

**A COMPOUND FOR PREPARING LIPID NANOPARTICLES
ENCAPSULATING AN AGENT, NANOPARTICLE COMPOSITION
COMPRISING SAID COMPOUND AND RELATED METHODS THEREOF**

5

TECHNICAL FIELD

The present disclosure relates broadly to a compound for preparing lipid nanoparticles encapsulating an agent and a method of preparing said compound.

10 The present disclosure also relates to a nanoparticle composition comprising said compound and related methods and uses.

BACKGROUND

15 Lipid nanoparticles are widely used in the delivery of therapeutic, prophylactic and/or biological agents (e.g., polynucleotides such as mRNA). However, a safe, stable and efficacious delivery system remains a challenge. Particularly, there have been reports of adverse health effects and cytotoxicity associated with the use of lipid nanoparticles for delivery.

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Currently, lipid nanoparticles have seen successful use in delivering Moderna's and Pfizer-BioNtech's mRNA Covid-19 vaccines, which have been approved by the United States Food and Drug Administration (US FDA) for human use. Both vaccines utilize SARS-CoV-2 mRNA as the antigen and lipids as the carrier. The lipids consist of 3 different types of lipids (ionizable lipid, PEG-lipid conjugate and helper lipid) and cholesterol. The lipids assemble with the mRNA to form nanoparticles that stimulate the immune cells for prophylactic response against the SARS-CoV-2 virus.

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However, the currently available formulations have several disadvantages and drawbacks, and are far from desirable. Firstly, the production of anti-lipid and

anti-PEG antibodies was evident with such formulations (due to use/presence of PEG-lipid conjugate), which can result in hypersensitivity and anaphylaxis in some subjects. Polyethylene glycol (PEG) has been reported to be a high-risk allergen found hidden in drug/food items. Binding of PEG to basophils through IgE can cause release of compounds that induce allergies and individuals may develop anaphylactic conditions from PEG present in medications. PEG has also been identified as the cause of accelerated blood clearance (ABC) phenomenon. In addition, the presence of anti-PEG antibodies in the body may reduce plasma half-life of mRNA LNPs and their vaccination efficacy.

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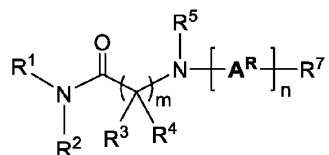
In view of the above, there is a need to address or at least ameliorate the above-mentioned problems. In particular, there is a need to provide a compound and/or nanoparticle composition for a cost efficient, substantially safe and stable, and/or efficacious delivery of therapeutic, prophylactic and/or biological agents.

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SUMMARY

In one aspect, there is provided a compound represented by general formula (1) for preparing lipid nanoparticles encapsulating a therapeutic, prophylactic and/or biological agent:

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(1)

wherein

A^{R} comprises a unit from a poly(amino acid);

R^1 and R^2 are each independently a hydrophobic group;

R^3 , R^4 , and R^5 are each independently H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl;

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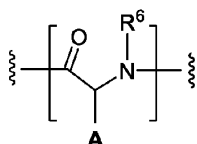
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R^7 is $-H$ or $-C(=O)R^8$, wherein R^8 is optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl or optionally substituted alkoxy;

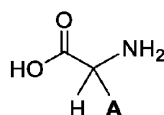
$m \geq 1$; and

5 $n \geq 1$.

In one embodiment, A^R comprises a structure that is represented by general formula (2), and wherein A comprises a hydrophilic organic group that is part of an amino acid represented by general formula (3):



(2)



(3)

10

wherein

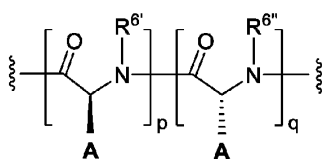
R^6 is H , optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl.

15 In one embodiment, the poly(amino acid) is selected from the group consisting of polyserine, polyglutamic acid and combinations thereof.

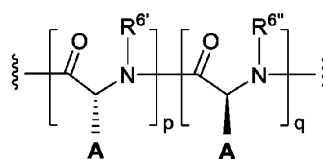
In one embodiment, A is selected from the group consisting of $-\text{CH}_2\text{OH}$, $-\text{CH}_2\text{CH}_2\text{C}(=\text{O})\text{OH}$, and combinations thereof.

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In one embodiment, A^R comprises a structure that is represented by general formula (2A) and/or (2B):



(2A)



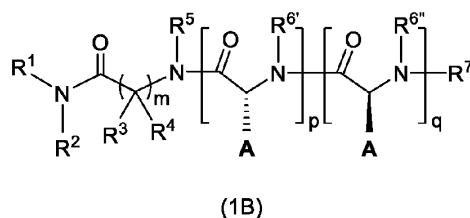
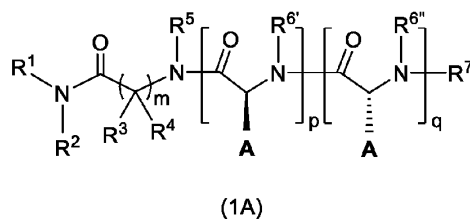
(2B)

wherein

$p + q = n$; and

$R^{6'} = R^{6''} = R^6$.

- 5 In one embodiment, the compound represented by general formula (1) comprises a structure that is represented by general formula (1A) and/or (1B):



wherein

- 10 $p + q = n$; and

$R^{6'} = R^{6''} = R^6$.

In one embodiment, $n \leq 60$.

- 15 In one embodiment, the hydrophobic group at R^1 and R^2 each independently comprises optionally substituted alkyl having at least 8 carbon atoms.

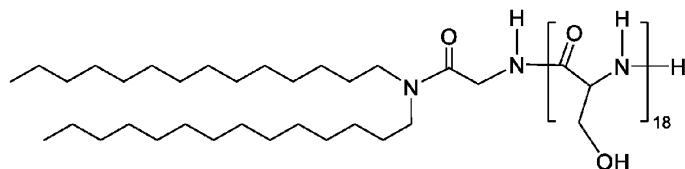
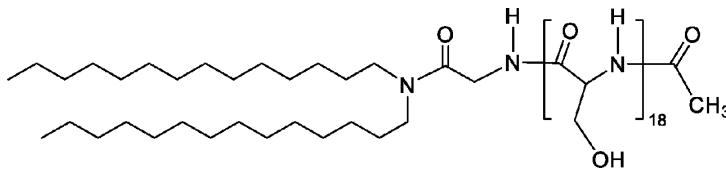
- 20 In one embodiment, the hydrophobic group at R^1 and R^2 each independently comprises optionally substituted alkyl having no more than 18 carbon atoms.

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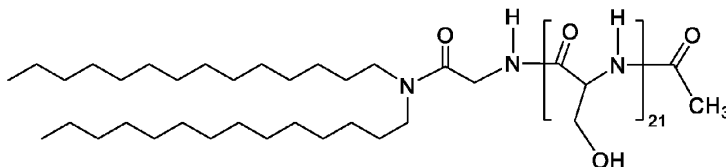
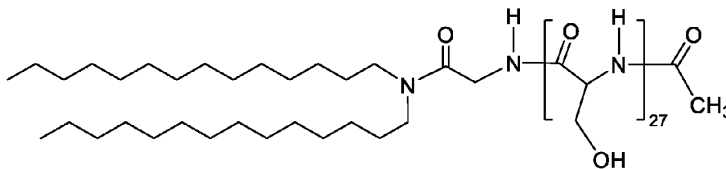
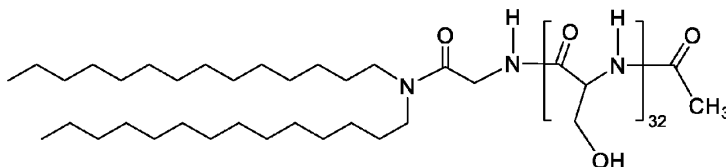
In one embodiment, R^3 , R^4 , R^5 , and R^6 are all H.

In one embodiment, the compound comprises a structure selected from one or more of the following:

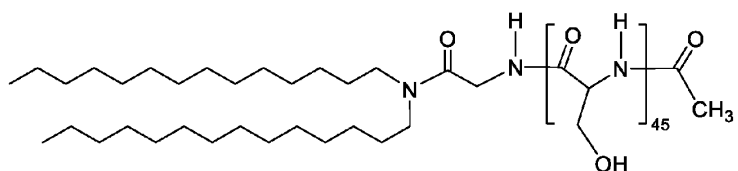
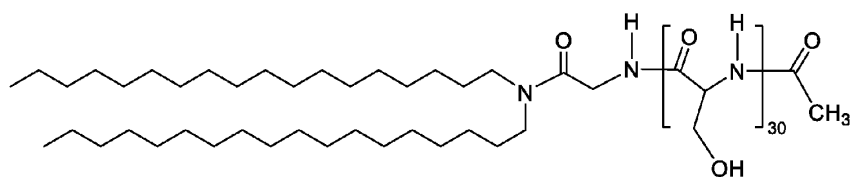
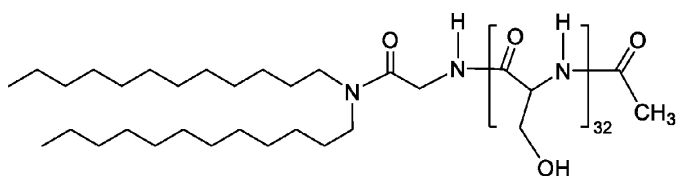
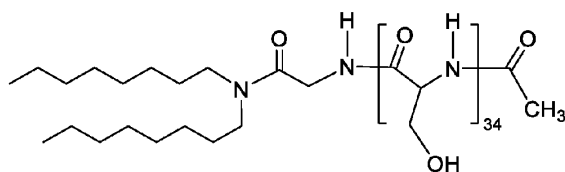
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**C14-pDLS1 (n = 18)****C14-pDLS2 (n = 18)**

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**C14-pDLS3 (n = 21)****C14-pDLS4 (n = 27)****C14-pDLS5 (n = 32)**

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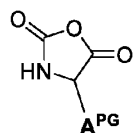
**C14-pDLS6 (n = 45)****C18-pDLS (n = 30)****C12-pDLS (n = 32)****C8-pDLS (n = 34).**

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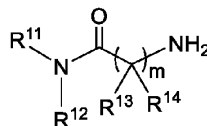
10 In another aspect, there is provided a method of preparing a compound as claimed in any one of the preceding claims, the method comprising:

- (i) polymerizing one or more N-carboxyanhydride (NCA) monomers represented by general formula (5) with a lipid initiator/molecule represented by general formula (6) to obtain a first intermediate compound represented by general formula (7):
- 15

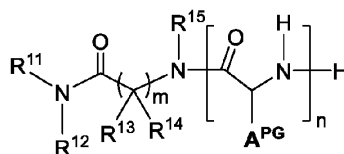
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(5)



(6)



(7)

wherein

A^{PG} represents **A** protected with one or more protecting groups, where **A** comprises a hydrophilic organic group that is part of an amino acid represented by general formula (3);

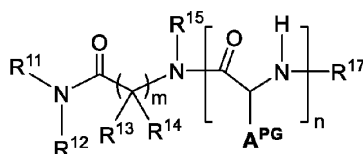
R¹¹ and R¹² are each independently a hydrophobic group;

R¹³, R¹⁴, and R¹⁵ are each independently H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl;

m ≥ 1; and

n ≥ 1;

- (ii) optionally reacting the first intermediate compound represented by general formula (7) with an acylating agent to obtain a second intermediate compound represented by general formula (8):



(8)

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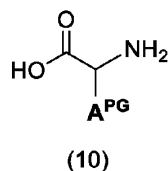
wherein R^{17} is $-H$ or $-C(=O)R^{18}$, wherein R^{18} is optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl or optionally substituted alkoxy;

- 5 (iii) deprotecting the first intermediate compound represented by general formula (7) or the second intermediate compound represented by general formula (8) to obtain the compound represented by general formula (1).

In one embodiment, the method further comprises, prior to step (i):

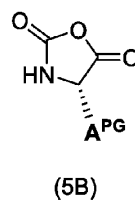
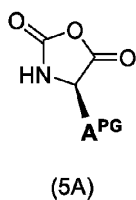
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- (a-i) reacting a protected amino acid represented by general formula (10) with a carbonylating agent to obtain a N-carboxyanhydride (NCA) monomers represented by general formula (5):



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In one embodiment, wherein the N-carboxyanhydride (NCA) monomer represented by general formula (5) comprises a structure represented by general formula (5A) and/or (5B):



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In one embodiment, the step (i) comprises mixing the general formula (5A) with the general formula (5B) at a molar ratio of from 1:5 to 5:1.

In one aspect, there is provided a nanoparticle composition for delivery of a therapeutic, prophylactic and/or biological agent, the nanoparticle composition comprising:

- a compound as disclosed herein; and
- 5 a therapeutic, prophylactic and/or biological agent.

In one embodiment, the composition further comprises:

- (a) ionizable lipid;
- (b) helper lipid; and
- 10 (c) cholesterol and/or derivatives thereof.

In one embodiment, wherein the ionizable lipid, helper lipid, cholesterol and/or derivatives thereof, and compound represented by general formula (1) are mixed at a mole ratio of 5 – 65 : 4 – 20 : 10 – 60 : 0.1 – 20.

- 15
- In one embodiment, the ionizable lipid is selected from ALC-0315, SM-102, Lipid 5, DLinDMA, D-Lin-MC2-DMA, DLin-MC3-DMA, D-Lin-MC4-DMA, Dlin-KC2-DMA, YSK05, AA3-Dlin, SSPalmM, SSPalmO-Phe, Lipid A9, L319, DODMA, CL1, BP Lipid 310, ATX-001, ATX-100, Lipid 2, 80-O16B, BP Lipid 309,
- 20 BP Lipid 307, 93-O17S, 93-O17O, NT1-O14B, 306-O12B-3, 306-O12B, 113-O16B, 306Oi10, 306Oi9-cis2, BAMEA-O16B, AI-28, 113-O12B, 98N12-5, Ckk-E12, OF-02, C12-200, BP Lipid 311, BP Lipid 308, BP Lipid 314, BP Lipid 312, LP01, TCL053, Lipid C24, BP Lipid 315, Lipid 29, 9A1P9, C13-112-tri-tail, C13-113-tri-tail, C13-112-tetra-tail, or C13-113-tetra-tail, C12-200 and combinations
- 25 thereof.

- In one embodiment, the helper lipid is selected from the group consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1-palmitoyl-2-
- 30

oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine,
5 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine,
10 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG), sphingomyelin and combinations thereof.

In one embodiment, the cholesterol and/or derivatives thereof is selected
15 from cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, avenasterol and combinations thereof.

In one embodiment, the nanoparticle composition comprises nanoparticles
having a N/P ratio from 1:1 to 50:1.

20

In one embodiment, the nanoparticle composition comprises nanoparticles
having an average particle size of no more than 300 nm.

In one embodiment, the nanoparticle composition comprises nanoparticles
25 having a zeta potential of from -20 mV to +20 mV.

In another aspect, there is provided a nanoparticle composition as
disclosed herein for use in medicine.

30 In another aspect, there is provided a nanoparticle composition as
disclosed herein for use in the treatment or prophylaxis of a disease, disorder or
condition in a subject in need thereof.

In another aspect, there is provided use of a nanoparticle composition as disclosed herein in the manufacture of a medicament for treatment or prophylaxis of a disease, disorder or condition in a subject in need thereof.

5 In another aspect, there is provided a method of treating or preventing a disease, disorder or condition in a subject in need thereof, the method comprising administering a therapeutically effective amount of the nanoparticle composition as disclosed herein to the subject.

10 In one embodiment, an immune response in the subject is to be induced through the administration of the nanoparticle composition thereto.

In one embodiment, the disease, disorder or condition is mediated by a coronavirus.

15

In one embodiment, the coronavirus is a SARS-CoV-2 coronavirus.

DEFINITIONS

20 The term "particle" as used herein broadly refers to a discrete entity or a discrete body. The particle described herein can include an organic, an inorganic, a composite particle or a biological particle. The particle used described herein may also be a macro-particle that is formed by an aggregate of a plurality of sub-particles or a fragment of a small object. The particle of the present disclosure
25 may be spherical, substantially spherical, or non-spherical, such as irregularly shaped particles or ellipsoidally shaped particles. The term "size" when used to refer to the particle broadly refers to the largest dimension of the particle. For example, the term "size" when used in the context of nanoparticle can refer to the diameter of the nanoparticle although it is not limited as such. In various
30 embodiments, when the particle is substantially spherical, the term "size" can refer to the diameter of the particle; or when the particle is substantially non-spherical, the term "size" can refer to the largest length of the particle.

The term "nano" as used herein is to be interpreted broadly to include dimensions in a nanoscale, *i.e.*, less than about 1000 nm, about 1 nm to less than about 1000 nm, about 1 nm to about 900 nm, about 1 nm to about 800 nm, about 1 nm to about 700 nm, about 1 nm to about 600 nm, about 1 nm to about 500 nm, about 1 nm to about 400 nm, about 1 nm to about 300 nm, about 1 nm to about 200 nm, or from about 1 nm to about 100 nm. Accordingly, the term "nanostructures", "nanoparticles", "nanomaterials" and the like as used herein may include structures that have at least one dimension in the range of no more than said range. The term "nanostructures", "nanoparticles", "nanomaterials" and the like as used herein may include structures that have at least one dimension that is no more than about 1,000 nm, no more than about 950 nm, no more than about 900 nm, no more than about 850 nm, no more than about 800 nm, no more than about 750 nm, no more than about 700 nm, no more than about 650 nm, no more than about 600 nm, no more than about 550 nm, no more than about 500 nm, no more than about 450 nm, no more than about 400 nm, no more than about 350 nm, no more than about 300 nm, no more than about 250 nm, no more than about 200 nm, no more than about 150 nm, no more about 100 nm, no more than about 90 nm, no more than about 80 nm, no more than about 70 nm, no more than about 60 nm, no more than about 50 nm, no more than about 40 nm, no more than about 30 nm, no more than about 20 nm, or no more than about 10 nm.

The term "micro" as used herein is to be interpreted broadly to include dimensions from about 1 micron to about 1000 microns, from about 1 micron to less than about 1000 microns, from about 1 micron to about 900 microns, from about 1 micron to about 800 microns, from about 1 micron to about 700 microns, from about 1 micron to about 600 microns, from about 1 micron to about 500 microns, from about 1 micron to about 400 microns, from about 1 micron to about 300 microns, from about 1 micron to about 200 microns, from about 1 micron to about 100 microns, or from about 1 micron to about 5 microns.

The term "treatment", "treat" and "therapy", and synonyms thereof as used herein refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) a medical condition, which includes but is not limited to diseases, symptoms and disorders.

5 A medical condition also includes a body's response to a disease or disorder, e.g., inflammation. Those in need of such treatment include those already with a medical condition as well as those prone to getting the medical condition or those in whom a medical condition is to be prevented.

10 As used herein, the term "therapeutically effective amount" of a compound is intended to refer to an amount that is sufficient or capable of preventing or at least slowing down (lessening) a medical condition, such as infectious diseases (e.g., dengue disease caused by dengue virus), respiratory illnesses (e.g., coronavirus caused by the SARS-CoV-2 virus or flu caused by influenza virus),
15 cancer, autoimmune diseases and cardiovascular diseases etc. Dosages and administration of compounds, compositions and formulations of the present disclosure may be determined by one of ordinary skill in the art of clinical pharmacology or pharmacokinetics. An effective amount of the active agent of the present disclosure to be employed therapeutically will depend, for example,
20 upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it may be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect.

25 The term "subject" is intended to broadly refer to any animal, such as a mammal, and including humans. Exemplary subjects include but are not limited to humans and non-human primates. The term "subject" as used herein also includes patients and non-patients. The term "patient" refers to individuals suffering or are likely to suffer from a medical condition such as infectious
30 diseases (e.g., coronavirus caused by the SARS-CoV-2 virus, dengue disease caused by dengue virus etc), while "non-patients" refer to individuals not suffering and are likely to not suffer from the medical condition. "Non-patients" include

healthy individuals, non-diseased individuals and/or an individual free from the medical condition. As used herein, the term "mammal" includes vertebrate such as a human or a large veterinary mammal (e.g., horses, cattle, deer, sheep, llamas, goats, pigs).

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The term "bond" refers to a linkage between atoms in a compound or molecule. The bond may be a single bond, a double bond, or a triple bond.

10 In the definitions of a number of substituents below, it is stated that "the group may be a terminal group or a bridging group". This is intended to signify that the use of the term is intended to encompass the situation where the group is a terminal group/moiety as well as the situation where the group is a linker between two other portions of the molecule. Using the term "alkyl" having 1 carbon atom as an example, it will be appreciated that when existing as a terminal
15 group, the term "alkyl" having 1 carbon atom may mean -CH_3 and when existing as a bridging group, the term "alkyl" having 1 carbon atom may mean $\text{-CH}_2\text{-}$ or the like.

The term "alkyl" as a group or part of a group refers to a straight or
20 branched aliphatic hydrocarbon group having 1 to 20 carbon atoms, 1 to 10 carbon atoms, 1 to 6 carbon atoms, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 carbon atoms. Examples of suitable straight and branched alkyl substituents include methyl, ethyl, n-propyl, 2-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, t-butyl, hexyl, amyl, 1,2-dimethylpropyl, 1,1-
25 dimethylpropyl, pentyl, isopentyl, hexyl, 4-methylpentyl, 1-methylpentyl, 2-methylpentyl, 3-methylpentyl, 2,2-dimethylbutyl, 3,3-dimethylbutyl, 1,2-dimethylbutyl, 1,3-dimethylbutyl, 1,2,2-trimethylpropyl, 1,1,2-trimethylpropyl, 2-ethylpentyl, 3-ethylpentyl, heptyl, 1-methylhexyl, 2,2-dimethylpentyl, 3,3-dimethylpentyl, 4,4-dimethylpentyl, 1,2-dimethylpentyl, 1,3-dimethylpentyl, 1,4-
30 dimethylpentyl, 1,2,3-trimethylbutyl, 1,1,2-trimethylbutyl, 1,1,3-trimethylbutyl, 5-methylheptyl, 1-methylheptyl, octyl, nonyl, decyl and the like. The group may be a terminal group or a bridging group.

The term "alkenyl" as a group or part of a group denotes an aliphatic hydrocarbon group containing at least one carbon-carbon double bond and which may be straight or branched having 2 to 20 carbon atoms, 2 to 10 carbon atoms, 2 to 6 carbon atoms, or 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 carbon atoms in the chain. The group may contain a plurality of double bonds and the orientation about each double bond is independently E or Z. Exemplary alkenyl groups include, but are not limited to, ethenyl, vinyl, allyl, 1-methylvinyl, 1-propenyl, 2-propenyl, 2-methyl-1-propenyl, 2-methyl-1-propenyl, 1-butenyl, 2-butenyl, 3-butenyl, 1,3-butadienyl, 1-pentenyl, 2-pentenyl, 3-pentenyl, 4-pentenyl, 1,3-pentadienyl, 2,4-pentadienyl, 1,4-pentadienyl, 3-methyl-2-butenyl, 1-hexenyl, 2-hexenyl, 3-hexenyl, 1,3-hexadienyl, 1,4-hexadienyl, 2-methylpentenyl, 1-heptenyl, 2-heptenyl, 3-heptenyl, 1-octenyl, 2-octenyl, 3-octenyl, 1-nonenyl, 2-nonenyl, 3-nonenyl, 1-decenyl, 2-decenyl, 3-decenyl and the like. The group may be a terminal group or a bridging group.

15

The term "alkynyl" as a group or part of a group denotes an aliphatic hydrocarbon group containing at least one carbon-carbon triple bond and which may be straight or branched having 2 to 20 carbon atoms, 2 to 10 carbon atoms, 2 to 6 carbon atoms, or 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 carbon atoms in the chain. The group may contain a plurality of triple bonds. Exemplary alkynyl groups include, but are not limited to, acetylenyl, propynyl, 1-butyne, 2-butyne, 3-butyne, 1-pentyne, 2-pentyne, 3-methyl-1-butyne, 4-pentyne, 1-hexyne, 2-hexyne, 5-hexyne, 1-heptyne, 2-heptyne, 6-heptyne, 1-octyne, 2-octyne, 7-octyne, 1-nonyne, 2-nonyne, 8-nonyne, 1-decynyl, 2-decynyl, 9-decynyl and the like. The group may be a terminal group or a bridging group.

25

The term "cyclic" as used herein broadly refers to a structure where one or more series of atoms are connected to form at least one ring. The term includes, but is not limited to, both saturated and unsaturated 5-membered and saturated and unsaturated 6-membered rings. Examples of groups having a cyclic structure include, but are not limited to, cyclopentane, cyclopentene,

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cyclohexane, cyclohexene, benzene and the like. The term "cyclic" as used herein includes "heterocyclic".

The term "heterocyclic" as used herein broadly refers to a structure where
5 two or more different kinds of atoms are connected to form at least one ring. For example, a heterocyclic ring may be formed by carbon atoms and at least another atom (i.e. heteroatom) selected from oxygen (O), nitrogen (N) or (NR) and sulfur (S), where R is independently a hydrogen or an organic group. The term also includes, but is not limited to, saturated and unsaturated 5-membered, and
10 saturated and unsaturated 6-membered rings. Examples of groups having a heterocyclic structure include, but are not limited to furan, thiophene, 1H-pyrrole, 2H-pyrrole, 1-pyrroline, 2-pyrroline, 3-pyrroline, 1-pyrazoline, 2-pyrazoline, 3-pyrazoline, 2-imidazoline, 3-imidazoline, 4-imidazoline, pyrazole, imidazole, oxazole, isoxazole, thiazole, isothiazole, 1,2,3-triazole, 1,2,4-triazole, 1,2,3-
15 oxadiazole, disubstituted 1,2,4-oxadiazole, 1,2,5-oxadiazole, 1,3,4-oxadiazole, 1,2,3-thiadiazole, 1,2,4-thiadiazole, 1,2,5-thiadiazole, 1,3,4-thiadiazole, tetrahydrofuran, tetrahydrothiophene, pyrrolidine, 1,3-dioxolane, 1,2-oxathiolane, 1,3-oxathiolane, pyrazolidine, imidazolidine, pyridine, pyridazine, pyrimidine, pyrazine, 1,2-oxazine, 1,3-oxazine, 1,4-oxazine, thiazine, 1,2,3-triazine, 1,2,4-
20 triazine, 1,3,5-triazine, 2H-pyran, 4H-pyran, 2-pyrone, 4-pyrone, 1,4-dioxin, 2H-thiopyran, 4H-thiopyran, tetrahydropyran, thiane, piperidine, 1,4-dioxane, 1,2-dithiane, 1,3-dithiane, 1,4-dithiane, 1,3,5-trithiane, piperazine, morpholine, thiomorpholine and the like.

