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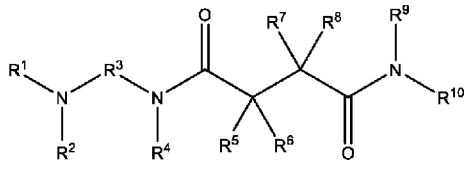
(54) Title: A NANOPARTICLE COMPOSITION AND RELATED METHODS THEREOF



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(57) Abstract: There is provided a nanoparticle composition comprising a compound represented by general formula (1) or ionized form thereof, wherein $R^1$, $R^2$, and $R^4$ to $R^8$ are each independently H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl, $R^3$ is optionally substituted alkylene, optionally substituted alkenylene, or optionally substituted alkynylene, and $R^9$ and $R^{10}$ are each independently a hydrophobic tail or contains at least one of the groups defined above for $R^4$ to $R^8$; a therapeutic, prophylactic, and/or biological agent that is encapsulated by the compound of general formula (1) to form nanoparticles; and a cryoprotectant. There is also provided a method of preparing said nanoparticle composition.

# A NANOPARTICLE COMPOSITION AND RELATED METHODS THEREOF

## TECHNICAL FIELD

The present disclosure relates broadly to a nanoparticle composition, a method of preparing said composition and related uses.

## BACKGROUND

Nanoparticles (NPs) have been used as carriers to deliver therapeutic, prophylactic and/or biological agents. In some applications, these NPs need to be further processed through processes such freeze-drying or lyophilization to extend its shelf life for transport or long-term storage.

One such example is the use of nanoparticles such as lipid nanoparticles as non-viral nanocarriers to deliver messenger ribonucleic acid (mRNA). mRNA is a large molecule with many negative charges, which is unable to enter cells to exert its biological functions. Therefore, in Pfizer/BioNTech and Moderna SARS-CoV-2 mRNA vaccines, lipids are used as the carrier. The lipids in Pfizer/BioNTech SARS-CoV-2 mRNA vaccine assemble with mRNA to form lipid nanoparticles (LNPs), which are taken up by immune cells via endocytosis to stimulate the immune cells.

However, thermal stability of these mRNA LNPs is challenging, which results in expensive cold-chain logistics. For example, current COVID-19 mRNA vaccines require storage at ultra cold temperatures and cold chain transport (-80°C and -20°C are required to store and transport Pfizer/BioNTech and Moderna SARS-CoV-2 mRNA vaccines, respectively). Therefore, cold-chain logistics may be mitigated by lyophilization of mRNA LNPs.

The principles underlying freeze-drying, or lyophilization, involve removing water from a frozen sample through sublimation and desorption processes. This

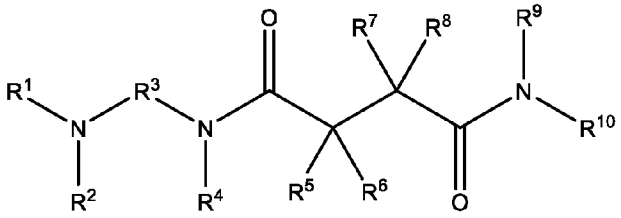
entails freezing the sample, followed by primary drying via sublimation and secondary drying via desorption. However, the freeze-drying process may generate various stresses that could destabilise the NPs. Examples of destabilization include NP aggregation (resulting in increased size), reorganization of lipid components within LNPs, and a decrease in the transfection efficiency of the LNPs.

In view of the above, there is a need to provide a nanoparticle composition and related methods that address or at least ameliorate the above-mentioned problems. In particular, there is a need to provide a nanoparticle composition and related methods that maintain the desired stability and efficacy in delivering the therapeutic, prophylactic and/or biological agents contained in the nanoparticle composition even after freeze-drying or lyophilization.

## SUMMARY

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

a compound represented by general formula (1) or ionized form thereof




(1)

wherein

$R^1$, $R^2$, and $R^4$ to $R^8$ are each independently H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl,

$R^3$ is optionally substituted alkylene, optionally substituted alkenylene, or optionally substituted alkynylene, and

$R^9$ and $R^{10}$ are each independently a hydrophobic tail or contains at least one of the groups defined above for $R^4$ to $R^8$;

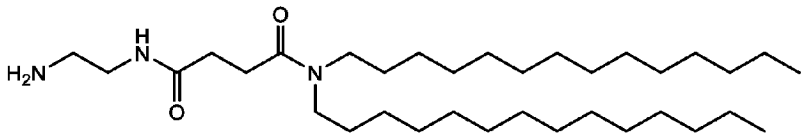
a therapeutic, prophylactic and/or biological agent that is encapsulated by the compound of general formula (1) to form nanoparticles; and

a cryoprotectant.

In one embodiment, $R^1$ and $R^2$ are each H; $R^3$ comprises alkylene; and $R^9$ and $R^{10}$ each independently comprises alkyl, where the alkyl contains at least 5 carbon atoms.

In one embodiment, $R^4$ to $R^8$ are each H.

In one embodiment, the compound is represented by formula (1A):



(1A)

In one embodiment, the therapeutic, prophylactic and/or biological agent comprises a nucleic acid.

In one embodiment, the nucleic acid comprises messenger ribonucleic acid (mRNA).

In one embodiment, the nanoparticle composition further comprises an ionizable lipid that is different from the compound of general formula (1).

In one embodiment, the ionizable lipid that is different from the compound of general formula (1) comprises ALC-0315.

In one embodiment, the ratio of the compound of general formula (1) to the ionizable lipid that is different from said compound is 1: 1 – 20.

In one embodiment, the composition further comprises neutral/helper lipid, sterol, and polyethylene glycol (PEG)-modified lipid.

In one embodiment, the compound of general formula (1), the neutral/helper lipid, the sterol, and the PEG-modified lipid are mixed at a molar ratio of 1 – 70 : 1 – 20 : 10 – 60 : 1 – 20.

In one embodiment, the neutral/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-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 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159), R-3-[(ω-methoxy-poly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxlpropyl-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.

In one embodiment, the cryoprotectant comprises a carbohydrate source selected from at least one of maltose, sucrose, trehalose, mannitol, glucose, fructose, lactose, galactose, ribose, xylose, mannose, arabinose, and combinations thereof.

In one embodiment, the cryoprotectant is present in an amount falling in the range of from 1 w/v% to 30 w/v% with respect to the total volume of the composition.

In one embodiment, the nanoparticle composition is in a lyophilized form or a freeze-thawed form.

In one embodiment, the therapeutic and/or prophylactic agent and/or biological agent is encapsulated at an encapsulation efficiency of at least 60%.

In one embodiment, the nanoparticles have a zeta potential in the range of from -10 mV to +10 mV.

In one embodiment, the nanoparticles have a polydispersity index (PDI) in the range of from 0.01 to 0.5.

In one embodiment, the nanoparticles have a particle size in the range of from 50 nm to 1200 nm.

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

In one aspect, there is provided use of a nanoparticle composition as disclosed herein in the manufacture of a medicament for inducing an immune response in a subject in need thereof.

In one aspect, there is provided the nanoparticle composition as disclosed herein for use in inducing an immune response in a subject in need thereof, wherein said nanoparticle composition or lyophilized nanoparticle composition is to be administered to the subject.

In one aspect, there is provided a method of inducing an immune response in a subject, the method comprising the step of administering to the subject a therapeutically effective amount of a nanoparticle composition as disclosed herein.

In one embodiment, the immune response is specific to a coronavirus or to cancer or to bacteria.

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

In one aspect, there is provided a method of preparing the nanoparticle composition as disclosed herein, the method comprising:

preparing an aqueous composition comprising a therapeutic and/or prophylactic agent and/or biological agent;

mixing the aqueous composition with the compound general formula (1), a helper lipid, a sterol, and a PEG-modified lipid to obtain

nanoparticles encapsulating the therapeutic and/or prophylactic agent and/or biological agent;

adding a cryoprotectant to the nanoparticles.

In one embodiment, the mixing step is carried out in the presence of a further ionizable lipid that is different from the compound of general formula (1).

In one embodiment, the compound of general formula (1) and the ionizable lipid that is different from said compound are mixed together in a ratio of 1: 1 - 20.

**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 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.

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/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), parasitic infections (i.e. diseases caused by parasite), respiratory diseases, or the like or combinations thereof.

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 suffering or are likely to suffer from a medical condition such as 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), parasitic infections (i.e. diseases caused by parasite), respiratory diseases, or the like or combinations thereof, while “non-patients” refer to individuals not suffering and are likely to not suffer from the medical condition. “Non-patients” include healthy individuals, non-diseased individuals and/or an individual free from the medical condition. As used herein, the term "mammal" includes vertebrate such as a human or a large veterinary mammal (e.g., horses, cattle, deer, sheep, llamas, goats, pigs).

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

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-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.

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

butynyl, 2-butynyl, 3-butynyl, 1-pentynyl, 2-pentynyl, 3-methyl-1-butynyl, 4-pentynyl, 1-hexynyl, 2-hexynyl, 5-hexynyl, 1-heptynyl, 2-heptynyl, 6-heptynyl, 1-octynyl, 2-octynyl, 7-octynyl, 1-nonynyl, 2-nonynyl, 8-nonynyl, 1-decynyl, 2-decynyl, 9-decynyl and the like. The group may be a terminal group or a bridging group.

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

The term "optionally substituted," when used to describe a chemical structure or moiety, refers to the chemical structure or moiety wherein one or more of its hydrogen atoms is optionally substituted with a chemical moiety or functional group such as alcohol, alkoxy, alkanoyloxy, alkoxycarbonyl, alkenyl, alkyl (e.g., methyl, ethyl, propyl, t-butyl), alkynyl, alkylcarbonyloxy (–OC(O)alkyl), amide (–C(O)NH–alkyl– or –alkylNHC(O)alkyl), amine (such as alkylamino, arylamino, arylalkylamino), aryl, aryloxy, azo, carbamoyl (–NHC(O)O–alkyl– or –OC(O)NH-alkyl), carbamyl (e.g., $CONH_2$, as well as CONH–alkyl, CONH–aryl, and CONH-arylalkyl), carboxyl, carboxylic acid, cyano, ester, ether (e.g., methoxy, ethoxy), halo, haloalkyl (e.g., $–CCl_3$, $–CF_3$, $–C(CF_3)_3$), heteroalkyl, isocyanate, isothiocyanate, nitrile, nitro, phosphodiester, sulfide, sulfonamido (e.g., $SO_2NH_2$), sulfone, sulfonyl (including alkylsulfonyl, arylsulfonyl and arylalkylsulfonyl), sulfoxide, thiol (e.g., sulfhydryl, thioether) or urea (–NHCONH-alkyl–).

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

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

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

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

Additionally, when describing some embodiments, the disclosure may have disclosed a method and/or process as a particular sequence of steps. However, unless otherwise required, it will be appreciated that the method or

process should not be limited to the particular sequence of steps disclosed. Other sequences of steps may be possible. The particular order of the steps disclosed herein should not be construed as undue limitations. Unless otherwise required, a method and/or process disclosed herein should not be limited to the steps being carried out in the order written. The sequence of steps may be varied and still remain within the scope of the disclosure.

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

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

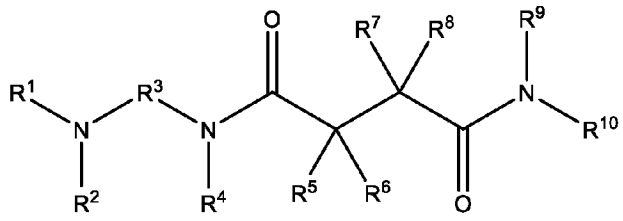
## DESCRIPTION OF EMBODIMENTS

Exemplary, non-limiting embodiments of a nanoparticle composition and related methods/uses thereto are disclosed hereinafter.

### NANOPARTICLE COMPOSITION

There is provided a nanoparticle composition for delivery of a therapeutic, prophylactic and/or biological agent, the nanoparticle composition comprising:

a compound represented by general formula (1) or ionized form thereof,



(1)

wherein

$R^1$, $R^2$, and $R^4$ to $R^8$ are each independently H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl,

$R^3$ is optionally substituted alkylene, optionally substituted alkenylene, or optionally substituted alkynylene, and

$R^9$ and $R^{10}$ are each independently a hydrophobic tail/chain/group or contains at least one of the groups defined above for $R^4$ to $R^8$;

a therapeutic, prophylactic and/or biological agent that is encapsulated by the compound of general formula (1) to form nanoparticles; and

a cryoprotectant.

In various embodiments, the compound of general formula (1) is part of an ionizable lipid component of the composition. In various embodiments, the compound of general formula (1) is part of the total lipid/lipid derivative component/content in the composition.

In various embodiments, $R^1$ and $R^2$ are each independently selected from 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-dimethylbutyl, 1,2-dimethylbutyl, 1,3-dimethylbutyl, 1,2,2-trimethylpropyl, 1,1,2-trimethylpropyl, 2-ethylpentyl, 3-ethylpentyl, heptyl, 1-methylhexyl, 2,2-

dimethylpentyl, 3,3-dimethylpentyl, 4,4-dimethylpentyl, 1,2-dimethylpentyl, 1,3-dimethylpentyl, 1,4-dimethylpentyl, 1,2,3-trimethylbutyl, 1,1,2-trimethylbutyl, 1,1,3-trimethylbutyl, 5-methylheptyl, 1-methylheptyl, octyl, nonyl, decyl, or the like or combinations thereof.

In various embodiments, $R^1$ and $R^2$ are both H. In such embodiments, $–NR^1R^2$ is $–NH_2$ or a primary amine group and the compound comprises a primary (1°) amine group (e.g., an ionizable primary amine group).

In various embodiments, either $R^1$ or $R^2$ is H. In such embodiments, $–NR^1R^2$ is $–NHR^2$ or $–NR^1H$ (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 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 and the compound comprises a tertiary (3°) amine group (e.g., an ionizable tertiary amine group).

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 a primary (1°) amine, i.e., when $R^1$ and $R^2$ are each H. 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 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., Severe Acute Respiratory Syndrome Coronavirus 2" (SARS-CoV-2) 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).

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 nanoparticle composition encapsulates a therapeutic and/or prophylactic agent and/or biological agent that is coupled/bonded/linked/bound to the nanoparticle composition. In various embodiments, $NR^1R^2$ is a group that is ionizable or capable of being ionized at a pH range of from 3 to physiological pH. 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 nanoparticle composition. The therapeutic and/or prophylactic agent and/or biological agent may be

coupled/bonded/linked/bound to the nanoparticle composition *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 nanoparticle composition.

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 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, 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 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 amine), $-NR^1R^2$ 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 $-NH_3^+$.

In various embodiments, for compounds (e.g., lipid compounds) that contain a primary amine group (e.g., when $R^1$ and $R^2$ are both H), 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 other physical interactions, 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).

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

In various embodiments, the 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 $R^9$ and $R^{10}$. In various embodiments, the hydrophobic tail/chain/group at $R^9$ and $R^{10}$ 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, $R^9$ and $R^{10}$ 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.

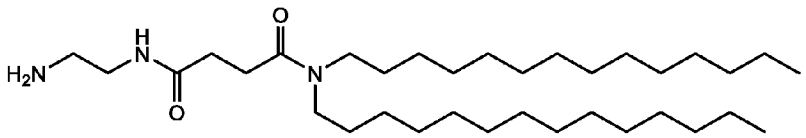
In various embodiments, $R^3$ comprises alkylene. In various embodiments, For example, $R^3$ may be selected from methylene, ethylene, n-propylene, isopropylene, n-butylene, isobutylene, sec-butylene, t-butylene, hexylene, amylene,1,2-dimethylpropylene,1,1-dimethylpropylene, pentylene, isopentylene, hexylene, 4-methylpentylene, 1-methylpentylene, 2-methylpentylene, 3-methylpentylene,2,2-dimethylbutylene,3,3-dimethylbutylene,1,2-dimethylbutylene,1,3-dimethylbutylene,1,2,2-trimethylpropylene,1,1,2-trimethylpropylene, 2-ethylpentylene, 3-ethylpentylene, heptylene, 1-methylhexylene,2,2-dimethylpentylene,3,3-dimethylpentylene,4,4-dimethylpentylene,1,2-dimethylpentylene,1,3-dimethylpentylene,1,4-dimethylpentylene,1,2,3-trimethylbutylene,1,1,2-trimethylbutylene,1,1,3-trimethylbutylene, 5-methylheptylene, 1-methylheptylene, octylene, nonylene, decylene, or the like or combinations thereof.

In various embodiments, $R^4$ to $R^8$ are each H.

In various embodiments, $R^9$ and $R^{10}$ may be identical or different. In various embodiments, $R^9$ and $R^{10}$ are not H at the same time or are both not H. For example, only one of $R^9$ and $R^{10}$ may be H (i.e., when $R^9$ is H, $R^{10}$ is not H or *vice versa*). In various embodiments, only one of $R^9$ and $R^{10}$ is a hydrophobic

tail/chain/group. For example, when $R^9$ is H, $R^{10}$ is a hydrophobic tail/chain/group, and vice versa. In various embodiments, both $R^9$ and $R^{10}$ are hydrophobic tails/chains/groups. For example, $R^9$ and $R^{10}$ each independently comprises alkyl, where the alkyl contains at least 5 carbon atoms. For example, $R^9$ and $R^{10}$ may each be independently selected from hexyl, amyl, 1,2-dimethylpropyl, 1,1-dimethylpropyl, pentyl, isopentyl, hexyl, 4-methylpentyl, 1-methylpentyl, 2-methylpentyl, 3-methylpentyl, 2,2-dimethylbutyl, 3,3-dimethylbutyl, 1,2-dimethylbutyl, 1,3-dimethylbutyl, 1,2,2-trimethylpropyl, 1,1,2-trimethylpropyl, 2-ethylpentyl, 3-ethylpentyl, heptyl, 1-methylhexyl, 2,2-dimethylpentyl, 3,3-dimethylpentyl, 4,4-dimethylpentyl, 1,2-dimethylpentyl, 1,3-dimethylpentyl, 1,4-dimethylpentyl, 1,2,3-trimethylbutyl, 1,1,2-trimethylbutyl, 1,1,3-trimethylbutyl, 5-methylheptyl, 1-methylheptyl, octyl, nonyl, decyl, or the like or combinations thereof.

In various embodiments, the compound is represented by formula (1A):

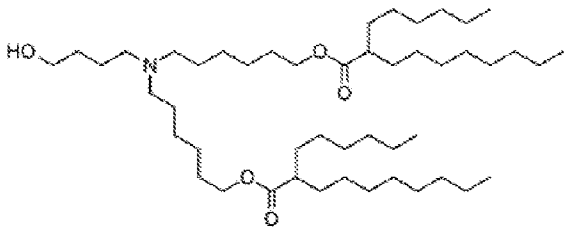


(1A)

In various embodiments, the nanoparticle composition further comprises an ionizable lipid that is different from the compound of general formula (1). In various embodiments, the ionizable lipid that is different from the compound of general formula (1) is part of an ionizable lipid component of the composition. In various embodiments, the ionizable lipid that is different from the compound of general formula (1) is part of the total lipid/lipid derivative content/component in the composition.

In various embodiments, the ionizable lipid comprises (4-hydroxybutyl)azanediyl]di(hexane-6,1-diyl) bis(2-hexyldecanoate) (ALC-0315), 8-[(2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino]-octanoic acid, 1-

octylnonyl ester (SM-102), (6Z,9Z,28Z,31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (D-Lin-MC3-DMA), heptadecan-9-yl (z)-n-(((4-(dimethylamino)butyl)thio)carbonyl)-n-(2-(non-2-en-1-yloxy)-2-oxoethyl)glycinate (Lipid 2,2 (8,8) 4C CH3), bis(2-butyloctyl) 10-(N-(3-(dimethylamino)propyl)nonanamido)nonadecanedioate (Lipid A9), 5-(dimethylamino)-pentanoic acid, (6Z)-1,2-di-(4Z)-4-decen-1-yl-6-dodecen-1-yl ester (CL1), 9Z,12Z-octadecadienoic acid, 3-[4,4-bis(octyloxy)-1-oxobutoxy]-2-[[[[3-(diethylamino)propoxy]carbonyl]oxy]methyl]propyl ester (LP01), ethyl 5,5-bis[(Z)-heptadec-8-enyl]-1-(3-pyrrolidin-1-ylpropyl)-2H-imidazole-2-carboxylate (A2-Iso5-2DC18), bis(2-(dodecyldisulfaneyl)ethyl) 3,3'-((3-methyl-9-oxo-10-oxa-13,14-dithia-3,6-diazahexacosyl)azanediyl)dipropionate (BAME-O16B), 2-(dioctylamino)ethyl nonyl hydrogen phosphate (9A1P9), 1,1′-[[2-[4-[2-[[2-[bis(2-hydroxydodecyl)amino]ethyl](2-hydroxydodecyl)amino]ethyl]-1-piperazinyl]ethyl]imino]bis-2-dodecanol (C12-200), 3,6-bis[4-[bis(2-hydroxydodecyl)amino]butyl]-2,5-piperazinedione (cKK-E12), 9Z,12Z-octadecadienoic acid, 1,1′,1″,1‴-[(3,6-dioxo-2,5-piperazinediyl)bis(4,1-butanediylnitrilodi-2,1-ethanediyl)] ester (OF-Deg-Lin), N1,N3,N5-tris[3-(didodecylamino)propyl]-1,3,5-benzenetricarboxamide (TT3), 9,9′,9″,9‴,9⁗,9⁗′-[1,3,5-benzenetriyltris(carbonylimino-3,1-propanediylnitrilo)]hexakis-nonanoic acid, 1,1′,1″,1‴,1⁗,1⁗′-hexakis(1-ethylhexyl) ester (FTT5), tetrakis(8-methylnonyl) 3,3',3",3"'-(((methylazanediyl)*bis*(propane-3,1-diyl))*bis*(azanetriyl))tetrapropionate ($306O_{i10}$).




Chemical structure of ALC-0315.

In various embodiments, the compound of general formula (1) and the ionizable lipid that is different from said compound are present in a molar ratio of 1: about 1 – 20, 1: about 1 – 19, 1: about 1 – 18, 1: about 1 – 17, 1: about 1 –

16, 1: about 1 – 15, 1: about 1 – 14, 1: about 1 – 13, 1: about 1 – 12, 1: about 1 – 11, 1: about 1 – 10, 1: about 1 – 9, 1: about 1 – 8, 1: about 1 – 7, 1: about 1 – 6, 1: about 1 – 5, 1: about 1 – 4, 1: about 1 – 3, 1: about 1 – 2, 1: 1, 1: 9, or 1: 4.

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

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.

In various embodiments, the cryoprotectant comprises a carbohydrate source. The carbohydrate source may be selected from at least one of maltose, sucrose, trehalose, mannitol, glucose, fructose, lactose, galactose, ribose, xylose, mannose, arabinose, or the like and combinations thereof.

In various embodiments, the cryoprotectant is present in an amount falling in the range of from about from about 1 w/v% to 30 w/v%, from about 2 w/v% to 29 w/v%, from about 3 w/v% to 28 w/v%, from about 4 w/v% to 27 w/v%, from about 5 w/v% to 26 w/v%, from about 6 w/v% to 25 w/v%, from about 7 w/v% to 24 w/v%, from about 8 w/v% to 23 w/v%, from about 9 w/v% to 22 w/v%, from about 10 w/v% to 21 w/v%, from about 11 w/v% to 20 w/v%, from about 12 w/v% to 19 w/v%, from about 13 w/v% to 18 w/v%, from about 14 w/v% to 17 w/v%, from about 15 w/v% to 16 w/v%, or about 15.5 w/v% with respect to the total volume of the composition. Advantageously, in various embodiments, the concentration of cryoprotectants used enables the composition to be suitable for *in vivo* applications. It will be appreciated that when the concentration of cryoprotectant with respect to the total volume of the composition is too high, the nanoparticle solution may be hypertonic and therefore unsuitable for *in vivo* applications.

In various embodiments, the nanoparticle composition is in a lyophilized/freeze-dried form or a freeze-thawed form. In various embodiments, the compound represented by general formula (1) may strengthen the binding of the therapeutic and/or prophylactic agent and/or biological agent to the nanoparticle composition, stabilizing the therapeutic and/or prophylactic agent and/or biological agent during the lyophilization process.

In various embodiments, the nanoparticle composition further comprises:

neutral/helper lipid;

sterol; and

polyethylene glycol (PEG)-modified lipid.

In various embodiments, the neutral/helper lipid, sterol, and PEG-modified lipid are part of the total lipid/lipid derivative content/component in the composition.

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

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 in 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 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, the neutral/helper lipid, the sterol, and the PEG-modified lipid are mixed/dissolved at a molar ratio of about 1 – 70 : 1 – 20 : 10 – 60 : 1 – 20. In various embodiments, when the ionizable lipid that is different from the compound represented by general formula (1) is absent, the compound or its ionized form thereof, neutral/helper lipid, sterol, and PEG-modified lipid may be mixed/dissolved at a molar ratio of about 40 – 60 : about 2 – 20 : about 30 – 50 : about 1 - 5. In various embodiments, when the ionizable lipid that is different from the compound represented by general formula (1) is present, said ionizable lipid, the compound represented by general formula (1) or its ionized form thereof, the neutral/helper lipid, the sterol, and the PEG-modified lipid are mixed/dissolved at a molar ratio of about 30 – 50: about 1 – 10 : about 2 – 20 : about 30 – 50 : about 1 – 5. In various embodiments, the compound represented by general formula (1) or its ionized form thereof, the neutral/helper lipid, the sterol, and the PEG-modified lipid are mixed/dissolved at a molar ratio of about 54.6: 7.9: 36.1: 1.4. In various embodiments, the ionizable lipid that is different from the compound represented by general formula (1), the compound represented by general formula (1) or its ionized form thereof, the neutral/helper lipid, the sterol, and the PEG-modified lipid are mixed/dissolved at a molar ratio of about 41.7: 4.6: 9.4: 42.7: 1.6. In various embodiments, the ionizable lipid that is different from the compound represented by general formula (1), the compound represented by general formula (1) or its ionized form thereof, the neutral/helper lipid, the sterol, and the PEG-modified lipid are mixed/dissolved at a molar ratio of about 37.0: 9.3: 9.4: 42.7: 1.6.

In various embodiments, when the nanoparticle composition comprises from about 1 mol% to about 60 mol%, from about 2 mol% to about 59 mol%, from about 3 mol% to about 58 mol%, or from about 4 mol% to about 57 mol%, from about 5 mol% to about 56 mol%, from about 6 mol% to about 55 mol%, from about 7 mol% to about 54 mol%, from about 8 mol% to about 53 mol%, from about 9 mol% to about 52 mol%, from about 10 mol% to about 51 mol%, from

about 11 mol% to about 50 mol%, from about 12 mol% to about 49 mol%, from about 13 mol% to about 48 mol%, from about 14 mol% to about 47 mol%, from about 15 mol% to about 46 mol%, from about 16 mol% to about 45 mol%, from about 17 mol% to about 44 mol%, from about 18 mol% to about 43 mol%, from about 19 mol% to about 42 mol%, from about 20 mol% to about 41 mol%, from about 21 mol% to about 40 mol%, from about 22 mol% to about 39 mol%, from about 23 mol% to about 38 mol%, from about 24 mol% to about 37 mol%, from about 25 mol% to about 36 mol%, from about 26 mol% to about 35 mol%, from about 27 mol% to about 34 mol%, from about 28 mol% to about 33 mol%, from about 29 mol% to about 32 mol%, from about 30 mol% to about 31 mol%, or about 30.5 mol% of the compound represented by general formula (1) with respect to the total amount of lipid/lipid derivatives present (e.g., compound of general formula (1), neutral/helper lipid, sterol, (PEG)-modified lipid and optionally the ionizable lipid that is different from formula (1)).

