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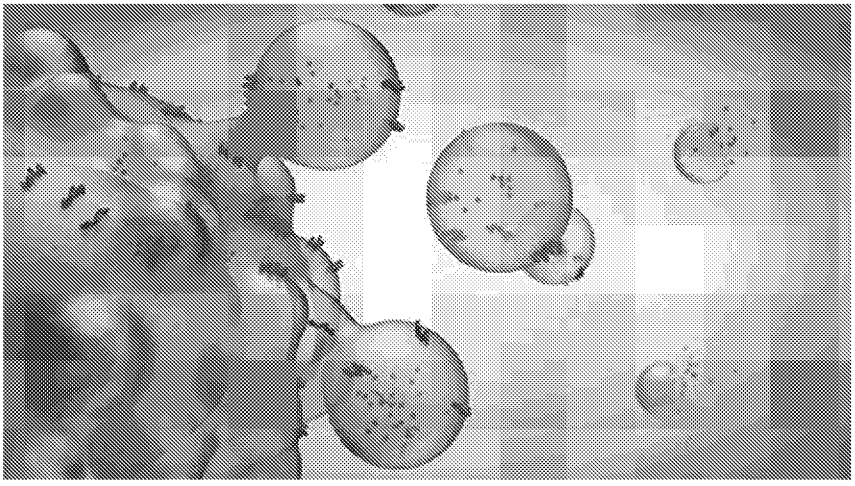

FIG. 1

(57) Abstract: The disclosure provides methods to chemically and photochemically initiate cell membrane blebbing to induce cell vesicle production, modifications thereof, and uses thereof, including for drug delivery, gene therapy, cell-free cell therapy, and molecular therapy.

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CHEMICALLY AND PHOTOCHEMICALLY INITIATED CELL MEMBRANE BLEBBING TO INDUCE CELL VESICLE PRODUCTION, MODIFICATIONS THEREOF, AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. §119 from Provisional Application Serial Nos. 62/765,034, 62/765,064, 62/765,102, and 62/765,063, which were filed on August 16, 2018, and from Provisional Application Serial No. 62/735,830, filed on September 24, 2018, the disclosures of which are incorporated herein by reference.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with Government support under Grant No. DGE-1321846, awarded by the National Science Foundation, and Grant No. 5T32AI7319-29, awarded by the National Institutes of Health. The Government has certain rights in the invention.

TECHNICAL FIELD

[0003] The disclosure provides methods to chemically and photochemically initiate cell membrane blebbing to induce cell vesicle production, modifications thereof, and uses thereof, including for drug delivery, gene therapy, cell-free cell therapy, and molecular therapy.

BACKGROUND

[0004] The field of drug delivery relies on nanometer scale carriers for effective delivery of cargo to a designated target site. These carriers play two roles: to protect cargo during transport and release cargo at the appropriate site without inducing immunogenic response. While many nano-carriers have been designed, issues with immunogenicity, toxicity, poor biocompatibility, and low efficacy of delivery still remain major challenges in the field.

[0005] Viral gene therapy is a method to directly target mutations at a molecular level. Its ability to selectively treat mutated cells offers opportunities for decreased off target effects and increased efficacy over traditional treatments. However, the major hurdle in this treatment is designing the ideal delivery vehicle, one that is effective but also safe. Viruses are effective at delivering genes into the nucleus of the cell, but are easily recognized by the immune system, which can lead to increased side effects and rapid clearance. Adeno-associated virus (AAV) has been used increasingly as a promising vector for viral gene therapy. AAV is a small, non-enveloped virus that can transduce both dividing and quiescent

cells, making it useful for many applications in viral gene therapy. The host's immune response to AAV is not a systemic response, and is limited to neutralizing antibodies, which leads to clearance, but no side effects. AAV is also nonpathogenic and therefore generally regarded as safe. Therefore, AAV has great potential in viral gene therapy if shielded from the immune system during transport.

[0006] Cancer cells survive and proliferate by several mechanisms including immune evasion. Immunotherapy overcomes this by activating the immune system to eliminate cancer cells. The most common strategy for activating the immune system against tumor cells is vaccination with tumor antigens. Previous studies have reported that bone marrow dendritic cells (BMDCs) pulsed with an antigen *ex vivo* are able to act as an anti-cancer vaccine, presenting an antigen *in vivo* and stimulating T cell response to eradicate tumor cells. However, intrinsic variability associated with whole-cell vaccine formulations has made them non-ideal candidates for immunotherapy.

SUMMARY

[0007] Extracellular vesicles (EVs) are cell membrane derived particles that show significant promise in the field of drug delivery as micro- and nano-scale carrier agents. Despite their high potential, EV-based therapeutics have been slow to progress to clinical trials due to issues with mass production. It was found herein that mass-scale production of micro and nanoscale cell vesicles could be induced by use of a sulfhydryl blocking agent or a photosensitizer that promotes cell blebbing. The techniques presented herein induce cell vesicle formation significantly faster, more efficiently, and in a higher yield than current vesicle production techniques.

[0008] Adeno-associated virus has become an increasingly popular vector for viral gene therapy. Free-AAV in the body, however, will be recognized by antibodies, resulting in clearance. Therefore, in order to use AAV, a shield or carrier is important. Under normal cellular processes, extracellular vesicles can incorporate AAV thereby shielding AAV in the host's body and providing for enhanced efficacy, however, the production yields of such vesicles is extremely low. The disclosure provides for methods and agents which overcome the forgoing challenges by (1) the use of an AIPCS2A photosensitizer and 670nm light, or (2) the use of sulfhydryl blocking agent to initiate mass-scale production of micro and nanoscale AAV-containing cell vesicles. The methods and techniques of the disclosure induce cell vesicle formation faster, more efficiently, and at higher yields compared to current AAV-containing-vesicle production techniques.

[0009] The methods of the disclosure also provide for modification of induced cell vesicles (ICVs) described herein by oxime ligation of aminooxy-functionalized molecules with aldehyde groups of oxidized sialylated glycoproteins on the cell surface of producer or host cells. As mammalian cells naturally display sialic acids on their surface of their cells, albeit at different levels (*e.g.*, particularly elevated sialic acid expression by cancer cells), the methods disclosed herein, by using bioorthogonal cell surface modification, generated molecularly functionalized cell vesicles in a simple, fast, and efficient way.

[0010] Antigen-pulsed bone marrow dendritic cells have shown promise as cancer vaccines. Variability in production of cell-based vaccines is problematic, and whole cell vaccines are challenging to store efficiently. Extracellular vesicles derived from immune cells are promising cancer vaccines, but extracellular vesicle-based immunotherapy has not progressed to clinical trials in part due to issues with heterogeneity. In the studies presented herein, cell vesicles induced from bone marrow dendritic cells using the methods of disclosure provided for antigenic cellular vesicles that could be locked at a specific maturation stage, or antigenic profile. The use of such antigenic cell vesicles could be used for cell-free therapies, including for cancer vaccination. It was shown herein, that antigenic mICVs produced by sulfhydryl blocking, maintained maturation characteristics of their parent cells, leading to enhanced immunotherapy outcomes. In tumor-challenged C57BL/6 mice, antigenic mICVs performed as well as whole cell therapy in terms of slowing tumor growth and improving survival while providing a safer, cell-free alternative.

[0011] As shown in the studies presented herein, the disclosure provides for a platform technology for the production of ICVs from cells using the blebbing methods and techniques described herein. In particular, the ICVs can be produced from nearly any type of cell, in any state of maturation or activation, in a highly efficient and rapid manner, and in mass scale. Further, the studies presented herein establish that the blebbing methods and techniques of the disclosure can be tailored to control the size of ICVs produced (e.g., from 10 nm to 10 μm) from the cells; and also demonstrate that the surfaces of the ICVs can be engineered using synthetic techniques (*e.g.*, bioorthogonal conjugation) to add additional functionalities to the ICVs, such as modifying the surface of the ICVs to comprise targeting and therapeutic moieties. The blebbing methods and techniques described herein thus allow for designing or tailoring ICVs to treat specific disease indications by customizing the ICVs with various surface proteins and/or cargoes. As such, an engineering biology approach can be used to iteratively design, build, and test 'customized' ICVs to treat specific diseases and

conditions by using synthetic biology to vary the surface composition of the ICVs and/or cargoes. These 'customized' ICVs would be expected to have higher potencies and efficacies for the targeted diseases and conditions than standardly used modalities. Moreover, these 'customized' ICVs can be used in any number of formulations to test the effectiveness of the 'customized' ICVs in any number of different types of disease models ranging from cancer to autoimmune disease to gene therapy, etc. Thus, the blebbing methods and techniques described herein provide tremendous flexibility to develop unique ICVs formulations that are highly specific and potent for a given disease indication.

In a particular embodiment, the disclosure provides a method to produce induced cell vesicles (ICVs) or antigenic ICVs, comprising: inducing cell vesicle production from cells by exposing or contacting the cells with a cell blebbing buffer which comprises a sulfhydryl blocking agent or a photosensitizer; wherein antigenic ICVs are produced from antigen presenting cells which can stimulate T-cell activation. In a further embodiment, the cells are from a mammal. In yet a further embodiment of any of the foregoing embodiments, the cells are from a human. In yet a further embodiment of any of the foregoing embodiments, the cells are from a human patient that has a disorder or disease that is to be treated with ICVs or antigenic ICVs produced therefrom. In yet a further embodiment of any of the foregoing embodiments, the cells are selected from epithelial cells, fibroblast cells, tumor cells, mast cells, T and B lymphocytes, dendritic cells, and Langerhans cells. In yet a further embodiment of any of the foregoing embodiments, the antigenic ICVs are produced from dendritic cells. In yet a further embodiment of any of the foregoing embodiments, the dendritic cells are bone marrow dendritic cells (BMDCs). In yet a further embodiment of any of the foregoing embodiments, wherein the BMDCs are immature BMDCs. In yet a further embodiment of any of the foregoing embodiments, wherein the BMDCs are mature BMDCs. In yet a further embodiment of any of the foregoing embodiments, wherein the induced cell vesicles comprise viruses, viral particles, or viral vectors, by being produced from cells comprising the same. In yet a further embodiment of any of the foregoing embodiments, wherein the viruses, viral particles, or viral vectors are selected from recombinant retroviruses, adenoviruses, adeno-associated viruses (AAV), alphaviruses, and lentiviruses. In yet a further embodiment of any of the foregoing embodiments, wherein the viruses, viral particles, or viral vectors are AAV. In yet a further embodiment of any of the foregoing embodiments, wherein the AAV expresses a heterologous transgene that is used for gene therapy. In yet a further embodiment of any of the foregoing embodiments, wherein the cell

blebbing buffer does not contain any small molecule redox reagents or reducing agents. In yet a further embodiment of any of the foregoing embodiments, wherein the cell blebbing buffer comprises a buffered balanced salt solution. In yet a further embodiment of any of the foregoing embodiments, wherein the buffered balanced salt solution selected from the group consisting of phosphate-buffered saline (PBS), Dulbecco's Phosphate-buffered saline (DPBS), Earle's Balanced Salt solution (EBSS), Hank's Balanced Salt Solution (HBSS), TRIS-buffered saline (TBS), and Ringer's balanced salt solution (RBSS). In yet a further embodiment of any of the foregoing embodiments, wherein the cell blebbing buffer comprises a 1X to 10X concentration/dilution of the buffered balanced salt solution. In yet a further embodiment of any of the foregoing embodiments, wherein the cell blebbing buffer comprises a 1X to 5X concentration/dilution of DPBS. In yet a further embodiment of any of the foregoing embodiments, wherein the cells are incubated in the cell blebbing buffer which comprises a sulfhydryl blocking agent for 0.5 h to 48 h. In yet a further embodiment of any of the foregoing embodiments, wherein the cells are incubated in the cell blebbing buffer for 1 h to 8 h at 37 °C. In yet a further embodiment of any of the foregoing embodiments, wherein the sulfhydryl blocking agent is selected from the group consisting of and *N*-ethylmaleimide, paraformaldehyde, mercury chloride, p-chloromercuribenzene sulfonic acid, auric chloride, *p*-chloromercuribenzoate, chlormerodrin, meralluride sodium, and iodoacetamide. In yet a further embodiment of any of the foregoing embodiments, wherein the sulfhydryl blocking agent is *N*-ethylmaleimide (NEM) or maleimide. In yet a further embodiment of any of the foregoing embodiments, wherein the cell blebbing buffer comprises from 1 mM to 10 mM of NEM. In yet a further embodiment of any of the foregoing embodiments, wherein the cell blebbing buffer consists essentially of 2 mM NEM in DPBS. In yet a further embodiment of any of the foregoing embodiments, wherein the cell blebbing buffer comprises paraformaldehyde. In yet a further embodiment of any of the foregoing embodiments, wherein the cell blebbing buffer comprises 25 mM of paraformaldehyde. In yet a further embodiment of any of the foregoing embodiments, wherein the cells are incubated with or exposed to a photosensitizer having a concentration of 0.5 ug/mL to 5.0 ug/mL. In yet a further embodiment of any of the foregoing embodiments, wherein the cells are incubated with or exposed to a photosensitizer having a concentration of 1.0 ug/mL. In yet a further embodiment of any of the foregoing embodiments, wherein the cells are exposed or incubated with the photosensitizer for 1 h to 48 h. In yet a further embodiment of any of the foregoing embodiments, wherein the cells are exposed to or incubated with the photosensitizer for 24 h

at 37° C. In yet a further embodiment of any of the foregoing embodiments, wherein the photosensitizer is a porphyrin, chlorin or a dye. In yet a further embodiment of any of the foregoing embodiments, wherein the photosensitizer is selected from $AlPcS_{2A}$, $AlPcS_4$, lutrin, 5-aminolevulinic acid (ALA), hypericin, silicon phthalocyanine zinc (II) phthalocyanine (ZnPc), silicon phthalocyanine, mono-L-aspartyl chlorin e6, benzoporphyrin derivative monoacid ring A, chlorin photosensitizer tin etiopurpurin, tetra(*m*-hydroxyphenyl)chlorin, lutetium texaphyrin, 9-acetoxy-2,7,12,17-tetrakis-(*β*-methoxyethyl)-porphycene, naphthalocyanines, Allumera®, Photofrin®, Visudyne®, Levulan®, Foscan®, Fospeg®, Metvix®, Hexvix®, Cysview® and Laserphyrin®, Antrin®, Photochlor®, Photosens®, Photrex®, Lumacan®, Cevira®, Visonac®, BF-200 ALA®, Amphinex® and Azadipyrromethenes. In yet a further embodiment of any of the foregoing embodiments, wherein the photosensitizer is $AlPcS_{2A}$. In yet a further embodiment of any of the foregoing embodiments, wherein the cells are washed with a buffered balanced salt solution one or more times, and taken up in the buffered balanced salt solution prior to exposure to light. In yet a further embodiment of any of the foregoing embodiments, wherein the buffered balanced salt solution selected from the group consisting of phosphate-buffered saline (PBS), Dulbecco's Phosphate-buffered saline (DPBS), Earle's Balanced Salt solution (EBSS), Hank's Balanced Salt Solution (HBSS), TRIS-buffered saline (TBS), and Ringer's balanced salt solution (RBSS). In yet a further embodiment of any of the foregoing embodiments, wherein the cells are taken up in 1X to 10X concentration/dilution of the buffered balanced salt solution prior to light exposure. In yet a further embodiment of any of the foregoing embodiments, wherein the cells are taken up in 1X DPBS prior to light exposure. In yet a further embodiment of any of the foregoing embodiments, wherein the cells are exposed to light for 1 min to 60 min. In yet a further embodiment of any of the foregoing embodiments, wherein the cells are exposed to light generated by a laser. In yet a further embodiment of any of the foregoing embodiments, wherein the cells are exposed to light having a wavelength from 600 nm to 850 nm that is generated by a laser. In yet a further embodiment of any of the foregoing embodiments, wherein the EB producing cells are incubated with or exposed to 1 ug/mL of $AlPcS_{2A}$ for 24 h at 37 °C, washed multiple times in 1X DPBS, taken up in 1X DPBS, and then exposed to light generated from a 670 nm laser for 1 min to 10 min. In yet a further embodiment of any of the foregoing embodiments, wherein the method further comprises the step of: purifying/isolating the ICVs or antigenic ICVs by using sucrose gradients. In yet a further embodiment of any of the foregoing embodiments, wherein the

method further comprises the step of: purifying/isolating the ICVs or the antigenic ICVs by: (i) removing cellular debris by centrifugation from 1,000 rpm to 2,500 rpm for 1 to 10 minutes; and (ii) recovering the ICVs or antigenic ICVs by centrifugation using 10,000 x g to 20,000 x g for 5 to 15 minutes; optionally, concentrating the recovered nanometer sized ICVs or antigenic nanometer sized ICVs by using concentrators with a pore size cut-off from 50 to 150 kDA. In yet a further embodiment of any of the foregoing embodiments, wherein the isolated ICVs or isolated antigenic ICVs have average diameters from 10 nm to 10,000 nm. In yet a further embodiment of any of the foregoing embodiments, wherein the isolated ICVs or isolated antigenic ICVs have average diameters from 150 nm to 5,000 nm. In yet a further embodiment of any of the foregoing embodiments, wherein the isolated ICVs or isolated antigenic ICVs have average diameters from 1000 nm to 5,000 nm. In yet a further embodiment of any of the foregoing embodiments, wherein the isolated ICVs or isolated antigenic ICVs comprise a cargo selected from biological molecules, therapeutic agents, prodrugs, gene silencing agents, chemotherapeutics, diagnostic agents, components of a gene therapy system and/or components of a gene editing system. In yet a further embodiment of any of the foregoing embodiments, wherein the isolated ICVs or isolated antigenic ICVs are loaded with the cargo by direct membrane penetration, chemical labeling and conjugation, electrostatic coating, adsorption, absorption, sonification, electroporation, use of pH gradients, or any combination thereof. In yet a further embodiment of any of the foregoing embodiments, wherein the isolated ICVs or isolated antigenic ICVs are loaded with the cargo by incubating isolated ICVs or isolated antigenic ICVs, or the cells used to produce ICVs or antigenic ICVs, with a cargo for a sufficient period of time to allow uptake or adsorption of the cargo by the ICVs, antigenic ICVs or by the cells. In yet a further embodiment of any of the foregoing embodiments, wherein the cells comprise or have been modified to comprise one or more functional moieties on the cell surface. In yet a further embodiment of any of the foregoing embodiments, wherein the one or more functional moieties are one or more targeting ligands. In yet a further embodiment of any of the foregoing embodiments, wherein the one or more targeting ligands direct the ICVs or antigenic ICVs to a certain cell, cell type, tissue type, tumor, or organ. In yet a further embodiment of any of the foregoing embodiments, wherein the one or more targeting ligands are an antibody or a single-chain variable fragment which binds to a tumor-specific antigen. In yet a further embodiment of any of the foregoing embodiments, wherein the tumor-specific antigen is selected from alphafetoprotein (AFP), carcinoembryonic antigen (CEA), CA-125, CA15-3, CA19-9, MUC-

1, epithelial tumor antigen (ETA), tyrosinase, melanoma-associated antigen (MAGE), abnormal products of ras or p53, CTAG1B, MAGEA1, and HER2/neu. In yet a further embodiment of any of the foregoing embodiments, wherein the cells have been bioorthogonally-conjugated to comprise one or more functional moieties. In yet a further embodiment of any of the foregoing embodiments, wherein the one or more functional moieties have been added to the surface of the cells by bioorthogonally-engineering, comprising: (1) treating sialic acid residues on the surface of the cells with an oxidizing agent to form aldehyde groups; then either step (2)(a) and (b), or step (3)(a) and (3)(b): (2)(a) ligating, linking or conjugating aminooxy-functionalized molecules to the surface of the cells by forming oxime bonds with the aldehyde groups; and (2)(b) inducing production of bioorthogonally-conjugated ICVs or bioorthogonally-conjugated antigenic ICVs by exposing or contacting the cells with the cell blebbing buffer which comprises a sulfhydryl blocking agent or a photosensitizer; or (3)(a) inducing production of ICVs or antigenic ICVs from the cells by exposing or contacting the cells with the cell blebbing buffer which comprises a sulfhydryl blocking agent or a photosensitizer; and (3)(b) producing bioorthogonally-conjugated ICVs or bioorthogonally-conjugated antigenic ICVs by ligating, linking or conjugating aminooxy-functionalized molecules to the surface of the ICVs by forming oxime bonds with the aldehyde groups. In yet a further embodiment of any of the foregoing embodiments, wherein the oxidizing agent is either sodium periodate or lead tetraacetate. In yet a further embodiment of any of the foregoing embodiments, wherein the cells are treated with 1 mM sodium periodate for 30 min at 4 °C. In yet a further embodiment of any of the foregoing embodiments, wherein the aminooxy-functionalized molecules comprise a detecting agent, and/or cell-, tumor-, or tissue-targeting ligands. In yet a further embodiment of any of the foregoing embodiments, wherein the detecting agent is an enhanced fluorophore-based dye. In yet a further embodiment of any of the foregoing embodiments, wherein the aminooxy-functionalized molecules are ligated, linked or conjugated to the aldehyde groups in the presence of a catalyst. In yet a further embodiment of any of the foregoing embodiments, wherein the catalyst is *p*-anisidine. In yet a further embodiment of any of the foregoing embodiments, wherein the aminooxy-functionalized molecules are ligated, linked or conjugated to the aldehyde groups in the presence of 10 mM *p*-anisidine for 90 min at 4 °C. In a particular embodiment, the disclosure also provides for bioorthogonally-conjugated ICVs produced by any of the foregoing embodiments. In yet a further embodiment of any of the foregoing embodiments, wherein the bioorthogonally-conjugated

ICVs comprise oxime-linked detecting agents. In yet a further embodiment of any of the foregoing embodiments, wherein the bioorthogonally-conjugated ICVs are loaded with one or more small molecule therapeutic compounds or agents. In a particular embodiment, the disclosure also provides for isolated antigenic ICVs produced by any of the foregoing embodiments. In yet a further embodiment of any of the foregoing embodiments, wherein the antigenic ICVs are loaded with one or more small molecule therapeutic compounds or agents. In a particular embodiment, the disclosure also provides for isolated ICVs produced by any of the foregoing embodiments. In a certain embodiment, the disclosure also provides for a pharmaceutical composition comprising the bioorthogonally-conjugated antigenic ICVs produced by any of the foregoing embodiments, the isolated antigenic ICVs produced by any of the foregoing embodiments, or the isolated ICVs produced by any of the foregoing embodiments; and a pharmaceutically acceptable carrier, excipient, and/or diluent. In another embodiment, the disclosure provides a method of stimulating an immune response to a cancer in a subject in need thereof, comprising: administering the pharmaceutical composition disclosed in any of the foregoing embodiments. In yet a further embodiment of any of the foregoing embodiments, wherein the cancer is selected from squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulvar cancer, thyroid cancer, hepatic carcinoma, leukemia and other lymphoproliferative disorders, and various types of head and neck cancers. In yet a further embodiment of any of the foregoing embodiments, wherein the pharmaceutical composition is administered by intravenous administration, intertumoral administration, intraperitoneal administration, intramuscular administration, intracoronary administration, intraarterial administration, subcutaneous administration, transdermal delivery, intratracheal administration, subcutaneous administration, intraarticular administration, intraventricular administration, inhalation, or intracerebral administration. In yet a further embodiment of any of the foregoing embodiments, wherein the pharmaceutical composition is administered to the subject concurrently or sequentially with one or more anticancer agents or chemotherapeutics. In another embodiment, the disclosure provides a method of treating a disease or disorder in a subject in need thereof, comprising: administering the pharmaceutical composition disclosed

in any of the foregoing embodiments. In another embodiment, the disclosure provides a method of stimulating an immune response to a cancer in a subject in need thereof, comprising: (a) obtaining antigen presenting cells; (b) pulsing the antigen presenting cells with an antigen associated with cancer cells; (c) inducing cell membrane blebbing by use of a sulfhydryl blocking agent; (d) collecting antigenic micrometer sized ICVs (mICVs) induced by cell membrane blebbing; and (e) administering said antigenic mICVs to the subject in need of immunotherapy. In yet a further embodiment of any of the foregoing embodiments, wherein the cancer is selected from squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, valvar cancer, thyroid cancer, hepatic carcinoma, leukemia and other lymphoproliferative disorders, and various types of head and neck cancers. In yet a further embodiment of any of the foregoing embodiments, wherein the antigen presenting cells are dendritic cells, macrophages, monocytes, Langerhans cells, B cells, genetically modified cells, and mesenchymal stem cells. In yet a further embodiment of any of the foregoing embodiments, wherein the antigen presenting cells are immature dendritic cells that are pulsed with an antigen to produce semimature or mature dendritic cells. In yet a further embodiment of any of the foregoing embodiments, where the immature dendritic cells are derived from bone marrow of a human subject. In yet a further embodiment of any of the foregoing embodiments, wherein the antigen presenting cells are obtained from the subject to be treated by immunotherapy. In yet a further embodiment of any of the foregoing embodiments, wherein the sulfhydryl blocking agent is paraformaldehyde. In yet a further embodiment of any of the foregoing embodiments, wherein the antigenic mICVs have diameters from 1 micrometer to 5 micrometers. In yet a further embodiment of any of the foregoing embodiments, wherein the antigenic mICVs are administered by intravenous administration, intertumoral administration, intraperitoneal administration, intramuscular administration, intracoronary administration, intraarterial administration, subcutaneous administration, transdermal delivery, intratracheal administration, subcutaneous administration, intraarticular administration, intraventricular administration, inhalation, or intracerebral administration. In yet a further embodiment of any of the foregoing embodiments, wherein the antigenic mICVs are administered concurrently or sequentially

with one or more anticancer agents or chemotherapeutics. A cell-free cell therapy comprising the pharmaceutical composition disclosed in any of the foregoing embodiments for treating a subject having a disease or disorder. In yet a further embodiment of any of the foregoing embodiments, wherein the subject has cancer. A vaccine comprising the pharmaceutical composition disclosed in any of the foregoing embodiments for prevention of an infection in a subject by an infectious agent. In yet a further embodiment of any of the foregoing embodiments, wherein the infectious agent is a bacterium, a virus, a fungus, or a protozoon. A therapeutic vaccine comprising the pharmaceutical composition disclosed in any of the foregoing embodiments for use in treating a subject having cancer.

DESCRIPTION OF DRAWINGS

[0012] **Figure 1** provides a diagram of the blebbing process induced by the chemical agents disclosed herein that can be used to induce cell vesicle formation, and the use of the induced cell vesicles for various applications, including cell-free based cell therapies.

[0013] **Figure 2** presents a photo of HeLa cells prior to treatment with *N*-ethylmaleimide.

[0014] **Figure 3** demonstrates that nanometer sized or micrometer sized ICVs that are induced from HeLa cells after treatment with *N*-ethylmaleimide. (Top Image) Micro scale ICVs are visible in the images as circular vesicles with a darker contrast compared with the cells. (Bottom Image) More micrometer sized ICVs are produced than nanometer sized ICVs post treatment of HeLa cells with *N*-ethylmaleimide.

[0015] **Figure 4** shows light microscope images of HeLa cells which were exposed to 2 mM NEM in 1X DPBS cell blebbing buffer for varying periods of time (1 h, 3 h, 5 h, 8 h, and 24 h).

[0016] **Figure 5** provides a graph presenting the size distribution of nanometer sized ICVs from **FIG. 4** using dynamic light scattering.

[0017] **Figure 6** show light microscope images of HeLa cells which were exposed to 2 mM NEM in 5X DPBS cell blebbing buffer for varying periods of time (1 h, 3 h, 5 h, 8 h, and 24 h).

[0018] **Figure 7** show light microscope images of HeLa cells which were exposed to 2 mM NEM in 4X DPBS cell blebbing buffer for varying periods of time (1 h, 3 h, 5 h, 8 h, and 24 h).

[0019] **Figure 8** show light microscope images of HeLa cells which were exposed to 2 mM NEM in 3X DPBS cell blebbing buffer for varying periods of time (1 h, 3 h, 5 h, 8 h, and 24 h).

[0020] **Figure 9** show light microscope images of HeLa cells which were exposed to 2 mM NEM in 2X DPBS cell blebbing buffer for varying periods of time (1 h, 3 h, 5 h, 8 h, and 24 h).

[0021] **Figure 10** show light microscope images of HeLa cells which were exposed to 2 mM NEM in 1X DPBS cell blebbing buffer for varying periods of time (1 h, 3 h, 5 h, 8 h, and 24 h).

[0022] **Figure 11** provides a transmission electron microscopy (TEM) image of nanometer sized ICVs produced by a method of the disclosure.

[0023] **Figure 12** shows a light microscope image of HeLa cells that were preincubated with photosensitive media prior to being subjected to light generated from a 670 nm laser.

[0024] **Figure 13** provides light microscope images of HeLa cells that were incubated with photosensitive media and subjected to light generated from a 670 nm laser for 10 minutes. Left photo: image of HeLa cells that was taken right after laser light exposure. Right photo: image of HeLa cells that was taken 18 hours after laser light exposure.

[0025] **Figure 14** provides light microscope images of HeLa cells that were incubated with photosensitive media and subjected to light generated from a 670 nm laser for 5 minutes. Left photo: image of HeLa cells that was taken right after laser light exposure. Right photo: image of HeLa cells that was taken 18 hours after laser light exposure.

[0026] **Figure 15** provides light microscope images of HeLa cells that were incubated with photosensitive media and subjected to light generated from a 670 nm laser for 2.5 minutes. Left photo: image of HeLa cells that was taken right after laser light exposure. Right photo: image of HeLa cells that was taken 18 hours after laser light exposure.

[0027] **Figure 16** provides light microscope images of HeLa cells that were incubated with photosensitive media and subjected to light generated from a 670 nm laser for 1.25 minutes. Left photo: image of HeLa cells that was taken right after laser light exposure. Right photo: image of HeLa cells that was taken 18 hours after laser light exposure.

[0028] **Figure 17** provides inverted light microscope images of micrometer sized induce cell vesicles produced by incubated with photosensitive media comprising the

photosensitizer $AlPcS_{2A}$ and subjected to light generated from a 670 nm laser for 10 min (left photo) or for 5 min (right photo).

[0029] **Figure 18** provides inverted light microscope images of micrometer sized induced cell vesicles produced from HeLa cells which were incubated with photosensitive media comprising the photosensitizer $AlPcS_{2A}$ and subjected to light generated from a 670 nm laser for 2.5 min (left photo) or for 1.25 min (right photo).

[0030] **Figure 19** provides dynamic light scattering (DLS) analysis of nanometer sized induced cell vesicles that were produced immediately after HeLa cells, which were incubated with photosensitive media comprising the photosensitizer $AlPcS_{2A}$, were subjected to light generated from a 670 nm laser for 10 min, 5 min, 2.5 min or 1.25 min.

[0031] **Figure 20** provides dynamic light scattering (DLS) analysis of nanometer sized induced cell vesicles that were produced 18 h after HeLa cells, which were incubated with photosensitive media comprising the photosensitizer $AlPcS_{2A}$, were subjected to light generated from a 670 nm laser for 10 min, 5 min, 2.5 min or 1.25 min.

[0032] **Figure 21** demonstrates the photo-initiated yield of nanometer sized induced cell vesicles and micrometer sized induced cell vesicles when the cells were exposed to light for 20 min without preincubation with a photosensitizer v. cells preincubated with a photosensitizer and exposed to light for 20 min.

[0033] **Figure 22** illustrates how adeno-associated virus replicates within cells. The virus can be released in three ways: directly in media, in cell vesicles, and through cell lysis and collection. The cell blebbing methods disclosed herein induce the mass scale production of AAV containing cell vesicles in a rapid and efficient manner.

[0034] **Figure 23** demonstrates that there is an increase in transduction of AAV when AAV encapsulated by vesicles, as denoted by AAV vexo. The presented experiment was performed in Hek293T and U87 glioblastoma cells. Figure is prior art from Maguire et al. (2012). Microvesicles-associated AAV vector as a novel gene delivery system. Molecular Therapy, 20(5), 960-971).

[0035] **Figure 24** provides for light microscope imaging and size analysis of photo-initiated induced AAV cell vesicles after 10 min (top), after 24 hours (middle) and NEM AAV cell vesicles (bottom). Micro scale ICVs are visible in these images as circular ICVs with darker contrast compared to cells.

[0036] **Figure 25** provides light microscope and fluorescent imaging of supernatant that was collected from virus producer cells without blebbing or lysis. Very weak fluorescence in the cells was observed.

[0037] **Figure 26** provides light microscope and fluorescent imaging of transduction tracking for free AAV from cells using a conventional method. As shown, AAVs were inactivated by anti-AAV antibodies.

[0038] **Figure 27** provides light microscope and fluorescent imaging of transduction tracking for *N*-ethylmaleimide (NEM) induced nano-sized cell vesicles containing AAV. As shown, there were no effects on AAVs by anti-AAV antibodies, thereby confirming encapsulation of AAVs in ICVs.

[0039] **Figure 28** provides light microscope and fluorescent imaging of transduction tracking for *N*-ethylmaleimide (NEM) induced micron-sized cell vesicles containing AAV. As shown, there were only negligible effects on AAVs by anti-AAV antibodies, thereby confirming encapsulation of AAVs in ICVs.

[0040] **Figure 29** provides light microscope and fluorescent imaging of transduction tracking for photoinitiated induced nano-sized cell vesicles containing AAV, post 10 min. As shown, there were only negligible effects on AAVs by anti-AAV antibodies, thereby confirming encapsulation of AAVs in ICVs.

[0041] **Figure 30** provides light microscope and fluorescent imaging of transduction tracking for photo-initiated induced micron-sized cell vesicles containing AAV, post 10 min. As shown, there were only negligible effects on AAVs by anti-AAV antibodies, thereby confirming encapsulation of AAVs in ICVs.

[0042] **Figure 31** provides light microscope and fluorescent imaging of transduction tracking for photo-initiated induced nano-sized cell vesicles containing AAV, post 24 h. As shown, there were only negligible effects on AAVs by anti-AAV antibodies, thereby confirming encapsulation of AAVs in ICVs.

[0043] **Figure 32** provides light microscope and fluorescent imaging of transduction tracking for photo-initiated induced micron-sized cell vesicles containing AAV, post 24 h. As shown, there were only negligible effects on AAVs by anti-AAV antibodies, thereby confirming encapsulation of AAVs in ICVs.

[0044] **Figure 33** provides light microscope and fluorescent imaging for supernatant collected from virus producer cells without blebbing or lysis. Anti-AAV antibodies affected transduction efficiency of the control supernatant.

[0045] **Figure 34** provides light microscope and fluorescent imaging for supernatant collected from virus producer cells using traditional vesicle formation conditions. Anti-AAV antibodies drastically reduced the transduction efficiency of traditionally produced AAV vesicles, suggesting that very few AAV are actually encapsulated in these vesicles.

[0046] **Figure 35A-B** provides free AAV titer and AAV containing ICVs titer from sucrose gradient fractions: **(A)** straight scale; **(B)** Log scale. The data shows a clear divide between where AAVs congregate in the sucrose gradient, with AAV fractions in 1-6, (most being in fractions 3-6) and AAV containing ICVs almost solely appearing in fraction 10. This clear division allows for separation of AAV containing ICVs from free AAV, as well as from cellular organelles and debris.

[0047] **Figure 36A-B** provides free AAV titer and AAV containing ICVs titer from sucrose gradient fractions: **(A)** straight scale; **(B)** Log scale. The data shows a clear divide between where AAVs congregate in the sucrose gradient, with AAV fractions in 1-7, (most being in fractions 2-6) and AAV containing ICVs appearing in fractions 10 to 12. This clear division allows for separation of AAV containing ICVs from free AAV, as well as from cellular organelles and debris.

[0048] **Figure 37** provides for the determination of titers of AAV stock and AAV from cell lysate treated with NEM. The goal of this experiment was to determine how NEM affected free AAVs versus intracellular AAVs (from producer cells). All titers appeared to be about the same, suggesting that NEM did not cause DNA damage. This data was also used to determine dosing for the next experiment (**FIG. 38**).

[0049] **Figure 38** provides quantitative results using AAV stock and AAV produced cell derived AAV treated with NEM. Stock AAV transduction was greatly affected by NEM, reducing the transduction efficiency after just 10 minutes, and leading to almost no transduction after incubation for 2 hours. However, AAV isolate from lysate showed that the cells had a protective effect. There was only a minimal drop in transduction until they had been exposed to NM for 4 hours, and the 8 hours timepoint only showed about half the transduction that the 0-minute timepoint showed. This demonstrated that any AAV that is free during the vesiculation process should be neutralized by the presence of NEM. It also demonstrates that cell membrane protects AAVs from NEM, and this should translate to the ICVs. Lastly, after seeing minimal DNA damage in the previous experiment, it is clear that the NEM affects protein, affecting the transduction efficiency of the AAVs.

[0050] **Figure 39** shows whether NEM inactivates stock AAV or AAV from producer cells using the time points of 0 min, 10 min, 30 min and 1 h. NEM was used at a dose of 5E09 GCs/mL. As shown, stock AAV transduction was greatly affected by NEM, reducing the transduction efficiency after just 10 minutes, while AAV from producer cells was not similarly affected.

[0051] **Figure 40** shows whether NEM inactivates stock AAV or AAV from producer cells using the time points of 2 h, 4 h, 6 h and 8 h. NEM was used at a dose of 5E09 GCs/mL. As shown, stock AAV transduction efficiency was greatly affected by NEM at all tested time points, while AAV from producer cells only showed a minimal drop in transduction efficiency until the lysate AAVs had been exposed to NEM for 4 hours, and the 8-hour timepoint only showed about half the transduction efficiency that the 0-minute timepoint showed.

[0052] **Figure 41A-B** shows the results of flow cytometry studies looking at whether **(A)** free AAV and AAV cells; and **(B)** photoinduced AAV containing ICVs were resistant to anti-AAV antibodies. Free AAVs were silenced by the neutralizing antibodies. Light induced cell vesicles were also silenced at a similar amount. This system appeared not to be protective from nAbs.

[0053] **Figure 42A-B** shows the results of flow cytometry studies looking at whether **(A)** free AAV or **(B)** 5 min photoinduced AAV containing ICVs were resistant to anti-AAV antibodies. NEM induced micrometer sized cell vesicles showed far greater transduction efficiency and were able to resist neutralization by neutralizing antibodies for AAV. The nanosized ICVs appeared to resist neutralization but showed insignificant transduction. Photo-induced cell vesicles showed a similar profile to the NEM induced cell vesicles, except the micro ICVs did show a response to the neutralizing antibody based on the concentration. However, they resisted being completely neutralized. This is due to the protective effect of the membrane around AAV containing ICVs that protected AAVs from neutralization.

[0054] **Figure 43** provides fluorescent images looking at whether micrometer sized AAV containing ICVs can protect from neutralizing antibodies. As shown, micrometer sized AAV containing ICVs were protected against neutralization by anti-AAV antibodies.

[0055] **Figure 44** provides fluorescent images looking at whether nanometer sized AAV containing ICVs can protect from neutralizing antibodies. As shown, nanometer sized AAV containing ICVs were susceptible to neutralization by anti-AAV antibodies.

[0056] **Figure 45** provides for the optimization of NEM incubation blebbing times. This data was not normalized by number of ICVs and therefore could also be representative of greater yields of AAV containing ICVs over time.

[0057] **Figure 46** provides a diagram for the bioorthogonal modification of vesicle-producing cells. Cells are modified by oxime ligation of aminooxy-functionalized molecules with aldehyde groups of oxidized sialylated glycoproteins. ICVs produced from the modified cells retain the bioorthogonally-conjugated molecules of interest on the ICV surface.

[0058] **Figure 47A-C** presents a bioorthogonally tethered retrovirus. **(A)** Production of functionalized retrovirus by bioorthogonally modifying the surface of virus producer cells whose sialylated glycoproteins (green) on the phospholipid cell membrane (purple) are oxidized to generate aldehyde groups. Aminooxy-functionalized molecules (yellow) are conjugated with aldehyde groups via oxime linkages. **(B)** Magnet-directed transduction of magnetically labeled retroviral particles that were produced from bioorthogonally engineered surface of 293GPG/EGFP retroviral producer cells. After one week of incubation with G418, selected cells were stained with 0.1% methylene blue in methanol for visualization. **(C)** Synthetically engineered retroviral particles for targeted transduction of folic acid receptor (FAR)-overexpressing cancer cells. The surface of 293GPG/EGFP retrovirus producing cells was bioorthogonally engineered as described in **(A)** using the aminooxy-activated ketal-PEG-FA molecules. The resulting FAR-targeting retroviral particles were incubated with FAR-overexpressing HeLa human cervical cancer cells and FAR-negative NIH 3T3 cells for 2 h. After 24 h of incubation, transduction efficiency was quantified by EGFP expressing cells by flow cytometry.

[0059] **Figure 48A-B** presents fluorescently labeled HeLa cells and HeLa cell-derived mICVs produced by sulfhydryl blocking, using biorthogonal conjugation of CF 488 on the cell surface. **(A)** Producer cells were treated with paraformaldehyde to induce cell vesicles, and **(B)** isolated mICVs were imaged using a confocal laser scanning microscope. Scale bar = 10 μm.

[0060] **Figure 49** provides a diagram of the activation of T cells with BMDC-derived ICVs. ICVs induced from BMDCs using the methods of the disclosure present the same antigens as parent cells. They are able to activate T cells *in vitro* and *in vivo* to induce anti-tumor immunity. BMDC-derived ICVs have utility for a broad range of biomedical applications since their antigens can be modified easily and they can also be loaded with additional therapeutic cargo.

[0061] **Figure 50A-B** indicates that BMDC-derived ICVs can be effectively produced by chemical crosslinking while maintaining antigen-presenting capabilities. **(A)** Inverted microscope image of SIINFEKL antigen presenting ICVs. **(B)** Results of an immunoassay using a fluorescently labeled anti-H-2kb antibody that binds to SINFEKL labelling.

[0062] **Figure 51** presents the results of a study looking at T cell activation using a β-Galactosidase (CPRG) assay and B3Z, a T cell hybridoma line. The greater absorbance in the case of B3Z exposure to antigen-presenting BMDC-derived ICVs indicates that antigen-presenting ICVs are able to activate T cells *in vitro*.

[0063] **Figure 52** presents the results of efficacy experiments in mice demonstrating that SINFEKL-presenting ICVs had greater efficacy at stimulating the CTL response then controls at a target ratio of 50:1 or 100:1.

[0064] **Figure 53** provides a diagram showing how bone marrow dendritic cells can be used to produce ICVs as a cancer vaccine.

[0065] **Figure 54** presents bright field microscope images of BMDC cells undergoing blebbing after 24 h using either 25 mM PFA (left panel) or 25 mM PFA with 2 mM DTT blebbing buffer (right panel). Bone marrow was isolated from C57BL/6 mice and differentiated into BMDCs and matured as previously described. Mature BMDCs were treated with 25mM PFA, or 25mM PFA with 2mM DTT, blebbing buffers for 24 hours, then observed by bright field microscope. Mature BMDC ICVs were prepared in PFA or PFA/DTT blebbing buffers, isolated, and compared for antigen presentation and T cell stimulation.

[0066] **Figure 55A-C** provides for the characterization of ICVs derived from dendritic cells. Immature or mature BMDCS were blebbed in 25 mM PFA blebbing buffer for 24 hours. Cells were imaged by bright field microscope **(A)**, then nano and micro-scale ICVs were isolated as previously described and analyzed by DLS or light microscope **(B)**, respectively. The sized of the ICVs were determined by using ImageJ analysis. BMDC and ICVs were labeled with fluorescent anti-CD11c and compared for CD11c presentation (marker of dendritic cells) by flow cytometry. As shown, BMDCs, when exposed to PFA blebbing buffer, induced cell vesicle formation **(A)**. **(B)** Both immature and mature dendritic cells produced similar sized micro and nano-scale ICVs. **(C)** BMDCs and ICVs exhibit similar presentation levels of CD11c, a dendritic cell marker.

[0067] **Figure 56** provides confocal images over time of mature BMDC blebbing. Bone marrow was isolated from C57BL/6 mice and differentiated into BMDCs and matured

as previously described. Mature BMDCs were stained with PKH26 (red membrane stain) and DAPI (blue nuclear stain) and exposed to PFA blebbing buffer. Cells and induced cell vesicle production were observed over time by confocal microscope imaging. Mature BMDCs produced larger ICVs over time, with complete use of cell membrane accomplished at 24 hours post-incubation.

[0068] **Figure 57** provides confocal images over time of immature BMDC blebbing. Bone marrow was isolated from C57BL/6 mice and differentiated into BMDCs as previously described. Immature BMDCs were stained with PKH26 (red membrane stain) and DAPI (blue nuclear stain) and exposed to PFA blebbing buffer. Cells and induced cell vesicle production dynamics were observed over time by confocal microscope imaging. Similar to mature BMDCs, immature BMDCs produced larger ICVs over time, with complete use of cell membrane accomplished at 24 hours post-incubation.

[0069] **Figure 58A-C** shows how the length of treatment of BMDCs with the blebbing methods disclosed herein influences ICV size. **(A)** Immature BMDCs or **(B)** mature BMDCs were treated in 25mM PFA blebbing buffer for 1, 6, 12, or 24 hours. **(A, B)** Nano- and **(C)** micro-scale ICVs were isolated as previously described and analyzed by DLS or light microscope imaging, respectively. To understand how ICV size was influenced by the time length of the blebbing treatment, immature or mature BMDCs were treated in 25 mM PFA blebbing buffer for 1, 6, 12, or 24 hours. **(A-C)** Both micro and nano ICVs showed similar size distributions over time. This indicated that larger ICVs created over time, as demonstrated by confocal microscopy data, are likely removed during cells and debris removal steps.

[0070] **Figure 59A-C** examines which blebbing method, paraformaldehyde (PFA) or paraformaldehyde + DTT, provides for ICVs having the best antigen presentation and T cell activation. **(A, B)** Mature BMDC ICVs were labeled with anti-SIINFEKL and analyzed by flow cytometry for antigen presentation. **(C)** Mature BMDC ICVs were tested for T cell activation by CPRG assay as previously described. **(A, B)** PFA and PFA/DTT ICVs showed similar levels of antigen presentation. While antigen presentation levels were similar, **(C)** PFA ICVs were capable of stimulating T cells by nearly 2-fold. This indicated that PFA was the preferred method for inducing cell vesicles from BMDs, as functionality of the ICVs were improved.

