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(54) **Title:** METHOD OF MAKING SYNTHETIC NANOPARTICLES FOR SPLENIC TARGETING

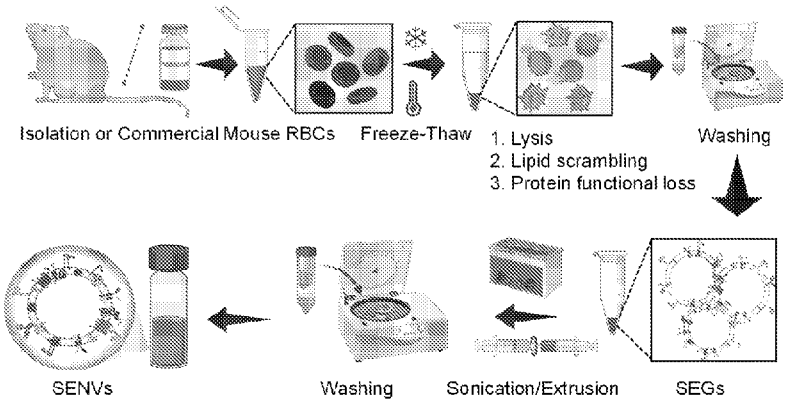

FIG. 1A

(57) **Abstract:** The present disclosure relates generally to nanovesicles that deliver therapeutic compositions to the spleen of a subject, including methods of making and methods of using thereof. For example, nanomaterials were rationally designed to exhibit splenic retention and immunomodulation for the treatment of various diseases. Significant uptake of these nanomaterials, e.g., engineered senescent erythrocyte-derived nanotheranostics (eSENTs), by immune cells of the spleen, including T and B cells, as well as monocytes and macrophages was observed. Further, when loaded with suberoylanilide hydroxamic acid (SAHA), the nanoagents exhibited a potent therapeutic effect. This approach may have far-reaching applicability for the treatment of both acute and chronic conditions where the immune responses are either stimulated or suppressed by the splenic (auto)immune milieu.

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METHOD OF MAKING SYNTHETIC NANOPARTICLES FOR SPLENIC TARGETING

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims benefit of U.S. Provisional Patent Application Serial No. 63/366,658, filed June 20, 2022, which is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

[0002] The instant application contains an electronic sequence listing. The contents of the electronic sequence listing (xml; H2695424.xml; Size: 1,999 bytes; and Date of Creation: June 12, 2023) is herein incorporated by reference in its entirety.

FIELD

[0003] The present disclosure relates generally to nanovesicles that deliver therapeutic compositions to the spleen of a subject, including methods of making and methods of using thereof. More particularly, the present disclosure relates to nanovesicles targeted to, and having increased retention in, the spleen.

BACKGROUND

[0004] The spleen is the largest secondary lymphoid organ in the body and has often been thought of as dispensable, yet it is an active regulator of the immune response.[1] The spleen is an important mediator of both adaptive and innate immunity. Given the architecture of splenic tissue, it has an increased likelihood of interactions between cells, in particular cognate lymphocytes and antigen-presenting cells.[2,3] The spleen acts as a primary remote organ that mainly responds to cardiac injury by providing various subsets of leukocytes, which modulates the immune responses by changing the expression of cytokines, growth factors, pathogen-associated molecular patterns, and other microenvironments mediators shifting macrophage polarization to a less inflammatory and more reparative phenotype.[4-7] Post-MI healing and outcome are divided into three phases – initiation, resolution, and progression. The initiation phase encompasses the initial minutes to hours or a few days of the acute inflammatory phase. The resolution phase lasts a few days to weeks and encompasses the reparative or resolving phase. Finally, the progression phase lasts weeks to months or years depending on the resolution phase, which, if defective, leads to left

ventricle dysfunction, chronic heart failure, and increased morbidity and mortality.[4] In the post-MI initiation phase, ~50% of leukocytes travel from the splenic reservoir through the circulation to the site of LV injury that generates an edematous inflammatory milieu.[8] Upon moving to injured tissue, these monocytes turn into macrophages, that not only respond to the acute inflammation but also prime the resolution phase through the production of multiple families of specialized pro-resolving mediators (SPMs).[9]

[0005] Histone deacetylases (HDACs) are evolutionarily conserved enzymes that operate by removing acetyl groups from histones and other protein regulatory factors, with functional consequences on chromatin remodeling and gene expression profiles.[7] Systemic HDAC inhibition (HDACi) via the intraperitoneal injection of suberoylanilide hydroxamic acid (SAHA), a pan-HDAC inhibitor, resulted in decreased inflammatory cytokines and Matrix metalloproteinases (MMPs), as well as earlier recruitment of reparative macrophages to the post-MI heart. Thus, systemic HDACi treatment correlates with improved ventricular function and remodeling. The precise molecular mechanism of HDACi and its relation to therapeutic cardiac remodeling is not well understood, however, targeted localization of HDACi to the post-MI spleen may be capable of modulating diseases both locally and systemically.[5]

[0006] Thus, there is a need to design nanomaterials capable of splenic retention and immunomodulation for the treatment of diseases in various organs, for example in the post-infarct heart.

[0007] The present disclosure is directed to overcoming these and other deficiencies in the art.

SUMMARY

[0008] In an aspect, provided is a method of delivering a composition to a spleen of a subject, including systemically administering the composition to the subject, wherein the composition includes a nanovesicle made by a process, the process including: (a) freezing erythrocytes, wherein freezing includes exposing the erythrocytes to a temperature of about 0 °C or lower, (b) thawing the erythrocytes, wherein thawing includes exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes, (c) sonicating the erythrocytes, wherein sonicating includes exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes, and (d) one of: (i) extruding the sonicated erythrocytes, wherein extruding includes successively

passing the sonicated erythrocytes through a series of filters each including a filter diameter, wherein each filter includes a larger filter diameter than a subsequent filter; and (ii) freeze-drying the sonicated erythrocytes to obtain a proteolipid powder, and reconstituting the proteolipid powder.

[0009] In an example, freeze-drying the sonicated erythrocytes includes mixing the sonicated erythrocytes with a cryoprotectant. In another example, reconstituting the proteolipid powder includes dissolving the proteolipid powder in an organic solvent and then mixing with an aqueous solvent.

[0010] In yet another example, one of (i) the sonicated erythrocytes are extruded in the presence of one or more added lipid; and (ii) the proteolipid powder is reconstituted in the presence of one or more added lipid. In still another example, one of (i) a ratio of weight of erythrocytes to weight of the one or more added lipid during the extruding is from about 1:24 to about 1:1; and (ii) a ratio of weight of proteolipid powder to weight of the one or more added lipid during reconstituting is from about 24:1 to about 5.3:1.

[0011] In a further example, at least one of the one or more added lipid is a natural lipid, a synthetic lipid, or a combination thereof. In yet a further example, the synthetic lipid includes a lipid attached to one or more targeting ligand. In still a further example, the one or more targeting ligand is a peptide, an antibody, or an aptamer, or a combination thereof. In an example, the one or more targeting ligand targets a cell type, wherein the cell type is selected from T cell, B cell, monocyte, macrophage, granulocyte, dendritic cell, endothelial cell, (myo)fibroblast, and cancer cell. In another example, the synthetic lipid is selected from 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol). In yet another example, the natural lipid is selected from a sphingolipid, a phosphatidylglycerol, a lysophosphatidylcholine, a phosphatidyl serine, a ceramide, an eicosanoid, a phosphoinositide, and a lysophosphatidic acid. In still another example, the process further includes encapsulating nanoparticles with the nanovesicles, wherein encapsulating includes one of (i) sonicating the nanovesicles in the presence of the nanoparticles and sonicating includes exposing the nanovesicles and the nanoparticles to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 2 minutes to about 3 minutes; and (ii) reconstituting the proteolipid powder in the presence of nanoparticles. In a further example, a ratio of weight of nanoparticles to weight of nanovesicles during the encapsulating is from about 1:1 to about 1:0.1. In yet a further example,

the nanoparticles include one or more of polymeric nanoparticles, carbon nanoparticles, composite nanoparticles, or inorganic nanoparticles, or a combination thereof.

[0012] In an example, the nanoparticles are selected from iron oxide, gold, silver, silica, nanoceria, quantum dots, up conversion nanoparticles, calcium phosphate, polystyrene, polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, chitosan, alginate, poly(methyl methacrylate), poly(β-amino ester), ethyl cellulose, semiconducting polymer nanoparticles, carbon nanotubes, nanographite, carbon nanohorn, carbon black nanoparticle, graphene, nanodiamond, and carbon dots, or a combination thereof. In another example, the process for making the nanovesicles further includes loading the nanovesicle with one or more therapeutic compound. In yet another example, the one or more therapeutic compound includes an antibiotic, an antifungal, an antigen, an immunosuppressant, a chemotherapeutic, a protein functional modulator, a pain management compound, an oligonucleotide, a gene editor, or a peptide, or a combination thereof. In still another example, at least one of the one or more therapeutic compound is selected from suberoylanilide hydroxamic acid, Vancomycin, Amphotericin B, Ketoconazole, a tumor peptide, a tumor antigen, a microbial protein, a peptide, a polysaccharide, an immunogenic peptide, a toxin, Dexamethasone, Cyclosporine A, Tacrolimus, Rapamycin, docetaxel, irinotecan, doxorubicin, gemcitabine, cisplatin, paclitaxel, CCG1423, GSK269962, MG-132, INCB8761, Bupivacaine, Ketamine, Morphine, RNA, DNA, CRISPR/Cas9, monoclonal antibodies, growth factors, and functional enzymes.

[0013] In an example, the subject has an autoimmune disorder, has a monocyte-mediated condition, or experienced a myocardial infarction, wherein the autoimmune disorder is chosen from Lupus, multiple sclerosis (MS), and psoriasis, and wherein the monocyte-mediated condition is chosen from atherosclerosis and ischemia. In another example, the administering is intravenously, intraperitoneally, enterally, by inhalation, intranasally, sublingually, intramuscularly, or subcutaneously. In yet another example, the subject experienced a myocardial infarction and the administering is within about two hours after the myocardial infarction. In still another example, the administering includes treating the subject for a myocardial infarction. In a further example, the treating includes reducing size of an infarct in the subject compared to infarct size of another individual who experienced a myocardial infarction and was not administered the nanovesicles. In yet a further example, the reducing is determined 72 hours after the myocardial infarction and includes an about 15% reduction in size.

[0014] In another aspect, provided is a method of preparing nanovesicles, including freezing erythrocytes, thawing the erythrocytes, sonicating the thawed erythrocytes, and one of (i) extruding the sonicated erythrocytes; and (ii) freeze-drying the sonicated erythrocytes to obtain a proteolipid powder, and reconstituting the proteolipid powder; wherein: freezing includes exposing the erythrocytes to a temperature of about 0 °C or lower; thawing includes exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes; sonicating includes exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes to form nanovesicles; extruding includes successively passing the sonicated erythrocytes through a series of filters each including a filter diameter, wherein each filter includes a larger filter diameter than a subsequent filter; freeze-drying the sonicated erythrocytes includes mixing the sonicated erythrocytes with a cryoprotectant; and reconstituting the proteolipid powder includes dissolving the proteolipid powder in an organic solvent and then mixing with an aqueous solvent.

[0015] In an example, one of (i) the sonicated erythrocytes are extruded in the presence of one or more added lipid; and (ii) the proteolipid powder is reconstituted in the presence of one or more added lipid. In another example, one of (i) a ratio of weight of erythrocytes to weight of the one or more added lipid during the extruding is from about 1:24 to about 1:1; and (ii) a ratio of weight of proteolipid powder to weight of the one or more added lipid during reconstituting is from about 24:1 to about 5.3:1.

[0016] In an example, at least one of the one or more added lipid is a natural lipid or a synthetic lipid. In another example, the synthetic lipid includes a lipid attached to one or more targeting ligand. In yet another example, the one or more targeting ligand is a peptide, an antibody, or an aptamer, or a combination thereof. In still another example, the one or more targeting ligand targets a cell type, wherein the cell type is selected from T cell, B cell, monocyte, macrophage, granulocyte, dendritic cell, endothelial cell, (myo)fibroblast, and cancer cell. In a further example, the synthetic lipid is selected from 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol). In yet a further example, the natural lipid is selected from a sphingolipid, a phosphatidylglycerol, a lysophosphatidylcholine, a phosphatidyl serine, a ceramide, an eicosanoid, a phosphoinositide, and a lysophosphatidic acid, or a combination thereof.

[0017] In an example, the process further includes encapsulating nanoparticles with the nanovesicles, wherein encapsulating includes one of (i) sonicating the nanovesicles in the presence of the nanoparticles and sonicating includes exposing the nanovesicles and the nanoparticles to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 2 minutes to about 3 minutes; and (ii) reconstituting the proteolipid powder in the presence of nanoparticles. In another example, a ratio of weight of nanoparticles to weight of nanovesicles during the encapsulating is from about 1:1 to about 1:0.1. In yet another example, the nanoparticles include one or more of polymeric nanoparticles, carbon nanoparticles, composite nanoparticles, or inorganic nanoparticles, or a combination thereof. In still another example, the nanoparticles are selected from iron oxide, gold, silver, silica, nanoceria, quantum dots, up conversion nanoparticles, calcium phosphate, polystyrene, polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, chitosan, alginate, poly(methyl methacrylate), poly(β-amino ester), ethyl cellulose, semiconducting polymer nanoparticles, carbon nanotubes, nanographite, carbon nanohorn, carbon black nanoparticle, graphene, nanodiamond, and carbon dots, or a combination thereof.

[0018] In an example, the process for making the nanovesicle further includes loading the nanovesicle with one or more therapeutic compound. In another example, the one or more therapeutic compound includes an antibiotic, an antifungal, an antigen, an immunosuppressant, a chemotherapeutic, a protein functional modulator, a pain manager, an oligonucleotide, a gene editor, or a peptide, or a combination thereof. In yet another example, at least one of the one or more therapeutic compound is selected from suberoylanilide hydroxamic acid, Vancomycin, Amphotericin B, Ketoconazole, a tumor peptide, a tumor antigen, a microbial protein, a peptide, a polysaccharide, an immunogenic peptide, a toxin, Dexamethasone, Cyclosporine A, Tacrolimus, Rapamycin, docetaxel, irinotecan, doxorubicin, gemcitabine, cisplatin, paclitaxel, CCG1423, GSK269962, MG-132, INCB8761, Bupivacaine, Ketamine, Morphine, RNA, DNA, CRISPR/Cas9, monoclonal antibodies, growth factors, and functional enzymes.

[0019] In another aspect, provided is a plurality of nanovesicles made by a process, wherein the process includes freezing erythrocytes, thawing the erythrocytes, sonicating the thawed erythrocytes, and one of (i) extruding the sonicated erythrocytes; and (ii) freeze-drying the sonicated erythrocytes to obtain a proteolipid powder, and reconstituting the proteolipid powder; wherein: freezing includes exposing the erythrocytes to a temperature of about 0 °C or lower; thawing includes exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C

for from about 20 minutes to about 30 minutes; sonicating includes exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes; extruding includes successively passing the sonicated erythrocytes through a series of filters each including a filter diameter, wherein each filter includes a larger filter diameter than a subsequent filter; freeze-drying the sonicated erythrocytes includes mixing the sonicated erythrocytes with a cryoprotectant; and reconstituting the proteolipid powder includes dissolving the proteolipid powder in an organic solvent and then mixing with an aqueous solvent.

BRIEF DESCRIPTION OF THE FIGURES

[0020] Some embodiments of the present disclosure will now be explained, with reference to the accompanied drawings, in which:

[0021] **FIGs. 1A-1G** depict the preparation and characterization of senescent erythrocyte-like nanovesicles (SENVs); **FIG. 1A** is a schematic diagram outlining the step-by-step preparation process of senescent erythrocyte-like nanovesicles (SENVs); **FIG. 1B** depicts a scanning electron micrograph showing healthy RBCs as compared to senescent-like erythrocyte ghosts (SEGs), with an inset showing the fresh RBCs and processed SEGs produced by the controlled freeze-thaw with sonication (cFTS) process; **FIG. 1C** depicts a transmission electron micrograph showing the SENVs (Scale bar ^{1}200, ^{2}100, ^{3}50, ^{4}25 nm respectively) with white arrows highlighting the multilayered nanovesicles; **FIG. 1D** depicts a size comparison of mouse RBCs, SEGs, and SENVs; **FIG. 1E** depicts a confocal microscopy demonstrating the presence of "don't eat me" signaling protein CD47 on the mouse RBC surface (CD47-FITC - green) and its loss of function on the SEGs surface (cell membranes are labeled with CyAm7 (red)); **FIG. 1F** depicts a flow cytometry analysis confirming the presence of CD47 on the mouse RBCs surface, compared to the SEGs; and **FIG. 1G** depicts a LC-MS-based proteomic analysis of SENVs and senescent-like erythrocytes(SEs) showing the cFT process concentrates the "eat me" signaling antigenic peptides on the SENVs surface;

[0022] **FIGs. 2A and 2B** depict the effect of controlled freeze-thaw (cFT) process on mouse RBCs; **FIG. 2A** depicts the cFT process (the SEGs formation process was monitored using KEYENCE BZ-X series all in one fluorescence microscope and the samples were labeled with CyAm7 (scale bar 50 μm)); **FIG. 2B** is a schematic of the changes induced by the cFT process;

[0023] **FIGs. 3A-3C** depict the proteomics analysis of senescent erythrocytes and senescent erythrocytes derived ghosts (SEGs); **FIG. 3A** depicts a LC-MS-based proteomic analysis of senescent-like erythrocytes (SEs) and senescent-like nanovesicles (SENVs) showing the cFT process-induced protein loss in the SENVs; **FIG. 3B** depicts the percentage of absolute protein amount measured as the sum of iBAQ intensities of proteins related with cellular compartment according to Proteomics Database (abbreviations as follows, CM: cell membrane, ICM: integral cell membrane, CK: cytoskeleton, CT: cytosol, GE: golgi and endoplasmic reticulum, RI: ribosome, PR: proteosome complex); **FIG. 3C** depicts a heat map comparing proteomics profiles in SENVs and SEGs (the Z-score was used to calculate statistical significance);

[0024] **FIGs. 4A and 4B** depict the cFT and cFTs processes degrading the "don't eat me" signaling protein CD47 on the SEGs; **FIG 4A** depicts confocal laser scanning microscopy images showing the presence of "don't eat me" signaling protein CD47 on the mouse RBC surface (CD47-FITC-green, scale bar 10 μm); **FIG 4B** depicts the loss of function of CD47 on the SEGs surface owing to the cFTS process (Scale bar 50 μm); CyAm7 (red) was used to fluorescently label the mouse RBCs and SEGs;

[0025] **FIGs. 5A-5G** depict the effect of the controlled free-thaw process on the erythrocyte membrane lipidomics; **FIG. 5A** is a lipidomics analysis heatmap of cFTS , shown in a white-green scale are all lipid species including > 0.1-mole percent (mol%) of lipids, with mole percentage encoded in green intensity (darkest = most abundant) and gray indicates species below the 0.1 mol% thresholds (including not detected); **FIG. 5B** is a series of pie charts showing the compiled compositions of the lipidomics analysis of the cFT and cFTS processes; **FIG. 5C** is a lipidomic heatmap of ceramide lipid species, shown in a red-blue scale are ceramide lipids species based on Z -Score encoded in red intensity (darkest = most abundant) and dark blue indicates species below the 0.1 mol% thresholds (including not detected); **FIG. 5D** depicts confocal microscopy-based live sequential images acquired at 0 minutes and 5 minutes demonstrating phosphatidylserine lipid externalization via the binding of FITC-labeled annexin-V; **FIG. 5E** is a schematic diagram outlining the fluorescent labeling of SENVs and senescent-like RBCs (sRBCs); **FIG. 5F** is an IVIS image of SENVs and sRBCs in the spleen; **FIG. 5G** is a quantitative fluorescence image analysis of SENVs and sRBCs in the spleen (n=3);

[0026] **FIG. 6** demonstrates the cFT- and cFTs-process induced migration of unsaturated lipids towards the exoplasmic leaflets of sRBCs;

[0027] **FIG. 7** is a series of confocal laser scanning microscopy images showing the cFT process-induced phosphatidylserine lipid membrane migration, visualized by annexin V-FITC staining (Scale bar 20 μm);

[0028] **FIGs. 8A-8H** is a schematic of the preparation and characterization of phosphatidylserine (PS) lipid engineered senescent erythrocyte-like nanovesicles coated nanotheranostics (eSENTs); **FIG. 8A**, is a schematic of the overall approach to designing and producing eSEGs and eSENTs; **FIG. 8B** is a series of cryogenic TEM micrographs showing the formation of heterogeneous eSEGs (Scale bar [1]100 nm, [2]50 nm, Color code of arrow was used to emphasize the diverse natures of eSEGs: green color represents heterogenous size of eSEGs; red color represents different shapes of eSEGs; blue color highlights the different degree of lipid layer thicknesses (Insets show the uni- and multilamellar nature of lipids vesicles (ULVs and MLVs)); **FIG. 8C** is an image of a unilamellar, spherical nanovesicles of eSEGs (Scale bar 50 nm); **FIG. 8D**, is a histogram demonstrating the thickness of cryo-TEM lipid layer as analyzed using Image J software (n=10); **FIGs. 8E and 8F**, show course-grained molecular dynamics (MD) simulations (1.5 μs) models based on the lipidomics analysis to predict the partitioning of PS in the upper leaflets (UL) and lower leaflets (LL) of RBCs; **FIG. 8G** is a negatively stained transmission electron microscopy images of core-shell hybrid nanostructure of eSENTs (Scale bar 100 nm); **FIG. 8H** is a series of confocal laser scanning fluorescence microscopy images of the core-shell hybrid nature of the materials produced by the sonication and extrusion process; the micrometer-sized CyAl5-labeled polymeric particles and CyAm7-labeled eSEGs were generated and then fused (Scale bar 20 μm); inset images show a representative schematic illustration of the core-shell hybrid structure of eSENTs;

[0029] **FIG. 9** depicts coarse-grained molecular dynamics simulations (1.5 μs) for side view (**left**) and top view (right) using three different PS lipid to SEGs lipid (w/w) ratios as denoted as +PS-eSENVs (24:1), ++PS-eSENVs (9.6:1), +++PS-eSENVs (5.3:1);

[0030] **FIG. 10** is a series of density maps of coarse-grained molecular dynamics simulations (1.5 μs) of PS lipid engineered-SENVs (eSENVs) forming from three different PS lipid to RBC lipid (w/w) ratios: +PS-eSENVs (24:1), ++PS-eSENVs (9.6:1), +++PS-eSENVs (5.3:1);

[0031] **FIG. 11** is a series of scanning electron microscopy images of the core-shell hybrid nature of eSEG coated materials; the micrometer sized PLGA core and eSEGs were generated and

then fused by the sonication process to produce coated materials (Scale bar 10 μm); representative schematic illustration of the core-shell hybrid structure of eSENTs;

[0032] **FIGs. 12A and 12B** depict in vitro eSENT uptake by RAW 246.7 cells; representative confocal laser scanning microscopy images demonstrates the eSENTs uptake by RAW 246.7 cells. (Scale bar 10 μm).

[0033] **FIGs. 13A-13O** depict the results of a series of investigative experiments concerning the splenic retention of eSENTs in naïve C57BL/6J mice; **FIG. 13A** is a schematic outlining the PS-dependent splenic accumulation of eSENTs; **FIG. 13B** depicts IVIS images showing that increases in PS doping result in increased splenic retention; **FIG. 13C** depicts the quantitative fluorescence image analysis of the PS-dependent splenic accumulation of eSENTs (t-test,*$P < 0.05$); **FIG. 13D** is a schematic representation of the comparative experiments between engineered (eSENTs), non-engineered (SENTs), and uncoated nanotheranostics (NTs); **FIG. 13E** depicts IVIS images show the comparative accumulation of eSENTs, SENTs, and uncoated nanotheranostics within the spleen; **FIG. 13F** depicts quantitative image analysis of the splenic accumulation of eSENTs versus SENTs, and uncoated nanotheranostics 48 h post-injection as a function of dose (3, 6, and 30 mg/kg, $n = 3$ mice/group, t-test,**$P < 0.05$); **FIG. 13G** is a schematic outline of the determination of the dose-dependent plasma clearance half-life of eSENTs; **FIG. 13H** depicts quantitative fluorescence analysis of the dose-dependent circulation half-life of eSENTs ($n = 3$ mice/group); **FIG. 13I** is a schematic representation of the dose-dependent splenic immunocyte specific uptake of eSENTs; **FIGs. 13J** and **13K** depict flow cytometric analysis of eSENT (6 and 30 mg/kg) uptake by splenocytes ($n = 3$ mice/group); **FIGs 13L-13O** is a series of representative images of immunofluorescent staining of spleen sections showing eSENT uptake by specific splenic immunocytes including B (CD20), T (CD3) and dendritic cells (CD11c), and macrophages (F4/80); respective antibodies – green, eSENTs – red, DAPI or Sytox Green-blue, fluorescent labeling, scale bar 200 μm;

[0034] **FIGs. 14A-14J** depict representative flow cytometry analysis of the splenocyte-specific accumulation of NTs;

[0035] **FIG. 15** depicts representative immunofluorescence analysis of the splenocyte-specific accumulation of eSENTs; immunofluorescent staining of spleen sections was performed using CD3, CD20, and F4/80 antibodies – green, eSENTs-Cy5 – red, DAPI, or Sytox Green - blue. Scale bar - 200 μm;

[0036] **FIG. 16** is a schematic outline of the in vivo experimental hypothesis of spleen-mediated cardiac immunomodulation;

[0037] **FIGs. 17A-17F** is a schematic of the investigation of the splenic immunocyte-mediated immunomodulatory potential of SAHA-eSENTs; **FIG. 17A** is a schematic representation of the overall experimental approach; **FIG. 17B** depicts flow cytometry-based quantitation of $CD11b^{+}Ly6C^{+}$ and $CD11b^{+}Ly6C^{+}NTs^{+}$ (cells that have engulfed CyAl5-eSENTS) within the spleens of CD1 mice receiving MI (cells/mg spleen tissue, no NTs (n=7), eSENTs alone (n=7), SAHA-eSENTs (n=8)); flow cytometry-based quantification of $CD11b^{+}F4/80^{+}$ and $CD11b^{+}F4/80^{+}NTs^{+}$ (cells that have engulfed CyAl5-eSENTs) in infarcted murine hearts (cells/mg infarct tissue) (**FIG 17C**) 24-h post (No NTs (n=7), eSENTs (n=8), eSENTs-SAHA (n=7)) and (**FIG. 17D**) 72-hr post-MI (No NTs (n=3), eSENTs (n=4) and eSENTs-SAHA (n=5); **FIG. 17E** depicts the quantitative assessment infarct area at 72-hr post-MI by TTC staining depicting a 14% decrease in infarct size in the treated group (No NTs (n=10), eSENTs (n=10), eSENTs-SAHA (n=9)); **FIG. 17F** depicts representative images of yellow/white region indicating an area of infarction at 72-hr post-MI; infarct areas were measured using ImageJ software; all data are represented as mean±SEM, *P value <0.05, **P value <0.005, and ***P value <0.0005 versus control as determined by Kruskal Wallis non-parametric analysis with Dunn's multiple comparison post-hoc test; and

[0038] **FIGs. 18A-18F** depict representative flow cytometry analysis; **FIGs. 18A and 18B** depict the intravenous injection of the immunomodulatory SAHA-eSENTs 2 hrs after MI significantly increased the number of $CD11b^{+}Ly6C^{+}$ monocytes/macrophages retained in the spleen; **FIGs. 18C and 18D** depict the number of $CD11b^{+}/F4/80^{+}$ macrophages present in the infarct at 24 hours post-MI were reduced by 3.5-fold when compared to the saline group; **FIGs. 18E and 18F** depicts this trend at 72-hours post-MI.

[0039] **FIGs. 19A and 19B** depict flow cytometry analysis of CD-47 specific antisense oligonucleotide delivery using eSENTs.

DETAILED DESCRIPTION

[0040] As disclosed herein, biomimetic nanotheranostics (NTs) using the spleen's recognition of senescent red blood cells (RBC) to affect enhanced retention of the materials, were generated.[8] These engineered senescent erythrocyte-like nanotheranostics (eSENTs) utilize flipped RBC membranes featuring unique scavenger signals including the externalization of phosphatidylserine

(PS), other oxidized lipids, and senescent cell antigens on their surface, as well as the loss of cell surface CD47, a "don't eat me" signal on healthy RBCs.[9-11] Engineered senescent erythrocyte-derived nanotheranostics (eSENTs) demonstrated significant uptake by the immune cells of the spleen including T and B cells, as well as monocytes and macrophages. These materials were extensively characterized and, in an example, their further ability to modulate the therapeutic response in a distant organ, in particular scar size after myocardial infarction (MI), was studied. When loaded with suberoylanilide hydroxamic acid (SAHA), as an example of cargo, the nanoagents exhibit a potent therapeutic effect, reducing infarct size by 14% at 72-hours post-myocardial infarction when given as a single intravenous dose 2 hours after injury. This approach may have far-reaching applicability for the treatment of both acute and chronic conditions where the immune responses are either stimulated or suppressed by the splenic (auto)immune milieu.

[0041] According to an aspect of the present invention, there is provided a method of preparing nanovesicles, including freezing erythrocytes, thawing the erythrocytes, sonicating the thawed erythrocytes, and extruding the sonicated erythrocytes, wherein freezing includes exposing the erythrocytes to a temperature of about 0 °C or lower, thawing includes exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes, sonicating includes exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes to form nanovesicles, and extruding includes successively passing the sonicated erythrocytes through a series of filters each including a filter diameter, wherein each filter includes a larger filter diameter than a subsequent filter.

[0042] In an example, the erythrocytes are frozen at a temperature of about 0 °C or lower, including all ranges, subranges, and values, therein, e.g., about 0 °C to about -20 °C, about -20 °C to about -40 °C, about -40 °C to about -60 °C, about -60 °C to about -80 °C, about -5 °C, about -10 °C, about -15 °C, about -20 °C, about -25 °C, about -30 °C, about -35 °C, about -40 °C, about -45 °C, about -50 °C, about -55 °C, about -60 °C, about -65 °C, about -70 °C, about -75 °C, about -80 °C, etc. In another example, during freezing the erythrocytes are exposed to the temperature of about 0 °C or lower for about 5 minutes or more, including all ranges, subranges, and values, therein, e.g., about 10 minutes, about 15 minutes, about 20 minutes, about 25 minutes, about 30 minutes, about 35 minutes, about 40 minutes, about 45 minutes, about 50 minutes, about 55 minutes, about 60 minutes, about 65 minutes, about 70 minutes, about 75 minutes, about 80

minutes, about 85 minutes, about 90 minutes, about 95 minutes, about 100 minutes, about 105 minutes, about 110 minutes, about 115 minutes, about 120 minutes, etc. In a further example, the frozen erythrocytes may stay at a freezing temperature of about 0 °C or lower for a prolonged duration before the thawing. For example, once frozen, the erythrocytes may remain frozen, by being stored or maintained at a temperature below 0 °C, including any one or more of the above-recited temperatures or temperature ranges below 0 °C, including without limitation about -20 °C or about -80 °C or any other freezing temperature, for up to hours, or up to days, or longer.

[0043] In an example, during thawing the erythrocytes are exposed to a temperature of from about 25 °C to about 60 °C, including all ranges, subranges, and values therein, e.g., about 25 °C to about 30 °C, about 30 °C to about 35 °C, about 35°C to about 40 °C, about 40 °C to about 45 °C, about 45 °C to about 50 °C, about 50 °C to about 55 °C about 55 °C to about 60 °C, about 25 °C to about 35 °C, about 35 °C to about 45 °C, about 45 °C to about 55 °C, about 25 °C to about 50 °C, about 50 °C to about 60 °C, about 25 °C, about 30 °C, about 35 °C, about 40 °C, about 45°C, about 50 °C, about 55 °C, about 60 °C. etc. In another example, the frozen erythrocytes are thawed at about 52 °C. In an example, during thawing the erythrocytes are exposed to the temperature for from about 20 minutes to about 30 minutes, including all ranges, subranges, and values therein, e.g., about 20 minutes to about 25 minutes, about 25 minutes to about 30 minutes, about 20 minutes, about 22 minutes, about 24 minutes, about 26 minutes, about 28 minutes, about 30 minutes, etc.

[0044] In an example, during sonicating, the thawed erythrocytes are exposed to ultrasonic frequencies of from about 20 kHz to about 40 kHz, including all ranges, subranges, and values therein, e.g., about 20 kHz to about 25 kHz, about 25 kHz to about 30 kHz, about 30 kHz, to about 35 kHz, about 35 kHz to about 40 kHz, about 20 kHz to about 30 kHz, about 30 kHz to about 40 kHz, about 20 kHz, about 25 kHz, about 30 kHz, about 35 kHz, about 40 kHz, etc. In another example, during sonicating, the thaw erythrocytes are exposed to ultrasonic frequencies for about 1 minute to about 3 minutes, including all ranges, subranges, and values therein, e.g., about 1 minute to about 1.5 minutes, about 1.5 minutes to about 2 minutes, about 2 minutes to about 2.5 minutes, about 2.5 minutes to about 3 minutes, about 1 minute to about 2 minutes, about 2 minutes to about 3 minutes, about 1 minute, about 2 minutes, about 3 minutes, etc.

[0045] In an example, the sonicated erythrocytes may be extruded in the presence of one or more added lipid. In another example, a ratio of weight of erythrocytes to weight of the one or more

added lipid during the extruding is from about 1:24 to about 1:1. Non-limiting examples of the one or more added lipid include cationic lipids, ionizable cationic lipids, helper zwitterionic lipids, stabilizing lipids, phosphatidyl glycerol and its modified derivatives, and PEG lipids and their derivatives. Non-limiting examples of cationic lipids include 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), 1,2-dioleoyloxy-3-[trimethylammonium]-propane (DOTAP), dimethyl-dioctadecyl ammonium bromide (DDAB), cetyl-trimethyl ammonium bromide (CTAB) and its derivatives. Non-limiting examples of helper zwitterionic lipids includes 1,2-dioleoyl-sn-glycerol-3 phosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), and lecitins and its derivatives. Non-limiting examples of stabilizing lipids include cholesterol and its derivatives. Non-limiting examples of PEG lipids and their derivatives include modified PEG lipids, and polyethylene glycol including polyethylene glycol-modified ceramides with variable fatty acid chain lengths, PEG-CerC8, C14, and C20, PEG-sterols such as Cholesterol-(polyethylene glycol-600), methoxy form of PEGs such as 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000], mPEG Glycerides such as (1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol) and distearoyl-rac-glycerol-methoxy polyethylene glycol, PEG-derivatized 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE-PEG(2000), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE-PEG(2000)), or 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG(2000)), (2-hexyldecanoate), 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide, and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000.

[0046] In yet another example, at least one of the one or more added lipid is a natural lipid or a synthetic lipid. In still a further example, the synthetic lipid is selected from 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol). In another further example, the natural lipid is selected from a sphingolipid, a phosphatidylglycerol, a lysophosphatidylcholine, a phosphatidyl serine, a ceramide, an eicosanoid, a phosphoinositide, and a lysophosphatidic acid, or a combination thereof.

[0047] In still another example, the synthetic lipid may include a lipid attached to one or more targeting ligand. In a further example, the one or more targeting ligand is a peptide or a protein, an antibody, an aptamer, a small molecule, or a combination thereof. In yet a further example, the one or more targeting ligand targets a cell type. Non-limiting examples of cells targeted by the targeting ligand include T cells, B cells, monocytes, macrophages, granulocytes, dendritic cells, endothelial

cells, (myo)fibroblasts, and/or cancers. Non-limiting examples of ligands that target T cells include antibodies, such as CD5, CD3, CD4, and CD8. Non-limiting examples of ligands that target B cells include antibodies, such as CD20 and CD5. Non-limiting examples of ligands that target macrophages include mannose. Non-limiting examples of ligands that target granulocytes include antibodies such as NIMP and neutrophil chemoattractant peptide. Non-limiting examples of ligands that target endothelial cells include antibodies such as anti-VCAM-1 and anti-ICAM-1, VLA-4 mimetic peptides, and LFA-1 mimetic peptide. Non-limiting examples of ligands that target (myo)fibroblasts include peptides to angiotensin II receptors.

[0048] In an example, the process may further include encapsulating nanoparticles with the nanovesicles, wherein encapsulating includes sonicating the nanovesicles in the presence of the nanoparticles and sonicating includes exposing the nanovesicles and the nanoparticles to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 2 minutes to about 3 minutes. In another example, a ratio of weight of nanoparticles to weight of nanovesicles during the encapsulating is from about 1:1 to about 1:0.1. In yet another example, the nanoparticles include one or more of polymeric nanoparticles, carbon nanoparticles, composite nanoparticles, or inorganic nanoparticles, or a combination thereof. In still another example, the nanoparticles are selected from iron oxide, gold, silver, silica, nanoceria, quantum dots, up conversion nanoparticles, calcium phosphate, polystyrene, polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, chitosan, alginate, poly(methyl methacrylate), poly(β-amino ester), ethyl cellulose, semiconducting polymer nanoparticles, carbon nanotubes, nanographite, carbon nanohorn, carbon black nanoparticle, graphene, nanodiamond, and carbon dots, or a combination thereof. Non-limiting examples of inorganic nanoparticles include iron oxide, gold, silver, silica, nanoceria, quantum dots, up conversion nanoparticles, and calcium phosphate. Non-limiting examples of polymeric nanoparticles include polystyrene, polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, chitosan, alginate, poly(methyl methacrylate), poly(β-amino ester), ethyl cellulose, and semiconducting polymer nanoparticles. Non-limiting examples of carbon nanoparticles include carbon nanotubes, nanographite, carbon nanohorn, carbon black nanoparticle, graphene, nanodiamond, and carbon dots. Composite nanoparticles are included of at least two different nanoparticles chosen from an inorganic nanoparticle, a polymeric nanoparticle, or a carbon nanoparticle.

