCUGGGCGCCCCCCCCCGGCUGAUCUGCGACAGCCGGGUGCU
GGAGCGGUACCUGCUGGAGGCCAAGGAGGCCGAGAACAUCACCACCGGCUGCGCCGAGC
ACUGCAGCCUGAACGAGAACAUCACCGUGCCCGACACCAAGGUGAACUUCUACGCCUGGA
AGCGGAUGGAGGUGGGCCAGCAGGCCGUGGAGGUGUGGCAGGGCCUGGCCCUGCUGAG
CGAGGCCGUGCUGCGGGGCCAGGCCCUGCUGGUGAACAGCAGCCAGCCCUGGGAGCCC
CUGCAGCUGCACGUGGACAAGGCCGUGAGCGGCCUGCGGAGCCUGACCACCCUGCUGCG
GGCCCUGGGCGCCCAGAAGGAGGCCAUCAGCCCCCCCGACGCCGCCAGCGCCGCCCCCC
UGCGGACCAUCACCGCCGACACCUUCCGGAAGCUGUUCCGGGUGUACAGCAACUUCCUG
CGGGGCAAGCUGAAGCUGUACACCGGCGAGGCCUGCCGGACCGGCGACCGGUGA

[0679] The coding portion of the FLuc mRNA (TriLink, CleanCap FLuc mRNA) used in this example is as follows:

AUGGAGGACGCCAAGAACAUCAAGAAGGGCCCCGCCCCCUUCUACCCCCUGGAGGACGG
CACCGCCGGCGAGCAGCUGCACAAGGCCAUGAAGCGGUACGCCCUGGUGCCCGGCACCA
UCGCCUUCACCGACGCCCACAUCGAGGUGGACAUCACCUACGCCGAGUACUUCGAGAUG
AGCGUGCGGCUGGCCGAGGCCAUGAAGCGGUACGGCCUGAACACCAACCACCGGAUCGU
GGUGUGCAGCGAGAACAGCCUGCAGUUCUUCAUGCCCGUGCUGGGCGCCCUGUUCAUCG
GCGUGGCCGUGGCCCCCGCCAACGACAUCUACAACGAGCGGGAGCUGCUGAACAGCAUG
GGCAUCAGCCAGCCCACCGUGGUGUUCGUGAGCAAGAAGGGCCUGCAGAAGAUCCUGAA

CGUGCAGAAGAAGCUGCCCAUCAUCCAGAAGAUCAUCAUCAUGGACAGCAAGACCGACUA
CCAGGGCUUCCAGAGCAUGUACACCUUCGUGACCAGCCACCUGCCCCCCGGCUUCAACG
AGUACGACUUCGUGCCCGAGAGCUUCGACCGGGACAAGACCAUCGCCCUGAUCAUGAACA
GCAGCGGCAGCACCGGCCUGCCCAAGGGCGUGGCCCUGCCCCACCGGACCGCCUGCGU
GCGGUUCAGCCACGCCCGGGACCCCAUCUUCGGCAACCAGAUCAUCCCCGACACCGCCA
UCCUGAGCGUGGUGCCCUUCCACCACGGCUUCGGCAUGUUCACCACCCUGGGCUACCUG
AUCUGCGGCUUCCGGGUGGUGCUGAUGUACCGGUUCGAGGAGGAGCUGUUCCUGCGGA
GCCUGCAGGACUACAAGAUCCAGAGCGCCCUGCUGGUGCCCACCCUGUUCAGCUUCUUC
GCCAAGAGCACCCUGAUCGACAAGUACGACCUGAGCAACCUGCACGAGAUCGCCAGCGGC
GGCGCCCCCCUGAGCAAGGAGGUGGGCGAGGCCGUGGCCAAGCGGUUCCACCUGCCCG
GCAUCCGGCAGGGCUACGGCCUGACCGAGACCACCAGCGCCAUCCUGAUCACCCCCGAG
GGCGACGACAAGCCCGGCGCCGUGGGCAAGGUGGUGCCCUUCUUCGAGGCCAAGGUGG
UGGACCUGGACACCGGCAAGACCCUGGGCGUGAACCAGCGGGGCGAGCUGUGCGUGCG
GGGCCCCAUGAUCAUGAGCGGCUACGUGAACAACCCCGAGGCCACCAACGCCCUGAUCG
ACAAGGACGGCUGGCUGCACAGCGGCGACAUCGCCUACUGGGACGAGGACGAGCACUUC
UUCAUCGUGGACCGGCUGAAGAGCCUGAUCAAGUACAAGGGCUACCAGGUGGCCCCCGC
CGAGCUGGAGAGCAUCCUGCUGCAGCACCCCAACAUCUUCGACGCCGGCGUGGCCGGCC
UGCCCGACGACGACGCCGGCGAGCUGCCCGCCGCCGUGGUGGUGCUGGAGCACGGCAA
GACCAUGACCGAGAAGGAGAUCGUGGACUACGUGGCCAGCCAGGUGACCACCGCCAAGA
AGCUGCGGGGCGGCGUGGUGUUCGUGGACGAGGUGCCCAAGGGCCUGACCGGCAAGCU
GGACGCCCGGAAGAUCCGGGAGAUCCUGAUCAAGGCCAAGAAGGGCGGCAAGAUCGCCG
UGUGA

Formulation and characterization

[0680] This example further describes the formulation of exemplary lemon LPMPs with ionizable lipids, sterols, and PEG lipids, to encapsulate an exemplary mRNA having gene-editing capabilities (e.g., 1:1 fLuc:hEPO) for LPMP / mRNA formulation, and LNPs formulated with ionizable lipids, sterols, and PEG lipids, to encapsulate an mRNA (e.g., 1:1 FLuc:hEPO) for LNP / mRNA formulation.

Repeated Intravenous Dosing at Various Concentrations

[0681] **LNP formulation** was prepared according to Example 2, composed of ionizable lipid:structural lipid:sterol:PEG-lipid at given molar ratios outlined in Table 7 and 9. Lipids were solubilized in ethanol. These lipids were mixed at the indicated molar ratios and diluted in ethanol (organic phase) to 12.5 mM total lipid concentration and the mRNA solution (aqueous phase) was prepared with RNAse-free water and 100 mM citrate buffer pH 3 for a final concentration of 50 mM citrate buffer.

[0682] Exemplary lemon **LPMP compositions** (recLemon LPMP) were prepared according to Example 2, composed of ionizable lipid:natural lipids:sterol:PEG-lipid at given molar ratios outlined in Tables 7 and 9. Lipids were solubilized and extracted in ethanol or chloroform:methanol (e.g, 4:1 DMF:methanol). Formulations were then handled as above.

[0683] Formulations were maintained at ionizable lipid to mRNA N:P ratio of 15:1 for each lipid listed in Table 7. Formulations were maintained at ionizable lipid to mRNA N:P ratio of 6:1 for each lipid listed in Table 9.

[0684] The lipid mix and mRNA solution were mixed at a 1:3 ratio by volume, respectively, on the NANOASSEMBLR® IGNITE™ (Precision Nanosystems) at a total flow rate of 9 or 14 mL/minute. The resulting formulations were then loaded into Slide-A-Lyzer G2/G3 dialysis cassettes (10k MWCO) and dialyzed against 1x PBS for 2 hours at room temperature. The PBS was refreshed, and the formulations were further dialyzed for at least 14 hours at 4^0C with gentle stirring. The dialyzed formulations were then collected and concentrated by tangential flow filtration (TFF) using Sartorius

VIVAFLOW® 50R cassettes (100k MWCO) at fixed feed flowrate of 150ml/minute. The TFF-concentrated formulations were then further concentrated by centrifugation at 3000xg using AMICON® Ultra centrifugation filters (100k MWCO). The concentrated formulations were characterized for size, polydispersity, and particle concentration using a Zetasizer Ultra (Malvern Panalytical) and for mRNA encapsulation efficiency using QUANT-IT™ RIBOGREEN® RNA Assay Kit (ThermoFisher Scientific).

Table 7. Characterizations of the LPMP/LNP formulations for repeated dosing

Formulation	Ionizable: Structural: Sterol: PEG-lipid Molar ratio	Ionizable	Structural	Sterol	PEG-lipid	Cargo	Extraction Method
LNP 2272	*50:10:38.5:1.5*	2272	DSPC	Chol	DMPE-PEG	1:1 fLuc:hEPO	-
recLemon LPMP 2272	*35:20:42.5:2.5*	2272	Naturally derived lemon lipid	Chol	DMPE-PEG	1:1 fLuc:hEPO	Ethanol or DMF:Methanol
LNP Lipid 5	*50:10:38.5:1.5*	Lipid 5	DSPC	Chol	DMG-PEG	1:1 fLuc:hEPO	-
LNP C12	*35:16:46.5:2.5*	C12-200	DOPE	Chol	DMPE-PEG	1:1 fLuc:hEPO	-
recLemon LPMP C12_EE	*35:20:42.5:2.5*	C12-200	Naturally derived lemon lipid	Chol	DMPE-PEG	1:1 fLuc:hEPO	Ethanol
recLemon LPMP C12_CM	*35:20:42.5:2.5*	C12-200	Naturally derived lemon lipid	Chol	DMPE-PEG	1:1 fLuc:hEPO	Chloroform: Methanol

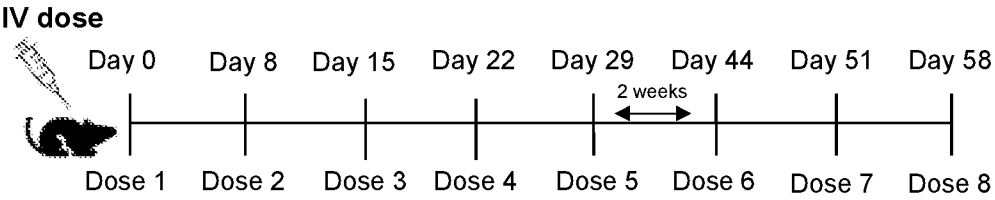
Bioluminescence screening

[0685] 8-9 week old Balb/c mice were utilized for bioluminescence-based screening efforts of the formulations provided in Table 7, with Lipid 5 acting as a positive control. Mice were obtained from Jackson Laboratories and allowed to acclimate for one week prior to manipulations. Animals were placed under a heat lamp for a few minutes before introducing them to a restraining chamber. The tail was wiped with alcohol pads (Fisher Scientific) and, for each LPMP or LNP composition described in Table 7, mice were bled 24h prior to a 100 µL of a composition containing either 0.5 mg/kg or 0.125 mg/kg mRNA (1:1 FLuc:hEPO) being injected intravenously using a 29G insulin syringe (Covidien). 4 hours post-dose, animals were bled and then injected with 200 µL of 15mg/mL D-Luciferin (GoldBio), and placed in set nose cones inside the IVIS Lumina LT imager (PerkinElmer). LivingImage software was utilized for imaging. Whole body bio-luminescence was captured at auto-exposure after which animals were removed from the IVIS. This was repeated for each dose following Scheme 2, and is displayed as average radiance. Mice were placed into a CO_2 chamber for euthanasia after either Dose 8 (medium dose, 0.5 mg/kg) or Dose 5 (low dose, 0.125 mg/kg). Cardiac puncture was performed on each animal after placing it in dorsal recumbency. Blood collection was performed using a 25G insulin syringe (BD). Once all blood samples were collected, tubes are spun at 2000G for 10 minutes using a tabletop centrifuge and plasma was aliquoted into individual Eppendorf tubes (Fisher

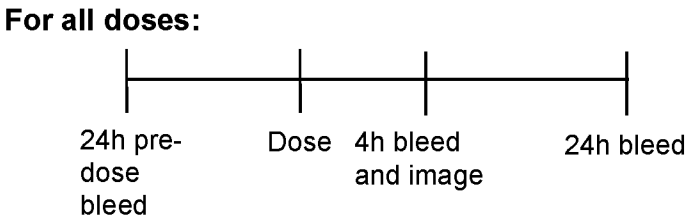
Scientific) and stored at -80 °C for subsequent EPO quantification. The EPO levels (pg/mL) in plasma were determined using EPO MSD kit (Meso Scale Diagnostics). Anti-PEG IgM and IgG antibody titers in plasma were determined using anti-PEG ELISAs.

Table 8

Particle ID	Number of animals	Medium Dose	Low Dose	Route Of Administration	Payload
LNP 2272	5	0.5 mg/kg	0.125 mg/kg	IV; 100uL	1:1 FLuc:hEPO
recLemon LPMP 2272	5	0.5 mg/kg	0.125 mg/kg	IV; 100uL	1:1 FLuc:hEPO
Lipid 5	5	0.5 mg/kg	0.125 mg/kg	IV; 100uL	1:1 FLuc:hEPO
C12 LNP	5	0.5 mg/kg	0.125 mg/kg	IV; 100uL	1:1 FLuc:hEPO
recLemon C12_EE	5	0.5 mg/kg	0.125 mg/kg	IV; 100uL	1:1 FLuc:hEPO
recLemon C12_CM	5	0.5 mg/kg	0.125 mg/kg	IV; 100uL	1:1 FLuc:hEPO



Scheme 2



Scheme 3

hEPO MSD Measurement.

[0686] The reagents used for measuring hEPO levels included:

MSD wash buffer (#R61AA-1) MSD EPO Kit (#K151VXK-2)

o MSD GOLD 96 Small Spot Streptavidin Plate o Diluent 100

o Diluent 3

o Diluent 43

o Calibrator 9

o Capture Ab

o Detection Ab

o MSD GOLD Read Buffer B

[0687] General procedure. The Plate was coated. 200 µL of biotinylated capture antibody was added

to 3.3 mL of Diluent 100 and was mixed by vortexing. 25 µL of the above solution was added to each well of the provided MSD GOLD Small Spot Streptavidin Plate. The plate was sealed overnight. The plate was washed 3 times with at least 150 µL/well of 1X MSD Wash Buffer.

[0688] Preparation of Calibrator Standards. The Calibrator vial(s) were brought to room temperature. Each vial of Calibrator was reconstituted by adding 250 µL of Diluent 43 to the glass vial, resulting in a 5× concentrated stock of the Calibrator. The reconstituted Calibrator was inverted at least 3 times, and equilibrated at room temperature for 15–30 minutes and then was vortexed briefly. Calibrator Standard 1 was prepared by adding 50 µL of the reconstituted Calibrator to 200 µL of Diluent 43 and vortexing. Calibrator Standard 2 was prepared by adding 75 µL of Calibrator Standard 1 to 225 µL of Diluent 43 and vortexing. The four-fold serial dilutions were repeated 5 additional times to generate a total of 7 Calibrator Standards. Mix by vortexing between each serial dilution. Diluent 43 was used as Calibrator Standard 8 (zero Calibrator).

[0689] Samples and Calibrators additions. 25 µL of Diluent 43 was added to each well. 25 µL of the prepared Calibrator Standard or sample was added to each well. The plate was sealed with an adhesive plate seal, and incubate at room temperature with shaking for 1 hour.

[0690] Preparation and addition of the Detection Antibody Solution. The detection antibody solution was provided as a 100× stock solution. The working solution was 1×. 60 µL of the supplied 100× detection antibody was added to 5940 µL of Diluent 3. The plate was washed 3 times with at least 150 µL/well of 1× MSD Wash Buffer. 50 µL of the Detection Antibody Solution prepared above was added to each well. The plate was sealed with an adhesive plate seal, and incubated at room temperature with shaking for 1 hour.

[0691] Sample reading. The plate was washed 3 times with at least 150 µL/well of 1× MSD Wash Buffer. 150 µL of MSD GOLD Read Buffer B was added to each well. The plate was analyzed on an MSD instrument to read the EPO level.

Anti-PEG Antibdy ELISA Measurement.

[0692] The reagents used for measuring anti-PEG antibody levels included:

o 96 well ELISA plate

o bicarbonate buffer 0.2M

o $mPEG_{20000}$-NH_2

o 0.1% CHAPS (w/v) wash buffer

o Blocking buffer

o mouse anti-PEG IgG or IgM for standards

o goat anti-mouse IgG or IgM detection antibodies

o TMB substrate

General procedure

[0693] On Day 1, the plate was coated. 0.1 M pH 9.4 bicarbonate buffer and the mPEG (1 mg/mL) in 0.1M buffer were prepared. Coating solution (1ug/mL) was created by adding 6 uL of stock into 6 mL of 0.1M bicarbonate buffer. 50 uL were added per well. The plate was sealed, and then incubated overnight at 4^0C.

[0694] On Day 2, the wash buffer was prepared by adding 1g CHAPS to 1L PBS. He liquid in the

plate was aspirated. The plate was washed 2 times with wash buffer and once with PBS. 150-200 µL/well of blocking buffer was added, allowed to sit for a minute, and then dumped from the plate. This was repeated twice more, and then the plate was washed once with PBS. Samples were prepared by diluting 1:50 in 1% StartingBlock. Standards were prepared by preparing a 500ng/mL solution of IgG or IgM and then serially diluting 3-fold to create 7 standards. Standard 8 is buffer alone (0 ng/mL). The plate was washed, and 100 uL sample or standard was added. The plate was incubated overnight at 4^0C.

[0695] On Day 3, detection antibodies were prepared by diluting anti-mouse IgG or IgM 1:5000 in dilution buffer or PBS. The plate was washed. 100 uL of detection antibody was added to the plate and incubated at room temperature on a shaker for 1-2 hours. The plate was washed. 100 uL TMB substrate was added and the plate was incubated for around 20 minutes at room temperature in the dark. The plate was then read at 640 nm. 50 uL of stop solution was added and the plates were read at 450 nm with wavelength correction at 540 or 570 nm.

[0696] As shown in Figure 8A, the mice given intravenous doses of LNP 2272, recLemon LPMP 2272, LNP Lipid 5, LNP C12, recLemon LPMP C12_EE, or recLemon LPMP_CM (0.5 mg/kg per dose; 1:1 mRNA FLuc:hEPO) over the course of all 8 doses showed comparable levels of average radiance to each other and throughout all doses. As shown in Figure 8B, the systemic levels of hEPO at pre-dose (0h), 4h post-dose, and 24h post-dose over the first five doses indicate sustained efficacy for the recLemon LPMP formulations and synthetic LNP formulations with various ionizable lipids. Antibody titers of anti-PEG IgM and IgG were determined for the first five doses and the results are shown in Figures 10A-10B. The results indicate an increase in titers with each successive dose, with a marked increase between doses 2 and 3. The mice administered with formulations containing the ionizable lipid 2272 showed a more moderate increase than the mice administered with formulations containing C12-200.

[0697] As shown in Figures 9A-9B, the mice given intravenous doses of LNP 2272, recLemon LPMP 2272, LNP Lipid 5, LNP C12, recLemon LPMP C12_EE, or recLemon LPMP_CM (0.125 mg/kg per dose; 1:1 mRNA FLuc:hEPO) over the course of all 5 doses show comparable levels of average radiance and hEPO concentrations to each other and throughout all doses, confirming the sustained efficacy of recLemon LPMP formulations and synthetic LNP formulations with various ionizable lipids at a lower dose.

Table 9. Formulation characterization

Formulation	Ionizable: Structural: Sterol: PEG-lipid Molar ratio	Ionizable	Structural	Sterol	PEG-lipid	Cargo	N:P Ratio
recLemon LPMP 2272	*35:20:42.5:2.5*	2272	Naturally derived lemon lipid	Chol	DMG-PEG2k	3:1 fLuc:hEPO	6
recLemon LPMP 2320	*35:20:42.5:2.5*	2320	Naturally derived lemon lipid	Chol	DMG-PEG2k	3:1 fLuc:hEPO	6

2320 LNP	*35:16:46.5:2.5*	2320	DOPE	Chol	DMG-PEG2k	3:1 fLuc:hEPO	6

[0698] In a further experiment, 8-9 week old Balb/c mice were utilized for bioluminescence-based screening efforts of the formulations provided in Table 7, with PBS and LNPs acting as controls. Animals were placed under a heat lamp for a few minutes before introducing them to a restraining chamber. The tail was wiped with alcohol pads (Fisher Scientific) and, for each experimental group, mice were bled 24h prior to a 100 µL of a composition containing either 1 mg/kg or 0.5 mg/kg mRNA (3:1 FLuc:hEPO) being injected intravenously using a 29G insulin syringe (Covidien). 4 hours post-dose, animals were bled and then injected with 200 µL of 15mg/mL D-Luciferin (GoldBio), and placed in set nose cones inside the IVIS Lumina LT imager (PerkinElmer). LivingImage software was utilized for imaging. Whole body bio-luminescence was captured at auto-exposure after which animals were removed from the IVIS. This was repeated for each dose given intravenously on Day 1, 8, 15, 22, and 29 and is displayed as average radiance. Mice were placed into a CO_2 chamber for euthanasia 24h after Dose 5 on Day 30. Cardiac puncture was performed on each animal after placing it in dorsal recumbency. Blood collection was performed using a 25G insulin syringe (BD). Once all blood samples were collected, tubes are spun at 2000G for 10 minutes using a tabletop centrifuge and plasma was aliquoted into individual Eppendorf tubes (Fisher Scientific) and stored at -80 °C for subsequent EPO quantification. The EPO levels (pg/mL) in plasma were determined using EPO MSD kit (Meso Scale Diagnostics). Anti-PEG IgM and IgG antibody titers in plasma were determined using anti-PEG ELISAs.

