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(54) **LIPID-COATED ALBUMIN NANOPARTICLE COMPOSITIONS AND METHODS OF MAKING AND METHOD OF USING THE SAME**

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(57) **ABSTRACT**

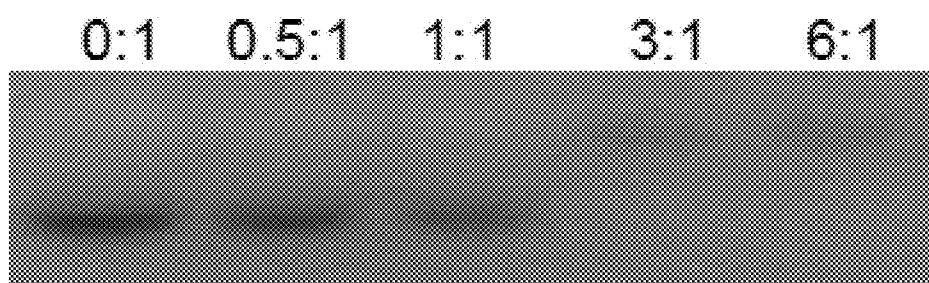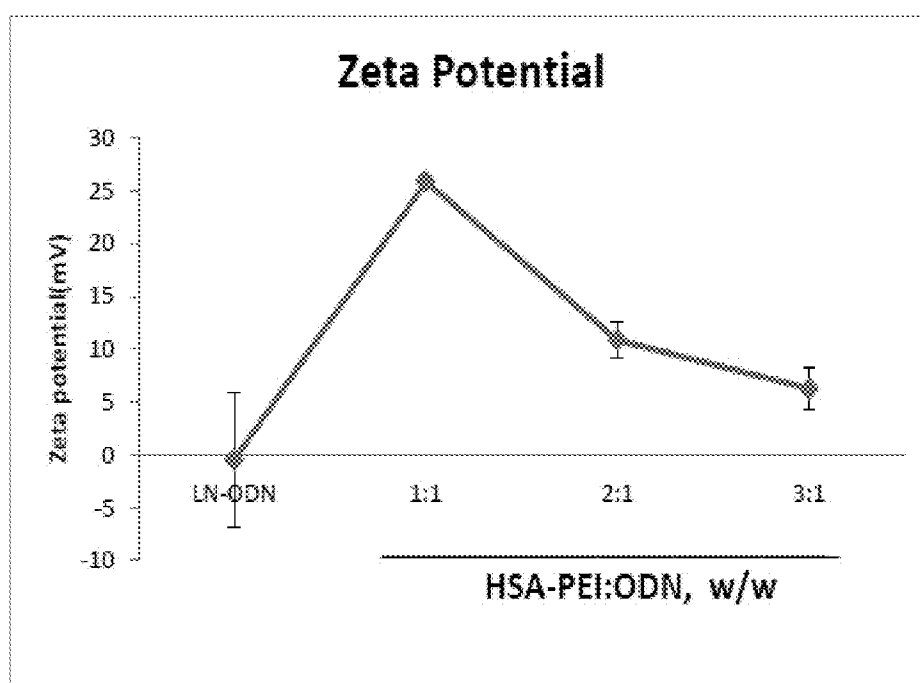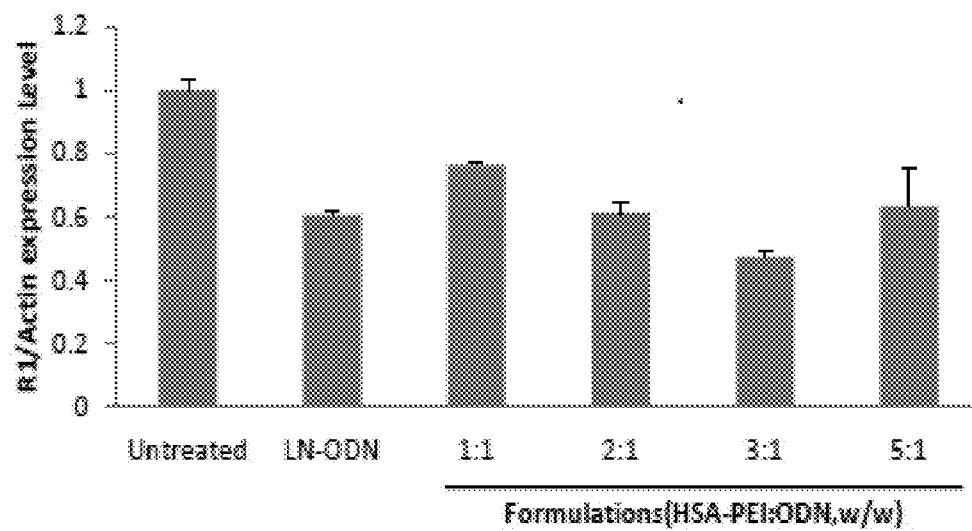Lipid nanoparticle formulations, methods of making, and methods of using same are disclosed.
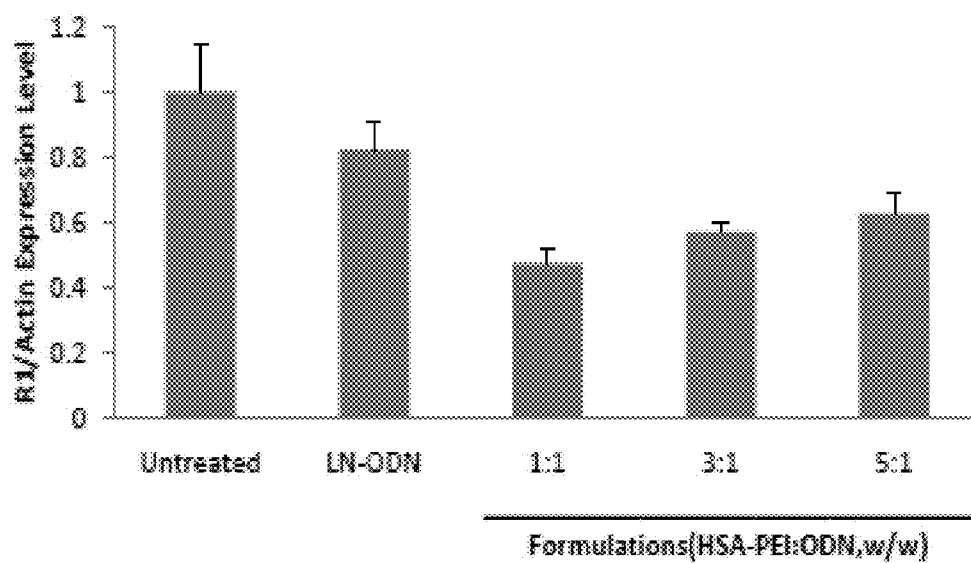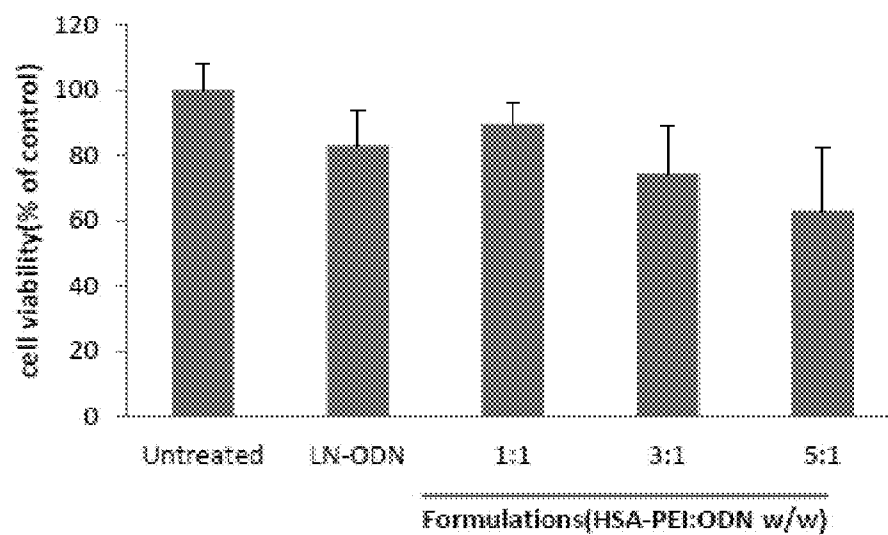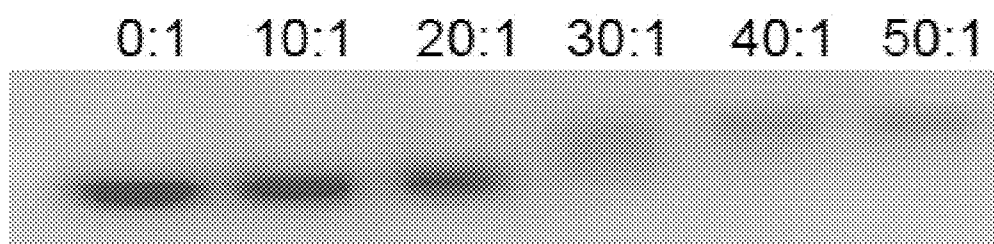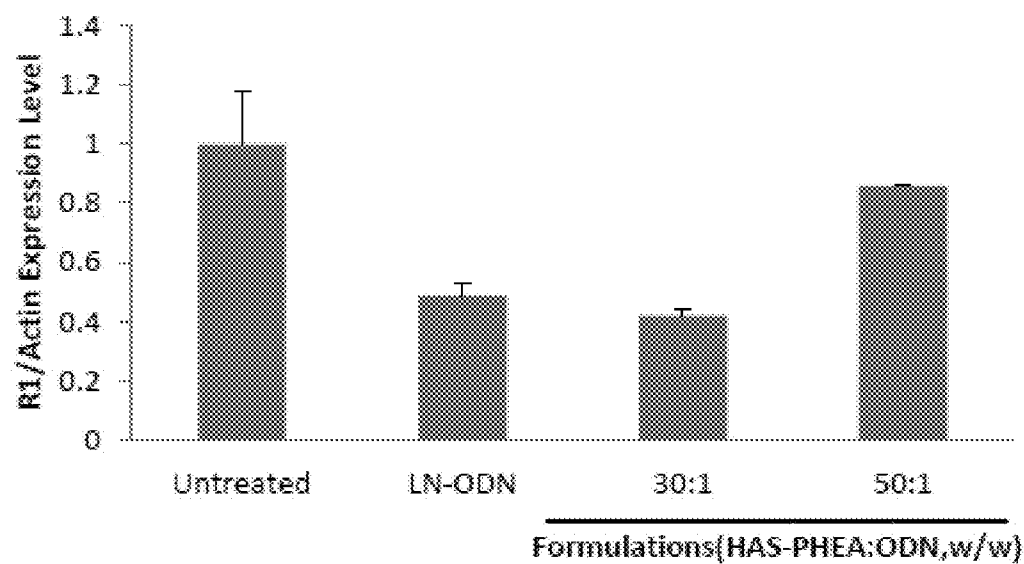
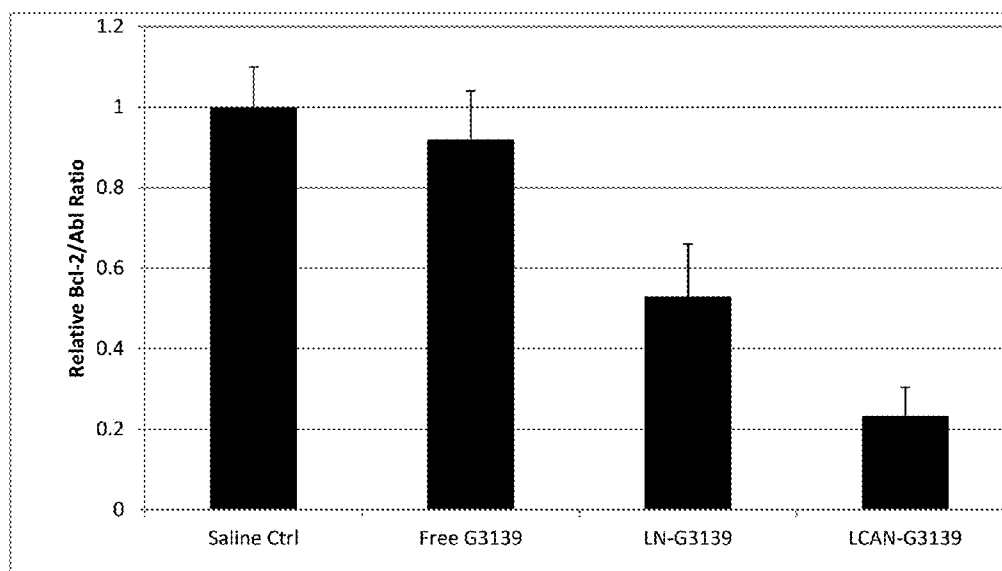0:1   0.5:1   1:1   3:1   6:1