25 The term "amine group" or the like is intended to broadly refer to a group containing $-NR_2$, where R is independently a hydrogen or an organic group. The group may be a terminal group or a bridging group.

The term "amide group" or the like is intended to broadly refer to a group
30 containing $-C(=O)NR_2$, where R is independently a hydrogen or an organic group. The group may be a terminal group or a bridging group.

The term "aryl" as a group or part of a group denotes (i) an optionally substituted monocyclic, or fused polycyclic, aromatic carbocycle (ring structure having ring atoms that are all carbon) preferably having from 5 to 20, or 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 carbon atoms per ring. Examples of aryl groups include but are not limited to phenyl, tolyl, xylyl, naphthyl, anthracenyl, phenanthrenyl, fluorenyl, indenyl or indanyl and the like.

The term "heteroaryl" as a group or part of a group refers to groups containing an aromatic ring (preferably a 5- or 6- membered aromatic ring) having one or more carbon atoms (for example 1 to 6 carbon atoms) in the ring replaced by a heteroatom. Suitable heteroatoms may include nitrogen (N) or (NH), oxygen (O) and sulfur (S). Examples of heteroaryl include but are not limited to thiophene, benzothiophene, benzofuran, benzimidazole, benzoxazole, benzothiazole, benzisothiazole, naphtha[2,3-b]thiophene, furan, isoindolizine, xantholene, phenoxatine, pyrrole, imidazole, pyrazole, pyridine, pyrazine, pyrimidine, pyridazine, tetrazole, indole, isoindole, 1H-indazole, purine, quinoline, isoquinoline, phthalazine, naphthyridine, quinoxaline, cinnoline, carbazole, phenantridine, acridine, phenazine, thiazole, isothiazole, phenothiazine, oxazole, isooxazole, furazane, phenoxazine, 2-, 3- or 4-pyridyl, 2-, 3-, 4-, 5-, or 8-quinolyl, 1-, 3-, 4-, or 5-isoquinolyl 1-, 2-, or 3-indolyl, and 2-, or 3-thienyl and the like. The group may be a terminal group or a bridging group.

The term "halogen" represents chlorine, fluorine, bromine or iodine. The term "halide" represents chloride, fluoride, bromide or iodide.

25

The term "optionally substituted," when used to describe a chemical structure or moiety, refers to the chemical structure or moiety wherein one or more of its hydrogen atoms is optionally substituted with a chemical moiety or functional group such as alcohol, alkoxy, alkanoyloxy, alkoxycarbonyl, alkenyl, alkyl (e.g., methyl, ethyl, propyl, t-butyl), alkynyl, alkylcarbonyloxy (–OC(O)alkyl), amide (–C(O)NH–alkyl– or –alkylNHC(O)alkyl), amine (such as alkylamino, arylamino, arylalkylamino), aryl, aryloxy, azo, carbamoyl

30

(-NHC(O)O-alkyl- or -OC(O)NH-alkyl), carbamyl (e.g., CONH₂, as well as CONH-alkyl, CONH-aryl, and CONH-arylalkyl), carboxyl, carboxylic acid, cyano, ester, ether (e.g., methoxy, ethoxy), halo, haloalkyl (e.g., -CCl₃, -CF₃, -C(CF₃)₃), heteroalkyl, isocyanate, isothiocyanate, nitrile, nitro, phosphodiester, sulfide, sulfonamido (e.g., SO₂NH₂), sulfone, sulfonyl (including alkylsulfonyl, arylsulfonyl and arylalkylsulfonyl), sulfoxide, thiol (e.g., sulfhydryl, thioether) or urea (-NHCONH-alkyl-).

The terms "coupled" or "connected" as used in this description are intended to cover both directly connected or connected through one or more intermediate means, unless otherwise stated.

The term "associated with", used herein when referring to two elements refers to a broad relationship between the two elements. The relationship includes, but is not limited to a physical, a chemical or a biological relationship. For example, when element A is associated with element B, elements A and B may be directly or indirectly attached to each other or element A may contain element B or vice versa.

The term "adjacent" used herein when referring to two elements refers to one element being in close proximity to another element and may be but is not limited to the elements contacting each other or may further include the elements being separated by one or more further elements disposed therebetween.

The term "and/or", e.g., "X and/or Y" is understood to mean either "X and Y" or "X or Y" and should be taken to provide explicit support for both meanings or for either meaning.

Further, in the description herein, the word "substantially" whenever used is understood to include, but not restricted to, "entirely" or "completely" and the like. In addition, terms such as "comprising", "comprise", and the like whenever used, are intended to be non-restricting descriptive language in that they broadly

include elements/components recited after such terms, in addition to other components not explicitly recited. For example, when "comprising" is used, reference to a "one" feature is also intended to be a reference to "at least one" of that feature. Terms such as "consisting", "consist", and the like, may in the appropriate context, be considered as a subset of terms such as "comprising", "comprise", and the like. Therefore, in embodiments disclosed herein using the terms such as "comprising", "comprise", and the like, it will be appreciated that these embodiments provide teaching for corresponding embodiments using terms such as "consisting", "consist", and the like. Further, terms such as "about", "approximately" and the like whenever used, typically means a reasonable variation, for example a variation of +/- 5% of the disclosed value, or a variance of 4% of the disclosed value, or a variance of 3% of the disclosed value, a variance of 2% of the disclosed value or a variance of 1% of the disclosed value.

Furthermore, in the description herein, certain values may be disclosed in a range. The values showing the end points of a range are intended to illustrate a preferred range. Whenever a range has been described, it is intended that the range covers and teaches all possible sub-ranges as well as individual numerical values within that range. That is, the end points of a range should not be interpreted as inflexible limitations. For example, a description of a range of 1% to 5% is intended to have specifically disclosed sub-ranges 1% to 2%, 1% to 3%, 1% to 4%, 2% to 3% etc., as well as individually, values within that range such as 1%, 2%, 3%, 4% and 5%. The intention of the above specific disclosure is applicable to any depth/breadth of a range.

Additionally, when describing some embodiments, the disclosure may have disclosed a method and/or process as a particular sequence of steps. However, unless otherwise required, it will be appreciated that the method or process should not be limited to the particular sequence of steps disclosed. Other sequences of steps may be possible. The particular order of the steps disclosed herein should not be construed as undue limitations. Unless otherwise required, a method and/or process disclosed herein should not be limited to the steps being

carried out in the order written. The sequence of steps may be varied and still remain within the scope of the disclosure.

Furthermore, it will be appreciated that while the present disclosure provides embodiments having one or more of the features/characteristics discussed herein, one or more of these features/characteristics may also be disclaimed in other alternative embodiments and the present disclosure provides support for such disclaimers and these associated alternative embodiments.

It will also be appreciated that where priority is claimed to an earlier application, the full contents of the earlier application is also taken to form part of the present disclosure and may serve as support for embodiments disclosed herein.

DESCRIPTION OF EMBODIMENTS

Exemplary, non-limiting embodiments of a compound for preparing lipid nanoparticles encapsulating an agent, a method of preparing said compound, a nanoparticle composition comprising said compound and related methods/uses thereto are disclosed hereinafter.

COMPOUND

There is provided a compound for preparing lipid nanoparticles. In various embodiments, the compound comprises one or more peptide units/blocks and/or derivative(s) thereof. For example, the compound may comprise one or more oligopeptides, polypeptides/poly(amino acids) and/or derivative(s) thereof. In various embodiments, the total number of peptide units/blocks and/or derivative(s) thereof (or total length of oligopeptides, polypeptides/poly(amino acids) and/or derivative(s) thereof) in the compound is adjustable as desired. Advantageously, in various embodiments, the compound is designed/configured to allow the hydrophilicity/hydrophobicity balance of said compound to be

customizable/tunable by the adjustment of the number of peptide units/blocks and/or derivative(s) thereof (or length of the oligopeptides, polypeptides/poly(amino acids) and/or derivative(s) thereof). In various embodiments, the compound comprises hydrophobic tail(s)/chain(s)/group(s).

5 For example, the compound may comprise one or more linear aliphatic, branched aliphatic and/or cyclic hydrocarbons. In various embodiments, the total length of hydrophobic tail(s)/chain(s)/group(s) in the compound is adjustable as desired. Advantageously, in various embodiments, the compound is also designed/configured to allow the hydrophilicity/ hydrophobicity balance of said

10 compound to be customizable/tunable by the adjustment of the length of the hydrophobic tail(s)/chain(s)/group(s). In various embodiments, the peptide units/blocks and/or derivative(s) thereof is/are hydrophilic. Advantageously, the presence of peptide units/blocks and/or derivatives thereof increases the hydrophilicity of the compound, and consequently solubility of the compound.

15 Even more advantageously, the structure of the compound allows for embodiments of the compound to be used/formulated into nanoparticles in a composition that may be used as an encapsulation/loading agent, delivery vehicle/system and/or transfection vehicle/system. In various embodiments, the design of the compound helps prevent non-specific protein absorption, particle

20 aggregation and controls the size of the nanoparticles formed. In various embodiments, embodiments of the compound help maintain colloidal stability (of the nanoparticles), and facilitate the condensation and encapsulating/loading of molecules/cargoes into the nanoparticle composition. In various embodiments, the compound is designed/configured to allow loading/encapsulation of one or

25 more types of molecules or cargoes. In various embodiments, the compound is also designed/configured to allow the loaded/encapsulated agent to be released from a composition containing said compound and/or subsequently delivered to a desired target (e.g., cell, cytosol, tissue or organ). The molecules/cargoes to be loaded/encapsulated onto/into/within a composition containing the compound

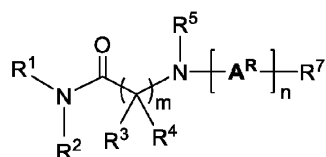
30 may include but is not limited to a therapeutic agent, a prophylactic agent, a biological agent or the like. In various embodiments, the molecules/cargoes to be loaded/encapsulated comprises a nucleic acid. For example, the

molecules/cargoes to be loaded/encapsulated may be a nucleic acid selected from ribonucleic acid (RNA), messenger ribonucleic acid (mRNA), microRNA (miRNA), small interfering ribonucleic acid (siRNA), deoxyribonucleic acid (DNA), plasmid deoxyribonucleic acid (pDNA), oligonucleotides such as antisense oligonucleotide or allele-specific oligonucleotides (ASO) or the like or combinations thereof. In various embodiments, the molecules/cargoes to be loaded/encapsulated comprises therapeutics, e.g., negatively-charged therapeutics. For example, the molecules/cargoes to be loaded/encapsulated may be therapeutics selected from drug molecule, vaccine (e.g., dengue vaccine, Covid-19 vaccine), or the like or combinations thereof. Advantageously, the compound is suitable for use in formulating into nanoparticles for encapsulating and/or delivering one or more therapeutic agent, prophylactic agent and/or biological agent to a desired target (e.g., subject, cell, cytosol, tissue or organ). Advantageously, the compound is suitable for use in a composition/nanoparticle composition for delivering one or more therapeutic agent, prophylactic agent and/or biological agent.

Accordingly, in various embodiments, there is also provided a carrier, nanocarrier or delivery system/vehicle comprising the compound.

20

In various embodiments, the compound comprises a structure that is represented by general formula (1):



(1)

wherein

25 **A^R** comprises a unit from a poly(amino acid)/polypeptide/oligopeptide;

R¹ and R² are each independently a hydrophobic tail/chain/group or contains at least linear aliphatic, branched aliphatic and/or cyclic hydrocarbons;

R^3 , R^4 , and R^5 are each independently H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl;

R^7 is $-H$ or $-C(=O)R^8$, wherein R^8 is optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl or optionally substituted alkoxy;

5 $m \geq 1$; and

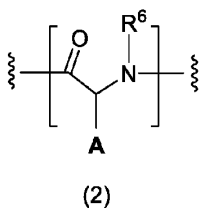
$n \geq 1$.

In various embodiments where $n \geq 2$, A^R may be same or different. For example, when $n = 20$ and there is a total of twenty A^R , each of the twenty A^R
 10 present in the structure represented by general formula (1) may be same or different from each other.

In various embodiments, the compound comprises a polypeptide/poly(amino acid)/oligopeptide and/or derivative(s) thereof, e.g., a
 15 block polypeptide and/or derivative(s) thereof. In various embodiments, the polypeptide/poly(amino acid)/oligopeptide and/or derivative(s) thereof component/part of the compound is represented by $-[A^R]_n-$.

In various embodiments, A^R comprises a repeating unit of a poly(amino acid) or a polypeptide or an oligopeptide. The repeating unit of a poly(amino acid) or a polypeptide or an oligopeptide A^R may be an amino acid residue, which is the remain/leftover of an amino acid when the amino acid reacts to form a peptide, along with removal of a water molecule (H_2O). In various embodiments,
 20 A^R comprises a structure that is represented by general formula (2):

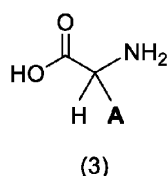
25



wherein

R^6 is H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl.

- 5 In various embodiments, **A** comprises a hydrophilic and/or polar moiety. In various embodiments, **A** comprises a side chain of an amino acid residue. In various embodiments, **A** comprises an organic group that is part of an amino acid represented by general formula (3):



10

In various embodiments, the amino acid comprises an α -amino acid. In various embodiments, α -amino acid comprises amino group ($-NH_2$) and carboxyl group ($-COOH$) that are both bound to the same carbon atom.

- 15 In various embodiments, the amino acid comprises one or more hydrophilic groups. For example, the amino acid may comprise one, two, three, four or five hydrophilic groups. The hydrophilic groups may be $-OH$, $-C(=O)OH$ and/or $-NR^xR^y$, where R^x and R^y are each independently selected from H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted
 20 alkynyl or combinations thereof. It will be appreciated that various hydrophilic and/or polar moieties may be used as **A** in embodiments of **A^R** represented as general formula (2) as long as the hydrophilic and/or polar moiety is capable of imparting hydrophilicity as a form of replacement of PEG.

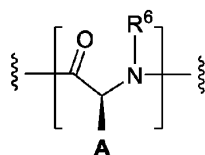
- 25 In various embodiments, the amino acid is selected from the group consisting of serine, threonine, aspartic acid, glutamic acid, asparagine, glutamine, the like, and combinations thereof. In various embodiments, **A** is selected from $-CH_2OH$, $-CH_2CH_2C(=O)OH$ or combinations thereof. In various

embodiments, the poly(amino acid) or polypeptide/oligopeptide is selected from the group consisting of polyserine, polyglutamic acid, the like, and combinations thereof.

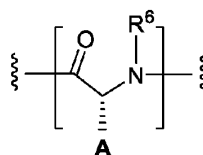
5 Advantageously, the presence of peptide units/blocks and/or derivatives thereof (or oligopeptides, polypeptides/poly(amino acids) and/or derivative(s) thereof) increases the hydrophilicity of the compound, and consequently solubility of the compound. Advantageously, in various embodiments, the presence of peptide units/blocks and/or derivatives thereof (or oligopeptides,
10 polypeptides/poly(amino acids) and/or derivative(s) thereof) in the compound eliminates the requirement of a hydrophilic polyethylene glycol (PEG) which is otherwise necessary in a conventional PEG-lipid conjugate for LNP formulations. In various embodiments, by eliminating the presence of polyethylene glycol (PEG) in the compound, embodiments of the compound and lipid nanoparticles
15 formed therefrom are not and/or avoid the possibility of being shielded by the long PEG chain. In various embodiments, the compound is substantially devoid of polyethylene glycol (PEG). In various embodiments, the compound is substantially devoid of polyethylene glycol (PEG)-modified lipid conjugates, polyethylene glycol (PEG)-modified lipid, PEGylated lipid, PEG-conjugated lipid,
20 PEG-lipid conjugate, and/or lipid modified with PEG. Advantageously, in various embodiments, the design of the structure of the compound allows said compound to be used, in lieu of or as a substitute/replacement for a conventional PEG-lipid conjugate (e.g., ALC-0159).

25 In various embodiments, **A^R**, i.e. the structure/repeating unit represented by general formula (2) may exist in the form of L-enantiomer (e.g., L-amino acid and derivatives thereof) and/or D-enantiomer (e.g., D-amino acid and derivatives thereof):

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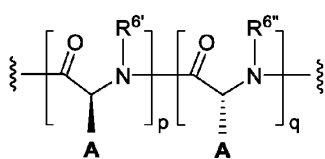
L-enantiomer



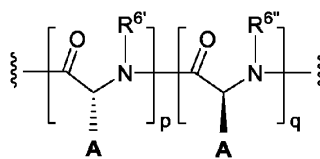
D-enantiomer

In various embodiments, $\mathbf{A}^R$ comprises L-amino acid and/or its derivatives thereof. In various embodiments, $\mathbf{A}^R$ comprises D-amino acid and/or its derivatives thereof. In various embodiments, $\mathbf{A}^R$ comprises a mixture of two enantiomers, namely L-amino acid and/or its derivatives thereof; and D-amino acid and/or its derivatives thereof. In various embodiments, the compound comprises L-enantiomers and/or D-enantiomers that are randomly distributed in/within the structure/repeating unit(s) of the compound.

In various embodiments, the compound comprises a mixture (e.g., racemic mixture of enantiomers) containing both L-enantiomers and D-enantiomers. For example, the compound/poly(amino acid)/polypeptide/oligopeptide may comprise repeating unit(s) or block(s) of both L-enantiomers and D-enantiomers (e.g., poly(D,L-amino acid or poly(L,D-amino acid)). In such embodiments, $\mathbf{A}^R$, i.e. the structure/repeating unit represented by general formula (2) comprises a structure that is represented by general formula (2A) and/or (2B):



(2A)



(2B)

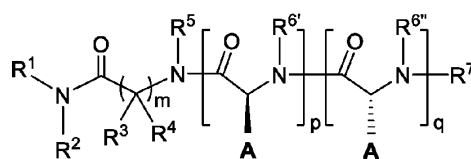
wherein

$$p + q = n;$$

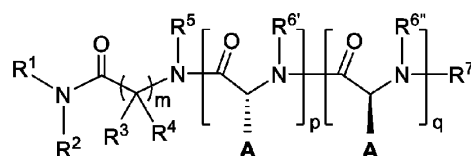
$$R^{6'} = R^{6''} = R^6; \text{ and}$$

A contain one or more features and/or share one or more properties that are similar to those described above (e.g., as defined in general formula (3)).

In various embodiments, the compound represented by general formula
 5 (1) comprises a structure that is represented by general formula (1A) and/or (1B):



(1A)



(1B)

wherein R^1 , R^2 , R^3 , R^4 , R^5 , $R^{6'}$, $R^{6''}$, R^7 , **A**, m , p , q contain one or more
 10 features and/or share one or more properties that are similar to those described above (e.g., as defined in general formula (1) and (3)).

It will be appreciated that, in various embodiments of the compound disclosed herein, L-enantiomers and D-enantiomers are randomly distributed
 15 in/within the structure/repeating unit(s) of the compound. In various embodiments, the compound/poly(amino acid)/polypeptide/oligopeptide is a random copolypeptide or a block copolypeptide.

Advantageously, in various embodiments, the compound is
 20 designed/configured to allow the hydrophilicity/hydrophobicity balance of said compound to be customizable by adjusting the length or hydrophobicity of R^1 and R^2 , and/or adjusting the number of peptide units/blocks and/or derivative(s) thereof (or length of the oligopeptides or polypeptides/poly(amino acids) and/or

derivative(s) thereof), that is, by adjusting/altering/tuning the value of n and/or controlling the degree of polymerization. It will be appreciated that in various embodiments, hydrophilicity of the compound may be increased by increasing the value of n and/or degree of polymerization, and vice versa.

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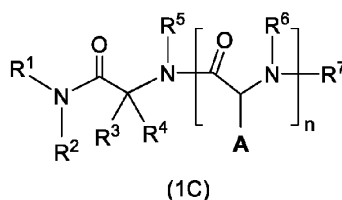
In various embodiments, n is an integer ≥ 1 . In various embodiments, $n \geq 1, n \geq 2, n \geq 3, n \geq 4, n \geq 5, n \geq 6, n \geq 7, n \geq 8, n \geq 9, n \geq 10, n \geq 11, n \geq 12, n \geq 13, n \geq 14, n \geq 15, n \geq 16, n \geq 17, n \geq 18, n \geq 19, n \geq 20, n \geq 21, n \geq 22, n \geq 23, n \geq 24, n \geq 25, n \geq 26, n \geq 27, n \geq 28, n \geq 29, n \geq 30, n \geq 31, n \geq 32,$
10 $n \geq 33, n \geq 34, n \geq 35, n \geq 36, n \geq 37, n \geq 38, n \geq 39, n \geq 40, n \geq 41, n \geq 42,$
 $n \geq 43, n \geq 44, n \geq 45, n \geq 46, n \geq 47, n \geq 48, n \geq 49, n \geq 50, n \geq 51, n \geq 52,$
 $n \geq 53, n \geq 54, n \geq 55, n \geq 56, n \geq 57, n \geq 58, n \geq 59,$ or $n \geq 60$. In some
embodiments, $n \leq 45, n \leq 44, n \leq 43, n \leq 42, n \leq 41, n \leq 40, n \leq 39, n \leq 38, n \leq$
15 $37, n \leq 36, n \leq 35, n \leq 34, n \leq 33$ or $n \leq 32$. For example, $1 \leq n \leq 60, 5 \leq n \leq 60,$
 $1 \leq n \leq 40, 1 \leq n \leq 32, 18 \leq n \leq 40,$ or $18 \leq n \leq 32$. In various embodiments, n is
from about 1 to about 60, from about 5 to about 60, from about 1 to about 40,
from about 1 to about 32, from about 18 to about 40, or from about 18 to about
32. In some embodiments, n is no more about 60, no more than about 50, no
more than about 48, no more about 45, no more than about 44, no more about
20 43, no more than about 42, no more about 41, no more than about 40, no more
about 39, no more than about 38, no more about 37, no more than about 36, no
more about 35, no more than about 34, no more about 33, no more than about
32, no more about 31, or no more than about 30.

25 Advantageously, in various embodiments, the compound is
designed/configured to allow the hydrophilicity/ hydrophobicity balance of said
compound to be customizable/tunable by the adjustment of the length of the
hydrophobic tail(s)/chain(s)/group(s) (e.g., the length of the hydrophobic
tail(s)/chain(s)/group(s) at R^1 and/or R^2). It will be appreciated that in various
30 embodiments, hydrophobicity of the compound may be increased by increasing
the length of hydrophobic tail(s)/chain(s)/group(s) at R^1 and/or R^2 , and vice versa.

In various embodiments, R^1 and R^2 are each independently a hydrophobic tail/chain/group, or contains at least linear aliphatic, branched aliphatic and/or cyclic hydrocarbons. In various embodiments, the hydrophobic tail/chain/group at R^1 and R^2 each independently comprises optionally substituted alkyl. In various
5 embodiments, the hydrophobic tail/chain/group at R^1 and R^2 each independently comprises unsaturated hydrocarbons such as an optionally substituted alkenyl. For example, R^1 and R^2 may contain one or more C=C double bond(s). The alkyl or alkenyl may have at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19,
10 at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 31, at least 32, at least 33, at least 34, at least 35, or at least 36 carbon atoms. For example, R^1 and R^2 may be each independently C_xH_{2x+1} or C_xH_{2x} , where x is an integer ≥ 8 , $x \geq 9$, $x \geq 10$, $x \geq 11$, $x \geq 12$, $x \geq 13$, $x \geq 14$, $x \geq 15$, $x \geq 16$, $x \geq 17$, $x \geq 18$, $x \geq 19$, $x \geq 20$, $x \geq 21$,
15 $x \geq 22$, $x \geq 23$, $x \geq 24$, $x \geq 25$, $x \geq 26$, $x \geq 27$, $x \geq 28$, $x \geq 29$, $x \geq 30$, $x \geq 31$, $x \geq 32$, $x \geq 33$, $x \geq 34$, $x \geq 35$, or $x \geq 36$. In some embodiments, the hydrophobic tail/chain/group at R^1 and R^2 each independently comprises alkyl or alkenyl having no more than 36 carbon atoms, no more than 35 carbon atoms, no more than 34 carbon atoms, no more than 33 carbon atoms, no more than 32 carbon
20 atoms, no more than 31 carbon atoms, no more than 30 carbon atoms, no more than 29 carbon atoms, no more than 28 carbon atoms, no more than 27 carbon atoms, no more than 26 carbon atoms, no more than 25 carbon atoms, no more than 24 carbon atoms, no more than 23 carbon atoms, no more than 22 carbon atoms, no more than 21 carbon atoms, no more than 20 carbon atoms, no more than 19 carbon atoms, or no more than 18 carbon atoms. For example, R^1 and R^2 may be each independently C_xH_{2x+1} or C_xH_{2x} , where $1 \leq x \leq 36$, $5 \leq x \leq 30$, $5 \leq x \leq 20$, or $8 \leq x \leq 18$. Advantageously, in various embodiments, the presence of hydrophobic parts/tails/chains/groups in the compound allows for ease of integration of the compound into the lipid domain of lipid nanoparticles (LNPs),
25 presenting the poly(amino acid)/polypeptide domain on the surface of the LNPs for stability and cell-targeting ability (e.g., immune cells-targeting ability).
30

In various embodiments, m is an integer ≥ 1 . In various embodiments, $m \geq 1$, $m \geq 2$, $m \geq 3$, $m \geq 4$, $m \geq 5$, $m \geq 6$, $m \geq 7$, $m \geq 8$, $m \geq 9$, $m \geq 10$, $m \geq 11$, $m \geq 12$, $m \geq 13$, $m \geq 14$, $m \geq 15$, $m \geq 16$, $m \geq 17$, $m \geq 18$, $m \geq 19$, $m \geq 20$, $m \geq 21$, $m \geq 22$, $m \geq 23$, $m \geq 24$, $m \geq 25$, $m \geq 26$, $m \geq 27$, $m \geq 28$, $m \geq 29$, $m \geq 30$, $m \geq 31$, $m \geq 32$, $m \geq 33$, $m \geq 34$, $m \geq 35$, $m \geq 36$, $m \geq 37$, $m \geq 38$, $m \geq 39$, $m \geq 40$, $m \geq 41$, $m \geq 42$, $m \geq 43$, $m \geq 44$, $m \geq 45$, $m \geq 46$, $m \geq 47$, $m \geq 48$, $m \geq 49$, or $m \geq 50$.