[0699] As shown in Figure 11A, the mice given intravenous doses of recLemon LPMP 2272 and recLemon LPMP 2320 (1 mg/kg per dose or 0.5 mg/kg; 3:1 mRNA FLuc:hEPO) over the course of all 5 doses show comparable levels of systemic hEPO concentration (pg/mL) post-dose of intravenous administration, compared to the tested LNPs at 1 mg/kg and 0.5 mg/kg (3:1 mRNA FLuc:hEPO). Figure 11B shows the whole body average radiance, indicating that the majority of the LPMPs at both doses performed well when compared to LNPs. Figure 11C shows an increase in anti-PEG IgG over all 5 doses, with recLemon LPMP 2272 comparable to 2320 LNP at both concentrations. Figure 11D indicates an increase in anti-PEG IgM across all experimental groups over the first three doses. Pre-dose bleeds from doses 4 and 5 were too sparse to test both anti-PEG IgG and IgM. Despite increases in anti-PEG antibodies, overall potency over all 5 doses was not impacted in LPMPs.

Repeated Subcutaneous Dosing

[0700] **LNP formulation** was prepared according to Example 2, composed of ionizable lipid:structural lipid:sterol:PEG-lipid at given molar ratios outlined in Table 10. Lipids were solubilized in ethanol. These lipids were mixed at the indicated molar ratios and diluted in ethanol (organic phase) to 12.5 mM total lipid concentration and the mRNA solution (aqueous phase) was prepared with RNAse-free water and 100 mM citrate buffer pH 3 for a final concentration of 50 mM citrate buffer.

[0701] Exemplary lemon **LPMP compositions** (recLemon LPMP) were prepared according to Example 2, composed of ionizable lipid:natural lipids:sterol:PEG-lipid at given molar ratios outlined in

Table 10. Lipids were solubilized and extracted in ethanol or chloroform:methanol (e.g, 4:1 DMF:methanol). Formulations were then handled as above.

[0702] Formulations were maintained at ionizable lipid to mRNA N:P ratio of 6:1 for each lipid listed in Table 10.

[0703] The lipid mix and mRNA solution were mixed at a 1:3 ratio by volume, respectively, on the NANOASSEMBLR® IGNITE™ (Precision Nanosystems) at a total flow rate of 14 mL/minute. The resulting formulations were then loaded into Slide-A-Lyzer G2 dialysis cassettes (10k MWCO) and dialyzed against 1x PBS for 2 hours at room temperature. The PBS was refreshed, and the formulations were further dialyzed for at least 14 hours at 4^0C with gentle stirring. The dialyzed formulations were then collected and concentrated by tangential flow filtration (TFF) using Sartorius VIVAFLOW® 50R cassettes (100k MWCO) at fixed feed flowrate of 150ml/minute. The TFF-concentrated formulations were then further concentrated by centrifugation at 3000xg using AMICON® Ultra centrifugation filters (100k MWCO). The concentrated formulations were characterized for size, polydispersity, and particle concentration using a Zetasizer Ultra (Malvern Panalytical) and for mRNA encapsulation efficiency using QUANT-IT™ RIBOGREEN® RNA Assay Kit (ThermoFisher Scientific).

Table 10. Characterizations of the LPMP/LNP formulations for repeated dosing

Formulation	Ionizable: Structural: Sterol: PEG-lipid Molar ratio	Ionizable	Structural	Sterol	PEG-lipid	Cargo	N:P Ratio
2272 LNP	*50:10:38.5:1.5*	2272	DSPC	Chol	DMG-PEG2k	1:1 fLuc:hEPO	6
recLemon LPMP 2272	*35:20:42.5:2.5*	2272	Naturally derived lemon lipid	Chol	DMG-PEG2k	1:1 fLuc:hEPO	6
2320 LNP	*35:16:46.5:2.5*	2320	DOPE	Chol	DMG-PEG2k	1:1 fLuc:hEPO	6
recLemon LPMP 2320	*35:20:42.5:2.5*	2320	Naturally derived lemon lipid	Chol	DMG-PEG2k	1:1 fLuc:hEPO	6
2439 LNP	*35:16:46.5:2.5*	2439	DOPE	Chol	DMG-PEG2k	1:1 fLuc:hEPO	6
recLemon LPMP 2439	*35:20:42.5:2.5*	2439	Naturally derived lemon lipid	Chol	DMG-PEG2k	1:1 fLuc:hEPO	6

Bioluminescence screening

[0704] 8-9 week old Balb/c mice were utilized for repeat dosing efforts of the formulations provided in Table 10, with LNPs acting as a positive control. Mice were obtained from Jackson Laboratories and allowed to acclimate for one week prior to manipulations. Animals were placed under a heat lamp for a few minutes before introducing them to a restraining chamber. The tail was wiped with alcohol pads (Fisher Scientific) and, for each LPMP or LNP composition described in Table 10, mice were bled 24h

prior to a 100 µL of a composition containing 0.2 mg/kg mRNA (1:1 FLuc:hEPO) being injected subcutaneously using a 29G insulin syringe (Covidien). 4 hours post-dose, animals were bled and then injected with 200 µL of 15mg/mL D-Luciferin (GoldBio), and placed in set nose cones inside the IVIS Lumina LT imager (PerkinElmer). LivingImage software was utilized for imaging. Whole body bio-luminescence was captured at auto-exposure after which animals were removed from the IVIS. This was repeated for each dose given subcutaneously on Day 1, 8, 15, 22, and 29, and is displayed as average radiance. Mice were placed into a CO_2 chamber for euthanasia 24h after Dose 5 on Day 30. Cardiac puncture was performed on each animal after placing it in dorsal recumbency. Blood collection was performed using a 25G insulin syringe (BD). Once all blood samples were collected, tubes are spun at 2000G for 10 minutes using a tabletop centrifuge and plasma was aliquoted into individual Eppendorf tubes (Fisher Scientific) and stored at -80 °C for subsequent EPO quantification. The EPO levels (pg/mL) in plasma were determined using EPO MSD kit (Meso Scale Diagnostics). Anti-PEG IgM and IgG antibody titers in plasma were determined using anti-PEG ELISAs.

[0705] As shown, in Figure 12A, whole body radiance indicates comparable performance from the LPMPs compared to LNPs, though there is some variation across all doses. Figures 12B-12C show an increase in anti-PEG antibodies across all treatment groups. Again, potency was not impacted by the increased anti-PEG antibodies.

Intravenous and Subcutaneous Comparison

[0706] Lipid nanoparticle (LNP) formulation was prepared according to Example 2, composed of ionizable lipid:structural lipid:sterol:PEG-lipid at given molar ratios outlined in Table 11. Lipids were solubilized in ethanol. These lipids were mixed at the indicated molar ratios and diluted in ethanol (organic phase) to 12.5 mM total lipid concentration and the mRNA solution (aqueous phase) was prepared with RNAse-free water and 100 mM citrate buffer pH 3 for a final concentration of 50 mM citrate buffer.

[0707] Exemplary lemon LPMP compositions (recLemon LPMP) were prepared according to Example 2, composed of ionizable lipid:natural lipids:sterol:PEG-lipid at given molar ratios outlined in Table 11. Lipids were solubilized and extracted in ethanol or chloroform:methanol (e.g, 4:1 DMF:methanol). Formulations were then handled as above.

[0708] Formulations were maintained at ionizable lipid to mRNA N:P ratio of 6:1 for each lipid listed in Table 11.

[0709] The lipid mix and mRNA solution were mixed at a 1:3 ratio by volume, respectively, on the NANOASSEMBLR® IGNITE™ (Precision Nanosystems) at a total flow rate of 14 mL/minute. The resulting formulations were then loaded into Slide-A-Lyzer G2 dialysis cassettes (10k MWCO) and dialyzed against 1x PBS for 2 hours at room temperature. The PBS was refreshed, and the formulations were further dialyzed for at least 14 hours at 4^{0}C with gentle stirring. The dialyzed formulations were then collected and concentrated by tangential flow filtration (TFF) using Sartorius VIVAFLOW® 50R cassettes (100k MWCO) at fixed feed flowrate of 150ml/minute. The TFF-concentrated formulations were then further concentrated by centrifugation at 3000xg using AMICON® Ultra centrifugation filters (100k MWCO). The concentrated formulations were

characterized for size, polydispersity, and particle concentration using a Zetasizer Ultra (Malvern Panalytical) and for mRNA encapsulation efficiency using QUANT-IT™ RIBOGREEN® RNA Assay Kit (ThermoFisher Scientific).

Table 11. Characterizations of the LPMP/LNP formulations for repeated dosing

Formulation	Ionizable: Structural: Sterol: PEG-lipid Molar ratio	Ionizable	Structural	Sterol	PEG-lipid	Cargo	N:P Ratio
recLemon LPMP 2272	*35:20:42.5:2.5*	2272	Naturally derived lemon lipid	Chol	DMG-PEG2k	1:1 fLuc:hEPO	6
2272 LNP	*50:10:38.5:1.5*	2272	DSPC	Chol	DMG-PEG2k	1:1 fLuc:hEPO	6

Bioluminescence screening

[0710] 8-9 week old Balb/c mice were utilized for repeat dosing efforts of the formulations provided in Table 11, with LNP and PBS acting as a control. Mice were obtained from Jackson Laboratories and allowed to acclimate for one week prior to manipulations. Animals were placed under a heat lamp for a few minutes before introducing them to a restraining chamber. The tail was wiped with alcohol pads (Fisher Scientific) and, for the LPMP and LNP composition described in Table 11, mice were bled 24h prior to a 100 µL of a composition containing 0.5 mg/kg mRNA (1:1 FLuc:hEPO) being injected either subcutaneously or intravenously using a 29G insulin syringe (Covidien). 4 hours post-dose, animals were bled and then injected with 200 µL of 15mg/mL D-Luciferin (GoldBio), and placed in set nose cones inside the IVIS Lumina LT imager (PerkinElmer). LivingImage software was utilized for imaging. Whole body bio-luminescence was captured at auto-exposure after which animals were removed from the IVIS. This was repeated for each dose given subcutaneously and intravenously on Day 1, 15, 29, 43, and 57, and is displayed as average radiance. Mice were placed into a CO_2 chamber for euthanasia 24h after Dose 5. Cardiac puncture was performed on each animal after placing it in dorsal recumbency. Blood collection was performed using a 25G insulin syringe (BD). Once all blood samples were collected, tubes are spun at 2000G for 10 minutes using a tabletop centrifuge and plasma was aliquoted into individual Eppendorf tubes (Fisher Scientific) and stored at -80 °C for subsequent EPO quantification. The EPO levels (pg/mL) in plasma were determined using EPO MSD kit (Meso Scale Diagnostics). Anti-PEG IgM and IgG antibody titers in plasma were determined using anti-PEG ELISAs.

[0711] As shown in Figure 13A and 13B, whole body radiance and hEPO concentration indicate that the delivery route can impact efficacy, with intravenous delivery outperforming subcutaneous delivery. Importantly, on average, the LPMPs formulations delivered by both routes (i.e. IV or SQ) showed higher radiance and hEPO concentration than the PBS control. Additionally, when comparing within a specific route of administration, LPMPs formulations had higher average radiance and higher hEPO concentration than the comparative LNPs formulations. Figures 13C-13D show a general increase in anti-PEG antibodies across all treatment groups.

[0712] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention. The disclosures of all patent and scientific literature cited herein are expressly incorporated in their entirety by reference. Other embodiments are within the claims.

What is claimed is:

1. A method for delivering a gene editing system to a subject in need thereof, the method comprising administering to the subject a RNA composition comprising:

one or more polynucleotides encoding one or more components of a gene editing system or one or more gene editing systems, formulated within

(a) a plurality of lipid nanoparticles (LNPs) comprising synthetic structural lipids and an ionizable lipid; or

(b) a lipid reconstructed natural messenger packs (LNMPs) comprising natural lipids and an ionizable lipid,

wherein the ionizable lipid has two or more of the characteristics listed below:

(i) at least 2 ionizable amines;

(ii) at least 3 lipid tails; wherein each of the lipid tails is at least 6 carbon atoms in length;

(iii) a pKa of about 4.5 to about 7.5;

(iv) an ionizable amine and a heteroorganic group separated by a chain of at least two atoms; and

(v) an N:P ratio of at least 3.

2. A method of gene editing, comprising:

contacting a cell with or administering into a subject a RNA composition comprising:

one or more polynucleotides encoding one or more components of a gene editing system or one or more gene editing systems, formulated within

(a) a plurality of lipid nanoparticles (LNPs) comprising synthetic structural lipids and an ionizable lipid; or

(b) a lipid reconstructed natural messenger packs (LNMPs) comprising natural lipids and an ionizable lipid,

wherein the ionizable lipid has two or more of the characteristics listed below:

(i) at least 2 ionizable amines;

(ii) at least 3 lipid tails; wherein each of the lipid tails is at least 6 carbon atoms in length;

(iii) a pKa of about 4.5 to about 7.5;

(iv) an ionizable amine and a heteroorganic group separated by a chain of at least two atoms; and

(v) an N:P ratio of at least 3,

wherein the one or more components of the gene editing systems or one or more gene editing systems are delivered to the cell or subject to modify the genome of the cell or the subject.

3. The method of claim 1 or 2, wherein the gene editing system is CRISPR-Cas gene editing system.

4. The method of any one of claims 1-3, wherein the RNA composition further comprises at least one template nucleic acid.

5. The method of claim 1 or 2, wherein the RNA composition is administered at least one time.

6. The method of claim 1 or 2, wherein the RNA composition is administered at least two times, at least three times, at least four times, at least five times, at least six times, at least seven times, at least eight times, at least nine times, at least ten times, at least fifteen times, at least twenty times, or more.

7. The method of claim 1 or 2, wherein the RNA composition is administered 2-8 times.

8. The method of claim 6 or 7, wherein the delivery of the gene editing system, or the result of gene editing improves upon multiple administrations.

9. The method of claim 1 or 2, wherein at least two RNA compositions are administered into the subject or contacted with the cell: a first RNA composition comprising a mRNA, and the second RNA composition comprising a guide RNA nucleic acid.

10. The method of claim 9, wherein the first and second RNA compositions are administered simultaneously.

11. The method of claim 9, wherein the first and second RNA compositions are administered sequentially.

12. The method of claim 1 or 2, wherein a single RNA composition is contacted with the cell or administered into the subject, wherein the single RNA composition comprises an mRNA and a guide RNA nucleic acid.

13. The method of claim 1 or 2, wherein the RNA composition is administered via oral, intravenous, intramuscular, intranasal, or subcutaneous route.

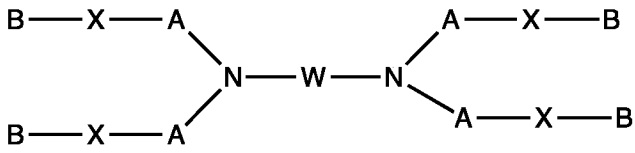
14. The method of claim 1 or 2, wherein the RNA composition is administered more than once, within at least one week, at least two weeks, at least three weeks, or at least four weeks between administrations.

15. The method of claim 1 or 2, wherein the RNA composition is formulated with b) LNMPs.

16. The method of claim 15, wherein the ionizable lipid is C12-200.

17. The method of claim 1 or 2, wherein the RNA composition is formulated with a) LNPs.

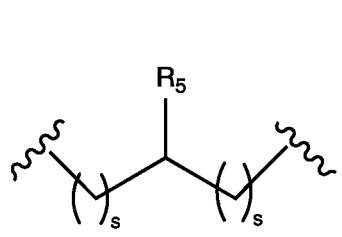
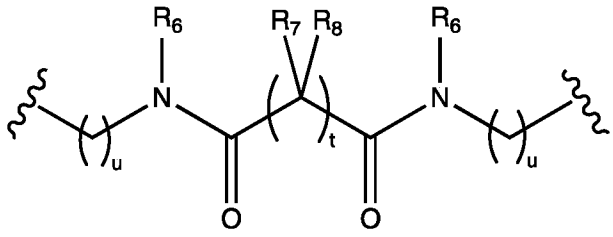
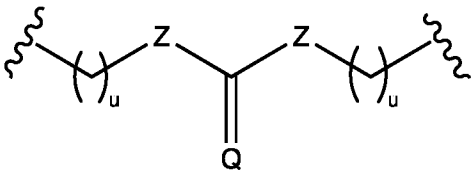
18. The method of claim 15 or 17, wherein the ionizable lipid is selected from one of the following groups of compounds:

i) a compound of formula  (I), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing, wherein:

each **A** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally substituted with heteroatom or substituted with OH, SH, or halogen;

each **B** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally substituted with heteroatom or substituted with OH, SH, or halogen;

each **X** is independently a biodegradable moiety; and

W is  ,  ; or  , wherein

$\mathbf{R_5}$ is OH, SH, $N\mathbf{R_{10}R_{11}}$;

each $\mathbf{R_6}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each $\mathbf{R_7}$ and each $\mathbf{R_8}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $N\mathbf{R_{10}R_{11}}$, wherein each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 alkyl, or $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring;

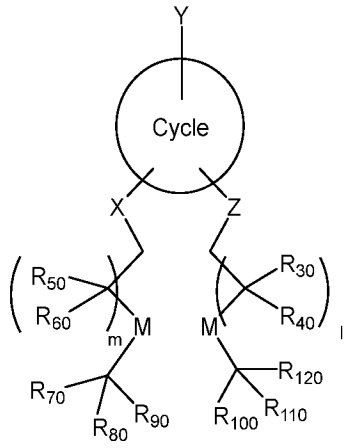
each **s** is independently 1, 2, 3, 4, or 5;

each **u** is independently 1, 2, 3, 4, or 5;

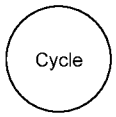
t is 1, 2, 3, 4 or 5;

each **Z** is independently absent, O, S, or $N\mathbf{R_{12}}$, wherein $\mathbf{R_{12}}$ is H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl, and

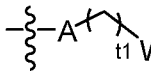
Q is O, S, or $N\mathbf{R_{13}}$, wherein each $\mathbf{R_{13}}$ is H, C_1-C_5 alkyl;



ii) a compound of formula (II), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing, wherein:



is cyclic or heterocyclic moiety;

Y is alkyl, hydroxy, hydroxyalkyl or 

;

A is absent, -O-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -N(R^7)C(O)N(R^7)-, -S-, -S-S-, or a bivalent heterocycle;

each of X and Z is independently absent, -O-, -CO-, -N(R^7)-, -O-alkylene-; -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, or -S-;

each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxy, hydroxyalkyl, or aminoalkyl;

each M is independently a biodegradable moiety;

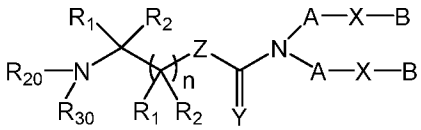
each of R_{30}, R_{40}, R_{50}, R_{60}, R_{70}, R_{80}, R_{90}, R_{100}, R_{110}, and R_{120} is independently H, C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with heteroatom or substituted with OH, SH, or halogen, or cycloalkyl or substituted cycloalkyl;

each of l and m is an integer from 1 to 10;

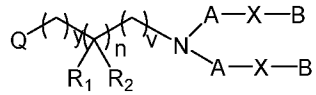
t1 is an integer from 0 to 10; and

W is hydroxyl, substituted or unsubstituted hydroxyalkyl, substituted or unsubstituted amino, substituted or unsubstituted aminocarbonyl, or substituted or unsubstituted heterocylyl or heteroaryl; and

iii) a compound of formula



(III) or



(V), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein:

R_{20} and **R_{30}** are each independently H, C_1-C_5 branched or unbranched alkyl, or C_2-C_5 branched or unbranched alkenyl, or **R_{20}** and **R_{30}** together with the adjacent N atom form a 3 to 7 membered cyclic ring, optionally substituted with **R^a**;

R^a is H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, or SH;

each R_1 and each R_2 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, OH, halogen, SH, or $NR_{10}R_{11}$, or

R_1 and R_2 are taken together to form a cyclic ring;

each R_{10} and R_{11} is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or R_{10} and R_{11} are taken together to form a heterocyclic ring;

n is 0, 1, 2, 3 or 4;

Y is O or S;

Z is absent, O, S, or $N(R_{12})(R_{12})$, wherein each R_{12} is independently H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl, provided that when Z is not absent, the adjacent R_1 and R_2 cannot be OH, $NR_{10}R_{11}$, or SH;

u is 0, 1, 2, 3, 4, 5, 6, 7, or 8;

v is 0, 1, 2, 3, or 4;

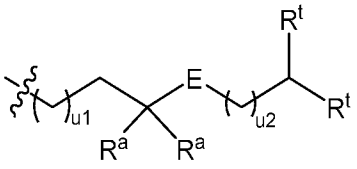
y is 0, 1, 2, 3, or 4;

each **A** is each independently C_1-C_{16} branched or unbranched alkyl, or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen;

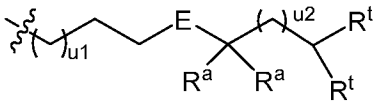
each **B** is each independently C_1-C_{16} branched or unbranched alkyl, or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen; and

each **X** is independently a biodegradable moiety; and

iv) a lipid comprising at least one head group and at least one tail group of formula (TI) or (TI')



(TI) or



(TI'), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing,

wherein:

E is each independently -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -C(O-R_{13})-O-, -C(O)O$(CH_2)_r$-, -C(O)N(R^7) $(CH_2)_r$-, -S-S-, or -C(O-R_{13})-O-$(CH_2)_r$-, wherein each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxyalkyl, or aminoalkyl;

R_{13} is branched or unbranched C_3-C_{10} alkyl;

r is 1, 2, 3, 4, or 5;

R^a is each independently C_1-C_5 alkyl, C_2-C_5 alkenyl, or C_2-C_5 alkynyl;

u1 and u2 are each independently 0, 1, 2, 3, 4, 5, 6, or 7;

R^t is each independently H, C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with heteroatom or substituted with OH, SH, or halogen, or cycloalkyl or substituted cycloalkyl;

 represents the bond connecting the tail group to the head group; and

wherein the lipid has a pKa from about 4 to about 8.