Fig. 1

Fig. 2

Fig. 3A

Fig. 3B

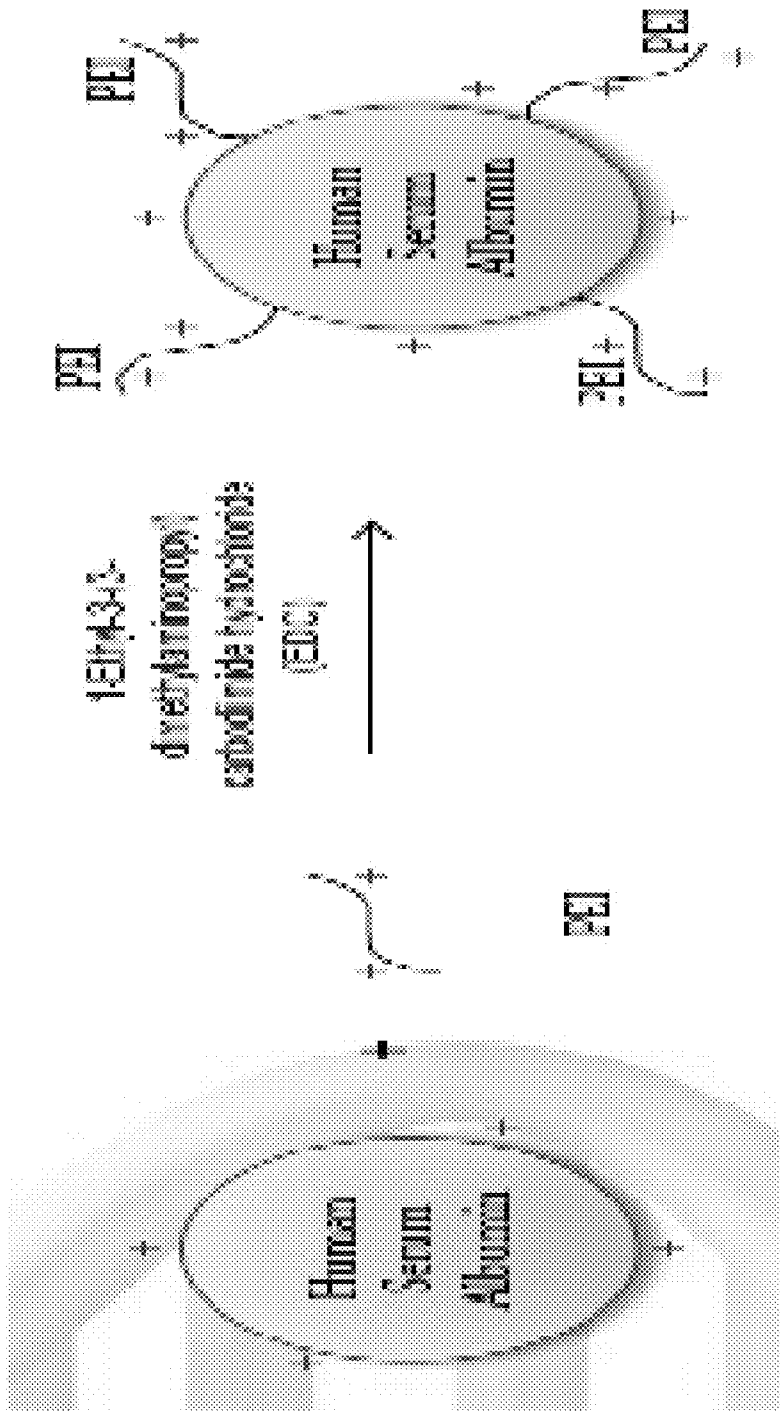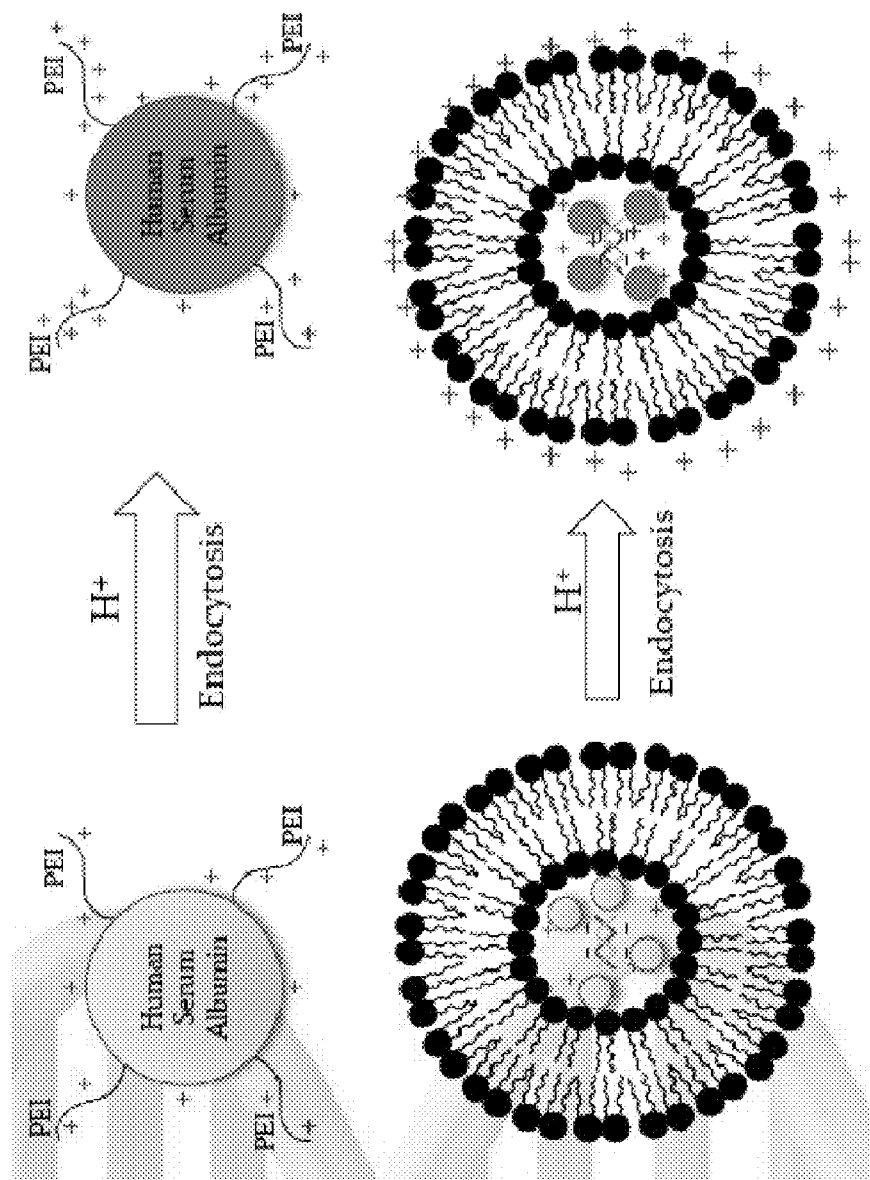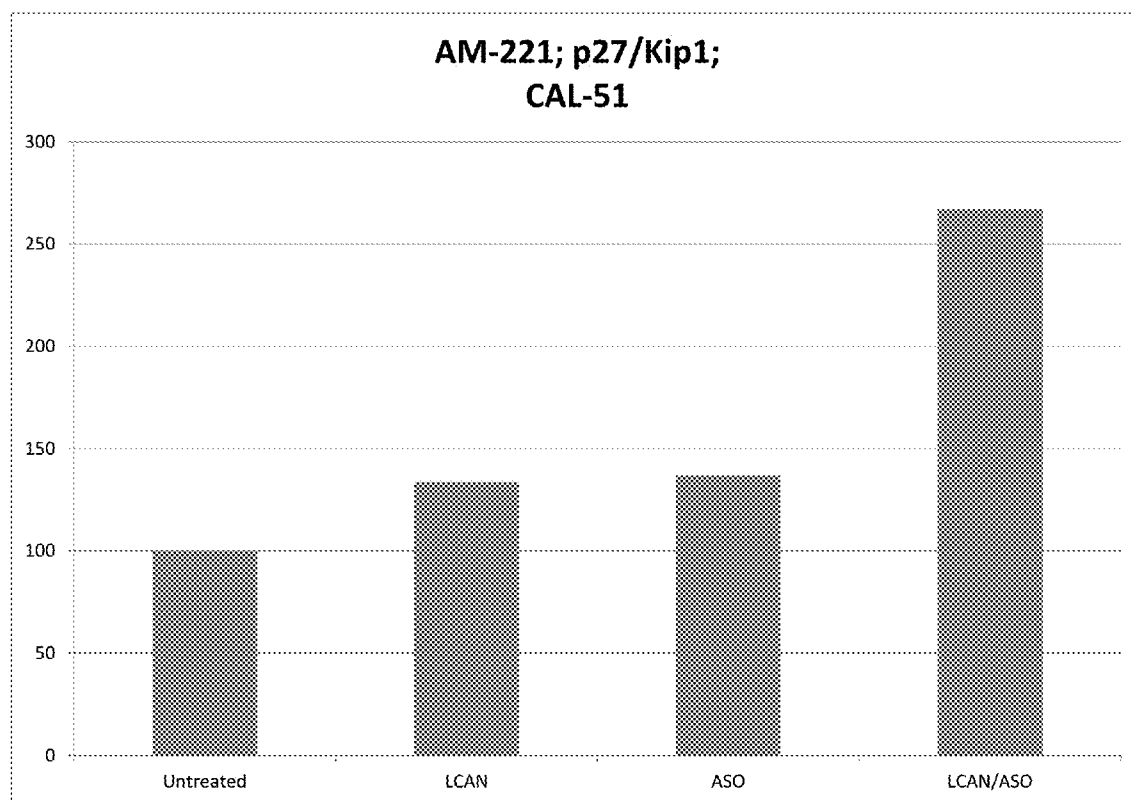Fig. 4

