

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

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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, there are only 2 mRNA Covid-19 vaccines from Moderna and Pfizer-BioNtech that 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
25 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.

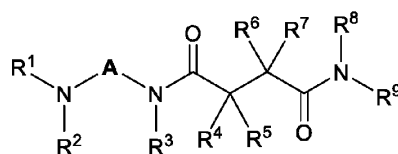
30 However, the currently available formulations have several disadvantages and drawbacks, and are far from desirable. Firstly, the ionizable lipids used in

Moderna and Pfizer-BioNTech mRNA vaccine formulations are extremely expensive. Next, the production of anti-lipid and anti-PEG antibodies was evident with such formulations, which can result in hypersensitivity and anaphylaxis in some subjects. Often, thermal stability is also a challenge for the mRNA formulations which are required to be stored under low temperature conditions, thereby resulting in expensive storage and transportation costs (due to cold-chain logistics requirements).

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.

SUMMARY

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



(1)

wherein
 NR¹R² is a group that is ionizable at a pH range of from 3 to physiological pH;
 A comprises a linear aliphatic, branched aliphatic and/or cyclic hydrocarbons optionally comprising one or more groups selected from -OH, -NR-, -O-, -O-C_xH_{2x}-O-, where x ≥ 1; and where R is H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl;
 R³, R⁴, R⁵, R⁶ and R⁷ are each independently H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl; and

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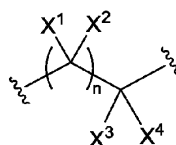
R^8 and R^9 are each independently hydrophobic group.

In one embodiment, R^1 and R^2 are each independently selected from the group consisting of H, optionally substituted alkyl, optionally substituted alkenyl,
 5 optionally substituted alkynyl, and combinations thereof.

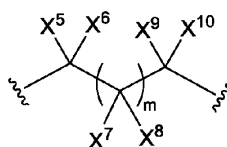
In one embodiment, R^1 and R^2 are both H and $-NR^1R^2$ is a primary amine group.

10 In one embodiment, the hydrophobic group at R^8 and R^9 each independently comprises optionally substituted alkyl.

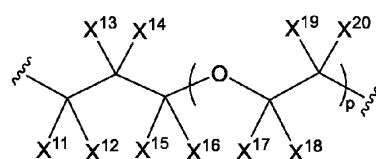
In one embodiment, **A** is selected from the following general formula (2), (3), (4) and/or (5):



(2)



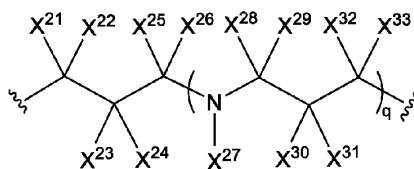
(3)



(4)

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(5)

wherein

X^1 to X^{31} are each independently selected from $-H$, $-OH$, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl;

5 $n \geq 1$; $m \geq 1$; $p \geq 1$; and $q \geq 1$.

In one embodiment, the compound is selected from the group consisting of DnO-EDEA, DD-EDEA, DTD-EDEA, HO-DnO-NH₂, HO-DD-NH₂, HO-DTD-NH₂, DnO-NH₂, DD-NH₂, DTD-NH₂, DMAPAPA-DTD and combinations thereof.

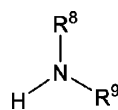
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In one embodiment, the compound is an ionized form of general formula (1), where $-NR^1R^2$ has been ionized to become a positively charged group.

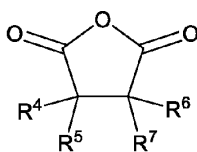
According to another aspect, there is provided a method of preparing a compound as disclosed herein, the method comprising:

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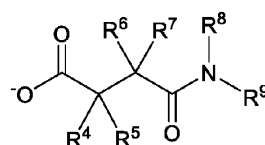
(a-i) reacting an amine compound represented by general formula (6) with a cyclic anhydride represented by general formula (7) to obtain a first intermediate compound comprising carboxylate group represented by general formula (8):



(6)



(7)



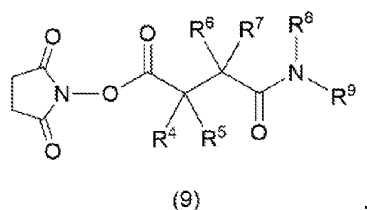
(8)

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(a-ii) reacting the first intermediate compound represented by general formula (8) with a N-hydroxysuccinimide (NHS) in the presence of a coupling agent to obtain a second intermediate compound comprising amide group represented by general formula (9):

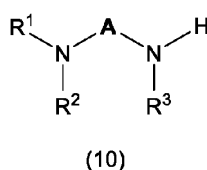
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;

(a-iii) reacting the second intermediate compound represented by general formula (9) with an amine compound represented by general formula (10) to obtain a compound represented by general formula (1):

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wherein

A comprises a linear aliphatic, branched aliphatic and/or cyclic hydrocarbons optionally comprising one or more groups selected from – OH, –NR–, –O–, –O–C_xH_{2x}–O–, where x ≥ 1; and where R is H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl;

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R¹, R², R³, R⁴, R⁵, R⁶ and R⁷ are each independently H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl; and

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R⁸ and R⁹ are each independently hydrophobic group; and

(a-iv) optionally ionizing –NR¹R² to become a positively charged group.

In one embodiment, the coupling agent comprises carbodiimide selected from the group consisting of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N,N'-dicyclohexylcarbodiimide (DCC), N,N'-Diisopropylcarbodiimide (DIC), and combinations thereof.

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According to another 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
- 10 a therapeutic, prophylactic and/or biological agent that is encapsulated in said compound as disclosed herein.

In one embodiment, the composition further comprises:

- (a) helper lipid;
- 15 (b) sterol; and
- (c) polyethylene glycol (PEG)-modified lipid.

In one embodiment, the compound represented by general formula (1), helper lipid, sterol, and PEG-modified lipid are mixed at a weight ratio of
20 10 – 50 : 2 – 20 : 4 – 30 : 1 – 15.

In one embodiment, the helper lipid is present in an amount of from 1 mol% to 20 mol%, the sterol is present in an amount of from 10 mol% to 50 mol%, and PEG-modified lipid is present in an amount of from 0.5 mol% to 10 mol%.

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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-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-

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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-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.

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

In one embodiment, the PEG-modified lipid is selected from PEG-modified phosphatidylethanolamines, PEG-modified phosphatidic acids, PEG-modified ceramides, PEG-modified dialkylamines, PEG-modified diacylglycerols, PEG-modified dialkylglycerols, or the like or combinations thereof. Examples of PEG-modified/PEGylated lipid include, but is not limited to, 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159), R-3-[(ω -methoxy-poly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxypropyl-3-amine (PEG-c-DOMG), 3-N-[(ω -methoxypoly (ethyleneglycol)2000)carbamoyl]-1,2-dimyristyloxypropylamine (PEG-S-DMG), PEG-DMPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[(polyethylene glycol)-methoxy] (sodium salt)), PEG-DPPC, PEG-DSPE lipid and combinations thereof.

In one embodiment, the nanoparticle composition comprises nanoparticles having a N/P ratio from 2:1 to 40:1.

In one embodiment, the nanoparticle composition comprises nanoparticles having an average particle size of from 20 nm to 200 nm.

In one embodiment, the nanoparticle composition comprises nanoparticles
5 having a zeta potential of from -15 mV to +20 mV in phosphate-buffered saline (PBS).

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

15 According to 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.

20 According to 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.

25 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.
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In one embodiment, the coronavirus is a SARS-CoV-2 coronavirus.

DEFINITIONS

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 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 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 200 nm, no more than about 150 nm, no more than 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, about 1 micron to less than about 1000 microns, about 1 micron to about 900 microns, about 1 micron to about 800 microns, about 1 micron to about 700 microns, about 1 micron to about 600 microns, about 1 micron to about 500 microns, about 1 micron to about 400 microns, about 1 micron to about 300 microns, about 1 micron to about 200 microns, about 1 micron to about 100 microns, or from about 1 micron to about 5 microns. In various embodiments, particles of about 5 microns or lesser may be useful for intranasal spray delivery.

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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. 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.

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, respiratory illnesses (e.g., coronavirus caused by the SARS-CoV-2 virus or flu caused by influenza virus). 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, 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.

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
5 suffering or are likely to suffer from a medical condition such as infectious diseases (e.g., coronavirus caused by the SARS-CoV-2 virus), 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,
10 the term "mammal" includes vertebrate such as a human or a large veterinary mammal (e.g., horses, cattle, deer, sheep, llamas, goats, pigs).

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.

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The term "alkyl" as a group or part of a group refers to a straight or 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
20 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-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-
25 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 and the like. The group may be a terminal group or a bridging group.

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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.

5 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-

10 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.

The term "alkynyl" as a group or part of a group denotes an aliphatic

15 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-

20 butynyl, 2-butyne, 3-butyne, 1-pentyne, 2-pentyne, 3-methyl-1-butyne, 4-pentyne, 1-hexynyl, 2-hexynyl, 5-hexynyl, 1-heptyne, 2-heptyne, 6-heptyne, 1-octynyl, 2-octynyl, 7-octynyl, 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.

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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

30 cyclic structure include, but are not limited to, cyclopentane, cyclopentene, 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 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 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-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-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.

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.

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The term "amide group" or the like is intended to broadly refer to a group 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,

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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.

5 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,
10 benzothiophene, benzofuran, benzimidazole, benzoxazole, benzothiazole, benzisothiazole, naphtha[2,3-b]thiophene, furan, isoindolizine, xantholene, phenoxazine, pyrrole, imidazole, pyrazole, pyridine, pyrazine, pyrimidine, pyridazine, tetrazole, indole, isoindole, 1H-indazole, purine, quinoline, isoquinoline, phthalazine, naphthyridine, quinoxaline, cinnoline, carbazole,
15 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.

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

 The term "optionally substituted," when used to describe a chemical structure or moiety, refers to the chemical structure or moiety wherein one or
25 more of its hydrogen atoms is optionally substituted with a chemical moiety or functional group such as alcohol, alkoxy, alkanoyloxy, alkoxy-carbonyl, 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–).

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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.

10 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
15 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
20 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.

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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
30 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

5 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

10 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

15 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

20 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.

25 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

30 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
5 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
10 herein.

DESCRIPTION OF EMBODIMENTS

Exemplary, non-limiting embodiments of a compound for preparing lipid
15 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

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There is provided a compound for preparing lipid nanoparticles. In various embodiments, the compound comprises one or more amine group(s) that is/are ionizable and/or capable of being ionized. The amine group may be selected from the group consisting of primary (1°) amine, secondary (2°) amine, tertiary (3°)
25 amine and combinations thereof. In various embodiments therefore, the compound is ionizable and/or capable of being ionized and/or exists in an ionized form at e.g. physiological pH. Advantageously, the ionizable property of the compound (due to presence of ionizable amine group) allows for embodiments of the compound to be used as an encapsulation/loading agent, delivery
30 vehicle/system and/or transfection vehicle/system. In various embodiments, the compound is designed/configured to allow loading/encapsulation of one or 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 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 the compound 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), small interfering ribonucleic acid (siRNA), deoxyribonucleic acid (DNA), plasmid deoxyribonucleic acid (pDNA), oligonucleotides such as antisense oligonucleotide (ASO) or the like or combinations thereof. In various embodiments, the molecules/cargoes to be loaded/encapsulated comprises therapeutics. For example, the molecules/cargoes to be loaded/encapsulated may be therapeutics selected from negatively charged therapeutics, drug molecule, vaccine (e.g., dengue vaccine etc) or the like or combinations thereof. Advantageously, the compound is suitable for use in 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).

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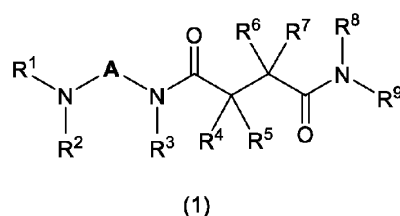
Accordingly, in various embodiments, there is also provided a carrier, nanocarrier or delivery system/vehicle comprising the compound or its ionized form thereof.

Advantageously, the compound is designed/configured to be ionizable at a pH range of from about 3 to about physiological pH, depending on the type or nature of the amine group(s). In various embodiments, the compound is capable of being ionized at a pH range of from about 3.0 to about 7.8, from about 3.1 to about 7.7, from about 3.2 to about 7.6, from about 3.3 to about 7.5, from about 3.4 to about 7.4, from about 3.5 to about 7.3, from about 3.6 to about 7.2, from about 3.7 to about 7.1, from about 3.8 to about 7.0, from about 3.9 to about 6.9, from about 4.0 to about 6.8, from about 4.1 to about 6.7, from about 4.2 to about

30

6.6, from about 4.3 to about 6.5, from about 4.4 to about 6.4, from about 4.5 to about 6.3, from about 4.6 to about 6.2, from about 4.7 to about 6.1, from about 4.8 to about 6.0, from about 4.9 to about 5.9, from about 5.0 to about 5.8, from about 5.1 to about 5.7, from about 5.2 to about 5.6, from about 5.3 to about 5.5, or about 5.4. In various embodiments where the compound comprises primary amine, the compound is capable of being ionized at physiological pH range of from about 7.00 to about 7.80, from about 7.05 to about 7.75, from about 7.10 to about 7.70, from about 7.15 to about 7.65, from about 7.20 to about 7.60, from about 7.25 to about 7.55, from about 7.30 to about 7.50, from about 7.35 to about 7.45, about 7.36, about 7.37, about 7.38, about 7.39, about 7.40, about 7.41, about 7.42, about 7.43, about 7.44, or about 7.45. In various embodiments where the compound comprises secondary amine and/or tertiary amine, the compound is capable of being ionized at a pH range of from about 3.0 to about 5.5, 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 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 compound comprises a structure that is represented by general formula (1) or an ionized form thereof:



wherein

NR¹R² is a group that is ionizable or capable of being ionized at a pH range of from 3 to physiological pH;

A comprises a linear aliphatic, branched aliphatic and/or cyclic hydrocarbons optionally comprising one or more groups selected from -OH, -NR-, -O-,

$-\text{O}-\text{C}_x\text{H}_{2x}-\text{O}-$, where $x \geq 1$; and where R is H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl;

R^3 , R^4 , R^5 , R^6 and R^7 are each independently H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl; and

- 5 R^8 and R^9 are each independently hydrophobic tail/chain/group or contains at least one of the groups defined above for R^3 - R^7 .

In various embodiments, x is an integer ≥ 1 . In various embodiments, x is ≥ 1 , ≥ 2 , ≥ 3 , ≥ 4 , ≥ 5 , ≥ 6 , ≥ 7 , ≥ 8 , ≥ 9 , ≥ 10 , ≥ 11 , ≥ 12 , ≥ 13 , ≥ 14 , ≥ 15 , ≥ 16 ,
 10 ≥ 17 , ≥ 18 , ≥ 19 , or ≤ 20 . Advantageously, when x is ≤ 20 (e.g. 1 to 20), the compound is not too hydrophobic. For example, **A** may comprise $-\text{O}-\text{CH}_2-\text{O}-$, $-\text{O}-\text{C}_2\text{H}_4-\text{O}-$, $-\text{O}-\text{C}_3\text{H}_6-\text{O}-$, $-\text{O}-\text{C}_4\text{H}_8-\text{O}-$ or $-\text{O}-\text{C}_5\text{H}_{10}-\text{O}-$.

In various embodiments, R^1 and R^2 are each independently selected from
 15 H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl. For example, R^1 and R^2 may be selected from methyl, ethyl, n-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-
 20 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
 25 or combinations thereof.

In various embodiments, R^1 and R^2 are both H. In such embodiments, $-\text{NR}^1\text{R}^2$ is $-\text{NH}_2$ or a primary amine group and the compound comprises a primary
 (1°) amine group (e.g., an ionizable primary amine group).

30

In various embodiments, either R^1 or R^2 is H. In such embodiments, $-\text{NR}^1\text{R}^2$ is $-\text{NHR}^2$ or $-\text{NR}^1\text{H}$ (i.e. secondary amine group) and the compound

comprises a secondary (2°) amine group (e.g., an ionizable secondary amine group).

In various embodiments, both R^1 and R^2 are not H. In various
5 embodiments, R^1 and R^2 are each independently selected from optionally
substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl or
combinations thereof. In such embodiments, $-NR^1R^2$ is a tertiary amine group
(such as that in DMAPAPA-DTD) and the compound comprises a tertiary (3°)
amine group (e.g., an ionizable tertiary amine group).

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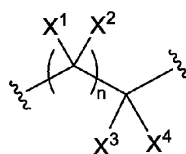
In various embodiments, the compound is in an ionized form, where
 $-NR^1R^2$ has been ionized to become a positively charged group. In various
embodiments, $-NR^1R^2$ is ionized/protonated at a pH range of from about 3 to
physiological pH (or about neutral pH) to become a positively charged
15 group/ion/cation. In various embodiments where the compound comprises
primary amine (i.e. $-NR^1R^2$ is a primary amine), $-NR^1R^2$ is ionized at
physiological pH (or about neutral pH) to become a positively charged
group/ion/cation. In various embodiments where the compound comprises
secondary and/or tertiary amine (e.g., $-NR^1R^2$ is a secondary and/or tertiary
20 amine such as DMAPAPA-DTD), $-NR^1R^2$ is ionized at a pH range of from about
3 to about 5, or about pH 4 to become a positively charged group/ion/cation. In
various embodiments, $-NR^1R^2$ is protonated to become $-NR^1R^2H^+$. For example,
when both R^1 and R^2 are H, then $-NR^1R^2$ may be protonated to become $-NH_3^+$.

25 In various embodiments, the compound comprises at least 1, at least 2, or
at least 3 ionizable amine groups selected from the group consisting of primary
(1°) amine, secondary (2°) amine, tertiary amine (3°), and combinations thereof.
For example, besides the ionizable group at NR^1R^2 , the compound may also
contain 1 or more, 2 or more, or 3 or more ionizable amine groups in **A**. The
30 encapsulation efficiency of a compound comprising secondary (2°) amine and/or
tertiary amine (3°) as the ionizable group(s) may be comparable to most
compounds comprising a primary amine group as the only ionizable group.