In various embodiments, $m = 1$. In such embodiments, the compound represented by general formula (1) comprises a structure that is represented by
 10 general formula (1C):



In various embodiments, the compound comprises a lipid compound. Accordingly, in various embodiments therefore, the term “compound” may
 15 comprise and/or may be used interchangeably with the terms “lipid polypeptide”, “lipid-polypeptide”, “polypeptide lipid”, “polypeptide-lipid”, “lipid-*block*-polypeptide”, or the like.

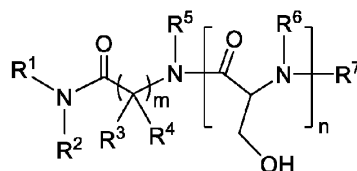
In various embodiments, R^3 , R^4 , R^5 and R^6 are each independently
 20 selected from H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl. In various embodiments, R^3 , R^4 , R^5 and R^6 are each independently optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl. For example, R^3 , R^4 , R^5 , R^6 and R^8 may be selected from methyl, ethyl, *n*-propyl, 2-propyl, isopropyl, *n*-butyl, isobutyl, *sec*-butyl, *t*-
 25 butyl, hexyl, amyl, 1,2-dimethylpropyl, 1,1-dimethylpropyl, pentyl, isopentyl, hexyl, 4-methylpentyl, 1-methylpentyl, 2-methylpentyl, 3-methylpentyl, 2,2-dimethylbutyl, 3,3-dimethylbutyl, 1,2-dimethylbutyl, 1,3-dimethylbutyl, 1,2,2-trimethylpropyl, 1,1,2-trimethylpropyl, 2-ethylpentyl, 3-ethylpentyl, heptyl, 1-

methylohexyl, 2,2-dimethylpentyl, 3,3-dimethylpentyl, 4,4-dimethylpentyl, 1,2-dimethylpentyl, 1,3-dimethylpentyl, 1,4-dimethylpentyl, 1,2,3-trimethylbutyl, 1,1,2-trimethylbutyl, 1,1,3-trimethylbutyl, 5-methylheptyl, 1-methylheptyl, octyl, nonyl, decyl, or the like or combinations thereof. In various embodiments, R³ and
5 R⁴ are H. In various embodiments, R⁵ is H. In various embodiments, R⁶ is H.

In various embodiments, R⁸ is optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl or optionally substituted alkoxy. For example, R⁸ may be selected from methyl, ethyl, n-propyl, 2-propyl, isopropyl,
10 n-butyl, isobutyl, sec-butyl, t-butyl, hexyl, amyl, 1,2-dimethylpropyl, 1,1-dimethylpropyl, pentyl, isopentyl, hexyl, 4-methylpentyl, 1-methylpentyl, 2-methylpentyl, 3-methylpentyl, 2,2-dimethylbutyl, 3,3-dimethylbutyl, 1,2-dimethylbutyl, 1,3-dimethylbutyl, 1,2,2-trimethylpropyl, 1,1,2-trimethylpropyl, 2-ethylpentyl, 3-ethylpentyl, heptyl, 1-methylhexyl, 2,2-dimethylpentyl, 3,3-
15 dimethylpentyl, 4,4-dimethylpentyl, 1,2-dimethylpentyl, 1,3-dimethylpentyl, 1,4-dimethylpentyl, 1,2,3-trimethylbutyl, 1,1,2-trimethylbutyl, 1,1,3-trimethylbutyl, 5-methylheptyl, 1-methylheptyl, octyl, nonyl, decyl, or the like or combinations thereof.

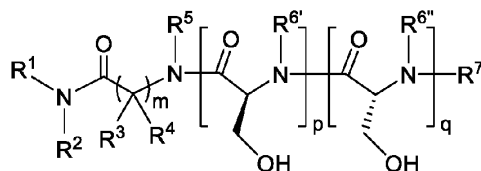
20 In various embodiments, the compound has a number average molecular weight (M_n) of from about 500 g/mol to about 10,000 g/mol, from about 750 g/mol to about 9,750 g/mol, from about 1,000 g/mol to about 9,500 g/mol, from about 1,250 g/mol to about 9,250 g/mol, from about 1,500 g/mol to about 9,000 g/mol, from about 1,750 g/mol to about 8,750 g/mol, from about 2,000 g/mol to about
25 8,500 g/mol, from about 2,250 g/mol to about 8,250 g/mol, from about 2,500 g/mol to about 8,000 g/mol, from about 2,750 g/mol to about 7,750 g/mol, from about 3,000 g/mol to about 7,500 g/mol, from about 3,250 g/mol to about 7,250 g/mol, from about 3,500 g/mol to about 7,000 g/mol, from about 3,750 g/mol to about 6,750 g/mol, from about 4,000 g/mol to about 6,500 g/mol, from about 4,250 g/mol to about
30 6,250 g/mol, from about 4,500 g/mol to about 6,000 g/mol, from about 4,750 g/mol to about 5,750 g/mol, or from about 5,000 g/mol to about 5,500 g/mol, or about 5,250 g/mol.

In various embodiments where the amino acid comprises serine, then **A** is $-\text{CH}_2\text{OH}$ and the compound is represented by general formula (4):

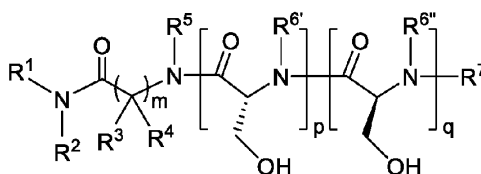


(4)

In various embodiments, the compound represented by general formula (4) may comprise a structure that is represented by general formula (4A) and/or (4B):

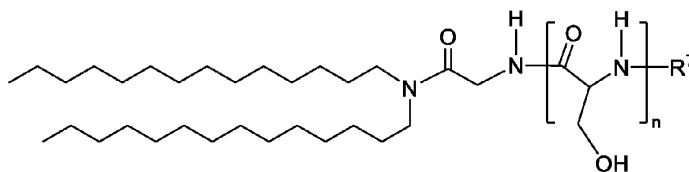


(4A)



(4B)

In various embodiments, there is provided a lipid nanoparticle (LNP) comprising a lipid composition comprising a lipid-poly(serine) represented by general formula (11):



(11)

wherein

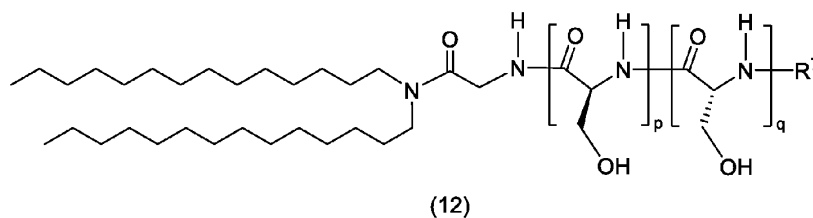
R^7 is H, $C(=O)CH_3$ or $C(=O)CH_2CH_3$;

n is 1 to 100 (e.g., 18, 21, 27, 32, 45); and

nucleic acid (e.g., mRNA) encapsulated in said lipid composition,

- 5 wherein the lipid composition is devoid of PEG.

In various embodiments, the LNP comprises repeating units/blocks of L- and D-enantiomers (i.e. lipid-*block*-poly(D,L-serine)) and is represented by general formula (12):



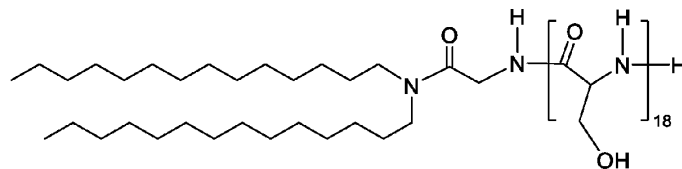
10

wherein

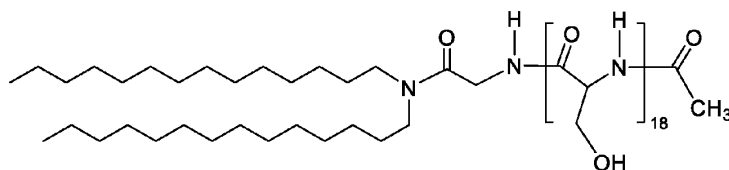
R^7 is H, $C(=O)CH_3$ or $C(=O)CH_2CH_3$;

p + q is 1 to 100 (e.g., 18, 21, 27, 32, 45).

- 15 In various embodiments, the compound comprises a structure selected from one or more of the following:

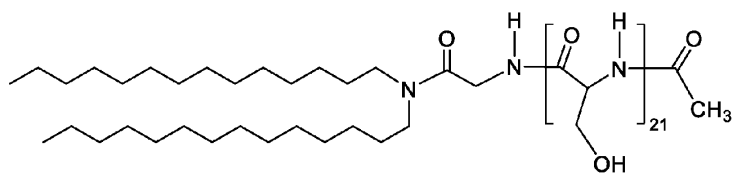
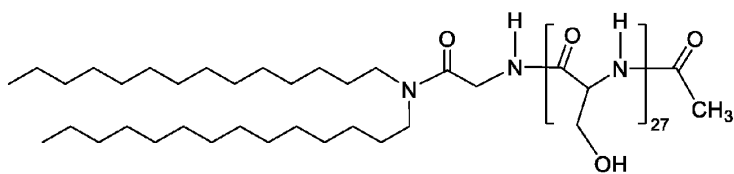
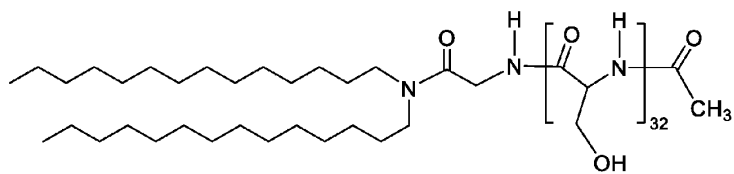
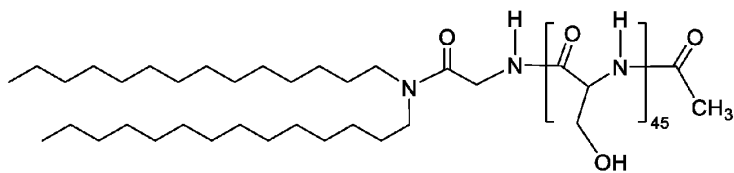
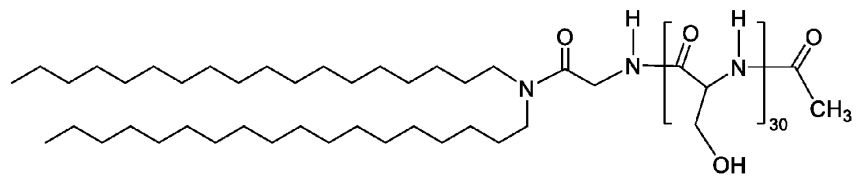


pDLS1 or C14-pDLS1 (n = 18)

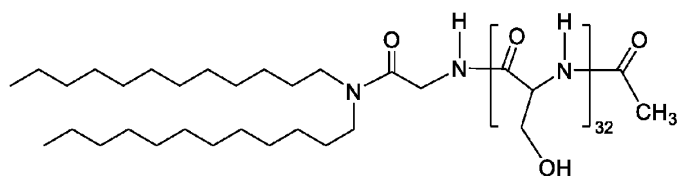
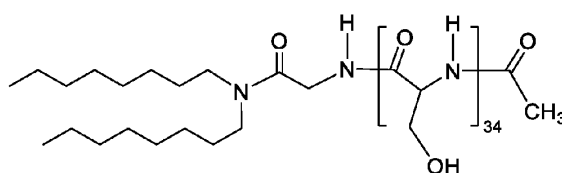


pDLS2 or C14-pDLS2 (n = 18)

20

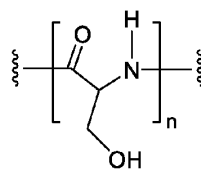
**pDLS3 or C14-pDLS3 (n = 21)****pDLS4 or C14-pDLS4 (n = 27)****pDLS5 or C14-pDLS5 (n = 32)****pDLS6 or C14-pDLS6 (n = 45)****C18-pDLS (n = 30)**

35

**C12-pDLS (n = 32)****C8-pDLS (n = 34)**

5

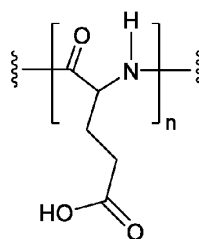
In various embodiments, polyserine in **C14-pDLS1**, **C14-pDLS2**, **C14-pDLS3**, **C14-pDLS4**, **C14-pDLS5**, **C14-pDLS6**, **C18-pDLS**, **C12-pDLS** and **C8-pDLS** are poly(D,L-serine) and may be replaced with poly(L-serine), poly(D-serine) and/or poly(L,D-serine). In various embodiments, polyserine in **C14-pDLS1**, **C14-pDLS2**, **C14-pDLS3**, **C14-pDLS4**, **C14-pDLS5**, **C14-pDLS6**, **C18-pDLS**, **C12-pDLS** and **C8-pDLS** may also be replaced with polyglutamic acid. In



various embodiments, one or more of

group(s) in **C14-pDLS1**,

C14-pDLS2, **C14-pDLS3**, **C14-pDLS4**, **C14-pDLS5**, **C14-pDLS6**, **C18-pDLS**,



C12-pDLS and **C8-pDLS** may be replaced with

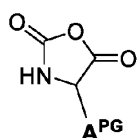
group(s).

15

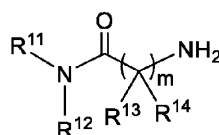
METHOD OF PREPARING COMPOUND

There is provided a method of preparing a compound represented by general formula (1) as disclosed herein, the method comprising:

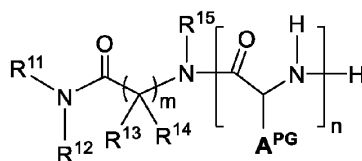
- 5 (i) polymerizing/reacting one or more N-carboxyanhydride (NCA) monomers represented by general formula (5) with a lipid initiator/molecule represented by general formula (6) to obtain a first intermediate compound represented by general formula (7):



(5)



(6)



(7)

wherein

A^{PG} represents **A** functionalized/protected with one or more protecting groups, where **A** comprises a hydrophilic organic group that is part of an amino acid represented by general formula (3);

R¹¹ and R¹² are each independently a hydrophobic group;

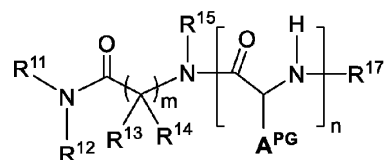
R¹³, R¹⁴, and R¹⁵ are each independently H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl;

m ≥ 1; and

n ≥ 1;

37

- (ii) optionally reacting the first intermediate compound represented by general formula (7) with an acylating agent to obtain a second intermediate compound represented by general formula (8):



(8)

wherein

R^{17} is $-H$ or $-C(=O)R^{18}$, where R^{18} is optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl or optionally substituted alkoxy;

- (iii) deprotecting the first intermediate compound represented by general formula (7) and/or the second intermediate compound represented by general formula (8) to obtain the compound represented by general formula (1).

In various embodiments, the polymerizing/reacting step (i) comprises ring opening polymerization of the NCA ring/group in the compound represented by general formula (5). It will be appreciated that in step (i), the one or more NCA monomers (i.e. represented by general formula (5)) may be added simultaneously to the initiator to polymerize and form random copolypeptides or may be added sequentially to the initiator to form block copolypeptides.

In various embodiments, the lipid initiator comprises a primary amine group.

In various embodiments, A^{PG} represents A being functionalized/protected with one or more protecting groups. In various embodiments, the protecting group

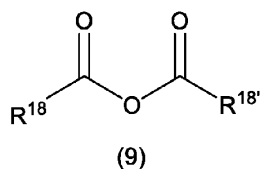
is benzyl, *tert*-butyl, the like, or combinations thereof. For example, one or more of hydroxyl group(s) (i.e. $-\text{OH}$) in **A** may be functionalized/protected with a protecting group and converted into $-\text{OBn}$ or $-\text{OtBu}$ in **A^{PG}**. In various embodiments, **A^{PG}** comprises **A** protected/functionalized with a benzyl (Bn) group, *tert*-butyl (*t*Bu) group, the like, or combinations thereof. For example, **A^{PG}** may be $-\text{CH}_2\text{O}-t\text{Bu}$, $-\text{CH}_2\text{O}-\text{Bn}$, $-\text{CH}(\text{CH}_3)\text{O}-t\text{Bu}$, or $-\text{CH}(\text{CH}_3)\text{O}-\text{Bn}$. In various embodiments, **A^{PG}** comprises $-\text{CH}_2\text{O}-\text{Bn}$:



In various embodiments, R^{11} to R^{17} contain one or more features and/or share one or more properties that are similar to those described respectively above for R^1 to R^7 . In various embodiments, w , m and n contain one or more features and/or share one or more properties that are similar to those described above.

In various embodiments, step (ii) comprises reacting the first intermediate compound represented by general formula (7) with an acylating agent to convert R^{17} from $-\text{H}$ into $-\text{C}(=\text{O})\text{R}^{18}$.

In various embodiments, the acylating agent (in step (ii)) is selected from acid anhydride (e.g., acetic anhydride (Ac_2O)), acid halide (e.g., acyl halide), *N*-hydroxysuccinimide (NHS) esters, imidoesters or the like or combinations thereof. In various embodiments, the acylating agent is represented by general formula (9):



wherein R^{18} and $R^{18'}$ are each independently optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl. In various embodiments, R^{18} and $R^{18'}$ are each independently selected from methyl, ethyl, *n*-propyl, 2-propyl, isopropyl, *n*-butyl, isobutyl, *sec*-butyl, *t*-butyl, hexyl, amyl, 1,2-dimethylpropyl, 1,1-dimethylpropyl, pentyl, isopentyl, hexyl, 4-methylpentyl, 1-methylpentyl, 2-methylpentyl, 3-methylpentyl, 2,2-dimethylbutyl, 3,3-dimethylbutyl, 1,2-dimethylbutyl, 1,3-dimethylbutyl, 1,2,2-trimethylpropyl, 1,1,2-trimethylpropyl, 2-ethylpentyl, 3-ethylpentyl, heptyl, 1-methylhexyl, 2,2-dimethylpentyl, 3,3-dimethylpentyl, 4,4-dimethylpentyl, 1,2-dimethylpentyl, 1,3-dimethylpentyl, 1,4-dimethylpentyl, 1,2,3-trimethylbutyl, 1,1,2-trimethylbutyl, 1,1,3-trimethylbutyl, 5-methylheptyl, 1-methylheptyl, octyl, nonyl, decyl, or the like or combinations thereof. In various embodiments, R^{18} and $R^{18'}$ contain one or more features and/or share one or more properties that are similar to those described respectively above for R^8 (e.g., as described in general formula (1)).

15

In various embodiments, the deprotecting/deprotection step (iii) of the first intermediate compound represented by general formula (7) and/or the second intermediate compound represented by general formula (8) comprises deprotecting/removing the one or more protecting groups (e.g., benzyl or *tert*-butyl) from the intermediate compound. In various embodiments, the deprotecting/deprotection step (iii) comprises subjecting the first intermediate compound represented by general formula (7) and/or the second intermediate compound represented by general formula (8) to acidic conditions. For example, the deprotection step (iii) may be carried out in the presence of one or more acids (e.g., trifluoroacetic acid (TFA), hydrobromic acid (HBr) and acetic acid (AcOH)).

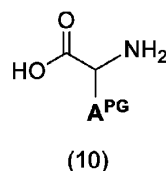
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In various embodiments, the NCA monomers are derived from a protected amino acid. In various embodiments, the method further comprises, prior to step (i):

30

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- (a-i) reacting a protected amino acid represented by general formula (10) with carbonylating agent to obtain an amino acid-*N*-carboxyanhydride (NCA) represented by general formula (5):



5

In various embodiments, the reacting step (a-i) comprises cyclization of the protected amino acid represented by general formula (10), e.g., to obtain a cyclic product, i.e. amino acid-*N*-carboxyanhydride (NCA) represented by general formula (5). In various embodiments, the carbonylating agent used in step (a-i) is selected from phosgene, diphosgene, triphosgene, the like, or combinations thereof. It will be appreciated that, in various embodiments, the protected amino acid represented by general formula (10) comprises –NH₂ and –COOH in order for ring cyclization to occur to obtain/make NCA prior to ring-opening polymerization.

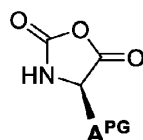
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In various embodiments, the polymerizing step (i) comprises suspending/dispersing/mixing/dissolving the one or more *N*-carboxyanhydride (NCA) monomers represented by general formula (5) with the lipid initiator/molecule represented by general formula (6) in a molar ratio of from about 5:1 to about 70:1, from about 5:1 to about 65:1, from about 5:1 to about 60:1, from about 10:1 to about 55:1, from about 15:1 to about 50:1, from about 20:1 to about 45:1, from about 25:1 to about 40:1, or from about 30:1 to about 35:1.

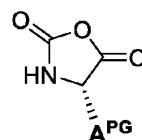
In various embodiments, the one or more *N*-carboxyanhydride (NCA) monomers (i.e. represented by general formula (5)) comprise a structure represented by general formula (5A) and/or (5B):

25

41



(5A)



(5B)

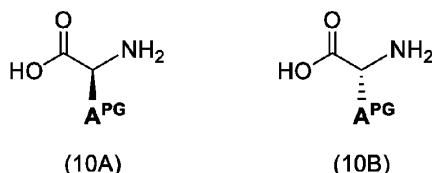
In various embodiments, **A^{PG}** contains one or more features and/or share one or more properties that are similar to those described above.

5

In various embodiments, the one or more N-carboxyanhydride (NCA) monomers comprise or may exist in the form of L-enantiomer (e.g., L-protected amino acid-NCA). In various embodiments, the one or more N-carboxyanhydride (NCA) monomers comprise or may exist in the form of D-enantiomer (e.g., D-protected amino acid-NCA). In various embodiments, the one or more N-carboxyanhydride (NCA) monomers comprise a mixture (e.g., a racemic mixture) of L-enantiomers and D-enantiomers. For example, the one or more N-carboxyanhydride (NCA) monomers may comprise or exist in the form of a mixture of L-protected amino acid-NCA and D-protected amino acid-NCA. In some embodiments, the NCA monomer comprises a racemic mixture, e.g., *o*-benzyl-L-serine-N-carboxyanhydride and *o*-benzyl-D-serine-N-carboxyanhydride. In various embodiments, the lipid-polypeptides are formed from a mixture (e.g., a racemic mixture) of L-serine-NCA and D-serine-NCA.

In various embodiments, general formula (5A) is mixed with general formula (5B) in step (i) at a molar ratio of from about 1:5 to about 5:1, from about 1:4 to about 4:1, from about 1:3 to about 3:1, from about 1:2 to about 2:1, or about 1:1. Advantageously, the method is designed to allow the hydrophilicity/hydrophobicity (or the value of *n* and/or degree of polymerization) of the compound to be adjustable/customizable/tunable by varying the amount of N-carboxyanhydride (NCA) monomers (e.g., general formula (5A) and/or general formula (5B)).

In various embodiments, the protected amino acid comprises a structure represented by general formula (10A) and/or (10B):



5

In various embodiments, A^{PG} contains one or more features and/or share one or more properties that are similar to those described above.

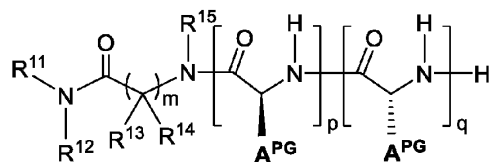
In various embodiments, the protected amino acid represented by general formula (10) comprises or may exist is in the form of L-enantiomer (e.g., L-protected amino acid). In various embodiments, the protected amino acid represented by general formula (10) comprises or may exist is in the form of D-enantiomer (e.g., D-protected amino acid). In various embodiments, the protected amino acid represented by general formula (10) comprises a mixture (e.g., a racemic mixture) of L-enantiomers and D-enantiomers. For example, the protected amino acid represented by general formula (10) may comprise or exist in the form of a mixture of L-protected amino acid and D-protected amino acid. In some embodiments, the protected amino acid comprises *o*-benzyl-L-serine and/or *o*-benzyl-D-serine.

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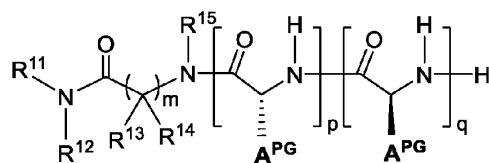
In various embodiments, the first intermediate compound represented by general formula (7) comprises repeating unit(s) or block(s) of L-enantiomers and/or D-enantiomers (e.g., poly(D-protected amino acid), poly(L-protected amino acid), poly(D,L-protected amino acid), or poly(L,D-protected amino acid)). For example, the first intermediate compound represented by general formula (7) may comprise a structure or exist in the form of a structure that is represented by general formula (7A) and/or (7B):

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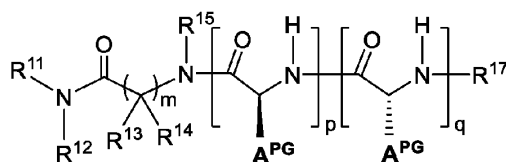


(7A)

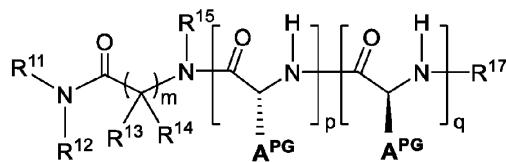


(7B)

In various embodiments, the second intermediate compound represented
 5 by general formula (8) comprises repeating unit(s) or block(s) of L-enantiomers
 and/or D-enantiomers (e.g., poly(D-protected amino acid), poly(L-protected
 amino acid), poly(D,L-protected amino acid), or poly(L,D-protected amino acid)).
 For example, the second intermediate compound represented by general formula
 (8) may comprise a structure or exist in the form of a structure that is represented
 10 by general formula (8A) and/or (8B):

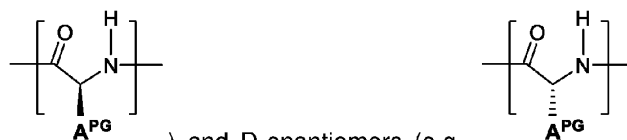


(8A)



(8B)

It will be appreciated that, in various embodiments of the first intermediate compound and/or second intermediate compound disclosed herein, the



L-enantiomers (e.g.,) and D-enantiomers (e.g.,) are randomly distributed in/within the structure/repeating unit of the compound.

- 5 In various embodiments therefore, the compound/poly(amino acid)/polypeptide/oligopeptide designed/prepared in accordance with various embodiments disclosed herein is a random copolypeptide or a block copolypeptide. In various embodiments, the lipid-poly(protected amino acid) comprises repeating units/blocks of
- 10 L- and D-enantiomers, i.e. lipid-*block*-poly(D,L-protected amino acid). In various embodiments, the lipid-poly(o-benzyl-serine) comprises repeating units/blocks of L- and D-enantiomers, i.e. lipid-*block*-poly(o-benzyl-D,L-serine).

