OPTIMIZED LINKERS

CROSS REFERENCE TO RELATED APPLICATIONS

[001] This PCT application claims the priority benefit of U.S. Provisional Application No. 63/150,523 filed February 17, 2021; which is incorporated herein by reference in its entirety.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY VIA EFS-WEB

[002] The content of the electronically submitted sequence listing (Name: 4000.125PC01_Seqlisting_ST25.txt, Size: 766,399 bytes; and Date of Creation: February 17, 2022) submitted in this application is incorporated herein by reference in its entirety.

TECHNICAL FIELD

[003] The present disclosure provides extracellular vesicles (EVs), *e.g.,* exosomes, which can be useful as an agent for the prophylaxis or treatment of cancer and other diseases comprising at least one biologically active molecule linked to the extracellular vesicle, *e.g.,* exosome, via an optimized linker and an anchoring moiety.

BACKGROUND

[004] Many bioactive compounds have potent biological activity that is of therapeutic interest. However, these compounds often exhibit toxicity in non-target organs. One way to limit exposure of non-target tissues is to chemically conjugate small molecules to affinity-based reagents such as antibodies, which can direct the therapeutic compound to specific cell types (Dosio, F. *et al.*, *Toxins (Basel) 3(7)*:848-883 (2011)), but this approach is limited by the number of molecules of the compound of interest that can be attached to an antibody (typically 2-6 molecules per antibody), and by the availability/existence of antibodies that specifically bind to targeted, relevant diseased/effector cells without binding to non-target cells. These two issues limit the use of antibody-drug conjugates (ADC) by decreasing potency and increasing systemic toxicity, respectively. Accordingly, there is a need for delivery systems with a higher payload than ADCs that can selectively target specific tissues or organs while at the same time limiting overall systemic exposure to the therapeutic compound.

[005] EVs, *e.g.*, exosomes, are important mediators of intercellular communication. They are also important biomarkers in the diagnosis and prognosis of many diseases, such as cancer. As drug delivery vehicles, EVs, *e.g.*, exosomes, offer many advantages over traditional drug delivery methods (*e.g.*, peptide immunization, DNA vaccines) as a new treatment modality in many therapeutic areas. However, despite its advantages, many EVs, *e.g.*, exosomes, have had limited clinical efficacy. For example, dendritic-cell derived exosomes (DEX) were investigated in a Phase II clinical trial as maintenance immunotherapy after first line chemotherapy in patients with inoperable non-small cell lung cancer (NSCLC). However, the trial was terminated because the primary endpoint (at least 50% of patients with progression-free survival (PFS) at 4 months after chemotherapy cessation) was not reached. Besse, B., *et al.*, *Oncoimmunology* 5(4):e1071008 (2015).

[006] Accordingly, new and more effective engineered-EVs, *e.g.*, exosomes, are necessary to better enable therapeutic use and other applications of EV-based technologies.

BRIEF SUMMARY

[007] The present disclosure provides an extracellular vesicle (EV) comprising a biologically active molecule (BAM) covalently linked to the EV via an anchoring moiety (AM) according to the formula [AM]-L_1-[SP_1]-L_2-[SP_2]-L_3-[BAM] (Formula 1), wherein: [AM] is the anchoring moiety; L_1 is a cleavable or non-cleavable linkage; L_2 and L_3 are optional cleavable or non-cleavable linkages; SP_1 is an optional first spacer; and, SP_2 is an optional second spacer.

[008] In some aspects, the anchoring moiety [AM] comprises a sterol, a lipid, a vitamin, a peptide, or a combination thereof and optionally a spacer. In some aspects, the optional spacer is an alkyl spacer. In some aspects, the alkyl spacer is C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14, or C15. In some aspects, the alkyl spacer is C6 or C8. In some aspects, the optional spacer is a glycol spacer. In some aspects, the glycol spacer has 2 (diethylene glycol), 3 (triethylene glycol), 4 (tetraethylene glycol; TEG), 5 (pentaethylene glycol), 6 (hexaethylene glycol; HEG), 7, 8, 9, 10, 11, 12, 13, 14, or 15 glycol units. In some aspects, the glycol spacer is tetraethylene glycol (TEG).

[009] In some aspects, the sterol is selected from the group consisting of cholesterol, ergosterol, 7-dehydrocholesterol, 24S-hydroxycholesterol, lanosterol, cycloartenol, fucosterol, saringosterol, campesterol, β-sitosterol, sitostanol, coprostanol, avenasterol, and stigmasterol. In some aspects, the sterol is cholesterol. In some aspects, the lipid is a fatty acid. In some aspects, the fatty acid is a straight chain fatty acid. In some aspects, the fatty acid is a straight chain fatty acid, a branched

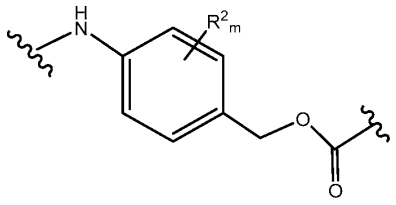
fatty acid, an unsaturated fatty acid, a monounsaturated fatty acid, a polyunsaturated fatty acid, a hydroxyl fatty acid, a polycarboxylic acid, or any combination thereof. In some aspects, the straight chain fatty acid is butyric acid, caproic acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, or stearic acid. In some aspects, the straight chain fatty acid is palmitic acid. In some aspects, the vitamin is vitamin E (tocopherol or tocotrienol), vitamin D, vitamin K, riboflavin, niacin, or pyridoxine. In some aspects, the vitamin is vitamin E (tocopherol or tocotrienol).

[0010] In some aspects, L_1 is a cleavable linkage comprising a phosphodiester bond or a non-cleavable linkage comprising a phosphorothioate bond. In some aspects, L_2 is an optional cleavable linkage comprising a phosphodiester bond or a non-cleavable linkage comprising a phosphorothioate bond. In some aspects, L_3 is an optional cleavable linkage comprising a phosphodiester bond or a non-cleavable linkage comprising a phosphorothioate bond. In some aspects, the SP_1 optional first spacer and/or the SP_2 an optional second spacer independently comprise an alkyl spacer, a glycol spacer, or a combination thereof. In some aspects, the alkyl spacer is C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14, or C15. In some aspects, the alkyl spacer is C3 or C6. In some aspects, the glycol spacer has 2 (diethylene glycol), 3 (triethylene glycol), 4 (tetraethylene glycol; TEG), 5 (pentaethylene glycol), 6 (hexaethylene glycol; HEG), 7, 8, 9, 10, 11, 12, 13, 14, or 15 glycol units. In some aspects, the glycol spacer is tetraethylene glycol (TEG) or hexaethylene glycol (HEG). In some aspects, each non-cleavable spacer is independently selected from alkyl, diethylene glycol, triethylene glycol, tetraethylene glycol (TEG), hexaethylene glycol (HEG), pentaethylene glycol, polyethylene glycol (PEG), glycerol, diglycerol, triglycerol, tetraglycerol (TG), pentaglycerol, a hexaglycerol (HG), polyglycerol (PG), succinimide, maleimide, or any combination thereof. In some aspects, the polyethylene glycol (PEG) is characterized by the formula R_1-(O-CH_2-CH_2)$_n$- or R_1-(O-CH_2-CH_2)n-O-, wherein R1 is hydrogen, methyl or ethyl and n is an integer between 1 and 15. In some aspects, the polyglycerol (PG) is characterized by the formula ((R_1—O—(CH_2—CHOH—CH_2O)$_n$—), wherein R_1 is hydrogen, methyl or ethyl, and n is an integer between 1 and 15.

[0011] In some aspects, the L_1, L_2, or L_3 cleavable linkage, or any combination thereof comprises a redox cleavable linker, a reactive oxygen species cleavable linker, a pH dependent cleavable linker, an enzymatic cleavable linker, a protease cleavable linker, an esterase cleavable linker, a phosphatase cleavable linker, a photoactivated cleavable linker, a self-immolative linker, or any combination thereof. In some aspects, the L_1, L_2, or L_3 cleavable linkage, or any combination thereof comprises a self-immolative linker. In some aspects, the L_1, L_2, or L_3 cleavable linkage, or any combination thereof comprises a cinnamyl group, a naphthyl group, a

biphenyl group, a heterocyclic ring, a homoaromatic group, coumarin, furan, thiophene, thiazole, oxazole, isoxazole, pyrrole, pyrazole, pyridine, imidazone, triazole, or any combination thereof.

[0012] In some aspects, the L_1, L_2, or L_3 cleavable linkage, or any combination thereof has the formula: -Aa-Yy- wherein each –A- is independently an amino acid unit or a combination thereof, a is independently an integer from 1 to 15; -Y- is a spacer unit, and y is 0, 1, or 2. In some aspects, -Aa- is a dipeptide, a tripeptide, a tetrapeptide, a pentapeptide, or a hexapeptide, or a combination thereof, wherein each dipeptide, tripeptide, tetrapeptide, pentapeptide, or hexapeptide in the combination can be the same or different.. In some aspects, a is 2 and –Aa- is selected from the group consisting of valine-alanine, valine-citrulline, phenylalanine-lysine, N-methylvaline-citrulline, cyclohexylalanine-lysine, glutamic acid-valine-citrulline, and beta-alanine-lysine. In some aspects, –Aa- is valine-alanine, valine-citrulline, or glutamic acid-valine-citrulline. In some aspects, y is 1. In some aspects, –Y- is a self-immolative spacer. In some aspects, –Yy- has the formula:



wherein each R_2 is independently C_{1-8} alkyl, -O-(C_{1-8} alkyl), halogen, nitro, or cyano; and m is an integer from 0 to 4. In some aspects, m is 0, 1, or 2. In some aspects, m is 0.

[0013] In some aspects, L_1, L_2, or L_3 cleavable linkage, or any combination thereof comprises valine-alanine-p-aminobenzylcarbamate or valine-citrulline-p-aminobenzylcarbamate. In some aspects, –Y- is a non self-immolative spacer. In some aspects, the non self-immolative spacer is –Gly- or –Gly-Gly-.

[0014] In some aspects, the anchoring moiety [AM] comprises a scaffold protein. In some aspects, the anchoring moiety [AM] and/or the scaffold moiety is Scaffold X. In some aspects, the Scaffold X is selected from the group consisting of prostaglandin F2 receptor negative regulator (the PTGFRN protein); basigin (the BSG protein); immunoglobulin superfamily member 2 (the IGSF2 protein); immunoglobulin superfamily member 3 (the IGSF3 protein); immunoglobulin superfamily member 8 (the IGSF8 protein); integrin beta-1 (the ITGB1 protein); integrin alpha-4 (the ITGA4 protein); 4F2 cell-surface antigen heavy chain (the SLC3A2 protein); a class of ATP transporter proteins (the ATP1A1, ATP1A2, ATP1A3, ATP1A4, ATP1B3, ATP2B1, ATP2B2, ATP2B3, ATP2B4 proteins); a functional fragment thereof; and any combination thereof. In some aspects, the Scaffold X is PTGFRN protein or a functional

fragment thereof. In some aspects, the Scaffold X comprises an amino acid sequence as set forth in SEQ ID NO:302. In some aspects, the Scaffold X comprises an amino acid sequence at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or about 100% identical to SEQ ID NO:302. In some aspects, the biologically active molecule [BAM] is linked via an anchoring moiety [AM] to the exterior surface of the EV. In some aspects, the biologically active molecule [BAM] is a polypeptide, a peptide, a polynucleotide (DNA and/or RNA), a chemical compound, or any combination thereof. In some aspects, the biologically active molecule [BAM] is a chemical compound. In some aspects, the chemical compound is a small molecule.

[0015] In some aspects, the biologically active molecule [BAM] comprises an antisense oligonucleotide (ASO), a siRNA, a miRNA, a shRNA, an mRNA, a nucleic acid, or any combination thereof. In some aspects, the biologically active molecule [BAM] comprises a peptide, a protein, an antibody or an antigen binding fragment thereof, or any combination thereof. In some aspects, the antigen binding fragment thereof comprises scFv, (scFv)2, Fab, Fab', F(ab')2, F(ab1)2, Fv, dAb, and Fd fragment, diabodys, antibody-related polypeptide, or any fragment thereof. In some aspects, the biologically active molecule [BAM] comprises an ASO. In some aspects, the ASO targets a transcript. In some aspects, the transcript is a STAT6 transcript, a CEBP/β transcript, a STAT3 transcript, a KRAS transcript, a NRAS transcript, an NLPR3 transcript, or any combination thereof. In some aspects, the EV is an exosome. In some aspects, the exosome is a native exosome. In some aspects, the exosome is a exosome overexpressing PTGFRN or a functional fragment thereof.

[0016] The present disclosure provides a pharmaceutical composition comprising an extracellular vesicle disclosed herein and a pharmaceutically acceptable carrier.

[0017] The present disclosure provides a kit comprising an EV or pharmaceutical composition disclosed herein and instructions for use.

[0018] The present disclosure provides a method of treating or preventing a disease or disorder in a subject in need thereof comprising administering an EV or pharmaceutical composition disclosed herein to the subject. In some aspects, the disease or disorder is a cancer, an inflammatory disorder, a neurodegenerative disorder, a central nervous disease, or a metabolic disease. In some aspects, the EV is administered intravenously, intraperitoneally, nasally, orally, intramuscularly, subcutaneously, parenterally, or intratumorally.

[0019] The present disclosure provides a method of attaching a biologically active molecule (BAM) to an EV, comprising linking an anchoring moiety (AM) to the EV, wherein the anchoring moiety (AM) is attached to the biologically active moiety (BAM) according to the

formula: [AM]-L_1-[SP_1]-L_2-[SP_2]-L_3-[BAM] wherein: [AM] is the anchoring moiety; L_1 is a cleavable or non-cleavable linkage; L_2 and L_3 are optional cleavable or non-cleavable linkages; SP_1 is an optional first spacer; and, SP_2 is an optional second spacer.

[0020] In some aspects, [AM] is cholesterol-C6, cholesterol-TEG, tocopherol-C8, tocopherol, of palmitate-C6. In some aspects, L_1, L_2, or L_3, or a combination thereof is a phosphodiester bond. In some aspects, SP_1 is C3, C6, TEG, or HEG. In some aspects, L_1, L_2, or L_3, or a combination thereofis a phosphorothioate bond. In some aspects, SP_2 is C3, C6, TEG or HEG. In some aspects, L_1, L_2, or L_3, or a combination thereof is a phosphorothioate bond. In some aspects, [BAM] is an antisense oligonucleotide (ASO).

[0021] The present disclosure provides a method of increasing the load density of a biologically active molecule (BAM) attached to an EV, comprising screening a library of anchoring moieties (AM) attached to the biologically active moiety (BAM) according to the formula: [AM]-L_1-[SP_1]-L_2-[SP_2]-L_3-[BAM] wherein: [AM] is the anchoring moiety; L_1 is a cleavable or non-cleavable linkage; L_2 and L_3 are optional cleavable or non-cleavable linkages; SP_1 is an optional first spacer; and, SP_2 is an optional second spacer.

[0022] In some aspects, [AM] is cholesterol-C6, cholesterol-TEG, tocopherol-C8, tocopherol, of palmitate-C6. In some aspects, L_1, L_2, or L_3, or a combination thereof is a phosphodiester bond. In some aspects, SP_1 is C3, C6, TEG, or HEG. In some aspects, L_1, L_2, or L_3, or a combination thereofis a phosphorothioate bond. In some aspects, SP_2 is C3, C6, TEG or HEG. In some aspects, L_1, L_2, or L_3, or a combination thereof is a phosphorothioate bond. In some aspects, [BAM] is an antisense oligonucleotide (ASO). In some aspects, the load density of a biologically active molecule (BAM) attached to an EV is increased at least about 1-fold, at least about 1.5-fold, at least about 2-fold, at least about 2.5-fold, at least about 3-fold, at last about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least 8-fold, at least about 9-fold, or at least about 10-fold.

[0023] The present disclosure provides an extracellular vesicle (EV) comprising an antisense oligonucleotide [ASO] covalently linked to the EV via an anchoring moiety [AM] according to the formula: [AM]-L_1-[SP_1]-L_2-[SP_2]-L_3-[ASO] wherein: [AM] is the anchoring moiety selected from the group consisting of cholesterol-C6, cholesterol-TEG, tocopherol-C8, tocopherol, and palmitate-C6; L_1 is a phosphodiesterase cleavable linkage; SP_1 is an optional first spacer selected from the group consisting of C3, C6, TEG and HEG; L_2 is optional phosphorothioate non-cleavable linkage; SP_2 is an optional second spacer selected from the group consisting of C3, C6, TEG, and HEG; and, L_3 is an optional phosphorothioate non-cleavable linkage.

[0024] In some aspects, the EV is an exosome. In some aspects, the exosome is a native exosome. In some aspects, the load density of ASO attached to the exosome is increased by at least about 1.5-fold. In some aspects, the anchoring moiety [AM] is cholesterol-C6. In some aspects, the average number of ASO molecules per exosome is 5032+/-386. In some aspects, the average number of ASO molecules per exosome is between about 4500 and about 5500. In some aspects, the average number of ASO molecules per exosome is between about 4500 and about 4600, between about 4600 and about 4700, between about 4700 and about 4800, between about 4800 and about 4900, between about 4900 and about 5000, between about 5000 and about 5100, between about 5100 and about 5200, between about 5200 and about 5300, between about 5300 and about 5400, or between about 5400 and about 5500. In some aspects, the average number of ASO molecules per exosome is at least about 4500, at least about 4600, at least about 4700, at least about 4800, at least about 4900, at least about 5000, at least about 5100, at least about 5200, at least about 5300, at least about 5400, or at least about 5500. In some aspects, the loading efficiency is 73% to 93%. In some aspects, the loading efficiency is between about 70% and about 95%. In some aspects, the loading efficiency is between about 70% and about 75%, between about 75% and about 80%, between about 80% and about 85%, between about 85% and about 90%, or between about 90% and about 95%. In some aspects, the loading efficiency is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%. In some aspects, the anchoring moiety [AM] is cholesterol-TEG. In some aspects, the average number of ASO molecules per exosome is 3991+/-490. In some aspects, the average number of ASO molecules per exosome is between about 3500 and about 4500. In some aspects, the average number of ASO molecules per exosome is between about 3500 and about 3600, between about 3600 and about 3700, between about 3700 and about 3800, between about 3800 and about 3900, between about 3900 and about 4000, between about 4000 and about 4100, between about 4100 and about 4200, between about 4200 and about 4300, between about 4300 and about 4400, or between about 4400 and about 4500. In some aspects, the average number of ASO molecules per exosome is at least about 3500, at least about 3600, at least about 3700, at least about 3800, at least about 3900, at least about 4000, at least about 4100, at least about 4200, at least about 4300, at least about 4400, or at least about 4500. In some aspects, the loading efficiency is 56% to 79%. In some aspects, the loading efficiency is between about 50% and about 85%. In some aspects, the loading efficiency is between about 50% and about 55%, between about 55% and about 60%, between about 60% and about 65%, between about 65% and about 70%, between about 70% and about 75%, between about 75% and about 80%, or between about 80% and about 85%. In some aspects, the loading efficiency is at least about 50%, at least

about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, or at least about 85%. In some aspects, the anchoring moiety [AM] is tocopherol-C8, tocopherol, or palmitate-C6. In some aspects, the average number of ASO molecules per exosome is 4241+/-722. In some aspects, the average number of ASO molecules per exosome is between about 3500 and about 5000. In some aspects, the average number of ASO molecules per exosome is between about 3500 and about 3600, between about 3600 and about 3700, between about 3700 and about 3800, between about 3800 and about 3900, between about 3900 and about 4000, between about 4000 and about 4100, between about 4100 and about 4200, between about 4200 and about 4300, between about 4300 and about 4400, between about 4400 and about 4500, between about 4500 and about 4600, between about 4600 and about 4700, between about 4700 and about 4800, between about 4800 and about 4900, or between about 4900 and about 5000. In some aspects, the average number of ASO molecules per exosome is at least about 3500, at least about 3600, at least about 3700, at least about 3800, at least about 3900, at least about 4000, at least about 4100, at least about 4200, at least about 4300, at least about 4400, at least about 4500, at least about 4600, at least about 4700, at least about 4800, at least about 4900, or at least about 5000. In some aspects, the loading efficiency is 57% to 73%. In some aspects, the loading efficiency is between about 50% and about 80%. In some aspects, the loading efficiency is between about 50% and about 55%, between about 55% and about 60%, between about 60% and about 65%, between about 65% and about 70%, between about 70% and about 75%, or between about 75% and about 80%. In some aspects, the loading efficiency is at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, or at least about 80%. In some aspects, the exosome is an exosome overexpressing PTGFRN. In some aspects, the load density of ASO attached to the exosome is increased by at least about 2-fold. In some aspects, the anchoring moiety [AM] is cholesterol-C6. In some aspects, the average number of ASO molecules per exosome is 2442+/-339. In some aspects, the average number of ASO molecules per exosome is between about 2000 and about 3000. In some aspects, the average number of ASO molecules per exosome is between about 2000 and about 2100, between about 2100 and about 2200, between about 2200 and about 2300, between about 2300 and about 2400, between about 2400 and about 2500, between about 2500 and about 2600, between about 2600 and about 2700, between about 2700 and about 2800, between about 2800 and about 2900, or between about 2900 and about 3000. In some aspects, the average number of ASO molecules per exosome is at least about 2000, at least about 2100, at least about 2200, at least about 2300, at least about 2400, at least about 2500, at least about 2600, at least about 2700, at least about 2800, at least about 2900, or at least about 3000. In some aspects, the loading efficiency is 27% to 46%.

In some aspects, the loading efficiency is between about 25% and about 50%. In some aspects, the loading efficiency is between about 25% and about 30%, between about 30% and about 35%, between about 35% and about 40%, between about 40% and about 45%, or between about 45% and about 50%. In some aspects, the loading efficiency is at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, or at least about 50%. In some aspects, the anchoring moiety [AM] is cholesterol-TEG. In some aspects, the average number of ASO molecules per exosome is 1728+/-264. In some aspects, the average number of ASO molecules per exosome is between about 1400 and about 2100. In some aspects, the average number of ASO molecules per exosome is between about 1400 and about 1500, between about 1500 and about 1600, between about 1600 and about 1700, between about 1700 and about 1800, between about 1800 and about 1900, between about 1900 and about 2000, or between about 2000 and about 2100. In some aspects, the average number of ASO molecules per exosome is at least about 1400, at least about 1500, at least about 1600, at least about 1700, at least about 1800, at least about 1900, at least about 2000, or at least about 2100. In some aspects, the loading efficiency is 19% to 33%. In some aspects, the loading efficiency is between about 15% and about 35%. In some aspects, the loading efficiency is between about 15% and about 20%, between about 20% and about 25%, between about 25% and about 30%, or between about 30% and about 35%. In some aspects, the loading efficiency is at least about 15%, at least about 20%, at least about 25%, at least about 30%, or at least about 35%. In some aspects, the anchoring moiety [AM] is tocopherol-C8, tocopherol, or palmitate-C6. In some aspects, the average number of ASO molecules per exosome is 2979+/-1006. In some aspects, the average number of ASO molecules per exosome is between about 1900 and about 4000. In some aspects, the average number of ASO molecules per exosome is between about 1900 and about 2000, between about 2000 and about 2100, between about 2100 and about 2200, between about 2200 and about 2300, between about 2300 and about 2400, between about 2400 and about 2500, between about 2500 and about 2600, between about 2600 and about 2700, between about 2700 and about 2800, between about 2800 and about 2900, between about 2900 and about 3000, between about 3000 and about 3100, between about 3100 and about 3200, between about 3200 and about 3300, between about 3300 and about 3400, between about 3400 and about 3500. between about 3500 and about 3600, between about 3600 and about 3700, between about 3700 and about 3800, between about 3800 and about 3900, or between about 3900 and about 4000. In some aspects, the average number of ASO molecules per exosome is at least about 1900, at least about 2000, at least about 2100, at least about 2200, at least about 2300, at least about 2400, at least about 2500, at least about 2600, at least about 2700, at least about 2800, at least about 2900, at least about 3000, at least about

3100, at least about 3200, at least about 3300, at least about 3400, at least about 3500, at least about 3600, at least about 3700, at least about 3800, at least about 3900, or at least about 4000. In some aspects, the loading efficiency is 37% to 68%. In some aspects, the loading efficiency is between about 30% and about 75%. In some aspects, the loading efficiency is between about 30% and about 35%, between about 35% and about 40%, between about 40% and about 45%, between about 45% and about 50%, between about 50% and about 55%, between about 55% and about 60%, between about 60% and about 65%, between about 65% and about 70%, or between about 70% and about 75%. In some aspects, the loading efficiency is at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%.

[0025] The present disclosure provides an exosome comprising an antisense oligonucleotide [ASO] covalently linked to the exosome via an anchoring moiety [AM] according to the formula: [AM]-L_1-[SP_1]-L_2-[SP_2]-L_3-[BAM] wherein [AM] is cholesterol-TEG; L_1 is a phosphodiesterase cleavable bond; SP_1 is C3; L_2 is a phosphorothioate non-cleavable bond; SP_2 is TEG; and, L_3 is a phosphorothioate non-cleavable bond.

[0026] In some aspects, the exosome is a native exosome. In some aspects, the average number of ASO molecules per exosome is about 4780. In some aspects, the average number of ASO molecules per exosome is between about 4500 and about 5000. In some aspects, the average number of ASO molecules per exosome is at least 4500. In some aspects, the loading efficiency is about 80%. In some aspects, n the loading efficiency is between about 70% and about 90%. In some aspects, the loading efficiency is at least about 70%. In some aspects, the exosome is an exosome overexpressing PTGFRN. In some aspects, the average number of ASO molecules per exosome is about 1659. In some aspects, the average number of ASO molecules per exosome is between about 1500 and about 2000. In some aspects, the average number of ASO molecules per exosome is at least about 1500. In some aspects, the loading efficiency is about 28%. In some aspects, the loading efficiency is between about 20% and about 35%. In some aspects, the loading efficiency is at least about 20%.

[0027] The present disclosure also provides an exosome comprising an antisense oligonucleotide [ASO] covalently linked to the exosome via an anchoring moiety [AM] according to the formula: [AM]-L_1-[SP_1]-L_2-[BAM] wherein [AM] is cholesterol-TEG; L_1 is a phosphodiesterase cleavable bond; SP_1 is TEG; and L_2 is a phosphorothioate non-cleavable bond.

[0028] In some aspects, the exosome is a native exosome. In some aspects, the average number of ASO molecules per exosome is about 4090. In some aspects, the average number of ASO molecules per exosome is between about 3500 and about 4500. In some aspects, the

average number of ASO molecules per exosome is at least 3500. In some aspects, the loading efficiency is about 68%. In some aspects, the loading efficiency is at least about 60%. In some aspects, the loading efficiency is between about 60% and about 70%. In some aspects, the exosome is an exosome overexpressing PTGFRN. In some aspects, the average number of ASO molecules per exosome is about 1890. In some aspects, the average number of ASO molecules per exosome is between about 1400 and about 2400. In some aspects, the average number of ASO molecules per exosome is at least about 1400. In some aspects, the loading efficiency is about 31%. In some aspects, the loading efficiency is between about 20% and about 40%. In some aspects, the loading efficiency is at least about 20%.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0029] **FIG. 1** is a schematic view showing the general structure of an exosome (left), an exemplary biologically active molecule (e.g., an oligonucleotide) connected to a ligand that allows the attachment to the exterior surface of the exosome via a linker (center), and how the biologically active molecule (e.g., an oligonucleotide) connected to a lipid anchor (e.g., cholesterol) via a linker can be attached to the membrane of the exosome (right).

[0030] **FIG. 2** is a schematic representation showing the general architecture of the constructs disclosed in the present application, comprising for example a membrane anchoring moiety (e.g., a lipid or a lipid plus a spacer), a biologically active molecule, and a linker or combination thereof. Note that the anchoring moiety may represent a complex molecule that contains hydrophobic domains.

[0031] **FIG. 3** shows exemplary membrane anchor lipid blocks suitable for solid phase synthesis.

[0032] **FIG. 4** shows exemplary spacer and combinations thereof that can be interspersed between an anchoring moiety (e.g., a lipid) and a biologically active molecule (e.g., an ASO). Stable or cleavable linkers can be included between each spacer. Also shown are spacer blocks suitable for solid phase synthesis.

[0033] **FIG. 5** shows different constructs comprising a lipid anchor (including associated linker where commercially available anchors include linker), combinations of spacers, and an ASO where FFLuc is an ASO sequence complementary to the mRNA for the firefly luciferase protein. The loading efficiencies, the number of ASO per exosome, and group averages for number of ASO per exosome are given when the different structures were loaded on native exosomes. The amount of ASO/EV is positively correlated with potency where potency is

determined to be the amount of knock-down of the expression of firefly luciferase stably expressed in a cell line.

[0034] **FIG. 6** shows different constructs comprising a lipid anchor (including associated linker where commercially available anchors include linker), combinations of spacers, and an ASO where FFLuc is an ASO sequence complementary to the mRNA for the firefly luciferase protein. The loading efficiencies, the number of ASO per exosome, and group averages for number of ASO per exosome are given when the different structures were loaded on exosomes with over-expressed PTGFRN. The amount of ASO/EV is positively correlated with potency where potency is determined to be the amount of knock-down of the expression of firefly luciferase stably expressed in a cell line.

[0035] **FIG. 7** show a statistical analysis of the contributions of exosome type, anchoring moiety (i.e., membrane anchor), spacer proximal to the vesicle surface (i.e., spacer 1), and spacer distal to the vesicle surface (i.e., spacer 2) to loading density (i.e. ASO/EV). In **FIG. 7**, native EVs and PrX EVs (over-expressing PTGFRN) were analyzed jointly. An integrated least squares model with $R^2 \geq 0.91$ was generated and demonstrated exosome type, membrane anchor, and spacer 1 to be statistically significant contributors ($p < 0.05$) to loading density. This shows that the linker technology can be applied in a platform manner across select ASO sequences with contributions of linker components largely independent of ASO sequence.

[0036] **FIGS. 8A** and **8B** show statistical models in which native EVs and PrX EVs were considered separaterly. To improve the accuracy of the statistical model, separate least squares models were generated for native exosomes (**FIG. 8A**; $R^2 \geq 0.95$) and exosomes over-expressing PTGFRN (**FIG. 8B**; PrX; $R^2 \geq 0.97$).

[0037] **FIG. 8C** shows the relative contributions of each structural property to the quantity of ASO molecules per exosome. The membrane anchor, tocopherol and the spacer 1, C3 provided the largest increase in loading density for both types of exosomes.

[0038] **FIGS. 9A, 9B,** and **9C** show the potency of specific anchor-spacer constructs on luciferase knockdown in native exosomes. The constructs used in the experiments are shown in **FIG. 9A. FIG. 9B** shows the potency of constructs T1-T9. **FIG. 9C** shows the potency of constructs C1-C9 and L1-L3. Arrows is FIG. 9A highligh the structures that provided the most luciferase knockdown.

[0039] **FIGS. 10A** and **10B** shows knock-down of the signal for luciferase protein native exosomes (**FIG. 10A**) or exosomes over-expressing PTGFRN (**FIG. 10B**) compared to the loading density achieved with the linker. Optimal linkers are defined by maximum ASO/EV loading and/or minimum normalized expression of firefly luciferase. Cholesterol and tocopherol-

c8 were shown to be the best anchoring moiety for association with native exosomes and exosomes with over-expressed PTGFRN, respectively. TEG and C3 provided the largest increase in potency of the options for spacer 1.

[0040] **FIG. 11** shows membrane anchors (lipids), linkers, and antisense oligonucleotides that were using to generate the Anchor-Linker-ASO constructs of Example 4. The number between parathesis after each each ASO name denotes the number of different linker structures evaluated.

[0041] **FIG. 12** shows the sequences of the ASOs in the experiments presented in Example 4. FFLUC and RLUC are named after the luminescent reporter genes targeted. The MYC and STAT6 ASO are named after the genes targeted by the ASO. Nb: LNA residues (including LNA-5MeC and LNA T/LNA-5MeU). Nm: 2'-O'MOE residues (including MOE-5MeC and MOE-T/MOED-5MeU). dN: DNA residues. (5MdC): 5-Methyl-dC. s: phosphonothioate backbone modification.

[0042] **FIG. 13A** shows a statistical model (R2 = 0.86) was generated to determine the contribution of different structural parameters to loading density on the exosomes. The structural parameters considered were ASO, Spacer 1, Spacer 2, and membrane anchor.

[0043] **FIG. 13B** shows the number of ASO constructs per exosome for each one of the tested linker constructs compared to the exoASO-STAT6 control (Chol-TEG-HEG linker).

[0044] **FIG. 13C** is a schematic representation of the relative impact of each linker component on loading density.

[0045] **FIGS. 14A-14E** show stability plots presenting the amount of ASO associated to exosomes loaded with structures (constructs) comprising STAT6 ASO after 2, 4, or 8 days of incubation in different buffers. The structures evaluated were Cholesterol-TEG-None-STAT6 (**FIG. 14A**); Cholesterol-C6-C3-STAT6 (**FIG. 14B**); Tocopherol-C8-TEG-STAT6 (**FIG. 14C**); Tocopherol-TEG-HEG-STAT6 (**FIG. 14D**); and Palmitate-C6-HEG-STAT6 (**FIG. 14E**).

[0046] **FIG. 15** provides a summary of stability and load density results. Stability was higher when exosomes were stored under high salt or high salt and sucrose conditions, independently of the temperature. Exosomes under high salt conditions without sucrose were more stable at high temperatures. Load density was lower under low salt conditions. ASO constructsd with cholesterol or tocopherol lipid anchors had higher load densities that linkers with palmitate. Under high salt and low temperature conditions, ASO constructs having a single spacer were less stable that ASO constructs having two spacers.

[0047] **FIG. 16** shows that under the salt, temperature, and sucrose conditions tested there were no changes in particle count.

[0048] **FIG. 17** shows that under the salt, temperature, and sucrose conditions tested there were no changes in particle size.

[0049] **FIG. 18** shows that for the exoASO-STAT6 control, the potency per exosome positively correlated with loading density.

[0050] **FIG. 19A** shows examples of lipid-linker-ASO structure designs with POI/PS variations. PO: phosphodiester; PSA: phosphorothioate.

[0051] **FIG. 19B** shows potency measured as IC50 values normalized based on ASO concentration (nM).

[0052] **FIG. 19C** shows potency measured as IC50 values normalized based on per exosome particle (p/mL).

[0053] **FIG. 20** shows the stability of exoASO with five different linkers. %Associated ASO loss is calculated based on formula: $\%\ Associated\ ASO\ loss=([ASO]_0-[ASO]_N)/[ASO]_0$, where $[ASO]_0$ is the total associated ASO concentration at day 0, $[ASO]_N$ is the associated ASO concentration at day N.

[0054] **FIG. 21** shows stability of exoASO with five different linkers as measured by particle concentration, which remained stable over 8 days at 4 °C.

[0055] **FIG. 22** shows stability of exoASO with five different linkers as measured by particle size, whivh remained stable over 8 days at 4 °C.

[0056] **FIG. 23A** shows examples of lipid-linker-ASO structure designs with Val-Cit cleavable linker mechanism in which the ASO is a STAT6 ASO.

[0057] **FIG. 23B** shows the potency of the constructs presented in FIG. 23A measured as IC50 values normalized based on ASO concentration (nM).

[0058] **FIG. 24** shows examples of lipid-linker-ASO design in which the ASO is an EGFP ASO.

[0059] **FIG. 25** shows an exemplary lipid-linker-ASO design with a redox cleavable linker mechanism in which the ASO is a STAT6 ASO

[0060] **FIG. 26** shows the potency of the construct presented in FIG. 25 measured as IC50 value normalized based on ASO concentration (nM).

[0061] **FIG. 27** shows a schematic representation of the loading, purification, and characterization processes for a lipid-linker-ASO constructr, in this case, an ASO targeting EGFP.

[0062] **FIG. 28** shows a schematic representation of the development of an EGFP splicing rescue assay. The 293AAV-pCB-2754 cell line was developed to evaluate the efficacy of EGFP and exoEGFP with various linkers. Point mutation at 654 results in the inability of fully

transcribing the EGFP; thus, no luminescence from EGFP or Nano Luciferase is observed. With the treatment with an ASO, EGFP was rescued, and therefore, both EGFP and Nano Luciferase signal was observed. The luminescence of Nano luciferase was employed for high-throughput quantification of efficacy. The modified sequence of the EGFP ASO is also shown Nm: 2'-O'MOE residues (including MOE-5MeC and MOE-T/MOED-5MeU); s: phosphonothioate backbone modification. The base sequence of the EGFP ASO is GCTATTACCTTAACCCAG (SEQ ID NO: 1093).

[0063] **FIG. 29** shows the characterization of the newly developed cell line used in the EGFP splicing rescue assay in terms of cell viability (CTG assay) and efficacy at three dose levels.

[0064] **FIG. 30** shows loading density and efficacy of exoASO-EGFP with various PO and non-cleavable PEG linkers using the EGFP splicing rescue assay. "Low" and "High" is a relative, qualitative reference to the loading density achieved in a paired set of experiments, where loading density was controlled through modulation of loading temperature and ASO concentration. The legend in the lower plot delineates the dose of ASO administered to each sample in the potency assay.

DETAILED DESCRIPTION

[0065] The present disclosure is directed to extracellular vesicles (EVs), *e.g.,* exosomes, comprising at least one biologically active molecule covalently linked to the EV, *e.g.,* exosome, via an optimized linker and an anchoring moiety and uses thereof. Non-limiting examples of the various aspects are shown in the present disclosure.

[0066] Before the present disclosure is described in greater detail, it is to be understood that this invention is not limited to the particular compositions or process steps described, as such can, of course, vary. As will be apparent to those of skill in the art upon reading this disclosure, each of the individual aspects described and illustrated herein has discrete components and features which can be readily separated from or combined with the features of any of the other several aspects without departing from the scope or spirit of the present invention. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

[0067] The headings provided herein are not limitations of the various aspects of the disclosure, which can be defined by reference to the specification as a whole. It is also to be understood that the terminology used herein is for the purpose of describing particular aspects

only, and is not intended to be limiting, since the scope of the present disclosure will be limited only by the appended claims.

[0068] Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

I. Definitions

[0069] In order that the present description can be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

[0070] It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, "a nucleotide sequence," is understood to represent one or more nucleotide sequences. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein. It is further noted that the claims can be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a negative limitation.

[0071] Furthermore, "and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term "and/or" as used in a phrase such as "A and/or B" herein is intended to include "A and B," "A or B," "A" (alone), and "B" (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following aspects: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[0072] It is understood that wherever aspects are described herein with the language "comprising," otherwise analogous aspects described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[0073] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure is related. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary of Biochemistry and Molecular Biology, Revised, 2000, Oxford University Press, provide one of skill with a general dictionary of many of the terms used in this disclosure.

[0074] Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Where a

range of values is recited, it is to be understood that each intervening integer value, and each fraction thereof, between the recited upper and lower limits of that range is also specifically disclosed, along with each subrange between such values. The upper and lower limits of any range can independently be included in or excluded from the range, and each range where either, neither or both limits are included is also encompassed within the disclosure. Thus, ranges recited herein are understood to be shorthand for all of the values within the range, inclusive of the recited endpoints. For example, a range of 1 to 10 is understood to include any number, combination of numbers, or sub-range from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10.

[0075] Where a value is explicitly recited, it is to be understood that values which are about the same quantity or amount as the recited value are also within the scope of the disclosure. Where a combination is disclosed, each subcombination of the elements of that combination is also specifically disclosed and is within the scope of the disclosure. Conversely, where different elements or groups of elements are individually disclosed, combinations thereof are also disclosed. Where any element of a disclosure is disclosed as having a plurality of alternatives, examples of that disclosure in which each alternative is excluded singly or in any combination with the other alternatives are also hereby disclosed; more than one element of a disclosure can have such exclusions, and all combinations of elements having such exclusions are hereby disclosed.

[0076] Nucleotides are referred to by their commonly accepted single-letter codes. Unless otherwise indicated, nucleotide sequences are written left to right in 5' to 3' orientation. Nucleotides are referred to herein by their commonly known one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Accordingly, A represents adenine, C represents cytosine, G represents guanine, T represents thymine, U represents uracil.

[0077] Amino acid sequences are written left to right in amino to carboxy orientation. Amino acids are referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission.

[0078] The term "about" is used herein to mean approximately, roughly, around, or in the regions of. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" can modify a numerical value above and below the stated value by a variance of, *e.g.*, 10 percent, up or down (higher or lower).

[0079] The terms "administration," "administering," and grammatical variants thereof refer to introducing a composition, such as an EV (*e.g.,* exosome) of the present disclosure, into a

subject via a pharmaceutically acceptable route. The introduction of a composition, such as an EV (*e.g.,* exosome) of the present disclosure, into a subject is by any suitable route, including intratumorally, orally, pulmonarily, intranasally, parenterally (intravenously, intra-arterially, intramuscularly, intraperitoneally, or subcutaneously), rectally, intralymphatically, intrathecally, periocularly or topically. Administration includes self-administration and the administration by another. A suitable route of administration allows the composition or the agent to perform its intended function. For example, if a suitable route is intravenous, the composition is administered by introducing the composition or agent into a vein of the subject.

[0080] As used herein, the term "agonist" refers to a molecule that binds to a receptor and activates the receptor to produce a biological response. Receptors can be activated by either an endogenous or an exogenous agonist. Non-limiting examples of endogenous agonist include hormones, neurotransmitters, and cyclic dinucleotides. Non-limiting examples of exogenous agonist include drugs, small molecules, and cyclic dinucleotides. The agonist can be a full, partial, or inverse agonist.

[0081] The term "amino acid substitution" refers to replacing an amino acid residue present in a parent or reference sequence (*e.g.,* a wild type sequence) with another amino acid residue. An amino acid can be substituted in a parent or reference sequence *(e.g.,* a wild type polypeptide sequence), for example, via chemical peptide synthesis or through recombinant methods known in the art. Accordingly, a reference to a "substitution at position X" refers to the substitution of an amino acid present at position X with an alternative amino acid residue. In some aspects, substitution patterns can be described according to the schema AnY, wherein A is the single letter code corresponding to the amino acid naturally or originally present at position n, and Y is the substituting amino acid residue. In other aspects, substitution patterns can be described according to the schema An(YZ), wherein A is the single letter code corresponding to the amino acid residue substituting the amino acid naturally or originally present at position n, and Y and Z are alternative substituting amino acid residues that can replace A.

[0082] As used herein, the term "antagonist" refers to a molecule that blocks or dampens an agonist mediated response rather than provoking a biological response itself upon bind to a receptor. Many antagonists achieve their potency by competing with endogenous ligands or substrates at structurally defined binding sites on the receptors. Non-limiting examples of antagonists include alpha blockers, beta-blocker, and calcium channel blockers. The antagonist can be a competitive, non-competitive, or uncompetitive antagonist.

[0083] As used herein, the term "antibody" encompasses an immunoglobulin whether natural or partly or wholly synthetically produced, and fragments thereof. The term also covers

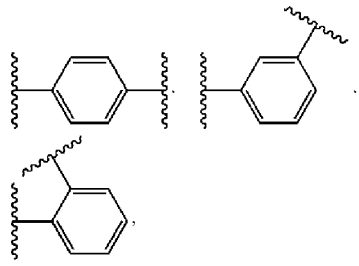
any protein having a binding domain that is homologous to an immunoglobulin binding domain. "Antibody" further includes a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. Use of the term antibody is meant to include whole antibodies, polyclonal, monoclonal and recombinant antibodies, fragments thereof, and further includes single-chain antibodies, humanized antibodies, murine antibodies, chimeric, mouse-human, mouse-primate, primate-human monoclonal antibodies, anti-idiotype antibodies, antibody fragments, such as, *e.g.*, scFv, $(scFv)_2$, Fab, Fab', and $F(ab')_2$, $F(ab1)_2$, Fv, dAb, and Fd fragments, diabodies, and antibody-related polypeptides. Antibody includes bispecific antibodies and multispecific antibodies so long as they exhibit the desired biological activity or function. In some aspects of the present disclosure, the biologically active molecule is an antibody or a molecule comprising an antigen binding fragment thereof.

[0084] The terms "antibody-drug conjugate" and "ADC" are used interchangeably and refer to an antibody linked, *e.g.,* covalently, to a therapeutic agent (sometimes referred to herein as agent, drug, or active pharmaceutical ingredient) or agents. In some aspects of the present disclosure, the biologically active molecule is an antibody-drug conjugate.

[0085] As used herein, the term "approximately," as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain aspects, the term "approximately" refers to a range of values that fall within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[0086] The term "aryl" refers to a carbocyclic aromatic group. Examples of aryl groups include, but are not limited to, phenyl, naphthyl and anthracenyl. A carbocyclic aromatic group can be unsubstituted or substituted with one or more groups including, but not limited to, $-C_{1-8}$ alkyl, -O-(C_{1-8} alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', $-C(O)NH_2$, -C(O)NHR', $-C(O)N(R')_2$-, -NHC(O)R', $-S(O)_2R'$, -S(O)R', -OH, -halogen, $-N_3$, $-NH_2$, -NH(R'), $-N(R')_2$ and –CN, wherein each R' is independently H, $-C_{1-8}$ alkyl, or aryl.

[0087] The term "arylene" refers to an aryl group which has two covalent bonds and can be in the ortho, meta, or para configurations as shown in the following structures:



in which the phenyl group can be unsubstituted or substituted with up to four groups including, but not limited to, -C_{1-8} alkyl, -O-(C_{1-8} alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -C(O)NH_2, -C(O)NHR', -C(O)N(R')$_2$-, -NHC(O)R', -S(O)$_2$R', -S(O)R', -OH, -halogen, -N_3, -NH_2, -NH(R'), -N(R')$_2$ and –CN, wherein each R' is independently H, -C_{1-8} alkyl, or aryl.

[0088] The term "biologically active molecule" as use herein refers to any molecule that can be attached to an EV, *e.g.,* exosome, via an anchoring moiety, wherein the molecule can have a therapeutic or prophylactic effect in a subject in need thereof, or be used for diagnostic purposes. Accordingly, by way of example, the term biologically active molecule includes proteins (*e.g.,* antibodies, proteins, polypeptides, and derivatives, fragments, and variants thereof), lipids and derivatives thereof, carbohydrates (*e.g.,* glycan portions in glycoproteins), or small molecules. In some aspects, the biologically active molecule is a radioisotope. In some aspects, the biologically active molecule is a detectable moiety, *e.g.,* a radionuclide, a fluorescent molecule, or a contrast agent. In some aspects, the biologically active molecule can be or can comprise a targeting moiety or a tropism moiety. In some aspects, the biologically active molecule can be or can comprise, for example, an affinity ligand such as biotin. In some aspects, the biologically active molecule can be or can comprise a moiety capable to improve a pharmacokinetic or pharmacodynamic property, for example, a moiety capable in increase plasma half-life, e.g., a PEG moiety.

[0089] The term "C_{1-8} alkyl" as used herein refers to a straight chain or branched, saturated hydrocarbon having from 1 to 8 carbon atoms. Representative "C_{1-8} alkyl" groups include, but are not limited to, methyl, ethyl, n-propyl, n-butyl, n-pentyl, n-hexyl, n-heptyl, n-octyl, isopropyl, sec-butyl, isobutyl, tert-butyl, isopentyl, and 2-methylbutyl.

[0090] The term "C_{1-10} alkylene" refers to a saturated, straight chain hydrocarbon group of the formula –(CH_2)$_{1-10}$-. Examples of C_{1-10} alkylene include methylene, ethylene, propylene, butylene, pentylene, hexylene, heptylene, octylene, nonylene, and decalene.

[0091] The term "C_{3-8} carbocycle" refers to a 3-, 4-, 5-, 6-, 7- or 8-membered saturated or unsaturated non-aromatic carbocyclic ring. Representative C_{3-8} carbocycles include, but are not

limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclopentadienyl, cyclohexyl, cyclohexenyl, 1,3-cyclohexadienyl, 1,4-cyclohexadienyl, cycloheptyl, 1,3-cycloheptadienyl, 1,3,5-cycloheptatrienyl, cyclooctyl, and -cyclooctadienyl. A C_{3-8} carbocycle group can be unsubstituted or substituted with one or more groups including, but not limited to, -C_{1-8} alkyl, -O-(C_{1-8} alkyl), aryl, -C(O)R', -OC(O)R', -C(O)OR', -$C(O)NH_2$, -C(O)NHR', -$C(O)N(R')_2$-, NHC(O)R', -$S(O)_2$R', -S(O)R', -OH, -halogen, -N_3, -NH_2, -NH(R'), -$N(R')_2$ and –CN, where each R' is independently H, -C_{1-8} alkyl, or aryl.

[0092] The term "C_{3-8} carbocyclo" refers to a C_{3-8} carbocycle group defined above wherein one or more of the carbocycle's hydrogen atoms is replaced with a bond.

[0093] The term "C_{3-8} heterocycle" refers to an aromatic or non-aromatic C_{3-8} carbocycle in which one to four of the ring carbon atoms are independently replaced with a heteroatom selected from the group consisting of O, S and N. Representative examples of a C_{3-8} heterocycle include, but are not limited to, benzofuranyl, benzothiophene, indolyl, benzopyrazolyl, coumarinyl, isoquinolinyl, pyrrolyl, thiophenyl, furanyl, thiazolyl, imidazolyl, pyrazolyl, triazolyl, quinolinyl, pyrimidinyl, pyridinyl, pyridonyl, pyrazinyl, pyridazinyl, isothiazolyl, isoxazolyl and tetrazolyl. A C_{3-8} heterocycle can be unsubstituted or substituted with up to seven groups including, but not limited to, -C_{1-8} alkyl, -O-(C_{1-8} alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -$C(O)NH_2$, -C(O)NHR', -$C(O)N(R')_2$, -NHC(O)R', -$S(O)_2$R', -S(O)R', -OH, -halogen, -N_3, -NH_2, -NH(R'), -$N(R')_2$, and –CN, wherein each R' is independently H, -C_{1-8} alkyl, or aryl.

[0094] The term "C_{3-8} heterocyclo" refers to a C_{3-8} heterocycle group defined above wherein one of the heterocycle group's hydrogen atoms is replaced with a bond. A C_{3-8} heterocyclo can be unsubstituted or substituted with up to six groups including, but not limited to, -C_{1-8} alkyl, -O-(C_{1-8} alkyl), -aryl, -C(O)R', -OC(O)R', -C(O)OR', -$C(O)NH_2$, -C(O)NHR', -$C(O)N(R')_2$, -NHC(O)R', -$S(O)_2$R', -S(O)R', -OH, -halogen, -N_3, -NH_2, -NH(R'), -$N(R')_2$ and –CN, wherein each R' is independently H, -C_{1-8} alkyl, or aryl.

[0095] A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, if an amino acid in a polypeptide is replaced with another amino acid from the same side chain family, the substitution is considered

to be conservative. In another aspect, a string of amino acids can be conservatively replaced with a structurally similar string that differs in order and/or composition of side chain family members.

[0096] As used herein, the term "conserved" refers to nucleotides or amino acid residues of a polynucleotide sequence or polypeptide sequence, respectively, that are those that occur unaltered in the same position of two or more sequences being compared. Nucleotides or amino acids that are relatively conserved are those that are conserved amongst more related sequences than nucleotides or amino acids appearing elsewhere in the sequences.

[0097] In some aspects, two or more sequences are said to be "completely conserved" or "identical" if they are 100% identical to one another. In some aspects, two or more sequences are said to be "highly conserved" if they are at least 70% identical, at least 80% identical, at least 90% identical, or at least 95% identical to one another. In some aspects, two or more sequences are said to be "highly conserved" if they are about 70% identical, about 80% identical, about 90% identical, about 95%, about 98%, or about 99% identical to one another. In some aspects, two or more sequences are said to be "conserved" if they are at least 30% identical, at least 40% identical, at least 50% identical, at least 60% identical, at least 70% identical, at least 80% identical, at least 90% identical, or at least 95% identical to one another. In some aspects, two or more sequences are said to be "conserved" if they are about 30% identical, about 40% identical, about 50% identical, about 60% identical, about 70% identical, about 80% identical, about 90% identical, about 95% identical, about 98% identical, or about 99% identical to one another. Conservation of sequence may apply to the entire length of a polynucleotide or polypeptide or may apply to a portion, region or feature thereof.