Fig. 5

Fig. 6

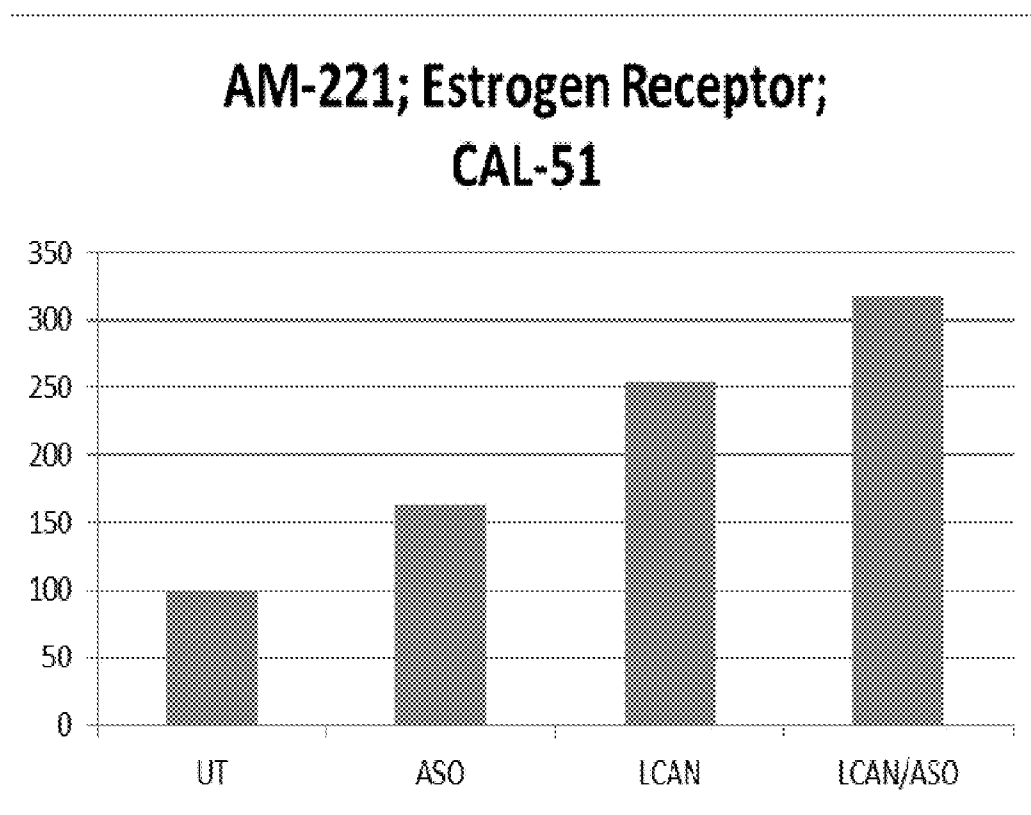Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fig. 11

LIPID-COATED ALBUMIN NANOPARTICLE COMPOSITIONS AND METHODS OF MAKING AND METHOD OF USING THE SAME

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application 61/650,729, filed May 23, 2012, and U.S. Provisional Application 61/784,892, filed Mar. 14, 2013, the disclosures of which are hereby incorporated by reference in their entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under Grant Numbers R01 CA135243, DK088076, and CA152969 awarded by the National Institutes of Health. The government has certain rights in the invention.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted via EFS-web and is hereby incorporated by reference in its entirety. The ASCII copy, created on May 22, 2013, is named 604_55043_SEQ_LIST_OSU-2013-246(2).txt, and is 1,476 bytes in size.

TECHNICAL FIELD

[0004] The present disclosure pertains to lipid nanoparticles (LNs) usable for the delivery of therapeutic compositions, including, but not limited to nucleic acids (NAs).

BACKGROUND OF THE INVENTION

[0005] A liposome is a vesicle composed of one or more lipid bilayers, capable of carrying hydrophilic molecules within an aqueous core or hydrophobic molecules within its lipid bilayer(s). As used herein, "Lipid nanoparticles" (LNs) is a general term to described lipid-based particles in the submicron range. LNs can have structural characteristics of liposomes and/or have alternative non-bilayer types of structures. Drug delivery by LNs via systemic route requires overcoming several physiological barriers. The reticuloendothelial system (RES) is responsible for clearance of LNs from the circulation. Once escaping the vasculature and reaching the target cell, LNs are typically taken up by endocytosis and must release the drug into the cytoplasm prior to degradation within acidic endosome conditions.

[0006] In particular, the delivery of such nucleic acids (NAs), including siRNA and other therapeutic oligonucleotides is a major technical challenge that has limited their potential for clinical translation.

[0007] The development of efficient delivery vehicles is a key to clinical translation of oligonucleotide (ON) therapeutics. It is desired that a LN formulation should be able to (1) protect the drug from enzymatic degradation; (2) traverse the capillary endothelium; (3) specifically reach the target cell type without causing excessive immunoactivation or off-target cytotoxicity; (4) promote endocytosis and endosomal release; and (5) form a stable formulation with high colloidal stability and long shelf-life.

SUMMARY OF THE INVENTION

[0008] Provided herein are LNs that encapsulate therapeutic oligonucleotides with high efficiency and fulfill physical and biological criteria for efficacious delivery. In certain embodiments, the LNs comprise hyper-cationized and/or pH-responsive HSA-polymer conjugates. In certain embodiments, the HSA-polymer conjugate comprises HSA-PEI or HSA-PEHA. The incorporation of hyper-cationized albumin-polymer conjugates (APC) increases the transfection efficiency of LN formulations. These LNs particles are also described herein as lipid-coated albumin nanoparticles (LCANs)

[0009] In a first aspect, provided herein is a lipid nanoparticle (LN) comprising at least one lipid and albumin conjugated to a positively charged polymer.

[0010] In certain embodiments, the LN comprises a hyper-cationized albumin-polycation conjugate (APC). In certain embodiments, the polycation comprises a polyamine selected from the group consisting of spermine, dispermine, trispermine, tetraspermine, oligospermine, thermine, spermidine, dispermidine, trispermidine, oligospermidine, putrescine, polylysine, polyarginine, a polyethylenimine of branched or linear type, and polyallylamine.

[0011] In certain embodiments, the positively-charged polymer consists essentially of a polyethylenimine.

[0012] In certain embodiments, the polyethylenimine has a molecular weight not greater than 50 kDa, or from about 200 Da to about 2000 Da. In certain embodiments, the positively-charged polymer comprises pentaethylenehexamine (PEHA) or tetraethylenepentamine (TEPA).

[0013] In certain embodiments, the LN comprises a polyethylenimine conjugated to human serum albumin.

[0014] In certain embodiments, the conjugation is via one or more cross linking agents. In certain embodiments, the LN comprises PEHA conjugated to HSA.

[0015] In certain embodiments, multiple PEHA molecules are linked to each HSA molecule. For example, in certain embodiments, between about two (2) and about twenty (2) PEHA molecules can be linked to each HSA molecule. In certain embodiments, eleven (11) PEHA molecules are linked to each HSA molecule.

[0016] In certain embodiments, the LN comprises a mixture of two or more low molecular weight polymers.

[0017] In certain embodiments, the at least one lipid comprises a cationic lipid, a neutral lipid, and a PEGylated lipid, with or without cholesterol.

[0018] In certain embodiments, at least one lipid comprises 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), L-α-phosphatidylcholine (SPC), and d-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS). In particular embodiments, the lipids are in a 25:70:5 molar ratio of DOTAP:SPC:TPGS.

[0019] In certain embodiments, the LN encapsulates molecules selected from nucleic acids, chemo therapeutic agents, or combinations thereof. In certain embodiments, the encapsulated molecules comprise a nucleic acid selected from plasmid DNAs, antisense oligonucleotides, miRs, anti-miRs, shRNAs, siRNAs, or combinations thereof. In certain embodiments, the encapsulation rate of therapeutic agents or nucleotides is 40% or higher.

[0020] In certain embodiments, the LN has a diameter under 300 nm, or under 200 nm, or about 98 nm.

[0021] In certain embodiments, the polymer is bound only to an external surface of the nanoparticle via direct connection or via a crosslinker.

[0022] In certain embodiments, the LN further comprises a polyethylene glycol-conjugated lipid. In certain embodiments, the polyethylene glycol-conjugated lipid is selected from the group consisting of polysorbate 80, TPGS, and mPEG-DSPE. In particular embodiments, the polyethylene glycol-conjugated lipid is present at a concentration less than about 15.0 molar percent.

[0023] In certain embodiments, the LN further comprises a ligand capable of binding to a target cell or a target molecule. In certain embodiments, the ligand is an antibody or an antibody fragment. In certain embodiments, the ligand is selected from cRGD, galatose-containing moieties, transferrin, folate, low density lipoprotein, or epidermal growth factors.

[0024] In another broad aspect, provided herein is a pharmaceutical composition comprising a lipid nanoparticle having at least one lipid and albumin conjugated to a positively charged polymer, and a pharmaceutically acceptable excipient.

[0025] In certain embodiments, the pharmaceutical composition is administered perorally, intravenously, intraperitoneally, subcutaneously, or transdermally. In particular embodiments, the pharmaceutical composition is prepared as an orally administered tablet, a sterile solution, a sterile suspension, a lyophilized powder, or a suppository.

[0026] In another broad aspect, provided herein is a method of making a lipid-coated albumin nanoparticle (LCAN). The method involves synthesizing a human serum albumin-pentaethylenehexamine (HSA-PEHA) conjugate; adding at least one lipid to the HSA-PEHA conjugate; adding a nucleic acid to the mixture of lipids and the HSA-PEHA conjugate to obtain an LCAN precursor; and subjecting the LCAN precursor to a dialysis or diafiltration step to make a lipid-coated albumin nanoparticle.

[0027] In certain embodiments, the at least one lipid comprises DOTAP, SPC, and TPGS at a 25:70:5 ratio. In certain embodiments, the nucleic acid is selected from pDNAs, antisense oligonucleotides, miRs, anti-miRs, shRNAs, siRNAs, or combinations thereof. Further provided herein is the product made from the described method.