- 15 In various embodiments, the polymerizing/reacting/deprotecting step (i), (ii), (iii), and/or (a-i) comprises one or more of the following steps: suspending, dispersing, mixing, stirring, dissolving, sonicating and/or ultrasonication.

- 20 In various embodiments, the polymerizing/reacting/deprotecting step (i), (ii), (iii), and/or (a-i) is/are performed in the presence of an organic solvent. In various embodiments, any organic solvent that effectively serves as a medium to contain the components of the reaction mixture (e.g., reactants/substrates) may be used in embodiments of the reaction mixture disclosed herein. In various embodiments, the organic solvent is capable of substantially dissolving the components present in the reaction mixture. The organic solvent may be an
- 25 organic solvent such as dichloromethane (DCM), tetrahydrofuran (THF), dimethylsulfoxide (DMSO), acetonitrile, ethyl acetate, dimethylformamide (DMF) or the like or combinations thereof. In various embodiments, the organic solvent may be provided in a dry or anhydrous form. For example, polymerizing/reacting step (i) may comprise suspending, dispersing, mixing, stirring, dissolving,

sonicating and/or ultrasonication the one or more N-carboxyanhydride (NCA) monomers represented by general formula (5) with a lipid initiator represented by general formula (6) in the presence of dry/anhydrous organic solvent. For example, reacting step (a-i) may comprise suspending, dispersing, mixing, stirring, dissolving, sonicating and/or ultrasonication the protected amino acid
5 represented by general formula (10) with a carbonylating agent in the presence of dry/anhydrous organic solvent.

In various embodiments, the polymerizing/reacting/deprotecting step (i),
10 (ii), (iii), and/or (a-i) is/are carried out under vacuum or in an inert atmosphere. For example, the step(s) of suspending, dispersing, mixing, stirring, dissolving, sonicating and/or ultrasonication may be performed in a glove box, in the presence of an inert gas such as argon or nitrogen, or in the absence of reactive gases such as oxygen (e.g., dissolved oxygen).

15

In various embodiments, the polymerizing/reacting/deprotecting step (i), (ii), (iii), and/or (a-i) is/are performed over a time duration of from about 1 hour to about 200 hours, from about 1 hour to about 190 hours, from about 10 hours to about 180 hours, from about 20 hours to about 170 hours, from about 30 hours
20 to about 160 hours, from about 40 hours to about 150 hours, from about 50 hours to about 140 hours, from about 60 hours to about 130 hours, from about 70 hours to about 120 hours, from about 80 hours to about 110 hours, from about 90 hours to about 100 hours, or about 95 hours. The polymerizing/reacting/deprotecting step (i), (ii), (iii), and/or (a-i) may also be performed over a time duration of from
25 about 1 hour to about 72 hours, from about 2 hours to about 60 hours, from about 3 hours to about 48 hours, from about 4 hours to about 36 hours, from about 5 hours to about 24 hours, or from about 6 hours to about 12 hours.

In various embodiments, the polymerizing/reacting/deprotecting step (i),
30 (ii), (iii), and/or (a-i) is/are performed at a temperature that is from about 10.0°C to about 100.0°C, from about 20.0°C to about 90.0°C, from about 30.0°C to about 80.0°C, from about 40.0°C to about 70.0°C, from about 50.0°C to about 60.0°C,

or about 55.0°C. In various embodiments, the polymerizing/reacting/deprotecting step (i), (ii), (iii), and/or (a-i) is/are performed at room temperature e.g., that is from about 20°C to about 30°C, about 21°C, about 22°C, about 23°C, about 24°C, about 25°C, about 26°C, about 27°C, about 28°C, about 29°C, or about 30°C.

5

In various embodiments, the polymerizing/reacting/deprotecting step (i), (ii), (iii), and/or (a-i) is/are optionally performed at a temperature that is from about -10°C to about 10°C, from about -9°C to about 9°C, from about -8°C to about 8°C, from about -7°C to about 7°C, from about -6°C to about 6°C, from about -5°C to about 5°C, from about -4°C to about 4°C, from about -3°C to about 3°C, from about -2°C to about 2°C, from about -1°C to about 1°C, or 0°C, e.g., to control reaction kinetics. For example, the reacting step may be performed in an ice bath.

In various embodiments, the deprotecting step (iii) is performed at a temperature that is from about -20.0°C to about 50.0°C, from about -10.0°C to about 40.0°C, from about 0.0°C to about 30.0°C, from about 10.0°C to about 20.0°C, or about 15.0°C. For example, the deprotection step may be performed in an ice bath.

In various embodiments, the method further comprises:

- (b-i) a step of isolating the first intermediate compound represented by general formula (7) after step (i);
- (b-ii) a step of isolating the second intermediate compound represented by general formula (8) after step (ii);
- (b-iii) a step of isolating the compound represented by general formula (1) after step (iii); and/or
- (b-iv) a step of isolating the N-carboxyanhydride (NCA) monomer represented by general formula (5) after step (a-i).

In various embodiments, the isolating step(s) comprises one or more of the following steps: re-dissolving, purifying, centrifuging, quenching, washing, precipitating, filtering, and/or recrystallizing the first intermediate compound

represented by general formula (7), the second intermediate compound represented by general formula (8), the compound represented by general formula (1), and/or the N-carboxyanhydride (NCA) monomer represented by general formula (5). In various embodiments, the isolating step is performed to
5 remove by-products from the first intermediate compound represented by general formula (7), the second intermediate compound represented by general formula (8), the compound represented by general formula (1), and/or the N-carboxyanhydride (NCA) monomer represented by general formula (5).

10 In various embodiments, the step(s) of purifying, centrifuging, quenching, recrystallizing and/or washing is/are performed at least 1 time, at least 2 times, at least 3 times, at least 4 times, at least 5 times, at least 6 times, at least 7 times, at least 8 times, at least 9 times, at least 10 times, at least 15 times, at least 20
15 times with a washing medium. In various embodiments, the step(s) of purifying, centrifuging, recrystallizing and/or washing is/are typically/usually repeated at least 3 times.

In various embodiments, the washing medium comprises aqueous medium/solutions such as salt solution, deionized water, acid or the like, or
20 combinations thereof. The salt solution may be bicarbonate salts such as sodium bicarbonate, chloride salts such as saturated sodium chloride (brine). The acid may be citric acid. In various embodiments, the salt solution comprises highly concentrated/saturated salt solution.

25 In various embodiments, the method further comprises one or more of the following post reaction steps: drying optionally under low temperature (e.g., freeze drying), under vacuum, and/or in an inert atmosphere.

In various embodiments, the step(s) of drying is performed in the presence
30 of a drying agent such as anhydrous sodium sulfate, anhydrous magnesium sulfate, anhydrous calcium sulfate, and anhydrous calcium chloride, the like or combinations thereof.

In various embodiments, the yield of the compound represented by general formula (1) is from about 1.0% to about 100.0%, from about 5.0% to about 99.0%, from about 10.0% to about 98.0%, from about 20.0% to about 97.0%, from about 30.0% to about 96.0%, from about 40.0% to about 95.0%,
5 from about 50.0% to about 90.0%, from about 55.0% to about 85.0%, from about 60.0% to about 80.0%, from about 65.0% to about 70.0%, or about 75.0%. The yield of the compound represented by general formula (1) may be from about 50.0% to about 98.0%.

10 Advantageously, embodiments of the method are straightforward to perform and/or have a low production/manufacturing cost (i.e. cost effective) as the reaction conditions are mild and do not require harsh and/or tedious step(s). Advantageously, embodiments of the method comprise simple purification steps (e.g., ease of isolation from by-products etc) and products are synthesized with
15 high yields. Advantageously, embodiments of the method are scalable and/or have substantially high scalability.

NANOPARTICLE COMPOSITION

20 Advantageously, in various embodiments, the design of the structure of the compound represented by general formula (1) allows said compound to be used, in lieu or in replacement/substitute of a conventional lipid-PEG conjugate (e.g., ALC-0159), in the formulation of nanoparticles in a composition. In various
25 embodiments, embodiments of the compound are capable of being formulated into nanoparticles in a composition. Advantageously, in various embodiments, the design of the compound represented by general formula (1) helps prevent non-specific protein absorption, particle aggregation and controls the size of the nanoparticles formed. In various embodiments, embodiments of the compound
30 represented by general formula (1) helps maintain colloidal stability (of the lipid nanoparticles), facilitate the condensation and encapsulating/loading of molecules/cargoes into the nanoparticle composition, and consequently deliver

nucleic acids (e.g., mRNAs) therefore enhancing vaccination or therapeutic efficiency.

The term “nanoparticles” may comprise and/or may be used interchangeably with the terms “lipid nanoparticles”, “encapsulated lipid nanoparticles”, “loaded lipid nanoparticles”, “LNPs”, “cell-targeting lipid nanoparticles”, “immune cells-targeting lipid nanoparticles”, or the like.

There is provided a nanoparticle composition comprising:

- (i) a compound represented by general formula (1) as disclosed herein; and
- (ii) a therapeutic agent, prophylactic agent and/or biological agent that is encapsulated/loaded in said composition.

Advantageously, the composition is suitable for use in the encapsulation, delivery and/or transfection of one or more therapeutic agent, prophylactic agent and/or biological agent e.g., to a desired target (such as subject, cell, cytosol, tissue or organ).

In various embodiments, the composition further comprises:

- (a) ionizable lipid;
- (b) neutral/helper lipid; and
- (c) cholesterol and/or derivatives thereof.

In various embodiments, the composition is substantially devoid of polyethylene glycol (PEG). In various embodiments, the composition is substantially devoid of polyethylene glycol (PEG)-modified lipid conjugates, polyethylene glycol (PEG)-modified lipid, PEGylated lipid, PEG-conjugated lipid, PEG-lipid conjugate and/or lipid modified with PEG. In various embodiments, the compound is a substitute/replacement for PEG-lipid conjugate.

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In various embodiments, the compound, ionizable lipid, neutral/helper lipid, and cholesterol and/or derivatives thereof are mixed/dissolved in an organic

solvent. In various embodiments, the formation of lipid nanoparticle comprises self-assembly of the lipid components and one or more types of molecules or cargoes. In various embodiments, any organic solvent that effectively serves as a medium to contain the lipid components may be used in embodiments of the
5 lipid materials disclosed herein. In various embodiments, the organic solvent is capable of substantially dissolving the components present in the mixture. The organic solvent may comprise methanol, ethanol, isopropanol, acetonitrile, ethyl acetate, tetrahydrofuran, dimethyl sulfoxide (DMSO), dimethylformamide (DMF) or the like or combinations thereof.

10

In various embodiments, the ionizable lipid, neutral/helper lipid, cholesterol and/or derivatives thereof, and compound represented by general formula (1) are mixed at a mole ratio of about 5 – 65 : about 4 – 20 : about 10 – 60 : about 0.1 – 20, about 10 – 60 : about 6 – 18 : about 15 – 55 : about 0.2 – 18, about 15 – 55 :
15 about 7 – 17 : about 20 – 50 : about 0.5 – 16, about 20 – 50 : about 8 – 16 : about 25 – 45 : about 1 – 14, about 25 – 45 : about 9 – 15 : about 30 – 40 : about 1 – , about 30 – 40 : about 10 – 14 : about 32 – 38 : about 4 – 12, or about 33 – 37 : about 11 – 13 : about 34 – 36 : about 6 – 10.

In various embodiments, the ionizable lipid includes, but is not limited to ALC-0315, SM-102, Lipid 5, DLinDMA, D-Lin-MC2-DMA, DLin-MC3-DMA, D-Lin-MC4-DMA, Dlin-KC2-DMA, YSK05, AA3-Dlin, SSPalmM, SSPalmO-Phe, Lipid A9, L319, DODMA, CL1, BP Lipid 310, ATX-001, ATX-100, Lipid 2, 80-O16B, BP Lipid 309, BP Lipid 307, 93-O17S, 93-O17O, NT1-O14B, 306-O12B-3, 306-
25 O12B, 113-O16B, 306Oi10, 306Oi9-cis2, BAMEA-O16B, AI-28, 113-O12B, 98N12-5, Ckk-E12, OF-02, C12-200, BP Lipid 311, BP Lipid 308, BP Lipid 314, BP Lipid 312, LP01, TCL053, Lipid C24, BP Lipid 315, Lipid 29, 9A1P9, C13-112-tri-tail, C13-113-tri-tail, C13-112-tetra-tail, or C13-113-tetra-tail, C12-200 and combinations thereof. It will be appreciated that any suitable ionizable lipid that
30 effectively modulates/adjusts/changes its charge depending on the environmental pH may be used in embodiments of the composition disclosed herein. Accordingly, any kind/type of ionizable lipids may be used with

embodiments of the compound disclosed herein (e.g., lipid-poly(D,L-serine), and is not limited to the list as recited hereinbefore.

In various embodiments, the composition comprises from about 5.0 mol% to about 65.0 mol%, from about 10.0 mol% to about 60.0 mol%, from about 15.0 mol% to about 55.0 mol%, from about 20.0 mol% to about 50.0 mol%, from about 25.0 mol% to about 45.0 mol%, from about 30.0 mol% to about 40.0 mol%, or about 35.0 mol% of the ionizable lipid. In various embodiments, the ionizable lipid is present in an amount of about 5 mol%, about 6 mol%, about 7 mol%, about 8 mol%, about 9 mol%, about 10 mol%, about 11 mol%, about 12 mol%, about 13 mol%, about 14 mol%, about 15 mol%, about 16 mol%, about 17 mol%, about 18 mol%, about 19 mol%, about 20 mol%, about 21 mol%, about 22 mol%, about 23 mol%, about 24 mol%, about 25 mol%, about 26 mol%, about 27 mol%, about 28 mol%, about 29 mol%, about 30 mol%, about 31 mol%, about 32 mol%, about 33 mol%, about 34 mol%, about 35 mol%, about 36 mol%, about 37 mol%, about 38 mol%, about 39 mol%, about 40 mol%, about 41 mol%, about 42 mol%, about 43 mol%, about 44 mol%, about 45 mol%, about 46 mol%, about 47 mol%, about 48 mol%, about 49 mol%, about 50 mol%, about 51 mol%, about 52 mol%, about 53 mol%, about 54 mol%, about 55 mol%, about 56 mol%, about 57 mol%, about 58 mol%, about 59 mol%, about 60 mol%, about 61 mol%, about 62 mol%, about 63 mol%, about 64 mol%, or about 65 mol% of the composition.

In various embodiments, the neutral/helper lipid comprises a phospholipid such as an unsaturated lipid. Examples of phospholipid includes, but are not limited to, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemPC), 1-

hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-didocosaheptaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoeoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosaheptaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG), sphingomyelin, and the like and combinations thereof.

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In various embodiments, the composition comprises from about 1.0 mol% to about 20.0 mol%, from about 1.5 mol% to about 19.5 mol%, from about 2.0 mol% to about 19.0 mol%, from about 2.5 mol% to about 18.5 mol%, from about 3.0 mol% to about 18.0 mol%, from about 3.5 mol% to about 17.5 mol%, from about 4.0 mol% to about 17.0 mol%, from about 4.5 mol% to about 16.5 mol%, from about 5.0 mol% to about 16.0 mol%, from about 5.5 mol% to about 15.5 mol%, from about 6.0 mol% to about 15.0 mol%, from about 6.5 mol% to about 14.5 mol%, from about 7.0 mol% to about 14.0 mol%, from about 7.5 mol% to about 13.5 mol%, from about 8.0 mol% to about 13.0 mol%, from about 8.5 mol% to about 12.5 mol%, from about 9.0 mol% to about 12.0 mol%, from about 9.5 mol% to about 11.5 mol%, from about 10.0 mol% to about 11.0 mol%, or about 10.5 of neutral/helper lipid. In various embodiments, the neutral/helper lipid is present in an amount of about 1 mol%, about 2 mol%, about 3 mol%, about 4 mol%, about 5 mol%, about 6 mol%, about 7 mol%, about 8 mol%, about 9 mol%, about 10 mol%, about 11 mol%, about 12 mol%, about 13 mol%, about 14 mol%, about 15 mol%, %, about 16 mol%, about 17 mol%, about 18 mol%, about 19 mol%, or about 20 mol% of the composition.

In various embodiments, the cholesterol and/or derivatives thereof includes, but is not limited to cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, avenasterol, or the like or combinations thereof.

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In various embodiments, the composition comprises from about 10.0 mol% to about 60.0 mol%, from about 15.0 mol% to about 55.0 mol%, from about 20.0 mol% to about 50.0 mol%, from about 25.0 mol% to about 45.0 mol%, from about 30.0 mol% to about 40.0 mol%, or about 35.0 mol% of the cholesterol and/or derivatives thereof. In various embodiments, the cholesterol and/or derivatives thereof is/are present in an amount of about 10 mol%, about 11 mol%, about 12 mol%, about 13 mol%, about 14 mol%, about 15 mol%, about 16 mol%, about 17 mol%, about 18 mol%, about 19 mol%, about 20 mol%, about 21 mol%, about 22 mol%, about 23 mol%, about 24 mol%, about 25 mol%, about 26 mol%, about 27 mol%, about 28 mol%, about 29 mol%, about 30 mol%, about 31 mol%, about 32 mol%, about 33 mol%, about 34 mol%, about 35 mol%, about 36 mol%, about 37 mol%, about 38 mol%, about 39 mol%, about 40 mol%, about 41 mol%, about 42 mol%, about 43 mol%, about 44 mol%, about 45 mol%, about 46 mol%, about 47 mol%, about 48 mol%, about 49 mol%, about 50 mol%, about 51 mol%, about 52 mol%, about 53 mol%, about 54 mol%, about 55 mol%, about 56 mol%, about 57 mol%, about 58 mol%, about 59 mol%, or about 60 mol% of the composition.

In various embodiments, the composition comprises from about 0.10 mol% to about 20.0 mol%, from about 0.20 mol% to about 19.0 mol%, from about 0.30 mol% to about 18.0 mol%, from about 0.40 mol% to about 17.0 mol%, from about 0.50 mol% to about 16.0 mol%, from about 0.60 mol% to about 15.0 mol%, from about 0.70 mol% to about 14.0 mol%, from about 0.80 mol% to about 13.0 mol%, from about 0.90 mol% to about 12.0 mol%, from about 1.0 mol% to about 11.0 mol%, from about 1.5 mol% to about 10.0 mol%, from about 2.0 mol% to about 9.5 mol%, from about 2.5 mol% to about 9.0 mol%, from about 3.0 mol% to about 8.5 mol%, from about 3.5 mol% to about 8.0 mol%, from about 4.0 mol% to about 7.5 mol%, from about 4.5 mol% to about 7.0 mol%, from about 5.0 mol% to about 6.5 mol%, or from about 5.5 mol% to about 6.0 mol% of compound represented by general formula (1). In various embodiments, the compound represented by general formula (1) is the major component of the composition and is present in an amount of about 0.1 mol%, about 0.2 mol%, about 0.3 mol%,

about 0.4 mol%, about 0.5 mol%, about 0.6 mol%, about 0.7 mol%, about 0.8 mol%, about 0.9 mol%, about 1.0 mol%, about 1.1 mol%, about 1.2 mol%, about 1.3 mol%, about 1.4 mol%, about 1.5%, about 1.6 mol%, about 1.7 mol%, about 1.8 mol%, about 1.9 mol%, about 2.0 mol%, about 2.1 mol%, about 2.2 mol%,
5 about 2.3 mol%, about 2.4 mol%, about 2.5 mol%, about 2.6 mol%, about 2.7 mol%, about 2.8 mol%, about 2.9 mol%, about 3.0 mol%, about 3.1 mol%, about 3.2 mol%, about 3.3 mol%, about 3.4 mol%, about 3.5 mol%, about 3.6 mol%, about 3.7 mol%, about 3.8 mol%, about 3.9 mol%, about 4.0 mol%, about 4.1 mol%, about 4.2 mol%, about 4.3 mol%, about 4.4 mol%, about 4.5 mol%, about
10 4.6 mol%, about 4.7 mol%, about 4.8 mol%, about 4.9 mol%, about 5.0 mol%, about 10.0 mol%, about 15.0 mol%, or about 20.0 mol% of the composition.

In various embodiments, the therapeutic agent, prophylactic agent and/or biological agent is provided in an aqueous buffer. The aqueous buffer may be
15 sodium acetate.

In various embodiments, the nanoparticle composition comprises nanoparticles formed from the compound represented by general formula (1).

20 NANOPARTICLES

There is provided nanoparticles (e.g., lipid nanoparticles) comprising:

- (i) the compound represented by general formula (1) as disclosed herein; and
- (ii) a therapeutic and/or prophylactic agent and/or biological agent that is
25 encapsulated/loaded/coupled/bonded/linked/bound in/to said nanoparticles.

In various embodiments, the nanoparticles have a N:P or N/P ratio (i.e. molar ratio of ionizable nitrogen atoms in the ionizable lipid to phosphate groups
30 in the therapeutic agent, prophylactic agent and/or biological agent (e.g., nucleic acid) is from about 1:1 to about 50:1. The nanoparticles may have a N:P or N/P

ratio that is from about 1:1 to about 50:1, about 2:1, about 5:1, about 10:1 about 15:1, about 20:1, about 25:1, about 30:1, about 35:1, about 40:1, or about 45:1.

It will be appreciated that in various embodiments, the optimal N/P ratio is dependent on the type of therapeutic and/or prophylactic agent and/or biological agent (e.g., a nucleic acid such as mRNA, siRNA, miRNA, pDNA and oligonucleotides). For example, the optimal N/P ratio may be different for siRNA, pDNA and oligonucleotides. In various embodiments, it will be appreciated that shorter nucleic acid therapeutics (e.g. siRNA) or prophylactic agents (e.g., mRNA) require more (i.e. a larger amount/concentration/volume of) ionizable lipids to encapsulate them into lipid nanoparticles. In various embodiments therefore, a N/P ratio of up to about 20:1 is used to encapsulate and deliver nucleic acid therapeutics (e.g. shorter nucleic acid therapeutics siRNA).

In various embodiments, the encapsulation/loading/binding efficiency/capacity of the therapeutic agent, prophylactic agent and/or biological agent in the composition/nanoparticles is at least about 10.0%, at least about 15.0%, at least about 20.0%, at least about 25.0%, at least about 30.0%, at least about 35.0%, at least about 40.0%, at least about 45.0%, at least about 50.0%, at least about 55.0%, at least about 60.0%, at least about 65.0%, at least about 70.0%, at least about 75.0%, at least about 80.0%, at least about 85.0%, at least about 90.0%, at least about 95.0%, at least about 96.0%, at least about 97.0%, at least about 98.0%, at least about 99.0%, at least about 99.5%, or at least about 99.9%.

In various embodiments, the nanoparticles have an encapsulation efficiency that is slightly lower, comparable to, no less or is higher than that of corresponding nanoparticles using ALC-0159 as the PEG-lipid conjugate under similar conditions. For example, the encapsulation efficiency may be at least about 50% of that of a corresponding nanoparticle using ALC-0159 as the PEG-lipid conjugate under similar conditions. In another example, the encapsulation efficiency may be at least about 1% to at least about 50% higher than that of

corresponding nanoparticles using ALC-0159 as the PEG-lipid conjugate under similar conditions.

In various embodiments, the cell/nucleic acid transfection efficiency (% of
5 the cells/nucleic acids that are transfected with the gene) of the composition/nanoparticles is at least about 1.0%, at least about 5.0%, at least about 10.0%, at least about 15.0%, at least about 20.0%, at least about 25.0%, at least about 30.0%, at least about 35.0%, at least about 40.0%, at least about 45.0%, at least about 50.0%, at least about 55.0%, at least about 60.0%, at least
10 about 65.0%, at least about 70.0%, at least about 75.0%, at least about 80.0%, at least about 85.0%, at least about 90.0%, at least about 95.0%, at least about 96.0%, at least about 97.0%, at least about 98.0%, at least about 99.0%, at least about 99.5%, or at least about 99.9%. In various embodiments, the cell transfection efficiency is not required to be 100%. For example, it will be
15 appreciated that vaccine applications may not need/require to transfect 100% cells in order to mediate an immune response, unlike in the case of cancer therapy applications.

In various embodiments, the nucleic acid transfection efficiency of the
20 therapeutic and/or prophylactic agent and/or biological agent in the composition/nanoparticle is greater than those using PEG-conjugated lipids (e.g., ALC-0159).

In various embodiments, the nanoparticles have a nucleic acid transfection
25 efficiency that is no less or higher than that of corresponding nanoparticles using ALC-0159 PEG-modified lipid under similar conditions. For example, the nucleic acid (e.g., mRNA) transfection efficiency may be at least 2 times, at least 3 times, at least 4 times, at least 5 times, at least 6 times, at least 7 times, at least 8 times, at least 9 times, at least 10 times, at least 15 times, at least about 20 times, at
30 least 25 times, at least 30 times, at least 35 times, at least 40 times, at least 45 times, at least 50 times, at least 55 times, at least 60 times, at least 65 times, at least 70 times, at least 75 times, at least 80 times, at least 85 times, at least 90

times, at least 95 times, or at least 100 times higher than that of corresponding nanoparticles using ALC-0159 PEG-modified lipid under similar conditions.

In various embodiments, the nanoparticles have a cell transfection efficiency that is comparable to, no less or is higher than that of corresponding nanoparticles using ALC-0159 as the PEG-lipid conjugate under similar conditions. For example, the cell transfection efficiency may be at least about 30% of that of a corresponding nanoparticle using ALC-0159 as the PEG-lipid conjugate under similar conditions. In another example, the cell transfection efficiency may be at least about 1% to at least about 50% higher than that of corresponding nanoparticles using ALC-0159 as the PEG-lipid conjugate under similar conditions.

In various embodiments, the transfection efficiency in certain cell lines is lower than that of corresponding nanoparticles using ALC-0159 as the PEG-lipid conjugate under similar conditions but still at the same order of magnitude. It will be appreciated that such level of gene transfection may still be applicable for gene therapy.

Advantageously, in various embodiments, the average or mean particle size (or diameter) nanoparticles are designed to be customizable/adjustable to suit a desired application.

