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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
06 November 2025 (06.11.2025)

WIPO | PCT

(10) International Publication Number
WO 2025/231114 A1

(51) International Patent Classification:
A61K 9/00 (2006.01) *A61K 9/51* (2006.01)

(21) International Application Number:
PCT/US2025/027062

(22) International Filing Date:
30 April 2025 (30.04.2025)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/641,376 01 May 2024 (01.05.2024) US
63/792,167 21 April 2025 (21.04.2025) US

(71) Applicant: **ACUITAS THERAPEUTICS, INC.** [CA/CA]; 6190 Agronomy Road, Suite 405, Vancouver, British Columbia V6T 1Z3 (CA).

(72) Inventors: **CHAMBERLAIN, Thomas C.**; 6190 Agronomy Road, Suite 405, Vancouver, British Columbia V6T 1Z3 (CA). **YAN, Fan**; 6190 Agronomy Road, Suite 405, Vancouver, British Columbia V6T 1Z3 (CA). **BLAGOJEVIC, Polina**; 6190 Agronomy Road, Suite 405, Vancouver, British Columbia V6T 1Z3 (CA). **STEPHENSON, Kyle B.**; 6190 Agronomy Road, Suite 405, Vancouver, British Columbia V6T 1Z3 (CA). **LIN, Paulo Jia Ching**; 6190 Agronomy Road, Suite 405, Vancouver, British Columbia V6T 1Z3 (CA). **ARNS, Stephen Paul**; 6190 Agronomy Road, Suite 405, Vancouver, British Columbia V6T 1Z3 (CA). **TAM, Ying K.**; 6190 Agronomy Road, Suite 405, Vancouver, British Columbia V6T 1Z3 (CA). **CHIKH, Ghania**; 6190 Agronomy Road, Suite 405, Vancouver, British Columbia V6T 1Z3 (CA).

(74) Agent: **LIGON, Toby J.** et al.; Seed Intellectual Property Law Group LLP, Suite 5400, 701 Fifth Avenue, Seattle, Washington 98104-7064 (US).

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

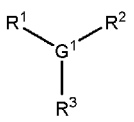
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB,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

Declarations under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(54) Title: METHOD OF USING LIPID NANOPARTICLES FOR INTRAMUSCULAR DELIVERY

(57) Abstract: Compounds are provided having the following Formula (I): or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof, wherein R^1, R^2, R^3, and G^1 are as defined herein. Use of the compounds as a component of lipid nanoparticle formulations for delivery of a therapeutic agent, compositions comprising the compounds and methods for their use and preparation are also provided.



(I)

WO 2025/231114 A1

METHOD OF USING LIPID NANOPARTICLES FOR INTRAMUSCULAR DELIVERY

BACKGROUND

Technical Field

The present disclosure generally relates to a novel delivery method that uses a combination of lipid components, such as cationic lipids, neutral lipids, cholesterol, and polymer conjugated lipids, to form lipid nanoparticles with oligonucleotides, to facilitate delivery of therapeutic nucleic acids (*e.g.*, oligonucleotides, messenger RNA) both *in vitro* and *in vivo*.

Description of Related Art

There are many challenges associated with the delivery of nucleic acids to achieve the desired response in a biological system. Nucleic acid-based therapeutics have enormous potential but there remains a need for more effective delivery of nucleic acids to appropriate sites within tissue or an organism to realize this potential. Therapeutic nucleic acids include, *e.g.*, messenger RNA (mRNA), antisense oligonucleotides, ribozymes, DNAzymes, plasmids, immune stimulating nucleic acids, antagomir, antimir, mimic, supermir, and aptamers. Some nucleic acids, such as mRNA or plasmids, can be used to achieve the expression of specific cellular products as would be useful in the treatment of, for example, diseases related to a deficiency of a protein or enzyme. The therapeutic applications of translatable nucleotide delivery are extremely broad as constructs can be synthesized to produce any chosen protein sequence, whether indigenous to the system. The expression products of the nucleic acid can augment existing levels of protein, replace missing or non-functional versions of a protein, or introduce a new protein and associated functionality in a cell or organism.

BRIEF SUMMARY

Some nucleic acids, such as miRNA inhibitors, can be used to achieve expression of specific cellular products that are regulated by miRNA as would be useful in the treatment of, for example, diseases related to deficiency of protein or enzyme. The therapeutic applications of miRNA inhibition are extremely broad as constructs can be synthesized to inhibit one or more miRNA that would in turn regulate the expression of mRNA products. The inhibition of endogenous miRNA can augment its downstream target endogenous protein expression and

restore proper function in a cell or organism as a means to treat disease associated to a specific miRNA or a group of miRNA.

Other nucleic acids can down-regulate intracellular levels of specific mRNA and, as a result, down-regulate the synthesis of the corresponding proteins through processes such as RNA interference (RNAi) or complementary binding of antisense RNA. The therapeutic applications of antisense oligonucleotide and RNAi are also extremely broad, since oligonucleotide constructs can be synthesized with any nucleotide sequence directed against a target mRNA. Targets may include mRNAs from normal cells, mRNAs associated with disease-states, such as cancer, and mRNAs of infectious agents, such as viruses. To date, antisense oligonucleotide constructs have shown the ability to specifically down-regulate target proteins through degradation of the cognate mRNA in both *in vitro* and *in vivo* models. In addition, antisense oligonucleotide constructs are currently being evaluated in clinical studies.

However, two problems are currently facing using oligonucleotides in therapeutic contexts. First, free RNAs are susceptible to nuclease digestion in plasma. Second, free RNAs have limited ability to gain access to the intracellular compartment where the relevant translation machinery resides. Lipid nanoparticles formed from lipid components, such as cationic lipids, neutral lipids, cholesterol, PEG, PEGylated lipids, and oligonucleotides have been used to block degradation of the RNAs in plasma and facilitate the cellular uptake of the oligonucleotides.

There remains a need for improved lipid nanoparticles compositions for the delivery of oligonucleotides. Preferably, these lipid nanoparticles would provide optimal drug to lipid ratios, protect the nucleic acid from degradation and clearance in serum, be suitable for intramuscular delivery, and provide intracellular delivery of the nucleic acid. In addition, these lipid-nucleic acid particles should be well-tolerated and provide an adequate therapeutic index, such that patient treatment at an effective dose of the nucleic acid is not associated with unacceptable toxicity and/or risk to the patient. The present disclosure provides these and related advantages. In brief, the present disclosure provides lipid compounds, including stereoisomers, pharmaceutically acceptable salts, or tautomers thereof, which can be used alone or in combination with other lipid components such as neutral lipids, charged lipids, steroids (including for example, all sterols) and/or their analogs, and/or polymer conjugated lipids to form lipid nanoparticles for the delivery of therapeutic agents. In some instances, the lipid nanoparticles are used to deliver nucleic acids such as antisense and/or messenger RNA. Methods for use of such lipid nanoparticles for treatment of various diseases or conditions, such as those caused by infectious entities and/or insufficiency of a protein, are also provided.

One embodiment provides a method of inducing an immune response in a subject, the method comprising intramuscular delivery of a lipid nanoparticle (LNP) to the subject, wherein the lipid nanoparticle comprises a nucleic acid that encodes an antigen and a cationic lipid, wherein:

a) the cationic lipid has a pKa ranging from 5.8 to 7.2;

b) the cationic lipid has a LogP value ranging from 12 to 25; and/or

c) the LNP has a spleen activity greater than 45 ng/g.

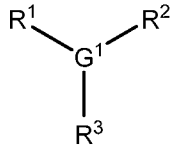
One embodiment provides a method of inducing an immune response in a subject, the method comprising intramuscular delivery of a lipid nanoparticle (LNP) to the subject, wherein the lipid nanoparticle comprises a nucleic acid that encodes an antigen and a cationic lipid, wherein:

a) the cationic lipid has a pKa from about 5.8 to about 7.2;

b) the cationic lipid has a LogP value from about 12 to about 25; and/or

c) the LNP has a spleen activity greater than about 45 ng/g.

Another embodiment provides a method of inducing an immune response in a subject, the method comprising intramuscular delivery of a lipid nanoparticle to the subject, wherein the lipid nanoparticle comprises a nucleic acid that encodes an antigen and a cationic lipid having the following structure of Formula (I):



or stereoisomer, tautomer, or salt thereof, wherein R^1, R^2, R^3, and G^1 are as defined herein.

Pharmaceutical compositions comprising one or more of the foregoing compounds of Formula (I) and a therapeutic agent are also provided. In some embodiments, the pharmaceutical compositions further comprise one or more components selected from neutral lipids, charged lipids, steroids, and polymer conjugated lipids. Such compositions are useful for formation of lipid nanoparticles for the delivery of the therapeutic agent.

In other embodiments, the present disclosure provides a method for administering a therapeutic agent to a patient in need thereof, the method comprising preparing a composition of lipid nanoparticles comprising the compound of Formula (I) and a therapeutic agent and delivering the composition to the patient. Such methods are useful for inducing expression of a protein in a subject, for example for expressing an antigen for purposes of vaccination or a gene editing protein.

These and other aspects of the disclosure will be apparent upon reference to the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

In the figures, identical reference numbers identify similar elements. The sizes and relative positions of elements in the figures are not necessarily drawn to scale. For example, the shapes of various elements and angles are not drawn to scale and some of these elements are enlarged and positioned to improve figure legibility. Further, the shapes of the elements as drawn are not intended to convey any information regarding the actual shape of the elements and have been solely selected for ease of recognition in the figures.

FIG. 1 shows results for a hemagglutination inhibition assay performed with representative cationic lipids at a dose of 0.2 μg. Results are further discussed in Example 3 herein.

FIG. 2 shows results for a hemagglutination inhibition assay performed with representative cationic lipids at a dose of 0.5 μg. Results are further discussed in Example 3 herein.

FIG. 3 shows values for the mean neutralizing antibody titer against H1N1 influenza (PR8 HA) for samples treated with LNPs prepared with the cationic lipids as indicated. The dosage used was 0.2 micrograms. Results further discussed in Example 3 herein.

FIG. 4 shows potency of identified cationic lipid formulations as measured by anti-RSV-Pre-F IgG. Changes are normalized to a composition prepared with Compound I-1. The dosage used was 0.2 micrograms. Results further discussed in Example 4 herein.

FIG. 5 shows potency of identified cationic lipid formulations as measured by neutralization titer FRNT for SARS-CoV-2 RBD. Changes are normalized to a composition prepared with Compound I-1. The dosage used was 0.5 micrograms. The combination of FIGs. 3-5 demonstrate the potency of identified lipid formulations with 3 different antigens and superiority compared to known lipids is antigen independent. Results further discussed in Example 5 herein.

FIG. 6 shows the effect of delivering a multivalent LNP (prepared using compound I-4) vaccine targeting H1N1 influenza (PR8), RSV (A2), and SARS-CoV-2 (WA-1) viruses. In all, the data indicates that the delivery performance of the multivalent LNPs was comparable to that of each respective monovalent LNP. The graphs (from left to right) show neutralizing antibody (nAb) titers in response to H1N1 influenza (0.2 μg), RSV-PreF (0.2 μg), and SARS-CoV-2 RBD

(0.5 μg). Titers were measured 14 days following prime / boost vaccination of BALB/c mice (10/group) with either monovalent, comixed trivalent, or co-formulated LNPs prepared with cationic lipid compound I-4.

FIG. 7A shows a biodistribution for luciferase expression (pg / total tissue) in organs harvested 4 hours after intramuscular administration to BALB/c mice (3/group) with 0.2 micrograms of Fluc mRNA-LNPs. The number above the bars indicates the ratio of lymph node + spleen to liver expression.

FIG. 7B also shows a biodistribution for luciferase expression after intramuscular administration to BALB/c mice (3/group) with 2 micrograms of Fluc mRNA-LNPs. Live whole-body imaging was performed at 4-, 24-, and 48-hours post dose. This graph illustrates the ratio of liver to injection site signal as determined from calculated area under the curve (AUC) from 0 to 48 hours.

Biodistribution data indicated higher secondary lymphoid exposure than liver expression compared to LNPs prepared with cationic lipid compound I-1.

Each LNP formulation shown in FIG. 7A and 7B were prepared with cationic lipids as follows:

LNP Formulation	Cationic Lipid
LNP 2	Compound I-2
LNP 3	Compound I-4
LNP 4	Compound I-5
LNP 5	Compound I-3
LNP 6	Compound I-6
LNP 16	Compound I-15
LNP 22	Compound I-19

DETAILED DESCRIPTION

In the following description, certain specific details are set forth to provide a thorough understanding of various embodiments of the disclosure. However, one skilled in the art will understand that the embodiments of the disclosure may be practiced without these details.

The present disclosure is based, in part, upon the discovery of a novel method for administering lipid nanoparticles that provides advantages when used for the *in vivo* delivery of an active or therapeutic agent such as a nucleic acid into a cell of a mammal. Embodiments of the present disclosure provide nucleic acid-lipid nanoparticle compositions comprising one or more cationic lipids described herein that provide increased activity of the nucleic acid and improved tolerability of the compositions *in vivo*, resulting in a significant increase in the

therapeutic index as compared to nucleic acid-lipid nanoparticle compositions previously described.

In certain embodiments, the present disclosure provides cationic lipids that enable the formulation of improved compositions for the *in vitro* and *in vivo* delivery of mRNA and/or other oligonucleotides. In some embodiments, these improved lipid nanoparticle compositions are useful for expression of protein encoded by mRNA. In other embodiments, these improved lipid nanoparticles compositions are useful for upregulation of endogenous protein expression by delivering miRNA inhibitors targeting one specific miRNA or a group of miRNA regulating one target mRNA or several mRNA. In other embodiments, these improved lipid nanoparticle compositions are useful for down-regulating (*e.g.*, silencing) the protein levels and/or mRNA levels of target genes. In some other embodiments, lipid nanoparticles are also useful for delivery of mRNA and plasmids for expression of transgenes. In yet other embodiments, the lipid nanoparticle compositions are useful for inducing a pharmacological effect resulting from expression of a protein, *e.g.*, increased production of red blood cells through the delivery of a suitable erythropoietin mRNA, or protection against infection through delivery of mRNA encoding for a suitable antibody.

The lipid nanoparticles and compositions of the present disclosure may be used for a variety of purposes, including the delivery of encapsulated or associated (*e.g.*, complexed) therapeutic agents such as nucleic acids to cells, both *in vitro* and *in vivo*. Accordingly, embodiments of the present disclosure provide methods of treating or preventing diseases or disorders in a subject in need thereof by contacting the subject with a lipid nanoparticle that encapsulates or is associated with a suitable therapeutic agent, wherein the lipid nanoparticle comprises one or more of the cationic lipids described herein.

As described herein, embodiments of the lipid nanoparticles of the present disclosure are particularly useful for the delivery of nucleic acids, including, *e.g.*, mRNA, antisense oligonucleotide, plasmid DNA, microRNA (miRNA), miRNA inhibitors (antagomirs/antimirs), messenger-RNA-interfering complementary RNA (micRNA), DNA, multivalent RNA, dicer substrate RNA, complementary DNA (cDNA), *etc*. Therefore, the lipid nanoparticles and compositions of the present disclosure may be used to induce expression of a desired protein both *in vitro* and *in vivo* by contacting cells with a lipid nanoparticle comprising one or more cationic lipids described herein, wherein the lipid nanoparticle encapsulates or is associated with a nucleic acid that is expressed to produce the desired protein (*e.g.*, a messenger RNA or plasmid encoding the desired protein). Alternatively, the lipid nanoparticles and compositions of the

present disclosure may be used to decrease the expression of target genes and proteins both *in vitro* and *in vivo* by contacting cells with a lipid nanoparticle comprising one or more cationic lipids described herein, wherein the lipid nanoparticle encapsulates or is associated with a nucleic acid that reduces target gene expression (*e.g.*, an antisense oligonucleotide or small interfering RNA (siRNA)). The lipid nanoparticles and compositions of the present disclosure may also be used for co-delivery of different nucleic acids (*e.g.*, mRNA and plasmid DNA) separately or in combination, such as may be useful to provide an effect requiring co-localization of different nucleic acids (*e.g.*, mRNA encoding for a suitable gene modifying enzyme and DNA segment(s) for incorporation into the host genome).

Nucleic acids for use with this disclosure may be prepared according to any available technique. For mRNA, the primary methodology of preparation is, but not limited to, enzymatic synthesis (also termed *in vitro* transcription) which currently represents the most efficient method to produce long sequence-specific mRNA. *In vitro* transcription describes a process of template-directed synthesis of RNA molecules from an engineered DNA template comprised of an upstream bacteriophage promoter sequence (*e.g.*, including but not limited to that from the T7, T3, and SP6 coliphage) linked to a downstream sequence encoding the gene of interest. Template DNA can be prepared for *in vitro* transcription from a number of sources with appropriate techniques which are well known in the art including, but not limited to, plasmid DNA and polymerase chain reaction amplification (see Linpinsel, J.L and Conn, G.L., General protocols for preparation of plasmid DNA template and Bowman, J.C., Azizi, B., Lenz, T.K., Ray, P., and Williams, L.D. in RNA *in vitro* transcription and RNA purification by denaturing PAGE in Recombinant and *in vitro* RNA syntheses Methods v. 941 Conn G.L. (ed), New York, N.Y. Humana Press, 2012).

Transcription of the RNA occurs *in vitro* using the linearized DNA template in the presence of the corresponding RNA polymerase and adenosine, guanosine, uridine, and cytidine ribonucleoside triphosphates (rNTPs) under conditions that support polymerase activity while minimizing potential degradation of the resultant mRNA transcripts. *In vitro* transcription can be performed using a variety of commercially available kits including, but not limited to RiboMax Large Scale RNA Production System (Promega), MegaScript Transcription kits (Life Technologies) as well as with commercially available reagents including RNA polymerases and rNTPs. The methodology for *in vitro* transcription of mRNA is well known in the art. (see, *e.g.*, Losick, R., 1972, *In vitro* transcription, Ann Rev Biochem v.41 409-46; Kamakaka, R. T. and Kraus, W. L. 2001. *In Vitro* Transcription. Current Protocols in Cell Biology. 2:11.6:11.6.1–

11.6.17; Beckert, B. And Masquida, B., (2010) Synthesis of RNA by *In Vitro* Transcription in RNA in Methods in Molecular Biology v. 703 (Neilson, H. Ed), New York, N.Y. Humana Press, 2010; Brunelle, J.L. and Green, R., 2013, Chapter Five – *In vitro* transcription from plasmid or PCR-amplified DNA, Methods in Enzymology v. 530, 101-114; all of which are incorporated herein by reference).

The desired *in vitro* transcribed mRNA is then purified from the undesired components of the transcription or associated reactions (including unincorporated rNTPs, protein enzyme, salts, short RNA oligos *etc.*). Techniques for the isolation of the mRNA transcripts are well known in the art. Well known procedures include phenol/chloroform extraction or precipitation with either alcohol (ethanol, isopropanol) in the presence of monovalent cations or lithium chloride. Additional, non-limiting examples of purification procedures which can be used include size exclusion chromatography (Lukavsky, P.J. and Puglisi, J.D., 2004, Large-scale preparation and purification of polyacrylamide-free RNA oligonucleotides, RNA v.10, 889-893), silica-based affinity chromatography and polyacrylamide gel electrophoresis (Bowman, J.C., Azizi, B., Lenz, T.K., Ray, P., and Williams, L.D. in RNA *in vitro* transcription and RNA purification by denaturing PAGE in Recombinant and *in vitro* RNA syntheses Methods v. 941 Conn G.L. (ed), New York, N.Y. Humana Press, 2012). Purification can be performed using a variety of commercially available kits including, but not limited to SV Total Isolation System (Promega) and *In Vitro* Transcription Cleanup and Concentration Kit (Norgen Biotek).

Furthermore, while reverse transcription can yield large quantities of mRNA, the products can contain several aberrant RNA impurities associated with undesired polymerase activity which may need to be removed from the full-length mRNA preparation. These include short RNAs that result from abortive transcription initiation as well as double-stranded RNA (dsRNA) generated by RNA-dependent RNA polymerase activity, RNA-primed transcription from RNA templates and self-complementary 3' extension. It has been demonstrated that these contaminants with dsRNA structures can lead to undesired immunostimulatory activity through interaction with various innate immune sensors in eukaryotic cells that function to recognize specific nucleic acid structures and induce potent immune responses. This in turn, can dramatically reduce mRNA translation since protein synthesis is reduced during the innate cellular immune response. Therefore, additional techniques to remove these dsRNA contaminants have been developed and are known in the art including but not limited to scalable HPLC purification (see, *e.g.,* Kariko, K., Muramatsu, H., Ludwig, J. And Weissman, D., 2011, Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and

improves translation of nucleoside-modified, protein-encoding mRNA, Nucl Acid Res, v. 39 e142; Weissman, D., Pardi, N., Muramatsu, H., and Kariko, K., HPLC Purification of *in vitro* transcribed long RNA in Synthetic Messenger RNA and Cell Metabolism Modulation in Methods in Molecular Biology v.969 (Rabinovich, P.H. Ed), 2013). HPLC purified mRNA has been reported to be translated at much greater levels, particularly in primary cells and *in vivo*.

A significant variety of modifications have been described in the art which are used to alter specific properties of *in vitro* transcribed mRNA and improve its utility. These include but are not limited to modifications to the 5' and 3' termini of the mRNA. Endogenous eukaryotic mRNA typically contains a cap structure on the 5′-end of a mature molecule which plays an important role in mediating binding of the mRNA Cap Binding Protein (CBP), which is in turn responsible for enhancing mRNA stability in the cell and efficiency of mRNA translation. Therefore, highest levels of protein expression are achieved with capped mRNA transcripts. The 5′-cap contains a 5′-5′-triphosphate linkage between the 5′-most nucleotide and guanine nucleotide. The conjugated guanine nucleotide is methylated at the N7 position. Additional modifications include methylation of the ultimate and penultimate most 5′-nucleotides on the 2′-hydroxyl group.

Multiple distinct cap structures can be used to generate the 5′-cap of *in vitro* transcribed synthetic mRNA. 5'-capping of synthetic mRNA can be performed co-transcriptionally with chemical cap analogs (*i.e.*, capping during *in vitro* transcription). For example, the Anti-Reverse Cap Analog (ARCA) cap contains a 5′-5′-triphosphate guanine-guanine linkage where one guanine contains an N7 methyl group as well as a 3′-O-methyl group. However, up to 20% of transcripts remain uncapped during this co-transcriptional process and the synthetic cap analog is not identical to the 5′-cap structure of an authentic cellular mRNA, potentially reducing translatability and cellular stability. Alternatively, synthetic mRNA molecules may also be enzymatically capped post-transcriptionally. These may generate a more authentic 5′-cap structure that more closely mimics, either structurally or functionally, the endogenous 5'-cap which have enhanced binding of cap binding proteins, increased half-life, reduced susceptibility to 5′ endonucleases and/or reduced 5′ decapping. Numerous synthetic 5'-cap analogs have been developed and are known in the art to enhance mRNA stability and translatability (see, *e.g.*, Grudzien-Nogalska, E., Kowalska, J., Su, W., Kuhn, A.N., Slepenkov, S.V., Darynkiewicz, E., Sahin, U., Jemielity, J., and Rhoads, R.E., Synthetic mRNAs with superior translation and stability properties in Synthetic Messenger RNA and Cell Metabolism Modulation in Methods in Molecular Biology v.969 (Rabinovich, P.H. Ed), 2013).

On the 3'-terminus, a long chain of adenine nucleotides (poly-A tail) is normally added to mRNA molecules during RNA processing. Immediately after transcription, the 3′ end of the transcript is cleaved to free a 3′ hydroxyl to which poly-A polymerase adds a chain of adenine nucleotides to the RNA in a process called polyadenylation. The poly-A tail has been extensively shown to enhance both translational efficiency and stability of mRNA (see Bernstein, P. and Ross, J., 1989, Poly (A), poly (A) binding protein and the regulation of mRNA stability, Trends Bio Sci v. 14 373-377; Guhaniyogi, J. And Brewer, G., 2001, Regulation of mRNA stability in mammalian cells, Gene, v. 265, 11-23; Dreyfus, M. And Regnier, P., 2002, The poly (A) tail of mRNAs: Bodyguard in eukaryotes, scavenger in bacteria, Cell, v.111, 611-613).

Poly (A) tailing of *in vitro* transcribed mRNA can be achieved using various approaches including, but not limited to, cloning of a poly (T) tract into the DNA template or by post-transcriptional addition using Poly (A) polymerase. The first case allows *in vitro* transcription of mRNA with poly (A) tails of defined length, depending on the size of the poly (T) tract, but requires additional manipulation of the template. The latter case involves the enzymatic addition of a poly (A) tail to *in vitro* transcribed mRNA using poly (A) polymerase which catalyzes the incorporation of adenine residues onto the 3'termini of RNA, requiring no additional manipulation of the DNA template, but results in mRNA with poly(A) tails of heterogenous length. 5'-capping and 3'-poly (A) tailing can be performed using a variety of commercially available kits including, but not limited to Poly (A) Polymerase Tailing kit (EpiCenter), mMESSAGE mMACHINE T7 Ultra kit and Poly (A) Tailing kit (Life Technologies) as well as with commercially available reagents, various ARCA caps, Poly (A) polymerase, *etc.*

In addition to 5' cap and 3' poly adenylation, other modifications of the *in vitro* transcripts have been reported to provide benefits as related to efficiency of translation and stability. It is well known in the art that pathogenic DNA and RNA can be recognized by a variety of sensors within eukaryotes and trigger potent innate immune responses. The ability to discriminate between pathogenic and self-DNA and RNA has been shown to be based, at least in part, on structure and nucleoside modifications since most nucleic acids from natural sources contain modified nucleosides In contrast, *in vitro* synthesized RNA lacks these modifications, thus rendering it immunostimulatory which in turn can inhibit effective mRNA translation as outlined above. The introduction of modified nucleosides into *in vitro* transcribed mRNA can be used to prevent recognition and activation of RNA sensors, thus mitigating this undesired immunostimulatory activity and enhancing translation capacity (see, *e.g.*, Kariko, K. And Weissman, D. 2007, Naturally occurring nucleoside modifications suppress the

immunostimulatory activity of RNA: implication for therapeutic RNA development, Curr Opin Drug Discov Devel, v.10 523-532; Pardi, N., Muramatsu, H., Weissman, D., Kariko, K., *In vitro* transcription of long RNA containing modified nucleosides in Synthetic Messenger RNA and Cell Metabolism Modulation in Methods in Molecular Biology v.969 (Rabinovich, P.H. Ed), 2013); Kariko, K., Muramatsu, H., Welsh, F.A., Ludwig, J., Kato, H., Akira, S., Weissman, D., 2008, Incorporation of Pseudouridine Into mRNA Yields Superior Nonimmunogenic Vector With Increased Translational Capacity and Biological Stability, Mol Ther v.16, 1833-1840. The modified nucleosides and nucleotides used in the synthesis of modified RNAs can be prepared monitored and utilized using general methods and procedures known in the art. A large variety of nucleoside modifications are available that may be incorporated alone or in combination with other modified nucleosides to some extent into the *in vitro* transcribed mRNA (see, *e.g.*, US Publication No. 2012/0251618). *In vitro* synthesis of nucleoside-modified mRNA has been reported to have reduced ability to activate immune sensors with a concomitant enhanced translational capacity.

Other components of mRNA which can be modified to provide benefit in terms of translatability and stability include the 5′ and 3' untranslated regions (UTR). Optimization of the UTRs (favorable 5' and 3' UTRs can be obtained from cellular or viral RNAs), either both or independently, have been shown to increase mRNA stability and translational efficiency of *in vitro* transcribed mRNA (see, *e.g.*, Pardi, N., Muramatsu, H., Weissman, D., Kariko, K., *In vitro* transcription of long RNA containing modified nucleosides in Synthetic Messenger RNA and Cell Metabolism Modulation in Methods in Molecular Biology v.969 (Rabinovich, P.H. Ed), 2013).

In addition to mRNA, other nucleic acid payloads may be used for this disclosure. For oligonucleotides, methods of preparation include but are not limited to chemical synthesis and enzymatic, chemical cleavage of a longer precursor, *in vitro* transcription as described above, *etc*. Methods of synthesizing DNA and RNA nucleotides are widely used and well known in the art (see, *e.g.*, Gait, M. J. (ed.)Oligonucleotide synthesis: a practical approach, Oxford [Oxfordshire], Washington, D.C.: IRL Press, 1984; and Herdewijn, P. (ed.) Oligonucleotide synthesis: methods and applications, Methods in Molecular Biology, v. 288 (Clifton, N.J.) Totowa, N.J.: Humana Press, 2005; both of which are incorporated herein by reference).

For plasmid DNA, preparation for use with this disclosure commonly utilizes but is not limited to expansion and isolation of the plasmid DNA *in vitro* in a liquid culture of bacteria containing the plasmid of interest. The presence of a gene in the plasmid of interest that encodes

resistance to a particular antibiotic (penicillin, kanamycin, *etc.*) allows those bacteria containing the plasmid of interest to selective grow in antibiotic-containing cultures. Methods of isolating plasmid DNA are widely used and well known in the art (see, *e.g.*, Heilig, J., Elbing, K. L. and Brent, R (2001) Large-Scale Preparation of Plasmid DNA. Current Protocols in Molecular Biology. 41:II:1.7:1.7.1–1.7.16; Rozkov, A., Larsson, B., Gillström, S., Björnestedt, R. and Schmidt, S. R. (2008), Large-scale production of endotoxin-free plasmids for transient expression in mammalian cell culture. Biotechnol. Bioeng., 99: 557–566; and US Patent No. 6,197,553). Plasmid isolation can be performed using a variety of commercially available kits including, but not limited to Plasmid Plus (Qiagen), GenJET plasmid MaxiPrep (Thermo) and PureYield MaxiPrep (Promega) kits as well as with commercially available reagents.

Various exemplary embodiments of the cationic lipids of the present disclosure, lipid nanoparticles and compositions used with the same, and uses to deliver active or therapeutic agents such as nucleic acids to modulate gene and protein expression, are described in further detail below.

As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

Unless the context requires otherwise, throughout the present specification and claims, the word "comprise" and variations thereof, such as, "comprises" and "comprising" are to be construed in an open and inclusive sense, that is, as "including, but not limited to".

Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment of the present disclosure. Thus, the appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this disclosure belongs. As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise.

The phrase "induce expression of a desired protein" refers to the ability of a nucleic acid to increase expression of the desired protein. To examine the extent of protein expression, a test sample (*e.g.*, a sample of cells in culture expressing the desired protein) or a test mammal (*e.g.*, a

mammal such as a human or an animal model such as a rodent (*e.g.*, mouse) or a non-human primate (*e.g.*, monkey) model) is contacted with a nucleic acid (*e.g.*, nucleic acid in combination with a lipid of the present disclosure). Expression of the desired protein in the test sample or test animal is compared to expression of the desired protein in a control sample (*e.g.*, a sample of cells in culture expressing the desired protein) or a control mammal (*e.g.*, a mammal such as a human or an animal model such as a rodent (*e.g.*, mouse) or non-human primate (*e.g.*, monkey) model) that is not contacted with or administered the nucleic acid. When the desired protein is present in a control sample or a control mammal, the expression of a desired protein in a control sample or a control mammal may be assigned a value of 1.0. In some embodiments, inducing expression of a desired protein is achieved when the ratio of desired protein expression in the test sample or the test mammal to the level of desired protein expression in the control sample or the control mammal is greater than 1, for example, about 1.1, 1.5, 2.0. 5.0 or 10.0. When a desired protein is not present in a control sample or a control mammal, inducing expression of a desired protein is achieved when any measurable level of the desired protein in the test sample or the test mammal is detected. One of ordinary skill in the art will understand appropriate assays to determine the level of protein expression in a sample, for example dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, and phenotypic assays, or assays based on reporter proteins that can produce fluorescence or luminescence under appropriate conditions.

The phrase "inhibiting expression of a target gene" refers to the ability of a nucleic acid to silence, reduce, or inhibit the expression of a target gene. To examine the extent of gene silencing, a test sample (*e.g.*, a sample of cells in culture expressing the target gene) or a test mammal (*e.g.*, a mammal such as a human or an animal model such as a rodent (*e.g.*, mouse) or a non-human primate (*e.g.*, monkey) model) is contacted with a nucleic acid that silences, reduces, or inhibits expression of the target gene. Expression of the target gene in the test sample or test animal is compared to expression of the target gene in a control sample (*e.g.*, a sample of cells in culture expressing the target gene) or a control mammal (*e.g.*, a mammal such as a human or an animal model such as a rodent (*e.g.*, mouse) or non-human primate (*e.g.*, monkey) model) that is not contacted with or administered the nucleic acid. The expression of the target gene in a control sample or a control mammal may be assigned a value of 100%. In particular embodiments, silencing, inhibition, or reduction of expression of a target gene is achieved when the level of target gene expression in the test sample or the test mammal relative to the level of target gene expression in the control sample or the control mammal is about 95%, 90%, 85%,

80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. In other words, the nucleic acids are capable of silencing, reducing, or inhibiting the expression of a target gene by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% in a test sample or a test mammal relative to the level of target gene expression in a control sample or a control mammal not contacted with or administered the nucleic acid. Suitable assays for determining the level of target gene expression include, without limitation, examination of protein or mRNA levels using techniques known to those of skill in the art, such as, *e.g.*, dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

An "effective amount" or "therapeutically effective amount" of an active agent or therapeutic agent such as a therapeutic nucleic acid is an amount sufficient to produce the desired effect or outcome, *e.g.*, an increase or inhibition of expression of a target sequence in comparison to the normal expression level detected in the absence of the nucleic acid. An increase in expression of a target sequence is achieved when any measurable level is detected in the case of an expression product that is not present in the absence of the nucleic acid. In the case where the expression product is present at some level prior to contact with the nucleic acid, an in increase in expression is achieved when the fold increase in value obtained with a nucleic acid such as mRNA relative to control is about 1.05, 1.1, 1.2, 1.3, 1.4, 1.5, 1.75, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 75, 100, 250, 500, 750, 1000, 5000, 10000 or greater. Inhibition of expression of a target gene or target sequence is achieved when the value obtained with a nucleic acid such as antisense oligonucleotide relative to the control is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays for measuring expression of a target gene or target sequence include, *e.g.*, examination of protein or RNA levels using techniques known to those of skill in the art such as dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, fluorescence or luminescence of suitable reporter proteins, as well as phenotypic assays known to those of skill in the art.

The term "nucleic acid" as used herein refers to a polymer containing at least two deoxyribonucleotides or ribonucleotides in either single- or double-stranded form and includes DNA, RNA, and hybrids thereof. DNA may be in the form of antisense molecules, plasmid DNA, cDNA, PCR products, or vectors. RNA may be in the form of small hairpin RNA (shRNA), messenger RNA (mRNA), antisense RNA, miRNA, micRNA, multivalent RNA, dicer

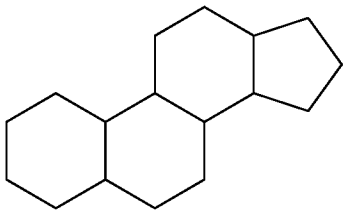
substrate RNA or viral RNA (vRNA), and combinations thereof. Nucleic acids include nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, and which have similar binding properties as the reference nucleic acid. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2′-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs). Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, single nucleotide polymorphisms, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res., 19:5081 (1991); Ohtsuka et al., J. Biol. Chem., 260:2605-2608 (1985); Rossolini et al., Mol. Cell. Probes, 8:91-98 (1994)). "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides.

The term "gene" refers to a nucleic acid (*e.g.*, DNA or RNA) sequence that comprises partial length or entire length coding sequences necessary to produce a polypeptide or precursor polypeptide.

"Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

The term "lipid" refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are generally characterized by being poorly soluble in water, but soluble in many organic solvents. They are usually divided into at least three classes: (1) "simple lipids," which include fats and oils as well as waxes; (2) "compound lipids," which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids.

A "steroid" is a compound comprising the following carbon skeleton:



Non-limiting examples of steroids include cholesterol, and the like.

A "cationic lipid" refers to a lipid capable of being positively charged. Exemplary cationic lipids include one or more amine group(s) which bear the positive charge. Preferred cationic lipids are ionizable such that they can exist in a positively charged or neutral form depending on pH. The ionization of the cationic lipid affects the surface charge of the lipid nanoparticle under different pH conditions. This charge state can influence plasma protein absorption, blood clearance and tissue distribution (Semple, S.C., et al., Adv. Drug Deliv Rev 32:3-17 (1998)) as well as the ability to form endosomolytic non-bilayer structures (Hafez, I.M., et al., Gene Ther 8:1188-1196 (2001)) critical to the intracellular delivery of nucleic acids.

The term "lipid nanoparticle" refers to particles having at least one dimension on the order of nanometers (*e.g.*, 1-1,000 nm) which include one or more of the compounds of Formula (I) or other specified cationic lipids. In some embodiments, lipid nanoparticles are included in a formulation that can be used to deliver an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, mRNA) to a target site of interest (*e.g.*, cell, tissue, organ, tumor, and the like). In some embodiments, the lipid nanoparticles of the disclosure comprise a nucleic acid. Such lipid nanoparticles typically comprise a compound of Formula (I) and one or more excipient selected from neutral lipids, charged lipids, steroids, and polymer conjugated lipids. In some embodiments, the active agent or therapeutic agent, such as a nucleic acid, may be encapsulated in the lipid portion of the lipid nanoparticle or an aqueous space enveloped by some or all the lipid portion of the lipid nanoparticle, thereby protecting it from enzymatic degradation or other undesirable effects induced by the mechanisms of the host organism or cells, *e.g.*, an adverse immune response.

In various embodiments, the lipid nanoparticles have a mean diameter of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm, and are substantially non-toxic. In certain embodiments, nucleic acids,

when present in the lipid nanoparticles, are resistant in aqueous solution to degradation with a nuclease. Lipid nanoparticles comprising nucleic acids and their method of preparation are disclosed in, *e.g.*, U.S. Patent Publication Nos. 2004/0142025, 2007/0042031 and PCT Pub. Nos. WO 2013/016058 and WO 2013/086373, the full disclosures of which are herein incorporated by reference in their entirety for all purposes.

