IMPROVED PROCESS OF PREPARING MRNA-LOADED LIPID NANOPARTICLES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional patent application Serial No. 62/847,837, filed May 14, 2019, which is hereby incorporated by reference in their entirety for all purposes.

BACKGROUND

[0002] Messenger RNA therapy (MRT) is becoming an increasingly important approach for the treatment of a variety of diseases. MRT involves administration of messenger RNA (mRNA) to a patient in need of the therapy for production of the protein encoded by the mRNA within the patient's body. Lipid nanoparticles are commonly used to encapsulate mRNA for efficient *in vivo* delivery of mRNA.

[0003] To improve lipid nanoparticle delivery, much effort has focused on identifying novel lipids or particular lipid compositions that can affect intracellular delivery and/or expression of mRNA, e.g., in various types of mammalian tissue, organs and/or cells (e.g., mammalian liver cells). However, these existing approaches are costly, time consuming and unpredictable.

SUMMARY

[0004] The present disclosure provides, among other things, further improved processes for preparing mRNA-loaded lipid nanoparticles (mRNA-LNPs). The disclosure is based on the surprising discovery that following a process of encapsulating messenger RNA (mRNA) in LNPs comprising mixing one or more lipids in a lipid solution with one or more mRNAs in an mRNA solution to form mRNA encapsulated within LNPs (mRNA-LNPs) in a LNP formation solution (e.g., Process A as further described below), the further steps of exchanging the LNP formation solution for a drug product formulation solution and heating the mRNA-LNPs in the drug

product formulation solution provide an unexpected benefit of significantly increasing the encapsulation efficiency of the mRNA-LNPs, i.e., the amount or percent of mRNA encapsulated within the LNPs (i.e., encapsulation rate or efficiency). The present disclosure is particularly useful for manufacturing mRNA-LNPs to have a higher encapsulation rate or efficiency as compared to conventional approaches.

[0005] As compared to conventional approaches, the inventive process described herein provides higher encapsulation efficiency and accordingly may provide higher potency and better efficacy of lipid nanoparticle delivered mRNA, thereby shifting the therapeutic index in a positive direction and providing additional advantages, such as lower cost, better patient compliance, and more patient friendly dosing regimens. mRNA-loaded lipid nanoparticle formulations provided by the present disclosure may be successfully delivered *in vivo* for more potent and efficacious protein expression via different routes of administration such as intravenous, intramuscular, intra-articular, intrathecal, inhalation (respiratory), subcutaneous, intravitreal, and ophthalmic.

[0006] This inventive process can be performed using a pump system and is therefore scalable, allowing for improved particle formation/formulation in amounts sufficient for, e.g., performance of clinical trials and/or commercial sale. Various pump systems may be used to practice the present disclosure including, but not limited to, pulse-less flow pumps, gear pumps, peristaltic pumps, and centrifugal pumps.

[0007] This inventive process results in superior encapsulation efficiency and homogeneous particle sizes.

[0008] Thus, in one aspect, the present disclosure provides a process of encapsulating messenger RNA (mRNA) in lipid nanoparticles (LNPs) comprising the steps of (a) mixing one or more lipids in a lipid solution with one or more mRNAs in an mRNA solution to form mRNA encapsulated within the LNPs (mRNA-LNPs) in a LNP formation solution; (b) exchanging the LNP formation solution for a drug product formulation solution to provide mRNA-LNP in a drug product formulation solution; and (c) heating the mRNA-LNP in the drug product formulation

solution, wherein the encapsulation efficiency of the mRNA-LNPs resulting from step (c) is greater than the encapsulation efficiency of the mRNA-LNPs resulting from step (b).

[0009] In some examples, in step (c) the drug product formulation solution is heated by applying heat from a heat source to the solution.