[0071] **Figure 60A-B** demonstrates that ICVs from BMDCs exhibit similar CD40 surface markers as parent dendritic cells. Immature BMDCs were matured in LPS for 0, 6,

and 12 hours to generate BMDCs at different stages of maturation. BMDCs from these time points were treated with 25 mM PFA blebbing buffers and micro ICVs were isolated as previously described. BMDCs from the varying time points and ICVs subsequently produced were labeled with fluorescent anti-CD40 (BMDC maturation marker) and analyzed by flow cytometry. BMDCs and ICVs from varying maturation states showed similar levels of **(A)** % CD40(+) maturation marker and **(B)** mean CD40(+) marker presentation as BMDC cells. This indicates that ICVs can have controlled levels of maturation, similar to their parent cells. However, unlike cells, ICVs cannot continue to undergo changes, locking their stage of maturation into place. This is important for controlling immune response *in vivo*.

[0072] **Figure 61** provides images of the CD40 expression on mBMDC-derived antigenic mICVs. BMDCs were cultured in RPMI supplemented 10% FBS and 20 ng/mL rmGM-CSF. On day 7 of culture, cells were incubated with 20 ng/mL lipopolysaccharide for 12 h to induce maturation. Cells were collected by centrifugation and plated in either DMEM without FBS (for exosome production) or DPBS with paraformaldehyde as described herein. After 12 h, the plates were washed and incubated with 10% anti-mouse CD11c antibody (Alexa Flour 488). The plates were then washed with DPBS and imaged.

[0073] **Figure 62A-D** demonstrates that ICVs exhibit similar CD80 and CD86 surface markers as parent dendritic cells. Immature BMDCs were matured in LPS for 0, 6, and 12 hours to generate BMDCs at different stages of maturation. BMDCs from these time points were treated with 25 mM PFA blebbing buffers and micro ICVs were isolated as previously described. BMDCs from the varying time points and ICVs subsequently produced were labeled with fluorescent (**A**, **B**) anti-CD80 or (**C**, **D**) anti-CD86 (BMDC costimulatory molecules) and analyzed by flow cytometry. Similar to CD40 presentation, ICVs produced from cells of varying stages of maturation presented increased levels of **(A)** % CD80(+) markers and **(B)** mean CD80(+) markers, as well as, **(C)** %CD86(+) markers and **(D)** mean CD86(+) markers, which mirrored those of the parent cells. This indicated that ICVs likely mirror many of the parent cell presentation qualities, with the unique feature that ICV presentation is locked and cannot undergo further changes.

[0074] **Figure 63A-B** demonstrates that ICVs exhibit similar antigen presentation to parent dendritic cells but are more stable. Immature BMDCs were matured in LPS for 0, 6, and 12 hours to generate BMDCs at different stages of maturation. BMDCs from these time points were treated with 25 mM PFA blebbing buffers and micro ICVs were isolated as previously described. BMDCs from the varying time points and ICVs subsequently produced

were labeled with fluorescent SIINFEKL (antigen) and analyzed by flow cytometry. **(A)** and **(B)** Similar to CD40, CD80, and CD86 presentation findings, BMDCs and ICVs from varying maturation states displayed similar levels of antigen presentation.

[0075] **Figure 64A-B** provides graphs looking at the expression of MHC1 from immature and mature BMDC. Immature and mature BMDCs were labeled with fluorescent anti-MHCI and compared for **(A)** % MHC1(+) cells, and **(B)** Mean MHC1(+) presentation by flow cytometry. In the literature, MHCI presentation has been reported as similar between immature and mature dendritic cell groups. Flow cytometry data indicated that while percentages of MHCI positive cells were similar, density of MHCI on mature cells was increased by nearly 2-fold. This indicated that mature BMDCs are superior at antigen presentation.

[0076] **Figure 65** provides images of a 12% agarose gel where mature MBDC and ICVs from mature BMDCs were lysed (lane 2 and lane 4, respectively) and run out on the gels for 100 V for 60 min. Mature BMDCs and ICVs display similar RNA profiles, with DNA from cells displaying at the top of the lane.

[0077] **Figure 66** provides images of a 12% agarose gel where ICVs from immature BMDCs or ICVs from mature BMDCs were lysed (lane 2 and lane 4, respectively), or ICVs from immature BMDCs or ICVs from mature BMDCs were lysed and further treated with RNase (lane 3 and lane 5, respectively) and run out on the gels for 100 V for 60 min. The ICVs showed similar RNA profiles irrespective of the maturation state of the DCs.

[0078] **Figure 67** provides "unsupervised" clustering analysis of single-cell RNA sequence analysis of particles having similarities in RNA content. 15 distinct clusters were indicated.

[0079] **Figure 68** provides a refined clustering analysis of single-cell RNA sequence analysis of particles having similarities in RNA content. Two main clusters of particles having similarities in RNA content were found. These RNAs were identified (see **FIG. 66**) and are known to be upregulated in immune cells during maturation/activation.

[0080] **Figure 69** provides a heat map matrix of BMDC ICV single cell sequence data. As indicated, genes of interest include GM42418, Fth1, AY036118, Saa3, Mt-Co2, Mt-Co1, Mt-Co3, Rpl19, Vezt, Snap91, Ctbs, Mon2, Hif1an, Nbas, Rock2, Rap1b, Tmed7, and Fbll1.

[0081] **Figure 70A-B** provides the results of PKH dosing and T-Cell stimulation on mature and immature BMDCs, and ICVs produced from mature and immature BMDCs. **(A)**

Comparing equivalent numbers of immature or mature BMDCs, mature BMDCs had nearly 3-fold greater surface area based on PKH membrane signal. **(B)** Mature BMDCs produced 3-fold the amount of ICVs. It is therefore likely that amount of ICVs produced is correlated with surface area of membrane.

[0082] **Figure 71** shows that ICVs produced from mature BMDCs have increased antigen presentation and stimulation ability than ICVs produced from immature BMDCs. A similar antigen presentation trend was seen with mature BMDCs and immature BDCs. Based on dosing by equivalent surface area, T cell stimulation showed the following trend: ICVs from mature BMDCs>mature BMDCs > immature BMDCs and ICVs from immature BMDCs. When dosing is based on surface area, there are more ICVs:T cells than BMDCs:T cells, generating greater likelihood of interactions and stimulation.

[0083] **Figure 72** presents images from Day 30 of the tumor challenge with mice by vaccine category. PBS, OVA, and SIINFEKL groups all rapidly developed tumors. Immature and mature BMDC vaccine groups developed small tumors by day 15 and went into complete remission. ICVs from immature BMDCs developed tumors at a slowed rate, indicating a low immune response, but not potent enough to prevent tumor growth. ICVs from mature BMDCs developed small tumors, larger than those of the cell vaccine group, and later went into total remission. This data suggests that immature BMDCs likely matured after administration in order to achieve a similar response to the mature BMDC group. This demonstrates the need for a cell-free vaccine, as cell maturation and state could not be accurately controlled, leading to unpredictability of the elicited the immune response. However, ICVs from immature BMDCs had a tolerant effect while ICVs from mature BMDCs performed similarly to mature BMDCs. This indicated that the controlled maturation state of the ICVs provided for a controllable immune response, imperative for a safe cancer vaccine.

[0084] **Figure 73A-B** provides graphs that go with the images presented on Figure 70. **(A)** ICVs from mature BMDCs had a similar anti-tumor effect as mature BMDC vaccine group. By contrast, PBS, OVA, and SIINFEKL groups all rapidly developed tumors. **(B)** All mice treated with mICV or BMDC were alive at day 50 of the tumor challenge. By contrast, all of the PBS, OVA, and SIINFEKL treated mice had died prior to day 35 of the tumor challenge.

DETAILED DESCRIPTION

[0085] As used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell vesicle" includes a plurality of such vesicles and reference to "the sulfhydryl blocking agent" includes reference to one or more sulfhydryl blocking agents and equivalents thereof known to those skilled in the art, and so forth.

[0086] Also, the use of "or" means "and/or" unless stated otherwise. Similarly, "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting.

[0087] It is to be further understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language "consisting essentially of" or "consisting of."

[0088] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although many methods and reagents are similar or equivalent to those described herein, the exemplary methods and materials are disclosed herein.

[0089] All publications mentioned herein are incorporated herein by reference in full for the purpose of describing and disclosing the methodologies, which might be used in connection with the description herein. Moreover, with respect to any term that is presented in one or more publications that is similar to, or identical with, a term that has been expressly defined in this disclosure, the definition of the term as expressly provided in this disclosure will control in all respects.

[0090] It should be understood that this disclosure is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such may vary. The terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present disclosure.

[0091] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used to described the present invention, in connection with percentages means ±1%.

[0092] The term "induced cell vesicle" or "ICV" as used herein refers to cell vesicles that have been induced to form from cells when the cells have been treated using the cell

blebbing methods of the disclosure (*e.g.*, see **FIG. 1**). Thus, an "ICV" is distinguishable from a "bleb" or "a cellular bleb" as the later terms refers to a bulge or protrusion of the plasma membrane of a cell, and from "extracellular vesicles" in that the production of ICVs are a direct result of using the cell blebbing methods of the disclosure, as opposed to naturally occurring production of extracellular vesicles from the cells. Moreover, the term "induced cell vesicle" or "ICV" refers to all forms of induced cell vesicles or ICVs disclosed herein, unless stated otherwise. Thus, the term "induced cell vesicle" or "ICV" includes antigenic induced cell vesicles, bioorthogonally engineered induced cell vesicles, AAV containing induced cell vesicles, etc.

[0093] The term "antigenic induced cell vesicle" or "antigenic ICV" as used herein, refers to an induced cell vesicle disclosed herein that comprises or has been modified to comprise an antigen on the induced cell vesicle surface which can be used to stimulate an immune response in a subject. Typically, an "antigenic induced cell vesicle" or "antigenic ICV" is produced from antigen presenting cells, like dendritic cells. Accordingly, an "antigenic induced cell vesicle" of the disclosure is ideally suited for cell-free immunotherapy and cell-free cancer vaccine applications.

[0094] The term "extracellular vesicle" as used herein, refers to any membrane-derived vesicle that is secreted by a cell. Extracellular vesicles can include a range of extracellular vesicles, including exosomes, microparticles and microvesicles, which are secreted by many cell types under both normal physiological and pathological conditions. An "extracellular vesicle" is distinguishable from an "induced cell vesicle", in that an "induced cell vesicle" directly results from using the cell blebbing methods disclosed herein, *i.e.*, an "induced cell vesicle" does not naturally form from a cell using a naturally occurring process, unless the cells is treated with the cell blebbing methods disclosed herein.

[0095] In a particular embodiment, the method and compositions described herein comprise induced cell vesicles that have an average diameter of 10 nm, 20 nm, 30 nm, 40 nm, 50 nm, 60 nm, 70 nm, 80 nm, 90 nm, 100 nm, 110 nm, 120 nm, 130 nm, 140 nm, 150 nm, 160 nm, 170 nm, 180 nm, 190 nm, 200 nm, 250 nm, 300 nm, 350 nm, 400 nm, 450 nm, 500 nm, 550 nm, 600 nm, 650 nm, 700 nm, 750 nm, 800 nm, 850 nm, 900 nm, 950 nm, 1000 nm, 1100 nm, 1200 nm, 1300 nm, 1400 nm, 1500 nm, 1600 nm, 1700 nm, 1800 nm, 1900 nm, 2000 nm, 2500 nm, 3000 nm, 3500 nm, 4000 nm, 5000 nm, 6000 nm, 7000 nm, 8000 nm, 9000 nm, 10,000 nm or any range that includes or is between any two of the foregoing values, including fractional increments thereof. In another embodiment, the methods

disclosed herein afford micrometer sized induced cell vesicles (*i.e.*, induced cell vesicles having a diameter greater than 1000 nm). In yet another embodiment, the methods disclosed herein afford both nanometer sized and micrometer sized induced cell vesicles (*i.e.*, induced cell vesicles having a diameter from 1 nm to greater than 1 μm). In a certain embodiment, the methods disclosed herein provide for isolation of induced cell vesicles having average diameters from 20 nm to 200 nm. In an alternate embodiment, the methods disclosed herein provide for the isolation of induced cell vesicles having average diameters from 20 nm to 500 nm. In another alternate embodiment, the methods disclosed herein provide for the isolation of induced cell vesicles having average diameters from about 500 nm to about 1000 nm. In yet another alternate embodiment, the methods disclosed herein provide for isolation of induced cell vesicles having average diameters from about 1000 nm to about 10000 nm.

[0096] The terms "micrometer sized induced cell vesicle", or "mICV" as used herein, all refer to induced cell vesicles having a dimeter in the micrometer size range. In a particular embodiment, the mICV has a diameter of 1 μm, 2 μm, 3 μm, 4 μm, 5 μm, 6 μm, 7 μm, 8 μm, 9 μm, 10 μm, or a range that includes or is between any two of the foregoing values, including fractional increments thereof.

[0097] The terms "blebbing", "plasma membrane blebbing" or "cell membrane blebbing" as used herein, all refer to cellular biological process that results from use of the methods disclosed herein which induce plasma membrane blebbing in cells. Typically, blebbing of the plasma membrane is a morphological feature of cells undergoing late stage apoptosis. A bleb is an irregular bulge in the plasma membrane of a cell caused by localized decoupling of the cytoskeleton from the plasma membrane. The bulge eventually separates from the parent plasma membrane taking part of the cytoplasm with it to form an extracellular vesicle. Blebbing is also involved in some normal cell processes, including cell locomotion and cell division. Cell blebbing can be manipulated by mechanical or chemical treatment. It can be induced following microtubule disassembly, by inhibition of actin polymerization, increasing membrane rigidity or inactivating myosin motors, and by modulating intracellular pressure. Blebbing can also be induced in response to various extracellular chemical stimuli, such as exposure to agents that bind up sulfhydryl groups (*i.e.*, sulfhydryl blocking agents), exposure to inhibitors of energy metabolism, or exposure to agents that cause ion deregulation.

[0098] The term "bioorthogonally-conjugated induced cell vesicle" as used herein, refers to an induced cell vesicle that surface has been modified by bioorthogonal chemistry.

Accordingly, an "bioorthogonally-conjugated induced cell vesicle" of the disclosure can be specifically tuned for certain applications, like detection or therapeutic delivery, based upon the surface modification. In a particular embodiment, the surface of the induced cell vesicle has been bioorthogonally modified by tethering or affixing aminooxy-functionalized molecules to aldehyde groups of oxidized sialylated glycoproteins found on the induced cell vesicle surface.

[0099] The term "chemical agent that induces blebbing" as used herein, refers to a small molecule compound that when administered induces plasma membrane blebbing in cells, usually by causing injuries to cells which result in changes to cytosolic calcium (Ca^{2+}) levels. Examples of chemical agents that can be used in the methods or compositions disclosed herein to induce blebbing include, agents that block or bind up sulfhydryl groups, such as mercury chloride, p-chloromercuribenzene sulfonic acid, auric chloride, *p*-chloromercuribenzoate, chlormerodrin, meralluride sodium, iodoacetamide, paraformaldehyde and *N*-ethylmaleimide; inhibitors of energy metabolism, such as carbonyl cyanide, m-chlorophenylhydrazone, trivalent arsenicals, potassium cyanate, and potassium cyanate and iodoacetate; agents that cause ion deregulation, such as ouabain, ionomycin, and A23187; and agents that induce oxidative stress, such as menadione. In a particular embodiment, the chemical agent that induces cell membrane blebbing is a sulfhydryl blocking agent.

[00100] The terms "cancer" and "cancerous" as used herein, refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Examples of cancer include, but are not limited to, carcinoma, lymphoma (*e.g.*, Hodgkin's and non-Hodgkin's lymphoma), blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulvar cancer, thyroid cancer, hepatic carcinoma, leukemia and other lymphoproliferative disorders, and various types of head and neck cancers.

[00101] The terms "cell proliferative disorder" and "proliferative disorder" as used herein, refer to disorders that are associated with some degree of abnormal cell proliferation.

In one embodiment, the cell proliferative disorder is a tumor. In one embodiment, the cell proliferative disorder is cancer.

[00102] The term “CRISPR-Cas system”, “CRISPRi system” or “CRISPR-Cpf1 system” as used herein refers to all the components that can be or are used to perform gene editing using a CRISPR gene editing system, including components, such as plasmids, guide RNAs (*e.g.*, crRNAs), Cas proteins (*e.g.*, Cas-3, Cas-9), and Cpf1 proteins.

[00103] The term "effective amount" as used herein, refers to an amount that is sufficient to produce at least a reproducibly detectable amount of the desired results. An effective amount will vary with the specific conditions and circumstances. Such an amount can be determined by the skilled practitioner for a given situation.

[00104] The term “immunotherapy” as used herein, refers to treatment that uses the subject’s own immune system to combat a disease or disorder, such as cancer. Immunotherapy includes treatment that works in different ways, such as boosting the subject’s immune system in a general way, or training the immune system to specifically attack disease or abnormal cells or tissue.

[00105] The term “cancer vaccine” as used herein, refers to an immunotherapy treatment that tries to stimulate the immune system to mount an attack against cancer cells in the body. A “cancer vaccine” may be made from antigenic ICVs made from a cancer subject’s own cells that are then administered to the same subject as a cancer vaccine. Alternatively, the cells used to make the antigenic ICVs may come from someone other than the cancer subject to be treated.

[00106] The term "inhibiting tumor cell growth or proliferation" as used herein, means decreasing a tumor cell's growth or proliferation by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100%, and includes inducing cell death in a cell or cells within a cell mass.

[00107] The terms "patient", "subject" and "individual" are used interchangeably herein, and refer to an animal, particularly a human, to whom treatment including prophylaxis treatment is provided. This includes human and non-human animals. The term "non-human animals" and "non-human mammals" are used interchangeably herein includes all vertebrates, *e.g.*, mammals, such as non-human primates, (particularly higher primates), sheep, dog, rodent (*e.g.*, mouse or rat), guinea pig, goat, pig, cat, rabbits, cows, and non-mammals such as chickens, amphibians, reptiles etc. In one embodiment, the subject is human. In another embodiment, the subject is an experimental animal or animal substitute as

a disease model. "Mammal" refers to any animal classified as a mammal, including humans, non-human primates, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, etc. Patient or subject includes any subset of the foregoing, e.g., all of the above, but excluding one or more groups or species such as humans, primates or rodents. A subject can be male or female. A subject can be a fully developed subject (*e.g.*, an adult) or a subject undergoing the developmental process (*e.g.*, a child, infant or fetus).

[00108] The term "photoinitiated induced cell vesicle" as used herein, refers to cells that have been incubated or exposed to a photosensitizer that induces the production of cell vesicles once exposed to light.

[00109] The term "photosensitizer" as used herein, refer to a molecule that produces a chemical change in another molecule in a photochemical process. Photosensitizers generally act by absorbing ultraviolet or visible region of electromagnetic radiation and transferring it to adjacent molecules. Photosensitizers usually have large de-localized π systems, which lower the energy of HOMO orbitals and its absorption of light might be able to ionize the molecule. Photosensitizers are a key part of photodynamic therapy (PDT) which is used to treat some cancers. They help to produce singlet oxygen to damage tumors. They can generally be divided into porphyrins, chlorophylls and dyes.

[00110] The term "purified" when used in reference to an induced cell vesicle disclosed herein, refers to the fact that it is removed from the majority of other cellular components from which it was generated or in which it is typically present in nature. The induced cell vesicles disclosed herein are typically prepared to the state where they are purified or semi-purified.

[00111] The term "sulfhydryl blocking agent" as used herein, refers to compound or reagent that interacts with cellular sulfhydryl groups so that the sulfhydryl groups are blocked or bound up by the sulfhydryl blocking agent, typically via alkylation or disulfide exchange reactions.

[00112] The term "therapeutically effective amount" as used herein, refers to an amount that is sufficient to affect a therapeutically significant reduction in one or more symptoms of the condition when administered to a typical subject who has the condition. A therapeutically significant reduction in a symptom is, *e.g.*, about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 100%, or more as compared to a control or non-treated subject.

[00113] The term "treat" or "treatment" as used herein, refers to a therapeutic treatment wherein the object is to eliminate or lessen symptoms. Beneficial or desired clinical results include, but are not limited to, elimination of symptoms, alleviation of symptoms, diminishment of extent of condition, stabilized (i.e., not worsening) state of condition, delay or slowing of progression of the condition.

[00114] Extracellular vesicles (EVs) are cell membrane-derived particles naturally formed and released by all cells. As they are derived from cells themselves, EVs exhibit similar anatomy. They are composed of a lipid bilayer and present and contain similar molecules as their parent cell but without organelles (mitochondria, nucleus, etc.). EVs can be categorized into two general groups for therapeutic use, exosomes and microvesicles. Exosomes are 20-100 nm in diameter formed by invagination of multivesicular bodies (MVB) and released by subsequent MVB fusion with the plasma membrane. Microvesicles are 100-1000 nm in diameter and are released by direct blebbing from the plasma membrane.

[00115] Extracellular vesicles also play major roles in cell signaling, cell communication, and in the pathology of disease. As such, EVs have been designed and evolved by nature to effectively transfer internalized material to cells, making them excellent candidates for bio-active drug delivery vehicles. Cells in culture naturally produce EVs but at a rate significantly below the requirements for therapeutic administration, which has been attempted to be mitigated by exposing cells to endosomal trafficking regulators, modified proteins, and external stressors. However, these time- and labor-intensive processes directly affect cellular activities and make it difficult to preserve the composition and biological functions of EVs at a desired cellular stage.

[00116] Extracellular vesicle-based therapeutics are currently hindered from clinical advancement due to issues in mass production. To address this challenge, exploration in increasing EV production has been pursued by methods including starvation, manipulating intracellular calcium levels, thermal stress, hypoxia, radiation, and micro-environmental pH. These production methods require between 12-48 hours to achieve significantly improved yields. The disclosure provides for techniques and methods that provide for high yields of induced cell vesicles (ICVs) in as little as a few minutes, producing both micro and nano-scale sized ICVs. For example, the $AlPcS_{2A}$ photoinitiated blebbing methods and techniques disclosed herein can induce cell vesicle production immediately after light exposure. Accordingly, the techniques and methods disclosed herein can induce cell vesicle production

faster, more efficiently, and at higher yields than standard extracellular vesicle production techniques.

[00117] Any cell which naturally secretes extracellular vesicles may be used to induce cell vesicles by using the cell blebbing methods and techniques disclosed herein. As such, nearly any type of cell can be used with the cell blebbing methods and techniques disclosed herein to induce cell vesicles. In a particular embodiment, the cells used to induce cell vesicle production are selected from epithelial cells, fibroblast cells, tumor cells and cells of the immune system (mast cells, T and B lymphocytes, dendritic cells, especially Langerhans cells). In one embodiment, the ICV producing cell is a eukaryotic cell comprising internal vesicles for secretion. In another embodiment the cell is an ICV producing cell that is available commercially from ATTC® or other suppliers. In a further embodiment the ICV producing cell is capable of exocytosis. In yet a further embodiment, the ICV producing cell has been modified genetically, e.g., by gene editing, gene knockout, gene therapy, homologous recombination, site directed mutagenesis, transfection with plasmids and vectors, and the like. In another embodiment, the ICV producing cell has been modified on its surface using the bioorthogonally conjugation methods disclosed herein, or any other surface engineering method known in the art. Cells that can be used to produce ICVs include, without limitation, skin fibroblasts, mast cells, T and B lymphocytes and dendritic cells (for example Langerhans cells), or cells derived from these cell types, and cells or cell lines modified by genetic engineering so as to render them capable of secreting ICVs. In a particular embodiment, the cell used to produce antigenic ICV is a dendritic cell. In further embodiment, the dendritic cell is a bone marrow derived dendritic cell.

[00118] In a particular embodiment, ICVs may be produced from a mammalian cell by contacting the mammalian cell with a chemical agent that induces blebbing. Examples of such chemical agents include, but are not limited to, photosensitizers, mercury chloride, p-chloromercuribenzene sulfonic acid, auric chloride, *p*-chloromercuribenzoate, chlormerodrin, meralluride sodium, iodoacetamide, maleimide, cyanide, m-chlorophenylhydrazone, trivalent arsenicals, potassium cyanate, and potassium cyanate and iodoacetate, glyoxal, acrolein, methacrolein, pyridoxal, *N*-ethylmaleimide (NEM), maleimide, chloromercuribenzoate, iodoacetate, potassium arsenite, sodium selenite, thimerosal (merthiolate), benzoyl peroxide, cadmium chloride, hydrogen peroxide, iodosobenzoic acid, meralluride sodium, (mercuhydrin), mercuric chloride, mercurous chloride, chlormerodrin (neohydrin), phenylhydrazine, potassium tellurite, sodium malonate, p-arsenosobenzoic acid, 5,5′-

diamino-2,2′-dimethyl arsenobenzene, *N*,*N*′-dimethylene sulfonate disodium salt, iodoacetamide, oxophenarsine (mapharsen), auric chloride, p-chloromercuribenzoic acid, p-chloromercuriphenyisullonic acid, cupric chloride, iodine merbromin (mercurochrome)porphyrindine, potassium permanganate, mersalyl (salyrgan), silver nitrate, strong silver protein (protargol), and uranyl acetate.

[00119] In a particular embodiment, ICVs are produced from a cell by contacting or exposing the cell to *N*-ethylmaleimide. In a further embodiment, the cell is incubated with a cell blebbing buffer that comprises or consists essentially of *N*-ethylmaleimide at a concentration of 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1.0 mM, 1.1 mM, 1.2 mM, 1.3 mM, 1.4 mM, 1.5 mM, 1.6 mM, 1.7 mM, 1.8 mM, 1.9 mM, 2.0 mM, 2.1 mM, 2.2 mM, 2.3 mM, 2.4 mM, 2.5 mM, 2.6 mM, 2.7 mM, 2.8 mM, 2.9 mM, 3.0 mM, 3.1 mM, 3.2 mM, 3.3 mM, 3.4 mM, 3.5 mM, 3.6 mM, 3.7 mM, 3.8 mM, 3.9 mM, 4.0 mM, 4.1 mM, 4.2 mM, 4.3 mM, 4.4 mM, 4.5 mM, 4.6 mM, 4.7 mM, 4.8 mM, 4.9 mM, 5.0 mM, 6.0 mM, 7.0 mM, 8.0 mM, 9.0 mM, 0.01 M, 0.1 M, 1.0 M, 1.5 M, 2.0 M, or a range that includes or is between any two of the foregoing concentrations, including fractional increments thereof. In a further embodiment, the cell blebbing buffer further comprises a buffered balanced salt solution. Examples of buffered saline solutions include but are not limited to, phosphate-buffered saline (PBS), Dulbecco's Phosphate-buffered saline (DPBS), Earle's Balanced Salt solution (EBSS), Hank's Balanced Salt Solution (HBSS), TRIS-buffered saline (TBS), and Ringer's balanced salt solution (RBSS). In a further embodiment, the cell blebbing buffer containing a sulfhydryl blocking agent or NEM further comprises a buffered balanced salt solution at a concentration/dilution of 0.5X, 0.6X, 0.7X, 0.8X, 0.9X, 1X, 2X, 3X, 4X, 5X, 6X, 7X, 8X, 9X, and 10X, or a range that includes or is between any two of the foregoing concentrations/dilutions, including fractional increments thereof. In yet a further embodiment, the cells are incubated in a cell blebbing buffer containing a sulfhydryl blocking agent or NEM for 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 10 h, 11 h, 12 h, 13 h, 14 h, 15 h, 16 h, 17 h, 18 h, 19 h, 20 h, 21 h, 22 h, 23 h, 24 h, 36 h, 48 h, 72 h or a range that includes or is between any two of the foregoing time points, including fractional values thereof.

[00120] In another embodiment, ICVs are produced from a cell by contacting or exposing the cell to paraformaldehyde (PFA). In an another embodiment, the cell is incubated with a cell blebbing buffer that comprises or consists essentially of PFA at a concentration of 10.0 mM, 12.0 mM, 14.0 mM, 15.0 mM, 16.0 mM, 18.0 mM, 20.0 mM, 21.0 mM, 22.0 mM,

23.0 mM, 24.0 mM, 25.0 mM, 26.0 mM, 27.0 mM, 28.0 mM, 29.0 mM, 30.0 mM, 32.0 mM, 34.0 mM, 35.0 mM, 40.0 mM, 45.0 mM, 50.0 mM, 100.0 mM, 200.00 mM or a range that includes or is between any two of the foregoing concentrations, including fractional values thereof. In a further embodiment, the cell blebbing buffer comprising PFA further comprises a buffered balanced salt solution. Examples of buffered saline solutions include but are not limited to, phosphate-buffered saline (PBS), Dulbecco's Phosphate-buffered saline (DPBS), Earle's Balanced Salt solution (EBSS), Hank's Balanced Salt Solution (HBSS), TRIS-buffered saline (TBS), and Ringer's balanced salt solution (RBSS). In a further embodiment, the cell blebbing buffer comprising PFA further comprises a buffered balanced salt solution at a concentration/dilution of 0.5X, 0.6X, 0.7X, 0.8X, 0.9X, 1X, 2X, 3X, 4X, 5X, 6X, 7X, 8X, 9X, and 10X, or a range that includes or is between any two of the foregoing concentrations/dilutions, including fractional values thereof. In a further embodiment, the cell blebbing buffer that comprises or consists essentially of PFA does not contain any small molecule redox reagents or reducing agents (*e.g.*, dithiothreitol (DTT)). In yet a further embodiment, the cells are incubated in a cell blebbing buffer containing PFA for 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 10 h, 11 h, 12 h, 13 h, 14 h, 15 h, 16 h, 17 h, 18 h, 19 h, 20 h, 21 h, 22 h, 23 h, 24 h, 36 h, 48 h, 72 h or a range that includes or is between any two of the foregoing time points, including fractional values thereof.

[00121] In a particular embodiment, ICVs may be produced from a cell by contacting the cell with a photosensitizer that induces blebbing. In the studies provided herein, HeLa cells were induced to undergo blebbing and ICV production by incubating or exposing the HeLa cells to a photosensitizer (*e.g.*, $AlPcS_{2A}$) and then exposing the cells to light. It was discovered that the size of generated ICVs was largely in the micrometer or nanometer size range. Further, it was shown herein that the methods of the disclosure efficiently produced nanometer/micrometer ICVs almost immediately upon light exposure without the need to add other chemical agents, like reducing agents (*e.g.*, DTT), agents that interact with sulfhydryl groups (*e.g.*, *N*-ethylmaleimide), inhibitors of energy metabolism (*e.g.*, cyanates), and agents that induce oxidative stress (*e.g.*, menadione). Moreover, the studies presented herein demonstrate that the size allocation of the ICVs could be controlled based upon length of the light exposure dose.

[00122] In a particular embodiment, ICVs may be produced from a cell by incubating or exposing the cell with a photosensitizer and then exposing the cell to light. Examples of such photosensitizers include, but are not limited to, $AlPcS_{2A}$, $AlPcS_4$, lutrin, 5-

aminolevulinic acid (ALA), hypericin, silicon phthalocyanine zinc (II) phthalocyanine (ZnPc), silicon phthalocyanine, mono-L-aspartyl chlorin e6, benzoporphyrin derivative monoacid ring A, chlorin photosensitizer tin etiopurpurin, tetra(*m*-hydroxyphenyl)chlorin, lutetium texaphyrin, 9-acetoxy-2,7,12,17-tetrakis-(*β*-methoxyethyl)-porphycene, naphthalocyanines, Allumera®, Photofrin®, Visudyne®, Levulan®, Foscan®, Fospeg®, Metvix®, Hexvix®, Cysview® and Laserphyrin®, Antrin®, Photochlor®, Photosens®, Photrex®, Lumacan®, Cevira®, Visonac®, BF-200 ALA®, Amphinex® and Azadipyrromethenes. In a further embodiment, ICVs are produced from a cell by incubating or exposing the cell with $AlPcS_{2A}$ and the exposing the cells to light having a wavelength from 600 nm to 850 nm. In a further embodiment, the cell is incubated in the dark with a photosensitive media that comprises or consists essentially of a photosensitizer (*e.g.*, $AlPcS_{2A}$) at a concentration of 0.1 ug/mL, 0.2 ug/mL, 0.3 ug/mL, 0.4 ug/mL, 0.5 ug/mL, 0.6 ug/mL, 0.7 ug/mL, 0.8 ug/mL, 0.9 ug/mL, 1.0 ug/mL, 1.1 ug/mL, 1.2 ug/mL, 1.3 ug/mL, 1.4 ug/mL, 1.5 ug/mL, 1.6 ug/mL, 1.7 ug/mL, 1.8 ug/mL, 1.9 ug/mL, 2.0 ug/mL, 2.5 ug/mL, 3.0 ug/mL, 3.5 ug/mL, 4.0 ug/mL, 4.5 ug/mL, 5.0 ug/mL, 10.0 ug/mL or a range that includes or is between any two of the foregoing concentrations, including fractional values thereof. In yet a further embodiment, the cell is incubated in the dark with a photosensitive media that comprises or consists essentially of a photosensitizer (*e.g.*, $AlPcS_{2A}$) for 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 10 h, 11 h, 12 h, 13 h, 14 h, 15 h, 16 h, 17 h, 18 h, 19 h, 20 h, 21 h, 22 h, 23 h, 24 h, 36 h, 48 h, 72 h or a range that includes or is between any two of the foregoing time points, including fractional values thereof. In another embodiment, the cells contacted with the photosensitizer are exposed to light for 1 min, 2 min, 3 min, 4 min, 5 min, 6 min, 7 min, 8 min, 9 min, 10 min, 12 min, 14 min, 15 min, 16 min, 18 min, 20 min, 25 min, 30 min, 35 min, 40 min, 45 min, 50 min, 55 min, 60 min, 90 min, 120 min, or a range that includes or is between any two of the foregoing timepoints, including fractional values thereof. In a further embodiment, the cells are exposed to light generated from a laser. In yet a further embodiment, the cells are exposed to light having a wavelength of 500 nm, 550 nm, 580 nm, 600 nm, 610 nm, 620 nm, 630 nm, 640 nm, 650 nm, 660 nm, 670 nm, 680 nm, 690 nm, 700 nm, 720 nm, 740 nm, 760 nm, 780 nm, 800 nm, 850 nm, 900 nm that is generated by a laser, or a range that includes or is between any two of the foregoing wavelengths, including fractional values thereof.

[00123] The disclosure further provides that the ICVs may be collected by any suitable means to separate ICVs from cells or cell debris. In some embodiments, to isolate ICVs,

cells can be removed by centrifugation at 1,000 to 2,500 rpm for 1 minutes, 1.5 minutes, 2 minutes, 2.5 minutes, 3 minutes, 3.5 minutes, 4 minutes, 4.5 minutes, 5 minutes, 5.5 minutes, 6 minutes, 6.5 minutes, 7 minutes, 7.5 minutes, 8 minutes, 8.5 minutes, 9 minutes, 9.5 minutes, 10 minutes, or a range that includes or is between any two of the forgoing timepoints, including fractional increments thereof, followed by removal of cell debris. Induce cell vesicles can then be recovered by centrifugation at 8,000 x g to 20,000 x g for 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 11 minutes, 12 minutes, 13 minutes, 14 minutes, or 15 minutes, or a range that includes or is between any two of the forgoing timepoints, including fractional increments thereof. ICVs can be further concentrated using concentrators with a size cutoff of 100 kDA or less. In a particular embodiment, ICVs can be concentrated with a 30 kDa Amicon® concentrator by centrifuging at 4,500 rpm for 15 minutes for one or more cycles. The disclosure also provides that the ICVs may be collected of further purified by use of sucrose gradients.

[00124] To increase the applications of the ICVs the surfaces of the ICVs may modified to comprise one or more functional moieties using the methods disclosed herein. Such functional moieties can provide for a variety of beneficial effects, including, but not limited to, easy purification and concentration of the ICVs; targeted delivery of the ICVs; controlled cellular uptake of the ICVs; monitoring of intracellular pathways using the ICVs; and molecular imaging of the ICVs. The functional moieties for ICVs may originate from the cells used to produce the ICVs. In such a case, the functional moieties may naturally occur on the surface of the cells that are treated with the blebbing methods disclosed herein. For example, when treated with TNF-α, human umbilical vein endothelial cells (HUVEC) overexpress ICAM-1 in the plasma membrane [J. Exp. Med. 177; 1277-1286 (1993)]. In monocytes treated with PMA (phorbol 12-myristate 13-acetate), the membrane protein LFA-1 is activated [J. Exp. Med. 163; 1132-1149 (1986)]. [Human Gene Therapy. 7(11); 1339-1346 (1996)] or other methods known in the art. Alternatively, the functional moieties for ICVs may result from recombinant expression of the functional moieties in the ICV producing cells. In this context, plasmid DNA, RNA or virus is introduced into cells [PNAS. 90 (18); 8392-8396 (1993)] using calcium phosphate precipitation [Current Protocols in Cell Biology 20.3.1-20.3.8 (2003)], lipofectamine mediation [PNAS. 84 (21); 7413-7417 (1987)], electroporation [Nucleic Acids Research. 15 (3) 1311-1326 (1987)], microinjection [Mol Cell Biol. 2(9); 1145-1154 (1982)], ultrasound mediation. The functional moieties may be anchored to the outer membrane surface of the cells, by use of anchoring motifs and the like,

such as the transmembrane domain of human platelet derived growth factor receptor; outer membrane proteins, lipoproteins, autotransporters. Use of cell anchoring motifs are known in the art and can be used to anchor the functional moieties (*e.g.*, receptor ligands, antigens, T cell stimulatory domains) to the cell surface (*e.g.*, see Cheng *et al.*, *J. Am. Chem. Soc.* 140:16413-16417 (2018)). Alternatively, the surface of the ICVs can be modified to comprise one or more functional moieties. Such modifications can result from coupling functional moieties to proteins or other biomolecules found on the surface of ICVs.

[00125] In another embodiment, the surface of the ICVs have been modified to comprise one or more functional moieties that are targeting ligands. Such targeting ligands may originate from the cell used to produce the ICVs, i.e., the cell naturally expresses the targeting ligand; or the targeting ligand may be recombinant expressed in the ICV producing cell; or the targeting ligand may be fixed to the ICV or ICV producing cell surface by use of the surface modification techniques disclosed herein or known in the art (see US10,308,942). In yet another embodiment, the targeting ligand may direct ICVs to, for example, a cell, cell type, tissue type or organ. For example, the targeting ligand may specifically or non-specifically bind with a molecule on the surface of a target cell. The targeting moiety can be a molecule with a specific affinity for a target cell. Targeting moieties can include antibodies or scFVs directed against a protein found on the surface of a target cell, or the ligand or a receptor-binding portion of a ligand for a molecule found on the surface of a target cell. For example, the targeting moiety can recognize a tumor-specific antigen. Examples of tumor-specific antigens include, but are not limited to, alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), CA-125, CA15-3, CA19-9, MUC-1, epithelial tumor antigen (ETA), tyrosinase, melanoma-associated antigen (MAGE), abnormal products of ras or p53, CTAG1B, MAGEA1, and HER2/neu. In a further embodiment, the target cells or tissue are also subjected to a second event. A second event is used, for example, to activate a conditionally therapeutic molecule. Such a second event can be exposure to a second molecule (*e.g.*, extracellularly or delivery intracellularly) such as a prodrug. In one embodiment the second molecule is administered to a subject locally to the relative location of the target cells within the subject (*e.g.*, injection into a tumor or organ). The second molecule may be a pro-drug or an activator of the therapeutic molecule, or a molecule involved in specific delivery of the therapeutic molecule to the target cell or tissue.

[00126] In a particular embodiment, the cellular membranes of ICVs can be modified to comprise one or more functional moieties by chemical conjugation methods, or by

expressing one or more functional moieties of interest using expression vectors, viral vectors, etc. in the ICV producing cells. In another embodiment, the disclosure also provides for modifying the surface of ICVs to comprise one or more functional moieties by using bioorthogonally conjugation. The bioorthogonally conjugation methods disclosed herein provide for facile introduction of desired functional moieties onto the membrane surface of the ICVs. Therefore, the fast, convenient, versatile, and efficient methods of the disclosure afford for synthetically functionalized ICVs that have great utility in a variety of applications, including, targeted delivery for therapeutics, imaging, and detection. Thus, the disclosure provides for the use of bioorthogonal chemistry for all-purpose surface modification of ICVs, and further that the bioorthogonal chemistry techniques can also be used with any ICV producing cell. The methods of the disclosure allow for rapid isolation of the ICVs via the one or more functional moieties, as well as custom tuning of the ICVs interactions with target cells, cellular uptake/intracellular processes, and monitoring the ICVs activities *in vitro* and *in vivo*.

[00127] In contrast to other surface modification techniques, the bioorthogonal approach avoids randomly introducing conjugates and disrupting envelope membrane and proteins. Applying simple chemistry at the cellular level resolves the problem of implementing difficult and laborious genetic alterations. The methods disclosed herein are highly efficient and specific under physiological conditions (*e.g.*, cell culture medium), which allows for modification of ICV producing cells, and the resultant ICVs, with desired molecules. As demonstrated using an ICV-like, enveloped viruses, the method of disclosure can be easily applied to bioorthogonally conjugated ICVs induced by sulfhydryl blocking.

[00128] The disclosure provides for techniques and methods that provide for high yields of bioorthogonally-conjugated ICVs in as little as a few hours, producing both micro and nano-scale sized bioorthogonally-conjugated ICVs. For example, use of the sulfhydryl blocking agent to induce cell membrane blebbing described herein, can initiate the production of bioorthogonally-conjugated ICVs in as little as 2 h to 5 h.

[00129] In the studies provided herein, bioorthogonal chemistry was used to efficiently modify ICVs through modifying the ICV producing cells. ICVs are derived from the cellular membrane during the budding process and carry the biochemical properties of the ICV producing cell membrane. In other words, bioorthogonal modification of the membranes of the ICV producing cells provides for bioorthogonal functionalized ICVs. Any of the ICV producing cells described herein may be used to make bioorthogonal functionalized ICVs.

In a particular, embodiment, the ICV producing cell comprises sialic acids on the surface of the cell. As shown in the experiments presented herein, sialic acids on the cell surface can be oxidized to aldehydes using oxidizing reagents, like sodium periodate or lead tetraacetate. Particular examples of oxidizing sialic acids on cells surfaces are described in Zeng *et al.*, *Nat Methods*, 2009, 6, 2017, which is incorporated herein in-full. By use of oxime ligation, aminooxy-functionalized molecules can be linked to the aldehydes made from oxidizing sialic acid residues on the cell surface using bioorthogonal chemistry. Specific aminooxy-functionalized molecules can be purchased commercially (*e.g.*, Sigma-Aldrich, ThermoFisher, etc.), or can be made *de-novo* using established methods in the art (*e.g.*, Peri *et al.* Tetrahedron 1998, 54, 12269; Carrasco *et al.*, *Tetrahedron Lett.* 2002, 43, 5727; Carrasco et al., *J. Org. Chem.* 2003, 68, 8853; Seo *et al.*, *Org. Lett.* 2009, 11, 5210; Matsubara et al., *Chem-Eur. J.* 2005, 11, 6974; Leung et al., *Carbohydr. Res.* 2009, 344, 570; Peri et al., *Chem. Commun.* 2002, 15041; Peri et al., *Chem-Eur. J.* 2004, 10, 1433; Bohorov et al., *Glycobiology* 2006, 16, 21C; Clo et al., *J. Org. Chem.* 2010, 540; and Carrasco *et al.*, *J. Org. Chem.* 2010, *75*, 5757).

[00130] The ICVs disclosed herein can be used to treat diseases which can be ameliorated by the delivery or the actions of the therapeutic molecules (*e.g.*, antisense oligonucleotides, CRISPR-Cas system, small molecule therapeutics, etc.) gene therapy or immunotherapy on the targeted disease-causing cells or tissue. In one embodiment, the disease involves or is caused by a genetic deficiency in the target cells. The molecule for which they are deficient (or encoding the molecule for which they are deficient) can be delivered to the appropriate cells via the ICVs disclosed herein.

[00131] In a particular embodiment, the ICVs of the disclosure can be used to deliver gene therapies components, such as a gene therapy viral vector. For example, in the studies presented herein, it was found that the methods of the disclosure allow for the packaging of viral particles into induced cell vesicles. Generally, when AAV replicates in a cell it can be released directly or remains in the cell. The disclosure, however, provides a third option, the encapsulation of AAV particles in induced cell vesicles (*e.g.*, see **FIG. 22**). ICVs that contain AAV can be isolated and purified using the methods disclosed herein, and then used as an effective delivery vehicle to targeted cells or tissue. Studies have shown that extracellular vesicle encapsulated AAV can increase the transduction of AAV into the target cell lines. Encapsulating AAV in this envelope has been shown to increase delivery to cells over naked AAV alone (*e.g.*, se **FIG. 23**). The extracellular vesicle associated AAVs were

shown to be more effective both *in vitro* and *in vivo*, owing to the natural role of extracellular vesicles as signaling agents. With vesicle encapsulating AAV, the capsid is blocked from recognition by the immune system. This masking reduces neutralization of the AAV, giving it a longer time to reach and infect the target cells. Despite this system's potential, extracellular vesicle-based therapeutics are currently hindered from clinical advancement due to issues in mass production. To address this challenge, exploration in increasing extracellular vesicle production has been pursued by methods including starvation, manipulating intracellular calcium levels, thermal stress, hypoxia, radiation, and micro-environmental pH. These production methods require between 12-48 hours to achieve significantly improved yields. So, while extracellular vesicles containing AAV are recognized as excellent candidates for viral gene therapy, the use of such agents in clinical trials has been prohibitive due to issues with mass production.

