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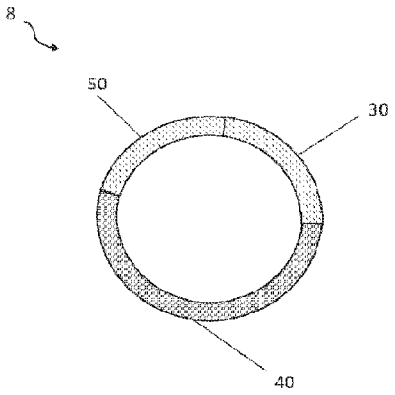

FIG. 88

(57) **Abrégé/Abstract:**
Circular RNA, along with related compositions and methods are described herein. In some embodiments, the inventive circular RNA comprises group I intron fragments, spacers, an IRES, duplex forming regions, and an expression sequence. In some embodiments, the expression sequence encodes an antigen. In some embodiments, circular RNA of the invention has improved expression, functional stability, immunogenicity, ease of manufacturing, and/or half-life when compared to linear RNA. In some embodiments, inventive methods and constructs result in improved circularization efficiency, splicing efficiency, and/or purity when compared to existing RNA circularization approaches.

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Abstract:

Circular RNA, along with related compositions and methods are described herein. In some embodiments, the inventive circular RNA comprises group I intron fragments, spacers, an IRES, duplex forming regions, and an expression sequence. In some embodiments, the expression sequence encodes an antigen. In some embodiments, circular RNA of the invention has improved expression, functional stability, immunogenicity, ease of manufacturing, and/or half-life when compared to linear RNA. In some embodiments, inventive methods and constructs result in improved circularization efficiency, splicing efficiency, and/or purity when compared to existing RNA circularization approaches.

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CIRCULAR RNA COMPOSITIONS AND METHODS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of, and priority to, U.S. Provisional Application No. 63/209,271, filed on June 10, 2021; and U.S. Provisional Application No. 63/311,923, filed on February 18, 2022, the contents of each of which are hereby incorporated by reference in their entirety for all purposes.

SEQUENCE LISTING

[0002] This application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on June 9, 2022, is named OBS_017_SL.txt and is 3,370,624 bytes in size.

BACKGROUND OF THE INVENTION

[0003] Conventional gene therapy involves the use of DNA for insertion of desired genetic information into host cells. The DNA introduced into the cell is usually integrated to a certain extent into the genome of one or more transfected cells, allowing for long-lasting action of the introduced genetic material in the host. While there may be substantial benefits to such sustained action, integration of exogenous DNA into a host genome may also have many deleterious effects. For example, it is possible that the introduced DNA will be inserted into an intact gene, resulting in a mutation which impedes or even totally eliminates the function of the endogenous gene. Thus, gene therapy with DNA may result in the impairment of a vital genetic function in the treated host, such as *e.g.*, elimination or deleteriously reduced production of an essential enzyme or interruption of a gene critical for the regulation of cell growth, resulting in unregulated or cancerous cell proliferation. In addition, with conventional DNA based gene therapy it is necessary for effective expression of the desired gene product to include a strong promoter sequence, which again may lead to undesirable changes in the regulation of normal gene expression in the cell. It is also possible that the DNA based genetic material will result in the induction of undesired anti-DNA antibodies, which in turn, may trigger a possibly fatal immune response. Gene therapy approaches using viral vectors can also result in an adverse immune response. In some circumstances, the viral vector may even integrate into the host genome. In addition, production of clinical grade viral

vectors is also expensive and time consuming. Targeting delivery of the introduced genetic material using viral vectors can also be difficult to control. Thus, while DNA based gene therapy has been evaluated for delivery of secreted proteins using viral vectors (U.S. Patent No. 6,066,626; U.S. Publication No. US2004/0110709), these approaches may be limited for these various reasons.

[0004] In contrast to DNA, the use of RNA as a gene therapy agent is substantially safer because RNA does not involve the risk of being stably integrated into the genome of the transfected cell, thus eliminating the concern that the introduced genetic material will disrupt the normal functioning of an essential gene, or cause a mutation that results in deleterious or oncogenic effects, and extraneous promoter sequences are not required for effective translation of the encoded protein, again avoiding possible deleterious side effects. In addition, it is not necessary for mRNA to enter the nucleus to perform its function, while DNA must overcome this major barrier.

[0005] Circular RNA is useful in the design and production of stable forms of RNA. The circularization of an RNA molecule provides an advantage to the study of RNA structure and function, especially in the case of molecules that are prone to folding in an inactive conformation (Wang and Ruffner, 1998). Circular RNA can also be particularly interesting and useful for *in vivo* applications, especially in the research area of RNA-based control of gene expression and therapeutics, including protein replacement therapy and vaccination.

[0006] Prior to this invention, there were three main techniques for making circularized RNA *in vitro*: the splint-mediated method, the permuted intron-exon method, and the RNA ligase-mediated method. However, the existing methodologies are limited by the size of RNA that can be circularized, thus limiting their therapeutic application. The present invention addresses this need by providing methods and compositions for the manufacture and optimization of circularized RNAs via engineering of the sequences for the DNA template, precursor linear RNA and ultimately the circular RNA along with methods of treating a subject in need using the invented circular RNA.

SUMMARY

[0007] Precursor RNAs, circular RNAs, and the related compositions and methods are described herein.

[0008] In one aspect, provided herein are precursor RNA polynucleotides comprising, in the

following order: a. a 5' enhanced intron element, b. a 5' enhanced exon element, c. a core functional element, d. a 3' enhanced exon element, and e. a 3' enhanced intron element, wherein the core functional element comprises, in the following order: i. a translation initiation element (TIE), ii. a coding element, and iii. optionally, a stop codon or a stop cassette.

[0009] In one aspect, provided herein are precursor RNA polynucleotides comprising, in the following order: a. a 5' enhanced intron element, b. a 5' enhanced exon element, c. a core functional element, d. a 3' enhanced exon element, and e. a 3' enhanced intron element wherein the core functional element comprises, in the following order: i. a coding region, ii. optionally, a stop codon or a stop cassette, and iii. a translation initiation element (TIE).

[0010] In one aspect, provided herein are precursor RNA polynucleotides comprising, in the following order: a. a 5' enhanced intron element, b. a 5' enhanced exon element, c. a core functional element, d. a 3' enhanced exon element, and e. a 3' enhanced intron element, wherein the core functional element comprises a noncoding element.

[0011] In some embodiments, the TIE comprises an untranslated region (UTR) or a fragment thereof, a aptamer complex or a fragment thereof, or a combination thereof.

[0012] In some embodiments, the UTR or fragment thereof is derived from a viral or eukaryotic messenger RNA. In some embodiments, the UTR or fragment thereof comprises a viral internal ribosome entry site (IRES) or eukaryotic IRES. In some embodiments, core functional element comprises two or more IRESs. In some embodiments, the core functional element comprises a TIE, a coding element, a termination sequence, optionally a spacer, a TIE, a coding element, and a termination sequence, wherein the TIE comprises an IRES. In some embodiments, the IRES comprises a sequence selected from SEQ ID NOs: 1-2983 and 3282-3287, or a fragment thereof. In some embodiments, the IRES comprises a sequence selected from SEQ ID NOs: 75, 77, 137, 532, 566, 582, 648, 680, 693, 752, 785, 787, 791, 793, 820, 823, 839, 840, 843, 852, 857, 861, 862, 863, 864, 871, 874, 876, 922, 959, 983, 984, 1015, 1017, 1023, 1026, 1031, 1041, 1047, 1059, 1068, 1134, 1168, 1169, 1171, 1177, 1178, 1179, 1180, 1189, 1192, 1193, 1198, 1216, 1218, 1230, 1263, 1276, 1280, 1282, 1284, 1287, 1346, 1354, 1364, 1367, 1370, 1432, 1438, 1440, 2285, 2465, 2601, 2615, 2616, 2617, 2618, 2627, 2667, 2681, 2742, 2746, 2758, 2777, 2778, 3282, 3283, 3286, and 3287, or a fragment thereof. In some embodiments, the IRES comprises one or more modified nucleotides compared to the wild-type viral IRES or eukaryotic IRES.

[0013] In some embodiments, the IRES is capable of facilitating expression of a protein encoded

by the precursor RNA in a cell. In some embodiments, the IRES is capable of facilitating expression of the protein, such that the expression level of the protein is comparable to or higher than when a control IRES is used. In some embodiments, the control IRES comprises the sequence of SEQ ID NO: 3282. In some embodiments, the IRES is derived from Enterovirus, Kobuvirus, Parechovirus, or Cardiovirus. In some embodiments, the IRES is derived from Enterovirus or Kobuvirus.

[0014] In some embodiments, the cell is a myotube. In some embodiments, the IRES is derived from Bopivirus, Oscivirus, Hunnivirus, Passerivirus, Mischivirus, Kobuvirus, Enterovirus, Cardiovirus, Salivirus, Rabovirus, Parechovirus, Gallivirus, or Sicinivirus. In some embodiments, the IRES is derived from Hunnivirus, Passerivirus, Kobuvirus, Bopivirus, or Enterovirus. In some embodiments, the IRES is derived from Enterovirus I, Enterovirus F, Enterovirus E, Enterovirus J, Enterovirus C, Enterovirus A, Enterovirus B, Aichivirus B, Parechovirus A, Cardiovirus F, Cardiovirus B, or Cardiovirus E. In some embodiments, the IRES comprises a sequence selected from SEQ ID NOs: 137, 580, 785, 791, 820, 922, 1041, 1047, 1068, 1168, 1169, 1171, 1177, 1178, 1179, 1180, 1189, 1192, 1263, 1276, 1280, 1282, 1284, 1287, 1354, 1356, 1432, 1436, 1439, 1440, 2285, 2667, 2746, 2777, 2778, 3283, and 3284.

[0015] In some embodiments, the cell is a hepatocyte. In some embodiments, the IRES is derived from Enterovirus, Bopivirus, Mischivirus, Gallivirus, Oscivirus, Cardiovirus, Kobuvirus, Rabovirus, Salivirus, Parechovirus, Hunnivirus, Tottorivirus, Passerivirus, Cosavirus, or Sicinivirus. In some embodiments, the IRES is derived from Enterovirus, Mischivirus, Kobuvirus, Bopivirus, or Gallivirus. In some embodiments, the IRES is derived from Enterovirus B, Enterovirus A, Enterovirus D, Enterovirus J, Enterovirus C, Rhinovirus B, Enterovirus H, Enterovirus I, Enterovirus E, Enterovirus F, Aichivirus B, Aichivirus A, Parechovirus A, Cardiovirus F, Cardiovirus E, or Cardiovirus B. In some embodiments, the IRES comprises a sequence selected from SEQ ID NOs: 137, 580, 648, 693, 752, 785, 791, 793, 820, 823, 839, 840, 861, 862, 863, 876, 922, 959, 983, 984, 1015, 1017, 1023, 1026, 1031, 1041, 1047, 1059, 1068, 1134, 1168, 1169, 1171, 1177, 1178, 1179, 1180, 1189, 1192, 1193, 1198, 1216, 1263, 1276, 1280, 1282, 1284, 1287, 1346, 1354, 1356, 1432, 1436, 1438, 1439, 1440, 2285, 2777, 2778, 3283, and 3284.

[0016] In some embodiments, the cell is a T cell. In some embodiments, the IRES is derived from Passerivirus, Bopivirus, Hunnivirus, Mischivirus, Enterovirus, Kobuvirus, Rabovirus,

Tottorivirus, Salivirus, Cardiovirus, Parechovirus, Megrivirus, Allexivirus, Oscivirus, or Shanbavirus. In some embodiments, the IRES is derived from Passerivirus, Hunnivirus, Mischivirus, Enterovirus, or Kobuvirus. In some embodiments, the IRES is derived from Enterovirus I, Enterovirus D, Enterovirus C, Enterovirus A, Enterovirus J, Enterovirus H, Aichivirus B, Parechovirus A, or Cardiovirus B. In some embodiments, the IRES comprises a sequence selected from SEQ ID NOs: 77, 787, 793, 820, 839, 840, 843, 852, 857, 861, 862, 863, 864, 871, 874, 876, 959, 1193, 1216, 1284, 1287, 1346, 1356, 1364, 1432, 1438, 1440, 2667, 2681, 2742, 2746, 2758, 3283, and 3284.

[0017] In some embodiments, the aptamer complex or a fragment thereof comprises a natural or synthetic aptamer sequence. In some embodiments, the aptamer complex or a fragment thereof comprises a sequence selected from SEQ ID NOs: 3266-3268. In some embodiments, the aptamer complex or a fragment thereof comprises more than one aptamer.

[0018] In some embodiments, the TIE comprises an UTR and an aptamer complex. In some embodiments, the UTR is located upstream to the aptamer complex. In some embodiments, the TIE further comprises an accessory element. In some embodiments, the accessory element comprises a miRNA binding site or a fragment thereof, a restriction site or a fragment thereof, an RNA editing motif or a fragment thereof, a zip code element or a fragment thereof, an RNA trafficking element or a fragment thereof, or a combination thereof. In some embodiments, the accessory element comprises a binding domain to an IRES transacting factor (ITAF). In some embodiments, the binding domain comprises a polyA region, a polyC region, a poly AC region, a polyprimidine tract, or a combination or variant thereof. In some embodiments, the ITAF comprises a poly(rC)-binding protein 1 (PCBP1), PCBP2, PCBP3, PCBP4, poly(A) -binding protein 1 (PABP1), polyprimidine-tract binding protein (PTB), Argonaute protein family member, HNRNPK (heterogeneous nuclear ribonucleoprotein K protein), or La protein, or a fragment or combination thereof.

[0019] In some embodiments, the coding element comprises a sequence encoding for a therapeutic protein. In some embodiments, the therapeutic protein comprises a chimeric protein. In some embodiments, the chimeric protein comprises a chimeric antigen receptor (CAR), T-cell receptor (TCR), B-cell receptor (BCR), immune cell activation or inhibitory receptor, recombinant fusion protein, chimeric mutant protein, or fusion protein, or a combination thereof. In some embodiments, the therapeutic protein comprises an antibody, nanobody, non-antibody protein,

immune modulatory ligand, receptor, structural protein, growth factor ligand or receptor, hormone or hormone receptor, transcription factor, checkpoint inhibitor or agonist, Fc fusion protein, anticoagulant, blood clotting factor, chaperone protein, antimicrobial protein, structural protein, biochemical enzyme, tight junction protein, mitochondrial stress response, cytoskeletal protein, metal-binding protein, or small molecule. In some embodiments, the immune modulatory ligand comprises an interferon, cytokine, chemokine, or interleukin. In some embodiments, the structural protein is a channel protein or nuclear pore protein.

[0020] In some embodiments, the noncoding element comprises more than one noncoding element. In some embodiments, the noncoding element comprises 50 to 15,000 nucleotides in length.

[0021] In some embodiments, the core functional element comprises a termination sequence. In some embodiments, the termination sequence is located at the 5' end of the 3' enhanced exon element. In some embodiments, the termination sequence is a stop codon. In some embodiments, termination sequence is a stop cassette. In some embodiments, the stop cassette comprises one or more stop codons in one or more frames. In some embodiments, each frame comprises a stop codon. In some embodiments, each frame comprises two or more stop codons.

[0022] In some embodiments, the 5' enhanced intron element comprises a 3' intron fragment. In some embodiments, the 3' intron fragment further comprises a first or a first and a second nucleotides of a 3' group I intron splice site dinucleotide. In some embodiments, the 3' intron fragment is located at the 3' end of the 5' enhanced intron element. In some embodiments, the group I intron comprises is derived from a bacterial phage, viral vector, organelle genome, nuclear rDNA gene. In some embodiments, the nuclear rDNA gene comprises a nuclear rDNA gene derived from a fungi, plant, or algae, or a fragment thereof.

[0023] In some embodiments, the 5' enhanced intron element comprises a leading untranslated sequence located at the 5' end. In some embodiments, the leading untranslated sequence comprises a spacer. In some embodiments, the leading untranslated sequence comprises the last nucleotide of a transcription start site. In some embodiments, the leading untranslated sequence comprises 1 to 100 additional nucleotides.

[0024] In some embodiments, the 5' enhanced intron element comprises a 5' affinity sequence. In some embodiments, the 5' affinity sequence comprises a polyA, polyAC, or polypyrimidine sequence. In some embodiments, the 5' affinity sequence comprises 10 to 100 nucleotides. In

some embodiments, the 5' enhanced intron element comprises a 5' external spacer sequence. In some embodiments, the 5' external spacer sequence is located between the 5' affinity sequence and the 3' intron fragment. In some embodiments, the 5' external spacer sequence has a length of about 6 to 60 nucleotides. In some embodiments, the 5' external spacer sequence comprises or consists of a sequence selected from SEQ ID NOs: 3094-3152.

[0025] In some embodiments, the 5' enhanced intron element comprises, in the following order: a. a leading untranslated sequence; b. a 5' affinity sequence; c. a 5' external spacer sequence; and d. a 3' intron fragment including the first nucleotide of a 3' Group I intron splice site; wherein the leading untranslated sequence comprises the last nucleotide of a transcription start site and 1 to 100 nucleotides.

[0026] In some embodiments, the 5' enhanced intron element comprises, in the following order: a. a leading untranslated sequence; b. a 5' external spacer sequence; c. a 5' affinity sequence; and d. a 3' intron fragment including the first nucleotide of a 3' group I splice site; wherein the leading untranslated sequence comprises the last nucleotide of a transcription start site and 1 to 100 nucleotide.

[0027] In some embodiments, the 5' enhanced intron element comprises, in the following order: a. a leading untranslated sequence; b. a 5' affinity sequence; c. a 5' external spacer sequence; and d. a 3' intron fragment including the first and second nucleotides of a 3' Group I intron splice site; wherein the leading untranslated sequence comprises the last nucleotide of a transcription start site and 1 to 100 nucleotides; and wherein the 5' enhanced exon element comprises a 3' exon fragment lacking the second nucleotide of a 3' group I splice site dinucleotide.

[0028] In some embodiments, the 5' enhanced intron element comprises, in the following order: a. a leading untranslated sequence; b. a 5' external spacer sequence; c. a 5' affinity sequence; and d. a 3' intron fragment including the first and second nucleotides of a 3' Group I splice site; wherein the leading untranslated sequence comprises the last nucleotide of a transcription start site and 1 to 100 nucleotide; and wherein the 5' enhanced exon element comprises a 3' exon fragment lacking the second nucleotide of a 3' group I splice site dinucleotide.

[0029] In some embodiments, the 5' enhanced exon element comprises a 3' exon fragment. In some embodiments, the 3' exon fragment further comprises the second nucleotide of a 3' group I intron splice site dinucleotide. In some embodiments, the 3' exon fragment comprises 1 to 100 natural nucleotides derived from a natural exon. In some embodiments, the natural exon derived

from a Group I intron containing gene or a fragment thereof. In some embodiments, the natural exon derived from an anabaena bacterium, T4 phage virus, twort bacteriophage, tetrahymena, or azoarcus bacterium.

[0030] In some embodiments, the 5' enhanced exon element comprises a 5' internal spacer sequence located downstream from the 3' exon fragment. In some embodiments, the 5' internal spacer sequence is about 6 to 60 nucleotides in length. In some embodiments, the 5' internal spacer sequence comprises or consists of a sequence selected from SEQ ID NOs: 3094-3152.

[0031] In some embodiments, the 5' enhanced exon element comprises in the following order: a. a 3' exon fragment including the second nucleotide of a 3' group I intron splice site dinucleotide; and b. a 5' internal spacer sequence, wherein the 3' exon fragment comprises 1 to 100 natural nucleotides derived from a natural exon.

[0032] In some embodiments, the 5' enhanced exon element comprises in the following order: a. a 3' exon fragment; and b. a 5' internal spacer sequence, wherein the 3' exon fragment comprises 1 to 100 natural nucleotides derived from a natural exon; and wherein the 5' enhanced intron element comprises a 3' intron fragment comprising the first and second nucleotides of a 3' group I splice site dinucleotide.

[0033] In some embodiments, the 3' enhanced exon element comprises a 5' exon fragment. In some embodiments, the 5' exon fragment comprises the first nucleotide of a 5' group I intron fragment. In some embodiments, the 5' exon fragment further comprises 1 to 100 nucleotides derived from a natural exon. In some embodiments, the natural exon is derived from a Group I intron containing gene or a fragment thereof.

[0034] In some embodiments, the 3' enhanced exon element comprises a 3' internal spacer sequence. In some embodiments, the 3' internal spacer sequence is located between the termination sequence and the 5' exon fragment. In some embodiments, the 3' internal spacer is about 6 to 60 nucleotides in length. In some embodiments, the 3' internal spacer comprises or consists of a sequence selected SEQ ID NOs: 3094-3152.

[0035] In some embodiments, the 3' enhanced exon element comprises: a. a 3' internal spacer sequence; and b. a 5' exon fragment including the first nucleotide of a 5' group I intron splice site dinucleotide, wherein the 5' exon fragment comprises 1 to 100 nucleotides derived from a natural exon.

[0036] In some embodiments, the 3' enhanced exon element comprises: a. a 3' internal spacer

sequence; and b. a 5' exon fragment, wherein the 5' exon fragment comprises 1 to 100 nucleotides derived from a natural exon; wherein the 3' enhanced intron element comprises a 5' intron fragment comprising the first and second nucleotide of a 5' group I intron splice site dinucleotide.

[0037] In some embodiments, the 3' enhanced intron element comprises a 5' intron fragment. In some embodiments, the 5' intron fragment comprises a second nucleotide of a 5' group I intron splice site dinucleotide.

[0038] In some embodiments, the 3' enhanced intron element comprises a trailing untranslated sequence located at the 3' end of the 5' intron. In some embodiments, the trailing untranslated sequence comprises 3 to12 nucleotides.

[0039] In some embodiments, the 3' enhanced intron fragment comprises a 3' external spacer sequence. In some embodiments, the 3' external spacer sequence is located between the 5' intron fragment and trailing untranslated sequence. In some embodiments, the 3' external spacer sequence has a length of 6 to 60 nucleotides in length. In some embodiments, the 3' external spacer sequence comprises or consists of a sequence selected from SEQ ID NOs: 3094-3152.

[0040] In some embodiments, the 3' enhanced intron element comprises a 3' affinity sequence. In some embodiments, the 3' affinity sequence is located between the 3' external spacer sequence and the trailing untranslated sequence. In some embodiments, the 3' affinity sequence comprises a polyA, poly AC, or polypyrimidine sequence. In some embodiments, the affinity sequence comprises 10 to 100 nucleotides.

[0041] In some embodiments, the 5' enhanced intron element further comprises a 5' external duplex sequence; wherein the 3' enhanced intron element further comprises a 3' external duplex sequence. In some embodiments, the 5' external duplex sequence and 3' external duplex sequence are fully or partially complementary to each other. In some embodiments, the 5' external duplex sequence comprises fully synthetic or partially synthetic nucleotides. In some embodiments, the 3' external duplex sequence comprises fully synthetic or partially synthetic nucleotides. In some embodiments, the 3' external duplex sequence is about 6 to about 50 nucleotides. In some embodiments, the 5' external duplex sequence is about 6 to about 50 nucleotides.

[0042] In some embodiments, the 5' enhanced exon element further comprises a 5' internal duplex sequence; wherein the 3' enhanced exon element further comprises a 3' internal duplex sequence. In some embodiments, the 5' internal duplex sequence and 3' internal duplex sequence are fully or partially complementary to each other. In some embodiments, the 5' internal duplex

sequence comprises fully synthetic or partially synthetic nucleotides. In some embodiments, the 3' internal duplex sequence comprises fully synthetic or partially synthetic nucleotides. In some embodiments, the 3' internal duplex sequence is about 6 to about 19 nucleotides. In some embodiments, the 5' internal duplex sequence is about 6 to about 19 nucleotides.

[0043] In some embodiments, the 3' enhanced intron fragment comprises in the following order: a. a 5' intron fragment including the second nucleotide of a 5' group I intron splice site dinucleotide; b. a 3' external spacer sequence; and c. a 3' affinity sequence.

[0044] In some embodiments, the 3' enhanced intron fragment comprises in the following order: a. a 5' intron fragment including the first and second nucleotide of a 5' group I intron splice site dinucleotide; b. a 3' external spacer sequence; and c. a 3' affinity sequence, wherein the 3' enhanced exon element comprises a 5' exon fragment lacking the first nucleotide of a 5' group I intron splice site dinucleotide.

[0045] In some embodiments, a provided precursor RNA polynucleotide comprises in the following order: a. a leading untranslated sequence; b. a 5' affinity sequence; c. 5' external duplex sequence; d. 5' spacer sequence; e. 3' intron fragment; f. 3' exon fragment; g. 5' internal duplex sequence; h. 5' internal spacer sequence; i. a translation initiation element; j. a coding element; k. a termination sequence; l. a 3' internal spacer sequence; m. a 3' internal duplex sequence; n. a 5' exon fragment; o. a 5' intron fragment; p. a 3' external duplex sequence; q. a 3' affinity sequence; and r. a trailing untranslated sequence.

[0046] In some embodiments, a provided precursor RNA polynucleotide comprises in the following order: a. a leading untranslated sequence; b. a 5' affinity sequence; c. a 5' external spacer sequence; d. a 3' intron fragment; e. a 3' exon fragment; f. a 5' internal duplex sequence; g. a 5' internal spacer sequence; h. a noncoding element; i. a 3' internal spacer sequence; j. a 3' internal duplex sequence; k. a 5' exon fragment; l. a 5' intron fragment; m. a 3' external spacer sequence; n. a 3' affinity sequence; and o. a trailing untranslated sequence.

[0047] In some embodiments, a provided precursor RNA polynucleotide comprises in the following order: a. a leading untranslated sequence; b. a 5' affinity sequence; c. a 5' external spacer sequence; d. a 3' intron fragment; e. a 3' exon fragment; f. a 5' internal duplex sequence; g. a 5' internal spacer sequence; h. a translation initiation element; i. a coding element; j. a termination sequence; k. a 3' internal spacer sequence; l. a 3' internal duplex sequence; m. a 5' exon fragment; n. a 5' intron fragment; o. a 3' external spacer sequence; and p. a 3' affinity sequence.

[0048] In some embodiments, a provided precursor RNA polynucleotide comprises in the following order: a. a leading untranslated sequence; b. a 5' affinity sequence; c. a 5' external spacer sequence; d. a 3' intron fragment; e. a 3' exon fragment; f. a 5' internal spacer sequence; g. a translation initiation element; h. a coding element; i. a termination sequence; j. a 3' internal spacer sequence; k. a 5' exon fragment; l. a 5' intron fragment; m. a 3' external spacer sequence; and n. a 3' affinity sequence.

[0049] In some embodiments, a provided precursor RNA polynucleotide comprises in the following order: a. a leading untranslated sequence; b. a 5' affinity sequence; c. a 5' external spacer sequence; d. a 3' intron fragment; e. a 3' exon fragment; f. a 5' internal spacer sequence; g. a noncoding element; h. a 3' internal spacer sequence; i. a 5' exon fragment; j. a 5' intron fragment; k. a 3' external spacer sequence; l. a 3' affinity sequence; and m. a trailing untranslated sequence.

[0050] In some embodiments, a provided precursor RNA polynucleotide comprises in the following order: a. a leading untranslated sequence; b. a 5' affinity sequence; c. 5' external duplex sequence; d. 5' spacer sequence; e. 3' intron fragment; f. 3' exon fragment; g. 5' internal duplex sequence; h. 5' internal spacer sequence; i. a termination sequence; j. a coding element; k. a translation initiation element; l. a 3' internal spacer sequence; m. a 3' internal duplex sequence; n. a 5' exon fragment; o. a 5' intron fragment; p. a 3' external duplex sequence; q. a 3' affinity sequence; and r. a trailing untranslated sequence.

[0051] In some embodiments, the coding element comprises two or more protein coding regions. In some embodiments, the precursor RNA polynucleotide comprises a polynucleotide sequence encoding a proteolytic cleavage site or a ribosomal stuttering element between the first and second expression sequence. In some embodiments, the ribosomal stuttering element is a self-cleaving spacer. In some embodiments, the precursor RNA polynucleotide comprises a polynucleotide sequence encoding 2A ribosomal stuttering peptide.

[0052] In some embodiments, the core functional element comprises two or more internal ribosome entry sites (IRESs). In some embodiments, the core functional element comprises a TIE, a coding element, a termination sequence, optionally a spacer, a TIE, a coding element, and a termination sequence, wherein the TIE comprises an IRES.

[0053] Also provided herein are circular RNA polynucleotides produced from the precursor RNA polynucleotides provided herein. In some embodiments, the precursor RNA polynucleotide is transcribed from a vector or DNA comprising a PCR product, a linearized plasmid, non-

linearized plasmid, linearized minicircle, a non-linearized minicircle, viral vector, cosmid, ceDNA, or an artificial chromosome. In some embodiments, the circular RNA polynucleotide consists of natural nucleotides. In some embodiments, the protein coding or non-coding sequence is codon optimized. In some embodiments, the circular RNA polynucleotide is from about 0.1 to about 15 kilobases in length. In some embodiments, the circular RNA polynucleotide is optimized to lack at least one microRNA binding site present in an equivalent pre-optimized polynucleotide. In some embodiments, the circular RNA polynucleotide is optimized to lack at least one RNA-editing susceptible site present in an equivalent pre-optimized polynucleotide. In some embodiments, the circular RNA polynucleotide has an in vivo duration of therapeutic effect in humans of at least 20 hours. In some embodiments, the circular RNA polynucleotide has a functional half -life of at least 6 hours. In some embodiments, the circular RNA polynucleotide has a duration of therapeutic effect in a human cell greater than or equal to that of an equivalent linear RNA polynucleotide comprising the same expression sequence. In some embodiments, the circular RNA polynucleotide has an in vivo duration of therapeutic effect in human greater than that of an equivalent linear RNA polynucleotide having the same expression sequence.

[0054] Also provided herein is a method of making a translation initiation element (TIE) comprising: a. obtaining a viral untranslated region (UTR); b. determining the functional unit of the UTR capable of binding to an initiation factor and/or initiating translation by progressively deleting sequence; c. removing non-functional units of the UTR; and optionally, modifying the ends of the UTR. In some embodiments, the modification of the ends of the UTR is about 1 percent to 75% of the viral UTR. In some embodiments, the functional unit of UTR is determined by deletion scanning from the 5' and 3' ends of the UTR or mutational scanning across the length of the UTR to identify important regions.

[0055] Also provided herein is a pharmaceutical composition comprising a circular RNA polynucleotide provided herein, a nanoparticle, and optionally, a targeting moiety operably connected to the nanoparticle. In some embodiments, the nanoparticle is a lipid nanoparticle, a core-shell nanoparticle, a biodegradable nanoparticle, a biodegradable lipid nanoparticle, a polymer nanoparticle, a polyplex or a biodegradable polymer nanoparticle. In some embodiments, the pharmaceutical composition comprises a targeting moiety, wherein the targeting moiety mediates receptor-mediated endocytosis, endosome fusion, or direct fusion into selected cells of a selected cell population or tissue in the absence of cell isolation or purification. In some

embodiments, the pharmaceutical composition comprises a targeting moiety operably connected to the nanoparticle. In some embodiments, the targeting moiety is a small molecule, scFv, nanobody, peptide, cyclic peptide, di or tri cyclic peptide, minibody, polynucleotide aptamer, engineered scaffold protein, heavy chain variable region, light chain variable region, or a fragment thereof. In some embodiments, less than 1%, by weight, of the polynucleotides in the composition are double stranded RNA, DNA splints, DNA template, or triphosphorylated RNA. In some embodiments, less than 1%, by weight, of the polynucleotides and proteins in the pharmaceutical composition are double stranded RNA, DNA splints, DNA template, triphosphorylated RNA, phosphatase proteins, protein ligases, RNA polymerases, and capping enzymes.

[0056] Also provided herein is a pharmaceutical composition comprising a circular RNA polynucleotide provided herein and a liposome, dendrimer, carbohydrate carrier, glycan nanomaterial, fusome, exosome, or a combination thereof.

[0057] Also provided herein is a pharmaceutical composition a circular RNA polynucleotide provided herein and a pharmaceutical salt, buffer, diluent or combination thereof.

[0058] Also provided herein is a method of treating a subject in need thereof comprising administering a therapeutically effective amount of a composition comprising the circular RNA polynucleotide provided herein, a nanoparticle, and optionally, a targeting moiety operably connected to the nanoparticle. In some embodiments, the targeting moiety is a small molecule, scFv, nanobody, peptide, cyclic peptide, di or tri cyclic peptide, minibody, heavy chain variable region, engineered scaffold protein, light chain variable region or fragment thereof. In some embodiments, the nanoparticle is a lipid nanoparticle, a core-shell nanoparticle, or a biodegradable nanoparticle. In some embodiments, the nanoparticle comprises one or more cationic lipids, ionizable lipids, or poly β-amino esters. In some embodiments, the nanoparticle comprises one or more non-cationic lipids. In some embodiments, the nanoparticle comprises one or more PEG-modified lipids, polyglutamic acid lipids, or hyaluronic acid lipids. In some embodiments, the nanoparticle comprises cholesterol. In some embodiments, the nanoparticle comprises arachidonic acid, leukotriene, or oleic acid. In some embodiments, the composition comprises a targeting moiety, wherein the targeting moiety mediates receptor-mediated endocytosis selectively into cells of a selected cell population in the absence of cell selection or purification. In some embodiments, the nanoparticle comprises more than one circular RNA polynucleotide. In some embodiments, the subject has a cancer selected from the group consisting of: acute myeloid leukemia (AML);

alveolar rhabdomyosarcoma; B cell malignancies; bladder cancer (e.g., bladder carcinoma); bone cancer; brain cancer (e.g., medulloblastoma and glioblastoma multiforme); breast cancer; cancer of the anus, anal canal, or anorectum; cancer of the eye; cancer of the intrahepatic bile duct; cancer of the joints; cancer of the neck; gallbladder cancer; cancer of the pleura; cancer of the nose, nasal cavity, or middle ear; cancer of the oral cavity; cancer of the vulva; chronic lymphocytic leukemia; chronic myeloid cancer; colon cancer; esophageal cancer, cervical cancer; fibrosarcoma; gastrointestinal carcinoid tumor; head and neck cancer (e.g., head and neck squamous cell carcinoma); Hodgkin lymphoma; hypopharynx cancer; kidney cancer; larynx cancer; leukemia; liquid tumors; lipoma; liver cancer; lung cancer (e.g., non-small cell lung carcinoma, lung adenocarcinoma, and small cell lung carcinoma); lymphoma; mesothelioma; mastocytoma; melanoma; multiple myeloma; nasopharynx cancer; non-Hodgkin lymphoma; B-chronic lymphocytic leukemia; hairy cell leukemia; Burkitt's lymphoma; ovarian cancer; pancreatic cancer; cancer of the peritoneum; cancer of the omentum; mesentery cancer; pharynx cancer; prostate cancer; rectal cancer; renal cancer; skin cancer; small intestine cancer; soft tissue cancer; solid tumors; synovial sarcoma; gastric cancer; teratoma; testicular cancer; thyroid cancer; and ureter cancer. In some embodiments, the subject has an autoimmune disorder selected from scleroderma, Grave's disease, Crohn's disease, Sjogren's disease, multiple sclerosis, Hashimoto's disease, psoriasis, myasthenia gravis, autoimmune polyendocrinopathy syndromes, Type I diabetes mellitus (TIDM), autoimmune gastritis, autoimmune uveoretinitis, polymyositis, colitis, thyroiditis, and the generalized autoimmune diseases typified by human Lupus.

[0059] Also provided herein is a eukaryotic cell comprising a circular RNA polynucleotide or pharmaceutical composition provided herein. In some embodiments, the eukaryotic cell is a human cell. In some embodiments, the eukaryotic cell is an immune cell. In some embodiments, the eukaryotic cell is a T cell, dendritic cell, macrophage, B cell, neutrophil, or basophil.

[0060] Also provided herein is a prokaryotic cell comprising a circular RNA polynucleotide provided herein.

[0061] In another aspect, provided herein are methods of purifying circular RNA, comprising hybridizing an oligonucleotide conjugated to a solid surface with an affinity sequence.

[0062] In some embodiments, one or more copies of the affinity sequence is present in a precursor RNA. In some embodiments, the precursor RNA is the precursor described herein. In some embodiments, the circular RNA is the circular RNA described herein. In some embodiments,

the affinity sequence is removed during formation of the circular RNA. In some embodiments, the method comprises separating the circular RNA from the precursor RNA.

[0063] In some embodiments, the affinity sequence comprises a polyA sequence. In some embodiments, the oligonucleotide that hybridizes to the affinity sequence is a deoxythymidine oligonucleotide. In some embodiments, the affinity sequence comprises a dedicated binding site (DBS). In some embodiments, the DBS comprises the nucleotide sequence of: of TATAATTCTACCCTATTGAGGCATTGACTA (SEQ ID NO: 3269). In some embodiments, the oligonucleotide that hybridizes to the affinity sequence comprises a sequence complementary to the DBS.

[0064] In another aspect, provided herein are methods of purifying circular RNA comprising: a. contacting a composition comprising linear RNA and circular RNA with a binding agent that preferentially binds to the linear RNA over the circular RNA; and b. separating RNA bound to the binding agent from RNA that is not bound to the binding agent.

[0065] In some embodiments, the binding agent is conjugated to a solid support. In some embodiments, the solid support comprises agarose, an agarose-derived resin, cellulose, a cellulose fiber, a magnetic bead, a high throughput microtiter plate, a non-agarose resin, a glass surface, a polymer surface, or a combination thereof. In some embodiments, the solid support comprises agarose or cellulose.

[0066] In some embodiments, the binding agent comprises an oligonucleotide that is complementary to a sequence present in the linear RNA and absent from the circular RNA. In some embodiments, the binding agent comprises an oligonucleotide that is 100% complementary to a sequence present in the linear RNA and absent from the circular RNA. In some embodiments, the sequence present in the linear RNA and absent from the circular RNA is an affinity sequence. In some embodiments, the sequence present in the linear RNA and absent from the circular RNA comprises a polyA sequence. In some embodiments, the binding agent comprises an oligonucleotide comprising a poly-deoxythymidine sequence. In some embodiments, the sequence present in the linear RNA and absent from the circular RNA comprises a DBS sequence. In some embodiments, the DBS sequence comprises the nucleotide sequence of: of TATAATTCTACCCTATTGAGGCATTGACTA (SEQ ID NO: 3269). In some embodiments, the sequence present in the linear RNA and absent from the circular RNA is 10-150 nucleotides in length. In some embodiments, the sequence present in the linear RNA and absent from the circular

RNA is 10-70 nucleotides in length. In some embodiments, the sequence present in the linear RNA and absent from the circular RNA is 20-30 nucleotides in length. In some embodiments, the sequence present in the linear RNA and absent from the circular RNA is present at two locations in the linear RNA. In some embodiments, the sequence present in the linear RNA and absent from the circular RNA is encoded into the linear RNA during transcription of the linear RNA. In some embodiments, the sequence present in the linear RNA and absent from the circular RNA is enzymatically added to the linear RNA. In some embodiments, the linear RNA does not comprise a methylguanylate cap. In some embodiments, the linear RNA comprises a precursor RNA or a fragment thereof.

[0067] In some embodiments, the precursor RNA is the precursor RNA described herein or a fragment thereof. In some embodiments, the precursor RNA is produced using in vitro transcription (IVT). In some embodiments, the fragment comprises an intron. In some embodiments, the linear RNA comprises a prematurely terminated RNA or RNA formed by abortive transcription.

[0068] In some embodiments, the circular RNA comprises the circular RNA described herein. In some embodiments, the circular RNA is produced using a method comprising splicing the precursor RNA. In some embodiments, the sequence present in the linear RNA and absent from the circular RNA is excised during the splicing. In some embodiments, the circular RNA is less than 6 kilobases in size.

[0069] In some embodiments, the separating comprises removing the unbound RNA from the solid support. In some embodiments, the removing comprises eluting the unbound RNA from the solid support.

[0070] In some embodiments, the method comprises heating the composition. In some embodiments, the method comprises buffer exchange. In some embodiments, buffer exchange is performed before the contacting. In some embodiments, buffer exchange is performed after the separating. In some embodiments, buffer exchange is performed before the contacting, and the resulting buffer comprises greater than 1 mM monovalent salt. In some embodiments, the monovalent salt is NaCl or KCl. In some embodiments, the resulting buffer comprises Tris. In some embodiments, the resulting buffer comprises EDTA. In some embodiments, buffer exchange is performed after the separating into storage buffer, wherein the storage buffer comprises 1mM sodium citrate, pH 6.5. In some embodiments, the method comprises filtering the circular RNA

after the separating.

BRIEF DESCRIPTION OF THE DRAWINGS

[0071] **FIG. 1** depicts luminescence in supernatants of HEK293 (**FIGs. 1A**, **1D**, and **1E**), HepG2 (**FIG. 1B**), or 1C1C7 (**FIG. 1C**) cells 24 hours after transfection with circular RNA comprising a *Gaussia* luciferase expression sequence and various IRES sequences.

[0072] **FIG. 2** depicts luminescence in supernatants of HEK293 (**FIG. 2A**), HepG2 (**FIG. 2B**), or 1C1C7 (**FIG. 2C**) cells 24 hours after transfection with circular RNA comprising a *Gaussia* luciferase expression sequence and various IRES sequences having different lengths.

[0073] **FIG. 3** depicts stability of select IRES constructs in HepG2 (**FIG. 3A**) or 1C1C7 (**FIG. 3B**) cells over 3 days as measured by luminescence.

[0074] **FIGs. 4A** and **4B** depict protein expression from select IRES constructs in Jurkat cells, as measured by luminescence from secreted *Gaussia* luciferase in cell supernatants.

[0075] **FIGs. 5A** and **5B** depict stability of select IRES constructs in Jurkat cells over 3 days as measured by luminescence.

[0076] **FIG. 6** depicts comparisons of 24 hour luminescence (**FIG. 6A**) or relative luminescence over 3 days (**FIG. 6B**) of modified linear, unpurified circular, or purified circular RNA encoding *Gaussia* luciferase.

[0077] **FIG. 7** depicts transcript induction of IFNγ (**FIG. 7A**), IL-6 (**FIG. 7B**), IL-2 (**FIG. 7C**), RIG-I (**FIG. 7D**), IFN-β1 (**FIG. 7E**), and TNFα (**FIG. 7F**) after electroporation of Jurkat cells with modified linear, unpurified circular, or purified circular RNA.

[0078] **FIG. 8** depicts a comparison of luminescence of circular RNA and modified linear RNA encoding *Gaussia* luciferase in human primary monocytes (**FIG. 8A**) and macrophages (**FIG. 8B** and **FIG. 8C**).

[0079] **FIG. 9** depicts relative luminescence over 3 days (**FIG. 9A**) in supernatant of primary T cells after transduction with circular RNA comprising a *Gaussia* luciferase expression sequence and varying IRES sequences or 24 hour luminescence (**FIG. 9B**).

[0080] **FIG. 10** depicts 24 hour luminescence in supernatant of primary T cells (FIG. 10A) after transduction with circular RNA or modified linear RNA comprising a gaussia luciferase expression sequence, or relative luminescence over 3 days (**FIG. 10B**), and 24 hour luminescence in PBMCs (**FIG. 10C**).

[0081] **FIG. 11** depicts HPLC chromatograms (**FIG. 11A**) and circularization efficiencies (FIG. 11B) of RNA constructs having different permutation sites.

[0082] **FIG. 12** depicts HPLC chromatograms (**FIG. 12A**) and circularization efficiencies (**FIG. 12B**) of RNA constructs having different introns and/or permutation sites.

[0083] **FIG. 13** depicts HPLC chromatograms (**FIG. 13A**) and circularization efficiencies (**FIG. 13B**) of 3 RNA constructs with or without homology arms.

[0084] **FIG. 14** depicts circularization efficiencies of 3 RNA constructs without homology arms or with homology arms having various lengths and GC content.

[0085] **FIG. 15A** and **15B** depict HPLC chromatograms showing the contribution of strong homology arms to improved splicing efficiency, the relationship between circularization efficiency and nicking in select constructs, and combinations of permutations sites and homology arms hypothesized to demonstrate improved circularization efficiency.

[0086] **FIG. 16** shows fluorescent images of T cells mock electroporated (left) or electroporated with circular RNA encoding a CAR (right) in co-cultured with Raji cells expressing GFP and firefly luciferase.

[0087] **FIG. 17** shows bright field (left), fluorescent (center), and overlay (right) images of T cells mock electroporated (top) or electroporated with circular RNA encoding a CAR (bottom) and co-cultured with Raji cells expressing GFP and firefly luciferase.

[0088] **FIG. 18** depicts specific lysis of Raji target cells by T cells mock electroporated or electroporated with circular RNA encoding different CAR sequences.

[0089] **FIG. 19** depicts luminescence in supernatants of Jurkat cells (left) or resting primary human CD3+ T cells (right) 24 hours after transduction with linear or circular RNA comprising a *Gaussia* luciferase expression sequence and varying IRES sequences (FIG. 19A), and relative luminescence over 3 days (**FIG. 19B**).

[0090] **FIG. 20** depicts transcript induction of IFN-β1 (**FIG. 20A**), RIG-I (**FIG. 20B**), IL-2 (**FIG. 20C**), IL-6 (**FIG. 20D**), IFNγ (**FIG. 20E**), and TNFα (**FIG. 20F**) after electroporation of human CD3+ T cells with modified linear, unpurified circular, or purified circular RNA.

[0091] **FIG. 21** depicts specific lysis of Raji target cells by human primary CD3+ T cells electroporated with circRNA encoding a CAR as determined by detection of firefly luminescence (**FIG. 21A**), and IFNγ transcript induction 24 hours after electroporation with different quantities of circular or linear RNA encoding a CAR sequence (**FIG. 21B**).

[0092] **FIG. 22** depicts specific lysis of target or non-target cells by human primary CD3+ T cells electroporated with circular or linear RNA encoding a CAR at different E:T ratios (**FIG. 22A** and **FIG. 22B**) as determined by detection of firefly luminescence.

[0093] **FIG. 23** depicts specific lysis of target cells by human CD3+ T cells electroporated with RNA encoding a CAR at 1, 3, 5, and 7 days post electroporation.

[0094] **FIG. 24** depicts specific lysis of target cells by human CD3+ T cells electroporated with circular RNA encoding a CD19 or BCMA targeted CAR.

[0095] **FIG. 25** depicts total Flux of organs harvested from CD-1 mice dosed with circular RNA encoding FLuc and formulated with 50% Lipid 10b-15, 10% DSPC, 1.5% PEG-DMG, and 38.5% cholesterol.

[0096] **FIG. 26** shows images highlighting the luminescence of organs harvested from CD-1 mice dosed with circular RNA encoding FLuc and formulated with 50% Lipid 10b-15, 10% DSPC, 1.5% PEG-DMG, and 38.5% cholesterol.

[0097] **FIG. 27** depicts molecular characterization of Lipids 10a-26 and 10a-27. **FIG. 27A** shows the proton nuclear magnetic resonance (NMR) spectrum of Lipid 10a-26. **FIG. 27B** shows the retention time of Lipid 10a-26 measured by liquid chromatography-mass spectrometry (LC-MS). **FIG. 27C** shows the mass spectrum of Lipid 10a-26. **FIG. 27D** shows the proton NMR spectrum of Lipid 10a-27. **FIG. 27E** shows the retention time of Lipid 10a-27 measured by LC-MS. **FIG. 27F** shows the mass spectrum of Lipid 10a-27.

[0098] **FIG. 28** depicts molecular characterization of Lipid 22-S14 and its synthetic intermediates. **FIG. 28A** depicts the NMR spectrum of 2-(tetradecylthio)ethan-1-ol. **FIG. 28B** depicts the NMR spectrum of 2-(tetradecylthio)ethyl acrylate. **FIG. 28C** depicts the NMR spectrum of bis(2-(tetradecylthio)ethyl) 3,3'-((3-(2-methyl-1H-imidazol-1-yl)propyl)azanediyl)dipropionate (Lipid 22-S14).

[0099] **FIG. 29** depicts the NMR spectrum of bis(2-(tetradecylthio)ethyl) 3,3'-((3-(1H-imidazol-1-yl)propyl)azanediyl)dipropionate (Lipid 93-S14).

[0100] **FIG. 30** depicts molecular characterization of heptadecan-9-yl 8-((3-(2-methyl-1H-imidazol-1-yl)propyl)(8-(nonyloxy)-8-oxooctyl)amino)octanoate (Lipid 10a-54). **FIG. 30A** shows the proton NMR spectrum of Lipid 10a-54. **FIG. 30B** shows the retention time of Lipid 10a-54measured by LC-MS. **FIG. 30C** shows the mass spectrum of Lipid 10a-54.

[0101] **FIG. 31** depicts molecular characterization of heptadecan-9-yl 8-((3-(1H-imidazol-1-

yl)propyl)(8-(nonyloxy)-8-oxooctyl)amino)octanoate (Lipid 10a-53). **FIG. 31A** shows the proton NMR spectrum of Lipid 10a-53. **FIG. 31B** shows the retention time of Lipid 10a-53 measured by LC-MS. **FIG. 31C** shows the mass spectrum of Lipid 10a-53.

[0102] **FIG. 32A** depicts total flux of spleen and liver harvested from CD-1 mice dosed with circular RNA encoding firefly luciferase (FLuc) and formulated with ionizable lipid of interest, DSPC, cholesterol, and DSPE-PEG 2000 (Avanti Polar Lipids Inc.) at a weight ratio of 16:1:4:1 or 62:4:33:1 molar ratio. **FIG. 32B** depicts average radiance for biodistribution of protein expression.

[0103] **FIG. 33A** depicts images highlighting the luminescence of organs harvested from CD-1 mice dosed with circular RNA encoding FLuc and formulated with ionizable Lipid 22-S14, DSPC, cholesterol, and DSPE-PEG 2000 (Avanti Polar Lipids Inc.) at a weight ratio of 16:1:4:1 or 62:4:33:1 molar ratio. **FIG. 33B** depicts whole body IVIS images of CD-1 mice dosed with circular RNA encoding FLuc and formulated with ionizable Lipid 22-S14, DSPC, cholesterol, and DSPE-PEG 2000 (Avanti Polar Lipids Inc.) at a weight ratio of 16:1:4:1 or 62:4:33:1 molar ratio.

[0104] **FIG. 34A** depicts images highlighting the luminescence of organs harvested from CD-1 mice dosed with circular RNA encoding FLuc and formulated with ionizable Lipid 93-S14, DSPC, cholesterol, and DSPE-PEG 2000 (Avanti Polar Lipids Inc.) at a weight ratio of 16:1:4:1 or 62:4:33:1 molar ratio. **FIG. 34B** depicts whole body IVIS images of CD-1 mice dosed with circular RNA encoding FLuc and formulated with ionizable Lipid 93-S14, DSPC, cholesterol, and DSPE-PEG 2000 (Avanti Polar Lipids Inc.) at a weight ratio of 16:1:4:1 or 62:4:33:1 molar ratio.

[0105] **FIG. 35A** depicts images highlighting the luminescence of organs harvested from CD-1 mice dosed with circular RNA encoding FLuc and formulated with ionizable Lipid 10a-26, DSPC, cholesterol, and DSPE-PEG 2000 (Avanti Polar Lipids Inc.) at a weight ratio of 16:1:4:1 or 62:4:33:1 molar ratio. **FIG. 35B** depicts whole body IVIS images of CD-1 mice dosed with circular RNA encoding FLuc and formulated with ionizable Lipid 10a-26, DSPC, cholesterol, and DSPE-PEG 2000 (Avanti Polar Lipids Inc.) at a weight ratio of 16:1:4:1 or 62:4:33:1 molar ratio.

[0106] **FIG. 36** depicts images highlighting the luminescence of organs harvested from c57BL/6J mice dosed with circular RNA encoding FLuc and encapsulated in lipid nanoparticles formed with Lipid 10b-15 (**FIG. 36A**), Lipid 10a-53 (**FIG. 36B**), or Lipid 10a-54 (**FIG. 36C**). PBS was used as control (**FIG. 36D**).

[0107] **FIGs. 37A** and **37B** depict relative luminescence in the lysates of human PBMCs after

24-hour incubation with testing lipid nanoparticles containing circular RNA encoding firefly luciferase.

[0108] **FIGs. 38** shows the expression of GFP (**FIG. 38A**) and CD19 CAR (**FIG. 38B**) in human PBMCs after incubating with testing lipid nanoparticle containing circular RNA encoding either GFP or CD19 CAR.

[0109] **FIGs. 39** depicts the expression of an anti-murine CD19 CAR in 1C1C7 cells lipotransfected with circular RNA comprising an anti-murine CD19 CAR expression sequence and varying IRES sequences.

[0110] **FIGs. 40** shows the cytotoxicity of an anti-murine CD19 CAR to murine T cells. The CD19 CAR is encoded by and expressed from a circular RNA, which is electroporated into the murine T cells.

[0111] **FIG. 41** depicts the B cell counts in peripheral blood (**FIGs. 41A** and **41B**) or spleen (**FIG. 41C**) in C57BL/6J mice injected every other day with testing lipid nanoparticles encapsulating a circular RNA encoding an anti-murine CD19 CAR.

[0112] **FIGs. 42A** and **42B** compares the expression level of an anti-human CD19 CAR expressed from a circular RNA with that expressed from a linear mRNA.

[0113] **FIGs. 43A** and **43B** compares the cytotoxic effect of an anti-human CD19 CAR expressed from a circular RNA with that expressed from a linear mRNA

[0114] **FIG. 44** depicts the cytotoxicity of two CARs (anti-human CD19 CAR and anti-human BCMA CAR) expressed from a single circular RNA in T cells.

[0115] **FIG. 45A** shows representative FACS plots with frequencies of tdTomato expression in various spleen immune cell subsets following treatment with LNPs formed with Lipid 10a-27 or 10a-26 or Lipid 10b-15. **FIG. 45B** shows the quantification of the proportion of myeloid cells, B cells, and T cells expressing tdTomato (mean + std. dev., n = 3), equivalent to the proportion of each cell population successfully transfected with Cre circular RNA. **FIG. 45C** illustrates the proportion of additional splenic immune cell populations, including NK cells, classical monocytes, nonclassical monocytes, neutrophils, and dendritic cells, expressing tdTomato after treatment with Lipids 27 and 26 (mean + std. dev., n = 3).

[0116] **FIG. 46A** depicts an exemplary RNA construct design with built-in polyA sequences in the introns. **FIG. 46B** shows the chromatography trace of unpurified circular RNA. **FIG. 46C** shows the chromatography trace of affinity-purified circular RNA. FIG. 46D shows the

immunogenicity of the circular RNAs prepared with varying in vitro transcription (IVT) conditions and purification methods. (Commercial = commercial IVT mix; Custom = customerized IVT mix; Aff = affinity purification; Enz = enzyme purification; GMP:GTP ratio = 8, 12.5, or 13.75).

[0117] **FIG. 47A** depicts an exemplary RNA construct design with a dedicated binding sequence of TATAATTCTACCCTATTGAGGCATTGACTA (SEQ ID NO: 3269) as an alternative to polyA for hybridization purification. **FIG. 47B** shows the chromatography trace of unpurified circular RNA. **FIG. 46C** shows the chromatography trace of affinity-purified circular RNA.

[0118] **FIG. 48A** shows the chromatography trace of unpurified circular RNA encoding dystrophin. **FIG. 48B** shows the chromatography trace of enzyme-purified circular RNA encoding dystrophin.

[0119] **FIG. 49** compares the expression (**FIG. 49A**) and stability (**FIG. 49B**) of purified circRNAs with different 5' spacers between the 3' intron fragment/5' internal duplex region and the IRES in Jurkat cells. (AC = only A and C were used in the spacer sequence; UC = only U and C were used in the spacer sequence.)

[0120] **FIG. 50** shows luminescence expression levels and stability of expression in primary T cells from circular RNAs containing the original or modified IRES elements indicated.

[0121] **FIG. 51** shows luminescence expression levels and stability of expression in HepG2 cells from circular RNAs containing the original or modified IRES elements indicated.

[0122] **FIG. 52** shows luminescence expression levels and stability of expression in 1C1C7 cells from circular RNAs containing the original or modified IRES elements indicated.