19. The method of claim 18, wherein the ionizable lipid is a compound in Table I, Table II, Table III, or Table IV.

20. The method of claim 19, wherein the ionizable lipid is 2272, 2320, 2439, 2356, 2243, 2431, 2455, 2454, 2424, 2433, 2425, 2275, 2220, or 2335.

21. An RNA composition for gene editing, comprising:

one or more polynucleotides encoding one or more components of a gene editing system or one or more gene editing systems,

formulated within a lipid reconstructed natural messenger packs (LNMPs) comprising natural lipids and an ionizable lipid, wherein the ionizable lipid has two or more of the characteristics listed below:

(i) at least 2 ionizable amines;

(ii) at least 3 lipid tails; wherein each of the lipid tails is at least 6 carbon atoms in length;

(iii) a pKa of about 4.5 to about 7.5;

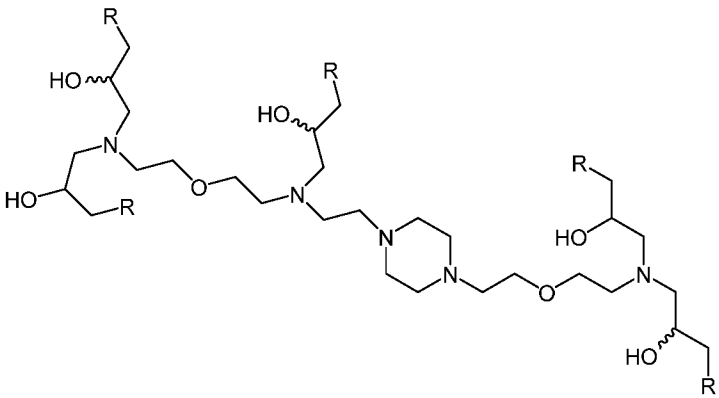
(iv) an ionizable amine and a heteroorganic group separated by a chain of at least two atoms; and

(v) an N:P ratio of at least 3.

22. The RNA composition of claim 21, wherein the ionizable lipid is selected from the group consisting of 1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl) (2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-200), MD1 (cKK-E12), OF2, EPC, ZA3-Ep10, TT3, LP01, 5A2-SC8, Lipid 5, SM-102 (Lipid H), and ALC-315.

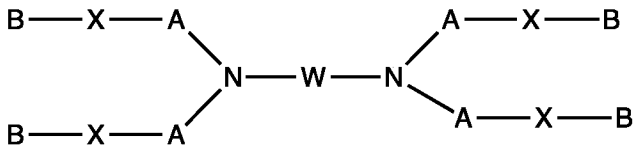
23. The RNA composition of claim 21, wherein the ionizable lipid is C12-200.

24. The RNA composition of claim 21, wherein the ionizable lipid is



(III), wherein R is C8-C14 alkyl group.

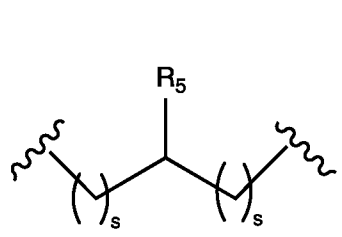
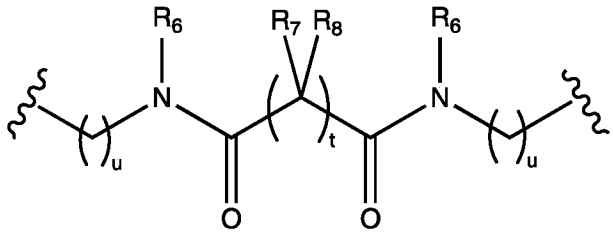
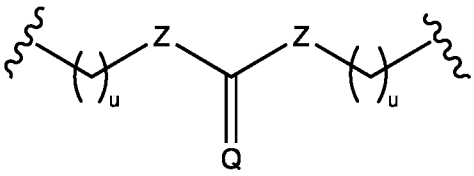
25. The RNA composition of claim 21, wherein the ionizable lipid is selected from one of the following groups of compounds:

i) a compound of formula  (I), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing, wherein:

each **A** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally substituted with heteroatom or substituted with OH, SH, or halogen;

each **B** is independently C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally substituted with heteroatom or substituted with OH, SH, or halogen;

each **X** is independently a biodegradable moiety; and

W is  ,  , or  , wherein

$\mathbf{R_5}$ is OH, SH, $N\mathbf{R_{10}R_{11}}$;

each $\mathbf{R_6}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each $\mathbf{R_7}$ and each $\mathbf{R_8}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $N\mathbf{R_{10}R_{11}}$, wherein each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 alkyl, or $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring;

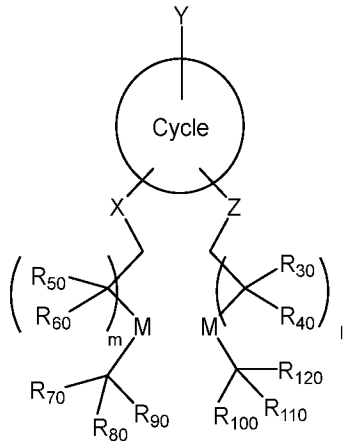
each **s** is independently 1, 2, 3, 4, or 5;

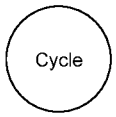
each **u** is independently 1, 2, 3, 4, or 5;

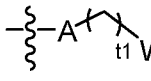
t is 1, 2, 3, 4 or 5;

each **Z** is independently absent, O, S, or $N\mathbf{R_{12}}$, wherein $\mathbf{R_{12}}$ is H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl, and

Q is O, S, or $N\mathbf{R_{13}}$, wherein each $\mathbf{R_{13}}$ is H, C_1-C_5 alkyl;

ii) a compound of formula  (II), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing, wherein:

 is cyclic or heterocyclic moiety;

Y is alkyl, hydroxy, hydroxyalkyl or  ;

A is absent, -O-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -N(R^7)C(O)N(R^7)-, -S-, -S-S-, or a bivalent heterocycle;

each of X and Z is independently absent, -O-, -CO-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, or -S-;

each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxy, hydroxyalkyl, or aminoalkyl;

each M is independently a biodegradable moiety;

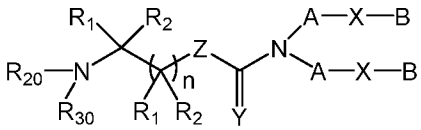
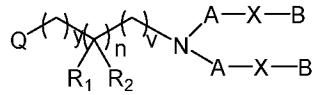
each of R_{30}, R_{40}, R_{50}, R_{60}, R_{70}, R_{80}, R_{90}, R_{100}, R_{110}, and R_{120} is independently H, C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with heteroatom or substituted with OH, SH, or halogen, or cycloalkyl or substituted cycloalkyl;

each of l and m is an integer from 1 to 10;

t1 is an integer from 0 to 10; and

W is hydroxyl, substituted or unsubstituted hydroxyalkyl, substituted or unsubstituted amino, substituted or unsubstituted aminocarbonyl, or substituted or unsubstituted heterocylyl or heteroaryl; and

iii) a compound of formula

 (III) or  (V), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein:

R_{20} and **R_{30}** are each independently H, C_1-C_5 branched or unbranched alkyl, or C_2-C_5 branched or unbranched alkenyl, or **R_{20}** and **R_{30}** together with the adjacent N atom form a 3 to 7 membered cyclic ring, optionally substituted with **R^a**;

R^a is H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, or SH;

each R_1 and each R_2 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, OH, halogen, SH, or $NR_{10}R_{11}$, or

R_1 and R_2 are taken together to form a cyclic ring;

each R_{10} and R_{11} is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or R_{10} and R_{11} are taken together to form a heterocyclic ring;

n is 0, 1, 2, 3 or 4;

Y is O or S;

Z is absent, O, S, or $N(R_{12})(R_{12})$, wherein each R_{12} is independently H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl, provided that when Z is not absent, the adjacent R_1 and R_2 cannot be OH, $NR_{10}R_{11}$, or SH;

u is 0, 1, 2, 3, 4, 5, 6, 7, or 8;

v is 0, 1, 2, 3, or 4;

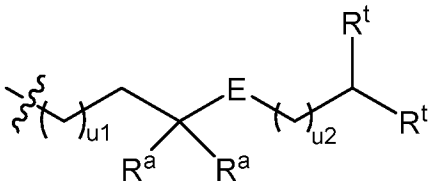
y is 0, 1, 2, 3, or 4;

each **A** is each independently C_1-C_{16} branched or unbranched alkyl, or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen;

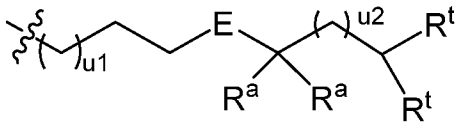
each **B** is each independently C_1-C_{16} branched or unbranched alkyl, or C_2-C_{16} branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or optionally substituted with OH, SH, or halogen; and

each **X** is independently a biodegradable moiety; and

iv) a lipid comprising at least one head group and at least one tail group of formula (TI) or (TI')



(TI) or



(TI'), a pharmaceutically acceptable salt thereof, or a stereoisomer of any of the foregoing,

wherein:

E is each independently -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -C(O-R_{13})-O-, -C(O)O$(CH_2)_r$-, -C(O)N(R^7) $(CH_2)_r$-, -S-S-, or -C(O-R_{13})-O-$(CH_2)_r$-, wherein each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxyalkyl, or aminoalkyl;

R_{13} is branched or unbranched C_3-C_{10} alkyl;

r is 1, 2, 3, 4, or 5;

R^a is each independently C_1-C_5 alkyl, C_2-C_5 alkenyl, or C_2-C_5 alkynyl;

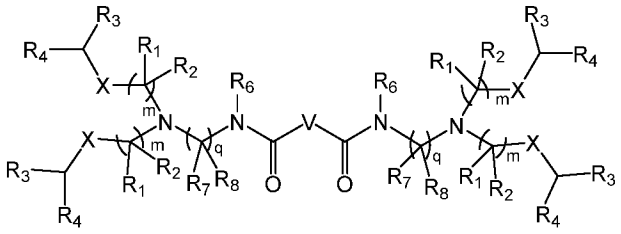
u1 and u2 are each independently 0, 1, 2, 3, 4, 5, 6, or 7;

R^t is each independently H, C_1-C_{16} branched or unbranched alkyl or C_1-C_{16} branched or unbranched alkenyl, optionally interrupted with heteroatom or substituted with OH, SH, or halogen, or cycloalkyl or substituted cycloalkyl;

 represents the bond connecting the tail group to the head group; and

wherein the lipid has a pKa from about 4 to about 8.

26. The RNA composition of claim 25, wherein the ionizable lipid is a compound of group i),



represented by a formula of (IX),

pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein:

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ is independently H, C_1-C_3 branched or unbranched alkyl, OH, halogen, SH, or $N\mathbf{R_{10}R_{11}}$, or

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ are independently taken together with the carbon atom(s) to which they are attached to form a cyclic ring;

each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 branched or unbranched alkyl, or $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring;

each $\mathbf{R_3}$ and each $\mathbf{R_4}$ is independently H, C_2-C_{14} branched or unbranched alkyl (e.g., C_3-C_{10} branched or unbranched alkyl), or C_3-C_{10} branched or unbranched alkenyl, provided that at least one of $\mathbf{R_3}$ and $\mathbf{R_4}$ is not H;

each **X** is independently a biodegradable moiety;

each **q** is independently 2, 3, 4,or 5;

V is branched or unbranched C_2-C_{10} alkylene, C_2-C_{10} alkenylene, C_2-C_{10} alkynylene, or C_2-C_{10} heteroalkylene, optionally substituted with one or more OH, SH, and/or halogen groups;

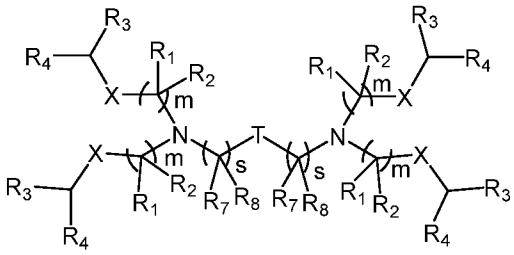
each $\mathbf{R_6}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each $\mathbf{R_7}$ and each $\mathbf{R_8}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $(CH_2)_v\mathbf{R_{17}}$, or $N\mathbf{R_{10}R_{11}}$, wherein each **v** is independently 0, 1, 2, 3, 4, or 5, and $\mathbf{R_{17}}$ is OH, SH, or $N(CH_3)_2$; and

each **m** is independently 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

27. The RNA composition of claim 26, wherein **V** is a branched or unbranched C_2-C_3 alkylene, and each $\mathbf{R_6}$ is independently H or methyl.

28. The RNA composition of claim 25, wherein the ionizable lipid is a compound of group i),

represented by a formula of  (XI), pharmaceutically acceptable salts thereof, and stereoisomers of any of the foregoing, wherein:

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ is independently H, C_1-C_3 branched or unbranched alkyl, OH, halogen, SH, or $N\mathbf{R_{10}R_{11}}$, or

each $\mathbf{R_1}$ and each $\mathbf{R_2}$ are independently taken together with the carbon atom(s) to which they are attached to form a cyclic ring;

each $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ is independently H, C_1-C_3 branched or unbranched alkyl, or $\mathbf{R_{10}}$ and $\mathbf{R_{11}}$ are taken together to form a heterocyclic ring;

each $\mathbf{R_3}$ and each $\mathbf{R_4}$ is independently H, C_2-C_{14} branched or unbranched alkyl (e.g., C_3-C_{10} branched or unbranched alkyl), or C_3-C_{10} branched or unbranched alkenyl, provided that at least one of $\mathbf{R_3}$ and $\mathbf{R_4}$ is not H;

each **X** is independently a biodegradable moiety;

each **s** is independently 1, 2, 3, 4, or 5;

T is –NHC(O)O-, –OC(O)NH-, or a divalent heterocyclic optionally substituted with one or more $-(CH_2)_vOH$, $-(CH_2)_vSH$, or $-(CH_2)_v$-halogen groups,

each $\mathbf{R_7}$ and each $\mathbf{R_8}$ is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, OH, SH, $(CH_2)_v\mathbf{R_{17}}$, or $N\mathbf{R_{10}R_{11}}$, wherein $\mathbf{R_{17}}$ is OH, SH, or $N(CH_3)_2$;

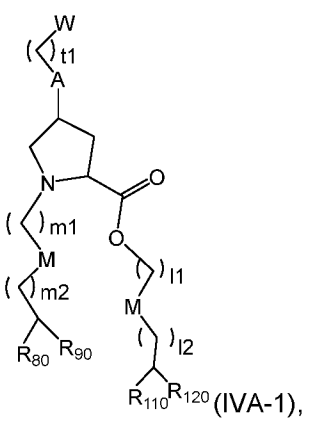
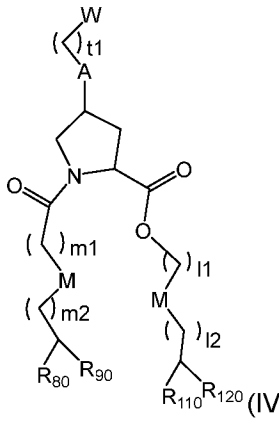
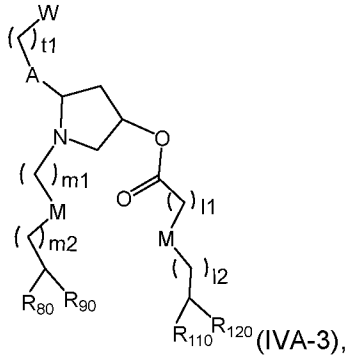
each **v** is independently 0, 1, 2, 3, 4, or 5; and

each **m** is independently 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

29. The RNA composition of claim 28, wherein T is a divalent piperazine or a divalent dioxopiperazine.

30. The RNA composition of any one of claims 26-29, wherein **X** is -OCO-, -COO-, -CONH-, or -NHCO-.

31. The RNA composition of claim 25, wherein the ionizable lipid is a compound of group ii), represented by one of the following formulas:


(IVA-1),

(IVA-2), or

(IVA-3),

wherein:

each m1 is independently an integer from 3 to 6,

each l1 is independently an integer from 4 to 8,

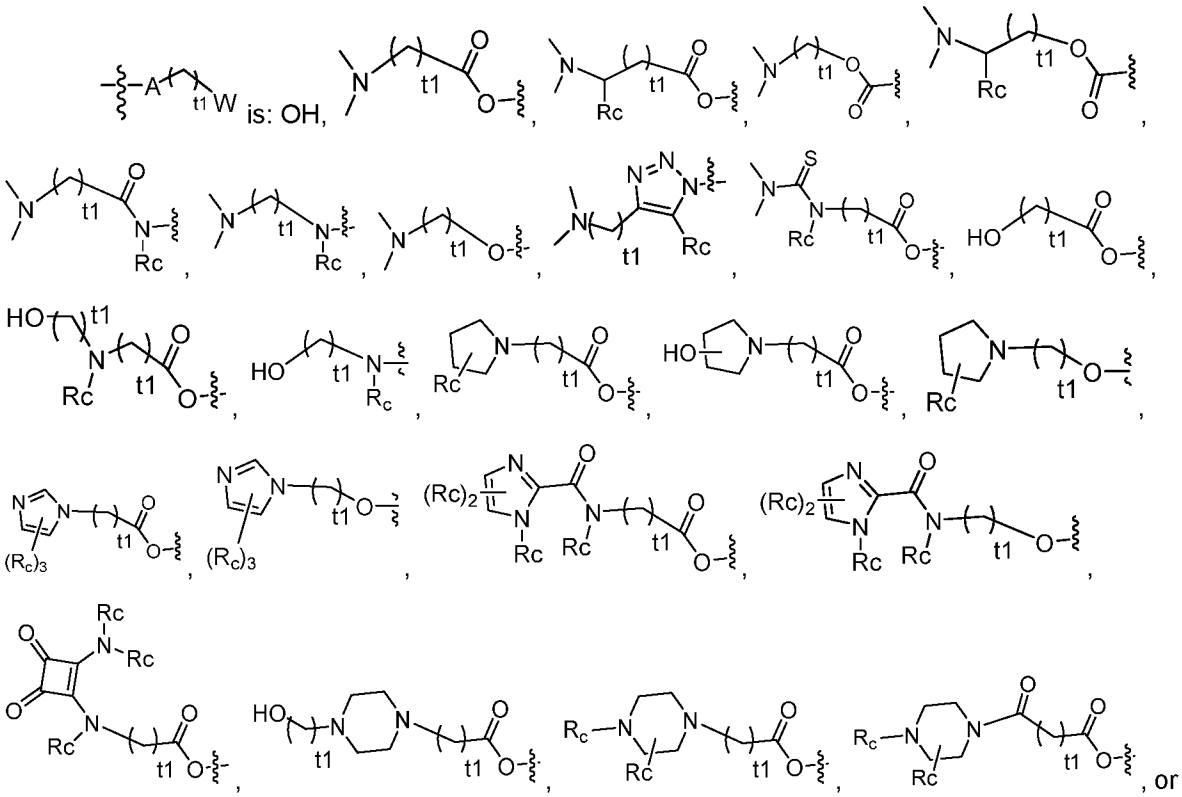
m2 and l2 are each independently an integer from 0 to 3,

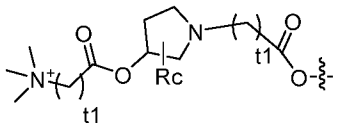
R_{80} and R_{90} are each independently unsubstituted C_5-C_8 alkyl or alkenyl; or R_{80} is H or unsubstituted C_1-C_4 alkyl or alkenyl, and R_{90} is unsubstituted C_5-C_{11} alkyl or alkenyl; and

R_{110} and R_{120} are each independently unsubstituted C_5-C_8 alkyl or alkenyl; or R_{110} is H or unsubstituted C_1-C_4 alkyl or alkenyl, and R_{120} is unsubstituted C_5-C_{11} alkyl or alkenyl.

32. The RNA composition of claim 31, wherein:

M is -OC(O)- or -C(O)O-;


, or



,

each R^c is independently H or C_1-C_3 alkyl;

each t1 is independently 1, 2, 3, or 4;

each of R_{80} and R_{90} is independently H or C_1-C_{12} branched or unbranched alkyl; and

each of R_{110} and R_{120} is independently H or C_1-C_{12} branched or unbranched alkyl, provided that at least one of R_{80} and R_{90} is not H, and at least one of R_{110} and R_{120} is not H.

33. The RNA composition of claim 25, wherein the ionizable lipid is a compound of group iii), wherein $\mathbf{R_1}$ and $\mathbf{R_2}$ are each H, or each $\mathbf{R_1}$ is H, and one of the $\mathbf{R_2}$ variables is OH; and X is –OC(O)- or –C(O)O-.

34. The RNA composition of claim 33, wherein the ionizable lipid is a compound of group iii), represented by formula III), wherein:

$\mathbf{R_{20}}$ and $\mathbf{R_{30}}$ are each independently H or C_1-C_3 branched or unbranched alkyl; or $\mathbf{R_{20}}$ and $\mathbf{R_{30}}$ together with the adjacent N atom form a 3 to 7 membered cyclic ring, optionally substituted with $\mathbf{R^a}$;

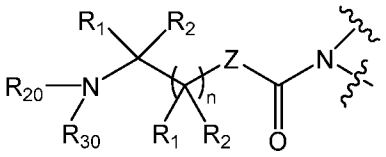
$\mathbf{R^a}$ is H or OH;

Z is absent, S, O, or NH; and

n is 0, 1, or 2.