[0049] In a further example, the process for making the nanovesicle may further include loading the nanovesicle with one or more therapeutic compound. In yet a further example, the one or more therapeutic compound includes an antibiotic, an antifungal, an antigen, an immunosuppressant, a chemotherapeutic, a protein functional modulator, a pain manager, an oligonucleotide, a gene editor, or a peptide, or a combination thereof. In still a further example, at least one of the one or more therapeutic compound is selected from suberoylanilide hydroxamic acid, Vancomycin, Amphotericin B, Ketoconazole, a tumor peptide, a tumor antigen, a microbial protein, a peptide, a polysaccharide, an immunogenic peptide, a toxin, Dexamethasone, Cyclosporine A, Tacrolimus, Rapamycin, docetaxel, irinotecan, doxorubicin, gemcitabine, cisplatin, paclitaxel, CCG1423, GSK269962, MG-132, INCB8761, Bupivacaine, Ketamine, Morphine, RNA, DNA, CRISPR/Cas9, monoclonal antibodies, growth factors, and functional enzymes. Non-limiting examples of antibiotics include Vancomycin, Amphotericin B. A non-limiting example of an antifungal includes Ketoconazole. Non-limiting examples of an antigen include a tumor peptide, a tumor antigen, a microbial protein, a peptide, a polysaccharide, an immunogenic peptide, and a toxin. Non-limiting examples of an immunosuppressive include Dexamethasone, Cyclosporine A, Tacrolimus, and Rapamycin. Non-limiting examples of a chemotherapeutic include docetaxel, irinotecan, doxorubicin, gemcitabine, cisplatin, and paclitaxel. Non-limiting examples of protein functional modulators include CCG1423, GSK269962, MG-132, INCB8761, and suberoylanilide hydroxamic acid (SAHA). Non-limiting examples of a pain manager include Bupivacaine, Ketamine, and Morphine. Non-limiting examples of oligonucleotides include RNA and DNA. A non-limiting example of a gene editor is CRISPR/Cas9. Non-limiting examples of a therapeutic protein includes monoclonal antibodies, growth factors, and functional enzymes.

[0050] The method of preparing nanovesicles, as disclosed above, may be used to synthesize compositions, which compositions may then be delivered to a spleen of a subject, such as by systemically administering the composition to the subject. Such composition may include a nanovesicle made by a process, the process including: freezing erythrocytes, thawing the erythrocytes, sonicating the thawed erythrocytes, and extruding the sonicated erythrocytes, wherein freezing includes exposing the erythrocytes to a temperature of about 0 °C or lower, thawing includes exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes, sonicating includes exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1

minute to about 3 minutes, and extruding includes successively passing the sonicated erythrocytes through a series of filters each including a filter diameter, wherein each filter includes a larger filter diameter than a subsequent filter.

[0051] In an example, the erythrocytes are frozen at a temperature of about 0 °C or lower, including all ranges, subranges, and values, therein, e.g., about 0 °C to about -20 °C, about -20 °C to about -40 °C, about -40 °C to about -60 °C, about -60 °C to about -80 °C, about -5 °C, about -10 °C, about -15 °C, about -20 °C, about -25 °C, about -30 °C, about -35 °C, about -40 °C, about -45 °C, about -50 °C, about -55 °C, about -60 °C, about -65 °C, about -70 °C, about -75 °C, about -80 °C, etc. In another example, during freezing the erythrocytes are exposed to the temperature of about 0 °C or lower for about 5 minutes or more, including all ranges, subranges, and values, therein, e.g., about 10 minutes, about 15 minutes, about 20 minutes, about 25 minutes, about 30 minutes, about 35 minutes, about 40 minutes, about 45 minutes, about 50 minutes, about 55 minutes, about 60 minutes, about 65 minutes, about 70 minutes, about 75 minutes, about 80 minutes, about 85 minutes, about 90 minutes, about 95 minutes, about 100 minutes, about 105 minutes, about 110 minutes, about 115 minutes, about 120 minutes, etc. In a further example, the frozen erythrocytes may stay at a freezing temperature of about 0 °C or lower for a prolonged duration before the thawing. For example, once frozen, the erythrocytes may remain frozen, by being stored or maintained at a temperature below 0 °C, including any one or more of the above-recited temperatures or temperature ranges below 0 °C, including without limitation about -20 °C or about -80 °C or any other freezing temperature, for up to hours, or up to days, or longer.

[0052] In an example, during thawing the erythrocytes are exposed to a temperature of from about 25 °C to about 60 °C, including all ranges, subranges, and values therein, e.g., about 25 °C to about 30 °C, about 30 °C to about 35 °C, about 35°C to about 40 °C, about 40 °C to about 45 °C, about 45 °C to about 50 °C, about 50 °C to about 55 °C about 55 °C to about 60 °C, about 25 °C to about 35 °C, about 35 °C to about 45 °C, about 45 °C to about 55 °C, about 25 °C to about 50 °C, about 50 °C to about 60 °C, about 25 °C, about 30 °C, about 35 °C, about 40 °C, about 45°C, about 50 °C, about 55 °C, about 60 °C. etc. In another example, the frozen erythrocytes are thawed at about 52 °C. In an example, during thawing the erythrocytes are exposed to the temperature for from about 20 minutes to about 30 minutes, including all ranges, subranges, and values therein, e.g., about 20 minutes to about 25 minutes, about 25 minutes to about 30 minutes,

about 20 minutes, about 22 minutes, about 24 minutes, about 26 minutes, about 28 minutes, about 30 minutes, etc.

[0053] In an example, during sonicating, the thawed erythrocytes are exposed to ultrasonic frequencies of from about 20 kHz to about 40 kHz, including all ranges, subranges, and values therein, e.g., about 20 kHz to about 25 kHz, about 25 kHz to about 30 kHz, about 30 kHz, to about 35 kHz, about 35 kHz to about 40 kHz, about 20 kHz to about 30 kHz, about 30 kHz to about 40 kHz, about 20 kHz, about 25 kHz, about 30 kHz, about 35 kHz, about 40 kHz, etc. In another example, during sonicating, the thaw erythrocytes are exposed to ultrasonic frequencies for about 1 minute to about 3 minutes, including all ranges, subranges, and values therein, e.g., about 1 minute to about 1.5 minutes, about 1.5 minutes to about 2 minutes, about 2 minutes to about 2.5 minutes, about 2.5 minutes to about 3 minutes, about 1 minute to about 2 minutes, about 2 minutes to about 3 minutes, about 1 minute, about 2 minutes, about 3 minutes, etc.

[0054] In an example, the subject may have an autoimmune disorder, a monocyte-mediated condition, or experienced a myocardial infarction, wherein the autoimmune disorder is chosen from Lupus, multiple sclerosis (MS), and psoriasis, and wherein the monocyte-mediated condition is chosen from atherosclerosis and ischemia. In another example, the administering is intravenously, intraperitoneally, enterally, by inhalation, intranasally, sublingually, intramuscularly, or subcutaneously. In yet another example, the subject experienced a myocardial infarction and the administering is within about two hours after the myocardial infarction.

[0055] In still another example, the administering may include treating the subject for a myocardial infarction. In a further example, the treating may include reducing size of an infarct in the subject compared to infarct size of another individual who experienced a myocardial infarction and was not administered the nanovesicles. In yet a further example, the reducing is determined 72 hours after the myocardial infarction and includes an about 15% reduction in size.

[0056] In yet another aspect, provided is a plurality of nanovesicles made by a process as disclosed above.

[0057] Features of the nanovesicles and methods disclosed above are also applicable to the nanovesicles and method disclosed below.

[0058] In a further aspect, provided is a method of preparing nanovesicles, including freezing erythrocytes, thawing the erythrocytes, sonicating the thawed erythrocytes, freeze-drying the sonicated erythrocytes to obtain a proteolipid powder, and reconstituting the proteolipid powder,

wherein freezing includes exposing the erythrocytes to a temperature of about 0 °C or lower, thawing includes exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes, and sonicating includes exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes.

[0059] In an example, the erythrocytes are frozen at a temperature of about 0 °C or lower, including all ranges, subranges, and values, therein, e.g., about 0 °C to about -20 °C, about -20 °C to about -40 °C, about -40 °C to about -60 °C, about -60 °C to about -80 °C, about -5 °C, about -10 °C, about -15 °C, about -20 °C, about -25 °C, about -30 °C, about -35 °C, about -40 °C, about -45 °C, about -50 °C, about -55 °C, about -60 °C, about -65 °C, about -70 °C, about -75 °C, about -80 °C, etc. In another example, during freezing the erythrocytes are exposed to the temperature of about 0 °C or lower for about 5 minutes or more, including all ranges, subranges, and values, therein, e.g., about 10 minutes, about 15 minutes, about 20 minutes, about 25 minutes, about 30 minutes, about 35 minutes, about 40 minutes, about 45 minutes, about 50 minutes, about 55 minutes, about 60 minutes, about 65 minutes, about 70 minutes, about 75 minutes, about 80 minutes, about 85 minutes, about 90 minutes, about 95 minutes, about 100 minutes, about 105 minutes, about 110 minutes, about 115 minutes, about 120 minutes, etc. In a further example, the frozen erythrocytes may stay at a freezing temperature of about 0 °C or lower for a prolonged duration before the thawing. For example, once frozen, the erythrocytes may remain frozen, by being stored or maintained at a temperature below 0 °C, including any one or more of the above-recited temperatures or temperature ranges below 0 °C, including without limitation about -20 °C or about -80 °C or any other freezing temperature, for up to hours, or up to days, or longer.

[0060] In an example, during thawing the erythrocytes are exposed to a temperature of from about 25 °C to about 60 °C, including all ranges, subranges, and values therein, e.g., about 25 °C to about 30 °C, about 30 °C to about 35 °C, about 35°C to about 40 °C, about 40 °C to about 45 °C, about 45 °C to about 50 °C, about 50 °C to about 55 °C about 55 °C to about 60 °C, about 25 °C to about 35 °C, about 35 °C to about 45 °C, about 45 °C to about 55 °C, about 25 °C to about 50 °C, about 50 °C to about 60 °C, about 25 °C, about 30 °C, about 35 °C, about 40 °C, about 45°C, about 50 °C, about 55 °C, about 60 °C. etc. In another example, the frozen erythrocytes are thawed at about 52 °C. In an example, during thawing the erythrocytes are exposed to the temperature for from about 20 minutes to about 30 minutes, including all ranges, subranges, and

values therein, e.g., about 20 minutes to about 25 minutes, about 25 minutes to about 30 minutes, about 20 minutes, about 22 minutes, about 24 minutes, about 26 minutes, about 28 minutes, about 30 minutes, etc.

[0061] In an example, during sonicating, the thawed erythrocytes are exposed to ultrasonic frequencies of from about 20 kHz to about 40 kHz, including all ranges, subranges, and values therein, e.g., about 20 kHz to about 25 kHz, about 25 kHz to about 30 kHz, about 30 kHz, to about 35 kHz, about 35 kHz to about 40 kHz, about 20 kHz to about 30 kHz, about 30 kHz to about 40 kHz, about 20 kHz, about 25 kHz, about 30 kHz, about 35 kHz, about 40 kHz, etc. In another example, during sonicating, the thaw erythrocytes are exposed to ultrasonic frequencies for about 1 minute to about 3 minutes, including all ranges, subranges, and values therein, e.g., about 1 minute to about 1.5 minutes, about 1.5 minutes to about 2 minutes, about 2 minutes to about 2.5 minutes, about 2.5 minutes to about 3 minutes, about 1 minute to about 2 minutes, about 2 minutes to about 3 minutes, about 1 minute, about 2 minutes, about 3 minutes, etc.

[0062] In an example, freeze-drying the sonicated erythrocytes may include mixing the sonicated erythrocytes with a cryoprotectant. In another example, reconstituting the proteolipid powder includes dissolving the proteolipid powder in an organic solvent and then mixing with an aqueous solvent.

[0063] In an example, the proteolipid powder is reconstituted in the presence of one or more added lipid. In another example, a ratio of weight of proteolipid powder to weight of the one or more added lipid during reconstituting is from about 24:1 to about 5.3:1. Non-limiting examples of the one or more added lipid include cationic lipids, ionizable cationic lipids, helper zwitterionic lipids, stabilizing lipids, phosphatidyl glycerol and its modified derivatives, and PEG lipids and their derivatives. Non-limiting examples of cationic lipids include 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), 1,2-dioleoyloxy-3-[trimethylammonium]-propane (DOTAP), dimethyl-dioctadecyl ammonium bromide (DDAB), cetyl-trimethyl ammonium bromide (CTAB) and its derivatives. Non-limiting examples of helper zwitterionic lipids includes 1,2-dioleoyl-sn-glycerol-3 phosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), and lecithins and its derivatives. Non-limiting examples of stabilizing lipids include cholesterol and its derivatives. Non-limiting examples of PEG lipids and their derivatives include modified PEG lipids PEG lipids, and polyethylene glycol including polyethyleneglycol-modified ceramides with variable fatty acid chain lengths, PEG-CerC8, C14, and C20, PEG-sterols such as

Cholesterol-(polyethylene glycol-600), methoxy form of PEGs such as 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000], mPEG Glycerides such as (1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol) and distearoyl-rac-glycerol-methoxy polyethylene glycol, PEG-derivatized 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE-PEG(2000), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE-PEG(2000)), or 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG(2000)), (2-hexyldecanoate), 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide, and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000.

[0064] In yet another example, at least one of the one or more added lipid is a natural lipid or a synthetic lipid. In still a further example, the synthetic lipid is selected from 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol). In another further example, the natural lipid is selected from a sphingolipid, a phosphatidylglycerol, a lysophosphatidylcholine, a phosphatidyl serine, a ceramide, an eicosanoid, a phosphoinositide, and a lysophosphatidic acid, or a combination thereof.

[0065] In still another example, the synthetic lipid includes a lipid attached to one or more targeting ligand. In a further example, the one or more targeting ligand is a peptide or a protein, an antibody, an aptamer, a small molecule, or a combination thereof. In yet a further example, the one or more targeting ligand targets a cell type. Non-limiting examples of cells targeted by the targeting ligand include T cells, B cells, monocytes, macrophages, granulocytes, dendritic cells, endothelial cells, (myo)fibroblasts, and/or cancers. Non-limiting examples of ligands that target T cells include antibodies, such as CD5, CD3, CD4, and CD8. Non-limiting examples of ligands that target B cells include antibodies, such as CD20 and CD5. Non-limiting examples of ligands that target macrophages include mannose. Non-limiting examples of ligands that target granulocytes include antibodies such as NIMP and neutrophil chemoattractant peptide. Non-limiting examples of ligands that target endothelial cells include antibodies such as anti-VCAM-1 and anti-ICAM-1, VLA-4 mimetic peptides, and LFA-1 mimetic peptide. Non-limiting examples of ligands that target (myo)fibroblasts include peptides to angiotensin II receptors.

[0066] In an example, the process may further include encapsulating nanoparticles with the nanovesicles, wherein encapsulating includes reconstituting the proteolipid powder in the presence of nanoparticles. In another example, a ratio of weight of nanoparticles to weight of nanovesicles during the encapsulating is from about 1:1 to about 1:0.1. In yet another example, the nanoparticles

include one or more of polymeric nanoparticles, carbon nanoparticles, composite nanoparticles, or inorganic nanoparticles, or a combination thereof. In still another example, the nanoparticles are selected from iron oxide, gold, silver, silica, nanoceria, quantum dots, up conversion nanoparticles, calcium phosphate, polystyrene, polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, chitosan, alginate, poly(methyl methacrylate), poly(β-amino ester), ethyl cellulose, semiconducting polymer nanoparticles, carbon nanotubes, nanographite, carbon nanohorn, carbon black nanoparticle, graphene, nanodiamond, and carbon dots, or a combination thereof. Non-limiting examples of inorganic nanoparticles include iron oxide, gold, silver, silica, nanoceria, quantum dots, up conversion nanoparticles, and calcium phosphate. Non-limiting examples of polymeric nanoparticles include polystyrene, polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, chitosan, alginate, poly(methyl methacrylate), poly(β-amino ester), ethyl cellulose, and semiconducting polymer nanoparticles. Non-limiting examples of carbon nanoparticles include carbon nanotubes, nanographite, carbon nanohorn, carbon black nanoparticle, graphene, nanodiamond, and carbon dots. Composite nanoparticles are included of at least two different nanoparticles chosen from an inorganic nanoparticle, a polymeric nanoparticle, or a carbon nanoparticle.

[0067] In a further example, a process for making the nanovesicle may further include loading the nanovesicle with one or more therapeutic compound. In yet a further example, one or more therapeutic compound may include an antibiotic, an antifungal, an antigen, an immunosuppressant, a chemotherapeutic, a protein functional modulator, a pain manager, an oligonucleotide, a gene editor, or a peptide, or a combination thereof. In still a further example, at least one of the one or more therapeutic compound is selected from suberoylanilide hydroxamic acid, Vancomycin, Amphotericin B, Ketoconazole, a tumor peptide, a tumor antigen, a microbial protein, a peptide, a polysaccharide, an immunogenic peptide, a toxin, Dexamethasone, Cyclosporine A, Tacrolimus, Rapamycin, docetaxel, irinotecan, doxorubicin, gemcitabine, cisplatin, paclitaxel, CCG1423, GSK269962, MG-132, INCB8761, Bupivacaine, Ketamine, Morphine, RNA, DNA, CRISPR/Cas9, monoclonal antibodies, growth factors, and functional enzymes. Non-limiting examples of antibiotics include Vancomycin, Amphotericin B. A non-limiting example of an antifungal includes Ketoconazole. Non-limiting examples of an antigen include a tumor peptide, a tumor antigen, a microbial protein, a peptide, a polysaccharide, an immunogenic peptide, and a toxin. Non-limiting examples of an immunosuppressive include Dexamethasone, Cyclosporine A,

Tacrolimus, and Rapamycin. Non-limiting examples of a chemotherapeutic include docetaxel, irinotecan, doxorubicin, gemcitabine, cisplatin, and paclitaxel. Non-limiting examples of protein functional modulators include CCG1423, GSK269962, MG-132, INCB8761, and suberoylanilide hydroxamic acid (SAHA). Non-limiting examples of a pain manager include Bupivacaine, Ketamine, and Morphine. Non-limiting examples of oligonucleotides include RNA and DNA. A non-limiting example of a gene editor is CRISPR/Cas9. Non-limiting examples of a therapeutic protein includes monoclonal antibodies, growth factors, and functional enzymes.

[0068] Nanovesicles made according to any example of a process as disclosed above may be used in a method of delivering a composition to a spleen of a subject, such as by systemically administering the composition to the subject. In an example the composition may include a nanovesicle made by a process, the process including: freezing erythrocytes, thawing the erythrocytes, sonicating the thawed erythrocytes, freeze-drying the sonicated erythrocytes to obtain a proteolipid powder, and reconstituting the proteolipid powder, wherein freezing includes exposing the erythrocytes to a temperature of about 0 °C or lower, thawing includes exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes, and sonicating includes exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes.

[0069] In an example, the erythrocytes are frozen at a temperature of about 0 °C or lower, including all ranges, subranges, and values, therein, e.g., about 0 °C to about -20 °C, about -20 °C to about -40 °C, about -40 °C to about -60 °C, about -60 °C to about -80 °C, about -5 °C, about -10 °C, about -15 °C, about -20 °C, about -25 °C, about -30 °C, about -35 °C, about -40 °C, about -45 °C, about -50 °C, about -55 °C, about -60 °C, about -65 °C, about -70 °C, about -75 °C, about -80 °C, etc. In another example, during freezing the erythrocytes are exposed to the temperature of about 0 °C or lower for about 5 minutes or more, including all ranges, subranges, and values, therein, e.g., about 10 minutes, about 15 minutes, about 20 minutes, about 25 minutes, about 30 minutes, about 35 minutes, about 40 minutes, about 45 minutes, about 50 minutes, about 55 minutes, about 60 minutes, about 65 minutes, about 70 minutes, about 75 minutes, about 80 minutes, about 85 minutes, about 90 minutes, about 95 minutes, about 100 minutes, about 105 minutes, about 110 minutes, about 115 minutes, about 120 minutes, etc. In a further example, the frozen erythrocytes may stay at a freezing temperature of about 0 °C or lower for a prolonged duration before the thawing. For example, once frozen, the erythrocytes may remain frozen, by

being stored or maintained at a temperature below 0 °C, including any one or more of the above-recited temperatures or temperature ranges below 0 °C, including without limitation about -20 °C or about -80 °C or any other freezing temperature, for up to hours, or up to days, or longer.

[0070] In an example, during thawing the erythrocytes are exposed to a temperature of from about 25 °C to about 60 °C, including all ranges, subranges, and values therein, e.g., about 25 °C to about 30 °C, about 30 °C to about 35 °C, about 35°C to about 40 °C, about 40 °C to about 45 °C, about 45 °C to about 50 °C, about 50 °C to about 55 °C about 55 °C to about 60 °C, about 25 °C to about 35 °C, about 35 °C to about 45 °C, about 45 °C to about 55 °C, about 25 °C to about 50 °C, about 50 °C to about 60 °C, about 25 °C, about 30 °C, about 35 °C, about 40 °C, about 45°C, about 50 °C, about 55 °C, about 60 °C. etc. In another example, the frozen erythrocytes are thawed at about 52 °C. In an example, during thawing the erythrocytes are exposed to the temperature for from about 20 minutes to about 30 minutes, including all ranges, subranges, and values therein, e.g., about 20 minutes to about 25 minutes, about 25 minutes to about 30 minutes, about 20 minutes, about 22 minutes, about 24 minutes, about 26 minutes, about 28 minutes, about 30 minutes, etc.

[0071] In an example, during sonicating, the thawed erythrocytes are exposed to ultrasonic frequencies of from about 20 kHz to about 40 kHz, including all ranges, subranges, and values therein, e.g., about 20 kHz to about 25 kHz, about 25 kHz to about 30 kHz, about 30 kHz, to about 35 kHz, about 35 kHz to about 40 kHz, about 20 kHz to about 30 kHz, about 30 kHz to about 40 kHz, about 20 kHz, about 25 kHz, about 30 kHz, about 35 kHz, about 40 kHz, etc. In another example, during sonicating, the thaw erythrocytes are exposed to ultrasonic frequencies for about 1 minute to about 3 minutes, including all ranges, subranges, and values therein, e.g., about 1 minute to about 1.5 minutes, about 1.5 minutes to about 2 minutes, about 2 minutes to about 2.5 minutes, about 2.5 minutes to about 3 minutes, about 1 minute to about 2 minutes, about 2 minutes to about 3 minutes, about 1 minute, about 2 minutes, about 3 minutes, etc.

[0072] In an example, the subject has an autoimmune disorder, has a monocyte-mediated condition, or experienced a myocardial infarction, wherein the autoimmune disorder is chosen from Lupus, multiple sclerosis (MS), and psoriasis, and wherein the monocyte-mediated condition is chosen from atherosclerosis and ischemia. In another example, the administering is intravenously, intraperitoneally, enterally, by inhalation, intranasally, sublingually,

intramuscularly, or subcutaneously. In yet another example, the subject experienced a myocardial infarction and the administering is within about two hours after the myocardial infarction.

[0073] In still another example, administering may include treating the subject for a myocardial infarction. In a further example, the treating includes reducing size of an infarct in the subject compared to infarct size of another individual who experienced a myocardial infarction and was not administered the nanovesicles. In yet a further example, the reducing is determined 72 hours after the myocardial infarction and includes an about 15% reduction in size.

[0074] In yet another aspect, provided is a plurality of nanovesicles made by a process as disclosed above.

[0075] As used herein, the term "about" followed by a numeral is intended to mean any amount varying from about 10% less than the recited amount to 10% more than the recited amount, such that, for example, "about 10 min" would be intended to mean "from 9 min to 11 min," for example.

EXAMPLES

[0076] **Preparation and characterization of nanovesicles**

[0077] Senescent erythrocyte-like ghosts (SEGs) were generated from mouse RBCs by a controlled freezing and thawing (cFT) process (FIG. 1A).[12] Erythrocytes, having a simple structure lacking nuclei and internal organelles, are highly susceptible to cFT that freezes the cellular water, causing osmotic stress and subsequently leading to cell shrinkage (FIG. 1B). Freezing temperatures below -20 °C promote the removal of water molecules associated with the phospholipid head groups, thereby inducing strong fluid-to-gel phase transitions of the erythrocyte lipids, resulting in intracellular ice formation.[13,14] When this is followed by rapid thawing at above 52 °C, hemolysis is accelerated inducing protein denaturation and enzyme inactivation that triggers phospholipid scrambling, thereby promoting the formation of heterogeneous SEGs (FIG. 1A, FIG. 1B, and FIG. 2A). A subsequent sonication process further reduces the size of SEGs, allowing the spontaneous formation of senescent erythrocytes like nanovesicles (SENVs) which was further confirmed by transmission electron microscopy (TEM) with negative staining (FIG. 1C) and dynamic light scattering (DLS) analysis (FIG. 1D).

[0078] Proteomics characterization of these SENVs revealed that a process combining cFT with sonication (cFTS) induces a significant loss of erythrocyte plasma membrane proteins (FIGs. 3A-3C) including CD47 (FIG. 1E, FIG. 1F, and FIG. 4) and concomitantly concentrates the "eat me" signaling antigenic peptides including band-3, stomatin and carbonic anhydrase 2, on the SENV

surface (FIG. 1G). Furthermore, lipidomics analysis revealed that the cFTS process interferes with the erythrocyte plasma membrane asymmetry, scrambling the membranes (FIGs. 5A-5C), and modulating the compositional diversity and acyl chain length of the lipids (FIG. 6), which is seen over various scales in sphingomyelins (SMs), phosphatidylcholines (PCs), phosphatidylserines (PS) and phosphatidylinositol's (PIs)[15,16]. This process also enhances the formation of immunomodulatory lipids (FIGs. 5B-5D) including lysophosphatidylcholine (LPC), in both the outer and inner membrane-associated lipid species.[17,18] Additional ceramide-specific lipidomics of the SENVs revealed that cFTS potentiates a membrane rearrangement process, inducing long-chain ceramide derivatives (FIG. 5C), which further enhanced the phosphatidylserine migration from the lower to the upper leaflets (FIG. 5D and FIG. 7).[19,20]

[0079] To correlate the properties of the material with biological activity, the cFTS-derived SENVs were fluorescently labeled with CyAm7 and injected into naïve C57BL/6J mice, with CyAm7-labeled senescent-like RBCs serving as a control (FIG. 5E and 5F). Forty-eight hours after injection, the animals were sacrificed and the organs were examined for splenic retention, with the SENVs displaying similar accumulation to the source senescent-like erythrocytes (FIG. 5E and 5F) illustrating how the surface characteristics of the materials are significantly more critical to splenic immunocyte recognition than size. Collectively, this data demonstrates that the proteolipids of SENVs afford both the "find me" and "eat me" signals promoting enhanced retention within the spleen (FIG. 2B).

[0080] PS lipid engineered SENVs (eSENVs) were produced by fusing SENVs with PS at three different PS lipid to SEG lipid ratios: +PS-eSENVs (24:1), ++PS-eSENVs (9.6:1), +++PS-eSENVs (5.3:1) (FIG. 8A).[23] Cryo-TEM analysis revealed the initial formation of heterogeneous PS-modified engineered SEGs (eSEGs, FIG. 8B), which were subsequently used to form unilamellar-spherical eSENVs (FIG. 8C) by an extrusion and sonication (ES) process.[24] To examine the effect of PS fusion with SENVs, coarse-grained molecular dynamics simulations (1.5 μs) were performed of the PS and SENVs fusion process, using the results of our lipidomics analysis to modify the input data for the RBC membrane bilayer as a starting point.[25] As shown in FIG. 8D and FIG. 9, the model predicted that given the composition of the bilayer, that the PS would partition in slightly greater quantities to the outer leaflet (10%), resulting in a 6% overall increase in outer leaflet PS at the highest doping percentage. Density maps and domain size

measurements (FIG. 8E, FIG. 8F, and FIG. 10), show that the domain size increased monotonously from about 1 nm to about 5 nm with an increasing concentration of PS in the simulated bilayer.

[0081] Each eSENVs was subsequently utilized to generate engineered senescent erythrocyte-like nanotheranostics (eSENTs) by the ES process (FIG. 8A). The eSENVs were coated upon fluorescently labeled poly(lactic-co-glycolic acid) nanoparticles using known strategies,[26,27], increasing the overall hydrodynamic diameter from about 104 nm to about 116 nm. TEM images demonstrated the formation of core-shell hybrid eSENTs featuring an 8-12 nm eSENV layer on the surface of the polymeric core (FIG. 8G). The process was also accomplished using micron sized PLGA nanoparticles to enable fluorescence microscopic visualization of the resulting materials, including the co-localization of the CyAm7-labeled proteolipids with the Cy5.5-labeled particles (FIG. 8H and FIG. 11). Preliminary cell uptake experiments with the eSENVs were also undertaken using RAW 264.7 murine macrophages, with the PS-doped materials demonstrating significant uptake by these model immune cells (FIG. 12).

[0082] **Study on the splenic retention of formulated nanomaterials in naïve C57BL/6J mice**

[0083] The splenic retention of the resultant eSENTs with differing PS to RBC lipid ratios was investigated in naïve C57BL/6J mice, with increases in fluorescence signal correlating with an increased degree of PS doping (FIGs. 13A-13C). It was next determined whether the observed splenic retention could be modulated by decreasing the dose of the material given, using the highest doped eSENTs versus the undoped or uncoated particles (FIGs. 13D-13F). At the lowest dose (3 mg/kg polymer) there was no significant difference between groups, whereas a doubling of the dose resulted in dramatically increased signal from the spleen in both coated groups (FIG. 13E and 13F). Only at the highest dose (30 mg/kg) did the eSENTs show a significant advantage over the control groups (FIG. 13E and 13F). Given the proposed mechanism of splenic accumulation, we hypothesized that the plasma elimination half-life would be relatively short, yet at this high dose, that was not observed ($t_{1/2}$= 7.8 h). To test whether a saturation effect was being observed, the particles were injected at half dose (15 mg/kg). Rapid plasma clearance ($t_{1/2}$ = 0.33 h, FIG. 13G and 13H) was observed, suggesting that for materials such as these, there may be an active, yet dynamic, process responsible for organ retention.

[0084] Assessment of the splenic accumulation on the cellular level was accomplished using flow cytometric and histological examination (FIG. 13I and FIG. 14). At the 6 mg/kg dose of particles, eSENTs were mainly localized to $F4/80^+$ macrophages (FIG. 13J), yet a 5-fold increase

in the injected dose (30 mg/kg), resulted in the material uptake switching mainly to $CD3^-$ T cells and $CD20^+$ B cells (FIG. 13K). Immunofluorescence microscopy further elucidated the intra-splenic accumulation of the particles as being mainly distributed throughout the marginal zone, yet with a minor amount escaping the meshes of the red pulp cords and distributing into the white pulp compartments (FIG. 13L-13N and FIG. 15). These results demonstrate that eSENTs can mimic the molecular proteo-lipids signals of dysfunctional and senescent erythrocytes. Further, the functional loss of CD47 and enhanced presence of PS lipids along with oxidized ceramide lipids on the eSENTs surfaces likely contributes to the enhanced retention and uptake of eSENTs by the various splenic immunocytes in the splenic subcellular compartments.

[0085] **Investigation of the splenic mediated immunomodulatory potential of developed nanomaterials in murine model of myocardial infarction**

[0086] To examine the potential immunomodulatory effect of spleen-localized nanomaterials, a murine model of myocardial infarction (MI) was utilized, as shown in FIG. 17A. The spleen serves as a reservoir for monocytes which migrate to damaged tissues in response to injury.[4,6] Inflammatory $Ly6C^{high}$ monocyte recruitment to the infarct area post-MI begins within the first 30 minutes of ischemic onset and peaks between 48-72 hours. These monocytes are predominantly recruited from the splenic reserve in a CCR2-dependent manner and differentiate into inflammatory macrophages upon reaching the injured tissue. These early inflammatory macrophages produce high levels of TNF-α, IL-1β, IL-6, MMPs, and reactive oxygen species. The number of $Ly6C^{low}$ monocytes recruited to the post-MI infarct area is initially negligible, slowly increases throughout the first week, and peaks at day 7. This recruitment changes the expression of cytokines, growth factors, pathogen-associated molecular patterns, and other microenvironment mediators shifting macrophage polarization to a less inflammatory and more reparative phenotype.[30,31] Systemic histone deacetylase (HDAC) inhibition via the intraperitoneal injection of suberoylanilide hydroxamic acid (SAHA), a pan-HDAC inhibitor, results in decreased inflammatory cytokines and MMPs, as well as earlier recruitment of reparative macrophages to the post-MI heart.[30,32] Importantly, this correlates with improved ventricular function and remodeling. If this observed effect is driven by the spleen, the localization of SAHA to this organ may serve to recapitulate the results from the systemic intervention (FIG. 16).

[0087] eSENTs containing SAHA within the polymeric core (SAHA-eSENT) were generated and their therapeutic efficacy examined, with non-drug-loaded particles (empty-eSENTS) and

saline injections servings as controls (FIG. 17A). Intravenous injection of the immunomodulatory SAHA-eSENTs 2 hours after MI significantly increased the number of $CD11b^{+}Ly6C^{-}$ monocytes/macrophages retained in the spleen (FIG. 17 and FIG. 18). Consistent with this, the number of $CD11b^{+}/F4/80^{+}$ macrophages present in the infarct at 24 hours post-MI were reduced by 3.5-fold when compared to the saline group (FIG. 17C and FIG. 18). While not significant, this trend continued at 72-hours post-MI (FIG. 17D and FIG. 18). Treatment with SAHA-eSENTs dramatically reduced infarct size by 14% at 72-hours post-MI when compared to empty-eSENTs or saline controls (FIG. 17E and 17F). Importantly, this significant reduction in ischemic damage was accomplished via a single injection of the SAHA-loaded eSENTs at a drug dose of 3 mg/kg, as compared to our previously reported 100 mg/kg/day required for a similar effect, where it was given systemically. Taken together, this study serves as the first of its kind to demonstrate the potential of the spleen-targeted inhibition of HDACs in the attenuation of post-MI ischemic damage and the promotion of infarct healing.

[0088] Hybrid nanoparticles bearing cell-derived coatings possess the ability to mimic the natural homing capabilities of their source materials.[8,27,33] The engineering of senescent erythrocyte membranes facilitates the retention of eSENTs within the spleen, enabling immunomodulation of the monocyte response in the setting of myocardial injury. Erythrocyte membranes and PLGA polymer, which have been used in clinics, were used as disclosed above, and therefore the disclosed nanomaterials can be readily translated from bedside to patients. As well, several other (auto)immune disorders have splenic manifestations, including systemic lupus erythematosus, for which there are no curative measures. Spleen-localized immunomodulation may play an important role in the provision of new therapeutic avenues for a wide range of diseases.

[0089] **Methods**

[0090] **Preparation of engineered senescent erythrocyte-like nanovesicles (SENVs) and engineered senescent erythrocyte-like nanovesicle-coated nanotheranostics (eSENTs).** The SENVs and eSENTs were prepared by a multistep process. First, the senescent erythrocyte-like ghosts (SEGs) were prepared from commercially purchased mouse RBCs (Fitzgerald Industries International, MA) by a controlled freezing (below -20 °C) and thawing (52 °C) process. Afterward, the size of the SEGs was reduced and reconstructed by sonication and extrusion, resulting in the formation of SENVs. The SENVs were further modified to produce engineered

senescent erythrocyte-like nanovesicles (eSENVs) by fusing the SENVs with additional "eat me" signaling phosphatidylserine lipids (PS) at three different PS to RBC lipid ratios (w/w, 24:1, 9.6:1, and 5.3:1). In parallel, amine-reactive near-infrared (NIR) fluorescent dyes (CyAm7 or CyAl5), which were provided by the MMRI chemistry core, were used to fluorescently label the eSENVs and poly(lactic-co-glycolic acid) (PLGA) polymers. The CyAl5-conjugated PLGA polymer was used for the preparation of control PLGA nanotheranostics (NTs) and the histone deacetylase inhibitor (HDAC) suberoylanilide hydroxamic acid (SAHA)-loaded nanotheranostic (SAHA-NTs). Finally, the eSENVs were coated on the control NTs or SAHA-loaded NTs via a sonication and extrusion process.[8,24,27]

[0091] Characterization of eSENVs and eSENTs. A Zetasizer NanoZS (Malvern Instruments, Malvern, UK) was used for the determination of all hydrodynamic diameters. The morphology of SEGs derived from the RBCs was initially visualized using scanning electron microscopy. 20 μl of RBC and SEG samples were fixed with 2% glutaraldehyde and dried with a series of ethanol and hexamethyldisilane (HMDS), and then imaged with a Phenom Pro/ProX tabletop scanning electron microscope (Phenom Pro/ProX, Phoenix, USA). Additionally, transmission electron microscopy images of the SEGs, eSEGs, and eSENTs were captured using a JEOL JSM-IT100-TEM (JEOL, Tokyo, Japan) at an accelerating voltage of 80-200 kV. The samples for TEM were prepared by adding 10 μL of SEGs, eSEGs, and eSENTs suspension was drop-cast on glow discharged copper grids with pure carbon support film and incubated for 10–15 min and then washed with ultrapure water. Finally, the sample grids were negatively stained with 1% uranyl acetate solution. The lipid vesicle morphology was analyzed by cryo-transmission electron microscopy (Tecnai 12 BioTwin). The grids were prepared by plunge-freezing on a Thermo Fisher Scientific Vitrobot Mk IV. Briefly, 3.5 μL of the sample was applied to a fresh plasma cleaned (PELCO easiGlow, Ted Pella) carbon coated Quantifoil R1.2/1.3 grid with a humidity setting of 100% and a temperature of 4-5 °C. The grids were imaged using a Philips Tecnai 12 BioTwin electron microscope equipped with a Gatan SC1000 CCD (Gatan, Inc, Pleasanton, CA), operated at 120 kV, under low dose conditions, with a 1-1.5s exposure time. Magnification ranges from 20,000-40,000x at the detector level. Additionally, the Keyence BZX700 (Keyance Corporation, Itasca, IL), an all-in-one fluorescence Microscope and Zeiss LSM-700 confocal fluorescence microscope (Oberkochen, Germany), and Zeiss Zen 8.1 software were used for further characterization of formulated materials.