[0010] In some examples, in step (c) the drug product formulation solution is heated by applying heat from a heat source to the solution and the solution is maintained at a temperature greater than ambient temperature for 5 seconds or more, 10 seconds or more, 20 seconds or more, 30 seconds or more, 40 seconds or more, 50 seconds or more, 1 minute or more, 2 minutes or more, 3 minutes or more 4 minute or more, 5 minutes or more, 10 minutes or more, 15 minutes or more, 20 minutes or more, 25 minutes or more, 30 minutes or more, 35 minutes or more, 40 minutes or more, 45 minutes or more, 50 minutes or more, 60 minutes or more, 70 minutes or more, 80 minutes or more, 90 minutes or more, 100 minutes or more or 120 minutes or more. In some examples, in step (c) the drug product formulation solution is heated by applying heat from a heat source to the solution and the solution is maintained at a temperature greater than ambient temperature for 120 minutes or less, 100 minutes or less, 90 minutes or less, 60 minutes or less, 45 minutes or less, 30 minutes or less, 25 minutes or less, 20 minutes or less, 15 minutes or less, 10 minutes or less, 5 minutes or less, 4 minutes or less, 3 minutes or less, 2 minutes or less, 1 minute or less, 50 seconds or less, 40 seconds or less, 30 seconds or less, 20 seconds or less, 10 seconds or less or 5 seconds or less. In some examples, in step (c) the drug product formulation solution is heated by applying heat from a heat source to the solution and the solution is maintained at a temperature greater than ambient temperature for between 10 and 20 minutes. In some examples, in step (c) the drug product formulation solution is heated by applying heat from a heat source to the solution and the solution is maintained at a temperature greater than ambient temperature for between 20 and 90 minutes. In some examples, in step (c) the drug product formulation solution is heated by applying heat from a heat source to the solution and the solution is maintained at a temperature greater than ambient temperature for between 30 and 60 minutes. In some examples, in step (c) the drug product formulation solution is heated by applying heat from a heat source to the solution and the solution is maintained at a temperature greater than ambient temperature for about 15 minutes. In some examples, the

temperature to which the drug product formulation is heated (or at which the drug product formulation solution is maintained) is or is greater than about 30 °C, 37 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, or 70 °C. In some examples, the temperature to which the drug product formulation solution is heated ranges from about 25-70 °C, about 30-70 °C, about 35-70 °C, about 40-70 °C, about 45-70 °C, about 50-70 °C, or about 60-70 °C. In some examples, the temperature greater than ambient temperature to which the drug product formulation solution is heated is about 65 °C.

[0011] In some examples, in step (a) the lipid nanoparticles are formed by mixing lipids dissolved in the lipid solution comprising ethanol with mRNA dissolved in an aqueous mRNA solution. In some examples, in step (a) the one or more lipids include one or more cationic lipids, one or more helper lipids, and one or more PEG-modified lipids (also referred to as PEG lipids). In some examples, the lipids also contain one or more cholesterol lipids. The mRNA-LNPs are formed by the mixing of the lipid solution and the mRNA solution. Accordingly, in some examples, the LNPs comprise one or more cationic lipids, one or more helper lipids, and one or more PEG lipids. In some examples, the LNPs also contain one or more cholesterol lipids.

[0012] In some examples, the one or more cationic lipids are selected from the group consisting of cKK-E12, OF-02, C12-200, MC3, DLinDMA, DLinkC2DMA, ICE (Imidazol-based), HGT5000, HGT5001, HGT4003, DODAC, DDAB, DMRIE, DOSPA, DOGS, DODAP, DODMA and DMDMA, DODAC, DLenDMA, DMRIE, CLinDMA, CpLinDMA, DMOBA, DOcarbDAP, DLinDAP, DLincarbDAP, DLinCDAP, KLin-K-DMA, DLin-K-XTC2-DMA, 3-(4-(bis(2-hydroxydodecyl)amino)butyl)-6-(4-((2-hydroxydodecyl)(2-hydroxyundecyl)amino)butyl)-1,4-dioxane-2,5-dione (Target 23), 3-(5-(bis(2-hydroxydodecyl)amino)pentan-2-yl)-6-(5-((2-hydroxydodecyl)(2-hydroxyundecyl)amino)pentan-2-yl)-1,4-dioxane-2,5-dione (Target 24), N1GL, N2GL, V1GL and combinations thereof.