35. The RNA composition of claim 33, wherein the ionizable lipid is a compound of group iii), represented by formula V),

36. The RNA composition of claim 25, wherein the ionizable lipid is a compound of group iv), comprising at least one head group and at least one tail group, wherein:
the tail group has a structure of formula (TI) (or TI'); and
the head group has a structure of one of the following formulas:

i)  (HA-I),

wherein:

R_{20} and R_{30} are each independently H, C_1-C_5 branched or unbranched alkyl, or C_2-C_5 branched or unbranched alkenyl, optionally interrupted with one or more heteroatoms or substituted with OH, SH, halogen, or cycloalkyl groups; or

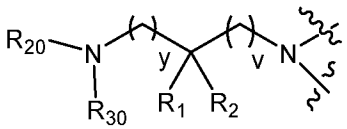
R_{20} and R_{30}, together with the adjacent N atom, form a 3 to 7 membered heterocylic or heteroaromatic ring containing one or more heteroatoms, optionally substituted with one or more OH, SH, halogen, alkyl, or cycloalkyl groups;

each of R_1 and R_2 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, OH, halogen, SH, or $NR_{10}R_{11}$; or R_1 and R_2 together form a cyclic ring;

each of R_{10} and R_{11} is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl; or R_{10} and R_{11} together form a heterocyclic ring;

n is 0, 1, 2, 3 or 4; and

Z is absent, O, S, or NR_{12}, wherein R_{12} is H or C_1-C_7 branched or unbranched alkyl; provided that when Z is not absent, the adjacent R_1 and R_2 cannot be OH, $NR_{10}R_{11}$, SH;

ii)  (HA-V).

wherein:

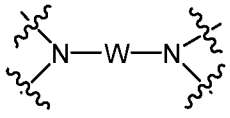
R_1 is H, C_1-C_3 alkyl, OH, halogen, SH, or $NR_{10}R_{11}$;

R_2 is OH, halogen, SH, or $NR_{10}R_{11}$; or R_1 and R_2 can be taken together to form a cyclic ring;

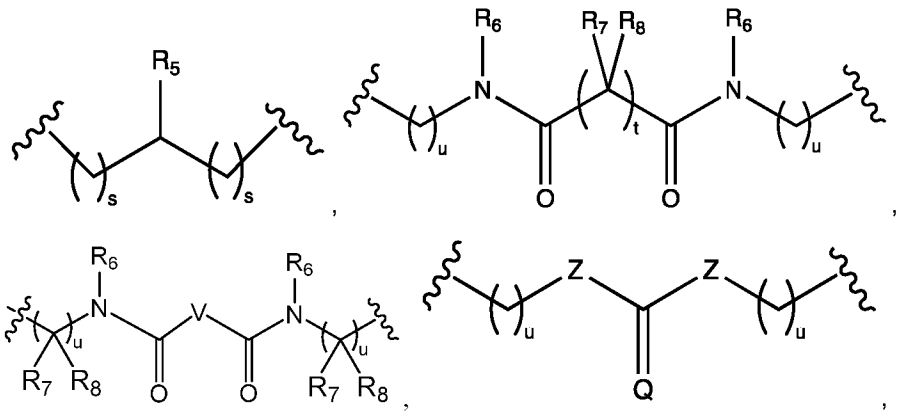
R_{10} and R_{11} are each independently H or C_1-C_3 alkyl; or R_{10} and R_{11} can be taken together to form a heterocyclic ring;

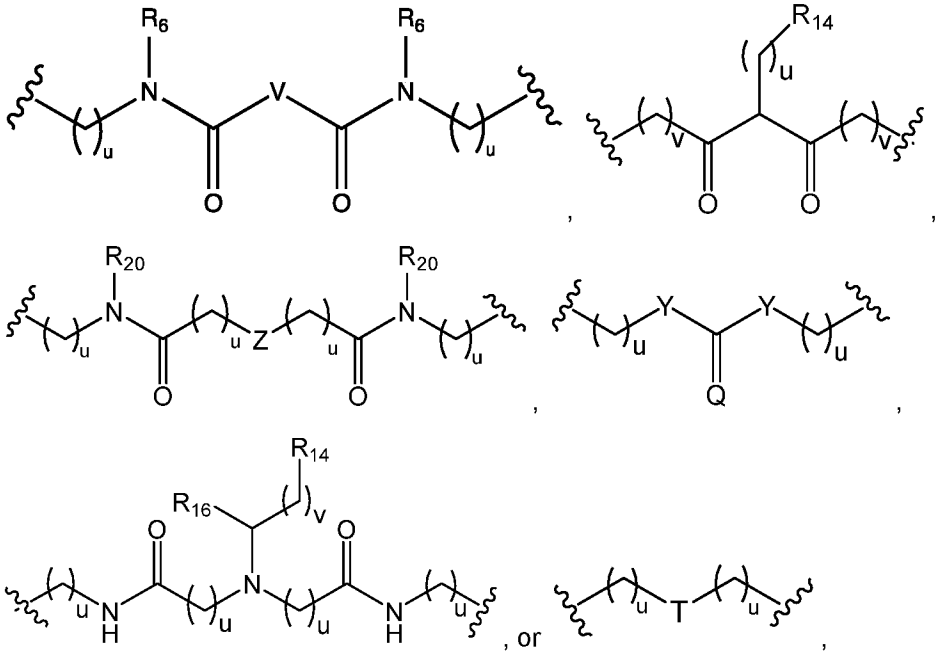
R_{20} and R_{30} are each independently H, C_1-C_5 branched or unbranched alkyl, C_2-C_5 branched or unbranched alkenyl; or R_{20} and R_{30} can be taken together to form a cyclic ring; and

each of v and y is independently 1, 2, 3, or 4;

iii)  (HB-I),

wherein W is





wherein

R_5 is OH, SH, $(CH_2)_sOH$, or $NR_{10}R_{11}$;

each R_6 is independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, or cycloalkyl;

each R_7 and R_8 are independently H, C_1-C_3 branched or unbranched alkyl, C_2-C_3 branched or unbranched alkenyl, halogen, $CH_2)_vOH$, $(CH_2)_vSH$, $(CH_2)_sN(CH_3)_2$, or $NR_{10}R_{11}$, wherein each R_{10} and R_{11} is independently H or C_1-C_3 alkyl, or R_{10} and R_{11} are taken together to form a heterocyclic ring; or R_7 and R_8 are taken together to form a ring;

each R_{20} is independently H, or C_1-C_3 branched or unbranched alkyl;

R_{14} is a heterocyclic, $NR_{10}R_{11}$, $C(O)NR_{10}R_{11}$, $NR_{10}C(O)NR_{10}R_{11}$, or $NR_{10}C(S)NR_{10}R_{11}$, wherein each R_{10} and R_{11} is independently H, C_1-C_3 alkyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloalkenyl, optionally substituted with one or more NH and/or oxo groups, or R_{10} and R_{11} are taken together to form a heterocyclic ring;

R_{16} is H, =O, =S, or CN;

each of s, u, and t is independently 1, 2, 3, 4, or 5;

each v is independently 0, 1, 2, 3, 4, or 5;

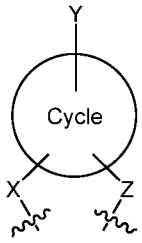
each Y is a divalent heterocyclic;

each Z is independently absent, O, S, or NR_{12}, wherein R_{12} is H, C_1-C_7 branched or unbranched alkyl, or C_2-C_7 branched or unbranched alkenyl;

Q is O, S, CH_2, or NR_{13}, wherein each R_{13} is H, C_1-C_5 alkyl;

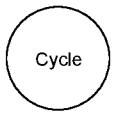
V is branched or unbranched C_2-C_{10} alkylene, C_2-C_{10} alkenylene, C_2-C_{10} alkynylene, or C_2-C_{10} heteroalkylene, optionally substituted with one or more OH, SH, and/or halogen groups; and

T is –NHC(O)O-, –OC(O)NH-, or a divalent heterocyclic; and

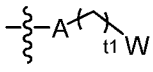
iv) 

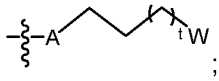
(HC-I),

wherein:



is cyclic or heterocyclic moiety;

Y is alkyl, hydroxy, hydroxyalkyl, 

, or 

;

A is absent, -O-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, -N(R^7)C(O)N(R^7)-, -S-, or -S-S-;

each of X and Z is independently absent, -O-, -C(O)-, -N(R^7)-, -O-alkylene-, -alkylene-O-, -OC(O)-, -C(O)O-, -N(R^7)C(O)-, -C(O)N(R^7)-, or -S-;

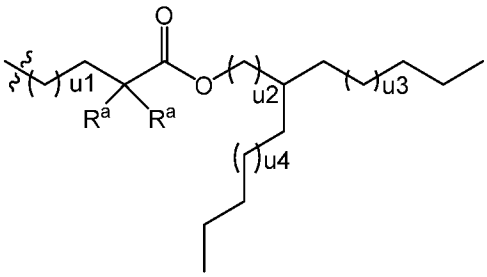
each R^7 is independently H, alkyl, alkenyl, cycloalkyl, hydroxy, alkoxy, hydroxyalkyl, alkylamino, alkylaminoalkyl, or aminoalkyl;

t1 is an integer from 0 to 10; and

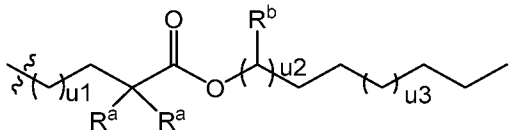
W is hydroxyl, substituted or unsubstituted hydroxyalkyl, substituted or unsubstituted amino, substituted or unsubstituted aminocarbonyl, or substituted or unsubstituted heterocyclyl or heteroaryl; and

wherein the lipid has a pKa from about 4 to about 8.

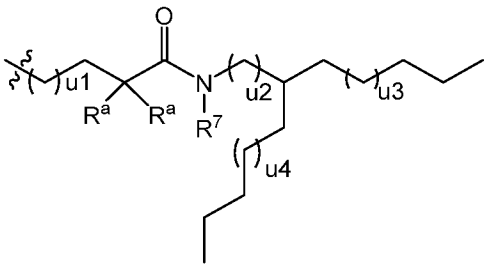
37. The RNA composition of claim 36, wherein the ionizable lipid is a compound of group iv), and wherein at least one tail group of the lipid has one of the following formulas:



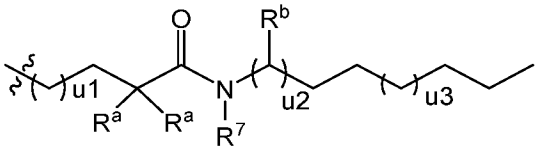
(TII),



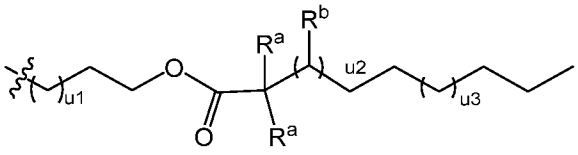
(TIII),



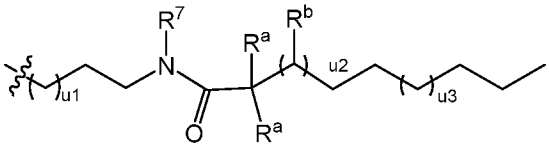
(TIV),



(TV),



(TII'), and



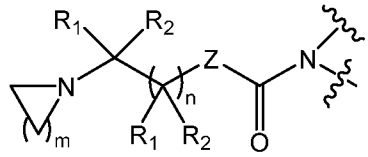
(TIII'), wherein:

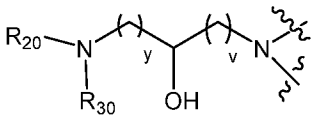
R^7 is each independently H or methyl;

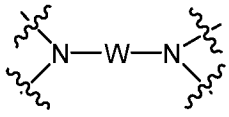
R^b is in each occasion independently H or C_1-C_4 alkyl; and

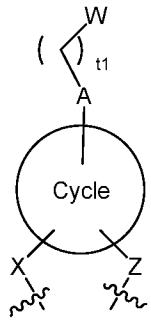
u3 and u4 are each independently 0, 1, 2, 3, 4, 5, 6, or 7; and

the head group has a structure of one of the following formulas:

i)  (HA-IA), wherein m is 1, 2, 3, 4, 5, 6, 7 or 8;

ii)  (HA-VI).

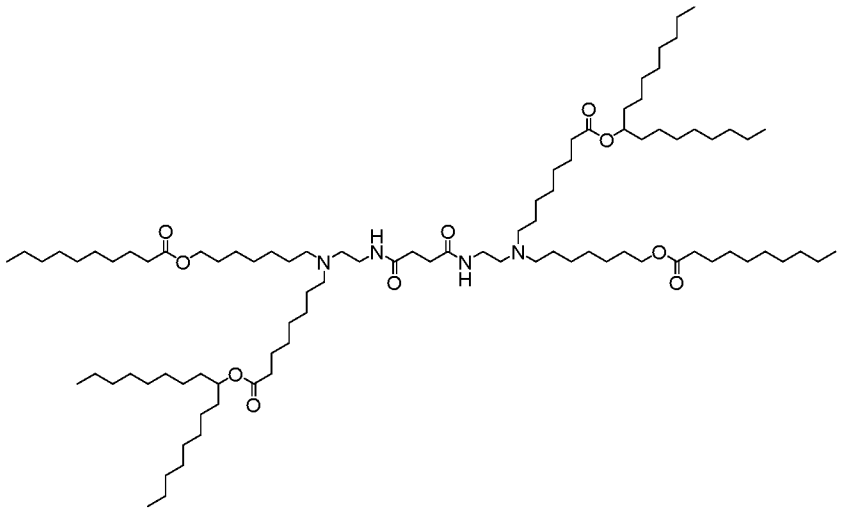
iii)  (HB-I), and

iv)  (HC-IB).

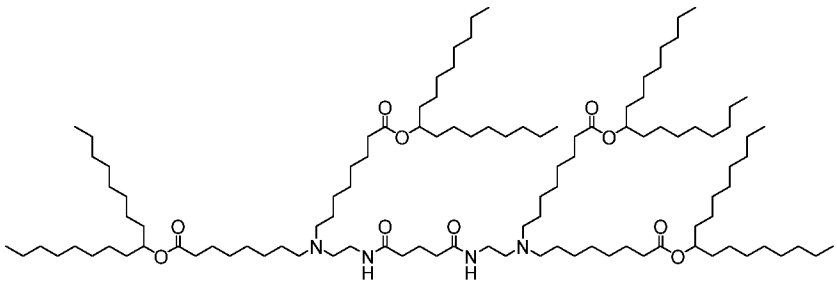
38. The RNA composition of claim 37, wherein at least one tail group has the structure of formula (TII), (TIII) TIV), (TV), (TII'), and/or (TIII'), wherein u1 is 3-5, u2 is 0-3, wherein u3 and u4 are each independently 1-7, and R^a is each independently methyl.

39. The RNA composition of claim 25, wherein the ionizable lipid is a compound in Table I, Table II, Table III, or Table IV.

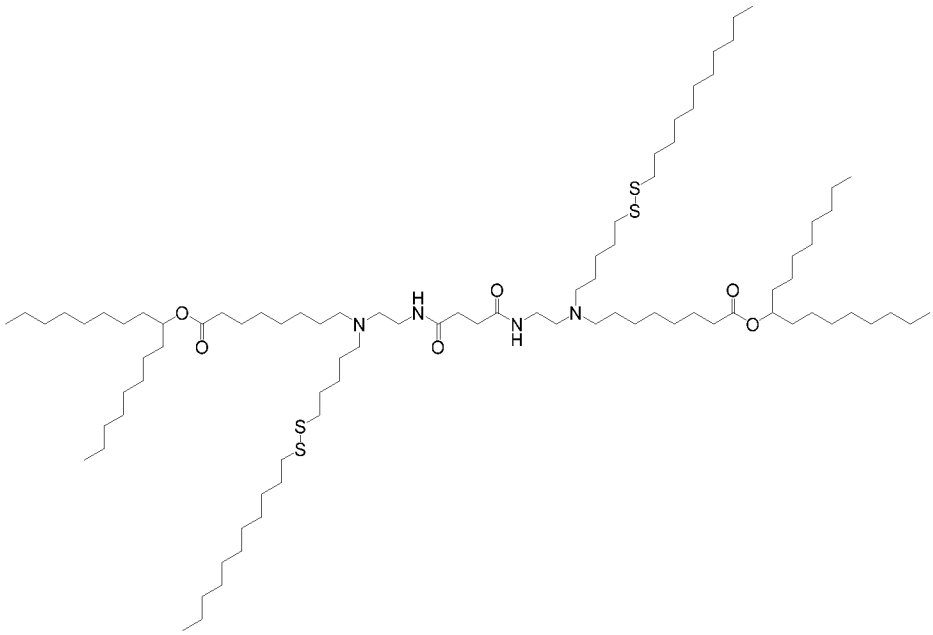
40. The RNA composition of claim 39, wherein the ionizable lipid is