As used herein, "lipid encapsulated" refers to a lipid nanoparticle that provides an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, mRNA), with full encapsulation, partial encapsulation, or both. In an embodiment, the nucleic acid (*e.g.*, mRNA) is fully encapsulated in the lipid nanoparticle.

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

The term "neutral lipid" refers to any of several lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, but are not limited to, phosphotidylcholines such as 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), phophatidylethanolamines such as 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), sphingomyelins (SM), ceramides, steroids such as sterols and their derivatives. Neutral lipids may be synthetic or naturally derived.

The term "charged lipid" refers to any of several lipid species that exist in either a positively charged or negatively charged form independent of the pH within a useful physiological range, *e.g.*, pH ~3 to pH ~9. Charged lipids may be synthetic or naturally derived. Examples of charged lipids include phosphatidylserines, phosphatidic acids, phosphatidylglycerols, phosphatidylinositols, sterol hemisuccinates, dialkyl trimethylammonium-propanes, (*e.g.*, DOTAP, DOTMA), dialkyl dimethylaminopropanes, ethyl phosphocholines, dimethylaminoethane carbamoyl sterols (*e.g.*, DC-Chol).

As used herein, the term "aqueous solution" refers to a composition comprising water.

"Serum-stable" in relation to nucleic acid-lipid nanoparticles means that the nucleotide is not significantly degraded after exposure to a serum or nuclease assay that would significantly

degrade free DNA or RNA. Suitable assays include, for example, a standard serum assay, a DNAse assay, or an RNAse assay.

"Systemic delivery," as used herein, refers to delivery of a therapeutic product that can result in a broad exposure of an active agent within an organism. Some techniques of administration can lead to the systemic delivery of certain agents, but not others. Systemic delivery means that a useful, preferably therapeutic, amount of an agent is exposed to most parts of the body. Systemic delivery of lipid nanoparticles can be by any means known in the art including, for example, intravenous, intraarterial, subcutaneous, and intraperitoneal delivery. In some embodiments, systemic delivery of lipid nanoparticles is by intravenous delivery.

"Local delivery," as used herein, refers to delivery of an active agent directly to a target site within an organism. For example, an agent can be locally delivered by direct injection into a disease site such as a tumor, other target site such as a site of inflammation, or a target organ such as the liver, heart, pancreas, kidney, and the like. Local delivery can also include topical applications or localized injection techniques such as intramuscular, subcutaneous, or intradermal injection. Local delivery does not preclude a systemic pharmacological effect.

"Alkyl" refers to a straight or branched hydrocarbon chain radical consisting solely of carbon and hydrogen atoms that is saturated (*i.e.,* contains no double and/or triple bonds), having from one to twenty-four carbon atoms (C_1-C_{24} alkyl), one to sixteen carbon atoms (C_1-C_{16} alkyl),one to twelve carbon atoms (C_1-C_{12} alkyl), six to twenty-four carbon atoms (C_6-C_{24} alkyl), one to eight carbon atoms (C_1-C_8 alkyl) or one to six carbon atoms (C_1-C_6 alkyl) and which is attached to the rest of the molecule by a single bond, *e.g.*, methyl, ethyl, n-propyl, 1-methylethyl (iso propyl), n-butyl, n-pentyl, 1,1-dimethylethyl (t-butyl), 3-methylhexyl, 2-methylhexyl, and the like. Unless stated otherwise specifically in the specification, an alkyl group is optionally substituted.

"Alkylhydroxyl" refers to an alkyl group, as defined herein, comprising at least one hydroxyl (OH) substituent.

"Alkenyl" refers to a straight or branched hydrocarbon chain radical consisting solely of carbon and hydrogen atoms that contains at least one carbon-carbon double, having from one to twenty-four carbon atoms (C_2-C_{24} alkenyl), one to twelve carbon atoms (C_2-C_{12} alkenyl), six to twenty-four carbon atoms (C_6-C_{24} alkenyl), two to sixteen carbon atoms (C_2-C_{16} alkenyl), four to twelve carbon atoms (C_4-C_{12} alkenyl), one to eight carbon atoms (C_2-C_8 alkenyl) or one to six carbon atoms (C_2-C_6 alkenyl) and which is attached to the rest of the molecule by a single bond, *e.g.*, ethenyl, n-propenyl, 1-methylethenyl, n-butenyl, n-pentenyl, 1,1-dimethylethenyl, 3-

methylhexenyl, 2-methylhexenyl, and the like. Unless stated otherwise specifically in the specification, an alkenyl group is optionally substituted.

"Alkynyl" refers to a straight or branched hydrocarbon chain radical consisting solely of carbon and hydrogen atoms that contains at least one carbon-carbon triple bond, having from one to twenty-four carbon atoms (C_2-C_{24} alkynyl), one to twelve carbon atoms (C_2-C_{12} alkynyl), one to eight carbon atoms (C_2-C_8 alkynyl) or one to six carbon atoms (C_2-C_6 alkynyl) and which is attached to the rest of the molecule by a single bond, *e.g.*, ethynyl, n-propynyl, 1-methylethynyl, n-butynyl, n-pentynyl, 1,1-dimethylethynyl, 3-methylhexynyl, 2-methylhexynyl, and the like. Unless stated otherwise specifically in the specification, an alkynyl group is optionally substituted.

"Alkylene" or "alkylene chain" refers to a straight or branched divalent saturated hydrocarbon chain linking the rest of the molecule to a radical group, consisting solely of carbon and hydrogen. In some embodiments, an alkylene chain has from one to twenty-four carbon atoms (C_1-C_{24} alkylene), one to fifteen carbon atoms (C_1-C_{15} alkylene),one to twelve carbon atoms (C_1-C_{12} alkylene), one to eight carbon atoms (C_1-C_8 alkylene), one to six carbon atoms (C_1-C_6 alkylene), four to six carbon atoms (C_4-C_6 alkylene),two to four carbon atoms (C_2-C_4 alkylene), one to two carbon atoms (C_1-C_2 alkylene), *e.g.*, methylene, ethylene, propylene, *n*-butylene, and the like. The alkylene chain is attached to the rest of the molecule through a single bond and to the radical group through a single bond. The points of attachment of the alkylene chain to the rest of the molecule and to the radical group can be through one carbon or any two carbons within the chain. Unless stated otherwise specifically in the specification, an alkylene chain is optionally substituted.

"Alkylene-cycloalkyl" refers to a radical of the formula -R_aR_b, wherein R_a is an alkylene, as defined herein, and R_b is a cycloalkyl, as defined herein. Unless stated otherwise specifically in the specification, an alkylene-cycloalkyl is optionally substituted.

"Alkenylene" or "alkenylene chain" refers to a straight or branched divalent hydrocarbon chain linking the rest of the molecule to a radical group, consisting solely of carbon and hydrogen and which comprises at least one carbon-carbon double bond. In some embodiments, an alkenylene chain has from two to twenty-four carbon atoms (C_2-C_{24} alkenylene), two to fifteen carbon atoms (C_2-C_{15} alkenylene), two to twelve carbon atoms (C_2-C_{12} alkenylene), two to eight carbon atoms (C_2-C_8 alkenylene), two to six carbon atoms (C_2-C_6 alkenylene), four to six carbon atoms (C_4-C_6 alkenylene),two to four carbon atoms (C_2-C_4 alkenylene), *e.g.*, ethenylene, propenylene, *n*-butenylene, and the like. The alkenylene chain is attached to the rest of the

molecule through a single bond and to the radical group through a single bond. The points of attachment of the alkenylene chain to the rest of the molecule and to the radical group can be through one carbon or any two carbons within the chain. Unless stated otherwise specifically in the specification, an alkenylene chain is optionally substituted.

"Alkenylene-cycloalkyl" refers to a radical of the formula $-R_aR_b$, wherein R_a is an alkenylene, as defined herein, and R_b is a cycloalkyl, as defined herein. Unless stated otherwise specifically in the specification, an alkenylene-cycloalkyl is optionally substituted.

"Cycloalkyl" or "carbocyclic ring" refers to a stable non aromatic monocyclic or polycyclic hydrocarbon radical consisting solely of carbon and hydrogen atoms, which may include fused or bridged ring systems, having from three to fifteen ring carbon atoms (C_3-C_{15}), from three to ten ring carbon atoms (C_3-C_{10}) or from three to eight ring carbon atoms (C_3-C_8), and which is saturated or unsaturated and attached to the rest of the molecule by a single bond. Monocyclic radicals include, for example, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl. Polycyclic radicals include, for example, adamantyl, norbornyl, decalinyl, 7,7-dimethyl bicyclo[2.2.1]heptanyl, and the like. Unless otherwise stated specifically in the specification, a cycloalkyl group is optionally substituted.

"Aryl" refers to a carbocyclic ring system radical comprising hydrogen, 6 to 18 carbon atoms and at least one aromatic ring. For purposes of this disclosure, the aryl radical is a monocyclic, bicyclic, tricyclic, or tetracyclic ring system, which may include fused or bridged ring systems. Aryl radicals include, but are not limited to, aryl radicals derived from aceanthrylene, acenaphthylene, acephenanthrylene, anthracene, azulene, benzene, chrysene, fluoranthene, fluorene, *as*-indacene, *s*-indacene, indane, indene, naphthalene, phenalene, phenanthrene, pleiadene, pyrene, and triphenylene.

"Arylalkyl" refers to a radical of the formula $-R_b$-R_c where R_b is an alkylene or alkenylene as defined above and R_c is one or more aryl radicals as defined above, for example, benzyl, diphenylmethyl and the like. Unless stated otherwise specifically in the specification, an arylalkyl group is optionally substituted.

"Heterocyclyl" or "heterocyclic ring" refers to a stable 3- to 18-membered non-aromatic ring radical having one to twelve ring carbon atoms (*e.g.*, two to twelve) and from one to six ring heteroatoms selected from the group consisting of nitrogen, oxygen, and sulfur. Unless stated otherwise specifically in the specification, the heterocyclyl radical is a monocyclic, bicyclic, tricyclic, or tetracyclic ring system, which may include fused, spirocyclic ("spiro-heterocyclyl") and/or bridged ring systems; and the nitrogen, carbon or sulfur atoms in the heterocyclyl radical

is optionally oxidized; the nitrogen atom is optionally quaternized; and the heterocyclyl radical is partially or fully saturated. Examples of such heterocyclyl radicals include, but are not limited to, dioxolanyl, thienyl[1,3]dithianyl, decahydroisoquinolyl, imidazolinyl, imidazolidinyl, isothiazolidinyl, isoxazolidinyl, morpholinyl, octahydroindolyl, octahydroisoindolyl, 2-oxopiperazinyl, 2-oxopiperidinyl, 2-oxopyrrolidinyl, oxazolidinyl, piperidinyl, piperazinyl, 4-piperidonyl, pyrrolidinyl, pyrazolidinyl, quinuclidinyl, thiazolidinyl, tetrahydrofuryl, trithianyl, tetrahydropyranyl, thiomorpholinyl, thiamorpholinyl, 1-oxo-thiomorpholinyl, and 1,1-dioxo-thiomorpholinyl. Unless stated otherwise specifically in the specification, a heterocyclyl group is optionally substituted.

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

"Optional" or "optionally" (*e.g.*, optionally substituted) means that the subsequently described event of circumstances may or may not occur, and that the description includes instances where said event or circumstance occurs and instances in which it does not. For example, "optionally substituted alkyl" means that the alkyl radical may or may not be substituted and that the description includes both substituted alkyl radicals and alkyl radicals having no substitution. In some embodiments, "optionally substituted" means a particular radical is substituted with one or more substituents selected from the group consisting of halo (*e.g.*, F, Cl, Br, and I), oxo (=O), hydroxyl (-OH), alkoxy (-OR^a, where R^a is C_1-C_{12} alkyl), cycloalkoxy (-OR^a, where R^a is C_3-C_8 cycloalkyl), carboxyl (-$OC(=O)R^a$ or –$C(=O)OR^a$, where R^a is H, C_1-C_{12} alkyl, or C_3-C_8 cycloalkyl), amine (-NR^aR^b, where R^a and R^b are each independently H, C_1-C_{12} alkyl, or C_3-C_8 cycloalkyl), C_1-C_{12} alkyl, and C_3-C_8 cycloalkyl.

In some embodiments, "optionally substituted" means substituted with one or more halo substituents. In some embodiments, "optionally substituted" means substituted with one or more oxo substituents. In some embodiments, "optionally substituted" means substituted with one or more hydroxyl substituents. In certain embodiments, "optionally substituted" means substituted with one or more alkoxy substituents. In some embodiments, "optionally substituted" means substituted with one or more cycloalkoxy substituents. In certain embodiments, "optionally substituted" means substituted with one or more carboxy substituents. In some embodiments, "optionally substituted" means substituted with one or more amine substituents. In certain embodiments, "optionally substituted" means substituted with one or more C_1-C_{12} alkyl substituents. In some embodiments, "optionally substituted" means substituted with one or more C_3-C_8 cycloalkyl substituents.

When a functional group is described as "optionally substituted," and in turn, substituents on the functional group are also "optionally substituted" and so on, for the purposes of this disclosure, such iterations are limited to five, preferably such iterations are limited to two. In some embodiments, such iterations are limited to one. In some embodiments, such iterations are limited to zero.

This disclosure is also meant to encompass all pharmaceutically acceptable cationic lipids (*e.g.*, compounds of Formula (I)) being isotopically labelled by having one or more atoms replaced by an atom having a different atomic mass or mass number. Examples of isotopes that can be incorporated into the disclosed compounds include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorous, fluorine, chlorine, and iodine, such as ^{2}H, ^{3}H, ^{11}C, ^{13}C, ^{14}C, ^{13}N, ^{15}N, ^{15}O, ^{17}O, ^{18}O, ^{31}P, ^{32}P, ^{35}S, ^{18}F, ^{36}Cl, ^{123}I, and ^{125}I, respectively. These radiolabeled compounds could be useful to help determine or measure the effectiveness of the compounds, by characterizing, for example, the site or mode of action, or binding affinity to pharmacologically important site of action. Certain isotopically labelled cationic lipids (*e.g.*, compounds of Formula (I)), for example, those incorporating a radioactive isotope, are useful in drug and/or substrate tissue distribution studies. The radioactive isotopes tritium, *i.e.*, ^{3}H, and carbon-14, *i.e.*, ^{14}C, are particularly useful for this purpose in view of their ease of incorporation and ready means of detection.

Substitution with heavier isotopes such as deuterium, *i.e.*, ^{2}H, may afford certain therapeutic advantages resulting from greater metabolic stability, for example, increased *in vivo* half-life or reduced dosage requirements, and hence may be preferred in some circumstances.

Substitution with positron emitting isotopes, such as ^{11}C, ^{18}F, ^{15}O and ^{13}N, can be useful in Positron Emission Topography (PET) studies for examining substrate receptor occupancy. Isotopically labeled cationic lipids (*e.g.*, compounds of Formula (I)) can generally be prepared by conventional techniques known to those skilled in the art or by processes analogous to those described in the Preparations and Examples as set out below using an appropriate isotopically labeled reagent in place of the non-labeled reagent previously employed.

This disclosure is also meant to encompass the *in vivo* metabolic products of the disclosed compounds. Such products may result from, for example, the oxidation, reduction, hydrolysis, amidation, esterification, and the like of the administered compound, primarily due to enzymatic processes. Accordingly, the disclosure includes compounds produced by a process comprising administering a compound of this disclosure to a mammal for a period sufficient to yield a metabolic product thereof. Such products are typically identified by administering a radiolabeled compound of the disclosure in a detectable dose to an animal, such as rat, mouse, guinea pig, monkey, or to human, allowing sufficient time for metabolism to occur, and isolating its conversion products from the urine, blood, or other biological samples.

"Stable compound" and "stable structure" are meant to indicate a compound that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and formulation into an efficacious therapeutic agent.

"Mammal" includes humans and both domestic animals such as laboratory animals and household pets (*e.g.*, cats, dogs, swine, cattle, sheep, goats, horses, rabbits), and non-domestic animals such as wildlife and the like.

"Pharmaceutically acceptable carrier, diluent or excipient" includes without limitation any adjuvant, carrier, excipient, glidant, sweetening agent, diluent, preservative, dye/colorant, flavor enhancer, surfactant, wetting agent, dispersing agent, suspending agent, stabilizer, isotonic agent, solvent, or emulsifier which has been approved by the United States Food and Drug Administration as being acceptable for use in humans or domestic animals.

"Pharmaceutically acceptable salt" includes both acid and base addition salts.

"Pharmaceutically acceptable acid addition salt" refers to those salts which retain the biological effectiveness and properties of the free bases, which are not biologically or otherwise undesirable, and which are formed with inorganic acids such as, but are not limited to, hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as, but not limited to, acetic acid, 2,2-dichloroacetic acid, adipic acid, alginic acid, ascorbic acid, aspartic acid, benzenesulfonic acid, benzoic acid, 4-acetamidobenzoic acid,

camphoric acid, camphor-10-sulfonic acid, capric acid, caproic acid, caprylic acid, carbonic acid, cinnamic acid, citric acid, cyclamic acid, dodecylsulfuric acid, ethane-1,2-disulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, formic acid, fumaric acid, galactaric acid, gentisic acid, glucoheptonic acid, gluconic acid, glucuronic acid, glutamic acid, glutaric acid, 2-oxo-glutaric acid, glycerophosphoric acid, glycolic acid, hippuric acid, isobutyric acid, lactic acid, lactobionic acid, lauric acid, maleic acid, malic acid, malonic acid, mandelic acid, methanesulfonic acid, mucic acid, naphthalene-1,5-disulfonic acid, naphthalene-2-sulfonic acid, 1-hydroxy-2-naphthoic acid, nicotinic acid, oleic acid, orotic acid, oxalic acid, palmitic acid, pamoic acid, propionic acid, pyroglutamic acid, pyruvic acid, salicylic acid, 4-aminosalicylic acid, sebacic acid, stearic acid, succinic acid, tartaric acid, thiocyanic acid, *p*-toluenesulfonic acid, trifluoroacetic acid, undecylenic acid, and the like.

"Pharmaceutically acceptable base addition salt" refers to those salts which retain the biological effectiveness and properties of the free acids, which are not biologically or otherwise undesirable. These salts are prepared from addition of an inorganic base or an organic base to the free acid. Salts derived from inorganic bases include, but are not limited to, the sodium, potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, aluminum salts and the like. Preferred inorganic salts are the ammonium, sodium, potassium, calcium, and magnesium salts. Salts derived from organic bases include, but are not limited to, salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as ammonia, isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, diethanolamine, ethanolamine, deanol, 2-dimethylaminoethanol, 2-diethylaminoethanol, dicyclohexylamine, lysine, arginine, histidine, caffeine, procaine, hydrabamine, choline, betaine, benethamine, benzathine, ethylenediamine, glucosamine, methylglucamine, theobromine, triethanolamine, tromethamine, purines, piperazine, piperidine, *N*-ethylpiperidine, polyamine resins and the like. Particularly preferred organic bases are isopropylamine, diethylamine, ethanolamine, trimethylamine, dicyclohexylamine, choline and caffeine.

Often crystallizations produce a solvate of the compound of the disclosure. As used herein, the term "solvate" refers to an aggregate that comprises one or more molecules of a compound of the disclosure with one or more molecules of solvent. The solvent may be water, in which case the solvate may be a hydrate. Alternatively, the solvent may be an organic solvent. Thus, the compounds of the present disclosure may exist as a hydrate, including a monohydrate, dihydrate, hemihydrate, sesquihydrate, trihydrate, tetrahydrate and the like, as well as the

corresponding solvated forms. The compound of the disclosure may be true solvates, while in other cases, the compound of the disclosure may merely retain adventitious water or be a mixture of water plus some adventitious solvent.

A "pharmaceutical composition" refers to a formulation of a compound of the disclosure and a medium generally accepted in the art for the delivery of the biologically active compound to mammals, *e.g.*, humans. Such a medium includes all pharmaceutically acceptable carriers, diluents, or excipients therefore.

"Treating" or "treatment" as used herein covers the treatment of the disease or condition of interest in a mammal, preferably a human, having the disease or condition of interest, and includes:

(i) preventing the disease or condition from occurring in a mammal, in particular, when such mammal is predisposed to the condition but has not yet been diagnosed as having it;

(ii) inhibiting the disease or condition, *i.e.*, arresting its development;

(iii) relieving the disease or condition, *i.e.*, causing regression of the disease or condition; or

(iv) relieving the symptoms resulting from the disease or condition, *i.e.*, relieving pain without addressing the underlying disease or condition. As used herein, the terms "disease" and "condition" may be used interchangeably or may be different in that the malady or condition may not have a known causative agent (so that etiology has not yet been worked out) and it is therefore not yet recognized as a disease but only as an undesirable condition or syndrome, wherein a more or less specific set of symptoms have been identified by clinicians.

The compounds of the disclosure, or their pharmaceutically acceptable salts may contain one or more asymmetric centers and may thus give rise to enantiomers, diastereomers, and other stereoisomeric forms that may be defined, in terms of absolute stereochemistry, as (*R*)- or (*S*)- or, as (D)- or (L)- for amino acids. The present disclosure is meant to include all such possible isomers, as well as their racemic and optically pure forms. Optically active (+) and (-), (*R*)- and (*S*)-, or (D)- and (L)- isomers may be prepared using chiral synthons or chiral reagents, or resolved using conventional techniques, for example, chromatography and fractional crystallization. Conventional techniques for the preparation/isolation of individual enantiomers include chiral synthesis from a suitable optically pure precursor or resolution of the racemate (or the racemate of a salt or derivative) using, for example, chiral high-pressure liquid chromatography (HPLC). When the compounds described herein contain olefinic double bonds or other centers of geometric asymmetry, and unless specified otherwise, it is intended that the

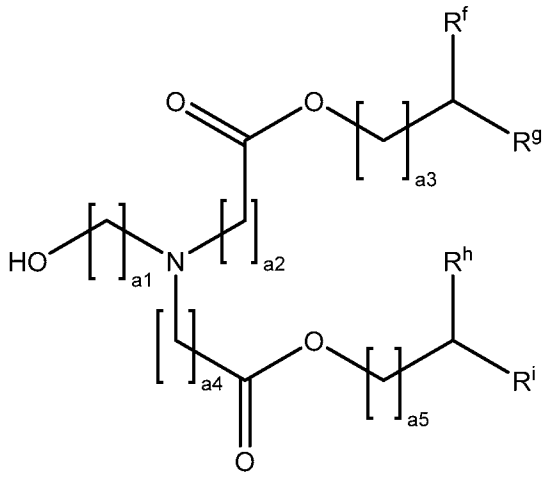
compounds include both E and Z geometric isomers. Likewise, all tautomeric forms are also intended to be included.

A "stereoisomer" refers to a compound made up of the same atoms bonded by the same bonds but having different three-dimensional structures, which are not interchangeable. The present disclosure contemplates various stereoisomers and mixtures thereof and includes "enantiomers", which refers to two stereoisomers whose molecules are non-superimposable mirror images of one another.

A "tautomer" refers to a proton shift from one atom of a molecule to another atom of the same molecule. The present disclosure includes tautomers of any said compounds.

Compounds

Some embodiments include the cationic lipids themselves. One embodiment provides a compound having the following Formula (A):



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

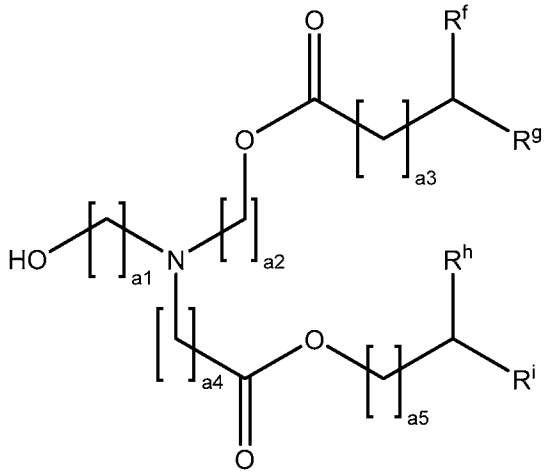
a1 is an integer from 1-12;

a2 and a4 are each independently an integer from 4-12;

a3 and a5 are each independently an integer from 0-3; and

R^f, R^g, R^h, and R^j are each independently a C_4-C_{12} alkyl, C_4-C_{12} alkenyl, or C_4-C_{12} alkynyl.

One embodiment provides a compound having the following Formula (B):



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

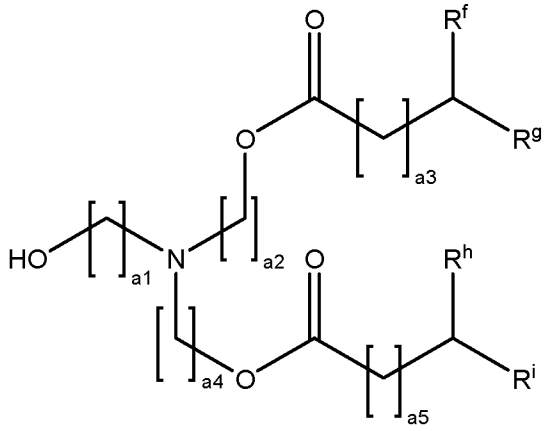
a1 is an integer from 1-12;

a2 and a4 are each independently an integer from 4-12;

a3 and a5 are each independently an integer from 0-3; and

R^f, R^g, R^h, and R^j are each independently a C_4-C_{12} alkyl, C_4-C_{12} alkenyl, or C_4-C_{12} alkynyl.

One embodiment provides a compound having the following Formula (C):



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

a1 is an integer from 1-12;

a2 and a4 are each independently an integer from 4-12;

a3 and a5 are each independently an integer from 0-3; and

R^f, R^g, R^h, and R^j are each independently a C_4-C_{12} alkyl, C_4-C_{12} alkenyl, or C_4-C_{12} alkynyl.

In some embodiments, a1 is 1, 2, 3, or 4. In some embodiments, a1 is 1, 2, 5, 6, 7, 8, 9, 10, 11, or 12. In certain embodiments, a1 is 4, 5, 6, 7, or 8. In some embodiments, a1 is 7, 8, 9, 10, 11, or 12.

In some embodiments, a2 is 4, 5, 6, 7, or 8. In certain embodiments, a2 is 7, 8, 9, 10, 11, or 12. In some embodiments, a4 is 4, 5, 6, 7, or 8. In certain embodiments, a4 is 7, 8, 9, 10, 11, or 12.

In some embodiments, a3 is 0 or 1. In some embodiments, a3 is 1, 2, or 3. In some embodiments, a5 is 0 or 1. In some embodiments, a5 is 1, 2, or 3.

In some embodiments, a3 and a5 are both 2 or 3.

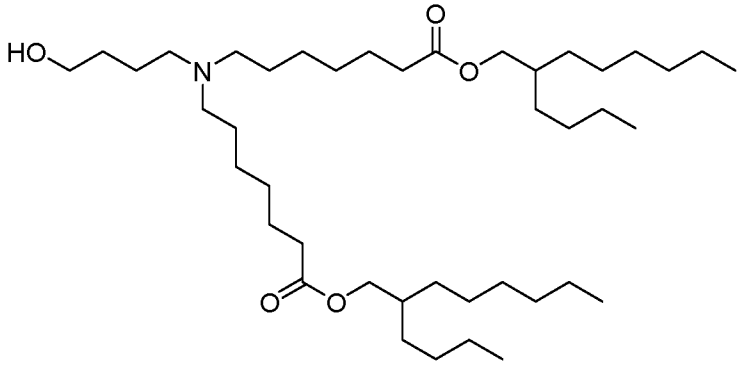
In some embodiments, R^f is C_4-C_{12} alkyl. In certain embodiments, R^f is C_4-C_{10} alkyl. In some embodiments, R^f is C_6-C_{12} alkyl. In certain embodiments, R^f is C_4 alkyl, C_6 alkyl, or C_8 alkyl. In some embodiments, R^f is C_4 alkyl. In some embodiments, R^f is C_6 alkyl. In some embodiments, R^f is C_8 alkyl.

In some embodiments, R^g is C_4-C_{12} alkyl. In certain embodiments, R^g is C_4-C_{10} alkyl. In some embodiments, R^g is C_6-C_{12} alkyl. In certain embodiments, R^g is C_4 alkyl, C_6 alkyl, or C_8 alkyl. In some embodiments, R^g is C_4 alkyl. In some embodiments, R^g is C_6 alkyl. In some embodiments, R^g is C_8 alkyl.

In some embodiments, R^h is C_4-C_{12} alkyl. In certain embodiments, R^h is C_4-C_{10} alkyl. In some embodiments, R^h is C_6-C_{12} alkyl. In certain embodiments, R^h is C_4 alkyl, C_6 alkyl, or C_8 alkyl. In some embodiments, R^h is C_4 alkyl. In some embodiments, R^h is C_6 alkyl. In some embodiments, R^h is C_8 alkyl.

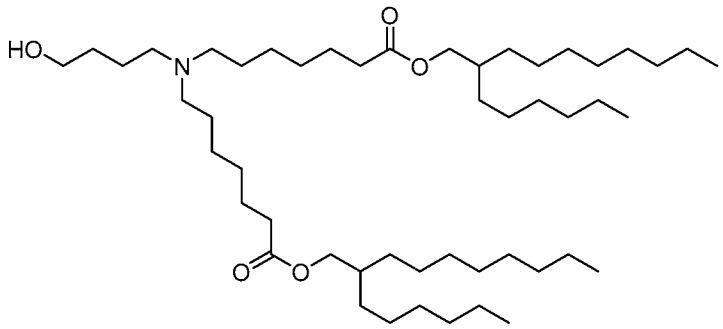
In some embodiments, R^i is C_4-C_{12} alkyl. In certain embodiments, R^i is C_4-C_{10} alkyl. In some embodiments, R^i is C_6-C_{12} alkyl. In certain embodiments, R^i is C_4 alkyl, C_6 alkyl, or C_8 alkyl. In some embodiments, R^i is C_4 alkyl. In some embodiments, R^i is C_6 alkyl. In some embodiments, R^i is C_8 alkyl.

For example, one embodiment provides a compound having the following structure:



or a salt, stereoisomer, or tautomer thereof.

One embodiment provides a compound having the following structure:



or a salt, stereoisomer, or tautomer thereof.

These compounds can be used in combination with other components (*e.g.*, one or more excipients selected from neutral lipids, steroids, and polymer conjugated lipids) to form a lipid nanoparticle. Additional embodiments describing lipid nanoparticles are detailed herein.

Methods

One embodiment provides a method of inducing an immune response in a subject, the method comprising intramuscular delivery of a lipid nanoparticle (LNP) to the subject, wherein the lipid nanoparticle comprises a nucleic acid that encodes an antigen and a cationic lipid, wherein:

a) the cationic lipid has a pKa ranging from 5.8 to 7.2;

b) the cationic lipid has a LogP value ranging from 12 to 25; and/or

c) the LNP has a spleen activity greater than 45 ng/g (*i.e.*, nanograms of luciferase per g of tissue – *e.g.*, at a dose of 0.3 mg, 0.5 mg, or 1.0 mg mRNA/kg and expressed as ng luciferase/g spleen).

One embodiment provides a method of inducing an immune response in a subject, the method comprising intramuscular delivery of a lipid nanoparticle (LNP) to the subject, wherein the lipid nanoparticle comprises a nucleic acid that encodes an antigen and a cationic lipid, wherein:

a) the cationic lipid has a pKa from about 5.8 to about 7.2;

b) the cationic lipid has a LogP value from about 12 to about 25; and/or

c) the LNP has a spleen activity greater than about 45 ng/g (*i.e.*, nanograms of luciferase per g of tissue – *e.g.*, at a dose of 0.3 mg, 0.5 mg, or 1.0 mg mRNA/kg and expressed as ng luciferase/g spleen).

In some embodiments, the cationic lipid has a pKa ranging from 5.8 to 7.2 and a LogP value ranging from 12 to 25. In some embodiments, the cationic lipid has a pKa ranging from 5.8 to 7.2 and the LNP has a ratio of spleen activity to liver activity of at least 1.3 to 1.

In some embodiments, the cationic lipid has a pKa from about 5.8 to about 7.2 and a LogP value from about 12 to about 25. In some embodiments, the cationic lipid has a pKa from about 5.8 to about 7.2 and the LNP has a ratio of spleen activity to liver activity of at least about 1.3 to about 1.

In some embodiments, the cationic lipid has a LogP value ranging from 12 to 25 and the LNP has a ratio of spleen activity to liver activity of at least 1.3 to 1. In some other embodiments, the cationic lipid has a pKa ranging from 5.8 to 7.2 and a LogP value ranging from 12 to 25 and the LNP has a ratio of spleen activity to liver activity of at least 1.3 to 1.

In some embodiments, the cationic lipid has a LogP value from about 12 to about 25 and the LNP has a ratio of spleen activity to liver activity of at least about 1.3 to about 1. In some other embodiments, the cationic lipid has a pKa from about 5.8 to about 7.2 and a LogP value from about 12 to about 25 and the LNP has a ratio of spleen activity to liver activity of at least about 1.3 to about 1.

In some embodiments, the cationic lipid has an ionizable headgroup. In certain embodiments, the cationic lipid has at least two alkyl tail groups comprising a C_4-C_{24} alkyl chain.

In certain embodiments, the pKa ranges from 5.8 to 7.4. In some embodiments, the pKa ranges from 5.8 to 6.2. In certain embodiments, the pKa ranges from 6.2 to 6.8. In some embodiments, the pKa ranges from 6.8 to 7.2. In certain embodiments, the pKa ranges from 7.2 to 7.4. In some embodiments the pKa ranges from 6.4 to 7.4. In some embodiments, the pKa ranges from 6.3 to 7.0. In certain embodiments, the pKa ranges from 6.3 to 7.1. In some embodiments, the pKa ranges from 6.4 to 7.2.

In certain embodiments, the pKa is from about 5.8 to about 7.4. In some embodiments, the pKa is from about 5.8 to about 6.2. In certain embodiments, the pKa is from about 6.2 to about 6.8. In some embodiments, the pKa is from about 6.8 to about 7.2. In certain embodiments, the pKa is from about 7.2 to about 7.4. In some embodiments the pKa is from about 6.4 to about 7.4. In some embodiments, the pKa is from about 6.3 to about 7.0. In certain embodiments, the pKa is from about 6.3 to about 7.1. In some embodiments, the pKa is from about 6.4 to about 7.2.

In some embodiments, the pKa of the cationic lipid is determined by sigmoidal best fit analysis applied to fluorescence data (*e.g.*, fluorescence intensity is measured at room temperature in a SLM Aminco Series 2 Luminescence Spectrophotometer using excitation and emission wavelengths of 321 nm and 445 nm. A sigmoidal best fit analysis is applied to the

fluorescence data and the pKa is measured as the pH giving rise to half-maximal fluorescence intensity, as described in PCT Publication No. WO 2015/1999952, which is hereby incorporated by reference for its disclosure of pKa determination).

In some embodiments, spleen activity is greater than 50 ng/g (*e.g.*, at a dose of 0.3 mg, 0.5 mg, or 1.0 mg mRNA/kg and expressed as ng luciferase/g spleen). In some embodiments, the spleen activity is greater than 125 ng/g. In certain embodiments, the spleen activity is greater than 200 ng/g, 400 ng/g, 500 ng/g, 1000 ng/g, or 1500 ng/g (*e.g.*, at a dose of 0.3 mg, 0.5 mg, or 1.0 mg mRNA/kg and expressed as ng luciferase/g spleen).

In some embodiments, spleen activity is greater than about 50 ng/g (*e.g.*, at a dose of 0.3 mg, 0.5 mg, or 1.0 mg mRNA/kg and expressed as ng luciferase/g spleen). In some embodiments, the spleen activity is greater than about 125 ng/g. In certain embodiments, the spleen activity is greater than about 200 ng/g, about 400 ng/g, 500 ng/g, 1000 ng/g, or 1500 ng/g (*e.g.*, at a dose of 0.3 mg, 0.5 mg, or 1.0 mg mRNA/kg and expressed as ng luciferase/g spleen).

In some embodiments, the spleen activity is greater than the spleen activity of an LNP prepared with Compound I-1. In some embodiments, the spleen activity is 1.05 times greater than the spleen activity of an LNP prepared with Compound I-1 (*i.e.*, if the spleen activity of the LNP prepared with Compound I-1 is 1, then the spleen activity of an LNP prepared according to the present disclosure is 1.05). In some embodiments, the spleen activity is 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.25, 2.5, 2.75, 3.0, 3.25, 3.5, 3.75, 4.0, 4.25, 4.5, 4.75, or 5.0 times greater than the spleen activity of an LNP prepared with Compound I-1 (*e.g.*, at a dose of 0.3 mg, 0.5 mg, or 1.0 mg mRNA/kg and expressed as ng luciferase/g spleen).

In some embodiments, the spleen activity is about 1.05 times greater than the spleen activity of an LNP prepared with Compound I-1 (*i.e.*, if the spleen activity of the LNP prepared with Compound I-1 is 1, then the spleen activity of an LNP prepared according to the present disclosure is about 1.05). In some embodiments, the spleen activity is about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.25, about 2.5, about 2.75, about 3.0, about 3.25, about 3.5, about 3.75, about 4.0, about 4.25, about 4.5, about 4.75, or about 5.0 times greater than the spleen activity of an LNP prepared with Compound I-1 (*e.g.*, at a dose of 0.3 mg, 0.5 mg, or 1.0 mg mRNA/kg and expressed as ng luciferase/g spleen).

In certain embodiments, spleen activity is as measured by luciferase mRNA *in vivo* evaluation studies were performed on 6–8-week-old female C57BL/6 mice (Charles River) or 8–10-week-old CD-1 mice (Charles River or Inotiv) (*e.g.*, as described in Example 2 herein). In

some embodiments, the spleen activity is measured upon dosing at 0.3 mg/kg. In certain embodiments, the spleen activity is measured upon dosing at 0.5 mg/kg. In some embodiments, the spleen activity is measured upon dosing at 1.0 mg/kg. In certain embodiments, the spleen activity is measured upon dosing at 0.1, 0.2, 0.4, 0.6, 0.7, 0.8, 0.9, 1.1, 1.2, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 7.5, or 10.0 mg/kg.