[00132] Provided herein are protocols and methods which avoid the foregoing drawbacks by inducing membrane blebbing by: (1) use of a photosensitizer (*e.g.*, AIPCS2A photosensitizer) and exposure to a certain wavelength of light (*e.g.*, 670nm light), and/or (2) use of a membrane blebbing inducing agent (*e.g.*, NEM or PAF) to initiate mass-scale production of micro and nanoscale AAV-containing ICVs. The techniques and methods disclosed herein provide for the quick and efficient AAV-containing ICV formation that is substantial faster and more efficient, with higher yields, to current AAV-containing-vesicle production techniques. For example, through the use of AIPCS2A, a clinical grade photosensitizing agent, can induce AAV-containing ICV production in just minutes and produces both micro and nano-scale AAV-containing ICVs. Other photosensitizing agents (*i.e.*, photosensitizers) that can be used to produce AAV-containing ICVs are further described herein. In another example presented herein, the use of chemical agent (e.g., NEM) can initiate AAV-containing ICVs secretion in 2-5 h. Other chemical agents, e.g., PAF, that can be used to produce AAV-containing ICVs from AAV producer cells are further described herein.

[00133] While AAV-containing ICVs are shown in the exemplary studies presented herein, it should be understood that the methods and compositions of the disclosure work equally well to produce ICVs that contain any type of viral particles, vectors or viruses. For example, the methods and compositions of the disclosure can be used to produce ICVs which comprise recombinant retroviruses, adenovirus, adeno-associated virus, alphavirus, or lentivirus.

[00134] In a particular embodiment, the disclosure provides for ICVs which comprise an adeno-associated virus (AAV). AAV is a tiny non- enveloped virus having a 25 nm capsid. No disease is known or has been shown to be associated with the wild type virus. AAV has a single-stranded DNA (ssDNA) genome. AAV has been shown to exhibit long-term episomal transgene expression, and AAV has demonstrated excellent transgene expression in the brain, particularly in neurons. Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.7 kb. An AAV vector such as that described in Tratschin *et al.*, *Mol. Cell. Biol.* 5:3251-3260 (1985) can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat *et al.*, *Proc. Natl. Acad. Sci. USA* 81 :6466-6470 (1984); Tratschin *et al.*, *Mol. Cell. Biol.* 4:2072-2081 (1985); Wondisford *et al.*, *Mol. Endocrinol.* 2:32-39 (1988); Tratschin *et al.*, *J. Virol.* 51 :611-619 (1984); and Flotte *et al.*, *J. Biol. Chem.* 268:3781-.3790 (1993). There are numerous alternative AAV variants (over 100 have been cloned), and AAV variants have been identified based on desirable characteristics. For example, AAV9 has been shown to efficiently cross the blood-brain barrier. Moreover, the AAV capsid can be genetically engineered to increase transduction efficient and selectivity, *e.g.*, biotinylated AAV vectors, directed molecular evolution, self-complementary AAV genomes and so on. Modified AAV have also been described, including AAV based on ancestral sequences; see, *e.g.*, US7906111; WO/2005/033321; WO2008027084, WO2014124282; WO2015054653; and WO2007127264. Other modified AAVs that have been described include chimeric nanoparticles (ChNPs) that have an AAV core that expresses a transgene that is surrounded by layer(s) of acid labile polymers that have embedded antisense oligonucleotides (*e.g.*, see Hong *et al.,* ACS Nano 10:8705-8716 (2016)) and Cho *et al.*, *Biomaterials* 2012, 33, 3316−3323). The compositions and methods disclosed herein is a platform technology, and as such the composition and methods disclosed herein can be used with all known AAVs, including the modified AAVs described in the literature.

[00135] Alternatively, the disclosure also provides for ICVs which comprise retroviruses. Retroviruses can be used as a recombinant gene delivery system for the transfer of exogenous genes *in vivo*, particularly into humans. Retroviruses provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of

retroviruses for viral gene therapy, and defective retroviruses are characterized for use in gene transfer for viral gene therapy purposes (for a review see Miller, *Blood* 76:271 (1990)). A replication defective retrovirus can be packaged into virions, which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells in vitro or in vivo with such viruses can be found in Ausubel, *et al*, eds., Current Protocols in Molecular Biology, Greene Publishing Associates, (1989), Sections 9.10-9.14, and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include Ψ&íρ, Ψ&ε, Ψ2 and ΨΑπι. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, in vitro and/or in vivo (see for example Eglitis, *et al.* (1985) *Science* 230: 1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury *et al.* (1991) Science 254: 1802-1805; van Beusechem *et al.* (1992) Proc. Natl. Acad. Sci. USA 89:7640-7644; Kay et al. (1992) Human Gene Therapy 3:641- 647; Dai *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89: 10892-10895; Hwu *et al.* (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868, 116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). By using the methods of the disclosure, one can produce ICVs which comprise retroviruses or retroviral particle or vectors, in a manner similar to producing ICVs which contain AAV as described herein.

[00136] In another embodiment, the disclosure also provides for ICVs which comprise adenovirus-derived vectors. The genome of an adenovirus can be manipulated, such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See, for example, Berkner *et al.*, *BioTechniques* 6:616 (1988); Rosenfeld *et al.*, Science 252:431-434 (1991); and Rosenfeld *et al.*, *Cell* 68: 143-155 (1992). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, or Ad7 etc.) are known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances, in that they are not capable of infecting non- dividing cells and can be used to infect a wide variety of cell types, including epithelial cells (Rosenfeld et al., (1992) supra). Furthermore, the virus

particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situ, where introduced DNA becomes integrated into the host genome (*e.g.*, retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner *et al.*, supra; Haj-Ahmand and Graham, J. Virol. 57:267 (1986). Alphaviruses can also be used. Alphaviruses are enveloped single stranded RNA viruses that have a broad host range, and when used in viral gene therapy protocols alphaviruses can provide high-level transient gene expression. Exemplary alphaviruses include the Semliki Forest virus (SFV), Sindbis virus (SIN) and Venezuelan Equine Encephalitis (VEE) virus, all of which have been genetically engineered to provide efficient replication-deficient and -competent expression vectors. Alphaviruses exhibit significant neurotropism, and so are useful for CNS- related diseases. See, *e.g.*, Lundstrom, Viruses. 2009 Jun; 1(1): 13-25; Lundstrom, Viruses. 2014 Jun; 6(6): 2392-2415; Lundstrom, *Curr Gene Ther.* 2001 May; l(l): 19- 29; Rayner *et al.*, *Rev Med Virol.* 2002 Sep-Oct; 12(5):279-96. By using the methods of the disclosure, one can produce ICVs which comprise adenovirus-derived particles, viruses or vectors, in a manner similar to producing ICVs which contain AAV as described herein.

[00137] The methods of the disclosure address some limitations of using viral vectors, such as AAV vectors, for gene transfer through the use of ICVs. For example, while microvesicles containing AAVs have been shown to be produced and outperform conventionally purified AAV vectors using standard culture conditions, the amount of microvesicles containing AAVs using standard culture conditions is a mere minor fraction of the total AAVs produced and released by the cultured cells (*e.g.*, see Maguire *et al.*, *Mol. Ther.* 20(5):960-971). In direct contrast, the methods disclosed herein, provided for mass production of ICVs which contain viruses, viral particles or viral vectors, by inducing blebbing in the cells that comprise a high percentage of the viruses, viral particles or viral vectors. Such cells that can be used in the blebbing methods are further described herein.

[00138] Cancer cells survive and proliferate by several mechanisms including immune evasion. Immunotherapy overcomes this by activating the immune system to eliminate cancer cells, and the most common strategy for activating the immune system against tumor cells is vaccination with tumor antigens. Previous studies have reported that bone marrow

dendritic cells (BMDCs) pulsed with antigen *ex vivo* are able to act as an anti-cancer vaccine, presenting antigen in vivo and stimulating T cell response to eradicate tumor cells. However, intrinsic variability associated with whole-cell vaccine formulations has made them non-ideal candidates for immunotherapy.

[00139] Extracellular vesicles (EVs) are promising cell-free vaccines for cancer therapy. Extracellular vesicles (EVs) are cell membrane-derived particles naturally formed and released by all cells. As they are derived from cells themselves, EVs exhibit similar anatomy. They are composed of a lipid bilayer and present and contain similar molecules as their parent cell but without organelles (mitochondria, nucleus, etc.). EVs can be categorized into two general groups for therapeutic use, exosomes and microvesicles. Exosomes are 20-100 nm in diameter formed by invagination of multivesicular bodies (MVB) and released by subsequent MVB fusion with the plasma membrane. Microvesicles are 100-1000 nm in diameter and are released by direct blebbing from the plasma membrane.

[00140] Naturally occurring EVs are intrinsically biocompatible, maintaining similar plasma membrane characteristics to their parent cells. EVs maintain the intrinsic biocompatible benefits of cell therapy while gaining advantage with better storability, minimized risk, and ability to tailor size based on desired application. Several groups have demonstrated EVs as effective carriers of therapeutic cargo *in vitro* and *in vivo*. Cells in culture naturally produce EVs but at a rate significantly below the requirements for therapeutic administration, which has been attempted to be mitigated by exposing cells to endosomal trafficking regulators, modified proteins, and external stressors. However, these time- and labor-intensive processes directly affect cellular activities and make it difficult to preserve the composition and biological functions of EVs at a desired cellular stage.

[00141] As noted above, extracellular vesicle-based therapeutics are currently hindered from clinical advancement due to issues in mass production. To address this challenge, exploration in increasing EV production has been pursued by methods including starvation, manipulating intracellular calcium levels, thermal stress, hypoxia, radiation, and micro-environmental pH. These production methods require between 12-48 hours to achieve significantly improved yields. In addition to production problems, high-levels of variability in EV formulations have also hindered use of EV therapies in clinical trials. Reducing variability associated with EVs is necessary to ensuring safety and therapeutic efficacy. Antigen-pulsed dendritic cell derived EVs are a promising cell-free option for cancer immunotherapy, but intrinsic issues with heterogeneity of EV populations has slowed their

progression to the clinic. Improving homogeneity, in particular maturation state of the parent cell and EV size, will be important for achieving effective immunotherapy.

[00142] The disclosure provides for techniques and methods that provide for high yields of antigen presenting or antigenic ICVs in as little as a few hours, producing both micro and nano-scale sized antigen presenting ICVs. For example, use of a sulfhydryl blocking agent described herein to induce blebbing, can induce cell vesicle production in as little as 2-5 h. Accordingly, the techniques and methods disclosed herein can induce fast, efficient and high yields of ICVs, which are simply not possible using current extracellular vesicle production techniques. It was further postulated herein that the blebbing methods of the disclosure would allow for control over the parent cell maturation state and improvement in homogeneity of the induced cell vesicles produced therefrom. As is shown herein, antigenic ICVs produced by the blebbing methods described herein maintained maturation characteristics of their parent cells and served as efficient cell-free cancer vaccines. Cells particularly suited for antigenic ICV production include, but are not limited to, epithelial cells, fibroblast cells, tumor cells and cells of the immune system (mast cells, T and B lymphocytes, dendritic cells, especially Langerhans cells). In a further embodiment the antigenic ICV producing cell is from a subject to be treated with an ICV disclosed herein. In yet a further embodiment, the antigenic ICV producing cell has been modified genetically, *e.g.*, by gene editing, gene knockout, gene therapy, homologous recombination, site directed mutagenesis, transfection with plasmids and vectors, and the like. In a particular embodiment, the antigenic ICV producing cell is a cell of the immune system, including but not limited to, mast cells, T and B lymphocytes and dendritic cells (for example Langerhans cells and bone marrow derived dendritic cells), or cells derived from these cell types, and cells or cell lines modified by genetic engineering so as to render them capable of secreting ICVs.

[00143] In a certain embodiment, the disclosure further provides that that the antigenic ICV producing cell is an 'immature', 'semimature' or 'mature' dendritic cell. Dendritic cells (DCs) are the sentinel antigen-presenting cells of the immune system; such that their productive interface with the dying cancer cells is crucial for proper communication of the "non-self" status of cancer cells to the adaptive immune system. Efficiency and the ultimate success of such a communication hinges upon the maturation status of the DCs, attained following their interaction with cancer cells. Immature DCs facilitate tolerance toward cancer cells (observed for many apoptotic inducers) while fully mature DCs can strongly

promote anticancer immunity if they secrete the correct combinations of cytokines [observed when DCs interact with cancer cells undergoing immunogenic cell death (ICD)]. However, an intermediate population of DC maturation, called semi-mature DCs exists, which can potentiate either tolerogenicity or pro-tumorigenic responses (as happens in the case of certain chemotherapeutics and agents exerting ambivalent immune reactions). Specific combinations of DC phenotypic markers, DC-derived cytokines/chemokines, dying cancer cell-derived danger signals, and other less characterized entities (*e.g.*, exosomes) can define the nature and evolution of the DC maturation state.

[00144] The initial reaction orchestrated by innate immune cells consists of capturing, as well as clearing up or destroying the source of injury, infection, or diseased cells, followed by wound healing and if required (in case of well discernable antigens) "priming" of the adaptive immune cells against antigens derived from the "non-self" diseased cells or pathogens. This adaptive immune cell priming helps to initiate more specific responses, directed against the acquired antigens and leading to the eradication of the antigen source. This in principle is also the basic theory behind anticancer immunity or anticancer immunosurveillance, where innate immune cells recognize the "non-self" tumor-associated antigens (TAAs) and prime adaptive immune cells (mainly T cells) against them. This leads to both: direct and indirect cancer killing, anticancer effector functions, production of anti-TAA antibodies and subsequent immunity capable of rejecting tumor cells possessing the corresponding TAAs. In this complex interplay, one may appreciate that the step of "priming" adaptive immune cells by innate immune cells against TAAs represents a crucial milestone that is completely dependent on the antigen-presenting and antigen-sensing capabilities of innate immune cells. While most innate immune cells (professional presenters) and certain cells of epithelial lineage (non-professional presenters) are capable of presenting antigens to the adaptive immune cells be it to varying degrees; yet the sentinel antigen-presenting cells (APCs) of the immune system are the DCs. DCs are the guardian APCs because they are both efficient at antigen-presenting and adaptive immune cell activation and also good at judging whether an entity possesses "self" or "non-self" antigens. The ability of DCs to present "non-self" TAAs properly to prime as well as to activate adaptive immune cells is an absolute pre-requisite for activation of potent anticancer immunity.

[00145] The disclosure provides that the antigenic ICV producing cells disclosed herein are, as indicated above, are antigen presenting cells (*e.g.*, dendritic cells). Alternatively, the ICV producing cells are cells that have been modified to present antigens,

e.g., by use of expression vectors, gene therapy, and the like. For example, tumor cells can be forced to present their own tumor antigens to the immune system, by use of expression vectors and gene therapy. Generation of dendritic cells is known in the art and may be performed according to methods described and incorporated by reference, specifically methodologies have been described for DCs generation, in which the DCs have been used in clinical trials of the following cancers: melanoma, soft tissue sarcoma, thyroid, glioma, multiple myeloma, lymphoma, leukemia, as well as liver, lung, ovarian, and pancreatic cancer.

[00146] In the studies provided herein, the ability of producer cells to secrete ICVs was exploited. DC cells were induced to undergo blebbing by use of a method disclosed herein. It was discovered that the size of generated antigenic ICVs were largely in the nanometer or micrometer size range (*e.g.*, from 0.2 μm to 10 μm). Further, it was shown herein that the methods of the disclosure efficiently produced nanometer/micrometer antigenic ICVs without the need to add other chemical agents, like reducing agents (*e.g.*, DTT). The foregoing is especially beneficial as the studies presented herein indicate that the use of DTT negatively affected antigen presentation of antigenic ICVs presented herein.

[00147] The antigenic ICVs disclosed herein can be used in immunotherapy applications, whereby administration of the antigenic ICVs can promote or induce a desired immune response in a subject in need of immunotherapy. For example, the antigenic ICVs disclosed herein represent an improved platform for cancer immunotherapy by being scalable, tunable size, tunable maturation rate, and having a cell-free aspect. More specifically, the disclosure provides a cell-free antigenic ICV-based immunotherapy that was developed by isolating ICVs (*e.g.*, mICVs or nICVs) from antigen-presenting dendritic cells harvested from bone marrow (BMDCs). By inducing cell membrane blebbing by using a sulfhydryl-blocking agent or p photosensitizer, antigen-presenting ICVs were produced from mature BMDCs that expressed CD11c, CD40, CD80, CD86 and H-2Kb bound to SIINFEKL. The antigenic ICVs of the disclosure demonstrated effective activation of T cell hybridomas *in vitro* and cytotoxic T lymphocytes *in vivo*. Immunization with cell-free antigenic ICVs derived from mature BMDCs resulted in slowed tumor growth and improved survival outcomes comparable to whole cell therapy. While the exemplary study presented herein used immunization against OVA-expressing cancer cells, it should be recognized that antigenic ICVs can be engineered to present specific cancer antigens and therefore have a range of potential for various types of cancer therapy. Moreover, the antigenic ICVs

disclosed herein can be used for suppression immunotherapies to dampen an abnormal immune response in autoimmune diseases or reduces a normal immune response to prevent rejection of transplanted organs or cells. Additionally, there is potential to develop antigenic ICVs as vaccines against infectious diseases, and as helminthic therapies.

[00148] The disclosure teaches means of utilizing antigenic ICVs for cancer immunotherapy. In one embodiment, the disclosure provides generation of therapeutic immune stimulating antigenic ICVs through the steps of: (a) obtaining immature dendritic cells (*e.g.*, bone marrow dendritic cells (BMDCs)); (b) pulsing dendritic cells with an antigen associated with cancer cells; (c) inducing cell membrane blebbing by use of a sulfhydryl blocking agent or a photosensitizer; (d) collecting antigenic ICVs that are produced from cell membrane blebbing; and (d) administering said antigenic ICVs to a subject in need of immunotherapy. In one embodiment said dendritic cells (DCs) are pulsed with antigen in the form of protein, peptide, altered peptide, or DNA or RNA transfection. Cancer associated proteins may be utilized for pulsing of DCs include, but are not limited to, oncogenic proteins, such as EGFRvIII, EGFR, HER-2, HER-3, HER-4, MET, cKit, PDGFR, Wnt, beta-catenin, K-ras, H-ras, N-ras, Raf, N-myc, c-myc, IGFR, IGFR, PI3K, and Akt; tumor suppressor proteins, such as BRCA1, BRCA2 and PTEN; cancer-related host receptors and proteins associated with angiogenesis such as VEGFR-2, VEGFR-1, Tie-2, TEM-1 and CD276. It is contemplated that all oncogenic proteins, tumor suppressor proteins, host-cell related receptors and microvesicle-associated molecules may be used, alone or in combination, in the methods, compositions and kits of the present disclosure. It is further contemplated that any oncogenic protein, and any combination of oncogenic proteins, which is determined to be mechanistically, diagnostically, prognostically or therapeutically important for cancer, may be used in the methods, compositions and kits of the present disclosure.

[00149] The disclosure further provides methods of delivering the ICVs disclosed herein to a subject in need of immunotherapy comprising, administering an effective amount of an antigenic ICV preparation or ICV preparation produced by a method disclosed herein to the subject. The administering can be local or systemic. For example, the antigenic ICV preparation or ICV preparation may be locally administered to a subject by injection, such as by injection into an organ or a tumor.

[00150] The disclosure also provides methods of elucidating an immune response in an immune cell, comprising: contacting the immune cell with an effective amount of an

antigenic ICV preparation produced by a method disclosed herein. In one embodiment, the immune cell is contacted *in vivo*. In a further embodiment, the immune cell is contacted within an organ or tumor. In yet a further embodiment, the antigenic ICV preparation is produced *ex vivo* from donor cells of a subject. In an alternate embodiment, the immune cell is contacted *in vitro* with antigenic ICV preparation.

[00151] In view of the foregoing, the methods disclosed in the application provide a foundational platform for the production of ICVs that can be rapidly and mass produced, and further, can be designed and tailored for use in specific applications such as for drug delivery, gene therapy, immunotherapy, cell-free cell therapy, and molecular therapy, and that such advantages cannot be recognized using currently available techniques. For example, by using an engineering biology approach, one can design ICVs that are customized with various surface proteins and/or cargoes to treat specific diseases or conditions.

[00152] In a particular embodiment, ICVs described herein can be used to deliver therapeutic molecules (*e.g.*, gene therapy components, antisense oligonucleotide, small molecule therapeutics, etc.) to a target cell or population of cells. Delivery is accomplished from contacting of the ICVs to the target cells (*e.g.*, tumor cells) or tissue. The target cells or tissue may be contacted *in vitro* or *in vivo*. *In vivo* delivery is accomplished by administration of the ICVs described herein to a subject by a route that results in contacting of the ICVs with the target cells or tissue. For therapeutic purposes, a therapeutically effective amount is administered such that an effective amount of the therapeutic molecule is delivered to the target cells or tissue. In a further embodiment, the targeted cell or tissue is contacted within an organ or tumor. In yet a further embodiment, the ICVs described herein are produced *ex vivo* from donor cells of a subject that is to be treated. In an alternate embodiment, the targeted cell or tissue is contacted *in vitro*. The ICVs can be loaded with different types of therapeutic molecules, including small molecule drugs, biological molecules, viruses, therapeutic agents, prodrugs, gene silencing agents, chemotherapeutics, diagnostic agents, and/or components of gene editing systems. Examples of biological molecules include, but are not limited to, nucleic acids (*e.g.*, DNA, RNA, mRNA, modified mRNA, small RNAs, siRNA, miRNA, genes, and transgenes), peptides/proteins (including antibodies, enzymes, transcription factors, etc.), viruses, hormones, carbohydrates, lipids, and vitamins. Examples of gene silencing agents, include siRNA, chRNAs, miRs, ribozymes, morpholinos, and esiRNAs. Examples of gene editing systems include, but are not limited to,

CRISPR-Cas systems, zinc finger nucleases, and TALENs. Examples of diagnostic agents, include but are not limited to, dyes and stains, radioactive tracers, and contrast agents.

[00153] In a particular embodiment, the ICVs are loaded with one or more small molecule therapeutic compounds or agents. In a further embodiment, the ICVs are loaded or co-administered with one or more anticancer agents or chemotherapeutics. Examples, of anticancer agents and chemotherapeutics that can be used with or loaded into the ICVs disclosed herein include, but are not limited to, alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and tiimethylolomelamine; acetogenins (*e.g.*, bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; vinca alkaloids; epipodophyllotoxins; antibiotics such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gammall and calicheamicin omegall; L-asparaginase; anthracenedione substituted urea; methyl hydrazine derivatives; dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic

acid analogs such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitiaerine; pentostatin; phenamet; pirarubicin; losoxantione; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2 2"-trichlorotiiethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, *e.g.*, TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE® Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Ill.), and TAXOTERE® (docetaxel) (Rhone-Poulenc Rorer, Antony, France); chloranbucil; GEMZAR® (gemcitabine); 6-thioguanine; mercaptopurine; methotrexate; platinum coordination complexes such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE® vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (e.g., CPT-11); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DFMO); retinoids such as retinoic acid; capecitabine; leucovorin (LV); irenotecan; adrenocortical suppressant; adrenocorticosteroids; progestins; estrogens; androgens; gonadotropin-releasing hormone analogs; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included anticancer agents are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON-toremifene; aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen

production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASL® exemestane, formestanie, fadrozole, RIVISOR® vorozole, FEMARA® letrozole, and ARTMIDEX® anastrozole; and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those which inhibit expression of genes in signaling pathways implicated in abherant cell proliferation, such as, for example, PKC-alpha, Ralf and H-Ras; ribozymes such as a VEGF-A expression inhibitor (*e.g.*, ANGIOZYME® ribozyme) and a HER2 expression inhibitor; vaccines such as gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; PROLEUKIN® rJL-2; LURTOTECAN® topoisomerase 1 inhibitor; ABARELLX® rmRH; antibodies such as trastuzumab and pharmaceutically acceptable salts, acids or derivatives of any of the above.

[00154] The disclosure further provides that the ICVs disclosed herein may be used to deliver biological molecules, therapeutic agents, prodrugs, gene silencing agents, chemotherapeutics, diagnostic agents, gene therapy agents and/or components of a gene editing system to a subject, or cells thereof. Delivery of the biological molecule may elicit a desired effect directly, such as an mRNA encoding a peptide for immediate transcription. Alternatively, delivery of the biological molecule may cause the desired tissue to generate the response, such that delivery of a transcription factor may activate an innate gene. Delivery of specific imaging agents may allow accumulation of dyes to a specific cell-type or tissue for imaging without background imaging signal being produced by neighboring tissue (*e.g.*, FRET sensing applications).

[00155] The disclosure also provides that the ICVs disclosed herein which comprise a cargo, *e.g.*, biological molecules, therapeutic agents, prodrugs, gene silencing agents, chemotherapeutics, diagnostic agents, and/or components of a gene editing system, prior to, concurrently with, after, or any combination thereof for the production of cargo loaded ICVs. For example, ICV producing cells may be loaded with the cargo, or alternatively the ICV producing cells may be induced to produce cargo like biomolecules. In embodiments where the ICV producing cells are loaded with the cargo, the cells may be incubated with the cargo in similar conditions as described within this disclosure or in the art to allow for the cell to uptake the cargo (*e.g.*, therapeutic agents) (*e.g.*, see Pascucci *et al., J. Control. Release* ***2014****, 192, 262-270;* Lv *et al., J. Biol. Chem.* ***2012****, 287, 15874-15885*, and US20180296483). In embodiments where the cells are loaded by inducing a cell to produce the cargo (*i.e.*,

biomolecules), the cell may be engineered to express or produce specific peptides, nucleic acids, or both peptides and nucleic acids with therapeutic properties (*e.g.*, see Tian *et al.*, *Biomaterials* 2014 35(7):2383-90). Therapeutic peptides may include small peptides, protein subunits, entire proteins, or any combination of the above. Therapeutic nucleic acids may include DNA or RNA, including genie sequences, plasmid DNA, tRNA, rRNA, mRNA, small RNAs, miRNA, siRNA, shRNA, crRNA, or any combination of nucleic acids produced within the cell. Additionally, ribonucleoproteins or any other form of protein-nucleic acid complex may be produced within a cell. In some embodiments, once the cells are loaded with the cargo, cell blebbing as described herein is used to induce cell vesicle formation. In other embodiments, cell blebbing may be induced during the loading of the cargo into the cells. Situations were cell blebbing may occur during loading may include where the cell produces the cargo (*i.e.*, biomolecules). Upon inducing cell blebbing, ICVs produced from the cargo loaded cells may contain the agents of interest.

[00156] ICVs produced in accordance with embodiments of the disclosure may also be loaded with the cargo via direct membrane penetration, chemical labeling and conjugation, electrostatic coating, adsorption, absorption, sonification, electroporation, use of pH gradients, or any combination thereof. For example, the ICVs can be loaded by sonification by the methods described in Kim *et al.* (*Nanomedicine* 2016 12(3):6550664). In another embodiment, the cargo can be loaded in ICVs by use of a pH gradient between the inside of and the outside of the ICV, *e.g.*, (i) increasing or decreasing the pH value inside of the ICV and/or (ii) increasing or decreasing the pH value outside of the ICV (*e.g.*, see US20180177725). Methods of formulating the pH gradient between the inside of and the outside of the vesicle composition are known to the skilled artisan.

[00157] Further, ICVs produced in accordance with certain embodiments of the disclosure may undergo multiple loading steps, such that some cargo may be loaded prior to cell blebbing, while additional cargo may be loaded during or after cell blebbing. Additionally, ICVs may be loaded with the cargo during cell blebbing, and further loaded with another cargo after cell blebbing. In a further embodiment, the ICVs may be loaded with a cargo as defined above by incubating the ICV producing cells or empty ICVs with a cargo as defined above having the concentration of 25 pg/mL, 50 pg/mL, 100 pg/mL, 200 pg/mL, 300 pg/mL, 400 pg/mL, 500 pg/mL, 600 pg/mL, 700 pg/mL, 800 pg/mL, 900 pg/ml, 1 ng/mL, 10 ng/mL, 100 ng/mL, 1 µg/mL, 10 ug/mL or any range that includes or is between any two of the foregoing concentrations, including fractional increments thereof.

Additionally, the incubation with the cargo may occur for 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 24 hours, 48 hours, or any range that includes or is between any two of the foregoing time points, including fractional increments thereof. Alternatively, the loading conditions may occur at a ratio of ICVs to a cargo as defined above of 1:20 to 20:1, 1:15 to 15:1, 12:1 to 1:12, 11:1 to 1:11, 10:1 to 1:10, 9:1 to 1:9, 8:1 to 1:8, 7:1 to 1:7, 6:1 to 1:6, 5:1 to 1:5, 4:1 to 1:4, 3:1 to 1:3, 2:1 to 1:2, 1.5:1 to 1:1.5, or 1:1. Additionally, the polydispersity of cargo-loaded ICVs may have a similar polydispersity index (PDI) as unloaded ICVs. As such, cargo-loaded ICVs may have a PDI of about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, or any range that includes or is between any two of the foregoing values, including fractional increments thereof.

[00158] In a further embodiment, a composition, such as a pharmaceutical composition, preparation or formulation may comprise a plurality of different types of ICVs, that differ, *e.g.*, in size, cargo, recombinant modifications or originate from different ICV producing cells. For example, the composition may comprise antigenic ICVs; and ICVs that are loaded with a therapeutic cargo, such as anticancer agents, or gene therapy components. Such gene therapy components are described more fully below, but can include AAVs that express a transgene (*e.g.*, a tumor suppressor gene, pro-apoptosis gene, etc.). Any number of combinations of different types of ICVs are possible using the methods disclosed herein. The disclosure further provides that the ICVs made by the methods disclosed herein can be used for cell-free cell therapies. For example, antigenic ICVs disclosed herein can be used as cell-free versions of the immunotherapy approach of adoptive cell transfer (ACT). In such a case, immune cells (mammalian T cells, DC cells, NK cells, neutrophils, macrophages, and stem cells (iPSCs included)) are first extracted from the patient, genetically modified, and cultured *in vitro*; then these cells are used to produce ICVs using the methods disclosed herein and the ICVs are administered to the same patient. The most well known and advanced form of ACT is CAR T-cell therapy. As such, it is envisioned that the antigenic ICVs disclosed herein can be used as cell-free version of CAR T-cell therapy. Additionally, any cell-free cell therapy that uses exosomes can similarly use ICVs. Examples of such cell-free cell therapies, include use of ICVs include to suppress T-cell activity by expressing PD-L1 (*e.g.*, see Poggio *et al.*, Cell 177(2):P414-427); use of ICVs to inhibit tumor growth and increase T cell infiltration by expressing a hyaluronidase (*e.g.*, see Hong et al., *Advanced Functional Materials* 28(5) (2018)); use of ICVs to redirect and activate cytotoxic T cells toward cancer cells by displaying antibodies to CD3 and EGFR using an anchoring motif

(*e.g.*, see Cheng *et al.*, Journal of American Chemical Society 140:16413:16417 (2018)). Additionally, the ICVs disclosed herein can be used as cell-free vaccines. Such ICVs can be loaded with biomolecules (*e.g.*, mRNA, DNA, proteins, etc.) that provide active acquired immunity to a particular disease or condition when the ICVs are administered. Such vaccines can be used to treat diseases caused by infectious agents, like disease causing bacteria, viruses, and protozoa; or used as therapies to treat cancer, herpes, HIV and Alzheimer's disease. Further, the ICVs disclosed herein can be designed to be multifunctional therapeutic cell free scaffolds, such that they can be used to have multiple and synergistic biological effects when administered, *e.g.*, the ICVs be loaded with multiple types of cargo to treat the same disease or disorder; may have a cargo that works in conjunction with a cell surface modification to treat a disease or disorder; may have multiple cell surface modifications that activate multiple cell types in treating a disease or disorder; etc.

[00159] The disclosure further provides for pharmaceutical compositions, preparation or formulations comprising an ICV preparation described herein for specified modes of administration. In one embodiment, an ICV preparation described herein is an active ingredient in a composition comprising a pharmaceutically acceptable carrier. Such a composition is referred to herein as a pharmaceutical composition. A "pharmaceutically acceptable carrier" means any pharmaceutically acceptable means to mix and/or deliver the targeted delivery composition to a subject. The term "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agents from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the composition and is compatible with administration to a subject, for example a human. Such compositions can be specifically formulated for administration via one or more of a number of routes, such as the routes of administration described herein. Supplementary active ingredients also can be incorporated into the compositions. When an agent, formulation or pharmaceutical composition described herein, is administered to a subject, preferably, a therapeutically effective amount is administered. As used herein, the term "therapeutically effective amount" refers to an amount that results in an improvement or remediation of the condition.

[00160] Administration of the pharmaceutical composition to a subject is by means which an ICV preparation contained therein will contact the target cell. The specific route

will depend upon certain variables such as the target cell and can be determined by the skilled practitioner. Suitable methods of administering an ICV preparation described herein to a patient include any route of *in vivo* administration that is suitable for delivering extracellular vesicles to a patient. The preferred routes of administration will be apparent to those of skill in the art, depending on the extracellular vesicle preparation's type of therapeutic molecule used, the target cell population, and the disease or condition experienced by the subject. Preferred methods of *in vivo* administration include, but are not limited to, intravenous administration, intertumoral administration, intraperitoneal administration, intramuscular administration, intracoronary administration, intraarterial administration (*e.g.*, into a carotid artery), subcutaneous administration, transdermal delivery, intratracheal administration, subcutaneous administration, intraarticular administration, intraventricular administration, inhalation (*e.g.*, aerosol), intracerebral, nasal, oral, pulmonary administration, impregnation of a catheter, and direct injection into a tissue. In an embodiment where the target immune cells are in or near a tumor, a preferred route of administration is by direct injection of antigenic ICVs into the tumor or tissue surrounding the tumor. For example, when the tumor is a breast tumor, the preferred methods of administration include impregnation of a catheter, and direct injection of antigenic ICVs into the tumor.

[00161] Intravenous, intraperitoneal, and intramuscular administrations can be performed using methods standard in the art. Aerosol (inhalation) delivery can also be performed using methods standard in the art (see, for example, Stribling *et al.*, *Proc. Natl. Acad. Sci. USA* 189: 11277-11281, 1992, which is incorporated herein by reference in its entirety). Oral delivery can be performed by complexing an ICV preparation of the disclosure to a carrier capable of withstanding degradation by digestive enzymes in the gut of an animal. Examples of such carriers, include plastic capsules or tablets, such as those known in the art.

[00162] One method of local administration is by direct injection. Direct injection techniques are particularly useful for administering ICVs to a cell or tissue that is accessible by surgery, and particularly, on or near the surface of the body. Administration of a composition locally within the area of a target cell refers to injecting the composition centimeters and preferably, millimeters from the target cell or tissue.

[00163] The appropriate dosage and treatment regimen for the methods of treatment described herein will vary with respect to the particular disease being treated, the ICVs being delivered, and the specific condition of the subject. The skilled practitioner is to determine

the amounts and frequency of administration on a case by case basis. In one embodiment, the administration is over a period of time until the desired effect (*e.g.*, reduction in symptoms is achieved). In a certain embodiment, administration is 1, 2, 3, 4, 5, 6, or 7 times per week. In a particular embodiment, administration is over a period of 1, 2, 3, 4, 5, 6, 7 ,8, 9, or 10 weeks. In another embodiment, administration is over a period of 2, 3, 4, 5, 6 or more months. In yet another embodiment, treatment is resumed following a period of remission.

[00164] For use in the therapeutic applications described herein, kits and articles of manufacture are also described herein. Such kits can comprise a carrier, package, or container that is compartmentalized to receive one or more containers such as vials, tubes, and the like, each of the container(s) comprising one of the separate elements to be used in a method described herein. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers can be formed from a variety of materials such as glass or plastic.

[00165] For example, the container(s) can comprise one or more ICV preparations described herein, optionally in a composition or in combination with another agent as disclosed herein. The container(s) optionally have a sterile access port (for example the container can be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). Such kits optionally comprise a compound disclosed herein with an identifying description or label or instructions relating to its use in the methods described herein.

[00166] A kit will typically comprise one or more additional containers, each with one or more of various materials (such as reagents, optionally in concentrated form, and/or devices) desirable from a commercial and user standpoint for use of a compound described herein. Non-limiting examples of such materials include, but are not limited to, buffers, diluents, filters, needles, syringes; carrier, package, container, vial and/or tube labels listing contents and/or instructions for use, and package inserts with instructions for use. A set of instructions will also typically be included.

[00167] A label can be on or associated with the container. A label can be on a container when letters, numbers or other characters forming the label are attached, molded or etched into the container itself; a label can be associated with a container when it is present within a receptacle or carrier that also holds the container, *e.g.*, as a package insert. A label can be used to indicate that the contents are to be used for a specific application. The label can also indicate directions for use of the contents, such as in the methods described herein.

[00168] The disclosure further provides that the methods and compositions described herein can be further defined by the following aspects (aspects 1 to 90):

1. A method to produce induced cell vesicles (ICVs) or antigenic ICVs, comprising:

inducing cell vesicle production from cells by exposing or contacting the cells with a cell blebbing buffer which comprises a sulfhydryl blocking agent or a photosensitizer

wherein antigenic ICVs are produced from antigen presenting cells which can stimulate T-cell activation.

2. The method of aspect 1, wherein the cells are from a mammal.

3. The method of aspect 1 or 2, wherein the cells are from a human.

4. The method of any one of the previous aspects, wherein the cells are from a human patient that has a disorder or disease that is to be treated with ICVs or antigenic ICVs produced therefrom.

5. The method of any one of the previous aspects, wherein the cells are selected from epithelial cells, fibroblast cells, tumor cells, mast cells, T and B lymphocytes, dendritic cells, and Langerhans cells.

6. The method of any one of the previous aspects, wherein the antigenic ICVs are produced from dendritic cells, preferably wherein the dendritic cells are conventional dendritic cells and/or plasmacytoid dendritic cell, more preferably wherein the dendritic cells are CD11c+ myeloid dendritic cells, CD 141+ myeloid dendritic cells, and/or CD 303+ plasmacytoid dendritic cells.

7. The method of aspect 6, wherein the dendritic cells are bone marrow dendritic cells (BMDCs).

8. The method of aspect 7, wherein the BMDCs are immature BMDCs, preferably wherein the immature BMDCs express CD11c surface markers.

9. The method of aspect 7, wherein the BMDCs are mature BMDCs, preferably wherein the mature BMDCs express CD80, CD83, and/or CD86 surface markers.

10. The method of any one of the previous aspects, wherein the induced cell vesicles comprise viruses, viral particles, or viral vectors, by being produced from cells comprising the same.

11. The method of aspect 10, wherein the viruses, viral particles, or viral vectors are selected from recombinant retroviruses, adenoviruses, adeno-associated viruses (AAV), alphaviruses, and lentiviruses.

12. The method of aspect 11, wherein the viruses, viral particles, or viral vectors are AAV, preferably wherein the AAV has serotype selected from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, or AAV12, more preferably where the AAV is AAV2 or AA8.

13. The method of aspect 12, wherein the AAV expresses a heterologous transgene that is used for gene therapy, preferably wherein the heterologous transgene expresses a fully functioning gene product that is not expressed, mis-expressed or mutated in a subject as a result of an inherited disorder or as a result of organ damage, more preferably wherein the heterologous transgene expresses Factor IX, RPE65, lipoprotein lipase, SCID-X1, IL2RG, telomerase, sarcoplasmic calcium ATPase, Ca^{2+} channel-binding domain 3, or angiotensin-converting enzyme 2 gene products.

14. The method of any one of the previous aspects, wherein the cell blebbing buffer does not contain any small molecule redox reagents or reducing agents, preferably wherein the cell blebbing buffer does not contain dithiothreitol (DTT).

15. The method of any one of the previous aspects, wherein the cell blebbing buffer comprises a buffered balanced salt solution.

16. The method of aspect 15, wherein the buffered balanced salt solution selected from the group consisting of phosphate-buffered saline (PBS), Dulbecco's Phosphate-buffered saline (DPBS), Earle's Balanced Salt solution (EBSS), Hank's Balanced Salt Solution (HBSS), TRIS-buffered saline (TBS), and Ringer's balanced salt solution (RBSS).

17. The method of aspect 15, wherein the cell blebbing buffer comprises 0.5X, 0.6X, 0.7X, 0.8X, 0.9X, 1X, 2X, 3X, 4X, 5X, 6X, 7X, 8X, 9X, or 10X concentration/dilution of the buffered balanced salt solution, or a range that includes or is between any two of the foregoing concentrations/dilutions, including fractional values thereof, preferably wherein the cell blebbing buffer comprises a concentration/dilution of the buffered balanced salt solution, preferably wherein the cell blebbing buffer comprises 1X to 10X concentration/dilution of the buffered balanced salt solution.

18. The method of aspect 15, wherein the cell blebbing buffer comprises 1X to 5X, 1X to 4X, 1X to 3X, 1X to 2X, 2X to 5X, 2X to 4X, 2X to 3X, 3X to 5X, or 3X to 4X, concentration/dilution of the buffered balanced salt solution, preferably wherein the buffered balanced salt solution is DPBS.

19. The method of any one of the previous aspects, wherein the cells are incubated in the cell blebbing buffer which comprises a sulfhydryl blocking agent for 0.5 h, 1 h, 2 h, 3

h, 4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 10 h, 11 h, 12 h, 13 h, 14 h, 15 h, 16 h, 17 h, 18 h, 19 h, 20 h, 21 h, 22 h, 23 h, 24 h, 36 h, 48 h, 72 h or a range that includes or is between any two of the foregoing time points, including fractional values thereof, more preferably wherein the cells are incubated in the cell blebbing buffer which comprises a sulfhydryl blocking agent for 1 h to 10 h, 2 h to 10 h, 3 h to 10 h, 4 h to 10 h, 5 h to 10 h, 6 h to 10 h, 7 h to 10 h, 8 h to 10 h, 1 h to 9 h, 2 h to 9 h, 3 h to 9 h, 4 h to 9 h, 5 h to 9 h, 6 h to 9 h, 7 h to 9 h, 8 h to 9 h, 1 h to 8 h, 2 h to 8 h, 3 h to 8 h, 4 h to 8 h, 5 h to 8 h, 6 h to 8 h, 7 h to 8 h, 1 h to 7 h, 2 h to 7 h, 3 h to 7 h, 4 h to 7 h, 5 h to 7 h, or 6 h to 7 h.

20. The method of aspect 19, wherein the cells are incubated in the cell blebbing buffer for 1 h to 8 h at about 37 °C.

21. The method of any one of the previous aspects, wherein the sulfhydryl blocking agent is selected from the group consisting of and *N*-ethylmaleimide, paraformaldehyde, mercury chloride, p-chloromercuribenzene sulfonic acid, auric chloride, *p*-chloromercuribenzoate, chlormerodrin, meralluride sodium, and iodoacetamide.

22. The method of aspect 21, wherein the sulfhydryl blocking agent is *N*-ethylmaleimide (NEM) or maleimide.

23. The method of aspect 22, wherein the cell blebbing buffer comprises NEM at a concentration of 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1.0 mM, 1.1 mM, 1.2 mM, 1.3 mM, 1.4 mM, 1.5 mM, 1.6 mM, 1.7 mM, 1.8 mM, 1.9 mM, 2.0 mM, 2.1 mM, 2.2 mM, 2.3 mM, 2.4 mM, 2.5 mM, 2.6 mM, 2.7 mM, 2.8 mM, 2.9 mM, 3.0 mM, 3.1 mM, 3.2 mM, 3.3 mM, 3.4 mM, 3.5 mM, 3.6 mM, 3.7 mM, 3.8 mM, 3.9 mM, 4.0 mM, 4.1 mM, 4.2 mM, 4.3 mM, 4.4 mM, 4.5 mM, 4.6 mM, 4.7 mM, 4.8 mM, 4.9 mM, 5.0 mM, 6.0 mM, 7.0 mM, 8.0 mM, 9.0 mM, 0.01 M, 0.1 M, 1.0 M, 1.5 M, 2.0 M, or a range that includes or is between any two of the foregoing concentrations, preferably wherein the cell blebbing buffer comprises NEM at a concentration of

1 mM to 10 mM, 1 mM to 9 mM, 1 mM to 8 mM, 1 mM to 7 mM, 1 mM to 6 mM, 1 mM to 5 mM, 1 mM to 4 mM, 1 mM to 3 mM, 1 mM to 2 mM, 2 mM to 10 mM, 2 mM to 9 mM, 2 mM to 8 mM, 2 mM to 7 mM, 2 mM to 6 mM, 2 mM to 5 mM, 2 mM to 4 mM, 2 mM to 3 mM, 3 mM to 10 mM, 3 mM to 9 mM, 3 mM to 8 mM, 3 mM to 7 mM, 3 mM to 6 mM, 3 mM to 5 mM, 3 mM to 4 mM, 4 mM to 10 mM, 4 mM to 9 mM, 4 mM to 8 mM, 4 mM to 7 mM, 4 mM to 6 mM, or 4 mM to 5 mM, more preferably wherein the cell blebbing buffer comprises NEM at a concentration from 1 mM to 10 mM.

24. The method of aspect 23, wherein the cell blebbing buffer consists essentially of 1 mM, 1.5 mM, 2 mM, 2.5 mM, or 3 mM NEM in DPBS, preferably wherein the cell blebbing buffer consists essentially of 2 mM NEM in DPBS.