[0092] **Proteomic Analysis.** The protein samples from senescent-like erythrocytes (SEs) and SENVs were prepared as mentioned earlier.[24] 100 μg of proteins were digested using the filter aided sample preparation (FASP) method.[34] In-solution proteins were reduced and denatured with dithiothreitol (DTT) and sodium dodecyl sulfate (SDS) (Fisher, BP2436), mixed with urea (Fisher, BP169) to 8 M, and concentrated on a 10 kilodalton (kDa) molecular weight cutoffs (MWCO) membrane filter (Pall, OD010C34). Cysteine residues were alkylated using iodoacetamide at room temperature in a dark location for 25 min. The proteins were rinsed with urea and ammonium bicarbonate solutions and digested overnight at 37°C using trypsin at a ratio of 1:100. The resulting peptides were recovered from the filtrate with the addition of 50 μL of 1% Trifluoroacetic acid (TFA). A 10-μg aliquot was desalted on 2-core MCX stop and go extraction tips (Stage Tips) (3M, 2241). The stage tips were activated with acetonitrile (ACN) followed by 3% ACN with 0.1% TFA. Next, samples were applied, followed by two washes with 3% ACN with 0.1% TFA, and one wash with 65% ACN with 0.1% TFA. Peptides were eluted with 75 μL of 65% ACN with 5% ammonium hydroxide (NH_4OH) (Millipore, 5.33003), and dried.

[0093] **LC-MS methods.** Samples were dissolved in 20 to 75 μL of water containing 2% ACN and 0.5% formic acid. Two μL (0.3 – 0.8 μg) were injected onto a pulled tip nano-LC column (New Objective, FS360-75-10-N) with 75 μm inner diameter packed to 25 cm with 3 μm, 120 Å, C18AQ particles (Dr. Maisch, r13.aq.0001). The column was maintained at 45°C with a column oven (Sonation GmbH, PRSO-V2). The peptides were separated using a 60-minute gradient from 3 – 28% ACN over 60 min, followed by a 7 min ramp to 85% ACN. The column was connected in line with an Orbitrap Lumos (Thermo) via a nanoelectrospray source operating at 2.2 kV. The mass spectrometer was operated in data-dependent top speed mode with a cycle time of 2.5 s. MS1 scans were collected at 120000 resolutions with an AGC target of 6.0E5 and a maximum injection time of 50 ms. Higher energy collisional dissociation (HCD) fragmentation was used followed by MS2 scans in the ion trap with automatic gain control (AGC) target 2.0E3 and 35 ms maximum injection time.

[0094] **Database searching and label-free quantification.** The MS data was searched using Byonic 2.11.0 (Protein Metrics, San Carlos, CA) against the M. Musculus Swiss-Prot reference proteome database (2019; 17,027 entries). Enzyme specificity for trypsin allows up to 2 missed cleavages. Precursor and product ion mass tolerances were 10 parts per million (ppm) and 0.6 Da, respectively. Cysteine carbamidomethylation was set as a fixed modification. Methionine

oxidation was set as a variable modification. The output was filtered using false discovery rates (FDR) set to 0.01. Quantitative information was extracted from MS1 spectra of all identified peptides using an in-house R script based on MSn base package as the AUC of the extracted ion current (XIC) of all remaining peptides after the alignment of the chromatographic runs.[35]

[0095] **Lipidomics analysis.** Glycerophospholipids and ceramide lipidomic analysis was performed by the Kansas Lipidomics Core and the Medical University of South Carolina to investigate the lipid scrambling during the SENVs preparation process. The SENVs were prepared as mentioned above and after processing, GPL and ceramide lipid extraction were performed as per the procedure mentioned earlier[16]. Lipid analysis data were acquired with a Xevo TQS electrospray ionization mass spectrometer in direct infusion mode at 30 uL per min at KLC and MUSC lipidomics core. Data were smoothed and centroided, exported to Excel files, and uploaded to Lipidome DB Data Calculation Environment (http://lipidome.bcf.ku.edu:8080/Lipidomics/index.jsp) for isotopic deconvolution and comparison to internal standards. Additionally, data were corrected to SPLASH standards (Avanti Polar Lipids, Alabaster AL). Furthermore, advanced analyses of endogenous bioactive sphingolipids were performed by the Analytical Unit in the Lipidomics Shared Resource at MUSC. Preparation of samples and endogenous bioactive sphingolipid lipidomics analyses were performed on a Thermo Fisher TSQ Quantum liquid chromatography/triple-stage quadrupole mass spectrometer system operating in a multiple reaction monitoring (MRM)–positive ionization mode as previously described.[36]

[0096] **Molecular Dynamics Simulation.** MD simulations were performed on a GPU accelerated computer workstation using GROMACS Version 5.1.4. The computer is equipped with a 40 Core central processing unit (CPU, Intel(R) Xeon(R) CPU E5-2630 v4 @ 2.20GHz), 130 GB random access memory (RAM) and three graphic processing units (GPU, 2 × NVIDIA 1080 TDI + 1 × GeForce GT 730). Three membrane models were designed using the CHARMMGUI MARTINI membrane builder (http://charmm-gui.org/)[1,2] and the MARTINI forcefield 2.2.[2] A model of a red blood cell membrane was designed using our measured lipidomic data and bases on a previously published model.[25] Each experimentally determined lipid species was mapped to available models in the Martini 2.2 force field: An error coefficient was calculated for every available model lipid describing the difference in the tail length and the difference in tail saturation between the model and the experimental lipid. The MARTINI lipid with the smallest

error value was then used for each experimental lipid respectively. The membrane's asymmetry, *i.e.*, the unequal distribution of lipids among both leaflets, was adjusted by using values for the compositional asymmetry published in previous coarse-grained plasma membrane simulations.[37] For instance, from all simulated PC lipids, 75 % were placed in the upper and 25 % were placed in the lower leaflet. Three SEGs: PS hybrid nanosystems were created corresponding to red blood cell membranes engineered with PS lipids to SEGs lipids ratios (w/w, 24:1, 9.6:1, and 5.3:1 respectively. Each system represents a membrane patch of approximately 26 × 26 nm with 1777 lipid molecules on each leaflet and 37 water molecules per lipid representing a fully hydrated state of the membrane (FIG. 9 and FIG. 10). We note that the total amount of lipid per simulated box was kept constants for all simulated systems. Simulations were equilibrated for 5 ns using an NPT ensemble (constant pressure and temperature) and then run for 1.5 μs. Only the final 500 ns were analyzed, after affirming the membrane had reached equilibrium by determining the area per lipid. All simulations used a 1 fs time step, a short-range van der Waal cutoff of 1.1 nm, a potential shift-verlet coulomb modifier and periodic boundary conditions were applied to all spatial directions. Neighbor lists were updated in intervals of 20 steps. The temperature coupling was controlled by a v-rescale thermostat at a constant pressure of 1 bar using Parrinello– Rahman semi-isotropic weak coupling (τ = 12 ps; compressibility β = 3 10−4 bar−1). The area per lipid was determined for all simulations by dividing the number of lipids in the upper and lower leaflet respectively by the average lateral area of the simulation box. PS lipid density maps were calculated using the gmx densmap function provided by GROMACS. For this purpose, the phosphate group of DOPS was indexed for each leaflet respectively and the density map was averaged over the last microsecond of the simulation. The domain size of PS-rich areas was determined by first binarizing the calculated density map using a threshold particle density of 0.3 and manually selecting 40 points on the edges of the observed clusters. The domain size was then calculated by measuring the distance between the points, respectively, and are graphed.

[0097] **In vivo biodistribution studies in healthy mice. All animal procedures complied with the guidelines of the MMRI Institutional Animal Care and Use Committee.** SENV and sRBC biodistribution were visualized and quantified on an IVIS Spectrum fluorescence imager (Perkin Elmer) with the proper excitation and emission filters to match the CyAm7 fluorescence profile. Similarly, eSENTs pharmacokinetics and biodistribution were visualized and quantified on an IVIS Spectrum fluorescence imager (Perkin Elmer) with the proper excitation and emission filters

to match the eSENTs fluorescence profile. For all in vivo experiments, the eSENTs were injected intravenously via the mouse tail vein at time = 0 at a concentration of 3 to 30 mg particle/kg body weight. For blood half-life studies, 20 μL of whole blood was collected and then mixed with an equal volume of anticoagulant ADC and loaded into a black clear bottom 96 well plate for each timepoint. For biodistribution studies, animals were sacrificed 48 h after injection of eSENTs. Tissues were then resected immediately imaged. Histological analyses and microscopic imaging were carried out on harvested tissues that were formalin-fixed and paraffin-embedded (FFPE) or flash-frozen fresh tissue. Hematoxylin and eosin staining was carried out on FFPE tissue. All micrographs were captured on a Nikon Ni-E upright epifluorescence/brightfield microscope at a total magnification of 200-400x. For immunofluorescence analysis, eSENT uptake and specific immune cells were visualized utilizing 6 μm fresh-frozen sections and antibodies: T cells (anti-CD3 ab: AF488 Anti-mouse- Biolegend cat:100212); B cells (CD20 ab: BV421anti-mouse-Biolegend cat:150405); macrophages (F480 ab: rat anti mouse-bio-rad ct:mca497ga). All stained sections were mounted in Dako fluorescence mounting media. Depending on secondary antibody fluorophores, nuclear staining was achieved with either DAPI or Sytox Green. Flow cytometry analysis of eSENT uptake in splenic cells was performed on a Symphony A3 FACS (BD). Single-cell suspensions of the spleen were prepared as previously described[38]. Briefly, freshly resected spleens were macerated in cold PBS and pushed through a 70 μm filter to produce a single-cell suspension. After which the samples underwent RBC lysis, and remaining cells were fixed and stained with a vital stain (ZombieYellow) and a cocktail of antibodies: CD3-AF488 (Biolegend 100212), CD20-BV421 (Biolegend 150405), CD11b-BV785 (Biolegend 101243), CD68-PE/Cy7 (Biolegend 137015), F4/80-PE (Biolegend 123109). Results were analyzed using Flowjo software (v10.7.1, BD).

[0098] **In vivo therapeutic efficacy in myocardial infarction (MI) mouse model.** The MI procedure was performed as reported in our earlier study.[30] Briefly, the left anterior descending coronary artery was permanently ligated on 12–15-week-old CD1 male mice. Mice were anesthetized (isoflurane, 2 %; O_2 2 L/min), intubated, and ventilated. A left thoracotomy was performed in the fourth intercostal space and the left anterior descending coronary artery was visualized and ligated just distal to its main bifurcation using 7.0 Prolene. MI was confirmed by left ventricular (LV) blanching and ST-segment elevation on the electrocardiogram. Mice were randomly assigned to the treatment cohort. Nanotheranostics loaded with or without SAHA were

administered in a saline suspension at a dose of 30 mg particle/kg body weight, 2 hrs post-MI via tail vein injection. Animals were euthanized at 24 or 72 hrs post-MI and the heart was excised for either 2,3,5-Triphenyl tetrazolium chloride (TTC) staining or flow cytometry. All the animal experiments were performed according to protocols approved by the Medical University of South Carolina Institutional Animal Care and Use Committee and by U.S. National Institute of Health (NIH) guidelines.

[0099] **TTC Staining and Infarct Measurement.** Upon excision, hearts were rinsed with PBS. The whole LV was cut into 2 mm slices using a heart matrix (Zivic Instruments, HSMA001-1). Slices were incubated in a 1% TTC (Sigma, T8877-100G) in PBS solution for 15 minutes at 37 °C, rinsed in PBS, and tissue section images were captured using an Epson Perfection 4490 Photo scanner. ImageJ v1.53 software was used to measure areas of infarct and total LV. Using the digital images, the total LV area and infarct area were manually traced and measured by ImageJ. Infarct size, as a percentage of total LV size, was calculated by dividing the sum of the infarct area of all sections by the sum of the total LV area of all sections and multiplying by 100.

[0100] **Cell preparation and flow cytometry.** Single-cell suspension was prepared from hearts and spleen as previously described.[39] Briefly, spleens were mashed with a syringe plunger through a 70 μm cell strainer and collected in ice-cold PBS. Cells were washed in PBS (300g, 10 minutes). Hearts were harvested after transcardial perfusion with 20 ml of PBS and infarct tissue was minced with fine scissors before digestion in a cocktail of 600 units/ml collagenase type II (Gibco™,17101-015) and 60 U/mL DNase I (Roche, 10104159001) in Hank's Buffered Saline Solution (with calcium chloride and magnesium chloride, Gibco, 14025-092) at 37°C. Digested tissues were triturated and filtered with a 30 μm pre-separation filter (Miltenyi, 130-041-407), washed (300 g,10 min), and resuspended in PEB buffer (Miltenyi, 130-091-222 and Miltenyi, 130-091-376). Heart and spleen samples were then incubated with Red Blood Cell Lysis Solution (Miltenyi, 130-094-183) to remove red blood cells, FcR-Blocking Reagent (Miltenyi, 130-092-575) to block non-specific reactions, and Live/Dead viability Dye (Invitrogen, L34959) to identify live cells. Finally, samples were stained with the following conjugated antibody panel: CD11b-FITC (BD Pharmingen, M1/70, 553310), F4/80-PE-Cy7 (BioLegend, BM8, 123114), and Ly6C (PE-conjugated, REA796, Miltenyi Biotec). Flow cytometry analysis was performed using MACSQuant Analyzer 10, results were analyzed using Flowlogic™ software (Invai technologies), and gates were set based on Fluorescence Minus One control.

[0101] **Statistical Analysis.** Student T-test was used for statistical testing in the physiochemical characterization of nano formulations and their biodistribution studies. Kruskal Wallis's non-parametric analysis with Dunn's multiple comparison post hoc test was used for statistical testing in cardiac infarct immunomodulation studies.

[0102] **Synthesis of antisense oligonucleotides (ASOs)**. Antisense oligonucleotides (ASO), which were designed to silence the synthesis of CD-47, were synthesized and encapsulated in nanovesicles of the present disclosure. The sequence 5' - AAGGACGTAGCCCAGCACTT - 3' (SEQ ID NO: 1) was synthesized with phosphorothioate bonds connecting the nucleotides.

[0103] **Synthesis of delivery vehicles.** PLGA and a cationic component (1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) – 18:1 TAP) were dissolved in dichloromethane. To this, ASO dissolved in TE buffer was added dropwise, and the solution was emulsified with a probe sonicator. The emulsified mixture was added to an aqueous solution containing the reconstituted proteolipid powder. This solution was also emulsified using the probe sonicator to generate a double emulsion, which was subsequently added to an aqueous solution of additional senescent erythrocyte-derived proteolipids and stirred for 4-5 hours to allow the organic solvent to evaporate and the particles to harden.

[0104] **In vitro assay.** Initial in vitro assays in 4T1 cells showed uptake of the synthesized materials. Flow cytometry was used to detect CD-47 using a fluorescein-labeled anti-CD47 antibody. A significant decrease in CD-47 levels was detected by flow cytometry.

[0105] **In vivo assay.** Naïve C57Bl/6 mice were treated with eSENTS containing either the CD47 silencing ASO (SEQ ID NO: 1), a scrambled control, or else remained uninjected. Seventy-two hours after injection, the mice were sacrificed and the spleen, lung, liver, and kidneys of each mouse were removed and digested to yield a single cell suspension. The single cell suspension was labeled with a fluorescein-modified anti-CD47 antibody and the fluorescence intensity per cell within each organ analyzed by flow cytometry. The resultant histograms generated for each organ were compared across groups (FIG. 19A). Now referring to FIG. 19B, depicted is a comparison of the relative CD-47 levels across groups in the spleen. The right peak is the spleen of a mouse that received the eSENT containing the control ASO, the middle peak is of a mouse that received the CD-47 silencing ASO, and the left peak is a group that did not receive the fluorescent anti-CD47 antibody. The leftward shift of the CD-47-silencing ASO treated population indicates a decrease in CD47 per cell. Thus, FIG. 19A and FIG. 19B show that the spleen-targeted materials

dramatically decrease the production of CD-47 in splenocytes, with negligible effects in other tissues.

[0106] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the disclosure. As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. It will be further understood that the terms "include" (and any form of include, such as "includes" and "including"), "have" (and any form of have, such as "has" and "having"), "include" (and any form of include, such as "includes" and "including"), "contain" (and any form contain, such as "contains" and "containing"), and any other grammatical variant thereof, are open-ended linking verbs. As a result, a method or article that "includes", "has", "includes" or "contains" one or more steps or elements possesses those one or more steps or elements, but is not limited to possessing only those one or more steps or elements. Likewise, a step of a method or an element of an article that "includes", "has", "includes" or "contains" one or more features possesses those one or more features, but is not limited to possessing only those one or more features.

[0107] Terms like "obtainable" or "definable" and "obtained" or "defined" are used interchangeably. This, for example, means that, unless the context clearly dictates otherwise, the term "obtained" does not mean to indicate that, for example, an embodiment must be obtained by, for example, the sequence of steps following the term "obtained" though such a limited understanding is always included by the terms "obtained" or "defined" as a preferred embodiment.

[0108] Approximating language, as used herein throughout disclosure, may be applied to modify any quantitative representation that could permissibly vary without resulting in a change in the basic function to which it is related. Accordingly, a value modified by a term or terms, such as "about" or "substantially," is not limited to the precise value specified. For example, these terms can refer to an amount that is within ±10% of the recited value, an amount that is within ±5% of the recited value, less than or equal to ±2%, an amount that is within ±1% of the recited value, an amount that is within ±0.5% of the recited value, an amount that is within ±0.2% of the recited value, an amount that is within ±0.1% of the recited value, or an amount that is within ±0.05% of the recited value. In some instances, the approximating language may correspond to the precision of an instrument for measuring the value.

[0109] All publications cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.

[0110] Subject matter incorporated by reference is not considered to be an alternative to any claim limitations, unless otherwise explicitly indicated.

[0111] Where one or more ranges are referred to throughout this specification, each range is intended to be a shorthand format for presenting information, where the range is understood to encompass each discrete point within the range as if the same were fully set forth herein.

[0112] While several aspects and embodiments of the present disclosure have been described and depicted herein, alternative aspects and embodiments may be affected by persons having ordinary skill in the art to accomplish the same objectives. Accordingly, this disclosure and the appended claims are intended to cover all such further and alternative aspects and embodiments as fall within the true spirit and scope of the present disclosure.

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[0153] Various non-limiting implementations of this disclosure are described below:

[0154] [Implementation A] A method of delivering a composition to a spleen of a subject, comprising systemically administering the composition to the subject, wherein the composition comprises a nanovesicle made by a process, the process comprising:

freezing erythrocytes, thawing the erythrocytes, sonicating the thawed erythrocytes, and extruding the sonicated erythrocytes, wherein

freezing comprises exposing the erythrocytes to a temperature of about 0 °C or lower,

thawing comprises exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes,

sonicating comprises exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes, and

extruding comprises successively passing the sonicated erythrocytes through a series of filters each comprising a filter diameter, wherein each filter comprises a larger filter diameter than a subsequent filter.

[0155] [Implementation B] A method according to Implementation [A] above, or according to other Implementations of the disclosure, wherein the sonicated erythrocytes are extruded in the presence of one or more added lipid.

[0156] [Implementation C] A method according to Implementation [A] or [B] above, or according to other Implementations of the disclosure, wherein a ratio of weight of erythrocytes to weight of the one or more added lipid during the extruding is from about 1:24 to about 1:1.

[0157] [Implementation D] A method according to Implementation [B] or [C] above, or according to other Implementations of the disclosure, wherein at least one of the one or more added lipid is a natural lipid or a synthetic lipid.

[0158] [Implementation E] A method according to Implementation [D] above, or according to other Implementations of the disclosure, wherein the synthetic lipid comprises a lipid attached to one or more targeting ligand.

[0159] [Implementation F] A method according to Implementation [E] above, or according to other Implementations of the disclosure, wherein the one or more targeting ligand is a peptide, an antibody, or an aptamer, or a combination thereof.

[0160] [Implementation G] A method according to Implementation [E] or [F] above, or according to other Implementations of the disclosure, wherein the one or more targeting ligand targets a cell type, wherein the cell type is selected from T cell, B cell, monocyte, macrophage, granulocyte, dendritic cell, endothelial cell, (myo)fibroblast, and cancer cell.

[0161] [Implementation H] A method according to any one of Implementations [D]-[G] above, or according to other Implementations of the disclosure, wherein the synthetic lipid is selected from 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol).

[0162] [Implementation I] A method according to Implementation [D] above, or according to other Implementations of the disclosure, wherein the natural lipid is selected from a sphingolipid, a phosphatidylglycerol, a lysophosphatidylcholine, a phosphatidyl serine, a ceramide, an eicosanoid, a phosphoinositide, and a lysophosphatidic acid.

[0163] [Implementation J] A method according to any one of Implementations [A]-[I] above, or according to other Implementations of the disclosure, wherein the process further comprises encapsulating nanoparticles with the nanovesicles, wherein encapsulating comprises sonicating the nanovesicles in the presence of the nanoparticles and sonicating comprises exposing the nanovesicles and the nanoparticles to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 2 minutes to about 3 minutes.

[0164] [Implementation K] A method according to Implementation [J] above, or according to other Implementations of the disclosure, wherein a ratio of weight of nanoparticles to weight of nanovesicles during the encapsulating is from about 1:1 to about 1:0.1.

[0165] [Implementation L] A method according to Implementation [J] or [K] above, or according to other Implementations of the disclosure, wherein the nanoparticles comprise one or more of polymeric nanoparticles, carbon nanoparticles, composite nanoparticles, or inorganic nanoparticles, or a combination thereof.

[0166] [Implementation M] A method according to Implementation [L] above, or according to other Implementations of the disclosure, wherein the nanoparticles are selected from iron oxide, gold, silver, silica, nanoceria, quantum dots, up conversion nanoparticles, calcium phosphate, polystyrene, polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, chitosan, alginate, poly(methyl methacrylate), poly(β-amino ester), ethyl cellulose, semiconducting polymer nanoparticles, carbon nanotubes, nanographite, carbon nanohorn, carbon black nanoparticle, graphene, nanodiamond, and carbon dots, or a combination thereof.

[0167] [Implementation N] A method according to any one of Implementations [A]-[M] above, or according to other Implementations of the disclosure, wherein the process for making the nanovesicles further comprises loading the nanovesicle with one or more therapeutic compound.

[0168] [Implementation O] A method according to Implementation [N] above, or according to other Implementations of the disclosure, wherein the one or more therapeutic compound comprises an antibiotic, an antifungal, an antigen, an immunosuppressant, a chemotherapeutic, a protein functional modulator, a pain management compound, an oligonucleotide, a gene editor, or a peptide, or a combination thereof.

[0169] [Implementation P] A method according to Implementation [N] or [O] above, or according to other Implementations of the disclosure, wherein at least one of the one or more

therapeutic compound is selected from suberoylanilide hydroxamic acid, Vancomycin, Amphotericin B, Ketoconazole, a tumor peptide, a tumor antigen, a microbial protein, a peptide, a polysaccharide, an immunogenic peptide, a toxin, Dexamethasone, Cyclosporine A, Tacrolimus, Rapamycin, docetaxel, irinotecan, doxorubicin, gemcitabine, cisplatin, paclitaxel, CCG1423, GSK269962, MG-132, INCB8761, Bupivacaine, Ketamine, Morphine, RNA, DNA, CRISPR/Cas9, monoclonal antibodies, growth factors, and functional enzymes.

[0170] [Implementation Q] A method according to any one of Implementations [A]-[P] above, or according to other Implementations of the disclosure, wherein the subject has an autoimmune disorder, has a monocyte-mediated condition, or experienced a myocardial infarction, wherein the autoimmune disorder is chosen from Lupus, multiple sclerosis (MS), and psoriasis, and wherein the monocyte-mediated condition is chosen from atherosclerosis and ischemia.

[0171] [Implementation R] A method according to any one of Implementations [A]-[Q] above, or according to other Implementations of the disclosure, wherein the administering is intravenously, intraperitoneally, enterally, by inhalation, intranasally, sublingually, intramuscularly, or subcutaneously.

[0172] [Implementation S] A method according to any one of Implementations [A]-[R] above, or according to other Implementations of the disclosure, wherein the subject experienced a myocardial infarction and the administering is within about two hours after the myocardial infarction.

[0173] [Implementation T] A method according to any one of Implementations [A]-[S] above, or according to other Implementations of the disclosure, wherein the administering comprises treating the subject for a myocardial infarction.

[0174] [Implementation U] A method according to Implementation [T] above, or according to other Implementations of the disclosure, wherein the treating comprises reducing size of an infarct in the subject compared to infarct size of another individual who experienced a myocardial infarction and was not administered the nanovesicles.

[0175] [Implementation V] A method according to Implementation [U] above, or according to other Implementations of the disclosure, wherein the reducing is determined 72 hours after the myocardial infarction and comprises an about 15% reduction in size.

[0176] [Implementation W] A method of preparing nanovesicles, comprising freezing erythrocytes, thawing the erythrocytes, sonicating the thawed erythrocytes, and extruding the sonicated erythrocytes, wherein

freezing comprises exposing the erythrocytes to a temperature of about 0 °C or lower,

thawing comprises exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes

sonicating comprises exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes to form nanovesicles, and

extruding comprises successively passing the sonicated erythrocytes through a series of filters each comprising a filter diameter, wherein each filter comprises a larger filter diameter than a subsequent filter.

[0177] [Implementation X] A method according to Implementation [W] above, or according to other Implementations of the disclosure, wherein the sonicated erythrocytes are extruded in the presence of one or more added lipid.

[0178] [Implementation Y] A method according to Implementation [X] above, or according to other Implementations of the disclosure, wherein a ratio of weight of erythrocytes to weight of the one or more added lipid during the extruding is from about 1:24 to about 1:1.

[0179] [Implementation Z] A method according to Implementation [X] or [Y] above, or according to other Implementations of the disclosure, wherein at least one of the one or more added lipid is a natural lipid or a synthetic lipid.

[0180] [Implementation AA] A method according to Implementation [Z] above, or according to other Implementations of the disclosure, wherein the synthetic lipid comprises a lipid attached to one or more targeting ligand.

[0181] [Implementation AB] A method according to Implementation [AA] above, or according to other Implementations of the disclosure, wherein the one or more targeting ligand is a peptide, an antibody, or an aptamer, or a combination thereof.

[0182] [Implementation AC] A method according to Implementation [AA] or [AB] above, or according to other Implementations of the disclosure, wherein the one or more targeting ligand

targets a cell type, wherein the cell type is selected from T cell, B cell, monocyte, macrophage, granulocyte, dendritic cell, endothelial cell, (myo)fibroblast, and cancer cell.

[0183] [Implementation AD] A method according to any one of Implementations [Z]-[AC] above, or according to other Implementations of the disclosure, wherein the synthetic lipid is selected from 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol).

[0184] [Implementation AE] A method according to Implementation [Z] above, or according to other Implementations of the disclosure, wherein the natural lipid is selected from a sphingolipid, a phosphatidylglycerol, a lysophosphatidylcholine, a phosphatidyl serine, a ceramide, an eicosanoid, a phosphoinositide, and a lysophosphatidic acid, or a combination thereof.

[0185] [Implementation AF] A method according to any one of Implementations [W]-[AC] above, or according to other Implementations of the disclosure, wherein the process further comprises encapsulating nanoparticles with the nanovesicles, wherein encapsulating comprises sonicating the nanovesicles in the presence of the nanoparticles and sonicating comprises exposing the nanovesicles and the nanoparticles to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 2 minutes to about 3 minutes.

[0186] [Implementation AG] A method according to Implementation [AF] above, or according to other Implementations of the disclosure, wherein a ratio of weight of nanoparticles to weight of nanovesicles during the encapsulating is from about 1:1 to about 1:0.1.

[0187] [Implementation AH] A method according to Implementation [AF] or [AG] above, or according to other Implementations of the disclosure, wherein the nanoparticles comprise one or more of polymeric nanoparticles, carbon nanoparticles, composite nanoparticles, or inorganic nanoparticles, or a combination thereof.

[0188] [Implementation AI] A method according to Implementation [AH] above, or according to other Implementations of the disclosure, wherein the nanoparticles are selected from iron oxide, gold, silver, silica, nanoceria, quantum dots, up conversion nanoparticles, calcium phosphate, polystyrene, polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, chitosan, alginate, poly(methyl methacrylate), poly(β-amino ester), ethyl cellulose, semiconducting polymer nanoparticles, carbon nanotubes, nanographite, carbon nanohorn, carbon black nanoparticle, graphene, nanodiamond, and carbon dots, or a combination thereof.

[0189] [Implementation AJ] A method according to any one of Implementations [W]-[AI] above, or according to other Implementations of the disclosure, wherein the process for making the nanovesicle further comprises loading the nanovesicle with one or more therapeutic compound.

[0190] [Implementation AK] A method according to Implementation [AJ] above, or according to other Implementations of the disclosure, wherein the one or more therapeutic compound comprises an antibiotic, an antifungal, an antigen, an immunosuppressant, a chemotherapeutic, a protein functional modulator, a pain manager, an oligonucleotide, a gene editor, or a peptide, or a combination thereof.

[0191] [Implementation AL] A method according to Implementation [AJ] or [AK] above, or according to other Implementations of the disclosure, wherein at least one of the one or more therapeutic compound is selected from suberoylanilide hydroxamic acid, Vancomycin, Amphotericin B, Ketoconazole, a tumor peptide, a tumor antigen, a microbial protein, a peptide, a polysaccharide, an immunogenic peptide, a toxin, Dexamethasone, Cyclosporine A, Tacrolimus, Rapamycin, docetaxel, irinotecan, doxorubicin, gemcitabine, cisplatin, paclitaxel, CCG1423, GSK269962, MG-132, INCB8761, Bupivacaine, Ketamine, Morphine, RNA, DNA, CRISPR/Cas9, monoclonal antibodies, growth factors, and functional enzymes.

[0192] [Implementation AM] A plurality of nanovesicles made by a process, wherein the process comprises freezing erythrocytes, thawing the erythrocytes, sonicating the thawed erythrocytes, and extruding the sonicated erythrocytes, wherein:

freezing comprises exposing the erythrocytes to a temperature of about 0 °C or lower;

thawing comprises exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes;

sonicating comprises exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes; and

extruding comprises successively passing the sonicated erythrocytes through a series of filters each comprising a filter diameter, wherein each filter comprises a larger filter diameter than a subsequent filter.

[0193] [Implementation AN] A method according to Implementation [AM] above, or according to other Implementations of the disclosure, wherein the sonicated erythrocytes are extruded in the presence of one or more added lipid.

[0194] [Implementation AO] A method according to Implementation [AN] above, or according to other Implementations of the disclosure, wherein a ratio of weight of erythrocytes to weight of the one or more added lipid during the extruding is from about 1:24 to about 1:1.

[0195] [Implementation AP] A method according to Implementation [AN] or [AO] above, or according to other Implementations of the disclosure, wherein at least one of the one or more added lipid is a natural lipid or a synthetic lipid.

[0196] [Implementation AQ] A method according to Implementation [AP] above, or according to other Implementations of the disclosure, wherein the synthetic lipid comprises a lipid attached to one or more targeting ligand.

[0197] [Implementation AR] A method according to Implementation [AQ] above, or according to other Implementations of the disclosure, wherein the one or more targeting ligand is a peptide, an antibody, or an aptamer, or a combination thereof.

[0198] [Implementation AS] A method according to Implementation [AQ] or [AR] above, or according to other Implementations of the disclosure, wherein the one or more targeting ligand targets a cell type, wherein the cell type is selected from T cell, B cell, monocyte, macrophage, granulocyte, dendritic cell, endothelial cell, (myo)fibroblast, and cancer cell.

[0199] [Implementation AT] A method according to any one of Implementations [AP]-[AS] above, or according to other Implementations of the disclosure, wherein the synthetic lipid is selected from 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol).

[0200] [Implementation AU] A method according to Implementation [AP] above, or according to other Implementations of the disclosure, wherein the natural lipid is selected from a sphingolipid, a phosphatidylglycerol, a lysophosphatidylcholine, a phosphatidyl serine, a ceramide, an eicosanoid, a phosphoinositide, and a lysophosphatidic acid.

[0201] [Implementation AV] A method according to any one of Implementations [AM]-[AU] above, or according to other Implementations of the disclosure, wherein the process further comprises encapsulating nanoparticles with the nanovesicles, wherein encapsulating comprises sonicating the nanovesicles in the presence of the nanoparticles and sonicating comprises

exposing the nanovesicles and the nanoparticles to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 2 minutes to about 3 minutes.

[0202] [Implementation AW] A method according to Implementation [AV] above, or according to other Implementations of the disclosure, wherein a ratio of weight of nanoparticles to weight of nanovesicles during the encapsulating is from about 1:1 to about 1:0.1.

[0203] [Implementation AX] A method according to Implementation [AV] or [AW] above, or according to other Implementations of the disclosure, wherein the nanoparticles comprise one or more of polymeric nanoparticles, carbon nanoparticles, composite nanoparticles, or inorganic nanoparticles, or a combination thereof.

[0204] [Implementation AY] A method according to Implementation [AX] above, or according to other Implementations of the disclosure, wherein the nanoparticles are selected from iron oxide, gold, silver, silica, nanoceria, quantum dots, up conversion nanoparticles, calcium phosphate, polystyrene, polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, chitosan, alginate, poly(methyl methacrylate), poly(β-amino ester), ethyl cellulose, semiconducting polymer nanoparticles, carbon nanotubes, nanographite, carbon nanohorn, carbon black nanoparticle, graphene, nanodiamond, and carbon dots, or a combination thereof.

[0205] [Implementation AZ] A method according to any one of Implementations [AM]-[AY] above, or according to other Implementations of the disclosure, wherein the process for making the nanovesicles further comprises loading the nanovesicle with one or more therapeutic compound.

[0206] [Implementation BA] A method according to Implementation [AZ] above, or according to other Implementations of the disclosure, wherein the one or more therapeutic compound comprises an antibiotic, an antifungal, an antigen, an immunosuppressant, a chemotherapeutic, a protein functional modulator, a pain manager, an oligonucleotide, a gene editor, or a peptide, or a combination thereof.

[0207] [Implementation BB] A method according to Implementation [AZ] or [BA] above, or according to other Implementations of the disclosure, wherein at least one of the one or more therapeutic compound is selected from suberoylanilide hydroxamic acid, Vancomycin, Amphotericin B, Ketoconazole, a tumor peptide, a tumor antigen, a microbial protein, a peptide, a polysaccharide, an immunogenic peptide, a toxin, Dexamethasone, Cyclosporine A, Tacrolimus, Rapamycin, docetaxel, irinotecan, doxorubicin, gemcitabine, cisplatin, paclitaxel,

CCG1423, GSK269962, MG-132, INCB8761, Bupivacaine, Ketamine, Morphine, RNA, DNA, CRISPR/Cas9, monoclonal antibodies, growth factors, and functional enzymes.

[0208] [Implementation BC] A method of delivering a composition to a spleen of a subject, comprising systemically administering the composition to the subject, wherein the composition comprises a nanovesicle made by a process, the process comprising:

freezing erythrocytes, thawing the erythrocytes, sonicating the thawed erythrocytes, freeze-drying the sonicated erythrocytes to obtain a proteolipid powder, and reconstituting the proteolipid powder, wherein

freezing comprises exposing the erythrocytes to a temperature of about 0 °C or lower,

thawing comprises exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes, and

sonicating comprises exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes.

[0209] [Implementation BD] A method according to Implementation [BC] above, or according to other Implementations of the disclosure, wherein freeze-drying the sonicated erythrocytes comprises mixing the sonicated erythrocytes with a cryoprotectant.

[0210] [Implementation BE] A method according to Implementation [BC] or [BD] above, or according to other Implementations of the disclosure, wherein reconstituting the proteolipid powder comprises dissolving the proteolipid powder in an organic solvent and then mixing with an aqueous solvent.

[0211] [Implementation BF] A method according to any one of Implementations [BC]-[BE] above, or according to other Implementations of the disclosure, wherein the proteolipid powder is reconstituted in the presence of one or more added lipid.

[0212] [Implementation BG] A method according to Implementation [BF] above, or according to other Implementations of the disclosure, wherein a ratio of weight of proteolipid powder to weight of the one or more added lipid during reconstituting is from about 24:1 to about 5.3:1.

[0213] [Implementation BH] A method according to Implementation [BF] or [BG] above, or according to other Implementations of the disclosure, wherein the one or more added lipid is a natural lipid or a synthetic lipid, or a combination thereof.

[0214] [Implementation BI] A method according to Implementation [BH] above, or according to other Implementations of the disclosure, wherein the synthetic lipid comprises a lipid attached to one or more targeting ligand.

[0215] [Implementation BJ] A method according to Implementation [BI] above, or according to other Implementations of the disclosure, wherein at least one of the one or more added lipid is a natural lipid or a synthetic lipid.

[0216] [Implementation BK] A method according to Implementation [BI] or [BJ] above, or according to other Implementations of the disclosure, wherein the one or more targeting ligand targets a cell type, wherein the cell type is selected from T cell, B cell, monocyte, macrophage, granulocyte, dendritic cell, endothelial cell, (myo)fibroblast, and cancer cell.

[0217] [Implementation BL] A method according to any one of Implementations [BI]-[BK] above, or according to other Implementations of the disclosure, wherein the synthetic lipid is selected from 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol).