In various embodiments, the nanoparticles have an average or mean particle size (or diameter) in the nano meter range. In various embodiments, the nanoparticles have an average or mean particle size (or diameter) of no more than about 300 nm, no more than about 250 nm, no more than about 200 nm, no more than about 150 nm, no more about 100 nm, no more than about 90 nm, no more than about 80 nm, no more than about 70 nm, no more than about 60 nm, no more than about 50 nm, no more than about 40 nm, no more than about 30 nm, no more than about 20 nm, or no more than about 10 nm. For example, the nanoparticle has an average particle size (or diameter) of from about 50.0 nm to

about 300.0 nm, from about 75.0 nm to about 250.0 nm, from about 100.0 nm to about 200.0 nm, about 105.0 nm, about 110.0 nm, about 115.0 nm, about 120.0 nm, about 125.0 nm, about 130.0 nm, about 135.0 nm, about 140.0 nm, about 145.0 nm, about 150.0 nm, about 155.0 nm, about 160.0 nm, about 165.0 nm, about 170.0 nm, about 175.0 nm, about 180.0 nm, about 185.0 nm, about 190.0 nm, about 195.0 nm, about 200.0 nm, or about 205.0 nm.

In various embodiments, the composition comprising the nanoparticles has a polydispersity index (PDI) of no more than about 0.50, no more than about 0.40, no more than about 0.30, or no more than about 0.20. For example, the nanoparticle has a polydispersity index (PDI) of from about 0.005 to about 0.50, from about 0.01 to about 0.40, or from about 0.05 to about 0.30. In various embodiments, the composition comprising the nanoparticles has a polydispersity index (PDI) of from about 0.01 to about 0.50, from about 0.0125 to about 0.45, from about 0.015 to about 0.40, from about 0.020 to about 0.35, from about 0.025 to about 0.30, from about 0.030 to about 0.25, from about 0.035 to about 0.20, from about 0.040 to about 0.15, from about 0.045 to about 0.10, from about 0.050 to about 0.095, from about 0.055 to about 0.090, from about 0.060 to about 0.085, from about 0.065 to about 0.080, or from about 0.070 to about 0.075. Advantageously, in various embodiments, the nanoparticles have a narrow particle size distribution (e.g., not more than about 0.2) and/or the nanoparticles or nanoparticle composition is relatively/substantially homogenous.

In various embodiments, the nanoparticles have a zeta potential of from about -20.0 mV to about +20.0 mV, from about -19.5 mV to about +19.5 mV, from about -19.0 mV to about +19.0 mV, from about -18.5 mV to about +18.5 mV, from about -18.0 mV to about +18.0 mV, from about -17.5 mV to about +17.5 mV, from about -17.0 mV to about +17.0 mV, from about -16.5 mV to about +16.5 mV, from about -16.0 mV to about +16.0 mV, from about -15.5 mV to about +15.5 mV, from about -15.0 mV to about +15.0 mV, from about -14.5 mV to about +14.5 mV, from about -14.0 mV to about +14.0 mV, from about -13.5 mV to about +13.5 mV, from about -13.0 mV to about +13.0 mV, from about -12.5 mV to about +12.5 mV, from

about -12.0 mV to about +12.0 mV, from about -11.5 mV to about +11.5 mV, from about -11.0 mV to about +11.0 mV, from about -10.5 mV to about +10.5 mV, from about -10.0 mV to about +10.0 mV, from about -9.5 mV to about +9.5 mV, from about -9.0 mV to about +9.0 mV, from about -8.5 mV to about +8.5 mV, from about -8.0 mV to about +8.0 mV, from about -7.5 mV to about +7.5 mV, from about -7.0 mV to about +7.0 mV, from about -6.5 mV to about +6.5 mV, from about -6.0 mV to about +6.0 mV, from about -5.5 mV to about +5.5 mV, from about -5.0 mV to about +5.0 mV, from about -4.5 mV to about +4.5 mV, from about -4.0 mV to about +4.0 mV, from about -3.5 mV to about +3.5 mV, from about -3.0 mV to about +3.0 mV, from about -2.5 mV to about +2.5 mV, from about -2.0 mV to about +2.0 mV, from about -1.5 mV to about +1.5 mV, from about -1.0 mV to about +1.0 mV, from about -0.5 mV to about +0.5 mV, or about +0.0 mV in saline (e.g., phosphate-buffered saline (PBS)) or in a physiological environment. Advantageously, in various embodiments, the nanoparticles have a substantially neutral surface charge or a negative charge or a positive charge, making the nanoparticles suitable/desirable for *in vivo* applications. In various embodiments, the nanoparticles with a negative charge may be used to target spleen and lymph nodes. In various embodiments, the nanoparticles with a positive charge may be used for local delivery of cargos across the mucus layer, e.g. nasal delivery. In various embodiments, the nanoparticles with a negative charge may be used to target the spleens or lymph nodes.

In various embodiments, the cell viability of the composition/nanoparticle is at least about 50.0%, at least about 60.0%, at least about 70.0%, at least about 80.0%, at least about 90.0%, at least about 95.0%, at least about 96.0%, at least about 97.0%, at least about 98.0%, at least about 99.0%, at least about 99.5%, or at least about 99.9%.

In various embodiments, the nanoparticle has a cell viability that is comparable or is no less or is higher than that of a corresponding nanoparticle using ALC-0519 as the PEG-lipid conjugate under similar conditions. For example, the cell viability may be at least comparable to that of a corresponding

nanoparticle using ALC-0519 as the PEG-lipid conjugate under similar conditions. Accordingly, in various embodiments, the nanoparticle comprises/possesses high cytocompatibility and/or negligible cytotoxicity.

5 In various embodiments, the composition/compound/nanoparticles is/are biocompatible, i.e. the composition/compound/nanoparticle is compatible with biological systems or parts of the biological systems without substantially or significantly eliciting an adverse physiological response such as a toxic reaction/response (e.g., cytotoxicity), an immune reaction/response, an allergic
10 reaction, an injury or the like when used on the human or animal body. In various embodiments, the composition/compound/nanoparticle is substantially devoid of substances that elicit an adverse physiological response. It will be appreciated that the composition/compound/nanoparticle may trigger/elicit an immune response (e.g., to enhance vaccination efficacy), and in such embodiments, the
15 composition/compound/nanoparticle is still considered to be biocompatible. Advantageously, the nanoparticles (e.g., lipid nanoparticles) are capable of binding therapeutic agent, prophylactic agent and/or biological agent (e.g., RNA) effectively and/or providing high transfection efficiency without causing/inducing substantial or any cytotoxicity.

20

METHOD OF PREPARING NANOPARTICLES

There is provided a method of preparing nanoparticles as disclosed herein, the method comprising:

- 25 (c-i) preparing an aqueous composition comprising therapeutic and/or prophylactic agent and/or biological agent;
- (c-ii) mixing the aqueous composition obtained from (c-i) with the composition as disclosed herein to obtain nanoparticles.

30 In various embodiments, the step (c-i) comprises mixing therapeutic and/or prophylactic agent and/or biological agent in an aqueous buffer. The

aqueous buffer may be sodium acetate, citrate buffer, phosphate buffer, glycine buffer solution, or the like or combinations thereof.

In various embodiments, the mixing step (c-i) is performed at a pH value
5 of from about 2.0 to about 6.0, from about 2.1 to about 5.9, from about 2.2 to about 5.8, from about 2.3 to about 5.7, from about 2.4 to about 5.6, from about 2.5 to about 5.5, from about 2.6 to about 5.4, from about 2.7 to about 5.3, from about 2.8 to about 5.2, from about 2.9 to about 5.1, from about 3.0 to about 5.0, from about 3.1 to about 4.9, from about 3.2 to about 4.8, from about 3.3 to about
10 4.7, from about 3.4 to about 4.6, from about 3.5 to about 4.5, from about 3.6 to about 4.4, from about 3.7 to about 4.3, from about 3.8 to about 4.2, from about 3.9 to about 4.1, or about 4.0.

In various embodiments, the composition as disclosed herein comprises
15 organic phase (e.g., ethanol). In various embodiments, the aqueous composition comprises aqueous phase. In various embodiments, the step (c-ii) comprises mixing the aqueous composition with the organic composition as disclosed herein at a volume ratio of the aqueous phase to organic phase from about 10:1 to about 1:1. For example, the aqueous phase may be mixed with the organic phase at a
20 volume ratio of from about 10:1 to about 1:1, at about 9:1, at about 8:1, at about 7:1, at about 6:1, at about 5:1, at about 4:1, at about 3:1, or at about 2:1.

In various embodiments, the step (c-ii) of mixing the aqueous composition with the organic composition comprises injecting (e.g., direct injecting) the
25 organic composition into the aqueous composition. In various embodiments, the step (c-ii) of mixing the aqueous composition with the organic composition comprises pipetting (e.g., rapid pipetting) the organic composition into the aqueous composition.

30 In various embodiments, the step (c-ii) of mixing the aqueous composition with the composition comprises micro-mixing, e.g., microfluidic mixing using a microfluidic device. The micro-mixing may be performed *via* passive mixing using

passive micromixers such as T-shaped or Y-shaped microfluidic mixers parallel lamination, sequential, focusing enhanced mixers or droplet micromixers. The micro-mixing may also be performed *via* active mixing using external forces such as pressure field, electrokinetic, dielectrophoretic, electrowetting, magneto-
5 hydrodynamic or ultrasound. Advantageously, as microfluidic mixing comprises mixing the two compositions (i.e. aqueous composition and composition disclosed herein) in a controlled manner and/or with a specified/fixed/controlled/precise mixing ratio, the interaction between the two compositions (e.g., between ionizable lipid and therapeutic, prophylactic and/or
10 biological agent) is regulated, thereby producing nanoparticles with a smaller particle size and/or with a narrow size distribution or homogeneity (e.g, smaller PDI).

In various embodiments, the method further comprises removing the
15 organic phase (e.g. ethanol). For example, removing the organic phase may include dialysis or filtration. Advantageously, removal of the organic phase through dialysis or filtration may solidify the LNPs and improve the encapsulation efficiency of the therapeutic and/or prophylactic agent and/or biological agent.

20 In various embodiments, there is also provided a carrier, nanocarrier or delivery system/vehicle comprising the composition/compound/nanoparticles as disclosed herein.

In various embodiments, there is also provided a vaccine composition
25 comprising the composition/compound/nanoparticles as disclosed herein.

In various embodiments, there is also provided a carrier, a nanocarrier, a delivery system/vehicle, a compound, a nanoparticle composition, nanoparticles (or lipid nanoparticles) disclosed herein for use in medicine (e.g., for the treatment
30 or prophylaxis of one or more of the diseases, disorders or conditions mentioned herein).

In various embodiments, there is also provided a carrier, a nanocarrier, a delivery system/vehicle, a compound, a nanoparticle composition, nanoparticles (or lipid nanoparticles) disclosed herein for use in the treatment or prophylaxis of a disease, disorder or condition, the use of said carrier, a nanocarrier, a delivery system/vehicle, a compound, a nanoparticle composition, nanoparticles (or lipid nanoparticles) in the manufacture of a medicament for the treatment or prophylaxis of a disease, disorder or condition and/or a method of treatment or prophylaxis of a disease, disorder or condition, comprising a step of administering (e.g. in a therapeutically effective amount of) said carrier, a nanocarrier, a delivery system/vehicle, a compound, a nanoparticle composition, nanoparticles (or lipid nanoparticles) to a subject (e.g., vertebrate such as a human or a large veterinary mammal (e.g., horses, cattle, deer, sheep, llamas, goats, pigs) in need thereof.

The disease, disorder or condition may be selected from the group consisting of infectious/contagious diseases, viral infections (i.e. diseases caused by virus), bacterial infections (i.e. diseases caused by bacteria), fungal infections (i.e. diseases caused by fungi), respiratory diseases or the like, cancer, cardiovascular diseases, skin disease or the like, or combinations thereof. In various embodiments, the disease, disorder or condition is mediated by an influenza virus (e.g., influenza A, B, C and/or D virus). For example, the disease may be influenza A, B, C or D such as H1N1, H3N2). In various embodiments, the disease, disorder or condition is mediated by a coronavirus (e.g., severe acute respiratory syndrome coronavirus such as SARS-CoV-2 or SARS-CoV-1). For example, the disease, disorder or condition may be SARS-CoV-2 coronavirus disease. In various embodiments, the disease, disorder or condition is mediated by a dengue virus (e.g., DEN-1, DEN-2, DEN-3, and/or DEN-4 virus). For example, the disease may be dengue disease.

In various embodiments, there is also provided a carrier, a nanocarrier, a delivery system/vehicle, a compound, a nanoparticle composition, nanoparticles (or lipid nanoparticles) disclosed herein for use in encapsulating and/or delivering a therapeutic, prophylactic and/or biological agent to a subject, cell, cytosol,

tissue or organ (e.g., a mammalian cell, cytosol, tissue or organ), the use of said carrier, a nanocarrier, a delivery system/vehicle, a compound, a nanoparticle composition, nanoparticles (or lipid nanoparticles) in the manufacture of a medicament for encapsulating and/or delivering a therapeutic, prophylactic and/or biological agent to a subject, cell, cytosol, tissue or organ (e.g., a mammalian cell, cytosol, tissue or organ), and/or a method of delivering a therapeutic, prophylactic and/or biological agent to a subject, cell, cytosol, tissue or organ (e.g., a mammalian cell, cytosol, tissue or organ), comprising a step of administering (e.g. in a therapeutically effective amount of) said carrier, a nanocarrier, a delivery system/vehicle, a compound, a nanoparticle composition, nanoparticles (or lipid nanoparticles) to a subject (e.g., vertebrate such as a human or a large veterinary mammal (e.g., horses, cattle, deer, sheep, llamas, goats, pigs)) in need thereof.

15 In various embodiments, there is also provided a carrier, a nanocarrier, a delivery system/vehicle, a compound, a nanoparticle composition, nanoparticles (or lipid nanoparticles) disclosed herein for use in inducing an immune response in a subject (e.g., vertebrate such as a human or a large veterinary mammal (e.g., horses, cattle, deer, sheep, llamas, goats, pigs)), the use of said carrier, a nanocarrier, a delivery system/vehicle, a compound, a nanoparticle composition, nanoparticles (or lipid nanoparticles) in the manufacture of a medicament for inducing an immune response in a subject, and/or a method of inducing an immune response in a subject, comprising a step of administering (e.g. in a therapeutically effective amount of) said carrier, a nanocarrier, a delivery system/vehicle, a compound, a nanoparticle composition, nanoparticles (or lipid nanoparticles) to a subject in need thereof. In various embodiments, an immune response in the subject is to be induced through the administration of the compound, a nanoparticle composition, nanoparticles (or lipid nanoparticles) thereto. In various embodiments, by inducing an immune response in the subject, the subject is protected against various diseases, disorders or conditions e.g., infectious/contagious diseases, viral infections (i.e. diseases caused by virus), bacterial infections (i.e. diseases caused by bacteria), fungal infections (i.e.

diseases caused by fungi), respiratory diseases or the like, or combinations thereof as mentioned herein. The carrier, nanocarrier, delivery system/vehicle, compound, nanoparticle composition, nanoparticles may be delivered to a subject in the form of or as a component of a vaccine. In various embodiments, the method of inducing an immune response is specific to a coronavirus such as SARS-CoV-2 virus.

In various embodiments, the disease, disorder or condition is mediated by an influenza virus (e.g., influenza A, B, C and/or D virus). For example, the disease may be influenza A, B, C or D such as H1N1, H3N2). In various embodiments, the disease, disorder or condition is mediated by a coronavirus (e.g., severe acute respiratory syndrome coronavirus such as SARS-CoV-2 or SARS-CoV-1). For example, the disease, disorder or condition may be SARS-CoV-2 coronavirus disease.

In various embodiments, the carrier, nanocarrier, delivery system/vehicle, compound, nanoparticle composition, nanoparticles prepared from embodiments of the method disclosed herein comprises one or more of the following characteristics or properties: broad applicability (e.g., can be used to encapsulate, deliver and/or transfect a wide range of therapeutic, prophylactic and/or biological reagents), nanosized, substantially neutral surface charge or negative surface charge, high encapsulation efficiency (e.g., > 80%), high transfection efficiency (e.g., > 80%), high stability, low toxicity (e.g., low cytotoxicity), low production/synthesis cost, therefore making them suitable for *in vivo* applications that require efficient cellular uptake and/or gene transfection.

In various embodiments, the present technology is different from those in the art that use cationic polymer or peptide to condense mRNA directly into nanoparticles. It will be appreciated by a person skilled in the art that such nanoparticles are extremely unstable in aqueous solution, difficult to store, transport and/or use *in vivo*. Furthermore, nanoassemblies of such nanoparticles

do not have a hydrophilic shell, and thereby leading to instability *in vivo*, in storage.

In various embodiments, the present technology is different from those in the art that conjugate lipid to polypeptide that is formed by using a small amine compound as initiator. It will be appreciated that such synthesis routes are impractical, tedious, and the resulting product is difficult to purify, therefore difficult to obtain a pure product. Furthermore, not every polypeptide chain contains a lipid in the product obtained from such synthesis routes.

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In contrast, the present technology comprises the use of lipid as an initiator to polymerize amino acid N-carboxyanhydride (NCA). That is, in various embodiments, lipid is attached to the final product using the lipid as an initiator. Advantageously, in various embodiments therefore, every molecule of the final product will contain the lipid. In various embodiments, the lipid nanoparticles of the present technology are different from those in the art at least in the length of the hydrocarbon chain and/or linker between the hydrophobic tails and the polypeptide.

In various embodiments, the present technology is different from those in the art that use lipid-block-hydrophilic polycarbonate. It will be appreciated by a person skilled in the art that lipid-block-hydrophilic homopolycarbonate leads to the formation of huge mRNA-loaded LNPs, and cannot be used as a substitute/replacement for lipid-PEG conjugate.

25

In various embodiments, the present technology comprises capping the primary amine group in the polypeptide. Advantageously, in various embodiments, the capping of the primary amine group in the polypeptide plays a pivotal role in providing high mRNA transfection efficiency (see for e.g., pDLS2 vs. pDLS1). Without being bound by theory, it is believed that the amine group interacts strongly with mRNA, leading to incomplete intracellular release of mRNA from the LNPs. Advantageously, in various embodiments, the capping of the

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primary amine group in the polypeptide allows embodiments of the present technology (e.g., lipid nanoparticles of the present technology) to be direct substitute/replacement of ALC-0159.

5 Advantageously, in various embodiments, the hydrophilicity/hydrophobicity balance is customizable/tunable/adjustable in the lipid-polypeptide or lipid-PEG conjugate of the present technology. Even more advantageously, the lipid nanoparticles of the present technology require a shorter chain length in the polypeptide (e.g., polyserine) than those in the art to
10 achieve a sufficient/appropriate/suitable degree/level of hydrophilicity for the desired application. In various embodiments, by using pDLS6 with $n = 45$ (which is similar to the length of PEG), mRNA transfection efficiency is significantly lower than pDLS2-5 with a shorter polypeptide chain. Without being bound by theory, it is believed that the hydrophilicity of polyserine is stronger than that of PEG as
15 each serine molecule contains two hydrophilic components (i.e. $-NH-$ and $-OH$), while each monomer of PEG only contains one hydrophilic component (i.e. $-O-$). Therefore, in various embodiments, a chain length of only 18-32 repeated units in polyserine is needed (as compared to 45-50 repeated units in PEG) to provide the sufficient hydrophilicity of the lipid-polypeptide conjugate.

20 In various embodiments, when the repeated units (or Degree of Polymerization - DP) are 45 or greater, the encapsulation efficiency of mRNA and mRNA transfection efficiency are significantly lower than those of lipid-poly(D,L-serine) with polypeptide's DP of 18-32. As an example, three lipid-poly(D,L-serine) are synthesized with different chain lengths of the lipid (C8, C12 and C18
25 in comparison with C14) in the presently disclosed invention. From the results (see e.g., Examples 3 and 4, Tables 14 to 15 and FIG. 24 to 25), there is a critical lipid length of C8. With this length of lipid (the product: C8-pDLS), lipid-poly(D,L-serine) did not form nanoparticles with mRNA (in micron meter size with wide size
30 distribution, PDI: 0.62). In contrast, C12-pDLS, C14-pDLS and C18-pDLS produced nanosized particles with narrow size distributions (PDI < 0.12).

Particularly, C18-pDLS yielded the highest transfection efficiency in both HeLa and macrophage cell lines, followed by C14-pDLS.

In various embodiments, the lipid composition of the present technology involves direct replacement of PEG-lipid conjugate with lipid-*block*-polypeptide in a lipid composition to achieve enhanced encapsulation efficiencies and mRNA transfection efficiencies. Such replacement and technical effects are not taught or expected from the prior art.

10 BRIEF DESCRIPTION OF FIGURES

FIG. 1 shows ^1H NMR of L-Ser-NCA (solvent, DMSO- d_6) in accordance with various embodiments disclosed herein.

15 FIG. 2 shows ^{13}C NMR of L-Ser-NCA (solvent, DMSO- d_6) in accordance with various embodiments disclosed herein.

FIG. 3 shows ^1H NMR of D-Ser-NCA (solvent, DMSO- d_6) in accordance with various embodiments disclosed herein.

20 FIG. 4 shows ^{13}C NMR of D-Ser-NCA (solvent, DMSO- d_6) in accordance with various embodiments disclosed herein.

FIG. 5 shows ^1H NMR of the lipid of 2-amino-N, N-ditetradecylacetamide (solvent, CDCl_3) in accordance with various embodiments disclosed herein.

FIG. 6 shows ^1H NMR of lipid-*block*-poly(*o*-benzyl-D, L-Serine) $_{18}$ (solvent, DMSO- d_6) in accordance with various embodiments disclosed herein.

30 FIG. 7 shows ^1H NMR of lipid-*block*-poly(*o*-benzyl-D, L-Serine) $_{21}$ (solvent, DMSO- d_6) in accordance with various embodiments disclosed herein.

FIG. 8 shows ^1H NMR of lipid-*block*-poly(*o*-benzyl-D, L-Serine)₂₇ (solvent, DMSO-d₆) in accordance with various embodiments disclosed herein.

FIG. 9 shows ^1H NMR of lipid-*block*-poly(*o*-benzyl-D, L-Serine)₃₂ (solvent, DMSO-d₆) in accordance with various embodiments disclosed herein.

FIG. 10 shows ^1H NMR of lipid-*block*-poly(*o*-benzyl-D, L-Serine)₄₅ (solvent, DMSO-d₆) in accordance with various embodiments disclosed herein.

FIG. 11 shows ^1H NMR of lipid-*block*-poly(D, L-Serine)₁₈ [pDLS2] (solvent, DMSO-d₆) in accordance with various embodiments disclosed herein.

FIG. 12 shows ^1H NMR of lipid-*block*-poly(D, L-Serine)₂₁ [pDLS3] (solvent, DMSO-d₆) in accordance with various embodiments disclosed herein.

FIG. 13 shows ^1H NMR of lipid-*block*-poly(D, L-Serine)₂₇ [pDLS4] (solvent, DMSO-d₆) in accordance with various embodiments disclosed herein.

FIG. 14 shows ^1H NMR of lipid-*block*-poly(D, L-Serine)₃₂ [pDLS5] (solvent, DMSO-d₆) in accordance with various embodiments disclosed herein.

FIG. 15 shows ^1H NMR of lipid-*block*-poly(D, L-Serine)₄₅ [pDLS6] (solvent, DMSO-d₆) in accordance with various embodiments disclosed herein.

FIG. 16 shows viability and mRNA transfection efficiency of HELA cells after 48-h transfection with mRNA LNPs in accordance with various embodiments disclosed herein. Statistical significance was determined using the Mann – Whitney test comparing ALC-0159 to other pDLS formulations at 1.0% mole ratio (** $p < 0.01$).

FIG. 17 shows viability and mRNA transfection efficiency of HELA cells after 48-h transfection with mRNA LNPs LNPs in accordance with various

embodiments disclosed herein. Statistical significance was determined using the Mann – Whitney test comparing ALC-0159 to other pDLS formulations at 1.2% mole ratio (** $p < 0.01$).

5 FIG. 18 shows viability and mRNA transfection efficiency of HELA cells after 48-h transfection with mRNA LNPs in accordance with various embodiments disclosed herein. Statistical significance was determined using the Mann – Whitney test comparing ALC-0159 to other pDLS formulations at 1.4% mole ratio (** $p < 0.01$).

10

FIG. 19 shows viability and mRNA transfection efficiency of HELA cells after 48-h transfection with mRNA LNPs in accordance with various embodiments disclosed herein. Statistical significance was determined using the Mann – Whitney test comparing ALC-0159 to other pDLS formulations at 1.6% mole ratio (** $p < 0.01$).

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FIG. 20 shows ^1H NMR of C18-lipid-*block*-poly(D, L-Serine)₃₀ [C18-pDLS] (solvent, DMSO- d_6) LNPs in accordance with various embodiments disclosed herein.

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FIG. 21 shows ^1H NMR of C14-lipid-*block*-poly(D, L-Serine)₃₀ [C14-pDLS or pDLS5] (solvent, DMSO- d_6) LNPs in accordance with various embodiments disclosed herein.

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FIG. 22 shows ^1H NMR of C12-lipid-*block*-poly(D, L-Serine)₃₂ [C12-pDLS] (solvent, DMSO- d_6) in accordance with various embodiments disclosed herein.

FIG. 23 shows ^1H NMR of C8-lipid-*block*-poly(D, L-Serine)₃₄ [C8-pDLS] (solvent, DMSO- d_6) in accordance with various embodiments disclosed herein.

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FIG. 24 shows viability and mRNA transfection efficiency of HeLa cells after 48-h transfection with mRNA LNPs of varying lipid tail length in accordance

with various embodiments disclosed herein. Statistical significance was determined using the Mann – Whitney test comparing ALC-0159 to other pDLS formulations at 1.0% mole ratio (** $p < 0.01$).

5 FIG. 25 shows viability and mRNA transfection efficiency of RAW264.7 cells after 48-h transfection with mRNA LNPs of varying lipid tail length in accordance with various embodiments disclosed herein. Statistical significance was determined using the Mann – Whitney test comparing ALC-0159 to other pDLS formulations at 1.0% mole ratio (** $p < 0.01$).

10

EXAMPLES

Example embodiments of the disclosure will be better understood and readily apparent to one of ordinary skill in the art from the following examples, tables and if applicable, in conjunction with the figures. It should be appreciated that other modifications related to structural, and/or chemical changes may be made without deviating from the scope of the invention. Example embodiments are not necessarily mutually exclusive as some may be combined with one or more embodiments to form new example embodiments. The example
15 embodiments should not be construed as limiting the scope of the disclosure.