[0028] In another broad aspect, provided herein is a method of diagnosing or treating a cancer or infectious disease. The method involves administering an effective amount of a pharmaceutical composition comprising at least one lipid, albumin conjugated to a positively-charged polymer, and a pharmaceutically acceptable excipient, to a patient in need thereof.

[0029] In another broad aspect, provided herein is a delivery system comprising at least one lipid and a macromolecule conjugated to a polymer, wherein the macromolecule forms an electrostatic complex with a nucleic acid.

[0030] In another broad aspect, provided herein is a method of using a lipid nanoparticle. The method involves encapsulating a nucleic acid in a lipid nanoparticle, wherein the lipid nanoparticle comprises albumin conjugated to a polymer, incorporating the lipid nanoparticle into a pharmaceutical composition, and administering the pharmaceutical composition to a patient in need thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] FIG. 1: Gel mobility shift analysis of HSA-PEI (600) (APC)-oligodeoxynucleotide (ODN) complexes at varying ODN-to-HSA-PEI(600) w/w ratios. LOR-2501, an ASO against ribonuclease reductase R1 (RNR R1) subunit (purchased from Alpha DNA) was used.

[0032] FIG. 2: Zeta potential of LN (LN)-HSA-PEI(600)-LOR-2501 (APC-ODN) complexes.

[0033] FIGS. 3A-B: Downregulation of RNR R1 mRNA expression by LOR-2501 in LCANs. The LCANs were prepared at varying APC concentrations under different media conditions: FIG. 3A displays serum-free media; FIG. 3B displays media containing 10% FBS. RNR R1 mRNA expression relative to actin was determined by RT-PCR where untreated KB cells served as a baseline for mRNA expression.

[0034] FIG. 4: Cell viability study of KB cells treated with LCAN-HSA-PEI(600)-LOR-2501 (APC) complex. Transfection was performed in serum-free media. Cell viabilities are expressed as a percentage relative to the mean viability of the untreated KB cells.

[0035] FIG. 5: Gel mobility shift analysis of HSA-PEHA-LOR-2501 (APC-ODN) complexes at varying ODN-to-APC w/w ratios. LOR-2501 was used as the ODN in this study.

[0036] FIG. 6: Downregulation of RNR R1 mRNA expression by LOR-2501 in LCANs. The LCANs were prepared at varying APC concentrations under serum-free conditions. RNR R1 mRNA expression relative to actin was determined by RT-PCR where untreated KB cells served as a baseline for mRNA expression.

[0037] FIG. 7: Bcl-2 down regulation in KB cells by lipid-coated albumin nanoparticle (LCAN)-G3139 as compared to LN-G3139.

[0038] FIG. 8: An example scheme for synthesizing an APC.

[0039] FIG. 9: The mechanism of action for hyper-cationized pH-responsive APCs.

[0040] FIG. 10: Upregulation of p27/kip1 mRNA by LCAN loaded with anti-miR-221 in CAL-51 breast cancer cells.

[0041] FIG. 11: Upregulation of estrogen mRNA by LCAN loaded with anti-miR-221 in CAL-51 cells. The estrogen receptor is a target of miR-221.

DETAILED DESCRIPTION OF THE INVENTION

[0042] Those of ordinary skill in the art will realize that the following detailed description of the embodiments is illustrative only and not intended to be in any way limiting. Other embodiments will readily suggest themselves to such skilled persons having the benefit of this disclosure. Reference to an "embodiment," "aspect," or "example" herein indicate that the embodiments of the invention so described may include a particular feature, structure, or characteristic, but not every embodiment necessarily includes the particular feature, structure, or characteristic. Further, repeated use of the phrase "in one embodiment" does not necessarily refer to the same embodiment, although it may.

[0043] Not all of the routine features of the implementations or processes described herein are shown and described. It will, of course, be appreciated that in the development of any such actual implementation, numerous implementation-specific decisions will be made in order to achieve the developer's specific goals, such as compliance with application-and business-related constraints, and that these specific goals will vary from one implementation to another and from one developer to another. Moreover, it will be appreciated that such a development effort might be complex and time-con-

3

suming, but would nevertheless be a routine undertaking for those of ordinary skill in the art having the benefit of this disclosure.

[0044] General Description

[0045] Nucleic acid (NA)-based therapies are being developed to promote or inhibit gene expression. As mutations in genes and changes in miRNA profile are believed to be the underlying cause of cancer and other diseases, NA-based agents can directly act upon the underlying etiology, maximizing therapeutic potential. Non-limiting examples of NA-based therapies include: plasmid DNA (pDNA), small interfering RNA (siRNA), small hairpin RNA (shRNA), microRNA (miR), mimic (mimetic), anti-miR/antagomiR/miR inhibitor, and antisense oligonucleotide (ASO). Until the development of the nanoparticle compositions described herein, the clinical translation of NA-based therapies faced several obstacles in their implementation since transporting NAs to their intracellular target was particularly challenging and since NAs are relatively unstable and subject to degradation by serum and cellular nucleases. Further, the high negative charges of NAs made it impossible for transport across the cell membrane, further limiting utility.

[0046] The LNs described herein provide a useful platform for the delivery of both traditional therapeutic compounds and NA-based therapies. Drugs formulated using LNs provide desirable pharmacokinetic (PK) properties in vivo, such as increased blood circulation time and increased accumulation at the site of solid tumors due to enhanced permeability and retention (EPR) effect. Moreover, in certain embodiments, the LNs may be surface-coated with polyethylene glycol to reduce opsonization of LNs by serum proteins and the resulting RES-mediated uptake, and/or coated with cell-specific ligands to provide targeted drug delivery.

[0047] It is desired that the zeta potential of LNs not be excessively positive or negative for systemic delivery. LNs with a highly positive charge tend to interact non-specifically with non-target cells, tissues, and circulating plasma proteins, and may cause cytotoxicity. Alternatively, LNs with a highly negative charge cannot effectively incorporate NAs, which are themselves negatively charged, and may trigger rapid RES-mediated clearance, reducing therapeutic efficacy. LNs with a neutral to moderate charge are best suited for in vivo drug and gene delivery.

[0048] In certain embodiments, the LNs described herein comprise hyper-cationized albumin-polymer conjugates (APCs). As used herein, the term "hyper-cationized" mean each polycation carries multiple positive charges. In particular embodiments, up to 20 polycations can be linked to each albumin molecule. These factors result in a much higher overall charge content for APCs compared to traditional cationized albumin, which typically comprises an albumin conjugate where carboxyl groups are replaced with single-positive-charge amine functional groups. Because of their high charge density, the APCs are able to very efficiently interact with polyanions such as an oligonucleotide particle, molecule, compound or formulation having multiple cations or positive charges.

[0049] The term "lipid nanoparticle" (LP) as used herein refers to a vesicle formed by one or more lipid components. The lipid components described herein may include cationic lipids. Cationic lipids are lipids that carry a net positive charge at any physiological pH. In certain embodiments, the

positive charge is used for association with negatively charged therapeutics such as ASOs via electrostatic interaction.

[0050] Suitable cationic lipids include, but are not limited to: 3β-[N—(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Chol); 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); 1,2-dioleoyl-3-dimethylammonium-propane (DODAP); dimethyldioctadecylammonium bromide salt (DDAB); 1,2-dilauroyl-sn-glycero-3-ethylphosphocholine chloride (DL-EPC); N-[1-(2,3-dioleyloxy)propyl]-N—N—N-trimethyl ammonium chloride (DOTMA); N-[1-(2,3-dioleyloxy)propyl]-N—N—N-dimethyl ammonium chloride (DODMA); N,N-dioctadecyl-N,N-dimethylammonium chloride (DODAC); N-(1-(2,3-dioleyloxy)propyl)-N-2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoracetate (DOSPA); 1,2-dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide (DMRIE); dioctadecylamidoglycylspermine (DOGS); neutral lipids conjugated to cationic modifying groups; and combinations thereof. In addition, a number of cationic lipids in available preparations could be used, such as LIPOFECTIN® (from GIBCO/BRL), LIPOFECTAMINE® (from GIBCO/MRL), siPORT NEOFX® (from Applied Biosystems), T RANSFECTAM® (from Promega), and TRANSFECTIN® (from Bio-Rad Laboratories, Inc.). The skilled practitioner will recognize that many more cationic lipids are suitable for inclusion in the LN formulations. In certain embodiments, the cationic lipids may be present at concentrations up to about 80.0 molar percent of total lipids in the formulation, or from about 5.0 to about 50.0 molar percent of the formulation.

[0051] In certain embodiments, the LN formulations presently disclosed may also include anionic lipids. Anionic lipids are lipids that carry a net negative charge at physiological pH. These anionic lipids, when combined with cationic lipids, are useful to reduce the overall surface charge of LNs and introduce pH-dependent disruption of the LN bilayer structure, facilitating nucleotide release by inducing nonlamellar phases at acidic pH or induce fusion with the cellular membrane.

[0052] Examples of suitable anionic lipids include, but are not limited to: fatty acids such as oleic, linoleic, and linolenic acids; cholesteryl hemisuccinate; 1,2-di-O-tetradecyl-sn-glycero-3-phospho-(1'-rac-glycerol) (diether PG); 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt); 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1-hexadecanoyl, 2-(9Z,12Z)-octadecadienoyl-sn-glycero-3-phosphate; 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DOPG); dioleoylphosphatidic acid (DOPA); and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS); anionic modifying groups conjugated to neutral lipids; and combinations thereof. The anionic lipids of the present disclosure are present at concentrations up to about 60.0 molar percent of the formulation, or from about 5.0 to about 25.0 molar percent of the formulation.

[0053] In certain embodiments, charged LNs are advantageous for transfection, but off-target effects such as cytotoxicity and RES-mediated uptake may occur. Hydrophilic molecules such as polyethylene glycol (PEG) may be conjugated to a lipid anchor and included in the LNs described herein to prevent LN aggregation or interaction with membranes. Hydrophilic polymers may be covalently bonded to lipid components or conjugated using crosslinking agents to functional groups such as amines.

4

[0054] Suitable conjugates of hydrophilic polymers include, but are not limited to: polyvinyl alcohol (PVA); polysorbate 80; 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-PEG2000 (DSPE-PEG2000); D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS); dimyristoylphosphatidylethanolamine-PEG2000 (DMPE-PEG2000); and dipalmitoylphosphatidylethanolamine-PEG2000 (DPPE-PEG2000). In certain embodiments, the hydrophilic polymer may be present at concentrations ranging from about 0 to about 15.0 molar percent of the formulation, or from about 5.0 to about 10.0 molar percent of the formulation. Also, in certain embodiments, the molecular weight of the PEG used is between about 100 and about 10,000 Da, or from about 100 to about 2,000 Da.

[0055] The LNs described herein may further comprise neutral and/or cholesterol lipids as helper lipids. These lipids are useful to stabilize the formulation, reduce elimination in vivo, or increase transfection efficiency. The LNs may be formulated in a solution of saccharides such as, but not limited to, glucose, sorbitol, sucrose, maltose, trehalose, lactose, cellubiose, raffinose, maltotriose, dextran, or combinations thereof, to promote lyostability and/or cryostability.