[0098] As used herein, the term "conventional EV protein" means a protein previously known to be enriched in EVs.

[0099] As used herein, the term "conventional exosome protein" means a protein previously known to be enriched in exosomes, including but is not limited to CD9, CD63, CD81, PDGFR, GPI anchor proteins, lactadherin LAMP2, and LAMP2B, a fragment thereof, or a peptide that binds thereto.

[00100] The term "derivative" as used herein refers to an EV, *e.g.,* exosome, component (*e.g.,* a protein, such as Scaffold X, a lipid, or a carbohydrate) or to a biologically active molecule (*e.g.,* a polypeptide, polynucleotide, lipid, carbohydrate, antibody or fragment thereof, PROTAC, etc.) that has been chemically modified to either introduce a reactive moiety (e.g., a phosphoramidite moiety).

[00101] The terms "excipient" and "carrier" are used interchangeably and refer to an inert substance added to a pharmaceutical composition to further facilitate administration of a compound.

[00102] As used herein, the terms "extracellular vesicle," "EV," and grammatical variants thereof, are used interchangeably and refer to a cell-derived vesicle comprising a membrane that encloses an internal space. Extracellular vesicles comprise all membrane-bound vesicles (*e.g.*, exosomes, nanovesicles) that have a smaller diameter than the cell from which they are derived. In some aspects, extracellular vesicles range in diameter from 20 nm to 1000 nm, and can comprise various macromolecular payload either within the internal space (*i.e.*, lumen), displayed on the external surface of the extracellular vesicle, and/or spanning the membrane. In some aspects, the payload can comprise nucleic acids, proteins, carbohydrates, lipids, small molecules, and/or combinations thereof. In certain aspects, an extracellular vehicle comprises a scaffold moiety. By way of example and without limitation, extracellular vesicles include apoptotic bodies, fragments of cells, vesicles derived from cells by direct or indirect manipulation (*e.g.*, by serial extrusion or treatment with alkaline solutions), vesiculated organelles, and vesicles produced by living cells (*e.g.*, by direct plasma membrane budding or fusion of the late endosome with the plasma membrane). Extracellular vesicles can be derived from a living or dead organism, explanted tissues or organs, prokaryotic or eukaryotic cells, and/or cultured cells. In some aspects, the extracellular vesicles are produced by cells that express one or more transgene products.

[00103] As used herein, the term "exosome" refers to an extracellular vesicle with a diameter between 20-300 nm (*e.g.*, between 40-200 nm). Exosomes comprise a membrane that encloses an internal space (*i.e.*, lumen), and, in some aspects, can be generated from a cell (*e.g.*, producer cell) by direct plasma membrane budding or by fusion of the late endosome with the plasma membrane. In certain aspects, an exosome comprises a scaffold moiety. As described *infra*, exosome can be derived from a producer cell, and isolated from the producer cell based on its size, density, biochemical parameters, or a combination thereof. In some aspects, the exosomes of the present disclosure are produced by cells that express one or more transgene products.

[00104] In some aspects, EVs, *e.g.,* exosomes, *e.g.,* nanovesicles, of the present disclosure are engineered by covalently linking at least one biologically active molecule (*e.g.,* a protein such as an antibody or ADC, a RNA or DNA such as an antisense oligonucleotide, a small molecule drug, a toxin) to the EV, *e.g.,* exosome, e.g., nanovesicle, via an anchoring moiety.

[00105] In some aspects, the EVs, *e.g.,* exosomes or nanovesicles, of the present disclosure can comprise various macromolecular payloads either within the internal space (*i.e.*, lumen), displayed on the external (exterior) surface or internal (luminal) surface of the EV, and/or spanning the membrane. In some aspects, the payload can comprise, *e.g.,* nucleic acids, proteins, carbohydrates, lipids, small molecules, and/or combinations thereof. In certain aspects, an EV, e.g, an exosome, comprises a scaffold moiety (*e.g.,* Scaffold X). EVs, *e.g.,* exosomes, can be derived from a living or dead organism, explanted tissues or organs, prokaryotic or eukaryotic cells, and/or cultured cells. In some aspects, the EVs, *e.g.,* exosomes, are produced by cells that express one or more transgene products. In other aspects, the EVs of the present disclosure are without limitation nanovesicles, microsomes, microvesicles, extracellular bodies, or apoptotic bodies.

[00106] A schematic view of the general structure of an exosome, an exemplary biologically active molecule (e.g., an oligonucleotide) connected to a ligand that allows the attachment to the exterior surface of the exosome via a linker, and how the biologically active molecule (e.g., an oligonucleotide) connected to a lipid anchor (e.g., cholesterol) via a linker can be attached to the membrane of the exosome, are shown in **FIG. 1**.

[00107] As used herein, the term "fragment" of a protein (e.g., a biologically active molecule such as a therapeutic protein, or a scaffold protein such as Scaffold X) refers to an amino acid sequence of a protein that is shorter than the naturally-occurring sequence, N- and/or C-terminally deleted or any part of the protein deleted in comparison to the naturally occurring protein.

[00108] As used herein, the term "functional fragment" refers to a protein fragment that retains protein function. Accordingly, in some aspects, a functional fragment of a Scaffold protein, *e.g.,* Scaffold X protein, retains the ability to anchor a biologically active molecule on the luminal surface or on the external surface of the EV, *e.g.,* exosome.

[00109] Whether a fragment is a functional fragment can be assessed by any art known methods to determine the protein content of EVs, *e.g.,* exosomes, including Western Blots, FACS analysis and fusions of the fragments with autofluorescent proteins like, *e.g.*, GFP. In certain aspects, a functional fragment of a Scaffold X protein retains, *e.g.,* at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or at least about 100% of the ability of the naturally occurring Scaffold X protein to anchor a biologically active molecule on the luminal or on the external surface of the EV, *e.g.,* exosome.

[00110] As used herein "anchoring" a biologically active molecule on the luminal or external surface of an EV (*e.g.,* exosome) of the present disclosure via a scaffold protein refers to

attaching covalently or non-covalently the biologically active molecule to the portion of the scaffold molecule located on the luminal or external surface of the EV (*e.g.,* exosome), respectively.

[00111] As used herein, the term "homology" refers to the overall relatedness between polymeric molecules, *e.g.* between nucleic acid molecules (*e.g.* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Generally, the term "homology" implies an evolutionary relationship between two molecules. Thus, two molecules that are homologous will have a common evolutionary ancestor. In the context of the present disclosure, the term homology encompasses both to identity and similarity.

[00112] In some aspects, polymeric molecules are considered to be "homologous" to one another if at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% of the monomers in the molecule are identical (exactly the same monomer) or are similar (conservative substitutions). The term "homologous" necessarily refers to a comparison between at least two sequences (polynucleotide or polypeptide sequences).

[00113] In the context of the present disclosure, substitutions (even when they are referred to as amino acid substitution) are conducted at the nucleic acid level, *i.e.,* substituting an amino acid residue with an alternative amino acid residue is conducted by substituting the codon encoding the first amino acid with a codon encoding the second amino acid.

[00114] As used herein, the term "identity" refers to the overall monomer conservation between polymeric molecules, *e.g.*, between polypeptide molecules or polynucleotide molecules (*e.g.* DNA molecules and/or RNA molecules). The term "identical" without any additional qualifiers, *e.g.,* protein A is identical to protein B, implies the sequences are 100% identical (100% sequence identity). Describing two sequences as, *e.g.,* "70% identical," is equivalent to describing them as having, *e.g.,* "70% sequence identity."

[00115] Calculation of the percent identity of two polypeptide sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second polypeptide sequences for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In certain aspects, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or 100% of the length of the reference sequence. The amino acids at corresponding amino acid positions are then compared.

[00116] When a position in the first sequence is occupied by the same amino acid as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions

shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm.

[00117] Suitable software programs are available from various sources, and for alignment of both protein and nucleotide sequences. One suitable program to determine percent sequence identity is bl2seq, part of the BLAST suite of program available from the U.S. government's National Center for Biotechnology Information BLAST web site (blast.ncbi.nlm.nih.gov). Bl2seq performs a comparison between two sequences using either the BLASTN or BLASTP algorithm. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. Other suitable programs are, *e.g.,* Needle, Stretcher, Water, or Matcher, part of the EMBOSS suite of bioinformatics programs and also available from the European Bioinformatics Institute (EBI) at www.ebi.ac.uk/Tools/psa.

[00118] Sequence alignments can be conducted using methods known in the art such as MAFFT, Clustal (ClustalW, Clustal X or Clustal Omega), MUSCLE, etc.

[00119] Different regions within a single polynucleotide or polypeptide target sequence that aligns with a polynucleotide or polypeptide reference sequence can each have their own percent sequence identity. It is noted that the percent sequence identity value is rounded to the nearest tenth. For example, 80.11, 80.12, 80.13, and 80.14 are rounded down to 80.1, while 80.15, 80.16, 80.17, 80.18, and 80.19 are rounded up to 80.2. It also is noted that the length value will always be an integer.

[00120] In certain aspects, the percentage identity (%ID) or of a first amino acid sequence (or nucleic acid sequence) to a second amino acid sequence (or nucleic acid sequence) is calculated as %ID = 100 x (Y/Z), where Y is the number of amino acid residues (or nucleobases) scored as identical matches in the alignment of the first and second sequences (as aligned by visual inspection or a particular sequence alignment program) and Z is the total number of residues in the second sequence. If the length of a first sequence is longer than the second sequence, the percent identity of the first sequence to the second sequence will be higher than the percent identity of the second sequence to the first sequence.

[00121] One skilled in the art will appreciate that the generation of a sequence alignment for the calculation of a percent sequence identity is not limited to binary sequence-sequence comparisons exclusively driven by primary sequence data. It will also be appreciated that sequence alignments can be generated by integrating sequence data with data from heterogeneous sources such as structural data *(e.g.,* crystallographic protein structures), functional data *(e.g.,*

location of mutations), or phylogenetic data. A suitable program that integrates heterogeneous data to generate a multiple sequence alignment is T-Coffee, available at www.tcoffee.org, and alternatively available, *e.g.,* from the EBI. It will also be appreciated that the final alignment used to calculate percent sequence identity can be curated either automatically or manually.

[00122] As used herein, the terms "isolated," "purified," "extracted," and grammatical variants thereof are used interchangeably and refer to the state of a preparation of desired EVs (*e.g.*, a plurality of EVs of known or unknown amount and/or concentration), that has undergone one or more processes of purification, *e.g.*, a selection or an enrichment of the desired EV, e.g., exosome, preparation. In some aspects, isolating or purifying as used herein is the process of removing, partially removing (*e.g.,* a fraction) of the EVs, *e.g.,* exosomes, from a sample containing producer cells. In some aspects, an isolated EV, *e.g.,* exosome, composition has no detectable undesired activity or, alternatively, the level or amount of the undesired activity is at or below an acceptable level or amount. In other aspects, an isolated EV, *e.g.,* exosome, composition has an amount and/or concentration of desired EVs, *e.g.,* exosomes, at or above an acceptable amount and/or concentration. In other aspects, the isolated EVs, *e.g.,* exosome, composition is enriched as compared to the starting material (*e.g.,* producer cell preparations) from which the composition is obtained. This enrichment can be by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.9%, at least about 99.99%, at least about 99.999%, at least about 99.9999%, or greater than 99.9999% as compared to the starting material. In some aspects, isolated EV, e.g. exosome, preparations are substantially free of residual biological products. In some aspects, the isolated EV, *e.g.,* exosome, preparations are 100% free, at least about 99% free, at least about 98% free, at least about 97% free, at least about 96% free, at least about 95% free, at least about 94% free, at least about 93% free, at least about 92% free, at least about 91% free, or at least about 90% free of any contaminating biological matter. Residual biological products can include abiotic materials (including chemicals) or unwanted nucleic acids, proteins, lipids, or metabolites. Substantially free of residual biological products can also mean that the EV, *e.g.,* exosome, composition contains no detectable producer cells and that only EVs, *e.g.,* exosomes, are detectable.

[00123] The terms "linked," "fused," and grammatical variants thereof are used interchangeably and refer to a first moiety, *e.g.,* a first amino acid sequence or nucleotide sequence, covalently or non-covalently joined to a second moiety, *e.g.,* a second amino acid sequence, nucleotide sequence, and/or a lipid (e.g., cholesterol), respectively. The first moiety

can be directly joined or juxtaposed to the second moiety or alternatively an intervening moiety can covalently join the first moiety to the second moiety. The term "linked" means not only a fusion of a first moiety to a second moiety at the C-terminus or the N-terminus, but also includes insertion of the whole first moiety (or the second moiety) into any two points, *e.g.,* amino acids, in the second moiety (or the first moiety, respectively). In one aspect, the first moiety is linked to a second moiety by a peptide bond or a linker. The first moiety can be linked to a second moiety by a phosphodiester bond or a linker. The linker can be a peptide or a polypeptide (for polypeptide chains) or a nucleotide or a nucleotide chain (for nucleotide chains) or any chemical moiety (for polypeptide or polynucleotide chains or any chemical molecules). The term "linked" is also indicated by a hyphen (-). In some aspects, a Scaffold X protein on an EV, *e.g.,* exosome, can be linked or fused to a biologically active molecule via a linker, spacer, or combination thereof.

[00124] The term "modified," when used in the context of EVs, *e.g.,* exosomes, described herein, refers to an alteration or engineering of an EV, *e.g.,* exosome and/or its producer cell, such that the modified EV, *e.g.,* exosome, is different from a naturally-occurring EV, *e.g.,* exosome. In some aspects, a modified EV, *e.g.,* exosome, described herein comprises a membrane that differs in composition of a protein, a lipid, a small molecular, a carbohydrate, *etc.* compared to the membrane of a naturally-occurring EV, *e.g.,* exosome. *E.g.*, the membrane comprises higher density or number of natural EV, *e.g.,* exosome, proteins and/or membrane comprises proteins that are not naturally found in EV, *e.g.,* exosomes. In certain aspects, such modifications to the membrane change the exterior surface of the EV, *e.g.,* exosome (*e.g.*, surface-engineered EVs and exosomes described herein).

[00125] As used herein the terms "modified protein" or "protein modification" refers to a protein having at least 15% identity to the non-mutant amino acid sequence of the protein. A modification of a protein includes a fragment or a variant of the protein. A modification of a protein can further include chemical, or physical modification to a fragment or a variant of the protein.

[00126] As used herein, the terms "modulate," "modify," and grammatical variants thereof, generally refer when applied to a specific concentration, level, expression, function or behavior, to the ability to alter, by increasing or decreasing, *e.g.*, directly or indirectly promoting/stimulating/up-regulating or interfering with/inhibiting/down-regulating the specific concentration, level, expression, function or behavior, such as, *e.g.*, to act as an antagonist or agonist. In some instances, a modulator can increase and/or decrease a certain concentration,

level, activity or function relative to a control, or relative to the average level of activity that would generally be expected or relative to a control level of activity.

[00127] As used herein, the term "nanovesicle" refers to an extracellular vesicle with a diameter between 20-250 nm (*e.g.*, between 30-150 nm) and is generated from a cell (*e.g.*, producer cell) by direct or indirect manipulation such that the nanovesicle would not be produced by the cell without the manipulation. Appropriate manipulations of the cell to produce the nanovesicles include but are not limited to serial extrusion, treatment with alkaline solutions, sonication, or combinations thereof. In some aspects, production of nanovesicles can result in the destruction of the producer cell. In some aspects, population of nanovesicles described herein are substantially free of vesicles that are derived from cells by way of direct budding from the plasma membrane or fusion of the late endosome with the plasma membrane. In certain aspects, a nanovesicle comprises a scaffold moiety, *e.g.,* Scaffold X. Nanovesicles, once derived from a producer cell, can be isolated from the producer cell based on its size, density, biochemical parameters, or a combination thereof.

[00128] As used herein, the term "payload" refers to a biologically active molecule (*e.g.,* a therapeutic agent) that acts on a target (*e.g.*, a target cell) that is contacted with the EV, *e.g.,* exosome, of the present disclosure. Non-limiting examples of payloads that can be introduced into an EV, *e.g.,* exosome, include therapeutic agents such as, nucleotides (*e.g.*, nucleotides comprising a detectable moiety or a toxin or that disrupt transcription), nucleic acids (*e.g.,* DNA or mRNA molecules that encode a polypeptide such as an enzyme, or RNA molecules that have regulatory function such as miRNA, dsDNA, lncRNA, and siRNA), amino acids (*e.g.,* amino acids comprising a detectable moiety or a toxin or that disrupt translation), polypeptides (*e.g.,* enzymes), lipids, carbohydrates, and small molecules (*e.g.,* small molecule drugs and toxins). In certain aspects, a payload comprises an antigen. As used herein, the term "antigen" refers to any agent that when introduced into a subject elicits an immune response (cellular or humoral) to itself. In some aspects, the payload molecules are covalently linked to the EV, *e.g.,* exosome, via linker, spacer, or combination thereof disclosed herein. In other aspects, a payload comprises an adjuvant.

[00129] The terms "pharmaceutically-acceptable carrier," "pharmaceutically-acceptable excipient," and grammatical variations thereof, encompass any of the agents approved by a regulatory agency of the U.S. Federal government or listed in the U.S. Pharmacopeia for use in animals, including humans, as well as any carrier or diluent that does not cause the production of undesirable physiological effects to a degree that prohibits administration of the composition to a subject and does not abrogate the biological activity and properties of the administered

compound. Included are excipients and carriers that are useful in preparing a pharmaceutical composition and are generally safe, non-toxic, and desirable.

[00130] As used herein, the term "pharmaceutical composition" refers to one or more of the compounds described herein, such as, *e.g.*, an EV, such as exosome of the present disclosure, mixed or intermingled with, or suspended in one or more other chemical components, such as pharmaceutically-acceptable carriers and excipients. One purpose of a pharmaceutical composition is to facilitate administration of preparations of EVs, *e.g.,* exosomes, to a subject.

[00131] The term "polynucleotide" as used herein refers to polymers of nucleotides of any length, including ribonucleotides, deoxyribonucleotides, analogs thereof, or mixtures thereof. This term refers to the primary structure of the molecule. Thus, the term includes triple-, double- and single-stranded deoxyribonucleic acid ("DNA"), as well as triple-, double- and single-stranded ribonucleic acid ("RNA"). It also includes modified, for example by alkylation, and/or by capping, and unmodified forms of the polynucleotide. More particularly, the term "polynucleotide" includes polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), including tRNA, rRNA, hRNA, siRNA and mRNA, whether spliced or unspliced, any other type of polynucleotide which is an N- or C-glycoside of a purine or pyrimidine base, and other polymers containing normucleotidic backbones, for example, polyamide (*e.g.,* peptide nucleic acids "PNAs") and polymorpholino polymers, and other synthetic sequence-specific nucleic acid polymers providing that the polymers contain nucleobases in a configuration which allows for base pairing and base stacking, such as is found in DNA and RNA. In some aspects of the present disclosure, the biologically active molecule attached to the EV, *e.g.,* exosome, via a linker, spacer, or combination thereof disclosed herein is a polynucleotide, *e.g.,* an antisense oligonucleotide. In particular aspects, the polynucleotide comprises an mRNA. In other aspect, the mRNA is a synthetic mRNA. In some aspects, the synthetic mRNA comprises at least one unnatural nucleobase. In some aspects, all nucleobases of a certain class have been replaced with unnatural nucleobases (*e.g.,* all uridines in a polynucleotide disclosed herein can be replaced with an unnatural nucleobase, *e.g.,* 5-methoxyuridine). In some aspects of the present disclosure, the biologically active molecule is a polynucleotide (e.g., an antisense oligonucleotide, ASO).

[00132] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer can comprise modified amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling

component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids such as homocysteine, ornithine, p-acetylphenylalanine, D-amino acids, and creatine), as well as other modifications known in the art. In some aspects of the present disclosure, the biologically active molecule attached to the EV, *e.g.,* exosome, via a linker, spacer, or combination thereof disclosed herein is a polypeptide, *e.g.,* an antibody or a derivative thereof such as an ADC, a PROTAC, a toxin, a fusion protein, or an enzyme.

[00133] The term "polypeptide," as used herein, refers to proteins, polypeptides, and peptides of any size, structure, or function. Polypeptides include gene products, naturally occurring polypeptides, synthetic polypeptides, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing. A polypeptide can be a single polypeptide or can be a multi-molecular complex such as a dimer, trimer or tetramer. They can also comprise single chain or multichain polypeptides. Most commonly disulfide linkages are found in multichain polypeptides. The term polypeptide can also apply to amino acid polymers in which one or more amino acid residues are an artificial chemical analogue of a corresponding naturally occurring amino acid. In some aspects, a "peptide" can be less than or equal to 50 amino acids long, *e.g.,* about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

[00134] The terms "prevent," "preventing," and variants thereof as used herein, refer partially or completely delaying onset of an disease, disorder and/or condition; partially or completely delaying onset of one or more symptoms, features, or clinical manifestations of a particular disease, disorder, and/or condition; partially or completely delaying onset of one or more symptoms, features, or manifestations of a particular disease, disorder, and/or condition; partially or completely delaying progression from a particular disease, disorder and/or condition; and/or decreasing the risk of developing pathology associated with the disease, disorder, and/or condition. In some aspects, preventing an outcome is achieved through prophylactic treatment.

[00135] As used herein, the term "producer cell" refers to a cell used for generating an EV, *e.g.,* exosome. A producer cell can be a cell cultured *in vitro,* or a cell *in vivo.* A producer cell includes, but not limited to, a cell known to be effective in generating EVs, *e.g.,* exosomes, *e.g.,* HEK293 cells, Chinese hamster ovary (CHO) cells, mesenchymal stem cells (MSCs), BJ human foreskin fibroblast cells, fHDF fibroblast cells, AGE.HN® neuronal precursor cells, CAP® amniocyte cells, adipose mesenchymal stem cells, RPTEC/TERT1 cells. In certain aspects, a producer cell is not an antigen-presenting cell. In some aspects, a producer cell is not a dendritic cell, a B cell, a mast cell, a macrophage, a neutrophil, Kupffer-Browicz cell, cell derived from any of these cells, or any combination thereof.

[00136] As used herein, "prophylactic" refers to a therapeutic or course of action used to prevent the onset of a disease or condition, or to prevent or delay a symptom associated with a disease or condition.

[00137] As used herein, a "prophylaxis" refers to a measure taken to maintain health and prevent or delay the onset of a bleeding episode, or to prevent or delay symptoms associated with a disease or condition.

[00138] A "recombinant" polypeptide or protein refers to a polypeptide or protein produced via recombinant DNA technology. Recombinantly produced polypeptides and proteins expressed in engineered host cells are considered isolated for the purpose of the disclosure, as are native or recombinant polypeptides which have been separated, fractionated, or partially or substantially purified by any suitable technique. The polypeptides disclosed herein can be recombinantly produced using methods known in the art. Alternatively, the proteins and peptides disclosed herein can be chemically synthesized. In some aspects of the present disclosure, the Scaffold X proteins present in EVs, *e.g.,* exosomes, are recombinantly produced by overexpressing the scaffold proteins in the producer cells, so that levels of scaffold proteins in the resulting EVs, *e.g.,* exosomes are significantly increased with respect to the levels of scaffold proteins present in EVs, *e.g.,* exosomes, of producer cells not overexpressing such scaffold proteins.

[00139] As used herein, the term "scaffold moiety" refers to a molecule, *e.g.,* a protein such as Scaffold X, that can be used to anchor a payload, *e.g.,* a biologically active molecule, to the EV, *e.g.,* exosome, e.g., on the external surface of the EV, *e.g.,* exosome. In certain aspects, a scaffold moiety comprises a synthetic molecule. In some aspects, a scaffold moiety comprises a non-polypeptide moiety. In other aspects, a scaffold moiety comprises, *e.g.,* a lipid, carbohydrate, protein, or combination thereof (*e.g.,* a glycoprotein or a proteolipid) that naturally exists in the EV, *e.g.,* exosome. In some aspects, a scaffold moiety comprises a lipid, carbohydrate, or protein that does not naturally exist in the EV, *e.g.,* exosome. In some aspects, a scaffold moiety comprises a lipid or carbohydrate which naturally exists in the EV, *e.g.,* exosome, but has been enriched in the EV, *e.g.,* exosome with respect to basal/native/wild type levels. In some aspects, a scaffold moiety comprises a protein which naturally exists in the EV, *e.g.,* exosome but has been enriched in the EV, *e.g.,* exosome, for example, by recombinant overexpression in the producer cell, with respect to basal/native/wild type levels. In certain aspects, a scaffold moiety is Scaffold X.

[00140] As used herein, the term "Scaffold X" refers to EV, *e.g.,* exosome, proteins that have been identified on the surface of EVs, *e.g.,* exosomes. *See, e.g.*, U.S. Pat. No. 10,195,290,

which is incorporated herein by reference in its entirety. Non-limiting examples of Scaffold X proteins include: prostaglandin F2 receptor negative regulator ("PTGFRN"); basigin ("BSG"); immunoglobulin superfamily member 2 ("IGSF2"); immunoglobulin superfamily member 3 ("IGSF3 "); immunoglobulin superfamily member 8 ("IGSF8"); integrin beta-1 ("ITGB1"); integrin alpha-4 ("ITGA4 "); 4F2 cell-surface antigen heavy chain ("SLC3A2"); and a class of ATP transporter proteins ("ATP1A1," "ATP1A2," "ATP1A3," "ATP1A4," "ATP1B3," "ATP2B1," "ATP2B2," "ATP2B3," "ATP2B"). In some aspects, a Scaffold X protein can be a whole protein or a fragment thereof (*e.g.*, functional fragment, *e.g.,* the smallest fragment that is capable of anchoring another moiety on the external surface or on the luminal surface of the EV, *e.g.,* exosome). In some aspects, a Scaffold X can anchor a biologically active molecule to the external surface or the lumen of the EV, e.g. an exosome. In some aspects of the present disclosure, a biologically active molecule can be covalently attached to a Scaffold X, e.g., via a linker, spacer, or combination thereof disclosed herein. Non-limiting examples of other scaffold moieties that can be used with the present disclosure include: aminopeptidase N (CD13); Neprilysin, AKA membrane metalloendopeptidase (MME); ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (ENPP1); Neuropilin-1 (NRP1); CD9, CD63, CD81, PDGFR, GPI anchor proteins, lactadherin, LAMP2, and LAMP2B.

[00141] As used herein, the term "similarity" refers to the overall relatedness between polymeric molecules, *e.g.* between polynucleotide molecules (*e.g.* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of percent similarity of polymeric molecules to one another can be performed in the same manner as a calculation of percent identity, except that calculation of percent similarity takes into account conservative substitutions as is understood in the art. It is understood that percentage of similarity is contingent on the comparison scale used, i.e., whether the amino acids are compared, *e.g.,* according to their evolutionary proximity, charge, volume, flexibility, polarity, hydrophobicity, aromaticity, isoelectric point, antigenicity, or combinations thereof.

[00142] Unless otherwise indicated, reference to a compound that has one or more stereocenters intends each stereoisomer, and all combinations of stereoisomers, thereof.

[00143] The terms "subject," "patient," "individual," and "host," and variants thereof are used interchangeably herein and refer to any mammalian subject, including without limitation, humans, domestic animals (*e.g.,* dogs, cats and the like), farm animals (*e.g.*, cows, sheep, pigs, horses and the like), and laboratory animals (*e.g.*, monkey, rats, mice, rabbits, guinea pigs and the like) for whom diagnosis, treatment, or therapy is desired, particularly humans. The methods described herein are applicable to both human therapy and veterinary applications.

[00144] As used herein, the term "substantially free" means that the sample comprising EVs, *e.g.,* exosomes, comprises less than 10% of macromolecules, *e.g.,* contaminants, by mass/volume (m/v) percentage concentration. Some fractions may contain less than 0.001%, less than 0.01%, less than 0.05%, less than 0.1%, less than 0.2%, less than 0.3%, less than 0.4%, less than 0.5%, less than 0.6%, less than 0.7%, less than 0.8%, less than 0.9%, less than 1%, less than 2%, less than 3%, less than 4%, less than 5%, less than 6%, less than 7%, less than 8%, less than 9%, or less than 10% (m/v) of macromolecules.

[00145] As used herein the term "surface-engineered EV" (*e.g.*, Scaffold X-engineered exosome) refers to an EV with the membrane or the surface of the EV modified in its composition so that the surface of the engineered EV is different from that of the EV prior to the modification or of the naturally occurring EV.

[00146] As used herein the term "surface-engineered exosome" (*e.g.*, Scaffold X-engineered exosome) refers to an exosome with the membrane or the surface of the exosome (external surface or luminal surface) modified in its composition so that the surface of the engineered exosome is different from that of the exosome prior to the modification or of the naturally occurring exosome.

[00147] The engineering can be on the surface of the EV, *e.g.,* exosome or in the membrane of the EV, *e.g.,* exosome, so that the surface of the EV, *e.g.,* exosome is changed. For example, the membrane can be modified in its composition of, *e.g.,* a protein, a lipid, a small molecule, a carbohydrate, or a combination thereof. The composition can be changed by a chemical, a physical, or a biological method or by being produced from a cell previously or concurrently modified by a chemical, a physical, or a biological method. Specifically, the composition can be changed by a genetic engineering or by being produced from a cell previously modified by genetic engineering. In some aspects, a surface-engineered EV, *e.g.,* exosome, comprises an exogenous protein (*i.e.*, a protein that the EV, *e.g.,* exosome, does not naturally express) or a fragment or variant thereof that can be exposed to the surface of the EV, *e.g.,* exosome or can be an anchoring point (attachment) for a moiety exposed on the surface of the EV, *e.g.,* exosome. In other aspects, a surface-engineered EV, *e.g.,* exosome comprises a higher expression (*e.g.,* higher number) of a natural EV, *e.g.,* exosome protein (*e.g.*, Scaffold X) or a fragment or variant thereof that can be exposed to the surface of the EV, *e.g.,* exosome or can be an anchoring point (attachment) for a moiety exposed on the surface of the EV, *e.g.,* exosome. In a specific aspect, a surface-engineered EV, *e.g.,* exosome, comprises the modification of one or more membrane components, *e.g.,* a protein such as Scaffold X, a lipid, a small molecule, a carbohydrate, or a combination thereof, wherein at least one of the components

is covalently attached to a biologically active molecule via a linker, spacer, or combination thereof disclosed herein.

[00148] As used herein the term "therapeutically effective amount" is the amount of reagent or pharmaceutical compound comprising an EV or exosome of the present disclosure that is sufficient to a produce a desired therapeutic effect, pharmacologic and/or physiologic effect on a subject in need thereof. A therapeutically effective amount can be a "prophylactically effective amount" as prophylaxis can be considered therapy.

[00149] The terms "treat," "treatment," or "treating," as used herein refers to, *e.g.*, the reduction in severity of a disease or condition; the reduction in the duration of a disease course; the amelioration or elimination of one or more symptoms associated with a disease or condition; the provision of beneficial effects to a subject with a disease or condition, without necessarily curing the disease or condition. The term also includes prophylaxis or prevention of a disease or condition or its symptoms thereof. In one aspect, the term "treating" or "treatment" means inducing an immune response in a subject against an antigen.

[00150] As used herein, the term "variant" of a molecule (*e.g.*, functional molecule, antigen, or Scaffold X) refers to a molecule that shares certain structural and functional identities with another molecule upon comparison by a method known in the art. For example, a variant of a protein can include a substitution, insertion, deletion, frame shift or rearrangement in another protein.

[00151] In some aspects, a variant of a Scaffold X or derivative comprises a Scaffold X variant having at least about 70% identity to the full-length, mature PTGFRN, BSG, IGSF2, IGSF3, IGSF8, ITGB1, ITGA4, SLC3A2, or ATP transporter proteins or a fragment (*e.g.*, functional fragment) of the PTGFRN, BSG, IGSF2, IGSF3, IGSF8, ITGB1, ITGA4, SLC3A2, or ATP transporter proteins.

[00152] In some aspects, the variant or variant of a fragment of Scaffold X protein disclosed herein, or derivatives thereof, retains the ability to be specifically targeted to EVs, *e.g.*, exosomes. In some aspects, the Scaffold X or Scaffold X derivative includes one or more mutations, for example, conservative amino acid substitutions.

[00153] Naturally occurring variants are called "allelic variants," and refer to one of several alternate forms of a gene occupying a given locus on a chromosome of an organism (Genes II, Lewin, B., ed., John Wiley & Sons, New York (1985)). These allelic variants can vary at either the polynucleotide and/or polypeptide level and are included in the present disclosure. Alternatively, non-naturally occurring variants can be produced by mutagenesis techniques or by direct synthesis.

[00154] Using known methods of protein engineering and recombinant DNA technology, variants can be generated to improve or alter the characteristics of the polypeptides. For instance, one or more amino acids can be deleted from the N-terminus or C-terminus of the secreted protein without substantial loss of biological function. Ron *et al.*, *J. Biol. Chem.* 268: 2984-2988 (1993), incorporated herein by reference in its entirety, reported variant KGF proteins having heparin binding activity even after deleting 3, 8, or 27 amino-terminal amino acid residues. Similarly, interferon gamma exhibited up to ten times higher activity after deleting 8-10 amino acid residues from the carboxy terminus of this protein. (Dobeli *et al., J. Biotechnology 7*:199-216 (1988), incorporated herein by reference in its entirety.)

[00155] Moreover, ample evidence demonstrates that variants often retain a biological activity similar to that of the naturally occurring protein. For example, Gayle and coworkers (*J. Biol. Chem 268*:22105-22111 (1993), incorporated herein by reference in its entirety) conducted extensive mutational analysis of human cytokine IL-1a. They used random mutagenesis to generate over 3,500 individual IL-1a mutants that averaged 2.5 amino acid changes per variant over the entire length of the molecule. Multiple mutations were examined at every possible amino acid position. The investigators found that "[m]ost of the molecule could be altered with little effect on either [binding or biological activity]." (See Abstract.) In fact, only 23 unique amino acid sequences, out of more than 3,500 nucleotide sequences examined, produced a protein that significantly differed in activity from wild-type.

[00156] As stated above, variants or derivatives include, *e.g.*, modified polypeptides. In some aspects, variants or derivatives of, *e.g.,* polypeptides, polynucleotides, lipids, glycoproteins, are the result of chemical modification and/or endogenous modification. In some aspects, variants or derivatives are the result of *in vivo* modification. In some aspects, variants or derivatives are the result of *in vitro* modification. In yet other aspects, variant or derivatives are the result of intracellular modification in producer cells.

[00157] Modifications present in variants and derivatives include, *e.g.*, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation (Mei *et al., Blood 116:*270-79 (2010), which is incorporated herein by reference in its entirety), proteolytic processing, phosphorylation, prenylation, racemization,

selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

[00158] In some aspects, Scaffold X can be modified at any convenient location. In some aspects, a biologically active molecule can be modified at any convenient location. In particular aspects of the present disclosure, an EV, *e.g.,* exosome, component (*e.g.,* a protein such as Scaffold X, a lipid, or a glycan) and/or a biologically active molecule (*e.g.,* an antibody or ADC, a PROTAC, a small molecule such as a cyclic dinucleotide, a toxin such as MMAE, a STING agonist, a tolerizing agent, or an antisense oligonucleotide) can be modified to yield a derivative comprising at least one linker, spacer, or combination thereof disclosed herein.

II. EVs (*e.g.,* exosomes) comprising optimized linkers

[00159] Extracellular vesicles (EVs) typically have 20 nm to 1000 nm in diameter; *e.g.,* exosomes, which are small extracellular vesicles, have typically 100-200 nm in diameter. EVs, *e.g.,* exosomes, are composed of a limiting lipid bilayer and a diverse set of proteins and nucleic acids (Maas, S.L.N., *et al.*, *Trends. Cell Biol. 27(3)*:172-188 (2017)). EVs, *e.g.,* exosomes, exhibit preferential uptake in discrete cell types and tissues, and their tropism can be directed by adding proteins to their surface that interact with receptors on the surface of target cells (Alvarez-Erviti, L., *et al.*, *Nat. Biotechnol. 29(4)*:341-345 (2011)).

[00160] Unlike antibodies, EVs (*e.g.,* exosomes) can accommodate large numbers of molecules attached to their surface, on the order of thousands to tens of thousands of molecules per EV (*e.g.,* exosome). EV (*e.g.,* exosome)-drug conjugates thus represent a platform to deliver a high concentration of therapeutic compounds to discrete cell types, while at the same time limiting overall systemic exposure to the compound, which in turn reduces off-target toxicity. The accommodation of larger numbers of molecules on the surface of EVs (e.g., exosomes) can be influenced, for example, by the type of biologically active molecule used (e.g., an antibody is bulkier than an antisense oligonucleotide), the type of membrane anchor used (e.g., a protein anchor is bulkier than a lipid or lipid plus spacer anchor), and the combinations of linkers and spacers connecting the biologically active molecule and the membrane anchor. In this respect, the present disclosure provides specific combinations of linkers and spacers connecting a biologically active molecule (e.g., an ASO) and a membrane anchor (e.g., a lipid), wherein the membrane anchor attaches the biologically active molecule to the surface of an EV (*e.g.,* exosome).

[00161] In some aspects, the present disclosure provides a "biologically active molecule" (BAM), e.g., an ASO, attached, e.g., covalently, to one or more anchoring moieties (AM) either

directly or indirectly, e.g., via one or more linker combinations. The anchoring moiety can insert into the lipid bilayer of an EV, e.g., an exosome, allowing the loading of the exosome with a BAM, e.g., an ASO. Currently, a predominant obstacle to the commercialization of exosomes as a delivery vehicle for polar BAMs, e.g., ASOs, is highly inefficient loading. As shown herein, this obstacle can be overcome by using specific linkers/spacer combinations (i.e., "optimized linkers") connecting the BAM to the AM prior to loading them into EVs, e.g., exosomes. Thus, as described herein, the use of optimized linkers facilitates the loading of BAMs, e.g., ASOs, onto EVs, e.g., exosomes.

[00162] The composition and methods of loading EVs (e.g., exosomes) with constructs comprising BAMs, e.g., ASOs, connected to AMs (e.g., lipids such as sterols) via optimized linkers set forth herein significantly improve loading efficiency and BAM density as compared to the loading efficiency and BAM density previously reported for introducing unmodified BAMs into EVs (e.g., exosomes) by, for example, electroporation or cationic lipid transfection. The compositions and methods disclosed herein also significantly improve the potency of EVs (e.g., exosomes) compared to the potencies previously reported when unmodified BAMs are introduced into EVs (e.g., exosomes) by, for example, electroporation or cationic lipid transfection.

[00163] A schematic representation showing the general architecture of the constructs disclosed in the present application, comprising for example a membrane anchoring moiety (e.g., a lipids or a lipid plus a spacer), a biologically active molecule, and a linker or combination thereof is shown in **FIG. 2**.

[00164] Examplary key building blocks for linkers of the present disclosure, e.g., membrane anchores (lipids), spacers and combinations thereof, as well as the compounds used for their chemical synthesis (e.g., phosphoramidites) are shown in **FIGS. 3** and **4**. Specific building blocks are also presented in **FIG. 11**. Exemplary constructs are shown, for example, in **FIG. 5**, **FIG. 6**, and **FIG. 9A**,

[00165] The present disclosure provides an extracellular vesicle (EV) comprising a biologically active molecule (BAM) covalently linked to the EV via an anchoring moiety (AM) according to the formula:

$$[AM]\text{-}L_1\text{-}[SP_1]\text{-}L_2\text{-}[SP_2]\text{-}L_3\text{-}[BAM] \quad \text{(Formula 1)}$$

wherein:

[AM] is the anchoring moiety;

L_1 is a cleavable or non-cleavable linkage,

L_2 and L_3 are optional cleavable or non-cleavable linkages;

SP_1 is an optional first spacer; and,

SP_2 is an optional second spacer.

[00166] As used herein the term "optimized linker" refers to a combination of structural elements comprising, e.g., "linkages" and "spacers" which connect an anchoring moiety [AM] and a biological active molecule [BAM]. These optimized linkers of the present disclosure allow loading biological active molecules (e.g., ASOs) more efficiently and in larger numbers onto the surface of EVs (e.g., exosome), than corresponding construct comprising the same anchoring moiety [AM] and biologically active molecule [BAM] in the absence of an optimized linker of the present disclosure. In other words, the constructs disclosed herein, e.g., a construct of Formula 1 [AM]-L_1-[SP_1]-L_2-[SP_2]-L_3-[BAM], results in (i) higher EV loading efficiency, (ii) higher number of [BAM] per EV, (iii) higher density of [BAM] per EV, (iv) higher [BAM] potency, or (v) any combination therein, with respect to a construct with the architecture [AM]-L_1-[BAM].

[00167] As used herein, the term "linkage" refers to any stable bond or chemical group connecting, e.g., an anchoring moiety [AM] and a spacer [SP], a spacer [SP] and a biologically active molecule [BAM], or an anchoring moiety [AM] and a biologically active molecule [BAM]. In constructs where more than an anchoring moiety [AM] or biologically active molecule [BAM] is present, a linkage can connect two anchoring moieties or two biologically active moieties. In some aspects, a "linkage" can be a bond, for example a phosphodiester (cleavable) or phosphorothioate (non-cleavable) bond. In other aspects, a "linkage" can comprise a linker, for example, cleavable or non-cleavable linkers. In some aspects, a linkage can comprise multiple linkers and bonds, which can respond to different stimuli such as pH, temperature, enzymes, etc.

[00168] The term "spacer" as used herein refers to a chemical moiety which is capable of covalently linking together two spaced moieties (*e.g.*, a biologically active molecule) into a normally stable dipartate molecule. Generally, spacers as not cleavable. For example, a spacer can be an alkyl chain, or a polymeric chain formed by example by glycol or glycerol units.

[00169] In some aspects, the "optimized linkers" of the present disclosure prevent the aggregation of biological moieties on the surface of the EV (e.g., exosome). In some aspects, the length of the optimized linker connecting an anchoring moiety [AM] and a biologically active molecule [BAM] is between about 2 nm and about 30 nm.

[00170] In some aspects, the length of the optimized linker is about 2 nm, about 3 nm, about 4 nm, about 5 nm, about 6 nm, about 7 nm, about 8 nm, about 9 nm, about 10 nm, about 11 nm, about 12 nm, about 13 nm, about 14 nm, about 15 nm, about 16 nm, about 17 nm, about 18

nm, about 19 nm, about 20 nm, about 21 nm, about 22 nm, about 23 nm, about 24 nm, about 25 nm, about 26 nm, about 27 nm, about 28 nm, about 29 nm, or about 30 nm. In some aspects, the length of the optimized linker is at least about 2 nm, at least about 3 nm, at least about 4 nm, at least about 5 nm, at least about 6 nm, at least about 7 nm, at least about 8 nm, at least about 9 nm, at least about 10 nm, at least about 11 nm, at least about 12 nm, at least about 13 nm, at least about 14 nm, at least about 15 nm, at least about 16 nm, at least about 17 nm, at least about 18 nm, at least about 19 nm, at least about 20 nm, at least about 21 nm, at least about 22 nm, at least about 23 nm, at least about 24 nm, at least about 25 nm, at least about 26 nm, at least about 27 nm, at least about 28 nm, at least about 29 nm, or at least about 30 nm. In some aspects, the length of the optimized linker is less than about 2 nm, less than about 3 nm, less than about 4 nm, less than about 5 nm, less than about 6 nm, less than about 7 nm, less than about 8 nm, less than about 9 nm, less than about 10 nm, less than about 11 nm, less than about 12 nm, less than about 13 nm, less than about 14 nm, less than about 15 nm, less than about 16 nm, less than about 17 nm, less than about 18 nm, less than about 19 nm, less than about 20 nm, less than about 21 nm, less than about 22 nm, less than about 23 nm, less than about 24 nm, less than about 25 nm, less than about 26 nm, less than about 27 nm, less than about 28 nm, less than about 29 nm, or less than about 30 nm.

[00171] In some aspects, the length of the optimized linker is about 2 nm to about 4 nm, about 3 nm to about 5 nm, about 4 nm to about 6 nm, about 5 nm to about 7 nm, about 6 nm to about 8 nm, about 7 nm to about 9 nm, about 8 nm to about 10 nm, about 9 nm to about 11 nm, about 10 nm to about 12 nm, about 11 nm to about 13 nm, about 12 nm to about 14 nm, about 13 nm to about 15 nm, about 14 nm to about 16 nm, about 15 nm to about 17 nm, about 16 nm to about 18 nm, about 17 nm to about 19 nm, about 18 nm to about 20 nm, about 19 nm to about 21 nm, about 20 nm to about 22 nm, about 21 nm to about 23 nm, about 22 nm to about 24 nm, about 23 nm to about 25 nm, about 24 nm to about 26 nm, about 25 nm to about 27 nm, about 26 nm to about 28 nm, about 27 nm to about 29 nm, about 28 nm to about 30 nm, about 2 nm to about 6 nm, about 4 nm to about 8 nm, about 6 nm to about 10 nm, about 8 nm to about 12 nm, about 10 nm to about 14 nm, about 12 nm to about 16 nm, about 14 nm to about 18 nm, about 16 nm to about 20 nm to about, about 18 nm to about 22 nm, about 20 nm to about 24 nm, about 22 nm to about 26 nm, about 24 nm to about 28 nm, about 26 nm to about 30 nm, about 2 nm to about 10 nm, about 4 nm to about 12 nm, about 6 nm to about 14 nm, about 8 nm to about 16 nm, about 10 nm to about 18 nm, about 12 nm to about 20 nm, about 14 nm to about 22 nm, about 16 nm to about 24 nm, about 18 nm to about 26 nm, about 20 nm to about 28 nm, about 22 nm to about 30 nm, about 2 nm to about 12 nm, about 4 nm to about 14 nm, about 6 nm to about 16 nm,

about 8 nm to about 18 nm, about 10 nm to about 20 nm, about 12 nm to about 22 nm, about 14 nm to about 24 nm, about 16 nm to about 26 nm, about 18 nm to about 28 nm, about 20 nm to about 30 nm, about 2 nm to about 5 nm, about 5 nm to about 10 nm, about 10 nm to about 15 nm, about 15 nm to about 20 nm, about 20 nm to about 25 nm, or about 25 nm to about 30 nm.

[00172] In some aspects, the anchoring moiety [AM] comprises a sterol, a lipid (e.g., a phospholipid), a vitamin, a peptide, or a combination thereof and optionally a linker or spacer. In general, the AM can comprise any hydrophobic moiety or combination thereof capable of inserting into the lipid bilayer of the EV (e.g., exosome), interacting electrostatically with the surface of the EV (e.g., exosome), or a combination thereof.

[00173] In some aspects, the anchoring moiety [AM] can comprise, e.g., a hydrophobic moiety (e.g., a lipid such as a sterol) and a spacer, wherein the spacer is an alkyl spacer, generally a linear alkyl spacer. In some aspects, the alkyl spacer attached to the hydrophobic moiety is selected from the group consisting of C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14 or C15, wherein C denotes a methyl unit (carbon unit) and the numeral indicates the number of methyl units (carbon units) in the alkyl spacer. In some aspects, the alkyl spacer comprises a single carbon unit (C1). In some aspects, the alkyl spacer is C2. In some aspects, the alkyl spacer is C3. In some aspects, the alkyl spacer is C4. In some aspects, the alkyl spacer is C5. In some aspects, the alkyl spacer is C6. In some aspects, the alkyl spacer is C7. In some aspects, the alkyl spacer is C8. In some aspects, the alkyl spacer is C9. In some aspects, the alkyl spacer is C10. In some aspects, the alkyl spacer is C11. In some aspects, the alkyl spacer is C12. In some aspects, the alkyl spacer is C13. In some aspects, the alkyl spacer is C14. In some aspects, the alkyl spacer is C15. In some aspects, the spacer attached to the sterol, a lipid, a vitamin, or peptide in the anchoring moiety [AM] is any molecule or combination thereof with a length equivalent to that of a C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14, or C15 linear alkyl spacer.

[00174] In some aspects, the anchoring moiety [AM] can comprise, e.g., a hydrophobic moiety (e.g., a lipid such as a sterol) and a spacer, wherein the spacer is a glycol spacer. In some aspects, the glycol spacer is selected from the group consisting of diethylene glycol, triethylene glycol, tetraethylene glycol (TEG), pentaethylene glycol, hexaethylene glycol (HEG), heptaethylene glycol, octaethylene glycol, nonaethylene glycol, or decaethylene glycol. In some aspects, the glycol spacer can comprise 11, 12, 13, 14 or 15 glycol units. In some aspects, the glycol spacer is HEG. In some aspects, the glycol spacer is TEG. In some aspects, the spacer attached to the sterol, a lipid, a vitamin, or peptide in the anchoring moiety [AM] is any molecule or combination thereof with a length equivalent to that of a diethylene glycol, triethylene glycol,

tetraethylene glycol (TEG), pentaethylene glycol, hexaethylene glycol (HEG), heptaethylene glycol, octaethylene glycol, nonaethylene glycol, or decaethylene glycol linker. In some aspects, the spacer attached to the sterol, a lipid, a vitamin, or peptide in the anchoring moiety [AM] is any molecule or combination thereof with a length equivalent to that of a glycol spacer with 11, 12, 13, 14, or 15 glycol units.

[00175] In some aspects, the anchoring moiety [AM] comprises a stero, e.g., a sterol is selected from the group consisting of cholesterol, ergosterol, 7-dehydrocholesterol, 24S-hydroxycholesterol, lanosterol, cycloartenol, fucosterol, saringosterol, campesterol, β-sitosterol, sitostanol, coprostanol, avenasterol, stigmasterol, or combinations thereof. In some specific aspects, the anchoring moiety [AM] comprises cholesterol.

[00176] In some aspects, the anchoring moiety [AM] comprises, consists, or consists essentially of a fatty acid, e.g., a straight chain fatty acid. In some aspects, the anchoring moiety [AM] comprises, consists, or consists essentially of a straight chain fatty, a branched fatty acid, an unsaturated fatty acid, a monounsaturated fatty acid, polyunsaturated fatty acid, a hydroxyl fatty acid, a polycarboxylic acid, or any combination thereof. For example, in some aspects, the straight chain fatty comprises, consists, or consists essentially of butyric acid, caproic acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid, or a combination thereof. In some specific aspects, the straight chain fatty acid is palmitic acid.

[00177] In some aspects, the anchoring moiety [AM] comprises, consists, or consists essentially of a phospholipid, e.g., a lecithin, a phosphatidyl choline, a phosphoinositol, a phosphosphingolipid, a phosphoethanolamine, a phosphatidic acid, or any combination thereof.

[00178] In some aspects, the anchoring moiety [AM] comprises, consists, or consists essentially of a vitamin, e.g., vitamin E (tocopherol or tocotrienol), vitamin D (e.g., vitamin D_2 or ergocalciferol, vitamin D_3 or cholecalciferol, or a combination thereof), vitamin K, riboflavin, niacin, pyridoxine, or a combination thereof.

[00179] In the context of the present disclosure, a combination of lipid moieties, e.g., different fatty acids, different sterols, different vitamins, or combinations thereof, in an anchoring moiety [AM] means that some of the constructs disclosed herein, e.g., constructs of Formula 1, in a population of constructs can have different lipid moieties. For example, for the same biologically molecule [BAM], some of the constructs of Formula 1 may have different anchoring moieties [AM], e.g., some may comprise a fatty acid, whereas other may comprise a vitamin or a sterol. Selecting combinations of constructs with different lipids moieties generally results in better packing on the lipids in the membrane on the EV (e.g., an exosome), which can result in higher loading efficiency and BAM density.