In various embodiments, when the ionizable lipid that is different from the compound represented by general formula (1) is absent, the nanoparticle composition comprises from about 40 mol% to about 60 mol%, from about 41 mol% to about 59 mol%, from about 42 mol% to about 58 mol%, from about 43 mol% to about 57 mol%, from about 44 mol% to about 56 mol%, from about 45 mol% to about 55 mol%, from about 46 mol% to about 54 mol%, from about 47 mol% to about 53 mol%, from about 48 mol% to about 52 mol%, from about 49 mol% to about 51 mol%, about 50 mol%, or about 54.6 mol% of the compound represented by general formula (1) with respect to the total amount of lipid/lipid derivatives present (e.g., compound of general formula (1), neutral/helper lipid, sterol, (PEG)-modified lipid).

In various embodiments, when the ionizable lipid that is different from the compound represented by general formula (1) is present, the nanoparticle composition comprises from about 1 mol% to about 10 mol%, from about 2 mol% to about 9 mol%, from about 3 mol% to about 8 mol%, from about 4 mol% to about 7 mol%, from about 5 mol% to about 6 mol%, about 5.5 mol%, about 4.6

mol%, or about 9.3 mol% of compound represented by general formula (1) and from about 30 mol% to about 50 mol%, from about 31 mol% to about 49 mol%, from about 32 mol% to about 48 mol%, from about 33 mol% to about 47 mol%, from about 34 mol% to about 46 mol%, from about 35 mol% to about 45 mol%, from about 36 mol% to about 44 mol%, from about 37 mol% to about 43 mol%, from about 38 mol% to about 42 mol%, from about 39 mol% to about 41 mol%, about 40 mol%, about 41.7 mol%, or about 37 mol% of the ionizable lipid that is different from the compound represented by general formula (1) with respect to the total amount of lipid/lipid derivatives present (e.g., compound of general formula (1), ionizable lipid that is different from formula (1), neutral/helper lipid, sterol, (PEG)-modified lipid).

In various embodiments, the compound represented by general formula (1) the ionizable lipid that is different from the compound represented by general formula (1) are the major components of the nanoparticle composition.

In various embodiments, the neutral/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-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 nanoparticle composition comprises from about 1 mol% to about 20 mol%, from about 2 mol% to about 19 mol%, from about 3 mol% to about 18 mol%, from about 4 mol% to about 17 mol%, from about 5 mol% to about 16 mol%, from about 6 mol% to about 15 mol%, from about 7 mol% to about 14 mol%, from about 8 mol% to about 13 mol%, from about 9 mol% to about 12 mol%, from about 10 mol% to about 11 mol%, about 10.5 mol%, about 7.9 mol%, or about 9.4 mol% of the neutral/helper lipid with respect to the total amount of lipid/lipid derivatives present (e.g., compound of general formula (1), neutral/helper lipid, sterol, (PEG)-modified lipid and optionally the ionizable lipid that is different from formula (1)).

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

In various embodiments, the nanoparticle composition comprises from about 20 mol% to about 60 mol%, from about 21 mol% to about 59 mol%, from about 22 mol% to about 58 mol%, from about 23 mol% to about 57 mol%, from about 24 mol% to about 56 mol%, from about 25 mol% to about 55 mol%, from about 26 mol% to about 54 mol%, from about 27 mol% to about 53 mol%, from about 28 mol% to about 52 mol%, from about 29 mol% to about 51 mol%, from about 30 mol% to about 50 mol%, from about 31 mol% to about 49 mol%, from about 32 mol% to about 48 mol%, from about 33 mol% to about 47 mol%, from about 34 mol% to about 46 mol%, from about 35 mol% to about 45 mol%, from about 36 mol% to about 44 mol%, from about 37 mol% to about 43 mol%, from about 38 mol% to about 42 mol%, from about 39 mol% to about 41 mol%, about 39.5 mol%, about 36.7 mol%, or about 36.1 mol% of the sterol with respect to the total amount of lipid/lipid derivatives present (e.g., compound of general formula

(1), neutral/helper lipid, sterol, (PEG)-modified lipid and optionally the ionizable lipid that is different from formula (1)).

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-methoxy-poly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxlpropyl-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 the like and combinations thereof.

In various embodiments, the nanoparticle composition comprises from about 1 mol% to about 5 mol%, from about 1 mol% to about 4 mol%, from about 1 mol% to about 3 mol%, from about 1 mol% to about 2 mol%, about 1.6 mol%, or about 1.4 mol% of the PEG-modified lipid with respect to the total amount of lipid/lipid derivatives present (e.g., compound of general formula (1), neutral/helper lipid, sterol, (PEG)-modified lipid and optionally the ionizable lipid that is different from formula (1)).

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 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, the therapeutic and/or prophylactic agent and/or biological agent is encapsulated at an encapsulation efficiency of at least about 20 %, at least about 25 %, at least about 30 %, at least about 35 %, at least about 40 %, at least about 45 %, at least about 50 %, at least about 55 %, at least about 60 %, at least about 65 %, at least about 70 %, at least about 75 %, at least about 80 %, at least about 85 %, at least about 90 %, at least about 95 %, or at least about 100 %.

In various embodiments, the nanoparticles have a zeta potential in the range of from about -10 mV to about +10 mV, from about -10 mV to about 10 mV, from about -9 mV to about 9 mV, from about -8 mV to about 8 mV, from about -7 mV to about 7 mV, from about -6 mV to about 6 mV, from about -5 mV to about 5 mV, from about -4 mV to about 4 mV, from about -3 mV to about 3 mV, from about -2 mV to about 2 mV, from about -1 mV to about 1 mV, or about 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.

In various embodiments, the nanoparticles have a polydispersity index (PDI) in the range of from about 0.01 to about 1.0, from about 0.05 to about 0.95, from about 0.1 to about 0.9, from about 0.15 to about 0.85, from about 0.2 to about 0.8, from about 0.25 to about 0.75, from about 0.3 to about 0.7, from about

0.35 to about 0.65, from about 0.4 to about 0.6, from about 0.45 to about 0.55, or about 0.5.

In various embodiments, the nanoparticles have a particle size (diameter) in the range of from about 50 nm to about 1200 nm, from about 100 nm to about 1150 nm, from about 150 nm to about 1100 nm, from about 200 nm to about 1050 nm, from about 250 nm to about 1000 nm, from about 300 nm to about 950 nm, from about 350 nm to about 900 nm, from about 400 nm to about 850 nm, from about 450 nm to about 800 nm, from about 500 nm to about 750 nm, from about 550 nm to about 700 nm, from about 600 nm to about 650 nm, or about 625 nm. In various embodiments, advantageously, nanoparticles with a larger size may be used as nasal spray formulations.

In various embodiments, the cell translation efficiency using FLuc mRNA is 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% of that mediated by the LNPs without lyophilization.

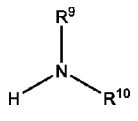
In various embodiments, the nanoparticles have a cell transfection efficiency that is comparable to or no less or higher than that of corresponding nanoparticles using solely ALC-0315 as the ionizable lipid under similar conditions. For example, the cell transfection efficiency may be at least about 40 %, at least about 50 %, at least about 60 %, at least about 70 %, at least about 80 %, at least about 90%, at least about 100% to about 800 %, at least from about 150 % to about 750 %, at least from about 200 % to about 700 %, at least from about 250 % to about 650 %, at least from about 300 % to about 600 %, at least from about 350 % to about 550 %, at least from about 400 % to about 500 %, or at least about 450 % of that of corresponding nanoparticles using ALC-0315 as the ionizable lipid under similar conditions.

In various embodiments, the nanoparticle composition is biocompatible, i.e., the nanoparticle composition 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 undesirable immune reaction/response, an injury or the like when used on the human or animal body. In various embodiments, the nanoparticle composition is substantially devoid of substances that elicit an adverse physiological response. Advantageously, the nanoparticles (e.g., lipid nanoparticles) in the nanoparticle composition 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.

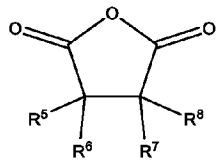
METHOD OF PREPARING COMPOUND AS REPRESENTED BY GENERAL FORMULA (1)

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

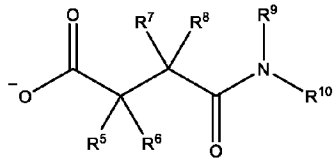
(a-i) reacting an amine compound represented by general formula (2) with a cyclic anhydride represented by general formula (3) to obtain a first intermediate compound comprising carboxylate group represented by general formula (4):



(2)



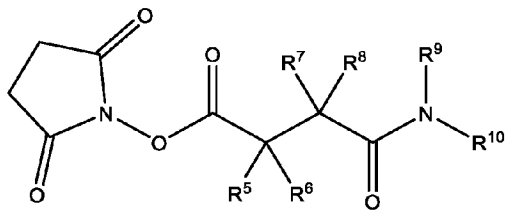
(3)



(4) ;

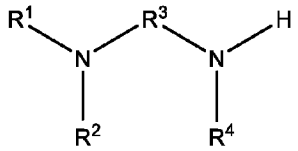
(a-ii) reacting the first intermediate compound represented by general formula (4) with a N-hydroxysuccinimide (NHS) in the presence of a coupling agent

to obtain a second intermediate compound comprising amide group represented by general formula (5):



(5) ;

(a-iii) reacting the second intermediate compound represented by general formula (5) with an amine compound represented by general formula (6) to obtain a compound represented by general formula (1):



(6)

wherein $R^1$ to $R^{10}$ 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

(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.

In various embodiments, the cyclic anhydride represented by general formula (3) 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 (6) 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 (6) with the second intermediate compound represented by general formula (5) 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 (6) with the second intermediate compound represented by general formula (5) 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 (6) 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 (6) and the second intermediate compound represented by general formula (5).

In various embodiments, the reacting step (a-iii) comprises adding the second intermediate compound represented by general formula (5) in a dropwise manner to the amine compound represented by general formula (6).

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 ultrasonicating.

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) 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 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 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 bicarbonate, chloride salts such as sodium chlorine (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 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.

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.

## METHOD OF PREPARING THE NANOPARTICLE COMPOSITION

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

preparing an aqueous composition comprising therapeutic and/or prophylactic agent and/or biological agent;

mixing the aqueous composition with the compound represented by general formula (1), a helper lipid, a sterol, and a PEG-modified lipid to obtain nanoparticles encapsulating the therapeutic and/or prophylactic agent and/or biological agent;

adding a cryoprotectant to the nanoparticles.

In various embodiments, the step of preparing the aqueous composition comprises mixing therapeutic and/or prophylactic agent and/or biological agent in an aqueous buffer. The aqueous buffer may be sodium acetate (10 mM).

In various embodiments, the mixing step is performed at a pH value of from about 2 to about 6, from about 2.5 to about 5.5, from about 3 to about 5, from about 3.5 to about 4.5, or about 4.

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 of mixing the aqueous composition with the composition 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.

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

In various embodiments, the step of mixing the aqueous composition with the composition is carried out in the presence of a further ionizable lipid that is different from the compound of general formula (1). In various embodiments, the compound of general formula (1) and the ionizable lipid that is different from said compound are mixed together in a molar ratio of 1: about 1 – 20, 1: about 1 – 19, 1: about 1 – 18, 1: about 1 – 17, 1: about 1 – 16, 1: about 1 – 15, 1: about 1 – 14, 1: about 1 – 13, 1: about 1 – 12, 1: about 1 – 11, 1: about 1 – 10, 1: about 1 – 9, 1: about 1 – 8, 1: about 1 – 7, 1: about 1 – 6, 1: about 1 – 5, 1: about 1 – 4, 1: about 1 – 3, 1: about 1 – 2, 1: 1, 1: 9, or 1: 4.

In various embodiments, the step 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-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 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).

In various embodiments, the method further comprises removing the organic phase (e.g., ethanol) and the unencapsulated components after the nanoparticles are formed following step (c-ii). For example, removing the organic phase may include diluting the resultant LNPs in 0.9% saline or PBS buffer or Tris buffer and subsequently subjecting the resultant LNPs to centrifugal ultrafiltration to remove residual organic solvents that are present. 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.

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

In various embodiments, there is also provided a vaccine composition comprising the nanoparticle composition as disclosed herein.

In various embodiments, there is also provided a carrier, a nanocarrier, a delivery system/vehicle, a nanoparticle composition 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).

In various embodiments, there is also provided a carrier, a nanocarrier, a delivery system/vehicle, a nanoparticle composition 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 nanoparticle composition in the manufacture of a medicament as therapeutics 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 nanoparticle composition to a subject (e.g., vertebrate such as a human or a large veterinary mammal (e.g., horses, cattle, deer, sheep, llamas, goats, pigs) in need thereof. The disease, disorder, or condition may be selected from the group consisting of infectious/contagious diseases, viral infections (i.e., diseases caused by virus), bacterial infections (i.e., diseases caused by bacteria), fungal infections (i.e., diseases caused by fungi), cancer, respiratory diseases or the like, or combinations thereof. In various embodiments, the types of diseases may vary depending on the therapeutic, prophylactic, and/or biological cargo encapsulated. 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 nanoparticle composition 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 nanoparticle composition 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 nanoparticle composition 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 nanoparticle composition 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 nanoparticle composition 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 nanoparticle composition to a subject in need thereof. In various embodiments, an immune response in the subject is to be induced through the administration of the nanoparticle composition 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), cancer, respiratory diseases or the like, or combinations thereof as mentioned herein. In various embodiments, the types of diseases may vary depending on the therapeutic, prophylactic, and/or biological cargo encapsulated. The carrier, nanocarrier, delivery system/vehicle, nanoparticle composition 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 a coronavirus (e.g., severe acute respiratory syndrome coronavirus such as

SARS-CoV-2 or SARS-CoV-1). For example, the disease, disorder or condition may be SARS-CoV-2 coronavirus disease.

In various embodiments, the carrier, nanocarrier, delivery system/vehicle, compound or ionized form thereof, nanoparticle composition, 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., > 20%), high transfection efficiency, 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.

## BRIEF DESCRIPTION OF FIGURES

FIG. 1 shows a three-phase diagram of water across various temperature and pressure conditions to elucidate the principle of freeze-drying/lyophilization. Water vaporizes from the samples through sublimation and desorption processes during the freeze-drying/lyophilization step, which involves freezing the sample followed by primary and secondary drying, as described in Example 1, part 1.5.

FIG. 2 shows the experimental setup 100 for the freeze-drying/lyophilization process in accordance with various embodiments disclosed herein. A sample 102 is connected to freeze-dryer 106 via vacuum pump tubing 104 and immersed in a bucket of dry ice 108 to maintain its temperature during the procedure.

FIG. 3 shows the freeze-dried/lyophilized mRNA LNP formulations with different formulations in accordance with various embodiments disclosed herein. All samples contain 20% sucrose and exhibit a spongy matrix appearance after lyophilization.

FIG. 4 is a gel electrophoresis graph showing the electrophoretic mobility of mRNA in LNPs made from ALC-0315 without and with sucrose at concentrations of 5 %, 10 %, and 20 % in accordance with various embodiments disclosed herein before freeze-drying. The electrophoresis was conducted at 100 V for 20 min. A DNA ladder, RiboRuler High Range RNA Ladder (Thermo Scientific) and free CleanCap® Firefly Luciferase mRNA (300 ng) were included as controls in the gel.

FIG. 5 is a gel electrophoresis graph showing the electrophoretic mobility of mRNA in LNPs made from ALC-0315 without and with sucrose at concentrations of 5 %, 10 %, and 20 % in accordance with various embodiments disclosed herein after 4 days of freeze-drying process (Groups 2 to 5). The electrophoretic mobility of mRNA in LNPs made from ALC-0315 without freeze-drying was also included in Lane 3 (Group 1). The electrophoresis was conducted at 100 V for 20 min. RiboRuler High Range RNA Ladder (Thermo Scientific) and free CleanCap® Firefly Luciferase mRNA (300 ng) were included as controls in the gel.

FIG. 6 is a gel electrophoresis graph showing the electrophoretic mobility of mRNA in LNPs made from DTD-$NH_2$ with sucrose in accordance with various embodiments disclosed herein before freeze-drying. The electrophoresis was conducted at 100 V for 20 min. A DNA ladder and free CleanCap® Firefly Luciferase mRNA (1000 ng and 500 ng) were included as controls in the gel.

FIG. 7 is a gel electrophoresis graph showing the electrophoretic mobility of mRNA in LNPs made from DTD-$NH_2$ without and with sucrose at concentrations of 5 %, 10 %, and 20 % in accordance with various embodiments disclosed herein after 4 days of freeze-drying process (Groups 2 to 5). The electrophoretic mobility of mRNA in LNPs made from DTD-$NH_2$ without freeze-drying was also included in Lane 3 (Group 1). The electrophoresis was conducted

at 100 V for 20 min. A DNA ladder and free CleanCap® Firefly Luciferase mRNA (300 ng) were included as controls in the gel.

FIG. 8 is a graph showing the encapsulation efficiency of mRNA in LNPs made from only ALC-0315 or only DTD-$NH_2$ without and with sucrose at concentrations of 5 %, 10 %, and 20 % in accordance with various embodiments disclosed herein after 4 days of freeze-drying process. The encapsulation efficiencies of mRNA in LNPs made from only ALC-0315 or only DTD-$NH_2$ without freeze-drying were also shown as controls.

FIG. 9 is a graph showing the cell viability of HeLa cells when incubated with mRNA in LNPs made from only ALC-0315 or only DTD-$NH_2$ without and with sucrose at concentrations of 5 %, 10 %, and 20 % in accordance with various embodiments disclosed herein after 4 days of freeze-drying process. The cell viability of untreated HeLa cells and of HeLa cells when incubated with mRNA in LNPs made from only ALC-0315 or only DTD-$NH_2$ without freeze-drying were also shown as controls.

FIG. 10 is a graph showing the mRNA transfection of HeLa cells treated with LNPs made from only ALC-0315 or only DTD-$NH_2$ without and with sucrose at concentrations of 5 %, 10 %, and 20 % in accordance with various embodiments disclosed herein after 4 days of freeze-drying process. The mRNA transfection of untreated HeLa cells as well as of HeLa cells treated with LNPs made from only ALC-0315 or only DTD-$NH_2$ without freeze-drying were also shown as controls.

FIG. 11 is a graph showing the encapsulation efficiency of mRNA in LNPs made from only ALC-0315 or only DTD-$NH_2$ without and with sucrose at concentrations of 5 %, 10 %, and 20 % in accordance with various embodiments disclosed herein after freeze-thawing or 1 day of freeze-drying process. The encapsulation efficiencies of mRNA in LNPs made from only ALC-0315 or only DTD-$NH_2$ without freeze-drying were also shown as controls.

FIG. 12 is a graph showing the cell viability of HeLa cells when incubated with mRNA in LNPs made from only ALC-0315 or only DTD-$NH_2$ without and with sucrose at concentrations of 5 %, 10 %, and 20 % in accordance with various embodiments disclosed herein after freeze-thawing or 1 day of freeze-drying process. The cell viability of untreated HeLa cells and of HeLa cells when incubated with mRNA in LNPs made from only ALC-0315 or only DTD-$NH_2$ without freeze-drying were also shown as controls.

FIG. 13 is a graph showing the mRNA transfection of HeLa cells treated with LNPs made from only ALC-0315 or only DTD-$NH_2$ without and with sucrose at concentrations of 5 %, 10 %, and 20 % in accordance with various embodiments disclosed herein after 4 days of freeze-drying process. The mRNA transfection of untreated HeLa cells as well as of HeLa cells treated with LNPs made from only ALC-0315 or only DTD-$NH_2$ without freeze-drying were also shown as controls.

FIG. 14 is a graph showing the particle size distribution of mRNA LNPs made from only ALC-0315 before freeze-drying in accordance with various embodiments disclosed herein.

FIG. 15 is a graph showing the particle size distribution of mRNA LNPs made from ALC-0315 and DTD-$NH_2$ at a molar ratio of 9: 1 before freeze-drying in accordance with various embodiments disclosed herein.

FIG. 16 is a graph showing the particle size distribution of mRNA LNPs made from ALC-0315 and DTD-$NH_2$ at a molar ratio of 8: 2 before freeze-drying in accordance with various embodiments disclosed herein.

FIG. 17 is a graph showing the particle size distribution of mRNA LNPs made from only ALC-0315 without sucrose without freeze-drying in accordance with various embodiments disclosed herein.

FIG. 18 is a graph showing the particle size distribution of mRNA LNPs made from only ALC-0315 with 20 % sucrose without freeze-drying in accordance with various embodiments disclosed herein.

FIG. 19 is a graph showing the particle size distribution of mRNA LNPs made from only ALC-0315 with 20 % sucrose after freeze-thawing in accordance with various embodiments disclosed herein.

FIG. 20 is a graph showing the particle size distribution of mRNA LNPs made from ALC-0315 and DTD-$NH_2$ at a molar ratio of 9: 1 after freeze-thawing in accordance with various embodiments disclosed herein.

FIG. 21 is a graph showing the particle size distribution of mRNA LNPs made from ALC-0315 and DTD-$NH_2$ at a molar ratio of 8: 2 after freeze-thawing in accordance with various embodiments disclosed herein.

FIG. 22 is a graph showing the particle size distribution of mRNA LNPs made from only ALC-0315 without sucrose after freeze-drying in accordance with various embodiments disclosed herein.

FIG. 23 is a graph showing the particle size distribution of mRNA LNPs made from ALC-0315 and DTD-$NH_2$ at a molar ratio of 9: 1 without sucrose after freeze-drying in accordance with various embodiments disclosed herein.

FIG. 24 is a graph showing the particle size distribution of mRNA LNPs made from ALC-0315 and DTD-$NH_2$ at a molar ratio of 8: 2 without sucrose after freeze-drying in accordance with various embodiments disclosed herein.

FIG. 25 is a graph showing the particle size distribution of mRNA LNPs made from only ALC-0315 with 20 % sucrose after freeze-drying in accordance with various embodiments disclosed herein.

FIG. 26 is a graph showing the particle size distribution of mRNA LNPs made from ALC-0315 and DTD-$NH_2$ at a molar ratio of 9: 1 with 20 % sucrose after freeze-drying in accordance with various embodiments disclosed herein.

FIG. 27 is a graph showing the particle size distribution of mRNA LNPs made from ALC-0315 and DTD-$NH_2$ at a molar ratio of 8: 2 with 20 % sucrose after freeze-drying in accordance with various embodiments disclosed herein.

FIG. 28 is a graph showing the luciferase expression mediated by different mRNA LNP formulations in Balb/c mice, in accordance with various embodiments disclosed herein. The in vivo study was conducted on Balb/c mice via intravenous injection (tail vein), each mouse received a dose of 1 μg mRNA/100 μL. Imaging was performed at 6 hours and 24 hours post-administration of mRNA LNPs. The ALC-0315 groups consisted of 6 mice before freeze-drying (lyophilization) and 7 mice after lyophilization while the ALC-0315:DTD-NH2 (9: 1) group consisted of 7 mice after lyophilization. A "Naïve" group served as the control group without any treatment.

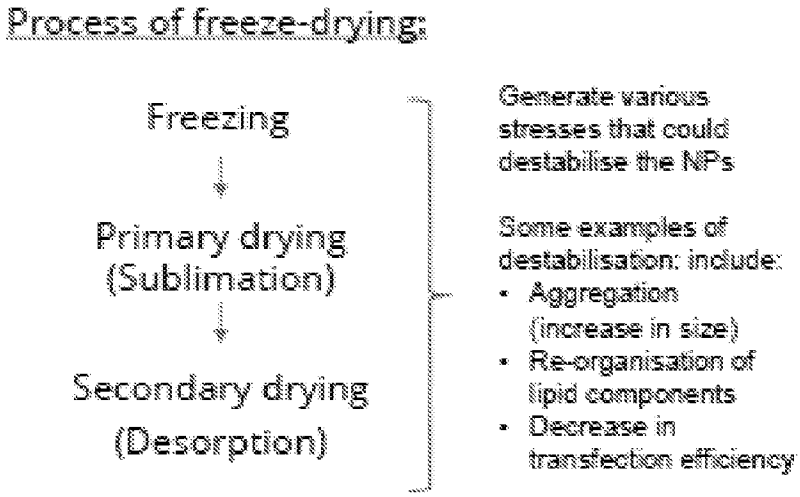
FIG. 29 shows $^1H$ NMR spectrum of DTD-$NH_2$ in $CD_3OD$, prepared in accordance with various embodiments of the method disclosed herein.

**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.

As shown in Scheme 1, freeze-drying/lyophilization procedures involves freezing the sample, followed by primary drying via sublimation and secondary drying via desorption to remove the water. However, the freeze-drying process may generate various stresses that could destabilise the NPs. Examples of

destabilization include NP aggregation (resulting in increased size), reorganization of lipid components within LNPs, and a decrease in the transfection efficiency of the LNPs.




Scheme 1. Fundamentals of freeze-drying, its procedural steps, and potential drawbacks.

The following examples demonstrate that nanoparticle compositions prepared in accordance with various embodiments disclosed herein are able to mitigate the negative effects typically experienced during freeze-drying/lyophilization to provide lyophilized/freeze dried compositions with enhanced stability.

It should be appreciated that other modifications related to structural, biological and/or chemical changes may be made without deviating from the scope of the disclosure. 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.

**Example 1: Materials and Methods**

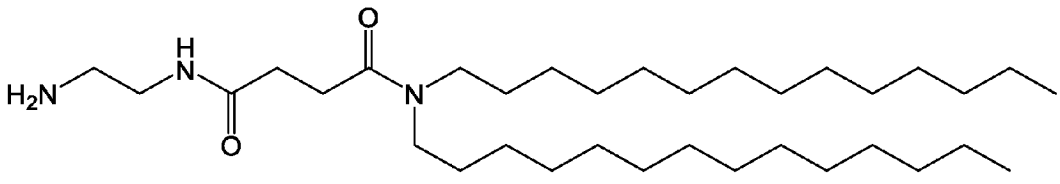
1.1. Materials

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, ALC-0315 and ALC-0159 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). AlamarBlue, gel loading buffer, and Pierce Firefly Luciferase Glow assay kit were purchased from Invitrogen (Waltham, MA, USA). Other reagents used were analytical grade.

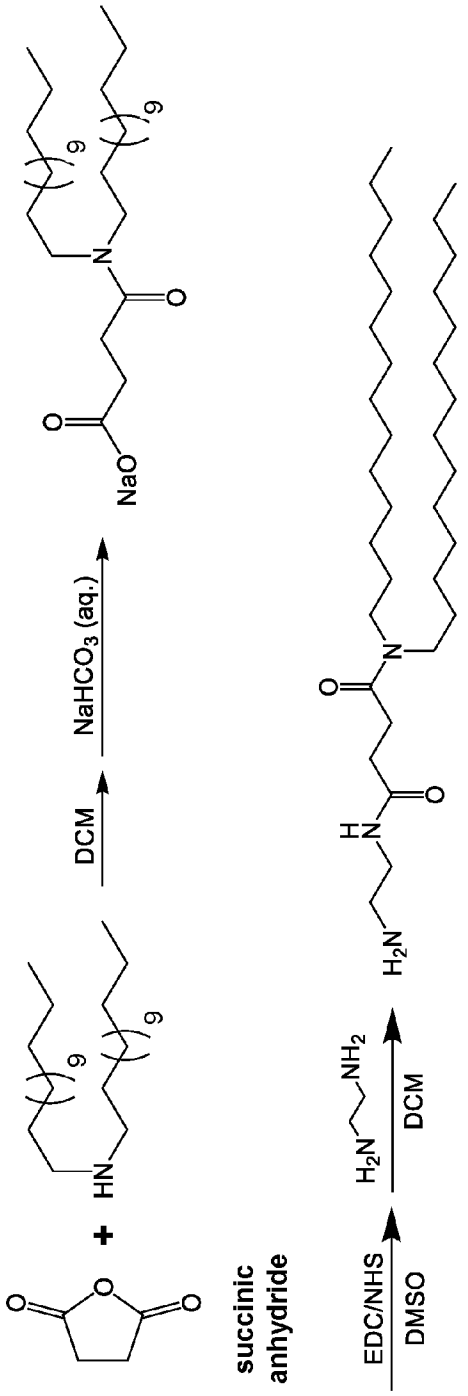
1.2. Synthesis of DTD-$NH_2$ (Scheme 2)

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 2.