[0013] In some examples, the one or more cationic lipids are amino lipids. Amino lipids suitable for use in the disclosure include those described in WO2017180917, which is hereby

incorporated by reference. Exemplary aminolipids in WO2017180917 include those described at paragraph [0744] such as DLin-MC3-DMA (MC3), (13Z,16Z)-N,N-dimethyl-3-nonyldocosa-13,16-dien-1-amine (L608), and Compound 18. Other amino lipids include Compound 2, Compound 23, Compound 27, Compound 10, and Compound 20. Further amino lipids suitable for use in the disclosure include those described in WO2017112865, which is hereby incorporated by reference. Exemplary amino lipids in WO2017112865 include a compound according to one of formulae (I), (Ial)-(Ia6), (lb), (II), (Ila), (III), (Ilia), (IV), (17-1), (19-1), (19-11), and (20-1), and compounds of paragraphs [00185], [00201], [0276]. In some examples, cationic lipids suitable for use in the disclosure include those described in WO2016118725, which is hereby incorporated by reference. Exemplary cationic lipids in WO2016118725 include those such as KL22 and KL25. In some examples, cationic lipids suitable for use in the disclosure include those described in WO2016118724, which is hereby incorporated by reference. Exemplary cationic lipids in WO2016118725 include those such as KL10, 1 ,2-dilinoleyloxy-N,N-dimethylaminopropane (DLin-DMA), and KL25.

[0014] In some examples, the one or more non-cationic lipids are selected from DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), DOPE (1,2-dioleyl-sn-glycero-3-phosphoethanolamine), DOPC (1,2-dioleyl-sn-glycero-3-phosphotidylcholine) DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine), DMPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine), DOPG (1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)).

[0015] In some examples, the one or more PEG-modified lipids comprise a poly(ethylene) glycol chain of up to 5 kDa in length covalently attached to a lipid with alkyl chain(s) of C_6-C_{20} length.

[0016] In some examples, following step (a) the mRNA-LNPs are purified by a Tangential Flow Filtration (TFF) process. In some examples, greater than about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the purified mRNA-LNPs have a size less than about 150 nm (e.g., less than about 145 nm, about 140 nm, about 135 nm, about 130 nm, about 125 nm, about 120 nm, about 115 nm, about 110 nm, about 105 nm,

about 100 nm, about 95 nm, about 90 nm, about 85 nm, about 80 nm, about 75 nm, about 70 nm, about 65 nm, about 60 nm, about 55 nm, or about 50 nm). In some examples, substantially all of the purified mRNA-LNPs have a size less than 150 nm (e.g., less than about 145 nm, about 140 nm, about 135 nm, about 130 nm, about 125 nm, about 120 nm, about 115 nm, about 110 nm, about 105 nm, about 100 nm, about 95 nm, about 90 nm, about 85 nm, about 80 nm, about 75 nm, about 70 nm, about 65 nm, about 60 nm, about 55 nm, or about 50 nm). In some examples, greater than about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the purified mRNA-LNPs have a size ranging from 50-150 nm. In some examples, substantially all of the purified mRNA-LNPs have a size ranging from 50-150 nm. In some examples, greater than about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the purified mRNA-LNPs have a size ranging from 80-150 nm. In some examples, substantially all of the purified nanoparticles have a size ranging from 80-150 nm.