[0218] [Implementation BM] A method according to Implementation [BL] above, or according to other Implementations of the disclosure, wherein the natural lipid is selected from a sphingolipid, a phosphatidylglycerol, a lysophosphatidylcholine, a phosphatidyl serine, a ceramide, an eicosanoid, a phosphoinositide, and a lysophosphatidic acid.

[0219] [Implementation BN] A method according to any one of Implementations [BC]-[BM] above, or according to other Implementations of the disclosure, wherein the process further comprises encapsulating nanoparticles with the nanovesicles, wherein encapsulating comprises reconstituting the proteolipid powder in the presence of nanoparticles.

[0220] [Implementation BO] A method according to Implementation [BN] above, or according to other Implementations of the disclosure, wherein a ratio of weight of nanoparticles to weight of nanovesicles during the encapsulating is from about 1:1 to about 1:0.1.

[0221] [Implementation BP] A method according to Implementation [BN] or [BO] above, or according to other Implementations of the disclosure, wherein the nanoparticles comprise one or more of polymeric nanoparticles, carbon nanoparticles, composite nanoparticles, or inorganic nanoparticles, or a combination thereof.

[0222] [Implementation BQ] A method according to Implementation [BP] above, or according to other Implementations of the disclosure, wherein the nanoparticles are selected from iron

oxide, gold, silver, silica, nanoceria, quantum dots, up conversion nanoparticles, calcium phosphate, polystyrene, polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, chitosan, alginate, poly(methyl methacrylate), poly(β-amino ester), ethyl cellulose, semiconducting polymer nanoparticles, carbon nanotubes, nanographite, carbon nanohorn, carbon black nanoparticle, graphene, nanodiamond, and carbon dots, or a combination thereof.

[0223] [Implementation BR] A method according to any one of Implementations [BC]-[BQ] above, or according to other Implementations of the disclosure, wherein the process for making the nanovesicles further comprises loading the nanovesicle with one or more therapeutic compound.

[0224] [Implementation BS] A method according to Implementation [BR] above, or according to other Implementations of the disclosure, wherein the one or more therapeutic compound comprises an antibiotic, an antifungal, an antigen, an immunosuppressant, a chemotherapeutic, a protein functional modulator, a pain manager, an oligonucleotide, a gene editor, or a peptide, or a combination thereof.

[0225] [Implementation BT] A method according to Implementation [BR] or [BS] above, or according to other Implementations of the disclosure, wherein at least one of the one or more therapeutic compound is selected from suberoylanilide hydroxamic acid, Vancomycin, Amphotericin B, Ketoconazole, a tumor peptide, a tumor antigen, a microbial protein, a peptide, a polysaccharide, an immunogenic peptide, a toxin, Dexamethasone, Cyclosporine A, Tacrolimus, Rapamycin, docetaxel, irinotecan, doxorubicin, gemcitabine, cisplatin, paclitaxel, CCG1423, GSK269962, MG-132, INCB8761, Bupivacaine, Ketamine, Morphine, RNA, DNA, CRISPR/Cas9, monoclonal antibodies, growth factors, and functional enzymes.

[0226] [Implementation BU] A method according to any one of Implementations [BC]-[BT] above, or according to other Implementations of the disclosure, wherein the subject has an autoimmune disorder, has a monocyte-mediated condition, or experienced a myocardial infarction, wherein the autoimmune disorder is chosen from Lupus, multiple sclerosis (MS), and psoriasis, and wherein the monocyte-mediated condition is chosen from atherosclerosis and ischemia.

[0227] [Implementation BV] A method according to any one of Implementations [BC]-[BU] above, or according to other Implementations of the disclosure, wherein the administering is

intravenously, intraperitoneally, enterally, by inhalation, intranasally, sublingually, intramuscularly, or subcutaneously.

[0228] [Implementation BW] A method according to any one of Implementations [BC]-[BV] above, or according to other Implementations of the disclosure, wherein the subject experienced a myocardial infarction and the administering is within about two hours after the myocardial infarction.

[0229] [Implementation BX] A method according to any one of Implementations [BC]-[BW] above, or according to other Implementations of the disclosure, wherein the administering comprises treating the subject for a myocardial infarction.

[0230] [Implementation BY] A method according to Implementation [BX] above, or according to other Implementations of the disclosure, wherein the treating comprises reducing size of an infarct in the subject compared to infarct size of another individual who experienced a myocardial infarction and was not administered the nanovesicles.

[0231] [Implementation BZ] A method according to Implementation [BY] above, or according to other Implementations of the disclosure, wherein the reducing is determined 72 hours after the myocardial infarction and comprises an about 15% reduction in size.

[0232] [Implementation CA] A method of preparing nanovesicles, comprising freezing erythrocytes, thawing the erythrocytes, sonicating the thawed erythrocytes, freeze-drying the sonicated erythrocytes to obtain a proteolipid powder, and reconstituting the proteolipid powder, wherein

freezing comprises exposing the erythrocytes to a temperature of about 0 °C or lower,

thawing comprises exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes, and

sonicating comprises exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes.

[0233] [Implementation CB] A method according to Implementation [CA] above, or according to other Implementations of the disclosure, wherein freeze-drying the sonicated erythrocytes comprises mixing the sonicated erythrocytes with a cryoprotectant.

[0234] [Implementation CC] A method according to Implementation [CA] or [CB] above, or according to other Implementations of the disclosure, wherein reconstituting the proteolipid

powder comprises dissolving the proteolipid powder in an organic solvent and then mixing with an aqueous solvent.

[0235] [Implementation CD] A method according to any one of Implementations [CA]-[CC] above, or according to other Implementations of the disclosure, wherein the proteolipid powder is reconstituted in the presence of one or more added lipid.

[0236] [Implementation CE] A method according to Implementation [CD] above, or according to other Implementations of the disclosure, wherein a ratio of weight of proteolipid powder to weight of the one or more added lipid during reconstituting is from about 24:1 to about 5.3:1.

[0237] [Implementation CF] A method according to Implementation [CD] or [CE] above, or according to other Implementations of the disclosure, wherein at least one of the one or more added lipid is a natural lipid or a synthetic lipid.

[0238] [Implementation CG] A method according to Implementation [CF] above, or according to other Implementations of the disclosure, wherein the synthetic lipid comprises a lipid attached to one or more targeting ligand.

[0239] [Implementation CH] A method according to Implementation [CG] above, or according to other Implementations of the disclosure, wherein the one or more targeting ligand is a peptide, an antibody, or an aptamer, or a combination thereof.

[0240] [Implementation CI] A method according to Implementation [CG] or [CH] above, or according to other Implementations of the disclosure, wherein the one or more targeting ligand targets a cell type, wherein the cell type is selected from T cell, B cell, monocyte, macrophage, granulocyte, dendritic cell, endothelial cell, (myo)fibroblast, and cancer cell.

[0241] [Implementation CJ] A method according to any one of Implementations [CF]-[CI] above, or according to other Implementations of the disclosure, wherein the synthetic lipid is selected from 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol).

[0242] [Implementation CK] A method according to Implementation [CF] above, or according to other Implementations of the disclosure, wherein the natural lipid is selected from a sphingolipid, a phosphatidylglycerol, a lysophosphatidylcholine, a phosphatidyl serine, a ceramide, an eicosanoid, a phosphoinositide, and a lysophosphatidic acid, or a combination thereof.

[0243] [Implementation CL] A method according to any one of Implementations [CC]-[CK] above, or according to other Implementations of the disclosure, wherein the process further comprises encapsulating nanoparticles with the nanovesicles, wherein encapsulating comprises reconstituting the proteolipid powder in the presence of nanoparticles.

[0244] [Implementation CM] A method according to Implementation [CL] above, or according to other Implementations of the disclosure, wherein a ratio of weight of nanoparticles to weight of nanovesicles during the encapsulating is from about 1:1 to about 1:0.1.

[0245] [Implementation CN] A method according to Implementation [CL] or [CM] above, or according to other Implementations of the disclosure, wherein the nanoparticles comprise one or more of polymeric nanoparticles, carbon nanoparticles, composite nanoparticles, or inorganic nanoparticles, or a combination thereof.

[0246] [Implementation CO] A method according to Implementation [CN] above, or according to other Implementations of the disclosure, wherein the nanoparticles are selected from iron oxide, gold, silver, silica, nanoceria, quantum dots, up conversion nanoparticles, calcium phosphate, polystyrene, polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, chitosan, alginate, poly(methyl methacrylate), poly(β-amino ester), ethyl cellulose, semiconducting polymer nanoparticles, carbon nanotubes, nanographite, carbon nanohorn, carbon black nanoparticle, graphene, nanodiamond, and carbon dots, or a combination thereof.

[0247] [Implementation CP] A method according to any one of Implementations [CC]-[CO] above, or according to other Implementations of the disclosure, wherein the process for making the nanovesicle further comprises loading the nanovesicle with one or more therapeutic compound.

[0248] [Implementation CQ] A method according to Implementation [CP] above, or according to other Implementations of the disclosure, wherein the one or more therapeutic compound comprises an antibiotic, an antifungal, an antigen, an immunosuppressant, a chemotherapeutic, a protein functional modulator, a pain manager, an oligonucleotide, a gene editor, or a peptide, or a combination thereof.

[0249] [Implementation CR] A method according to Implementation [CP] or [CQ] above, or according to other Implementations of the disclosure, wherein at least one of the one or more therapeutic compound is selected from suberoylanilide hydroxamic acid, Vancomycin, Amphotericin B, Ketoconazole, a tumor peptide, a tumor antigen, a microbial protein, a peptide,

a polysaccharide, an immunogenic peptide, a toxin, Dexamethasone, Cyclosporine A, Tacrolimus, Rapamycin, docetaxel, irinotecan, doxorubicin, gemcitabine, cisplatin, paclitaxel, CCG1423, GSK269962, MG-132, INCB8761, Bupivacaine, Ketamine, Morphine, RNA, DNA, CRISPR/Cas9, monoclonal antibodies, growth factors, and functional enzymes.

[0250] [Implementation CS] A plurality of nanovesicles made by a process, wherein the process comprises freezing erythrocytes, thawing the erythrocytes, sonicating the thawed erythrocytes, freeze-drying the sonicated erythrocytes to obtain a proteolipid powder, and reconstituting the proteolipid powder, wherein

freezing comprises exposing the erythrocytes to a temperature of about 0 °C or lower,

thawing comprises exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes, and

sonicating comprises exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes.

[0251] [Implementation CT] A method according to Implementation [CS] above, or according to other Implementations of the disclosure, wherein freeze-drying the sonicated erythrocytes comprises mixing the sonicated erythrocytes with a cryoprotectant.

[0252] [Implementation CU] A method according to Implementation [CS] or [CT] above, or according to other Implementations of the disclosure, wherein reconstituting the proteolipid powder comprises dissolving the proteolipid powder in an organic solvent and then mixing with an aqueous solvent.

[0253] [Implementation CV] A method according to any one of Implementations [CS]-[CU] above, or according to other Implementations of the disclosure, wherein the proteolipid powder is reconstituted in the presence of one or more added lipid.

[0254] [Implementation CW] A method according to Implementation [CV] above, or according to other Implementations of the disclosure, wherein a ratio of weight of proteolipid powder to weight of the one or more added lipid during reconstituting is from about 24:1 to about 5.3:1.

[0255] [Implementation CX] A method according to Implementation [CV] or [CW] above, or according to other Implementations of the disclosure, wherein at least one of the one or more added lipid is a natural lipid or a synthetic lipid.

[0256] [Implementation CY] A method according to Implementation [CX] above, or according to other Implementations of the disclosure, wherein the synthetic lipid comprises a lipid attached to one or more targeting ligand.

[0257] [Implementation CZ] A method according to Implementation [CY] above, or according to other Implementations of the disclosure, wherein the one or more targeting ligand is a peptide, an antibody, or an aptamer, or a combination thereof.

[0258] [Implementation DA] A method according to Implementation [CY] or [CZ] above, or according to other Implementations of the disclosure, wherein the one or more targeting ligand targets a cell type, wherein the cell type is selected from T cell, B cell, monocyte, macrophage, granulocyte, dendritic cell, endothelial cell, (myo)fibroblast, and cancer cell.

[0259] [Implementation DB] A method according to any one of Implementations [CX]-[DA] above, or according to other Implementations of the disclosure, wherein the synthetic lipid is selected from 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol).

[0260] [Implementation DC] A method according to Implementation [DB] above, or according to other Implementations of the disclosure, wherein the natural lipid is selected from a sphingolipid, a phosphatidylglycerol, a lysophosphatidylcholine, a phosphatidyl serine, a ceramide, an eicosanoid, a phosphoinositide, and a lysophosphatidic acid.

[0261] [Implementation DD] A method according to any one of Implementations [CS]-[DC] above, or according to other Implementations of the disclosure, wherein the process further comprises encapsulating nanoparticles with the nanovesicles, wherein encapsulating comprises reconstituting the proteolipid powder in the presence of nanoparticles.

[0262] [Implementation DE] A method according to Implementation [DD] above, or according to other Implementations of the disclosure, wherein a ratio of weight of nanoparticles to weight of nanovesicles during the encapsulating is from about 1:1 to about 1:0.1.

[0263] [Implementation DF] A method according to Implementation [DD] or [DE] above, or according to other Implementations of the disclosure, wherein the nanoparticles comprise one or more of polymeric nanoparticles, carbon nanoparticles, composite nanoparticles, or inorganic nanoparticles, or a combination thereof.

[0264] [Implementation DG] A method according to Implementation [DF] above, or according to other Implementations of the disclosure, wherein the nanoparticles are selected from iron

oxide, gold, silver, silica, nanoceria, quantum dots, up conversion nanoparticles, calcium phosphate, polystyrene, polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, chitosan, alginate, poly(methyl methacrylate), poly(β-amino ester), ethyl cellulose, semiconducting polymer nanoparticles, carbon nanotubes, nanographite, carbon nanohorn, carbon black nanoparticle, graphene, nanodiamond, and carbon dots, or a combination thereof.

[0265] [Implementation DH] A method according to any one of Implementations [CS]-[DG] above, or according to other Implementations of the disclosure, wherein the process for making the nanovesicles further comprises loading the nanovesicle with one or more therapeutic compound.

[0266] [Implementation DI] A method according to Implementation [DH] above, or according to other Implementations of the disclosure, wherein the one or more therapeutic compound comprises an antibiotic, an antifungal, an antigen, an immunosuppressant, a chemotherapeutic, a protein functional modulator, a pain manager, an oligonucleotide, a gene editor, or a peptide, or a combination thereof.

[0267] [Implementation DJ] A method according to Implementation [DH] or [DI] above, or according to other Implementations of the disclosure, wherein at least one of the one or more therapeutic compound is selected from suberoylanilide hydroxamic acid, Vancomycin, Amphotericin B, Ketoconazole, a tumor peptide, a tumor antigen, a microbial protein, a peptide, a polysaccharide, an immunogenic peptide, a toxin, Dexamethasone, Cyclosporine A, Tacrolimus, Rapamycin, docetaxel, irinotecan, doxorubicin, gemcitabine, cisplatin, paclitaxel, CCG1423, GSK269962, MG-132, INCB8761, Bupivacaine, Ketamine, Morphine, RNA, DNA, CRISPR/Cas9, monoclonal antibodies, growth factors, and functional enzymes.

CLAIMS

What is claimed is:

1. A method of delivering a composition to a spleen of a subject, comprising systemically administering the composition to the subject, wherein the composition comprises a nanovesicle made by a process, the process comprising:

freezing erythrocytes, wherein freezing comprises exposing the erythrocytes to a temperature of about 0 °C or lower,

thawing the erythrocytes, wherein thawing comprises exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes,

sonicating the erythrocytes, wherein sonicating comprises exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes, and

one of

(i) extruding the sonicated erythrocytes, wherein extruding comprises successively passing the sonicated erythrocytes through a series of filters each comprising a filter diameter, wherein each filter comprises a larger filter diameter than a subsequent filter; and

(ii) freeze-drying the sonicated erythrocytes to obtain a proteolipid powder, and reconstituting the proteolipid powder.

2. The method of claim 1, wherein freeze-drying the sonicated erythrocytes comprises mixing the sonicated erythrocytes with a cryoprotectant.

3. The method of claim 1 or 2, wherein reconstituting the proteolipid powder comprises dissolving the proteolipid powder in an organic solvent and then mixing with an aqueous solvent.

4. The method of any one of claims 1 through 3, wherein one of

(i) the sonicated erythrocytes are extruded in the presence of one or more added lipid; and

(ii) the proteolipid powder is reconstituted in the presence of one or more added lipid.

5. The method of any one of claims 1 through 4, wherein one of

(i) a ratio of weight of erythrocytes to weight of the one or more added lipid during the extruding is from about 1:24 to about 1:1; and

(ii) a ratio of weight of proteolipid powder to weight of the one or more added lipid during reconstituting is from about 24:1 to about 5.3:1.

6. The method of claim 4 or 5, wherein at least one of the one or more added lipid is a natural lipid, a synthetic lipid, or a combination thereof.

7. The method of claim 6, wherein the synthetic lipid comprises a lipid attached to one or more targeting ligand.

8. The method of claim 7, wherein the one or more targeting ligand is a peptide, an antibody, or an aptamer, or a combination thereof.

9. The method of claim 7 or 8, wherein the one or more targeting ligand targets a cell type, wherein the cell type is selected from T cell, B cell, monocyte, macrophage, granulocyte, dendritic cell, endothelial cell, (myo)fibroblast, and cancer cell.

10. The method of any one of claims 6 through 9, wherein the synthetic lipid is selected from 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol).

11. The method of claim 6, wherein the natural lipid is selected from a sphingolipid, a phosphatidylglycerol, a lysophosphatidylcholine, a phosphatidyl serine, a ceramide, an eicosanoid, a phosphoinositide, and a lysophosphatidic acid.

12. The method of any one of claims 1 through 11, wherein the process further comprises encapsulating nanoparticles with the nanovesicles, wherein encapsulating comprises one of

(i) sonicating the nanovesicles in the presence of the nanoparticles and sonicating comprises exposing the nanovesicles and the nanoparticles to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 2 minutes to about 3 minutes; and

(ii) reconstituting the proteolipid powder in the presence of nanoparticles.

13. The method of claim 12, wherein a ratio of weight of nanoparticles to weight of nanovesicles during the encapsulating is from about 1:1 to about 1:0.1.

14. The method of claim 12 or 13, wherein the nanoparticles comprise one or more of polymeric nanoparticles, carbon nanoparticles, composite nanoparticles, or inorganic nanoparticles, or a combination thereof.

15. The method of claim 14, wherein the nanoparticles are selected from iron oxide, gold, silver, silica, nanoceria, quantum dots, up conversion nanoparticles, calcium phosphate, polystyrene, polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, chitosan, alginate, poly(methyl methacrylate), poly(β-amino ester), ethyl cellulose, semiconducting polymer nanoparticles, carbon nanotubes, nanographite, carbon nanohorn, carbon black nanoparticle, graphene, nanodiamond, and carbon dots, or a combination thereof.

16. The method of any one of claims 1 through 15, wherein the process for making the nanovesicles further comprises loading the nanovesicle with one or more therapeutic compound.

17. The method of claim 16, wherein the one or more therapeutic compound comprises an antibiotic, an antifungal, an antigen, an immunosuppressant, a chemotherapeutic, a protein functional modulator, a pain management compound, an oligonucleotide, a gene editor, or a peptide, or a combination thereof.

18. The method of claim 16 or 17, wherein at least one of the one or more therapeutic compound is selected from suberoylanilide hydroxamic acid, Vancomycin, Amphotericin B, Ketoconazole, a tumor peptide, a tumor antigen, a microbial protein, a peptide, a polysaccharide, an immunogenic peptide, a toxin, Dexamethasone, Cyclosporine A, Tacrolimus, Rapamycin, docetaxel, irinotecan, doxorubicin, gemcitabine, cisplatin, paclitaxel, CCG1423, GSK269962, MG-132, INCB8761, Bupivacaine, Ketamine, Morphine, RNA, DNA, CRISPR/Cas9, monoclonal antibodies, growth factors, and functional enzymes.

19. The method of any one of claims 1 through 18, wherein the subject has an autoimmune disorder, has a monocyte-mediated condition, or experienced a myocardial infarction, wherein the autoimmune disorder is chosen from Lupus, multiple sclerosis (MS), and psoriasis, and wherein the monocyte-mediated condition is chosen from atherosclerosis and ischemia.

20. The method of any one of claims 1 through 19, wherein the administering is intravenously, intraperitoneally, enterally, by inhalation, intranasally, sublingually, intramuscularly, or subcutaneously.

21. The method of any one of claims 1 through 20, wherein the subject experienced a myocardial infarction and the administering is within about two hours after the myocardial infarction.

22. The method of any one of claims 1 through 21, wherein the administering comprises treating the subject for a myocardial infarction.

23. The method of claim 22 wherein the treating comprises reducing size of an infarct in the subject compared to infarct size of another individual who experienced a myocardial infarction and was not administered the nanovesicles.

24. The method of claim 23, wherein the reducing is determined 72 hours after the myocardial infarction and comprises an about 15% reduction in size.

25. A method of preparing nanovesicles, comprising freezing erythrocytes, thawing the erythrocytes, sonicating the thawed erythrocytes, and one of

(i) extruding the sonicated erythrocytes; and

(ii) freeze-drying the sonicated erythrocytes to obtain a proteolipid powder, and reconstituting the proteolipid powder;

wherein

freezing comprises exposing the erythrocytes to a temperature of about 0 °C or lower;

thawing comprises exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes;

sonicating comprises exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes to form nanovesicles;

extruding comprises successively passing the sonicated erythrocytes through a series of filters each comprising a filter diameter, wherein each filter comprises a larger filter diameter than a subsequent filter;

freeze-drying the sonicated erythrocytes comprises mixing the sonicated erythrocytes with a cryoprotectant; and

reconstituting the proteolipid powder comprises dissolving the proteolipid powder in an organic solvent and then mixing with an aqueous solvent.

26. The method of claim 25, wherein one of

(i) the sonicated erythrocytes are extruded in the presence of one or more added lipid; and

(ii) the proteolipid powder is reconstituted in the presence of one or more added lipid.

27. The method of claim 26, wherein one of

(i) a ratio of weight of erythrocytes to weight of the one or more added lipid during the extruding is from about 1:24 to about 1:1; and

(ii) a ratio of weight of proteolipid powder to weight of the one or more added lipid during reconstituting is from about 24:1 to about 5.3:1.

28. The method of claim 26 or 27, wherein at least one of the one or more added lipid is a natural lipid or a synthetic lipid.

29. The method of claim 28, wherein the synthetic lipid comprises a lipid attached to one or more targeting ligand.

30. The method of claim 29, wherein the one or more targeting ligand is a peptide, an antibody, or an aptamer, or a combination thereof.

31. The method of claim 29 or 30, wherein the one or more targeting ligand targets a cell type, wherein the cell type is selected from T cell, B cell, monocyte, macrophage, granulocyte, dendritic cell, endothelial cell, (myo)fibroblast, and cancer cell.

32. The method of any one of claims 28 through 31, wherein the synthetic lipid is selected from 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol).

33. The method of claim 28, wherein the natural lipid is selected from a sphingolipid, a phosphatidylglycerol, a lysophosphatidylcholine, a phosphatidyl serine, a ceramide, an eicosanoid, a phosphoinositide, and a lysophosphatidic acid, or a combination thereof.

34. The method of any one of claims 25 through 33, wherein the process further comprises encapsulating nanoparticles with the nanovesicles, wherein encapsulating comprises one of

(i) sonicating the nanovesicles in the presence of the nanoparticles and sonicating comprises exposing the nanovesicles and the nanoparticles to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 2 minutes to about 3 minutes; and

(ii) reconstituting the proteolipid powder in the presence of nanoparticles.

35. The method of claim 34, wherein a ratio of weight of nanoparticles to weight of nanovesicles during the encapsulating is from about 1:1 to about 1:0.1.

36. The method of claim 34 or 35, wherein the nanoparticles comprise one or more of polymeric nanoparticles, carbon nanoparticles, composite nanoparticles, or inorganic nanoparticles, or a combination thereof.

37. The method of claim 36, wherein the nanoparticles are selected from iron oxide, gold, silver, silica, nanoceria, quantum dots, up conversion nanoparticles, calcium phosphate, polystyrene, polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, chitosan, alginate, poly(methyl methacrylate), poly(β-amino ester), ethyl cellulose, semiconducting polymer nanoparticles, carbon nanotubes, nanographite, carbon nanohorn, carbon black nanoparticle, graphene, nanodiamond, and carbon dots, or a combination thereof.

38. The method of any one of claims 25 through 37, wherein the process for making the nanovesicle further comprises loading the nanovesicle with one or more therapeutic compound.

39. The method of claim 38, wherein the one or more therapeutic compound comprises an antibiotic, an antifungal, an antigen, an immunosuppressant, a chemotherapeutic, a protein functional modulator, a pain manager, an oligonucleotide, a gene editor, or a peptide, or a combination thereof.

40. The method of claim 38 or 39, wherein at least one of the one or more therapeutic compound is selected from suberoylanilide hydroxamic acid, Vancomycin, Amphotericin B, Ketoconazole, a tumor peptide, a tumor antigen, a microbial protein, a peptide, a polysaccharide, an immunogenic peptide, a toxin, Dexamethasone, Cyclosporine A, Tacrolimus, Rapamycin, docetaxel, irinotecan, doxorubicin, gemcitabine, cisplatin, paclitaxel, CCG1423, GSK269962, MG-132, INCB8761, Bupivacaine, Ketamine, Morphine, RNA, DNA, CRISPR/Cas9, monoclonal antibodies, growth factors, and functional enzymes.

41. A plurality of nanovesicles made by a process, wherein the process comprises freezing erythrocytes, thawing the erythrocytes, sonicating the thawed erythrocytes, and one of

(i) extruding the sonicated erythrocytes; and

(ii) freeze-drying the sonicated erythrocytes to obtain a proteolipid powder, and reconstituting the proteolipid powder;

wherein:

freezing comprises exposing the erythrocytes to a temperature of about 0 °C or lower;

thawing comprises exposing the erythrocytes to a temperature of from about 25 °C to about 60 °C for from about 20 minutes to about 30 minutes;

sonicating comprises exposing the thawed erythrocytes to ultrasonic frequencies of from about 20 kHz to about 40 kHz for from about 1 minute to about 3 minutes;

extruding comprises successively passing the sonicated erythrocytes through a series of filters each comprising a filter diameter, wherein each filter comprises a larger filter diameter than a subsequent filter;

freeze-drying the sonicated erythrocytes comprises mixing the sonicated erythrocytes with a cryoprotectant; and

reconstituting the proteolipid powder comprises dissolving the proteolipid powder in an organic solvent and then mixing with an aqueous solvent.

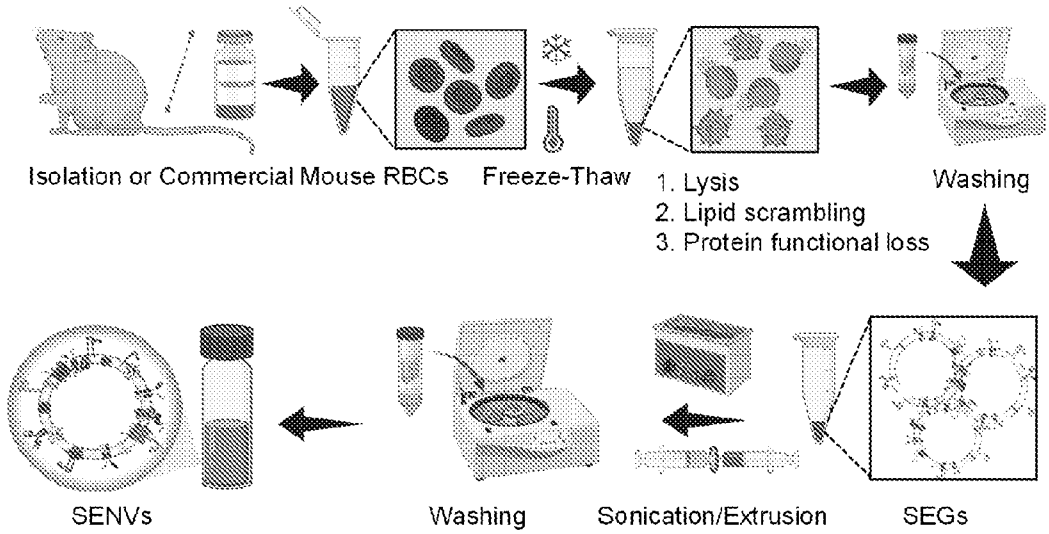
Isolation or Commercial Mouse RBCs
Freeze-Thaw
1. Lysis
2. Lipid scrambling
3. Protein functional loss
Washing
SENVs
Washing
Sonication/Extrusion
SEGs

FIG. 1A

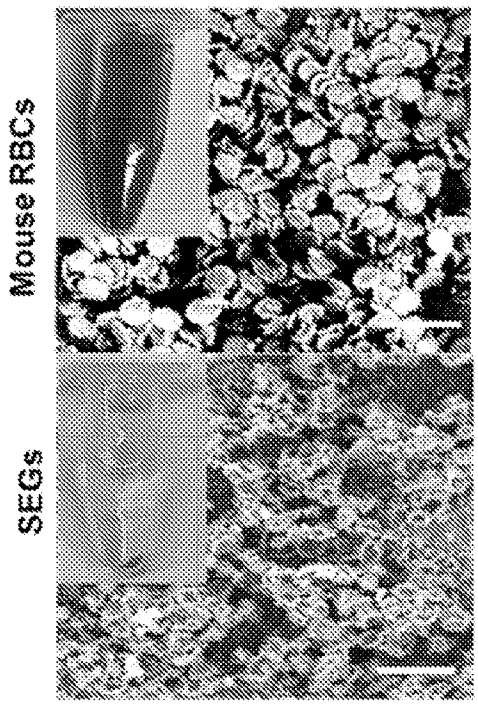
Mouse RBCs
SEGs

FIG. 1B

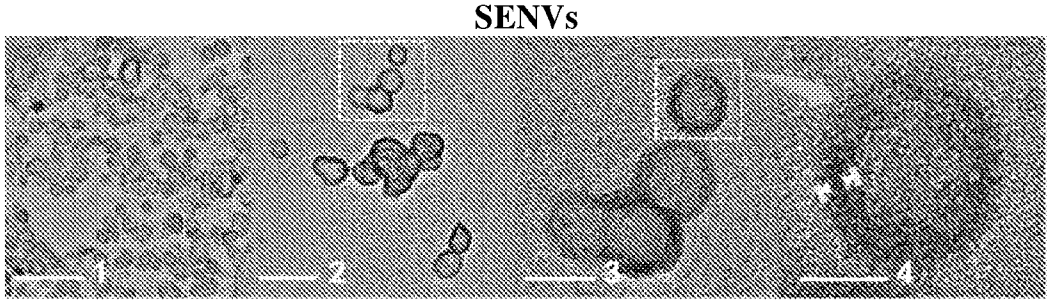
SENVs
1
2
3
4

FIG. 1C

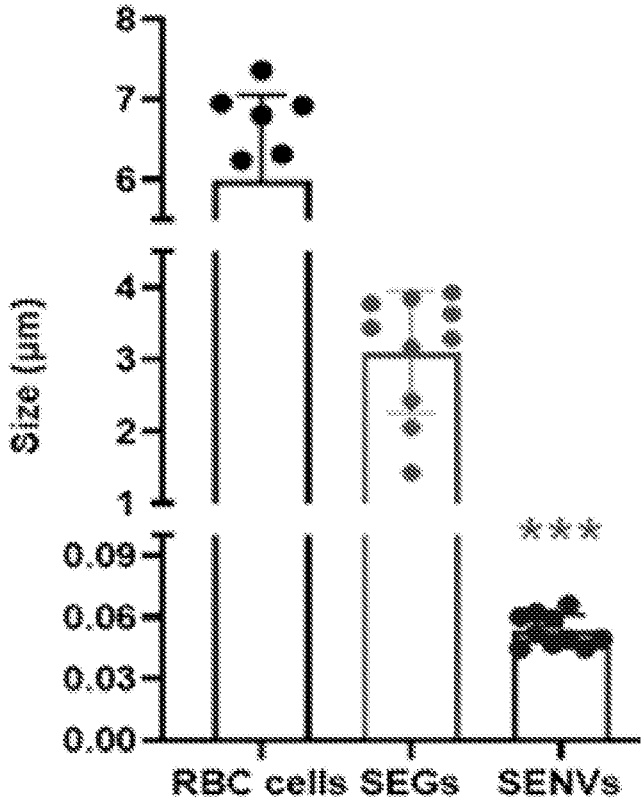
Size (μm)
8
7
6
4
3
2
1
0.09
0.06
0.03
0.00
***
RBC cells
SEGs
SENVs

FIG. 1D

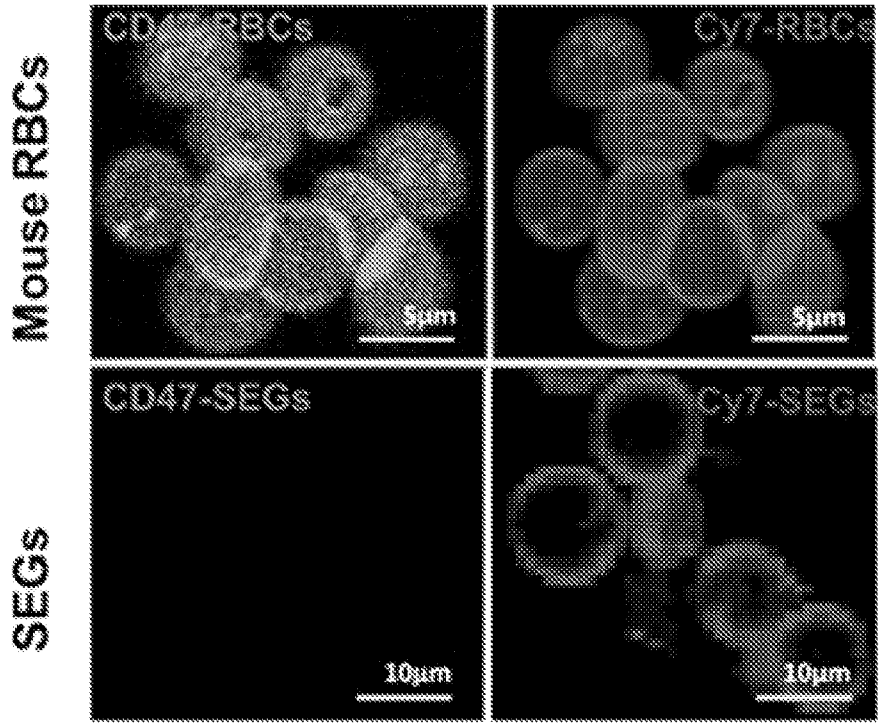
Mouse RBCs
SEGs
Cy7-RBCs
5μm
5μm
CD47-SEGs
Cy7-SEGs
10μm
10μm

FIG. 1E

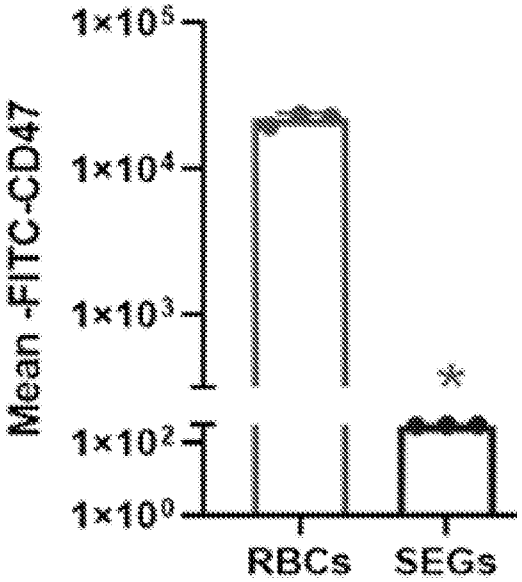
Mean -FITC-CD47
1×10⁵
1×10⁴
1×10³
1×10²
1×10⁰
*
RBCs
SEGs

FIG. 1F

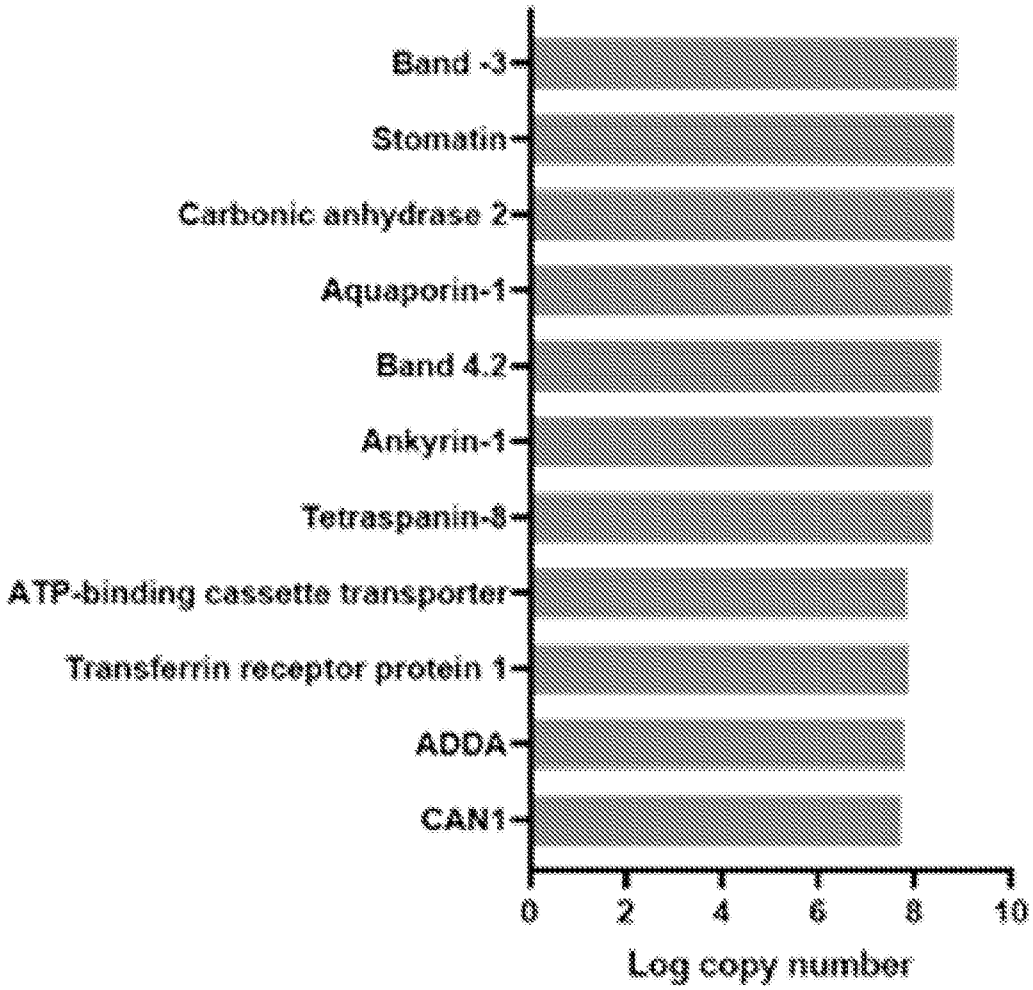
Band -3
Stomatin
Carbonic anhydrase 2
Aquaporin-1
Band 4.2
Ankyrin-1
Tetraspanin-8
ATP-binding cassette transporter
Transferrin receptor protein 1
ADDA
CAN1
0
2
4
6
8
10
Log copy number

