(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 September 2024 (19.09.2024)

(10) International Publication Number
WO 2024/192257 A2

(51) International Patent Classification:
A61K 47/69 (2017.01) *A61K 31/711* (2006.01)

(21) International Application Number:
PCT/US2024/019965

(22) International Filing Date:
14 March 2024 (14.03.2024)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/490,150 14 March 2023 (14.03.2023) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

WO 2024/192257 A2

(54) Title: MRNA THERAPEUTICS FOR DISORDERS OF VASCULAR PERMEABILITY

(57) Abstract: The present invention relates to compositions and methods for pulmonary targeted delivery of VE-cadherin and methods of use thereof for treating or preventing vascular permeability disorders, such as acute respiratory stress syndrome (ARDS).

mRNA THERAPEUTICS FOR DISORDERS OF VASCULAR PERMEABILITY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 63/490,150, filed March 14, 2023, which is incorporated by reference herein in its entirety.

BACKGROUND OF THE INVENTION

Vascular permeability occurs in a variety of diseases. Examples of vascular permeability disorders include acute lung (such as acute respiratory distress syndrome; ARDS), pulmonary edema, asthma, ventilator-induced lung injury, ischemic stroke, traumatic brain injury, viral infections (e.g., viral hemorrhagic fever, SARS-CoV2, etc.), diabetes mellitus, diabetic kidney disease, acute kidney injury, chronic kidney disease, diabetic macular edema, proliferative diabetic retinopathy, and others. One such example, ARDS, is an acute inflammation of the lungs' alveoli (air sacs) that leads to hypoxemia and death (40% mortality). It is caused by a variety of inflammatory stimuli (sepsis, pneumonia, aspiration of gastric contents into lungs, trauma). While ARDS usually produces 190,000 annual US cases, recent cases have been significantly increased due to COVID-19 (Rubenfeld, G. D., et al., 2005, New England Journal of Medicine, 353:1685-1693).

Unfortunately, there are no specific drug therapies for ARDS (non-COVID), despite over 50 years of study and more than 30 large clinical trials (Shaw, T. D., et al., 2019, Expert Opinion on Emerging Drugs, 24(1):29-41).

RNA-based agents are emerging as potential therapeutic options distinct from DNA-based gene therapy approaches. For example, mRNA, which does not integrate into host genome nor require nuclear delivery, offers transient translation of needed sequence in cells (Weissman & Kariko Mol. Ther. 2015, 23, 1416–1417). While RNA-based therapies are still in their infancy, there are currently more than 30 clinical trials registered for mRNA-based cancer therapeutics and vaccines (Pardi, et al. J. Control. Release 2015, 217, 345–351). Like all drugs and especially biotherapeutics, delivery of mRNA is a major challenge for most organs except liver (Shuvaev, et al., J. Control. Release 2015, 219, 576–595). Drug delivery systems (DDS) including lipid

nanoparticles (LNPs) are employed to pack RNA and protect cargo en route to the site of action (Kauffman, et al., J. Control. Release 2016, 240, 227–234). However, targeted delivery and effect of RNA in organs and tissues of interest remains a formidable barrier for the biomedical translation and utility of this class of agents.

5 Thus, there is a need in the art for improved compositions and methods for treating ARDS. The present invention satisfies this unmet need.

SUMMARY OF THE INVENTION

In one embodiment, the invention relates to a composition comprising a delivery vehicle conjugated to a targeting domain, wherein the delivery vehicle comprises or encapsulates an mRNA molecule encoding vascular endothelial (VE)-cadherin, truncated VE-cadherin, neural (N)-cadherin, claudin-5, occludin, zonula occludens-1 (ZO-1), or sphingosine-1-phosphate receptor 1 (S1PR1), and further wherein the targeting domain specifically binds to a cell surface molecule on an endothelial cell (e.g., a endothelial marker) of the pulmonary system, wherein the marker is selected from the group consisting of platelet-endothelial cell adhesion molecule 1 (PECAM-1), intercellular adhesion molecule 1 (ICAM-1), angiotensin-converting enzyme (ACE), plasmalemma vesicle-associated protein 1 (PV1), P-selectin, E-selectin, and VE-cadherin.

In one embodiment, the delivery vehicle is selected from the group consisting of a lipid nanoparticle, a liposome, and a micelle. In one embodiment, the delivery vehicle is a lipid nanoparticle.

In one embodiment, the mRNA molecule encoding VE-cadherin, truncated VE-cadherin, N-cadherin, claudin-5, occludin, zonula occludens-1 (ZO-1), or sphingosine-1-phosphate receptor 1 (S1PR1) is encapsulated in the lipid nanoparticle.

In one embodiment, the mRNA molecule encoding VE-cadherin, truncated VE-cadherin, N-cadherin, claudin-5, occludin, zonula occludens-1 (ZO-1), or sphingosine-1-phosphate receptor 1 (S1PR1) is a nucleoside modified mRNA molecule.

In one embodiment, the targeting domain specifically binds to platelet-endothelial cell adhesion molecule-1 (PECAM-1).

In one embodiment, the composition comprises a lipid nanoparticle comprising a nucleoside modified mRNA molecule encoding VE-cadherin, wherein the lipid nanoparticle is conjugated to a PECAM-1 targeting domain.

In one embodiment, the PECAM-1 targeting domain comprises an anti-PECAM-1 antibody.

In one embodiment, the invention relates to a method of treating or preventing a vascular permeability disorder in a subject, the method comprising administering to the subject a composition comprising a delivery vehicle conjugated to a targeting domain, wherein the delivery vehicle comprises or encapsulates an mRNA molecule encoding vascular endothelial (VE)-cadherin, and further wherein the targeting domain specifically binds to a cell surface molecule on an endothelial cell (e.g.,, an endothelial marker) of the pulmonary system, wherein the cell surface molecule is platelet-endothelial cell adhesion molecule 1 (PECAM-1).

In one embodiment, the method further comprises administering an anti-inflammatory cytokine.

In one embodiment, the vascular permeability disorder is selected from the group consisting of acute lung injury, pulmonary edema, asthma, ventilator-induced lung injury, ischemic stroke, traumatic brain injury, vascular permeability associated with a viral infection, diabetes mellitus, diabetic kidney disease, acute kidney injury, chronic kidney disease, diabetic macular edema, and proliferative diabetic retinopathy. In one embodiment, the acute lung injury is acute respiratory distress syndrome (ARDS).

In one embodiment, the invention relates to a method of treating or preventing endothelial dysfunction in a subject in need thereof, the method comprising administering to the subject a composition comprising a delivery vehicle conjugated to a targeting domain, wherein the delivery vehicle comprises or encapsulates an mRNA molecule encoding vascular endothelial (VE)-cadherin, and further wherein the targeting domain specifically binds to a cell surface molecule on an endothelial cell (e.g.,, an endothelial marker) of the pulmonary system, wherein the cell surface molecule is platelet-endothelial cell adhesion molecule 1 (PECAM-1).

In one embodiment, the method further comprises administering an anti-inflammatory cytokine, or an mRNA molecule encoding an anti-inflammatory cytokine.

In one embodiment, the subject has a vascular permeability disease or disorder selected from the group consisting of acute lung injury, pulmonary edema, asthma, ventilator-induced lung injury, ischemic stroke, traumatic brain injury, viral infection, diabetes mellitus, diabetic kidney disease, acute kidney injury, chronic kidney disease, diabetic macular edema, and proliferative diabetic retinopathy. In one embodiment, the acute lung injury is ARDS.

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description of embodiments of the invention will be better understood when read in conjunction with the appended drawings. It should be
5 understood that the invention is not limited to the precise arrangements and instrumentalities of the embodiments shown in the drawings.

Figure 1, comprising Figure 1A and Figure 1B, depict representative experimental results demonstrating that VE-cadherin encoded by PECAM-mRNA-LNPs enhance endothelial barrier function *in vitro*. Figure 1A depicts a representative Western
10 blot showing that VE-cadherin protein expression is driven by PECAM-mRNA-LNPs encoding VE-cadherin in 293T cells *in vitro*. Figure 1B depicts representative experimental results demonstrating that endothelial barrier function is restored by PECAM-mRNA-LNPs encoding VE-cadherin. Human umbilical vein endothelial cells (HUVECs) were plated and their transendothelial impedance measured over 24 hours.
15 Data is plotted such that increasing values on the y-axis indicating an increase in barrier function.

Figure 2 depicts representative widefield fluorescent imaging of blood vessels from mice treated with CD31-targeted mRNA-LNPs. Twenty-four hours after treatment with the mRNA-LNPs mice were anesthetized, mounted, and their ears cleared
20 of fur. Histamine dihydrochloride was applied transcutaneously to the ventral surface of the ear through injection and the ears imaged every minute.

DETAILED DESCRIPTION

The present invention relates to compositions having a delivery vehicle
25 conjugated to a targeting domain, wherein the delivery agent comprises at least one

agent. In some embodiments, the targeting domain specifically binds to a cell surface molecule on an endothelial cell (e.g.,, an endothelial marker). For example, in some embodiments, the targeting domain directs the vehicle to the vasculature or to a specific region of the vasculature. In certain embodiments, the targeting domains direct the
5 vehicle to the pulmonary vasculature.

In some embodiments, the delivery vehicle serves to deliver a therapeutic agent to the pulmonary vasculature. In some embodiments, the therapeutic agent is a nucleic acid molecule encoding VE-cadherin. In some embodiments, the therapeutic agent is an mRNA molecule encoding VE-cadherin.

10 The present invention also relates to methods of treating vascular permeability disorders using the compositions described herein. Examples of vascular permeability disorders include, but are not limited to, pulmonary edema, asthma, ventilator-induced lung injury, ischemic stroke, traumatic brain injury, viral infections including, but not limited to, viral hemorrhagic fevers and SARS-CoV2, diabetes
15 mellitus, diabetic kidney disease, acute kidney injury, chronic kidney disease, diabetic macular edema, proliferative diabetic retinopathy, and others. In some embodiments, the invention provides a method for treating ARDS.

Definitions

20 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 invention belongs.

As used herein, each of the following terms has the meaning associated with it in this section.

25 The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

"About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20%,
30 ±10%, ±5%, ±1%, or ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

The term "antibody," as used herein, refers to an immunoglobulin molecule, which specifically binds with an antigen or epitope. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and $F(ab)_2$, as well as single chain antibodies and humanized antibodies (Harlow et al., 1999, In: Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, In: Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426).

The term "antibody fragment" refers to a portion of an intact antibody and refers to the antigenic determining variable regions of an intact antibody. Examples of antibody fragments include, but are not limited to, Fab, Fab', F(ab')2, and Fv fragments, linear antibodies, scFv antibodies, and multispecific antibodies formed from antibody fragments.

An "antibody heavy chain," as used herein, refers to the larger of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations.

An "antibody light chain," as used herein, refers to the smaller of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations. k and l light chains refer to the two major antibody light chain isotypes.

By the term "synthetic antibody" as used herein, is meant an antibody, which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art. The term should also be construed to mean an antibody, which has been generated by the synthesis of an RNA molecule encoding the antibody. The RNA

molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the RNA has been obtained by transcribing DNA (synthetic or cloned) or other technology, which is available and well known in the art.

A "disease" is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate. In contrast, a "disorder" in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal's state of health.

An "effective amount" as used herein, means an amount which provides a therapeutic or prophylactic benefit.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

"Expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) RNA, and viruses (e.g., lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

"Homologous" refers to the sequence similarity or sequence identity between two polypeptides or between two nucleic acid molecules. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by
5 adenine, then the molecules are homologous at that position. The percent of homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences divided by the number of positions compared X 100. For example, if 6 of 10 of the positions in two sequences are matched or homologous then the two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC
10 and TATGGC share 50% homology. Generally, a comparison is made when two sequences are aligned to give maximum homology.

"Isolated" means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not "isolated," but the same nucleic acid or peptide partially or completely separated from the coexisting
15 materials of its natural state is "isolated." An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

In the context of the present invention, the following abbreviations for the commonly occurring nucleosides (nucleobase bound to ribose or deoxyribose sugar via
20 N-glycosidic linkage) are used. "A" refers to adenosine, "C" refers to cytidine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that
25 encodes a protein or an RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

By the term "modulating," as used herein, is meant mediating a detectable increase or decrease in the level of a response in a subject compared with the level of a response in the subject in the absence of a treatment or compound, and/or compared with
30 the level of a response in an otherwise identical but untreated subject. The term encompasses perturbing and/or affecting a native signal or response thereby mediating a

beneficial therapeutic response in a subject. In some embodiments, the subject is a human.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns. In addition, the nucleotide sequence may contain modified nucleosides that are capable of being translated by translational machinery in a cell. For example, an mRNA where all of the uridines have been replaced with pseudouridine, 1-methyl pseudouridine, or another modified nucleoside.

The term "operably linked" refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. For example, a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA or RNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

The terms "patient," "subject," "individual," and the like are used interchangeably herein, and refer to any animal, or cells thereof whether in vitro or in situ, amenable to the methods described herein. In certain non-limiting embodiments, the patient, subject or individual is a human.

The term "polynucleotide" as used herein is defined as a chain of nucleotides. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the general knowledge that nucleic acids are polynucleotides, which can be hydrolyzed into the monomeric "nucleotides." The monomeric nucleotides can be hydrolyzed into nucleosides. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any means available in the art, including, without limitation, recombinant means, i.e., the cloning of nucleic acid sequences from a recombinant library or a cell genome, using ordinary cloning technology and PCR™, and the like, and by synthetic means.

In certain instances, the polynucleotide or nucleic acid of the invention is a "nucleoside-modified nucleic acid," which refers to a nucleic acid comprising at least one modified nucleoside. A "modified nucleoside" refers to a nucleoside with a modification. For example, over one hundred different nucleoside modifications have been identified in
5 RNA (Rozenski, et al., 1999, The RNA Modification Database: 1999 update. Nucl Acids Res 27: 196-197).

In certain embodiments, "pseudouridine" refers, in another embodiment, to m^1acp^3Y (1-methyl-3-(3-amino-3-carboxypropyl) pseudouridine. In another embodiment, the term refers to m^1Y (1-methylpseudouridine). In another embodiment,
10 the term refers to Ym (2'-O-methylpseudouridine. In another embodiment, the term refers to m^5D (5-methyldihydrouridine). In another embodiment, the term refers to m^3Y (3-methylpseudouridine). In another embodiment, the term refers to a pseudouridine moiety that is not further modified. In another embodiment, the term refers to a monophosphate, diphosphate, or triphosphate of any of the above pseudouridines. In another embodiment,
15 the term refers to any other pseudouridine known in the art. Each possibility represents a separate embodiment of the present invention.

As used herein, the terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and
20 no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are
25 referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination
30 thereof.

The term "promoter" as used herein is defined as a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence. For example, the promoter that is recognized by bacteriophage RNA polymerase and is used to generate the mRNA by in vitro transcription.

By the term "specifically binds," as used herein with respect to an affinity ligand, in particular, an antibody, is meant an antibody which recognizes a specific antigen, but does not substantially recognize or bind other molecules in a sample. For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more other species. But, such cross-species reactivity does not itself alter the classification of an antibody as specific. In another example, an antibody that specifically binds to an antigen may also bind to different allelic forms of the antigen. However, such cross reactivity does not itself alter the classification of an antibody as specific. In some instances, the terms "specific binding" or "specifically binding," can be used in reference to the interaction of an antibody, a protein, or a peptide with a second chemical species, to mean that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope "A", the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled "A" and the antibody, will reduce the amount of labeled A bound to the antibody.

The term "therapeutic" as used herein means a treatment and/or prophylaxis. A therapeutic effect is obtained by suppression, diminution, remission, or eradication of at least one sign or symptom of a disease or disorder.

The term "therapeutically effective amount" refers to the amount of the subject compound that will elicit the biological or medical response of a tissue, system, or subject that is being sought by the researcher, veterinarian, medical doctor or other clinician. The term "therapeutically effective amount" includes that amount of a compound that, when administered, is sufficient to prevent development of, or alleviate to some extent, one or more of the signs or symptoms of the disorder or disease being

treated. The therapeutically effective amount will vary depending on the compound, the disease and its severity and the age, weight, etc., of the subject to be treated.

To "treat" a disease as the term is used herein, means to reduce the frequency or severity of at least one sign or symptom of a disease or disorder experienced by a subject.

The term "transfected" or "transformed" or "transduced" as used herein refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A "transfected" or "transformed" or "transduced" cell is one which has been transfected, transformed or transduced with exogenous nucleic acid. The cell includes the primary subject cell and its progeny.

The phrase "under transcriptional control" or "operatively linked" as used herein means that the promoter is in the correct location and orientation in relation to a polynucleotide to control the initiation of transcription by RNA polymerase and expression of the polynucleotide.

A "vector" is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

"Alkyl" refers to a straight or branched hydrocarbon chain radical consisting solely of carbon and hydrogen atoms, which is saturated or unsaturated (i.e., contains one or more double and/or triple bonds), having from one to twenty-four carbon atoms (C_1-C_{24} alkyl), one to twelve carbon atoms (C_1-C_{12} alkyl), one to eight carbon atoms (C_1-C_8 alkyl) or one to six carbon atoms (C_1-C_6 alkyl) and which is attached to the rest of the molecule by a single bond, e.g., methyl, ethyl, n-propyl, 1-methylethyl (iso propyl), n-butyl, n-pentyl, 1,1-dimethylethyl (t-butyl), 3-methylhexyl, 2-methylhexyl,

ethenyl, prop-1-enyl, but-1-enyl, pent-1-enyl, penta-1,4-dienyl, ethynyl, propynyl, butynyl, pentynyl, hexynyl, and the like. Unless specifically stated otherwise, an alkyl group is optionally substituted.

"Alkylene" or "alkylene chain" refers to a straight or branched divalent hydrocarbon chain linking the rest of the molecule to a radical group, consisting solely of carbon and hydrogen, which is saturated or unsaturated (*i.e.*, contains one or more double (alkenylene) and/or triple bonds (alkynylene)), and having, for example, from one to twenty-four carbon atoms (C_1-C_{24} alkylene), one to fifteen carbon atoms (C_1-C_{15} alkylene),one to twelve carbon atoms (C_1-C_{12} alkylene), one to eight carbon atoms (C_1-C_8 alkylene), one to six carbon atoms (C_1-C_6 alkylene), two to four carbon atoms (C_2-C_4 alkylene), one to two carbon atoms (C_1-C_2 alkylene), *e.g.*, methylene, ethylene, propylene, *n*-butylene, ethenylene, propenylene, *n*-butenylene, propynylene, *n*-butynylene, and the like. The alkylene chain is attached to the rest of the molecule through a single or double bond and to the radical group through a single or double bond. The points of attachment of the alkylene chain to the rest of the molecule and to the radical group can be through one carbon or any two carbons within the chain. Unless stated otherwise specifically in the specification, an alkylene chain may be optionally substituted.

"Cycloalkyl" or "carbocyclic ring" refers to a stable non aromatic monocyclic or polycyclic hydrocarbon radical consisting solely of carbon and hydrogen atoms, which may include fused or bridged ring systems, having from three to fifteen carbon atoms, and which is saturated or unsaturated and attached to the rest of the molecule by a single bond. In some embodiments the fused or bridged ring system has from three to ten carbon atoms. Monocyclic radicals include, for example, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl. Polycyclic radicals include, for example, adamantyl, norbornyl, decalinyl, 7,7 dimethyl bicyclo[2.2.1]heptanyl, and the like. Unless specifically stated otherwise, a cycloalkyl group is optionally substituted.

"Cycloalkylene" is a divalent cycloalkyl group. Unless otherwise stated specifically in the specification, a cycloalkylene group may be optionally substituted.

"Heterocyclyl" or "heterocyclic ring" refers to a stable 3- to 18-membered non-aromatic ring radical which consists of two to twelve carbon atoms and from one to six heteroatoms selected from the group consisting of nitrogen, oxygen and sulfur. Unless stated otherwise specifically in the specification, the heterocyclyl radical may be a monocyclic, bicyclic, tricyclic or tetracyclic ring system, which may include fused or bridged ring systems; and the nitrogen, carbon or sulfur atoms in the heterocyclyl radical may be optionally oxidized; the nitrogen atom may be optionally quaternized; and the heterocyclyl radical may be partially or fully saturated. Examples of such heterocyclyl radicals include, but are not limited to, dioxolanyl, thienyl[1,3]dithianyl, decahydroisoquinolyl, imidazolinyl, imidazolidinyl, isothiazolidinyl, isoxazolidinyl, morpholinyl, octahydroindolyl, octahydroisoindolyl, 2-oxopiperazinyl, 2-oxopiperidinyl, 2-oxopyrrolidinyl, oxazolidinyl, piperidinyl, piperazinyl, 4-piperidonyl, pyrrolidinyl, pyrazolidinyl, quinuclidinyl, thiazolidinyl, tetrahydrofuryl, trithianyl, tetrahydropyranyl, thiomorpholinyl, thiamorpholinyl, 1-oxo-thiomorpholinyl, and 1,1-dioxo-thiomorpholinyl. Unless specifically stated otherwise, a heterocyclyl group may be optionally substituted.

The term "substituted" used herein means any of the above groups (e.g., alkyl, cycloalkyl or heterocyclyl) wherein at least one hydrogen atom is replaced by a bond to a non-hydrogen atoms such as, but not limited to: a halogen atom such as F, Cl, Br, and I; oxo groups (=O); hydroxyl groups (-OH); alkoxy groups ($-OR^a$, where R^a is C_1-C_{12} alkyl or cycloalkyl); carboxyl groups ($-OC(=O)R^a$ or $–C(=O)OR^a$, where R^a is H, C_1-C_{12} alkyl or cycloalkyl); amine groups ($-NR^aR^b$, where R^a and R^b are each independently H, C_1-C_{12} alkyl or cycloalkyl); C_1-C_{12} alkyl groups; and cycloalkyl groups. In some embodiments the substituent is a C_1-C_{12} alkyl group. In other embodiments, the substituent is a cycloalkyl group. In other embodiments, the substituent is a halo group, such as fluoro. In other embodiments, the substituent is a oxo group. In other embodiments, the substituent is a hydroxyl group. In other embodiments, the substituent is an alkoxy group. In other embodiments, the substituent is a carboxyl group. In other embodiments, the substituent is an amine group.

"Optional" or "optionally" (e.g., optionally substituted) means that the subsequently described event of circumstances may or may not occur, and that the

description includes instances where said event or circumstance occurs and instances in which it does not. For example, "optionally substituted alkyl" means that the alkyl radical may or may not be substituted and that the description includes both substituted alkyl radicals and alkyl radicals having no substitution.

5 Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual 10 numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

15

Description

The present invention relates to compositions and methods for pulmonary targeted delivery of a therapeutic agent for treating a respiratory disease or disorder in a subject. In one aspect, the present invention relates to composition comprising a delivery 20 vehicle conjugated to a targeting domain. In some embodiments, the delivery vehicle comprises at least one agent, such as a therapeutic agent. In some embodiments, the delivery vehicle comprises RNA, including but not limited to mRNA, and nucleoside-modified RNA.

In various embodiments, the targeting domain binds to a cell surface 25 molecule of a cell related to the vasculature, such as an endothelial cell. For example, in various embodiments, the targeting domain binds to a molecule selected from the group including, but not limited to, platelet-endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), angiotensin-converting enzyme (ACE), plasmalemma vesicle-associated protein 30 (PV1), P-selectin, E-selectin, and VE cadherin.

In some embodiments, the targeting domain binds to PECAM-1, or ICAM-1, thereby directing the composition to the pulmonary vasculature.

In some embodiments, the composition comprises a delivery vehicle conjugated to a targeting domain that binds PECAM-1, thereby directing the composition to the pulmonary vasculature.

In various embodiments, the delivery vehicle comprises an mRNA encoding a therapeutic agent for pulmonary delivery. In certain embodiments, the therapeutic agent is VE-cadherin, truncated VE-cadherin, N-cadherin, claudin-5, occludin, zonula occludens-1 (ZO-1), or sphingosine-1-phosphate receptor 1 (S1PR1).

The present invention also relates in part to methods of treating conditions related to the vasculature in subjects in need thereof, the method comprising the administration of a composition including a delivery vehicle conjugated to a targeting domain.

In various embodiments, the invention provides a method for treating a pulmonary condition by targeting the composition to the pulmonary vasculature. Exemplary pulmonary conditions include, but are not limited to, acute lung injury (e.g., ARDS), pulmonary ischemia including organ transplantation, pulmonary embolism, pulmonary edema, pulmonary hypertension, fibrosis, infection, inflammation, emphysema, and cancer.

In some embodiments, the invention provides a method for preventing endothelial dysfunction in a subject in need thereof. In some embodiments, the invention provides a method for treating a disease or disorder associated with vascular permeability. Examples of vascular permeability disorders include, but are not limited to, pulmonary edema, asthma, ventilator-induced lung injury, ischemic stroke, traumatic brain injury, viral infections including, but not limited to, viral hemorrhagic fevers and SARS-CoV2, diabetes mellitus, diabetic kidney disease, acute kidney injury, chronic kidney disease, diabetic macular edema, proliferative diabetic retinopathy, and others. In some embodiments, the invention provides a method for treating ARDS.

<u>Delivery Vehicle</u>

In some embodiments, the delivery vehicle is a colloidal dispersion system, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle in vitro and in vivo is a liposome (e.g., an artificial membrane vesicle).

The use of lipid formulations is contemplated for the introduction of the at least one agent into a host cell (in vitro, ex vivo or in vivo). In another aspect, the at least one agent may be associated with a lipid. The at least one agent associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/nucleic acid or lipid/expression vector associated compositions are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming aggregates that are not uniform in size or shape. Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes.

Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine ("DMPC") can be obtained from Sigma, St. Louis, MO; dicetyl phosphate ("DCP") can be obtained from K & K Laboratories (Plainview, NY); cholesterol ("Chol") can be obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20 °C. Chloroform is used as the only solvent since it is more readily evaporated than methanol. "Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the

generation of enclosed lipid bilayers or aggregates. Liposomes can be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh et al., 1991 Glycobiology 5: 505-10). However, compositions that have different structures in solution than the normal vesicular structure are also encompassed. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine-agent complexes.

In some embodiments, delivery of the at least one agent comprises any suitable delivery method, including exemplary delivery methods described elsewhere herein. In certain embodiments, delivery of the at least one agent to a subject comprises mixing the at least one agent with a transfection reagent prior to the step of contacting. In another embodiment, a method of the present invention further comprises administering the at least one agent together with the transfection reagent. In another embodiment, the transfection reagent is a cationic lipid reagent.

In another embodiment, the transfection reagent is a lipid-based transfection reagent. In another embodiment, the transfection reagent is a protein-based transfection reagent. In another embodiment, the transfection reagent is a polyethyleneimine based transfection reagent. In another embodiment, the transfection reagent is calcium phosphate. In another embodiment, the transfection reagent is Lipofectin®, Lipofectamine®, or TransIT®. In another embodiment, the transfection reagent is any other transfection reagent known in the art.

In another embodiment, the transfection reagent forms a liposome. Liposomes, in another embodiment, increase intracellular stability, increase uptake efficiency and improve biological activity. In another embodiment, liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. In some embodiments, the liposomes comprise an internal aqueous space for entrapping water-soluble compounds. In another embodiment, liposomes can deliver the at least one agent to cells in an active form.

In some embodiments, the composition comprises a lipid nanoparticle (LNP) and at least one agent.

The term "lipid nanoparticle" refers to a particle having at least one dimension on the order of nanometers (e.g., 1-1,000 nm) which includes one or more lipids. In various embodiments, the particle includes a lipid of Formula (I), (II) or (III). In some embodiments, lipid nanoparticles are included in a formulation comprising at least one agent as described herein. In some embodiments, such lipid nanoparticles comprise a cationic lipid (e.g., a lipid of Formula (I), (II) or (III)) and one or more excipient selected from neutral lipids, charged lipids, steroids and polymer conjugated lipids (e.g., a pegylated lipid such as a pegylated lipid of structure (IV), such as compound IVa). In some embodiments, the at least one agent is encapsulated in the lipid portion of the lipid nanoparticle or an aqueous space enveloped by some or all of the lipid portion of the lipid nanoparticle, thereby protecting it from enzymatic degradation or other undesirable effects induced by the mechanisms of the host organism or cells e.g. an adverse immune response.