25. The method of aspect 21, wherein the cell blebbing buffer comprises paraformaldehyde.

26. The method of aspect 25, wherein the cell blebbing buffer comprises paraformaldehyde at a concentration of 10.0 mM, 12.0 mM, 14.0 mM, 15.0 mM, 16.0 mM, 18.0 mM, 20.0 mM, 21.0 mM, 22.0 mM, 23.0 mM, 24.0 mM, 25.0 mM, 26.0 mM, 27.0 mM, 28.0 mM, 29.0 mM, 30.0 mM, 32.0 mM, 34.0 mM, 35.0 mM, 40.0 mM, 45.0 mM, 50.0 mM, 100.0 mM, 200.00 mM or a range that includes or is between any two of the foregoing concentrations, including fractional values thereof, preferably wherein the cell blebbing buffer comprises paraformaldehyde at a concentration of 10.0 mM to 50.0 mM, 10.0 mM to 45.0 mM, 10.0 mM to 40.0 mM, 10.0 mM to 35.0 mM, 10.0 mM to 34.0 mM, 10.0 mM to 33.0 mM, 10.0 mM to 32.0 mM, 10.0 mM to 31.0 mM, 10.0 mM to 30.0 mM, 10.0 mM to 29.0 mM, 10.0 mM to 28.0 mM, 10.0 mM to 27.0 mM, 10.0 mM to 26.0 mM, 10.0 mM to 25.0 mM, 15.0 mM to 50.0 mM, 15.0 mM to 45.0 mM, 15.0 mM to 40.0 mM, 15.0 mM to 35.0 mM, 15.0 mM to 34.0 mM, 15.0 mM to 33.0 mM, 15.0 mM to 32.0 mM, 15.0 mM to 31.0 mM, 15.0 mM to 30.0 mM, 15.0 mM to 29.0 mM, 15.0 mM to 28.0 mM, 15.0 mM to 27.0 mM, 15.0 mM to 26.0 mM, 15.0 mM to 25.0 mM, 18.0 mM to 40.0 mM, 18.0 mM to 35.0 mM, 18.0 mM to 34.0 mM, 18.0 mM to 33.0 mM, 18.0 mM to 32.0 mM, 18.0 mM to 31.0 mM, 18.0 mM to 30.0 mM, 18.0 mM to 29.0 mM, 18.0 mM to 28.0 mM, 18.0 mM to 27.0 mM, 18.0 mM to 26.0 mM, 18.0 mM to 25.0 mM, 20.0 mM to 34.0 mM, 20.0 mM to 33.0 mM, 20.0 mM to 32.0 mM, 20.0 mM to 31.0 mM, 20.0 mM to 30.0 mM, 20.0 mM to 29.0 mM, 20.0 mM to 28.0 mM, 20.0 mM to 27.0 mM, 20.0 mM to 26.0 mM, or 20.0 mM to 25.0 mM, more preferably wherein the cell blebbing buffer comprises 25 mM of paraformaldehyde.

27. The method of any one of the previous aspects, wherein the cells are incubated with or exposed to a photosensitizer having a concentration of 0.1 ug/mL, 0.2 ug/mL, 0.3 ug/mL, 0.4 ug/mL, 0.5 ug/mL, 0.6 ug/mL, 0.7 ug/mL, 0.8 ug/mL, 0.9 ug/mL, 1.0 ug/mL, 1.1 ug/mL, 1.2 ug/mL, 1.3 ug/mL, 1.4 ug/mL, 1.5 ug/mL, 1.6 ug/mL, 1.7 ug/mL, 1.8 ug/mL, 1.9 ug/mL, 2.0 ug/mL, 2.5 ug/mL, 3.0 ug/mL, 3.5 ug/mL, 4.0 ug/mL, 4.5 ug/mL, 5.0 ug/mL, 10.0 ug/mL or a range that includes or is between any two of the foregoing concentrations, including fractional values thereof, preferably wherein the cells are incubated with or

exposed to a photosensitizer having a concentration of 0.1 ug/mL to 5.0 ug/mL, 0.1 ug/mL to 4.5 ug/mL, 0.1 ug/mL to 4.0 ug/mL, 0.1 ug/mL to 3.5 ug/mL, 0.1 ug/mL to 3.0 ug/mL, 0.1 ug/mL to 2.5 ug/mL, 0.1 ug/mL to 2.0 ug/mL, 0.1 ug/mL to 1.6 ug/mL, 0.1 ug/mL to 1.5 ug/mL, 0.1 ug/mL to 1.4 ug/mL, 0.1 ug/mL to 1.3 ug/mL, 0.1 ug/mL to 1.2 ug/mL, 0.1 ug/mL to 1.1 ug/mL, 0.3 ug/mL to 5.0 ug/mL, 0.3 ug/mL to 4.5 ug/mL, 0.3 ug/mL to 4.0 ug/mL, 0.3 ug/mL to 3.5 ug/mL, 0.3 ug/mL to 3.0 ug/mL, 0.3 ug/mL to 2.5 ug/mL, 0.3 ug/mL to 2.0 ug/mL, 0.3 ug/mL to 1.6 ug/mL, 0.3 ug/mL to 1.5 ug/mL, 0.3 ug/mL to 1.4 ug/mL, 0.3 ug/mL to 1.3 ug/mL, 0.3 ug/mL to 1.2 ug/mL, 0.3 ug/mL to 1.1 ug/mL, 0.5 ug/mL to 5.0 ug/mL, 0.5 ug/mL to 4.5 ug/mL, 0.5 ug/mL to 4.0 ug/mL, 0.5 ug/mL to 3.5 ug/mL, 0.5 ug/mL to 3.0 ug/mL, 0.5 ug/mL to 2.5 ug/mL, 0.5 ug/mL to 2.0 ug/mL, 0.5 ug/mL to 1.6 ug/mL, 0.5 ug/mL to 1.5 ug/mL, 0.5 ug/mL to 1.4 ug/mL, 0.5 ug/mL to 1.3 ug/mL, 0.5 ug/mL to 1.2 ug/mL, or 0.5 ug/mL to 1.1 ug/mL, more preferably wherein the cells are incubated with or exposed to a photosensitizer having a concentration of 0.5 ug/mL to 5.0 ug/mL.

28. The method of aspect 27, wherein the cells are incubated with or exposed to a photosensitizer having a concentration of 1.0 ug/mL.

29. The method of any one of the previous aspects, wherein the cells are exposed or incubated with the photosensitizer for 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 10 h, 11 h, 12 h, 13 h, 14 h, 15 h, 16 h, 17 h, 18 h, 19 h, 20 h, 21 h, 22 h, 23 h, 24 h, 36 h, 48 h, 72 h or a range that includes or is between any two of the foregoing time points, including fractional values thereof, more preferably wherein the cells are incubated with the photosensitizer for 1 h to 48 h, 2 h to 48 h, 4 h to 48 h, 6 h to 48 h, 8 h to 48 h, 10 h to 48 h, 12 h to 48 h, 14 h to 48 h, 16 h to 48 h, 18 h to 48 h, 20 h to 48 h, 22 h to 48 h, 24 h to 48 h, 1 h to 36 h, 2 h to 36 h, 4 h to 36 h, 6 h to 36 h, 8 h to 36 h, 10 h to 36 h, 12 h to 36 h, 14 h to 36 h, 16 h to 36 h, 18 h to 36 h, 20 h to 36 h, 22 h to 36 h, 24 h to 36 h, 1 h to 24 h, 2 h to 24 h, 4 h to 24 h, 6 h to 24 h, 8 h to 24 h, 10 h to 24 h, 12 h to 24 h, 14 h to 24 h, 16 h to 24 h, 18 h to 24 h, 20 h to 24 h, or 22 h to 24 h, preferably wherein the cells are incubated with the photosensitizer for 1 h to 48 h.

30. The method of aspect 29, wherein the cells are exposed to or incubated with the photosensitizer for 24 h at 37 °C.

31. The method of any one of the previous aspects, wherein the photosensitizer is a porphyrin, chlorin or a dye.

32. The method of any one of the previous aspects, wherein the photosensitizer is selected from $AlPcS_{2A}$, $AlPcS_4$, lutrin, 5-aminolevulinic acid (ALA), hypericin, silicon phthalocyanine zinc (II) phthalocyanine (ZnPc), silicon phthalocyanine, mono-L-aspartyl chlorin e6, benzoporphyrin derivative monoacid ring A, chlorin photosensitizer tin etiopurpurin, tetra(*m*-hydroxyphenyl)chlorin, lutetium texaphyrin, 9-acetoxy-2,7,12,17-tetrakis-(β-methoxyethyl)-porphycene, naphthalocyanines, Allumera®, Photofrin®, Visudyne®, Levulan®, Foscan®, Fospeg®, Metvix®, Hexvix®, Cysview® and Laserphyrin®, Antrin®, Photochlor®, Photosens®, Photrex®, Lumacan®, Cevira®, Visonac®, BF-200 ALA®, Amphinex® and Azadipyrromethenes.

33. The method of aspect 32, wherein the photosensitizer is $AlPcS_{2A}$.

34. The method of aspect 29, wherein the cells are washed with a buffered balanced salt solution one or more times, and taken up in the buffered balanced salt solution prior to exposure to light.

35. The method of aspect 34, wherein the buffered balanced salt solution selected from the group consisting of phosphate-buffered saline (PBS), Dulbecco's Phosphate-buffered saline (DPBS), Earle's Balanced Salt solution (EBSS), Hank's Balanced Salt Solution (HBSS), TRIS-buffered saline (TBS), and Ringer's balanced salt solution (RBSS).

36. The method of aspect 34, wherein the cells are taken up in 0.5X, 0.6X, 0.7X, 0.8X, 0.9X, 1X, 2X, 3X, 4X, 5X, 6X, 7X, 8X, 9X, or 10X concentration/dilution of the buffered balanced salt solution, or a range that includes or is between any two of the foregoing concentrations/dilutions, preferably wherein the cells are taken up in 1X to 10X concentration/dilution of the buffered balanced salt solution prior to light exposure.

37. The method of aspect 34, wherein the cells are taken up in 1X DPBS prior to light exposure.

38. The method of aspect 34, wherein the cells are exposed to light for 1 min, 2 min, 3 min, 4 min, 5 min, 6 min, 7 min, 8 min, 9 min, 10 min, 12 min, 14 min, 15 min, 16 min, 18 min, 20 min, 25 min, 30 min, 35 min, 40 min, 45 min, 50 min, 55 min, 60 min, 90 min, 120 min, or a range that includes or is between any two of the foregoing timepoints, including fractional values thereof, preferably wherein the cells are exposed to light for 1 min to 60 min, 1 min to 55 min, 1 min to 50 min, 1 min to 45 min, 1 min to 40 min, 1 min to 35 min, 1 min to 30 min, 1 min to 25 min, 1 min to 20 min, 1 min to 15 min, 1 min to 14 min, 1 min to 13 min, 1 min to 12 min, 1 min to 11 min, 1 min to 10 min, 2 min to 60 min, 2 min to 55 min, 2 min to 50 min, 2 min to 45 min, 2 min to 40 min, 2 min to 35 min, 2 min to 30 min,

2 min to 25 min, 2 min to 20 min, 2 min to 15 min, 2 min to 14 min, 2 min to 13 min, 2 min to 12 min, 2 min to 11 min, 2 min to 10 min, 5 min to 60 min, 5 min to 55 min, 5 min to 50 min, 5 min to 45 min, 5 min to 40 min, 5 min to 35 min, 5 min to 30 min, 5 min to 25 min, 5 min to 20 min, 5 min to 15 min, 5 min to 14 min, 5 min to 13 min, 5 min to 12 min, 5 min to 11 min, or 5 min to 10 min, more preferably wherein the cells are exposed to light for 1 min to 60 min.

39. The method of aspect 38, wherein the cells are exposed to light generated by a laser.

40. The method of aspect 39, wherein the cells are exposed to light having a wavelength

of 500 nm, 550 nm, 580 nm, 600 nm, 610 nm, 620 nm, 630 nm, 640 nm, 650 nm, 660 nm, 670 nm, 680 nm, 690 nm, 700 nm, 720 nm, 740 nm, 760 nm, 780 nm, 800 nm, 850 nm, 900 nm that is generated by a laser, or a range that includes or is between any two of the foregoing wavelengths, including fractional values thereof, preferably wherein the cells are exposed to light having a wavelength of 550 nm to 850 nm, 580 nm to 850 nm, 600 nm to 850 nm, 610 nm to 850 nm, 620 nm to 850 nm, 630 nm to 850 nm, 640 nm to 850 nm, 650 nm to 850 nm, 660 nm to 850 nm, 670 nm to 850 nm, 680 nm to 850 nm, 690 nm to 850 nm, 700 nm to 850 nm, 550 nm to 700 nm, 580 nm to 700 nm, 600 nm to 700 nm, 610 nm to 700 nm, 620 nm to 700 nm, 630 nm to 700 nm, 640 nm to 700 nm, 650 nm to 700 nm, 660 nm to 700 nm, or 670 nm to 700 nm, that is generated by a laser, more preferably wherein the cells are exposed to light having a wavelength from 600 nm to 850 nm that is generated by a laser.

41. The method of aspect 33, wherein the EB producing cells are incubated with or exposed to 1 ug/mL of $AlPcS_{2A}$ for 24 h at 37 °C, washed multiple times in 1X DPBS, taken up in 1X DPBS, and then exposed to light generated from a 670 nm laser for 1 min to 10 min.

42. The method of any one of the previous aspects, wherein the method further comprises the step of:

purifying/isolating the ICVs or antigenic ICVs by using sucrose gradients.

43. The method of any one of the previous aspects, wherein the method further comprises the step of:

purifying/isolating the ICVs or the antigenic ICVs by:

(i) removing cellular debris by centrifugation from 1,000 rpm to 2,500 rpm for 1 to 10 minutes, preferably by centrifugation from 1,000 rpm to 2,000 rpm for 4 to 8 minutes, more preferably by centrifugation from 1,000 rpm to 1,500 rpm for 5 to 6 minutes; and

(ii) recovering the ICVs or antigenic ICVs by centrifugation using 10,000 x g to 20,000 x g for 5 to 15 minutes, preferably by centrifugation using 14,000 x g to 18,000 x g for 8 to 12 minutes, more preferably by centrifugation using 15,000 x g to 17,000 x g for 9 to 11 min;

optionally, concentrating the recovered nanometer sized ICVs or nanometer sized antigenic ICVs by using concentrators with a pore size cut-off from 50 to 150 kDA, preferably with a pore size cut-off of 100 kDA.

44. The method of aspect 43, wherein the isolated ICVs or isolated antigenic ICVs have average diameters of 10 nm, 20 nm, 30 nm, 40 nm, 50 nm, 60 nm, 70 nm, 80 nm, 90 nm, 100 nm, 110 nm, 120 nm, 130 nm, 140 nm, 150 nm, 160 nm, 170 nm, 180 nm, 190 nm, 200 nm, 250 nm, 300 nm, 350 nm, 400 nm, 450 nm, 500 nm, 550 nm, 600 nm, 650 nm, 700 nm, 750 nm, 800 nm, 850 nm, 900 nm, 950 nm, 1000 nm, 1100 nm, 1200 nm, 1300 nm, 1400 nm, 1500 nm, 1600 nm, 1700 nm, 1800 nm, 1900 nm, 2000 nm, 2500 nm, 3000 nm, 3500 nm, 4000 nm, 5000 nm, 6000 nm, 7000 nm, 8000 nm, 9000 nm, 10,000 nm or any range that includes or is between any two of the foregoing values, including fractional increments thereof, preferably the isolated ICVs or isolated antigenic ICVs have average diameters of 10 nm to 10,000 nm, 20 nm to 10,000 nm, 30 nm to 10,000 nm, 40 nm to 10,000 nm, 50 nm to 10,000 nm, 60 nm to 10,000 nm, 70 nm to 10,000 nm, 80 nm to 10,000 nm, 90 nm to 10,000 nm, 100 nm to 10,000 nm, 110 nm to 10,000 nm, 120 nm to 10,000 nm, 130 nm to 10,000 nm, 140 nm to 10,000 nm, 150 nm to 10,000 nm, 160 nm to 10,000 nm, 170 nm to 10,000 nm, 180 nm to 10,000 nm, 190 nm to 10,000 nm, 200 nm to 10,000 nm, 250 nm to 10,000 nm, 300 nm to 10,000 nm, 350 nm to 10,000 nm, 400 nm to 10,000 nm, 450 nm to 10,000 nm, 500 nm to 10,000 nm, 550 nm to 10,000 nm, 600 nm to 10,000 nm, 650 nm to 10,000 nm, 700 nm to 10,000 nm, 750 nm to 10,000 nm, 800 nm to 10,000 nm, 850 nm to 10,000 nm, 900 nm to 10,000 nm, 950 nm to 10,000 nm, 1000 nm to 10,000 nm, 10 nm to 5000 nm, 20 nm to 5000 nm, 30 nm to 5000 nm, 40 nm to 5000 nm, 50 nm to 5000 nm, 60 nm to 5000 nm, 70 nm to 5000 nm, 80 nm to 5000 nm, 90 nm to 5000 nm, 100 nm to 5000 nm, 110 nm to 5000 nm, 120 nm to 5000 nm, 130 nm to 5000 nm, 140 nm to 5000 nm, 150 nm to 5000 nm, 160 nm to 5000 nm, 170 nm to 5000 nm, 180 nm to 5000 nm, 190 nm to 5000 nm, 200 nm to 5000 nm, 250 nm to 5000 nm, 300 nm to 5000 nm, 350 nm to 5000 nm,

400 nm to 5000 nm, 450 nm to 5000 nm, 500 nm to 5000 nm, 550 nm to 5000 nm, 600 nm to 5000 nm, 650 nm to 5000 nm, 700 nm to 5000 nm, 750 nm to 5000 nm, 800 nm to 5000 nm, 850 nm to 5000 nm, 900 nm to 5000 nm, 950 nm to 5000 nm, or 1000 nm to 5000 nm, more preferably the isolated ICVs or isolated antigenic ICVs have average diameters from 10 nm to 10,000 nm.

45. The method of aspect 44, wherein the isolated ICVs or isolated antigenic ICVs have average diameters from 150 nm to 5,000 nm.

46. The method of aspect 45, wherein the isolated ICVs or isolated antigenic ICVs have average diameters from 1000 nm to 5,000 nm.

47. The method of aspect 43, wherein the isolated ICVs or isolated antigenic ICVs comprise a cargo selected from biological molecules, therapeutic agents, prodrugs, gene silencing agents, chemotherapeutics, diagnostic agents, components of a gene therapy system and/or components of a gene editing system, preferably wherein the biological molecules are selected from nucleic acids, peptides/proteins, viruses, hormones, carbohydrates, lipids, and vitamins, preferably where the gene silencing agents are selected from siRNA, chRNAs, miRs, ribozymes, morpholinos, and esiRNAs, preferably wherein the components of a gene editing system are selected from CRISPR-Cas systems, zinc finger nucleases, and TALENs.

48. The method of aspect 47, wherein the isolated ICVs or isolated antigenic ICVs are loaded with the cargo by direct membrane penetration, chemical labeling and conjugation, electrostatic coating, adsorption, absorption, sonification, electroporation, use of pH gradients, or any combination thereof.

49. The method of aspect 47, wherein the isolated ICVs or isolated antigenic ICVs are loaded with the cargo by incubating isolated ICVs or isolated antigenic ICVs, or the cells used to produce ICVs or antigenic ICVs,

50. The method of any one of the previous aspects, wherein the cells comprise or have been modified to comprise one or more functional moieties on the cell surface.

51. The method of aspect 40, wherein the one or more functional moieties are one or more targeting ligands.

52. The method of aspect 51, wherein the one or more targeting ligands direct the ICVs or antigenic ICVs to a certain cell, cell type, tissue type, tumor, or organ.

53. The method of aspect 51, wherein the one or more targeting ligands are an antibody or a single-chain variable fragment which binds to a tumor-specific antigen.

54. The method of aspect 51, wherein the tumor-specific antigen is selected from

alphafetoprotein (AFP), carcinoembryonic antigen (CEA), CA-125, CA15-3, CA19-9, MUC-1, epithelial tumor antigen (ETA), tyrosinase, melanoma-associated antigen (MAGE), abnormal products of ras or p53, CTAG1B, MAGEA1, and HER2/neu.

55. The method of aspect 50, wherein the cells have been bioorthogonally-conjugated to comprise one or more functional moieties.

56. The method of aspect 55, wherein the one or more functional moieties have been added to the surface of the cells by bioorthogonally-conjugation, comprising:

(1) treating sialic acid residues on the surface of the cells with an oxidizing agent to form aldehyde groups; then either step (2)(a) and (b), or step (3)(a) and (3)(b):

(2)(a) ligating, linking or conjugating aminooxy-functionalized molecules to the surface of the cells by forming oxime bonds with the aldehyde groups; and

(2)(b) inducing production of bioorthogonally-conjugated ICVs or bioorthogonally-conjugated antigenic ICVs by exposing or contacting the cells with the cell blebbing buffer which comprises a sulfhydryl blocking agent or a photosensitizer;

or

(3)(a) inducing production of ICVs or antigenic ICVs from the cells by exposing or contacting the cells with the cell blebbing buffer which comprises a sulfhydryl blocking agent or a photosensitizer; and

(3)(b) producing bioorthogonally-conjugated ICVs or bioorthogonally-conjugated antigenic ICVs by ligating, linking or conjugating aminooxy-functionalized molecules to the surface of the ICVs by forming oxime bonds with the aldehyde groups.

57. The method of aspect 56, wherein the oxidizing agent is either sodium periodate or lead tetraacetate.

58. The method of aspect 57, wherein the cells are treated with 1 mM sodium periodate for 30 min at 4 °C.

59. The method of aspect 56, wherein the aminooxy-functionalized molecules comprise a detecting agent, and/or cell-, tumor-, or tissue-targeting ligands.

60. The method of aspect 59, wherein the detecting agent is an enhanced fluorophore-based dye.

61. The method of aspect 56, wherein the aminooxy-functionalized molecules are ligated, linked or conjugated to the aldehyde groups in the presence of a catalyst.

62. The method of aspect 56, wherein the catalyst is *p*-anisidine.

63. The method of aspect 62, wherein the aminooxy-functionalized molecules are ligated, linked or conjugated to the aldehyde groups in the presence of 10 mM *p*-anisidine for 90 min at 4 °C.

64. Bioorthogonally-conjugated ICVs produced by the method of any one of aspects 55 to 63.

65. The bioorthogonally-conjugated ICVs of aspect 64, wherein the bioorthogonally-conjugated ICVs comprise oxime-linked detecting agents.

66. The bioorthogonally-conjugated ICVs of aspect 64, wherein the bioorthogonally-conjugated ICVs are loaded with one or more small molecule therapeutic compounds or agents.

67. Isolated antigenic ICVs produced by the method of any one of aspects 1 to 55.

68. The isolated antigenic ICVs of aspect 67, wherein the antigenic ICVs are loaded with one or more small molecule therapeutic compounds or agents.

69. Isolated ICVs produced by the method of any one of aspects 1 to 55.

70. A pharmaceutical composition comprising the bioorthogonally-conjugated antigenic ICVs of aspect 66, the isolated antigenic ICVs of aspect 67, or the isolated ICVs of aspect 69; and a pharmaceutically acceptable carrier, excipient, and/or diluent.

71. A method of stimulating an immune response to a cancer in a subject in need thereof, comprising:

administering the pharmaceutical composition of aspect 70 to the subject.

72. The method of aspect 71, wherein the cancer is selected from squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulvar cancer, thyroid cancer, hepatic carcinoma, leukemia and other lymphoproliferative disorders, and various types of head and neck cancers.

73. The method of aspect 71, wherein the pharmaceutical composition is administered by intravenous administration, intertumoral administration, intraperitoneal administration, intramuscular administration, intracoronary administration, intraarterial administration, subcutaneous administration, transdermal delivery, intratracheal

administration, subcutaneous administration, intraarticular administration, intraventricular administration, inhalation, or intracerebral administration.

74. The method of aspect 71, wherein the pharmaceutical composition is administered to the subject concurrently or sequentially with one or more anticancer agents or chemotherapeutics.

75. A method of treating a disease or disorder in a subject in need thereof, comprising:

administering the pharmaceutical composition of aspect 70.

76. A method of stimulating an immune response to a cancer in a subject in need thereof, comprising:

(a) obtaining antigen presenting cells;

(b) pulsing the antigen presenting cells with an antigen associated with cancer cells;

(c) inducing cell membrane blebbing by use of a sulfhydryl blocking agent;

(d) collecting antigenic micrometer sized ICVs (mICVs) induced by cell membrane blebbing; and

(e) administering said antigenic mICVs to the subject in need of immunotherapy.

77. The method of aspect 76, wherein the cancer is selected from squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, valvar cancer, thyroid cancer, hepatic carcinoma, leukemia and other lymphoproliferative disorders, and various types of head and neck cancers.

78. The method of aspect 76, wherein the antigen presenting cells are dendritic cells, macrophages, monocytes, Langerhans cells, B cells, genetically modified cells, and mesenchymal stem cells.

79. The method of aspect 77, wherein the antigen presenting cells are immature dendritic cells that are pulsed with an antigen to produce semimature or mature dendritic cells.

80. The method of aspect 79, where the immature dendritic cells are derived from bone marrow of a human subject.

81. The method of aspect 76, wherein the antigen presenting cells are obtained from the subject to be treated by immunotherapy.

82. The method of aspect 76, wherein the sulfhydryl blocking agent is paraformaldehyde.

83. The method of aspect 76, wherein the antigenic mICVs have diameters from 1 micrometer to 5 micrometers.

84. The method of aspect 76, wherein the antigenic mICVs are administered by intravenous administration, intertumoral administration, intraperitoneal administration, intramuscular administration, intracoronary administration, intraarterial administration, subcutaneous administration, transdermal delivery, intratracheal administration, subcutaneous administration, intraarticular administration, intraventricular administration, inhalation, or intracerebral administration, preferably wherein the antigenic mICVs are administered by intravenous administration, or intertumoral administration.

85. The method of aspect 76, wherein the antigenic mICVs are administered concurrently or sequentially with one or more anticancer agents or chemotherapeutics.

86. A cell-free cell therapy comprising the pharmaceutical composition of aspect 69 for use in treating a subject having a disease or disorder.

87. The cell-free cell therapy of aspect 86, wherein the subject has cancer.

88. A vaccine comprising the pharmaceutical composition of aspect 70 for prevention of an infection in a subject by an infectious agent.

89. The vaccine of aspect 88, wherein the infectious agent is a bacterium, a virus, a fungus, or a protozoon.

90. A therapeutic vaccine comprising the pharmaceutical composition of aspect 70 for use in treating a subject having cancer.

[00169] The following examples are intended to illustrate but not limit the disclosure. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

EXAMPLES

[00170] ***Cell culture.*** HeLa cells were obtained from American Type Culture Collection (ATCC) and cultured in DMEM (Thermo Fisher Scientific, Waltham, MA), 10%

FBS (Gemini Bio Products, West Sacramento, CA) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA). A mouse lymphoma cell line (EL4) was obtained from ATCC and grown in DMEM (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS (Gemini Bio Products, West Sacramento, CA) and 1% penicillin streptomycin (Thermo Fisher Scientific, Waltham, MA). An OVA expressing derivative of EL4 (E.G7-OVA) was obtained from the American Type Culture Collection (ATCC) and grown in RPMI (Thermo Fisher Scientific, Waltham, MA) supplemented with 0.40 mg/mL Geneticin (Thermo Fisher Scientific, Waltham, MA), 10% FBS (Gemini Bio Products, West Sacramento, CA) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA). Obtained B3Z $CD8^+$ T hybridomas were grown in RPMI (Thermo Fisher Scientific, Waltham, MA) supplemented with 1 mM sodium pyruvate (Thermo Fisher Scientific, Waltham, MA), 2 mM L-glutamine (Thermo Fisher Scientific, Waltham, MA), 0.055 mM 2-mercaptoethaol (Thermo Fisher Scientific, Waltham, MA), 10% FBS (Gemini Bio Products, West Sacramento, CA) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA). Cells were incubated at 37 °C with 5% CO_2 and 100% humidity.

Example 1

[00171] ***Protocol for N-ethylmaleimide induced cell vesicle production:*** HeLa cells were plated at 70% confluency (6 x 10^6 cells / 100 mm cell culture plate) and incubated for 4 h at 37 °C and 5% CO_2. Media was removed and cells were washed three times with 10 mL of 1X DPBS. Then, 2 mM *N*-ethylmaleimide in 6 mL of 1X DPBS was added to each 100 mm plate and plates were incubated at 37 °C and 5% CO_2 for 24 h. Cells were imaged using a standard inverted light microscope and DPBS solution containing ICVs were collected, isolated, and analyzed by dynamic light scattering (DLS) for size.

[00172] ***Protocol for nano- and microscale induce cell vesicle isolation:*** Cells and debris were removed by centrifugation at 1200 rpm for 5.5 min, repeated three times, each time collecting supernatant into a clean tube. Then, mICVs were isolated from the supernatant by centrifugation at 16,000 x g for 10 min. After the first spin, supernatant was collected for nICV isolation while micro ICV pellets were washed with 1x DPBS. This was repeated two additional times, discarding supernatant and washing the mICV pellet with 1x DPBS. nICVs were isolated using 100 kDa Amicon® ultrafiltration at 3300 x g for 15 min, repeated three times with 1x DPBS washes. Final mICVs and nICVs collected were suspended in 100 uL 1x DPBS and then characterized.

[00173] ***Characterization of mICVs and nICVs:*** mICVs and nICVs were imaged using a bright field microscope and inverted light microscope. The sizing of produced ICVs were measured by using dynamic light scattering (DLS). Use of transmission electron microscopy can also be used to size the produced ICVs.

[00174] ***Time course study looking at nICV production by use a cell blebbing buffer comprising NEM over time.*** In order to better understand the timing for nICV production, a time course study was performed using a cell blebbing buffer comprising *N*-ethylmaleimide (NEM). In the experiment, HeLa cells were exposed to a cell blebbing buffer comprising 2 mM NEM in 1X DPBS for varying periods of time (1 h, 3 h, 5 h, 8 h, and 24) and imaged by light microscope (*e.g.*, see **FIG. 4**). Then, mICVs were removed and nICVs were analyzed for size distribution by dynamic light scattering (*e.g.*, see **FIG. 5** or by TEM (*e.g.*, see **FIG. 11**). It was found that the cell blebbing buffer comprising NEM induced the formation of nICVs at each time point.

[00175] ***Time course study looking at the effects of osmotic pressure in conjunction with a cell blebbing buffer comprising NEM on inducing cell vesicle production over time.*** HeLa cells were plated at 70% confluence in 24-well plates and subjected to a cell blebbing buffer comprising NEM prepared in 1X, 2X, 3X, 4X, or 5X DPBS. Cells were imaged and vesicles were collected at 1 h, 3 h, 5 h, or 8 h after addition of a quenching solution for NEM (*e.g.*, see **FIGs. 6** to **10**). Based on imaging, a trend of decreasing mICV size was seen by going from 5X to 1X DPBS, indicating that osmotic pressure could potentially play a significant role in assisting in size control. Additionally, a trend of increased mICV size over time was seen within each DPBS concentration, indicating that the time of collection can also play a role in size control of the mICVs. It is expected that nICVs will follow a similar trend, as all vesicles are induced by the same mechanism and membrane blebbing.

Example 2

[00176] ***Protocol for Photoinitiated induced cell vesicle production:*** HeLa cells were plated at 70% confluency (6×10^6 cells / 100 mm cell culture plate) and incubated for 4 h at 37 °C and 5% CO_2. Cells were then imaged to confirm adherence to the plate and, in the dark, media was changed to media containing 1 ug/mL of the photosensitizer aluminum disulfonated phthalocyanine ($AlPcS_{2A}$) (10 mL of photosensitizer media per 100 mm each plate). Plates were wrapped in tinfoil and allowed to incubate for 24 h at 37 °C and 5% CO_2. In the dark, the photosensitive media was removed and cells were washed three times with 10 mL of 1X DPBS. Then, 6 mL of 1X DPBS was added to each 100 mm plate and the plates

were subjected to a 670 nm laser for varying time doses (10 min, 5 min, 2.5 min, 1.25 min). Cells were imaged using a standard inverted light microscope, and the DPBS solution containing ICVs was collected. 6 mL of fresh 1X DPBS was added to each 100 mm plate and the plates were incubated at 37 °C and 5% CO_2 for 18 h to investigate ICV formation effects over time. Cells were imaged using a standard inverted light microscope and the DPBS solution containing ICVs was collected.

[00177] ***Protocol for nano- and microscale induced cell vesicle isolation:*** Cells and debris were removed by centrifugation at 1200 rpm for 5.5 min, repeated three times, each time collecting supernatant into a clean tube. Then, mICVs were isolated from the supernatant by centrifugation at 16,000 x g for 10 min. After the first spin, supernatant was collected for nICV isolation while micro ICV pellets were washed with 1x DPBS. This was repeated two additional times, discarding supernatant and washing the mICV pellet with 1x DPBS. nICVs were isolated using 100 kDa Amicon® ultrafiltration at 3300 x g for 15 min, repeated three times with 1x DPBS washes. Final mICVs and nICVs collected were suspended in 100 uL 1x DPBS and then characterized.

[00178] ***Characterization of mICVs and nICVs:*** mICVs and nICVs were imaged using a bright field microscope and inverted light microscope. The sizing of produced ICVs were measured by using dynamic light scattering (DLS). Use of transmission electron microscopy can also be used to size the produced ICVs.

[00179] ***Effects of the duration of light exposure on $AlPcS_{2A}$ photoinitiated induced cell vesicle production from HeLa cells.*** In order to better understand the effects of the duration of light exposure on ICV production, a time course study was performed using a 670 nm laser with varying time doses (10 min, 5 min, 2.5 min, and 1.25 min). In the experiment, HeLa cells were first incubated with media containing 1 ug/mL of the photosensitizer $AlPcS_{2A}$ for 24 h, the cells were washed and taken up in 1X DPBS, and then exposed to light from a 670 nm laser.

[00180] It was found that for all of tested light exposure doses (10 min, 5 min, 2.5 min, and 1.25 min), mICVs were produced 18 h after post light exposure. Moreover, it was found that mICVs were produced immediately after 10 min and 5 min light exposure doses (*e.g.*, see **FIGs. 13-16**; see also **FIG. 17** and **FIG. 18**).

[00181] In regards to nICVs, it was found with dynamic light scattering that all light exposure doses (10 min, 5 min, 2.5 min, and 1.25 min) provided for nICVs with average diameters between 10-600 nm immediately after photoinitiation (*e.g.*, see **FIG. 19**) and after

18 h post photoinitiation, averages diameters between 40 to 1000 nm (*e.g.*, see **FIG. 20**). Moreover, it was observed that there were clear differences in the size allocation of the nICVs based upon the light exposure dose (*e.g.*, see **FIG. 19** and **FIG. 20**). In control experiment, HeLa cells were incubated with a vehicle control or with media containing 1 ug/mL of the photosensitizer $AlPcS_{2A}$ for 24 h and then exposed to light. It was found that incubation of the cells with the photosensitizer $AlPcS_{2A}$ prior to light exposure greatly increased the yield of nICVs and mICVs v. just exposing the cells to light alone (*e.g.*, see **FIG. 21**).

Example 3

[00182] ***Protocol for AAV Producer Cell Production:*** To create ICVs loaded with AAV, it was found that it was important for the AAV producer cells to reach a level of about 70% confluence when vesiculation is induced in order to maximize vesicle yield. For production purposes, cells were seeded at ~15% confluence, and after 24 hours, the triple plasmid transfection was delivered to cells. After an additional 24 hours, media was exchanged for fresh media. After a 1-hour incubation, a full dose of the triple plasmid transfection was applied to each plate. After 24 hours, media was exchanged for fresh media. Then, after another 24 hours (48 hours after the second treatment) the cells were either collected and lysed for AAV isolation or treated with the vesiculation components. Three 10 cm plates comprising 1.8×10^7 of cells were used for each vesiculation treatment. In the studies the AAV produced was serotype 2 expression GFP.

[00183] ***Supernatant collected from AAV producer cells without blebbing or lysis.*** Producer cells were prepared and triple plasmid transfection was performed in order to make AAV. 48 hours after production began, cells were washed with PBS then DMEM media with FBS was added to the cells. After 24 hours the DMEM was collected and isolated through 100 kDa Amicon filtration (washed three times). This is essentially the control supernatant. As shown in **FIG 33**, anti-AAV antibodies affected transduction efficiency of the control supernatant.

[00184] ***Supernatant collected from virus producer cells with traditional extracellular vesicle conditions.*** Producer cells were prepared and triple plasmid transfection was performed in order to make AAV. 48 hours after production began, cells were washed with PBS then DMEM media without FBS was added to the cells. After 24 hours the DMEM was collected and isolated through 100 kDa Amicon filtration (washed three times). This is the "natural extracellular vesicle production" control. As shown in **FIG. 34**, anti-AAV

antibodies drastically reduced the transduction efficiency of traditionally produced AAV extracellular vesicles, suggesting that very few AAV are actually encapsulated in these extracellular vesicles.

[00185] ***Protocol for the production of induced cell vesicles containing AAV:***

(1) AIPCS2A Photosensitizer-Initiated Production of ICVs containing AAVs. Adeno-associated virus producer cells were grown to 70% confluence. Media was then changed to media containing 1 ug/mL AIPCS2A photosensitizer (10 mL of photosensitizer media per 100 mm each plate). Plates were wrapped in tinfoil and allowed to incubate for 24 h at 37 °C and 5% CO_2. In the dark, photosensitive media was removed and cells were washed three times with 10 mL of 1X DPBS. Then, 6 mL of 1X DPBS was added to each 100 mm plate and plates were subjected to a 670 nm laser for 10 min or approximately 3 Joules of energy. Cells were then imaged using a standard inverted light microscope, and the DPBS solution containing ICVs was collected. 6 mL of fresh 1x DPBS was added to each 100 mm plate and plates were incubated at 37 °C and 5% CO_2 for 24 h to investigate ICV formation effects over time. Cells were imaged using a standard inverted light microscope and DPBS solution containing ICVs was collected.

(2) N-ethylmaleimide Initiated Production of ICVs containing AAVs. Adeno-associated virus producer cells were grown to 70% confluence. Media was removed and cells were washed three times with 10 mL of 1x DPBS. Then, 2 mM *N*-ethylmaleimide in 6 mL of 1X DPBS was added to each 100 mm plate. The plates were incubated at 37 °C and 5% CO_2 for 24 h. Cells were then imaged using a standard inverted light microscope and DPBS solution containing ICVs was collected. Free-AAV was also exposed to identical vesicle production and isolation methods and included as controls.

[00186] ***Optimizing NEM Incubation Times for AAV production:*** HEK293T cells were blebbed in NEM blebbing buffer for 1, 3, 5, 8, or 24 hours. Micro and nano ICVs were isolated as previously described, lysed, and analyzed by QPCR for AAV content. QPCR data demonstrated that nano ICVs generated over time contained the same amount of AAV while micro ICVs contained the most AAV at 8-24 hours. This data was not normalized by number of ICVs and therefore could also be representative of greater yields of ICVs containing AAV over time (see **FIG. 45**).

[00187] ***Protocol for isolation of ICVs containing AAVs:*** Cells and debris were removed by centrifugation at 1200 rpm for 5.5 min, repeated three times, each time collecting supernatant and moving to a clean tube. Then, micro ICVs were isolated from the supernatant

by centrifugation at 16,000 x g for 10 min. After the first spin, supernatant was collected for nano ICV isolation while micro ICV pellets were washed with 1X DPBS. This was repeated two additional times, discarding supernatant and washing micro ICV pellet with 1X DPBS. Nano ICVs were isolated using 100 kDa Amicon filtration at 3300 x g for 15 min, repeated three times with 1X DPBS washes. Final micro ICVs and nano ICVs collected were suspended in 100 uL 1X DPBS and then characterized.

[00188] ***Characterization of Micro and Nano-scale ICVs containing AAVs:*** Light microscope images of AAV producer cells were acquired. ICVs were then isolated and analyzed by dynamic light scattering (DLS) measurements for size. As shown in **FIG 24,** there was quite a divergence in the size of induce cell vesicles produced using the various blebbing conditions, with the largest induced cell vesicles being produced by using NEM or photoinitiation for 10 minutes. As shown in the images for **FIG. 25**, only weak fluorescence was seen in the supernatant from virus producer cells without blebbing. While free AAVs were inactivated by anti-AAV antibodies (see **FIG. 26**), there was only minimal inactivation by anti-AAV antibodies either nanometer sized ICVs containing AAVs or micrometer sized ICVs containing AAVs from using NEM (see **FIG. 27** and **FIG. 28**). Similar minimal inactivation by anti-AAV antibodies for ICVs containing AAVs produced by using photoinitiation, post 10 min (see **FIG. 29** and **FIG. 30**) and post 24 h. (see **FIG. 31** and **FIG. 32**).

[00189] ***Protocol for ICVs containing AAVs purification using Sucrose gradients.*** Hek293T cells were plated at 15% confluency, 24 hours later they were given a triple plasmid transfection to produce AAV. After 48 hours incubation, cells were either lysed and AAV collected for the AAV group, or treated with NEM to produce ICVs containing AAVs. These were collected after 8 hours and NEM was removed through filtration. AAVs from lysate and ICVs containing AAVs were separately run on a sucrose gradient and ultracentrifuged to determine which fractions contain free AAVs and which contain ICVs containing AAVs. These were then tittered through qPCR.

[00190] AAV from lysate and ICVs containing AAVs were treated with DNase solution for 15 minutes at 37C to digest any free DNA, then samples were heated to 95 °C for 10 minutes to inactivate DNase. Then samples were treated with a viral lysate solution that lyses the virus and the ICV membrane. Samples were then prepared using qPCR Adeno-Associated Virus Titration (Titer) Kit from Applied Biological Materials Inc. according to kit

instructions. Samples were run for qPCR. Resulting data was analyzed according to kit instructions.

[00191] The data shows a clear divide between where AAVs congregate in the sucrose gradient, with AAV fractions in 1-6, (most being in fractions 3-6) and ICVs containing AAVs being mostly in fraction 10 (e.g., **FIG. 35A-B**) or fractions 10 to 12 (e.g., **FIG. 36. A-B**). This clear divide between where each species is found, allows for purification of ICVs containing AAVs from any free AAV that may appear in solution, as well as removing any organelles, nucleic acids, and extra protein.

[00192] ***Determining titers of AAV stock and AAV from cell lysate (treated with NEM).*** The goal of this experiment was to determine how NEM affected free AAVs versus intracellular AAVs (from producer cells). For the stock group, AAVs purchased from abm® (AAV2-GFP). 6e10 GCs of AAV was incubated in NEM for various timepoints (0 minutes, 10 minutes, 30 minutes, 1 hours, 2 hours, 4 hours, 6 hours, and 8 hours). There titer was determined using qPCR to determine if there was any DNA damage and to find dosing. For the from lysate group, hek293T cells were plated at 15% confluency, 24 hours later they were given a triple plasmid transfection to produce AAV. After 48 hours incubation, cells were lysed and AAV was collected and purified. The resulting AAVs were treated with DNase solution for 15 minutes at 37C to digest any free DNA, then samples were heated to 95C for 10 minutes to inactivate DNase. Then samples were treated with a viral lysate solution that lyses the virus and the ICV membrane. Samples were then prepared using qPCR Adeno-Associated Virus Titration (Titer) Kit from Applied Biological Materials Inc according to kit instructions. Samples were run for qPCR. Resulting data was analyzed according to kit instructions. All titers appeared to be about the same, suggesting that NEM did not cause DNA damage. This data was also used to determine dosing for the next experiment.

[00193] ***Quantitative results using AAV stock and AAV producer cell derived AAV treated with NEM.*** Stock AAV transduction was greatly affected by NEM, reducing the transduction efficiency after just 10 minutes, and leading to almost no transduction after incubation for 2 hours. However, the AAV isolate from lysate showed that the cells had a protective effect (see **FIG. 38A-B**). There was only a minimal drop in transduction until they had been exposed to NM for 4 hours, and the 8 hours timepoint only showed about half the transduction that the 0-minute timepoint showed (see **FIGs. 39** and **40**). This demonstrated that any AAV that is free during the blebbing process should be neutralized by the presence of NEM. It also demonstrates that the cell membrane protects AAVs from NEM, and this

should translate to the ICVs. Lastly, after seeing minimal DNA damage in a previous experiment, it is clear that the NEM affects protein, affecting the transduction efficiency of the AAVs.

[00194] ***Flow cytometry on cells treated with AAV from stock and lysate and photo-induced cell vesicles (5 minutes).*** Hek293T cells were plated at 15% confluency, 24 hours later they were given a triple plasmid transfection to produce AAV. After 48 hours incubation, AAV cells groups were lysed and AAV was purified or a photo-initiator was delivered and exposed to a laser. After 5 minutes these were collected and the nICVs and mICVs were separated according to size. Stock AAV was purchased from Applied Biological Materials Inc. HeLa cells were plated and after 24 hours 20 uL of the resulting AAV or ICVs containing AAVs were delivered to each well, with antibody dilutions of 1:16,000, 1:4,000, and 1:1,000 (Ab:AAV). After three days these cells were imaged and run through flow cytometry to analyze fluorescence. Free AAVs were silenced by the neutralizing antibodies (see **FIG. 41A**). Light induced ICVs containing AAVs were also silenced at a similar amount (see **FIG. 41B**). This system appeared not to be protective from nAbs.

[00195] ***Flow cytometry on cells treated with NEM initiated ICVs containing AAVs and cells treated with photoinitiated ICVs containing AAVs (24 hours) demonstrating their resistance to nABs.*** Hek293T cells were plated at 15% confluency, 24 hours later they were given a triple plasmid transfection to produce AAV. After 48 hours incubation, cells were treated with NEM or a photo-initiator and exposed to a laser. After 24 hours these were collected and the nICVs and mICS containing AAVs. were separated according to size. HeLa cells were plated and after 24 hours 20 uL of the resulting ICVs were delivered to each well, with antibody dilutions of 1:16,000, 1:4,000, and 1:1,000 (Ab:AAV). After three days these cells were imaged and run through flow cytometry to analyze fluorescence.

[00196] NEM initiated ICVs containing AAVs showed far greater transduction efficiency and were able to resist neutralization by neutralizing antibodies for AAV. The nICVs containing AAVs appeared to resist neutralization but showed insignificant transduction (see **FIG. 42A**). Photoinitiated ICVs containing AAVs showed a similar profile to the NEM initiated ICVs containing AAVs, except the mICVs containing AAVs did show a response to the neutralizing antibody based on the concertation (see **FIG. 42B**). However, they resisted being completely neutralized. This is due to the protective effect of the membrane around ICVs protecting AAVs from neutralization.