FIG. 1G

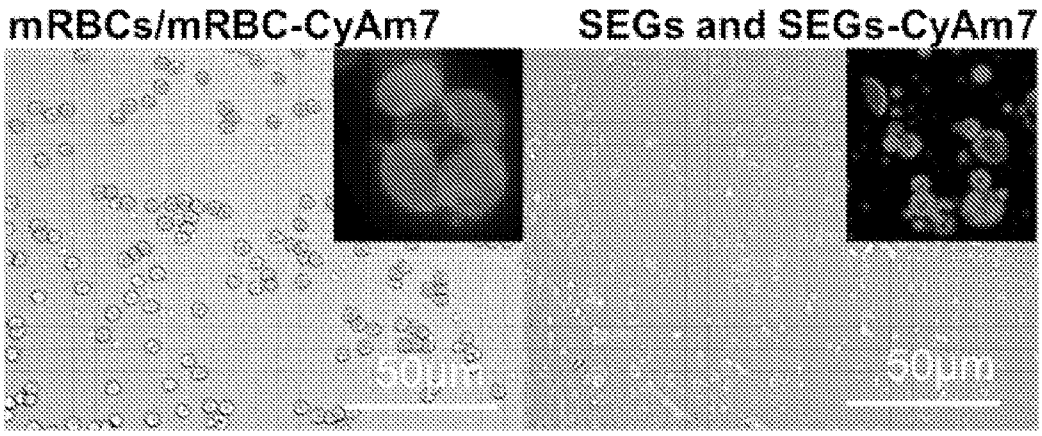

FIG. 2A

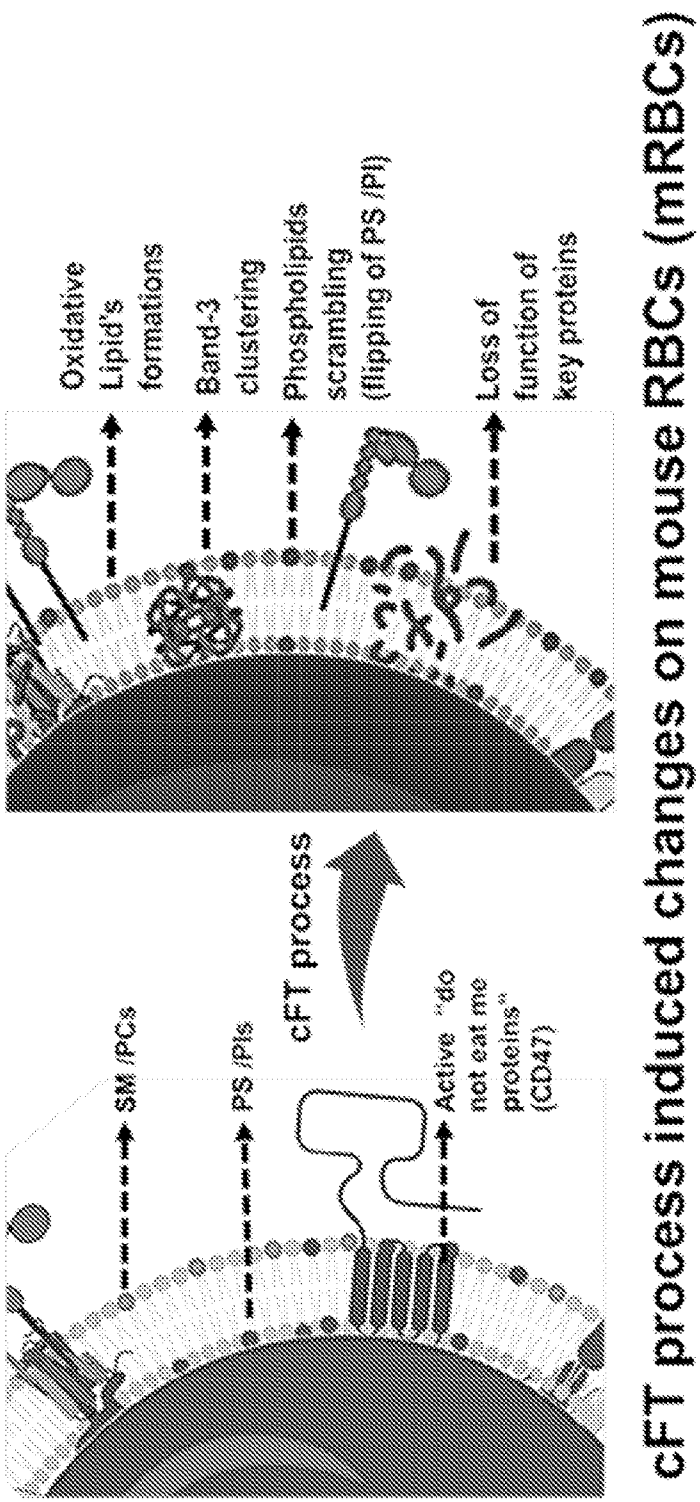
cFT process induced changes on mouse RBCs (mRBCs)
SM /PCs
PS /PIs
Active "do not eat me proteins" (CD47)
cFT process
Oxidative Lipid's formations
Band-3 clustering
Phospholipids scrambling (flipping of PS /PI)
Loss of function of key proteins

FIG. 2B

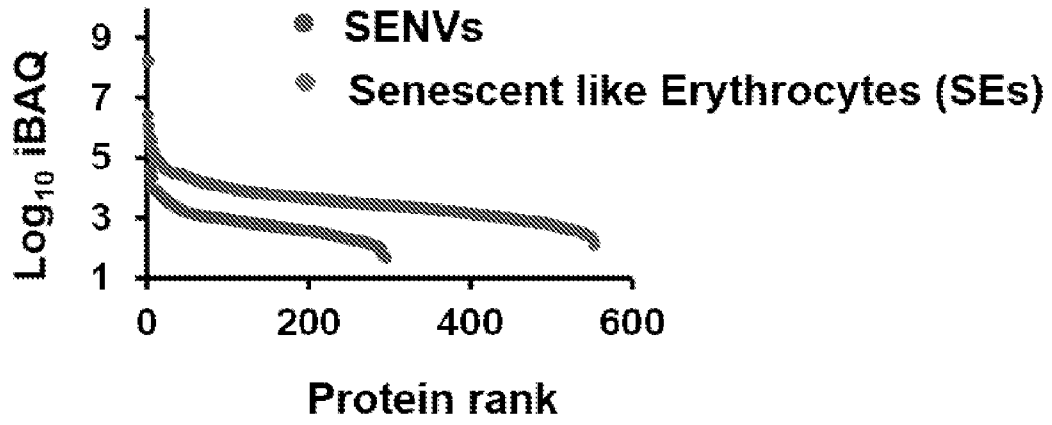
SENVs
Senescent like Erythrocytes (SEs)
Log10 iBAQ
9
7
5
3
1
0
200
400
600
Protein rank

FIG. 3A

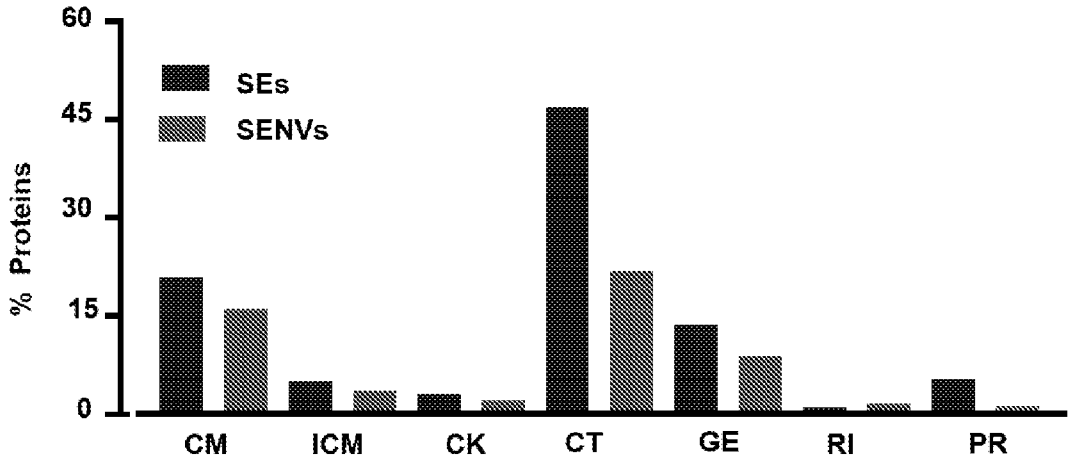
60
45
30
15
0
% Proteins
SEs
SENVs
CM
ICM
CK
CT
GE
RI
PR

FIG. 3B

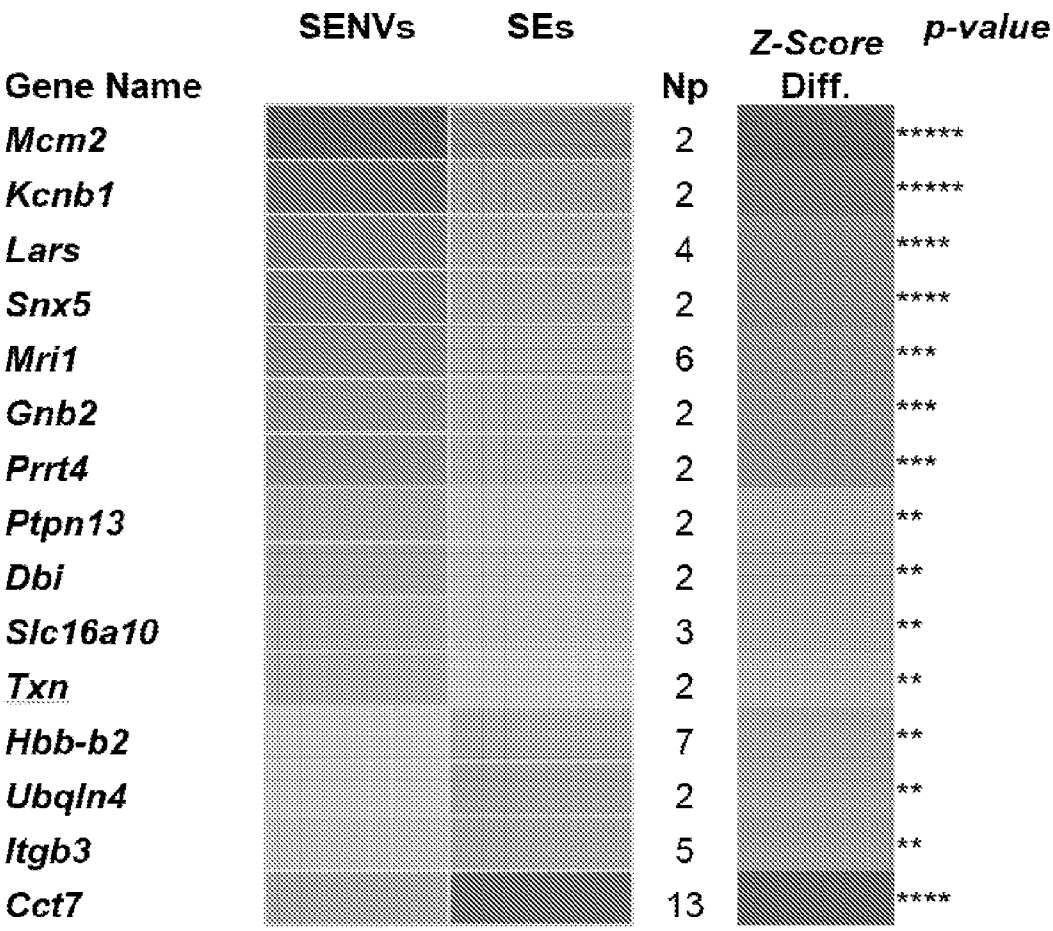
SENVs
SEs
Z-Score
p-value
Gene Name
Np
Diff.
Mcm2 2 *****
Kcnb1 2 *****
Lars 4 ****
Snx5 2 ****
Mri1 6 ***
Gnb2 2 ***
Prrt4 2 ***
Ptpn13 2 **
Dbi 2 **
Slc16a10 3 **
Txn 2 **
Hbb-b2 7 **
Ubqln4 2 **
Itgb3 5 **
Cct7 13 ****

FIG. 3C

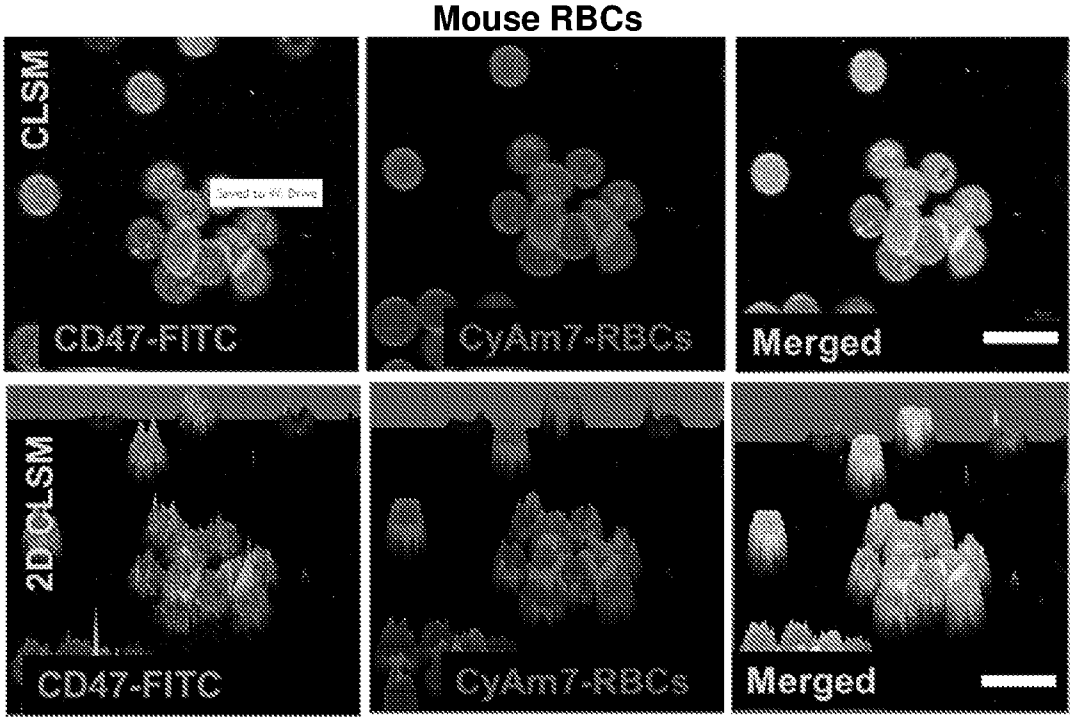
Mouse RBCs
CLSM
CD47-FITC
CyAm7-RBCs
Merged
2D CLSM
CD47-FITC
CyAm7-RBCs
Merged

FIG. 4A

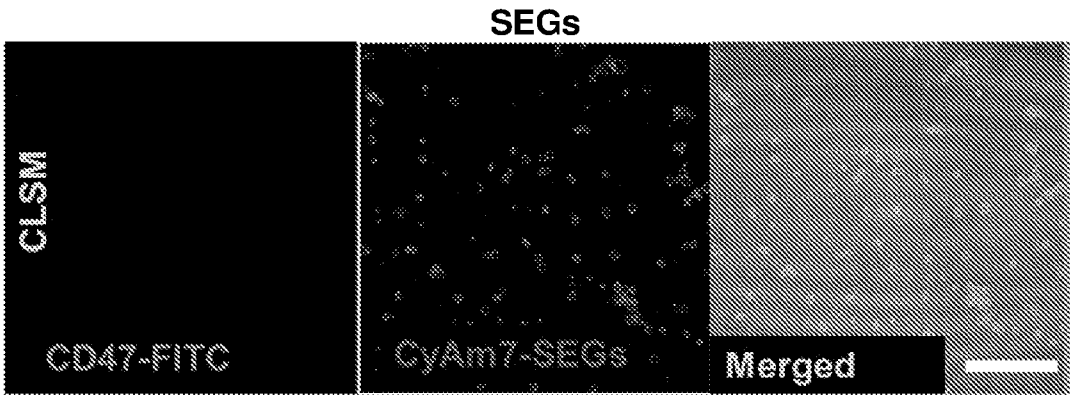
SEGs
CLSM
CD47-FITC
CyAm7-SEGs
Merged

FIG. 4B

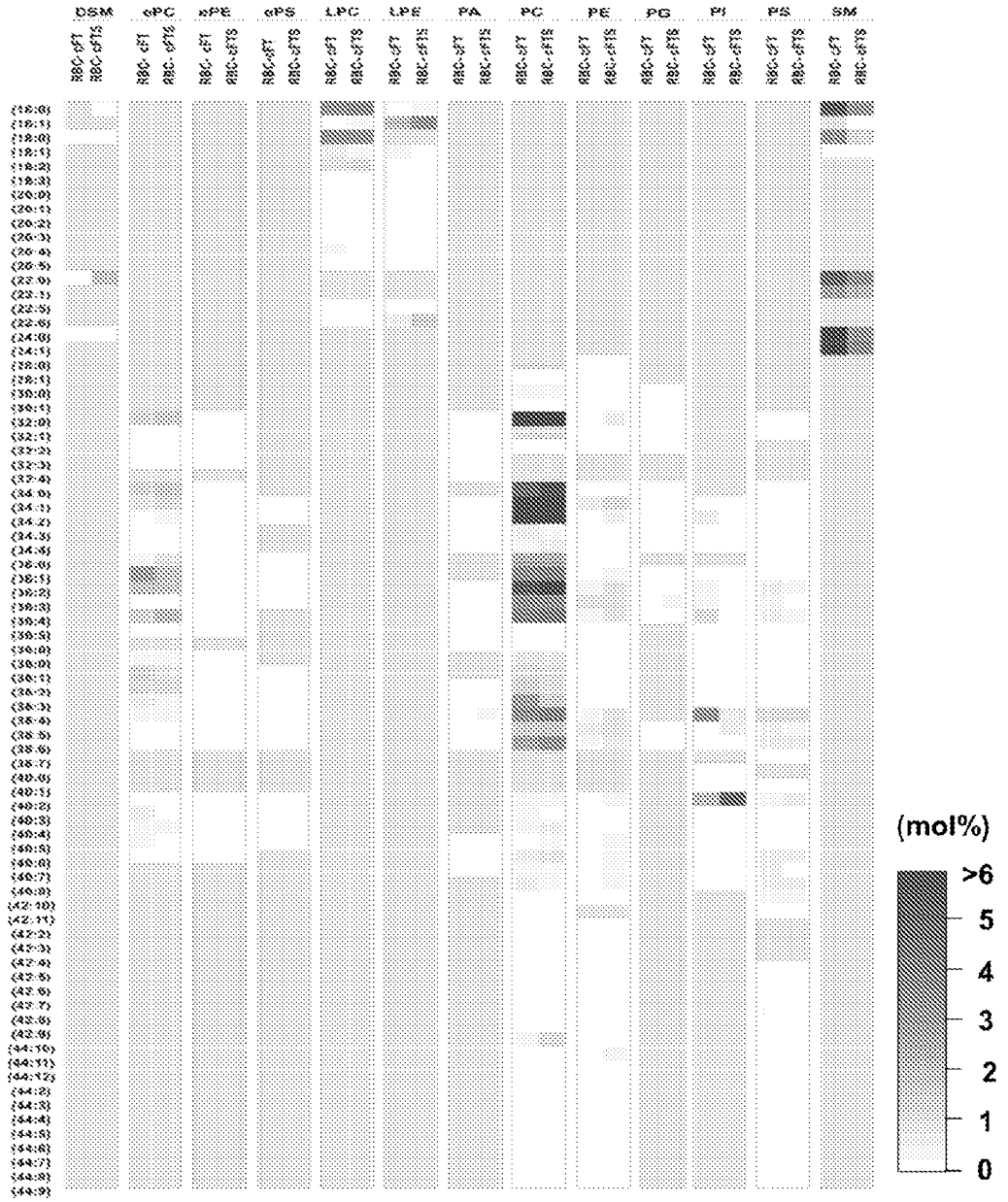

FIG. 5A

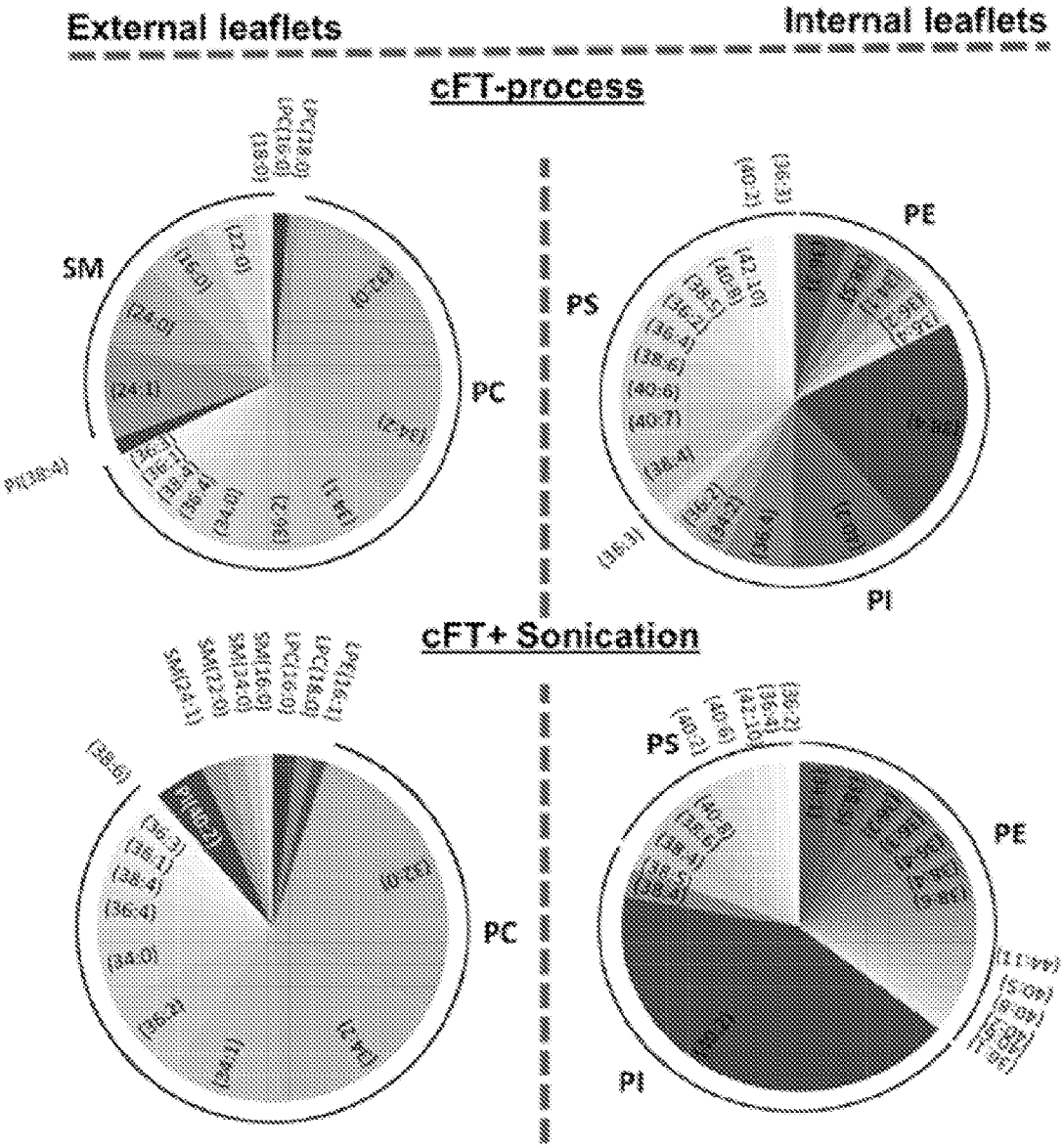
External leaflets
Internal leaflets
cFT-process
SM
PC
PS
PE
PI
cFT+ Sonication
SM
PC
PS
PE
PI

FIG. 5B

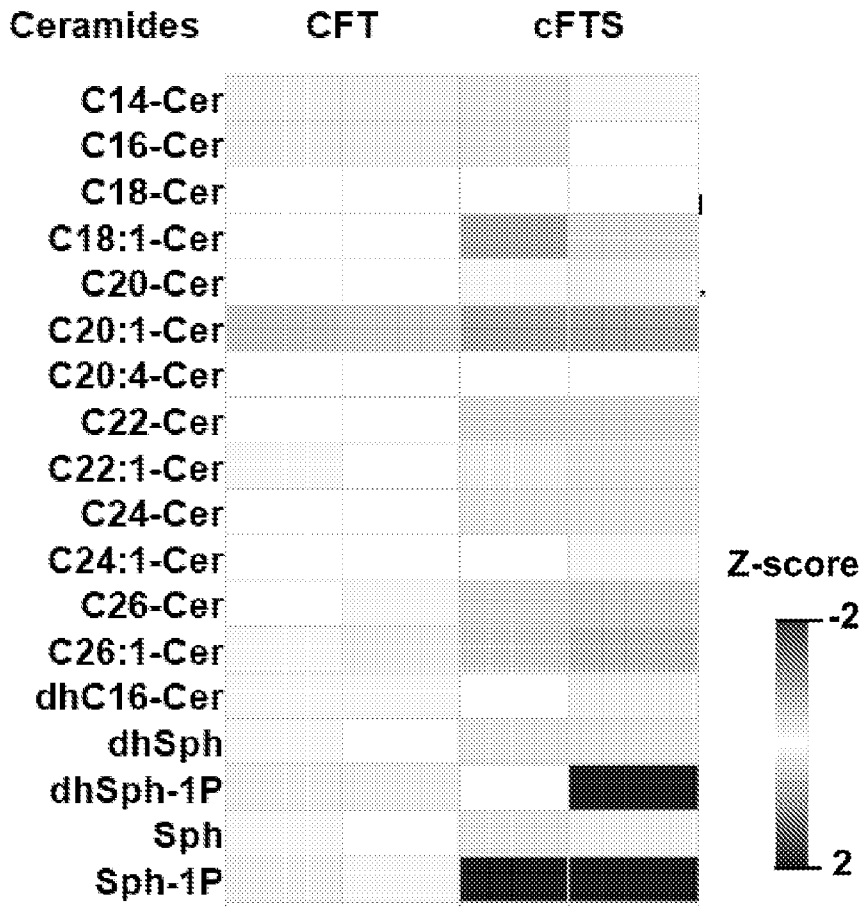
Ceramides
CFT
cFTS
C14-Cer
C16-Cer
C18-Cer
C18:1-Cer
C20-Cer
C20:1-Cer
C20:4-Cer
C22-Cer
C22:1-Cer
C24-Cer
C24:1-Cer
C26-Cer
C26:1-Cer
dhC16-Cer
dhSph
dhSph-1P
Sph
Sph-1P
Z-score
-2
2

FIG. 5C

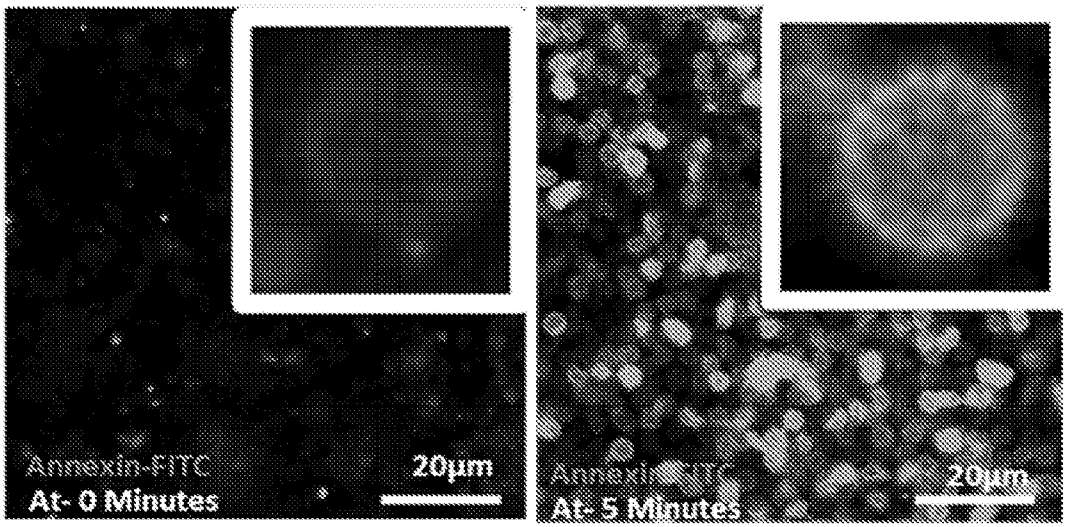
Annexin-FITC
At- 0 Minutes
20µm
Annexin-FITC
At- 5 Minutes
20µm

FIG. 5D

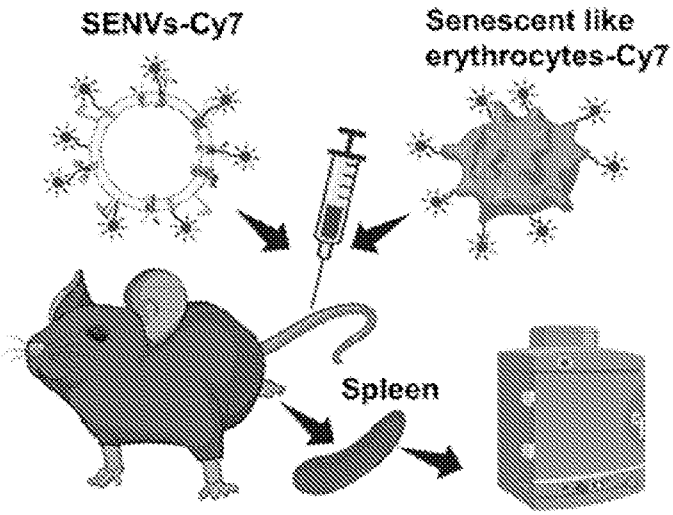
SENVs-Cy7
Senescent like erythrocytes-Cy7
Spleen

FIG. 5E

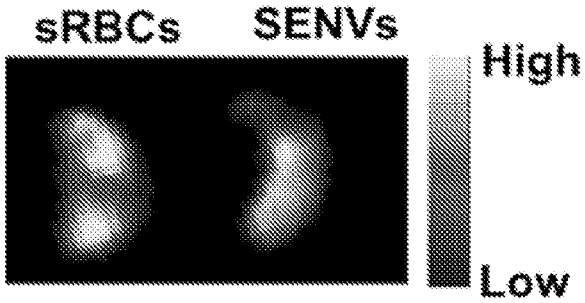
sRBCs
SENVs
High
Low

FIG. 5F

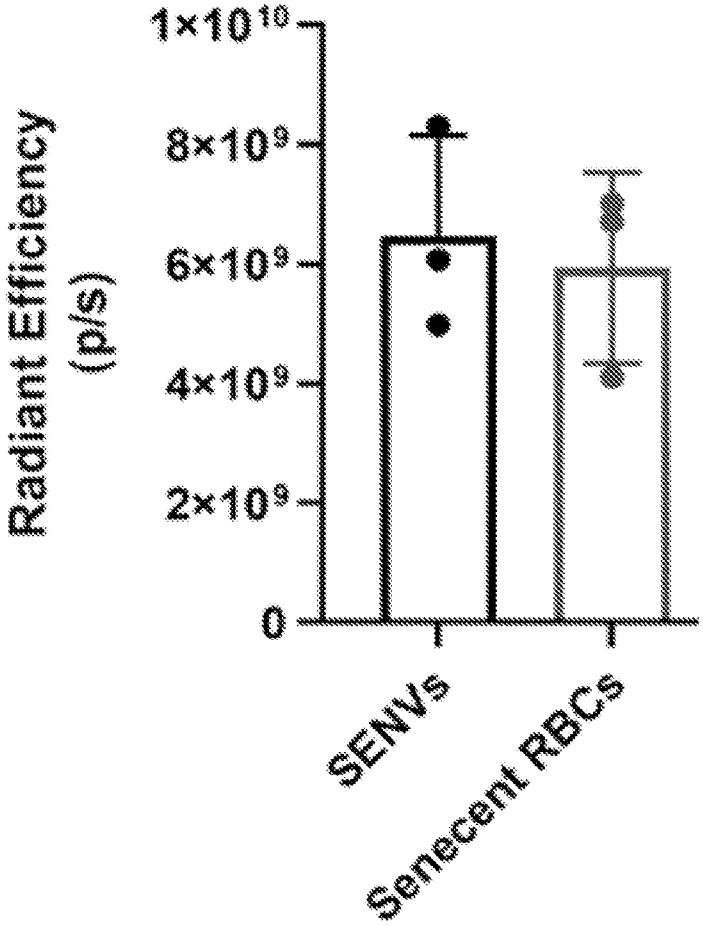
Radiant Efficiency (p/s)
1×10^10
8×10^9
6×10^9
4×10^9
2×10^9
0
SENVs
Senecent RBCs

FIG. 5G

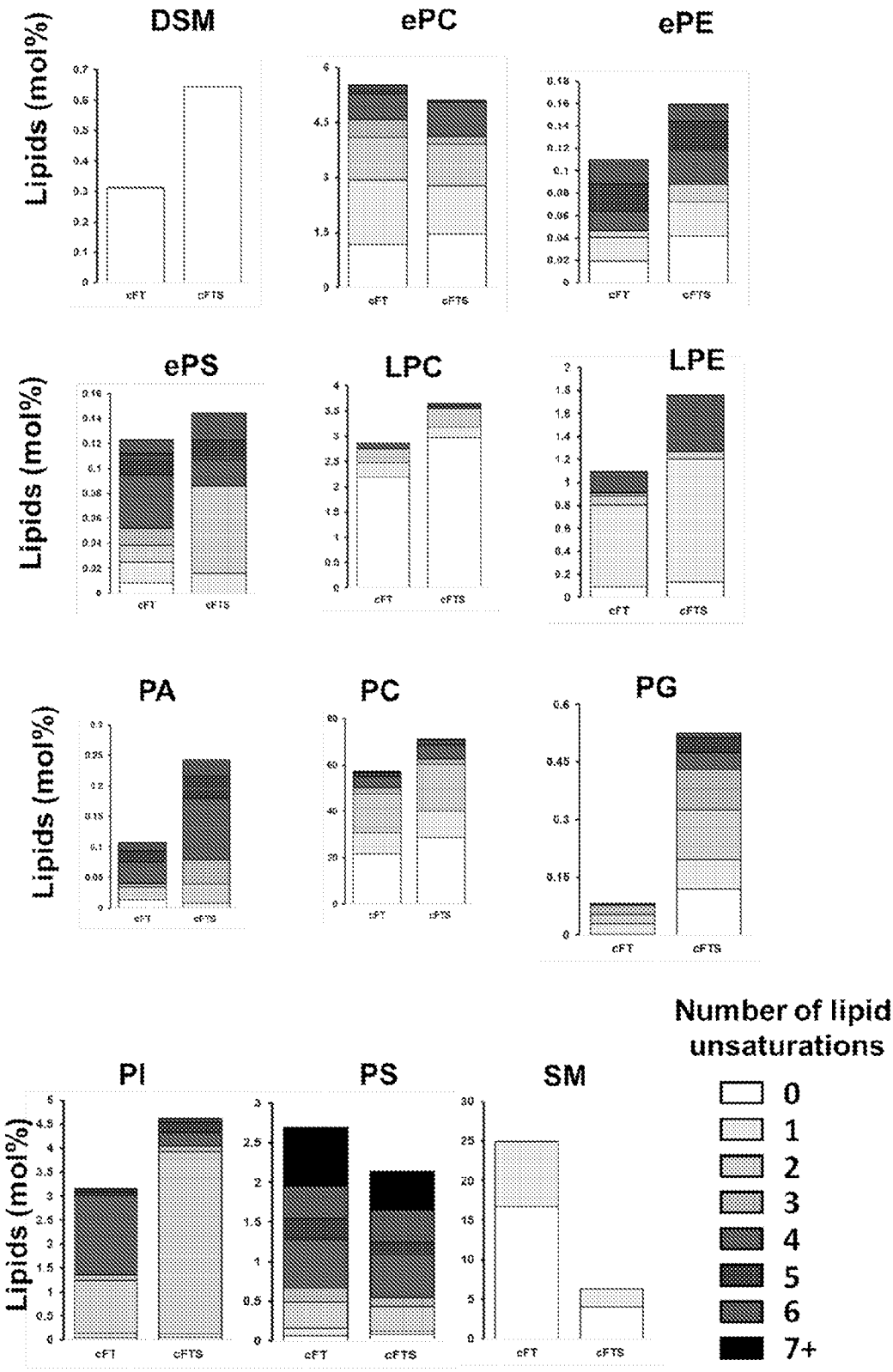
DSM
ePC
ePE
ePS
LPC
LPE
PA
PC
PG
PI
PS
SM
Lipids (mol%)
cFT
cFTS
Number of lipid unsaturations
0
1
2
3
4
5
6
7+