In various embodiments, the lipid nanoparticles have a mean diameter of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm. In some embodiments, the lipid nanoparticles have a mean diameter of about 83 nm. In some embodiments, the lipid nanoparticles have a mean diameter of about 102 nm. In some embodiments, the lipid nanoparticles have a mean diameter of about 103 nm. In some embodiments, the lipid nanoparticles are substantially non-toxic. In certain embodiments, the at least one agent, when present in the lipid nanoparticles, is resistant in aqueous solution to degradation by intra- or intercellular enzymes.

The LNP may comprise any lipid capable of forming a particle to which the at least one agent is attached, or in which the at least one agent is encapsulated. The

term "lipid" refers to a group of organic compounds that are derivatives of fatty acids (e.g., esters) and are generally characterized by being insoluble in water but soluble in many organic solvents. Lipids are usually divided in at least three classes: (1) "simple lipids" which include fats and oils as well as waxes; (2) "compound lipids" which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids.

In some embodiments, the LNP comprises one or more cationic lipids, and one or more stabilizing lipids. Stabilizing lipids include neutral lipids and pegylated lipids.

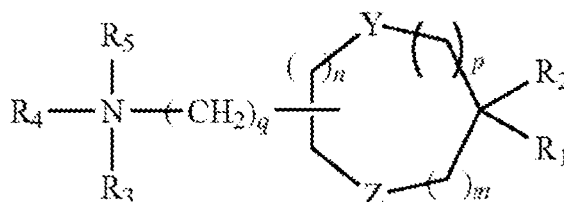
In some embodiments, the LNP comprises a cationic lipid. As used herein, the term "cationic lipid" refers to a lipid that is cationic or becomes cationic (protonated) as the pH is lowered below the pK of the ionizable group of the lipid, but is progressively more neutral at higher pH values. At pH values below the pK, the lipid is then able to associate with negatively charged nucleic acids. In certain embodiments, the cationic lipid comprises a zwitterionic lipid that assumes a positive charge on pH decrease.

In certain embodiments, the cationic lipid comprises any of a number of lipid species which carry a net positive charge at a selective pH, such as physiological pH. Such lipids include, but are not limited to, N,N-dioleyl-N,N-dimethylammonium chloride (DODAC); N-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA); N,N-distearyl-N,N-dimethylammonium bromide (DDAB); N-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP); 3-(N—(N′,N′-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1-(2,3-dioleoyloxy)propyl)-N-2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoracetate (DOSPA), dioctadecylamidoglycyl carboxyspermine (DOGS), 1,2-dioleoyl-3-dimethylammonium propane (DODAP), N,N-dimethyl-2,3-dioleoyloxy)propylamine (DODMA), and N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE). Additionally, a number of commercial preparations of cationic lipids are available which can be used in the present invention. These include, for example, LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and 1,2-dioleoyl-sn-3-phosphoethanolamine (DOPE), from GIBCO/BRL, Grand Island, N.Y.); LIPOFECTAMINE® (commercially available cationic liposomes comprising N-(1-(2,3-dioleyloxy)propyl)-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium

trifluoroacetate (DOSPA) and (DOPE), from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic lipids comprising dioctadecylamidoglycyl carboxyspermine (DOGS) in ethanol from Promega Corp., Madison, Wis.). The following lipids are cationic and have a positive charge at below physiological pH:
5 DODAP, DODMA, DMDMA, 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA).

In some embodiments, the cationic lipid is an amino lipid. Suitable amino lipids useful in the invention include those described in WO 2012/016184, incorporated herein by reference in its entirety. Representative amino lipids include, but are not limited
10 to, 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt
15 (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanediol (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), and 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA).

Suitable amino lipids include those having the formula:



20

wherein R_1 and R_2 are either the same or different and independently optionally substituted C_{10}-C_{24} alkyl, optionally substituted C_{10}-C_{24} alkenyl, optionally substituted C_{10}-C_{24} alkynyl, or optionally substituted C_{10}-C_{24} acyl;

R_3 and R_4 are either the same or different and independently optionally
25 substituted C_1-C_6 alkyl, optionally substituted C_2-C_6 alkenyl, or optionally substituted C_2-

C_6 alkynyl or R_3 and R_4 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms chosen from nitrogen and oxygen;

R_5 is either absent or present and when present is hydrogen or C_1-C_6 alkyl;

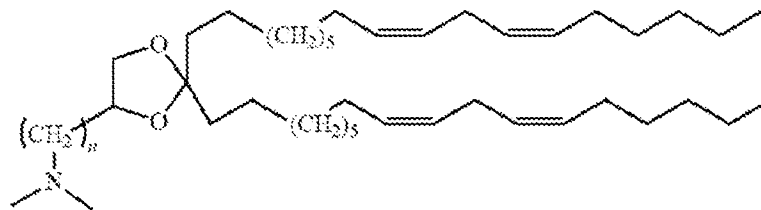
m, n, and p are either the same or different and independently either 0 or 1 with the proviso that m, n, and p are not simultaneously 0;

q is 0, 1, 2, 3, or 4; and

Y and Z are either the same or different and independently O, S, or NH.

In some embodiments, R_1 and R_2 are each linoleyl, and the amino lipid is a dilinoleyl amino lipid. In some embodiments, the amino lipid is a dilinoleyl amino lipid.

A representative useful dilinoleyl amino lipid has the formula:

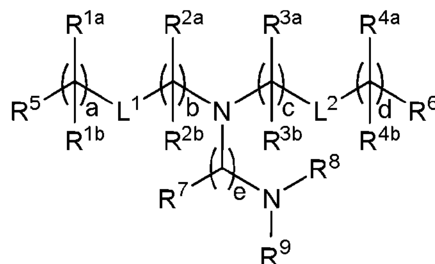


DLin-K-DMA

wherein n is 0, 1, 2, 3, or 4.

In some embodiments, the cationic lipid is a DLin-K-DMA. In some embodiments, the cationic lipid is DLin-KC2-DMA (DLin-K-DMA above, wherein n is 2).

In some embodiments, the cationic lipid component of the LNPs has the structure of Formula (I):



(I)

or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein:

L^1 and L^2 are each independently -O(C=O)-, -(C=O)O- or a carbon-carbon double bond;

R^{1a} and R^{1b} are, at each occurrence, independently either (a) H or C_1-C_{12} alkyl, or (b) R^{1a} is H or C_1-C_{12} alkyl, and R^{1b} together with the carbon atom to which it is
5 bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{2a} and R^{2b} are, at each occurrence, independently either (a) H or C_1-C_{12} alkyl, or (b) R^{2a} is H or C_1-C_{12} alkyl, and R^{2b} together with the carbon atom to which it is bound is taken together with an adjacent R^{2b} and the carbon atom to which it is bound to
10 form a carbon-carbon double bond;

R^{3a} and R^{3b} are, at each occurrence, independently either (a) H or C_1-C_{12} alkyl, or (b) R^{3a} is H or C_1-C_{12} alkyl, and R^{3b} together with the carbon atom to which it is bound is taken together with an adjacent R^{3b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

15 R^{4a} and R^{4b} are, at each occurrence, independently either (a) H or C_1-C_{12} alkyl, or (b) R^{4a} is H or C_1-C_{12} alkyl, and R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^5 and R^6 are each independently methyl or cycloalkyl;

20 R^7 is, at each occurrence, independently H or C_1-C_{12} alkyl;

R^8 and R^9 are each independently C_1-C_{12} alkyl; or R^8 and R^9, together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring comprising one nitrogen atom;

a and d are each independently an integer from 0 to 24;

25 b and c are each independently an integer from 1 to 24; and

e is 1 or 2.

In certain embodiments of Formula (I), at least one of R^{1a}, R^{2a}, R^{3a} or R^{4a} is C_1-C_{12} alkyl, or at least one of L^1 or L^2 is –O(C=O)- or –(C=O)O-. In other embodiments, R^{1a} and R^{1b} are not isopropyl when a is 6 or n-butyl when a is 8.

In still further embodiments of Formula (I), at least one of R^{1a}, R^{2a}, R^{3a} or R^{4a} is C_1-C_{12} alkyl, or at least one of L^1 or L^2 is -O(C=O)- or -(C=O)O-; and

R^{1a} and R^{1b} are not isopropyl when a is 6 or n-butyl when a is 8.

In other embodiments of Formula (I), R^8 and R^9 are each independently unsubstituted C_1-C_{12} alkyl; or R^8 and R^9, together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring comprising one nitrogen atom;

In certain embodiments of Formula (I), any one of L^1 or L^2 may be -O(C=O)- or a carbon-carbon double bond. L^1 and L^2 may each be -O(C=O)- or may each be a carbon-carbon double bond.

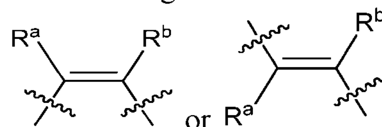
In some embodiments of Formula (I), one of L^1 or L^2 is -O(C=O)-. In other embodiments, both L^1 and L^2 are -O(C=O)-.

In some embodiments of Formula (I), one of L^1 or L^2 is -(C=O)O-. In other embodiments, both L^1 and L^2 are -(C=O)O-.

In some other embodiments of Formula (I), one of L^1 or L^2 is a carbon-carbon double bond. In other embodiments, both L^1 and L^2 are a carbon-carbon double bond.

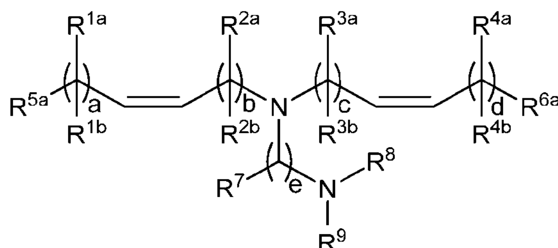
In still other embodiments of Formula (I), one of L^1 or L^2 is -O(C=O)- and the other of L^1 or L^2 is -(C=O)O-. In more embodiments, one of L^1 or L^2 is -O(C=O)- and the other of L^1 or L^2 is a carbon-carbon double bond. In yet more embodiments, one of L^1 or L^2 is -(C=O)O- and the other of L^1 or L^2 is a carbon-carbon double bond.

It is understood that "carbon-carbon" double bond, as used throughout the specification, refers to one of the following structures:



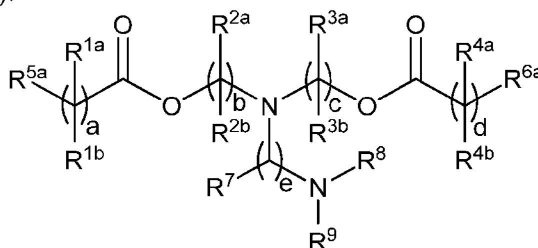
wherein R^a and R^b are, at each occurrence, independently H or a substituent. For example, in some embodiments R^a and R^b are, at each occurrence, independently H, C_1-C_{12} alkyl or cycloalkyl, for example H or C_1-C_{12} alkyl.

In other embodiments, the lipid compounds of Formula (I) have the following structure (Ia):



(Ia)

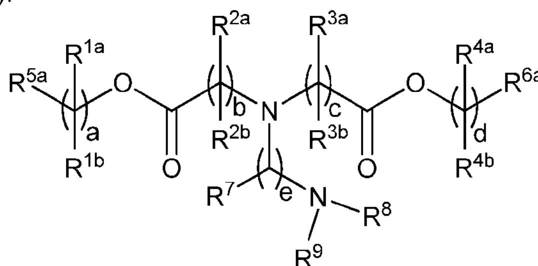
In other embodiments, the lipid compounds of Formula (I) have the following structure (Ib):



5

(Ib)

In yet other embodiments, the lipid compounds of Formula (I) have the following structure (Ic):



10 (Ic)

In certain embodiments of the lipid compound of Formula (I), a, b, c and d are each independently an integer from 2 to 12 or an integer from 4 to 12. In other embodiments, a, b, c and d are each independently an integer from 8 to 12 or 5 to 9. In some certain embodiments, a is 0. In some embodiments, a is 1. In other embodiments, a
15 is 2. In more embodiments, a is 3. In yet other embodiments, a is 4. In some embodiments, a is 5. In other embodiments, a is 6. In more embodiments, a is 7. In yet other embodiments, a is 8. In some embodiments, a is 9. In other embodiments, a is 10. In more embodiments, a is 11. In yet other embodiments, a is 12. In some embodiments, a is

13. In other embodiments, a is 14. In more embodiments, a is 15. In yet other embodiments, a is 16.

In some other embodiments of Formula (I), b is 1. In other embodiments, b is 2. In more embodiments, b is 3. In yet other embodiments, b is 4. In some embodiments, b is 5. In other embodiments, b is 6. In more embodiments, b is 7. In yet other embodiments, b is 8. In some embodiments, b is 9. In other embodiments, b is 10. In more embodiments, b is 11. In yet other embodiments, b is 12. In some embodiments, b is 13. In other embodiments, b is 14. In more embodiments, b is 15. In yet other embodiments, b is 16.

In some more embodiments of Formula (I), c is 1. In other embodiments, c is 2. In more embodiments, c is 3. In yet other embodiments, c is 4. In some embodiments, c is 5. In other embodiments, c is 6. In more embodiments, c is 7. In yet other embodiments, c is 8. In some embodiments, c is 9. In other embodiments, c is 10. In more embodiments, c is 11. In yet other embodiments, c is 12. In some embodiments, c is 13. In other embodiments, c is 14. In more embodiments, c is 15. In yet other embodiments, c is 16.

In some certain other embodiments of Formula (I), d is 0. In some embodiments, d is 1. In other embodiments, d is 2. In more embodiments, d is 3. In yet other embodiments, d is 4. In some embodiments, d is 5. In other embodiments, d is 6. In more embodiments, d is 7. In yet other embodiments, d is 8. In some embodiments, d is 9. In other embodiments, d is 10. In more embodiments, d is 11. In yet other embodiments, d is 12. In some embodiments, d is 13. In other embodiments, d is 14. In more embodiments, d is 15. In yet other embodiments, d is 16.

In some other various embodiments of Formula (I), a and d are the same. In some other embodiments, b and c are the same. In some other specific embodiments, a and d are the same and b and c are the same.

The sum of a and b and the sum of c and d in Formula (I) are factors which may be varied to obtain a lipid of Formula (I) having the desired properties. In some embodiments, a and b are chosen such that their sum is an integer ranging from 14 to 24. In other embodiments, c and d are chosen such that their sum is an integer ranging

from 14 to 24. In further embodiment, the sum of a and b and the sum of c and d are the same. For example, in some embodiments the sum of a and b and the sum of c and d are both the same integer which may range from 14 to 24. In still more embodiments, a. b, c and d are selected such the sum of a and b and the sum of c and d is 12 or greater.

5 In some embodiments of Formula (I), e is 1. In other embodiments, e is 2.

The substituents at R^{1a}, R^{2a}, R^{3a} and R^{4a} of Formula (I) are not particularly limited. In certain embodiments R^{1a}, R^{2a}, R^{3a} and R^{4a} are H at each occurrence. In certain other embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_{12} alkyl. In certain other embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_8 alkyl. In certain other
10 embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_6 alkyl. In some of the foregoing embodiments, the C_1-C_8 alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

In certain embodiments of Formula (I), R^{1a}, R^{1b}, R^{4a} and R^{4b} are C_1-C_{12} alkyl at each occurrence.

15 In further embodiments of Formula (I), at least one of R^{1b}, R^{2b}, R^{3b} and R^{4b} is H or R^{1b}, R^{2b}, R^{3b} and R^{4b} are H at each occurrence.

In certain embodiments of Formula (I), R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond. In other embodiments of the foregoing
20 R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

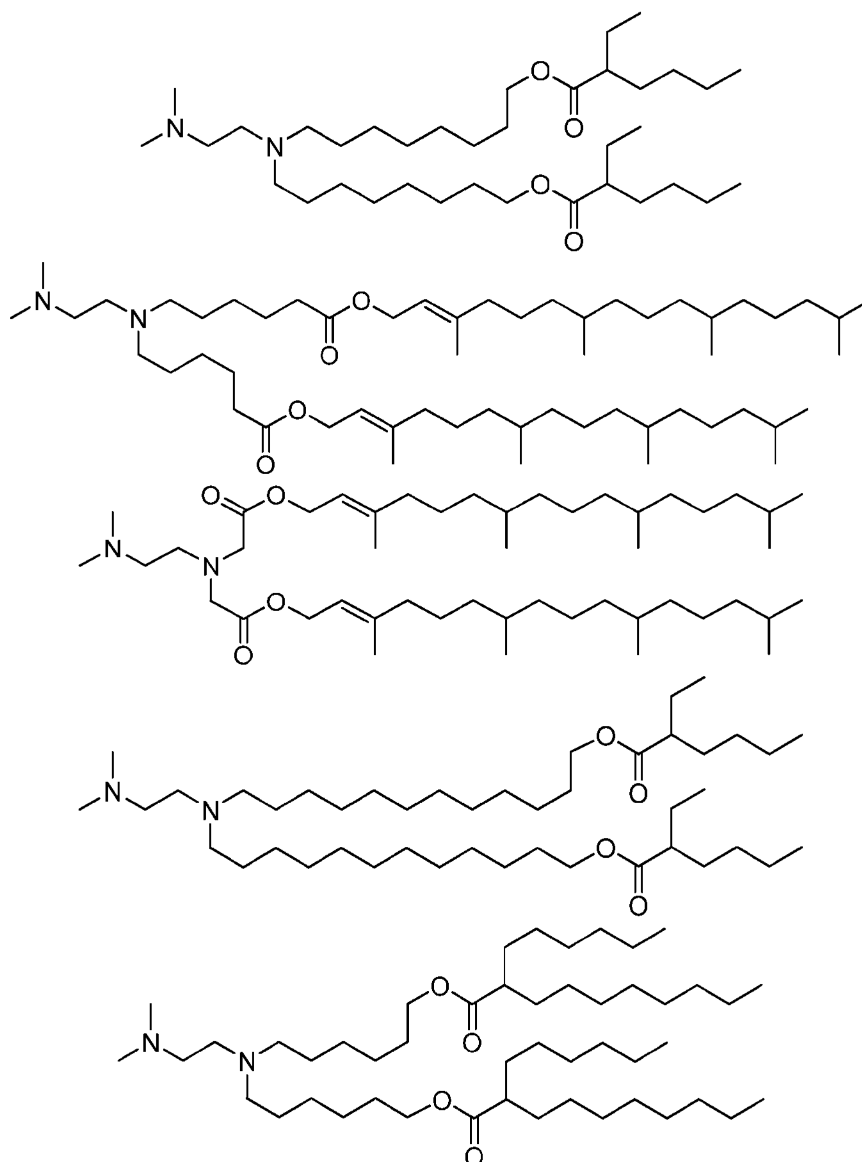
The substituents at R^5 and R^6 of Formula (I) are not particularly limited in the foregoing embodiments. In certain embodiments one or both of R^5 or R^6 is methyl. In certain other embodiments one or both of R^5 or R^6 is cycloalkyl for example cyclohexyl.
25 In these embodiments the cycloalkyl may be substituted or not substituted. In certain other embodiments the cycloalkyl is substituted with C_1-C_{12} alkyl, for example tert-butyl.

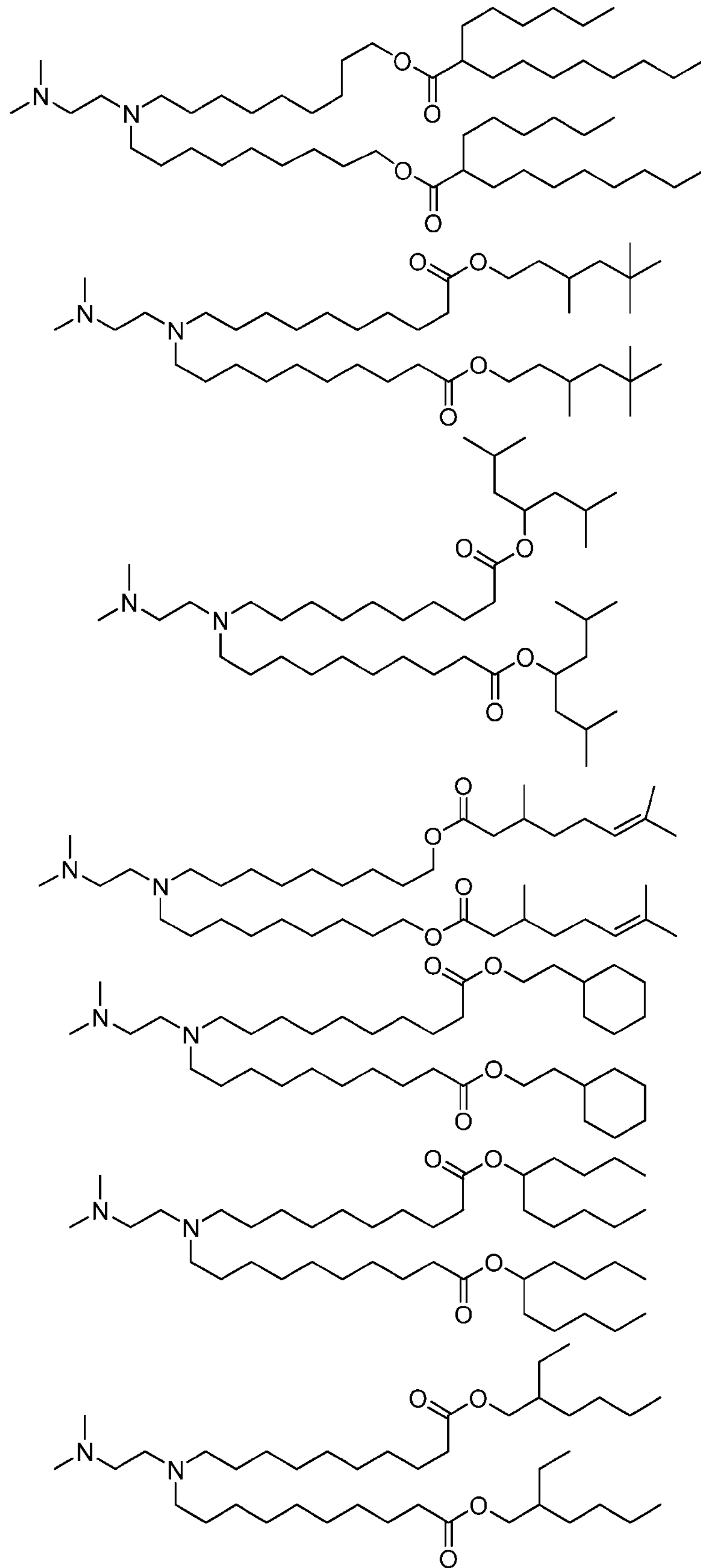
The substituents at R^7 are not particularly limited in the foregoing embodiments of Formula (I). In certain embodiments at least one R^7 is H. In some other embodiments, R^7 is H at each occurrence. In certain other embodiments R^7 is C_1-C_{12}
30 alkyl.

In certain other of the foregoing embodiments of Formula (I), one of R^8 or R^9 is methyl. In other embodiments, both R^8 and R^9 are methyl.

In some different embodiments of Formula (I), R^8 and R^9, together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring. In some embodiments of the foregoing, R^8 and R^9, together with the nitrogen atom to which they are attached, form a 5-membered heterocyclic ring, for example a pyrrolidinyl ring.