[0056] Neutral lipids have zero net charge at physiological pH. One or a combination of several neutral lipids may be included in any LN formulation disclosed herein.

[0057] Suitable neutral lipids include, but are not limited to: phosphatidylcholine (PC, e.g., DSPC, DPPC, DOPC, DMPC, soyPC, eggPC, HSPC), phosphatidylethanolamine (PE, e.g., DOPE, DSPE, DPPE, DSPE, DMPE), ceramide, cerebrosides, sphingomyelin, cephalin, cholesterol, diacylglycerol, glycosylated diacylglycerols, prenols, lysosomal PLA2 substrates, N-acylglycines, and combinations thereof.

[0058] Other suitable lipids include, but are not limited to: phosphatidic acid, (PG, e.g., DSPG, DMPG, DPPG), and lysophosphatidylethanolamine; sterols such as cholesterol, demosterol, sitosterol, zymosterol, diosgenin, lanostenol, stigmasterol, lathosterol, and dehydroepiandrosterone; and sphingolipids such as sphingosines, ceramides, sphingomyelin, gangliosides, glycosphingolipids, phosphosphingolipids, phytoshingosine; and combinations thereof.

[0059] The LN formulations described herein may further comprise fusogenic lipids or fusogenic coatings to promote membrane fusion. Examples of suitable fusogenic lipids include, but are not limited to, glyceryl mono-oleate, oleic acid, palmitoleic acid, phosphatidic acid, phosphoinositol 4,5-bisphosphate (PIP$_2$), and combinations thereof.

[0060] The LN formulations described here may further comprise cationic polymers or conjugates of cationic polymers. Cationic polymers or conjugates thereof may be used alone or in combination with lipid nanocarriers. Suitable cationic polymers include, but are not limited to: polyethylenimine (PEI); pentaethylenehexamine (PEHA); spermine; spermidine; poly(L-lysine); poly(amido amine) (PAMAM) dendrimers; polypropyleneiminie dendrimers; poly(2-dimethylamino ethyl)-methacrylate (pDMAEMA); chitosan; tris (2-aminoethyl)amine and its methylated erivatives; and combinations thereof. In certain embodiments, the chain length and branching are important considerations for the implementation of polymeric delivery systems. High molecular weight polymers such as PEI (MW 25,000) are useful as transfection agents, but suffer from cytotoxicity. Low molecular weight PEI (MW 600) does not cause cytotoxicity, but is limited due to its inability to facilitate stable condensation with NAs. As described herein the conjugation of low

molecular weight polymers to a larger molecule such as albumin is a thus a useful method of increasing activity of electrostatic complexation with NA condensation while lowing cytotoxicity of LN formulations.

[0061] Anionic polymers may be incorporated into the LN formulations presently disclosed as well. Suitable anionic polymers include, but are not limited to: poly(propylacrylic acid) (PPAA); poly(glutamic acid) (PGA); alginates; dextran derivatives; xanthans; derivatized polymers; and combinations thereof.

[0062] In certain embodiments, the LN formulation includes conjugates of polymers. The conjugates may be crosslinked to targeting agents, lipophilic moieties, proteins, or other molecules that increase the overall therapeutic efficacy. Suitable crosslinking agents include, but are not limited to: N-succinimidyl 3-[2-pyridyldithio]-propionate (SPDP); dimethyl 3,3'-dithiobispropionimidate (DTBP); dicyclohexylcarbodiimide (DCC); diisopropyl carbodiimide (DIC); 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC); N-hydroxysulfosuccinimide (Sulfo-NHS); N'—N'-carbonyldiimidazole (CDI); N-ethyl-5-phenylisoxazolium-3'sulfonate (Woodward's reagent K); and combinations thereof.

[0063] The addition of targeting agents to the LN provides increased efficacy over passive targeting approaches. Targeting involves incorporation of specific targeting moieties such as, but not limited to, ligands or antibodies against cell surface receptors, peptides, lipoproteins, glycoproteins, hormones, vitamins, antibodies, antibody fragments, and conjugates or combinations of these moieties.

[0064] In certain embodiments, the maximization of targeting efficiency includes the surface coating of the LN with the appropriate targeting moiety rather than pre-mixing of the targeting ligand with other components, which results in partial encapsulation of the targeting agent, rendering it inaccessible to the cellular target. This method optimizes interaction with cell surface receptors.

[0065] It is to be understood that targeting agents may be either directly incorporated into the LN during synthesis or added in a subsequent step. Functional groups on the targeting moiety as well as specifications of the therapeutic application (e.g., degradable linkage) dictate the appropriate means of incorporation into the LN. For example, targeting moieties that do not have lipophilic regions cannot insert into the lipid bilayer of the LN directly and require prior conjugation to a lipid anchor before insertion or must form an electrostatic complex with the LNs.

[0066] Also, under certain circumstances, a targeting ligand cannot directly connect to a lipophilic anchor. In these circumstances, a molecular bridge in the form of a crosslinking agent may be utilized to facilitate the interaction. In certain embodiments, it is advantageous to use a crosslinking agent if steric restrictions of the anchored targeting moiety prevent sufficient interaction with the intended physiological target. Additionally, if the targeting moiety is only functional under certain orientations (e.g., monoclonal antibody), linking to a lipid anchor via crosslinking agent is beneficial. In certain embodiments, other methods of bioconjugation may be used to link targeting agents to LNs. Reducible or hydrolysable linkages may be applied to prevent accumulation of the formulation in vivo and the related cytotoxicity.

[0067] Various methods of LN preparation are suitable to synthesize the LNs of the present disclosure. For example, ethanol dilution, freeze-thaw, thin film hydration, sonication, extrusion, high pressure homogenization, detergent dialysis,

microfluidization, tangential flow diafiltration, sterile filtration, and/or lyophilization may be utilized. Additionally, several methods may be employed to decrease the size of the LNs. For example, homogenization may be conducted on any devices suitable for lipid homogenization such as an Avestin Emulsiflex C5®. Extrusion may be conducted on a Lipex Biomembrane extruder using a polycarbonate membrane of appropriate pore size (0.05 to 0.2 μm). Multiple particle size reduction cycles may be conducted to minimize size variation within the sample. The resultant LNs may then be passed through a size exclusion column such as Sepharose CL4B or processed by tangential flow diafiltration to purify the LNs.

[0068] Any embodiment of the LNs described herein may further include ethanol in the preparation process. The incorporation of about 30-50% ethanol in LN formulations destabilizes the lipid bilayer and promotes electrostatic interactions among charged moieties such as cationic lipids with anionic ASO and siRNA. LNs prepared in high ethanol solution are diluted before administration. Alternatively, ethanol may be removed by dialysis or diafiltration, which also removes non-encapsulated NA.

[0069] In certain embodiment, it is desirable that the LNs be sterilized. This may be achieved by passing of the LNs through a 0.2 or 0.22 μm sterile filter with or without prefiltration.

[0070] Physical characterization of the LNs can be carried through many methods. For example, dynamic light scattering (DLS) or atomic force microscopy (AFM) can be used to determine the average diameter and its standard deviation. In certain embodiments, it is especially desirable that the LNs have about a 200 nm diameter, or less. Zeta potential measurement via zeta potentiometer is useful in determining the relative stability of particles. Both dynamic light scattering analysis and zeta potential analysis may be conducted with diluted samples in deionized water or appropriate buffer solution. Cryogenic transmission electron microscopy (Cryo-TEM) and scanning electron microscopy (SEM) may be used to determine the detailed morphology of LNs.

[0071] The LNs described herein are stable under refrigeration for several months. LNs requiring extended periods of time between synthesis and administration may be lyophilized using standard procedures. A cryoprotectant such as 10% sucrose may be added to the LN suspension prior to freezing to maintain the integrity of the formulation during lyophilization. Freeze drying of LN formulations is recommended for long term stability.

[0072] In certain embodiments, the LCANs described herein have a diameter of less than 300 nm, and, in particular embodiments, between about 50 and about 200 nm in mean diameter. These LNs show enhanced transfection and reduced cytotoxicity, especially under high serum conditions found during systemic administration. The LNs are useful in a wide range of current therapeutic agents and systems, have high serum stability, and can be designed for targeted delivery with high transfection efficiency.

[0073] Albumin-Polymer Conjugates (APCs)

[0074] The utilization of cationic polymers as transfection agents alone and in conjunction with lipids in LNs often benefits transfection efficiency. One polymeric transfection agent is high molecular weight polyethylenimine (PEI), a large polymer with a molecular weight of ~25 kDa. While PEI25K has been used to deliver pDNA to cells, cytotoxicity has limited its use in vivo. Less toxic, low molecular weight

PEI (MW ~600 kDa) has also been investigated, but this has shown diminished ability to interact with and deliver NAs.

[0075] Provided herein are hyper-cationized albumin-polymer conjugates (APCs), which do not have any of the deficiencies of the aforementioned polymers. APCs may either be used alone to deliver agents such as pDNA or combined with lipid-based formulations to deliver agents such as ASOs and siRNA. Albumin also possesses endosomal lytic activity due to its hydrophobic core, which upon conformational change can be exposed and can induce bilayer disruption or membrane fusion. In some embodiments, such as the HSA-PEI600 conjugate, the APC has an ionization profile that is responsive to pH change. The charge density is increased at endosomal pH, which is acidic.

[0076] In one embodiment, an APC is combined with a cationic lipid combination to assemble a cationic lipid-APC-NA nanoparticle, sometimes herein called LCAN. In another embodiment, an APC is combined with an anionic lipid combination to assemble a lipid-APC-NA nanoparticle. In certain embodiments, the LNs comprise hyper-cationized APCs. These LNs have high transfection efficiency without additional cytotoxicity. An example scheme for synthesizing an APC is shown in FIG. 8. The mechanism of action for hyper-cationized pH-responsive APCs is shown in FIG. 9.

[0077] Also provided herein are macromolecules conjugated to polymers, such as positively charged polymers. In one embodiment, a low molecular weight pH-sensitive polymer (polyethylenimine MW 600, PEI600) is conjugated to human serum albumin (HSA) via cross linking agents, resulting in a hyper-cationized pH-responsive APC. The addition of HSA-PEI600 conjugates to LNs significantly increases downregulation of RRM1 (aka RNR R1) with ASO LOR-2501 (purchased from Alpha DNA) in the presence of serum without substantial cytotoxicity in KB cells (a subline of HeLa).

[0078] In another embodiment, a low molecular weight pentaethylenehexamine (PEHA) is conjugated to HSA via cross linking agents, resulting in a hyper-cationized pH-responsive APC. This particular formulation, referred to as a lipid-coated albumin nanoparticle (LCAN), is especially useful for the delivery of oligonucleotides, such as antisense ODNs, pDNAs, siRNAs, shRNAs, miRs, and anti-miRs. Without wishing to be bound by theory, it is believed HSA-PEHA improves the stability and biological activity of the nanoparticles. In certain embodiments, the lipids in this formulation are DOTAP, SPC, and TPGS, at a ratio of 25:70:5 (mol/mol).