[0123] **FIG. 53** shows luminescence expression levels and stability of expression in HepG2 cells from circular RNAs containing IRES elements with untranslated regions (UTRs) inserted or hybrid IRES elements. "Scr" means Scrambled, which was used as a control.

[0124] **FIG. 54** shows luminescence expression levels and stability of expression in 1C1C7 cells from circular RNAs containing an IRES and variable stop codon cassettes operably linked to a gaussia luciferase coding sequence.

[0125] **FIG. 55** shows luminescence expression levels and stability of expression in 1C1C7 cells from circular RNAs containing an IRES and variable untranslated regions (UTRs) inserted before the start codon of a gaussian luciferase coding sequence.

[0126] **FIG. 56** shows expression levels of human erythropoietin (hEPO) in Huh7 cells from circular RNAs containing two miR-122 target sites downstream from the hEPO coding sequence.

[0127] **FIG. 57** shows luminescence expression levels in SupT1 cells (from a human T cell tumor line) and MV4-11 cells (from a human macrophage line) from LNPs transfected with circular RNAs encoding for Firefly luciferase *in vitro*.

[0128] **FIG. 58** shows a comparison of transfected primary human T cells LNPs containing circular RNAs dependency of ApoE based on the different helper lipid, PEG lipid, and ionizable lipid:phosphate ratio formulations.

[0129] **FIG. 59** shows uptake of LNP containing circular RNAs encoding eGFP into activated primary human T cells with or without the aid of ApoE3.

[0130] **FIG. 60** shows immune cell expression from a LNP containing circular RNA encoding for a Cre fluorescent protein in a Cre reporter mouse model.

[0131] **FIG. 61** shows immune cell expression of mOX40L in wildtype mice following intravenous injection of LNPs that have been transfected with circular RNAs encoding mOX40L.

[0132] **FIG. 62** shows single dose of mOX40L in LNPs transfected with circular RNAs capable of expressing mOX40L. **FIGs. 62A** and **62B** provide percent of mOX40L expression in splenic T cells, CD4+ T cells, CD8+ T cells, B cells, NK cells, dendritic cells, and other myloid cells. **FIG. 62C** provides mouse weight change 24 hours after transfection.

[0133] **FIG. 63** shows B cell depletion of LNPs transfected intravenously with circular RNAs in mice. **FIG. 63A** quantifies B cell depletion through B220+ B cells of live, CD45+ immune cells and **FIG. 63B** compares B cell depletion of B220+ B cells of live, CD45+ immune cells in comparison to luciferase expressing circular RNAs. **FIG. 63C** provides B cell weight gain of the transfected cells.

[0134] **FIG. 64** shows CAR expression levels in the peripheral blood (**FIG. 64A)** and spleen (**FIG. 64B**) when treated with LNP encapsulating circular RNA that expresses anti-CD19 CAR. Anti-CD20 (aCD20) and circular RNA encoding luciferase (oLuc) were used for comparison.

[0135] **FIG. 65** shows the overall frequency of anti-CD19 CAR expression, the frequency of anti-CD19 CAR expression on the surface of cells and effect on anti-tumor response of IRES specific circular RNA encoding anti-CD19 CARs on T-cells. **FIG. 65A** shows anti-CD19 CAR geometric mean florescence intensity, **FIG. 65B** shows percentage of anti-CD19 CAR expression, and **FIG. 65C** shows the percentage target cell lysis performed by the anti-CD19 CAR. (CK = Caprine Kobuvirus; AP = Apodemus Picornavirus; CK* = Caprine Kobuvirus with codon optimization; PV = Parabovirus; SV = Salivirus.)

[0136] **FIG. 66** shows CAR expression levels of A20 FLuc target cells when treated with IRES specific circular RNA constructs.

[0137] **FIG. 67** shows luminescence expression levels for cytosolic (**FIG. 67A**) and surface (**FIG. 67B**) proteins from circular RNA in primary human T-cells.

[0138] **FIG. 68** shows luminescence expression in human T-cells when treated with IRES specific circular constructs. Expression in circular RNA constructs were compared to linear mRNA. **FIG. 68A**, **FIG. 68B**, and **FIG. 68G** provide *Gaussia* luciferase expression in multiple donor cells. **FIG. 68C**, **FIG. 68D**, **FIG. 68E**, and **FIG. 68F** provides firefly luciferase expression in multiple donor cells.

[0139] **FIG. 69** shows anti-CD19 CAR (**FIG. 69A** and **FIG. 69B**) and anti-BCMA CAR (**FIG. 68B**) expression in human T-cells following treatment of a lipid nanoparticle encompassing a circular RNA that encodes either an anti-CD19 or anti-BCMA CAR to a firefly luciferase expressing K562 cell.

[0140] **FIG. 70** shows anti-CD19 CAR expression levels resulting from delivery via electroporation *in vitro* of a circular RNA encoding an anti-CD19 CAR in a specific antigen-dependent manner. **FIG. 70A** shows Nalm6 cell lysing with an anti-CD19 CAR. **FIG. 70B** shows K562 cell lysing with an anti-CD19 CAR.

[0141] **FIG. 71** shows transfection of LNP mediated by use of ApoE3 in solutions containing LNP and circular RNA expressing green fluorescence protein (GFP). **FIG. 71A** showed the live-dead results. **FIG. 71B**, **FIG. 71C**, **FIG. 71D**, and **FIG. 71E** provide the frequency of expression for multiple donors.

[0142] **FIG. 72A**, **FIG. 72B**, **FIG. 72C**, **FIG. 72D**, **FIG. 72E**, **FIG. 72F**, **FIG. 72G**, **FIG. 72H**, **FIG. 72I**, **FIG. 72J**, **FIG. 72K**, and **FIG. 72L** show total flux and precent expression for varying lipid formulations. See Example 74.

[0143] **FIG. 73** shows circularization efficiency of an RNA molecule encoding a stabilized (double proline mutant) SARS-CoV2 spike protein. **FIG. 73A** shows the in vitro transcription product of the ~4.5kb SARS-CoV2 spike-encoding circRNA. **FIG. 73B** shows a histogram of spike protein surface expression via flow cytometry after transfection of spike-encoding circRNA into 293 cells. Transfected 293 cells were stained 24 hours after transfection with CR3022 primary antibody and APC-labeled secondary antibody. **FIG. 73C** shows a flow cytometry plot of spike protein surface expression on 293 cells after transfection of spike-encoding circRNA. Transfected

293 cells were stained 24 hours after transfection with CR3022 primary antibody and APC-labeled secondary antibody.

[0144] **FIG. 74** provides multiple controlled adjuvant strategies. CircRNA as indicated on the figure entails an unpurified sense circular RNA splicing reaction using GTP as an indicator molecule *in vitro*. 3p-circRNA entails a purified sense circular RNA as well as a purified antisense circular RNA mixed containing triphosphorylated 5' termini. **FIG. 74A** shows IFN-β Induction *in vitro* in wild type and MAVS knockout A549 cells and **FIG. 74B** shows *in vivo* cytokine response to formulated circRNA generated using the indicated strategy.

[0145] **FIG. 75** illustrates an intramuscular delivery of LNP containing circular RNA constructs. **FIG. 75A** provides a live whole body flux post a 6 hour period and **75B** provides whole body IVIS 6 hours following a 1 μg dose of the LNP-circular RNA construct. **FIG. 75C** provides an *ex vivo* expression distribution over a 24-hour period.

[0146] **FIG. 76** illustrates expression of multiple circular RNAs from a single lipid formulation. **FIG. 76A** provides hEPO titers from a single and mixed set of LNP containing circular RNA constructs, while **FIG. 76B** provides total flux of bioluminescence expression from single or mixed set of LNP containing circular RNA constructs.

[0147] **FIG. 77** illustrates SARS-CoV2 spike protein expression of circular RNA encoding spike SARS-CoV2 proteins. **FIG. 77A** shows frequency of spike CoV2 expression; **FIG. 77B** shows geometric mean fluorescence intensity (gMFI) of the spike CoV2 expression; and **FIG. 77C** compares gMFI expression of the construct to the frequency of expression.

[0148] **FIG. 78** depicts a general sequence construct of a linear RNA polynucleotide precursor (**10**). The sequence as provided is illustrated in a 5' to 3' order of a 5' enhanced intron element (**20**), a 5' enhanced exon element (**30**), a core functional element (**40**), a 3' enhanced exon element (**50**) and a 3' enhanced intron element (**60**).

[0149] **FIG. 79** depicts various exemplary iterations of the 5' enhanced exon element (**20**). As illustrated, one iteration of the 5' enhanced exon element (**20**) comprises in a 5' to 3' order in the following order: a leading untranslated sequence (**21**), a 5' affinity tag (**22**), a 5' external duplex region (**24**), a 5' external spacer (**26**), and a 3' intron fragment (**28**).

[0150] **FIG. 80** depicts various exemplary iterations of the 5' enhanced exon element (**30**). As illustrated, one iteration of the 5' enhanced exon element (**30**) comprises in a 5' to 3' order: a 3' exon fragment (**32**), a 5' internal duplex region (**34**), and a 5' internal spacer (**36**).

[0151] **FIG. 81** depicts various exemplary iterations of the core functional element (**40**). As illustrated, one iteration of the core functional element (**40**) comprises a TIE (**42**), a coding region (**46**) and a stop region (*e.g.*, a stop codon or stop cassette) (**48**). Another iteration is illustrated to show the core functional element (**47**) comprising a noncoding region (**47**).

[0152] **FIG. 82** depicts various exemplary iterations of the 3' enhanced exon element (**50**). As illustrated, one of the iterations of the 3' enhanced exon element (**50**) comprises, in the following 5' to 3' order: a 3' internal spacer (**52**), a 3' internal duplex region (**54**), and a 5' exon fragment (**56**).

[0153] **FIG. 83** depicts various exemplary iterations of the 3' enhanced intron element (**60**). As illustrated, one of the iterations of the 3' enhanced intron element (**60**) comprises, in the following order, a 5' intron fragment (**62**), a 3' external spacer (**64**), a 3' external duplex region (**66**), a 3' affinity tag (**68**) and a terminal untranslated sequence (**69**).

[0154] **FIG. 84** depicts various exemplary iterations a translation initiation element (TIE) (**42**). TIE (**42**) sequence as illustrated in one iteration is solely an IRES (**43**). In another iteration, the TIE (**42**) is an aptamer (**44**). In two different iterations, the TIE (**42**) is an aptamer (**44**) and IRES (**43**) combination. In another iteration, the TIE (**42**) is an aptamer complex (**45**).

[0155] **FIG. 85** illustrates an exemplary linear RNA polynucleotide precursor (**10**) comprising in the following 5' to 3' order, a leading untranslated sequence (**21**), a 5' affinity tag (**22**), a 5' external duplex region (**24**), a 5' external spacer (**26**), a 3' intron fragment (**28**), a 3' exon fragment (**32**), a 5' internal duplex region (**34**), a 5' internal spacer (**36**), a TIE (**42**), a coding element (**46**), a stop region (**48**), a 3' internal spacer (**52**), a 3' internal duplex region (**54**), a 5' exon fragment (**56**), a 5' intron fragment (**62**), a 3' external spacer (**64**), a 3' external duplex region (**66**), a 3' affinity tag (**68**) and a terminal untranslated sequence (**69**).

[0156] **FIG. 86** illustrates an exemplary linear RNA polynucleotide precursor (**10**) comprising in the following 5' to 3' order, a leading untranslated sequence (**21**), a 5' affinity tag (**22**), a 5' external duplex region (**24**), a 5' external spacer (**26**), a 3' intron fragment (**28**), a 3' exon fragment (**32**), a 5' internal duplex region (**34**), a 5' internal spacer (**36**), a coding element (**46**), a stop region (**48**), a TIE (**42**), a 3' internal spacer (**52**), a 3' internal duplex region (**54**), a 5' exon fragment (**56**), a 5' intron fragment (**62**), a 3' external spacer (**64**), a 3' external duplex region (**66**), a 3' affinity tag (**68**) and a terminal untranslated sequence (**69**).

[0157] **FIG. 87** illustrates an exemplary linear RNA polynucleotide precursor (**10**) comprising

in the following 5' to 3' order, a leading untranslated sequence (**21**), a 5' affinity tag (**22**), a 5' external duplex region (**24**), a 5' external spacer (**26**), a 3' intron fragment (**28**), a 3' exon fragment (**32**), a 5' internal duplex region (**34**), a 5' internal spacer (**36**), a noncoding element (**47**), a 3' internal spacer (**52**), a 3' internal duplex region (**54**), a 5' exon fragment (**56**), a 5' intron fragment (**62**), a 3' external spacer (**64**), a 3' external duplex region (**66**), a 3' affinity tag (**68**) and a terminal untranslated sequence (**69**).

[0158] **FIG. 88** illustrates the general circular RNA (**8**) structure formed post splicing. The circular RNA as depicted includes a 5' exon element (**30**), a core functional element (**40**) and a 3' exon element (**50**).

[0159] **FIG. 89** illustrates the various ways an accessory element (**70**) (*e.g.*, a miRNA binding site) may be included in a linear RNA polynucleotide. **FIG. 89A** shows a linear RNA polynucleotide comprising an accessory element (**70**) at the spacer regions. **FIG. 89B** shows a linear RNA polynucleotide comprising an accessory element (**70**) located between each of the external duplex regions and the exon fragments. **FIG. 89C** depicts an accessory element (**70**) within a spacer. **FIG. 89D** illustrates various iterations of an accessory element (**70**) located within the core functional element. **FIG. 89E** illustrates an accessory element (**70**) located within an internal ribosome entry site (IRES).

[0160] **FIG. 90** illustrates a screening of a LNP formulated with circular RNA encoding firefly luciferase and having a TIE in primary human (**FIG. 90A**), mouse (**FIG. 90B**), and cynomolgus monkey (**FIG. 90C**) hepatocyte with varying dosages *in vitro*.

[0161] **FIG. 91A-C** illustrates a screening of a LNP formulated with circular RNA encoding firefly luciferase and having a TIE, in primary human hepatocyte from three different donors with varying dosages *in vitro*.

[0162] **FIG. 92** illustrates *in vitro* expression of LNP formulated with circular RNA encoding for GFP and having a TIE, in HeLa, HEK293, and HUH7 human cell models.

[0163] **FIG.93** illustrates *in vitro* expression of LNP formulated with circular RNAs encoding a GFO protein and having a TIE, in primary human hepatocytes.

[0164] **FIG. 94** illustrates *in vitro* expression of circular RNA encoding firefly luciferase and having a TIE, in mouse myoblast (**FIG. 94A**) and primary human muscle myoblast (**FIG. 94B**) cells.

[0165] **FIG. 95** illustrates *in vitro* expression of circular RNA encoding for firefly luciferase and

having a TIE, in myoblasts and differentiated primary human skeletal muscle myotubes. **FIG. 95A** provides the data related to cells received from human donor 1; **FIG. 95B** provides the data related to cell received from human donor 2.

[0166] **FIG. 96** illustrates cell-free *in vitro* translation of circular RNA of variable sizes. In **FIG. 96A** circular RNA encoding for firefly luciferase and linear mRNA encoding for firefly luciferase was tested for expression. In **FIG. 96B**, human and mouse cells were given circular RNAs encoding for ATP7B proteins. Some of the circular RNAs tested were codon optimized. Circular RNA expressing firefly luciferase was used for comparison.

[0167] **FIG. 97** shows an exemplary RNA circularization process. The schematic shown in **FIG. 97A** depicts an autocatalytic circularization process. Briefly, precursor RNA molecules containing intron segments and accessory elements that enhance circularization efficiency undergo splicing, resulting in a synthetic circular RNA and two excised intron/accessory sequence segments (spliced out intron segments/fragments). Some circularized RNA (oRNA) is nicked during synthesis. **FIG. 97B** shows an exemplary chromatogram showing peak residence of different species after size exclusion HPLC analysis.

[0168] **FIG. 98** depicts an exemplary negative selection purification method for circular RNA molecules such as oRNA. Oligonucleotides that are complementary to sequences present in the precursor RNA (such as the intron segments or external accessory regions) but not the oRNA are bound to a solid support, such as a bead. oRNA preparations are washed over the bead; precursor RNA, partially spliced RNA, incomplete transcripts, and post-splicing intron segments bind to the oligonucleotide under certain buffer conditions while oRNA and nicked oRNA flow through. Flowthrough is collected for further processing.

[0169] **FIG. 99A** and **FIG. 99B** depict an exemplary negative selection purification method for circular RNA molecule such as oRNA. The schematic shown in **FIG. 99A** depicts enzymatic polyadenylation of in vitro transcription reaction products containing oRNA and linear RNA, resulting in polyadenylation of only the linear RNA. The mixture of linear and circular RNA is washed over beads conjugated to deoxythymidine oligonucleotides ("Oligo dT") under specific buffer conditions. Polyadenylated linear RNA anneals to the beads while oRNA flows through for collection. **FIG. 99B** shows exemplary SEC-HPLC chromatograms of in vitro transcription (IVT) reaction products prior to polyadenylation and purification (left panel) and of the eluant following polyadenylation using E. coli polyA polymerase and purification with oligo-dT beads in binding

buffer (right panel).

[0170] **FIG. 100A** and **FIG. 100B** depict an exemplary circular RNA enzymatic purification method. In this method, oRNA is synthesized by IVT in the presence of excess GMP and is autocatalytically spliced during the process. The resulting reaction products are digested with Xrn1 (a 5' to 3' exonuclease requiring a 5' terminal monophosphate) and RNase R (a 3' to 5' exonuclease) to remove non-circular RNA molecules. **FIG. 100A** shows such Xrn1 and RNaseR digestion of linear RNA. **FIG. 100B** shows exemplary SEC-HPLC chromatograms of IVT reaction products prior to enzymatic digestion (left pane) and of the final, enzymatically purified material (right panel).

[0171] **FIG. 101A** and **FIG. 101B** show induction of RIG-1 and IFNB1 RNA expression, markers of immune stimulation, following transfection of the cells with the various RNA preparations indicated. All RNA preparations except for the commerically available 3phpRNA were produced using in vitro transcription and circularization of RNA comprising an Anabaena permuted intron, GLuc reading frame, strong homology arms, 5' and 3' spacers, and a CVB3 IRES. RIG-1 and IFNB1 RNA expression was measured using RT-qPCR. In FIG. 101, "IVT" indicates an unpurified reaction mixture; "+GMP" indicates an unpurified reaction mixture in which the in vitro transcription was performed in the presence of 12.5-fold GMP relative to GTP; "+HPLC" indicates a reaction mixture purified by HPLC; "+HPLC/GMP" indicates a reaction mixture purified by HPLC in which the in vitro transcription was performed in the presence of 12.5-fold GMP relative to GTP; "3phpRNA" indicates a positive control comprising a triphosphate hairpin RNA (tlrl-hprna, Invivogen); and "mock" indicates a preparation containing no RNA. **FIG. 101A** shows immune stimulation of HeLa cells, and **FIG. 101B** shows immune stimulation of A594 cells.

[0172] **FIG. 102A** and **FIG. 102B** shows anti-CD19 CAR expression levels resulting from *in vitro* delivery via electroporation of various circular RNA encoding chimeric antigen receptors in human T cells. **FIG. 102A** provides representative dot plots from FACs analysis of human T cell expression of CD19-41BBζ, CD19-CD28ζ, HER2-41BBζ, and HER2-CD28ζ CARs. **FIG. 102B** depicts cumulative data for the MFI of CD19-41BBζ, CD19-CD28ζ, HER2-41BBζ, and HER2-CD28ζ expression collected via fluorescence-activated cell sorting (FACS).

[0173] **FIGs. 103A-103C** illustrate cytotoxic response to tumor cells upon electroporation of T cells with circular RNA encoding CD19-41BBζ and CD19-CD28ζ and subsequent co-culture with

tumor cells. **FIG. 103A** provides the % specific lysis of tumor cells after coculture with T cells expressing oRNA encoding CD19-41BBζ, CD19-CD28ζ, HER2-41BBζ, and HER2-CD28ζ CARs in comparison to T cells expressing a circular RNA encoding mOX40L. **FIGs. 103B** and **103C** depict IFN- g and IL-2 cytokine in pg/mL, respectively, secreted by T cells expressing the listed oRNA as compared to a circular RNA encoding mOX40L after co-cultured with tumor cells.

[0174] **FIG. 104A** and **FIG. 104B** show *in vivo* mOX40L expression in the splenic and peripheral blood T cells of humanized mice following intravenous administration of LNP formulated with circular RNAs encoding mOX40L. LNPs were formulated with either PBS (indicated as "vehicle" in said figure), or LNP-oRNA constructs formulated with lipid 10b-15 (Table 10b, Lipid 15), 10a-27 (Table 10a, Lipid 27), or 10a-26 (Table 10a, Lipid 26). **FIG. 104A** depicts mOX40L detection in T cells in the spleen of the humanized mice. **FIG. 104B** depicts mOX40L detection in T cells in the peripheral blood of the humanized mice.

[0175] **FIG. 105** illustrates B cell aplasia in humanized mice after intravenous administration of LNP formulated with circular RNA encoding anti-CD19 chimeric antigen receptor (CAR). Representative FACS dot plots from the peripheral blood of untreated animals (left) and treated animals (right) show the percentage of B cells post 6 days from intravenous administration.

[0176] **FIG. 106A** and **FIG. 106B** show % killing of Nalm6 tumor cells after co-culture with LNP-oRNA encoding CAR or control (**FIG. 106A**) and chimeric antigen receptor (CAR) surface expression (**FIG. 106B**) following *in vitro* transfection of LNP-circular RNA (oRNA) encoding CD19-41BBζ or CD19-CD28ζ CARs. **FIG. 106A** illustrates killing of Nalm6 tumor cells after co-culture of T cells transfected with LNP-oRNA constructs encoding CARs of CD19-41BBζ and CD19-CD28ζ CARs along with HER2-41BBz, HER2-CD28z, or the control LNP-oRNA mOX40L. **FIG. 106B** provides mean fluorescence intensity (MFI) of the CAR surface expression on T cells treated with the LNP-oRNA CAR constructs.

[0177] **FIG. 107** depicts antigen-dependent tumor regression measured by total flux (in photons/sec) following dosing of mice with either PBS, PBMC, LNP-oRNA encoding for mOx40L, LNP-oRNA encoding for CD19-41BBζ ("CD19-41BBζ isCAR"), oRNA encoding for and CD19-CD28ζ ("CD19-CD28ζ isCAR"), LNP-oRNA encoding for HER2-41BBz CAR ("HER2-41BBz isCAR"), or LNP-oRNA encoding for HER2-CD28z CAR ("HER2-CD28z isCAR"). PBS and PBMC solutions lacking oRNAs were used as negative control.

[0178] **FIG. 108A**, **FIG. 108B**, and **FIG. 108C** depict the correlation between IRES activities

in myotubes and hepatocytes or myotubes and T cells. Each data point indicates the mean expression value of a circular RNA containing a IRES in front of a Gaussia luciferase coding region, wherein each IRES comprises a sequence selected from SEQ ID NOs: 1-2983 and 3282-3287 or a fragment thereof. Circular RNAs containing the IRESs were synthesized in an array format and formulated into LNPs before being transfected into activated primary human T cells, primary human myotubes, and primary human hepatocytes. All data points are normalized to a positive control IRES (SEQ ID NO: 3282).

[0179] **FIG. 109A**, **FIG. 109B**, and **FIG. 109C** depict IRES activities in hepatocytes (**FIG. 109A**), myotubes (**FIG. 109B**), and T cells (**FIG. 109C**) relative to IRESs commonly used (EMCV, CVB3). Each data point indicates the mean expression value of a circular RNA containing a IRES in front of a Gaussia luciferase coding region, wherein each IRES comprises a sequence selected from SEQ ID NOs: 1-2983 and 3282-3287 or a fragment thereof. Circular RNAs containing the IRESs were synthesized in an array format and formulated into LNPs before being transfected into activated primary human T cells, primary human myotubes, and primary human hepatocytes. All data points are normalized to a positive control IRES (SEQ ID NO: 3282).

DETAILED DESCRIPTION

[0180] The present invention provides, among other things, methods and compositions for treating an autoimmune disorder, deficiency disease, or cancer based on circular RNA therapy. In particular, the present invention provides methods for treating an autoimmune disorder, deficiency disease, or cancer by administering to a subject in need of treatment a composition comprising a circular RNA encoding at least one therapeutic protein at an effective dose and an administration interval such that at least one symptom or feature of the relevant disease or disorder is reduced in intensity, severity, or frequency or is delayed in onset.

[0181] As disclosed herein, the improved circular RNA therapy, along with associated compositions and methods, allows for increased circular RNA stability, expression, and prolonged half-life, among other things. In some embodiments, the inventive circular RNA is transcribed from a linear RNA polynucleotide construct comprising enhanced intron elements, enhanced exon elements, and a core functional element. The enhanced intron element, in some embodiments, comprises post splicing group I intron fragments, spacers, duplex sequences, affinity sequences, and unique untranslated sequences that allows for optimal circularization. In some embodiments,

the enhanced exon element comprises an exon fragment, spacers and duplex sequences to aid with the circularization process and for maintaining stability of the circular RNA post circularization. Within the same embodiments, the core functional element includes the essential elements for protein translation of a translation initiation element (TIE), a coding or noncoding element, and a termination sequence (*e.g.*, a stop codon or stop cassette). Together, the enhanced intron elements, enhanced exon elements, and core functional element comprising a coding element provides an optimal circular RNA polynucleotide for encoding a therapeutic protein. In one embodiment, the enhanced intron elements, enhanced exon elements, and core functional element comprising a noncoding element provides an optimal circular RNA polynucleotide for triggering an immune system as an adjuvant.

[0182] Also disclosed herein is a DNA template (*e.g.*, a vector) for making circular RNA. In some embodiments, the DNA template comprises a 3' enhanced intron fragment, a 3' enhanced exon fragment, a core functional element, a 5' enhanced exon fragment, and a 5' enhanced intron fragment. In some embodiments, these elements are positioned in the DNA template in the above order.

[0183] Additional embodiments include circular RNA polynucleotides, including circular RNA polynucleotides (*e.g.*, a circular RNA comprising 3' enhanced exon element, a core functional element, and a 5' enhanced exon element) made using the DNA template provided herein, compositions comprising such circular RNA, cells comprising such circular RNA, methods of using and making such DNA template, circular RNA, compositions and cells.

[0184] In some embodiments, provided herein are methods comprising administration of circular RNA polynucleotides provided herein into cells for therapy or production of useful proteins. In some embodiments, the method is advantageous in providing the production of a desired polypeptide inside eukaryotic cells with a longer half-life than linear RNA, due to the resistance of the circular RNA to ribonucleases.

[0185] Circular RNA polynucleotides lack the free ends necessary for exonuclease-mediated degradation, causing them to be resistant to several mechanisms of RNA degradation and granting extended half-lives when compared to an equivalent linear RNA. Circularization may allow for the stabilization of RNA polynucleotides that generally suffer from short half-lives and may improve the overall efficacy of exogenous mRNA in a variety of applications. In an embodiment, the functional half-life of the circular RNA polynucleotides provided herein in eukaryotic cells

(*e.g.*, mammalian cells, such as human cells) as assessed by protein synthesis is at least 20 hours (*e.g.*, at least 80 hours).

[0186] Various aspects of the invention are described in detail in the following sections. The use of sections is not meant to limit the invention. Each section can apply to any aspect of the invention. In this application, the use of "or" means "and/or" unless stated otherwise.

1. DEFINITIONS

[0187] Linear nucleic acid molecules are said to have a "**5'-terminus**" (or "**5' end**") and a "**3'-terminus**" (or "**3' end**") because nucleic acid phosphodiester linkages occur at the 5' carbon and 3' carbon of the sugar moieties of the substituent mononucleotides. The end nucleotide of a polynucleotide at which a new linkage would be to a 5' carbon is its 5' terminal nucleotide. The end nucleotide of a polynucleotide at which a new linkage would be to a 3' carbon is its 3' terminal nucleotide. A "**terminal nucleotide**," as used herein, is the nucleotide at the end position of the 3'- or 5'-terminus.

[0188] As used herein, the term "**3' group I intron fragment**" refers to a sequence with 75% or higher similarity to the 3'-proximal end of a natural group I intron including the splice site dinucleotide and optionally a stretch of natural exon sequence. As used herein, the term "**5' group I intron fragment**" refers to a sequence with 75% or higher similarity to the 5'-proximal end of a natural group I intron including the splice site dinucleotide and optionally a stretch of natural exon sequence. As used herein, the term "**permutation site**" refers to the site in a group I intron where a cut is made prior to permutation of the intron. This cut generates 3' and 5' group I intron fragments that are permuted to be on either side of a stretch of precursor RNA to be circularized.

[0189] As used herein, the singular forms "**a**," "**an**," and "**the**" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a cell" includes combinations of two or more cells, or entire cultures of cells; reference to "a polynucleotide" includes, as a practical matter, many copies of that polynucleotide.

[0190] Unless specifically stated or obvious from context, as used herein, the term "**about**," is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. "About" can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.09%, 0.08%, 0.07%, 0.06%, 0.05%, 0.04%, 0.03%, 0.02%, or 0.01% of the stated value. Unless otherwise clear from the context, all numerical values provided herein are modified by the term "about."

[0191] As used herein, an "**affinity sequence**" or "**affinity tag**" is a region of a polynucleotide sequence ranging from one (1) nucleotide to hundreds or thousands of nucleotides containing a repeated set of nucleotides for the purposes of aiding purification of a polynucleotide sequence. For example, an affinity sequence may comprise, but is not limited to, a polyA or polyAC sequence. In some embodiments, affinity tags are used in purification methods, referred to herein as "affinity-purification," in which selective binding of a binding agent to molecules comprising an affinity tag facilitates separation from molecules that do not comprise an affinity tag. In some embodiments, an affinity-purification method is a "negative selection" purification method, in which unwanted species, such as linear RNA, are selectively bound and removed and wanted species, such as circular RNA, are eluted and separated from unwanted species.

[0192] An "**anti-tumor effect**" as used herein, refers to a biological effect that may present as a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in tumor cell proliferation, a decrease in the number of metastases, an increase in overall or progression-free survival, an increase in life expectancy, or amelioration of various physiological symptoms associated with the tumor. An anti-tumor effect may also refer to the prevention of the occurrence of a tumor, e.g., a vaccine.

[0193] An "**antigen**" refers to any molecule that provokes an immune response or is capable of being bound by an antibody or an antigen binding molecule. The immune response may involve either antibody production, or the activation of specific immunologically -competent cells, or both. A person of skill in the art would readily understand that any macromolecule, including virtually all proteins or peptides, may serve as an antigen. An antigen may be endogenously expressed, i.e. expressed by genomic DNA, or may be recombinantly expressed. An antigen may be specific to a certain tissue, such as a cancer cell, or it may be broadly expressed. In addition, fragments of larger molecules may act as antigens. In some embodiments, antigens are tumor antigens.

[0194] An "**antigen binding molecule**," "**antigen binding portion**," or "**antibody fragment**" refers to any molecule that specifically binds to a desired antigen. In some embodiments, an antigen binding molecule comprises the antigen binding parts (e.g., CDRs) of an antibody or antibody-like molecule. An antigen binding molecule may include the antigenic complementarity determining regions (CDRs). Examples of antibody fragments include, but are not limited to, Fab, Fab', F(ab')2, Fv fragments, dAb, linear antibodies, scFv antibodies, and multispecific antibodies formed from antigen binding molecules. Peptibodies (i.e. Fc fusion molecules comprising peptide

binding domains) are another example of suitable antigen binding molecules. In some embodiments, the antigen binding molecule binds to an antigen on a tumor cell. In some embodiments, the antigen binding molecule binds to an antigen on a cell involved in a hyperproliferative disease or to a viral or bacterial antigen. In some embodiments, the antigen binding molecule binds to BCMA. In further embodiments, the antigen binding molecule is an antibody fragment, including one or more of the complementarity determining regions (CDRs) thereof, that specifically binds to the antigen. In further embodiments, the antigen binding molecule is a single chain variable fragment (scFv). In some embodiments, the antigen binding molecule comprises or consists of avimers.

[0195] The term "**antibody**" (Ab) includes, without limitation, a glycoprotein immunoglobulin which binds specifically to an antigen. In general, an antibody may comprise at least two heavy (H) chains and two light (L) chains interconnected by disulfide bonds, or an antigen-binding molecule thereof. Each H chain may comprise a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain constant region can comprise three constant domains, CH1, CH2 and CH3. Each light chain can comprise a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region can comprise one constant domain, CL. The VH and VL regions may be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL may comprise three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the Abs may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component of the classical complement system. Antibodies may include, for example, monoclonal antibodies, recombinantly produced antibodies, monospecific antibodies, multispecific antibodies (including bispecific antibodies), human antibodies, engineered antibodies, humanized antibodies, chimeric antibodies, immunoglobulins, synthetic antibodies, tetrameric antibodies comprising two heavy chain and two light chain molecules, an antibody light chain monomer, an antibody heavy chain monomer, an antibody light chain dimer, an antibody heavy chain dimer, an antibody light chain-antibody heavy chain pair, intrabodies, antibody fusions (sometimes referred to herein as

"antibody conjugates"), heteroconjugate antibodies, single domain antibodies, monovalent antibodies, single chain antibodies or single-chain variable fragments (scFv), camelized antibodies, affybodies, Fab fragments, F(ab')2 fragments, disulfide-linked variable fragments (sdFv), anti-idiotypic (anti-id) antibodies (including, e.g., anti-anti-Id antibodies), minibodies, domain antibodies, synthetic antibodies (sometimes referred to herein as "antibody mimetics"), and antigen-binding fragments of any of the above. In some embodiments, antibodies described herein refer to polyclonal antibody populations.

[0196] An immunoglobulin may derive from any of the commonly known isotypes, including but not limited to IgA, secretory IgA, IgG and IgM. IgG subclasses are also well known to those in the art and include but are not limited to human IgG1, IgG2, IgG3 and IgG4. "Isotype" refers to the Ab class or subclass (e.g., IgM or IgG1) that is encoded by the heavy chain constant region genes. The term "antibody" includes, by way of example, both naturally occurring and non-naturally occurring Abs; monoclonal and polyclonal Abs; chimeric and humanized Abs; human or nonhuman Abs; wholly synthetic Abs; and single chain Abs. A nonhuman Ab may be humanized by recombinant methods to reduce its immunogenicity in humans. Where not expressly stated, and unless the context indicates otherwise, the term "antibody" also includes an antigen-binding fragment or an antigen-binding portion of any of the aforementioned immunoglobulins, and includes a monovalent and a divalent fragment or portion, and a single chain Ab.

[0197] A number of definitions of the CDRs are commonly in use: Kabat numbering, Chothia numbering, AbM numbering, or contact numbering. The AbM definition is a compromise between the two used by Oxford Molecular's AbM antibody modelling software. The contact definition is based on an analysis of the available complex crystal structures. The term "Kabat numbering" and like terms are recognized in the art and refer to a system of numbering amino acid residues in the heavy and light chain variable regions of an antibody, or an antigen-binding molecule thereof. In certain aspects, the CDRs of an antibody may be determined according to the Kabat numbering system (see, e.g., Kabat EA & Wu TT (1971) Ann NY Acad Sci 190: 382-391 and Kabat EA et al., (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). Using the Kabat numbering system, CDRs within an antibody heavy chain molecule are typically present at amino acid positions 31 to 35, which optionally may include one or two additional amino acids, following 35 (referred to in the Kabat numbering scheme as 35A and 35B) (CDR1), amino acid positions 50 to 65 (CDR2),

and amino acid positions 95 to 102 (CDR3). Using the Kabat numbering system, CDRs within an antibody light chain molecule are typically present at amino acid positions 24 to 34 (CDR1), amino acid positions 50 to 56 (CDR2), and amino acid positions 89 to 97 (CDR3). In a specific embodiment, the CDRs of the antibodies described herein have been determined according to the Kabat numbering scheme. In certain aspects, the CDRs of an antibody may be determined according to the Chothia numbering scheme, which refers to the location of immunoglobulin structural loops (see, e.g., Chothia C & Lesk AM, (1987), J Mol Biol 196: 901-917; Al-Lazikani B et al, (1997) J Mol Biol 273: 927-948; Chothia C et al., (1992) J Mol Biol 227: 799-817; Tramontano A et al, (1990) J Mol Biol 215(1): 175- 82; and U.S. Patent No. 7,709,226). Typically, when using the Kabat numbering convention, the Chothia CDR-H1 loop is present at heavy chain amino acids 26 to 32, 33, or 34, the Chothia CDR-H2 loop is present at heavy chain amino acids 52 to 56, and the Chothia CDR-H3 loop is present at heavy chain amino acids 95 to 102, while the Chothia CDR-L1 loop is present at light chain amino acids 24 to 34, the Chothia CDR-L2 loop is present at light chain amino acids 50 to 56, and the Chothia CDR-L3 loop is present at light chain amino acids 89 to 97. The end of the Chothia CDR-HI loop when numbered using the Kabat numbering convention varies between H32 and H34 depending on the length of the loop (this is because the Kabat numbering scheme places the insertions at H35A and H35B; if neither 35A nor 35B is present, the loop ends at 32; if only 35A is present, the loop ends at 33; if both 35A and 35B are present, the loop ends at 34). In a specific embodiment, the CDRs of the antibodies described herein have been determined according to the Chothia numbering scheme.

[0198] As used herein, the term "**variable region**" or "**variable domain**" is used interchangeably and are common in the art. The variable region typically refers to a portion of an antibody, generally, a portion of a light or heavy chain, typically about the amino-terminal 110 to 120 amino acids in the mature heavy chain and about 90 to 115 amino acids in the mature light chain, which differ extensively in sequence among antibodies and are used in the binding and specificity of a particular antibody for its particular antigen. The variability in sequence is concentrated in those regions called complementarity determining regions (CDRs) while the more highly conserved regions in the variable domain are called framework regions (FR). Without wishing to be bound by any particular mechanism or theory, it is believed that the CDRs of the light and heavy chains are primarily responsible for the interaction and specificity of the antibody with antigen. In some embodiments, the variable region is a human variable region. In some

embodiments, the variable region comprises rodent or murine CDRs and human framework regions (FRs). In particular embodiments, the variable region is a primate (e.g., non-human primate) variable region. In some embodiments, the variable region comprises rodent or murine CDRs and primate (e.g., non-human primate) framework regions (FRs). The terms "**VL**" and "**VL domain**" are used interchangeably to refer to the light chain variable region of an antibody or an antigen-binding molecule thereof. The terms "**VH**" and "**VH domain**" are used interchangeably to refer to the heavy chain variable region of an antibody or an antigen-binding molecule thereof.

[0199] As used herein, the terms "**constant region**" and "**constant domain**" are interchangeable and have a meaning common in the art. The constant region is an antibody portion, e.g., a carboxyl terminal portion of a light and/or heavy chain which is not directly involved in binding of an antibody to antigen but which may exhibit various effector functions, such as interaction with the Fc receptor. The constant region of an immunoglobulin molecule generally has a more conserved amino acid sequence relative to an immunoglobulin variable domain.

[0200] As used herein, "**aptamer**" refers in general to either an oligonucleotide of a single defined sequence or a mixture of said nucleotides, wherein the mixture retains the properties of binding specifically to the target molecule (*e.g.*, eukaryotic initiation factor, 40S ribosome, polyC binding protein, polyA binding protein, polypyrimidine tract-binding protein, argonaute protein family, Heterogeneous nuclear ribonucleoprotein K and La and related RNA-binding protein). Thus, as used herein "aptamer" denotes both singular and plural sequences of nucleotides, as defined hereinabove. The term "aptamer" is meant to refer to a single- or double-stranded nucleic acid which is capable of binding to a protein or other molecule. In general, aptamers preferably comprise about 10 to about 100 nucleotides, preferably about 15 to about 40 nucleotides, more preferably about 20 to about 40 nucleotides, in that oligonucleotides of a length that falls within these ranges are readily prepared by conventional techniques. Optionally, aptamers can further comprise a minimum of approximately 6 nucleotides, preferably 10, and more preferably 14 or 15 nucleotides, that are necessary to effect specific binding.

[0201] As used herein, "**autoimmunity**" is defined as persistent and progressive immune reactions to non-infectious self-antigens, as distinct from infectious non self-antigens from bacterial, viral, fungal, or parasitic organisms which invade and persist within mammals and humans. Autoimmune conditions include scleroderma, Grave's disease, Crohn's disease, Sjorgen's disease, multiple sclerosis, Hashimoto's disease, psoriasis, myasthenia gravis, autoimmune

polyendocrinopathy syndromes, Type I diabetes mellitus (TIDM), autoimmune gastritis, autoimmune uveoretinitis, polymyositis, colitis, and thyroiditis, as well as in the generalized autoimmune diseases typified by human Lupus. "**Autoantigen**" or "**self-antigen**" as used herein refers to an antigen or epitope which is native to the mammal and which is immunogenic in said mammal.

[0202] The term "**autologous**" refers to any material derived from the same individual to which it is later to be re-introduced. For example, the engineered autologous cell therapy (eACT™) method described herein involves collection of lymphocytes from a patient, which are then engineered to express, e.g., a CAR construct, and then administered back to the same patient. The term "**allogeneic**" refers to any material derived from one individual which is then introduced to another individual of the same species, e.g., allogeneic T cell transplantation.

[0203] "**Binding affinity**" generally refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1 : 1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y may generally be represented by the dissociation constant (K_D or K_d). Affinity may be measured and/or expressed in a number of ways known in the art, including, but not limited to, equilibrium dissociation constant (K_D), and equilibrium association constant (K_A or K_a). The K_D is calculated from the quotient of k_{off}/k_{on}, whereas K_A is calculated from the quotient of k_{on}/k_{off}. k_{on} refers to the association rate constant of, e.g., an antibody to an antigen, and k_{off} refers to the dissociation of, e.g., an antibody to an antigen. The k_{on} and k_{off} may be determined by techniques known to one of ordinary skill in the art, such as BIACORE® or KinExA.

[0204] As used herein, the terms "immunospecifically binds," "immunospecifically recognizes," "specifically binds," and "specifically recognizes" are analogous terms in the context of antibodies and refer to molecules that bind to an antigen (e.g., epitope or immune complex) as such binding is understood by one skilled in the art. For example, a molecule that specifically binds to an antigen may bind to other peptides or polypeptides, generally with lower affinity as determined by, e.g., immunoassays, BIACORE®, KinExA 3000 instrument (Sapidyne Instruments, Boise, ID), or other assays known in the art. In a specific embodiment, molecules that specifically bind to an antigen bind to the antigen with a K_A that is at least 2 logs, 2.5 logs, 3 logs, 4 logs or greater than

the K_A when the molecules bind to another antigen.

[0205] As used herein, "**bicistronic RNA**" refers to a polynucleotide that includes two expression sequences coding for two distinct proteins. These expression sequences can be separated by a nucleotide sequence encoding a cleavable peptide such as a protease cleavage site. They can also be separated by a ribosomal skipping element.

[0206] A "**cancer**" refers to a broad group of various diseases characterized by the uncontrolled growth of abnormal cells in the body. Unregulated cell division and growth results in the formation of malignant tumors that invade neighboring tissues and may also metastasize to distant parts of the body through the lymphatic system or bloodstream. A "cancer" or "cancer tissue" may include a tumor. Examples of cancers that may be treated by the methods disclosed herein include, but are not limited to, cancers of the immune system including lymphoma, leukemia, myeloma, and other leukocyte malignancies. In some embodiments, the methods disclosed herein may be used to reduce the tumor size of a tumor derived from, for example , bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, uterine cancer, multiple myeloma, Hodgkin's Disease, non-Hodgkin's lymphoma (NHL), primary mediastinal large B cell lymphoma (PMBC), diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL), transformed follicular lymphoma, splenic marginal zone lymphoma (SMZL), cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, cancer of the urethra, cancer of the penis, chronic or acute leukemia, acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia (ALL) (including non T cell ALL), chronic lymphocytic leukemia (CLL), solid tumors of childhood, lymphocytic lymphoma, cancer of the bladder, cancer of the kidney or ureter, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, epidermoid cancer, squamous cell cancer, T cell lymphoma, environmentally induced cancers including those induced by asbestos, other B cell malignancies, and combinations of said cancers. In some embodiments, the methods disclosed herein may be used to reduce the tumor size of a tumor derived from, for example, sarcomas and carcinomas, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, Kaposi's sarcoma, sarcoma of soft tissue, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic

cancer, breast cancer, ovarian cancer, prostate cancer, hepatocellular carcinomna, lung cancer, colorectal cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma (for example adenocarcinoma of the pancreas, colon, ovary, lung, breast, stomach, prostate, cervix, or esophagus), sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, bladder carcinoma, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, carcinoma of the renal pelvis, CNS tumors (such as a glioma, astrocytoma, medulloblastoma, craniopharyogioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma and retinoblastoma). The particular cancer may be responsive to chemo- or radiation therapy or the cancer may be refractory. A refractory cancer refers to a cancer that is not amenable to surgical intervention and the cancer is either initially unresponsive to chemo- or radiation therapy or the cancer becomes unresponsive over time.

[0207] As used herein, the terms "**circRNA**," "**circular polyribonucleotide**," "**circular RNA**," "**circularized RNA**," and "**oRNA**" are used interchangeably and refer to a polyribonucleotide that forms a circular structure through covalent bonds. As used herein, such terms also include preparations comprising circRNAs.

[0208] As used herein, the term "**circularization efficiency**" refers to a measurement of the rate of formation of amount of resultant circular polyribonucleotide as compared to its linear starting material.

[0209] The expression sequences in the polynucleotide construct may be separated by a "**cleavage site**" sequence which enables polypeptides encoded by the expression sequences, once translated, to be expressed separately by the cell. A "**self-cleaving peptide**" refers to a peptide which is translated without a peptide bond between two adjacent amino acids, or functions such that when the polypeptide comprising the proteins and the self-cleaving peptide is produced, it is immediately cleaved or separated into distinct and discrete first and second polypeptides without the need for any external cleavage activity.

[0210] As used herein, "**coding element**," "**coding sequence**," "**coding nucleic acid**," or "**coding region**" is region located within the expression sequence and encodings for one or more proteins or polypeptides (*e.g.*, therapeutic protein). As used herein, a "**noncoding element**,"

"**noncoding sequence**," "**non-coding nucleic acid**," or "**noncoding nucleic acid**" is a region located within the expression sequence. This sequence, but itself does not encode for a protein or polypeptide, but may have other regulatory functions, including but not limited, allow the overall polynucleotide to act as a biomarker or adjuvant to a specific cell.

[0211] As used herein, a "**conservative**" amino acid substitution is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). In some embodiments, one or more amino acid residues within a CDR(s) or within a framework region(s) of an antibody or antigen-binding molecule thereof may be replaced with an amino acid residue with a similar side chain.

[0212] A "**costimulatory ligand**," as used herein, includes a molecule on an antigen presenting cell that specifically binds a cognate co-stimulatory molecule on a T cell. Binding of the costimulatory ligand provides a signal that mediates a T cell response, including, but not limited to, proliferation, activation, differentiation, and the like. A costimulatory ligand induces a signal that is in addition to the primary signal provided by a stimulatory molecule, for instance, by binding of a T cell receptor (TCR)/CD3 complex with a major histocompatibility complex (MHC) molecule loaded with peptide. A co-stimulatory ligand may include, but is not limited to, 3/TR6, 4-IBB ligand, agonist or antibody that binds Toll-like receptor, B7-1 (CD80), B7-2 (CD86), CD30 ligand, CD40, CD7, CD70, CD83, herpes virus entry mediator (HVEM), human leukocyte antigen G (HLA-G), ILT4, immunoglobulin-like transcript (ILT) 3, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (ICAM), ligand that specifically binds with B7-H3, lymphotoxin beta receptor, MHC class I chain-related protein A (MICA), MHC class I chain-related protein B (MICB), OX40 ligand, PD-L2, or programmed death (PD) LI. A co-stimulatory ligand includes, without limitation, an antibody that specifically binds with a co-stimulatory molecule present on a T cell, such as, but not limited to, 4-1BB, B7-H3, CD2, CD27, CD28, CD30, CD40, CD7, ICOS, ligand that specifically binds with CD83, lymphocyte function- associated

antigen-1 (LFA-1), natural killer cell receptor C (NKG2C), OX40, PD-1, or tumor necrosis factor superfamily member 14 (TNFSF14 or LIGHT).

[0213] A "**costimulatory molecule**" is a cognate binding partner on a T cell that specifically binds with a costimulatory ligand, thereby mediating a costimulatory response by the T cell, such as, but not limited to, proliferation. Costimulatory molecules include, but are not limited to, 4-1BB/CD137, B7-H3, BAFFR, BLAME (SLAMF8), BTLA, CD 33, CD 45, CD100 (SEMA4D), CD103, CD134, CD137, CD154, CD16, CD160 (BY55), CD 18, CD19, CD19a, CD2, CD22, CD247, CD27, CD276 (B7-H3), CD28, CD29, CD3 (alpha; beta; delta; epsilon; gamma; zeta), CD30, CD37, CD4, CD4, CD40, CD49a, CD49D, CD49f, CD5, CD64, CD69, CD7, CD80, CD83 ligand, CD84, CD86, CD8alpha, CD8beta, CD9, CD96 (Tactile), CD1- la, CDl-lb, CDl-lc, CDl-ld, CDS, CEACAM1, CRT AM, DAP-10, DNAM1 (CD226), Fc gamma receptor, GADS, GITR, HVEM (LIGHTR), IA4, ICAM-1, ICAM-1, ICOS, Ig alpha (CD79a), IL2R beta, IL2R gamma, IL7R alpha, integrin, ITGA4, ITGA4, ITGA6, IT GAD, ITGAE, ITGAL, ITGAM, ITGAX, ITGB2, ITGB7, ITGB1, KIRDS2, LAT, LFA-1, LFA-1, LIGHT, LIGHT (tumor necrosis factor superfamily member 14; TNFSF14), LTBR, Ly9 (CD229), lymphocyte function-associated antigen-1 (LFA-1 (CD1 la/CD18), MHC class I molecule, NKG2C, NKG2D, NKp30, NKp44, NKp46, NKp80 (KLRF1), OX40, PAG/Cbp, PD-1, PSGL1, SELPLG (CD162), signaling lymphocytic activation molecule, SLAM (SLAMF1; CD150; IPO-3), SLAMF4 (CD244; 2B4), SLAMF6 (NTB-A; Lyl08), SLAMF7, SLP-76, TNF, TNFr, TNFR2, Toll ligand receptor, TRANCE/RANKL, VLA1, or VLA-6, or fragments, truncations, or combinations thereof.

[0214] A "**costimulatory signal**," as used herein, refers to a signal, which in combination with a primary signal, such as TCR/CD3 ligation, leads to a T cell response, such as, but not limited to, proliferation and/or upregulation or down regulation of key molecules.

[0215] As used herein, an antigen binding molecule, an antibody, or an antigen binding molecule thereof "**cross-competes**" with a reference antibody or an antigen binding molecule thereof if the interaction between an antigen and the first binding molecule, an antibody, or an antigen binding molecule thereof blocks, limits, inhibits, or otherwise reduces the ability of the reference binding molecule, reference antibody, or an antigen binding molecule thereof to interact with the antigen. Cross competition may be complete, e.g., binding of the binding molecule to the antigen completely blocks the ability of the reference binding molecule to bind the antigen, or it may be partial, e.g., binding of the binding molecule to the antigen reduces the ability of the reference

binding molecule to bind the antigen. In some embodiments, an antigen binding molecule that cross-competes with a reference antigen binding molecule binds the same or an overlapping epitope as the reference antigen binding molecule. In other embodiments, the antigen binding molecule that cross-competes with a reference antigen binding molecule binds a different epitope as the reference antigen binding molecule. Numerous types of competitive binding assays may be used to determine if one antigen binding molecule competes with another, for example: solid phase direct or indirect radioimmunoassay (RIA); solid phase direct or indirect enzyme immunoassay (EIA); sandwich competition assay (Stahli et al., 1983, Methods in Enzymology 9:242-253); solid phase direct biotin-avidin EIA (Kirkland et al., 1986, J. Immunol. 137:3614-3619); solid phase direct labeled assay, solid phase direct labeled sandwich assay (Harlow and Lane, 1988, Antibodies, A Laboratory Manual, Cold Spring Harbor Press); solid phase direct label RIA using 1-125 label (Morel et al., 1988, Molec. Immunol. 25:7-15); solid phase direct biotin-avidin EIA (Cheung, et al., 1990, Virology 176:546-552); and direct labeled RIA (Moldenhauer et al., 1990, Scand. J. Immunol. 32:77-82).

[0216] A "**cytokine**," as used herein, refers to a non-antibody protein that is released by one cell in response to contact with a specific antigen, wherein the cytokine interacts with a second cell to mediate a response in the second cell. A cytokine may be endogenously expressed by a cell or administered to a subject. Cytokines may be released by immune cells, including macrophages, B cells, T cells, neutrophils, dendritic cells, eosinophils and mast cells to propagate an immune response. Cytokines may induce various responses in the recipient cell. Cytokines may include homeostatic cytokines, chemokines, pro- inflammatory cytokines, effectors, and acute-phase proteins. For example, homeostatic cytokines, including interleukin (IL) 7 and IL-15, promote immune cell survival and proliferation, and pro- inflammatory cytokines may promote an inflammatory response. Examples of homeostatic cytokines include, but are not limited to, IL-2, IL-4, IL-5, IL-7, IL-10, IL-12p40, IL-12p70, IL-15, and interferon (IFN) gamma. Examples of pro-inflammatory cytokines include, but are not limited to, IL-la, IL-lb, IL- 6, IL-13, IL-17a, IL-23, IL-27, tumor necrosis factor (TNF)-alpha, TNF-beta, fibroblast growth factor (FGF) 2, granulocyte macrophage colony-stimulating factor (GM-CSF), soluble intercellular adhesion molecule 1 (sICAM-1), soluble vascular adhesion molecule 1 (sVCAM-1), vascular endothelial growth factor (VEGF), VEGF-C, VEGF-D, and placental growth factor (PLGF). Examples of effectors include, but are not limited to, granzyme A, granzyme B, soluble Fas ligand (sFasL),

TGF-beta, IL-35, and perforin. Examples of acute phase-proteins include, but are not limited to, C-reactive protein (CRP) and serum amyloid A (SAA).

[0217] By "**co-administering**" is meant administering a therapeutic agent provided herein in conjunction with one or more additional therapeutic agents sufficiently close in time such that the therapeutic agent provided herein can enhance the effect of the one or more additional therapeutic agents, or vice versa.

[0218] As used herein, the term "**co-formulate**" refers to a nanoparticle formulation comprising two or more nucleic acids or a nucleic acid and other active drug substance. Typically, the ratios are equimolar or defined in the ratiometric amount of the two or more nucleic acids or the nucleic acid and other active drug substance.

[0219] The terms "**deoxyribonucleic acid**" and "**DNA**" as used herein mean a polymer composed of deoxyribonucleotides. The terms "**ribonucleic acid**" and "**RNA**" as used herein mean a polymer composed of ribonucleotides.

[0220] As used herein, the term "**DNA template**" refers to a DNA sequence capable of transcribing a linear RNA polynucleotide. For example, but not intending to be limiting, a DNA template may include a DNA vector, PCR product or plasmid.

[0221] As used herein, the terms "**duplexed**," "**double-stranded**," and "**hybridized**" are used interchangeably and refer to double-stranded nucleic acids formed by hybridization of two single strands of nucleic acids containing complementary sequences. Sequences of the two single-stranded nucleic acids can be fully complementary or partially complementary. In some embodiments, a nucleic acid provided herein may be fully double-stranded or partially double-stranded. In most cases, genomic DNA is double-stranded.

[0222] As used herein, two "**duplex sequences**," "**duplex forming sequences**," "**duplex region**," "**duplex forming regions**," "**homology arms**," or "**homology regions**," complement, or are complementary, fully or partially, to one another when the two regions share a sufficient level of sequence identity to one another's reverse complement to act as substrates for a hybridization reaction. In some embodiments, two duplex forming sequences are thermodynamically favored to cross-pair in a sequence specific interaction. As used herein, polynucleotide sequences have "**homology**" when they are either identical or share sequence identity to a reverse complement or "complementary" sequence. The percent sequence identity between a homology region and a counterpart homology region's reverse complement can be any percent of sequence identity that

allows for hybridization to occur. In some embodiments, an internal duplex forming region of a polynucleotide disclosed herein is capable of forming a duplex with another internal duplex forming region and does not form a duplex with an external duplex forming region.