In various different embodiments, exemplary lipid of Formula (I) can include

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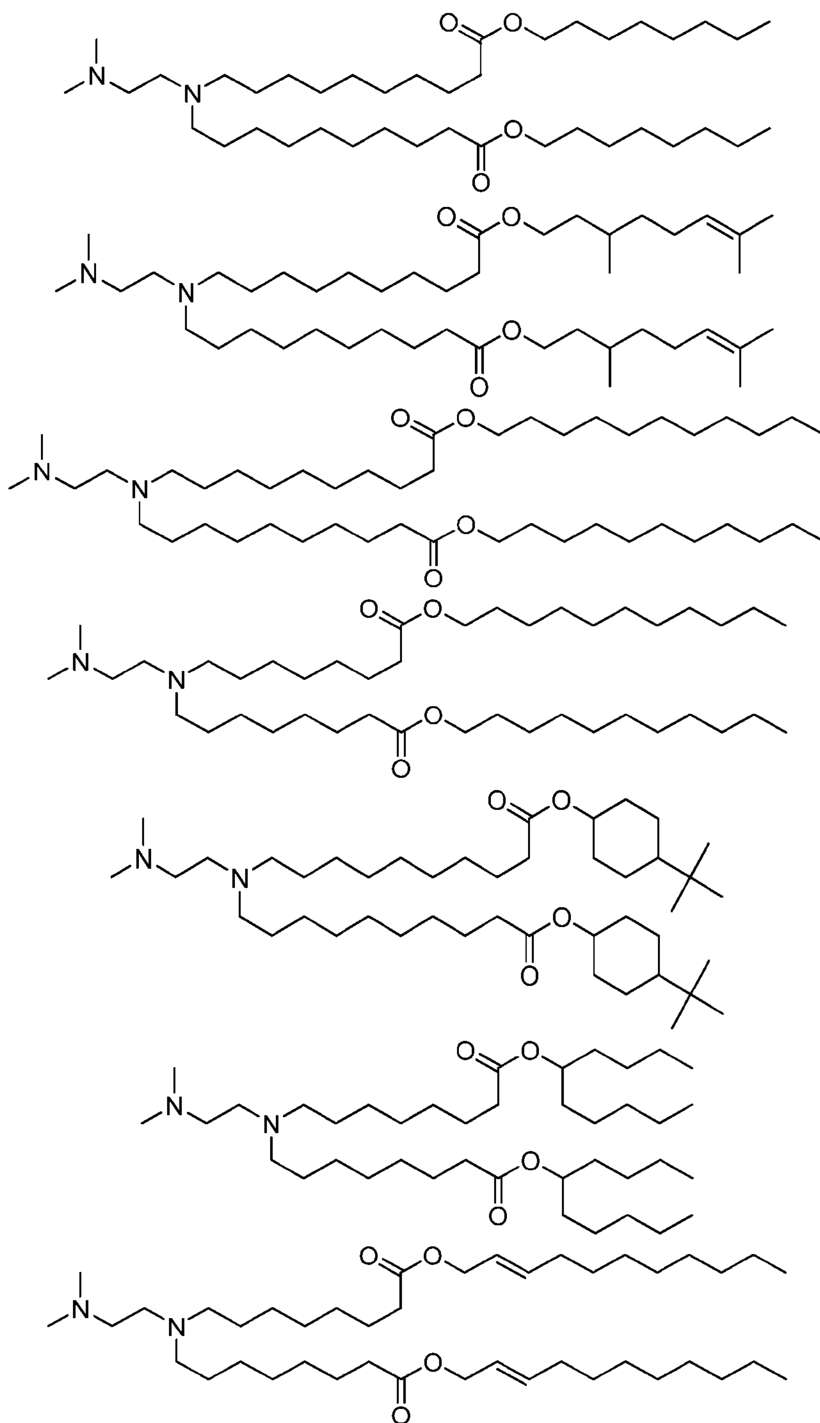
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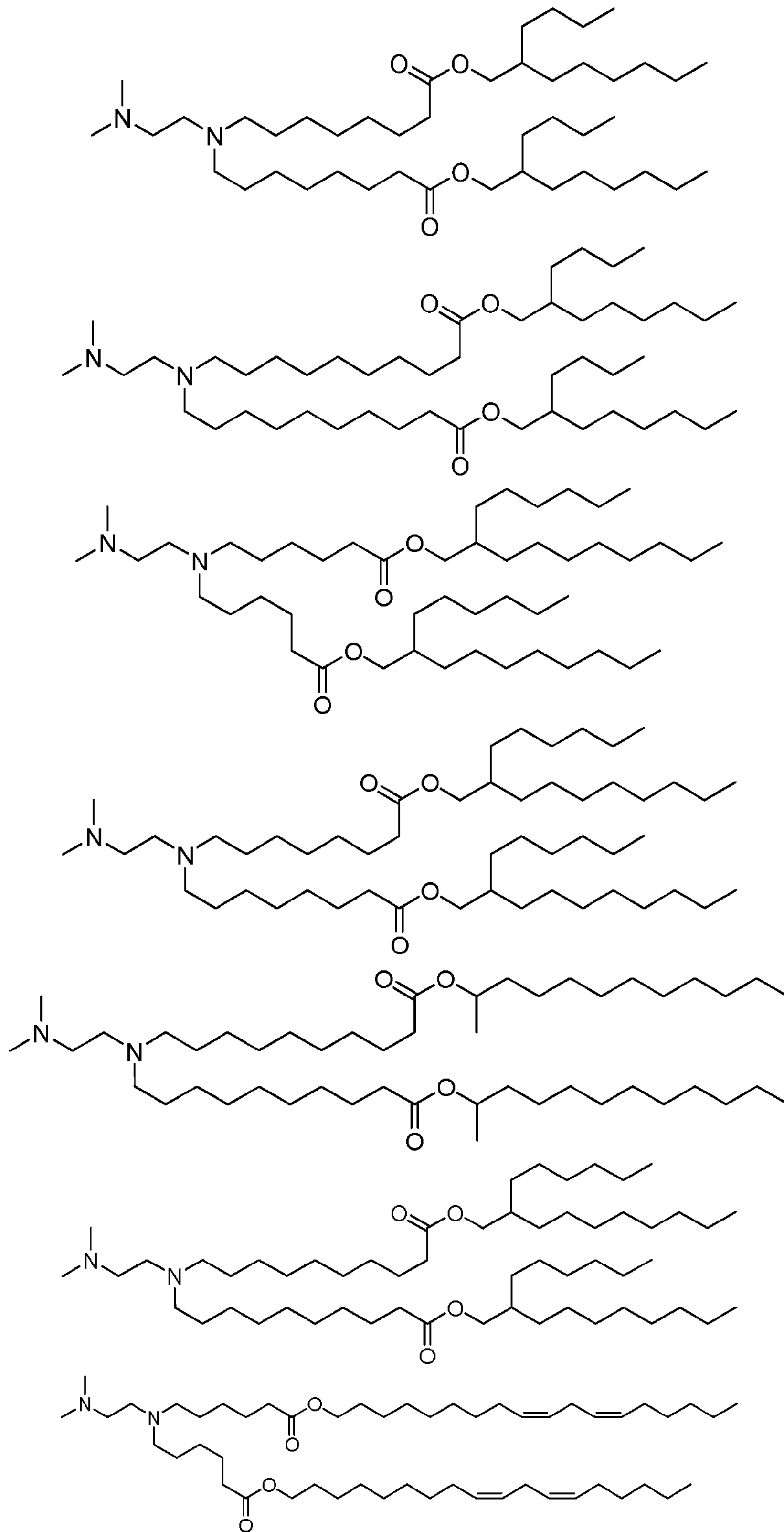
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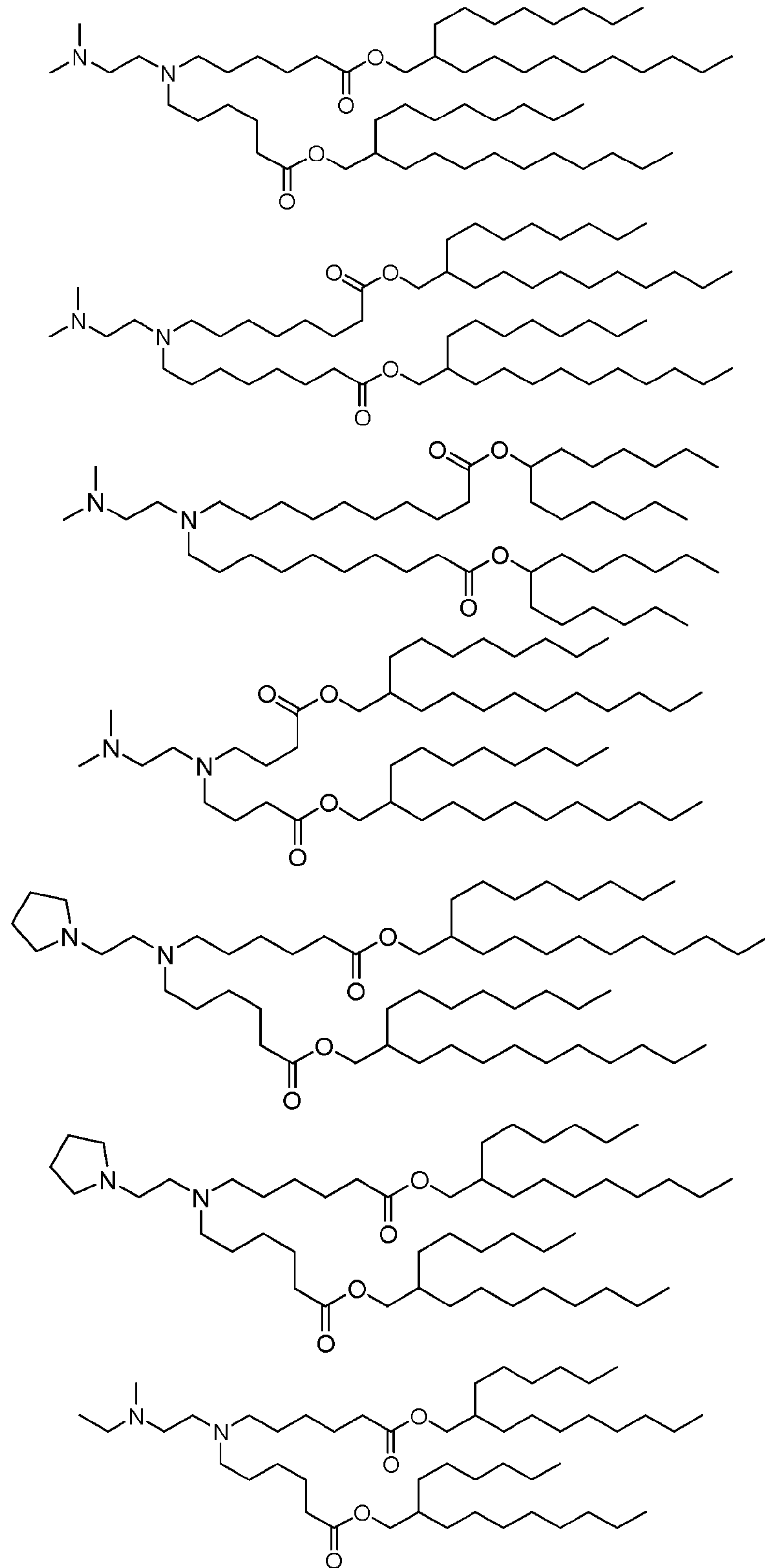
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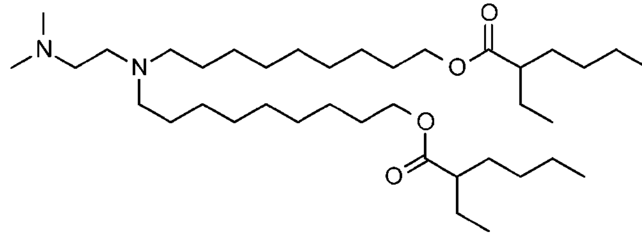
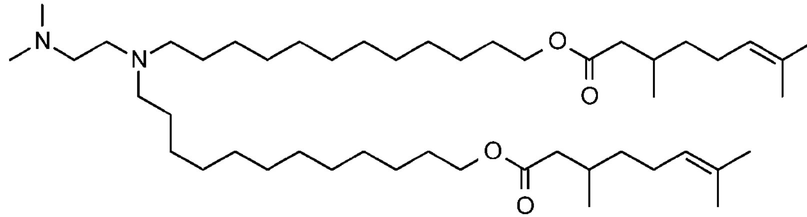
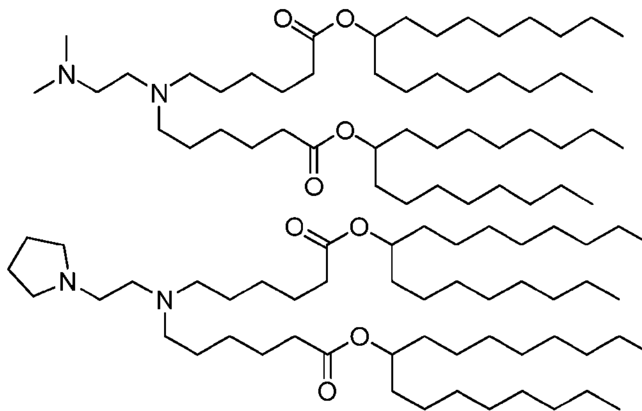
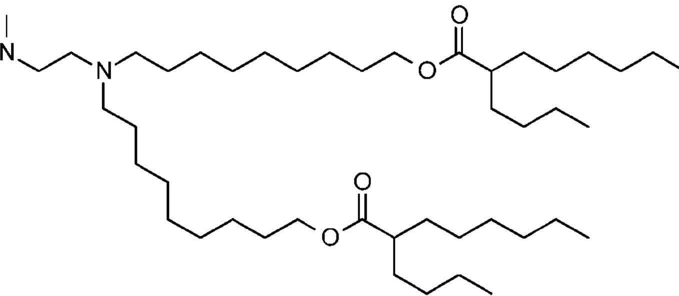
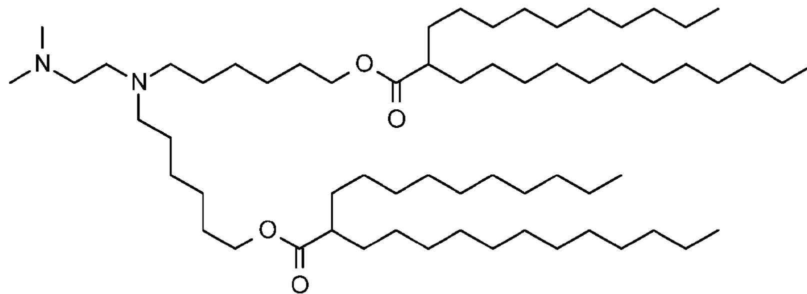
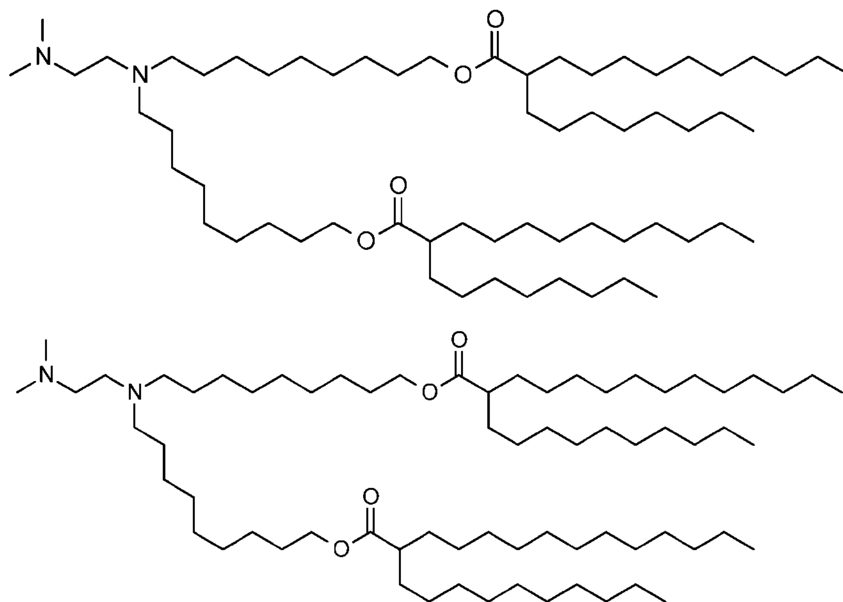






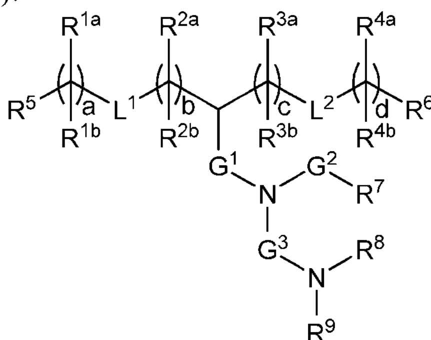






In some embodiments, the LNPs comprise a lipid of Formula (I), at least one agent, and one or more excipients selected from neutral lipids, steroids and pegylated lipids. In some embodiments the lipid of Formula (I) is compound I-5. In some
5 embodiments the lipid of Formula (I) is compound I-6.

In some other embodiments, the cationic lipid component of the LNPs has the structure of Formula (II):



(II)

10 or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein:

L^1 and L^2 are each independently -O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -S(O)$_x$-, -S-S-, -C(=O)S-, -SC(=O)-, -NR^{a}C(=O)-, -C(=O)NR^a-, -NR^{a}C(=O)NR^a,

-OC(=O)NR^a-, -NR^{a}C(=O)O-, or a direct bond;

G^1 is C_1-C_2 alkylene, –(C=O)- , -O(C=O)-, -SC(=O)-, -NR^{a}C(=O)- or a direct bond;

G^2 is –C(=O)- , -(C=O)O-, -C(=O)S-, -C(=O)NR^a or a direct bond;

5 G^3 is C_1-C_6 alkylene;

R^a is H or C_1-C_{12} alkyl;

R^{1a} and R^{1b} are, at each occurrence, independently either: (a) H or C_1-C_{12} alkyl; or (b) R^{1a} is H or C_1-C_{12} alkyl, and R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to

10 form a carbon-carbon double bond;

R^{2a} and R^{2b} are, at each occurrence, independently either: (a) H or C_1-C_{12} alkyl; or (b) R^{2a} is H or C_1-C_{12} alkyl, and R^{2b} together with the carbon atom to which it is bound is taken together with an adjacent R^{2b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

15 R^{3a} and R^{3b} are, at each occurrence, independently either: (a) H or C_1-C_{12} alkyl; or (b) R^{3a} is H or C_1-C_{12} alkyl, and R^{3b} together with the carbon atom to which it is bound is taken together with an adjacent R^{3b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{4a} and R^{4b} are, at each occurrence, independently either: (a) H or C_1-C_{12}

20 alkyl; or (b) R^{4a} is H or C_1-C_{12} alkyl, and R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^5 and R^6 are each independently H or methyl;

R^7 is C_4-C_{20} alkyl;

25 R^8 and R^9 are each independently C_1-C_{12} alkyl; or R^8 and R^9, together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring;

a, b, c and d are each independently an integer from 1 to 24; and

x is 0, 1 or 2.

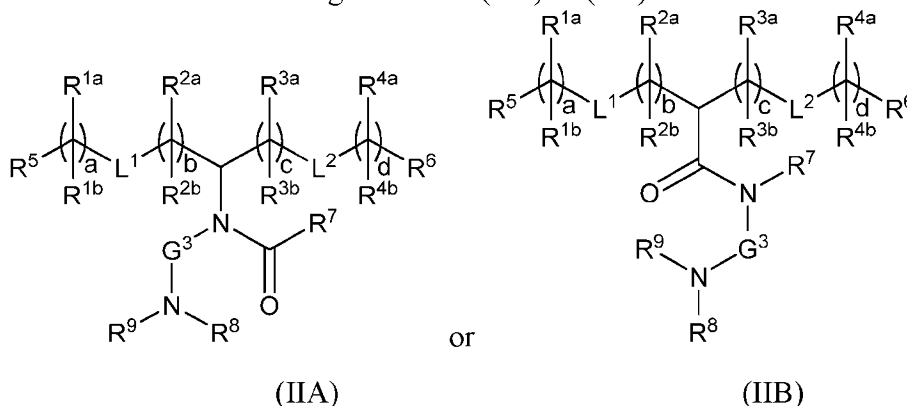
30 In some embodiments of Formula (II), L^1 and L^2 are each independently

–O(C=O)-, -(C=O)O- or a direct bond. In other embodiments, G^1 and G^2 are each independently -(C=O)- or a direct bond. In some different embodiments, L^1 and L^2 are each independently –O(C=O)-, -(C=O)O- or a direct bond; and G^1 and G^2 are each independently –(C=O)- or a direct bond.

5 In some different embodiments of Formula (II), L^1 and L^2 are each independently -C(=O)-, -O-, $-S(O)_x-$, -S-S-, -C(=O)S-, -SC(=O)-, $-NR^a-$, $-NR^aC(=O)-$, $-C(=O)NR^a-$, $-NR^aC(=O)NR^a$, $-OC(=O)NR^a-$, $-NR^aC(=O)O-$, $-NR^aS(O)_xNR^a-$, $-NR^aS(O)_x-$ or $-S(O)_xNR^a-$.

In other of the foregoing embodiments of Formula (II), the lipid

10 compound has one of the following structures (IIA) or (IIB):



(IIA) (IIB)

In some embodiments of Formula (II), the lipid compound has structure (IIA). In other embodiments, the lipid compound has structure (IIB).

15 In any of the foregoing embodiments of Formula (II), one of L^1 or L^2 is -O(C=O)-. For example, in some embodiments each of L^1 and L^2 are -O(C=O)-.

In some different embodiments of Formula (II), one of L^1 or L^2 is -(C=O)O-. For example, in some embodiments each of L^1 and L^2 is -(C=O)O-.

In different embodiments of Formula (II), one of L^1 or L^2 is a direct bond.

20 As used herein, a “direct bond” means the group (e.g., L^1 or L^2) is absent. For example, in some embodiments each of L^1 and L^2 is a direct bond.

In other different embodiments of Formula (II), for at least one occurrence of R^{1a} and R^{1b}, R^{1a} is H or C_1-C_{12} alkyl, and R^{1b} together with the carbon atom to which it

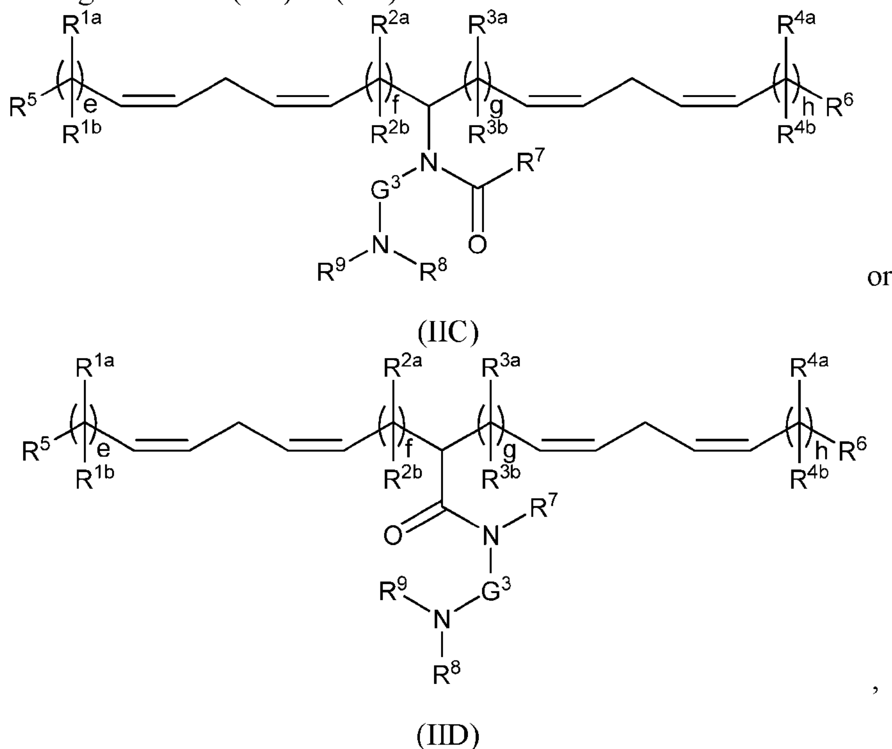
is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

In still other different embodiments of Formula (II), for at least one occurrence of R^{4a} and R^{4b}, R^{4a} is H or C_1-C_{12} alkyl, and R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

In more embodiments of Formula (II), for at least one occurrence of R^{2a} and R^{2b}, R^{2a} is H or C_1-C_{12} alkyl, and R^{2b} together with the carbon atom to which it is bound is taken together with an adjacent R^{2b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

In other different embodiments of Formula (II), for at least one occurrence of R^{3a} and R^{3b}, R^{3a} is H or C_1-C_{12} alkyl, and R^{3b} together with the carbon atom to which it is bound is taken together with an adjacent R^{3b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

In various other embodiments of Formula (II), the lipid compound has one of the following structures (IIC) or (IID):



wherein e, f, g and h are each independently an integer from 1 to 12.

In some embodiments of Formula (II), the lipid compound has structure (IIC). In other embodiments, the lipid compound has structure (IID).

In various embodiments of structures (IIC) or (IID), e, f, g and h are each
5 independently an integer from 4 to 10.

In certain embodiments of Formula (II), a, b, c and d are each independently an integer from 2 to 12 or an integer from 4 to 12. In other embodiments, a, b, c and d are each independently an integer from 8 to 12 or 5 to 9. In some certain embodiments, a is 0. In some embodiments, a is 1. In other embodiments, a is 2. In more
10 embodiments, a is 3. In yet other embodiments, a is 4. In some embodiments, a is 5. In other embodiments, a is 6. In more embodiments, a is 7. In yet other embodiments, a is 8. In some embodiments, a is 9. In other embodiments, a is 10. In more embodiments, a is 11. In yet other embodiments, a is 12. In some embodiments, a is 13. In other embodiments, a is 14. In more embodiments, a is 15. In yet other embodiments, a is 16.

15 In some embodiments of Formula (II), b is 1. In other embodiments, b is 2. In more embodiments, b is 3. In yet other embodiments, b is 4. In some embodiments, b is 5. In other embodiments, b is 6. In more embodiments, b is 7. In yet other embodiments, b is 8. In some embodiments, b is 9. In other embodiments, b is 10. In more embodiments, b is 11. In yet other embodiments, b is 12. In some embodiments, b
20 is 13. In other embodiments, b is 14. In more embodiments, b is 15. In yet other embodiments, b is 16.

In some embodiments of Formula (II), c is 1. In other embodiments, c is 2. In more embodiments, c is 3. In yet other embodiments, c is 4. In some embodiments, c is 5. In other embodiments, c is 6. In more embodiments, c is 7. In yet other embodiments, c
25 is 8. In some embodiments, c is 9. In other embodiments, c is 10. In more embodiments, c is 11. In yet other embodiments, c is 12. In some embodiments, c is 13. In other embodiments, c is 14. In more embodiments, c is 15. In yet other embodiments, c is 16.

In some certain embodiments of Formula (II), d is 0. In some embodiments, d is 1. In other embodiments, d is 2. In more embodiments, d is 3. In yet
30 other embodiments, d is 4. In some embodiments, d is 5. In other embodiments, d is 6. In

more embodiments, d is 7. In yet other embodiments, d is 8. In some embodiments, d is 9. In other embodiments, d is 10. In more embodiments, d is 11. In yet other embodiments, d is 12. In some embodiments, d is 13. In other embodiments, d is 14. In more embodiments, d is 15. In yet other embodiments, d is 16.

5 In some embodiments of Formula (II), e is 1. In other embodiments, e is 2. In more embodiments, e is 3. In yet other embodiments, e is 4. In some embodiments, e is 5. In other embodiments, e is 6. In more embodiments, e is 7. In yet other embodiments, e is 8. In some embodiments, e is 9. In other embodiments, e is 10. In more embodiments, e is 11. In yet other embodiments, e is 12.

10 In some embodiments of Formula (II), f is 1. In other embodiments, f is 2. In more embodiments, f is 3. In yet other embodiments, f is 4. In some embodiments, f is 5. In other embodiments, f is 6. In more embodiments, f is 7. In yet other embodiments, f is 8. In some embodiments, f is 9. In other embodiments, f is 10. In more embodiments, f is 11. In yet other embodiments, f is 12.

15 In some embodiments of Formula (II), g is 1. In other embodiments, g is 2. In more embodiments, g is 3. In yet other embodiments, g is 4. In some embodiments, g is 5. In other embodiments, g is 6. In more embodiments, g is 7. In yet other embodiments, g is 8. In some embodiments, g is 9. In other embodiments, g is 10. In more embodiments, g is 11. In yet other embodiments, g is 12.

20 In some embodiments of Formula (II), h is 1. In other embodiments, e is 2. In more embodiments, h is 3. In yet other embodiments, h is 4. In some embodiments, e is 5. In other embodiments, h is 6. In more embodiments, h is 7. In yet other embodiments, h is 8. In some embodiments, h is 9. In other embodiments, h is 10. In more embodiments, h is 11. In yet other embodiments, h is 12.

25 In some other various embodiments of Formula (II), a and d are the same. In some other embodiments, b and c are the same. In some other specific embodiments and a and d are the same and b and c are the same.

The sum of a and b and the sum of c and d of Formula (II) are factors which may be varied to obtain a lipid having the desired properties. In some

30 embodiments, a and b are chosen such that their sum is an integer ranging from 14 to 24.

In other embodiments, c and d are chosen such that their sum is an integer ranging from 14 to 24. In further embodiment, the sum of a and b and the sum of c and d are the same. For example, in some embodiments the sum of a and b and the sum of c and d are both the same integer which may range from 14 to 24. In still more embodiments, a. b, c and d
5 are selected such that the sum of a and b and the sum of c and d is 12 or greater.

The substituents at R^{1a}, R^{2a}, R^{3a} and R^{4a} of Formula (II) are not particularly limited. In some embodiments, at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is H. In certain embodiments R^{1a}, R^{2a}, R^{3a} and R^{4a} are H at each occurrence. In certain other embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_{12} alkyl. In certain other
10 embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_8 alkyl. In certain other embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_6 alkyl. In some of the foregoing embodiments, the C_1-C_8 alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

In certain embodiments of Formula (II), R^{1a}, R^{1b}, R^{4a} and R^{4b} are C_1-C_{12}
15 alkyl at each occurrence.

In further embodiments of Formula (II), at least one of R^{1b}, R^{2b}, R^{3b} and R^{4b} is H or R^{1b}, R^{2b}, R^{3b} and R^{4b} are H at each occurrence.

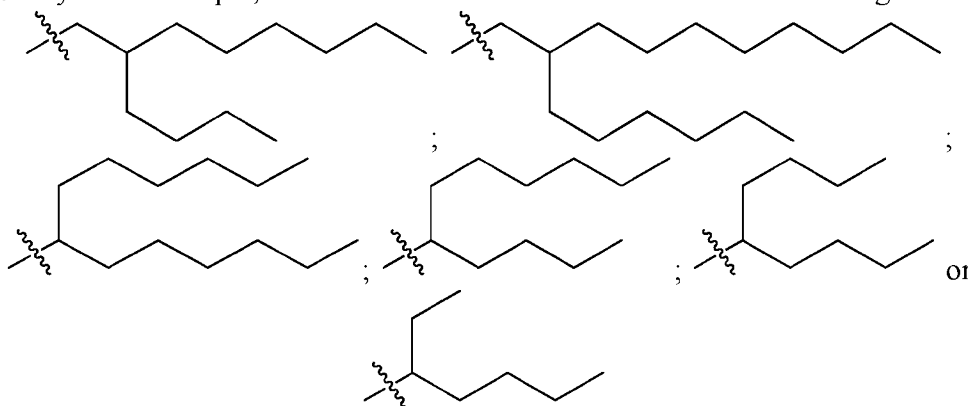
In certain embodiments of Formula (II), R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which
20 it is bound to form a carbon-carbon double bond. In other embodiments of the foregoing R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

The substituents at R^5 and R^6 of Formula (II) are not particularly limited in the foregoing embodiments. In certain embodiments one of R^5 or R^6 is methyl. In other
25 embodiments each of R^5 or R^6 is methyl.