The synthesis method of DTD-$NH_2$ is given below as a typical example.




Chemical structure of DTD-$NH_2$.




**Scheme 1.** Chemical structure and synthetic procedures of DTD-$NH_2$.

**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 $N_2$ 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). $^1$H NMR (400 MHz, $CDCl_3$, 22 °C): $\delta$ 3.31 (dt, 4H, -CON(C*H*$_2$-)$_2$), 2.67 (s, 4H, -C*H*$_2$C*H*$_2$COONa), 1.53 (m, br, 4H, -CON(CH$_2$C*H*$_2$-)$_2$), 1.26 (s, 44H, -(C*H*$_2$)$_{11}$CH$_3$), 0.88 (t, 6H, -C*H*$_3$).

**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). $^1$H NMR (400 MHz, $CDCl_3$, 22 °C): $\delta$ 3.30 (dt, 4H, -CON(C*H*$_2$-)$_2$), 3.01 (t, 2H, -C*H*$_2$- of NHS), 2.83 (s, 4H, -COC*H*$_2$C*H*$_2$CON-), 2.72 (t, 2H, -C*H*$_2$- of NHS), 1.52 (m, br, 4H, -CON(CH$_2$C*H*$_2$-)$_2$), 1.26 (s, 44H, -(C*H*$_2$)$_{11}$CH$_3$), 0.88 (t, 6H, -C*H*$_3$).

**Synthesis of DTD-NH$_2$:** 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-$NH_2$ as off-white sticky solid (63% yield). $^1H$ NMR (400 MHz, $CD_3OD$, 22 °C): $\delta$ 2.80 (t, 2H, -$CH_2NH_2$), 2.69 (m, 2H, -NHCO$CH_2CH_2$CON-), 2.48 (t, 2H, -NHCO$CH_2CH_2$CON-), 1.62 (m, br, 2H, -CON($CH_2CH_2$-)$_2$), 1.52 (m, br, 2H, -CON($CH_2CH_2$-)$_2$), 1.29 (s, 44H, -$(CH_2)_{11}CH_3$), 0.90 (t, 6H, -$CH_3$).

1.3. Nuclear Magnetic Resonance Spectroscopy (NMR)

DTD-$NH_2$ was characterized by nuclear magnetic resonance spectroscopy (NMR). $^1H$-NMR spectra of the DTD-$NH_2$ and its precursors 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 ($\delta$ = 3.31 ppm for $CD_3OD$).

1.4. Fabrication of mRNA-loaded Lipid Nanoparticles (mRNA LNPs)

CleanCap® Firefly Luciferase mRNA was purchased from Trilink Biotechnologies and was encapsulated in all LNP formulations. LNPs were prepared in a controlled mixing process between the mRNA-containing aqueous phase and ethanolic lipid mixture using the NanoAssemblr Ignite microfluidic system (Precision NanoSystems Inc.), at a volume ratio of 3:1, 12 mL/min flow rate. In the aqueous phase, mRNA was diluted in 10 mM sodium acetate buffer, pH 4. The lipid mixture consists of the ionizable lipid ALC-0315 (MedChem Express) or DTD-$NH_2$ (produced in-house; MW = 551; structure illustrated in Figure 1), cholesterol (MedChem Express), helper lipid 1,2-distearoyl-sn-glycero-3-phosphorylcholine (DSPC; MedChem Express), and PEG-lipid ALC-0159 (MedChem Express) in a molar ratio of 46.4 : 9.3 : 42.7 : 1.6 for ALC-0315 LNPs,

and a molar ratio of 54.6 : 7.9 : 36.1 : 1.4 for DTD-$NH_2$ LNPs respectively. N/P ratio of 6 between the ionizable lipid and mRNA was used throughout the study. Removal of ethanol and unencapsulated components were achieved by diluting the resultant LNPs in 0.9% saline and subsequent centrifugal ultrafiltration using Vivaspin Ultrafiltration Units (MWCO: 30,000 Da, PES; Satorius).

1.5. Preparation of Lyophilized mRNA-Loaded Lipid Nanoparticles (mRNA LNPs) with Cryoprotectants and Reconstitution of Lyophilized mRNA LNPs

LNPs were mixed with sucrose and/or maltose to achieve various %w/v (0%, 5%, 10% and 20%), and were subsequently frozen in glass vials at -80°C overnight. Frozen LNP samples were subjected to lyophilization for 24 h or 96 h at -48°C, 0.021 mBar using the Labconco benchtop freeze-dryer. Lyophilized LNP samples were reconstituted for 1 h with nuclease-free water, using the same volume prior to lyophilization. LNP samples subjected to freeze-thaw were thawed at 4°C on the same day when the lyophilized LNP samples were reconstituted. All LNP samples were stored at 4°C before usage.

1.6. mRNA Encapsulation Efficiency (EE) and Concentration

mRNA encapsulation efficiency and concentration in each LNP sample were determined using the Quant-iT RiboGreen RNA assay (Invitrogen). Briefly, LNPs were diluted with 1x TE buffer in the presence and absence of 5% Triton X-100, and incubated at 37°C for 20 min. Fluorescence intensity was measured using a microplate reader (Tecan Spark Multimode Microplate Reader) at 485 nm excitation and 528nm emission wavelengths. mRNA concentrations were obtained by means of a standard curve plotted using linear regression of fluorescence intensity against the concentration of mRNA standards. mRNA encapsulation efficiency was determined using the following equation:

$$Encapsulation\ Efficiency\ (\%) = \frac{([Concentration]total - [Concentration]unencapsulated)}{(Concentration)total} \times 100\%,$$

where $(Concentration)_{total}$ refers to calculated mRNA concentration of LNPs in presence of 5% Triton X-100 and $(Concentration)_{unencapsulated}$ refers to calculated mRNA concentration of LNPs in absence of 5% Triton X-100.

Visualization of mRNA encapsulation for each LNP sample was performed using 1% agarose gel containing GelStar™ Nucleic Acid Gel Stain (Lonza) and electrophoresis was conducted at 100 V for 20 min. Equal volumes of each LNP sample were loaded onto the gel after mixing with RNA Gel Loading Dye (Thermo Scientific). RiboRuler High Range RNA Ladder (Thermo Scientific) and free CleanCap® Firefly Luciferase mRNA (300 ng) were included as controls in the gel.

1.7. Dynamic Light Scattering (DLS)

LNP samples were diluted in 0.9% saline, and particle sizes and polydispersity (PDI) were measured in polystyrene cuvettes *via* dynamic light scattering using the Zetasizer Ultra (Malvern Panalytical). Measurements were obtained from 3 runs of 20 readings each. LNP diameters are reported as mean z-average ± standard deviation and PDI are reported as mean ± standard deviation.

1.8. Surface Zeta Potential

Surface zeta potential measurements of LNP samples were also obtained using the Zetasizer Ultra in disposable folded capillary cells (Malvern Panalytical), diluted with 0.9% saline. Zeta potential measurements were obtained from 3 runs of 20 readings each, and are reported as mean ± standard deviation.

1.9. Cell Culture and Treatment with mRNA LNPs and Lyophilized mRNA LNPs

HeLa cells were cultured in 1x Dulbecco's Modified Eagle Medium (high D-glucose, L- Glutamine, no sodium pyruvate; Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) and 1% Penicillin/Streptomycin (Gibco) and were maintained at 37°C, 5% $CO_2$ in an incubator. For LNP treatment, HeLa cells were seeded onto 96-well microplates one day prior to treatment at a seeding density of 10,000 cells per well in complete growth media and incubated at 37°C overnight. Old culture media from each well was replaced by fresh culture media containing each LNP sample at a dose of 100 ng mRNA per well. mRNA concentrations of the LNP samples were determined prior to each cellular treatment. Culture media in the control wells were replaced with fresh culture media without LNPs. HeLa cells were incubated at 37°C with 5% $CO_2$ for 48 h after LNP treatment.

1.10. *In Vitro* Cytotoxicity of the mRNA LNPs and Lyophilized mRNA LNPs

Cellular viability was assessed using the alamarBlue® Cell Viability Reagent (Invitrogen) after 48 h of LNP treatment. Briefly, LNPs-containing media was aspirated after 48 h of incubation and cells were stained with the alamarBlue reagent (diluted to 1x with complete growth media) for 2 h. Fluorescence intensity measurements were obtained using the microplate reader (Tecan Spark Multimode Microplate Reader) at 570 nm excitation and 600 nm emission wavelengths. Cellular viability was calculated using the following equation:

$$Cellular\ viability\ (\%) = \frac{(Fluorescence)treated}{(Fluorescence)control} \times 100\%,$$

where $(Fluorescence)_{treated}$ refers to the fluorescence intensity of LNPs-treated cells and $(Fluorescence)_{control}$ refers to the fluorescence intensity of control cells (without LNP treatment). Calculated cellular viability percentages are reported as mean ± the standard deviation, with 5 replicates for each experimental condition.

1.11. *In Vitro* mRNA Transfection Efficiency

Transfection efficiency of the LNP-treated cells was determined using the Pierce® Firefly Luciferase Glow Assay (Thermo Scientific) after LNP treatment. The assay working reagent was prepared by mixing the cell lysis buffer (diluted to 1× using 1× PBS, pH 7.4) with the Firefly Glow Assay Buffer in a 1:1 v/v ratio, and D-Luciferin was added to a final concentration of 0.3 mg/ml. LNPs-containing media was aspirated and assay working reagent was applied onto the cells, such that 30 µg of D-Luciferin was added into each well. Samples were incubated with the assay working reagent for 10 minutes at 37°C protected from light, and luminescence intensity measurements were obtained using the microplate reader (Tecan Spark Multimode Microplate Reader) after 20 s of shaking, with an integration time of 1000 ms. Luminescence intensities are reported as mean ± the standard deviation, with 5 replicates for each experimental condition.

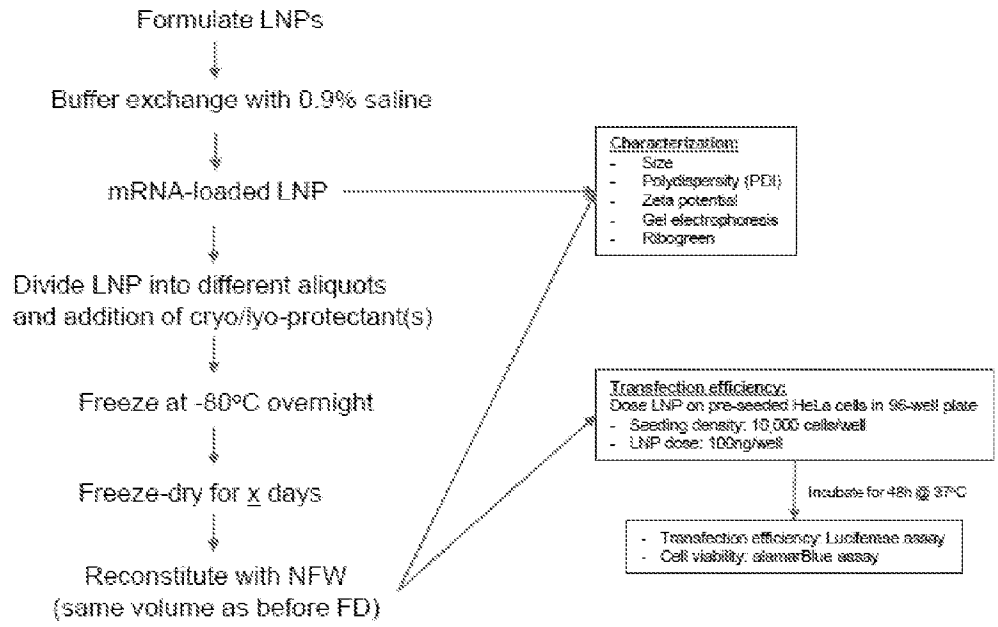
1.12. Statistical Analysis

Statistical analysis was conducted using the GraphPad Prism v.9.0.0 (GraphPad Software). Statistical significance was determined by Welch's t-test for pairwise comparisons.

1.13. General Flow of Experiment

As depicted in Scheme 3 below, the experiments conducted in Example 1 above can be summarized as follows. Firstly, LNPs were formulated and buffer-exchanged with 0.9% saline. Subsequently, mRNA-loaded LNPs were synthesized, divided into different aliquots, and added with cryo- or lyoprotectants. Before the addition of cryo- or lyoprotectants, the mRNA-loaded LNPs were characterized for particle size, PDI, and zeta potential using gel electrophoresis and the Quant-iT RiboGreen RNA assay. Afterwards, the mixtures were frozen at -80°C overnight and freeze-dried for varying durations in accordance with the various embodiments as disclosed herein. The freeze-dried samples were then

reconstituted with nuclease-free water to their original volume before freeze-dying. Subsequent to reconstitution, characterization as previously described was conducted, and transfection efficiency and cell viability were assessed using HeLa cells.




Scheme 3. General Flow of Experiment (NFW: nuclease-free water; FD: freeze-dry).

**Example 2: Preparation and Characterization of mRNA-Loaded Lipid Nanoparticles (mRNA LNPs) Comprising either ALC-0315 or DTD-$NH_2$ Before and After One Day or Four Days of Lyophilization**

The mRNA LNPs were made using a microfluidic device and preparation conditions are listed in **Tables 1** and **2**. The major component is the ionizable lipid. The mRNA LNPs formed from ALC-0315 and DTD-$NH_2$ had an average size of 85.6 and 101.5 nm, respectively, narrow size distribution and a zeta potential close to neutral (**Tables 3** and **4**), which is desirable for *in vivo* application. After 6 days of storage at 4°C, the size of mRNA LNPs made from either ALC-0315 or DTD-$NH_2$ remained unchanged (**Tables 3** and **4**). After 4 days of lyophilization process, the size

of mRNA LNPs made from either ALC-0315 or DTD-$NH_2$ became significantly larger (ALC-0315 LNPs: 85.6 nm *vs.* 556.6-846.3 nm; DTD-$NH_2$ LNPs: 101.5 nm *vs.* 413.5-885.7 nm) with wide size distribution as evidenced by increased PDI (**Tables 3** and **4**) although the cryoprotectant sucrose was used at 5-20%.

**Table 1.** Preparation conditions of mRNA LNPs (total volume of 4.0 mL) from ALC-0315.

| Lipid | Molecular Weight (g/moL) | Stock Concentration (mg/mL) | Amount added (mg) | Volume (mL) | Molar percentage (%) |
|---|---|---|---|---|---|
| ALC-0159 | 766.29 | 20 | 2.760 | 0.138 | 46.3 |
| DSPC | 790.15 | 10 | 0.572 | 0.057 | 9.4 |
| Cholsterol | 386.65 | 20 | 1.280 | 0.064 | 42.7 |
| ALC-0159 | 2450.00 | 3 | 0.305 | 0.102 | 1.6 |
| Ethanol | - | - | - | 0.639 | - |
| Total: | - | - | 4.917 | 1.000 | 100.0 |

- Total volume for aqueous (3 mL) and organic phase (1 mL) was 4.0 mL
- N/P ratio = 6
- Amount of fLuc mRNA (Trilink) added = 0.2 mg
- 10 mM sodium acetate (pH 4.0) was used to dilute mRNA

**Table 2.** Preparation conditions of mRNA LNPs (total volume of 4.0 mL) from DTD-$NH_2$.

| Lipid | Molecular Weight (g/moL) | Stock Concentration (mg/mL) | Amount added (mg) | Volume (mL) | Molar percentage (%) |
|---|---|---|---|---|---|
| DTD-$NH_2$ | 551.00 | 4 | 2.760 | 0.690 | 54.6 |
| DSPC | 790.15 | 10 | 0.572 | 0.057 | 7.9 |
| Cholesterol | 386.65 | 20 | 1.280 | 0.064 | 36.1 |
| ALC-0159 | 2450.00 | 3 | 0.305 | 0.102 | 1.4 |
| Ethanol | - | - | - | 0.087 | - |
| Total: | - | - | 4.917 | 1.000 | 100.0 |

- Total volume for aqueous (3 mL) and organic phase (1 mL) was 4.0 mL
- N/P ratio = 6
- Amount of fLuc mRNA (Trilink) added = 0.28 mg
- 10 mM sodium acetate (pH 4.0) was used to dilute mRNA

**Table 3.** Particle size, size distribution, and zeta potential of mRNA LNPs made from ALC-0315 before and after 4 days of freeze-drying (lyophilization). The samples with FD indicate they are lyophilized samples, and the rest are samples without lyophilization.

| ALC-0315 | | | | |
|---|---|---|---|---|
| **Condition** | **Sample** | **Size (nm)** | **PDI** | **Zeta potential** |
| Before freeze-dry | 0% sucrose | 85.64 ± 2.92 | 0.20 ± 0.01 | -0.44 ± 0.74 |
| | 5% sucrose | 85.43 ± 1.16 | 0.16 ± 0.01 | -0.15 ± 0.43 |
| | 10% sucrose | 86.98 ± 0.98 | 0.17 ± 0.03 | -0.19 ± 0.24 |
| | 20% sucrose | 92.15 ± 1.63 | 0.17 ± 0.01 | -0.79 ± 0.33 |
| After freeze-dry | 4 deg, 0% | 86.05 ± 0.78 | 0.19 ± 0.01 | -0.46 ± 0.44 |
| | 0% sucrose + FD | 458.80 ± 27.49 | 0.48 ± 0.03 | -4.46 ± 1.34 |
| | 5% sucrose + FD | 556.60 ± 4.36 | 0.23 ± 0.03 | -0.83 ± 0.56 |
| | 10% sucrose + FD | 846.30 ± 17.18 | 0.23 ± 0.05 | -1.04 ± 0.42 |
| | 20% sucrose + FD | 655.00 ± 14.18 | 0.36 ± 0.03 | -2.63 ± 0.11 |

**Table 4.** Particle size, size distribution, and zeta potential of mRNA LNPs made from DTD-$NH_2$ before and after 4 days of freeze-drying (lyophilization). The samples with FD indicate they are lyophilized samples, and the rest are samples without lyophilization.

| DTD-$NH_2$ | | | | |
|---|---|---|---|---|
| **Condition** | **Sample** | **Size (nm)** | **PDI** | **Zeta potential** |
| Before freeze-dry | 0% sucrose | 101.50 +1.75 | 0.22 ± 0.01 | 0.76 ± 0.67 |
| | 5% sucrose | 101.70 ± 1.22 | 0.20 ± 0.02 | 2.80 ± 0.60 |
| | 10% sucrose | 100.70 ± 1.81 | 0.19 ± 0.00 | 2.08 ± 0.47 |
| | 20% sucrose | 104.90 ± 1.90 | 0.23 ± 0.02 | 5.26 ± 0.56 |
| After freeze-dry | 4 deg, 0% | 99.99 ± 1.20 | 0.20 ± 0.02 | 1.84 ± 0.85 |
| | 0% sucrose + FD | 2229.00 ± 222.10 | 0.55 ± 0.09 | 2.89 ± 0.54 |
| | 5% sucrose + FD | 885.70 ± 41.22 | 0.56 ± 0.05 | 1.98 ± 0.25 |
| | 10% sucrose + FD | 413.50 ± 7.67 | 0.17 ± 0.01 | 3.60 ± 0.79 |
| | 20% sucrose + FD | 525.70 ± 17.73 | 0.44 ± 0.06 | 2.79 ± 0.37 |

mRNA binding efficiency of the mRNA LNPs before and after 4 days of lyophilisation was evaluated using the gel retardation assay. As shown in **FIG. 4**, the retardation of mRNA movement from ALC-0315 LNPs was observed with and without sucrose, suggesting good encapsulation of mRNA in the LNPs. However, free mRNA molecules were observed in **FIG. 5** after 4 days of lyophilisation, suggesting that mRNA was not well encapsulated inside the LNPs after the lyophilisation process. Some free mRNA molecules were also observed in the LNPs after 8 days of storage at 4°C (**FIG. 5**), suggesting loss of mRNA stability to a certain degree. These phenomena were not observed for mRNA in the LNPs made from DTD-$NH_2$ (**FIG. 6**), demonstrating mRNA stability in the LNPs after 4 days of lyophilisation or 6 days of storage at 4°C.

It is noted that the process of freezing-thawing in the presence of 10% sucrose or 10% maltose or the mixture of 10% sucrose and 10% maltose led to larger particle size and wider size distribution of mRNA LNPs made from ALC-0315 (85 nm *vs.* 92.6-218 nm, PDI: 0.05 *vs.* 0.1-0.35, **Table 5**). Since 4 days of freeze-drying led to large particle size and wide size distribution with or without the cryoprotectant, the freeze-drying process was shortened to one day. After one day of lyophilization without the use of sucrose, the size of mRNA LNPs made from either ALC-0315 or DTD-$NH_2$ became larger with wide size distribution (high PDI values) due to aggregation, whereas the zeta potential remained unchanged (**Tables 5** and **6**). However, the use of the cryoprotectant sucrose reduced aggregation caused by one day of freeze-drying, leading to smaller particle size with narrow size distribution. The size of mRNA LNPs after freeze-drying and re-dispersing in saline was dependent on the content of sucrose used, and an increased sucrose content led to smaller size. The contents of 5%-20% are desirable for mRNA LNPs formed from either ALC-0315 or DTD-$NH_2$ as the particle size was between 145 nm and 254 nm with narrow size distribution (PDI: 0.16-0.30) (**Tables 5** and **6**).

**Table 5.** Particle size, size distribution and zeta potential of mRNA LNPs made from ALC-0315 before and after 1 day of freeze-drying (lyophilization) (FT: freeze-thaw; FD: freeze-dry).

| Condition | Sample | Size (nm) | PDI | Zeta potential (mV) |
|---|---|---|---|---|
| Before freeze-drying | 0% sucrose | 85.3 ± 0.6 | 0.05 ± 0.01 | 0.23 ± 0.93 |
| | 5% sucrose | 86.4 ± 0.8 | 0.06 ± 0.01 | -0.42 ± 1.30 |
| | 10% sucrose | 86.9 ± 0.7 | 0.05 ± 0.02 | 1.09 ± 0.83 |
| | 20% sucrose | 88.6 ± 0.8 | 0.06 ± 0.01 | 0.34 ± 2.70 |
| | 10% maltose | 113.3 ± 0.5 | 0.15 ± 0.02 | -1.54 ± 1.11 |
| | 10% sucrose+10% maltose | 114.6 ± 0.8 | 0.15 ± 0.02 | 0.63 ± 0.24 |
| After freeze-drying | 4 deg, 0% | 85.2 ± 0.7 | 0.08 ± 0.02 | -1.65 ± 0.21 |
| | FT, 10% sucrose | 92.6 ± 0.6 | 0.10 ± 0.00 | -0.14 ± 1.00 |
| | FT, 10% maltose | 218.2 ± 7.7 | 0.35 ± 0.04 | -0.28 ± 16.49 |
| | FT, 10% sucrose+10% maltose | 136.3 ± 1.5 | 0.19 ± 0.02 | 1.86 ± 0.42 |
| | FD, 0% sucrose | 292.6 ± 82.8 | 0.37 ± 0.02 | -0.57 ± 1.85 |
| | FD, 5% sucrose | 191.5 ± 5.9 | 0.21 ± 0.03 | 0.62 ± 0.84 |
| | FD, 10% sucrose | 186.3 ± 3.5 | 0.22 ± 0.02 | 0.10 ± 0.34 |
| | FD, 20% sucrose | 145.5 ± 0.2 | 0.16 ± 0.02 | 0.95 ± 0.90 |

**Table 6.** Particle size, size distribution and zeta potential of mRNA LNPs made from DTD-$NH_2$ before and after 1 day of freeze-drying (lyophilization) (FT: freeze-thaw; FD: freeze-dry).

| Condition | Sample | Size (nm) | PDI | Zeta potential (mV) |
|---|---|---|---|---|
| Before freeze-drying | 0% sucrose | 84.2 ± 0.4 | 0.21 ± 0.02 | 2.87±2.52 |
| | 5% sucrose | 84.2 ± 0.7 | 0.21 ± 0.02 | 2.22±1.53 |
| | 10% sucrose | 85.4 ± 0.3 | 0.22 ± 0.01 | 3.57 ± 0.98 |
| | 20% sucrose | 86.7 ± 0.8 | 0.22 ± 0.01 | 3.33 ± 0.09 |
| After freeze-drying | 4 deg, 0% | 83.5 ± 1.2 | 0.22 ± 0.01 | 3.27 ± 0.69 |
| | FD, 0% sucrose | 1478 ± 362 | 0.87±0.11 | 2.28 ± 0.01 |
| | FD, 5% sucrose | 254.0 ± 8.7 | 0.30 ± 0.02 | 2.54 ± 1.22 |
| | FD, 10% sucrose | 223.6 ± 7.6 | 0.28 ± 0.06 | 3.74 ± 0.80 |
| | FD, 20% sucrose | 151.6 ± 1.7 | 0.19 ± 0.02 | 3.50 ± 1.31 |

The encapsulation efficiency (EE) of mRNA, which was measured just after the mRNA LNPs were made from the microfluidic device, was 85.6% and 94.0% for ALC-0315 and DTD-$NH_2$ LNPs, respectively (**Table 7**). After purification of the mRNA LNPs to remove ethanol, EE of mRNA was determined to be 90.9% and 97.4% for ALC-0315 LNPs and DTD-$NH_2$ LNPs, respectively (**Table 7**). After the freeze-thaw process, the presence of 10% sucrose did not affect the stability of mRNA in ALC-0315 LNPs, while the presence of 10% maltose reduced the stability of mRNA, leading to lower EE of mRNA in ALC-0315 LNPs. After one day of freeze-drying process, EE of mRNA in ALC-0315 LNPs decreased from 90.9% to 40.7%-57.5% when 5%, 10% or 20% sucrose was used, while EE of mRNA in DTD-$NH_2$ LNPs was comparable (88%-90% for 5-20% sucrose) before and after one day of freeze-drying process (**Table 7**). The reduction in EE of mRNA in ALC-0315 LNPs might be due to degradation of mRNA and/or because mRNA was pulled out from the core of LNPs to the surface of LNPs during the freeze-drying process. In contrast, DTD-$NH_2$ LNPs were able to stabilize mRNA during one day of freeze-drying process in the presence of 5%, 10% or 20% sucrose.

