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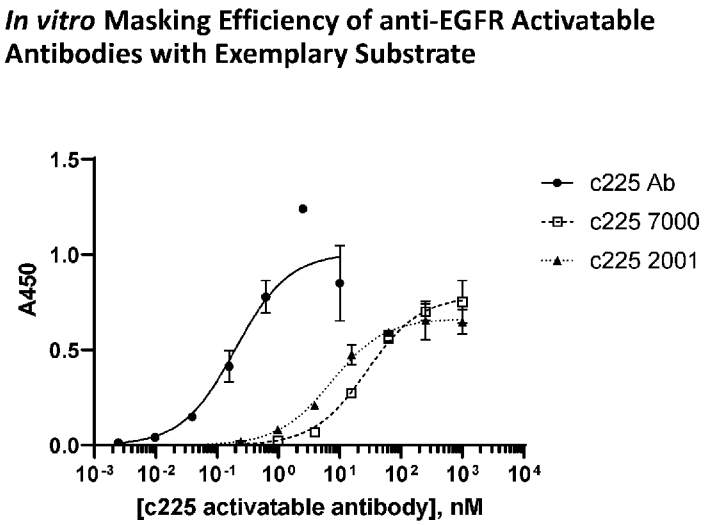

FIG. 1

(57) Abstract: Isolated polypeptides that include a cleavable moiety that is a substrate for at least one protease (e.g., MMP) are disclosed. Activatable molecules including the isolated polypeptides are disclosed. Methods of making and using the isolated polypeptides and activatable molecules including the isolated polypeptides in a variety of therapeutic, diagnostic, and prophylactic applications are disclosed.

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[Continued on next page]

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PROTEASE-CLEAVABLE MOIETIES AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. provisional application no. 63/370,021 filed August 1, 2022, the contents of which are incorporated herein in their entireties by reference thereto.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in XML format and is hereby incorporated by reference in its entirety. Said XML copy, created on July 25, 2023 is named CYTX-092-PCT_SL.xml and is 110,592 bytes in size.

TECHNICAL FIELD

[0003] The present disclosure generally relates to polypeptides that include a cleavable moiety that is a substrate for at least one protease (e.g., a matrix metalloproteinase (MMP)), and to methods of making and using the polypeptides and activatable molecules in a variety of therapeutic, diagnostic, and prophylactic applications.

BACKGROUND

[0004] Proteases are enzymes that catalyze the hydrolysis of peptide bonds between amino acid residues. Some proteases are known to break specific peptide bonds based on the presence of a particular amino acid sequence within a protein. Proteases occur naturally in all organisms and are involved in a variety of physiological reactions from simple degradation to highly regulated pathways. Some proteases break specific peptide bonds based on the presence of a particular amino acid sequence within a protein while some amino acid sequences are resistant to cleavage by particular proteases.

[0005] Accordingly, there exists a need to identify new substrates for proteases and to use these substrates in a variety of therapeutic, diagnostic and prophylactic applications.

SUMMARY OF THE INVENTION

[0006] In one aspect, the present disclosure provides an isolated polypeptide comprising a cleavable moiety (CM) that comprises the amino acid sequence AIALY (SEQ ID NO: 5), wherein the CM is a substrate for a protease. In some embodiments, the present disclosure provides an isolated polypeptide comprising a cleavable moiety (CM) that comprises the

amino acid sequence AIALYA (SEQ ID NO: 2), wherein the CM is a substrate for a protease. In some embodiments, the CM comprises the amino acid sequence AIALYAD (SEQ ID NO: 1).

[0007] In another aspect, the present disclosure provides an isolated polypeptide comprising a cleavable moiety (CM) that comprises an amino acid sequence selected from SEQ ID NOs: 1-14, wherein the CM is a substrate for a protease.

[0008] According to the present disclosures, the isolated polypeptide is a molecule in which cleavage of the CM by a protease results in a part or component of the molecule being separated from the remainder of the molecule. In some aspects of the present disclosure, cleavage of the CM by a protease activates the molecule. In some aspects, the isolated polypeptide is a molecule in which multiple proteases cleave the CM. In some aspects, the isolated polypeptide is a molecule in which MMP2 cleaves the CM. In some aspects, the isolated polypeptide is a molecule in which MMP9 cleaves the CM. In some aspects, the isolated polypeptide is a molecule in which MMP14 cleaves the CM. In some aspects, the isolated polypeptide is a molecule in which two or all of MMP2, MMP9, and MMP14 cleave the CM. In some aspects, the isolated polypeptide is a molecule in which the % cleavability of the CM is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%, or 100%, e.g., at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%, or 100% cleavable by any one of MMP2, MMP9, and MMP14 or any two of MMP2, MMP9, and MMP14 or each of MMP2, MMP9, and MMP14. According to the present disclosures, the isolated polypeptide is a molecule that has high *in vivo* stability such that it is not cleaved in plasma as demonstrated by less than 60%, less than 50%, less than 40%, or less than 25% in vivo activation following 7 days of administration *in vivo*. According to embodiments of the present disclosures, the isolated polypeptide is a molecule comprising a CM that has a k_{cat}/K_M ($M^{-1}s^{-1}$) of greater than 5×10^2 M^{-1} s^{-1}.

[0009] In some embodiments, the isolated polypeptide is an activatable molecule and further comprises an "active moiety" (AM) that specifically binds a target. In some embodiments, the AM is a therapeutic macromolecule. In some embodiments, the AM is an antibody or antigen binding fragment thereof. In some embodiments, the antibody is a full-length antibody. single-chain variable fragment (scFv), diabody (a noncovalent dimer of scFv), single chain antibody (scab), a VHH, a domain antibody (dAb) or single domain antibody (nanobody, e.g., single domain heavy chain antibody, single domain light chain

antibody). In some embodiments, the antibody is a monoclonal antibody, single chain antibody, Fab fragment, $F(ab')_2$ fragment, single-chain variable fragment (scFv), diabody (a noncovalent dimer of scFv), single chain antibody (scab), a VHH, a domain antibody (dAb) or single domain antibody (nanobody, e.g., single domain heavy chain antibody, single domain light chain antibody). According to some embodiments of the present disclosures, the isolated polypeptide is an activatable molecule that has high *in vivo* stability such that it is not cleaved in plasma as demonstrated by less than 25% in vivo activation following 7 days of administration *in vivo* (e.g., as exemplified in Example 3). According to some embodiments of the present disclosures, the isolated polypeptide is an activatable molecule that has masking efficiency of 25x, 40x, 41x, 50x, 75x, 100x, 150x, 200x, or higher (e.g., as exemplified in Example 4). According to the present disclosures, the activatable molecule is activated by one, two, or all of MMP2, MMP9, and MMP14. According to the present disclosures, the activatable molecule is activated to an extent of having a cleavability percentage of at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%, or 100%, e.g., at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%, or 100% cleavable by any one of MMP2, MMP9, and MMP14 or any two of MMP2, MMP9, and MMP14 or each of MMP2, MMP9, and MMP14. In some embodiments, the AM is a cytokine. In some embodiments, the AM is a chimeric antigen receptor. In some aspects, the AM is a drug or agent, e.g., a therapeutic, imaging, or diagnostic agent.

[0010] In some embodiments, the AM is coupled to the CM. In some embodiments, the AM is directly coupled to the CM. In some embodiments, the AM is indirectly coupled to the CM via a linking peptide. In some embodiments, the AM is indirectly coupled to the CM via one or more components of the activatable protein.

[0011] In some embodiments, the isolated polypeptide further comprises a masking moiety (MM). In some embodiments, the MM has a dissociation constant for binding to the AM that is greater than the dissociation constant of the AM for binding to the target. In some embodiments, the MM does not interfere or compete with the AM for binding to the target in in the activated molecule (i.e., following cleavage of the CM by a protease). In some embodiments, the MM is 2 to 40 amino acids in length. In some embodiments, the MM does not bind the AM, but still interferes with AM's binding to its binding partner through non-specific interactions. In some embodiments, the MM is a steric mask. In some embodiments, the MM is a protein. In some embodiments, the MM is coupled to the CM such that the

isolated polypeptide comprises the structural arrangement from N-terminus to C-terminus as follows: MM-CM-AM or AM-CM-MM. In some embodiments, the MM is coupled directly to the CM.

[0012] In some embodiments, the MM is coupled indirectly to the CM via a linking peptide. In some embodiments, the isolated polypeptide comprises a linking peptide (LP) and wherein the isolated polypeptide has a structural arrangement from N-terminus to C-terminus as follows: MM-LP-CM-AM or MM-CM-LP-AM. In some embodiments, the isolated polypeptide comprises a first linking peptide (LP1) and a second linking peptide (LP2), and wherein the isolated polypeptide has a structural arrangement from N-terminus to C-terminus as follows: MM-LP1-CM-LP2-AM or AM-LP2-CM-LP1-MM. In some embodiments, the LP1 and LP2 are not identical to each other. In some embodiments, the LP1 and LP2 are identical to each other. In some embodiments, each of LP1 and LP2 is a peptide of 1 to 20 amino acids in length.

[0013] In general, in each embodiment herein, unless otherwise stated, a polypeptide may comprise one or more optional linkers between each of the elements listed, and such linkers may be 1 to 30, 6 to 29, 7 to 28, 8 to 27, 9 to 26, 10 to 25, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, or 27 amino acids in length.

[0014] In some embodiments, the CM is a substrate for a matrix metalloproteinase (MMP). In some embodiments, the MMP is 2 MMP9, or MMP14. In some embodiments, the k_{cat}/K_M of the CM by MMP2 cleavage is at least 1×10^3 $M^{-1}s^{-1}$. In some embodiments, the k_{cat}/K_M of the CM by MMP2 cleavage is at least 1×10^4 $M^{-1}s^{-1}$. In some embodiments, the k_{cat}/K_M of the CM by MMP9 cleavage is at least 1×10^2 $M^{-1}s^{-1}$. In some embodiments, the k_{cat}/K_M of the CM by MMP9 cleavage is at least 1×10^3 $M^{-1}s^{-1}$. In some embodiments, the k_{cat}/K_M of the CM by MMP14 cleavage is at least 1×10^2 $M^{-1}s^{-1}$. In some embodiments, the k_{cat}/K_M of the CM by MMP14 cleavage is at least 1×10^3 $M^{-1}s^{-1}$.

[0015] In another aspect, the present disclosure provides an isolated polypeptide comprising a cleavable moiety (CM) comprising an amino acid sequence with one-amino acid or two-amino acid mutation(s) of any one of SEQ ID NOs: 1-14, wherein the CM is a substrate for a protease. For example, the mutations may include substitution between any one of lysine, arginine, and histidine residues. In certain aspects, the present disclosure may include substitution of any arginine in the disclosed sequences with a lysine. In other aspects, the present disclosure also includes substitution of any arginine in the disclosed sequences

with an amino acid that is not lysine. For example, the mutations may include substitution between any one of alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, and tryptophan residues. For example, the mutations may include substitution between any one of glycine, asparagine, glutamine, cysteine, serine, threonine, and tyrosine residues. For example, the mutations may include substitution between any one of arginine, asparagine, aspartate, glutamine, glutamate, histidine, lysine, serine, and threonine residues. For example, the mutations may include substitution between any one of alanine, cysteine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine and valine residues. For example, the mutations may include substitution between any one of serine and threonine residues. For example, the mutations may include substitution between any one of asparagine and glutamine residues. For example, the mutations may include substitution between any one of alanine, valine, leucine and isoleucine residues. For example, the mutations may include substitution between any one of phenylalanine, tryptophan, and tyrosine residues.

[0016] In another aspect, the present disclosure provides a polypeptide complex comprising one or more of the isolated polypeptides comprising the CMs disclosed herein. In some aspects, the complex comprises one or more of the isolated polypeptides of the present disclosure bound to a second isolated polypeptide, e.g., via protein-protein affinity interactions, hydrophobic interactions, disulfide linkage(s), cross-link(s), covalent bond(s), chemical linkage(s), or any other type of binding between two polypeptides.

[0017] In another aspect, the present disclosure provides a conjugated polypeptide comprising the isolated polypeptide herein conjugated to an agent. In some embodiments, the agent is conjugated to the isolated polypeptide via a conjugating linker. In some embodiments, the conjugating linker is cleavable. In some embodiments, the conjugating linker is non-cleavable. In some embodiments, the conjugating linker comprises an amino acid sequence selected from SEQ ID NOs: 1-14. In some embodiments, the agent is a toxin, a microtubule inhibitor, a nucleic acid damaging agent, a dolastatin, an auristatin, a maytansinoid, a duocarmycin, a calicheamicin, or a combination thereof.

[0018] In another aspect, the present disclosure provides a composition comprising the isolated polypeptide herein, the polypeptide complex, or the conjugated polypeptide herein, and a carrier. In some embodiments, the carrier is a pharmaceutically acceptable carrier. In some embodiments, the composition comprises an additional agent. In some embodiments, the additional agent is a therapeutic, imaging, or diagnostic agent.

[0019] In each of the foregoing embodiments, and unless otherwise stated, the polypeptide may comprise, e.g., one or more optional linkers between each of the elements listed. In some embodiments, a linker is a peptide having a length of 5 to 30, 6 to 29, 7 to 28, 8 to 27, 9 to 26, 10 to 25, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 amino acids. In the disclosed structural arrangements in the foregoing paragraphs and throughout this disclosure, one or more linkers may optionally be present between the elements. Further, this disclosure also contemplates and includes activatable proteins in which any one or more of the disclosed elements optionally directly abut each other such that there are no linkers or other amino acid sequences between the elements.

[0020] In another aspect, the present disclosure provides an isolated nucleic acid molecule encoding the isolated polypeptide herein.

[0021] In another aspect, the present disclosure provides a vector comprising the isolated nucleic acid molecule herein.

[0022] In another aspect, the present disclosure provides a cell comprising the isolated nucleic acid molecule or the vector herein.

[0023] In another aspect, the present disclosure provides a method of manufacturing an activatable molecule that contains a cleavable moiety (CM), the method comprising expressing and recovering a polypeptide comprising the isolated polypeptide herein.

[0024] In another aspect, the present disclosure provides a method of treating, alleviating a symptom of, or delaying the progression of a disease or disorder in a subject, comprising administering a therapeutically effective amount of the isolated polypeptide, the polypeptide complex, the conjugated polypeptide, or the composition herein to the subject. In some embodiments, the disease is a cancer, an infection, an inflammatory disorder, a cardiovascular disorder, a neurodegenerative disorder, or an autoimmune disorder.

[0025] In another aspect, the present disclosure provides a method of detecting or diagnosing a disease or health condition of a subject, comprising: contacting the isolated polypeptide, the polypeptide complex, the conjugated polypeptide, or the composition herein with a sample from the subject; and measuring a level of cleavage of the isolated polypeptide, thereby detecting or diagnosing the disease or health condition of the subject. In some embodiments, the disease is a cancer, an infection, an inflammatory disorder, a cardiovascular disorder, a neurodegenerative disorder, or an autoimmune disorder.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] An understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention may be utilized, and the accompanying drawings of which:

[0027] **FIG. 1** is a graph showing the *in vitro* masking efficiency of an exemplary anti-EGFR activatable antibody of the present disclosure. These exemplary results show that the substrate affected the masking efficiency of the prodomain of the activatable antibody.

[0028] **FIG. 2A** shows the effects of exemplary activatable antibodies on tumor regression in mice. The mean tumor volume ± SEM was plotted for each measured time point following administration of the exemplary activatable antibodies or with cetuximab or immunoglobulin (IVIG) control. **FIG. 2B** shows intra-tumoral activation of the activatable antibodies.

[0029] **FIGs. 3A-3C** show activation of exemplary activatable antibodies in patient-derived tumor samples (cholangiocarcinoma in Fig.3A, pancreatic cancer in Fig. 3B, and TNBC in Fig. 3C).

[0030] **FIG. 4** shows activation of exemplary activatable antibodies in patient-derived acute myeloid leukemia tumor (AML) samples.

DETAILED DESCRIPTION OF THE INVENTION

[0031] Proteases play a critical role in the homeostasis of healthy tissues but are known to be dysregulated within diseases, including cancer and autoimmune disorders (Vasiljeva et al. "The multifaceted roles of tumor-associated proteases and harnessing their activity for prodrug activation," Biol. Chem. 2019 Apr 22). This dysregulation of protease activity provides new opportunities for the development of protease-activatable therapeutic molecules, which are preferentially activated in the local tissue microenvironment. These therapeutics have demonstrated a greater therapeutic window and safety profile with less on-target toxicities occurring in healthy tissues. Hence, there is a need for identification of substrates that act as cleavage recognition sites for proteases that are found to be dysregulated in disease tissues. These substrates or cleavable moieties (CM) may have multiple cleavage sites for leveraging the activities of multiple disease-associated proteases.

[0032] Understanding the substrate cleavage profile and using these substrates as tools for activation in a specific disease or cancer type will enable the development of new therapeutic protease-activatable molecules. Fine tuning the therapeutic-activatable molecules by using protease substrates with unique cleavage profiles will allow for treatment options for a broader spectrum of patients while offering an improved therapeutic index. For example, "omics" studies have demonstrated the distribution of numerous matrix metalloproteases (MMPs) across numerous cancer types and differences in the expression of the MMPs compared to normal tissues (Gobin et al. "A pan-cancer perspective of matrix metalloproteases (MMP) gene expression profile and their diagnostic/prognostic potential," BMC Cancer. 2019 Jun 14; 19(1):581), highlighting the need for appropriate cleavable moiety selection. Indeed, the first protease-activatable antibodies were designed using MMP substrates (Bleuez et al., "Exploiting protease activation for therapy," Drug Discovery Today, 2022 Jun;27(6):1743-1754).

[0033] The present disclosure provides polypeptides comprising a cleavable moiety (CM) that is a substrate for at least one protease, e.g., an MMP. In some aspects, the CMs herein are cleaved in a diseased tissue (e.g., tumor tissue) but less in a healthy tissue. These CMs are useful in a variety of therapeutic, diagnostic and prophylactic applications. In some embodiments, the CM-containing polypeptides are activatable molecules and further comprise an active moiety (AM) that specifically binds a target. For example, the AM may be a therapeutic protein, a therapeutic agent, an imaging agent, a diagnostic agent, an antibody or antigen-binding fragment, a cytokine, chimeric antigen receptor or other molecules used in therapeutic and diagnostic applications.

[0034] Also provided herein are related compositions, kits, nucleic acids, vectors, and recombinant cells, as well as related methods, including methods of using and methods of producing any of the CM-containing polypeptides described herein.

DEFINITIONS

[0035] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present disclosure; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references

mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

[0036] The terms "a" and "an" refer to one or more (i.e., at least one) of the grammatical object of the article. By way of example, "a cell" encompasses one or more cells.

[0037] As used herein, the terms "about" and "approximately," when used to modify an amount specified in a numeric value or range, indicate that the numeric value as well as reasonable deviations from the value known to the skilled person in the art. For example ± 20%, ± 10%, or ± 5%, are within the intended meaning of the recited value where appropriate.

[0038] Concentrations, amounts, and other numerical data may be expressed or presented herein in a range format. It is to be understood that such a range format is used merely for convenience and brevity and thus should be interpreted flexibly to include not only the numerical values explicitly recited as the limits of the range, but also to include all the individual numerical values or sub-ranges encompassed within that range as if each numerical value and sub-range is explicitly recited. As an illustration, a numerical range of "about 0.01 to 2.0" should be interpreted to include not only the explicitly recited values of about 0.01 to about 2.0, but also include individual values and sub-ranges within the indicated range. Thus, included in this numerical range are individual values such as 0.5, 0.7, and 1.5, and sub-ranges such as from 0.5 to 1.7, 0.7 to 1.5, and from 1.0 to 1.5, etc. Furthermore, such an interpretation should apply regardless of the breadth of the range or the characteristics being described. Additionally, it is noted that all percentages are in weight, unless specified otherwise.

[0039] In understanding the scope of the present disclosure, the terms "including" or "comprising" and their derivatives, as used herein, are intended to be open ended terms that specify the presence of the stated features, elements, components, groups, integers, and/or steps, but do not exclude the presence of other unstated features, elements, components, groups, integers and/or steps. The foregoing also applies to words having similar meanings such as the terms "including", "having" and their derivatives. The term "consisting" and its derivatives, as used herein, are intended to be closed terms that specify the presence of the stated features, elements, components, groups, integers, and/or steps, but exclude the presence of other unstated features, elements, components, groups, integers and/or steps. The term "consisting essentially of," as used herein, is intended to specify the presence of the stated features, elements, components, groups, integers, and/or steps as well as those that do not materially affect the basic and novel characteristic(s) of features, elements, components,

groups, integers, and/or steps. It is understood that reference to any one of these transition terms (i.e. "comprising," "consisting," or "consisting essentially") provides direct support for replacement to any of the other transition term not specifically used. For example, amending a term from "comprising" to "consisting essentially of" or "consisting of" would find direct support due to this definition for any elements disclosed throughout this disclosure. Based on this definition, any element disclosed herein or incorporated by reference may be included in or excluded from the claimed invention.

[0040] As used herein, a plurality of compounds, elements, or steps may be presented in a common list for convenience. However, these lists should be construed as though each member of the list is individually identified as a separate and unique member. Thus, no individual member of such list should be construed as a *de facto* equivalent of any other member of the same list solely based on their presentation in a common group without indications to the contrary.

[0041] The term "exemplary" is used herein to mean serving as an example, instance, or illustration. Any aspect or design described herein as "exemplary" is not necessarily to be construed as preferred or advantageous over other aspects or designs. Rather, use of the word exemplary is intended to present concepts in a more concrete fashion.

[0042] Furthermore, certain molecules, constructs, compositions, elements, moieties, excipients, disorders, conditions, properties, steps, or the like may be discussed in the context of one specific embodiment or aspect or in a separate paragraph or section of this disclosure. It is understood that this is merely for convenience and brevity, and any such disclosure is equally applicable to and intended to be combined with any other embodiments or aspects found anywhere in the present disclosure and claims, which all form the application and claimed invention at the filing date. For example, a list of constructs, molecules, method steps, kits, or compositions described with respect to a construct, molecule, isolated polypeptide, activatable molecule, composition, or method is intended to and does find direct support for embodiments related to constructs, molecules, isolated polypeptides, activatable molecules, compositions, formulations, and methods described in any other part of this disclosure, even if those method steps, active agents, kits, or compositions are not re-listed in the context or section of that embodiment or aspect.

[0043] The term "isolated polynucleotide" as used herein shall mean a polynucleotide of genomic, cDNA, RNA, mRNA, or synthetic origin or some combination thereof, which by

virtue of its origin the "isolated polynucleotide" (1) is not associated with all or a portion of a polynucleotide in which the "isolated polynucleotide" is found in nature, (2) is operably linked to a polynucleotide which it is not linked to in nature, and/or (3) does not occur in nature as part of a larger sequence. In some embodiments, polynucleotides include the nucleic acid molecules encoding heavy chain immunoglobulin molecules, and nucleic acid molecules encoding light chain immunoglobulin molecules.

[0044] The term "isolated polypeptide" as used herein refers a polypeptide that is present in a form other than that found in nature. An "isolated polypeptide" as used herein may be encoded by cDNA, recombinant RNA, recombinant DNA, messenger RNA, or a polynucleotide of synthetic origin or some combination thereof. By virtue of its origin, or source of derivation, the "isolated polypeptide" (1) is not in a naturally occurring organism (e.g., is not an endogenous polypeptide of a naturally occurring organism) and (2) is present in a form not found in nature. In some aspects, the "isolated polypeptide" is expressed by a cell from a different species. In some aspects, the "isolated polypeptide" is a therapeutic protein or a diagnostic protein and not a naturally occurring protein. For example, as used herein, the "isolated polypeptide" is not a plant protein or a protein naturally occurring in bacteria or other natural organisms. The term isolated polypeptide includes and provides support for activatable molecules including activatable macromolecules, activatable polypeptides, activatable antibodies, activatable cytokines, and the like. The term isolated polypeptide includes and provides support for activatable molecules in which cleavage of the CM activates the molecule.

[0045] The term "polypeptide" is used herein as a generic term to refer to a native protein, fragments, or analogs of a polypeptide sequence. Hence, proteins, protein fragments, and analogs are species of the polypeptide genus. In some embodiments, polypeptides in accordance with the disclosure comprise the heavy chain immunoglobulin, and the light chain immunoglobulin molecules, as well as antibody molecules formed by combinations comprising the heavy chain immunoglobulin molecules with light chain immunoglobulin molecules, such as kappa light chain immunoglobulin molecules, and vice versa, as well as fragments and analogs thereof.

[0046] As discussed herein, minor variations in the amino acid sequences of polypeptides are contemplated as being encompassed by the present disclosure, providing that the variations in the amino acid sequence maintain at least 75%, in some embodiments, at least

80%, at least 90%, at least 95%, and in some embodiments, at least 99% identity to the amino acid sequence that is not varied. In particular, conservative amino acid substitutions are contemplated. Conservative substitutions include those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are generally divided into families: (1) acidic amino acids are aspartate, glutamate; (2) basic amino acids are lysine, arginine, histidine; (3) non-polar amino acids are alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar amino acids are glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. The hydrophilic amino acids include arginine, asparagine, aspartate, glutamine, glutamate, histidine, lysine, serine, and threonine. The hydrophobic amino acids include alanine, cysteine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine and valine. Other families of amino acids include (i) serine and threonine, which are the aliphatic-hydroxy family; (ii) asparagine and glutamine, which are the amide containing family; (iii) alanine, valine, leucine and isoleucine, which are the aliphatic family; and (iv) phenylalanine, tryptophan, and tyrosine, which are the aromatic family. For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the binding or properties of the resulting molecule, especially if the replacement does not involve an amino acid within a framework site. Whether an amino acid change results in a functional peptide can readily be determined by assaying the specific activity of the polypeptide derivative. Assays are described in detail herein. Fragments or analogs of antibodies or immunoglobulin molecules can be readily prepared by those of ordinary skill in the art. Suitable amino- and carboxyl-termini of fragments or analogs occur near boundaries of functional domains. Structural and functional domains can be identified by comparison of the nucleotide and/or amino acid sequence data to public or proprietary sequence databases. In some embodiments, computerized comparison methods are used to identify sequence motifs or predicted protein conformation domains that occur in other proteins of known structure and/or function. Methods to identify protein sequences that fold into a known three-dimensional structure are known, e.g., as described in Bowie *et al.* Science 253:164 (1991). Thus, the foregoing examples demonstrate that those of skill in the art can recognize sequence motifs and

structural conformations that may be used to define structural and functional domains in accordance with the disclosure.

[0047] Suitable amino acid substitutions include those that: (1) alter susceptibility to proteolysis, (2) reduce susceptibility to oxidation, (3) alter binding affinity for forming protein complexes, (4) alter binding affinities, and (5) confer or modify other physicochemical or functional properties of such analogs. Analogs can include various muteins of a sequence other than the naturally-occurring peptide sequence. For example, single or multiple amino acid substitutions (for example, conservative amino acid substitutions) may be made in the naturally- occurring sequence (for example, in the portion of the polypeptide outside the domain(s) forming intermolecular contacts. A conservative amino acid substitution should not substantially change the structural characteristics of the parent sequence (*e.g.*, a replacement amino acid should not tend to break a helix that occurs in the parent sequence, or disrupt other types of secondary structure that characterizes the parent sequence). Examples of art-recognized polypeptide secondary and tertiary structures are described in Proteins, Structures and Molecular Principles (Creighton, Ed., W. H. Freeman and Company, New York (1984)); Introduction to Protein Structure (C. Branden and J. Tooze, eds., Garland Publishing, New York, N.Y. (1991)); and Thornton et at. Nature 354:105 (1991).

[0048] The term "sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. Included within the usage of the term "sample," therefore, is blood and a fraction or component of blood including blood serum, blood plasma, or lymph.

[0049] The term "therapeutic macromolecule" refers to any protein or nucleic acid that may be administered to a subject and have a therapeutic effect. In some embodiments, the therapeutic macromolecule may be a therapeutic polynucleotide or therapeutic polypeptide, i.e., a polynucleotide or polynucleotide that may be used in therapy.

[0050] As generally provided herein, an activatable molecule may comprise MM-CM construct(s), also referred to herein as a prodomain. Accordingly, as used herein, the term "prodomain" refers to a polypeptide domain comprising a masking moiety (MM) and a cleavable moiety (CM). In some embodiments, the MM and the CM are separated by a linker, referred to herein as LP1. In some embodiments, the prodomain comprises a linker (referred to herein as LP2) that links the CM of the prodomain to the active moiety (AM) in an activatable molecule. In some embodiments, the prodomain comprises a linker between the

MM and the CM and a linker between the CM and the AM. In some embodiments, the MM and the CM are not separated by a linker. In certain embodiments, a prodomain comprises one of the following formulas (where the formulas below represents amino acid sequences in either N- to C-terminal direction or C- to N-terminal direction): MM-LP1-CM, MM-CM-LP2, MM-LP1-CM-LP2, or MM-CM. As used herein and unless otherwise stated, each dash (-) between the components of the activatable molecule represents either a direct linkage or indirect linkage via one or more linking peptides.

CLEAVABLE MOIETIES

[0051] Proteases are involved in the control of numerous physiological processes, and their dysregulation has been identified in a number of pathologies, such as, for example, oncological, cardiovascular, autoimmune, and neurodegenerative diseases. *See, e.g.*, O. Vasiljeva, et al., "Monitoring protease activity in biological tissues using antibody prodrugs as sensing probes," *Scientific Reports*, 10, 5894 (2020); O. Erster, et al., "Site-specific targeting of antibody activity in vivo mediated by disease-associated proteases," *J. Control Release*, 161(3):804-812 (2012); L. Desnoyers, et al., "Tumor-specific activation of an EGFR-targeting probody enhances therapeutic index," *Science Translational Medicine*, 5(207):207ra144 (2013); and B. Turk "Targeting proteases: successes, failures and future prospects" Nature Reviews Drug Discovery, 5 (2006). Protease-activated antibodies have been described in the literature that are activated by native proteases which are more prevalently active in, for example, tumor tissue, and the like, when compared to normal tissue. These prodrugs have incorporated within their structure, a protease substrate that releases active drug following exposure to the appropriate protease and its subsequent cleavage. What appears evident, however, is that the profile of dysregulated protease activity in diseased tissue may differ from one type of disease tissue/disorder to another. Thus, it is desirable to have a collection of substrates that target a variety of different protease activity profiles.

[0052] In some aspects, the present disclosure provides cleavable moieties that exhibit enhanced cleavability to a matrix metalloproteinase (MMP), e.g., MMP2, MMP9, or MMP14. In certain aspects, the cleavable moieties are selectively cleavable by certain proteases (e.g., an MMP), but have reduced or no cleavability by another protease. In some aspects, resistance of cleavable moieties to protease cleavage in healthy tissue may reduce systemic toxicities by limiting binding of the activatable molecule to target that also may be present in healthy tissues. Therefore, cleavable moieties with selective cleavability by some proteases and

resistance to other proteases have the potential to demonstrate a greater therapeutic window and safety profile with less on-target toxicities occurring in healthy tissues.

[0053] In a specific aspect, the present disclosure provides polypeptides (e.g., isolated polypeptides) comprising a cleavable moiety (CM). A CM is a polypeptide that comprises a substrate for a sequence-specific protease. In some aspects, the present disclosure provides polypeptides and polypeptide complexes comprising a CM and an active moiety.

[0054] In some embodiments, the CM comprises the amino acid sequence of SEQ ID NO: 1. In some embodiments, the CM comprises the amino acid sequence of SEQ ID NO: 2. In some embodiments, the CM comprises the amino acid sequence of SEQ ID NO: 3. In some embodiments, the CM comprises the amino acid sequence of SEQ ID NO: 4. In some embodiments, the CM comprises the amino acid sequence of SEQ ID NO: 5. In some embodiments, the CM comprises the amino acid sequence of SEQ ID NO: 6. In some embodiments, the CM comprises the amino acid sequence of SEQ ID NO: 7. In some embodiments, the CM comprises the amino acid sequence of SEQ ID NO: 8. In some embodiments, the CM comprises the amino acid sequence of SEQ ID NO: 9. In some embodiments, the CM comprises the amino acid sequence of SEQ ID NO: 10. In some embodiments, the CM comprises the amino acid sequence of SEQ ID NO: 11. In some embodiments, the CM comprises the amino acid sequence of SEQ ID NO: 12. In some embodiments, the CM comprises the amino acid sequence of SEQ ID NO: 13. In some embodiments, the CM comprises the amino acid sequence of SEQ ID NO: 14.