The following examples describe the development of a series of lipid-*b*-hydrophilic polypeptide as replacement of PEG-lipid conjugate. The lipid-*b*-polypeptide (pDLS) is formed from ring-opening polymerization of L-Serine NCA and D-Serine NCA using a lipid containing a primary amine group as an initiator.
25 The length of the polypeptide is varied to tune hydrophilicity/hydrophobicity of pDLS. mRNA LNPs encapsulated using the pDLS as a replacement for ALC-0159 showed nanosize, narrow size distribution ($PDI < 0.2$) and greater encapsulation efficiency compared to LNPs formulated with ALC-0159. Transfection efficiencies of mRNA LNPs formulated with pDLS with the optimal length of polypeptide are greater than mRNA LNPs formulated with ALC-0159. The pDLS can serve as a viable replacement for ALC-0159 in the delivery of
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mRNA, potentially reducing the risk of allergic reactions caused by the PEG-lipid conjugate, prolonging the plasma half-life of mRNA LNPs and thus enhancing vaccination efficacy.

5 Advantageously, embodiments of the lipid-*block*- polypeptides disclosed herein are designed to replace/substitute conventional PEG-lipid conjugate such as ALC-0159. The present application demonstrated the use of a series of poly D, L-Serine (pDLS) as a replacement for PEG-conjugated lipids in mRNA lipid nanoparticles (LNPs) at varying mole contents. Encapsulated mRNA with lipid-
10 *block*- polypeptides designed in accordance with various embodiments disclosed herein showed nanosize (< 200nm) and good size distribution (PDI < 0.2) with near neutral zeta potential. mRNA LNPs with lipid-*block*-polypeptides designed in accordance with various embodiments disclosed herein also showed greater encapsulation and *in vitro* transfection efficiency at negligible cytotoxicity.

15

Example 1: Materials and Methods

1.1. Materials

Chemical reagents for the synthesis of the lipids were purchased from
20 Sigma-Aldrich and used as received unless otherwise noted. 1,2-Distearoyl-sn-glycerol-3-phosphocholine (DSPC), cholesterol and ALC-0315 were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Sodium acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Triton®-X100, Tris-EDTA, and VivoGlo Luciferin (In Vivo Grade) were purchased from Promega (Madison,
25 WI, USA). Alamar Blue and Pierce Firefly Luciferase Glow assay kit were purchased from Invitrogen (Waltham, MA, USA). Other reagents used were of analytical grade.

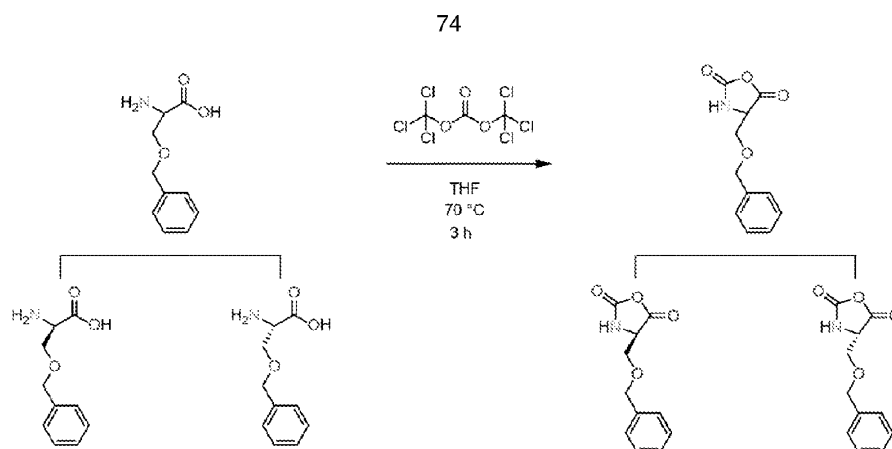
1.2. Synthesis of o-benzyl-L-serine-N-carboxyanhydride (L-Ser-NCA) 30 (Scheme 1)

Synthesis strategy for the L-Ser-NCA is showed in **Scheme 1**. General synthetic method for L-Ser-NCA: o-benzyl-L-serine (6.0 g, 2.53 mmol) was

suspended in 100 mL of dry tetrahydrofuran (THF) and then triphosgene (3.4 g) was added under N₂. The mixture was stirred at 70 °C under a flow of N₂ for 3 h. After the reaction mixture was cooled down to room temperature, the crude product was precipitated by pouring the mixture solution into hexane (800 mL), collected by filtration. The resulting crude product was purified by recrystallizing with THF/hexane mixture for three times. The yield of L-Ser-NCA was 67%. The structure of L-Ser-NCA was verified by ¹H NMR and ¹³C NMR spectra (FIG. 1 and FIG. 2).

10 **1.3. Synthesis of o-benzyl-D-serine-N-carboxyanhydride (D-Ser-NCA)**
(Scheme 1)

Synthesis strategy for the D-Ser-NCA is showed in **Scheme 1**. General synthetic method for D-Ser-NCA: o-benzyl-D-serine (6.0 g, 2.53 mmol) was suspended in 100 mL of dry tetrahydrofuran (THF) and then triphosgene (3.4 g) was added under N₂. The mixture was stirred at 70 °C under a flow of N₂ for 3 h. After the reaction mixture was cooled down to room temperature, the crude product was precipitated by pouring the mixture solution into hexane (800 mL), collected by filtration. The resulting crude product was purified by recrystallizing with THF/hexane mixture for three times. The yield of D-Ser-NCA was 62%. The structure of D-Ser-NCA was verified by ¹H NMR and ¹³C NMR spectra (FIG. 3 and FIG. 4).



Scheme 1. Synthesis strategy for serine-N-carboxyanhydride (Ser-NCA).

1.4. Synthesis of the lipid of 2-amino-N, N-ditetradecylacetamide

tert-Butyl (2-(ditetradecylamino)-2-oxoethyl)carbamate was first synthesized. Boc-glycine (350.3 mg, 2.0 mmol) and ditetradecylamine (819.6 mg, 2.0 mmol) were dissolved in dry dichloromethane (DCM) (40 mL). 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (910.5 mg, 2.4 mmol) and N-ethyl-N-(1-methylethyl)-2-propanamine (DIPEA) (646.1 mg, 5.0 mmol) were added to the solution. The reaction mixture was stirred for 24 h at room temperature under N₂. The mixture was diluted with 50 mL of DCM, and washed with 7% citric acid, brine and H₂O. The resulting organic layer was collected and dried over anhydrous MgSO₄. DCM was evaporated in vacuo to yield crude product. The resulting crude product was purified by flash silica gel column chromatography (Hexane:Diethylether 8:2, v/v), obtaining the final product as a yellow oil. The yield of the compound was 85%.

2-Amino-N, N-ditetradecylacetamide was then synthesized. *tert*-Butyl (2-(ditetradecylamino)-2-oxoethyl)carbamate was dissolved in 5 mL of anhydrous DCM, to which was added 4 mL of trifluoroacetic acid (TFA). The mixture was stirred for 2 h under N₂ atmosphere. The solvents were evaporated in vacuo to yield crude product. The resulting crude product was dissolved in 20 mL of DCM,

and then 20 mL of NaHCO₃ aqueous solution (10%) was added. The mixture was stirred for 12 h under N₂ atmosphere. The organic layer was collected and dried over anhydrous MgSO₄. DCM was evaporated in vacuo. The resulting product was dried under vacuum to obtain a white powder. The yield of the compound
5 was 95%. The structure of product was verified by ¹H NMR spectrum (FIG. 5).

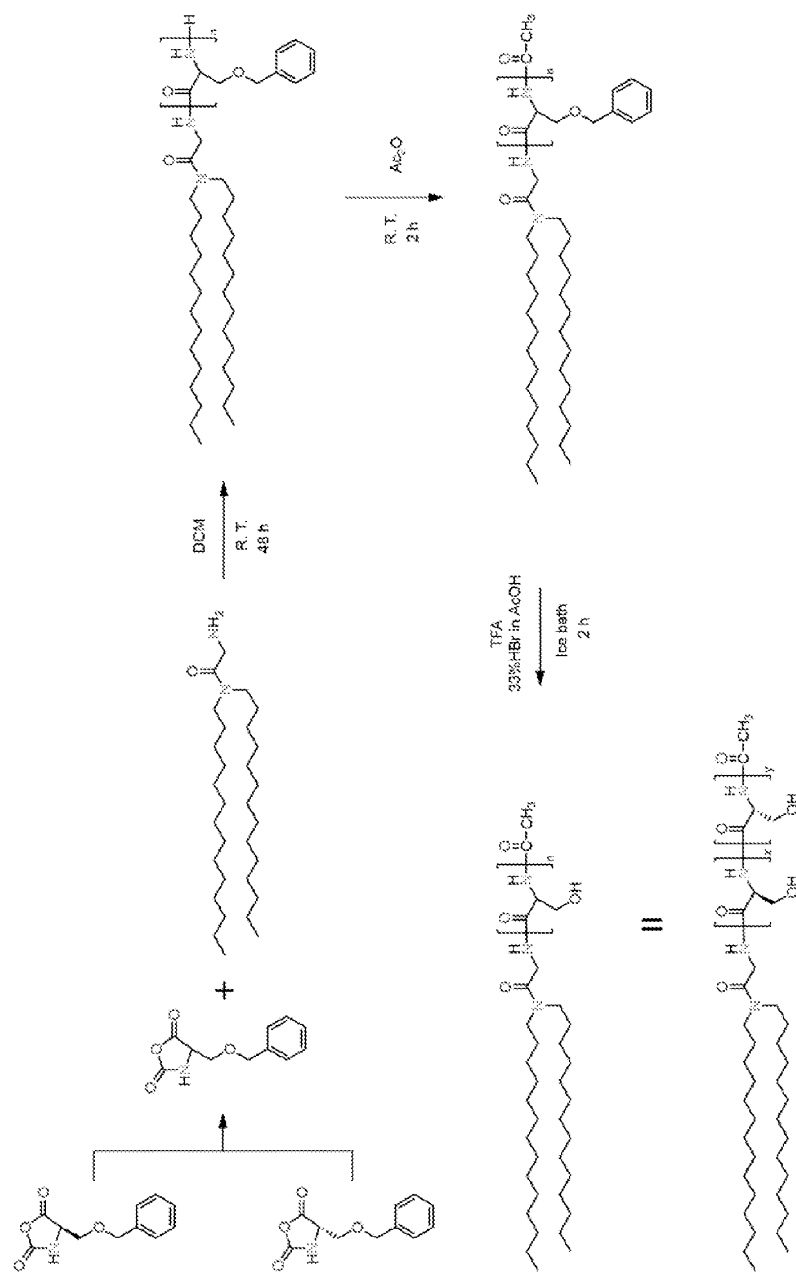
1.5. Synthesis of lipid-*block*-poly(D, L-Serine)

Synthesis strategy for lipid-*block*-poly(D, L-Serine) [lipid-poly(D, L-Ser)] is showed in **Scheme 2**. Lipid-*block*-poly(o-benzyl-D, L-serine) [lipid-poly(o-benzyl-
10 D, L-Ser)] was first synthesized. General synthetic method for lipid-poly(o-benzyl-D, L-Ser): In the glove box, 2-amino-N, N-ditetradecylacetamide (46.6 mg, 0.1 mmol), L-Ser-NCA (221 mg, 1.0 mmol) and D-Ser-NCA (221 mg, 1.0 mmol) were dissolved in 40 mL of anhydrous DCM. The mixture was stirred for 48 h at room temperature in the glove box. Then, 3.0 mL of acetic anhydride was added and
15 the reaction was continued for 2 h. The crude product was precipitated by pouring the mixture solution into glacial ether (300 mL), collected by centrifugation. The resulting crude product was purified by dissolving with DCM and precipitated by pouring the solution into glacial ether. The resulting product was dried under vacuum. The number of polymerization unit for lipid-poly(o-benzyl-D, L-Ser)
20 (protected pDLS) can be adjusted by varying the amount of L-Ser-NCA and D-Ser-NCA. The yields of the protected polypeptides for pDLS2, pDLS3, pDLS4, pDLS5 and pDLS6, were 58%, 63%, 70%, 72% and 78%, respectively. The typical structure of lipid-poly(o-benzyl-D, L-Ser) was verified by ¹H NMR (FIG. 6, FIG. 7, FIG. 8, FIG. 9, FIG. 10 and FIG. 11). The reactant feed ratios for the
25 synthesis of lipid-*block*-poly(o-benzyl-D, L-Serine) are listed in **Table 1**.

Table 1. Reactant feed ratios of lipid-*block*-poly(o-benzyl-D, L-Serine).

Lipid- polypeptide conjugate	Initiator	Monomer 1	Monomer 2	Solvent	
	Lipid (mmol)	L-Ser-NCA (mmol)	D-Ser-NCA (mmol)	DCM (mL)	Reaction time (h)
pDLS1	0.1	1.0	1.0	40	48
pDLS2	0.1	1.5	1.5	40	48
pDLS3	0.1	2.0	2.0	40	48
pDLS4	0.1	2.5	2.5	40	48
pDLS5	0.1	3.0	3.0	40	48
pDLS6	0.1	4.0	4.0	40	48

The deprotection of Lipid-*block*-poly(o-benzyl-D, L-Ser) was then performed to yield lipid-poly(D, L-Ser). General synthetic method for lipid-poly(D, L-Ser) (pDLS): 1.0 g of lipid-poly(o-benzyl-D, L-Ser) was dissolved in 10 mL of TFA, to which was added 3 mL of 33% HBr in acetic acid. The mixture was stirred in ice bath for 2 h. The solvents were removed in vacuo. The resulting crude product was suspended in 30 mL of methanol and then precipitated by pouring the mixture solution into glacial ether (300 mL), collected by centrifugation. The crude product was purified by suspending it in methanol and precipitated by pouring the solution into glacial ether. The obtained product was dried under vacuum. The crude lipid-poly(D, L-Ser) was purified by dialysis with deionized water. The final lipid-poly(D, L-Ser) was obtained by freeze-drying under vacuum. The yields of the deprotected lipid-polypeptide for pDLS2, pDLS3, pDLS4, pDLS5 and pDLS6 were 55%, 58%, 64%, 75% and 87%. The typical structure of lipid-poly(D, L-Ser) was verified by ¹H NMR (FIG. 11, FIG. 12, FIG. 13, FIG. 14 and FIG. 15). Characteristics of the lipid-poly(D, L-Serine) conjugates (pDLS) are listed in **Table 2**.



Scheme 2. Synthesis strategy for lipid-block-poly(D, L-Serine) (pDLS).

Table 2. Characteristics of the lipid-poly(D, L-Serine) (pDLS).

Lipid-polypeptide	<i>Degree of polymerization (n)</i>	<i>Lipid-polypeptide end-group</i>	M_n^{NMR} [g mol ⁻¹]
pDLS1	18	-NH ₂	2033.1
pDLS2	18	-CO-CH ₃	2076.3
pDLS3	21	-CO-CH ₃	2337.5
pDLS4	27	-CO-CH ₃	2860.0
pDLS5	32	-CO-CH ₃	3295.4
pDLS6	45	-CO-CH ₃	4427.4

1.6. Formulation of mRNA-loaded lipid nanoparticles (mRNA LNPs)

5 mRNA LNPs were prepared through manual mixing and the varying mole ratios used are displayed in **Table 3**. The dose of mRNA used in each formulation was standardized to 10 µg. Additionally, the ratio between the ionizable lipid and the mRNA was standardized through an N/P ratio of 6.

10 As a replacement for lipid-PEG conjugate (ALC-0159), lipid-pDLS conjugates were made with varying PDLS length. Lipid-pDLS was mixed with other lipids to make mRNA LNPs at different mole contents of Lipid-pDLS to optimize mRNA LNPs. The mole contents of the other lipids used was adjusted to account for the difference between the mole content of the pDLS compound and the mole content of ALC-0159 (1.6%).

20 During the preparation of the mRNA LNPs, two separate phases were mixed together manually. The organic phase consisted of organic phase (the mixture of lipids dissolved in ethanol to achieve a final volume of 50 µL). The aqueous phase consisted of 140 µL of 10 mM sodium acetate solution at pH 4 containing 1 mg/mL firefly luciferase mRNA (TriLink Biotechnologies) to achieve a final volume of 150 µL. The organic phase was added into the aqueous phase and mixed thoroughly through rapid pipetting. The mRNA LNP solution was then

allowed to incubate at room temperature for 30 minutes to allow for encapsulation and self-assembly of LNPs.

Table 3. Mole ratio (content) of lipids used in formulations.

Lipid	ALC-0315	DSPC	Cholesterol	ALC-0159	pDLS
Molecular weight (g/mol)	766.3	790.2	386.7	2481.0	Refer to Table 2
Mole ratio – ALC-0159 (%)	46.3	9.4	42.7	1.6	–
Mole ratio – pDLS (%)	46.7	9.5	43.1	-	0.8%
	46.6	9.5	42.0		1.0%
	46.5	9.4	42.9		1.2%
	46.4	9.4	42.8		1.4%
	46.3	9.4	42.7		1.6%
Stock concentration (mg/mL)	20.0	10.0	10.0	3.0	10.0

5

1.7. Assessing encapsulation efficiency of mRNA in LNPs

In order to assess the efficacy of the varying lipid mixtures in encapsulating mRNA, the Quant-it™ RiboGreen RNA Assay Kit (Invitrogen, Waltham, MA, USA) was used to determine the encapsulation efficiencies of the formulated mRNA LNPs. The RiboGreen RNA reagent was diluted 200 times with either Tris-EDTA buffer containing 5% Triton-X100 or Tris-EDTA buffer only. 90 µL of the buffer solution containing the RiboGreen reagent was then added to 10 µL of the mRNA LNP sample and incubated at 37°C for 20 min in a black 96-well plate. Following that, the fluorescence intensity of the wells of the plate was read using a microplate reader (Tecan, Männedorf, Switzerland) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The values obtained were then used to calculate the encapsulation efficiencies of mRNA in LNPs based on the equation as follows.

10

15

$$\text{Encapsulation Efficiency} = \frac{\text{Conc}_{\text{Trit}} - \text{Conc}_{\text{TE}}}{\text{Conc}_{\text{Trit}}} \times 100$$

where $\text{Conc}_{\text{Trit}}$ is the concentration of mRNA obtained by adding the mRNA LNPs to 5% Triton-X100 diluted in Tris-EDTA buffer, while Conc_{TE} is the concentration of the respective mRNA obtained by adding the mRNA LNPs to Tris-EDTA buffer without Triton-X100.

1.8. Characterization of mRNA LNPs

The size, polydispersity index (PDI), and zeta potential of the mRNA LNPs were also characterized using a Zetasizer (Malvern, UK). The size and PDI were obtained through dynamic light scattering (DLS) by diluting 25 μL of the mRNA LNPs sample with saline solution to achieve a final volume of 500 μL . The sample was measured three times at 25°C with 20 runs each time. The sample was also measured at 1.68 s per run. The surface zeta potential of the mRNA LNPs was measured by diluting 25 μL of the sample with saline solution to reach a final volume of 1 mL. The samples were also measured three times at 25°C with 20 runs each time when the samples were measured.

1.9. Cell culturing and dosing of HELA cells with mRNA LNPs

To test the cytotoxicity and transfection efficiency of the mRNA LNPs, HELA cells were cultured and dosed with the mRNA LNPs. The HELA cell line was cultured in DMEM media containing 10% Fetal Bovine Serum (FBS) (v/v) and 1% Penicillin/Streptomycin (v/v). The cells were allowed to incubate at 37°C with 5% CO_2 in an incubator (Thermo Fisher, Waltham, MA, USA). The cells were then seeded at 10,000 cells per well in a white 96-well plate for testing mRNA transfection efficiency and in a black 96-well plate for cytotoxicity evaluation of mRNA LNPs. The amount of mRNA LNPs added to each well was standardized to a dose of 100 ng of mRNA per well. After dosing, the 96-well plates were incubated for 48 hours before assessing cell viability and transfection efficiency.

1.10. In vitro viability of HELA cells after incubation with mRNA LNPs

Following 48 hours of incubation, the old media containing the mRNA LNPs was removed from the wells of the black 96-well plate. 100 μ L of solution containing fresh DMEM media with 10% Alamar Blue reagent was added into each well. The plate was then incubated for 2 h at 37°C. Fluorescence intensity was then measured using the microplate reader (Tecan, Männedorf, Switzerland) at an excitation wavelength of 570 nm and an emission wavelength of 600 nm. The cell viability of the treated wells was then expressed as a percentage relative to the negative control wells that did not receive any treatment.

1.11. In vitro transfection efficiency of mRNA LNPs in HELA cells after incubation with mRNA LNPs

Transfection efficiency of mRNA LNPs in HELA cells was measured after 48 hours of incubation by removing media containing mRNA LNPs and replaced with 100 μ L of cell lysis buffer/D-luciferin mixture. The mixture contained 50 μ L of 2x cell lysis buffer diluted with phosphate-buffered saline (PBS) and 50 μ L of 100x D-luciferin diluted with Firefly Assay Buffer. The cells were incubated with the mixture for 10 min at 37°C to allow for cell lysis and signal stabilization. Subsequently, luminescence intensity was read using the microplate reader (Tecan, Männedorf, Switzerland) at an exposure time of 1000 ms.

Example 2: Results and Discussion**2.1. Synthesis of pDLS**

The lipid-*block*-poly(D, L-Serine) (pDLS) was synthesized *via* the ring-opening polymerization (ROP) of *o*-benzyl-L-serine-N-carboxyanhydride and *o*-benzyl-D-serine-N-carboxyanhydride (**Scheme 1**) using the lipid of 2-amino-N, N-ditetradecylacetamide as the initiator, followed by the deprotection of lipid-*block*-poly(*o*-benzyl-D, L-Serine) in TFA and HBr/CH₃COOH (**Scheme 2**). The successful synthesis of D-Ser-NCA and L-Ser-NCA was confirmed by ¹H and ¹³C NMR spectra (FIG. 1, FIG. 2, FIG. 3 and FIG. 4), and that of 2-amino-N, N-ditetradecylacetamide was also verified by ¹H NMR spectrum (FIG. 5). The

structures of lipid-*block*-poly(*o*-benzyl-D, L-Serine)_n were confirmed by ¹H NMR spectroscopy. FIG. 6, FIG. 7, FIG. 8, FIG. 9 and FIG. 10 clearly showed the peaks of **k-o** (-PhH-) from polypeptide and **a** (-CH₃) from lipid at δ 7.24 and 0.84 ppm, respectively, suggested successful synthesis of lipid-poly(*o*-benzyl-D, L-Serine) conjugates. The deprotection of lipid-*block*-poly(*o*-benzyl-D, L-Serine) to form lipid-*block*-poly(D, L-Serine) (pDLS) was also verified by ¹H NMR spectroscopy. As shown in FIG. 11, FIG. 12, FIG. 13, FIG. 14 and FIG. 15, the disappearance of peaks **k-o** (-PhH-) and **e** (Ph-CH₂-) at δ 7.24 and 4.41 ppm, respectively, indicated successful deprotection of lipid-*block*-poly(*o*-benzyl-D, L-Serine). The degrees of polymerization (DPs) of serine in pDLS was adjusted by varying the amount of L-Ser-NCA and D-Ser-NCA. The DPs of serine in lipid-poly(D, L-Serine) were determined by the integration area (peak **g** of the polypeptide; peak **a** of the lipid) (FIG. 11, FIG. 12, FIG. 13, FIG. 14 and FIG. 15). The lipid-poly(D, L-Serine) with different DPs including 18, 21, 27, 32 and 45, were synthesized and denoted as pDLS2, pDLS3, pDLS4, pDLS5 and pDLS6, respectively. Compared to pDLS2, the synthesis process of pDLS1 did not add acetic anhydride to shield the terminal amino group.

2.2. Size, size distribution (PDI), and zeta potential of mRNA LNPs

Using the Zetasizer (Malvern, UK), the size, PDI, and zeta potential of the mRNA LNPs were determined and displayed in **Tables 4 – 7**. All formulations yielded small particle sizes of around 200 nm or below and a narrow size distribution at a PDI < 0.2. Furthermore, all formulations yielded a near neutral zeta potential (or neutral surface charge) at < ±6 mV, which is desirable/ideal for *in vivo* application.

Table 4. Characteristics of manually formulated mRNA LNPs at pDLS mole ratio of 1.0%.

Formulation	Mole ratio (%)	Diameter (nm)	PDI	Zeta potential (mV)
pDLS1	1.0	192 ± 1	0.039 ± 0.032	1.23 ± 1.14
pDLS2		147 ± 1	0.066 ± 0.012	2.91 ± 2.58
pDLS3		168 ± 1	0.098 ± 0.013	4.27 ± 0.54
pDLS4		178 ± 2	0.152 ± 0.031	4.56 ± 0.96
pDLS5		174 ± 2	0.081 ± 0.015	3.76 ± 0.53
pDLS6		126 ± 1	0.179 ± 0.031	3.09 ± 0.84

Table 5. Characteristics of manually formulated mRNA LNPs at pDLS mole ratio of 1.2%.

Formulation	Mole ratio (%)	Diameter (nm)	PDI	Zeta potential (mV)
pDLS1	1.2	203 ± 2	0.063 ± 0.019	2.12 ± 0.89
pDLS2		142 ± 1	0.092 ± 0.013	1.15 ± 0.29
pDLS3		167 ± 2	0.106 ± 0.031	4.33 ± 1.02
pDLS4		184 ± 2	0.189 ± 0.024	5.59 ± 0.52
pDLS5		175 ± 2	0.139 ± 0.024	3.92 ± 0.26
pDLS6		126 ± 2	0.142 ± 0.025	3.03 ± 0.39

Table 6. Characteristics of manually formulated mRNA LNPs at pDLS mole ratio of 1.4%.

Formulation	Mole ratio (%)	Diameter (nm)	PDI	Zeta potential (mV)
pDLS1	1.4	185 ± 1	0.014 ± 0.010	3.06 ± 1.78
pDLS2		157 ± 3	0.054 ± 0.046	2.45 ± 1.15
pDLS3		170 ± 1	0.125 ± 0.017	3.93 ± 1.58
pDLS4		180 ± 1	0.139 ± 0.015	3.93 ± 1.03
pDLS5		172 ± 2	0.112 ± 0.021	4.06 ± 0.54
pDLS6		135 ± 1	0.149 ± 0.008	1.70 ± 2.01

Table 7. Characteristics of manually formulated mRNA LNPs at pDLS mole ratio of 1.6%.