[0223] As used herein, the term "**encode**" refers broadly to any process whereby the information in a polymeric macromolecule is used to direct the production of a second molecule that is different from the first. The second molecule may have a chemical structure that is different from the chemical nature of the first molecule. For example, a DNA template (e.g., a DNA vector) may encode a RNA polynucleotide; a precursor RNA polynucleotide (e.g., a linear precursor RNA polynucleotide) may encode a mature RNA polynucleotide (e.g., a circular RNA polynucleotide).

[0224] As used herein, "endogenous" means a substance that is native to, i.e., naturally originated from, a biological system (e.g., an organism, a tissue, or a cell). For example, in some embodiments, a "endogenous polynucleotide" is normally expressed in a cell or tissue. In some embodiments, a polynucleotide is still considered endogenous if the control sequences, such as a promoter or enhancer sequences which activate transcription or translation, have been altered through recombinant techniques. As used herein, the term "**heterologous**" means from any source other than naturally occurring sequences.

[0225] As used herein, an "**endonuclease site**" refers to a stretch of nucleotides within a polynucleotide that is capable of being recognized and cleaved by an endonuclease protein.

[0226] An "**eukaryotic initiation factor**" or "**eIF**" refers to a protein or protein complex used in assembling an initiator tRNA, 40S and 60S ribosomal subunits required for initiating eukaryotic translation.

[0227] As used herein, an "**epitope**" is a term in the art and refers to a localized region of an antigen to which an antibody may specifically bind. An epitope may be, for example, contiguous amino acids of a polypeptide (linear or contiguous epitope) or an epitope can, for example, come together from two or more non-contiguous regions of a polypeptide or polypeptides (conformational, non-linear, discontinuous, or non-contiguous epitope). In some embodiments, the epitope to which an antibody binds may be determined by, e.g., NMR spectroscopy, X-ray diffraction crystallography studies, ELISA assays, hydrogen/deuterium exchange coupled with mass spectrometry (e.g., liquid chromatography electrospray mass spectrometry), array -based oligo-peptide scanning assays, and/or mutagenesis mapping (e.g., site- directed mutagenesis mapping). For X-ray crystallography, crystallization may be accomplished using any of the known

methods in the art (e.g., Giege R et al., (1994) Acta Crystallogr D Biol Crystallogr 50(Pt 4): 339-350; McPherson A (1990) Eur J Biochem 189: 1-23; Chayen NE (1997) Structure 5: 1269- 1274; McPherson A (1976) J Biol Chem 251: 6300-6303). Antibody: antigen crystals may be studied using well known X-ray diffraction techniques and may be refined using computer software such as X- PLOR (Yale University, 1992, distributed by Molecular Simulations, Inc.; see e.g. Meth Enzymol (1985) volumes 114 & 115, eds Wyckoff HW et al.; U.S. Patent Publication No. 2004/0014194), and BUSTER (Bricogne G (1993) Acta Crystallogr D Biol Crystallogr 49(Pt 1): 37-60; Bricogne G (1997) Meth Enzymol 276A: 361-423, ed Carter CW; Roversi P et al., (2000) Acta Crystallogr D Biol Crystallogr 56(Pt 10): 1316-1323).

[0228] As used herein, the term "**expression sequence**" refers to a nucleic acid sequence that encodes a product, e.g., a peptide or polypeptide, regulatory nucleic acid, or non-coding nucleic acid. An exemplary expression sequence that codes for a peptide or polypeptide can comprise a plurality of nucleotide triads, each of which can code for an amino acid and is termed as a "**codon**."

[0229] As used herein, a "**fusion protein**" is a protein with at least two domains that are encoded by separate genes that have been joined to transcribe for a single peptide.

[0230] The term "**genetically engineered**" or "**engineered**" refers to a method of modifying the genome of a cell, including, but not limited to, deleting a coding or non-coding region or a portion thereof or inserting a coding region or a portion thereof. In some embodiments, the cell that is modified is a lymphocyte, e.g., a T cell, which may either be obtained from a patient or a donor. The cell may be modified to express an exogenous construct, such as, e.g., a chimeric antigen receptor (CAR) or a T cell receptor (TCR), which is incorporated into the cell's genome.

[0231] An "**immune response**" refers to the action of a cell of the immune system (for example, T lymphocytes, B lymphocytes, natural killer (NK) cells, macrophages, eosinophils, mast cells, dendritic cells and neutrophils) and soluble macromolecules produced by any of these cells or the liver (including Abs, cytokines, and complement) that results in selective targeting, binding to, damage to, destruction of, and/or elimination from a vertebrate's body of invading pathogens, cells or tissues infected with pathogens, cancerous or other abnormal cells, or, in cases of autoimmunity or pathological inflammation, normal human cells or tissues.

[0232] As used herein, the term "**immunogenic**" or "**immunostimulatory**" refers to a potential to induce an immune response to a substance. An immune response may be induced when an immune system of an organism or a certain type of immune cells is exposed to an immunogenic

substance. The term "**non-immunogenic**" refers to a lack of or absence of an immune response above a detectable threshold to a substance. No immune response is detected when an immune system of an organism or a certain type of immune cells is exposed to a non-immunogenic substance. In some embodiments, a non-immunogenic circular polyribonucleotide as provided herein, does not induce an immune response above a pre-determined threshold when measured by an immunogenicity assay. In some embodiments, no innate immune response is detected when an immune system of an organism or a certain type of immune cells is exposed to a non-immunogenic circular polyribonucleotide as provided herein. In some embodiments, no adaptive immune response is detected when an immune system of an organism or a certain type of immune cell is exposed to a non-immunogenic circular polyribonucleotide as provided herein.

[0233] As used herein, an "**internal ribosome entry site**" or "**IRES**" refers to an RNA sequence or structural element ranging in size from 10 nt to 1000 nt or more , capable of initiating translation of a polypeptide in the absence of a typical RNA cap structure. An IRES is typically about 500 nt to about 700 nt in length.

[0234] "**Isolated**" or "**purified**" generally refers to isolation of a substance (for example, in some embodiments, a compound, a polynucleotide, a protein, a polypeptide, a polynucleotide composition, or a polypeptide composition) such that the substance comprises a significant percent (e.g., greater than 1%, greater than 2%, greater than 5%, greater than 10%, greater than 20%, greater than 50%, or more, usually up to about 90%-100%) of the sample in which it resides. In certain embodiments, a substantially purified component comprises at least 50, 60, 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% of the sample. In additional embodiments, a substantially purified component comprises about, 80%-85%, or 90%-95%, 95-99%, 96-99%, 97-99%, or 95-100% of the sample. Techniques for purifying polynucleotides and polypeptides of interest are well-known in the art and include, for example, ion-exchange chromatography, affinity chromatography and sedimentation according to density. Generally, a substance is purified when it exists in a sample in an amount, relative to other components of the sample, that is more than as it is found naturally.

[0235] As used herein, a "**leading untranslated sequence**" is a region of polynucleotide sequences ranging from 1 nucleotide to hundreds of nucleotides located at the upmost 5' end of a polynucleotide sequence. The sequences can be defined or can be random. An leading untranslated sequence is non-coding. As used herein, a "**terminal untranslated sequence**" is a region of polynucleotide sequences ranging from 1 nucleotide to hundreds of nucleotides located

at the downmost 3' end of a polynucleotide sequence. The sequences can be defined or can be random. A terminal untranslated sequence is non-coding.

[0236] The term "**lymphocyte**" as used herein includes natural killer (NK) cells, T cells, or B cells. NK cells are a type of cytotoxic (cell toxic) lymphocyte that represent a major component of the innate immune system. NK cells reject tumors and cells infected by viruses. It works through the process of apoptosis or programmed cell death. They were termed "natural killers" because they do not require activation in order to kill cells. T cells play a major role in cell-mediated-immunity (no antibody involvement). T cell receptors (TCR) differentiate T cells from other lymphocyte types. The thymus, a specialized organ of the immune system, is the primary site for T cell maturation. There are numerous types of T cells, including: helper T cells (e.g., CD4+ cells), cytotoxic T cells (also known as TC, cytotoxic T lymphocytes, CTL, T-killer cells, cytolytic T cells, CD8+ T cells or killer T cells), memory T cells ((i) stem memory cells (TSCM), like naive cells, are CD45RO-, CCR7+, CD45RA+, CD62L+ (L- selectin), CD27+, CD28+ and IL-7Ra+, but also express large amounts of CD95, IL-2R, CXCR3, and LFA-1, and show numerous functional attributes distinctive of memory cells); (ii) central memory cells (TCM) express L-selectin and CCR7, they secrete IL-2, but not IFNγ or IL-4, and (iii) effector memory cells (TEM), however, do not express L-selectin or CCR7 but produce effector cytokines like IFNγ and IL-4), regulatory T cells (Tregs, suppressor T cells, or CD4+CD25+ or CD4+ FoxP3+ regulatory T cells), natural killer T cells (NKT) and gamma delta T cells. B-cells, on the other hand, play a principal role in humoral immunity (with antibody involvement). B-cells make antibodies, are capable of acting as antigen-presenting cells (APCs) and turn into memory B-cells and plasma cells, both short-lived and long-lived, after activation by antigen interaction. In mammals, immature B-cells are formed in the bone marrow.

[0237] As used herein, a "**miRNA site**" refers to a stretch of nucleotides within a polynucleotide that is capable of forming a duplex with at least 8 nucleotides of a natural miRNA sequence.

[0238] As used herein, a "**neoantigen**" refers to a class of tumor antigens which arises from tumor-specific mutations in an expressed protein.

[0239] The term "**nucleotide**" refers to a ribonucleotide, a deoxyribonucleotide, a modified form thereof, or an analog thereof. Nucleotides include species that comprise purines, e.g., adenine, hypoxanthine, guanine, and their derivatives and analogs, as well as pyrimidines, e.g., cytosine, uracil, thymine, and their derivatives and analogs. Nucleotide analogs include nucleotides having

modifications in the chemical structure of the base, sugar and/or phosphate, including, but not limited to, 5'-position pyrimidine modifications, 8'-position purine modifications, modifications at cytosine exocyclic amines, and substitution of 5-bromo-uracil; and 2'-position sugar modifications, including but not limited to, sugar-modified ribonucleotides in which the 2'-OH is replaced by a group such as an H, OR, R, halo, SH, SR, NH_2, NHR, NR_2, or CN, wherein R is an alkyl moiety as defined herein. Nucleotide analogs are also meant to include nucleotides with bases such as inosine, queuosine, xanthine; sugars such as 2'-methyl ribose; non-natural phosphodiester linkages such as methylphosphonate, phosphorothioate and peptide linkages. Nucleotide analogs include 5-methoxyuridine, 1-methylpseudouridine, and 6-methyladenosine.

[0240] All nucleotide sequences disclosed herein can represent an RNA sequence or a corresponding DNA sequence. It is understood that deoxythymidine (dT or T) in a DNA is transcribed into a uridine (U) in an RNA. As such, "T" and "U" are used interchangeably herein in nucleotide sequences.

[0241] The terms "**nucleic acid**" and "**polynucleotide**" are used interchangeably herein to describe a polymer of any length, e.g., greater than about 2 bases, greater than about 10 bases, greater than about 100 bases, greater than about 500 bases, greater than 1000 bases, or up to about 10,000 or more bases, composed of nucleotides, e.g., deoxyribonucleotides or ribonucleotides, and may be produced enzymatically or synthetically (e.g., as described in U.S. Pat. No. 5,948,902 and the references cited therein), which can hybridize with naturally occurring nucleic acids in a sequence specific manner analogous to that of two naturally occurring nucleic acids, e.g., can participate in Watson-Crick base pairing interactions. An "oligonucleotide" is a polynucleotide comprising fewer than 1000 nucleotides, such as a polynucleotide comprising fewer than 500 nucleotides or fewer than 100 nucleotides. Naturally occurring nucleic acids are comprised of nucleotides, including guanine, cytosine, adenine, thymine, and uracil containing nucleotides (G, C, A, T, and U respectively). As used herein, "polyA" means a polynucleotide or a portion of a polynucleotide consisting of nucleotides comprising adenine. As used herein, "polyT" means a polynucleotide or a portion of a polynucleotide consisting of nucleotides comprising thymine. As used herein, "polyAC" means a polynucleotide or a portion of a polynucleotide consisting of nucleotides comprising adenine or cytosine.

[0242] As used herein, the term "**ribosomal skipping element**" refers to a nucleotide sequence encoding a short peptide sequence capable of causing generation of two peptide chains from

translation of one RNA molecule. While not wishing to be bound by theory, it is hypothesized that ribosomal skipping elements function by (1) terminating translation of the first peptide chain and re-initiating translation of the second peptide chain; or (2) cleavage of a peptide bond in the peptide sequence encoded by the ribosomai skipping element by an intrinsic protease activity of the encoded peptide, or by another protease in the environment (e.g., cytosol).

[0243] The term "**sequence identity**," as used herein, refer to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity" may be calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, I) or the identical amino acid residue (e.g., Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. Included are nucleotides and polypeptides having at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to any of the reference sequences described herein, typically where the polypeptide variant maintains at least one biological activity of the reference polypeptide.

[0244] As used herein, a "**spacer**" refers to a region of a polynucleotide sequence ranging from 1 nucleotide to hundreds or thousands of nucleotides separating two other elements along a polynucleotide sequence. The sequences can be defined or can be random. A spacer is typically non-coding. In some embodiments, spacers include duplex regions.

[0245] As used herein, the term "**splice site**" refers to a dinucleotide that is partially or fully included in a group I intron and between which a phosphodiester bond is cleaved during RNA circularization.

[0246] As used herein, "**structured**" with regard to RNA refers to an RNA sequence that is predicted by the RNAFold software or similar predictive tools to form a structure (e.g., a hairpin loop) with itself or other sequences in the same RNA molecule. As used herein, "**unstructured**" with regard to RNA refers to an RNA sequence that is not predicted by RNA structure predictive tools to form a structure (e.g., a hairpin loop) with itself or other sequences in the same RNA molecule. In some embodiments, unstructured RNA can be functionally characterized using

nuclease protection assays.

[0247] As used herein, the term "**therapeutic protein**" refers to any protein that, when administered to a subject directly or indirectly in the form of a translated nucleic acid, has a therapeutic, diagnostic, and/or prophylactic effect and/or elicits a desired biological and/or pharmacological effect.

[0248] "**Transcription**" means the formation or synthesis of an RNA molecule by an RNA polymerase using a DNA molecule as a template. The invention is not limited with respect to the RNA polymerase that is used for transcription. For example, in some embodiments, a T7-type RNA polymerase can be used.

[0249] "**Translation**" means the formation of a polypeptide molecule by a ribosome based upon an RNA template. As used herein, the term "**translation efficiency**" refers to a rate or amount of protein or peptide production from a ribonucleotide transcript. In some embodiments, translation efficiency can be expressed as amount of protein or peptide produced per given amount of transcript that codes for the protein or peptide.

[0250] As used herein, the terms "**transfect**" or "**transfection**" refer to the intracellular introduction of one or more encapsulated materials (e.g., nucleic acids and/or polynucleotides) into a cell, or preferably into a target cell. The term "**transfection efficiency**" refers to the relative amount of such encapsulated material (e.g., polynucleotides) up-taken by, introduced into and/or expressed by the target cell which is subject to transfection. In some embodiments, transfection efficiency may be estimated by the amount of a reporter polynucleotide product produced by the target cells following transfection. In some embodiments, a transfer vehicle has high transfection efficiency. In some embodiments, a transfer vehicle has at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% transfection efficiency.

[0251] As used herein, "**transfer vehicle**" includes any of the standard pharmaceutical carriers, diluents, excipients, and the like, which are generally intended for use in connection with the administration of biologically active agents, including nucleic acids. In certain embodiments of the present invention, the transfer vehicles (e.g., lipid nanoparticles) are prepared to encapsulate one or more materials or therapeutic agents (e.g., circRNA). The process of incorporating a desired therapeutic agent (e.g., circRNA) into a transfer vehicle is referred to herein as or "loading" or "encapsulating" (Lasic, et al., FEBS Lett., 312: 255-258, 1992). The transfer vehicle-loaded or -encapsulated materials (e.g., circRNA) may be completely or partially located in the interior space

of the transfer vehicle, within a bilayer membrane of the transfer vehicle, or associated with the exterior surface of the transfer vehicle.

[0252] The terms "**treat**," and "**prevent**" as well as words stemming therefrom, as used herein, do not necessarily imply 100% or complete treatment or prevention. Rather, there are varying degrees of treatment or prevention of which one of ordinary skill in the art recognizes as having a potential benefit or therapeutic effect. The treatment or prevention provided by the method disclosed herein can include treatment or prevention of one or more conditions or symptoms of the disease. Also, for purposes herein, "prevention" can encompass delaying the onset of the disease, or a symptom or condition thereof.

[0253] The α and β chains of αβ TCR's are generally regarded as each having two domains or regions, namely variable and constant domains/regions. The variable domain consists of a concatenation of variable regions and joining regions. In the present specification and claims, the term "**TCR alpha variable domain**" therefore refers to the concatenation of TRAV and TRAJ regions, and the term TCR alpha constant domain refers to the extracellular TRAC region, or to a C-terminal truncated TRAC sequence. Likewise, the term "**TCR beta variable domain**" refers to the concatenation of TRBV and TRBD/TRBJ regions, and the term TCR beta constant domain refers to the extracellular TRBC region, or to a C-terminal truncated TRBC sequence.

[0254] As used herein, the terms "**upstream**" and "**downstream**" refer to relative positions of genetic code, e.g., nucleotides, sequence elements, in polynucleotide sequences. In some embodiments, in an RNA polynucleotide, upstream is toward the 5' end of the polynucleotide and downstream is toward the 3' end. In some embodiments, in a DNA polynucleotide, upstream is toward the 5' end of the coding strand for the gene in question and downstream is toward the 3' end.

[0255] As used herein, a "**vaccine**" refers to a composition for generating immunity for the prophylaxis and/or treatment of diseases. Accordingly, vaccines are medicaments which comprise antigens and are intended to be used in humans or animals for generating specific defense and protective substances upon administration to the human or animal.

A. LIPID DEFINITIONS

[0256] As used herein, the phrase "**biodegradable lipid**" or "**degradable lipid**" refers to any of a number of lipid species that are broken down in a host environment on the order of minutes,

hours, or days ideally making them less toxic and unlikely to accumulate in a host over time. Common modifications to lipids include ester bonds, and disulfide bonds among others to increase the biodegradability of a lipid.

[0257] As used herein, the phrase "**biodegradable PEG lipid**" or "**degradable PEG lipid**" refers to any of a number of lipid species where the PEG molecules are cleaved from the lipid in a host environment on the order of minutes, hours, or days ideally making them less immunogenic. Common modifications to PEG lipids include ester bonds, and disulfide bonds among others to increase the biodegradability of a lipid.

[0258] As used herein, the term "**cationic lipid**" or "**ionizable lipid**" refers to any of a number of lipid species that carry a net positive charge at a selected pH, such as physiological pH 4 and a neutral charge at other pHs such as physiological pH 7.

[0259] As used herein, the term "**PEG**" means any polyethylene glycol or other polyalkylene ether polymer.

[0260] As generally defined herein, a "**PEG-OH lipid**" (also referred to herein as "hydroxy-PEGylated lipid") is a PEGylated lipid having one or more hydroxyl (–OH) groups on the lipid.

[0261] As used herein, a "**phospholipid**" is a lipid that includes a phosphate moiety and one or more carbon chains, such as unsaturated fatty acid chains.

[0262] As used herein, the term "**structural lipid**" refers to sterols and also to lipids containing sterol moieties. As defined herein, "**sterols**" are a subgroup of steroids consisting of steroid alcohols.

[0263] The terms "**head-group**" and "**tail-group**," when used herein to describe the compounds (e.g., lipids) of the present invention, and in particular functional groups that are comprised in such compounds, are used for ease of reference to describe the orientation of such compounds or of one or more functional groups relative to other functional groups. For example, in certain embodiments, a hydrophilic head-group (e.g., guanidinium) is bound (e.g., by one or more of hydrogen-bonds, van der Waals' forces, ionic interactions and covalent bonds) to a cleavable functional group (e.g., a disulfide group), which in turn is bound to a hydrophobic tail-group (e.g., cholesterol). In certain embodiments, the compounds disclosed herein comprise, for example, at least one hydrophilic head-group and at least one hydrophobic tail-group, each bound to at least one cleavable group, thereby rendering such compounds amphiphilic.

[0264] As used herein, the term "**amphiphilic**" means the ability to dissolve in both polar (e.g.,

water) and non-polar (e.g., lipid) environments. For example, in certain embodiments, the compounds (e.g., lipids) disclosed herein comprise at least one lipophilic tail-group (e.g., cholesterol or a C_{6-20} alkyl) and at least one hydrophilic head-group (e.g., imidazole), each bound to a cleavable group (e.g., disulfide).

[0265] As used herein, the term "**hydrophilic**" is used to indicate in qualitative terms that a functional group is water-preferring, and typically such groups are water-soluble. For example, disclosed herein are compounds (e.g., ionizable lipids) that comprise a cleavable group (e.g., a disulfide (S—S) group) bound to one or more hydrophilic groups (e.g., a hydrophilic head-group), wherein such hydrophilic groups comprise or are selected from the group consisting of imidazole, guanidinium, amino, imine, enamine, an optionally-substituted alkyl amino (e.g., an alkyl amino such as dimethylamino) and pyridyl.

[0266] As used herein, the term "**hydrophobic**" is used to indicate in qualitative terms that a functional group is water-avoiding, and typically such groups are not water soluble. In certain embodiments, at least one of the functional groups of moieties that comprise the compounds disclosed herein is hydrophobic in nature (e.g., a hydrophobic tail-group comprising a naturally occurring lipid such as cholesterol). For example, disclosed herein are compounds (e.g., ionizable lipids) that comprise a cleavable functional group (e.g., a disulfide (S—S) group) bound to one or more hydrophobic groups, wherein such hydrophobic groups may comprise, or may be selected from, one or more naturally occurring lipids such as cholesterol, an optionally substituted, variably saturated or unsaturated C_6-C_{20} alkyl, and/or an optionally substituted, variably saturated or unsaturated C_6-C_{20} acyl.

[0267] As used herein, the term "**liposome**" generally refers to a vesicle composed of lipids (e.g., amphiphilic lipids) arranged in one or more spherical bilayer or bilayers. Such liposomes may be unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior that contains the encapsulated circRNA to be delivered to one or more target cells, tissues and organs.

[0268] As used herein, the phrase "**lipid nanoparticle**" refers to a transfer vehicle comprising one or more cationic or ionizable lipids, stabilizing lipids, structural lipids, and helper lipids.

[0269] In certain embodiments, the compositions described herein comprise one or more liposomes or lipid nanoparticles. Examples of suitable lipids (e.g., ionizable lipids) that may be used to form the liposomes and lipid nanoparticles contemplated include one or more of the

compounds disclosed herein (e.g., HGT4001, HGT4002, HGT4003, HGT4004 and/or HGT4005). Such liposomes and lipid nanoparticles may also comprise additional ionizable lipids such as C12-200, DLin-KC2-DMA, and/or HGT5001, helper lipids, structural lipids, PEG-modified lipids, MC3, DLinDMA, DLinkC2DMA, cKK-E12, ICE, HGT5000, DODAC, DDAB, DMRIE, DOSPA, DOGS, DODAP, DODMA, DMDMA, DODAC, DLenDMA, DMRIE, CLinDMA, CpLinDMA, DMOBA, DOcarbDAP, DLinDAP, DLincarbDAP, DLinCDAP, KLin-K-DMA, DLin-K-XTC2-DMA, HGT4003, and combinations thereof.

[0270] In some embodiments, a lipid, e.g., an ionizable lipid, disclosed herein comprises one or more cleavable groups. The terms "cleave" and "cleavable" are used herein to mean that one or more chemical bonds (e.g., one or more of covalent bonds, hydrogen-bonds, van der Waals' forces and/or ionic interactions) between atoms in or adjacent to the subject functional group are broken (e.g., hydrolyzed) or are capable of being broken upon exposure to selected conditions (e.g., upon exposure to enzymatic conditions). In certain embodiments, the cleavable group is a disulfide functional group, and in particular embodiments is a disulfide group that is capable of being cleaved upon exposure to selected biological conditions (e.g., intracellular conditions). In certain embodiments, the cleavable group is an ester functional group that is capable of being cleaved upon exposure to selected biological conditions. For example, the disulfide groups may be cleaved enzymatically or by a hydrolysis, oxidation or reduction reaction. Upon cleavage of such disulfide functional group, the one or more functional moieties or groups (e.g., one or more of a head-group and/or a tail-group) that are bound thereto may be liberated. Exemplary cleavable groups may include, but are not limited to, disulfide groups, ester groups, ether groups, and any derivatives thereof (e.g., alkyl and aryl esters). In certain embodiments, the cleavable group is not an ester group or an ether group. In some embodiments, a cleavable group is bound (e.g., bound by one or more of hydrogen-bonds, van der Waals' forces, ionic interactions and covalent bonds) to one or more functional moieties or groups (e.g., at least one head-group and at least one tail-group). In certain embodiments, at least one of the functional moieties or groups is hydrophilic (e.g., a hydrophilic head-group comprising one or more of imidazole, guanidinium, amino, imine, enamine, optionally-substituted alkyl amino and pyridyl).

B. CHEMICAL DEFINITIONS

[0271] When describing the invention, which may include compounds and pharmaceutically

acceptable salts thereof, pharmaceutical compositions containing such compounds and methods of using such compounds and compositions, the following terms, if present, have the following meanings unless otherwise indicated. It should also be understood that when described herein any of the moieties defined forth below may be substituted with a variety of substituents, and that the respective definitions are intended to include such substituted moieties within their scope as set out below. Unless otherwise stated, the term "substituted" is to be defined as set out below. It should be further understood that the terms "groups" and "radicals" can be considered interchangeable when used herein.

[0272] Compound described herein may also comprise one or more isotopic substitutions. For example, H may be in any isotopic form, including ^{1}H, ^{2}H (D or deuterium), and ^{3}H (T or tritium); C may be in any isotopic form, including ^{12}C, ^{13}C, and ^{14}C; O may be in any isotopic form, including ^{16}O and ^{18}O; F may be in any isotopic form, including ^{18}F and ^{19}F; and the like.

[0273] When a range of values is listed, it is intended to encompass each value and sub–range within the range. For example, "C_{1-6} alkyl" is intended to encompass, C_1, C_2, C_3, C_4, C_5, C_6, C_{1-6}, C_{1-5}, C_{1-4}, C_{1-3}, C_{1-2}, C_{2-6}, C_{2-5}, C_{2-4}, C_{2-3}, C_{3-6}, C_{3-5}, C_{3-4}, C_{4-6}, C_{4-5}, and C_{5-6} alkyl.

[0274] As used herein, the term "**alkyl**" refers to both straight and branched chain C_{1-40} hydrocarbons (e.g., C_{6-20} hydrocarbons), and include both saturated and unsaturated hydrocarbons. In certain embodiments, the alkyl may comprise one or more cyclic alkyls and/or one or more heteroatoms such as oxygen, nitrogen, or sulfur and may optionally be substituted with substituents (e.g., one or more of alkyl, halo, alkoxyl, hydroxy, amino, aryl, ether, ester or amide). In certain embodiments, a contemplated alkyl includes (9Z,12Z)-octadeca-9,12-dien. The use of designations such as, for example, "C_{6-20}" is intended to refer to an alkyl (e.g., straight or branched chain and inclusive of alkenes and alkyls) having the recited range carbon atoms. In some embodiments, an alkyl group has 1 to 10 carbon atoms ("C_{1-10} alkyl"). In some embodiments, an alkyl group has 1 to 9 carbon atoms ("C_{1-9} alkyl"). In some embodiments, an alkyl group has 1 to 8 carbon atoms ("C_{1-8} alkyl"). In some embodiments, an alkyl group has 1 to 7 carbon atoms ("C_{1-7} alkyl"). In some embodiments, an alkyl group has 1 to 6 carbon atoms ("C_{1-6} alkyl"). In some embodiments, an alkyl group has 1 to 5 carbon atoms ("C_{1-5} alkyl"). In some embodiments, an alkyl group has 1 to 4 carbon atoms ("C_{1-4} alkyl"). In some embodiments, an alkyl group has 1 to 3 carbon atoms ("C_{1-3} alkyl"). In some embodiments, an alkyl group has 1 to 2 carbon atoms ("C_{1-2} alkyl"). In some embodiments, an alkyl group has 1 carbon atom ("C_1 alkyl"). Examples of C_{1-}

$_6$ alkyl groups include methyl, ethyl, propyl, isopropyl, butyl, isobutyl, pentyl, hexyl, and the like.

[0275] As used herein, "**alkenyl**" refers to a radical of a straight–chain or branched hydrocarbon group having from 2 to 20 carbon atoms, one or more carbon–carbon double bonds (e.g., 1, 2, 3, or 4 carbon–carbon double bonds), and optionally one or more carbon–carbon triple bonds (e.g., 1, 2, 3, or 4 carbon–carbon triple bonds) ("C_{2-20} alkenyl"). In certain embodiments, alkenyl does not contain any triple bonds. In some embodiments, an alkenyl group has 2 to 10 carbon atoms ("C_{2-10} alkenyl"). In some embodiments, an alkenyl group has 2 to 9 carbon atoms ("C_{2-9} alkenyl"). In some embodiments, an alkenyl group has 2 to 8 carbon atoms ("C_{2-8} alkenyl"). In some embodiments, an alkenyl group has 2 to 7 carbon atoms ("C_{2-7} alkenyl"). In some embodiments, an alkenyl group has 2 to 6 carbon atoms ("C_{2-6} alkenyl"). In some embodiments, an alkenyl group has 2 to 5 carbon atoms ("C_{2-5} alkenyl"). In some embodiments, an alkenyl group has 2 to 4 carbon atoms ("C_{2-4} alkenyl"). In some embodiments, an alkenyl group has 2 to 3 carbon atoms ("C_{2-3} alkenyl"). In some embodiments, an alkenyl group has 2 carbon atoms ("C_2 alkenyl"). The one or more carbon–carbon double bonds can be internal (such as in 2–butenyl) or terminal (such as in 1–butenyl). Examples of C_{2-4} alkenyl groups include ethenyl (C_2), 1–propenyl (C_3), 2–propenyl (C_3), 1–butenyl (C_4), 2–butenyl (C_4), butadienyl (C_4), and the like. Examples of C_{2-6} alkenyl groups include the aforementioned C_{2-4} alkenyl groups as well as pentenyl (C_5), pentadienyl (C_5), hexenyl (C_6), and the like. Additional examples of alkenyl include heptenyl (C_7), octenyl (C_8), octatrienyl (C_8), and the like.

[0276] As used herein, "**alkynyl**" refers to a radical of a straight–chain or branched hydrocarbon group having from 2 to 20 carbon atoms, one or more carbon–carbon triple bonds (e.g., 1, 2, 3, or 4 carbon–carbon triple bonds), and optionally one or more carbon–carbon double bonds (e.g., 1, 2, 3, or 4 carbon–carbon double bonds) ("C_{2-20} alkynyl"). In certain embodiments, alkynyl does not contain any double bonds. In some embodiments, an alkynyl group has 2 to 10 carbon atoms ("C_{2-10} alkynyl"). In some embodiments, an alkynyl group has 2 to 9 carbon atoms ("C_{2-9} alkynyl"). In some embodiments, an alkynyl group has 2 to 8 carbon atoms ("C_{2-8} alkynyl"). In some embodiments, an alkynyl group has 2 to 7 carbon atoms ("C_{2-7} alkynyl"). In some embodiments, an alkynyl group has 2 to 6 carbon atoms ("C_{2-6} alkynyl"). In some embodiments, an alkynyl group has 2 to 5 carbon atoms ("C_{2-5} alkynyl"). In some embodiments, an alkynyl group has 2 to 4 carbon atoms ("C_{2-4} alkynyl"). In some embodiments, an alkynyl group has 2 to 3 carbon atoms ("C_{2-3} alkynyl"). In some embodiments, an alkynyl group has 2 carbon atoms ("C_2

alkynyl"). The one or more carbon–carbon triple bonds can be internal (such as in 2–butynyl) or terminal (such as in 1–butynyl). Examples of C_{2-4} alkynyl groups include, without limitation, ethynyl (C_2), 1–propynyl (C_3), 2–propynyl (C_3), 1–butynyl (C_4), 2–butynyl (C_4), and the like. Examples of C_{2-6} alkenyl groups include the aforementioned C_{2-4} alkynyl groups as well as pentynyl (C_5), hexynyl (C_6), and the like. Additional examples of alkynyl include heptynyl (C_7), octynyl (C_8), and the like.

[0277] As used herein, "**alkylene**," "**alkenylene**," and "**alkynylene**," refer to a divalent radical of an alkyl, alkenyl, and alkynyl group respectively. When a range or number of carbons is provided for a particular "alkylene," "alkenylene," or "alkynylene" group, it is understood that the range or number refers to the range or number of carbons in the linear carbon divalent chain. "Alkylene," "alkenylene," and "alkynylene" groups may be substituted or unsubstituted with one or more substituents as described herein.

[0278] The term "**alkoxy**," as used herein, refers to an alkyl group which is attached to another moiety via an oxygen atom (–O(alkyl)). Non-limiting examples include e.g., methoxy, ethoxy, propoxy, and butoxy.

[0279] As used herein, the term "**aryl**" refers to aromatic groups (e.g., monocyclic, bicyclic and tricyclic structures) containing six to ten carbons in the ring portion. The aryl groups may be optionally substituted through available carbon atoms and in certain embodiments may include one or more heteroatoms such as oxygen, nitrogen or sulfur. In some embodiments, an aryl group has six ring carbon atoms ("C_6 aryl"; e.g., phenyl). In some embodiments, an aryl group has ten ring carbon atoms ("C_{10} aryl"; e.g., naphthyl such as 1–naphthyl and 2–naphthyl).

[0280] The term "**cycloalkyl**" refers to a monovalent saturated cyclic, bicyclic, or bridged cyclic (e.g., adamantyl) hydrocarbon group of 3-12, 3-8, 4-8, or 4-6 carbons, referred to herein, e.g., as "C_{4-8} cycloalkyl," derived from a cycloalkane. Exemplary cycloalkyl groups include, but are not limited to, cyclohexanes, cyclopentanes, cyclobutanes and cyclopropanes.

[0281] As used herein, "**cyano**" refers to –CN.

[0282] As used herein, "**heteroaryl**" refers to a radical of a 5–10 membered monocyclic or bicyclic 4n+2 aromatic ring system (e.g., having 6 or 10 electrons shared in a cyclic array) having ring carbon atoms and 1–4 ring heteroatoms provided in the aromatic ring system, wherein each heteroatom is independently selected from nitrogen, oxygen and sulfur ("5–10 membered heteroaryl"). In heteroaryl groups that contain one or more nitrogen atoms, the point of attachment

can be a carbon or nitrogen atom, as valency permits. Heteroaryl bicyclic ring systems can include one or more heteroatoms in one or both rings. "Heteroaryl" includes ring systems wherein the heteroaryl ring, as defined above, is fused with one or more carbocyclyl or heterocyclyl groups wherein the point of attachment is on the heteroaryl ring, and in such instances, the number of ring members continue to designate the number of ring members in the heteroaryl ring system. "Heteroaryl" also includes ring systems wherein the heteroaryl ring, as defined above, is fused with one or more aryl groups wherein the point of attachment is either on the aryl or heteroaryl ring, and in such instances, the number of ring members designates the number of ring members in the fused (aryl/heteroaryl) ring system. Bicyclic heteroaryl groups wherein one ring does not contain a heteroatom (e.g., indolyl, quinolinyl, carbazolyl, and the like) the point of attachment can be on either ring, i.e., either the ring bearing a heteroatom (e.g., 2–indolyl) or the ring that does not contain a heteroatom (e.g., 5–indolyl).

[0283] As used herein, "**heterocyclyl**" or "**heterocyclic**" refers to a radical of a 3– to 10–membered non–aromatic ring system having ring carbon atoms and 1 to 4 ring heteroatoms, wherein each heteroatom is independently selected from nitrogen, oxygen, sulfur, boron, phosphorus, and silicon ("3–10 membered heterocyclyl"). In heterocyclyl groups that contain one or more nitrogen atoms, the point of attachment can be a carbon or nitrogen atom, as valency permits. A heterocyclyl group can either be monocyclic ("monocyclic heterocyclyl") or a fused, bridged or spiro ring system such as a bicyclic system ("bicyclic heterocyclyl"), and can be saturated or can be partially unsaturated. Heterocyclyl bicyclic ring systems can include one or more heteroatoms in one or both rings. "Heterocyclyl" also includes ring systems wherein the heterocyclyl ring, as defined above, is fused with one or more carbocyclyl groups wherein the point of attachment is either on the carbocyclyl or heterocyclyl ring, or ring systems wherein the heterocyclyl ring, as defined above, is fused with one or more aryl or heteroaryl groups, wherein the point of attachment is on the heterocyclyl ring, and in such instances, the number of ring members continue to designate the number of ring members in the heterocyclyl ring system. The terms "heterocycle," "heterocyclyl," "heterocyclyl ring," "heterocyclic group," "heterocyclic moiety," and "heterocyclic radical," may be used interchangeably.

[0284] The terms "**halo**" and "**halogen**" as used herein refer to an atom selected from fluorine (fluoro, F), chlorine (chloro, Cl), bromine (bromo, Br), and iodine (iodo, I). In certain embodiments, the halo group is either fluoro or chloro.

[0285] As used herein, "**oxo**" refers to –C=O.

[0286] In general, the term "**substituted**", whether preceded by the term "optionally" or not, means that at least one hydrogen present on a group (e.g., a carbon or nitrogen atom) is replaced with a permissible substituent, e.g., a substituent which upon substitution results in a stable compound, e.g., a compound which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, or other reaction. Unless otherwise indicated, a "substituted" group has a substituent at one or more substitutable positions of the group, and when more than one position in any given structure is substituted, the substituent is either the same or different at each position.

[0287] As used herein, "**pharmaceutically acceptable salt**" refers to those salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response and the like, and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well known in the art. For example, Berge et al., describes pharmaceutically acceptable salts in detail in J. Pharmaceutical Sciences (1977) 66:1–19. Pharmaceutically acceptable salts of the compounds of this invention include those derived from suitable inorganic and organic acids and bases. Examples of pharmaceutically acceptable, nontoxic acid addition salts are salts of an amino group formed with inorganic acids such as hydrochloric acid, hydrobromic acid, phosphoric acid, sulfuric acid and perchloric acid or with organic acids such as acetic acid, oxalic acid, maleic acid, tartaric acid, citric acid, succinic acid or malonic acid or by using other methods used in the art such as ion exchange. Other pharmaceutically acceptable salts include adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, formate, fumarate, glucoheptonate, glycerophosphate, gluconate, hemisulfate, heptanoate, hexanoate, hydroiodide, 2–hydroxy–ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2–naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3–phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, p–toluenesulfonate, undecanoate, valerate salts, and the like. Pharmaceutically acceptable salts derived from appropriate bases include alkali metal, alkaline earth metal, ammonium and N+(C1–4alkyl)4 salts. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like. Further

pharmaceutically acceptable salts include, when appropriate, nontoxic ammonium, quaternary ammonium, and amine cations formed using counterions such as halide, hydroxide, carboxylate, sulfate, phosphate, nitrate, lower alkyl sulfonate, and aryl sulfonate.

[0288] In typical embodiments, the present invention is intended to encompass the compounds disclosed herein, and the pharmaceutically acceptable salts, pharmaceutically acceptable esters, tautomeric forms, polymorphs, and prodrugs of such compounds. In some embodiments, the present invention includes a pharmaceutically acceptable addition salt, a pharmaceutically acceptable ester, a solvate (e.g., hydrate) of an addition salt, a tautomeric form, a polymorph, an enantiomer, a mixture of enantiomers, a stereoisomer or mixture of stereoisomers (pure or as a racemic or non-racemic mixture) of a compound described herein.

[0289] Compounds described herein can comprise one or more asymmetric centers, and thus can exist in various isomeric forms, e.g., enantiomers and/or diastereomers. For example, the compounds described herein can be in the form of an individual enantiomer, diastereomer or geometric isomer, or can be in the form of a mixture of stereoisomers, including racemic mixtures and mixtures enriched in one or more stereoisomer. Isomers can be isolated from mixtures by methods known to those skilled in the art, including chiral high pressure liquid chromatography (HPLC) and the formation and crystallization of chiral salts; or preferred isomers can be prepared by asymmetric syntheses. See, for example, Jacques et al., Enantiomers, Racemates and Resolutions (Wiley Interscience, New York, 1981); Wilen et al., Tetrahedron 33:2725 (1977); Eliel, Stereochemistry of Carbon Compounds (McGraw–Hill, NY, 1962); and Wilen, Tables of Resolving Agents and Optical Resolutions p. 268 (E.L. Eliel, Ed., Univ. of Notre Dame Press, Notre Dame, IN 1972). The invention additionally encompasses compounds described herein as individual isomers substantially free of other isomers, and alternatively, as mixtures of various isomers.

[0290] In certain embodiments, the compounds (e.g., ionizable lipids) and the transfer vehicles (e.g., lipid nanoparticles) of which such compounds are a component exhibit an enhanced (e.g., increased) ability to transfect one or more target cells. Accordingly, also provided herein are methods of transfecting one or more target cells. Such methods generally comprise the step of contacting the one or more target cells with the compounds and/or pharmaceutical compositions disclosed herein such that the one or more target cells are transfected with the circular RNA encapsulated therein.

[0291] It is to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive. Unless defined herein and below in the reminder of the specification, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains.

2. DNA TEMPLATE, PRECUSOR RNA & CIRCULAR RNA

[0292] According to the present invention, transcription of a DNA template provided herein (*e.g.*, comprising a 3' enhanced intron element, 3' enhanced exon element, a core functional element, a 5' enhanced exon element, and a 5' enhanced intron element) results in formation of a precursor linear RNA polynucleotide capable of circularizing. In some embodiments, this DNA template comprises a vector, PCR product, plasmid, minicircle DNA, cosmid, artificial chromosome, complementary DNA (cDNA), extrachromosomal DNA (ecDNA), or a fragment therein. In certain embodiments, the minicircle DNA may be linearized or non-linearized. In certain embodiments, the plasmid may be linearized or non-linearized. In some embodiments, the DNA template may be single-stranded. In other embodiments, the DNA template may be double-stranded. In some embodiments, the DNA template comprises in whole or in part from a viral, bacterial or eukaryotic vector.

[0293] The present invention, as provided herein, comprises a DNA template that shares the same sequence as the precursor linear RNA polynucleotide prior to splicing of the precursor linear RNA polynucleotide (*e.g.*, a 3' enhanced intron element, a 3' enhanced exon element, a core functional element, and a 5' enhanced exon element, a 5' enhanced intron element). In some embodiments, said linear precursor RNA polynucleotide undergoes splicing leading to the removal of the 3' enhanced intron element and 5' enhanced intron element during the process of circularization. In some embodiments, the resulting circular RNA polynucleotide lacks a 3' enhanced intron fragment and a 5' enhanced intron fragment, but maintains a 3' enhanced exon fragment, a core functional element, and a 5' enhanced exon element.

[0294] In some embodiments, the precursor linear RNA polynucleotide circularizes when incubated in the presence of one or more guanosine nucleotides or nucleoside (*e.g.*, GTP) and a divalent cation (*e.g.*, Mg^{2+}). In some embodiments, the 3' enhanced exon element, 5' enhanced exon element, and/or core functional element in whole or in part promotes the circularization of

the precursor linear RNA polynucleotide to form the circular RNA polynucleotide provided herein.

[0295] In certain embodiments, circular RNA provided herein is produced inside a cell. In some embodiments, precursor RNA is transcribed using a DNA template (*e.g.*, in some embodiments, using a vector provided herein) in the cytoplasm by a bacteriophage RNA polymerase, or in the nucleus by host RNA polymerase II and then circularized.

[0296] In certain embodiments, the circular RNA provided herein is injected into an animal (*e.g.*, a human), such that a polypeptide encoded by the circular RNA molecule is expressed inside the animal.

[0297] In some embodiments, the DNA (*e.g.*, vector), linear RNA (*e.g.*, precursor RNA), and/or circular RNA polynucleotide provided herein is between 300 and 10000, 400 and 9000, 500 and 8000, 600 and 7000, 700 and 6000, 800 and 5000, 900 and 5000, 1000 and 5000, 1100 and 5000, 1200 and 5000, 1300 and 5000, 1400 and 5000, and/or 1500 and 5000 nucleotides in length. In some embodiments, the polynucleotide is at least 300 nt, 400 nt, 500 nt, 600 nt, 700 nt, 800 nt, 900 nt, 1000 nt, 1100 nt, 1200 nt, 1300 nt, 1400 nt, 1500 nt, 2000 nt, 2500 nt, 3000 nt, 3500 nt, 4000 nt, 4500 nt, or 5000 nt in length. In some embodiments, the polynucleotide is no more than 3000 nt, 3500 nt, 4000 nt, 4500 nt, 5000 nt, 6000 nt, 7000 nt, 8000 nt, 9000 nt, or 10000 nt in length. In some embodiments, the length of a DNA, linear RNA, and/or circular RNA polynucleotide provided herein is about 300 nt, 400 nt, 500 nt, 600 nt, 700 nt, 800 nt, 900 nt, 1000 nt, 1100 nt, 1200 nt, 1300 nt, 1400 nt, 1500 nt, 2000 nt, 2500 nt, 3000 nt, 3500 nt, 4000 nt, 4500 nt, 5000 nt, 6000 nt, 7000 nt, 8000 nt, 9000 nt, or 10000 nt.

[0298] In some embodiments, the circular RNA provided herein has higher functional stability than mRNA comprising the same expression sequence. In some embodiments, the circular RNA provided herein has higher functional stability than mRNA comprising the same expression sequence, 5moU modifications, an optimized UTR, a cap, and/or a polyA tail.

[0299] In some embodiments, the circular RNA polynucleotide provided herein has a functional half-life of at least 5 hours, 10 hours, 15 hours, 20 hours. 30 hours, 40 hours, 50 hours, 60 hours, 70 hours or 80 hours. In some embodiments, the circular RNA polynucleotide provided herein has a functional half-life of 5-80, 10-70, 15-60, and/or 20-50 hours. In some embodiments, the circular RNA polynucleotide provided herein has a functional half-life greater than (*e.g.*, at least 1.5-fold greater than, at least 2-fold greater than) that of an equivalent linear RNA polynucleotide encoding the same protein. In some embodiments, functional half-life can be assessed through the detection

of functional protein synthesis.

[0300] In some embodiments, the circular RNA polynucleotide provided herein has a half-life of at least 5 hours, 10 hours, 15 hours, 20 hours. 30 hours, 40 hours, 50 hours, 60 hours, 70 hours or 80 hours. In some embodiments, the circular RNA polynucleotide provided herein has a half-life of 5-80, 10-70, 15-60, and/or 20-50 hours. In some embodiments, the circular RNA polynucleotide provided herein has a half-life greater than (*e.g.*, at least 1.5-fold greater than, at least 2-fold greater than) that of an equivalent linear RNA polynucleotide encoding the same protein. In some embodiments, the circular RNA polynucleotide, or pharmaceutical composition thereof, has a functional half-life in a human cell greater than or equal to that of a pre-determined threshold value. In some embodiments the functional half-life is determined by a functional protein assay. For example in some embodiments, the functional half-life is determined by an in vitro luciferase assay, wherein the activity of Gaussia luciferase (GLuc) is measured in the media of human cells (e.g. HepG2) expressing the circular RNA polynucleotide every 1, 2, 6, 12, or 24 hours over 1, 2, 3, 4, 5, 6, 7, or 14 days. In other embodiments, the functional half-life is determined by an in vivo assay, wherein levels of a protein encoded by the expression sequence of the circular RNA polynucleotide are measured in patient serum or tissue samples every 1, 2, 6, 12, or 24 hours over 1, 2, 3, 4, 5, 6, 7, or 14 days. In some embodiments, the pre-determined threshold value is the functional half-life of a reference linear RNA polynucleotide comprising the same expression sequence as the circular RNA polynucleotide.

[0301] In some embodiments, the circular RNA provided herein may have a higher magnitude of expression than equivalent linear mRNA, *e.g.*, a higher magnitude of expression 24 hours after administration of RNA to cells. In some embodiments, the circular RNA provided herein has a higher magnitude of expression than mRNA comprising the same expression sequence, 5moU modifications, an optimized UTR, a cap, and/or a polyA tail.

[0302] In some embodiments, the circular RNA provided herein may be less immunogenic than an equivalent mRNA when exposed to an immune system of an organism or a certain type of immune cell. In some embodiments, the circular RNA provided herein is associated with modulated production of cytokines when exposed to an immune system of an organism or a certain type of immune cell. For example, in some embodiments, the circular RNA provided herein is associated with reduced production of IFN-β1, RIG-I, IL-2, IL-6, IFNγ, and/or TNFα when exposed to an immune system of an organism or a certain type of immune cell as compared to

mRNA comprising the same expression sequence. In some embodiments, the circular RNA provided herein is associated with less IFN-β1, RIG-I, IL-2, IL-6, IFNγ, and/or TNFα transcript induction when exposed to an immune system of an organism or a certain type of immune cell as compared to mRNA comprising the same expression sequence. In some embodiments, the circular RNA provided herein is less immunogenic than mRNA comprising the same expression sequence. In some embodiments, the circular RNA provided herein is less immunogenic than mRNA comprising the same expression sequence, 5moU modifications, an optimized UTR, a cap, and/or a polyA tail.

[0303] In certain embodiments, the circular RNA provided herein can be transfected into a cell as is, or can be transfected in DNA vector form and transcribed in the cell. Transcription of circular RNA from a transfected DNA vector can be via added polymerases or polymerases encoded by nucleic acids transfected into the cell, or preferably via endogenous polymerases.

A. ENHANCED INTRON ELEMENTS & ENHANCED EXON ELEMENTS

[0304] Polynucleotides provided herein may comprise one or more enhance intron elements and/or one or more enhanced exon elements. In some embodiments, the enhanced intron elements and enhanced exon elements may comprise spacers, duplex regions, affinity sequences, intron fragments, exon fragments, and/or various untranslated elements. These sequences within the enhanced intron elements or enhanced exon elements are arranged to optimize circularization or protein expression.

a. SPACER

[0305] In some embodiments, a provided polynucleotide (e.g., a DNA template, a precursor RNA polynucleotide, or a circular RNA polynucleotide) comprises one or more spacers. In certain embodiments, the polynucleotide comprises a first (5') and/or a second (3') spacer. In some embodiments, the polynucleotide (e.g., DNA template or precursor linear RNA polynucleotide) comprises one or more spacers in the enhanced intron elements. In some embodiments, the polynucleotide (e.g., DNA template, precursor linear RNA polynucleotide, or a circular RNA polynucleotide) comprises one or more spacers in the enhanced exon elements. In certain embodiments, the polynucleotide comprises a spacer in the 3' enhanced intron fragment and a spacer in the 5' enhanced intron fragment. In certain embodiments, the polynucleotide comprises a spacer in the 3' enhanced exon fragment and another spacer in the 5' enhanced exon fragment to

aid with circularization or protein expression due to symmetry created in the overall sequence.

[0306] In some embodiments, including a spacer between the 3' group I intron fragment and the core functional element may conserve secondary structures in those regions by preventing them from interacting, thus increasing splicing efficiency. In some embodiments, the first (between 3' group I intron fragment and core functional element) and second (between the two expression sequences and core functional element) spacers comprise additional base pairing regions that are predicted to base pair with each other and not to the first and second duplex regions. In other embodiments, the first (between 3' group I intron fragment and core functional element) and second (between the one of the core functional element and 5' group I intron fragment) spacers comprise additional base pairing regions that are predicted to base pair with each other and not to the first and second duplex regions. In some embodiments, such spacer base pairing brings the group I intron fragments in close proximity to each other, further increasing splicing efficiency. Additionally, in some embodiments, the combination of base pairing between the first and second duplex regions, and separately, base pairing between the first and second spacers, promotes the formation of a splicing bubble containing the group I intron fragments flanked by adjacent regions of base pairing. Typical spacers are contiguous sequences with one or more of the following qualities: 1) predicted to avoid interfering with proximal structures, for example, the IRES, expression sequence, aptamer, or intron; 2) is at least 7 nt long and no longer than 100 nt; 3) is located after and adjacent to the 3' intron fragment and/or before and adjacent to the 5' intron fragment; and 4) contains one or more of the following: a) an unstructured region at least 5 nt long, b) a region of base pairing at least 5 nt long to a distal sequence, including another spacer, and c) a structured region at least 7 nt long limited in scope to the sequence of the spacer. Spacers may have several regions, including an unstructured region, a base pairing region, a hairpin/structured region, and combinations thereof. In an embodiment, the spacer has a structured region with high GC content. In an embodiment, a region within a spacer base pairs with another region within the same spacer. In an embodiment, a region within a spacer base pairs with a region within another spacer. In an embodiment, a spacer comprises one or more hairpin structures. In an embodiment, a spacer comprises one or more hairpin structures with a stem of 4 to 12 nucleotides and a loop of 2 to 10 nucleotides. In an embodiment, there is an additional spacer between the 3' group I intron fragment and the core functional element. In an embodiment, this additional spacer prevents the structured regions of the IRES or aptamer of a TIE from interfering with the folding of the 3' group

I intron fragment or reduces the extent to which this occurs. In some embodiments, the 5' spacer sequence is at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25 or 30 nucleotides in length. In some embodiments, the 5' spacer sequence is no more than 100, 90, 80, 70, 60, 50, 45, 40, 35 or 30 nucleotides in length. In some embodiments the 5' spacer sequence is between 5 and 50, 10 and 50, 20 and 50, 20 and 40, and/or 25 and 35 nucleotides in length. In certain embodiments, the 5' spacer sequence is 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides in length. In one embodiment, the 5' spacer sequence is a polyA sequence. In another embodiment, the 5' spacer sequence is a polyAC sequence. In one embodiment, a spacer comprises about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% polyAC content. In one embodiment, a spacer comprises about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% polypyrimidine (C/T or C/U) content.

b. DUPLEX REGION

[0307] In some embodiments, a provided polynucleotide (e.g., a DNA template, a precursor linear RNA polynucleotide, or a circular RNA polynucleotide provided herein comprise one or more duplex regions. In some embodiments, the polynucleotide comprises a first (5') duplex region and a second (3') duplex region. In certain embodiments, the polynucleotide comprises a 5' external duplex region located within the 3' enhanced intron fragment and a 3' external duplex region located within the 5' enhanced intron fragment. In some embodiments, the polynucleotide comprise a 5' internal duplex region located within the 3' enhanced exon fragment and a 3' internal duplex region located within the 5' enhanced exon fragment. In some embodiments, the polynucleotide comprises a 5' external duplex region, 5' internal duplex region, a 3' internal duplex region, and a 3' external duplex region.

[0308] In certain embodiments, the first and second duplex regions may form perfect or imperfect duplexes. Thus, in certain embodiments at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the first and second duplex regions may be base paired with one another. In some embodiments, the duplex regions are predicted to have less than 50% (*e.g.*, less than 45%, less than 40%, less than 35%, less than 30%, less than 25%) base pairing with unintended sequences in the RNA (*e.g.*, non-duplex region sequences). In some embodiments, including such duplex regions on the ends of the precursor RNA strand, and adjacent or very close to the group I intron fragment, bring the group I intron fragments in close proximity to each other,

increasing splicing efficiency. In some embodiments, the duplex regions are 3 to 100 nucleotides in length (*e.g.*, 3-75 nucleotides in length, 3-50 nucleotides in length, 20-50 nucleotides in length, 35-50 nucleotides in length, 5-25 nucleotides in length, 9-19 nucleotides in length). In some embodiments, the duplex regions are about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides in length. In some embodiments, the duplex regions have a length of about 9 to about 50 nucleotides. In one embodiment, the duplex regions have a length of about 9 to about 19 nucleotides. In some embodiments, the duplex regions have a length of about 20 to about 40 nucleotides. In certain embodiments, the duplex regions have a length of about 30 nucleotides.