**Table 7.** Encapsulation efficiency (EE%) of mRNA in ALC-0315 and DTD-$NH_2$ LNPs (FT: freeze-thaw; FD: freeze-dry).

**Before freeze-drying**

| Sample | Original EE % | Final EE % |
|---|---|---|
| ALC-0315 | 85.6 ± 0.3 | 94.9 ± 0.2 |
| DTD-NH2 | 94.0 ± 0.3 | 97.2 ± 0.2 |

**After freeze-drying**

| Sample | | Final EE % |
|---|---|---|
| ALC-0315 | 4 deg, 0% | 90.9 ± 0.3 |
| | FT, 10% sucrose | 90.5 ± 0.7 |
| | FT, 10% maltose | 51.9 ± 1.1 |
| | FT, 10% sucrose+10% maltose | 77.5 ± 0.1 |
| | FD, 0% sucrose | 22.2 ± 18.6 |
| | FD, 5% sucrose | 45.3 ± 2.9 |
| | FD, 10% sucrose | 40.7 ± 0.7 |
| | FD, 20% sucrose | 57.5 ± 2.4 |
| DTD-NH2 | 4 deg, 0% | 97.4 ± 0.2 |
| | FD, 0% sucrose | 88.4 ± 0.1 |
| | FD, 5% sucrose | 87.7 ± 0.0 |
| | FD, 10% sucrose | 90.3 ± 0.6 |
| | FD, 20% sucrose | 92.2 ± 0.0 |

As shown in **Table 8**, the use of 20% sucrose protected mRNA stability in ALC-0315 LNPs after one day of freeze-drying process, maintaining 55% mRNA transfection efficiency, relative to mRNA-loaded ALC-0315 LNPs without freeze-drying. The use of 10% sucrose in one day of freeze-drying process maintained 67% mRNA transfection efficiency of mRNA-loaded DTD-$NH_2$ LNPs, relative to mRNA-loaded ALC-0315 LNPs without freeze-drying. When 5% sucrose was used in mRNA-loaded DTD-$NH_2$ LNPs during the freeze-drying process, the transfection efficiency was similar to that of mRNA-loaded DTD-$NH_2$ LNPs without freeze-drying (after freeze-drying: 170514 *vs.* before freeze-drying: 167626). These results demonstrate the importance of using the cryoprotectant sucrose and shorter freeze-drying time for mRNA-loaded LNPs made from either ALC-0315 or DTD-$NH_2$. In addition, the use of DTD-$NH_2$ gave better protection of mRNA in LNPs during the freeze-drying process.

**Table 8.** Viability of HeLa cells and mRNA transfection efficiency in HeLa cells after 48 h of incubation with various mRNA LNPs (FT: freeze-thaw; FD: freeze-dry).

| Sample | Dose (ng/well) | Cell viability (%) | Luminescence intensity (RLU) |
|---|---|---|---|
| **ALC-0315** | | | |
| Control | - | 100.0 ± 1.7 | 40 ± 7 |
| 4 deg, 0% sucrose | 100 | 99.0 ± 1.1 | 7192 ± 793 |
| FT, 10% sucrose | 100 | 99.9 ± 1.7 | 5855 ± 471 |
| FT, 10% maltose | 100 | 98.6 ± 1.3 | 4729 ± 155 |
| FT, 10% sucrose + 10% maltose | 100 | 98.2 ± 1.3 | 6509 ± 478 |
| FD, 0% sucrose | 100 | 98.9 ± 1.9 | 3439 ± 160 |
| FD, 5% sucrose | 100 | 98.7 ± 1.0 | 1605 ± 188 |
| FD, 10% sucrose | 100 | 98.6 ± 1.4 | 2351 ± 280 |
| FD, 20% sucrose | 100 | 96.4 ± 1.1 | 3941 ± 497 |
| **DTD-NH2** | | | |
| Control | - | 100.0 ± 1.1 | 409 ± 33 |
| 4 deg, 0% sucrose | 100 | 105.7 ± 1.1 | 167626 ± 7592 |
| FD, 0% sucrose | 100 | 104.9 ± 2.3 | 12271 ± 537 |
| FD, 5% sucrose | 100 | 105.9 ± 0.7 | 170514 ± 8396 |
| FD, 10% sucrose | 100 | 105.4 ± 1.8 | 112216 ± 6656 |
| FD, 20% sucrose | 100 | 104.7 ± 0.7 | 74431 ± 2790 |

Summary of Example 2

The use of sucrose at 5%, 10% or 20% protected the stability of mRNA in LNPs made from either ALC-0315 or DTD-$NH_2$ during one day of freeze-drying process. The particle size of the mRNA lipids after one day of lyophilization process remained less than 250 nm, depending on the amount of sucrose used. 10-20% sucrose gave smaller size of freeze-dried mRNA LNPs. In addition, it was discovered that the cryoprotectant sucrose (10-20%) gave better protection of mRNA stability during one day of lyophilization process for the mRNA-loaded ALC-0315 LNPs, yielding ~54-55% transfection efficiency as compared to the

mRNA LNPs before lyophilisation. The stability of mRNA in LNPs made from DTD-$NH_2$ remains after one day of lyophilisation process when 5-20% sucrose is used, leading to comparable transfection efficiency at the same order of magnitude as compared to the mRNA LNPs before lyophilisation.

After 4 days of lyophilization, mRNA in the ALC-0315 LNPs that were made from the lipids used in Pfizer/BioNTech's mRNA vaccine formulation lost its stability although 5%-20% sucrose was used as cryoprotectant, leading to no transfection efficiency. When the lipid DTD-$NH_2$ as the ionizable lipid and 5% or 20% sucrose were used during 4 days of lyophilisation process, mRNA stability in DTD-$NH_2$ LNPs was fully protected, leading to no loss in transfection efficiency. Four days of lyophilization process led to large particle size with wide size distribution of mRNA LNPs made from either ALC-0315 or DTD-$NH_2$ even in the presence of sucrose.

Therefore, it can be seen that the use of the compound of general formula (1) (e.g., in the form of DTD-$NH_2$) in combination with a cryoprotectant (e.g., in the form of sucrose) provided better stability of mRNA during the freeze-drying process.

**Example 3: Preparation and Characterization of mRNA-Loaded Lipid Nanoparticles (mRNA LNPs) Comprising either ALC-0315 or ALC-0315 with DTD-$NH_2$ Before and After Lyophilization**

The mRNA LNPs were made using a microfluidic device and preparation conditions are listed in **Tables 9** to **11**. Samples with 20% sucrose had a spongy matrix appearance after lyophilization as depicted in **FIG. 3**. All freeze-dried samples were subsequently reconstituted to form opalescent solutions that are well-dissolved. **Table 12** presents the characteristics of the mRNA LNPs, including particle size, PDI, zeta potential, and final encapsulation efficiency. **FIGS. 14** to **18** display the particle size distribution of mRNA LNPs made from ALC-0315 or a mixture

of ALC-0315 and DTD-$NH_2$ in mole ratios of 9:1 and 8:2. As shown in **FIGS. 14** to **16**, the particle size distribution showed uniformity (single peak) before freeze-drying/lyophilization. However, aggregation of mRNA LNPs occurred after lyophilization in the absence of a cryoprotectant, indicated by multiple peaks in **FIGS. 22** to **24**. Conversely, no aggregation was observed in the presence of 20% sucrose after lyophilization, as demonstrated by the single peak in **FIGS. 25** to **27**.

**Table 9.** Preparation conditions of mRNA LNPs (total volume of 2.0 mL) from ALC-0315.

| Lipid | Molecular Weight (g/moL) | Stock Concentration (mg/mL) | Amount added (mg) | Volume (µL) | Molar ratio |
|---|---|---|---|---|---|
| ALC-0315 | 766.29 | 20 | 1.380 | 69.00 | 46.3 |
| DSPC | 790.15 | 10 | 0.286 | 28.60 | 9.4 |
| Cholesterol | 386.65 | 20 | 0.640 | 32.00 | 42.7 |
| ALC-0159 | 2450.00 | 3 | 0.152 | 50.77 | 1.6 |
| Ethanol | - | - | - | 319.63 | - |
| Total | - | - | 2.458 | 500.00 | 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
- LNPs were prepared by microfluidics

**Table 10.** Preparation conditions of mRNA LNPs (total volume of 2.0 mL) from ALC-0315 and DTD-$NH_2$ in a molar ratio of 9: 1.

| Lipid | Molecular Weight (g/moL) | Stock Concentration (mg/mL) | Amount added (mg) | Volume (µL) | Molar ratio |
|---|---|---|---|---|---|
| ALC-0315 | 766.29 | 20 | 1.219 | 60.94 | 41.7 |
| DTD-$NH_2$ | 551.00 | 4 | 0.097 | 24.34 | 4.6 |
| DSPC | 790.15 | 10 | 0.284 | 28.35 | 9.4 |
| Cholesterol | 386.65 | 20 | 0.630 | 31.51 | 42.7 |
| ALC-0159 | 2450.00 | 3 | 0.152 | 50.51 | 1.6 |
| Ethanol | - | - | - | 304.34 | - |
| Total | - | - | 2.381 | 500.00 | 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
- LNPs were prepared by microfluidics

**Table 11.** Preparation conditions of mRNA LNPs (total volume of 2.0 mL) from ALC-0315 and DTD-$NH_2$ in a molar ratio of 8: 2.

| Lipid | Molecular Weight (g/moL) | Stock Concentration (mg/mL) | Amount added (mg) | Volume (µL) | Molar ratio |
|---|---|---|---|---|---|
| ALC-0315 | 766.29 | 20 | 1.083 | 54.17 | 37.0 |
| DTD-$NH_2$ | 551.00 | 4 | 0.195 | 48.69 | 9.3 |
| DSPC | 790.15 | 10 | 0.284 | 28.35 | 9.4 |
| Cholesterol | 386.65 | 20 | 0.630 | 31.51 | 42.7 |
| ALC-0159 | 2450.00 | 3 | 0.152 | 50.51 | 1.6 |
| Ethanol | - | - | - | 286.77 | - |
| Total | - | - | 2.343 | 500.00 | 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
- LNPs were prepared by microfluidics

**Table 12.** Characterizations of mRNA LNPs (total volume of 2.0 mL) from only ALC-0315 or ALC-0315 and DTD-$NH_2$ in a molar ratio of 9: 1 or 8: 2 (particle size, PDI, zeta potential, final encapsulation efficiency).

| Condition | Sample | Conditions | Size (nm) | PDI | Zeta potential | Original EE % | Final EE % | mRNA concentration (ng/μl) |
|---|---|---|---|---|---|---|---|---|
| Before FD | ALC0315 | 0% sucrose | 62 ± 0 | 0.11 ± 0.02 | -0.34 ± 0.38 | 86.5 ± 0.2 | 95.3 ± 0.2 | 199.7 ± 4.9 |
| | ALC:DTD (9:1) | 0% sucrose | 63 ± 1 | 0.14 ± 0.01 | -0.67 ± 2.69 | 86.8 ± 0.1 | 93.9 ± 0.4 | 198.2 ± 1.4 |
| | ALC:DTD (8:2) | 0% sucrose | 63 ± 0 | 0.16 ± 0.01 | -0.99 ± 0.42 | 87.1 ± 1.0 | 94.2 ± 1.3 | 175.0 ± 4.9 |
| After FT/FD | ALC0315 (w/o FD) | 0% sucrose | 65 ± 0 | 0.14 ± 0.01 | -1.38 ± 1.23 | - | 95.6 ± 0.4 | 36.0 ± 0.0 |
| | ALC0315 (w/o FD) | 20% sucrose | 65 ± 1 | 0.11 ± 0.02 | 0.18 ± 1.22 | - | 95.1 ± 0.3 | 27.5 ± 0.9 |
| | ALC0315 | FT, 20% sucrose | 76 ± 1 | 0.21 ± 0.04 | 2.03 ± 1.31 | - | 87.4 ± 0.4 | 28.3 ± 0.3 |
| | ALC0315 | FD, 0% sucrose | 1065 ± 823 | 0.74 ± 0.23 | 2.01 ± 0.81 | - | 14.3 ± 7.4 | 11.5 ± 0.0 |
| | ALC0315 | FD, 20% sucrose | 143 ± 0 | 0.12 ± 0.02 | -2.10 ± 0.74 | - | 79.6 ± 0.4 | 22.3 ± 0.5 |
| | ALC:DTD (9:1) | FT, 20% sucrose | 73 ± 1 | 0.19 ± 0.04 | 1.09 ± 1.50 | - | 89.0 ± 1.0 | 37.6 ± 0.4 |
| | ALC:DTD (9:1) | FD, 0% sucrose | 325 ± 12 | 0.43 ± 0.04 | 1.58 ± 0.94 | - | 27.7 ± 15.1 | 30.6 ± 2.2 |
| | ALC:DTD (9:1) | FD, 20% sucrose | 155 ± 4 | 0.08 ± 0.07 | -0.91 ± 0.53 | - | 56.7 ± 1.0 | 27.7 ± 0.2 |
| | ALC:DTD (8:2) | FT, 20% sucrose | 76 ± 1 | 0.28 ± 0.02 | 0.35 ± 1.51 | - | 91.2 ± 0.1 | 23.4 ± 0.2 |
| | ALC:DTD (8:2) | FD, 0% sucrose | 544 ± 237 | 0.56 ± 0.09 | 0.04 ± 1.72 | - | 19.6 ± 28.3 | 6.1 ± 0.4 |
| | ALC:DTD (8:2) | FD, 20% sucrose | 184 ± 1 | 0.08 ± 0.02 | 0.39 ± 0.83 | - | 52.4 ± 0.2 | 16.6 ± 0.1 |

- FT: Freeze-thaw; FD: freeze-dry; ALC: ALC-0315; DTD: DTD-$NH_2$.

Furthermore, in the *in vitro* study conducted using HeLa cells, Table 13 demonstrates that the inclusion of sucrose as a cryoprotectant in mRNA LNPs and the lyophilization process do not affect cell viability. Additionally, it is evident from Table 13 that mixing ALC-0315 with DTD-$NH_2$ in mRNA LNPs helps preserve greater mRNA transfection efficiency. Specifically, the luminescence intensities after lyophilization for mRNA LNPs containing both ALC-0315 and DTD-$NH_2$ at molar ratios of 9:1 (4031 RLU) and 8:2 (6203 RLU) with 20% sucrose as a cryoprotectant are higher than those of mRNA LNPs containing only ALC-0315 (1267 RLU) with 20% sucrose as a cryoprotectant.

In the *in vivo* study conducted on Balb/c mice via intravenous injection (tail vein), each mouse received a dose of 1 μg mRNA/100 μL. Imaging was performed at 6 hours and 24 hours post-administration of mRNA LNPs. The ALC-0315 groups consisted of 6 mice before freeze-drying (lyophilization) and 7 mice after lyophilization. Additionally, the ALC-0315:DTD-$NH_2$ (9: 1) group consisted of 7 mice after lyophilization. A "Naïve" group served as the control group without any treatment. It was observed that mixing ALC-0315 with DTD-$NH_2$ in mRNA LNPs preserved significantly higher mRNA transfection efficiency in mice compared to using ALC-0315 alone. Specifically, the total flux after lyophilization for mRNA LNPs containing both ALC-0315 and DTD-$NH_2$ at molar ratios of 9:1 is higher than that of mRNA LNPs containing only ALC-0315, as illustrated in **FIG. 28**.

**Table 13.** Viability of HeLa cells and mRNA transfection efficiency in HeLa cells after 48 h of incubation with various mRNA LNPs (FT: freeze-thaw; FD: freeze-dry/lyophilization).

| Sample | Group | Dose (ng/well) | Cell viability (%) | Luminescence intensity (RLU) |
|---|---|---|---|---|
| Control | - | - | 100.0 ± 1.5 | 229 ± 52 |
| ALC-0315 | 4 deg, 0% | 100 | 101.1 ± 1.7 | 1567 ± 194 |
| | FT, 20% sucrose | 100 | 103.2 ± 0.8 | 2081 ± 163 |
| | FD, 0% sucrose | 100 | 106.5 ± 1.9 | 555 ± 14 |
| | FD, 20% sucrose | 100 | 101.3 ± 2.1 | 1267 ± 77 |
| ALC-0315:DTD-$NH_2$ (9:1) | FT, 20% sucrose | 100 | 100.1 ± 1.7 | 2222 ± 195 |
| | FD, 0% sucrose | 100 | 106.6 ± 1.4 | 874 ± 67 |
| | FD, 20% sucrose | 100 | 104.1 ± 2.2 | 4031 ± 182 |
| ALC-0315:DTD-$NH_2$ (8:2) | FT, 20% sucrose | 100 | 98.9 ± 2.5 | 5327 ± 179 |
| | FD, 0% sucrose | 100 | 101.5 ± 3.6 | 828 ± 52 |
| | FD, 20% sucrose | 100 | 101.8 ± 1.7 | 6203 ± 420 |

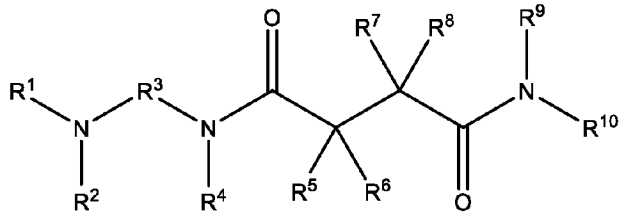
- FT: freeze-thaw; FD: freeze-dry (lyophilization)
- HeLa cells, 48 h incubation

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 nanoparticle composition for delivery of a therapeutic, prophylactic and/or biological agent, the nanoparticle composition comprising:

a compound represented by general formula (1) or ionized form thereof



(1)

wherein

$R^1$, $R^2$, and $R^4$ to $R^8$ are each independently H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl,

$R^3$ is optionally substituted alkylene, optionally substituted alkenylene, or optionally substituted alkynylene, and

$R^9$ and $R^{10}$ are each independently a hydrophobic tail or contains at least one of the groups defined above for $R^4$ to $R^8$;

a therapeutic, prophylactic and/or biological agent that is encapsulated by the compound of general formula (1) to form nanoparticles; and

a cryoprotectant.

2. The nanoparticle composition of claim 1, wherein

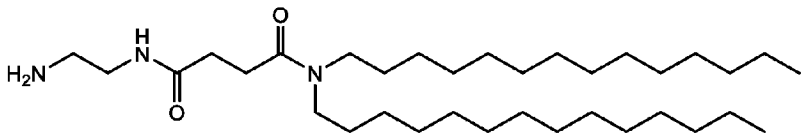
$R^1$ and $R^2$ are each H;

$R^3$ comprises alkylene; and

$R^9$ and $R^{10}$ each independently comprises alkyl, where the alkyl contains at least 5 carbon atoms.

3. The nanoparticle composition of claim 1 or 2, wherein $R^4$ to $R^8$ are each H.

4. The nanoparticle composition of any one of the preceding claims, wherein the compound is represented by formula (1A):



(1A)

5. The nanoparticle composition of any one of the preceding claims, wherein the therapeutic, prophylactic and/or biological agent comprises a nucleic acid.

6. The nanoparticle composition of claim 5, wherein the nucleic acid comprises messenger ribonucleic acid (mRNA).

7. The nanoparticle composition of any one of the preceding claims, wherein the nanoparticle composition further comprises an ionizable lipid that is different from the compound of general formula (1).

8. The nanoparticle composition of claim 7, wherein the ionizable lipid that is different from the compound of general formula (1) comprises ALC-0315.

9. The nanoparticle composition of any one of claims 7 to 8, wherein the ratio of the compound of general formula (1) to the ionizable lipid that is different from said compound is 1: 1 – 20.

10. The nanoparticle composition of any one of the preceding claims, wherein the composition further comprises:

neutral/helper lipid;

sterol; and

polyethylene glycol (PEG)-modified lipid.

11. The nanoparticle composition of claim 10, wherein the compound of general formula (1), the neutral/helper lipid, the sterol, and the PEG-modified lipid are mixed at a molar ratio of 1 – 70 : 1 – 20 : 10 – 60 : 1 – 20.

12. The nanoparticle composition of any one of claims 10 to 11, wherein the neutral/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-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.

13. The nanoparticle composition of any one of claims 10 to 12, wherein the sterol is selected from cholesterol, fecosterol, sitosterol, ergosterol,

campesterol, stigmasterol, brassicasterol, avenasterol and combinations thereof.

14. The nanoparticle composition of any one of claims 10 to 13, wherein the PEG-modified lipid is selected from 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159), R-3-[(ω-methoxy-poly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxlpropyl-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.

15. The nanoparticle composition of any one of the preceding claims, wherein the cryoprotectant comprises a carbohydrate source selected from at least one of maltose, sucrose, trehalose, mannitol, glucose, fructose, lactose, galactose, ribose, xylose, mannose, arabinose, and combinations thereof.

16. The nanoparticle composition of any one of the preceding claims, wherein the cryoprotectant is present in an amount falling in the range of from 1 w/v% to 30 w/v% with respect to the total volume of the composition.

17. The nanoparticle composition of any one of the preceding claims, wherein the nanoparticle composition is in a lyophilized form or a freeze-thawed form.

18. The nanoparticle composition of claim 17, wherein the therapeutic and/or prophylactic agent and/or biological agent is encapsulated at an encapsulation efficiency of at least 60%.

19. The nanoparticle composition of any one of claims 17 to 18, wherein the nanoparticles have a zeta potential in the range of from -10 mV to +10 mV.

20. The nanoparticle composition of any one of claims 17 to 19, wherein the nanoparticles have a polydispersity index (PDI) in the range of from 0.01 to 0.5.

21. The nanoparticle composition of any one of claims 17 to 20, wherein the nanoparticles have a particle size in the range of from 50 nm to 1200 nm.

22. The nanoparticle composition as claimed in any one of the preceding claims for use in medicine.

23. Use of a nanoparticle composition as claimed in any one of the preceding claims in the manufacture of a medicament for inducing an immune response in a subject in need thereof.

24. The nanoparticle composition as claimed in any one of the preceding claims for use in inducing an immune response in a subject in need thereof, wherein said nanoparticle composition or lyophilized nanoparticle composition is to be administered to the subject.

25. A method of inducing an immune response in a subject, the method comprising the step of administering to the subject a therapeutically effective amount of a nanoparticle composition as claimed in any one of the preceding claims.

26. The use of claim 23, the nanoparticle composition of claim 24 or the method of claim 25, wherein the immune response is specific to a coronavirus or to cancer or to bacteria.

27. The use, the nanoparticle composition or the method of claim 26, wherein the coronavirus is a SARS-CoV-2 coronavirus.

28. A method of preparing the nanoparticle composition as claimed in claim 1, the method comprising:

preparing an aqueous composition comprising a therapeutic and/or prophylactic agent and/or biological agent;

mixing the aqueous composition with the compound general formula (1), a helper lipid, a sterol, and a PEG-modified lipid to obtain nanoparticles encapsulating the therapeutic and/or prophylactic agent and/or biological agent;

adding a cryoprotectant to the nanoparticles.

29. The method of claim 28, wherein the mixing step is carried out in the presence of a further ionizable lipid that is different from the compound of general formula (1).

30. The method of claim 29, wherein the compound of general formula (1) and the ionizable lipid that is different from said compound are mixed together in a ratio of 1: 1 - 20.

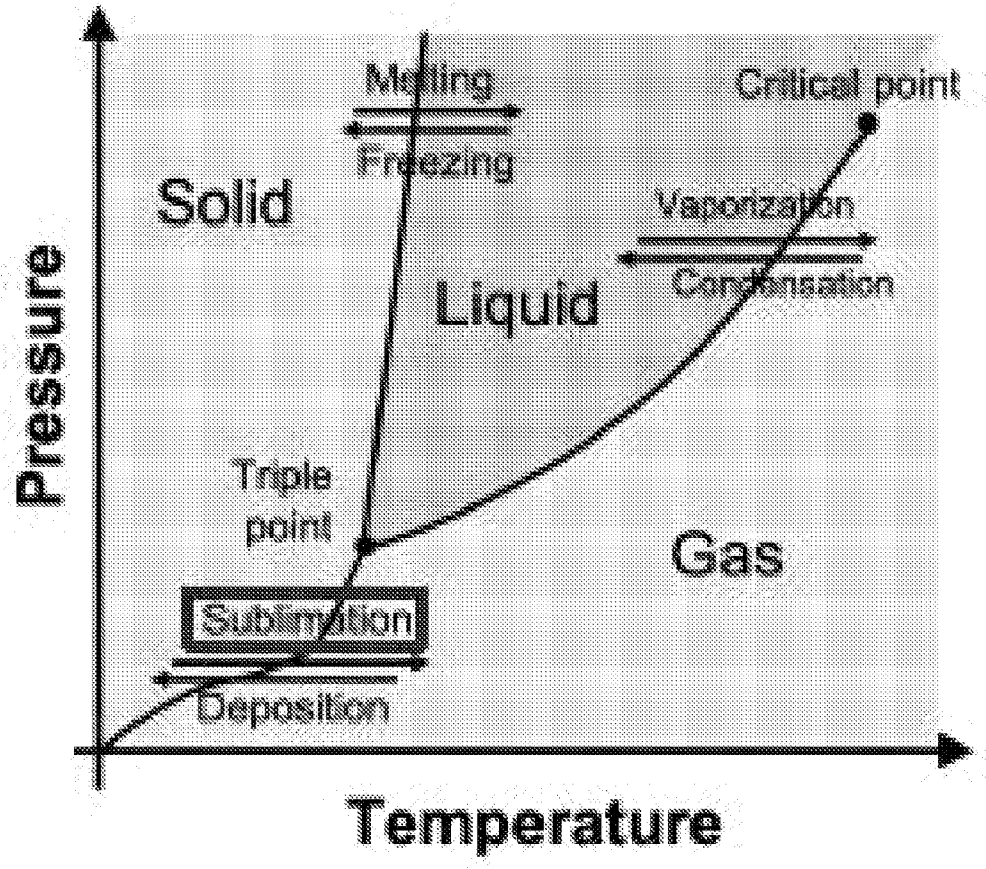

Melting
Freezing
Critical point
Solid
Vaporization
Condensation
Liquid
Pressure
Triple
point
Gas
Sublimation
Deposition
Temperature