[00197] ***Protocol for AAV and ICVs containing AAVs Dosing and Transduction Tracking:*** ICVs containing AAVs and free AAVs were introduced to HeLa cells using 20% of the final volume for each well (40 μL of sample into 100 μL of media). Wells were also treated with no neutralizing antibodies, or antibodies with dilutions in the final volume (*i.e.*, media, sample, and antibody solution). The wells comprised a ratio of virus particles to antibodies of 1:1,000, 1:4,000, and 1:16,000 (virus particles: antibody). After 24 h, the media was switched for fresh media. Images were taken every day after transduction, with the contained images coming from day four post-transduction (*e.g.*, **FIG. 43** and **FIG 44**).

Example 4

[00198] ***Synthetically modifying retroviral surface using bioorthogonal ligation.*** Both enveloped virus (e.g., influenza virus, retrovirus, and herpes virus) and ICVs are wrapped by phospholipid bilayer membrane that is derived from producer cells. Therefore, enveloped viruses are ICR-like, biologically active nanomaterials and molecular modification of enveloped viral surface is a good model for engineering ICRs. In order to explore the possibility of synthetically modifying retroviral surface, a highly efficient and specific conjugation under biological conditions ("bioorthogonal" ligation) was used. Aminooxy-functionalized molecules can be oxime ligated with aldehyde groups of oxidized sialylated glycoproteins by use of sodium periodate. Since mammalian cells, including retroviral producing cells, display sialic acids on their surface, bioorthogonal cell surface modification generates chemically functionalized retroviral particles in a simple, fast, and efficient way (*e.g.*, see **FIG. 46**).

[00199] 293GPG/EGFP cells producing EGFP-expressing VSV-G pseudotyped Molony murine leukemia viruses (MoMLVs) were treated with 1 mM sodium periodate to oxidize the sialic acid on the glycosylated membrane proteins and the resulting aldehyde groups were conjugated with aminooxy-activated biotin in the presence of 10 mM *p*-anisidine. The retroviral supernatant collected at 24 h after bioorthogonal biotinylation of virus producing cells was conjugated with anti-biotin magnetic particles at the pre-determined optimized ratio. The magnetically labeled retroviral particles were incubated with NIH 3T3 cells on a tissue culture dish where a patterned magnet was applied underneath. Transduced cells (neomycin-resistant cells) survived and were selected to proliferate for one week before visualization with methylene blue staining.

[00200] The results clearly demonstrated that significantly more cells were transduced in the areas where magnetic forces were applied than other areas without magnetic forces

(*e.g.*, see **FIG. 47B**). This confirms that most magnetically labeled retroviral particles were directed to transduce the cells where the external magnetic force was applied. Therefore, magnetically labeled retroviral particles can be used for targeted gene delivery by applying magnetic forces in desired target tissues for localization of significant quantities of viral particles. Magnetic particles are also potent contrast agents for magnetic resonance imaging (MRI).

[00201] Magnetically labeling retroviral particles by bioorthogonally functionalizing virus producing cell surfaces is a promising approach to prepare novel viral vectors for gene delivery in combination with *in vivo* imaging of vector biodistribution by MRI. The retrovirus producing cells were also bioorthogonally modified with a synthetic molecule (aminooxy-ketal-PEG-folic acid [FA])) that is designed to protect the virus during circulation (PEG) but specifically bind to folic acid receptor (FAR)-expressing cancer cells (*e.g.*, see **FIG. 47C**). FARs are overexpressed by many kinds of cancer cells. The molecules were also designed to cleave (ketal linkage) when the modified virus reaches the mildly acidic endosome/lysosome for full exposure of the VSV-G envelope proteins for rapid release into the cytoplasm upon membrane fusion of viral and endosomal membrane. The resulting viral particles were significantly more efficient in transducing FAR-overexpressing HeLa human cervical cancer cells than FAR-negative NIH 3T3 mouse fibroblasts. Thus, confirming the successful, synthetic engineering of retroviral particles for targeted transduction. The results shown in **FIG.47** demonstrate that the surfaces of virus producing cells were bioorthogonally functionalized to produce retroviral particles carrying functionalized envelope for magnet-directed transduction and cancer cell-targeted transduction.

[00202] ***Bioorthogonal modification of ICVs:*** In furtherance of the evidence presented in **FIG. 47**, experiments were performed to confirm whether bioorthogonally labeled cells would produce ICVs with the same modification (*e.g.*, see **FIG. 48**). As a simple and convenient model, HeLa cells were treated with sodium periodate for sialic group oxidation and conjugated with CF488 as described earlier. The cells were very efficiently labeled with fluorescence (*e.g.*, see **FIG. 48A**) and the mICVs produced by sulfhydryl-blocking produced from the CF488-labeled HeLa were also fluorescent (*e.g.*, see **FIG. 48B**). These results confirmed ICVs can be easily engineered by bioorthogonally modifying the surface of the producer cells.

Example 5

[00203] ***Bone-marrow isolation and bone marrow dendritic cell (BMDC) culture.*** Bone marrow was isolated from femurs of 12-week of C57BL/6 mice (Charles River Laboratories, Wilmington, MA). After mice were euthanized, the femurs were isolated and the bone marrow flushed out with a 25-guage needle using RPMI (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS (Gemini Bio Products, West Sacramento, CA). Red blood cells were lysed using Red Blood Cell Lysis Buffer (Thermo Fisher Scientific, Waltham, MA). Remaining cells were cultured in RPMI (Thermo Fisher Scientific, Waltham, MA) supplemented 10% FBS (Gemini Bio Products, West Sacramento, CA) and 20 ng/mL rmGM-CSF (R&D Systems, Minneapolis, MN). On day 7 of culture, the percentage of BMDCs in the population was assessed with anti-mouse CD11c antibody (BioLegend, San Diego, CA). Cells were then incubated with 20 ng/mL lipopolysaccharide (Sigma Aldrich, St. Louis, MO) for 24 h to induce maturation. The percentage of mature BMDCs (mBMDCs) in the population was assessed with anti-mouse CD40 antibody (BioLegend, San Diego, CA).

[00204] ***Preparation and isolation of SIINFEKL-presenting mICVs.*** Immature BMDCs (imBMDCs) were incubated with 50 μM SIINFEKL for 1 h prior to maturation. 10^6 BMDCs/mL in the culture media were centrifuged at 200 x g and for 5.5 min and the cell pellet was resuspended and incubated with 25 mM paraformaldehyde (Thermo Fisher Scientific, Waltham, MA) in DPBS for 12 h at 37 °C with 5% CO_2. To isolate SIINFEKL-presenting mICVs, cells in the cell blebbing buffer were removed by centrifugation at 200 x g for 5 min at room temperature followed by concentration of SIINFEKL-presenting mICVs at 9,300 x g for 10 min at room temperature. SIINFEKL-presenting mICVs were washed three times with 10 mL of DPBS. SIINFEKL-presentation was assessed with anti-mouse H-2Kb bound to SIINFEKL antibody (BioLegend, San Diego, CA). As shown in **FIG. 50A-B**, large numbers of SIINFEKL-presenting mICVs can be produced by using the foregoing method.

[00205] ***T cell hybridoma activation assay.*** T cell activation was assessed using a β-Galactosidase (CPRG) assay and B3Z, T cell hybridoma line. 10 μL of SIINFEKL-presenting mICVs derived from BMDCs in DPBS were incubated with 30,000 B3Z cells in 100 μL/well of RPMI supplemented with 10% FBS for 24 h at 37 °C with 5% CO_2. After 24 h, the plate was spun down and the supernatant removed. Cells were resuspended in CPRG buffer consisting of 90% DPBS, 10% NP-40 (Sigma Aldrich, St. Louis, MO), and 0.6 mg/mL chlorophenol red-β-D-galactopyranoside (Sigma Aldrich, St. Louis, MO) and incubated at room temperature for 12 h. The assay was assessed by measuring absorbance at 595 nm compared to set standards of B3Z cells incubated with BMDCs and known SIINFEKL

concentration. As shown in **FIG. 51**, the greater absorbance in the case of B3Z exposure to antigen-presenting BMDC-derived mICVs indicates that antigen-presenting mICVs are able to activate T cells *in vitro*.

[00206] ***Cytotoxic T lymphocyte assay.*** In order to evaluate whether immunization with antigen-presenting microvesicles could induced cytotoxic T lymphocyte activation in vivo, a cytotoxic T lymphocyte (CTL) assay was completed. 12 wk.-old C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were vaccinated by *s.c.* injection with DPBS, OVA protein, SIINFEKL peptide, SIINFEKL-presenting BDMCs, or SIINFEKL-presenting BMDC mICVs. With the exception of the control (DPBS) group, each group received vaccinations that were equivalent to a dosage of 100 μL of 50 μM SIINFEKL as quantified by CPRG assay. Vaccinations were given twice 14 days apart. 7 days after the second injection, mice were sacrificed, spleen cells were isolated, and spleen cells were incubated in various E:T with PKH26 (Sigma Aldrich, St. Louis, MO)-stained E.G7-OVA cells. After 4 h, cells were stained with 1 mM YoPro1 (Thermo Fisher Scientific, Waltham, MA) and viability of the EG7.OVA cells was assessed at two effector to target ratios, 50:1 and 100:1 by flow cytometry. As shown in **FIG. 52**, antigen-presenting microvesicles show the greatest efficacy at stimulation of CTL response at the 50:1 E:T ratio. At the larger E:T ratio, the difference is less drastic. This may be a result of overloading target cells with effector cells.

[00207] ***Tumor challenge study.*** 12 wk.-old C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were vaccinated by *s.c.* injection with DPBS, OVA protein, SIINFEKL peptide, SIINFEKL-presenting BDMCs, or SIINFEKL-presenting BMDC mICVs. With the exception of the control (DPBS) group, each group received vaccinations that were equivalent to a dosage of 100 μL of 50 μM SIINFEKL as quantified by CPRG assay. Vaccinations were given twice 14 days apart. 7 days after the second injection, mice were injected *s.c.* with 300,000 E.G7-OVA cells in the right flank. Tumor growth was monitored by measuring the tumor size with calipers every 48 h.

[00208] ***Production and characterization of antigenic mICVs derived from BMDCs.*** mICVs were efficiently produced from BMDCs in culture by blocking sulfhydryl groups with paraformaldehyde (PFA) or with PFA/DTT (*e.g.*, see **FIG. 54**). Sulfhydryl-blocking led to rapid ICV production (*e.g.*, see **FIG. 55A**). This is favorable to natural vesicle production, which requires several days of cell culture and results in significant levels of impurities including proteins and cell debris. The resulting antigenic mICVs were isolated by centrifugation and range in size from 0.05 μm to 5 μm (*e.g.*, see **FIG. 55B**). Although

previous studies have primarily focused on using nano-scale exosomes as vaccines, the studies presented herein assess vesicles of various discrete size ranges. Antigenic mICVs were assessed for CD11c, a specific marker of bone marrow derived dendritic cells (BMDCs). Culture of cells in granulocyte-macrophage colony-stimulating factor (GM-CSF) led to relatively high levels of CD11c expression on antigenic mICVs (*e.g.*, see **FIG. 55C**). The percentage of CD11c positive antigenic mICVs agrees with the percentage of CD11c positive cells from which they were derived. This is an indication that antigenic mICVs maintain surface protein expression of their parent cells. Although other studies have shown that extracellular vesicles express proteins found on their parent cells, this is the first study to show that ICVs produced via sulfhydryl-blocking maintain a surface protein expressed on their parent cells. The antigenic ICVs were also assessed to see if the antigen presentation was similar to parent dendritic cells. It was found that the antigenic ICVs had similar antigen presentation to parent dendritic cells but with superior stability (*e.g.*, see **FIG. 63A-B**).

[00209] ***Determining whether the PFA and PFA/DTT blebbing agents interfere with antigenic presentation and T-cell stimulation***. It was further determined as to whether use of chemical agents to induce cell blebbing of antigen-presenting cells, impaired antigens and other proteins expressed on the surface of the ICVs. While antigen presentation levels were similar using both PFA and PFA/DTT, it was further found that ICVs produced using PFA were capable of stimulating T cells by nearly 2-fold over ICVs produced using PFA/DTT (*e.g.*, see **FIG. 56A-C**). This indicated that PFA was the preferred method for inducing BMDC ICV formation, as functionality of ICVs was improved.

[00210] ***Control of maturation properties of antigenic mICVs.*** While immature BMDCs (imBMDCs) are known to induce tolerance, mature BMDCs (mBMDCs) prime immune cells, and studies have shown that ICVs derived from mBMDCs to be more effective at stimulating T cells. As shown in **FIG. 57**, mature BMDCs were stained with PKH26 (red membrane stain) and DAPI (blue nuclear stain) and exposed to PFA blebbing buffer. Cells and induced cell vesicle production were observed over time by confocal microscope imaging. Mature BMDCs produced larger ICVs over time, with complete use of cell membrane accomplished at 24 hours post-incubation. Similar blebbing dynamics were seen when immature BMDCs were tracked (*e.g.*, see **FIG. 57**). Further, both micro and nano ICVs from mature BMDCs and immature BMDCs showed similar size distributions over time (*e.g.*, see **FIG. 58A-C**).

[00211] With the aim of producing antigenic mICVs from a range of immature to mature BMDCs, the cells were assessed for CD40, a costimulatory molecule that is upregulated in mBMDCs. Immature BMDCs were matured in 20 ng/mL lipopolysaccharide (LPS) for 0, 6, and 12 hours to generate BMDCs at different stages of maturation. BMDCs from these time points were blebbed in 25 mM PFA blebbing buffers and mICVs were isolated as previously described. BMDCs from the varying time points and ICVs subsequently produced were labeled with fluorescent anti-CD40 (BMDC maturation marker) and analyzed by flow cytometry. BMDCs and ICVs from varying maturation states showed similar levels of CD40 presentation, a BMDC maturation marker (*e.g.*, see **FIG. 60A-B**). This indicated that ICVs can have controlled levels of maturation, similar to their parent cells. However, unlike cells, ICVs cannot continue to undergo changes, locking their stage of maturation into place. Additionally, imaging confirmed that antigenic mICVs from mBMDCs expressed CD40 unlike naturally occurring extracellular vesicles (*e.g.*, see **FIG. 61**). Since it has been shown that mBMDCs are more favorable for applications in immunotherapy, this result is of particular interest, demonstrating that sulfhydryl-blocking offers a means to achieve "mature" antigenic mICVs. It is believed that this is the first report demonstrating precise control over ICV properties based on cell stage.

[00212] The antigenic mICVs from a range of immature to mature BMDCs were further assessed for costimulatory marker presentation of CD-80 and CD-86. Immature BMDCs were matured in LPS for 0, 6, and 12 hours to generate BMDCs at different stages of maturation. BMDCs from these time points were blebbed in 25mM PFA blebbing buffers and mICVs were isolated as previously described. BMDCs from the varying time points and ICVs subsequently produced were labeled with fluorescent anti-CD80 or anti-CD86 (BMDC costimulatory molecules) and analyzed by flow cytometry. Similar to CD40 presentation, ICVs produced from cells of varying stages of maturation presented increased levels of co-stimulatory molecules that mirrored those of the parent cells (*e.g.*, see **FIG. 62A-D**). This indicated that ICVs likely mirror many of the parent cell presentation qualities, with the unique feature that ICV presentation is locked and cannot undergo further changes.

[00213] The antigenic mICVs from a range of immature to mature BMDCs were also assessed for MHC1 expression. Immature BMDCs were matured in LPS for 0, 6, and 12 hours to generate BMDCs at different stages of maturation. BMDCs from these time points were blebbed in 25mM PFA blebbing buffers and mICVs were isolated as previously described. Immature and mature BMDCs were labeled with fluorescent anti-MHC1 and

compared for MHC1 presentation by flow cytometry. It was found that while percentages of MHC1 positive cells were similar between mature BMDCs and immature BMDC's, density of MHC1 on mature cells was increased by nearly 2-fold (*e.g.*, see **FIG. 64A-B**). This indicated that mature BMDCs are superior at antigen presentation.

[00214] ***Comparing the molecular contents of BMDCs, ICVs derived from mature BMDCs, and ICVs derived from immature BMDCs***. Mature BMDCs or BMDC derived ICVs were lysed in RIPA buffer, vortexed, and run on 12% agarose gels at 100 V for 60 min, then visualized by UV lamp (*e.g.*, see **FIG. 65**). It was found that mature BMDCs and ICVs display similar RNA profiles, with DNA from the cells displaying at the top of the lane. When ICVs derived from mature BMDCs, and ICVs derived from immature BMDCs were compared on 12% agarose gels after lyses in RIPA buffer with or without RNase treatment, it was found that immature and mature ICVs showed similar RNA profiles (*e.g.*, see **FIG. 66**).

[00215] ***Identification of RNA from ICVs produced from mature BMDCs using singe-cell RNA sequencing.*** To further investigate ICV contents, were analyzed by single-cell RNA sequencing analysis. Clusters of particles by similarity in RNA content showed two main clusters (e.g., see **FIG. 67B**). The main contents of the heat maps are identified in **FIG. 68**. Many identified RNAs are known to be upregulated in immune cells during maturation/activation. In particular, the following 19 genes of interest were identified:

GM42418: Predicted non-coding gene for C57BL/6 mice.

Fth1 (Ferritin heavy chain 1): Protein coding gene; Gene for cell oxidoreductase, proliferation, establishment of localization and homeostasis processes; Found in mitochondria and vacuole.

AY036118: Non-coding RNA; Involved in cell proliferation, immune system process and system development.

Saa3 (Serum amyloid A 3): Protein coding gene; For signaling receptor binding, protein metabolic process, response to stimulus, signaling; Found in extracellular region.

Mt-Co2 (Mitochondrially encoded cytochrome c oxidase II): Protein coding gene; For oxidoreductase, carbohydrate derivative metabolism, cell death; Found in mitochondria and organelle envelope.

Mt-Co1 (Mitochondrially encoded cytochrome c oxidase I): Protein coding gene; For oxidoreductase and transporter, establishment of localization and response to stimulus; Found in mitochondrion and organelle envelope.

Mt-Co3 (Mitochondrially encoded cytochrome c oxidase III): Protein coding gene; For oxidoreductase, transporter, cellular component organization; Found in mitochondria and organelle envelope.

Ubc (Ubiquitin C): Protein coding gene; For protein metabolic process; Found in nucleus

Rpl19 (Ribosomal protein L19): Protein coding gene; For RNA binding, protein metabolic processing; Found in cytosol, non-membrane bounded, synapse.

Vezt (Vezatin): Adherens junction transmembrane protein; protein coding gene; For cytoskeletal protein binding; Found in cell projection, cytoplasmic vesicle, cytosol, nucleus, organelle lumen, plasma membrane.

Snap91 (Synaptosomal-associated protein 91): Protein coding gene; For protein lipid binding, cell differentiation, cell component organization, establishment of localization, signaling, system development; Found in cell projection, cytoplasmic vesicle, cytosol, endosome, plasma membrane, synapse.

Ctbs (Chitiobiase): Protein coding gene; For carbohydrate derivative binding, hydrolase, carbohydrate derivative metabolism; Found in vacuole.

Mon2 (MON2 homolog): regulator of endosome to Golgi trafficking; Protein coding gene; For establishment of localization.

Hif1an (Hypoxia-inducible factor 1): Protein coding gene; For oxidoreductase, signaling receptor binding, cell differentiation, nucleic acid-templated transcription, protein metabolic process, response to stimulus, signaling; Found in cytosol, nucleus, organelle lumen.

Nbas (Neuroblastoma amplified sequence): Protein coding gene; For establishment of localization; Found in the endoplasmic reticulum.

Rock2 (Rho-associated coiled-coil containing protein kinase 2): Protein coding gene; For carbohydrate derivative binding, cell death, cell differentiation, cellular component organization, protein metabolic process, response to stimulus, signaling, system development; Found in cytoskeleton, cytosol, non-membrane-bounded organelle, nucleus, plasma membrane, synapse.

Rap1b (RAS related protein 1b): Protein coding gene; For carbohydrate derivative binding, hydrolase, cell differentiation, cell proliferation, cell component organization, establishment of localization; Found in cytosol, non-membrane bound organelle, plasma membrane.

Tmed7 (Transmembrane p24 trafficking protein 7): Protein coding gene; For cell component organization, establishment of localization; Found in cytoplasmic vesicle, ER, Golgi.

Fbll1 (Fibrillarin-like 1): Protein coding gene; For RNA binding, transferase, cell component organization, protein metabolic process; Found in non-membrane-bounded organelle, nucleus, organelle lumen.

[00216] ***Dosing and T-Cell stimulation between groups of ICVs from immature BMDCs, immature BMDCs, ICVs from mature BMDCs, and mature BMDCs.*** A dosing protocol based on equivalent surface area of materials was developed for comparisons between groups of ICVs from immature BMDCs, immature BMDCs, ICVs from mature BMDCs, and mature BMDCs. BMDCs were labeled with PKH26, a red fluorescent membrane stain, lysed and read by plate reader for fluorescent intensity. BMDCs were also labeled and blebbed in 25 mM PFA blebbing buffer for 24 hours. mICVs were collected, lysed, and read for fluorescent signal by plate reader.

[00217] Comparing equivalent numbers of immature or mature BMDCs, mature BMDCs had nearly 3-fold greater surface area based on PKH membrane signal (e.g. see **FIG 70A-B**). Furthermore, mature BMDCs produced 3-fold the amount of ICVs. It is therefore likely that amount of ICVs produced is correlated with surface area of membrane.

[00218] Based on equivalent PKH signal, and therefore material surface area, immature or mature BMDCs and ICVs produced therefrom were dosed to T cells and evaluated for T cell stimulation ability by CPRG assay. Based on dosing by equivalent surface area, T cell stimulation had the following trend: ICVs from mature BMDCs>mature BMDCs>immature BMDCs and ICVs from immature BMDCs (*e.g.*, see **FIG. 71**). When dosing based on surface area, there are more ICVs from BMDCs:T cells than BMDCs:T cells, generating greater likelihood of interactions and stimulation.

[00219] ***Protective immunity in tumor challenge study by mICVs vaccination.*** While ICVs have a variety of benefits including ease of production, size and lack of whole-cell components, it was important to investigate whether antigenic ICVs had efficacy in generating protective immunity in a tumor challenge scenario. While mature BMDCs are known to induce protective immunity, antigenic ICVs produced by sulfhydryl blocking have not previously been assessed for this potential.

[00220] Mice received a single vaccination followed by a booster vaccination two weeks later). Seven days after completion of a vaccination schedule. mice were challenged with E.G7-OVA cells and tumor growth was assessed over four weeks. **FIG. 72** provides images from Day 30 of the tumor challenge with mice by vaccine category. PBS, OVA, and SIINFEKL groups all rapidly developed tumors. Immature and mature BMDC vaccine

groups developed small tumors by day 15 and went into complete remission. ICVs from immature BMDCs developed tumors at a slowed rate, indicating a low immune response, but not potent enough to prevent tumor growth. ICVs from mature BMDCs developed small tumors, larger than those of the cell vaccine group, and later went into total remission. This data suggests that immature cells likely matured after administration in order to achieve a similar response to the mature cell group. This demonstrates the need for a cell-free vaccine, as cell maturation and state and therefore resulting immune response could not be accurately controlled. However, ICVs from immature BMDCs had a tolerant effect while ICVs from mature BMDCs performed similarly to mature cells. So, while antigenic ICVs were found to be as effective as BMDCs in generating protective immunity in a tumor challenge scenario (*e.g.*, see **FIG. 73A**), antigenic ICVs exhibit notable technical features over BMDCs, such as scalability, size, and cell-free aspect. The foregoing tumor challenge result indicates promise for use of antigenic ICVs as effective cancer vaccines. Beyond reducing tumor growth, antigenic ICVs also performed as well as BMDCs in increasing survival rates (*e.g.*, see **FIG. 73B**). BMDC or antigenic ICV treatment led to complete remission in half of the animals treated.

[00221] It will be understood that various modifications may be made without departing from the spirit and scope of this disclosure. Accordingly, other embodiments are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method to produce induced cell vesicles (ICVs) or antigenic ICVs, comprising:

inducing cell vesicle production from cells by exposing or contacting the cells with a cell blebbing buffer which comprises a sulfhydryl blocking agent or a photosensitizer;

wherein antigenic ICVs are produced from antigen presenting cells which can stimulate T-cell activation.

2. The method of claim 1, wherein the cells are from a mammal.

3. The method of claim 2, wherein the cells are from a human.

4. The method of claim 3, wherein the cells are from a human patient that has a disorder or disease that is to be treated with ICVs or antigenic ICVs produced therefrom.

5. The method of claim 1, wherein the cells are selected from epithelial cells, fibroblast cells, tumor cells, mast cells, T and B lymphocytes, dendritic cells, and Langerhans cells.

6. The method of claim 1, wherein the antigenic ICVs are produced from dendritic cells.

7. The method of claim 6, wherein the dendritic cells are bone marrow dendritic cells (BMDCs).

8. The method of claim 7, wherein the BMDCs are immature BMDCs.

9. The method of claim 7, wherein the BMDCs are mature BMDCs.

10. The method of claim 1, wherein the induced cell vesicles comprise viruses, viral particles, or viral vectors, by being produced from cells comprising the same.

11. The method of claim 10, wherein the viruses, viral particles, or viral vectors are selected from recombinant retroviruses, adenoviruses, adeno-associated viruses (AAV), alphaviruses, and lentiviruses.

12. The method of claim 11, wherein the viruses, viral particles, or viral vectors are AAV.

13. The method of claim 12, wherein the AAV expresses a heterologous transgene that is used for gene therapy.

14. The method of claim 1, wherein the cell blebbing buffer does not contain any small molecule redox reagents or reducing agents.

15. The method of claim 1, wherein the cell blebbing buffer comprises a buffered balanced salt solution.

16. The method of claim 15, wherein the buffered balanced salt solution selected from the group consisting of phosphate-buffered saline (PBS), Dulbecco's Phosphate-buffered saline (DPBS), Earle's Balanced Salt solution (EBSS), Hank's Balanced Salt Solution (HBSS), TRIS-buffered saline (TBS), and Ringer's balanced salt solution (RBSS).

17. The method of claim 15, wherein the cell blebbing buffer comprises a 1X to 10X concentration/dilution of the buffered balanced salt solution.

18. The method of claim 15, wherein the cell blebbing buffer comprises a 1X to 5X concentration/dilution of DPBS.

19. The method of claim 1, wherein the cells are incubated in the cell blebbing buffer which comprises a sulfhydryl blocking agent for 0.5 h to 48 h.

20. The method of claim 19, wherein the cells are incubated in the cell blebbing buffer for 1 h to 8 h at 37 °C.

21. The method of claim 1, wherein the sulfhydryl blocking agent is selected from the group consisting of and *N*-ethylmaleimide, paraformaldehyde, mercury chloride, p-chloromercuribenzene sulfonic acid, auric chloride, *p*-chloromercuribenzoate, chlormerodrin, meralluride sodium, and iodoacetamide.

22. The method of claim 21, wherein the sulfhydryl blocking agent is *N*-ethylmaleimide (NEM) or maleimide.

23. The method of claim 22, wherein the cell blebbing buffer comprises from 1 mM to 10 mM of NEM.

24. The method of claim 23, wherein the cell blebbing buffer consists essentially of 2 mM NEM in DPBS.

25. The method of claim 21, wherein the cell blebbing buffer comprises paraformaldehyde.

26. The method of claim 25, wherein the cell blebbing buffer comprises 25 mM of paraformaldehyde.

27. The method of claim 1, wherein the cells are incubated with or exposed to a photosensitizer having a concentration of 0.5 ug/mL to 5.0 ug/mL.

28. The method of claim 27, wherein the cells are incubated with or exposed to a photosensitizer having a concentration of 1.0 ug/mL.

29. The method of claim 1, wherein the cells are exposed or incubated with the photosensitizer for 1 h to 48 h.

30. The method of claim 29, wherein the cells are exposed to or incubated with the photosensitizer for 24 h at 37° C.

31. The method of claim 1, wherein the photosensitizer is a porphyrin, chlorin or a dye.

32. The method of claim 1, wherein the photosensitizer is selected from $AlPcS_{2A}$, $AlPcS_4$, lutrin, 5-aminolevulinic acid (ALA), hypericin, silicon phthalocyanine zinc (II) phthalocyanine (ZnPc), silicon phthalocyanine, mono-L-aspartyl chlorin e6, benzoporphyrin derivative monoacid ring A, chlorin photosensitizer tin etiopurpurin, tetra(*m*-

hydroxyphenyl)chlorin, lutetium texaphyrin, 9-acetoxy-2,7,12,17-tetrakis-(β-methoxyethyl)-porphycene, naphthalocyanines, Allumera®, Photofrin®, Visudyne®, Levulan®, Foscan®, Fospeg®, Metvix®, Hexvix®, Cysview® and Laserphyrin®, Antrin®, Photochlor®, Photosens®, Photrex®, Lumacan®, Cevira®, Visonac®, BF-200 ALA®, Amphinex® and Azadipyrromethenes.

33. The method of claim 32, wherein the photosensitizer is $AlPcS_{2A}$.

34. The method of claim 29, wherein the cells are washed with a buffered balanced salt solution one or more times, and taken up in the buffered balanced salt solution prior to exposure to light.

35. The method of claim 34, wherein the buffered balanced salt solution selected from the group consisting of phosphate-buffered saline (PBS), Dulbecco's Phosphate-buffered saline (DPBS), Earle's Balanced Salt solution (EBSS), Hank's Balanced Salt Solution (HBSS), TRIS-buffered saline (TBS), and Ringer's balanced salt solution (RBSS).

36. The method of claim 34, wherein the cells are taken up in 1X to 10X concentration/dilution of the buffered balanced salt solution prior to light exposure.

37. The method of claim 34, wherein the cells are taken up in 1X DPBS prior to light exposure.

38. The method of claim 34, wherein the cells are exposed to light for 1 min to 60 min.

39. The method of claim 38, wherein the cells are exposed to light generated by a laser.

40. The method of claim 39, wherein the cells are exposed to light having a wavelength from 600 nm to 850 nm that is generated by a laser.

41. The method of claim 33, wherein the EB producing cells are incubated with or exposed to 1 ug/mL of $AlPcS_{2A}$ for 24 h at 37 °C, washed multiple times in 1X DPBS, taken up in 1X DPBS, and then exposed to light generated from a 670 nm laser for 1 min to 10 min.

42. The method of claim 1, wherein the method further comprises the step of:

purifying/isolating the ICVs or antigenic ICVs by using sucrose gradients.

43. The method of claim 1, wherein the method further comprises the step of:

purifying/isolating the ICVs or the antigenic ICVs by:

(i) removing cellular debris by centrifugation from 1,000 rpm to 2,500 rpm for 1 to 10 minutes; and

(ii) recovering the ICVs or antigenic ICVs by centrifugation using 10,000 x g to 20,000 x g for 5 to 15 minutes;

optionally, concentrating the recovered nanometer sized ICVs or antigenic nanometer sized ICVs by using concentrators with a pore size cut-off from 50 to 150 kDA.

44. The method of claim 43, wherein the isolated ICVs or isolated antigenic ICVs have average diameters from 10 nm to 10,000 nm.

45. The method of claim 44, wherein the isolated ICVs or isolated antigenic ICVs have average diameters from 150 nm to 5,000 nm.

46. The method of claim 45, wherein the isolated ICVs or isolated antigenic ICVs have average diameters from 1000 nm to 5,000 nm.

47. The method of claim 43, wherein the isolated ICVs or isolated antigenic ICVs comprise a cargo selected from biological molecules, therapeutic agents, prodrugs, gene silencing agents, chemotherapeutics, diagnostic agents, components of a gene therapy system and/or components of a gene editing system.

48. The method of claim 47, wherein the isolated ICVs or isolated antigenic ICVs are loaded with the cargo by direct membrane penetration, chemical labeling and conjugation, electrostatic coating, adsorption, absorption, sonification, electroporation, use of pH gradients, or any combination thereof.

49. The method of claim 48, wherein the isolated ICVs or isolated antigenic ICVs are loaded with the cargo by incubating isolated ICVs or isolated antigenic ICVs, or the cells used to produce ICVs or antigenic ICVs, with a cargo for a sufficient period of time to allow uptake or adsorption of the cargo by the ICVs, antigenic ICVs or by the cells.

50. The method of claim 1, wherein the cells comprise or have been modified to comprise one or more functional moieties on the cell surface.

51. The method of claim 50, wherein the one or more functional moieties are one or more targeting ligands.

52. The method of claim 51, wherein the one or more targeting ligands direct the ICVs or antigenic ICVs to a certain cell, cell type, tissue type, tumor, or organ.

53. The method of claim 51, wherein the one or more targeting ligands are an antibody or a single-chain variable fragment which binds to a tumor-specific antigen.

54. The method of claim 51, wherein the tumor-specific antigen is selected from alphafetoprotein (AFP), carcinoembryonic antigen (CEA), CA-125, CA15-3, CA19-9, MUC-1, epithelial tumor antigen (ETA), tyrosinase, melanoma-associated antigen (MAGE), abnormal products of ras or p53, CTAG1B, MAGEA1, and HER2/neu.

55. The method of claim 50, wherein the cells have been bioorthogonally-conjugated to comprise one or more functional moieties.

56. The method of claim 55, wherein the one or more functional moieties have been added to the surface of the cells by bioorthogonally-engineering, comprising:

(1) treating sialic acid residues on the surface of the cells with an oxidizing agent to form aldehyde groups; then either step (2)(a) and (b), or step (3)(a) and (3)(b):

(2)(a) ligating, linking or conjugating aminooxy-functionalized molecules to the surface of the cells by forming oxime bonds with the aldehyde groups; and

(2)(b) inducing production of bioorthogonally-conjugated ICVs or bioorthogonally-conjugated antigenic ICVs by exposing or contacting the cells with the cell blebbing buffer which comprises a sulfhydryl blocking agent or a photosensitizer;
or

(3)(a) inducing production of ICVs or antigenic ICVs from the cells by exposing or contacting the cells with the cell blebbing buffer which comprises a sulfhydryl blocking agent or a photosensitizer; and

(3)(b) producing bioorthogonally-conjugated ICVs or bioorthogonally-conjugated antigenic ICVs by ligating, linking or conjugating aminooxy-functionalized molecules to the surface of the ICVs by forming oxime bonds with the aldehyde groups.

57. The method of claim 56, wherein the oxidizing agent is either sodium periodate or lead tetraacetate.

58. The method of claim 57, wherein the cells are treated with 1 mM sodium periodate for 30 min at 4 °C.

59. The method of claim 56, wherein the aminooxy-functionalized molecules comprise a detecting agent, and/or cell-, tumor-, or tissue-targeting ligands.

60. The method of claim 59, wherein the detecting agent is an enhanced fluorophore-based dye.

61. The method of claim 56, wherein the aminooxy-functionalized molecules are ligated, linked or conjugated to the aldehyde groups in the presence of a catalyst.

62. The method of claim 56, wherein the catalyst is *p*-anisidine.

63. The method of claim 62, wherein the aminooxy-functionalized molecules are ligated, linked or conjugated to the aldehyde groups in the presence of 10 mM *p*-anisidine for 90 min at 4 °C.

64. Bioorthogonally-conjugated ICVs produced by the method of claim 56.

65. The bioorthogonally-conjugated ICVs of claim 64, wherein the bioorthogonally-conjugated ICVs comprise oxime-linked detecting agents.

66. The bioorthogonally-conjugated ICVs of claim 64, wherein the bioorthogonally-conjugated ICVs are loaded with one or more small molecule therapeutic compounds or agents.

67. Isolated antigenic ICVs produced by the method of claim 43.

68. The isolated antigenic ICVs of claim 67, wherein the antigenic ICVs are loaded with one or more small molecule therapeutic compounds or agents.

69. Isolated ICVs produced by the method of claim 48.

70. A pharmaceutical composition comprising the bioorthogonally-conjugated antigenic ICVs of claim 66, the isolated antigenic ICVs of claim 67, or the isolated ICVs of claim 69; and a pharmaceutically acceptable carrier, excipient, and/or diluent.

71 A method of stimulating an immune response to a cancer in a subject in need thereof, comprising:

administering the pharmaceutical composition of claim 70 to the subject.

72. The method of claim 71, wherein the cancer is selected from squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulvar cancer, thyroid cancer, hepatic carcinoma, leukemia and other lymphoproliferative disorders, and various types of head and neck cancers.

73. The method of claim 71, wherein the pharmaceutical composition is administered by intravenous administration, intertumoral administration, intraperitoneal administration, intramuscular administration, intracoronary administration, intraarterial administration, subcutaneous administration, transdermal delivery, intratracheal administration, subcutaneous administration, intraarticular administration, intraventricular administration, inhalation, or intracerebral administration.

74. The method of claim 71, wherein the pharmaceutical composition is administered to the subject concurrently or sequentially with one or more anticancer agents or chemotherapeutics.

75. A method of treating a disease or disorder in a subject in need thereof, comprising:
administering the pharmaceutical composition of claim 70.

76. A method of stimulating an immune response to a cancer in a subject in need thereof, comprising:

(a) obtaining antigen presenting cells;

(b) pulsing the antigen presenting cells with an antigen associated with cancer cells;

(c) inducing cell membrane blebbing by use of a sulfhydryl blocking agent;

(d) collecting antigenic micrometer sized ICVs (mICVs) induced by cell membrane blebbing; and

(e) administering said antigenic mICVs to the subject in need of immunotherapy.

77. The method of claim 76, wherein the cancer is selected from squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, valvar cancer, thyroid cancer, hepatic carcinoma, leukemia and other lymphoproliferative disorders, and various types of head and neck cancers.

78. The method of claim 76, wherein the antigen presenting cells are dendritic cells, macrophages, monocytes, Langerhans cells, B cells, genetically modified cells, and mesenchymal stem cells.

79. The method of claim 78, wherein the antigen presenting cells are immature dendritic cells that are pulsed with an antigen to produce semimature or mature dendritic cells.

80. The method of claim 79, where the immature dendritic cells are derived from bone marrow of a human subject.

81. The method of claim 76, wherein the antigen presenting cells are obtained from the subject to be treated by immunotherapy.

82. The method of claim 76, wherein the sulfhydryl blocking agent is paraformaldehyde.

83. The method of claim 76, wherein the antigenic mICVs have diameters from 1 micrometer to 5 micrometers.

84. The method of claim 76, wherein the antigenic mICVs are administered by intravenous administration, intertumoral administration, intraperitoneal administration, intramuscular administration, intracoronary administration, intraarterial administration, subcutaneous administration, transdermal delivery, intratracheal administration, subcutaneous administration, intraarticular administration, intraventricular administration, inhalation, or intracerebral administration.

85. The method of claim 76, wherein the antigenic mICVs are administered concurrently or sequentially with one or more anticancer agents or chemotherapeutics.

86. A cell-free cell therapy comprising the pharmaceutical composition of claim 70 for use in treating a subject having a disease or disorder.

87. The cell-free cell therapy of claim 86, wherein the subject has cancer.

88. A vaccine comprising the pharmaceutical composition of claim 70 for prevention of an infection in a subject by an infectious agent.

89. The vaccine of claim 88, wherein the infectious agent is a bacterium, a virus, a fungus, or a protozoon.

90. A therapeutic vaccine comprising the pharmaceutical composition of claim 70 for use in treating a subject having cancer.