[0055] In some embodiments, the CM comprises a combination, a C-terminal truncation variant, a C-terminal extension variant, an N-terminal truncation variant, or an N-terminal extension variant of the amino acid sequences of any one of SEQ ID NOs: 1-14. Truncation variants of the aforementioned amino acid sequences that are suitable for use in a CM may be any that retain the recognition site for the corresponding protease. These include C-terminal and/or N-terminal truncation variants comprising at least 1, 2, 3, 4, 5, or more contiguous amino acids of the above-described amino acid sequences that retain a recognition site for a protease. In certain embodiments, the truncation variant comprises a C-terminal deletion and/or an N-terminal deletion of one amino acid residue from an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-14. Extension variants of the aforementioned amino acid sequences that are suitable for use in a CM may be any that have one or more (e.g., 1, 2, 3, 4, 5 or more) additional amino acids and retain the recognition site

for the corresponding protease. In some examples, the additional amino acids are coupled to the C-terminus of the aforementioned amino acid sequences. In some examples, the additional amino acids are coupled to the N-terminus of the aforementioned amino acid sequences. In some examples, the extension variants may comprise additional amino acids coupled to both the C-terminus and the N-terminus of the aforementioned amino acid sequences. In some instances, the C-terminus or N-terminus extension variants can have a C-terminal glycine or an N-terminal serine amino acid.

[0056] In some embodiments, the CM comprises one, two, three, four, five, six or more amino acids in addition to the amino acid sequence of any one of SEQ ID NOs: 1-14. In some examples, the CM comprises one, two, three, four, five, six or more additional amino acids at the N-terminus of the amino acid sequence of any one of SEQ ID NOs: 1-14. In some examples, the CM comprises one, two, three, four, five, six or more additional amino acids at the C-terminus of the amino acid sequence of any one of SEQ ID NOs: 1-14. In some examples, the CM comprises one, two, three, four, five, six or more additional amino acids at the N-terminus, and one, two, three, four, five, six or more additional amino acids at the C-terminus of the amino acid sequence of any one of SEQ ID NOs: 1-14.

[0057] In some embodiments, the CM comprises a sequence with mutation(s) of one or more amino acid of the amino acid sequence of any one of SEQ ID NOs: 1-14. For example, the CM comprises a sequence with one-amino acid, two-amino acid, three-amino acid, four-amino acid, or five-amino acid mutation(s) of the amino acid sequence of any one of SEQ ID NOs: 1-14. In some embodiments, the CM comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-14 and having one conservative substitution.

[0058] In some embodiments, the CM consists of the amino acid sequence of SEQ ID NO: 1. In some embodiments, the CM consists of the amino acid sequence of SEQ ID NO: 2. In some embodiments, the CM consists of the amino acid sequence of SEQ ID NO: 3. In some embodiments, the CM consists of the amino acid sequence of SEQ ID NO: 4. In some embodiments, the CM consists of the amino acid sequence of SEQ ID NO: 5. In some embodiments, the CM consists of the amino acid sequence of SEQ ID NO: 6. In some embodiments, the CM consists of the amino acid sequence of SEQ ID NO: 7. In some embodiments, the CM consists of the amino acid sequence of SEQ ID NO: 8. In some embodiments, the CM consists of the amino acid sequence of SEQ ID NO: 9. In some embodiments, the CM consists of the amino acid sequence of SEQ ID NO: 10. In some

embodiments, the CM consists of the amino acid sequence of SEQ ID NO: 11. In some embodiments, the CM consists of the amino acid sequence of SEQ ID NO: 12. In some embodiments, the CM consists of the amino acid sequence of SEQ ID NO: 13. In some embodiments, the CM consists of the amino acid sequence of SEQ ID NO: 14.

[0059] In some embodiments, the CM consists of a sequence with mutation(s) of one or more amino acid of the amino acid sequence of any one of SEQ ID NOs: 1-14. For example, the CM consists of a sequence with one-amino acid, two-amino acid, three-amino acid, four-amino acid, or five-amino acid mutation(s) of the amino acid sequence of any one of SEQ ID NOs: 1-14.

[0060] In some embodiments, the CM comprises a total of 3 amino acids to 25 amino acids. For example, the CM may comprise a total of 3 to 25, 3 to 20, 3 to 15, 3 to 10, 3 to 5, 5 to 25, 5 to 20, 5 to 15, 5 to 10, 10 to 25, 10 to 20, 10 to 15, 15 to 25, 15 to 20, or 20 to 25 amino acids. In some embodiments, the CM consists of a total of 3 amino acids to 25 amino acids. For example, the CM may consist of a total of 3 to 25, 3 to 20, 3 to 15, 3 to 10, 3 to 5, 5 to 25, 5 to 20, 5 to 15, 5 to 10, 10 to 25, 10 to 20, 10 to 15, 15 to 25, 15 to 20, or 20 to 25 amino acids.

[0061] The CM may be specifically cleaved by a protease (e.g., by an MMP such as MMP2, MMP9, or MMP14) at a desired rate. The rate may be measured as substrate cleavage kinetics (k_{cat}/K_M) as disclosed in WO2016118629, which is incorporated by reference in its entirety. In brief, k_{cat} is the turnover number and describes how many substrate molecules are transformed into products per unit time by a protease. The K_M value describes the affinity of the substrate to the active site of the protease. The k_{cat}/K_M ratio provides a measurement of cleavability of the substrate by the protease. In general, the greater the ratio, the higher the rate of cleavability is; conversely, the lower the ratio, the slower the rate of cleavability is. The k_{cat}/K_M values may be determined with the following equation

$$\frac{k_{cat}}{K_M} = -\ln(1 - C)/(t*p)$$

where C is product conversion, t is time (s), and p is protease concentration (M), which assumes that the substrate concentration is below the K_M and in excess of the protease concentration.

[0062] In some embodiments, the CM is cleaved by an MMP at a rate that has a k_{cat}/K_M value from 1×10 to 1×10^6 $M^{-1}s^{-1}$, e.g., from 1×10 to 5×10, from 5×10 to 1×10^2, from 1×10^2 to 5×10^2, from 5×10^2 to 1×10^3, from 1×10^3 to 5×10^3, from 5×10^3 to 1×10^4, from 1×10^4 to 5×10^4, from 5×10^4 to 1×10^5, from 1×10^5 to 5×10^5, or from 5×10^5 to 1×10^6 $M^{-1}s^{-1}$. In some embodiments, the CM is cleaved by an MMP at a rate that has a k_{cat}/K_M value of at least 1×10, at least 5×10, at least 1×10^2, at least 5×10^2, at least 1×10^3, 5×10^3, at least 1×10^4, at least 5×10^4, at least 1×10^5, at least 5×10^5, or at least 1×10^6.

[0063] In some embodiments, the CM is cleaved by MMP2 at a rate that has a k_{cat}/K_M value from 1×10 to 1×10^6 $M^{-1}s^{-1}$, e.g., from 1×10 to 5×10, from 5×10 to 1×10^2, from 1×10^2 to 5×10^2, from 5×10^2 to 1×10^3, from 1×10^3 to 5×10^3, from 5×10^3 to 1×10^4, from 1×10^4 to 5×10^4, from 5×10^4 to 1×10^5, from 1×10^5 to 5×10^5, or from 5×10^5 to 1×10^6 $M^{-1}s^{-1}$. In some embodiments, the CM is cleaved by MMP2 at a rate that has a k_{cat}/K_M value of at least 1×10, at least 5×10, at least 1×10^2, at least 5×10^2, at least 1×10^3, 5×10^3, at least 1×10^4, at least 5×10^4, at least 1×10^5, at least 5×10^5, or at least 1×10^6.

[0064] In some embodiments, the CM is cleaved by MMP9 at a rate that has a k_{cat}/K_M value from 1×10 to 1×10^6 $M^{-1}s^{-1}$, e.g., from 1×10 to 5×10, from 5×10 to 1×10^2, from 1×10^2 to 5×10^2, from 5×10^2 to 1×10^3, from 1×10^3 to 5×10^3, from 5×10^3 to 1×10^4, from 1×10^4 to 5×10^4, from 5×10^4 to 1×10^5, from 1×10^5 to 5×10^5, or from 5×10^5 to 1×10^6 $M^{-1}s^{-1}$. In some embodiments, the CM is cleaved by MMP9 at a rate that has a k_{cat}/K_M value of at least 1×10, at least 5×10, at least 1×10^2, at least 5×10^2, at least 1×10^3, 5×10^3, at least 1×10^4, at least 5×10^4, at least 1×10^5, at least 5×10^5, or at least 1×10^6.

[0065] In some embodiments, the CM is cleaved by MMP14 at a rate that has a k_{cat}/K_M value from 1×10 to 1×10^6 $M^{-1}s^{-1}$, e.g., from 1×10 to 5×10, from 5×10 to 1×10^2, from 1×10^2 to 5×10^2, from 5×10^2 to 1×10^3, from 1×10^3 to 5×10^3, from 5×10^3 to 1×10^4, from 1×10^4 to 5×10^4, from 5×10^4 to 1×10^5, from 1×10^5 to 5×10^5, or from 5×10^5 to 1×10^6 $M^{-1}s^{-1}$. In some embodiments, the CM is cleaved by MMP14 at a rate that has a k_{cat}/K_M value of at least 1×10, at least 5×10, at least 1×10^2, at least 5×10^2, at least 1×10^3, 5×10^3, at least 1×10^4, at least 5×10^4, at least 1×10^5, at least 5×10^5, or at least 1×10^6.

[0066] In some embodiments, the cleavability of the CMs are presented as the percentage of the fraction of cleaved CMs (or polypeptides comprising the CMs), e.g., as determined in a capillary electrophoresis assay described Example 2. In some examples, the cleavability of the CM by a protease (e.g., an MMP) is at least 50%, at least 60%, at least 70%, at least 80%,

at least 90%, or at least 95%, or 100%. In some examples, the cleavability of the CM by MMP2 is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%, or 100% when 500nM activatable antibody c225 containing a prodomain with the CM being tested was incubated with 10 nM of MMP2 for 1.5 hours at 37°C. In some examples, the cleavability of the CM by MMP9 is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%, or 100% when 500nM activatable antibody c225 containing a prodomain with the CM being tested was incubated with 10 nM of MMP9 for 1.5 hours at 37°C. In some examples, the cleavability of the CM by MMP14 is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%, or 100% when 500nM activatable antibody c225 containing a prodomain with the CM being tested was incubated with 10 nM of MMP14 for 1.5 hours at 37°C.

[0067] In some embodiments, for specific cleavage by an enzyme, contact between the enzyme and CM is made. When a CM-containing polypeptide (e.g., activatable molecule comprising an AM coupled to a MM and a CM) is in the presence of target and sufficient protease activity, the CM can be cleaved. Sufficient protease activity refers to the ability of the protease to access the CM and effect cleavage.

[0068] In some embodiments, a CM according to the present disclosure and a reference polypeptide can be cleaved by the same protease (e.g., an MMP), but the CM according to the present disclosure has reduced cleavage or resistance to cleavage (e.g., by a different protease(s) than an MMP2, MMP9, and MMP14) in certain tissues *in situ* compared to a reference polypeptide. For example, the cleavage (e.g., by a different protease than MMP such as MMP2, MMP9, or MMP14) in a tissue or a sample comprising a tissue or cell *in situ* of the CM may be less than 99%, less than 95%, 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 5%, or less than 1% compared to the cleavage of the reference polypeptide. A CM that is resistant to cleavage by a protease, or a sample or tissue comprising a protease, refers to (i) a CM in which no peptide bond is hydrolyzed by the protease, or no peptide bond is hydrolyzed when incubated in the sample or tissue comprising the protease, or (ii) a CM in which a reduced level of peptide bond is hydrolyzed by the protease, or reduced level of peptide bond is hydrolyzed when incubated in the sample or tissue comprising the protease, compared to a reference CM.

[0069] In some embodiments, the CM is cleavable by more than one proteases. For example, the CM may be cleaved by one or more MMPs (e.g., MMP2, MMP9, and/or MMP14) and by a second or multiple additional proteases. Examples of the additional protease could be any one or more of the following proteases: a disintegrin and metalloprotease (ADAM), an ADAM-like, or a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS, such as, for example, ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17/TACE, ADAMDEC1, ADAMTS1, ADAMTS4, ADAMTS5); an aspartate protease (such as, for example, BACE, Renin, and the like); an aspartic cathepsin (such as, for example, Cathepsin D, Cathepsin E, and the like); a caspase (such as, for example, Caspase 1, Caspase 2, Caspase 3, Caspase 4, Caspase 5, Caspase 6, Caspase 7, Caspase 8, Caspase 9, Caspase 10, Caspase 14, and the like); a cysteine cathepsin (such as, for example, Cathepsin B, Cathepsin C, Cathepsin K, Cathepsin L, Cathepsin S, Cathepsin V/L2, Cathepsin X/Z/P); a cysteine proteinase (such as, for example, Cruzipain, Legumain, Otubain-2, and the like); a kallikrein-related peptidase (KLK) (such as, for example, KLK4, KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13, KLK14, and the like); a metalloproteinase (such as, for example, Meprin, Neprilysin, prostate-specific membrane antigen (PSMA), bone morphogenetic protein 1 (BMP-1), and the like); a matrix metalloproteinase (MMP, such as, for example, MMP1, MMP2, MMP3, MMP7, MMP8, MMP9, MMP10, MMP11, MMP12, MMP13, MMP14, MMP15, MMP16, MMP17, MMP19, MMP20, MMP23, MMP24, MMP26, MMP27, and the like); a serine protease (such as, for example, activated protein C, Cathepsin A, Cathepsin G, Chymase, a coagulation factor protease (such as, for example, FVIIa, FIXa, FXa, FXIa, FXIIa, and the like); elastase, granzyme B, Guanidinobenzoatase, HtrA1, proteinase 3, neutrophil elastase, neutrophil serine protease 4 (NSP4), Lactoferrin, Marapsin, NS3/4A, PACE4, Plasmin, prostate-specific antigen (PSA), tissue plasminogen activator (tPA), Thrombin, Tryptase, urokinase-type plasminogen activator (uPA), a Type II transmembrane Serine Protease (TTSP) (such as, for example, DESC1, DPP-4, FAP, Hepsin, Matriptase-2, MT-SP1/Matriptase, TMPRSS2, TMPRSS3, TMPRSS4, TMPRSS5, TMPRSS6, TMPRSS7, TMPRSS8, TMPRSS9, TMPRSS10, TMPRSS11, and the like), and the like. Specific substrates are described, for example, in WO 2010/081173, WO 2015/048329, WO 2015/116933, and WO 2016/118629, each of which is incorporated herein by reference in its entirety.

ACTIVATABLE MOLECULES

[0070] In some embodiments, the polypeptide or polypeptide complex comprising a CM is an activatable molecule. The activatable molecule may comprise an active moiety (AM) that specifically binds a target. The AM may be coupled to the CM. In some embodiments, the activatable molecule comprises a masking moiety (MM) coupled with the AM via the CM.

[0071] The coupling of two components in a polypeptide or polypeptide complex (e.g., an activatable molecule) may be direct or indirect. When the two components are coupled directly, the amino acid residue at the C-terminus of a component forms a peptide bond with the amino acid residue at the N-terminus of the other component. When the two components are coupled indirectly, there is a stretch of amino acids between the two components. In some examples, the two components of a polypeptide may be indirectly coupled via one or more other components in the polypeptide, i.e., the one or more other components are between the two coupled components. For indirectly coupling or linking via another component, the one or more other components may be a linker, AM(s), CM(s), MM(s), or any combination thereof.

[0072] As used herein, the term "activatable molecule" refers to a molecule that comprises at least one set of MM, CM, and AM and which exhibits attenuated binding to a target as compared to the binding of a counterpart "activated" molecule comprising the same AM to the same target. The terms "activated molecule," and "cleaved activatable molecule," are used interchangeably herein to refer to the AM-containing cleavage product that is generated after exposure of the activatable molecule to a CM-specific protease (i.e., after cleavage of the CM by at least one protease). In some embodiments, a cleaved activatable molecule may lack a MM due to cleavage of the CM (e.g., by a protease), resulting in release of the MM.

[0073] An AM may be any polypeptide that specifically binds a target. In some examples, the AM may be a therapeutic macromolecule. In some examples, the AM may be an antibody or an antigen-binding fragment. In some examples, the AM may be an antineoplastic macromolecule. In some examples, the AM may be a cytokine. In some examples, the AM may be a chimeric antigen receptor.

[0074] In some examples, the AM may be a diagnostic macromolecule. For example, the diagnostic macromolecule may be a diagnostic polypeptide having 3 to 30, 5 to 25, 7 to 20,

or 9 to 15 amino acids in length. Such diagnostic polypeptide may be used, in non-limiting aspects, e.g., for testing cleavage in tissues, and/or assessment of the tissue microenvironment.

[0075] As used herein, the terms "specific binding" and "specifically binds" refer to the non-covalent interactions of the type that occur between an AM and its target, e.g., an immunoglobulin molecule and an antigen or a cytokine and its receptor, for which the AM is specific. The strength or affinity of binding interactions can be expressed in terms of the dissociation constant (K_d) of the interaction, wherein a smaller K_d represents a greater affinity. Unless indicated otherwise, as used herein, "affinity" refers to intrinsic binding affinity, which reflects a 1:1 interaction between members of an AM and its target. Affinity can be measured by common methods known in the art, including those described herein. Affinity can be determined, for example, using surface plasmon resonance (SPR) technology (e.g., BIACORE®) or biolayer interferometry (e.g., FORTEBIO®). Additional methods for determining the affinity for an AM and its target are known in the art. Immunological binding properties of selected polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and geometric parameters that equally influence the rate in both directions. Thus, both the "on rate constant" (K_{on}) and the "off rate constant" (K_{off}) can be determined by calculation of the concentrations and the actual rates of association and dissociation. (*See* Nature 361:186-87 (1993)). The ratio of K_{off} /K_{on} enables the cancellation of all parameters not related to affinity, and is equal to the dissociation constant K_d. (*See, generally*, Davies et al. (1990) Annual Rev Biochem 59:439-473). As used herein, a statement that an AM "specifically binds" to its target refers to an AM that binds its target with a dissociation constant (K_d) of less than 100 μM (e.g., less than 5 μM or 10 μM). In some examples, the AM specifically binds its target with a K_d of about 0.01 nM to about 500 nM. In some examples, an AM is said to specifically bind the target, when the equilibrium binding constant (K_d) is ≤1 μM, in some embodiments ≤ 100 nM, in some embodiments ≤ 10 nM, and in some embodiments ≤ 100 pM to about 1 pM, as measured by assays such as radioligand binding assays or similar assays known to those skilled in the art.

[0076] In general, an activatable molecule may be designed by selecting an AM of interest and constructing the remainder of the activatable molecule so that, when conformationally constrained, the MM provides for masking of the AM or reduction of binding of the AM to its target. Structural design criteria can be to be taken into account to provide for this functional feature.

[0077] Activatable molecules may be provided in a variety of structural configurations. Exemplary formulas for activatable molecules are provided below. It is contemplated that the N- to C-terminal order of the AM, MM and CM may be reversed within an activatable molecule. For example, activatable molecules can be represented by the following formulas (in order from an amino (N) terminal region to carboxyl (C) terminal region):

MM-CM-AM

AM-CM-MM

As used herein and unless otherwise stated, each dash (-) between the components of the activatable molecule represents either a direct linkage or indirect linkage via one or more linkers. It should be noted that although MM and CM are indicated as distinct components in the formulas above, in all exemplary embodiments (including formulae) disclosed herein it is contemplated that the amino acid sequences of the MM and the CM may overlap, *e.g.*, such that the CM is completely or partially contained within the MM. In addition, the formulas above provide for additional amino acid sequences that may be positioned N-terminal or C-terminal to the activatable molecules components. Examples include targeting moieties (e.g., a ligand for a receptor of a cell present in a target tissue) and half-life extending moieties.

[0078] In some embodiments, MM, CM, and/or AM are coupled indirectly via one or more linkers (e.g., a linking peptide (LP)). For example, an activatable molecule may comprise one of the following formulae (in order from an amino (N) terminal region to carboxyl (C) terminal region):

MM-LP-CM-AM

MM-CM-LP-AM

MM-LP1-CM-LP2-AM

AM-LP-CM-MM

AM-CM-LP-MM

AM-LP2-CM-LP1-MM

wherein LP1 and LP2 are two linking peptides. In some examples, the LP1 and LP2 are identical to each other. In some examples, the LP1 and LP2 are not identical to each other.

[0079] In some embodiments, the activatable molecule comprise a plurality of CMs, at least one of which comprises the sequence of any of SEQ ID NOs: 1-14. For example, the CM comprising the sequence of any of SEQ ID NOs: 1-14 may be engineered into a longer cleavage substrate that has a plurality of CMs. Examples of the additional CM(s) in the activatable molecule that are not the CM comprising the sequence of any of SEQ ID NOs: 1-14 include those described in WO 2010/081173, WO2021207669, WO2021207657, WO2021142029, WO2021061867, WO2020252349, WO2020252358, WO2020236679, WO2020176672, WO2020118109, WO2020092881, WO2020086665, WO2019213444, WO2019183218, WO2019173771, WO2019165143, WO2019075405, WO2019046652, WO2019018828, WO2019014586, WO2018222949, WO2018165619, WO2018085555, WO2017011580, WO2016179335, WO2016179285, WO2016179257, WO2016149201, WO2016014974, which are incorporated herein by reference in their entireties for all purposes. In some examples, one or more of the additional CMs may be cleavable by legumain. In some examples, the CM cleavable by legumain may comprise a sequence of any of SEQ ID NO: 1-14 and an Asparagine (Asn) residue at the N-terminus or C-terminus.

[0080] In some embodiments, the substrate comprises CM1 cleavable by a first protease, and CM2 cleavable by a second protease. In some embodiments, the substrate comprises CM1 cleavable by a first protease, CM2 cleavable by a second protease, and CM3 cleavable by a third protease. In some embodiments, the substrate comprises CM1 cleavable by a first protease, CM2 cleavable by a second protease, CM3 cleavable by a third protease, and CM4 cleavable by a fourth protease.

[0081] In some embodiments, the activatable molecule comprises a structural arrangement from N-terminus to C-terminus as follows: MM-CM1-CM2-AM, MM-CM2-CM1-AM, AM-CM1-CM2-MM, or AM-CM2-CM1-MM, MM-CM2-CM1-CM3-AM, MM-CM1-CM2-CM3-AM, MM-CM1-CM3-CM2-AM, MM-CM3-CM1-CM2-AM, or MM-CM3-CM2-CM1-AM. Likewise, a CM4 may be inserted any position between the MM and AM.

[0082] In some embodiments, the activatable molecule comprises a linking peptide (LP) and wherein the activatable molecule has a structural arrangement from N-terminus to C-terminus as follows: MM-LP-CM1-CM2-AM, MM-CM1-CM2-LP-AM, MM-LP-CM2-

CM1-AM, MM-CM2-CM1-LP-AM, MM-LP-CM2-CM1-CM3-AM, MM-LP-CM1-CM2-CM3-AM, MM-LP-CM1-CM3-CM2-AM, MM-LP-CM3-CM1-CM2-AM, MM-LP-CM3-CM2-CM1-AM, MM-CM2-CM1-CM3-LP-AM, MM-CM1-CM2-CM3-LP-AM, MM-CM1-CM3-CM2-LP-AM, MM-CM3-CM1-CM2-LP-AM, or MM-CM3-CM2-CM1-LP-AM. Likewise, a CM4 may be inserted any position between the MM and AM.

[0083] In some embodiments, the activatable molecule comprises a first linking peptide (LP1) and a second linking peptide (LP2), and wherein the activatable molecule has a structural arrangement from N-terminus to C-terminus as follows: MM- LP1-CM1-CM2-LP2-AM, MM-LP1-CM2-CM1-LP2-AM, AM-LP2-CM1-CM2-LP1-MM, or AM-LP2-CM2-CM1-LP1-MM, MM-LP1-CM2-CM1-CM3-LP2-AM, MM-LP1-CM1-CM2-CM3-LP2-AM, MM-LP1-CM1-CM3-CM2-LP2-AM, MM-LP1-CM3-CM1-CM2-LP2-AM, MM-LP1-CM3-CM2-CM1-LP2-AM, MM-LP2-CM2-CM1-CM3-LP1-AM, MM-LP2-CM1-CM2-CM3-LP1-AM, MM-LP2-CM1-CM3-CM2-LP1-AM, MM-LP2-CM3-CM1-CM2-LP1-AM, or MM-LP2-CM3-CM2-CM1-LP1-AM. Likewise, a CM4 may be inserted any position between the MM and AM.

[0084] In some embodiments, the activatable molecule comprises an additional linking peptide (LP3) and wherein the activatable molecule has a structural arrangement from N-terminus to C-terminus as follows: MM-LP-CM1-LP3-CM2-AM, MM-CM1-LP3-CM2-LP-AM, MM-LP-CM2-LP3-CM1-AM, MM-CM2-LP3-CM1-LP-AM, MM-LP-CM2-LP3-CM1-CM3-AM, MM-LP-CM1-LP3-CM2-CM3-AM, MM-LP-CM1-LP3-CM3-CM2-AM, MM-LP-CM3-LP3-CM1-CM2-AM, MM-LP-CM3-LP3-CM2-CM1-AM, MM-CM2-LP3-CM1-CM3-LP-AM, MM-CM1-LP3-CM2-CM3-LP-AM, MM-CM1-LP3-CM3-CM2-LP-AM, MM-CM3-LP3-CM1-CM2-LP-AM, MM-CM3-LP3-CM2-CM1-LP-AM, MM-LP-CM2-CM1-LP3-CM3-AM, MM-LP-CM1-CM2-LP3-CM3-AM, MM-LP-CM1-CM3-LP3-CM2-AM, MM-LP-CM3-CM1-LP3-CM2-AM, MM-LP-CM3-CM2-LP3-CM1-AM, MM-CM2-CM1-LP3-CM3-LP-AM, MM-CM1-CM2-LP3-CM3-LP-AM, MM-CM1-CM3-LP3-CM2-LP-AM, MM-CM3-CM1-LP3-CM2-LP-AM, MM-CM3-CM2-LP3-CM1-LP-AM,MM-LP1-CM1-LP3-CM2-LP2-AM, MM-LP1-CM2-LP3-CM1-LP2-AM, AM-LP1-CM1-LP3-CM2-LP2-MM, or AM-LP1-CM2-LP3-CM1-LP2-MM, MM-LP1-CM2-LP3-CM1-CM3-LP2-AM, MM-LP1-CM1-LP3-CM2-CM3-LP2-AM, MM-LP1-CM1-LP3-CM3-CM2-LP2-AM, MM-LP1-CM3-LP3-CM1-CM2-LP2-AM, MM-LP1-CM3-LP3-CM2-CM1-LP2-AM, MM-LP2-CM2-LP3-CM1-CM3-LP1-AM, MM-LP2-CM1-LP3-

CM2-CM3-LP1-AM, MM-LP2-CM1-LP3-CM3-CM2-LP1-AM, MM-LP2-CM3-LP3-CM1-CM2-LP1-AM, or MM-LP2-CM3-LP3-CM2-CM1-LP1-AM. Likewise, a CM4 may be inserted any position between the MM and AM.

[0085] In some embodiments, the activatable molecule has a structural arrangement from N-terminus to C-terminus as follows: MM-LP-CM2-LP3-CM1-LP4-CM3-AM, MM-LP-CM1-LP3-CM2-LP4-CM3-AM, MM-LP-CM1-LP3-CM3-LP4-CM2-AM, MM-LP-CM3-LP3-CM1-LP4-CM2-AM, MM-LP-CM3-LP3-CM2-LP4-CM1-AM, MM-CM2-LP3-CM1-LP4-CM3-LP-AM, MM-CM1-LP3-CM2-LP4-CM3-LP-AM, MM-CM1-LP3-CM3-LP4-CM2-LP-AM, MM-CM3-LP3-CM1-LP4-CM2-LP-AM, MM-CM3-LP3-CM2-LP4-CM1-LP-AM, MM-LP-CM2-LP4-CM1-LP3-CM3-AM, MM-LP-CM1-LP4-CM2-LP3-CM3-AM, MM-LP-CM1-LP4-CM3-LP3-CM2-AM, MM-LP-CM3-LP4-CM1-LP3-CM2-AM, MM-LP-CM3-LP4-CM2-LP3-CM1-AM, MM-CM2-LP4-CM1-LP3-CM3-LP-AM, MM-CM1-LP4-CM2-LP3-CM3-LP-AM, MM-CM1-LP4-CM3-LP3-CM2-LP-AM, MM-CM3-LP4-CM1-LP3-CM2-LP-AM, MM-CM3-LP4-CM2-LP3-CM1-LP-AM, MM-LP1-CM2-LP3-CM1-LP4-CM3-LP2-AM, MM-LP1-CM1-LP3-CM2-LP4-CM3-LP2-AM, MM-LP1-CM1-LP3-CM3-LP4-CM2-LP2-AM, MM-LP1-CM3-LP3-CM1-LP4-CM2-LP2-AM, MM-LP1-CM3-LP3-CM2-LP4-CM1-LP2-AM, MM-LP2-CM2-LP3-CM1-LP4-CM3-LP1-AM, MM-LP2-CM1-LP3-CM2-LP4-CM3-LP1-AM, MM-LP2-CM1-LP3-CM3-LP4-CM2-LP1-AM, MM-LP2-CM3-LP3-CM1-LP4-CM2-LP1-AM, or MM-LP2-CM3-LP3-CM2-LP4-CM1-LP1-AM. Likewise, a CM4 may be inserted any position between the MM and AM.

[0086] In some embodiments, in the above structural arrangements, the CM1 comprises a sequence of any one of SEQ ID NOs: 1-14. Alternatively or additionally, in some embodiments, in the above structural arrangements, the CM2 comprises a sequence of any one of SEQ ID NOs: 1-14. Alternatively or additionally, in some embodiments, in the above structural arrangements, the CM3 comprises a sequence of any one of SEQ ID NOs: 1-14.

[0087] In some embodiments where the activatable molecule comprises a plurality of CMs, at least a portion of a first CM overlaps with at least a portion of a second CM in the substrate, such that one or more amino acids in the substrate belongs to both CMs. For example, a substrate with the sequence $X_1X_2X_3X_4X_5X_6$ (each X is an amino acid), may comprise overlapping CM1 and CM2, in which CM1 is $X_1X_2X_3X_4$ and CM2 is $X_3X_4X_5X_6$.

[0088] In some embodiments where the activatable molecule comprises a plurality of CMs, two CMs do not overlap in amino acid sequences such that no amino acid in the

substrate belongs to both CMs. For example, a substrate with the sequence $X_1X_2X_3X_4X_5X_6X_7X_8$ (each X is an amino acid) may comprise non-overlapping CM1 and CM2, in which CM1 is $X_1X_2X_3X_4$ and CM2 is $X_5X_6X_7X_8$. In some embodiments, the non-overlapping CM1 and CM2 are coupled directly. In some embodiments, the non-overlapping CM1 and CM2 are coupled indirectly (e.g., via a linking peptide).

[0089] In some embodiments, two CMs, e.g., CM1 and CM2, in a substrate have a structural arrangement from N-terminus to C-terminus as CM1-CM2. In some embodiments, two CMs, e.g., CM1 and the CM2 in a substrate have a structural arrangement from N-terminus to C-terminus as CM2-CM1. As used herein, the CM1 and CM2 in the formula CM1-CM2 or CM2-CM1 may be overlapping CM1 and CM2, non-overlapping CM1 and CM2 coupled directly, or non-overlapping CM1 and CM2 coupled indirectly (e.g., via a linking peptide).