[00180] In some aspects, the anchoring moiety [AM] comprises a vitamin. In some aspects, the anchoring moiety [AM] consists of a vitamin. In some aspects, the anchoring moiety [AM] comprises a vitamin and an alkyl spacer. In some aspects, the anchoring moiety [AM] consists of a vitamin and an alkyl spacer. In some aspects, the anchoring moiety [AM] comprises tocopherol and an alkyl spacer. In some aspects, the anchoring moiety [AM] consists of tocopherol and an alkyl spacer. In some aspects, the anchoring moiety [AM] comprises a vitamin and an octyl (C8) alkyl spacer. In some aspects, the anchoring moiety [AM] consists of a vitamin and an octyl (C8) alkyl spacer. In some aspects, the anchoring moiety [AM] comprises tocopherol and an octyl (C8) alkyl spacer. In some aspects, the anchoring moiety [AM] consists of tocopherol and an octyl (C8) alkyl spacer. In some aspects, the anchoring moiety [AM] comprises tocopherol. In some aspects, the anchoring moiety [AM] consists of tocopherol.

[00181] In some aspects, the anchoring moiety [AM] comprises a fatty acid and an alkyl spacer. In some aspects, the anchoring moiety [AM] consists of a fatty acid and an alkyl spacer. In some aspects, the anchoring moiety [AM] comprises a palmitate and an alkyl spacer. In some aspects, the anchoring moiety [AM] consists of palmitate and an alkyl spacer. In some aspects, the anchoring moiety [AM] comprises a fatty acid and a hexyl (C6) alkyl spacer. In some aspects, the anchoring moiety [AM] consists of a fatty acid and a hexyl (C6) alkyl spacer. In some aspects, the anchoring moiety [AM] comprises palmitate and a hexyl (C6) alkyl spacer. In some aspects, the anchoring moiety [AM] consists of palmitate and a hexyl (C6) alkyl spacer.

[00182] In some aspects, the anchoring moiety [AM] comprises a sterol and a glycol spacer. In some aspects, the anchoring moiety [AM] consists of a sterol and a glycol spacer. In some aspects, the anchoring moiety [AM] comprises cholesterol and a glycol spacer. In some aspects, the anchoring moiety [AM] consists of cholesterol and a glycol spacer. In some aspects, the anchoring moiety [AM] comprises a sterol and a TEG glycol spacer. In some aspects, the anchoring moiety [AM] consists of a sterol and a TEG glycol spacer. In some aspects, the anchoring moiety [AM] comprises cholesterol and a TEG glycol spacer. In some aspects, the anchoring moiety [AM] consists of cholesterol and a TEG glycol spacer. In some aspects, the anchoring moiety [AM] comprises a sterol and an alkyl spacer. In some aspects, the anchoring moiety [AM] consists of a sterol and an alkyl spacer. In some aspects, the anchoring moiety [AM] comprises a sterol and a hexyl (C6) alkyl spacer. In some aspects, the anchoring moiety [AM] consists of a sterol and a hexyl (C6) alkyl spacer. In some aspects, the anchoring moiety [AM] comprises cholesterol and an alkyl spacer. In some aspects, the anchoring moiety [AM] consists of cholesterol and an alkyl spacer. In some aspects, the anchoring moiety [AM]

comprises cholesterol and a hexyl (C6) alkyl spacer. In some aspects, the anchoring moiety [AM] consists of cholesterol and a hexyl (C6) alkyl spacer.

[00183] In some aspects, L_1 is a cleavable linkage comprising a phosphodiester bond. In some aspects, L_1 is a non-cleavable linkage comprising a phosphorothioate bond. In some aspects, L_2 is an optional cleavable linkage comprising a phosphodiester bond. In some aspects, L_2 is an optional non-cleavable linkage comprising a phosphorothioate bond. In some aspects, L_3 is an optional cleavable linkage comprising a phosphodiester bond. In some aspects, L_3 is an optional non-cleavable linkage comprising a phosphorothioate bond.

[00184] In some aspects, L_1 is a phosphodiester bond. In some aspects, L_1 is a phosphorothioate bond. In some aspects, L_2 is a phosphodiester bond. In some aspects, L_2 is a phosphorothioate bond. In some aspects, L_3 is a phosphodiester bond. In some aspects, L_3 is a phosphorothioate bond.

[00185] In some aspects, each of L_1, L_2, or L_3 independently comprises a phosphodiester or phosphorothioate bond, and one or more cleavable or non-cleavable linkers or spacers disclosed herein.

[00186] In some aspects, the SP_1 optional first spacer and/or the SP_2 optional second spacer independently comprise a spacer (e.g., an alkyl spacer or a glycol spacer), or a combination thereof. In some aspects, the SP_1 optional first spacer comprises or consists of an alkyl spacer which is C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14, or C15. In some aspects, SP_1 is a C3 or C6 alkyl spacer. In some aspects, the SP_2 optional first spacer comprises or consists of an alkyl spacer which is C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14, or C15. In some aspects, SP_2 is a C3 or C6 alkyl spacer.

[00187] In some aspects, the SP_1 optional first spacer comprises or consists of a glycol spacer which has 2 (diethylene glycol), 3 (triethylene glycol), 4 (tetraethylene glycol; TEG), 5 (pentaethylene glycol), 6 (hexaethylene glycol; HEG), 7, 8, 9, 10, 11, 12, 13, 14, or 15 glycol units. In some aspects, S_1 is a tetraethylene glycol (TEG) or hexaethylene glycol (HEG) glycol spacer. In some aspects, the SP_2 optional first spacer comprises or consists of a glycol spacer which has 2 (diethylene glycol), 3 (triethylene glycol), 4 (tetraethylene glycol; TEG), 5 (pentaethylene glycol), 6 (hexaethylene glycol; HEG), 7, 8, 9, 10, 11, 12, 13, 14, or 15 glycol units. In some aspects, S_2 is a tetraethylene glycol (TEG) or hexaethylene glycol (HEG) glycol spacer.

[00188] In some aspects, the SP_1 spacer comprises an alkyl spacer and the SP_2 spacer also comprises an alkyl spacer. In some aspects, the SP_1 spacer comprises a glycol spacer and the SP_2 spacer also comprises a glycol spacer. In some aspects, the SP_1 spacer comprises an alkyl spacer

and the SP_2 spacer comprises a glycol spacer. In some aspects, the SP_1 spacer comprises a glycol spacer and the SP_2 spacer comprises an alkyl spacer. In some aspects, the SP_1 spacer consists of an alkyl spacer and the SP_2 spacer also consists of an alkyl spacer. In some aspects, the SP_1 spacer consists of a glycol spacer and the SP_2 spacer also consists of a glycol spacer. In some aspects, the SP_1 spacer consists of an alkyl spacer and the SP_2 spacer consists of a glycol spacer. In some aspects, the SP_1 spacer consists of a glycol spacer and the SP_2 spacer consists of an alkyl spacer.

[00189] In some aspects, each non-cleavable spacer is independently selected from the group consisting of alkyl (e.g., C2, C3, C4, C5, C6, C7 or C8), glycol (e.g., diethylene glycol, triethylene glycol, tetraethylene glycol (TEG), hexaethylene glycol (HEG), pentaethylene glycol, polyethylene glycol (PEG)), glycerol (e.g., diglycerol, triglycerol, tetraglycerol (TG), pentaglycerol, a hexaglycerol (HG), polyglycerol (PG)), succinimide, maleimide, or any combination thereof.

[00190] In some aspects, a spacer in an anchoring moiety [AM], a first (proximal) spacer [SP_1], a second (distal) spacer [SP_2], or any combination thereof can comprise or consist of a polyethylene glycol (PEG) characterized by the formula R_1-(O-CH_2-CH_2)$_n$- or R_1-(O-CH_2-CH_2)$_n$-O-, wherein R_1 is hydrogen, methyl or ethyl and n is an integer between 1 and 15. Thus, in some aspects, n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15. These PEG can be described as PEG_1, PEG_2, PEG_3, PEG_4, PEG_5, PEG_6, PEG_7, PEG_8, PEG_9, PEG_{10}, PEG_{11}, PEG_{12}, PEG_{13}, PEG_{14} or PEG_{15}, or PEG_1-O-, PEG_2-O-, PEG_3-O-, PEG_4-O-, PEG_5-O-, PEG_6-O-, PEG_7-O-, PEG_8-O-, PEG_9-O-, PEG_{10}-O-, PEG_{11}-O-, PEG_{12}-O-, PEG_{13}-O-, PEG_{14}-O- or PEG_{15}-O-, respectively.

[00191] In some aspects, a spacer in an anchoring moirty [AM], a first (proximal) spacer [SP_1], a second (distal) spacer [SP_2], or any combination thereof can comprise or consist of a polyglycerol (PG) characterized by the formula ((R$_1$—O—(CH_2—CHOH—CH_2O)$_n$—), wherein R_1 is hydrogen, methyl or ethyl, and n is an integer between 1 and 15. Thus, in some aspects, n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15. These PEG can be described as PG_1, PG_2, PG_3, PG_4, PG_5, PG_6, PG_7, PG_8, PG_9, PG_{10}, PG_{11}, PG_{12}, PG_{13}, PG_{14} or PG_{15}.

[00192] In some aspects, an L_1, L_2, or L_3 linkage, or any combination thereof can comprise or consist of a cleavable linkage comprising a redox cleavable linker, a reactive oxygen species cleavable linker, a pH dependent cleavable linker, an enzymatic cleavable linker, a protease cleavable linker, an esterase cleavable linker, a phosphatase cleavable linker, a photoactivated cleavable linker, a self-immolative linker, or any combination thereof. In some aspects, the L_1 linkage comprise a cleavable linkage comprising a redox cleavable linker, a reactive oxygen

species cleavable linker, a pH dependent cleavable linker, an enzymatic cleavable linker, a protease cleavable linker, an esterase cleavable linker, a phosphatase cleavable linker, a photoactivated cleavable linker, a self-immolative linker, or any combination thereof. In some aspects, the L_1 linkage consists of a cleavable linkage comprising a redox cleavable linker, a reactive oxygen species cleavable linker, a pH dependent cleavable linker, an enzymatic cleavable linker, a protease cleavable linker, an esterase cleavable linker, a phosphatase cleavable linker, a photoactivated cleavable linker, a self-immolative linker, or any combination thereof. In some aspects, the L_2 linkage comprise a cleavable linkage comprising a redox cleavable linker, a reactive oxygen species cleavable linker, a pH dependent cleavable linker, an enzymatic cleavable linker, a protease cleavable linker, an esterase cleavable linker, a phosphatase cleavable linker, a photoactivated cleavable linker, a self-immolative linker, or any combination thereof. In some aspects, the L_2 linkage consists of a cleavable linkage comprising a redox cleavable linker, a reactive oxygen species cleavable linker, a pH dependent cleavable linker, an enzymatic cleavable linker, a protease cleavable linker, an esterase cleavable linker, a phosphatase cleavable linker, a photoactivated cleavable linker, a self-immolative linker, or any combination thereof. In some aspects, the L_3 linkage comprise a cleavable linkage comprising a redox cleavable linker, a reactive oxygen species cleavable linker, a pH dependent cleavable linker, an enzymatic cleavable linker, a protease cleavable linker, an esterase cleavable linker, a phosphatase cleavable linker, a photoactivated cleavable linker, a self-immolative linker, or any combination thereof. In some aspects, the L_3 linkage consists of a cleavable linkage comprising a redox cleavable linker, a reactive oxygen species cleavable linker, a pH dependent cleavable linker, an enzymatic cleavable linker, a protease cleavable linker, an esterase cleavable linker, a phosphatase cleavable linker, a photoactivated cleavable linker, a self-immolative linker, or any combination thereof.

[00193] In some aspects, a cleavable linkage disclosed herein, e.g., an L_1, L_2, or L_3 linkage or any combination thereof comprises or consists of a self-immolative linker (e.g., p-aminobenzyl carbamate, pABC). In some aspects, L_1 comprises a self-immolative linker (e.g., p-aminobenzyl carbamate, pABC). In some aspects, L_1 consists of a self-immolative linker (e.g., p-aminobenzyl carbamate, pABC). In some aspects, L_2 comprises a self-immolative linker (e.g., p-aminobenzyl carbamate, pABC). In some aspects, L_2 consists of a self-immolative linker (e.g., p-aminobenzyl carbamate, pABC). In some aspects, L_3 comprises a self-immolative linker (e.g., p-aminobenzyl carbamate, pABC). In some aspects, L_3 consists of a self-immolative linker (e.g., p-aminobenzyl carbamate, pABC).

[00194] In some aspects, a cleavable linkage disclosed herein, e.g., an L_1, L_2, or L_3 linkage or any combination thereof comprises or consists of a cinnamyl group, a naphthyl group, a

biphenyl group, a heterocyclic ring, a homoaromatic group, coumarin, furan, thiophene, thiazole, oxazole, isoxazole, pyrrole, pyrazole, pyridine, imidazone, triazole, or any combination thereof. In some aspects, L_1 comprises a cleavable linker selected from the group consisting of a cinnamyl group, a naphthyl group, a biphenyl group, a heterocyclic ring, a homoaromatic group, coumarin, furan, thiophene, thiazole, oxazole, isoxazole, pyrrole, pyrazole, pyridine, imidazone, triazole, or any combination thereof. In some aspects, L_1 consists of a cleavable linker selected from the group consisting of a cinnamyl group, a naphthyl group, a biphenyl group, a heterocyclic ring, a homoaromatic group, coumarin, furan, thiophene, thiazole, oxazole, isoxazole, pyrrole, pyrazole, pyridine, imidazone, triazole, or any combination thereof. In some aspects, L_2 comprises a cleavable linker selected from the group consisting of a cinnamyl group, a naphthyl group, a biphenyl group, a heterocyclic ring, a homoaromatic group, coumarin, furan, thiophene, thiazole, oxazole, isoxazole, pyrrole, pyrazole, pyridine, imidazone, triazole, or any combination thereof. In some aspects, L_2 consists of a cleavable linker selected from the group consisting of a cinnamyl group, a naphthyl group, a biphenyl group, a heterocyclic ring, a homoaromatic group, coumarin, furan, thiophene, thiazole, oxazole, isoxazole, pyrrole, pyrazole, pyridine, imidazone, triazole, or any combination thereof. In some aspects, L_3 comprises a cleavable linker selected from the group consisting of a cinnamyl group, a naphthyl group, a biphenyl group, a heterocyclic ring, a homoaromatic group, coumarin, furan, thiophene, thiazole, oxazole, isoxazole, pyrrole, pyrazole, pyridine, imidazone, triazole, or any combination thereof. In some aspects, L_3 consists of a cleavable linker selected from the group consisting of a cinnamyl group, a naphthyl group, a biphenyl group, a heterocyclic ring, a homoaromatic group, coumarin, furan, thiophene, thiazole, oxazole, isoxazole, pyrrole, pyrazole, pyridine, imidazone, triazole, or any combination thereof.

[00195] In some aspects, a cleavable linkage disclosed herein, e.g., an L_1, L_2, or L_3 linkage or any combination thereof comprises or consists of a linkage (generally a dipeptide or tripeptide linker) having the formula:

-Aa-Yy-

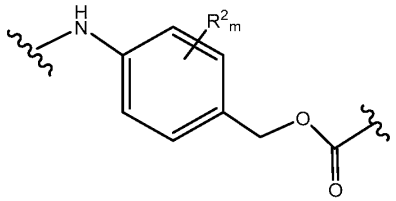
wherein each –A- is independently an amino acid unit or a combination thereof, a is independently an integer from 1 to 15; -Y- is a spacer unit, and y is 0, 1, or 2. In some aspects, L_1 comprises or consists of a -Aa-Yy- linker described above. In some aspects, L_2 comprises or consists of a -Aa-Yy- linker described above. In some aspects, L_3 comprises or consists of a -Aa-Yy- linker described above.

[00196] In some aspects -Aa- is a dipeptide, a tripeptide, a tetrapeptide, a pentapeptide, or a hexapeptide. In some aspects wherein a is 2 (i.e., a dipeptide cleavable linker) –Aa- is selected

from the group consisting of valine-alanine, valine-citrulline, phenylalanine-lysine, N-methylvaline-citrulline, cyclohexylalanine-lysine, and beta-alanine-lysine. In some aspects wherein a is 3 (i.e., a tripeptide cleavable linker) –Aa- is glutamic acid-valine-citrulline. In some aspects, y is 1, i.e., the cleavable linker of formula -Aa-Yy- comprises a single spacer. In some aspects, the cleavable linker of formula -Aa-Yy- can comprises more than spacer, e.g., two spacers. In some aspects, the two spacers are the same. In other aspects, the two spacers are different. In some aspects, the single spacer can be cleavable or non-cleavable. In some aspects, the first spacer (proximal to Aa) can be cleavable and the second spacer (distal to Aa) can be non-cleavable. In some aspects, the first spacer (proximal to Aa) can be non-cleavable and the second spacer (distal to Aa) can be cleavable. In some aspects, both spacers can be non-cleavable. In some aspects, both spacer can be cleavable.

[00197] In some aspects, a –Y- spacer can be a self-immolative spacer, e.g., p-aminobenzylcarbamate (pABC). The term "self-immolative spacer" as used herein refers to a spacer that will spontaneously separate from a first moiety (e.g., a biologically active molecule, linkage, spacer, or anchoring moiety) if its bond to a second moiety (e.g., a biologically active molecule, linkage, spacer, or anchoring moiety) is cleaved.

[00198] In some aspects, –Yy- has the formula:



wherein each R_2 is independently C_{1-8} alkyl, -O-(C_{1-8} alkyl), halogen, nitro, or cyano; and m is an integer from 0 to 4. In some aspects, m is 0, 1, or 2. In some aspects, m is 0.

[00199] In some aspects, a cleavable linkage disclosed herein, e.g., an L_1, L_2, or L_3 linkage or any combination thereof comprises or consists of valine-alanine-p-aminobenzylcarbamate or valine-citrulline-p-aminobenzylcarbamate.

[00200] In some aspects, –Y- is a non self-immolative spacer, e.g., a peptide spacer. Peptide spacers are generally Gly polymers or Gly/Ser polymers. In some aspects, the –Y-peptide spacer comprises or consists of –Gly- or –Gly-Gly-. Other peptide spacers such as the (Gly_4Ser)n spacer are disclosed more in detail below.

[00201] In some aspects, in addition or instead of a lipid, a vitamin, or a sterol, an anchoring moiety [AM] can comprise a scaffold protein (e.g., a Scaffold X protein such as PTGFRN or a fragment thereof), or a binding molecule which can bind to a scaffold protein

present in the EV (e.g., exosome) membrane, for example, an antibody or a binding portion thereof that can specifically bind to PTGRN natively or recombinantly expressed on the surface of the EV (e.g., exosome). Thus, in some aspects, the anchoring moiety [AM] and/or the scaffold moiety is Scaffold X.

[00202] In some aspects, the Scaffold X is selected from the group consisting of prostaglandin F2 receptor negative regulator (the PTGFRN protein); basigin (the BSG protein); immunoglobulin superfamily member 2 (the IGSF2 protein); immunoglobulin superfamily member 3 (the IGSF3 protein); immunoglobulin superfamily member 8 (the IGSF8 protein); integrin beta-1 (the ITGB1 protein); integrin alpha-4 (the ITGA4 protein); 4F2 cell-surface antigen heavy chain (the SLC3A2 protein); a class of ATP transporter proteins (the ATP1A1, ATP1A2, ATP1A3, ATP1A4, ATP1B3, ATP2B1, ATP2B2, ATP2B3, ATP2B4 proteins); a functional fragment thereof; and any combination thereof. In some aspects, the Scaffold X is PTGFRN protein or a functional fragment thereof. In some aspects, the Scaffold X comprises an amino acid sequence as set forth in SEQ ID NO:302. In some aspects, the Scaffold X comprises an amino acid sequence at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO:302. Different Scaffold X proteins are disclosed in detail below.

[00203] In some aspects, the biologically active molecule [BAM] is attached via an anchoring moiety [AM] to the exterior surface of the EV, wherein the [AM] and [BAM] are connected by an optimized linker disclosed herein (i.e., a linker comprising the specific architecture, components, and length constraint disclosed above). In some aspects, the biologically active molecule [BAM] comprises or consists of a polypeptide, a peptide, a polynucleotide (DNA and/or RNA), a chemical compound, or any combination thereof.

[00204] In some aspects, the biologically active molecule [BAM] comprises a chemical compound, e.g., a small molecule drug. In some aspects, the biologically active molecule [BAM] comprises an antisense oligonucleotide (ASO), a siRNA, a miRNA, a shRNA, an mRNA, a nucleic acid, an antimirs, an RNA decoy, or any combination thereof. In some aspects, the ASO is a gapmer. Specific nucleic acid biologically active molecules, e.g., ASOs against specific targets such as NLRP3, STAT6, CEBP/β, STAT3, NRas, KRAS, or Pmp22 are disclosed in detail below.

[00205] In some aspects, the biologically active molecule [BAM] comprises or consists of a peptide, a protein, an antibody or an antigen binding portion thereof, or any combination

thereof. In some aspects, the antigen binding portion thereof comprises scFv, (scFv)2, Fab, Fab', F(ab')2, F(ab1)2, Fv, dAb, and Fd fragment, diabodys, antibody-related polypeptide, or any fragment thereof. In some aspects, the antibody or antigen binding portion thereof can bind to a Protein X protein present in the membrane of the EV (e.g., exosome).

[00206] In some aspects, biologically active molecule [BAM] comprises or consist of an ASO. In some aspects, the ASO can target pre-mRNA or a mature mRNA, including protein coding regions (exons), non coding regions (e.g., 5' or 3' unstranslated regions, or introns), intron-exon junctions, or regulatory regions (e.g., promoters). In some aspects, the ASO targets a protein transcript, e.g., a STAT6 transcript, a CEBP/β transcript, a STAT3 transcript, a KRAS transcript, a NRAS transcript, an NLPR3 transcript, or any combination thereof. Detailed descriptions of ASOs directed to specific targets are provided below.

[00207] In some aspects of the present disclosure, the EV is an exosome, for example, a native exosome, or an exosome overexpressing a recombinant protein, e.g., a Scaffold X protein such as PTGFRN or a functional portion thereof. Types of exosomes, including different sizes, compositions, or methods of manufacture are disclosed in detail below.

[00208] The present disclosure provides also pharmaceutical compositions comprising the EVs (extracellular vesicles) disclosed herein (e.g., exosomes), which comprise constructs such as the construct of Formula 1 comprising an optimized linker of the present disclosure, and a pharmaceutically acceptable carrier. Also provides is a kit comprising any EV or pharmaceutical composition disclosed herein, comprising constructs such as the construct of Formula 1, which comprise an optimized linker of the present disclosure and instructions for use.

[00209] The present disclose provides a method of treating or preventing a disease or disorder in a subject in need thereof comprising administering an EV (e.g., exosome) of the present disclosure or pharmaceutical composition comprising such EV (e.g., exosome) to the subject. In some aspects, the disease or disorder is a cancer, an inflammatory disorder, a neurodegenerative disorder, a central nervous disease, or a metabolic disease. In some aspects, an EV (e.g., exosome) disclosed herein can be administered intravenously, intraperitoneally, nasally, orally, intramuscularly, subcutaneously, parenterally, intrathecally, peritumorally, intraocularly, or intratumorally.

[00210] The present disclosure provides a method of attaching a biologically active molecule [BAM] to an EV (e.g., an exosome) comprising linking an anchoring moiety [AM] to the EV, wherein the anchoring moiety [AM] is attached to the biologically active moiety [BAM] according to the formula:

$$[AM]\text{-}L_1\text{-}[SP_1]\text{-}L_2\text{-}[SP_2]\text{-}L_3\text{-}[BAM]$$

wherein [AM] is the anchoring moiety; L_1 is a cleavable or non-cleavable linkage; L_2 and L_3 are optional cleavable or non-cleavable linkages; SP_1 is an optional first spacer; and, SP_2 is an optional second spacer. In some aspects, [AM] is cholesterol-C6, cholesterol-TEG, tocopherol-C8, tocopherol, of palmitate-C6. In some aspects, L_1 is a phosphodiester bond. In some aspects, SP_1 is C3, C6, TEG, or HEG. In some aspects, L_2 is a phosphorothioate bond. In some aspects, SP_2 is C3, C6, TEG or HEG. In some aspects, L_3 is a phosphorothioate bond. In some aspects, [BAM] is an antisense oligonucleotide (ASO), e.g., a gapmer. In some aspects, the length of the optimized linker connecting [AM] and [BAM] is about 5 nm to about 10 nm, about 10 nm to about 15 nm, about 15 nm to about 20 nm, about 20 nm to about 25 nm, or about 25 to about 30 nm.

[00211] The present disclosure also provides a method of increasing the load density of a biologically active molecule (BAM) attached to an EV, comprising screening a library of anchoring moieties (AM) attached to the biologically active moiety (BAM) according to the formula:

$$[AM]\text{-}L_1\text{-}[SP_1]\text{-}L_2\text{-}[SP_2]\text{-}L_3\text{-}[BAM]$$

wherein [AM] is the anchoring moiety; L_1 is a cleavable or non-cleavable linkage; L_2 and L_3 are optional cleavable or non-cleavable linkages; SP_1 is an optional first spacer; and, SP_2 is an optional second spacer. In some aspects, [AM] is cholesterol-C6, cholesterol-TEG, tocopherol-C8, tocopherol, of palmitate-C6. In some aspects, L_1 is a phosphodiester bond. In some aspects, SP_1 is C3, C6, TEG, or HEG. In some aspects, L_2 is a phosphorothioate bond. In some aspects, SP_2 is C3, C6, TEG or HEG. In some aspects, L_3 is a phosphorothioate bond. In [BAM] is an antisense oligonucleotide (ASO). In some aspects, the length of the optimized linker connecting [AM] and [BAM] is about 5 nm to about 10 nm, about 10 nm to about 15 nm, about 15 nm to about 20 nm, about 20 nm to about 25 nm, or about 25 to about 30 nm. In some aspects, the load density of a biologically active molecule [BAM] attached to an EV, e.g., an exosome, is increased at least about 1-fold, at least about 1.5-fold, at least about 2-fold, at least about 2.5-fold, at least about 3-fold, at least about 3.5-fold, at last about 4-fold, at least about 4.5-fold, at least about 5-fold, at least about 5.5-fold, at least about 6-fold, at least about 6.5-fold, at least about 7-fold, at least about 7.5-fold, at least about 8-fold, at least about 8.5-fold, at least about 9-fold, at least about 9.5-fold, or at least about 10-fold with respect to a control. In some aspects, the control is a corresponding construct lacking the optimized linker. For example, in some aspects, the control for a construct with the structure $[AM]\text{-}L_1\text{-}[SP_1]\text{-}L_2\text{-}[SP_2]\text{-}L_3\text{-}[BAM]$ is a control construct with the structure [AM]-[BAM].

[00212] An extracellular vesicle (EV) comprising an antisense oligonucleotide [ASO] covalently linked to the EV via an anchoring moiety [AM] according to the formula:

$$[AM]\text{-}L_1\text{-}[SP_1]\text{-}L_2\text{-}[SP_2]\text{-}L_3\text{-}[ASO]$$

wherein [AM] is the anchoring moiety selected from the group consisting of cholesterol-C6, cholesterol-TEG, tocopherol-C8, tocopherol, and palmitate-C6; L_1 is a phosphodiesterase cleavable linkage; SP_1 is an optional first spacer selected from the group consisting of C3, C6, TEG and HEG; L_2 is optional phosphorothioate non-cleavable linkage; SP_2 is an optional second spacer selected from the group consisting of C3, C6, TEG, and HEG; and, L_3 is an optional phosphorothioate non-cleavable linkage. In some aspects, the EV is an exosome, e.g., a native exosome or a recombinant exosome. In some aspects wherein the exosome is a native exosome, the load density of ASO attached to the exosome is increased by at least about 1.5-fold with respect to a control (see above). In some aspects wherein the exosome is an exosome overexpressing PTGFRN the load density of ASO attached to the exosome is increased by at least about 2-fold with respect to a control, e.g., a construct without an optimized linker such as [AM]-[BAM].

[00213] In some aspects wherein the exosome is a native exosome and the anchoring moiety [AM] is cholesterol-C6. In some aspects, the average number of ASO molecules per native exosome is 5032+/-386. In some aspects, the average number of ASO molecules per native exosome is between about 4500 and about 5500. In some aspects, the average number of ASO molecules per native exosome is between about 4500 and about 4600, between about 4600 and about 4700, between about 4700 and about 4800, between about 4800 and about 4900, between about 4900 and about 5000, between about 5000 and about 5100, between about 5100 and about 5200, between about 5200 and about 5300, between about 5300 and about 5400, or between about 5400 and about 5500. In some aspects, the average number of ASO molecules per native exosome is at least about 4500, at least about 4600, at least about 4700, at least about 4800, at least about 4900, at least about 5000, at least about 5100, at least about 5200, at least about 5300, at least about 5400, or at least about 5500. In some aspects, the loading efficiency of the native exosome is 73% to 93%. In some aspects, the loading efficiency of the native exosome is between about 70% and about 95%. In some aspects, the loading efficiency of the native exosome is between about 70% and about 75%, between about 75% and about 80%, between about 80% and about 85%, between about 85% and about 90%, or between about 90% and about 95%. In some aspects, the loading efficiency of the native exosome is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%.

[00214] In some aspects wherein the exosome is a native exosome and the anchoring moiety [AM] is cholesterol-TEG, the average number of ASO molecules per exosome is 3991+/-490. In some aspects, the average number of ASO molecules per native exosome is between about 3500 and about 4500. In some aspects, the average number of ASO molecules per native exosome is between about 3500 and about 3600, between about 3600 and about 3700, between about 3700 and about 3800, between about 3800 and about 3900, between about 3900 and about 4000, between about 4000 and about 4100, between about 4100 and about 4200, between about 4200 and about 4300, between about 4300 and about 4400, or between about 4400 and about 4500. In some aspects, the average number of ASO molecules per native exosome is at least about 3500, at least about 3600, at least about 3700, at least about 3800, at least about 3900, at least about 4000, at least about 4100, at least about 4200, at least about 4300, at least about 4400, or at least about 4500. In some aspects, the loading efficiency of the native exosome is 56% to 79%. In some aspects, the loading efficiency of the native exosome is between about 50% and about 85%. In some aspects, the loading efficiency of the native exosome is between about 50% and about 55%, between about 55% and about 60%, between about 60% and about 65%, between about 65% and about 70%, between about 70% and about 75%, between about 75% and about 80%, or between about 80% and about 85%. In some aspects, the loading efficiency of the native exosome is at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, or at least about 85%.

[00215] In some aspects wherein the exosome is a native exosome and the anchoring moiety [AM] is tocopherol-C8, tocopherol, or palmitate-C6, the average number of ASO molecules per native exosome is 4241+/-722. In some aspect, the average number of ASO molecules per native exosome is between about 3500 and about 5000. In some aspect, the average number of ASO molecules per native exosome is between about 3500 and about 3600, between about 3600 and about 3700, between about 3700 and about 3800, between about 3800 and about 3900, between about 3900 and about 4000, between about 4000 and about 4100, between about 4100 and about 4200, between about 4200 and about 4300, between about 4300 and about 4400, between about 4400 and about 4500, between about 4500 and about 4600, between about 4600 and about 4700, between about 4700 and about 4800, between about 4800 and about 4900, or between about 4900 and about 5000. In some aspects, the average number of ASO molecules per native exosome is at least about 3500, at least about 3600, at least about 3700, at least about 3800, at least about 3900, at least about 4000, at least about 4100, at least about 4200, at least about 4300, at least about 4400, at least about 4500, at least about 4600, at least about 4700, at least about 4800, at least about 4900, or at least about 5000. In some aspects,

the loading efficiency of the native exosome is 57% to 73%. In some aspects, the loading efficiency of the native exosome is between about 50% and about 80%. In some aspects, the loading efficiency of the native exosome is between about 50% and about 55%, between about 55% and about 60%, between about 60% and about 65%, between about 65% and about 70%, between about 70% and about 75%, or between about 75% and about 80%. In some aspects, the loading efficiency of the native exosome is at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, or at least about 80%. In some aspects, the average number of ASO molecules per native exosome is more than 5000, more than 6000, more than 7000, more than 8000, more than 9000, more than 10,000, more than 11,000, more than 12,000, more than 13,000, more than 14,000, more than 15,000, more than 16,000, more than 17,000, more than 18,000, more than 19,000, or more than 20,000. In some aspects, the average number of ASO molecules per exosome is between about 5000 and about 6,000, about 6,000 and about 7,000, about 7,000 and about 8,000, about 8,000 and about 9,000, about 9,000 and about 10,000, about 10,000 and about 11,000, about 11,000 and about 12,000 about 12,000 and about 13,000, about 13,000 and about 14,000, about 14,000 and about 15,000, about 15,000 and about 16,000, about 16,000 and about 17,000, about 17,000 and about 18,000, about 18,000 and about 19,000, or about 19,0000 and about 20,000.

[00216] In some aspects wherein the exosome is a Scaffold-X exosome and the anchoring moiety [AM] is cholesterol-C6, the average number of ASO molecules per exosome is 2442+/-339. In some aspects, the average number of ASO molecules per Scaffold-X exosome is between about 2000 and about 3000. In some aspects, the average number of ASO molecules per Scaffold-X exosome is between about 2000 and about 2100, between about 2100 and about 2200, between about 2200 and about 2300, between about 2300 and about 2400, between about 2400 and about 2500, between about 2500 and about 2600, between about 2600 and about 2700, between about 2700 and about 2800, between about 2800 and about 2900, or between about 2900 and about 3000. In some aspects, the average number of ASO molecules per Scaffold-X exosome is at least about 2000, at least about 2100, at least about 2200, at least about 2300, at least about 2400, at least about 2500, at least about 2600, at least about 2700, at least about 2800, at least about 2900, or at least about 3000. In some aspects, the loading efficiency of the Scaffold-X exosome is 27% to 46%. In some aspects, the loading efficiency of the Scaffold-X exosome is between about 25% and about 50%. In some aspects, the loading efficiency of the Scaffold-X exosome is between about 25% and about 30%, between about 30% and about 35%, between about 35% and about 40%, between about 40% and about 45%, or between about 45% and about 50%. In some aspects, the loading efficiency of the Scaffold-X exosome is at least about 25%, at

least about 30%, at least about 35%, at least about 40%, at least about 45%, or at least about 50%.

[00217] In some aspects wherein the exosome is a Scaffold-X exosome and the anchoring moiety [AM] is cholesterol-TEG, the average number of ASO molecules per exosome is 1728+/-264. In some aspects, the average number of ASO molecules per Scaffold-X exosome is between about 1400 and about 2100. In some aspects, the average number of ASO molecules per Scaffold-X exosome is between about 1400 and about 1500, between about 1500 and about 1600, between about 1600 and about 1700, between about 1700 and about 1800, between about 1800 and about 1900, between about 1900 and about 2000, or between about 2000 and about 2100. In some aspects, the average number of ASO molecules per Scaffold-X exosome is at least about 1400, at least about 1500, at least about 1600, at least about 1700, at least about 1800, at least about 1900, at least about 2000, or at least about 2100. In some aspects, the loading efficiency of the Scaffold-X exosome is 19% to 33%. In some aspects, the loading efficiency of the Scaffold-X exosome is between about 15% and about 35%. In some aspects, the loading efficiency of the Scaffold-X exosome is between about 15% and about 20%, between about 20% and about 25%, between about 25% and about 30%, or between about 30% and about 35%. In some aspects, the loading efficiency of the Scaffold-X exosome is at least about 15%, at least about 20%, at least about 25%, at least about 30%, or at least about 35%. In some aspects wherein the exosome is a Scaffold-X exosome and the anchoring moiety [AM] is tocopherol-C8, tocopherol, or palmitate-C6, the average number of ASO molecules per exosome is 2979+/-1006. In some aspects, the average number of ASO molecules per Scaffold-X exosome is between about 1900 and about 4000. In some aspects, the average number of ASO molecules per Scaffold-X exosome is between about 1900 and about 2000, between about 2000 and about 2100, between about 2100 and about 2200, between about 2200 and about 2300, between about 2300 and about 2400, between about 2400 and about 2500, between about 2500 and about 2600, between about 2600 and about 2700, between about 2700 and about 2800, between about 2800 and about 2900, between about 2900 and about 3000, between about 3000 and about 3100, between about 3100 and about 3200, between about 3200 and about 3300, between about 3300 and about 3400, between about 3400 and about 3500. between about 3500 and about 3600, between about 3600 and about 3700, between about 3700 and about 3800, between about 3800 and about 3900, or between about 3900 and about 4000. In some aspects, the average number of ASO molecules per Scaffold-X exosome is at least about 1900, at least about 2000, at least about 2100, at least about 2200, at least about 2300, at least about 2400, at least about 2500, at least about 2600, at least about 2700, at least about 2800, at least about 2900, at least about 3000, at least about 3100, at

least about 3200, at least about 3300, at least about 3400, at least about 3500, at least about 3600, at least about 3700, at least about 3800, at least about 3900, or at least about 4000. In some aspects, the loading efficiency of the Scaffold-X exosome is 37% to 68%. the loading efficiency is between about 30% and about 75%. In some aspects, the loading efficiency is between about 30% and about 35%, between about 35% and about 40%, between about 40% and about 45%, between about 45% and about 50%, between about 50% and about 55%, between about 55% and about 60%, between about 60% and about 65%, between about 65% and about 70%, or between about 70% and about 75%. In some aspects, the loading efficiency of the Scaffold-X exosome is at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%.

[00218] In some aspects, the average number of ASO molecules per Scaffold-X exosome is more than 5000, more than 6000, more than 7000, more than 8000, more than 9000, more than 10,000, more than 11,000, more than 12,000, more than 13,000, more than 14,000, more than 15,000, more than 16,000, more than 17,000, more than 18,000, more than 19,000, or more than 20,000. In some aspects, the average number of ASO molecules per Scaffold-X exosome is between about 5000 and about 6,000, about 6,000 and about 7,000, about 7,000 and about 8,000, about 8,000 and about 9,000, about 9,000 and about 10,000, about 10,000 and about 11,000, about 11,000 and about 12,000 about 12,000 and about 13,000, about 13,000 and about 14,000, about 14,000 and about 15,000, about 15,000 and about 16,000, about 16,000 and about 17,000, about 17,000 and about 18,000, about 18,000 and about 19,000, or about 19,0000 and about 20,000

[00219] The present disclosure also provides an exosome comprising an antisense oligonucleotide [ASO] covalently linked to the exosome via an anchoring moiety [AM] according to the formula:

$$[AM]\text{-}L_1\text{-}[SP_1]\text{-}L_2\text{-}[SP_2]\text{-}L_3\text{-}[BAM]$$

wherein [AM] is cholesterol-TEG; L_1 is a phosphodiesterase cleavable bond; SP_1 is C3; L_2 is a phosphorothioate non-cleavable bond; SP_2 is TEG; and, L_3 is a phosphorothioate non-cleavable bond. When the exosome is a native exosome the average number of ASO molecules per exosome is at least 4500, for example, between about 4500 and about 5000 (e.g., about 4780), and the loading efficiency is at least about 70%, for example, between about 70% and about 90% (e.g., about 80%). When the exosome is an exosome overexpressing PTGFRN, the average number of ASO molecules per exosome is at least about 1500, for example, between about 1500

and about 2000 (e.g., about 1659) and the loading efficiency is at least about 20%, for example, between about 20% and about 35% (e.g., about 28%).

[00220] The present disclosure also provides an exosome comprising an antisense oligonucleotide [ASO] covalently linked to the exosome via an anchoring moiety [AM] according to the formula:

$$[AM]\text{-}L_1\text{-}[SP_1]\text{-}L_2\text{-}[BAM]$$

wherein [AM] is cholesterol-TEG; L_1 is a phosphodiesterase cleavable bond; SP_1 is TEG; and L_2 is a phosphorothioate non-cleavable bond. When the exosome is a native exosome, the average number of ASO molecules per exosome is at least about 3500, for example, between about 3500 and about 4500 (e.g., about 4090), and the loading efficiency is at least about 60%, for example, between about 60% and about 70% (e.g., about 68%). When the exosome is an exosome overexpressing PTGFRN, the average number of ASO molecules per exosome is at least about 1400, for example, between about 1400 and about 2400 (e.g., about 1890) and the loading efficiency is at least about 20%, for example, between about 20% and about 40% (e.g., about 31%).

[00221] Specific optimized linker combinations of the present disclosure are presented, e.g., in **FIG. 4**, **FIG. 5**, **FIG. 9A**, **FIG. 11**, and **FIG. 13**.

[00222] In some aspects the present disclosure, provides an exosome comprising a biologically active molecule [BAM] covalently linked to the exosome via an anchoring moiety [AM] according to the formula: [AM]-[SP1]-L1-[SP2]-[BAM], wherein [AM] is a lipid anchor, [SP1] is a first spacer (proximal with respect to the exosome surface), [SP2] is a second spacer (distal with respect to the exosome surface), [BAM] is a biologically active molecule disclosed herein, and [SP1] and [SP2] are linked by a cleavable linker. In some aspects, the cleavable linker is a phosphodiester cleavable linker. In some aspects, the lipid is selected from the group consisting of palmitic acid, cholesterol, tocopherol, and 16:0 Cyanur (cyanuric chloride) ethanolamine (PE). In some aspects, Spacer 1 is selected from the group consisting of C3, C6, C8, TEG, and None. In some aspects, Spacer 2 is selected from the group consisting of C3, TEG, HEG and None. None indicates that there is no Spacer 1 or Spacer 2 in the construct.

[00223] In some aspects the present disclosure, provides an exosome comprising an antisense oligonucleotide [ASO] covalently linked to the exosome via an anchoring moiety [AM] according to the formula: [AM]-[SP1]-L1-[SP2]-[ASO], wherein [AM] is a lipid anchor, [SP1] is a first spacer (proximal with respect to the exosome surface), [SP2] is a second spacer (distal with respect to the exosome surface), [ASO] is an antisense oligonucleotide, and [SP1] and [SP2] are linked by a cleavable linker. In some aspects, the cleavable linker is a phosphodiester

cleavable linker. In some aspects, the lipid is selected from the group consisting of palmitic acid, cholesterol, tocopherol, and 16:0 Cyanur (cyanuric chloride) PE. In some aspects, Spacer 1 is selected from the group consisting oa C3, C6, C8, TEG, and None. In some aspects, Spacer 2 is selected from the group consisting of C3, TEG, HEG and None.

[00224] In some aspects, the present disclosure provides a membrane anchoring construct according to the formula [AM]-[SP1]-L1-[SP2]-, wherein [AM] is a lipid anchor, [SP1] is a first spacer (proximal with respect to the membrane of a vesicle, e.g., an exosome), [SP2] is a second spacer (distal with respect to the membrane of a vesicle, e.g., an exosome), and [SP1] and [SP2] are linked by a cleavable linker. In some aspects, the cleavable linker is a phosphodiester cleavable linker. In some aspects, the lipid is selected from the group consisting of palmitic acid, cholesterol, tocopherol, and 16:0 Cyanur (cyanuric chloride) PE. In some aspects, Spacer 1 is selected from the group consisting oa C3, C6, C8, TEG, and None. In some aspects, Spacer 2 is selected from the group consisting of C3, TEG, HEG and None.

[00225] In some aspects, the present disclosure provides a construct selected from the group consisting of tocopherol-C8-TEG-[ASO], tocopherol-TEG-None-[ASO], cholesterol-TEG-C3-[ASO], cholesterol-C6-C3-[ASO], cholesterol-TEG-HEG-[ASO], cholesterol-TEG-None-[ASO], and palmitate-C6-HEG-[ASO].

[00226] In some aspects, the present disclosure provides an exosome comprising a membrane-anchored construct selected from the group consisting of tocopherol-C8-TEG-[ASO], tocopherol-TEG-None-[ASO], cholesterol-TEG-C3-[ASO], cholesterol-C6-C3-[ASO], cholesterol-TEG-HEG-[ASO], cholesterol-TEG-None-[ASO], and palmitate-C6-HEG-[ASO].

[00227] In some aspects, the present disclosure provides a membrane anchoring construct selected from the group consisting of tocopherol-C8-TEG, tocopherol-TEG-None, cholesterol-TEG-C3, cholesterol-C6-C3, cholesterol-TEG-HEG, cholesterol-TEG-None, and palmitate-C6-HEG. In some aspects, the membrane anchoring construct comprises, consists, or consists essentially of tocopherol-C8-TEG. In some aspects, the membrane anchoring construct comprises, consists, or consists essentially of tocopherol-TEG-None. In some aspects, the membrane anchoring construct comprises, consists, or consists essentially of cholesterol-TEG-C3. In some aspects, the membrane anchoring construct comprises, consists, or consists essentially of cholesterol-C6-C3. In some aspects, the membrane anchoring construct comprises, consists, or consists essentially of cholesterol-TEG-HEG. In some aspects, the membrane anchoring construct comprises, consists, or consists essentially of cholesterol-TEG-None. In some aspects, the membrane anchoring construct comprises, consists, or consists essentially of palmitate-C6-HEG.

[00228] In some aspects, the present disclosure provides an exosome comprising a biologically active molecule covalently linked to the surface of the exosome via a membrane anchoring construct selected from the group consisting of tocopherol-C8-TEG, tocopherol-TEG-None, cholesterol-TEG-C3, cholesterol-C6-C3, cholesterol-TEG-HEG, cholesterol-TEG-None, and palmitate-C6-HEG. In some aspects, the membrane anchoring construct comprises, consists, or consists essentially of tocopherol-C8-TEG. In some aspects, the membrane anchoring construct comprises, consists, or consists essentially of tocopherol-TEG-None. In some aspects, the membrane anchoring construct comprises, consists, or consists essentially of cholesterol-TEG-C3. In some aspects, the membrane anchoring construct comprises, consists, or consists essentially of cholesterol-C6-C3. In some aspects, the membrane anchoring construct comprises, consists, or consists essentially of cholesterol-TEG-HEG. In some aspects, the membrane anchoring construct comprises, consists, or consists essentially of cholesterol-TEG-None. In some aspects, the membrane anchoring construct comprises, consists, or consists essentially of palmitate-C6-HEG.

[00229] In some aspects, the exosomes disclosed herein can be prepared and/or stored under conditions that preserve the stability of the exosome and/or promote higher load density. In some aspects, an exosome comprising an ASO attached to its surface via a membrane anchoring construct selected from the group consisting of tocopherol-C8-TEG, tocopherol-TEG-None, cholesterol-TEG-C3, cholesterol-C6-C3, cholesterol-TEG-HEG, cholesterol-TEG-None, and palmitate-C6-HEG, can be maintained in a low salt buffer (e.g., comprising about 50 mM NaCl) for about 2, 4, 6 or 8 days. In some aspects, an exosome comprising an ASO attached to its surface via a membrane anchoring construct selected from the group consisting of tocopherol-C8-TEG, tocopherol-TEG-None, cholesterol-TEG-C3, cholesterol-C6-C3, cholesterol-TEG-HEG, cholesterol-TEG-None, and palmitate-C6-HEG, can be maintained in a high salt buffer (e.g., comprising about 150 mM NaCl) for about 2, 4, 6 or 8 days. In some aspects, an exosome comprising an ASO attached to its surface via a membrane anchoring construct selected from the group consisting of tocopherol-C8-TEG, tocopherol-TEG-None, cholesterol-TEG-C3, cholesterol-C6-C3, cholesterol-TEG-HEG, cholesterol-TEG-None, and palmitate-C6-HEG, can be maintained in a high salt buffer (e.g., comprising about 150 mM NaCl) further comprising sucrose for about 2, 4, 6 or 8 days. In some aspects, an exosome comprising an ASO attached to its surface via a membrane anchoring construct selected from the group consisting of tocopherol-C8-TEG, tocopherol-TEG-None, cholesterol-TEG-C3, cholesterol-C6-C3, cholesterol-TEG-HEG, cholesterol-TEG-None, and palmitate-C6-HEG, can be maintained in a low salt buffer,

high salt buffer, or high salt buffer (e.g., comprising about 150 mM NaCl) further comprising sucrose for about 2, 4, 6 or 8 days, at either 4°C or 25°C.

[00230] The present disclosure also provides a method to increase the loading density of an exosome comprising loading the exosome under high salt conditions (e.g., using a high salt buffer comprising about 150 mM NaCl). In some aspects, the exosome is loaded with an ASO attached (e.g., covalently attached) to a membrane anchoring construct selected, for example, from the group consisting of tocopherol-C8-TEG, tocopherol-TEG-None, cholesterol-TEG-C3, cholesterol-C6-C3, cholesterol-TEG-HEG, cholesterol-TEG-None, and palmitate-C6-HEG.

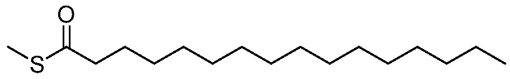
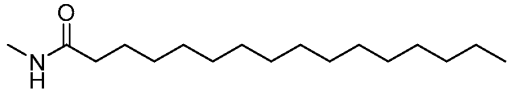
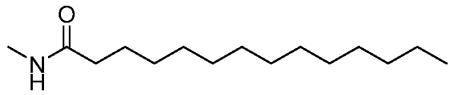
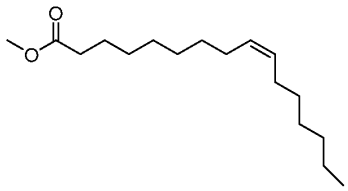
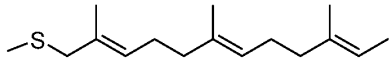
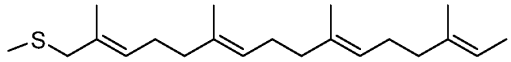
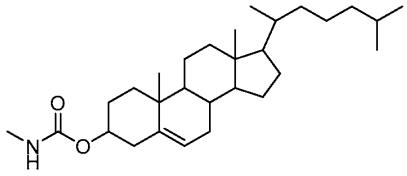
Anchoring moieties [AM]

[00231] The constructs disclosed herein, e.g., constructs of Formula 1 comprise at least one anchoring moiety [AM] that anchor at least one biologically active molecule [BAM] to the surface of an EV, e.g., an exosome, via an optimized liner of the present disclosure. Suitable anchoring moieties [AM] capable of anchoring a biologically active molecule [BAM] to the surface of an EV, e.g., an exosome, comprise for example sterols (e.g., cholesterol), lipids, phospholipids, lysophospholipids, fatty acids, fat-soluble vitamins, scaffolding moieties (e.g., Protein X), or combinations thereof as described in detail below.

[00232] In some aspects, the anchoring moiety [AM] comprises or consists of a lipid. A lipid anchoring moiety [AM] can comprise any lipid known in the art, *e.g.*, palmitic acid or glycosylphosphatidylinositols. In some aspects, the lipid, is a fatty acid, phosphatide, phospholipid (e.g., phosphatidyl choline, phosphatidyl serine, or phosphatidyl ethanolamine), or analogue thereof (e.g. phosphatidylcholine, lecithin, phosphatidylethanolamine, cephalin, or phosphatidylserine or analogue or portion thereof, such as a partially hydrolyzed portion thereof).

[00233] Generally, anchoring moieties are chemically attached, e.g., via solid phase synthesis. However, an anchoring moiety [AM] can be attached to a biologically active molecule [BAM] enzymatically. The anchoring moiety [AM] can be conjugated to a biologically active molecule [BAM] directly or indirectly via a linker or spacer combination, at any chemically feasible location, e.g., at the 5′ and/or 3′ end of a nucleotide sequence, e.g., an ASO. In one aspect, the anchoring moiety [AM] is conjugated only to the 3′ end of the biologically active molecule [BAM], directly or indirectly via an optimized linker disclosed herein. In one aspect, the anchoring moiety [AM] is conjugated only to the 5′ end of a nucleotide sequence, e.g., an ASO. In one aspect, the anchoring moiety [AM] is conjugated at a location which is not the 3′ end or 5’ end of a nucleotide sequence, e.g., an ASO.

[00234] In some aspects, anchoring moieties [AM] of the present disclosure can comprise any of the hydrophobic group modifications disclosed below:

Modification	*Modifying Group*
S-Palmitoylation	
N-Palmitoylation	
N-Myristoylation	
O-Acylation	
Farnesylation	
Geranylgeranylation	
Cholesterol	

[00235] In some aspects, an anchoring moiety of the present disclosure can comprise two or more types of anchoring moieties disclosed herein. For example, in some aspects, an anchoring moiety can comprise two lipids, e.g., a phospholipids and a fatty acid, or two phospholipids, or two fatty acids, or a lipid and a vitamin, or cholesterol and a vitamin, etc. which taken together have 6-30 carbon atoms (i.e., an equivalent carbon number (ECN) of 6-30).