[0079] Applications

[0080] Depending on the application, the LNs disclosed herein may be designed to favor characteristics such as increased loading of NAs, increased serum stability, reduced RES-mediated uptake, targeted delivery, or pH sensitive release within the endosome. Because of the varied nature of LN formulations, any one of the several methods provided herein may be used to achieve a particular therapeutic aim. Cationic lipids, anionic lipids, polyalkenes, neutral lipids, fusogenic lipids, cationic polymers, anionic polymers, polymer conjugates, peptides, targeting moieties, and combinations thereof may be utilized to meet specific aims.

[0081] The LNs described herein can be used as platforms for therapeutic delivery of oligonucleotide (ON) therapeutics, such as cDNA, siRNA, shRNA, miRNA, anti-miR, and antisense ODN. These therapeutics are useful to manage a wide variety of diseases such as various types of cancers, leuke-

mias, viral infections, and other diseases. For instance, targeting moieties such as cyclic-RGD, folate, transferrin, or antibodies greatly enhance activity by enabling targeted drug delivery. A number of tumors overexpress receptors on their cell surface. Non-limiting examples of suitable targeting moieties include transferrin (Tf), folate, low density lipoprotein (LDL), and epidermal growth factors. In addition, tumor vascular endothelium markers such as alpha-v-beta-3 integrin and prostate-specific membrane antigen (PSMA) are valuable as targets for LNs. In certain embodiments, LN formulations having particles measuring about 300 nm or less in diameter with a zeta potential of less than 50 mV and an encapsulation efficiency of greater than 20.0% are useful for NA delivery.

[0082] Implementation of embodiments of the LN formulations described herein alone or in combination with one another synergizes with current paradigms of LN design.

[0083] A wide spectrum of therapeutic agents may be used in conjunction with the LNs described herein. Non-limiting examples of such therapeutic agents include antineoplastic agents, anti-infective agents, local anesthetics, anti-allergics, antianemics, angiogenesis-inhibitors, beta-adrenergic blockers, calcium channel antagonists, anti-hypertensive agents, anti-depressants, anti-convulsants, anti-bacterial, anti-fungal, anti-viral, anti-rheumatics, anthelminthics, antiparasitic agents, corticosteroids, hormones, hormone antagonists, immunomodulators, neurotransmitter antagonists, anti-diabetic agents, anti-epileptics, anti-hemmorhagics, anti-hypertonics, antiglaucoma agents, immunomodulatory cytokines, sedatives, chemokines, vitamins, toxins, narcotics, imaging agents, and combinations thereof.

[0084] NA-based therapeutic agents are highly applicable to the LN formulations of the present disclosure. Examples of such NA-based therapeutic agents include, but are not limited to: pDNA, siRNA, miRNA, anti-miRNA, ASO, and combinations thereof. To protect from serum nucleases and to stabilize the therapeutic agent, modifications to the substituent NA base units and/or phosphodiester linker can be made. Such modifications include, but are not limited to: backbone modifications (e.g., phosphorothioate linkages); 2' modifications (e.g., 2'-O-methyl substituted bases); zwitterionic modifications (6'-aminohexy modified ODNs); the addition of a terminal lipophilic moiety (e.g., fatty acids, cholesterol, or cholesterol derivatives); and combinations thereof. The modified sequences synergize with the LN formulations disclosed herein. For example, addition of a 3'-cholesterol to an ODN supplies stability to a LN complex by adding lipophilic interaction in a system otherwise primarily held together by electrostatic interaction during synthesis. In addition, this lipophilic attachment promotes cell permeation by localizing the ODN to the outer leaflet of the cell membrane.

[0085] Depending on the therapeutic application, the LNs described herein may be administered by the following methods: peroral, parenteral, intravenous, intramuscular, subcutaneous, intraperitoneal, transdermal, intratumoral, intraarterial, systemic, or convection-enhanced delivery. In particular embodiments, the LNs are delivered intravenously, intramuscularly, subcutaneously, or intratumorally. Subsequent dosing with different or similar LNs may use alternative routes of administration.

[0086] Pharmaceutical compositions of the present disclosure comprise an effective amount of a LN formulation disclosed herein, and/or additional agents, dissolved or dispersed in a pharmaceutically acceptable carrier. The phrases

"pharmaceutical" or "pharmacologically acceptable" refers to molecular entities and compositions that produce no adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human. The preparation of a pharmaceutical composition that contains at least one compound or additional active ingredient will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington's Pharmaceutical Sciences, 2003, incorporated herein by reference. Moreover, for animal (and human) administration, it will be understood that LN preparations should meet sterility, pyrogenicity, and general safety and purity standards as required by FDA Office of Biological Standards.

[0087] A composition disclosed herein may comprise different types of carriers depending on whether it is to be administered in solid, liquid or aerosol form, and whether it need to be sterile for such routes of administration as injection. Compositions disclosed herein can be administered intravenously, intradermally, transdermally, intrathecally, intraarterially, intraperitoneally, intranasally, intravaginally, intrarectally, topically, intramuscularly, subcutaneously, mucosally, in utero, orally, topically, locally, via inhalation (e.g., aerosol inhalation), by injection, by infusion, by continuous infusion, by localized perfusion bathing target cells directly, via a catheter, via a lavage, in cremes, in lipid compositions (e.g., liposomes), or by other method or any combination of the forgoing as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 2003, incorporated herein by reference).

[0088] The actual dosage amount of a composition disclosed herein administered to an animal or human patient can be determined by physical and physiological factors such as body weight or surface area, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. Depending upon the dosage and the route of administration, the number of administrations of a preferred dosage and/or an effective amount may vary according to the response of the subject. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose (s) for the individual subject.

[0089] In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1% of an active compound. Naturally, the amount of active compound (s) in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[0090] In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about

500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight of active pharmaceutical ingredient (API), etc., can be administered, based on the numbers described above.

[0091] In certain embodiments, a composition herein and/or additional agents is formulated to be administered via an alimentary route. Alimentary routes include all possible routes of administration in which the composition is in direct contact with the alimentary tract. Specifically, the pharmaceutical compositions disclosed herein may be administered orally, buccally, rectally, or sublingually. As such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier.

[0092] In further embodiments, a composition described herein may be administered via a parenteral route. As used herein, the term "parenteral" includes routes that bypass the alimentary tract. Specifically, the pharmaceutical compositions disclosed herein may be administered, for example but not limited to, intravenously, intradermally, intramuscularly, intraarterially, intrathecally, subcutaneous, or intraperitoneally (U.S. Pat. Nos. 6,753,514, 6,613,308, 5,466,468, 5,543,158; 5,641,515; and 5,399,363 are each specifically incorporated herein by reference in their entirety).

[0093] Solutions of the compositions disclosed herein as free bases or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U.S. Pat. No. 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy injectability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (i.e., glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, 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. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include agents to achieve isotonicity, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption such as, for example, aluminum monostearate or gelatin.

[0094] For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solu-

tions are especially suitable for intravenous, intramuscular, subcutaneous, and intraperitoneal administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety, and purity standards as required by FDA Office of Biologics standards.

[0095] Sterile injectable solutions are prepared by incorporating the compositions in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by sterilization. Generally, dispersions are prepared by incorporating the various sterilized compositions into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, some methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. A powdered composition is combined with a liquid carrier such as, e.g., water or a saline solution, with or without a stabilizing agent.

[0096] In other embodiments, the compositions may be formulated for administration via various miscellaneous routes, for example, topical (i.e., transdermal) administration, mucosal administration (intranasal, vaginal, etc.) and/or via inhalation.

[0097] In certain embodiments, the compositions may be delivered by eye drops, intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering compositions directly to the lungs via nasal aerosol sprays has been described in U.S. Pat. Nos. 5,756,353 and 5,804,212 (each specifically incorporated herein by reference in their entirety). Likewise, the delivery of drugs using intranasal microparticle resins (Takenaga et al., 1998) and lysophosphatidyl-glycerol compounds (U.S. Pat. No. 5,725,871, specifically incorporated herein by reference in its entirety) are also well-known in the pharmaceutical arts and could be employed to deliver the compositions described herein. Likewise, transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U.S. Pat. No. 5,780,045 (specifically incorporated herein by reference in its entirety), and could be employed to deliver the compositions described herein.

[0098] It is further envisioned the compositions disclosed herein may be delivered via an aerosol. The term aerosol refers to a colloidal system of finely divided solid or liquid particles dispersed in a liquefied or pressurized gas propellant. The typical aerosol for inhalation consists of a suspension of active ingredients in liquid propellant or a mixture of liquid propellant and a suitable solvent. Suitable propellants include hydrocarbons and hydrocarbon ethers. Suitable containers will vary according to the pressure requirements of the

propellant. Administration of the aerosol will vary according to subject's age, weight and the severity and response of the symptoms.

EXAMPLES

[0099] Certain embodiments of the present invention are defined in the Examples herein. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

Example 1

[0100] HSA (25%) was purchased from Octapharma. PEHA was purchased from Sigma-Aldrich. A stock solution of PEHA, pH adjusted to 8.0 with M HCl was prepared. HSA was combined with 500× of PEHA. Then 80× of the 1-ethyl-3-[3dimethylaminopropyl]carbodiimide hydrochloride (EDC) (from Fisher Scientific) was added to the solution under stiffing. The reaction proceeded at room temperature for >4 h. The produce HSA-PEHA was purified by gel filtration chromatography on a PD-10 desalting column or by dialysis using a MWCO 10,000 membrane to remove unreacted PEHA and byproducts. Protein concentration of the product was determined by a BCA protein assay. The molecular weight of the HSA-PEHA conjugate was determined by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MADLI TOF MS). On average, there were 11 PEHA linked to each HSA based on the result showing m/z of 66405.756. The product can be stored at 4° C., frozen, or lyophilized.

Example 2

[0101] Lipids 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (Avanti Polar Lipids), L-α-phosphatidylcholine derived from soybean (SPC) (Avanti Polar Lipids), and d-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS) (Eastman Chemical) were dissolved in ethanol. Lipids were combined at 25:70:5 (mol/mol). Briefly, 3.15, 8.83, and 0.63 μmol of DOTAP, SPC, and TPGS, respectively, were combined in 4 mL of ethanol. This was then added into 3 mg of HSA-PEHA dissolved in 4 mL of 20 mM HEPES buffer (pH 7.4), followed by 1 mg of an ASO against bcl-2, G3139 (purchased from Alpha DNA) in 2 mL of 20 mM HEPES buffer to form a mixture containing 40% ethanol. This was then dialyzed against HEPES buffer using a MWCO 10K Slide-A-Lyzer cassette to remove ethanol and free G3139. The product, lipid-coated albumin nanoparticle (LCAN)-G3139, was concentrated to 2 mg/mL ODN concentration by diafiltration, 10% sucrose was added into the product, and the product was sterile filtered through a 0.2 μm filter. The LCAN-G3139 was stable at 4° C., and was frozen or lyophilized for long term storage. The particle size of LCAN was determined by NICOMP 370 particle size analyzer. The zeta potential was determined on a zetaPALS instrument. Drug loading efficiency was determined by Oligreen ssDNA quantitation reagents. LCAN particles were found to be <200 nm in diameter, had a zeta potential of +20~+40 mV and a G3139 encapsulation efficiency of greater than >60%. The same process was used to synthesize ligand-conjugated LCANs by

incorporating lipid-derivatized ligands into the lipid components during nanoparticle synthesis. Possible ligands include transferring, folate, cRGD, or antibodies.