[0309] In other embodiments, the polynucleotide does not comprise of any duplex regions to optimize translation or circularization.

c. AFFINITY SEQUENCE

[0310] As provided herein, a provided polynucleotide (e.g., a DNA template, a precursor linear RNA polynucleotide, or a circular RNA polynucleotide) may comprise an affinity sequence (or affinity tag). In some embodiments, the affinity tag is located in the 3' enhanced intron element. In some embodiments, the affinity tag is located in the 5' enhanced intron element. In some embodiments, both (3' and 5') enhanced intron elements each comprise an affinity tag. In one embodiment, an affinity tag of the 3' enhanced intron element is the length as an affinity tag in the 5' enhanced intron element. In some embodiments, an affinity tag of the 3' enhanced intron element is the same sequence as an affinity tag in the 5' enhanced intron element. In some embodiments, the affinity sequence is placed to optimize oligo-dT purification.

[0311] In some embodiments, the one or more affinity tags present in a precursor linear RNA polynucleotide are removed upon circularization. See, for example, **FIG. 97A** and **FIG. 97B**. In some embodiments, affinity tags are added to remaining linear RNA after circularization of RNA is performed. In some such embodiments, the affinity tags are added enzymatically to linear RNA. The presence of one or more affinity tags in linear RNA and their absence from circular RNA can facilitate purification of circular RNA. In some embodiments, such purification is performed using a negative selection or affinity-purification method. In some embodiments, such purification is performed using a binding agent that preferentially or specifically binds to the affinity tag.

[0312] In some embodiments, an affinity tag comprises a polyA sequence. In some embodiments

the polyA sequence is at least 15, 30, or 60 nucleotides long. In some embodiments, the affinity tag comprising a polyA sequence is present in two places in a precursor linear RNA. In some embodiments, one or both polyA sequences are 15-50 nucleotides long. In some embodiments, one or both polyA sequences are 20-25 nucleotides long. In some embodiments, the polyA sequence(s) is removed upon circularization. Thus, an oligonucleotide hybridizing with the polyA sequence, such as a deoxythymidine oligonucleotide (oligo(dT)) conjugated to a solid surface (*e.g.*, a resin), can be used to separate circular RNA from its precursor RNA.

[0313] In some embodiments, an affinity tag comprises a sequence that is absent from the circular RNA product. In some such embodiments, the sequence that is absent from the circular RNA product is a dedicated binding site (DBS). In some embodiments, the DBS is an unstructured sequence, i.e., a sequence that does not form a defined structural element, such as a hairpin loop, contiguous dsRNA region, or triple helix. In some embodiments, the DBS sequence forms a random coil. In some embodiments, the DBS comprises at least 25% GC content, at least 50% GC content, at least 75% GC content, or at least 100% GC content. In some embodiments, the DBS comprises at least 25% AC content, at least 50% AC content, at least 75% AC content, or 100% AC content. In some embodiments, the DBS is at least 15, 30, or 60 nucleotides long. In some embodiments, the affinity tag comprising a DBS is present in two places in a precursor linear RNA. In some embodiments, the DBS sequences are each independently 15-50 nucleotides long. In some embodiments, the DBS sequences are each independently 20-25 nucleotides long.

[0314] In some embodiments, the DBS sequence(s) is removed upon circularization. Thus, binding agents comprising oligonucleotides comprising a sequence that is complementary to the DBS can be used to facilitate purification of circular RNA. For example, the binding agent may comprise an oligonucleotide complementary to a DBS conjugated to a solid surface (*e.g.*, a resin).

[0315] In some embodiments, an affinity sequence or other type of affinity handle, such as biotin, is added to linear RNA by ligation. In some embodiments, an oligonucleotide comprising an affinity sequence is ligated to the linear RNA. In some embodiments, an oligonucleotide conjugated to an affinity handle is ligated to the linear RNA. In some embodiments, a solution comprising the linear RNA ligated to the affinity sequence or handle and the circular RNA that does not comprise an affinity sequence or handle are contacted with a binding agent comprising a solid support conjugated to an oligonucleotide complementary to the affinity sequence or to a binding partner of the affinity handle, such that the linear RNA binds to the binding agent, and the

circular RNA is eluted or separated from the solid support.

[0316] Any purification method for circular RNA described herein may comprise one or more buffer exchange steps. In some embodiments, buffer exchange is performed after in vitro transcription (IVT) and before additional purification steps. In some such embodiments, the IVT reaction solution is buffer exchanged into a buffer comprising Tris. In some embodiments, the IVT reaction solution is buffer exchanged into a buffer comprising greater than 1 mM or greater than 10 mM one or more monovalent salts, such as NaCl or KCl, and optionally comprising EDTA. In some embodiments, buffer exchange is performed after purification of circular RNA is complete. In some embodiments, buffer exchange is performed after IVT and after purification of circular RNA. In some embodiments, the buffer exchange that is performed after purification of circular RNA comprises exchange of the circular RNA into water or storage buffer. In some embodiments, the storage buffer comprises 1mM sodium citrate, pH 6.5.

[0317] In certain embodiments, the 3' enhanced intron element comprises a leading untranslated sequence. In some embodiments, the leading untranslated sequence is a the 5' end of the 3' enhanced intron fragment. In some embodiments, the leading untranslated sequence comprises of the last nucleotide of a transcription start site (TSS). In some embodiments, the TSS is chosen from a viral, bacterial, or eukaryotic DNA template. In one embodiment, the leading untranslated sequence comprise the last nucleotide of a TSS and 0 to 100 additional nucleotides. In some embodiments, the TSS is a terminal spacer. In one embodiment, the leading untranslated sequence contains a guanosine at the 5' end upon translation of an RNA T7 polymerase.

[0318] In certain embodiments, the 5' enhanced intron element comprises a trailing untranslated sequence. In some embodiments, the 5' trailing untranslated sequence is located at the 3' end of the 5' enhanced intron element. In some embodiments, the trailing untranslated sequence is a partial restriction digest sequence. In one embodiment, the trailing untranslated sequence is in whole or in part a restriction digest site used to linearize the DNA template. In some embodiments, the restriction digest site is in whole or in part from a natural viral, bacterial or eukaryotic DNA template. In some embodiments, the trailing untranslated sequence is a terminal restriction site fragment.

d. ENHANCED INTRON FRAGMENTS

[0319] In some embodiments, the 3' enhanced intron element and 5' enhanced intron element each comprise an intron fragment. In certain embodiments, a 3' intron fragment is a contiguous

sequence at least 75% homologous (*e.g.*, at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% homologous) to a 3' proximal fragment of a natural group I intron including the 3' splice site dinucleotide. Typically, a 5' intron fragment is a contiguous sequence at least 75% homologous (*e.g.*, at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% homologous) to a 5' proximal fragment of a natural group I intron including the 5' splice site dinucleotide. In some embodiments, the 3' intron fragment includes the first nucleotide of a 3' group I splice site dinucleotide. In some embodiments, the 5' intron fragment includes the first nucleotide of a 5' group I splice site dinucleotide. In other embodiments, the 3' intron fragment includes the first and second nucleotides of a 3' group I intron fragment splice site dinucleotide; and the 5' intron fragment includes the first and second nucleotides of a 3' group I intron fragment dinucleotide.

e. ENHANCED EXON FRAGMENTS

[0320] In certain embodiments, a provided polynucleotide (e.g., a DNA template, a linear precursor RNA polynucleotide, or a circular RNA polynucleotide) comprises an enhanced exon fragment. In some embodiments, following a 5' to 3' order, the 3' enhanced exon element is located upstream to core functional element. In some embodiments, following a 5' to 3' order, the 5' enhanced intron element is located downstream to the core functional element.

[0321] According to the present invention, the 3' enhanced exon element and 5' enhanced exon element each comprise an exon fragment. In some embodiments, the 3' enhanced exon element comprises a 3' exon fragment. In some embodiments, the 5' enhanced exon element comprises a 5' exon fragment. In certain embodiments, as provided herein, the 3' exon fragment and 5' exon fragment each comprises a group I intron fragment and 1 to 100 nucleotides of an exon sequence. In certain embodiments, a 3' intron fragment is a contiguous sequence at least 75% homologous (*e.g.*, at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% homologous) to a 3' proximal fragment of a natural group I intron including the 3' splice site dinucleotide. Typically, a 5' group I intron fragment is a contiguous sequence at least 75% homologous (*e.g.*, at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% homologous) to a 5' proximal fragment of a natural group I intron including the 5' splice site dinucleotide. In some embodiments, the 3' exon fragment comprises a second nucleotide of a 3' group I intron splice site dinucleotide and 1 to 100 nucleotides of an exon sequence. In some embodiments, the 5' exon fragment comprises the first nucleotide of a 5' group I intron splice site

dinucleotide and 1 to 100 nucleotides of an exon sequence. In some embodiments, the exon sequence comprises in part or in whole from a naturally occurring exon sequence from a virus, bacterium or eukaryotic DNA vector. In other embodiments, the exon sequence further comprises a synthetic, genetically modified (*e.g.*, containing modified nucleotide), or other engineered exon sequence.

[0322] In one embodiment, where the 3' intron fragment comprises both nucleotides of a 3' group I splice site dinucleotide and the 5' intron fragment comprises both nucleotides of a 5' group I splice site dinucleotide, the exon fragments located within the 5' enhanced exon element and 3' enhanced exon element does not comprise of a group I splice site dinucleotide.

f. EXAMPLAR PERMUTATION OF THE ENHANCED INTRON ELEMENTS & ENHANCED EXON ELEMENTS

[0323] For means of example and not intended to be limiting, in some embodiment, a 3' enhanced intron element comprises in the following 5' to 3' order: a leading untranslated sequence, a 5' affinity tag, an optional 5' external duplex region, a 5' external spacer, and a 3' intron fragment. In same embodiments, the 3' enhanced exon element comprises in the following 5' to 3' order: a 3' exon fragment, an optional 5' internal duplex region, an optional 5' internal duplex region, and a 5' internal spacer. In the same embodiments, the 5' enhanced exon element comprises in the following 5' to 3' order: a 3' internal spacer, an optional 3' internal duplex region, and a 5' exon fragment. In still the same embodiments, the 3' enhanced intron element comprises in the following 5' to 3' order: a 5' intron fragment, a 3' external spacer, an optional 3' external duplex region, a 3' affinity tag, and a trailing untranslated sequence.

B. CORE FUNCTIONAL ELEMENT

[0324] In some embodiments, a provided polynucleotide (e.g., a DNA template, a linear precursor RNA polynucleotide, or a circular RNA polynucleotide) comprises a core functional element. In some embodiments, the core functional element comprises a coding or noncoding element. In certain embodiments, the core functional element may contain both a coding and noncoding element. In some embodiments, the core functional element further comprises translation initiation element (TIE) upstream to the coding or noncoding element. In some embodiments, the core functional element comprises a termination element. In some embodiments, the termination element is located downstream to the TIE and coding element. In

some embodiments, the termination element is located downstream to the coding element but upstream to the TIE. In certain embodiments, where the coding element comprises a noncoding region, a core functional element lacks a TIE and/or a termination element.

a. CODING OR NONCODING ELEMENT

[0325] In some embodiments, the polynucleotides provided herein comprise coding or noncoding element or a combination of both. In some embodiments, the coding element comprises an expression sequence. In some embodiments, the coding element encodes at least one therapeutic protein.

[0326] In some embodiments, a provided circular RNA encodes two or more polypeptides. In some embodiments, the circular RNA is a bicistronic RNA. The sequences encoding the two or more polypeptides can be separated by a ribosomal skipping element or a nucleotide sequence encoding a protease cleavage site. In certain embodiments, the ribosomai skipping element encodes thosea-asigna virus 2A peptide (T2A), porcine teschovirus-1 2 A peptide (P2A), foot-and-mouth disease virus 2 A peptide (F2A), equine rhinitis A vims 2A peptide (E2A), cytoplasmic polyhedrosis vims 2A peptide (BmCPV 2A), or flacherie vims of B. mori 2A peptide (BmIFV 2A).

b. TRANSLATION INITIATION ELEMENT (TIE)

[0327] As provided herein in some embodiments, the core functional element comprises at least one translation initiation element (TIE). TIEs are designed to allow translation efficiency of an encoded protein. Thus, optimal core functional elements comprising only of noncoding elements lack any TIEs. In some embodiments, core functional elements comprising one or more coding element will further comprise one or more TIEs.

[0328] In some embodiments, a TIE comprises an untranslated region (UTR). In certain embodiments, the TIE provided herein comprise an internal ribosome entry site (IRES). Inclusion of an IRES permits the translation of one or more open reading frames from a circular RNA (*e.g.*, open reading frames that form the expression sequences). The IRES element attracts a eukaryotic ribosomal translation initiation complex and promotes translation initiation. See, *e.g.*, Kaufman *et al.*, Nuc. Acids Res. (1991) 19:4485-4490; Gurtu *et al.*, Biochem. Biophys. Res. Comm. (1996) 229:295-298; Rees *et al.*, BioTechniques (1996) 20: 102-110; Kobayashi *et al.*, BioTechniques (1996) 21 :399-402; and Mosser *et al.*, BioTechniques 1997 22 150-161.

i. NATURAL TIES: VIRAL, & EUKARYOTIC/CELLULAR INTERNAL RIBOSOME ENTRY SITE (IRES)

[0329] A multitude of IRES sequences are available and include sequences derived from a wide variety of viruses, such as from leader sequences of picornaviruses such as the encephalomyocarditis virus (EMCV) UTR (Jang *et al.*, J. Virol. (1989) 63: 1651-1660), the polio leader sequence, the hepatitis A virus leader, the hepatitis C virus IRES, human rhinovirus type 2 IRES (Dobrikova *et al.*, Proc. Natl. Acad. Sci. (2003) 100(25): 15125- 15130), an IRES element from the foot and mouth disease virus (Ramesh *et al.*, Nucl. Acid Res. (1996) 24:2697-2700), a giardiavirus IRES (Garlapati *et al.*, J. Biol. Chem. (2004) 279(5):3389-3397), and the like.

[0330] Different IRES sequences have varying ability to drive protein expression, and the ability of any particular identified or predicted IRES sequence to drive protein expression from linear mRNA or circular RNA constructs is unknown and unpredictable. In certain embodiments, potential IRES sequences can be bioinformatically identified based on sequence positions in viral sequences. However, the activity of such sequences has been previously uncharacterized. As demonstrated herein, such IRES sequences may have differing protein expression capability depending on cell type, for example in T cells, liver cells, or muscle cells. In some embodiments, the novel IRES sequences described herein may have at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or 100 fold increased expression in a particular cell type compared to previously described EMCV IRES sequences.

[0331] In some embodiments, for driving protein expression, a provided circular RNA comprises an IRES operably linked to a protein coding sequence. In some embodiments, the IRES comprises a sequence selected from SEQ ID NOs: 1-2983 and 3282-3287 or a fragment thereof. In some embodiments, the the IRES comprises a sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence selected from SEQ ID NOs: 1-2983 and 3282-3287. In some embodiments, the circular RNA disclosed herein comprises an IRES sequence at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a sequence selected from SEQ ID NOs: 1-2983 and 3282-3287. In some embodiments, the circular RNA disclosed herein comprises an IRES sequence selected from SEQ ID NOs: 1-2983 and 3282-3287 or a fragment thereof. Modifications of IRES and accessory sequences are disclosed herein to increase or reduce IRES activities, for example, by truncating the 5' and/or 3' ends of the IRES, adding a spacer 5' to the IRES, modifying the 6 nucleotides 5' to the translation initiation site (Kozak sequence), modification of alternative translation initiation sites, and creating

chimeric/hybrid IRES sequences. In some embodiments, the IRES sequence in the circular RNA disclosed herein comprises one or more of these modifications relative to a native IRES (*e.g.*, SEQ ID NOs: 1-2983 and 3282-3287).

[0332] In some embodiments, the IRES is an Aalivirus, Ailurivirus, Ampivirus, Anativirus, Aphthovirus, Aquamavirus, Avihepatovirus, Avisivirus, Boosepivirus, Bopivirus, Caecilivirus, Cardiovirus, Cosavirus, Crahelivirus, Crohivirus, Danipivirus, Dicipivirus, Diresapivirus, Enterovirus, Erbovirus, Felipivirus, Fipivirus, Gallivirus, Gruhelivirus, Grusopivirus, Harkavirus, Hemipivirus, Hepatovirus, Hunnivirus, Kobuvirus, Kunsagivirus, Limnipivirus, Livupivirus, Ludopivirus, Malagasivirus, Marsupivirus, Megrivirus, Mischivirus, Mosavirus, Mupivirus, Myrropivirus, Orivirus, Oscivirus, Parabovirus, Parechovirus, Pasivirus, Passerivirus, Pemapivirus, Poecivirus, Potamipivirus, Pygoscepivirus, Rabovirus, Rafivirus, Rajidapivirus, Rohelivirus, Rosavirus, Sakobuvirus, Salivirus, Sapelovirus, Senecavirus, Shanbavirus, Sicinivirus, Symapivirus, Teschovirus, Torchivirus, Tottorivirus, Tremovirus, Tropivirus, Hepacivirus, Pegivirus, Pestivirus, Flavivirus IRES.

[0333] In some embodiments, the IRES is an IRES sequence of Taura syndrome virus, Triatoma virus, Theiler's encephalomyelitis virus, Simian Virus 40, Solenopsis invicta virus 1, Rhopalosiphum padi virus, Reticuloendotheliosis virus, Human poliovirus 1, Plautia stali intestine virus, Kashmir bee virus, Human rhinovirus 2, Homalodisca coagulata virus- 1, Human Immunodeficiency Virus type 1, , Himetobi P virus, Hepatitis C virus, Hepatitis A virus, Hepatitis GB virus , Foot and mouth disease virus, Human enterovirus 71, Equine rhinitis virus, Ectropis obliqua picorna-like virus, Encephalomyocarditis virus, Drosophila C Virus, Human coxsackievirus B3, Crucifer tobamovirus, Cricket paralysis virus, Bovine viral diarrhea virus 1, Black Queen Cell Virus, Aphid lethal paralysis virus, Avian encephalomyelitis virus, Acute bee paralysis virus, Hibiscus chlorotic ringspot virus, Classical swine fever virus, Human FGF2, Human SFTPA1, Human AML1/RUNX1, Drosophila antennapedia, Human AQP4, Human AT1R, Human BAG-1, Human BCL2, Human BiP, Human c-IAP1, Human c-myc, Human eIF4G, Mouse NDST4L, Human LEF1, Mouse HIF1 alpha, Human n.myc, Mouse Gtx, Human p27kipl, Human PDGF2/c-sis, Human p53, Human Pim-1, Mouse Rbm3, Drosophila reaper, Canine Scamper, Drosophila Ubx, Human UNR, Mouse UtrA, Human VEGF-A, Human XIAP, Drosophila hairless, S. cerevisiae TFIID, S. cerevisiae YAP1, tobacco etch virus, turnip crinkle virus, EMCV-A, EMCV-B, EMCV-Bf, EMCV-Cf, EMCV pEC9, Picobirnavirus, HCV QC64,

Human Cosavirus E/D, Human Cosavirus F, Human Cosavirus JMY, Rhinovirus NAT001, HRV14, HRV89, HRVC-02, HRV-A21, Salivirus A SH1, Salivirus FHB, Salivirus NG-J1, Human Parechovirus 1, Crohivirus B, Yc-3, Rosavirus M-7, Shanbavirus A, Pasivirus A, Pasivirus A 2, Echovirus E14, Human Parechovirus 5, Aichi Virus, Hepatitis A Virus HA16, Phopivirus, CVA10, Enterovirus C, Enterovirus D, Enterovirus J, Human Pegivirus 2, GBV-C GT110, GBV-C K1737, GBV-C Iowa, Pegivirus A 1220, Pasivirus A 3, Sapelovirus, Rosavirus B, Bakunsa Virus, Tremovirus A, Swine Pasivirus 1, PLV-CHN, Pasivirus A, Sicinivirus, Hepacivirus K, Hepacivirus A, BVDV1, Border Disease Virus, BVDV2, CSFV-PK15C, SF573 Dicistrovirus, Hubei Picorna-like Virus, CRPV, Salivirus A BN5, Salivirus A BN2, Salivirus A 02394, Salivirus A GUT, Salivirus A CH, Salivirus A SZ1, Salivirus FHB, CVB3, CVB1, Echovirus 7, CVB5, EVA71, CVA3, CVA12, EV24 or an aptamer to eIF4G.

[0334] In some embodiments, the IRES comprises in whole or in part from a eukaryotic or cellular IRES. In certain embodiments, the IRES is from a human gene, where the human gene is ABCF1, ABCG1, ACAD10, ACOT7, ACSS3, ACTG2, ADCYAP1, ADK, AGTR1, AHCYL2, AHI1, AKAP8L, AKR1A1, ALDH3A1, ALDOA, ALG13, AMMECR1L, ANGPTL4, ANK3, AOC3, AP4B1, AP4E1, APAF1, APBB1, APC, APH1A, APOBEC3D, APOM, APP, AQP4, ARHGAP36, ARL13B, ARMC8, ARMCX6, ARPC1A, ARPC2, ARRDC3, ASAP1, ASB3, ASB5, ASCL1, ASMTL, ATF2, ATF3, ATG4A, ATP5B, ATP6V0A1, ATXN3, AURKA, AURKA, AURKA, AURKA, B3GALNT1, B3GNTL1, B4GALT3, BAAT, BAG1, BAIAP2, BAIAP2L2, BAZ2A, BBX, BCAR1, BCL2, BCS1L, BET1, BID, BIRC2, BPGM, BPIFA2, BRINP2, BSG, BTN3A2, C12orf43, C14orf93, C17orf62, C1orf226, C21orf62, C2orf15, C4BPB, C4orf22, C9orf84, CACNA1A, CALCOCO2, CAPN11, CASP12, CASP8AP2, CAV1, CBX5, CCDC120, CCDC17, CCDC186, CCDC51, CCN1, CCND1, CCNT1, CD2BP2, CD9, CDC25C, CDC42, CDC7, CDCA7L, CDIP1, CDK1, CDK11A, CDKN1B, CEACAM7, CEP295NL, CFLAR, CHCHD7, CHIA, CHIC1, CHMP2A, CHRNA2, CLCN3, CLEC12A, CLEC7A, CLECL1, CLRN1, CMSS1, CNIH1, CNR1, CNTN5, COG4, COMMD1, COMMD5, CPEB1, CPS1, CRACR2B, CRBN, CREM, CRYBG1, CSDE1, CSF2RA, CSNK2A1, CSTF3, CTCFL, CTH, CTNNA3, CTNNB1, CTNNB1, CTNND1, CTSL, CUTA, CXCR5, CYB5R3, CYP24A1, CYP3A5, DAG1, DAP3, DAP5, DAXX, DCAF4, DCAF7, DCLRE1A, DCP1A, DCTN1, DCTN2, DDX19B, DDX46, DEFB123, DGKA, DGKD, DHRS4, DHX15, DIO3, DLG1, DLL4, DMD UTR, DMD ex5, DMKN, DNAH6, DNAL4, DUSP13, DUSP19, DYNC1I2, DYNLRB2,

DYRK1A, ECI2, ECT2, EIF1AD, EIF2B4, EIF4G1, EIF4G2, EIF4G3, ELANE, ELOVL6, ELP5, EMCN, ENO1, EPB41, ERMN, ERVV-1, ESRRG, ETFB, ETFBKMT, ETV1, ETV4, EXD1, EXT1, EZH2, FAM111B, FAM157A, FAM213A, FBXO25, FBXO9, FBXW7, FCMR, FGF1, FGF1, FGF1A, FGF2, FGF2, FGF-9, FHL5, FMR1, FN1, FOXP1, FTH1, FUBP1, G3BP1, GABBR1, GALC, GART, GAS7, gastrin, GATA1, GATA4, GFM2, GHR, GJB2, GLI1, GLRA2, GMNN, GPAT3, GPATCH3, GPR137, GPR34, GPR55, GPR89A, GPRASP1, GRAP2, GSDMB, GSTO2, GTF2B, GTF2H4, GUCY1B2, HAX1, HCST, HIGD1A, HIGD1B, HIPK1, HIST1H1C, HIST1H3H, HK1, HLA-DRB4, HMBS, HMGA1, HNRNPC, HOPX, HOXA2, HOXA3, HPCAL1, HR, HSP90AB1, HSPA1A, HSPA4L, HSPA5, HYPK, IFFO1, IFT74, IFT81, IGF1, IGF1R, IGF1R, IGF2, IL11, IL17RE, IL1RL1, IL1RN, IL32, IL6, ILF2, ILVBL, INSR, INTS13, IP6K1, ITGA4, ITGAE, KCNE4, KERA, KIAA0355, KIAA0895L, KIAA1324, KIAA1522, KIAA1683, KIF2C, KIZ, KLHL31, KLK7, KRR1, KRT14, KRT17, KRT33A, KRT6A, KRTAP10-2, KRTAP13-3, KRTAP13-4, KRTAP5-11, KRTCAP2, LACRT, LAMB1, LAMB3, LANCL1, LBX2, LCAT, LDHA, LDHAL6A, LEF1, LINC-PINT, LMO3, LRRC4C, LRRC7, LRTOMT, LSM5, LTB4R, LYRM1, LYRM2, MAGEA11, MAGEA8, MAGEB1, MAGEB16, MAGEB3, MAPT, MARS, MC1R, MCCC1, METTL12, METTL7A, MGC16025, MGC16025, MIA2, MIA2, MITF, MKLN1, MNT, MORF4L2, MPD6, MRFAP1, MRPL21, MRPS12, MSI2, MSLN, MSN, MT2A, MTFR1L, MTMR2, MTRR, MTUS1, MYB, MYC, MYCL, MYCN, MYL10, MYL3, MYLK, MYO1A, MYT2, MZB1, NAP1L1, NAV1, NBAS, NCF2, NDRG1, NDST2, NDUFA7, NDUFB11, NDUFC1, NDUFS1, NEDD4L, NFAT5, NFE2L2, NFE2L2, NFIA, NHEJ1, NHP2, NIT1, NKRF, NME1-NME2, NPAT, NR3C1, NRBF2, NRF1, NTRK2, NUDCD1, NXF2, NXT2, ODC1, ODF2, OPTN, OR10R2, OR11L1, OR2M2, OR2M3, OR2M5, OR2T10, OR4C15, OR4F17, OR4F5, OR5H1, OR5K1, OR6C3, OR6C75, OR6N1, OR7G2, p53, P2RY4, PAN2, PAQR6, PARP4, PARP9, PC, PCBP4, PCDHGC3, PCLAF, PDGFB, PDZRN4, PELO, PEMT, PEX2, PFKM, PGBD4, PGLYRP3, PHLDA2, PHTF1, PI4KB, PIGC, PIM1, PKD2L1, PKM, PLCB4, PLD3, PLEKHA1, PLEKHB1, PLS3, PML, PNMA5, PNN, POC1A, POC1B, POLD2, POLD4, POU5F1, PPIG, PQBP1, PRAME, PRPF4, PRR11, PRRT1, PRSS8, PSMA2, PSMA3, PSMA4, PSMD11, PSMD4, PSMD6, PSME3, PSMG3, PTBP3, PTCH1, PTHLH, PTPRD, PUS7L, PVRIG, QPRT, RAB27A, RAB7B, RABGGTB, RAET1E, RALGDS, RALYL, RARB, RCVRN, REG3G, RFC5, RGL4, RGS19, RGS3, RHD, RINL, RIPOR2, RITA1, RMDN2, RNASE1, RNASE4, RNF4, RPA2, RPL17, RPL21, RPL26L1, RPL28, RPL29, RPL41,

RPL9, RPS11, RPS13, RPS14, RRBP1, RSU1, RTP2, RUNX1, RUNX1T1, RUNX1T1, RUNX2, RUSC1, RXRG, S100A13, S100A4, SAT1, SCHIP1, SCMH1, SEC14L1, SEMA4A, SERPINA1, SERPINB4, SERTAD3, SFTPD, SH3D19, SHC1, SHMT1, SHPRH, SIM1, SIRT5, SLC11A2, SLC12A4, SLC16A1, SLC25A3, SLC26A9, SLC5A11, SLC6A12, SLC6A19, SLC7A1, SLFN11, SLIRP, SMAD5, SMARCAD1, SMN1, SNCA, SNRNP200, SNRPB2, SNX12, SOD1, SOX13, SOX5, SP8, SPARCL1, SPATA12, SPATA31C2, SPN, SPOP, SQSTM1, SRBD1, SRC, SREBF1, SRPK2, SSB, SSB, SSBP1, ST3GAL6, STAB1, STAMBP, STAU1, STAU1, STAU1, STAU1, STAU1, STK16, STK24, STK38, STMN1, STX7, SULT2B1, SYK, SYNPR, TAF1C, TAGLN, TANK, TAS2R40, TBC1D15, TBXAS1, TCF4, TDGF1, TDP2, TDRD3, TDRD5, TESK2, THAP6, THBD, THTPA, TIAM2, TKFC, TKTL1, TLR10, TM9SF2, TMC6, TMCO2, TMED10, TMEM116, TMEM126A, TMEM159, TMEM208, TMEM230, TMEM67, TMPRSS13, TMUB2, TNFSF4, TNIP3, TP53, TP53, TP73, TRAF1, TRAK1, TRIM31, TRIM6, TRMT1, TRMT2B, TRPM7, TRPM8, TSPEAR, TTC39B, TTLL11, TUBB6, TXLNB, TXNIP, TXNL1, TXNRD1, TYROBP, U2AF1, UBA1, UBE2D3, UBE2I, UBE2L3, UBE2V1, UBE2V2, UMPS, UNG, UPP2, USMG5, USP18, UTP14A, UTRN, UTS2, VDR, VEGFA, VEGFA, VEPH1, VIPAS39, VPS29, VSIG10L, WDHD1, WDR12, WDR4, WDR45, WDYHV1, WRAP53, XIAP, XPNPEP3, YAP1, YWHAZ, YY1AP1, ZBTB32, ZNF146, ZNF250, ZNF385A, ZNF408, ZNF410, ZNF423, ZNF43, ZNF502, ZNF512, ZNF513, ZNF580, ZNF609, ZNF707, or ZNRD1.

ii. SYNTHETIC TIES: APTAMER COMPLEXES, MODIFIED NUCLEOTIDES, IRES VARIANTS & OTHER ENGINEERED TIES

[0335] As contemplated herein, in certain embodiments, a translation initiation element (TIE) comprises a synthetic TIE. In some embodiments, a synthetic TIE comprises aptamer complexes, synthetic IRES or other engineered TIES capable of initiating translation of a linear RNA or circular RNA polynucleotide.

[0336] In some embodiments, one or more aptamer sequences is capable of binding to a component of a eukaryotic initiation factor to either enhance or initiate translation. In some embodiments, aptamer may be used to enhance translation *in vivo* and *in vitro* by promoting specific eukaryotic initiation factors (eIF) (*e.g.*, aptamer in WO 2019/081383 A1 is capable of binding to eukaryotic initiation factor 4F (eIF4F). In some embodiments, the aptamer or a complex of aptamers may be capable of binding to EIF4G, EIF4E, EIF4A, EIF4B, EIF3, EIF2, EIF5, EIF1, EIF1A, 40S ribosome, PCBP1 (polyC binding protein), PCBP2, PCBP3, PCBP4, PABP1 (polyA

binding protein), PTB, Argonaute protein family, HNRNPK (heterogeneous nuclear ribonucleoprotein K), or La protein.

c. TERMINATION SEQUENCE

[0337] In certain embodiments, the core functional element comprises a termination sequence. In some embodiments, the termination sequence comprises a stop codon. In one embodiment, the termination sequence comprises a stop cassette. In some embodiments, the stop cassette comprises at least 2 stop codons. In some embodiments, the stop cassette comprises at least 2 frames of stop codons. In the same embodiment, the frames of the stop codons in a stop cassette each comprise 1, 2 or more stop codons. In some embodiments, the stop cassette comprises a LoxP or a RoxStopRox, or frt-flanked stop cassette. In the same embodiment, the stop cassette comprises a lox-stop-lox stop cassette.

C. VARIANTS

[0338] In certain embodiments, a provided polynucleotide (e.g., a DNA template, a precursor RNA polynucleotide, or a circular RNA polynucleotide) comprises modified nucleotides and/or modified nucleosides. In some embodiments, the modified nucleoside is m^5C (5-methylcytidine). In another embodiment, the modified nucleoside is m^5U (5-methyluridine). In another embodiment, the modified nucleoside is m^6A (N^6-methyladenosine). In another embodiment, the modified nucleoside is s^2U (2-thiouridine). In another embodiment, the modified nucleoside is Ψ (pseudouridine). In another embodiment, the modified nucleoside is Um (2′-O-methyluridine). In other embodiments, the modified nucleoside is m^1A (1-methyladenosine); m^2A (2-methyladenosine); Am (2'-O-methyladenosine); ms^2m^6A (2-methylthio-N^6-methyladenosine); i^6A (N^6-isopentenyladenosine); ms^2i6A (2-methylthio-N^6 isopentenyladenosine); io^6A (N^6-(cis-hydroxyisopentenyl)adenosine); ms^2io^6A (2-methylthio-N^6-(cis-hydroxyisopentenyl)adenosine); g^6A (N^6-glycinylcarbamoyladenosine); t^6A (N^6-threonylcarbamoyladenosine); ms^2t^6A (2-methylthio-N^6-threonyl carbamoyladenosine); m^6t^6A (N^6-methyl-N^6-threonylcarbamoyladenosine); hn^6A(N^6-hydroxynorvalylcarbamoyladenosine); ms^2hn^6A (2-methylthio-N^6-hydroxynorvalyl carbamoyladenosine); Ar(p) (2'-O-ribosyladenosine (phosphate)); I (inosine); m^1I (1-methylinosine); m^1Im (1,2'-O-dimethylinosine); m^3C (3-methylcytidine); Cm (2'-O-methylcytidine); s^2C (2-thiocytidine); ac^4C (N^4-acetylcytidine); f^5C (5-formylcytidine); m^5Cm (5,2′-O-dimethylcytidine); ac^4Cm (N^4-acetyl-2'-O-methylcytidine); k^2C

(lysidine); m^1G (1-methylguanosine); m^2G (N^2-methylguanosine); m^7G (7-methylguanosine); Gm (2′-O-methylguanosine); $m^2{}_2G$ (N^2,N^2-dimethylguanosine); m^2Gm (N^2,2'-O-dimethylguanosine); $m^2{}_2Gm$ (N^2,N^2,2'-O-trimethylguanosine); Gr(p) (2'-O-ribosylguanosine(phosphate)); yW (wybutosine); o_2yW (peroxywybutosine); OHyW (hydroxywybutosine); OHyW* (undermodified hydroxywybutosine); imG (wyosine); mimG (methylwyosine); Q (queuosine); oQ (epoxyqueuosine); galQ (galactosyl-queuosine); manQ (mannosyl-queuosine); $preQ_0$ (7-cyano-7-deazaguanosine); $preQ_1$ (7-aminomethyl-7-deazaguanosine); G^+ (archaeosine); D (dihydrouridine); m^5Um (5,2'-O-dimethyluridine); s^4U (4-thiouridine); m^5s^2U (5-methyl-2-thiouridine); s^2Um (2-thio-2'-O-methyluridine); acp^3U (3-(3-amino-3-carboxypropyl)uridine); ho^5U (5-hydroxyuridine); mo^5U (5-methoxyuridine); cmo^5U (uridine 5-oxyacetic acid); $mcmo^5U$ (uridine 5-oxyacetic acid methyl ester); chm^5U (5-(carboxyhydroxymethyl)uridine)); $mchm^5U$ (5-(carboxyhydroxymethyl)uridine methyl ester); mcm^5U (5-methoxycarbonylmethyluridine); mcm^5Um (5-methoxycarbonylmethyl-2'-O-methyluridine); mcm^5s^2U (5-methoxycarbonylmethyl-2-thiouridine); nm^5S^2U (5-aminomethyl-2-thiouridine); mnm^5U (5-methylaminomethyluridine); mnm^5s^2U (5-methylaminomethyl-2-thiouridine); mnm^5se^2U (5-methylaminomethyl-2-selenouridine); ncm^5U (5-carbamoylmethyluridine); ncm^5Um (5-carbamoylmethyl-2′-O-methyluridine); $cmnm^5U$ (5-carboxymethylaminomethyluridine); $cmnm^5Um$ (5-carboxymethylaminomethyl-2′-O-methyluridine); $cmnm^5s^2U$ (5-carboxymethylaminomethyl-2-thiouridine); $m^6{}_2A$ (N^6,N^6-dimethyladenosine); Im (2'-O-methylinosine); m^4C (N^4-methylcytidine); m^4Cm (N^4,2'-O-dimethylcytidine); hm^5C (5-hydroxymethylcytidine); m^3U (3-methyluridine); cm^5U (5-carboxymethyluridine); m^6Am (N^6,2'-O-dimethyladenosine); $m^6{}_2Am$ (N^6,N^6,O-2'-trimethyladenosine); $m^{2,7}G$ (N^2,7-dimethylguanosine); $m^{2,2,7}G$ (N^2,N^2,7-trimethylguanosine); m^3Um (3,2'-O-dimethyluridine); m^5D (5-methyldihydrouridine); f^5Cm (5-formyl-2'-O-methylcytidine); m^1Gm (1,2'-O-dimethylguanosine); m^1Am (1,2'-O-dimethyladenosine); $\tau m\ ^5U$ (5-taurinomethyluridine); τm^5s^2U (5-taurinomethyl-2-thiouridine)); imG-14 (4-demethylwyosine); imG2 (isowyosine); or ac^6A (N^6-acetyladenosine).

[0339] In some embodiments, the modified nucleoside may include a compound selected from the group of: pyridin-4-one ribonucleoside, 5-aza-uridine, 2-thio-5-aza-uridine, 2-thiouridine, 4-thio-pseudouridine, 2-thio-pseudouridine, 5-hydroxyuridine, 3-methyluridine, 5-carboxymethyl-uridine, 1-carboxymethyl-pseudouridine, 5-propynyl-uridine, 1-propynyl-pseudouridine, 5-

taurinomethyluridine, 1-taurinomethyl-pseudouridine, 5-taurinomethyl-2-thio-uridine, 1-taurinomethyl-4-thio-uridine, 5-methyl-uridine, 1-methyl-pseudouridine, 4-thio-1-methyl-pseudouridine, 2-thio-1-methyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-1-deaza-pseudouridine, dihydrouridine, dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-dihydropseudouridine, 2-methoxyuridine, 2-methoxy-4-thio-uridine, 4-methoxy-pseudouridine, 4-m ethoxy-2-thio-pseudouridine, 5-aza-cytidine, pseudoisocytidine, 3-methyl-cytidine, N4-acetylcytidine, 5-formylcytidine, N4-methylcytidine, 5-hydroxymethylcytidine, 1-methyl-pseudoisocytidine, pyrrolo-cytidine, pyrrolo-pseudoisocytidine, 2-thio-cytidine, 2-thio-5-methyl-cytidine, 4-thio-pseudoisocytidine, 4-thio-1-methyl-pseudoisocytidine, 4-thio-1-methyl-1-deaza-pseudoisocytidine, 1-methyl-1-deaza-pseudoisocytidine, zebularine, 5-aza-zebularine, 5-methyl-zebularine, 5-aza-2-thio-zebularine, 2-thio-zebularine, 2-methoxy-cytidine, 2-methoxy-5-methyl-cytidine, 4-methoxy-pseudoisocytidine, 4-methoxy-1-methyl-pseudoisocytidine, 2-aminopurine, 2, 6-diaminopurine, 7-deaza-adenine, 7-deaza-8-aza-adenine, 7-deaza-2-aminopurine, 7-deaza-8-aza-2-aminopurine, 7-deaza-2,6-diaminopurine, 7-deaza-8-aza-2,6-diaminopurine, 1-methyladenosine, N6-methyladenosine, N6-isopentenyladenosine, N6-(cis-hydroxyisopentenyl)adenosine, 2-methylthio-N6-(cis-hydroxyisopentenyl) adenosine, N6-glycinylcarbamoyladenosine, N6-threonylcarbamoyladenosine, 2-methylthio-N6-threonyl carbamoyladenosine, N6,N6-dimethyladenosine, 7-methyladenine, 2-methylthio-adenine, 2-methoxy-adenine, inosine, 1-methyl-inosine, wyosine, wybutosine, 7-deaza-guanosine, 7-deaza-8-aza-guanosine, 6-thio-guanosine, 6-thio-7-deaza-guanosine, 6-thio-7-deaza-8-aza-guanosine, 7-methyl-guanosine, 6-thio-7-methyl-guanosine, 7-methylinosine, 6-methoxy-guanosine, 1-methylguanosine, N2-methylguanosine, N2,N2-dimethylguanosine, 8-oxo-guanosine, 7-methyl-8-oxo-guanosine, 1-methyl-6-thio-guanosine, N2-methyl-6-thio-guanosine, and N2,N2-dimethyl-6-thio-guanosine. In another embodiment, the modifications are independently selected from the group consisting of 5-methylcytosine, pseudouridine and 1-methylpseudouridine.

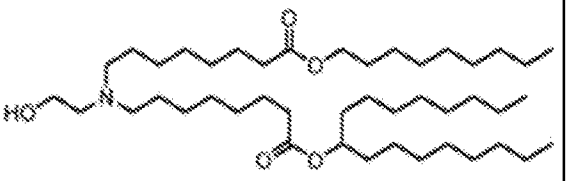
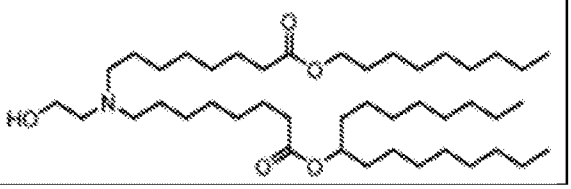
[0340] In some embodiments, the modified ribonucleosides include 5-methylcytidine, 5-methoxyuridine, 1-methyl-pseudouridine, N6-methyladenosine, and/or pseudouridine. In some embodiments, such modified nucleosides provide additional stability and resistance to immune activation.

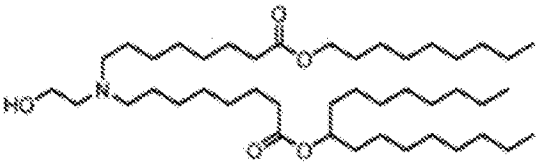
[0341] In particular embodiments, polynucleotides may be codon-optimized. A codon optimized sequence may be one in which codons in a polynucleotide encoding a polypeptide have been

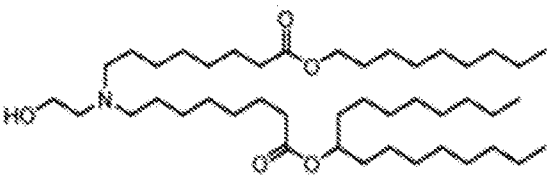
substituted in order to increase the expression, stability and/or activity of the polypeptide. Factors that influence codon optimization include, but are not limited to one or more of: (i) variation of codon biases between two or more organisms or genes or synthetically constructed bias tables, (ii) variation in the degree of codon bias within an organism, gene, or set of genes, (iii) systematic variation of codons including context, (iv) variation of codons according to their decoding tRNAs, (v) variation of codons according to GC %, either overall or in one position of the triplet, (vi) variation in degree of similarity to a reference sequence for example a naturally occurring sequence, (vii) variation in the codon frequency cutoff, (viii) structural properties of mRNAs transcribed from the DNA sequence, (ix) prior knowledge about the function of the DNA sequences upon which design of the codon substitution set is to be based, and/or (x) systematic variation of codon sets for each amino acid. In some embodiments, a codon optimized polynucleotide may minimize ribozyme collisions and/or limit structural interference between the expression sequence and the core functional element.

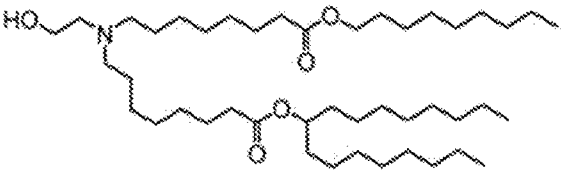
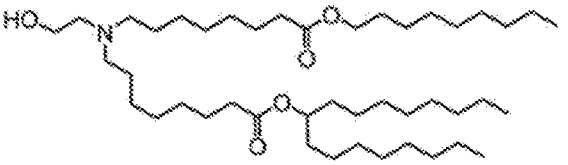
3. PAYLOADS

[0342] In some embodiments, the expression sequence encodes a therapeutic protein. In some embodiments, the therapeutic protein is selected from the proteins listed in the following table.

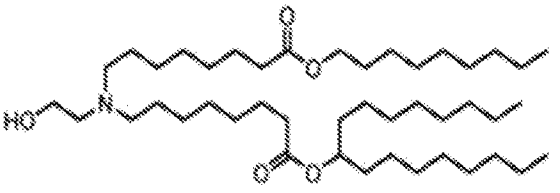
Payload	**Sequence**	**Target cell / organ**	**Preferred delivery formulation**
CD19 CAR	Any of sequences 309-314	T cells	 (50 mol %) DSPC (10 mol %) Beta-sitosterol (28.5% mol %) Cholesterol (10 mol %) PEG DMG (1.5 mol %)
BCMA CAR*	MALPVTALLLPLALLL HAARPDIVLTQSPASLA VSLGERATINCRASESV SVIGAHLIHWYQQKPG QPPKLLIYLASNLETGV	T cells	

	PARFSGSGSGTDFTLTIS SLQAEDAAIYYCLQSRI FPRTFGQGTKLEIKGST SGSGKPGSGEGSTKGQ VQLVQSGSELKKPGAS VKVSCKASGYTFTDYSI NWVRQAPGQGLEWMG WINTETREPAYAYDFR GRFVFSLDTSVSTAYLQ ISSLKAEDTAVYYCAR DYSYAMDYWGQGTLV TVSSAAATTTPAPRPPT PAPTIASQPLSLRPEACR PAAGGAVHTRGLDFAC DIYIWAPLAGTCGVLLL SLVITLYCKRGRKKLLY IFKQPFMRPVQTTQEED GCSCRFPEEEEGGCELR VKFSRSADAPAYQQGQ NQLYNELNLGRREEYD VLDKRRGRDPEMGGKP RRKNPQEGLYNELQKD KMAEAYSEIGMKGERR RGKGHDGLYQGLSTAT KDTYDALHMQALPPR (SEQ ID NO: 3270) *The BCMA CAR may be chosen from any of the anti-BCMA CARs disclosed in US Patent Application US 2021/0128618A1		(50 mol %) DSPC (10 mol %) Beta-sitosterol (28.5% mol %) Cholesterol (10 mol %) PEG DMG (1.5 mol %)
MAGE-A4 TCR	TCR alpha chain: KNQVEQSPQSLIILEGK NCTLQCNYTVSPFSNLR WYKQDTGRGPVSLTIM TFSENTKSNGRYTATLD ADTKQSSLHITASQLSD SASYICVVNHSGGSYIP TFGRGTSLIVHPYIQKP DPAVYQLRDSKSSDKS VCLFTDFDSQTNVSQSK DSDVYITDKTVLDMRS MDFKSNSAVAWSNKS DFACANAFNNSIIPEDT	T cells	 (50 mol %) DSPC (10 mol %) Beta-sitosterol (28.5% mol %) Cholesterol (10 mol %) PEG DMG (1.5 mol %)

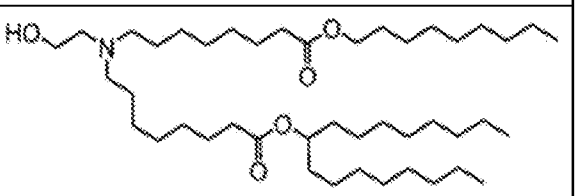
	FFPSPESS (SEQ ID NO: 3271) TCR beta chain: DVKVTQSSRYLVKRTG EKVFLECVQDMDHEN MFWYRQDPGLGLRLIY FSYDVKMKEKGDIPEG YSVSREKKERFSLILES ASTNQTSMYLCASSFL MTSGDPYEQYFGPGTR LTVTEDLKNVFPPEVA VFEPSEAEISHTQKATL VCLATGFYPDHVELSW WVNGKEVHSGVSTDPQ PLKEQPALNDSRYCLSS RLRVSATFWQNPRNHF RCQVQFYGLSENDEWT QDRAKPVTQIVSAEAW GRAD (SEQ ID NO: 3272)		
NY-ESO TCR	TCRalpha extracellular sequence MQEVTQIPAALSVPEGE NLVLNCSFTDSAIYNLQ WFRQDPGKGLTSLLLIQ SSQREQTSGRLNASLDK SSGRSTLYIAASQPGDS ATYLCAVRPTSGGSYIP TFGRGTSLIVHPY (SEQ ID NO: 3273) TCRbeta extracellular sequence MGVTQTPKFQVLKTGQ SMTLQCAQDMNHEYM SWYRQDPGMGLRLIHY SVGAGITDQGEVPNGY NVSRSTTEDFPLRLLSA APSQTSVYFCASSYVG NTGELFFGEGSRLTVL (SEQ ID NO: 3274)	T cells	 (50 mol %) DSPC (10 mol %) Beta-sitosterol (28.5% mol %) Cholesterol (10 mol %) PEG DMG (1.5 mol %)
EPO	APPRLICDSRVLERYLL EAKEAENITTGCAEHCS LNENITVPDTKVNFYA WKRMEVGQQAVEVW QGLALLSEAVLRGQAL	Kidney or bone marrow	

	LVNSSQPWEPLQLHVD KAVSGLRSLTTLLRALG AQKEAISPPDAASAAPL RTITADTFRKLFRVYSN FLRGKLKLYTGEACRT GDR (SEQ ID NO: 3275)		
PAH	MSTAVLENPGLGRKLS DFGQETSYIEDNCNQN GAISLIFSLKEEVGALA KVLRLFEENDVNLTHIE SRPSRLKKDEYEFFTHL DKRSLPALTNIIKILRHD IGATVHELSRDKKKDT VPWFPRTIQELDRFANQ ILSYGAELDADHPGFKD PVYRARRKQFADIAYN YRHGQPIPRVEYMEEE KKTWGTVFKTLKSLYK THACYEYNHIFPLLEKY CGFHEDNIPQLEDVSQF LQTCTGFRLRPVAGLLS SRDFLGGLAFRVFHCT QYIRHGSKPMYTPEPDI CHELLGHVPLFSDRSFA QFSQEIGLASLGAPDEY IEKLATIYWFTVEFGLC KQGDSIKAYGAGLLSSF GELQYCLSEKPKLLPLE LEKTAIQNYTVTEFQPL YYVAESFNDAKEKVRN FAATIPRPFSVRYDPYT QRIEVLDNTQQLKILAD SINSEIGILCSALQKIK (SEQ ID NO: 3257)	Hepatic cells	 (50 mol %) DSPC (10 mol %) Cholesterol (38.5% mol %) PEG-DMG (1.5%) OR MC3 (50 mol %) DSPC (10 mol %) Cholesterol (38.5% mol %) PEG-DMG (1.5%)
CPS1	LSVKAQTAHIVLEDGT KMKGYSFGHPSSVAGE VVFNTGLGGYPEAITDP AYKGQILTMANPIIGNG GAPDTTALDELGLSKY LESNGIKVSGLLVLDYS KDYNHWLATKSLGQW LQEEKVPAIYGVDTRM LTKIIRDKGTMLGKIEF EGQPVDFVDPNKQNLI AEVSTKDVKVYGKGNP TKVVAVDCGIKNNVIR	Hepatic cells	 (50 mol %) DSPC (10 mol %) Cholesterol (38.5% mol %) PEG-DMG (1.5%) OR

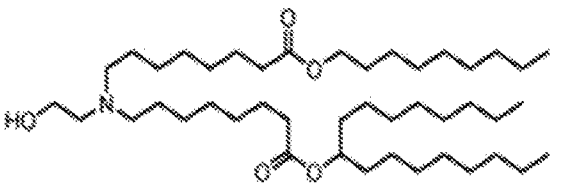
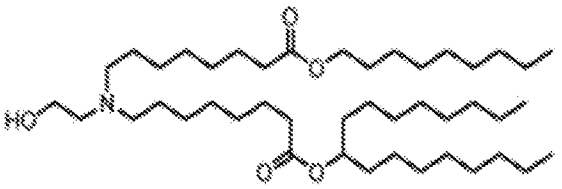
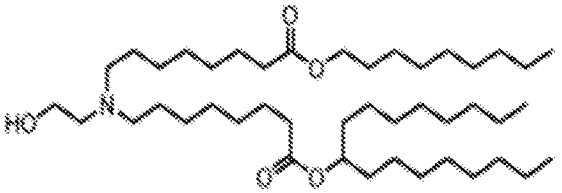
	LLVKRGAEVHLVPWN HDFTKMEYDGILIAGGP GNPALAEPLIQNVRKIL ESDRKEPLFGISTGNLIT GLAAGAKTYKMSMAN RGQNQPVLNITNKQAFI TAQNHGYALDNTLPAG WKPLFVNVNDQTNEGI MHESKPFFAVQFHPEV TPGPIDTEYLFDSFFSLI KKGKATTITSVLPKPAL VASRVEVSKVLILGSGG LSIGQAGEFDYSGSQAV KAMKEENVKTVLMNP NIASVQTNEVGLKQAD TVYFLPITPQFVTEVIKA EQPDGLILGMGGQTAL NCGVELFKRGVLKEYG VKVLGTSVESIMATED RQLFSDKLNEINEKIAPS FAVESIEDALKAADTIG YPVMIRSAYALGGLGS GICPNRETLMDLSTKAF AMTNQILVEKSVTGWK EIEYEVVRDADDNCVT VCNMENVDAMGVHTG DSVVVAPAQTLSNAEF QMLRRTSINVVRHLGIV GECNIQFALHPTSMEYC IIEVNARLSRSSALASK ATGYPLAFIAAKIALGIP LPEIKNVVSGKTSACFE PSLDYMVTKIPRWDLD RFHGTSSRIGSSMKSVG EVMAIGRTFEESFQKAL RMCHPSIEGFTPRLPMN KEWPSNLDLRKELSEPS STRIYAIAKAIDDNMSL DEIEKLTYIDKWFLYK MRDILNMEKTLKGLNS ESMTEETLKRAKEIGFS DKQISKCLGLTEAQTRE LRLKKNIHPWVKQIDTL AAEYPSVTNYLYVTYN GQEHDVNFDDHGMMV LGCGPYHIGSSVEFDW		MC3 (50 mol %) DSPC (10 mol %) Cholesterol (38.5% mol %) PEG-DMG (1.5%)

	CAVSSIRTLRQLGKKTV VVNCNPETVSTDFDEC DKLYFEELSLERILDIYH QEACGGCIISVGGQIPN NLAVPLYKNGVKIMGT SPLQIDRAEDRSIFSAVL DELKVAQAPWKAVNT LNEALEFAKSVDYPCLL RPSYVLSGSAMNVVFS EDEMKKFLEEATRVSQ EHPVVLTKFVEGAREV EMDAVGKDGRVISHAI SEHVEDAGVHSGDATL MLPTQTISQGAIEKVKD ATRKIAKAFAISGPFNV QFLVKGNDVLVIECNL RASRSFPFVSKTLGVDF IDVATKVMIGENVDEK HLPTLDHPIIPADYVAIK APMFSWPRLRDADPILR CEMASTGEVACFGEGI HTAFLKAMLSTGFKIPQ KGILIGIQQSFRPRFLGV AEQLHNEGFKLFATEA TSDWLNANNVPATPVA WPSQEGQNPSLSSIRKLI RDGSIDLVINLPNNNTK FVHDNYVIRRTAVDSGI PLLTNFQVTKLFAEAV QKSRKVDSKSLFHYRQ YSAGKAA (SEQ ID NO: 3276)		
Cas9	MKRNYILGLDIGITSVG YGIIDYETRDVIDAGVR LFKEANVENNEGRRSK RGARRLKRRRRHRIQR VKKLLFDYNLLTDHSE LSGINPYEARVKGLSQK LSEEEFSAALLHLAKRR GVHNVNEVEEDTGNEL STKEQISRNSKALEEKY VAELQLERLKKDGEVR GSINRFKTSDYVKEAK QLLKVQKAYHQLDQSF IDTYIDLLETRRTYYEG PGEGSPFGWKDIKEWY	Immune cells	 (50 mol %) DSPC (10 mol %) Beta-sitosterol (28.5% mol %) Cholesterol (10 mol %) PEG DMG (1.5 mol %)