FIG. 1

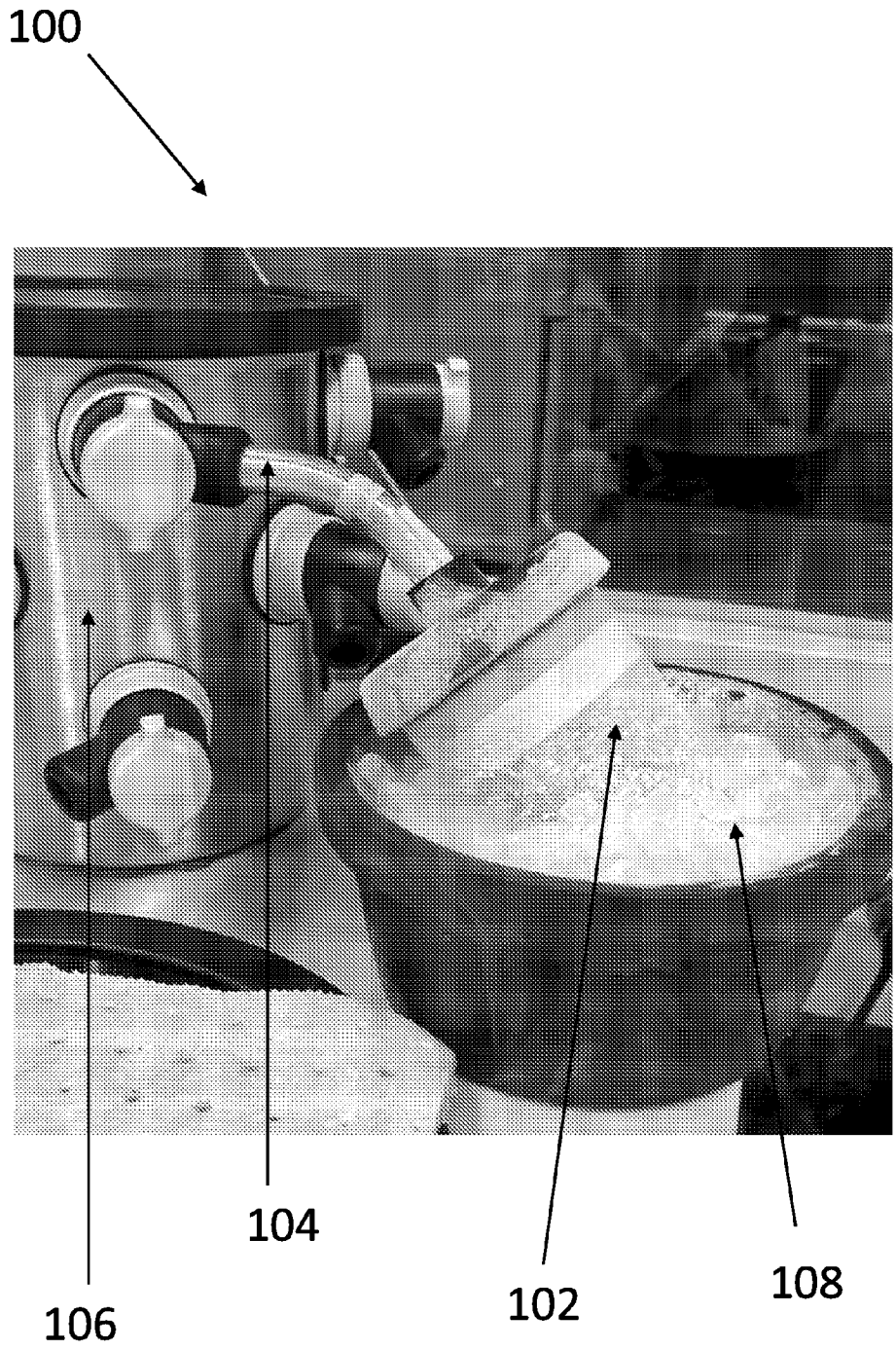



FIG. 2

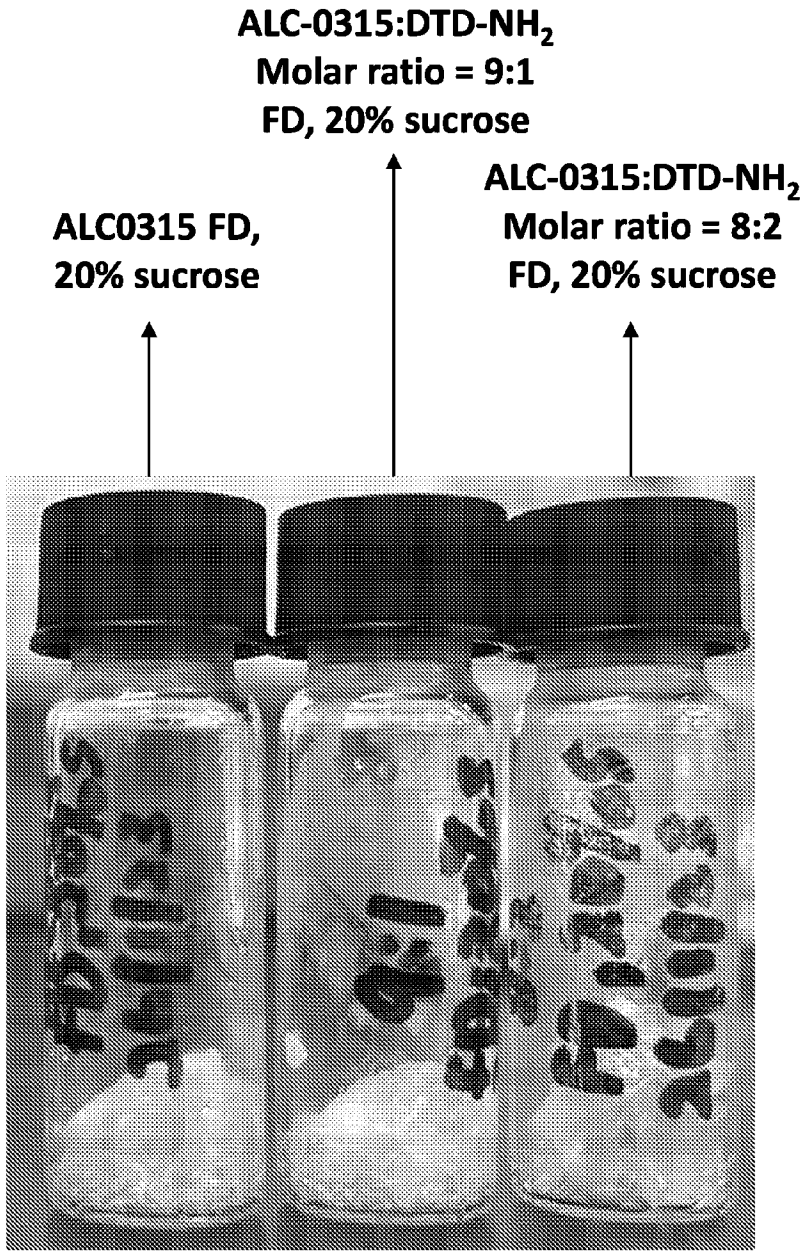

ALC-0315:DTD-NH2
Molar ratio = 9:1
FD, 20% sucrose
ALC0315 FD,
20% sucrose
ALC-0315:DTD-NH2
Molar ratio = 8:2
FD, 20% sucrose

FIG. 3

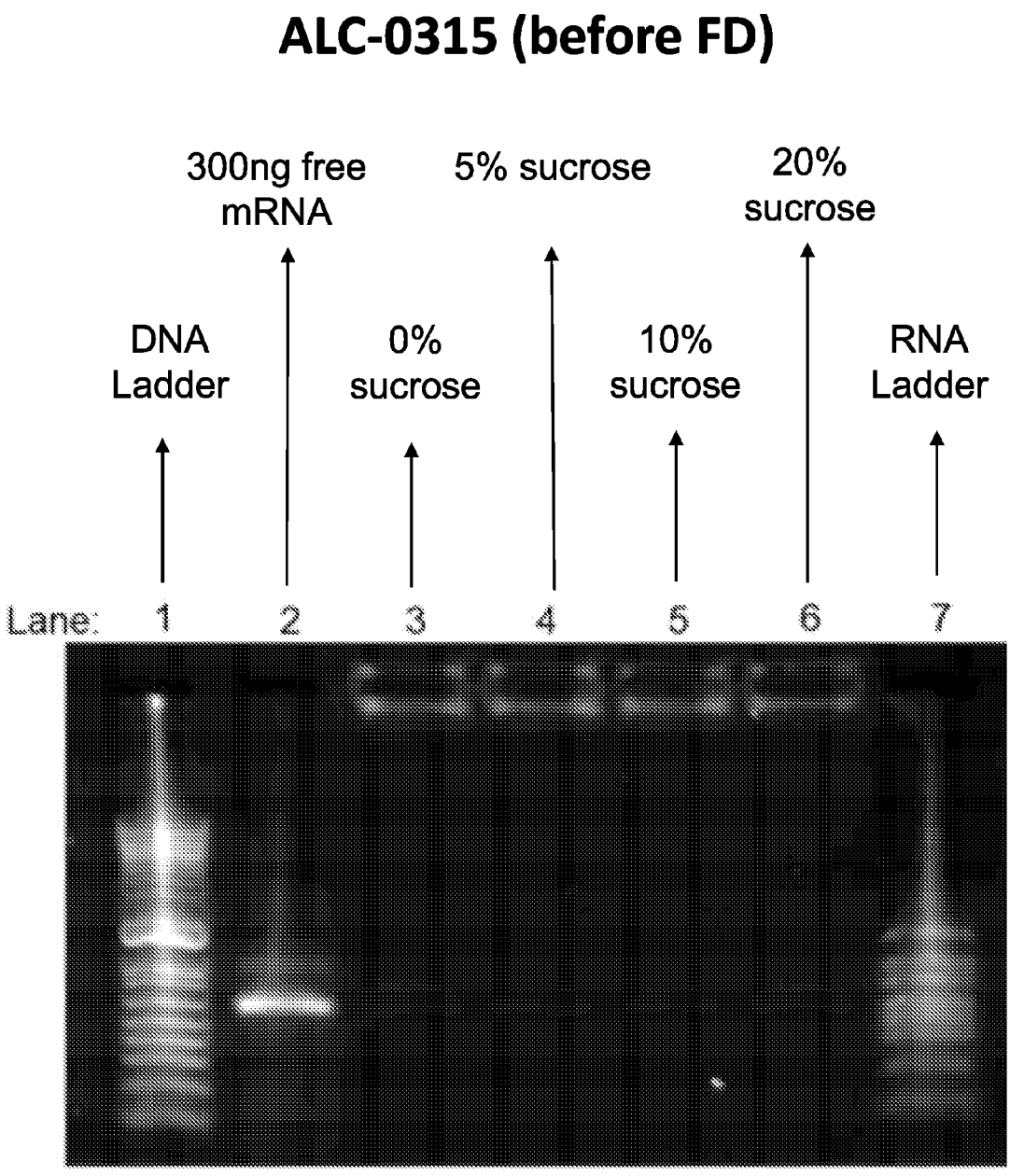

ALC-0315 (before FD)
300ng free mRNA
5% sucrose
20% sucrose
DNA Ladder
0% sucrose
10% sucrose
RNA Ladder
Lane: 1 2 3 4 5 6 7

FIG. 4

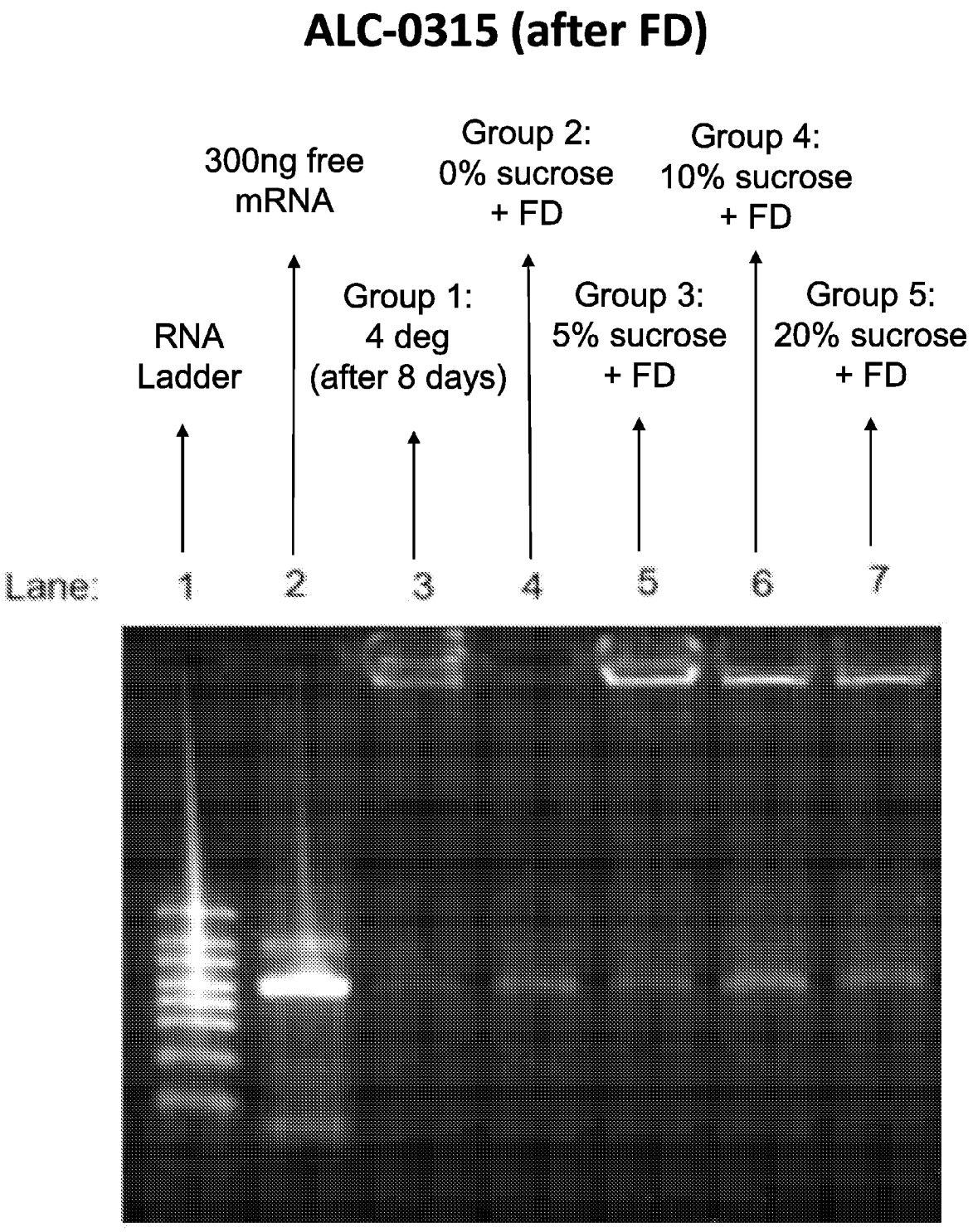

ALC-0315 (after FD)
RNA Ladder
300ng free mRNA
Group 1: 4 deg (after 8 days)
Group 2: 0% sucrose + FD
Group 3: 5% sucrose + FD
Group 4: 10% sucrose + FD
Group 5: 20% sucrose + FD
Lane:
1
2
3
4
5
6
7

FIG. 5

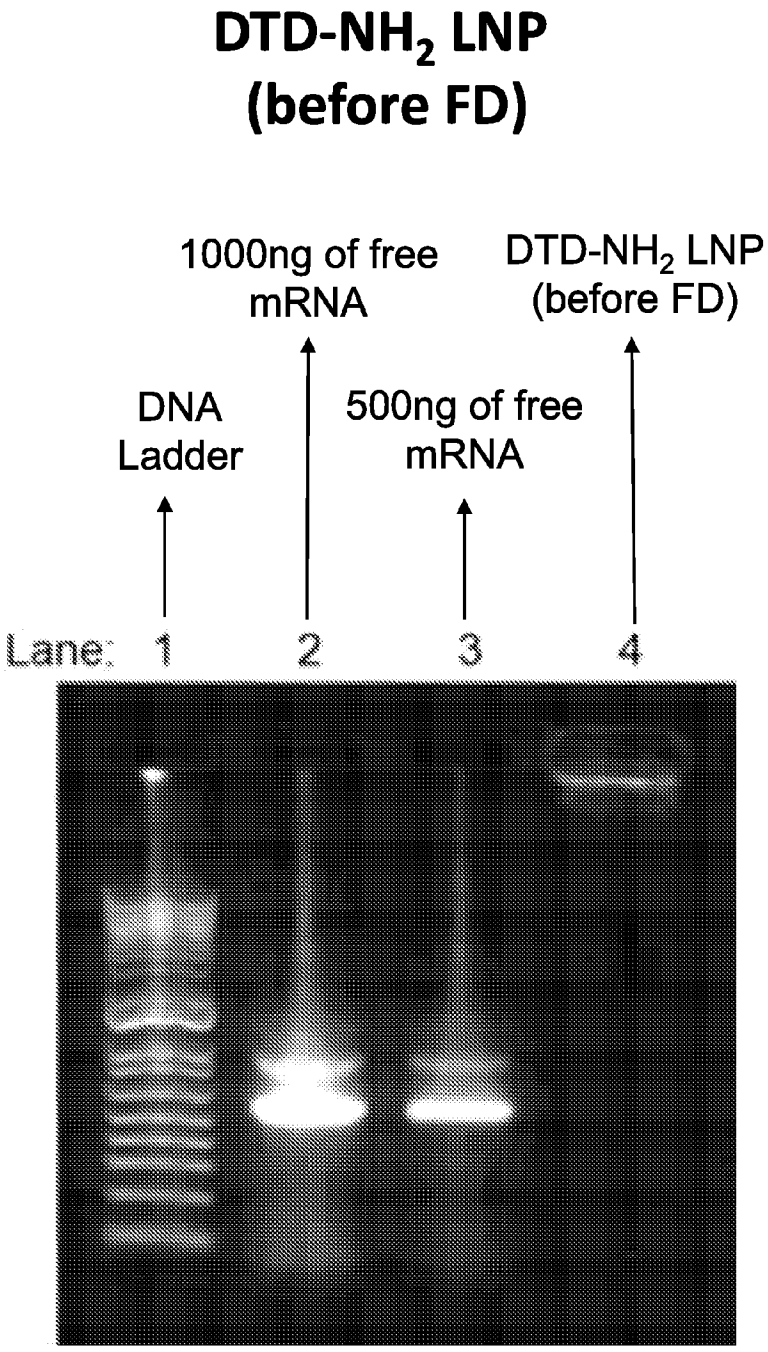

DTD-NH2 LNP
(before FD)
1000ng of free
mRNA
DTD-NH2 LNP
(before FD)
DNA
Ladder
500ng of free
mRNA
Lane: 1
2
3
4

FIG. 6

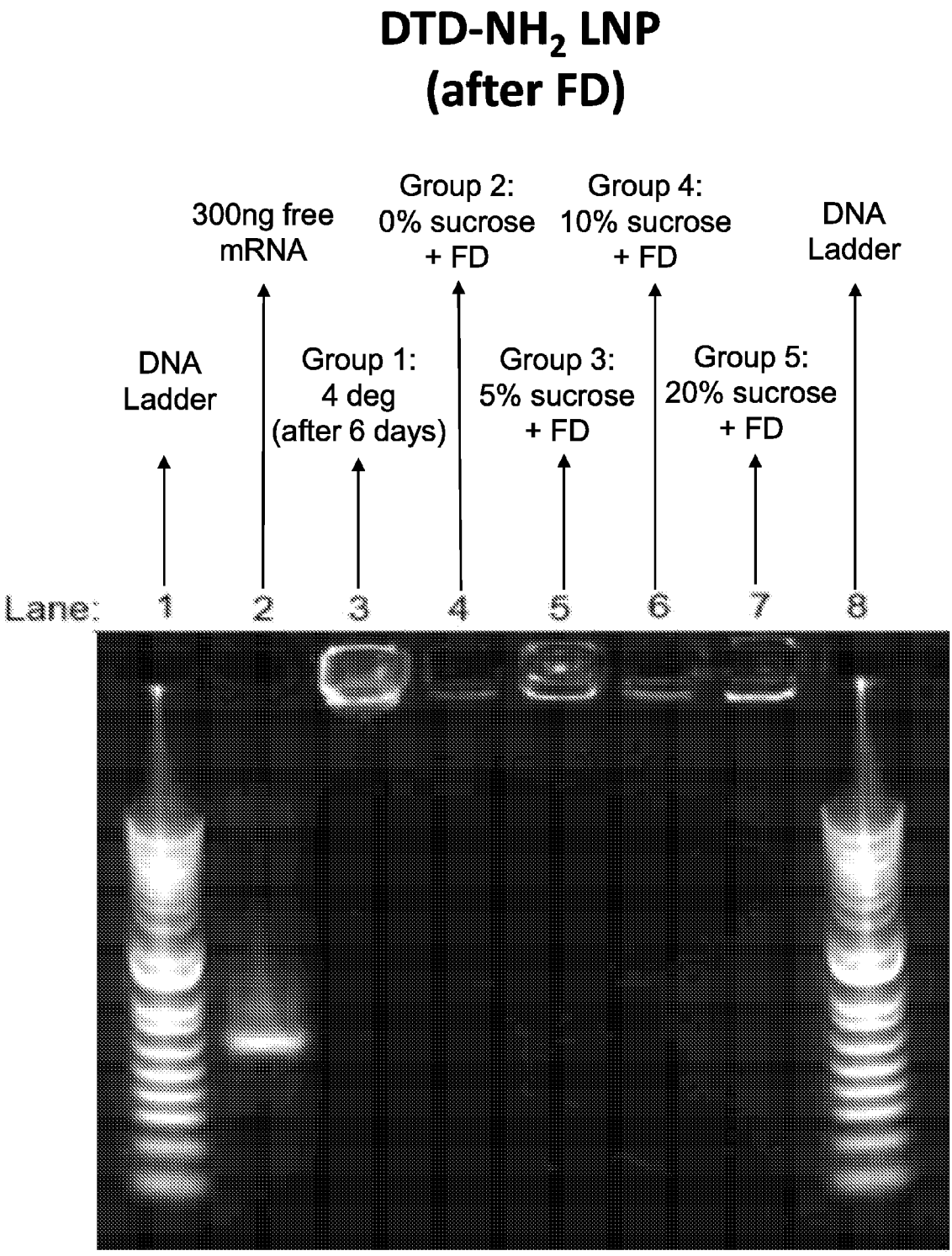

DTD-NH2 LNP
(after FD)
DNA Ladder
300ng free mRNA
Group 1: 4 deg (after 6 days)
Group 2: 0% sucrose + FD
Group 3: 5% sucrose + FD
Group 4: 10% sucrose + FD
Group 5: 20% sucrose + FD
DNA Ladder
Lane: 1 2 3 4 5 6 7 8

FIG. 7

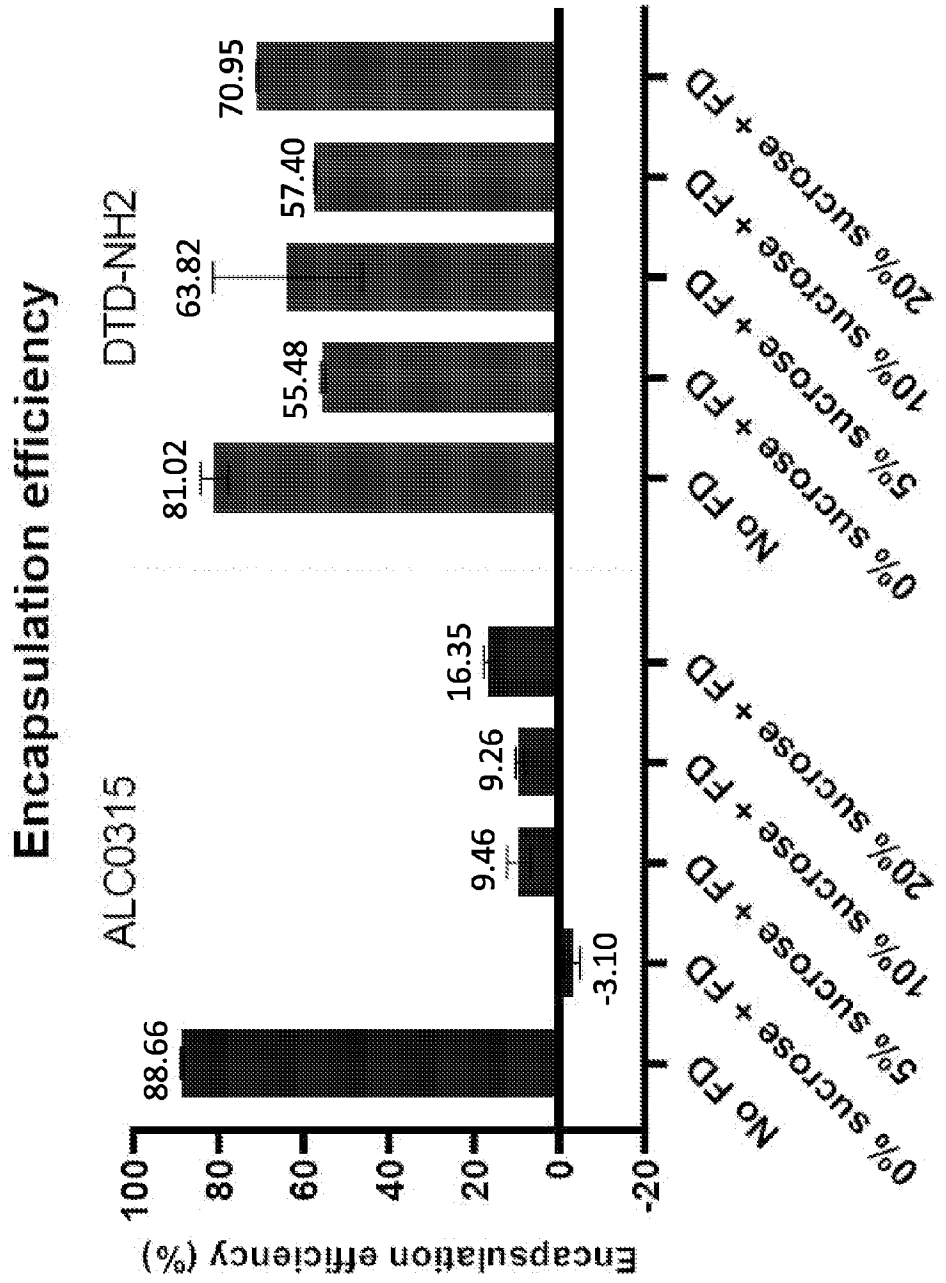

Encapsulation efficiency
ALC0315
DTD-NH2
Encapsulation efficiency (%)
100
80
60
40
20
0
-20
88.66
-3.10
9.46
9.26
16.35
81.02
55.48
63.82
57.40
70.95
No FD
0% sucrose + FD
5% sucrose + FD
10% sucrose + FD
20% sucrose + FD
No FD
0% sucrose + FD
5% sucrose + FD
10% sucrose + FD
20% sucrose + FD

FIG. 8

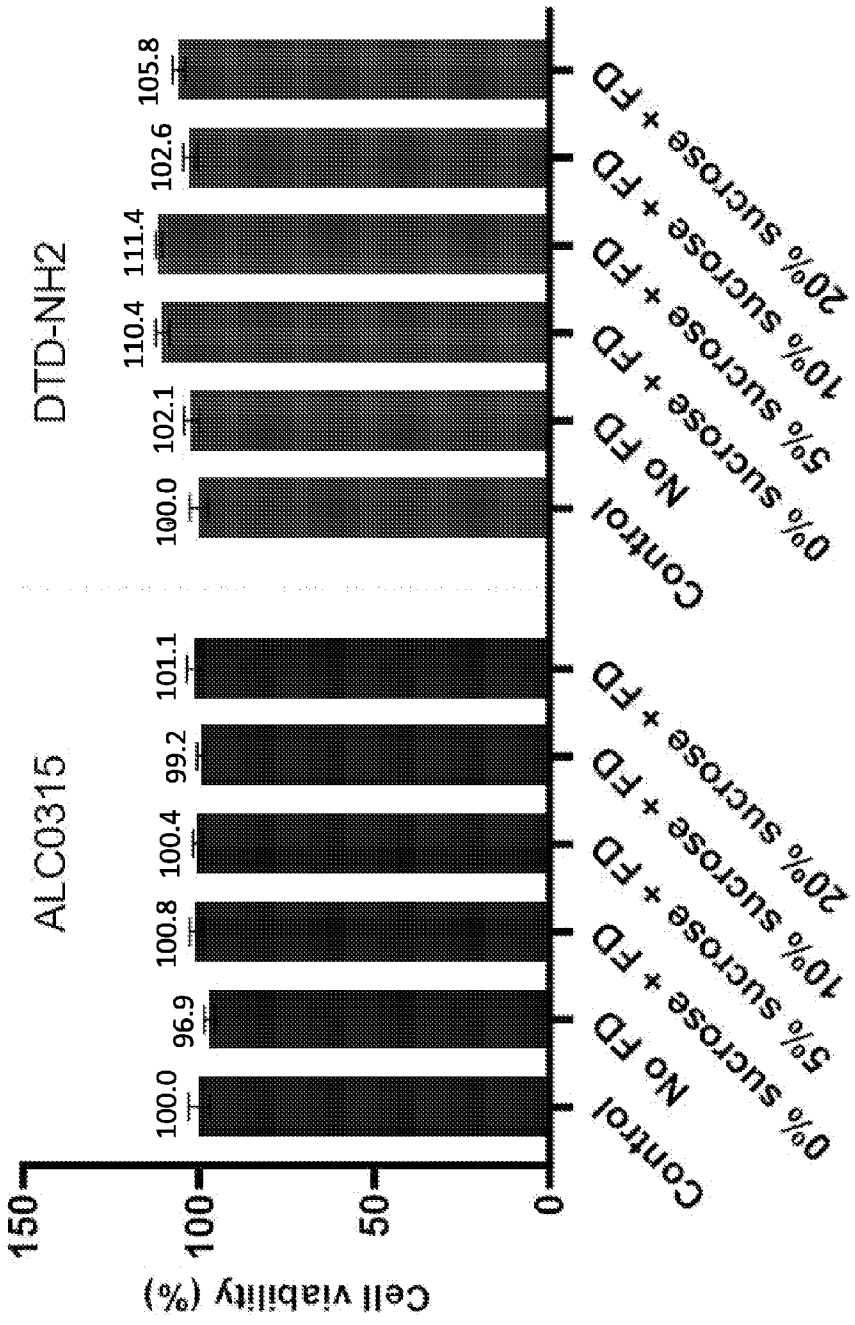

Cell viability
ALC0315
DTD-NH2
Cell viability (%)
150
100
50
0
100.0
96.9
100.8
100.4
99.2
101.1
100.0
102.1
110.4
111.4
102.6
105.8
Control
No FD
0% sucrose + FD
5% sucrose + FD
10% sucrose + FD
20% sucrose + FD
Control
No FD
0% sucrose + FD
5% sucrose + FD
10% sucrose + FD
20% sucrose + FD