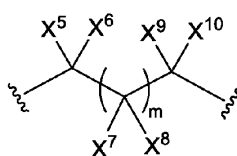
In various embodiments, the compound comprises a lipid compound. The term "compound" may comprise and/or may be used interchangeably with the terms "lipid", "lipid compound", "ionizable lipid", "ionizable lipid compound", "cationic lipid compound", "ionizable cationic lipid compound" or the like. In various embodiments, the compound is amphiphilic/amphipathic and comprises hydrophilic and hydrophobic parts. In various embodiments, the lipid part of the compound is hydrophobic, while groups such as the amine and/or hydroxyl groups in the compound are hydrophilic. In various embodiments, the compound comprises hydrophilic part(s) at the amine groups (e.g., ionizable NR^1R^2). In various embodiments, the compound comprises hydrophobic parts/tails/chains/groups at both R^8 and R^9 . In various embodiments, the hydrophobic tail/chain/group at R^8 and R^9 each independently comprises optionally substituted alkyl. The alkyl may have at least 5, at least 6, at least 7, 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, or at least 20 carbon atoms. For example, R^8 and R^9 may be each independently C_yH_{2y+1} , where $y \geq 5$, $y \geq 6$, $y \geq 7$, $y \geq 8$, $y \geq 9$, $y \geq 10$, $y \geq 11$, $y \geq 12$, $y \geq 13$, $y \geq 14$, $y \geq 15$, $y \geq 16$, $y \geq 17$, $y \geq 18$, $y \geq 19$, or $y \geq 20$. Advantageously, in various embodiments, the presence of hydrophobic parts/tails/chains/groups in the compound aids in imparting improved cellular uptake and/or transfection, thereby leading to a higher and/or better transfection efficiency.

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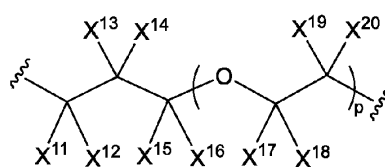
In various embodiments, **A** is selected from the following general formula (2), (3), (4) or (5):



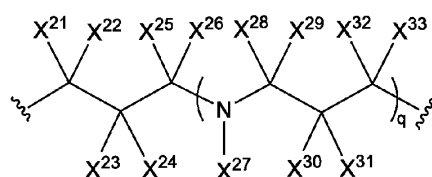
(2)



(3)



(4)



(5)

5

wherein

X¹ to X³¹ are each independently selected from -H, -OH, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl;

n ≥ 1; m ≥ 1; p ≥ 1; and q ≥ 1.

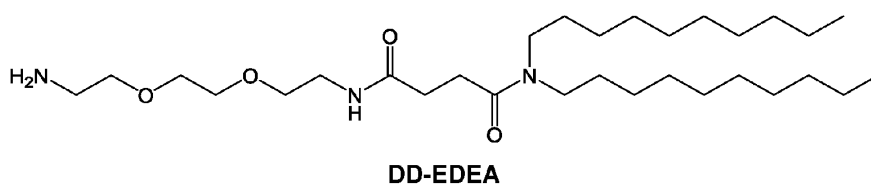
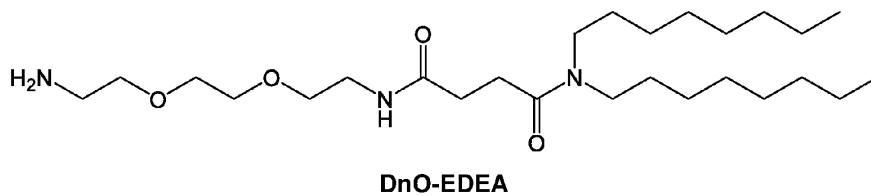
In various embodiments, n is an integer ≥ 1 . In various embodiments, n is $\geq 1, \geq 2, \geq 3, \geq 4, \geq 5, \geq 6, \geq 7, \geq 8, \geq 9, \geq 10, \geq 11, \geq 12, \geq 13, \geq 14, \geq 15, \geq 16, \geq 17, \geq 18, \geq 19$, or ≤ 20 .

5 In various embodiments, m is an integer ≥ 1 . In various embodiments, m is $\geq 1, \geq 2, \geq 3, \geq 4, \geq 5, \geq 6, \geq 7, \geq 8, \geq 9, \geq 10, \geq 11, \geq 12, \geq 13, \geq 14, \geq 15, \geq 16, \geq 17, \geq 18, \geq 19$, or ≤ 20 .

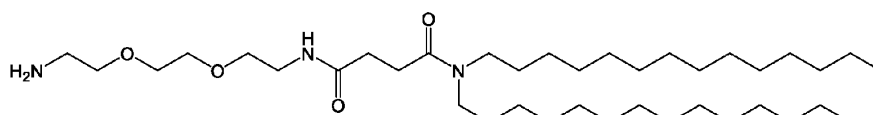
In various embodiments, p is an integer ≥ 1 . In various embodiments, p is
10 $\geq 1, \geq 2, \geq 3, \geq 4, \geq 5, \geq 6, \geq 7, \geq 8, \geq 9, \geq 10, \geq 11, \geq 12, \geq 13, \geq 14, \geq 15, \geq 16, \geq 17, \geq 18, \geq 19$, or ≤ 20 .

In various embodiments, q is an integer ≥ 1 . In various embodiments, q is
15 $\geq 1, \geq 2, \geq 3, \geq 4, \geq 5, \geq 6, \geq 7, \geq 8, \geq 9, \geq 10, \geq 11, \geq 12, \geq 13, \geq 14, \geq 15, \geq 16, \geq 17, \geq 18, \geq 19$, or ≤ 20 .

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

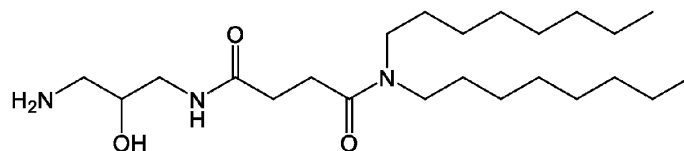
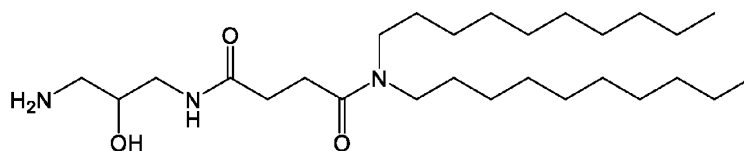


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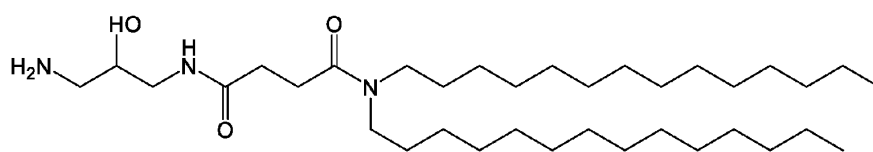


DTD-EDEA

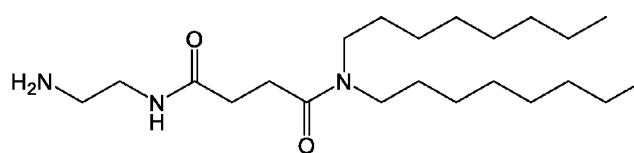
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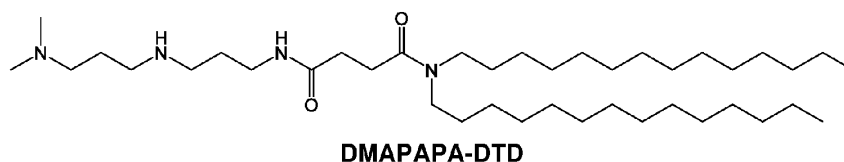
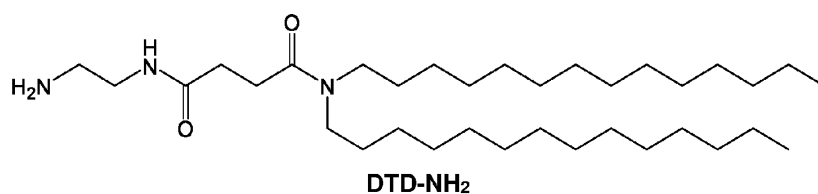
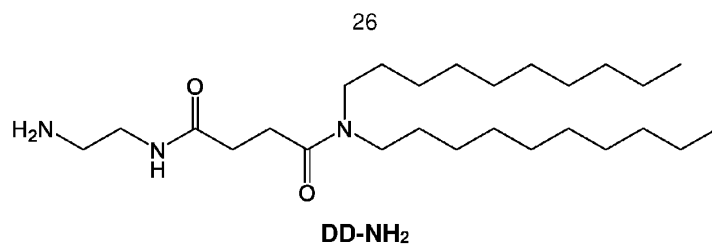
HO-DnO-NH₂HO-DD-NH₂

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HO-DTD-NH₂

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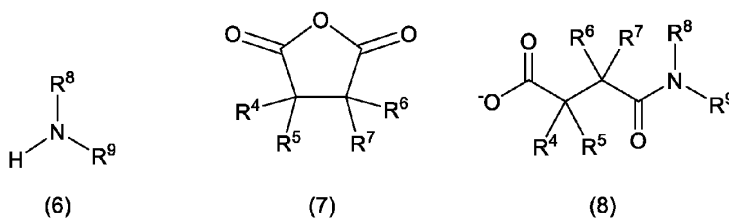
DnO-NH₂



10 METHOD OF PREPARING COMPOUND

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

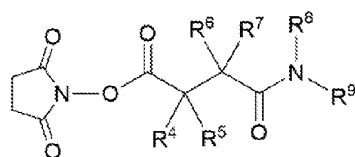
- (a-i) reacting an amine compound represented by general formula (6) with a cyclic anhydride represented by general formula (7) to obtain a first intermediate compound comprising carboxylate group represented by general formula (8):



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- (a-ii) reacting the first intermediate compound represented by general formula (8) with a N-hydroxysuccinimide (NHS) in the presence of a coupling agent to obtain a second intermediate compound comprising amide group represented by general formula (9):

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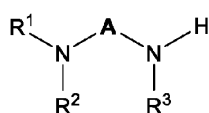


(9)

;

- (a-iii) reacting the second intermediate compound represented by general formula (9) with an amine compound represented by general formula (10) to obtain a compound represented by general formula (1):

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(10)

wherein R^1 to R^9 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 (1)); and

15

- (a-iv) optionally ionizing $-NR^1R^2$ to become a positively charged group.

Advantageously, embodiments of the method are straightforward to perform and have a low production/manufacturing cost (i.e. cost effective) as they may be carried out simply in 3 synthetic/reaction steps. It will be appreciated that currently available or known methods require at least 5 or at least 6 synthetic/reaction steps to produce ionizable lipid compounds in the art. Advantageously, embodiments of the method are scalable and/or have substantially high scalability.

20

In various embodiments, the cyclic anhydride represented by general formula (7) comprises succinic anhydride or the like.

In various embodiments, the coupling agent comprises carbodiimide. For example, the coupling agent may be 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N,N'-dicyclohexylcarbodiimide (DCC), N,N'-Diisopropylcarbodiimide (DIC), or the like or combinations thereof.

In various embodiments, the amine compound represented by general formula (10) comprises ethylenediamine (EDA), N,N-dimethyldipropylenetriamine, or the like or combinations thereof.

In various embodiments, step (a-iii) comprises reacting an amine compound represented by general formula (10) with the second intermediate compound represented by general formula (9) in a molar ratio of from about 1:1 to about 5:1. The step (a-iii) may comprise reacting an amine compound represented by general formula (10) with the second intermediate compound represented by general formula (9) in a molar ratio of from about 1:1 to about 5:1, about 1:1, about 2:1, about 3:1, about 4:1 or about 5:1. It will be appreciated that the amine compound represented by general formula (10) may be provided in excess (e.g., slight excess) to ensure that a desired molar ratio is achieved between the amine compound represented by general formula (10) and the second intermediate compound represented by general formula (9).

25

In various embodiments, the reacting step (a-iii) comprises adding the second intermediate compound represented by general formula (9) in a dropwise manner to the amine compound represented by general formula (10).

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

30

In various embodiments, the reacting step (a-i), (a-ii) and/or (a-iii) 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 a dry or anhydrous organic solvent such as dry or anhydrous dichloromethane (DCM).

In various embodiments, the reacting step (a-i), (a-ii) and/or (a-iii) is/are carried out in an inert atmosphere. For example, the step(s) of dispersing, mixing and/or stirring may be performed 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).

In various embodiments, the reacting step (a-i), (a-ii) and/or (a-iii) is/are performed over a time duration of from 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 reacting step (a-i) and/or (a-ii) are optionally 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.

In various embodiments, the reacting step (a-iii) is optionally performed at a temperature that is from about -30°C to about -80°C, from about -35°C to about -75°C, from about -40°C to about -70°C, from about -45°C to about -65°C, from about -50°C to about -60°C, or about -55°C, e.g., to control reaction kinetics. For example, the reacting step may be performed in a dry ice bath.

In various embodiments, the method further comprises:

- (b-i) a step of isolating the first intermediate compound after step (a-i);
- (b-ii) a step of isolating the second intermediate compound after step (a-ii); and
- (b-iii) a step of isolating the compound represented by general formula (1)
5 after step (a-iii).

In various embodiments, the isolating step(s) comprises one or more of the following steps: re-dissolving, purifying, centrifuging, quenching, washing, precipitating and/or recrystallizing the first intermediate compound, the second
10 intermediate compound and/or the compound represented by general formula (1). The step(s) of purifying, centrifuging, quenching and/or washing may be repeated 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 times with a washing medium. In various
15 embodiments, the isolating step is performed to remove by-products from the first intermediate compound, the second intermediate compound and/or the compound represented by general formula (1). In various embodiments, the washing medium comprises aqueous medium/solutions such as salt solution or deionized water. The salt solution may be bicarbonate salts such as sodium
20 bicarbonate, chloride salts such as sodium chloride (brine). In various embodiments, the salt solution comprises highly concentrated/saturated salt solution.

In various embodiments, the method further comprises one or more of the
25 following post reaction steps: drying the first intermediate compound, the second intermediate compound and/or the compound represented by general formula (1), optionally under low temperature (e.g., freeze drying), under vacuum. The step(s) of drying may be performed in the presence of a drying agent such as magnesium sulfate, sodium sulfate, calcium chloride or combinations thereof.

30

In various embodiments, step (a-iv) is present and is performed at a pH range of from about 3 to about physiological pH. In various embodiments, step

(a-iv) is performed at a pH range of from about 3.0 to about 7.8, from about 3.1 to about 7.7, from about 3.2 to about 7.6, from about 3.3 to about 7.5, from about 3.4 to about 7.4, from about 3.5 to about 7.3, from about 3.6 to about 7.2, from about 3.7 to about 7.1, from about 3.8 to about 7.0, from about 3.9 to about 6.9, from about 4.0 to about 6.8, from about 4.1 to about 6.7, from about 4.2 to about 6.6, from about 4.3 to about 6.5, from about 4.4 to about 6.4, from about 4.5 to about 6.3, from about 4.6 to about 6.2, from about 4.7 to about 6.1, from about 4.8 to about 6.0, from about 4.9 to about 5.9, from about 5.0 to about 5.8, from about 5.1 to about 5.7, from about 5.2 to about 5.6, from about 5.3 to about 5.5, or about 5.4.

NANOPARTICLE COMPOSITION

Advantageously, in various embodiments, the ionizable property of the compound represented by general formula (1) (due to presence of ionizable amine group) allows for the condensation and encapsulation/loading of molecules/cargoes into embodiments of the compound, thereby forming nanoparticles in a composition. In various embodiments, embodiments of the compound are capable of forming nanoparticles in a composition. In various embodiments, in the presence of a composition comprising molecules/cargoes (e.g., therapeutic agent, prophylactic agent and/or biological agent), the one or more amine group(s) in the compound (e.g., ionized/protonated amine group(s) in the lipid compound) condenses and encapsulates/loads the molecules/cargoes into the compound to form nanoparticles (e.g., lipid nanoparticles (LNPs)) in the composition.

The term "nanoparticles" may comprise and/or may be used interchangeably with the terms "lipid nanoparticles", "encapsulated lipid nanoparticles", "loaded lipid nanoparticles", "LNPs" or the like.

There is provided a nanoparticle composition comprising:

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

In various embodiments, the compound represented by general formula (1) is capable of being ionized (e.g., protonated) at a pH range of from 3 to physiological pH (or neutral pH) such that the composition encapsulates a therapeutic and/or prophylactic agent and/or biological agent that is coupled/bonded/linked/bound to the composition/nanoparticles. In various embodiments, the compound represented by general formula (1) is capable of being ionized (e.g., protonated) at physiological pH (or neutral pH) such that the composition encapsulates a therapeutic and/or prophylactic agent and/or biological agent that is coupled/bonded/linked/bound to the composition/nanoparticles. The therapeutic and/or prophylactic agent and/or biological agent may be coupled/bonded/linked/bound to the composition/nanoparticles *via* electrostatic interaction and/or other physical interactions. In various embodiments, the therapeutic and/or prophylactic agent and/or biological agent is electrostatically and/or physically coupled/bonded/linked/bound to the composition/nanoparticle.

In various embodiments, for compounds (e.g., lipid compounds) that contain a primary amine group (e.g., DnO-NH₂, DD-NH₂, DTD-NH₂, HO-DnO-NH₂, HO-DD-NH₂, HO-DTD-NH₂, DnO-EDEA, DD-EDEA, DTD-EDEA), these lipids are protonated readily in water, leading to a positively charged molecule that condenses molecules/cargoes (e.g., nucleic acid such as mRNA) into lipid nanoparticles (LNPs) through electrostatic and/or physical interaction, forming encapsulated LNPs (e.g., mRNA LNPs). For example, after being taken up by cells *via* endocytosis, mRNA LNPs may enter the endolysosomes where they

fuse with endolysosomal membrane, leading to release of the encapsulated mRNA into cytosol for transfection (e.g., gene transfection).

In various embodiments, for compounds (e.g., lipid compounds) that
5 contains secondary and/or tertiary amine groups (e.g., DMAPAPA-DTD), the lipids are protonated at about pH 4.0, at which the encapsulated LNPs (e.g., mRNA LNPs) are made. For example, such lipids may carry positive charges at pH 4.0, and condense mRNA into LNPs. The mRNA LNPs may then be taken up
10 by cells *via* endocytosis, and then enter endolysosomes, where the secondary and/or tertiary amine groups can absorb protons in the endolysosomes, breaking down the endolysosomal membrane and releasing mRNA into the cytosol for transfection (e.g., gene transfection).