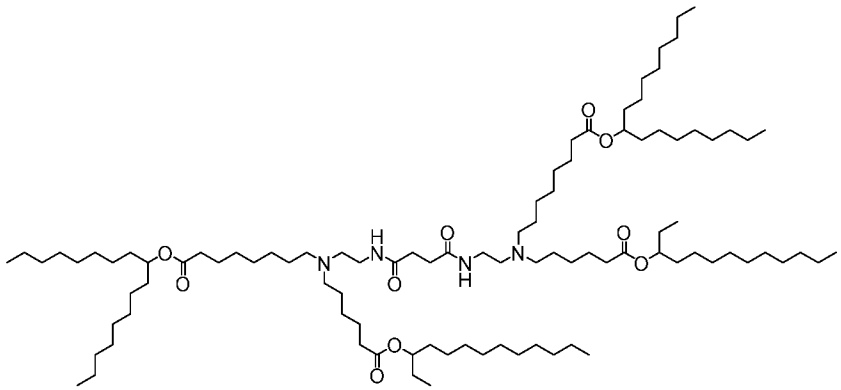
(2272),



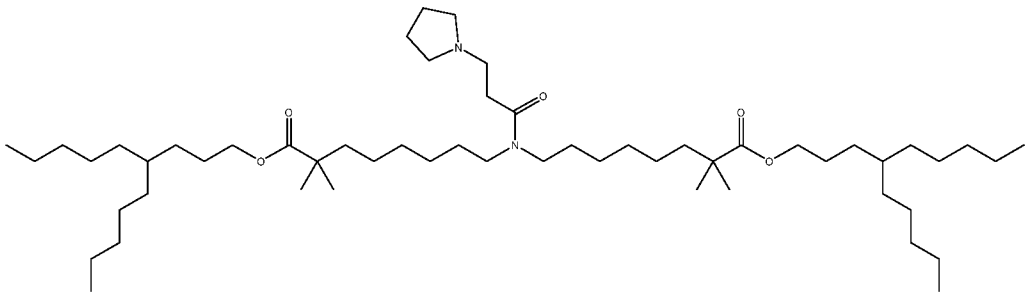
(2320),



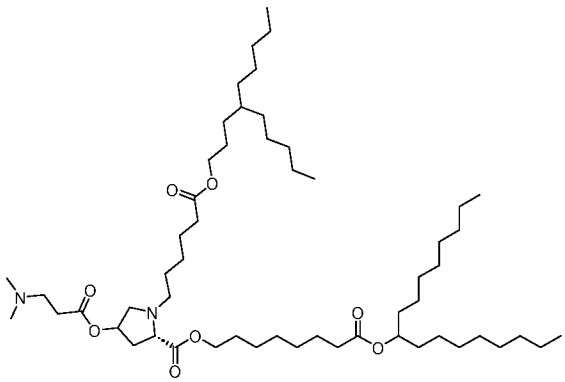
(2439),



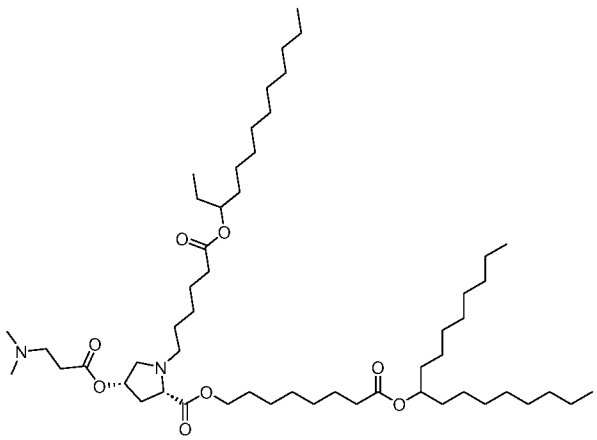
(2356),



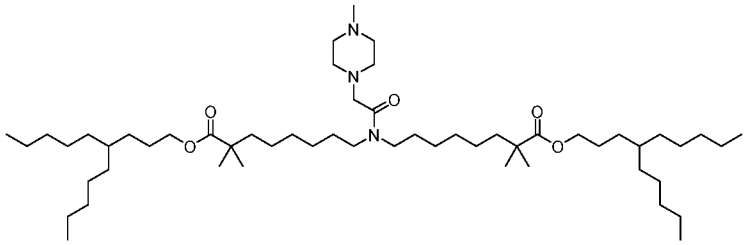
(2243),



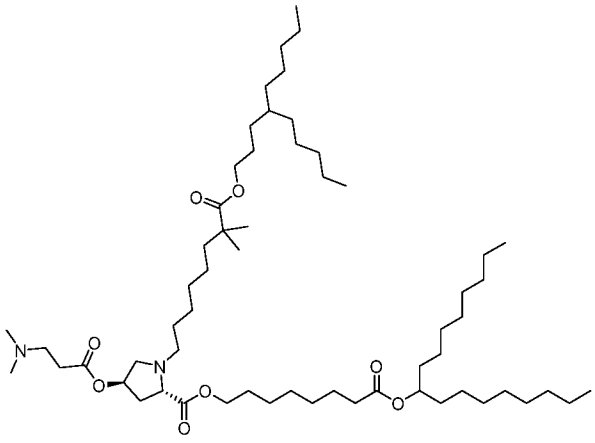
(2431),



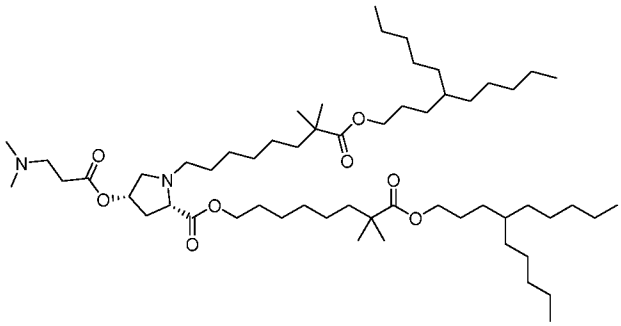
(2455),



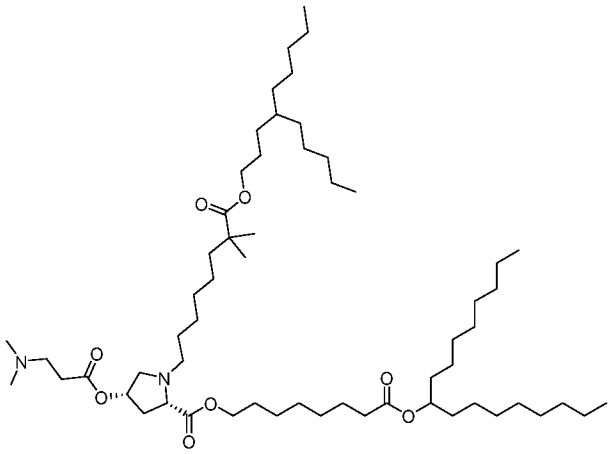
(2454),



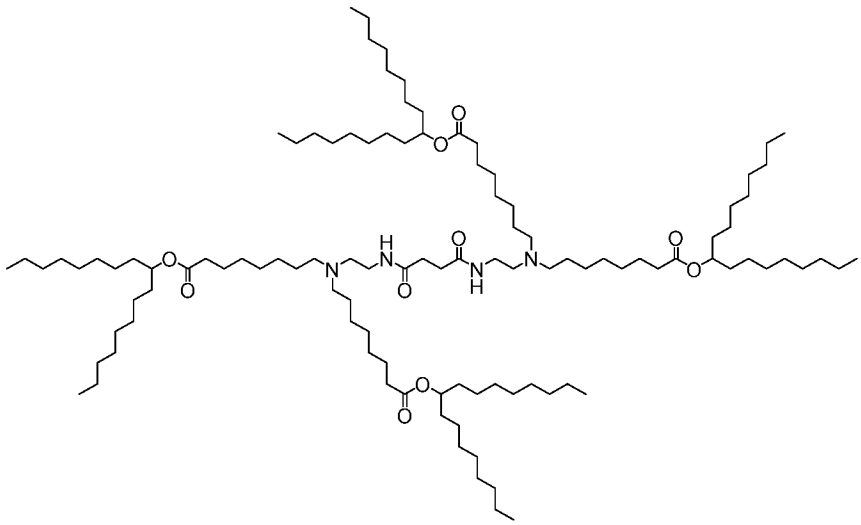
(2424),



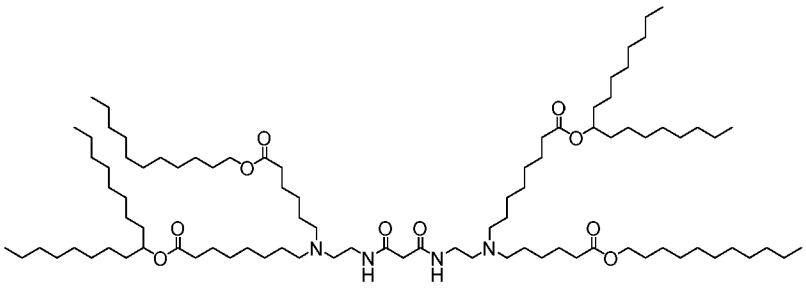
(2433),



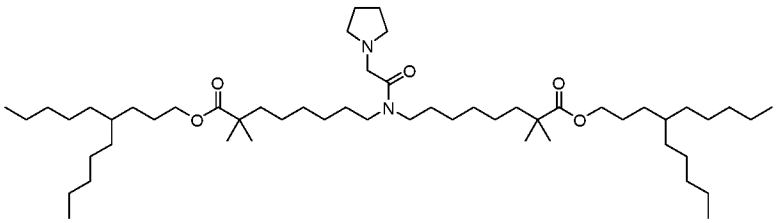
(2425),



(2275),



(2220), or



(2335).

41. The RNA composition of claim 21, wherein the natural lipids are extracted from lemon or algae.

42. The RNA composition of claim 21, wherein the LNMPs further comprise a sterol and a polyethylene glycol (PEG)-lipid conjugate.

43. The RNA composition of claim 42, wherein the sterol is cholesterol.

44. The RNA composition of claim 42, wherein the PEG lipid conjugate comprises a PEG-2k.

45. The RNA composition of claim 42, wherein the PEG lipid conjugate is a PEG-DMG or PEG-PE.

46. The RNA composition of claim 42, wherein the PEG lipid conjugate is a PEG2k-DMG or PEG-2k-PE.

47. The RNA composition of claim 21, wherein the LNMP comprises:
about 20 mol% to about 50 mol% of the ionizable lipid,
about 20 mol% to about 60 mol% of the natural lipids,
about 7 mol% to about 50 mol% of the sterol, and
about 0.5 mol% to about 3 mol% of the polyethylene glycol (PEG)-lipid conjugate.

48. The RNA composition of claim 47, wherein the LNMPs comprise ionizable lipid:natural lipids:sterol:PEG-lipid at a molar ratio of about 35:50:12.5:2.5, about 35:20:42.5:2.5, about 35:16:46.5:2.5, about 50:10:38.5:1.5, or about 50:20:28.5:1.5.

49. The RNA composition of claim 47, wherein the amount of the PEG lipid conjugate is about 1.5-2.5 mol%.

50. The RNA composition of claim 47, wherein the amount of the ionizable lipid is about 30-50 mol%.

51. The RNA composition of claim 50, wherein the amount of the ionizable lipid is about 50 or 35 mol%.

52. The RNA composition of claim 47, wherein the N/P ratio is 6 ± 1.

53. The RNA composition of claim 47, wherein the N/P ratio is 3 ± 1.

54. The RNA composition of claim 47, wherein the N/P ratio is 15 ± 1.

55. The RNA composition of any one of the preceding claims, wherein the gene editing system comprises an RNA-guided DNA-binding agent.

56. The RNA composition of claim 55, wherein the gene editing system is CRISPR-Cas gene editing system.

57. The RNA composition of claim 55, wherein the one or more polynucleotides comprise an mRNA or modified mRNA.

58. The RNA composition of claim 55, wherein the RNA-guided DNA-binding agent is a Cas nuclease mRNA.

59. The RNA composition of claim 58, wherein the Cas nuclease mRNA is a Class II Cas nuclease mRNA.

60. The RNA composition of claim 59, wherein the Class II Cas nuclease is a Cas9 nuclease mRNA.

61. The RNA composition of claim 55, wherein the one or more polynucleotides comprise a gRNA or modified gRNA.

62. The RNA composition of claim 55, wherein the one or more polynucleotides comprise a gRNA and a Class II Cas nuclease mRNA.

63. The RNA composition of claim 55, wherein the one or more polynucleotides comprise an RNA

comprising an open reading frame encoding an RNA-guided DNA-binding agent, wherein the open reading frame has a uridine content ranging from its minimum uridine content to 150% of the minimum uridine content.

64. The RNA composition of claim 63, wherein the one or more polynucleotides comprise an mRNA comprising an open reading frame encoding an RNA-guided DNA-binding agent, wherein the open reading frame has a uridine dinucleotide content ranging from its minimum uridine dinucleotide content to 150% of the minimum uridine dinucleotide content.

65. The RNA composition of claim 61 or 62, wherein the gRNA is a dual-guide RNA (dgRNA) or an sgRNA.

66. The RNA composition of claim 61, wherein the gRNA is a modified gRNA comprising a modification selected from the group consisting of 2'-O-methyl (2'-O-Me) modified nucleotide, a phosphorothioate (PS) bond between nucleotides, and a 2'-fiuoro (2'-F) modified nucleotide.

67. The RNA composition of claim 61, wherein the gRNA is a modified gRNA comprising a modification at one or more of the first five nucleotides at the 5' end or the 3' end.

68. The RNA composition of claim 61, wherein the gRNA is a modified gRNA comprising PS bonds between the first four nucleotides or the last four nucleotides.

69. The RNA composition of any of claims 66-68, wherein the modified gRNA further comprises 2'-O-Me modified nucleotides at the first three nucleotides at the 5' end or the 3' end.

70. The RNA composition of claim 62, wherein the gRNA and Class 2 Cas nuclease mRNA are present in a ratio ranging from about 10:1 to about 1:10 by weight.

71. The RNA composition of claim 62, wherein the gRNA and Class 2 Cas nuclease mRNA are present in a ratio ranging from about 5:1 to about 1:5 by weight.

72. The RNA composition of claim 62, wherein the gRNA and Class 2 Cas nuclease mRNA are present in a ratio ranging from about 2:1 to about 1:2 by weight.

73. The RNA composition of claim 62, wherein the gRNA and Class 2 Cas nuclease mRNA are present in a ratio of about 2:1 by weight or about 1:1 by weight.