FIG. 9

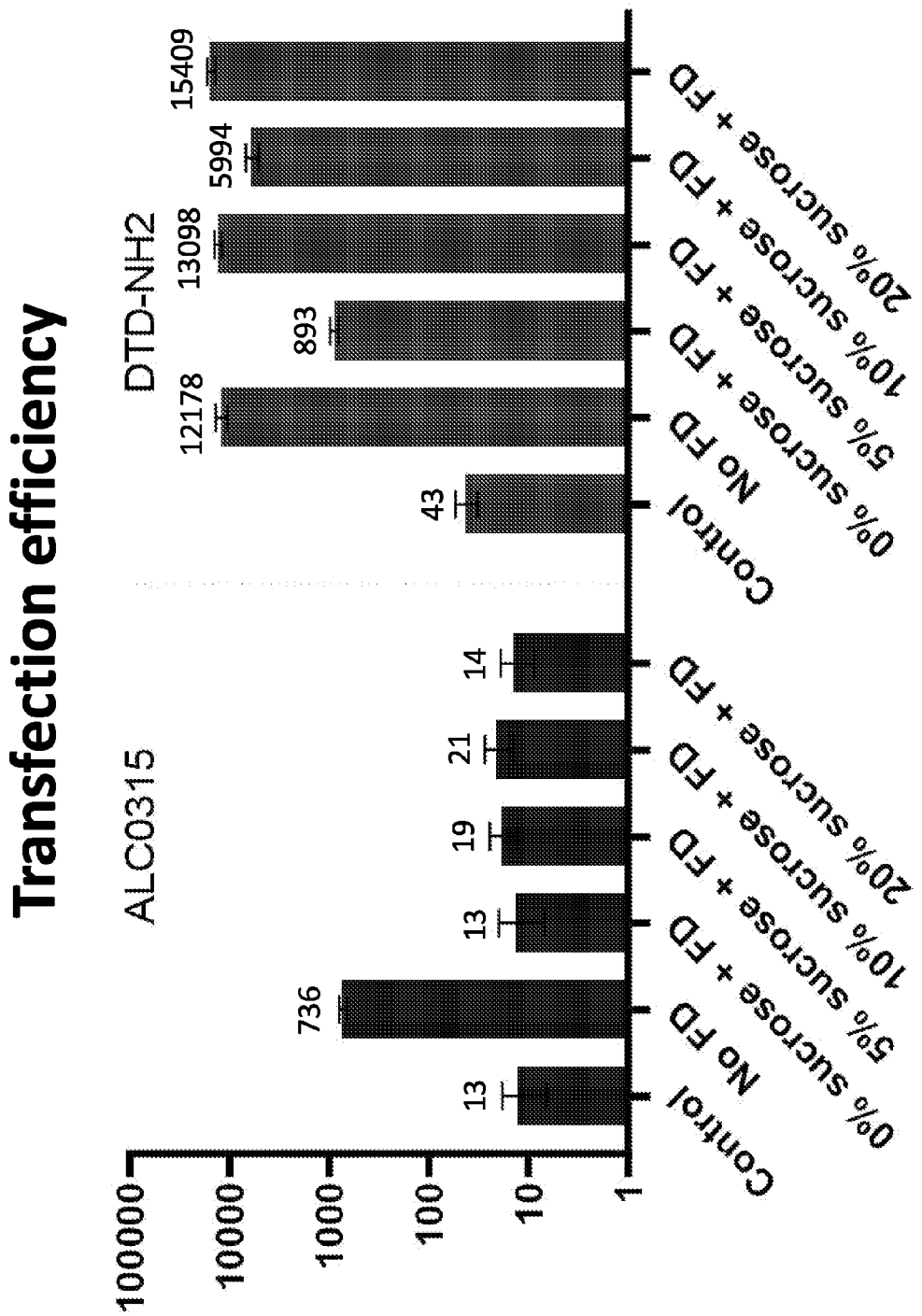

Transfection efficiency
ALC0315
DTD-NH2
100000
10000
1000
100
10
1
13
736
13
19
21
14
43
12178
893
13098
5994
15409
Control
No FD
5% sucrose + FD
10% sucrose + FD
20% sucrose + FD
0% sucrose + FD
Control
No FD
5% sucrose + FD
10% sucrose + FD
20% sucrose + FD
0% sucrose + FD

FIG. 10

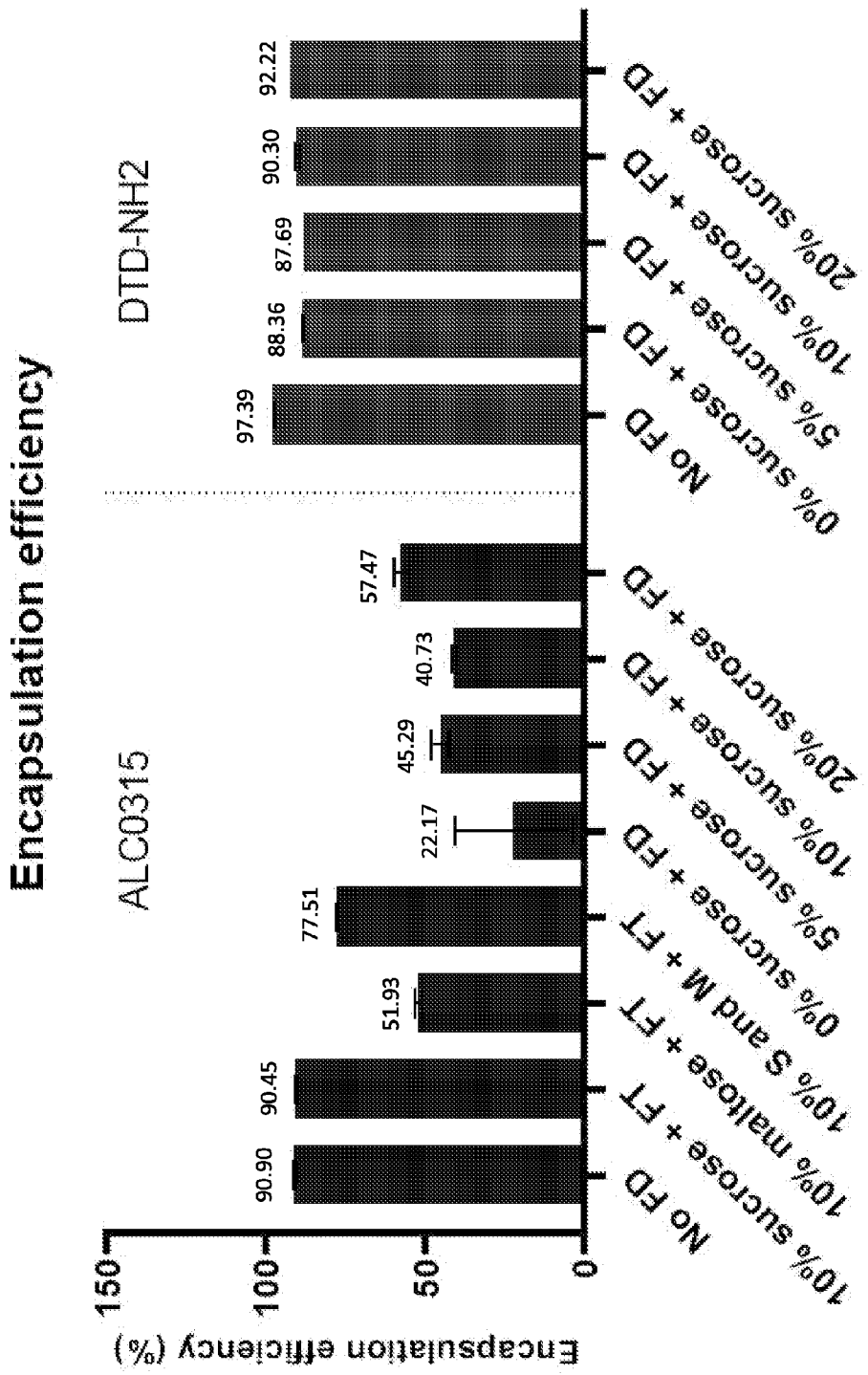

Encapsulation efficiency
ALC0315
DTD-NH2
Encapsulation efficiency (%)
150
100
50
0
90.90
90.45
51.93
77.51
22.17
45.29
40.73
57.47
97.39
88.36
87.69
90.30
92.22
No FD
10% sucrose + FT
10% maltose + FT
0% 5 and M + FT
0% sucrose + FD
5% sucrose + FD
10% sucrose + FD
20% sucrose + FD
No FD
0% sucrose + FD
5% sucrose + FD
10% sucrose + FD
20% sucrose + FD

FIG. 11

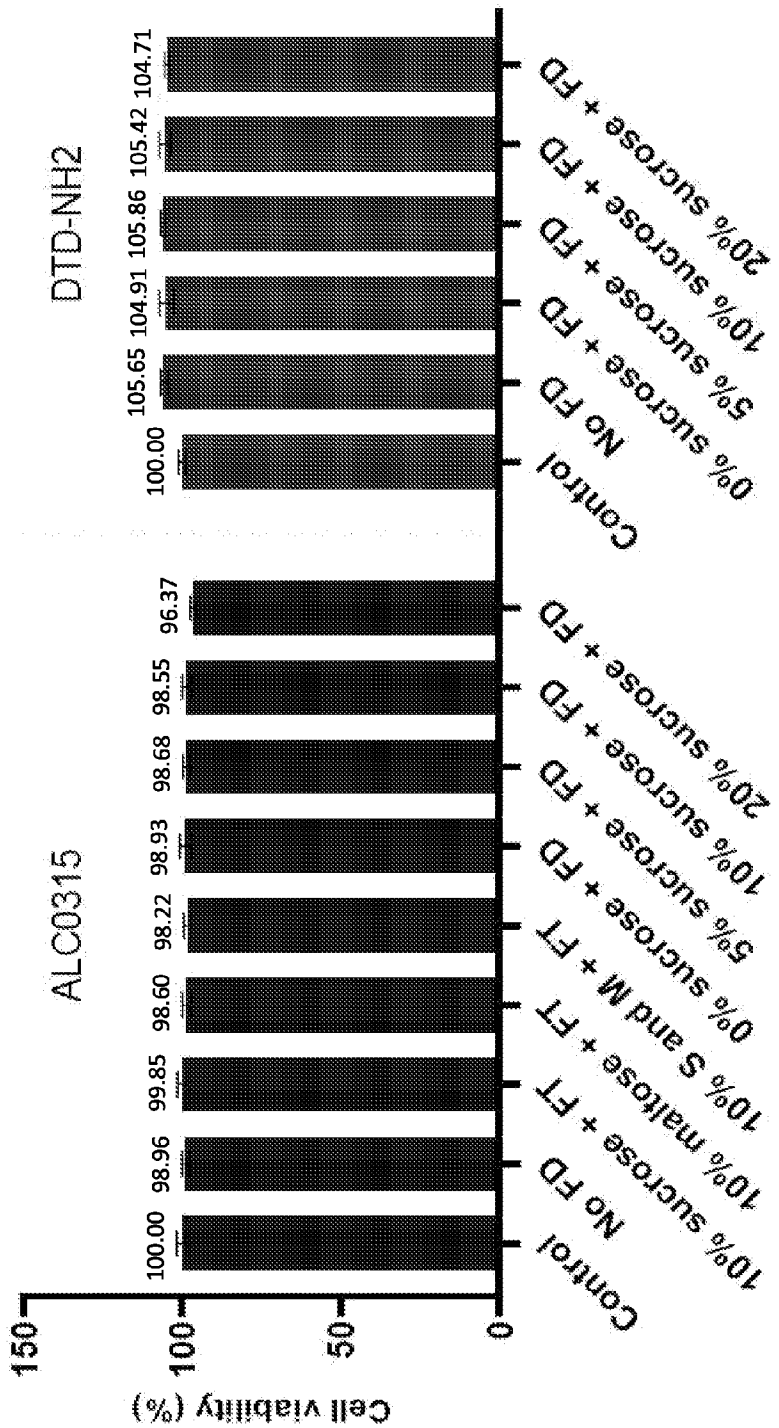

Cell viability
ALC0315
DTD-NH2
Cell viability (%)
150
100
50
0
100.00
98.96
99.85
98.60
98.22
98.93
98.68
98.55
96.37
105.65
104.91
105.86
105.42
104.71
Control
No FD
10% sucrose + FT
10% maltose + FT
10% S and M + FT
0% sucrose + FD
5% sucrose + FD
10% sucrose + FD
20% sucrose + FD
Control
No FD
0% sucrose + FD
5% sucrose + FD
10% sucrose + FD
20% sucrose + FD

FIG. 12

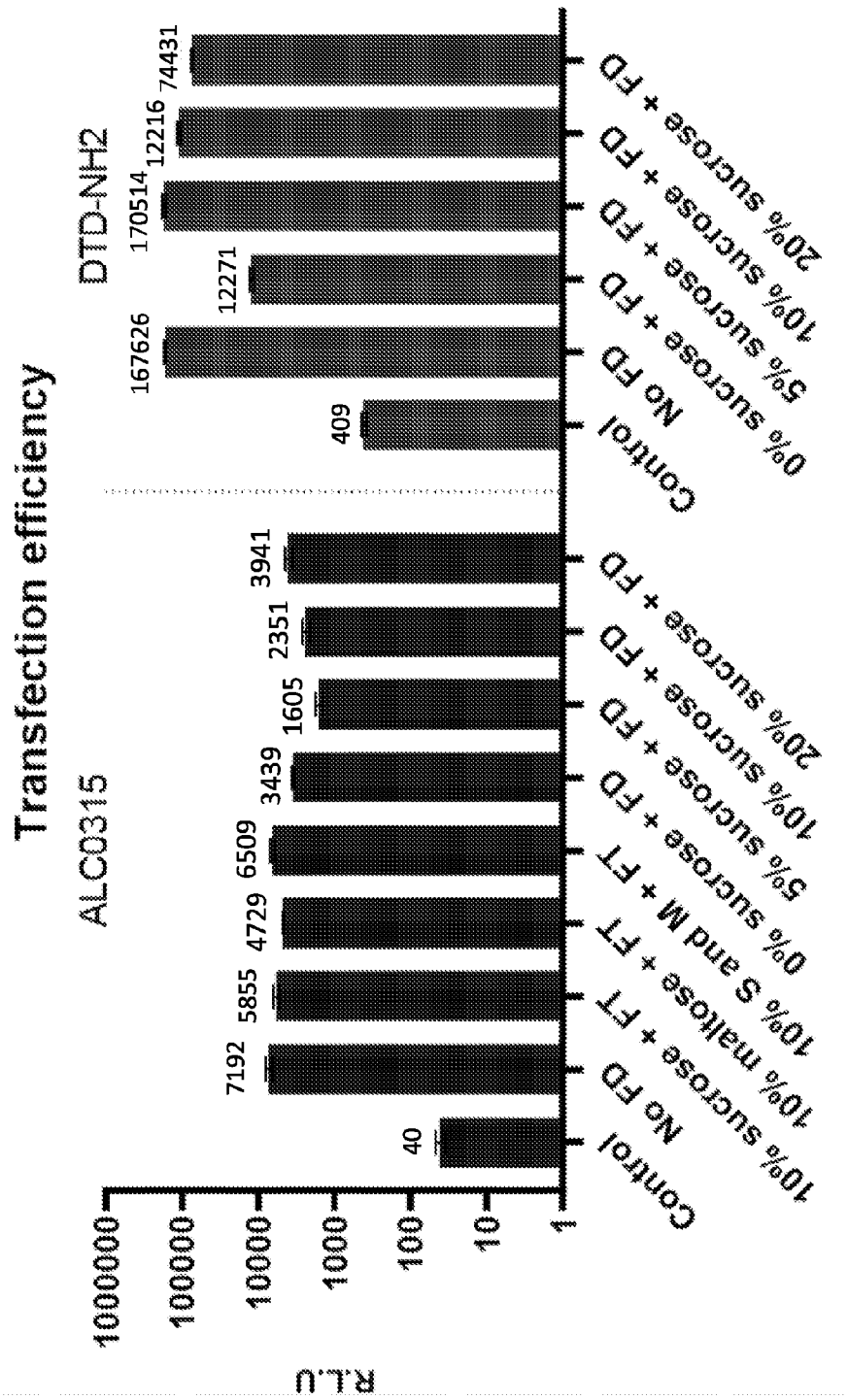

Transfection efficiency
ALC0315
DTD-NH2
R.L.U
1000000
100000
10000
1000
100
10
1
40
7192
5855
4729
6509
3439
1605
2351
3941
409
167626
12271
170514
12216
74431
Control
No FD
10% sucrose + FT
10% maltose + FT
0% S and M + FT
5% sucrose + FD
10% sucrose + FD
20% sucrose + FD
Control
No FD
0% sucrose + FD
5% sucrose + FD
10% sucrose + FD
20% sucrose + FD

FIG. 13

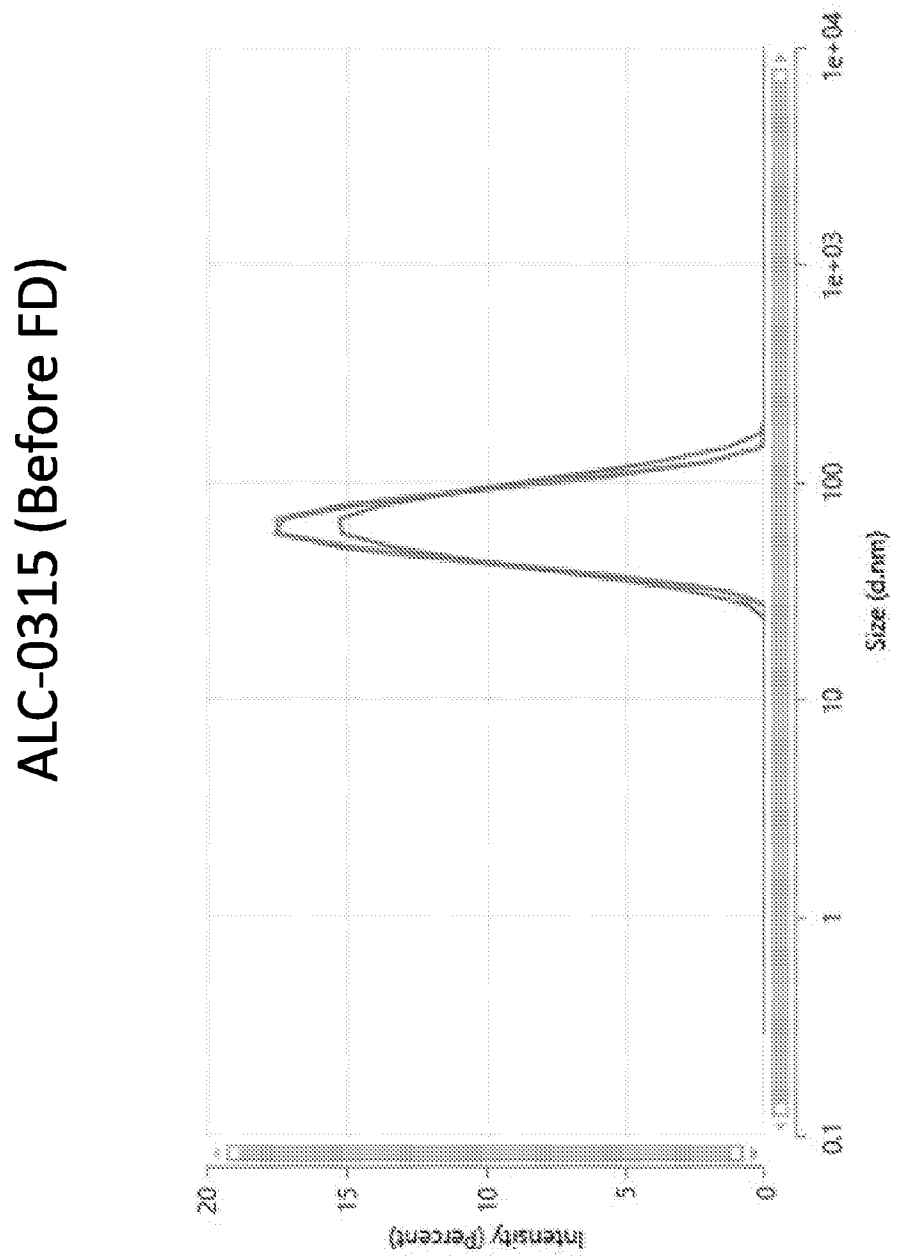

ALC-0315 (Before FD)
Intensity (Percent)
20
15
10
5
0
0.1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG. 14

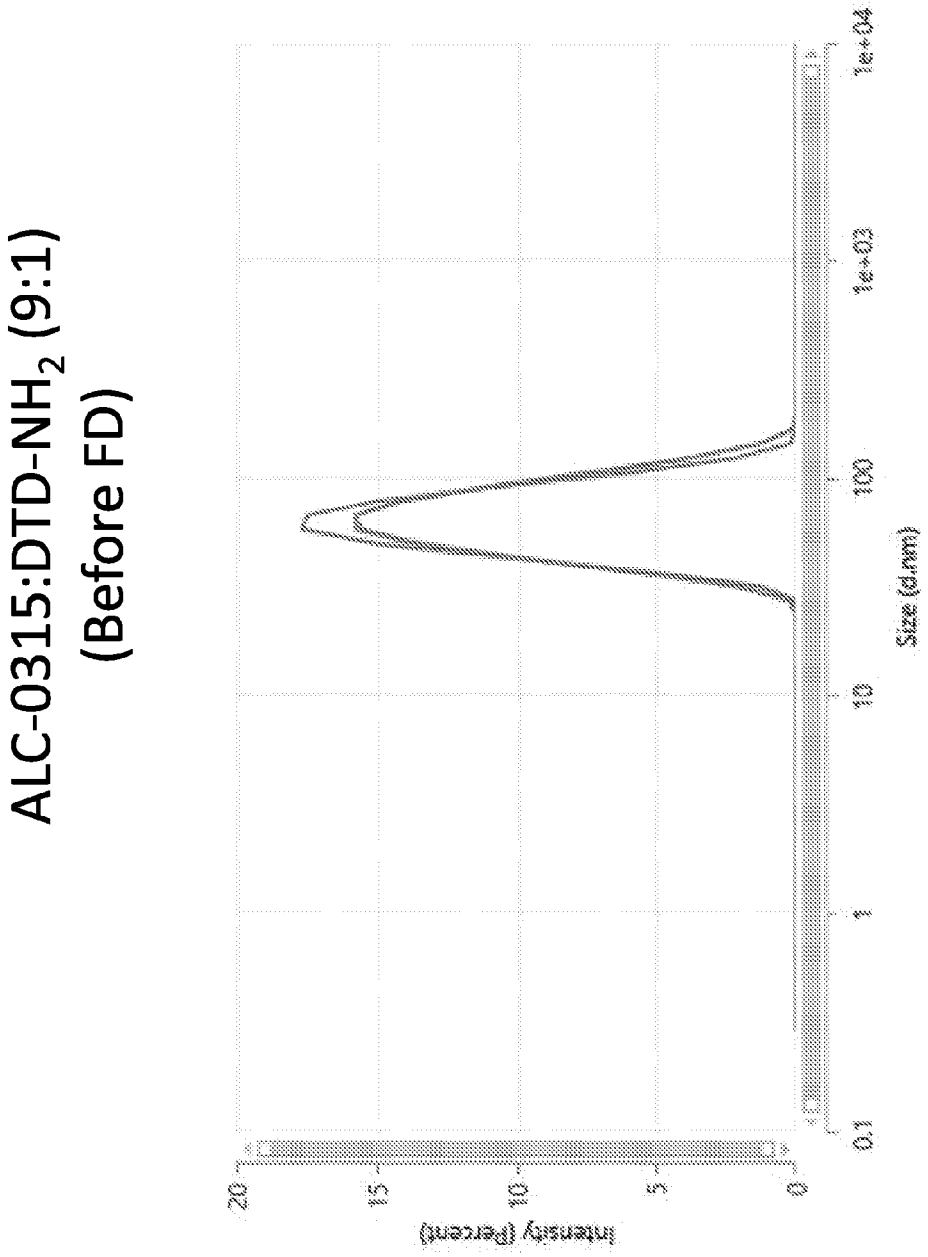

ALC-0315:DTD-NH2 (9:1)
(Before FD)
Intensity (Percent)
0
5
10
15
20
Size (d.nm)
0.1
1
10
100
1e+03
1e+04