Formulation	Mole ratio (%)	Diameter (nm)	PDI	Zeta potential (mV)
ALC-0159	1.6	104 ± 1	0.140 ± 0.021	0.79 ± 2.41
pDLS1		167 ± 1	0.017 ± 0.009	0.16 ± 0.89
pDLS2		160 ± 2	0.090 ± 0.028	2.72 ± 0.76
pDLS3		153 ± 2	0.119 ± 0.008	4.83 ± 0.37
pDLS4		165 ± 1	0.166 ± 0.047	3.72 ± 1.69
pDLS5		165 ± 2	0.116 ± 0.003	5.52 ± 0.57
pDLS6		133 ± 1	0.156 ± 0.007	1.18 ± 2.23

2.3. Encapsulation efficiency of mRNA in LNPs

5 The encapsulation efficiency of the mRNA LNPs was determined through the RiboGreen Assay and shown in **Tables 8 – 11**. All formulations containing pDLS showed an encapsulation efficiency greater than that of the formulation which employed lipid-PEG conjugate (ALC-0159) (17.5%). This suggests that pDLS provide a better alternative to encapsulating mRNA through manual mixing, allowing for a reduction in the wastage of mRNA as a greater amount of mRNA can be encapsulated in LNPs if pDLS are used. Of the different pDLS compounds used, pDLS6 showed the lowest encapsulation efficiency. This could possibly be explained by the larger length of polypeptide in the pDLS6 compounds (48 subunits), resulting in poorer hydrophilicity/hydrophobicity balance in pDLS6 and then lower encapsulation efficiency of mRNA.

Table 8. Encapsulation efficiencies of mRNA LNPs at mole ratio of 1.0%.

Lipid	Mole ratio (%)	Encapsulation efficiency (%)
pDLS1	1.0	71.7 ± 1.3
pDLS2		70.2 ± 0.7
pDLS3		62.5 ± 0.0
pDLS4		62.5 ± 2.3
pDLS5		69.3 ± 0.1

pDLS6	26.9 ± 2.8
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Table 9. Encapsulation efficiencies of mRNA LNPs at mole ratio of 1.2%.

Lipid	Mole ratio (%)	Encapsulation efficiency (%)
pDLS1	1.2	77.9 ± 1.2
pDLS2		74.5 ± 3.3
pDLS3		67.5 ± 1.9
pDLS4		52.3 ± 7.4
pDLS5		63.2 ± 0.4
pDLS6		18.6 ± 1.5

Table 10. Encapsulation efficiencies of mRNA LNPs at mole ratio of 1.4%.

Lipid	Mole ratio (%)	Encapsulation efficiency (%)
pDLS1	1.4	70.4 ± 3.3
pDLS2		81.4 ± 0.2
pDLS3		69.3 ± 0.4
pDLS4		60.0 ± 1.0
pDLS5		71.3 ± 0.4
pDLS6		29.2 ± 0.2

5

Table 11. Encapsulation efficiencies of mRNA LNPs at mole ratio of 1.6%.

Lipid	Mole ratio (%)	Encapsulation efficiency (%)
ALC-0159	1.6	17.5 ± 6.0
pDLS1		74.2 ± 0.1
pDLS2		66.8 ± 1.3
pDLS3		56.9 ± 1.3
pDLS4		67.8 ± 0.3
pDLS5		57.6 ± 4.1
pDLS6		23.7 ± 5.2

2.4. *In vitro* cytocompatibility and transfection efficiency of mRNA LNPs in HELA cells

To determine the cytotoxicity and transfection efficiency of pDLS compounds as a replacement for ALC-0159, HELA cells were dosed with the formulated mRNA LNPs. The results for the cell viability and luminescence intensity assays are displayed in FIG. 16, FIG. 17, FIG. 18 and FIG. 19. All formulations showed negligible cytotoxicity. Furthermore, except for pDLS1, all other pDLS formulations showed significantly greater transfection efficiency compared to ALC-0159. This suggests that pDLS as a replacement for ALC-0159 is able to achieve greater transfection efficiency of mRNA in HELA cells with mRNA LNPs. pDLS1 mediated lower mRNA transfection efficiency than pDLS2 possibly due to the presence of a primary amine group in the polypeptide in pDLS1, which might bind mRNA too strongly to release it in the cytosol. In addition, pDLS2-pDLS5 induced greater mRNA transfection efficiency than pDLS6 of a longer hydrophilic polypeptide (45 subunits), indicating that there is a balance between hydrophobicity and hydrophilicity to achieve an optimal transfection efficiency.

Example 3: Materials and Methods

3.1. Materials

Chemical reagents for the synthesis of the lipids were purchased from Sigma-Aldrich and used as received unless otherwise noted. 1,2-Distearoyl-sn-glycerol-3-phosphocholine (DSPC), cholesterol and ALC-0315 were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Sodium acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Triton®-X100 and Tris-EDTA were purchased from Promega (Madison, WI, USA). Alamar Blue and Pierce Firefly Luciferase Glow assay kit were purchased from Invitrogen (Waltham, MA, USA). Other reagents used were of analytical grade.

3.2. Synthesis of C18-lipid-*block*-poly(D, L-Serine)

Synthesis strategy for C18-lipid-*block*-poly(D, L-Serine) [C18-lipid-poly(D, L-Ser)] is showed in **Scheme 3**. C18-Lipid-*block*-poly(o-benzyl-D, L-serine) [C18-lipid-poly(o-benzyl-D, L-Ser)] was first synthesized. General synthetic method for

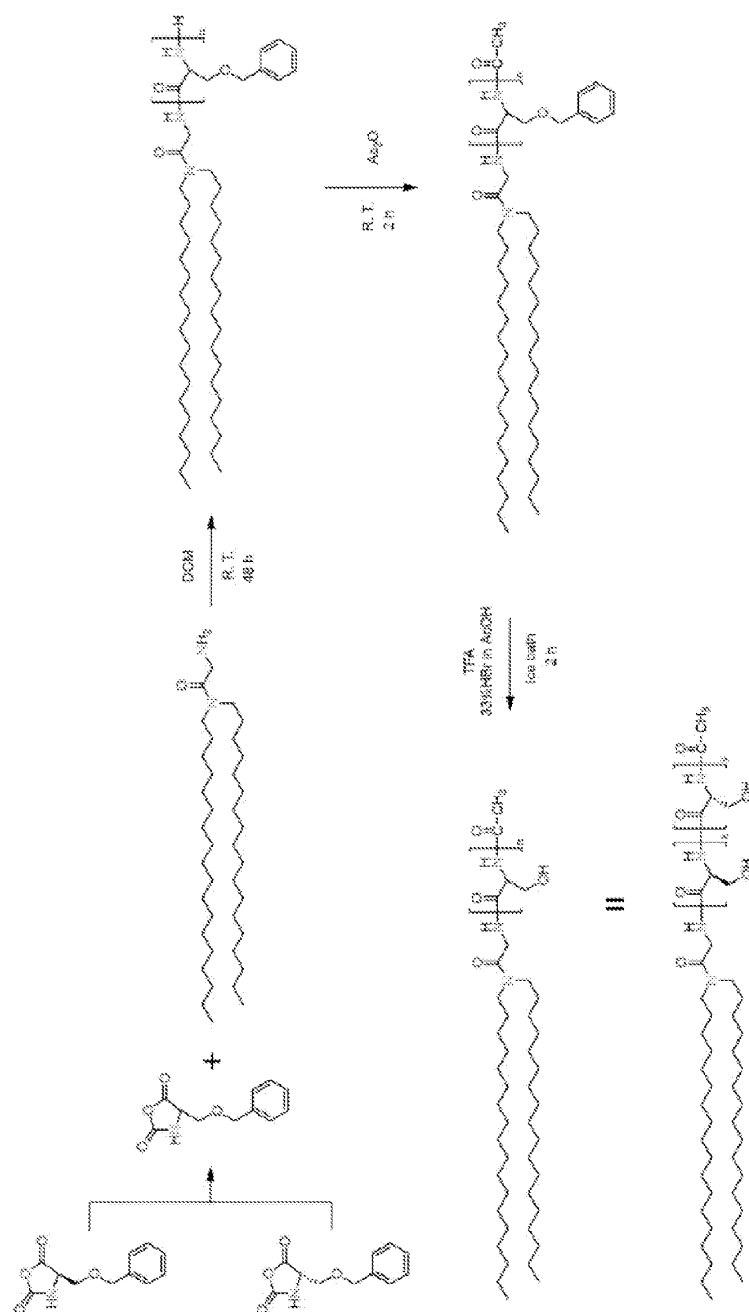
5 C18-lipid-poly(o-benzyl-D, L-Ser): In the glove box, 2-amino-N, N-dioctadecylacetamide (57.9 mg, 0.1 mmol), L-Ser-NCA (663 mg, 1.0 mmol) and D-Ser-NCA (663 mg, 1.0 mmol) were dissolved in 40 mL of anhydrous DCM. The mixture was stirred for 48 h at room temperature in the glove box. Then, 3.0 mL of acetic anhydride was added and the reaction was continued for 2 h. The crude

10 product was precipitated by pouring the mixture solution into glacial ether (300 mL), collected by centrifugation. The resulting crude product was purified by dissolving with DCM and precipitated by pouring the solution into glacial ether. The resulting product was dried under vacuum.

15 The deprotection of C18-lipid-*block*-poly(o-benzyl-D, L-Ser) was then performed to yield C18-lipid-poly(D, L-Ser). General synthetic method for C18-lipid-poly(D, L-Ser) (C18-pDLS): 1.0 g of C18-lipid-poly(o-benzyl-D, L-Ser) was dissolved in 10 mL of TFA, to which was added 3 mL of 33% HBr in acetic acid. The mixture was stirred in ice bath for 2 h. The solvents were removed in vacuo.

20 The resulting crude product was suspended in 30 mL of methanol and then precipitated by pouring the mixture solution into glacial ether (300 mL), collected by centrifugation. The crude product was purified by suspending it in methanol and precipitated by pouring the solution into glacial ether. The obtained product was dried under vacuum. The crude C18-lipid-poly(D, L-Ser) was purified by

25 dialysis with deionized water. The final C18-lipid-poly(D, L-Ser) was obtained by freeze-drying under vacuum. The yields of the deprotected lipid-polypeptide for C18-pDLS was 85%. The typical structure of C18-lipid-poly(D, L-Ser) was verified by ¹H NMR (FIG. 20). Characteristics of the C18-lipid-poly(D, L-Serine) conjugates (C18-pDLS) are listed in **Table 12**.

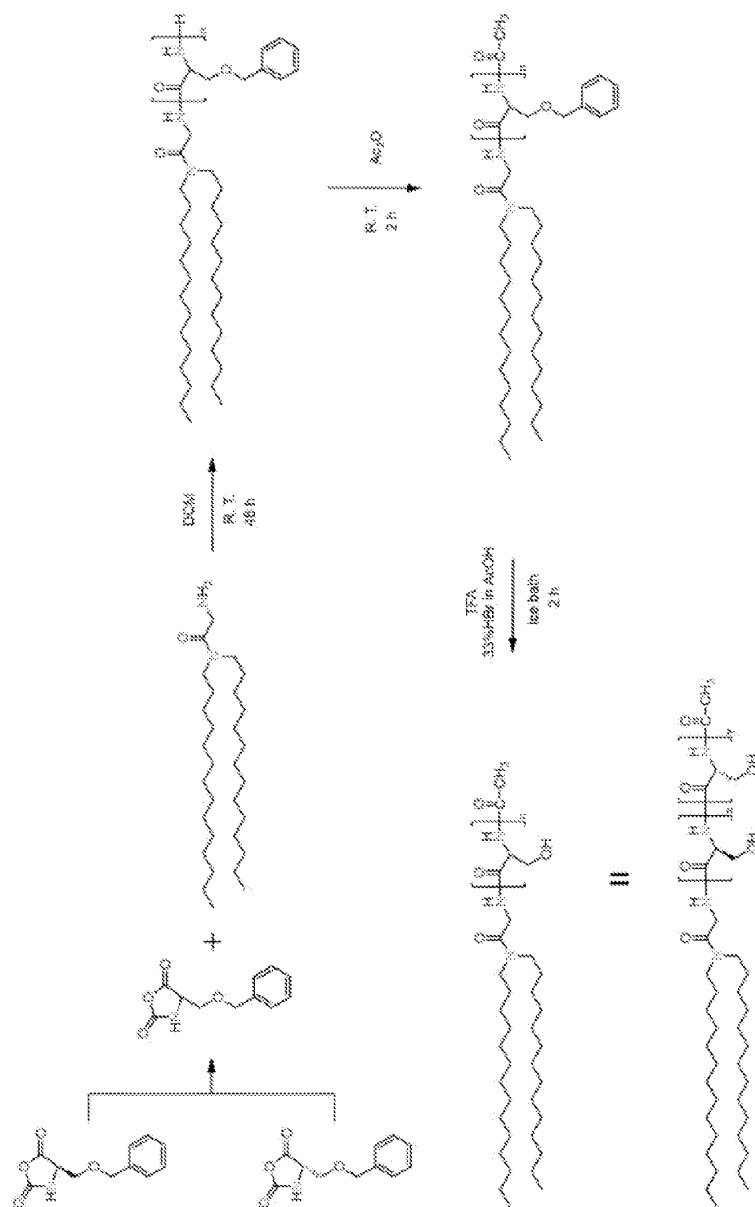


Scheme 3. Synthesis strategy for C18-lipid-block-poly(D,L-Serine) (C18-pDLS).

3.3. Synthesis of C14-lipid-*block*-poly(D, L-Serine)

Synthesis strategy for C14-lipid-*block*-poly(D, L-Serine) [C14-lipid-poly(D, L-Ser)] is showed in **Scheme 4**. C14-Lipid-*block*-poly(o-benzyl-D, L-serine) [C14-lipid-poly(o-benzyl-D, L-Ser)] was first synthesized. General synthetic
5 method for C14-lipid-poly(o-benzyl-D, L-Ser): In the glove box, 2-amino-N, N-ditetradecylacetamide (46.6 mg, 0.1 mmol), L-Ser-NCA (663 mg, 1.0 mmol) and D-Ser-NCA (663 mg, 1.0 mmol) were dissolved in 40 mL of anhydrous DCM. The mixture was stirred for 48 h at room temperature in the glove box. Then, 3.0 mL of acetic anhydride was added and the reaction was continued for 2 h. The
10 crude product was precipitated by pouring the mixture solution into glacial ether (300 mL), collected by centrifugation. The resulting crude product was purified by dissolving with DCM and precipitated by pouring the solution into glacial ether. The resulting product was dried under vacuum.

15 The deprotection of C14-lipid-*block*-poly(o-benzyl-D, L-Ser) was then performed to yield lipid-poly(D, L-Ser). General synthetic method for C14-lipid-poly(D, L-Ser) (C14-pDLS): 1.0 g of C14-lipid-poly(o-benzyl-D, L-Ser) was dissolved in 10 mL of TFA, to which was added 3 mL of 33% HBr in acetic acid. The mixture was stirred in ice bath for 2 h. The solvents were removed in vacuo.
20 The resulting crude product was suspended in 30 mL of methanol and then precipitated by pouring the mixture solution into glacial ether (300 mL), collected by centrifugation. The crude product was purified by suspending it in methanol and precipitated by pouring the solution into glacial ether. The obtained product was dried under vacuum. The crude C14-lipid-poly(D, L-Ser) was purified by
25 dialysis with deionized water. The final C18-lipid-poly(D, L-Ser) was obtained by freeze-drying under vacuum. The yields of the deprotected lipid-polypeptide for C14-pDLS was 82%. The typical structure of C14-lipid-poly(D, L-Ser) was verified by ¹H NMR (FIG. 21). Characteristics of the C14-lipid-poly(D, L-Serine) conjugates (C14-pDLS) are listed in **Table 12**.

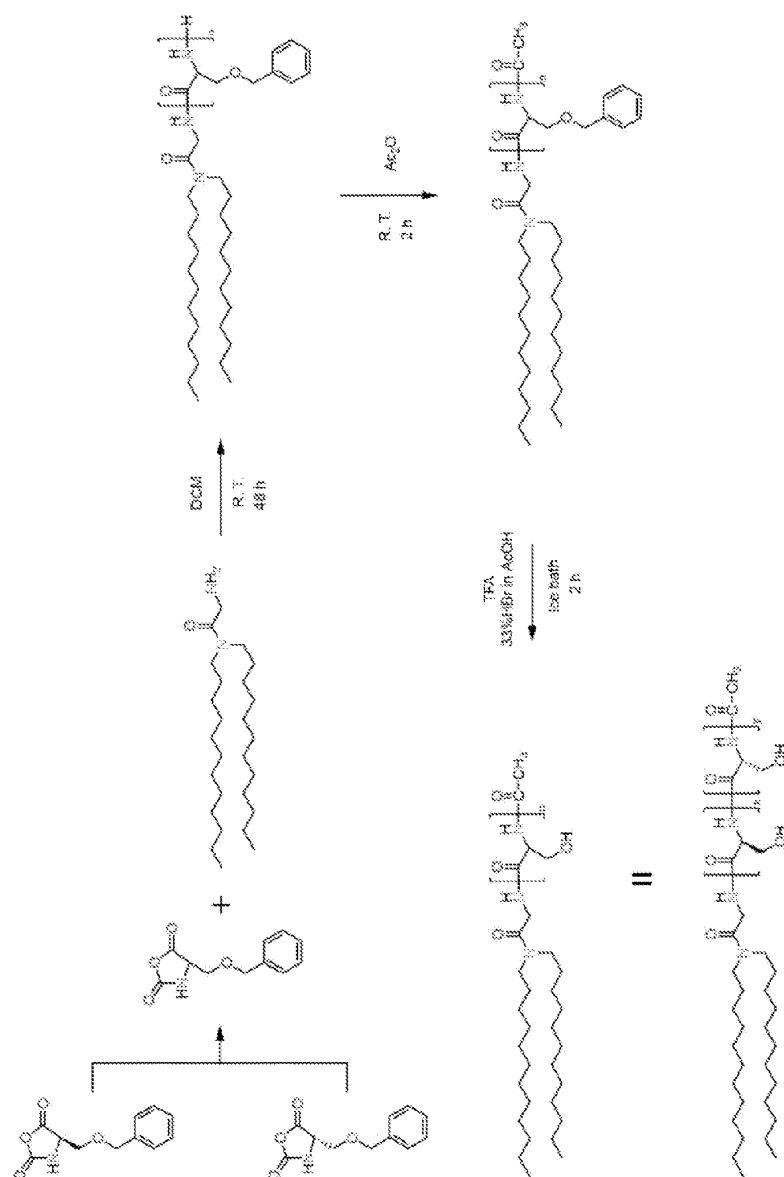


Scheme 4. Synthesis strategy for C14-lipid-block-poly(D,L-Serine) (C14-pDLS).

3.4. Synthesis of C12-lipid-*block*-poly(D, L-Serine)

Synthesis strategy for C12-lipid-*block*-poly(D, L-Serine) [C12-lipid-poly(D, L-Ser)] is shown in **Scheme 5**. C12-Lipid-*block*-poly(o-benzyl-D, L-serine) [C12-lipid-poly(o-benzyl-D, L-Ser)] was first synthesized. General synthetic method for
5 C12-lipid-poly(o-benzyl-D, L-Ser): In the glove box, 2-amino-N,N-didodecylacetamide (41 mg, 0.1 mmol), L-Ser-NCA (663 mg, 1.0 mmol) and D-Ser-NCA (663 mg, 1.0 mmol) were dissolved in 40 mL of anhydrous DCM. The mixture was stirred for 48 h at room temperature in the glove box. Then, 3.0 mL of acetic anhydride was added and the reaction was continued for 2 h. The crude
10 product was precipitated by pouring the mixture solution into glacial ether (300 mL), collected by centrifugation. The resulting crude product was purified by dissolving with DCM and precipitated by pouring the solution into glacial ether. The resulting product was dried under vacuum.

15 The deprotection of C12-lipid-*block*-poly(o-benzyl-D, L-Ser) was then performed to yield C12-lipid-poly(D, L-Ser). General synthetic method for C12-lipid-poly(D, L-Ser) (C12-pDLS): 1.0 g of C12-lipid-poly(o-benzyl-D, L-Ser) was dissolved in 10 mL of TFA, to which was added 3 mL of 33% HBr in acetic acid. The mixture was stirred in ice bath for 2 h. The solvents were removed in vacuo.
20 The resulting crude product was suspended in 30 mL of methanol and then precipitated by pouring the mixture solution into glacial ether (300 mL), collected by centrifugation. The crude product was purified by suspending it in methanol and precipitated by pouring the solution into glacial ether. The obtained product was dried under vacuum. The crude C12-lipid-poly(D, L-Ser) was purified by
25 dialysis with deionized water. The final C12-lipid-poly(D, L-Ser) was obtained by freeze-drying under vacuum. The yields of the deprotected lipid-polypeptide for C12-pDLS was 80%. The typical structure of C12-lipid-poly(D, L-Ser) was verified by ¹H NMR (FIG. 22). Characteristics of the C12-lipid-poly(D, L-Serine) conjugates (C12-pDLS) are listed in **Table 12**.

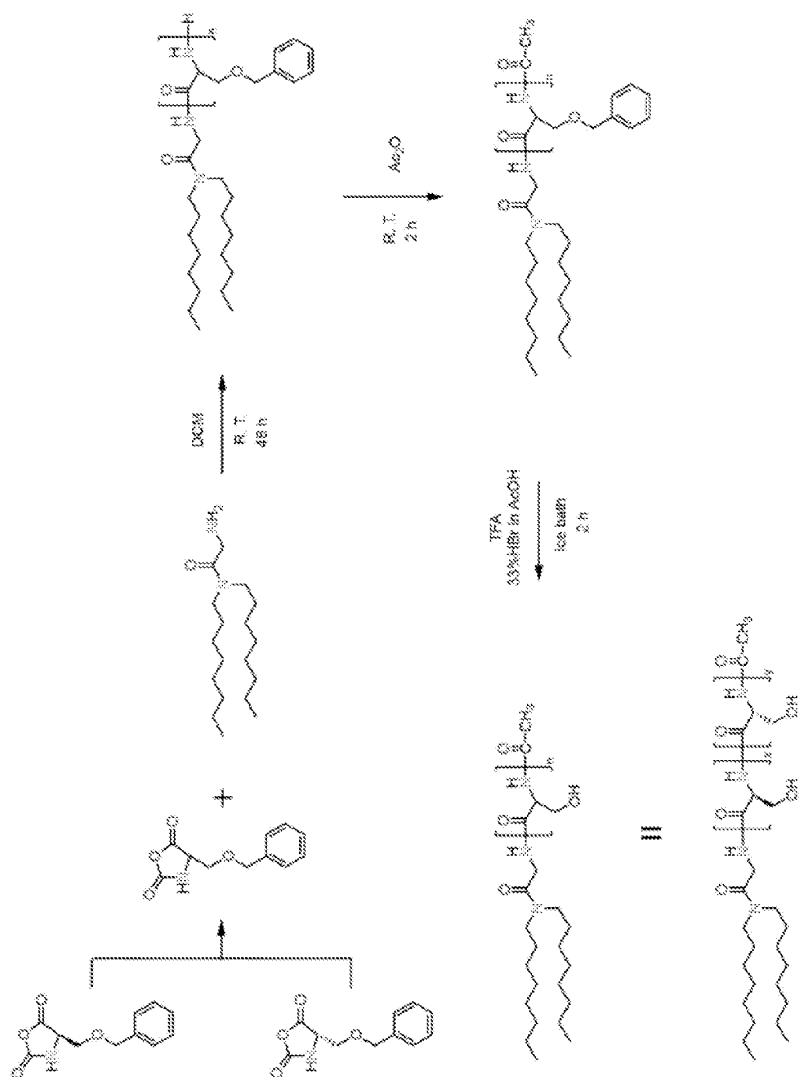


Scheme 5. Synthesis strategy for C12-lipid-block-poly(D,L-Serine) (C12-pDLS).

3.5. Synthesis of C8-lipid-*block*-poly(D, L-Serine)

Synthesis strategy for C8-lipid-*block*-poly(D, L-Serine) [C8-lipid-poly(D, L-Ser)] is showed in **Scheme 6**. C8-Lipid-*block*-poly(o-benzyl-D, L-serine) [C8-lipid-poly(o-benzyl-D, L-Ser)] was first synthesized. General synthetic method for C8-lipid-poly(o-benzyl-D, L-Ser): In the glove box, 2-amino-N,N-dioctylacetamide (29.8 mg, 0.1 mmol), L-Ser-NCA (663 mg, 1.0 mmol) and D-Ser-NCA (663 mg, 1.0 mmol) were dissolved in 40 mL of anhydrous DCM. The mixture was stirred for 48 h at room temperature in the glove box. Then, 3.0 mL of acetic anhydride was added and the reaction was continued for 2 h. The crude product was precipitated by pouring the mixture solution into glacial ether (300 mL), collected by centrifugation. The resulting crude product was purified by dissolving with DCM and precipitated by pouring the solution into glacial ether. The resulting product was dried under vacuum.

The deprotection of C8-lipid-*block*-poly(o-benzyl-D, L-Ser) was then performed to yield C8-lipid-poly(D, L-Ser). General synthetic method for C8-lipid-poly(D, L-Ser) (C8-pDLS): 1.0 g of C8-lipid-poly(o-benzyl-D, L-Ser) was dissolved in 10 mL of TFA, to which was added 3 mL of 33% HBr in acetic acid. The mixture was stirred in ice bath for 2 h. The solvents were removed in vacuo. The resulting crude product was suspended in 30 mL of methanol and then precipitated by pouring the mixture solution into glacial ether (300 mL), collected by centrifugation. The crude product was purified by suspending it in methanol and precipitated by pouring the solution into glacial ether. The obtained product was dried under vacuum. The crude C8-lipid-poly(D, L-Ser) was purified by dialysis with deionized water. The final C8-lipid-poly(D, L-Ser) was obtained by freeze-drying under vacuum. The yields of the deprotected lipid-polypeptide for C8-pDLS was 86%. The typical structure of C8-lipid-poly(D, L-Ser) was verified by ¹H NMR (FIG. 23). Characteristics of the C8-lipid-poly(D, L-Serine) conjugates (C8-pDLS) are listed in **Table 12**.



Scheme 6. Synthesis strategy for C8-lipid-block-poly(D, L-Serine) (C8-pDLS).

Table 12. Characteristics of the lipid-poly(D, L-Serine) with alkyl chain length.

Lipid-polypeptide	Degree of polymerization (n)	Lipid- polypeptide end-group	M_n^{NMR} [g mol ⁻¹]
C18-pDLS	30	-CO-CH ₃	3191
C14-pDLS	32	-CO-CH ₃	3253
C12-pDLS	32	-CO-CH ₃	3197
C8-pDLS	34	-CO-CH ₃	3259

3.6. Formulation of mRNA-loaded lipid nanoparticles (mRNA LNPs)

mRNA LNPs were prepared through manual mixing and the varying mole ratios used are displayed in **Table 13**. The dose of mRNA used in each formulation was standardized to 10 µg. Additionally, the ratio between the ionizable lipid and the mRNA was standardized through an N/P ratio of 6.