	EMLMGHCTYFPEELRS VKYAYNADLYNALND LNNLVITRDENEKLEYY EKFQIIENVFKQKKKPT LKQIAKEILVNEEDIKG YRVTSTGKPEFTNLKV YHDIKDITARKEIIENAE LLDQIAKILTIYQSSEDI QEELTNLNSELTQEEIE QISNLKGYTGTHNLSLK AINLILDELWHTNDNQI AIFNRLKLVPKKVDLSQ QKEIPTTLVDDFILSPVV KRSFIQSIKVINAIIKKY GLPNDIIIELAREKNSKD AQKMINEMQKRNRQT NERIEEIIRTTGKENAKY LIEKIKLHDMQEGKCLY SLEAIPLEDLLNNPFNY EVDHIIPRSVSFDNSFNN KVLVKQEENSKKGNRT PFQYLSSSDSKISYETFK KHILNLAKGKGRISKTK KEYLLEERDINRFSVQK DFINRNLVDTRYATRG LMNLLRSYFRVNNLDV KVKSINGGFTSFLRRK WKFKKERNKGYKHHA EDALIIANADFIFKEWK KLDKAKKVMENQMFE EKQAESMPEIETEQEYK EIFITPHQIKHIKDFKDY KYSHRVDKKPNRELIN DTLYSTRKDDKGNTLI VNNLNGLYDKDNDKL KKLINKSPEKLLMYHH DPQTYQKLKLIMEQYG DEKNPLYKYYEETGNY LTKYSKKDNGPVIKKIK YYGNKLNAHLDITDDY PNSRNKVVKLSLKPYR FDVYLDNGVYKFVTVK NLDVIKKENYYEVNSK CYEEAKKLKKISNQAEF IASFYNNDLIKINGELY RVIGVNNDLLNRIEVN		

	MIDITYREYLENMNDK RPPRIIKTIASKTQSIKK YSTDILGNLYEVKSKK HPQIIKKG (SEQ ID NO: 3277)		
ADAM TS13	AAGGILHLELLVAVGP DVFQAHQEDTERYVLT NLNIGAELLRDPSLGAQ FRVHLVKMVILTEPEG APNITANLTSSLLSVCG WSQTINPEDDTDPGHA DLVLYITRFDLELPDGN RQVRGVTQLGGACSPT WSCLITEDTGFDLGVTI AHEIGHSFGLEHDGAPG SGCGPSGHVMASDGAA PRAGLAWSPCSRRQLL SLLSAGRARCVWDPPR PQPGSAGHPPDAQPGL YYSANEQCRVAFGPKA VACTFAREHLDMCQAL SCHTDPLDQSSCSRLLV PLLDGTECGVEKWCSK GRCRSLVELTPIAAVHG RWSSWGPRSPCSRSCG GGVVTRRRQCNNPRPA FGGRACVGADLQAEM CNTQACEKTQLEFMSQ QCARTDGQPLRSSPGG ASFYHWGAAVPHSQG DALCRHMCRAIGESFIM KRGDSFLDGTRCMPSG PREDGTLSLCVSGSCRT FGCDGRMDSQQVWDR CQVCGGDNSTCSPRKG SFTAGRAREYVTFLTVT PNLTSVYIANHRPLFTH LAVRIGGRYVVAGKMS ISPNTTYPSLLEDGRVE YRVALTEDRLPRLEEIRI WGPLQEDADIQVYRRY GEEYGNLTRPDITFTYF QPKPRQAWVWAAVRG PCSVSCGAGLRWVNYS CLDQARKELVETVQCQ GSQQPPAWPEACVLEP	Hepatic cells	 (50 mol %) DSPC (10 mol %) Cholesterol (38.5% mol %) PEG-DMG (1.5%) OR MC3 (50 mol %) DSPC (10 mol %) Cholesterol (38.5% mol %) PEG-DMG (1.5%)

	CPPYWAVGDFGPCSAS CGGGLRERPVRCVEAQ GSLLKTLPPARCRAGA QQPAVALETCNPQPCP ARWEVSEPSSCTSAGG AGLALENETCVPGADG LEAPVTEGPGSVDEKLP APEPCVGMSCPPGWGH LDATSAGEKAPSPWGSI RTGAQAAHVWTPAAG SCSVSCGRGLMELRFLC MDSALRVPVQEELCGL ASKPGSRREVCQAVPC PARWQYKLAACSVSCG RGVVRRILYCARAHGE DDGEEILLDTQCQGLPR PEPQEACSLEPCPPRWK VMSLGPCSASCGLGTA RRSVACVQLDQGQDVE VDEAACAALVRPEASV PCLIADCTYRWHVGTW MECSVSCGDGIQRRRD TCLGPQAQAPVPADFC QHLPKPVTVRGCWAGP CVGQGTPSLVPHEEAA APGRTTATPAGASLEW SQARGLLFSPAPQPRRL LPGPQENSVQSSACGR QHLEPTGTIDMRGPGQ ADCAVAIGRPLGEVVT LRVLESSLNCSAGDML LLWGRLTWRKMCRKL LDMTFSSKTNTLVVRQ RCGRPGGGVLLRYGSQ LAPETFYRECDMQLFG PWGEIVSPSLSPATSNA GGCRLFINVAPHARIAI HALATNMGAGTEGAN ASYILIRDTHSLRTTAFH GQQVLYWESESSQAEM EFSEGFLKAQASLRGQ YWTLQSWVPEMQDPQ SWKGKEGT (SEQ ID NO: 3278)		

FOXP3	MPNPRPGKPSAPSLALG PSPGASPSWRAAPKAS DLLGARGPGGTFQGRD LRGGAHASSSSLNPMPP SQLQLPTLPLVMVAPSG ARLGPLPHLQALLQDR PHFMHQLSTVDAHART PVLQVHPLESPAMISLT PPTTATGVFSLKARPGL PPGINVASLEWVSREPA LLCTFPNPSAPRKDSTL SAVPQSSYPLLANGVC KWPGCEKVFEEPEDFL KHCQADHLLDEKGRA QCLLQREMVQSLEQQL VLEKEKLSAMQAHLAG KMALTKASSVASSDKG SCCIVAAGSQGPVVPA WSGPREAPDSLFAVRR HLWGSHGNSTFPEFLH NMDYFKFHNMRPPFTY ATLIRWAILEAPEKQRT LNEIYHWFTRMFAFFR NHPATWKNAIRHNLSL HKCFVRVESEKGAVWT VDELEFRKKRSQRPSRC SNPTPGP (SEQ ID NO: 3187)	Immune cells	 (50 mol %) DSPC (10 mol %) Beta-sitosterol (28.5% mol %) Cholesterol (10 mol %) PEG DMG (1.5 mol %)
IL-10	SPGQGTQSENSCTHFPG NLPNMLRDLRDAFSRV KTFFQMKDQLDNLLLK ESLLEDFKGYLGCQALS EMIQFYLEEVMPQAEN QDPDIKAHVNSLGENL KTLRLRLRRCHRFLPCE NKSKAVEQVKNAFNKL QEKGIYKAMSEFDIFIN YIEAYMTMKIRN (SEQ ID NO: 3181)	Immune cells	 (50 mol %) DSPC (10 mol %) Beta-sitosterol (28.5% mol %) Cholesterol (10 mol %) PEG DMG (1.5 mol %)
IL-2	APTSSSTKKTQLQLEHL LLDLQMILNGINNYKNP KLTRMLTFKFYMPKKA TELKHLQCLEEELKPLE EVLNLAQSKNFHLRPR DLISNINVIVLELKGSET	Immune cells	

	TFMCEYADETATIVEFL NRWITFCQSIISTLT (SEQ ID NO: 3177)		(50 mol %) DSPC (10 mol %) Beta-sitosterol (28.5% mol %) Cholesterol (10 mol %) PEG DMG (1.5 mol %)

[0343] In some embodiments, the expression sequence encodes a therapeutic protein. In some embodiments, the expression sequence encodes a cytokine, *e.g.*, IL-12p70, IL-15, IL-2, IL-18, IL-21, IFN-α, IFN- β, IL-10, TGF-beta, IL-4, or IL-35, or a functional fragment thereof. In some embodiments, the expression sequence encodes an immune checkpoint inhibitor. In some embodiments, the expression sequence encodes an agonist (*e.g.*, a TNFR family member such as CD137L, OX40L, ICOSL, LIGHT, or CD70). In some embodiments, the expression sequence encodes a chimeric antigen receptor. In some embodiments, the expression sequence encodes an inhibitory receptor agonist (*e.g.*, PDL1, PDL2, Galectin-9, VISTA, B7H4, or MHCII) or inhibitory receptor (e.g., PD1, CTLA4, TIGIT, LAG3, or TIM3). In some embodiments, the expression sequence encodes an inhibitory receptor antagonist. In some embodiments, the expression sequence encodes one or more TCR chains (alpha and beta chains or gamma and delta chains). In some embodiments, the expression sequence encodes a secreted T cell or immune cell engager (*e.g.*, a bispecific antibody such as BiTE, targeting, *e.g.*, CD3, CD137, or CD28 and a tumor-expressed protein e.g., CD19, CD20, or BCMA etc.). In some embodiments, the expression sequence encodes a transcription factor (*e.g.*, *FOXP3*, *HELIOS*, *TOX1*, *or TOX2*). In some embodiments, the expression sequence encodes an immunosuppressive enzyme (*e.g.*, IDO or CD39/CD73). In some embodiments, the expression sequence encodes a GvHD (*e.g.*, anti-HLA-A2 CAR-Tregs).

[0344] In some embodiments, a polynucleotide encodes a protein that is made up of subunits that are encoded by more than one gene. For example, the protein may be a heterodimer, wherein each chain or subunit of the protein is encoded by a separate gene. It is possible that more than one circRNA molecule is delivered in the transfer vehicle and each circRNA encodes a separate subunit of the protein. Alternatively, a single circRNA may be engineered to encode more than one subunit. In certain embodiments, separate circRNA molecules encoding the individual subunits may be administered in separate transfer vehicles.

A. ANTIGEN-RECOGNITION RECEPTORS

a. CHIMERIC ANTIGEN RECEPTORS (CARS)

[0345] In some embodiments, a provided RNA polynucleotide encodes one or more chimeric antigen receptors (CARs or CAR-Ts). CARs are genetically-engineered receptors. These engineered receptors may be inserted into and expressed by immune cells, including T cells via circular RNA as described herein. With a CAR, a single receptor may be programmed to both recognize a specific antigen and, when bound to that antigen, activate the immune cell to attack and destroy the cell bearing that antigen. When these antigens exist on tumor cells, an immune cell that expresses the CAR may target and kill the tumor cell. In some embodiments, the CAR encoded by the polynucleotide comprises (i) an antigen-binding molecule that specifically binds to a target antigen, (ii) a hinge domain, a transmembrane domain, and an intracellular domain, and (iii) an activating domain.

[0346] In some embodiments, an orientation of the CARs in accordance with the disclosure comprises an antigen binding domain (such as an scFv) in tandem with a costimulatory domain and an activating domain. The costimulatory domain may comprise one or more of an extracellular portion, a transmembrane portion, and an intracellular portion. In other embodiments, multiple costimulatory domains may be utilized in tandem.

i. Antigen binding domain

[0347] CARs may be engineered to bind to an antigen (such as a cell-surface antigen) by incorporating an antigen binding molecule that interacts with that targeted antigen. In some embodiments, the antigen binding molecule is an antibody fragment thereof, *e.g.*, one or more single chain antibody fragment (scFv). An scFv is a single chain antibody fragment having the variable regions of the heavy and light chains of an antibody linked together. See U.S. Patent Nos. 7,741,465, and 6,319,494 as well as Eshhar *et al.*, Cancer Immunol Immunotherapy (1997) 45: 131-136. An scFv retains the parent antibody's ability to specifically interact with target antigen. scFvs are useful in chimeric antigen receptors because they may be engineered to be expressed as part of a single chain along with the other CAR components. *Id.* See also Krause *et al.*, J. Exp. Med., Volume 188, No. 4, 1998 (619-626); Finney *et al.*, Journal of Immunology, 1998, 161 : 2791-2797. It will be appreciated that the antigen binding molecule is typically contained within the extracellular portion of the CAR such that it is capable of recognizing and binding to the antigen of interest. Bispecific and multispecific CARs are contemplated within the scope of the

invention, with specificity to more than one target of interest.

[0348] In some embodiments, the antigen binding molecule comprises a single chain, wherein the heavy chain variable region and the light chain variable region are connected by a linker. In some embodiments, the VH is located at the N terminus of the linker and the VL is located at the C terminus of the linker. In other embodiments, the VL is located at the N terminus of the linker and the VH is located at the C terminus of the linker. In some embodiments, the linker comprises at least about 5, at least about 8, at least about 10, at least about 13, at least about 15, at least about 18, at least about 20, at least about 25, at least about 30, at least about 35, at least about 40, at least about 45, at least about 50, at least about 60, at least about 70, at least about 80, at least about 90, or at least about 100 amino acids.

[0349] In some embodiments, the antigen binding molecule comprises a nanobody. In some embodiments, the antigen binding molecule comprises a DARPin. In some embodiments, the antigen binding molecule comprises an anticalin or other synthetic protein capable of specific binding to target protein.

[0350] In some embodiments, the CAR comprises an antigen binding domain specific for an antigen selected from the group CD19, CD123, CD22, CD30, CD171, CS-1, C-type lectin-like molecule-1, CD33, epidermal growth factor receptor variant III (EGFRvIII), ganglioside G2 (GD2), ganglioside GD3, TNF receptor family member B cell maturation (BCMA), Tn antigen ((Tn Ag) or (GalNAca-Ser/Thr)), prostate-specific membrane antigen (PSMA), Receptor tyrosine kinase-like orphan receptor 1 (ROR1), Fms-Like Tyrosine Kinase 3 (FLT3), Tumor-associated glycoprotein 72 (TAG72), CD38, CD44v6, Carcinoembryonic antigen (CEA), Epithelial cell adhesion molecule (EPCAM), B7H3 (CD276), KIT (CD117), Interleukin-13 receptor subunit alpha-2, mesothelin, Interleukin 11 receptor alpha (IL-11Ra), prostate stem cell antigen (PSCA), Protease Serine 21, vascular endothelial growth factor receptor 2 (VEGFR2), Lewis(Y) antigen, CD24, Platelet-derived growth factor receptor beta (PDGFR-beta), Stage-specific embryonic antigen-4 (SSEA-4), CD20, Folate receptor alpha, HER2, HER3, Mucin 1, cell surface associated (MUC1), epidermal growth factor receptor (EGFR), neural cell adhesion molecule (NCAM), Prostase, prostatic acid phosphatase (PAP), elongation factor 2 mutated (ELF2M), Ephrin B2, fibroblast activation protein alpha (FAP), insulin-like growth factor 1 receptor (IGF-I receptor), carbonic anhydrase IX (CAIX), Proteasome (Prosome, Macropain) Subunit, Beta Type, 9 (LMP2), glycoprotein 100 (gp100), oncogene fusion protein consisting of breakpoint cluster region (BCR)

and Abelson murine leukemia viral oncogene homolog 1 (Abl) (bcr-abl), tyrosinase, ephrin type-A receptor 2 (EphA2), Fucosyl GM1, sialyl Lewis adhesion molecule (sLe), ganglioside GM3, transglutaminase 5 (TGS5), high molecular weight-melanoma-associated antigen (HMWMAA), o-acetyl-GD2 ganglioside (OAcGD2), Folate receptor beta, tumor endothelial marker 1 (TEM1/CD248), tumor endothelial marker 7-related (TEM7R), claudin 6 (CLDN6), thyroid stimulating hormone receptor (TSHR), G protein-coupled receptor class C group 5, member D (GPRC5D), chromosome X open reading frame 61 (CXORF61), CD97, CD179a, anaplastic lymphoma kinase (ALK), Polysialic acid, placenta-specific 1 (PLAC1), hexasaccharide portion of globoH glycoceramide (GloboH), mammary gland differentiation antigen (NY-BR-1), uroplakin 2 (UPK2), Hepatitis A virus cellular receptor 1 (HAVCR1), adrenoceptor beta 3 (ADRB3), pannexin 3 (PANX3), G protein-coupled receptor 20 (GPR20), lymphocyte antigen 6 complex, locus K 9 (LY6K), Olfactory receptor 51E2 (OR51E2), TCR Gamma Alternate Reading Frame Protein (TARP), Wilms tumor protein (WT1), Cancer/testis antigen 1 (NY-ESO-1), Cancer/testis antigen 2 (LAGE-1a), MAGE family members (including MAGE-A1, MAGE-A3 and MAGE-A4), ETS translocation-variant gene 6, located on chromosome 12p (ETV6-AML), sperm protein 17 (SPA17), X Antigen Family, Member 1A (XAGE1), angiopoietin-binding cell surface receptor 2 (Tie 2), melanoma cancer testis antigen-1 (MAD-CT-1), melanoma cancer testis antigen-2 (MAD-CT-2), Fos-related antigen 1, tumor protein p53 (p53), p53 mutant, prostein, surviving, telomerase, prostate carcinoma tumor antigen-1, melanoma antigen recognized by T cells 1, Rat sarcoma (Ras) mutant, human Telomerase reverse transcriptase (hTERT), sarcoma translocation breakpoints, melanoma inhibitor of apoptosis (ML-IAP), ERG (transmembrane protease, serine 2 (TMPRSS2) ETS fusion gene), N-Acetyl glucosaminyl-transferase V (NA17), paired box protein Pax-3 (PAX3), Androgen receptor, Cyclin B1, v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN), Ras Homolog Family Member C (RhoC), Tyrosinase-related protein 2 (TRP-2), Cytochrome P450 1B1 (CYP1B1), CCCTC-Binding Factor (Zinc Finger Protein)-Like, Squamous Cell Carcinoma Antigen Recognized By T Cells 3 (SART3), Paired box protein Pax-5 (PAX5), proacrosin binding protein sp32 (OY-TES1), lymphocyte-specific protein tyrosine kinase (LCK), A kinase anchor protein 4 (AKAP-4), synovial sarcoma, X breakpoint 2 (SSX2), Receptor for Advanced Glycation Endproducts (RAGE-1), renal ubiquitous 1 (RU1), renal ubiquitous 2 (RU2), legumain, human papilloma virus E6 (HPV E6), human papilloma virus E7 (HPV E7), intestinal carboxyl esterase, heat shock protein 70-2 mutated

(mut hsp70-2), CD79a, CD79b, CD72, Leukocyte-associated immunoglobulin-like receptor 1 (LAIR1), Fc fragment of IgA receptor (FCAR or CD89), Leukocyte immunoglobulin-like receptor subfamily A member 2 (LILRA2), CD300 molecule-like family member f (CD300LF), C-type lectin domain family 12 member A (CLEC12A), bone marrow stromal cell antigen 2 (BST2), EGF-like module-containing mucin-like hormone receptor-like 2 (EMR2), lymphocyte antigen 75 (LY75), Glypican-3 (GPC3), Fc receptor-like 5 (FCRL5), MUC16, 5T4, 8H9, $\alpha v\beta\theta$ integrin, $\alpha v\beta 6$ integrin, alphafetoprotein (AFP), B7-H6, ca-125, CA9, CD44, CD44v7/8, CD52, E-cadherin, EMA (epithelial membrane antigen), epithelial glycoprotein-2 (EGP-2), epithelial glycoprotein-40 (EGP-40), ErbB4, epithelial tumor antigen (ETA), folate binding protein (FBP), kinase insert domain receptor (KDR), k-light chain, L1 cell adhesion molecule, MUC18, NKG2D, oncofetal antigen (h5T4), tumor/testis-antigen 1B, GAGE, GAGE-1, BAGE, SCP-1, CTZ9, SAGE, CAGE, CT10, MART-1, immunoglobulin lambda-like polypeptide 1 (IGLL1), Hepatitis B Surface Antigen Binding Protein (HBsAg), viral capsid antigen (VCA), early antigen (EA), EBV nuclear antigen (EBNA), HHV-6 p41 early antigen, HHV-6B U94 latent antigen, HHV-6B p98 late antigen , cytomegalovirus (CMV) antigen, large T antigen, small T antigen, adenovirus antigen, respiratory syncytial virus (RSV) antigen, haemagglutinin (HA), neuraminidase (NA), parainfluenza type 1 antigen, parainfluenza type 2 antigen, parainfluenza type 3 antigen, parainfluenza type 4 antigen, Human Metapneumovirus (HMPV) antigen, hepatitis C virus (HCV) core antigen, HIV p24 antigen, human T-cell lympotrophic virus (HTLV-1) antigen, Merkel cell polyoma virus small T antigen, Merkel cell polyoma virus large T antigen, Kaposi sarcoma-associated herpesvirus (KSHV) lytic nuclear antigen and KSHV latent nuclear antigen. In some embodiments, an antigen binding domain comprises an amino acid sequence selected from SEQ ID NOs: 3165-3176.

ii. Hinge / spacer domain

[0351] In some embodiments, a CAR of the instant disclosure comprises a hinge or spacer domain. In some embodiments, the hinge/spacer domain may comprise a truncated hinge/spacer domain (THD) the THD domain is a truncated version of a complete hinge/spacer domain ("CHD"). In some embodiments, an extracellular domain is from or derived from (*e.g.*, comprises all or a fragment of) ErbB2, glycophorin A (GpA), CD2, CD3 delta, CD3 epsilon, CD3 gamma, CD4, CD7, CD8a, CD8[T CDl la (IT GAL), CDl lb (IT GAM), CDl lc (ITGAX), CDl ld (IT GAD),

CD18 (ITGB2), CD19 (B4), CD27 (TNFRSF7), CD28, CD28T, CD29 (ITGB1), CD30 (TNFRSF8), CD40 (TNFRSF5), CD48 (SLAMF2), CD49a (ITGA1), CD49d (ITGA4), CD49f (ITGA6), CD66a (CEACAM1), CD66b (CEACAM8), CD66c (CEACAM6), CD66d (CEACAM3), CD66e (CEACAM5), CD69 (CLEC2), CD79A (B-cell antigen receptor complex-associated alpha chain), CD79B (B-cell antigen receptor complex-associated beta chain), CD84 (SLAMF5), CD96 (Tactile), CD100 (SEMA4D), CD103 (ITGAE), CD134 (0X40), CD137 (4-1BB), CD150 (SLAMF1), CD158A (KIR2DL1), CD158B1 (KIR2DL2), CD158B2 (KIR2DL3), CD158C (KIR3DP1), CD158D (KIRDL4), CD158F1 (KIR2DL5A), CD158F2 (KIR2DL5B), CD158K (KIR3DL2), CD160 (BY55), CD162 (SELPLG), CD226 (DNAM1), CD229 (SLAMF3), CD244 (SLAMF4), CD247 (CD3-zeta), CD258 (LIGHT), CD268 (BAFFR), CD270 (TNFSF14), CD272 (BTLA), CD276 (B7-H3), CD279 (PD-1), CD314 (NKG2D), CD319 (SLAMF7), CD335 (NK-p46), CD336 (NK-p44), CD337 (NK-p30), CD352 (SLAMF6), CD353 (SLAMF8), CD355 (CRT AM), CD357 (TNFRSF18), inducible T cell co-stimulator (ICOS), LFA-1 (CDl la/CD18), NKG2C, DAP-10, ICAM-1, NKp80 (KLRF1), IL-2R beta, IL-2R gamma, IL-7R alpha, LFA-1, SLAMF9, LAT, GADS (GrpL), SLP-76 (LCP2), PAG1/CBP, a CD83 ligand, Fc gamma receptor, MHC class 1 molecule, MHC class 2 molecule, a TNF receptor protein, an immunoglobulin protein, a cytokine receptor, an integrin, activating NK cell receptors, a Toll ligand receptor, and fragments or combinations thereof. A hinge or spacer domain may be derived either from a natural or from a synthetic source.

[0352] In some embodiments, a hinge or spacer domain is positioned between an antigen binding molecule (*e.g.*, an scFv) and a transmembrane domain. In this orientation, the hinge/spacer domain provides distance between the antigen binding molecule and the surface of a cell membrane on which the CAR is expressed. In some embodiments, a hinge or spacer domain is from or derived from an immunoglobulin. In some embodiments, a hinge or spacer domain is selected from the hinge/spacer regions of IgGl, IgG2, IgG3, IgG4, IgA, IgD, IgE, IgM, or a fragment thereof. In some embodiments, a hinge or spacer domain comprises, is from, or is derived from the hinge/spacer region of CD8 alpha. In some embodiments, a hinge or spacer domain comprises, is from, or is derived from the hinge/spacer region of CD28. In some embodiments, a hinge or spacer domain comprises a fragment of the hinge/spacer region of CD8 alpha or a fragment of the hinge/spacer region of CD28, wherein the fragment is anything less than the whole hinge/spacer region. In some embodiments, the fragment of the CD8 alpha hinge/spacer region or the fragment

of the CD28 hinge/spacer region comprises an amino acid sequence that excludes at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or at least 20 amino acids at the N-terminus or C-Terminus, or both, of the CD8 alpha hinge/spacer region, or of the CD28 hinge/spacer region.

iii. Transmembrane domain

[0353] The CAR of the present disclosure may further comprise a transmembrane domain and/or an intracellular signaling domain. The transmembrane domain may be designed to be fused to the extracellular domain of the CAR. It may similarly be fused to the intracellular domain of the CAR. In some embodiments, the transmembrane domain that naturally is associated with one of the domains in a CAR is used. In some instances, the transmembrane domain may be selected or modified (e.g., by an amino acid substitution) to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex. The transmembrane domain may be derived either from a natural or from a synthetic source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein.

[0354] Transmembrane regions may be derived from (*i.e.* comprise) a receptor tyrosine kinase (*e.g.*, ErbB2), glycophorin A (GpA), 4-1BB/CD137, activating NK cell receptors, an immunoglobulin protein, B7-H3, BAFFR, BFAME (SEAMF8), BTEA, CD100 (SEMA4D), CD103, CD160 (BY55), CD18, CD19, CD19a, CD2, CD247, CD27, CD276 (B7-H3), CD28, CD29, CD3 delta, CD3 epsilon, CD3 gamma, CD30, CD4, CD40, CD49a, CD49D, CD49f, CD69, CD7, CD84, CD8alpha, CD8beta, CD96 (Tactile), CD1 la, CD1 lb, CD1 lc, CD1 Id, CDS, CEACAM1, CRT AM, cytokine receptor, DAP-10, DNAM1 (CD226), Fc gamma receptor, GADS, GITR, HVEM (EIGHTR), IA4, ICAM-1, ICAM-1, Ig alpha (CD79a), IE-2R beta, IE-2R gamma, IE-7R alpha, inducible T cell costimulator (ICOS), integrins, ITGA4, ITGA4, ITGA6, IT GAD, ITGAE, ITGAE, IT GAM, ITGAX, ITGB2, ITGB7, ITGB1, KIRDS2, EAT, LFA-1, LFA-1, a ligand that specifically binds with CD83, LIGHT, LIGHT, LTBR, Ly9 (CD229), lymphocyte function-associated antigen-1 (LFA-1; CDl-la/CD18), MHC class 1 molecule, NKG2C, NKG2D, NKp30, NKp44, NKp46, NKp80 (KLRF1), OX-40, PAG/Cbp, programmed death-1 (PD-1), PSGL1, SELPLG (CD162), Signaling Lymphocytic Activation Molecules (SLAM proteins), SLAM (SLAMF1; CD150; IPO-3), SLAMF4 (CD244; 2B4), SLAMF6 (NTB-A; Lyl08),

SLAMF7, SLP-76, TNF receptor proteins, TNFR2, TNFSF14, a Toll ligand receptor, TRANCE/RANKL, VLA1, or VLA-6, or a fragment, truncation, or a combination thereof.

[0355] In some embodiments, suitable intracellular signaling domain include, but are not limited to, activating Macrophage/Myeloid cell receptors CSFR1, MYD88, CD14, TIE2, TLR4, CR3, CD64, TREM2, DAP10, DAP12, CD169, DECTIN1, CD206, CD47, CD163, CD36, MARCO, TIM4, MERTK, F4/80, CD91, C1QR, LOX-1, CD68, SRA, BAI-1, ABCA7, CD36, CD31, Lactoferrin, or a fragment, truncation, or combination thereof.

[0356] In some embodiments, a receptor tyrosine kinase may be derived from (*e.g.*, comprise) Insulin receptor (InsR), Insulin-like growth factor I receptor (IGF1R), Insulin receptor-related receptor (IRR), platelet derived growth factor receptor alpha (PDGFRa), platelet derived growth factor receptor beta (PDGFRfi). KIT proto-oncogene receptor tyrosine kinase (Kit), colony stimulating factor 1 receptor (CSFR), fms related tyrosine kinase 3 (FLT3), fms related tyrosine kinase 1 (VEGFR-1), kinase insert domain receptor (VEGFR-2), fms related tyrosine kinase 4 (VEGFR-3), fibroblast growth factor receptor 1 (FGFR1), fibroblast growth factor receptor 2 (FGFR2), fibroblast growth factor receptor 3 (FGFR3), fibroblast growth factor receptor 4 (FGFR4), protein tyrosine kinase 7 (CCK4), neurotrophic receptor tyrosine kinase 1 (trkA), neurotrophic receptor tyrosine kinase 2 (trkB), neurotrophic receptor tyrosine kinase 3 (trkC), receptor tyrosine kinase like orphan receptor 1 (ROR1), receptor tyrosine kinase like orphan receptor 2 (ROR2), muscle associated receptor tyrosine kinase (MuSK), MET proto-oncogene, receptor tyrosine kinase (MET), macrophage stimulating 1 receptor (Ron), AXL receptor tyrosine kinase (Axl), TYR03 protein tyrosine kinase (Tyro3), MER proto-oncogene, tyrosine kinase (Mer), tyrosine kinase with immunoglobulin like and EGF like domains 1 (TIE1), TEK receptor tyrosine kinase (TIE2), EPH receptor A1 (EphAl), EPH receptor A2 (EphA2), (EPH receptor A3) EphA3, EPH receptor A4 (EphA4), EPH receptor A5 (EphA5), EPH receptor A6 (EphA6), EPH receptor A7 (EphA7), EPH receptor A8 (EphA8), EPH receptor A10 (EphAlO), EPH receptor B1 (EphBl), EPH receptor B2 (EphB2), EPH receptor B3 (EphB3), EPH receptor B4 (EphB4), EPH receptor B6 (EphB6), ret proto oncogene (Ret), receptor-like tyrosine kinase (RYK), discoidin domain receptor tyrosine kinase 1 (DDR1), discoidin domain receptor tyrosine kinase 2 (DDR2), c-ros oncogene 1, receptor tyrosine kinase (ROS), apoptosis associated tyrosine kinase (Lmrl), lemur tyrosine kinase 2 (Lmr2), lemur tyrosine kinase 3 (Lmr3), leukocyte receptor tyrosine kinase (LTK), ALK receptor tyrosine kinase (ALK), or serine/threonine/tyrosine kinase 1 (STYK1).

iv. Costimulatory Domain

[0357] In certain embodiments, the CAR comprises a costimulatory domain. In some embodiments, the costimulatory domain comprises 4-1BB (CD137), CD28, or both, and/or an intracellular T cell signaling domain. In a preferred embodiment, the costimulatory domain is human CD28, human 4-1BB, or both, and the intracellular T cell signaling domain is human CD3 zeta (ζ). 4-1BB, CD28, CD3 zeta may comprise less than the whole 4-1BB, CD28 or CD3 zeta, respectively. Chimeric antigen receptors may incorporate costimulatory (signaling) domains to increase their potency. See U.S. Patent Nos. 7,741,465, and 6,319,494, as well as Krause *et al.* and Finney *et al.* (*supra*), Song *et al.*, Blood 119:696-706 (2012); Kalos *et al.*, Sci Transl. Med. 3:95 (2011); Porter *et al.*, N. Engl. J. Med. 365:725-33 (2011), and Gross *et al.*, Amur. Rev. Pharmacol. Toxicol. 56:59-83 (2016).

[0358] In some embodiments, a costimulatory domain comprises the amino acid sequence of SEQ ID NO: 3162 or 3164.

v. Intracellular signalling domain

[0359] The intracellular (signaling) domain of the engineered T cells disclosed herein may provide signaling to an activating domain, which then activates at least one of the normal effector functions of the immune cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity including the secretion of cytokines.

[0360] In some embodiments, suitable intracellular signaling domain include (*e.g.*, comprise), but are not limited to 4-1BB/CD137, activating NK cell receptors, an Immunoglobulin protein, B7-H3, BAFFR, BLAME (SLAMF8), BTLA, CD100 (SEMA4D), CD103, CD160 (BY55), CD18, CD19, CD 19a, CD2, CD247, CD27, CD276 (B7-H3), CD28, CD29, CD3 delta, CD3 epsilon, CD3 gamma, CD30, CD4, CD40, CD49a, CD49D, CD49f, CD69, CD7, CD84, CD8alpha, CD8beta, CD96 (Tactile), CD1 la, CD1 lb, CD1 lc, CD1 1d, CDS, CEACAM1, CRT AM, cytokine receptor, DAP-10, DNAM1 (CD226), Fc gamma receptor, GADS, GITR, HVEM (LIGHTR), IA4, ICAM-1, Ig alpha (CD79a), IL-2R beta, IL-2R gamma, IL-7R alpha, inducible T cell costimulator (ICOS), integrins, ITGA4, ITGA6, ITGAD, ITGAE, ITGAL, ITGAM, ITGAX, ITGB2, ITGB7, ITGB1, KIRDS2, LAT, LFA-1, ligand that specifically binds with CD83, LIGHT, LTBR, Ly9 (CD229), Lyl08, lymphocyte function-associated antigen- 1 (LFA-1; CDl-la/CD18), MHC class 1 molecule, NKG2C, NKG2D, NKp30, NKp44, NKp46, NKp80 (KLRF1), OX-40, PAG/Cbp, programmed death-1 (PD-1), PSGL1, SELPLG (CD162), Signaling Lymphocytic

Activation Molecules (SLAM proteins), SLAM (SLAMF1; CD150; IPO-3), SLAMF4 (CD244; 2B4), SLAMF6 (NTB-A), SLAMF7, SLP-76, TNF receptor proteins, TNFR2, TNFSF14, a Toll ligand receptor, TRANCE/RANKL, VLA1, or VLA-6, or a fragment, truncation, or a combination thereof.

[0361] CD3 is an element of the T cell receptor on native T cells, and has been shown to be an important intracellular activating element in CARs. In some embodiments, the CD3 is CD3 zeta. In some embodiments, the activating domain comprises an amino acid sequence at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to the polypeptide sequence of SEQ ID NO: 3163.

b. T-CELL RECEPTORS (TCR)

[0362] In some embodiments, a provided circular RNA polynucleotide encodes a T-cell receptor. TCRs are described using the International Immunogenetics (IMGT) TCR nomenclature, and links to the IMGT public database of TCR sequences. Native alpha-beta heterodimeric TCRs have an alpha chain and a beta chain. Broadly, each chain may comprise variable, joining and constant regions, and the beta chain also usually contains a short diversity region between the variable and joining regions, but this diversity region is often considered as part of the joining region. Each variable region may comprise three CDRs (Complementarity Determining Regions) embedded in a framework sequence, one being the hypervariable region named CDR3. There are several types of alpha chain variable (Vα) regions and several types of beta chain variable (Vβ) regions distinguished by their framework, CDR1 and CDR2 sequences, and by a partly defined CDR3 sequence. The Vα types are referred to in IMGT nomenclature by a unique TRAV number. Thus "TRAV21" defines a TCR Vα region having unique framework and CDR1 and CDR2 sequences, and a CDR3 sequence which is partly defined by an amino acid sequence which is preserved from TCR to TCR but which also includes an amino acid sequence which varies from TCR to TCR. In the same way, "TRBV5-1" defines a TCR Vβ region having unique framework and CDR1 and CDR2 sequences, but with only a partly defined CDR3 sequence.

[0363] The joining regions of the TCR are similarly defined by the unique IMGT TRAJ and TRBJ nomenclature, and the constant regions by the IMGT TRAC and TRBC nomenclature.

[0364] The beta chain diversity region is referred to in IMGT nomenclature by the abbreviation

TRBD, and, as mentioned, the concatenated TRBD/TRBJ regions are often considered together as the joining region.

[0365] The unique sequences defined by the IMGT nomenclature are widely known and accessible to those working in the TCR field. For example, they can be found in the IMGT public database. The "T cell Receptor Factsbook", (2001) LeFranc and LeFranc, Academic Press, ISBN 0-12-441352-8 also discloses sequences defined by the IMGT nomenclature, but because of its publication date and consequent time-lag, the information therein sometimes needs to be confirmed by reference to the IMGT database.

[0366] Native TCRs exist in heterodimeric αβ or γδ forms. However, recombinant TCRs consisting of αα or ββ homodimers have previously been shown to bind to peptide MHC molecules. Therefore, the TCR of the invention may be a heterodimeric αβ TCR or may be an αα or ββ homodimeric TCR.

[0367] For use in adoptive therapy, an αβ heterodimeric TCR may, for example, be transfected as full length chains having both cytoplasmic and transmembrane domains. In certain embodiments TCRs of the invention may have an introduced disulfide bond between residues of the respective constant domains, as described, for example, in WO 2006/000830.

[0368] TCRs of the invention, particularly alpha-beta heterodimeric TCRs, may comprise an alpha chain TRAC constant domain sequence and/or a beta chain TRBC1 or TRBC2 constant domain sequence. The alpha and beta chain constant domain sequences may be modified by truncation or substitution to delete the native disulfide bond between Cys4 of exon 2 of TRAC and Cys2 of exon 2 of TRBC1 or TRBC2. The alpha and/or beta chain constant domain sequence(s) may also be modified by substitution of cysteine residues for Thr 48 of TRAC and Ser 57 of TRBC1 or TRBC2, the said cysteines forming a disulfide bond between the alpha and beta constant domains of the TCR.

[0369] Binding affinity (inversely proportional to the equilibrium constant K_D) and binding half-life (expressed as T½) can be determined by any appropriate method. It will be appreciated that doubling the affinity of a TCR results in halving the K_D. T½ is calculated as ln 2 divided by the off-rate (koff). So doubling of T½ results in a halving in koff. K_D and koff values for TCRs are usually measured for soluble forms of the TCR, *i.e.* those forms which are truncated to remove cytoplasmic and transmembrane domain residues. Therefore it is to be understood that a given TCR has an improved binding affinity for, and/or a binding half-life for the parental TCR if a

soluble form of that TCR has the said characteristics. Preferably the binding affinity or binding half-life of a given TCR is measured several times, for example 3 or more times, using the same assay protocol, and an average of the results is taken.

[0370] Since the TCRs of the invention have utility in adoptive therapy, the invention includes a non-naturally occurring and/or purified and/or or engineered cell, especially a T-cell, presenting a TCR of the invention. There are a number of methods suitable for the transfection of T cells with nucleic acid (such as DNA, cDNA or RNA) encoding the TCRs of the invention (see for example Robbins *et al.*, (2008) J Immunol. 180: 6116-6131). T cells expressing the TCRs of the invention will be suitable for use in adoptive therapy-based treatment of cancers such as those of the pancreas and liver. As will be known to those skilled in the art, there are a number of suitable methods by which adoptive therapy can be carried out (see for example Rosenberg *et al.*, (2008) Nat Rev Cancer 8(4): 299-308).

[0371] As is well-known in the art TCRs of the invention may be subject to post-translational modifications when expressed by transfected cells. Glycosylation is one such modification, which may comprise the covalent attachment of oligosaccharide moieties to defined amino acids in the TCR chain. For example, asparagine residues, or serine/threonine residues are well-known locations for oligosaccharide attachment. The glycosylation status of a particular protein depends on a number of factors, including protein sequence, protein conformation and the availability of certain enzymes. Furthermore, glycosylation status (i.e oligosaccharide type, covalent linkage and total number of attachments) can influence protein function. Therefore, when producing recombinant proteins, controlling glycosylation is often desirable. Glycosylation of transfected TCRs may be controlled by mutations of the transfected gene (Kuball J *et al.* (2009), J Exp Med 206(2):463-475). Such mutations are also encompassed in this invention.

[0372] A TCR may be specific for an antigen in the group MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-A13, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, BAGE-1, RAGE-1, LB33/MUM-1, PRAME, NAG, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (AGE-B4), tyrosinase, brain glycogen phosphorylase, Melan-A, MAGE-C1, MAGE-C2, NY-ESO-1, LAGE-1, SSX-1, SSX-2(HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1, CT-7, alpha-actinin-4, Bcr-Abl fusion protein, Casp-8, beta-catenin, cdc27, cdk4, cdkn2a, coa-1, dek-can fusion protein, EF2, ETV6-AML1 fusion

protein, LDLR-fucosyltransferaseAS fusion protein, HLA-A2, HLA-A11, hsp70-2, KIAAO205, Mart2, Mum-2, and 3, neo-PAP, myosin class I, OS-9, pml-RARa fusion protein, PTPRK, K-ras, N-ras, Triosephosphate isomeras, GnTV, Herv-K-mel, Lage-1, Mage-C2, NA-88, Lage-2, SP17, and TRP2-Int2, (MART-I), gp100 (Pmel 17), TRP-1, TRP-2, MAGE-1, MAGE-3, p15(58), CEA, NY-ESO (LAGE), SCP-1, Hom/Mel-40, p53, H-Ras, HER-2/neu, BCR-ABL, E2A-PRL, H4-RET, IGH-IGK, MYL-RAR, Epstein Barr virus antigens, EBNA, human papillomavirus (HPV) antigens E6 and E7, TSP-180, MAGE-4, MAGE-5, MAGE-6, p185erbB2, p180erbB-3, c-met, nm-23H1, PSA, TAG-72-4, CA 19-9, CA 72-4, CAM 17.1, NuMa, K-ras, beta-catenin, CDK4, Mum-1, p16, TAGE, PSMA, PSCA, CT7, telomerase, 43-9F, 5T4, 791Tgp72, α-fetoprotein, 13HCG, BCA225, BTAA, CA 125, CA 15-3 (CA 27.29\BCAA), CA 195, CA 242, CA-50, CAM43, CD68\KP1, CO-029, FGF-5, G250, Ga733 (EpCAM), HTgp-175, M344, MA-50, MG7-Ag, MOV18, NB\170K, NY-CO-1, RCAS1, SDCCAG16, TA-90 (Mac-2 binding protein\cyclophilin C-associated protein), TAAL6, TAG72, TLP, and TPS.

c. B-CELL RECEPTORS (BCR)

[0373] In some embodiments, a provided circular RNA polynucleotide encodes one or more B-cell receptors (BCRs). BCRs (or B-cell antigen receptors) are immunoglobulin molecules that form a type I transmembrane protein on the surface of a B cell. A BCR is capable of transmitting activatory signal into a B cell following recognition of a specific antigen. Prior to binding of a B cell to an antigen, the BCR will remain in an unstimulated or "resting" stage. Binding of an antigen to a BCR leads to signaling that initiates a humoral immune response.

[0374] A BCR is expressed by mature B cells. These B cells work with immunoglobulins (Igs) in recognizing and tagging pathogens. The typical BCR comprises a membrane-bound immunoglobulin (*e.g.*, mIgA, mIgD, mIgE, mIgG, and mIgM), along with associated and Igα/Igβ (CD79a/CD79b) heterodimers (α/β). These membrane-bound immunoglobulins are tetramers consisting of two identical heavy and two light chains. Within the BCR, the membrane bound immunoglobulins is capable of responding to antigen binding by signal transmission across the plasma membrane leading to B cell activation and consequently clonal expansion and specific antibody production (Friess M *et al.* (2018), Front. Immunol. 2947(9)). The Igα/Igβ heterodimers is responsible for transducing signals to the cell interior.

[0375] A Igα/Igβ heterodimer signaling relies on the presence of immunoreceptor tyrosine-based activation motifs (ITAMs) located on each of the cytosolic tails of the heterodimers. ITAMs

comprise two tyrosine residues separated by 9-12 amino acids (*e.g.*, tyrosine, leucine, and/or valine). Upon binding of an antigen, the tyrosine of the BCR's ITAMs become phosphorylated by Src-family tyrosine kinases Blk, Fyn, or Lyn (Janeway C *et al.*, Immunobiology: The Immune System in Health and Disease (Garland Science, 5th ed. 2001)).

d. OTHER CHIMERIC PROTEINS

[0376] In addition to the chimeric proteins provided above, the circular RNA polynucleotide may encode for a various number of other chimeric proteins available in the art. The chimeric proteins may include recombinant fusion proteins, chimeric mutant protein, or other fusion proteins.

B. IMMUNE MODULATORY LIGANDS

[0377] In some embodiments, the circular RNA polynucleotide encodes for an immune modulatory ligand. In certain embodiments, the immune modulatory ligand may be immunostimulatory; while in other embodiments, the immune modulatory ligand may be immunosuppressive.

a. CYTOKINES: INTERFERON, CHEMOKINES, INTERLEUKINS, GROWTH FACTOR & OTHERS

[0378] In some embodiments, the circular RNA polynucleotide encodes for a cytokine. In some embodiments, the cytokine comprises a chemokine, interferon, interleukin, lymphokine, and tumor necrosis factor. Chemokines are chemotactic cytokine produced by a variety of cell types in acute and chronic inflammation that mobilizes and activates white blood cells. An interferon comprises a family of secreted α-helical cytokines induced in response to specific extracellular molecules through stimulation of TLRs (Borden, Molecular Basis of Cancer (Fourth Edition) 2015). Interleukins are cytokines expressed by leukocytes.

[0379] Descriptions and/or amino acid sequences of IL-2, IL-7, IL-10, IL-12, IL-15, IL-18, IL-27β, IFNγ, and/or TGFβ1 are provided herein and at the www.uniprot.org database at accession numbers: P60568 (IL-2), P29459 (IL-12A), P29460 (IL-12B), P13232 (IL-7), P22301 (IL-10), P40933 (IL-15), Q14116 (IL-18), Q14213 (IL-27β), P01579 (IFNγ), and/or P01137 (TGFβ1).

C. TRANSCRIPTION FACTORS

[0380] Regulatory T cells (Treg) are important in maintaining homeostasis, controlling the magnitude and duration of the inflammatory response, and in preventing autoimmune and allergic

responses.

[0381] In general, Tregs are thought to be mainly involved in suppressing immune responses, functioning in part as a "self-check" for the immune system to prevent excessive reactions. In particular, Tregs are involved in maintaining tolerance to self-antigens, harmless agents such as pollen or food, and abrogating autoimmune disease.

[0382] Tregs are found throughout the body including, without limitation, the gut, skin, lung, and liver. Additionally, Treg cells may also be found in certain compartments of the body that are not directly exposed to the external environment such as the spleen, lymph nodes, and even adipose tissue. Each of these Treg cell populations is known or suspected to have one or more unique features and additional information may be found in Lehtimaki and Lahesmaa, Regulatory T cells control immune responses through their non-redundant tissue specific features, 2013, FRONTIERS IN IMMUNOL., 4(294): 1-10, the disclosure of which is hereby incorporated in its entirety.

[0383] Typically, Tregs are known to require TGF-β and IL-2 for proper activation and development. Tregs, expressing abundant amounts of the IL-2 receptor (IL-2R), are reliant on IL-2 produced by activated T cells. Tregs are known to produce both IL-10 and TGF-β, both potent immune suppressive cytokines. Additionally, Tregs are known to inhibit the ability of antigen presenting cells (APCs) to stimulate T cells. One proposed mechanism for APC inhibition is via CTLA-4, which is expressed by Foxp3+ Tregs. It is thought that CTLA-4 may bind to B7 molecules on APCs and either block these molecules or remove them by causing internalization resulting in reduced availability of B7 and an inability to provide adequate co-stimulation for immune responses. Additional discussion regarding the origin, differentiation and function of Tregs may be found in Dhamne *et al.*, Peripheral and thymic Foxp3+ regulatory T cells in search of origin, distinction, and function, 2013, Frontiers in Immunol., 4 (253): 1-11, the disclosure of which is hereby incorporated in its entirety.

D. CHECKPOINT INHIBITORS & AGONISTS

[0384] As provided herein, in certain embodiments, the coding element of the circular RNA encodes for one or more checkpoint inhibitors or agonists.

[0385] In some embodiments, the immune checkpoint inhibitor is an inhibitor of Programmed Death-Ligand 1 (PD-L1, also known as B7-H1, CD274), Programmed Death 1 (PD-1), CTLA-4,

PD-L2 (B7-DC, CD273), LAG3, TIM3, 2B4, A2aR, B7H1, B7H3, B7H4, BTLA, CD2, CD27, CD28, CD30, CD40, CD70, CD80, CD86, CD137, CD160, CD226, CD276, DR3, GAL9, GITR, HAVCR2, HVEM, IDO1, IDO2, ICOS (inducible T cell costimulator), KIR, LAIR1, LIGHT, MARCO (macrophage receptor with collageneous structure), PS (phosphatidylserine), OX-40, SLAM, TIGHT, VISTA, VTCN1, or any combinations thereof. In some embodiments, the immune checkpoint inhibitor is an inhibitor of IDO1, CTLA4, PD-1, LAG3, PD-L1, TIM3, or combinations thereof. In some embodiments, the immune checkpoint inhibitor is an inhibitor of PD-L1. In some embodiments, the immune checkpoint inhibitor is an inhibitor of PD-1. In some embodiments, the immune checkpoint inhibitor is an inhibitor of CTLA-4. In some embodiments, the immune checkpoint inhibitor is an inhibitor of LAG3. In some embodiments, the immune checkpoint inhibitor is an inhibitor of TIM3. In some embodiments, the immune checkpoint inhibitor is an inhibitor of IDO1.

[0386] As described herein, at least in one aspect, the invention encompasses the use of immune checkpoint antagonists. Such immune checkpoint antagonists include antagonists of immune checkpoint molecules such as Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), Programmed Cell Death Protein 1 (PD-1), Programmed Death-Ligand 1 (PDL-1), Lymphocyte-activation gene 3 (LAG-3), and T-cell immunoglobulin and mucin domain 3 (TIM-3). An antagonist of CTLA-4, PD-1, PDL-1, LAG-3, or TIM-3 interferes with CTLA-4, PD-1, PDL-1, LAG-3, or TIM-3 function, respectively. Such antagonists of CTLA-4, PD-1, PDL-1, LAG-3, and TIM-3 can include antibodies which specifically bind to CTLA-4, PD-1, PDL-1, LAG-3, and TIM-3, respectively and inhibit and/or block biological activity and function.

E. OTHERS

[0387] In some embodiments, the payload encoded within one or more of the coding elements is a hormone, FC fusion protein, anticoagulant, blood clotting factor, protein associated with deficiencies and genetic disease, a chaperone protein, an antimicrobial protein, an enzyme (*e.g.*, metabolic enzyme), a structural protein (*e.g.*, a channel or nuclear pore protein), protein variant, small molecule, antibody, nanobody, an engineered non-body antibody, or a combination thereof.

4. ADDITIONAL ACCESSORY ELEMENTS (SEQUENCE ELEMENTS)

[0388] As described in this invention, the polynucleotide (e.g., circular RNA polynucleotide, linear RNA polynucleotide, and/or DNA template) may further comprise of accessory elements.

In certain embodiments, these accessory elements may be included within the sequences of the circular RNA, linear RNA polynucleotide and/or DNA template for enhancing circularization, translation or both. Accessory elements are sequences, in certain embodiments that are located with specificity between or within the enhanced intron elements, enhanced exon elements, or core functional element of the respective polynucleotide. As an example, but not intended to be limiting, an accessory element includes, a IRES transacting factor region, a miRNA binding site, a restriction site, an RNA editing region, a structural or sequence element, a granule site, a zip code element, an RNA trafficking element or another specialized sequence as found in the art that enhances promotes circularization and/or translation of the protein encoded within the circular RNA polynucleotide.

A. IRES TRANSACTING FACTORS

[0389] In certain embodiments, the accessory element comprises an IRES transacting factor (ITAF) region. In some embodiments, the IRES transacting factor region modulates the initiation of translation through binding to PCBP1 - PCBP4 (polyC binding protein), PABP1 (polyA binding protein), PTB (polyprimidine tract binding), Argonaute protein family, HNRNPK (Heterogeneous nuclear ribonucleoprotein K protein), or La protein. In some embodiments, the IRES transacting factor region comprises a polyA, polyC, polyAC, or polyprimidine track.

[0390] In some embodiments, the ITAF region is located within the core functional element. In some embodiments, the ITAF region is located within the TIE.

B. miRNA BINDING SITES

[0391] In certain embodiments, the accessory element comprises a miRNA binding site. In some embodiments the miRNA binding site is located within the 5' enhanced intron element, 5' enhanced exon element, core functional element, 3' enhanced exon element, and/or 3' enhanced intron element.

[0392] In some embodiments, wherein the miRNA binding site is located within the spacer within the enhanced intron element or enhanced exon element. In certain embodiments, the miRNA binding site comprises the entire spacer regions.

[0393] In some embodiments, the 5' enhanced intron element and 3' enhanced intron elements each comprise identical miRNA binding sites. In another embodiment, the miRNA binding site

of the 5' enhanced intron element comprises a different, in length or nucleotides, miRNA binding site than the 3' enhanced intron element. In one embodiment, the 5' enhanced exon element and 3' enhanced exon element comprise identical miRNA binding sites. In other embodiments, the 5' enhanced exon element and 3' enhanced exon element comprises different, in length or nucleotides, miRNA binding sites.

[0394] In some embodiments, the miRNA binding sites are located adjacent to each other within the circular RNA polynucleotide, linear RNA polynucleotide precursor, and/or DNA template. In certain embodiments, the first nucleotide of one of the miRNA binding sites follows the first nucleotide last nucleotide of the second miRNA binding site.

[0395] In some embodiments, the miRNA binding site is located within a translation initiation element (TIE) of a core functional element. In one embodiment, the miRNA binding site is located before, trailing or within an internal ribosome entry site (IRES). In another embodiment, the miRNA binding site is located before, trailing, or within an aptamer complex.

[0396] Incorporation of miRNA sequences within a circular RNA molecule can permit tissue-specific expression of a coding sequence within a core functional element. For example, in a circular RNA intended to express a protein in immune cells, miRNA binding sequences resulting in expression suppression in tissues such as the liver or kidney may be desired. Such miRNA binding sequences may be selected based on the cell or tissue expression of miRNAs.

[0397] The unique sequences defined by the miRNA nomenclature are widely known and accessible to those working in the microRNA field. For example, they can be found in the miRDB public database.

5. PRODUCTION OF POLYNUCLEOTIDES

[0398] The DNA templates provided herein can be made using standard techniques of molecular biology. For example, the various elements of the vectors provided herein can be obtained using recombinant methods, such as by screening cDNA and genomic libraries from cells, or by deriving the polynucleotides from a DNA template known to include the same.

[0399] The various elements of the DNA template provided herein can also be produced synthetically, rather than cloned, based on the known sequences. The complete sequence can be assembled from overlapping oligonucleotides prepared by standard methods and assembled into the complete sequence. See, *e.g.*, Edge, Nature (1981) 292:756; Nambair *et al.*, Science (1984) 223 : 1299; and Jay *et al.*, J. Biol. Chem. (1984) 259:631 1.

[0400] Thus, particular nucleotide sequences can be obtained from DNA template harboring the desired sequences or synthesized completely, or in part, using various oligonucleotide synthesis techniques known in the art, such as site-directed mutagenesis and polymerase chain reaction (PCR) techniques where appropriate. One method of obtaining nucleotide sequences encoding the desired DNA template elements is by annealing complementary sets of overlapping synthetic oligonucleotides produced in a conventional, automated polynucleotide synthesizer, followed by ligation with an appropriate DNA ligase and amplification of the ligated nucleotide sequence via PCR. See, *e.g.*, Jayaraman *et al.*, Proc. Natl. Acad. Sci. USA (1991) 88:4084-4088. Additionally, oligonucleotide-directed synthesis (Jones *et al.*, Nature (1986) 54:75-82), oligonucleotide directed mutagenesis of preexisting nucleotide regions (Riechmann *et al.*, Nature (1988) 332:323-327 and Verhoeyen *et al.*, Science (1988) 239: 1534-1536), and enzymatic filling-in of gapped oligonucleotides using T4 DNA polymerase (Queen *et al.*, Proc. Natl. Acad. Sci. USA (1989) 86: 10029-10033) can be used.