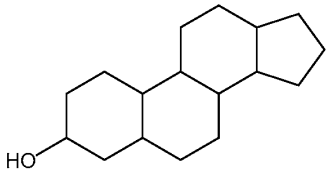
Anchoring moieties: Cholesterol and other sterols

[00236] In some aspects, the anchoring moiety [AM] comprises a sterol, steroid, hopanoid, hydroxysteroid, secosteroid, or analog thereof with lipophilic properties. In some aspects, the anchoring moiety comprises a sterol, such as a phytosterol, mycosterol, or zoosterol. Exemplary zoosterols include cholesterol and 24S-hydroxycholesterol; exemplary phytosterols include ergosterol (mycosterol), campesterol, sitosterol, and stigmasterol. In some aspects, the sterol is selected from ergosterol, 7-dehydrocholesterol, cholesterol, 24S-hydroxycholesterol, lanosterol, cycloartenol, fucosterol, saringosterol, campesterol, β-sitosterol, sitostanol, coprostanol,

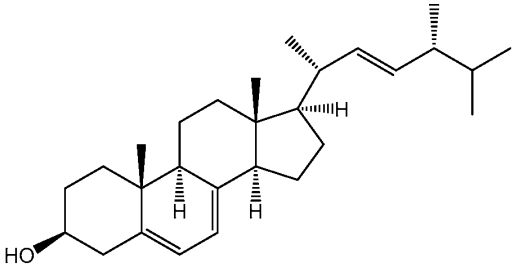
avenasterol, or stigmasterol. Sterols may be found either as free sterols, acylated (sterol esters), alkylated (steryl alkyl ethers), sulfated (sterol sulfate), or linked to a glycoside moiety (steryl glycosides), which can be itself acylated (acylated sterol glycosides).

[00237] In some aspects, the anchoring moiety [AM] comprises a steroid. In some aspects, the steroid is selected from dihydrotestosterone, uvaol, hecigenin, diosgenin, progesterone, or cortisol.

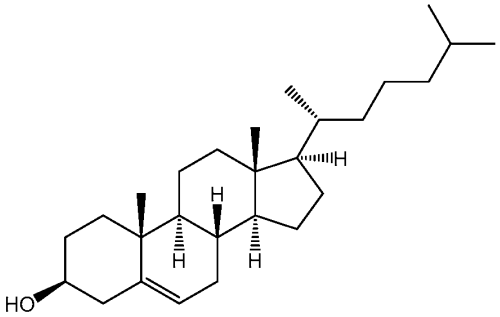
[00238] For example, sterols may be attached (via solid phase synthesis or conjugation), e.g., to a spacer [SP] via the available —OH group of the sterol. Exemplary sterols have the general skeleton shown below:



[00239] As a further example, ergosterol has the structure below:



[00240] Cholesterol has the structure below:



[00241] In some aspects, the sterol or steroid is a phosphoramidite. In some aspects, the sterol or steroid is a phosphoramidite comprising a linker, e.g., an alkyl (C3, C6 or C8) or a glycol linker (e.g., TEG). In some aspects, the sterol/steroid-phosphoramidite, or sterol/steroid-linker-phosphoramidite is attached to a spacer [SP] or to a biologically active molecule [BAM] via solid phase synthesis, for example, via a cleavable (e.g., phosphodiester) or non-cleavable (e.g., phosphorothioate) linkage.

Anchoring moieties: Fatty acids

[00242] In some aspects, the anchoring moiety [AM] comprises a fatty acid. In some aspects, the fatty acid is a short-chain, medium-chain, or long-chain fatty acid. In some aspects, the fatty acid is a saturated fatty acid. In some aspects, the fatty acid is an unsaturated fatty acid. In some aspects, the fatty acid is a monounsaturated fatty acid. In some aspects, the fatty acid is a polyunsaturated fatty acid, such as an ω-3 (omega-3) or ω-6 (omega-6) fatty acid.

[00243] In some aspects, the lipid, e.g., fatty acid, has a C_2-C_{18} chain. In some aspects, the lipid, e.g., fatty acid, has a C_2, C_3, C_4, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, C_{17}, or C_{18} chain. In some aspects, the fatty acid, has a C_2 chain. In some aspects, the fatty acid, has a C_3 chain. In some aspects, the fatty acid, has a C_4 chain. In some aspects, the fatty acid, has a C_5 chain. In some aspects, the fatty acid, has a C_6 chain. In some aspects, the fatty acid, has a C_7 chain. In some aspects, the fatty acid, has a C_8 chain. In some aspects, the fatty acid, has a C_9 chain. In some aspects, the fatty acid, has a C_{10} chain. In some aspects, the fatty acid, has a C_{11} chain. In some aspects, the fatty acid, has a C_{12} chain. In some aspects, the fatty acid, has a C_{13} chain. In some aspects, the fatty acid, has a C_{14} chain. In some aspects, the fatty acid, has a C_{15} chain. In some aspects, the fatty acid, has a C_{16} chain. In some aspects, the fatty acid, has a C_{17} chain. In some aspects, the fatty acid, has a C_{18} chain.

[00244] In some aspects, the fatty acid, has a C_4-C_{18} chain. In some aspects, the fatty acid, has a C_2-C_3, C_2-C_4, C_2-C_5, C_2-C_6, C_2-C_7, C_2-C_8, C_2-C_9, C_2-C_{10}, C_2-C_{11}, C_2-C_{12}, C_2-C_{13}, C_2-C_{14}, C_2-C_{15}, C_2-C_{16}, C_2-C_{17}, C_2-C_{18}, C_3-C_4, C_3-C_5, C_3-C_6, C_3-C_7, C_3-C_8, C_3-C_9, C_3-C_{10}, C_3-C_{11}, C_3-C_{12}, C_3-C_{13}, C_3-C_{14}, C_3-C_{15}, C_3-C_{16}, C_3-C_{17}, C_3-C_{18}, C_4-C_5, C_4-C_6, C_4-C_7, C_4-C_8, C_4-C_9, C_4-C_{10}, C_4-C_{11}, C_4-C_{12}, C_4-C_{13}, C_4-C_{14}, C_4-C_{15}, C_4-C_{16}, C_4-C_{17}, C_4-C_{18}, C_5-C_6, C_5-C_7, C_5-C_8, C_5-C_9, C_5-C_{10}, C_5-C_{11}, C_5-C_{12}, C_5-C_{13}, C_5-C_{14}, C_5-C_{15}, C_5-C_{16}, C_5-C_{17}, C_5-C_{18}, C_6-C_7, C_6-C_8, C_6-C_9, C_6-C_{10}, C_6-C_{11}, C_6-C_{12}, C_6-C_{13}, C_6-C_{14}, C_6-C_{15}, C_6-C_{16}, C_6-C_{17}, C_6-C_{18}, C_7-C_8, C_7-C_9, C_7-C_{10}, C_7-C_{11}, C_7-C_{12}, C_7-C_{13}, C_7-C_{14}, C_7-C_{15}, C_7-C_{16}, C_7-C_{17}, C_7-C_{18}, C_8-C_9, C_8-C_{10}, C_8-C_{11}, C_8-C_{12}, C_8-C_{13}, C_8-C_{14}, C_8-C_{15}, C_8-C_{16}, C_8-C_{17}, C_8-C_{18}, C_9-C_{10}, C_9-C_{11}, C_9-C_{12}, C_9-C_{13}, C_9-C_{14}, C_9-C_{15}, C_9-C_{16}, C_9-C_{17}, C_9-C_{18}, C_{10}-C_{11}, C_{10}-C_{12}, C_{10}-C_{13}, C_{10}-C_{14}, C_{10}-C_{15}, C_{10}-C_{16}, C_{10}-C_{17}, C_{10}-C_{18}, C_{11}-C_{12}, C_{11}-C_{13}, C_{11}-C_{14}, C_{11}-C_{15}, C_{11}-C_{16}, C_{11}-C_{17}, C_{11}-C_{18}, C_{12}-C_{13}, C_{12}-C_{14}, C_{12}-C_{15}, C_{12}-C_{16}, C_{12}-C_{17}, C_{12}-C_{18}, C_{13}-C_{14}, C_{13}-C_{15}, C_{13}-C_{16}, C_{13}-C_{17}, C_{13}-C_{18}, C_{14}-C_{15}, C_{14}-C_{16}, C_{14}-C_{17}, C_{14}-C_{18}, C_{15}-C_{16}, C_{15}-C_{17}, C_{15}-C_{18}, C_{16}-C_{17}, C_{16}-C_{18}, or C_{17}-C_{18} chain.

[00245] In some aspects, the anchoring moiety [AM] comprises two fatty acids, each of which is independently selected from a fatty acid having a chain with any one of the foregoing ranges or numbers of carbon atoms. In some aspects, one of the fatty acids is independently a

fatty acid with a C_2-C_3, C_2-C_4, C_2-C_5, C_2-C_6, C_2-C_7, C_2-C_8, C_2-C_9, C_2-C_{10}, C_2-C_{11}, C_2-C_{12}, C_2-C_{13}, C_2-C_{14}, C_2-C_{15}, C_2-C_{16}, C_2-C_{17}, C_2-C_{18}, C_3-C_4, C_3-C_5, C_3-C_6, C_3-C_7, C_3-C_8, C_3-C_9, C_3-C_{10}, C_3-C_{11}, C_3-C_{12}, C_3-C_{13}, C_3-C_{14}, C_3-C_{15}, C_3-C_{16}, C_3-C_{17}, C_3-C_{18}, C_4-C_5, C_4-C_6, C_4-C_7, C_4-C_8, C_4-C_9, C_4-C_{10}, C_4-C_{11}, C_4-C_{12}, C_4-C_{13}, C_4-C_{14}, C_4-C_{15}, C_4-C_{16}, C_4-C_{17}, C_4-C_{18}, C_5-C_6, C_5-C_7, C_5-C_8, C_5-C_9, C_5-C_{10}, C_5-C_{11}, C_5-C_{12}, C_5-C_{13}, C_5-C_{14}, C_5-C_{15}, C_5-C_{16}, C_5-C_{17}, C_5-C_{18}, C_6-C_7, C_6-C_8, C_6-C_9, C_6-C_{10}, C_6-C_{11}, C_6-C_{12}, C_6-C_{13}, C_6-C_{14}, C_6-C_{15}, C_6-C_{16}, C_6-C_{17}, C_6-C_{18}, C_7-C_8, C_7-C_9, C_7-C_{10}, C_7-C_{11}, C_7-C_{12}, C_7-C_{13}, C_7-C_{14}, C_7-C_{15}, C_7-C_{16}, C_7-C_{17}, C_7-C_{18}, C_8-C_9, C_8-C_{10}, C_8-C_{11}, C_8-C_{12}, C_8-C_{13}, C_8-C_{14}, C_8-C_{15}, C_8-C_{16}, C_8-C_{17}, C_8-C_{18}, C_9-C_{10}, C_9-C_{11}, C_9-C_{12}, C_9-C_{13}, C_9-C_{14}, C_9-C_{15}, C_9-C_{16}, C_9-C_{17}, C_9-C_{18}, C_{10}-C_{11}, C_{10}-C_{12}, C_{10}-C_{13}, C_{10}-C_{14}, C_{10}-C_{15}, C_{10}-C_{16}, C_{10}-C_{17}, C_{10}-C_{18}, C_{11}-C_{12}, C_{11}-C_{13}, C_{11}-C_{14}, C_{11}-C_{15}, C_{11}-C_{16}, C_{11}-C_{17}, C_{11}-C_{18}, C_{12}-C_{13}, C_{12}-C_{14}, C_{12}-C_{15}, C_{12}-C_{16}, C_{12}-C_{17}, C_{12}-C_{18}, C_{13}-C_{14}, C_{13}-C_{15}, C_{13}-C_{16}, C_{13}-C_{17}, C_{13}-C_{18}, C_{14}-C_{15}, C_{14}-C_{16}, C_{14}-C_{17}, C_{14}-C_{18}, C_{15}-C_{16}, C_{15}-C_{17}, C_{15}-C_{18}, C_{16}-C_{17}, C_{16}-C_{18}, or C_{17}-C_{18} chain and the other one is independently a fatty acid with a C_2-C_3, C_2-C_4, C_2-C_5, C_2-C_6, C_2-C_7, C_2-C_8, C_2-C_9, C_2-C_{10}, C_2-C_{11}, C_2-C_{12}, C_2-C_{13}, C_2-C_{14}, C_2-C_{15}, C_2-C_{16}, C_2-C_{17}, C_2-C_{18}, C_3-C_4, C_3-C_5, C_3-C_6, C_3-C_7, C_3-C_8, C_3-C_9, C_3-C_{10}, C_3-C_{11}, C_3-C_{12}, C_3-C_{13}, C_3-C_{14}, C_3-C_{15}, C_3-C_{16}, C_3-C_{17}, C_3-C_{18}, C_4-C_5, C_4-C_6, C_4-C_7, C_4-C_8, C_4-C_9, C_4-C_{10}, C_4-C_{11}, C_4-C_{12}, C_4-C_{13}, C_4-C_{14}, C_4-C_{15}, C_4-C_{16}, C_4-C_{17}, C_4-C_{18}, C_5-C_6, C_5-C_7, C_5-C_8, C_5-C_9, C_5-C_{10}, C_5-C_{11}, C_5-C_{12}, C_5-C_{13}, C_5-C_{14}, C_5-C_{15}, C_5-C_{16}, C_5-C_{17}, C_5-C_{18}, C_6-C_7, C_6-C_8, C_6-C_9, C_6-C_{10}, C_6-C_{11}, C_6-C_{12}, C_6-C_{13}, C_6-C_{14}, C_6-C_{15}, C_6-C_{16}, C_6-C_{17}, C_6-C_{18}, C_7-C_8, C_7-C_9, C_7-C_{10}, C_7-C_{11}, C_7-C_{12}, C_7-C_{13}, C_7-C_{14}, C_7-C_{15}, C_7-C_{16}, C_7-C_{17}, C_7-C_{18}, C_8-C_9, C_8-C_{10}, C_8-C_{11}, C_8-C_{12}, C_8-C_{13}, C_8-C_{14}, C_8-C_{15}, C_8-C_{16}, C_8-C_{17}, C_8-C_{18}, C_9-C_{10}, C_9-C_{11}, C_9-C_{12}, C_9-C_{13}, C_9-C_{14}, C_9-C_{15}, C_9-C_{16}, C_9-C_{17}, C_9-C_{18}, C_{10}-C_{11}, C_{10}-C_{12}, C_{10}-C_{13}, C_{10}-C_{14}, C_{10}-C_{15}, C_{10}-C_{16}, C_{10}-C_{17}, C_{10}-C_{18}, C_{11}-C_{12}, C_{11}-C_{13}, C_{11}-C_{14}, C_{11}-C_{15}, C_{11}-C_{16}, C_{11}-C_{17}, C_{11}-C_{18}, C_{12}-C_{13}, C_{12}-C_{14}, C_{12}-C_{15}, C_{12}-C_{16}, C_{12}-C_{17}, C_{12}-C_{18}, C_{13}-C_{14}, C_{13}-C_{15}, C_{13}-C_{16}, C_{13}-C_{17}, C_{13}-C_{18}, C_{14}-C_{15}, C_{14}-C_{16}, C_{14}-C_{17}, C_{14}-C_{18}, C_{15}-C_{16}, C_{15}-C_{17}, C_{15}-C_{18}, C_{16}-C_{17}, C_{16}-C_{18}, or C_{17}-C_{18} chain. In some aspects, each fatty acid independently has a chain of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 or 18 carbon atoms.

[00246] Suitable fatty acids include saturated straight-chain fatty acids, saturated branched fatty acids, unsaturated fatty acids, hydroxy fatty acids, and polycarboxylic acids.

[00247] Examples of useful saturated straight-chain fatty acids include those having an even number of carbon atoms, such as butyric acid (C4), caproic acid (C6), caprylic acid (C8), capric acid (C10), lauric acid (C12), myristic acid (C14), palmitic acid (C16), or stearic acid (C18), and those having an odd number of carbon atoms, such as propionic acid (C3), n-valeric acid (C5), enanthic acid (C7), pelargonic acid (C9), hendecanoic acid (C11), tridecanoic acid (C13), pentadecanoic acid (C15), or heptadecanoic acid (C17).

[00248] Examples of suitable saturated branched fatty acids include isobutyric acid, isocaproic acid, isocaprylic acid, isocapric acid, isolauric acid, 11-methyldodecanoic acid, isomyristic acid, 13-methyl-tetradecanoic acid, isopalmitic acid, 15-methyl-hexadecanoic acid, or isostearic acid. Suitable saturated odd-carbon branched fatty acids include anteiso fatty acids terminating with an isobutyl group, such as 6-methyl-octanoic acid, 8-methyl-decanoic acid, 10-methyl-dodecanoic acid, 12-methyl-tetradecanoic acid, or 14-methyl-hexadecanoic acid.

[00249] Examples of suitable unsaturated fatty acids include 4-decenoic acid, caproleic acid, 4-dodecenoic acid, 5-dodecenoic acid, lauroleic acid, 4-tetradecenoic acid, 5-tetradecenoic acid, 9-tetradecenoic acid, palmitoleic acid, 6-octadecenoic acid, oleic acid, and the like.

[00250] Examples of suitable hydroxy fatty acids include α-hydroxylauric acid, α-hydroxymyristic acid, α-hydroxypalmitic acid, α-hydroxystearic acid, ω-hydroxylauric acid, α-hydroxyarachic acid, 9-hydroxy-12-octadecenoic acid, ricinoleic acid, 9-hydroxy-trans-10,12-octadecadienic acid, 9,10-dihydroxystearic acid, 12-hydroxystearic acid and the like.

[00251] Examples of suitable polycarboxylic acids include oxalic acid, malonic acid, succinic acid, glutaric acid, adipic acid, pimelic acid, suberic acid, azelaic acid, sebacic acid, D,L-malic acid, and the like.

[00252] In some aspects, each fatty acid is independently selected from propionic acid, butyric acid, valeric acid, caproic acid, enanthic acid, caprylic acid, pelargonic acid, capric acid, undecylic acid, lauric acid, tridecylic acid, myristic acid, pentadecylic acid, palmitic acid, margaric acid, or stearic acid.

[00253] In some aspects, each fatty acid is independently selected from α-linolenic acid, stearidonic acid, eicosapentaenoic acid, docosahexaenoic acid, linoleic acid, gamma-linoleic acid, dihomo-gamma-linoleic acid, arachidonic acid, docosatetraenoic acid, palmitoleic acid, vaccenic acid, paullinic acid, oleic acid, elaidic acid, bosseopentaenoic acid, sardine acid, or another monounsaturated or polyunsaturated fatty acid.

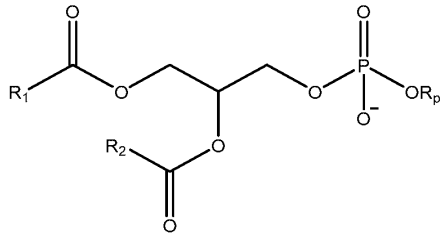
[00254] In some aspects, one or both of the fatty acids is an essential fatty acid. In view of the beneficial health effects of certain essential fatty acids, the therapeutic benefits of disclosed therapeutic-loaded exosomes may be increased by including such fatty acids in the therapeutic agent. In some aspects, the essential fatty acid is an n-6 or n-3 essential fatty acid selected from the group consisting of linolenic acid, gamma-linolenic acid, dihomo-gamma-linolenic acid, arachidonic acid, adrenic acid, docosapentaenoic n-6 acid, alpha-linolenic acid, or stearidonic acid.

[00255] Fatty acid chains differ greatly in the length of their chains and may be categorized according to chain length, e.g. as short to very long. Short-chain fatty acids (SCFA)

are fatty acids with chains of about five or less carbons (e.g. butyric acid). In some aspects, the fatty acid is a SCFA. Medium-chain fatty acids (MCFA) include fatty acids with chains of about 6-12 carbons, which can form medium-chain triglycerides. In some aspects, the fatty acid is a MCFA. Long-chain fatty acids (LCFA) include fatty acids with chains of 13-21 carbons. In some aspects, the fatty acid is a LCFA. In some aspects, the fatty acid is a LCFA.

Anchoring moieties: Phospholipids

[00256] In some aspects, the anchoring moiety [AM] comprises a phospholipid. Phospholipids are a class of lipids that are a major component of all cell membranes. They can form lipid bilayers because of their amphiphilic characteristic. The structure of the phospholipid molecule generally consists of two hydrophobic fatty acid "tails" and a hydrophilic "head" consisting of a phosphate group. For example, a phospholipid can be a lipid according to the following formula:



in which R_p represents a phospholipid moiety and R_1 and R_2 represent fatty acid moieties with or without unsaturation that may be the same or different.

[00257] A phospholipid moiety may be selected, for example, from the non-limiting group consisting of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl serine, phosphatidic acid, 2 lysophosphatidyl choline, and a sphingomyelin.

[00258] Particular phospholipids may facilitate fusion to a lipid bilayer, e.g., the lipid bilayer of an exosomal membrane. For example, a cationic phospholipid may interact with one or more negatively charged phospholipids of a membrane. Fusion of a phospholipid to a membrane may allow one or more elements of a lipid-containing composition to bind to the membrane or to pass through the membrane.

[00259] A fatty acid moiety may be selected, for example, from the non-limiting group consisting of lauric acid, myristic acid, myristoleic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, or linoleic acid.

[00260] The phospholipids using as anchoring moieties in the present disclosure can be natural or non-natural phospholipids. Non-natural phospholipid species including natural species

with modifications and substitutions including branching, oxidation, cyclization, and alkynes are also contemplated. For example, a phospholipid may be functionalized with or cross-linked to one or more alkynes (e.g., an alkenyl group in which one or more double bonds is replaced with a triple bond). Under appropriate reaction conditions, an alkyne group may undergo a copper-catalyzed cycloaddition upon exposure to an azide.

[00261] Phospholipids include, but are not limited to, glycerophospholipids such as phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines, phosphatidylinositols, phosphatidy glycerols, and phosphatidic acids. Examples of phospholipids that can be used in the anchoring moieties disclosed herein include phosphatidylethanolamines (e.g., dilauroylphosphatidyl ethanolamine, dimyristoylphosphatidyl ethanolamine, dipalmitoylphosphatidyl ethanolamine, distearoylphosphatidyl ethanolamine, dioleoylphosphatidyl ethanolamine, 1-palmitoyl-2-oleylphosphatidyl ethanolamine, 1-oleyl-2-palmitoylphosphatidyl ethanolamine, and dierucoylphosphatidyl ethanolamine), phosphatidyl glycerols (e.g., dilauroylphosphatidyl glycerol, dimyristoylphosphatidyl glycerol, dipalmitoylphosphatidyl glycerol, distearoylphosphatidyl glycerol, dioleoylphosphatidyl glycerol, 1-palmitoyl-2-oleyl-phosphatidyl glycerol, 1-oleyl-2-palmitoyl-phosphatidyl glycerol, and dierucoylphosphatidyl glycerol); phosphatidyl serines (e.g., such as dilauroylphosphatidyl serine, dimyristoylphosphatidyl serine, dipalmitoylphosphatidyl serine, distearoylphosphatidyl serine, dioleoylphosphatidyl serine, 1-palmitoyl-2-oleyl-phosphatidyl serine, 1-oleyl-2-palmitoyl-phosphatidyl serine, and dierucoylphosphatidyl serine); phosphatidic acids (e.g., dilauroylphosphatidic acid, dimyristoylphosphatidic acid, dipalmitoylphosphatidic acid, distearoylphosphatidic acid, dioleoylphosphatidic acid, 1-palmitoyl-2-oleylphosphatidic acid, 1-oleyl-2-palmitoyl-phosphatidic acid, and dierucoylphosphatidic acid); and phosphatidyl inositols (e.g., dilauroylphosphatidyl inositol, dimyristoylphosphatidyl inositol, dipalmitoylphosphatidyl inositol, distearoylphosphatidyl inositol, dioleoylphosphatidyl inositol, 1-palmitoyl-2-oleyl-phosphatidyl inositol, 1-oleyl-2-palmitoyl-phosphatidyl inositol, and dierucoylphosphatidyl inositol.

[00262] Phospholipids may be of a symmetric or an asymmetric type. As used herein, the term "symmetric phospholipid" includes glycerophospholipids having matching fatty acid moieties and sphingolipids in which the variable fatty acid moiety and the hydrocarbon chain of the sphingosine backbone include a comparable number of carbon atoms. As used herein, the term "asymmetric phospholipid" includes lysolipids, glycerophospholipids having different fatty acid moieties (e.g., fatty acid moieties with different numbers of carbon atoms and/or unsaturations (e.g., double bonds)), and sphingolipids in which the variable fatty acid moiety and

the hydrocarbon chain of the sphingosine backbone include a dissimilar number of carbon atoms (e.g., the variable fatty acid moiety include at least two more carbon atoms than the hydrocarbon chain or at least two fewer carbon atoms than the hydrocarbon chain).

[00263] In some aspects, the anchoring moiety [AM] comprises phospholipid, e.g., a symmetric phospholipid, with 10 carbons (C10), twelve carbons (C12), fourteen carbons (C14), sixteen carbons (C16) or eighteen carbons (C18). In some aspects, the anchoring moiety [AM] comprises a symmetric phospholipid with fourteen carbons (C14). In some aspects, the anchoring moiety [AM] comprises a symmetric phospholipid with sixteen carbons (C16). In some aspects, the anchoring moiety [AM] comprises a symmetric phospholipid with eighteen carbons (C18). In some aspects, the phospholipid in phosphatidyl ethanolamine (PE). Accordingly, in some aspects, the anchoring moiety [AM] comprises a C14 PE. In some aspects, the anchoring moiety [AM] comprises a C16 PE. In some aspects, the anchoring moiety [AM] comprises a C18 PE. In some aspects, the acyl chains of the PE contain no insaturations. Accordingly, in some aspects, the anchoring moiety [AM] comprises a C14:0 PE, a C16:0 PE, or a C18:0 PE.

[00264] In some aspects, the anchoring moiety [AM] comprises a phospholipid comprising a cyanuric acid (cyanur) group. In some aspects, the phospholipid comprising a cyanuric acid group is a PE. In some aspects, the phospholipid comprising a cyanuric acid group is a C14:0 PE, a C16:0 PE, or a C18:0 PE. In a particular aspect, the phospholipid comprising a cyanuric acid group is a C16:0 Cyanur PE as shown in **FIG. 11**.

[00265] In some aspects, the anchoring moiety [AM] comprises at least one symmetric phospholipid. Symmetric phospholipids may be selected from the non-limiting group consisting of 1,2 dipropionyl sn-glycero 3 phosphocholine (03:0 PC), 1,2 dibutyryl sn glycero 3 phosphocholine (04:0 PC), 1,2 dipentanoyl sn glycero 3 phosphocholine (05:0 PC), 1,2 dihexanoyl sn glycero 3 phosphocholine (06:0 PC), 1,2 diheptanoyl sn glycero 3 phosphocholine (07:0 PC), 1,2 dioctanoyl sn glycero 3 phosphocholine (08:0 PC), 1,2 dinonanoyl sn glycero 3 phosphocholine (09:0 PC), 1,2 didecanoyl sn glycero 3 phosphocholine (10:0 PC), 1,2 diundecanoyl sn glycero 3 phosphocholine (11:0 PC, DUPC), 1,2 dilauroyl sn glycero 3 phosphocholine (12:0 PC), 1,2 ditridecanoyl sn glycero 3 phosphocholine (13:0 PC), 1,2 dimyristoyl sn glycero 3 phosphocholine (14:0 PC, DMPC), 1,2 dipentadecanoyl sn glycero 3 phosphocholine (15:0 PC), 1,2 dipalmitoyl sn glycero 3 phosphocholine (16:0 PC, DPPC), 1,2 diphytanoyl sn glycero 3 phosphocholine (4ME 16:0 PC), 1,2 diheptadecanoyl sn glycero 3 phosphocholine (17:0 PC), 1,2 distearoyl sn glycero 3 phosphocholine (18:0 PC, DSPC), 1,2 dimyristelaidoyl sn glycero 3 phosphocholine (14:1 (Δ9-Trans) PC), 1,2 dipalmitoleoyl sn glycero 3 phosphocholine (16:1 (Δ9-Cis) PC), 1,2 dipalmitelaidoyl sn glycero 3 phosphocholine

(16:1 (Δ9-Trans) PC), 1,2 dipetroselenoyl sn glycero 3 phosphocholine (18:1 (Δ6-Cis) PC), 1,2 dioleoyl sn glycero 3 phosphocholine (18:1 (Δ9-Cis) PC, DOPC), 1,2 dielaidoyl sn glycero 3 phosphocholine (18:1 (Δ9-Trans) PC), 1,2 dilinoleoyl sn glycero 3 phosphocholine (18:2 (Cis) PC, DLPC), 1,2 dilinolenoyl sn glycero 3 phosphocholine (18:3 (Cis) PC, DLnPC), 1,2 dihexanoyl sn glycero 3 phosphoethanolamine (06:0 PE), 1,2 dioctanoyl sn glycero 3 phosphoethanolamine (08:0 PE), 1,2 didecanoyl sn glycero 3 phosphoethanolamine (10:0 PE), 1,2 dilauroyl sn glycero 3 phosphoethanolamine (12:0 PE), 1,2 dimyristoyl sn glycero 3 phosphoethanolamine (14:0 PE), 1,2 dipentadecanoyl sn glycero 3 phosphoethanolamine (15:0 PE), 1,2 dipalmitoyl sn glycero 3 phosphoethanolamine (16:0 PE), 1,2 diphytanoyl sn glycero 3 phosphoethanolamine (4ME 16:0 PE), 1,2 diheptadecanoyl sn glycero 3 phosphoethanolamine (17:0 PE), 1,2 distearoyl sn glycero 3 phosphoethanolamine (18:0 PE, DSPE), 1,2 dipalmitoleoyl sn glycero 3 phosphoethanolamine (16:1 PE), 1,2 dioleoyl sn glycero 3 phosphoethanolamine (18:1 (Δ9-Cis) PE, DOPE), 1,2 dielaidoyl sn glycero 3 phosphoethanolamine (18:1 (Δ9-Trans) PE), 1,2 dilinoleoyl sn glycero 3 phosphoethanolamine (18:2 PE, DLPE), 1,2 dilinolenoyl sn glycero 3 phosphoethanolamine (18:3 PE, DLnPE), 1,2 di O octadecenyl sn glycero 3 phosphocholine (18:0 Diether PC), 1,2 dioleoyl sn glycero 3 phospho rac (1 glycerol) sodium salt (DOPG), and any combination thereof.

[00266] In some aspects, the anchoring moiety [AM] comprises at least one symmetric phospholipid selected from the non-limiting group consisting of DLPC, DMPC, DOPC, DPPC, DSPC, DUPC, 18:0 Diether PC, DLnPC, DAPC, DHAPC, DOPE, 4ME 16:0 PE, DSPE, DLPE,DLnPE, DAPE, DHAPE, DOPG, and any combination thereof.

[00267] In some aspects, the anchoring moiety [AM] comprises at least one asymmetric phospholipid. Asymmetric phospholipids may be selected from the non-limiting group consisting of 1 myristoyl 2 palmitoyl sn glycero 3 phosphocholine (14:0-16:0 PC, MPPC), 1 myristoyl 2 stearoyl sn glycero 3 phosphocholine (14:0-18:0 PC, MSPC), 1 palmitoyl 2 acetyl sn glycero 3 phosphocholine (16:0-02:0 PC), 1 palmitoyl 2 myristoyl sn glycero 3 phosphocholine (16:0-14:0 PC, PMPC), 1 palmitoyl 2 stearoyl sn glycero 3 phosphocholine (16:0-18:0 PC, PSPC), 1 palmitoyl 2 oleoyl sn glycero 3 phosphocholine (16:0-18:1 PC, POPC), 1 palmitoyl 2 linoleoyl sn glycero 3 phosphocholine (16:0-18:2 PC, PLPC), 1 palmitoyl 2 arachidonoyl sn glycero 3 phosphocholine (16:0-20:4 PC), 1 palmitoyl 2 docosahexaenoyl sn glycero 3 phosphocholine (14:0-22:6 PC), 1 stearoyl 2 myristoyl sn glycero 3 phosphocholine (18:0-14:0 PC, SMPC), 1 stearoyl 2 palmitoyl sn glycero 3 phosphocholine (18:0-16:0 PC, SPPC), 1 stearoyl 2 oleoyl sn glycero 3 phosphocholine (18:0-18:1 PC, SOPC), 1 stearoyl 2 linoleoyl sn glycero 3 phosphocholine (18:0-18:2 PC), 1 stearoyl 2 arachidonoyl sn glycero 3 phosphocholine (18:0-

20:4 PC), 1 stearoyl 2 docosahexaenoyl sn glycero 3 phosphocholine (18:0-22:6 PC), 1 oleoyl 2 myristoyl sn glycero 3 phosphocholine (18:1-14:0 PC, OMPC), 1 oleoyl 2 palmitoyl sn glycero 3 phosphocholine (18:1-16:0 PC, OPPC), 1 oleoyl 2 stearoyl sn glycero 3 phosphocholine (18:1-18:0 PC, OSPC), 1 palmitoyl 2 oleoyl sn glycero 3 phosphoethanolamine (16:0-18:1 PE, POPE), 1 palmitoyl 2 linoleoyl sn glycero 3 phosphoethanolamine (16:0-18:2 PE), 1 palmitoyl 2 arachidonoyl sn glycero 3 phosphoethanolamine (16:0-20:4 PE), 1 palmitoyl 2 docosahexaenoyl sn glycero 3 phosphoethanolamine (16:0-22:6 PE), 1 stearoyl 2 oleoyl sn glycero 3 phosphoethanolamine (18:0-18:1 PE), 1 stearoyl 2 linoleoyl sn glycero 3 phosphoethanolamine (18:0-18:2 PE), 1 stearoyl 2 arachidonoyl sn glycero 3 phosphoethanolamine (18:0-20:4 PE), 1 stearoyl 2 docosahexaenoyl sn glycero 3 phosphoethanolamine (18:0-22:6 PE), 1 oleoyl 2 cholesterylhemisuccinoyl sn glycero 3 phosphocholine (OChemsPC), and any combination thereof.

[00268] To provide more remarkable nuclease resistance, cellular uptake efficiency, and a more remarkable RNA interference effect, phosphatidylethanolamines may be used as anchoring moieties, for example, dimyristoylphosphatidyl ethanolamine, dipalmitoylphosphatidyl ethanolamine, 1-palmitoyl-2-oleyl-phosphatidyl ethanolamine, and dioleoylphosphatidyl ethanolamine.

Anchoring moieties: Lysolipids (e.g., lysophospholipids)

[00269] In some aspects, the anchoring moiety [AM] comprises a lysolipid, e.g., a lysophospholipid. Lysolipids are derivatives of a lipid in which one or both fatty acyl chains have been removed, generally by hydrolysis. Lysophospholipids are derivatives of a phospholipid in which one or both fatty acyl chains have been removed by hydrolysis.

[00270] In some aspects, the anchoring moiety comprises any of the phospholipids disclosed above, in which one or both acyl chains have been removed via hydrolysis, and therefore the resulting lysophospholipid comprises one or no fatty acid acyl chain.

[00271] In some aspects, the anchoring moiety comprises a lysoglycerophospholipid, a lysoglycosphingoliopid, a lysophosphatidylcholine, a lysophosphatidylethanolamine, a lysophosphatidylinositol, or a lysophosphatidylserine.

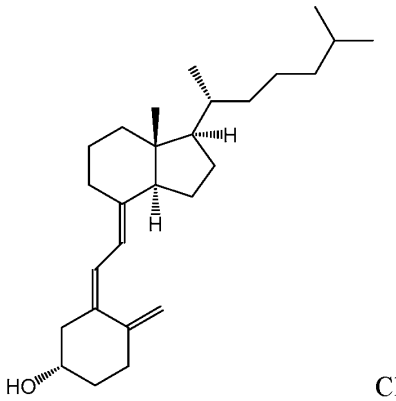
[00272] In some aspect, the anchoring moiety [AM] comprises a lysolipid selected from the non-limiting group consisting of 1 hexanoyl 2 hydroxy sn glycero 3 phosphocholine (06:0 Lyso PC), 1 heptanoyl 2 hydroxy sn glycero 3 phosphocholine (07:0 Lyso PC), 1 octanoyl 2 hydroxy sn glycero 3 phosphocholine (08:0 Lyso PC), 1 nonanoyl 2 hydroxy sn glycero 3 phosphocholine (09:0 Lyso PC), 1 decanoyl 2 hydroxy sn glycero 3 phosphocholine (10:0 Lyso

PC), 1 undecanoyl 2 hydroxy sn glycero 3 phosphocholine (11:0 Lyso PC), 1 lauroyl 2 hydroxy sn glycero 3 phosphocholine (12:0 Lyso PC), 1 tridecanoyl 2 hydroxy sn glycero 3 phosphocholine (13:0 Lyso PC), 1 myristoyl 2 hydroxy sn glycero 3 phosphocholine (14:0 Lyso PC), 1 pentadecanoyl 2 hydroxy sn glycero 3 phosphocholine (15:0 Lyso PC), 1 palmitoyl 2 hydroxy sn glycero 3 phosphocholine (16:0 Lyso PC), 1 heptadecanoyl 2 hydroxy sn glycero 3 phosphocholine (17:0 Lyso PC), 1 stearoyl 2 hydroxy sn glycero 3 phosphocholine (18:0 Lyso PC), or 1 oleoyl 2 hydroxy sn glycero 3 phosphocholine (18:1 Lyso PC), 1 myristoyl 2 hydroxy sn glycero 3 phosphoethanolamine (14:0 Lyso PE), 1 palmitoyl 2 hydroxy sn glycero 3 phosphoethanolamine (16:0 Lyso PE), 1 stearoyl 2 hydroxy sn glycero 3 phosphoethanolamine (18:0 Lyso PE), 1 oleoyl 2 hydroxy sn glycero 3 phosphoethanolamine (18:1 Lyso PE), 1 hexadecyl sn glycero 3 phosphocholine (C16 Lyso PC), and any combination thereof.

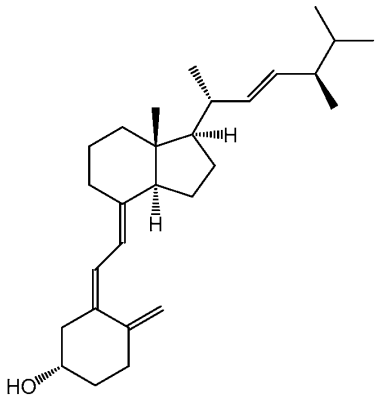
Anchoring moieties: Vitamins

[00273] In some aspects, the anchoring moiety [AM] comprises a lipophilic vitamin, e.g., vitamin A, vitamin E, vitamin K, vitamin D, folic acid, vitamin B_2 (riboflavin), vitamin B_3 (niacin), or vitamin B_6 (pyridoxine).

[00274] In some aspects, the anchoring moiety [AM] comprises or consists of vitamin D. Vitamin D is a group of fat-soluble secosteroids responsible for increasing intestinal absorption of calcium, magnesium, and phosphate, and many other biological effectsIn humans, the most important compounds in this group are vitamin D_3 (also known as cholecalciferol) and vitamin D_2 (ergocalciferol).

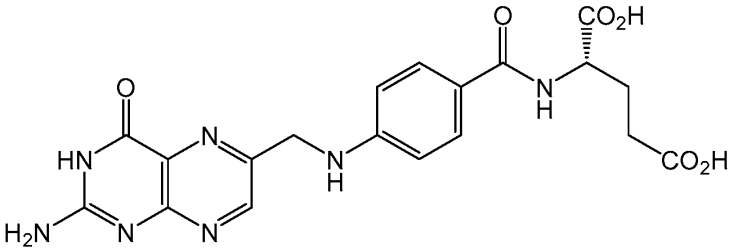


Cholecalciferol

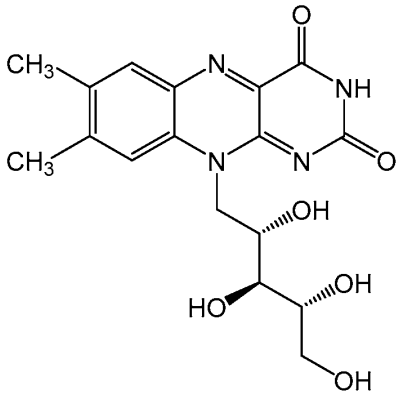


Ergocalciferol

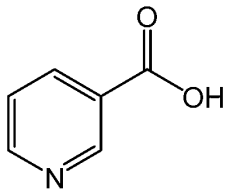
[00275] In some aspects, the anchoring moiety [AM] comprises or consists of vitamin B9 (folic acid).



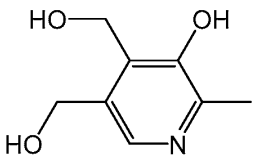
[00276] In some aspects, the anchoring moiety [AM] comprises or consists of vitamin B_2 (riboflavin).



[00277] In some aspects, the anchoring moiety [AM] comprises or consists of vitamin B_3 (niacin)

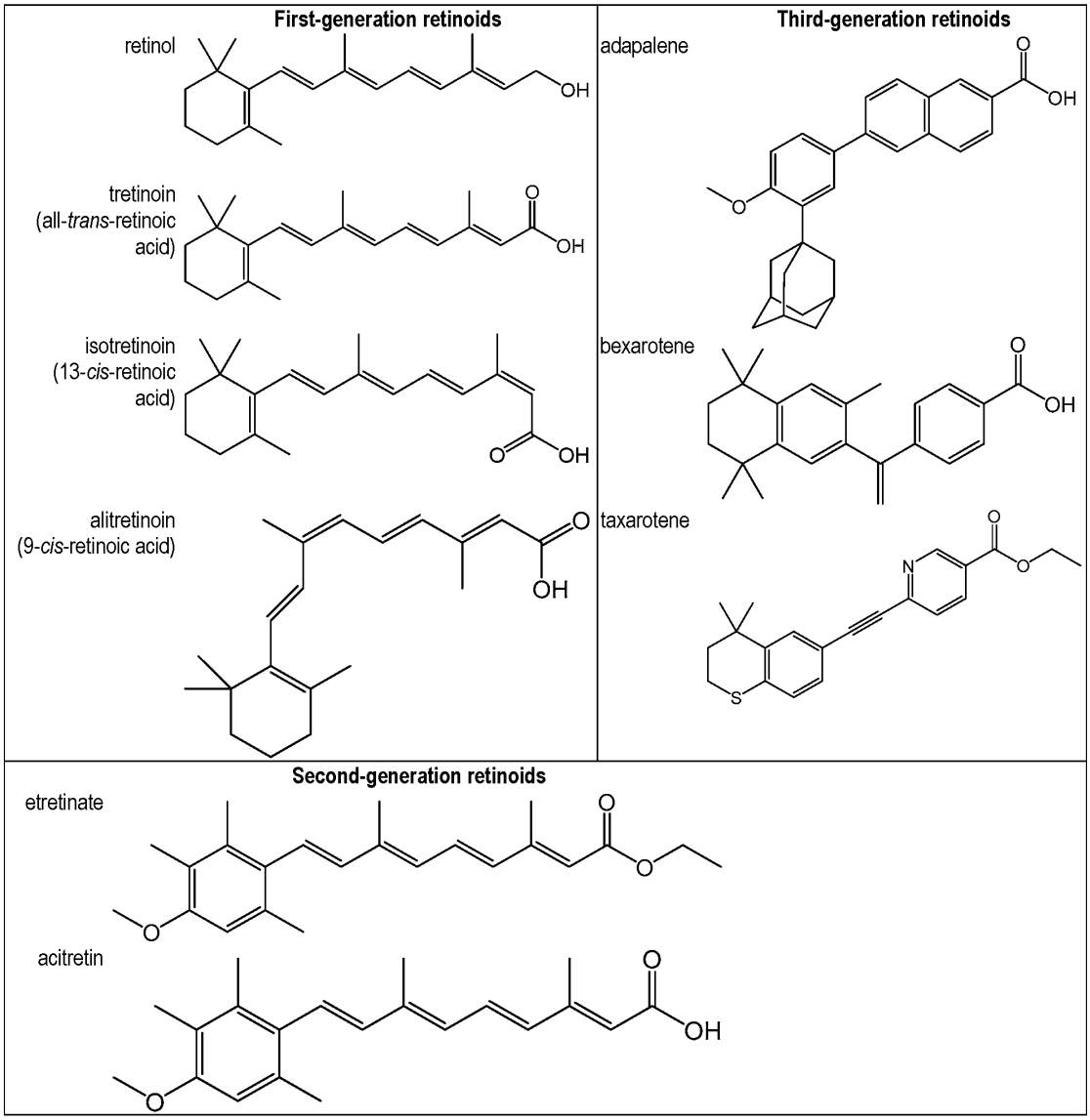


[00278] In some aspects, the anchoring moiety [AM] comprises or consists of vitamin B_6 (pyridoxine).

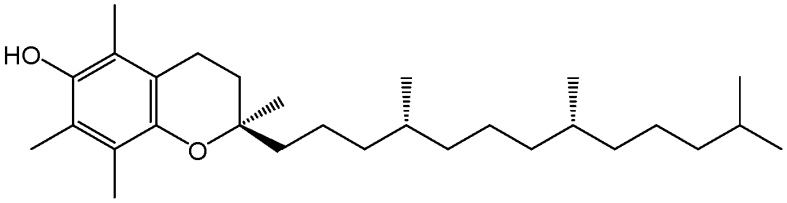


[00279] In some aspects, the anchoring moiety [AM] comprises or consists of vitamin A. Vitamin A is a group of unsaturated nutritional organic compounds that includes retinol, retinal, retinoic acid, and several provitamin A carotenoids (most notably beta-carotene). In some aspects, the anchoring moiety comprises retinol. In some aspects, the anchoring moiety comprises a retinoid. Retinoids are a class of chemical compounds that are vitamers of vitamin A or are chemically related to it. In some aspects, the anchoring moiety comprises a first generation

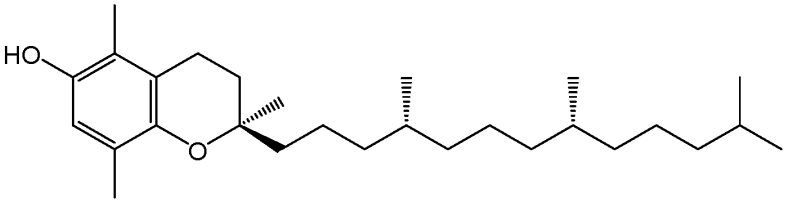
retinoid (e.g., retinol, tretinoin, isotreatinoin, or alitretinoin), a second-generation retinoid (e.g., etretinate or acitretin), a third-generation retinoid (e.g., adapalene, bexarotene, or tazarotene), or any combination thereof.



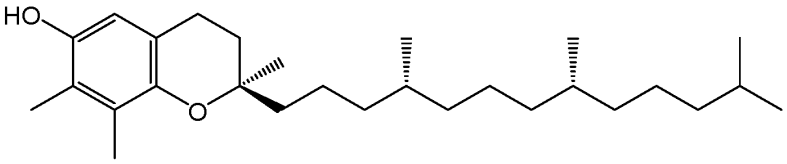
[00280] In some aspects, the anchoring moiety [AM] comprises or consists of vitamin E. Tocopherols are a class of methylated phenols many of which have vitamin E activity. Thus, in some aspects, the anchoring moiety comprises alpha-tocopherol, beta-tocopherol, gamma-tocopherol, delta-tocopherol, or a combination thereof.



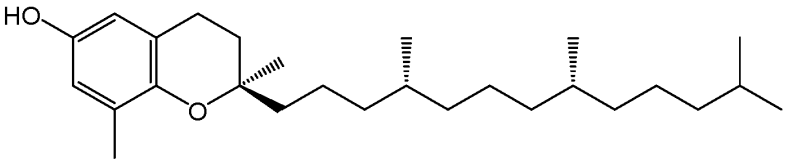
Alpha tocopherol



Beta tocopherol

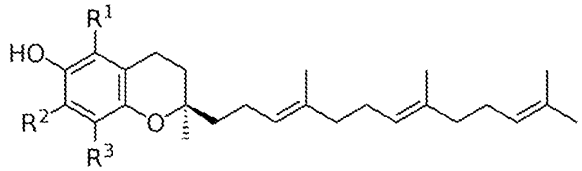


Gamma tocopherol



Delta tocopherol

[00281] Tocotrienols also have vitamin E activity. The critical chemical structural difference between tocotrienols and tocopherols is that tocotrienols have unsaturated isoprenoid side chain with three carbon-carbon double bonds versus saturated side chains for tocopherols. In some aspects, the anchoring moiety comprises alpha-tocotrienol, beta-tocotrienol, gamma-tocotrienol, delta-tocotrienol, or a combination thereof. Tocotrienols can be represented by the formula below



alpha(α)-Tocotrienol: R1 = Me, R2 = Me, R3 = Me;

beta(β)-Tocotrienol: R1 = Me, R2 = H, R3= Me;

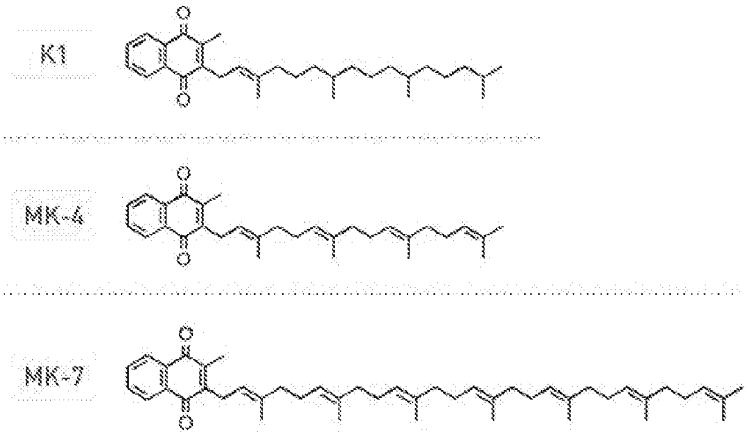
gamma(γ)-Tocotrienol: R1 = H, R2 = Me, R3= Me;

delta(δ)-Tocotrienol: R1 = H, R2 = H, R3= Me.

[00282] In some aspects, the anchoring moiety [AM] comprises or consists of vitamin K. Chemically, the vitamin K family comprises 2-methyl-1.4-naphthoquinone (3-) derivatives. Vitamin K includes two natural vitamers: vitamin K_1 and vitamin K_2. The structure of vitamin K_1

(also known as phytonadione, phylloquinone, or (E)-phytonadione) is marked by the presence of a phytyl group. The structures of vitamin K_2 (menaquinones) are marked by the polyisoprenyl side chain present in the molecule that can contain six to 13 isoprenyl units. Thus, vitamin K_2 consists of a number of related chemical subtypes, with differing lengths of carbon side chains made of isoprenoid groups of atoms. MK-4 is the most common form of vitamin K_2. Long chain forms, such as MK-7, MK-8 and MK-9 are predominant in fermented foods. Longer chain forms of vitamin K_2 such as MK-10 to MK-13 are synthesized by bacteria, but they are not well absorbed and have little biological function. In addition to the natural forms of vitamin K, there is a number of synthetic forms of vitamin K such as vitamin K_3 (menadione; 2-methylnaphthalene-1,4-dione), vitamin K_4, and vitamin K_5.

[00283] Accordingly, in some aspects, the anchoring moiety comprises vitamin K_1, K_2 (e.g., MK-4, MK-5, MK-6, MK-7, MK-8, MK-9, MK-10, MK-11, MK-12, or MK-13), K_3, K_4, K_5, or any combination thereof.