74. The RNA composition of claim 55, further comprising at least one template nucleic acid.

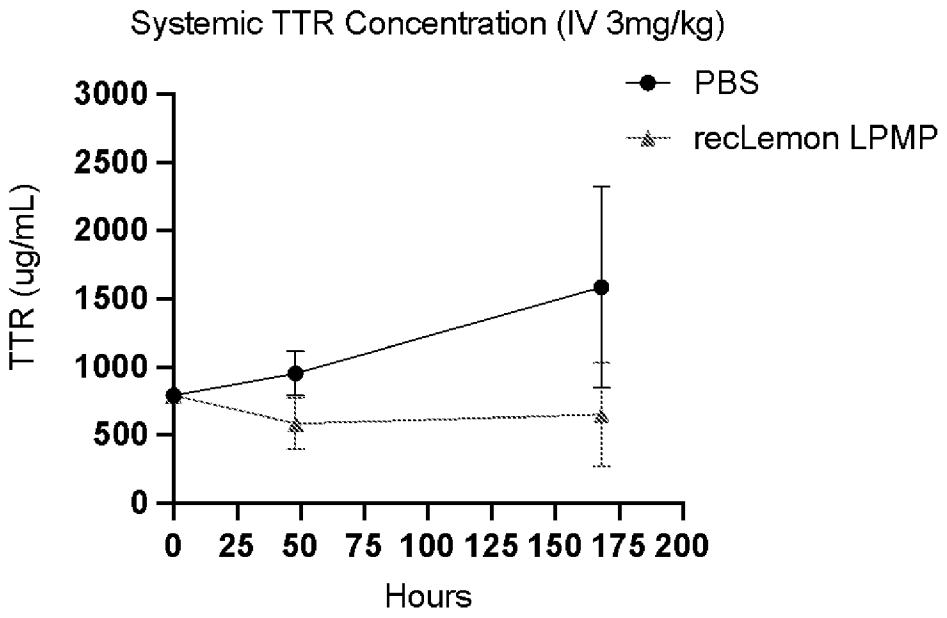


Figure 1A

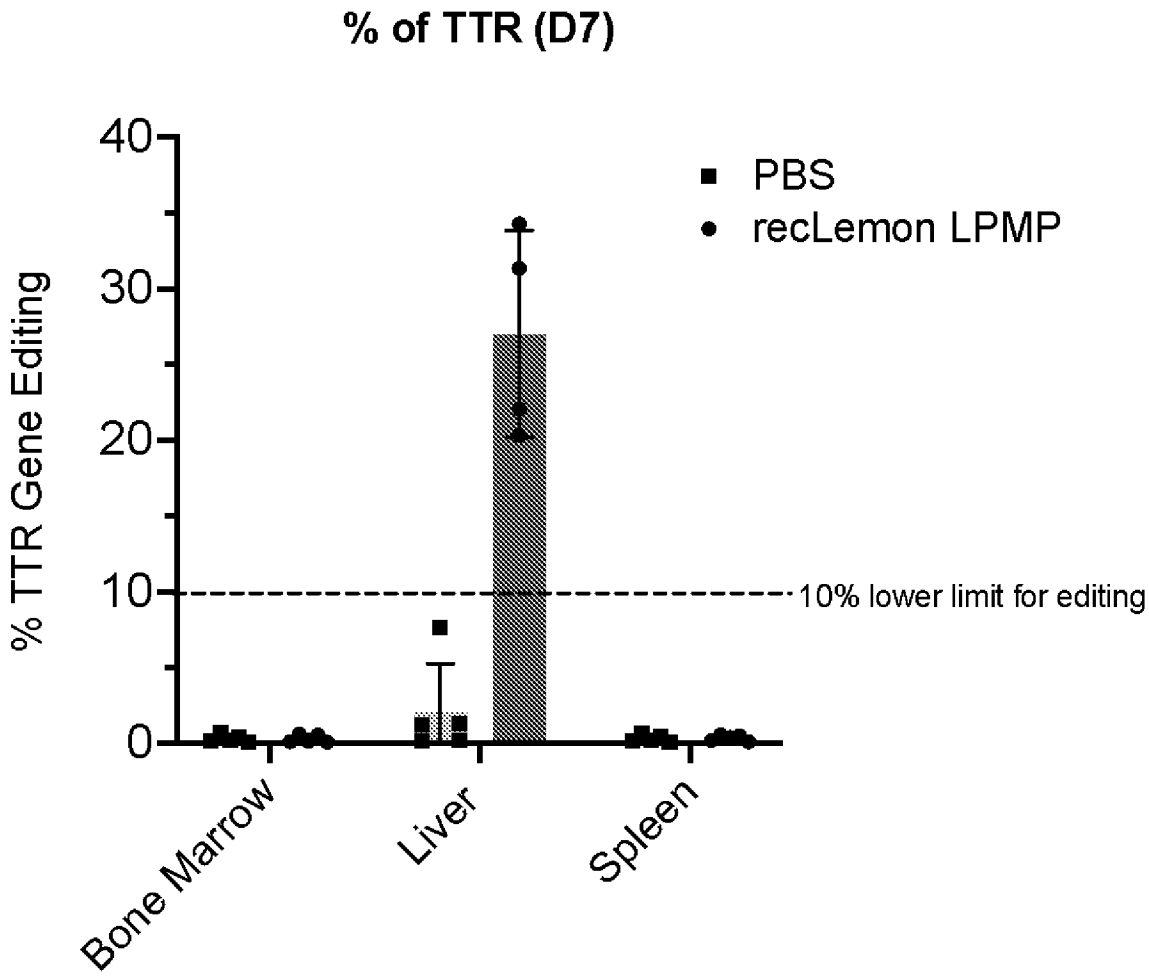


Figure 1B

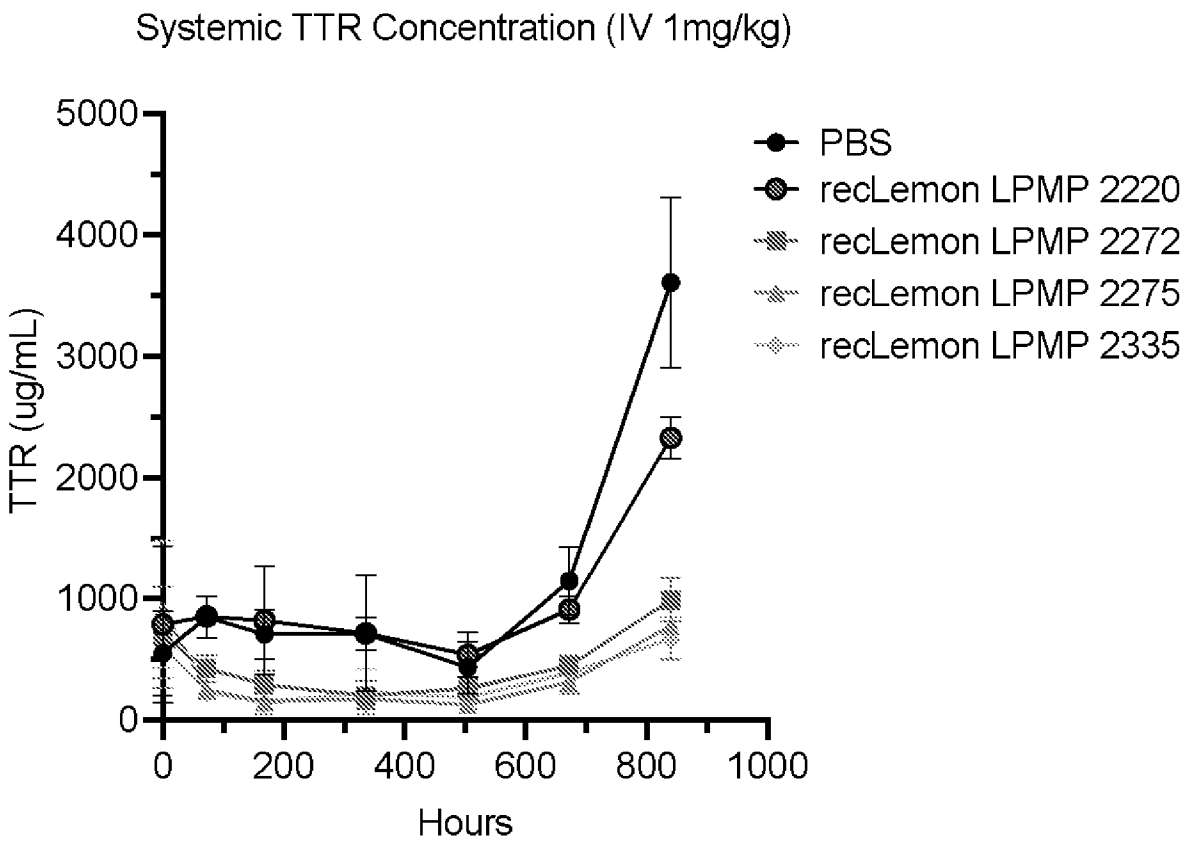


Figure 2A

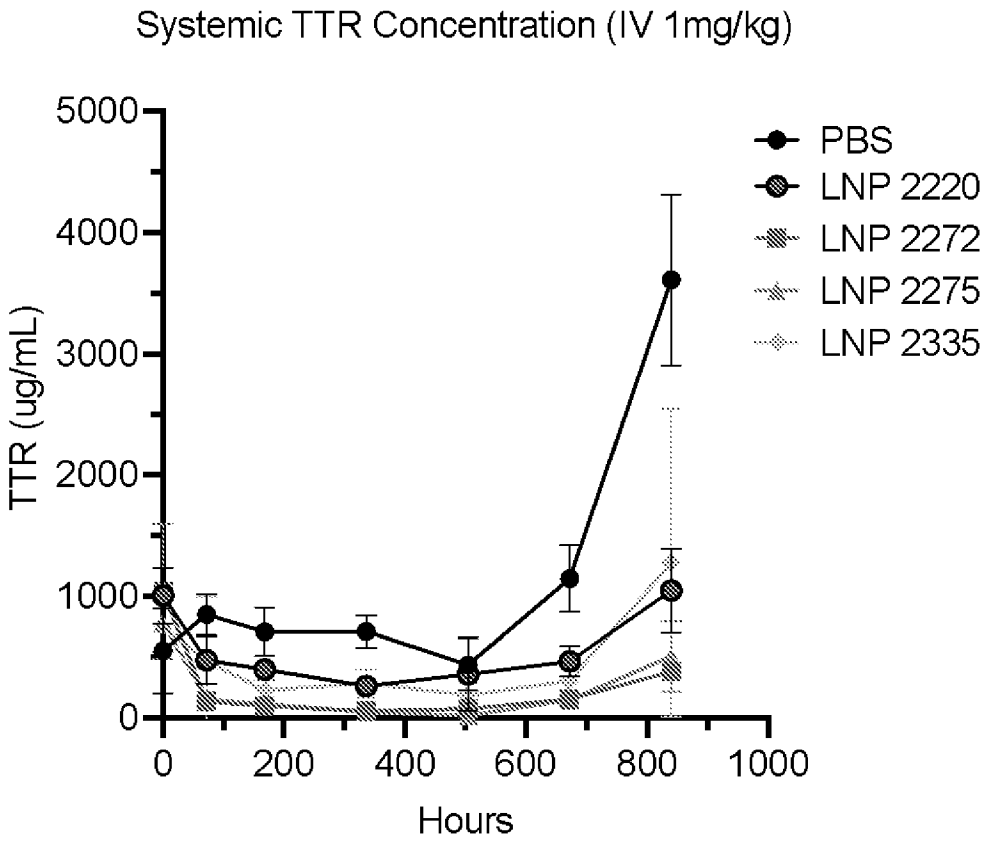


Figure 2B

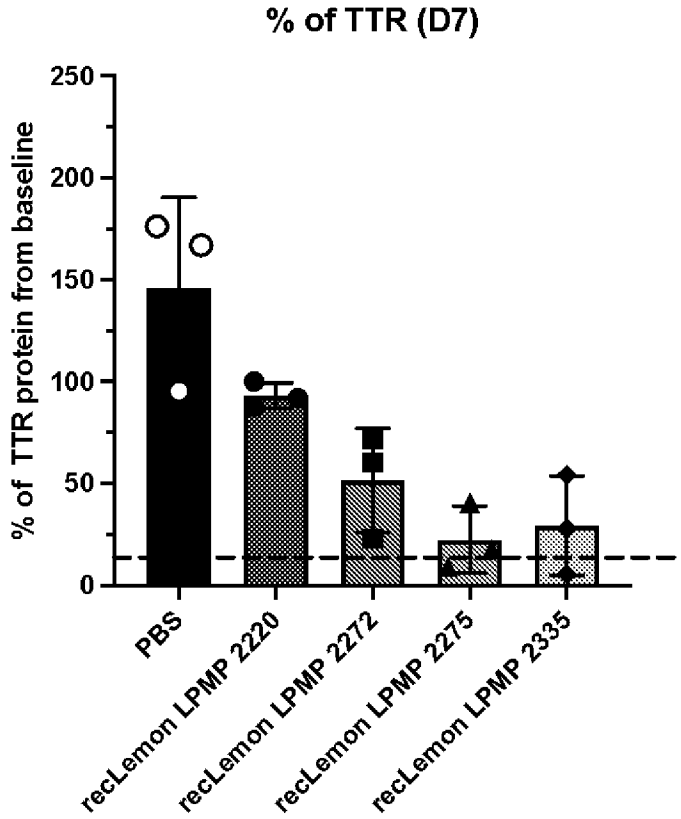


Figure 3A

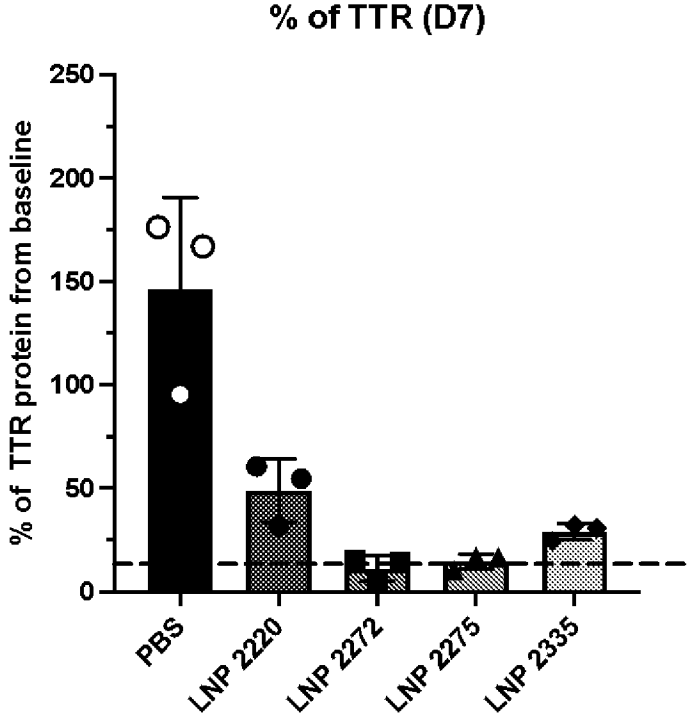


Figure 3B

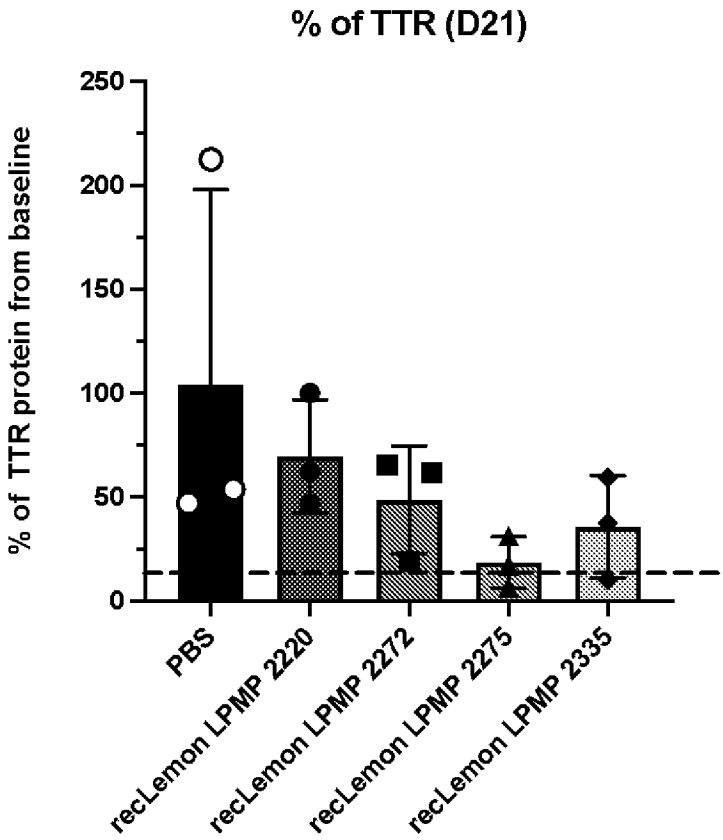


Figure 4A

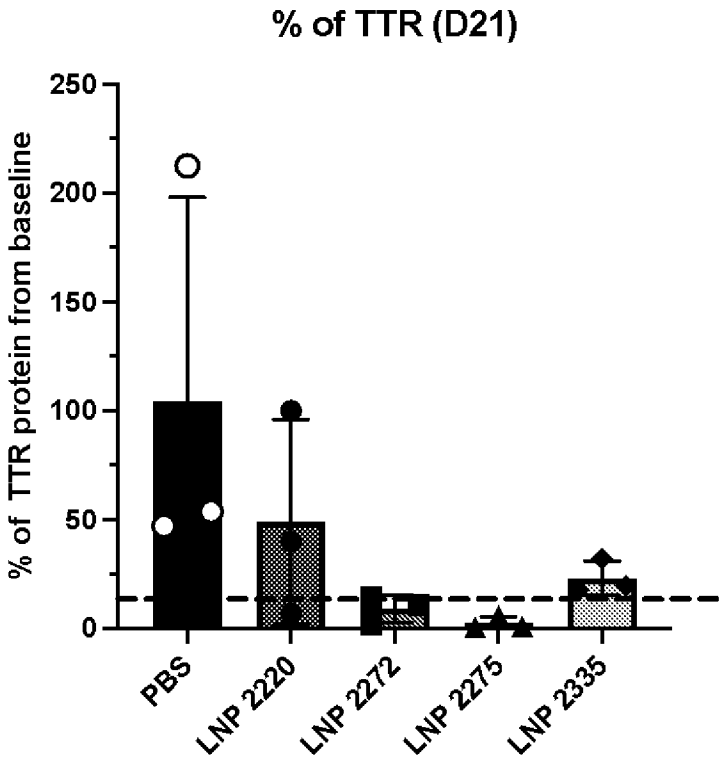


Figure 4B

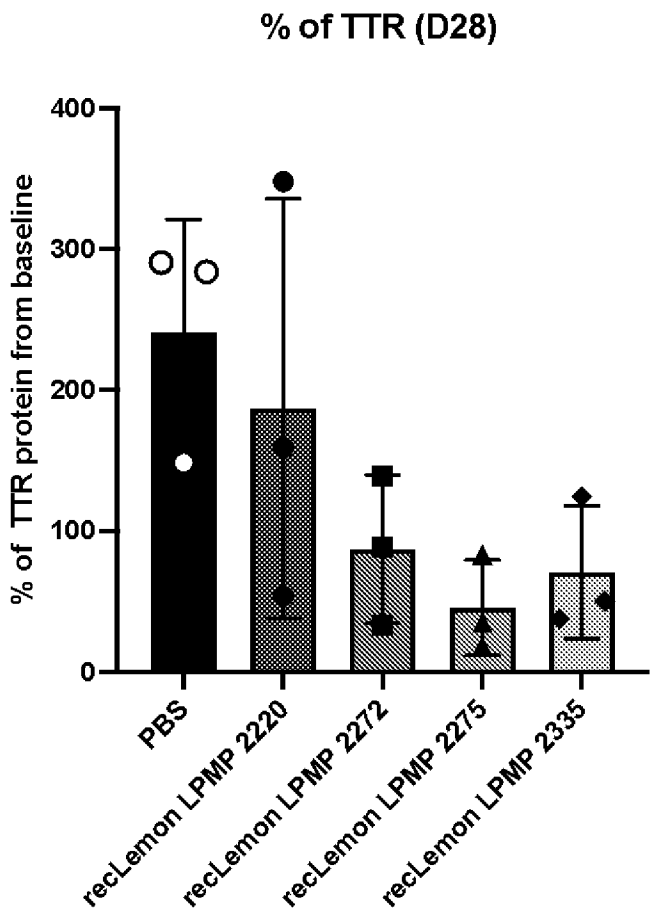


Figure 5A

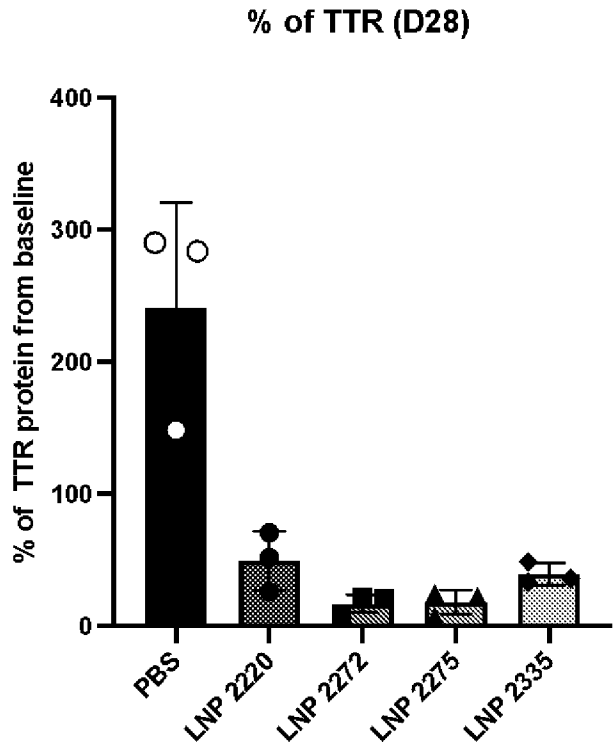


Figure 5B

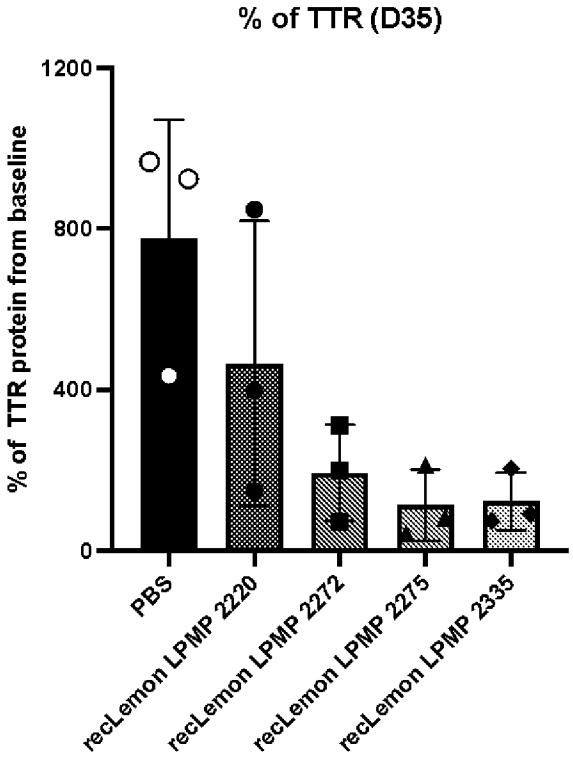


Figure 6A

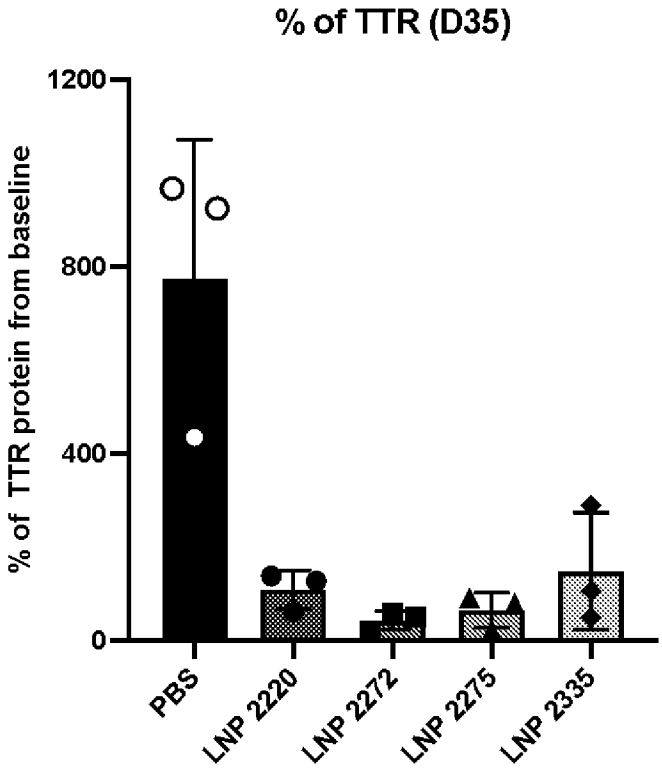


Figure 6B

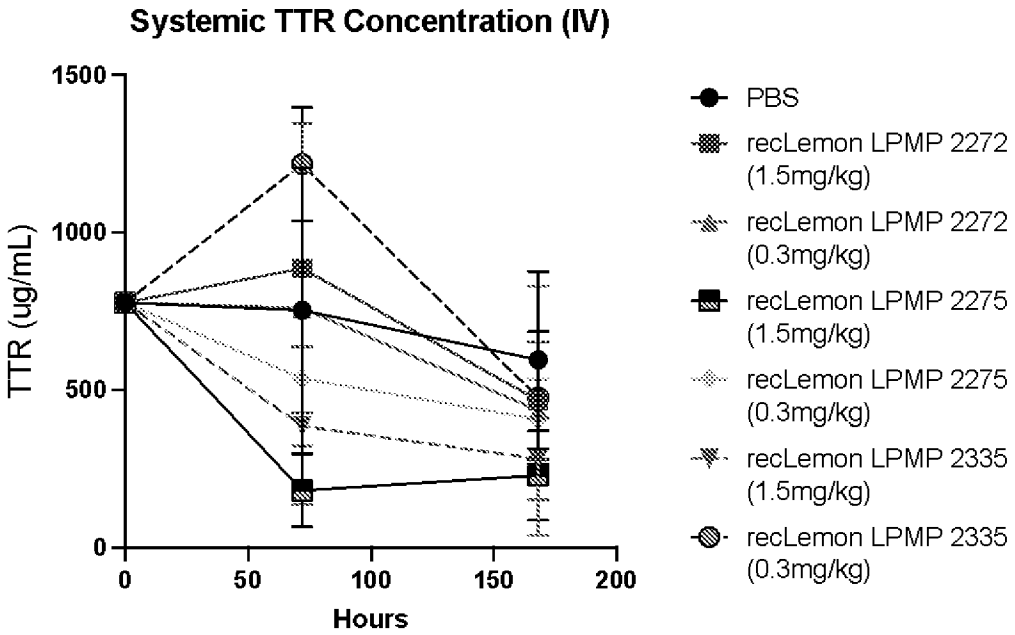


Figure 7A

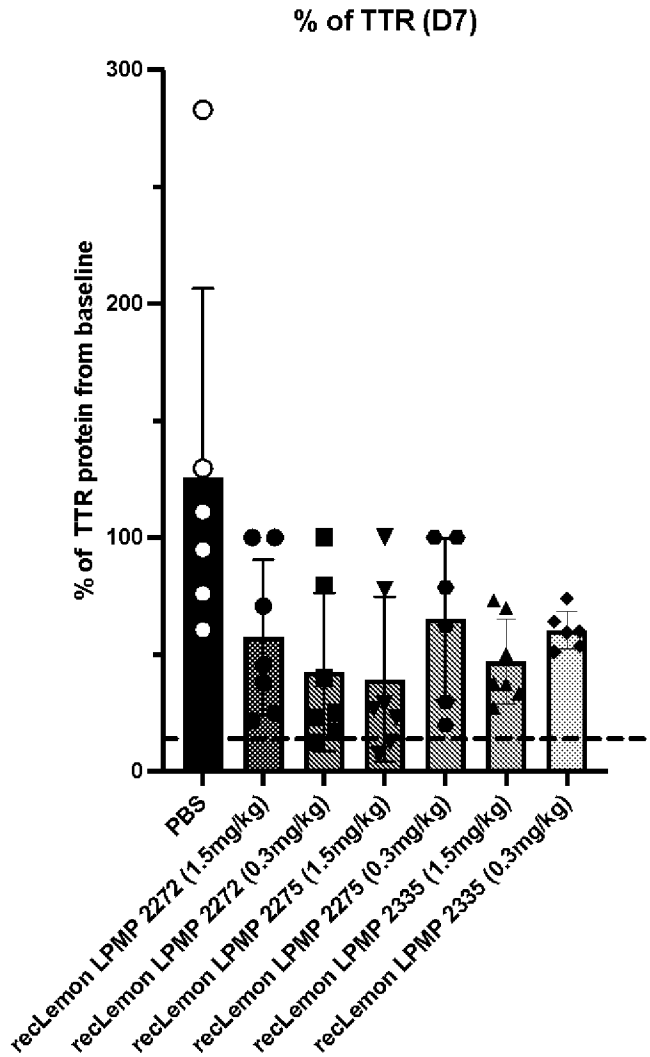


Figure 7B

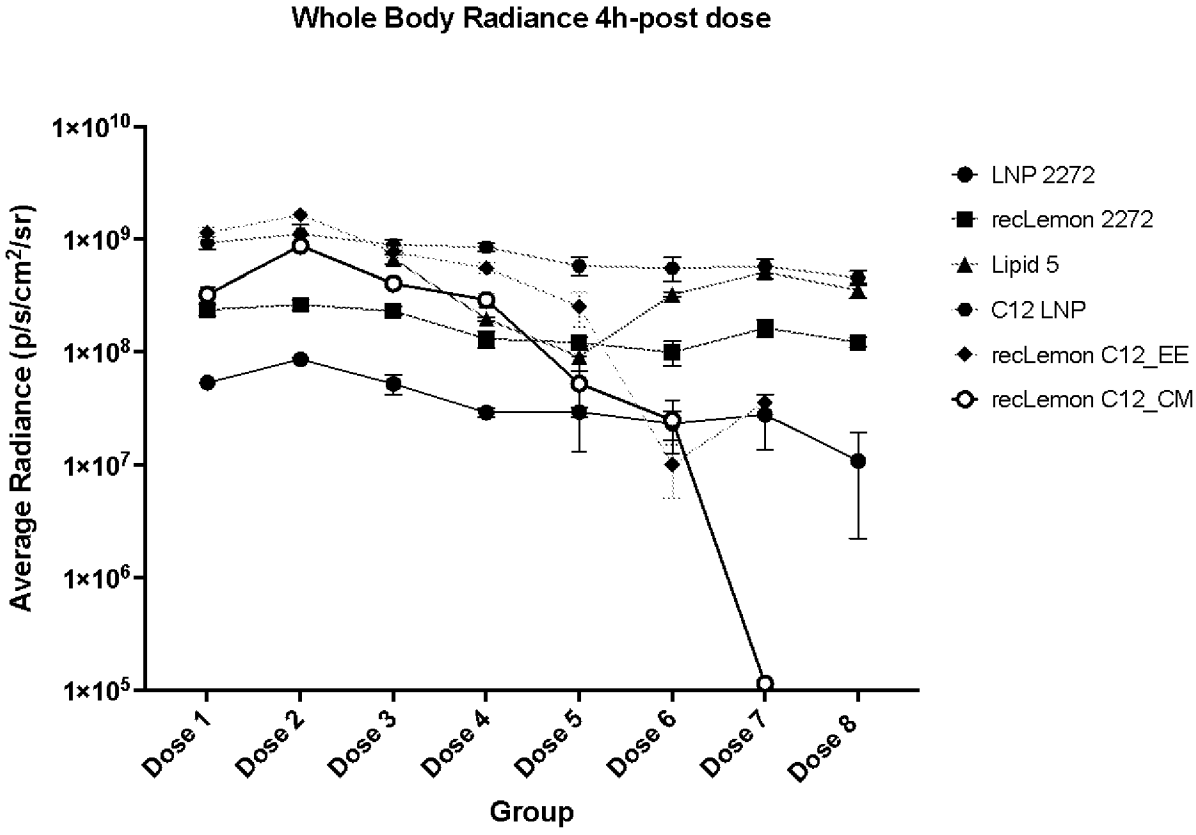


Figure 8A

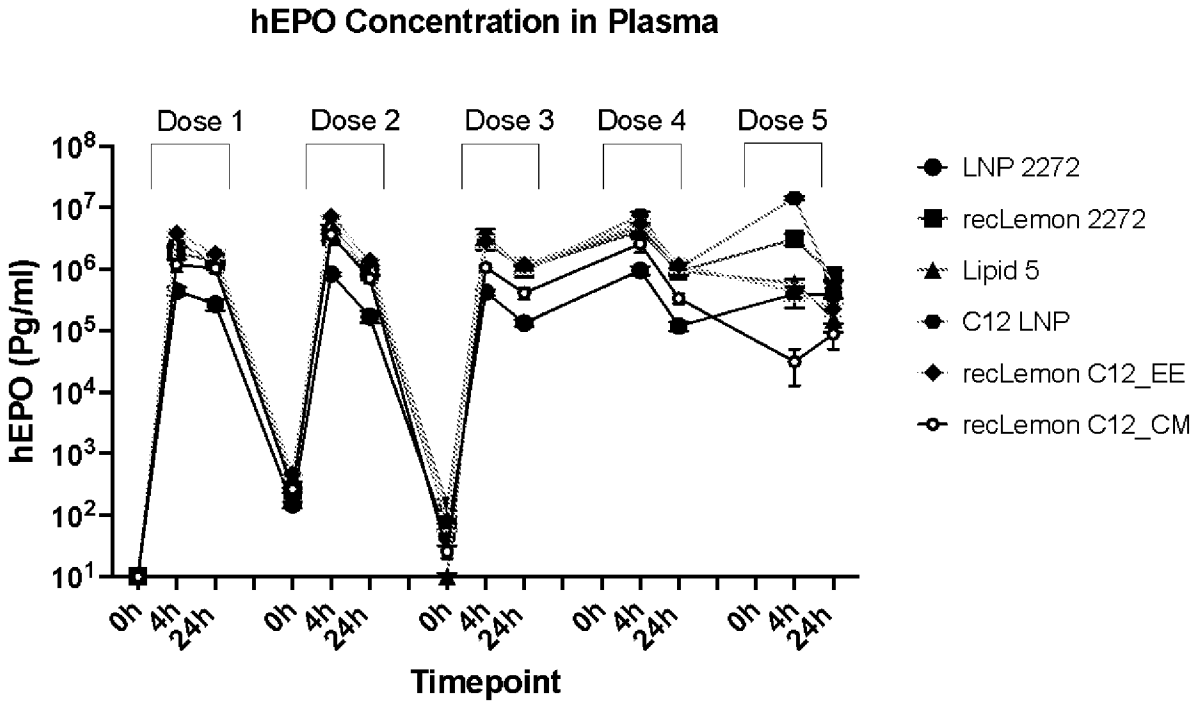


Figure 8B

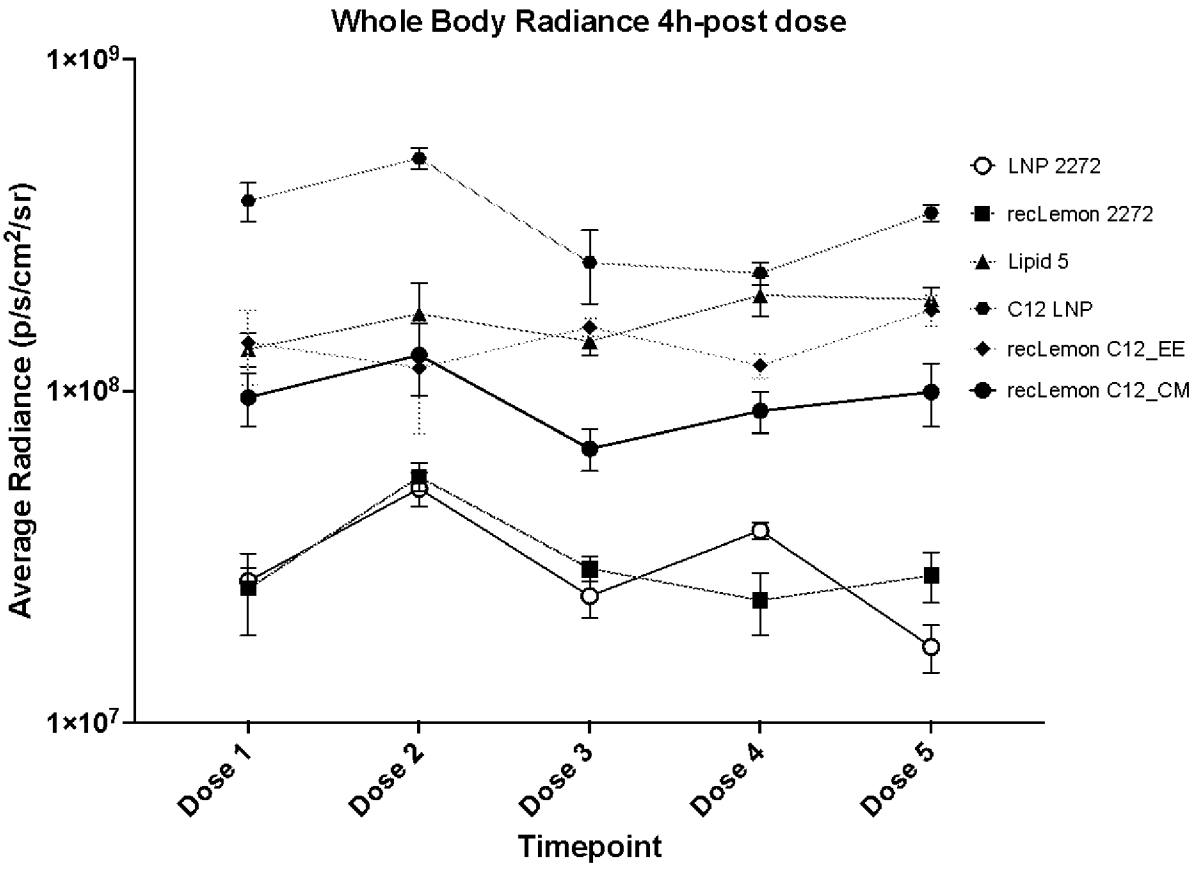


Figure 9A

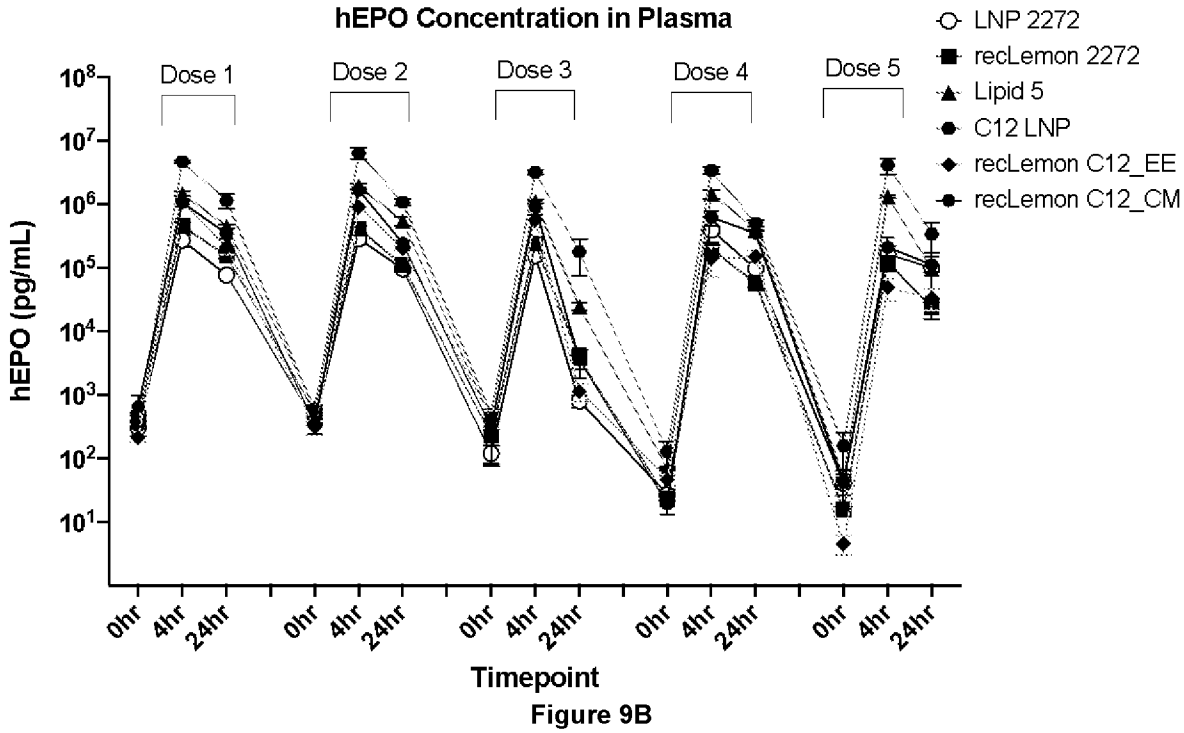


Figure 9B

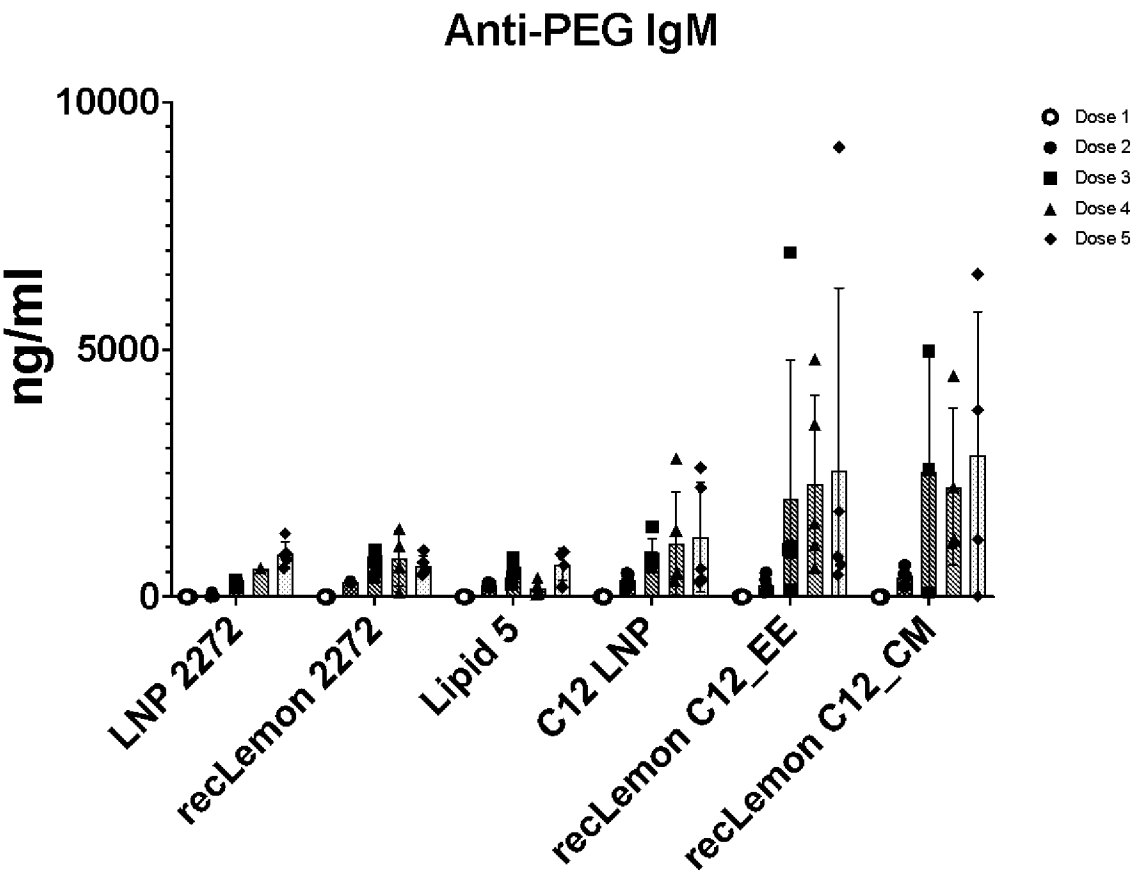


Figure 10A

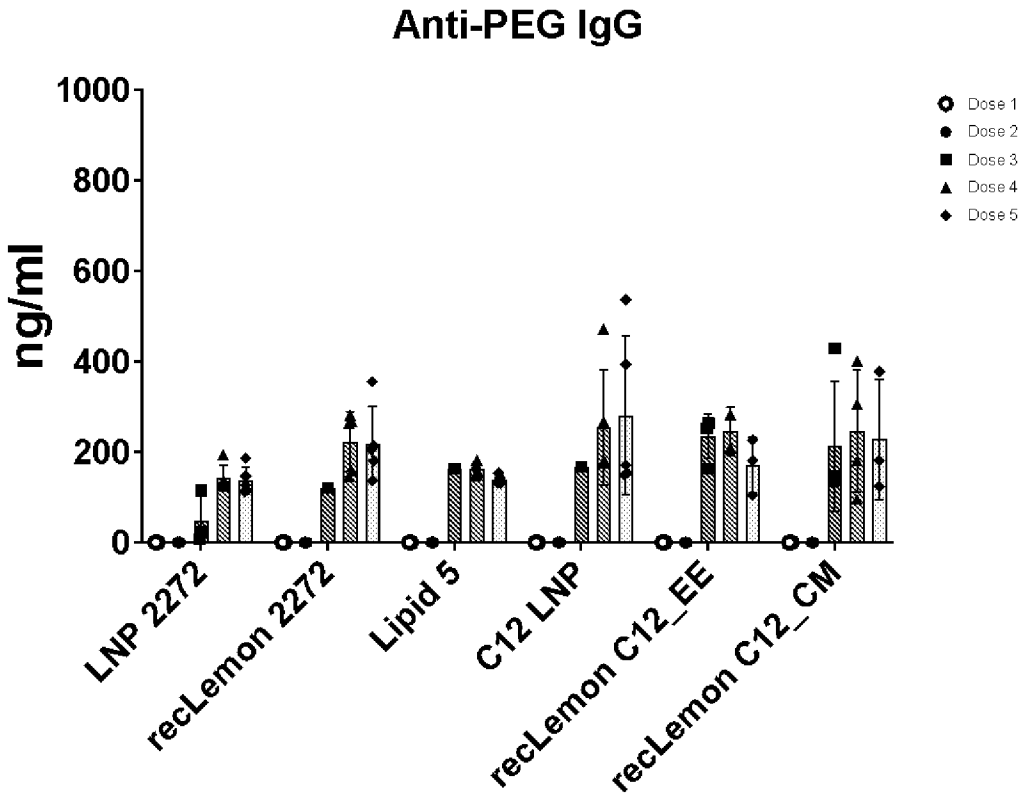


Figure 10B

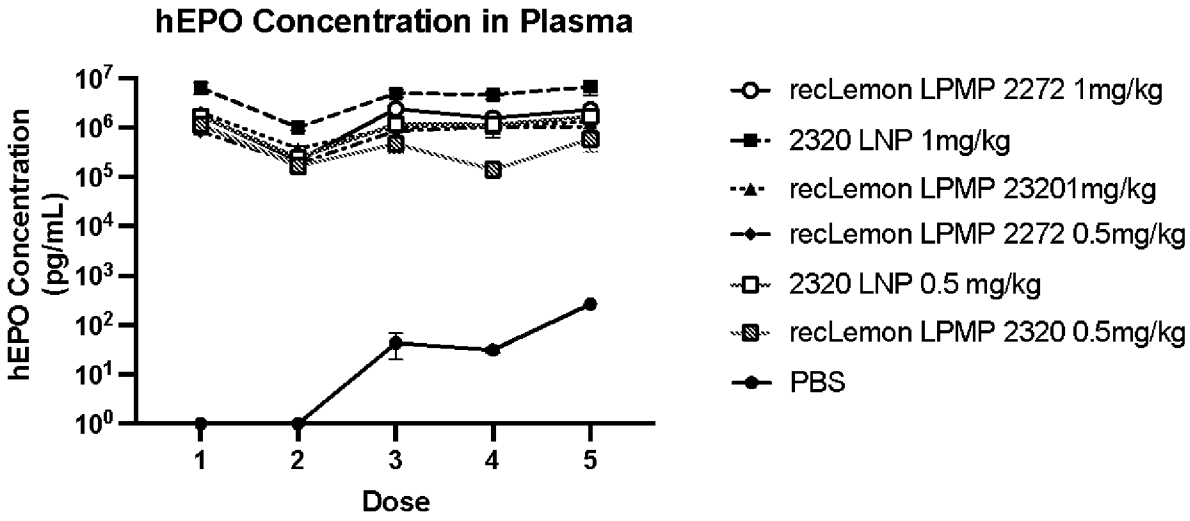


Figure 11A

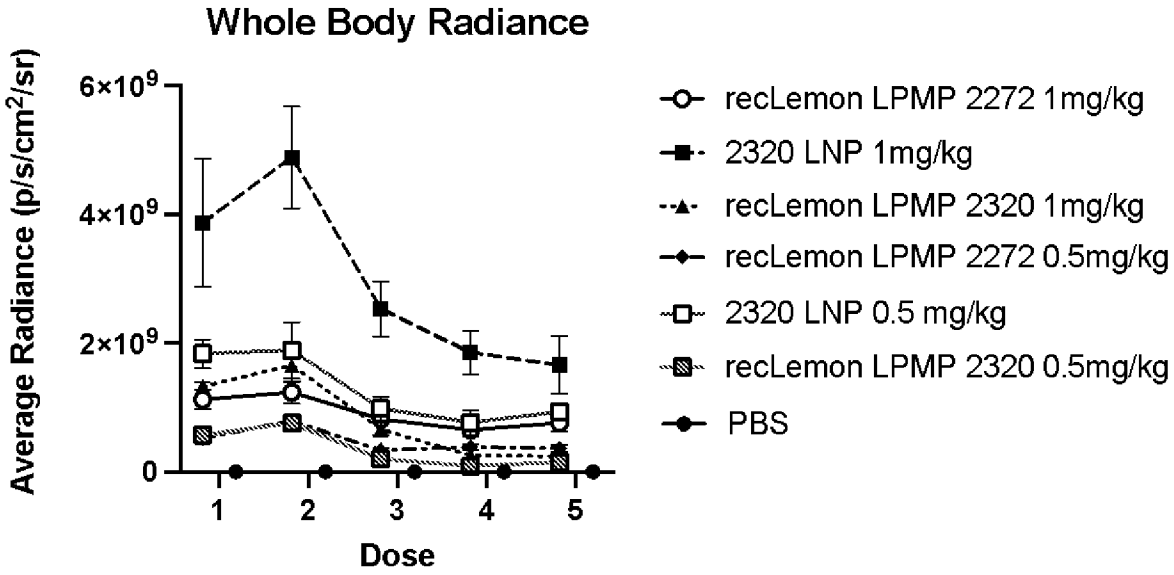


Figure 11B

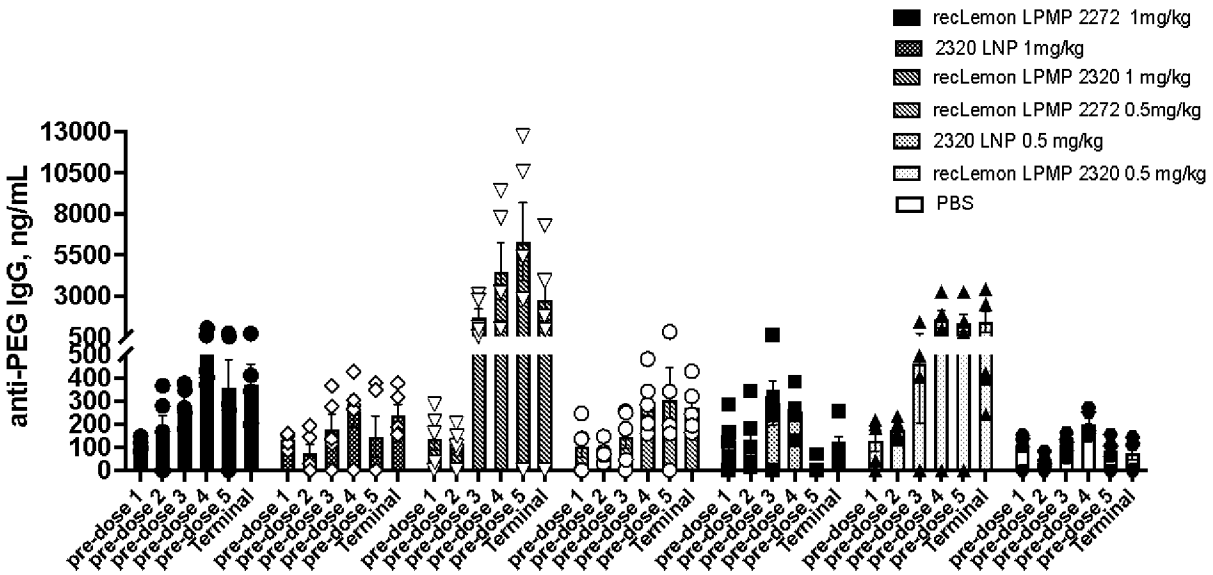


Figure 11C

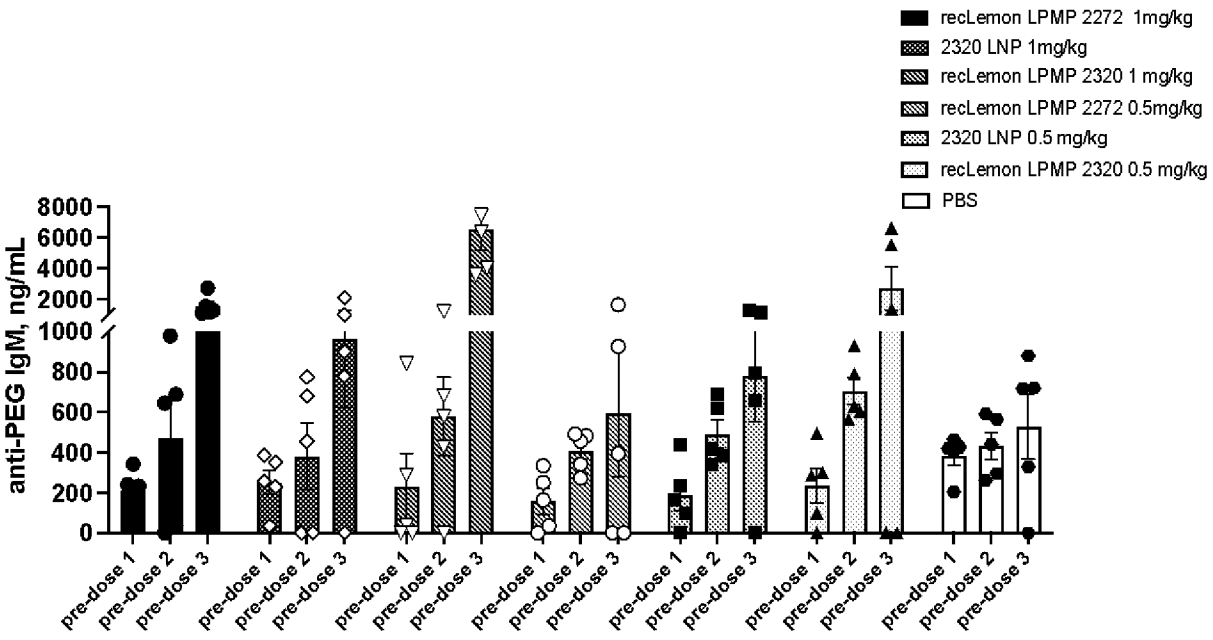


Figure 11D

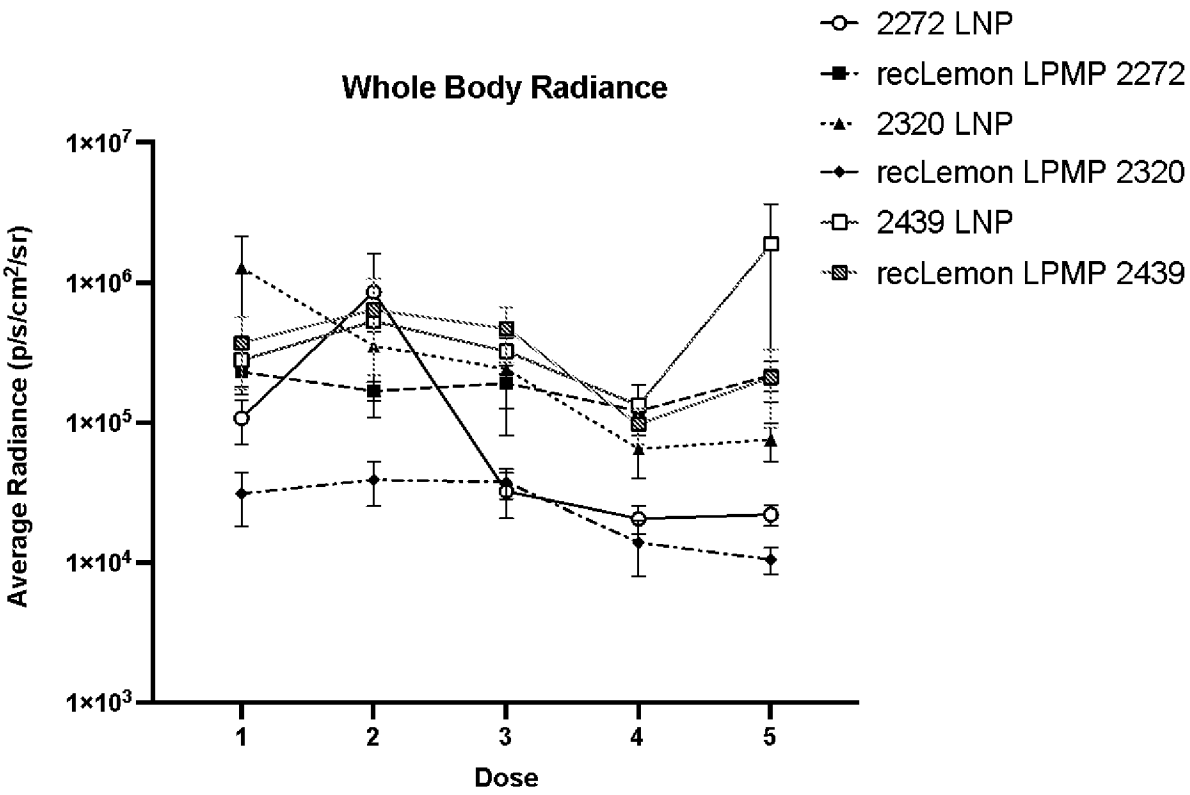

Whole Body Radiance