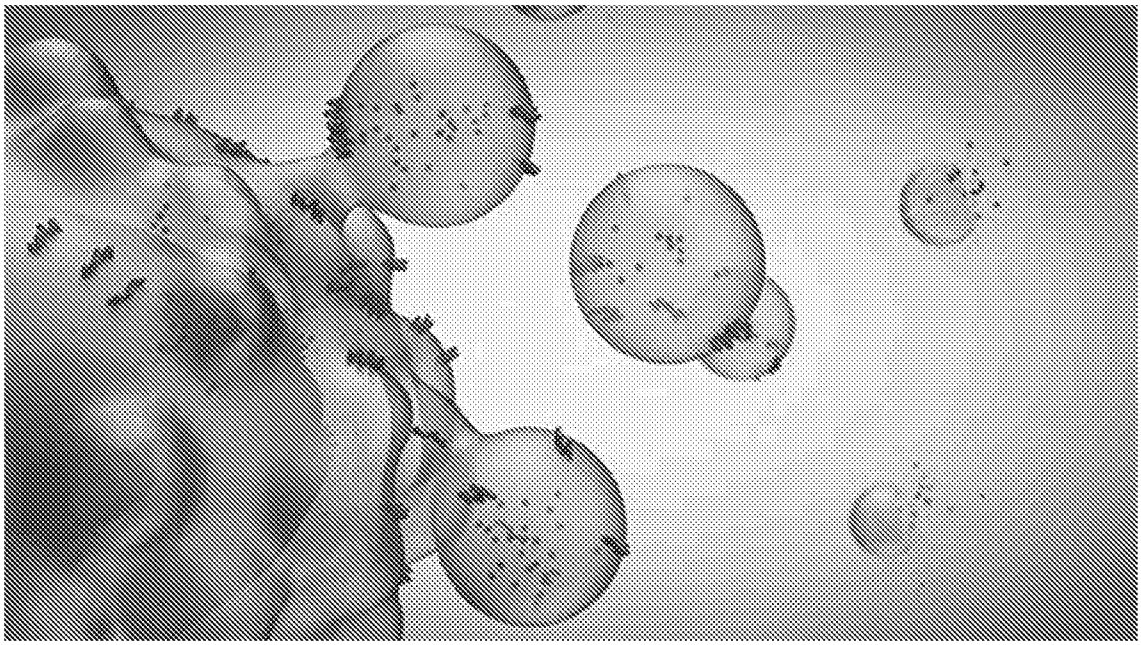

FIG. 1

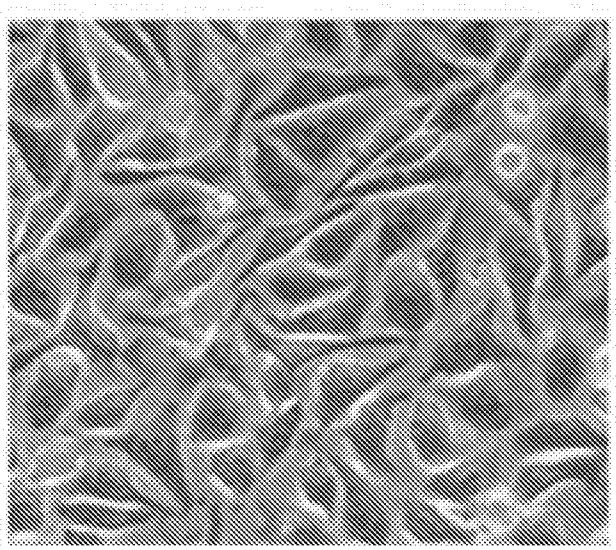

FIG. 2

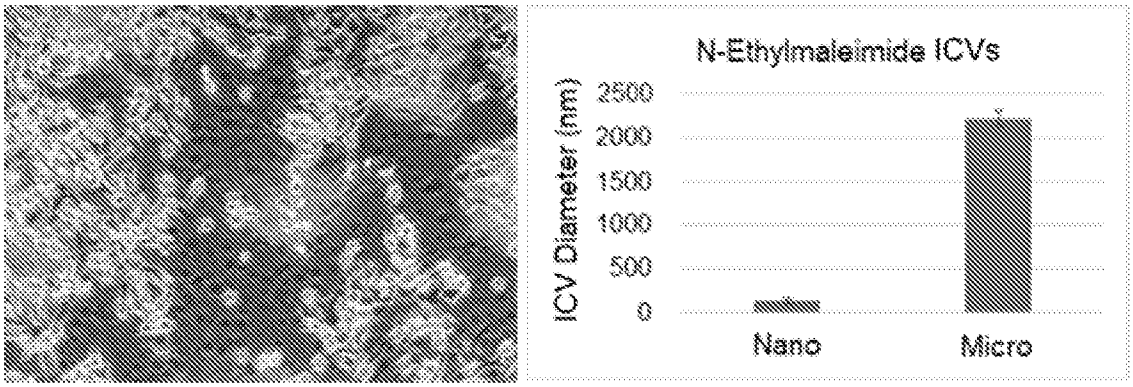

FIG. 3

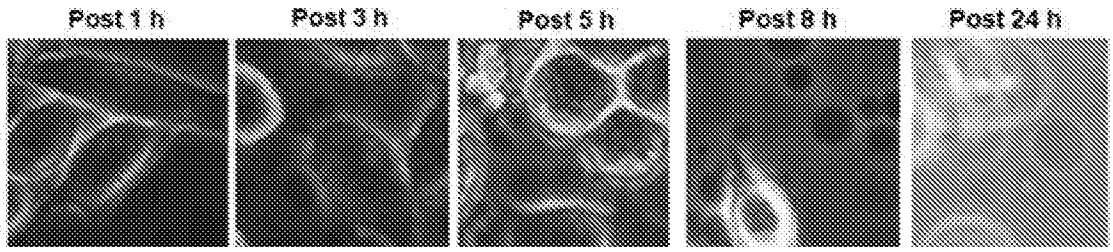

FIG. 4

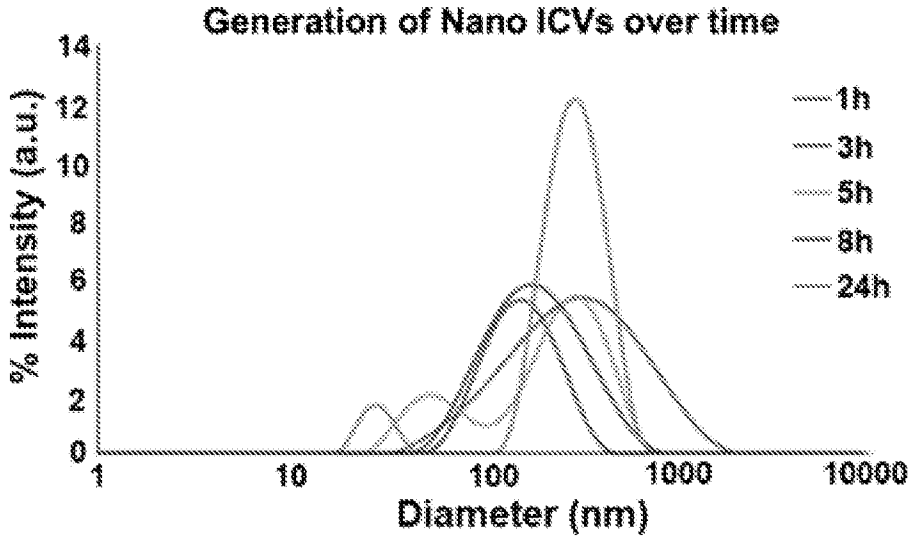

FIG. 5

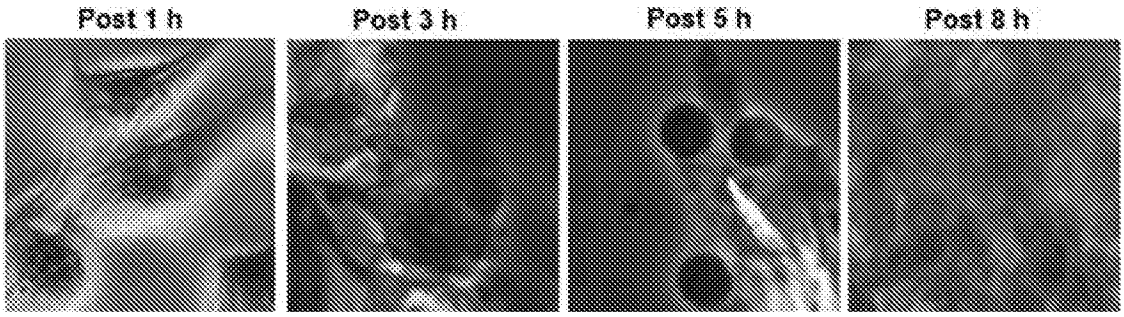

FIG. 6

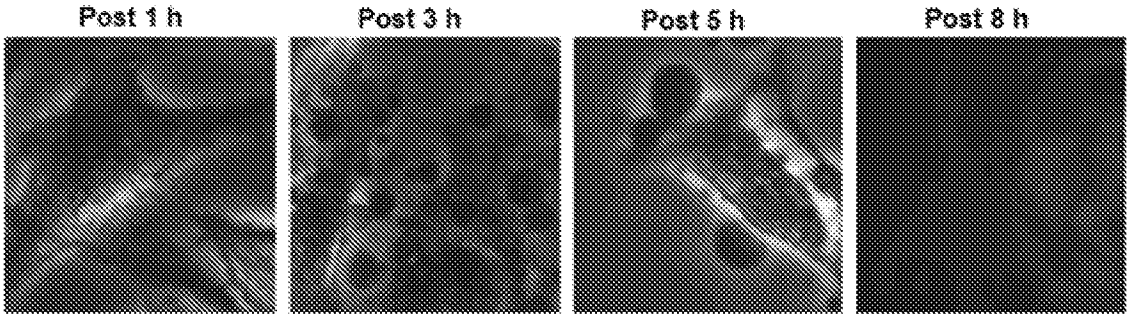

FIG. 7

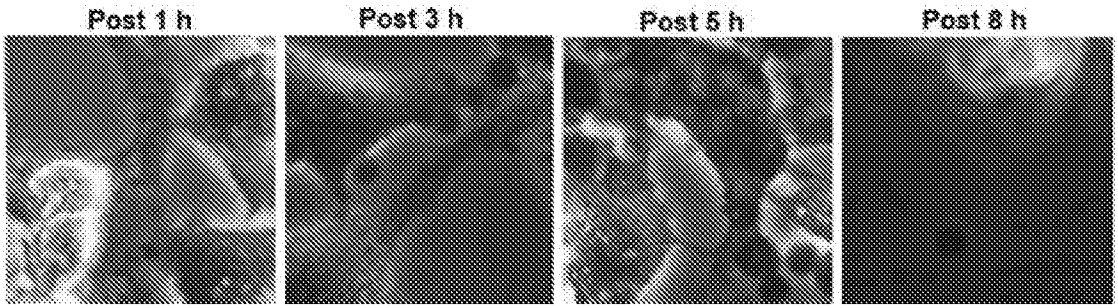

FIG. 8

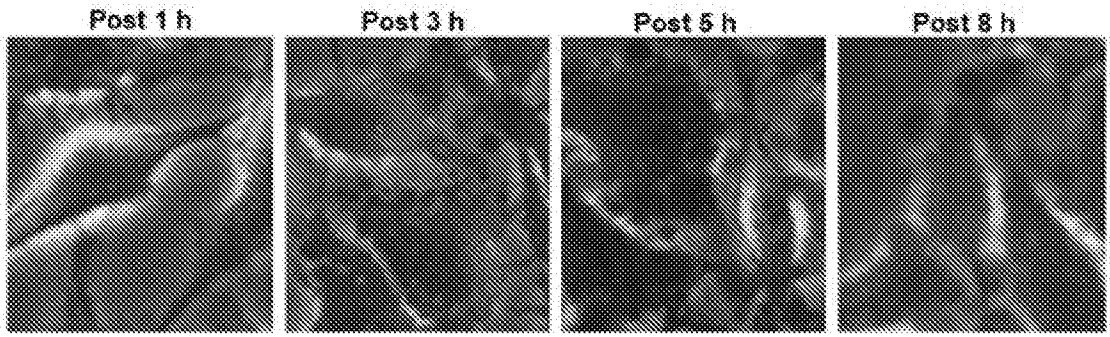

FIG. 9

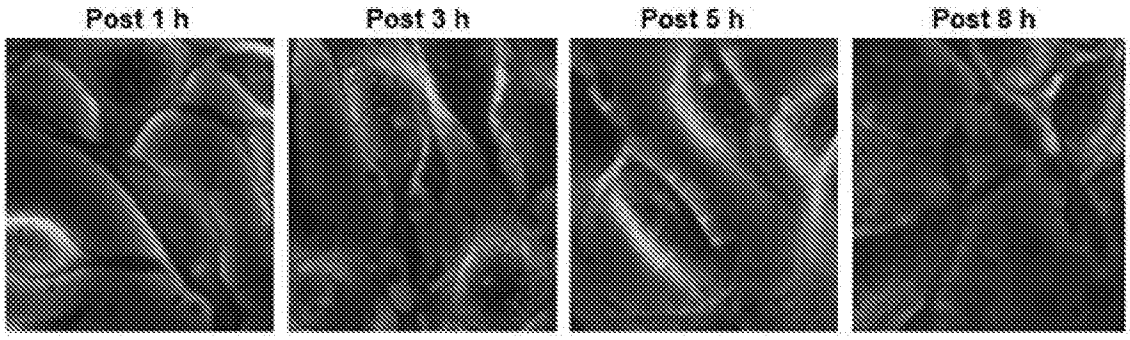

FIG. 10

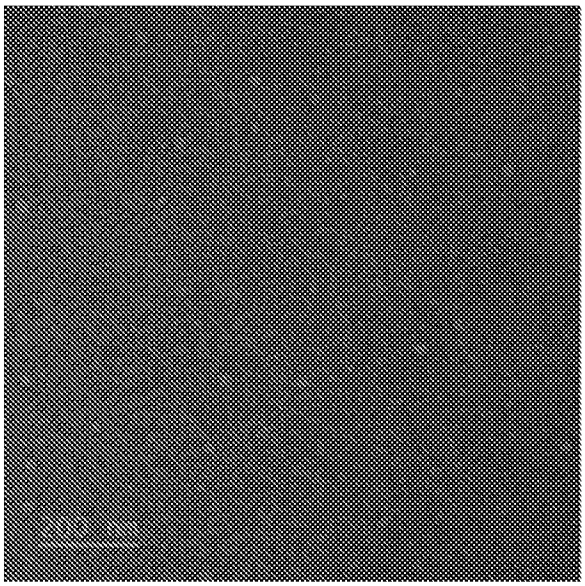

FIG. 11

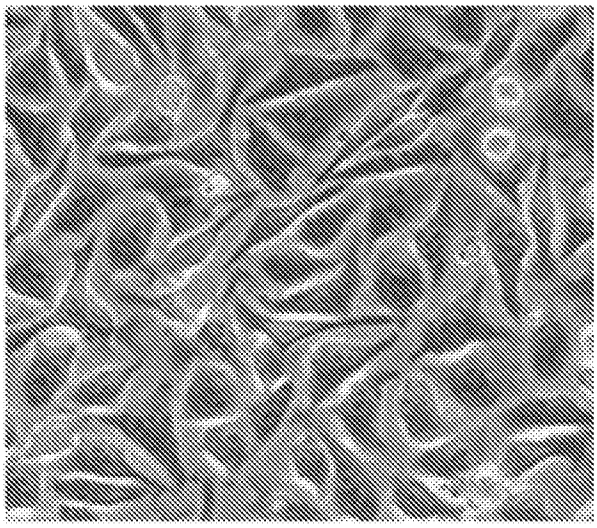

FIG. 12

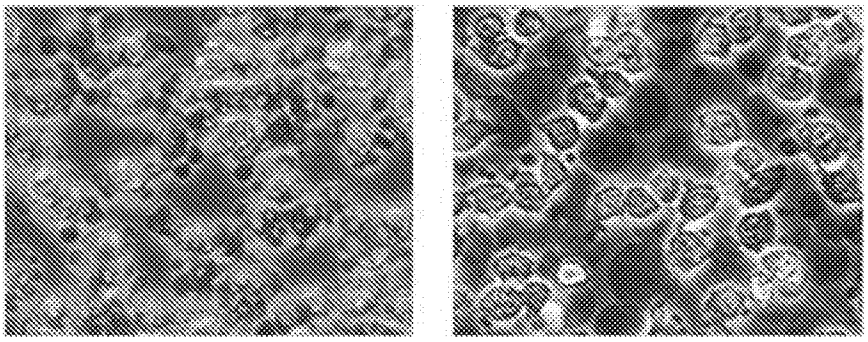

FIG. 13

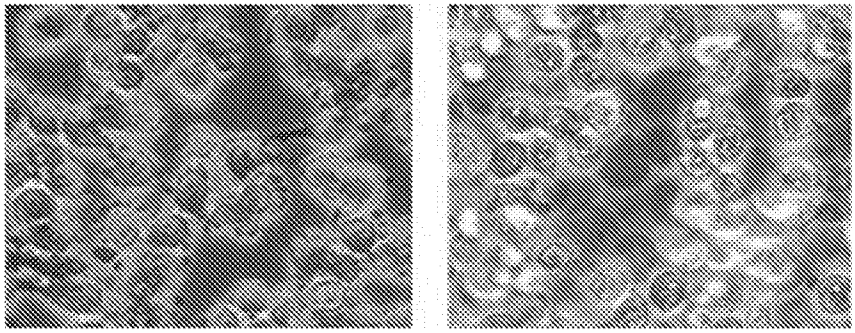

FIG. 14

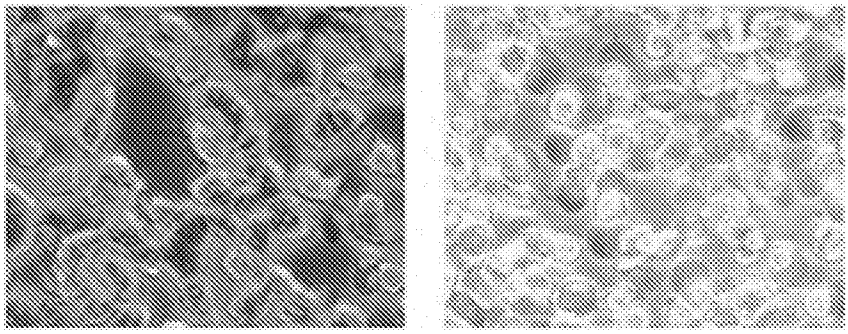

FIG. 15

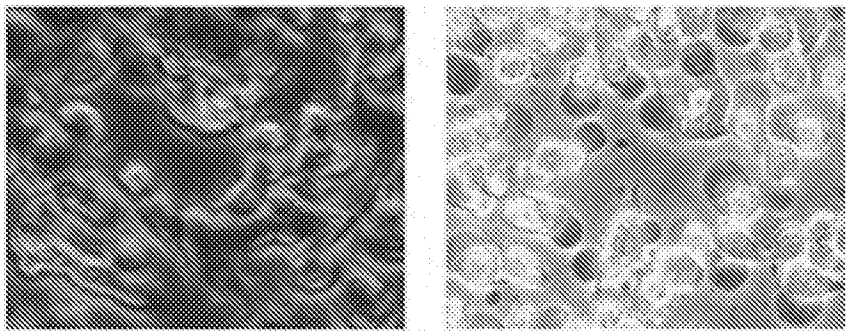

FIG. 16

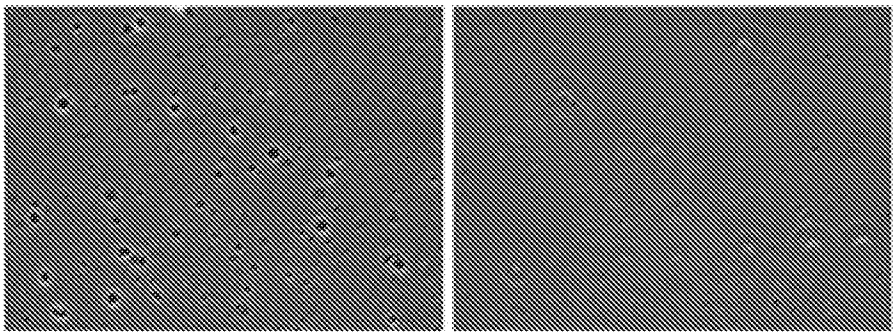

FIG. 17

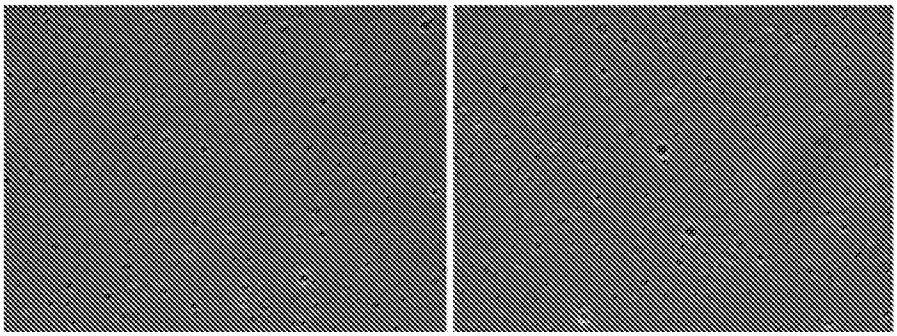

FIG. 18

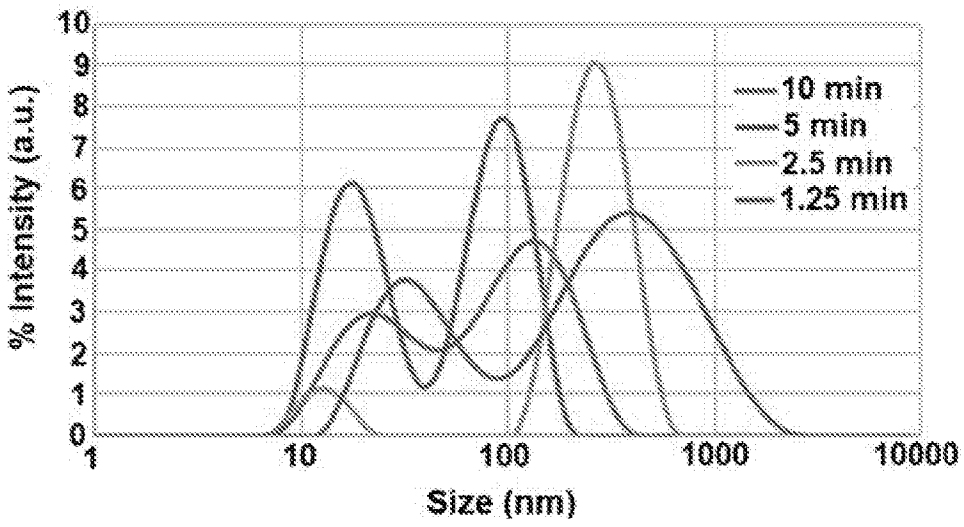

FIG. 19

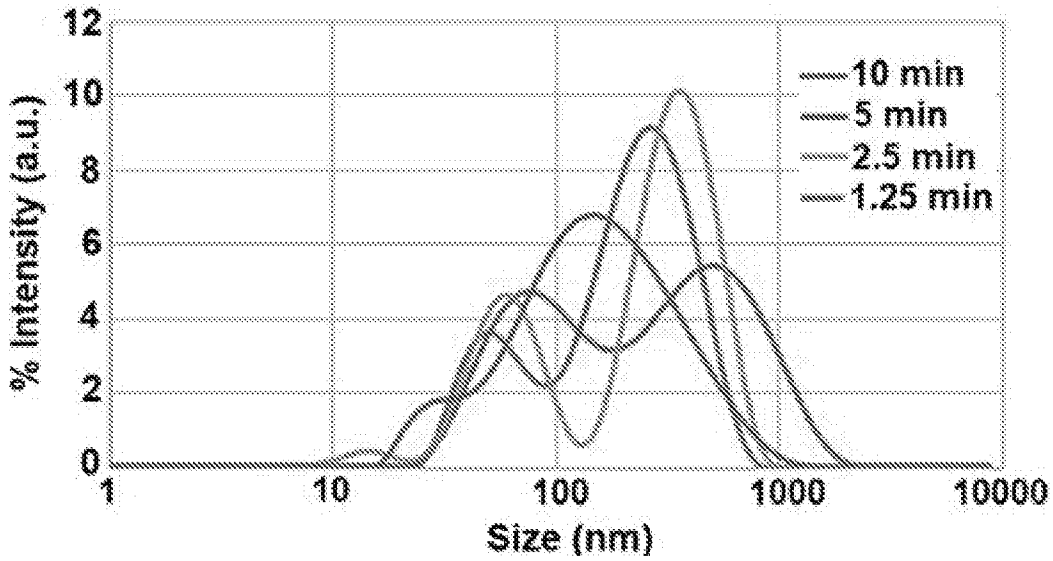
% Intensity (a.u.)
12
10
8
6
4
2
0
1
10
100
1000
10000
Size (nm)
10 min
5 min
2.5 min
1.25 min

FIG. 20

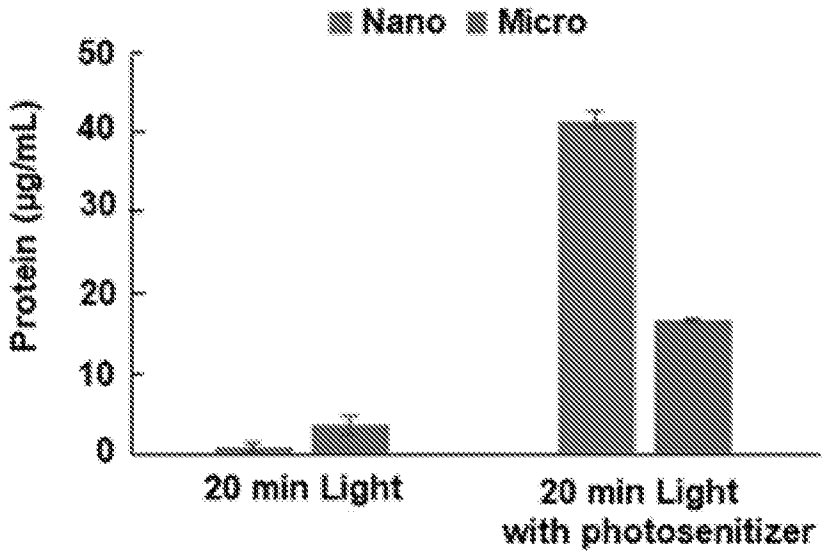
Nano
Micro
Protein (µg/mL)
50
40
30
20
10
0
20 min Light
20 min Light
with photosenitizer

FIG. 21

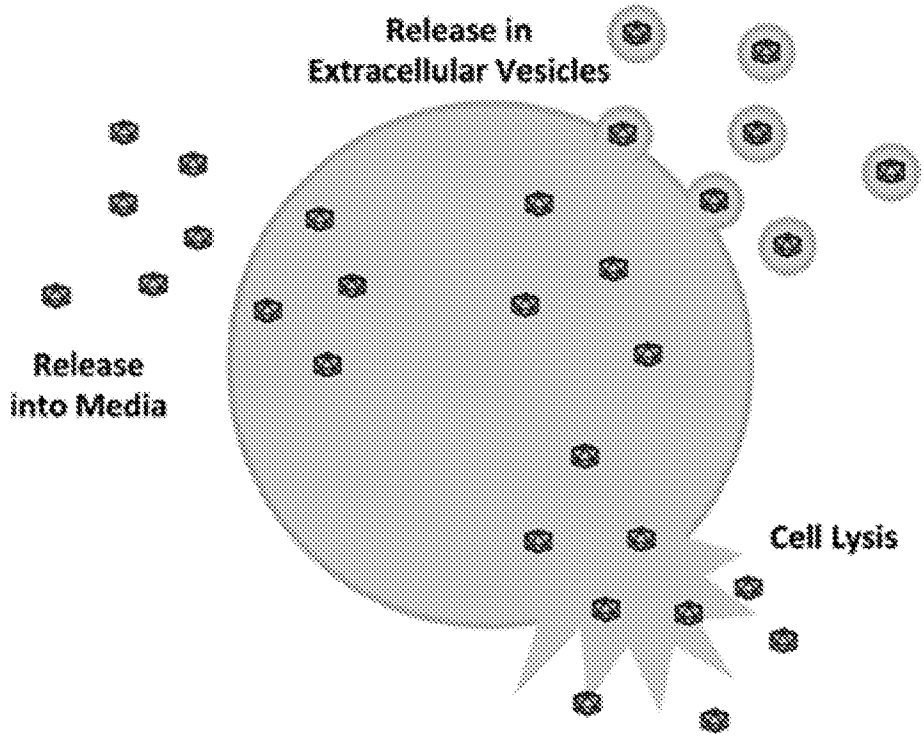
Release in
Extracellular Vesicles
Release
into Media
Cell Lysis

FIG. 22

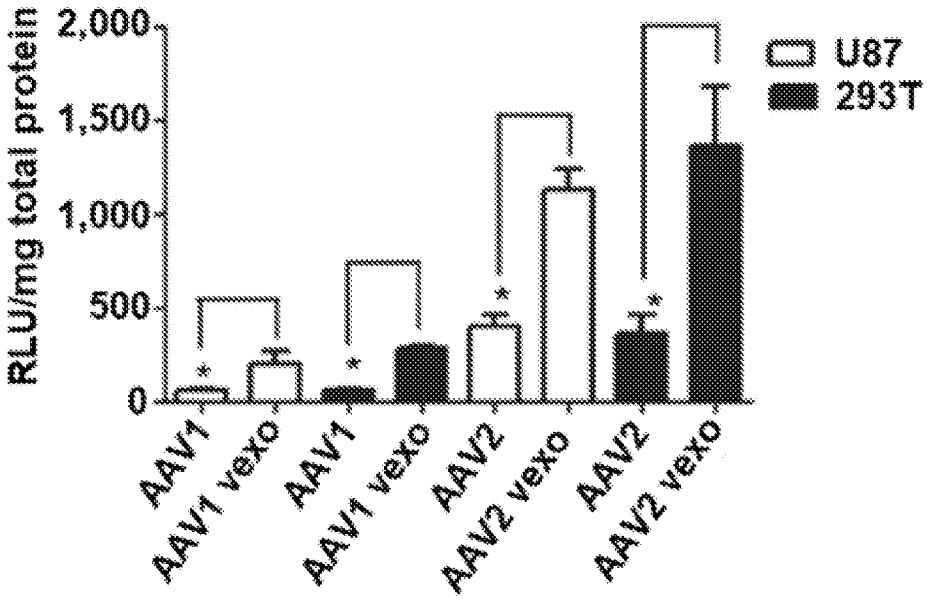
RLU/mg total protein
2,000
1,500
1,000
500
0
U87
293T
*
AAV1
AAV1 vexo
AAV1
AAV1 vexo
AAV2
AAV2 vexo
AAV2
AAV2 vexo

FIG. 23

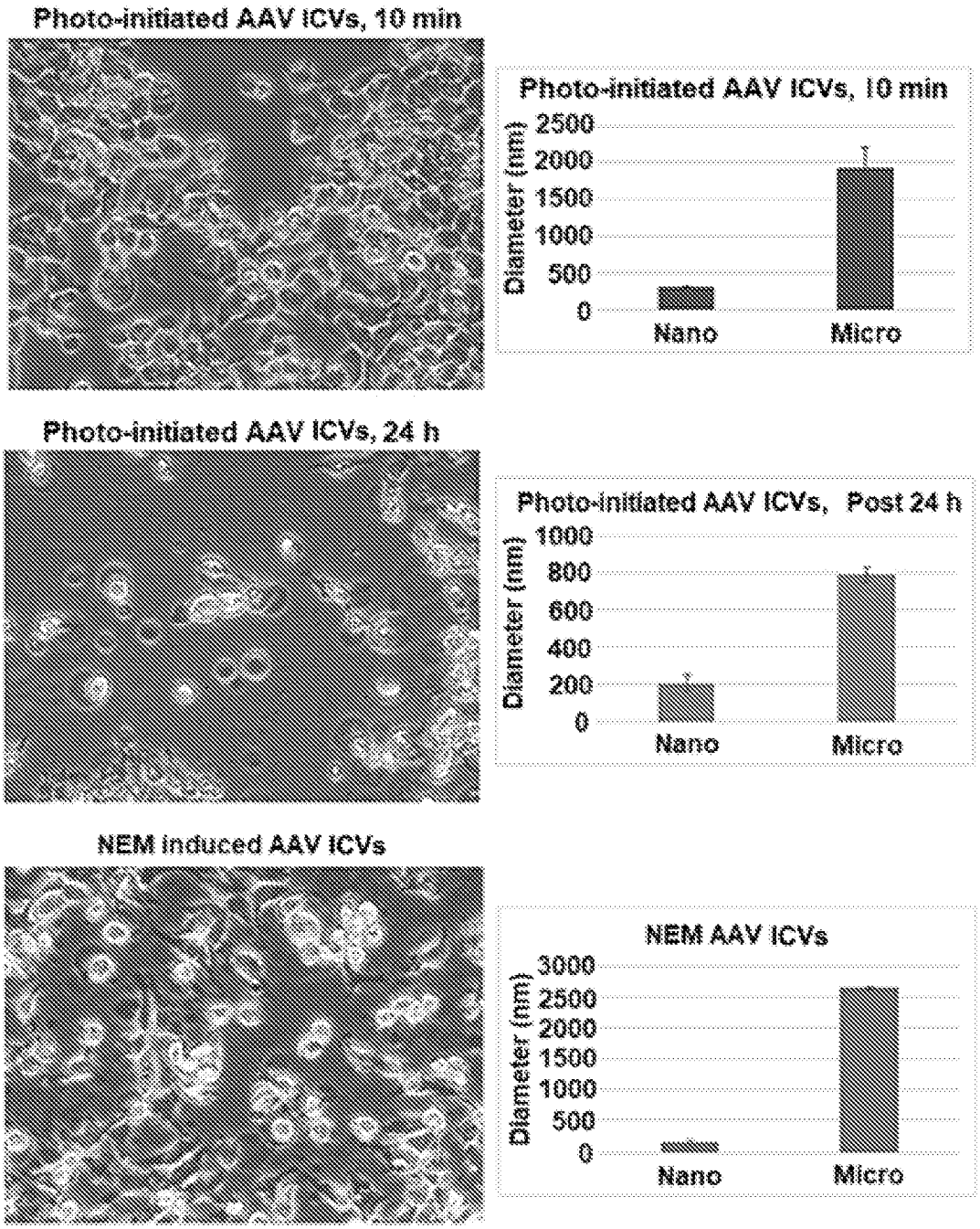
Photo-initiated AAV ICVs, 10 min
Photo-initiated AAV ICVs, 10 min
Diameter (nm)
2500
2000
1500
1000
500
0
Nano
Micro
Photo-initiated AAV ICVs, 24 h
Photo-initiated AAV ICVs, Post 24 h
Diameter (nm)
1000
800
600
400
200
0
Nano
Micro
NEM induced AAV ICVs
NEM AAV ICVs
Diameter (nm)
3000
2500
2000
1500
1000
500
0
Nano
Micro