[0017] In some examples, a process according to the present disclosure results in an encapsulation efficiency following step (c) that is improved by at least 5% or more over the encapsulation efficiency following step (b). In some examples, a process according to the present disclosure results in an encapsulation efficiency following step (c) that is improved by at least 10% or more over the encapsulation efficiency following step (b). In some examples, a process according to the present disclosure results in an encapsulation efficiency following step (c) that is improved by at least 15% or more over the encapsulation efficiency following step (b). In some examples, a process according to the present disclosure results in an encapsulation efficiency following step (c) that is improved by at least 20% or more over the encapsulation efficiency following step (b). In some examples, a process according to the present disclosure results in an encapsulation efficiency following step (c) that is improved by at least 25% or more over the encapsulation efficiency following step (b).

[0018] In some examples, a process according to the present disclosure improves the encapsulation amount by 5% encapsulation or more from the encapsulation following step (b) to the encapsulation following step (c). In some examples, a process according to the present disclosure improves the encapsulation amount by 10% encapsulation or more from the encapsulation following step (b) to the encapsulation following step (c). In some examples, a

process according to the present disclosure improves the encapsulation amount by 15% encapsulation or more from the encapsulation following step (b) to the encapsulation following step (c). In some examples, a process according to the present disclosure improves the encapsulation amount by 20% encapsulation or more from the encapsulation following step (b) to the encapsulation following step (c). In some examples, a process according to the present disclosure improves the encapsulation amount by 25% encapsulation or more from the encapsulation following step (b) to the encapsulation following step (c).

[0019] In some examples, a process according to the present disclosure results in greater than about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% recovery of mRNA following step (c).

[0020] In some examples, a process according to the present disclosure results in an encapsulation rate following step (c) of greater than about 90%, 95%, 96%, 97%, 98%, or 99%. In some examples, a process according to the present disclosure results in greater than about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% recovery of mRNA following step (c).

[0021] In some examples, the lipid solution and the mRNA solution are mixed using a pump system. In some examples, the pump system comprises a pulse-less flow pump. In some examples, the pump system is a gear pump. In some examples, a suitable pump is a peristaltic pump. In some examples, a suitable pump is a centrifugal pump. In some examples, the process using a pump system is performed at large scale. For example, in some examples, the process includes using pumps as described herein to mix a solution of at least about 1 mg, 5 mg, 10 mg, 50 mg, 100 mg, 500 mg, or 1000 mg of mRNA with a lipid solution comprising one or more cationic lipids, one or more helper lipids and one or more PEG-modified lipids. In some examples, the process of mixing the lipid solution and the mRNA solution provides a composition according to the present disclosure that contains at least about 1 mg, 5mg, 10 mg, 50 mg, 100 mg, 500 mg, or 1000 mg of encapsulated mRNA following step (c).

[0022] In some examples, the lipid solution is mixed at a flow rate ranging from about 25-75 ml/minute, about 75-200 ml/minute, about 200-350 ml/minute, about 350-500 ml/minute,

about 500-650 ml/minute, about 650-850 ml/minute, or about 850-1000 ml/minute. In some examples, the lipid solution is mixed at a flow rate of about 50 ml/minute, about 100 ml/minute, about 150 ml/minute, about 200 ml/minute, about 250 ml/minute, about 300 ml/minute, about 350 ml/minute, about 400 ml/minute, about 450 ml/minute, about 500 ml/minute, about 550 ml/minute, about 600 ml/minute, about 650 ml/minute, about 700 ml/minute, about 750 ml/minute, about 800 ml/minute, about 850 ml/minute, about 900 ml/minute, about 950 ml/minute, or about 1000 ml/minute.

[0023] In some examples, the mRNA solution is mixed at a flow rate ranging from about 25-75 ml/minute, about 75-200 ml/minute, about 200-350 ml/minute, about 350-500 ml/minute, about 500-650 ml/minute, about 650-850 ml/minute, or about 850-1000 ml/minute. In some examples, the mRNA solution is mixed at a flow rate of about 50 ml/minute, about 100 ml/minute, about 150 ml/minute, about 200 ml/minute, about 250 ml/minute, about 300 ml/minute, about 350 ml/minute, about 400 ml/minute, about 450 ml/minute, about 500 ml/minute, about 550 ml/minute, about 600 ml/minute, about 650 ml/minute, about 700 ml/minute, about 750 ml/minute, about 800 ml/minute, about 850 ml/minute, about 900 ml/minute, about 950 ml/minute, or about 1000 ml/minute.