In some embodiments, when comparing the activity of LNPs the only difference is the cationic lipid. That is, an LNP prepared with Compound I-1 vs. an LNP with a cationic lipid of the present disclosure would only differ in the identity of the cationic lipid (e.g., ratios of other components, molar concentrations, *etc*. would all remain the same)

In some embodiments, the lipid nanoparticle has a ratio of spleen activity to liver activity of at least 1.3. In certain embodiments, the ratio of spleen activity to liver activity is greater than 1.5 to 1. In some embodiments, the ratio of spleen activity to liver activity is greater than 1.7 to 1. In some embodiments, the ratio of spleen activity to liver activity is greater than 1.8 to 1. In certain embodiments, the ratio of spleen activity to liver activity is greater than 2.0 to 1. In some embodiments, the ratio of spleen activity to liver activity is greater than 5.0 to 1. In certain embodiments, the ratio of spleen activity to liver activity is greater than 6.5 to 1.

In some embodiments, the lipid nanoparticle has a ratio of spleen activity to liver activity of at least about 1.3. In certain embodiments, the ratio of spleen activity to liver activity is greater than about 1.5 to about 1. In some embodiments, the ratio of spleen activity to liver activity is greater than about 1.7 to about 1. In some embodiments, the ratio of spleen activity to liver activity is greater than about 1.8 to about 1. In certain embodiments, the ratio of spleen activity to liver activity is greater than about 2.0 to about 1. In some embodiments, the ratio of spleen activity to liver activity is greater than about 5.0 to about 1. In certain embodiments, the ratio of spleen activity to liver activity is greater than about 6.5 to about 1.

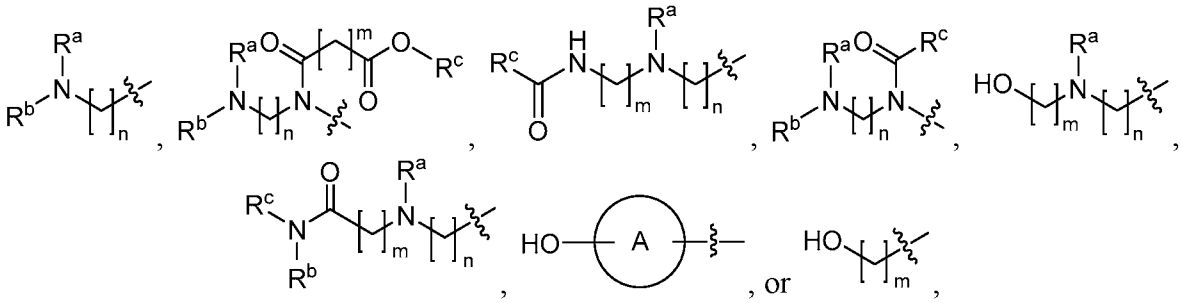
In certain embodiments, the cationic lipid has a LogP ranging from 12 to 25. In some embodiments, the cationic lipid has a LogP ranging from 13 to 15, from 15 to 17, from 17 to 19, from 19 to 21, from 21 to 23, or from 23 to 25. In certain embodiments, the cationic lipid has a LogP ranging from 12 to 14, from 14 to 16, from 16 to 18, from 18 to 20, from 20 to 22, or from 22 to 24.

In certain embodiments, the cationic lipid has a LogP from about 12 to about 25. In some embodiments, the cationic lipid has a LogP from about 13 to about 15, from about 15 to about 17, from about 17 to about 19, from about 19 to about 21, from about 21 to about 23, or from about 23 to about 25. In certain embodiments, the cationic lipid has a LogP from about 12 to

about 14, from about 14 to about 16, from about 16 to about 18, from about 18 to about 20, from about 20 to about 22, or from about 22 to about 24.

In some embodiments, LogP is as measured by methods known in the art (see, *e.g.*, Bharate, S. et al. Determining Partition Coefficient (Log P), Distribution Coefficient (Log D) and Ionization Constant (pKa) in Early Drug Discovery, *Comb. Chem. High Throughput Screen*, (2016)19(6):461-9).

In certain embodiments, the ionizable headgroup has one of the following structures:



wherein:

ring A is a C_4-C_8 cycloalkyl;

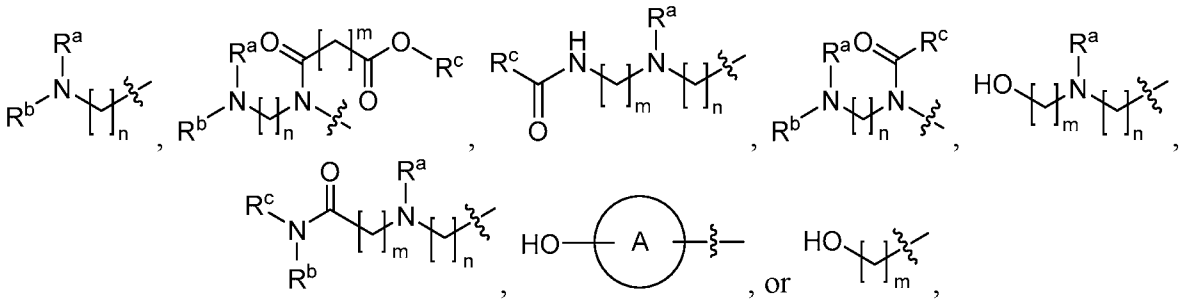
R^a and R^b are each independently C_1-C_4 alkyl;

R^c is C_1-C_{14} alkyl or C_1-C_8 haloalkyl ;

n is an integer ranging from 1 to 6; and

m is an integer ranging from 1 to 12.

In certain embodiments, the ionizable headgroup has one of the following structures:



wherein:

ring A is a C_4-C_8 cycloalkyl;

R^a and R^b are each independently C_1-C_6 alkyl, or R^a and R^b join with the nitrogen to which they are attached to form a 3-8 membered heterocyclyl (*e.g.*, having from 1-4 heteroatoms selected from N, O, and S and from 2-7 carbon atoms);

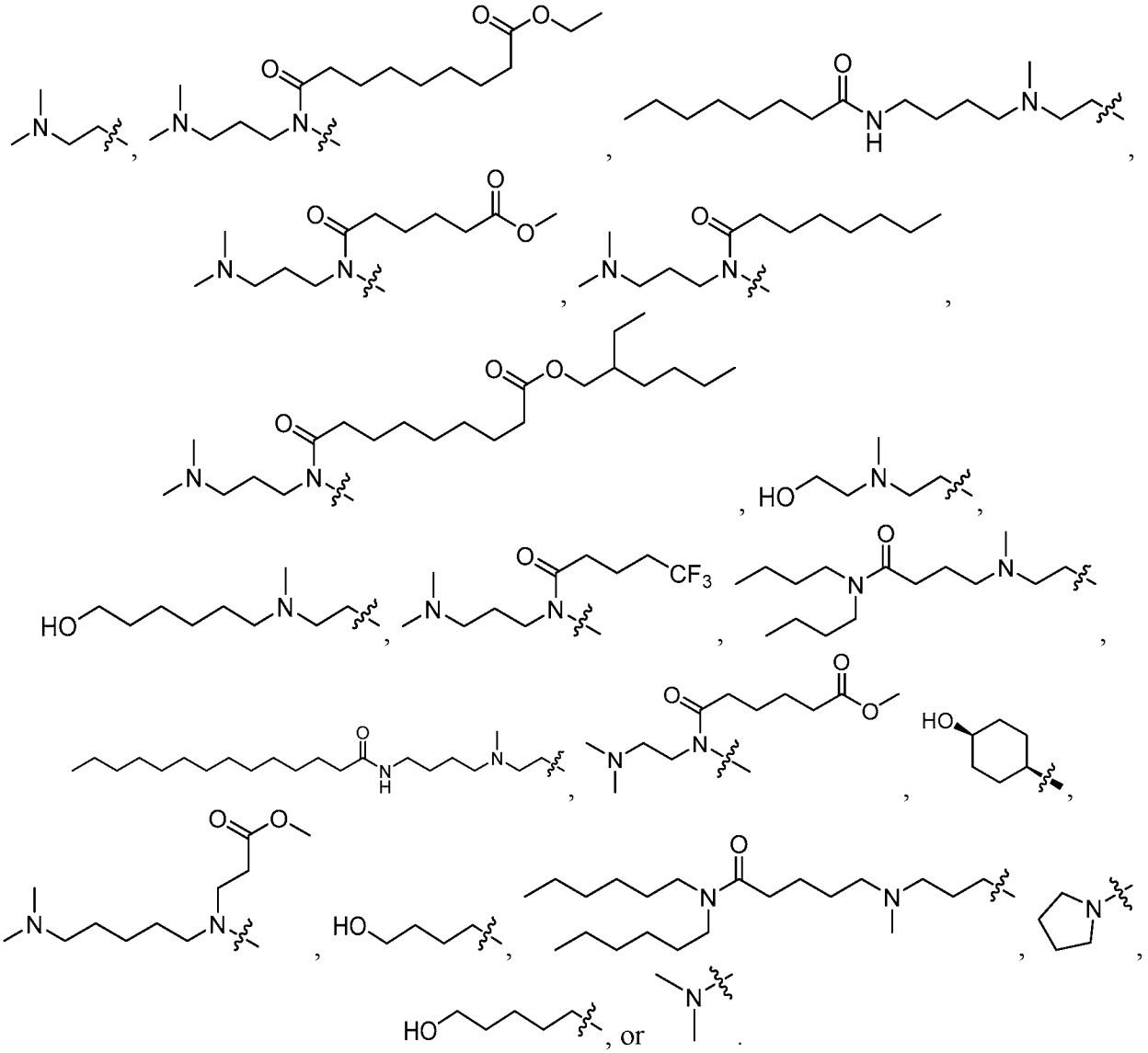
R^c is C_1-C_{14} alkyl or C_1-C_8 haloalkyl;

n is an integer from 0 to 6; and

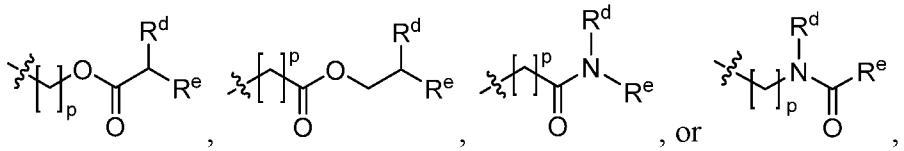
m is an integer from 1 to 12.

In some embodiments, R^a and R^b are both methyl. In some embodiments, R^a and R^b join with the nitrogen to which they are attached to form pyrrolidinyl. In certain embodiments, R^a and

R^b are both methyl or R^a and R^b join with the nitrogen to which they are attached to form pyrrolidinyl. In some embodiments, the ionizable headgroup has one of the following structures:



In some embodiments, the two alkyl tail groups each independently have one of the following structures:

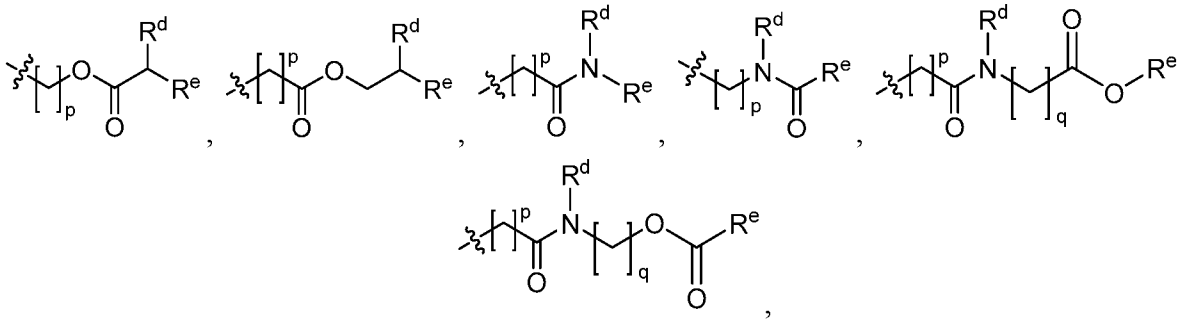


wherein:

each occurrence of R^d and R^e are independently C_4-C_{18} alkyl; and

p is an integer ranging from 4 to 12.

In some embodiments, the two alkyl tail groups each independently have one of the following structures:



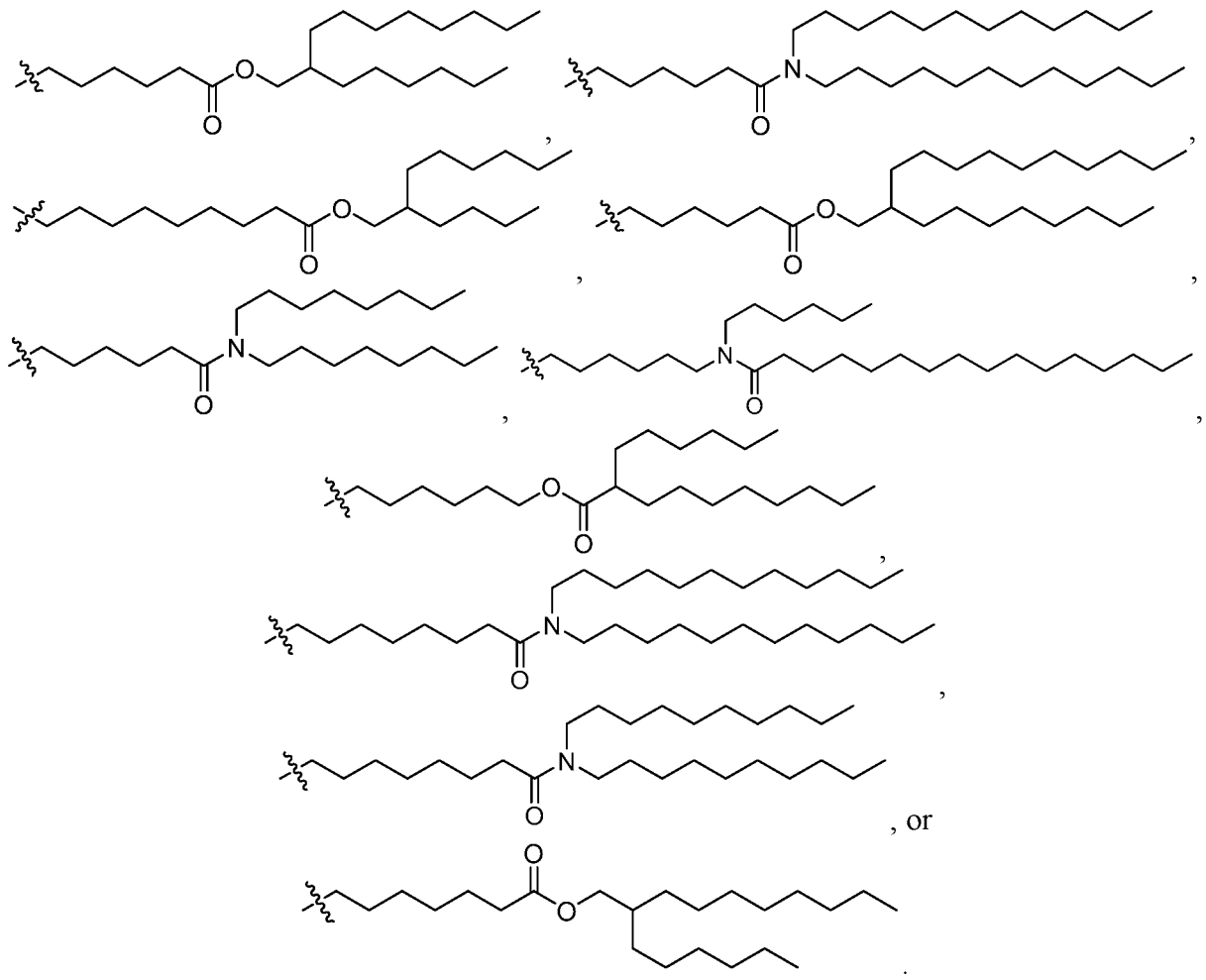
wherein:

each occurrence of R^d and R^e are independently C_4-C_{18} alkyl;

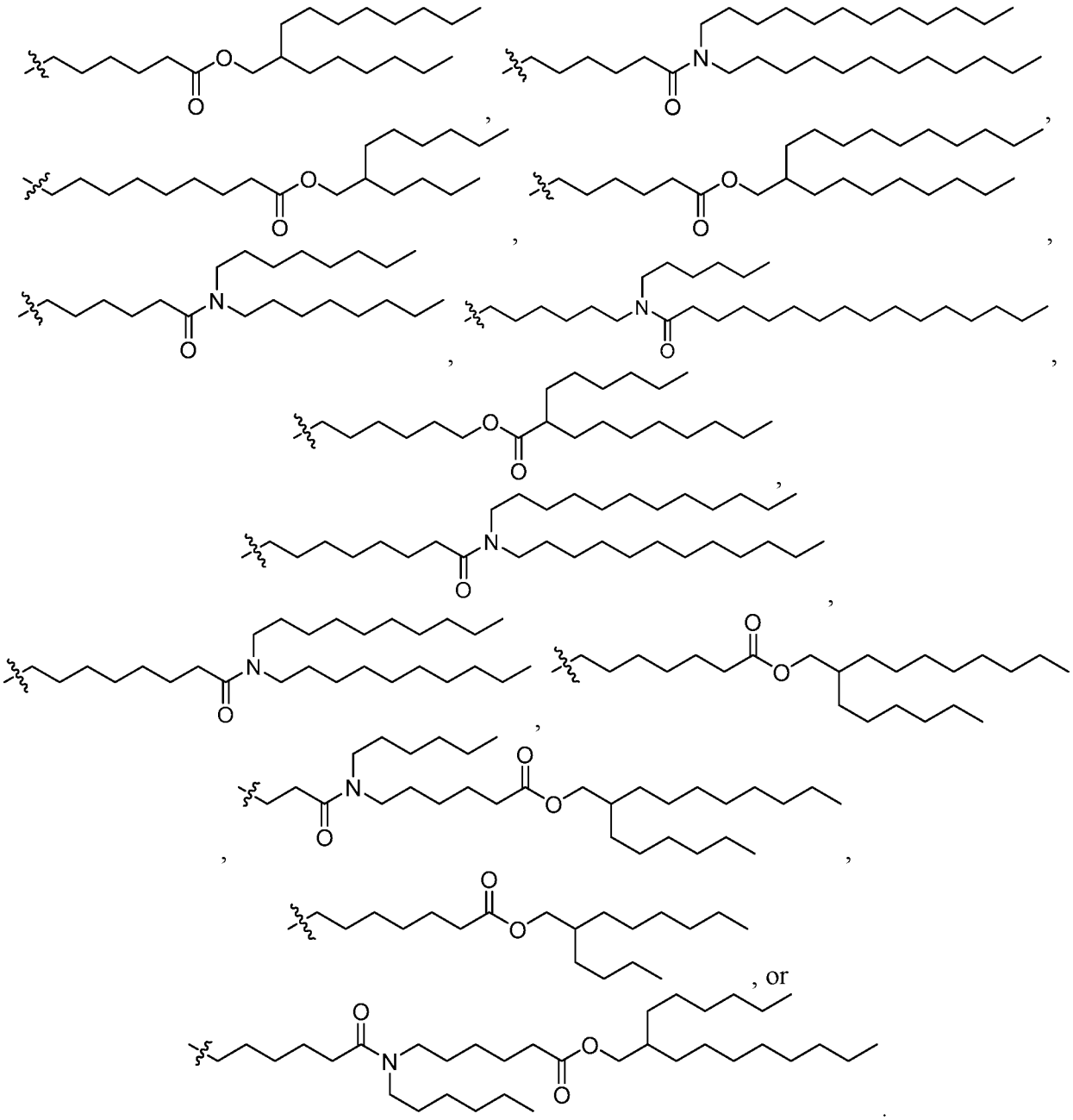
each occurrence of p is independently an integer from 2 to 12; and

each occurrence of q is independently an integer from 4 to 12.

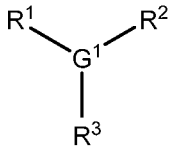
In certain embodiments, the two alkyl tail groups each independently have one of the following structures:



In certain embodiments, the two alkyl tail groups each independently have one of the following structures:



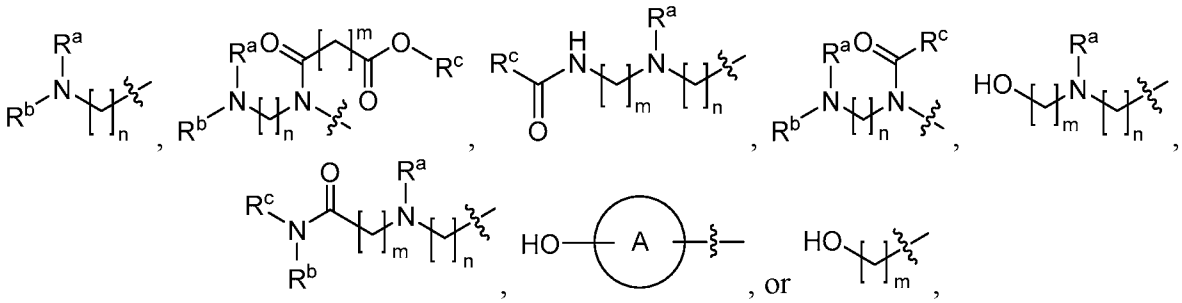
One embodiment provides a method of inducing an immune response in a subject, the method comprising intramuscular delivery of a lipid nanoparticle to the subject, wherein the lipid nanoparticle comprises a nucleic acid that encodes an antigen and a cationic lipid having the following structure of Formula (I):



or stereoisomer, tautomer, or salt thereof, wherein:

G^1 is N or CH;

R^1 is $-CH_3$ or has one of the following structures:



wherein:

ring A is a C_4-C_8 cycloalkyl;

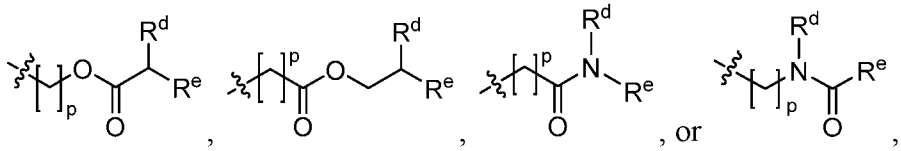
R^a and R^b are each independently C_1-C_4 alkyl;

R^c is C_1-C_{14} alkyl or C_1-C_8 haloalkyl ;

n is an integer ranging from 1 to 6; and

m is an integer ranging from 1 to 12;

R^2 and R^3 each independently have one of the following structures:

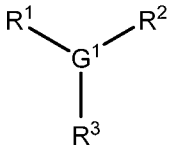


wherein:

each occurrence of R^d and R^e are independently C_4-C_{18} alkyl; and

p is an integer ranging from 4 to 12.

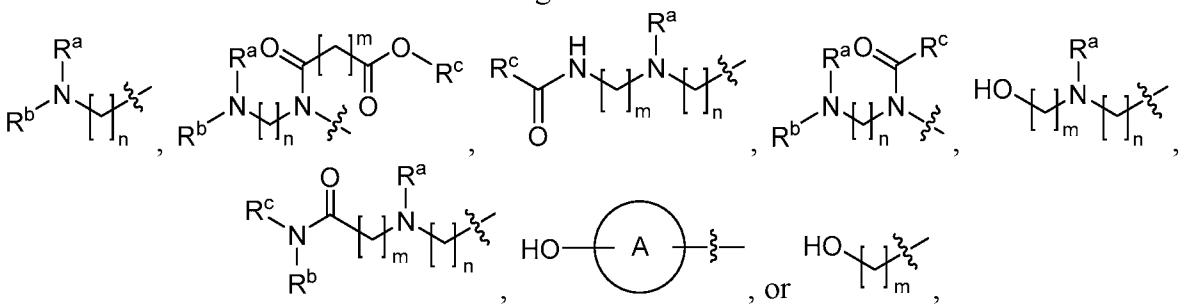
One embodiment provides a method of inducing an immune response in a subject, the method comprising intramuscular delivery of a lipid nanoparticle to the subject, wherein the lipid nanoparticle comprises a nucleic acid that encodes an antigen and a cationic lipid having the following structure of Formula (I):



or stereoisomer, tautomer, or salt thereof, wherein:

G^1 is N or CH;

R^1 is -CH_3 or has one of the following structures:



wherein:

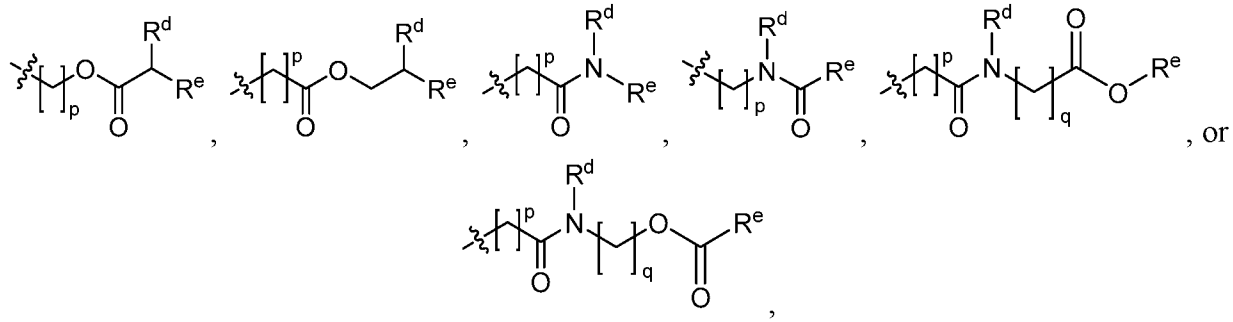
ring A is a C_4-C_8 cycloalkyl;

R^a and R^b are each independently C_1-C_6 alkyl, or R^a and R^b join together with the nitrogen to which they are attached to form a 3-8 membered heterocyclyl (*e.g.*, having from 1-4 heteroatoms selected from N, O, and S and from 2-7 carbon atoms);

R^c is C_1-C_{14} alkyl or C_1-C_8 haloalkyl;

n is an integer from 0 to 6; and

m is an integer from 1 to 12;



wherein:

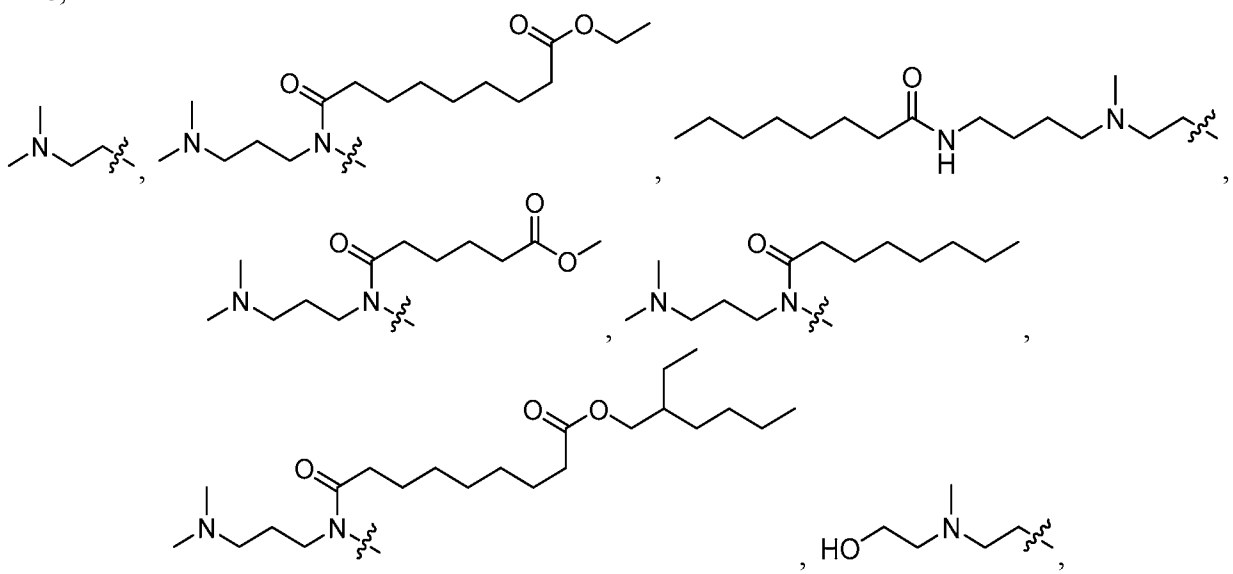
each occurrence of R^d and R^e are independently C_4-C_{18} alkyl;

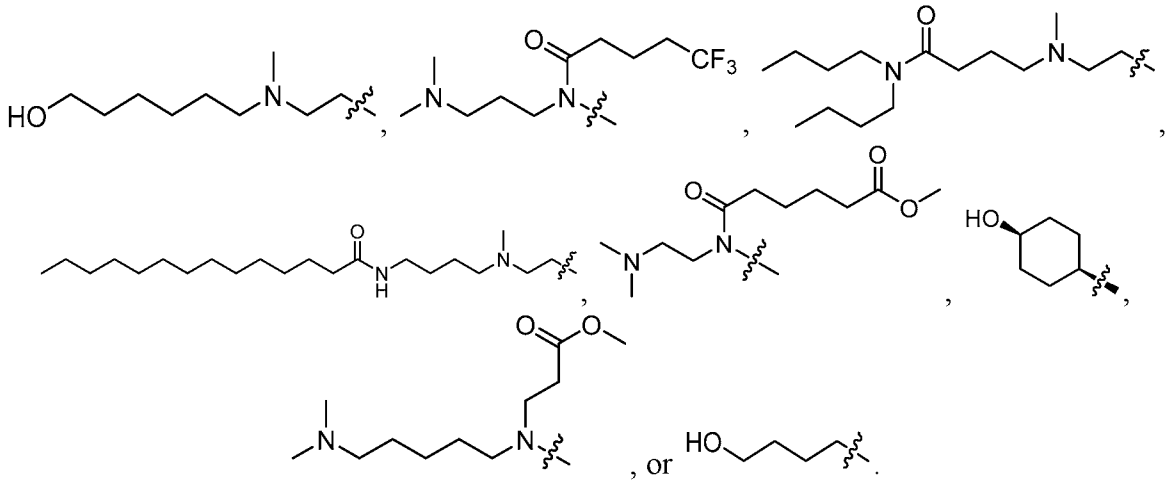
each occurrence of p is independently an integer from 2 to 12; and

each occurrence of q is independently an integer from 4 to 12.

In some embodiments, R^a and R^b are both methyl. In some embodiments, R^a and R^b join with the nitrogen to which they are attached to form pyrrolidinyl. In certain embodiments, R^a and R^b are both methyl or R^a and R^b join with the nitrogen to which they are attached to form pyrrolidinyl. In certain embodiments, R^1 has one of the following structures:

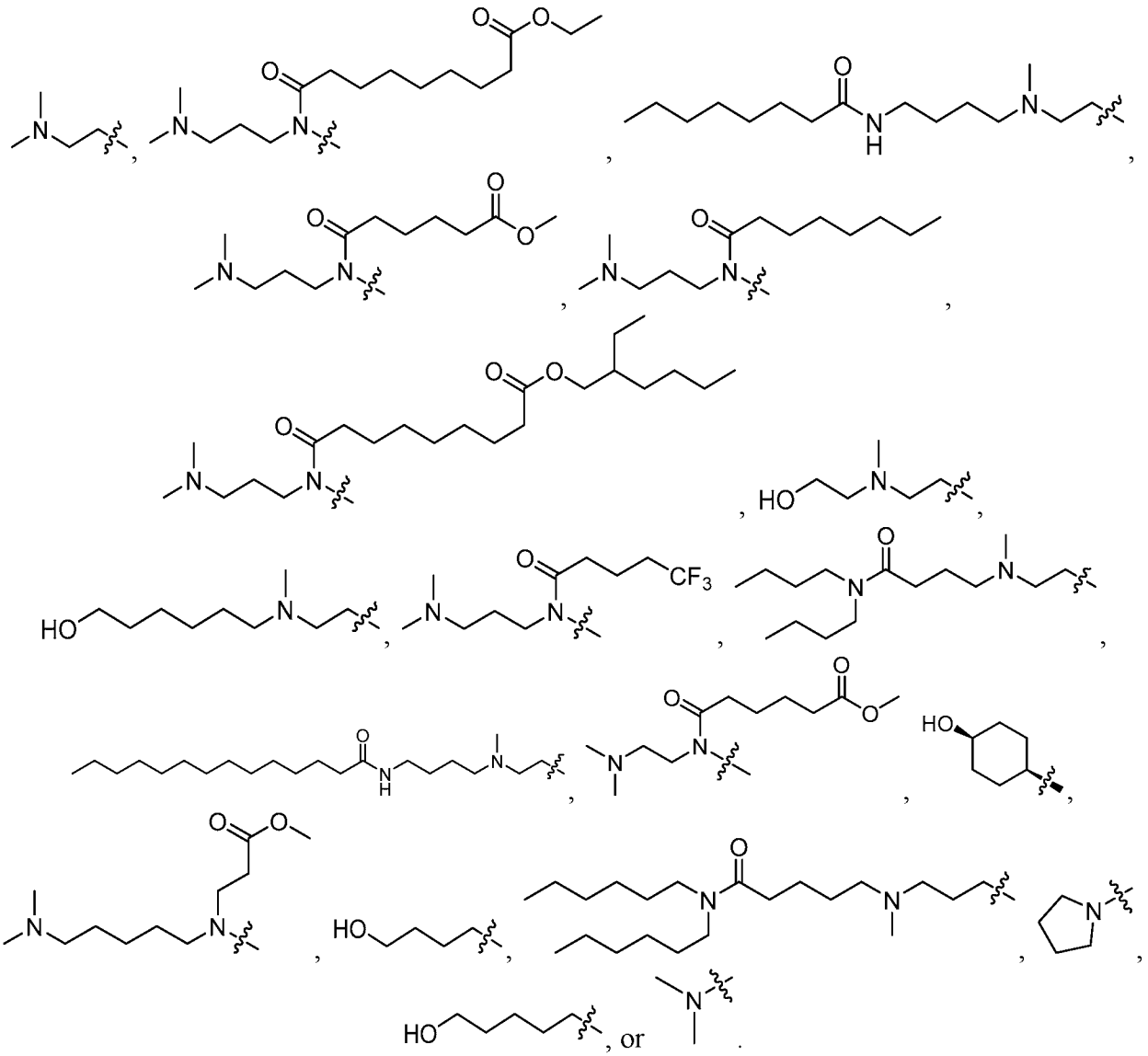
$-CH_3$,



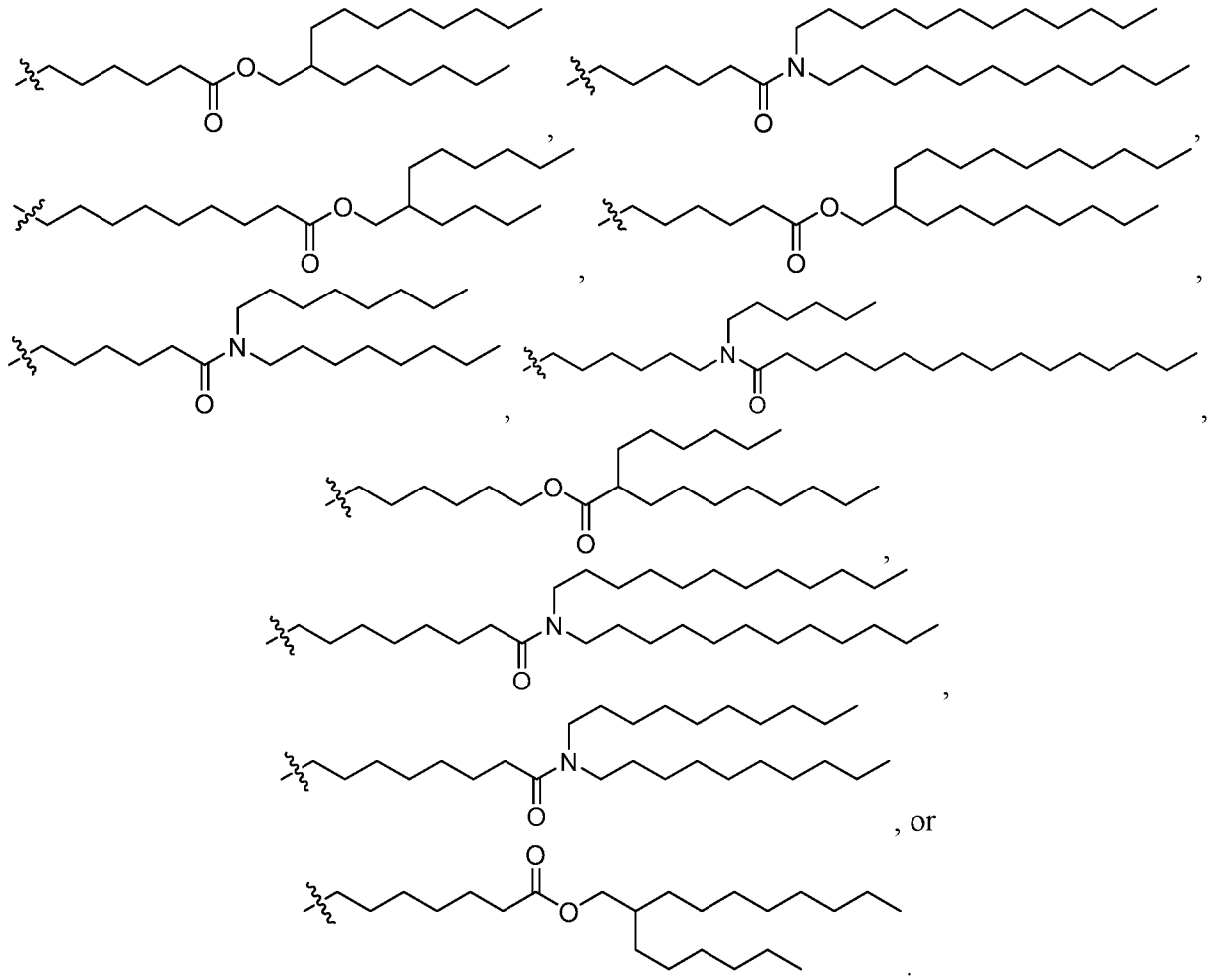


In some embodiments, R^a and R^b are both methyl. In certain embodiments, R^1 has one of the following structures:

-CH_3,



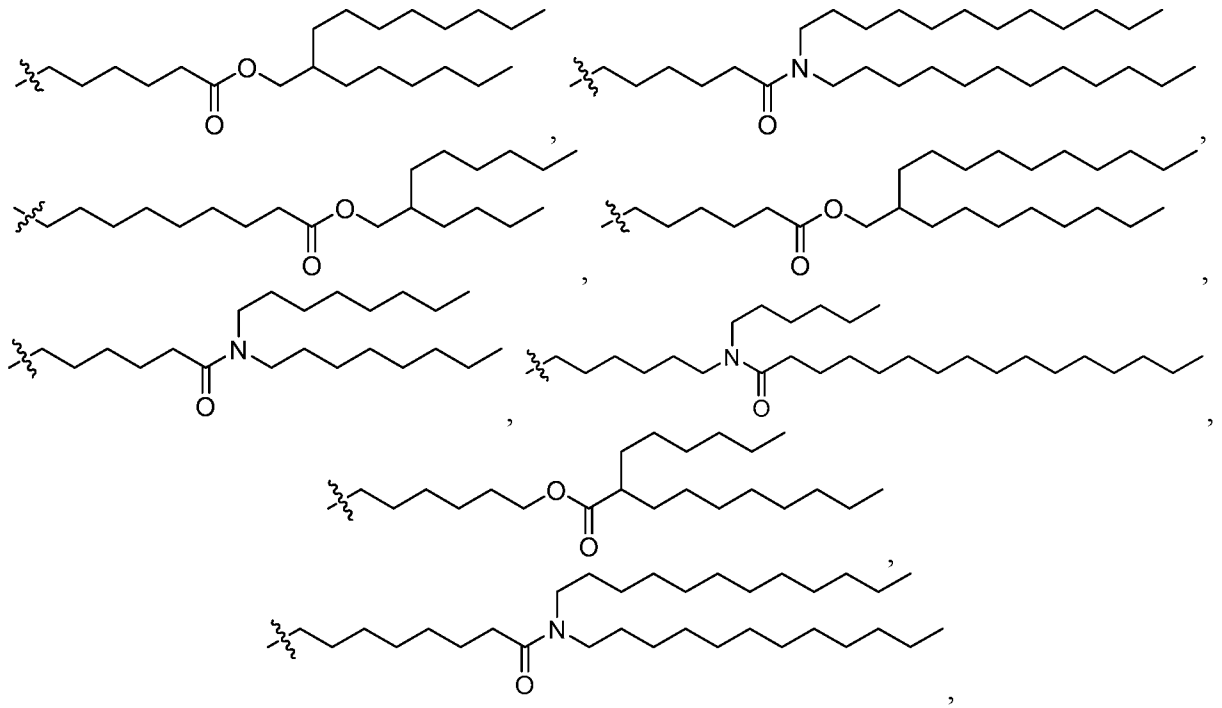
In some embodiments, R^2 and R^3 each independently have one of the following structures:

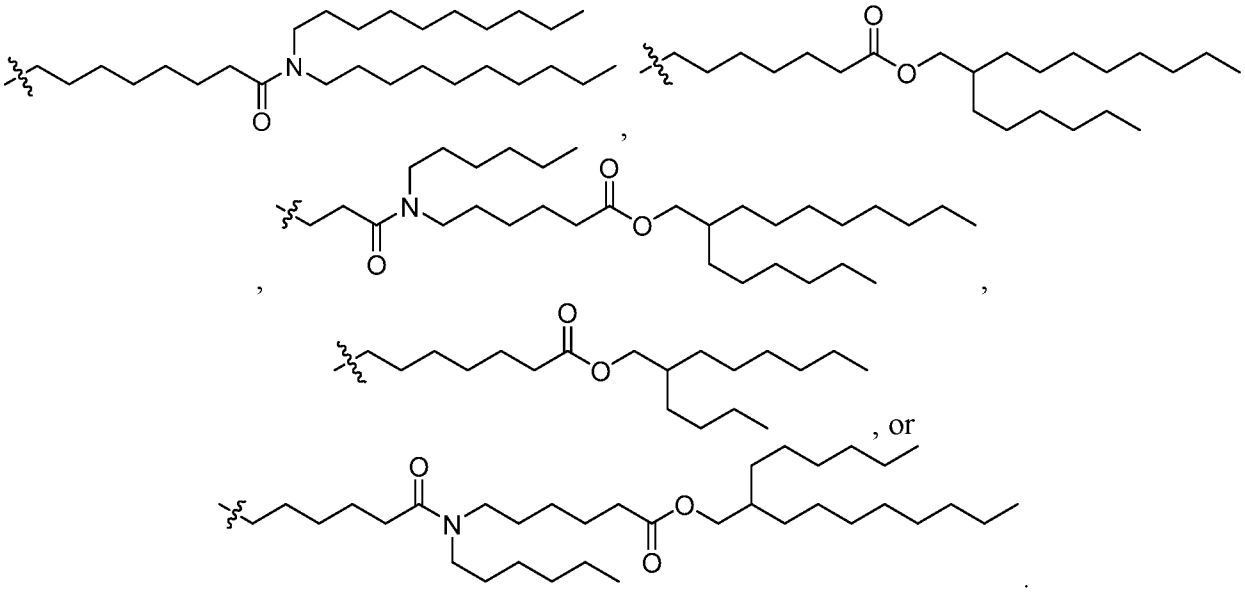


, or

.

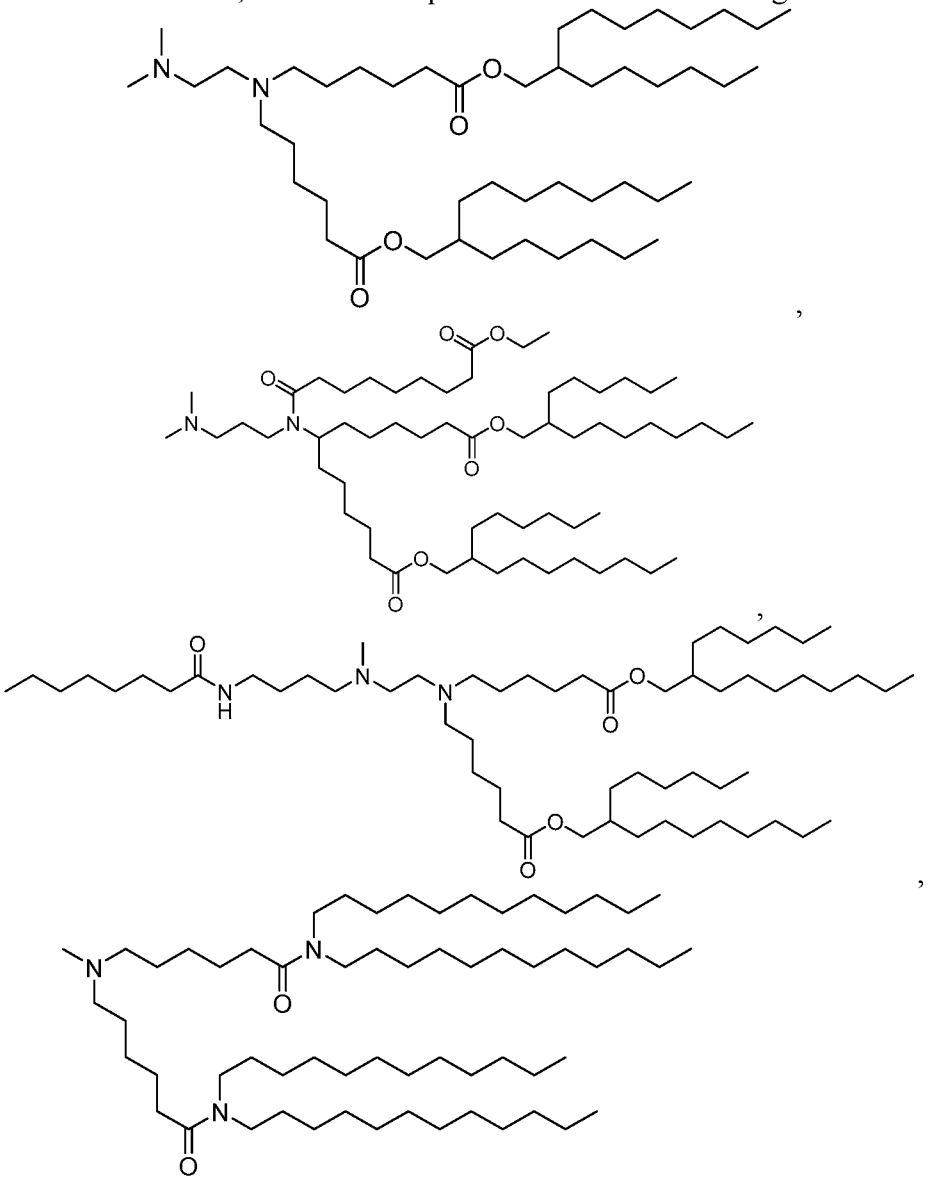
In some embodiments, R^2 and R^3 each independently have one of the following structures:

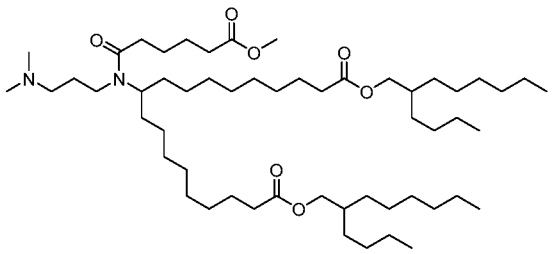




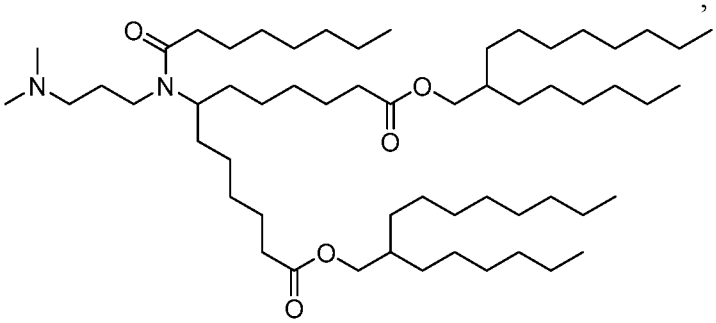
, or

In certain embodiments, the cationic lipid has one of the following structures:

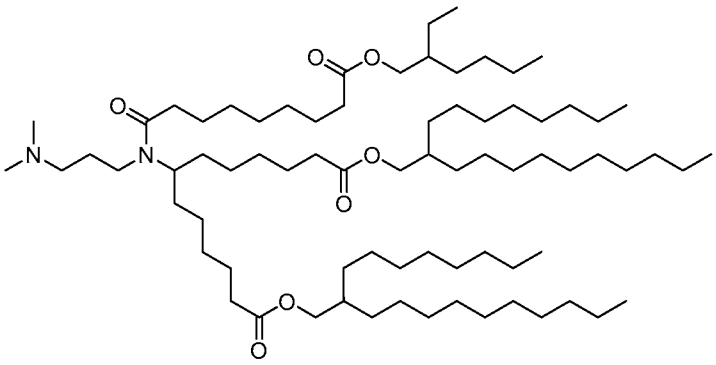




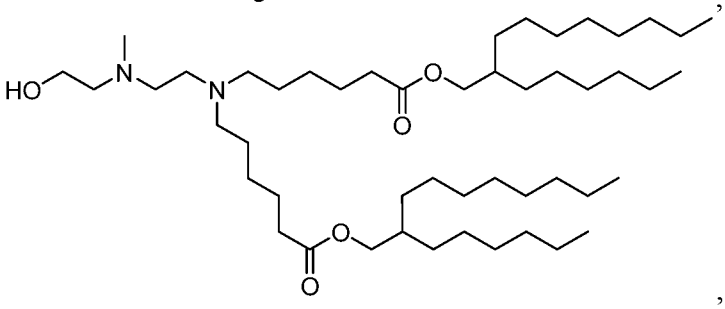
,



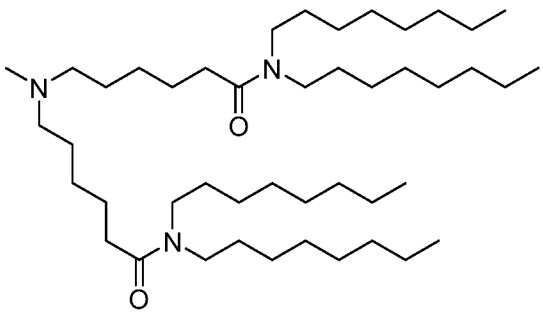
,



,

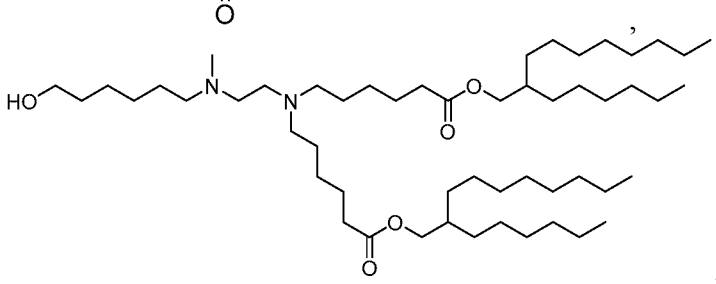


,

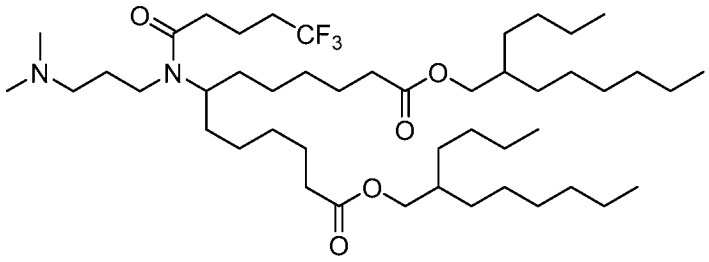


5

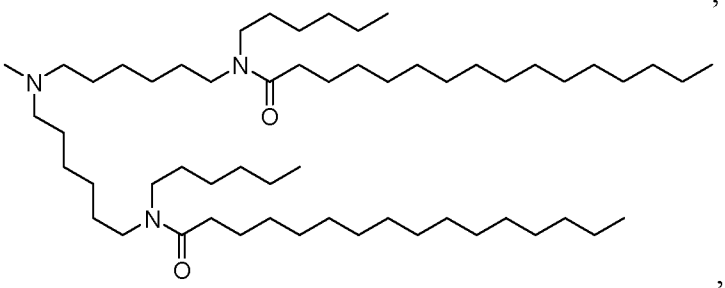
,



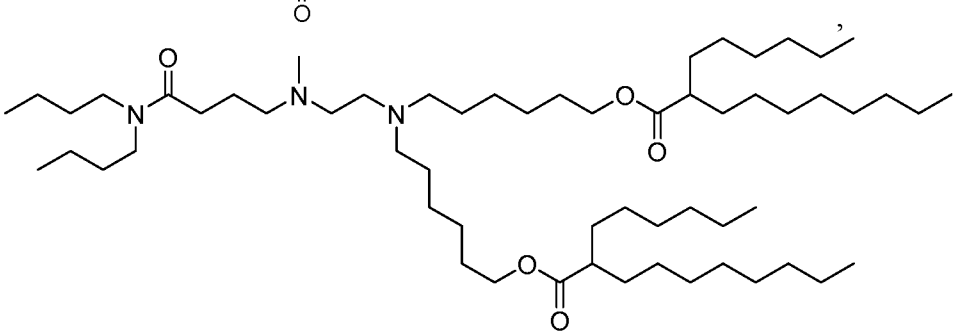
,



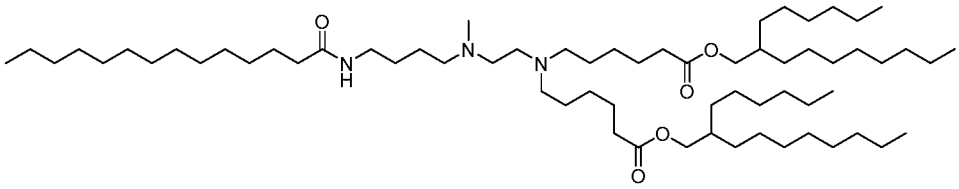
,



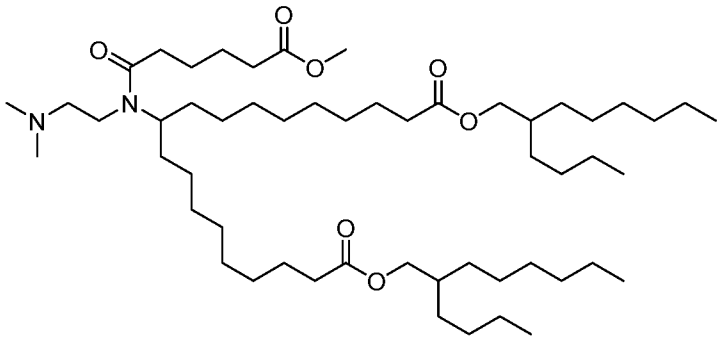
,



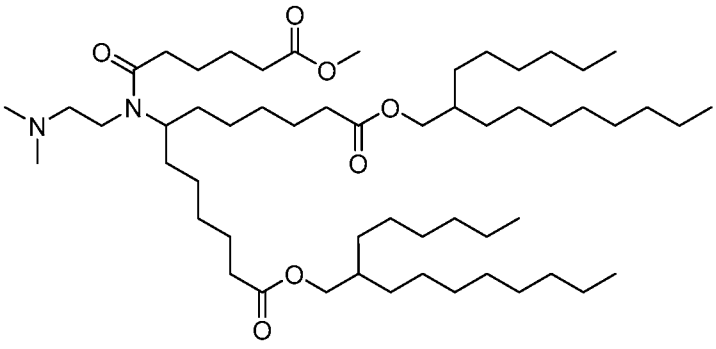
,



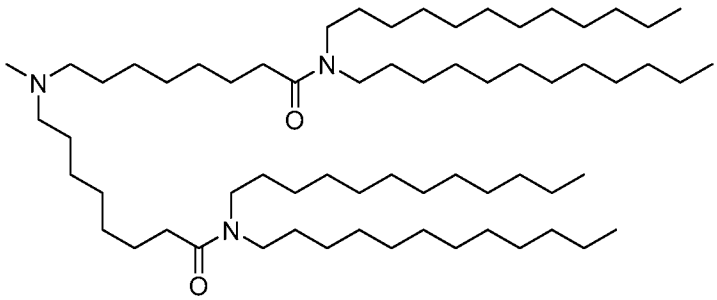
,

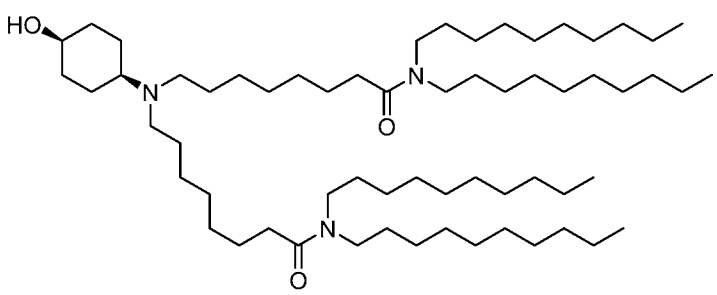


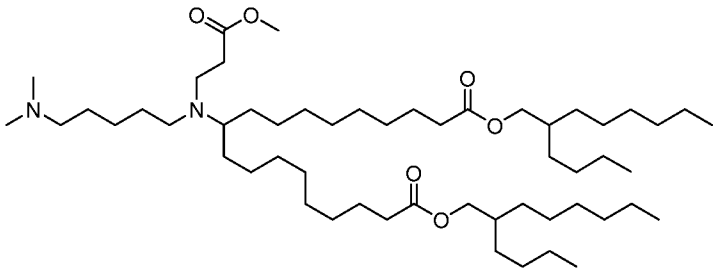
5 ,

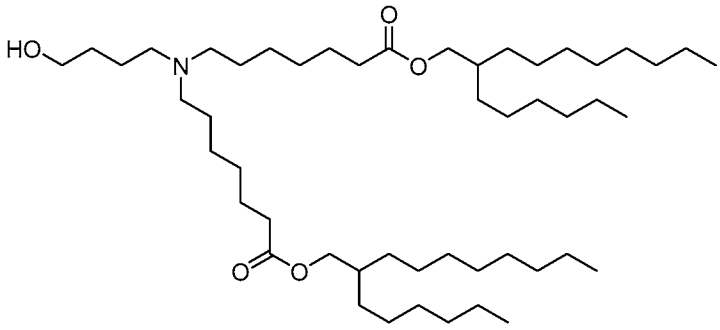


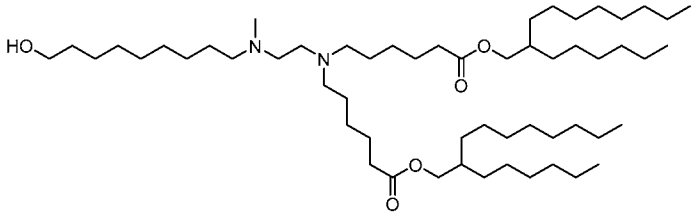
,


,

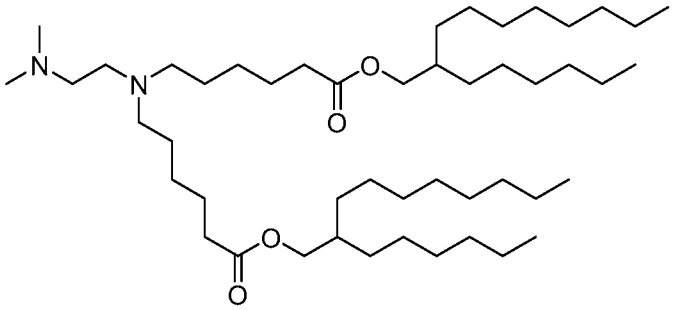

,


,

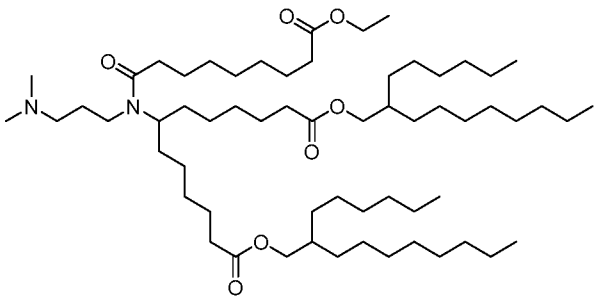

or


.

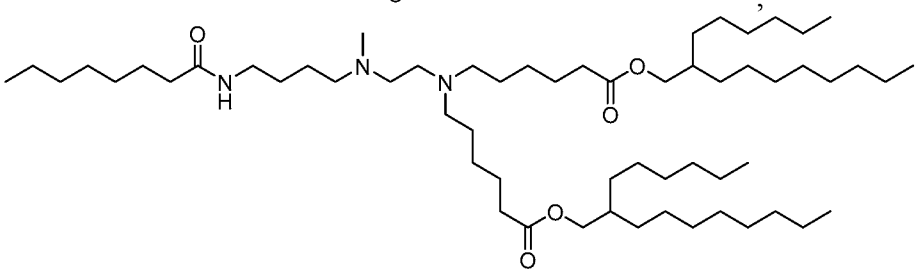
In certain embodiments, the cationic lipid has one of the following structures:



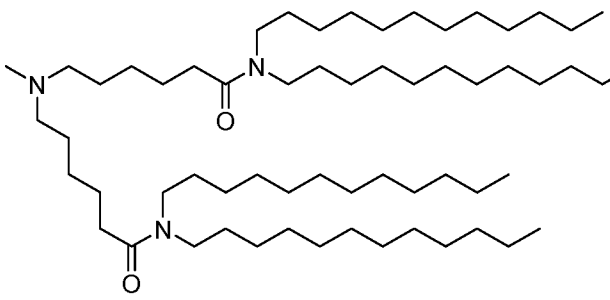
,



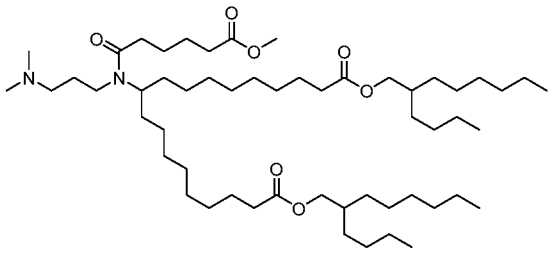
,



,

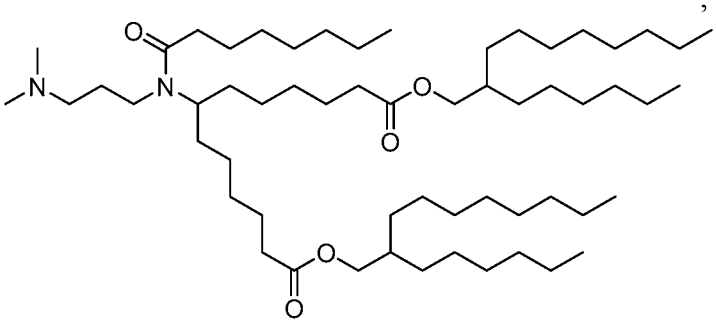


,

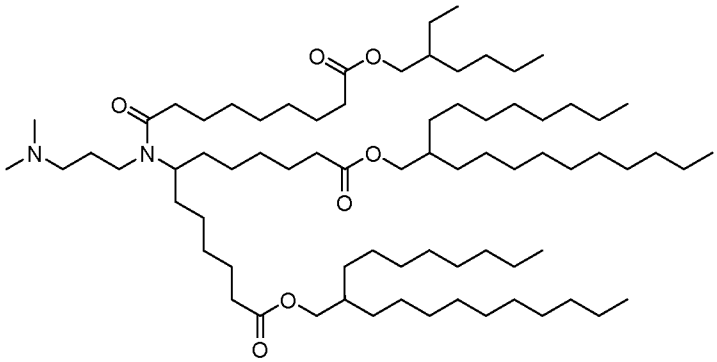


5

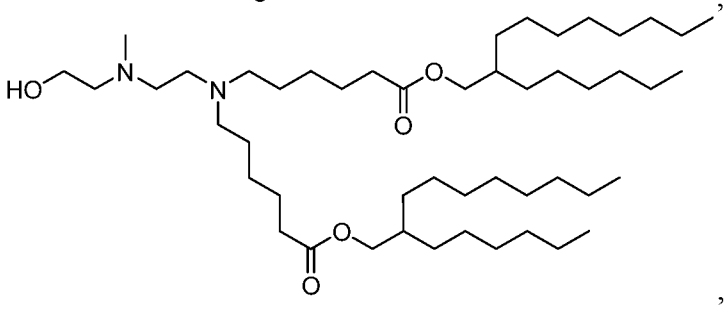
,



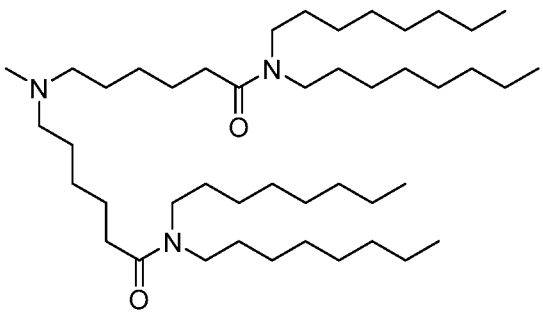
,



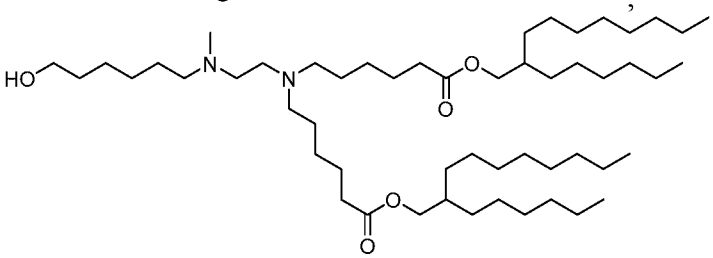
,



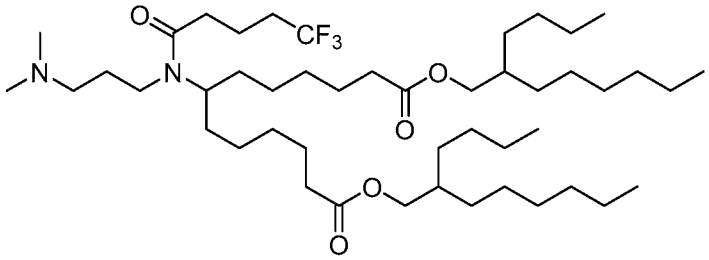
,



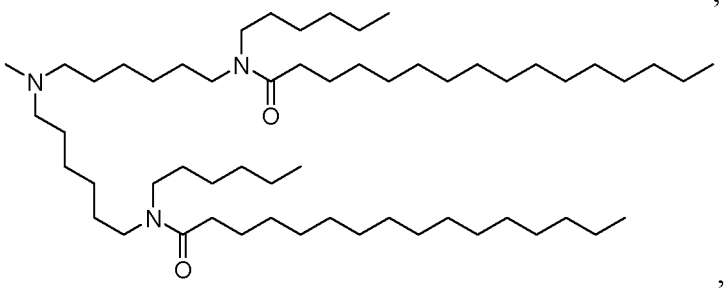
,



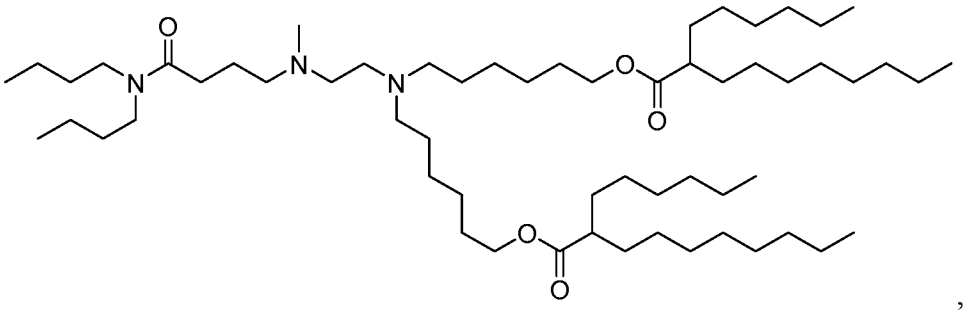
,



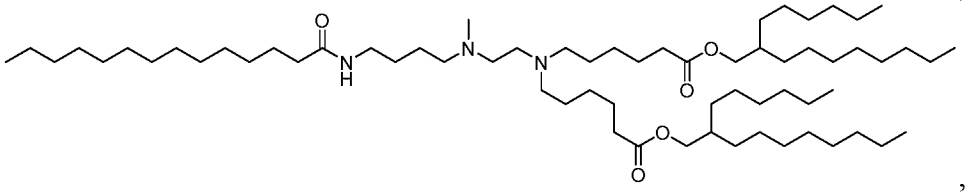
,



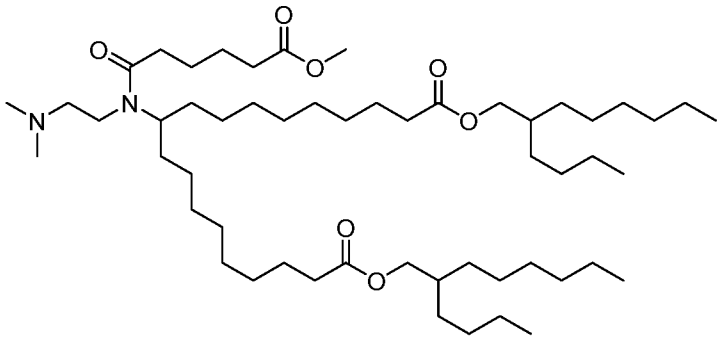
,



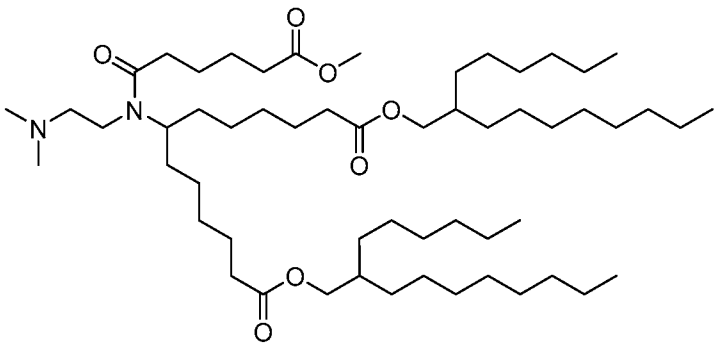
,



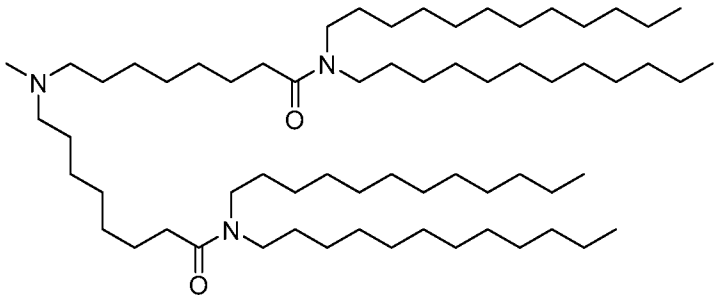
,



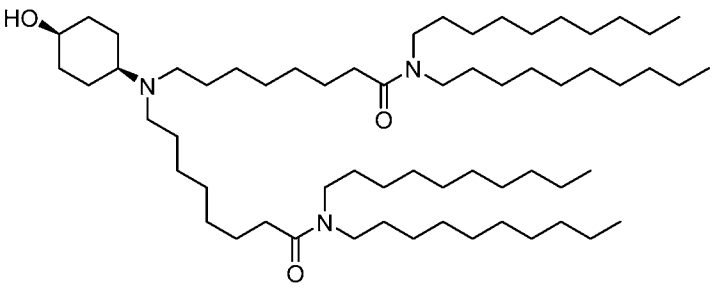
,



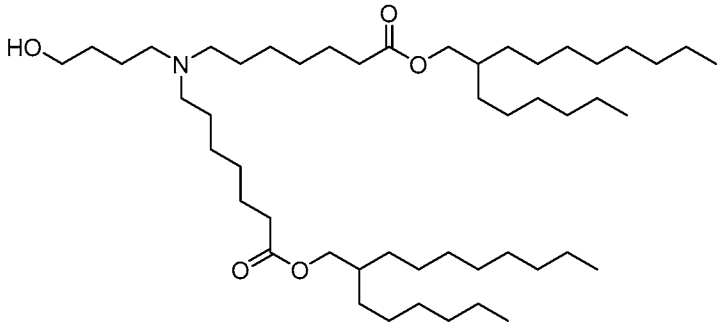
,



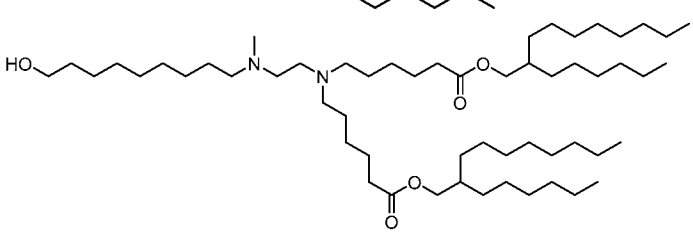
,



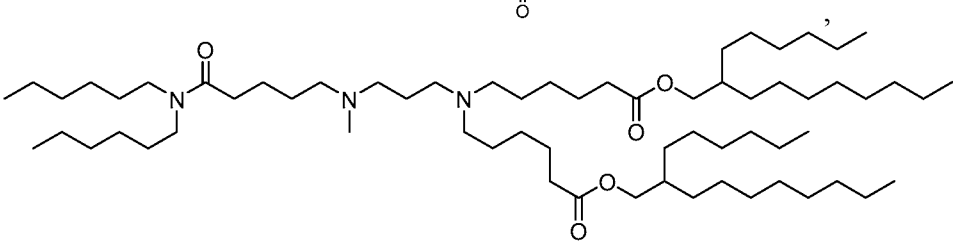
,



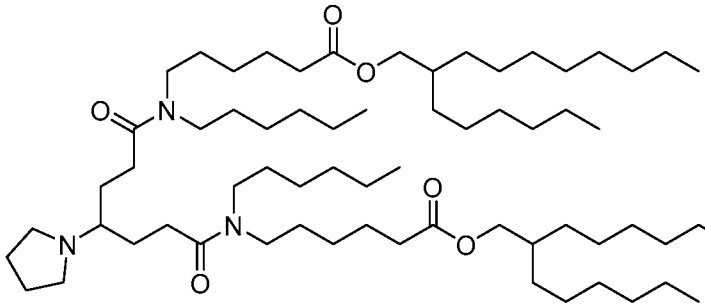
,



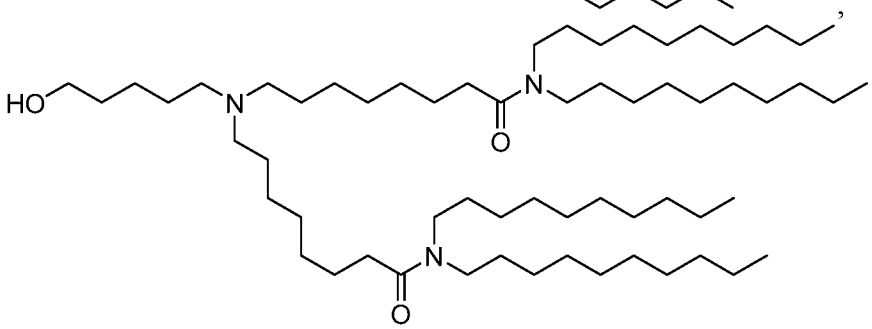
,



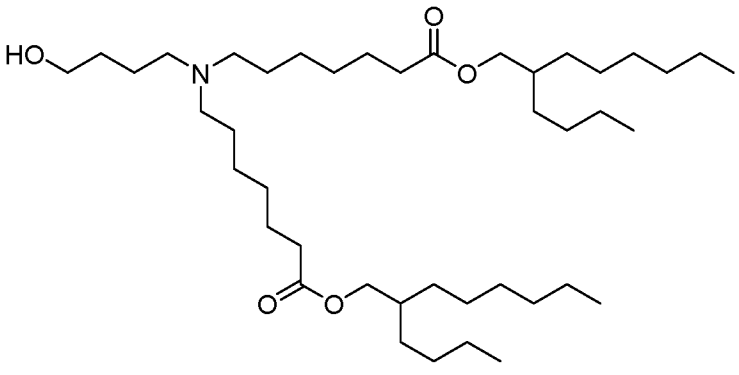
,



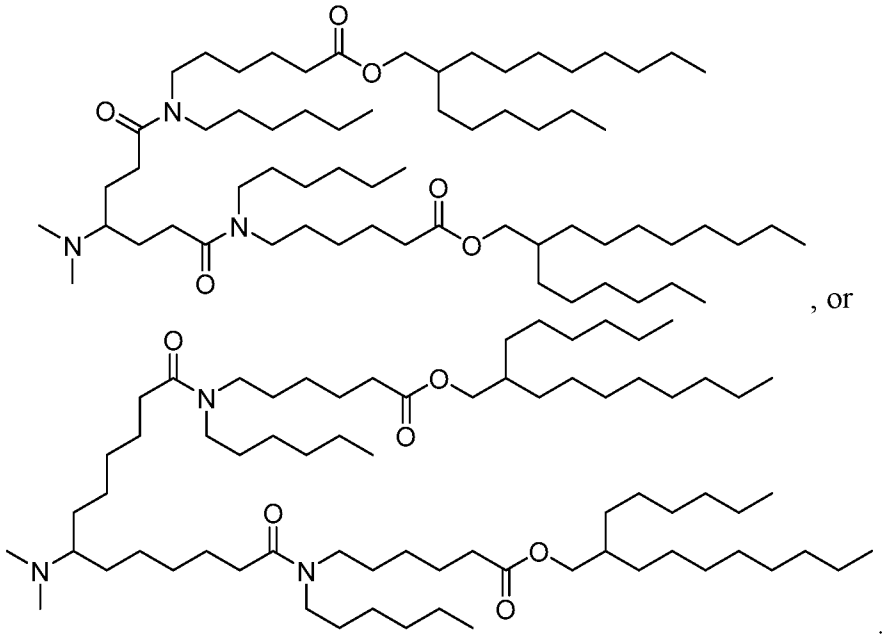
,



,



,



, or

In some embodiments, the lipid nanoparticle comprises a therapeutic agent. In certain embodiments, the therapeutic agent comprises a nucleic acid. In some embodiments, the nucleic acid is selected from antisense and messenger RNA.