[0090] In some embodiments, two CMs, e.g., CM2 or CM4 and CM3 or CM4, in a substrate have a structural arrangement from N-terminus to C-terminus as CM2-CM3, CM2-CM4 or CM3-CM4. In some embodiments, two CMs, e.g., CM2 and the CM3 in a substrate have a structural arrangement from N-terminus to C-terminus as CM3-CM2 or CM4-CM2 or CM4-CM3. As used herein, the CM2 and CM3 in the formula CM2-CM3 or CM3-CM2 may be overlapping CM2 and CM3, non-overlapping CM2 and CM3 coupled directly, or non-overlapping CM2 and CM3 coupled indirectly (e.g., via a linking peptide). As used herein, the CM2 and CM4 in the formula CM2-CM4 or CM4-CM2 may be overlapping CM2 and CM4, non-overlapping CM2 and CM4 coupled directly, or non-overlapping CM2 and CM4 coupled indirectly (e.g., via a linking peptide). As used herein, the CM4 and CM3 in the formula CM4-CM3 or CM3-CM4 may be overlapping CM4 and CM3, non-overlapping CM4and CM3 coupled directly, or non-overlapping CM4 and CM3 coupled indirectly (e.g., via a linking peptide).

Antibodies and Antigen-Binding Fragments

[0091] In some embodiments, the AM is an antibody or antigen-binding fragment thereof. The term "antibody" is used herein in its broadest sense and includes certain types of immunoglobulin molecules that include one or more target-binding domains that specifically bind an antigen or epitope. Examples of antibodies include intact antibodies (e.g., intact immunoglobulins), antibody fragments, bispecific, and multi-specific antibodies. One

example of a target-binding domain is formed by a V_H -V_L dimer. Additional examples of an antibody are described herein. Additional examples of an antibody are known in the art.

[0092] A "light chain" includes one variable domain (VL) and one constant domain (CL). There are two different light chains termed kappa or lambda. A "heavy chain" consists of one variable domain (VH) and three constant region domains (CH1, CH2, CH3). There are five main heavy-chain classes or isotypes, some of which have several subtypes, and these determine the functional activity of an antibody molecule. The five major classes of immunoglobulin are immunoglobulin M (IgM), immunoglobulin D (IgD), immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin E (IgE). IgG is by far the most abundant immunoglobulin and has several subclasses (IgG1, IgG2, IgG3, and IgG4 in humans).

[0093] In some embodiments, the antigen-binding fragment is a Fab fragment, a $F(ab')_2$ fragment, a scFv, a scAb, a dAb, a single domain heavy chain antibody, or a single domain light chain antibody. Additional examples of the antigen-binding fragments include a VH domain, a VHH domain, a VNAR domain, and a single chain fragment variable (scFv), BiTE or a component thereof, a $(scFv)_2$, a NANOBODY®, a nanobody-HSA, VHH-scAb, a VHH-Fab, a Dual scFab, a $F(ab')_2$, a diabody, a CROSSMAB®, a DAF (two-in-one), a DAE (four-in-one), a DUTAMAB®, a DT- IgG, a knobs-in-holes common light chain, a knobs-in-holes assembly, a charge pair, a Fab-arm exchange, a SEEDbody, a LUZ-Y, a FcAb, a kl-body, an orthogonal Fab, a DVD-IgG, a IgG(H)-scFv, a scFv-(H)IgG, IgG(L)-scFv, scFv-(L)IgG, IgG(L,H)-Fv, IgG(H)-V, V(H)-IgG, IgG(L)-V, V(L)-IgG, KIH IgG-scFab, 2scFv-IgG, IgG-2scFv, scFv4-Ig, ZYBODY™, DVI-IgG, Diabody-CH3, a triple body, a miniantibody, a minibody, a TriBi minibody, scFv-CH3 KIH , Fab-scFv, a F(ab')2-scFv2, a scFv-KIH, a Fab-scFv-Fc, a tetravaient HCAb, a scDiabody-Fc, a Diabody-Fc, a tandem scFv-Fc, a VHH-Fc, a tandem VHH-Fc, a L'HH-Fc KiH, a Fab- VHH-Fc, an Intrabody, a dock and lock, an ImmTAC® (immune-mobilizing monoclonal TCRs (T cell receptors) against cancer), an IgG-IgG conjugate, a Cov-X-Body, a scFvl- PEG-scFv2, an Adnectin, a DARPin®, a fibronectin, an IgG, an IgM, an IgA, an IgE, an IgD, a DEP conjugate, TMEAbody™, SAFEbody®, TRITAC® , or SHIELD antibody.

[0094] A "fragment antigen binding" (Fab) includes a complete light chain paired with the VH domain and the CH1 domain of a heavy chain. A $F(ab')_2$ fragment is formed when an antibody is cleaved by pepsin (or otherwise truncated) below the hinge region, in which case

the two fragment target-binding domains (Fabs) of the antibody molecule remain linked. A $F(ab')_2$ fragment contains two complete light chains paired with the two VH and CH1 domains of the heavy chains joined together by the hinge region. A "fragment crystallizable" (Fc) fragment (also referred to herein as F_C domain) corresponds to the paired CH2 and CH3 domains and is the part of the antibody molecule that interacts with effector molecules and cells. The functional differences between heavy-chain isotypes lie mainly in the Fc fragment. A "single chain fragment variable" (scFv) contains only the variable domain of a light chain (VL) linked by a stretch of peptide to a variable domain of a heavy chain (VH). The name single-chain Fv is derived from Fragment variable. A "hinge region" or "interdomain" is flexible amino acid stretch that joins or links the Fab fragment to the Fc domain. A "synthetic hinge region" is an amino acid sequence that joins or links a Fab fragment to an Fc domain.

[0095] An "Fv" fragment includes a non-covalently-linked dimer of one heavy chain variable domain and one light chain variable domain. A "dual variable domain immunoglobulin G" or "DVD-IgG" refers to multivalent and multispecific target-binding proteins as described, e.g., in DiGiammarino et al., Methods Mol. Biol. 899:145-156, 2012, Jakob et al., MABs 5:358-363, 2013; and U.S, Patent Nos. 7,612,181; 8,258,268; 8,586,714; 8,716,450; 8,722,855; 8,735,546; and 8,822,645, each of which is incorporated by reference in its entirety. Examples of DARTs are described in, e.g., Garber, Nature Reviews Drug Discovery 13:799-801, 2014.

[0096] A VHH domain is a single monomeric variable antibody domain that can be found in camelids. A VNAR domain is a single monomeric variable antibody domain that can be found in cartilaginous fish. Non-limiting aspects of VHH domains and VNAR domains are described in, e.g., Cromie et al., Curr. Top. Med. Chem. 15:2543-2557, 2016; De Genst et al. Dev. Comp. Immunol. 30:187-198, 2006; De Meyer et al, Trends Biotechnol 32:263-270, 2014; Kijanka et al., Nanomedicine 10:161-174, 2015; Kovaleva et al., Expert. Opin. Biol. Ther. 14: 1527-1539, 2014; Krah et al., Immunopharmacol. Immunotoxicol. 38:21-28, 2016; Mujic-Delic et al., Trends Pharmacol. Sci. 35:247-255, 2014; Muyldermans, J. Biotechnol. 74:277-302, 2001, Muyldermans et al., Trends Biocheni. Sci. 26:230-235, 2001; Muyldermans, Ann. Rev. Biochem. 82:775-797, 2013; Rahbarizadeh et al., Immunol, invest. 40:299-338, 2011; Van Audenhove et al., EBioMedicine 8:40-48, 2016; Van Bockstaele et al., Curr. Opin. Investig. Drugs 10:1212-1224, 2009; Vincke et al. Methods Mol, Biol,

911:15-26, 2012; and Wesolowski et al. Med. Microbiol. Immunol. 198:157-174, 2009, each of which is incorporated by reference herein in its entirety.

[0097] In some embodiments, the AM may be a mouse, rat, rabbit, goat, camel, donkey, primate, human, or humanized or chimeric polypeptide. In one example, the AM may be a human polypeptide. In one example, the AM may be a humanized (e.g., fully humanized) polypeptide.

[0098] The term "humanized" refer to an AM having an amino acid sequence that includes VH and VL region sequences from a reference protein raised in a non-human species (e.g., a mouse), but also includes modifications in those sequences relative to the reference protein intended to render them more "human-like," i.e., more similar to human germline variable sequences. In some embodiments, a "humanized" AM is one that immunospecifically binds an antigen of interest and that has a framework (FR) region having substantially the amino acid sequence as that of a human protein, and a complementary determining region (CDR) having substantially the amino acid sequence as that of a non-human protein contains humanized VH and VL regions.

[0099] The term "human polypeptide" is intended to include AMs having variable and constant regions generated, assembled, or derived from human immunoglobulin sequences. In some embodiments, an AM may be considered to be "human" even though its amino acid sequence include residues or elements not encoded by human germline immunoglobulin sequences (e.g., include sequence variations, for example that may (originally) have been introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo), e.g., in one or more CDRs.

[0100] Examples of antibodies and antigen-binding fragments include those binding to cell surface receptors and secreted binding proteins (*e.g.*, growth factors), soluble enzymes, structural proteins (*e.g.* collagen, fibronectin) and the like, or an extracellular target (e.g., an extracellular protein target). In some embodiments, antibodies and antigen-binding fragments are designed for cellular uptake and are activatable inside a cell.

[0101] Examples of antibodies and antigen-binding fragments include those in Example 1, e.g., those comprising a light chain comprising a sequence selected from one of SEQ ID NOs: 73, 74, 83, 85, 87, 89, and 91, and a heavy chain comprising a sequence selected from one of SEQ ID NOs: 84, 86, 88, and 92.

Multispecific Activatable Antibodies

[0102] In some embodiments, the activatable antibodies are multispecific activatable antibodies. In some examples, the multispecific activatable antibodies herein recognize two or more different antigens or epitopes and that include at least one masking moiety (MM) linked to at least one antigen- or epitope-binding domain of the multispecific antibody such that coupling of the MM reduces the ability of the antigen- or epitope-binding domain to bind its target. In some embodiments, the MM is coupled to the antigen- or epitope-binding domain of the multispecific antibody via a cleavable moiety (CM) that functions as a substrate for at least one protease, e.g., an MMP. The activatable multispecific antibodies provided herein are stable in circulation, activated at intended sites of therapy and/or diagnosis but not in normal, i.e., healthy tissue, and, when activated, exhibit binding to a target that is at least comparable to the corresponding, unmodified multispecific antibody.

[0103] The multispecific activatable molecules may be used to target a first and a second target tissues. In one embodiment, the first and second target tissues are spatially separated, for example, at different sites in the organism. In one embodiment, the first and second target tissues are the same tissue temporally separated, for example the same tissue at two different points in time, for example the first time point is when the tissue is an early stage tumor, and the second time point is when the tissue is a late stage tumor.

[0104] In some embodiments, the multispecific activatable antibody includes a first antibody or antigen-binding fragment thereof (AB1) that binds a first target, where the AB1 is coupled to a masking moiety (MM1) such that coupling of the MM1 reduces the ability of the AB1 to bind the first target, and the multispecific activatable antibody includes a second antibody or antigen-binding fragment thereof (AB2) that binds a second target, where the AB2 is coupled to a masking moiety (MM2) such that coupling of the MM2 reduces the ability of the AB2 to bind the second target. In some embodiments, AB1 is coupled to MM1 via CM1, and AB2 is coupled to MM2 via CM2. In some embodiments, there are linking peptides between AB1 and CM1, between CM1 and MM1, between AB2 and CM2, and/or between CM2 and MM2. In some embodiments, AB1 is directly coupled to CM1, CM1 is directly coupled to MM1, AB2 is directly coupled to CM2, and/or CM2 is directly coupled to MM2.

[0105] For example, the multispecific activatable antibodies can be represented by the following formulas (in order from an amino (N) terminal region to carboxyl (C) terminal region):

MM1-CM1-AB1 : MM2-CM2-AB2

AB1-CM1-MM1 : MM2-CM2-AB2

AB1-CM1-MM1 : AB2-CM2-MM2

wherein ":" separates two polypeptides, which may be two independent polypeptides on two different molecules, or two polypeptides on the same molecule (e.g., two polypeptide chains of the same protein). As used herein and unless otherwise stated, each dash (-) between the components of the activatable molecule represents either a direct linkage or indirect linkage via one or more linking peptides.

[0106] In some embodiments, the multispecific activatable antibodies are designed to engage immune effector cells, also referred to herein as immune-effector cell engaging multispecific activatable antibodies. In some embodiments, the multispecific activatable antibodies are designed to engage leukocytes, also referred to herein as leukocyte engaging multispecific activatable antibodies. In some embodiments, the multispecific activatable antibodies are designed to engage T cells, also referred to herein as T-cell engaging multispecific activatable antibodies. In some embodiments, the multispecific activatable antibodies engage a surface antigen on a leukocyte, such as on a T cell, on a natural killer (NK) cell, on a myeloid mononuclear cell, on a macrophage, and/or on another immune effector cell. In some embodiments, the immune effector cell is a leukocyte. In some embodiments, the immune effector cell is a T cell. In some embodiments, the immune effector cell is a NK cell. In some embodiments, the immune effector cell is a mononuclear cell, such as a myeloid mononuclear cell. In some embodiments, the multispecific activatable antibodies are designed to bind or otherwise interact with more than one target and/or more than one epitope, also referred to herein as multi-antigen targeting activatable antibodies. As used herein, the terms "target" and "antigen" are used interchangeably.

[0107] In some embodiments, immune effector cell engaging multispecific activatable antibodies of the disclosure include a targeting antibody or antigen-binding fragment thereof and an immune effector cell engaging antibody or antigen-binding portion thereof, where at least one of the targeting antibody or antigen-binding fragment thereof and/or the immune effector cell engaging antibody or antigen-binding portion thereof is masked.

[0108] In some embodiments, the non-immune effector cell engaging antibody is a cancer targeting antibody. In some embodiments the non-immune cell effector antibody is an IgG. In some embodiments the immune effector cell engaging antibody is a scFv. In some embodiments the targeting antibody (e.g., non-immune cell effector antibody) is an IgG and the immune effector cell engaging antibody is a scFv. In some embodiments, the immune effector cell is a leukocyte. In some embodiments, the immune effector cell is a T cell. In some embodiments, the immune effector cell is a NK cell. In some embodiments, the immune effector cell is a myeloid mononuclear cell.

[0109] In some embodiments of an immune effector cell engaging multispecific activatable antibody, one antigen is typically an antigen present on the surface of a tumor cell or other cell type associated with disease, and another antigen is typically a stimulatory or inhibitory receptor present on the surface of a T-cell, natural killer (NK) cell, myeloid mononuclear cell, macrophage, and/or other immune effector cell.

[0110] One embodiment of the disclosure is a multispecific activatable antibody that is activatable in a cancer microenvironment and that includes an antibody, for example an IgG or scFv, directed to a tumor target and an agonist antibody, for example an IgG or scFv, directed to a co-stimulatory receptor expressed on the surface of an activated T cell or NK cell, wherein at least one of the cancer target antibody and/or agonist antibody is masked. In this embodiment, the multispecific activatable antibody, once activated by tumor-associated proteases, effectively crosslinks and activates the T cell or NK cell expressed co-stimulatory receptors in a tumor-dependent manner to enhance the activity of T cells that are responding to any tumor antigen via their endogenous T cell antigen or NK-activating receptors. The activation-dependent nature of these T cell or NK cell costimulatory receptors focuses the activity of the activated multispecific activatable antibody to tumor-specific T cells, without activating all T cells independent of their antigen specificity. In one embodiment, at least the co-stimulatory receptor antibody of the multispecific activatable antibody is masked to prevent activation of auto-reactive T cells that may be present in tissues that also express the antigen recognized by the tumor target-directed antibody in the multispecific activatable antibody, but whose activity is restricted by lack of co-receptor engagement.

[0111] One embodiment of the disclosure is a multispecific activatable antibody that is activatable in a disease characterized by T cell overstimulation, such as an autoimmune disease or inflammatory disease microenvironment. Such a multispecific activatable antibody

includes an antibody, for example an IgG or scFv, directed to a target comprising a surface antigen expressed in a tissue targeted by a T cell in autoimmune or inflammatory disease and an antibody, for example an IgG or scFv, directed to an inhibitory receptor expressed on the surface of a T cell or NK cell, wherein at least one of the disease tissue target antibody and/or T cell inhibitory receptor antibody is masked. Examples of a tissue antigen targeted by T cells in autoimmune disease include a surface antigen expressed on myelin or nerve cells in multiple sclerosis or a surface antigen expressed on pancreatic islet cells in Type 1 diabetes. In this embodiment, the multispecific activatable antibody when localized in the tissue under autoimmune attack or inflammation is activated and co-engages the T cell or NK cell inhibitory receptor to suppress the activity of autoreactive T cells responding to any disease tissue-targeted antigens via their endogenous TCR or activating receptors. In one embodiment, at least one or multiple antibodies are masked to prevent suppression of T cell responses in non-disease tissues where the target antigen may also be expressed.

[0112] In some embodiments, the multi-antigen targeting antibodies and/or multi-antigen targeting activatable antibodies include at least a first antibody or antigen-binding fragment thereof that binds a first target and/or first epitope and a second antibody or antigen-binding fragment thereof that binds a second target and/or a second epitope. In some embodiments, the multi-antigen targeting antibodies and/or multi-antigen targeting activatable antibodies bind two or more different targets. In some embodiments, the multi-antigen targeting antibodies and/or multi-antigen targeting activatable antibodies bind two or more different epitopes on the same target. In some embodiments, the multi-antigen targeting antibodies and/or multi-antigen targeting activatable antibodies bind a combination of two or more different targets and two or more different epitopes on the same target.

Masking Moieties (MMs)

[0113] The activatable molecules herein may comprise one or more masking moieties (MMs) capable of interfering with the binding of the AMs to the target. A masking moiety in an activatable molecule "masks" or reduces or otherwise inhibits the binding of the activatable molecule to its target. In some embodiments, the coupling of an AM (e.g., an antibody or fragment thereof, or other therapeutic or diagnostic protein) with an MM may inhibit the ability of the AM to specifically bind its target by means of inhibition known in the art (e.g., structural change, competition for antigen-binding domain, and the like). In some embodiments, the coupling of an AM with an MM may effect a structural change that reduces

or inhibits the ability of the AM to specifically bind its target. In some embodiments, the coupling of a protein comprising an AM with an MM sterically blocks, reduces or inhibits the ability of the AM to specifically bind its target and or epitope. In some embodiments, when an activatable molecule is not activated, the MM prevents the AM from target binding; but when the activatable molecule is activated (when the CM is cleaved by a protease), the MM does not substantially or significantly interfere with the AM's binding to the target.

[0114] An MM may be coupled to an AM (e.g., an antibody or fragment thereof, or other therapeutic or diagnostic protein) via the CM described herein, either directly or indirectly (e.g., via one or more linkers described herein). Alternatively, an MM interfering with the target binding of an AM may be coupled, either directly or indirectly, to a component of the activatable molecule that is not the AM. For example, the MM may be coupled, either directly or indirectly, to a different AM. In another example, the MM may be coupled, either directly or indirectly, with a half-life extending moiety (EM). In either case, in the tertiary or quaternary structure of the activatable structure, the MM may be in a position (e.g., proximal to the AM to be masked) that allows the MM to mask the AM.

[0115] In some embodiments, an MM interacts with the AM, thus reducing or inhibiting the interaction between the AM and its binding partner. In some embodiments, the MM comprises at least a partial or complete amino acid sequence of a naturally occurring binding partner of the AM. The term "naturally occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses or bacteria) that can be isolated from a source in nature and that has not been intentionally modified by man in the laboratory or otherwise is naturally occurring.

[0116] For example, the MM may be a fragment of a naturally occurring binding partner. The fragment may retain at least 95%, at least 90%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 40%, at least 30%, at least 25%, or at least 20% nucleic acid or amino acid sequence homology to the naturally occurring binding partner. In some embodiments, the MM is a cognate peptide of the AM. For example, the MM may comprise a sequence of the AM's epitope or a fragment thereof.

[0117] In some embodiments, the MM comprises an amino acid sequence that is not naturally occurring or does not contain the amino acid sequence of a naturally occurring binding partner or target protein. In certain embodiments, the MM is not a natural binding

partner of the AM. In some embodiments, the MM does not comprise a subsequence of more than 4, 5, 6, 7, 8, 9 or 10 consecutive amino acid residues of a natural binding partner of the AM. The MM may be a modified binding partner for the AM which contains amino acid changes that decrease affinity and/or avidity of binding to the AM. In some embodiments, the MM contains no or substantially no nucleic acid or amino acid homology to the AM's natural binding partner. In other embodiments the MM has no more than 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, or 80% homology to the natural binding partner of the AM.

[0118] In some embodiments, the MM is a polypeptide that binds to the AM. In some examples, the MM may be an antibody or antibody fragment (e.g., a Fab fragment, a $F(ab')_2$ fragment, a scFv, a scAb, a dAb, a single domain heavy chain antibody, and a single domain light chain antibody) that binds to the AM such that interrupts the AM's binding to its target. In some examples, the MM may be a ligand, a receptor, a fragment thereof (e.g., an extracellular domain of a receptor) of the AM that binds to the AM and interrupts the AM's binding to its target. In some examples, when the AM is an antibody or antibody fragment thereof, the MM may be an anti-idiotypic antibody or fragment thereof (e.g., scFv) that binds to the idiotype of the AM. In some examples, the MM may be a cytokine or a receptor for a cytokine. In some examples, the MM may have an amino acid sequence that is at least 85% identical to a cytokine or to a receptor for a cytokine.

[0119] In some embodiments, the MM does not bind the AM, but still interferes with AM's binding to its binding partner through non-specific interactions such as steric hindrance. For example, the MM may be positioned in the activatable molecule such that the tertiary or quaternary structure of the activatable molecule allows the MM to mask the AM through charge-based interaction, thereby holding the MM in place to interfere with binding partner access to the AM. Examples of such MMs include an albumin, e.g., human serum albumin (HSA), a fragment crystallizable (Fc) domain, an antibody constant domain (e.g., CH domains), a polymer (e.g., branched or multi-armed polyethylene glycol (PEG)), a latency associated protein (LAP), and any polypeptide or other moieties that sterically interfere AM-target interactions. In some examples, the MM may recruit a large protein binding partner that sterically interfere AM-target interactions. For example, the MM may be an antibody or a fragment thereof that binds to serum albumin.

[0120] Examples of suitable masking moieties include the full-length or a AM-binding fragment or mutein of a cognate receptor of the AM, and AM-binding antibodies and fragment thereof, e.g., a polyclonal antibody, a recombinant antibody, a human antibody, a humanized antibody a single chain variable fragment (scFv), single-domain antibody such as a heavy chain variable domain (VH), a light chain variable domain (VL), a variable domain of camelid-type nanobody (VHH), a dAb and the like. Other exemplary antigen-binding domain that bind the AM can also be used as an MM include non-immunoglobulin proteins that mimic antibody binding and/or structure such as, anticalins, affilins, affibody molecules, affimers, affitins, alphabodies, avimers, DARPins, fynomers, kunitz domain peptides, monobodies, and binding domains based on other engineered scaffolds such as SpA, GroEL, fibronectin, lipocallin and CTLA4 scaffolds. As another example, a peptide that is modified by conjugation to a water-soluble polymer, such as PEG, can sterically inhibit or prevent binding of the cytokine to its receptor. Antibodies and antigen-binding domains that bind to, for example, a protein with a long serum half- life such as HSA, immunoglobulin or transferrin, or to a receptor that is recycled to the plasma membrane, such as FcRn or transferrin receptor, can also inhibit the cytokine, particularly when bound to their antigen. In some embodiments, the MMs (e.g., those sterically interfere with the AM-target interaction) can also function as half-life extending elements.

[0121] In some embodiments, the MM may have a dissociation constant for binding to the AM that is no more than the dissociation constant of the AM to the target. In some embodiments, the MM does not interfere or compete with the AM for binding to the target in in the activated molecule (i.e., following cleavage of the CM by a protease).

[0122] The structural properties of the MMs may be selected according to factors such as the minimum amino acid sequence required for interference with the AM binding to target, the target protein-protein binding pair of interest, the size of the AM, the presence or absence of linkers, and the like.

[0123] In some embodiments, the MM may be unique for the coupled AM. Examples of MMs include MMs that were specifically screened to bind a binding domain of the AM or fragment thereof (e.g., affinity masks). Methods for screening MMs to obtain MMs unique for the AM and those that specifically and/or selectively bind a binding domain of a binding partner/target are provided herein and can include protein display methods.

[0124] As used herein, the term "masking efficiency" refers to the activity (e.g., EC_{50}) of the activatable molecule divided by the activity of a control molecule, wherein the control molecule may be either cleavage product of the activatable molecule (i.e., the activated molecule) or the AM used in the activatable molecule. An activatable molecule having a reduced level of an AM activity may have a masking efficiency that is greater than 10. In some embodiments, the activatable molecules described herein have a masking efficiency that is greater than 10, 100, 1000, or 5000.

[0125] In some embodiments, the MM is a polypeptide of about 2 to 50 amino acids in length. For example, the MM may be a polypeptide of from 2 to 40, from 2 to 30, from 2 to 20, from 2 to 10, from 5 to 15, from 10 to 20, from 15 to 25, from 20 to 30, from 25 to 35, from 30 to 40, from 35 to 45, from 40 to 50 amino acids in length. For example, the MM may be a polypeptide with 2, 3, 4, 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, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acids in length. In some examples, the MM may be a polypeptide of more than 50 amino acids in length, e.g., 100, 200, 300, 400, 500, 600, 700, 800, or more amino acids. In some embodiments, the MM is a steric mask.

[0126] In some embodiments, in an activatable molecule with an AM and an interfering MM, in the presence of the target of an AM, there is no binding or substantially no binding of the AM to the target, or no more than 0.001%, 0.01%, 0.1%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, or 50% binding of the AM to its target, as compared to the binding of an counterpart molecule without the interfering MM, for at least 0.1, 0.5, 1, 2, 4, 6, 8, 12, 28, 24, 30, 36, 48, 60, 72, 84, or 96 hours, or 5, 10, 15, 30, 45, 60, 90, 120, 150, or 180 days, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months when measured in vitro immunoabsorbant assay, e.g., as described in US20200308243A1.

[0127] The binding affinity of the AM towards the target or binding partner with an interfering MM may be at least 5, 10, 25, 50, 100, 250, 500, 1,000, 2,500, 5,000, 10,000, 50,000, 100,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000 times lower than the binding affinity of the AM towards its binding partner without an interfering MM, or between 5-10, 10-100, 10-1,000, 10-10,000, 10-100,000, 10-1,000,000, 10-10,000,000, 100-1,000, 100-10,000, 100-100,000, 100-1,000,000, 100-10,000,000, 1,000-10,000, 1,000-100,000, 1,000-1,000,000, 1000-10,000,000, 10,000-100,000, 10,000-1,000,000, 10,000-

10,000,000, 100,000-1,000,000, or 100,000-10,000,000 times lower than the binding affinity of the AM towards its binding partner when there is no interfering MM.

[0128] The dissociation constant (K_d) of the MM towards the AM it masks, may be greater than the dissociation constant of the AM towards the target. The dissociation constant of the MM towards the masked AM may be at least 5, 10, 25, 50, 100, 250, 500, 1,000, 2,500, 5,000, 10,000, 100,000, 1,000,000 or even 10,000,000 times greater than the dissociation constant of the AM towards the target. Conversely, the binding affinity of the MM towards the masked AM may be lower than the binding affinity of the AM towards the target. The binding affinity of MM towards the AM may be at least 5, 10, 25, 50, 100, 250, 500, 1,000, 2,500, 5,000, 10,000, 100,000, 1,000,000 or even 10,000,000 times lower than the binding affinity of the AM towards the target.

[0129] In some embodiments, the K_d of the activatable molecule comprising an MM and a CM towards the AM's target is at least 5, 10, 25, 50, 100, 250, 500, 1,000, 2,500, 5,000, 10,000, 50,000, 100,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000 or greater, or between 5-10, 10-100, 10-1,000, 10-10,000, 10-100,000, 10-1,000,000, 10-10,000,000, 100-1,000, 100-10,000, 100-100,000, 100-1,000,000, 100-10,000,000, 1,000-10,000, 1,000-100,000, 1,000-1,000,000, 1000-10,000,000, 10,000-100,000, 10,000-1,000,000, 10,000-10,000,000, 100,000-1,000,000, or 100,000-10,000,000 times greater than the K_d of a counterpart molecule that is substantially the same as the activatable molecule but does not comprise the MM or CM towards the AM's target. Conversely, the binding affinity of the activatable molecule comprising an MM and a CM towards the AM's target is at least 5, 10, 25, 50, 100, 250, 500, 1,000, 2,500, 5,000, 10,000, 50,000, 100,000, 500,000, 1,000,000, 5,000,000, 10,000,000, 50,000,000 or greater, or between 5-10, 10-100, 10-1,000, 10-10,000, 10-100,000, 10-1,000,000, 10-10,000,000, 100-1,000, 100-10,000, 100-100,000, 100-1,000,000, 100-10,000,000, 1,000-10,000, 1,000-100,000, 1,000-1,000,000, 1000-10,000,000, 10,000-100,000, 10,000-1,000,000, 10,000-10,000,000, 100,000-1,000,000, or 100,000-10,000,000 times lower than the binding affinity of a counterpart molecule that is substantially the same as the activatable molecule but does not comprise the MM or CM towards the AM's target.

[0130] In some embodiments, when the AM is coupled with an MM and is in the presence of the target, the specific binding of the AM to its target is reduced or inhibited, as compared to the specific binding of the AM not coupled with the MM. When compared to the binding

of the AM not coupled with the MM to the target, the target-binding ability of the AM coupled with the MM may be reduced by at least 50%, 60%, 70%, 80%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% for at least 2, 4, 6, 8, 12, 28, 24, 30, 36, 48, 60, 72, 84, or 96 hours, or 5, 10, 15, 30, 45, 60, 90, 120, 150, or 180 days, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months or more when measured *in vivo* or in an *in vitro* assay.

[0131] In some embodiments, the MM comprises a non-binding steric moiety (NB) that does not bind the AM but is able to interfere the binding between the AM and its target via steric hindrance. In some embodiments, the MM comprises a binding partner (BP) for a NB, where the BP recruits or otherwise attracts the NB to the activatable molecule.

[0132] In some embodiments, the MM contains genetically encoded or genetically non-encoded amino acid(s). Examples of genetically non-encoded amino acids include D-amino acids, β-amino acids, and γ-amino acids. In specific embodiments, the MMs contain no more than 50%, 40%, 30%, 20%, 15%, 10%, 5% or 1% of genetically non-encoded amino acids.

[0133] In some embodiments, once released from the activatable molecule and in a free state, the MM may have a biological activity or a therapeutic effect, such as binding capability. For example, the free peptide may bind with the same or a different binding partner. In certain embodiments, the free MM may exert a therapeutic effect, providing a secondary function to the compositions disclosed herein. In some embodiments, once uncoupled from the activatable molecule and in a free state, the MM may advantageously not exhibit biological activity. For example, in some embodiments the MM after cleavage from the activatable molecule does not elicit an immune response in the subject.

[0134] Suitable MMs may be identified and/or further optimized through a screening procedure from a library of candidate activatable molecule having variable MMs. For example, an AM and a CM may be selected to provide for a desired enzyme/target combination, and the amino acid sequence of the MM can be identified by the screening procedure described below to identify an MM that provides for an activatable phenotype. For example, a random peptide library (e.g., of peptides comprising 2 to 40 amino acids or more) may be used in the screening methods disclosed herein to identify a suitable MM.