FIG. 24

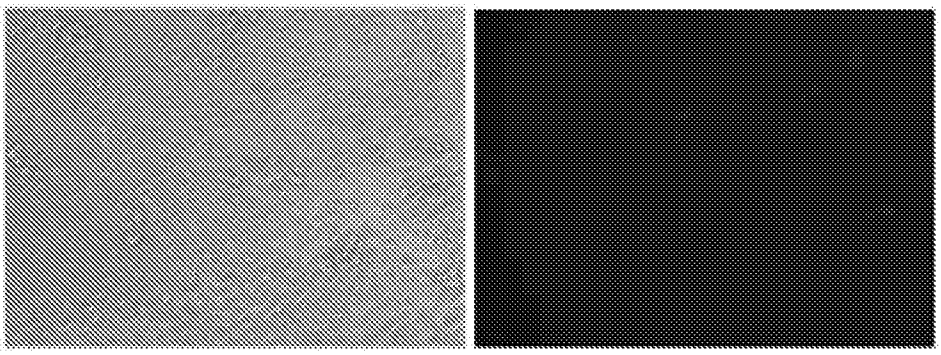

FIG. 25

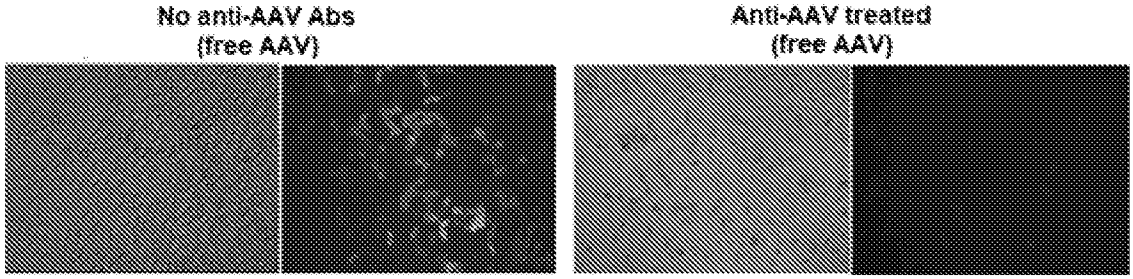

FIG. 26

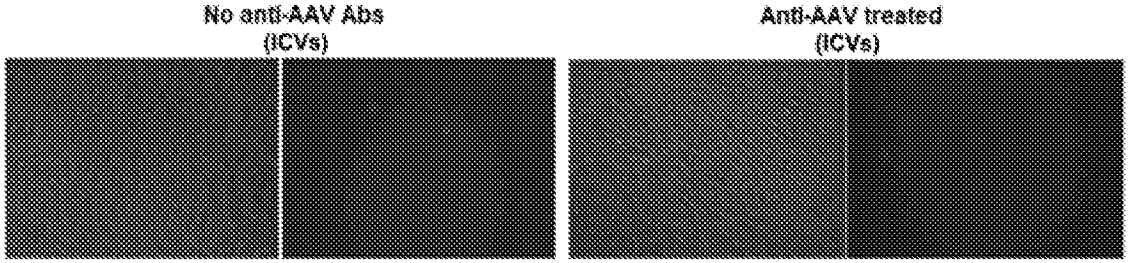

FIG. 27

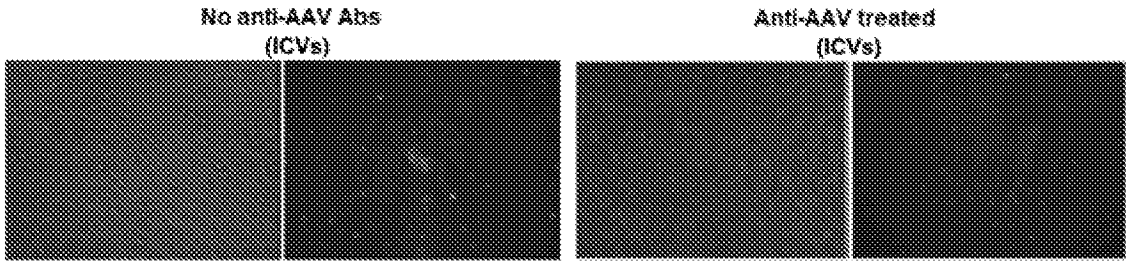

FIG. 28

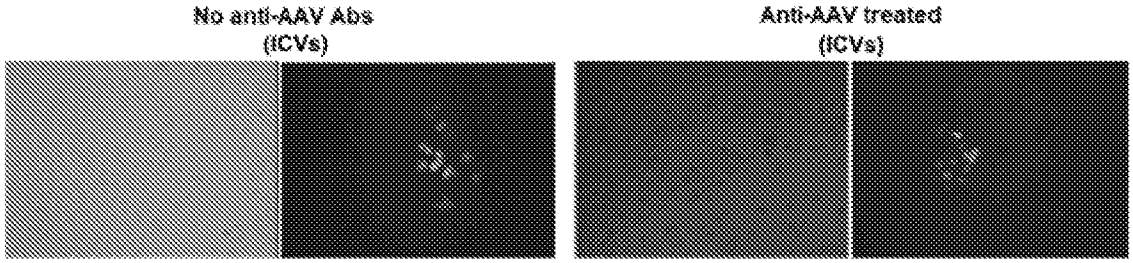

FIG. 29

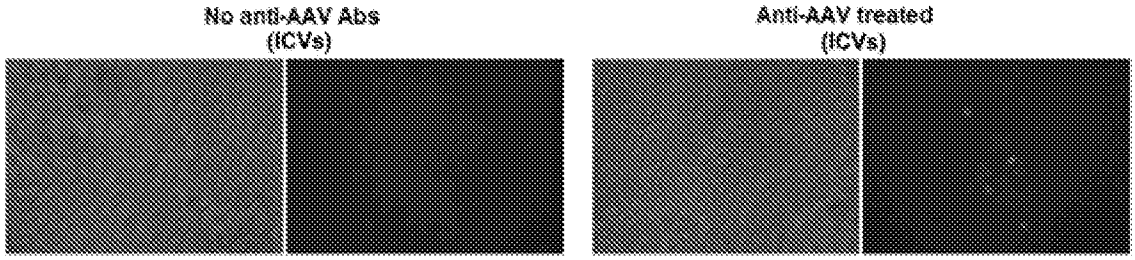

FIG. 30

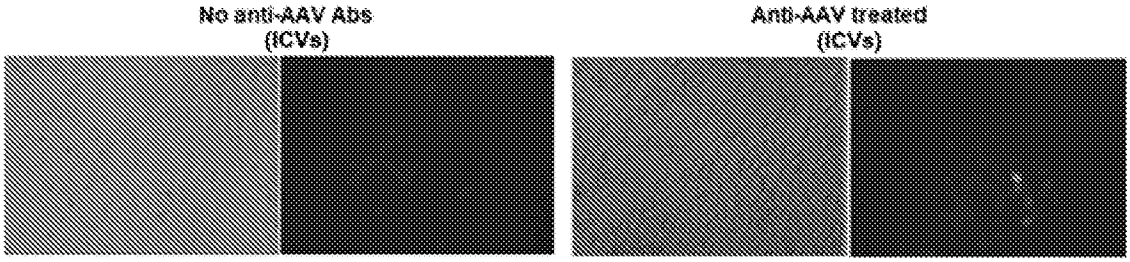

FIG. 31

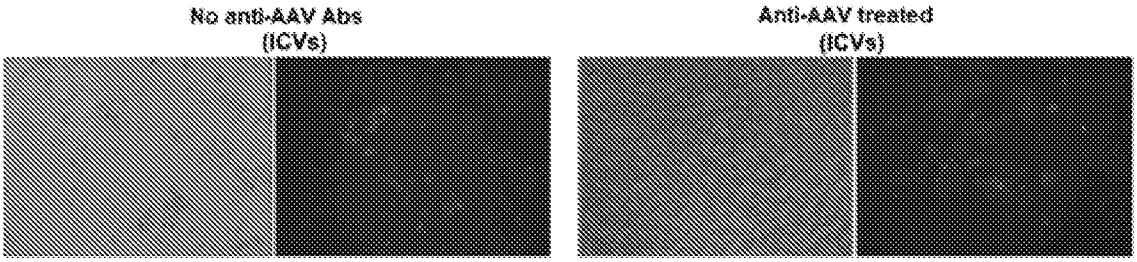

FIG. 32

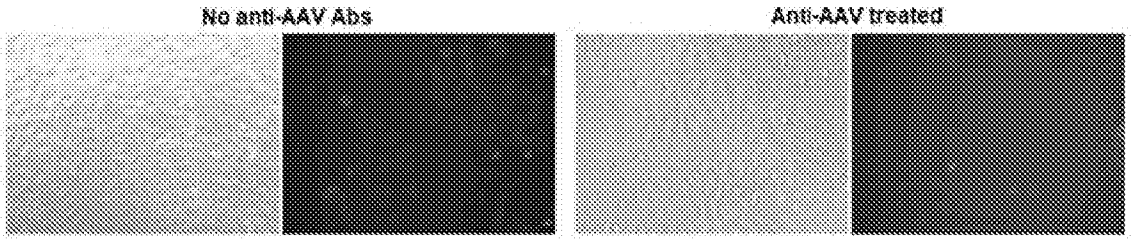

FIG. 33

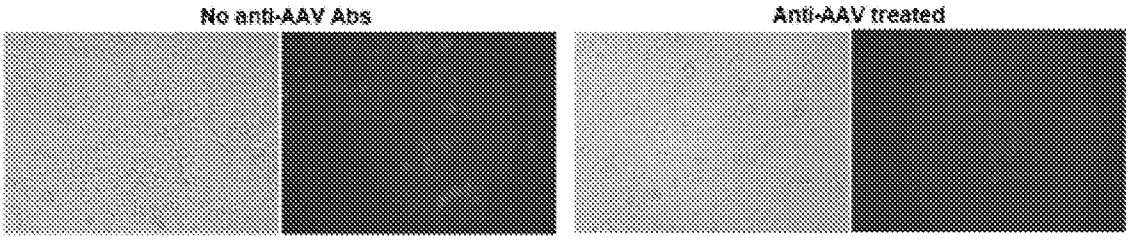

FIG. 34

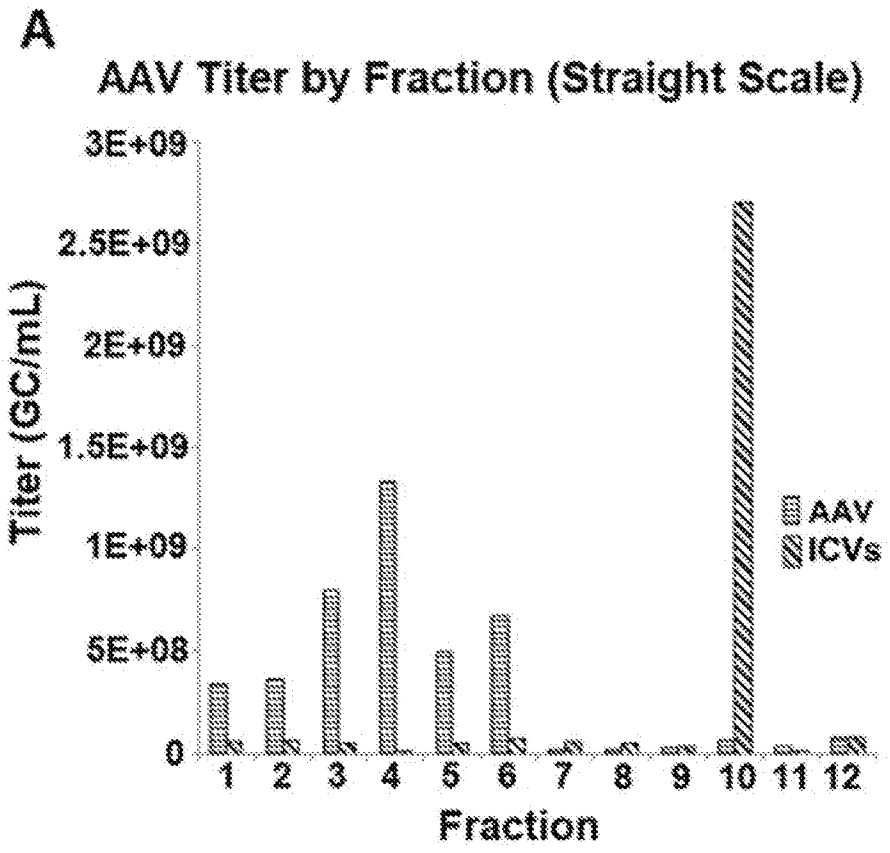

FIG. 35A

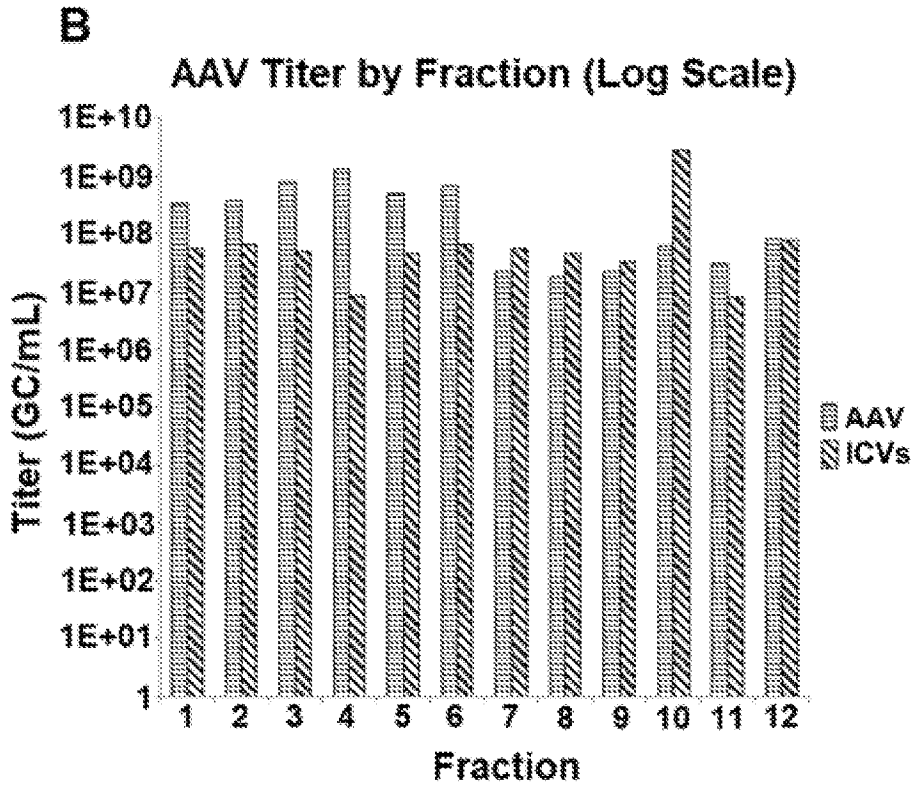

FIG. 35B

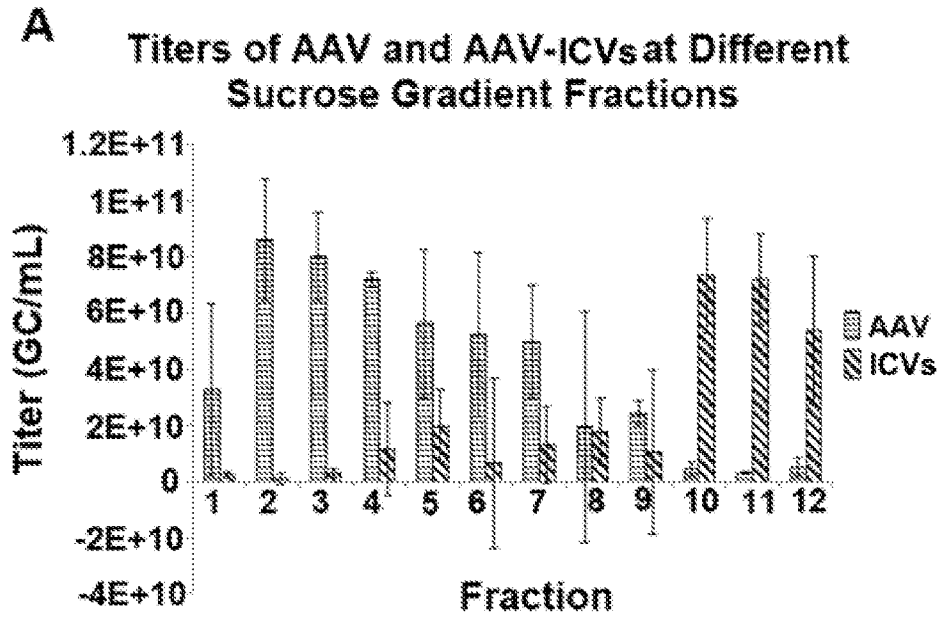

FIG. 36A

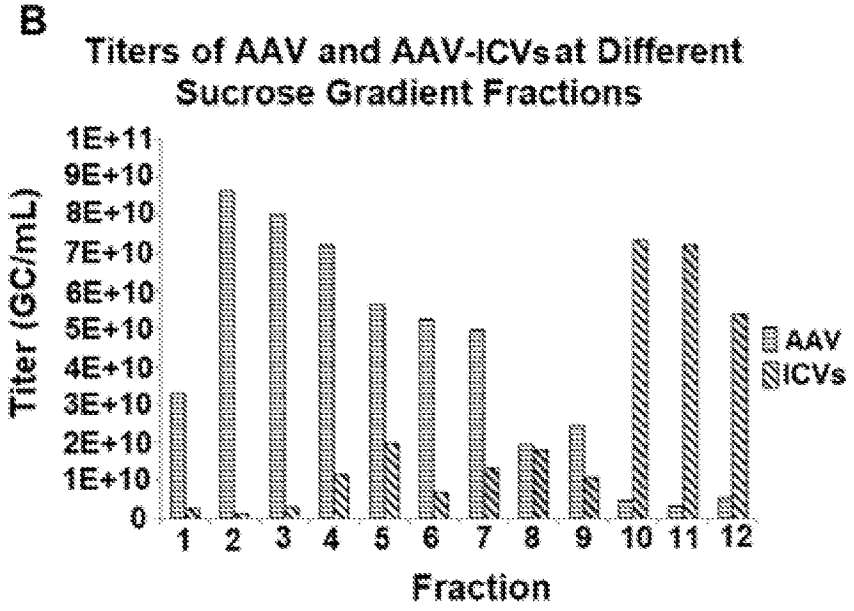

FIG. 36B

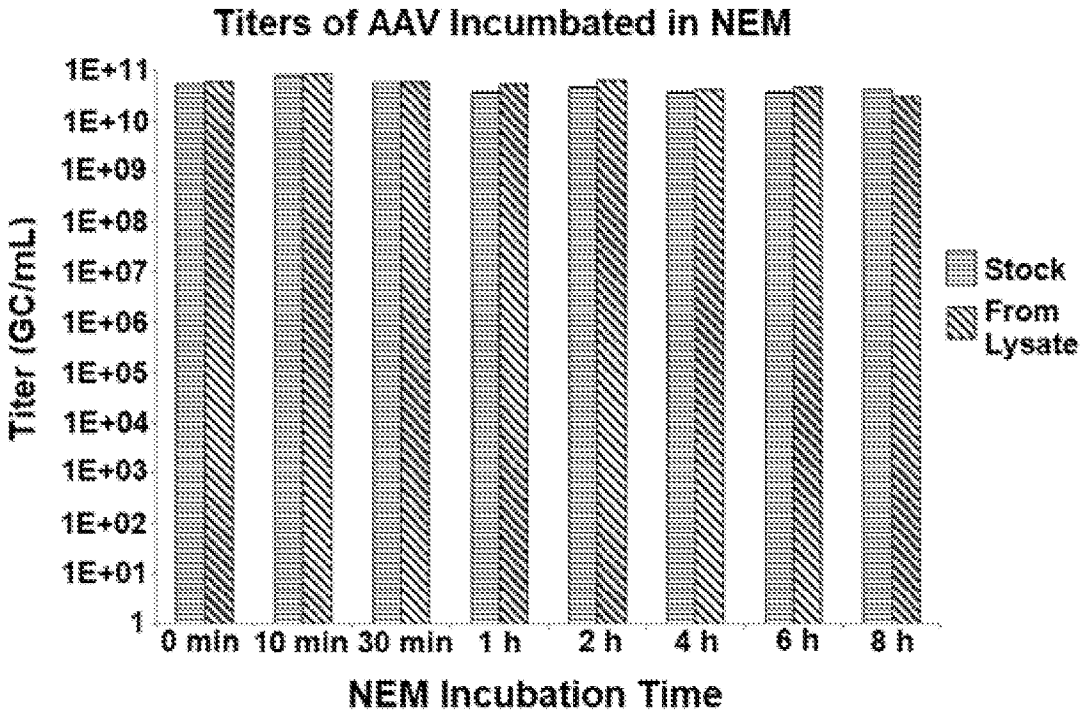

FIG. 37

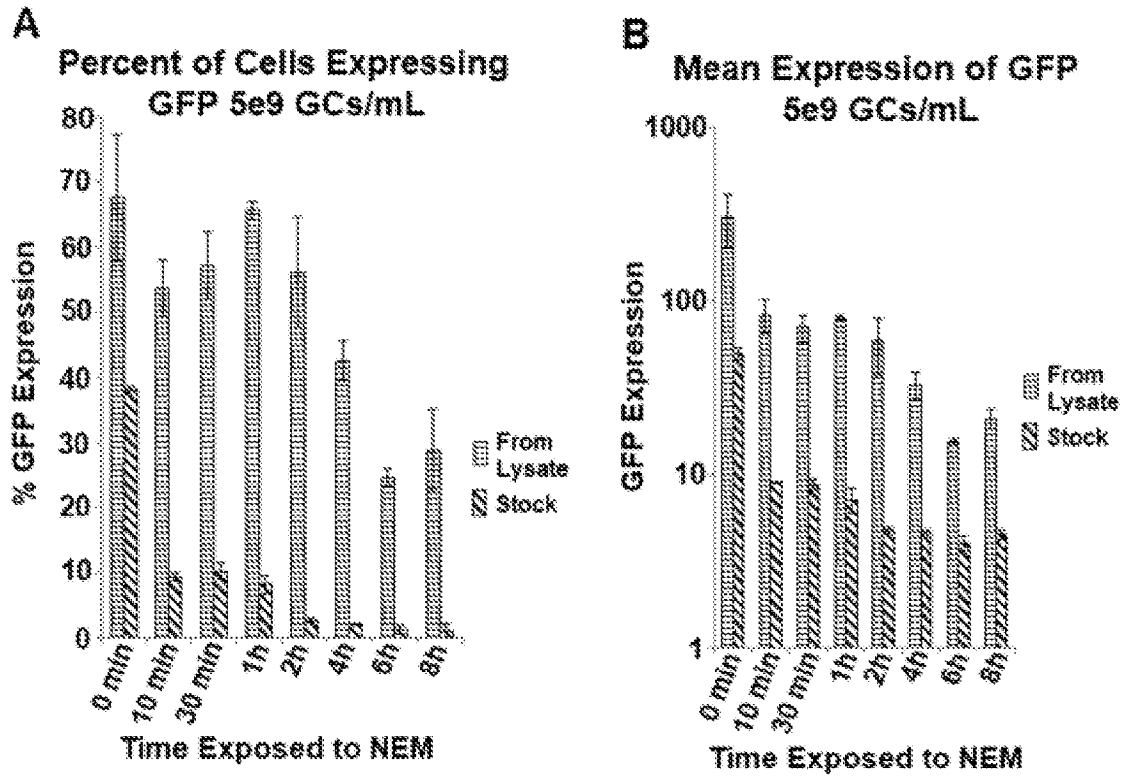

FIG. 38A-B

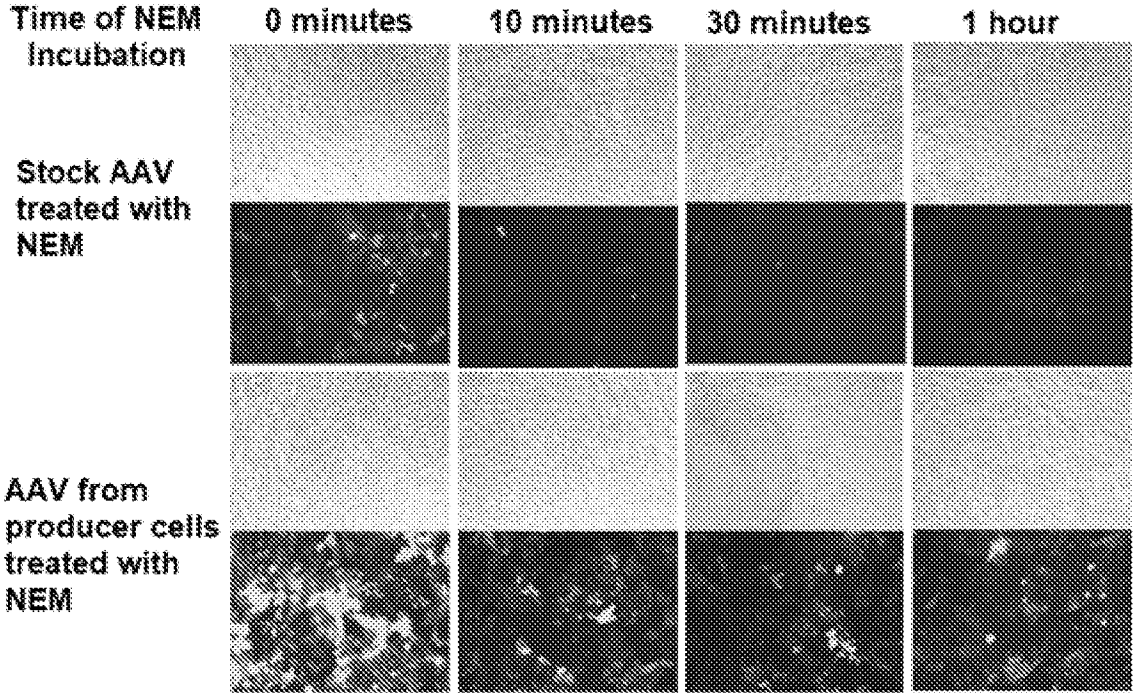

FIG. 39

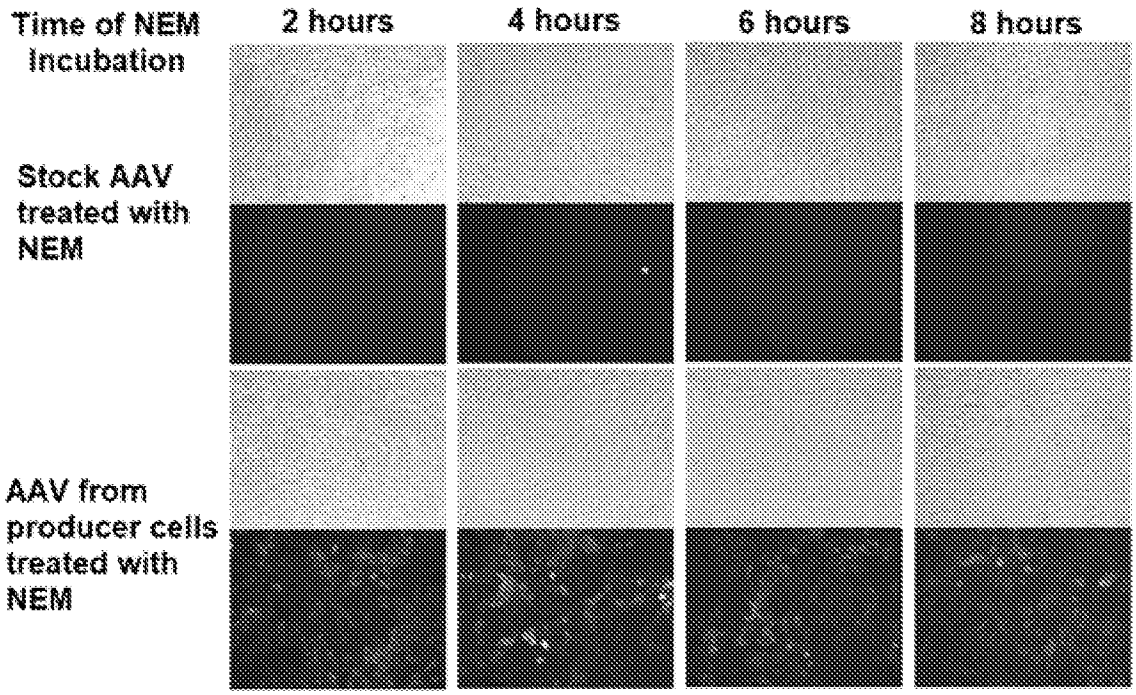

FIG. 40

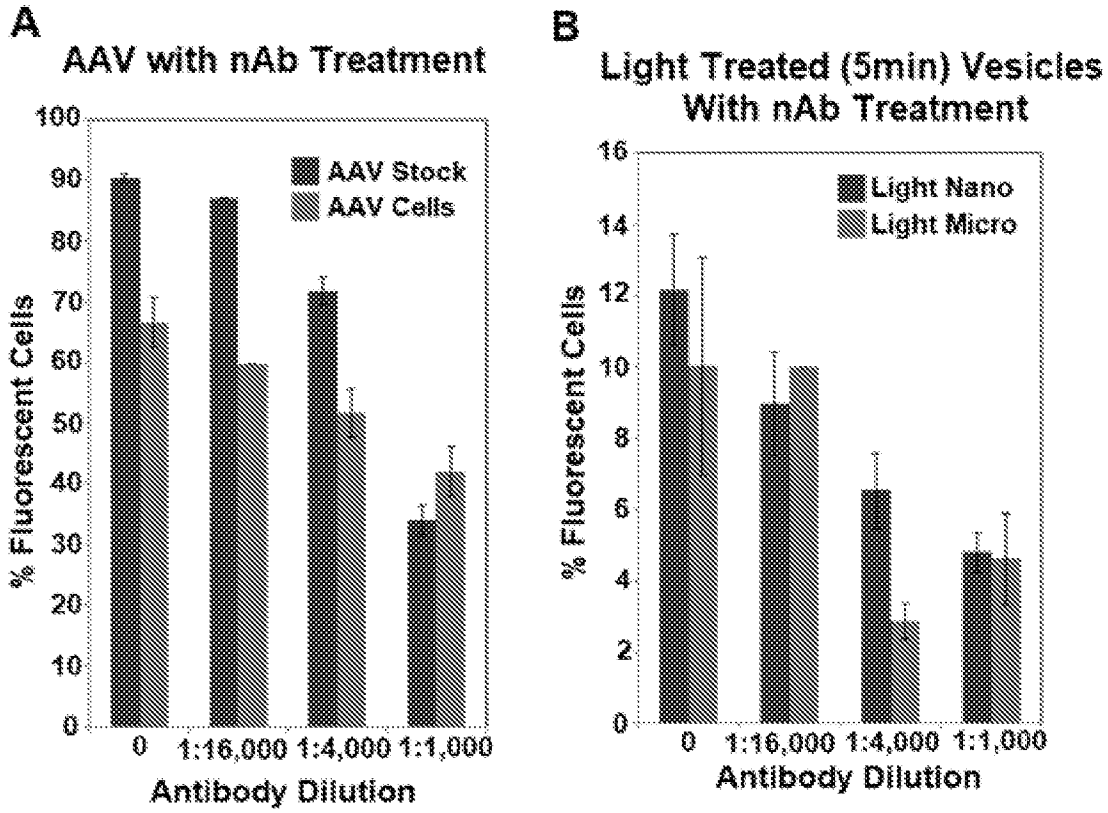

FIG. 41A-B

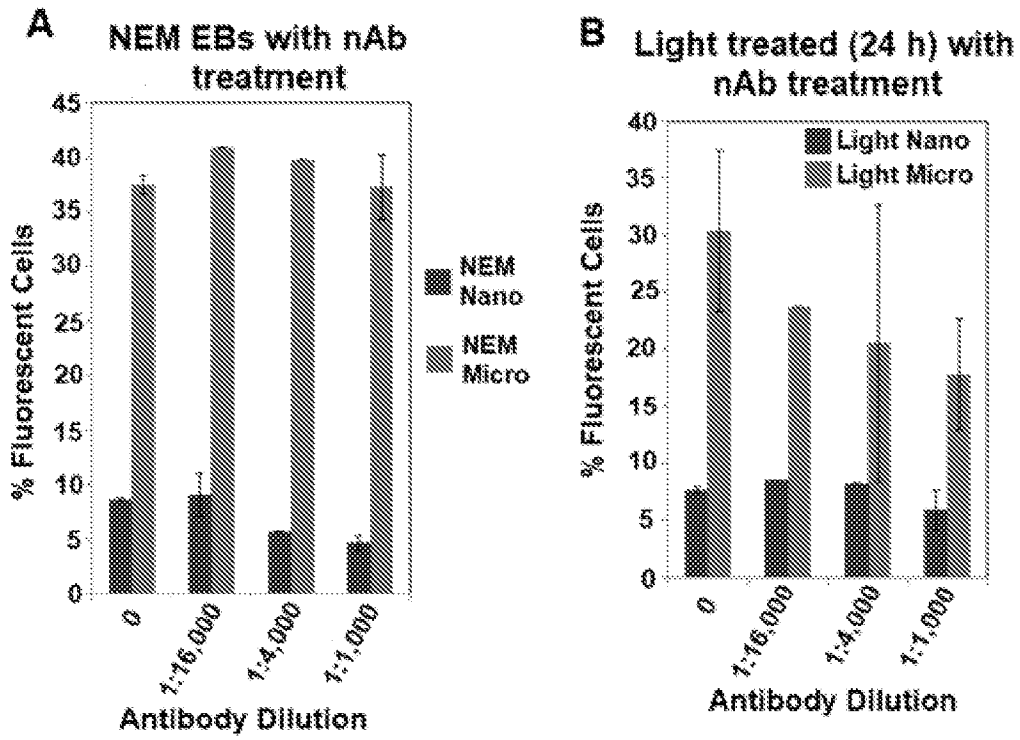

FIG. 42A-B

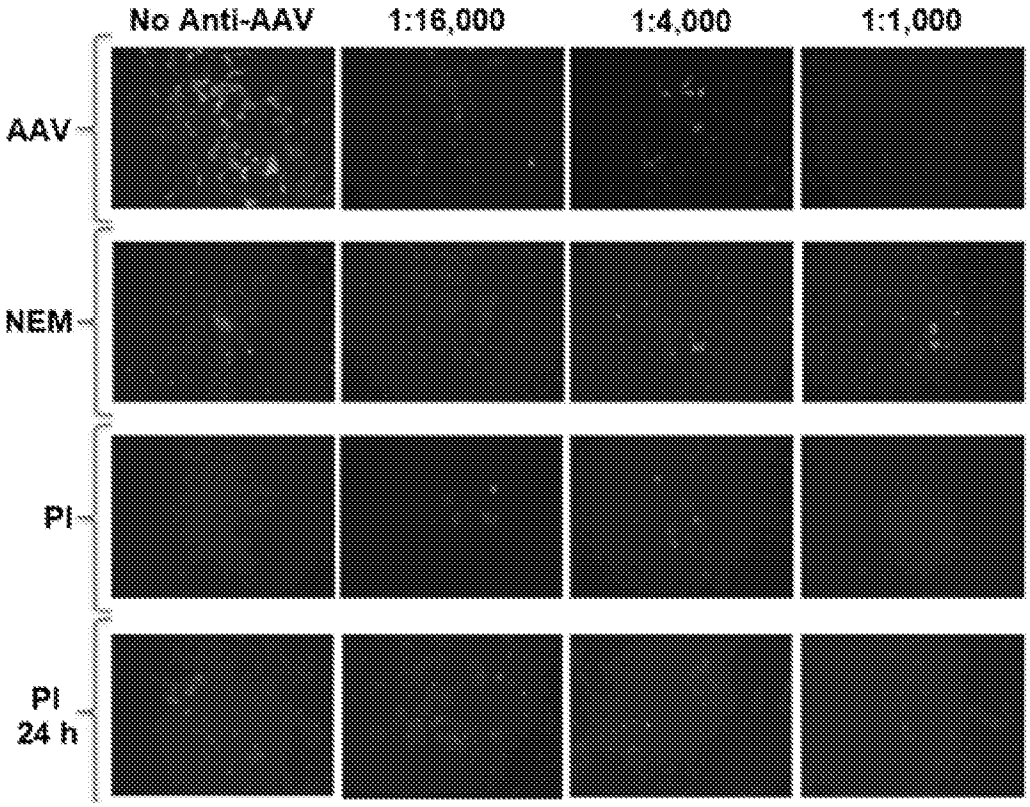

FIG. 43

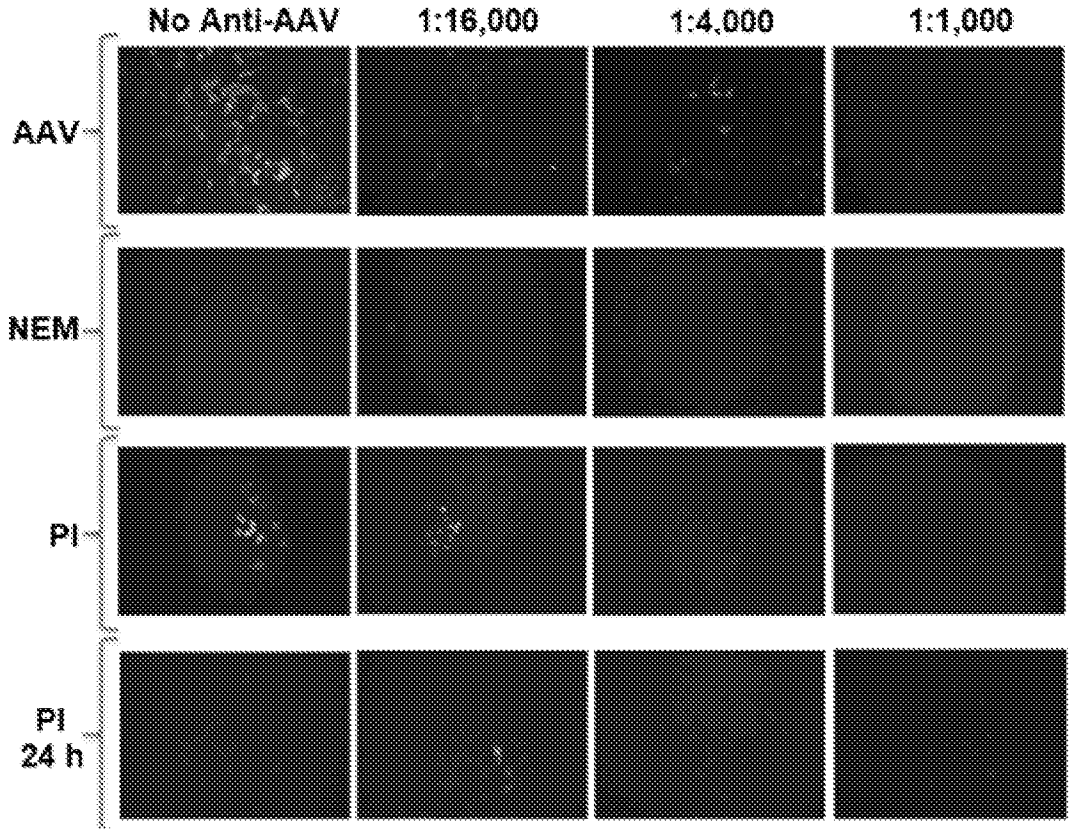
No Anti-AAV
1:16,000
1:4,000
1:1,000
AAV
NEM
PI
PI
24 h

FIG. 44

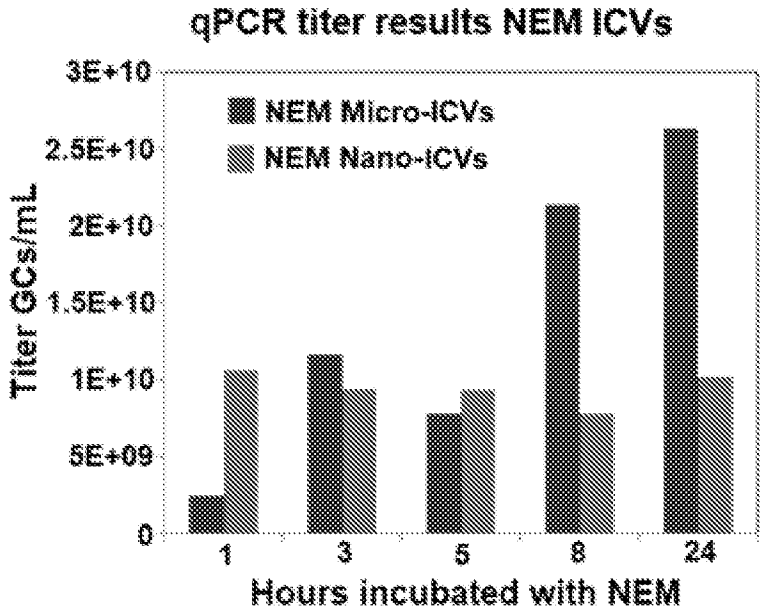
qPCR titer results NEM ICVs
NEM Micro-ICVs
NEM Nano-ICVs
Titer GCs/mL
3E+10
2.5E+10
2E+10
1.5E+10
1E+10
5E+09
0
1
3
5
8
24
Hours incubated with NEM

FIG. 45

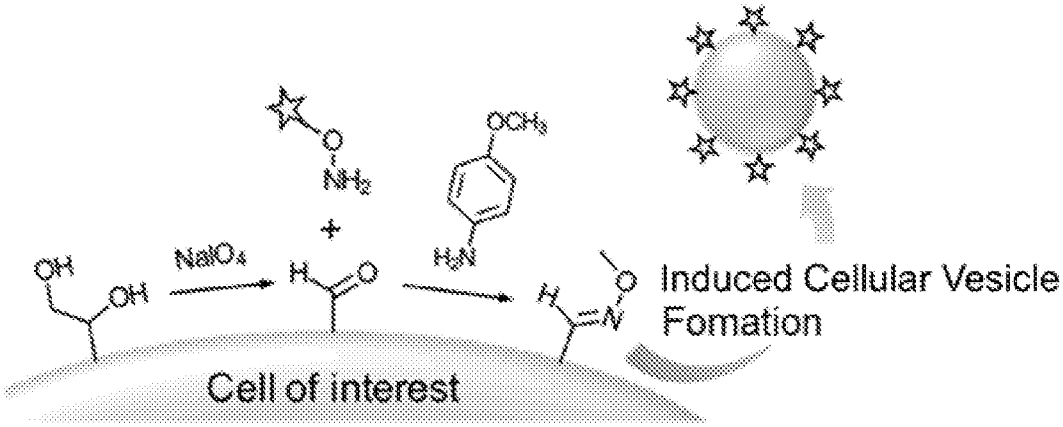

FIG. 46

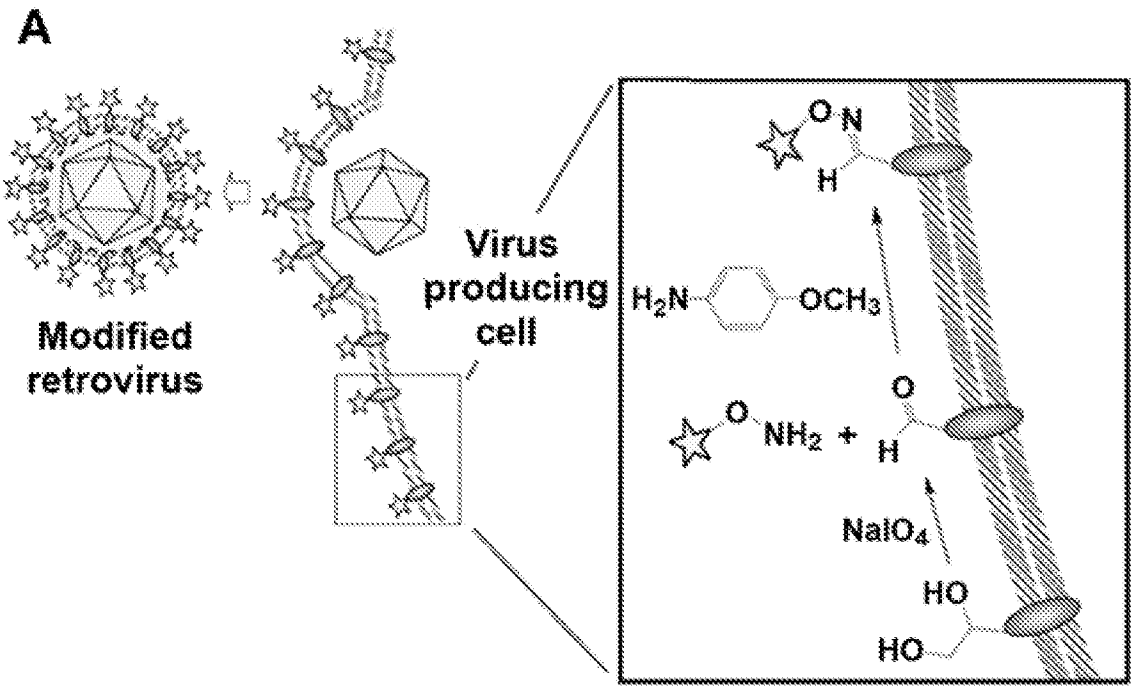

FIG. 47A

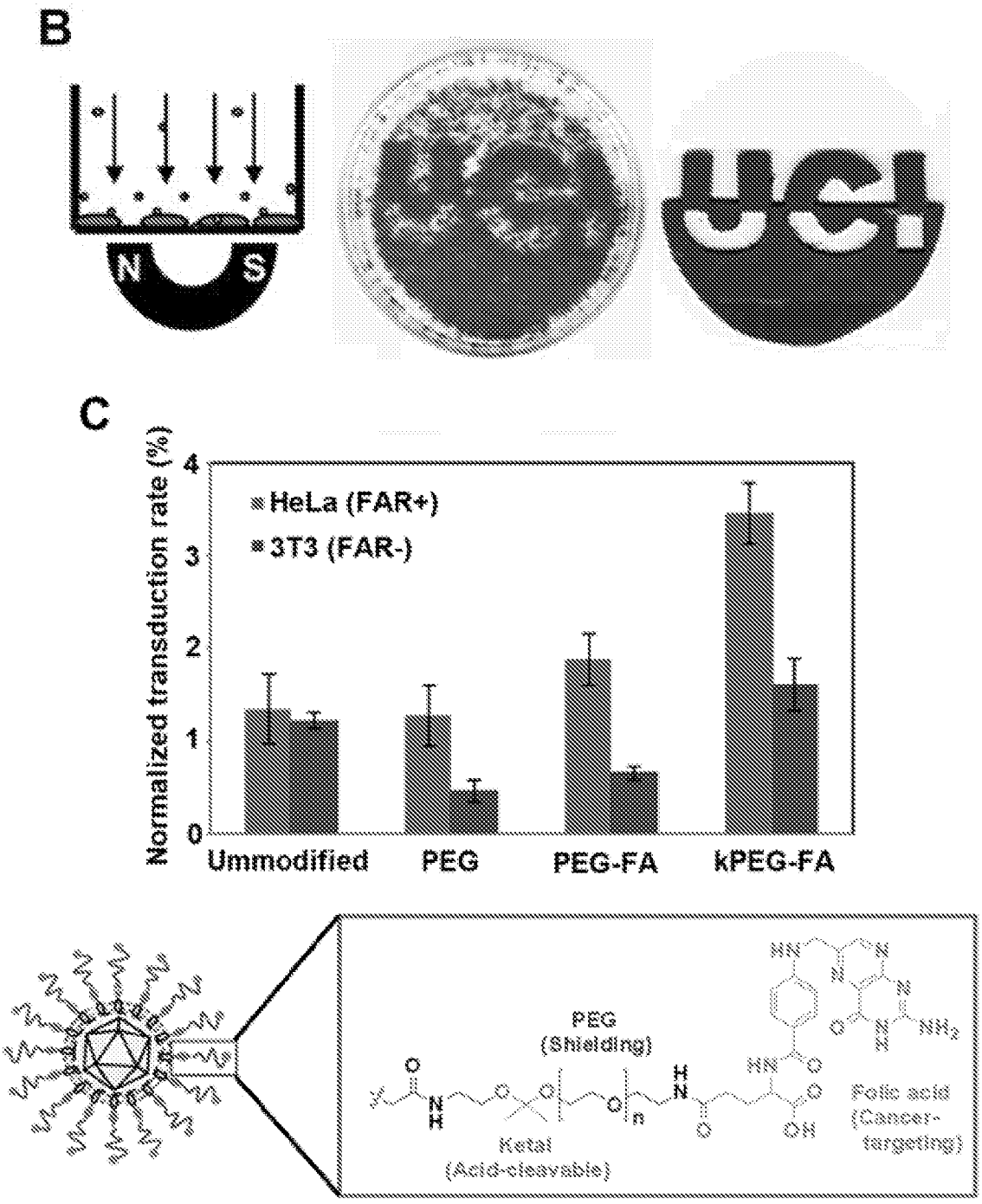
B
N
S
C
Normalized transduction rate (%)
0
1
2
3
4
HeLa (FAR+)
3T3 (FAR-)
Ummodified
PEG
PEG-FA
kPEG-FA
PEG
(Shielding)
Ketal
(Acid-cleavable)
Folic acid
(Cancer-
targeting)

FIG. 47B-C

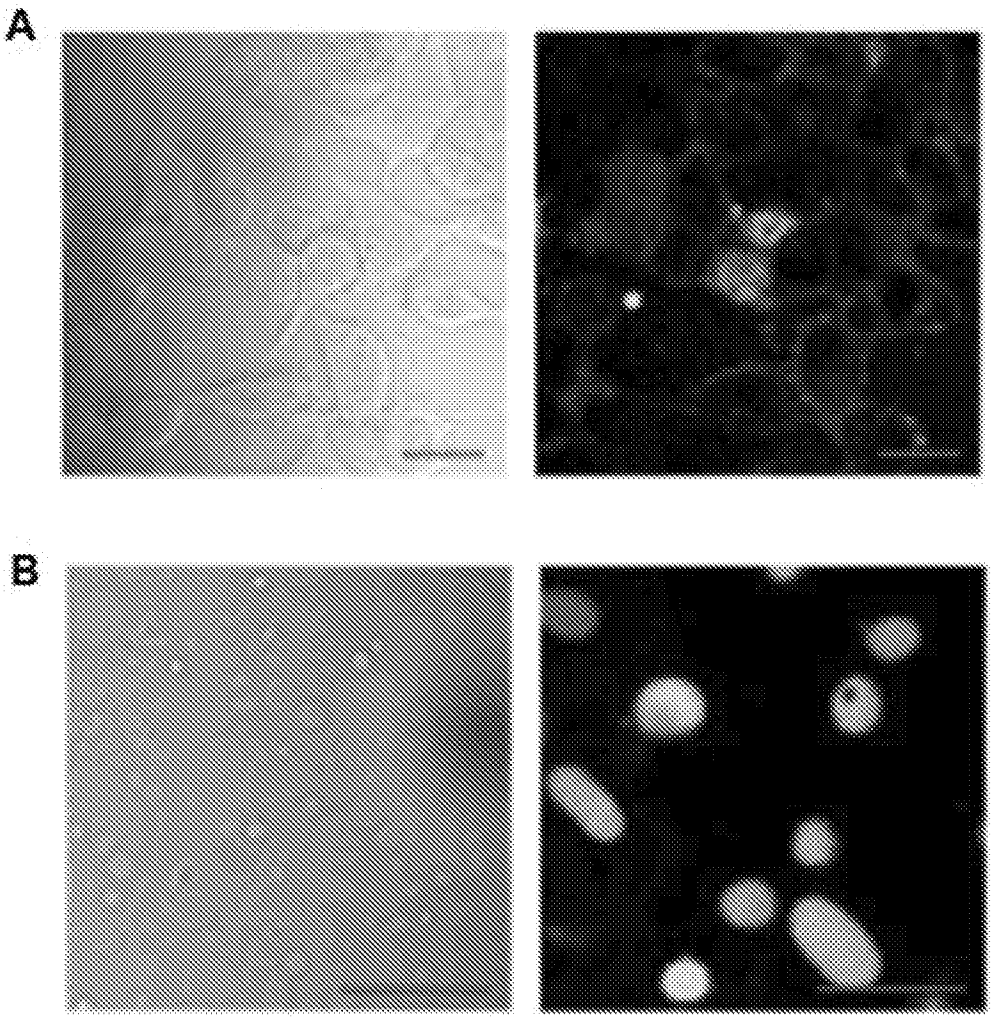

FIG. 48A-B

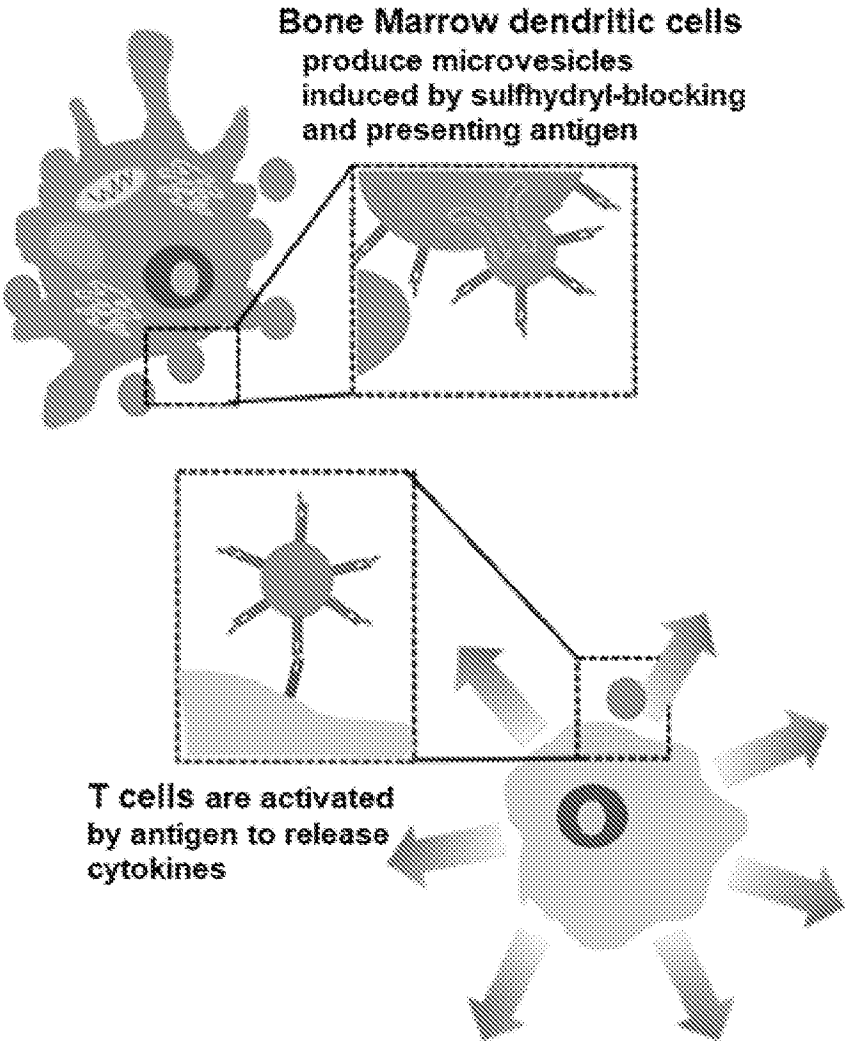

FIG. 49

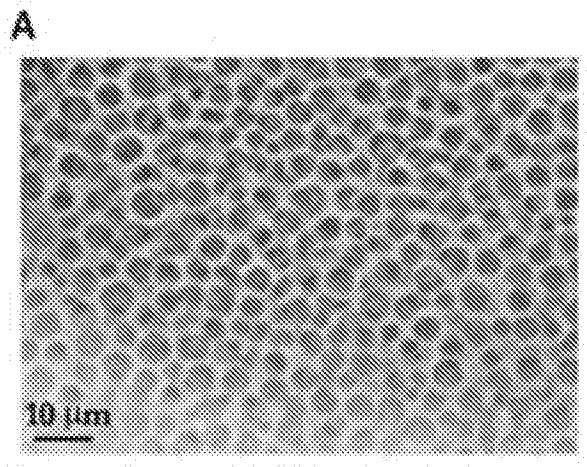

FIG. 50A

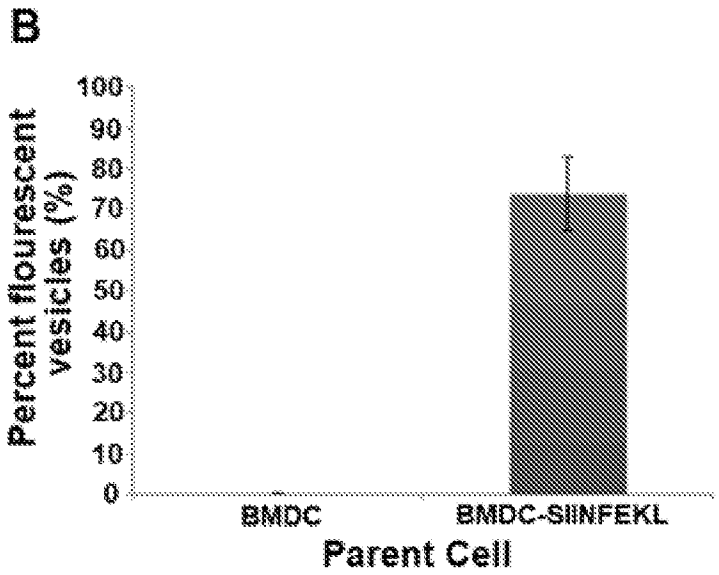

FIG. 50B

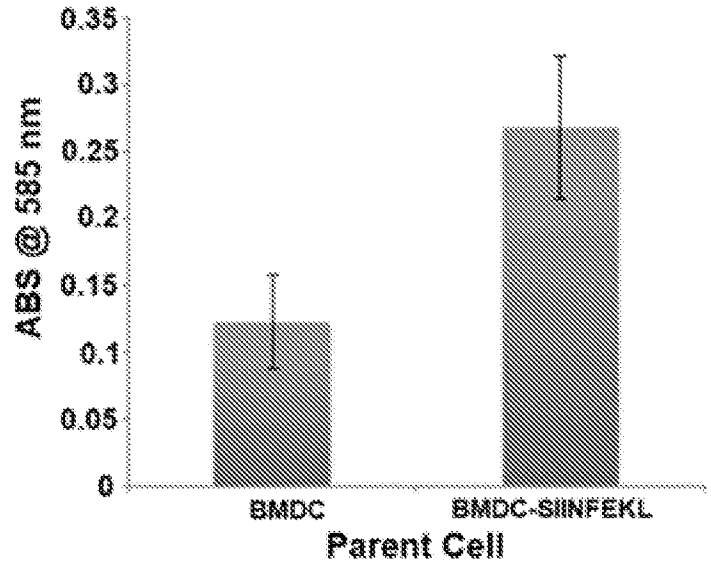

FIG. 51

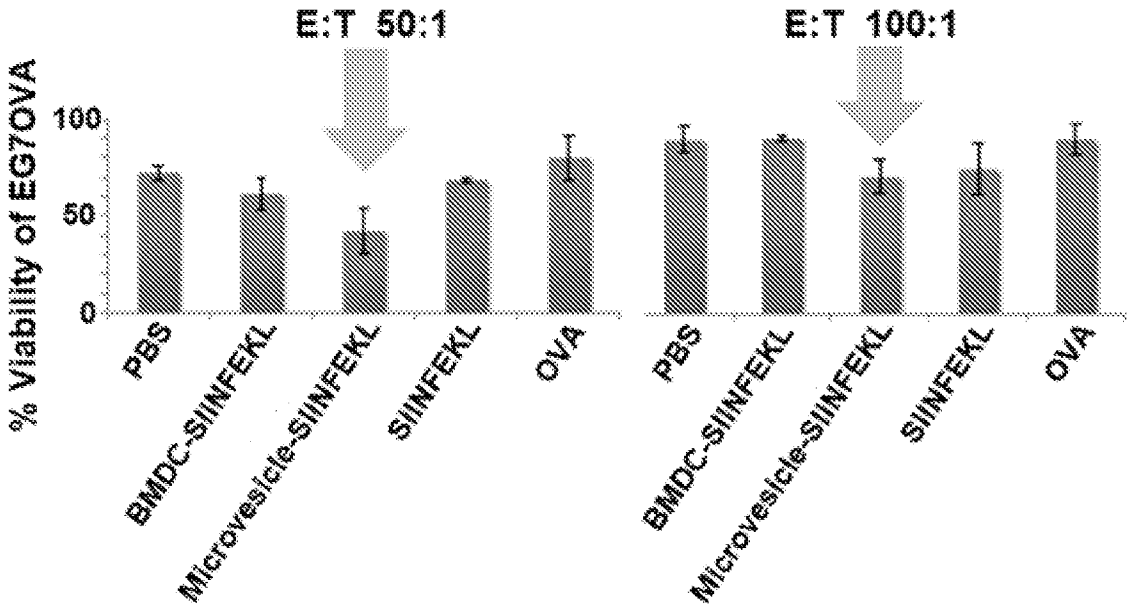
E:T 50:1
E:T 100:1
% Viability of EG7OVA
100
50
0
PBS
BMDC-SIINFEKL
Microvesicle-SIINFEKL
SIINFEKL
OVA
PBS
BMDC-SIINFEKL
Microvesicle-SIINFEKL
SIINFEKL
OVA

FIG. 52

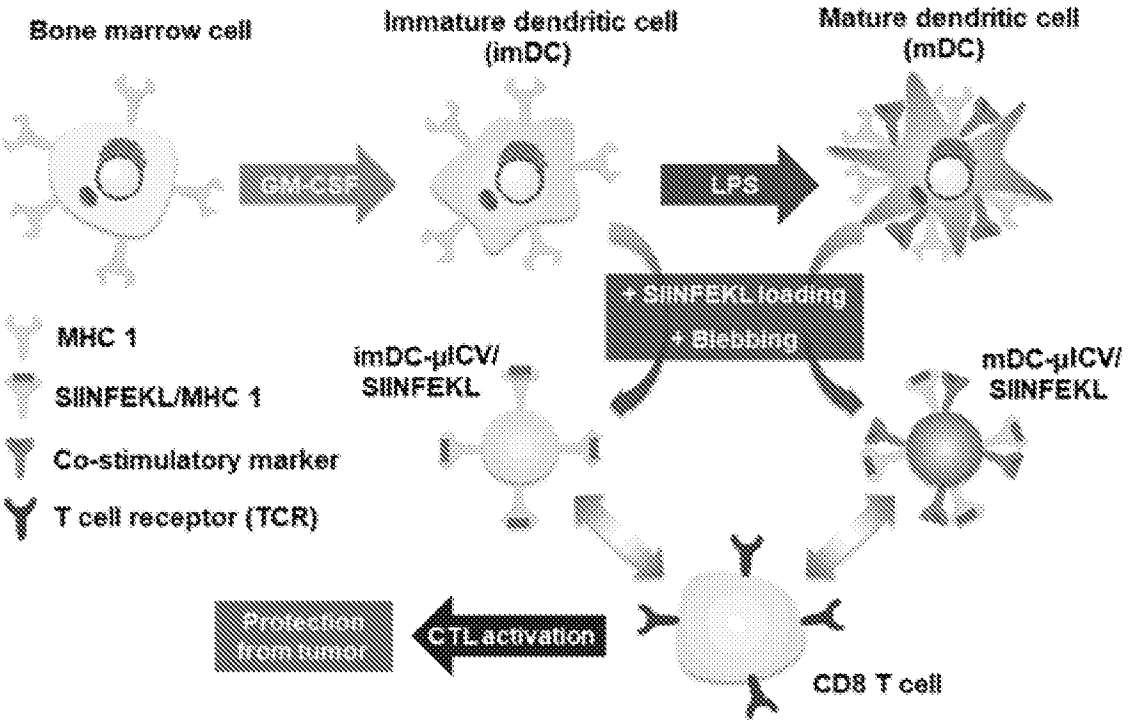
Bone marrow cell
Immature dendritic cell (imDC)
Mature dendritic cell (mDC)
GM-CSF
LPS
+ SIINFEKL loading
+ Blebbing
MHC 1
SIINFEKL/MHC 1
Co-stimulatory marker
T cell receptor (TCR)
imDC-μICV/ SIINFEKL
mDC-μICV/ SIINFEKL
Protection from tumor
CTL activation
CD8 T cell