Average Radiance (p/s/cm²/sr)
1×10⁷
1×10⁶
1×10⁵
1×10⁴
1×10³
1
2
3
4
5
Dose
2272 LNP
recLemon LPMP 2272
2320 LNP
recLemon LPMP 2320
2439 LNP
recLemon LPMP 2439

Figure 12A

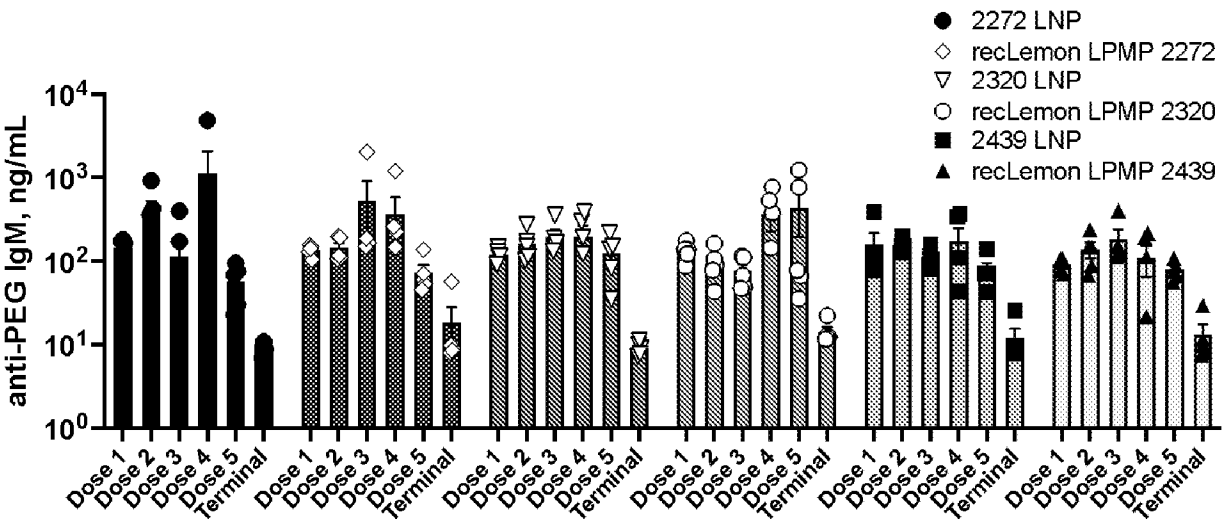


Figure 12B

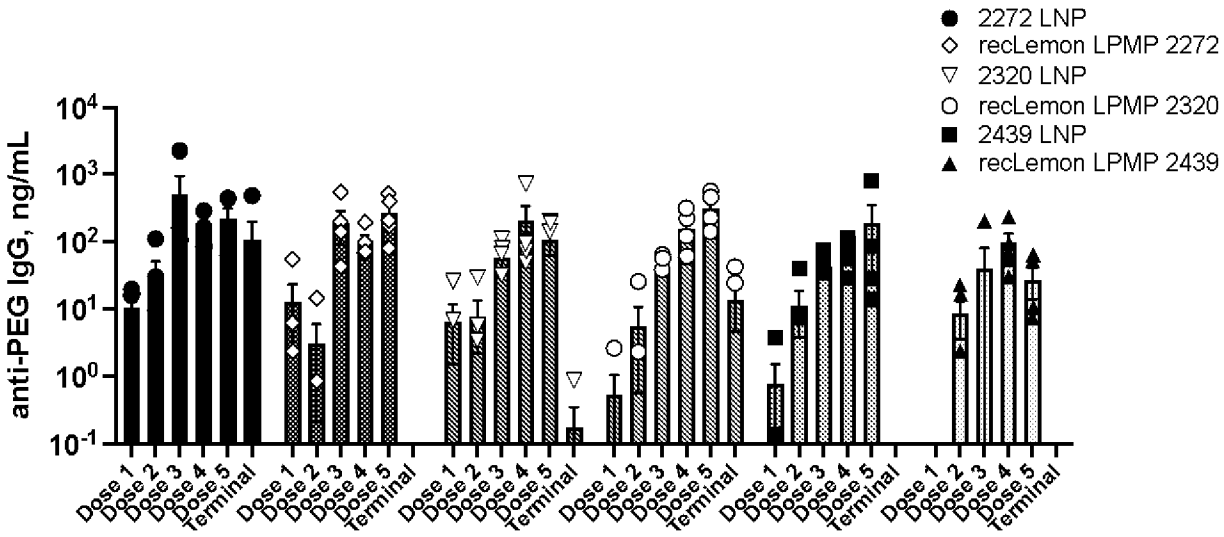


Figure 12C

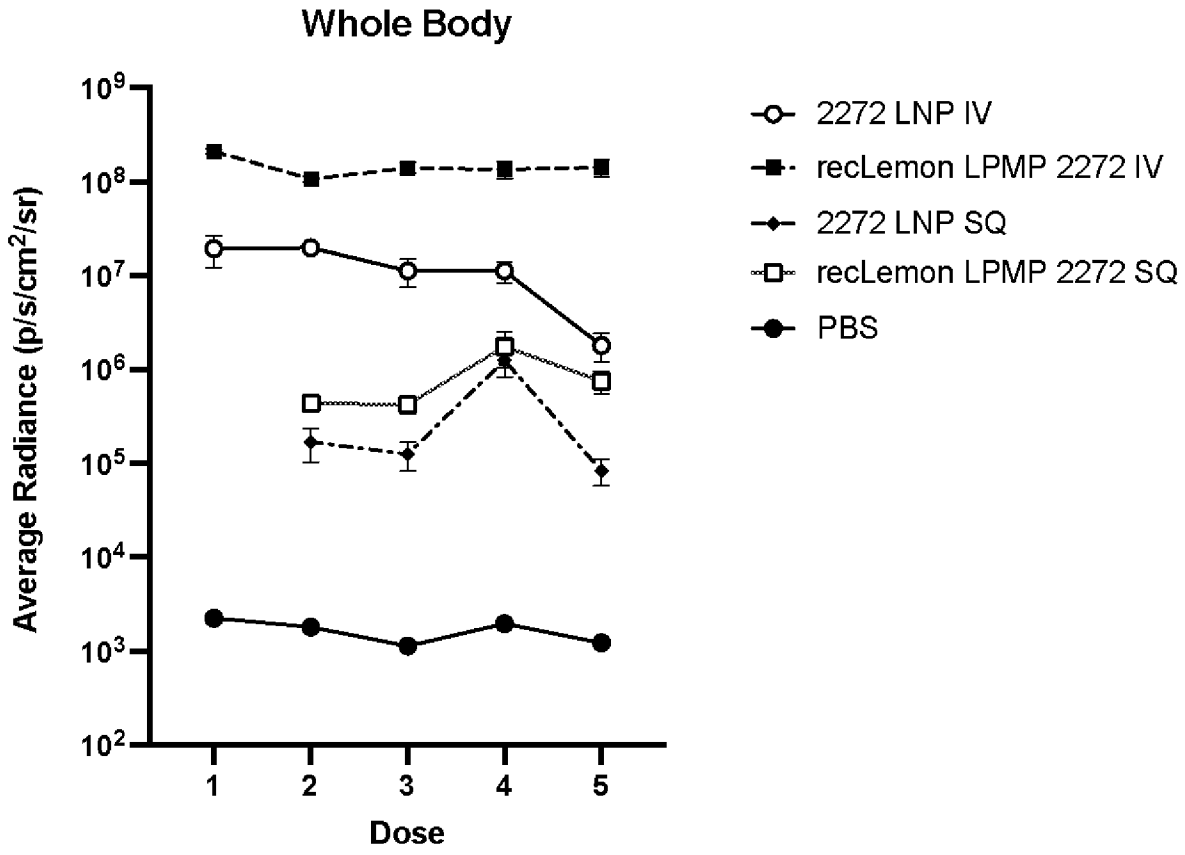
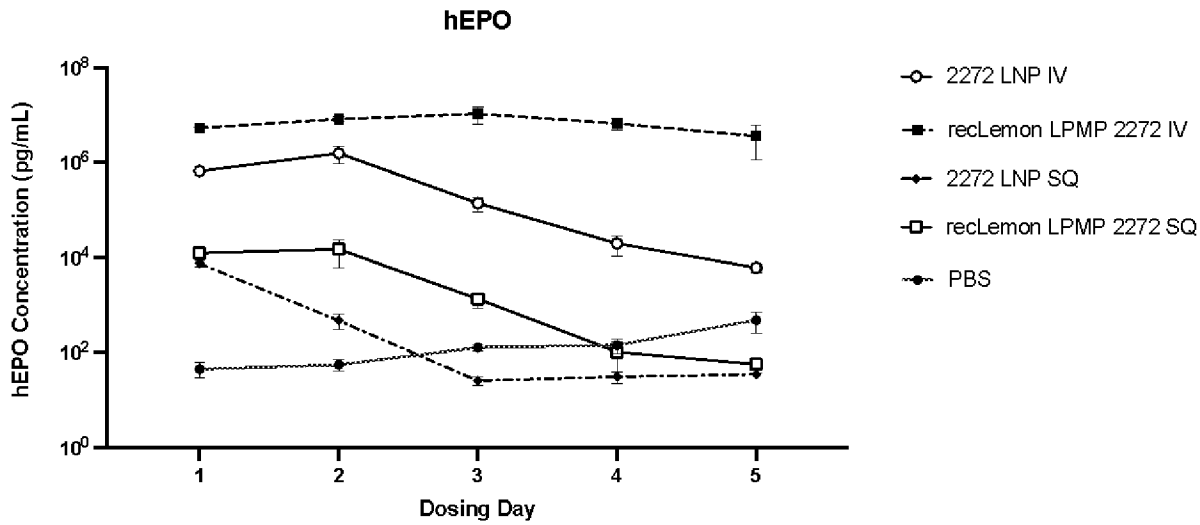


Figure 13A



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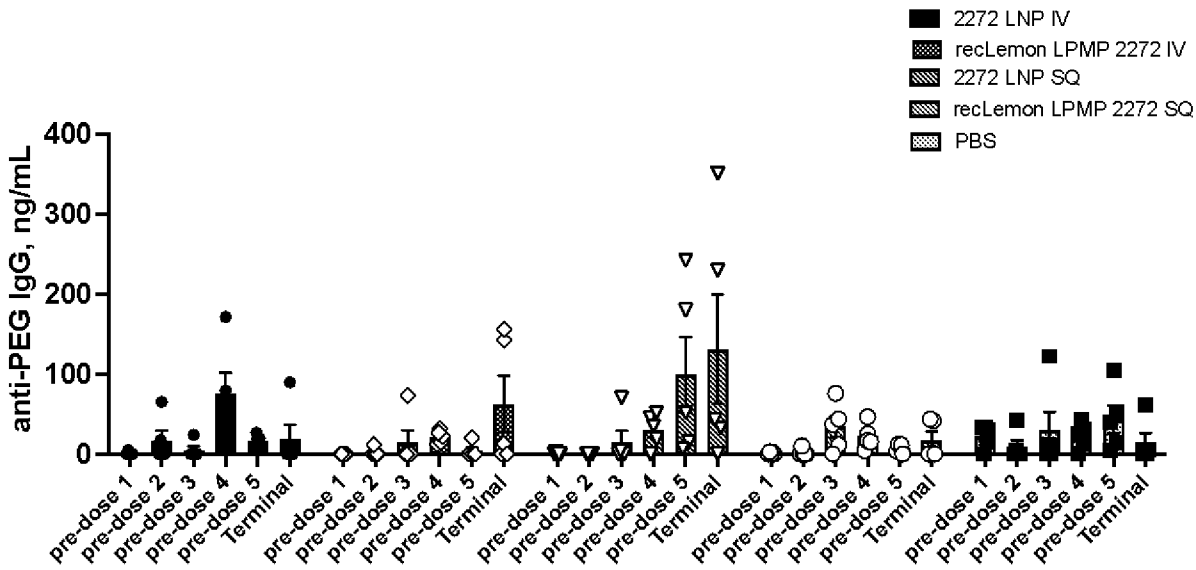

Figure 13C

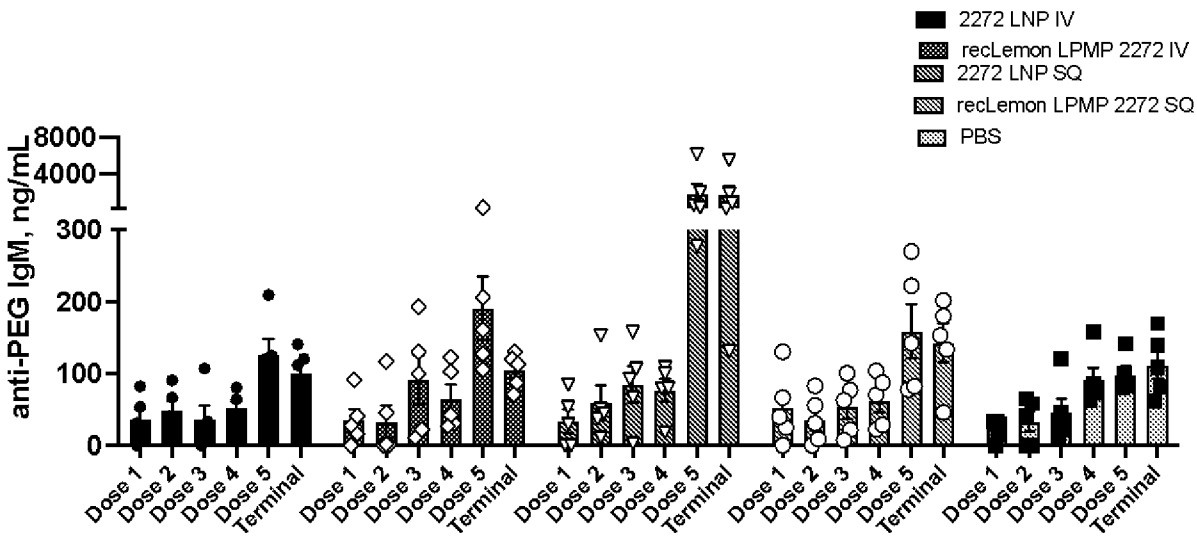

Figure 13D

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2023/037077

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/51 C12N9/16 C12N15/88
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2021/041301 A1 (FLAGSHIP PIONEERING INNOVATIONS VI LLC [US]) 4 March 2021 (2021-03-04) cited in the application pages 58-66; examples 9, 10	1-74
Y	JONATHAN D. FINN ET AL: "A Single Administration of CRISPR/Cas9 Lipid Nanoparticles Achieves Robust and Persistent In Vivo Genome Editing", CELL REPORTS, vol. 22, no. 9, 1 February 2018 (2018-02-01), pages 2227-2235, XP55527484, US ISSN: 2211-1247, DOI: 10.1016/j.celrep.2018.02.014 the whole document	1-74

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[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 February 2024	27/02/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bilang, Jürg

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2023/037077