[0135] In some embodiments, MMs with specific binding affinity for an AM may be identified through a screening procedure that includes providing a library of peptide scaffolds comprising candidate MMs wherein each scaffold is made up of a transmembrane protein and the candidate MM. The library may then be contacted with an entire or portion of a protein

such as a full length protein, a naturally occurring protein fragment, or a non-naturally occurring fragment containing a protein (also capable of binding the binding partner of interest), and identifying one or more candidate MMs having detectably bound protein. The screening may be performed by one more rounds of magnetic-activated sorting (MACS) or fluorescence-activated sorting (FACS), as well as determination of the binding affinity of MM towards the AM and subsequent determination of the masking efficiency, e.g., as described in WO2009025846 and US20200308243A1, which are incorporated herein by reference in their entireties.

[0136] Examples of suitable MMs are disclosed in WO2021207657, WO2021142029, WO2021061867, WO2020252349, WO2020252358, WO2020236679, WO2020176672, WO2020118109, WO2020092881, WO2020086665, WO2019213444, WO2019183218, WO2019173771, WO2019165143, WO2019075405, WO2019046652, WO2019018828, WO2019014586, WO2018222949, WO2018165619, WO2018085555, WO2017011580, WO2016179335, WO2016179285, WO2016179257, WO2016149201, and WO2016014974, which are incorporated herein by reference in their entireties.

[0137] In some embodiments, the AM in an activatable molecule is an antibody or antigen-binding fragment that specifically binds EGFR. In some examples, such an activatable molecule comprises an MM that comprises the amino acid sequence of SEQ ID NO: 81. In some examples, such an activatable molecule comprises an MM that comprises the amino acid sequence of SEQ ID NO: 82. In some examples, such an activatable molecule comprises an MM that consists of the amino acid sequence of SEQ ID NO: 81. In some examples, such an activatable molecule comprises an MM that consists of the amino acid sequence of SEQ ID NO: 82.

[0138] In some aspects, the present disclosure includes an activatable antibody comprising an anti-EGFR antibody coupled directly or indirectly to a CM, wherein the CM is directly or indirectly coupled to an MM that comprises or consists of the amino acid sequence of SEQ ID NO: 82.

Linkers

[0139] The activatable molecules may comprise one or more linkers. The linkers may be linking peptides that comprise a stretch of amino acid sequence that link two components in the activatable molecule. The linkers may be non-cleavable by any protease. In some embodiments, one or more linkers may be introduced into the activatable molecule to provide

flexibility at one or more of the junctions between domains, between moieties, between moieties and domains, or at any other junctions where a linker would be beneficial. In some embodiments, where the activatable molecule is provided as a conformationally constrained construct, a flexible linker may be inserted to facilitate formation and maintenance of a structure in the activatable molecule. Any of the linkers described herein may provide the desired flexibility to facilitate the inhibition of the binding of a target, or to facilitate cleavage of a CM by a protease. In some embodiments, linkers included in the activatable molecule may be all or partially flexible, such that the linker can include a flexible linker as well as one or more portions that confer less flexible structure to provide for a desired activatable molecule. Some linkers may include cysteine residues, which may form disulfide bonds and reduce flexibility of the construct.

[0140] In some embodiments, a linker coupled to an MM may have a length that allows the MM to be in a position in the tertiary or quaternary to effectively mask an AM, (e.g., proximal to the AM to be masked) that allows the MM to mask the AM.

[0141] In most instances, the linker's length may be determined by counting, in a N- to C- direction, the number of amino acids from the N-terminus of the linker adjacent to the C-terminal amino acid of the preceding component, to the C-terminus of the linker adjacent to the N-terminal amino acid of the following component (i.e., where the linker length does not include either the C-terminal amino acid of the preceding component or the N-terminal amino acid of the following component).

[0142] In some embodiments, a linker may include a total of 1 to 50, 1 to 40, 1 to 30, 1 to 25 (e.g., 1 to 24, 1 to 22, 1 to 20, 1 to 18, 1 to 16, 1 to 15, 1 to 14, 1 to 12, 1 to 10, 1 to 8, 1 to 6, 1 to 5, 1 to 4, 1 to 3, 1 to 2, 2 to 25, 2 to 24, 2 to 22, 2 to 20, 2 to 18, 2 to 16, 2 to 15, 2 to 14, 2 to 12, 2 to 10, 2 to 8, 2 to 6, 2 to 5, 2 to 4, 2 to 3, 4 to 25, 4 to 24, 4 to 22, 4 to 20, 4 to 18, 4 to 16, 4 to 15, 4 to 14, 4 to 12, 4 to 10, 4 to 8, 4 to 6, 4 to 5, 5 to 25, 5 to 24, 5 to 22, 5 to 20, 5 to 18, 5 to 16, 5 to 15, 5 to 14, 5 to 12, 5 to 10, 5 to 8, 5 to 6, 6 to 25, 6 to 24, 6 to 22, 6 to 20, 6 to 18, 6 to 16, 6 to 15, 6 to 14, 6 to 12, 6 to 10, 6 to 8, 8 to 25, 8 to 24, 8 to 22, 8 to 20, 8 to 18, 8 to 16, 8 to 15, 8 to 14, 8 to 12, 8 to 10, 10 to 25, 10 to 24, 10 to 22, 10 to 20, 10 to 18, 10 to 16, 10 to 15, 10 to 14, 10 to 12, 12 to 25, 12 to 24, 12 to 22, 12 to 20, 12 to 18, 12 to 16, 12 to 15, 12 to 14, 14 to 25, 14 to 24, 14 to 22, 14 to 20, 14 to 18, 14 to 16, 14 to 15, 15 to 25, 15 to 24, 15 to 22, 15 to 20, 15 to 18, 15 to 16, 16 to 25, 16 to 24, 16 to 22, 16 to 20, 16 to 18, 18 to 25, 18 to 24, 18 to 22, 18 to 20, 20 to 25, 20 to 24, 20 to 22, 22 to 25,

22 to 24, or 24 to 25 amino acids). In some embodiments, the linker may include a total of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acids.

[0143] In some embodiments, a linker may be rich in glycine (Gly or G) residues. In some embodiments, the linker may be rich in serine (Ser or S) residues. In some embodiments, the linker may be rich in glycine and serine residues. In some embodiments, the linker may have one or more glycine-serine residue pairs (GS) (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more GS pairs).

[0144] In some embodiments, the linker may have one or more Gly-Gly-Gly-Ser (GGGS) (SEQ ID NO: 102) sequences (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more GGGS (SEQ ID NO: 102) sequences). In some embodiments, the linker may have one or more Gly-Gly-Gly-Gly-Ser (GGGGS) (SEQ ID NO: 108) sequences (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more GGGGS (SEQ ID NO: 108) sequences). In some embodiments, the linker may have one or more Gly-Gly-Ser-Gly (GGSG) (SEQ ID NO: 95) sequences (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more GGSG (SEQ ID NO: 95) sequences). Examples of the linkers may include glycine polymers (G)n, glycine-serine polymers (including, for example, (GS)n, (GGS)n, (GSGGS)n (SEQ ID NO: 159) and (GGGS)n (SEQ ID NO: 102), where n is an integer of at least one), glycine-alanine polymers, alanine-serine polymers, and other flexible linkers known in the art. Glycine and glycine-serine polymers may be relatively unstructured, and therefore may be able to serve as a neutral link between components. Glycine accesses significantly more phi-psi space than even alanine, and is much less restricted than residues with longer side chains (see Scheraga, Rev. Computational Chem. 11173-142 (1992)). Exemplary flexible linkers include one of or a combination of one or more of: GGSG (SEQ ID NO: 95), GGSGG (SEQ ID NO: 96), GSGSG (SEQ ID NO: 97), GSGGG (SEQ ID NO: 98), GGGSG (SEQ ID NO: 99), GSSSG (SEQ ID NO: 100), GSSGGSGGSGG (SEQ ID NO: 101), GGGS (SEQ ID NO: 102), GGGSGGGS (SEQ ID NO: 103), GGGSGGGSGGGS (SEQ ID NO: 104), GGGGSGGGGSGGGGS (SEQ ID NO: 105), GGGGSGGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 106), GGGGSGGGGS (SEQ ID NO: 107), GGGGS (SEQ ID NO: 108), GS, GGGGSGS (SEQ ID NO: 109), GGGGSGGGGSGGGGSGS (SEQ ID NO: 110), GGSLDPKGGGGS (SEQ ID NO: 111), PKSCDKTHTCPPCPAPELLG (SEQ ID NO: 112), SKYGPPCPPCPAPEFLG (SEQ ID NO: 113), GKSSGSGSESKS (SEQ ID NO: 114), GSTSGSGKSSEGKG (SEQ ID NO: 115), GSTSGSGKSSEGSGSTKG (SEQ ID NO: 116), GSTSGSGKPGSGEGSTKG (SEQ ID NO: 117), GSTSGSGKPGSSEGST (SEQ ID NO:

118), GGGSSGGS (SEQ ID NO: 119), GGGGSGGGGSS (SEQ ID NO: 120), GGGSSGGSGGSSGGS (SEQ ID NO: 121), and GSTSGSGKPGSSEGST (SEQ ID NO: 122).

[0145] Examples of linkers may further include a sequence that is at least 70% identical (e.g., at least 72%, at least 74%, at least 75%, at least 76%, at least 78%, at least 80%, at least 82%, at least 84%, at least 85%, at least 86%, at least 88%, at least 90%, at least 92%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the example linkers described herein. An ordinarily skilled artisan will recognize that design of an activatable molecules can include linkers that are all or partially flexible, such that the linker can include a flexible linker as well as one or more portions that confer less flexible structure to provide for a desired activatable molecules structure.

[0146] In some embodiments, an activatable molecule may include one, two, three, four, five, six, seven, eight, nine, or ten linker sequence(s) (e.g., the same or different linker sequences of any of the exemplary linker sequences described herein or known in the art). In some embodiments, a linker may comprise sulfo-SIAB, SMPB, and sulfo-SMPB, wherein the linkers react with primary amines sulfhydryls.

Half-life extending moieties (EMs)

[0147] The activatable molecule may further comprise a half-life extending moiety (EM). In some examples, the half-life extending moiety may be a serum half-life extending moiety, i.e., capable of extending the serum half-life of the molecule attached to the EM.

[0148] In some examples, the EM may comprise a fragment crystallizable region (Fc domain) of an antibody. For example, the EM may be the Fc domain of an IgG (e.g., IgG1, IgG2, IgG3, or IgG4). In some examples, the EM may comprise a dimer formed by two Fc domains. The Fc domain may be a wild type peptide or a mutant. For example, the EM may comprise a dimer formed by two Fc domain mutants. In such cases, the two Fc domain mutants may be a Fc domain hole mutant and a Fc domain knob mutant. The knob and hole mutants may interact with each other to facilitate the dimerization of the two Fc domains. In some embodiments, the knob and hole mutants may comprise one or more amino acid modifications within the interface between two Fc domains (e.g., in the CH3 domain). In one example, the modifications comprise amino acid substitution T366W and optionally the amino acid substitution S354C in one IgG Fc domain and the amino acid substitutions T366S,

L368A, Y407V and optionally Y349C in the other IgG Fc domain (numbering according to EU numbering system). Example of Fc mutants also include SEQ ID NOs: 123-124.

[0149] Examples of the Fc domain mutants also include those described in U.S. Pat. Nos. 7,695,936, which is incorporated herein by reference in its entirety. In one example, the modifications comprise amino acid substitution T366Y in one IgG Fc domain, and the amino acid substitutions Y407T in the other IgG Fc domain. In one example, the modifications comprise amino acid substitution T366W in one IgG Fc domain, and the amino acid substitutions Y407A in the other IgG Fc domain. In one example, the modifications comprise amino acid substitution F405A in one IgG Fc domain, and the amino acid substitutions T394W in the other IgG Fc domain. In one example, the modifications comprise amino acid substitution T366Y and F405A in one IgG Fc domain, and the amino acid substitutions T394W and Y407T in the other IgG Fc domain. In one example, the modifications comprise amino acid substitution T366W and F405W in one IgG Fc domain, and the amino acid substitutions T394S and Y407A in the other IgG Fc domain. In one example, the modifications comprise amino acid substitution F405W and Y407A in one IgG Fc domain, and the amino acid substitutions T366W and T394S in the other IgG Fc domain. In one example, the modifications comprise amino acid substitution F405W in one IgG Fc domain, and the amino acid substitutions T394S in the other IgG Fc domain. The mutation positions in the Fc domains are numbered according to EU numbering system. The IgG Fc domain may be comprise a sequence of SEQ ID NOs: 125-128 (IgG1, IgG2, IgG3 or IgG4). In these sequences, amino acids 1-107 correspond to EU numbering 341-447.

[0150] In some examples, the Fc domains mutants may have reduced effector function. Examples of such Fc domains include those disclosed in in US20190135943, which incorporated herein by reference in its entirety.

[0151] Further examples of EMs include immunoglobulin (e.g., IgG), serum albumin (e.g., human serum albumin (HSA), hexa-hat GST (glutathione S-transferase) glutathione affinity, Calmodulin-binding peptide (CBP), Strep-tag, Cellulose Binding Domain, Maltose Binding Protein, S-Peptide Tag, Chitin Binding Tag, Immuno-reactive Epitopes, Epitope Tags, E2Tag, HA Epitope Tag, Myc Epitope, FLAG Epitope, AU1 and AU5 Epitopes, Glu-Glu Epitope, KT3 Epitope, IRS Epitope, Btag Epitope, Protein Kinase-C Epitope, and VSV Epitope.

[0152] In some embodiments, the serum half-life of an activatable molecule comprising an EM is longer than that of a counterpart molecule that is substantially the same as the activatable molecule but does not comprise the EM, e.g., the pK of the activatable molecule is longer than that of the reference molecule. In some examples, the activatable molecule with an EM may have a serum half-life that is at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 2-fold, 4-fold, 6-fold, 8-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold longer than the serum half-life of the reference counterpart molecule. In some embodiments, the serum half-life of the activatable molecule with an EM may be at least 15 days, 12 days, 11 days, 10 days, 9 days, 8 days, 7 days, 6 days, 5 days, 4 days, 3 days, 2 days, 1 day, 20 hours, 18 hours, 16 hours, 14 hours, 12 hours, 10 hours, 8 hours, 6 hours, 4 hours, 3 hours, 2 hours, or 1 hour when administered to an organism.

Conjugation Agents

[0153] In some aspects, the present disclosure provides conjugated polypeptides. In some embodiments, a conjugated polypeptide comprises a CM-containing polypeptide herein conjugated to one or more agent, e.g., a targeting moiety to facilitate delivery to a cell or tissue of interest, a therapeutic agent (e.g., an antineoplastic agent such as chemotherapeutic or anti-neoplastic agent), a toxin, or a fragment thereof. The agents may be conjugated to a component of the activatable molecules. In some embodiments, the conjugated polypeptide is an antibody-drug conjugate (ADC), which comprises an antibody or antigen-binding fragment thereof conjugated with a drug. In some examples, the antibody or antigen-binding fragment thereof may be conjugated with the drug via a CM disclosed herein. In some examples, the antibody or antigen-binding fragment thereof may be an activatable antibody or antigen-binding fragment thereof (e.g., coupled with a MM via a CM), which is further conjugated with a drug (e.g., via a cleavable or non-cleavable conjugating linker).

[0154] The term "agent" is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials. Examples of the agent include toxin, a microtubule inhibitor, a nucleic acid damaging agent, a dolastatin, an auristatin, a maytansinoid, a duocarmycin, a calicheamicin, or a combination thereof.

[0155] In some embodiments, the activatable molecule is conjugated to a cytotoxic agent, e.g., a toxin (e.g., an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof) or a radioactive isotope.

[0156] Examples of cytotoxic agents include that can be conjugated to the activatable molecules dolastatins and derivatives thereof (e.g., auristatin E, AFP, monomethyl auristatin D (MMAD), monomethyl auristatin F (MMAF), monomethyl auristatin E (MMAE), desmethyl auristatin E (DMAE), auristatin F, desmethyl auristatin F (DMAF), dolastatin 16 (DmJ), dolastatin 16 (Dpv), auristatin derivatives (e.g., auristatin tyramine, auristatin quinolone), maytansinoids (e.g., DM-1, DM-4), maytansinoid derivatives, duocarmycin, alpha-amanitin, turbostatin, phenstatin, hydroxyphenstatin, spongistatin 5, spongistatin 7, halistatin 1, halistatin 2, halistatin 3, halocomstatin, pyrrolobenzimidazoles (PBI), cibrostatin6, doxaliform, cemadotin analogue (CemCH2-SH), Pseudomonas toxin A (PES8) variant, Pseudomonase toxin A (ZZ-PE38) variant, ZJ-101, anthracycline, doxorubicin, daunorubicin, bryostatin, camptothecin, 7-substituted campothecin, 11-difluoromethylenedioxycamptothecin, combretastatins, debromoaplysiatoxin, KahaMide-F, discodermolide, and Ecteinascidins. In some embodiments, the agent is DM1 or DM4. In some embodiments, the agent is a duocarmycin or derivative thereof. In some embodiments, the agent is a calicheamicin or derivative thereof. In some embodiments, the agent is a pyrrolobenzodiazepine.

[0157] Examples of enzymatically active toxins that can be conjugated to the activatable molecules include diphtheria toxin, exotoxin A chain from Pseudomonas aeruginosa, ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleuriies fordii proteins, dianfhin proteins, Phytolaca Americana proteins (e.g., PAPI, PAPII, and PAP-8), momordica charantia inhibitor, curcin, crotirs, sapaonaria officinalis inhibitor, geionin, mitogeliin, restrictocin, phenomycin, neomycin, and tricothecenes. A variety of radionuclides are available for the production of radioconjugated molecules. Examples of radionuclides include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y, and ^{186}Re.

[0158] Examples of anti-neoplastics that can be conjugated to the activatable molecules include: adriamycin, cerubidine, bleomycin, alkeran, velban, oncovin, fluorouracil, methotrexate, thiotepa, bisantrene, novantrone, thioguanine, procarabizine, and cytarabine.

[0159] Examples of antivirals that can be conjugated to the activatable molecules include acyclovir, vira A, and symmetrel. Examples of antifungals that can be conjugated to the activatable molecules include: nystatin. Examples of detection reagents that can be conjugated to the activatable molecules include: fluorescein and derivatives thereof, fluorescein isothiocyanate (FITC). Examples of antibacterials that can be conjugated to the

activatable molecules include: aminoglycosides, streptomycin, neomycin, kanamycin, amikacin, gentamicin, and tobramycin. Examples of 3beta,16beta,17alpha-trihydroxycholest-5-en-22-one 16-O-(2-O-4-methoxybenzoyl-beta-D-xylopyranosyl)-(1-->3)-(2-O-acetyl-alpha-L-arabinopyranoside) (OSW-1) that can be conjugated to the activatable molecules include: s-nitrobenzyloxycarbonyl derivatives of O6-benzylguanine, toposisomerase inhibitors, hemiasterlin, cephalotaxine, homoharringionine, pyrrol obenzodiazepine dimers (PBDs), functionalized pyrrolobenzodiazepenes, calcicheamicins, podophyiitoxins, taxanes, and vinca alkoids. Examples of radiopharmaceuticals that can be conjugated to the activatable molecules include: ^{123}I , ^{89}Zr, ^{125}I, ^{131}I, ^{201}T1, ^{62}Cu, ^{18}F, ^{68}Ga, ^{13}N, ^{15}O, ^{38}K, ^{82}Rb, ^{111}In, ^{133}Xe, ^{11}C, and ^{99m}Tc (Technetium). Examples of heavy metals that can be conjugated to the activatable molecules include: barium, gold, and platinum. Examples of anti-mycoplasmals that can be conjugated to the activatable molecules include: tylosine, spectinomycin, streptomycin B, ampicillin, sulfanilamide, polymyxin, and chloramphenicol.

[0160] In some embodiments, the agent is a nucleic acid damaging agent, such as a DNA alkylator or DNA intercalator, or other DNA damaging agent.

[0161] Additional examples of the agents that can be conjugated include those in Table 1 below.

Table 1: Exemplary Pharmaceutical Agents for Conjugation

CYTOTOXIC AGENTS	
Auristatins	Turbostatin
Auristatin E	Phenstatins
Monomethyl auristatin D (MMAD)	Hydroxyphenstatin
Monomethyl auristatin E (MMAE)	Spongistatin 5
Desmethyl auristatin E (DMAE)	Spongistatin 7
Auristatin F	Halistatin 1
Monomethyl auristatin F (MMAF)	Halistatin 2
Desmethyl auristatin F (DMAF)	Halistatin 3
Auristatin derivatives, *e.g.*, amides thereof	Modified Bryostatins
Auristatin tyramine	Halocomstatins
Auristatin quinoline	Pyrrolobenzimidazoles (PBI)
Dolastatins	Cibrostatin6
Dolastatin derivatives	Doxaliform
Dolastatin 16 DmJ	Anthracyclins analogues
Dolastatin 16 Dpv	
Maytansinoids, *e.g.* DM-1; DM-4	
Maytansinoid derivatives	Cemadotin analogue (CemCH2-SH)
Duocarmycin	Pseudomonas toxin A (PE38) variant

Duocarmycin derivatives	Pseudomonas toxin A (ZZ-PE38) variant
Alpha-amanitin	ZJ-101
Anthracyclines	OSW-1
Doxorubicin	4-Nitrobenzyloxycarbonyl Derivatives of O6-Benzylguanine
Daunorubicin	Topoisomerase inhibitors
Bryostatins	Hemiasterlin
Camptothecin	Cephalotaxine
Camptothecin derivatives	Homoharringtonine
7-substituted Camptothecin	Pyrrolobenzodiazepine dimers (PBDs)
10, 11-Difluoromethylenedioxycamptothecin	Functionalized pyrrolobenzodiazepenes
Combretastatins	Calicheamicins
Debromoaplysiatoxin	Podophyllotoxins
Kahalalide-F	Taxanes
Discodermolide	Vinca alkaloids
Ecteinascidins	
	CONJUGATABLE DETECTION REAGENTS
ANTIVIRALS	Fluorescein and derivatives thereof
Acyclovir	Fluorescein isothiocyanate (FITC)
Vira A	
Symmetrel	**RADIOPHARMACEUTICALS**
	^{125}I
ANTIFUNGALS	^{131}I
Nystatin	^{89}Zr
	^{111}In
ADDITIONAL ANTI-NEOPLASTICS	^{123}I
Adriamycin	^{131}I
Cerubidine	^{99}mTc (Technetium)
Bleomycin	^{201}Tl
Alkeran	^{133}Xe
Velban	^{11}C
Oncovin	^{62}Cu
Fluorouracil	^{18}F
Methotrexate	^{68}Ga
Thiotepa	^{13}N
Bisantrene	^{15}O
Novantrone	^{38}K
Thioguanine	^{82}Rb
Procarabizine	
Cytarabine	
	HEAVY METALS
ANTI-BACTERIALS	Barium
Aminoglycosides	Gold
Streptomycin	Platinum

Neomycin	
Kanamycin	**ANTI-MYCOPLASMALS**
Amikacin	Tylosine
Gentamicin	Spectinomycin
Tobramycin	
Streptomycin B	Nanoparticles
Spectinomycin	
Ampicillin	
Sulfanilamide	
Polymyxin	
Chloramphenicol	

[0162] In some embodiments, the activatable molecule comprises a signal peptide. If comprising multiple polypeptides, the activatable molecule may comprise multiple signal peptides, e.g., one signal peptide for each of the multiple polypeptides. A signal peptide may be a peptide (e.g., 10-30 amino acids long) present at a terminus (e.g., the N-terminus or C-terminus) of a newly synthesized proteins that are destined toward the secretory pathway. In some embodiments, the signal peptide may be conjugated to the activatable molecule via a spacer. In some embodiments, the spacer may be conjugated to the activatable molecule in the absence of a signal peptide.

[0163] Those of ordinary skill in the art will recognize that a large variety of possible agents may be conjugated to any of the activatable molecules described herein. The agents may be conjugated to another component of the activatable molecule by a conjugating linker. Conjugation may include any chemical reaction that binds the two molecules so long as the activatable molecule and the other moiety retain their respective activities. Conjugation may include many chemical mechanisms, e.g., covalent binding, affinity binding, intercalation, coordinate binding, and complexation. In some embodiments, the binding may be covalent binding. Covalent binding may be achieved either by direct condensation of existing side chains or by the incorporation of external bridging molecules. Many bivalent or polyvalent linking agents may be useful in conjugating any of the activatable molecules described herein. For example, conjugation may include organic compounds, such as thioesters, carbodiimides, succinimide esters, glutaraldehyde, diazobenzenes, and hexamethylene diamines. In some embodiments, the activatable molecules may include, or otherwise introduce, one or more non-natural amino acid residues to provide suitable sites for conjugation.

[0164] In some embodiments, an agent is attached by disulfide bonds (e.g., disulfide bonds on a cysteine molecule) to the activatable molecule. Since many cancers naturally release high levels of glutathione, a reducing agent, glutathione present in the cancerous tissue microenvironment can reduce the disulfide bonds, and subsequently release the agent at the site of delivery.

[0165] In some embodiments, when the agent binds its target in the presence of complement within the target site (e.g., diseased tissue (e.g., cancerous tissue)), the amide or ester bond attaching the agent to the linker is cleaved, resulting in the release of the agent in its activated form. These agents when administered to a subject, may accomplish delivery and release of the agent at the target site (e.g., diseased tissue (e.g., cancerous tissue)). These agents may be effective for the in vivo delivery of any of the agents described herein.

[0166] In some embodiments, the one or more agents is conjugated to a component of the activatable molecule (e.g., AM) via a conjugating linker. The conjugating linker may be a peptide or chemical moiety linking the agent and the activatable molecule. In some examples, the conjugating linker may be cleavable (e.g., by an enzyme such as a protease). In some examples, the conjugating linker may be non-cleavable (e.g., cannot be cleaved by an enzyme such as a protease). In some embodiments, the conjugating linker may be non-cleavable by enzymes of the complement system. In some embodiments, two or more conjugating linkers are present. The two or more conjugating linkers may be the same, i.e., cleavable or non-cleavable. The two or more conjugating linkers may be different, i.e., at least one cleavable and at least one non-cleavable. For example, the agent may be released without complement activation since complement activation ultimately lyses the target cell. In such embodiments, the conjugate and/or agent is to be delivered to the target cell (e.g., hormones, enzymes, corticosteroids, neurotransmitters, or genes). Furthermore, the conjugating linker may be mildly susceptible to cleavage by serum proteases, and the conjugate and/or agent is released slowly at the target site.

[0167] In some embodiments, the agent is conjugated to a component of the activatable molecule via a maleimide caproyl-valine-citrulline linker or a maleimide PEG-valine-citrulline linker. In some embodiments, the agent is conjugated to a component of the activatable molecule via a maleimide caproyl-valine-citrulline linker. In some embodiments, the agent is conjugated to a component of the activatable molecule via a maleimide PEG-valine-citrulline linker. In some embodiments, the agent is monomethyl auristatin D

(MMAD) conjugated to a component of the activatable molecule via a maleimide PEG-valine-citrulline-para-aminobenzyloxycarbonyl linker, and this linker payload construct is vc-MMAD. In some embodiments, the agent is monomethyl auristatin E (MMAE) conjugated to a component of the activatable molecule via a maleimide PEG-valine-citrulline-para-aminobenzyloxycarbonyl linker, and this linker payload construct is vc-MMAE.

[0168] In some embodiments, the agent may be designed such that the agent is delivered to the target site (e.g., disease tissue (e.g., cancerous tissue)) but the conjugate and/or agent is not released.

[0169] In some embodiments, the agent may be attached to an AM either directly or via amino acids (e.g., D-amino acids), peptides, thiol-containing moieties, or other organic compounds that may be modified to include functional groups that can subsequently be utilized in attachment to AM by methods described herein.

[0170] In some embodiments, an activatable molecule includes at least one point of conjugation for an agent. In some embodiments, all possible points of conjugation are available for conjugation to an agent. In some embodiments, the one or more points of conjugation may include sulfur atoms involved in disulfide bonds, sulfur atoms involved in interchain disulfide bonds, sulfur atoms involved in interchain sulfide bonds but not sulfur atoms involved in intrachain disulfide bonds, and/or sulfur atoms of cysteine or other amino acid residues containing a sulfur atom. In such cases, residues may occur naturally in the protein construct structure or may be incorporated into the protein construct using methods including site-directed mutagenesis, chemical conversion, or mis-incorporation of non-natural amino acids.

[0171] The present disclosure also provides methods and materials for preparing an activatable molecule with one or more conjugated agents. In some embodiments, an activatable molecule may be modified to include one or more interchain disulfide bonds. For example, disulfide bonds may undergo reduction following exposure to a reducing agent such as, without limitation, TCEP, DTT, or β-mercaptoethanol. In some cases, the reduction of the disulfide bonds may be only partial. As used herein, the term partial reduction refers to situations where an activatable molecule is contacted with a reducing agent and a fraction of all possible sites of conjugation undergo reduction (e.g., not all disulfide bonds are reduced). In some embodiments, an activatable molecule may be partially reduced following contact with a reducing agent if less than 99%, (e.g., less than 98%, 97%, 96%, 95%, 90%, 85%,

80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10% or 5%) of all possible sites of conjugation are reduced. In some embodiments, the activatable molecule having a reduction in one or more interchain disulfide bonds may be conjugated to a drug reactive with free thiols.

[0172] The present disclosure also provides methods and materials for conjugating a therapeutic agent to a particular location on an activatable molecule. In some embodiments, an activatable molecule may be modified so that the therapeutic agents can be conjugated to the activatable molecule at particular locations on the activatable molecule. For example, an activatable molecule may be partially reduced in a manner that facilitates conjugation to the activatable molecule. In such cases, partial reduction of the activatable molecule may occur in a manner that conjugation sites in the activatable molecule are not reduced. In some embodiments, the conjugation site(s) on the activatable molecule may be selected to facilitate conjugation of an agent at a particular location on the protein construct. Various factors can influence the "level of reduction" of the activatable molecule upon treatment with a reducing agent. For example, without limitation, the ratio of reducing agent to activatable molecule, length of incubation, incubation temperature, and/or pH of the reducing reaction solution can require optimization in order to achieve partial reduction of the activatable molecule with the methods and materials described herein. Any appropriate combination of factors (e.g., ratio of reducing agent to activatable molecule, the length and temperature of incubation with reducing agent, and/or pH of reducing agent) may be used to achieve partial reduction of the activatable molecule (e.g., general reduction of possible conjugation sites or reduction at specific conjugation sites).

[0173] An effective ratio of reducing agent to activatable molecule can be any ratio that at least partially (i.e., partially or fully) reduces the activatable molecule in a manner that allows conjugation to an agent (e.g., general reduction of possible conjugation sites or reduction at specific conjugation sites). In some embodiments, the ratio of reducing agent to activatable molecule may be in a range from about 20:1 to 1:1, from 10:1 to 1:1, from 9:1 to 1:1, from 8:1 to 1:1, from 7:1 to 1:1, from 6:1 to 1:1, from 5:1 to 1:1, from 4:1 to 1:1, from 3:1 to 1:1, from 2:1 to 1:1, from 20:1 to 1:1.5, from 10:1 to 1:1.5, from 9:1 to 1:1.5, from 8:1 to 1:1.5, from 7:1 to 1:1.5, from 6:1 to 1:1.5, from 5:1 to 1:1.5, from 4:1 to 1:1.5, from 3:1 to 1:1.5, from 2:1 to 1:1.5, from 1.5:1 to 1:1.5, or from 1:1 to 1:1.5.

[0174] An effective incubation time and temperature for treating an activatable molecule with a reducing agent may be any time and temperature that at least partially reduces the activatable molecule in a manner that allows conjugation of an agent to an activatable molecule (e.g., general reduction of possible conjugation sites or reduction at specific conjugation sites). In some embodiments, the incubation time and temperature for treating an activatable molecule may be in a range from about 1 hour at 37 °C to about 12 hours at 37 °C (or any subranges therein).

[0175] An effective pH for a reduction reaction for treating an activatable molecule with a reducing agent can be any pH that at least partially reduces the activatable molecule in a manner that allows conjugation of the activatable molecule to an agent (e.g., general reduction of possible conjugation sites or reduction at specific conjugation sites).