[00284] Chemically, the vitamin K family comprises 2-methyl-1,4-naphthoquinone (3-) derivatives. Vitamin K includes two natural vitamers: vitamin K1 (phylloquinone) and vitamin K2 (menaquinone). Vitamin K2, in turn, consists of a number of related chemical subtypes, with differing lengths of carbon side chains made of isoprenoid groups of atoms. The two most studied ones are menaquinone-4 (MK-4) and menaquinone-7 (MK-7). Thus, in some aspects, the vitamin K is MK-4, MK-5, or a combination thereof.

Scaffold Moieties

[00285] In some aspects, the anchoring moiety [AM] comprise a scaffolding moiety, e.g., a Scaffold X protein (e.g., PTGFR or a fragment disclosed), that can be inserted in the membrane

of the EV (e.g., exosome), or a molecule capable of interacting via affinity (e.g., an antibody or binding portion thereof) with a scaffold moiety, e.g., a Scaffold X protein (e.g., PTGFR or a fragment disclosed), present in the membrane of the EV (e.g., exosome) natively or recombinantly expressed. In some aspects, one or more scaffold moieties can be CD47, CD55, CD49, CD40, CD133, CD59, glypican-1, CD9, CD63, CD81, integrins, selectins, lectins, cadherins, other similar polypeptides known to those of skill in the art, or any combination thereof. Non-limiting examples of other scaffold moieties that can be used with the present disclosure include: aminopeptidase N (CD13); Neprilysin, AKA membrane metalloendopeptidase (MME); ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (ENPP1); Neuropilin-1 (NRP1); or any combination thereof.

[00286] In other aspects, one or more scaffold moieties are expressed in the membrane of the EVs (*e.g.,* exosomes) by recombinantly expressing the scaffold moieties in the producer cells. The EVs (*e.g.,* exosomes) obtained from the producer cells can be further modified to be conjugated to a maleimide moiety or to a linker. In other aspects, the scaffold moiety, e.g., Scaffold X, is deglycosylated. In some aspects, the scaffold moiety, e.g., Scaffold X, is highly glycosylated, *e.g.,* higher than naturally-occurring Scaffold X under the same condition.

Scaffold Moieties: Scaffold X

[00287] In some aspects, the anchoring moiety [AM] can comprise a scaffold moiety disclosed herein, *e.g.*, Scaffold X, *e.g.,* PTGFRN, BSG, IGSF2, IGSF3, IGSF8, ITGB1, ITGA4, SLC3A2, ATP transporter, or a fragment or a variant thereof. In other aspects, the anchoring moiety [AM] can comprise a ligand (e.g., an antibody or binding portion thereof) than can specifically bind to a scaffold moiety disclosed herein, *e.g.*, Scaffold X, *e.g.,* PTGFRN, BSG, IGSF2, IGSF3, IGSF8, ITGB1, ITGA4, SLC3A2, ATP transporter, or a fragment or a variant thereof.

[00288] In some aspects, surface (*e.g.,* Scaffold X)-engineered EVs (*e.g.,* exosomes), i.e., EVs overexpressing a scaffold protein (e.g., PTGFRN) demonstrate superior characteristics compared to natives EVs (*e.g.,* exosomes) not overexpressing the scaffold protein (e.g., PTGFRN). For example, surface (*e.g.,* Scaffold X)-engineered EVs (e.g., exosomes) can contain more scaffold proteins on their external surface than naturally occurring EVs (*e.g.,* exosomes). Accordingly, surface (*e.g.,* Scaffold X)-engineered EVs (*e.g.,* exosomes) can have greater, more specific, or more controlled biological activity compared to naturally occurring EVs (*e.g.,* exosomes).

[00289] In some aspects, the Scaffold X comprises Prostaglandin F2 receptor negative regulator (the PTGFRN polypeptide). The PTGFRN polypeptide can be also referred to as CD9 partner 1 (CD9P-1), Glu-Trp-Ile EWI motif-containing protein F (EWI-F), Prostaglandin F2-alpha receptor regulatory protein, Prostaglandin F2-alpha receptor-associated protein, or CD315.

[00290] Non-limiting examples of other Scaffold X proteins can be found at US Patent No. US10195290B1, issued Feb. 5, 2019, which is incorporated by reference in its entirety.

Linkages and Spacers

[00291] The optimized linkers of the present disclosure comprise combinations of linkages, e.g., cleavable or non-cleavable bonds (e.g., phosphodiester or phosphorothioate bonds, respectively) and cleavable or non-cleavable linkers, and spacers. The main function of a combination of linkages and spacers is to provide the optimal spacing between the anchoring moiety or moieties [AM] and the biologically active molecule or molecules [BAM]. For example, in the case of an ASO, a goal of a combination of linker and spacers it to reduce steric hindrances and position the ASO so it can interact with a target nucleic acid, e.g., a mRNA or a miRNA. Other goals are to increase the loading efficiency of the EV (e.g., an exosome), increase the number of BAM per EV, increase the surface density of BAM on the EV, increase the potency of the BAM after incorporate to the EV, or any combination thereof. In the context of the present disclosure the term "linker combination" refers to the combination of linkages and spacer that constitutes an optimizer liker disclosed herein.

[00292] Some linkers may be susceptible to cleavage ("cleavable linker") thereby facilitating release of the biologically active molecule [BAM]. Thus, in some aspects, a linker combination disclosed herein can comprise a cleavable linker. Such cleavable linkers may be susceptible, for example, to acid-induced cleavage, photo-induced cleavage, peptidase-induced cleavage, esterase-induced cleavage, and disulfide bond cleavage, at conditions under which the biologically active molecule remains active. Alternatively, linkers may be substantially resistant to cleavage ("non-cleavable linker"). In some aspects, the cleavable linker comprises a spacer. In some aspects the spacer is PEG.

[00293] In some aspects, a linker combination comprises at least 2, at least 3, at least 4, at least 5, or at least 6 or more different linkers disclosed herein. In some aspects, linkers in a linker combination can be linked by an ester bond (e.g., phosphodiester or phosphorothioate ester).

[00294] In some aspects, the linker is direct bond between an anchoring moiety [AM] and a biologically active molecule [BAM], e.g., an ASO.

Non-cleavable linkers

[00295] In some aspects, the linker combination comprises a "non-cleavable liker." Non-cleavable linkers are any chemical moiety capable of linking two or more components of a construct disclosed herein, e.g., a construct of Formula 1. E.g., the non-cleavable linker can link a anchoring moiety [AM] and a spacer [SP], a first spacer [SP] to a second spacer [SP], or a spacer [SP] to a biologically active molecule [BAM]. Non-cleavable linkers are substantially resistant to acid-induced cleavage, photo-induced cleavage, peptidase-induced cleavage, esterase-induced cleavage and disulfide bond cleavage.

[00296] Furthermore, non-cleavable refers to the ability of the chemical bond in the linker or adjoining to the linker to withstand cleavage induced by an acid, photolabile-cleaving agent, a peptidase, an esterase, or a chemical or physiological compound that cleaves a disulfide bond, at conditions under which a cyclic dinucleotide and/or the antibody does not lose its activity.

[00297] In some aspects, the linker combination comprises a non-cleavable linker comprising, e.g., tetraethylene glycol (TEG), hexaethylene glycol (HEG), polyethylene glycol (PEG), succinimide, or any combination thereof. In some aspects, the non-cleavable linker comprises a spacer unit to link the biologically active molecule to the non-cleavable linker.

[00298] In some aspects, one or more non-cleavable linkers comprise smaller units (e.g., HEG, TEG, glycerol, C2 to C12 alkyl, and the like) linked together. In one aspect, the linkage is an ester linkage (e.g., phosphodiester or phosphorothioate ester) or other linkage.

Non-cleavable linkers/Spacers: Ethylene Glycols (HEG, TEG, PEG)

[00299] In some aspects, the linker combination comprises a non-cleavable linker, wherein the non-cleavable linker comprises a polyethylene glycol (PEG) characterized by a formula R^3-$(O\text{-}CH_2\text{-}CH_2)_n$- or R^3-$(0\text{-}CH_2\text{-}CH_2)_n$-O- with R^3 being hydrogen, methyl or ethyl and n having a value from 2 to 200. In some aspects, the linker comprises a spacer, wherein the spacer is PEG.

[00300] In some aspects, the PEG linker is an oligo-ethylene glycol, e.g., diethylene glycol, triethylene glycol, tetra ethylene glycol (TEG), pentaethylene glycol, or a hexaethylene glycol (HEG) linker.

[00301] In some aspects, n has a value of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15. In some aspects, n is between 2 and 10, between 10 and 15. In some specific aspects, n has a value from 2 to 5, from 5 to 10, or from 10 to 15. In some aspects, the PEG is a branched PEG. Branched PEGs have three to ten PEG chains emanating from a central core group.

[00302] In certain aspects, the PEG moiety is a monodisperse polyethylene glycol. In the context of the present disclosure, a monodisperse polyethylene glycol (mdPEG) is a PEG that has

a single, defined chain length and molecular weight. mdPEGs are typically generated by separation from the polymerization mixture by chromatography. In certain formulae, a monodisperse PEG moiety is assigned the abbreviation mdPEG.

[00303] In some aspects, the PEG is a Star PEG. Star PEGs have 10 to 15 PEG chains emanating from a central core group. In some aspects, the PEG is a Comb PEGs. Comb PEGs have multiple PEG chains normally grafted onto a polymer backbone.

[00304] In some aspects, a linker combination of the present disclosure can comprise several PEG linkers, e.g., a cleavable linker flanked by PEG, HEG, or TEG linkers. In some aspects, the linker combination comprises more than one (HEG) and/or (TEG) units, wherein each unit is connected, e.g., via a phosphate ester linker, a phosphorothioate ester linkage, or a combination thereof, and wherein the total number of carbon units in the linker is C15 or less.

Non-cleavable linkers/Spacers: Glycerol and Polyglycerols (PG)

[00305] In some aspects, the linker combination comprises a non-cleavable linker comprising a glycerol unit or a polyglycerol (PG) described by the formula ((R3—O—(CH_2—CHOH—CH_2O)n—) with R3 being hydrogen, methyl or ethyl, and n having a value from 1 to 15. In some aspects, n has a value from 1 to 5. In some aspects, n has a value from 5 to 10. In some aspects, n has a value from 10 to 15.

[00306] In some aspects, the PG linker is a diglycerol, triglycerol, tetraglycerol (TG), pentaglycerol, or a hexaglycerol (HG) linker. In some aspects, n has a value of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15. In some aspects, n is between 2 and 5, between 5 and 10, or between 10 and 15.

[00307] In some alternatives of these aspects, n has a value from 1 to 15. In some aspects, the heterologous moiety is a branched polyglycerol described by the formula (R^3—O—(CH_2—$CHOR^5$—CH_2—O)n—) with R^5 being hydrogen or a linear glycerol chain described by the formula (R^3—O—(CH_2—CHOH—CH_2—O)n—) and R^3 being hydrogen, methyl or ethyl. In some aspects, the heterologous moiety is a hyperbranched polyglycerol described by the formula (R^3—O—(CH_2—$CHOR^5$—CH_2—O)n—) with R^5 being hydrogen or a glycerol chain described by the formula (R^3—O—(CH_2—$CHOR^6$—CH_2—O)n—), with R^6 being hydrogen or a glycerol chain described by the formula (R^3—O—(CH_2—$CHOR^7$—CH_2—O)n—), with R^7 being hydrogen or a linear glycerol chain described by the formula (R^3—O—(CH_2—CHOH—CH_2—O)n—) and R^3 being hydrogen, methyl or ethyl. Hyperbranched glycerol and methods for its synthesis are described in Oudshorn et al. (2006) Biomaterials 27:5471-5479; Wilms et al. (20100 Acc. Chem. Res. 43, 129-41, and references cited therein.

[00308] In some aspects, the linker combination comprises more than one (glycerol), and/or (HG) and/or (TG) units wherein and each unit is connected, e.g., via a phosphate ester linker, a phosphorothioate ester linkage, or a combination thereof, and wherein the total number of carbon units in the linker is C15 or less.

Non-cleavable linkers: Aliphatic (Alkyl) linkers

[00309] In some aspects, the linker combination comprises at least one aliphatic (alkyl) linker, e.g., propyl, butyl, hexyl, or C2-C15 alkyl, such as C2-C10 alkyl or C2-C6 alkyl.

[00310] In some aspects, the linker combination comprises an alkyl chain, e.g., an unsubstituted alkyl. In some aspects, the linker combination comprises an substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, arylalkyl, arylalkenyl, arylalkynyl, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, heterocyclylalkyl, heterocyclylalkenyl, heterocyclylalkynyl, aryl, heteroaryl, heterocyclyl, cycloalkyl, cycloalkenyl, alkylarylalkyl, alkylarylalkenyl, alkylarylalkynyl, alkenylarylalkyl, alkenylarylalkenyl, alkenylarylalkynyl, alkynylarylalkyl, alkynylarylalkenyl, alkynylarylalkynyl, alkylheteroarylalkyl, alkylheteroarylalkyl, alkylheteroarylalkenyl, alkylheteroarylalkynyl, alkenylheteroarylalkyl, alkenylheteroarylalkenyl, alkenylheteroarylalkynyl, alkynylheteroarylalkyl, alkynylheteroarylalkenyl, alkynylheteroarylalkynyl, alkylheterocyclylalkyl, alkylheterocyclylalkenyl, alkylheterocyclylalkynyl, alkenylheterocyclylalkyl, alkenylheterocyclylalkenyl, or alkenylheterocyclylalkynyl.

[00311] Optionally these components are substituted. Substituents include alcohol, alkoxy (such as methoxy, ethoxy, and propoxy), straight or branched chain alkyl (such as C1-C15 alkyl), amine, aminoalkyl (such as amino C1-C15 alkyl), phosphoramidite, phosphate, phosphoramidate, phosphorodithioate, thiophosphate, hydrazide, hydrazine, halogen, (such as F, Cl, Br, or I), amide, alkylamide (such as amide C1-C15 alkyl), carboxylic acid, carboxylic ester, carboxylic anhydride, carboxylic acid halide, ether, sulfonyl halide, imidate ester, isocyanate, isothiocyanate, haloformate, carboduimide adduct, aldehydes, ketone, sulfhydryl, haloacetyl, alkyl halide, alkyl sulfonate, C(=O)CH=CHC(=O) (maleimide), thioether, cyano, sugar (such as mannose, galactose, and glucose), α,β-unsaturated carbonyl, alkyl mercurial, or α,β-unsaturated sulfone.

[00312] The term "alkyl," by itself or as part of another substituent, means, unless otherwise stated, a straight or branched chain hydrocarbon radical having the number of carbon atoms designated (*e.g.*, C_1-C_{10} means one to ten carbon atoms). Typically, an alkyl group will have from 1 to 15 carbon atoms, for example having from 1 to 10 carbon atoms, from 1 to 8

carbon atoms or from 1 to 6 carbon atoms. A “lower alkyl” group is an alkyl group having from 1 to 4 carbon atoms. The term "alkyl" includes di- and multivalent radicals. For example, the term "alkyl" includes "alkylene" wherever appropriate, *e.g.*, when the formula indicates that the alkyl group is divalent or when substituents are joined to form a ring. Examples of alkyl radicals include, but are not limited to, methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *tert*-butyl, *iso*-butyl, *sec*-butyl, as well as homologs and isomers of, for example, *n*-pentyl, *n*-hexyl, *n*-heptyl and *n*-octyl.

[00313] The term "alkylene" by itself or as part of another substituent means a divalent (diradical) alkyl group, wherein alkyl is defined herein. "Alkylene" is exemplified, but not limited, by $-CH_2CH_2CH_2CH_2-$. Typically, an "alkylene" group will have from 1 to 15 carbon atoms, for example, having 10 or fewer carbon atoms (*e.g.*, 1 to 8 or 1 to 6 carbon atoms). A "lower alkylene" group is an alkylene group having from 1 to 4 carbon atoms.

[00314] The term "alkenyl" by itself or as part of another substituent refers to a straight or branched chain hydrocarbon radical having from 2 to 15 carbon atoms and at least one double bond. A typical alkenyl group has from 2 to 10 carbon atoms and at least one double bond. In one aspect, alkenyl groups have from 2 to 8 carbon atoms or from 2 to 6 carbon atoms and from 1 to 3 double bonds. Exemplary alkenyl groups include vinyl, 2-propenyl, 1-but-3-enyl, crotyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), 2-isopentenyl, 1-pent-3-enyl, 1-hex-5-enyl and the like.

[00315] The term "alkynyl" by itself or as part of another substituent refers to a straight or branched chain, unsaturated or polyunsaturated hydrocarbon radical having from 2 to 15 carbon atoms and at least one triple bond. A typical "alkynyl" group has from 2 to 10 carbon atoms and at least one triple bond. In one aspect of the disclosure, alkynyl groups have from 2 to 6 carbon atoms and at least one triple bond. Exemplary alkynyl groups include prop-1-ynyl, prop-2-ynyl (*i.e.*, propargyl), ethynyl and 3-butynyl.

[00316] The terms "alkoxy," "alkylamino" and "alkylthio" (or thioalkoxy) are used in their conventional sense, and refer to alkyl groups that are attached to the remainder of the molecule via an oxygen atom, an amino group, or a sulfur atom, respectively.

[00317] The term "heteroalkyl," by itself or in combination with another term, means a stable, straight or branched chain hydrocarbon radical consisting of the stated number of carbon atoms (*e.g.*, C_2-C_{10}, or C_2-C_8) and at least one heteroatom chosen, *e.g.*, from N, O, S, Si, B and P (in one aspect, N, O and S), wherein the nitrogen, sulfur and phosphorus atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. The heteroatom(s) is/are placed at any interior position of the heteroalkyl group. Examples of heteroalkyl groups include, but are

not limited to, -CH_2-CH_2-O-CH_3, -CH_2-CH_2-NH-CH_3, -CH_2-CH_2-N(CH_3)-CH_3, -CH_2-S-CH_2-CH_3, -CH_2-CH_2-S(O)-CH_3, -CH_2-CH_2-S(O)$_2$-CH_3, -CH=CH-O-CH_3, -CH_2-Si(CH_3)$_3$, -CH_2-CH=N-OCH_3, and -CH=CH-N(CH_3)-CH_3. Up to two heteroatoms can be consecutive, such as, for example, -CH_2-NH-OCH_3 and –CH_2-O-Si(CH_3)$_3$.

[00318] Similarly, the term "heteroalkylene" by itself or as part of another substituent means a divalent radical derived from heteroalkyl, as exemplified, but not limited by, -CH_2-CH_2-S-CH_2-CH_2- and –CH_2-S-CH_2-CH_2-NH-CH_2-. Typically, a heteroalkyl group will have from 3 to 24 atoms (carbon and heteroatoms, excluding hydrogen) (3- to 24-membered heteroalkyl). In another example, the heteroalkyl group has a total of 3 to 10 atoms (3- to 10-membered heteroalkyl) or from 3 to 8 atoms (3- to 8-membered heteroalkyl). The term "heteroalkyl" includes "heteroalkylene" wherever appropriate, *e.g.*, when the formula indicates that the heteroalkyl group is divalent or when substituents are joined to form a ring.

[00319] The term "cycloalkyl" by itself or in combination with other terms, represents a saturated or unsaturated, non-aromatic carbocyclic radical having from 3 to 24 carbon atoms, for example, having from 3 to 12 carbon atoms (*e.g.*, C_3-C_8 cycloalkyl or C_3-C_6 cycloalkyl). Examples of cycloalkyl include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl and the like. The term "cycloalkyl" also includes bridged, polycyclic (*e.g.*, bicyclic) structures, such as norbornyl, adamantyl and bicyclo[2.2.1]heptyl. The "cycloalkyl" group can be fused to at least one (*e.g.*, 1 to 3) other ring selected from aryl (*e.g.*, phenyl), heteroaryl (*e.g.*, pyridyl) and non-aromatic (*e.g.*, carbocyclic or heterocyclic) rings. When the "cycloalkyl" group includes a fused aryl, heteroaryl or heterocyclic ring, then the "cycloalkyl" group is attached to the remainder of the molecule via the carbocyclic ring.

[00320] The term "heterocycloalkyl," "heterocyclic," "heterocycle," or "heterocyclyl," by itself or in combination with other terms, represents a carbocyclic, non-aromatic ring (*e.g.*, 3- to 8-membered ring and for example, 4-, 5-, 6- or 7-membered ring) containing at least one and up to 5 heteroatoms selected from, *e.g.*, N, O, S, Si, B and P (for example, N, O and S), wherein the nitrogen, sulfur and phosphorus atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized (*e.g.*, from 1 to 4 heteroatoms selected from nitrogen, oxygen and sulfur), or a fused ring system of 4- to 8-membered rings, containing at least one and up to 10 heteroatoms (*e.g.*, from 1 to 5 heteroatoms selected from N, O and S) in stable combinations known to those of skill in the art. Exemplary heterocycloalkyl groups include a fused phenyl ring. When the "heterocyclic" group includes a fused aryl, heteroaryl or cycloalkyl ring, then the

"heterocyclic" group is attached to the remainder of the molecule via a heterocycle. A heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule.

[00321] Exemplary heterocycloalkyl or heterocyclic groups of the present disclosure include morpholinyl, thiomorpholinyl, thiomorpholinyl S-oxide, thiomorpholinyl S,S-dioxide, piperazinyl, homopiperazinyl, pyrrolidinyl, pyrrolinyl, imidazolidinyl, tetrahydropyranyl, piperidinyl, tetrahydrofuranyl, tetrahydrothienyl, piperidinyl, homopiperidinyl, homomorpholinyl, homothiomorpholinyl, homothiomorpholinyl S,S-dioxide, oxazolidinonyl, dihydropyrazolyl, dihydropyrrolyl, dihydropyrazolyl, dihydropyridyl, dihydropyrimidinyl, dihydrofuryl, dihydropyranyl, tetrahydrothienyl S-oxide, tetrahydrothienyl S,S-dioxide, homothiomorpholinyl S-oxide, 1-(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1-piperazinyl, 2-piperazinyl, and the like.

[00322] By "aryl" is meant a 5-, 6- or 7-membered, aromatic carbocyclic group having a single ring (*e.g.*, phenyl) or being fused to other aromatic or non-aromatic rings (*e.g.*, from 1 to 3 other rings). When the "aryl" group includes a non-aromatic ring (such as in 1,2,3,4-tetrahydronaphthyl) or heteroaryl group then the "aryl" group is bonded to the remainder of the molecule via an aryl ring (*e.g.*, a phenyl ring). The aryl group is optionally substituted (*e.g.*, with 1 to 5 substituents described herein). In one example, the aryl group has from 6 to 10 carbon atoms. Non-limiting examples of aryl groups include phenyl, 1-naphthyl, 2-naphthyl, quinoline, indanyl, indenyl, dihydronaphthyl, fluorenyl, tetralinyl, benzo[d][1,3]dioxolyl or 6,7,8,9-tetrahydro-5H-benzo[a]cycloheptenyl. In one aspects, the aryl group is selected from phenyl, benzo[d][1,3]dioxolyl and naphthyl. The aryl group, in yet another aspect, is phenyl.

[00323] The term "arylalkyl" or "aralkyl" is meant to include those radicals in which an aryl group or heteroaryl group is attached to an alkyl group to create the radicals -alkyl-aryl and -alkyl-heteroaryl, wherein alkyl, aryl and heteroaryl are defined herein. Exemplary "arylalkyl" or "aralkyl" groups include benzyl, phenethyl, pyridylmethyl and the like.

[00324] By "aryloxy" is meant the group -O-aryl, where aryl is as defined herein. In one example, the aryl portion of the aryloxy group is phenyl or naphthyl. The aryl portion of the aryloxy group, in one aspect, is phenyl.

[00325] The term "heteroaryl" or "heteroaromatic" refers to a polyunsaturated, 5-, 6- or 7-membered aromatic moiety containing at least one heteroatom (*e.g.*, 1 to 5 heteroatoms, such as 1-3 heteroatoms) selected from N, O, S, Si and B (for example, N, O and S), wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. The "heteroaryl" group can be a single ring or be fused to other aryl, heteroaryl, cycloalkyl or

heterocycloalkyl rings (*e.g.*, from 1 to 3 other rings). When the "heteroaryl" group includes a fused aryl, cycloalkyl or heterocycloalkyl ring, then the "heteroaryl" group is attached to the remainder of the molecule via the heteroaryl ring. A heteroaryl group can be attached to the remainder of the molecule through a carbon- or heteroatom.

[00326] In one example, the heteroaryl group has from 4 to 10 carbon atoms and from 1 to 5 heteroatoms selected from O, S and N. Non-limiting examples of heteroaryl groups include pyridyl, pyrimidinyl, quinolinyl, benzothienyl, indolyl, indolinyl, pyridazinyl, pyrazinyl, isoindolyl, isoquinolyl, quinazolinyl, quinoxalinyl, phthalazinyl, imidazolyl, isoxazolyl, pyrazolyl, oxazolyl, thiazolyl, indolizinyl, indazolyl, benzothiazolyl, benzimidazolyl, benzofuranyl, furanyl, thienyl, pyrrolyl, oxadiazolyl, thiadiazolyl, triazolyl, tetrazolyl, isothiazolyl, naphthyridinyl, isochromanyl, chromanyl, tetrahydroisoquinolinyl, isoindolinyl, isobenzotetrahydrofuranyl, isobenzotetrahydrothienyl, isobenzothienyl, benzoxazolyl, pyridopyridyl, benzotetrahydrofuranyl, benzotetrahydrothienyl, purinyl, benzodioxolyl, triazinyl, pteridinyl, benzothiazolyl, imidazopyridyl, imidazothiazolyl, dihydrobenzisoxazinyl, benzisoxazinyl, benzoxazinyl, dihydrobenzisothiazinyl, benzopyranyl, benzothiopyranyl, chromonyl, chromanonyl, pyridyl-N-oxide, tetrahydroquinolinyl, dihydroquinolinyl, dihydroquinolinonyl, dihydroisoquinolinonyl, dihydrocoumarinyl, dihydroisocoumarinyl, isoindolinonyl, benzodioxanyl, benzoxazolinonyl, pyrrolyl N-oxide, pyrimidinyl N-oxide, pyridazinyl N-oxide, pyrazinyl N-oxide, quinolinyl N-oxide, indolyl N-oxide, indolinyl N-oxide, isoquinolyl N-oxide, quinazolinyl N-oxide, quinoxalinyl N-oxide, phthalazinyl N-oxide, imidazolyl N-oxide, isoxazolyl N-oxide, oxazolyl N-oxide, thiazolyl N-oxide, indolizinyl N-oxide, indazolyl N-oxide, benzothiazolyl N-oxide, benzimidazolyl N-oxide, pyrrolyl N-oxide, oxadiazolyl N-oxide, thiadiazolyl N-oxide, triazolyl N-oxide, tetrazolyl N-oxide, benzothiopyranyl S-oxide, benzothiopyranyl S,S-dioxide. Exemplary heteroaryl groups include imidazolyl, pyrazolyl, thiadiazolyl, triazolyl, isoxazolyl, isothiazolyl, imidazolyl, thiazolyl, oxadiazolyl, and pyridyl. Other exemplary heteroaryl groups include 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, pyridin-4-yl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalinyl, 5-quinoxalinyl, 3-quinolyl, and 6-quinolyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable aryl group substituents described below.

[00327] Examples of aliphatic linkers include the following structures: —O—CO—O—; —NH—CO—O—; —NH—CO—NH—; —NH—$(CH_2)_{n1}$—; —S—$(CH_2)_{n1}$—; —CO—$(CH_2)_{n1}$—CO—; —CO—$(CH_2)_{n1}$—NH—; —NH—$(CH_2)_{n1}$—NH—; —CO—NH—$(CH_2)_{n1}$—NH—CO—; —C(=S)—NH—$(CH_2)_{n1}$—NH—CO—; —C(=S)—NH—$(CH_2)_{n1}$—NH—C—(=S)—; —CO—O—$(CH_2)_{n1}$—O—CO—; —C(=S)—O—$(CH_2)_{n1}$—O—CO—; —C(=S)—O—$(CH_2)_{n1}$—O—C—(=S)—; —CO—NH—$(CH_2)_{n1}$—O—CO—; —C(=S)—NH—$(CH_2)_{n1}$—O—CO—; —C(=S)—NH—$(CH_2)_{n1}$—O—C—(=S)—; —CO—NH—$(CH_2)_{n1}$—O—CO—; —C(=S)—NH—$(CH_2)_{n1}$—CO—; —C(=S)—O—$(CH_2)_{n1}$—NH—CO—; —C(=S)—NH—$(CH_2)_{n1}$—O—C—(=S)—; —NH—$(CH_2CH_2O)_{n2}$—CH(CH_2OH)—; —NH—$(CH_2CH_2O)_{n2}$—CH_2—; —NH—$(CH_2CH_2O)_{n2}$—CH_2—CO—; —O—$(CH_2)_{n3}$—S—S—$(CH_2)_{n4}$—O—P(=O)$_2$—; —CO—$(CH_2)_{n3}$—O—CO—NH—$(CH_2)_{n4}$—; —CO—$(CH_2)_{n3}$—CO—NH—$(CH_2)_{n4}$—; —$(CH2)_{n1}$NH—; —C(O)$(CH2)_{n1}$NH—; —C(O)—$(CH2)_{n1}$-C(O)—; —C(O)—$(CH2)_{n1}$-C(O)O—; —C(O)—O—; —C(O)—$(CH2)_{n1}$-NH—C(O)—; —C(O)—$(CH2)_{n1}$—; —C(O)—NH—; —C(O)—; —$(CH2)_{n1}$-C(O)—; —$(CH2)_{n1}$-C(O)O—; —$(CH2)_{n1}$—; — $(CH2)_{n1}$-NH—C(O)—; wherein n1 is an integer between 1 and 15 (e.g., 2 to 20, or 2 to 12); n2 is an integer between 1 and 15 (e.g., 1 to 10, or 1 to 6); n3 and n4 may be the same or different and are an integer between 1 and 15 (e.g., 1 to 10, or 1 to 6), and wherein the total number of carbon units in the linker is C15 or less.

[00328] In some aspects, the linker combination comprises (C3)n, (C4)n, (C5)n, (C6)n, (C7)n, or (C8)n, or a combination thereof, wherein n is an integer between 1 and 5, wherein the total number of carbon units in the linker is C15 or less, and each unit is connected, e.g., via a phosphate ester linker, a phosphorothioate ester linkage, or a combination thereof.

Cleavable linkers

[00329] In some aspects, different components of a construct disclosed herein, e.g., a construct of Formula 1, can be linked by a cleavable linker. The term cleavable linker refers to a linker comprising at least one linkage or chemical bond that can be broken or cleaved. As used herein, the term cleave refers to the breaking of one or more chemical bonds in a relatively large molecule in a manner that produces two or more relatively smaller molecules. Cleavage may be mediated, e.g., by a nuclease, peptidase, protease, phosphatase, oxidase, or reductase, for example, or by specific physicochemical conditions, e.g., redox environment, pH, presence of reactive oxygen species, or specific wavelengths of light.

[00330] In some aspects, the term "cleavable," as used herein, refers, e.g., to rapidly degradable linkers, such as, e.g., phosphodiester and disulfides, while the term "non-cleavable" refers, e.g., to more stable linkages, such as, e.g., nuclease-resistant phosphorothioates.

[00331] In some aspects, the cleavable linker is a dinucleotide or trinucleotide linker, a disulfide, an imine, a thioketal, a val-cit dipeptide, or any combination thereof.

[00332] In some aspects, the cleavable linker comprises valine-alanine-p-aminobenzylcarbamate or valine-citrulline-p-aminobenzylcarbamate.

Cleavable linkers: Redox cleavable linkers

[00333] In some aspects, the linker combination comprises a redox cleavable linker. As a non-limiting example, one type of cleavable linker is a redox cleavable linking group that is cleaved upon reduction or upon oxidation. In some aspects, the redox cleavable linker contains a disulfide bond, i.e., it is a disulfide cleavable linker. Redox cleavable linkers can be reduced, e.g., by intracellular mercaptans, oxidases, or reductases.

Cleavable linkers: Reactive Oxygen Species (ROS) cleavable linkers

[00334] In some aspects, the linker combination can comprise a cleavable linker which may be cleaved by a reactive oxygen species (ROS), such as superoxide (Of) or hydrogen peroxide (H_2O_2), generated, e.g., by inflammation processes such as activated neutrophils. In some aspects, the ROS cleavable linker is a thioketal cleavable linker. See, e.g., U.S. Pat. 8,354,455B2, which is herein incorporated by reference in its entirety.

Cleavable linkers: pH dependent cleavable linkers

[00335] In some aspects, the linker is an "acid labile linker" comprising an acid cleavable linking group, which is a linking group that is selectively cleaved under acidic conditions (pH<7).

[00336] As a non-limiting example, the acid cleavable linking group is cleaved in an acidic environment, e.g., about 6.0, 5.5, 5.0 or less. In some aspects, the pH is about 6.5 or less. In some aspects, the linker is cleaved by an agent such as an enzyme that can act as a general acid, e.g., a peptidase (which may be substrate specific) or a phosphatase. Within cells, certain low pH organelles, such as endosomes and lysosomes, can provide a cleaving environment to the acid cleavable linking group. Although the pH of human serum is 7.4, the average pH in cells is slightly lower, ranging from about 7.1 to 7.3. Endosomes also have an acidic pH, ranging from

5.5 to 6.0, and lysosomes are about 5.0 at an even more acidic pH. Accordingly, pH dependent cleavable linkers are sometimes called endosomically labile linkers in the art.

[00337] The acid cleavable group may have the general formula -C=NN-, C(O)O, or -OC(O). In another non-limiting example, when the carbon attached to the ester oxygen (alkoxy group) is attached to an aryl group, a substituted alkyl group, or a tertiary alkyl group such as dimethyl pentyl or t-butyl, for example. Examples of acid cleavable linking groups include, but are not limited to amine, imine, amino ester, benzoic imine, diortho ester, polyphosphoester, polyphosphazene, acetal, vinyl ether, hydrazone, cis-aconitate, hydrazide, thiocarbamoyl, imizine, azidomethyl-methylmaleic anhydride, thiopropionate, a masked endosomolytic agent, a citraconyl group, or any combination thereof. Disulfide linkages are also susceptible to pH.

[00338] In some aspects, the linker comprises a low pH-labile hydrazone bond. Such acid-labile bonds have been extensively used in the field of conjugates, e.g., antibody-drug conjugates. See, for example, Zhou et al (2011) Biomacromolecules 12:1460-7; Yuan et al (2008) Acta Biomater. 4:1024-37; Zhang et al (2007) Acta Biomater. 6:838-50; Yang et al (2007) J. Pharmacol. Exp. Ther. 321:462-8; Reddy et al (2006) Cancer Chemother. Pharmacol. 58:229-36; Doronina et al (2003) Nature Biotechnol. 21:778-84.

[00339] In certain aspects, the linker comprises a low pH-labile bond selected from the following: ketals that are labile in acidic environments (e.g., pH less than 7, greater than about 4) to form a diol and a ketone; acetals that are labile in acidic environments (e.g., pH less than 7, greater than about 4) to form a diol and an aldehyde; imines or iminiums that are labile in acidic environments (e.g., pH less than 7, greater than about 4) to form an amine and an aldehyde or a ketone; silicon-oxygen-carbon linkages that are labile under acidic condition; silicon-nitrogen (silazane) linkages; silicon-carbon linkages (e.g., arylsilanes, vinylsilanes, and allylsilanes); maleamates (amide bonds synthesized from maleic anhydride derivatives and amines); ortho esters; hydrazones; activated carboxylic acid derivatives (e.g., esters, amides) designed to undergo acid catalyzed hydrolysis); or vinyl ethers.

[00340] Further examples may be found in U.S. Pat. Nos. 9,790,494B2 and 8,137,695B2, the contents of which are incorporated herein by reference in their entireties.

Cleavable linkers: Enzymatic cleavable linkers

[00341] In some aspects, the linker combination can comprise a linker cleavable by intracellular or extracellular enzymes, e.g., proteases, esterases, nucleases, amidades. The range of enzymes that can cleave a specific linker in a linker combination depends on the specific bonds and chemical structure of the linker. Accordingly, peptidic linkers can be cleaved, e.g., by

peptidades, linkers containing ester linkages can be cleaved, e.g., by esterases; linkers containing amide linkages can be cleaved, e.g., by amidases; etc.

Cleavable linkers: Enzymatic cleavable linkers: Protease cleavable linkers

[00342] In some aspects, the linker combination comprises a protease cleavable linker, i.e., a linker that can be cleaved by an endogenous protease. Only certain peptides are readily cleaved inside or outside cells. See, e.g., Trout et al., 79 Proc. Natl. Acad. Sci. USA, 626-629 (1982) and Umemoto et al. 43 Int. J. Cancer, 677-684 (1989). Cleavable linkers can contain cleavable sites composed of α-amino acid units and peptidic bonds, which chemically are amide bonds between the carboxylate of one amino acid and the amino group of a second amino acid. Other amide bonds, such as the bond between a carboxylate and the α-amino acid group of lysine, are understood not to be peptidic bonds and are considered non-cleavable.

[00343] In some aspects, the protease-cleavable linker comprises a cleavage site for a protease, *e.g.,* neprilysin (CALLA or CDlO), thimet oligopeptidase (TOP), leukotriene A4 hydrolase, endothelin converting enzymes, ste24 protease, neurolysin, mitochondrial intermediate peptidase, interstitial collagenases, collagenases, stromelysins, macrophage elastase, matrilysin, gelatinases, meprins, procollagen C- endopeptidases, procollagen N-endopeptidases, ADAMs and ADAMTs metalloproteinases, myelin associated metalloproteinases, enamelysin, tumor necrosis factor α-converting enzyme, insulysin, nardilysin, mitochondrial processing peptidase, magnolysin, dactylysin-like metalloproteases, neutrophil collagenase, matrix metallopeptidases, membrane-type matrix metalloproteinases, SP2 endopeptidase, prostate specific antigen (PSA), plasmin, urokinase, human fibroblast activation protein (FAPα), trypsin, chymotrypsins, caldecrin, pancreatic elastases, pancreatic endopeptidase, enteropeptidase, leukocyte elastase, myeloblasts, chymases, tryptase, granzyme, stratum corneum chymotryptic enzyme, acrosin, kallikreins, complement components and factors, alternative-complement pathway c3/c5 convertase, mannose- binding protein-associated serine protease, coagulation factors, thrombin, protein c, u and t-type plasminogen activator, cathepsin G, hepsin, prostasin, hepatocyte growth factor- activating endopeptidase, subtilisin/kexin type proprotein convertases, furin, proprotein convertases, prolyl peptidases, acylaminoacyl peptidase, peptidyl-glycaminase, signal peptidase, n-terminal nucleophile aminohydrolases, 20s proteasome, γ-glutamyl transpeptidase, mitochondrial endopeptidase, mitochondrial endopeptidase Ia, htra2 peptidase, matriptase, site 1 protease, legumain, cathepsins, cysteine cathepsins, calpains, ubiquitin isopeptidase T, caspases, glycosylphosphatidylinositoliprotein transamidase, cancer procoagulant, prohormone thiol protease, γ-Glutamyl hydrolase, bleomycin hydrolase, seprase,

cathepsin B, cathepsin D, cathepsin L, cathepsin M, proteinase K, pepsins, chymosyn, gastricsin, renin, yapsin and/or mapsins, Prostate-Specific antigen (PSA), or any Asp-N, Glu-C, Lys-C or Arg-C proteases in general. See, *e.g.,* Cancer Res. 77(24):7027-7037 (2017), which is herein incorporated by reference in its entirety.

[00344] In some aspects, the cleavable linker component comprises a peptide comprising one to ten amino acid residues. In these aspects, the peptide allows for cleavage of the linker by a protease, thereby facilitating release of the biologically active molecule upon exposure to intracellular proteases, such as lysosomal enzymes (Doronina et al. (2003) Nat. Biotechnol. 21:778-784). Exemplary peptides include, but are not limited to, dipeptides, tripeptides, tetrapeptides, pentapeptides, and hexapeptides.

[00345] A peptide may comprise naturally-occurring and/or non-natural amino acid residues. The term "naturally-occurring amino acid" refer to Ala, Asp, Cys, Glu, Phe, Gly, His, He, Lys, Leu, Met, Asn, Pro, Gin, Arg, Ser, Thr, Val, Trp, and Tyr. "Non-natural amino acids" (i.e., amino acids do not occur naturally) include, by way of non-limiting example, homoserine, homoarginine, citrulline, phenylglycine, taurine, iodotyrosine, seleno- cysteine, norleucine ("Nle"), norvaline ("Nva"), beta-alanine, L- or D-naphthalanine, ornithine ("Orn"), and the like. Peptides can be designed and optimized for enzymatic cleavage by a particular enzyme, for example, a tumor-associated protease, cathepsin B, C and D, or a plasmin protease.

[00346] Amino acids also include the D-forms of natural and non-natural amino acids. "D-" designates an amino acid having the "D" (dextrorotary) configuration, as opposed to the configuration in the naturally occurring ("L-") amino acids. Natural and non-natural amino acids can be purchased commercially (Sigma Chemical Co., Advanced Chemtech) or synthesized using methods known in the art. Exemplary dipeptides include, but are not limited to, valine-alanine, valine-citrulline, phenylalanine-lysine, N-methyl-valine-citrulline, cyclohexylalanine-lysine, and beta-alanine-lysine. Exemplary tripeptides include, but are not limited to, glycine-valine-citrulline (gly-val-cit) and glycine-glycine-glycine (gly-gly-gly).

Cleavable linkers: Enzymatic cleavable linkers: Esterase cleavable linkers

[00347] Some linkers are cleaved by esterases ("esterase cleavable linkers"). Only certain esters can be cleaved by esterases and amidases present inside or outside of cells. Esters are formed by the condensation of a carboxylic acid and an alcohol. Simple esters are esters produced with simple alcohols, such as aliphatic alcohols, and small cyclic and small aromatic alcohols. Examples of ester-based cleavable linking groups include, but are not limited to, esters

of alkylene, alkenylene and alkynylene groups. The ester cleavable linking group has the general formula -C(O)O- or -OC (O)-.

Cleavable linkers: Enzymatic cleavable linkers: Phosphatase cleavable linkers

[00348] In some aspects, a linker combination can include a phosphate-based cleavable linking group is cleaved by an agent that degrades or hydrolyzes phosphate groups. An example of an agent that cleaves intracellular phosphate groups is an enzyme such as intracellular phosphatase. Examples of phosphate-based linking groups are —O—P(O)(OR_k)—O—, —O—P(S)(OR_k)—O—, —O—P(S)(SR_k)— O-, -S-P(O)(OR_k)-O-, -O-P(O)(OR_k)-S-, -S-P(O)(OR_k)-S-, -O-P(S)(OR_k)-S-, -SP (S)(OR_k)-O-, -OP(O)(R_k)-O-, -OP(S)(R_k)-O-, -SP(O)(R_k)-O-, -SP(S)(R_k)-O-, -SP(O)(R_k)-S-, or -OP(S)(R_k)-S-. In various aspects, R_k is any of the following: NH_2, BH_3, CH_3, C_{1-6} alkyl, C_{6-10} aryl, C_{1-6} alkoxy and C_{6-10} aryl-oxy. In some aspects, C_{1-6} alkyl and C_{6-10} aryl are unsubstituted. Further non-limiting examples are -O-P(O)(OH)-O-, -O-P(S)(OH)-O-, -O-P(S)(SH)-O-, -S-P(O)(OH)-O-, O-P(O)(OH)-S-, -S-P(O)(OH)-S-, -O-P(S)(OH)-S-, -S-P(S)(OH)-O-, -O-P(O)(H)-O-, -O-P(S)(H)-O-, -S-P(O)(H)-O-, -SP(S)(H)-O-, -SP(O)(H)-S-, -OP(S)(H)-S-, or -O-P(O)(OH)-O-.

Cleavable linkers: Photoactivated cleavable linkers

[00349] In some aspects, the combination linker comprises a photoactivated cleavable linker, e.g., a nitrobenzyl linker or a linker comprising a nitrobenzyl reactive group.

Cleavable linkers: Self-immolative linker

[00350] In some aspects, the linker combination comprises a self-immolative linker. In some aspects, the self-immolative linker in the EV (*e.g.,* exosome) of the present disclosure undergoes 1,4 elimination after the enzymatic cleavage of the protease-cleavable linker. In some aspects, the self-immolative linker in the EV (*e.g.,* exosome) of the present disclosure undergoes 1,6 elimination after the enzymatic cleavage of the protease-cleavable linker. In some aspects, the self-immolative linker is, e.g., a p-aminobenzyl (pAB) derivative, such as a p-aminobenzyl carbamate (pABC), a p-amino benzyl ether (PABE), a p-amino benzyl carbonate, or a combination thereof. In certain aspects, the self-immolative linker comprises an aromatic group. In some aspects, the aromatic group is selected from the group consisting of benzyl, cinnamyl, naphthyl, and biphenyl. In some aspects, the aromatic group is heterocyclic. In other aspects, the aromatic group comprises at least one substituent. In some aspects, the at least one substituent is selected from the group consisting of F, Cl, I, Br, OH, methyl, methoxy, NO_2, NH_2, NO^{3+},

$NHCOCH_3$, $N(CH_3)_2$, $NHCOCF_3$, alkyl, haloalkyl, C_1-C_8 alkylhalide, carboxylate, sulfate, sulfamate, and sulfonate. In other aspects, at least one C in the aromatic group is substituted with N, O, or C-R*, wherein R* is independently selected from H, F, Cl, I, Br, OH, methyl, methoxy, NO_2, NH_2, NO^{3+}, $NHCOCH_3$, $N(CH_3)_2$, $NHCOCF_3$, alkyl, haloalkyl, C_1-C_8 alkylhalide, carboxylate, sulfate, sulfamate, and sulfonate.

[00351] In some aspects, the self-immolative linker comprises an aminobenzyl carbamate group (e.g., para-aminobenzyl carbamate), an aminobenzyl ether group, or an aminobenzyl carbonate group. In one aspect, the self-immolative linker is p-amino benzyl carbamate (pABC). pABC is the most efficient and most widespread connector linkage for self-immolative site-specific prodrug activation (see, *e.g.*, Carl *et al.* J. Med. Chem. 24:479-480 (1981); WO 1981/001145; Rautio et la, Nature Rev. Drug Disc. 7:255-270 (2008); Simplicio *et al.*, Molecules 13:519-547 (2008)).

[00352] In some aspects, the self-immolative linker connects a biologically active molecule (*e.g.*, an ASO) to a protease-cleavable substrate (e.g, Val-Cit). In specific aspects, the carbamate group of a pABC self-immolative linker is connected to an amino group of a biologically active molecule (*e.g.*, ASO), and the amino group of the pABC self-immolative linker is connected to a protease-cleavable substrate.

[00353] The aromatic ring of the aminobenzyl group can optionally be substituted with one or more (*e.g.*, R_1 and/or R_2) substituents on the aromatic ring, which replace a hydrogen that is otherwise attached to one of the four non-substituted carbons that form the ring. As used herein, the symbol "R_X" (*e.g.*, R_1, R_2, R_3, R_4) is a general abbreviation that represents a substituent group as described herein. Substituent groups can improve the self-immolative ability of the p-aminobenzyl group (Hay *et al.*, J. Chem Soc., Perkin Trans. 1:2759-2770 (1999); see also, Sykes *et al.* J. Chem. Soc., Perkin Trans. 1:1601-1608 (2000)).

[00354] Self-immolative elimination can take place, *e.g.*, via 1,4 elimination, 1,6 elimination (*e.g.*, pABC), 1,8 elimination (*e.g.*, p-amino-cinnamyl alcohol), β-elimination, cyclisation-elimination (*e.g.*, 4-aminobutanol ester and ethylenediamines), cyclization/lactonization, cyclization/lactolization, *etc.* See, *e.g.*, Singh *et al.* Curr. Med. Chem. 15:1802-1826 (2008); Greenwald *et al.* J. Med. Chem. 43:475-487 (2000).

[00355] In some aspects, the self-immolative linker can comprise, *e.g.*, cinnamyl, naphthyl, or biphenyl groups (see, *e.g.*, Blencowe *et al.* Polym. Chem. 2:773-790 (2011)). In some aspects, the self-immolative linker comprises a heterocyclic ring (see., *e.g.*, U.S. Patent Nos. 7,375,078; 7,754,681). Numerous homoaromatic (see, *e.g.*, Carl *et al.* J. Med. Chem. 24:479

(1981); Senter *et al.* J. Org. Chem. 55:2975 (1990); Taylor *et al.* J. Org. Chem. 43:1197 (1978); Andrianomenjanahary *et al.* Bioorg. Med. Chem. Lett. 2:1903 (1992)), and coumarin (see, *e.g.*, Weinstein *et al.* Chem. Commun. 46:553 (2010)), furan, thiophene, thiazole, oxazole, isoxazole, pyrrole, pyrazole (see, *e.g.*, Hay *et al.* J. Med. Chem. 46:5533 (2003)), pyridine (see, *e.g.*, Perry-Feigenbaum *et al.* Org. Biomol. Chem. 7:4825 (2009)), imidazone (see, *e.g.*, Nailor *et al.* Bioorg. Med. Chem. Lett. Z:1267 (1999); Hay and Denny, Tetrahedron Lett. 38:8425 (1997)), and triazole (see, *e.g.*, Bertrand and Gesson, J. Org. Chem. 72:3596 (2007)) based heteroaromatic groups that are self-immolative under both aqueous and physiological conditions are known in the art. See also, U.S. Pat Nos. 7,691,962; 7,091,186; U.S. Pat. Publ. Nos. US2006/0269480; US2010/0092496; US2010/0145036; US2003/0130189; US2005/0256030)

[00356] In some aspects, a linker combination disclosed herein comprises more than one self-immolative linker in tandem, *e.g.*, two or more pABC units. See, *e.g.*, de Groot *et al.* J. Org. Chem. 66:8815-8830 (2001). In some aspects, a linker combination disclosed herein can comprise a self-immolative linker (*e.g.*, a p-aminobenzylalcohol or a hemithioaminal derivative of p-carboxybenzaldehyde or glyoxilic acid) linked to a fluorigenic probe (see, *e.g.*, Meyer *et al.* Org. Biomol. Chem. 8:1777-1780 (2010)).

[00357] Where substituent groups in the self-immolative linker s are specified by their conventional chemical formulae, written from left to right, they equally encompass the chemically identical substituents, which would result from writing the structure from right to left. For example, "-CH_2O-" is intended to also recite "-OCH_2-".

[00358] Substituent groups in self-immolative, for example, R_1 and/or R_2 substituents in a p-aminobenzyl self-immolative linker as discuss above can include, *e.g.*, alkyl, alkylene, alkenyl, alkynyl, alkoxy, alkylamino, alkylthio, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, arylalkyl, aryloxy, heteroaryl, *etc.* When a compound of the present disclosure includes more than one substituent, then each of the substituents is independently chosen.

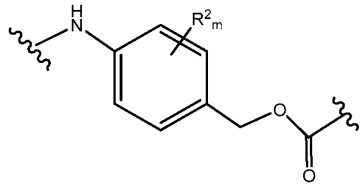
[00359] In some specific aspects, the self-immolative linker is attached to cleavable peptide linker has the following formula, the combination having the following formula:

$$-A_a-Y_y-$$

wherein each –A- is independently an amino acid unit or a combination thereof, a is independently an integer from 1 to 12; and -Y- is a self-immolative spacer, and y is 1, or 2. In some aspects, -A_a- is a dipeptide, a tripeptide, a tetrapeptide, a pentapeptide, or a hexapeptide. In some aspects, –A_a- is selected from the group consisting of valine-alanine, valine-citrulline,

phenylalanine-lysine, N-methylvaline-citrulline, cyclohexylalanine-lysine, and beta-alanine-lysine. In some aspects, $-A_a-$ is valine-alanine or valine-citrulline.

[00360] In some aspects, the self-immolative linker $-Y_y-$ has the following formula:



wherein each R^2 is independently C_{1-8} alkyl, -O-(C_{1-8} alkyl), halogen, nitro, or cyano; and m is an integer from 0 to 4. In some aspects, m is 0, 1, or 2. In some aspects, m is 0.