[0401] The precursor RNA provided herein can be generated by incubating a DNA template provided herein under conditions permissive of transcription of the precursor RNA encoded by the DNA template. For example, in some embodiments a precursor RNA is synthesized by incubating a DNA template provided herein that comprises an RNA polymerase promoter upstream of its 5' duplex sequence and/or expression sequences with a compatible RNA polymerase enzyme under conditions permissive of *in vitro* transcription. In some embodiments, the DNA template is incubated inside of a cell by a bacteriophage RNA polymerase or in the nucleus of a cell by host RNA polymerase II.

[0402] In certain embodiments, provided herein is a method of generating precursor RNA by performing *in vitro* transcription using a DNA template provided herein as a template (*e.g.*, a vector provided herein with an RNA polymerase promoter positioned upstream of the 5' duplex region).

[0403] In certain embodiments, the resulting precursor RNA can be used to generate circular RNA (*e.g.*, a circular RNA polynucleotide provided herein) by incubating it in the presence of magnesium ions and guanosine nucleotide or nucleoside at a temperature at which RNA circularization occurs (*e.g.*, between 20 °C and 60 °C).

[0404] Thus, in certain embodiments provided herein is a method of making circular RNA. In certain embodiments, the method comprises synthesizing precursor RNA by transcription (*e.g.*, run-off transcription) using a vector provided herein (*e.g.*, a 5' enhanced intron element, a 5'

enhanced exon element, a core functional element, a 3' enhanced exon element, and a 3' enhanced intron element) as a template, and incubating the resulting precursor RNA in the presence of divalent cations (*e.g.*, magnesium ions) and GTP such that it circularizes to form circular RNA. In some embodiments, the precursor RNA disclosed herein is capable of circularizing in the absence of magnesium ions and GTP and/or without the step of incubation with magnesium ions and GTP. It has been discovered that circular RNA has reduced immunogenicity relative to a corresponding mRNA, at least partially because the mRNA contains an immunogenic 5' cap. When transcribing a DNA vector from certain promoters (*e.g.*, a T7 promoter) to produce a precursor RNA, it is understood that the 5' end of the precursor RNA is G. To reduce the immunogenicity of a circular RNA composition that contains a low level of contaminant linear mRNA, an excess of GMP relative to GTP can be provided during transcription such that most transcripts contain a 5' GMP, which cannot be capped. Therefore, in some embodiments, transcription is carried out in the presence of an excess of GMP. In some embodiments, transcription is carried out where the ratio of GMP concentration to GTP concentration is within the range of about 3:1 to about 15:1, for example, about 3:1 to about 10:1, about 3:1 to about 5:1, about 3:1, about 4:1, or about 5:1.

[0405] In some embodiments, a composition comprising circular RNA has been purified. Circular RNA may be purified by any known method commonly used in the art, such as column chromatography, gel filtration chromatography, and size exclusion chromatography. In some embodiments, purification comprises one or more of the following steps: phosphatase treatment, HPLC size exclusion purification, and RNase R digestion. In some embodiments, purification comprises the following steps in order: RNase R digestion, phosphatase treatment, and HPLC size exclusion purification. In some embodiments, purification comprises reverse phase HPLC. In some embodiments, a purified composition contains less double stranded RNA, DNA splints, triphosphorylated RNA, phosphatase proteins, protein ligases, capping enzymes and/or nicked RNA than unpurified RNA. In some embodiments, purification of circular RNA comprises an affinity-purification or negative selection method described herein. In some embodiments, purification of circular RNA comprises separation of linear RNA from circular RNA using oligonucleotides that are complementary to a sequence in the linear RNA but are not complementary to a sequence in the circular RNA. In some embodiments, a purified composition is less immunogenic than an unpurified composition. In some embodiments, immune cells

exposed to a purified composition produce less TNFα, RIG-I, IL-2, IL-6, IFNγ, and/or a type 1 interferon, *e.g.*, IFN-β1, than immune cells exposed to an unpurified composition.

6. OVERVIEW OF TRANSFER VEHICLE & OTHER DELIVERY MECHANISMS

A. IONIZABLE LIPIDS

[0406] In certain embodiments, disclosed herein are ionizable lipids that may be used as a component of a transfer vehicle to facilitate or enhance the delivery and release of circular RNA to one or more target cells (*e.g.*, by permeating or fusing with the lipid membranes of such target cells). In certain embodiments, an ionizable lipid comprises one or more cleavable functional groups (*e.g.*, a disulfide) that allow, for example, a hydrophilic functional head-group to dissociate from a lipophilic functional tail-group of the compound (*e.g.*, upon exposure to oxidative, reducing or acidic conditions), thereby facilitating a phase transition in the lipid bilayer of the one or more target cells.

[0407] In some embodiments, an ionizable lipid is a lipid as described in international patent application PCT/US2018/058555.

[0408] In some of embodiments, a cationic lipid has the following formula:

wherein:

R_1 and R_2 are either the same or different and independently optionally substituted C_{10}-C_{24} alkyl, optionally substituted C_{10}-C_{24} alkenyl, optionally substituted C_{10}-C_{24} alkynyl, or optionally substituted C_{10}-C_{24} acyl;

R_3 and R_4 are either the same or different and independently optionally substituted C_1-C_6 alkyl, optionally substituted C_2-C_6 alkenyl, or optionally substituted C_2-C_6 alkynyl or R_3 and R_4 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms chosen from nitrogen and oxygen;

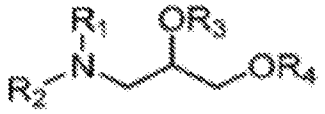
R_5 is either absent or present and when present is hydrogen or C_1-C_6 alkyl; m, n, and p are either the same or different and independently either 0 or 1 with the proviso that m, n, and p are not simultaneously 0; q is 0, 1, 2, 3, or 4; and

Y and Z are either the same or different and independently O, S, or NH .

[0409] In one embodiment, R_1 and R_2 are each linoleyl, and the amino lipid is a dilinoleyl amino lipid.

[0410] In one embodiment, the amino lipid is a dilinoleyl amino lipid.

[0411] In various other embodiments, a cationic lipid has the following structure:



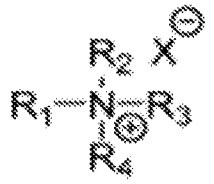
or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein:

R_1 and R_2 are each independently selected from the group consisting of H and C_1-C_3 alkyls; and

R_3 and R_4 are each independently an alkyl group having from about 10 to about 20 carbon atoms, wherein at least one of R_3 and R_4 comprises at least two sites of unsaturation.

[0412] In some embodiments, R_3 and R_4 are each independently selected from dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In an embodiment, R_3 and R_4 and are both linoleyl. In some embodiments, R_3 and/or R_4 may comprise at least three sites of unsaturation (*e.g.*, R_3 and/or R_4 may be, for example, dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl).

[0413] In some embodiments, a cationic lipid has the following structure:



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

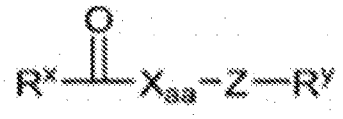
R_1 and R_2 are each independently selected from H and C_1-C_3 alkyls;

R_3 and R_4 are each independently an alkyl group having from about 10 to about 20 carbon atoms, wherein at least one of R_3 and R_4 comprises at least two sites of unsaturation.

[0414] In one embodiment, R_3 and R_4 are the same, for example, in some embodiments R_3 and R_4 are both linoleyl (C_{18}-alkyl). In another embodiment, R_3 and R_4 are different, for example, in some embodiments, R_3 is tetradectrienyl (C_{14}-alkyl) and R_4 is linoleyl (C_{18}-alkyl). In a preferred embodiment, the cationic lipid(s) of the present invention are symmetrical, *i.e.*, R_3 and R_4 are the

same. In another preferred embodiment, both R_3 and R_4 comprise at least two sites of unsaturation. In some embodiments, R_3 and R_4 are each independently selected from dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In an embodiment, R_3 and R_4 are both linoleyl. In some embodiments, R_3 and/or R_4 comprise at least three sites of unsaturation and are each independently selected from dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl.

[0415] In various embodiments, a cationic lipid has the formula:



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

X_{aa} is a D- or L-amino acid residue having the formula $–NR^N–CR^1R^2–C(C=O)–$, or a peptide or a peptide of amino acid residues having the formula $–\{NR^N–CR^1R^2–C(C=O)\}_n–$, wherein n is an integer from 2 to 20;

R^1 is independently, for each occurrence, a non-hydrogen or a substituted or unsubstituted side chain of an amino acid;

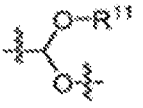
R^2 and R^N are independently, for each occurrence, hydrogen, an organic group consisting of carbon, oxygen, nitrogen, sulfur, and hydrogen atoms, or any combination of the foregoing, and having from 1 to 20 carbon atoms, $C_{(1-5)}$alkyl, cycloalkyl, cycloalkylalkyl, $C_{(1-5)}$alkenyl, $C_{(1-5)}$alkynyl, $C_{(1-5)}$alkanoyl, $C_{(1-5)}$alkanoyloxy, $C_{(1-5)}$alkoxy, $C_{(1-5)}$alkoxy- $C_{(1-5)}$alkyl, $C_{(1-5)}$alkoxy- $C_{(1-5)}$alkoxy, $C_{(1-5)}$alkyl-amino- $C_{(1-5)}$alkyl-, $C_{(1-5)}$dialkyl-amino- $C_{(1-5)}$alkyl-, nitro-$C_{(1-5)}$alkyl, cyano-$C_{(1-5)}$alkyl, aryl-$C_{(1-5)}$alkyl, 4-biphenyl-$C_{(1-5)}$alkyl, carboxyl, or hydroxyl;

Z is –NH–, –O–, –S–, $–CH_2S–$, $–CH_2S(O)–$, or an organic linker consisting of 1-40 atoms selected from hydrogen, carbon, oxygen, nitrogen, and sulfur atoms (preferably, Z is –NH– or –O–);

R^x and R^y are, independently, (i) a lipophilic tail derived from a lipid (which can be naturally occurring or synthetic), *e.g.*, a phospholipid, a glycolipid, a triacylglycerol, a glycerophospholipid, a sphingolipid, a ceramide, a sphingomyelin, a cerebroside, or a ganglioside, wherein the tail optionally includes a steroid; (ii) an amino acid terminal group selected from hydrogen, hydroxyl, amino, and an organic protecting group; or (iii) a substituted or unsubstituted $C_{(3-22)}$alkyl, $C_{(6-}$

$_{12)}$cycloalkyl, $C_{(6-12)}$cycloalkyl- $C_{(3-22)}$alkyl, $C_{(3-22)}$alkenyl, $C_{(3-22)}$alkynyl, $C_{(3-22)}$alkoxy, or $C_{(6-12)}$-alkoxy $C_{(3-22)}$alkyl;

[0416] In some embodiments, one of R^x and R^y is a lipophilic tail as defined above and the other is an amino acid terminal group. In some embodiments, both R^x and R^y are lipophilic tails.

[0417] In some embodiments, at least one of R^x and R^y is interrupted by one or more biodegradable groups (*e.g.*, –OC(O)–, –C(O)O–, –SC(O)–, –C(O)S–, –OC(S)–, –C(S)O–, –S–S–, –C(O)(NR^5)–, –N(R^5)C(O)–, –C(S)(NR^5)–, –N(R^5)C(O)–, –N(R^5)C(O)N(R^5)–, –OC(O)O–, –OSi(R^5)$_2$O–, –C(O)(CR^3R^4)C(O)O–, –OC(O)(CR^3R^4)C(O)–, or  .

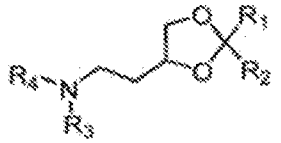
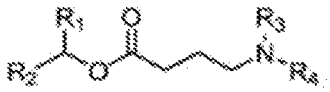
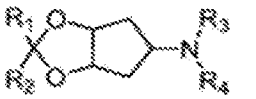
[0418] In some embodiments, R^{11} is a C_2-C_8alkyl or alkenyl.

[0419] In some embodiments, each occurrence of R^5 is, independently, H or alkyl.

[0420] In some embodiments, each occurrence of R^3 and R^4 are, independently H, halogen, OH, alkyl, alkoxy, $–NH_2$, alkylamino, or dialkylamino; or R_3 and R_4, together with the carbon atom to which they are directly attached, form a cycloalkyl group. In some particular embodiments, each occurrence of R^3 and R^4 are, independently H or C_1-C_4alkyl.

[0421] In some embodiments, R^x and R^y each, independently, have one or more carbon-carbon double bonds.

[0422] In some embodiments, the cationic lipid is one of the following:

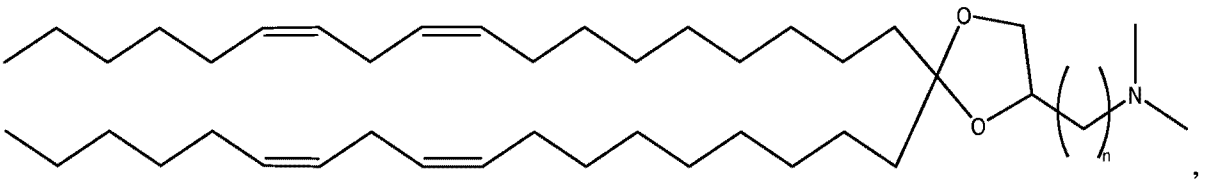

; 
; or  ,

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

R_1 and R_2 are each independently alkyl, alkenyl, or alkynyl, each of which can optionally substituted;

R_3 and R_4 are each independently a C_1-C_6 alkyl, or R_3 and R_4 are taken together to form an optionally substituted heterocyclic ring.

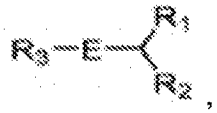
[0423] A representative useful dilinoleyl amino lipid has the formula:



wherein n is 0, 1, 2, 3, or 4 .

[0424] In one embodiment, a cationic lipid is DLin-K-DMA. In one embodiment, a cationic lipid is DLin-KC2-DMA (DLin-K-DMA above, wherein n is 2).

[0425] In one embodiment, a cationic lipid has the following structure:



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

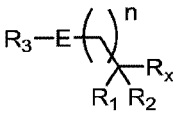
R_1 and R_2 are each independently for each occurrence optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkenyl, optionally substituted C_{10}-C_{30} alkynyl or optionally substituted C_{10}-C_{30} acyl;

R_3 is H, optionally substituted C_2-C_{10} alkyl, optionally substituted C_2-C_{10} alkenyl, optionally substituted C_2-C_{10} alkylyl, alkylhetrocycle, alkylpbosphate, alkylphosphorothioate, alkylphosphorodithioate, alkylphosphonate, alkylamine, hydroxyalkyl, ω-aminoalkyl, ω-(substituted)aminoalkyl, ω-phosphoalkyl, ω-thiophosphoalkyl, optionally substituted polyethylene glycol (PEG, mw 100-40K), optionally substituted mPEG (mw 120-40K), heteroaryl, or heterocycle, or a linker ligand, for example, in some embodiments, R_3 is $(CH_3)_2N(CH_2)_n$–, wherein n is 1, 2, 3 or 4;

E is O, S, N(Q), C(O), OC(O), C(O)O, N(Q)C(O), C(O)N(Q), (Q)N(CO)O, O(CO)N(Q), S(O), $NS(O)_2N(Q)$, $S(O)_2$, $N(Q)S(O)_2$, SS, O=N, aryl, heteroaryl, cyclic or heterocycle, for example -C(O)O, wherein – is a point of connection to R_3; and

Q is H, alkyl, ω-aminoalkyl, ω-(substituted)aminoalkyl, ω-phosphoalkyl or ω-thiophosphoalkyl.

In one specific embodiment, the cationic lipid of Embodiments 1, 2, 3, 4 or 5 has the following structure:



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

E is O, S, N(Q), C(O), N(Q)C(O), C(O)N(Q), (Q)N(CO)O, O(CO)N(Q), S(O), $NS(O)_2N(Q)$, $S(O)_2$, $N(Q)S(O)_2$, SS, O=N, aryl, heteroaryl, cyclic or heterocycle;

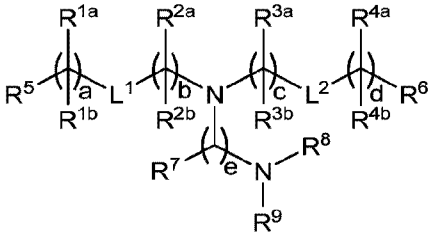
Q is H, alkyl, ω-amninoalkyl, ω-(substituted)amninoalky, ω-phosphoalkyl or ω-thiophosphoalkyl;

R_1 and R_2 and R_x are each independently for each occurrence H, optionally substituted C_1-C_{10} alkyl, optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkenyl, optionally substituted C_{10}-C_{30} alkynyl, optionally substituted C_{10}-C_{30} acyl, or linker-ligand, provided that at least one of R_1, R_2 and R_x is not H;

R_3 is H, optionally substituted C_1-C_{10} alkyl, optionally substituted C_2-C_{10} alkenyl, optionally substituted C_2-C_{10} alkynyl, alkylhetrocycle, alkylphosphate, alkylphosphorothioate, alkylphosphorodithioate, alkylphosphonate, alkylamine, hydroxyalkyl, ω-aminoalkyl, ω-(substituted)aminoalkyl, ω-phosphoalkyl, ω-thiophosphoalkyl, optionally substituted polyethylene glycol (PEG, mw 100-40K), optionally substituted mPEG (mw 120-40K), heteroaryl, or heterocycle, or linker-ligand; and

n is 0, 1, 2, or 3.

In one embodiment, the cationic lipid of Embodiments 1, 2, 3, 4 or 5 has the structure of Formula I:



I

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

one of L^1 or L^2 is –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -$S(O)_x$-, -S-S-, -C(=O)S-, SC(=O)-, -$NR^aC(=O)$-, -$C(=O)NR^a$-, $NR^aC(=O)NR^a$-, -$OC(=O)NR^a$- or -$NR^aC(=O)O$-, and the other of L^1 or L^2 is –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -$S(O)_x$-, -S-S-, -C(=O)S-, SC(=O)-, -$NR^aC(=O)$-, -$C(=O)NR^a$-, ,$NR^aC(=O)NR^a$-, -$OC(=O)NR^a$- or -$NR^aC(=O)O$- or a direct bond;

R^a is H or C_1-C_{12} alkyl;

R^{1a} and R^{1b} are, at each occurrence, independently either (a) H or C_1-C_{12} alkyl, or (b) R^{1a} is H or C_1-C_{12} alkyl, and R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{2a} and R^{2b} are, at each occurrence, independently either (a) H or C_1-C_{12} alkyl, or (b) R^{2a} is H or C_1-C_{12} alkyl, and R^{2b} together with the carbon atom to which it is bound is taken together with an adjacent R^{2b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{3a} and R^{3b} are, at each occurrence, independently either (a) H or C_1-C_{12} alkyl, or (b) R^{3a} is H or C_1-C_{12} alkyl, and R^{3b} together with the carbon atom to which it is bound is taken together with an adjacent R^{3b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{4a} and R^{4b} are, at each occurrence, independently either (a) H or C_1-C_{12} alkyl, or (b) R^{4a} is H or C_1-C_{12} alkyl, and R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^5 and R^6 are each independently methyl or cycloalkyl;

R^7 is, at each occurrence, independently H or C_1-C_{12} alkyl;

R^8 and R^9 are each independently unsubstituted C_1-C_{12} alkyl; or R^8 and R^9, together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring comprising one nitrogen atom;

a and d are each independently an integer from 0 to 24;

b and c are each independently an integer from 1 to 24;

e is 1 or 2; and

x is 0, 1 or 2.

In some embodiments of Formula I, L^1 and L^2 are independently –O(C=O)- or -(C=O)O-.

In certain embodiments of Formula I, at least one of R^{1a}, R^{2a}, R^{3a} or R^{4a} is C_1-C_{12} alkyl, or at least one of L^1 or L^2 is –O(C=O)- or –(C=O)O-. In other embodiments, R^{1a} and R^{1b} are not isopropyl when a is 6 or n-butyl when a is 8.

In still further embodiments of Formula I, at least one of R^{1a}, R^{2a}, R^{3a} or R^{4a} is C_1-C_{12} alkyl, or at least one of L^1 or L^2 is –O(C=O)– or –(C=O)O–; and

R^{1a} and R^{1b} are not isopropyl when a is 6 or n-butyl when a is 8.

In other embodiments of Formula I, R^8 and R^9 are each independently unsubstituted C_1-C_{12} alkyl; or R^8 and R^9, together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring comprising one nitrogen atom;

In certain embodiments of Formula I, any one of L^1 or L^2 may be –O(C=O)– or a carbon-carbon double bond. L^1 and L^2 may each be –O(C=O)– or may each be a carbon-carbon double bond.

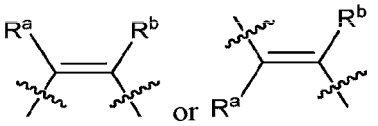
In some embodiments of Formula I, one of L^1 or L^2 is –O(C=O)–. In other embodiments, both L^1 and L^2 are –O(C=O)–.

In some embodiments of Formula I, one of L^1 or L^2 is –(C=O)O–. In other embodiments, both L^1 and L^2 are –(C=O)O–.

In some other embodiments of Formula I, one of L^1 or L^2 is a carbon-carbon double bond. In other embodiments, both L^1 and L^2 are a carbon-carbon double bond.

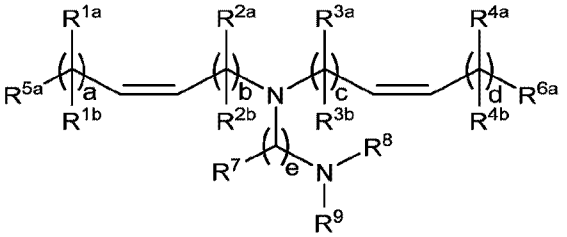
In still other embodiments of Formula I, one of L^1 or L^2 is –O(C=O)– and the other of L^1 or L^2 is –(C=O)O–. In more embodiments, one of L^1 or L^2 is –O(C=O)– and the other of L^1 or L^2 is a carbon-carbon double bond. In yet more embodiments, one of L^1 or L^2 is –(C=O)O– and the other of L^1 or L^2 is a carbon-carbon double bond.

It is understood that "carbon-carbon" double bond, as used throughout the specification, refers to one of the following structures:



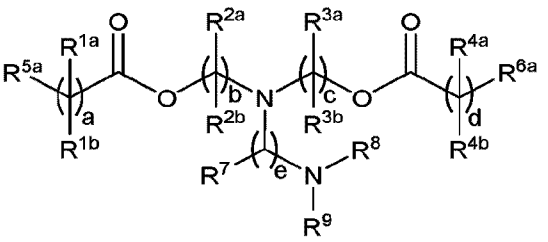
wherein R^a and R^b are, at each occurrence, independently H or a substituent. For example, in some embodiments R^a and R^b are, at each occurrence, independently H, C_1-C_{12} alkyl or cycloalkyl, for example H or C_1-C_{12} alkyl.

In other embodiments, the lipid compounds of Formula I have the following Formula (Ia):



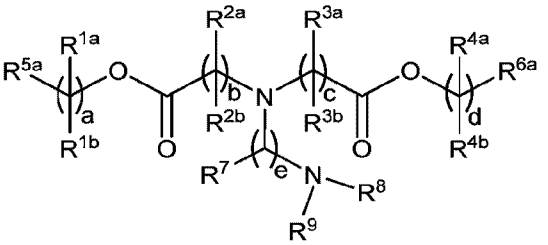
(Ia)

In other embodiments, the lipid compounds of Formula I have the following Formula (Ib):



(Ib)

In yet other embodiments, the lipid compounds of Formula I have the following Formula (Ic):



(Ic)

In certain embodiments of the lipid compound of Formula I, a, b, c and d are each independently an integer from 2 to 12 or an integer from 4 to 12. In other embodiments, a, b, c and d are each independently an integer from 8 to 12 or 5 to 9. In some certain embodiments, a is 0. In some embodiments, a is 1. In other embodiments, a is 2. In more embodiments, a is 3. In yet other embodiments, a is 4. In some embodiments, a is 5. In other embodiments, a is 6. In more embodiments, a is 7. In yet other embodiments, a is 8. In some embodiments, a is 9. In other embodiments, a is 10. In more embodiments, a is 11. In yet other embodiments, a is 12. In some embodiments, a is 13. In other embodiments, a is 14. In more embodiments, a is 15. In yet other embodiments, a is 16.

In some other embodiments of Formula I, b is 1. In other embodiments, b is 2. In more embodiments, b is 3. In yet other embodiments, b is 4. In some embodiments, b is 5. In other embodiments, b is 6. In more embodiments, b is 7. In yet other embodiments, b is 8. In some embodiments, b is 9. In other embodiments, b is 10. In more embodiments, b is 11. In yet other embodiments, b is 12. In some

embodiments, b is 13. In other embodiments, b is 14. In more embodiments, b is 15. In yet other embodiments, b is 16.

In some more embodiments of Formula I, c is 1. In other embodiments, c is 2. In more embodiments, c is 3. In yet other embodiments, c is 4. In some embodiments, c is 5. In other embodiments, c is 6. In more embodiments, c is 7. In yet other embodiments, c is 8. In some embodiments, c is 9. In other embodiments, c is 10. In more embodiments, c is 11. In yet other embodiments, c is 12. In some embodiments, c is 13. In other embodiments, c is 14. In more embodiments, c is 15. In yet other embodiments, c is 16.

In some certain other embodiments of Formula I, d is 0. In some embodiments, d is 1. In other embodiments, d is 2. In more embodiments, d is 3. In yet other embodiments, d is 4. In some embodiments, d is 5. In other embodiments, d is 6. In more embodiments, d is 7. In yet other embodiments, d is 8. In some embodiments, d is 9. In other embodiments, d is 10. In more embodiments, d is 11. In yet other embodiments, d is 12. In some embodiments, d is 13. In other embodiments, d is 14. In more embodiments, d is 15. In yet other embodiments, d is 16.

In some other various embodiments of Formula I, a and d are the same. In some other embodiments, b and c are the same. In some other specific embodiments, a and d are the same and b and c are the same.

The sum of a and b and the sum of c and d in Formula I are factors which may be varied to obtain a lipid of formula I having the desired properties. In one embodiment, a and b are chosen such that their sum is an integer ranging from 14 to 24. In other embodiments, c and d are chosen such that their sum is an integer ranging from 14 to 24. In further embodiment, the sum of a and b and the sum of c and d are the same. For example, in some embodiments the sum of a and b and the sum of c and d are both the same integer which may range from 14 to 24. In still more embodiments, a. b, c and d are selected such the sum of a and b and the sum of c and d is 12 or greater.

In some embodiments of Formula I, e is 1. In other embodiments, e is 2.

The substituents at R^{1a}, R^{2a}, R^{3a} and R^{4a} of Formula I are not particularly limited. In certain embodiments R^{1a}, R^{2a}, R^{3a} and R^{4a} are H at each occurrence. In

certain other embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_{12} alkyl. In certain other embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_8 alkyl. In certain other embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_6 alkyl. In some of the foregoing embodiments, the C_1-C_8 alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

In certain embodiments of Formula I, R^{1a}, R^{1b}, R^{4a} and R^{4b} are C_1-C_{12} alkyl at each occurrence.

In further embodiments of Formula I, at least one of R^{1b}, R^{2b}, R^{3b} and R^{4b} is H or R^{1b}, R^{2b}, R^{3b} and R^{4b} are H at each occurrence.

In certain embodiments of Formula I, R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond. In other embodiments of the foregoing R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

The substituents at R^5 and R^6 of Formula I are not particularly limited in the foregoing embodiments. In certain embodiments one or both of R^5 or R^6 is methyl. In certain other embodiments one or both of R^5 or R^6 is cycloalkyl for example cyclohexyl. In these embodiments the cycloalkyl may be substituted or not substituted. In certain other embodiments the cycloalkyl is substituted with C_1-C_{12} alkyl, for example tert-butyl.

The substituents at R^7 are not particularly limited in the foregoing embodiments of Formula I. In certain embodiments at least one R^7 is H. In some other embodiments, R^7 is H at each occurrence. In certain other embodiments R^7 is C_1-C_{12} alkyl.

In certain other of the foregoing embodiments of Formula I, one of R^8 or R^9 is methyl. In other embodiments, both R^8 and R^9 are methyl.

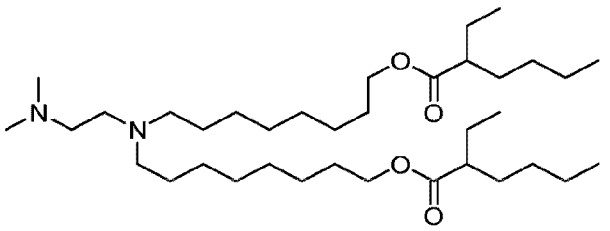
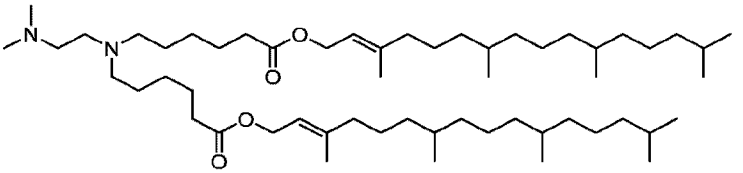
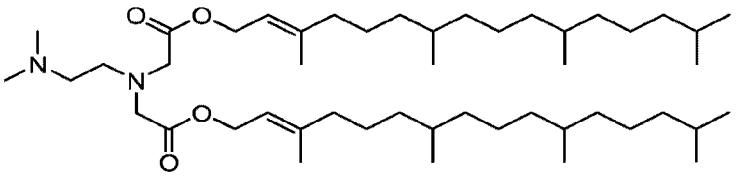
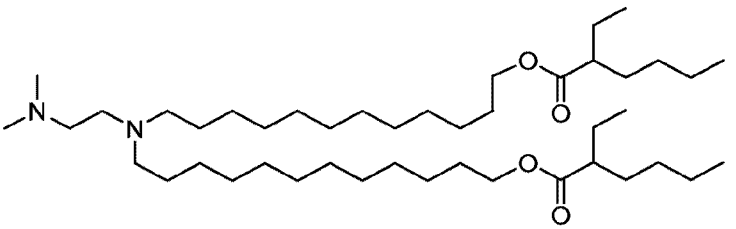
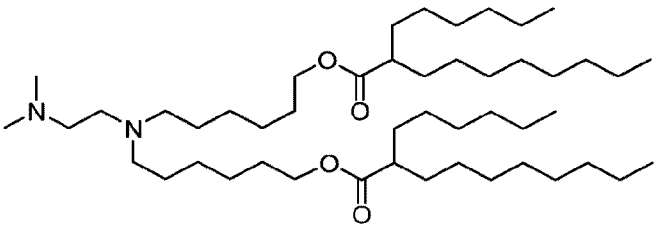
In some different embodiments of Formula I, R^8 and R^9, together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring. In some embodiments of the foregoing, R^8 and R^9, together with the nitrogen

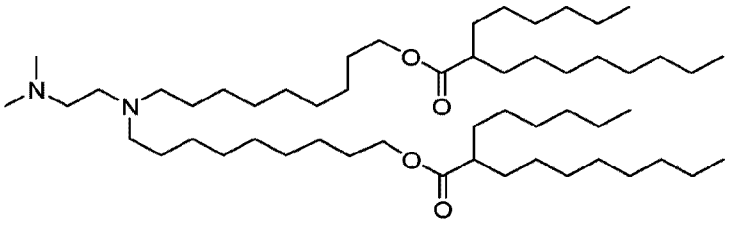
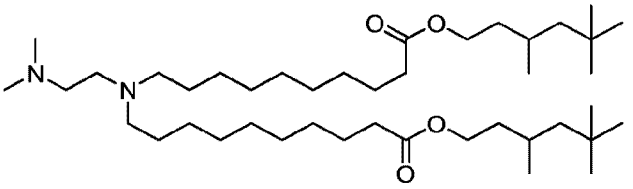
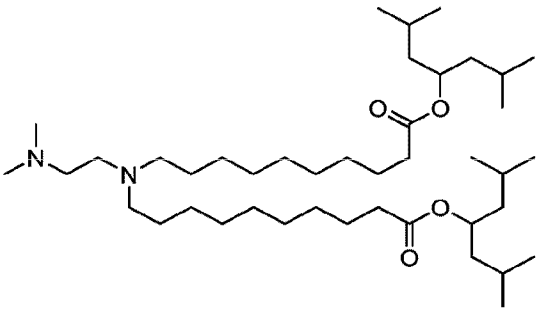
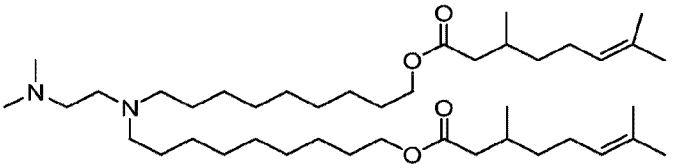
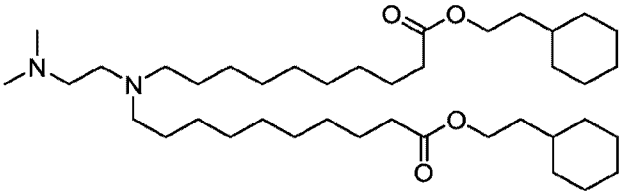
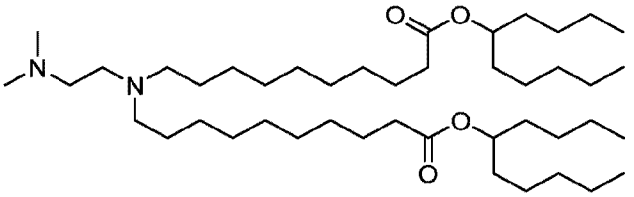
atom to which they are attached, form a 5-membered heterocyclic ring, for example a pyrrolidinyl ring.

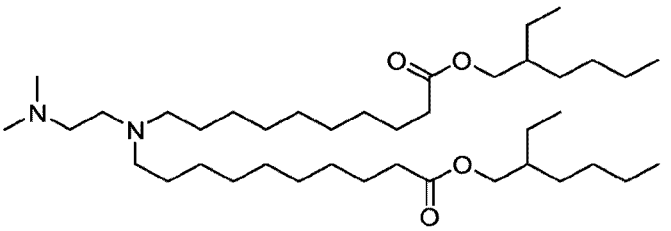
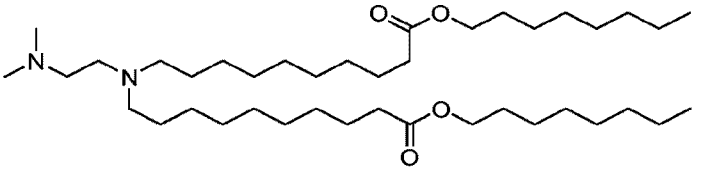
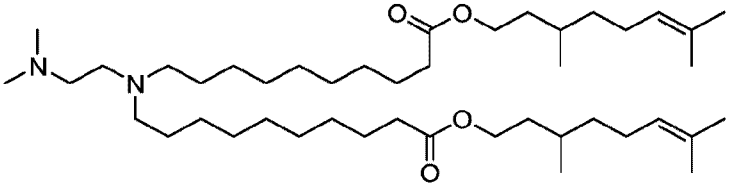
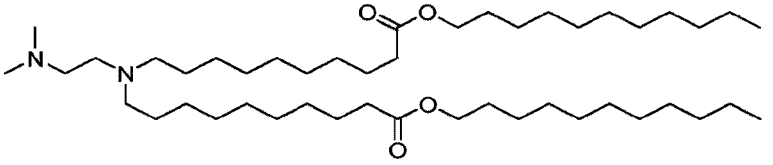
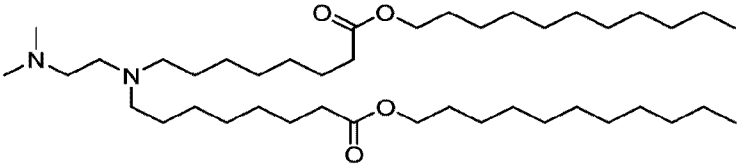
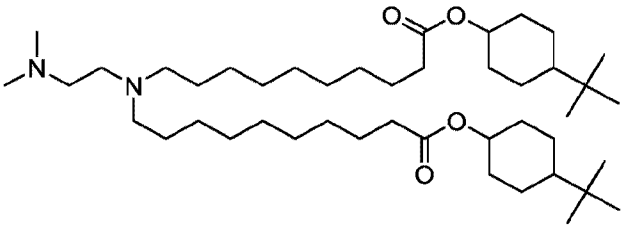
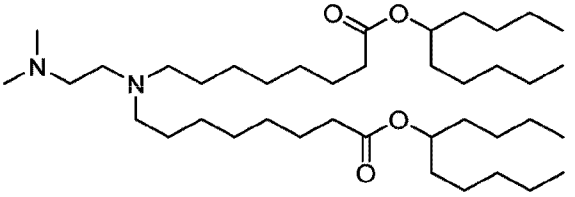
In some embodiments of Embodiment 3, the first and second cationic lipids are each, independently selected from a lipid of Formula I.

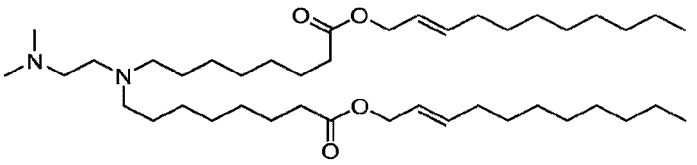
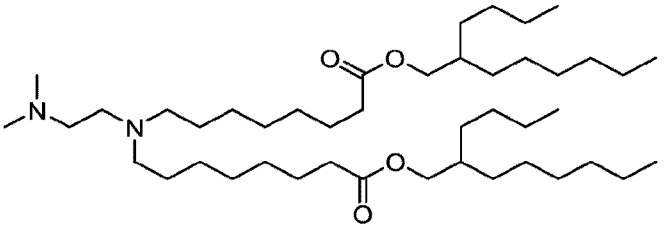
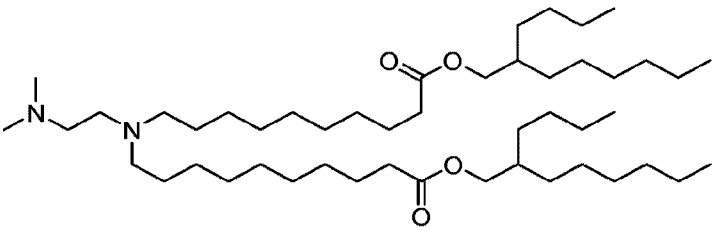
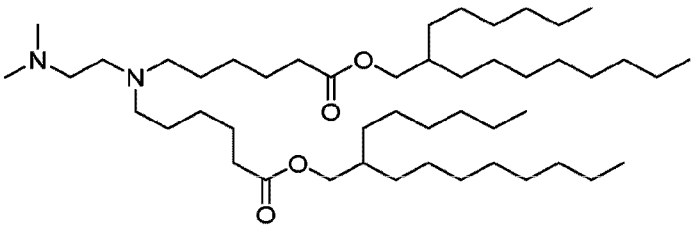
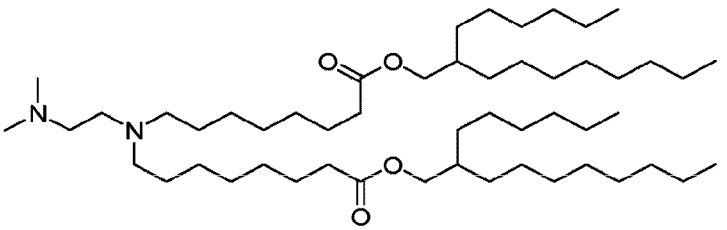
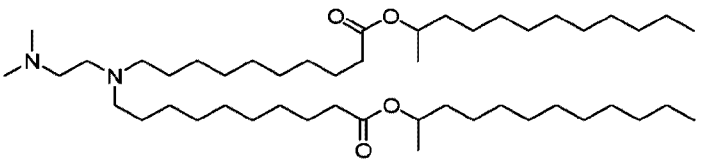
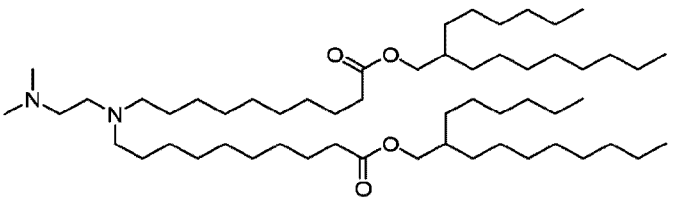
In various different embodiments, the lipid of Formula I has one of the structures set forth in Table 1 below.

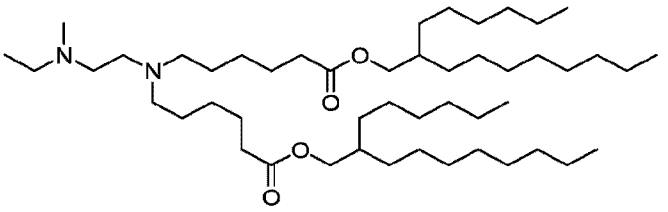
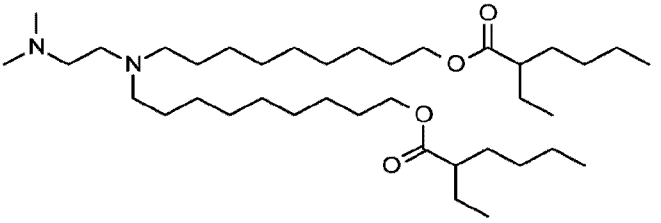
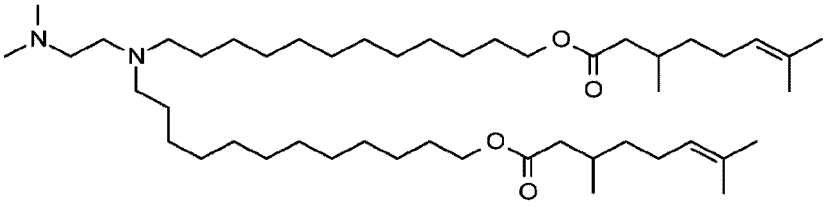
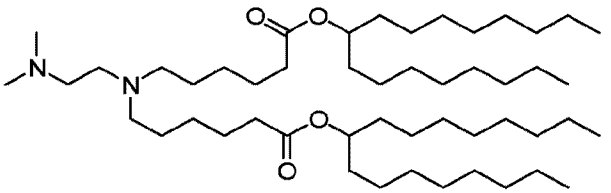
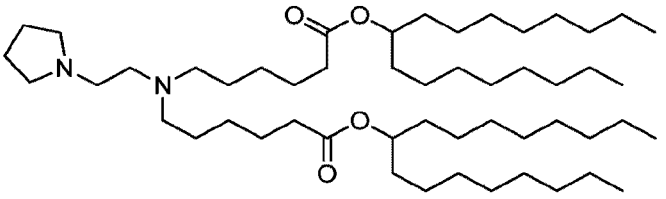
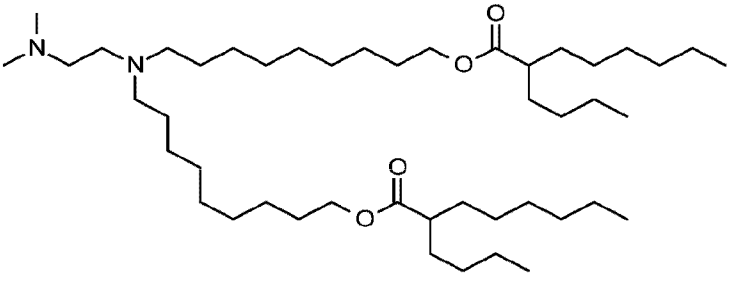
Table 1: Representative Lipids of Formula I

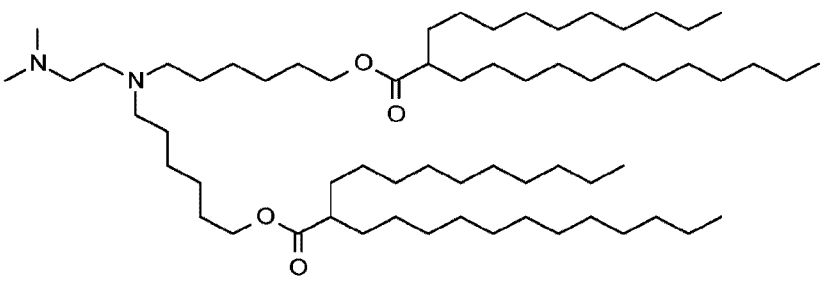
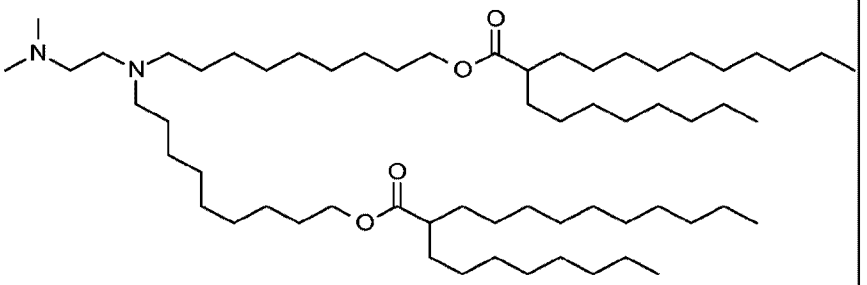
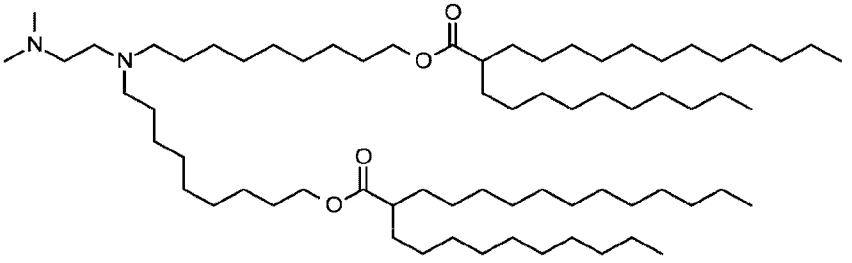
No.	Structure	pKa
I-1		-
I-2		5.64
I-3		7.15
I-4		6.43
I-5		6.28

No.	Structure	pKa
I-6		6.12
I-7		-
I-8		-
I-9		-
I-10		-
I-11		6.36

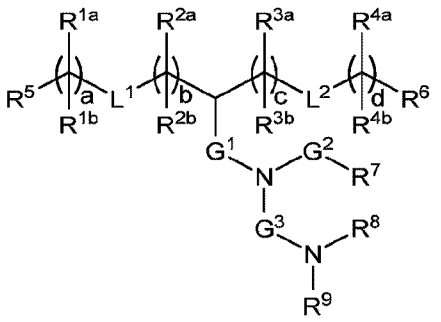
No.	Structure	pKa
I-12		-
I-13		6.51
I-14		-
I-15		6.30
I-16		6.63
I-17		-
I-18		-

No.	Structure	pKa
I-19		6.72
I-20		6.44
I-21		6.28
I-22		6.53
I-23		6.24
I-24		6.28
I-25		6.20

No.	Structure	pKa
I-33		6.27
I-34		-
I-35		6.21
I-36		-
I-37		-
I-38		6.24

No.	Structure	pKa
I-39		5.82
I-40		6.38
I-41		5.91

In some embodiments, the cationic lipid of Embodiments 1, 2, 3, 4 or 5 has a structure of Formula II:



II

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

one of L^1 or L^2 is –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -$S(O)_x$-, -S-S-, -C(=O)S-, SC(=O)-, -$NR^aC(=O)$-, -$C(=O)NR^a$-, $NR^aC(=O)NR^a$-, -$OC(=O)NR^a$- or -$NR^aC(=O)O$-, and the other of L^1 or L^2 is –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -$S(O)_x$-, -S-S-, -C(=O)S-, SC(=O)-, -$NR^aC(=O)$-, -$C(=O)NR^a$-, ,$NR^aC(=O)NR^a$-, -$OC(=O)NR^a$- or -$NR^aC(=O)O$- or a direct bond;

G^1 is C_1-C_2 alkylene, -(C=O)-, -O(C=O)-, -SC(=O)-, -$NR^aC(=O)$- or a direct bond;

G^2 is –C(=O)-, -(C=O)O-, -C(=O)S-, -$C(=O)NR^a$- or a direct bond;

G^3 is C_1-C_6 alkylene;

R^a is H or C_1-C_{12} alkyl;

R^{1a} and R^{1b} are, at each occurrence, independently either: (a) H or C_1-C_{12} alkyl; or (b) R^{1a} is H or C_1-C_{12} alkyl, and R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{2a} and R^{2b} are, at each occurrence, independently either: (a) H or C_1-C_{12} alkyl; or (b) R^{2a} is H or C_1-C_{12} alkyl, and R^{2b} together with the carbon atom to which it is bound is taken together with an adjacent R^{2b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{3a} and R^{3b} are, at each occurrence, independently either (a): H or C_1-C_{12} alkyl; or (b) R^{3a} is H or C_1-C_{12} alkyl and R^{3b} together with the carbon atom to which it

is bound is taken together with an adjacent R^{3b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{4a} and R^{4b} are, at each occurrence, independently either: (a) H or C_1-C_{12} alkyl; or (b) R^{4a} is H or C_1-C_{12} alkyl, and R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^5 and R^6 are each independently H or methyl;

R^7 is C_4-C_{20} alkyl;

R^8 and R^9 are each independently C_1-C_{12} alkyl; or R^8 and R^9, together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring;

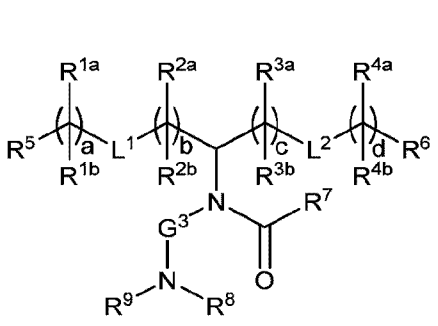
a, b, c and d are each independently an integer from 1 to 24; and

x is 0, 1 or 2.

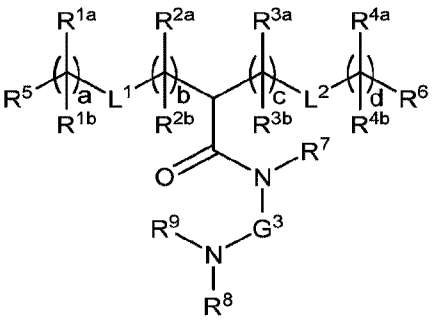
In some embodiments of Formula (II), L^1 and L^2 are each independently –O(C=O)-, -(C=O)O- or a direct bond. In other embodiments, G^1 and G^2 are each independently -(C=O)- or a direct bond. In some different embodiments, L^1 and L^2 are each independently –O(C=O)-, -(C=O)O- or a direct bond; and G^1 and G^2 are each independently –(C=O)- or a direct bond.

In some different embodiments of Formula (II), L^1 and L^2 are each independently -C(=O)-, -O-, -$S(O)_x$-, -S-S-, -C(=O)S-, -SC(=O)-, -NR^a-, -NR^aC(=O)-, -C(=O)NR^a-, -NR^aC(=O)NR^a, -OC(=O)NR^a-, -NR^aC(=O)O-, -$NR^aS(O)_xNR^a$-, -$NR^aS(O)_x$- or -$S(O)_xNR^a$-.

In other of the foregoing embodiments of Formula (II), the lipid compound has one of the following Formulae (IIA) or (IIB):



or



(IIA) (IIB)

In some embodiments of Formula (II), the lipid compound has Formula (IIA). In other embodiments, the lipid compound has Formula (IIB).

In any of the foregoing embodiments of Formula (II), one of L^1 or L^2 is -O(C=O)-. For example, in some embodiments each of L^1 and L^2 are -O(C=O)-.

In some different embodiments of Formula (II), one of L^1 or L^2 is -(C=O)O-. For example, in some embodiments each of L^1 and L^2 is -(C=O)O-.

In different embodiments of Formula (II), one of L^1 or L^2 is a direct bond. As used herein, a "direct bond" means the group (*e.g.*, L^1 or L^2) is absent. For example, in some embodiments each of L^1 and L^2 is a direct bond.

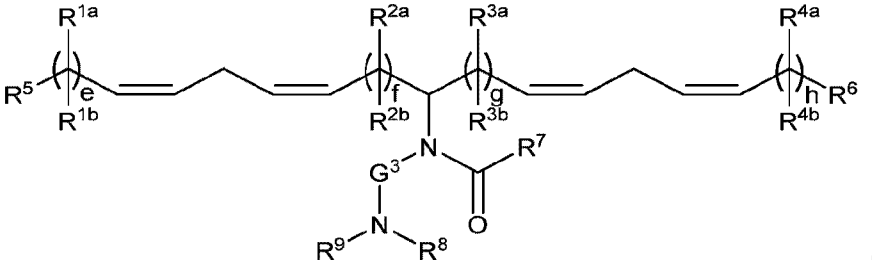
In other different embodiments of Formula (II), for at least one occurrence of R^{1a} and R^{1b}, R^{1a} is H or C_1-C_{12} alkyl, and R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

In still other different embodiments of Formula (II), for at least one occurrence of R^{4a} and R^{4b}, R^{4a} is H or C_1-C_{12} alkyl, and R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

In more embodiments of Formula (II), for at least one occurrence of R^{2a} and R^{2b}, R^{2a} is H or C_1-C_{12} alkyl, and R^{2b} together with the carbon atom to which it is bound is taken together with an adjacent R^{2b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

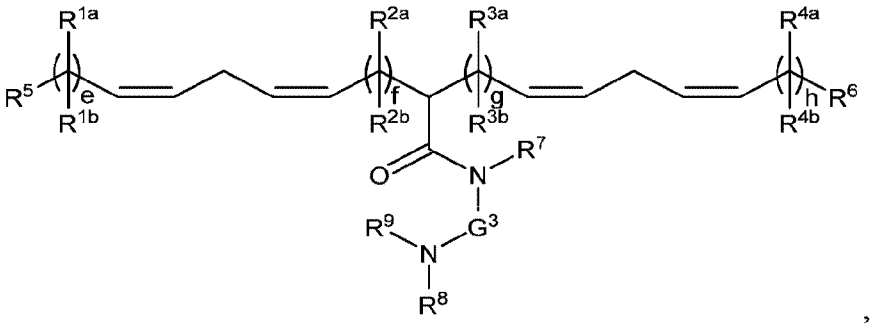
In other different embodiments of Formula (II), for at least one occurrence of R^{3a} and R^{3b}, R^{3a} is H or C_1-C_{12} alkyl, and R^{3b} together with the carbon atom to which it is bound is taken together with an adjacent R^{3b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

In various other embodiments of Formula (II), the lipid compound has one of the following Formulae (IIC) or (IID):



or

(IIC)



,

(IID)

wherein e, f, g and h are each independently an integer from 1 to 12.

In some embodiments of Formula (II), the lipid compound has Formula (IIC). In other embodiments, the lipid compound has Formula (IID).

In various embodiments of Formulae (IIC) or (IID), e, f, g and h are each independently an integer from 4 to 10.

In certain embodiments of Formula (II), a, b, c and d are each independently an integer from 2 to 12 or an integer from 4 to 12. In other embodiments, a, b, c and d are each independently an integer from 8 to 12 or 5 to 9. In some certain embodiments, a is 0. In some embodiments, a is 1. In other embodiments,

a is 2. In more embodiments, a is 3. In yet other embodiments, a is 4. In some embodiments, a is 5. In other embodiments, a is 6. In more embodiments, a is 7. In yet other embodiments, a is 8. In some embodiments, a is 9. In other embodiments, a is 10. In more embodiments, a is 11. In yet other embodiments, a is 12. In some embodiments, a is 13. In other embodiments, a is 14. In more embodiments, a is 15. In yet other embodiments, a is 16.

In some embodiments of Formula (II), b is 1. In other embodiments, b is 2. In more embodiments, b is 3. In yet other embodiments, b is 4. In some embodiments, b is 5. In other embodiments, b is 6. In more embodiments, b is 7. In yet other embodiments, b is 8. In some embodiments, b is 9. In other embodiments, b is 10. In more embodiments, b is 11. In yet other embodiments, b is 12. In some embodiments, b is 13. In other embodiments, b is 14. In more embodiments, b is 15. In yet other embodiments, b is 16.