Advantageously, the composition is suitable for use in the encapsulation,
15 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:

- 20 (a) neutral/helper lipid;
(b) sterol; and
(c) polyethylene glycol (PEG)-modified lipid.

The term "polyethylene glycol (PEG)-modified lipid" may comprise and/or
25 may be used interchangeably with the terms "PEGylated lipid" and "lipid modified with PEG".

In various embodiments, the compound or its ionized form thereof,
neutral/helper lipid, sterol, and PEG-modified lipid are mixed/dissolved in an
30 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 mixture. The organic solvent may comprise ethanol, isopropanol, acetonitrile, ethyl acetate, methanol, tetrahydrofuran, dimethyl sulfoxide, dimethylformamide or the like or combinations thereof.

In various embodiments, the compound represented by general formula (1) or its ionized form thereof, neutral/helper lipid, sterol, and PEG-modified lipid are mixed/dissolved at a weight ratio of about 10 – 50 : about 2 – 20 : about 4 – 30 : about 1 – 15. For example, the compound or its ionized form thereof, neutral/helper lipid, sterol, and PEG-modified lipid may be mixed at a weight ratio of about 10 – 50 : about 2 – 20 : about 4 – 30 : about 1 – 15, about 15 – 45 : about 4 – 18 : about 8 – 26 : about 3 – 13, about 20 – 40 : about 6 – 16 : about 12 – 22 : about 5 – 11, about 25 – 35 : about 8 – 14 : about 16 – 18 : about 7 – 9, or about 30 : about 11 : about 17 : about 8. In various embodiments, the compound represented by general formula (1) or its ionized form thereof, neutral/helper lipid, sterol, and PEG-modified lipid are mixed/dissolved at a weight ratio of about 28: 6: 13: 3.

In various embodiments, the composition comprises from about 10.0 mol% to about 75.0 mol%, from about 15.0 mol% to about 70.0 mol%, from about 20.0 mol% to about 65.0 mol%, from about 25.0 mol% to about 60.0 mol%, from about 30.0 mol% to about 55.0 mol%, from about 35.0 mol% to about 50.0 mol%, or from about 40.0 mol% to about 45.0 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 present in an amount of no less than about 30 wt%, no less than about 31 wt%, no less than about 32 wt%, no less than about 33 wt%, no less than about 34 wt%, no less than about 35 wt%, no less than about 36 wt%, no less than about 37 wt%, no less than about 38 wt%, no less than about 39 wt%, no less than about 40 wt%, no less than about 41 wt%, no less than about 42 wt%, no less than about 43 wt%, no less than about 44 wt%, no less than about 45 wt%, no less than about

46 wt%, no less than about 47 wt%, no less than about 48 wt%, no less than about 49 wt%, no less than about 50 wt%, no less than about 51 wt%, no less than about 52 wt%, no less than about 53 wt%, no less than about 54 wt%, no less than about 55 wt%, or no less than about 56 wt%, or no less than about 57 wt%, or no less than about 58 wt%, no less than about 59 wt%, or no less than about 60 wt% 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-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 the like and combinations thereof.

In various embodiments, the composition comprises from about 1.0 mol% to about 20.0 mol%, from about 2.0 mol% to about 19.0 mol%, from about 3.0 mol% to about 18.0 mol%, from about 4.0 mol% to about 17.0 mol%, from about 5.0 mol% to about 16.0 mol%, from about 6.0 mol% to about 15.0 mol%, from about 7.0 mol% to about 14.0 mol%, from about 8.0 mol% to about 13.0 mol%,

from about 9.0 mol% to about 12.0 mol%, from about 10.0 mol% to about 11.0 mol%, or about 10.5 mol% of neutral/helper lipid. In various embodiments, the neutral/helper lipid is present in an amount of no less than about 3 wt%, no less than about 4 wt%, no less than about 5 wt%, no less than about 6 wt%, no less than about 7 wt%, no less than about 8 wt%, no less than about 9 wt%, no less than about 10 wt%, or no less than about 11 wt%, or no less than about 12 wt%, or no less than about 13 wt%, or no less than about 14 wt%, or no less than about 15 wt% of the composition.

10 In various embodiments, the sterol is selected from cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, avenasterol, or the like or combinations thereof.

In various embodiments, the composition comprises from about 10.0 mol% to about 50.0 mol%, from about 15.0 mol% to about 47.5 mol%, from about 17.5 mol% to about 45.0 mol%, from about 20.0 mol% to about 42.5 mol%, from about 22.5 mol% to about 40.0 mol%, from about 25.0 mol% to about 37.5 mol%, from about 27.5 mol% to about 35.0 mol%, or from about 30.0 mol% to about 32.5 mol% of sterol. In various embodiments, the sterol is present in an amount of no less than about 10 wt%, no less than about 11 wt%, no less than about 12 wt%, no less than about 13 wt%, no less than about 14 wt%, no less than about 15 wt%, no less than about 16 wt%, no less than about 17 wt%, no less than about 18 wt%, no less than about 19 wt%, no less than about 20 wt%, no less than about 21 wt%, no less than about 22 wt%, no less than about 23 wt%, no less than about 24 wt%, no less than about 25 wt%, no less than about 26 wt%, no less than about 27 wt%, no less than about 28 wt%, no less than about 29 wt%, no less than about 30 wt%, no less than about 31 wt%, no less than about 32 wt%, no less than about 33 wt%, no less than about 34 wt%, no less than about 35 wt%, no less than about 36 wt%, no less than about 37 wt%, no less than about 38 wt%, no less than about 39 wt%, or no less than about 40 wt% of the composition.

In various embodiments, the PEG-modified lipid is selected from PEG-modified phosphatidylethanolamines, PEG-modified phosphatidic acids, PEG-modified ceramides, PEG-modified dialkylamines, PEG-modified diacylglycerols, PEG-modified dialkylglycerols, or the like or combinations thereof. Examples of PEG-modified/PEGylated lipid include, but is not limited to, 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159), R-3-[(ω -methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxypropyl-3-amine (PEG-c-DOMG), 3-N-[(ω -methoxypoly(ethyleneglycol)2000)carbamoyl]-1,2-dimyristyloxy-propylamine (PEG-S-DMG), PEG-DMPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[(polyethylene glycol)-methoxy] (sodium salt)), PEG-DPPC, PEG-DSPE lipid, or the like or combinations thereof. In various embodiments, the composition comprises from about 0.5 mol% to about 10.0 mol%, from about 1.0 mol% to about 9.5 mol%, from about 1.5 mol% to about 9.0 mol%, from about 2.0 mol% to about 8.5 mol%, from about 2.5 mol% to about 8.0 mol%, from about 3.0 mol% to about 7.5 mol%, from about 3.5 mol% to about 7.0 mol%, from about 4.0 mol% to about 6.5 mol%, from about 4.5 mol% to about 6.0 mol%, or from about 5.0 mol% to about 5.5 mol% of PEG-modified lipid. In various embodiments, the PEG-modified lipid is present in an amount of no less than about 0.5 wt%, no less than about 0.6 wt%, no less than about 0.7 wt%, no less than about 0.8 wt%, no less than about 0.9 wt%, no less than about 1 wt%, no less than about 2 wt%, no less than about 3 wt%, no less than about 4 wt%, no less than about 5 wt%, no less than about 6 wt%, no less than about 7 wt%, or no less than about 8 wt%, or no less than about 9 wt%, or no less than about 10 wt%, or no less than about 11 wt%, or no less than about 12 wt% 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 sodium acetate.

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

5 NANOPARTICLES

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

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

In various embodiments, the nanoparticles have a N:P or N/P ratio (i.e. molar ratio of ionizable nitrogen atoms in the compound (e.g., ionizable lipid compound) to phosphate groups in the therapeutic agent, prophylactic agent and/or biological agent (e.g., nucleic acid) is from about 2:1 to about 40:1. The nanoparticles may have a N:P or N/P ratio that is from about 2:1 to about 40:1, from about 3:1 to about 39:1, from about 4:1 to about 38:1, from about 5:1 to about 37:1, from about 6:1 to about 36:1, from about 7:1 to about 35:1, from about 8:1 to about 34:1, from about 9:1 to about 33:1, from about 10:1 to about 32:1, from about 11:1 to about 31:1, from about 12:1 to about 30:1, from about 13:1 to about 29:1, from about 14:1 to about 28:1, from about 15:1 to about 27:1, from about 16:1 to about 26:1, from about 17:1 to about 25:1, from about 18:1 to about 24:1, from about 19:1 to about 23:1, from about 20:1 to about 22:1, or about 21:1.

In various embodiments, it will be appreciated that shorter nucleic acid therapeutics (e.g. siRNA) or prophylactic agents 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 40:1 is used to encapsulate and deliver nucleic acid therapeutics (e.g. shorter nucleic acid therapeutics siRNA) or prophylactic agents 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 20.0%, at least about 30.0%, at least about 40.0%, 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%,
5 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
10 efficiency that is slightly lower, comparable to or no less or higher than that of corresponding nanoparticles using ALC-0315 as the ionizable lipid under similar conditions. For example, the encapsulation efficiency may be at least about 50% of that of a corresponding nanoparticles using ALC-0315 as the ionizable lipid under similar conditions. In another example, the encapsulation efficiency may
15 be at least about 1% to at least about 50% higher than that of corresponding nanoparticles using ALC-0315 as the ionizable lipid under similar conditions.

In various embodiments, the cell transfection efficiency (% of the cells that are transfected with the gene) of the composition/nanoparticles is at least about
20 5.0%, at least about 10.0%, at least about 20.0%, at least about 30.0%, at least about 40.0%, 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%, at least about 99.9%, or about 100%. In various embodiments, the
25 cell transfection efficiency is not required to be 100%. For example, it will be 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.

30 In various embodiments, the nanoparticles have a cell transfection efficiency that is comparable to or no less or higher than that of corresponding nanoparticles using ALC-0315 as the ionizable lipid under similar conditions. For

example, the cell transfection efficiency may be at least about 30% of that of a corresponding nanoparticles using ALC-0315 as the ionizable lipid under similar conditions. In another example, the cell transfection efficiency may be at least about 50% to at least about 1,000% higher than that of corresponding nanoparticles using ALC-0315 as the ionizable lipid under similar conditions.

In various embodiments, the transfection efficiency in certain cell lines is lower than that of corresponding nanoparticles using ALC-0315 as the ionizable lipid 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.

In various embodiments, the nanoparticles have an average or mean particle size (or diameter) of from about 20.0 nm to about 200.0 nm, from about 30.0 nm to about 190.0 nm, from about 40.0 nm to about 180.0 nm, from about 50.0 nm to about 170.0 nm, from about 60.0 nm to about 160.0 nm, from about 70.0 nm to about 150.0 nm, from about 80.0 nm to about 140.0 nm, from about 90.0 nm to about 130.0 nm, from about 100.0 nm to about 120.0 nm, or about 110.0 nm.

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 and/or the nanoparticles or nanoparticle composition is relatively/substantially homogenous.

In various embodiments, the nanoparticles have a zeta potential of from about -15.0 mV to about +20.0 mV, from about -14.0 mV to about +19.0 mV, from about -13.0 mV to about +18.0 mV, from about -12.0 mV to about +17.0 mV, from about -11.0 mV to about +16.0 mV, from about -10.0 mV to about +15.0 mV, from about -9.0 mV to about +14.0 mV, from about -8.0 mV to about +13.0 mV, from about -7.0 mV to about +12.0 mV, from about -6.0 mV to about +11.0 mV, from about -5.0 mV to about +10.0 mV, from about -4.0 mV to about +9.0 mV, from about -3.0 mV to about +8.0 mV, from about -2.0 mV to about +7.0 mV, from about -1.0 mV to about +6.0 mV, from about 0 mV to about +5.0 mV, from about +1.0 mV to about +4.0 mV, or from about +2.0 mV to about +3.0 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, making the nanoparticles suitable/desirable for *in vivo* applications.

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In various embodiments, the ionizable lipid/nanoparticles has/have an average or mean molecular weight of from about 100.0 g/mol to about 2,000.0 g/mol, from about 200.0 g/mol to about 1,900.0 g/mol, from about 300.0 g/mol to about 1,800 g/mol, from about 400.0 g/mol to about 1,700.0 g/mol, from about 500.0 g/mol to about 1,600.0 g/mol, from about 600.0 g/mol to about 1,500.0 g/mol, from about 700.0 g/mol to about 1,400.0 g/mol, from about 800.0 g/mol to about 1,300.0 g/mol, from about 900.0 g/mol to about 1,200.0 g/mol, or from about 1,000.0 g/mol to about 1,100 g/mol.

25

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 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. Advantageously, the nanoparticles

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(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.

5 METHOD OF PREPARING NANOPARTICLES

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

- 10 (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.

15 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.

20 In various embodiments, the mixing step (c-i) is performed at a pH value of from about 2.5 to about 6.5, from about 2.6 to about 6.4, from about 2.7 to about 6.3, from about 2.8 to about 6.2, from about 2.9 to about 6.1, from about 3.0 to about 6.0, from about 3.1 to about 5.9, from about 3.2 to about 5.8, from about 3.3 to about 5.7, from about 3.4 to about 5.6, from about 3.5 to about 5.5, from about 3.6 to about 5.4, from about 3.7 to about 5.3, from about 3.8 to about 5.2, from about 3.9 to about 5.1, from about 4.0 to about 5.0, from about 4.1 to about 4.9, from about 4.2 to about 4.8, from about 4.3 to about 4.7, from about 4.4 to about 4.6, or about 4.5.

30 In various embodiments, the composition as disclosed herein comprises 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 composition as described 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 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.

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

10 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-
15 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 mixing ratio, the interaction between the two compositions (e.g., between
20 ionizable lipid and therapeutic, prophylactic and/or 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).

25 In various embodiments, the method further comprises removing the organic phase (e.g. ethanol). For example, removing the organic phase may include dialysing the nanoparticles to remove residual organic solvents that are present. Advantageously, removal of the organic phase through dialysis may improve the encapsulation efficiency of the therapeutic and/or prophylactic agent and/or biological agent.

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

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

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

- 15 In various embodiments, there is also provided a carrier, a nanocarrier, a delivery system/vehicle, a compound or ionized form thereof, 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 or ionized form thereof, a nanoparticle composition, nanoparticles (or lipid nanoparticles) in the
20 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 or ionized form thereof, a nanoparticle composition, nanoparticles (or
25 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
30 (i.e. diseases caused by fungi), respiratory diseases 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, there is also provided a carrier, a nanocarrier, a delivery system/vehicle, a compound or ionized form thereof, 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 or ionized form thereof, 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 or ionized form thereof, 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.

In various embodiments, there is also provided a carrier, a nanocarrier, a delivery system/vehicle, a compound or ionized form thereof, 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 or ionized form thereof, 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 or ionized form thereof, 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 or ionized form thereof, 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 or ionized form thereof, nanoparticle composition, nanoparticles may be delivered to a subject in the form of or as a component of a vaccine.

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.

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In various embodiments, the carrier, nanocarrier, delivery system/vehicle, compound or ionized form thereof, 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, high encapsulation efficiency (e.g., > 80%), high transfection efficiency

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(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.

5 In various embodiments, the compound comprises primary amine groups only. Accordingly, in various embodiments, the compound is substantially devoid of ionizable secondary (2°) amine groups and/or tertiary (3°) amine groups. Advantageously, in various embodiments, the primary amine acts as a binding group to condense molecules/cargoes (e.g., mRNA) through electrostatic
10 interaction into lipid nanoparticles (LNPs). In various embodiments, the release of molecules/cargoes (e.g., mRNA) into the cytosol from the endosome is through fusion of the lipid with the endosomal membrane. Such technical effects with only primary amine are not shown or expected from the prior art.

15 In various embodiments, the ionizable lipid compounds of the present technology are different from ionizable lipid compounds in the art that contain a tertiary amine and/or a secondary amine group, as an ionizable lipid. In various embodiments, the ionizable lipid compounds of the present application are substantially devoid of a tertiary amine and/or a secondary amine group. Since
20 embodiments of the presently disclosed ionizable lipid compounds (e.g., DTD-NH₂ and HO-DTD-NH₂) do not contain any tertiary amine and/or a secondary amine group, a person skilled in the art would not easily think of using them as an ionizable lipid for gene delivery. It is understood that this is the first report of use of a lipid which does not contain a tertiary amine or secondary amine group,
25 as an ionizable lipid.

 In various embodiments, the ionizable lipid compounds of the present technology are different from ionizable lipid compounds in the art that contain a guanidine group (i.e. one does not contain a primary amine group). Furthermore,
30 it has been found through experiments performed by the inventors that guanidinium-functionalized polycarbonates are unable to transfect mRNA in cells.

In various embodiments, the ionizable lipid compounds disclosed herein are different from known ionizable lipids used for siRNA delivery, which specifically contain a tertiary amine group or guanidinium group. In contrast, embodiments of the ionizable lipid disclosed herein comprise DTD-NH₂ and/or
5 ionizable lipids that contain a primary amine group as the only ionizable group for making lipid nanoparticles for nucleic acid delivery, which is not previously known.

In various embodiments disclosed herein, DTD-NH₂ and the other lipids that contain a primary amine group as the only ionizable group (e.g., DD-NH₂,
10 HO-DnO-NH₂, HO-DD-NH₂, HO-DTD-NH₂, DnO-EDEA, DD-EDEA and DTD-EDEA) was directly used to make lipid nanoparticles for delivery of mRNA and DNA and promising results were obtained. This finding is surprising as the precursor is not expected to work well based on the common general knowledge in the field of nucleic acid delivery.

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It will be appreciated that various embodiments of the ionizable lipids disclosed herein are different from those known in the art relating to ligand conjugated oligonucleotide for targeted delivery of the oligonucleotide.

20 BRIEF DESCRIPTION OF FIGURES

FIG. 1 shows ¹H NMR spectrum of DTD-NH₂ prepared in accordance with various embodiments of the method disclosed herein in CD₃OD.

25 FIG. 2 shows ¹H NMR spectrum of DMAPAPA-DTD prepared in accordance with various embodiments of the method disclosed herein in CD₃OD.

FIG. 3 shows luciferase expression mediated by different mRNA LNP formulations in HeLa cell lines, in accordance with various embodiments
30 disclosed herein. Cells in each well of a 96-well plate were incubated with 100 ng of each mRNA LNP formulation for 48 h. Luciferase expression was quantified by the addition of D-luciferin to the cell lysate and the luminescence intensity was

measured. In HeLa cells, 1st batch of ALC-0315 LNPs were used. Statistical significance was calculated using Mann-Whitney test between ALC-0315 formulation against the other LNP formulations (* $p < 0.05$).