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YAN JINGYUE ET AL: "Harnessing lipid nanoparticles for efficient CRISPR delivery", BIOMATERIALS SCIENCE, vol. 9, no. 18, 14 September 2021 (2021-09-14), pages 6001-6011, XP093041709, GB ISSN: 2047-4830, DOI: 10.1039/D1BM00537E Retrieved from the Internet: URL:https://pubs.rsc.org/en/content/articlepdf/2021/bm/d1bm00537e> the whole document tables 1, 2	1-74
A	HAN XUEXIANG ET AL: "An ionizable lipid toolbox for RNA delivery", NATURE COMMUNICATIONS, vol. 12, no. 1, 1 December 2021 (2021-12-01), XP055938542, DOI: 10.1038/s41467-021-27493-0 Retrieved from the Internet: URL:https://www.nature.com/articles/s41467-021-27493-0.pdf> the whole document	1-74
A	HOU XUCHENG ET AL: "Lipid nanoparticles for mRNA delivery", NATURE REVIEWS MATERIALS, 10 August 2021 (2021-08-10), XP055861763, ISSN: 2058-8437, DOI: 10.1038/s41578-021-00358-0 the whole document	1-74
X,P	WO 2023/122080 A1 (SENDA BIOSCIENCES INC [US]) 29 June 2023 (2023-06-29) the whole document paragraph [0312]	1-74

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2023/037077

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2021041301 A1	04-03-2021	AU 2020340290 A1	07-04-2022
		BR 112022003021 A2	19-07-2022
		CA 3151814 A1	04-03-2021
		CN 114727959 A	08-07-2022
		EP 4017479 A1	29-06-2022
		JP 2022545275 A	26-10-2022
		US 2022304930 A1	29-09-2022
		WO 2021041301 A1	04-03-2021
WO 2023122080 A1	29-06-2023	NONE	