FIG. 6

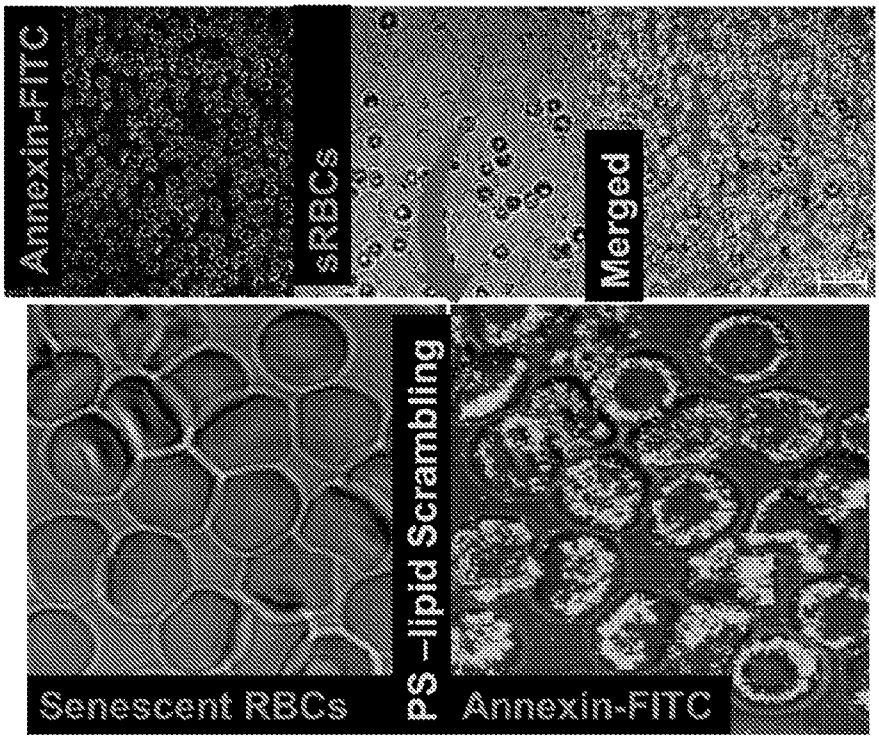
Annexin-FITC
sRBCs
Merged
Senescent RBCs
PS –lipid Scrambling
Annexin-FITC

FIG. 7

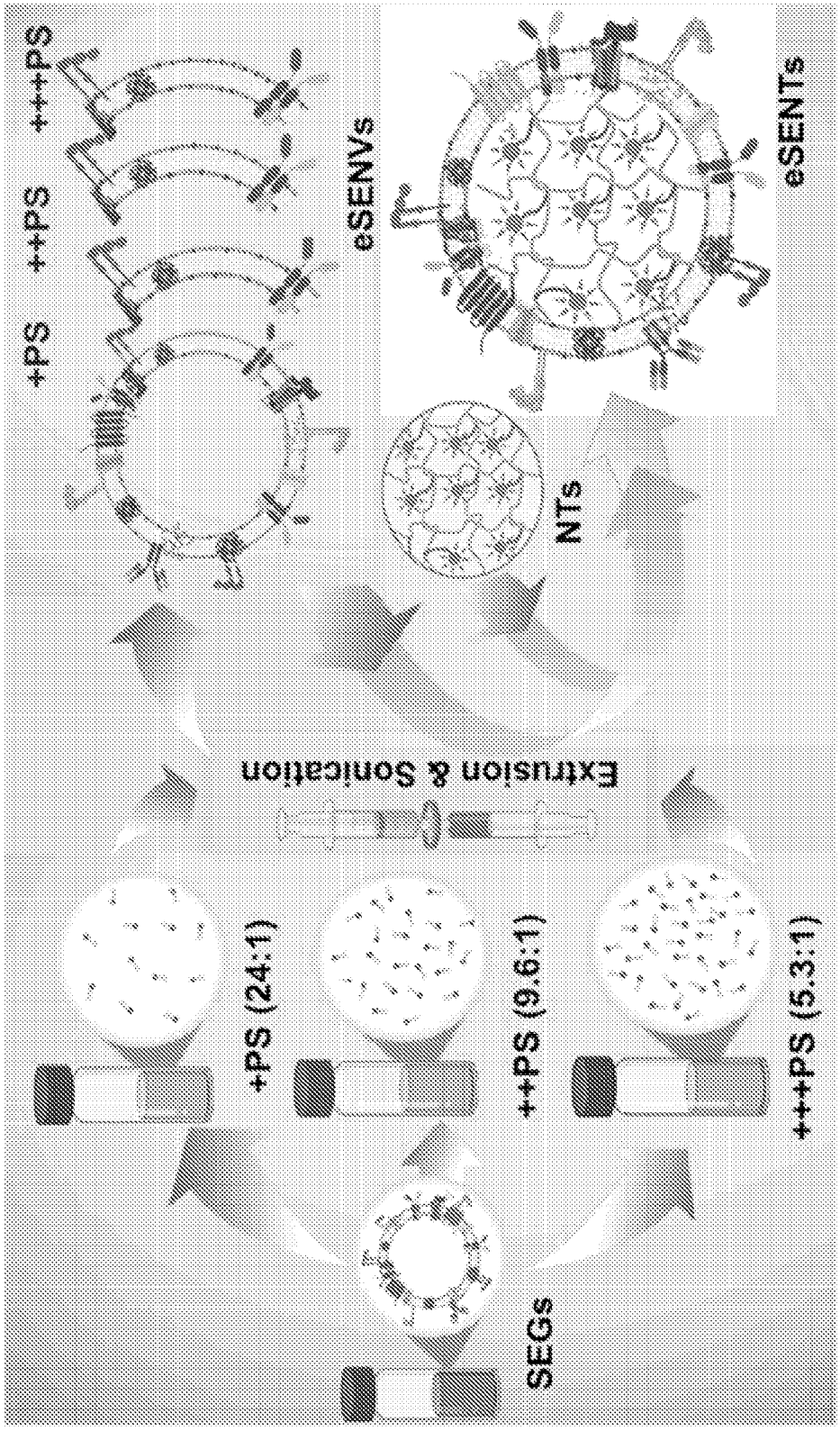
+PS
++PS
+++PS
eSENVs
eSENTs
NTs
Extrusion & Sonication
+PS (24:1)
++PS (9.6:1)
+++PS (5.3:1)
SEGs

FIG. 8A

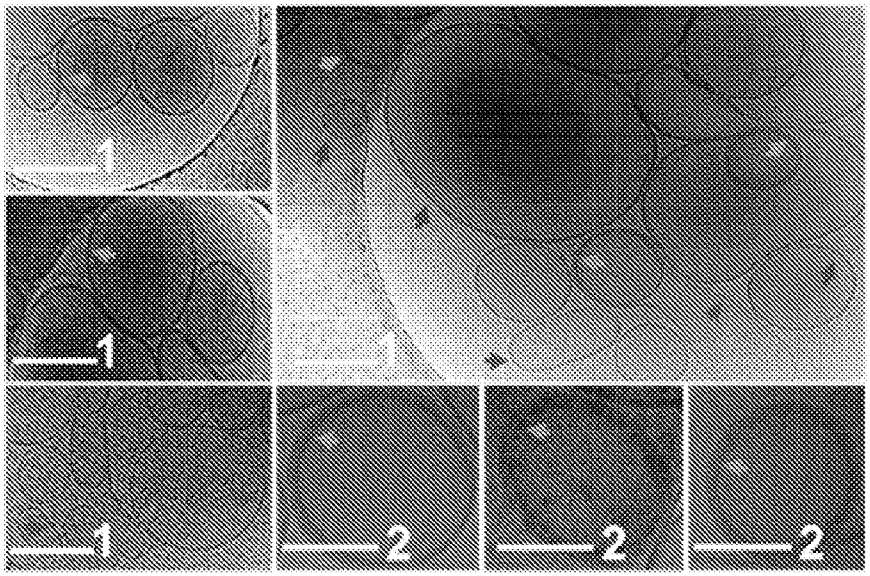

FIG. 8B

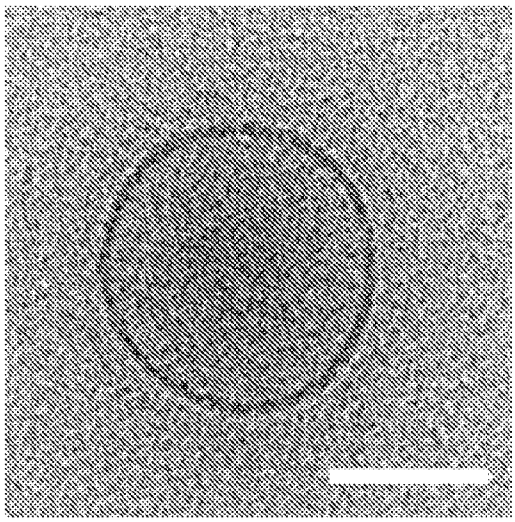

FIG. 8C

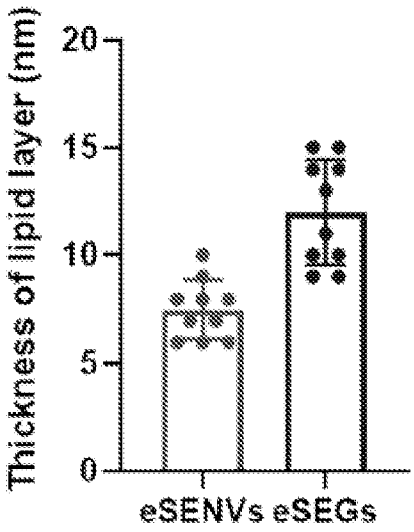

FIG. 8D

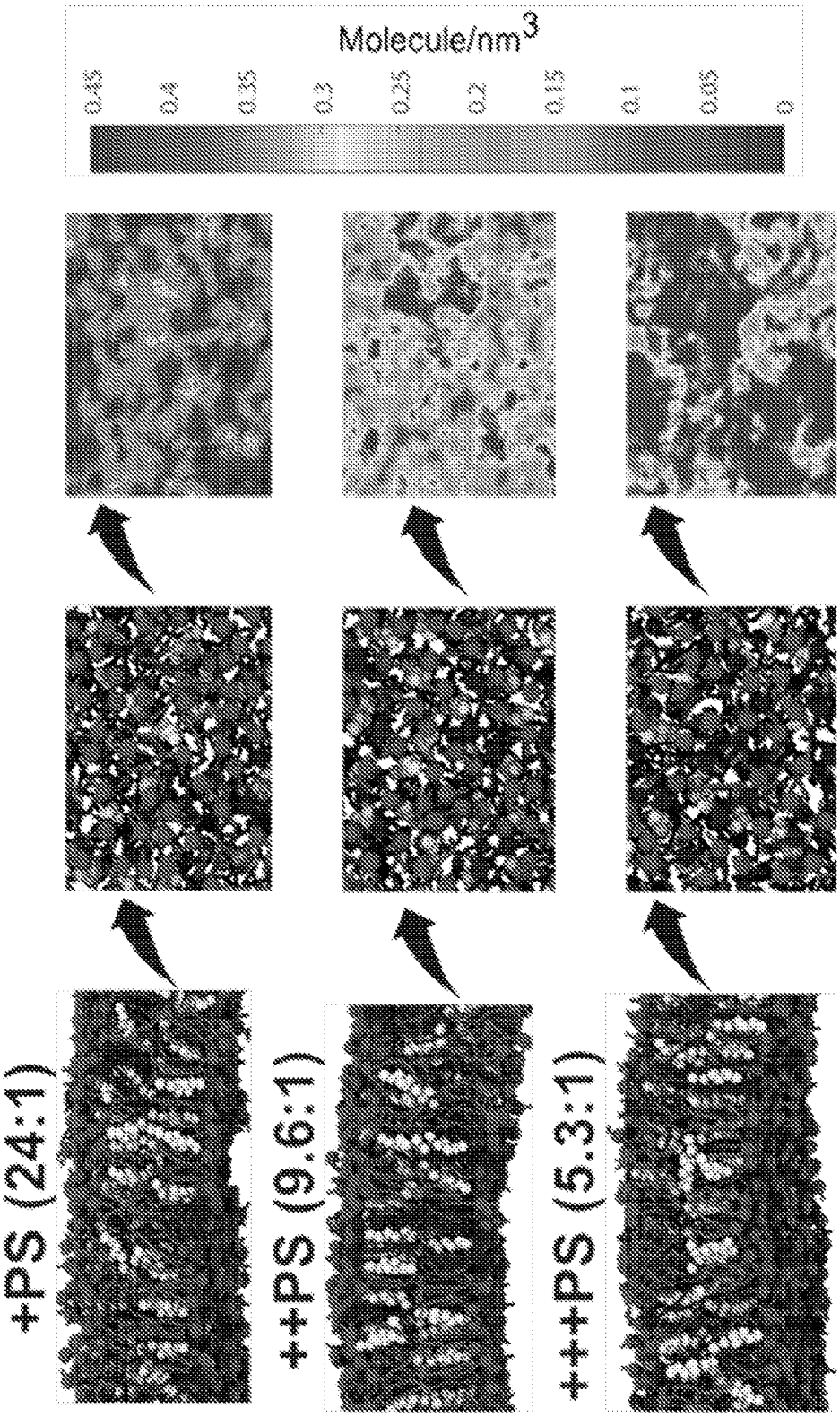
Molecule/nm3
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
+PS (24:1)
++PS (9.6:1)
+++PS (5.3:1)

FIG. 8E

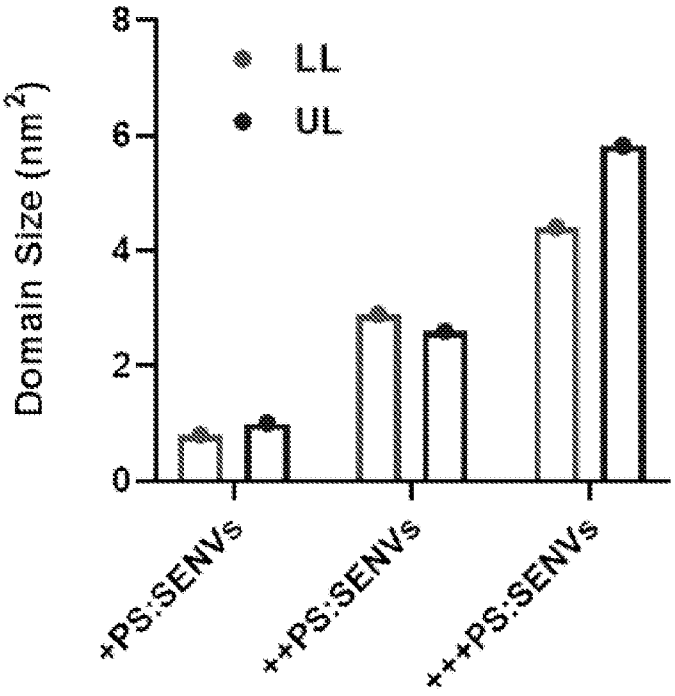

FIG. 8F

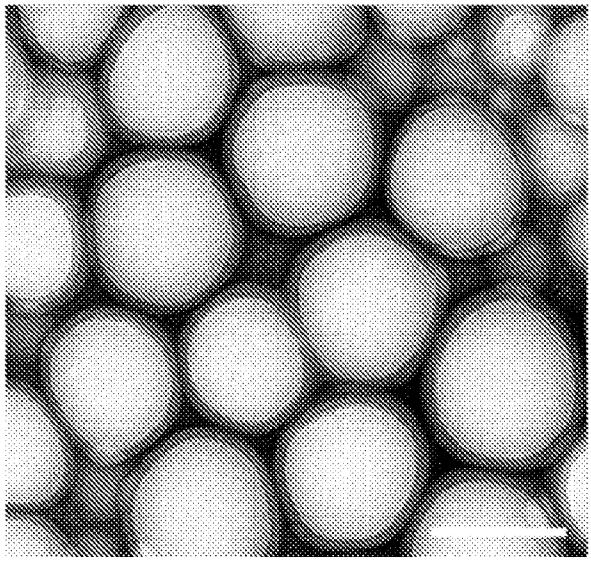

FIG. 8G

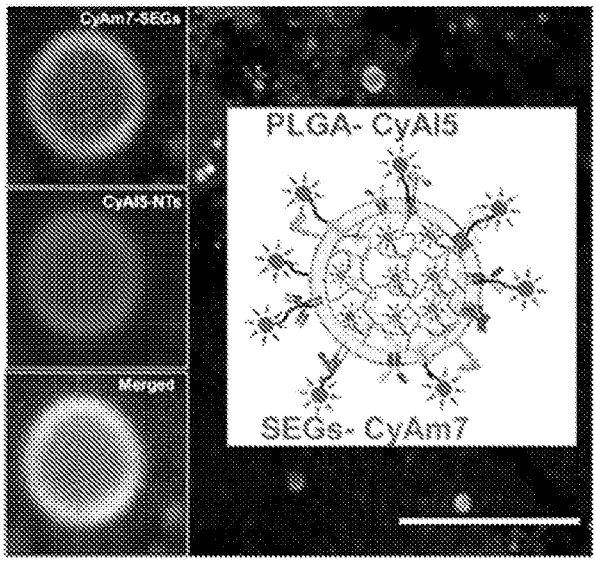

FIG. 8H

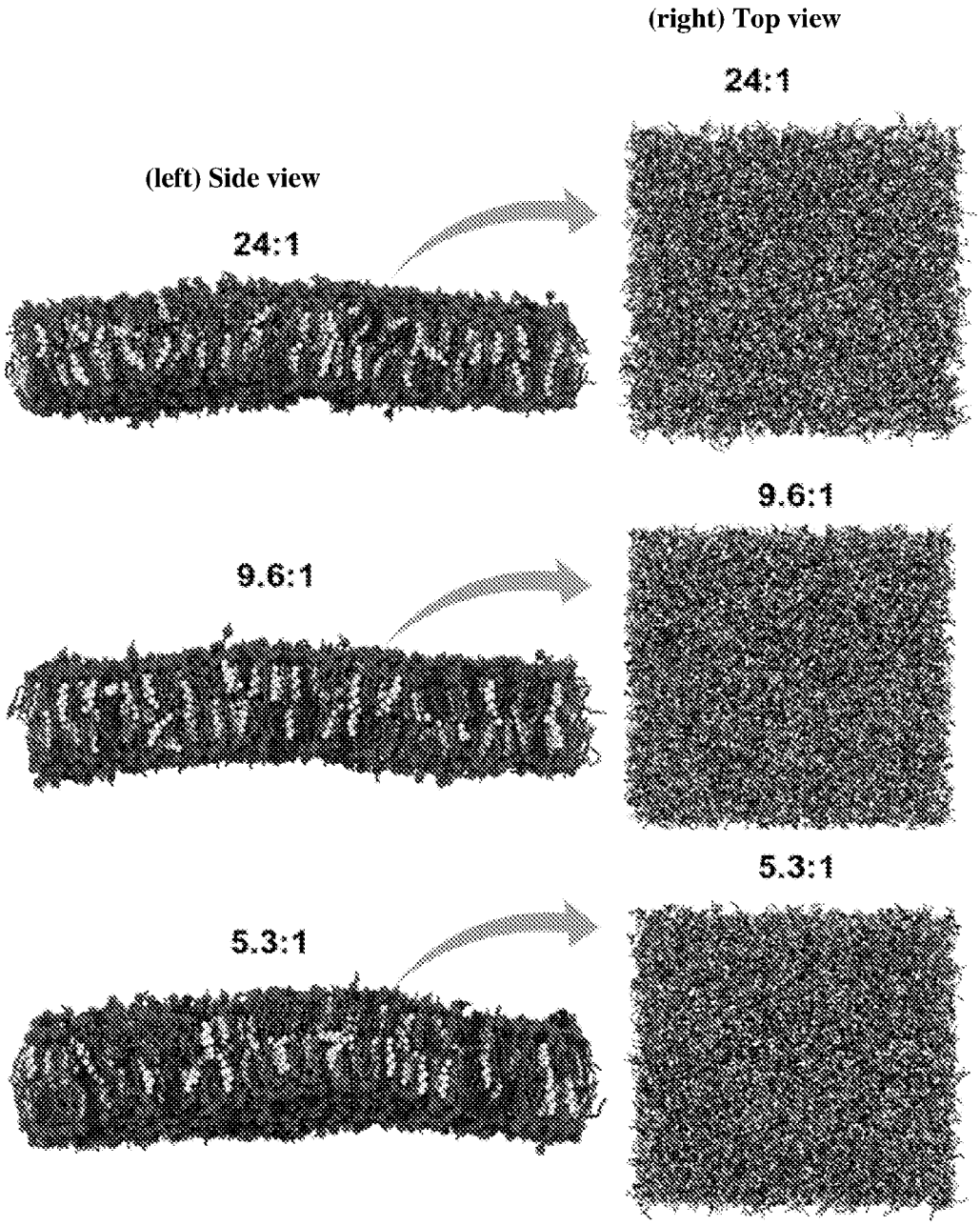

FIG. 9

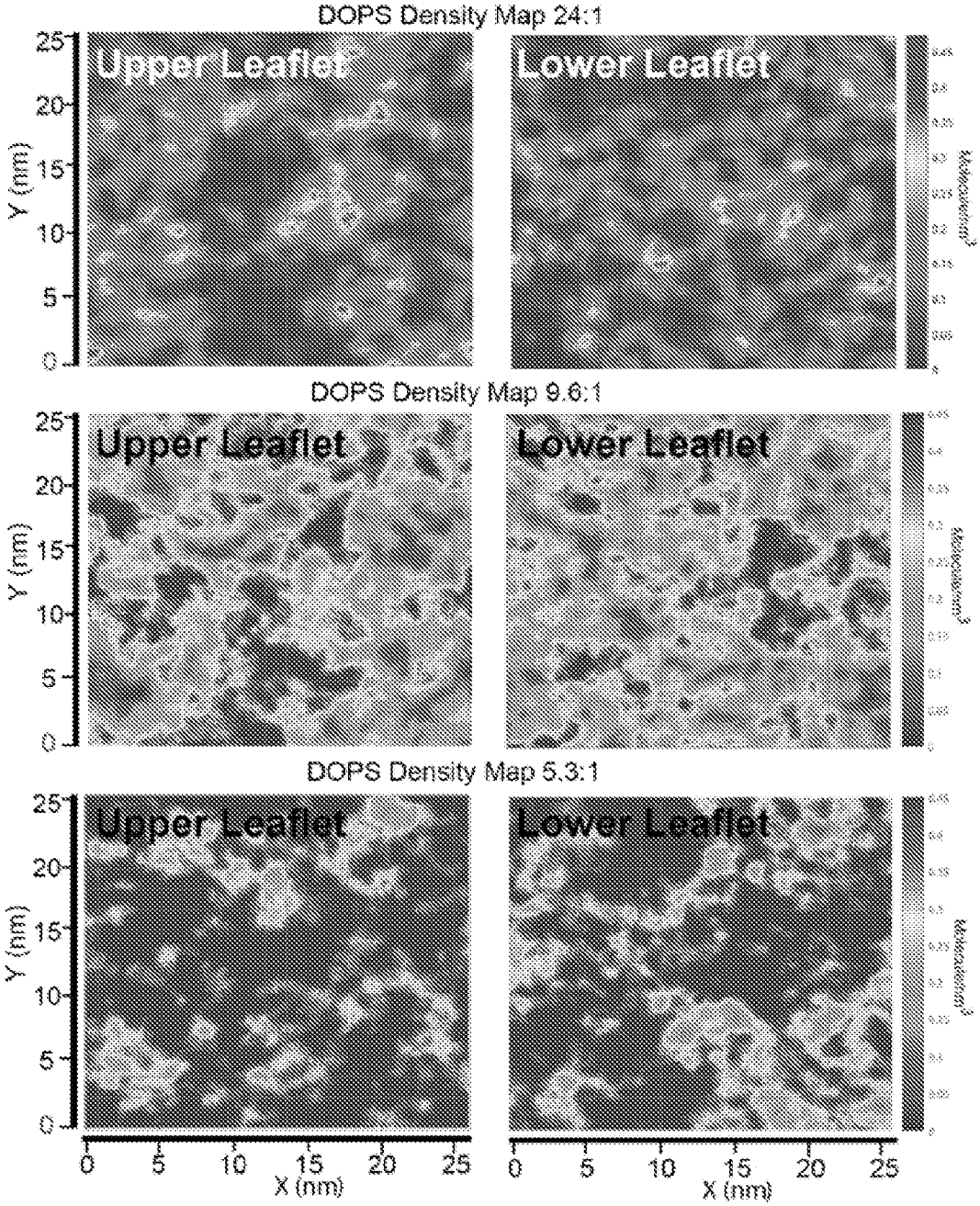
DOPS Density Map 24:1
Upper Leaflet
Lower Leaflet
DOPS Density Map 9.6:1
Upper Leaflet
Lower Leaflet
DOPS Density Map 5.3:1
Upper Leaflet
Lower Leaflet
Y (nm)
X (nm)
0
5
10
15
20
25

FIG. 10

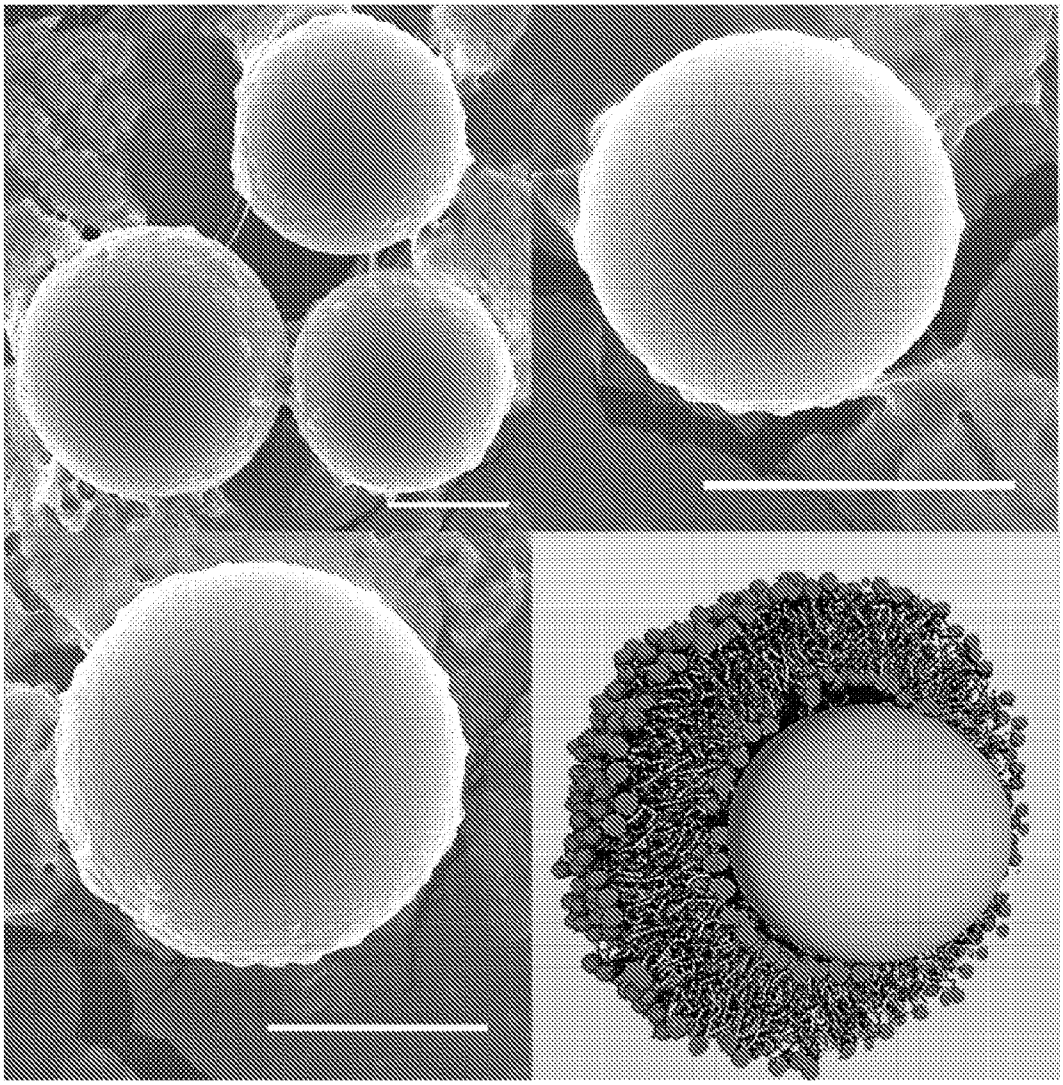

FIG. 11

FIG. 12 (top)

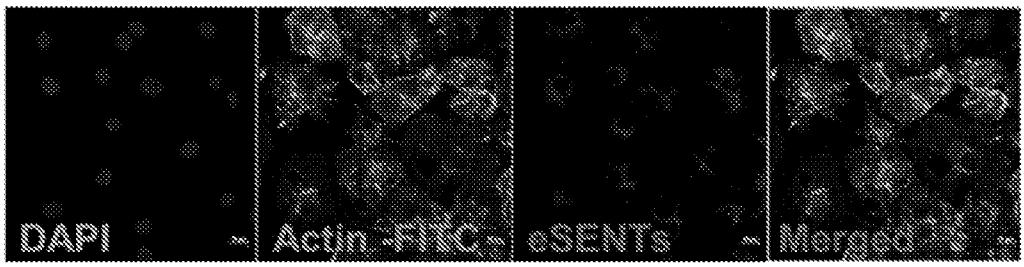

FIG. 12 (bottom)