As a replacement for lipid-PEG conjugate (ALC-0159), lipid-pDLS conjugates were made with varying PDLS length. Lipid-pDLS was mixed with other lipids to make mRNA LNPs at a mole content of 1.6%.

During the preparation of the mRNA LNPs, two separate phases were mixed together manually. The organic phase consisted of organic phase (the mixture of lipids dissolved in ethanol to achieve a final volume of 50 µL). The aqueous phase consisted of 140 µL of 10 mM sodium acetate solution at pH 4 containing 1 mg/mL firefly luciferase mRNA (TriLink Biotechnologies) to achieve a final volume of 150 µL. The organic phase was added into the aqueous phase and mixed thoroughly through rapid pipetting. The mRNA LNP solution was then allowed to incubate at room temperature for 30 minutes to allow for encapsulation and self-assembly of LNPs.

Table 13. Mole ratio (content) of lipids used in formulations.

Lipid	ALC-0315	DSPC	Cholesterol	ALC-0159	pDLS
					Refer to
Molecular weight (g/mol)	766.3	790.2	386.7	2481.0	Table 12
Mole ratio (%)	46.3	9.4	42.7	1.6	–
Stock concentration (mg/mL)	20.0	10.0	10.0	3.0	5.0

3.7. Assessing encapsulation efficiency of mRNA in LNPs

In order to assess the efficacy of the varying lipid mixtures in encapsulating mRNA, the Quant-it™ RiboGreen RNA Assay Kit (Invitrogen, Waltham, MA, USA) was used to determine the encapsulation efficiencies of the formulated mRNA LNPs. The RiboGreen RNA reagent was diluted 200 times with either Tris-EDTA buffer containing 5% Triton-X100 or Tris-EDTA buffer only. 90 µL of the buffer solution containing the RiboGreen reagent was then added to 10 µL of the mRNA LNP sample and incubated at 37°C for 20 min in a black 96-well plate. Following that, the fluorescence intensity of the wells of the plate was read using a microplate reader (Tecan, Männedorf, Switzerland) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The values obtained were then used to calculate the encapsulation efficiencies of mRNA in LNPs based on the equation as follows.

$$\text{Encapsulation Efficiency} = \frac{\text{Conc}_{\text{Trit}} - \text{Conc}_{\text{TE}}}{\text{Conc}_{\text{Trit}}} \times 100$$

where $\text{Conc}_{\text{Trit}}$ is the concentration of mRNA obtained by adding the mRNA LNPs to 5% Triton-X100 diluted in Tris-EDTA buffer, while Conc_{TE} is the concentration of the respective mRNA obtained by adding the mRNA LNPs to Tris-EDTA buffer without Triton-X100.

3.8. Characterization of mRNA LNPs

The size, polydispersity index (PDI), and zeta potential of the mRNA LNPs were also characterized using a Zetasizer (Malvern, UK). The size and PDI were obtained through dynamic light scattering (DLS) by diluting 25 μ L of the mRNA LNPs sample with saline solution to achieve a final volume of 500 μ L. The sample was measured three times at 25°C with 20 runs each time. The sample was also measured at 1.68 s per run. The surface zeta potential of the mRNA LNPs was measured by diluting 25 μ L of the sample with saline solution to reach a final volume of 1 mL. The samples were also measured three times at 25°C with 20 runs each time when the samples were measured.

3.9. Cell culturing and dosing of HeLa and RAW264.7 cells with mRNA LNPs

To test the cytotoxicity and transfection efficiency of the mRNA LNPs, HeLa and RAW264.7 cells were cultured and dosed with the mRNA LNPs. The HeLa and RAW264.7 cell line was cultured in DMEM media containing 10% Fetal Bovine Serum (FBS) (v/v) and 1% Penicillin/Streptomycin (v/v). The cells were allowed to incubate at 37°C with 5% CO₂ in an incubator (Thermo Fisher, Waltham, MA, USA). The cells were then seeded at 10,000 cells per well in a white 96-well plate for testing mRNA transfection efficiency and in a black 96-well plate for cytotoxicity evaluation of mRNA LNPs. The amount of mRNA LNPs added to each well was standardized to a dose of 100 ng of mRNA per well. After dosing, the 96-well plates were incubated for 48 hours before assessing cell viability and transfection efficiency.

3.10. In vitro viability of HeLa and RAW264.7 cells after incubation with mRNA LNPs

Following 48 hours of incubation, the old media containing the mRNA LNPs was removed from the wells of the black 96-well plate. 100 μ L of solution containing fresh DMEM media with 10% Alamar Blue reagent was added into each well. The plate was then incubated for 2 h at 37°C. Fluorescence intensity was then measured using the microplate reader (Tecan, Männedorf, Switzerland)

at an excitation wavelength of 570 nm and an emission wavelength of 600 nm. The cell viability of the treated wells was then expressed as a percentage relative to the negative control wells that did not receive any treatment.

5 **3.11. In vitro transfection efficiency of mRNA LNPs in HeLa and RAW264.7 cells after incubation with mRNA LNPs**

Transfection efficiency of mRNA LNPs in HeLa and RAW264.7 cells was measured after 48 hours of incubation by removing media containing mRNA LNPs and replaced with 100 μ L of cell lysis buffer/D-luciferin mixture. The
10 mixture contained 50 μ L of 2x cell lysis buffer diluted with phosphate-buffered saline (PBS) and 50 μ L of 100x D-luciferin diluted with Firefly Assay Buffer. The cells were incubated with the mixture for 10 min at 37°C to allow for cell lysis and signal stabilization. Subsequently, luminescence intensity was read using the microplate reader (Tecan, Männedorf, Switzerland) at an exposure time of 1000
15 ms.

Example 4: Results and Discussion

4.1. Synthesis of pDLS with different lipid alkyl chain tail length

20 The lipid-*block*-poly(D, L-Serine) with different lipid alkyl chain tail length was synthesized *via* the ring-opening polymerization (ROP) of *o*-benzyl-L-serine-N-carboxyanhydride and *o*-benzyl-D-serine-N-carboxyanhydride using the lipid with different alkyl chain tail length as the initiator, followed by the deprotection of lipid-*block*-poly(*o*-benzyl-D, L-Serine) in TFA and
25 HBr/CH₃COOH (**Schemes 3, 4, 5 and 6**). The lipid-poly(D, L-Serine) with different lipid alkyl chain tail length including 18, 14, 12 and 8, were synthesized and denoted as C18-pDLS, C14-pDLS, C12-pDLS and C8-pDLS, respectively. The successful synthesis of C18-pDLS, C14-pDLS, C12-pDLS and C8-pDLS were verified by ¹H NMR spectrum (FIG. 20, FIG. 21, FIG. 22 and FIG. 23).
30 Characteristics of the lipid-poly(D, L-Serine) with different lipid alkyl chain tail length conjugates are listed in **Table 12**.

4.2. Size, size distribution (PDI), and zeta potential of mRNA LNPs

Using the Zetasizer (Malvern, UK), the size, PDI, and zeta potential of the mRNA LNPs were determined and displayed in **Table 14**. Most formulations yielded small particle sizes of around 200 nm or below and a narrow size distribution at a PDI < 0.2, except for the LNP formulations which included C8-pDLS, showing a size of 1166 nm and a PDI of 0.620. This suggests that there is a lower limit of the lipid tail length before formulation of stable nanoparticles is no longer possible. Furthermore, all formulations yielded a near neutral zeta potential at < ± 4 mV, which is desirable/ideal for *in vivo* application.

Table 14. Characteristics of manually formulated mRNA LNPs at pDLS mole ratio of 1.6% with varying lipid tail length.

Formulation	Mole ratio (%)	Diameter (nm)	PDI	Zeta potential (mV)
ALC-0159		106 $\pm$ 1	0.076 $\pm$ 0.059	0.51 $\pm$ 1.17
C8-pDLS		1166 $\pm$ 67	0.620 $\pm$ 0.028	0.78 $\pm$ 1.16
C12-pDLS	1.6	140 $\pm$ 2	0.113 $\pm$ 0.016	0.76 $\pm$ 0.85
C14-pDLS		168 $\pm$ 2	0.119 $\pm$ 0.035	3.31 $\pm$ 0.00
C18-pDLS		173 $\pm$ 3	0.081 $\pm$ 0.048	-2.91 $\pm$ 0.00

4.2. Encapsulation efficiency of mRNA in LNPs

The encapsulation efficiency of the mRNA LNPs was determined through the RiboGreen Assay and shown in **Table 15**. All formulations containing pDLS showed an encapsulation efficiency greater than that of the formulation which employed lipid-PEG conjugate (ALC-0159) (71.8%). This suggests that pDLS provide a better alternative to encapsulating mRNA through manual mixing, allowing for a reduction in the wastage of mRNA as a greater amount of mRNA can be encapsulated in LNPs if pDLS are used. Of the different pDLS compounds used, C18-pDLS showed the lowest encapsulation efficiency. This suggests that encapsulation efficiency starts to drop after an upper limit of lipid tail length. Taken together with the results of the physiochemical properties of the tested LNPs, the results elucidate a range of viable lipid tail length in which stable lipid

nanoparticles can be formed. This range could possibly be greater than C8 and lower than C18.

Table 15. Encapsulation efficiencies of mRNA LNPs at mole ratio of 1.6% with varying lipid tail length.

Lipid	Mole ratio (%)	Encapsulation efficiency (%)
ALC-0159	1.6	71.8 ± 0.4
C8-pDLS		86.4 ± 0.1
C12-pDLS		80.5 ± 0.3
C14-pDLS		82.1 ± 0.9
C18-pDLS		73.4 ± 0.2

5

4.4. In vitro cytocompatibility and transfection efficiency of mRNA LNPs in HeLa and RAW264.7 cells

To determine the cytotoxicity and transfection efficiency of pDLS compounds as a replacement for ALC-0159, HeLa and RAW264.7 cells were dosed with the formulated mRNA LNPs. The results for the cell viability and luminescence intensity assays are displayed in FIG. 24 and FIG. 25. All formulations showed negligible cytotoxicity. Furthermore, except for C12-pDLS, all other pDLS formulations showed significantly greater transfection efficiency compared to ALC-0159. The results also seem to highlight a trend of longer lipid tails resulting in greater transfection efficiencies. C8-pDLS does not appear to fit this trend possible due to its larger size, resulting in the aggregation of nanoparticles to form a microparticle that would deliver a greater dose of mRNA upon its transfection of the cell. The results suggest that pDLS as a replacement for ALC-0159 is able to achieve greater transfection efficiency of mRNA in HeLa and RAW264.7 cells with mRNA LNPs, reaching transfection efficiencies 2x in HeLa cells and 20x in RAW264.7 cells greater than ALC-0159 in the case of C18-pDLS.

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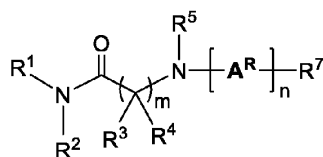
Example 5: Summary

The mRNA LNPs formulated with pDLS compounds to replace ALC-0159 showed nanosize (< 200 nm), narrow size distribution ($PDI < 0.2$), and near neutral zeta potentials ($< \pm 10$ mV). Furthermore, pDLS formulations yielded greater encapsulation efficiencies compared to ALC0159 formulation and showed significantly greater mRNA transfection efficiencies with negligible cytotoxicity in both HeLa and RAW264.7 cells. These results demonstrate that pDLS can serve as a replacement that surpasses ALC-0159 in terms of efficiency in encapsulating mRNA to reduce mRNA wastage and transfecting cells with mRNA to produce a greater amount of proteins at a similar dose. The results also indicate a viable range of lipid tail length by increasing the tail length to achieve greater transfection efficiencies while keeping it below an upper limit to maintain an acceptable encapsulation efficiency. Applied in a clinical context, this can potentially reduce the risk of immunogenic reactions caused by the PEG-conjugated lipid ALC-0159. Commercial use of mRNA LNPs formulated with pDLS may yield mRNA vaccines that address allergic reactions induced by the current mRNA vaccines available in the market.

It will be appreciated by a person skilled in the art that other variations and/or modifications may be made to the embodiments disclosed herein without departing from the spirit or scope of the disclosure as broadly described. For example, in the description herein, features of different exemplary embodiments may be mixed, combined, interchanged, incorporated, adopted, modified, included etc. or the like across different exemplary embodiments. The present embodiments are, therefore, to be considered in all respects to be illustrative and not restrictive.

CLAIMS

1. A compound represented by general formula (1) for preparing lipid nanoparticles encapsulating a therapeutic, prophylactic and/or biological agent:



(1)

wherein

A^R comprises a unit from a poly(amino acid);

R¹ and **R²** are each independently a hydrophobic group;

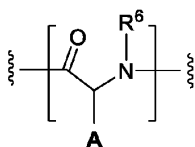
R³, **R⁴**, and **R⁵** are each independently H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl;

R⁷ is **−H** or **−C(=O)R⁸**, wherein **R⁸** is optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl or optionally substituted alkoxy;

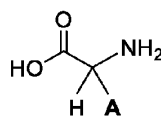
m ≥ 1; and

n ≥ 1.

2. The compound of claim 1, wherein **A^R** comprises a structure that is represented by general formula (2), and wherein **A** comprises a hydrophilic organic group that is part of an amino acid represented by general formula (3):



(2)

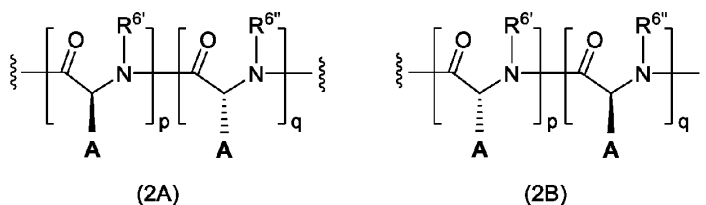


(3)

wherein

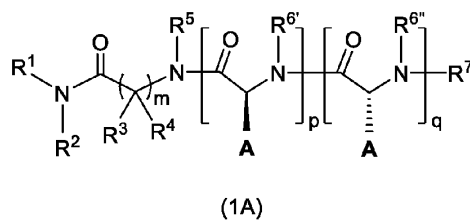
R^6 is H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl.

3. The compound of any one of the preceding claims, wherein the poly(amino acid) is selected from the group consisting of polyserine, polyglutamic acid and combinations thereof.
4. The compound of any one of claims 2 to 3, wherein **A** is selected from the group consisting of $-\text{CH}_2\text{OH}$, $-\text{CH}_2\text{CH}_2\text{C}(=\text{O})\text{OH}$, and combinations thereof.
5. The compound of any one of the preceding claims, wherein $\mathbf{A}^R$ comprises a structure that is represented by general formula (2A) and/or (2B):

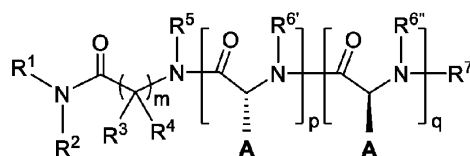


wherein
 $p + q = n$; and
 $R^{6'} = R^{6''} = R^6$.

6. The compound of any one of the preceding claims, wherein the compound represented by general formula (1) comprises a structure that is represented by general formula (1A) and/or (1B):



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(1B)

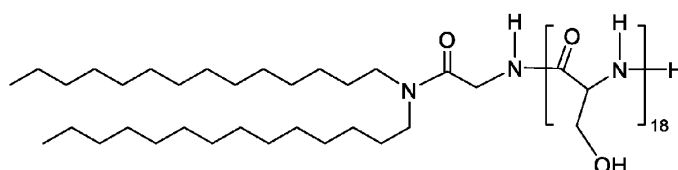
wherein

$$p + q = n; \text{ and}$$
$$R^{6'} = R^{6''} = R^6.$$

5

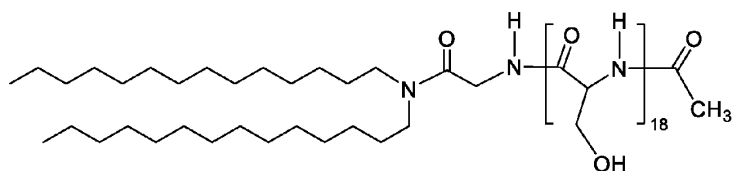
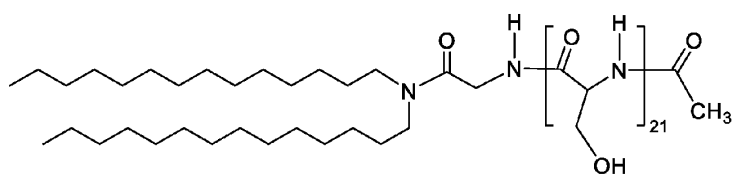
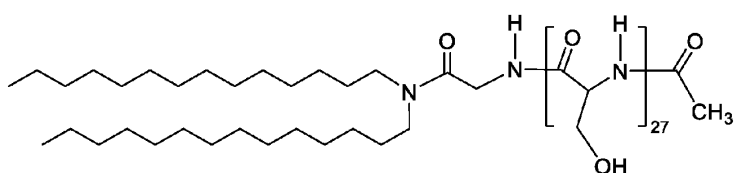
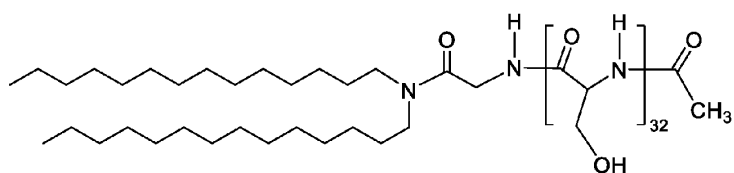
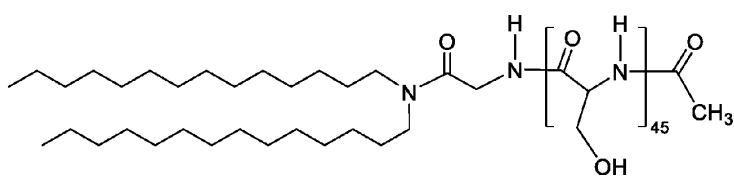
7. The compound of any one of the preceding claims, wherein $n \leq 60$.
8. The compound of any one of the preceding claims, wherein the hydrophobic group at R^1 and R^2 each independently comprises optionally substituted alkyl having at least 8 carbon atoms.
9. The compound of any one of the preceding claims, wherein the hydrophobic group at R^1 and R^2 each independently comprises optionally substituted alkyl having no more than 18 carbon atoms.
10. The compound of any one of the preceding claims, wherein R^3 , R^4 , R^5 , and R^6 are all H.
11. The compound of any one of the preceding claims, wherein the compound comprises a structure selected from one or more of the following:

20

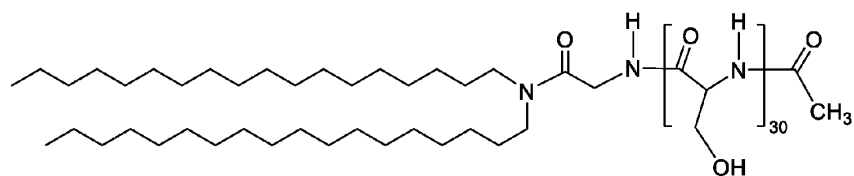
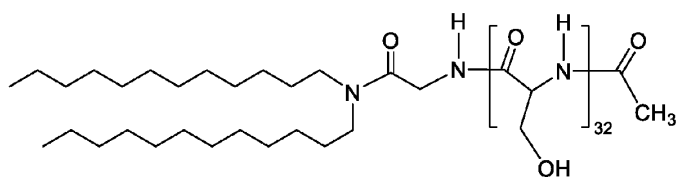
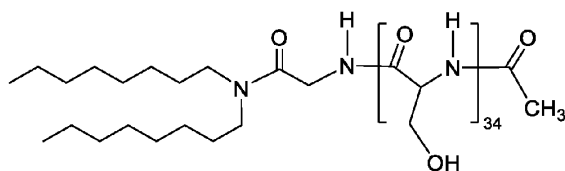


C14-pDLS1 (n = 18)

105

**C14-pDLS2 (n = 18)****C14-pDLS3 (n = 21)****C14-pDLS4 (n = 27)****C14-pDLS5 (n = 32)****C14-pDLS6 (n = 45)**

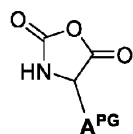
106

**C18-pDLS (n = 30)****C12-pDLS (n = 32)****C8-pDLS (n = 34)**

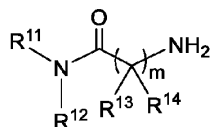
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12. A method of preparing a compound as claimed in any one of the preceding claims, the method comprising:

- 10 (i) polymerizing one or more N-carboxyanhydride (NCA) monomers represented by general formula (5) with a lipid initiator/molecule represented by general formula (6) to obtain a first intermediate compound represented by general formula (7):



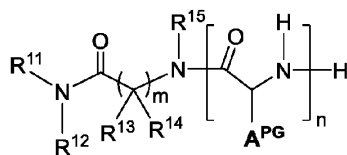
(5)



(6)

15

107



(7)

wherein

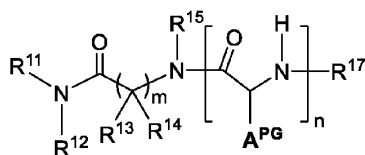
A^{PG} represents **A** protected with one or more protecting groups, where **A** comprises a hydrophilic organic group that is part of an amino acid represented by general formula (3);

R¹¹ and R¹² are each independently a hydrophobic group;

R¹³, R¹⁴, and R¹⁵ are each independently H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl;

 $m \geq 1$; and
$$n \geq 1;$$

(ii) optionally reacting the first intermediate compound represented by general formula (7) with an acylating agent to obtain a second intermediate compound represented by general formula (8):



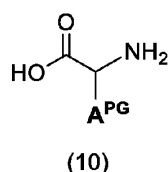
(8)

wherein R¹⁷ is -H or -C(=O)R¹⁸, wherein R¹⁸ is optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl or optionally substituted alkoxy;

(iii) deprotecting the first intermediate compound represented by general formula (7) or the second intermediate compound represented by

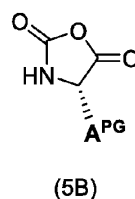
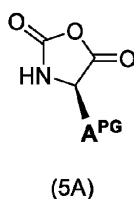
general formula (8) to obtain the compound represented by general formula (1).

13. The method of claim 12, wherein the method further comprises, prior to step (i):
- 5 (a-i) reacting a protected amino acid represented by general formula (10) with a carbonylating agent to obtain a N-carboxyanhydride (NCA) monomers represented by general formula (5):



10

14. The method of any one of claims 12 to 13, wherein the N-carboxyanhydride (NCA) monomer represented by general formula (5) comprises a structure represented by general formula (5A) and/or (5B):



15

15. The method of claim 14, wherein the step (i) comprises mixing the general formula (5A) with the general formula (5B) at a molar ratio of from 1:5 to 5:1.
- 20 16. A nanoparticle composition for delivery of a therapeutic, prophylactic and/or biological agent, the nanoparticle composition comprising:
- a compound as claimed in any one of claims 1 to 11; and
 - a therapeutic, prophylactic and/or biological agent.

17. The nanoparticle composition of claim 16, wherein the composition further comprises:
 - (a) ionizable lipid;
 - (b) helper lipid; and
 - 5 (c) cholesterol and/or derivatives thereof.

18. The nanoparticle composition of claim 17, wherein the ionizable lipid, helper lipid, cholesterol and/or derivatives thereof, and compound represented by general formula (1) are mixed at a mole ratio of 5 – 65 :
 10 4 – 20 : 10 – 60 : 0.1 – 20.

19. The nanoparticle composition of any one of claims 17 to 18, wherein the ionizable lipid is selected from ALC-0315, SM-102, Lipid 5, DLinDMA, D-Lin-MC2-DMA, DLin-MC3-DMA, D-Lin-MC4-DMA, Dlin-KC2-DMA,
 15 YSK05, AA3-Dlin, SSPalmM, SSPalmO-Phe, Lipid A9, L319, DODMA, CL1, BP Lipid 310, ATX-001, ATX-100, Lipid 2, 80-O16B, BP Lipid 309, BP Lipid 307, 93-O17S, 93-O17O, NT1-O14B, 306-O12B-3, 306-O12B, 113-O16B, 306Oi10, 306Oi9-cis2, BAMEA-O16B, AI-28, 113-O12B, 98N12-5, Ckk-E12, OF-02, C12-200, BP Lipid 311, BP Lipid 308, BP Lipid
 20 314, BP Lipid 312, LP01, TCL053, Lipid C24, BP Lipid 315, Lipid 29, 9A1P9, C13-112-tri-tail, C13-113-tri-tail, C13-112-tetra-tail, or C13-113-tetra-tail, C12-200 and combinations thereof.

20. The nanoparticle composition of any one of claims 17 to 19, wherein the helper lipid is selected from the group consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-
 30

- phosphocholine (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG), sphingomyelin and combinations thereof.
21. The nanoparticle composition of any one of claims 17 to 20, wherein the cholesterol and/or derivatives thereof is selected from cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, avenasterol and combinations thereof.
22. The nanoparticle composition of any one of claims 16 to 21, wherein the nanoparticle composition comprises nanoparticles having a N/P ratio from 1:1 to 50:1.
23. The nanoparticle composition of any one of claims 16 to 22, wherein the nanoparticle composition comprises nanoparticles having an average particle size of no more than 300 nm.
24. The nanoparticle composition of any one of claims 16 to 23, wherein the nanoparticle composition comprises nanoparticles having a zeta potential of from -20 mV to +20 mV.

25. The nanoparticle composition as claimed in any one of claims 16 to 24 for use in medicine.
26. The nanoparticle composition as claimed in any one of claims 16 to 24 for use in the treatment or prophylaxis of a disease, disorder or condition in a subject in need thereof.
27. Use of a nanoparticle composition as claimed in any one of claims 16 to 24 in the manufacture of a medicament for treatment or prophylaxis of a disease, disorder or condition in a subject in need thereof.
28. A method of treating or preventing a disease, disorder or condition in a subject in need thereof, the method comprising administering a therapeutically effective amount of the nanoparticle composition as claimed in any one of claims 16 to 24 to the subject.
29. The nanoparticle composition of claim 26, the use of claim 27 or the method of claim 28, wherein an immune response in the subject is to be induced through the administration of the nanoparticle composition thereto.
30. The nanoparticle composition of claim 26, the use of claim 27 or the method of claim 28, wherein the disease, disorder or condition is mediated by a coronavirus.
31. The nanoparticle composition, the use or the method of claim 30, wherein the coronavirus is a SARS-CoV-2 coronavirus.