FIG. 15

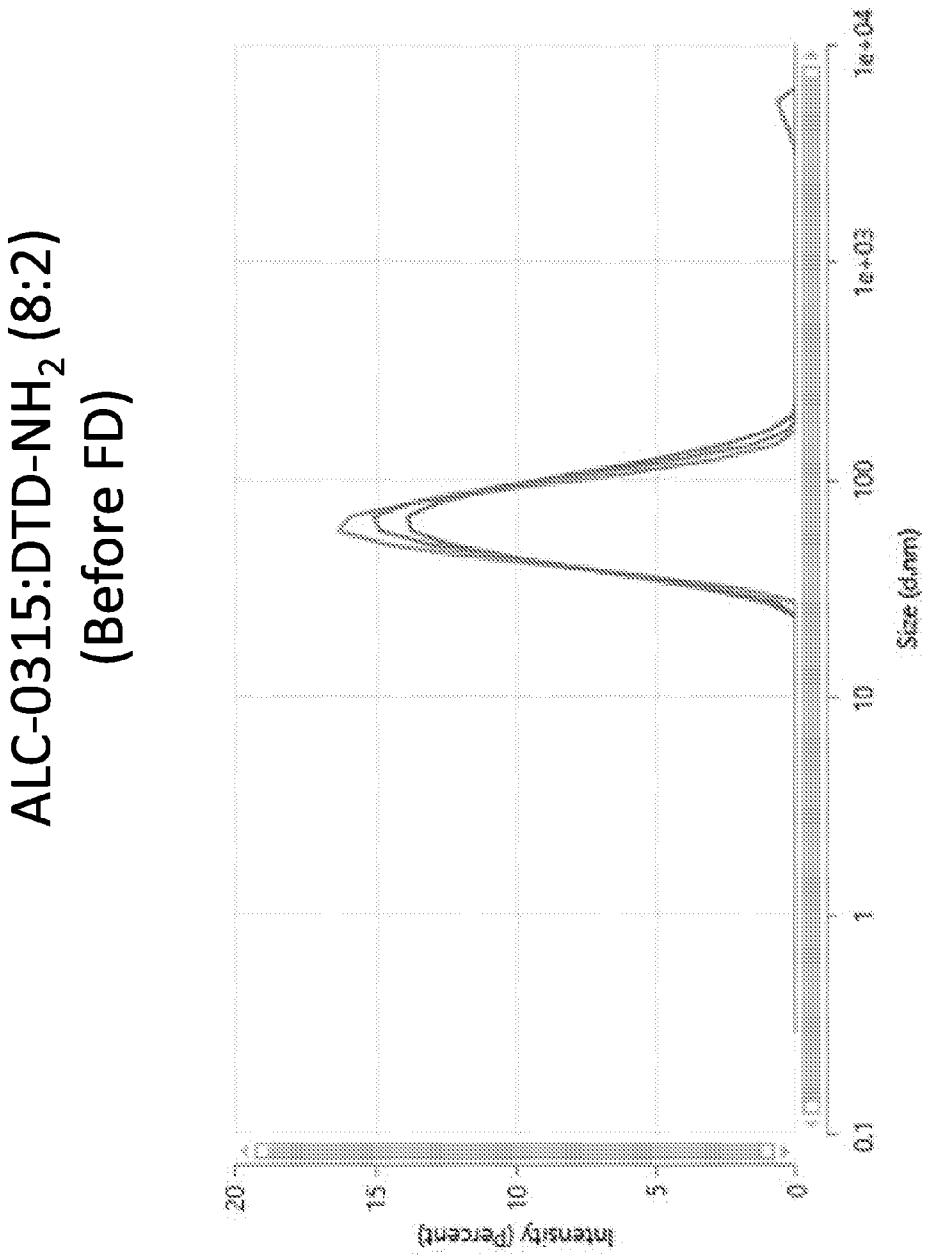

ALC-0315:DTD-NH2 (8:2)
(Before FD)
Intensity (Percent)
0
5
10
15
20
Size (d.nm)
0.1
1
10
100
1e+03
1e+04

FIG. 16

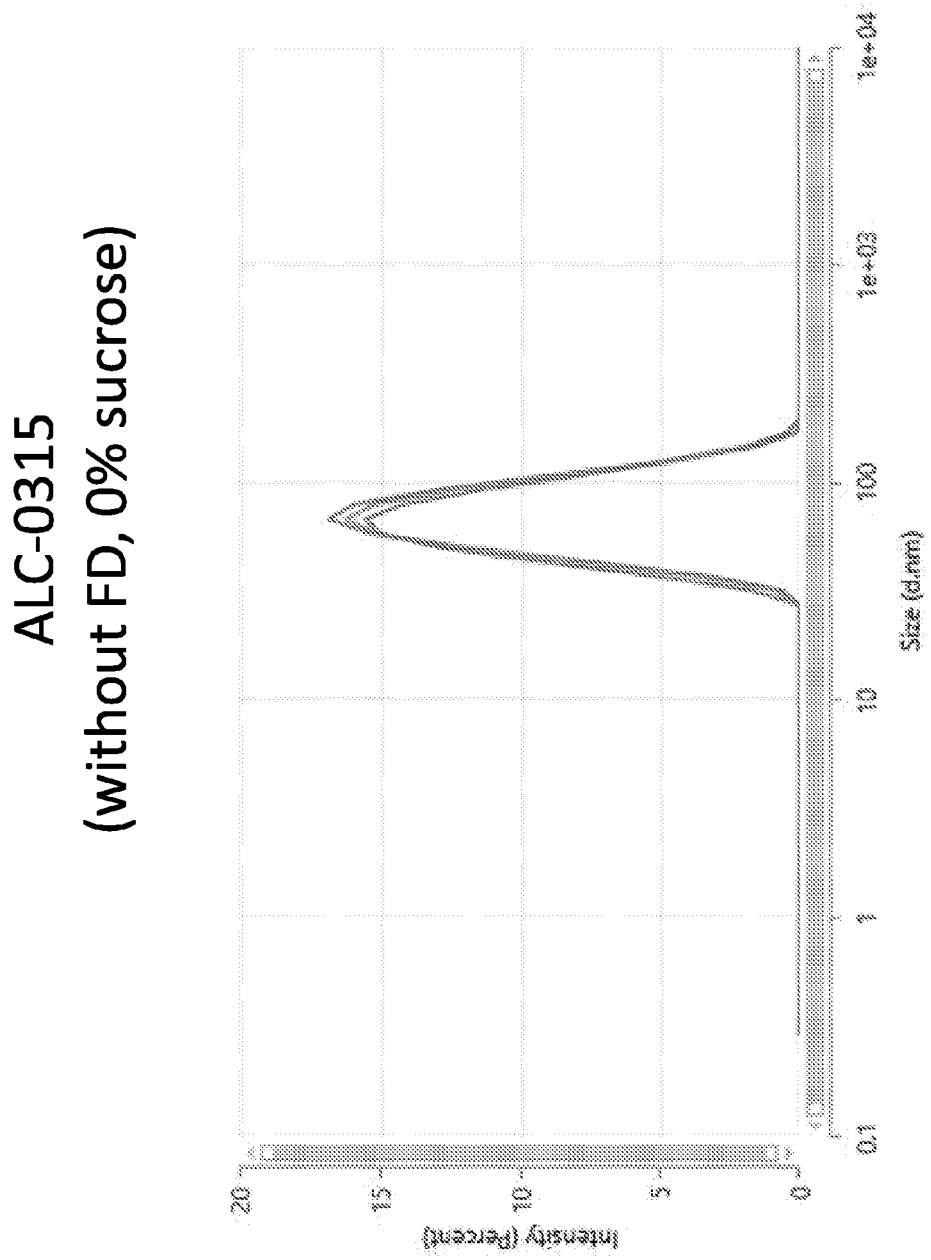

ALC-0315
(without FD, 0% sucrose)
Intensity (Percent)
0
5
10
15
20
Size (d.nm)
0.1
1
10
100
1e+03
1e+04

FIG. 17

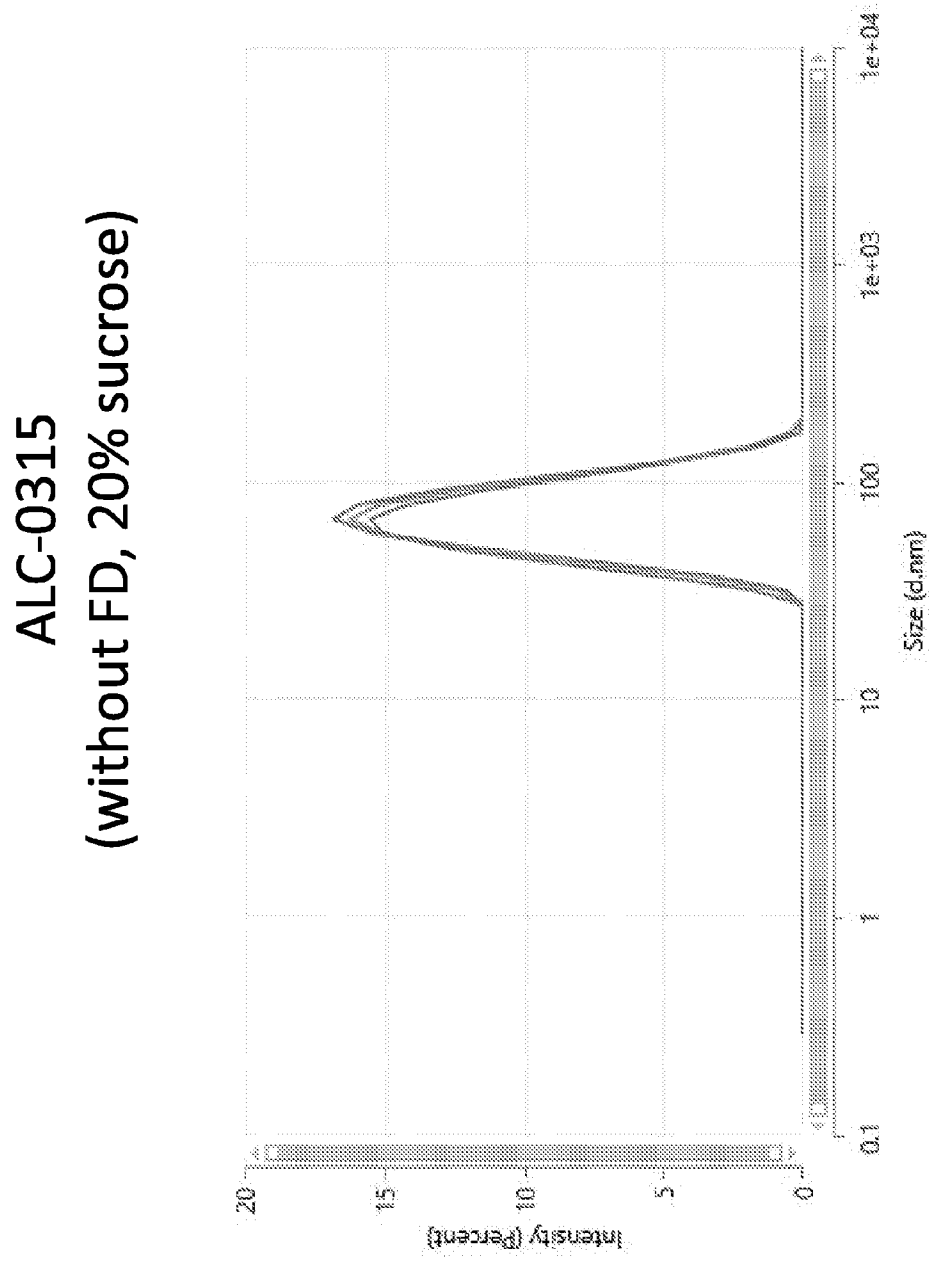

ALC-0315
(without FD, 20% sucrose)
Intensity (Percent)
20
15
10
5
0
0.1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG. 18

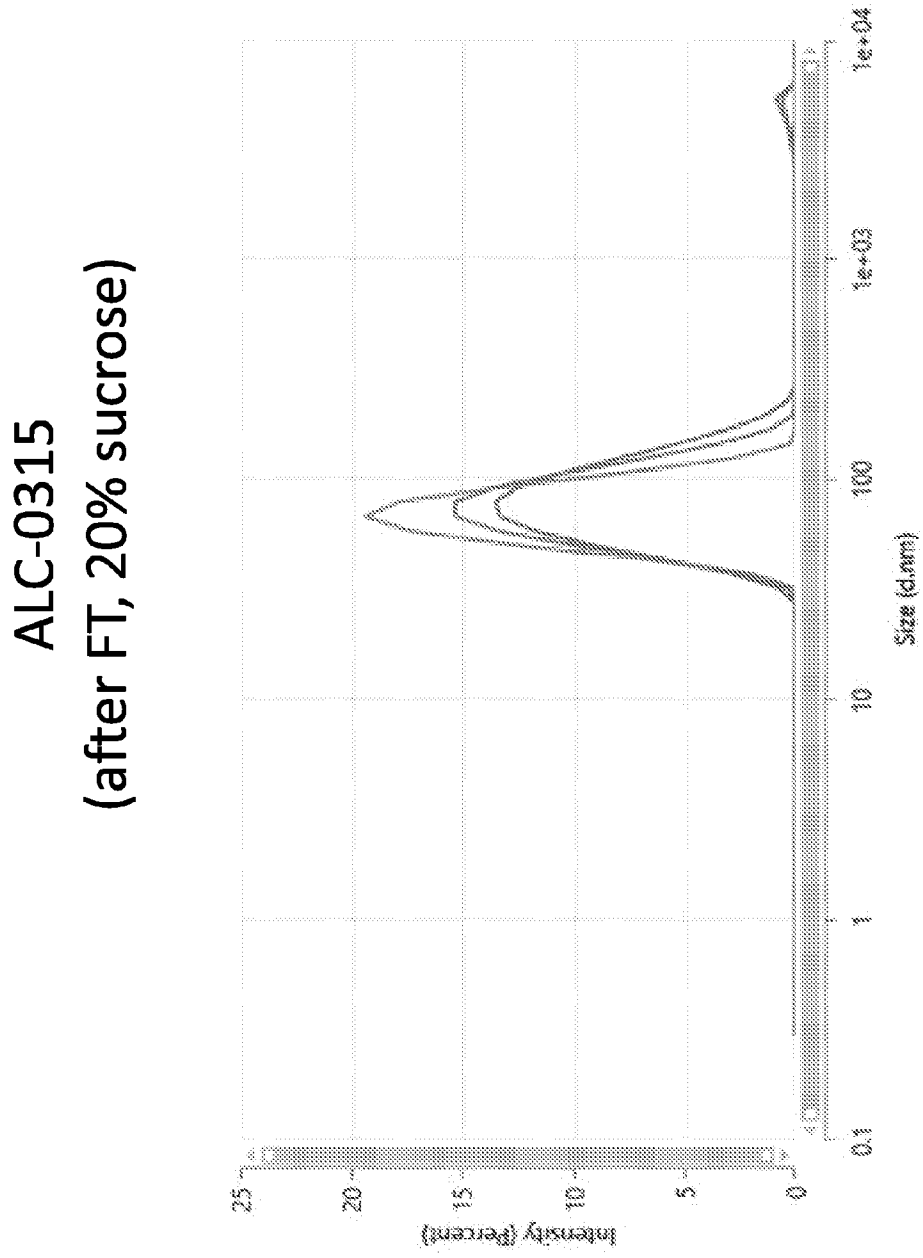

ALC-0315
(after FT, 20% sucrose)
Intensity (Percent)
25
20
15
10
5
0
0.1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG. 19

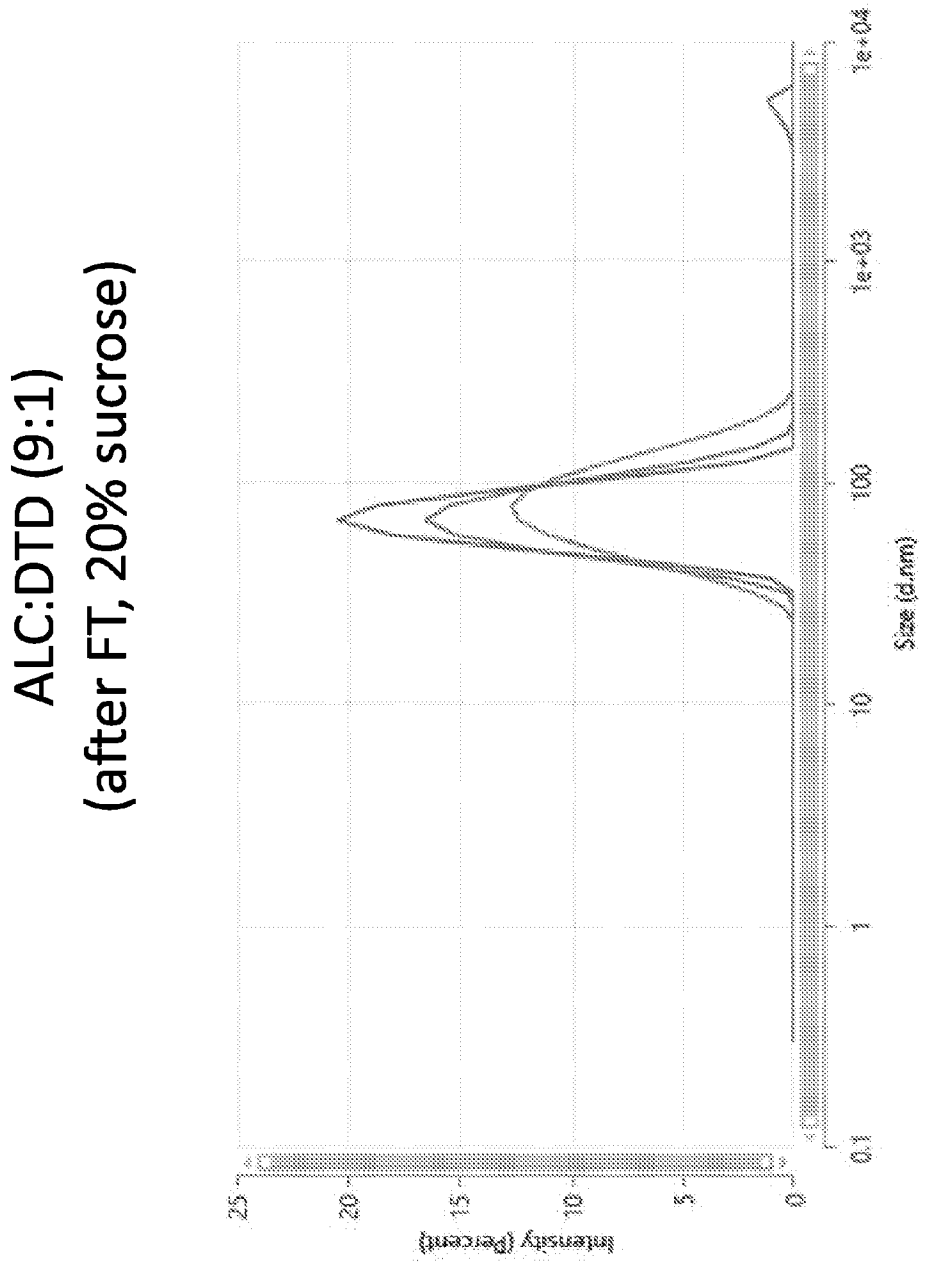

ALC:DTD (9:1)
(after FT, 20% sucrose)
Intensity (Percent)
25
20
15
10
5
0
0.1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG. 20

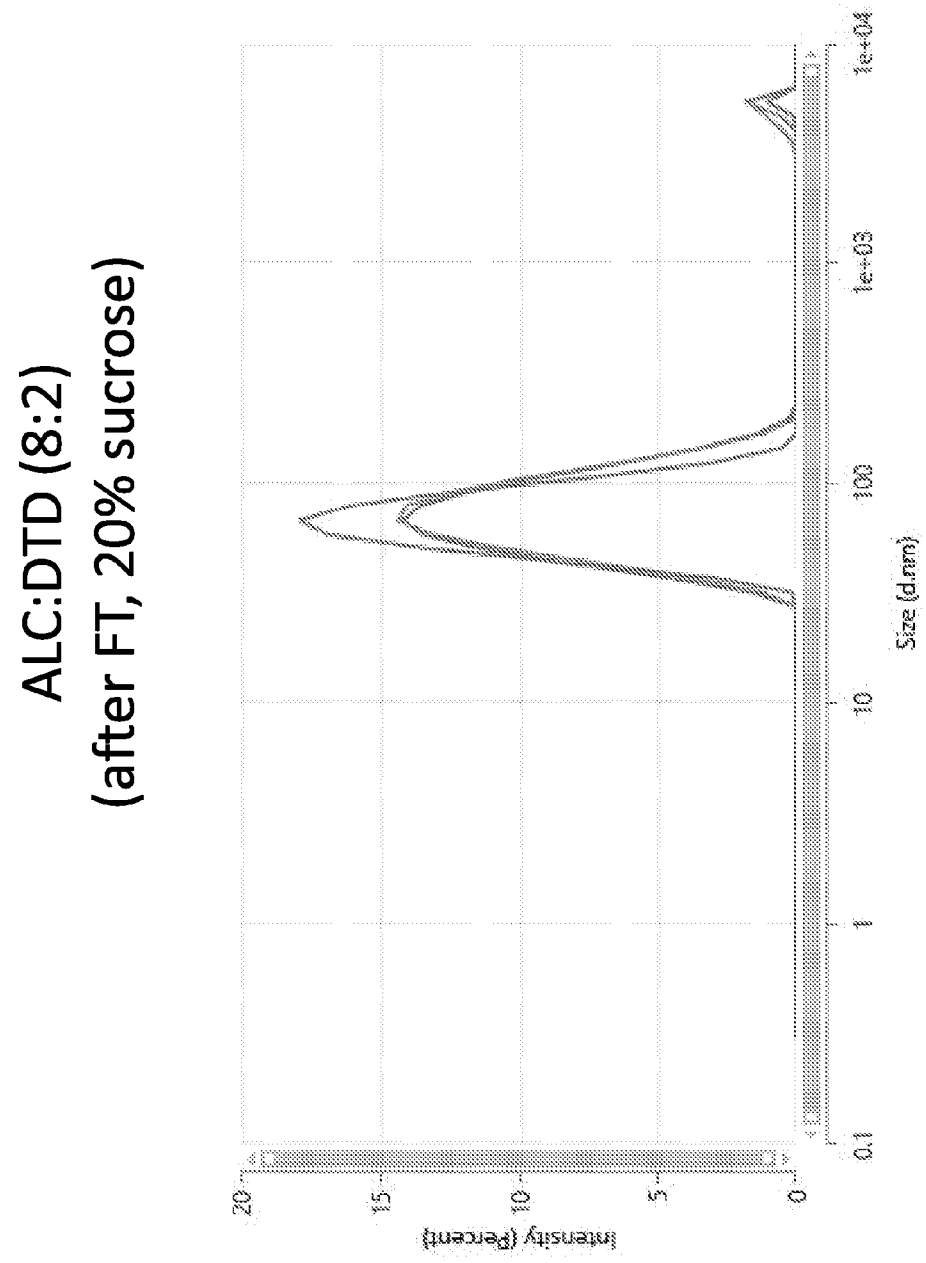

ALC:DTD (8:2)
(after FT, 20% sucrose)
Intensity (Percent)
20
15
10
5
0
0.1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG. 21

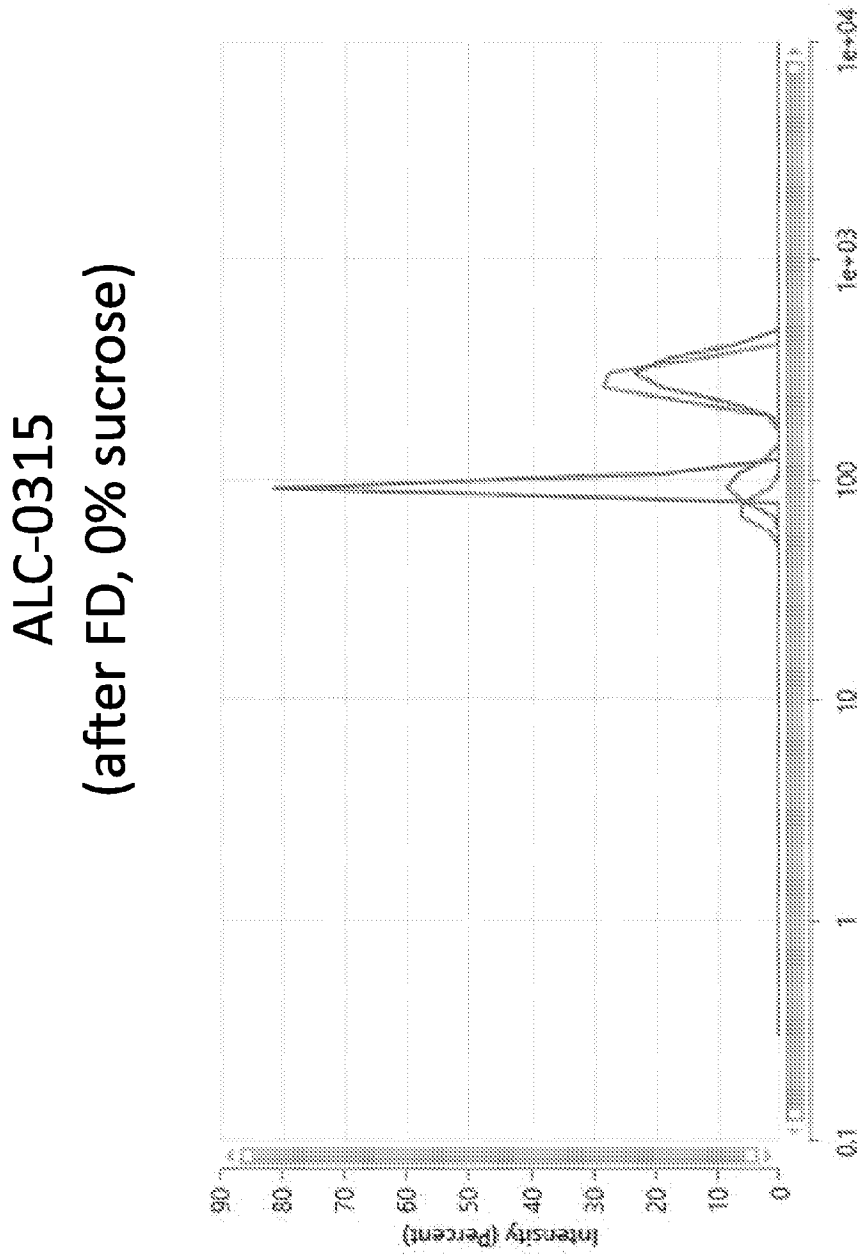

ALC-0315
(after FD, 0% sucrose)
Intensity (Percent)
0
10
20
30
40
50
60
70
80
90
0.1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG. 22

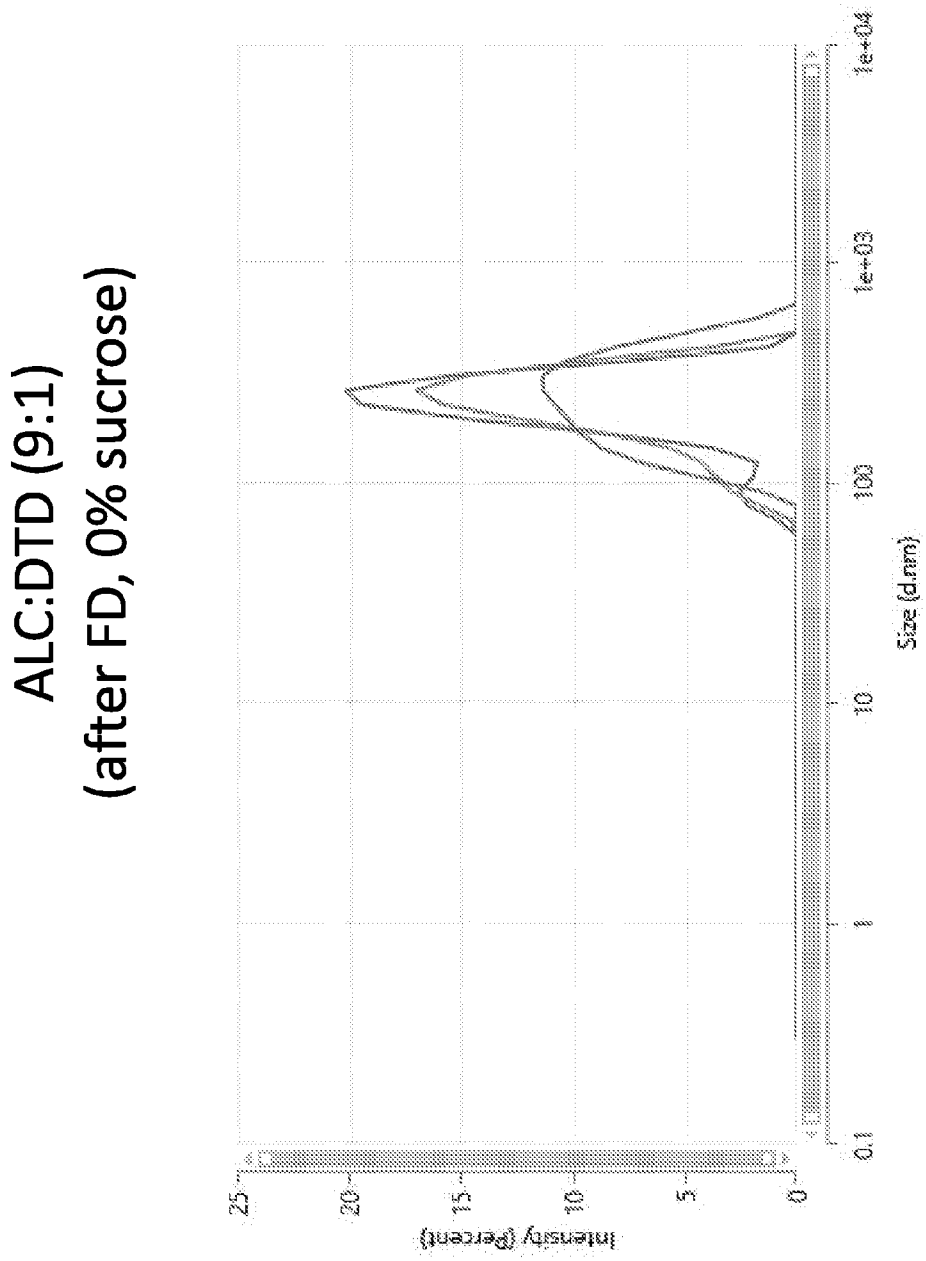

ALC:DTD (9:1)
(after FD, 0% sucrose)
Intensity (Percent)
25
20
15
10
5
0
0.1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG. 23