In certain embodiments, greater than 75% of the nucleic acid is encapsulated within the lipid nanoparticles. In some embodiments, greater than 85% of the nucleic acid is encapsulated within the lipid nanoparticles. In certain embodiments, greater than 90% of the nucleic acid is encapsulated within the lipid nanoparticles. In some embodiments, greater than 95% of the nucleic acid is encapsulated within the lipid nanoparticles. In certain embodiments, greater than 97% of the nucleic acid is encapsulated within the lipid nanoparticles. In some embodiments, greater than 98% of the nucleic acid is encapsulated within the lipid nanoparticles.

In some embodiments, the lipid nanoparticle further comprises one or more excipient selected from neutral lipids, steroids, and polymer conjugated lipids.

In some embodiments, the lipid nanoparticle comprises between 35-50 wt% of the cationic lipid. In certain embodiments, the lipid nanoparticle comprises between 45-50 wt% of the cationic lipid. In some embodiments, the lipid nanoparticle comprises 47.5 wt% of the cationic lipid. In certain embodiments, the lipid nanoparticle comprises between 35-40 wt% of the cationic lipid. In some embodiments, the lipid nanoparticle comprises 37.5 wt% of the cationic lipid.

In certain embodiments, the lipid nanoparticle comprises one or more neutral lipids selected from DSPC, DPPC, DMPC, DOPC, POPC, DOPE, and SM. In some embodiments, the neutral lipid is DSPC. In certain embodiments, the molar ratio of the cationic lipid to the neutral

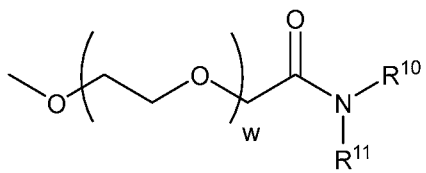
lipid ranges from about 2:1 to about 8:1. In certain embodiments, the molar ratio of the cationic lipid to the neutral lipid is from about 2:1 to about 8:1.

In some embodiments, the steroid is cholesterol. In certain embodiments, the molar ratio of the cationic lipid to cholesterol ranges from 5:1 to 1:1 or from 2:1 to 1:1. In some embodiments, the steroid is cholesterol. In certain embodiments, the molar ratio of the cationic lipid to cholesterol is from 5:1 to 1:1 or from 2:1 to 1:1.

In certain embodiments, the polymer conjugated lipid is a pegylated lipid. In some embodiments, the lipid nanoparticle comprises between 1.5-2.8 wt% of the pegylated lipid. In certain embodiments, the lipid nanoparticle comprises 1.8 wt% of the pegylated lipid. In some embodiments, the lipid nanoparticle comprises 2.5 wt% of the pegylated lipid. In certain embodiments, the molar ratio of the cationic lipid to pegylated lipid ranges from about 100:1 to about 20:1 or from about 100:1 to about 10:1. In certain embodiments, the molar ratio of the cationic lipid to pegylated lipid is from about 100:1 to about 20:1 or from about 100:1 to about 10:1.

In certain embodiments, the polymer conjugated lipid is a pegylated lipid. In various embodiments, the polymer conjugated lipid is a pegylated lipid. For example, some embodiments include a pegylated diacylglycerol (PEG-DAG) such as 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG), a pegylated phosphatidylethanoloamine (PEG-PE), a PEG succinate diacylglycerol (PEG-S-DAG) such as 4-O-(2',3'-di(tetradecanoyloxy)propyl-1-O-(ω-methoxy(polyethoxy)ethyl)butanedioate (PEG-S-DMG), a pegylated ceramide (PEG-cer), or a PEG dialkoxypropylcarbamate such as ω-methoxy(polyethoxy)ethyl-N-(2,3-di(tetradecanoxy)propyl)carbamate or 2,3-di(tetradecanoxy)propyl-N-(ω-methoxy(polyethoxy)ethyl)carbamate.

In certain embodiments, the pegylated lipid has the following structure:



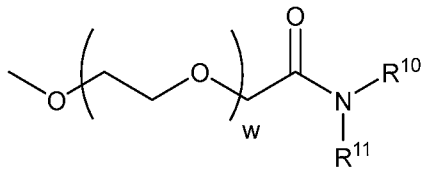
or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof, wherein:

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

w ranges from 30 to 60 (*e.g.*, w is an integer from 30 to 60).

In certain embodiments, R^{10} and R^{11} are each independently straight alkyl chain containing from 12 to 16 carbon atoms. In some embodiments, w is 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, or 55. In certain embodiments, w is 40 to 50. In some embodiments, w is 43, 44, 45, 46, 47, or 48.

In certain embodiments, a lipid nanoparticle or composition comprises a plurality of pegylated lipids having the following structure:



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

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

the average value of w for the plurality ranges from 30 to 60 (*e.g.*, the average value of w for the plurality is from 30 to 60).

Synthesis of pegylated lipids can be found in US Patent No. 9,738,593, the disclosure of which is hereby incorporated by reference.

In some embodiments, the lipid nanoparticle diameter (*i.e.*, particle size) is as determined by quasi-elastic light scattering. In some embodiments, the lipid nanoparticle has a diameter between 39-75 nanometers. In certain embodiments, the lipid nanoparticle has a diameter between 39-45 nanometers. In some embodiments, the lipid nanoparticle has a diameter between 45-50 nanometers. In certain embodiments, the lipid nanoparticle has a diameter between 50-55 nanometers. In some embodiments, the lipid nanoparticle has a diameter between 55-60 nanometers. In certain embodiments, the lipid nanoparticle has a diameter between 60-65 nanometers. In some embodiments, the lipid nanoparticle has a diameter between 65-70 nanometers. In certain embodiments, the lipid nanoparticle has a diameter between 70-75 nanometers. In some embodiments, the lipid nanoparticle has a diameter greater than 70 nanometers. In some embodiments, the lipid nanoparticle has a diameter between about 40-100 nanometers.

In some embodiments, the lipid nanoparticle has a Polydispersity Index (PDI) between 0.01 to 0.2. In certain embodiments, the lipid nanoparticle has a Polydispersity Index (PDI) between 0.01 to 0.05. In some embodiments, the lipid nanoparticle has a Polydispersity Index (PDI) between 0.05 to 0.1. In certain embodiments, the lipid nanoparticle has a Polydispersity

Index (PDI) between 0.1 to 0.15. In some embodiments, the lipid nanoparticle has a Polydispersity Index (PDI) between 0.15 to 0.2.

In some embodiments, the lipid nanoparticle has a Polydispersity Index (PDI) between about 0.01 to about 0.2. In certain embodiments, the lipid nanoparticle has a Polydispersity Index (PDI) between about 0.01 to about 0.05. In some embodiments, the lipid nanoparticle has a Polydispersity Index (PDI) between about 0.05 to about 0.1. In certain embodiments, the lipid nanoparticle has a Polydispersity Index (PDI) between about 0.1 to about 0.15. In some embodiments, the lipid nanoparticle has a Polydispersity Index (PDI) between about 0.15 to about 0.2.

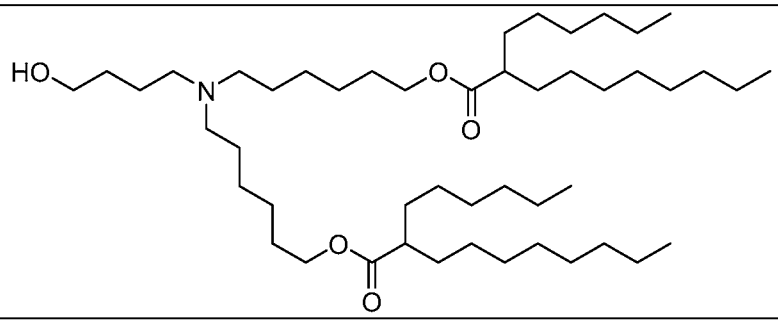
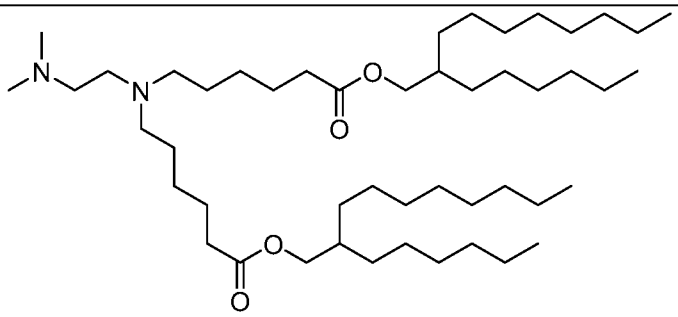
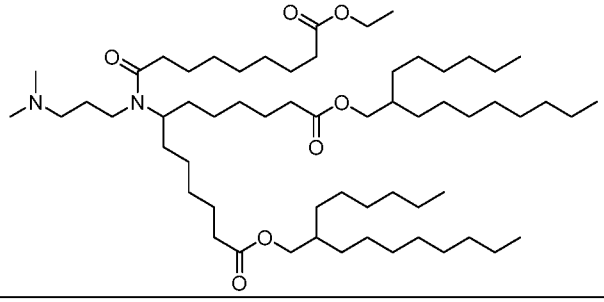
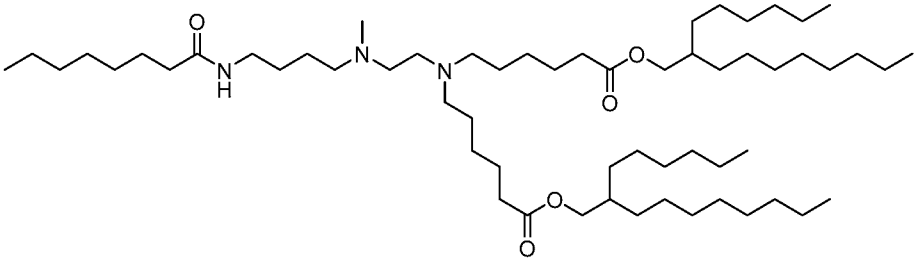
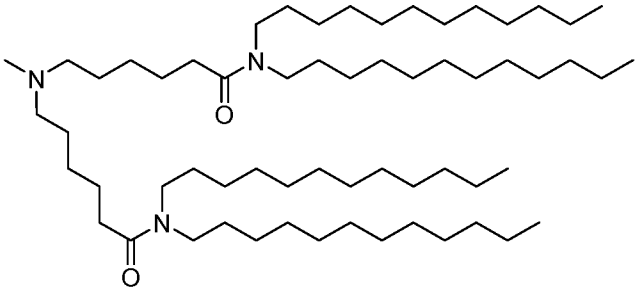
In certain embodiments, the lipid nanoparticle is delivered as part of an aqueous composition. In some embodiments, the aqueous composition further comprises a first buffer. In some embodiments, the first buffer is 25 mM acetate buffer, 50 mM citrate buffer, 25 mM phosphate buffer, or combinations thereof.

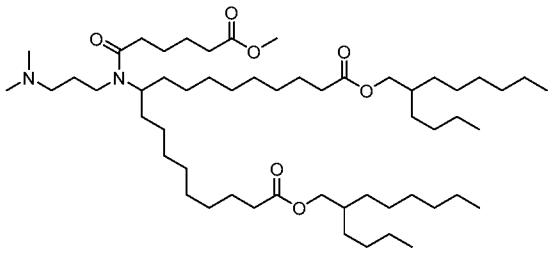
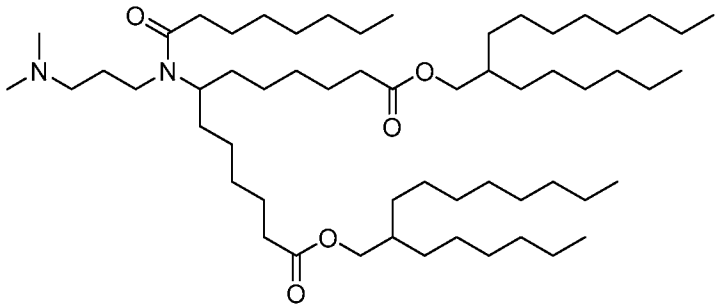
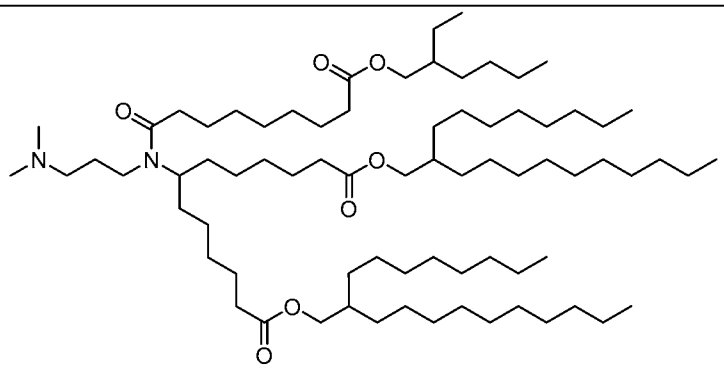
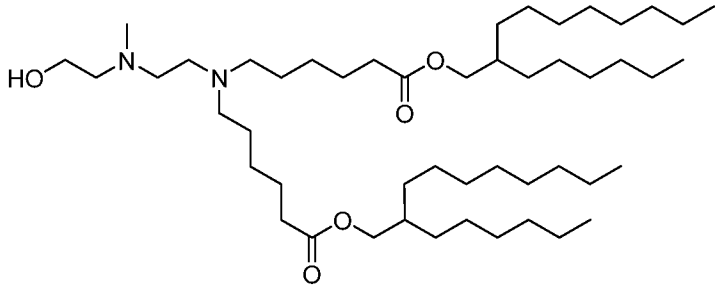
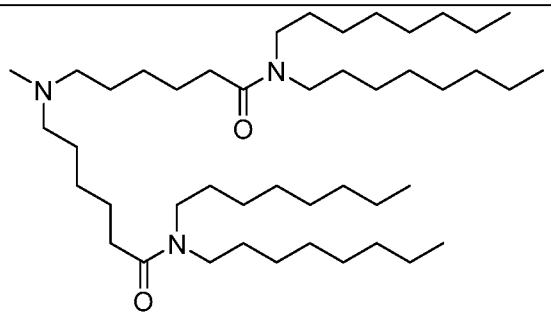
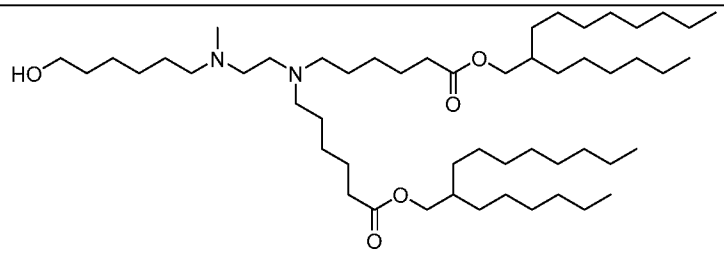
In some embodiments, the first buffer is 20-30 mM acetate buffer. In certain embodiments, the first buffer has a pH of 3.75-6.25. In some embodiments, the first buffer has a pH of 4. In certain embodiments, the first buffer has a pH of 5.5. In some embodiments, the first buffer has a pH of 6. In certain embodiments, the first buffer is 45-55 mM citrate buffer. In some embodiments, the first buffer is 50 mM citrate buffer. In certain embodiments, the first buffer has a pH of 3.75-4.25. In some embodiments, the first buffer has a pH of 4. In certain embodiments, the first buffer has a pH of 5.5. In some embodiments, the first buffer has a pH of 6. In certain embodiments, the first buffer is 25 mM phosphate buffer. In some embodiments, the first buffer has a pH of 5.75-6.25. In certain embodiments, the first buffer has a pH of 6.

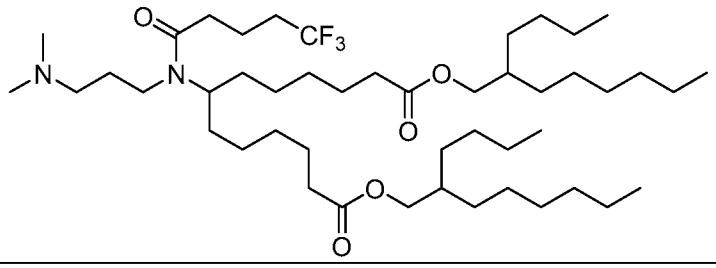
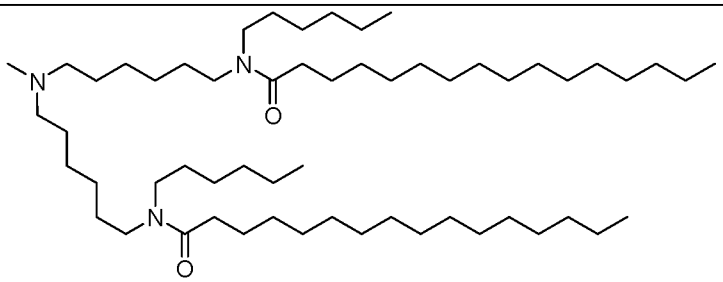
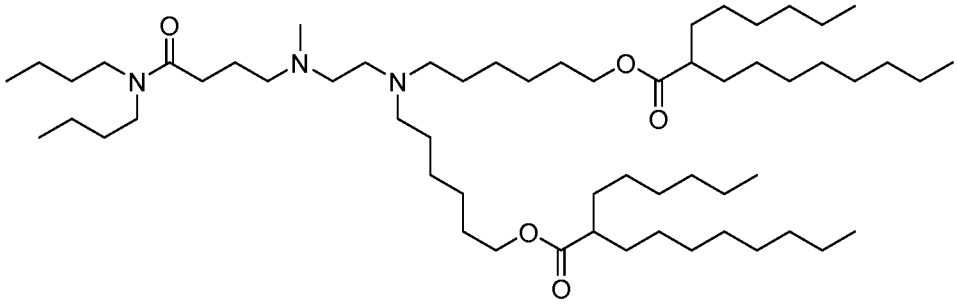
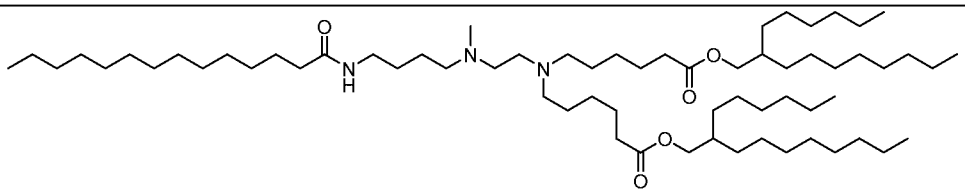
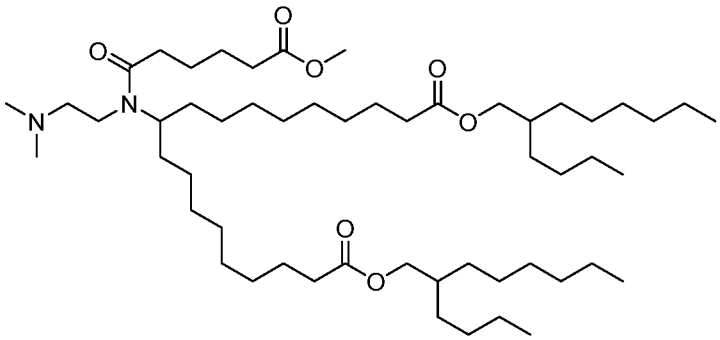
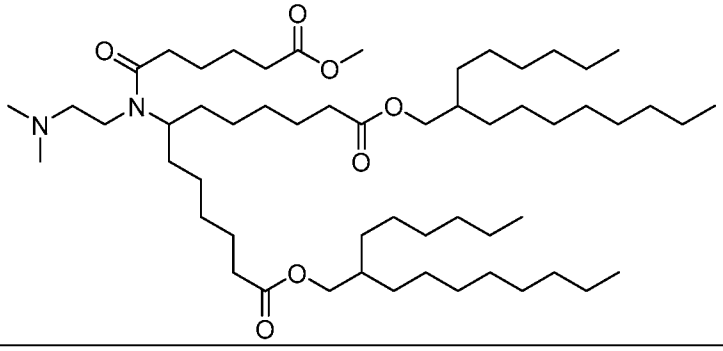
In some embodiments, the first buffer is about 20-30 mM acetate buffer. In certain embodiments, the first buffer has a pH of about 3.75-6.25. In some embodiments, the first buffer has a pH of about 4. In certain embodiments, the first buffer has a pH of about 5.5. In some embodiments, the first buffer has a pH of about 6. In certain embodiments, the first buffer is about 45-55 mM citrate buffer. In some embodiments, the first buffer is about 50 mM citrate buffer. In certain embodiments, the first buffer has a pH of about 3.75-4.25. In some embodiments, the first buffer has a pH of about 4. In certain embodiments, the first buffer has a pH of about 5.5. In some embodiments, the first buffer has a pH of about 6. In certain embodiments, the first buffer is about 25 mM phosphate buffer. In some embodiments, the first buffer has a pH of about 5.75-6.25. In certain embodiments, the first buffer has a pH of about 6.

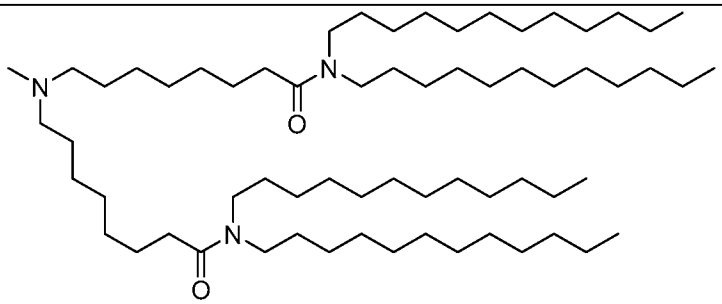
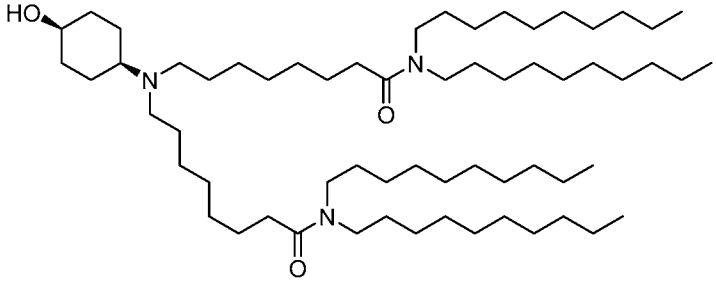
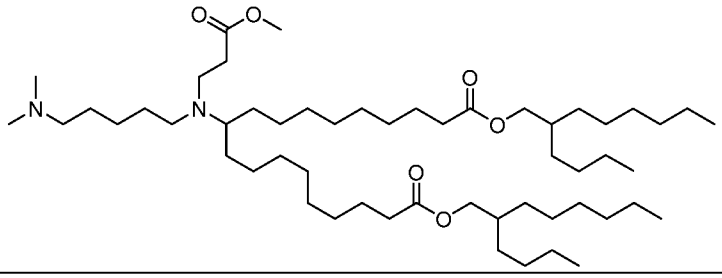
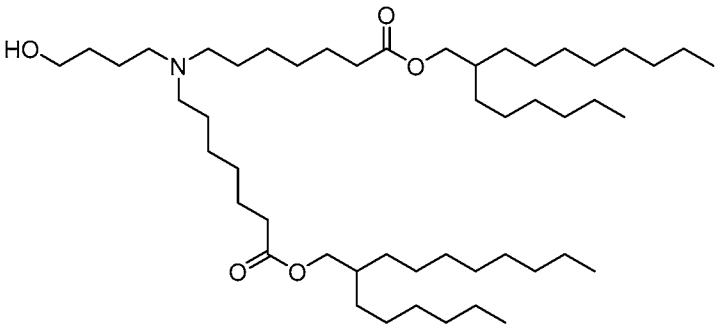
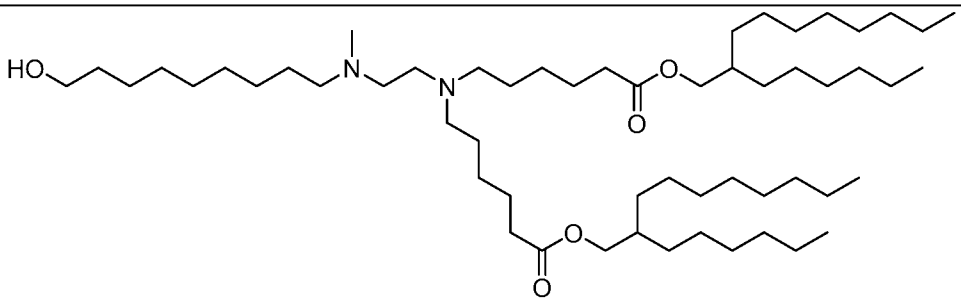
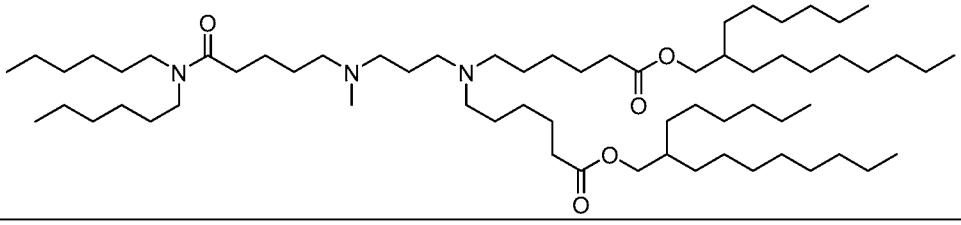
In various embodiments, the cationic lipid has one of the structures set forth in Table 1 below.

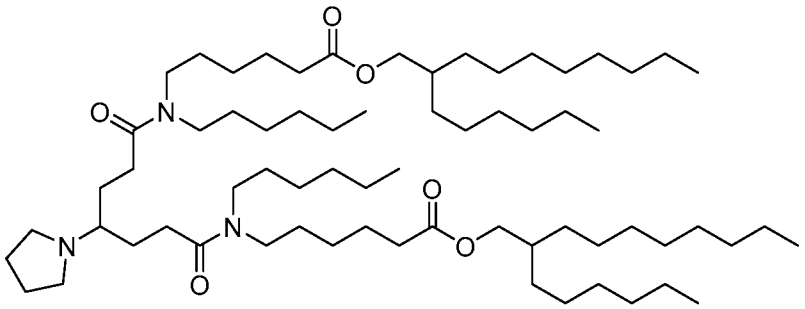
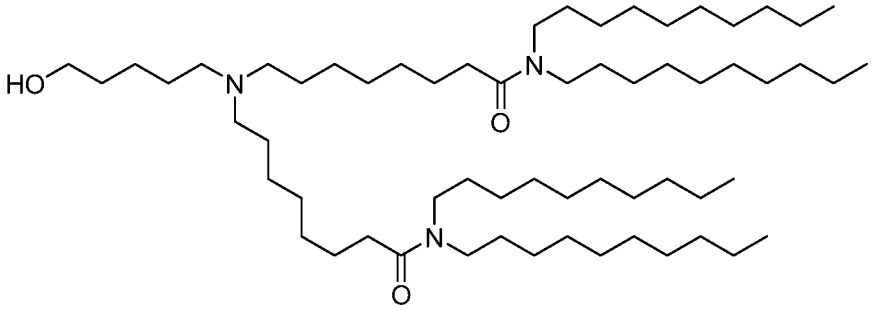
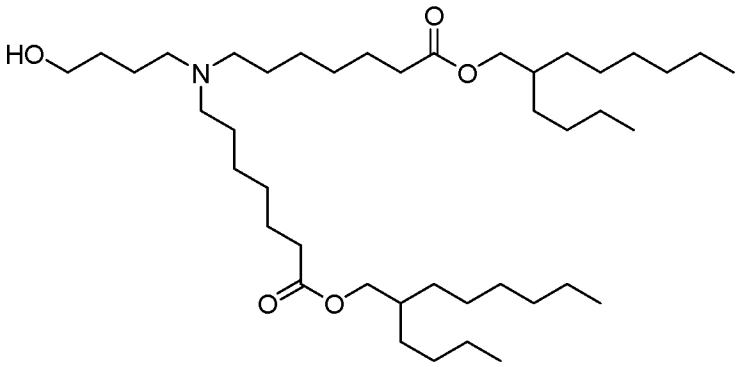
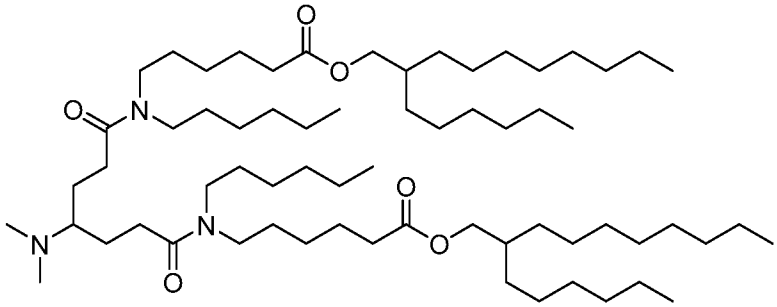
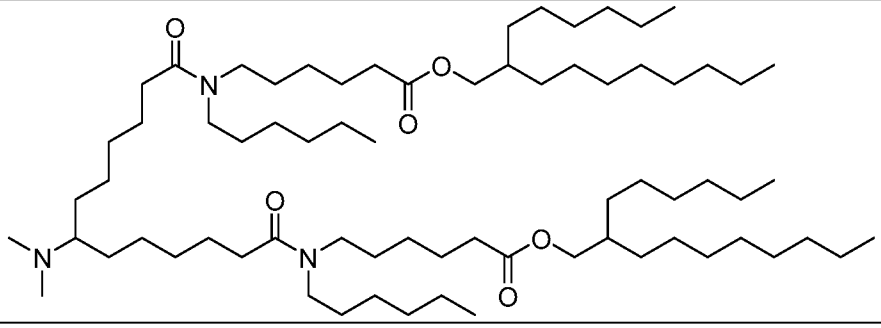
Table 1. Representative Cationic Lipids

No.	pKa LogP	Structure
I-1	6.09 14.8	
I-2	6.54 14.1	
I-3	6.57 16.3	
I-4	6.64 16.9	
I-5	6.67 19.8	

No.	pKa LogP	Structure
I-6	6.80 13.9	
I-7	6.45 16.6	
I-8	6.25 22.1	
I-9	6.91 13.9	
I-10	6.76 13.2	
I-11	6.69 15.3	

No.	pKa LogP	Structure
I-12	6.78 12.2	
I-13	6.83 18.1	
I-14	6.13 16.9	
I-15	6.70 19.4	
I-16	5.96 13.7	
I-17	6.25 14.5	

No.	pKa LogP	Structure
I-18	6.17 21.4	
I-19	6.58 19.0	
I-20	7.13 10.7	
I-21	6.49 15.5	
I-22	6.17 16.5	
I-23	6.34 19.2	

No.	pKa LogP	Structure
I-24	6.74 19.5	
I-25	6.54 18.3	
I-26	6.44 12.4	
I-27	6.36 15.1	
I-28	6.69 18.6	

It is understood that any embodiment of the compounds of Formula (I), as set forth above, and any specific substituent and/or variable in the compound Formula (I), as set forth above, may be independently combined with other embodiments and/or substituents and/or

variables of compounds of Formula (I) to form embodiments of the disclosure not specifically set forth above. In addition, in the event that a list of substituents and/or variables is listed for any particular R group in a particular embodiment and/or claim, it is understood that each individual substituent and/or variable may be deleted from the particular embodiment and/or claim and that the remaining list of substituents and/or variables will be considered to be within the scope of embodiments of the disclosure.

It is understood that in the present description, combinations of substituents and/or variables of the depicted formulae are permissible only if such contributions result in stable compounds.

For the purposes of administration, the compounds of the present disclosure (typically in the form of lipid nanoparticles in combination with a therapeutic agent) may be administered as a raw chemical or may be formulated as pharmaceutical compositions. Pharmaceutical compositions of the present disclosure comprise an LNP and one or more pharmaceutically acceptable carrier, diluent, or excipient. The cationic lipid is present in the composition in an amount which is effective to form a lipid nanoparticle and deliver the therapeutic agent, *e.g.*, for treating a particular disease or condition of interest. Appropriate concentrations and dosages can be readily determined by one skilled in the art.

Administration of the compositions of the disclosure can be carried out via any of the accepted modes of administration of agents for serving similar utilities. The pharmaceutical compositions of the disclosure may be formulated into preparations in solid, semi-solid, liquid, or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suspensions, suppositories, injections, inhalants, gels, microspheres, and aerosols. Typical routes of administering such pharmaceutical compositions include, without limitation, oral, topical, transdermal, inhalation, parenteral, sublingual, buccal, rectal, vaginal, and intranasal. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intradermal, intrasternal injection or infusion techniques. Pharmaceutical compositions of the disclosure are formulated to allow the active ingredients contained therein to be bioavailable upon administration of the composition to a patient. Compositions that will be administered to a subject or patient take the form of one or more dosage units, where for example, a tablet may be a single dosage unit, and a container of a compound of the disclosure in aerosol form may hold a plurality of dosage units. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see *Remington: The Science and Practice of Pharmacy*, 20th Edition (Philadelphia College of Pharmacy and Science, 2000). The composition

to be administered will, in any event, contain a therapeutically effective amount of a compound of the disclosure, or a pharmaceutically acceptable salt thereof, for treatment of a disease or condition of interest in accordance with the teachings of this disclosure.

A pharmaceutical composition of the disclosure may be in the form of a solid or liquid. In one aspect, the carrier(s) are particulate, so that the compositions are, for example, in tablet or powder form. The carrier(s) may be liquid, with the compositions being, for example, an oral syrup, injectable liquid, or an aerosol, which is useful in, for example, inhalatory administration.

When intended for oral administration, the pharmaceutical composition is preferably in either solid or liquid form, where semi-solid, semi-liquid, suspension and gel forms are included within the forms considered herein as either solid or liquid.

As a solid composition for oral administration, the pharmaceutical composition may be formulated into a powder, granule, compressed tablet, pill, capsule, chewing gum, wafer, or the like form. Such a solid composition will typically contain one or more inert diluents or edible carriers. In addition, one or more of the following may be present: binders such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, gum tragacanth or gelatin; excipients such as starch, lactose or dextrins, disintegrating agents such as alginic acid, sodium alginate, Primogel, corn starch and the like; lubricants such as magnesium stearate or Sterotex; glidants such as colloidal silicon dioxide; sweetening agents such as sucrose or saccharin; a flavoring agent such as peppermint, methyl salicylate or orange flavoring; and a coloring agent.

When the pharmaceutical composition is in the form of a capsule, for example, a gelatin capsule, it may contain, in addition to materials of the above type, a liquid carrier such as polyethylene glycol or oil.