FIG. 53

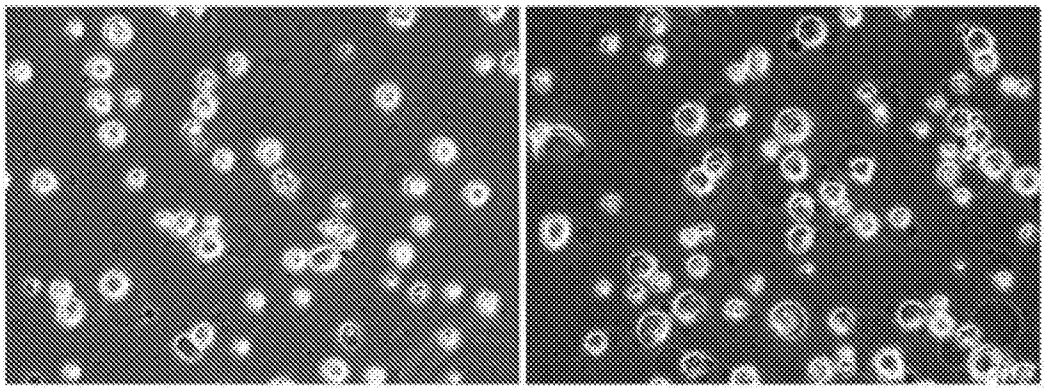

FIG. 54

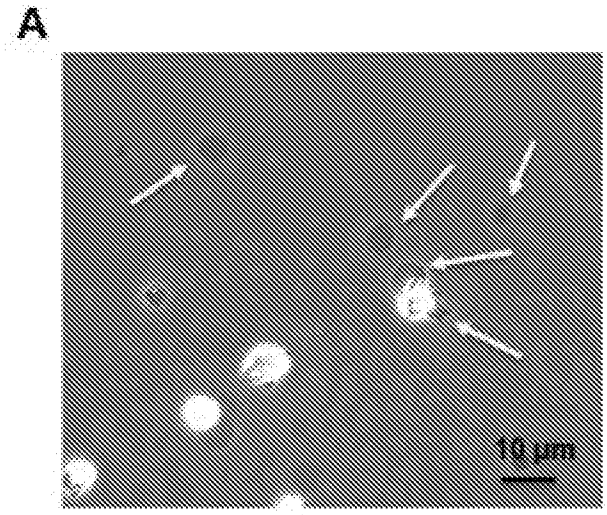

FIG. 55A

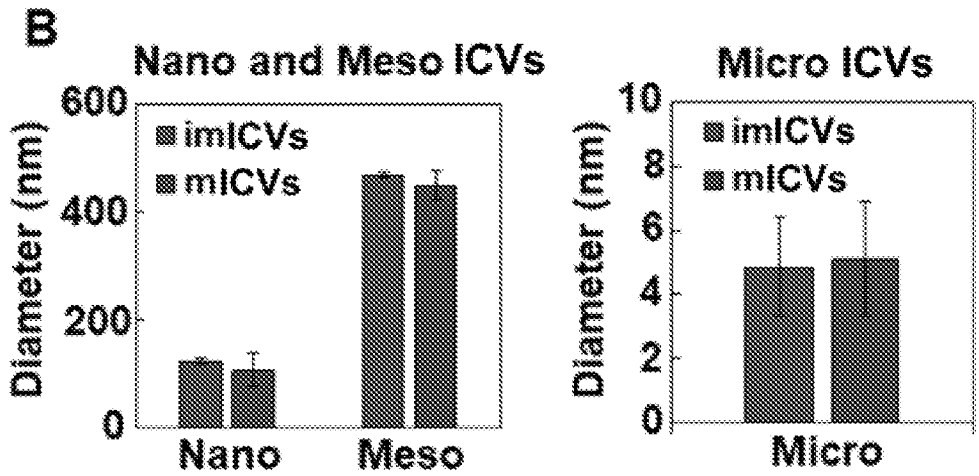

FIG. 55B

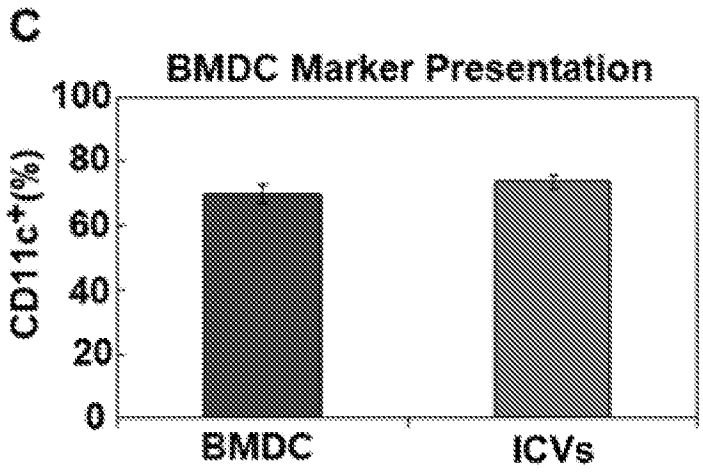

FIG. 55C

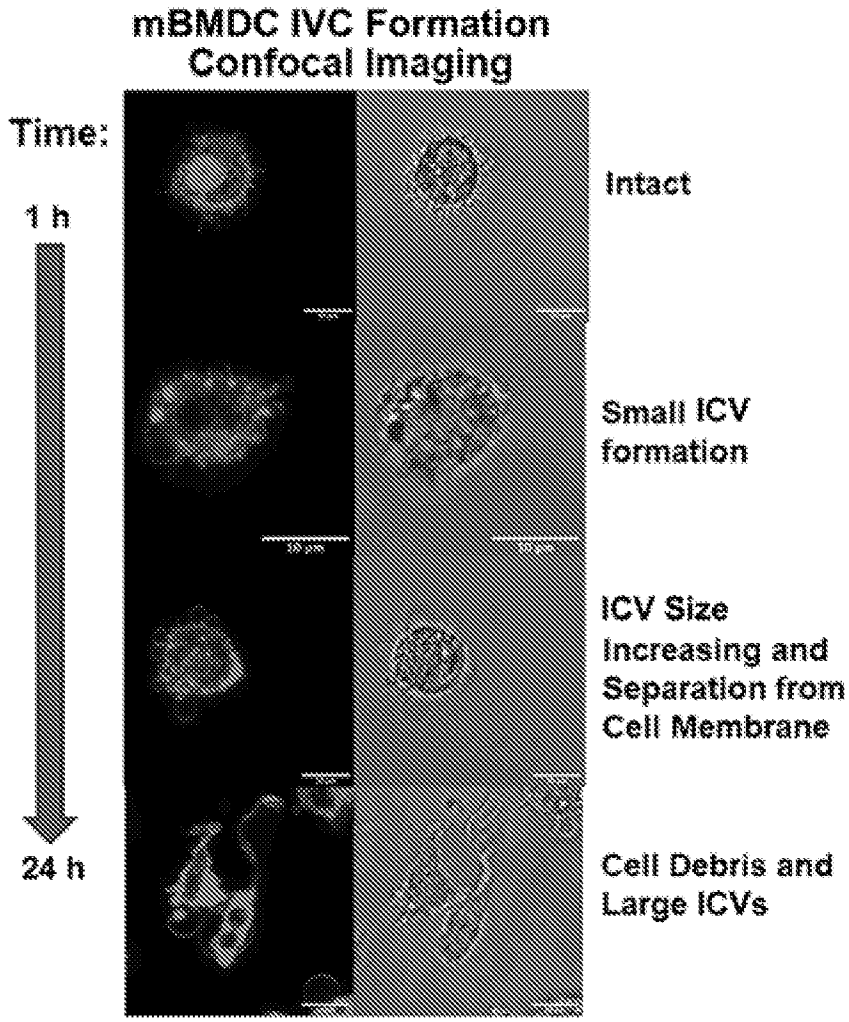

FIG. 56

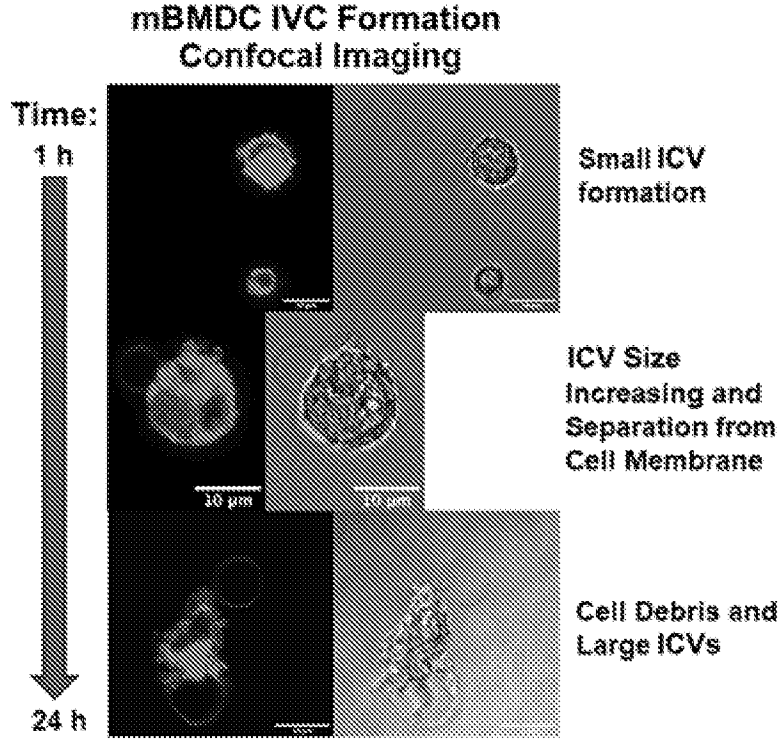

FIG. 57

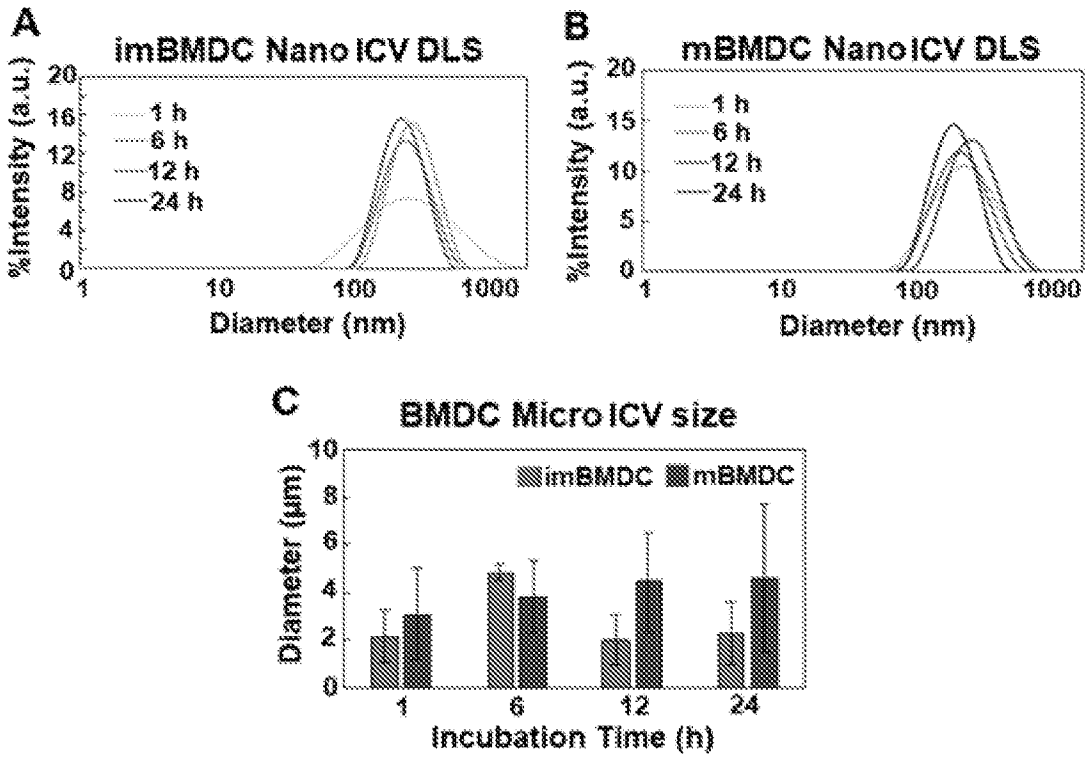

FIG. 58A-C

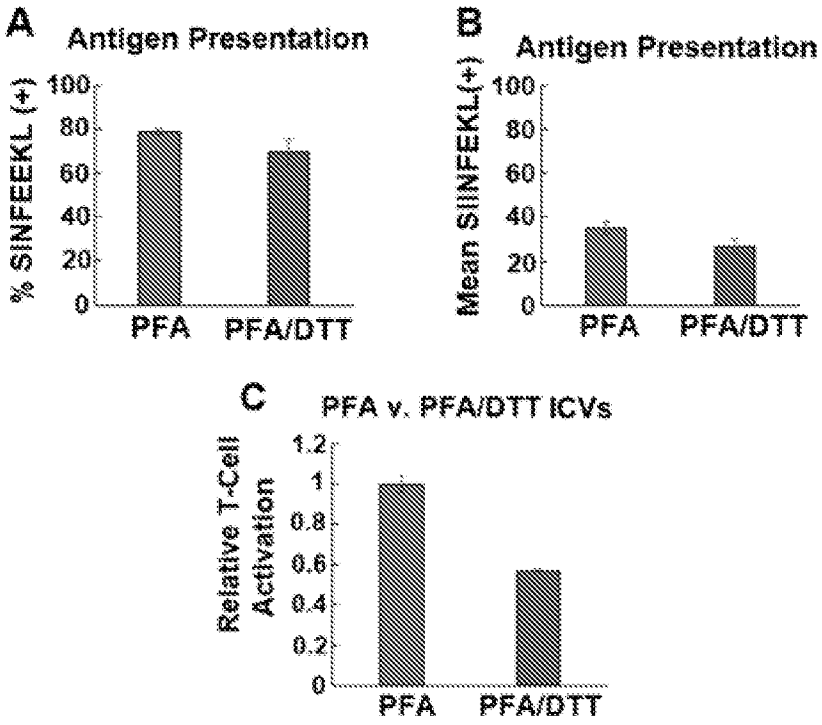

FIG. 59A-C

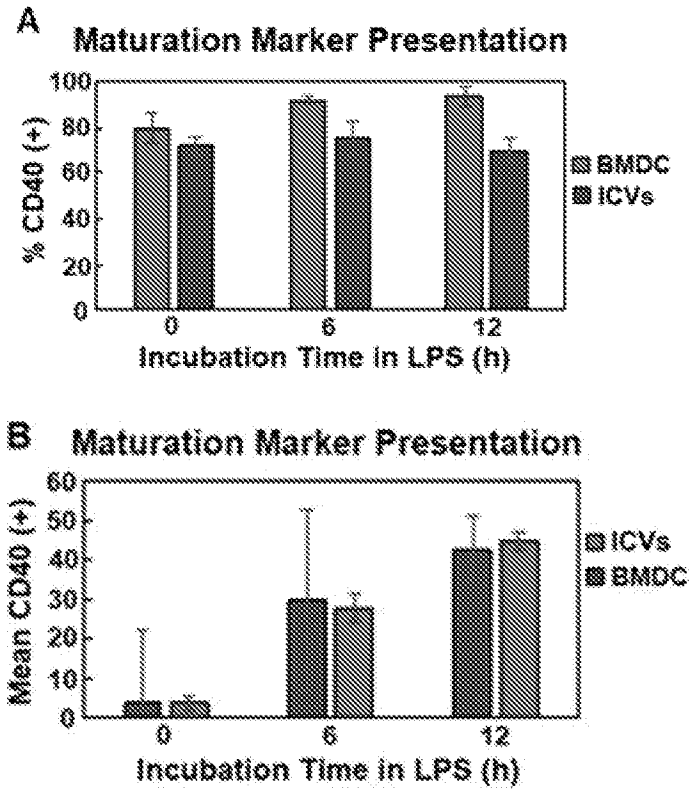

FIG. 60A-B

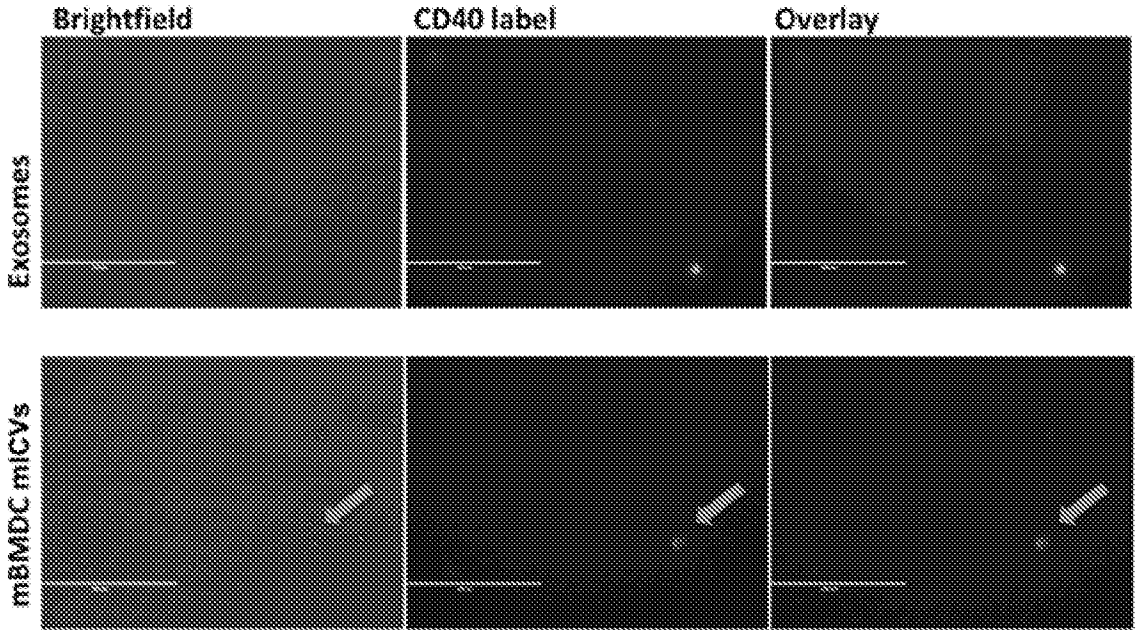

FIG. 61

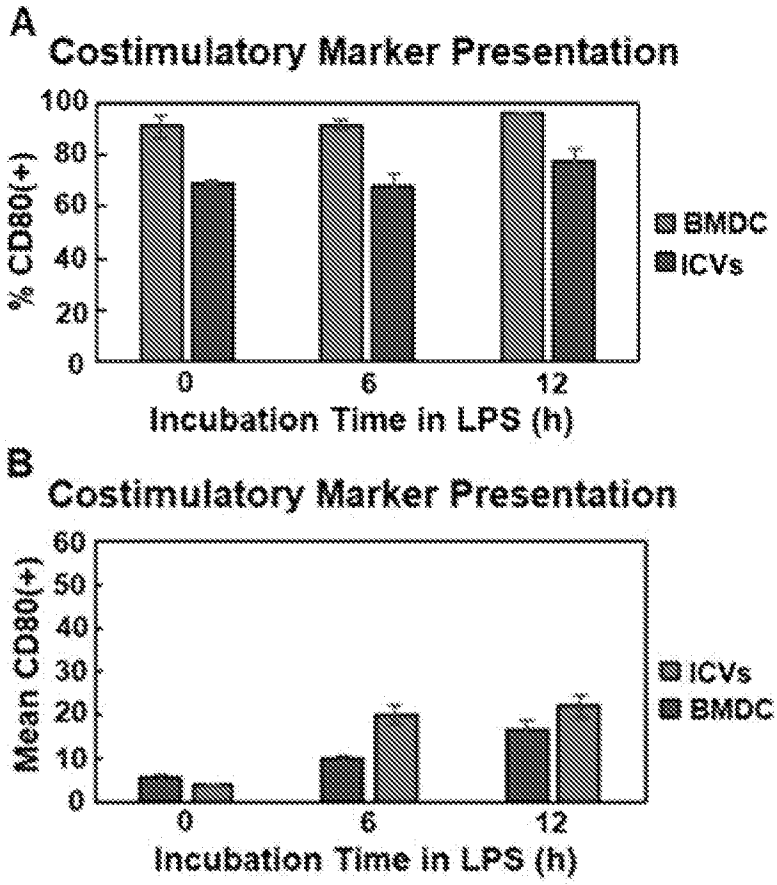

FIG. 62A-B

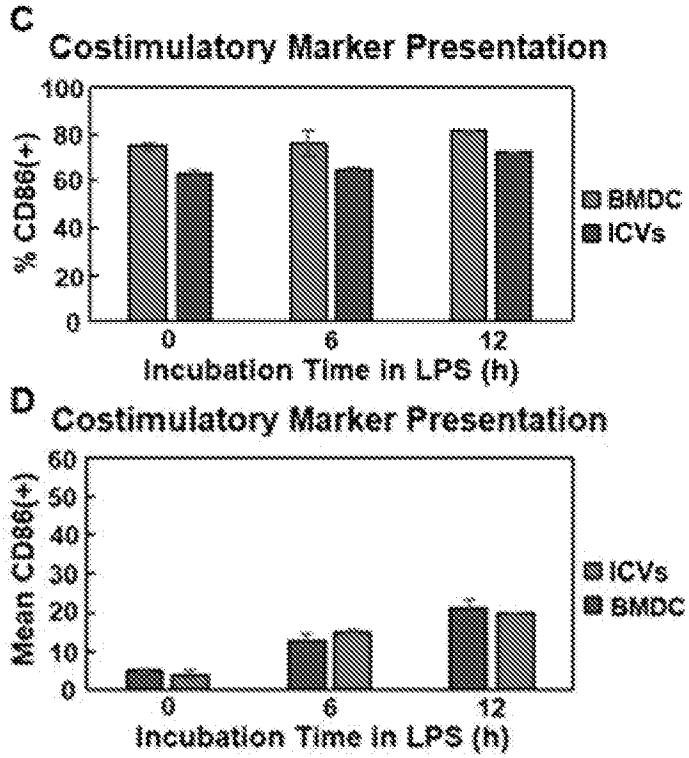

FIG. 62C-D

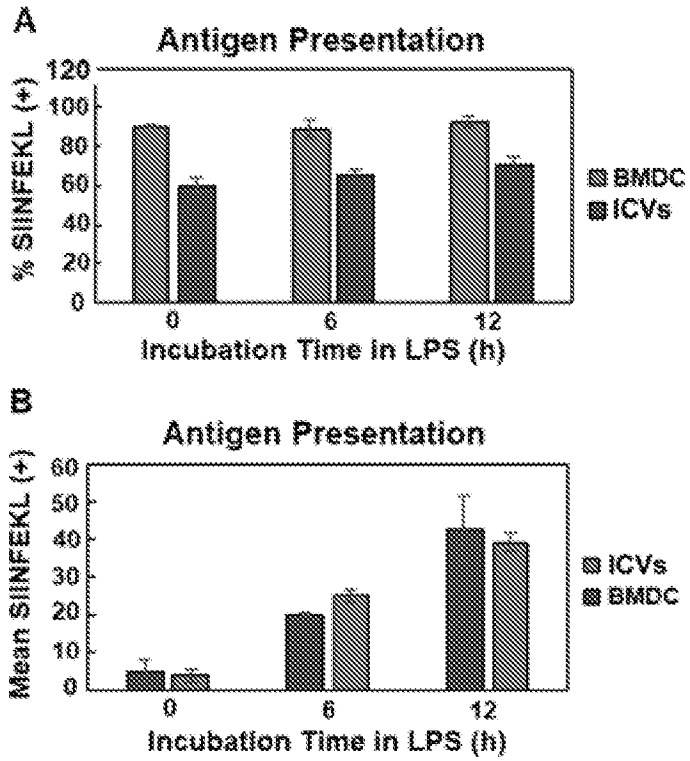

FIG. 63A-B

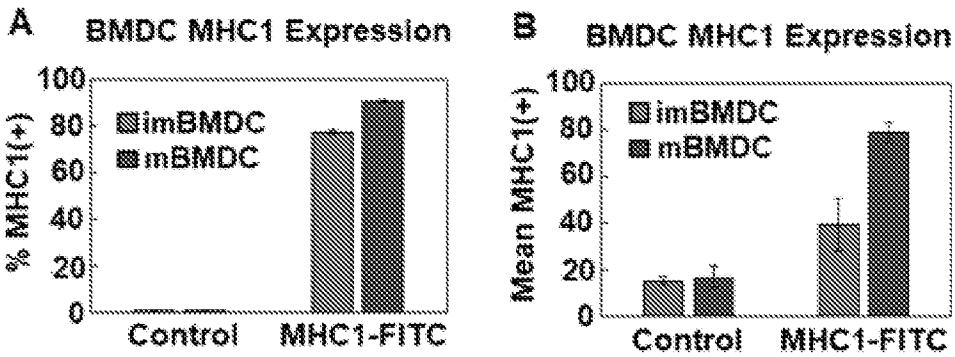

FIG. 64A-B

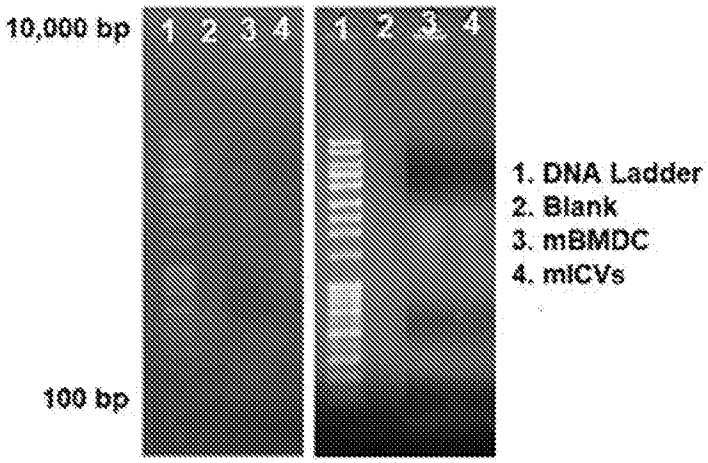

FIG. 65

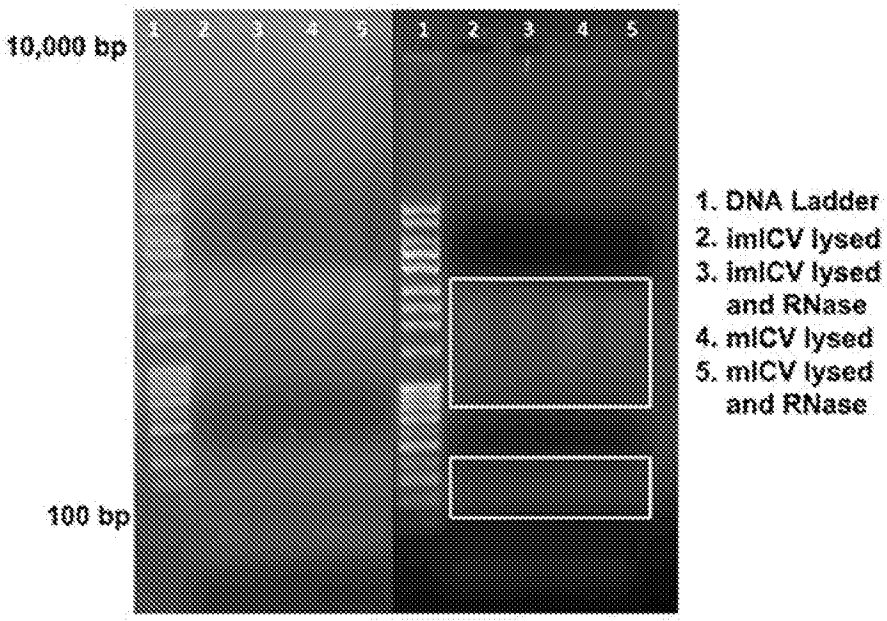

FIG. 66

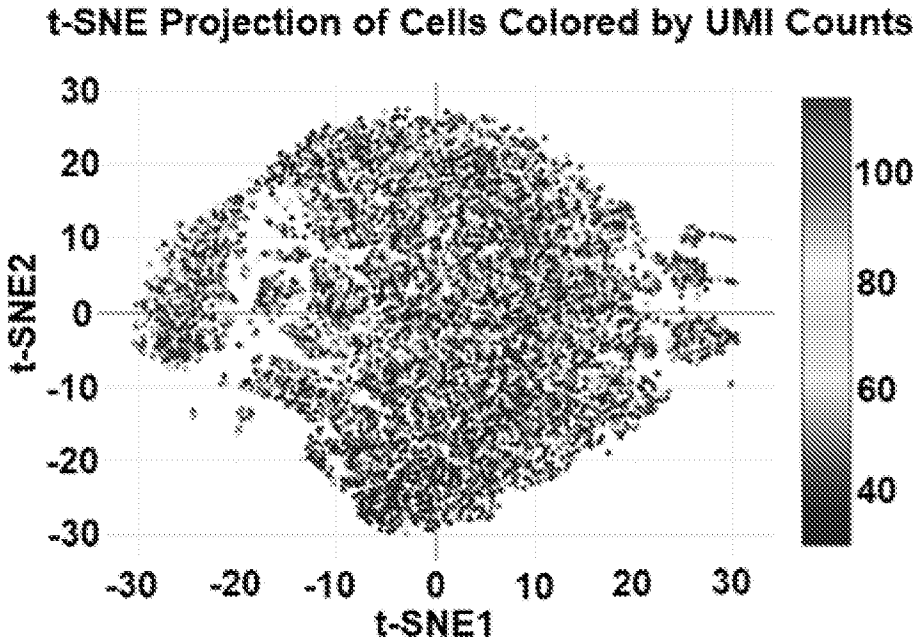
t-SNE Projection of Cells Colored by UMI Counts
30
20
10
0
-10
-20
-30
t-SNE2
-30 -20 -10 0 10 20 30
t-SNE1
100
80
60
40

FIG. 67

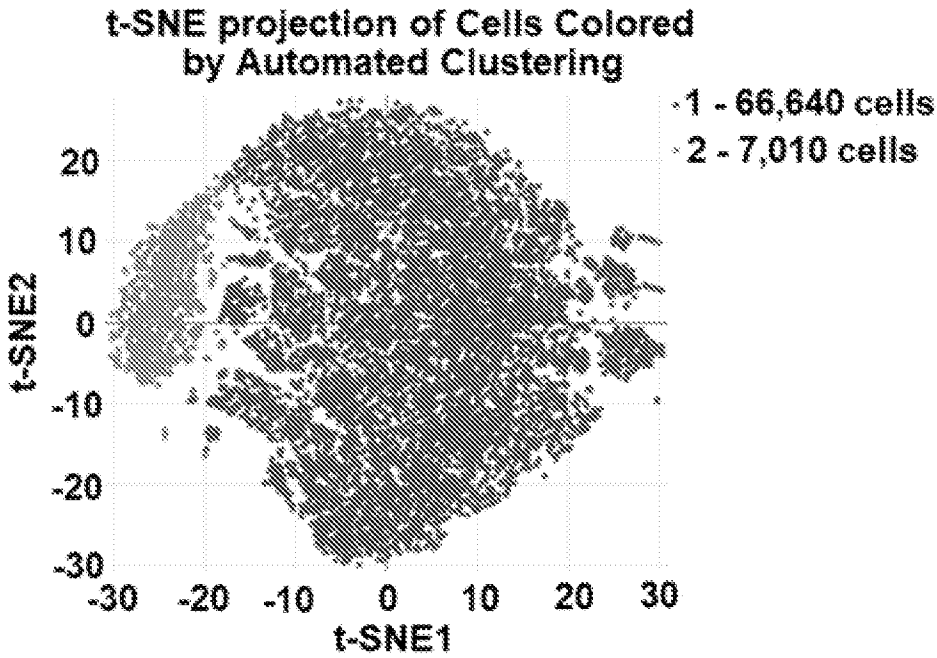
t-SNE projection of Cells Colored by Automated Clustering
1 - 66,640 cells
2 - 7,010 cells
20
10
0
-10
-20
-30
t-SNE2
-30 -20 -10 0 10 20 30
t-SNE1

FIG. 68

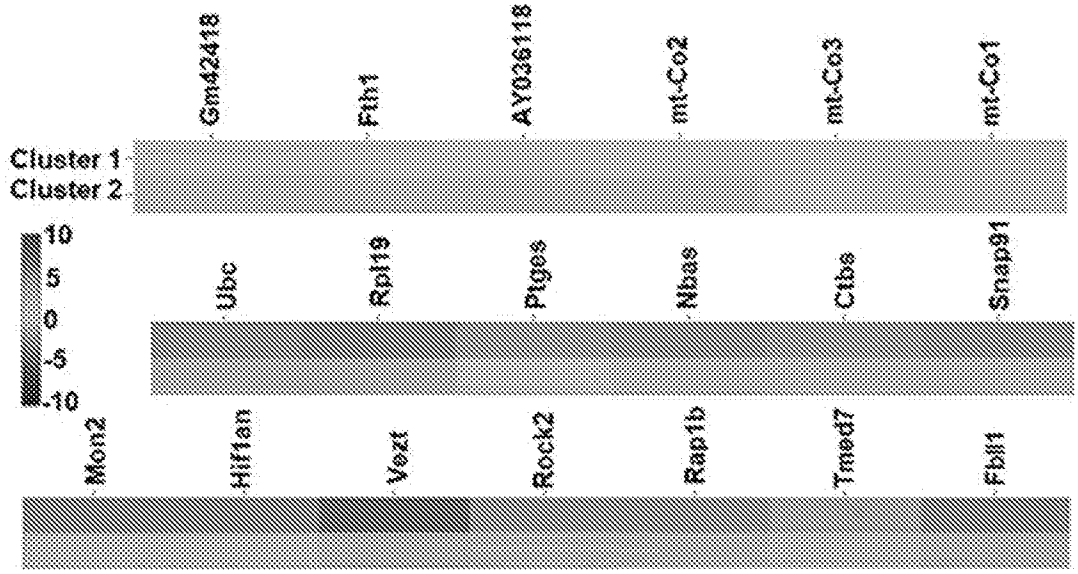

FIG. 69

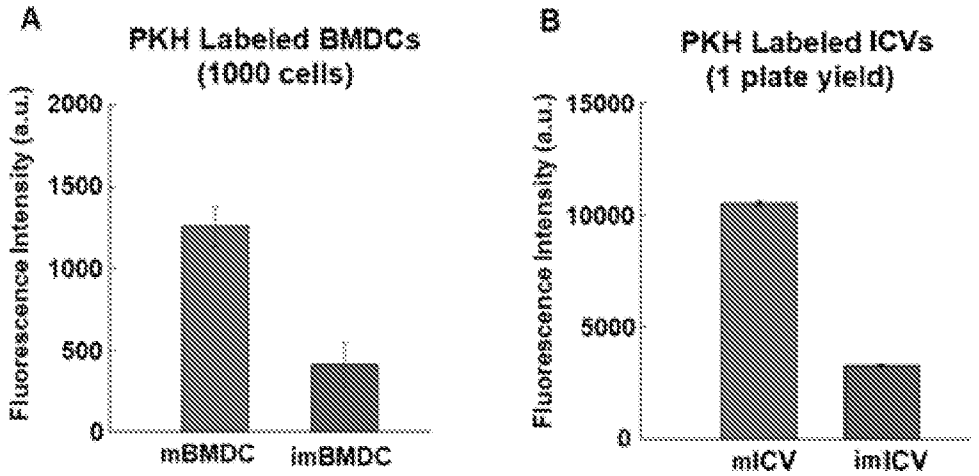

FIG. 70A-B

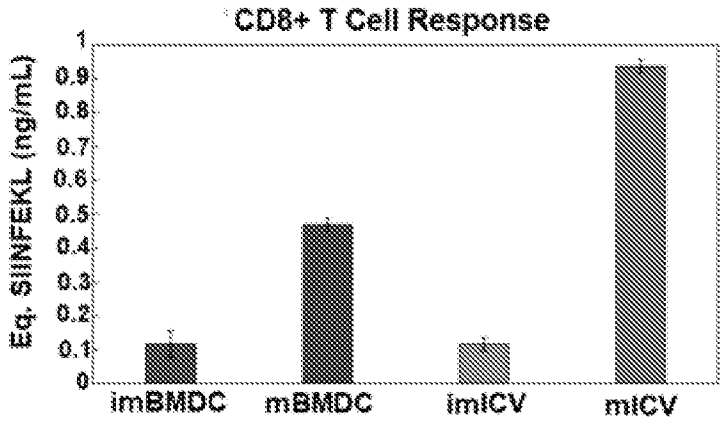

FIG. 71

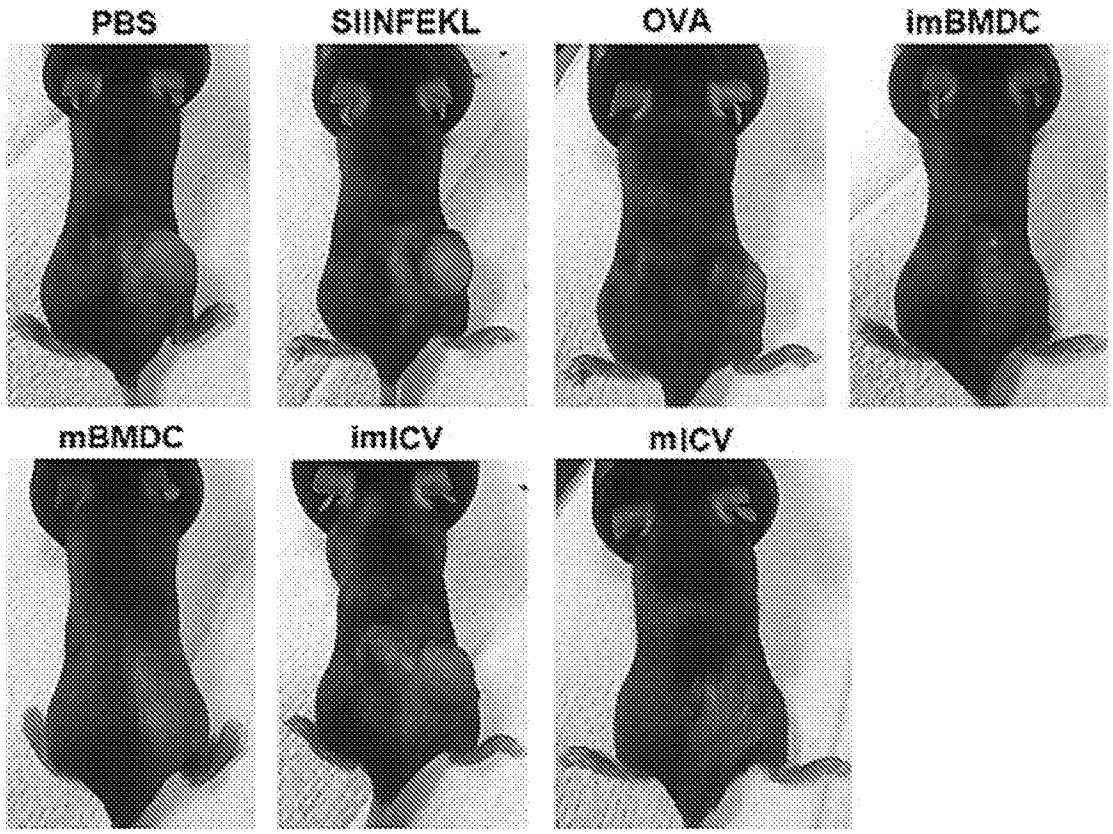

FIG. 72

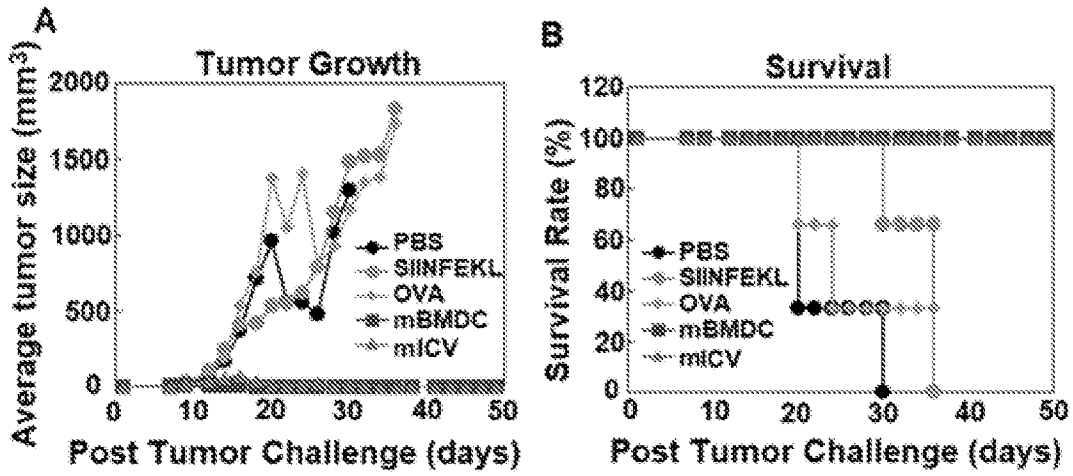

FIG. 73A-B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/46968

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 5/0781, 5/0783, 5/0784; A61N 5/067; C07K 16/28 (2019.01)

CPC - C12N 5/0635, 5/0636, 5/0637, 5/0638, 5/0639, 5/064; A61N 5/062; A61K 41/0057, 41/0071; C07K 16/28

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)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2018/102608 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 07 June 2018; paragraphs [0006], [0019]-[0020], [0077], [0080], [0082]-[0083], [0107], [0114], [0122]-[0123], [0125], [0139], [0141]-[0142], [0145], [0148], [0182]; claims 1, 6, 10-11, 19, 24-25, 27-33, 38; figure 13B	1-9, 15-21, 25-26, 43-52, 67-69, 70/67, 70/69, 71/70/67, 71/70/69, 72/71/70/67, 72/71/70/69, 73/71/70/67, 73/71/70/69, 74/71/70/67, 74/71/70/69, 75/70/67, 75/70/69, 76-85, 86/70/67, 86/70/69, 87/86/70/67, 87/86/70/69, 88/70/67, 88/70/69, 90/70/67, 90/70/69 --- 10-14, 22-24, 27-42, 53-66, 70/66, 71/70/66, 72/71/70/66, 73/71/70/66, 74/71/70/66, 75/70/66, 86/70/66, 87/86/70/66, 88/70/66, 89/88/70/66-67, 89/88/70/69, 90/70/66
Y	WO 1997/03703 A1 (RHONE-POULENC RORER PHARMACEUTICALS INC.) 06 February 1997; page 7, lines 2-7; page 63, line 39 – page 64, line 2	10-13

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"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
11 October 2019 (11.10.2019)	29 OCT 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300

Form PCT/ISA/210 (second sheet) (July 2019)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/46968

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2007/0274953 A1 (BERG, K et al.) 29 November 2007; figure 3; paragraphs [0070], [0129], [0144], [0146]-[0147]; claims 6, 11	14, 27-31, 34-41
Y	(SCOTT, RE et al.) Plasma Membrane Vesiculation in 3T3 and SV3T3 Cells. Journal of Cell Science. 1979; Vol. 35; pages 229-243; abstract; page 232, paragraph 4 – page 233, paragraph 1; table 1	22-24
Y	(MATHEWS, MS et al.) Photochemical Internalization of Bleomycin for Glioma Treatment. Journal of Biomedical Optics. May 2012; Vol. 17, No. 5; pages 058001-1-9; page 058001-2, column 1, paragraph 4; DOI: 10.1117/1.JBO.17.5.058001	32-33, 41
Y	WO 2013/158203 A1 (LIFE TECHNOLOGIES CORPORATION) 24 October 2013; paragraph [0094]	42
Y	WO 2006/087637 A2 (HELLENIC PASTEUR INSTITUTE) 24 August 2006; claim 1	53-54
Y	(ZENG, Y et al.) High Efficiency Labeling of Glycoproteins on Living Cells. Nature Methods. 22 February 2009; Vol. 6, No. 3; pages 1-7; abstract; page 2, paragraph 4; page 3, paragraph 5; figures 1-2; DOI: 10.1038/nmeth.1305	55-66, 70/66, 71/70/66, 72/71/70/66, 73/71/70/66, 74/71/70/66, 75/70/66, 86/70/66, 87/86/70/66, 88/70/66, 89/88/70/66, 90/70/66
Y	(PARK, KD et al.) Useful Tools for Biomolecule Isolation, Detection, and Identification: Acylhydrazone-Based Cleavable Linkers. Chemistry and Biology. 31 July 2009; Vol. 16, No. 7; pages 763-772; page 765, column 1, paragraph 2; page 765, column 2, paragraph 2; DOI: 10.1016/j.chembiol.2009.06.005	61-63
Y	(DUDEK, AM et al.) Immature, Semi-Mature, and Fully Mature Dendritic Cells: Toward a DC-Cancer Cells Interface that Augments Anticancer Immunity. Frontiers in Immunology. 11 December 2013; Vol. 4, No. 438; pages 1-14; abstract; DOI: 10.3389/fimmu.2013.00438	79-80
Y	WO 2016/193422 A1 (INNOVEX THERAPEUTICS, SL et al.) 08 December 2016; claim 2	89/88/70/66-67, 89/88/70/69