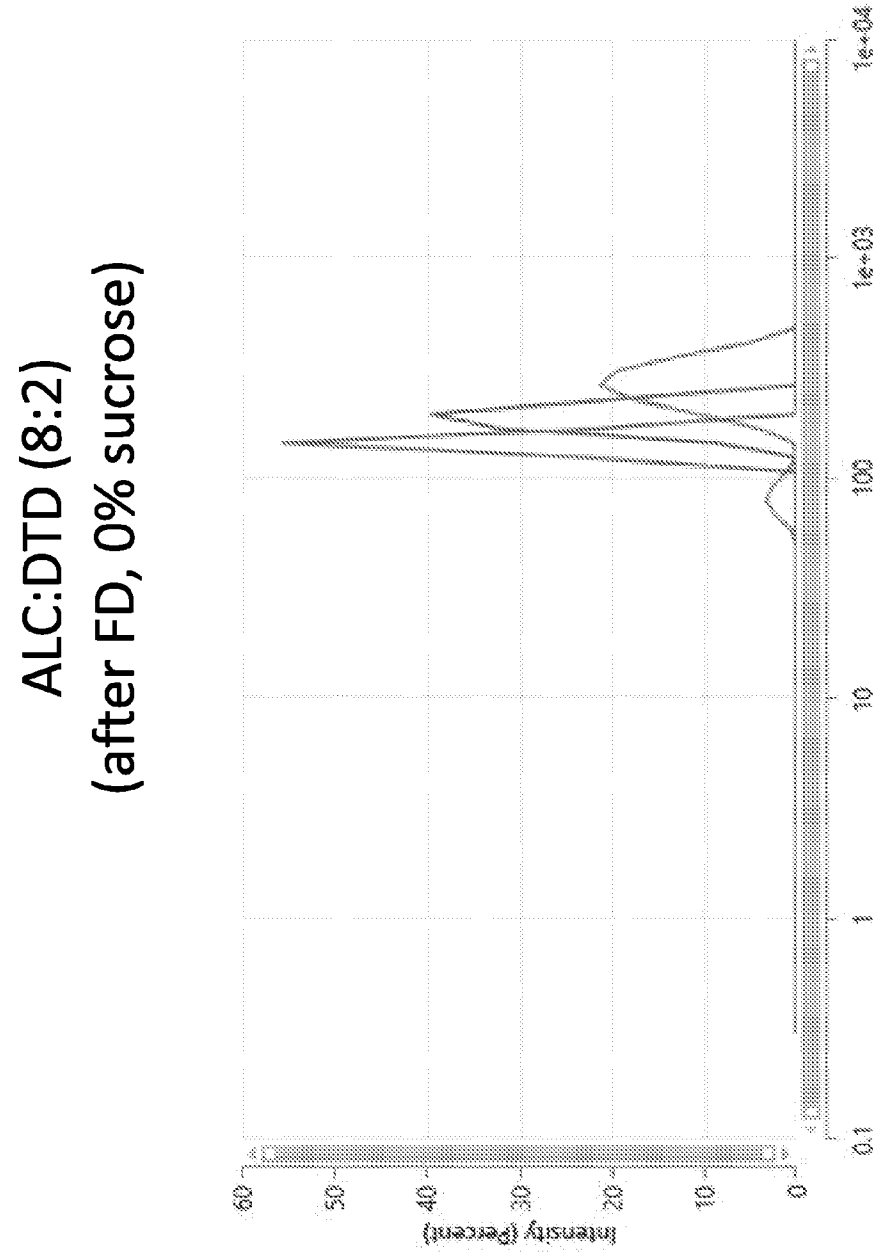

ALC:DTD (8:2)
(after FD, 0% sucrose)
Intensity (Percent)
0
10
20
30
40
50
60
0.1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG. 24

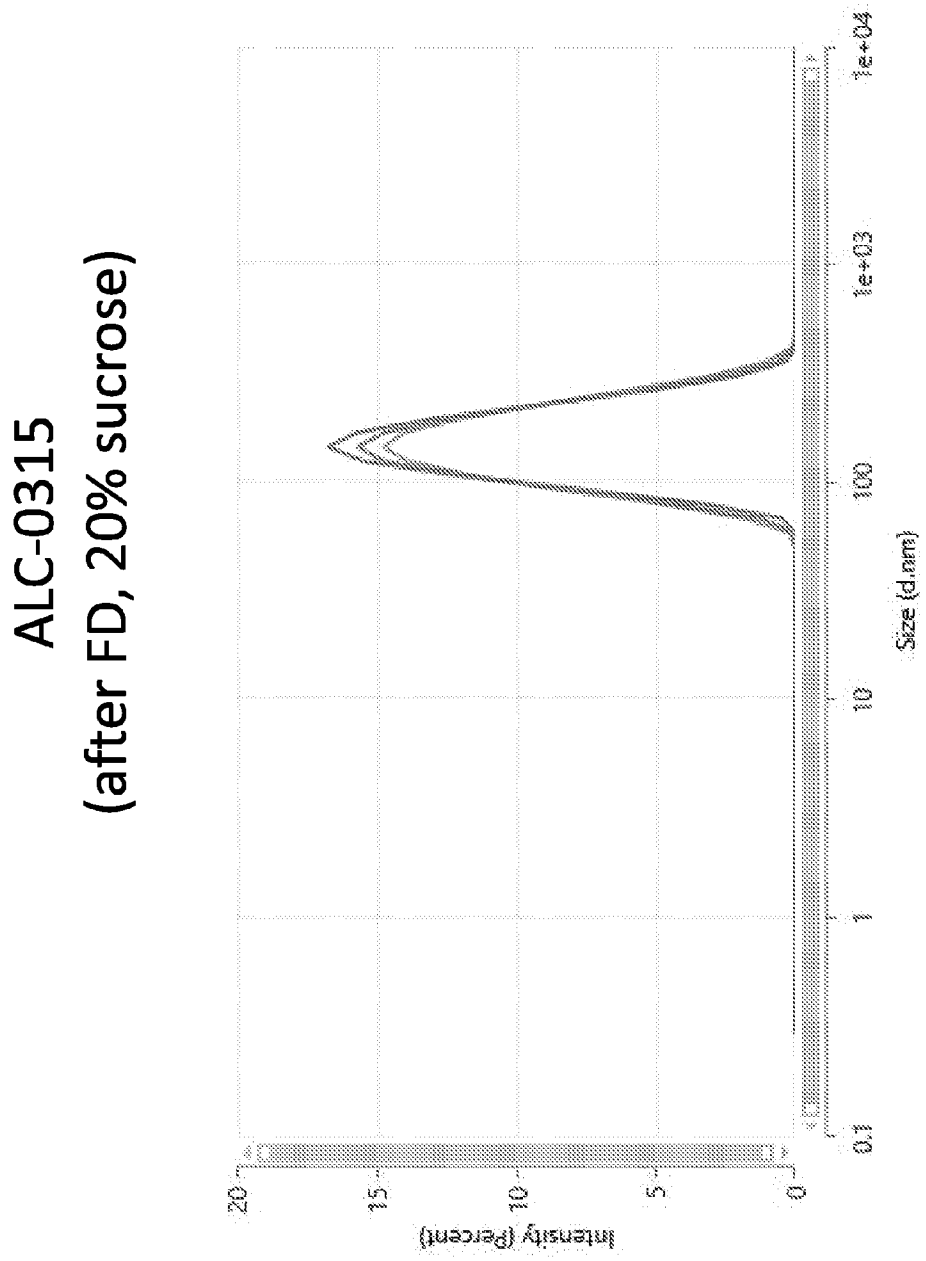

ALC-0315
(after FD, 20% sucrose)
Intensity (Percent)
20
15
10
5
0
0.1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG. 25

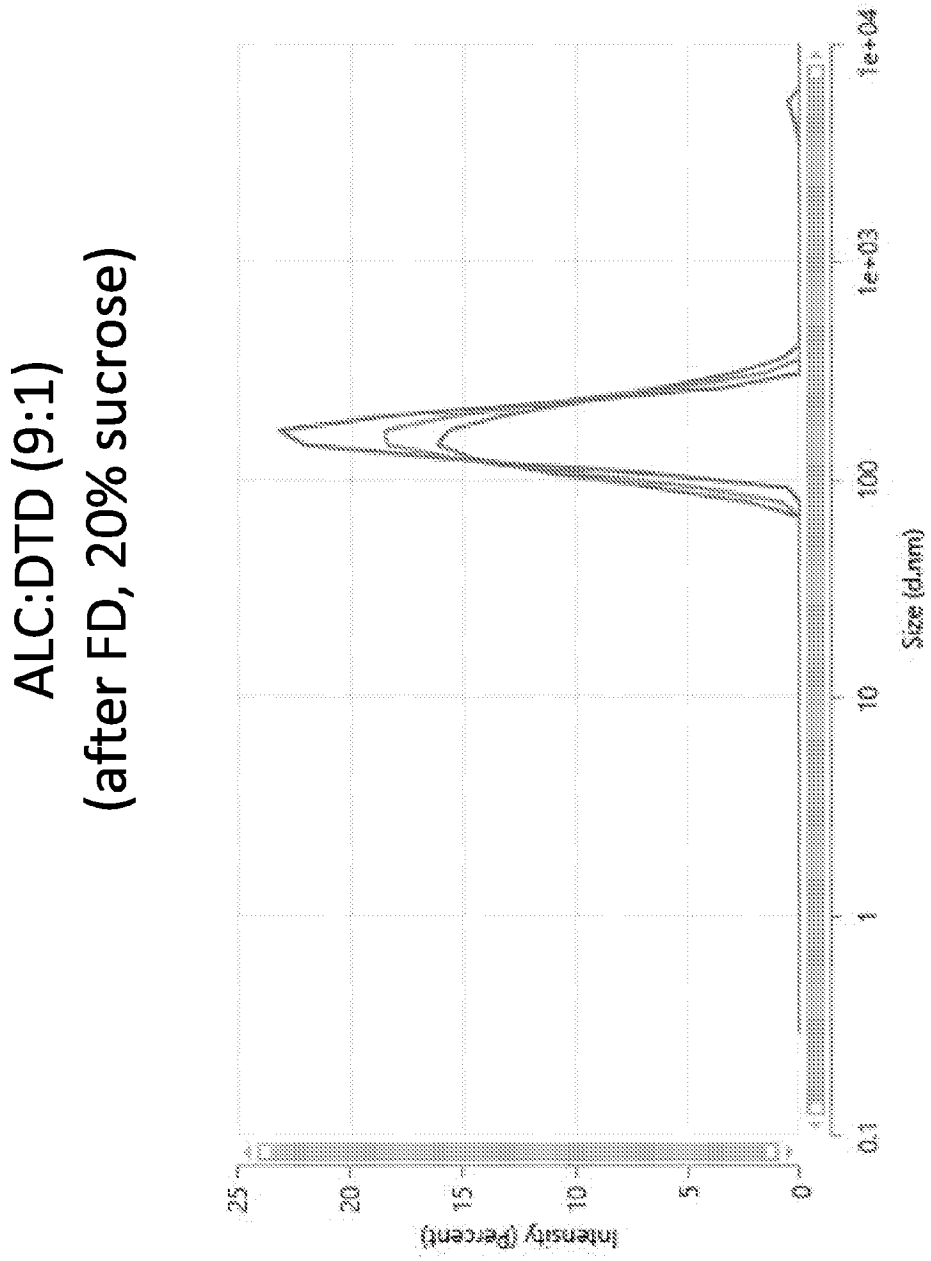

ALC:DTD (9:1)
(after FD, 20% sucrose)
Intensity (Percent)
0
5
10
15
20
25
0.1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG. 26

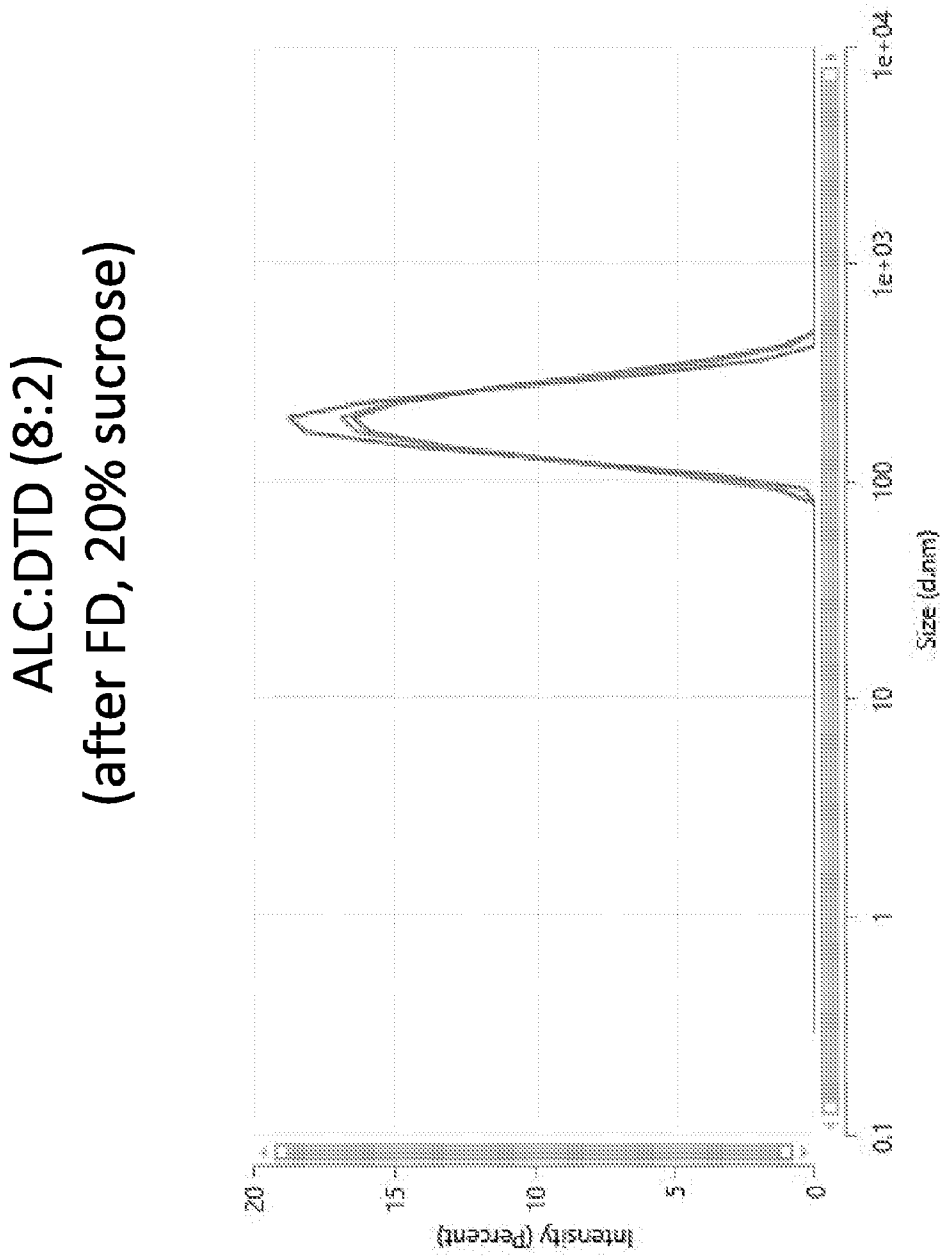

ALC:DTD (8:2)
(after FD, 20% sucrose)
Intensity (Percent)
20
15
10
5
0
0.1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG. 27

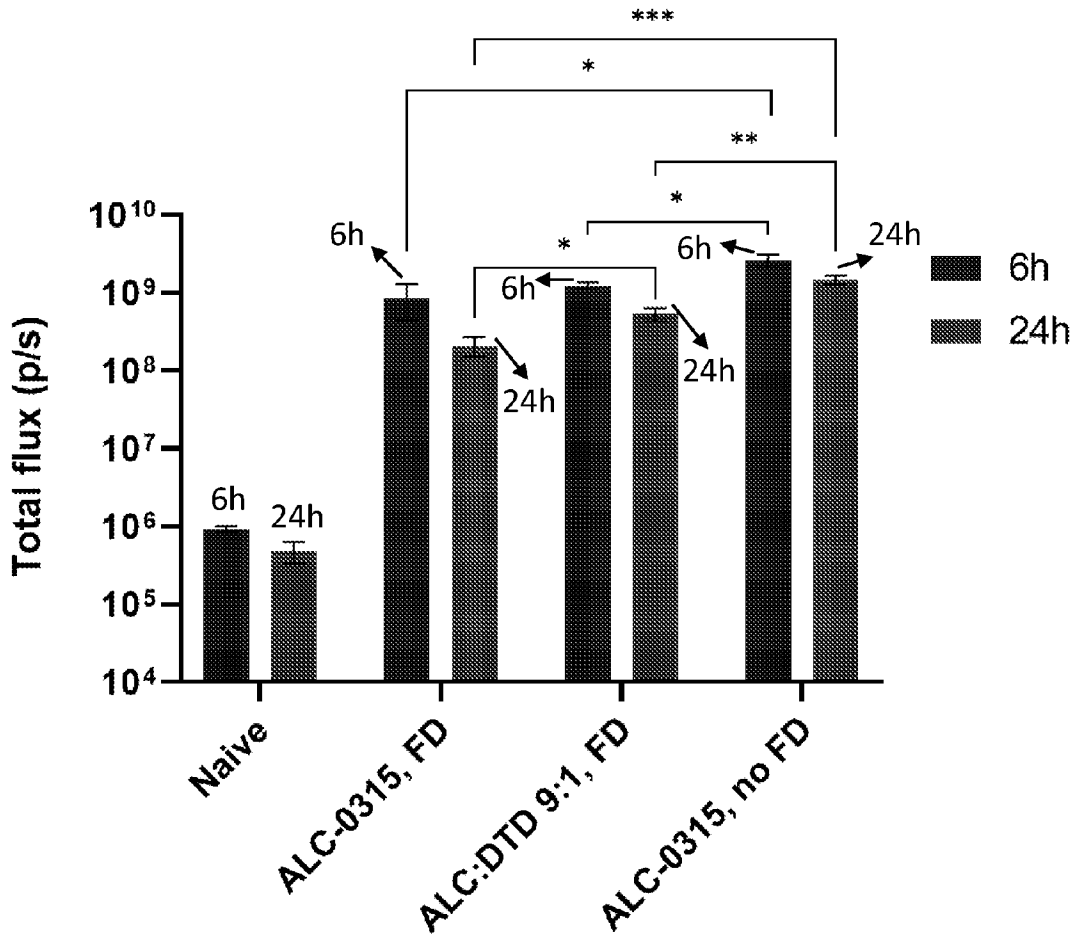

***
*
**
*
*
6h
6h
6h
24h
24h
24h
6h
24h
Total flux (p/s)
10^10
10^9
10^8
10^7
10^6
10^5
10^4
6h
24h
Naive
ALC-0315, FD
ALC:DTD 9:1, FD
ALC-0315, no FD

FIG. 28

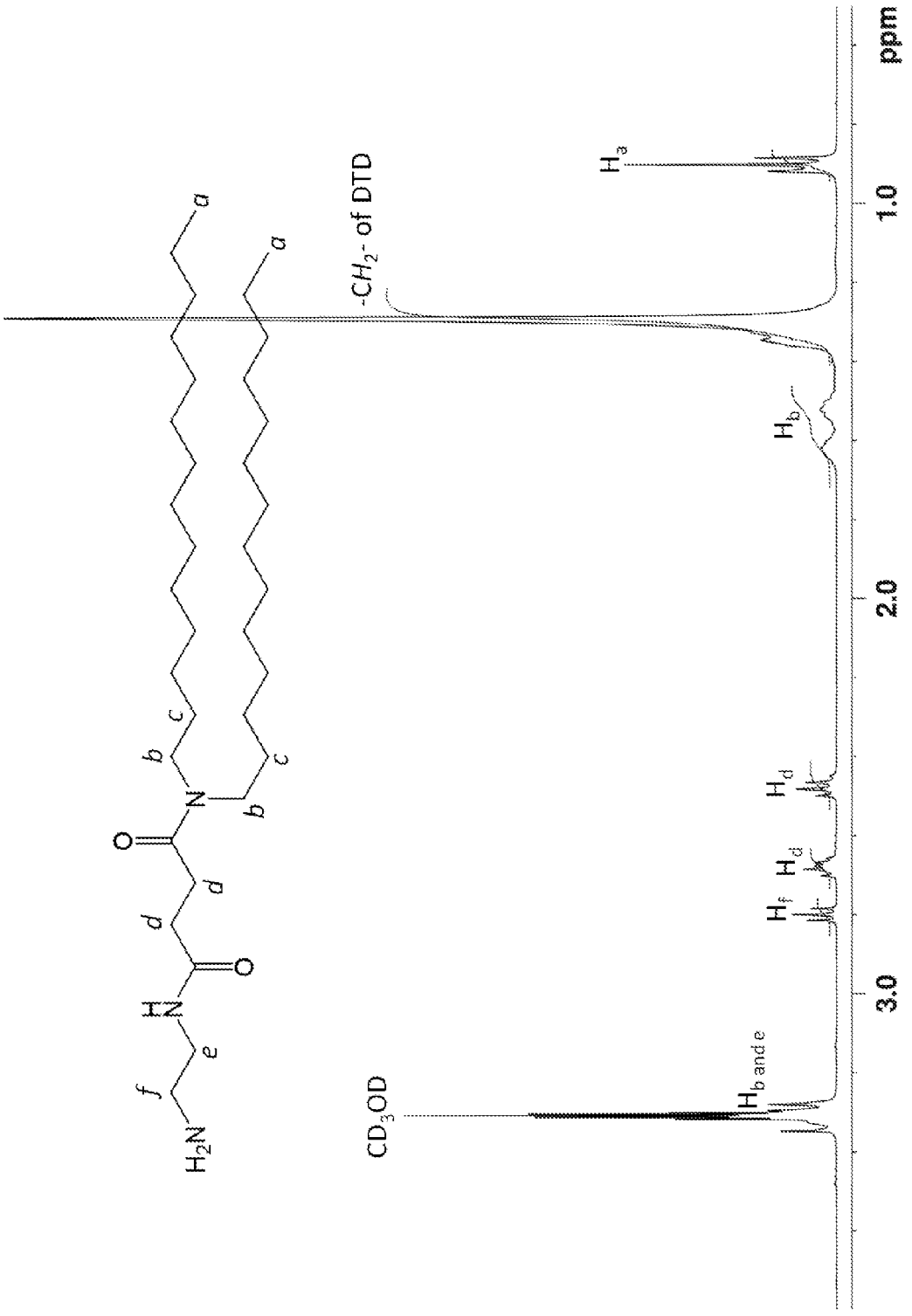

ppm
1.0
2.0
3.0
$H_a$
$-CH_2-$ of DTD
$H_b$
$H_d$
$H_d$
$H_f$
$H_{b\ and\ e}$
$CD_3OD$
a
a
b
c
b
c
N
O
d
d
O
H
N
e
f
$H_2N$

FIG. 29

# INTERNATIONAL SEARCH REPORT

International application No.
**PCT/SG2024/050368**

## A. CLASSIFICATION OF SUBJECT MATTER

See Supplemental Box

According to International Patent Classification (IPC)

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS REGISTRY, CAplus and Internet: Chemical structural search, ionizable lipid, lipid nanoparticle, nucleic acid delivery and related term

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2023/091787 A1 (SENDA BIOSCIENCES, INC.) 25 May 2023<br>The 9th para at page 4; the 5th para at page 8; sections "Lipid Nanoparticle | 1, 3 and 5-30 |
| A | Composition" and "Neutral Lipids" at pages 64-66; section "Lipid conjugates" at pages 68-73; the 2nd and 6th para at page 77; the 2nd para at page 78; the 1st para at page 85; the 1st para at page 89; Examples 5 and 6 | 2 and 4 |
| A | XU, Y. ET AL., Rational design and combinatorial chemistry of ionizable lipids for RNA delivery. *Journal of Materials Chemistry B*, 5 June 2023, Vol. 11, No. 28, pages 6527-6539<br>[Retrieved on 2024-08-05] <DOI: 10.1039/D3TB00649B><br>Whole document, particularly, Fig. 1; the 4th para of the left column at page 6529 | 1-30 |
| A | SUN, D. ET AL., Structure and Function of Cationic and Ionizable Lipids for Nucleic Acid Delivery. *Pharmaceutical Research*, 4 January 2023, Vol. 40, No. 1, pages 27-46<br>[Retrieved on 2024-08-05] <DOI: 10.1007/S11095-022-03460-2><br>Whole document, particularly, the 1st para of the right column at page 40; section "Ionizable Lipids for mRNA LNP" at page 41 | 1-30 |

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | HOU, X. ET AL., Lipid nanoparticles for mRNA delivery. *Nature Reviews Materials*, 10 August 2021, Vol. 6, pages 1078-1094<br>[Retrieved on 2024-08-05] <DOI: 10.1038/S41578-021-00358-0><br>Whole document, particularly, section “Ionizable lipids” at pages 1081 and 1082; section “Stability and storage” at page 1086; Fig. 2 | 1-30 |
| A | DONG, Y. ET AL., Bisethylnorspermine Lipopolyamine as Potential Delivery Vector for Combination Drug/Gene Anticancer Therapies. *Pharmaceutical Research*, 25 June 2010, Vol. 27, No. 9, pages 1927-1938 (NIH Public Access Author Manuscript in PMC)<br>[Retrieved on 2024-08-05] <DOI: 10.1007/S11095-010-0197-4><br>Whole document, particularly, abstract; Fig. 2 in the author manuscript in PMC | |

# INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
**PCT/SG2024/050368**

*Note: This Annex lists known patent family members relating to the patent documents cited in this International Search Report. This Authority is in no way liable for these particulars which are merely given for the purpose of information.*

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| WO 2023/091787 A1 | 25/05/2023 | CA 3238758 A1<br>AU 2022391677 A1 | 25/05/2023<br>06/06/2024 |

# INTERNATIONAL SEARCH REPORT

International application No.
**PCT/SG2024/050368**

**Supplemental Box**
**(Classification of Subject Matter)**

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***A61K 47/28 (2006.01)***
***A61P 31/14 (2006.01)***
***A61P 31/04 (2006.01)***
***A61P 35/00 (2006.01)***