5 FIG. 4 shows luciferase expression mediated by different mRNA LNP formulations in HEK293 cell lines, in accordance with various embodiments disclosed herein. Cells in each well of a 96-well plate were incubated with 100 ng of each mRNA LNP formulation for 48 h. Luciferase expression was quantified by the addition of D-luciferin to the cell lysate and the luminescence intensity was
10 measured. 2nd batch of ALC-0315 LNPs were used in HEK293 cells. Statistical significance was calculated using Mann-Whitney test between ALC-0315 formulation against the other LNP formulations (* $p < 0.05$).

 FIG. 5 shows cell viability after 48 h of incubation with the various mRNA
15 LNP formulations in accordance with various embodiments disclosed herein. Statistical significance was calculated using Mann-Whitney test between ALC-0315 formulation against the other LNP formulations (* $p < 0.05$, ** $p < 0.01$). In HeLa cells, 1st batch of ALC-0315 LNPs were used.

20 FIG. 6 shows cell viability after 48 h of incubation with the various mRNA LNP formulations in accordance with various embodiments disclosed herein. Statistical significance was calculated using Mann-Whitney test between ALC-0315 formulation against the other LNP formulations (* $p < 0.05$, ** $p < 0.01$). 2nd batch of ALC-0315 LNPs were used in HEK293 cells.

25 FIG. 7 shows cell viability of HeLa cells when incubated with mRNA LNPs in accordance with various embodiments disclosed herein. Statistical significance was calculated using Mann-Whitney test between ALC-0315 formulation against the other LNP formulations (* $p < 0.05$, ** $p < 0.01$).

30 FIG. 8 shows luciferase expression of HeLa cells when incubated with mRNA LNPs in accordance with various embodiments disclosed herein.

Statistical significance was calculated using Mann-Whitney test between ALC-0315 formulation against the other LNP formulations (* $p < 0.05$, ** $p < 0.01$).

FIG. 9 shows cell viability of HEK293 cells when incubated with pDNA LNPs in accordance with various embodiments disclosed herein. Statistical significance was calculated using Mann-Whitney test between ALC-0315 formulation against the other LNP formulations (** $p < 0.01$).

FIG. 10 shows luciferase expression of HEK293 cells when incubated with pDNA LNPs in accordance with various embodiments disclosed herein. Statistical significance was calculated using Mann-Whitney test between ALC-0315 formulation against the other LNP formulations (** $p < 0.01$).

FIG. 11 is a graph showing the particle size and size distribution of pDNA-loaded LNPs made from ALC-0315 (comparative example). The measurements were recorded on Day 0.

FIG. 12 is a graph showing the particle size and size distribution of pDNA-loaded LNPs made from DTD-NH₂ in accordance with various embodiments disclosed herein. The measurements were recorded on Day 0.

FIG. 13 is a graph showing the particle size and size distribution of pDNA-loaded LNPs made from ALC-0315 (comparative example). The measurements were recorded after 6 days of storage.

FIG. 14 is a graph showing the particle size and size distribution of pDNA-loaded LNPs made from DTD-NH₂ in accordance with various embodiments disclosed herein. The measurements were recorded after 6 days of storage.

FIG. 15 shows the results obtained from agarose gel electrophoresis experiments performed on pDNA LNPs made from DTD-NH₂ in accordance with various embodiments disclosed herein. ALC0315 was used as a comparative

example. Both ALC-0315 and DTD-NH₂ LNPs bind pDNA strongly. Triton is able to break down the LNPs to release pDNA for measurement of pDNA encapsulation efficiency.

5 FIG. 16 is a fluorescence intensity (RFU) vs. GFP fluorescence graph showing transfection efficiency of pDNA LNPs made from DTD-NH₂ in accordance with various embodiments disclosed herein after 48 hours of incubation in HeLa cells and in HepG2 cells. ALC0315 was used as a comparative example.

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FIG. 17 shows confocal microscopic images captured of a control after 48 hours of incubation in HeLa cells. Scale bar = 50 μm.

15 FIG. 18 shows confocal microscopic images captured of pDNA LNPs made from ALC0315 (comparative example) after 48 hours of incubation in HeLa cells. Scale bar = 50 μm.

20 FIG. 19 shows confocal microscopic images captured of pDNA LNPs made from DTD-NH₂ in accordance with various embodiments disclosed herein after 48 hours of incubation in HeLa cells. Scale bar = 50 μm.

FIG. 20 shows confocal microscopic images captured of a control after 48 hours of incubation in HepG2 cells. Scale bar = 50 μm.

25 FIG. 21 shows confocal microscopic images captured of pDNA LNPs made from ALC0315 (comparative example) after 48 hours of incubation in HepG2 cells. Scale bar = 50 μm.

30 FIG. 22 shows confocal microscopic images captured of pDNA LNPs made from DTD-NH₂ in accordance with various embodiments disclosed herein after 48 hours of incubation in HepG2 cells. Scale bar = 50 μm.

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 embodiments should not be construed as limiting the scope of the disclosure.

The following examples describe the development of a lipid that comprises a primary amine group, its composition, formation of RNA and DNA lipid nanoparticles (LNPs) and gene transfection. Advantageously, the present application has shown that, unlike known ionizable lipids in the art (e.g., those used in Moderna and Pfizer-BioNTech mRNA vaccine formulations), the primary amine group in embodiments of the lipid disclosed herein is ionized in the physiological environment or neutral pH, and condenses mRNA into lipid nanoparticles with > 90% encapsulation efficiency. Even more advantageously, the mRNA and pDNA transfection efficiency of the LNPs designed in accordance with various embodiments disclosed herein is significantly higher than that of mRNA or pDNA LNPs that are made from a conventional ionizable lipid ALC-0315 currently used in Pfizer-BioNTech's mRNA vaccine formulation. In addition, advantageously, treatment with mRNA and pDNA LNPs designed in accordance with various embodiments disclosed herein does not induce any cytotoxicity. In addition, the synthesis of the ionizable lipids in accordance with various embodiments disclosed herein requires less than the 5-6 steps needed to make the ionizable lipid ALC-0315 of the art, which will significantly reduce the manufacturing cost (e.g. 3 steps).

Example 1: Materials and Methods1.1. Materials

Chemical reagents for the synthesis of the lipids were purchased from Sigma-Aldrich and used as received unless otherwise noted. Ditetradecylamine (DTDA) was bought from Ambeed, Inc (Arlington Heights, IL, USA). 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). Agarose and Tris-Acetate-EDTA were purchased from 1st Base (Singapore). GelStar Nucleic Acid Gel Stain was purchased from Lonza (Basel, Switzerland). Triton®-X100 and Tris-EDTA were purchased from Promega (Madison, WI, USA). Alamar Blue, gel loading buffer, and Pierce Firefly Luciferase Glow assay kit were purchased from Invitrogen (Waltham, MA, USA). Other reagents used were analytical grade.

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1.2. Synthesis of DTD-NH₂ and DMAPAPA-DTD (Scheme 1)

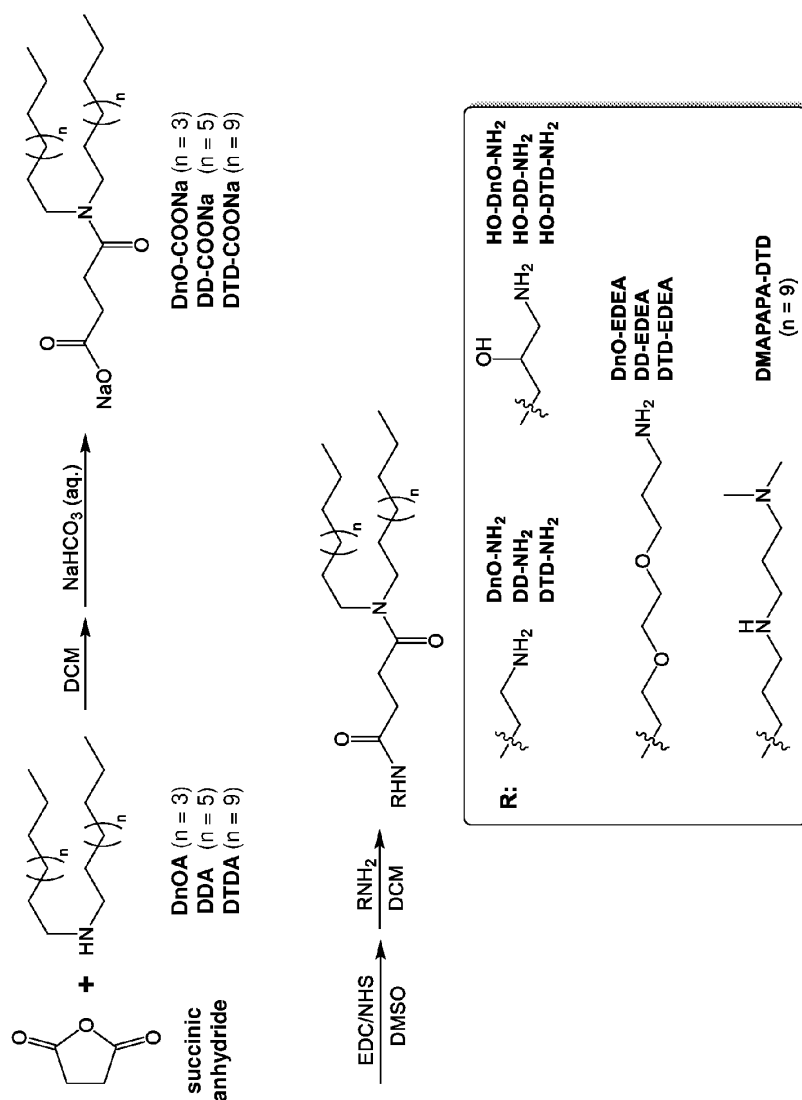
An exemplary overall scheme for the synthesis of a compound represented by general formula (1) or ionized form thereof in accordance with various embodiments disclosed herein is provided in Scheme 1.

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The synthesis method of DTD-NH₂ is given below as a typical example and the analogous synthesis of DMAPAP-DTA is also provided below as a subsidiary example.

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Scheme 1. Chemical structure and synthetic procedures of ionizable lipids.

Synthesis of DTD-COONa: In a 500 mL three-neck round bottom flask, ditetradecylamine (DTDA, 3.44 g, 8 mmol), succinic anhydride (2.4 g, 24 mmol) and triethylamine (5.6 mL, 40 mmol) were dissolved in 300 mL of dry DCM and the reaction solution was allowed to stir overnight under N₂ atmosphere. Then, the solution was transferred to a 1 L separation funnel, and washed with sodium

bicarbonate saturated solution (100 mL) for three times. The organic phase was dried over MgSO_4 for 4-5 hours. Finally, the solution was filtered by suction filtration, the filtrate was concentrated to dryness and dried *in vacuo*, giving DTD-COONa as off-white solid (93% yield). ^1H NMR (400 MHz, CDCl_3 , 22 °C): δ 3.31 (dt, 4H, $-\text{CON}(\text{CH}_2)_2$), 2.67 (s, 4H, $-\text{CH}_2\text{CH}_2\text{COONa}$), 1.53 (m, br, 4H, $-\text{CON}(\text{CH}_2\text{CH}_2)_2$), 1.26 (s, 44H, $-(\text{CH}_2)_{11}\text{CH}_3$), 0.88 (t, 6H, $-\text{CH}_3$).

Synthesis of DTD-NHS: In a 100 mL three-neck round bottom flask, DTD-COONa (4.85 g, 9.13 mmol) and *N*-hydroxysuccinimide (NHS, 2.88 g, 25 mmol) were dissolved in 60 mL of dry DCM, followed by adding 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC•HCl, 3.83 g, 20 mmol). The solution was allowed to stir overnight under N_2 atmosphere. Then, it was concentrated to dryness, and the residue was re-dissolved in the mixture of THF and de-ionized (DI) water (v/v = 1:1). Another 300 mL of DI water was added to precipitate the product in ice bath, centrifuged and washed with cold DI water for three times. Finally, the wet solid was freeze-dried, giving DTD-NHS as white powder (83% yield). ^1H NMR (400 MHz, CDCl_3 , 22 °C): δ 3.30 (dt, 4H, $-\text{CON}(\text{CH}_2)_2$), 3.01 (t, 2H, $-\text{CH}_2-$ of NHS), 2.83 (s, 4H, $-\text{COCH}_2\text{CH}_2\text{CON}-$), 2.72 (t, 2H, $-\text{CH}_2-$ of NHS), 1.52 (m, br, 4H, $-\text{CON}(\text{CH}_2\text{CH}_2)_2$), 1.26 (s, 44H, $-(\text{CH}_2)_{11}\text{CH}_3$), 0.88 (t, 6H, $-\text{CH}_3$).

Synthesis of DTD-NH₂: In a 100 mL single-neck round bottom flask, ethylenediamine (EDA, 53 μL , g, 0.79 mmol) was dissolved in 15 mL of dry DCM, and the solution was allowed to cool down in dry ice bath for 30 min under N_2 atmosphere. To this EDA solution, DTD-NHS (0.455 g, 0.75 mmol) was dissolved in 15 mL of DCM and the solution was added dropwise. Dry ice bath was then removed and the reaction solution continued to stir for another 2 hrs. The mixture was transferred to a 250 mL separation funnel and 70 mL of DCM was added. The solution was washed with brine (20 mL) for three times, and dried over MgSO_4 overnight. Finally, the mixture was filtered by suction filtration, the filtrate was concentrated to dryness and dried *in vacuo*, giving DTD-NH₂ as off-white sticky solid (63% yield). ^1H NMR (400 MHz, CD_3OD , 22 °C): δ 2.80 (t, 2H, -

CH_2NH_2), 2.69 (m, 2H, $-\text{NHCOCH}_2\text{CH}_2\text{CON}-$), 2.48 (t, 2H, $-\text{NHCOCH}_2\text{CH}_2\text{CON}-$), 1.62 (m, br, 2H, $-\text{CON}(\text{CH}_2\text{CH}_2-)_2$), 1.52 (m, br, 2H, $-\text{CON}(\text{CH}_2\text{CH}_2-)_2$), 1.29 (s, 44H, $-(\text{CH}_2)_{11}\text{CH}_3$), 0.90 (t, 6H, $-\text{CH}_3$).

- 5 Similarly, the following ionizable lipids were synthesized:

DnO-NH₂ Yield: 66%; ¹H NMR (400 MHz, CD₃OD, 22 °C): δ 2.75 (t, 2H, $-\text{CH}_2\text{NH}_2$), 2.68 (t, 2H, $-\text{NHCOCH}_2\text{CH}_2\text{CON}-$), 2.48 (t, 2H, $-\text{NHCOCH}_2\text{CH}_2\text{CON}-$), 1.62 (m, br, 2H, $-\text{CON}(\text{CH}_2\text{CH}_2-)_2$), 1.52 (m, br, 2H, $-\text{CON}(\text{CH}_2\text{CH}_2-)_2$), 1.30 (m, 20H, $-(\text{CH}_2)_5\text{CH}_3$), 0.90 (t, 6H, $-\text{CH}_3$).

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DD-NH₂ Yield: 63%; ¹H NMR (400 MHz, CD₃OD, 22 °C): δ 2.77 (t, 2H, $-\text{CH}_2\text{NH}_2$), 2.68 (t, 2H, $-\text{NHCOCH}_2\text{CH}_2\text{CON}-$), 2.48 (t, 2H, $-\text{NHCOCH}_2\text{CH}_2\text{CON}-$), 1.62 (m, br, 2H, $-\text{CON}(\text{CH}_2\text{CH}_2-)_2$), 1.52 (m, br, 2H, $-\text{CON}(\text{CH}_2\text{CH}_2-)_2$), 1.29 (m, 28H, $-(\text{CH}_2)_7\text{CH}_3$), 0.90 (t, 6H, $-\text{CH}_3$).

15

HO-DnO-NH₂ Yield: 73%; ¹H NMR (400 MHz, CD₃OD, 22 °C): δ 3.73 (m, 1H, $-\text{OH}$), 2.67 (t, 2H, $-\text{NHCOCH}_2\text{CH}_2\text{CON}-$), 2.50 (t, 2H, $-\text{NHCOCH}_2\text{CH}_2\text{CON}-$), 1.62 (m, br, 2H, $-\text{CON}(\text{CH}_2\text{CH}_2-)_2$), 1.52 (m, br, 2H, $-\text{CON}(\text{CH}_2\text{CH}_2-)_2$), 1.30 (m, 20H, $-(\text{CH}_2)_5\text{CH}_3$), 0.90 (m, 6H, $-\text{CH}_3$).

20

HO-DD-NH₂ Yield: 64%; ¹H NMR (400 MHz, CD₃OD, 22 °C): δ 3.73 (m, 1H, $-\text{OH}$), 2.67 (t, 2H, $-\text{NHCOCH}_2\text{CH}_2\text{CON}-$), 2.50 (t, 2H, $-\text{NHCOCH}_2\text{CH}_2\text{CON}-$), 1.62 (m, br, 2H, $-\text{CON}(\text{CH}_2\text{CH}_2-)_2$), 1.52 (m, br, 2H, $-\text{CON}(\text{CH}_2\text{CH}_2-)_2$), 1.29 (m, 28H, $-(\text{CH}_2)_7\text{CH}_3$), 0.90 (m, 6H, $-\text{CH}_3$).

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HO-DTD-NH₂ Yield: 86%; ¹H NMR (400 MHz, CD₃OD, 22 °C): δ 3.73 (m, 1H, $-\text{OH}$), 3.23 (t, 2H, $-\text{CH}_2\text{NH}_2$), 2.68 (t, 2H, $-\text{NHCOCH}_2\text{CH}_2\text{CON}-$), 2.50 (t, 2H, $-\text{NHCOCH}_2\text{CH}_2\text{CON}-$), 1.62 (m, br, 2H, $-\text{CON}(\text{CH}_2\text{CH}_2-)_2$), 1.52 (m, br, 2H, $-\text{CON}(\text{CH}_2\text{CH}_2-)_2$), 1.29 (m, 44H, $-(\text{CH}_2)_{11}\text{CH}_3$), 0.90 (t, 6H, $-\text{CH}_3$).