[0024] In some examples, the lipid solution includes a non-aqueous solvent such as an organic solvent. In some examples, the lipid solution includes an alcohol. In some examples, the lipid solution includes ethanol. In some examples, a process according to the present disclosure includes a step of first dissolving the one or lipids in the lipid solution. In some examples, a process according to the present disclosure includes a step of first dissolving the one or lipids in the lipid solution comprising ethanol.

[0025] In some examples, the mRNA solution is an aqueous solution. In some examples, the mRNA solution comprises citrate. In some examples, the mRNA solution is a citrate buffer. In some examples, a process according to the present disclosure includes a step of first dissolving the mRNA in the aqueous solution. In some examples, a process according to the present disclosure includes a step of first dissolving the mRNA in the aqueous solution comprising citrate.

[0026] In some examples, a process according to the present disclosure includes a step of mixing a lipid solution comprising lipids in ethanol with a mRNA buffer comprising mRNA dissolved in citrate buffer. In some examples, the LNP formation solution comprises ethanol and citrate.

[0027] In some examples, a process according to the present disclosure includes a step of first generating an mRNA solution by mixing a citrate buffer with an mRNA stock solution. In certain examples, a suitable citrate buffer contains about 10 mM citrate, about 150 mM NaCl, pH of about 4.5. In some examples, a suitable mRNA stock solution contains the mRNA at a concentration at or greater than about 1 mg/ml, about 10 mg/ml, about 50 mg/ml, or about 100 mg/ml.

[0028] In some examples, the citrate buffer is mixed at a flow rate ranging between about 100-300 ml/minute, 300-600 ml/minute, 600-1200 ml/minute, 1200-2400 ml/minute, 2400-3600 ml/minute, 3600-4800 ml/minute, or 4800-6000 ml/minute. In some examples, the citrate buffer is mixed at a flow rate of about 220 ml/minute, about 600 ml/minute, about 1200 ml/minute, about 2400 ml/minute, about 3600 ml/minute, about 4800 ml/minute, or about 6000 ml/minute.

[0029] In some examples, the mRNA stock solution is mixed at a flow rate ranging between about 10-30 ml/minute, about 30-60 ml/minute, about 60-120 ml/minute, about 120-240 ml/minute, about 240-360 ml/minute, about 360-480 ml/minute, or about 480-600 ml/minute. In some examples, the mRNA stock solution is mixed at a flow rate of about 20 ml/minute, about 40 ml/minute, about 60 ml/minute, about 80 ml/minute, about 100 ml/minute, about 200 ml/minute, about 300 ml/minute, about 400 ml/minute, about 500 ml/minute, or about 600 ml/minute.

[0030] In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising pharmaceutically acceptable excipients, including, but not limited to, a cryoprotectant. In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising pharmaceutically acceptable excipients, including, but not limited to, a sugar. In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising pharmaceutically acceptable excipients, including, but not limited to, one or

more of trehalose, sucrose, mannose, lactose, and mannitol. In some examples, in step (b) the drug product formulation solution comprises trehalose. In some examples, in step (b) the drug product formulation solution comprises sucrose. In some examples, in step (b) the drug product formulation solution comprises mannose. In some examples, in step (b) the drug product formulation solution comprises lactose. In some examples, in step (b) the drug product formulation solution comprises mannitol. In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising 5% to 20% weight to volume of a sugar, such as of trehalose, sucrose, mannose, lactose, and mannitol. In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising 5% to 20% weight to volume of trehalose. In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising 5% to 20% weight to volume of sucrose. In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising 5% to 20% weight to volume of mannose. In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising 5% to 20% weight to volume of lactose. In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising 5% to 20% weight to volume of mannitol. In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising about 10% weight to volume of a sugar, such as of trehalose, sucrose, mannose, lactose, and mannitol. In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising about 10% weight to volume of trehalose. In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising about 10% weight to volume of sucrose. In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising about 10% weight to volume of mannose. In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising about 10% weight to volume of lactose. In some examples, in step (b) the drug product formulation solution is an aqueous solution comprising about 10% weight to volume of mannitol.