The substituents at R^7 of Formula (II) are not particularly limited in the foregoing embodiments. In certain embodiments R^7 is C_6-C_{16} alkyl. In some other embodiments, R^7 is C_6-C_9 alkyl. In some of these embodiments, R^7 is substituted with -(C=O)OR^b, –O(C=O)R^b, -C(=O)R^b, -OR^b, -S(O)$_x$$R^b$, -S-S$R^b$, -C(=O)S$R^b$,
30 -SC(=O)R^b, -N$R^a$$R^b$, -N$R^a$C(=O)$R^b$, -C(=O)N$R^a$$R^b$, -N$R^a$C(=O)N$R^a$$R^b$,

-OC(=O)NR^{a}R^b, -NR^{a}C(=O)OR^b, -NR^{a}S(O)$_x$NR^{a}R^b, -NR^{a}S(O)$_x$R^b or -S(O)$_x$NR^{a}R^b, wherein: R^a is H or C_1-C_{12} alkyl; R^b is C_1-C_{15} alkyl; and x is 0, 1 or 2. For example, in some embodiments R^7 is substituted with -(C=O)OR^b or –O(C=O)R^b.

In various of the foregoing embodiments of Formula (II), R^b is branched
5 C_1-C_{15} alkyl. For example, in some embodiments R^b has one of the following structures:

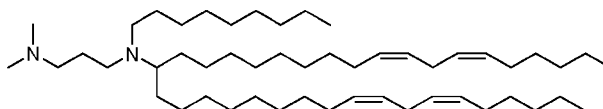


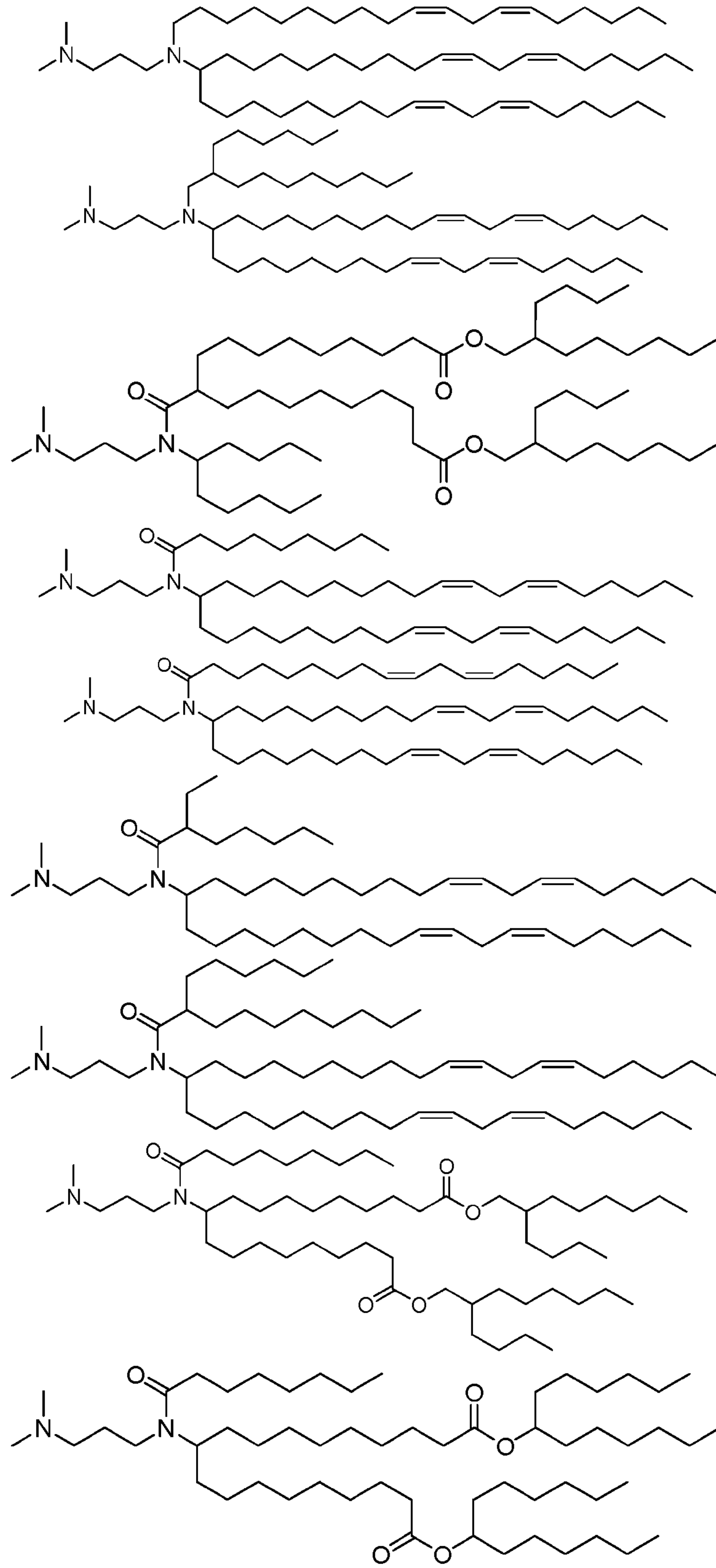
In certain other of the foregoing embodiments of Formula (II), one of R^8
10 or R^9 is methyl. In other embodiments, both R^8 and R^9 are methyl.

In some different embodiments of Formula (II), R^8 and R^9, together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring. In some embodiments of the foregoing, R^8 and R^9, together with the nitrogen atom to which they are attached, form a 5-membered heterocyclic ring, for example a
15 pyrrolidinyl ring. In some different embodiments of the foregoing, R^8 and R^9, together with the nitrogen atom to which they are attached, form a 6-membered heterocyclic ring, for example a piperazinyl ring.

In still other embodiments of the foregoing lipids of Formula (II), G^3 is C_2-C_4 alkylene, for example C_3 alkylene.

20 In various different embodiments, the lipid compound has one of the following structures:

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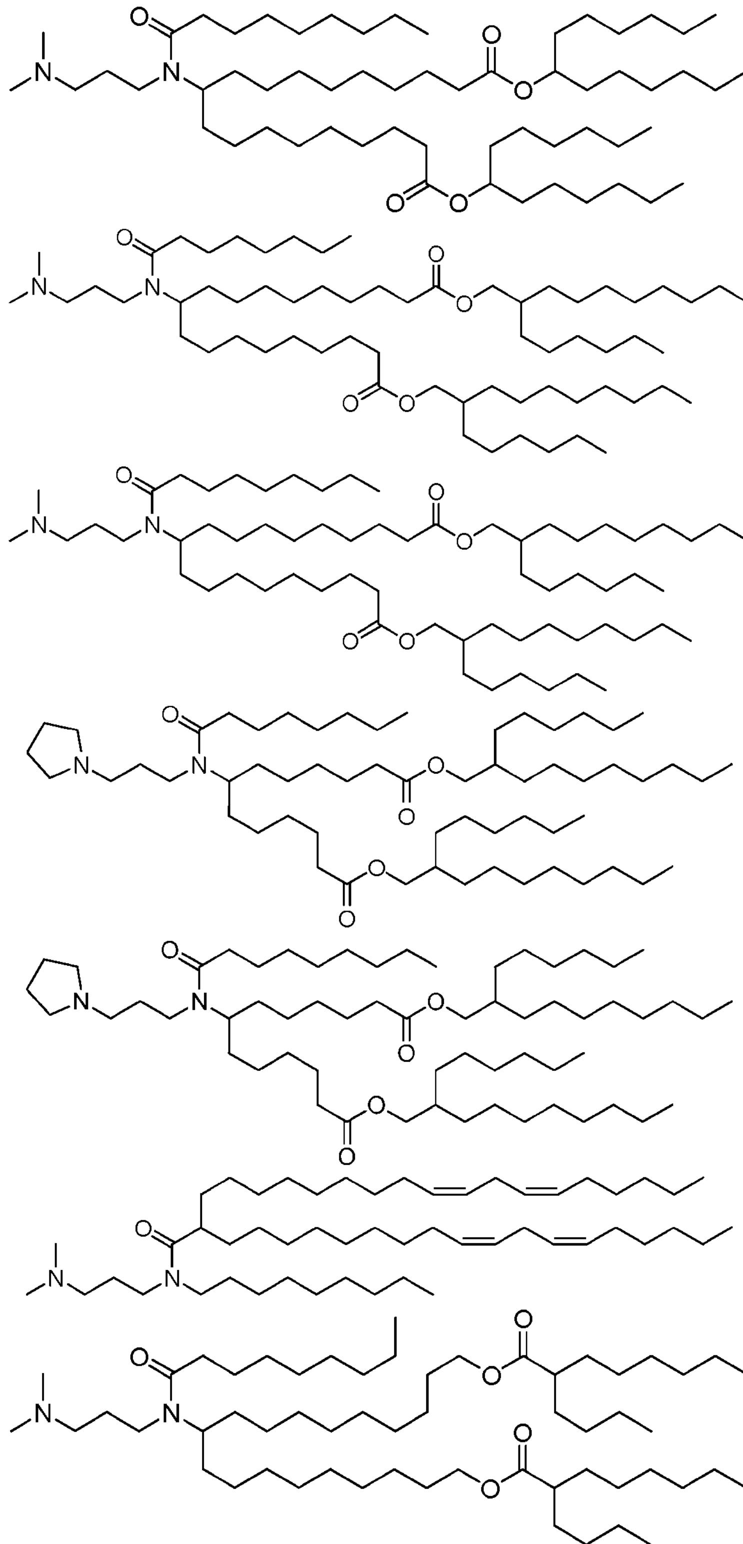

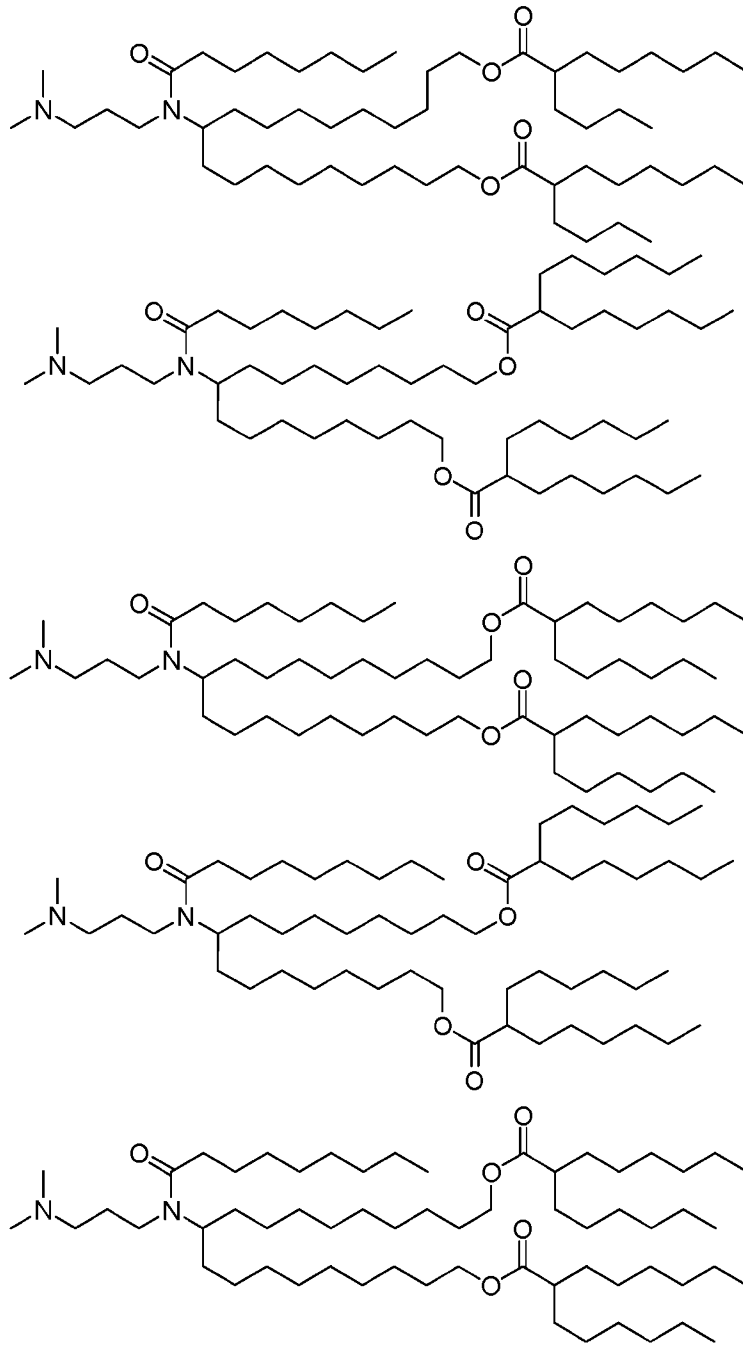
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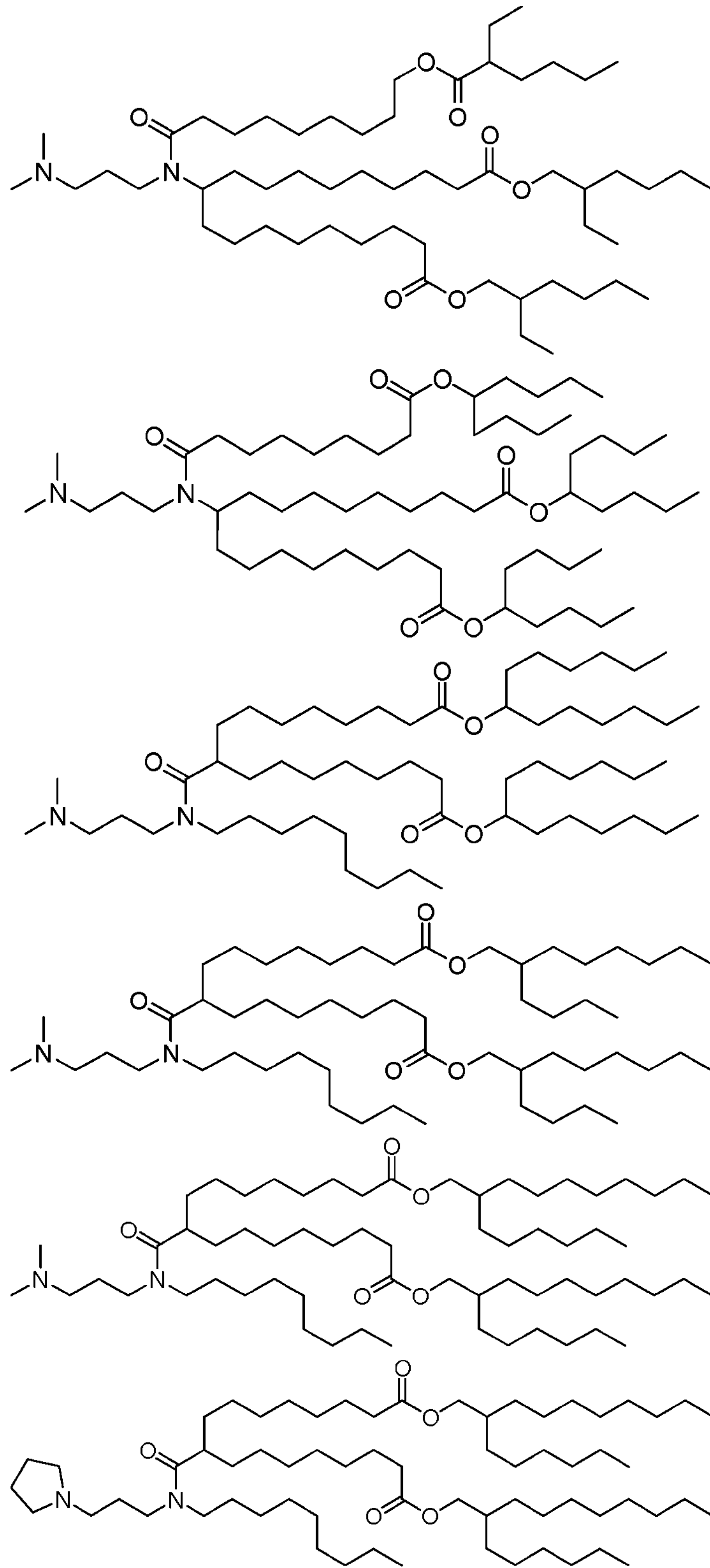
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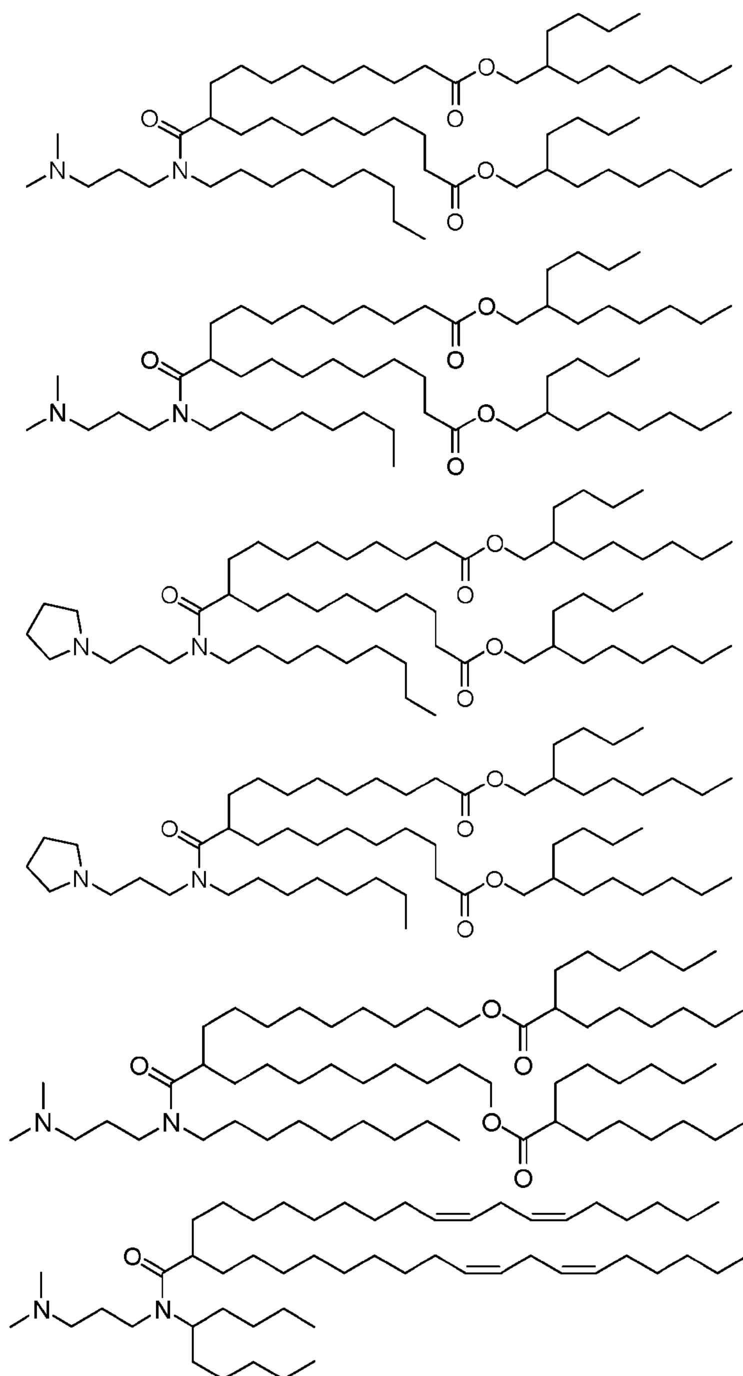
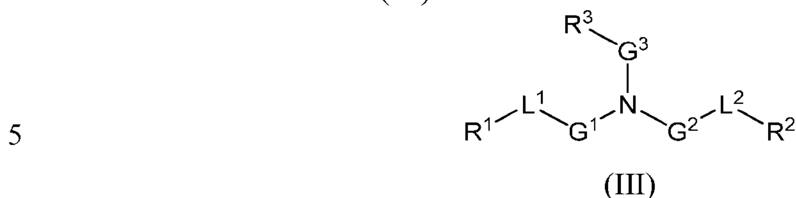


In some embodiments, the LNPs comprise a lipid of Formula (II), at least one agent, and one or more excipient selected from neutral lipids, steroids and pegylated lipids. In some embodiments, the lipid of Formula (II) is compound II-9. In some
5 embodiments, the lipid of Formula (II) is compound II-10. In some embodiments, the

lipid of Formula (II) is compound II-11. In some embodiments, the lipid of Formula (II) is compound II-12. In some embodiments, the lipid of Formula (II) is compound II-32.

In some other embodiments, the cationic lipid component of the LNPs has the structure of Formula (III):



(III)

or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein:

one of L^1 or L^2 is –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, $-S(O)_x-$, -S-S-, -C(=O)S-, SC(=O)-, $-NR^aC(=O)-$, $-C(=O)NR^a-$, $NR^aC(=O)NR^a-$, $-OC(=O)NR^a-$ or $-NR^aC(=O)O-$, and the other of L^1 or L^2 is –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, $-S(O)_x-$, -S-S-, -C(=O)S-, SC(=O)-, $-NR^aC(=O)-$, $-C(=O)NR^a-$, ,$NR^aC(=O)NR^a-$, $-OC(=O)NR^a-$ or $-NR^aC(=O)O-$ or a direct bond;

G^1 and G^2 are each independently unsubstituted C_1-C_{12} alkylene or C_1-C_{12} alkenylene;

G^3 is C_1-C_{24} alkylene, C_1-C_{24} alkenylene, C_3-C_8 cycloalkylene, C_3-C_8 cycloalkenylene;

R^a is H or C_1-C_{12} alkyl;

R^1 and R^2 are each independently C_6-C_{24} alkyl or C_6-C_{24} alkenyl;

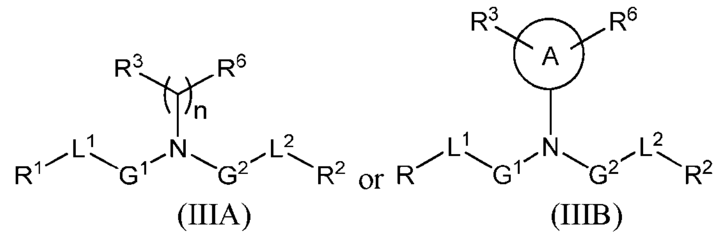
R^3 is H, OR^5, CN, $-C(=O)OR^4$, $-OC(=O)R^4$ or $–NR^5C(=O)R^4$;

R^4 is C_1-C_{12} alkyl;

R^5 is H or C_1-C_6 alkyl; and

x is 0, 1 or 2.

In some of the foregoing embodiments of Formula (III), the lipid has one of the following structures (IIIA) or (IIIB):



wherein:

A is a 3 to 8-membered cycloalkyl or cycloalkylene ring;

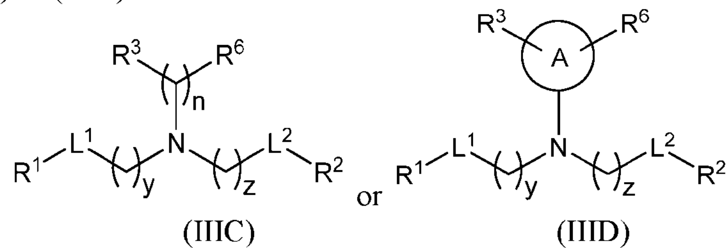
5 R^6 is, at each occurrence, independently H, OH or C_1-C_{24} alkyl;

n is an integer ranging from 1 to 15.

In some of the foregoing embodiments of Formula (III), the lipid has structure (IIIA), and in other embodiments, the lipid has structure (IIIB).

In other embodiments of Formula (III), the lipid has one of the following

10 structures (IIIC) or (IIID):



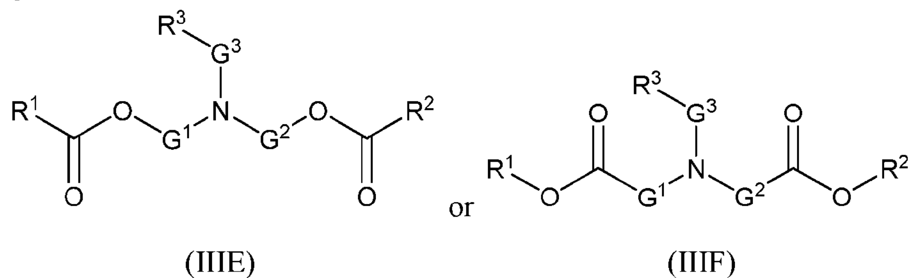
wherein y and z are each independently integers ranging from 1 to 12.

In any of the foregoing embodiments of Formula (III), one of L^1 or L^2

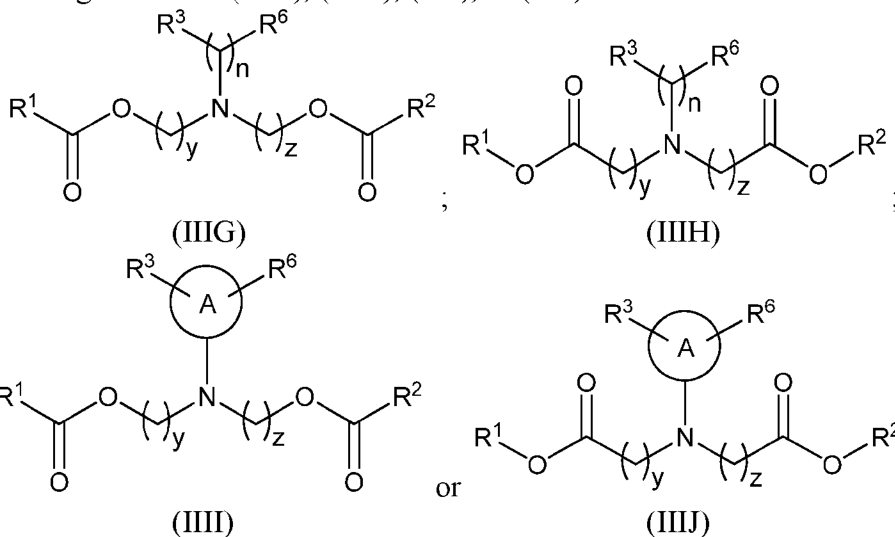
15 is -O(C=O)-. For example, in some embodiments each of L^1 and L^2 are -O(C=O)-. In some different embodiments of any of the foregoing, L^1 and L^2 are each independently -(C=O)O- or -O(C=O)-. For example, in some embodiments each of L^1 and L^2 is -(C=O)O-.

In some different embodiments of Formula (III), the lipid has one of the

20 following structures (IIIE) or (IIIF):



In some of the foregoing embodiments of Formula (III), the lipid has one of the following structures (IIIG), (IIIH), (IIII), or (IIIJ):



5

In some of the foregoing embodiments of Formula (III), n is an integer ranging from 2 to 12, for example from 2 to 8 or from 2 to 4. For example, in some embodiments, n is 3, 4, 5 or 6. In some embodiments, n is 3. In some embodiments, n is 4. In some embodiments, n is 5. In some embodiments, n is 6.

10

In some other of the foregoing embodiments of Formula (III), y and z are each independently an integer ranging from 2 to 10. For example, in some embodiments, y and z are each independently an integer ranging from 4 to 9 or from 4 to 6.

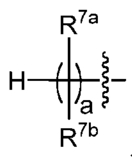
In some of the foregoing embodiments of Formula (III), R^6 is H. In other of the foregoing embodiments, R^6 is C_1-C_{24} alkyl. In other embodiments, R^6 is OH.

15

In some embodiments of Formula (III), G^3 is unsubstituted. In other embodiments, G3 is substituted. In various different embodiments, G^3 is linear C_1-C_{24} alkylene or linear C_1-C_{24} alkenylene.