30

DnO-EDEA Yield: 76%; ¹H NMR (400 MHz, CD₃OD, 22 °C): δ 3.63 (m, 4H, $-\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2-$), 3.55 (m, 4H, $-\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2-$), 3.35 (t, 2H,

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-CH₂NHCO-), 2.85 (t, 2H, -CH₂NH₂), 2.65 (t, 2H, -NHCOCH₂CH₂CON-), 2.49 (t, 2H, -NHCOCH₂CH₂CON-), 1.62 (m, br, 2H, -CON(CH₂CH₂)₂), 1.52 (m, br, 2H, -CON(CH₂CH₂)₂), 1.30 (m, 20H, -(CH₂)₅CH₃), 0.90 (m, 6H, -CH₃).

- 5 **DD-EDEA** Yield: 72%; ¹H NMR (400 MHz, CD₃OD, 22 °C): δ 3.64 (m, 4H, -CH₂OCH₂CH₂OCH₂-), 3.56 (m, 4H, -CH₂OCH₂CH₂OCH₂-), 3.36 (t, 2H, -CH₂NHCO-), 2.87 (t, 2H, -CH₂NH₂), 2.65 (t, 2H, -NHCOCH₂CH₂CON-), 2.49 (t, 2H, -NHCOCH₂CH₂CON-), 1.62 (m, br, 2H, -CON(CH₂CH₂)₂), 1.52 (m, br, 2H, -CON(CH₂CH₂)₂), 1.29 (m, 28H, -(CH₂)₇CH₃), 0.90 (m, 6H, -CH₃).

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DTD-EDEA Yield: 59%; ¹H NMR (400 MHz, CD₃OD, 22 °C): δ 3.64 (m, 4H, -CH₂OCH₂CH₂OCH₂-), 3.58 (m, 4H, -CH₂OCH₂CH₂OCH₂-), 3.36 (t, 2H, -CH₂NHCO-), 2.91 (t, 2H, -CH₂NH₂), 2.65 (t, 2H, -NHCOCH₂CH₂CON-), 2.49 (t, 2H, -NHCOCH₂CH₂CON-), 1.62 (m, br, 2H, -CON(CH₂CH₂)₂), 1.52 (m, br, 2H, -CON(CH₂CH₂)₂), 1.29 (m, 44H, -(CH₂)₁₁CH₃), 0.90 (t, 6H, -CH₃).

15

DMA-PAPA-DTD Yield: 51%; ¹H NMR (400 MHz, CD₃OD, 22 °C): δ 2.72 (t, 4H, -CH₂NHCH₂- of DMA-PAPA), 2.67 (t, 2H, -NHCOCH₂CH₂CON-), 2.47 (t, 2H, -NHCOCH₂CH₂CON-), 2.42 (t, 2H, -CH₂N(CH₃)₂), 2.27 (s, 6H, -N(CH₃)₂), 1.74 (m, 2H, -CH₂CH₂N(CH₃)₂), 1.57 (m, br, 4H, -CON(CH₂CH₂)₂), 1.29 (s, 44H, -(CH₂)₁₁CH₃), 0.90 (t, 6H, -CH₃).

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1.3. Nuclear Magnetic Resonance Spectroscopy (NMR)

¹H-NMR spectra of the lipids were recorded on a Bruker Advance 400 NMR spectrometer (400 MHz) under the conditions of ambient temperature, an acquisition time of 3.2 s, a pulse repetition time of 2.0 s, a 30° pulse width, 5208-Hz spectral width, and 32 K data points. Chemical shifts were referenced based on the respective solvent peaks (δ = 3.31 ppm for CD₃OD).

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1.4. Preparation of mRNA-loaded lipid nanoparticles (mRNA LNPs)

mRNA LNPs were prepared by using a microfluidic device (NanoAssemblr[®] Ignite[™], Precision Nanosystem, Vancouver, CA, USA). Lipids were dissolved in ethanol at a weight ratio of 28:6:13:3 (ionizable lipid, DSPC, cholesterol and ALC-0159) to form the organic phase. The aqueous phase was formed by dissolving firefly luciferase mRNA (Trilink Biotechnologies) in 10 mM sodium acetate solution (pH = 4) with a volume ratio of 3:1 (aqueous:organic). mRNA LNPs were formed by mixing the aqueous phase and the organic phase at a flow ratio of 3:1 (1.5 mL:0.5 mL), and the total flow rate was 12 mL/min. The resulting mRNA LNPs were diluted 20 times with 0.9% saline immediately. Then, the mRNA LNPs suspension was concentrated using a Vivaspin[®] 20 ultracentrifuge tube with a molecular weight cut-off of 30,000 Da (Sartorius, Goettingen, Germany) at 4°C, 2,500 rcf and 30 min. The concentrated mRNA LNPs were collected and stored at 4°C for future use.

1.5. In vitro high-throughput screening

For high-throughput screening of ionizable lipid candidates, mRNA and pDNA LNPs were prepared by direct injection of the ethanolic organic phase into the nucleic acid-containing aqueous phase. The organic phase consists of a lipid library mixed with DSPC, cholesterol and ALC-0159 in a weight ratio of 28:6:13:3. The aqueous phase contains the firefly luciferase mRNA (Trilink Biotechnologies) or pGL4.51 *luc2*/CMV/Neo vector pDNA (Promega) dissolved in 10 mM sodium acetate buffer, pH 4. The amount of mRNA and pDNA was adjusted accordingly to each lipid candidate to achieve N/P ratio 6 in all formulations. The organic phase and aqueous phase were gently mixed and incubated at room temperature for 30 minutes for LNP formation, before using it for downstream analysis.

1.6. Encapsulation Efficiency

The mRNA and pDNA encapsulation efficiency of LNPs was determined by using Quant-it[™] RiboGreen RNA Assay Kit (Invitrogen, Waltham, MA, USA) and Quant-it[™] PicoGreen DNA Assay Kit (Invitrogen, Waltham, MA, USA), respectively. The mRNA and pDNA LNPs suspension was diluted 5 times with

Tri-EDTA buffer. RiboGreen and PicoGreen stock solution was diluted 200 times with Tri-EDTA buffer or 5% Triton-X100 in Tri-EDTA buffer. 90 μ L of the RiboGreen and PicoGreen solution with or without Triton-X100 was added in 10 μ L of the diluted mRNA and pDNA LNPs suspension, respectively, which was
 5 incubated at 37 °C for 20 min. Then the fluorescence intensity was recorded using a microplate reader (Tecan, Männedorf, Switzerland) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm for the RiboGreen assay and at an excitation wavelength of 480 nm and an emission wavelength of 520 nm for the PicoGreen assay. The encapsulation efficiency of mRNA and pDNA was
 10 calculated according to the following equation:

$$\text{Encapsulation efficiency} = \frac{C_{tx100} - C_{TE}}{C_{tx100}} \times 100\%$$

where C_{tx100} was the mRNA or pDNA concentration measured using the 5% Triton-X100 in the Tri-EDTA buffer, and C_{TE} was the mRNA or pDNA concentration measured using the Tri-EDTA buffer.

15

1.7. Dynamic Light Scattering (DLS)

The size of mRNA and pDNA LNPs was determined by DLS using a Zetasizer (Malvern, UK). 50 μ L of the mRNA and pDNA LNPs suspension were diluted with saline to 1 mL. The size of the mRNA and pDNA LNPs was measured
 20 at 25 °C for 10 s each run and 11 runs for 1 measurement. The average size of every sample was obtained with 3 measurements.

1.8. Surface Zeta Potential

The surface zeta potential of the mRNA and pDNA LNPs was measured
 25 by using a Zetasizer (Malvern, UK). The samples were diluted with saline. The zeta potential was measured at 25 °C. The average zeta potential was obtained after 3 measurements.

1.9. mRNA Binding Analysis via Gel Electrophoresis Assay

30 10 μ L of mRNA LNPs suspension containing 300 ng of mRNA were mixed with 2 μ L of gel loading dye. The mixture was loaded into 1% agarose gel

containing 0.02% of GelStar Nucleic Acid Gel Stain. Then the gel was run at 100 mV for 20 min in Tris-Acetate-EDTA buffer. Finally, the gel was recorded with a gel imaging system (iBright 1500, Invitrogen, Waltham, MA, USA).

5 1.10. Cell Culture and Treatment with mRNA and pDNA LNPs

HELA and HEK293 cells were cultured in DMEM supplemented with 10% FBS (V/V), and 1% Penicillin/Streptomycin (V/V). All cells were maintained in the incubator at 37 °C with 5% CO₂ (Thermo Fisher, Waltham, MA, USA). HELA, HEPG2, and HEK293 cells were seeded in black/white 96-well plates at the
10 density of 10,000 cells per well, respectively. After the cells were attached to plates overnight, the cell culture medium in the plates was replaced with 100 µL of fresh medium containing the mRNA and pDNA LNPs at the final mRNA and pDNA concentration of 100 ng per well, and the cells were incubated for 48 h.

15 1.11. In Vitro Cytotoxicity of the mRNA and pDNA LNPs

The medium was removed after 48 h of incubation. 100 µL of fresh medium containing 10% Alamar Blue reagent was added to each well and incubated for another 2 h. The fluorescence intensity was measured with the microplate reader (Tecan, Männedorf, Switzerland) at an excitation wavelength of 560 nm and an
20 emission wavelength of 590 nm. The cell viability was calculated based on the negative control group without treatment.

1.12. In vitro mRNA and pDNA Transfection Efficiency

The medium was removed after 48 h of incubation. 50 µL of lysis buffer
25 and 50 µL of D-luciferin in firefly glow assay buffer (60 µg/mL) were added to each well and incubated for 10 min for cell lysis and signal stabilization. The luminescence intensity was read by using the microplate reader (Tecan, Männedorf, Switzerland) with an exposure time of 1000 ms. The transfection efficacy was expressed as luminescence units/well.

Example 2: Preparation and Characterization of Primary Amine-containing Ionizable Lipid

The preparation of the lipid started from synthesis of DTD-COONa, which was made by conjugation of succinic anhydride to ditetradecylamine (DTDA). DTD-NHS was then obtained by reacting DTD-COONa with *N*-hydroxysuccinimide (NHS) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC•HCl) as a coupling reagent. Finally, the ionizable lipids were made by substitution of DTD-NHS with ethylenediamine (EDA) or *N,N*-dimethyldipropylenetriamine (DMAPAPA) (**Scheme 1**). To ensure the molar ratio of amine to DTD in the lipids is 1:1, the amines with a slight excess amount (the feed molar ratio of amines to DTD-NHS is 1.05:1) in DCM solution were cooled in dry ice bath, followed by adding DTD-NHS solution dropwise. Excess amines and NHS produced in the substitution reaction were removed by washing the reaction solution with brine.

By referencing the integrated intensities of relevant resonances attributed from protons of the methylene group adjacent to amino group at 2.69 ppm with respect to hydrogens of the methyl groups on both DTD chain ends at 0.92 ppm (FIG. 1), ¹H NMR results indicated the molar ratio of amine to DTD in DTD-NH₂ lipid is 1:1. Similarly, the composition of DMAPAPA-DTD lipid was determined by proton NMR spectrum (FIG. 2) and the molar ratio of amine to DTD in the lipid was 1:1.

Example 3: Preparation and Characterization of mRNA-loaded Lipid Nanoparticles (mRNA LNPs) using Microfluidic Mixing

The mRNA LNPs were made using a microfluidic device and preparation conditions are listed in **Tables 1 - 4**. The major component is the ionizable lipid. As listed in **Table 5**, the mRNA LNPs made using the lipid candidates yielded particle sizes of <100 nm, except for DMAPAPA-DTD (139 nm). The polydispersity of the mRNA LNPs were < 0.2 for most lipid candidates, except for

DMAPAPA-DTD (PDI = 0.347) and DD-EDEA (PDI = 0.21). All the mRNA LNPs formulations had near-neutral zeta potentials. Taken together, these characteristics are desirable for *in vivo* applications.

5 **Table 1.** Preparation conditions of ALC-0315 mRNA-LNPs.

Lipid	Molecular Wt. (g/mol)	Stock concentration in ethanol (mg/mL)	Volume (mL)	Amount added (mg)
ALC-0315	766.29	20	0.0689	1.38
DSPC	790.15	10	0.0288	0.29
Cholesterol	386.65	20	0.0320	0.64
ALC-0159	2450.00	3	0.0508	0.15
Total	-	-	Ethanol added to reach 0.5 mL	2.46

Table 2. Preparation conditions of DTD-NH₂ mRNA-LNPs.

Lipid	Molecular Wt. (g/mol)	Stock concentration in ethanol (mg/mL)	Volume (mL)	Amount added (mg)
DTD-NH ₂	551.00	4	0.3450	1.38
DSPC	790.15	10	0.0288	0.29
Cholesterol	386.65	20	0.0320	0.64
ALC-0159	2450.00	3	0.0508	0.15
Total	-	-	Ethanol added to reach 0.5 mL	2.46

Table 3. Preparation conditions of DMAPAPA-DTD mRNA-LNPs.

Lipid	Molecular Wt. (g/mol)	Stock concentration in ethanol (mg/mL)	Volume (mL)	Amount added (mg)
DMAPAPA-DTD	650	4	0.345	1.38
DSPC	790.15	10	0.0288	0.288
Cholesterol	386.65	20	0.0320	0.64
ALC-0159	2450	3	0.0508	0.152
Total	-	-	Ethanol added to reach 0.5 mL	2.460

Table 4. Preparation conditions of lipid-EDEA mRNA-LNPs.

Lipid	Molecular Wt. (g/mol)	Stock concentration in ethanol (mg/mL)	Volume (mL)	Amount added (mg)
DnO-EDEA; DD-EDEA; DTD-EDEA	471.00; 527.00; 639.00	10	0.0690	0.69
DSPC	790.15	10	0.0143	0.14
Cholesterol	386.65	20	0.0160	0.32
ALC-0159	2450.00	3	0.0254	0.08
Total	-	-	Ethanol added to reach 0.25 mL	1.23

5

Table 5. Particle size, size distribution and zeta potential of mRNA LNPs.

Formulation	Diameter (nm)	PDI	Zeta Potential (mV)
ALC-0315	84.2 ± 0.3	0.04 ± 0.02	-2.15 ± 1.30
DTD-NH ₂	84.7 ± 0.6	0.17 ± 0.02	1.77 ± 0.80
DMAPAPA-DTD	139 ± 5	0.347 ± 0.012	-5.27 ± 3.28
DnO-EDEA	95.3 ± 1.1	0.17 ± 0.06	-2.94 ± 2.45
DD-EDEA	73.3 ± 0.5	0.21 ± 0.00	4.80 ± 1.57
DTD-EDEA	84.6 ± 0.1	0.17 ± 0.00	7.00 ± 1.35

Effective mRNA binding by ionizable lipid is an important first step for the formation of stable and compact mRNA LNPs that are critical for efficient cellular uptake and transfection. mRNA binding efficiency of the various mRNA LNP formulations was evaluated using the RiboGreen RNA assay. The encapsulation efficiency of the mRNA LNPs were calculated by the percentage of encapsulated mRNA over the total mRNA content (encapsulated and unencapsulated). As shown in **Table 6**, the encapsulation efficiency of the mRNA LNPs made from the lipid candidates were all > 80% (except for DnO-EDEA mRNA LNP), which are comparable with the ALC-0315 formulation. This demonstrates that the ionizable lipids with primary amines were able to condense mRNA in the LNP formulation, without affecting its particle size.

Table 6. Encapsulation efficiency of mRNA LNPs.

Formulation	Encapsulation Efficiency (%)
ALC-0315 (1 st Batch)	65.27 ± 0.67
ALC-0315 (2 nd Batch)	85.60 ± 0.31
DTD-NH ₂	88.99 ± 0.02
DMAPAPA-DTD	97.90 ± 0.01
DnO-EDEA	60.23 ± 14.29
DD-EDEA	95.62 ± 0.06
DTD-EDEA	96.29 ± 0.06

As shown in FIG. 3 and FIG. 4, the gene transfection efficiency of mRNA LNPs made from DTD-NH₂ were about 7 times and 4 times higher than those made from ALC-0315. mRNA DMAPAPA-DTD LNPs induced hundreds to one thousand times lower transfection efficiency than mRNA DTD-NH₂ LNPs. In addition, DTD-EDEA mRNA LNP showed comparable transfection efficiency to ALC0315 mRNA LNP in HeLa cells. Among the mRNA LNPs made with the EDEA lipids, DTD-EDEA showed the best transfection efficiency for both cell lines, demonstrating that a longer lipid tail may favor cellular transfection for this lipid series. Under the same transfection conditions, no significant cellular cytotoxicity in both HeLa and HEK293 cells was observed for all mRNA LNP formulations tested after 48 h of incubation (FIG. 5 and FIG. 6).

Example 4: High-throughput Screening of Other Ionizable Lipids for mRNA LNPs

For high-throughput screening of subsequent ionizable lipids, the mRNA LNPs were prepared by direct injection of lipids-containing organic phase into the mRNA-containing aqueous phase. Different ionizable lipids were added to the organic phase (**Tables 7 and 8**) and the amount of mRNA added was adjusted to achieve N/P ratio of 6 for all formulations. This method provides higher

efficiency and is more cost-efficient when screening for potential lipid candidates for lipid nanoparticles.

The ALC-0315 mRNA LNPs made using the direct injection method had
5 larger particle sizes and PDI (**Table 9**) than when it was made using the
microfluidic device (**Table 5**). This could be due to the difference in mixing
conditions between direct injection and microfluidic mixing. During microfluidic
mixing, the organic phase and the aqueous phase are mixed in a controlled
manner, with a specified mixing ratio of 1:3 (organic phase:aqueous phase). This
10 ensures that constant amounts of lipids and mRNA are interacting with each
other, allowing efficient mRNA condensation with the charged ionizable lipid as it
forms the LNPs. As these factors were not controlled for the direct injection
method, the interaction between the ionizable lipid and mRNA happens more
spontaneously and may not be maximised, resulting in slightly larger particle
15 sizes and PDI.