[00361] In some aspects, the cleavable linker is a cleavable peptide liker (e.g., a dipeptide linker) comprising a self-immolative linker, for example, valine-alanine-p-aminobenzylcarbamate or valine-citrulline-p-aminobenzylcarbamate.

Peptide linkers

[00362] In some aspects, the term "linker" refers to a peptide or polypeptide sequence (e.g., a synthetic peptide or polypeptide sequence) or to a non-polypeptide, e.g., an alkyl chain. In some aspects, two or more linkers can be linked in tandem. Generally, linkers provide flexibility or prevent/ameliorate steric hindrances. Linkers are not typically cleaved; however, in certain aspects, such cleavage can be desirable. Accordingly, in some aspects a linker can comprise one or more protease-cleavable sites, which can be located within the sequence of the linker or flanking the linker at either end of the linker sequence.

[00363] In some aspects, the linker is a peptide linker. In some aspects, the peptide linker can comprise at least about two, at least about three, at least about four, at least about five, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35, at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95, or at least about 100 amino acids.

[00364] In some aspects, the linker is a glycine/serine linker. In some aspects, the peptide linker is glycine/serine linker according to the formula [(Gly)n-Ser]m where n is any integer from 1 to 100 and m is any integer from 1 to 100. In other aspects, the glycine/serine linker is according to the formula [(Gly)x-Sery]z wherein x in an integer from 1 to 4, y is 0 or 1, and z is an integer from 1 to 50. In some aspects, the peptide linker comprises the sequence Gn, where n can be an integer from 1 to 100. In some aspects, the peptide linker can comprise the sequence

(GlyAla)n, wherein n is an integer between 1 and 100. In other aspects, the peptide linker can comprise the sequence (GlyGlySer)n, wherein n is an integer between 1 and 100.

[00365] In some aspects, the peptide linker is synthetic, i.e., non-naturally occurring. In one aspect, a peptide linker includes peptides (or polypeptides) (e.g., natural or non-naturally occurring peptides) which comprise an amino acid sequence that links or genetically fuses a first linear sequence of amino acids to a second linear sequence of amino acids to which it is not naturally linked or genetically fused in nature. For example, in one aspect the peptide linker can comprise non-naturally occurring polypeptides which are modified forms of naturally occurring polypeptides (e.g., comprising a mutation such as an addition, substitution or deletion).

[00366] In other aspects, the peptide linker can comprise non-naturally occurring amino acids. In yet other aspects, the peptide linker can comprise naturally occurring amino acids occurring in a linear sequence that does not occur in nature. In still other aspects, the peptide linker can comprise a naturally occurring polypeptide sequence.

Biologically Active Molecule [BAM]

[00367] In some aspects, an EV (*e.g.,* exosome) disclosed herein is capable of delivering a biologically active molecule [BAM] attached to the EV, *e.g.,* exosome, via an anchoring moiety [AM], wherein the anchoring moiety [AM] is connected to the biologically active molecule [BAM] via an optimized linker disclosed herein. The biologically active molecule [BAM] is an agent that acts on a target (*e.g.,* a target cell). Contacting can occur *in vitro* or in a subject. Non-limiting examples of biologically active molecules [BAM] that can attached to an EV (*e.g.,* exosome) as described in the present disclosure include agents such as, nucleotides (*e.g.,* nucleotides comprising a detectable moiety or a toxin or that disrupt transcription), nucleic acids (*e.g.,* DNA or mRNA molecules that encode a polypeptide such as an enzyme, or RNA molecules that have regulatory function such as miRNA, dsDNA, lncRNA, mRNA, siRNA, or ASO), amino acids (*e.g.,* amino acids comprising a detectable moiety or a toxin that disrupt translation), polypeptides (*e.g.,* enzymes), lipids, carbohydrates, and small molecules (*e.g.,* small molecule drugs and toxins).

[00368] In some aspects, an EV (*e.g.,* exosome) disclosed herein can comprise more than one biologically active molecule [BAM] attached, e.g., using the constructs disclosed herein. For example, in some aspects, the EV (*e.g.,* exosome) can comprise multiple populations of constructs of the present disclosure (e.g., a construct of Formula 1), wherein each population of constructs carry a different biologically active molecule [BAM].

[00369] Accordingly, in some aspects a population of EVs, e.g., exosomes, of the present disclosed can comprise a plurality of construct as exemplified below.

$[AM_1]$-(optimized $linker_1$)-$[BAM_1]$
$[AM_2]$-(optimized $linker_2$)-$[BAM_2]$
...
$[AM_n]$-(optimized linkern)-$[BAM_n]$

wherein $[AM_1]..[AM_n]$ can be the same or different anchoring moieties, (optimized $linker_1$)..(optimized $linker_n$) can be the same or different optimized linkers, and $[BAM_1]..[BAM_n]$ can be the same of different biologically active molecules.

[00370] In some aspects, the EVs (*e.g.,* exosomes) of the present disclosure can comprise constructs comprising optimized linkers disclosed herein (e.g., constructs of Formula 1), wherein each construct carries more than one biologically active molecule [BAM], as exemplified below.

[AM]-(optimized linker)-$[BAM_1]..$[BAMn]

wherein $[BAM_1]..$[BAMn] can be the same of different biologically active molecules.

[00371] In some aspects, a construct comprising an optimized liner disclosed herein can comprise multiple biologically active molecules in a non-linear arrangement as exemplified below, e.g., arrangement comprising two, three, or more biologically active moieties is an branched arrangement.

[BAM1]
[AM]-(optimized linker)---|
[BAM2]

[BAM1]
[AM]-(optimized linker)---|--- [BAM2]
[BAM3]

[00372] In some aspects, the EV, e.g., exosome of the present disclosure can comprise populations of constructs comprising any of the topological arrangements disclosed above and combinations thereof.

[00373] In some aspects, the biologically active molecule [BAM] targets a tumor antigen. Non-limiting examples of tumor antigens include: alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), epithelial tumor antigen (ETA), mucin 1 (MUC1), Tn-MUC1, mucin 16 (MUC16), tyrosinase, melanoma-associated antigen (MAGE), tumor protein p53 (p53), CD4, CD8, CD45, CD80, CD86, programmed death ligand 1 (PD-L1), programmed death ligand 2 (PD-L2), NY-ESO-1, PSMA, TAG-72, HER2, GD2, cMET, EGFR, Mesothelin, VEGFR, alpha-folate receptor, CE7R, IL-3, Cancer-testis antigen (CTA), MART-1 gp100, TNF-related apoptosis-inducing ligand, or combinations thereof.

[00374] In some aspects, the biologically active molecule [BAM] is targeting moiety, e.g., an antibody or binding portion thereof or a ligand, that specifically binds to a marker on a muscle cell. In some aspects, the muscle cell is a smooth muscle cell. In some aspects, the muscle cell is a skeletal muscle cell. In some aspects, the muscle cell is a cardiac muscle cell. In some aspects, the marker on the muscle cell is selected from alpha-smooth muscle actin, VE-cadherin, caldesmon/CALD1, calponin 1, hexim 1, histamine H2 R; motilin R/GPR38, transgelin/TAGLN, and any combination thereof. In some aspects, the marker on the muscle cell is selected from alpha-sarcoglycan, beta-sarcoglycan,calpain inhibitors, creatine kinase MM/CKMM, eIF5A, enolase 2/neuron-specific enolase, epsilon-sarcoglycan, FABP3/H-FABP, GDF-8/Myostatin, GDF-11/GDF-8, integrin alpha 7, integrin alpha 7 beta 1, integrin beta 1/CD29, MCAM/CD146, MyoD, myogenin, myosin light chain kinase inhibitors, NCAM-1/CD56, troponin I, and any combination thereof. In some aspects, the marker on the muscle cell is myosin heavy chain, myosin light chain, or a combination thereof.

[00375] In some aspects, the biologically active molecule [BAM] is a small molecule. In some aspects, the small molecule is a proteolysis-targeting chimera (PROTAC). In some aspects, the biologically active molecule [BAM] comprises a nucleotide, wherein the nucleotide is a stimulator of interferon genes protein (STING) agonist. STING is a cytosolic sensor of cyclic dinucleotides that is typically produced by bacteria. Upon activation, it leads to the production of type I interferons and initiates an immune response

[00376] In some aspects, the STING agonist comprises a cyclic nucleotide STING agonist or a non-cyclic dinucleotide STING agonist. Cyclic purine dinucleotides such as, but not limited to, cGMP, cyclic di-GMP (c-di-GMP), cAMP, cyclic di-AMP (c-di-AMP), cyclic-GMP-AMP (cGAMP), cyclic di-IMP (c-di-IMP), cyclic AMP-IMP (cAIMP), and any analogue thereof, are known to stimulate or enhance an immune or inflammation response in a patient. The CDNs may have 2'2', 2'3', 2'5', 3'3', or 3'5' bonds linking the cyclic dinucleotides, or any combination thereof. Cyclic purine dinucleotides may be modified via standard organic chemistry techniques

to produce analogues of purine dinucleotides. Suitable purine dinucleotides include, but are not limited to, adenine, guanine, inosine, hypoxanthine, xanthine, isoguanine, or any other appropriate purine dinucleotide known in the art. The cyclic dinucleotides may be modified analogues. Any suitable modification known in the art may be used, including, but not limited to, phosphorothioate, biphosphorothioate, fluorinate, and difluorinate modifications. Non cyclic dinucleotide agonists may also be used, such as 5,6-Dimethylxanthenone-4-acetic acid (DMXAA), or any other non-cyclic dinucleotide agonist known in the art.

[00377] It is contemplated that any STING agonist may be used as a biologically active molecule [BAM]. Among the STING agonists are DMXAA, STING agonist-1, ML RR-S2 CDA, ML RR-S2c-di-GMP, ML-RR-S2 cGAMP, 2'3'-c-di-AM(PS)2, 2'3'-cGAMP, 2'3'-cGAMPdFHS, 3'3'-cGAMP, 3'3'-cGAMPdFSH, cAIMP, cAIM(PS)2, 3'3'-cAIMP, 3'3'-cAIMPdFSH, 2'2'-cGAMP, 2'3'-cGAM(PS)2, 3'3'-cGAMP, c-di-AMP, 2'3'-c-di-AMP, 2'3'-c-di-AM(PS)2, c-di-GMP, 2'3'-c-di-GMP, c-di-IMP, c-di-UMP or any combination thereof. In a specific aspect, the STING agonist is 3'3'-cAIMPdFSH, alternatively named 3-3 cAIMPdFSH. Additional STING agonists known in the art may also be used.

[00378] In some aspects, the biologically active molecule [BAM] is an antibody or antigen binding fragment thereof. In some aspects, the biologically active molecule [BAM] is an antibody-drug conjugate (ADC). In some aspects, the biologically active molecule [BAM] is a small molecule comprising a synthetic antineoplastic agent (*e.g.,* monomethyl auristatin E (MMAE) (vedotin)), a cytokine release inhibitor (*e.g.,* MCC950), an mTOR inhibitor (e.g., rapamycin and its analogs (rapalogs)), an autotaxin inhibitor (*e.g.,* PAT409 or PAT505), a lysophosphatidic acid receptor antagonist (*e.g.,* BMS-986020), a STING antagonist (*e.g.*, CL656), or any combination thereof.). In some aspects, the biologically active molecule [BAM] is a fusogenic peptide.

[00379] In some aspects, the biologically active molecule [BAM] comprises an antisense oligonucleotide (ASO). In some aspects, the ASO targets various genes (transcripts) expressed in vivo. In some aspects, a biologically active molecule of the present disclosure comprises morpholino backbone structures disclosed in U.S. Pat. No. 5,034,506, which is herein incorporated by reference in its entirety.).

[00380] In some aspects, the biologically active molecule [BAM] targets macrophages. In other aspects, the biologically active molecule induces macrophage polarization. Macrophage polarization is a process by which macrophages adopt different functional programs in response to the signals from their microenvironment. This ability is connected to their multiple roles in the

organism: they are powerful effector cells of innate immune system, but also important in removal of cellular debris, embryonic development and tissue repair.

[00381] By simplified classification, macrophage phenotype has been divided into 2 groups: M1 (classically activated macrophages) and M2 (alternatively activated macrophages). This broad classification was based on in vitro studies, in which cultured macrophages were treated with molecules that stimulated their phenotype switching to particular state. In addition to chemical stimulation, it has been shown that the stiffness of the underlying substrate a macrophage is grown on can direct polarization state, functional roles and migration mode. M1 macrophages were described as the pro-inflammatory type, important in direct host-defense against pathogens, such as phagocytosis and secretion of pro-inflammatory cytokines and microbicidal molecules. M2 macrophages were described to have quite the opposite function: regulation of the resolution phase of inflammation and the repair of damaged tissues. Later, more extensive in vitro and ex vivo studies have shown that macrophage phenotypes are much more diverse, overlapping with each other in terms of gene expression and function, revealing that these many hybrid states form a continuum of activation states which depend on the microenvironment. Moreover, in vivo, there is a high diversity in gene expression profile between different populations of tissue macrophages. Macrophage activation spectrum is thus considered to be wider, involving complex regulatory pathway to response to plethora of different signals from the environment. The diversity of macrophage phenotypes still remains to be fully characterized *in vivo*.

[00382] The imbalance of the macrophage types is related to a number of immunity-related diseases. For example, increased M1/M2 ratio may correlate with development of inflammatory bowel disease, as well as obesity in mice. On the other side, *in vitro* experiments implicated M2 macrophages as the primary mediators of tissue fibrosis. Several studies have associated the fibrotic profile of M2 macrophages with the pathogenesis of systemic sclerosis. Non-limiting examples of the macrophage targeting biologically active molecules are: PI3Kγ (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma), RIP1 (Receptor Interacting Protein (RIP) kinase 1, RIPK1), HIF-1α (Hypoxia-inducible factor 1-alpha), AHR1 (Adhesion and hyphal regulator 1), miR146a, miR155, IRF4 (Interferon regulatory factor 4), PPARγ (Peroxisome proliferator-activated receptor gamma), IL-4RA (Interleukin-4 receptor subunit alpha), TLR8 (Toll-like receptor 8), and TGF-β1 (Transforming growth factor beta-1 proprotein)

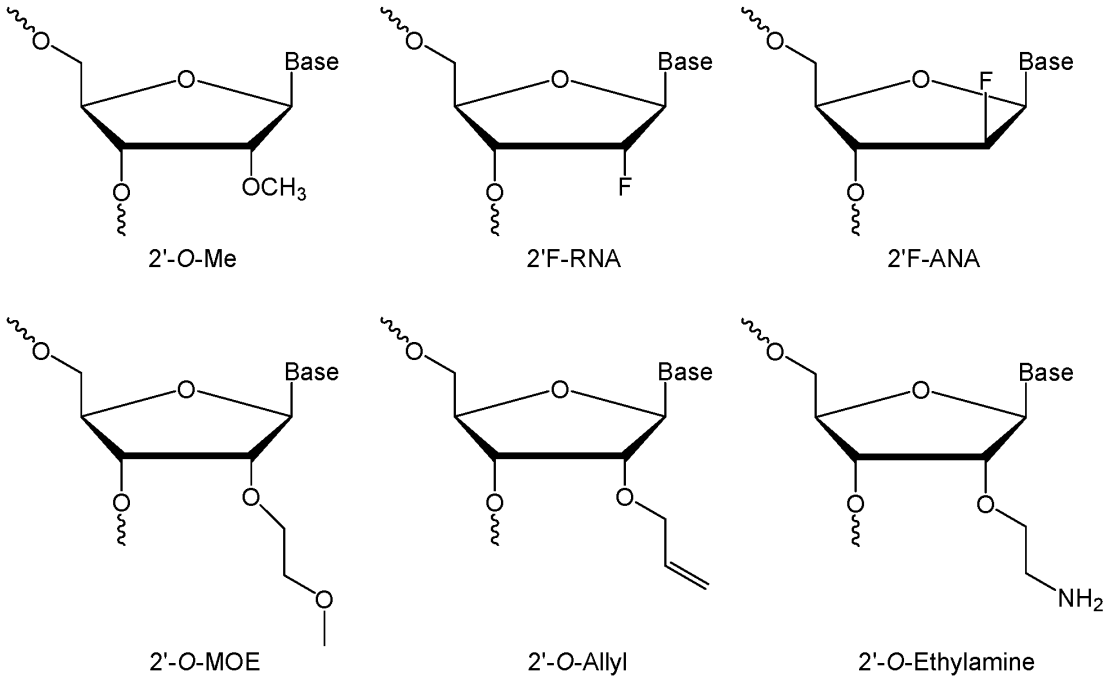
[00383] In some aspects, the BAM comprises an ASO (e.g., an ASO targeting NLRP3, STAT6, CEBP/β, STAT3, NRas, KRAS, Pmp22 disclosed below). In some aspects, the ASO is a

gapmer, a mixmer, or a totalmer. In some aspects, the ASO (e.g., an ASO targeting NLRP3, STAT6, CEBP/β, STAT3, NRas, KRAS, Pmp22 disclosed below) can comprise one or more nucleosides which have a modified sugar moiety, *i.e.* a modification of the sugar moiety when compared to the ribose sugar moiety found in DNA and RNA. Numerous nucleosides with modification of the ribose sugar moiety have been made, primarily with the aim of improving certain properties of oligonucleotides, such as affinity and/or nuclease resistance.

[00384] Such modifications include those where the ribose ring structure is modified, *e.g.* by replacement with a hexose ring (HNA), or a bicyclic ring, which typically have a biradical bridge between the C2' and C4' carbons on the ribose ring (LNA), or an unlinked ribose ring which typically lacks a bond between the C2' and C3' carbons (*e.g.,* UNA). Other sugar modified nucleosides include, for example, bicyclohexose nucleic acids (WO2011/017521) or tricyclic nucleic acids (WO2013/154798). Modified nucleosides also include nucleosides where the sugar moiety is replaced with a non-sugar moiety, for example in the case of peptide nucleic acids (PNA), or morpholino nucleic acids.

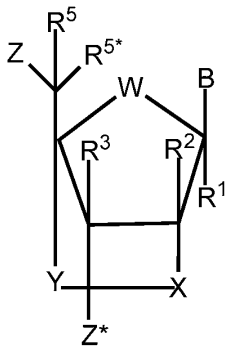
[00385] Sugar modifications also include modifications made via altering the substituent groups on the ribose ring to groups other than hydrogen, or the 2'-OH group naturally found in RNA nucleosides. Substituents may, for example be introduced at the 2', 3', 4', or 5' positions. Nucleosides with modified sugar moieties also include 2' modified nucleosides, such as 2' substituted nucleosides. Indeed, much focus has been spent on developing 2' substituted nucleosides, and numerous 2' substituted nucleosides have been found to have beneficial properties when incorporated into oligonucleotides, such as enhanced nucleoside resistance and enhanced affinity.

[00386] A 2' sugar modified nucleoside is a nucleoside which has a substituent other than H or –OH at the 2' position (2' substituted nucleoside) or comprises a 2' linked biradical, and includes 2' substituted nucleosides and LNA (2' – 4' biradical bridged) nucleosides. For example, the 2' modified sugar may provide enhanced binding affinity (*e.g.,* affinity enhancing 2' sugar modified nucleoside) and/or increased nuclease resistance to the oligonucleotide. Examples of 2' substituted modified nucleosides are 2'-O-alkyl-RNA, 2'-O-methyl-RNA, 2'-alkoxy-RNA, 2'-O-methoxyethyl-RNA (MOE), 2'-amino-DNA, 2'-Fluoro-RNA, 2'-Fluro-DNA, arabino nucleic acids (ANA), and 2'-Fluoro-ANA nucleoside. For further examples, please *see, e.g.*, Freier & Altmann; *Nucl. Acid Res.*, 1997, 25, 4429-4443; Uhlmann, *Curr. Opinion in Drug Development*, 2000, 3(2), 293-213; and Deleavey and Damha, Chemistry and Biology 2012, 19, 937. Below are illustrations of some 2' substituted modified nucleosides.



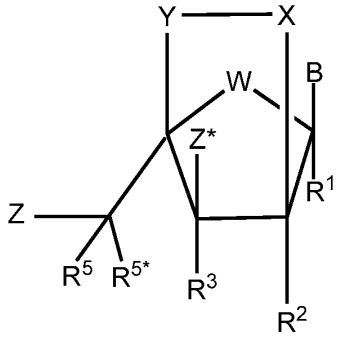
[00387] LNA nucleosides are modified nucleosides which comprise a linker group (referred to as a biradical or a bridge) between C2' and C4' of the ribose sugar ring of a nucleoside (*i.e.*, 2'-4' bridge), which restricts or locks the conformation of the ribose ring. These nucleosides are also termed bridged nucleic acid or bicyclic nucleic acid (BNA) in the literature. The locking of the conformation of the ribose is associated with an enhanced affinity of hybridization (duplex stabilization) when the LNA is incorporated into an oligonucleotide for a complementary RNA or DNA molecule. This can be routinely determined by measuring the melting temperature of the oligonucleotide/complement duplex.

[00388] Non limiting, exemplary LNA nucleosides are disclosed in WO99/014226, WO00/66604, WO98/039352, WO2004/046160, WO00/047599, WO2007/134181, WO2010/077578, WO2010/036698, WO2007/090071, WO2009/006478, WO2011/156202, WO2008/154401, WO2009/067647, WO2008/150729, Morita *et al.*, *Bioorganic & Med.Chem. Lett.* 12, 73-76, Seth *et al.*, *J. Org. Chem.* 2010, Vol 75(5) pp. 1569-81, or Mitsuoka *et al.*, *Nucleic Acids Research* 2009, 37(4), 1225-1238, all of which are herein incorporated by reference in their entireties. In some aspects, the modified nucleoside or the LNA nucleosides of an ASO of the disclosure has a general structure of the Formula I or Formula II:



β-D

or



α-L

Formula I Formula II

wherein

W is selected from -O-, -S-, -N(R^a)-, -C(R^aR^b)-, in particular –O-;

B is a nucleobase or a modified nucleobase moiety;

Z is an internucleoside linkage to an adjacent nucleoside or a 5'-terminal group;

Z* is an internucleoside linkage to an adjacent nucleoside or a 3'-terminal group;

R^1, R^2, R^3, R^5 and R^{5*} are independently selected from hydrogen, halogen, alkyl, alkenyl, alkynyl, hydroxy, alkoxy, alkoxyalkyl, alkenyloxy, carboxyl, alkoxycarbonyl, alkylcarbonyl, formyl, azide, heterocycle and aryl; and

X, Y, R^a and R^b are as defined herein.

ASO targeting NLRP3

[00389] In some aspects, the biologically active molecule [BAM] is an anti-*NLRP3* ASO. NLRP3 (*NLRP3*) is also known as NLR family pyrin domain containing 3. Unless indicated otherwise, the term "NLRP3," as used herein, can refer to NLRP3 from one or more species (*e.g.*, humans, non-human primates, dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, and bears). The sequence for the human NLRP3 gene can be found under publicly available GenBank Accession Number NC_000001.11:247416156-247449108. The human *NLRP3* gene is found at chromosome location 1q44 at 247,416,156-247,449,108. The sequence for the human *NLRP3* pre-mRNA transcript (SEQ ID NO: 1) corresponds to the reverse complement of residues 247,416,156-247,449,108 of chromosome 1q44. The *NLRP3* mRNA sequence (GenBank Accession No. NM_001079821.2) is provided in SEQ ID NO: 3, except that the nucleotide "t" in SEQ ID NO: 3 is shown as "u" in the mRNA. The sequence for human NLRP3 protein can be found under publicly available Accession Numbers: Q96P20, (canonical sequence, SEQ ID NO: 2), Q96P20-2 (SEQ ID NO: 4), Q96P20-3 (SEQ ID NO: 5), Q96P20-4 (SEQ ID NO: 6), Q96P20-5 (SEQ ID NO: 7), and Q96P20-6 (SEQ ID NO: 8), each of which is incorporated by

reference herein in its entirety. The anti-*NLRP3* ASOs of the present disclosure can be designed to reduce or inhibit expression of the natural variants of the NLRP3 protein.

[00390] An example of a target nucleic acid sequence of the anti-*NLRP3* ASOs is *NLRP3* pre-mRNA. SEQ ID NO: 1 represents a human *NLRP3* genomic sequence (*i.e.*, reverse complement of nucleotides 247,416,156-247,449,108 of chromosome 1q44). SEQ ID NO: 1 is identical to a *NLRP3* pre-mRNA sequence except that nucleotide "t" in SEQ ID NO: 1 is shown as "u" in pre-mRNA. In certain aspects, the "target nucleic acid" comprises an intron of a NLRP3 protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In other aspects, the target nucleic acid comprises an exon region of a NLRP3 protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In yet other aspects, the target nucleic acid comprises an exon-intron junction of a NLRP3 protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. The human NLRP3 protein sequence encoded by the *NLRP3* pre-mRNA is shown as SEQ ID NO: 3. In other aspects, the target nucleic acid comprises an untranslated region of a NLRP3 protein-encoding nucleic acids or naturally occurring variants thereof, *e.g.*, 5' UTR, 3' UTR, or both.

[00391] In some aspects, an anti-*NLRP3* ASO of the disclosure hybridizes to a region within the introns of a *NLRP3* transcript, *e.g.*, SEQ ID NO: 1. In certain aspects, an anti-*NLRP3* ASO of the disclosure hybridizes to a region within the exons of a *NLRP3* transcript, *e.g.*, SEQ ID NO: 1. In other aspects, an anti-*NLRP3* ASO of the disclosure hybridizes to a region within the exon-intron junction of a *NLRP3* transcript, *e.g.*, SEQ ID NO: 1. In some aspects, an anti-*NLRP3* ASO of the disclosure hybridizes to a region within a *NLRP3* transcript (*e.g.*, an intron, exon, or exon-intron junction), *e.g.*, SEQ ID NO: 1, wherein the anti-*NLRP3* ASO has a gapmer design.

[00392] In some aspects, the anti-*NLRP3* ASO targets a mRNA encoding a particular isoform of NLRP3 protein (*e.g.*, Isoform 1). In some aspects, the anti-*NLRP3* ASO targets all isoforms of NLRP3 protein. In other aspects, the anti-*NLRP3* ASO targets two isoforms (*e.g.*, Isoform 1 and Isoform 2, Isoform 3 and Isoform 4, and Isoform 5 and Isoform 6) of NLRP3 protein.

[00393] In some aspects, the nucleotide sequence of the anti-*NLRP3* ASOs of the disclosure or the contiguous nucleotide sequence has at least about 80% sequence identity to a sequence selected from SEQ ID NOs: 101 to 200, such as at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96% sequence identity, at least about 97% sequence identity, at

least about 98% sequence identity, at least about 99% sequence identity, such as about 100% sequence identity (homologous).

[00394] In some aspects, the anti-*NLRP3* ASO (or contiguous nucleotide portion thereof) is selected from, or comprises, one of the sequences selected from the group consisting of SEQ ID NOs: 101 to 200 or a region of at least 10 contiguous nucleotides thereof, wherein the anti-*NLRP3* ASO (or contiguous nucleotide portion thereof) can optionally comprise one, two, three, or four mismatches when compared to the corresponding *NLRP3* transcript.

[00395] In some aspects, the anti-*NLRP3* ASO comprises a sequence selected from the group consisting of SEQ ID NO: 101-200.

[00396] In some aspects, the anti-*NLRP3* ASO comprises a sequence as set forth in any one of SEQ ID NOs: 101 to 200. In some aspects, the anti-*NLRP3* ASO comprises or consists of a sequence at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to a sequence set forth in SEQ ID NOs: 101 to 200. In some aspects, the anti-*NLRP3* ASO (or contiguous nucleotide portion thereof) is selected from, or comprises, one of the sequences selected from the group consisting of SEQ ID NOs: 101 to 200 or a region of at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 contiguous nucleotides thereof. In some aspects, the anti-*NLRP3* ASO (or contiguous nucleotide portion thereof) is selected from, or comprises, one of the sequences selected from the group consisting of SEQ ID NOs: 101 to 200 or a region of at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 contiguous nucleotides thereof, wherein the anti-*NLRP3* ASO (or contiguous nucleotide portion thereof) can optionally comprise one, two, three, or four mismatches when compared to the corresponding *NLRP3* transcript. In some aspects, the anti-*NLRP3* ASO (or contiguous nucleotide portion thereof) is selected from, or comprises, one of the sequences selected from the group consisting of SEQ ID NOs: 101 to 200 except for 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 substitutions, wherein the substituted ASO can bind to the *NLRP3* transcript. In some aspects, the anti-*NLRP3* ASO (or contiguous nucleotide portion thereof) is selected from, or comprises, one of the sequences selected from the group consisting of SEQ ID NOs: 101 to 200 or a region of at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 contiguous nucleotides thereof, wherein the anti-*NLRP3* ASO (or contiguous nucleotide portion thereof) can optionally comprise one, two, three, or four additional 5' and/or 3' nucleotides complementary to the corresponding *NLRP3* transcript.

[00397] In some aspects, binding of an anti-*NLRP3* ASO targeting a *NLRP3* transcript disclosed herein to a mRNA transcript encoding *NLRP3* can reduce expression levels and/or activity levels of *NLRP3*.

[00398] In some aspects, any anti-*NLRP3* ASO described herein can be part of an EV (e.g., exosome) of the present disclosure, i.e., an EV (e.g., exosome) a comprising construct comprising an optimized linker disclosed herein, e.g., a construct of Formula 1 where the biologically active moiety [BAM] is an anti-*NLRP3* ASO described herein or a combination thereof.

ASO targeting STAT6

[00399] In some aspects, the biologically active molecule [BAM] is an anti-*STAT6* ASO. STAT6 (*STAT6*) is also known as signal transducer and activator of transcription 6. Unless indicated otherwise, the term "STAT6," as used herein, can refer to STAT6 from one or more species (*e.g.*, humans, non-human primates, dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, and bears).

[00400] The sequence for the human *STAT6* pre-mRNA transcript (SEQ ID NO: 11) corresponds to the reverse complement of residues 57111413-57095404, complement, of chromosome 12q13.3. The *STAT6* mRNA sequence (GenBank Accession No. NM_001178078.1) is provided in SEQ ID NO: 13, except that the nucleotide "t" in SEQ ID NO: 13 is shown as "u" in the mRNA. The sequence for human STAT6 protein can be found under publicly available Accession Numbers: P42226-1, (canonical sequence, SEQ ID NO: 12), P42226-2 (SEQ ID NO: 14), and P42226-3 (SEQ ID NO: 15), each of which is incorporated by reference herein in its entirety.

[00401] Natural variants of the human *STAT6* gene product are known. For example, natural variants of human STAT6 protein can contain one or more amino acid substitutions selected from: M118R, D419N, and any combination thereof. Additional variants of human STAT6 protein resulting from alternative splicing are also known in the art. STAT6 Isoform 2 (identifier: P42226-2 at UniProt) differs from the canonical sequence (SEQ ID NO: 13) as follows: deletion of residues 1-174 and substitution of $_{175}$PSE$_{177}$ with $_{175}$MEQ$_{177}$ relative to SEQ ID NO: 13. The sequence of STAT6 Isoform 3 (identifier: P42226-3) differs from the canonical sequence (SEQ ID NO: 13) as follows: deletion of residues 1-110 relative to SEQ ID NO: 13. Therefore, the anti-*STAT6* ASOs of the present disclosure can be designed to reduce or inhibit expression of the natural variants of the STAT6 protein.

[00402] An example of a target nucleic acid sequence of the anti-*STAT6* ASOs is *STAT6* pre-mRNA. SEQ ID NO: 11 represents a human *STAT6* genomic sequence (*i.e.*, reverse complement of nucleotides 57111413-57095404, complement, of chromosome 12q13.3). SEQ ID NO: 11 is identical to a *STAT6* pre-mRNA sequence except that nucleotide "t" in SEQ ID NO: 11 is shown as "u" in pre-mRNA. In certain aspects, the "target nucleic acid" comprises an intron of a STAT6 protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In other aspects, the target nucleic acid comprises an exon region of a STAT6 protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In yet other aspects, the target nucleic acid comprises an exon-intron junction of a STAT6 protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. The human STAT6 protein sequence encoded by the *STAT6* pre-mRNA is shown as SEQ ID NO: 13. In other aspects, the target nucleic acid comprises an untranslated region of a STAT6 protein-encoding nucleic acids or naturally occurring variants thereof, *e.g.*, 5' UTR, 3' UTR, or both.

[00403] In some aspects, an anti-*STAT6* ASO of the disclosure hybridizes to a region within the introns of a *STAT6* transcript, *e.g.*, SEQ ID NO: 11. In certain aspects, an anti-*STAT6* ASO of the disclosure hybridizes to a region within the exons of a *STAT6* transcript, *e.g.*, SEQ ID NO: 11. In other aspects, an anti-*STAT6* ASO of the disclosure hybridizes to a region within the exon-intron junction of a *STAT6* transcript, *e.g.*, SEQ ID NO: 11. In some aspects, an anti-*STAT6* ASO of the disclosure hybridizes to a region within a *STAT6* transcript (*e.g.,* an intron, exon, or exon-intron junction), *e.g.*, SEQ ID NO: 11, wherein the anti-*STAT6* ASO has a gapmer design.

[00404] In some aspects, the anti-*STAT6* ASO targets a mRNA encoding a particular isoform of STAT6 protein (*e.g.*, Isoform 1). In some aspects, the ASO targets all isoforms of STAT6 protein. In other aspects, the anti-*STAT6* ASO targets two isoforms (*e.g.*, Isoform 1 and Isoform 2, Isoform 1 and Isoform 3, or Isoform 2 and Isoform 3) of STAT6 protein.

[00405] In some aspects, a payload of the disclosure (*e.g.*, ASO) hybridizes to a region within the introns of a *STAT6* transcript. In certain aspects, the payload hybridizes to a region within the exons of a *STAT6* transcript. In some aspects, the payload hybridizes to a region within the exon-intron junction of a *STAT6* transcript. In some aspects, the payload hybridizes to a region within a *STAT6* transcript (*e.g.,* an intron, exon, or exon-intron junction). A non-limiting example of a payload (*e.g.*, ASO) that can specifically target a region of a *STAT6* transcript.

[00406] In some aspects, binding of an anti-*STAT6* ASO targeting a *STAT6* transcript disclosed herein to a mRNA transcript encoding *STAT6* can reduce expression levels and/or activity levels of *STAT6*.

[00407] In some aspects, any anti-*STAT6* ASO described herein can be part of an EV (e.g., exosome) of the present disclosure, i.e., an EV (e.g., exosome) a comprising construct comprising an optimized linker disclosed herein, e.g., a construct of Formula 1 where the biologically active moiety [BAM] is an anti-*STAT6* ASO described herein or a combination thereof.

[00408] In some aspects, an anti-STAT6 ASO of the present disclosure comprises the base sequence of SEQ ID NO: 1091. In some aspects, an anti-STAT6 ASO of the present disclosure comprises the STAT 6 ASO sequence shown in **FIG. 12**.

ASO targeting MYC

[00409] In some aspects, the biologically active molecule [BAM] is an anti *MYC* ASO. Unless indicated otherwise, the term "MYC," as used herein, can refer to MYC from one or more species (*e.g.*, humans, non-human primates, dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, and bears).

[00410] In some aspects, an anti-MYC ASO of the present disclosure comprises the base sequence of SEQ ID NO: 1092. In some aspects, and anti-MYC ASO of the present disclosure comprises the MYC ASO sequence shown in **FIG. 12**.

ASO targeting CEBP/β

[00411] In some aspects, the biologically active molecule [BAM] is an anti- *CEBP/β* ASO. Unless indicated otherwise, the term "CEBP/β," as used herein, can refer to CEBP/β from one or more species (*e.g.*, humans, non-human primates, dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, and bears).

[00412] The sequence for the human *CEBP/β* gene can be found under publicly available GenBank Accession Number NC_000020.11 (50190583..50192690). The human *CEBP/β* gene is found at chromosome location 20q13.13 at 50190583-50192690.

[00413] The sequence for the human *CEBP/β* pre-mRNA transcript (SEQ ID NO: 21) corresponds to the reverse complement of residues 50190583-50192690 of chromosome 20q13.13. The *CEBP/β* mRNA sequence (GenBank Accession No. NM_001285878.1) is provided in SEQ ID NO: 23, except that the nucleotide "t" in SEQ ID NO: 23 is shown as "u" in the mRNA. The sequence for human CEBP/β protein can be found under publicly available

Accession Numbers: P17676, (canonical sequence, SEQ ID NO: 22), P17676-2 (SEQ ID NO: 24), and P17676-3 (SEQ ID NO: 25), each of which is incorporated by reference herein in its entirety.

[00414] Natural variants of the human *CEBP/β* gene product are known. For example, natural variants of human CEBP/β protein can contain one or more amino acid substitutions selected from: A241P, A253G, G195S, and any combination thereof. Additional variants of human CEBP/β protein resulting from alternative splicing are also known in the art. CEBP/β Isoform 2 (identifier: P17676-2 at UniProt) differs from the canonical sequence (SEQ ID NO: 23) as follows: deletion of residues 1-23 relative to SEQ ID NO: 23. The sequence of CEBP/β Isoform 3 (identifier: P17676-3) differs from the canonical sequence (SEQ ID NO: 23) as follows: deletion of residues 1-198 relative to SEQ ID NO: 23. Therefore, the anti-*CEBPb* ASOs of the present disclosure can be designed to reduce or inhibit expression of the natural variants of the protein.

[00415] An example of a target nucleic acid sequence of the anti-*CEBPb* ASOs is *CEBP/β* pre-mRNA. SEQ ID NO: 21 represents a human *CEBP/β* genomic sequence (*i.e.*, reverse complement of nucleotides 50190583-50192690 of chromosome 20q13.13). SEQ ID NO: 21 is identical to a *CEBP/β* pre-mRNA sequence except that nucleotide "t" in SEQ ID NO: 21 is shown as "u" in pre-mRNA. In certain aspects, the "target nucleic acid" comprises an intron of a CEBP/β protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In other aspects, the target nucleic acid comprises an exon region of a CEBP/β protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In yet other aspects, the target nucleic acid comprises an exon-intron junction of a CEBP/β protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. The human CEBP/β protein sequence encoded by the *CEBP/β* pre-mRNA is shown as SEQ ID NO: 23. In other aspects, the target nucleic acid comprises an untranslated region of a CEBP/β protein-encoding nucleic acids or naturally occurring variants thereof, *e.g.*, 5' UTR, 3' UTR, or both.

[00416] In some aspects, an anti-*CEBPb* ASO of the disclosure hybridizes to a region within the introns of a *CEBP/β* transcript, *e.g.*, SEQ ID NO: 21. In certain aspects, an anti-*CEBPb* ASO of the disclosure hybridizes to a region within the exons of a *CEBP/β* transcript, *e.g.*, SEQ ID NO: 21. In other aspects, an anti-*CEBPb* ASO of the disclosure hybridizes to a region within the exon-intron junction of a *CEBP/β* transcript, *e.g.*, SEQ ID NO: 21. In some aspects, an anti-*CEBPb* ASO of the disclosure hybridizes to a region within a *CEBP/β* transcript

(*e.g.*, an intron, exon, or exon-intron junction), *e.g.*, SEQ ID NO: 21, wherein the anti-*CEBPb* ASO has a gapmer design.

[00417] In some aspects, the anti-*CEBPb* ASO targets a mRNA encoding a particular isoform of CEBP/β protein (*e.g.*, Isoform 1). In some aspects, the anti-*CEBPb* ASO targets all isoforms of CEBP/β protein. In other aspects, the anti-*CEBPb* ASO targets two isoforms (*e.g.*, Isoform 1 and Isoform 2, Isoform 1 and Isoform 3, or Isoform 2 and Isoform 3) of CEBP/β protein.

[00418] In some aspects, binding of an anti-*CEBPb* ASO targeting a *CEBPb* transcript disclosed herein to a mRNA transcript encoding *CEBPb* can reduce expression levels and/or activity levels of *CEBPb*.

[00419] In some aspects, any anti-*CEBPb* ASO described herein can be part of an EV (e.g., exosome) of the present disclosure, i.e., an EV (e.g., exosome) a comprising construct comprising an optimized linker disclosed herein, e.g., a construct of Formula 1 where the biologically active moiety [BAM] is an anti-*CEBPb* ASO described herein or a combination thereof.

ASO targeting STAT3

[00420] In some aspects, the biologically active molecule [BAM] is an anti-*STAT3* ASO. Signal Transducer and Activator of Transcription 3 (STAT3) is a signal transducer and activator of transcription that transmits signals from cell surface receptors to the nucleus. STAT3 is frequently hyperactivated in many human cancers.

[00421] Signal transducer and activator of transcription 3 (STAT3) is known in the art by various names. Such names include: DNA-binding protein APRF, and acute-phase response factor. The mRNA encoding human STAT3 can be found at Genbank Accession Number NM_003150.3, and is represented by the sequence (SEQ ID NO: 43).

[00422] Natural variants of the human *STAT3* gene product are known. Therefore, the ASOs of the present disclosure can be designed to reduce or inhibit expression of the natural variants of the STAT3 protein.

[00423] SEQ ID NO: 41 is identical to a *STAT3* pre-mRNA sequence except that nucleotide "t" in SEQ ID NO: 41 is shown as "u" in pre-mRNA. In certain aspects, the "target nucleic acid" comprises an intron of a STAT3 protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In other aspects, the target nucleic acid comprises an exon region of a STAT3 protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-

mRNA. In yet other aspects, the target nucleic acid comprises an exon-intron junction of a STAT3 protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. The human STAT3 protein sequence encoded by the *STAT3* pre-mRNA is shown as SEQ ID NO: 42. In other aspects, the target nucleic acid comprises an untranslated region of a STAT3 protein-encoding nucleic acids or naturally occurring variants thereof, *e.g.*, 5' UTR, 3' UTR, or both.

[00424] In yet other aspects, the target nucleic acid comprises an exon-intron junction of a STAT3 protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. The human STAT3 protein sequence encoded by the *STAT3* pre-mRNA is shown as SEQ ID NO: 43. In other aspects, the target nucleic acid comprises an untranslated region of a STAT3 protein-encoding nucleic acids or naturally occurring variants thereof, *e.g.*, 5' UTR, 3' UTR, or both.

[00425] In some aspects, an anti-*STAT3* ASO of the disclosure hybridizes to a region within the introns of a *STAT3* transcript, *e.g.*, SEQ ID NO: 41 or SEQ ID NO: 43. In certain aspects, an anti-*STAT3* ASO of the disclosure hybridizes to a region within the exons of a *STAT3* transcript, *e.g.*, SEQ ID NO: 41 or SEQ ID NO: 43. In other aspects, an anti-*STAT3* ASO of the disclosure hybridizes to a region within the exon-intron junction of a *STAT3* transcript, *e.g.*, SEQ ID NO: 41 or SEQ ID NO: 43. In some aspects, an anti-*STAT3* ASO of the disclosure hybridizes to a region within a *STAT3* transcript (*e.g.*, an intron, exon, or exon-intron junction), *e.g.*, SEQ ID NO: 41 or SEQ ID NO: 43, wherein the anti-*STAT3* ASO has a gapmer design.

[00426] In some aspects, the anti-*STAT3* ASO targets a mRNA encoding a particular isoform of STAT3 protein (*e.g.*, Isoform 1). In some aspects, the ASO targets all isoforms of STAT3 protein. In other aspects, the ASO targets two isoforms (*e.g.*, Isoform 1 (UniProt ID: P40763-1) and Isoform 2 (UniProt ID: P40763-2), Isoform 2 and Isoform 3 (UniProt ID: P40763-3) of STAT3 protein.

[00427] In some aspects, an anti-*STAT3* ASO of the disclosure hybridizes to a region within the introns of a *STAT3* transcript, *e.g.*, SEQ ID NO: 41 or SEQ ID NO: 43. In certain aspects, an anti-*STAT3* ASO of the disclosure hybridizes to a region within the exons of a *STAT3* transcript, *e.g.*, SEQ ID NO: 41 or SEQ ID NO: 43. In other aspects, an anti-*STAT3* ASO of the disclosure hybridizes to a region within the exon-intron junction of a *STAT3* transcript, *e.g.*, SEQ ID NO: 41 or SEQ ID NO: 43. In some aspects, an anti-*STAT3* ASO of the disclosure hybridizes to a region within a *STAT3* transcript (*e.g.*, an intron, exon, or exon-intron junction), *e.g.*, SEQ ID NO: 41 or SEQ ID NO: 43, wherein the ASO has a gapmer design.

[00428] In some aspects, the anti-*STAT3* ASO of the present disclosure hybridizes to multiple target regions within the *STAT3* transcript (*e.g.*, genomic sequence, SEQ ID NO: 41). In some aspects, the ASO hybridizes to two different target regions within the *STAT3* transcript. In some aspects, the anti-*STAT3* ASO hybridizes to three different target regions within the *STAT3* transcript. In some aspects, the anti-*STAT3* ASOs that hybridizes to multiple regions within the *STAT3* transcript (*e.g.*, genomic sequence, SEQ ID NO: 41) are more potent (*e.g.*, having lower EC50) at reducing *STAT3* expression compared to anti-*STAT3* ASOs that hybridizes to a single region within the *STAT3* transcript (*e.g.*, genomic sequence, SEQ ID NO: 41).

[00429] The anti-*STAT3* ASOs of the disclosure comprise a contiguous nucleotide sequence which corresponds to the complement of a region of *STAT3* transcript, *e.g.*, a nucleotide sequence corresponding to SEQ ID NO: 41.

[00430] In some aspects, binding of an anti-*STAT3* ASO targeting a *STAT3* transcript disclosed herein to a mRNA transcript encoding *STAT3* can reduce expression levels and/or activity levels of *STAT3*.

[00431] In some aspects, any anti-*STAT3* ASO described herein can be part of an EV (e.g., exosome) of the present disclosure, i.e., an EV (e.g., exosome) a comprising construct comprising an optimized linker disclosed herein, e.g., a construct of Formula 1 where the biologically active moiety [BAM] is an anti-*STAT3* ASO described herein or a combination thereof.

ASO targeting NRAS

[00432] In some aspects, the biologically active molecule [BAM] is an anti-*NRas* ASO. *NRas* is an oncogene encoding a membrane protein that shuttles between the Golgi apparatus and the plasma membrane. *NRas*-encoding genomic DNA can be found at Chromosomal position 1p13.2 (*i.e.*, nucleotides 5001 to 17438 of GenBank Accession No. NG_007572). Specifically, a combination of time-lapse microscopy and photobleaching techniques have revealed that in the absence of palmitoylation, GFP-tagged N-Ras undergoes rapid exchange between the cytosol and ER/Golgi membranes, and that wild-type GFP-N-Ras is recycled to the Golgi complex by a nonvesicular mechanism. N-ras mutations have been described in melanoma, thyroid carcinoma, teratocarcinoma, fibrosarcoma, neuroblastoma, rhabdomyosarcoma, Burkitt lymphoma, acute promyelocytic leukemia, T cell leukemia, and chronic myelogenous leukemia. Oncogenic N-Ras can induce acute myeloid leukemia (AML) or chronic myelomonocytic leukemia (CMML)–like disease in mice.

[00433] Neuroblastoma RAS viral oncogene (*NRas)* is known in the art by various names. Such names include: GTPase NRas, N-ras protein part 4, neuroblastoma RAS viral (v-ras) oncogene homolog neuroblastoma RAS viral oncogene homolog, transforming protein N-Ras, and v-ras neuroblastoma RAS viral oncogene homolog.

[00434] The NRAS gene provides instructions for making a protein called N-Ras that is involved primarily in regulating cell division. The mRNA sequence encoding human NRAS can be found at NCBI Reference sequence NM_002524.5 and is represented by the coding sequence (SEQ ID NO: 53).

[00435] Natural variants of the human *NRas* gene product are known. For example, natural variants of human NRas protein can contain one or more amino acid substitutions selected from: G12D, G13D, T50I, G60E, and any combinations thereof. Additional variants of human NRas protein resulting from alternative splicing are also known in the art, such as: G13R, Q61K, Q61R, and P34L. Therefore, the anti-*NRas* ASOs of the present disclosure can be designed to reduce or inhibit expression of the natural variants of the STAT3 protein.

[00436] SEQ ID NO: 51 is identical to a *NRas* pre-mRNA sequence except that nucleotide "t" in SEQ ID NO: 51 is shown as "u" in pre-mRNA. In certain aspects, the "target nucleic acid" comprises an intron of a NRas protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In other aspects, the target nucleic acid comprises an exon region of a NRas protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In yet other aspects, the target nucleic acid comprises an exon-intron junction of a NRas protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. The human NRas protein sequence encoded by the *NRas* pre-mRNA is shown as SEQ ID NO: 52. In other aspects, the target nucleic acid comprises an untranslated region of a NRas protein-encoding nucleic acids or naturally occurring variants thereof, *e.g.*, 5' UTR, 3' UTR, or both.

[00437] In certain aspects, the anti-*NRas* ASOs of the disclosure also are capable of down-regulating (*e.g.,* reducing or removing) expression of the *NRas* mRNA or protein. In this regard, the anti-*NRas* ASO of the disclosure can affect indirect inhibition of NRas protein through the reduction in *NRas* mRNA levels, typically in a mammalian cell, such as a human cell, such as a tumor cell. In particular, the present disclosure is directed to anti-*NRas* ASOs that target one or more regions of the *NRas* pre-mRNA (*e.g.*, intron regions, exon regions, and/or exon-intron junction regions). Unless indicated otherwise, the term "NRas," as used herein, can refer to NRas

from one or more species (*e.g.*, humans, non-human primates, dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, and bears).

[00438] In some aspects, an anti-*NRas* ASO of the disclosure hybridizes to a region within the introns of a *NRAS* transcript, *e.g.*, SEQ ID NO: 51 or SEQ ID NO: 53. In certain aspects, an ASO of the disclosure hybridizes to a region within the exons of a *NRAS* transcript, *e.g.*, SEQ ID NO: 51 or SEQ ID NO: 53. In other aspects, an ASO of the disclosure hybridizes to a region within the exon-intron junction of a *NRAS* transcript, *e.g.*, SEQ ID NO: 51 or SEQ ID NO: 53. In some aspects, an anti-*NRas* ASO of the disclosure hybridizes to a region within a *NRAS* transcript (*e.g.,* an intron, exon, or exon-intron junction), *e.g.*, SEQ ID NO: 51 or SEQ ID NO: 53, wherein the ASO has a gapmer design.

[00439] In some aspects, the anti-*NRas* ASO of the present disclosure hybridizes to multiple target regions within the *NRas* transcript (*e.g.*, genomic sequence, SEQ ID NO: 51). In some aspects, the anti-*NRas* ASO hybridizes to two different target regions within the *NRas* transcript. In some aspects, the anti-*NRas* ASO hybridizes to three different target regions within the *NRas* transcript. In some aspects, the anti-*NRas* ASOs that hybridizes to multiple regions within the *NRas* transcript (*e.g.*, genomic sequence, SEQ ID NO: 51) are more potent (*e.g.*, having lower EC50) at reducing *NRas* expression compared to anti-*NRas* ASOs that hybridizes to a single region within the *NRas* transcript (*e.g.*, genomic sequence, SEQ ID NO: 51).

[00440] In some aspects, the ASO targets a mRNA encoding a particular isoform of NRAS protein (*e.g.*, Isoform 1, NCBI ID: NP_001229821.1). In some aspects, the ASO targets all isoforms of NRas protein. In other aspects, the ASO targets two isoforms (*e.g.*, Isoform 1 and Isoform 2 (NCBI ID:NP_009089.4), Isoform 2 and Isoform 3(NCBI ID: NP_001123995), and Isoform 3 and Isoform 4(NCBI ID: NP_001229820.1)) of NRas protein.

[00441] The anti-*NRas* ASOs of the disclosure comprise a contiguous nucleotide sequence which corresponds to the complement of a region of *NRas* transcript, *e.g.*, a nucleotide sequence corresponding to SEQ ID NO: 51.