In some other foregoing embodiments of Formula (III), R^1 or R^2, or both, is C_6-C_{24} alkenyl. For example, in some embodiments, R^1 and R^2 each, independently have the following structure:

20



wherein:

R^{7a} and R^{7b} are, at each occurrence, independently H or C_1-C_{12} alkyl; and

a is an integer from 2 to 12,

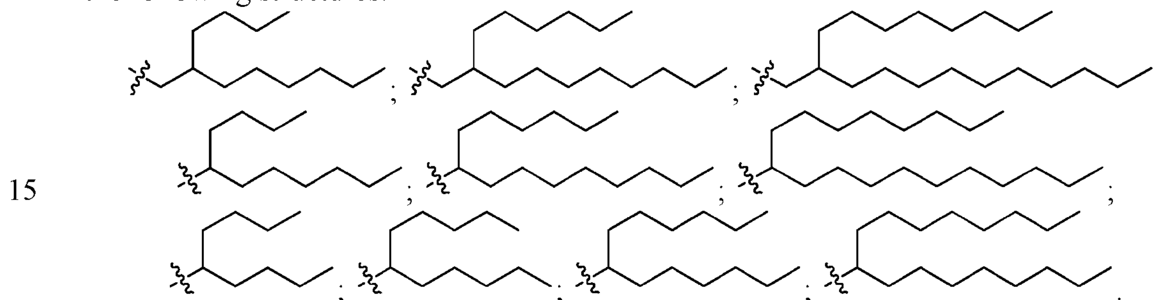
wherein R^{7a}, R^{7b} and a are each selected such that R^1 and R^2 each independently comprise

5 from 6 to 20 carbon atoms. For example, in some embodiments a is an integer ranging from 5 to 9 or from 8 to 12.

In some of the foregoing embodiments of Formula (III), at least one occurrence of R^{7a} is H. For example, in some embodiments, R^{7a} is H at each occurrence. In other different embodiments of the foregoing, at least one occurrence of R^{7b} is C_1-C_8

10 alkyl. For example, in some embodiments, C_1-C_8 alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

In different embodiments of Formula (III), R^1 or R^2, or both, has one of the following structures:



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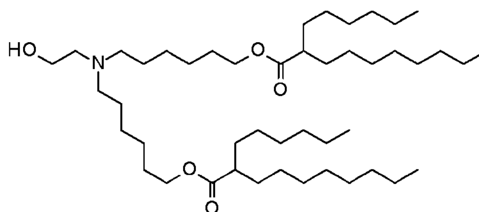
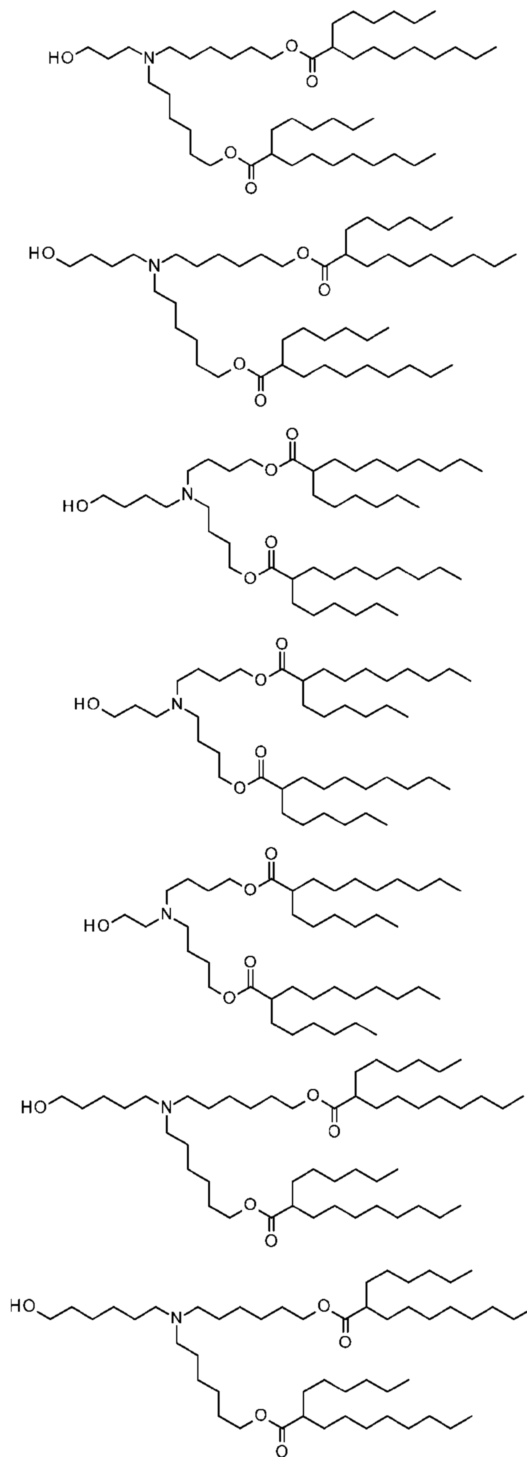
In some of the foregoing embodiments of Formula (III), R^3 is OH, CN, -C(=O)OR^4, -OC(=O)R^4 or –NHC(=O)R^4. In some embodiments, R^4 is methyl or ethyl.

20 In various different embodiments, the cationic lipid of Formula (III) has one of the following structures:

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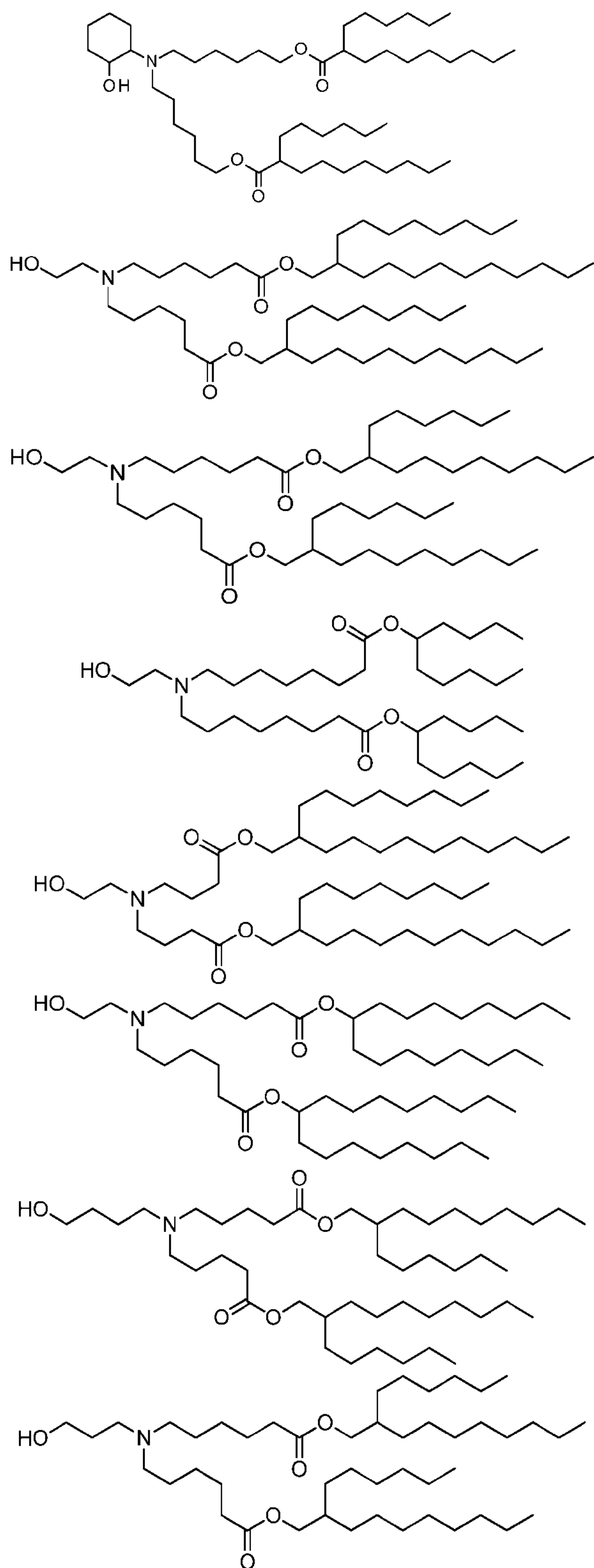

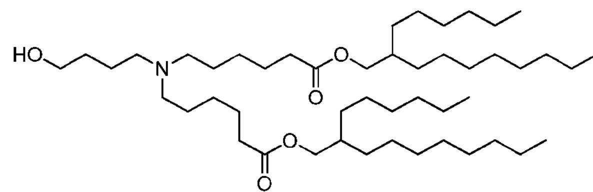
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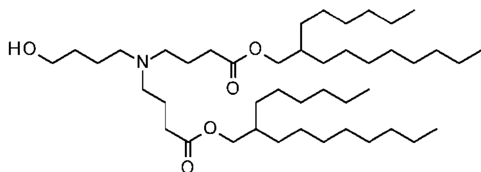
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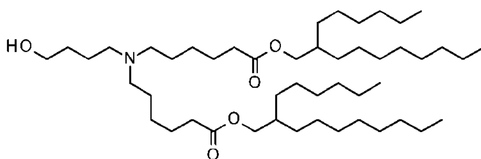
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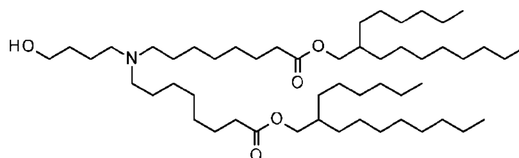
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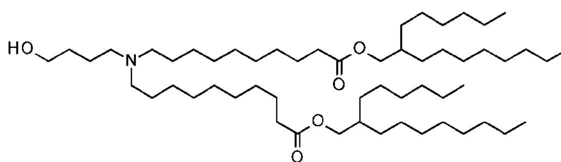
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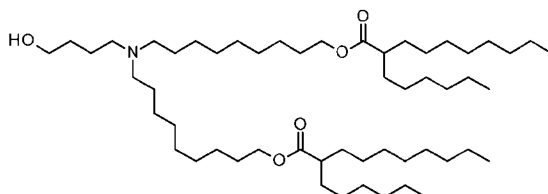
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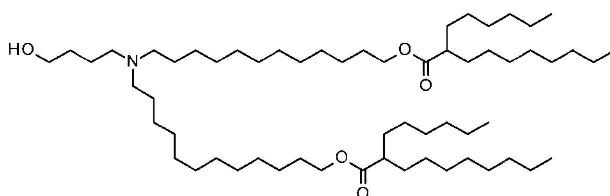
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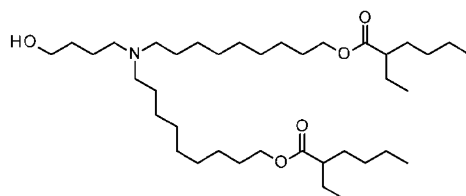
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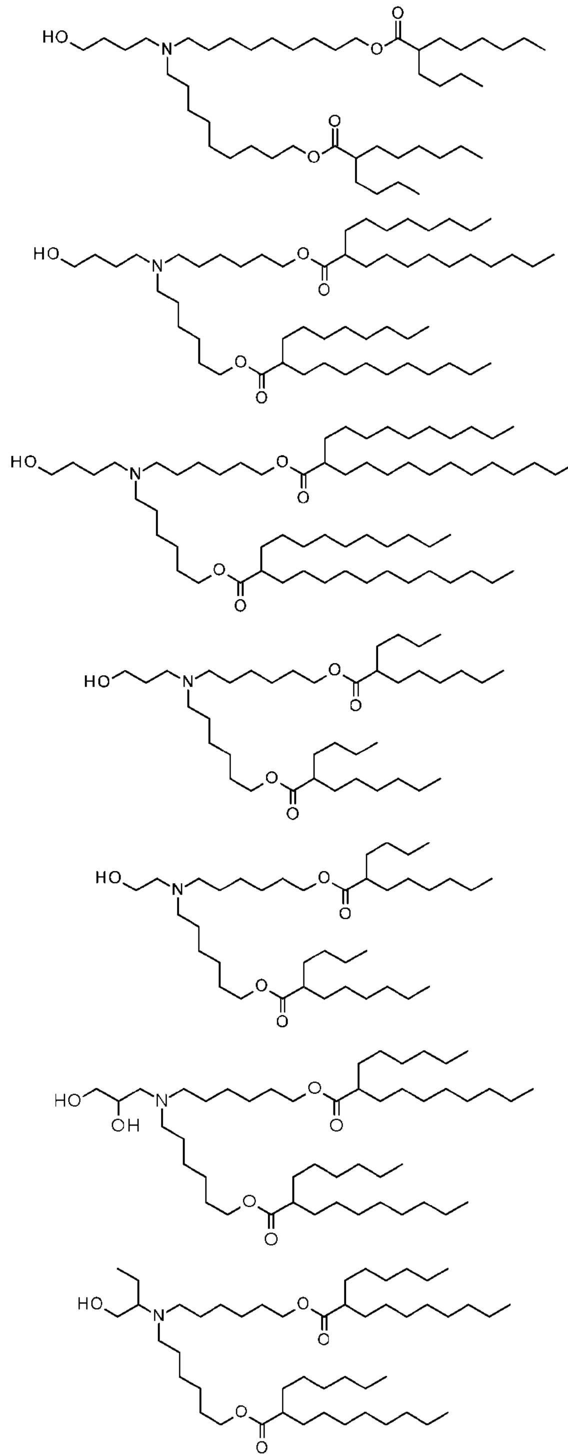
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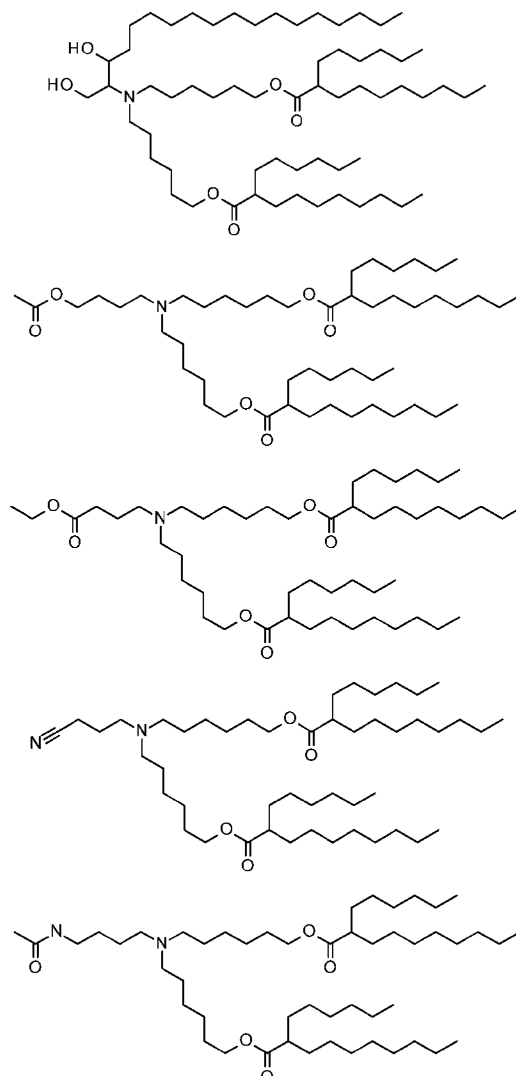


In some embodiments, the LNPs comprise a lipid of Formula (III), at least one agent, and one or more excipient selected from neutral lipids, steroids and pegylated lipids. In some embodiments, the lipid of Formula (III) is compound III-3. In some embodiments, the lipid of Formula (III) is compound III-7.

In certain embodiments, the cationic lipid is present in the LNP in an amount from about 30 to about 95 mole percent. In some embodiments, the cationic lipid is present in the LNP in an amount from about 30 to about 70 mole percent. In some embodiments, the cationic lipid is present in the LNP in an amount from about 40 to about 60 mole percent. In some embodiments, the cationic lipid is present in the LNP in

an amount of about 50 mole percent. In some embodiments, the LNP comprises only cationic lipids.

In certain embodiments, the LNP comprises one or more additional lipids which stabilize the formation of particles during their formation.

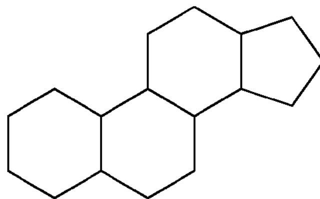
5 Suitable stabilizing lipids include neutral lipids and anionic lipids.

The term “neutral lipid” refers to any one of a number of lipid species that exist in either an uncharged or neutral zwitterionic form at physiological pH. Representative neutral lipids include diacylphosphatidylcholines, diacylphosphatidylethanolamines, ceramides, sphingomyelins, dihydro sphingomyelins,
10 cephalins, and cerebrosides.

Exemplary neutral lipids include, for example, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE),
15 palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE) and dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1-trans PE, 1-stearioyl-2-oleoyl-
20 phosphatidyethanol amine (SOPE), and 1,2-dielaidoyl-sn-glycero-3-phophoethanolamine (transDOPE). In some embodiments, the neutral lipid is 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC).

In some embodiments, the LNPs comprise a neutral lipid selected from DSPC, DPPC, DMPC, DOPC, POPC, DOPE and SM. In various embodiments, the molar
25 ratio of the cationic lipid (e.g., lipid of Formula (I)) to the neutral lipid ranges from about 2:1 to about 8:1.

In various embodiments, the LNPs further comprise a steroid or steroid analogue. A “steroid” is a compound comprising the following carbon skeleton:



In certain embodiments, the steroid or steroid analogue is cholesterol. In some of these embodiments, the molar ratio of the cationic lipid (e.g., lipid of Formula (I)) to cholesterol ranges from about 2:1 to 1:1.

5 The term "anionic lipid" refers to any lipid that is negatively charged at physiological pH. These lipids include phosphatidylglycerol, cardiolipin, diacylphosphatidylserine, diacylphosphatidic acid, N-dodecanoylphosphatidylethanolamines, N-succinylphosphatidylethanolamines, N-glutarylphosphatidylethanolamines, lysylphosphatidylglycerols,
10 palmitoyloleyolphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

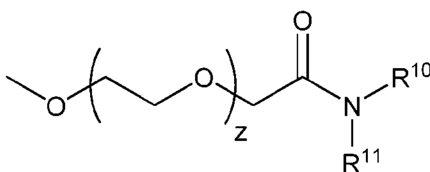
In certain embodiments, the LNP comprises glycolipids (e.g., monosialoganglioside GM_1). In certain embodiments, the LNP comprises a sterol, such as cholesterol.

15 In some embodiments, the LNPs comprise a polymer conjugated lipid. The term "polymer conjugated lipid" refers to a molecule comprising both a lipid portion and a polymer portion. An example of a polymer conjugated lipid is a pegylated lipid. The term "pegylated lipid" refers to a molecule comprising both a lipid portion and a polyethylene glycol portion. Pegylated lipids are known in the art and include
20 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-s- DMG) and the like.

In certain embodiments, the LNP comprises an additional, stabilizing - lipid which is a polyethylene glycol-lipid (pegylated lipid). Suitable polyethylene glycol-lipids include PEG-modified phosphatidylethanolamine, PEG-modified phosphatidic
25 acid, PEG-modified ceramides (e.g., PEG-CerC14 or PEG-CerC20), PEG-modified dialkylamines, PEG-modified diacylglycerols, PEG-modified dialkylglycerols. Representative polyethylene glycol-lipids include PEG-c-DOMG, PEG-c-DMA, and PEG-s-DMG. In some embodiments, the polyethylene glycol-lipid is N-[(methoxy

poly(ethylene glycol)$_{2000}$)carbamyl]-1,2-dimyristyloxlpropyl-3-amine (PEG-c-DMA). In some embodiments, the polyethylene glycol-lipid is PEG-c-DOMG). In other embodiments, the LNPs comprise a pegylated diacylglycerol (PEG-DAG) such as 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG), a pegylated phosphatidylethanoloamine (PEG-PE), a PEG succinate diacylglycerol (PEG-S-DAG) such as 4-O-(2',3'-di(tetradecanoyloxy)propyl-1-O-(ω-methoxy(polyethoxy)ethyl)butanedioate (PEG-S-DMG), a pegylated ceramide (PEG-cer), or a PEG dialkoxypropylcarbamate such as ωmethoxy(polyethoxy)ethyl-N-(2,3-di(tetradecanoxy)propyl)carbamate or 2,3-di(tetradecanoxy)propyl-N-(ω-methoxy(polyethoxy)ethyl)carbamate. In various embodiments, the molar ratio of the cationic lipid to the pegylated lipid ranges from about 100:1 to about 25:1.

In some embodiments, the LNPs comprise a pegylated lipid having the following structure (IV):



(IV)

or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein:

R^{10} and R^{11} are each independently a straight or branched, saturated or unsaturated alkyl chain containing from 10 to 30 carbon atoms, wherein the alkyl chain is optionally interrupted by one or more ester bonds; and

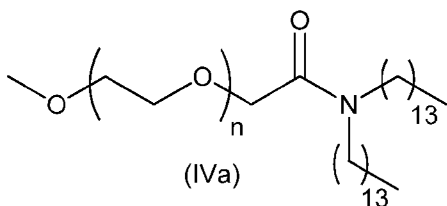
z has mean value ranging from 30 to 60.

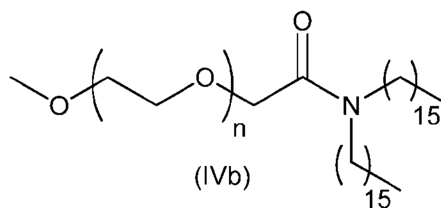
In some of the foregoing embodiments of the pegylated lipid (IV), R^{10} and R^{11} are not both n-octadecyl when z is 42. In some other embodiments, R^{10} and R^{11} are each independently a straight or branched, saturated or unsaturated alkyl chain containing from 10 to 18 carbon atoms. In some embodiments, R^{10} and R^{11} are each independently a straight or branched, saturated or unsaturated alkyl chain containing from 12 to 16 carbon atoms. In some embodiments, R^{10} and R^{11} are each independently a straight or branched, saturated or unsaturated alkyl chain containing 12 carbon atoms. In some embodiments, R^{10} and R^{11} are each independently a straight or branched, saturated or unsaturated alkyl

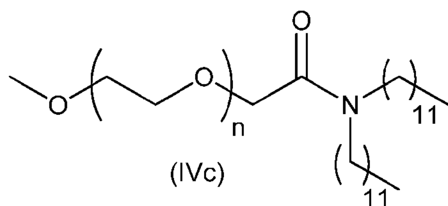
chain containing 14 carbon atoms. In other embodiments, R^{10} and R^{11} are each independently a straight or branched, saturated or unsaturated alkyl chain containing 16 carbon atoms. In still more embodiments, R^{10} and R^{11} are each independently a straight or branched, saturated or unsaturated alkyl chain containing 18 carbon atoms. In still other
5 embodiments, R^{10} is a straight or branched, saturated or unsaturated alkyl chain containing 12 carbon atoms and R^{11} is a straight or branched, saturated or unsaturated alkyl chain containing 14 carbon atoms.

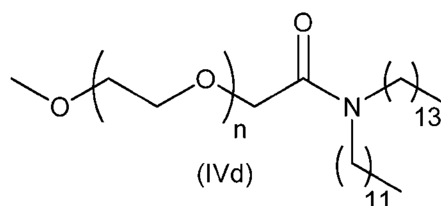
In various embodiments, z spans a range that is selected such that the PEG portion of (II) has an average molecular weight of about 400 to about 6000 g/mol. In
10 some embodiments, the average z is about 45.

In other embodiments, the pegylated lipid has one of the following structures:









wherein n is an integer selected such that the average molecular weight of the pegylated
15 lipid is about 2500 g/mol.

In certain embodiments, the additional lipid is present in the LNP in an amount from about 1 to about 10 mole percent. In some embodiments, the additional lipid is present in the LNP in an amount from about 1 to about 5 mole percent. In some embodiments, the additional lipid is present in the LNP in about 1 mole percent or about
20 1.5 mole percent.

In some embodiments, the LNPs comprise a lipid of Formula (I), a nucleoside-modified RNA, a neutral lipid, a steroid and a pegylated lipid. In some

embodiments the lipid of Formula (I) is compound I-6. In different embodiments, the neutral lipid is DSPC. In other embodiments, the steroid is cholesterol. In still different embodiments, the pegylated lipid is compound IVa.

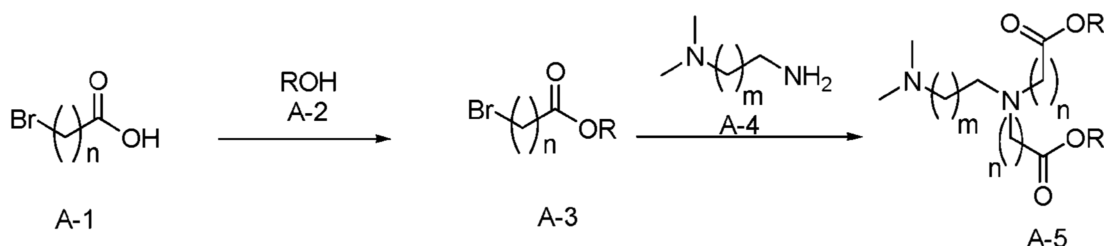
In certain embodiments, the LNP comprises one or more targeting moieties that targets the LNP to a cell or cell population. For example, in some embodiments, the targeting domain is a ligand which directs the LNP to a receptor found on a cell surface.

In certain embodiments, the LNP comprises one or more internalization domains. For example, in some embodiments, the LNP comprises one or more domains which bind to a cell to induce the internalization of the LNP. For example, in some embodiments, the one or more internalization domains bind to a receptor found on a cell surface to induce receptor-mediated uptake of the LNP. In certain embodiments, the LNP is capable of binding a biomolecule in vivo, where the LNP-bound biomolecule can then be recognized by a cell-surface receptor to induce internalization. For example, in some embodiments, the LNP binds systemic ApoE, which leads to the uptake of the LNP and associated cargo.

Other exemplary LNPs and their manufacture are described in the art, for example in U.S. Patent Application Publication No. US20120276209, Semple et al., 2010, Nat Biotechnol., 28(2):172-176; Akinc et al., 2010, Mol Ther., 18(7): 1357-1364; Basha et al., 2011, Mol Ther, 19(12): 2186-2200; Leung et al., 2012, J Phys Chem C Nanomater Interfaces, 116(34): 18440-18450; Lee et al., 2012, Int J Cancer., 131(5): E781-90; Belliveau et al., 2012, Mol Ther nucleic Acids, 1: e37; Jayaraman et al., 2012, Angew Chem Int Ed Engl., 51(34): 8529-8533; Mui et al., 2013, Mol Ther Nucleic Acids. 2, e139; Maier et al., 2013, Mol Ther., 21(8): 1570-1578; and Tam et al., 2013, Nanomedicine, 9(5): 665-74, each of which are incorporated by reference in their entirety.

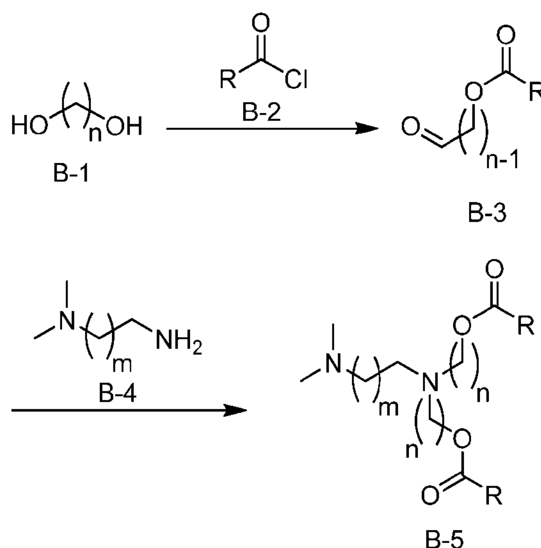
The following Reaction Schemes illustrate methods to make lipids of Formula (I), (II) or (III).

GENERAL REACTION SCHEME 1



Embodiments of the lipid of Formula (I) (e.g., compound A-5) can be prepared according to General Reaction Scheme 1 (“Method A”), wherein R is a
5 saturated or unsaturated C_1-C_{24} alkyl or saturated or unsaturated cycloalkyl, m is 0 or 1 and n is an integer from 1 to 24. Referring to General Reaction Scheme 1, compounds of structure A-1 can be purchased from commercial sources or prepared according to methods familiar to one of ordinary skill in the art. A mixture of A-1, A-2 and DMAP is treated with DCC to give the bromide A-3. A mixture of the bromide A-3, a base (e.g.,
10 N,N-diisopropylethylamine) and the N,N-dimethyldiamine A-4 is heated at a temperature and time sufficient to produce A-5 after any necessarily workup and or purification step.