[0176] When a partially-reduced activatable molecule is contacted with an agent containing thiols, the agent may conjugate to the interchain thiols in the activatable molecule. An agent can be modified in a manner to include thiols using a thiol-containing reagent (e.g., cysteine or N-acetyl cysteine). For example, the activatable molecule can be partially reduced following incubation with reducing agent (e.g., TEPC) for about 1 hour at about 37 °C at a desired ratio of reducing agent to activatable molecule. An effective ratio of reducing agent to activatable molecule may be any ratio that partially reduces at least two interchain disulfide bonds located in the activatable molecule in a manner that allows conjugation of a thiol-containing agent (e.g., general reduction of possible conjugation sites or reduction at specific conjugation sites).

[0177] In some embodiments, an activatable molecule may be reduced by a reducing agent in a manner that avoids reducing any intrachain disulfide bonds. In some embodiments of, an activatable molecule may be reduced by a reducing agent in a manner that avoids reducing any intrachain disulfide bonds and reduces at least one interchain disulfide bond.

[0178] In some embodiments, the agent may be a detectable moiety such as, for example, a label or other marker. For example, the agent may be or include a radiolabeled amino acid, one or more biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or calorimetric methods), one or more radioisotopes or radionuclides, one or more fluorescent labels, one or more enzymatic labels, and/or one or more chemiluminescent agents. In some embodiments, detectable moieties may be attached by spacer molecules. In some

embodiments, the detectable label may include an imaging agent, a contrasting agent, an enzyme, a fluorescent label, a chromophore, a dye, one or more metal ions, or a ligand-based label. In some embodiments, the imaging agent may comprise a radioisotope. In some embodiments, the radioisotope may be indium or technetium. In some embodiments, the contrasting agent may comprise iodine, gadolinium or iron oxide. In some embodiments, the enzyme may comprise horseradish peroxidase, alkaline phosphatase, or β-galactosidase. In some embodiments, the fluorescent label may comprise yellow fluorescent protein (YFP), cyan fluorescent protein (CFP), green fluorescent protein (GFP), modified red fluorescent protein (mRFP), red fluorescent protein tdimer2 (RFP tdimer2), HCRED, or a europium derivative. In some embodiments, the luminescent label may comprise an N- methylacrydium derivative. In some embodiments, the label may comprise an Alexa Fluor® label, such as Alex Fluor® 680 or Alexa Fluor® 750. In some embodiments, the ligand-based label may comprise biotin, avidin, streptavidin or one or more haptens.

[0179] Further examples of detectable labels also include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include 125I, 131I, 35S or 3H.

[0180] In some embodiments, the agent may be conjugated to the activatable molecule using a carbohydrate moiety, sulfhydryl group, amino group, or carboxylate group. In some embodiments, the agent may be conjugated to the activatable molecule via a linker and/or a CM described herein. In some embodiments, the agent may be conjugated to a cysteine or a lysine in the activatable molecule. In some embodiments, the agent may be conjugated to another residue of the activatable molecule, such as those residues disclosed herein.

[0181] In some embodiments, a variety of bifunctional protein-coupling agents may be used to conjugate the agent to the activatable molecule including N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters

(e.g., dimethyl adipimidate HCL), active esters (e.g., disuccinimidyl suberate), aldehydes (e.g., glutareldehyde), bis-azido compounds (e.g., bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (e.g., bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (e.g., tolyene 2,6-diisocyanate), and bis-active fluorine compounds (e.g., 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science 238: 1098 (1987). In some embodiments, a carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) chelating agent can be used to conjugate a radionucleotide to the activatable molecule. (See, e.g., WO94/11026).

[0182] Suitable conjugating linkers also include those described in the literature. (See, for example, Ramakrishnan, S. et al., Cancer Res. 44:201-208 (1984) describing use of MBS (M-maleimidobenzoyl-N-hydroxysuccinimide ester). See also, U.S. Patent No. 5,030,719, describing use of halogenated acetyl hydrazide derivative coupled to an activatable molecule by way of an oligopeptide. In some embodiments, suitable conjugating linkers include: (i) EDC (1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride; (ii) SMPT (4-succinimidyloxycarbonyl-alpha-methyl-alpha-(2-pridyl-dithio)-toluene (Pierce Chem. Co., Cat. (21558G); (iii) SPDP (succinimidyl-6 [3-(2-pyridyldithio) propionamido] hexanoate (Pierce Chem. Co., Cat #21651G); (iv) Sulfo-LC-SPDP (sulfosuccinimidyl 6 [3-(2-pyridyldithio)-propianamide] hexanoate (Pierce Chem. Co. Cat. #2165-G); and (v) sulfo-NHS (N-hydroxysulfo-succinimide: Pierce Chem. Co., Cat. #24510) conjugated to EDC. Additional example agents include SMCC, sulfo-SMCC, SPDB, and sulfo-SPDB.

[0183] Exemplary conjugating linkers for attachment to reduced activatable molecules include those having certain reactive groups capable of reaction with a sulfhydryl group of a reduced antibody or fragment. Such reactive groups include reactive haloalkyl groups (including, for example, haloacetyl groups), p-mercuribenzoate groups and groups capable of Michael-type addition reactions (including, for example, maleimides and groups of the type described by Mitra and Lawton, 1979, J. Amer. Chem. Soc. 101: 3097-3110).

[0184] Exemplary conjugating linkers for attachment to neither oxidized nor reduced activatable molecules include those having certain functional groups capable of reaction with the primary amino groups present in unmodified lysine residues in the activatable molecules. Such reactive groups include NHS carboxylic or carbonic esters, sulfo-NHS carboxylic or carbonic esters, 4-nitrophenyl carboxylic or carbonic esters, pentafluorophenyl carboxylic or

carbonic esters, acyl imidazoles, isocyanates, and isothiocyanates, and other dehydrating agents utilized for carboxamide formation. In these instances, the functional groups present in the suitable conjugating linkers include primary and secondary amines, hydrazines, hydroxylamines, and hydrazides.

[0185] The agent may be attached to the conjugating linker before or after the conjugating linker is attached to the activatable molecule. In certain applications it may be desirable to first produce an activatable molecule-conjugating linker intermediate in which the conjugating linker is free of an associated agent. Depending upon the particular application, a specific agent may then be covalently attached to the conjugating linker. In some embodiments, the AM is first attached to the MM, CM and associated linking peptides and then attached to the conjugating linker for conjugation purposes.

[0186] In specific embodiments, branched conjugating linkers that have multiple sites for attachment of agents are utilized. For multiple site conjugating linkers, a single covalent attachment to an activatable molecule may result in an activatable molecule-linker intermediate capable of binding an agent at a number of sites. The sites may be aldehyde or sulfhydryl groups or any chemical site to which agents can be attached.

[0187] In some embodiments, higher specific activity (or higher ratio of agents to activatable molecule) can be achieved by attachment of a single site conjugating linker at a plurality of sites on the activatable molecule. This plurality of sites may be introduced into the activatable molecule by either of two methods. First, one may generate multiple aldehyde groups and/or sulfhydryl groups in the same activatable molecule. Second, one may attach to an aldehyde or sulfhydryl of the activatable molecule a branched conjugating linker having multiple functional sites for subsequent attachment to conjugating linkers. The functional sites of the branched conjugating linker or multiple site conjugating linker may be aldehyde or sulfhydryl groups, or may be any chemical site to which conjugating linkers may be attached. Still higher specific activities may be obtained by combining these two approaches, that is, attaching multiple site conjugating linkers at several sites on the activatable molecule.

[0188] Peptide conjugating linkers that are susceptible to cleavage by enzymes of the complement system, such as but not limited to u-plasminogen activator, tissue plasminogen activator, trypsin, plasmin, or another enzyme having proteolytic activity may be used in one embodiment of the present disclosure. According to one method of the present disclosure, an agent is attached via a conjugating linker susceptible to cleavage by complement. The

antibody is selected from a class that can activate complement. The antibody-agent conjugate, thus, activates the complement cascade and releases the agent at the target site. According to another method of the present disclosure, an agent is attached via a conjugating linker susceptible to cleavage by enzymes having a proteolytic activity such as a u-plasminogen activator, a tissue plasminogen activator, plasmin, or trypsin. These cleavable conjugating linkers are useful in conjugated activatable molecules that include an extracellular toxin, *e.g.*, by way of non-limiting example, any of the extracellular toxins shown in Table 1.

[0189] Non-limiting examples of cleavable linker sequences include any cleavable sequence disclosed herein or incorporated herein by reference as well as the exemplary sequences provided in Table 2.

Table 2: Exemplary Conjugating Linker Sequences for Conjugation

Types of Cleavable Sequences	Amino Acid Sequence
Plasmin cleavable sequences	
Pro-urokinase	PRFKIIGG (SEQ ID NO: 129)
	PRFRIIGG (SEQ ID NO: 130)
TGFβ	SSRHRRALD (SEQ ID NO: 131)
Plasminogen	RKSSIIIRMRDVVL (SEQ ID NO: 132)
Staphylokinase	SSSFDKGKYKKGDDA (SEQ ID NO: 133)
	SSSFDKGKYKRGDDA (SEQ ID NO: 134)
Factor Xa cleavable sequences	IEGR (SEQ ID NO: 135)
	IDGR (SEQ ID NO: 136)
	GGSIDGR (SEQ ID NO: 137)
MMP cleavable sequences	
Gelatinase A	PLGLWA (SEQ ID NO: 138)
Collagenase cleavable sequences	
Calf skin collagen (α1(I) chain)	GPQGIAGQ (SEQ ID NO: 139)
Calf skin collagen (α2(I) chain)	GPQGLLGA (SEQ ID NO: 140)
Bovine cartilage collagen (α1(II) chain)	GIAGQ (SEQ ID NO: 141)
Human liver collagen (α1(III) chain)	GPLGIAGI (SEQ ID NO: 142)
Human α_2M	GPEGLRVG (SEQ ID NO: 143)
Human PZP	YGAGLGVV (SEQ ID NO:144)
	AGLGVVER (SEQ ID NO: 145)
	AGLGISST (SEQ ID NO: 146)
Rat α_1M	EPQALAMS (SEQ ID NO: 147)
	QALAMSAI (SEQ ID NO: 148)
Rat α_2M	AAYHLVSQ (SEQ ID NO: 149)
	MDAFLESS (SEQ ID NO: 150)
Rat α_1I_3(2J)	ESLPVVAV (SEQ ID NO: 151)

Rat α_1I_3(27J)	SAPAVESE (SEQ ID NO: 152)
Human fibroblast collagenase	DVAQFVLT (SEQ ID NO: 153)
(autolytic cleavages)	VAQFVLTE (SEQ ID NO: 154)
	AQFVLTEG (SEQ ID NO: 155)
	PVQPIGPQ (SEQ ID NO:156)

[0190] In addition, the agents may be attached via disulfide bonds (for example, the disulfide bonds on a cysteine molecule) to the activatable molecule. Since many tumors naturally release high levels of glutathione (a reducing agent) this can reduce the disulfide bonds with subsequent release of the agent at the site of delivery. In some embodiments, the reducing agent that would modify a CM would also modify the conjugating linker of the conjugated activatable molecule.

[0191] In some embodiments, it may be necessary to construct the conjugating linker in such a way as to optimize the spacing between the agent and the activatable molecule. This may be accomplished by use of a conjugating linker of the general structure:

$$W - (CH_2)n - Q$$

wherein

W is either -NH-CH_2- or -CH_2-;

Q is an amino acid, a polypeptide having between 2 to 20 amino acids; and

n is an integer from 0 to 20.

[0192] In some embodiments, the conjugating linker may comprise a spacer element and a cleavable element. The spacer element serves to position the cleavable element away from the core of the activatable molecule such that the cleavable element is more accessible to the enzyme responsible for cleavage. Certain of the branched linkers described above may serve as spacer elements.

[0193] Throughout this discussion, it should be understood that attachment of the conjugating linker to the agent (or of spacer element to cleavable element, or cleavable element to agent) need not be by a particular mode of attachment or reaction. Any reaction providing a product of suitable stability and biological compatibility is acceptable.

[0194] In some embodiments, when release of an agent is desired, an activatable molecule that is an antibody of a class that can activate complement is used. The resulting conjugate retains both the ability to bind antigen and activate the complement cascade. Thus, according to this embodiment of the present disclosure, an agent is joined to one end of the cleavable

conjugating linker or cleavable element and the other end of the conjugating linker group is attached to a specific site on the activatable molecule. For example, if the agent has a hydroxyl group or an amino group, it may be attached to the carboxyl terminus of a peptide, amino acid or other suitably chosen conjugating linker via an ester or amide bond, respectively. For example, such agents may be attached to the linker peptide via a carbodimide reaction. If the agent contains functional groups that would interfere with attachment to the conjugating linker, these interfering functional groups can be blocked before attachment and deblocked once the product conjugate or intermediate is made. The opposite or amino terminus of the linker is then used either directly or after further modification for binding to an activatable molecule that is capable of activating complement.

[0195] Conjugating linkers (or spacer elements of conjugating linkers) may be of any desired length, one end of which can be covalently attached to specific sites on the activatable molecule. The other end of the conjugating linker or spacer element may be attached to an amino acid or peptide conjugating linker.

[0196] Thus when these conjugates bind antigen in the presence of complement the amide or ester bond that attaches the agent to the linker will be cleaved, resulting in release of the agent in its active form. These conjugates, when administered to a subject, will accomplish delivery and release of the agent at the target site, and are particularly effective for the in vivo delivery of pharmaceutical agents, antibiotics, antimetabolites, antiproliferative agents and the like.

[0197] In some embodiments, release of the agent without complement activation is desired since activation of the complement cascade will ultimately lyse the target cell. Hence, this approach is useful when delivery and release of the agent should be accomplished without killing the target cell. Such is the goal when delivery of cell mediators such as hormones, enzymes, corticosteroids, neurotransmitters, genes or enzymes to target cells is desired. These conjugates may be prepared by attaching the agent to an activatable molecule that is not capable of activating complement via a linker that is mildly susceptible to cleavage by serum proteases. When this conjugate is administered to an individual, antigen-antibody complexes will form quickly whereas cleavage of the agent will occur slowly, thus resulting in release of the compound at the target site.

[0198] In some embodiments, the activatable molecule may be conjugated to one or more therapeutic agents using certain biochemical cross-linkers. Cross-linking reagents form

molecular bridges that tie together functional groups of two different molecules. To link two different proteins in a step-wise manner, hetero-bifunctional cross-linkers can be used that eliminate unwanted homopolymer formation.

[0199] Peptidyl conjugating linkers cleavable by lysosomal proteases are also useful, for example, Val-Cit, Val-Ala or other dipeptides. In addition, acid-labile conjugating linkers cleavable in the low-pH environment of the lysosome may be used, for example: bis-sialyl ether. Other suitable conjugating linkers include cathepsin-labile substrates, particularly those that show optimal function at an acidic pH.

[0200] Exemplary hetero-bifunctional cross-linkers are referenced in Table 3.

Table 3: Exemplary Hetero-Bifunctional Cross-Linkers

HETERO-BIFUNCTIONAL CROSS-LINKERS			
Linker	Reactive Toward	Advantages and Applications	Spacer Arm Length after cross-linking (Angstroms)
SMPT	Primary amines Sulfhydryls	Greater stability	11.2 Å
SPDP	Primary amines Sulfhydryls	Thiolation Cleavable cross-linking	6.8 Å
LC-SPDP	Primary amines Sulfhydryls	Extended spacer arm	15.6 Å
Sulfo-LC-SPDP	Primary amines Sulfhydryls	Extender spacer arm Water-soluble	15.6 Å
SMCC	Primary amines Sulfhydryls	Stable maleimide reactive group Enzyme-antibody conjugation Hapten-carrier protein conjugation	11.6 Å
Sulfo-SMCC	Primary amines Sulfhydryls	Stable maleimide reactive group Water-soluble Enzyme-antibody conjugation	11.6 Å
MBS	Primary amines Sulfhydryls	Enzyme-antibody conjugation Hapten-carrier protein conjugation	9.9 Å
Sulfo-MBS	Primary amines	Water-soluble	9.9 Å

SIAB	Sulfhydryls Primary amines	Enzyme-antibody conjugation	10.6 Å
Sulfo-SIAB	Sulfhydryls Primary amines	Water-soluble	10.6 Å
SMPB	Sulfhydryls Primary amines	Extended spacer arm	14.5 Å
Sulfo-SMPB	Sulfhydryls Primary amines	Enzyme-antibody conjugation Extended spacer arm	14.5 Å
EDE/Sulfo-NHS	Sulfhydryls Primary amines	Water-soluble Hapten-Carrier conjugation	0
ABH	Carboxyl groups Carbohydrates Nonselective	Reacts with sugar groups	11.9 Å

[0201] In some embodiments, the agent may be designed so that the agent is delivered to the target but not released. This may be accomplished by attaching an agent to an activatable molecule either directly or via a non-cleavable conjugating linker.

[0202] These non-cleavable conjugating linkers may include amino acids, peptides, D-amino acids or other organic compounds that may be modified to include functional groups that can subsequently be utilized in attachment to activatable molecules by the methods described herein.

[0203] In some embodiments, a compound may be attached to activatable molecules that do not activate complement. When using activatable molecules that are incapable of complement activation, this attachment may be accomplished using conjugating linkers that are susceptible to cleavage by activated complement or using linkers that are not susceptible to cleavage by activated complement.

[0204] The CM-containing polypeptides disclosed herein can also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., Proc. Natl. Acad. Sci. USA, 82: 3688 (1985); Hwang et al., Proc. Natl Acad. Sci. USA, 77: 4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556. Particularly useful liposomes can be generated by the reverse-phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol, and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of

defined pore size to yield liposomes with the desired diameter. A component of an activatable molecule can be conjugated to the liposomes as described in Martin et al., J. Biol. Chem., 257: 286-288 (1982) via a disulfide-interchange reaction.

[0205] The agents described above may contain components that have different attributes, thus leading to conjugates with differing physio-chemical properties. For example, sulfo-NHS esters of alkyl carboxylates are more stable than sulfo-NHS esters of aromatic carboxylates. NHS-ester containing linkers are less soluble than sulfo-NHS esters. Further, the SMPT contains a sterically-hindered disulfide bond, and can form conjugates with increased stability. Disulfide linkages, are in general, less stable than other linkages because the disulfide linkage is cleaved in vitro, resulting in less conjugate available. Sulfo-NHS, in particular, can enhance the stability of carbodimide couplings. Carbodimide couplings (such as EDC) when used in conjunction with sulfo-NHS, forms esters that are more resistant to hydrolysis than the carbodimide coupling reaction alone.

[0206] Those of ordinary skill in the art will recognize that a large variety of possible agents can be conjugated to the activatable molecule of the disclosure. (See, for example, "Conjugate Vaccines", Contributions to Microbiology and Immunology, J. M. Cruse and R. E. Lewis, Jr (eds), Carger Press, New York, (1989), the entire contents of which are incorporated herein by reference). In general, an effective conjugation of an agent (e.g., cytotoxic agent) to an activatable molecule can be accomplished by any chemical reaction that will bind the agent to the activatable molecule while also allowing the agent and the activatable molecule to retain functionality.

NUCLEIC ACIDS AND VECTORS

[0207] In some aspects, the present disclosure further provides nucleic acids comprising sequences that encode the CM-containing polypeptides and polypeptide complexes (e.g., activatable molecules) herein, or components or fragment thereof. The nucleic acids may comprise coding sequences for the CMs. The nucleic acids may further comprise coding sequences for other components in an activatable molecule, e.g., the AMs, the MMs, the EM and/or the linker(s). In cases where the activatable molecule comprises multiple polypeptides, the nucleic acids may comprise coding sequences for the multiple polypeptides. In some examples, the coding sequence for one of the polypeptides is comprised in a nucleic acid molecule, and the coding sequence for another one of the polypeptides is comprised in another

nucleic acid molecule. In some examples, the coding sequences for two or more of the multiple polypeptides are comprised in the same nucleic acid molecule.

[0208] Unless otherwise specified, a "nucleic acid sequence encoding a protein" includes all nucleotide sequences that are degenerate versions of each other and thus encode the same amino acid sequence. The term "nucleic acid" refers to a deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), or a combination thereof, in either a single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses complementary sequences as well as the sequence explicitly indicated. In some embodiments, the nucleic acid is DNA. In some embodiments, the nucleic acid is RNA.

[0209] Modifications may be introduced into a nucleotide sequence by standard techniques known in the art, such as site-directed mutagenesis and polymerase chain reaction (PCR)-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include: amino acids with acidic side chains (e.g., aspartate and glutamate), amino acids with basic side chains (e.g., lysine, arginine, and histidine), non-polar amino acids (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, and tryptophan), uncharged polar amino acids (e.g., glycine, asparagine, glutamine, cysteine, serine, threonine and tyrosine), hydrophilic amino acids (e.g., arginine, asparagine, aspartate, glutamine, glutamate, histidine, lysine, serine, and threonine), hydrophobic amino acids (e.g., alanine, cysteine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine, and valine). Other families of amino acids include: aliphatic-hydroxy amino acids (e.g., serine and threonine), amide family (e.g., asparagine and glutamine), alphatic family (e.g., alanine, valine, leucine and isoleucine), and aromatic family (e.g., phenylalanine, tryptophan, and tyrosine).

[0210] The present disclosure further provides vectors and sets of vectors comprising any of the nucleic acids described herein. One skilled in the art will be capable of selecting suitable vectors or sets of vectors (e.g., expression vectors) for making any of the activatable molecules described herein, and using the vectors or sets of vectors to express any of the activatable molecules described herein. For example, in selecting a vector or a set of vectors,

the type of cell may be selected such that the vector(s) may need to be able to integrate into a chromosome of the cell and/or replicate in it. Example vectors that can be used to produce an activatable molecule are also described herein. As used herein, the term "vector" refers to a polynucleotide capable of inducing the expression of a recombinant protein (e.g., a first or second monomer) in a cell (e.g., any of the cells described herein). A "vector" is able to deliver nucleic acids and fragments thereof into a host cell, and includes regulatory sequences (e.g., promoter, enhancer, poly(A) signal). Exogenous polynucleotides may be inserted into the expression vector in order to be expressed. The term "vector" also includes artificial chromosomes, plasmids, retroviruses, and baculovirus vectors.

[0211] Methods for constructing suitable vectors that comprise any of the nucleic acids described herein, and suitable for transforming cells (e.g., mammalian cells) are well-known in the art. See, e.g., Sambrook et al., Eds. "Molecular Cloning: A Laboratory Manual," 2nd Ed., Cold Spring Harbor Press, 1989 and Ausubel et al., Eds. "Current Protocols in Molecular Biology," Current Protocols, 1993.

[0212] Examples of vectors include plasmids, transposons, cosmids, and viral vectors (e.g., any adenoviral vectors (e.g., pSV or pCMV vectors), adeno-associated virus (AAV) vectors, lentivirus vectors, and retroviral vectors), and any Gateway® vectors. A vector may, for example, include sufficient cis-acting elements for expression; other elements for expression may be supplied by the host mammalian cell or in an in vitro expression system. Skilled practitioners will be capable of selecting suitable vectors and mammalian cells for making any activatable molecule described herein.

[0213] In some embodiments, the CM-containing polypeptides may be made biosynthetically using recombinant DNA technology and expression in eukaryotic or prokaryotic species.

CELLS

[0214] In some aspects, the present disclosure provides recombinant host cells comprising any of the vectors or nucleic acids described herein. The cells may be used to produce the CM-containing polypeptides (e.g., activatable molecules) described herein. In some embodiments, the cell may be an animal cell, a mammalian cell (e.g., a human cell), a rodent cell (e.g., a mouse cell, a rat cell, a hamster cell, or a guinea pig cell), a non-human primate cell, an insect cell, a bacterial cell, a fungal cell, or a plant cell. In some embodiments, the cell may be a eukaryotic cell. As used herein, the term "eukaryotic cell" refers to a cell

having a distinct, membrane-bound nucleus. Such cells may include, for example, mammalian (e.g., rodent, non-human primate, or human), insect, fungal, or plant cells. In some embodiments, the eukaryotic cell is a yeast cell, such as Saccharomyces cerevisiae. In some embodiments, the eukaryotic cell is a higher eukaryote, such as mammalian, avian, plant, or insect cells. Non-limiting examples of mammalian cells include Chinese hamster ovary (CHO) cells and human embryonic kidney cells (e.g., HEK293 cells). In some embodiments, the cell may be a prokaryotic cell, e.g., an E coli cell.

[0215] Methods of introducing nucleic acids and vectors (e.g., any of the vectors or any of the sets of vectors described herein) into a cell are known in the art. Examples of methods that can be used to introducing a nucleic acid into a cell include: lipofection, transfection, calcium phosphate transfection, cationic polymer transfection, viral transduction (e.g., adenoviral transduction, lentiviral transduction), nanoparticle transfection, and electroporation.

[0216] In some embodiments, the introducing step includes introducing into a cell a vector (e.g., any of the vectors or sets of vectors described herein) including a nucleic acid encoding the monomers that make up any activatable molecule described herein.

COMPOSITIONS AND KITS

[0217] The present disclosure also provides compositions and kits comprising the CM-containing polypeptides (e.g., activatable molecules or conjugated polypeptides) described herein. The compositions and kits may further comprise one or more excipients, carriers, reagents, instructions needed for the use of the activatable molecules.

[0218] In some embodiments, the compositions may be pharmaceutical compositions, which comprise the CM-containing polypeptides, derivatives, fragments, analogs and homologs thereof. The pharmaceutical compositions may comprise the CM-containing and a pharmaceutically acceptable carrier. As used herein, the term "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field, which is incorporated herein by reference. Suitable examples of such carriers or diluents include water, saline, ringer's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents

for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

[0219] A pharmaceutical composition may be formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (e.g., topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application may include one or more of the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH may be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. In some, any of the activatable molecules described herein are prepared with carriers that protect against rapid elimination from the body, e.g., sustained and controlled release formulations, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, e.g., ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, polylactic-co-glycolic acid, and polylactic acid. Methods for preparation of such pharmaceutical compositions and formulations are apparent to those skilled in the art. For example, the activatable molecules may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles, and nanocapsules) or in macroemulsions.

[0220] Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the CM-containing polypeptides, which matrices are in the form of shaped articles,

e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides, copolymers of L-glutamic acid and y ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers (e.g., injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods.

[0221] In some embodiments, pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor® EL (CAS No. 61791-12-6) (BASF, Parsippany, N.J.), which is a mixture of polyoxyethylated triglycerides, by reacting castor oil with ethylene oxide in a molar ratio of 1 : 35, that acts as a nonionic surfactant, or phosphate buffered saline (PBS). The composition may be sterile and should be fluid and of a viscosity that facilitates easy syringeability. It may be stable under the conditions of manufacture and storage and preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier may be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. For dispersed particulate compositions, the proper fluidity can be maintained, for example, by the use of a coating on the particles such as lecithin, and by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In some embodiments, the pharmaceutical compositions may further comprise one or more antibacterial and/or antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In some embodiments, isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, and the like, as well as salts, such as, for example, sodium chloride and the like may be included in the composition. Prolonged absorption of the injectable compositions may be brought about by including in the composition an agent that delays absorption, for example, aluminum monostearate and gelatin.

[0222] In some embodiments, the pharmaceutical composition may comprise a sterile injectable solution. Sterile injectable solutions may be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions may be prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0223] In some embodiments, the pharmaceutical composition may comprise an oral composition. Oral compositions may include an inert diluent or an edible carrier. They may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions may also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials may be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primojel®(sodium starch glycolate), or corn starch; a lubricant such as magnesium stearate; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

[0224] In some embodiments, the pharmaceutical composition may be formulized for administration by inhalation. For example, the compounds may be delivered in the form of an aerosol spray from pressured container or dispenser that contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

[0225] In some embodiments, the pharmaceutical composition may be formulized for systemic administration. For example, systemic administration may be by intravenous, as well by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated may be used in the formulation. Such

penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration may be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds may be formulated into ointments, salves, gels, or creams as generally known in the art.

[0226] In some embodiments, the pharmaceutical composition may be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

[0227] In one embodiment, the pharmaceutical composition may be prepared with carriers that protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers may be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, polylactic-co-glycolic acid and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art.

[0228] It may be advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the disclosure may be dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

[0229] In some embodiments, the compositions (e.g., pharmaceutical compositions) may be included in a container, vial, syringe, injector pen, pack, or dispenser, optionally together with instructions for administration.

[0230] Also provided herein are kits that include any of the CM-containing polypeptides (e.g., activatable molecules or conjugated polypeptides) described herein, any of the compositions that include any of the polypeptides described herein, or any of the pharmaceutical compositions that include any of the polypeptides described herein. Also provided are kits that include one or more second therapeutic agent(s) in addition to a

polypeptide described herein. The second therapeutic agent(s) may be provided in a dosage administration form that is separate from the polypeptides herein. Alternatively, the second therapeutic agent(s) may be formulated together with the polypeptides herein.

[0231] Any of the kits described herein can include instructions for using any of the compositions (e.g., pharmaceutical compositions) and/or any of the CM-containing polypeptides (e.g., activatable molecules or conjugated polypeptides) described herein. In some embodiments, the kits can include instructions for performing any of the methods described herein. In some embodiments, the kits can include at least one dose of any of the compositions (e.g., pharmaceutical compositions) described herein. In some embodiments, the kits can provide a syringe for administering any of the pharmaceutical compositions described herein.

[0232] Also provided herein are CM-containing polypeptides (e.g., activatable molecules or conjugated polypeptides) produced by any of the methods described herein. Also provided are compositions (e.g., pharmaceutical compositions) that comprise any of the polypeptides produced by any of the methods described herein. Also provided herein are kits that include at least one dose of any of the compositions (e.g., pharmaceutical compositions) described herein.

METHODS OF PRODUCING CM-CONTAINING POLYPEPTIDES

[0233] Provided herein are methods of producing the CM-containing polypeptides (e.g., activatable molecules or conjugated polypeptides) described herein that include: (a) culturing any of the recombinant host cells described herein in a liquid culture medium under conditions sufficient to produce the CM-containing polypeptides; and (b) recovering the CM-containing polypeptides from the host cell and/or the liquid culture medium.

[0234] Methods of culturing cells are well known in the art. In some embodiments, cells may be maintained in vitro under conditions that favor cell proliferation, cell differentiation and cell growth. For example, the recombinant cells may be cultured by contacting a cell (e.g., any of the cells described herein) with a cell culture medium that includes the necessary growth factors and supplements sufficient to support cell viability and growth.

[0235] In some embodiments, the method may further include isolating the recovered CM-containing polypeptides (e.g., activatable molecules or conjugated polypeptides). The isolation of the CM-containing polypeptides may be performed using any separation or purification technique for separating protein species, e.g., affinity tag-based protein

purification (e.g., polyhistidine (His) tag, glutathione-S-transferase tag, and the like), ammonium sulfate precipitation, polyethylene glycol precipitation, size exclusion chromatography, ligand-affinity chromatography (e.g., Protein A chromatography), ion-exchange chromatography (e.g., anion or cation), hydrophobic interaction chromatography, and the like.

[0236] Compositions and methods described herein may involve use of non-reducing or partially-reducing conditions that allow disulfide bonds to form between the MM and the AM of the activatable molecules.

[0237] In some embodiments, the method further includes formulating the isolated polypeptides into a pharmaceutical composition. Various formulations are known in the art and are described herein. Any isolated polypeptides described herein can be formulated for any route of administration (e.g., intravenous, intratumoral, subcutaneous, intradermal, oral (e.g., inhalation), transdermal (e.g., topical), transmucosal, or intramuscular).