As observed from **Table 9**, mRNA-LNPs made with the lipid candidates
yielded particle sizes of about 120 – 170 nm, with all their PDI being < 0.2. The
zeta potential of all the formulations were mostly near neutral (± 10 mV). The
20 encapsulation efficiencies of all the lipid candidates were significantly higher than
ALC-0315 mRNA LNP (**Table 10**). The low encapsulation efficiency of ALC-0315
mRNA LNPs could be attributed to the method of formulation. Nevertheless, ALC-
0315 mRNA LNPs still exhibited mRNA transfection in HeLa cells (FIG. 7 and FIG.
8). This could possibly be due to the mRNA being loosely bound on the LNP
25 surface instead of being encapsulated within the internal milieu of the LNPs,
which could still transfect cells. Out of all the formulations tested, DTD-NH₂ and
HO-DTD-NH₂ mRNA LNPs outperformed ALC-0315 mRNA LNPs in terms of
luciferase mRNA transfection in HeLa cells (FIG. 7 and FIG. 8). The cell viability
of HeLa cells incubated with DTD-NH₂ and HO-DTD-NH₂ formulations also
30 showed little to no cellular cytotoxicity (FIG. 7 and FIG. 8). The particle and
cellular transfection characteristics of DTD-NH₂ and HO-DTD-NH₂ LNPs may be
further improved if formulated using microfluidic mixing.

Table 7. Preparation conditions of mRNA LNPs for high-throughput screening.

Lipid	Molecular Wt. (g/mol)	Stock concentration in ethanol (mg/mL)	Volume (μL)	Amount added (mg)
Lipid candidate	Refer to Table 8	20 (ALC-0315); 4 (-NH ₂ lipids); 10 (HO- lipids)	6.90 (ALC-0315); 34.50 (-NH ₂ lipids); 13.80 (HO- lipids)	0.1380
DSPC	790.15	10	2.86	0.0286
Cholesterol	386.65	20	3.20	0.0640
ALC-0159	2450.00	3	5.08	0.0152
Total	-	-	Ethanol added to reach 50 μL	0.2458

Table 8. Molecular weight of lipid candidates.

Lipid candidate	Molecular Wt. (g/mol)
ALC-0315	766.29
DTD-NH ₂	551.00
DnO-NH ₂	383.00
DD-NH ₂	439.00
HO-DTD-NH ₂	581.00
HO-DnO-NH ₂	413.00
HO-DD-NH ₂	469.00

Table 9. Particle size, size distribution and zeta potential of mRNA LNPs from high-throughput screening.

Formulation	Diameter (nm)	PDI	Zeta Potential (mV)
ALC0315	142.9 ± 16.6	0.14 ± 0.03	2.40 ± 3.86
DTD-NH ₂	124.9 ± 3.4	0.15 ± 0.03	11.32 ± 1.34
DnO-NH ₂	174.0 ± 17.4	0.15 ± 0.04	-7.54 ± 3.88
DD-NH ₂	134.6 ± 6.7	0.25 ± 0.06	7.40 ± 3.78
HO-DTD-NH ₂	149.3 ± 6.6	0.15 ± 0.01	1.75 ± 1.77
HO-DnO-NH ₂	171.1 ± 6.9	0.14 ± 0.02	-7.42 ± 0.93
HO-DD-NH ₂	156.8 ± 1.0	0.10 ± 0.01	-0.38 ± 1.32

5 **Table 10.** Encapsulation efficiency of mRNA LNPs made using direct injection method.

Formulation	Encapsulation Efficiency (%)
ALC-0315	58.8 ± 18.0
DTD-NH ₂	95.2 ± 1.3
DnO-NH ₂	67.8 ± 2.4
DD-NH ₂	93.0 ± 0.8
HO-DTD-NH ₂	89.9 ± 2.7
HO-DnO-NH ₂	60.1 ± 5.5
HO-DD-NH ₂	77.6 ± 7.1

Example 5: High-throughput Screening of Ionizable Lipids for pDNA LNPs

10 The high-throughput screening of ionizable lipids for pDNA LNPs was performed similar to that of mRNA LNPs. The aforementioned ionizable lipids were added to the organic phase (**Table 11**) and the amount of pDNA added was adjusted to achieve N/P ratio of 6 for all formulations.

As observed from **Table 12**, pDNA LNPs made with the lipid candidates showed particle sizes ranging 80 – 160 nm, with all their PDI being < 0.2. The zeta potential of all the formulations were mostly near neutral (± 10 mV), with the exception of HO-DnO-NH₂ pDNA LNPs that yielded a zeta potential of -12.87 mV.

5 The encapsulation efficiencies of all the lipid candidates were higher than ALC-0315 pDNA LNP (**Table 13**). The differences in the results for particle size, zeta potential, and encapsulation efficiency between pDNA LNPs and mRNA LNPs could possibly be explained by the different sizes of nucleic acid cargoes and their respective binding efficiencies to the ionizable lipid. Similar to the high-

10 throughput screening of the mRNA LNPs, DTD-NH₂ and HO-DTD-NH₂ pDNA LNPs greatly outperformed ALC-0315 pDNA LNPs in terms of pDNA transfection in HEK293 cells (FIG. 9 and FIG. 10). The viability of HEK293 cells incubated with DTD-NH₂ and HO-DTD-NH₂ formulations also showed little to no cellular cytotoxicity (FIG. 9 and FIG. 10). Likewise, the particle and cellular transfection

15 characteristics of DTD-NH₂ and HO-DTD-NH₂ LNPs may be further improved if formulated using microfluidic mixing.

Table 11. Preparation conditions of pDNA LNPs for high-throughput screening.

Lipid	Molecular Wt. (g/mol)	Stock concentration in ethanol (mg/mL)	Volume (μL)	Amount added (mg)
Lipid candidate	Refer to Table 8	20 (ALC-0315); 4 (-NH ₂ lipids); 10 (HO- lipids)	6.90 (ALC-0315); 34.50 (-NH ₂ lipids); 13.80 (HO- lipids)	0.1380
DSPC	790.15	10	2.86	0.0286
Cholesterol	386.65	20	3.20	0.0640
ALC-0159	2450.00	3	5.08	0.0152
Total	-	-	Ethanol added to reach 50 μL	0.2458

Table 12. Particle size, size distribution and zeta potential of pDNA LNPs from high-throughput screening.

Formulation	Diameter (nm)	PDI	Zeta Potential (mV)
ALC0315	97.27 ± 0.54	0.13 ± 0.02	-0.40 ± 0.64
DTD-NH ₂	113.80 ± 1.39	0.13 ± 0.01	3.07 ± 1.40
DnO-NH ₂	106.00 ± 1.66	0.18 ± 0.01	-7.61 ± 1.93
DD-NH ₂	86.42 ± 1.71	0.18 ± 0.02	6.56 ± 1.64
HO-DTD-NH ₂	95.26 ± 0.94	0.17 ± 0.02	3.19 ± 1.82
HO-DnO-NH ₂	156.20 ± 1.38	0.13 ± 0.02	-12.87 ± 1.59
HO-DD-NH ₂	87.26 ± 0.60	0.13 ± 0.01	0.98 ± 1.22

Table 13. Encapsulation efficiency of pDNA LNPs made using the direct injection method.

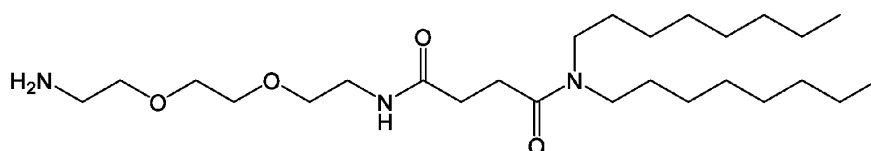
Formulation	Encapsulation Efficiency (%)
ALC-0315	74.0 ± 1.9
DTD-NH ₂	88.3 ± 0.2
DnO-NH ₂	92.8 ± 0.1
DD-NH ₂	90.4 ± 0.3
HO-DTD-NH ₂	89.0 ± 0.4
HO-DnO-NH ₂	84.6 ± 1.1
HO-DD-NH ₂	93.7 ± 0.1

Example 6: Summary

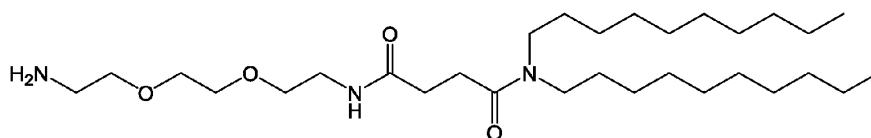
The ionizable lipid DTD-NH₂ was successfully designed and made through 3 synthetic steps. The synthesis of the new ionizable lipid HO-DTD-NH₂ was also straightforward. The use of these lipids led to the formation of mRNA and pDNA LNPs with ≥80% encapsulation efficiency of mRNA and pDNA, nanosize and neutral surface charge, which are desirable properties for *in vivo* application. The mRNA and pDNA LNPs made from DTD-NH₂ or HO-DTD-NH₂ mediated

significantly higher transfection efficiency in the cell lines tested than the mRNA and pDNA LNPs made from the commercial lipid ALC-0315 that is used in Pfizer-BioNTech mRNA vaccine formulation, without causing any cytotoxicity. These LNPs have great potential for use as nanocarriers to deliver mRNA vaccine or therapeutics, and pDNA. They may also be used to deliver other nucleic acid therapeutics.

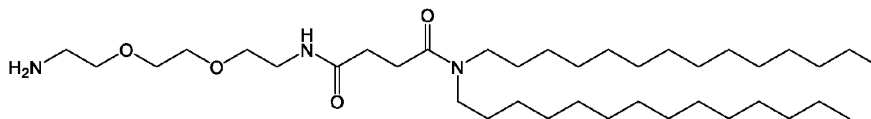
Example 7: Structures of Examples of Ionizable Lipids



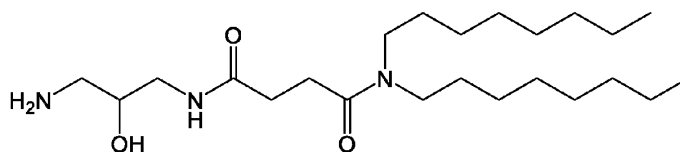
Chemical structure of **DnO-EDEA** (M = 471; N% = 3.0%)



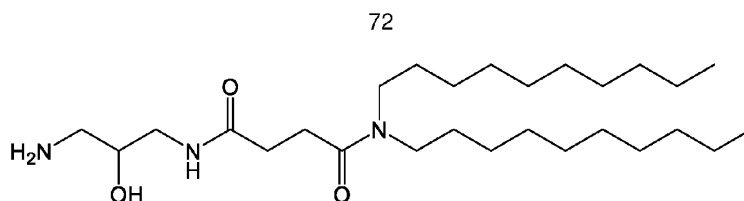
Chemical structure of **DD-EDEA** (M = 527; N% = 2.7%)



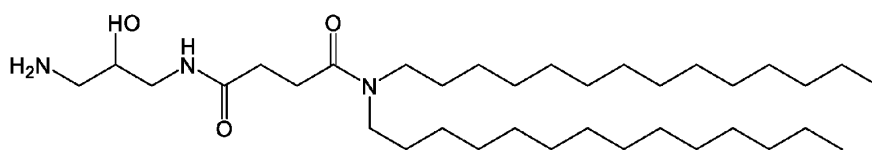
Chemical structure of **DTD-EDEA** (M = 639; N% = 2.2%)



Chemical structure of **HO-DnO-NH₂_3rd** (M = 413, N% = 3.4%)

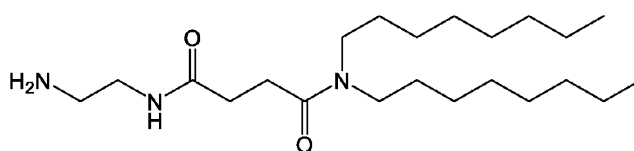


Chemical structure of **HO-DD-NH₂_3rd** (M = 469, N% = 3.0%)



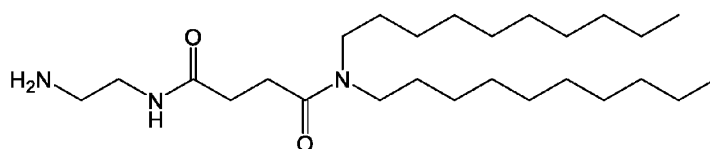
5

Chemical structure of **HO-DTD-NH₂_5th** (M = 581, N% = 2.4%)



Chemical structure of **DnO-NH₂_2nd** (M = 383; N% = 3.7%)

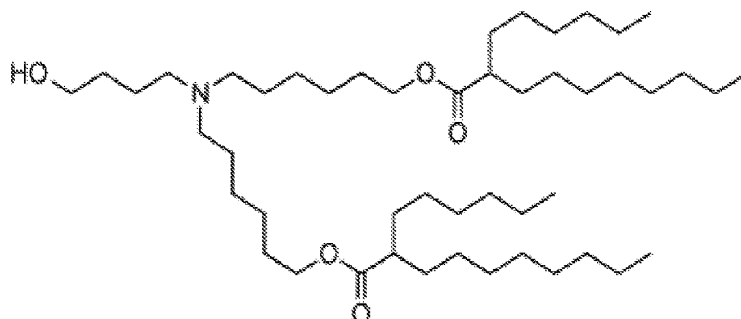
10



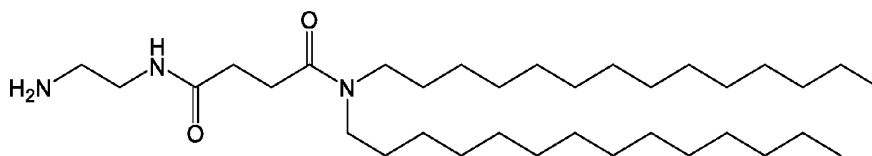
Chemical structure of **DD-NH₂_2nd** (M = 439; N% = 3.2%)

15

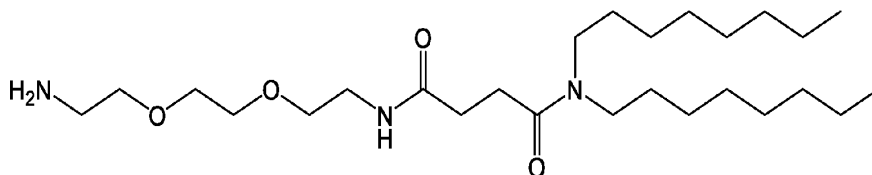
73

Example 8: mRNA LNPs Formulations

Chemical structure of ALC-0315 referred to in Table 14

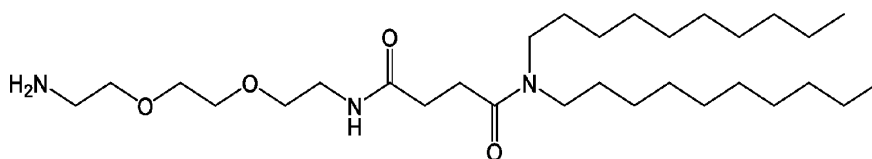


5

Chemical structure of DTD-NH₂ referred to in Table 15

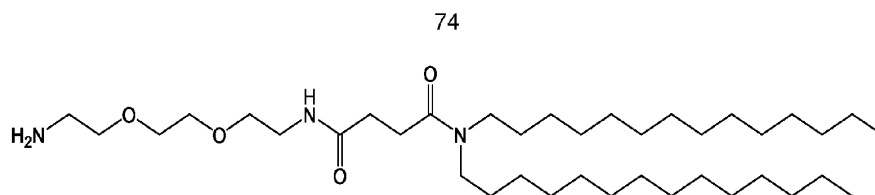
10

Chemical structure of DnO-EDEA referred to in Table 16



15

Chemical structure of DD-EDEA referred to in Table 17



Chemical structure of DTD-EDEA referred to in Table 18

5 8.1. Characterization of mRNA LNPs: Size, PDI, Zeta Potential

The size, PDI and zeta potential of mRNA LNPs were measured on Day 0 and after 8 days of storage. The characterization results are provided in **Table 19**. As shown in **Table 19**, the size of mRNA LNPs made from DnO-EDEA, DD-EDEA or DTD-EDEA is comparable with that of mRNA LNPs made from DTD-NH₂ or ALC-0315. mRNA LNPs made from DD-EDEA or DTD-EDEA were stable over 8 days of storage as evidenced by comparable particle size and size distribution (PDI), while mRNA LNPs made from DnO-EDEA were unstable as evidenced by the increase in size after 8 days of storage.