GENERAL REACTION SCHEME 2

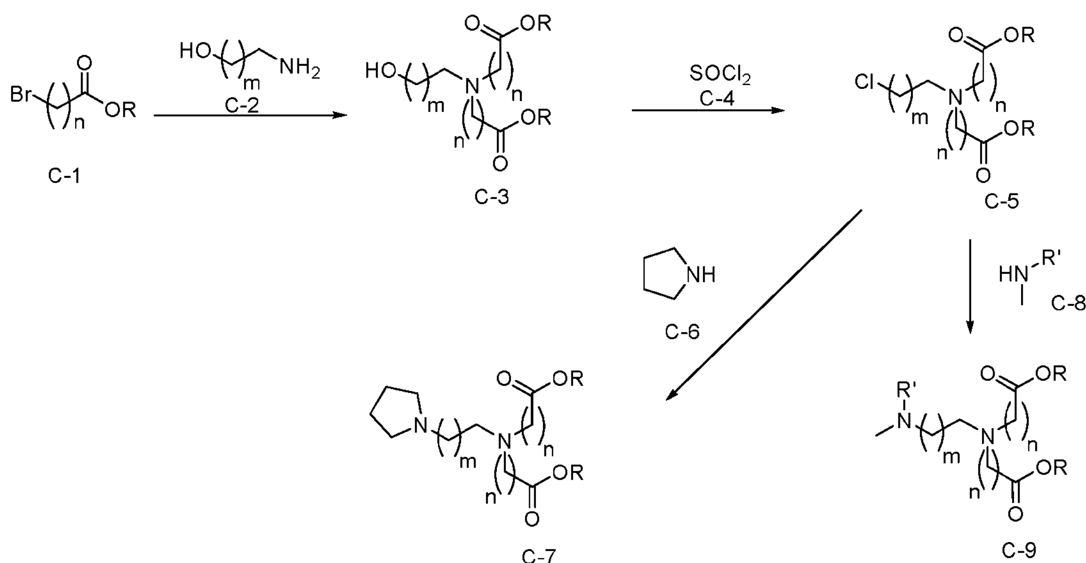


Other embodiments of the compound of Formula (I) (e.g., compound B-5)
15 can be prepared according to General Reaction Scheme 2 (“Method B”), wherein R is a saturated or unsaturated C_1-C_{24} alkyl or saturated or unsaturated cycloalkyl, m is 0 or 1 and n is an integer from 1 to 24. As shown in General Reaction Scheme 2, compounds of structure B-1 can be purchased from commercial sources or prepared according to

methods familiar to one of ordinary skill in the art. A solution of B-1 (1 equivalent) is treated with acid chloride B-2 (1 equivalent) and a base (e.g., triethylamine). The crude product is treated with an oxidizing agent (e.g., pyridinum chlorochromate) and intermediate product B-3 is recovered. A solution of crude B-3, an acid (e.g., acetic acid),
5 and N,N-dimethylaminoamine B-4 is then treated with a reducing agent (e.g., sodium triacetoxyborohydride) to obtain B-5 after any necessary work up and/or purification.

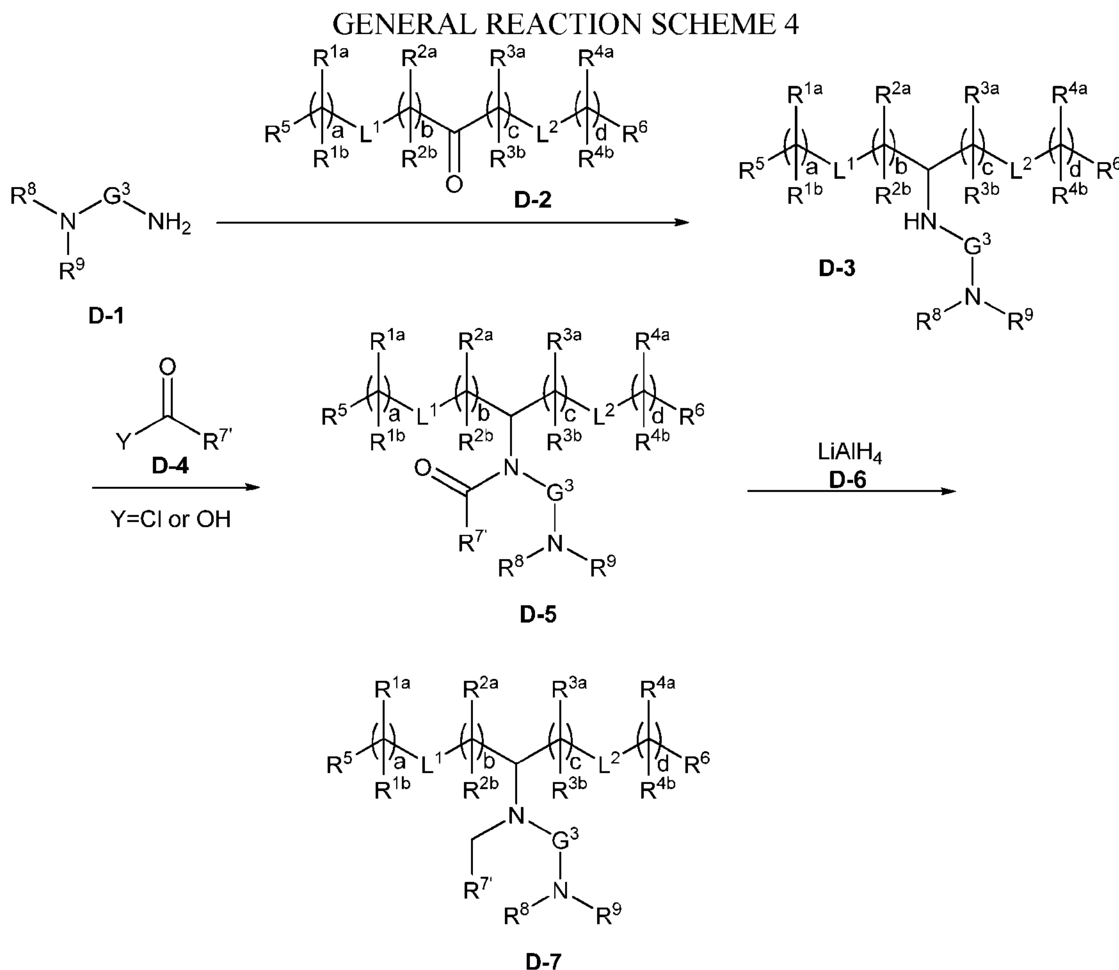
It should be noted that although starting materials A-1 and B-1 are depicted above as including only saturated methylene carbons, starting materials which include carbon-carbon double bonds may also be employed for preparation of compounds
10 which include carbon-carbon double bonds.

GENERAL REACTION SCHEME 3



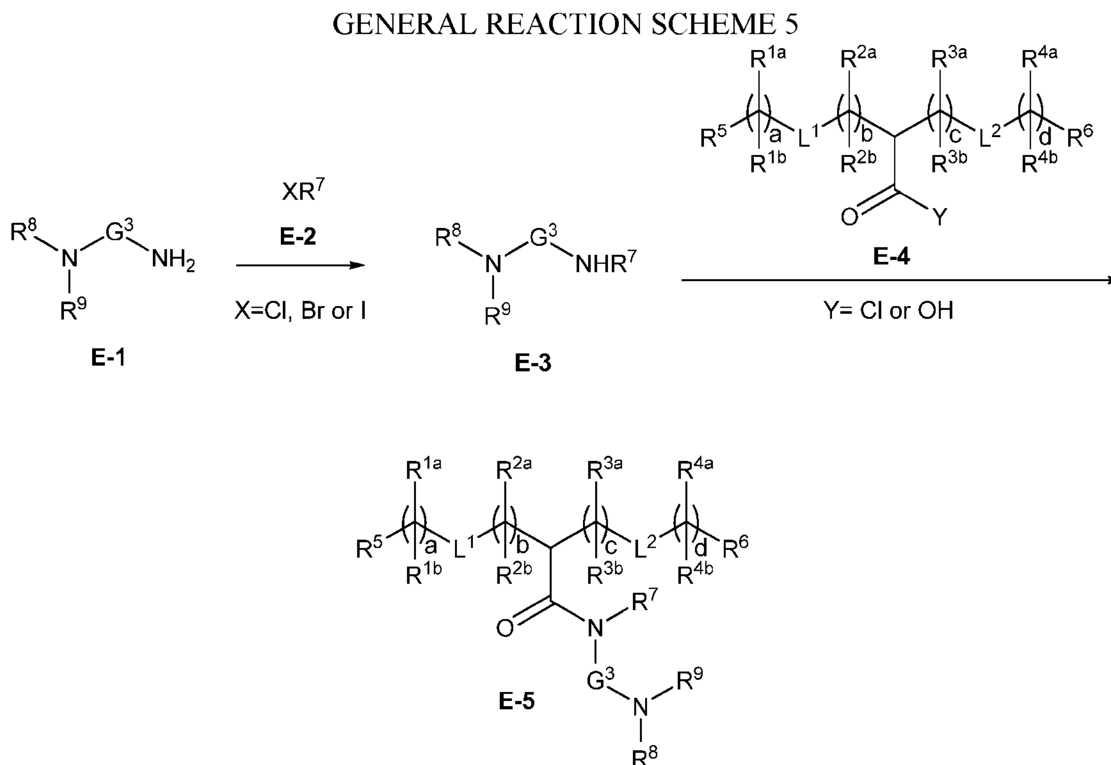
Different embodiments of the lipid of Formula (I) (e.g., compound C-7 or C9) can be prepared according to General Reaction Scheme 3 (“Method C”), wherein R
15 is a saturated or unsaturated C_1-C_{24} alkyl or saturated or unsaturated cycloalkyl, m is 0 or 1 and n is an integer from 1 to 24. Referring to General Reaction Scheme 3, compounds of structure C-1 can be purchased from commercial sources or prepared according to methods familiar to one of ordinary skill in the art.

GENERAL REACTION SCHEME 4



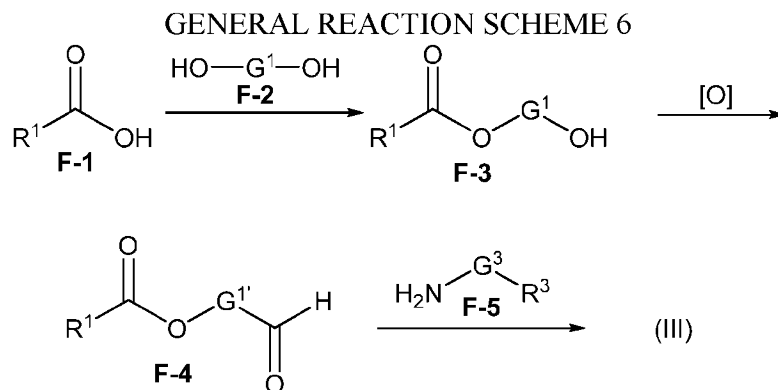
Embodiments of the compound of Formula (II) (e.g., compounds D-5 and D-7) can be prepared according to General Reaction Scheme 4 (“Method D”), wherein R^{1a}, R^{1b}, R^{2a}, R^{2b}, R^{3a}, R^{3b}, R^{4a}, R^{4b}, R^5, R^6, R^8, R^9, L^1, L^2, G^1, G^2, G^3, a, b, c and d are as defined herein, and $R^{7'}$ represents R^7 or a C_3-C_{19} alkyl. Referring to General Reaction Scheme 1, compounds of structure D-1 and D-2 can be purchased from commercial sources or prepared according to methods familiar to one of ordinary skill in the art. A solution of D-1 and D-2 is treated with a reducing agent (e.g., sodium triacetoxyborohydride) to obtain D-3 after any necessary work up. A solution of D-3 and a base (e.g. trimethylamine, DMAP) is treated with acyl chloride D-4 (or carboxylic acid and DCC) to obtain D-5 after any necessary work up and/or purification. D-5 can be reduced with LiAlH4 D-6 to give D-7 after any necessary work up and/or purification.

GENERAL REACTION SCHEME 5



Embodiments of the lipid of Formula (II) (e.g., compound E-5) can be prepared according to General Reaction Scheme 5 ("Method E"), wherein R^{1a}, R^{1b}, R^{2a}, R^{2b}, R^{3a}, R^{3b}, R^{4a}, R^{4b}, R^5, R^6, R^7, R^8, R^9, L^1, L^2, G^3, a, b, c and d are as defined herein. Referring to General Reaction Scheme 2, compounds of structure E-1 and E-2 can be purchased from commercial sources or prepared according to methods familiar to one of ordinary skill in the art. A mixture of E-1 (in excess), E-2 and a base (e.g., potassium carbonate) is heated to obtain E-3 after any necessary work up. A solution of E-3 and a base (e.g. trimethylamine, DMAP) is treated with acyl chloride E-4 (or carboxylic acid and DCC) to obtain E-5 after any necessary work up and/or purification.

GENERAL REACTION SCHEME 6



General Reaction Scheme 6 provides an exemplary method (Method F) for preparation of Lipids of Formula (III). G^1, G^3, R^1 and R^3 in General Reaction Scheme 6 are as defined herein for Formula (III), and G1' refers to a one-carbon shorter homologue of G1. Compounds of structure F-1 are purchased or prepared according to methods known in the art. Reaction of F-1 with diol F-2 under appropriate condensation conditions (e.g., DCC) yields ester/alcohol F-3, which can then be oxidized (e.g., PCC) to aldehyde F-4. Reaction of F-4 with amine F-5 under reductive amination conditions yields a lipid of Formula (III).

It should be noted that various alternative strategies for preparation of lipids of Formula (III) are available to those of ordinary skill in the art. For example, other lipids of Formula (III) wherein L^1 and L^2 are other than ester can be prepared according to analogous methods using the appropriate starting material. Further, General Reaction Scheme 6 depicts preparation of a lipids of Formula (III), wherein G^1 and G^2 are the same; however, this is not a required aspect of the invention and modifications to the above reaction scheme are possible to yield compounds wherein G^1 and G^2 are different.

It will be appreciated by those skilled in the art that in the process described herein the functional groups of intermediate compounds may need to be protected by suitable protecting groups. Such functional groups include hydroxy, amino, mercapto and carboxylic acid. Suitable protecting groups for hydroxy include trialkylsilyl or diarylalkylsilyl (for example, *t*-butyldimethylsilyl, *t*-butyldiphenylsilyl or trimethylsilyl), tetrahydropyranyl, benzyl, and the like. Suitable protecting groups for amino, amidino and guanidino include *t*-butoxycarbonyl, benzyloxycarbonyl, and the

like. Suitable protecting groups for mercapto include -C(O)-R″ (where R″ is alkyl, aryl or arylalkyl), *p*-methoxybenzyl, trityl and the like. Suitable protecting groups for carboxylic acid include alkyl, aryl or arylalkyl esters. Protecting groups may be added or removed in accordance with standard techniques, which are known to one skilled in the art and as described herein. The use of protecting groups is described in detail in Green, T.W. and P.G.M. Wutz, *Protective Groups in Organic Synthesis* (1999), 3rd Ed., Wiley. As one of skill in the art would appreciate, the protecting group may also be a polymer resin such as a Wang resin, Rink resin or a 2-chlorotrityl-chloride resin.

Agents

In some embodiments, the delivery vehicle comprises at least one agent. In some embodiments, the agent is a therapeutic agent. In some embodiments, the therapeutic agent is VE-cadherin, truncated VE-cadherin, N-cadherin, claudin-5, occludin, zonula occludens-1 (ZO-1), or sphingosine-1-phosphate receptor 1 (S1PR1) or a nucleic acid molecule encoding VE-cadherin, truncated VE-cadherin, N-cadherin, claudin-5, occludin, zonula occludens-1 (ZO-1), or sphingosine-1-phosphate receptor 1 (S1PR1).

In some embodiments, the therapeutic agent is an isolated nucleic acid molecule. In certain embodiments, the isolated nucleic acid molecule is a DNA molecule or an RNA molecule. In certain embodiments, the isolated nucleic acid molecule is a cDNA, mRNA, siRNA, shRNA or miRNA molecule. In some embodiments, the isolated nucleic acid molecule encodes a therapeutic peptide such as a cadherin. In some embodiments, the isolated nucleic acid molecule encodes VE-cadherin, truncated VE-cadherin, N-cadherin, claudin-5, occludin, zonula occludens-1 (ZO-1), or sphingosine-1-phosphate receptor 1 (S1PR1).

In some embodiments, the nucleic acid comprises a promoter/regulatory sequence such that the nucleic acid is capable of directing expression of the nucleic acid. Thus, the invention encompasses expression vectors and methods for the introduction of exogenous nucleic acid into cells with concomitant expression of the exogenous nucleic acid in the cells such as those described, for example, in Sambrook et al. (2012, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York),

and in Ausubel et al. (1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York) and as described elsewhere herein.

In order to assess the expression of the mRNA, an expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification of expressing cells from the population of cells sought to be transfected or infected using a the delivery vehicle of the invention. In other embodiments, the selectable marker may be carried on a separate piece of DNA and also be contained within the delivery vehicle. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers are known in the art and include, for example, antibiotic-resistance genes, such as neomycin resistance and the like.

Therefore, in one aspect, the delivery vehicle may contain a vector, comprising the nucleotide sequence or the construct to be delivered. The choice of the vector will depend on the host cell in which it is to be subsequently introduced. In a particular embodiment, the vector of the invention is an expression vector. Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells. In specific embodiments, the expression vector is selected from the group consisting of a viral vector, a bacterial vector and a mammalian cell vector. Prokaryote- and/or eukaryote-vector based systems can be employed for use with the present invention to produce polynucleotides, or their cognate polypeptides. Many such systems are commercially and widely available.

By way of illustration, a vector in which the nucleic acid sequence is introduced can be a plasmid, which is or is not integrated in the genome of a host cell when it is introduced in the cell. Illustrative, non-limiting examples of vectors in which the nucleotide sequence of the invention or the gene construct of the invention can be inserted include a tet-on inducible vector for expression in eukaryote cells.

The vector may be obtained by conventional methods known by persons skilled in the art (Sambrook et al., 2012). In a particular embodiment, the vector is a vector useful for transforming animal cells.

In some embodiments, the recombinant expression vectors may also contain nucleic acid molecules which encode a peptide or peptidomimetic.

A promoter may be one naturally associated with a gene or polynucleotide sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a polynucleotide sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding polynucleotide segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a polynucleotide sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a polynucleotide sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not "naturally occurring," i.e., containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (U.S. Patent 4,683,202, U.S. Patent 5,928,906). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type, organelle, and organism chosen for expression. Those of skill in the art of molecular biology generally know how to use promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook et al. (2012). The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high-level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

The recombinant expression vectors may also contain a selectable marker gene, which facilitates the selection of host cells. Suitable selectable marker genes are

genes encoding proteins such as G418 and hygromycin, which confer resistance to certain drugs, β-galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin. In some embodiments, the immunoglobulin is IgG. The selectable markers may be introduced on a separate vector from the nucleic acid of interest.

In vitro transcribed RNA

In some embodiments, the composition of the invention comprises in vitro transcribed (IVT) RNA. In some embodiments, the composition of the invention comprises in vitro transcribed (IVT) RNA encoding a therapeutic protein. In some embodiments, the composition of the invention comprises IVT RNA encoding a plurality of therapeutic proteins. In some embodiments, the IVT RNA encodes one or more selected from the group consisting of VE-cadherin, truncated VE-cadherin, N-cadherin, claudin-5, occludin, zonula occludens-1 (ZO-1), and sphingosine-1-phosphate receptor 1 (S1PR1).

In some embodiments, an IVT RNA can be introduced to a cell as a form of transient transfection. The RNA is produced by in vitro transcription using a plasmid DNA template generated synthetically. DNA of interest from any source can be directly converted by PCR into a template for in vitro mRNA synthesis using appropriate primers and RNA polymerase. The source of the DNA can be, for example, genomic DNA, plasmid DNA, phage DNA, cDNA, synthetic DNA sequence or any other appropriate source of DNA. In some embodiments, the desired template for in vitro transcription is a therapeutic protein, as described elsewhere herein.

In some embodiments, the DNA to be used for PCR contains an open reading frame. The DNA can be from a naturally occurring DNA sequence from the genome of an organism. In some embodiments, the DNA is a full-length gene of interest of a portion of a gene. The gene can include some or all of the 5' and/or 3' untranslated regions (UTRs). The gene can include exons and introns. In some embodiments, the DNA to be used for PCR is a human gene. In another embodiment, the DNA to be used for PCR is a human gene including the 5' and 3' UTRs. In another embodiment, the DNA to be used for PCR is a gene from a pathogenic or commensal organism, including

bacteria, viruses, parasites, and fungi. In another embodiment, the DNA to be used for PCR is from a pathogenic or commensal organism, including bacteria, viruses, parasites, and fungi, including the 5' and 3' UTRs. The DNA can alternatively be an artificial DNA sequence that is not normally expressed in a naturally occurring organism. An exemplary artificial DNA sequence is one that contains portions of genes that are ligated together to form an open reading frame that encodes a fusion protein. The portions of DNA that are ligated together can be from a single organism or from more than one organism.

Genes that can be used as sources of DNA for PCR include genes that encode polypeptides that induce or enhance an adaptive immune response in an organism. In some embodiments, the genes are genes which are useful for a short-term treatment, or where there are safety concerns regarding dosage or the expressed gene.

In various embodiments, a plasmid is used to generate a template for in vitro transcription of RNA which is used for transfection.

Chemical structures with the ability to promote stability and/or translation efficiency may also be used. In some embodiments, the RNA has 5' and 3' UTRs. In some embodiments, the 5' UTR is between zero and 3000 nucleotides in length. The length of 5' and 3' UTR sequences to be added to the coding region can be altered by different methods, including, but not limited to, designing primers for PCR that anneal to different regions of the UTRs. Using this approach, one of ordinary skill in the art can modify the 5' and 3' UTR lengths required to achieve optimal translation efficiency following transfection of the transcribed RNA.

The 5' and 3' UTRs can be the naturally occurring, endogenous 5' and 3' UTRs for the gene of interest. Alternatively, UTR sequences that are not endogenous to the gene of interest can be added by incorporating the UTR sequences into the forward and reverse primers or by any other modifications of the template. The use of UTR sequences that are not endogenous to the gene of interest can be useful for modifying the stability and/or translation efficiency of the RNA. For example, it is known that AU-rich elements in 3' UTR sequences can decrease the stability of RNA. Therefore, 3' UTRs can be selected or designed to increase the stability of the transcribed RNA based on properties of UTRs that are well known in the art.

In some embodiments, the 5' UTR can contain the Kozak sequence of the endogenous gene. Alternatively, when a 5' UTR that is not endogenous to the gene of interest is being added by PCR as described above, a consensus Kozak sequence can be redesigned by adding the 5' UTR sequence. Kozak sequences can increase the efficiency of translation of some RNA transcripts, but does not appear to be required for all RNAs to enable efficient translation. The requirement for Kozak sequences for many RNAs is known in the art. In other embodiments the 5' UTR can be derived from an RNA virus whose RNA genome is stable in cells. In other embodiments various nucleotide analogues can be used in the 3' or 5' UTR to impede exonuclease degradation of the RNA.

To enable synthesis of RNA from a DNA template without the need for gene cloning, a promoter of transcription should be attached to the DNA template upstream of the sequence to be transcribed. When a sequence that functions as a promoter for an RNA polymerase is added to the 5' end of the forward primer, the RNA polymerase promoter becomes incorporated into the PCR product upstream of the open reading frame that is to be transcribed. In some embodiments, the promoter is a T7 RNA polymerase promoter, as described elsewhere herein. Other useful promoters include, but are not limited to, T3 and SP6 RNA polymerase promoters. Consensus nucleotide sequences for T7, T3 and SP6 promoters are known in the art.

In some embodiments, the RNA has both a cap on the 5' end and a 3' poly(A) tail which determine ribosome binding, initiation of translation and stability mRNA in the cell. On a circular DNA template, for instance, plasmid DNA, RNA polymerase produces a long concatameric product which is not suitable for expression in eukaryotic cells. The transcription of plasmid DNA linearized at the end of the 3' UTR results in normal sized RNA which is effective in eukaryotic transfection when it is polyadenylated after transcription.

On a linear DNA template, phage T7 RNA polymerase can extend the 3' end of the transcript beyond the last base of the template (Schenborn and Mierendorf, Nuc Acids Res., 13:6223-36 (1985); Nacheva and Berzal-Herranz, Eur. J. Biochem., 270:1485-65 (2003).

The conventional method of integration of polyA/T stretches into a DNA template is molecular cloning. However, polyA/T sequence integrated into plasmid DNA can cause plasmid instability, which can be ameliorated through the use of recombination incompetent bacterial cells for plasmid propagation.

5 Poly(A) tails of RNAs can be further extended following in vitro transcription with the use of a poly(A) polymerase, such as E. coli polyA polymerase (E-PAP) or yeast polyA polymerase. In some embodiments, increasing the length of a poly(A) tail from 100 nucleotides to between 300 and 400 nucleotides results in about a two-fold increase in the translation efficiency of the RNA. Additionally, the attachment 10 of different chemical groups to the 3' end can increase RNA stability. Such attachment can contain modified/artificial nucleotides, aptamers and other compounds. For example, ATP analogs can be incorporated into the poly(A) tail using poly(A) polymerase. ATP analogs can further increase the stability of the RNA.

5' caps also provide stability to RNA molecules. In some embodiments, 15 RNAs produced by the methods to include a 5' cap1 structure. Such cap1 structure can be generated using Vaccinia capping enzyme and 2'-O-methyltransferase enzymes (CellScript, Madison, WI). Alternatively, 5' cap is provided using techniques known in the art and described herein (Cougot, et al., Trends in Biochem. Sci., 29:436-444 (2001); Stepinski, et al., RNA, 7:1468-95 (2001); Elango, et al., Biochim. Biophys. Res. 20 Commun., 330:958-966 (2005)).

Nucleoside-modified RNA

In some embodiments, the composition of the present invention comprises a nucleoside-modified nucleic acid. In some embodiments, the composition of the 25 invention comprises a nucleoside-modified RNA encoding a therapeutic protein. In some embodiments, the composition of the envention comprises a nucleoside-modified RNA encoding a plurality of therapeutic proteins. In some embodiments, the nucleoside-modified RNA encodes one or more selected from VE-cadherin, truncated VE-cadherin, N-cadherin, claudin-5, occludin, zonula occludens-1 (ZO-1), or sphingosine-1-phosphate 30 receptor 1 (S1PR1).

For example, in some embodiments, the composition comprises a nucleoside-modified RNA. In some embodiments, the composition comprises a nucleoside-modified mRNA. Nucleoside-modified mRNA molecules have particular advantages over non-modified mRNA, including for example, increased stability, low or absent innate immunogenicity, and enhanced translation. Nucleoside-modified mRNA useful in the present invention is further described in U.S. Patent No. 8,278,036, which is incorporated by reference herein in its entirety.

In certain embodiments, nucleoside-modified mRNA does not activate any pathophysiologic pathways, translates very efficiently and almost immediately following delivery, and serve as templates for continuous protein production in vivo lasting for several days (Kariko et al., 2008, Mol Ther 16:1833-1840; Kariko et al., 2012, Mol Ther 20:948-953). The amount of mRNA required to exert a physiological effect is small and that makes it applicable for human therapy.