METHODS OF USING CM-CONTAINING POLYPEPTIDES

[0238] In some aspects, the present disclosure further provides methods of using the CM-containing polypeptides herein. In some embodiments, the present disclosure provides methods of the treating a disease (e.g., a cancer (e.g., any of the cancers described herein)) in a subject including administering a therapeutically effective amount of any of the polypeptides (e.g., activatable molecules or conjugated polypeptides) described herein to the subject. In some embodiments, the disclosure provides methods of preventing, delaying the progression of, treating, alleviating a symptom of, or otherwise ameliorating disease in a subject by administering a therapeutically effective amount of an polypeptides (e.g., activatable molecules or conjugated polypeptides) described herein to a subject in need thereof. The term "treatment" refers to ameliorating at least one symptom of a disorder. In some embodiments, the disorder being treated may be a cancer or autoimmune disease or to ameliorate at least one symptom of a cancer or autoimmune disease. As used herein, the term "subject" refers to any mammal. In some embodiments, the subject is a feline (e.g., a cat), a canine (e.g., a dog), an equine (e.g., a horse), a rabbit, a pig, a rodent (e.g., a mouse, a rat, a hamster or a guinea pig), a non-human primate (e.g., a simian (e.g., a monkey (e.g., a baboon, a marmoset), or an ape (e.g., a chimpanzee, a gorilla, an orangutan, or a gibbon)), or a human. In some embodiments, the subject is a human. The terms subject and patient are used

interchangeably herein. In some embodiments, the subject has been previously identified or diagnosed as having the disease (e.g., cancer (e.g., any of the cancers described herein)).

[0239] A therapeutically effective amount of a CM-containing polypeptide (e.g., activatable molecule or conjugated polypeptide) of the disclosure relates generally to the amount needed to achieve a therapeutic objective. As noted above, this may be a binding interaction between the AM and its target that, in certain cases, interferes with the functioning of the targets. The amount required to be administered will furthermore depend on the binding affinity of the polypeptides for its specific target, and will also depend on the rate at which an administered polypeptide is depleted from the free volume other subject to which it is administered. Common ranges for therapeutically effective dosing of a polypeptides of the disclosure may be, by way of non-limiting example, from about 0.001, 0.01, 0.1, 0.3, 0.5, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 mg/kg body weight or higher. The structure of the polypeptides of the present disclosure makes it possible to reduce the dosage of the polypeptide that is administered to a subject compared to conventional activatable molecules and compared to conventional antibodies. For example, the administered dose on a unit dosage basis or total dosage over a dosage regimen period may be reduced by 10, 20, 30, 40, or 50% compared to the corresponding dose of a corresponding conventional therapeutic molecules.

[0240] Common dosing frequencies may range, for example, from once or twice daily, weekly, biweekly, or monthly.

[0241] Efficaciousness of treatment is determined in association with any known method for diagnosing or treating the particular disorder. Methods for the screening of polypeptides that possess the desired specificity include, but are not limited to, enzyme linked immunosorbent assay (ELISA) and other immunologically mediated techniques known within the art.

[0242] In another embodiment, a polypeptide directed two or more targets are used in methods known within the art relating to the localization and/or quantitation of the targets (e.g., for use in measuring levels of one or more of the targets within appropriate physiological samples, for use in diagnostic methods, for use in imaging the protein, and the like). In a given embodiment, a polypeptide directed two or more targets, or a derivative, fragment, analog or homolog thereof, that contain the antibody derived antigen binding domain, are utilized as pharmacologically active compounds (referred to hereinafter as "Therapeutics").

[0243] The CM-containing polypeptides used in any of the embodiments of these methods and uses may be administered at any stage of the disease. For example, such a polypeptide may be administered to a patient suffering cancer of any stage, from early to metastatic. In some embodiments, the CM-containing polypeptides and formulations thereof may be administered to a subject suffering from or susceptible to a disease or disorder associated with aberrant target expression and/or activity.

[0244] A subject suffering from or susceptible to a disease or disorder associated with aberrant target expression and/or activity may be identified using any of a variety of methods known in the art. For example, subjects suffering from cancer or other neoplastic condition may be identified using any of a variety of clinical and/or laboratory tests such as, physical examination and blood, urine and/or stool analysis to evaluate health status. For example, subjects suffering from inflammation and/or an inflammatory disorder may be identified using any of a variety of clinical and/or laboratory tests such as physical examination and/or bodily fluid analysis, e.g., blood, urine and/or stool analysis, to evaluate health status.

[0245] In some embodiments, administration of a polypeptide to a patient suffering from a disease or disorder associated with aberrant target expression and/or activity may be considered successful if any of a variety of laboratory or clinical objectives is achieved. For example, administration of a polypeptide to a patient suffering from a disease or disorder associated with aberrant target expression and/or activity may be considered successful if one or more of the symptoms associated with the disease or disorder is alleviated, reduced, inhibited or does not progress to a further, i.e., worse, state. Administration of a polypeptide to a patient suffering from a disease or disorder associated with aberrant target expression and/or activity may be considered successful if the disease or disorder enters remission or does not progress to a further, i.e., worse, state.

[0246] As used herein, the term "treat" includes reducing the severity, frequency or the number of one or more (e.g., 1, 2, 3, 4, or 5) symptoms or signs of a disease (e.g., a cancer (e.g., any of the cancers described herein)) in the subject (e.g., any of the subjects described herein). In some embodiments where the disease is cancer, treating results in reducing cancer growth, inhibiting cancer progression, inhibiting cancer metastasis, or reducing the risk of cancer recurrence in a subject having cancer.

[0247] In some embodiments, the CM comprises a substrate for a protease that is active, *e.g.*, upregulated or otherwise unregulated, in a disease condition or diseased tissue.

Exemplary disease conditions include, for example, a cancer (*e.g.*, where the diseased tissue is a tumor tissue) and an inflammatory or autoimmune condition (*e.g.*, where the diseased tissue is inflamed tissue). In some embodiments, the CM comprises a substrate for an extracellular protease. In some embodiments, the CM comprises a substrate for an intracellular protease. In some embodiments, the CM is a substrate for an intracellular protease and an extracellular protease. In some embodiments, the disease may be a cancer. In some embodiments, the subject may have been identified or diagnosed as having a cancer. Examples of cancer include: solid tumor, hematological tumor, sarcoma, a leukemia (e.g., hairy cell leukemia, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), stomach cancer, urothelial carcinoma, lung cancer, renal cell carcinoma, gastric and esophageal cancer, pancreatic cancer, prostate cancer, brain cancer, colon cancer, bone cancer, lung cancer, breast cancer, colorectal cancer, ovarian cancer, non-small cell lung carcinoma (NSCLC), squamous cell head and neck carcinoma, endometrial cancer, bladder cancer, cervical cancer, and liver cancer. Metastases of the aforementioned cancers may also be treated or prevented in accordance with the methods described herein.

[0248] In some embodiments, the disease may be an autoimmune disease or condition. In some embodiments, the subject may have been identified or diagnosed as having an autoimmune disease or condition or is at heightened risk of developing an autoimmune disease or condition. Examples of autoimmune diseases include Type 1 diabetes, Rheumatoid arthritis (RA), Psoriasis/psoriatic arthritis, Multiple sclerosis, Systemic lupus erythematosus, Inflammatory bowel disease (e.g., Crohn's disease, ulcerative colitis), chronic inflammation, or transplant rejection (e.g., in kidney, liver, or heart transplantation), autoimmune diseases, infectious disease, chronic inflammation, or transplant rejection. In some embodiments, the disease is a cardiovascular disorder. In some embodiments, the disease is a neurodegenerative disorder.

[0249] In some embodiments, the methods herein may result in a reduction in the number, severity, or frequency of one or more symptoms of the cancer in the subject (e.g., as compared to the number, severity, or frequency of the one or more symptoms of the cancer in the subject prior to treatment).

[0250] The methods may further comprise administering to a subject one or more additional agents. In some embodiments, the CM-containing polypeptides (e.g., activatable

molecules or conjugated polypeptides) may be administered during and/or after treatment in combination with one or more additional agents. In some embodiments, the polypeptide may be formulated into a single therapeutic composition, and the polypeptide and additional agent(s) may be administered simultaneously. Alternatively, the polypeptide and additional agent(s) may be separate from each other, e.g., each is formulated into a separate therapeutic composition, and the polypeptide and the additional agent are administered simultaneously, or the polypeptide and the additional agent are administered at different times during a treatment regimen. For example, the polypeptide may be administered prior to the administration of the additional agent, subsequent to the administration of the additional agent, or in an alternating fashion. The polypeptide and additional agent(s) may be administered in single doses or in multiple doses.

[0251] One of more of the polypeptides herein may be co-formulated with, and/or co-administered with, one or more anti-inflammatory drugs, immunosuppressants, or metabolic or enzymatic inhibitors. In some embodiments, one or more polypeptides herein may be combined with one or more polypeptides of other types.

[0252] The present disclosure also provides methods of detecting presence or absence of a cleaving agent and/or the target in a subject or a sample. Such methods may comprise (i) contacting a subject or biological sample with an activatable molecule, wherein the activatable molecule includes a detectable label that is positioned on a portion of the activatable molecule that is released following cleavage of the CM and (ii) measuring a level of activated molecule in the subject or biological sample, wherein a detectable level of activated molecule in the subject or biological sample indicates that the cleaving agent, the target or both the cleaving agent and the target are absent and/or not sufficiently present in the subject or biological sample, such that the target binding and/or protease cleavage of the activatable molecule cannot be detected in the subject or biological sample, and wherein a reduced detectable level of activated molecule in the subject or biological sample indicates that the cleaving agent and the target are present in the subject or biological sample.

[0253] Such detection methods may be adapted to also provide for detection of the presence or absence of a target that is capable of binding the AM of the activatable molecules when cleaved. Thus, the assays can be adapted to assess the presence or absence of a cleaving agent and the presence or absence of a target of interest. The presence or absence of the cleaving agent can be detected by the presence of and/or an increase in detectable label of the

activatable molecules as described above, and the presence or absence of the target can be detected by detection of a target-AM complex *e.g.*, by use of a detectably labeled anti-target antibody.

[0254] In some embodiments, activatable molecules are also useful in *in situ* imaging for the validation of activatable molecule activation, *e.g.*, by protease cleavage, and binding to a particular target. *In situ* imaging is a technique that enables localization of proteolytic activity and target in biological samples such as cell cultures or tissue sections. Using this technique, it is possible to confirm both binding to a given target and proteolytic activity based on the presence of a detectable label (e.g., a fluorescent label).

[0255] These techniques are useful with any frozen cells or tissue derived from a disease site (*e.g.* tumor tissue) or healthy tissues. These techniques are also useful with fresh cell or tissue samples.

[0256] In these techniques, an activatable molecule may be labeled with a detectable label. The detectable label may be a fluorescent dye, (e.g. a fluorophore, Fluorescein Isothiocyanate (FITC), Rhodamine Isothiocyanate (TRITC), an Alexa Fluor® label), a near infrared (NIR) dye (e.g., Qdot® nanocrystals), a colloidal metal, a hapten, a radioactive marker, biotin and an amplification reagent such as streptavidin, or an enzyme (e.g. horseradish peroxidase or alkaline phosphatase).

[0257] Detection of the label in a sample that has been incubated with the labeled, activatable molecule indicates that the sample contains the target and contains a protease that is specific for the CM of the activatable molecule. In some embodiments, the presence of the protease can be confirmed using broad spectrum protease inhibitors such as those described herein, and/or by using an agent that is specific for the protease, for example, an antibody such as A11, which is specific for the protease matriptase and inhibits the proteolytic activity of matriptase; see e.g., International Publication Number WO 2010/129609, published 11 November 2010. The same approach of using broad spectrum protease inhibitors such as those described herein, and/or by using a more selective inhibitory agent can be used to identify a protease that is specific for the CM of the activatable molecule. In some embodiments, the presence of the target can be confirmed using an agent that is specific for the target, *e.g.*, another antibody, or the detectable label can be competed with unlabeled target. In some embodiments, unlabeled activatable molecule may be used, with detection by a labeled secondary antibody or more complex detection system.

[0258] Similar techniques are also useful for *in vivo* imaging where detection of the fluorescent signal in a subject, *e.g.*, a mammal, including a human, indicates that the disease site contains the target and contains a protease that is specific for the CM of the activatable molecule.

[0259] These techniques are also useful in kits and/or as reagents for the detection, identification or characterization of protease activity in a variety of cells, tissues, and organisms based on the protease-specific CM in the activatable molecule.

[0260] A reduced level of detectable label may be, for example, a reduction of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or a reduction of substantially 100%. In some embodiments, the detectable label may be conjugated to a component of the polypeptide, e.g., the AM. In some embodiments, measuring the level of polypeptide in the subject or sample may be accomplished using a secondary reagent that specifically binds the activated protein, wherein the reagent comprises a detectable label. The secondary reagent may be an antibody comprising a detectable label.

[0261] In some embodiments, the CM-containing polypeptides may also be useful in the detection of the target in patient samples and accordingly are useful as diagnostics. For example, the polypeptides may be used in in vitro assays, e.g., ELISA, to detect target levels in a patient sample. For example, a polypeptide may be immobilized on a solid support (e.g., the well(s) of a microtiter plate). The immobilized polypeptide may serve as a capture protein for any target that may be present in a test sample. Prior to contacting the immobilized polypeptide with a patient sample, the solid support may be rinsed and treated with a blocking agent such as milk protein or albumin to prevent nonspecific adsorption of the analyte.

[0262] In some embodiments, based on the results obtained using the polypeptides in an in vitro diagnostic assay, the stage of a disease in a subject may be determined based on expression levels of the target protein (e.g., antigen). For a given disease, samples of blood may be taken from subjects diagnosed as being at various stages in the progression of the disease, and/or at various points in the therapeutic treatment of the disease. Using a population of samples that provides statistically significant results for each stage of progression or therapy, a range of concentrations of the target protein (e.g., antigen) that may be considered characteristic of each stage is designated.

[0263] Polypeptides herein may also be used in diagnostic and/or imaging methods. In some embodiments, such methods may be in vitro methods. In some embodiments, such methods may be in vivo methods. In some embodiments, such methods may be in situ methods. In some embodiments, such methods may be ex vivo methods. For example, polypeptides having a CM may be used to detect the presence or absence of an enzyme capable of cleaving the CM. Such polypeptides may be used in diagnostics, which can include in vivo detection (e.g., qualitative or quantitative) of enzyme activity (or, in some embodiments, an environment of increased reduction potential such as that which can provide for reduction of a disulfide bond) through measured accumulation of activated antibodies (i.e., antibodies resulting from cleavage of a polypeptide) in a given cell or tissue of a given host organism. Such accumulation of activated proteins indicates not only that the tissue expresses enzymatic activity (or an increased reduction potential depending on the nature of the CM) but also that the tissue expresses target to which the activated protein binds. In some examples, the polypeptides may be used for detecting protease activity with an assay that does not rely on target binding, e.g., a quantitative ex vivo zymography (QZ) assay as described in Howng et al., "Novel Ex Vivo Zymography Approach for Assessment of Protease Activity in Tissues with Activatable Antibodies," Pharmaceutics. 2021 Sep 2; 13(9):1390, which is incorporated by reference herein in its entirety.

[0264] For example, the CM may be selected to be a protease substrate for a protease found at the site of a tumor, at the site of a viral or bacterial infection at a biologically confined site (e.g., such as in an abscess, in an organ, and the like), and the like. The AM may be one that binds a target protein (e.g., antigen). Using methods familiar to one skilled in the art, a detectable label (e.g., a fluorescent label or radioactive label or radiotracer) may be conjugated to an AM or other region of a polypeptide. Suitable detectable labels may be discussed in the context of the above screening methods and additional specific examples are provided below. Using an AM specific to a protein or peptide of the disease state, along with a protease whose activity is elevated in the disease tissue of interest, polypeptides may exhibit an increased rate of binding to disease tissue relative to tissues where the CM specific enzyme is not present at a detectable level or is present at a lower level than in disease tissue or is inactive (e.g., in zymogen form or in complex with an inhibitor). Since small proteins and peptides are rapidly cleared from the blood by the renal filtration system, and because the enzyme specific for the CM is not present at a detectable level (or is present at lower levels

in non-disease tissues or is present in inactive conformation), accumulation of activated protein in the disease tissue may be enhanced relative to non-disease tissues.

[0265] In some embodiments, the CM-containing polypeptides may be useful for in vivo imaging where detection of the fluorescent signal in a subject, e.g., a mammal, including a human, indicates that the disease site contains the target and contains a protease that is specific for the CM of the polypeptide. The in vivo imaging may be used to identify or otherwise refine a patient population suitable for treatment with a polypeptide of the disclosure. For example, patients that test positive for both the target and a protease that cleaves the substrate in the CM of the polypeptide being tested (e.g., accumulate activated proteins at the disease site) are identified as suitable candidates for treatment with such a polypeptide comprising such a CM. Likewise, patients that test negative may be identified as suitable candidates for another form of therapy (i.e., not suitable for treatment with the polypeptide being tested). In some embodiments, such patients that test negative with respect to a first polypeptide can be tested with other polypeptides comprising different CMs until a suitable polypeptide for treatment is identified (e.g., a polypeptide comprising a CM that is cleaved by the patient at the site of disease).

[0266] In some embodiments, in situ imaging may be useful in methods to identify which patients to treat. For example, in in situ imaging, the polypeptides may be used to screen patient samples to identify those patients having the appropriate protease(s) and target(s) at the appropriate location, e.g., at a tumor site. In some embodiments, in situ imaging is used to identify or otherwise refine a patient population suitable for treatment with a polypeptide of the disclosure. For example, patients that test positive for both the target and a protease that cleaves the substrate in the CM of the polypeptide being tested (e.g., accumulate activated antibodies at the disease site) are identified as suitable candidates for treatment with such a polypeptide comprising such a CM. Likewise, patients that test negative for either or both of the target and the protease that cleaves the CM used in the polypeptide being tested using these methods are identified as suitable candidates for another form of therapy (i.e., not suitable for treatment with the polypeptide being tested). In some embodiments, such patients that test negative with respect to a first polypeptide can be tested with other polypeptides comprising different CMs until a suitable polypeptide for treatment is identified (e.g., a polypeptide comprising a CM that is cleaved by the patient at the site of disease).

[0267] The present application also provides aspects and embodiments as set forth in the following numbered Statements:

[0268] Statement 1. An isolated polypeptide comprising a cleavable moiety (CM) that comprises the amino acid sequence AIALY (SEQ ID NO: 5), wherein the CM is a substrate for a protease. According to the present disclosures, the isolated polypeptide is a molecule in which cleavage of the CM by a protease results in a part or component of the molecule being separated from the remainder of the molecule. In some aspects of the present disclosure, cleavage of the CM by a protease activates the molecule. In some aspects, the isolated polypeptide is a molecule in which multiple proteases cleave the CM. In some aspects, the isolated polypeptide is a molecule in which MMP2 cleaves the CM. In some aspects, the isolated polypeptide is a molecule in which MMP9 cleaves the CM. In some aspects, the isolated polypeptide is a molecule in which MMP14 cleaves the CM. In some aspects, the isolated polypeptide is a molecule in which two or all of MMP2, MMP9, and MMP14 cleave the CM. In some aspects, the isolated polypeptide is a molecule in which the % cleavability of the CM is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%, or 100%, e.g., at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%, or 100% cleavable by any one of MMP2, MMP9, and MMP14 or any two of MMP2, MMP9, and MMP14 or each of MMP2, MMP9, and MMP14. According to the present disclosures, the isolated polypeptide is a molecule that has high *in vivo* stability such that it is not cleaved in plasma as demonstrated by less than 60%, less than 50%, less than 40%, or less than 25% in vivo activation following 7 days of administration *in vivo*. According to embodiments of the present disclosures, the isolated polypeptide is a molecule comprising a CM that has a k_{cat}/K_M ($M^{-1}s^{-1}$) of greater than 5.0×10^2 M^{-1} s^{-1}.

[0269] Statement 2. The isolated polypeptide of Statement 1, wherein the CM comprises the amino acid sequence AIALYA (SEQ ID NO: 2).

[0270] Statement 3. The isolated polypeptide of Statement 1, wherein the CM comprises the amino acid sequence AIALYAD (SEQ ID NO: 1).

[0271] Statement 4. An isolated polypeptide comprising a cleavable moiety (CM) that comprises an amino acid sequence selected from SEQ ID NOs: 1-14, wherein the CM is a substrate for a protease. According to the present disclosures, the isolated polypeptide is a molecule in which cleavage of the CM by a protease results in a part or component of the molecule being separated from the remainder of the molecule. In some aspects of the present

disclosure, cleavage of the CM by a protease activates the molecule. In some aspects, the isolated polypeptide is a molecule in which multiple proteases cleave the CM. In some aspects, the isolated polypeptide is a molecule in which MMP2 cleaves the CM. In some aspects, the isolated polypeptide is a molecule in which MMP9 cleaves the CM. In some aspects, the isolated polypeptide is a molecule in which MMP14 cleaves the CM. In some aspects, the isolated polypeptide is a molecule in which two or all of MMP2, MMP9, and MMP14 cleave the CM. In some aspects, the isolated polypeptide is a molecule in which the % cleavability of the CM is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%, or 100%, e.g., at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%, or 100% cleavable by any one of MMP2, MMP9, and MMP14 or any two of MMP2, MMP9, and MMP14 or each of MMP2, MMP9, and MMP14. According to the present disclosures, the isolated polypeptide is a molecule that has high *in vivo* stability such that it is not cleaved in plasma as demonstrated by less than 60%, less than 50%, less than 40%, or less than 25% in vivo activation following 7 days of administration *in vivo*. According to embodiments of the present disclosures, the isolated polypeptide is a molecule comprising a CM that has a k_{cat}/K_M ($M^{-1}s^{-1}$) of greater than 5.0×10^2 M^{-1} s^{-1}.

[0272] Statement 5. The isolated polypeptide of any one of Statements 1-4, wherein the isolated polypeptide is an activatable molecule and further comprises an active moiety (AM) that specifically binds a target. According to the present disclosures, the isolated polypeptide may be an activatable molecule that has high *in vivo* stability such that it is not cleaved in plasma as demonstrated by less than 25% in vivo activation following 7 days of administration *in vivo*. According to the present disclosures, the isolated polypeptide may be an activatable molecule that has masking efficiency of 25x, 40x, 41x, 50x, 75x, 100x, 150x, 200x, or higher. According to the present disclosures, the activatable molecule may be activated by one, two, or all of MMP2, MMP9, and MMP14. According to the present disclosures, the activatable molecule may be activated to an extent of having a cleavability percentage of at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%, or 100%, e.g., at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%, or 100% cleavable by any one of MMP2, MMP9, and MMP14or any two of MMP2, MMP9, and MMP14 or each of MMP2, MMP9, and MMP14. In some aspects, the isolated polypeptide is an activatable molecule in which the % cleavability of the CM is at least 50%, 65%, and up to 100% in the presence of MMP2. In some aspects, the isolated

polypeptide is an activatable molecule in which the % cleavability of the CM is improved by 2x or more over the % cleavability of SEQ ID NO: 78 (see Example 2). According to the present disclosures, the activatable molecule exhibits attenuated binding to a target as compared to the binding of a counterpart "activated" molecule comprising the same active moiety (AM) to the same target.

[0273] Statement 6. The isolated polypeptide of Statement 5, wherein the AM is an antibody or antigen binding fragment thereof.

[0274] Statement 7. The isolated polypeptide of Statement 6, wherein the antigen binding fragment is selected from the group consisting of a Fab fragment, a $F(ab')_2$ fragment, a scFv, a scAb, a dAb, a single domain heavy chain antibody, and a single domain light chain antibody.

[0275] Statement 8. The isolated polypeptide of Statement 5, wherein the AM is a therapeutic macromolecule.

[0276] Statement 9. The isolated polypeptide of Statement 5, wherein the AM is a cytokine.

[0277] Statement 10. The isolated polypeptide of Statement 5, wherein the AM is a chimeric antigen receptor.

[0278] Statement 11. The isolated polypeptide of any one or combination of Statements 5-10, wherein the AM is coupled to the CM.

[0279] Statement 12. The isolated polypeptide of Statement 11, wherein the AM is directly coupled to the CM.

[0280] Statement 13. The isolated polypeptide of Statement 11, wherein the AM is indirectly coupled to the CM via a linking peptide.

[0281] Statement 14. The isolated polypeptide of any one or combination of Statements 5-13, further comprising a masking moiety (MM).

[0282] Statement 15. The isolated polypeptide of Statement 14, wherein the MM has a dissociation constant for binding to the AM that is greater than the dissociation constant of the AM for binding to the target.

[0283] Statement 16. The isolated polypeptide of Statement 14 or 15, wherein the MM is 2 to 40 amino acids in length.

[0284] Statement 17. The isolated polypeptide of any one or combination of Statements 14-16, wherein the MM interferes with AM's binding to its binding partner through non-

specific interactions such as steric hindrance, optionally wherein the MM is positioned in the activatable molecule such that the tertiary or quaternary structure of the activatable molecule allows the MM to mask the AM through charge-based interaction, optionally wherein the MM is an albumin, e.g., human serum albumin (HSA), a fragment crystallizable (Fc) domain, an antibody constant domain (e.g., CH domains), a polymer (e.g., branched or multi-armed polyethylene glycol (PEG)), a latency associated protein (LAP), and any polypeptide or other moieties that sterically interfere AM-target interactions, optionally wherein the MM may recruit a large protein binding partner that sterically interfere AM-target interactions, optionally wherein the MM is an antibody or a fragment thereof that binds to an albumin, optionally wherein the MM comprises a full-length or a AM-binding fragment or mutein of a cognate receptor of the AM, and AM-binding antibodies and fragment thereof, e.g., a polyclonal antibody, a recombinant antibody, a human antibody, a humanized antibody a single chain variable fragment (scFv), single-domain antibody such as a heavy chain variable domain (VH), a light chain variable domain (VL), a variable domain of camelid-type nanobody (VHH), or a dAb, optionally wherein the MM is a non-immunoglobulin proteins that mimic antibody binding and/or structure such as, anticalins, affilins, affibody molecules, affimers, affitins, alphabodies, avimers, DARPins, fynomers, kunitz domain peptides, monobodies, and binding domains based on other engineered scaffolds such as SpA, GroEL, fibronectin, lipocallin and CTLA4 scaffolds, optionally wherein the MM is a peptide that is modified by conjugation to a water-soluble polymer, such as a polyalkylene glycol, e.g., a polyethylene glycol (PEG), optionally wherein the MM is an antibody or antigen-binding domain that binds to a protein with a long serum half- life such as HSA, immunoglobulin or transferrin, or to a receptor that is recycled to the plasma membrane, such as FcRn or a transferrin receptor.

[0285] Statement 18. The isolated polypeptide of any one or combination of Statements 14-17, wherein the MM is coupled to the CM such that the isolated polypeptide comprises the structural arrangement from N-terminus to C-terminus as follows: MM-CM-AM or AM-CM-MM.

[0286] Statement 19. The isolated polypeptide of Statement 18, wherein the MM is coupled directly to the CM.

[0287] Statement 20. The isolated polypeptide of Statement 18, wherein the MM is coupled indirectly to the CM via a linking peptide.

[0288] Statement 21. The isolated polypeptide of any one or combination of Statements 14-20, wherein the isolated polypeptide comprises a first linking peptide (LP1) and a second linking peptide (LP2), and wherein the isolated polypeptide has the structural arrangement from N-terminus to C-terminus as follows: MM-LP1-CM-AM, MM-CM-LP1-AM, MM-LP1-CM-LP2-AM or AM-LP2-CM-LP1-MM.

[0289] Statement 22. The isolated polypeptide of Statement 21, wherein the LP1 and LP2 are not identical to each other.

[0290] Statement 23. The isolated polypeptide of Statement 21, wherein the LP1 and LP2 are identical to each other.

[0291] Statement 24. The isolated polypeptide of any one of Statements 21-23, wherein each of LP1 and LP2 is a peptide of 1 to 20 amino acids in length.

[0292] Statement 25. The isolated polypeptide of any one or combination of Statements 1-24, wherein the CM is a substrate for a matrix metalloproteinase (MMP).

[0293] Statement 26. The isolated polypeptide of Statement 25, wherein the MMP is MMP2, MMP9, or MMP14. The isolated polypeptide of Statement 25, wherein the MMP is MMP2. The isolated polypeptide of Statement 25, wherein the MMP is MMP9. The isolated polypeptide of Statement 25, wherein the MMP is MMP14.

[0294] Statement 27. The isolated polypeptide of Statement 26, wherein the k_{cat}/K_M of the CM by MMP2 cleavage is at least 1×10^3 $M^{-1}s^{-1}$ optionally at 37°C in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl, 0.05% (w/v) Brij-35.

[0295] Statement 28. The isolated polypeptide of Statement 26, wherein the k_{cat}/K_M of the CM by MMP2 cleavage is at least 1×10^4 $M^{-1}s^{-1}$, optionally at 37°C in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl, 0.05% (w/v) Brij-35.

[0296] Statement 29. The isolated polypeptide of any one or combination of Statements 26-28, wherein the k_{cat}/K_M of the CM by MMP9 cleavage is at least 1×10^2 $M^{-1}s^{-1}$, optionally at 37°C in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl, 0.05% (w/v) Brij-35.

[0297] Statement 30. The isolated polypeptide of any one or combination of Statements 26-29, wherein the k_{cat}/K_M of the CM by MMP9 cleavage is at least 1×10^3 $M^{-1}s^{-1}$, optionally at 37°C in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl, 0.05% (w/v) Brij-35.

[0298] Statement 31. The isolated polypeptide of any one or combination of Statements 25-30, wherein the k_{cat}/K_M of the CM by MMP14 cleavage is at least 1×10^2 $M^{-1}s^{-1}$, optionally

at 37°C in 50 mM HEPES (pH 6.8), 10 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.05% (w/v) Brij-35 for MMP14.

[0299] Statement 32. The isolated polypeptide of any one or combination of Statements 26-31, wherein the k_{cat}/K_M of the CM by MMP14 cleavage is at least 1×10^3 $M^{-1}s^{-1}$, optionally at 37°C in 50 mM HEPES (pH 6.8), 10 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.05% (w/v) Brij-35 for MMP14.

[0300] Statement 33. The isolated polypeptide of any one or combination of Statements 5-32, wherein the AM is an antibody or antigen-binding fragment that binds EGFR and the MM comprises the amino acid sequence of SEQ ID NO: 82.

[0301] Statement 34. An isolated polypeptide comprising an antibody or antigen-binding fragment thereof that binds EGFR (AB), a masking moiety (MM) comprising the SEQ ID NO: 82, and a cleavable moiety (CM), wherein AB is coupled with the MM via the CM.

[0302] Statement 35. An isolated polypeptide comprising a cleavable moiety (CM) comprising an amino acid sequence with one-amino acid mutation of any one of SEQ ID NOs: 1-14, wherein the CM is a substrate for a protease.

[0303] Statement 36. An isolated polypeptide comprising a cleavable moiety (CM) comprising an amino acid sequence with two-amino acid mutations of any one of SEQ ID NOs: 1-14, wherein the CM is a substrate for a protease.

[0304] Statement 37. An isolated polypeptide comprising a cleavable moiety (CM) comprising an amino acid sequence with three-amino acid mutations of any one of SEQ ID NOs: 1-14, wherein the CM is a substrate for a protease.

[0305] Statement 38. An isolated polypeptide comprising a cleavable moiety (CM) comprising an amino acid sequence with four-amino acid mutations of any one of SEQ ID NOs: 1-14, wherein the CM is a substrate for a protease.

[0306] Statement 39. The isolated polypeptide of any one or combination of Statements 1-38, further comprising one or more additional CMs, optionally wherein at least a portion of a first CM overlaps with at least a portion of a second CM in the substrate, such that one or more amino acids belong to both CMs.

[0307] Statement 40. A polypeptide complex comprising one or more of the isolated polypeptides of any one or combination of Statements 1-39.

[0308] Statement 41. A conjugated polypeptide comprising the isolated polypeptide of any one or combination of Statements 1-39 conjugated to an agent.

[0309] Statement 42. The conjugated polypeptide of Statement 41, wherein the agent is conjugated to the isolated polypeptide via a conjugating linker.