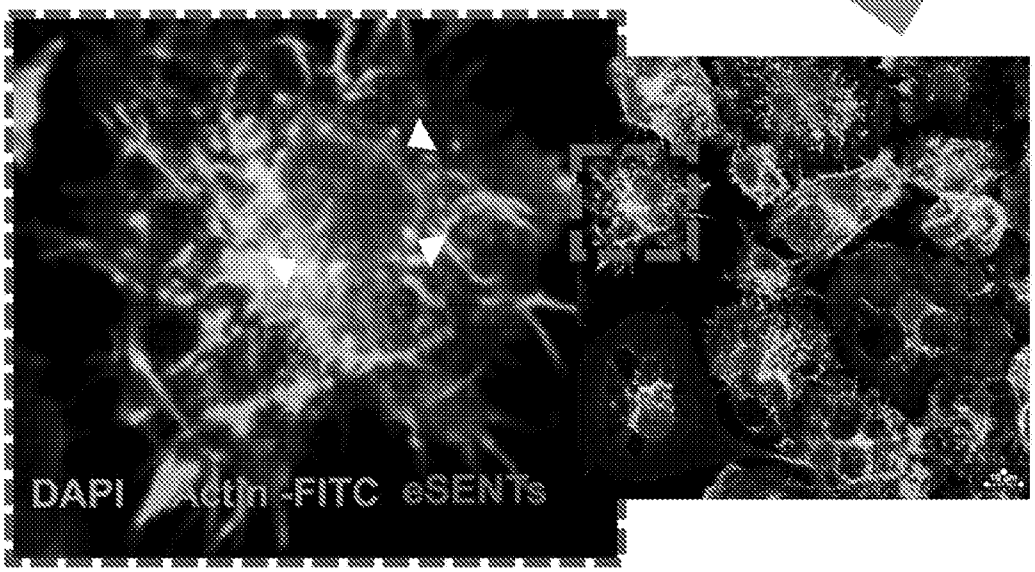

FIG. 12

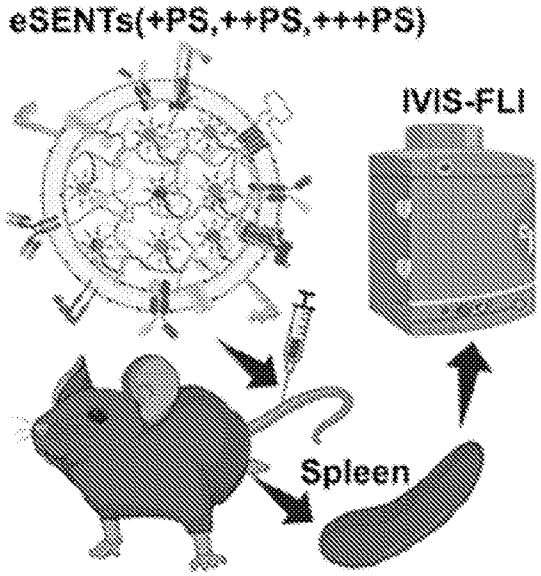

FIG. 13A

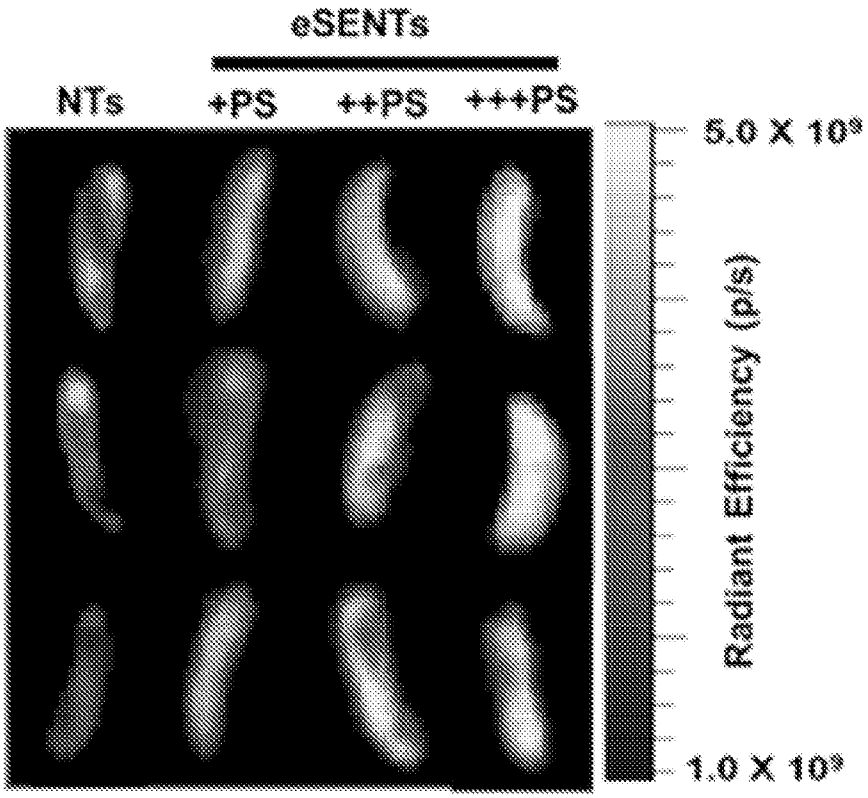

FIG. 13B

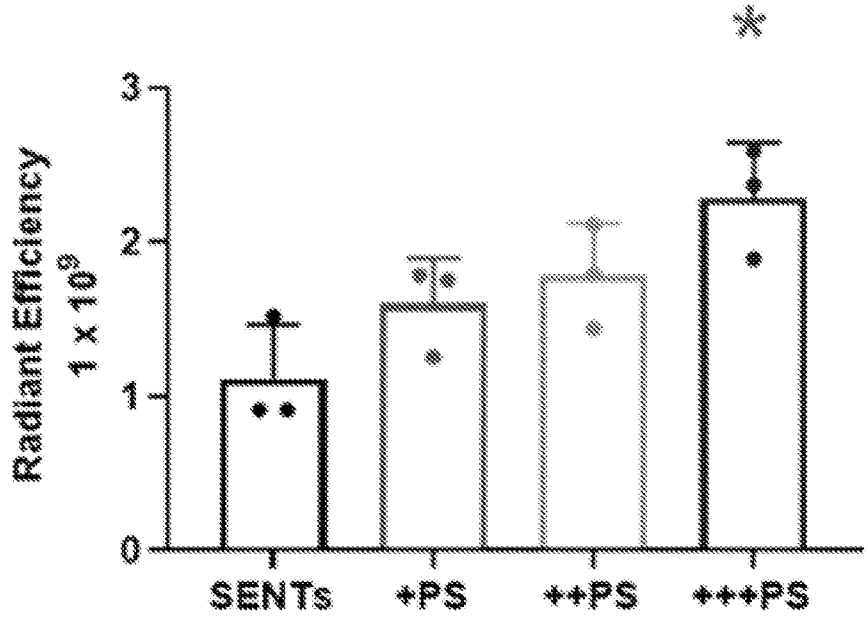

FIG. 13C

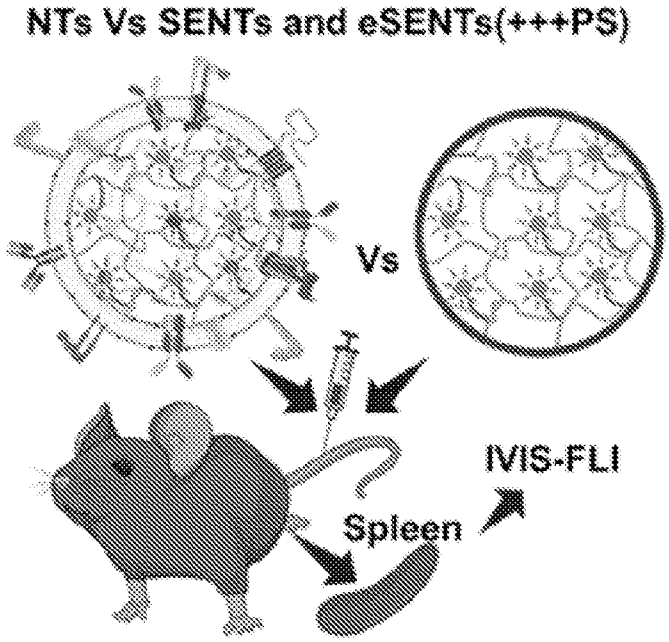

FIG. 13D

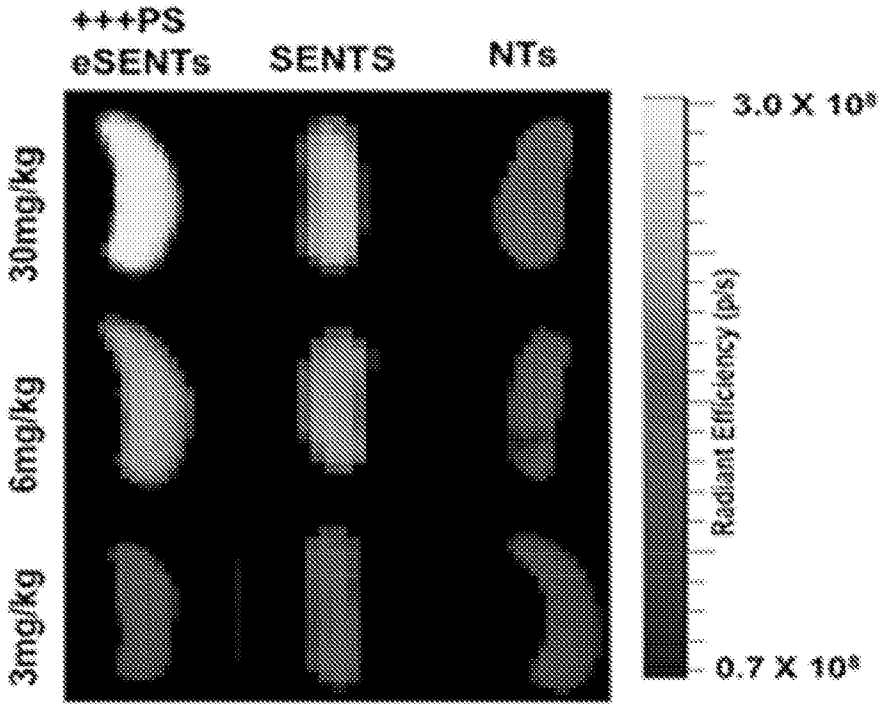

FIG. 13E

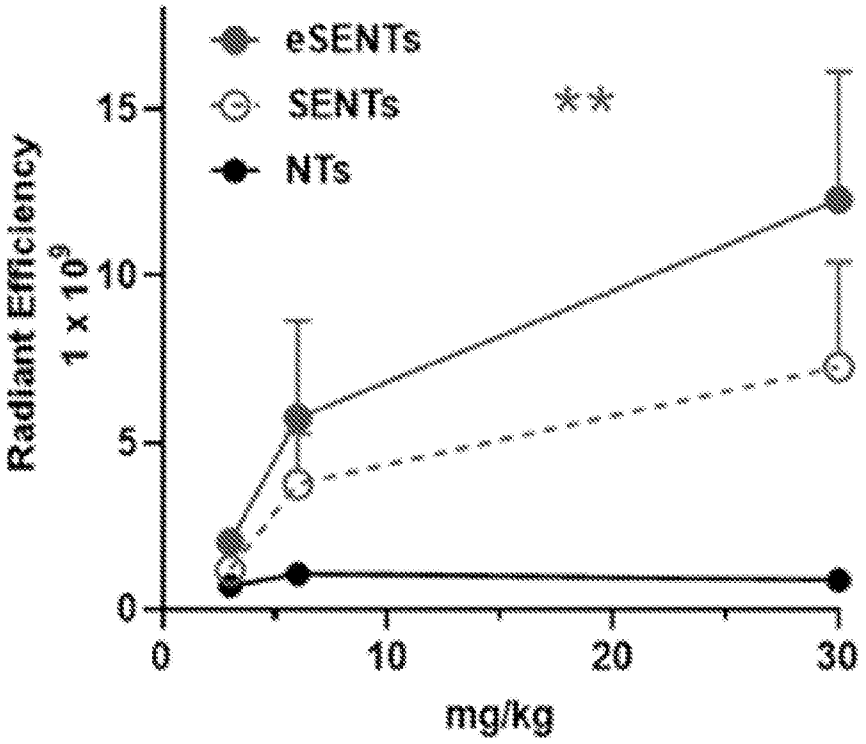

FIG. 13F

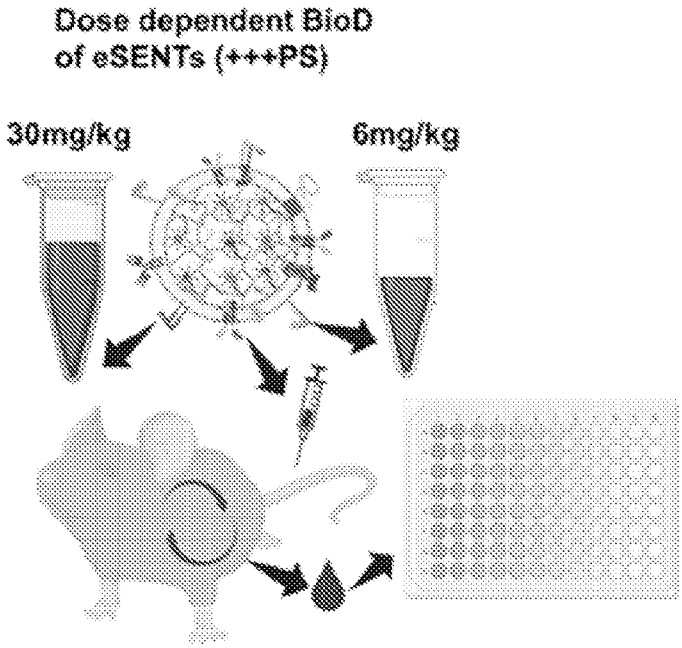

FIG. 13G

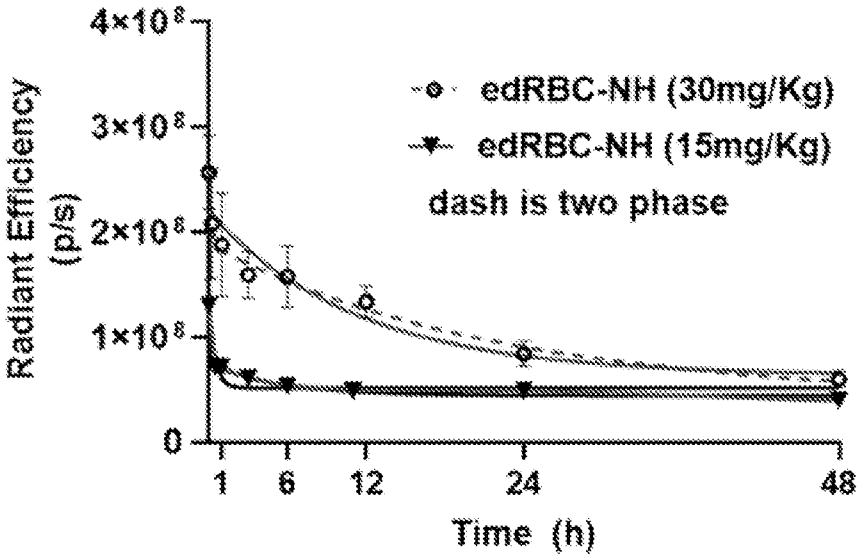

FIG. 13H

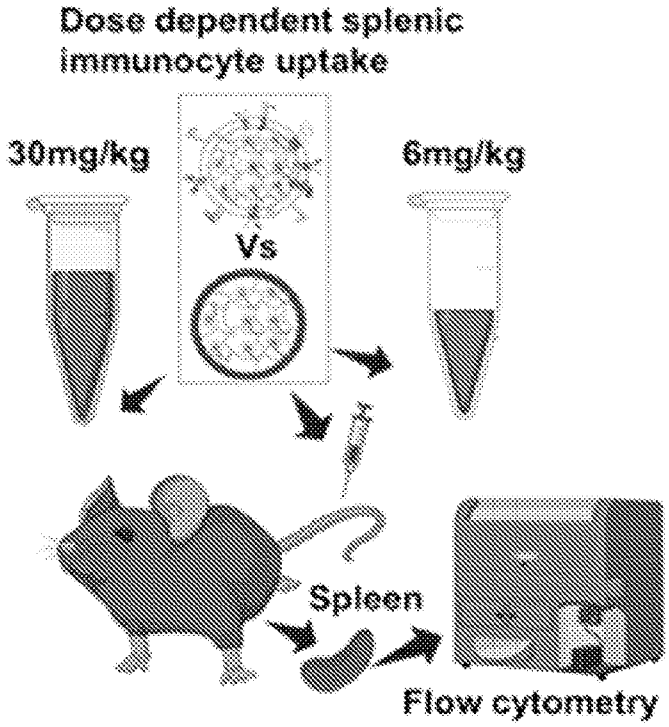

FIG. 13I

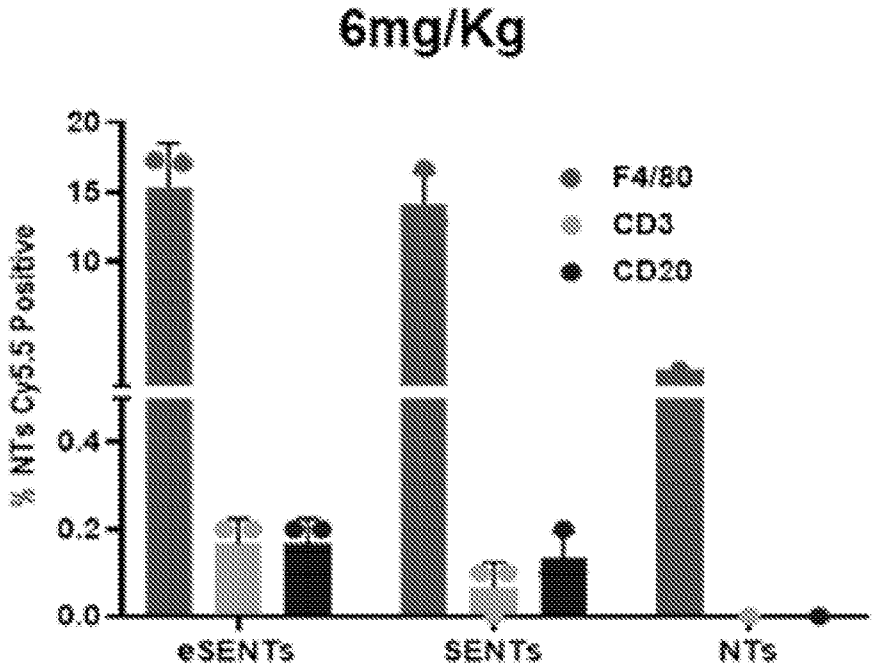

FIG. 13J

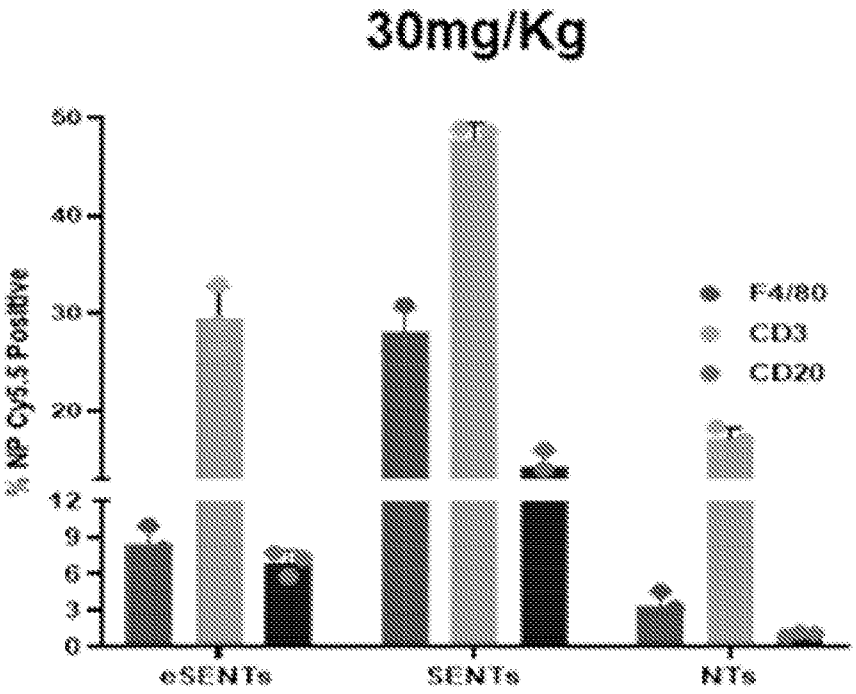
30mg/Kg
% MP Cy5.5 Positive
50
40
30
20
12
9
6
3
0
F4/80
CD3
CD20
eSENTs
SENTs
NTs

FIG. 13K

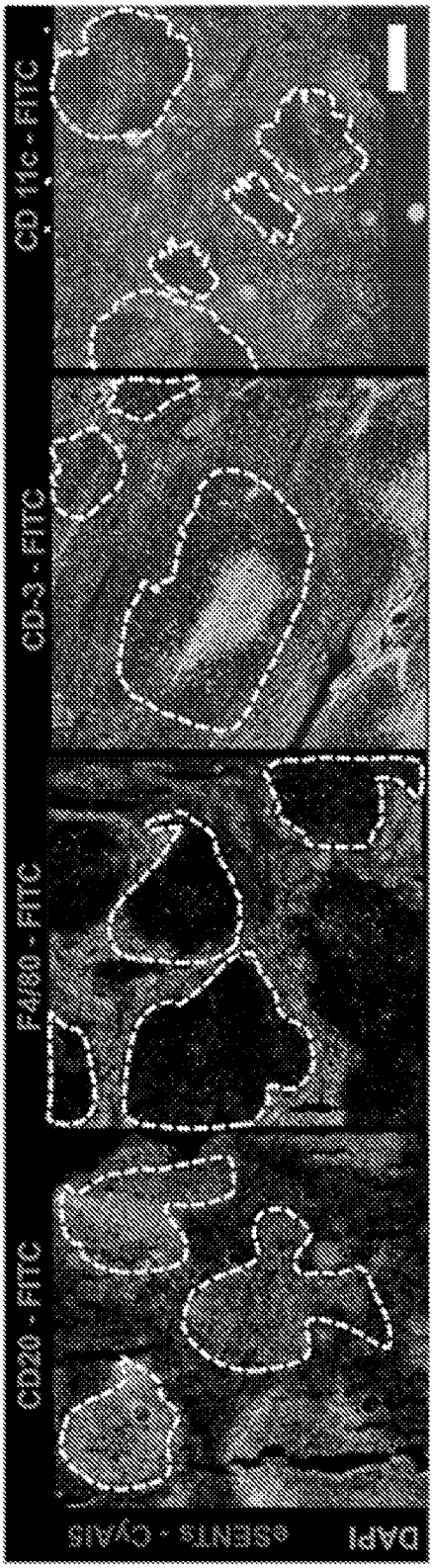

FIG. 13L FIG. 13M FIG. 13N FIG. 13O

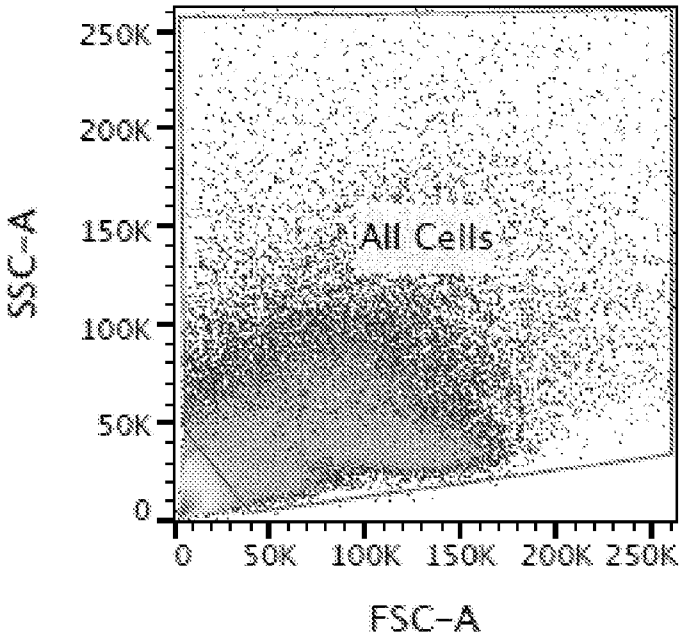

FIG. 14A

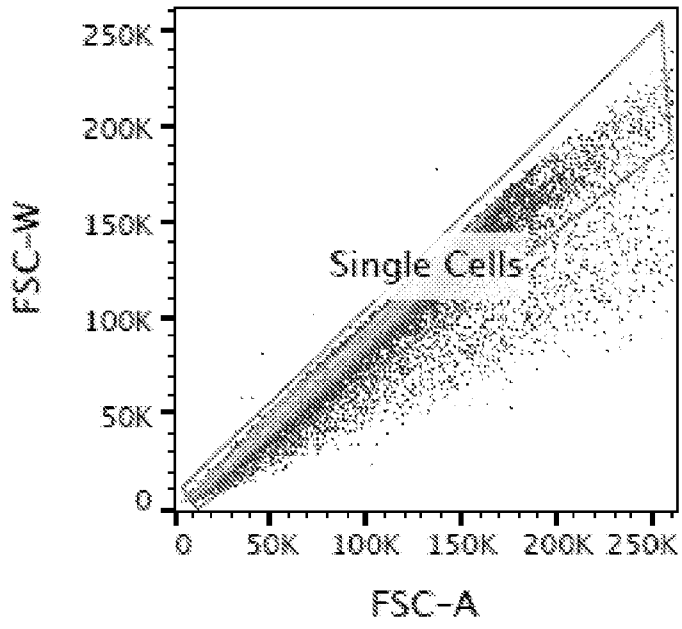

FIG. 14B

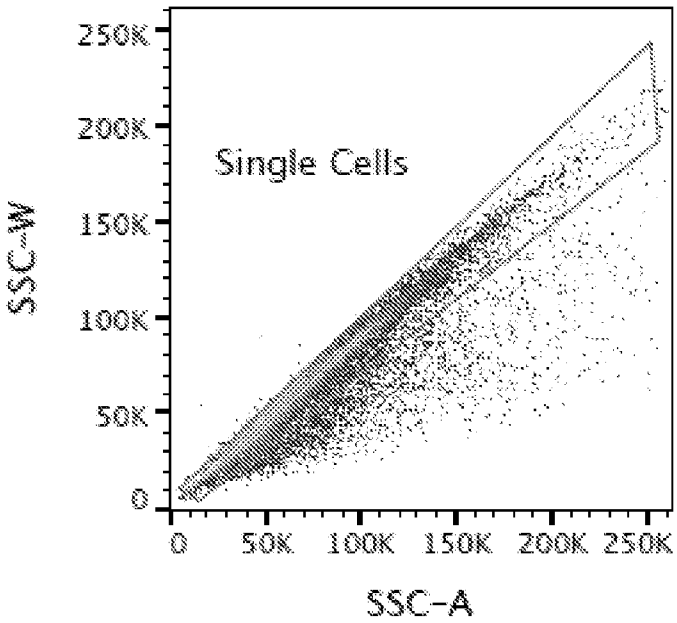

FIG. 14C

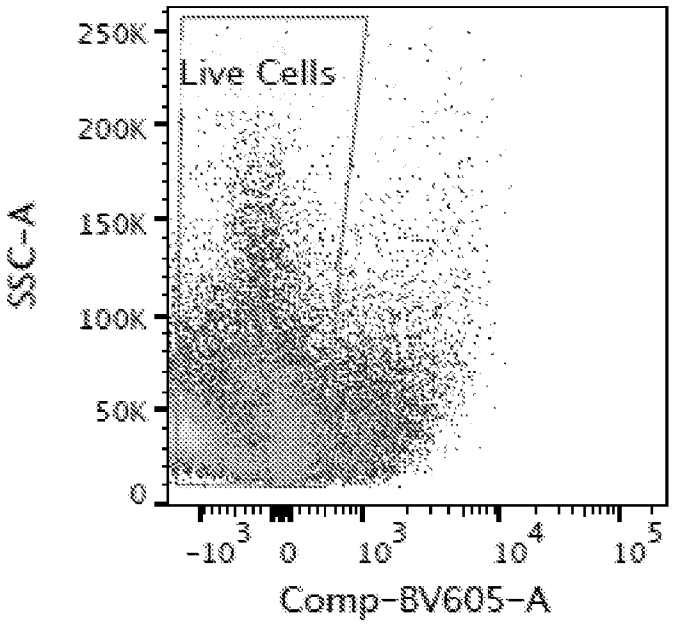

FIG. 14D

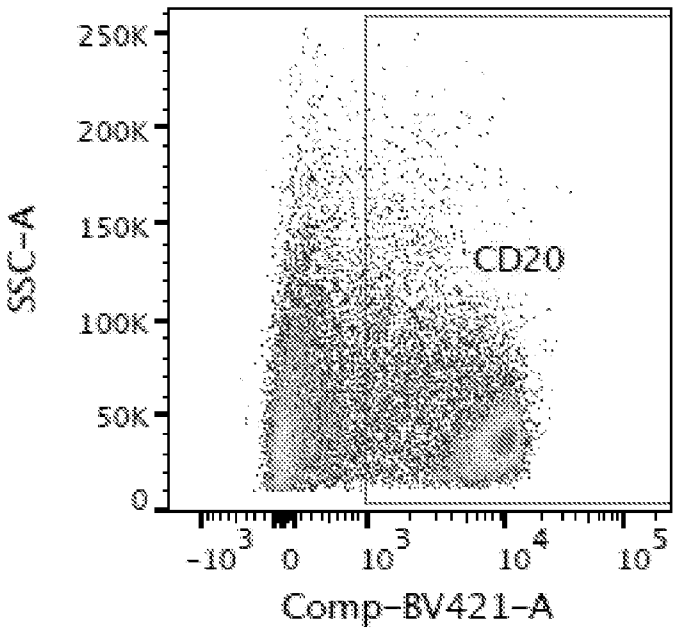

FIG. 14E

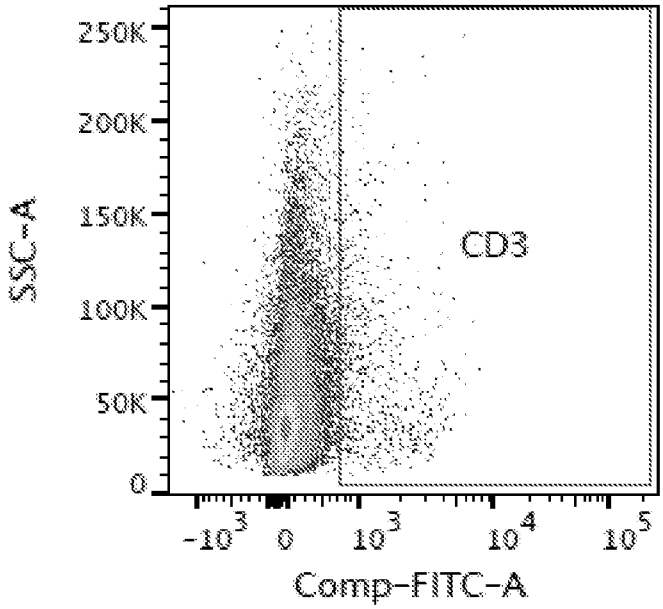

FIG. 14F

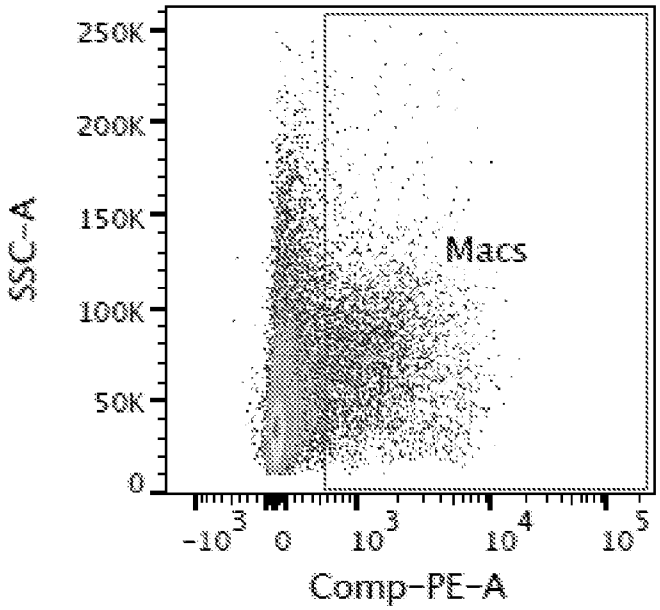

FIG. 14G

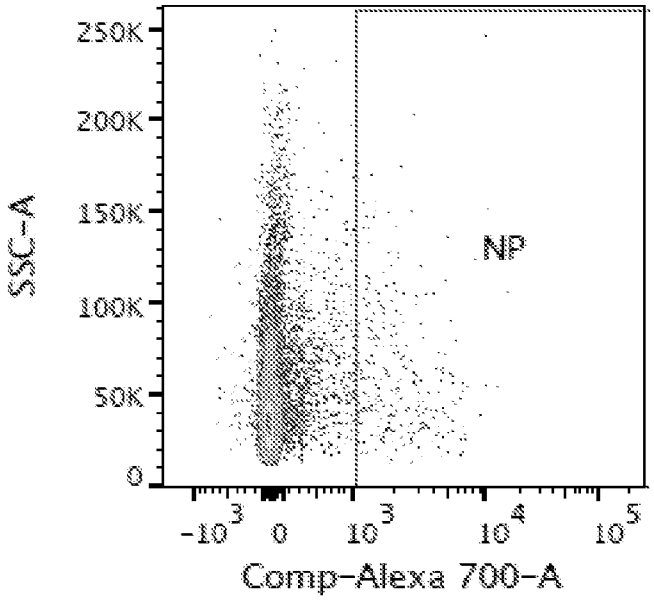

FIG. 14H

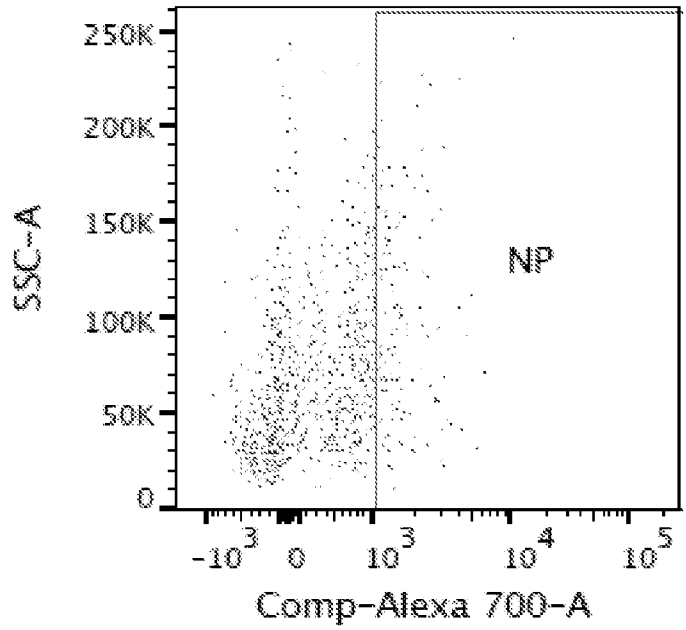

FIG. 14I

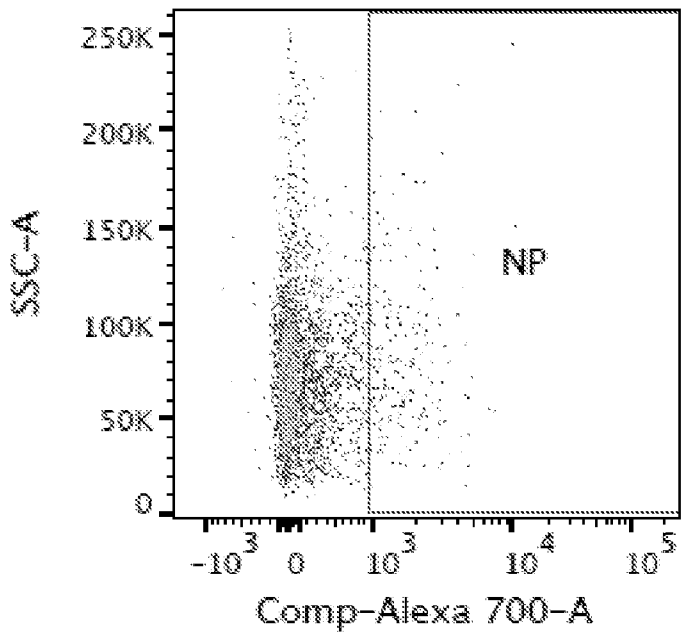

FIG. 14J

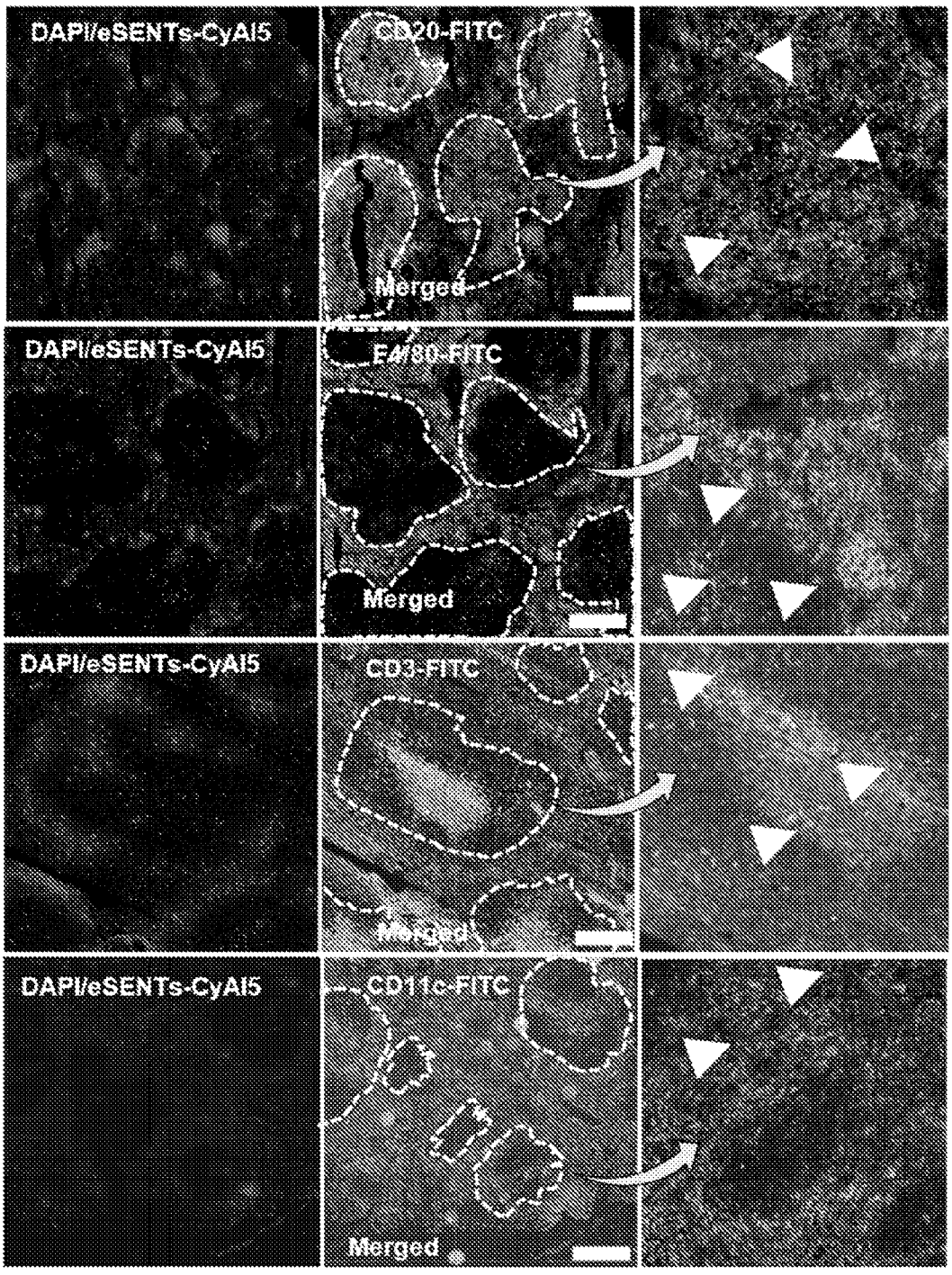
DAPI/eSENTs-CyAI5
CD20-FITC
Merged
DAPI/eSENTs-CyAI5
F4/80-FITC
Merged
DAPI/eSENTs-CyAI5
CD3-FITC
Merged
DAPI/eSENTs-CyAI5
CD11c-FITC
Merged

FIG. 15

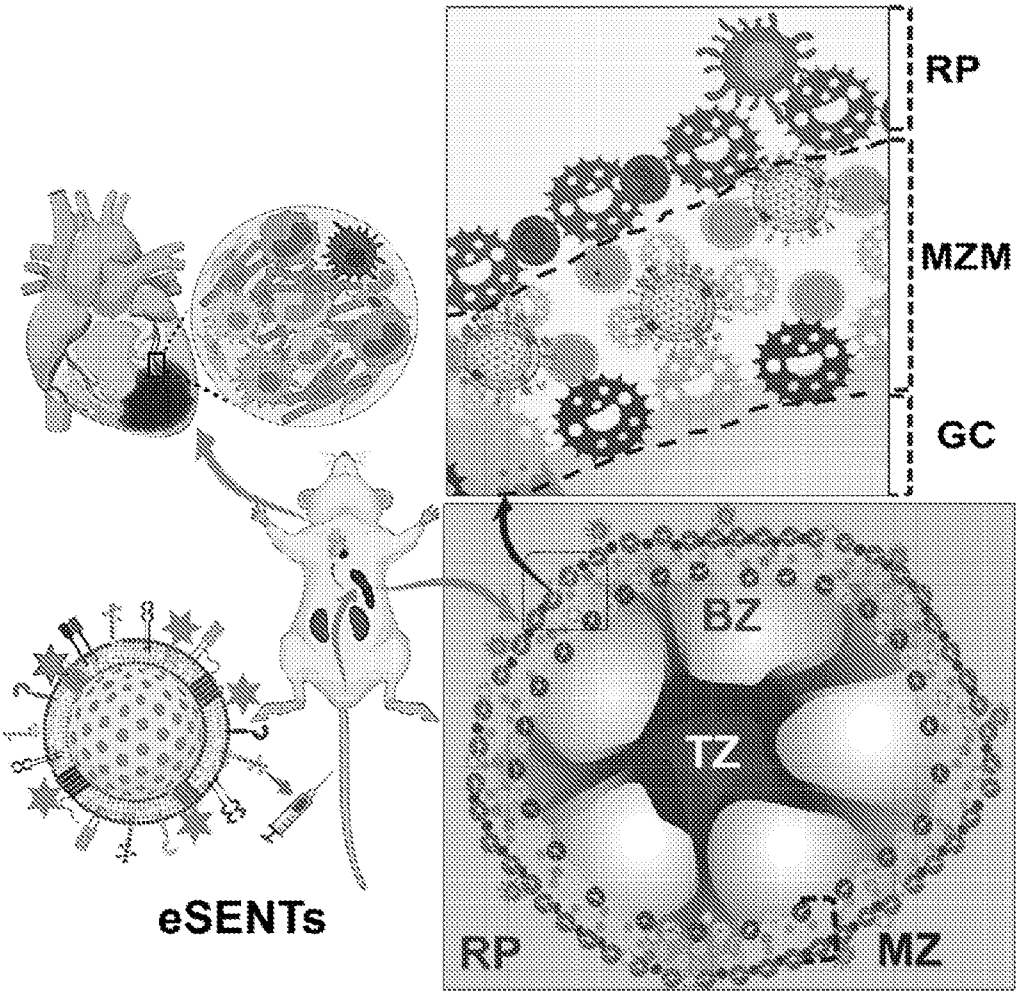

FIG. 16

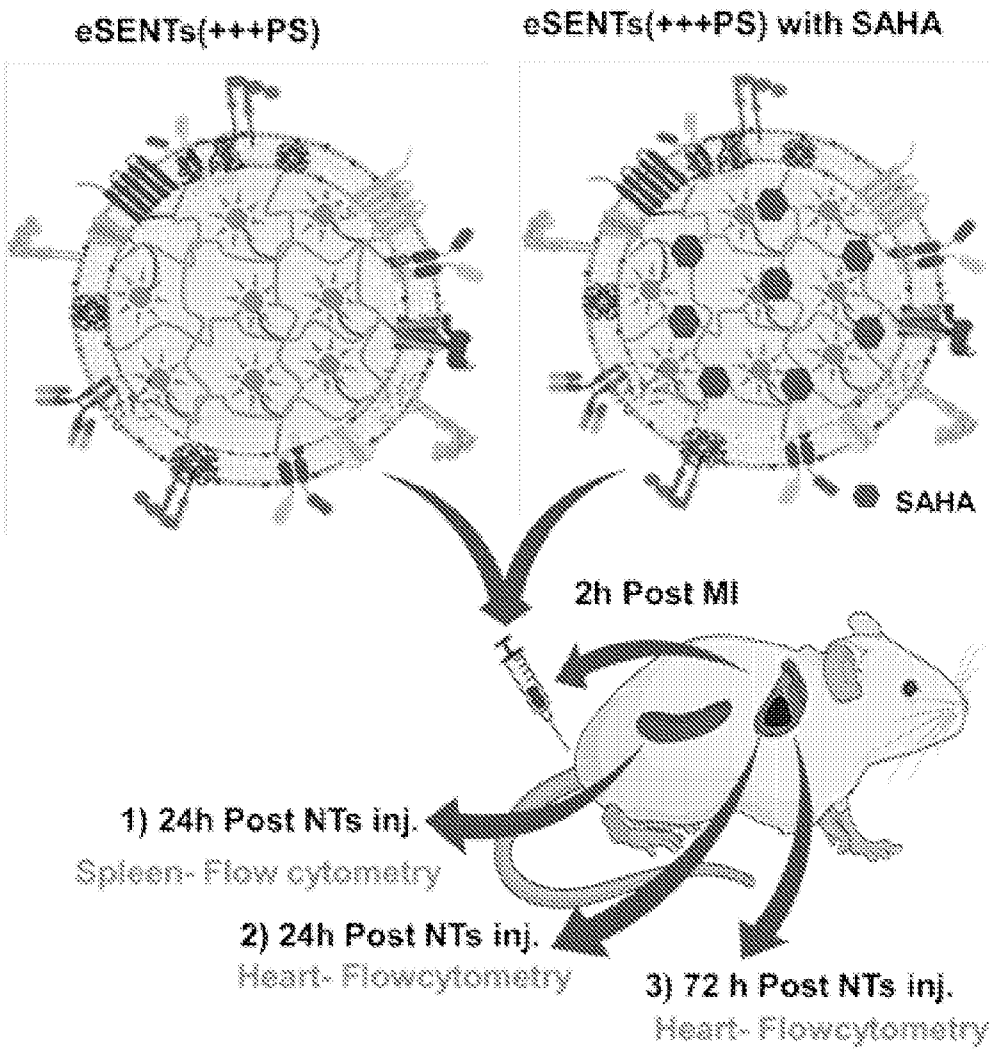
eSENTs(+++PS)
eSENTs(+++PS) with SAHA
SAHA
2h Post MI
1) 24h Post NTs inj.
Spleen- Flow cytometry
2) 24h Post NTs inj.
Heart- Flowcytometry
3) 72 h Post NTs inj.
Heart- Flowcytometry