In certain instances, expressing a protein by delivering the encoding mRNA has many benefits over methods that use protein, plasmid DNA or viral vectors. During mRNA transfection, the coding sequence of the desired protein is the only substance delivered to cells, thus avoiding all the side effects associated with plasmid backbones, viral genes, and viral proteins. More importantly, unlike DNA- and viral-based vectors, the mRNA does not carry the risk of being incorporated into the genome and protein production starts immediately after mRNA delivery. For example, high levels of circulating proteins have been measured within 15 to 30 minutes of in vivo injection of the encoding mRNA. In certain embodiments, using mRNA rather than the protein also has many advantages. Half-lives of proteins in the circulation are often short, thus protein treatment would need frequent dosing, while mRNA provides a template for continuous protein production for several days. Purification of proteins is problematic and they can contain aggregates and other impurities that cause adverse effects (Kromminga and Schellekens, 2005, Ann NY Acad Sci 1050:257-265).

In certain embodiments, the nucleoside-modified RNA comprises the naturally occurring modified-nucleoside pseudouridine. In certain embodiments, inclusion of pseudouridine makes the mRNA more stable, non-immunogenic, and highly translatable (Kariko et al., 2008, Mol Ther 16:1833-1840; Anderson et al., 2010, Nucleic

Acids Res 38:5884-5892; Anderson et al., 2011, Nucleic Acids Research 39:9329-9338; Kariko et al., 2011, Nucleic Acids Research 39:e142; Kariko et al., 2012, Mol Ther 20:948-953; Kariko et al., 2005, Immunity 23:165-175).

It has been demonstrated that the presence of modified nucleosides, including pseudouridines in RNA suppress their innate immunogenicity (Kariko et al., 2005, Immunity 23:165-175). Further, protein-encoding, in vitro-transcribed RNA containing pseudouridine can be translated more efficiently than RNA containing no or other modified nucleosides (Kariko et al., 2008, Mol Ther 16:1833-1840). Subsequently, it is shown that the presence of pseudouridine improves the stability of RNA (Anderson et al., 2011, Nucleic Acids Research 39:9329-9338) and abates both activation of PKR and inhibition of translation (Anderson et al., 2010, Nucleic Acids Res 38:5884-5892). A preparative HPLC purification procedure has been established that was critical to obtain pseudouridine-containing RNA that has superior translational potential and no innate immunogenicity (Kariko et al., 2011, Nucleic Acids Research 39:e142). Administering HPLC-purified, pseudourine-containing RNA coding for erythropoietin into mice and macaques resulted in a significant increase of serum EPO levels (Kariko et al., 2012, Mol Ther 20:948-953), thus confirming that pseudouridine-containing mRNA is suitable for in vivo protein therapy.

The present invention encompasses RNA, oligoribonucleotide, and polyribonucleotide molecules comprising pseudouridine or a modified nucleoside. In certain embodiments, the composition comprises an isolated nucleic acid, wherein the nucleic acid comprises a pseudouridine or a modified nucleoside. In certain embodiments, the composition comprises a vector, comprising an isolated nucleic acid, wherein the nucleic acid comprises a pseudouridine or a modified nucleoside.

In some embodiments, the nucleoside-modified RNA of the invention is IVT RNA, as described elsewhere herein. For example, in certain embodiments, the nucleoside-modified RNA is synthesized by T7 phage RNA polymerase. In another embodiment, the nucleoside-modified mRNA is synthesized by SP6 phage RNA polymerase. In another embodiment, the nucleoside-modified RNA is synthesized by T3 phage RNA polymerase.

In some embodiments, the modified nucleoside is $m^1acp^3\Psi$ (1-methyl-3-(3-amino-3-carboxypropyl) pseudouridine. In another embodiment, the modified nucleoside is $m^1\Psi$ (1-methylpseudouridine). In another embodiment, the modified nucleoside is Ψm (2'-O-methylpseudouridine. In another embodiment, the modified nucleoside is m^5D (5-methyldihydrouridine). In another embodiment, the modified nucleoside is $m^3\Psi$ (3-methylpseudouridine). In another embodiment, the modified nucleoside is a pseudouridine moiety that is not further modified. In another embodiment, the modified nucleoside is a monophosphate, diphosphate, or triphosphate of any of the above pseudouridines. In another embodiment, the modified nucleoside is any other pseudouridine-like nucleoside known in the art.

In another embodiment, the nucleoside that is modified in the nucleoside-modified RNA the present invention is uridine (U). In another embodiment, the modified nucleoside is cytidine (C). In another embodiment, the modified nucleoside is adenosine (A). In another embodiment, the modified nucleoside is guanosine (G).

In another embodiment, the modified nucleoside of the present invention is m^5C (5-methylcytidine). In another embodiment, the modified nucleoside is m^5U (5-methyluridine). In another embodiment, the modified nucleoside is m^6A (N^6-methyladenosine). In another embodiment, the modified nucleoside is s^2U (2-thiouridine). In another embodiment, the modified nucleoside is Ψ (pseudouridine). In another embodiment, the modified nucleoside is Um (2'-O-methyluridine).

In other embodiments, the modified nucleoside is m^1A (1-methyladenosine); m^2A (2-methyladenosine); Am (2'-O-methyladenosine); ms^2m^6A (2-methylthio-N^6-methyladenosine); i^6A (N^6-isopentenyladenosine); ms^2i6A (2-methylthio-N^6isopentenyladenosine); io^6A (N^6-(cis-hydroxyisopentenyl)adenosine); ms^2io^6A (2-methylthio-N^6-(cis-hydroxyisopentenyl) adenosine); g^6A (N^6-glycinylcarbamoyladenosine); t^6A (N^6-threonylcarbamoyladenosine); ms^2t^6A (2-methylthio-N^6-threonyl carbamoyladenosine); m^6t^6A (N^6-methyl-N^6-threonylcarbamoyladenosine); hn^6A(N^6-hydroxynorvalylcarbamoyladenosine); ms^2hn^6A (2-methylthio-N^6-hydroxynorvalyl carbamoyladenosine); Ar(p) (2'-O-ribosyladenosine (phosphate)); I (inosine); m^1I (1-methylinosine); m^1Im (1,2'-O-dimethylinosine); m^3C (3-methylcytidine); Cm (2'-O-methylcytidine); s^2C (2-thiocytidine); ac^4C (N^4-

acetylcytidine); f^{5}C (5-formylcytidine); m^{5}Cm (5,2'-O-dimethylcytidine); ac^{4}Cm (N^{4}-acetyl-2'-O-methylcytidine); k^{2}C (lysidine); m^{1}G (1-methylguanosine); m^{2}G (N^{2}-methylguanosine); m^{7}G (7-methylguanosine); Gm (2'-O-methylguanosine); m$^{2}_{2}$G (N^{2},N^{2}-dimethylguanosine); m^{2}Gm (N^{2},2'-O-dimethylguanosine); m$^{2}_{2}$Gm (N^{2},N^{2},2'-O-trimethylguanosine); Gr(p) (2'-O-ribosylguanosine (phosphate)); yW (wybutosine); o$_{2}$yW (peroxywybutosine); OHyW (hydroxywybutosine); OHyW* (undermodified hydroxywybutosine); imG (wyosine); mimG (methylwyosine); Q (queuosine); oQ (epoxyqueuosine); galQ (galactosyl-queuosine); manQ (mannosyl-queuosine); preQ$_{0}$ (7-cyano-7-deazaguanosine); preQ$_{1}$ (7-aminomethyl-7-deazaguanosine); G^{+} (archaeosine); D (dihydrouridine); m^{5}Um (5,2'-O-dimethyluridine); s^{4}U (4-thiouridine); m^{5}s^{2}U (5-methyl-2-thiouridine); s^{2}Um (2-thio-2'-O-methyluridine); acp^{3}U (3-(3-amino-3-carboxypropyl)uridine); ho^{5}U (5-hydroxyuridine); mo^{5}U (5-methoxyuridine); cmo^{5}U (uridine 5-oxyacetic acid); mcmo^{5}U (uridine 5-oxyacetic acid methyl ester); chm^{5}U (5-(carboxyhydroxymethyl)uridine)); mchm^{5}U (5-(carboxyhydroxymethyl)uridine methyl ester); mcm^{5}U (5-methoxycarbonylmethyluridine); mcm^{5}Um (5-methoxycarbonylmethyl-2'-O-methyluridine); mcm^{5}s^{2}U (5-methoxycarbonylmethyl-2-thiouridine); nm^{5}s^{2}U (5-aminomethyl-2-thiouridine); mnm^{5}U (5-methylaminomethyluridine); mnm^{5}s^{2}U (5-methylaminomethyl-2-thiouridine); mnm^{5}se^{2}U (5-methylaminomethyl-2-selenouridine); ncm^{5}U (5-carbamoylmethyluridine); ncm^{5}Um (5-carbamoylmethyl-2'-O-methyluridine); cmnm^{5}U (5-carboxymethylaminomethyluridine); cmnm^{5}Um (5-carboxymethylaminomethyl-2'-O-methyluridine); cmnm^{5}s^{2}U (5-carboxymethylaminomethyl-2-thiouridine); m$^{6}_{2}$A (N^{6},N^{6}-dimethyladenosine); Im (2'-O-methylinosine); m^{4}C (N^{4}-methylcytidine); m^{4}Cm (N^{4},2'-O-dimethylcytidine); hm^{5}C (5-hydroxymethylcytidine); m^{3}U (3-methyluridine); cm^{5}U (5-carboxymethyluridine); m^{6}Am (N^{6},2'-O-dimethyladenosine); m$^{6}_{2}$Am (N^{6},N^{6},O-2'-trimethyladenosine); m^{2,7}G (N^{2},7-dimethylguanosine); m^{2,2,7}G (N^{2},N^{2},7-trimethylguanosine); m^{3}Um (3,2'-O-dimethyluridine); m^{5}D (5-methyldihydrouridine); f^{5}Cm (5-formyl-2'-O-methylcytidine); m^{1}Gm (1,2'-O-dimethylguanosine); m^{1}Am (1,2'-O-dimethyladenosine); τm^{5}U (5-taurinomethyluridine); τm^{5}s^{2}U (5-taurinomethyl-2-thiouridine)); imG-14 (4-demethylwyosine); imG2 (isowyosine); or ac^{6}A (N^{6}-acetyladenosine).

In another embodiment, a nucleoside-modified RNA of the present invention comprises a combination of 2 or more of the above modifications. In another embodiment, the nucleoside-modified RNA comprises a combination of 3 or more of the above modifications. In another embodiment, the nucleoside-modified RNA comprises a combination of more than 3 of the above modifications.

In another embodiment, between 0.1% and 100% of the residues in the nucleoside-modified of the present invention are modified (e.g. either by the presence of pseudouridine or a modified nucleoside base). In another embodiment, 0.1% of the residues are modified. In another embodiment, the fraction of modified residues is 0.2%. In another embodiment, the fraction is 0.3%. In another embodiment, the fraction is 0.4%. In another embodiment, the fraction is 0.5%. In another embodiment, the fraction is 0.6%. In another embodiment, the fraction is 0.8%. In another embodiment, the fraction is 1%. In another embodiment, the fraction is 1.5%. In another embodiment, the fraction is 2%. In another embodiment, the fraction is 2.5%. In another embodiment, the fraction is 3%. In another embodiment, the fraction is 4%. In another embodiment, the fraction is 5%. In another embodiment, the fraction is 6%. In another embodiment, the fraction is 8%. In another embodiment, the fraction is 10%. In another embodiment, the fraction is 12%. In another embodiment, the fraction is 14%. In another embodiment, the fraction is 16%. In another embodiment, the fraction is 18%. In another embodiment, the fraction is 20%. In another embodiment, the fraction is 25%. In another embodiment, the fraction is 30%. In another embodiment, the fraction is 35%. In another embodiment, the fraction is 40%. In another embodiment, the fraction is 45%. In another embodiment, the fraction is 50%. In another embodiment, the fraction is 60%. In another embodiment, the fraction is 70%. In another embodiment, the fraction is 80%. In another embodiment, the fraction is 90%. In another embodiment, the fraction is 100%.

In another embodiment, the fraction is less than 5%. In another embodiment, the fraction is less than 3%. In another embodiment, the fraction is less than 1%. In another embodiment, the fraction is less than 2%. In another embodiment, the fraction is less than 4%. In another embodiment, the fraction is less than 6%. In another embodiment, the fraction is less than 8%. In another embodiment, the fraction is less than 10%. In another embodiment, the fraction is less than 12%. In another embodiment, the

fraction is less than 15%. In another embodiment, the fraction is less than 20%. In another embodiment, the fraction is less than 30%. In another embodiment, the fraction is less than 40%. In another embodiment, the fraction is less than 50%. In another embodiment, the fraction is less than 60%. In another embodiment, the fraction is less than 70%.

In another embodiment, 0.1% of the residues of a given nucleoside (i.e., uridine, cytidine, guanosine, or adenosine) are modified. In another embodiment, the fraction of the given nucleotide that is modified is 0.2%. In another embodiment, the fraction is 0.3%. In another embodiment, the fraction is 0.4%. In another embodiment, the fraction is 0.5%. In another embodiment, the fraction is 0.6%. In another embodiment, the fraction is 0.8%. In another embodiment, the fraction is 1%. In another embodiment, the fraction is 1.5%. In another embodiment, the fraction is 2%. In another embodiment, the fraction is 2.5%. In another embodiment, the fraction is 3%. In another embodiment, the fraction is 4%. In another embodiment, the fraction is 5%. In another embodiment, the fraction is 6%. In another embodiment, the fraction is 8%. In another embodiment, the fraction is 10%. In another embodiment, the fraction is 12%. In another embodiment, the fraction is 14%. In another embodiment, the fraction is 16%. In another embodiment, the fraction is 18%. In another embodiment, the fraction is 20%. In another embodiment, the fraction is 25%. In another embodiment, the fraction is 30%. In another embodiment, the fraction is 35%. In another embodiment, the fraction is 40%. In another embodiment, the fraction is 45%. In another embodiment, the fraction is 50%. In another embodiment, the fraction is 60%. In another embodiment, the fraction is 70%. In another embodiment, the fraction is 80%. In another embodiment, the fraction is 90%. In another embodiment, the fraction is 100%.

In another embodiment, the fraction of the given nucleotide that is modified is less than 8%. In another embodiment, the fraction is less than 10%. In another embodiment, the fraction is less than 5%. In another embodiment, the fraction is less than 3%. In another embodiment, the fraction is less than 1%. In another embodiment, the fraction is less than 2%. In another embodiment, the fraction is less than 4%. In another embodiment, the fraction is less than 6%. In another embodiment, the fraction is less than 12%. In another embodiment, the fraction is less than 15%. In

another embodiment, the fraction is less than 20%. In another embodiment, the fraction is less than 30%. In another embodiment, the fraction is less than 40%. In another embodiment, the fraction is less than 50%. In another embodiment, the fraction is less than 60%. In another embodiment, the fraction is less than 70%.

5 In another embodiment, a nucleoside-modified RNA of the present invention is translated in the cell more efficiently than an unmodified RNA molecule with the same sequence. In another embodiment, the nucleoside-modified RNA exhibits enhanced ability to be translated by a target cell. In another embodiment, translation is enhanced by a factor of 2-fold relative to its unmodified counterpart. In another 10 embodiment, translation is enhanced by a 3-fold factor. In another embodiment, translation is enhanced by a 5-fold factor. In another embodiment, translation is enhanced by a 7-fold factor. In another embodiment, translation is enhanced by a 10-fold factor. In another embodiment, translation is enhanced by a 15-fold factor. In another embodiment, translation is enhanced by a 20-fold factor. In another embodiment, translation is 15 enhanced by a 50-fold factor. In another embodiment, translation is enhanced by a 100-fold factor. In another embodiment, translation is enhanced by a 200-fold factor. In another embodiment, translation is enhanced by a 500-fold factor. In another embodiment, translation is enhanced by a 1000-fold factor. In another embodiment, translation is enhanced by a 2000-fold factor. In another embodiment, the factor is 10- 20 1000-fold. In another embodiment, the factor is 10-100-fold. In another embodiment, the factor is 10-200-fold. In another embodiment, the factor is 10-300-fold. In another embodiment, the factor is 10-500-fold. In another embodiment, the factor is 20-1000-fold. In another embodiment, the factor is 30-1000-fold. In another embodiment, the factor is 50-1000-fold. In another embodiment, the factor is 100-1000-fold. In another 25 embodiment, the factor is 200-1000-fold. In another embodiment, translation is enhanced by any other significant amount or range of amounts.

Polypeptide therapeutic agents

In other related aspects, the therapeutic agent includes an isolated peptide, 30 or a variant thereof. The variants of the polypeptide therapeutic agents may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-

conserved amino acid residue and such substituted amino acid residue may or may not be one encoded by the genetic code, (ii) one in which there are one or more modified amino acid residues, e.g., residues that are modified by the attachment of substituent groups, (iii) one in which the polypeptide is an alternative splice variant of the polypeptide of the present invention, (iv) fragments of the polypeptides and/or (v) one in which the polypeptide is fused with another polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification (for example, His-tag) or for detection (for example, Sv5 epitope tag). The fragments include polypeptides generated via proteolytic cleavage (including multi-site proteolysis) of an original sequence. Variants may be post-translationally, or chemically modified. Such variants are deemed to be within the scope of those skilled in the art from the teaching herein. In some embodiments, the polypeptide therapeutic agent comprises VE-cadherin, truncated VE-cadherin, N-cadherin, claudin-5, occludin, zonula occludens-1 (ZO-1), or sphingosine-1-phosphate receptor 1 (S1PR1) or a variant thereof.

Combinations

In some embodiments, the composition of the present invention comprises a combination of agents, or a combination of delivery vehicles for delivery of a combination of agents. In some embodiments, the invention relates to compositions and methods for delivery of a combination of a nucleoside modified mRNA molecule encoding VE-cadherin, truncated VE-cadherin, N-cadherin, claudin-5, occludin, zonula occludens-1 (ZO-1), or sphingosine-1-phosphate receptor 1 (S1PR1) and a nucleoside modified mRNA molecule encoding one or more anti-inflammatory cytokine. Exemplary anti-inflammatory cytokines that can be delivered include, but are not limited to, IL-10, IL-1RA, IL-4, IL-6, IL-11, or IL-13.

In certain embodiments, a composition comprising a combination of agents described herein has an additive effect, wherein the overall effect of the combination is approximately equal to the sum of the effects of each individual agent. In other embodiments, a composition comprising a combination of agents described herein has a synergistic effect, wherein the overall effect of the combination is greater than the sum of the effects of each individual agent.

A composition comprising a combination of agents comprises individual agents in any suitable ratio. For example, in some embodiments, the composition comprises a 1:1 ratio of two individual agents. However, the combination is not limited to any particular ratio. Rather any ratio that is shown to be effective is encompassed.

5

Conjugation

In various embodiments of the invention, the delivery vehicle is conjugated to a targeting domain. Exemplary methods of conjugation can include, but are not limited to, covalent bonds, electrostatic interactions, and hydrophobic ("van der
10 Waals") interactions. In some embodiments, the conjugation is a reversible conjugation, such that the delivery vehicle can be disassociated from the targeting domain upon exposure to certain conditions or chemical agents. In another embodiment, the conjugation is an irreversible conjugation, such that under normal conditions the delivery vehicle does not dissociate from the targeting domain.

15 In some embodiments, the conjugation comprises a covalent bond between an activated polymer conjugated lipid and the targeting domain. The term "activated polymer conjugated lipid" refers to a molecule comprising a lipid portion and a polymer portion that has been activated via functionalization of a polymer conjugated lipid with a first coupling group. In some embodiments, the activated polymer conjugated lipid
20 comprises a first coupling group capable of reacting with a second coupling group. In some embodiments, the activated polymer conjugated lipid is an activated pegylated lipid. In some embodiments, the first coupling group is bound to the lipid portion of the pegylated lipid. In another embodiment, the first coupling group is bound to the polyethylene glycol portion of the pegylated lipid. In some embodiments, the second
25 functional group is covalently attached to the targeting domain.

The first coupling group and second coupling group can be any functional groups known to those of skill in the art to together form a covalent bond, for example under mild reaction conditions or physiological conditions. In some embodiments, the first coupling group or second coupling group are selected from the group consisting of
30 maleimides, N-hydroxysuccinimide (NHS) esters, carbodiimides, hydrazide, pentafluorophenyl (PFP) esters, phosphines, hydroxymethyl phosphines, psoralen,

imidoesters, pyridyl disulfide, isocyanates, vinyl sulfones, alpha-haloacetyls, aryl azides, acyl azides, alkyl azides, diazirines, benzophenone, epoxides, carbonates, anhydrides, sulfonyl chlorides, cyclooctyne, aldehydes, and sulfhydryl groups. In some embodiments, the first coupling group or second coupling group is selected from the group consisiting
5 of free amines (–NH_2), free sulfhydryl groups (–SH), free hydroxide groups (–OH), carboxylates, hydrazides, and alkoxyamines. In some embodiments, the first coupling group is a functional group that is reactive toward sulfhydryl groups, such as maleimide, pyridyl disulfide, or a haloacetyl. In some embodiments, the first coupling group is a maleimide.

10 In some embodiments, the second coupling group is a sulfhydryl group. The sulfhydryl group can be installed on the targeting domain using any method known to those of skill in the art. In some embodiments, the sulfhydryl group is present on a free cysteine residue. In some embodiments, the sulfhydryl group is revealed via reduction of a disulfide on the targeting domain, such as through reaction with 2-mercaptoethylamine.
15 In some embodiments, the sulfhydryl group is installed via a chemical reaction, such as the reaction between a free amine and 2-iminothilane or N-succinimidyl S-acetylthioacetate (SATA).

In some embodiments, the polymer conjugated lipid and targeting domain are functionalized with groups used in “click” chemistry. Bioorthogonal “click”
20 chemistry comprises the reaction between a functional group with a 1,3-dipole, such as an azide, a nitrile oxide, a nitrone, an isocyanide, and the link, with an alkene or an alkyne dipolarophiles. Exemplary dipolarophiles include any strained cycloalkenes and cycloalkynes known to those of skill in the art, including, but not limited to, cyclooctynes, dibenzocyclooctynes, monofluorinated cyclcooctynes, difluorinated
25 cyclooctynes, and biarylazacyclooctynone

Targeting Domain

In some embodiments, the composition comprises a targeting domain that directs the delivery vehicle to a site. In some embodiments, the site is a site in need of the
30 agent comprised within the delivery vehicle. The targeting domain may comprise a nucleic acid, peptide, antibody, small molecule, organic molecule, inorganic molecule,

glycan, sugar, hormone, and the like that targets the particle to a site in particular need of the therapeutic agent. In certain embodiments, the particle comprises multivalent targeting, wherein the particle comprises multiple targeting mechanisms described herein. In certain embodiments, the targeting domain of the delivery vehicle specifically binds to a target associated with a site in need of an agent comprised within the delivery vehicle. For example, the targeting domain may be chosen to recognize a ligand that acts as a cell surface marker on target cells associated with a particular disease state. Such a target can be a protein, protein fragment, antigen, or other biomolecule that is associated with the targeted site. In some embodiments, the targeting domain is an affinity ligand which specifically binds to a target. In certain embodiments, the target (e.g. antigen) associated with a site in need of a treatment with an agent. In some embodiments, the targeting domain may be co-polymerized with the composition comprising the delivery vehicle. In some embodiments, the targeting domain may be covalently attached to the composition comprising the delivery vehicle, such as through a chemical reaction between the targeting domain and the composition comprising the delivery vehicle. In some embodiments, the targeting domain is an additive in the delivery vehicle. Targeting domains of the instant invention include, but are not limited to, antibodies, antibody fragments, proteins, peptides, and nucleic acids.

In various embodiments, the targeting domain binds to a cell surface molecule of a vascular endothelial cell. Exemplary cell surface molecules include, but are not limited to, ICAM-1, PECAM-1, VCAM-1, ACE, APP, PV1, P-selectin, E-selectin, and VE-cadherin. In various embodiments, the targeting domain binds to a cell surface molecule of a pulmonary endothelial cell that is upregulated during inflammation or endothelial activation. In various embodiments, the targeting domain binds to PECAM-1 (or CD31).

Peptides

In some embodiments, the targeting domain of the invention comprises a peptide. In certain embodiments, the peptide targeting domain specifically binds to a target of interest.

The peptide of the present invention may be made using chemical methods. For example, peptides can be synthesized by solid phase techniques (Roberge J Y et al (1995) Science 269: 202-204), cleaved from the resin, and purified by preparative high performance liquid chromatography. Automated synthesis may be achieved, for example, using the ABI 431 A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer.

The peptide may alternatively be made by recombinant means or by cleavage from a longer polypeptide. The composition of a peptide may be confirmed by amino acid analysis or sequencing.

The variants of the peptides according to the present invention may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue and such substituted amino acid residue may or may not be one encoded by the genetic code, (ii) one in which there are one or more modified amino acid residues, e.g., residues that are modified by the attachment of substituent groups, (iii) one in which the peptide is an alternative splice variant of the peptide of the present invention, (iv) fragments of the peptides and/or (v) one in which the peptide is fused with another peptide, such as a leader or secretory sequence or a sequence which is employed for purification (for example, His-tag) or for detection (for example, Sv5 epitope tag). The fragments include peptides generated via proteolytic cleavage (including multi-site proteolysis) of an original sequence. Variants may be post-translationally, or chemically modified. Such variants are deemed to be within the scope of those skilled in the art from the teaching herein.

As known in the art the "similarity" between two peptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one peptide to a sequence of a second peptide. Variants are defined to include peptide sequences different from the original sequence. In some embodiments, the variant is different from the original sequence in less than 40% of residues per segment of interest and at the same time sufficiently identical to the original sequence to preserve the functionality of the original sequence. In some embodiments, the variant is different from the original sequence in less than 25% of residues per segment of interest and at the same time sufficiently identical to the original sequence to preserve the functionality of the

original sequence. In some embodiments, the variant is different from the original sequence in less than 10% of residues per segment of interest and at the same time sufficiently identical to the original sequence to preserve the functionality of the original sequence. In some embodiments, the variant is different from the original sequence in just a few residues per segment of interest and at the same time sufficiently identical to the original sequence to preserve the functionality of the original sequence. The present invention includes amino acid sequences that are at least 60%, 65%, 70%, 72%, 74%, 76%, 78%, 80%, 90%, or 95% similar or identical to the original amino acid sequence. The degree of identity between two peptides is determined using computer algorithms and methods that are widely known for the persons skilled in the art. In some embodiments, the identity between two amino acid sequences is determined by using the BLASTP algorithm [BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894, Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990)].

The peptides of the invention can be post-translationally modified. For example, post-translational modifications that fall within the scope of the present invention include signal peptide cleavage, glycosylation, acetylation, isoprenylation, proteolysis, myristoylation, protein folding and proteolytic processing, etc. Some modifications or processing events require introduction of additional biological machinery. For example, processing events, such as signal peptide cleavage and core glycosylation, are examined by adding canine microsomal membranes or Xenopus egg extracts (U.S. Pat. No. 6,103,489) to a standard translation reaction.

The peptides of the invention may include unnatural amino acids formed by post-translational modification or by introducing unnatural amino acids during translation.

Nucleic acids

In some embodiments, the targeting domain of the invention comprises an isolated nucleic acid, including for example a DNA oligonucleotide and a RNA oligonucleotide. In certain embodiments, the nucleic acid targeting domain specifically binds to a target of interest. For example, in some embodiments, the nucleic acid comprises a nucleotide sequence that specifically binds to a target of interest.

The nucleotide sequences of a nucleic acid targeting domain can alternatively comprise sequence variations with respect to the original nucleotide sequences, for example, substitutions, insertions and/or deletions of one or more nucleotides, with the condition that the resulting nucleic acid functions as the original and
5 specifically binds to the target of interest.

In the sense used in this description, a nucleotide sequence is "substantially homologous" to any of the nucleotide sequences describe herein when its nucleotide sequence has a degree of identity with respect to the nucleotide sequence of at least 60%, at least 70%, at least 85%, or at least 95%. Other examples of possible
10 modifications include the insertion of one or more nucleotides in the sequence, the addition of one or more nucleotides in any of the ends of the sequence, or the deletion of one or more nucleotides in any end or inside the sequence. The degree of identity between two polynucleotides is determined using computer algorithms and methods that are widely known for the persons skilled in the art. In some embodiments, the identity
15 between two amino acid sequences is determined by using the BLASTN algorithm [BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894, Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990)].