[0310] Statement 43. The conjugated polypeptide of Statement 42, wherein the conjugating linker is cleavable.

[0311] Statement 44. The conjugated polypeptide of Statement 42, wherein the conjugating linker is non-cleavable.

[0312] Statement 45. The conjugated polypeptide of Statement 43, wherein the conjugating linker comprises an amino acid sequence selected from SEQ ID NOs: 1-14.

[0313] Statement 46. The conjugated polypeptide of any one or combination of Statements 41-45, wherein the agent is a toxin, a microtubule inhibitor, a nucleic acid damaging agent, a dolastatin, an auristatin, a maytansinoid, a duocarmycin, a calicheamicin, or a combination thereof.

[0314] Statement 47. A composition comprising the isolated polypeptide of any one or combination of Statements 1-39, the polypeptide complex of Statement 40, or the conjugated polypeptide of any one or combination of Statements 41-46, and a carrier.

[0315] Statement 48. The composition of Statement 47, wherein the carrier is a pharmaceutically acceptable carrier.

[0316] Statement 49. The composition of Statement 47 or 48, comprising an additional agent.

[0317] Statement 50. The composition of Statement 49, wherein the additional agent is a therapeutic agent.

[0318] Statement 51. An isolated nucleic acid molecule encoding the isolated polypeptide of any one or combination of Statements 1-39.

[0319] Statement 52. A vector comprising the isolated nucleic acid molecule of Statement 51.

[0320] Statement 53. A cell comprising the isolated polypeptide of any one or combination of Statements 1-39 or the isolated nucleic acid molecule of Statement 50 or the vector of Statement 52.

[0321] Statement 54. A method of manufacturing an isolated polypeptide or an activatable molecule that contains a cleavable moiety (CM), the method comprising expressing and recovering a polypeptide comprising the isolated polypeptide of any one or

combination of Statements 1-39, optionally wherein the polypeptide is an activatable molecule.

[0322] Statement 55. A method of treating, alleviating a symptom of, or delaying the progression of a disease or disorder in a subject, comprising administering a therapeutically effective amount of the isolated polypeptide of any one or combination of Statements 1-39, the polypeptide complex of Statement 40, the conjugated polypeptide of any one or combination of Statements 41-46, the composition of any one of Statements 47-50 to the subject, the nucleic acid molecule of Statement 51, the vector of Statement 52, or the cell of Statement 53. The isolated polypeptide of any one or combination of Statements 1-39, the polypeptide complex of Statement 40, the conjugated polypeptide of any one or combination of Statements 41-46, the composition of any one of Statements 47-50 to the subject, the nucleic acid molecule of Statement 51, the vector of Statement 52, or the cell of Statement 53 for use as a medicament or for use in therapy, optionally for treating a cancer, an infection, an inflammatory disorder, a cardiovascular disorder, a neurodegenerative disorder, or an autoimmune disorder, optionally with an additional agent which is optionally a therapeutic agent. The isolated polypeptide of any one or combination of Statements 1-39, the polypeptide complex of Statement 40, the conjugated polypeptide of any one or combination of Statements 41-46, the composition of any one of Statements 47-50 to the subject, the nucleic acid molecule of Statement 51, the vector of Statement 52, or the cell of Statement 53 for treating a cancer. The isolated polypeptide of any one or combination of Statements 1-39, the polypeptide complex of Statement 40, the conjugated polypeptide of any one or combination of Statements 41-46, the composition of any one of Statements 47-50 to the subject, the nucleic acid molecule of Statement 51, the vector of Statement 52, or the cell of Statement 53 for treating an infection. The isolated polypeptide of any one or combination of Statements 1-39, the polypeptide complex of Statement 40, the conjugated polypeptide of any one or combination of Statements 41-46, the composition of any one of Statements 47-50 to the subject, the nucleic acid molecule of Statement 51, the vector of Statement 52, or the cell of Statement 53 for treating an inflammatory disorder. The isolated polypeptide of any one or combination of Statements 1-39, the polypeptide complex of Statement 40, the conjugated polypeptide of any one or combination of Statements 41-46, the composition of any one of Statements 47-50 to the subject, the nucleic acid molecule of Statement 51, the vector of Statement 52, or the cell of Statement 53 for treating a cardiovascular disorder. The

isolated polypeptide of any one or combination of Statements 1-39, the polypeptide complex of Statement 40, the conjugated polypeptide of any one or combination of Statements 41-46, the composition of any one of Statements 47-50 to the subject, the nucleic acid molecule of Statement 51, the vector of Statement 52, or the cell of Statement 53 for treating a neurodegenerative disorder. The isolated polypeptide of any one or combination of Statements 1-39, the polypeptide complex of Statement 40, the conjugated polypeptide of any one or combination of Statements 41-46, the composition of any one of Statements 47-50 to the subject, the nucleic acid molecule of Statement 51, the vector of Statement 52, or the cell of Statement 53 for treating an autoimmune disorder.

[0323] Statement 56. The method of Statement 55, wherein the disease is a cancer, an infection, an inflammatory disorder, a cardiovascular disorder, a neurodegenerative disorder, or an autoimmune disorder.

[0324] Statement 57. A kit comprising the isolated polypeptide of any one or combination of Statements 1-39, the polypeptide complex of Statement 40, the conjugated polypeptide of any one or combination of Statements 41-46, or the composition of any one of Statements 47-50.

[0325] Statement 58. The use of the isolated polypeptide of any one or combination of Statements 1-39, the polypeptide complex of Statement 40, the conjugated polypeptide of any one or combination of Statements 41-46, or the composition of any one of Statements 47-50 for the manufacture of a medicament for the treatment of a disease or disorder.

[0326] Statement 59. The use of Statement 58, wherein the disease or disorder is a cancer, an infection, an inflammatory disorder, a cardiovascular disorder, a neurodegenerative disorder, or an autoimmune disorder.

[0327] Statement 60. A method of detecting or diagnosing a disease or health condition of a subject, comprising: contacting the isolated polypeptide of any one or combination of Statements 1-39, the polypeptide complex of Statement 40, the conjugated polypeptide of any one or combination of Statements 41-46, or the composition of any one of Statements 47-50 with a sample from the subject; and measuring a level of cleavage of the isolated polypeptide, thereby detecting or diagnosing the disease or health condition of the subject.

[0328] Statement 61. The method of Statement 60, wherein the disease is a cancer, an infection, an inflammatory disorder, a cardiovascular disorder, a neurodegenerative disorder, or an autoimmune disorder.

EXAMPLES

EXAMPLE 1: Activatable Antibodies and Matrix Metalloprotease (MMP) Cleavable Moieties

[0329] The studies provided herein describe exemplary CMs that are matrix metalloprotease (MMP) substrates and exemplary activatable antibodies that include the exemplary CMs.

[0330] Exemplary activatable antibodies were constructed such that each one includes one of the CMs listed in Table 4. The exemplary activatable antibodies, the sequences of which are listed in Table 5, include an antibody or antigen binding fragment thereof (AB) that is based on a mouse/human chimeric monoclonal antibody that specifically binds to epidermal growth factor receptor (EGFR). The exemplary activatable antibodies also include a prodomain coupled to the N-terminus of the light chain of the AB. Each prodomain includes a masking moiety (MM) and a cleavable moiety (CM), and the CM includes at least one MMP substrate sequence of Table 4.

Table 4: Exemplary CMs with Matrix Metalloprotease (MMP) Substrates

Name	Sequence	SEQ ID NO:
7000	AIALYAD	1
7001	AIALYA	2

Table 5: Activatable Antibodies

Molecule Name	Light chain EGFR Mask	Light chain CM	Protein description (Light chain / Heavy chain)
CX-122	CISPRGCPDGPYVMY (SEQ ID NO: 81)	2001 (SEQ ID NO: 78)	C225v5 3954 2001 (SEQ ID NO: 83 / SEQ ID NO: 84)
AA w/5007	CISPRGCPDGPYVMY (SEQ ID NO: 81)	5007 (SEQ ID NO: 80)	C225v5 3954 5007 (SEQ ID NO: 87 / SEQ ID NO: 88)
CX-229	CISPRGCLDGPYVMY (SEQ ID NO: 82)	3001 (SEQ ID NO: 79)	C225v5 3954 3001 (SEQ ID NO: 89/ SEQ ID NO: 84)
CTX-028	CISPRGCPDGPYVMY (SEQ ID NO: 81)	1001 (SEQ ID NO: 75)	C225v5 3954 1001 (SEQ ID NO: 91 / SEQ ID NO: 92)
AA w/7000	CISPRGCPDGPYVMY	7000 (SEQ ID NO: 1)	C225v5 3954 7000

	(SEQ ID NO: 81)		(SEQ ID NO: 73 / SEQ ID NO: 84)
ProC1589	CISPRGCPDGPYVM Y (SEQ ID NO: 81)	7001 (SEQ ID NO: 2)	C225v5 3954 7001 (SEQ ID NO: 74 / SEQ ID NO:84)

EXAMPLE 2: *In Vitro* Cleavability of Activatable Antibodies with Exemplary CMs

[0331] The studies provided herein evaluate the *in vitro* cleavability of activatable antibodies containing exemplary CMs cleavable by a matrix metalloprotease (MMP).

[0332] The cleavability of the activatable antibodies having the CMs of the present disclosure, along with control CMs 2001 (WO2016/118629) and 1001 (WO2016/048329), was measured in the presence of MMP2, MMP9, and MMP14. Each activatable antibody (500 nM) was incubated with 10 nM of indicated single protease for 1.5 hours at 37°C. Human recombinant proteases were purchased from R&D Systems: MMP2 (catalog No:902-MP), MMP9 (catalog No:911-MP), and MMP14 (catalog No:918-MP). MMPs were activated according to the manufacturer's instructions. Protease concentrations were determined by active site titration. Activity assays were performed in the following buffers: 50 mM Tris-HCl, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% (w/v) Brij-35, pH 7.5 for MMP2 and MMP9. Activity assays were performed for MMP14 using 50 mM HEPES (pH 6.8), 10 mM $CaCl_2$, 0.5 mM $MgCl_2$. Following incubation, the presence of cleavage product was determined by capillary electrophoresis immunoassay for each protease enzyme using the Wes™ Western Blot instrument (Protein Simple). For capillary electrophoresis immunoassays, the A110UK goat anti-human IgG antibody (American Qualex) and an anti-goat secondary antibody (Jackson ImmunoResearch) were used. The fraction of cleaved activatable antibody was determined by quantifying the fraction of the higher mobility polypeptide corresponding to the cleaved activatable antibody using the Compass software (Protein Simple). The fraction of activatable antibody, and hence the CM that was cleaved by each particular protease, is presented as a "cleavability percentage" in Table 6A.

[0333] The exemplary results of Table 6A show that CM 7000 (AIALYAD, SEQ ID NO: 1) and CM 7001 (AIALYA, SEQ ID NO: 2) exhibited a percent activation of greater than 10 percent for MMP9 and greater than 50 percent for MMP2.

[0334] In addition, an exemplary study was performed to determine the cleavability kinetics (*i.e.*, k_{cat}/K_M (M^{-1} s^{-1})) of the indicated CMs with the indicated protease enzymes. The results of this *in vitro* study are summarized in Table 6B.

[0335] The results of Table 6B show that the substrates of the present disclosure showed a range of cleavability by MMP2, MMP9, and MMP14 enzymes. These exemplary results also show that CM 7000 (AIALYAD, SEQ ID NO: 1) had a k_{cat}/K_M (M^{-1} s^{-1}) of greater than 1 x 10^3 $M^{-1}s^{-1}$ for *in vitro* cleavability with MMP9 and MMP2. These exemplary results also show that CM 7001 (AIALYA, SEQ ID NO: 2) had a k_{cat}/K_M (M^{-1} s^{-1}) of greater than 1 x 10^3 $M^{-1}s^{-1}$ for *in vitro* cleavability with MMP2, MMP9, and MMP14. These exemplary results also show that CMs 7000 and 7001 had a k_{cat}/K_M (M^{-1} s^{-1}) of greater than 1 x 10^4 $M^{-1}s^{-1}$ for *in vitro* cleavability with MMP2.

Table 6A: *In Vitro* Activation of Activatable Antibodies with Exemplary CMs

CM of Activatable Antibody	Substrate	Cleavability (%) 1.5h		
		MMP2	MMP9	MMP14
2001	ISSGLLSGRSDNH (SEQ ID NO: 78)	32.9	-	56.3
7000	AIALYAD (SEQ ID NO: 1)	54.4	16.0	7.5
7001	AIALYA (SEQ ID NO: 2)	65.3	17.3	17.0

Note: "-" indicates below limit of quantification.

Table 6B: *In Vitro* Activation of Activatable Antibodies with Exemplary CMs

CM of Activatable Antibody	Substrate (SEQ ID NO)	k_{cat}/K_M (M^{-1} s^{-1})		
		MMP2	MMP9	MMP14
2001	ISSGLLSGRSDNH (SEQ ID NO: 78)	5.08 E+03	-	1.21 E+04
1001	ISSGLLSS (SEQ ID NO: 75)	6.4 E+03	-	7.3 E+03
7000	AIALYAD (SEQ ID NO: 1)	1.10 E+04	2.00 E+03	8.47 E+02
7001	AIALYA (SEQ ID NO: 2)	1.62 E+04	2.73	2.16

			E+03	E+03

Note: "-" indicates below limit of quantification.

EXAMPLE 3: *In Vivo* Stability of Activatable Antibodies with Exemplary CMs

[0336] The study provided herein evaluates the in vivo stability of activatable antibodies of the present disclosure with the CMs 7000 (AIALYAD; SEQ ID NO: 1) and 7001 (AIALYA; SEQ ID NO: 2).

[0337] This exemplary study measured the stability of activatable antibodies containing substrates of the present disclosure by administering a dose of the activatable antibodies to mice, and then measuring the cleaved activatable antibody in the plasma by capillary electrophoresis immunoassay. The stability was compared to activatable antibodies with control CMs 2001, 3001, and 5007.

[0338] In this study, nu/nu mice of about 7-8 weeks of age were administered intraperitoneally with the indicated test article at a dosage of 10 mg/kg. After 7 days following the administration, terminal blood was collected by cardiac puncture and processed to plasma within 1 hour of collection. The collected sample was diluted 1:50 in phosphate-buffered saline solution and denatured and analyzed using the Wes™ Western Blot protocol (Protein Simple) using the A110UK goat anti-human IgG antibody (American Qualex) and an anti-goat secondary antibody (Jackson ImmunoResearch). The fraction of cleaved activatable antibody was determined by quantifying the fraction of the higher mobility polypeptide corresponding to the cleaved activatable antibody using the Compass software (Protein Simple). The results of these exemplary assays are summarized in Table 7.

[0339] These exemplary results showed that activatable antibodies with CM 7001 and CM 7000 demonstrate a comparable *in vivo* stability to activatable antibodies that have the control CM 2001 (ISSGLLSGRSDNH; SEQ ID NO: 78) and improved *in vivo* stability compared to control CMs 5007 (APRSALAHGLF; SEQ ID NO: 80) and 3001 (AVGLLAPPGGLSGRSDNH; SEQ ID NO: 79).

Table 7: *In Vivo* Stability of Activatable Antibodies with Exemplary CMs

CM of Activatable Antibody	CM (SEQ ID NO)	*In Vivo* % Activation (nu/nu, day 7)
2001	ISSGLLSGRSDNH (SEQ ID NO: 78)	32.1

3001	AVGLLAPPGGLSGRSDNH (SEQ ID NO: 79)	63.0
5007	APRSALAHGLF (SEQ ID NO: 80)	56.2
7000	AIALYAD (SEQ ID NO: 1)	37.0
7001	AIALYA (SEQ ID NO: 2)	38.2

EXAMPLE 4: Masking Efficiency of Activatable Antibodies with Exemplary CMs

[0340] The study provided herein evaluates the *in vitro* masking efficiency of activatable antibodies of the present disclosure with the CM 7000 (AIALYAD; SEQ ID NO: 1).

[0341] In this study, a solid-phase binding assay (ELISA) was used to demonstrate the binding affinity of anti-EGFR activatable antibodies that include CMs of the present disclosure to recombinant EGFR. The binding affinity to EGFR of the activatable antibodies with the indicated CM of the present disclosure was measured and compared to the unmasked control c225v5 antibody (SEQ ID NOs: 157 and 158). A summary of these exemplary results is shown in FIG. 1 and Table 8.

[0342] These exemplary results showed that CM 7000 (SEQ ID NO: 1) had an effect by increasing the apparent masking efficiency of the masking moiety in the activatable antibody compared to the activatable antibody with the control CM 2001 (ISSGLLSGRSDNH SEQ ID NO: 78).

Table 8. *In Vitro* Binding Activity and Masking Efficiency of Activatable Antibodies

Test Article	K_D in nM	Masking Efficiency
C225 7000	27.79	139x
C225 2001	7.30	37x
C225 Ab	0.20	1x

EXAMPLE 5: *In Vivo* Efficacy of Anti-EGFR Activatable Antibodies with Exemplary CMs

[0343] The study provided herein evaluates the *in vivo* efficacy of activatable antibodies of the present disclosure with the CM 7000 (AIALYAD; SEQ ID NO: 1) using a mouse H292 (human lung cancer cell line) xenograft model.

[0344] In this study, H292 (human lung cancer-derived cell line) subcutaneous xenograft tumors in female nu/nu mice of 6-8 weeks of age were grown to an average volume of 124-210 mm^3. The H292 cell line is responsive to the anti-EGFR antibody cetuximab. The mice were then randomized into groups of 8 mice each and each group was dosed intraperitoneally on day 1 with 9 mg/kg of the indicated test article. The mean tumor volume ± SEM was plotted for each time point following administration of the test article, as shown in FIG. 2A. Each mouse was treated with activated antibodies with either CM 7000 or the control CM 2001, or with cetuximab or immunoglobulin (IVIG) control.

[0345] An intra-tumoral activation assay was performed using the indicated activatable antibodies as shown in FIG. 2B. Tumors were collected from the mice 7 days after dosing. The tumor tissue was lysed with immunoprecipitation buffer (Pierce) containing HALT protease inhibitor cocktail (Thermo Fisher) and EDTA and lysed using the Barocycler (Pressure Bioscience). The sample was analyzed using the Wes™ Western Blot instrument (Protein Simple) with the A110UK goat anti-human IgG antibody (American Qualex) and an anti-goat secondary antibody (Jackson ImmunoResearch). The fraction of cleaved activatable antibody was determined by quantifying the fraction of the higher mobility polypeptide corresponding to the cleaved activatable antibody. The results of these exemplary assays are summarized in FIG. 2B.

[0346] As shown in FIG. 2A, the activatable antibody with CM 7000 demonstrated an *in vivo* efficacy that is higher than the activatable antibody with the control CM 2001, and similar to cetuximab, which lacks a prodomain.

[0347] As shown in FIG. 2B, the activatable antibody with CM 7000 was cleaved by proteases present in the tumor resulting in activation of the antibody.

EXAMPLE 6: Evaluation of Protease Activity in Patient-Derived Tumor Samples

[0348] Protease activities in patient-derived tumor samples were evaluated using the quantitative zymography (QZ) assay. See Howng, B. et al. "Novel Ex Vivo Zymography Approach for Assessment of Protease Activity in Tissues with Activatable Antibodies," Pharmaceutics 2021, 13(9), 1390. Tumor tissue samples from cholangiocarinoma, pancreatic, and triple negative breast cancer (TNBC) patients were analyzed utilizing activatable antibodies of the present disclosure with the CM 7000 (AIALYAD; SEQ ID NO: 1).

[0349] The protease activity in the tumor sections of 12 μm thickness was assessed. A hydrophobic barrier was drawn around the tissue sample to maintain liquid on the tissue using an ImmEdge Hydrophobic Barrier Pen (Vector Laboratories), and the slides were then incubated with 40 μg/mL unmasked control c225v5 antibody in buffer consisting of 150 mM Tris HCl pH 7.4, 5 mM $CaCl_2$ 100 μM $ZnCl_2$, and 0.005% Tween-20 (QZ assay buffer) for 30 minutes at room temperature. An equal volume of AF647-labeled c225 antibodies prepared at 40 μg/mL in QZ buffer were then added directly onto the tissue containing the buffer to form a mixture and incubated at a final concentration of 20 μg/mL in a humidified chamber at 37°C for 48 hours.

[0350] Following 48 hours of incubation, supernatants were collected from each incubated mixture and transferred into a well of a 96-well PCR plate for assay by capillary electrophoresis. Each supernatant sample was mixed with Pico Sample Buffer (Perkin Elmer) containing 2-beta-mercaptoethanol at four parts sample and one part of Pico Sample Buffer and then heated at 95°C for 10 minutes. The composition of each supernatant sample was then assessed using the LabChip GXII Touch (Perkin Elmer) with the HT Pico Protein Express 100 protocol (Perkin Elmer). Protein Express Assay LabChips (Perkin Elmer #760499) were set up using the protocol of the Protein Pico Assay Reagent Kit (Perkin Elmer #760498). The fraction of cleaved activatable antibody in the tumor tissue supernatants was determined by quantifying the fraction of the higher mobility polypeptide corresponding to the cleaved activatable antibody using the LabChip GX Reviewer software (Perkin Elmer).

[0351] As shown in FIGs. 3A-3C, higher LC activation is observed in the activatable antibody with CM 7000 compared to the activatable antibodies with the control CMs 2001, 5007, or 3001 in cholangiocarinoma tumor tissue. Comparable levels of LC activation are observed for the activatable antibody with CM 7000 and the activatable antibodies with the control CMs 2001, 5007, and 3001 in pancreatic cancer tumor tissue. The levels of LC activation with CM 7000 was comparable with the control CMs 2001 and 5007 in TNBC tumor tissue.

EXAMPLE 7: *In situ* Activation of Exemplary Anti-EGFR Activatable Antibodies in Patient-Derived Acute Myeloid Leukemia "Liquid Tumor" Samples

[0352] The study provided herein evaluates the *in situ* cleavage of exemplary activatable antibodies of the present disclosure with the CMs 7000 (AIALYAD; SEQ ID NO: 1) and 7001 (AIALYA; SEQ ID NO: 2) by human acute myeloid leukemia (AML) samples using

the QZ assay. Frozen AML bone marrow mononuclear cells (BMMCs) were purchased from Discovery Life Sciences. The cells were plated at a density of 83,000 cells per well in serum-free RPMI media and incubated for 30 min at room temperature with an equal volume of 80 µg/mL unmasked c225v5 antibody prepared in serum-free RPMI media. An equal volume of AF647-labeled c225 activatable antibodies prepared at 40 µg/mL in serum-free RPMI media were then added to form a mixture and incubated at a final concentration of 20 µg/mL at 37°C for 24 hours. Cells were pelleted through centrifugation for 5 min at 300 x *g*. Supernatants were collected from each incubated mixture and transferred into a well of a 96-well PCR plate for assay by capillary electrophoresis. Each supernatant sample was mixed with Pico Sample Buffer (Perkin Elmer) containing 2-beta-mercaptoethanol at four parts sample and one part of Pico Sample Buffer and then heated at 95°C for 10 minutes. Substrate cleavage was measured by capillary electrophoresis using a LabChip® GXII Touch™ system (Perkin Elmer) with the HT Pico Protein Express 100 protocol (Perkin Elmer). Protein Express Assay LabChips (Perkin Elmer #760499) were set up using the protocol of the Protein Pico Assay Reagent Kit (Perkin Elmer #760498). The fraction of cleaved activatable antibody in AML BMMC supernatants was determined by quantifying the fraction of the higher mobility polypeptide corresponding to the cleaved activatable antibody using the LabChip® GX Reviewer software (Perkin Elmer).

[0353] As shown in FIG. 4, high LC activation is observed in the activatable antibodies with CMs 7000 and 7001 compared to the activatable antibodies with the control CMs 2001, 5007, or 3001 in AML BMMCs.

EXAMPLE 8: *In Vitro* Cleavability of Exemplary CM in a Peptide Probe Cleavage Assay

[0354] The study provided herein evaluated the cleavability kinetics (i.e., pM/s and k_{cat}/K_M ($M^{-1}s^{-1}$)) of CMs with matrix metalloprotease (MMP) 2 (MMP2), MMP9, and MMP14. The CM AIALY (SEQ ID NO: 5) was presented in an internally quenched peptide probe format, rather than included in an activatable antibody format. In the internally quenched probe, the CM sequence was positioned between a 7-methoxycoumarin-4-acetyl (MCA) fluorophore and a 2,4-dinitrophenyl (DNP) quencher such that cleavage of the CM sequence produces a fluorescence signal. The probe was of the following design: (MCA)-Gly-Ser-Ala-Ile-Ala-Leu-Tyr-Gly-Gly-Ser-Lys(DNP)-*D*-Arg (SEQ ID NO: 160). The cleavage rates (pM/s) were measured using 20 µM internally quenched peptide probe and 20

nM MMP2, MMP9, or MMP14. Cleavability kinetics (i.e., pM/s and k_{cat}/K_M ($M^{-1}s^{-1}$)) were determined in 96- or 384-well plate format at 37°C in the following buffers: 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl, 0.05% (w/v) Brij-35 for MMP2 and MMP9 or 50 mM HEPES (pH 6.8), 10 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.05% (w/v) Brij-35 for MMP14. Cleavability kinetics were determined on an Infinite 200 PRO (Tecan) multimode plate reader using a fluorescence excitation wavelength of 320 nm and an emission wavelength of 405 nm.

[0355] Table 9 provides exemplary probe cleavage rates (pM/s) of the exemplary CM AIALY (SEQ ID NO: 5) with MMP2, MMP9, and MMP14. Table 10 provides exemplary k_{cat}/K_M (M^{-1} s^{-1}) values of the exemplary CM AIALY (SEQ ID NO: 5) with MMP2, MMP9, and MMP14.

Table 9. *In Vitro* Activation of Peptide Probes with Exemplary CMs (pM/s)

Probe CM Sequence	Probe Cleavage (pM/s)		
	MMP2	MMP9	MMP14
AIALY (SEQ ID No: 5)	135	87.7	145

Table 10. *In Vitro* Activation of Peptide Probes with Exemplary CMs (k_{cat}/K_M)

Probe CM Sequence	Probe Cleavage (k_{cat}/K_M)		
	MMP2	MMP9	MMP14
AIALY (SEQ ID No: 5)	3.9×10^4	4.1×10^4	4.8×10^3

[0356] These exemplary results show that CM AIALY (SEQ ID NO: 5) is cleavable by the MMPs MMP2, MMP9, MMP14, with a k_{cat}/K_M ($M^{-1}s^{-1}$) of greater than 4.0×10^3 M^{-1} s^{-1}. These results further demonstrate AIALY (SEQ ID NO: 5) to be a minimal core required for MMP cleavage by the CMs of the present disclosure.

Table 11. Exemplary sequences

SEQ ID NO	Notes	Sequences
1	7000	AIALYAD
2	7001	AIALYA

3	7002	AIALYADG
4	7003	AIALYAG
5	7004	AIALY
6	7005	AIAL
7	7006	SAIALYAD
8	7007	SAIALYA
9	7008	RSAIALYAD
10	7009	RSAIALYA
11	7012	AIALYAH
12	7010	AIALYADH
13	7011	AIALYADHQ
14	7013	AIALYAHQ
15-72	Intentionally left blank	
73	AA w/7000 light chain	QGQSGQG**CISPRGCPDGPYVMY**GGGSSGGS**AIALYA D**GGGSQILLTQSPVILSVSPGERVSFSCRASQSIGTNIH WYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTDFTLS INSVESEDIADYYCQQNNNWPTTFGAGTKLELKRTVA APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWK VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYE KHKVYACEVTHQGLSSPVTKSFNRGEC
	AA w/7000 heavy chain	same as SEQ ID NO:84
74	ProC1589 - Light chain	QGQSGQG**CISPRGCPDGPYVMY**GGGSSGGS**AIALYA** GGGSQILLTQSPVILSVSPGERVSFSCRASQSIGTNIHW YQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTDFTLSI NSVESEDIADYYCQQNNNWPTTFGAGTKLELKRTVAA PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEK HKVYACEVTHQGLSSPVTKSFNRGEC
	ProC1589 - heavy chain	same as SEQ ID NO:84
75	1001	ISSGLLSS
76	1004	AVGLLAPP
77	0001	LSGRSDNH
78	2001	ISSGLLSGRSDNH
79	3001	AVGLLAPPGGLSGRSDNH
80	5007	APRSALAHGLF
81	EGFR Mask	CISPRGCPDGPYVMY
82	EGFR Mask	CISPRGC**L**DGPYVMY

83	CX-122 - light chain	QGQSGQ**CISPRGCPDGPYVMY**GSSGGSGGSGGSG**ISS GLLSGRSDNH**GSSGTQILLTQSPVILSVSPGERVSFSCR ASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPSRFSG SGSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGAGT KLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYP REAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
84	CX-122 - heavy chain	QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWV RQSPGKGLEWLGVIWSGGNTDYNTPFTSRLSINKDNS KSQVFFKMNSLQSQDTAIYYCARALTYYDYEFAYWG QGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS VVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK
85	CX-023 - light chain	QGQSGQ**CISPRGCPDGPYVMY**GSSGGSGGSGGSG**LS GRSDNH**GSSGTQILLTQSPVILSVSPGERVSFSCRASQSI GTNIHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSG TDFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLEL KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREA KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
86	CX-023 - heavy chain	QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWV RQSPGKGLEWLGVIWSGGNTDYNTPFTSRLSINKDNS KSQVFFKMNSLQSQDTAIYYCARALTYYDYEFAYWG QGTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS VVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK
87	AA w/5007 - light chain	QGQSGQ**CISPRGCPDGPYVMY**GGGSSGGS**APRSALA HGLF**GGGSQILLTQSPVILSVSPGERVSFSCRASQSIGT NIHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTDF TLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELKRT VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQ WKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKA DYEKHKVYACEVTHQGLSSPVTKSFNRGEC

88	AA w/5007 - heavy chain	QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWV RQSPGKGLEWLGVIWSGGNTDYNTPFTSRLSINKDNS KSQVFFKMNSLQSQDTAIYYCARALTYYDYEFAYWG QGTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS VVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK
89	CX-229 - light chain	QGQSGQ**CISPRGCLDGPYVMY**GSSGGSGGSGGSG**AV GLLAPPGGLSGRSDNH**GSSGTQILLTQSPVILSVSPGE RVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESIS GIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNNWP TTFGAGTKLELKRTVAAPSVFIFPPSDEQLKSGTASVV CLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSK DSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTK SFNRGEC
	CX-229 – heavy chain	Same as SEQ ID NO: 84
90	Intentionally left blank	
91	CTX-028 - light chain	QGQSGQ**CISPRGCPDGPYVMY**GSSGGSGGSGGSG**ISS GLLSS**GSSGTQILLTQSPVILSVSPGERVSFSCRASQSIG TNIHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGT DFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELK RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLS KADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
92	CTX-028 - heavy chain	QGQSGQCISPRGCPDGPYVMYGSSGGSGGSGGSGISSG LLSSGSSGTQILLTQSPVILSVSPGERVSFSCRASQSIGT NIHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTDF TLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELKRT VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQ WKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKA DYEKHKVYACEVTHQGLSSPVTKSFNRGEC
93	Intentionally left blank	
94	Intentionally left blank	
95	Linking peptide	GGSG
96	Linking peptide	GGSGG
97	Linking peptide	GSGSG

98	Linking peptide	GSGGG
99	Linking peptide	GGGSG
100	Linking peptide	GSSSG
101	Linking peptide	GSSGGSGGSGG
102	Linking peptide	GGGS
103	Linking peptide	GGGSGGGS
104	Linking peptide	GGGSGGGSGGGS
105	Linking peptide	GGGGSGGGGSGGGGS
106	Linking peptide	GGGGSGGGGSGGGGSGGGGSGGGGS
107	Linking peptide	GGGGSGGGGS
108	Linking peptide	GGGGS
109	Linking peptide	GGGGSGS
110	Linking peptide	GGGGSGGGGSGGGGSGS
111	Linking peptide	GGSLDPKGGGGS
112	Linking peptide	PKSCDKTHTCPPCPAPELLG
113	Linking peptide	SKYGPPCPPCPAPEFLG
114	Linking peptide	GKSSGSGSESKS
115	Linking peptide	GSTSGSGKSSEGKG
116	Linking peptide	GSTSGSGKSSEGSGSTKG
117	Linking peptide	GSTSGSGKPGSGEGSTKG
118	Linking peptide	GSTSGSGKPGSSEGST
119	Linking peptide	GGGSSGGS
120	Linking peptide	GGGGSGGGGSS
121	Linking peptide	GGGSSGGSGGSSGGS
122	Linking peptide	GSTSGSGKPGSSEGST
123	Fc domain hole mutant	ESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREE QFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPS SIEKTISKAKGQPREPQVCTLPPSQEEMTKNQVSLSCA VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL VSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLS LSLG *Note: the Fc may further comprise a lysine residue (K) at the C-terminus.
124	Fc domain knob mutant	GSSKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTP EVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPRE EQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLP SSIEKTISKAKGQPREPQVYTLPPCQEEMTKNQVSLWC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSL SLSLG* *Note: the Fc may further comprise a lysine residue (K) at the C-terminus.