[0031] In some examples, one or both of a non-aqueous solvent, such as ethanol, and citrate are absent (i.e., below detectable levels) from the drug product formulation solution. In some examples, citrate is absent (i.e., below detectable levels) from the drug product formulation

solution. In some examples, ethanol is absent (i.e., below detectable levels) from the drug product formulation solution. In some examples, the drug product formulation solution comprises ethanol, but not citrate (i.e., below detectable levels). In some examples, the drug product formulation solution comprises citrate, but not ethanol (i.e., below detectable levels). In some examples, the drug product formulation solution includes only residual citrate. In some examples, the drug product formulation solution includes only residual non-aqueous solvent, such as ethanol. In some examples, the drug product formulation solution contains less than about 10mM (e.g., less than about 9mM, about 8mM, about 7mM, about 6mM, about 5mM, about 4mM, about 3mM, about 2mM, or about1mM) of citrate. In some examples, the drug product formulation solution contains less than about 25% (e.g., less than about 20%, about 15%, about 10%, about 5%, about 4%, about 3%, about 2%, or about 1%) of non-aqueous solvents, such as ethanol. In some examples, the drug product formulation solution does not require any further downstream processing (e.g., buffer exchange and/or further purification steps) prior to lyophilization. In some examples, the drug product formulation solution does not require any further downstream processing (e.g., buffer exchange and/or further purification steps) prior to administration to a subject.

[0032] In some examples, the drug product formulation solution has a pH between pH 4.5 and pH 7.5. In some examples, the drug product formulation solution has a pH between pH 5.0 and pH 7.0. In some examples, the drug product formulation solution has a pH between pH 5.5 and pH 7.0. In some examples, the drug product formulation solution has a pH above pH 4.5. In some examples, the drug product formulation solution has a pH above pH 5.0. In some examples, the drug product formulation solution has a pH above pH 5.5. In some examples, the drug product formulation solution has a pH above pH 6.0. In some examples, the drug product formulation solution has a pH above pH 6.5.

[0033] In some examples, the present disclosure is used to encapsulate mRNA containing one or more modified nucleotides. In some examples, one or more nucleotides is modified to a pseudouridine. In some examples, one or more nucleotides is modified to a 5-methylcytidine. In some examples, the present disclosure is used to encapsulate mRNA that is unmodified.

[0034] In yet another example, the present disclosure provides a method of delivering mRNA for *in vivo* protein production comprising administering into a subject a composition of lipid nanoparticles encapsulating mRNA generated by the process described herein, wherein the mRNA encodes one or more protein(s) or peptide(s) of interest.

[0035] In this application, the use of "or" means "and/or" unless stated otherwise. As used in this disclosure, the term "comprise" and variations of the term, such as "comprising" and "comprises," are not intended to exclude other additives, components, integers or steps. As used in this application, the terms "about" and "approximately" are used as equivalents. Both terms are meant to cover any normal fluctuations appreciated by one of ordinary skill in the relevant art.

[0036] Other features, objects, and advantages of the present disclosure are apparent in the detailed description, drawings and claims that follow. It should be understood, however, that the detailed description, the drawings, and the claims, while indicating examples of the present disclosure, are given by way of illustration only, not limitation. Various changes and modifications within the scope of the disclosure will become apparent to those skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037] The drawings are for illustration purposes only and not for limitation.