Example 3

[0102] HSA-PEHA conjugate was synthesized as follows. HSA (25%) was purchased from Octapharma. PEHA was dissolved in water and the pH was adjusted to 8.0 with HCl. A 500-fold excess of PEHA was added to the HSA solution, followed by 80-fold excess of EDC under stiffing. The reaction proceeded at room temperature for 4 hr. The product was purified and concentrated by tangential flow diafiltration on a MicroKros cartridge with MW of 10,000 against water. The product protein concentration was determined by BCA protein assay and the PEHA content in the product was determined by TNBS amine content assay using a PEHA-based standard curve. The PEHA-HSA ratio was calculated based on surplus in amine content relative to unmodified HSA and found to be 10.5:1. SDS-PAGE analysis showed that the conjugate migrated as a single band, indicating lack of intermolecular crosslinking in the HSA-PEHA product.

[0103] G3139 is an 18-mer phosphorothioate ASO against bcl-2. G3139 purchased from AlphaDNA was dissolved in mM HEPES, pH 7.4. DOTAP/SPC/TPGS at 25:70:5 (m/m) was dissolved in ethanol and added to HSA-PEHA diluted in 20 mM HEPES, followed by the G3139 solution, resulting in a final ethanol concentration of 40% and ODN:HSA-PEHA: Total lipids ratio of 1:3:10 (wt/wt). This resulted in formulation of colloidal complexes, which were precursors to LCAN. This was then diluted 4× by water and subjected to tangential flow diafiltration on a MicroKros cartridge with MWCO of 30,000 against 5 mM HEPES buffer, pH 7.4. Then, sucrose was added to the product (10% final concentration). The LCAN-G3139 was then sterile filtered using a 0.2 μm filter and stored frozen at −20° C. The product was 2 mg/mL in G3139 concentration, determined by OliGreen assay. The percent recovery of G3139 in the product was 67%. The particle size of LCAN was analyzed on a NICOMP Particle Sizer Model 370 (Particle Sizing Systems, Santa Barbara, Calif.). A volume-weighted Gaussian distribution analysis was used to determine the mean particle diameter and size distribution. The zeta potential (4) was determined on a Zeta-PALS (Brookhaven Instruments Corp., Worcestershire, N.Y.). All measurements were carried out in triplicate. The particle size was 98±40 nm and the zeta potential was +23 mV.

[0104] The product LCAN-G3139 was analyzed for bcl-2 down regulation in KB (human carcinoma) cells. KB cells were plated in 6-well plates at a density of 2×10^4 cells/cm² 24 h prior to transfection in RPMI 1640 (Life Technologies) medium containing 10% FBS and 1% antibiotics. The medium was removed and replaced with various G3139 ODN formulations in RPMI 1640 culture medium at G3139 concentration of 1 μM. The control LN-G3139 is a LN formulation with the composition of DOTAP/SPC/TPGS at 25:70:5 (m/m) without the addition of HSA-PEHA and otherwise prepared by the same method as LCAN. After 4 h at 37° C., the transfection medium was removed and cells were washed three times with PBS. Fresh medium was then added to the cells. At 48 h after the transfection, the cells were harvested. Briefly, total RNA was extracted using Trizol reagent (Invitrogen) and cDNA was synthesized by incubating RNA with random hexamer primer (Perkin Elmer, Boston, Mass.), and then with reverse transcriptase (Invitrogen), reaction buffer,