[00442] In some aspects, binding of an anti-*NRas* ASO targeting a *NRas* transcript disclosed herein to a mRNA transcript encoding *NRas* can reduce expression levels and/or activity levels of *NRas.*

[00443] In some aspects, any anti-*NRas* ASO described herein can be part of an EV (e.g., exosome) of the present disclosure, i.e., an EV (e.g., exosome) a comprising construct comprising an optimized linker disclosed herein, e.g., a construct of Formula 1 where the biologically active moiety [BAM] is an anti-*NRas* ASO described herein or a combination thereof.

ASO targeting KRAS

[00444] In some aspects, the biologically active molecule [BAM] is an anti-*KRAS* ASO. The sequence for the human *KRAS* gene can be found at chromosomal location 12p12.1 and under publicly available GenBank Accession Number NC_000012 (25,204,789 – 25,250,936). The genomic sequence for human wild-type *KRAS* transcript corresponds to the reverse complement of residues 25,204,789 – 25,250,936 of NC_000012 (SEQ ID NO: 35). The *KRAS G12D* genomic sequence provided in SEQ ID NO: 31 differs from SEQ ID NO: 35 in that it has a guanine to adenine substitution at nucleotide position 5,587. An exemplary *KRAS G12D* mRNA sequence is provided in SEQ ID NO: 33, except that the nucleotide "t" in SEQ ID NO: 33 is shown as "u" in the mRNA. The *KRAS G12D* mRNA provided in SEQ ID NO: 33 differs from the wild-type mRNA sequence (*e.g.*, GenBank Accession No. NM_004985.5; SEQ ID NO: 37) in that it has a guanine to adenine substitution at nucleotide position 225. The sequence for human KRAS protein can be found under publicly available Accession Numbers: P01116 (canonical sequence), A8K8Z5, B0LPF9, P01118, and Q96D10, each of which is incorporated by reference herein in its entirety.

[00445] There are two isoforms of the human KRAS protein (P01116), resulting from alternative splicing. Isoform 2A (Accession Number: P01116-1; SEQ ID NO: 38) is the canonical sequence. It is also known as K-Ras4A. Isoform 2B (Accession Number: P01116-2; also known as K-Ras4B; SEQ ID NO: 36) differs from the canonical sequence as follows: (i) 151-153: RVE → GVD; and (ii) 165-189: QYRLKKISKEEKTPGCVKIKKCIIM (SEQ ID NO:599) → KHKEKMSKDGKKKKKKSKTKCVIM (SEQ ID NO:600). In some aspects, anti-*KRAS* ASOs disclosed herein can reduce or inhibit expression of KRAS protein Isoform 2A, Isoform 2B, or both.

[00446] Natural variants of the human *KRAS* gene product are known. For example, natural variants of human KRAS protein can contain one or more amino acid substitutions selected from: K5E, K5N, G10GG, G10V, G12A, G12C, G12F, G12I, G12L, G12R, G12S, G12V, G13C, G13D, G13E, G13R, G13V, V14I, L19F, T20M, Q22E, Q22H, Q22K, Q22R, Q25H, N26Y, F28L, E31K, D33E, P34L, P34Q, P34R, I36M, R41K, D57N, T58I, A59T, G60D, G60R, G60S, G60V, Q61A, Q61H, Q61K, Q61L, Q61P, Q61R, E63K, S65N, R68S, Y71H, T74A, L79I, R97I, Q99E, M111L, K117N, K117R, D119G, S122F, T144P, A146P, A146T, A146V, K147E, K147T, R149K, L159S, I163S, R164Q, I183N, I84M, or combinations thereof. Natural variants that are specific to KRAS protein Isoform 2B contain one or more amino acid substitutions selected from: V152G, D153V, F156I, F156L, or combinations thereof. The anti-

KRAS ASOs of the present disclosure can be designed to reduce or inhibit expression of one or more of the variants of the KRAS protein (*e.g.*, any variants known in the art). In some aspects, a KRAS mutant has an amino acid substitution of G12D. In some aspects, the anti-*KRAS* ASOs of the present disclosure target one or more KRAS mutants. In other aspects, a KRAS mutant that the anti-*KRAS* ASOs target is KRAS G12D (SEQ ID NO: 32). Exemplary sequences for *KRAS G12D* mRNA and KRAS G12D protein are provided in SEQ ID NO: 33 and SEQ ID NO: 32.

[00447] In some aspects, a target nucleic acid sequence of an anti-*KRAS* ASO disclosed herein comprises one or more regions of a *KRAS* pre-mRNA. For example, SEQ ID NO: 31 (described above) is identical to a *KRAS* pre-mRNA sequence except that nucleotide "t" in SEQ ID NO: 31 is shown as "u" in the pre-mRNA. As used herein, the term "target nucleic acid sequence" refers to a nucleic acid sequence that is complementary to an anti-*KRAS* ASO disclosed herein. In certain aspects, the target nucleic acid sequence comprises an exon region of a KRAS protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In some aspects, the target nucleic acid sequence comprises an intron of a KRAS protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In further aspects, the target nucleic acid sequence comprises an exon-intron junction of a KRAS protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In some aspects, for example, when used in research or diagnostics, the target nucleic acid can be a cDNA or a synthetic oligonucleotide derived from DNA or RNA nucleic acid targets described herein. In some aspects, the target nucleic acid comprises an untranslated region of a KRAS protein-encoding nucleic acids or naturally occurring variants thereof, *e.g.*, 5' UTR, 3' UTR, or both.

[00448] Accordingly, in some aspects, an anti-*KRAS* ASO disclosed herein hybridizes to an exon region of a *KRAS* transcript, *e.g.*, SEQ ID NO: 31 or SEQ ID NO: 33. In some aspects, an anti-*KRAS* ASO of the present disclosure hybridizes to an intron region of a *KRAS* transcript, *e.g.*, SEQ ID NO: 31. In some aspects, an anti-*KRAS* ASO hybridizes to an exon-intron junction of a *KRAS* transcript, *e.g.*, SEQ ID NO: 31. In some aspects, an anti-*KRAS* ASO of the present disclosure hybridizes to a region within a *KRAS* transcript (*e.g.*, an intron, exon, or exon-intron junction), *e.g.*, SEQ ID NO: 31.

[00449] In some aspects, a target nucleic sequence of the ASOs disclosed herein is a *KRAS* mRNA, *e.g.*, SEQ ID NO: 33. Accordingly, in certain aspects, an anti-*KRAS* ASO disclosed herein can hybridize to one or more regions of a *KRAS* mRNA. In some aspects, anti-*KRAS* ASOs of the present disclosure target mRNA encoding a particular isoform of KRAS protein. In

certain aspects, anti-*KRAS* ASOs disclosed herein can target all isoforms of KRAS protein, including any variants thereof (*e.g.*, those described herein). In some aspects, a KRAS protein that can be targeted by anti-*KRAS* ASOs of the present disclosure comprises a G12D amino acid substitution.

[00450] In some aspects, binding of an anti-*KRAS* ASO targeting a *KRAS* transcript disclosed herein to a mRNA transcript encoding *KRAS* can reduce expression levels and/or activity levels of *KRAS*.

[00451] In some aspects, any anti-*KRAS* ASO described herein can be part of an EV (e.g., exosome) of the present disclosure, i.e., an EV (e.g., exosome) a comprising construct comprising an optimized linker disclosed herein, e.g., a construct of Formula 1 where the biologically active moiety [BAM] is an anti-*KRAS* ASO described herein or a combination thereof.

ASO targeting Pmp22

[00452] In some aspects, the biologically active molecule [BAM] is an anti-*Pmp22* ASO. Peripheral myelin protein 22 (PMP22) is also known as growth arrest-specific protein 3 (GAS-3), is encoded by the PMP22 gene. PMP22 is a 22 kDa transmembrane glycoprotein made up of 160 amino acids, and is mainly expressed in the Schwann cells of the peripheral nervous system. Schwann cells show high expression of PMP22, where it can constitute 2-5% of total protein content in compact myelin. Compact myelin is the bulk of the peripheral neuron's myelin sheath, a protective fatty layer that provides electrical insulation for the neuronal axon. The level of PMP22 expression is relatively low in the central nervous system of adults.

[00453] PMP22 plays an essential role in the formation and maintenance of compact myelin. When Schwann cells come into contact with a neuronal axon, expression of PMP22 is significantly up-regulated, whereas PMP22 is down-regulated during axonal degeneration or transection. PMP22 has shown association with zonula-occludens 1 and occludin, proteins that are involved in adhesion with other cells and the extracellular matrix, and also support functioning of myelin. Along with cell adhesion function, PMP22 is also up-regulated during Schwann cell proliferation, suggesting a role in cell-cycle regulation. PMP22 is detectable in non-neural tissues, where its expression has been shown to serve as growth-arrest-specific (gas-3) function.

[00454] Improper gene dosage of the PMP22 gene can cause aberrant protein synthesis and function of myelin sheath. Since the components of myelin are stoichiometrically set, any irregular expression of a component can cause destabilization of myelin and neuropathic

disorders. Alterations of PMP22 gene expression are associated with a variety of neuropathies, such as Charcot–Marie–Tooth type 1A (CMT1A), Dejerine–Sottas disease, and Hereditary Neuropathy with Liability to Pressure Palsy (HNPP). Too much PMP22 (e.g. caused by gene duplication) results in CMT1A. Gene duplication of PMP22 is the most common genetic cause of CMT where the overproduction of PMP22 results in defects in multiple signaling pathways and dysfunction of transcriptional factors like KNOX20, SOX10 and EGR2.

[00455] The sequence for the human *PMP22* gene can be found under publicly available as NCBI RefSeq Acc. No. NM_000304. Alternative RefSeq mRNA transcripts have accession numbers NM_001281455, NM-001281456, NM-153321, and NM_153322, respectively. The human *PMP22* gene is found at chromosome location 17p12 at 15,229,777-15,265,326.

[00456] The sequence for the human *PMP22* pre-mRNA transcript (SEQ ID NO: 264) corresponds to the reverse complement of residues 15,229,777-15,265,326, of chromosome location 17p12. The *PMP22* mRNA sequence (GenBank Accession No. NM_000304.4) is provided in SEQ ID NO: 58. The sequence for human PMP22 protein can be found under publicly available Uniprot Accession Number Q01453 (canonical sequence, SEQ ID NO: 60). Potential PMP22 isoforms have Uniprot Accession Numbers A8MU75, J3KQW0, A0A2R8Y5L5, J3KT36, and J3QS08, respectively. The publicly available contents of the database entries corresponding to accession numbers disclosed herein are incorporated by reference in their entireties.

[00457] The anti-*PMP22* ASOs of the present disclosure can be designed to reduce or inhibit expression of the natural variants of the PMP22 protein.

[00458] An example of a target nucleic acid sequence of the anti-*PMP22* ASOs is *PMP22* pre-mRNA. SEQ ID NO: 58 represents a human *PMP22* genomic sequence (*i.e.*, reverse complement of nucleotides 15,229,777-15,265,326, complement, of chromosome 17p12). SEQ ID NO: 58 is identical to a *PMP22* pre-mRNA sequence except that nucleotide "t" in SEQ ID NO: 58 is shown as "u" in pre-mRNA.

[00459] In some aspects, the anti-*PMP22* ASO comprises a contiguous nucleotide sequence of 10 to 30 nucleotides in length that is complementary to a nucleic acid sequence within nucleotides 1 to 1828 of a *PMP22* transcript corresponding to a nucleotide sequence as set forth in SEQ ID NO: 264 (PMP22 full mRNA transcript) or nucleotides 208 to 690 of a *PMP22* transcript corresponding to a nucleotide sequence as set forth in SEQ ID NO: 59 (PMP22 coding sequence).

[00460] In some aspects, the contiguous nucleotide sequence is at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100% complementary to the nucleic

acid sequence within the *PMP22* transcript. In some aspects, the anti-*PMP22* ASO is capable of reducing PMP22 protein expression in a human cell (e.g., a Schwan cell), wherein the human cell expresses the PMP22 protein.

[00461] In some aspects, the PMP22 protein expression is reduced by at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100% compared to PMP22 protein expression in a human cell that is not exposed to the anti-*PMP22* ASO.

[00462] In some aspects, the anti-*PMP22* ASO is capable of reducing a level of *PMP22* mRNA in a human cell (e.g., an immune cell), wherein the human cell expresses the *PMP22* mRNA. In some aspects, the level of *PMP22* mRNA is reduced by at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100% compared to the level of the *PMP22* mRNA in a human cell that is not exposed to the anti-*PMP22* ASO.

[00463] In certain aspects, the target nucleic acid comprises an intron of a PMP22 protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In other aspects, the target nucleic acid comprises an exon region of a PMP22 protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In yet other aspects, the target nucleic acid comprises an exon-intron junction of a PMP22 protein-encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, *e.g.*, pre-mRNA. In some aspects, for example when used in research or diagnostics the target nucleic acid can be a cDNA or a synthetic oligonucleotide derived from the above DNA or RNA nucleic acid targets. The human PMP22 coding sequence (CDS) is shows as SEQ ID NO: 59, and protein sequence encoded by the coding sequence in the *PMP22* pre-mRNA is shown as SEQ ID NO: 60. In other aspects, the target nucleic acid comprises an untranslated region of a PMP22 protein-encoding nucleic acids or naturally occurring variants thereof, *e.g.*, 5' UTR, 3' UTR, or both.

[00464] In some aspects, an anti-*PMP22* ASO of the disclosure hybridizes to a region within the introns of a *PMP22* transcript, *e.g.*, SEQ ID NO: 58. In certain aspects, an ASO of the disclosure hybridizes to a region within the exons of a *PMP22* transcript, *e.g.*, SEQ ID NO: 58. In other aspects, an anti-*PMP22* ASO of the disclosure hybridizes to a region within the exon-intron junction of a *PMP22* transcript, *e.g.*, SEQ ID NO: 58.

[00465] In some aspects, any anti-*PMP22* ASO described herein can be part of an EV (e.g., exosome) of the present disclosure, i.e., an EV (e.g., exosome) a comprising construct comprising an optimized linker disclosed herein, e.g., a construct of Formula 1 where the biologically active moiety [BAM] is an anti-*PMP22* ASO described herein or a combination thereof.

Extracellular Vesicle (EV), e.g., Exosome

[00466] The constructs comprising an optimized linker disclose herein, e.g., the constructs of Formula 1, can comprise at least one anchoring moiety [AM] that attaches the construct to an Extracellular Vesicle (EV), e.g., an exosome. The EVs (*e.g.,* exosomes) of the present disclosure can have a diameter between about 20 and about 300 nm. In certain aspects, an EV (*e.g.,* exosome) of the present disclosure has a diameter between about 20 to about 290 nm, about 20 to about 280 nm, about 20 to about 270 nm, about 20 to about 260 nm, about 20 to about 250 nm, about 20 to about 240 nm, about 20 to about 230 nm, about 20 to about 220 nm, about 20 to about 210 nm, about 20 to about 200 nm, about 20 to about 190 nm, about 20 to about 180 nm, about 20 to about 170 nm, about 20 to about 160 nm, about 20 to about 150 nm, about 20 to about 140 nm, about 20 to about 130 nm, about 20 to about 120 nm, about 20 to about 110 nm, about 20 to about 100 nm, about 20 to about 90 nm, about 20 to about 80 nm, about 20 to about 70 nm, about 20 to about 60 nm, about 20 to about 50 nm, about 20 to about 40 nm, or about 20 to about 30 nm. The size of the EV (*e.g.,* exosome) described herein can be measured according to methods known in the art.

[00467] EVs (*e.g.,* exosomes) of the present disclosure comprise a bi-lipid membrane ("exosome membrane" or "EV membrane"), comprising an interior surface (luminal surface) and an exterior surface. The interior surface faces the inner core of the EV (*e.g.,* exosome), i.e., the lumen of the EV.

[00468] The EV or exosome membrane comprises lipids and fatty acids. Exemplary lipids comprise phospholipids, glycolipids, fatty acids, sphingolipids, phosphoglycerides, sterols, cholesterols, and phosphatidylserines. The EV or exosome membrane comprises an inner leaflet and an outer leaflet. The composition of the inner and outer leaflet can be determined by transbilayer distribution assays known in the art, *see, e.g.*, Kuypers *et al., Biohim Biophys Acta* 1985 819:170.

[00469] In some aspects, the composition of the outer leaflet is between about 70% and about 90% choline phospholipids, between about 0% and about 15% acidic phospholipids, and between about 5% and about 30% phosphatidylethanolamine. In some aspects, the composition

of the inner leaflet is between about 15% and about 40% choline phospholipids, between about 10% and about 50% acidic phospholipids, and between about 30% and about 60% phosphatidylethanolamine. In some aspects, the EV or exosome membrane comprises one or more polysaccharides, such as glycan. Glycans on the surface of the EV or exosomes can serve as an attachment to a maleimide moiety or a linker that connect the glycan and a maleimide moiety. The glycan can be present on one or more proteins on the surface of an EV (*e.g.,* exosome), for example, a Scaffold X, such as a PTGFRN polypeptide, or on the lipid membrane of the EV (*e.g.,* exosome). Glycans can be modified to have thiofucose that can serve as a functional group for attaching a maleimide moiety to the glycans. In some aspects, the Scaffold X can be modified to express a high number of glycan to allow additional attachments on the EV (*e.g.,* exosome).

Methods of Making

[00470] EVs (*e.g.,* exosomes) of the present disclosure can be produced by chemical synthesis, recombinant DNA technology, biochemical or enzymatic fragmentation of larger molecules, combinations of the foregoing or by any other method. In one aspect, the present disclosure provides a method of attaching a biologically active molecule to an EV (*e.g.,* exosome) via an optimized link disclosed herein, e.g., via solid phase synthesis or conjugation.

[00471] **Exosome production**: In some aspects, EVs disclosed herein (*e.g.*, exosomes) can be produced from a cell grown *in vitro* or a body fluid of a subject. When exosomes are produced from *in vitro* cell culture, various producer cells, *e.g.,* HEK293 cells, CHO cells, and MSCs, can be used. In certain aspects, the producer cell is HEK293 cells. In some aspects, a producer cell is not a dendritic cell, macrophage, B cell, mast cell, neutrophil, Kupffer-Browicz cell, cell derived from any of these cells, or any combination thereof.

[00472] Human embryonic kidney 293 cells, also often referred to as HEK 293, HEK-293, 293 cells, or less precisely as HEK cells, are a specific cell line originally derived from human embryonic kidney cells grown in tissue culture.

[00473] HEK 293 cells were generated in 1973 by transfection of cultures of normal human embryonic kidney cells with sheared adenovirus 5 DNA in Alex van der Eb's laboratory in Leiden, the Netherlands. The cells were cultured and transfected by adenovirus. Subsequent analysis has shown that the transformation was brought about by inserting ~4.5 kilobases from the left arm of the viral genome, which became incorporated into human chromosome 19.

[00474] A comprehensive study of the genomes and transcriptomes of HEK 293 and five derivative cell lines compared the HEK 293 transcriptome with that of human kidney, adrenal,

pituitary and central nervous tissue. The HEK 293 pattern most closely resembled that of adrenal cells, which have many neuronal properties. HEK 293 cells have a complex karyotype, exhibiting two or more copies of each chromosome and with a modal chromosome number of 64. They are described as hypotriploid, containing less than three times the number of chromosomes of a haploid human gamete. Chromosomal abnormalities include a total of three copies of the X chromosome and four copies of chromosome 17 and chromosome 22. Variants of HEK293 cells useful to produce EVs include, but are not limited to, HEK 293F, HEK 293FT, and HEK 293T.

[00475] **Solid-phase synthesis**: Solid phase synthesis known in the art may additionally or alternatively be employed to generate the constructs disclosed in the present application. In some aspects, two or more components of an optimized linker disclosed herein can be attached to each other (e.g., concatenated) using solid phase synthesis. For example, an ASO can be synthesized, and diferent spacers or combinations thereof can be added to the ASO via conventional synthetic steps. Examplary phosphoramidite spacers (e.g., C3-phosphoramidite, TEG-phosphoramidite, and HEG-phosphoramidite) are presented in **FIG. 4**. In some aspects, the spacer or combination of spacers can be further extended via synthesis to incorporate the membrane anchor moiety, as exemplified in **FIG. 3**, which shows phosphoramidites that can be used to generate the optimized linkers of the present disclosure via solid phase synthesis such as octyl-tocopherol phosphoramidite, tocopherol phosphoramidite, palmitate-C6 phosphoramidite, cholesterol-TEG phosphoramidite or or cholesterol-C6 phosphoramidite. Suitable solid phase techniques, including automated synthesis techniques, are described, e.g., in F. Eckstein (ed.), Oligonucleotides and Analogues, a Practical Approach, Oxford University Press, New York (1991) and Toy, P.H.; Lam, Y (ed.), Solid-Phase Organic synthesis, concepts, Strategies, and Applications, John Wiley & Sons, Inc. New Jersey (2012).

[00476] **Conjugation**: In some aspects, two or more components of an optimized linker disclosed herein can be attached to each other (e.g., concatenated) using conjugation. Besides amine-reactive compounds, those having chemical groups that form bonds with sulfhydryls (–SH) are the most common crosslinkers and modification reagents for protein and other bioconjugate techniques. Sulfhydryls, also called thiols, exist in proteins in the side-chain of cysteine (Cys, C) amino acids. Pairs of cysteine sulfhydryl groups are often linked by disulfide bonds (–S–S–) within or between polypeptide chains as the basis of native tertiary or quaternary protein structure. Typically, only free or reduced sulfhydryl groups (–SH) [rather than sulfur atoms in disulfide bonds] are available for reaction with thiol-reactive compounds.

[00477] Sulfhydryl groups are useful targets for protein conjugation and labeling. First, sulfhydryls are present in most proteins but are not as numerous as primary amines; thus,

crosslinking via sulfhydryl groups is more selective and precise. Second, sulfhydryl groups in proteins are often involved in disulfide bonds, so crosslinking at these sites typically does not significantly modify the underlying protein structure or block binding sites. Third, the number of available (i.e., free) sulfhydryl groups can be easily controlled or modified; they can be generated by reduction of native disulfide bonds, or they can be introduced into molecules through reaction with primary amines using sulfhydryl-addition reagents, such as 2-iminothiolane (Traut's Reagent), SATA, SATP, or SAT(PEG). Finally, combining sulfhydryl-reactive groups with amine-reactive groups to make heterobifunctional crosslinkers provides greater flexibility and control over crosslinking procedures. For example, using 3-maleimido-propionic NHS ester, which contains a maleimide group and an NHS ester, the NHS ester can be used to label the primary amines (-NH2) of proteins, amine-modified oligonucleotides, and other amine-containing molecules. The maleimide group will react with a thiol group to form a covalent bond, enabling the connection of biomolecule with a thiol.

[00478] The maleimide group reacts specifically with sulfhydryl groups when the pH of the reaction mixture is between 6.5 and 7.5; the result is formation of a stable thioether linkage that is not reversible (i.e., the bond cannot be cleaved with reducing agents). In more alkaline conditions (pH >8.5), the reaction favors primary amines and also increases the rate of hydrolysis of the maleimide group to a non-reactive maleamic acid. Maleimides do not react with tyrosines, histidines or methionines.

[00479] Thiol-containing compounds, such as dithiothreitol (DTT) and beta-mercaptoethanol (BME), must be excluded from reaction buffers used with maleimides because they will compete for coupling sites. For example, if DTT were used to reduce disulfides in a protein to make sulfhydryl groups available for conjugation, the DTT would have to be thoroughly removed using a desalting column before initiating the maleimide reaction. Interestingly, the disulfide-reducing agent TCEP does not contain thiols and does not have to be removed before reactions involving maleimide reagents.

[00480] Excess maleimides can be quenched at the end of a reaction by adding free thiols. EDTA can be included in the coupling buffer to chelate stray divalent metals that otherwise promote oxidation of sulfhydryls (non-reactive).

[00481] In one aspect, the linking comprises treating the EV (*e.g.,* exosome) with a reducing agent. Suitable reducing agents include, for example, TCEP (Tris(2-carboxyethyl)phosphine), DTT (dithiothreitol), BME (2-mercaptoethanol), a thiolating agent, and any combination thereof. The thiolating agent can comprise, *e.g.,* Traut's reagent (2-iminothiolane).

[00482] After the treatment with the reducing agent, the linking reaction further comprises bringing the reduced EV (*e.g.,* exosome) in contact with the maleimide moiety. In one aspect, the maleimide moiety is linked to a biologically active molecule prior to the linking to the EV (*e.g.,* exosome). In some aspects, the maleimide moiety is further attached to a linker to connect the maleimide moiety to the biologically active molecule. Accordingly, in some aspects, one or more linkers or spacers are interposed between the maleimide moiety and the biologically active molecule.

[00483] Any of the anchoring moieties [AM], spacer [SP] or spacer combinations, or biologically active molecules [BAM] disclosed herein can be conjugated to a reactive moiety, e.g., an amino reactive moiety (e.g., NHS-ester, p-nitrophenol, isothiocyanate, isocyanate, or aldehyde), a thiol reactive moiety (e.g., acrylate, maleimide, or pyridyl disulfide), a hydroxy reactive moiety (e.g., isothiocyanate or isocyanate), a carboxylic acid reactive moiety (e.g., epoxide), or an azide reactive moiety (e.g., alkyne).

[00484] Exemplary reactive moieties that can be used to covalent bind two components disclosed herein (e.g., an anchoring moiety [AM] and a spacer [SP], two spacers [SP], a spacer [SP] and a biologally active molecule [BAM], or an anchoring moiety [AM] and a biologically acitve moiety [BAM]) via chemical conjugation include, e.g., N-succinimidyl-3-(2-pyridyldithio)propionate, N-4-maleimide butyric acid, S-(2-pyridyldithio)cysteamine, iodoacetoxysuccinimide, N-(4-maleimidebutyryl oxy)succinimide, N-[5-(3′-maleimide propylamide)-1-carboxypentyl]iminodiacetic acid, N-(5-aminopentyl)iminodiacetic acid, and 1′-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite). Bifunctional linkers (linkers containing two functional groups) are also usable.

[00485] In some aspects, an anchoring moiety [AM], spacer [SP], or biologically active molecule [BAM] can comprise a terminal oxyamino group, e.g., —ONH_2, a hydrazino group, —$NHNH_2$, a mercapto group (i.e., SH or thiol), or an olefin (e.g., $CH{=}CH_2$). In some aspects, an anchoring moiety [AM], spacer [SP], or biologically active molecule [BAM] can comprise an electrophilic moiety, e.g., at a terminal position, e.g., an aldehyde, alkyl halide, mesylate, tosylate, nosylate, or brosylate, or an activated carboxylic acid ester, e.g. an NHS ester, a phosphoramidite, or a pentafluorophenyl ester. In some aspects, a covalent bond can be formed by coupling a nucleophilic group of a ligand, e.g., a hydroxyl, a thiol or amino group, with an electrophilic group The present invention is amenable to all manner of reactive groups and reactive moieties including but not limited to those known in the art.

[00486] The term "protecting group," as used herein, refers to a labile chemical moiety which is known in the art to protect reactive groups including without limitation, hydroxyl,

amino and thiol groups, against undesired reactions during synthetic procedures. Protecting groups are typically used selectively and/or orthogonally to protect sites during reactions at other reactive sites and can then be removed to leave the unprotected group as is or available for further reactions. Protecting groups as known in the art are described generally in Greene and Wuts, *Protective Groups in Organic Synthesis,* 3rd edition, John Wiley & Sons, New York (1999).

[00487] Additionally, the various synthetic steps may be performed in an alternate sequence or order to give the desired compounds. Synthetic chemistry transformations and protecting group methodologies (protection and deprotection) useful in synthesizing the compounds described herein are known in the art and include, for example, those such as described in R. Larock, Comprehensive Organic Transformations, VCH Publishers (1989); T. W. Greene and P. G. M. Wuts, Protective Groups in Organic Synthesis, 2d. Ed., John Wiley and Sons (1991); L. Fieser and M. Fieser, Fieser and Fieser's Reagents for Organic Synthesis, John Wiley and Sons (1994); and L. Paquette, ed., Encyclopedia of Reagents for Organic Synthesis, John Wiley and Sons (1995), and subsequent editions thereof.

[00488] **Amine reactive moieties:** In some aspects, the reactive moiety is an amine reactive moiety. As used herein the term "amine reactive moiety" refers to a chemical group which can react with a reactive group having an amino moiety, e.g., primary amines. Exemplary amine reactive moieties are N-hydroxysuccinimide esters (NHS-ester), p-nitrophenol, isothiocyanate, isocyanate, and aldehyde. Alternative reactive moieties that react with primary amines are also well known in the art. In some aspects, an amine reactive moiety can be attached to a terminal position of an anchoring moiety [AM], spacer [SP], or biologically active molecule [BAM] of the present disclosure. In some aspects, the amine reactive moiety is a NHS-ester. Typically, a NHS-ester reactive moiety reacts with a primary amine of a reactive group to yield a stable amide bond and N-hydroxysuccinimide (NHS). In some aspects, the amine reactive moiety is a p-nitrophenol group. Typically, a p-nitrophenol reactive moiety is an activated carbamate that reacts with a primary amine of a reactive group to yield a stable carbamate moiety and p-nitrophenol. In some aspects, the amine reactive moiety is an isothiocyanate. Typically, an isothiocyanate reacts with a primary amine of a reactive group to yield a stable thiourea moiety. In some aspects, the amine reactive moiety is an isocyanate. Typically, an isocyanate reacts with a primary amine of a reactive group to yield a stable urea moiety. In some aspects, amine the reactive moiety is an aldehyde. Typically, aldehydes react with primary amines to form Schiff bases which can be further reduced to form a covalent bond through reductive amination.

[00489] **Thiol reactive moieties**: In some aspects, the reactive moiety is a thiol reactive moiety. As used herein the term "thiol reactive moiety" refers to a chemical group which can react with a reactive group having a thiol moiety (or mercapto group). Exemplary thiol reactive moieties are acrylates, maleimides, and pyridyl disulfides. Alternative reactive moieties that react with thiols are also well known in the art. In some aspects, a thiol reactive moiety can be attached to a terminal position of an anchoring moiety [AM], spacer [SP], or biologically active molecule [BAM] of the present disclosure. In some aspects, the thiol reactive moiety is an acrylate. Typically, acrylates react with thiols at the carbon β to the carbonyl of the acrylate to form a stable sulfide bond. In some aspects, the thiol reactive moiety is a maleimide. Typically, maleimides react with thiols at either the carbon β or the carbonyls to form a stable sulfide bond. In some aspects, the thiol reactive moiety is a pyridyl disulfide. Typically, pyridyl disulfides react with thiols at the sulfur atom β to the pyridyl to form a stable disulfide bond and pyridine-2-thione.

[00490] **Hydroxy reactive moieties**: In some aspects, the reactive moiety is a hydroxyl reactive moiety. As used herein the term "hydroxyl reactive moiety" refers to a chemical group which can react with a reactive group having a hydroxyl moiety. Exemplary hydroxyl reactive moieties are isothiocyanates and isocyanates. Alternative reactive moieties that react with hydroxyl moieties are also well known in the art. In some aspects, a hydroxyl reactive moiety can be attached to a terminal position of an anchoring moiety [AM], spacer [SP], or biologically active molecule [BAM] of the present disclosure. In some aspects, the hydroxyl reactive moiety is an isothiocyanate. Typically, an isothiocyanate reacts with a hydroxyl of a reactive group to yield a stable carbamothioate moiety. In some aspects, amine the reactive moiety is an isocyanate. Typically, an isocyanate reacts with a hydroxyl of a reactive group to yield a stable carbamate moiety.

[00491] **Carboxylic acid reactive moieties**: In some aspects, the reactive moiety is a carboxylic acid reactive moiety. As used herein the term "carboxylic acid reactive moiety" refers to a chemical group which can react with a reactive group having a carboxylic acid moiety. An exemplary carboxylic acid reactive moiety is an epoxide. Alternative reactive moieties that react with carboxylic acid moieties are also well known in the art. In some aspects, a carboxylic acid reactive moiety can be attached to a terminal position of an anchoring moiety [AM], spacer [SP], or biologically active molecule [BAM] of the present disclosure. In some aspects, the carboxylic acid reactive moiety is an epoxide. Typically, an epoxide reacts with the carboxylic acid of a reactive group at either of the carbon atoms of the epoxide to form a 2-hydroxyethyl acetate moiety.

[00492] **Azide reactive moieties:** In some aspects, the reactive moiety is an azide reactive moiety. As used herein the term "azide reactive moiety" refers to a chemical group which can react with a reactive group having an azide moiety. An exemplary azide reactive moiety is an alkyne. Alternative reactive moieties that react with azide moieties are also well known in the art. In some aspects, a carboxylic acid reactive moiety can be attached to a terminal position of an anchoring moiety [AM], spacer [SP], or biologically active molecule [BAM] of the present disclosure. In some aspects, the azide reactive moiety is an alkyne. Typically, an alkyne reacts with the azide of a reactive group through a 1,3-dipolar cycloaddition reaction, also referred to "click chemistry," to form a 1,2,3-triazole moiety.

Therapeutic Uses

[00493] The present disclosure provides methods of treating a disease or condition is a subject in need thereof comprising administering a composition comprising EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, to the subject. The present disclosure also provides methods of preventing or ameliorating the symptoms of a disease or condition is a subject in need thereof comprising administering a composition comprising EVs (*e.g.,* exosomes) of the present disclosure to the subject. The present disclosure also provides methods to diagnose a disease or condition in a subject in need thereof comprising administering a composition comprising EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, to the subject.

[00494] In one aspect, the disease or disorder is a cancer, an inflammatory disease, a neurodegenerative disorder, a central nervous disease or a metabolic disease.

[00495] Present disclosure also provides methods of preventing and/or treating a disease or disorder in a subject in need thereof, comprising administering an EV (*e.g.,* exosome) of the present disclosure, i.e., an EV comprising an optimized linker disclosed herein, to the subject. In some aspects, a disease or disorder that can be treated with the present methods comprises a cancer, graft-versus-host disease (GvHD), autoimmune disease, infectious diseases, or fibrotic diseases. In some aspects, the treatment is prophylactic. In other aspects, the EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, are used to induce an immune response. In other aspects, the EVs (*e.g.,* exosomes) for the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, are used to vaccinate a subject.

[00496] In some aspects, the disease or disorder is a cancer. When administered to a subject with a cancer, in certain aspects, EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs

comprising an optimized linker disclosed herein, can up-regulate an immune response and enhance the tumor targeting of the subject's immune system. In some aspects, the cancer being treated is characterized by infiltration of leukocytes (T-cells, B-cells, macrophages, dendritic cells, monocytes) into the tumor microenvironment, or so-called "hot tumors" or "inflammatory tumors." In some aspects, the cancer being treated is characterized by low levels or undetectable levels of leukocyte infiltration into the tumor microenvironment, or so-called "cold tumors" or "non-inflammatory tumors." In some aspects, an EV (*e.g.,* exosome) is administered in an amount and for a time sufficient to convert a "cold tumor" into a "hot tumor, " i.e., said administering results in the infiltration of leukocytes (such as T-cells) into the tumor microenvironment. In certain aspects, cancer comprises bladder cancer, cervical cancer, renal cell cancer, testicular cancer, colorectal cancer, lung cancer, head and neck cancer, and ovarian, lymphoma, liver cancer, glioblastoma, melanoma, myeloma, leukemia, pancreatic cancers, or combinations thereof. In other aspects, the terms "distal tumor" or "distant tumor" refer to a tumor that has spread from the original (or primary) tumor to distant organs or distant tissues, *e.g.,* lymph nodes. In some aspects, the EVs (*e.g.,* exosomes) of the disclosure, i.e., EVs comprising an optimized linker disclosed herein, can treat a tumor after the metastatic spread.

[00497] In some aspects, the disease or disorder is a graft-versus-host disease (GvHD). In some aspects, the disease or disorder that can be treated with the present disclosure is an autoimmune disease. Non-limiting examples of autoimmune diseases include: multiple sclerosis, peripheral neuritis, Sjogren's syndrome, rheumatoid arthritis, alopecia, autoimmune pancreatitis, Behcet's disease, bullous pemphigoid, celiac disease, Devic's disease (neuromyelitis optica), glomerulonephritis, IgA nephropathy, assorted vasculitides, scleroderma, diabetes, arteritis, vitiligo, ulcerative colitis, irritable bowel syndrome, psoriasis, uveitis, systemic lupus erythematosus, and combinations thereof.

[00498] In some aspects, the disease or disorder is an infectious disease. In certain aspects, the disease or disorder is an oncogenic virus. In some aspects, infectious diseases that can be treated with the present disclosure includes, but not limited to, human Gamma herpes virus 4 (Epstein Barr virus), influenza A virus, influenza B virus, cytomegalovirus, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, HIV-1, HIV-2, corona viruses (*e.g.,* MERS-CoV and SARS CoV), filoviruses (*e.g.,* Marburg and Ebola), Streptococcus pyogenes, *Streptococcus pneumoniae*, *Plasmodia* species (*e.g., vivax* and *falciparum*), Chikunga virus, human Papilloma virus (HPV), hepatitis B, hepatitis C, human herpes virus 8, herpes simplex virus 2 (HSV2), *Klebsiella sp.*, *Pseudomonas aeruginosa*, *Enterococcus sp.*, *Proteus sp.*,

Enterobacter sp., *Actinobacter sp.*, coagulase-negative staphylococci (CoNS), *Mycoplasma sp.*, or combinations thereof.

[00499] In some aspects, a disease or disorder that can be treated with the present methods comprises a Pompe disease, Gaucher, a lysosomal storage disorder, mucovicidosis, cystic fibrosis, Duchenne and Becker muscular dystrophy, transthyretin amyloidosis, hemophilia A, hemophilia B, adenosine-deaminase deficiency, Leber's congenital amaurosis, X-linked adrenoleukodystrophy, metachromatic leukodystrophy, OTC deficiency, glycogen storage disease 1A, Criggler-Najjar syndrome, primary hyperoxaluria type 1, acute intermittent porphyria, phenylketonuria, familial hypercholesterolemia, mucopolysaccharidosis type VI, α1 antitrypsin deficiency, Retts Syndrome, Dravet Syndrome, Angelman Syndrome, DM1 disease, Fragile X disease, Huntingtons Disease, Friedreichs ataxia, CMT disease (also known as Charcot-Marie-Tooth disease, hereditary motor and sensory neuropathy (HMSN), or peroneal muscular atrophy), CMT1X disease, catecholaminergic polymorphic ventricular tachycardia, spinocerebellar ataxia type 3 (SCA3) disease, limb-girdle muscular dystrophy, or a hypercholesterolemia. In some aspects, the treatment is prophylactic.

[00500] In some aspects, the disease or disorder is a neurodegenerative disease. In some aspects, the neurodegenerative disease is selected from Alzheimer's disease, Parkinson's disease, prion disease, motor neuron disease, Huntington's disease, spinocerebellar ataxia, spinal muscular atrophy, and any combination thereof.

[00501] In certain aspects, the disease or disorder comprises a muscular dystrophy. In some aspects, the muscular dystrophy is selected from Duchenne type muscular dystrophy (DMD), myotonic muscular dystrophy, facioscapulohumeral muscular dystrophy (FSHD), congenital muscular dystrophy, limb-girdle muscular dystrophy (including, but not limited to, LGMD2B, LGMD2D, LGNMD2L, LGMD2C, LGMD2E and LGMD2A), and any combination thereof.

[00502] In some aspects, the disease or disorder is selected from AADC deficiency (CNS), ADA-SCID, Alpha-1 antitrypsin deficiency, β-thalassemia (severe sickle cell), Cancer (head and neck squamous cell), Niemman-Pick Type C Disease, Cerebral ALD, Choroideremia, Congestive heart failure, Cystic Fibrosis, Duchenne muscular dystrophy (DMD), Fabry disease, Glaucoma, Glioma (cancer), Hemophilia A, Hemophilia B, HoFH (hypercholesterolemia), Huntington's Disease, Lipoprotein lipase deficiency, Leber hereditary optic neuropathy (LHON), Metachromatic leukodystrophy, MPS I (Hurler syndrome), MPS II (Hunter's syndrome), MPS III (Sanfilippo Syndrome), Parkinson's disease, Pompe Disease, Recessive Dystrophic

Epidermolysis Bullosa, RPE65 deficiency (vision loss), Spinal Muscular Atrophy (SMA I), Wet AMD (retinal disease), Wiskott Aldrich syndrome (WAS), Mucopolysaccharidosis type IIIA (MPS IIIA), X-linked myotubular myopathy, X-linked retinitis pigmentosa, and any combination thereof.

[00503] In some aspects, the EVs (*e.g.,* exosomes) are administered intravenously to the circulatory system of the subject. In some aspects, the EVs (*e.g.,* exosomes) are infused in suitable liquid and administered into a vein of the subject. In some aspects, the EVs (*e.g.,* exosomes) are administered intra-arterialy to the circulatory system of the subject. In some aspects, the EVs (*e.g.,* exosomes) are infused in suitable liquid and administered into an artery of the subject. In some aspects, the EVs (*e.g.,* exosomes) are administered to the subject by intrathecal administration. In some aspects, the EVs (*e.g.,* exosomes) are administered via an injection into the spinal canal, or into the subarachnoid space so that it reaches the cerebrospinal fluid (CSF). In some aspects, the EVs (*e.g.,* exosomes) are administered intratumorally into one or more tumors of the subject. In some aspects, the EVs (*e.g.,* exosomes) are administered to the subject by intranasal administration. In some aspects, the EVs (*e.g.,* exosomes) can be insufflated through the nose in a form of either topical administration or systemic administration. In certain aspects, the EVs (*e.g.,* exosomes) are administered as nasal spray. In some aspects, the EVs (*e.g.,* exosomes) are administered to the subject by intraperitoneal administration. In some aspects, the EVs (*e.g.,* exosomes) are infused in suitable liquid and injected into the peritoneum of the subject. In some aspects, the intraperitoneal administration results in distribution of the EVs (*e.g.,* exosomes) to the lymphatics. In some aspects, the intraperitoneal administration results in distribution of the EVs (*e.g.,* exosomes) to the thymus, spleen, and/or bone marrow. In some aspects, the intraperitoneal administration results in distribution of the EVs (*e.g.,* exosomes) to one or more lymph nodes. In some aspects, the intraperitoneal administration results in distribution of the EVs (*e.g.,* exosomes) to one or more of the cervical lymph node, the inguinal lymph node, the mediastinal lymph node, or the sternal lymph node. In some aspects, the intraperitoneal administration results in distribution of the EVs (*e.g.,* exosomes) to the pancreas. In some aspects, the EVs (*e.g.,* exosomes) are administered to the subject by periocular administration. In some aspects, the EVs (*e.g.,* exosomes) are injected into the periocular tissues. Periocular drug administration includes the routes of subconjunctival, anterior sub-Tenon's, posterior sub-Tenon's, and retrobulbar administration.

Pharmaceutical Compositions and Methods of Administration

[00504] The present disclosure also provides pharmaceutical compositions comprising EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, that are suitable for administration to a subject. The pharmaceutical compositions generally comprise a plurality of EVs (*e.g.,* exosomes) comprising a biologically active molecule covalently linked to the plurality of EVs (*e.g.,* exosomes) via an optimized linker disclosed herein and a pharmaceutically-acceptable excipient or carrier in a form suitable for administration to a subject. Pharmaceutically acceptable excipients or carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions comprising a plurality of EVs (*e.g.,* exosomes). (See, *e.g.*, Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa. 18th ed. (1990)). The pharmaceutical compositions are generally formulated sterile and in full compliance with all Good Manufacturing Practice (GMP) regulations of the U.S. Food and Drug Administration. In some aspects, the pharmaceutical composition comprises one or more chemical compounds, such as for example, small molecules covalently linked to an EV (*e.g.,* exosome) of the present disclosure, i.e., an EV comprising an optimized linker disclosed herein,.

[00505] In some aspects, a pharmaceutical composition comprises one or more therapeutic agents and an EV (*e.g.,* exosome) of the present disclosure, i.e., an EV comprising an optimized linker disclosed herein. In certain aspects, the EVs (*e.g.,* exosomes) are co-administered with of one or more additional therapeutic agents, in a pharmaceutically acceptable carrier. In some aspects, the pharmaceutical composition comprising the EV (*e.g.,* exosome) is administered prior to administration of the additional therapeutic agents. In other aspects, the pharmaceutical composition comprising the EV (*e.g.,* exosome) is administered after the administration of the additional therapeutic agents. In further aspects, the pharmaceutical composition comprising the EV (*e.g.,* exosome) is administered concurrently with the additional therapeutic agents.

[00506] The present disclosure provides pharmaceutical compositions comprising an EV (*e.g.,* exosome) of the present disclosure, i.e., an EV comprising an optimized linker disclosed herein, having the desired degree of purity, and a pharmaceutically acceptable carrier or excipient, in a form suitable for administration to a subject. Pharmaceutically acceptable excipients or carriers can be determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions comprising a plurality of extracellular vesicles. (*See*, *e.g.*, Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa. 21st ed. (2005)). The pharmaceutical compositions are generally formulated sterile

and in full compliance with all Good Manufacturing Practice (GMP) regulations of the U.S. Food and Drug Administration.

[00507] In some aspects, a pharmaceutical composition comprises one or more therapeutic agents and an EV (*e.g.,* exosome) of the present disclosure, i.e., an EV comprising an optimized linker disclosed herein. In certain aspects, the EVs (*e.g.,* exosomes) are co-administered with of one or more additional therapeutic agents, in a pharmaceutically acceptable carrier. In some aspects, the pharmaceutical composition comprising the EV (*e.g.,* exosome) is administered prior to administration of the additional therapeutic agents. In other aspects, the pharmaceutical composition comprising the EV (*e.g.,* exosome) is administered after the administration of the additional therapeutic agents. In further aspects, the pharmaceutical composition comprising the EV (*e.g.,* exosome) is administered concurrently with the additional therapeutic agents.

[00508] Acceptable carriers, excipients, or stabilizers are nontoxic to recipients (*e.g.*, animals or humans) at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.*, Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

[00509] Examples of carriers or diluents include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. The use of such media and compounds for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or compound is incompatible with the extracellular vesicles described herein, use thereof in the compositions is contemplated. Supplementary therapeutic agents can also be incorporated into the compositions. Typically, a pharmaceutical composition is formulated to be compatible with its intended route of administration. The EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, can be administered by parenteral, topical, intravenous, oral, subcutaneous, intra-arterial, intradermal, transdermal, rectal, intracranial, intraperitoneal, intranasal, intratumoral, intrathecal, intraocular, intramuscular

route or as inhalants. In certain aspects, the pharmaceutical composition comprising EVs (*e.g.,* exosomes) is administered intravenously, *e.g.* by injection. The EVs (*e.g.,* exosomes) can optionally be administered in combination with other therapeutic agents that are at least partly effective in treating the disease, disorder or condition for which the EVs (*e.g.,* exosomes) are intended.

[00510] Solutions or suspensions can include the following components: a sterile diluent such as water, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial compounds such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating compounds such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates, and compounds for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[00511] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (if water soluble) or dispersions and sterile powders. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). The composition is generally sterile and fluid to the extent that easy syringeability exists. The carrier can be a solvent or dispersion medium containing, *e.g.*, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, *e.g.*, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal compounds, *e.g.*, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. If desired, isotonic compounds, *e.g.*, sugars, polyalcohols such as mannitol, sorbitol, and sodium chloride can be added to the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition a compound which delays absorption, *e.g.*, aluminum monostearate and gelatin.

[00512] Sterile injectable solutions can be prepared by incorporating the EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, in an effective amount and in an appropriate solvent with one or a combination of ingredients enumerated herein, as desired. Generally, dispersions are prepared by incorporating the EVs (*e.g.,* exosomes) into a sterile vehicle that contains a basic dispersion medium and any desired other ingredients. In the case of sterile powders for the preparation of sterile injectable solutions,

methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The EVs (*e.g.,* exosomes) can be administered in the form of a depot injection or implant preparation which can be formulated in such a manner to permit a sustained or pulsatile release of the EVs (*e.g.,* exosomes).

[00513] Systemic administration of compositions comprising EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, can also be by transmucosal means. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, *e.g.*, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of, *e.g.*, nasal sprays.

[00514] In certain aspects the pharmaceutical composition comprising EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, is administered intravenously into a subject that would benefit from the pharmaceutical composition. In certain other aspects, the composition is administered to the lymphatic system, *e.g.*, by intralymphatic injection or by intranodal injection (*see e.g.*, Senti *et al.*, PNAS 105(46): 17908 (2008)), or by intramuscular injection, by subcutaneous administration, by intratumoral injection, by direct injection into the thymus, or into the liver.

[00515] In certain aspects, the pharmaceutical composition comprising EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, is administered as a liquid suspension. In certain aspects, the pharmaceutical composition is administered as a formulation that is capable of forming a depot following administration. In certain preferred aspects, the depot slowly releases the EVs (*e.g.,* exosomes) into circulation, or remains in depot form.

[00516] Typically, pharmaceutically-acceptable compositions are highly purified to be free of contaminants, are biocompatible and not toxic, and are suited to administration to a subject. If water is a constituent of the carrier, the water is highly purified and processed to be free of contaminants, *e.g.*, endotoxins.

[00517] The pharmaceutically-acceptable carrier can be lactose, dextrose, sucrose, sorbitol, mannitol, starch, gum acacia, calcium phosphate, alginates, gelatin, calcium silicate, micro-crystalline cellulose, polyvinylpyrrolidone, cellulose, water, syrup, methyl cellulose, methylhydroxy benzoate, propylhydroxy benzoate, talc, magnesium stearate, and/or mineral oil, but is not limited thereto. The pharmaceutical composition can further include a lubricant, a

wetting agent, a sweetener, a flavor enhancer, an emulsifying agent, a suspension agent, and/or a preservative.

[00518] The pharmaceutical compositions described herein comprise EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, and optionally a pharmaceutically active or therapeutic agent. The therapeutic agent can be a biological agent, a small molecule agent, or a nucleic acid agent.

[00519] Dosage forms are provided that comprise a pharmaceutical composition comprising EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein. In some aspects, the dosage form is formulated as a liquid suspension for intravenous injection. In some aspects, the dosage form is formulated as a liquid suspension for intratumoral injection.

[00520] In certain aspects, the preparation of EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, is subjected to radiation, *e.g.*, X rays, gamma rays, beta particles, alpha particles, neutrons, protons, elemental nuclei, UV rays in order to damage residual replication-competent nucleic acids.

[00521] In certain aspects, the preparation of EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, is subjected to gamma irradiation using an irradiation dose of more than 1, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, or more than 100 kGy.

[00522] In certain aspects, the preparation of EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, is subjected to X-ray irradiation using an irradiation dose of more than 0.1, 0.5, 1, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, or

[00523] The EVs (*e.g.,* exosomes) of the present disclosure may be used concurrently with other drugs. To be specific, the EVs (*e.g.,* exosomes) of the present disclosure may be used together with medicaments such as hormonal therapeutic agents, chemotherapeutic agents, immunotherapeutic agents, medicaments inhibiting the action of cell growth factors or cell growth factor receptors and the like.

Kits

[00524] The present disclosure also provides kits, or products of manufacture comprising one or more EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, and optionally instructions for use. In some aspects, the kit, or product of

manufacture contains a pharmaceutical composition described herein which comprises at least one EV (*e.g.,* exosome) of the present disclosure, i.e., an EV comprising an optimized linker disclosed herein, and instructions for use. In some aspects, the kit, or product of manufacture comprises at least one EV (*e.g.,* exosome) of the present disclosure, i.e., an EV comprising an optimized linker disclosed herein, or a pharmaceutical composition comprising the EVs (*e.g.,* exosomes) in one or more containers. One skilled in the art will readily recognize that the EVs (*e.g.,* exosomes) of the present disclosure, pharmaceutical composition comprising the EVs (*e.g.,* exosomes) of the present disclosure, i.e., EVs comprising an optimized linker disclosed herein, or combinations thereof can be readily incorporated into one of the established kit formats which are well known in the art.