[0038] **FIG. 1** shows a schematic of an conventional LNP-mRNA encapsulation process (Process A) that involves mixing mRNA dissolved in an aqueous mRNA solution with lipids dissolved in a lipid solution using a pump system to generate mRNA-LNPs in a LNP formation

solution and then exchanging the LNP formation solution for a drug product formulation solution.

[0039] **FIG. 2** shows a schematic of an exemplary LNP-mRNA encapsulation process of the present invention that involves mixing mRNA dissolved in an aqueous mRNA solution with lipids dissolved in a lipid solution using a pump system to generate mRNA-LNPs in a LNP formation solution, then exchanging the LNP formation solution for a drug product formulation solution, and then heating the drug product formulation solution to increase encapsulation of mRNA in the LNPs.

[0040] **FIG. 3** shows the difference in encapsulation before and after a final step of heating mRNA-LNPs in drug product formulation solution, for twelve different mRNA-LNPs tested.

[0041] **FIG. 4** shows the difference in encapsulation before and after a final step of heating mRNA-LNPs in drug product formulation solution, for thirteen different mRNA-LNPs tested.

[0042] **FIG. 5** shows exemplary graph of protein expression after pulmonary administration of mRNA encapsulated in lipid nanoparticles prepared by Process A after a heating step.

DEFINITIONS

[0043] In order for the present invention to be more readily understood, certain terms are first defined below. Additional definitions for the following terms and other terms are set forth throughout the specification.

[0044] *Alkyl*: As used herein, “alkyl” refers to a radical of a straight-chain or branched saturated hydrocarbon group having from 1 to 20 carbon atoms (“C_{1-20} alkyl”). In some embodiments, an alkyl group has 1 to 3 carbon atoms (“C_{1-3} alkyl”). Examples of C_{1-3} alkyl groups include methyl (C_1), ethyl (C_2), n-propyl (C_3), and isopropyl (C_3). In some embodiments, an alkyl group has 8 to 12 carbon atoms ("C_{8-12} alkyl"). Examples of C_{8-12} alkyl

CLAIMS

We claim:

1. A process of encapsulating messenger RNA (mRNA) in lipid nanoparticles (LNPs) comprising the steps of:

(a) mixing one or more lipids in a lipid solution with one or more mRNAs in an mRNA solution to form mRNA encapsulated within the LNPs (mRNA-LNPs) in a lipid nanoparticle (LNP) formation solution;

(b) exchanging the LNP formation solution for a drug product formulation solution to provide mRNA-LNP in a drug product formulation solution; and

(c) heating the mRNA-LNP in the drug product formulation solution;

wherein the encapsulation efficiency of the mRNA-LNPs resulting from step (c) is greater than the encapsulation efficiency of the mRNA-LNPs resulting from step (b).

2. The process according to claim 1, wherein in step (a) the one or more lipids include one or more cationic lipids, one or more helper lipids, and one or more PEG-modified lipids.

3. The process according to claim 2, wherein the lipids further comprise one or more cholesterol lipids (e.g., cholesterol).

4. The process according to any one of the preceding claims, wherein in step (a) the one or more cationic lipids are selected from cKK-E12, OF-02, C12-200, MC3, DLinDMA, DLinkC2DMA, ICE (Imidazol-based), HGT5000, HGT5001, HGT4001, HGT4002, HGT4003, HGT4004, HGT4005, DODAC, DDAB, DMRIE, DOSPA, DOGS, DODAP, DODMA and DMDMA, DODAC, DLenDMA, DMRIE, CLinDMA, CpLinDMA, DMOBA, DOcarbDAP, DLinDAP, DLincarbDAP, DLinCDAP, KLin-K-DMA, DLin-K-XTC2-DMA, 3-(4-(bis(2-hydroxydodecyl)amino)butyl)-6-(4-((2-hydroxydodecyl)(2-hydroxyundecyl)amino)butyl)-1,4-dioxane-2,5-dione (Target 23), 3-(5-(bis(2-hydroxydodecyl)amino)pentan-2-yl)-6-(5-((2-hydroxydodecyl)(2-hydroxyundecyl)amino)pentan-2-yl)-1,4-dioxane-2,5-dione (Target 24), N1GL, N2GL, V1GL, and combinations thereof.