125	Human IgG1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCP APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAV EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPG* *Note: the Fc may further comprise a lysine residue (K) at the C-terminus.
126	Human IgG2	ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFG TQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPAPPV AGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV QFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVV HQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREP QVYTLPPSREEMTKNQVSLTCLVKGFYPSDISVEWESN GQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPG* *Note: the Fc may further comprise a lysine residue (K) at the C-terminus.
127	Human IgG3	ASTKGPSVFPLAPCSRSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYTCNVNHKPSNTKVDKRVELKTPLGDTTHTCPR CPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCPEPKSCDTP PPCPRCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVQFKWYVDGVEVHNAKTKPREEQYNS TFRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT ISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY PSDIAVEWESSGQPENNYNTTPPMLDSDGSFFLYSKLT VDKSRWQQGNIFSCSVMHEALHNRFTQKSLSLSPG* *Note: the Fc may further comprise a lysine residue (K) at the C-terminus.
128	Human IgG4	ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG TKTYTCNVDHKPSNTKVDKRVESKYGPPCPSCPAPEFL GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEV QFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREP QVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEG NVFSCSVMHEALHNHYTQKSLSLSLG* *Note: the Fc may further comprise a lysine residue (K) at the C-terminus.
129		PRFKIIGG
130		PRFRIIGG

131		SSRHRRALD
132		RKSSIIIRMRDVVL
133		SSSFDKGKYKKGDDA
134		SSSFDKGKYKRGDDA
135		IEGR
136		IDGR
137		GGSIDGR
138		PLGLWA
139		GPQGIAGQ
140		GPQGLLGA
141		GIAGQ
142		GPLGIAGI
143		GPEGLRVG
144		YGAGLGVV
145		AGLGVVER
146		AGLGISST
147		EPQALAMS
148		QALAMSAI
149		AAYHLVSQ
150		MDAFLESS
151		ESLPVVAV
152		SAPAVESE
153		DVAQFVLT
154		VAQFVLTE
155		AQFVLTEG
156		PVQPIGPQ
157	Unmasked control c225v5 antibody heavy chain	QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWV RQSPGKGLEWLGVIWSGGNTDYNTPFTSRLSINKDNS KSQVFFKMNSLQSQDTAIYYCARALTYYDYEFAYWG QGTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS VVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK
158	Unmasked control c225v5 antibody light chain	QILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQR TNGSPRLLIKYASESISGIPSRFSGSGSGTDFTLSINSVES EDIADYYCQQNNNWPTTFGAGTKLELKRTVAAPSVFI FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNAL

		QSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVY ACEVTHQGLSSPVTKSFNRGEC

OTHER EMBODIMENTS

[0357] It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

[0358] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition section headings, the materials, methods, and examples are illustrative only and not intended to be limiting.

WHAT IS CLAIMED IS:

1. An isolated polypeptide comprising a cleavable moiety (CM) that comprises the amino acid sequence AIALY (SEQ ID NO: 5), wherein the CM is a substrate for a protease.

2. The isolated polypeptide of claim 1, wherein the CM comprises the amino acid sequence AIALYA (SEQ ID NO: 2) or AIALYAD (SEQ ID NO: 1).

3. An isolated polypeptide comprising a cleavable moiety (CM) that comprises an amino acid sequence selected from SEQ ID NOs: 1-14, wherein the CM is a substrate for a protease.

4. An isolated polypeptide comprising a cleavable moiety (CM) comprising an amino acid sequence with one-amino acid or two-amino acid mutation(s) in any one of SEQ ID NOs: 1-14, wherein the CM is a substrate for a protease.

5. The isolated polypeptide of any one of claims 1-4, wherein the isolated polypeptide is an activatable molecule and further comprises an active moiety (AM) that specifically binds a target.

6. The isolated polypeptide of claim 5, wherein the AM is an antibody or antigen binding fragment thereof.

7. The isolated polypeptide of claim 6, wherein the antigen binding fragment is selected from the group consisting of a Fab fragment, a $F(ab')_2$ fragment, a scFv, a scAb, a dAb, a single domain heavy chain antibody, and a single domain light chain antibody.

8. The isolated polypeptide of claim 5, wherein the AM is a therapeutic macromolecule.

9. The isolated polypeptide of claim 5, wherein the AM is a cytokine or a chimeric antigen receptor.

10. The isolated polypeptide of any one of claims 5-9, wherein the AM is coupled to the CM.

11. The isolated polypeptide of claim 10, wherein the AM is directly coupled to the CM.

12. The isolated polypeptide of claim 10, wherein the AM is indirectly coupled to the CM via a linking peptide.

13. The isolated polypeptide of any one of claims 5-12, further comprising a masking moiety (MM).

14. The isolated polypeptide of claim 13, wherein the MM has a dissociation constant for binding to the AM that is greater than the dissociation constant of the AM for binding to the target.

15. The isolated polypeptide of claim 13 or 14, wherein the MM is 2 to 40 amino acids in length.

16. The isolated polypeptide of any one of claims 13-15, wherein the MM is coupled to the CM such that the isolated polypeptide comprises the structural arrangement from N-terminus to C-terminus as follows: MM-CM-AM or AM-CM-MM.

17. The isolated polypeptide of claim 16, wherein the MM is coupled directly to the CM.

18. The isolated polypeptide of claim 16, wherein the MM is coupled indirectly to the CM via a linking peptide.

19. The isolated polypeptide of any one of claims 13-16 and 18, wherein the isolated polypeptide comprises a first linking peptide (LP1) and a second linking peptide

(LP2), and wherein the isolated polypeptide has the structural arrangement from N-terminus to C-terminus as follows: MM-LP1-CM-LP2-AM or AM-LP2-CM-LP1-MM.

20. The isolated polypeptide of claim 19, wherein the LP1 and LP2 are not identical to each other.

21. The isolated polypeptide of claim 19, wherein the LP1 and LP2 are identical to each other.

22. The isolated polypeptide of any one of claims 19-21, wherein each of LP1 and LP2 is a peptide of 1 to 20 amino acids in length.

23. The isolated polypeptide of any one of claims 1-22, wherein the CM is a substrate for a matrix metalloproteinase (MMP).

24. The isolated polypeptide of claim 23, wherein the MMP is MMP2, MMP9, or MMP14.

25. The isolated polypeptide of claim 24, wherein the k_{cat}/K_M of the CM by MMP2 cleavage is at least 1×10^3 $M^{-1}s^{-1}$.

26. The isolated polypeptide of claim 24, wherein the k_{cat}/K_M of the CM by MMP2 cleavage is at least 1×10^4 $M^{-1}s^{-1}$.

27. The isolated polypeptide of any one of claims 24-26, wherein the k_{cat}/K_M of the CM by MMP9 cleavage is at least 1×10^2 $M^{-1}s^{-1}$.

28. The isolated polypeptide of any one of claims 24-26, wherein the k_{cat}/K_M of the CM by MMP9 cleavage is at least 1×10^3 $M^{-1}s^{-1}$.

29. The isolated polypeptide of any one of claims 24-28, wherein the k_{cat}/K_M of the CM by MMP14 cleavage is at least 1×10^2 $M^{-1}s^{-1}$.

30. The isolated polypeptide of any one of claims 24-29, wherein the k_{cat}/K_M of the CM by MMP14 cleavage is at least 1×10^3 $M^{-1}s^{-1}$.

31. The isolated polypeptide of any one of claims 1-30, further comprising one or more additional cleavable moieties, optionally wherein at least a portion of the CM overlaps with at least a portion of a second cleavable moiety.

32. A polypeptide complex comprising one or more of the isolated polypeptides of any one of claims 1-31 bound to a second isolated polypeptide.

33. A conjugated polypeptide comprising the isolated polypeptide of any one of claims 1-31 conjugated to an agent.

34. The conjugated polypeptide of claim 33, wherein the agent is conjugated to the isolated polypeptide via a conjugating linker.

35. The conjugated polypeptide of claim 34, wherein the conjugating linker is cleavable.

36. The conjugated polypeptide of claim 34, wherein the conjugating linker is non-cleavable.

37. The conjugated polypeptide of claim 34, wherein the conjugating linker comprises an amino acid sequence selected from SEQ ID NOs: 1-14.

38. The conjugated polypeptide of any one of claims 33-37, wherein the agent is a toxin, a microtubule inhibitor, a nucleic acid damaging agent, a dolastatin, an auristatin, a maytansinoid, a duocarmycin, a calicheamicin, or a combination thereof.

39. A composition comprising the isolated polypeptide of any one of claims 1-31, the polypeptide complex of claim 32, or the conjugated polypeptide of any one of claims 33-38, and a carrier.

40. The composition of claim 39, wherein the carrier is a pharmaceutically acceptable carrier.

41. The composition of claim 39 or 40, comprising an additional agent.

42. The composition of claim 41, wherein the additional agent is a therapeutic agent.

43. An isolated nucleic acid molecule encoding the isolated polypeptide of any one of claims 1-31.

44. A vector comprising the isolated nucleic acid molecule of claim 43.

45. A cell comprising the isolated polypeptide of any one of claims 1-31, the isolated nucleic acid molecule of claim 43 or the vector of claim 44.

46. A method of manufacturing an activatable molecule that contains a cleavable moiety (CM), the method comprising expressing and recovering an activatable molecule comprising the isolated polypeptide of any one of claims 1-31.

47. A method of treating, alleviating a symptom of, or delaying the progression of a disease or disorder in a subject, comprising administering a therapeutically effective amount of the isolated polypeptide of any one of claims 1-31, the polypeptide complex of claim 32, or the conjugated polypeptide of any one of claims 33-38, the composition of any one of claims 39-42, the nucleic acid molecule of claim 43, the vector of claim 44, or the cell of claim 45 to the subject.

48. The method of claim 47, wherein the disease is a cancer, an infection, an inflammatory disorder, a cardiovascular disorder, a neurodegenerative disorder, or an autoimmune disorder.

49. The isolated polypeptide of any one of claims 1-31, the polypeptide complex of claim 32, or the conjugated polypeptide of any one of claims 33-38, the composition

of any one of claims 39-42, the nucleic acid molecule of claim 43, the vector of claim 44, or the cell of claim 45 for use as a medicament or for use in therapy, optionally for treating a cancer, an infection, an inflammatory disorder, a cardiovascular disorder, a neurodegenerative disorder, or an autoimmune disorder, optionally with an additional agent which is optionally a therapeutic agent.

50. A method of detecting or diagnosing a disease or health condition of a subject, comprising:

contacting the isolated polypeptide of any one of claims 1-31, the polypeptide complex of claim 32, or the conjugated polypeptide of any one of claims 33-38, or the composition of any one of claims 39-42 with a sample from the subject; and

measuring a level of cleavage of the isolated polypeptide, thereby detecting or diagnosing the disease or health condition of the subject.

51. The method of claim 49, wherein the disease is a cancer, an infection, an inflammatory disorder, a cardiovascular disorder, a neurodegenerative disorder, or an autoimmune disorder.

***In vitro* Masking Efficiency of anti-EGFR Activatable Antibodies with Exemplary Substrate**

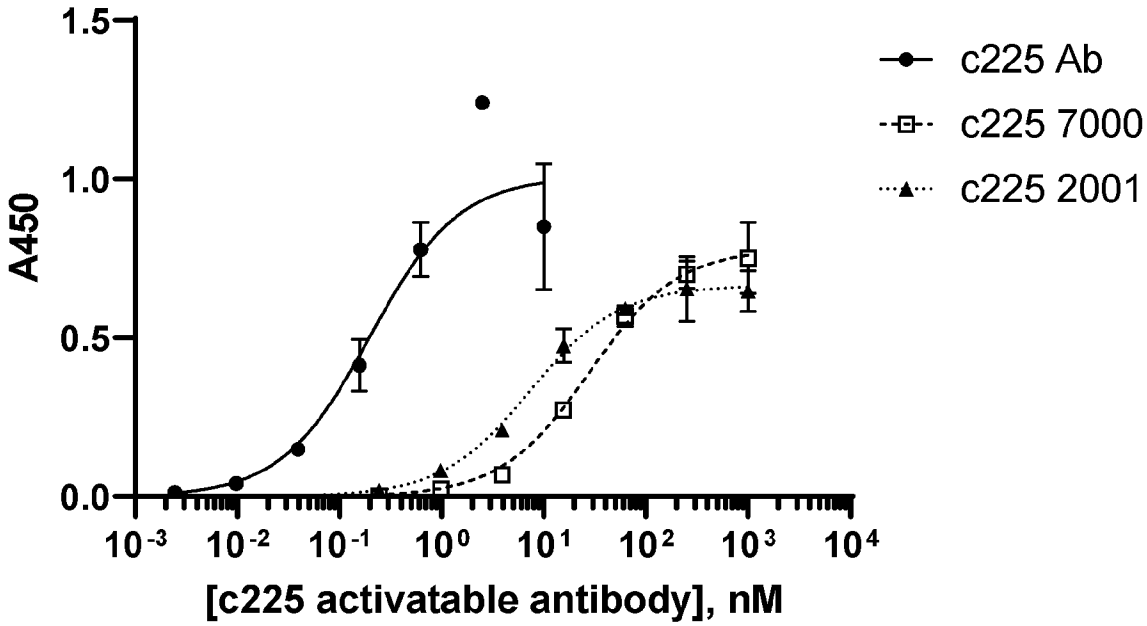

FIG. 1

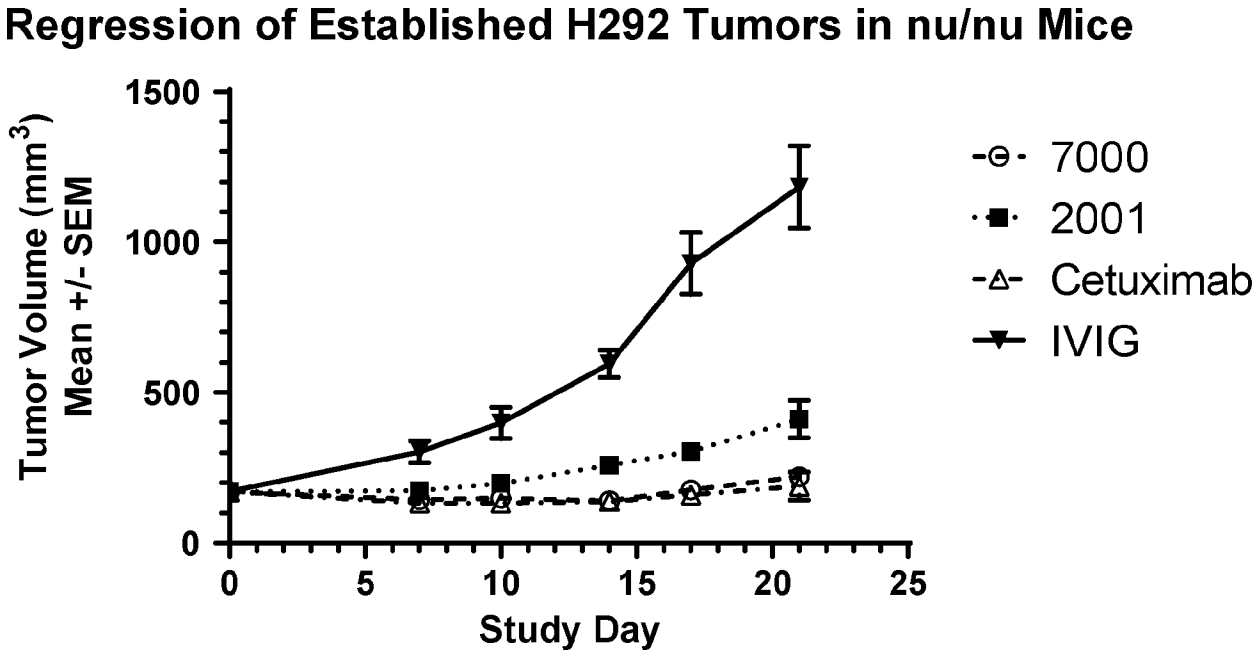
Regression of Established H292 Tumors in nu/nu Mice
Tumor Volume (mm3)
Mean +/- SEM
1500
1000
500
0
0
5
10
15
20
25
Study Day
7000
2001
Cetuximab
IVIG

FIG. 2A

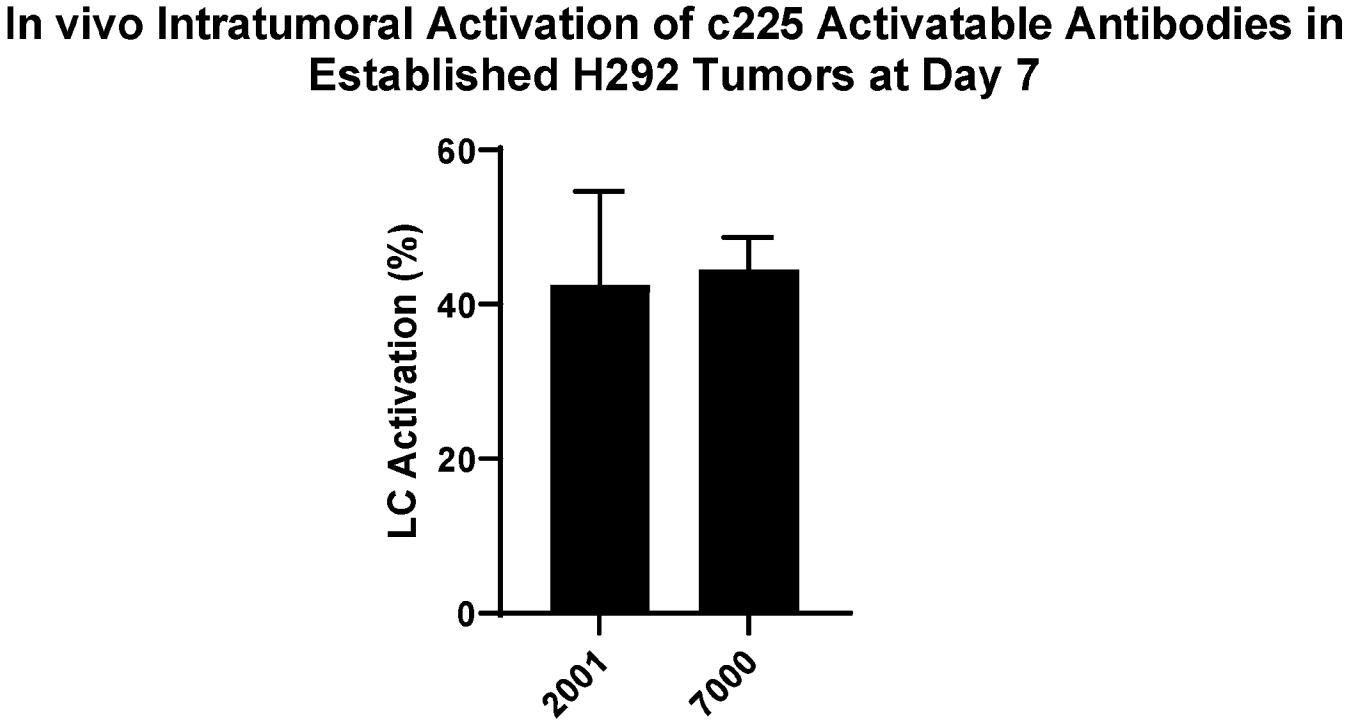
In vivo Intratumoral Activation of c225 Activatable Antibodies in Established H292 Tumors at Day 7
LC Activation (%)
60
40
20
0
2001
7000

FIG. 2B

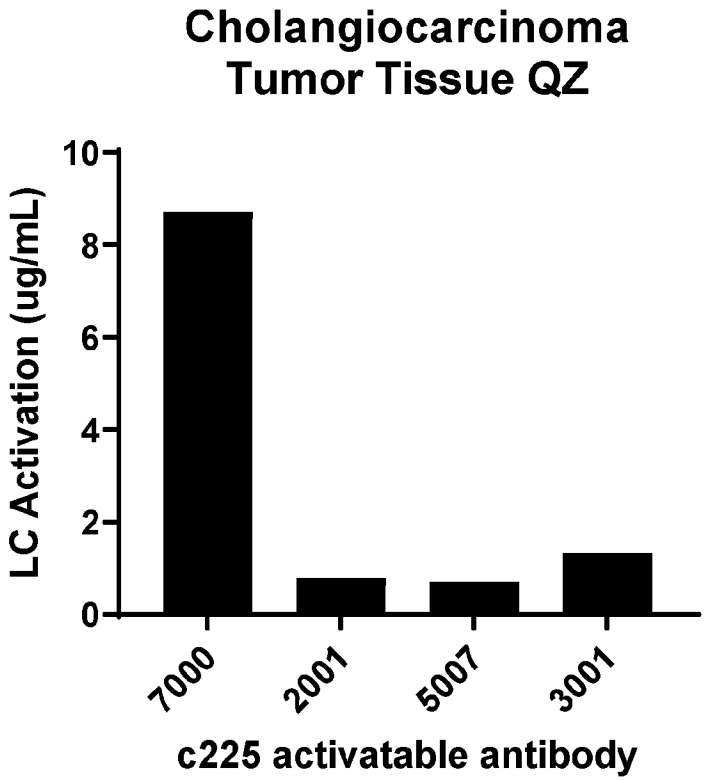
Cholangiocarcinoma
Tumor Tissue QZ
LC Activation (ug/mL)
10
8
6
4
2
0
7000
2001
5007
3001
c225 activatable antibody

FIG. 3A

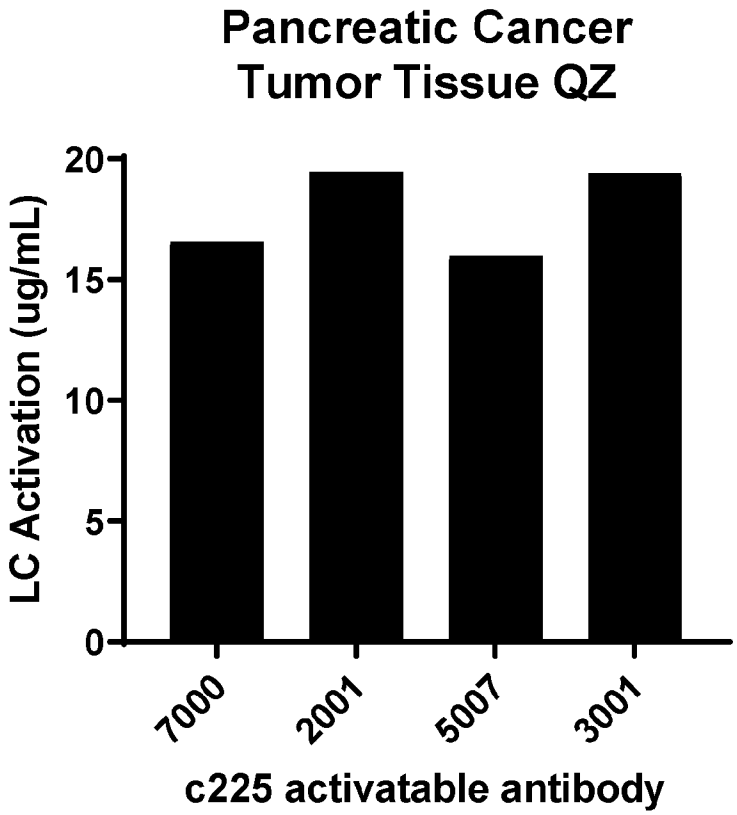
Pancreatic Cancer
Tumor Tissue QZ
LC Activation (ug/mL)
20
15
10
5
0
7000
2001
5007
3001
c225 activatable antibody

FIG. 3B

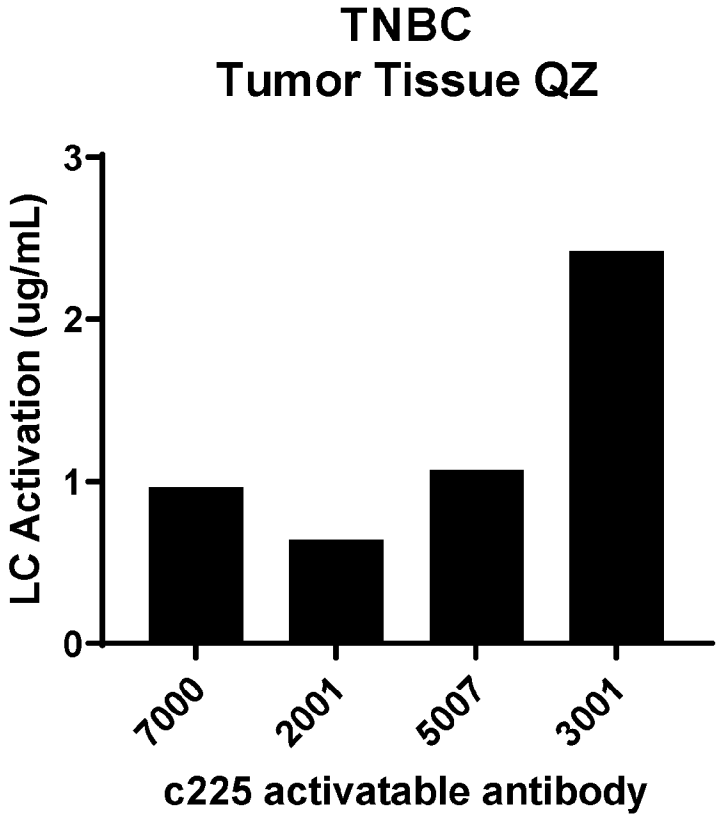
TNBC
Tumor Tissue QZ
LC Activation (ug/mL)
0
1
2
3
7000
2001
5007
3001
c225 activatable antibody

FIG. 3C

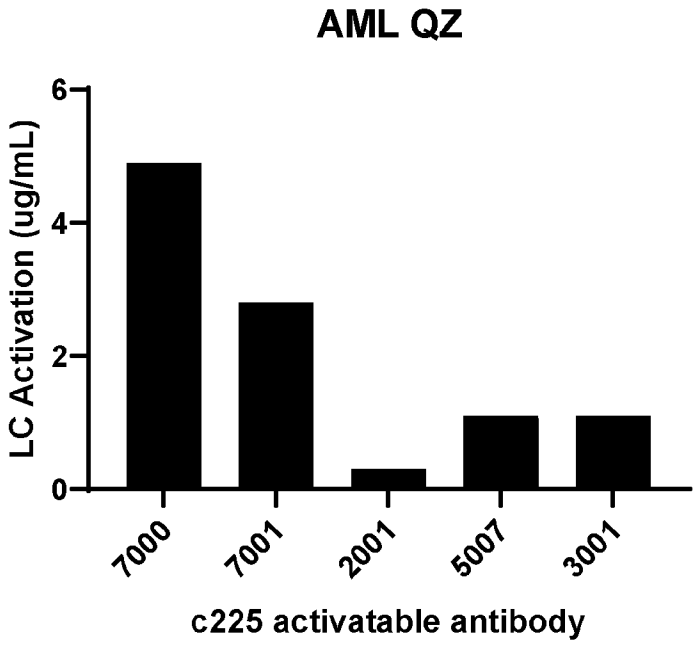
AML QZ
LC Activation (ug/mL)
0
2
4
6
7000
7001
2001
5007
3001
c225 activatable antibody

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2023/071301

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/00 A61K38/17 C12N9/64 C12N15/62
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 C07K

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, WPI Data, EMBL, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE UniProt [Online] 22 November 2017 (2017-11-22), "SubName: Full=Ribosomal-protein-alanine acetyltransferase {ECO:0000313¦EMBL:OUW57190.1};", XP93095919, retrieved from EBI accession no. UNIPROT:A0A1Z9LPE2 Database accession no. A0A1Z9LPE2 sequence ----- -/--	1-4, 23-31, 43-45

[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
30 October 2023	13/11/2023

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	Kools, Patrick

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE UniProt [Online] 16 October 2019 (2019-10-16), "RecName: Full=Phosphoribosylglycinamide formyltransferase {ECO:0000256|HAMAP-Rule:MF_01930}; EC=2.1.2.2 {ECO:0000256|HAMAP-Rule:MF_01930}; AltName: Full=5'-phosphoribosylglycinamide transformylase {ECO:0000256|HAMAP-Rule:MF_01930}; AltName: Full=GAR transformylase {ECO:0000256|HAMAP-Rule:MF_01930};", XP93095967, retrieved from EBI accession no. UNIPROT:A0A518GXK2 Database accession no. A0A518GXK2 sequence ----- DATABASE UniProt [Online]	1-4, 23-31, 43-45
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE UniProt [Online] 31 July 2019 (2019-07-31), "RecName: Full=DUF2730 family protein {ECO:0008006|Google:ProtNLM};", XP93095984, retrieved from EBI accession no. UNIPROT:A0A4V2F418 Database accession no. A0A4V2F418 sequence ----- DATABASE UniProt [Online] 29 September 2021 (2021-09-29), "RecName: Full=histidine kinase {ECO:0000256|ARBA:ARBA00012438}; EC=2.7.13.3 {ECO:0000256|ARBA:ARBA00012438};", XP93095987, retrieved from EBI accession no. UNIPROT:A0A838VSQ7 Database accession no. A0A838VSQ7 sequence -----	1-4, 23-31, 43-45 1-4, 23-31, 43-45
X	WO 2020/118109 A2 (CYTOMX THERAPEUTICS INC [US]) 11 June 2020 (2020-06-11) cited in the application Whole document, especially the claims, sequences on page 2 and 3. -----	1-51
X	WO 2016/118629 A1 (CYTOMX THERAPEUTICS INC [US]) 28 July 2016 (2016-07-28) cited in the application Whole document, especially the claims. -----	1-51
X	WO 2015/048329 A2 (CYTOMX THERAPEUTICS INC [US]) 2 April 2015 (2015-04-02) cited in the application Whole document, especially the claims. -----	1-51
A	ELTER ADRIAN ET AL: "Protease-Activation of Fc-Masked Therapeutic Antibodies to Alleviate Off-Tumor Cytotoxicity", FRONTIERS IN IMMUNOLOGY, vol. 12, 3 August 2021 (2021-08-03), XP093048541, DOI: 10.3389/fimmu.2021.715719 the whole document ----- -/--	1-51

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LUCCHI ROBERTA ET AL: "The Masking Game: Design of Activatable Antibodies and Mimetics for Selective Therapeutics and Cell Control", ACS CENTRAL SCIENCE, vol. 7, no. 5, 26 April 2021 (2021-04-26), pages 724-738, XP055827094, ISSN: 2374-7943, DOI: 10.1021/acscentsci.0c01448 the whole document	1-51
A	MUSKAN KUKREJA: "The high throughput multiplexed peptide-centric profiling illustrates both the substrate cleavage redundancy and specificity in the MMP family", CHEM BIOL, vol. 22, no. 8, 20 August 2015 (2015-08-20), pages 1122-1133, XP055666051, DOI: 10.1016/j.chembiol.2015.07.008 the whole document	1-51

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2023/071301

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2023/071301

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