Antibodies

20 In some embodiments, the targeting domain of the invention comprises an antibody, or antibody fragment. In certain embodiments, the antibody targeting domain specifically binds to a target of interest. Such antibodies include polyclonal antibodies, monoclonal antibodies, Fab and single chain Fv (scFv) fragments thereof, bispecific antibodies, heteroconjugates, human and humanized antibodies.

25 The antibodies may be intact monoclonal or polyclonal antibodies, and immunologically active fragments (e.g., a Fab or (Fab)2 fragment), an antibody heavy chain, an antibody light chain, humanized antibodies, a genetically engineered single chain Fv molecule (Ladner et al, U.S. Pat. No. 4,946,778), or a chimeric antibody, for example, an antibody which contains the binding specificity of a murine antibody, but in
30 which the remaining portions are of human origin. Antibodies including monoclonal and

polyclonal antibodies, fragments and chimeras, may be prepared using methods known to those skilled in the art.

Such antibodies may be produced in a variety of ways, including hybridoma cultures, recombinant expression in bacteria or mammalian cell cultures, and recombinant expression in transgenic animals. The choice of manufacturing methodology depends on several factors including the antibody structure desired, the importance of carbohydrate moieties on the antibodies, ease of culturing and purification, and cost. Many different antibody structures may be generated using standard expression technology, including full-length antibodies, antibody fragments, such as Fab and Fv fragments, as well as chimeric antibodies comprising components from different species. Antibody fragments of small size, such as Fab and Fv fragments, having no effector functions and limited pharmokinetic activity may be generated in a bacterial expression system. Single chain Fv fragments show low immunogenicity.

In some embodiments, the targeting domain of the instant invention is an antibody that specifically binds to endothelial cells lining vascular lumen. Exemplary targets include, but are not limited to, platelet-endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), angiotensin-converting enzyme (ACE), aminopeptidase P (APP), plasmalemma vesicle protein-1 (PV1), P-selectin, E-selectin, VE-cadherin.

In some embodiments, the targeting domain is an antibody which specifically binds to PECAM-1. In some embodiments, the targeting domain is an antibody which specifically binds to ICAM-1. In some embodiments, the targeting domain is an antibody which specifically binds to ACE, APP, PV1, P-selectin, E-selectin, or VE-cadherin.

Exemplary antibodies or antibody fragments that bind to an endothelial cell marker described herein and thus may be used as a targeting domain are well known in the art. An exemplary antibody that binds to PECAM-1 is Ab62 (Centocor). An exemplary antibody that binds to PECAM-1 are those produced from hybridoma clones clone 390. An exemplary antibody that binds to ICAM includes those produced from hybridoma clones of YN1/1.7.4 (ATCC® CRL-1878™). An exemplary antibody that

binds to VCAM includes those produced from hybridoma clones of M/K-2.7 (ATCC® CRL-1909™).

Therapeutic Methods

5 The present invention also provides methods of delivering at least one agent to endothelial cells lining vascular lumen. In certain embodiments, the method is used to treat or prevent a disease or disorder in a subject associated with inflammation in the lungs. Exemplary diseases or disorders include, but are not limited to, acute lung injury (including ARDS), pulmonary edema, asthma, ventilator-induced lung injury,
10 ischemic stroke, traumatic brain injury, viral infections (e.g., viral hemorrhagic fevers, SARS-CoV2, etc.), diabetes mellitus, diabetic kidney disease, acute kidney injury, chronic kidney disease, diabetic macular edema, and proliferative diabetic retinopathy.

It will be appreciated by one of skill in the art, when armed with the present disclosure including the methods detailed herein, that the invention is not limited
15 to treatment of diseases or disorders that are already established. Particularly, the disease or disorder need not have manifested to the point of detriment to the subject; indeed, the disease or disorder need not be detected in a subject before treatment is administered. That is, significant signs or symptoms of diseases or disorders do not have to occur before the present invention may provide benefit. Therefore, the present invention
20 includes a method for preventing diseases or disorders, in that a composition, as discussed previously elsewhere herein, can be administered to a subject prior to the onset of diseases or disorders, thereby preventing diseases or disorders.

One of skill in the art, when armed with the disclosure herein, would appreciate that the prevention of a disease or disorder, encompasses administering to a
25 subject a composition as a preventative measure against the development of, or progression of, a disease or disorder. As more fully discussed elsewhere herein, methods of modulating the level or activity of a gene, or gene product, encompass a wide plethora of techniques for modulating not only the level and activity of polypeptide gene products, but also for modulating expression of a nucleic acid, including either transcription,
30 translation, or both.

The invention encompasses delivery of a delivery vehicle, comprising at least one agent, conjugated to a targeting domain. To practice the methods of the invention; the skilled artisan would understand, based on the disclosure provided herein, how to formulate and administer the appropriate composition to a subject. The present
5 invention is not limited to any particular method of administration or treatment regimen.

One of skill in the art will appreciate that the compositions of the invention can be administered singly or in any combination. Further, the compositions of the invention can be administered singly or in any combination in a temporal sense, in that they may be administered concurrently, or before, and/or after each other. One of
10 ordinary skill in the art will appreciate, based on the disclosure provided herein, that the compositions of the invention can be used to prevent or to treat a disease or disorder, and that a composition can be used alone or in any combination with another composition to affect a therapeutic result. In various embodiments, any of the compositions of the invention described herein can be administered alone or in combination with other
15 modulators of other molecules associated with diseases or disorders.

In some embodiments, the invention includes a method comprising administering a combination of compositions described herein. In certain embodiments, the method has an additive effect, wherein the overall effect of the administering a combination of compositions is approximately equal to the sum of the effects of
20 administering each individual inhibitor. In other embodiments, the method has a synergistic effect, wherein the overall effect of administering a combination of compositions is greater than the sum of the effects of administering each individual composition.

The method comprises administering a combination of composition in any
25 suitable ratio. For example, in some embodiments, the method comprises administering two individual compositions at a 1:1 ratio. However, the method is not limited to any particular ratio. Rather any ratio that is shown to be effective is encompassed.

Pharmaceutical Compositions

30 The formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of

pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with a carrier or one or more other accessory ingredients, and then, if necessary or desirable, shaping or packaging the product into a desired single- or multi-dose unit.

5 Although the description of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for ethical administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to animals of all sorts. Modification of pharmaceutical compositions suitable for administration to humans in 10 order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions of the invention is contemplated include, but are not limited to, humans and other primates, mammals including 15 commercially relevant mammals such as non-human primates, cattle, pigs, horses, sheep, cats, and dogs.

Pharmaceutical compositions that are useful in the methods of the invention may be prepared, packaged, or sold in formulations suitable for ophthalmic, oral, rectal, vaginal, parenteral, topical, pulmonary, intranasal, buccal, intravenous, 20 intracerebroventricular, intradermal, intramuscular, or another route of administration. Other contemplated formulations include projected nanoparticles, liposomal preparations, resealed erythrocytes containing the active ingredient, and immunogenic-based formulations.

A pharmaceutical composition of the invention may be prepared, 25 packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a “unit dose” is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject or a convenient fraction of such a dosage such as, for example, 30 one-half or one-third of such a dosage.

The relative amounts of the active ingredient, the pharmaceutically acceptable carrier, and any additional ingredients in a pharmaceutical composition of the invention will vary, depending upon the identity, size, and condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100% (w/w) active ingredient.

In addition to the active ingredient, a pharmaceutical composition of the invention may further comprise one or more additional pharmaceutically active agents.

Controlled- or sustained-release formulations of a pharmaceutical composition of the invention may be made using conventional technology.

As used herein, "parenteral administration" of a pharmaceutical composition includes any route of administration characterized by physical breaching of a tissue of a subject and administration of the pharmaceutical composition through the breach in the tissue. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, intraocular, intravitreal, subcutaneous, intraperitoneal, intramuscular, intradermal, intrasternal injection, intratumoral, intravenous, intracerebroventricular and kidney dialytic infusion techniques.

Formulations of a pharmaceutical composition suitable for parenteral administration comprise the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules or in multi-dose containers containing a preservative. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing,

or dispersing agents. In some embodiments of a formulation for parenteral administration, the active ingredient is provided in dry (i.e., powder or granular) form for reconstitution with a suitable vehicle (e.g., sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition.

5 The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations may be 10 prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form, in a 15 liposomal preparation, or as a component of a biodegradable polymer systems. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

A pharmaceutical composition of the invention may be prepared, 20 packaged, or sold in a formulation suitable for pulmonary administration via the buccal cavity. Such a formulation may comprise dry particles which comprise the active ingredient and which have a diameter in the range from about 0.5 to about 7 nanometers. In some embodiments, dry particles which comprise the active ingredient have a diameter in the range from about 1 to about 6 nanometers. Such compositions are conveniently in 25 the form of dry powders for administration using a device comprising a dry powder reservoir to which a stream of propellant may be directed to disperse the powder or using a self-propelling solvent/powder-dispensing container such as a device comprising the active ingredient dissolved or suspended in a low-boiling propellant in a sealed container. In some embodiments, such powders comprise particles wherein at least 98% of the 30 particles by weight have a diameter greater than 0.5 nanometers and at least 95% of the particles by number have a diameter less than 7 nanometers. In some embodiments, at

least 95% of the particles by weight have a diameter greater than 1 nanometer and at least 90% of the particles by number have a diameter less than 6 nanometers. In some embodiments, dry powder compositions include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form.

5 Low boiling propellants generally include liquid propellants having a boiling point of below 65°F at atmospheric pressure. Generally the propellant may constitute 50 to 99.9% (w/w) of the composition, and the active ingredient may constitute 0.1 to 20% (w/w) of the composition. The propellant may further comprise additional ingredients such as a liquid non-ionic or solid anionic surfactant or a solid diluent. In 10 some embodiments, any additional ingredients have a particle size of the same order as particles comprising the active ingredient.

Formulations of a pharmaceutical composition suitable for parenteral administration comprise the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may 15 be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules or in multi-dose containers containing a preservative. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable 20 sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In some embodiments of a formulation for parenteral administration, the active ingredient is provided in dry (i.e., powder or granular) form for reconstitution with a suitable vehicle (e.g., sterile pyrogen-free water) prior to parenteral 25 administration of the reconstituted composition.

The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, 30 or suspending agents described herein. Such sterile injectable formulations may be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or

1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations that are useful include those that comprise the active ingredient in microcrystalline form, in a
5 liposomal preparation, or as a component of a biodegradable polymer system. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

As used herein, "additional ingredients" include, but are not limited to,
10 one or more of the following: excipients; surface active agents; dispersing agents; inert diluents; granulating and disintegrating agents; binding agents; lubricating agents; sweetening agents; flavoring agents; coloring agents; preservatives; physiologically degradable compositions such as gelatin; aqueous vehicles and solvents; oily vehicles and solvents; suspending agents; dispersing or wetting agents; emulsifying agents,
15 demulcents; buffers; salts; thickening agents; fillers; emulsifying agents; antioxidants; antibiotics; antifungal agents; stabilizing agents; and pharmaceutically acceptable polymeric or hydrophobic materials. Other "additional ingredients" which may be included in the pharmaceutical compositions of the invention are known in the art and described, for example in Remington's Pharmaceutical Sciences (1985, Genaro, ed.,
20 Mack Publishing Co., Easton, PA), which is incorporated herein by reference.

EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only,
25 and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Without further description, it is believed that one of ordinary skill in the
30 art can, using the preceding description and the following illustrative examples, make and utilize the present invention and practice the claimed methods. The following working

examples therefore are not to be construed as limiting in any way the remainder of the disclosure.

Example 1: VE-Cadherin mRNA Administration by mRNA-LNPs Targeting Endothelial Cells Ameliorates Alveolar Capillary Leak

PECAM mRNA-LNPs robustly drive endothelial expression of the encoded protein. PECAM-mRNA-LNPs encoding VE-cadherin were administered to 293T cells and protein expression examined. Western blotting of cell extract demonstrated successful expression of VE-cadherin in cells treated with PECAM-mRNA-LNPs encoding VE-cadherin but not in cells treated with LNPs encoding luciferase or control cells (Figure 1A). To examine the ability of PECAM-mRNA-LNPs encoding VE-cadherin to enhance endothelial barrier function, human umbilical vein endothelial cells (HUVECs) were plated and treated with lipopolysaccharide (LPS) to disrupt the endothelial barrier, similar to what happens in sepsis-related pneumonia. The HUVECs were then treated with PECAM-mRNA-LNPs encoding luciferase, untargeted mRNA-LNPs encoding VE-cadherin, or PECAM-mRNA-LNPs encoding VE-cadherin or left untreated as a control. Transendothelial impedance (a measure of barrier function) was measured using an xCELLigence Real-Time Cell Analysis device (Figure 1B). Endothelial cells treated with PECAM-mRNA-LNPs encoding VE-cadherin had a faster and more complete return of barrier function in response to LPS compared to all three alternative samples.

In-vivo reduction of endothelial leakage by targeted delivery of VE-cadherin mRNA

To examine the ability of targeted mRNA-LNPs encoding VE-cadherin to reduce endothelial leakage, male and female mice were injected with 10 μg/mouse of CD31-mRNA-LNPs encoding VE-cadherin. After 24 hours, mice were anesthetized and maintained at 37 °C on a mounting stage, where ear fur was removed with clippers and Nair™ hair remover and the ears cleaned with Dulbecco's phosphate-buffered saline (DPBS). Mice were then given 150 kDa dextran-FITC in 100 μL DPBS via IV, with male mice being given 1.5× more dextran-FITC to accommodate for an approximately 50% greater body weight. Histamine dihydrochloride was then transcutaneously applied to the

ventral surface of the ear by injection, after which the ears were examined by widefield fluorescent imaging every minute for 10 minutes. A noticeable decrease in leakage of dextran-FITC was observed in the ears of mice that had previously been treated with mRNA-LNPs, demonstrating their ability to protect against endothelial leakage (Figure 5 2).

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific embodiments, it is 10 apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. A composition comprising a delivery vehicle conjugated to a targeting domain, wherein the delivery vehicle comprises or encapsulates an mRNA molecule encoding vascular endothelial (VE)-cadherin, truncated VE-cadherin, neural (N)-cadherin, claudin-5, occludin, zonula occludens-1 (ZO-1), or sphingosine-1-phosphate receptor 1 (S1PR1), and further wherein the targeting domain specifically binds to cell surface molecule on an endothelial cell of the pulmonary system, wherein the cell surface molecule is selected from the group consisting of platelet-endothelial cell adhesion molecule 1 (PECAM-1), intercellular adhesion molecule 1 (ICAM-1), angiotensin-converting enzyme (ACE), plasmalemma vesicle-associated protein 1 (PV1), P-selectin, E-selectin, and VE-cadherin.

2. The composition of claim 1, wherein the delivery vehicle is selected from the group consisting of a lipid nanoparticle, a liposome, and a micelle.

3. The composition of claim 2, wherein the delivery vehicle is a lipid nanoparticle.

4. The composition of claim 3, wherein the mRNA molecule encoding VE-cadherin, truncated VE-cadherin, N-cadherin, claudin-5, occludin, zonula occludens-1 (ZO-1), or sphingosine-1-phosphate receptor 1 (S1PR1) is encapsulated in the lipid nanoparticle.

5. The composition of claim 3, wherein the mRNA molecule encoding VE-cadherin, truncated VE-cadherin, N-cadherin, claudin-5, occludin, zonula occludens-1 (ZO-1), or sphingosine-1-phosphate receptor 1 (S1PR1) is a nucleoside modified mRNA molecule.

6. The composition of claim 1, wherein the targeting domain specifically binds to platelet-endothelial cell adhesion molecule-1 (PECAM-1).

7. The composition of claim 1, comprising a lipid nanoparticle comprising a nucleoside modified mRNA molecule encoding VE-cadherin, wherein the lipid nanoparticle is conjugated to a PECAM-1 targeting domain.

8. The composition of claim 7, wherein the PECAM-1 targeting domain comprises an anti-PECAM-1 antibody.

9. A method of treating or preventing a vascular permeability disorder in a subject, the method comprising administering to the subject the composition of claim 7.

10. The method of claim 9, further comprising administering an anti-inflammatory cytokine.

11. The method of claim 9, wherein the vascular permeability disorder is selected from the group consisting of acute lung injury, pulmonary edema, asthma, ventilator-induced lung injury, ischemic stroke, traumatic brain injury, vascular permeability associated with a viral infection, diabetes mellitus, diabetic kidney disease, acute kidney injury, chronic kidney disease, diabetic macular edema, and proliferative diabetic retinopathy.

12. The method of claim 11, wherein the acute lung injury is acute respiratory distress syndrome (ARDS).

13. A method of treating or preventing endothelial dysfunction in a subject in need thereof, the method comprising administering to the subject the composition of claim 7.

14. The method of claim 13, further comprising administering an anti-inflammatory cytokine, or an mRNA molecule encoding an anti-inflammatory cytokine.

15. The method of claim 13, wherein the subject has a vascular permeability disease or disorder selected from the group consisting of acute lung injury, pulmonary edema, asthma, ventilator-induced lung injury, ischemic stroke, traumatic brain injury, viral infection, diabetes mellitus, diabetic kidney disease, acute kidney injury, chronic kidney disease, diabetic macular edema, and proliferative diabetic retinopathy.

16. The method of claim 15, wherein the acute lung injury is ARDS.

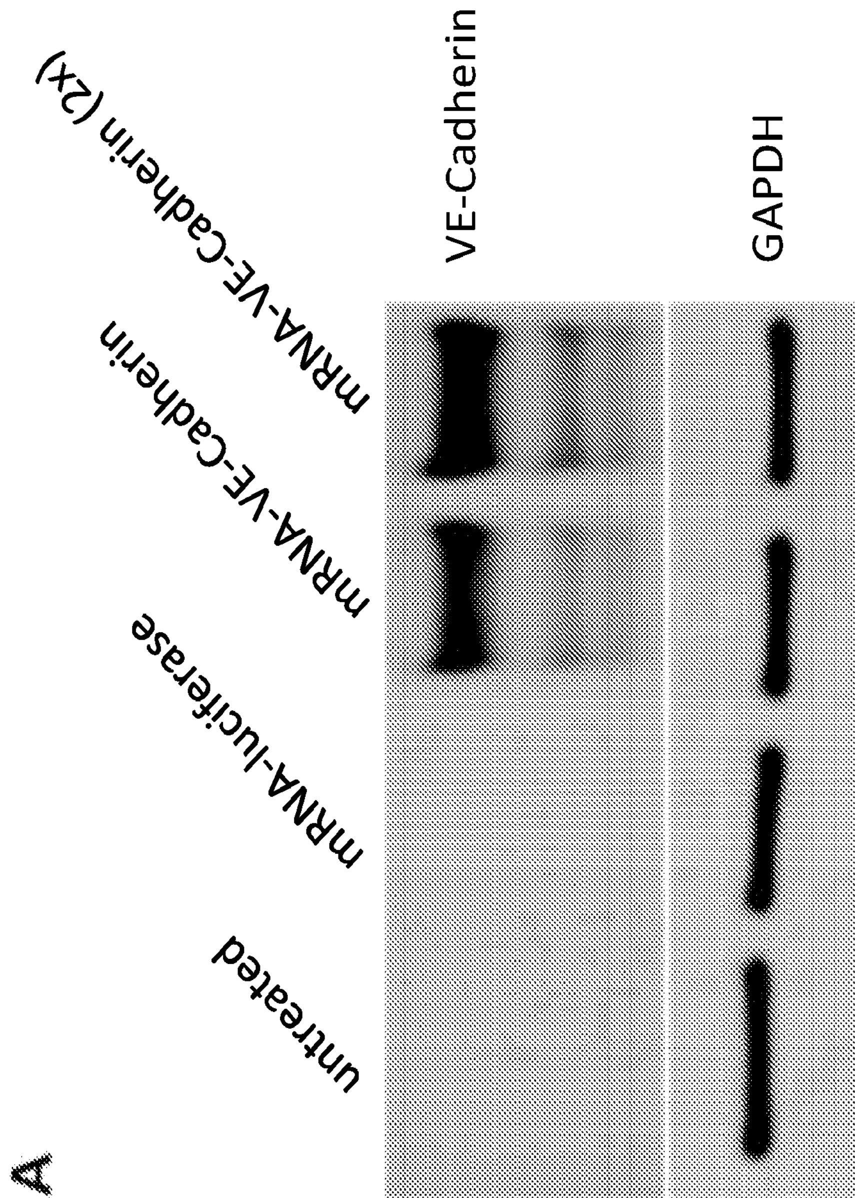


Fig. 1A

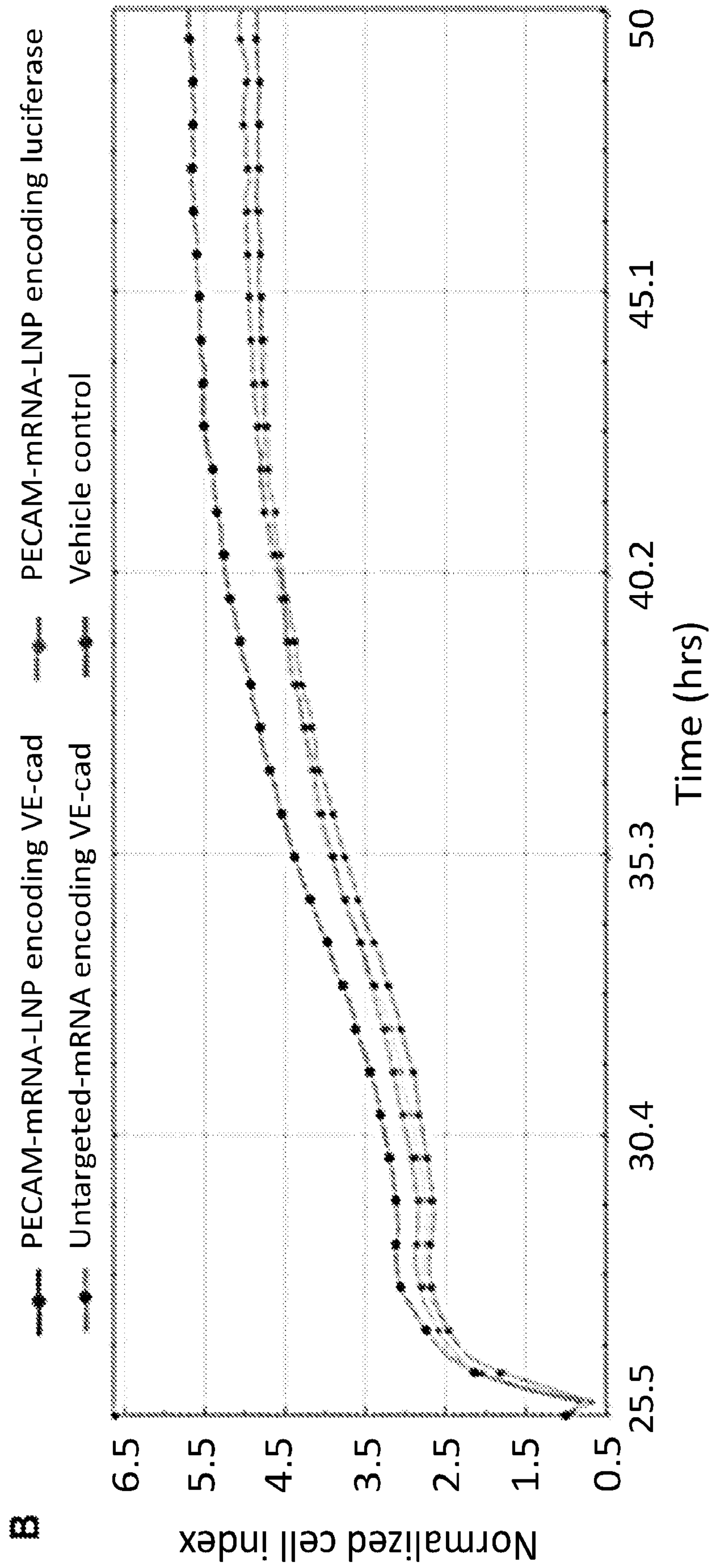


Fig. 1B

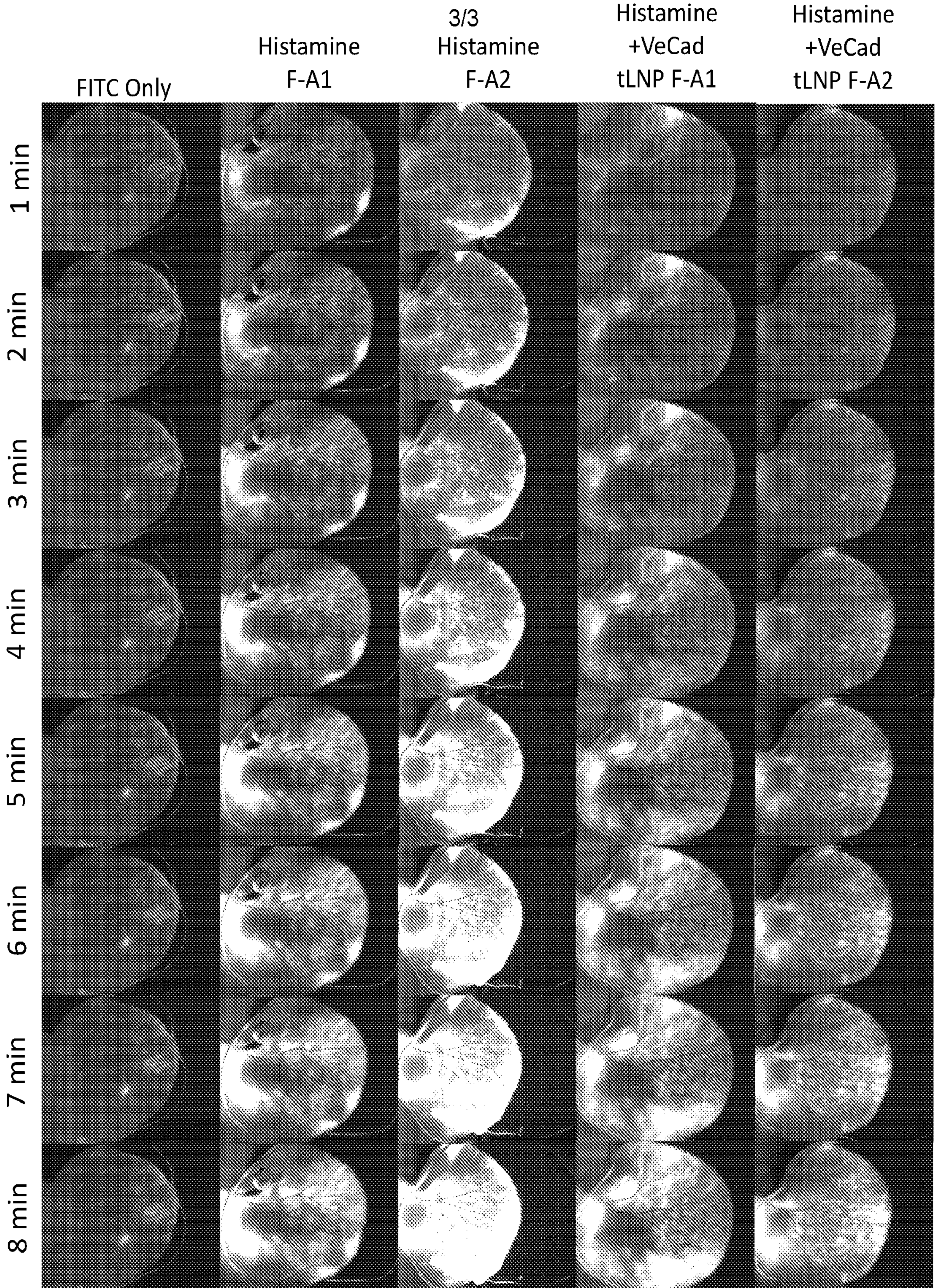


Fig. 2