In some embodiments of Formula (II), c is 1. In other embodiments, c is 2. In more embodiments, c is 3. In yet other embodiments, c is 4. In some embodiments, c is 5. In other embodiments, c is 6. In more embodiments, c is 7. In yet other embodiments, c is 8. In some embodiments, c is 9. In other embodiments, c is 10. In more embodiments, c is 11. In yet other embodiments, c is 12. In some embodiments, c is 13. In other embodiments, c is 14. In more embodiments, c is 15. In yet other embodiments, c is 16.

In some certain embodiments of Formula (II), d is 0. In some embodiments, d is 1. In other embodiments, d is 2. In more embodiments, d is 3. In yet other embodiments, d is 4. In some embodiments, d is 5. In other embodiments, d is 6. In more embodiments, d is 7. In yet other embodiments, d is 8. In some embodiments, d is 9. In other embodiments, d is 10. In more embodiments, d is 11. In yet other embodiments, d is 12. In some embodiments, d is 13. In other embodiments, d is 14. In more embodiments, d is 15. In yet other embodiments, d is 16.

In some embodiments of Formula (II), e is 1. In other embodiments, e is 2. In more embodiments, e is 3. In yet other embodiments, e is 4. In some embodiments, e is 5. In other embodiments, e is 6. In more embodiments, e is 7. In yet

other embodiments, e is 8. In some embodiments, e is 9. In other embodiments, e is 10. In more embodiments, e is 11. In yet other embodiments, e is 12.

In some embodiments of Formula (II), f is 1. In other embodiments, f is 2. In more embodiments, f is 3. In yet other embodiments, f is 4. In some embodiments, f is 5. In other embodiments, f is 6. In more embodiments, f is 7. In yet other embodiments, f is 8. In some embodiments, f is 9. In other embodiments, f is 10. In more embodiments, f is 11. In yet other embodiments, f is 12.

In some embodiments of Formula (II), g is 1. In other embodiments, g is 2. In more embodiments, g is 3. In yet other embodiments, g is 4. In some embodiments, g is 5. In other embodiments, g is 6. In more embodiments, g is 7. In yet other embodiments, g is 8. In some embodiments, g is 9. In other embodiments, g is 10. In more embodiments, g is 11. In yet other embodiments, g is 12.

In some embodiments of Formula (II), h is 1. In other embodiments, e is 2. In more embodiments, h is 3. In yet other embodiments, h is 4. In some embodiments, e is 5. In other embodiments, h is 6. In more embodiments, h is 7. In yet other embodiments, h is 8. In some embodiments, h is 9. In other embodiments, h is 10. In more embodiments, h is 11. In yet other embodiments, h is 12.

In some other various embodiments of Formula (II), a and d are the same. In some other embodiments, b and c are the same. In some other specific embodiments and a and d are the same and b and c are the same.

The sum of a and b and the sum of c and d of Formula (II) are factors which may be varied to obtain a lipid having the desired properties. In one embodiment, a and b are chosen such that their sum is an integer ranging from 14 to 24. In other embodiments, c and d are chosen such that their sum is an integer ranging from 14 to 24. In further embodiment, the sum of a and b and the sum of c and d are the same. For example, in some embodiments the sum of a and b and the sum of c and d are both the same integer which may range from 14 to 24. In still more embodiments, a. b, c and d are selected such that the sum of a and b and the sum of c and d is 12 or greater.

The substituents at R^{1a}, R^{2a}, R^{3a} and R^{4a} of Formula (II) are not particularly limited. In some embodiments, at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is H. In certain embodiments R^{1a}, R^{2a}, R^{3a} and R^{4a} are H at each occurrence. In certain other embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_{12} alkyl. In certain other embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_8 alkyl. In certain other embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_6 alkyl. In some of the foregoing embodiments, the C_1-C_8 alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

In certain embodiments of Formula (II), R^{1a}, R^{1b}, R^{4a} and R^{4b} are C_1-C_{12} alkyl at each occurrence.

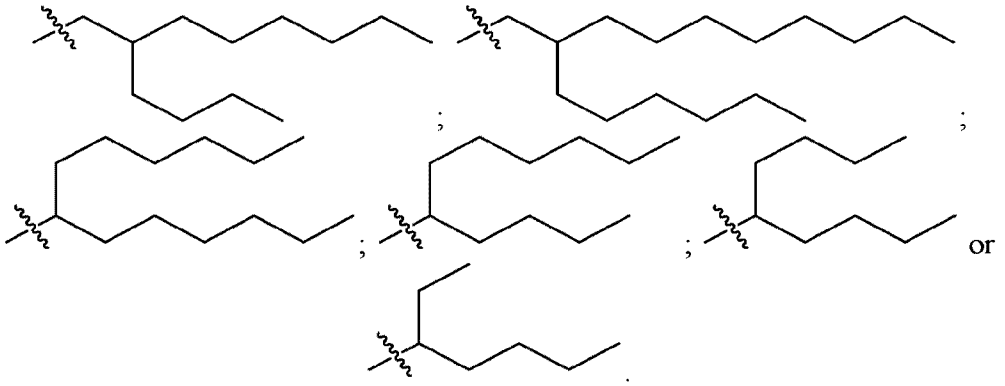
In further embodiments of Formula (II), at least one of R^{1b}, R^{2b}, R^{3b} and R^{4b} is H or R^{1b}, R^{2b}, R^{3b} and R^{4b} are H at each occurrence.

In certain embodiments of Formula (II), R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond. In other embodiments of the foregoing R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

The substituents at R^5 and R^6 of Formula (II) are not particularly limited in the foregoing embodiments. In certain embodiments one of R^5 or R^6 is methyl. In other embodiments each of R^5 or R^6 is methyl.

The substituents at R^7 of Formula (II) are not particularly limited in the foregoing embodiments. In certain embodiments R^7 is C_6-C_{16} alkyl. In some other embodiments, R^7 is C_6-C_9 alkyl. In some of these embodiments, R^7 is substituted with -(C=O)OR^b, –O(C=O)R^b, -C(=O)R^b, -OR^b, -$S(O)_xR^b$, -S-SR^b, -C(=O)SR^b, -SC(=O)R^b, -NR^aR^b, -$NR^aC(=O)R^b$, -C(=O)NR^aR^b, -$NR^aC(=O)NR^aR^b$, -OC(=O)NR^aR^b, -$NR^aC(=O)OR^b$, -$NR^aS(O)_xNR^aR^b$, -$NR^aS(O)_xR^b$ or -$S(O)_xNR^aR^b$, wherein: R^a is H or C_1-C_{12} alkyl; R^b is C_1-C_{15} alkyl; and x is 0, 1 or 2. For example, in some embodiments R^7 is substituted with -(C=O)OR^b or –O(C=O)R^b.

In some of the foregoing embodiments of Formula (II), R^b is branched C_1-C_{16} alkyl. For example, in some embodiments R^b has one of the following structures:



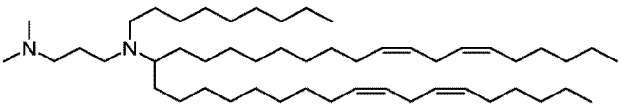
In certain other of the foregoing embodiments of Formula (II), one of R^8 or R^9 is methyl. In other embodiments, both R^8 and R^9 are methyl.

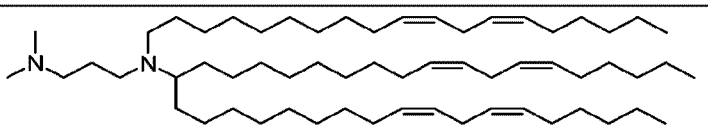
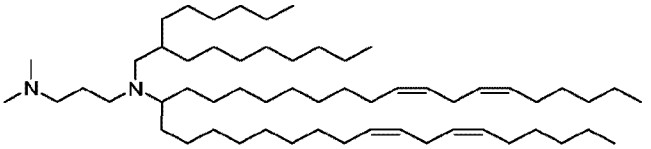
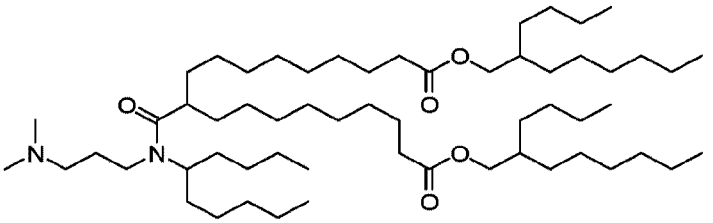
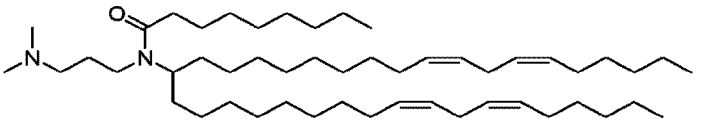
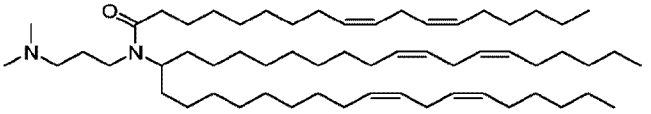
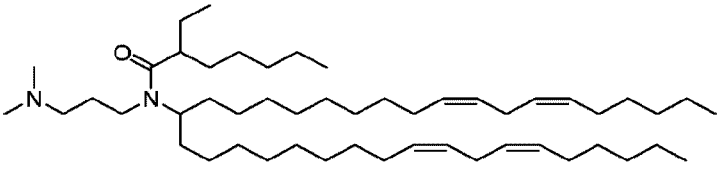
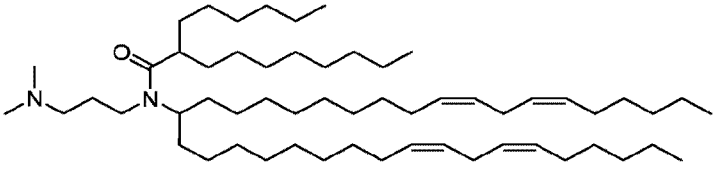
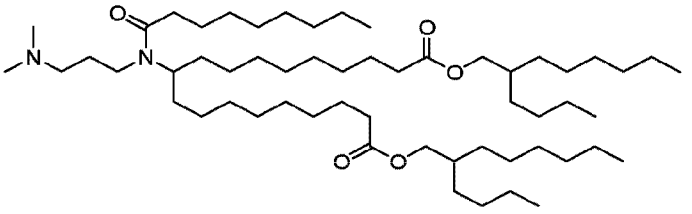
In some different embodiments of Formula (II), R^8 and R^9, together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring. In some embodiments of the foregoing, R^8 and R^9, together with the nitrogen atom to which they are attached, form a 5-membered heterocyclic ring, for example a pyrrolidinyl ring. In some different embodiments of the foregoing, R^8 and R^9, together with the nitrogen atom to which they are attached, form a 6-membered heterocyclic ring, for example a piperazinyl ring.

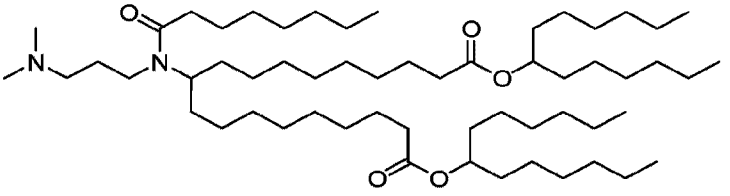
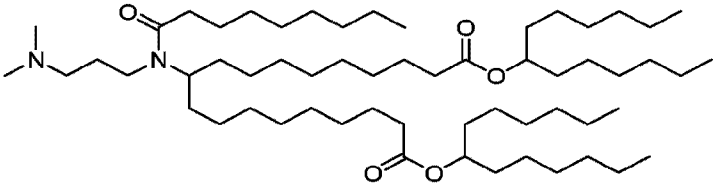
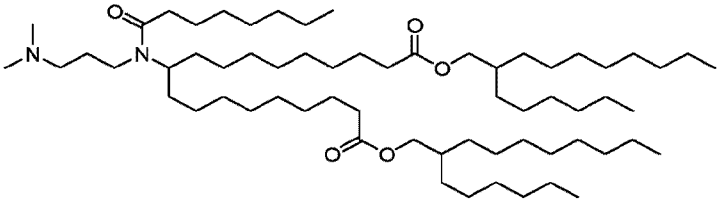
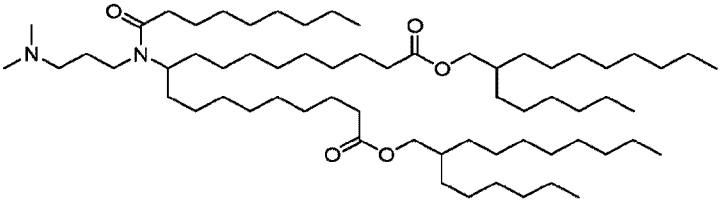
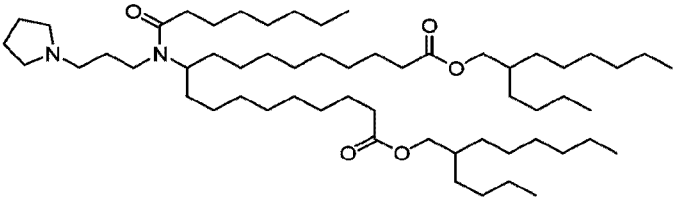
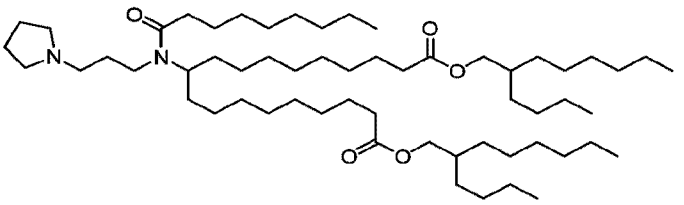
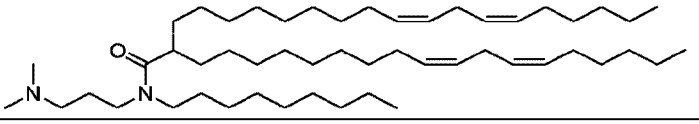
In certain embodiments of Embodiment 3, the first and second cationic lipids are each, independently selected from a lipid of Formula II.

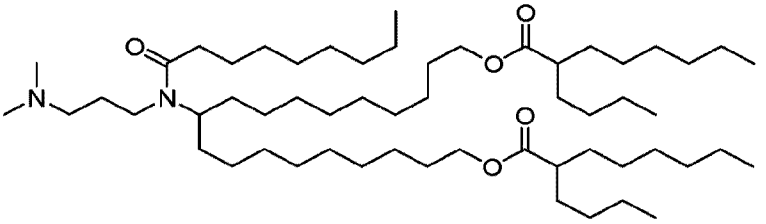
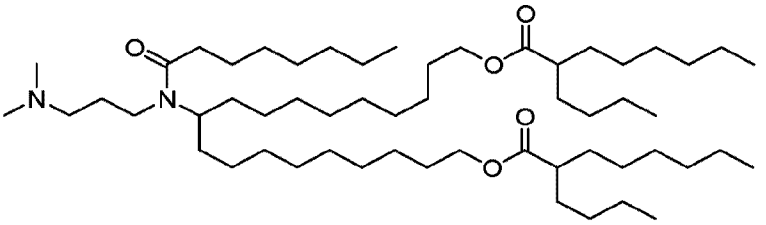
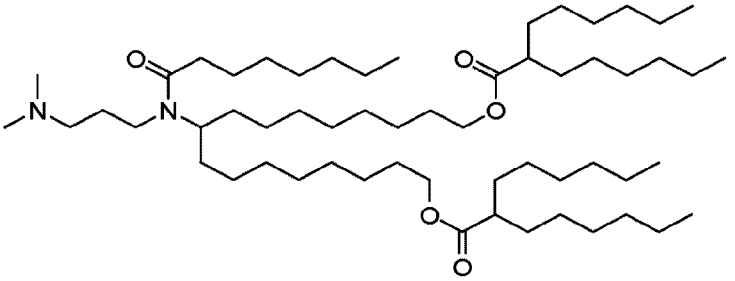
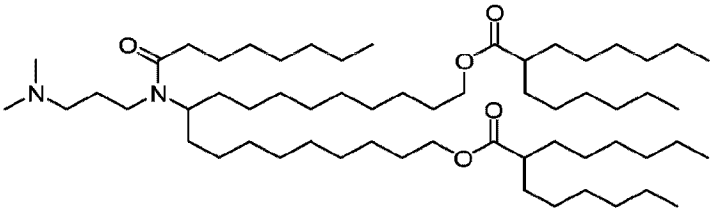
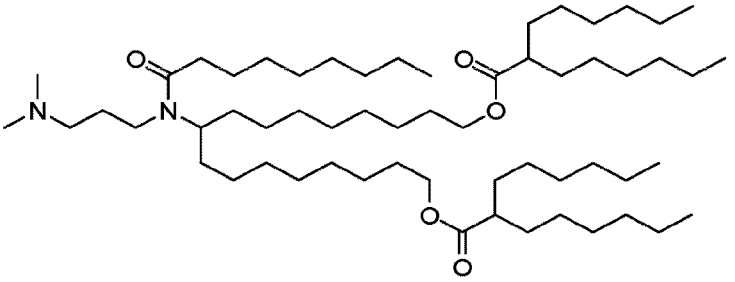
In still other embodiments of the foregoing lipids of Formula (II), G^3 is C_2-C_4 alkylene, for example C_3 alkylene. In various different embodiments, the lipid compound has one of the structures set forth in Table 2 below

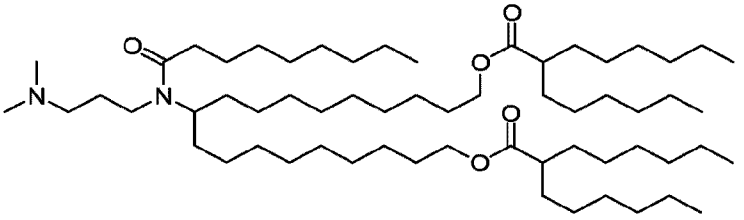
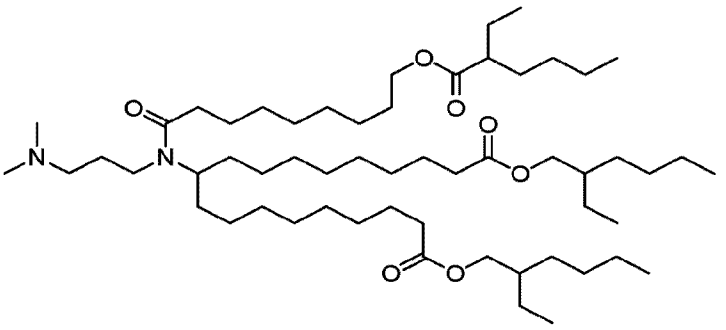
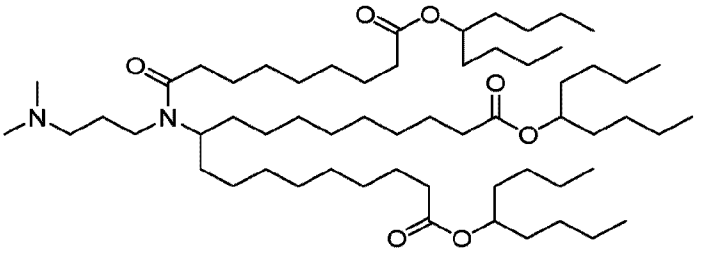
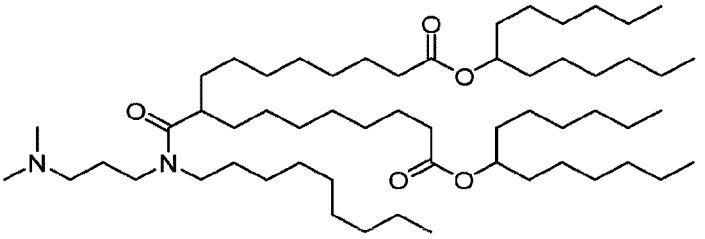
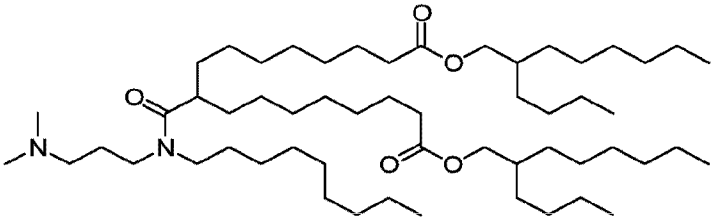
Table 2: Representative Lipids of Formula (II)

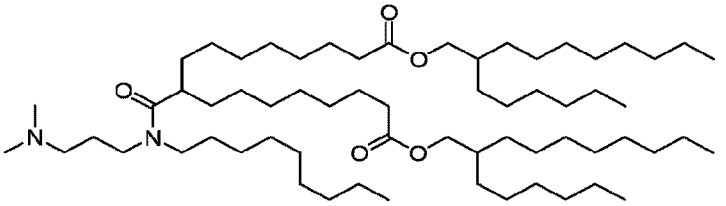
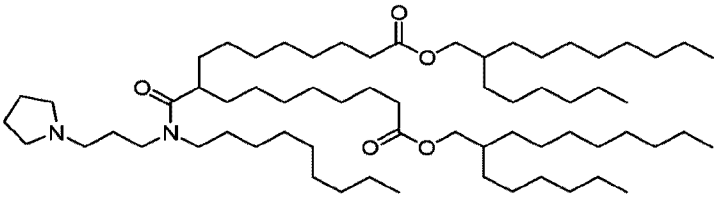
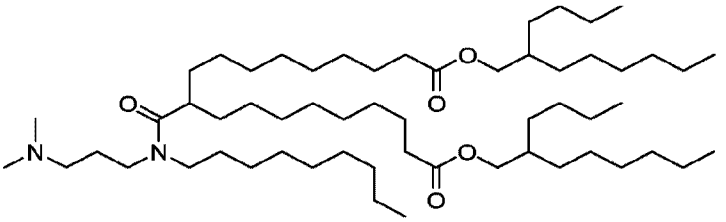
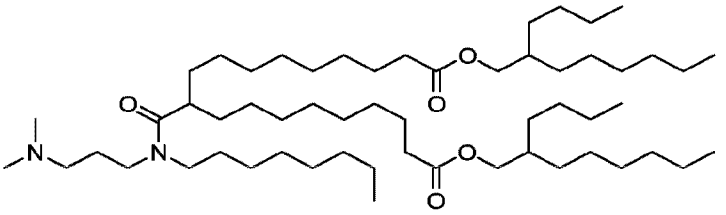
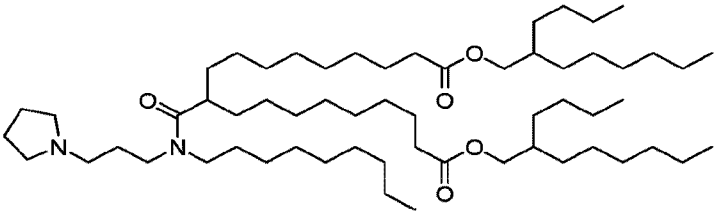
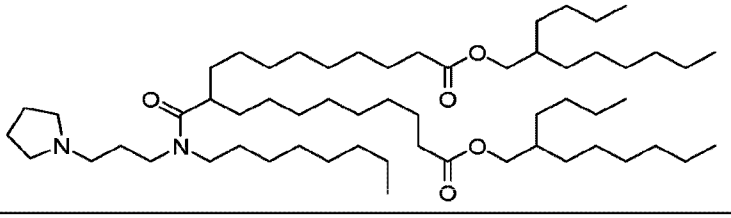
No.	Structure	pKa
II-1		5.64

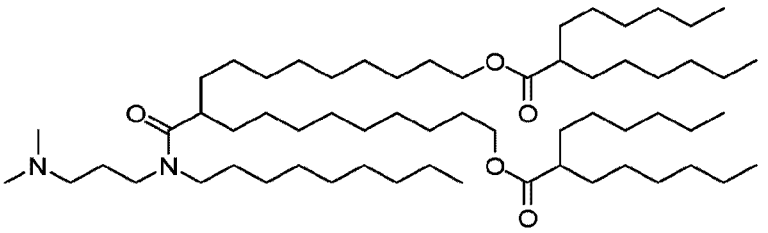
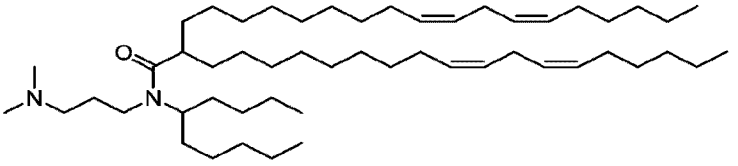
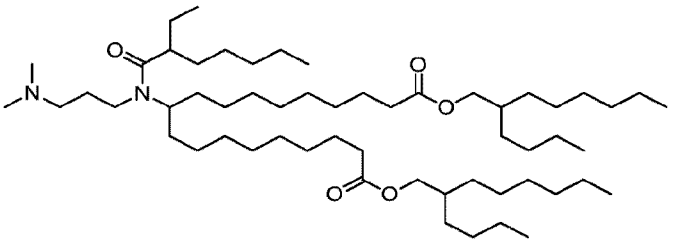
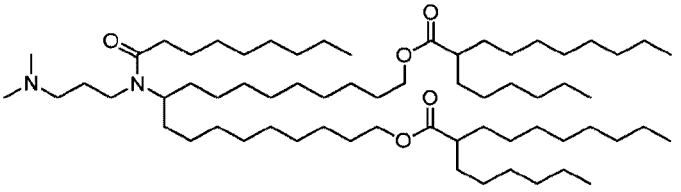
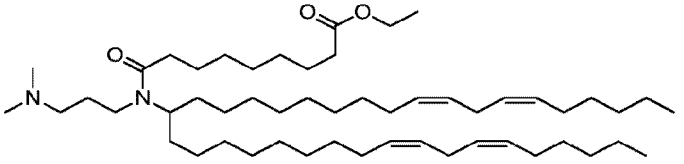
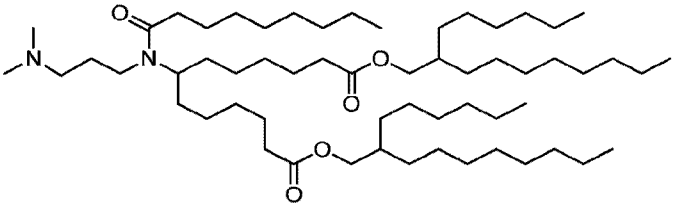
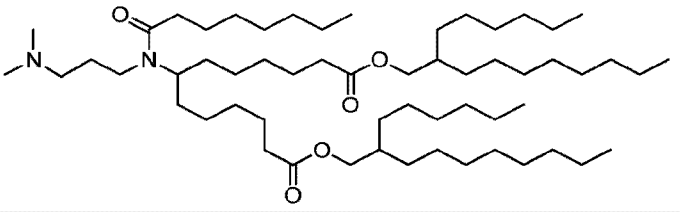
No.	Structure	pKa
II-2		-
II-3		-
II-4		-
II-5		6.27
II-6		6.14
II-7		5.93
II-8		5.35
II-9		6.27

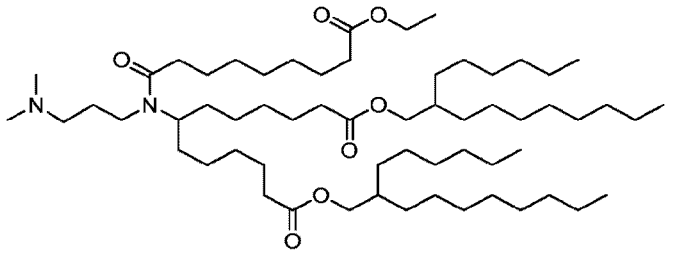
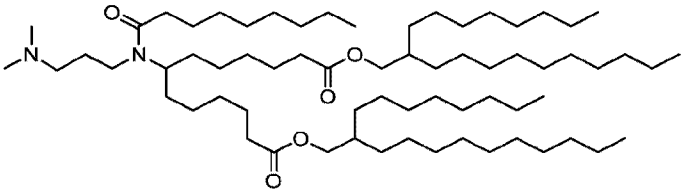
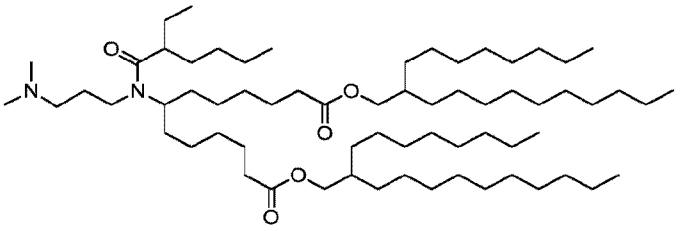
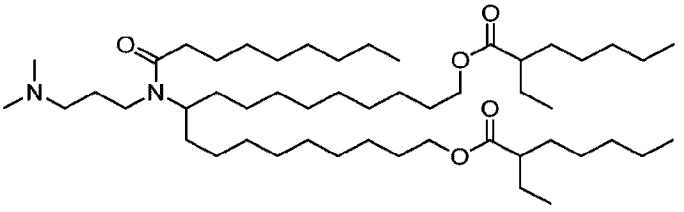
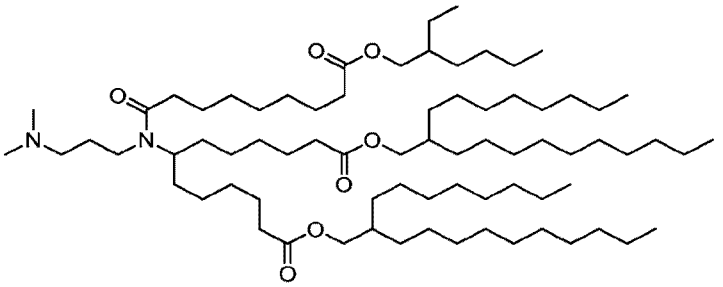
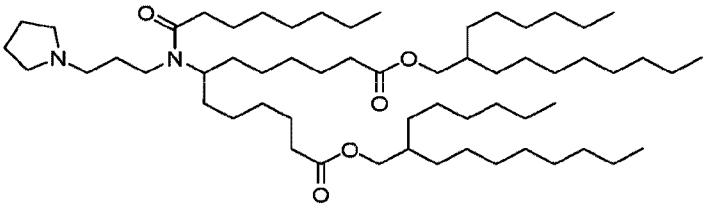
No.	Structure	pKa
II-10		6.16
II-11		6.13
II-12		6.21
II-13		6.22
II-14		6.33
II-15		6.32
II-16		6.37

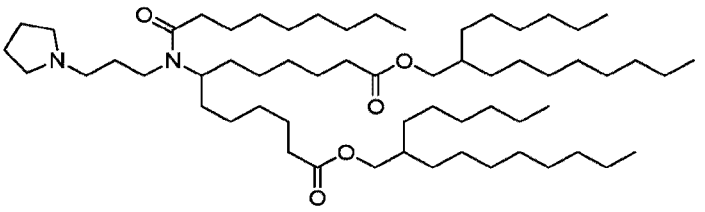
No.	Structure	pKa
II-17		6.27
II-18		-
II-19		-
II-20		-
II-21		-

No.	Structure	pKa
II-22		-
II-23		-
II-24		6.14
II-25		-
II-26		-

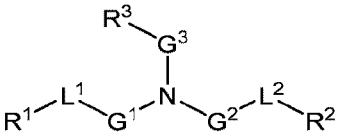
No.	Structure	pKa
II-27		-
II-28		-
II-29		-
II-30		-
II-31		-
II-32		-

No.	Structure	pKa
II-33		-
II-34		-
II-35		5.97
II-36		6.13
II-37		5.61
II-38		6.45
II-39		6.45

No.	Structure	pKa
II-40		6.57
II-41		-
II-42		-
II-43		-
II-44		-
II-45		-

No.	Structure	pKa
II-46		-

In some other embodiments, the cationic lipid of Embodiments 1, 2, 3, 4 or 5 has a structure of Formula III:



III

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

one of L^1 or L^2 is –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -S(O)$_x$-, -S-S-, -C(=O)S-, SC(=O)-, -NR^{a}C(=O)-, -C(=O)NR^a-, NR^{a}C(=O)NR^a-, -OC(=O)NR^a- or -NR^{a}C(=O)O-, and the other of L^1 or L^2 is –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -S(O)$_x$-, -S-S-, -C(=O)S-, SC(=O)-, -NR^{a}C(=O)-, -C(=O)NR^a-, ,NR^{a}C(=O)NR^a-, -OC(=O)NR^a- or -NR^{a}C(=O)O- or a direct bond;

G^1 and G^2 are each independently unsubstituted C_1-C_{12} alkylene or C_1-C_{12} alkenylene;

G^3 is C_1-C_{24} alkylene, C_1-C_{24} alkenylene, C_3-C_8 cycloalkylene, C_3-C_8 cycloalkenylene;

R^a is H or C_1-C_{12} alkyl;

R^1 and R^2 are each independently C_6-C_{24} alkyl or C_6-C_{24} alkenyl;

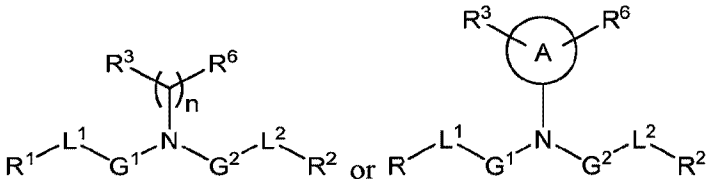
R^3 is H, OR^5, CN, -C(=O)OR^4, -OC(=O)R^4 or –NR^{5}C(=O)R^4;

R^4 is C_1-C_{12} alkyl;

R^5 is H or C_1-C_6 alkyl; and

x is 0, 1 or 2.

In some of the foregoing embodiments of Formula (III), the lipid has one of the following Formulae (IIIA) or (IIIB):



(IIIA) (IIIB)

wherein:

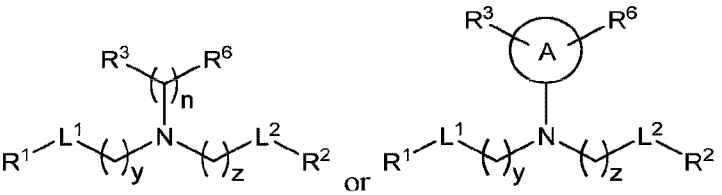
A is a 3 to 8-membered cycloalkyl or cycloalkylene ring;

R^6 is, at each occurrence, independently H, OH or C_1-C_{24} alkyl;

n is an integer ranging from 1 to 15.

In some of the foregoing embodiments of Formula (III), the lipid has Formula (IIIA), and in other embodiments, the lipid has Formula (IIIB).

In other embodiments of Formula (III), the lipid has one of the following Formulae (IIIC) or (IIID):

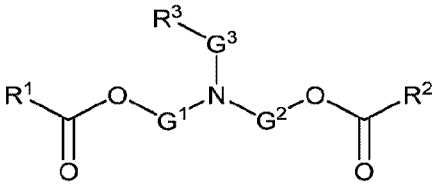


(IIIC) (IIID)

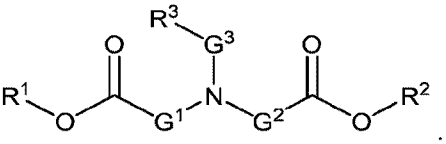
wherein y and z are each independently integers ranging from 1 to 12.

In any of the foregoing embodiments of Formula (III), one of L^1 or L^2 is -O(C=O)-. For example, in some embodiments each of L^1 and L^2 are -O(C=O)-. In some different embodiments of any of the foregoing, L^1 and L^2 are each independently -(C=O)O- or -O(C=O)-. For example, in some embodiments each of L^1 and L^2 is -(C=O)O-.

In some different embodiments of Formula (III), the lipid has one of the following Formulae (IIIE) or (IIIF):

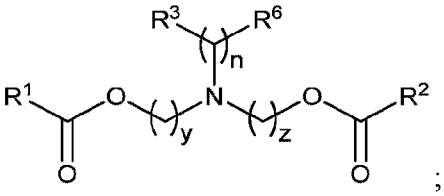


or

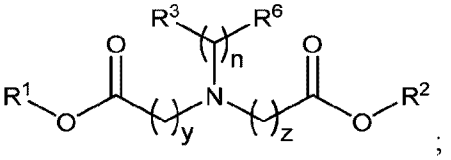


(IIIE) (IIIF)

In some of the foregoing embodiments of Formula (III), the lipid has one of the following Formulae (IIIG), (IIIH), (III), or (IIIJ):

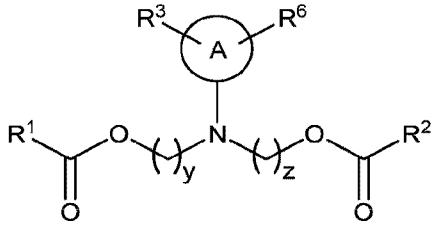


;

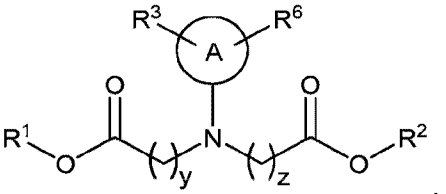


;

(IIIG) (IIIH)



or



(III) (IIIJ)

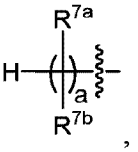
In some of the foregoing embodiments of Formula (III), n is an integer ranging from 2 to 12, for example from 2 to 8 or from 2 to 4. For example, in some embodiments, n is 3, 4, 5 or 6. In some embodiments, n is 3. In some embodiments, n is 4. In some embodiments, n is 5. In some embodiments, n is 6.

In some other of the foregoing embodiments of Formula (III), y and z are each independently an integer ranging from 2 to 10. For example, in some embodiments, y and z are each independently an integer ranging from 4 to 9 or from 4 to 6.

In some of the foregoing embodiments of Formula (III), R^6 is H. In other of the foregoing embodiments, R^6 is C_1-C_{24} alkyl. In other embodiments, R^6 is OH.

In some embodiments of Formula (III), G^3 is unsubstituted. In other embodiments, G3 is substituted. In various different embodiments, G^3 is linear C_1-C_{24} alkylene or linear C_1-C_{24} alkenylene.

In some other foregoing embodiments of Formula (III), R^1 or R^2, or both, is C_6-C_{24} alkenyl. For example, in some embodiments, R^1 and R^2 each, independently have the following structure:



,

wherein:

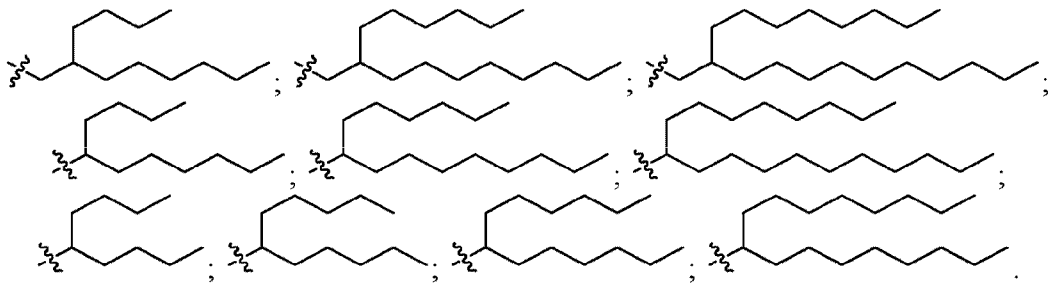
R^{7a} and R^{7b} are, at each occurrence, independently H or C_1-C_{12} alkyl; and

a is an integer from 2 to 12,

wherein R^{7a}, R^{7b} and a are each selected such that R^1 and R^2 each independently comprise from 6 to 20 carbon atoms. For example, in some embodiments a is an integer ranging from 5 to 9 or from 8 to 12.

In some of the foregoing embodiments of Formula (III), at least one occurrence of R^{7a} is H. For example, in some embodiments, R^{7a} is H at each occurrence. In other different embodiments of the foregoing, at least one occurrence of R^{7b} is C_1-C_8 alkyl. For example, in some embodiments, C_1-C_8 alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

In different embodiments of Formula (III), R^1 or R^2, or both, has one of the following structures:

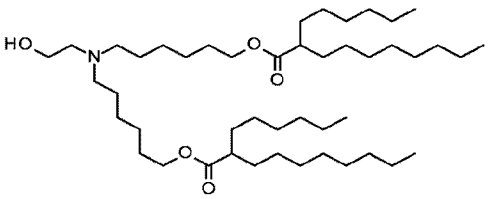
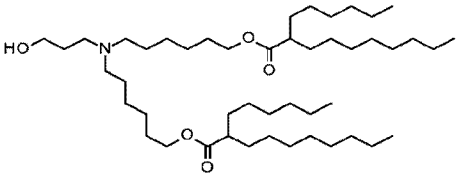
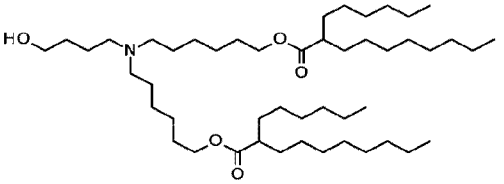
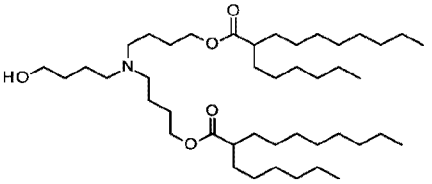

.

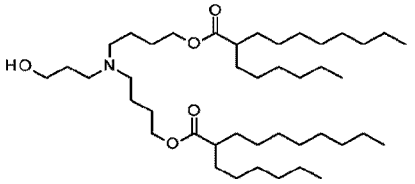
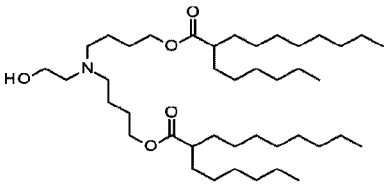
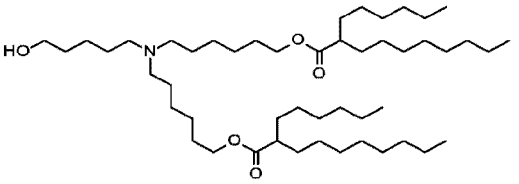
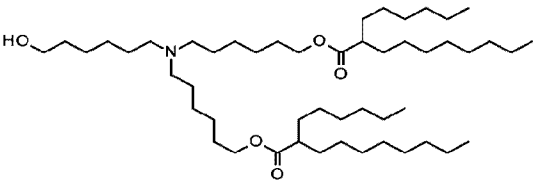
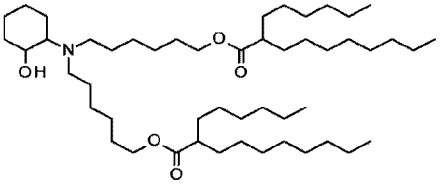
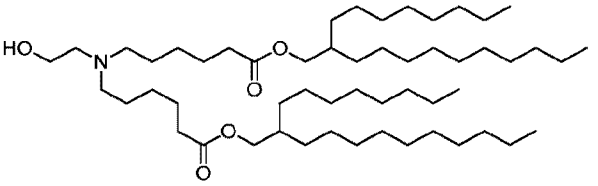
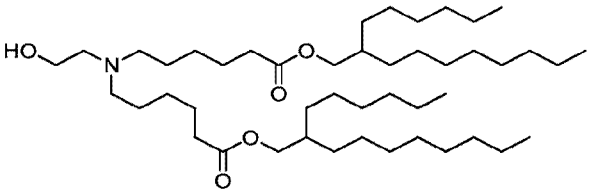
In some of the foregoing embodiments of Formula (III), R^3 is OH, CN, -C(=O)OR^4, -OC(=O)R^4 or –NHC(=O)R^4. In some embodiments, R^4 is methyl or ethyl.

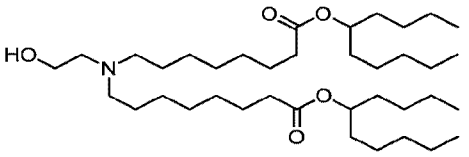
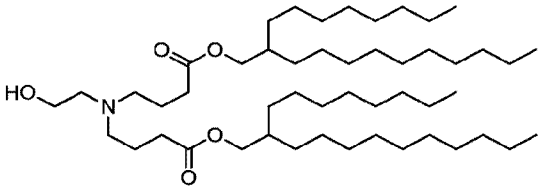
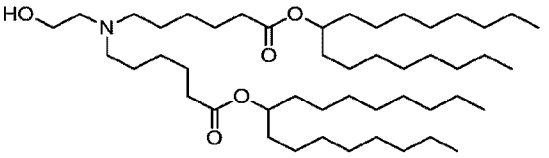
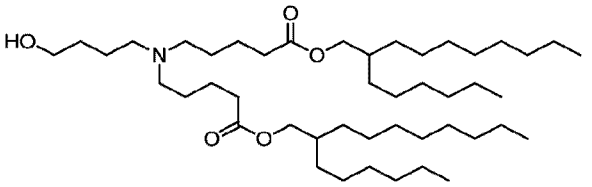
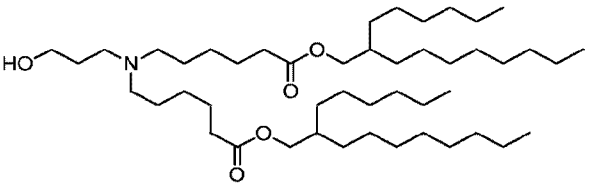
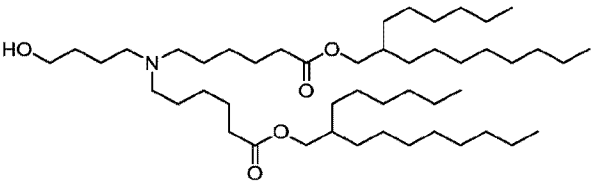
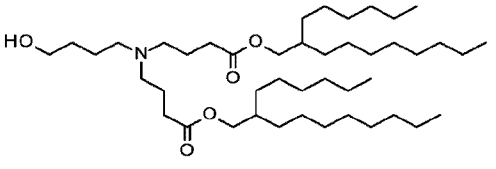
In some specific embodiments of Embodiment 3, the first and second cationic lipids are each, independently selected from a lipid of Formula III.

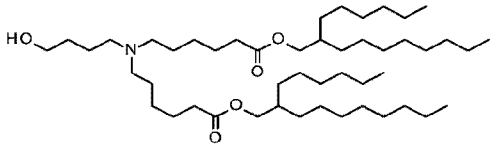
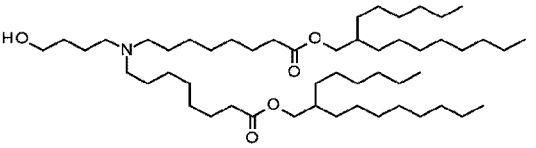
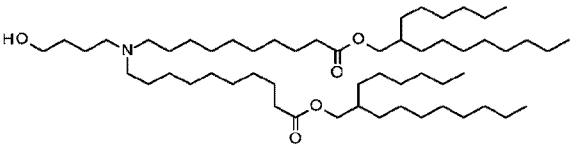
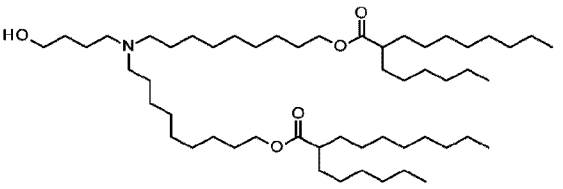
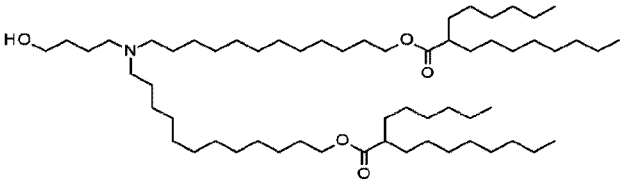
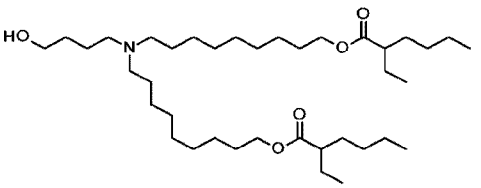
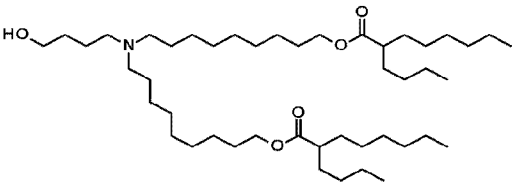
In various different embodiments, a cationic lipid of any one of the disclosed embodiments (*e.g.*, the cationic lipid, the first cationic lipid, the second cationic lipid) of Formula (III) has one of the structures set forth in Table 3 below.

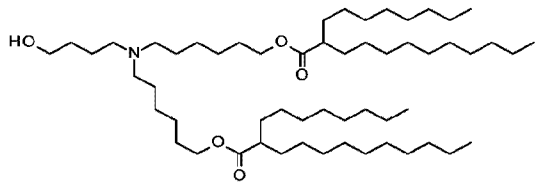
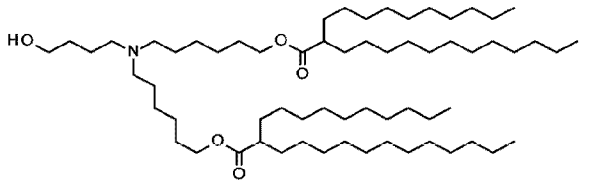
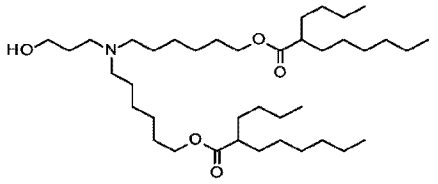
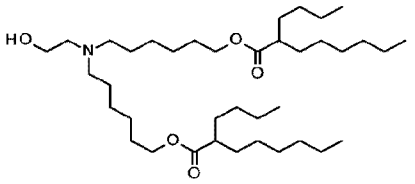
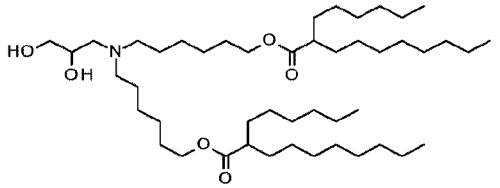
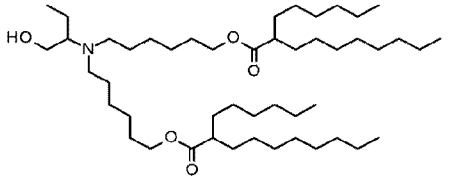
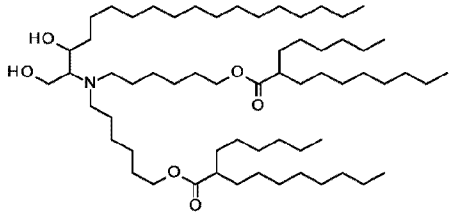
Table 3: Representative Compounds of Formula (III)

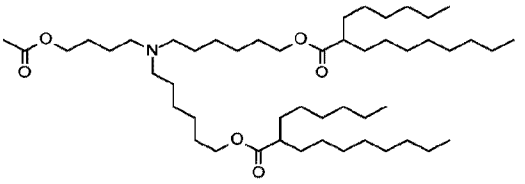
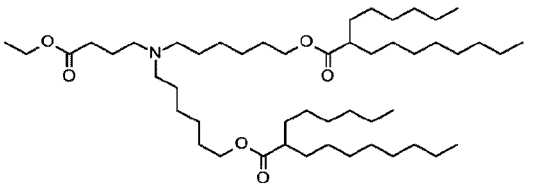
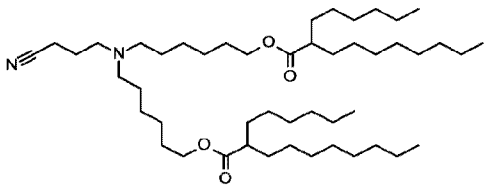
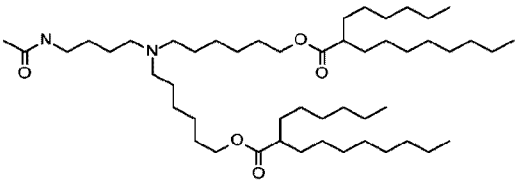
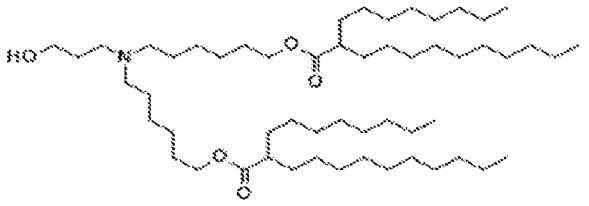
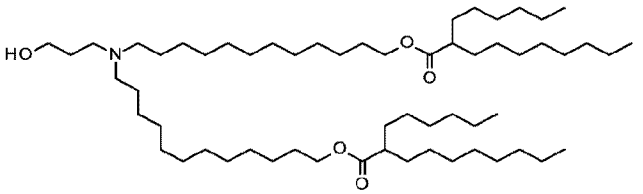
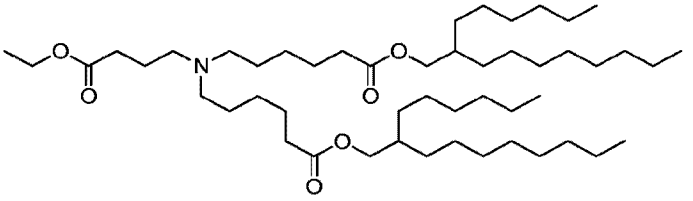
No.	Structure	pKa
III-1		5.89
III-2		6.05
III-3		6.09
III-4		5.60

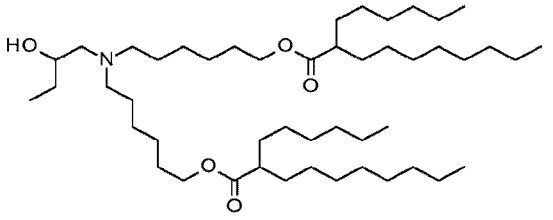
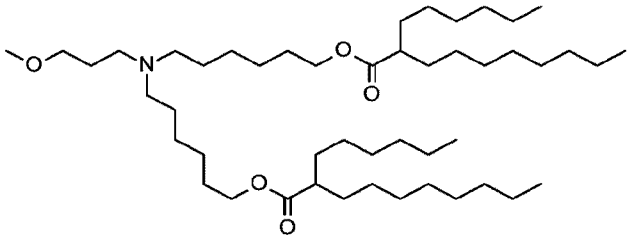
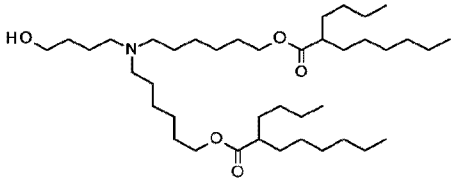
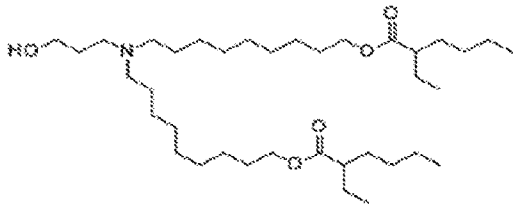
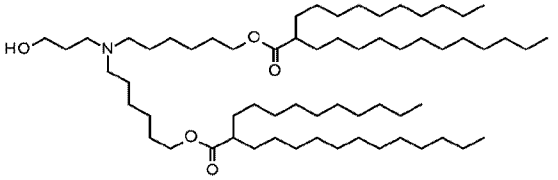
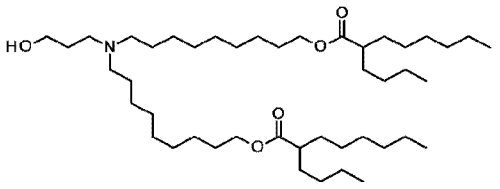
No.	Structure	pKa
III-5		5.59
III-6		5.42
III-7		6.11
III-8		5.84
III-9		-
III-10		-
III-11		-

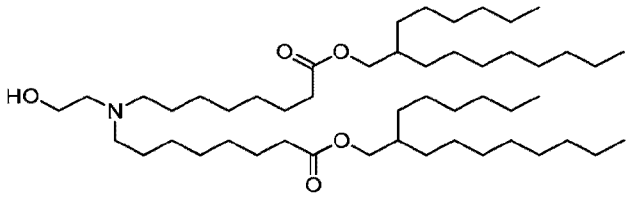
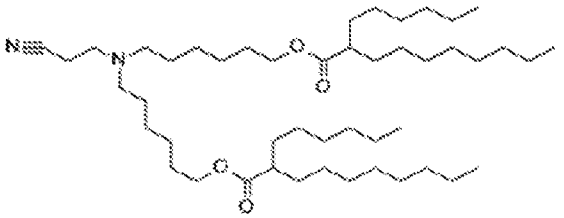
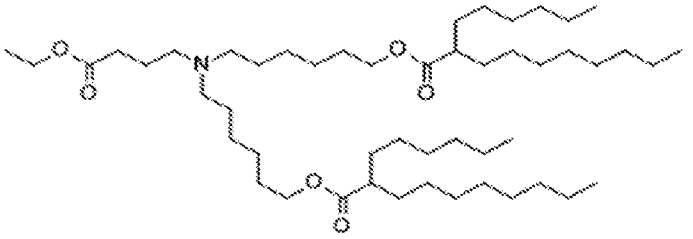
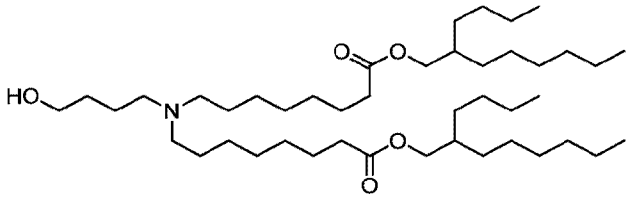
No.	Structure	pKa
III-12		-
III-13		-
III-14		-
III-15		6.14
III-16		6.31
III-17		6.28
III-18		-

No.	Structure	pKa
III-19		-
III-20		6.36
III-21		-
III-22		6.10
III-23		5.98
III-24		-
III-25		6.22

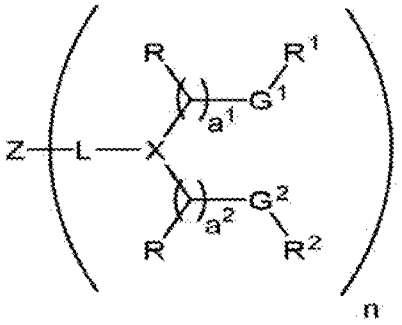
No.	Structure	pKa
III-26		5.84
III-27		5.77
III-28		-
III-29		-
III-30		6.09
III-31		-
III-32		-

No.	Structure	pKa
III-33		-
III-34		-
III-35		-
III-36		-
III-37		-
III-38		-
III-39		-

No.	Structure	pKa
III-40		-
III-41		-
III-42		-
III-43		-
III-44		-
III-45		-

No.	Structure	pKa
III-46		-
III-47		-
III-48		-
III-49		-

In one embodiment, the cationic lipid of any one of Embodiments 1, 2, 3, 4 or 5 has a structure of Formula (IV):



(IV)

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

one of G^1 or G^2 is, at each occurrence, –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -$S(O)_y$-, -S-S-, -C(=O)S-, SC(=O)-, -N(R^a)C(=O)-, -C(=O)N(R^a)-, -N(R^a)C(=O)N(R^a)-, -OC(=O)N(R^a)- or -N(R^a)C(=O)O-, and the other of G^1 or G^2 is, at each occurrence, –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -$S(O)_y$-, -S-S-, -C(=O)S-, -SC(=O)-, -N(R^a)C(=O)-, -C(=O)N(R^a)-, -N(R^a)C(=O)N(R^a)-, -OC(=O)N(R^a)- or –N(R^a)C(=O)O- or a direct bond;

L is, at each occurrence, ~O(C=O)-, wherein ~ represents a covalent bond to X;

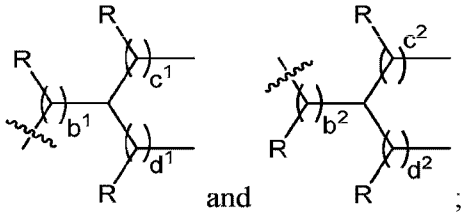
X is CR^a;

Z is alkyl, cycloalkyl or a monovalent moiety comprising at least one polar functional group when n is 1; or Z is alkylene, cycloalkylene or a polyvalent moiety comprising at least one polar functional group when n is greater than 1;

R^a is, at each occurrence, independently H, C_1-C_{12} alkyl, C_1-C_{12} hydroxylalkyl, C_1-C_{12} aminoalkyl, C_1-C_{12} alkylaminylalkyl, C_1-C_{12} alkoxyalkyl, C_1-C_{12} alkoxycarbonyl, C_1-C_{12} alkylcarbonyloxy, C_1-C_{12} alkylcarbonyloxyalkyl or C_1-C_{12} alkylcarbonyl;

R is, at each occurrence, independently either: (a) H or C_1-C_{12} alkyl; or (b) R together with the carbon atom to which it is bound is taken together with an adjacent R and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^1 and R^2 have, at each occurrence, the following structure, respectively:



R^1 R^2

a^1 and a^2 are, at each occurrence, independently an integer from 3 to 12;

b^1 and b^2 are, at each occurrence, independently 0 or 1;

c^1 and c^2 are, at each occurrence, independently an integer from 5 to 10;

d^1 and d^2 are, at each occurrence, independently an integer from 5 to 10;

y is, at each occurrence, independently an integer from 0 to 2; and

n is an integer from 1 to 6,

wherein each alkyl, alkylene, hydroxylalkyl, aminoalkyl, alkylaminylalkyl, alkoxyalkyl, alkoxycarbonyl, alkylcarbonyloxy, alkylcarbonyloxyalkyl and alkylcarbonyl is optionally substituted with one or more substituent.