The pharmaceutical composition may be in the form of a liquid, for example, an elixir, syrup, solution, emulsion, or suspension. The liquid may be for oral administration or for delivery by injection, as two examples. When intended for oral administration, preferred composition contain, in addition to the present compounds, one or more of a sweetening agent, preservatives, dye/colorant and flavor enhancer. In a composition intended to be administered by injection, one or more of a surfactant, preservative, wetting agent, dispersing agent, suspending agent, buffer, stabilizer, and isotonic agent may be included.

The liquid pharmaceutical compositions of the disclosure, whether they be solutions, suspensions or other like form, may include one or more of the following adjuvants: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or diglycerides which may

serve as the solvent or suspending medium, polyethylene glycols, glycerin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose; agents to act as cryoprotectants such as sucrose or trehalose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Physiological saline is a preferred adjuvant. An injectable pharmaceutical composition is preferably sterile.

A liquid pharmaceutical composition of the disclosure intended for either parenteral or oral administration should contain an amount of a compound of the disclosure such that a suitable dosage will be obtained.

The pharmaceutical composition of the disclosure may be intended for topical administration, in which case the carrier may suitably comprise a solution, emulsion, ointment or gel base. The base, for example, may comprise one or more of the following: petrolatum, lanolin, polyethylene glycols, bee wax, mineral oil, diluents such as water and alcohol, and emulsifiers and stabilizers. Thickening agents may be present in a pharmaceutical composition for topical administration. If intended for transdermal administration, the composition may include a transdermal patch or iontophoresis device.

The pharmaceutical composition of the disclosure may be intended for rectal administration, in the form, for example, of a suppository, which will melt in the rectum and release the drug. The composition for rectal administration may contain an oleaginous base as a suitable nonirritating excipient. Such bases include, without limitation, lanolin, cocoa butter and polyethylene glycol.

The pharmaceutical composition of the disclosure may include various materials, which modify the physical form of a solid or liquid dosage unit. For example, the composition may include materials that form a coating shell around the active ingredients. The materials that form the coating shell are typically inert, and may be selected from, for example, sugar, shellac, and other enteric coating agents. Alternatively, the active ingredients may be encased in a gelatin capsule.

The pharmaceutical composition of the disclosure in solid or liquid form may include an agent that binds to the compound of the disclosure and thereby assists in the delivery of the compound. Suitable agents that may act in this capacity include a monoclonal or polyclonal antibody, or a protein.

The pharmaceutical composition of the disclosure may consist of dosage units that can be administered as an aerosol. The term aerosol is used to denote a variety of systems ranging from those of colloidal nature to systems consisting of pressurized packages. Delivery may be by a liquefied or compressed gas or by a suitable pump system that dispenses the active ingredients. Aerosols of compounds of the disclosure may be delivered in single phase, bi-phasic, or tri-phasic systems to deliver the active ingredient(s). Delivery of the aerosol includes the necessary container, activators, valves, sub-containers, and the like, which together may form a kit. One skilled in the art, without undue experimentation may determine preferred aerosols.

The pharmaceutical compositions of the disclosure may be prepared by methodology well known in the pharmaceutical art. For example, a pharmaceutical composition intended to be administered by injection can be prepared by combining the lipid nanoparticles of the disclosure with sterile, distilled water or other carrier to form a solution. A surfactant may be added to facilitate the formation of a homogeneous solution or suspension. Surfactants are compounds that non-covalently interact with the compound of the disclosure to facilitate dissolution or homogeneous suspension of the compound in the aqueous delivery system.

The compositions of the disclosure, or their pharmaceutically acceptable salts, are administered in a therapeutically effective amount, which will vary depending upon a variety of factors including the activity of the specific therapeutic agent employed; the metabolic stability and length of action of the therapeutic agent; the age, body weight, general health, sex, and diet of the patient; the mode and time of administration; the rate of excretion; the drug combination; the severity of the particular disorder or condition; and the subject undergoing therapy.

Compositions of the disclosure may also be administered simultaneously with, prior to, or after administration of one or more other therapeutic agents. Such combination therapy includes administration of a single pharmaceutical dosage formulation of a composition of the disclosure and one or more additional active agents, as well as administration of the composition of the disclosure and each active agent in its own separate pharmaceutical dosage formulation. For example, a composition of the disclosure and the other active agent can be administered to the patient together in a single oral dosage composition such as a tablet or capsule, or each agent administered in separate oral dosage formulations. Where separate dosage formulations are used, the compounds of the disclosure and one or more additional active agents can be administered at essentially the same time, *i.e.,* concurrently, or at separately staggered times, *i.e.,* sequentially; combination therapy is understood to include all these regimens.

Preparation methods for the above compounds and compositions are described herein below and/or known in the art, for example in PCT Publication Nos. WO 2015/199952; WO 2017/004143; WO 2017/075531; WO 2017/117528; WO 2018/191657; WO 2018/107026; WO 2018/200943; WO 2018/078053; WO 2019/036000; WO 2019/036028; WO 2019/036030; WO 2019/036008; WO 2020/061426; WO 2020/081938; WO 2020/146805; WO 2021/030701; WO 2022/016070; WO 2023/114944; WO 2023/114937; WO 2023/114943; WO 2023/250427; and WO 2024/054843.

It will be appreciated by those skilled in the art that in the process described herein the functional groups of intermediate compounds may need to be protected by suitable protecting groups. Such functional groups include hydroxy, amino, mercapto and carboxylic acid. Suitable protecting groups for hydroxy include trialkylsilyl or diarylalkylsilyl (for example, *t*-butyldimethylsilyl, *t*-butyldiphenylsilyl or trimethylsilyl), tetrahydropyranyl, benzyl, and the like. Suitable protecting groups for amino, amidino and guanidino include *t*-butoxycarbonyl, benzyloxycarbonyl, and the like. Suitable protecting groups for mercapto include -C(O)-R″ (where R″ is alkyl, aryl or arylalkyl), *p*-methoxybenzyl, trityl and the like. Suitable protecting groups for carboxylic acid include alkyl, aryl or arylalkyl esters. Protecting groups may be added or removed in accordance with standard techniques, which are known to one skilled in the art and as described herein. The use of protecting groups is described in detail in Green, T.W. and P.G.M. Wutz, *Protective Groups in Organic Synthesis* (1999), 3rd Ed., Wiley. As one of skill in the art would appreciate, the protecting group may also be a polymer resin such as a Wang resin, Rink resin or a 2-chlorotrityl-chloride resin.

It will also be appreciated by those skilled in the art, although such protected derivatives of compounds of this disclosure may not possess pharmacological activity as such, they may be administered to a mammal and thereafter metabolized in the body to form compounds of the disclosure which are pharmacologically active. Such derivatives may therefore be described as "prodrugs". All prodrugs of compounds of this disclosure are included within the scope of the disclosure.

Furthermore, all compounds of the disclosure which exist in free base or acid form can be converted to their pharmaceutically acceptable salts by treatment with the appropriate inorganic or organic base or acid by methods known to one skilled in the art. Salts of the compounds of the disclosure can be converted to their free base or acid form by standard techniques.

It is understood that one skilled in the art may be able to make compounds of the present disclosure by methods known to those skilled in the art or by combining various methods known

to one skilled in the art. In general, starting components for synthesizing compounds of the present disclosure may be obtained from sources such as Sigma Aldrich, Lancaster Synthesis, Inc., Maybridge, Matrix Scientific, TCI, and Fluorochem USA, *etc.* or synthesized according to sources known to those skilled in the art (see, *e.g.*, Advanced Organic Chemistry: Reactions, Mechanisms, and Structure, 5th edition (Wiley, December 2000)) or prepared as described in this disclosure.

EXAMPLE 1

PREPARATION OF LIPID NANOPARTICLES

A compound of the present disclosure, DSPC, cholesterol, and pegylated lipid are solubilized in ethanol at desirable molar percentages (*e.g.*, 47.5:10:40.7:1.8). Lipid nanoparticles (LNP) are prepared at a total lipid to mRNA weight ratio of approximately 10:1 to 30:1. The mRNA is diluted to 0.2 mg/mL in 10 to 50 mM citrate buffer, pH 4 to 6 or 10 to 25 mM acetate buffer, pH 4 to 6. Syringe pumps are used to mix the ethanolic lipid solution with the mRNA aqueous solution at a ratio of about 1:5 to 1:3 (vol/vol) with total flow rates above 15 mL/min. The ethanol is then removed, and the external buffer is replaced with PBS by dialysis. Finally, the lipid nanoparticles are filtered through a 0.2 μm pore sterile filter. Lipid nanoparticle particle size is determined using quasi-elastic light scattering via a Nicomp 370 submicron particle sizer (Santa Barbara, CA). Alternatively, particle size can also be as determined by quasi-elastic light scattering using a Malvern Zetasizer Nano ZS (Malvern, UK).

Formulation Characterization:

Compound No.	Encapsulation (%)	Particle Diameter (nm)	Poly-dispersity
I-1	95%	70	0.076
I-2	98%	76	0.090
I-4	98%	78	0.019
I-5	98%	79	0.118
I-3	98%	80	0.047
I-6	94%	81	0.099

EXAMPLE 2

LUCIFERASE mRNA IN VIVO EVALUATION USING LIPID NANOPARTICLE COMPOSITIONS

Luciferase mRNA *in vivo* evaluation studies were performed on 6–8-week-old female C57BL/6 mice (Charles River) or 8–10-week-old CD-1 mice (Charles River or Inotiv). Varying doses of mRNA-lipid nanoparticle, formulated according to standard methods as described herein in Example 1, were administered systemically via tail vein injection. Animals were euthanized at specific time points (i.e., four hours) post-administration. Liver and spleen were collected in pre-weighed tubes, and weights determined. Approximately 50 mg of liver was dissected for analyses in 2 mL FastPrep tubes (MP Biomedicals, Solon OH). A ¼ inch ceramic sphere (MP Biomedicals) was added to each tube and 500-750 µL of Glo Lysis Buffer – GLB (Promega, Madison WI) equilibrated to room temperature was added to liver tissue. Liver tissues were homogenized with the FastPrep24 instrument (MP Biomedicals) at 2 × 6.0 m/s for 15 seconds. The homogenate was incubated at room temperature for five minutes prior to a 1:4 to 1:6 dilution in GLB and assessed using the SteadyGlo Luciferase assay system (Promega). Specifically, 50 µL of diluted tissue homogenate was reacted with 50 µL of SteadyGlo substrate, shaken for 10 seconds followed by five-minute incubation and then luminescence quantitated using a FilterMax F5 Microplate Reader (Molecular Devices, USA). The amount of protein assayed was determined by using the BCA protein assay kit (Pierce, Rockford, IL). Relative luminescence units (RLU) were then normalized to total µg protein or weight (g) of tissue assayed. To convert RLU to ng luciferase a standard curve was generated with QuantiLum Recombinant Luciferase (Promega). The activity was compared at a doses of 0.3, 0.5, and/or 1.0 mg mRNA/kg and expressed as ng luciferase/g liver and ng luciferase/g spleen.

Cmp. No.	Liver Luc @ 0.3 mg/kg (ng/g)	Liver Luc @ 0.5 mg/kg (ng/g)	Liver Luc @ 1.0 mg/kg (ng/g)	Spleen Luc @ 0.3 mg/kg (ng/g)	Spleen Luc @ 0.5 mg/kg (ng/g)	Spleen Luc @ 1.0 mg/kg (ng/g)
I-1	1275 ± 410		10643 ± 1858	45 ± 11		121 ± 13
I-2	2126 ± 294		14486 ± 6607	42 ± 12		220 ± 104
I-3	753 ± 156	1928 ± 1268	2203 ± 1555	253 ± 95	228 ± 126	444 ± 152

Cmp. No.	Liver Luc @ 0.3 mg/kg (ng/g)	Liver Luc @ 0.5 mg/kg (ng/g)	Liver Luc @ 1.0 mg/kg (ng/g)	Spleen Luc @ 0.3 mg/kg (ng/g)	Spleen Luc @ 0.5 mg/kg (ng/g)	Spleen Luc @ 1.0 mg/kg (ng/g)
I-4	5497 ± 1457	8113 ± 2889	13160 ± 5805	217 ± 30	310 ± 124	449 ± 102
I-6		669 ± 575			148 ± 37	
I-7	653 ± 132		3821 ± 1047	34 ± 7		121 ± 42
I-8	426 ± 174		5094 ± 988	10 ± 4		37 ± 13
I-11		16 ± 14			0 ± 0	
I-12		1007 ± 671			358 ± 230	
I-13	3529 ± 1135		17110 ± 10331	300 ± 100		1592 ± 965
I-14	16109 ± 2653		56927 ± 16125	471 ± 166		748 ± 301
I-15		1092 ± 365			31 ± 6	
I-16	7023 ± 3006	1745 ± 681	21357 ± 15325	1138 ± 389	248 ± 162	1680 ± 1164
I-17	8210 ± 2527		58913 ± 13963	378 ± 165		1207 ± 307
I-22	10441 ± 5777	1597 ± 456	44725 ± 14170	47 ± 3	288 ± 259	643 ± 506

EXAMPLE 3

IMMUNOGENICITY FOLLOWING INTRAMUSCULAR (I.M.) ADMINISTRATION OF MRNA-LNPS ENCODING INFLUENZA A HA

BALB/c mice (n=8-10 per group) were administered 30 µL of mRNA-LNP encoding Influenza A/Puerto Rico/8/1934 Hemagglutinin (HA) by intramuscular (I.M.) injection into the quadriceps at either 0.2 µg or 0.5 µg mRNA dose levels on Day 0 and Day 14. Blood samples were collected on Day -1, Day 13, and at the terminal bleed on Day 28. Serum was isolated and analyzed for immunogenicity by Hemagglutination Inhibition (HAI) assay.

Hemagglutination Inhibition (HAI) Assay

The samples were diluted 1:4 with Receptor Destroying Enzyme II (RDE) to inactivate non-specific inhibitors of hemagglutination present in sera. Samples were incubated for 18 hours at 37°C followed by further incubation for 30 minutes at 56°C to inactivate the enzyme. RDE-treated samples were diluted 1:10 in 0.85% NaCl. A 1% suspension of turkey red blood cells (TRBCs) was prepared in PBS. Non-specific agglutinins were detected in samples by incubating RDE-treated sera (25 uL), 25 uL PBS and 50 uL 1% TRBC suspension for 30 min at room temperature. If RBCs settle completely in serum-containing wells then serum sample is acceptable for use in the HAI assay.

Verified RDE-treated sera samples were serially diluted in duplicate two-fold in PBS for a total of 12 dilutions ranging from 20 – 40960. Inactivated IFV-A/PR/8/34 (Charles River Laboratories, #10100782) was prepared to a concentration equating to 4 hemagglutinin (HA) units and incubated with sera in a 1:1 ratio for 30 minutes at room temperature. The serum/antigen mixture was incubated with the 1% TRBC suspension in a 1:1 ratio for 30 minutes at room temperature. TRBCs only and antigen only controls were included.

Plates were then analyzed and scored. Each well was examined for the presence of hemagglutination. Inhibition of hemagglutination indicates the presence of HA-specific antibodies. For each serum sample, the highest dilution that completely inhibited hemagglutination was recorded as the HA titer.

0.2 µg dose:

Compound No.	Day 13		Day 28	
	HAI GMT	Fold Change vs. Compound I-1	HAI GMT	Fold Change vs. Compound I-1
I-1	29	-	131	-
I-2	68	2.3	1096	8.4
I-4	37	1.3	975	7.4
I-5	30	1.0	916	7.0
I-3	30	1.0	916	7.0
I-6	41	1.4	1595	12.2

0.5 µg dose:

Compound No.	Day 13		Day 28	
	HAI GMT	Fold Change vs. Compound I-1	HAI GMT	Fold Change vs. Compound I-1
I-1	60	-	1531	-
I-2	89	1.5	1885	1.2
I-4	124	2.1	1446	0.9
I-5	63	1.1	1488	1.0
I-3	53	0.9	1295	0.8
I-6	55	0.9	1729	1.1

An additional lipid screening using serum HAI titers 14 days following prime/boost vaccination of BALB/c mice (10/group) was performed with 0.2 μg PR8 HA mRNA-LNP. The results of the screening are presented in the table below (see also, *e.g.*, FIG. 3).

Cationic Lipid Compound No.	Mean HAI Titer
I-1	408
I-2	1155
I-3	1045
I-4	1337
I-5	1273
I-6	1177
I-7	953
I-9	1665
I-11	1184
I-12	1288
I-13	1296
I-14	800
I-15	1784
I-16	736
I-17	832
I-19	1888
I-20	357
I-21	1104
I-22	896
I-23	1760
I-24	1664
I-25	1632
I-26	1368
I-27	1288
I-28	1280

EXAMPLE 4

IMMUNOGENICITY FOLLOWING INTRAMUSCULAR (I.M.) ADMINISTRATION OF MRNA-LNPS ENCODING RSV PRE-F

BALB/c mice (n = 5–10 per group) were administered 30 μL of mRNA-LNP formulation encoding the Respiratory Syncytial Virus (RSV) Pre-Fusion (Pre-F) protein via intramuscular injection into the quadriceps at a dose of 0.2 μg mRNA on Day 0 and Day 14. Blood samples were collected on Day -1, Day 13, and at terminal bleed on Day 28. Serum was isolated and analyzed.

Neutralizing antibody responses were evaluated by FRNT assay. HEp-2 cells were seeded in 96-well plates at 2×10^4 cells per well and incubated overnight at 37°C in 5% CO_2.

The following day, serum samples were serially diluted 3-fold in serum-free medium to generate 12 dilutions ranging from 1:25 to 1:4,428,675. RSV A2 virus was diluted to yield a consistent number of foci upon infection of HEp-2 cells. The diluted virus was added to the serially diluted serum samples and incubated for 1 hour at 37°C. The virus-serum mixtures were then transferred to the HEp-2 cells, and viral infection was allowed to proceed for 2 hours at 37°C in 5% CO_2. After this incubation, the medium was replaced, and the cells were further incubated for 48 hours. Cells were subsequently fixed and stained to detect viral foci. For each serum sample, the highest dilution that completely inhibited viral infection (absence of foci) was recorded as the FRNT titer. No detectable FRNT titer (at 100% neutralization cutoff) was observed with Compound-I. Therefore, relative immunogenicity for RSV was assessed by quantification of IgG binding antibody levels specific to RSV.

IgG binding antibody levels were determined using the Mesoscale Diagnostics (MSD) V-PLEX Respiratory Panel 4 kit, following the manufacturer's instructions. Mouse sera were diluted 1:500,000. Standard curves were generated using commercial anti-RSV Pre-F IgG antibodies, and IgG concentrations were interpolated accordingly. Mean antibody concentrations were used to determine the fold change ranking relative to Compound I-1. The results of the screening are presented in the table below (see also, *e.g.*, FIG. 4).

Cationic Lipid Compound No.	Study #	FRNT (100%) Mean Titer	IgG Mean Titer (ng/mL)	IgG Mean Titer Fold Change vs Compound I-1 within study
I-1	1	0	49642	1
I-4	1	75	262259	5.3
I-2	2	76.25	463901	8.8
I-1	2	0	52727	1
I-3	2	57.5	590680	11.2
I-6	2	67.5	432487	8.2
I-5	2	57.5	551974	10.5

EXAMPLE 5

IMMUNOGENICITY FOLLOWING INTRAMUSCULAR (I.M.) ADMINISTRATION OF MRNA-LNPS ENCODING SARS-COV-2 RBD

BALB/c mice (n=5–8 per group) were injected intramuscularly into the quadriceps muscle with 30 µL of mRNA-LNP formulation encoding the SARS-CoV-2 Receptor Binding Domain (RBD) at a dose of 0.5 µg mRNA on Day 0 and Day 14. Blood samples were collected

on Day -1, Day 13, and terminally on Day 28. Serum was isolated and assessed for immunogenicity using a Foci Reduction Neutralization Test (FRNT) assay.

For the FRNT assay, VERO-TMPRSS2 cells were seeded in 96-well plates at 2×10^4 cells per well and incubated overnight at 37°C in 5% CO_2. On the following day, serum samples were serially diluted 3-fold in serum-free medium to generate 12 dilutions ranging from 1:25 to 1:4,428,675. SARS-CoV-2 virus was diluted to achieve a consistent number of foci per well upon infection of VERO-TMPRSS2 cells. The diluted virus was incubated with the serially diluted serum samples for 1 hour at 37°C. The virus-serum mixtures were then added to VERO-TMPRSS2 cells and incubated for 24 hours at 37°C in 5% CO_2. Following infection, cells were fixed and stained to visualize viral foci. For each serum sample, the highest dilution that completely inhibited viral infection (absence of foci) was recorded as the FRNT titer. Mean FRNT titers were used to determine fold change ranking relative to Compound I-1. The results of the screening are presented in the table below (see also, *e.g.*, FIG. 5).

Cationic Lipid Compound No.	Study #	Mean Titer	Fold Change vs Compound I-1 within study
I-2	3	381	7.9
I-1	4	2.5	1.0
I-1	3	48.4	1.0
I-3	3	208	4.3
I-4	4	37.5	15.0
I-6	3	164	3.4
I-5	3	253	5.2

EXAMPLE 6

IMMUNOGENICITY FOLLOWING INTRAMUSCULAR ADMINISTRATION OF MULTIVALENT LNP-MRNA ENCODING INFLUENZA HA, RSV PRE-F, AND SARS-COV-2 RBD

BALB/c mice (n = 10 per group) were administered 30 μL of LNP-mRNA formulation encoding Influenza A/Puerto Rico/8/1934 Hemagglutinin (HA; 0.2 μg), RSV Pre-F (0.2 μg), or SARS-CoV-2 RBD (0.5 μg) in either monovalent format or in co-mixed or co-formulated trivalent formats by intramuscular injection into the quadriceps on Day 0 and Day 14. Terminal bleeds were collected on Day 28, and sera were analyzed.

Immunogenicity was assessed using Hemagglutination Inhibition (HAI) assay for Influenza HA and FRNT assays for RSV Pre-F and SARS-CoV-2 RBD (see FIG. 6).

* In the co-mixed format, separately formulated mRNA-LNP formulations were mixed. In the co-formulated format, mRNAs encoding different antigens were combined prior to LNP formulation, resulting in LNPs encapsulating multiple mRNA species.

EXAMPLE 7

LNP BIODISTRIBUTION FOLLOWING INTRAMUSCULAR (I.M.) ADMINISTRATION OF MRNA-LNP ENCODING FIREFLY LUCIFERASE (FLUC)

BALB/c mice (n = 3 per group) were injected intramuscularly into the quadriceps with 30 µL of LNP-mRNA encoding Firefly Luciferase (Fluc) at a 0.2 µg mRNA dose. Four hours post-dose, mice were terminated and tissues including inguinal and popliteal lymph nodes, spleen, and liver were collected. Organs were homogenized, and luciferase expression was measured using the Steady-Glo Luciferase Assay (Promega). Data is expressed as picograms (pg) of luciferase per total tissue, and the ratio of luciferase expression in lymph nodes + spleen relative to liver was determined. The results are presented in the table below (see also, *e.g.*, FIG. 7A).

	Luciferase (pg/total tissue)					Ratio of fLuc Expression (Combined Lymph Nodes + Spleen vs. Liver)
	Inguinal LN	Popliteal LN	LN Combined	Spleen	Liver	
I-1*	0	3416	3416	65	4812	0.72
LNP 2	421	314	735	61	4004	0.20
LNP 3	3090	2912	6002	222	3314	1.88
LNP 4	749	3362	4111	71	1048	3.99
LNP 5	665	91	756	89	1291	0.65
LNP 6	142	1809	1951	207	689	3.13
LNP 16	28	1114	1142	22	592	1.97
LNP 22	119	3604	3723	75	1130	3.36

* LNP formulation prepared with Compound I-1

Live whole-body imaging was performed on mice at 4-, 24-, and 48-hour post-dose. Average radiance (photons/second/cm^2/steradian) at the injection site and liver regions was quantified. The area under the curve (AUC) was calculated for each region over time, and the ratio of liver to injection site signal was determined. The results are presented in the tables below (see also, *e.g.*, FIG. 7B).

	AUC_{0-48hr}			
	I-1*	LNP 2	LNP 3	LNP 4
Liver	7.35×10^8	3.67×10^8	3.97×10^8	1.00×10^8
Injection Site	1.85×10^8	1.66×10^8	2.81×10^8	2.57×10^8
Liver to Injection Site Ratio	4.0	2.2	1.4	0.4

	AUC_{0-48hr}			
	LNP 5	LNP 6	LNP 16	LNP 22
Liver	6.98×10^8	1.49×10^8	1.97×10^8	8.85×10^8
Injection Site	4.85×10^8	3.47×10^8	1.01×10^8	5.06×10^8
Liver to Injection Site Ratio	1.4	0.4	1.9	1.7

* LNP formulation prepared with Compound I-1

EXAMPLE 8

IMMUNOGLOBULIN G (IGG) MRNA IN VIVO EVALUATION USING LIPID NANOPARTICLE COMPOSITIONS

A cationic lipid (*e.g.*, a compound from Table 1), DSPC, cholesterol and a pegylated lipid are solubilized in ethanol at a molar ratio of 47.5:10:40.7:1.8. Lipid nanoparticles (LNP) are prepared at a total lipid to mRNA weight ratio of approximately 10:1 to 40:1. Briefly, the mRNA is diluted to 0.2 mg/mL in 10 to 50 mM citrate buffer, pH 4 to 6 or 10 to 25 mM acetate buffer, pH 4 to 6. Syringe pumps are used to mix the ethanolic lipid solution with the mRNA aqueous solution at a ratio of about 1:5 to 1:3 (vol/vol) with total flow rates above 15 mL/min. The ethanol is then removed, and the external buffer is replaced with PBS by dialysis. Finally, the lipid nanoparticles are filtered through a 0.2 μm pore sterile filter.

Studies are performed in 6–8-week-old CD-1/ICR mice (Envigo, Charles River, or Inotiv) according to guidelines established by an institutional animal care committee (ACC) and the Canadian Council on Animal Care (CCAC). Varying doses of mRNA-lipid nanoparticle are systemically administered by tail vein injection and animals euthanized at a specific time point (e.g., 24 hours) post-administration. The whole blood is collected, and the serum subsequentially separated by centrifuging the tubes of the whole blood at 2000× g for 10 minutes at 4 °C and stored at -80 °C until use for analysis.

For immunoglobulin G (IgG) ELISA (Life Diagnostics Human IgG ELISA kit), the serum samples are diluted at 100 to 20,000 folds with 1x diluent solution. 100 μL of diluted serum is dispensed into anti-human IgG coated 96-well plate in duplicate alongside human IgG standards and incubated in a plate shaker at 150 rpm at 25 °C for 45 minutes. The wells are washed 5 times with 1x wash solution using a plate washer (400 μL/well). 100 μL of HRP

conjugate is added into each well and incubated in a plate shaker at the same condition above. The wells are washed 5 times again with 1x wash solution using a plate washer (400 µL/well). 100 µL of TMB reagent is added into each well and incubated in a plate shaker at the same condition above. The reaction is stopped by adding 100 µL of Stop solution to each well. The absorbance is read at 450 nm (A450) with a microplate reader. The amount of human IgG in mouse serum is determined by plotting A450 values for the assay standard against human IgG concentration.

The activity was determined by measuring the amount of human IgG in mouse serum 24 hours after administration, following dosing at 1.0 or 0.3 mg mRNA/kg. Results are expressed as µg IgG/mL serum.

Cationic Lipid No.	Serum IgG (ug/mL) @ 0.3 mg/kg	Serum IgG (ug/mL) @ 1.0 mg/kg
I-5	8.22 ± 0.84	47.68 ± 5.26
I-18	16.72 ± 5.38	101.96 ± 4.12
I-19	97.57 ± 19.26	257.42 ± 91.86
I-21	10.16 ± 4.47	-
I-23	42.4 ± 11.7	120.6 ± 31.7
I-24	65.8 ± 22.75	270.3 ± 98.37
I-25	48.78 ± 6.88	220.38 ± 62.57
I-26	38.07 ± 15.77	-
I-27	50.94 ± 10.25	137.3 ± 70.80
I-28	31.05 ± 12.47	-

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification, including U.S. Provisional Application Nos. 63/641,376, filed on May 1, 2024, and 63/792,167, filed on April 21, 2025, are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications, and publications to provide yet further embodiments.

These and other changes can be made to the embodiments considering the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

CLAIMS

1. A method of inducing an immune response in a subject, the method comprising intramuscular delivery of a lipid nanoparticle (LNP) to the subject, wherein the lipid nanoparticle comprises a nucleic acid that encodes an antigen and a cationic lipid, wherein:

a) the cationic lipid has a pKa from about 5.8 to about 7.2;

b) the cationic lipid has a LogP value from about 12 to about 25; and/or

c) the LNP has a spleen activity greater than about 45 ng/g.

2. The method of claim 1, wherein the cationic lipid has an ionizable headgroup.

3. The method of any one of claims 1-2, wherein the cationic lipid has at least two alkyl tail groups comprising a C_6-C_{24} alkyl chain.

4. The method of any one of claims 1-3, wherein the pKa is from about 6.4 to about 7.2.

5. The method of any one of claims 1-3, wherein the pKa is from about 5.8 to about 7.4.

6. The method of any one of claims 1-3, wherein the pKa is from about 6.4 to about 7.4.

7. The method of any one of claims 1-3, wherein the pKa is from about 6.3 to about 7.0.

8. The method of any one of claims 1-3, wherein the pKa is from about 6.3 to about 7.1.

9. The method of any one of claims 1-3, wherein the pKa is from about 5.8 to about 6.2.

10. The method of any one of claims 1-3, wherein the pKa is from about 6.2 to about 6.8.

11. The method of any one of claims 1-3, wherein the pKa is from about 6.8 to about 7.2.

12. The method of any one of claims 1-11, wherein the cationic lipid has a LogP from about 12 to about 25.

13. The method of any one of claims 1-11, wherein the cationic lipid has a LogP from about 13 to about 15, from about 15 to about 17, from about 17 to about 19, from about 19 to about 21, from about 21 to about 23, or from about 23 to about 25.

14. The method of any one of claims 1-11, wherein the cationic lipid has a LogP from about 12 to about 14, from about 14 to about 16, from about 16 to about 18, from about 18 to about 20, from about 20 to about 22, or from about 22 to about 24.

15. The method of any one of claim 1-14, wherein the spleen activity is greater than about 50 ng/ g.

16. The method of any one of claims 1-15, wherein the spleen activity is greater than about 125 ng/ g.

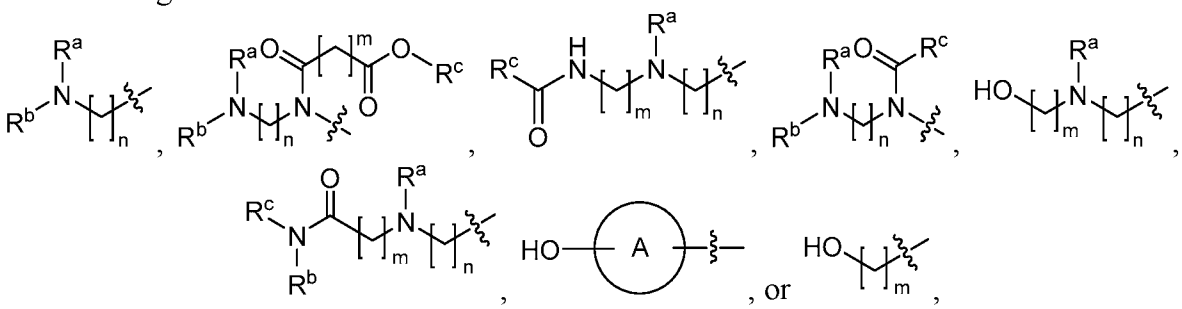
17. The method of any one of claims 1-16, wherein the spleen activity is greater than about 200 ng/ g.

18. The method of any one of claims 1-17, wherein the spleen activity is greater than about 400 ng/ g.

19. The method of any one of claims 1-18, wherein the spleen activity is about 1.05 times greater than the spleen activity of an LNP prepared with Compound I-1.

20. The method of any one of claims 1-18, wherein the spleen activity is about 1.5 times greater than the spleen activity of an LNP prepared with Compound I-1.

21. The method of any one of claims 2-20, wherein the ionizable headgroup has one of the following structures:



wherein:

ring A is a C_4-C_8 cycloalkyl;

R^a and R^b are each independently C_1-C_6 alkyl, or R^a and R^b join with the nitrogen to which they are attached to form a 3-8 membered heterocyclyl;

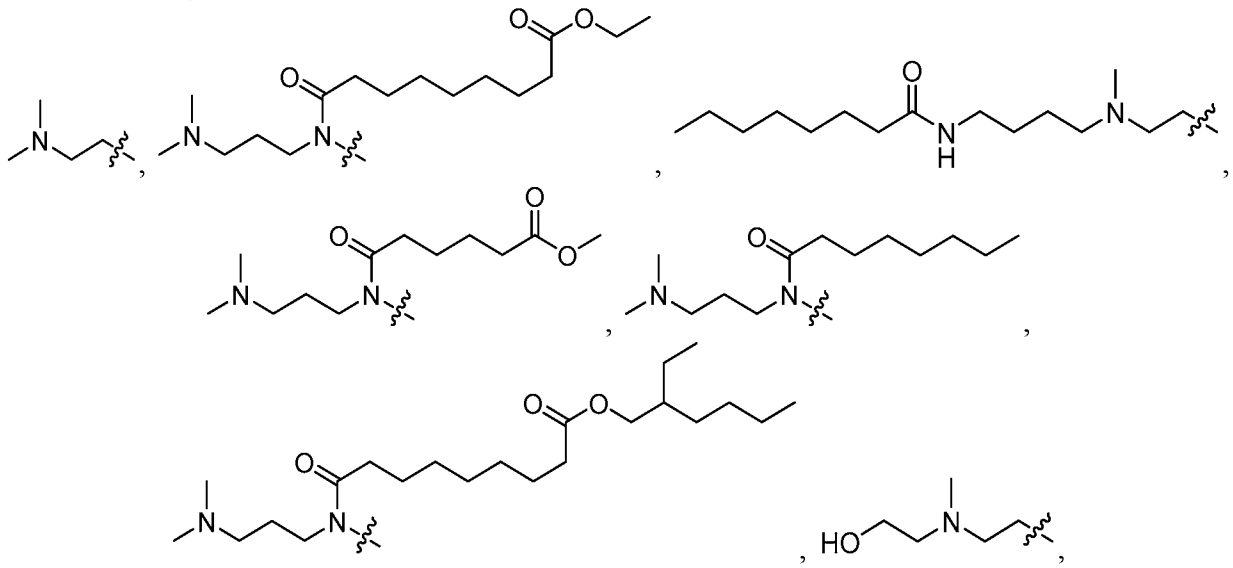
R^c is C_1-C_{14} alkyl or C_1-C_8 haloalkyl;

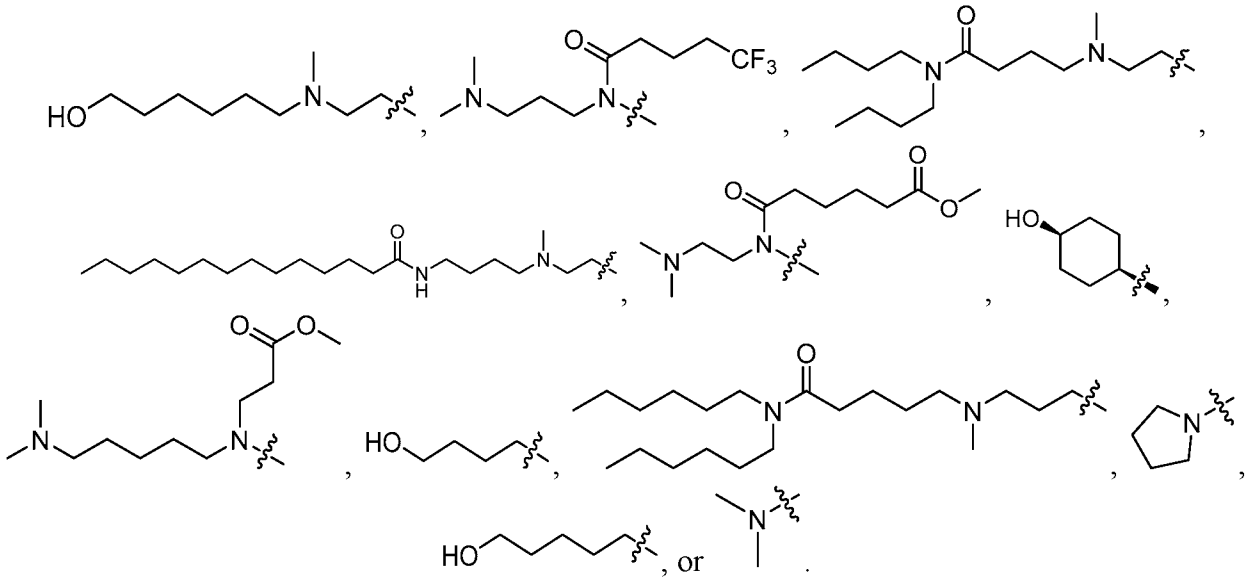
n is an integer from 0 to 6; and

m is an integer from 1 to 12.

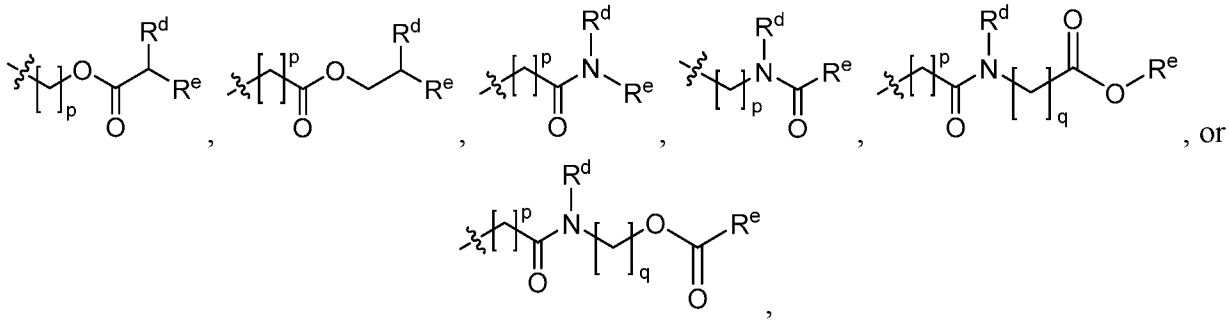
22. The method of claim 21, wherein R^a and R^b are both methyl or R^a and R^b join with the nitrogen to which they are attached to form pyrrolidinyl.