5. The process according to any one of claims 2-4, wherein in step (a) the one or more helper lipids are selected from distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-l-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), 1,2-dierucoyl-sn-glycero-3-phosphoethanolamine (DEPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1-trans PE, l-stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE), and combinations thereof.

6. The process according to claim 1, wherein in step (a) the one or more PEG-modified lipids comprise a polyethylene glycol chain of up to 2kDa, up to 3 kDa, up to 4kDa or up to 5 kDa in length covalently attached to a lipid with alkyl chain(s) of C_6-C_{20} length.

7. The process according to any one of the preceding claims, wherein the lipid component of the lipid solution consists of:

(a) a cationic lipid,
(b) a helper lipid,
(c) a cholesterol-based lipid, and
(d) a PEG-modified lipid.

8. The process according to claim 8, wherein the molar ratio of the cationic lipid to helper lipid to cholesterol-based lipid to PEG-modified lipid is 20–50:25–35:20–50:1–5.

9. The process according to any one of claims 1-6, wherein the lipid component of the lipid solution consists of:

(a) cationic lipid,

(b) a helper lipid,

(c) a PEG-modified lipid.

10. The process according to claim 9, wherein the cationic lipid is a cholesterol-based or imidazol-based cationic lipid.

11. The process according to claim 9 or 10, wherein the molar ratio of the cationic lipid to helper lipid to PEG-modified lipid is 55–65:30–40:1–15.

12. The process according to any one of the preceding claims, wherein the mRNA encodes for a protein or peptide.

13. The process according to any one of the preceding claims, wherein in step (c) the drug product formulation solution is heated by applying heat from a heat source to the solution and the solution is maintained at a temperature greater than ambient temperature for between 10 and 20 minutes.

14. The process according to claim 13, wherein, the temperature greater than ambient temperature is 60-70°C.

15. The process according to any one of the preceding claims, wherein the encapsulation efficiency following step (c) provides at least 5% or more over the encapsulation efficiency following step (b).

16. The process according to any one of the preceding claims, wherein the encapsulation efficiency following step (c) is improved by at least 10% or more from the encapsulation efficiency following step (b).

17. The process according to any one of the preceding claims, wherein in step (a) the lipid solution comprises lipids dissolved in ethanol.

18. The process according to any one of the preceding claims, wherein in step (a) the mRNA solution comprises mRNA dissolved in citrate buffer.

19. The process according to any one of the preceding claims, wherein the drug product formulation solution is an aqueous solution comprising pharmaceutically acceptable excipients comprising a cryoprotectant.

20. The process according to any one of the preceding claims, wherein the drug product formulation solution is an aqueous solution comprising sugar.

21. The process according to claim 20, wherein the sugar is selected from the group consisting of one or more of trehalose, sucrose, mannose, lactose, and mannitol.

22. The process according to claim 21, wherein the sugar comprises trehalose.

23. The process according to any one of the preceding claims, wherein in step (b) the drug product formulation solution is an aqueous solution comprising about 10% weight to volume of trehalose.

24. The process according to any one of the preceding claims, wherein both ethanol and citrate are absent from the drug product formulation solution.

25. The process according to any one of the preceding claims, wherein the lipid solution comprises ethanol, the mRNA solution comprises citrate, and both ethanol and citrate are absent from the drug product formulation solution.

26. The process according to any one of the preceding claims, wherein the mRNA solution has a pH less than pH 5.0.

27. The process according to any one of the preceding claims, wherein the drug product formulation solution has a pH between pH 5.0 and pH 7.0.