[00525] In some aspects, the kit, or product of manufacture comprises EVs (*e.g.,* exosomes) one or more biologically active molecules, reagents to covalently attach the one or more biologically active molecules to the EVs (*e.g.,* exosomes) via an optimized linker disclosed herein, e.g., via conjugation or solid phase synthesis, and instructions to conduct the reaction to covalently attach the one or more biologically active molecules to the EVs (*e.g.,* exosomes) via an optimized linker disclosed herein.

[00526] In some aspects, the kit comprises reagents to conjugate a biologically active molecule to an EV (*e.g.,* exosome) via an optimized linker disclosed herein, e.g., via conjugation (e.g., maleimide chemistry) or solid phase synthesis, and instructions to conduct the conjugation.

[00527] The practice of the present disclosure will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Sambrook et al., ed. (1989) Molecular Cloning A Laboratory Manual (2nd ed.; Cold Spring Harbor Laboratory Press); Sambrook et al., ed. (1992) Molecular Cloning: A Laboratory Manual, (Cold Springs Harbor Laboratory, NY); D. N. Glover ed., (1985) DNA Cloning, Volumes I and II; Gait, ed. (1984) Oligonucleotide Synthesis; Mullis et al. U.S. Pat. No. 4,683,195; Hames and Higgins, eds. (1984) Nucleic Acid Hybridization; Hames and Higgins, eds. (1984) Transcription And Translation; Freshney (1987) Culture Of Animal Cells (Alan R. Liss, Inc.); Immobilized Cells And Enzymes (IRL Press) (1986); Perbal (1984) A Practical Guide To Molecular Cloning; the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Miller and Calos eds. (1987) Gene Transfer Vectors For Mammalian Cells, (Cold Spring Harbor Laboratory); Wu et al., eds., Methods In Enzymology, Vols. 154 and 155; Mayer and Walker, eds. (1987) Immunochemical Methods In Cell And Molecular Biology (Academic Press, London); Weir and Blackwell, eds., (1986)

Handbook Of Experimental Immunology, Volumes I-IV; Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986);); Crooke, Antisense drug Technology: Principles, Strategies and Applications, 2nd Ed. CRC Press (2007) and in Ausubel et al. (1989) Current Protocols in Molecular Biology (John Wiley and Sons, Baltimore, Md.).

[00528] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. Database entries and electronic publications disclosed in the present disclosure are incorporated by reference in their entireties. The version of the database entry or electronic publication incorporated by reference in the present application is the most recent version of the database entry or electronic publication that was publicly available at the time the present application was filed. The database entries corresponding to gene or protein identifiers (e.g., genes or proteins identified by an accession number or database identifier of a public database such as Genbank, Refseq, or Uniprot) disclosed in the present application are incorporated by reference in their entireties. The gene or protein-related incorporated information is not limited to the sequence data contained in the database entry. The information incorporated by reference ncludes the entire contents of the database entry in the most recent version of the database that was publicly available at the time the present application was filed. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Examples

[00529] The following examples are provided for illustrative purposes only, and are not to be construed as limiting the scope or content of the invention in any way. The practice of the current invention will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, T.E. Creighton, Proteins: Structures and Molecular Properties (W.H. Freeman and Company, 1993); Green & Sambrook et al., Molecular Cloning: A Laboratory Manual, 4th Edition (Cold Spring Harbor Laboratory Press, 2012); Colowick & Kaplan, Methods in Enzymology (Academic Press); Remington: The Science and Practice of Pharmacy, 22nd Edition (Pharmaceutical Press, 2012); Sundberg & Carey, Advanced Organic Chemistry: Parts A and B, 5th Edition (Springer, 2007).

Example 1

Exosome Generation

[00530] To generate exosomes described herein, human embryonic kidney (HEK) cell line (HEK293SF) were used. Native cells were used to generate "native exosomes" and cells stably transfected with the protein, prostaglandin F2 receptor negative regulator (PTGFRN) were used to generate "Protein X exosomes". Non-transfected or transfected HEK293SF cells were grown to high density in chemically defined medium for 7 days.

[00531] Conditioned cell culture media wass then collected and centrifuged at 300 – 800 x g for 5 minutes at room temperature to remove cells and large debris. Media supernatant wass supplemented with 100 U/mL BENZONASE® and incubated at 37 °C for 1 hour in a water bath. Supernatant was collected and centrifuged at 16,000 x g for 30 minutes at 4 °C to remove residual cell debris and other large contaminants. Supernatant was then ultracentrifuged at 133,900 x g for 3 hours at 4 °C to pellet the exosomes. Supernatant was discarded and any residual media wa aspirated from the bottom of the tube. The pellet was resuspended in 200 – 1000 µL PBS (-Ca -Mg).

[00532] To further enrich exosome populations, the pellet was processed via density gradient purification (sucrose or OPTIPREP™). The gradient was spun at 200,000 x g for 16 hours at 4 °C in a 12 mL Ultra-Clear (344059) tube placed in a SW 41 Ti rotor to separate the exosome fraction.

[00533] The exosome layer was gently removed from the top layer and diluted in ~32.5 mL PBS in a 38.5 mL Ultra-Clear (344058) tube and ultracentrifuged again at 133,900 x g for 3 hours at 4 °C to pellet the purified exosomes. The resulting pellet was resuspended in a minimal volume of PBS (~200 µL) and stored at 4 °C.

[00534] For OPTIPREP™ gradient, a 3-tier sterile gradient was prepared with equal volumes of 10%, 30%, and 45% OPTIPREP™ in a 12 mL Ultra-Clear (344059) tube for a SW 41 Ti rotor. The pellet was added to the OPTIPREP™ gradient and ultracentrifuged at 200,000 x g for 16 hours at 4 °C to separate the exosome fraction. The exosome layer was then be gently collected from the top ~3 mL of the tube.

[00535] The exosome fraction was diluted in ~32 mL PBS in a 38.5 mL Ultra-Clear (344058) tube and ultracentrifuged at 133,900 x g for 3 hours at 4 °C to pellet the purified exosomes. The pelleted exosomes were then resuspended in a minimal volume of PBS (~200 µL) and stored at 4°C until ready to be used.

EXAMPLE 2

ASO Loading on Exosomes

[00536] 10 uL of Exosomes at $1x10^{13}$ P/mL were mixed with 10 uL of ASO (100 uM). 980 uL of PBS were added and then ultracentrifuged at 100k g, 20 min). The top 500 uL of supernatant ("supernatant") were removed. The pellet was decanted and resuspended in 500 uL PBS ("pellet"). "Loading Efficiency" was calculated as the ratio between ASO in the pellet and he total amound of ASO, i.e., ASO in supernatant plus ASO in pellet.

[00537] An ASO loading process in exemplified in **FIG. 27**.

EXAMPLE 3

Effect of ASO linker structure on potency and amount loaded in engineered or native exosomes

[00538] The effect of ASO linker structure on amount of ASO loaded in engineered or native exosomes, as well as the effect of ASO linker structure on ASO potency were evaluated.

[00539] Loading effectiveness of a fixed ASO sequence conjugated to different linkers was evaluated in engineered exosomes. Exosomes over-expressing PTGFRN (Protein X exosomes) were produced in HEK293 cells and purified. ASO payloads were loaded on to the surface of exosomes by mixing. Accordingly, exosomes at a concentration of $1x10^{13}$ P/mL were mixed 1:1 with 100 uM ASO with sequence for firefly luciferase (FFLuc) that had been conjugated to different linker structures. The loading efficiency was calculated as the percentage of starting ASOs that became coupled to the exosomes.

[00540] **FIG. 6** shows the structures of the constructs used. Constructs C1 to C9 used a cholesterol-C6 lipid anchor. Constructs T1 to T9 used a cholesterol-TEG lipid anchor. Construct L1 used a tocopherol-C8 lipid anchors whereas constructs L2 and L3 used a tocopherol palmitate-C6 lipid anchor. Other linker components indicated in **FIG**. 6 are: phosphodiester (PO), phosphorothioate (PS), hexamethylene (C6), trimethylene (C3), triethylene glycol (TEG), and hexaethylene glycol (HEG).

[00541] The results indicated that the mount of ASO molecules loaded per engineered exosome was affected by linker structure (**FIG. 6**). In general, loading efficiency was higher for constructs with the cholesterol-C6 anchor than for constructs with the cholesterol-TEG anchort. The highest loading efficacy, 68.78%, was observed for constructs with a tocopherol-C8 lipid anchor, corresponding to a load of 4,127 ASO units per exosome.

[00542] The loading efficiency of the same fixed ASO sequence conjugated to different linkers evaluated in **FIG. 5** using engineered exosomes was also evaluated using native

exosomes. See **FIG. 5**. Native exosomes were produced in HEK293 cells and purified. Exosomes at a concentration of $1x10^{13}$ P/mL were mixed 1:1 with 100 uM ASO with a sequence for firefly luciferase that had been conjugated to different linker structures. The loading efficiency was calculated as the percentage of starting ASOs that became coupled to the exosomes. As in the case of engineered exosomes, the amount of ASO molecules loaded per native exosome wass affected by linker structure (**FIG. 5**). Again, constructs with a cholesterol–C6 lipid anchor showed higher loading efficacy than constructs with a cholesterol-TEG lipid anchor. Loading efficacies over 90% were observed for constructs C2 and C3 (93.6% and 90.67%), corresponding to 5,616 and 5,440 ASO units per exosome, respectively. Thus, the maximum load efficacies and number of ASO per exosome were significantly higher in native exosomes than in engineered exosomes overexpressing PTGFRN (**FIG. 6**).

[00543] Predictive models for load density were developed based on ASO linker structure (**FIG. 7** and **FIG. 8**). The models show that load density depends, in decreasing order of importance, exosome > membrane anchor (AM) > proximal EV spacer (SP1) ~ distal EV spacer (SP2). The SP1 contribution was more important than the SP2 contribution in protein X exosome. On the other hand, the SP2 contribution was higher than the SP1 contribution in native exosomes.

[00544] The fold increases in load density as a function of linker modification were consistent across exosomes, i.e., in native and protein X exosomes. The best performing membrane anchors were tocopherol and cholesterol. The best S1 spacers were C3 and TEG, which were significantly better than HEG.

[00545] Use of the tocopherol-C8 membrane anchor resulted in exceptional exosome loading, but not a comparable increase in potency.

[00546] Stable H1299 cells were generated that constitutively express firefly luciferase protein. Native exosomes were loaded with ASO complementary to the mRNA for firefly luciferase protein, which when added to the cells, results in knock-down of the signal for luciferase protein. The linker structures described in **FIG. 9A** were comparatively evaluated to determine the effect on potency (**FIG. 9B**: T1-T9; **FIG. 9C**: C1-C9, L1-L3) with arrows highlighting the structures that provided the most luciferase knockdown (**FIG. 9A**, **9B**). The relative amounts of ASO-loaded exosomes that were added to the cells were varied as indicated by the relative "multiplicity of infection" ratios provided in the legend. The knockdown for each linker structure (C1-C9, T1-T9, L1-L3) was normalized by the amount of ASO in the T7 sample (control). Structures T2, T4, and C2-C5 providing the best increase in potency, with a $\geq$ 2-fold increase in potency achieved through optimization of the linker.

[00547] Stable H1299 cells were generated that constitutively express firefly luciferase protein. Native exosomes (**FIG. 10A**) or exosomes over-expressing PTGFRN (**FIG. 10B**) were loaded with ASO complementary to the mRNA for firefly luciferase protein, which when added to the cells, results in knock-down of the signal for luciferase protein. The minimum normalized expression of luciferase (indicating maximum knockdown) was determined for each ASO linker and compared to the loading density achieved with the linker. Optimal linkers are defined by maximum ASO/EV loading and/or minimum normalized expression of firefly luciferase. For this ASO sequence, cholesterol and tocopherol-c8 were shown to be the best anchoring moiety for association with native exosomes and exosomes with over-expressed PTGFRN, respectively. TEG and C3 provided the largest increase in potency of the options for spacer 1.

EXAMPLE 4

Correlation of loading-density, stability, and potenty with ASO-linker-lipid structures

[00548] Using the same ASO sequence, different linker chemistries were evaluated for the purpose of attaching an oligonucleotide (i.e. an anti-sense oligonucleotide (ASO)) to either native exosomes or exosomes with overexpressed PTGFRN. Five different membrane anchors were employed: cholesterol, palmitate, tocopherol, 18:1 PDP PE and 16:0 Cyanur PE. To these membrane anchors, ASOs were conjugated via linear linkers comprised of one or more of the following components were conjugated: trimethylene (C3), hexamethylene (C6), octylethylene (C8), triethyleneglycol (TEG), and hexaethylene glycol (HEG). The bonds between the linkers were phosphodiester bonds. The components used for the design of the ASO-linker-lipid constructs are shown in **FIG. 11**.

[00549] **FIG. 12** shows the sequences of the modified ASOs used in the experiments disclosed in the current example. The ASOs are named after the targeted genes. Two ASOs, named FFLUC and RLUC, target ther FFLUC and RLUC luminescent gene reporters. Two ASOs, named STAT6 and MYC, target the STAT6 and MYC therapeutic genes. The base sequences of the FFLUC, RLUC, STAT6, and MYC ASOs are respectively 5'-TCGAAGTACTCGGCGTAGGT-3' (SEQ ID NO: 1089), 5'-AACCCAGGGTCGGACTCGAT-3' (SEQ ID NO: 1090), 5′-GCAAGATCCCGGATTCGGTC-3′ (SEQ ID NO: 1091), and 5'-GACGTGGCACCTCTTGAGGA-3' (SEQ ID NO: 1092), which were modified as showin in **FIG. 12**.

[00550] **Correlation of loading-density with ASO-linker-lipid structures**: An optimal linker design will yield high loading density (amount of ASO loaded per EV) to maximize the potency per exosome and high potency per ASO molecule. ASO payload were combinatorially designed with four different oligonucleotide sequences and up to 14 different linker structures. These payloads were then loaded on exosomes with overexpressed PTGFRN and the loading density was determined. A statistical model (R2 = 0.86) was generated to determine the contribution of different structural parameters to loading density on the exosomes. The statistical model is shown in **FIG. 13A**.

[00551] The linker screen revealed that the ASO sequence, membrane anchor, and spacer 1 (the linker proximal to the exosome surface) had a significant effect on loading density. Each lipid has a different partition coefficient that defines the equilibrium amount of lipid that can stably reside in the hydrophobic membrane. The hybrid linear and cyclic hydrophobic structure may provide improved ASO loading because the alkyl chain allows the aromatic ring to access a greater interior volume of the phospholipid bilayer. Lipid composition can affect the membrane fluidity, potentially mediated, e.g., by induced dipole interactions, dipole interactions, and/or charge interactions. Finally, self-association of the lipids in the aqueous loading buffer may lead to differences in performance across the structures. Similarly, different ASO sequences adopt slightly different conformations and self-associate and/or associate with the exosome surfaces in a sequence-dependent manner.

[00552] The linker screen revealed loading density was significantly affected by the presence of spacer 1, the linker proximal to the exosome. Conversely, spacer 2, the linker that was farthest from the exosome surface did not have a significant effect, suggesting that further increasing the spacer length beyond that provided by spacer 1 does not offer a meaningful advantage for loading. These findings suggest that alterations to the configuration, conformation, or orientation in the area immediately adjacent to the membrane were critical in linker design. Hydrophobic linkers such as C3, C6 and C8 are more hydrophobic than the hydrophilic linkers such as TEG and HEG and thus, more energetically favorable to associate with and penetrate the hydrophobic interior of the membrane. By acting as a hydrophobic tether and increasing the nonpolar volume of the membrane that the membrane anchor could access, the linkers can increase the loading density of the payload. A hydrophilic spacer 1 may improve the thermodynamic binding equilibria in the region surrounding the highly charged and hydrophilic phosphate head groups and exosome exterior surface. Increasing the linker length will increase the spacing of ASOs and reduce the electrostatic repulsion of the anionic ASOs. Beyond a certain distance from the exosome surface, the benefit becomes insignificant.

[00553] In summary, all parts of the payload structures have an impact on their loading density to a different degree, but a trend is maintained across ASO structures (see **FIGs. 13B** and **13C**). The main factor is the ASO used, followed by the proximal EV spacer (Spacer 1), the membrane anchor (Lipid), and the distal EV spacer (Spacer 2).

[00554] **Stability of exoASOs**: The loading of ASO-linker-lipid relies on the hydrophobic interaction between the lipid moiety and the phospholipid bilayer of exosomes. To examine the resilience of this lipid association, we evaluated five combinations of linker/lipid structures with a range of buffer conditions to determine effect on stability of the associations. The experimental conditions used are summarized in the table below.

Structures	Buffer conditions	Time	Temperature	Characterization method
Cholesterol-TEG-None-STAT6; Cholesterol-C6-C3-STAT6; Tocopherol-C8-TEG-STAT6; Tocopherol-TEG-HEG-STAT6; Palmitate-C6-HEG-STAT6.	20 mM Phosphate, 50 mM NaCl, pH 7 (low salt); 20 mM Phosphate, 150 mM NaCl, pH 7 (high salt); 20 mM Phosphate, 150 mM NaCl, sucrose, pH 7 (sucrose).	0 day; 2 days; 4 days; 8 days	25 °C; 4 °C	Ribogreen: ASO concentration; Nanoparticle tracking analysis: particle count; Dynamic light scattering: particle size

HEG:hexaethylene glycol spacer, TEG: tetratethylene glycol spacer, C3: trimethylene; C6: hexamethylene, none: indicates no additional spacer was included at that location; STAT6: ASO sequence that knocks down expression of signal transducer and activator of transcription 6

[00555] The ASO-linker-lipid was loaded on exosomes and maintained in the three buffer conditions at two different temperatures for 8 days as described in previous table. The amount of exosome-associated ASO, referred to as "ASO (uM)" in **FIGS. 13A-13E** was assessed by subtracting the dissociated free ASO from the total ASO content.

[00556] The effect of the use of phosphodioester or phosphorothioate linkages in the exoASO-STAT6 Control (Chol-TEG-HEG) shown in **FIG. 13B** is presented in **FIGS.** 19A-19C. The highest potency, quantitated as ASO concentration (nM) or exosome psarticles (p/mL), was observed when both linkers were phosphorothioatre (PS). The lowest potency was observed when the linkage closest to the lipid anchoring moiety was phosphodiester and the distal linkage was phosphorothioate.

[00557] In general, buffers with increased NaCl resulted in a higher loading density. The loading density between high salt and low salt buffer condition varied with linker-lipid structures. The concentration of exosome associated ASO in three different buffer conditions remained

stable over 8 days at both 25°C and 4°C. See **FIG. 15**. In addition, the particle count (**FIG. 16**) and particle size (**FIG. 17**) did not change. See also **FIGS. 20-22**. The data indicated that ASOs with diverse lipid/linker structures were stable under the tested buffer conditions, time range and temperature, properties necessary for successful implementation in GMP manufacturing and use in clinical trials.

[00558] **Potency of ExoASOs**: **FIG. 18** shows that for the exoASO-STAT6 control, the potency per exosome positively correlated with loading density. However, among the different linker structures tested, there was no obvious correlation between potency and loading density. In other words, across linker structures, higher loading density did not necessarily result in higher potentcy. Among the linkers tested, three displayed lower IC_{50} values (i.e., higher potency). These linkers were Chol-TEG, Chol-C6-C3, and Toco-C8-TEG.

[00559] In addition to the non-cleavable constructs disclosed above, other constructs using cleavable linkers were tested, e.g., Val-Cit cleavable linkers. Examples of lipid-linker-ASO structure designs with a Val-Cit cleavable linker mechanism in which the ASO is a STAT6 ASO are shown in **FIG. 23A**. The potency of the constructs described in **FIG. 23A** is shown in **FIG. 23B**.

[00560] Another example of cleavable linker that was also tested were redox linkers. **FIG. 25** shows an exemplary lipid-linker-ASO design with a redox cleavable linker mechanism in which the ASO is a STAT6 ASO, and its potency is shown in **FIG. 26**.

EXAMPLE 5

EGFP splicing rescue assay

[00561] An EGFP splicing rescue assay was developed to characterize the linker constructs disclosed herein. The 293AAV-pCB-2754 cell line was developed to evaluate the efficacy of EGFP and exoEGFP with various linkers. Point mutation at 654 results in the inability of fully transcribing the EGFP; thus, no luminescence from EGFP or Nano Luciferase is observed. With the treatment with an ASO, EGFP was rescued, and therefore, both EGFP and Nano Luciferase signal was observed. The luminescence of Nano luciferase was employed for high-throughput quantification of efficacy. **FIG. 28** shows a schematic representation of the development of the EGFP splicing rescue assay. The modified sequence of the EGFP ASO used in the test is also shown in **FIG. 28**. The base sequence of the EGFP ASO is GCTATTACCTTAACCCAG (SEQ ID NO: 1093). Examples of lipid-linker-ASO design for testing in the EGFP assay, i.e., constructs in which the ASO was the modified EGFP ASO

presented in **FIG. 28** are shown in **FIG. 24**. The linkers tested included both cleavable and non-cleavable linkers. The loading, purification, and characterization processes for a lipid-linker-EGFP constructs used in the EGFP rescue assay are shown in **FIG. 27**.

[00562] The newly developed cell line used in the EGFP splicing rescue assay was characterized in terms of cell viability (CTG assay) and efficacy at three dose levels. See **FIG. 29**.

[00563] **FIG. 30** shows loading density and efficacy of exoASO-EGFP with various PO and non-cleavable PEG linkers using the EGFP splicing rescue assay. "Low" and "High" is a relative, qualitative reference to the loading density achieved in a paired set of experiments, where loading density was controlled through modulation of loading temperature and ASO concentration.

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[00564] It is to be appreciated that the Detailed Description section, and not the Summary and Abstract sections, is intended to be used to interpret the claims. The Summary and Abstract sections may set forth one or more but not all exemplary aspects of the present disclosure as contemplated by the inventor(s), and thus, are not intended to limit the present disclosure and the appended claims in any way.

[00565] The present disclosure has been described above with the aid of functional building blocks illustrating the implementation of specified functions and relationships thereof. The boundaries of these functional building blocks have been arbitrarily defined herein for the convenience of the description. Alternate boundaries can be defined so long as the specified functions and relationships thereof are appropriately performed.

[00566] The foregoing description of the specific aspects will so fully reveal the general nature of the disclosure that others can, by applying knowledge within the skill of the art, readily modify and/or adapt for various applications such specific aspects, without undue experimentation, without departing from the general concept of the present disclosure. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed aspects, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance.

[00567] The breadth and scope of the present disclosure should not be limited by any of the above-described exemplary aspects, but should be defined only in accordance with the following claims and their equivalents.

[00568] The contents of all cited references (including literature references, patents, patent applications, and websites) that may be cited throughout this application are hereby expressly incorporated by reference in their entirety for any purpose, as are the references cited therein.

WHAT IS CLAIMED IS:

1. An extracellular vesicle (EV) comprising a biologically active molecule (BAM) covalently linked to the EV via an anchoring moiety (AM) according to the formula:

$$[AM]\text{-}L_1\text{-}[SP_1]\text{-}L_2\text{-}[SP_2]\text{-}L_3\text{-}[BAM] \quad \text{(Formula 1)}$$

wherein:

[AM] is the anchoring moiety;

L_1 is a cleavable or non-cleavable linkage;

L_2 and L_3 are optional cleavable or non-cleavable linkages;

SP_1 is an optional first spacer; and,

SP_2 is an optional second spacer.

2. The extracellular vesicle of claim 1, wherein the anchoring moiety [AM] comprises a sterol, a lipid, a vitamin, a peptide, or a combination thereof and optionally a spacer.

3. The extracellular vesicle of claim 2, wherein the optional spacer is an alkyl spacer.

4. The extracellular vesicle of claim 3, wherein the alkyl spacer is C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14, or C15.

5. The extracellular vesicle of claim 3, wherein the alkyl spacer is C6 or C8.

6. The extracellular vesicle of claim 2, wherein the optional spacer is a glycol spacer.

7. The extracellular vesicle of claim 5, wherein the glycol spacer has 2 (diethylene glycol), 3 (triethylene glycol), 4 (tetraethylene glycol; TEG), 5 (pentaethylene glycol), 6 (hexaethylene glycol; HEG), 7, 8, 9, 10, 11, 12, 13, 14, or 15 glycol units.

8. The extracellular vesicle of claim 5, wherein the glycol spacer is tetraethylene glycol (TEG).

9. The extracellular vesicle of claim 2, wherein the sterol is selected from the group consisting of cholesterol, ergosterol, 7-dehydrocholesterol, 24S-hydroxycholesterol, lanosterol, cycloartenol, fucosterol, saringosterol, campesterol, β-sitosterol, sitostanol, coprostanol, avenasterol, and stigmasterol.

10. The extracellular vesicle of claim 2, wherein the sterol is cholesterol.

11. The extracellular vesicle of claim 2, wherein the lipid is a fatty acid.

12. The extracellular vesicle of claim 11, wherein the fatty acid is a straight chain fatty acid.

13. The extracellular vesicle of claim 11, wherein the fatty acid is a straight chain fatty acid, a branched fatty acid, an unsaturated fatty acid, a monounsaturated fatty acid, a polyunsaturated fatty acid, a hydroxyl fatty acid, a polycarboxylic acid, or any combination thereof.

14. The extracellular vesicle of claim 12, wherein the straight chain fatty acid is butyric acid, caproic acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, or stearic acid.

15. The extracellular vesicle of claim 12, wherein the straight chain fatty acid is palmitic acid.

16. The extracellular vesicle of claim 2, wherein the vitamin is vitamin E (tocopherol or tocotrienol), vitamin D, vitamin K, riboflavin, niacin, or pyridoxine.

17. The extracellular vesicle of claim 2, wherein the vitamin is vitamin E (tocopherol or tocotrienol).

18. The extracellular vesicle of anyone of claims 1-17, wherein L_1 is a cleavable linkage comprising a phosphodiester bond or a non-cleavable linkage comprising a phosphorothioate bond.

19. The extracellular vesicle of anyone of claims 1-18, wherein L_2 is an optional cleavable linkage comprising a phosphodiester bond or a non-cleavable linkage comprising a phosphorothioate bond.

20. The extracellular vesicle of anyone of claims 1-19, wherein L_3 is an optional cleavable linkage comprising a phosphodiester bond or a non-cleavable linkage comprising a phosphorothioate bond.

21. The extracellular vesicle of anyone of claims 1-20, wherein the SP_1 optional first spacer and/or the SP_2 an optional second spacer independently comprise an alkyl spacer, a glycol spacer, or a combination thereof.

22. The extracellular vesicle of claim 21, wherein the alkyl spacer is C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14, or C15.

23. The extracellular vesicle of claim 21, wherein the alkyl spacer is C3 or C6.

24. The extracellular vesicle of claim 21, wherein the glycol spacer has 2 (diethylene glycol), 3 (triethylene glycol), 4 (tetraethylene glycol; TEG), 5 (pentaethylene glycol), 6 (hexaethylene glycol; HEG), 7, 8, 9, 10, 11, 12, 13, 14, or 15 glycol units.

25. The extracellular vesicle of claim 21, wherein the glycol spacer is tetraethylene glycol (TEG) or hexaethylene glycol (HEG).

26. The extracellular vesicle of any one of claims 1 to 25, wherein each non-cleavable spacer is independently selected from alkyl, diethylene glycol, triethylene glycol, tetraethylene glycol (TEG), hexaethylene glycol (HEG), pentaethylene glycol, polyethylene glycol (PEG), glycerol, diglycerol, triglycerol, tetraglycerol (TG), pentaglycerol, a hexaglycerol (HG), polyglycerol (PG), succinimide, maleimide, or any combination thereof.

27. The extracellular vesicle of claim 26, wherein the polyethylene glycol (PEG) is characterized by the formula R_1-(O-CH_2-CH_2)$_n$- or R_1-(O-CH_2-CH_2)n-O-, wherein R1 is hydrogen, methyl or ethyl and n is an integer between 1 and 15.

28. The extracellular vesicle of claim 26, wherein the polyglycerol (PG) is characterized by the formula ((R_1—O—(CH_2—CHOH—CH_2O)$_n$—), wherein R_1 is hydrogen, methyl or ethyl, and n is an integer between 1 and 15.

29. The extracellular vesicle of anyone of claims 1 to 28, wherein the L_1, L_2, or L_3 cleavable linkage, or any combination thereof, comprises a redox cleavable linker, a reactive oxygen species cleavable linker, a pH dependent cleavable linker, an enzymatic cleavable linker, a protease cleavable linker, an esterase cleavable linker, a phosphatase cleavable linker, a photoactivated cleavable linker, a self-immolative linker, or any combination thereof.

30. The extracellular vesicle of anyone of claims 1 to 29, wherein the L_1, L_2, or L_3 cleavable linkage, or any combination thereof, comprises a self-immolative linker.

31. The extracellular vesicle of any one of claims 1 to 30, wherein the L_1, L_2, or L_3 cleavable linkage, or any combination thereof, comprises a cinnamyl group, a naphthyl group, a biphenyl group, a heterocyclic ring, a homoaromatic group, coumarin, furan, thiophene, thiazole, oxazole, isoxazole, pyrrole, pyrazole, pyridine, imidazone, triazole, or any combination thereof.

32. The extracellular vesicle of any one of claims 1 to 31, wherein the L_1, L_2, or L_3 cleavable linkage, or any combination thereof, has the formula:

-Aa-Yy-

wherein each –A- is independently an amino acid unit or a combination thereof, a is independently an integer from 1 to 15; -Y- is a spacer unit, and y is 0, 1, or 2.

33. The extracellular vesicle of claim 32, wherein -Aa- is a dipeptide, a tripeptide, a tetrapeptide, a pentapeptide, or a hexapeptide, or a combination thereof, wherein each dipeptide, tripeptide, tetrapeptide, pentapeptide, or hexapeptide in the combination can be the same or different.

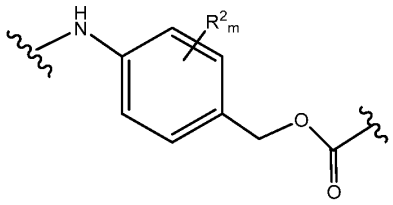
34. The extracellular vesicle of claim 33, wherein a is 2 and –Aa- is selected from the group consisting of valine-alanine, valine-citrulline, phenylalanine-lysine, N-methylvaline-citrulline, cyclohexylalanine-lysine, glutamic acid-valine-citrulline, and beta-alanine-lysine.

35. The extracellular vesicle of claim 33, wherein said –Aa- is valine-alanine, valine-citrulline, or glutamic acid-valine-citrulline.

36. The extracellular vesicle of any one of claims 31 to 35, wherein y is 1.

37. The extracellular vesicle of any one of claims 31 to 36, wherein –Y- is a self-immolative spacer.

38. The extracellular vesicle of claim 37, wherein –Yy- has the formula:



wherein each R_2 is independently C_{1-8} alkyl, -O-(C_{1-8} alkyl), halogen, nitro, or cyano; and m is an integer from 0 to 4.

39. The extracellular vesicle of claim 38, wherein m is 0, 1, or 2.

40. The extracellular vesicle of claim 38, wherein m is 0.

41. The extracellular vesicle of any one of claims 31 to 40, wherein the L_1, L_2, or L_3 cleavable linkage, or any combination thereof, comprises valine-alanine-p-aminobenzylcarbamate or valine-citrulline-p-aminobenzylcarbamate.

42. The extracellular vesicle of any one of claims 31 to 36, wherein –Y- is a non self-immolative spacer.

43. The extracellular vesicle of claim 42, wherein the non self-immolative spacer is –Gly- or –Gly-Gly-.

44. The extracellular vesicle of any one of claims 1 to 43, wherein the anchoring moiety [AM] comprises a scaffold protein.

45. The extracellular vesicle of claim 44, wherein the anchoring moiety [AM] and/or the scaffold moiety is Scaffold X.

46. The extracellular vesicle of claim 45, wherein the Scaffold X is selected from the group consisting of prostaglandin F2 receptor negative regulator (the PTGFRN protein); basigin (the BSG protein); immunoglobulin superfamily member 2 (the IGSF2 protein); immunoglobulin superfamily member 3 (the IGSF3 protein); immunoglobulin superfamily member 8 (the IGSF8 protein); integrin beta-1 (the ITGB1 protein); integrin alpha-4 (the ITGA4 protein); 4F2 cell-surface antigen heavy chain (the SLC3A2 protein); a class of ATP transporter proteins (the ATP1A1, ATP1A2, ATP1A3, ATP1A4, ATP1B3, ATP2B1, ATP2B2, ATP2B3, ATP2B4 proteins); a functional fragment thereof; and any combination thereof.

47. The extracellular vesicle of claim 45, wherein the Scaffold X is PTGFRN protein or a functional fragment thereof.

48. The extracellular vesicle of claim 45, wherein the Scaffold X comprises an amino acid sequence as set forth in SEQ ID NO:302.

49. The extracellular vesicle of claim 45, wherein the Scaffold X comprises an amino acid sequence at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or about 100% identical to SEQ ID NO:302.

50. The extracellular vesicle of any one of claims 1 to 49, wherein the biologically active molecule [BAM] is linked via an anchoring moiety [AM] to the exterior surface of the EV.

51. The extracellular vesicle of any one of claims 1 to 50, wherein the biologically active molecule is a polypeptide, a peptide, a polynucleotide (DNA and/or RNA), a chemical compound, or any combination thereof.

52. The extracellular vesicle of claim 51, wherein the biologically active molecule is a chemical compound.

53. The extracellular vesicle of claim 52, wherein the chemical compound is a small molecule.

54. The extracellular vesicle of any one of claims 1 to 53, wherein the biologically active molecule comprises an antisense oligonucleotide (ASO), a siRNA, a miRNA, a shRNA, an mRNA a nucleic acid, or any combination thereof.

55. The extracellular vesicle of any one of claims 1 to 54, wherein the biologically active molecule comprises a peptide, a protein, an antibody or an antigen binding fragment thereof, or any combination thereof.

56. The extracellular vesicle of claim 55, wherein the antigen binding fragment thereof comprises scFv, (scFv)2, Fab, Fab', F(ab')2, F(ab1)2, Fv, dAb, and Fd fragment, diabodys, antibody-related polypeptide, or any fragment thereof.

57. The extracellular vesicle of claim 54, wherein the biologically active molecule comprises an ASO.

58. The extracellular vesicle of claim 57, wherein the ASO targets a transcript.

59. The extracellular vesicle of claim 58, wherein the transcript is a STAT6 transcript, a CEBP/β transcript, a STAT3 transcript, a KRAS transcript, a NRAS transcript, an NLPR3 transcript, or any combination thereof.

60. The extracellular vesicle of any one of claims 1 to 59, wherein the EV is an exosome.

61. The extracellular vesicle of claim 60, wherein the exosome is a native exosome.

62. The extracellular vesicle of claim 60, wherein the exosome is an exosome overexpressing PTGFRN or a functional fragment thereof.

63. A pharmaceutical composition comprising the extracellular vesicle of any one of claims 1 to 62 and a pharmaceutically acceptable carrier.

64. A kit comprising the EV of any one of claim 1 to 62 or the pharmaceutical composition of claim 63 and instructions for use.

65. A method of treating or preventing a disease or disorder in a subject in need thereof comprising administering the EV of any one of claim 1 to 62 or the pharmaceutical composition of claim 63 to the subject.

66. The method of claim 65, wherein the disease or disorder is a cancer, an inflammatory disorder, a neurodegenerative disorder, a central nervous disease, or a metabolic disease.

67. The method of claim 65 or 66, wherein the EV is administered intravenously, intraperitoneally, nasally, orally, intramuscularly, subcutaneously, parenterally, or intratumorally.

68. A method of attaching a biologically active molecule (BAM) to an EV, comprising linking an anchoring moiety (AM) to the EV, wherein the anchoring moiety (AM) is attached to the biologically active moiety (BAM) according to the formula:

$$[AM]\text{-}L_1\text{-}[SP_1]\text{-}L_2\text{-}[SP_2]\text{-}L_3\text{-}[BAM]$$

wherein:
[AM] is the anchoring moiety;
L_1 is a cleavable or non-cleavable linkage;
L_2 and L_3 are optional cleavable or non-cleavable linkages;
SP_1 is an optional first spacer; and,
SP_2 is an optional second spacer.

69. The method of claim 68, wherein [AM] is cholesterol-C6, cholesterol-TEG, tocopherol-C8, tocopherol, of palmitate-C6.

70. The method of claim 68 or 69, wherein L_1, L_2, L_3, or a combination thereof is a phosphodiester bond.

71. The method of any one of claims 68 to 70, wherein SP_1 is C3, C6, TEG, or HEG.

72. The method of any one of claims 68 to 71, wherein L_1, L_2, L_3, or a combination thereof is a phosphorothioate bond.

73. The method of any one of claims 68 to 72, wherein SP_2 is C3, C6, TEG or HEG.

74. The method of any one of claims 68 to 73, wherein L_1, L_2, L_3, or a combination thereof is a phosphorothioate bond.

75. The method of any one of claims 68 to 74, wherein [BAM] is an antisense oligonucleotide (ASO).

76. A method of increasing the load density of a biologically active molecule (BAM) attached to an EV, comprising screening a library of anchoring moieties (AM) attached to the biologically active moiety (BAM) according to the formula:

$$[AM]\text{-}L_1\text{-}[SP_1]\text{-}L_2\text{-}[SP_2]\text{-}L_3\text{-}[BAM]$$

wherein:
[AM] is the anchoring moiety;
L_1 is a cleavable or non-cleavable linkage;
L_2 and L_3 are optional cleavable or non-cleavable linkages;
SP_1 is an optional first spacer; and,
SP_2 is an optional second spacer.

77. The method of claim 76, wherein [AM] is cholesterol-C6, cholesterol-TEG, tocopherol-C8, tocopherol, of palmitate-C6.

78. The method of claim 76 or 77, wherein L_1, L_2, L_3, or a combination thereof is a phosphodiester bond.

79. The method of any one of claims 76 to 78, wherein SP_1 is C3, C6, TEG, or HEG.

80. The method of any one of claims 76 to 79, wherein L_1, L_2, L_3, or a combination thereof is a phosphorothioate bond.

81. The method of any one of claims 76 to 80, wherein SP_2 is C3, C6, TEG or HEG.

82. The method of any one of claims 76 to 81, wherein L_1, L_2, L_3, or a combination thereof is a phosphorothioate bond.

83. The method of any one of claims 76 to 82, wherein [BAM] is an antisense oligonucleotide (ASO).

84. The method of any of claims 76 to 83, wherein the load density of a biologically active molecule (BAM) attached to an EV is increased at least about 1-fold, at least about 1.5-fold, at least about 2-fold, at least about 2.5-fold, at least about 3-fold, at last about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least 8-fold, at least about 9-fold, or at least about 10-fold.

85. An extracellular vesicle (EV) comprising an antisense oligonucleotide [ASO] covalently linked to the EV via an anchoring moiety [AM] according to the formula:

$$[AM]\text{-}L_1\text{-}[SP_1]\text{-}L_2\text{-}[SP_2]\text{-}L_3\text{-}[ASO]$$

wherein:
[AM] is the anchoring moiety selected from the group consisting of cholesterol-C6, cholesterol-TEG, tocopherol-C8, tocopherol, and palmitate-C6;
L_1 is a phosphodiesterase cleavable linkage;
SP_1 is an optional first spacer selected from the group consisting of C3, C6, TEG and HEG;
L_2 is optional phosphorothioate non-cleavable linkage;
SP_2 is an optional second spacer selected from the group consisting of C3, C6, TEG, and HEG; and,
L_3 is an optional phosphorothioate non-cleavable linkage.

85. The EV of claim 84, wherein the EV is an exosome.

86. The EV of claim 85, wherein the exosome is a native exosome.

87. The EV of claim 86, where the load density of ASO attached to the exosome is increased by at least about 1.5-fold.

88. The EV of claim 86, wherein the anchoring moiety [AM] is cholesterol-C6.

89. The EV of claim 88, wherein the average number of ASO molecules per exosome is 5032+/-386.

90. The EV of claim 88, wherein the average number of ASO molecules per exosome is between about 4500 and about 5500.

91. The EV of claim 88, wherein the average number of ASO molecules per exosome is between about 4500 and about 4600, between about 4600 and about 4700, between about 4700 and about 4800, between about 4800 and about 4900, between about 4900 and about 5000, between about 5000 and about 5100, between about 5100 and about 5200, between about 5200 and about 5300, between about 5300 and about 5400, or between about 5400 and about 5500.

92. The EV of claim 88, wherein the average number of ASO molecules per exosome is at least about 4500, at least about 4600, at least about 4700, at least about 4800, at least about 4900, at least about 5000, at least about 5100, at least about 5200, at least about 5300, at least about 5400, or at least about 5500.

93. The EV of claim 88, wherein the loading efficiency is 73% to 93%.

94. The EV of claim 88, wherein the loading efficiency is between about 70% and about 95%.

95. The EV of claim 88, wherein the loading efficiency is between about 70% and about 75%, between about 75% and about 80%, between about 80% and about 85%, between about 85% and about 90%, or between about 90% and about 95%.

96. The EV of claim 88, wherein the loading efficiency is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%.

97. The EV of claim 86, wherein the anchoring moiety [AM] is cholesterol-TEG.

98. The EV of claim 93, wherein the average number of ASO molecules per exosome is 3991+/-490.

99. The EV of claim 97, wherein the average number of ASO molecules per exosome is between about 3500 and about 4500.

100. The EV of claim 97, wherein the average number of ASO molecules per exosome is between about 3500 and about 3600, between about 3600 and about 3700, between about 3700 and about 3800, between about 3800 and about 3900, between about 3900 and about 4000, between about 4000 and about 4100, between about 4100 and about 4200, between about 4200 and about 4300, between about 4300 and about 4400, or between about 4400 and about 4500.

101. The EV of claim 97, wherein the average number of ASO molecules per exosome is at least about 3500, at least about 3600, at least about 3700, at least about 3800, at least about 3900, at least about 4000, at least about 4100, at least about 4200, at least about 4300, at least about 4400, or at least about 4500.

102. The EV of claim 97, wherein the loading efficiency is 56% to 79%.

103. The EV of claim 97, wherein the loading efficiency is between about 50% and about 85%.

104. The EV of claim 97, wherein the loading efficiency is between about 50% and about 55%, between about 55% and about 60%, between about 60% and about 65%, between about 65% and about 70%, between about 70% and about 75%, between about 75% and about 80%, or between about 80% and about 85%.

105. The EV of claim 97, wherein the loading efficiency is at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, or at least about 85%.

106. The EV of claim 86, wherein the anchoring moiety [AM] is tocopherol-C8, tocopherol, or palmitate-C6.

107. The EV of claim 106, wherein the average number of ASO molecules per exosome is 4241+/-722.

108. The EV of claim 106, wherein the average number of ASO molecules per exosome is between about 3500 and about 5000.

109. The EV of claim 106, wherein the average number of ASO molecules per exosome is between about 3500 and about 3600, between about 3600 and about 3700, between about 3700 and about 3800, between about 3800 and about 3900, between about 3900 and about 4000, between about 4000 and about 4100, between about 4100 and about 4200, between about 4200 and about 4300, between about 4300 and about 4400, between about 4400 and about 4500, between about 4500 and about 4600, between about 4600 and about 4700, between about 4700 and about 4800, between about 4800 and about 4900, or between about 4900 and about 5000.

110. The EV of claim 106, wherein the average number of ASO molecules per exosome is at least about 3500, at least about 3600, at least about 3700, at least about 3800, at least about 3900, at least about 4000, at least about 4100, at least about 4200, at least about 4300, at least about 4400, at least about 4500, at least about 4600, at least about 4700, at least about 4800, at least about 4900, or at least about 5000.

111. The EV of claim 106, wherein the loading efficiency is 57% to 73%.

112. The EV of claim 106, wherein the loading efficiency is between about 50% and about 80%.

113. The EV of claim 106, wherein the loading efficiency is between about 50% and about 55%, between about 55% and about 60%, between about 60% and about 65%, between about 65% and about 70%, between about 70% and about 75%, or between about 75% and about 80%.

114. The EV of claim 106, wherein the loading efficiency is at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, or at least about 80%.

115. The EV of claim 84, wherein the exosome is an exosome overexpressing PTGFRN.

116. The EV of claim 115, where the load density of ASO attached to the exosome is increased by at least about 2-fold.

117. The EV of claim 115, wherein the anchoring moiety [AM] is cholesterol-C6.

118. The EV of claim 117, wherein the average number of ASO molecules per exosome is 2442+/-339.

119. The EV of claim 117, wherein the average number of ASO molecules per exosome is between about 2000 and about 3000.

120. The EV of claim 117, wherein the average number of ASO molecules per exosome is between about 2000 and about 2100, between about 2100 and about 2200, between about 2200 and about 2300, between about 2300 and about 2400, between about 2400 and about 2500, between about 2500 and about 2600, between about 2600 and about 2700, between about 2700 and about 2800, between about 2800 and about 2900, or between about 2900 and about 3000.

121. The EV of claim 117, wherein the average number of ASO molecules per exosome is at least about 2000, at least about 2100, at least about 2200, at least about 2300, at least about 2400, at least about 2500, at least about 2600, at least about 2700, at least about 2800, at least about 2900, or at least about 3000.

122. The EV of claim 117, wherein the loading efficiency is 27% to 46%.

123. The EV of claim 117, wherein the loading efficiency is between about 25% and about 50%.

124. The EV of claim 117, wherein the loading efficiency is between about 25% and about 30%, between about 30% and about 35%, between about 35% and about 40%, between about 40% and about 45%, or between about 45% and about 50%.

125. The EV of claim 117, wherein the loading efficiency is at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, or at least about 50%.

126. The EV of claim 115, wherein the anchoring moiety [AM] is cholesterol-TEG.

127. The EV of claim 126, wherein the average number of ASO molecules per exosome is 1728+/-264.

128. The EV of claim 126, wherein the average number of ASO molecules per exosome is between about 1400 and about 2100.

129. The EV of claim 126, wherein the average number of ASO molecules per exosome is between about 1400 and about 1500, between about 1500 and about 1600, between about 1600 and about 1700, between about 1700 and about 1800, between about 1800 and about 1900, between about 1900 and about 2000, or between about 2000 and about 2100.

130. The EV of claim 126, wherein the average number of ASO molecules per exosome is at least about 1400, at least about 1500, at least about 1600, at least about 1700, at least about 1800, at least about 1900, at least about 2000, or at least about 2100.

131. The EV of claim 126, wherein the loading efficiency is 19% to 33%.

132. The EV of claim 126, wherein the loading efficiency is between about 15% and about 35%.

133. The EV of claim 126, wherein the loading efficiency is between about 15% and about 20%, between about 20% and about 25%, between about 25% and about 30%, or between about 30% and about 35%.

134. The EV of claim 126, wherein the loading efficiency is at least about 15%, at least about 20%, at least about 25%, at least about 30%, or at least about 35%.

135. The EV of claim 115, wherein the anchoring moiety [AM] is tocopherol-C8, tocopherol, or palmitate-C6.

136. The EV of claim 135, wherein the average number of ASO molecules per exosome is 2979+/-1006.

137. The EV of claim 135, wherein the average number of ASO molecules per exosome is between about 1900 and about 4000.

138. The EV of claim 135, wherein the average number of ASO molecules per exosome is between about 1900 and about 2000, between about 2000 and about 2100, between about 2100 and about 2200, between about 2200 and about 2300, between about 2300 and about 2400, between about 2400 and about 2500, between about 2500 and about 2600, between about 2600 and about 2700, between about 2700 and about 2800, between about 2800 and about 2900, between about 2900 and about 3000, between about 3000 and about 3100, between about 3100 and about 3200, between about 3200 and about 3300, between about 3300 and about 3400, between about 3400 and about 3500. between about 3500 and about 3600, between about 3600 and about 3700, between about 3700 and about 3800, between about 3800 and about 3900, or between about 3900 and about 4000.

139. The EV of claim 135, wherein the average number of ASO molecules per exosome is at least about 1900, at least about 2000, at least about 2100, at least about 2200, at least about 2300, at least about 2400, at least about 2500, at least about 2600, at least about 2700, at least about 2800, at least about 2900, at least about 3000, at least about 3100, at least about 3200, at least about 3300, at least about 3400, at least about 3500, at least about 3600, at least about 3700, at least about 3800, at least about 3900, or at least about 4000.

140. The EV of claim 135, wherein the loading efficiency is 37% to 68%.

141. The EV of claim 135, wherein the loading efficiency is between about 30% and about 75%.

142. The EV of claim 135, wherein the loading efficiency is between about 30% and about 35%, between about 35% and about 40%, between about 40% and about 45%, between about 45% and about 50%, between about 50% and about 55%, between about 55% and about 60%, between about 60% and about 65%, between about 65% and about 70%, or between about 70% and about 75%.

143. The EV of claim 135, wherein the loading efficiency is at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%.

144. An exosome comprising an antisense oligonucleotide [ASO] covalently linked to the exosome via an anchoring moiety [AM] according to the formula:

$$[AM]\text{-}L_1\text{-}[SP_1]\text{-}L_2\text{-}[SP_2]\text{-}L_3\text{-}[BAM]$$

wherein [AM] is cholesterol-TEG; L_1 is a phosphodiesterase cleavable bond; SP_1 is C3; L_2 is a phosphorothioate non-cleavable bond; SP_2 is TEG; and, L_3 is a phosphorothioate non-cleavable bond.

145. The exosome of claim 144, wherein the exosome is a native exosome.

146. The exosome of claim 145, wherein the average number of ASO molecules per exosome is about 4780.

147. The exosome of claim 145, wherein the average number of ASO molecules per exosome is between about 4500 and about 5000.

148. The exosome of claim 145, wherein the average number of ASO molecules per exosome is at least 4500.

149. The exosome of claim 145, wherein the loading efficiency is about 80%.

150. The exosome of claim 145, wherein the loading efficiency is between about 70% and about 90%.

151. The exosome of claim 145, wherein the loading efficiency is at least about 70%.

152. The exosome of claim 144, wherein the exosome is an exosome overexpressing PTGFRN.

153. The exosome of claim 152, wherein the average number of ASO molecules per exosome is about 1659.

154. The exosome of claim 152, wherein the average number of ASO molecules per exosome is between about 1500 and about 2000.

155. The exosome of claim 152, wherein the average number of ASO molecules per exosome is at least about 1500.

156. The exosome of claim 152, wherein the loading efficiency is about 28%.

157. The exosome of claim 152, wherein the loading efficiency is between about 20% and about 35%.

158. The exosome of claim 152, wherein the loading efficiency is at least about 20%.

159. An exosome comprising an antisense oligonucleotide [ASO] covalently linked to the exosome via an anchoring moiety [AM] according to the formula:

$$[AM]\text{-}L_1\text{-}[SP_1]\text{-}L_2\text{-}[BAM]$$

wherein [AM] is cholesterol-TEG; L_1 is a phosphodiesterase cleavable bond; SP_1 is TEG; and L_2 is a phosphorothioate non-cleavable bond.

160. The exosome of claim 159, wherein the exosome is a native exosome.

161. The exosome of claim 160, wherein the average number of ASO molecules per exosome is about 4090.

162. The exosome of claim 160, wherein the average number of ASO molecules per exosome is between about 3500 and about 4500.

163. The exosome of claim 160, wherein the average number of ASO molecules per exosome is at least 3500.

164. The exosome of claim 160, wherein the loading efficiency is about 68%.

165. The exosome of claim 160, wherein the loading efficiency is at least about 60%.

166. The exosome of claim 160, wherein the loading efficiency is between about 60% and about 70%.

167. The exosome of claim 159, wherein the exosome is an exosome overexpressing PTGFRN.

168. The exosome of claim 167, wherein the average number of ASO molecules per exosome is about 1890.

169. The exosome of claim 167, wherein the average number of ASO molecules per exosome is between about 1400 and about 2400.

170. The exosome of claim 167, wherein the average number of ASO molecules per exosome is at least about 1400.

171. The exosome of claim 167, wherein the loading efficiency is about 31%.

172. The exosome of claim 167, wherein the loading efficiency is between about 20% and about 40%.

173. The exosome of claim 167, wherein the loading efficiency is at least about 20%.