23. The method of any one of claims 2-22, wherein the ionizable headgroup has one of the following structures:





24. The method of any one of claims 3-23, wherein the two alkyl tail groups each independently have one of the following structures:



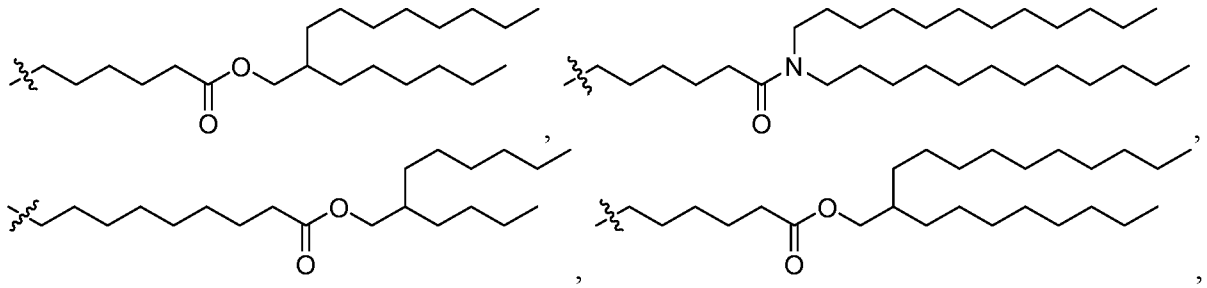
wherein:

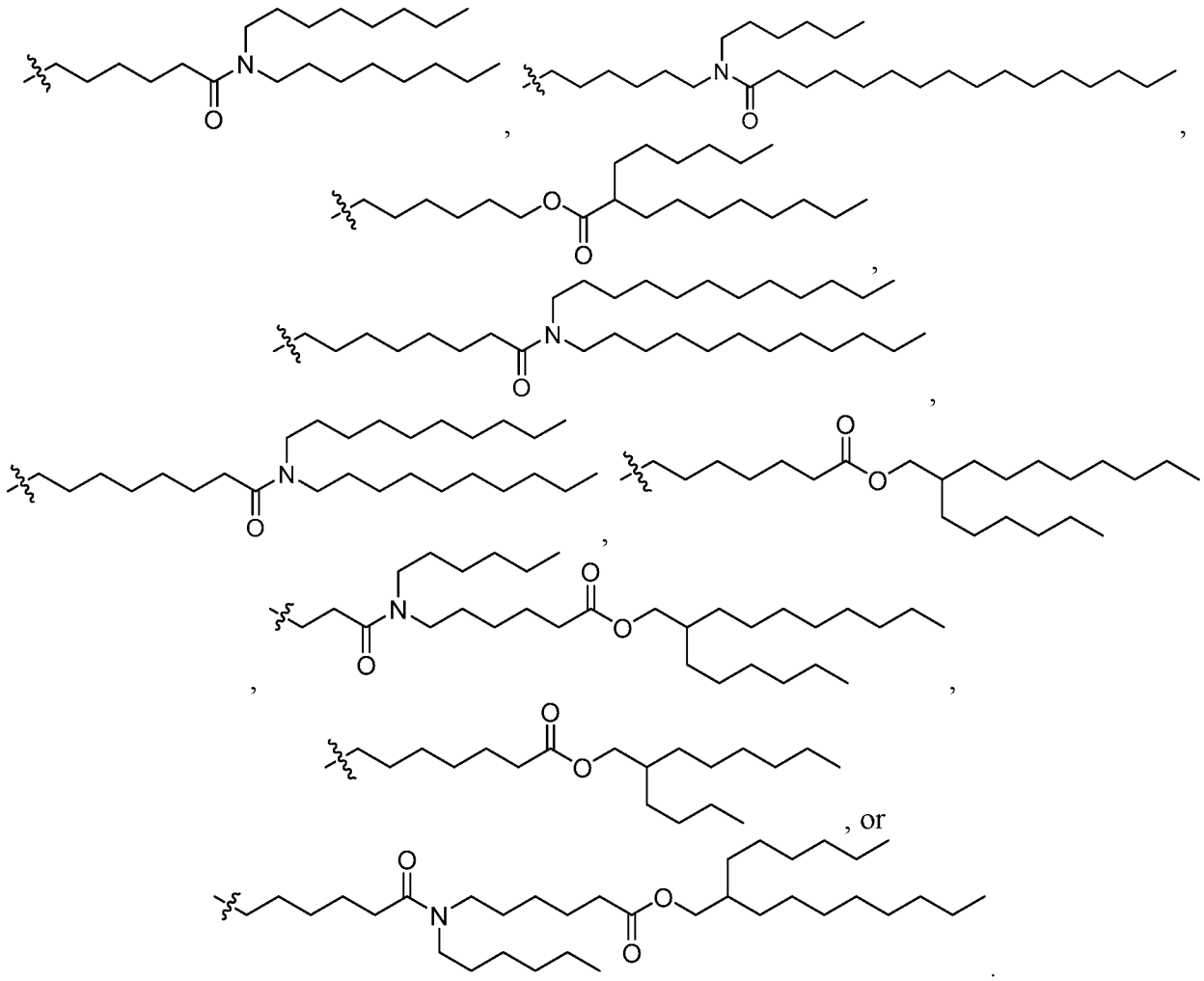
each occurrence of R^d and R^e are independently C_4-C_{18} alkyl;

each occurrence of p is independently an integer from 2 to 12; and

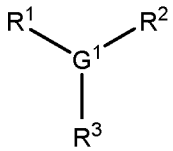
each occurrence of q is independently an integer from 2 to 12.

25. The method of any one of claims 3-24, wherein the two alkyl tail groups each independently have one of the following structures:





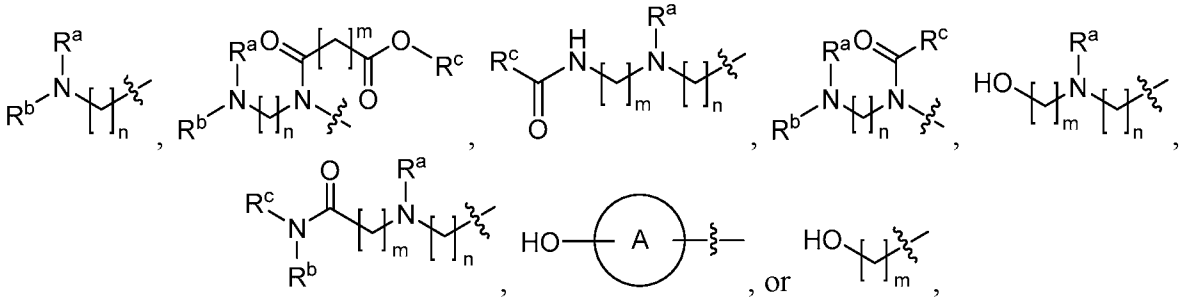
26. A method of inducing an immune response in a subject, the method comprising intramuscular delivery of a lipid nanoparticle (LNP) to the subject, wherein the LNP comprises a nucleic acid that encodes an antigen and a cationic lipid having the following structure of Formula (I):



or stereoisomer, tautomer, or salt thereof, wherein:

G^1 is N or CH;

R^1 is $-CH_3$ or has one of the following structures:



wherein:

ring A is a C_4-C_8 cycloalkyl;

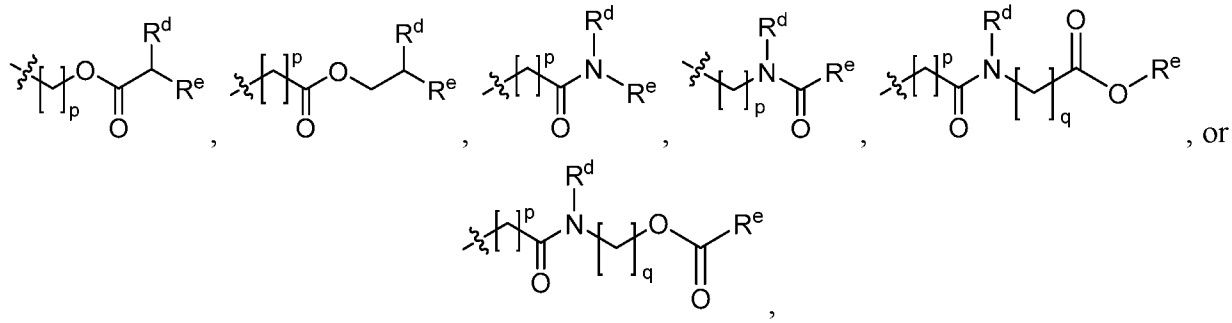
R^a and R^b are each independently C_1-C_6 alkyl, or R^a and R^b join together with the nitrogen to which they are attached to form a 3-8 membered heterocyclyl;

R^c is C_1-C_{14} alkyl or C_1-C_8 haloalkyl;

n is an integer from 0 to 6; and

m is an integer from 1 to 12;

R^2 and R^3 each independently have one of the following structures:



wherein:

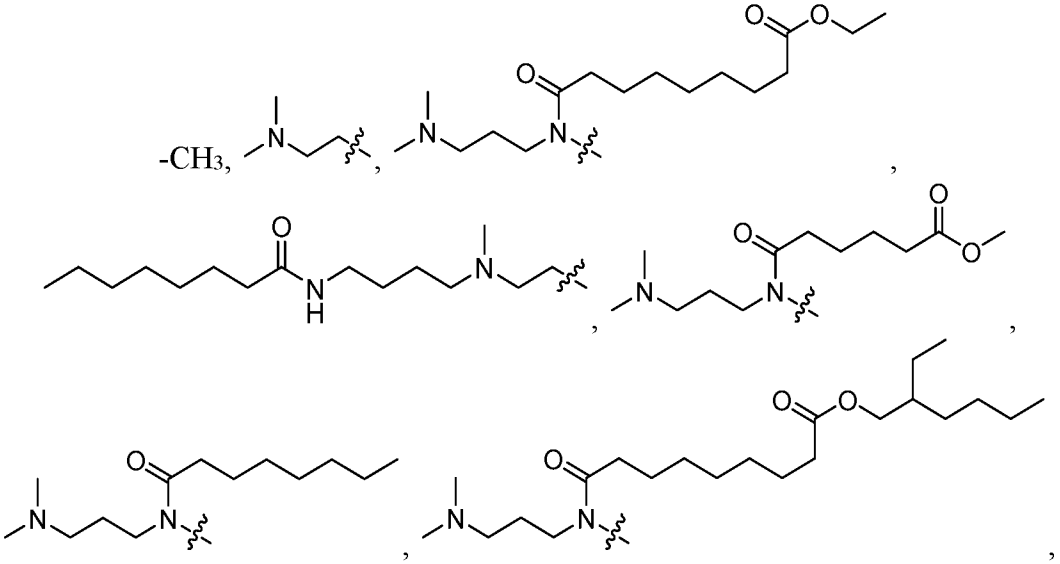
each occurrence of R^d and R^e are independently C_4-C_{18} alkyl;

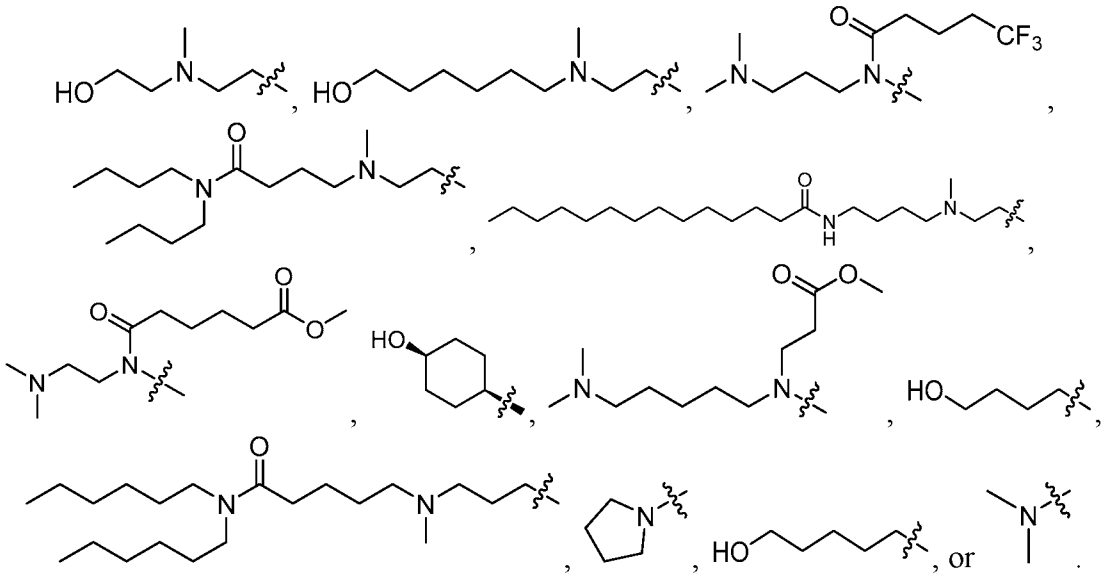
each occurrence of p is independently an integer from 2 to 12; and

each occurrence of q is independently an integer from 4 to 12.

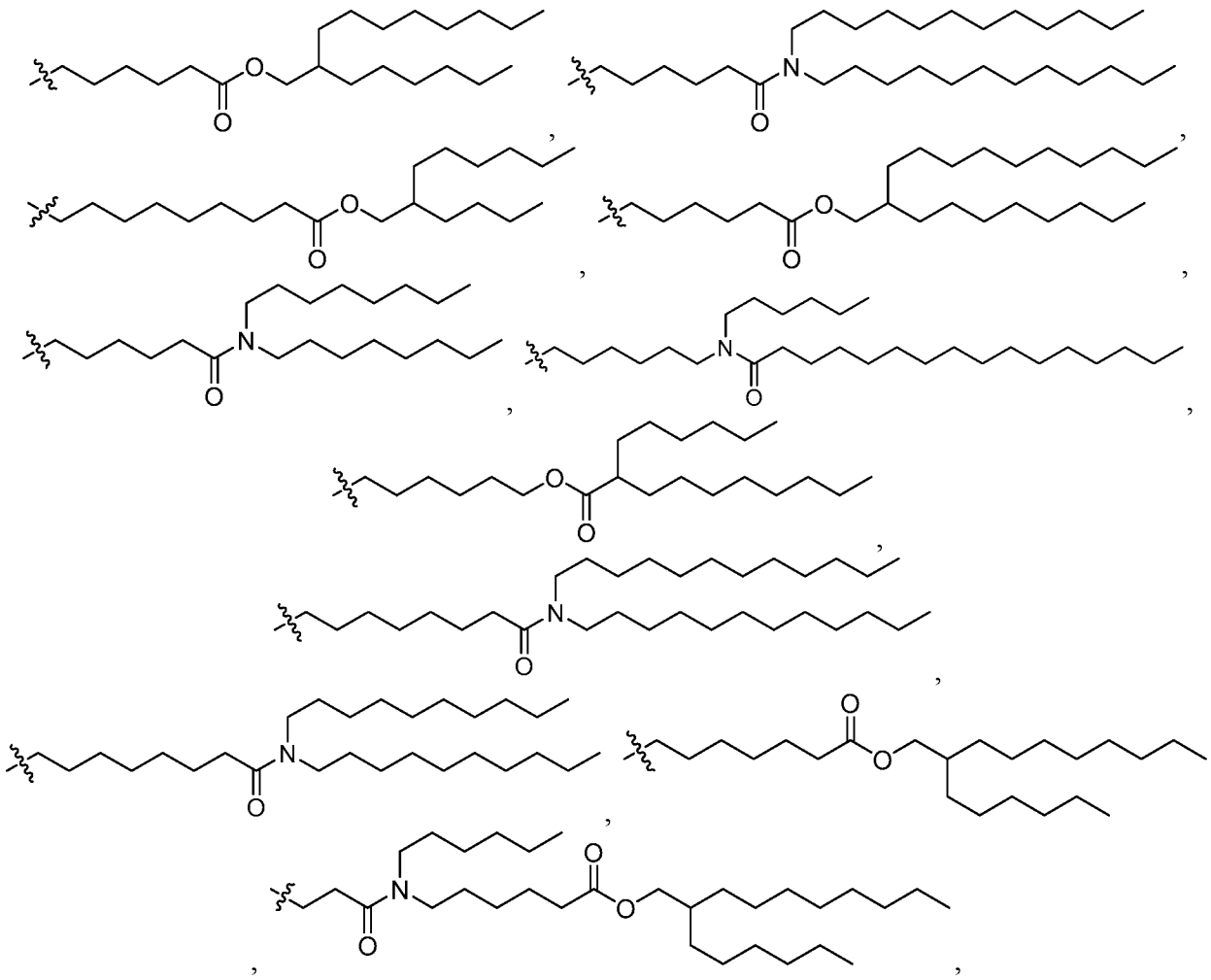
27. The method of claim 26, wherein R^a and R^b are both methyl or R^a and R^b join with the nitrogen to which they are attached to form pyrrolidinyl.

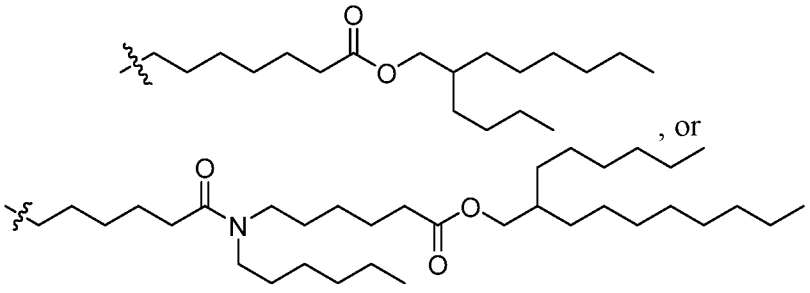
28. The method of claim 26, wherein R^1 has one of the following structures:





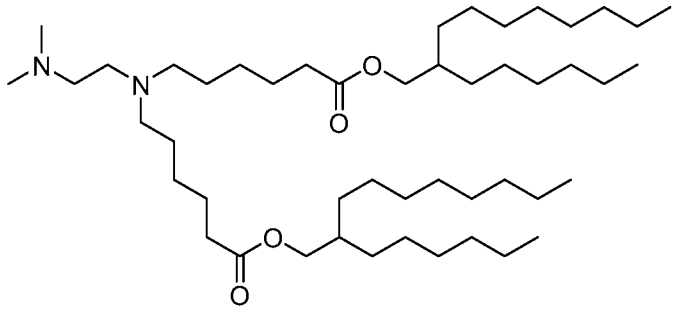
29. The method of any one of claims 26-28, wherein R^2 and R^3 each independently have one of the following structures:



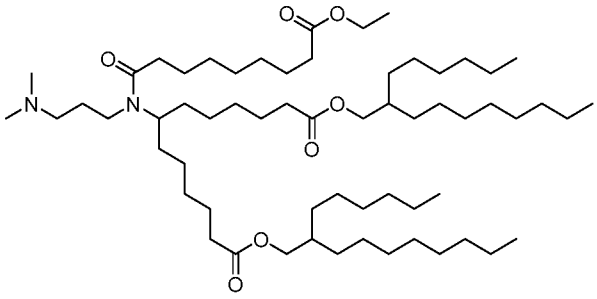


, or

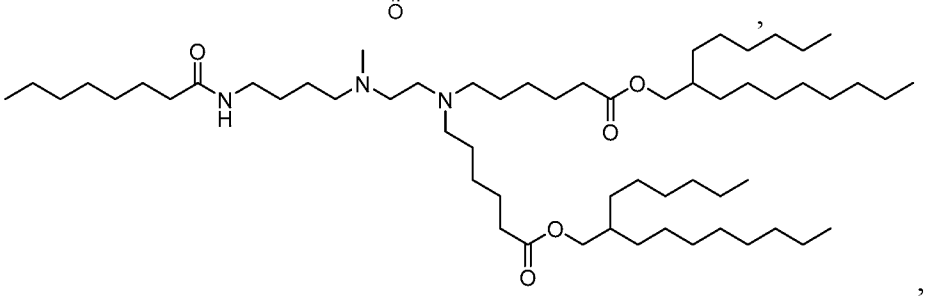
30. The method of any one of claims 1-29, wherein the cationic lipid has one of the following structures:



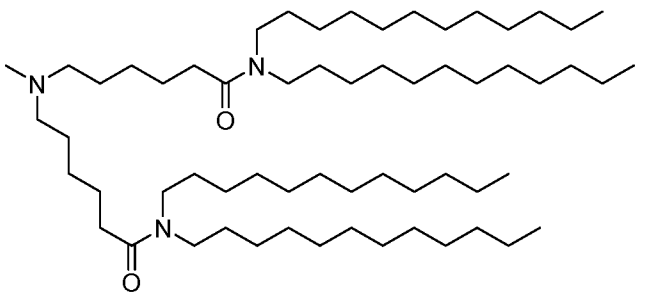
,



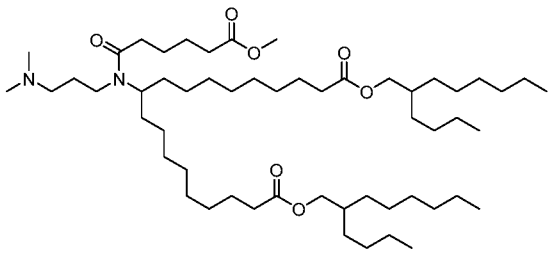
,

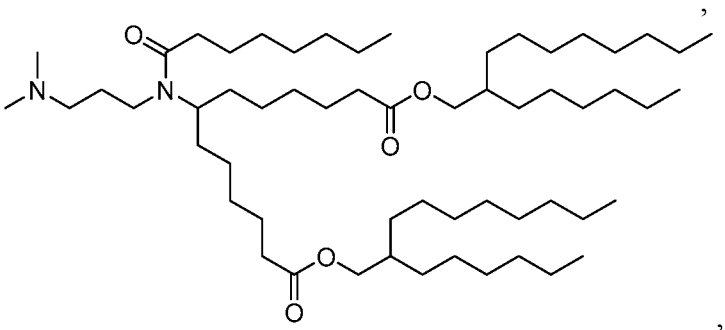


,

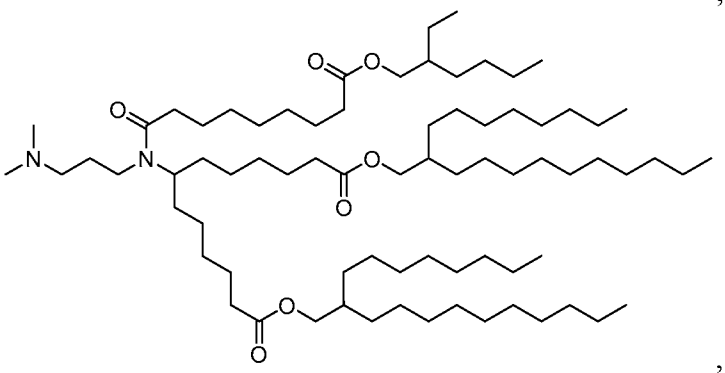


,

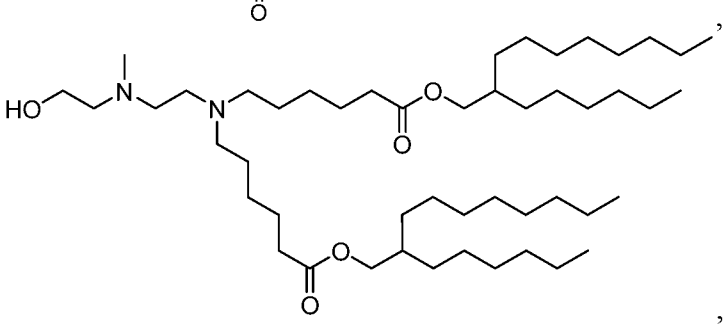




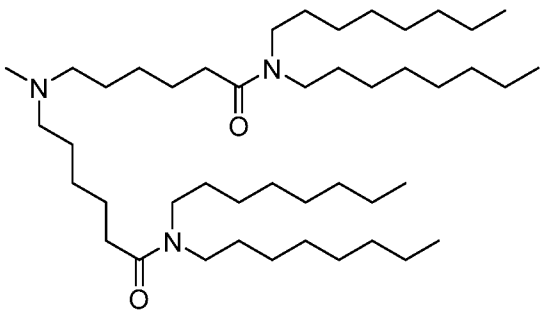
,



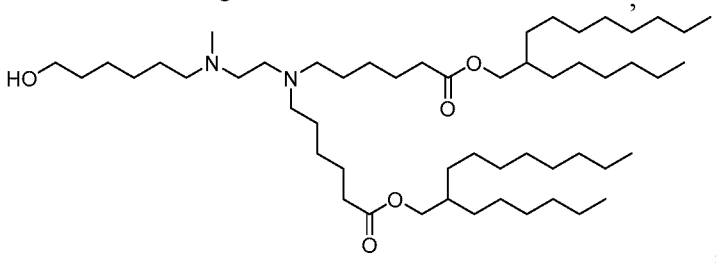
,



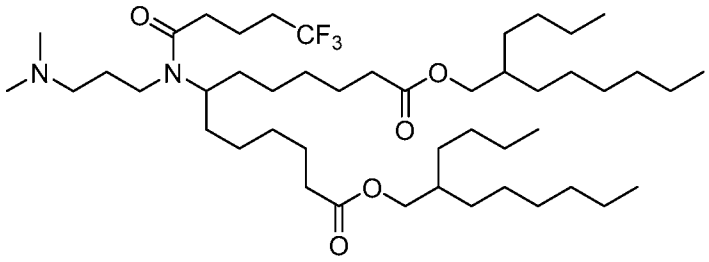
,



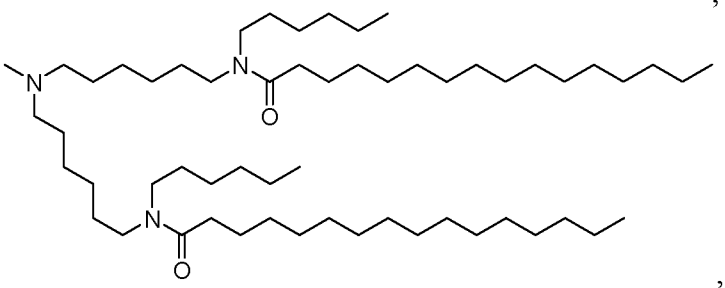
,



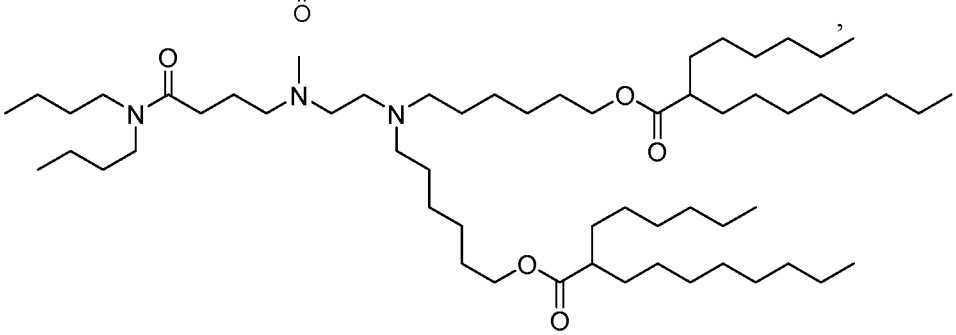
,



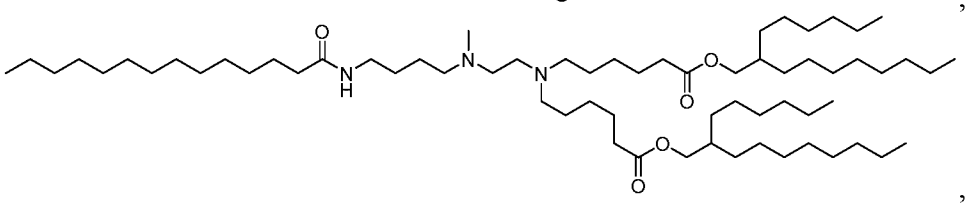
,



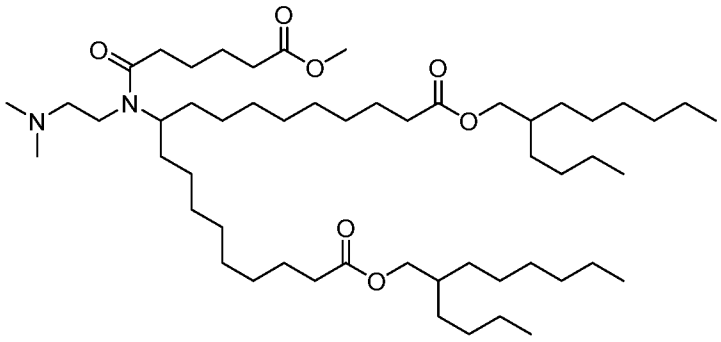
,



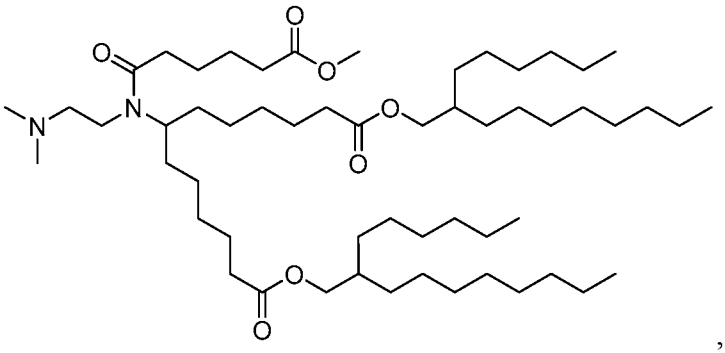
,



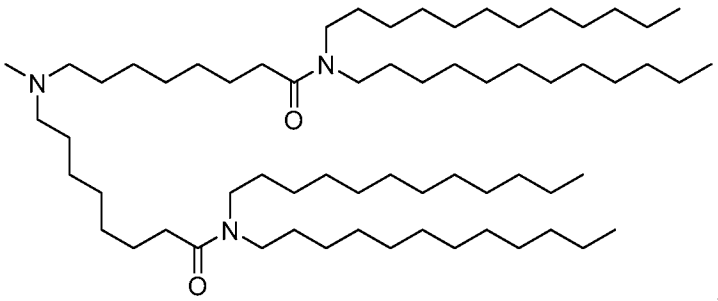
,



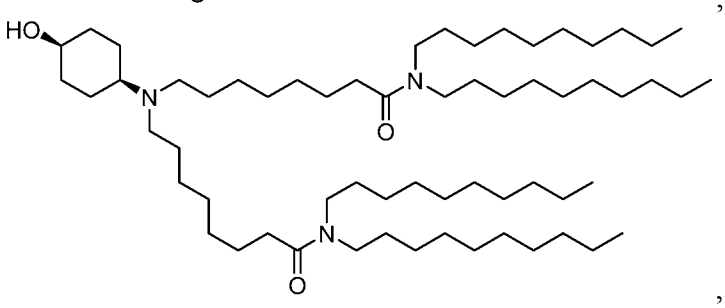
,



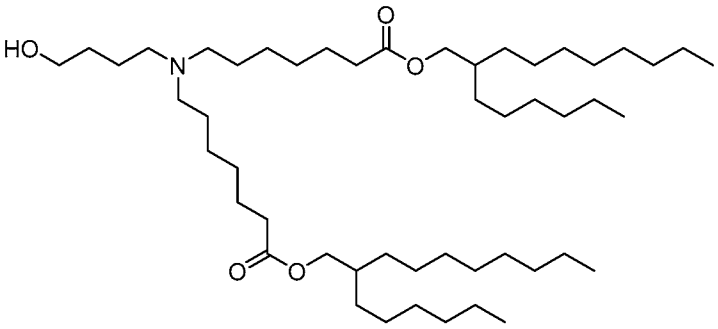
,



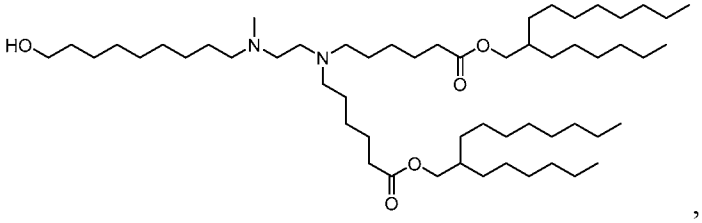
,



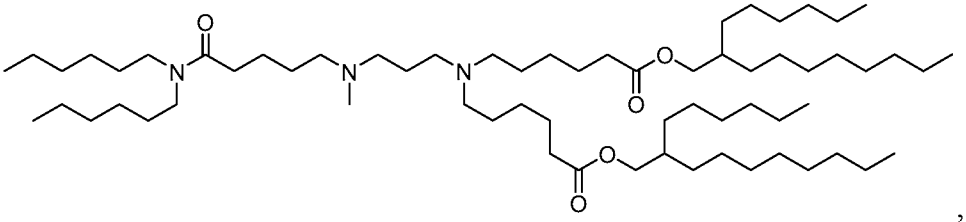
,



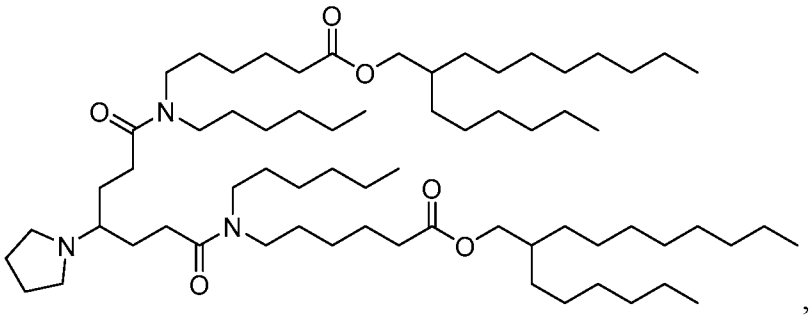
,



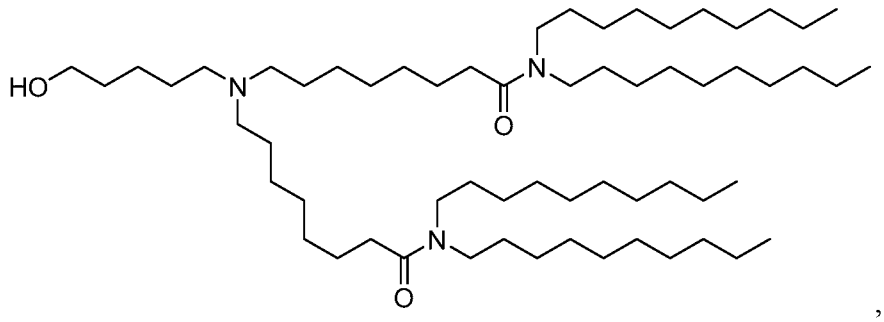
,



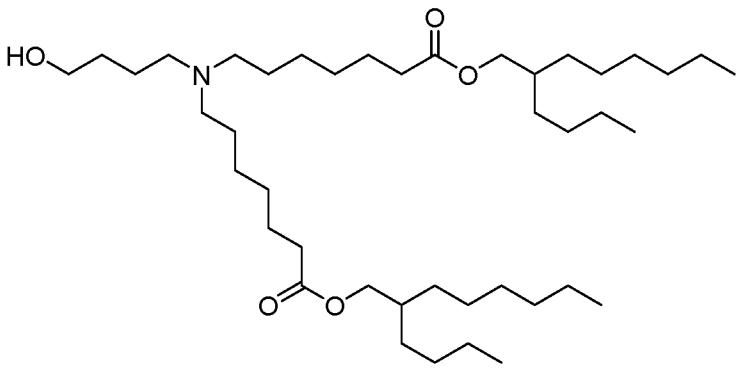
,



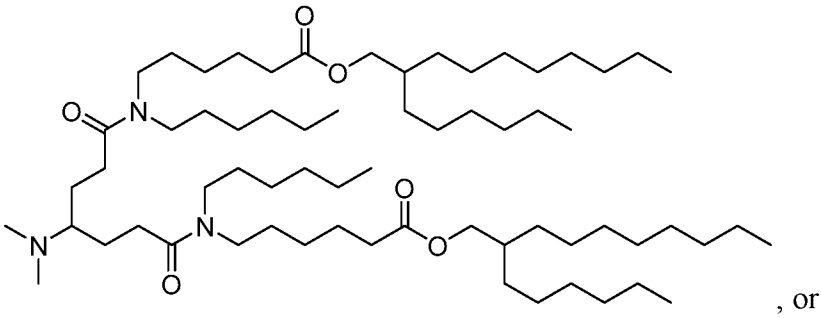
,



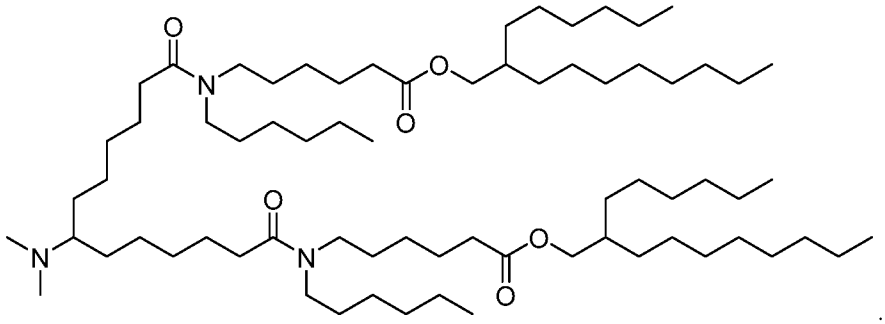
,



,



, or



.