FIG. 17A

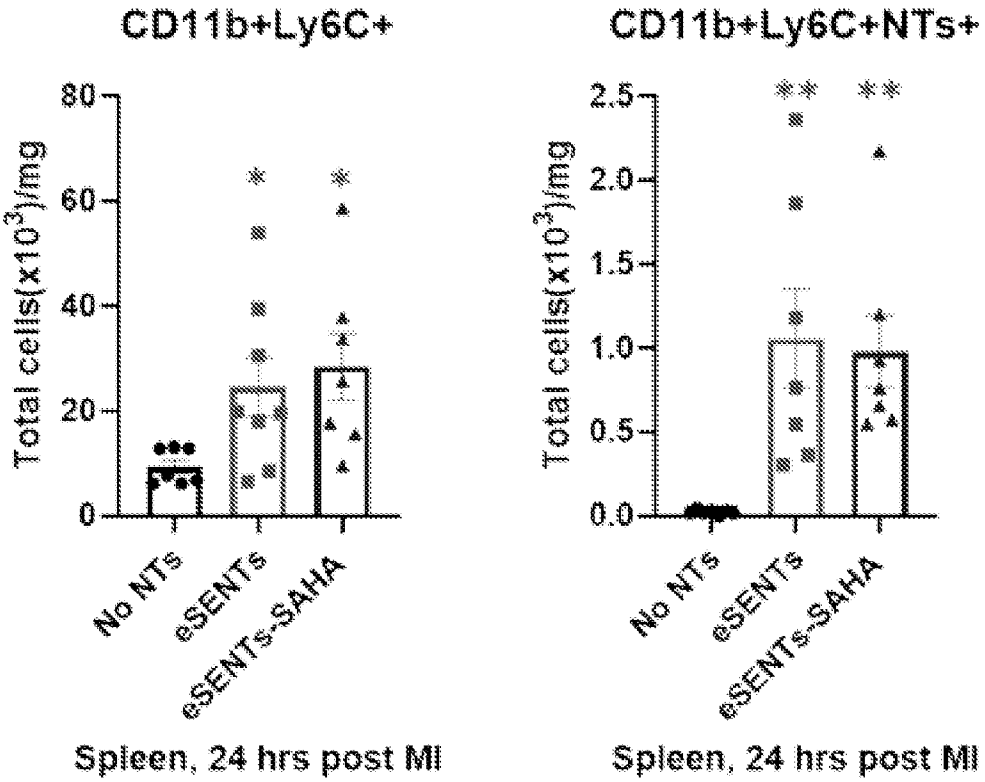

FIG. 17B

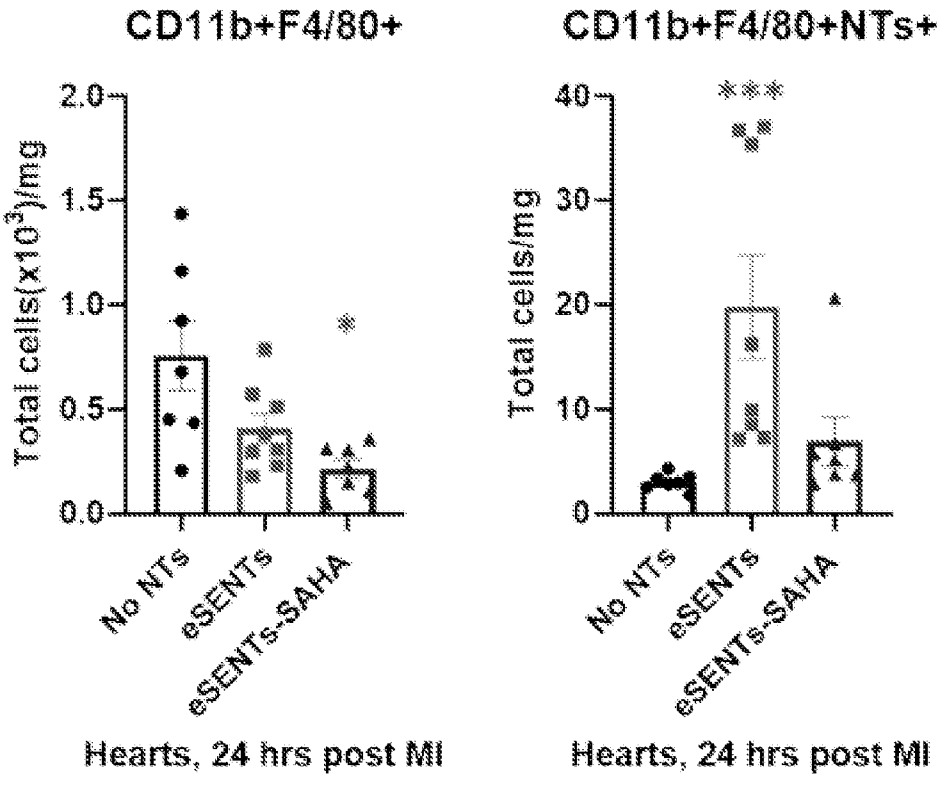

FIG. 17C

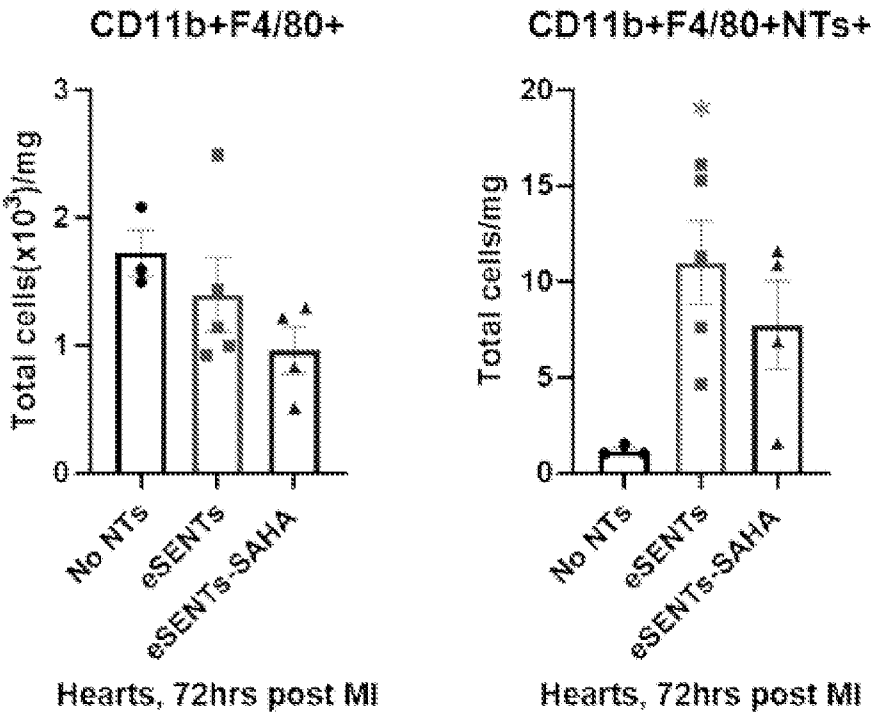

FIG. 17D

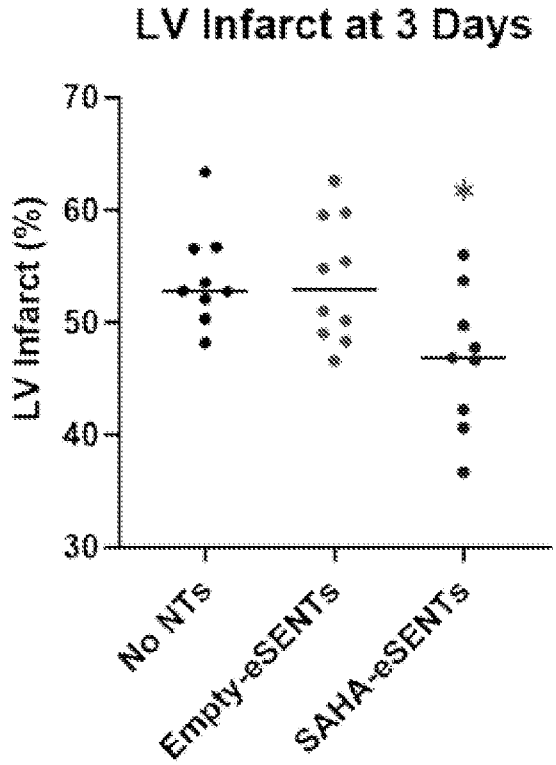

FIG. 17E

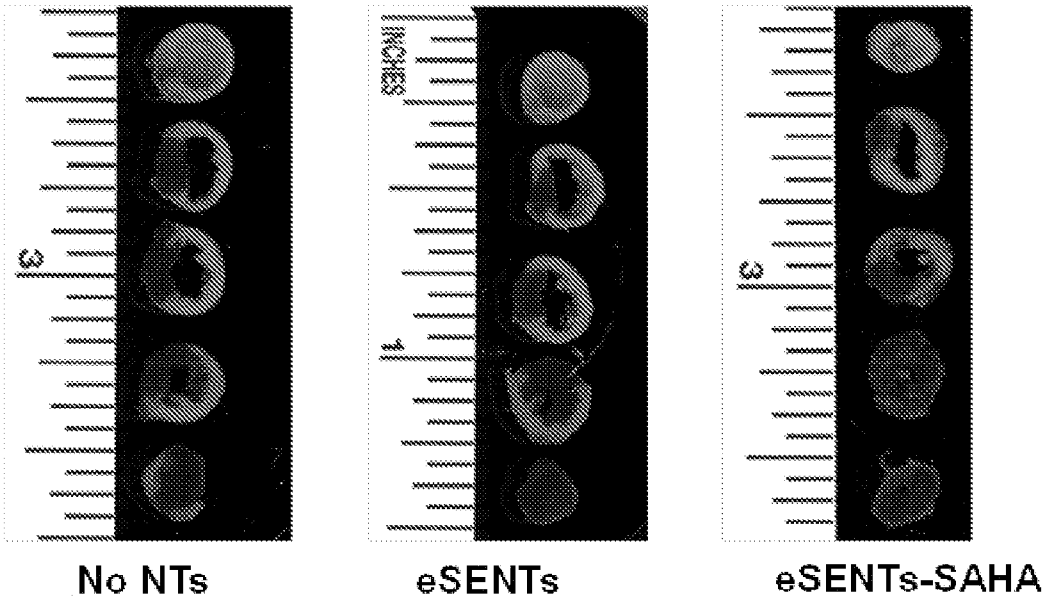

FIG. 17F

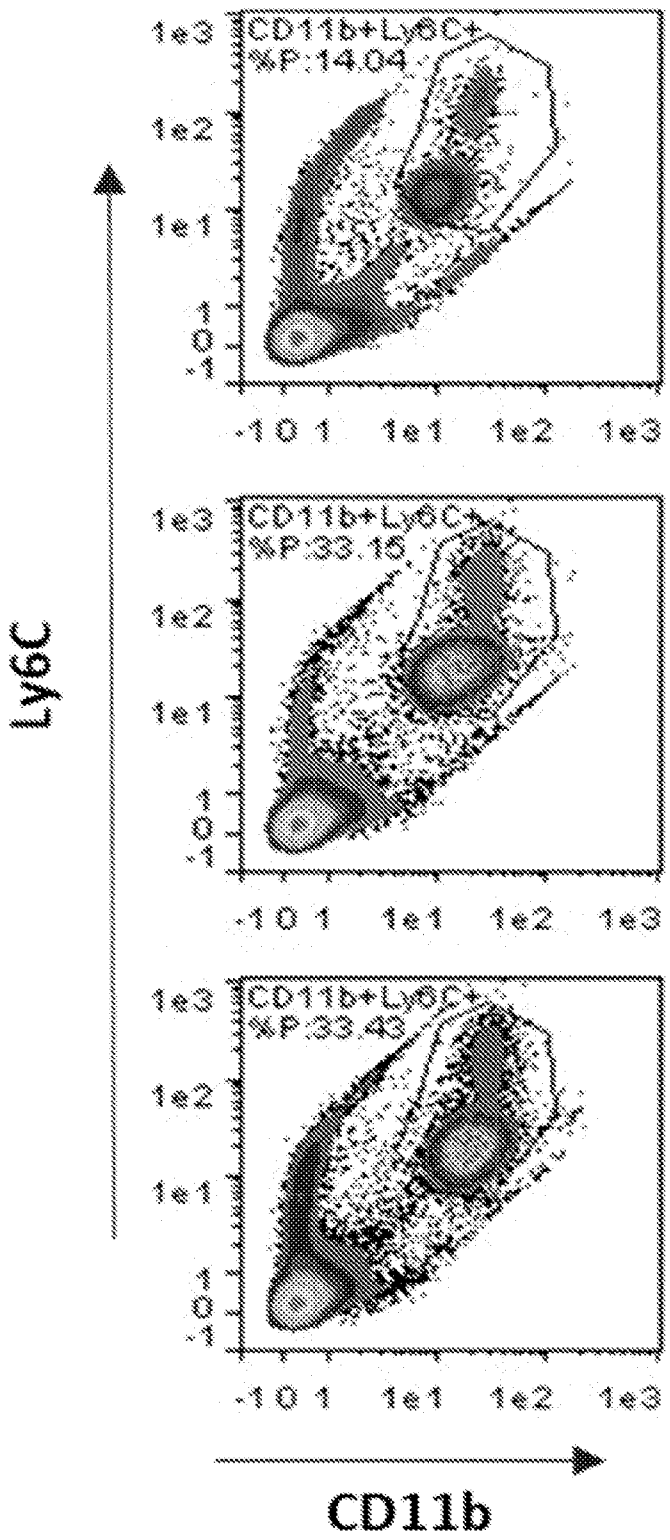
CD11b+Ly6C+
%P:14.04
CD11b+Ly6C+
%P:33.15
CD11b+Ly6C+
%P:33.43
1e3
1e2
1e1
0
-1
-1 0 1 1e1 1e2 1e3
Ly6C
CD11b

FIG. 18A

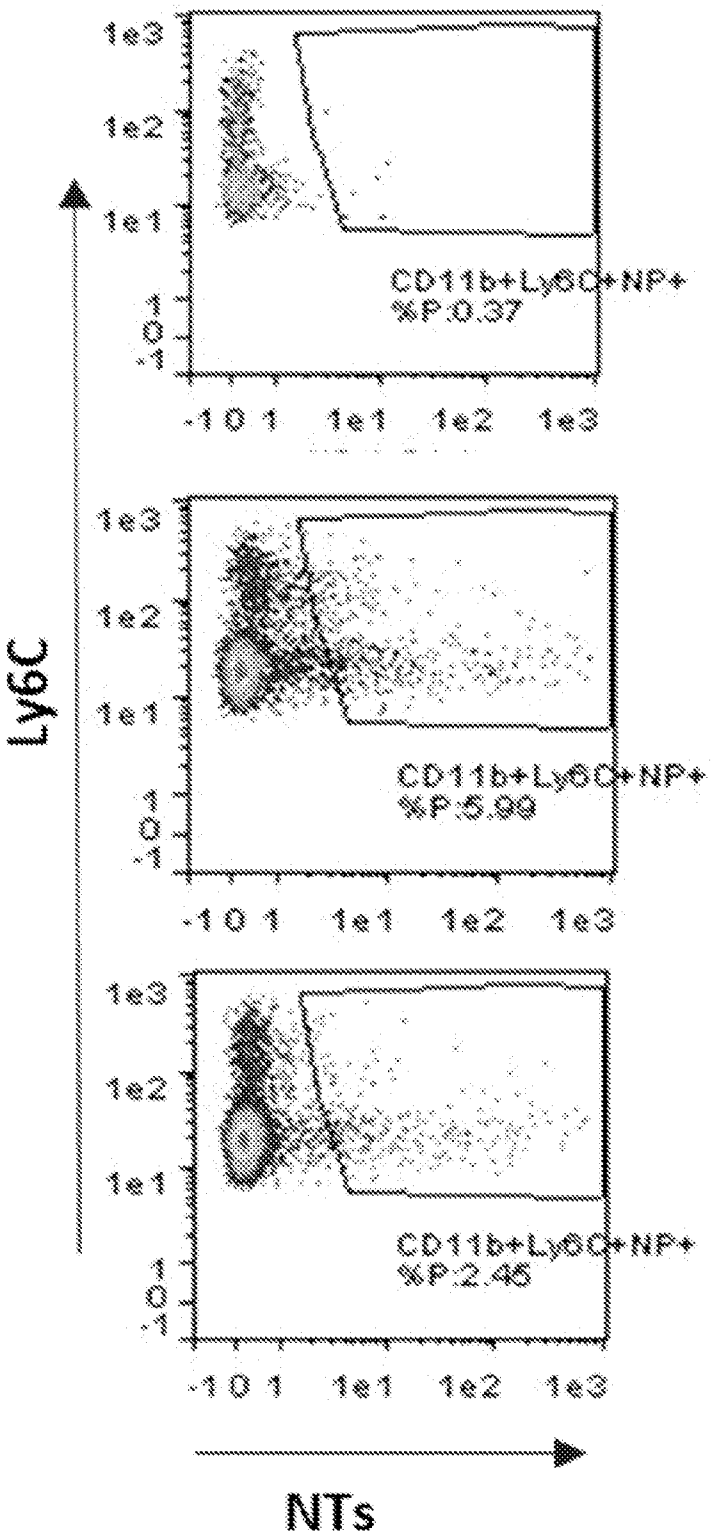
Ly6C
NTs
CD11b+Ly6C+NP+
%P:0.37
CD11b+Ly6C+NP+
%P:5.90
CD11b+Ly6C+NP+
%P:2.46
1e3
1e2
1e1
-1 0 1
1e1
1e2
1e3

FIG 18B

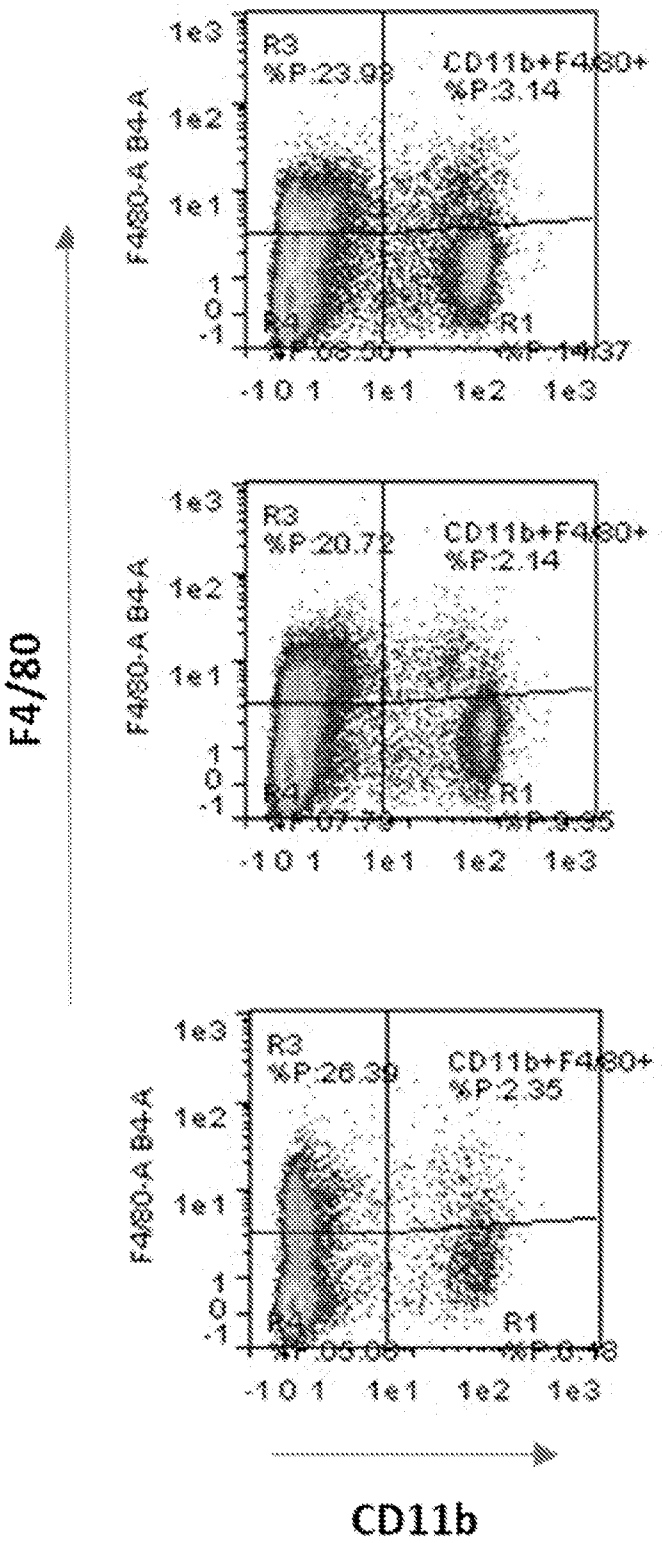
F4/80
CD11b
F480-A B4-A
R3
%P:23.99
CD11b+F480+
%P:3.14
R1
%P:14.37
%P:20.72
%P:2.14
%P:9.35
%P:26.30
%P:2.35
%P:0.13
-1 0 1
1e1
1e2
1e3

FIG. 18C

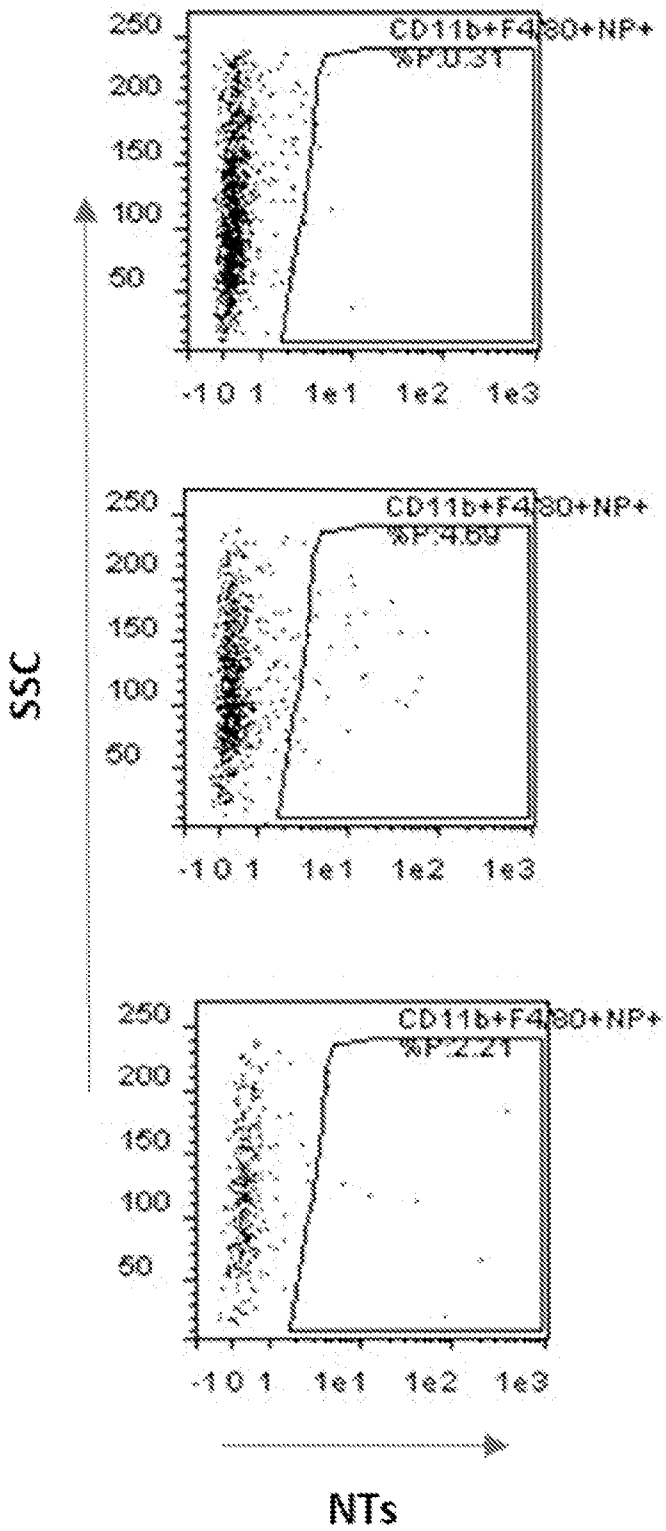

FIG. 18D

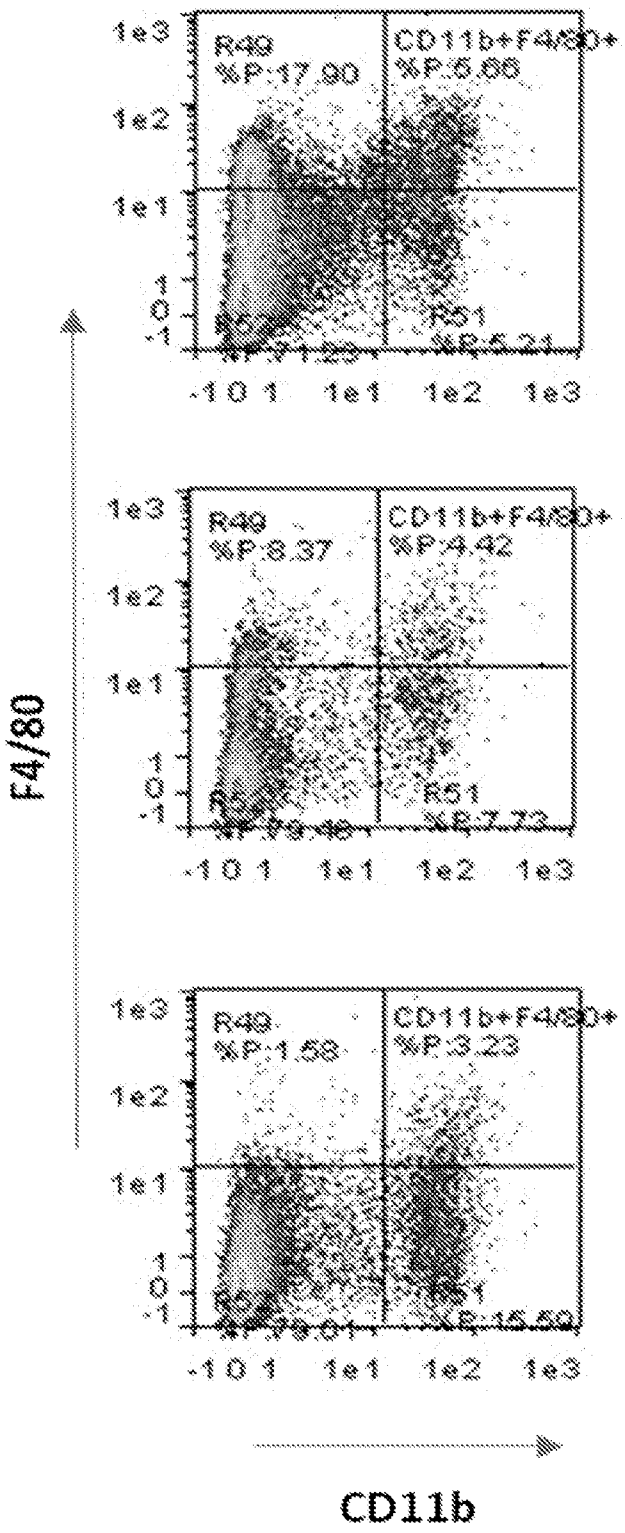
R49
%P:17.90
CD11b+F4/80+
%P:6.66
R51
%P:5.21
R49
%P:8.37
CD11b+F4/80+
%P:4.42
R51
%P:7.72
R49
%P:1.58
CD11b+F4/80+
%P:3.23
R51
%P:15.69
1e3
1e2
1e1
1
0
-1
-1 0 1 1e1 1e2 1e3
F4/80
CD11b

FIG. 18E

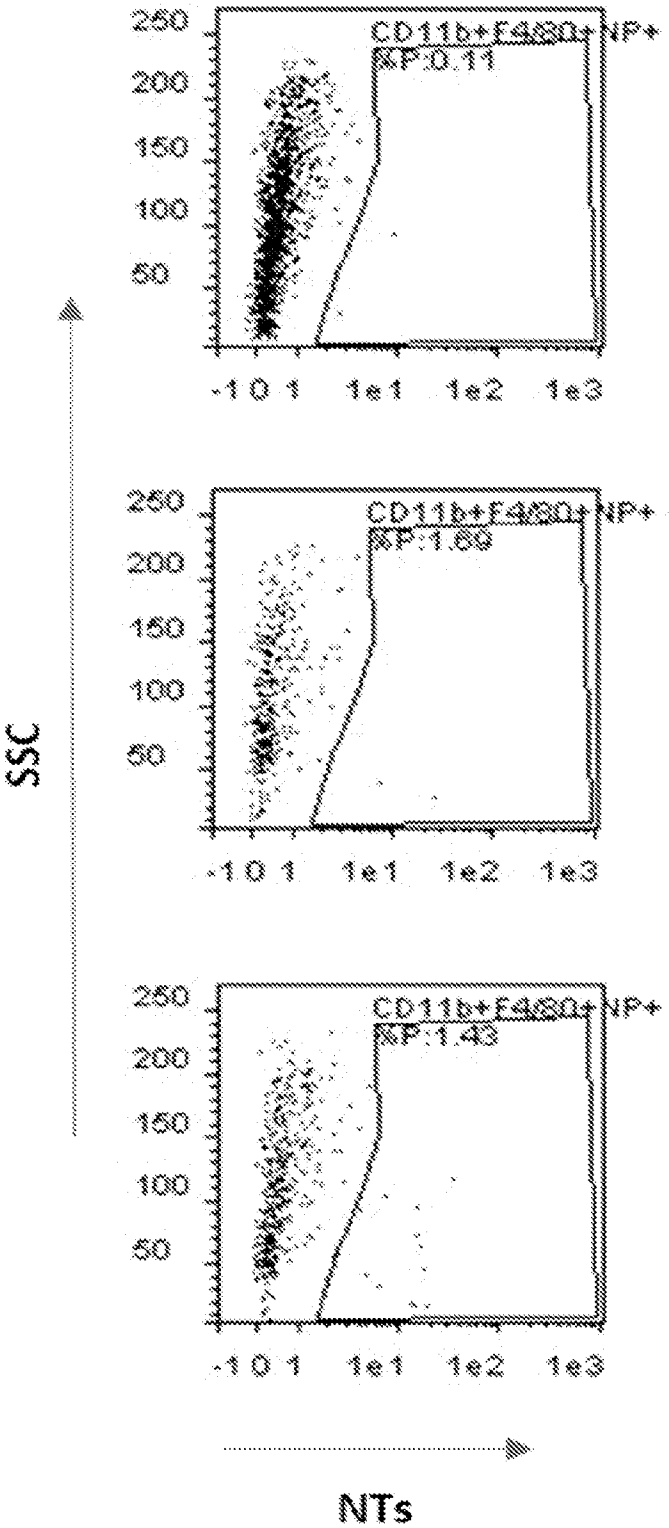
CD11b+F480+NP+
R:0.11
CD11b+F480+NP+
R:1.60
CD11b+F480+NP+
R:1.43
250
200
150
100
50
-1 0 1
1e1
1e2
1e3
SSC
NTs

FIG. 18F

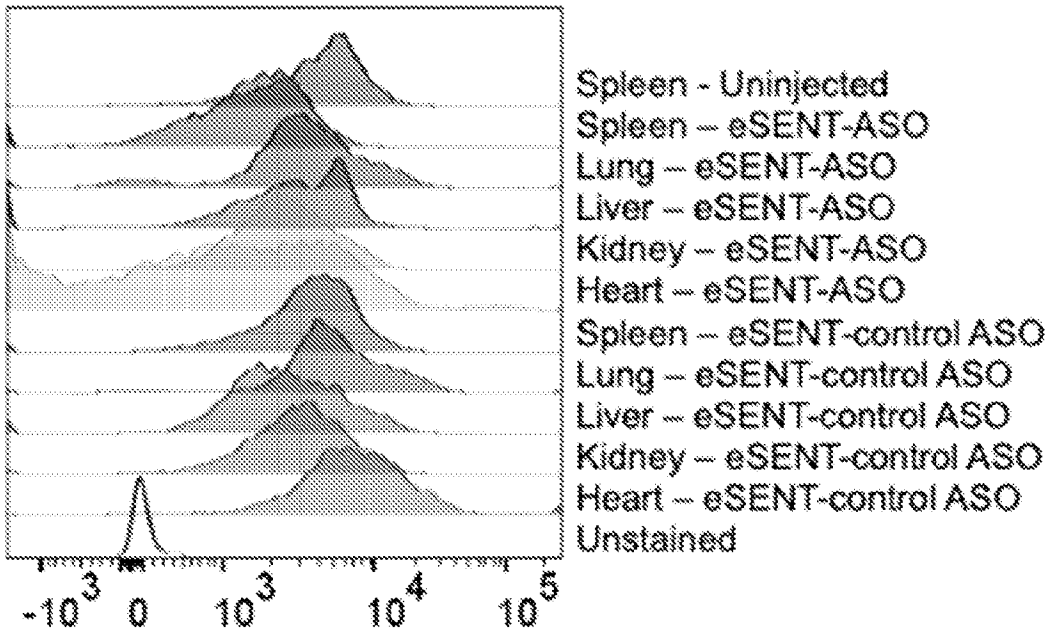

FIG. 19A

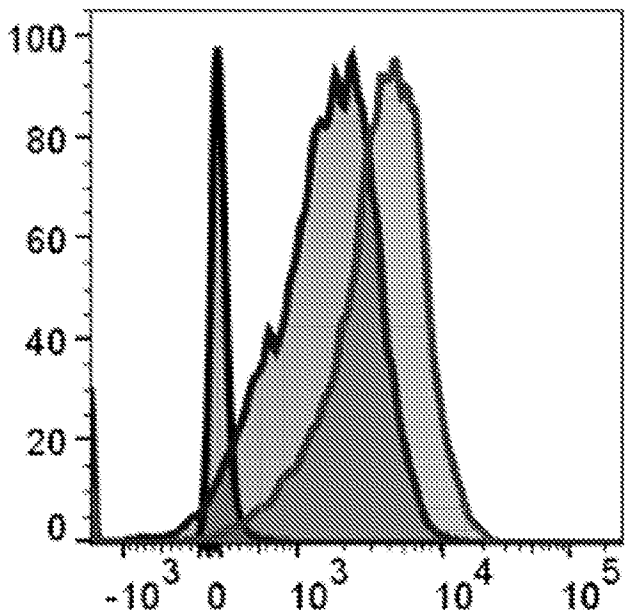

FIG. 19B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2023/068664

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/51 A61K9/127 A61K9/00 A61K9/19
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	CN 109 394 733 A (UNIV SHANGHAI JIAOTONG) 1 March 2019 (2019-03-01) page 1, paragraphs 0002, 0003 examples 2, 13	1-3,16, 17,19-24 4-11,13, 25-41
X	CN 111 184 700 A (UNIV CHONGQING) 22 May 2020 (2020-05-22) paragraphs [0004] - [0011] example 1	1,12, 14-20
Y	CN 108 355 139 B (UNIV HENAN) 18 December 2020 (2020-12-18) page 9, paragraph 0104 - page 10, paragraph 0108	4-11,13, 25-41

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 October 2023	19/10/2023

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Weiss, Marie-France

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

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2023/068664

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

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

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

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

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

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2023/068664

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 109394733 A	01-03-2019	NONE	
CN 111184700 A	22-05-2020	NONE	
CN 108355139 B	18-12-2020	NONE	