Table 14. LNP formulation (ALC-0315)

Lipid	Molecular Weight (g/mol)	Stock Concentration (mg/mL)	Amount added (mg)	Volume (mL)	Molar ratio
ALC-0315	766.29	20	1.3800	0.0690	46.3
DSPC	790.15	10	0.2860	0.0286	9.4
Cholesterol	386.65	20	0.6400	0.0320	42.7
ALC-0159	2450.00	3	0.1500	0.0508	1.6
Ethanol	-	-	-	0.3196	-
Total:	-	-	2.4583	0.5000	100.0

- Total volume for aqueous (1.5mL) and organic phase (0.5mL) was 2.0 mL
- N/P ratio = 6
- Amount of fLuc mRNA (Trilink) added = 0.1 mg
- 10mM sodium acetate (pH 4.0) was used to dilute mRNA

Table 15. LNP formulation (DTD-NH₂)

Lipid	Molecular Weight (g/mol)	Stock Concentration (mg/mL)	Amount added (mg)	Volume (mL)	Molar ratio
DTD-NH ₂ (5 th – 1 st)	551.00	4	1.3800	0.3450	54.6
DSPC	790.15	10	0.2860	0.0286	7.9
Cholesterol	386.65	20	0.6400	0.0320	36.1
ALC-0159	2450.00	3	0.1500	0.0508	1.4
Ethanol	-	-	-	0.0436	-
Total:	-	-	2.4583	0.5000	100.0

- Total volume for aqueous (1.5mL) and organic phase (0.5mL) was 2.0 mL
- N/P ratio = 6
- Amount of fLuc mRNA (Trilink) added = 0.14 mg
- 10mM sodium acetate (pH 4.0) was used to dilute mRNA
- Use of DTD-NH₂, which relies only on a primary amine group, as the ionizable lipid in LNPs for gene delivery

Table 16. LNP formulation (DnO-EDEA)

Lipid	Molecular Weight (g/mol)	Stock Concentration (mg/mL)	Amount added (mg)	Volume (mL)	Molar ratio
DnO-EDEA	471	10	0.69	0.0690	58.5
DSPC	790.15	10	0.143	0.0143	7.2
Cholesterol	386.65	20	0.32	0.0160	33.0
ALC-0159	2450	3	0.08	0.0254	1.2
Ethanol	-	-	-	0.1253	-
Total:	-	-	1.22915	0.2500	100

- Total volume for aqueous (0.75mL) and organic phase (0.25mL) was 1.0 mL
- N/P ratio = 6
- Amount of fluc mRNA (Trilink) added = 0.0813 mg
- 10mM sodium acetate (pH 4.0) was used to dilute mRNA
- New ionizable lipid

Table 17. LNP formulation (DD-EDEA)

Lipid	Molecular Weight (g/mol)	Stock Concentration (mg/mL)	Amount added (mg)	Volume (mL)	Molar ratio
DD-EDEA	527	10	0.69	0.0690	55.7
DSPC	790.15	10	0.143	0.0143	7.7
Cholesterol	386.65	20	0.32	0.0160	35.2
ALC-0159	2450	3	0.08	0.0254	1.3
Ethanol	-	-	-	0.1253	-
Total:	-	-	1.22915	0.2500	100

- Total volume for aqueous (0.75mL) and organic phase (0.25mL) was 1.0 mL
- N/P ratio = 6
- Amount of fluc mRNA (Trilink) added = 0.0727 mg
- 10mM sodium acetate (pH 4.0) was used to dilute mRNA
- New ionizable lipid

Table 18. LNP formulation (DTD-EDEA)

Lipid	Molecular Weight (g/mol)	Stock Concentration (mg/mL)	Amount added (mg)	Volume (mL)	Molar ratio
DTD-EDEA	639	10	0.69	0.0690	50.9
DSPC	790.15	10	0.143	0.0143	8.5
Cholesterol	386.65	20	0.32	0.0160	39.0
ALC-0159	2450	3	0.08	0.0254	1.5
Ethanol	-	-	-	0.1253	-
Total:	-	-	1.22915	0.2500	100

- Total volume for aqueous (0.75mL) and organic phase (0.25mL) was 1.0 mL
- N/P ratio = 6
- Amount of fluc mRNA (Trilink) added = 0.06 mg
- 10mM sodium acetate (pH 4.0) was used to dilute mRNA
- New ionizable lipid

Table 19. LNPs characterization

Day	Sample	Size (nm)	PDI	Zeta potential
0	ALC-0315	84.2 ± 0.3	0.04 ± 0.02	-2.15 ± 1.30
	DTD-NH ₂	84.7 ± 0.6	0.17 ± 0.02	1.77 ± 0.80
0	DnO-EDEA	95.3 ± 1.1	0.17 ± 0.06	-2.94 ± 2.45
	DD-EDEA	73.3 ± 0.5	0.21 ± 0.00	4.80 ± 1.57
	DTD-EDEA	84.6 ± 0.1	0.17 ± 0.00	7.00 ± 1.35
8	DnO-EDEA	111.8 ± 5.7	0.30 ± 0.04	-7.79 ± 0.97
	DD-EDEA	76.1 ± 0.0	0.22 ± 0.01	5.67 ± 0.70
	DTD-EDEA	85.6 ± 0.9	0.17 ± 0.01	9.01 ± 1.66

8.2. Encapsulation Efficiency of mRNA LNPs

Table 20 shows the percentage encapsulation efficiency of the mRNA LNPs. As shown in **Table 20**, mRNA LNPs made from DD-EDEA or DTD-EDEA as the ionizable lipid has comparable encapsulation efficiency as the mRNA LNPs made from DTD-NH₂. Use of DD-EDEA or DTD-EDEA as the ionizable lipid led to greater encapsulation efficiency than use of DnO-EDEA with the shorter lipid tails.

Table 20. Encapsulation Efficiency (EE)

Sample	Original Encapsulation Efficiency (%)	Final Encapsulation Efficiency (%)
ALC-0315	65.3 ± 0.7	88.2 ± 0.5
DTD-NH ₂	89.0 ± 0.0	96.5 ± 0.2
DnO-EDEA	60.2 ± 14.3	76.4 ± 0.6
DD-EDEA	95.6 ± 0.1	94.8 ± 0.3
DTD-EDEA	96.3 ± 0.1	92.7 ± 0.4

- Total volume for aqueous (0.75mL) and organic phase (0.25mL) was 1.0 mL
- N/P ratio = 6
- Amount of fLuc mRNA (Trilink) added = 0.06 mg
- 10mM sodium acetate (pH 4.0) was used to dilute mRNA
- New ionizable lipid

In Table 20, "Original" encapsulation efficiency refers to before the mRNA LNPs were dialyzed to remove residual ethanol and "Final" encapsulation efficiency refers to after the mRNA LNPs were dialyzed to remove residual ethanol.

8.3. Transfection – Cell Viability and Luminescence Intensity of mRNA LNPs

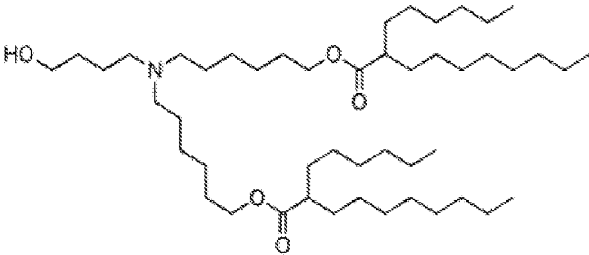
Table 21 shows cell viability and luminescence intensity results obtained for the mRNA LNPs after 48 hours of incubation. As shown in **Table 21**, all mRNA LNPs were cytocompatible without inducing cytotoxicity towards HeLa cells. Among DnO-EDEA, DD-EDEA and DTD-EDEA, DTD-EDEA gave the highest mRNA transfection efficiency in HeLa cells. Although the mRNA LNPs made from DTD-EDEA induced lower transfection efficiency, their transfection efficiency was comparable with that of the mRNA LNPs made from ALC-0351.

Table 21. Transfection in HeLa cells (48 hours)

Sample	Dose (ng/well)	Cell viability (%)	Luminescence intensity (RLU)
Control	-	100.0 ± 2.8	129 ± 26
ALC-0315	100	107.3 ± 2.2	2869 ± 762
DTD-NH ₂	100	107.3 ± 1.6	20032 ± 5791
DnO-EDEA	100	107.6 ± 2.7	237 ± 45
DD-EDEA	100	111.5 ± 1.9	195 ± 27
DTD-EDEA	100	101.0 ± 5.9	2347 ± 529

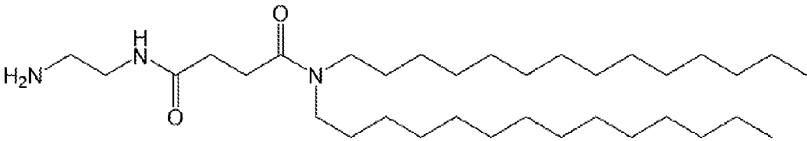
- Control refers to cells without LNP transfection (i.e. incubation with culture media without LNP)

Example 9: mRNA LNPs Formulations Made Manually



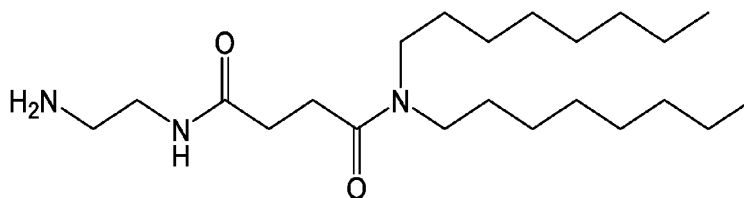
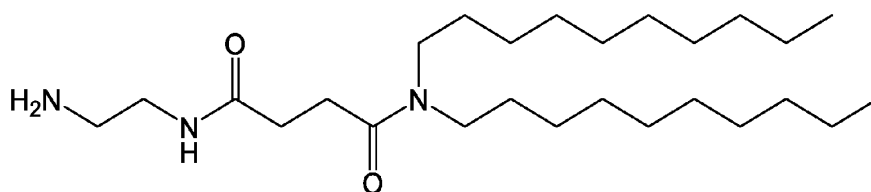
5

Chemical Structure of ALC-0315 referred to in Table 22

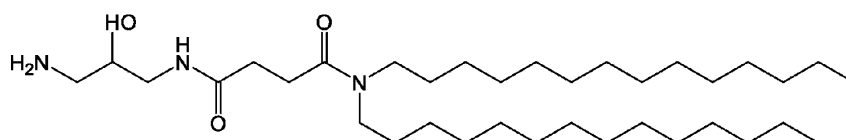
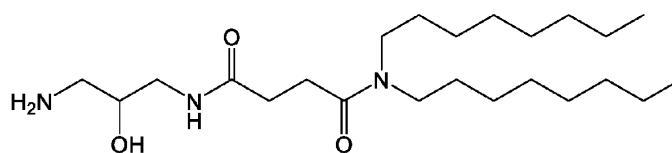


Chemical Structure of DTD-NH₂ referred to in Table 23

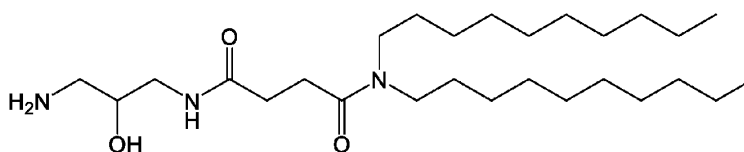
83

Chemical Structure of DnO-NH₂ referred to in Table 24

5

Chemical Structure of DD-NH₂ referred to in Table 25Chemical Structure of HO-DTD-NH₂ referred to in Table 26

10

Chemical Structure of HO-DnO-NH₂ referred to in Table 27Chemical Structure of HO-DD-NH₂ referred to in Table 28

9.1. Characterization and Encapsulation Efficiency (EE) of mRNA LNPs

The size, PDI and zeta potential of mRNA LNPs were measured on Day 0 and after 1 day of storage. The characterization results (i.e. size, PDI and zeta potential) of mRNA LNPs are provided in **Table 29**. Percentage encapsulation efficiency (EE) of the mRNA LNPs is also provided in **Table 29**.

As shown in **Table 29**, except for ALC-0315 and HO-DnO-NH₂, most lipids made manually led to high encapsulation efficiency (~80-90%), comparable to that of LNPs made using a microfluidic device. Particle sizes of the LNPs made manually are bigger than those made using the microfluidic device. However, the sizes are still below 200nm. The mRNA LNPs are stable over one day of storage at 4°C except for DnO-NH₂ LNPs (increase in size and PDI).

9.2. Transfection – Cell Viability and Luminescence Intensity of mRNA LNPs

Table 30 shows cell viability and luminescence intensity results obtained for the mRNA LNPs after 48 hours of incubation. As shown in **Table 30**, similar to mRNA LNPs made using the microfluidic device, the mRNA DTD-NH₂ LNPs made manually induced about 18 times higher mRNA transfection efficiency in HeLa cells than mRNA ALC-0315 LNPs. mRNA HO-DTD-NH₂ LNPs induced similarly high transfection efficiency as DTD-NH₂ did. mRNA LNPs made from the rest of the ionizable lipids did not induce significant transfection efficiency as compared to the control.

Table 22. LNP formulation (ALC-0315)

Lipid	Molecular Weight (g/mol)	Stock Concentration (mg/mL)	Amount added (mg)	Volume (μL)	Molar ratio
ALC-0315	766.29	20	0.1380	6.90	46.4
DSPC	790.15	10	0.0286	2.86	9.3
Cholesterol	386.65	20	0.0640	3.20	42.7
ALC-0159	2450	3	0.0152	5.08	1.6
Ethanol	-	-	-	31.96	-
Total:	-	-	0.2458	50.00	100

- Total volume for aqueous (0.15mL) and organic phase (0.05mL) was 0.2 mL
- N/P ratio = 6
- Amount of fluc mRNA (Trilink) added = 0.01 mg
- 10mM sodium acetate (pH 4.0) was used to dilute mRNA

Table 23. LNP formulation (DTD-NH₂)

Lipid	Molecular Weight (g/mol)	Stock Concentration (mg/mL)	Amount added (mg)	Volume (μL)	Molar ratio
DTD-NH ₂	551	4	0.1380	34.50	54.6
DSPC	790.15	10	0.0286	2.86	7.9
Cholesterol	386.65	20	0.0640	3.20	36.1
ALC-0159	2450	3	0.0152	5.08	1.4
Ethanol	-	-	-	4.36	-
Total:	-	-	0.2458	50.00	100

- Total volume for aqueous (0.15mL) and organic phase (0.05mL) was 0.2 mL
- N/P ratio = 6
- Amount of fLuc mRNA (Trilink) added = 0.014 mg
- 10mM sodium acetate (pH 4.0) was used to dilute mRNA

Table 24. LNP formulation (DnO-NH₂)

Lipid	Molecular Weight (g/mol)	Stock Concentration (mg/mL)	Amount added (mg)	Volume (μL)	Molar ratio
DnO-NH ₂	383	4	0.1380	34.50	63.4
DSPC	790.15	10	0.0286	2.86	6.4
Cholesterol	386.65	20	0.0640	3.20	29.1
ALC-0159	2450	3	0.0152	5.08	1.1
Ethanol	-	-	-	4.36	-
Total:	-	-	0.2458	50.00	100

- Total volume for aqueous (0.15mL) and organic phase (0.05mL) was 0.2 mL
- N/P ratio = 6
- Amount of fLuc mRNA (Trilink) added = 0.02 mg
- 10mM sodium acetate (pH 4.0) was used to dilute mRNA

Table 25. LNP formulation (DD-NH₂)

Lipid	Molecular Weight (g/mol)	Stock Concentration (mg/mL)	Amount added (mg)	Volume (μL)	Molar ratio
DD-NH ₂	439	4	0.1380	34.50	60.2
DSPC	790.15	10	0.0286	2.86	6.9
Cholesterol	386.65	20	0.0640	3.20	31.7
ALC-0159	2450	3	0.0152	5.08	1.2
Ethanol	-	-	-	4.36	-
Total:	-	-	0.2458	50.00	100

- Total volume for aqueous (0.15mL) and organic phase (0.05mL) was 0.2 mL
- N/P ratio = 6
- Amount of fLuc mRNA (Trilink) added = 0.0175 mg
- 10mM sodium acetate (pH 4.0) was used to dilute mRNA

Table 26. LNP formulation (HO-DTD-NH₂)

Lipid	Molecular Weight (g/mol)	Stock Concentration (mg/mL)	Amount added (mg)	Volume (μL)	Molar ratio
HO-DTD-NH ₂	581	10	0.1380	13.80	53.3
DSPC	790.15	10	0.0286	2.86	8.1
Cholesterol	386.65	20	0.0640	3.20	37.2
ALC-0159	2450	3	0.0152	5.08	1.4
Ethanol	-	-	-	25.06	-
Total:	-	-	0.2458	50.00	100

- Total volume for aqueous (0.15mL) and organic phase (0.05mL) was 0.2 mL
- N/P ratio = 6
- Amount of fLuc mRNA (Trilink) added = 0.0132 mg
- 10mM sodium acetate (pH 4.0) was used to dilute mRNA

Table 27. LNP formulation (HO-DnO-NH₂)

Lipid	Molecular Weight (g/mol)	Stock Concentration (mg/mL)	Amount added (mg)	Volume (μL)	Molar ratio
HO-DnO-NH ₂	413	10	0.1380	13.80	61.6
DSPC	790.15	10	0.0286	2.86	6.7
Cholesterol	386.65	20	0.0640	3.20	30.5
ALC-0159	2450	3	0.0152	5.08	1.1
Ethanol	-	-	-	25.06	-
Total:	-	-	0.2458	50.00	100

- Total volume for aqueous (0.15mL) and organic phase (0.05mL) was 0.2 mL
- N/P ratio = 6
- Amount of fLuc mRNA (Trilink) added = 0.0185 mg
- 10mM sodium acetate (pH 4.0) was used to dilute mRNA

Table 28. LNP formulation (HO-DD-NH₂)

Lipid	Molecular Weight (g/mol)	Stock Concentration (mg/mL)	Amount added (mg)	Volume (μL)	Molar ratio
HO-DD-NH ₂	469	10	0.1380	13.80	58.6
DSPC	790.15	10	0.0286	2.86	7.2
Cholesterol	386.65	20	0.0640	3.20	33.0
ALC-0159	2450	3	0.0152	5.08	1.2
Ethanol	-	-	-	25.06	-
Total:	-	-	0.2458	50.00	100

- Total volume for aqueous (0.15mL) and organic phase (0.05mL) was 0.2 mL
- N/P ratio = 6
- Amount of fLuc mRNA (Trilink) added = 0.0163 mg
- 10mM sodium acetate (pH 4.0) was used to dilute mRNA

Table 29. LNP Characterization and Encapsulation Efficiency

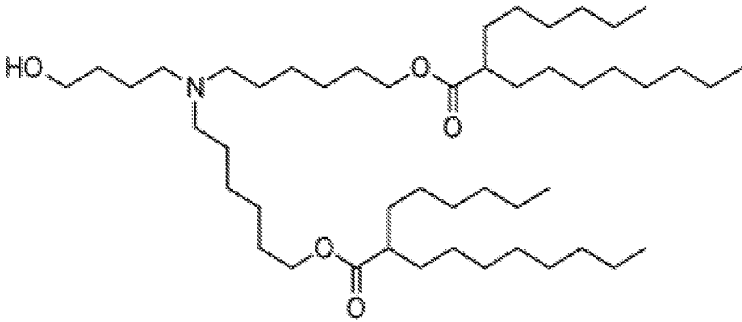
Day	Sample	Size (nm)	PDI	Zeta potential	EE %	mRNA concentration in LNP (ng/μL)
0	ALC-0315	127.50 ± 2.68	0.17 ± 0.03	3.27 ± 1.34	15.74 ± 2.65	48.67 ± 1.84
	DTD-NH ₂	154.50 ± 1.76	0.12 ± 0.02	1.82 ± 0.90	81.66 ± 2.00	54.91 ± 0.23
	DnO-NH ₂	152.90 ± 0.85	0.18 ± 0.01	-0.37 ± 1.09	79.01 ± 0.65	76.92 ± 1.72
	DD-NH ₂	126.60 ± 0.60	0.19 ± 0.02	14.40 ± 1.09	96.04 ± 0.21	73.67 ± 2.51
	HO-DTD-NH ₂	149.20 ± 1.08	0.19 ± 0.01	1.50 ± 0.28	80.00 ± 0.92	67.62 ± 3.22
	HO-DnO-NH ₂	172.50 ± 0.35	0.15 ± 0.01	-4.80 ± 3.03	27.05 ± 3.45	73.55 ± 2.26
1	HO-DD-NH ₂	156.60 ± 2.83	0.12 ± 0.03	0.45 ± 1.05	78.49 ± 5.15	65.42 ± 2.89
	ALC0315	138.10 ± 1.49	0.13 ± 0.01	1.31 ± 1.44	-	-
	DTD-NH ₂	155.80 ± 2.80	0.12 ± 0.03	2.79 ± 1.60	-	-
	DnO-NH ₂	191.70 ± 4.04	0.26 ± 0.01	0.58 ± 0.96	-	-
	DD-NH ₂	130.70 ± 1.12	0.19 ± 0.01	13.42 ± 0.90	-	-
	HO-DTD-NH ₂	150.10 ± 1.09	0.18 ± 0.01	3.20 ± 1.24	-	-
	HO-DnO-NH ₂	166.50 ± 1.19	0.15 ± 0.02	-2.90 ± 1.59	-	-
	HO-DD-NH ₂	153.80 ± 1.63	0.14 ± 0.02	0.26 ± 3.35	-	-

Table 30. Transfection in HeLa cells (48 hours)

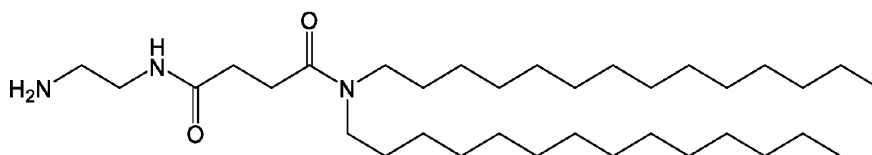
Sample	Dose (ng/well)	Cell viability (%)	Luminescence intensity (RLU)	Relative to ALC-0315
Control	-	100.0 ± 1.7	502 ± 59	10.0%
ALC-0315	100	99.8 ± 1.9	5002 ± 324	100.0%
DTD-NH ₂	100	101.7 ± 0.7	91619 ± 36743	1831.8%
DnO-NH ₂	100	86.3 ± 11.3	400 ± 40	8.0%
DD-NH ₂	100	49.0 ± 0.5	351 ± 51	7.0%
HO-DD-NH ₂	100	93.4 ± 2.8	101630 ± 39817	2031.9%
HO-DnO-NH ₂	100	93.3 ± 1.3	360 ± 48	7.2%
HO-DD-NH ₂	100	69.2 ± 15.7	967 ± 1192	19.3%

- Control refers to cells without LNP transfection (i.e. incubation with culture media without LNPs).