dithiothreitol, dNTPs and RNAsin, followed by incubation at 42° C. for 60 minutes and 94° C. for 5 minutes in a thermal cycler (Applied Biosystems, Foster City, Calif.). The resulting cDNA was amplified by real-time PCR (ABI Prism 7700 Sequence Detection System, Applied Biosystems) using bcl-2 primers and probes:

```
forward primer
                              [SEQ ID NO. 1]
CCCTGTGGATGACTGAGTACCTG;

reverse primer
                              [SEQ ID NO. 2]
CCAGCCTCCGTTATCCTGG;
and,

probe
                              [SEQ ID NO. 3])
CCGGCACCTGCACACCTGGA.
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[0105] Housekeeping gene ABL mRNAs were also amplified concurrently and Bcl-2 mRNA was normalized to ABL mRNA levels.

[0106] The results are displayed in FIG. **7**. These results showed that LCAN-G3139 was much more effective in Bcl-2 down regulation than LN-G3139, a typical LN formulation of G3139 that does not contain HSA-PEHA. These data showed that LCAN-G3139 is a superior composition to most LNs and can be used to deliver antisense ASOs and other oligonucleotide drugs, such as siRNA, miR mimics, and anti-miR oligos.

Example 4

[0107] Low molecular weight PEI(600) was used in this example. Alternative low molecular weight polymers, such as pentaethylenehexamine (PEHA), may also be conjugated using similar techniques.

[0108] HSA-PEI conjugates were produced using EDC. 42 mg HSA and 188 mg PEI (MW=600) (molar ratio HSA:PEI 1:500) were combined in HBS to a total volume of 2.0 mL in a small vial, adjusted to pH 8.0. Due to the highly alkaline nature of PEI, a PEI stock solution was titrated to pH 8.0 prior to mixing. EDC was allowed to equilibrate to room temperature before adding 9.60 mg EDC (80 fold molar excess relative to HSA), slowly to a stirring solution of HSA and PEI. The mixture was reacted for 1 h at room temperature with stirring. pH was maintained at ~9.0 over the course of the reaction. The product was passed through a PD-10 column to remove unreacted reagents. Gel mobility shift analysis was conducted to determine the DNA condensation efficiency of the HSA-PEI conjugate.

[0109] In an alternative conjugate method that introduces a reducible liner, HSA-PEI conjugates were produced using Traut's reagent. 42 mg HSA and 188 mg PEI (MW=600) (molar ratio HSA:PEI 1:500) were combined in HBS to a total volume of 2.0 mL in a small vial, adjusted to pH 8.0. Due to the highly alkaline nature of PEI, a PEI stock solution was titrated to pH 8.0 prior to mixing. 3.5 mg Traut's reagent (40 fold molar excess relative to HSA) was dissolved in 10 μL DMSO and added slowly to a stiffing solution of HSA and PEI. The mixture was reacted for 2 h at room temperature with stiffing. A pH of 8.0 was maintained over the course of the reaction. Disulfide crosslinking resulting in oxidation of sulfhydryls led to the formation of an HSA-PEI conjugate. This product was purified by passing through a PD-10 column

to remove unreacted reagents. Gel mobility shift analysis was conducted to determine the DNA condensation efficiency of the conjugate.

[0110] In yet another method of synthesis, HSA-PEI conjugates were produced using SPDP. 42 mg HSA, activated by Traut's reagent, and 188 mg PEI (MW=600) activated by SPDP (molar ratio HSA:PEI 1:500) were combined in HBS to a total volume of 2.0 mL in a small vial, adjusted to pH 8.0. This resulted in the formation of albumin-PEI conjugate via disulfide linkages. Gel mobility shift analysis was conducted to determine the condensation efficiency of the conjugate.

[0111] Albumin-PEI conjugates were produced using DTBP. 42 mg HSA and 188 mg PEI (MW=600) (molar ratio HSA:PEI 1:500) were combined in HBS to a total volume of 2.0 mL in a small vial, adjusted to pH 8.0. Due to the highly alkaline nature of PEI, a PEI stock solution was titrated to pH 8.0 prior to mixing. 3.9 mg DTBP (20 fold molar excess relative to HSA) was dissolved in 10 μL DMSO and added slowly to a stirring solution of HSA and PEI. The mixture was reacted overnight at room temperature with stirring. A pH of 8.0 was maintained over the course of the reaction and the product was passed through a PD-10 column to remove unreacted reagents. Gel mobility shift analysis was conducted to determine the condensation efficiency of the conjugate.

Example 5

[0112] LNs containing HSA-PEI conjugates were produced. HSA-PEI at various w/w ratios (0, 0.5, 1, 3, 6:1, HSA:ODN w/w) were combined with ODN LOR-2501 (0.2 μM) (purchased from Alpha DNA) to find the optimal retardation ratio using gel mobility shift analysis. Retardation occurred at 3:1 (HSA:ODN w/w) (FIG. **1**). DDAB, CHOL, and TPGS lipid stocks dissolved in 100% ethanol were combined at a molar ratio of 60:35:5. 100 μL lipid mixture in ethanol was added to 900 μL 1×PBS buffer as to form empty LNs in 10% ethanol. The HSA-PEI/ODN complex was then combined with the empty LNs to form LCANs. The formulation was briefly vortexed and allowed to stand for 15 m at room temperature before transfection into KB cells. Zeta potential analysis was completed on the LCAN containing HSA-PEI and ODN LOR-2501 (purchased from Alpha DNA) at the ratio LN:HSA:ODN=10:1, 2, 3:1. The concentration of ODN used was 0.2 μM (FIG. **2**). All LCANs containing HSA-PEI exhibited a positive charge ranging between 5 and 25 mV. LCANs without HSA-PEI were neutrally charged. FIG. **3** displays the downregulation of RNR R1 mRNA expression by LOR-2501 in LCANs.

[0113] KB cells, grown in RPMI 1640 medium at 37° C. under 5% CO_2 atmosphere, were plated 24 h prior to transfection at a density of 3.0×10^5 cells per well in a 6-well plate. Cells were grown to approximately 80% confluency and the serum-containing media was removed. Cells were transfected with 1000 μL transfection media and treated for 4 h. Transfection occurred in the presence of 0% and 10% serum-containing RPMI 1640 media. Experiments were performed with 3 replicates. After treatment was completed, cells were washed with 1×PBS and serum-containing RPMI 1640 was restored. At 48 h after treatment was completed, cells were analyzed for RNR R1 expression levels by RT-PCR with actin as a housekeeping gene. Results are shown in FIG. **2**. Under serum-free conditions, the 1:3, ODN:HSA LCAN formulation showed the greatest transfection efficacy. Conversely, in 10% serum, the 1:1 ODN:HSA LCAN formulation was the most efficacious. Cell viability 48 h after treatment was

assessed by MTT assay (FIG. **4**). A similar experiment involving conjugation of PEHA-to-albumin was completed and showed similar transfection activity (FIGS. **7** and **8**).

Example 6

[0114] LCAN for delivery of anti-miR-221 into CAL-51 breast cancer cells was studied.

[0115] LCAN (using HSA-PEI based APC) were prepared as described above. CAL-51 (triple negative breast cancer) cells were plated 24 h prior to transfection in a 6-well plate at a density of 2×10^4 cells/cm^2 in DMEM/F12 media supplemented with 1% penicillin/streptomycin and 10% FBS. LCAN was combined with anti-miR-221 (100 nM) to gauge its ability to upregulate the downstream targets of miR-221, p27/Kip1 and the estrogen receptor alpha (ERα). CAL-52 cells were transfected in the presence of 20% serum. Treatment was allowed to proceed for 4 h at which time the transfection medium was removed and replaced with fresh media (supplemented with 10% FBS). Cells were allowed to proliferate for an additional 44 h before the start of RT-PCR. RNA from cells was extracted with TRIzol Reagent (Life Technologies) and cDNA was generated by SuperScript® III First-Strand Synthesis System (Life Technologies) per the manufacturer's instructions. RT-PCR was then performed using SYBR green (Life Technologies) and primers for p27/kip1 (Alpha DNA) and ERα:

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forward:
                                  [SEQ ID NO. 4]
5'CGGAGCACGGGGACGGGTATC-3',

reverse:
                                  [SEQ ID NO. 5])
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```

[0116] β-actin was used as a control. As demonstrated in FIG. **10** and FIG. **11**, LCAN/anti-miR-221 led to moderate increases in p27/Kip1 expression and slight increases in ERα expression.

Example 7

[0117] HSA-PEHA conjugates were synthesized at a relatively large scale. The HSA:PEHA:EDC molar ratio used during synthesis was 1:1500:200 (mol/mol). 5 g PEHA (MW 232.37, technical grade) was dissolved in 80 mL of ddH$_2$O and then adjusted to pH 8.0 using 1 M HCl. 1 g (4 mL) of HSA (25%, Octapharma)) and then 562.5 mg of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC, dissolved in DMSO) were added into the PEHA solution under stirring. The reaction continued for 3 h at room temperature. The mixture was then dialyzed using MWCO 10,000 Spectrum membrane against ddH$_2$O at 4° C. The buffer was replaced every 3-4 h until amines from PEHA became undetectable by the standard ninhydrin or TNBS amine essay in the external buffer at the 3 h time point at the end of the dialysis cycle. For further scaled-up synthesis, the dialysis procedure can be replaced by tangential flow diafiltration, e.g., using a Millipore Pellicon cassette system or a Spectropor hollowfiber system. This method can also be used to concentrate the product to a desirable concentration. The product can be passed through a 0.22 µm sterile filter into a sterile container and stored at 4° C. For long-term storage, the product can be stored at −20° C. The product can also be lyophilized.

[0118] The product protein concentration was determined using BCA protein assay. The amine content of the HSA-PEHA conjugate was determined by TNBS assay or MALDI-TOF MS based on change in molecular weight relative to HSA. Gel permeation chromatography combined with amine TNBS assay is used to demonstrate the lack of crosslinked HSA and the absence of free PEHA in the product. Due to the modest cost of the reagents used, the yield of the reaction is not critical. The purity of the product is expected to be very high. Exact product specifications can be defined based on PEHA-to-HSA ratio and higher limits of crosslinked HSA and free PEHA in the final product.

[0119] Certain embodiments of the formulations and methods disclosed herein are defined in the above examples. It should be understood that these examples, while indicating particular embodiments of the invention, are given by way of illustration only. From the above discussion and these examples, one skilled in the art can ascertain the essential characteristics of this disclosure, and without departing from the spirit and scope thereof, can make various changes and modifications to adapt the compositions and methods described herein to various usages and conditions. Various changes may be made and equivalents may be substituted for elements thereof without departing from the essential scope of the disclosure. In addition, many modifications may be made to adapt a particular situation or material to the teachings of the disclosure without departing from the essential scope thereof.

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What is claimed is:

1. A lipid nanoparticle comprising, at least one lipid, and at least one albumin-polymer conjugate (APC), wherein the polymer comprises at least one positively-charged polymer.

2. The lipid nanoparticle of claim 1, wherein the APC comprises a hyper-cationized albumin-polycation conjugate.

3. The lipid nanoparticle of claim 1, wherein the lipid nanoparticle has a zeta potential of about 0 to about +40 mV.

4. The lipid nanoparticle of claim 1, wherein the APC is capable of binding at least one oligonucleotide.

5. The lipid nanoparticle of claim 1, wherein the positively-charged polymer comprises a polyamine selected from the group consisting of: spermine, dispermine, trispermine, tetraspermine, oligospermine, thermine, spermidine, dispermidine, trispermidine, oligospermidine, putrescine, polylysine, polyarginine, a polyethylenimine of branched or linear type, and polyallylamine.

6. The lipid nanoparticle of claim 1, wherein the at least one positively-charged polymer comprises a polyethylenimine.

7. The lipid nanoparticle of claim 6, wherein the polyethylenimine has a molecular weight not greater than 50 kDa, or has a molecular weight of from about 200 to about 2000 Da.

8. The lipid nanoparticle of claim 6, wherein the polyethylenimine is conjugated to human serum albumin.

9. The lipid nanoparticle of claim 1, wherein the at least one positively-charged polymer comprises tetraethylenepentamine (TEPA).

10. The lipid nanoparticle of claim 1, wherein the positively-charged polymer comprises a mixture of two or more low molecular weight polymers.

11. The lipid nanoparticle of claim 1, wherein the albumin is conjugated to the at least one positively-charged polymer via one or more cross linking agents.

12. The lipid nanoparticle of claim 1, wherein the lipid nanoparticle comprises pentaethylenehexamine (PEHA) conjugated to human serum albumin (HSA).

13. The lipid nanoparticle of claim 12, wherein an average of about eleven (11) PEHA molecules are linked to each HSA molecule.

14. The lipid nanoparticle of claim 1, wherein the at least one lipid comprises one or more of: a cationic lipid, a neutral lipid, a PEGylated lipid, and cholesterol.

15. The lipid nanoparticle of claim 1, wherein the at least one lipid comprises 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), L-α-phosphatidylcholine (SPC), and d-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS).

16. The lipid nanoparticle of claim 15, wherein the lipids are in a 25:70:5 molar ratio of DOTAP:SPC:TPGS.

17. The lipid nanoparticle of claim 1, wherein the lipid nanoparticle encapsulates at least one molecule selected from nucleic acids, chemotherapeutic agents, and combinations thereof.

18. The lipid nanoparticle of claim 17, wherein the encapsulated molecule comprises a nucleic acid selected from plasmid DNAs, antisense oligonucleotides, miRs, anti-miRs, shRNAs, siRNAs, and combinations thereof.

19. The lipid nanoparticle of claim 17, wherein the encapsulated molecule comprises a therapeutic agent selected from: antineoplastic agents, anti-infective agents, local anesthetics, anti-allergics, antianemics, angiogenesis, inhibitors, beta-adrenergic blockers, calcium channel antagonists, antihypertensive agents, anti-depressants, anti-convulsants, antibacterial, anti-fungal, anti-viral, anti-rheumatics, anthelminithics, antiparasitic agents, corticosteroids, hormones, hormone antagonists, immunomodulators, neurotransmitter antagonists, anti-diabetic agents, anti-epileptics, anti-hemmorhagics, anti-hypertonics, antiglaucoma agents, immunomodulatory cytokines, sedatives, chemokines, vitamins, toxins, narcotics, imaging agents, and combinations thereof.

20. The lipid nanoparticle of claim 17, wherein the encapsulated molecule comprises a nucleic acid therapeutic agent.

21. The lipid nanoparticle of claim 20, wherein the nucleic acid therapeutic agent is selected from: pDNA, siRNA, miRNA, anti-miRNA, ASO, and combinations thereof.

22. The lipid nanoparticle of claim 20, wherein the nucleic acid therapeutic agent is stabilized by modifications to substituent NA base units, by phosphorothioate substitution of phosphodiester linkers, and/or by 2'-O-methylation of the ribose units.

23. The lipid nanoparticle of claim 1, wherein the lipid nanoparticle has a diameter under about 300 nm

24. The lipid nanoparticle of claim 1, wherein the lipid nanoparticle has a diameter under about 200 nm.

25. The lipid nanoparticle of claim 1, wherein the polymer is bound to an external surface of the lipid via direct connection or via a linker.

26. The lipid nanoparticle of claim 16, wherein the lipid nanoparticle has an encapsulation efficiency of the molecule of at least about 40% or higher.

27. The lipid nanoparticle of claim 1, further including a polyethylene glycol-conjugated lipid.

28. The lipid nanoparticle of claim 27, wherein the polyethylene glycol-conjugated lipid comprises one or more of: polysorbate 80, TPGS and mPEG-DSPE.

29. The lipid nanoparticle of claim 27, wherein the polyethylene glycol-conjugated lipid is present at a concentration less than about 15.0 molar percent.

30. The lipid nanoparticle of claim 1, further comprising a ligand capable of binding to a target cell or a target molecule on a cell surface.

31. The lipid nanoparticle of claim 30, wherein the ligand is an antibody or an antibody fragment.

32. The lipid nanoparticle of claim 30, wherein the ligand is selected from cRGD, galatose-containing moieties, transferrin, folate, low density lipoprotein, or epidermal growth factors.

33. A pharmaceutical composition comprising the lipid nanoparticle of claim 1 and a pharmaceutically acceptable excipient.

34. The pharmaceutical composition of claim 33, wherein the pharmaceutical composition is administered perorally, intravenously, intraperitoneally, subcutaneously, or transdermally.

35. The pharmaceutical composition of claim 33, wherein the pharmaceutical composition is prepared as an orally administered tablet, an inhalant, or a suppository.

36. The pharmaceutical composition of claim 33, wherein the pharmaceutical composition is prepared as a sterile solution, a sterile suspension, or a lyophilized powder.

37. A method of making a lipid nanoparticle, the method comprising:
adding at least one lipid to a human serum albumin-penta-ethylenehexamine (HSA-PEHA) conjugate to form a mixture;
adding at least one therapeutic molecule to the mixture; and
subjecting the mixture to a dialysis or diafiltration step to make a lipid nanoparticle.

38. The method of claim 37, wherein the at least one lipid comprises DOTAP, SPC, and TPGS at a 25:70:5 ratio.

39. The method of claim 37, wherein the therapeutic molecule is selected from pDNAs, antisense oligonucleotides, miRs, anti-miRs, shRNAs, siRNAs, or combinations thereof.

40. The product made from the method of claim 39.

41. A method treating a disorder, the method comprising administering an effective amount of a pharmaceutical composition of claim 33 to a subject in need thereof.

42. A delivery system comprising:
a. at least one lipid; and
b. a macromolecule conjugated to a polymer;
c. wherein the macromolecule encapsulate a nucleic acid.

43. A method of using a lipid nanoparticle, comprising:
a. encapsulating a nucleic acid in a lipid nanoparticle, wherein the lipid nanoparticle comprises albumin conjugated to a polymer;
b. incorporating the lipid nanoparticle into a pharmaceutical composition; and
c. administering the pharmaceutical composition to a patient in need thereof.

* * * * *