31. The method of any one of claims 1-30, wherein the nucleic acid is a messenger RNA.

32. The method of any one of claims 1-31, wherein greater than 75% of the nucleic acid is encapsulated within the lipid nanoparticles.

33. The method of any one of claims 1-32, wherein greater than 85% of the nucleic acid is encapsulated within the lipid nanoparticles.

34. The method of any one of claims 1-33, wherein greater than 90% of the nucleic acid is encapsulated within the lipid nanoparticles.

35. The method of any one of claims 1-34, wherein greater than 95% of the nucleic acid is encapsulated within the lipid nanoparticles.

36. The method of any one of claims 1-35, wherein greater than 97% of the nucleic acid is encapsulated within the lipid nanoparticles.

37. The method of any one of claims 1-36, wherein greater than 98% of the nucleic acid is encapsulated within the lipid nanoparticles.

38. The method of any one of claims 1-37, wherein the lipid nanoparticle further comprises one or more excipient selected from neutral lipids, steroids, and polymer conjugated lipids.

39. The method of any one of claims 1-38, wherein the lipid nanoparticle comprises between 35-50 wt% of the cationic lipid.

40. The method of any one of claims 1-39, wherein the lipid nanoparticle comprises between 45-50 wt% of the cationic lipid.

41. The method of any one of claims 1-40, wherein the lipid nanoparticle comprises 47.5 wt% of the cationic lipid.

42. The method of any one of claims 1-38, wherein the lipid nanoparticle comprises between 35-40 wt% of the cationic lipid.

43. The method of any one of claims 1-38, wherein the lipid nanoparticle comprises 37.5 wt% of the cationic lipid.

44. The method of any one of claims 1-43, wherein the lipid nanoparticle comprises one or more neutral lipids selected from DSPC, DPPC, DMPC, DOPC, POPC, DOPE, and SM.

45. The method of any one of claims 44, wherein the neutral lipid is DSPC.

46. The method of any one of claims 38-45, wherein the molar ratio of the cationic lipid to the neutral lipid from about 2:1 to about 8:1.

47. The method of any one of claims 38-46, wherein the steroid is cholesterol.

48. The method of claim 47, wherein the molar ratio of the cationic lipid to cholesterol from about 5:1 to about 1:1 or from about 2:1 to about 1:1.

49. The method of any one of claims 38-48, wherein the polymer conjugated lipid is a pegylated lipid.

50. The method of any one of claims 1-49, wherein the lipid nanoparticle comprises between 1.5-2.8 wt% of a pegylated lipid.

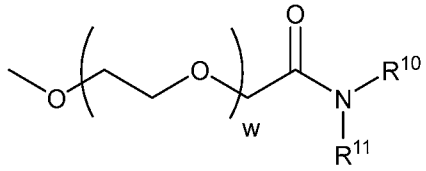
51. The method of any one of claims 1-50, wherein the lipid nanoparticle comprises 1.8 wt% of a pegylated lipid.

52. The method of any one of claims 1-50, wherein the lipid nanoparticle comprises 2.5 wt% of a pegylated lipid.

53. The method of any one of claims 49-52, wherein the molar ratio of the cationic lipid to pegylated lipid from about 100:1 to about 20:1 or from about 100:1 to about 10:1.

54. The method of any one of claims 49-53, wherein the pegylated lipid is PEG-DAG, PEG-PE, PEG-S-DAG, PEG-cer, or a PEG dialkyoxypropylcarbamate.

55. The method of any one of claims 49-53, wherein the pegylated lipid has the following structure:



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

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

w is an integer from 30 to 60.

56. The method of claim 55, wherein R^{10} and R^{11} are each independently straight alkyl chain containing from 12 to 16 carbon atoms.

57. The method of any one of claims 55-56, wherein w is 40 to 50.

58. The method of any one of claims 1-57, wherein the lipid nanoparticle has a diameter between 40-100 nanometers.

59. The method of any one of claims 1-58, wherein the lipid nanoparticle has a diameter between 39-45 nanometers.

60. The method of any one of claims 1-58, wherein the lipid nanoparticle has a diameter between 45-50 nanometers.

61. The method of any one of claims 1-58, wherein the lipid nanoparticle has a diameter between 50-55 nanometers.

62. The method of any one of claims 1-58, wherein the lipid nanoparticle has a diameter between 55-60 nanometers.

63. The method of any one of claims 1-58, wherein the lipid nanoparticle has a diameter between 60-65 nanometers.

64. The method of any one of claims 1-58, wherein the lipid nanoparticle has a diameter between 65-70 nanometers.

65. The method of any one of claims 1-58, wherein the lipid nanoparticle has a diameter between 70-75 nanometers.

66. The method of any one of claims 1-58, wherein the lipid nanoparticle has a diameter greater than 70 nanometers.

67. The method of any one of claims 1-66, wherein the lipid nanoparticle has a Polydispersity Index (PDI) between about 0.01 to about 0.2.

68. The method of any one of claims 1-67, wherein the lipid nanoparticle has a Polydispersity Index (PDI) between about 0.01 to about 0.05.

69. The method of any one of claims 1-67, wherein the lipid nanoparticle has a Polydispersity Index (PDI) between about 0.05 to about 0.1.

70. The method of any one of claims 1-67, wherein the lipid nanoparticle has a Polydispersity Index (PDI) between about 0.1 to about 0.15.

71. The method of any one of claims 1-67, wherein the lipid nanoparticle has a Polydispersity Index (PDI) between about 0.15 to about 0.2.

72. The method of any one of claims 1-71, wherein the lipid nanoparticle is delivered as part of an aqueous composition.

73. The method of claim 72, wherein the aqueous composition further comprises a first buffer.

74. The method of claim 73, wherein the first buffer is 25 mM acetate buffer, 50 mM citrate buffer, 25 mM phosphate buffer, or combinations thereof.

75. The method of any one of claims 73-74, wherein the first buffer is about 20-30 mM acetate buffer.

76. The method of any one of claims 73-75, wherein the first buffer has a pH of about 3.75-6.25.

77. The method of any one of claims 73-76, wherein the first buffer has a pH of about 4.

78. The method of any one of claims 73-76, wherein the first buffer has a pH of about 5.5.

79. The method of any one of claims 73-76, wherein the first buffer has a pH of about 6.

80. The method of any one of claims 73-74, wherein the first buffer is about 45-55 mM citrate buffer.

81. The method of any one of claims 73-74, wherein the first buffer is about 50 mM citrate buffer.

82. The method of claim 81, wherein the first buffer has a pH of about 3.75-4.25.

83. The method of any one of claims 81-82, wherein the first buffer has a pH of about 4.

84. The method of any one of claims 81-82, wherein the first buffer has a pH of about 5.5.

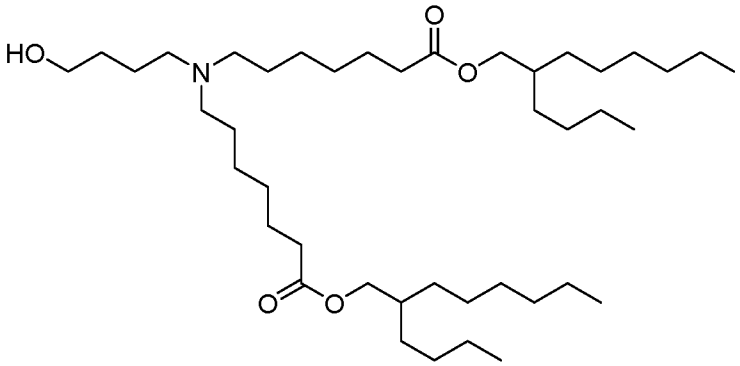
85. The method of any one of claims 81-82, wherein the first buffer has a pH of about 6.

86. The method of any one of claims 73-74, wherein the first buffer is about 25 mM phosphate buffer.

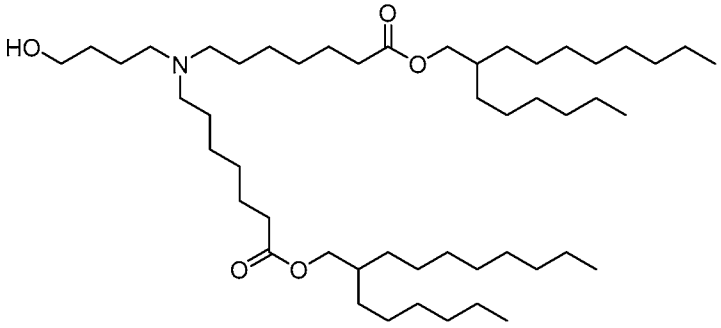
87. The method of claim 86, wherein the first buffer has a pH of about 5.75-6.25.

88. The method of any one of claims 86-87, wherein the first buffer has a pH of about 6.

89. A compound having one of the following structures:



or



or a salt, stereoisomer, or tautomer thereof.

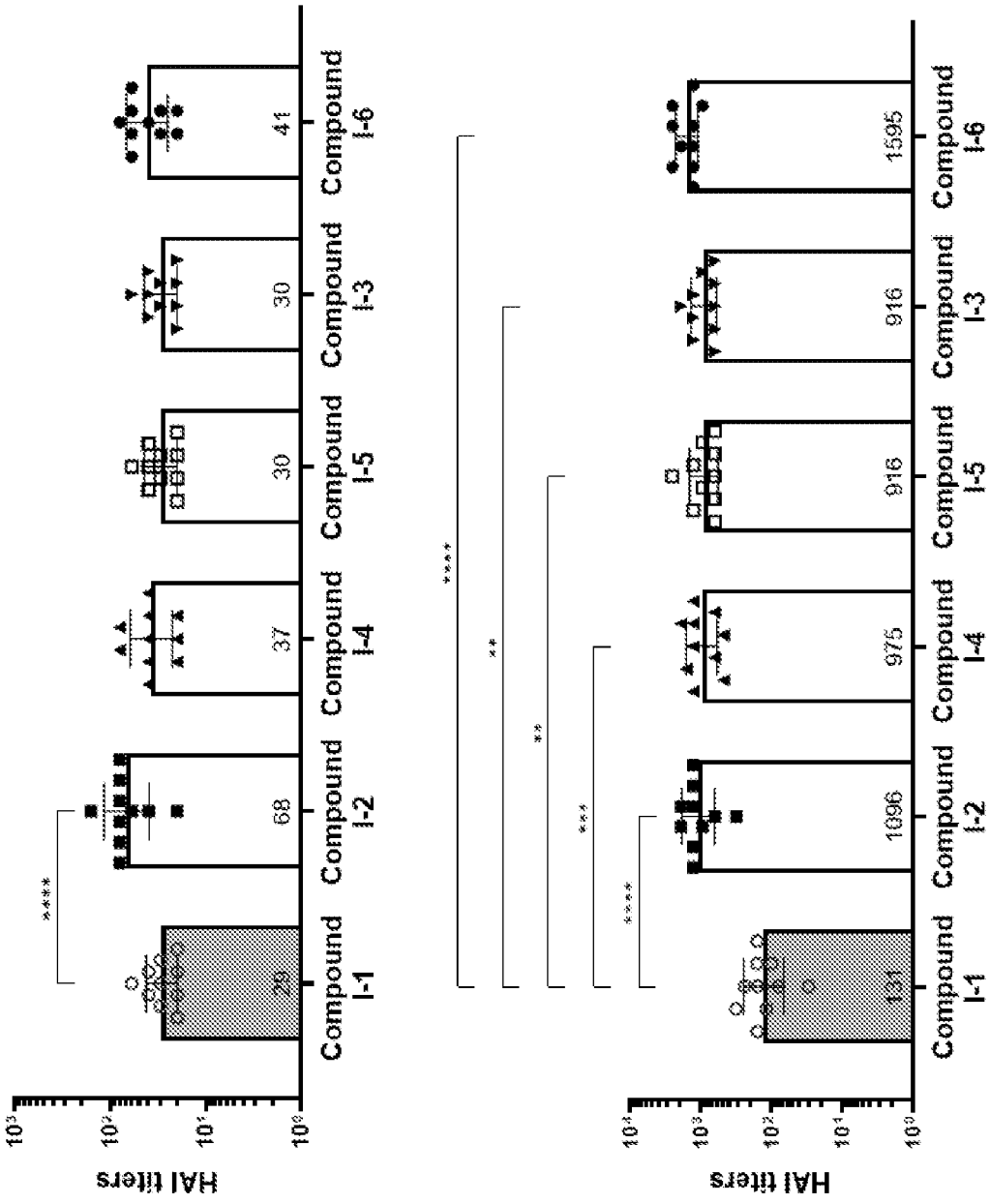

HAI titers
Compound I-1
Compound I-2
Compound I-4
Compound I-5
Compound I-3
Compound I-6
20
68
37
30
30
41
HAI titers
Compound I-1
Compound I-2
Compound I-4
Compound I-5
Compound I-3
Compound I-6
131
1096
975
916
916
1595

Fig. 1

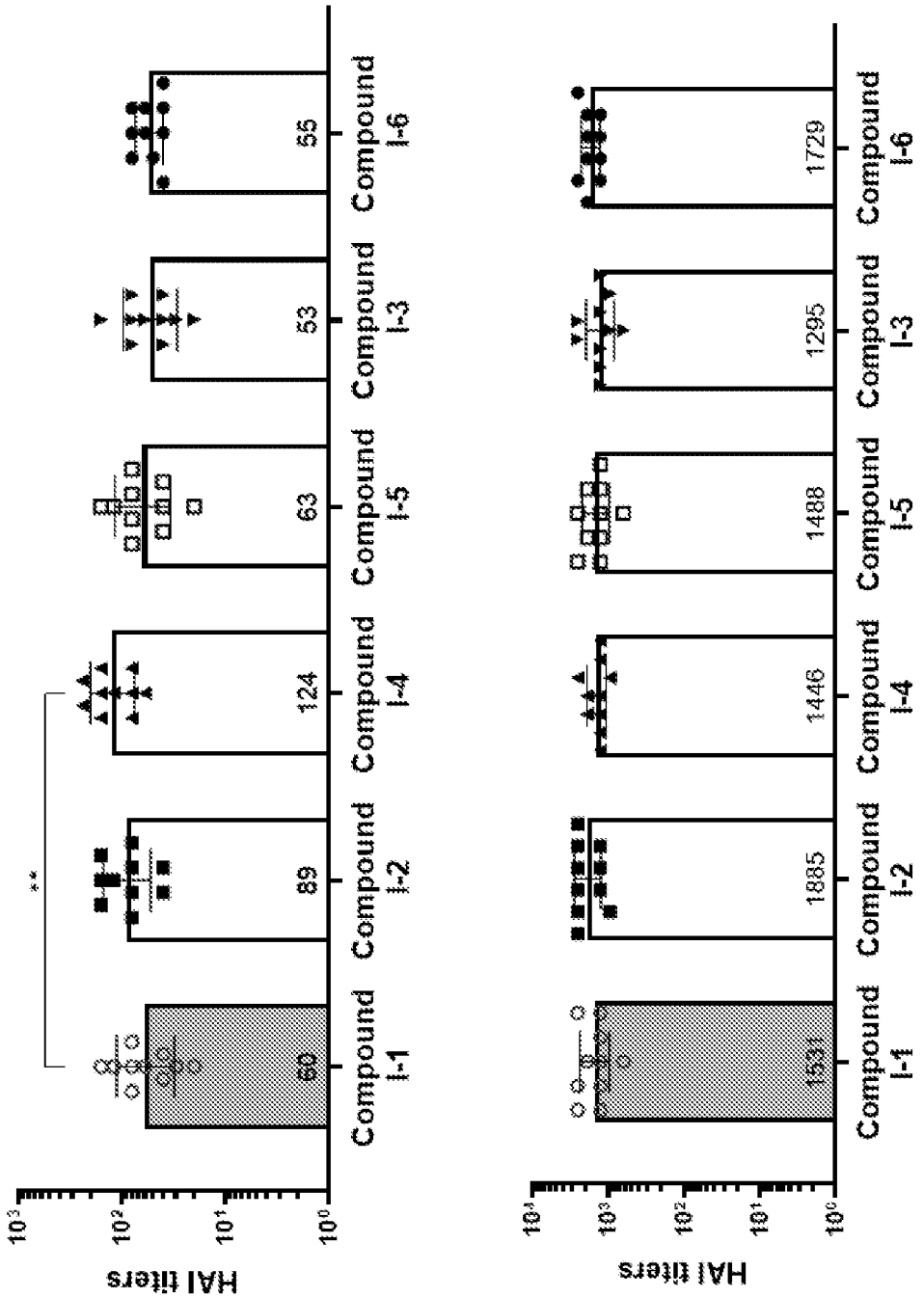

HAI titers
10^3
10^2
10^1
10^0
**
60
89
124
63
53
55
1531
1885
1446
1488
1295
1729
Compound I-1
Compound I-2
Compound I-4
Compound I-5
Compound I-3
Compound I-6

Fig. 2

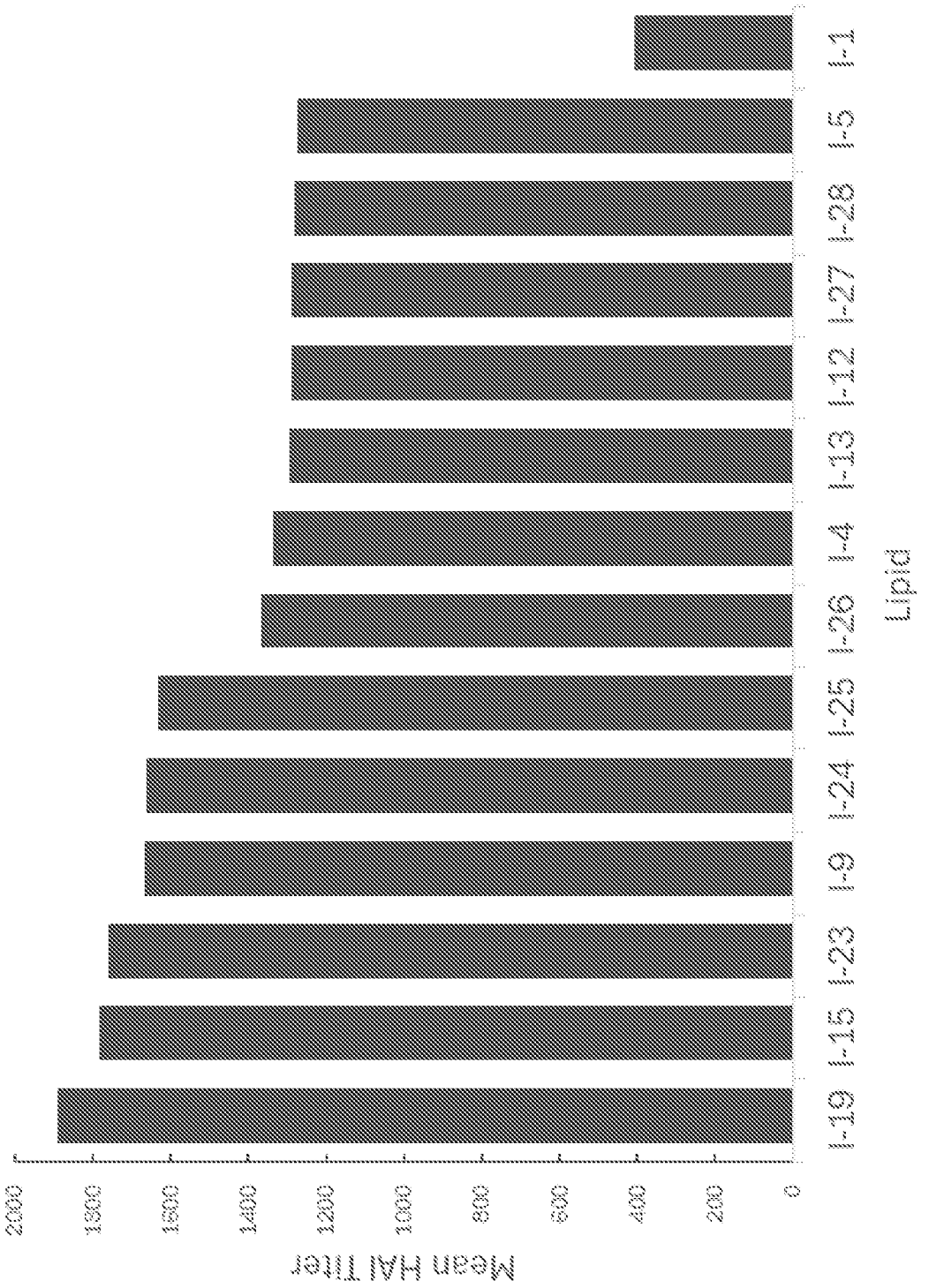

Mean HAI Titer
2000
1800
1600
1400
1200
1000
800
600
400
200
0
I-19
I-15
I-23
I-9
I-24
I-25
I-26
I-4
I-13
I-12
I-27
I-28
I-5
I-1
Lipid

Fig. 3

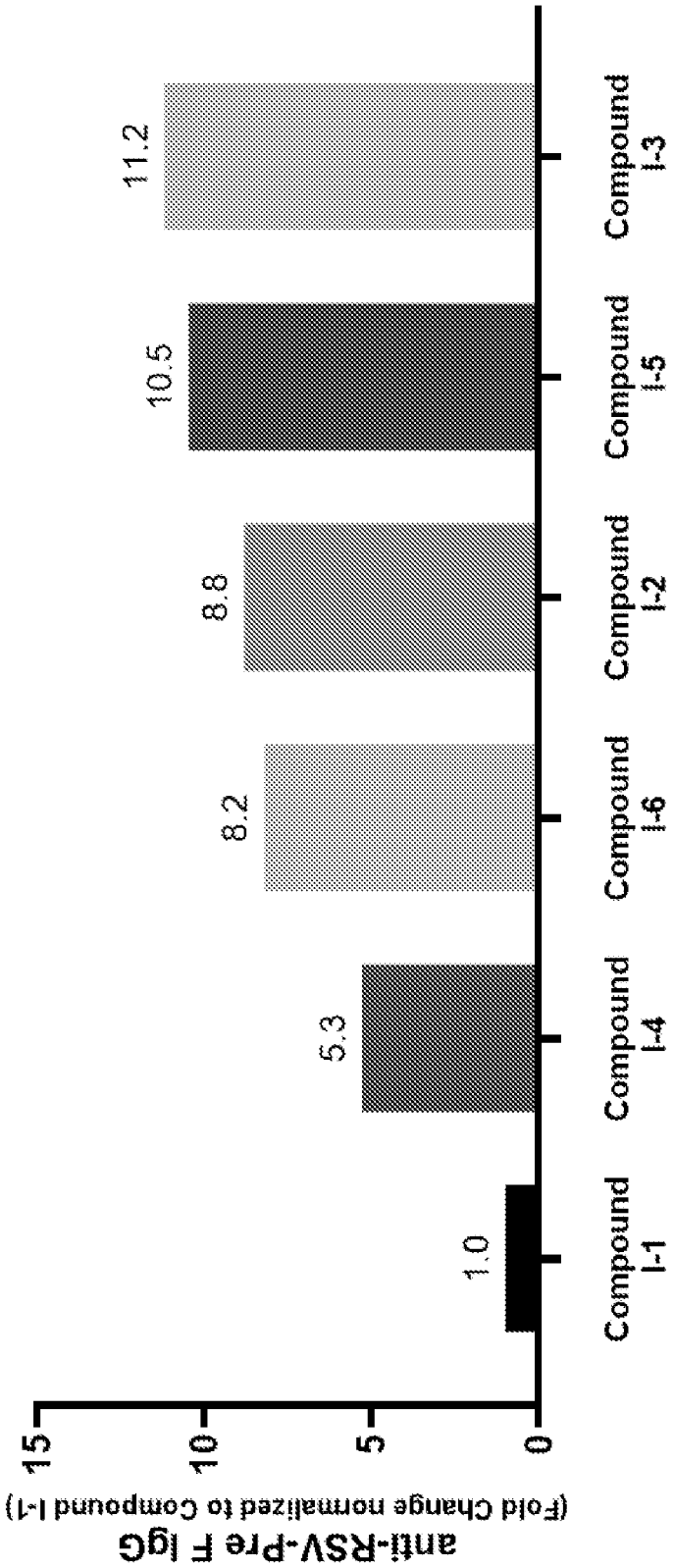

anti-RSV-Pre F IgG
(Fold Change normalized to Compound I-1)
0
5
10
15
1.0
5.3
8.2
8.8
10.5
11.2
Compound I-1
Compound I-4
Compound I-6
Compound I-2
Compound I-5
Compound I-3

Fig. 4

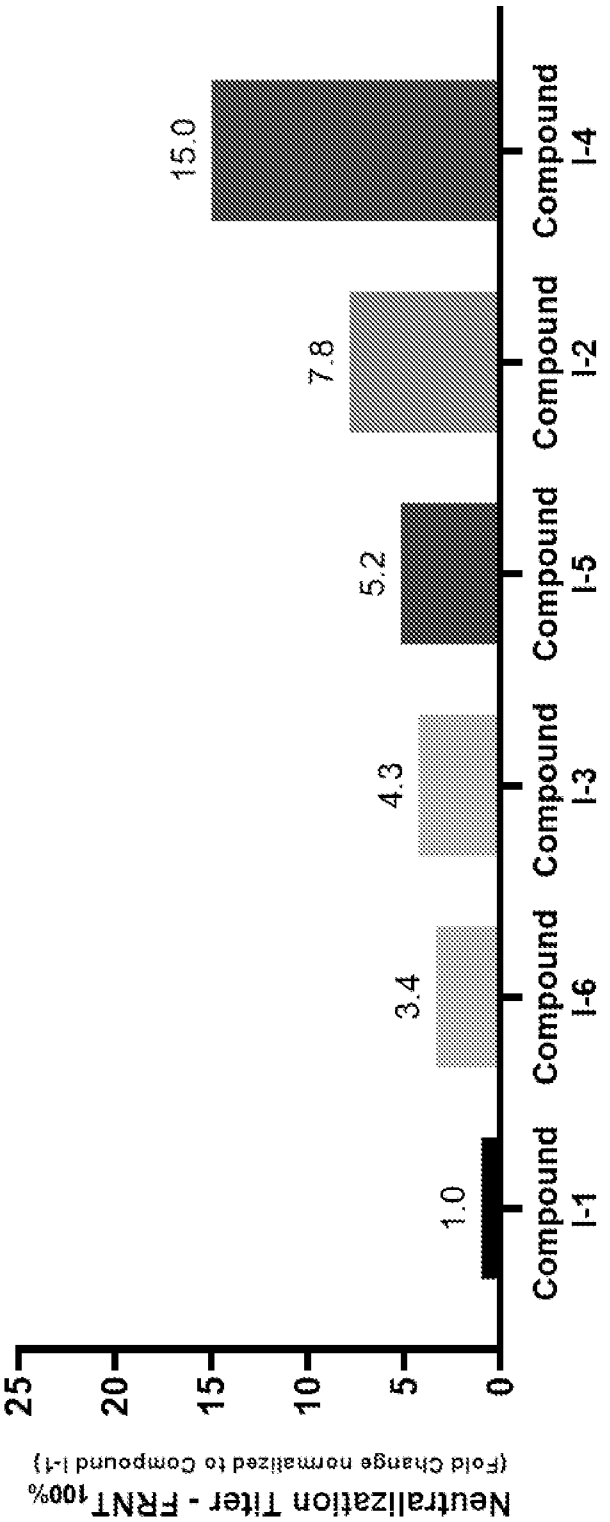

Neutralization Titer - FRNT100%
(Fold Change normalized to Compound I-1)
25
20
15
10
5
0
1.0
3.4
4.3
5.2
7.8
15.0
Compound I-1
Compound I-6
Compound I-3
Compound I-5
Compound I-2
Compound I-4

Fig. 5

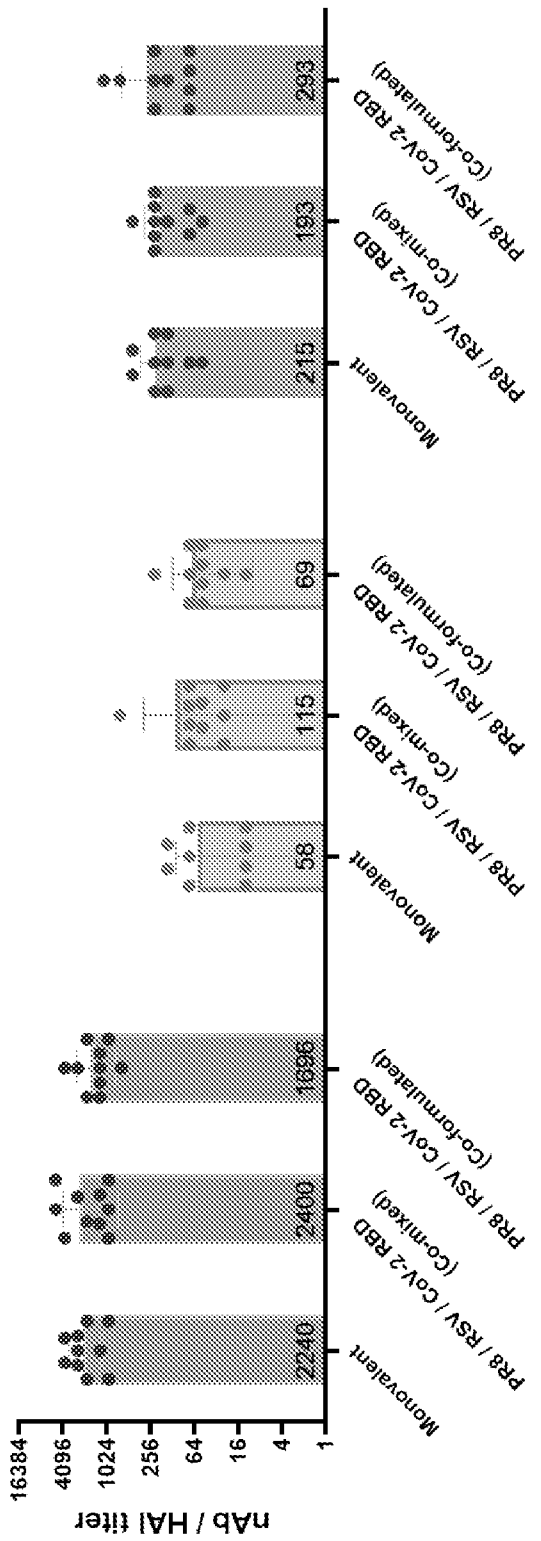

PR8 HA (0.2 ug)
RSV-Pre F (0.2 ug)
SARS-CoV-2 RBD (0.5 ug)
nAb / HAI titer
16384
4096
1024
256
64
16
4
1
2240
2400
1696
58
115
69
215
193
293
Monovalent
PR8 / RSV / CoV-2 RBD (Co-mixed)
PR8 / RSV / CoV-2 RBD (Co-formulated)

Fig. 6

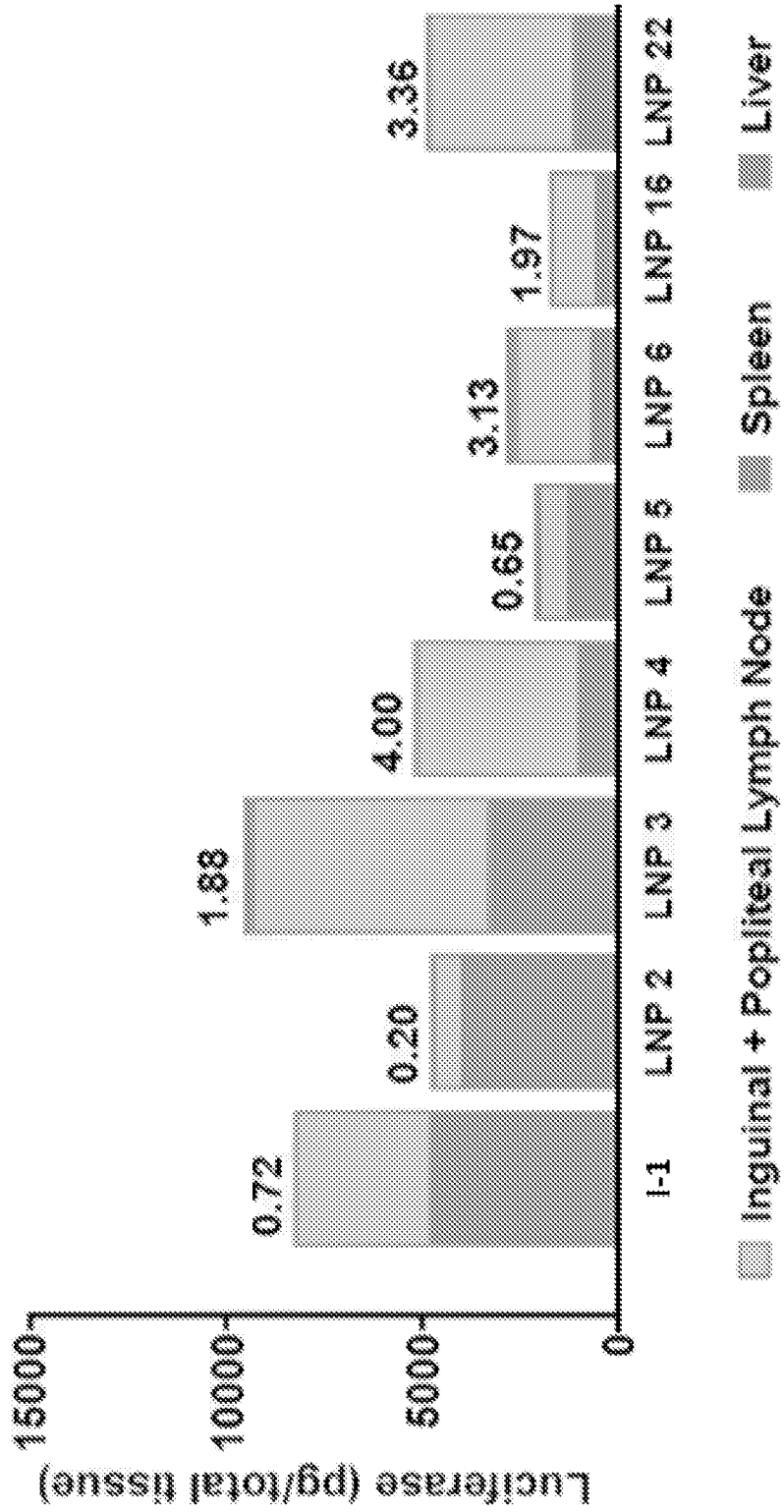

Luciferase (pg/total tissue)
15000
10000
5000
0
0.72
0.20
1.88
4.00
0.65
3.13
1.97
3.36
I-1
LNP 2
LNP 3
LNP 4
LNP 5
LNP 6
LNP 16
LNP 22
Inguinal + Popliteal Lymph Node
Spleen
Liver

Fig. 7A

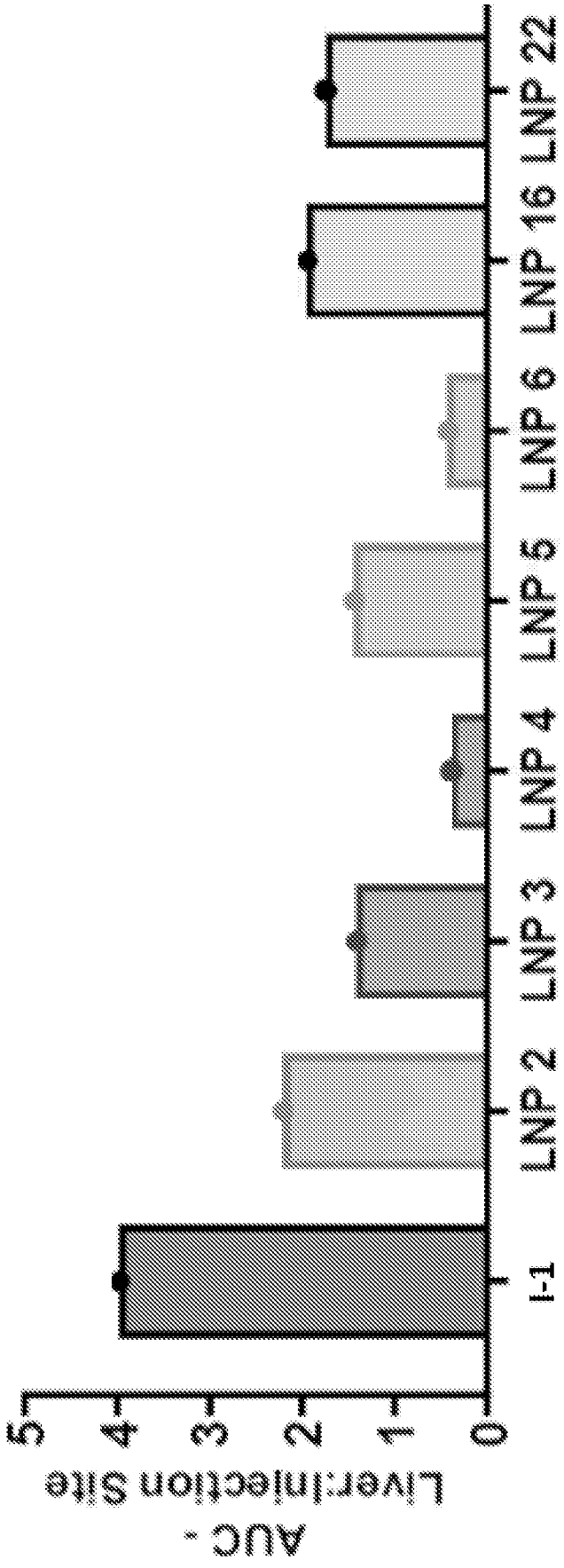

AUC -
Liver:Injection Site
0
1
2
3
4
5
I-1
LNP 2
LNP 3
LNP 4
LNP 5
LNP 6
LNP 16
LNP 22

Fig. 7B