Example 10: Plasmid DNA Delivery



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Chemical Structure of DTD-NH₂ referred to in Table 32

10.1. Characterization of pDNA-loaded LNPs

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The size, PDI and zeta potential of pDNA-loaded LNPs were measured on Day 0 and after 6 days of storage. The characterization results (i.e. size, PDI and zeta potential) of pDNA-loaded LNPs are provided in **Table 33**. Percentage encapsulation efficiency (EE) of the pDNA-loaded LNPs is also provided in **Table**

10 **33**.

As shown in Table 33, pDNA-loaded LNPs made from DTD-NH₂ have an average size of around 80 nm. pDNA-loaded LNPs made from DTD-NH₂ were stable after 6 days of storage at 4°C as evidenced by the consistent particle size, size distribution (PDI) and zeta potential.

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The particle size and size distribution measured on Day 0 for pDNA-loaded LNPs made respectively from ALC-0315 and DTD-NH₂ are shown respectively in FIG. 11 and FIG. 12.

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The particle size and size distribution measured after 6 days of storage for pDNA-loaded LNPs made respectively from ALC-0315 and DTD-NH₂ are shown in FIG. 13 and FIG. 14.

25 10.2. Encapsulation Efficiency of pDNA LNPs

Table 34 shows the percentage encapsulation efficiency of pDNA LNPs on Day 0, and after 6 days of storage. As shown, DTD-NH₂ LNPs gave a

comparable encapsulation efficiency of pDNA as compared to ALC-0315. The encapsulation efficiency of pDNA in DTD-NH₂ LNPs was not changed after 6 days of storage at 4°C, demonstrating stability of the pDNA-loaded DTD-NH₂ LNPs.

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10.3. Agarose Gel Electrophoresis

FIG. 15 shows the results obtained from agarose gel electrophoresis experiments performed on pDNA LNPs made from DTD-NH₂ and ALC0315. As shown, pDNA was well encapsulated in DTD-NH₂ and ALC-0315 LNPs as evidenced by the absence of free pDNA.

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10.4. Transfection - Cell Viability and Fluorescence Intensity of pDNA LNPs

Table 35 shows cell viability and fluorescence intensity results obtained for pDNA LNPs after 48 hours of incubation in HeLa cells. **Table 36** shows cell viability and fluorescence intensity results obtained for pDNA LNPs after 48 hours of incubation in HEPG2 cells.

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FIG. 16 is a fluorescence intensity (RFU) vs. GFP fluorescence graph showing transfection efficiency of pDNA LNPs made respectively from DTD-NH₂ and ALC0315 after 48 hours of incubation in HeLa cells and in HepG2 cells. As shown in FIG. 16, pDNA transfection efficiency was significantly stronger in both HeLa and HepG2 cell lines when mediated by DTD-NH₂ LNPs as compared to ALC-0315 LNPs.

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FIG. 17, FIG. 18 and FIG. 19 show confocal microscopic images captured after 48 hours of incubation in HeLa cells. As shown, pDNA transfection efficiency was significantly stronger in HeLa cells when mediated by DTD-NH₂ LNPs as compared to ALC-0315 LNPs.

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FIG. 20, FIG. 21 and FIG. 22 show confocal microscopic images captured after 48 hours of incubation in HepG2 cells. As shown, pDNA transfection efficiency was significantly stronger in HepG2 cells when mediated by DTD-NH₂ LNPs as compared to ALC-0315 LNPs.

Table 31. LNPs formulation for pDNA (ALC-0315)

Lipid	Molecular Weight (g/mol)	Stock Concentration (mg/mL)	Amount added (mg)	Volume (mL)	Molar ratio
ALC-0315	766.29	20	1.4060	0.0703	46.3
DSPC	790.15	10	0.2910	0.0291	9.4
Cholesterol	386.65	20	0.6520	0.0326	42.7
ALC-0159	2450.00	3	0.1570	0.0524	1.6
Ethanol	-	-	-	0.3156	-
Total:	-	-	2.5070	0.5000	100.0

- Total volume for aqueous (1.5mL) and organic phase (0.5mL) was 2.0 mL
- N/P ratio = 6
- Amount of GFP pDNA (SinoBio) added = 0.1 mg
- 10mM sodium acetate (pH 4.0) was used to dilute pDNA

Table 32. LNPs formulation for pDNA (DTD-NH₂)

Lipid	Molecular Weight (g/mol)	Stock Concentration (mg/mL)	Amount added (mg)	Volume (mL)	Molar ratio
DTD-NH ₂	551.00	4	1.4060	0.3515	54.6
DSPC	790.15	10	0.2910	0.0291	7.9
Cholesterol	386.65	20	0.6520	0.0326	36.1
ALC-0159	2450.00	3	0.1570	0.0524	1.4
Ethanol	-	-	-	0.0344	-
Total:	-	-	2.5070	0.5000	100.0

- Total volume for aqueous (1.5mL) and organic phase (0.5mL) was 2.0 mL
- N/P ratio = 6
- Amount of GFP pDNA (SinoBio) added = 0.139 mg
- 10mM sodium acetate (pH 4.0) was used to dilute pDNA

Table 33. LNPs characterization (pDNA)

Day	Sample	Size (nm)	PDI	Zeta potential	Date measured
0	ALC-0315	103.0 ± 0.77	0.08 ± 0.02	0.19 ± 3.43	5/10/2022
	ALC-0315	85.6 ± 0.6	0.04 ± 0.09	0.74 ± 0.47	11/10/2022
	DTD-NH ₂	79.6 ± 0.4	0.07 ± 0.02	2.82 ± 0.75	5/10/2022
	DTD-NH ₂	82.6 ± 0.2	0.11 ± 0.01	3.81 ± 1.16	11/10/2022

Table 34. Encapsulation Efficiency (pDNA)

Sample	Date	Original Encapsulation Efficiency (%)	Final Encapsulation Efficiency (%)
ALC-0315	5/10/2022	84.3 ± 6.1	84.1 ± 3.6
ALC-0315	11/10/2022	81.4 ± 1.2	89.1 ± 0.0
DTD-NH ₂	5/10/2022	78.8 ± 3.3	93.1 ± 0.0
DTD-NH ₂	11/10/2022	86.4 ± 1.1	90.3 ± 1.9

Table 35. Transfection in HeLa cells (48 hours)

Sample	Dose (ng/well)	Cell viability (%)	Fluorescence intensity (RFU)	Relative to control
Control	-	100.0 ± 2.0	26866 ± 3076	100.00%
ALC-0315	200	95.7 ± 0.9	23772 ± 874	88.5%
DTD-NH ₂	200	69.9 ± 2.4	31594 ± 1438	117.6%

- Control refers to cells without LNP transfection (i.e. incubation with culture media without LNPs).

Table 36. Transfection in HEPG2 cells (48 hours)

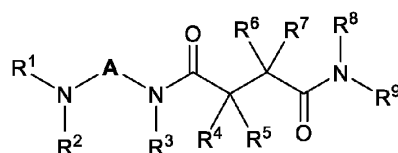
Sample	Dose (ng/well)	Cell viability (%)	Fluorescence intensity (RFU)	Relative to control
Control	-	100.0 ± 2.0	23869 ± 626	100.0%
ALC-0315	200	95.65 ± 0.93	23018 ± 843	96.4%
DTD-NH ₂	200	69.89 ± 2.39	28426 ± 1351	119.1%

- Control refers to cells without LNP transfection (i.e. incubation with culture media without LNPs).

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) or ionized forms thereof
for preparing lipid nanoparticles encapsulating a therapeutic, prophylactic
5 and/or biological agent:



(1)

wherein

- NR¹R² is a group that is ionizable at a pH range of from 3 to physiological
10 pH;

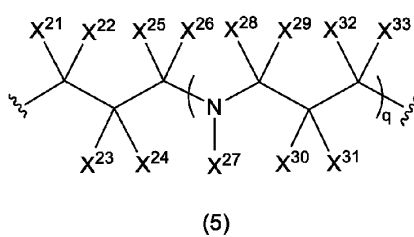
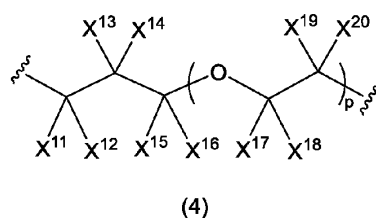
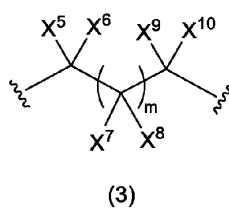
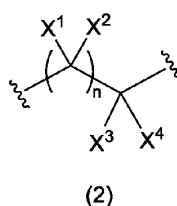
A comprises a linear aliphatic, branched aliphatic and/or cyclic
hydrocarbons optionally comprising one or more groups selected from
–OH, –NR–, –O–, –O–C_xH_{2x}–O–, where x ≥ 1; and where R is H, optionally
substituted alkyl, optionally substituted alkenyl or optionally substituted
15 alkynyl;

R³, R⁴, R⁵, R⁶ and R⁷ are each independently H, optionally substituted
alkyl, optionally substituted alkenyl or optionally substituted alkynyl; and
R⁸ and R⁹ are each independently hydrophobic group.

- 20 2. The compound of claim 1, wherein R¹ and R² are each independently
selected from the group consisting of H, optionally substituted alkyl,
optionally substituted alkenyl, optionally substituted alkynyl, and
combinations thereof.
- 25 3. The compound of any one of the preceding claims, wherein R¹ and R² are
both H and –NR¹R² is a primary amine group.

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4. The compound of any one of the preceding claims, wherein the hydrophobic group at R⁸ and R⁹ each independently comprises optionally substituted alkyl.
5. The compound of any one of the preceding claims, wherein **A** is selected from the following general formula (2), (3), (4) and/or (5):

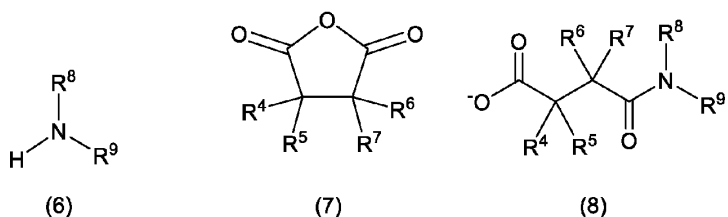


wherein

X^1 to X^{31} are each independently selected from $-H$, $-OH$, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl;

$n \geq 1$; $m \geq 1$; $p \geq 1$; and $q \geq 1$.

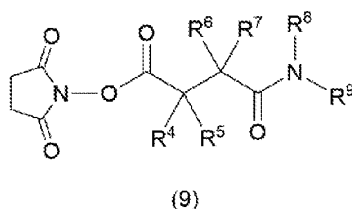
6. The compound of any one of the preceding claims, wherein the compound is selected from the group consisting of DnO-EDEA, DD-EDEA, DTD-EDEA, HO-DnO-NH₂, HO-DD-NH₂, HO-DTD-NH₂, DnO-NH₂, DD-NH₂, DTD-NH₂, DMAPAPA-DTD and combinations thereof.
7. The compound of any one of the preceding claims, wherein the compound is an ionized form of general formula (1), where $-NR^1R^2$ has been ionized to become a positively charged group.
8. A method of preparing a compound as claimed in any one of the preceding claims, the method comprising:
 - (a-i) reacting an amine compound represented by general formula (6) with a cyclic anhydride represented by general formula (7) to obtain a first intermediate compound comprising carboxylate group represented by general formula (8):



- (a-ii) reacting the first intermediate compound represented by general formula (8) with a N-hydroxysuccinimide (NHS) in the presence of

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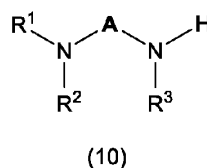
a coupling agent to obtain a second intermediate compound comprising amide group represented by general formula (9):



;

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(a-iii) reacting the second intermediate compound represented by general formula (9) with an amine compound represented by general formula (10) to obtain a compound represented by general formula (1):



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wherein

A comprises a linear aliphatic, branched aliphatic and/or cyclic hydrocarbons optionally comprising one or more groups selected from $-\text{OH}$, $-\text{NR}-$, $-\text{O}-$, $-\text{O}-\text{C}_x\text{H}_{2x}-\text{O}-$, where $x \geq 1$; and where R is H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl;

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R^1 , R^2 , R^3 , R^4 , R^5 , R^6 and R^7 are each independently H, optionally substituted alkyl, optionally substituted alkenyl or optionally substituted alkynyl; and

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R^8 and R^9 are each independently hydrophobic group; and

(a-iv) optionally ionizing $-\text{NR}^1\text{R}^2$ to become a positively charged group.

9. The method of claim 8, wherein the coupling agent comprises carbodiimide selected from the group consisting of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N,N'-dicyclohexylcarbodiimide (DCC), N,N'-Diisopropylcarbodiimide (DIC), and combinations thereof.
10. 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 7; and
a therapeutic, prophylactic and/or biological agent that is encapsulated in said compound as claimed in any one of the preceding claims 1 to 7.
11. The nanoparticle composition of claim 10, wherein the composition further comprises:
(a) helper lipid;
(b) sterol; and
(c) polyethylene glycol (PEG)-modified lipid.
12. The nanoparticle composition of claim 11, wherein the compound represented by general formula (1), helper lipid, sterol, and PEG-modified lipid are mixed at a weight ratio of 10 – 50 : 2 – 20 : 4 – 30 : 1 – 15.
13. The nanoparticle composition of any one of claims 11 to 12, wherein the helper lipid is present in an amount of from 1 mol% to 20 mol%, the sterol is present in an amount of from 10 mol% to 50 mol%, and PEG-modified lipid is present in an amount of from 0.5 mol% to 10 mol%.
14. The nanoparticle composition of any one of claims 11 to 13, 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-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-dilinoeoyl-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.

15. The nanoparticle composition of any one of claims 11 to 14, wherein the sterol is selected from cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, avenasterol and combinations thereof.

16. The nanoparticle composition of any one of claims 11 to 15, wherein the PEG-modified lipid is selected from PEG-modified phosphatidylethanolamines, PEG-modified phosphatidic acids, PEG-modified ceramides, PEG-modified dialkylamines, PEG-modified diacylglycerols, PEG-modified dialkylglycerols, or the like or combinations thereof. Examples of PEG-modified/PEGylated lipid include, but is not limited to, 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159), R-3-[(ω-methoxy-poly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxypropyl-3-amine (PEG-c-DOMG), 3-N-[(ω-methoxypoly

(ethyleneglycol)2000)carbamoyl]-1,2-dimyristyloxy-propylamine (PEG-S-DMG), PEG-DMPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[(polyethylene glycol)-methoxy] (sodium salt)), PEG-DPPC, PEG-DSPE lipid and combinations thereof.

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17. The nanoparticle composition of any one of claims 10 to 16, wherein the nanoparticle composition comprises nanoparticles having a N/P ratio from 2:1 to 40:1.
- 10 18. The nanoparticle composition of any one of claims 10 to 17, wherein the nanoparticle composition comprises nanoparticles having an average particle size of from 20 nm to 200 nm.
- 15 19. The nanoparticle composition of any one of claims 10 to 18, wherein the nanoparticle composition comprises nanoparticles having a zeta potential of from -15 mV to +20 mV in phosphate-buffered saline (PBS).
- 20 20. The nanoparticle composition as claimed in any one of claims 10 to 19 for use in medicine.
- 20 21. The nanoparticle composition as claimed in any one of claims 10 to 19 for use in the treatment or prophylaxis of a disease, disorder or condition in a subject in need thereof.
- 25 22. Use of a nanoparticle composition as claimed in any one of claims 10 to 19 in the manufacture of a medicament for treatment or prophylaxis of a disease, disorder or condition in a subject in need thereof.
- 30 23. 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 10 to 19 to the subject.

24. The nanoparticle composition of claim 21, the use of claim 22 or the method of claim 23, wherein an immune response in the subject is to be induced through the administration of the nanoparticle composition thereto.
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25. The nanoparticle composition of claim 21, the use of claim 22 or the method of claim 23, wherein the disease, disorder or condition is mediated by a coronavirus.
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26. The nanoparticle composition, the use or the method of claim 25, wherein the coronavirus is a SARS-CoV-2 coronavirus.