In some embodiments of Formula (IV), G^1 and G^2 are each independently

-O(C=O)- or -(C=O)O-.

In other embodiments of Formula (IV), X is CH.

In different embodiments of Formula (IV), the sum of $a^1 + b^1 + c^1$ or the sum of $a^2 + b^2 + c^2$ is an integer from 12 to 26.

In still other embodiments of Formula (IV), a^1 and a^2 are independently an integer from 3 to 10. For example, in some embodiments a^1 and a^2 are independently an integer from 4 to 9.

In various embodiments of Formula (IV), b^1 and b^2 are 0. In different embodiments, b^1 and b^2 are 1.

In more embodiments of Formula (IV), c^1, c^2, d^1 and d^2 are independently an integer from 6 to 8.

In other embodiments of Formula (IV), c^1 and c^2 are, at each occurrence, independently an integer from 6 to 10, and d^1 and d^2 are, at each occurrence, independently an integer from 6 to 10.

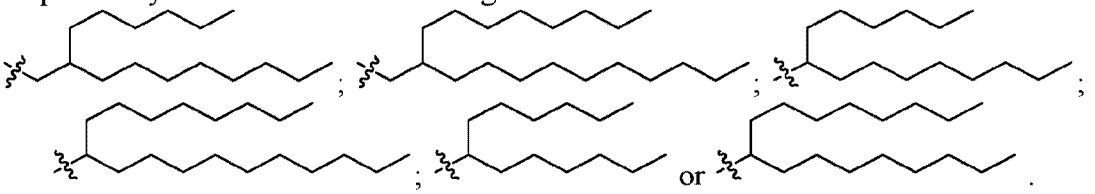
In other embodiments of Formula (IV), c^1 and c^2 are, at each occurrence, independently an integer from 5 to 9, and d^1 and d^2 are, at each occurrence, independently an integer from 5 to 9.

In more embodiments of Formula (IV), Z is alkyl, cycloalkyl or a monovalent moiety comprising at least one polar functional group when n is 1. In other embodiments, Z is alkyl.

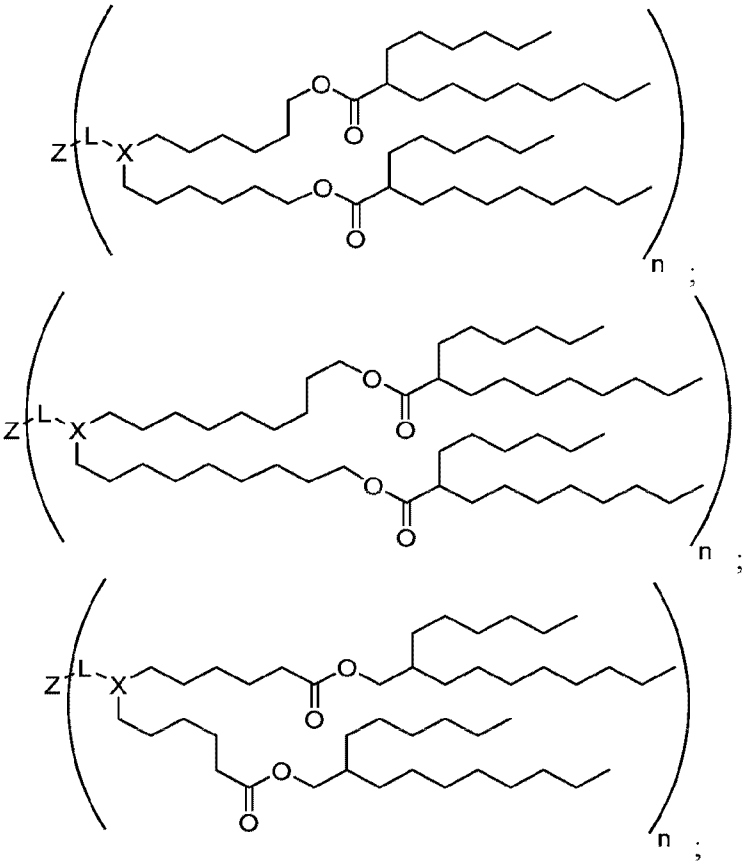
In various embodiments of the foregoing Formula (IV), R is, at each occurrence, independently either: (a) H or methyl; or (b) R together with the carbon

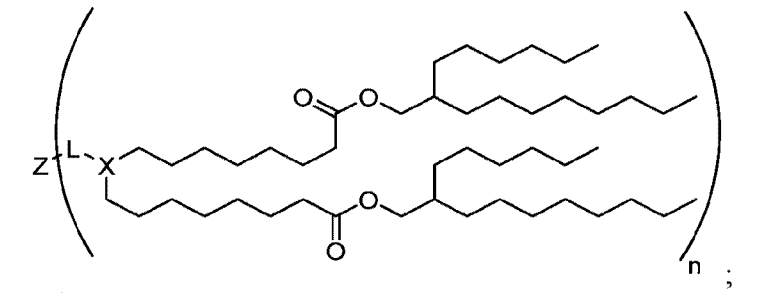
atom to which it is bound is taken together with an adjacent R and the carbon atom to which it is bound to form a carbon-carbon double bond. In certain embodiments, each R is H. In other embodiments at least one R together with the carbon atom to which it is bound is taken together with an adjacent R and the carbon atom to which it is bound to form a carbon-carbon double bond.

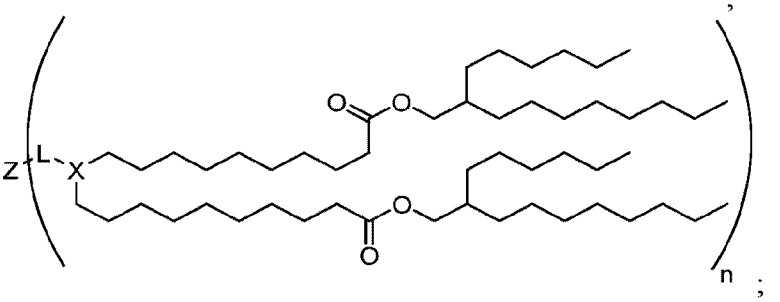
In other embodiments of the compound of Formula (IV), R^1 and R^2 independently have one of the following structures:

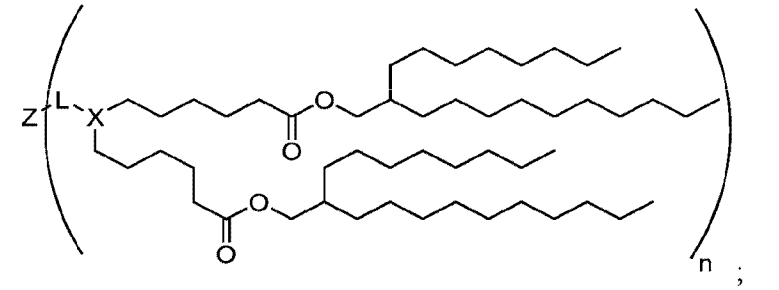


In certain embodiments of Formula (IV), the compound has one of the following structures:




Z
L
X
O
O
O
O
O
n
;


Z
L
X
O
O
O
O
O
n
;


Z
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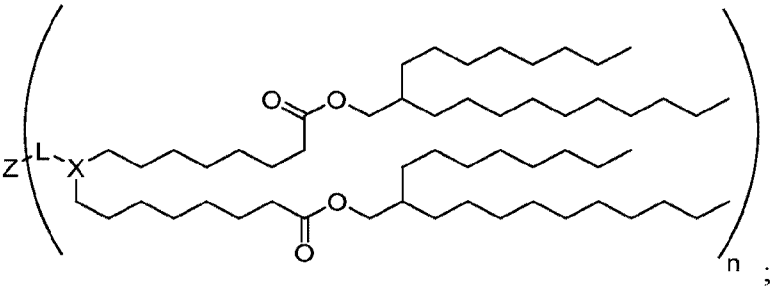
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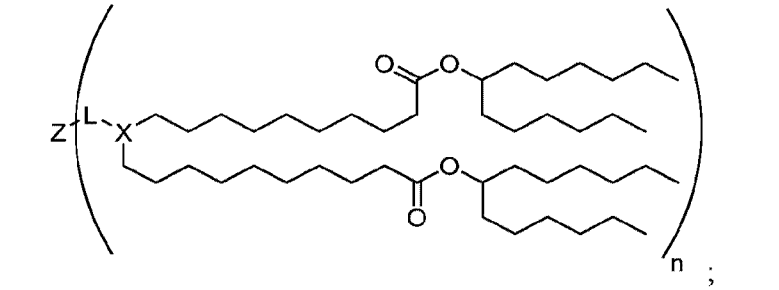
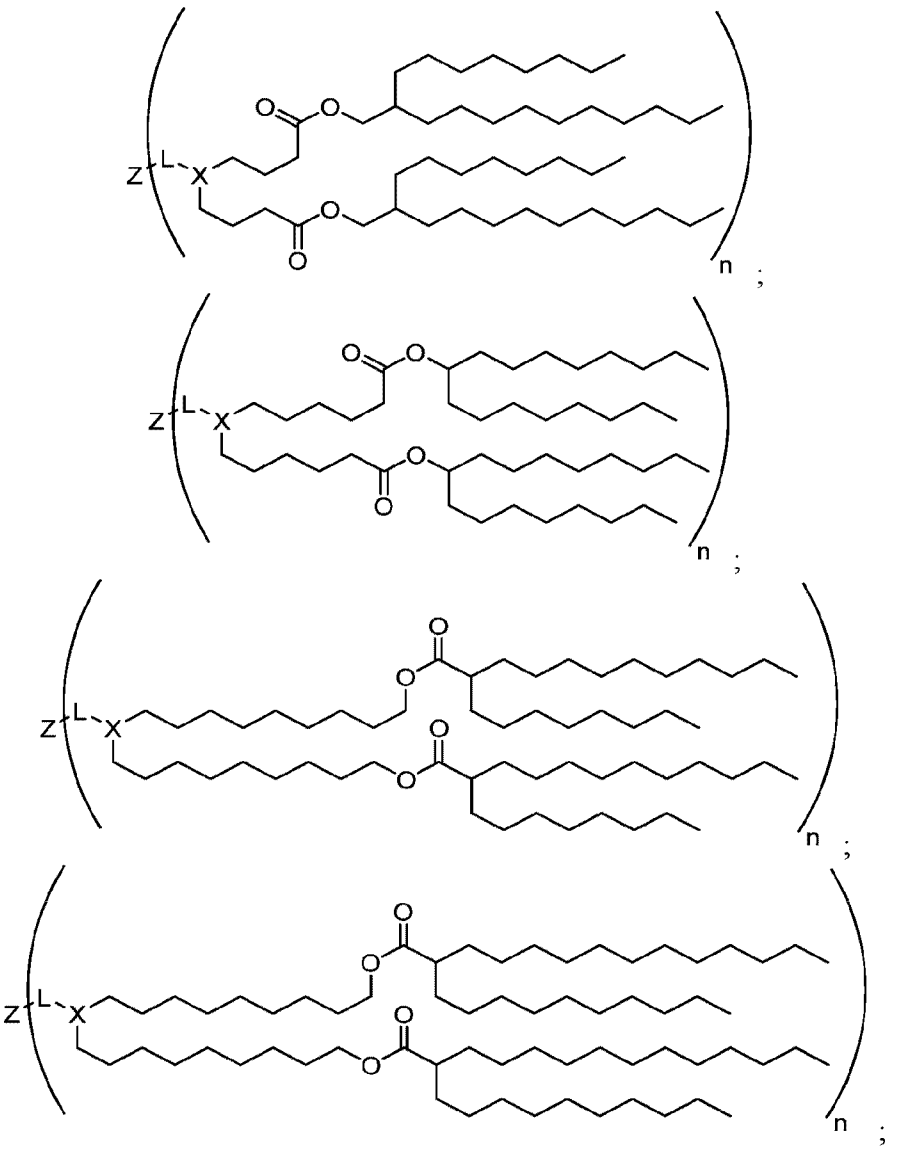
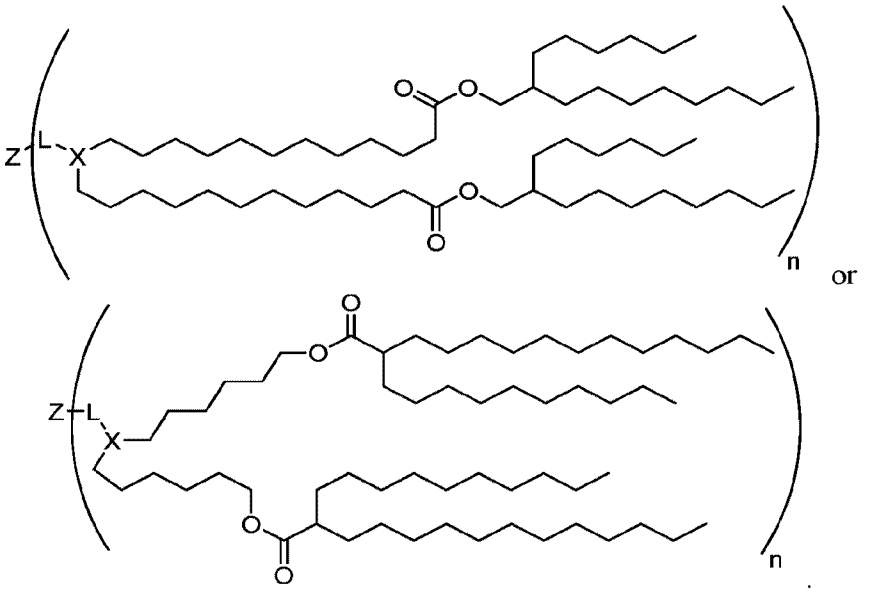
n
;

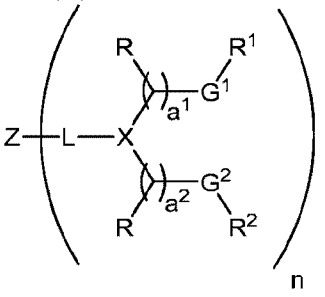

Z
L
X
O
O
O
O
O
n
;





or

In still different embodiments the cationic lipid of Embodiments 1, 2, 3, 4 or 5 has the structure of Formula (V):



(V)

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

one of G^1 or G^2 is, at each occurrence, –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -$S(O)_y$-, -S-S-, -C(=O)S-, SC(=O)-, -N(R^a)C(=O)-, -C(=O)N(R^a)-, -N(R^a)C(=O)N(R^a)-, -OC(=O)N(R^a)- or -N(R^a)C(=O)O-, and the other of G^1 or G^2 is, at each occurrence, -O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -$S(O)_y$-, -S-S-, -C(=O)S-, -SC(=O)-, -N(R^a)C(=O)-, -C(=O)N(R^a)-, -N(R^a)C(=O)N(R^a)-, -OC(=O)N(R^a)- or –N(R^a)C(=O)O- or a direct bond;

L is, at each occurrence, ~O(C=O)-, wherein ~ represents a covalent bond to X;

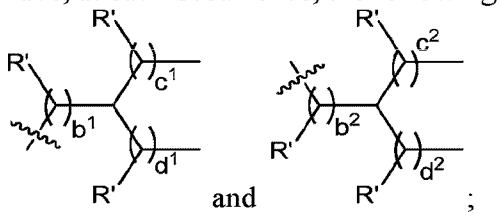
X is CR^a;

Z is alkyl, cycloalkyl or a monovalent moiety comprising at least one polar functional group when n is 1; or Z is alkylene, cycloalkylene or a polyvalent moiety comprising at least one polar functional group when n is greater than 1;

R^a is, at each occurrence, independently H, C_1-C_{12} alkyl, C_1-C_{12} hydroxylalkyl, C_1-C_{12} aminoalkyl, C_1-C_{12} alkylaminylalkyl, C_1-C_{12} alkoxyalkyl, C_1-C_{12} alkoxycarbonyl, C_1-C_{12} alkylcarbonyloxy, C_1-C_{12} alkylcarbonyloxyalkyl or C_1-C_{12} alkylcarbonyl;

R is, at each occurrence, independently either: (a) H or C_1-C_{12} alkyl; or (b) R together with the carbon atom to which it is bound is taken together with an adjacent R and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^1 and R^2 have, at each occurrence, the following structure, respectively:



R^1 R^2 ;

R' is, at each occurrence, independently H or C_1-C_{12} alkyl;

a^1 and a^2 are, at each occurrence, independently an integer from 3 to 12;

b^1 and b^2 are, at each occurrence, independently 0 or 1;

c^1 and c^2 are, at each occurrence, independently an integer from 2 to 12;

d^1 and d^2 are, at each occurrence, independently an integer from 2 to 12;

y is, at each occurrence, independently an integer from 0 to 2; and

n is an integer from 1 to 6,

wherein a^1, a^2, c^1, c^2, d^1 and d^2 are selected such that the sum of $a^1+c^1+d^1$ is an integer from 18 to 30, and the sum of $a^2+c^2+d^2$ is an integer from 18 to 30, and wherein each alkyl, alkylene, hydroxylalkyl, aminoalkyl, alkylaminylalkyl, alkoxyalkyl, alkoxycarbonyl, alkylcarbonyloxy, alkylcarbonyloxyalkyl and alkylcarbonyl is optionally substituted with one or more substituent.

In certain embodiments of Formula (V), G^1 and G^2 are each independently

-O(C=O)- or -(C=O)O-.

In other embodiments of Formula (V), X is CH.

In some embodiments of Formula (V), the sum of $a^1+c^1+d^1$ is an integer from 20 to 30, and the sum of $a^2+c^2+d^2$ is an integer from 18 to 30. In other embodiments, the sum of $a^1+c^1+d^1$ is an integer from 20 to 30, and the sum of $a^2+c^2+d^2$ is an integer from 20 to 30. In more embodiments of Formula (V), the sum of $a^1 + b^1 + c^1$ or the sum of $a^2 + b^2 + c^2$ is an integer from 12 to 26. In other embodiments, a^1, a^2, c^1, c^2, d^1 and d^2 are selected such that the sum of $a^1+c^1+d^1$ is an integer from 18 to 28, and the sum of $a^2+c^2+d^2$ is an integer from 18 to 28,

In still other embodiments of Formula (V), a^1 and a^2 are independently an integer from 3 to 10, for example an integer from 4 to 9.

In yet other embodiments of Formula (V), b^1 and b^2 are 0. In different embodiments b^1 and b^2 are 1.

In certain other embodiments of Formula (V), c^1, c^2, d^1 and d^2 are independently an integer from 6 to 8.

In different other embodiments of Formula (V), Z is alkyl or a monovalent moiety comprising at least one polar functional group when n is 1; or Z is alkylene or a polyvalent moiety comprising at least one polar functional group when n is greater than 1.

In more embodiments of Formula (V), Z is alkyl, cycloalkyl or a monovalent moiety comprising at least one polar functional group when n is 1. In other embodiments, Z is alkyl.

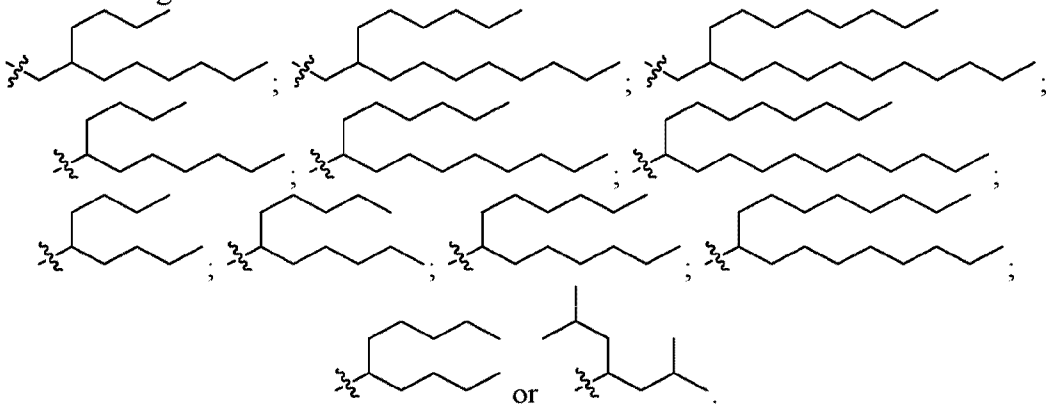
In other different embodiments of Formula (V), R is, at each occurrence, independently either: (a) H or methyl; or (b) R together with the carbon atom to which it is bound is taken together with an adjacent R and the carbon atom to which it is bound to form a carbon-carbon double bond. For example in some embodiments each R is H. In other embodiments at least one R together with the carbon atom to which it

is bound is taken together with an adjacent R and the carbon atom to which it is bound to form a carbon-carbon double bond.

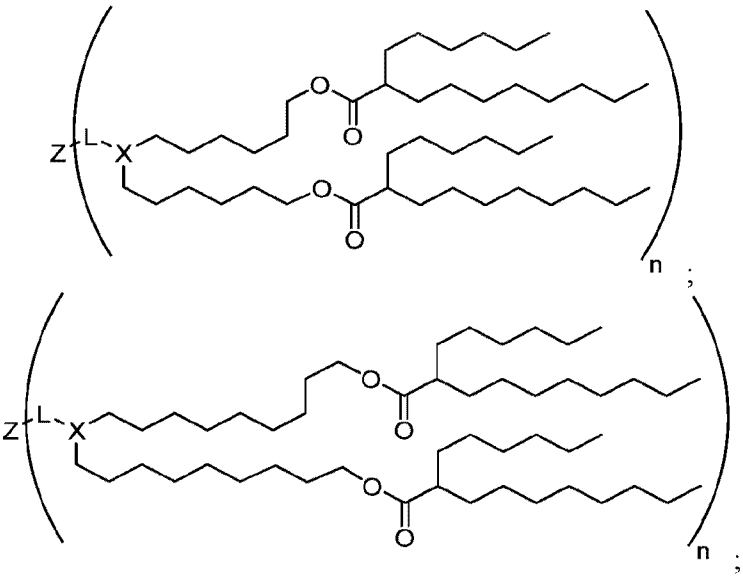
In more embodiments, each R′ is H.

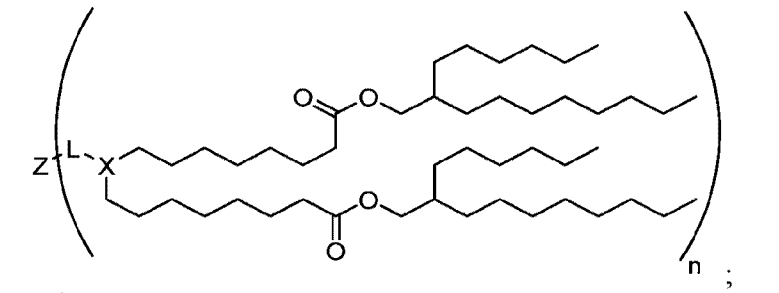
In certain embodiments of Formula (V), the sum of $a^1+c^1+d^1$ is an integer from 20 to 25, and the sum of $a^2+c^2+d^2$ is an integer from 20 to 25.

In other embodiments of Formula (V), R^1 and R^2 independently have one of the following structures:



In more embodiments of Formula (V), the compound has one of the following structures:




Z
L
X
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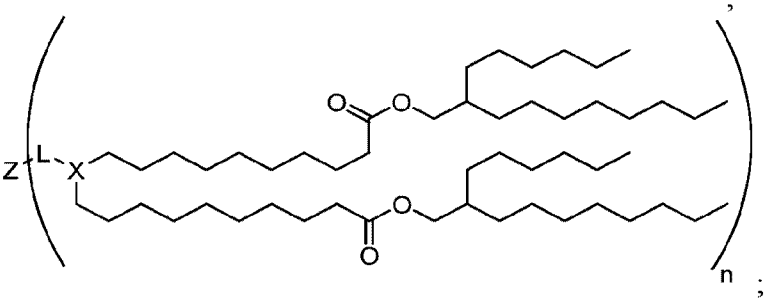
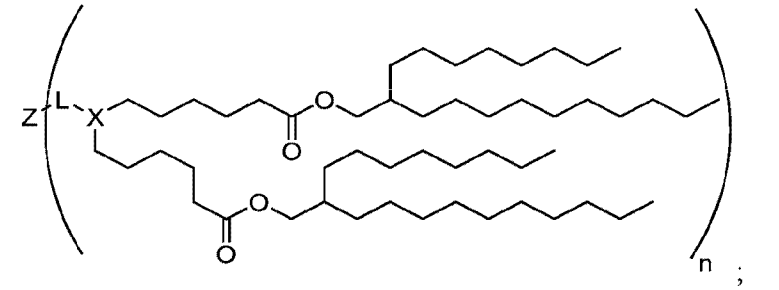
O
O
n
;


Z
L
X
O
O
O
O
O
n
;


Z
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X
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O
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n
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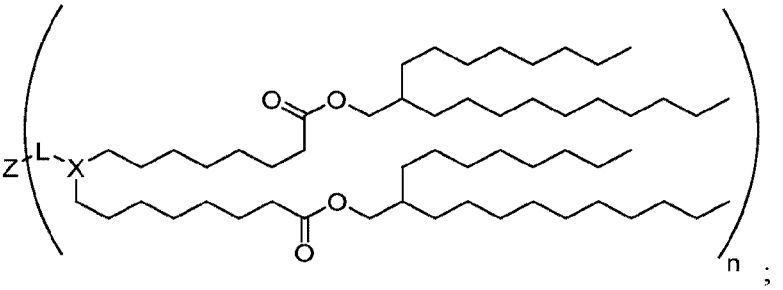
Z
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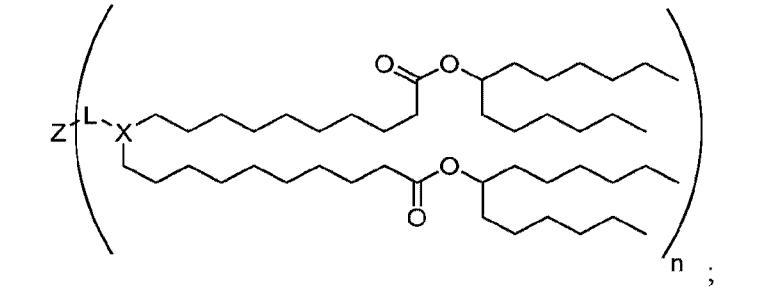
Z
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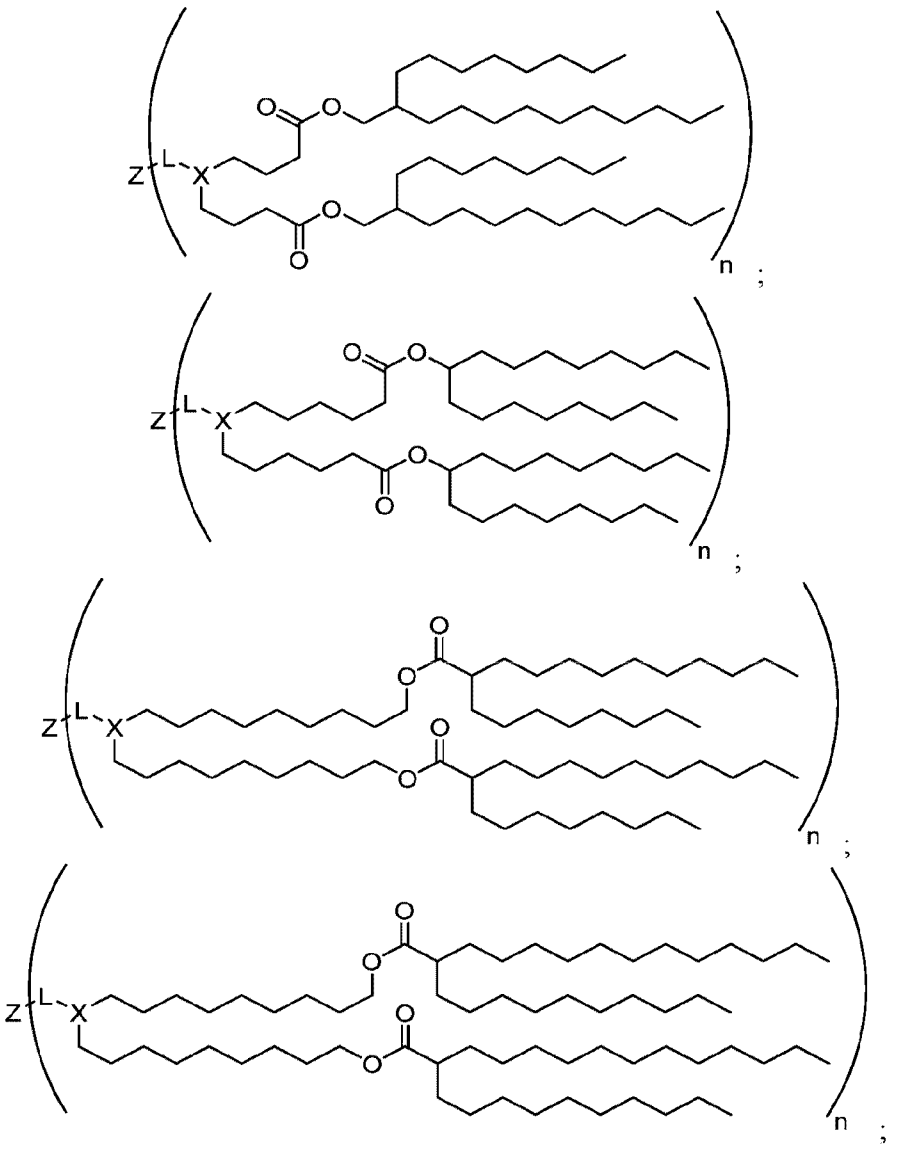
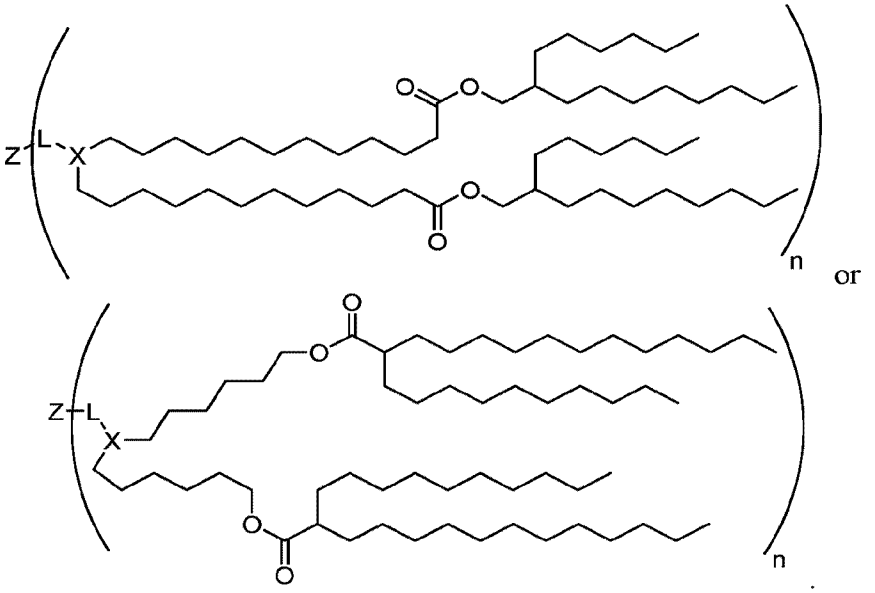




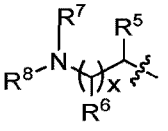
In any of the foregoing embodiments of Formula (IV) or (V), n is 1. In other of the foregoing embodiments of Formula (IV) or (V), n is greater than 1.

In more of any of the foregoing embodiments of Formula (IV) or (V), Z is a mono- or polyvalent moiety comprising at least one polar functional group. In some embodiments, Z is a monovalent moiety comprising at least one polar functional group. In other embodiments, Z is a polyvalent moiety comprising at least one polar functional group.

In more of any of the foregoing embodiments of Formula (IV) or (V), the polar functional group is a hydroxyl, alkoxy, ester, cyano, amide, amino, alkylaminyl, heterocyclyl or heteroaryl functional group.

In any of the foregoing embodiments of Formula (IV) or (V), Z is hydroxyl, hydroxylalkyl, alkoxyalkyl, amino, aminoalkyl, alkylaminyl, alkylaminylalkyl, heterocyclyl or heterocyclylalkyl.

In some other embodiments of Formula (IV) or (V), Z has the following structure:



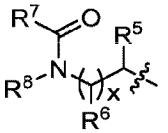
wherein:

R^5 and R^6 are independently H or C_1-C_6 alkyl;

R^7 and R^8 are independently H or C_1-C_6 alkyl or R^7 and R^8, together with the nitrogen atom to which they are attached, join to form a 3-7 membered heterocyclic ring; and

x is an integer from 0 to 6.

In still different embodiments of Formula (IV) or (V), Z has the following structure:



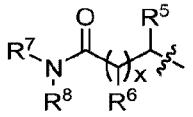
wherein:

R^5 and R^6 are independently H or C_1-C_6 alkyl;

R^7 and R^8 are independently H or C_1-C_6 alkyl or R^7 and R^8, together with the nitrogen atom to which they are attached, join to form a 3-7 membered heterocyclic ring; and

x is an integer from 0 to 6.

In still different embodiments of formula (IV) or (V), Z has the following structure:



wherein:

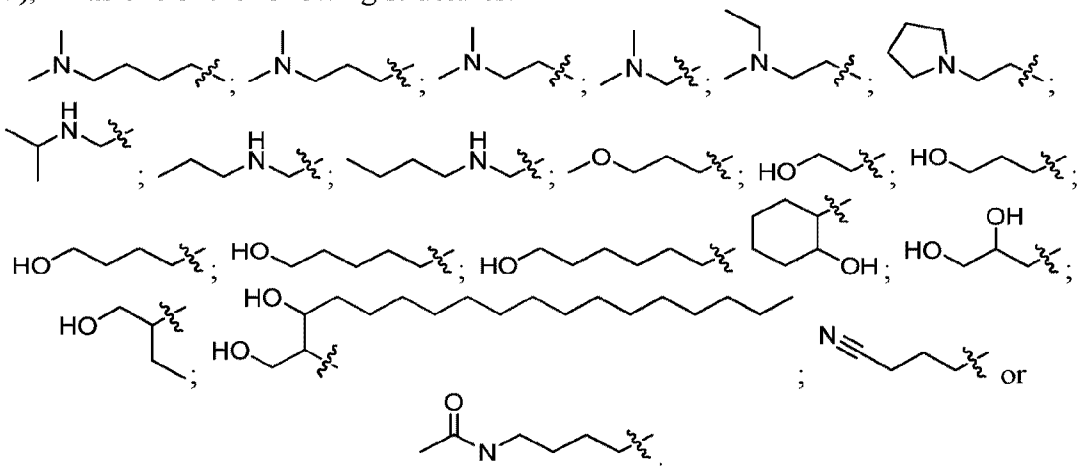
R^5 and R^6 are independently H or C_1-C_6 alkyl;

R^7 and R^8 are independently H or C_1-C_6 alkyl or R^7 and R^8, together with the nitrogen atom to which they are attached, join to form a 3-7 membered heterocyclic ring; and

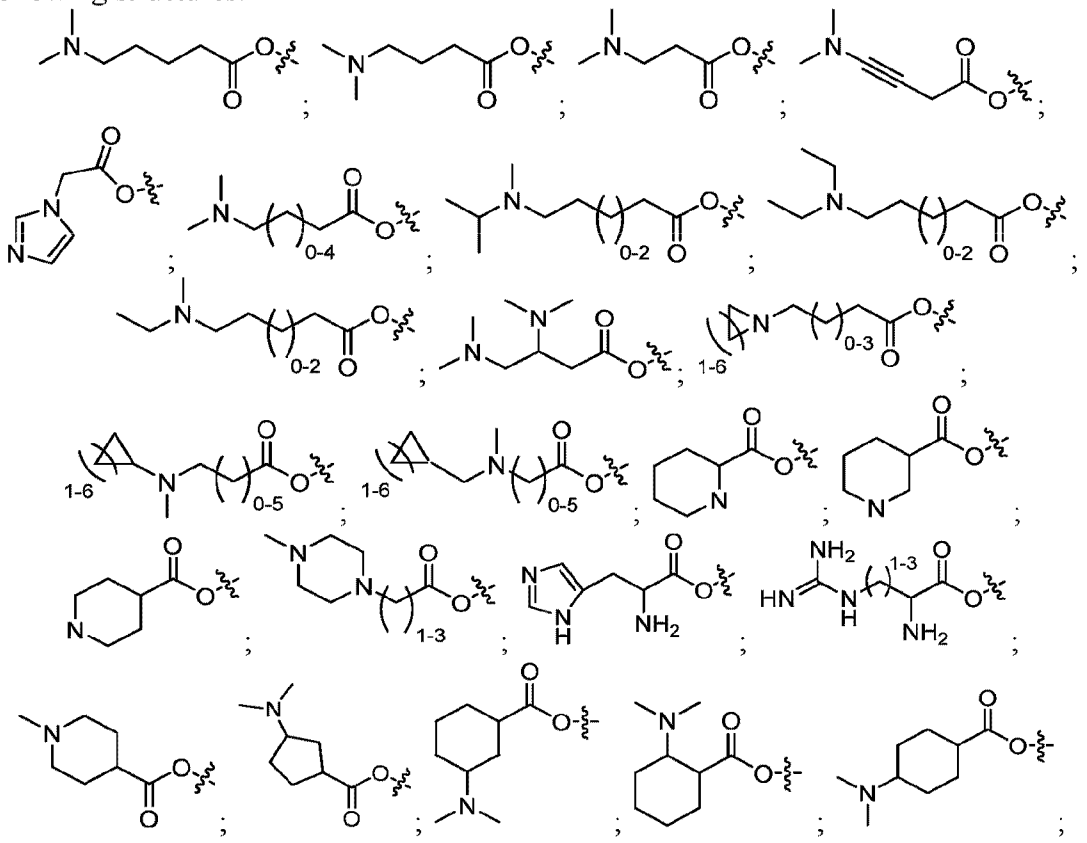
x is an integer from 0 to 6.

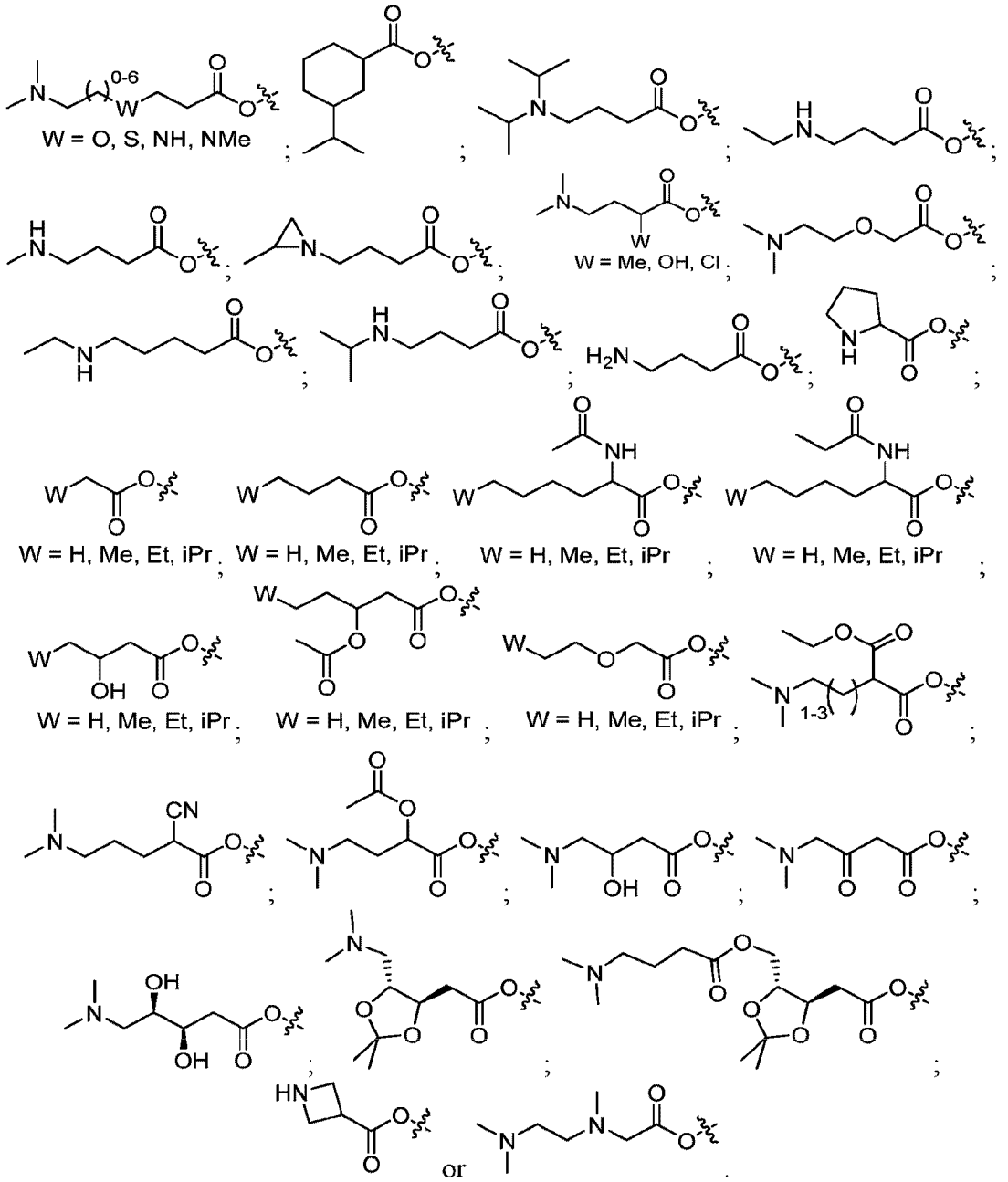
In some other embodiments of Formula (IV) or (V), Z is hydroxylalkyl, cyanoalkyl or an alkyl substituted with one or more ester or amide groups.

For example, in any of the foregoing embodiments of Formula (IV) or (V), Z has one of the following structures:

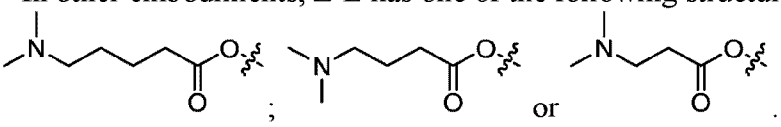


In other embodiments of Formula (IV) or (V), Z-L has one of the following structures:

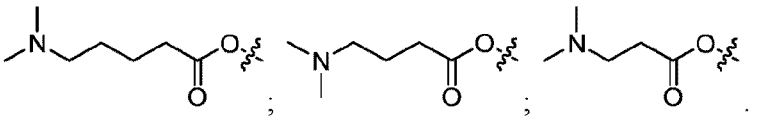




In other embodiments, Z-L has one of the following structures:

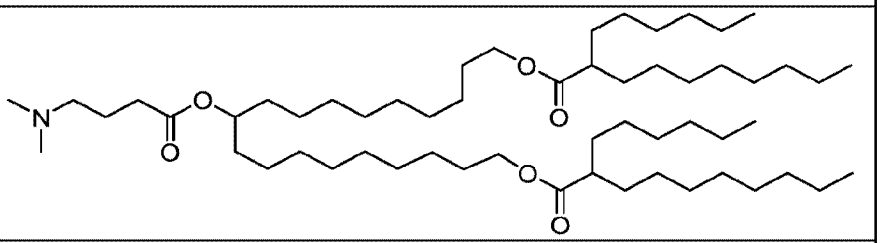
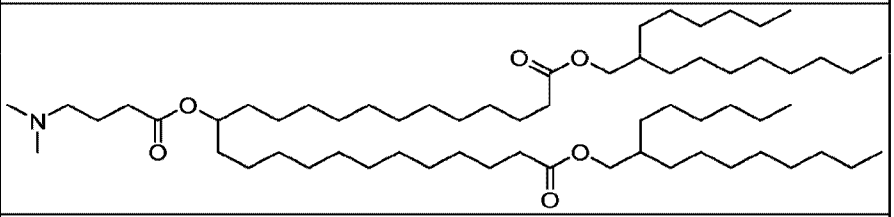
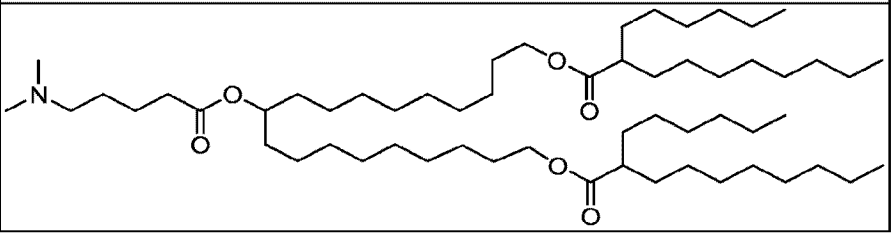


In still other embodiments, X is CH and Z-L has one of the following structures:

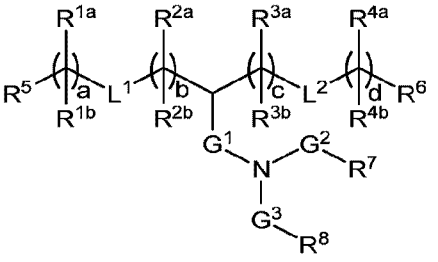


In various different embodiments, a cationic lipid of any one Embodiments 1, 2, 3, 4 or 5 has one of the structures set forth in Table 4 below.

Table 4: Representative Compounds of Formula (IV) or (V)

No.	Structure
IV-1	
IV-2	
IV-3	

In one embodiment, the cationic lipid is a compound having the following structure (VI):



(VI)

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

L^1 and L^2 are each independently -O(C=O)-, -(C=O)O-, -C(=O)-, -O-, $-S(O)_x$-, -S-S-, -C(=O)S-, -SC(=O)-, $-NR^aC(=O)$-, $-C(=O)NR^a$-, $-NR^aC(=O)NR^a$-, $-OC(=O)NR^a$-, $-NR^aC(=O)O$- or a direct bond;

G^1 is C_1-C_2 alkylene, -(C=O)-, -O(C=O)-, -SC(=O)-, $-NR^aC(=O)$- or a direct bond;

G^2 is –C(=O)-, -(C=O)O-, -C(=O)S-, $-C(=O)NR^a$- or a direct bond;

G^3 is C_1-C_6 alkylene;

R^a is H or C_1-C_{12} alkyl;

R^{1a} and R^{1b} are, at each occurrence, independently either: (a) H or C_1-C_{12} alkyl; or (b) R^{1a} is H or C_1-C_{12} alkyl, and R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{2a} and R^{2b} are, at each occurrence, independently either: (a) H or C_1-C_{12} alkyl; or (b) R^{2a} is H or C_1-C_{12} alkyl, and R^{2b} together with the carbon atom to which it is bound is taken together with an adjacent R^{2b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{3a} and R^{3b} are, at each occurrence, independently either (a): H or C_1-C_{12} alkyl; or (b) R^{3a} is H or C_1-C_{12} alkyl, and R^{3b} together with the carbon atom to which it is bound is taken together with an adjacent R^{3b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{4a} and R^{4b} are, at each occurrence, independently either: (a) H or C_1-C_{12} alkyl; or (b) R^{4a} is H or C_1-C_{12} alkyl, and R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^5 and R^6 are each independently H or methyl;

R^7 is H or C_1-C_{20} alkyl;

R^8 is OH, $-N(R^9)(C=O)R^{10}$, $-(C=O)NR^9R^{10}$, $-NR^9R^{10}$, $-(C=O)OR^{11}$ or $-O(C=O)R^{11}$, provided that G^3 is C_4-C_6 alkylene when R^8 is $-NR^9R^{10}$,

R^9 and R^{10} are each independently H or C_1-C_{12} alkyl;

R^{11} is aralkyl;

a, b, c and d are each independently an integer from 1 to 24; and

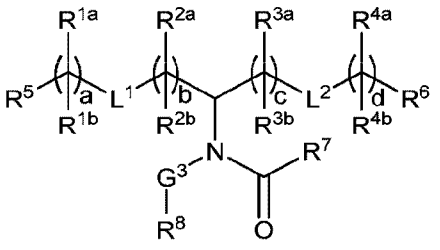
x is 0, 1 or 2,

wherein each alkyl, alkylene and aralkyl is optionally substituted.

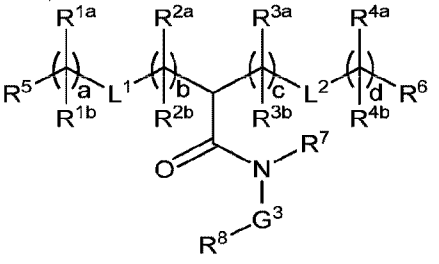
In some embodiments of structure (VI), L^1 and L^2 are each independently -O(C=O)-, -(C=O)O- or a direct bond. In other embodiments, G^1 and G^2 are each independently -(C=O)- or a direct bond. In some different embodiments, L^1 and L^2 are each independently -O(C=O)-, -(C=O)O- or a direct bond; and G^1 and G^2 are each independently - (C=O)- or a direct bond.

In some different embodiments of structure (VI), L^1 and L^2 are each independently -C(=O)-, -O-, -$S(O)_x$-, -S-S-, -C(=O)S-, -SC(=O)-, -NR^a-, -$NR^aC(=O)$-, -$C(=O)NR^a$-, -$NR^aC(=O)NR^a$, -$OC(=O)NR^a$-, -$NR^aC(=O)O$-, -$NR^aS(O)_xNR^a$-, -$NR^aS(O)_x$- or -$S(O)_xNR^a$-.

In other of the foregoing embodiments of structure (VI), the compound has one of the following structures (VIA) or (VIB):



or



(VIA) (VIB)

In some embodiments, the compound has structure (VIA). In other embodiments, the compound has structure (VIB).

In any of the foregoing embodiments of structure (VI), one of L^1 or L^2 is -O(C=O)-. For example, in some embodiments each of L^1 and L^2 are -O(C=O)-.

In some different embodiments of any of the foregoing, one of L^1 or L^2 is -(C=O)O-. For example, in some embodiments each of L^1 and L^2 is -(C=O)O-.

In different embodiments of structure (VI), one of L^1 or L^2 is a direct bond. As used herein, a "direct bond" means the group (*e.g.*, L^1 or L^2) is absent. For example, in some embodiments each of L^1 and L^2 is a direct bond.

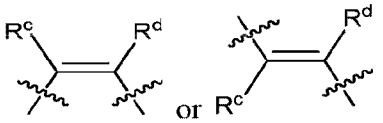
In other different embodiments of the foregoing, for at least one occurrence of R^{1a} and R^{1b}, R^{1a} is H or C_1-C_{12} alkyl, and R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

In still other different embodiments of structure (VI), for at least one occurrence of R^{4a} and R^{4b}, R^{4a} is H or C_1-C_{12} alkyl, and R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

In more embodiments of structure (VI), for at least one occurrence of R^{2a} and R^{2b}, R^{2a} is H or C_1-C_{12} alkyl, and R^{2b} together with the carbon atom to which it is bound is taken together with an adjacent R^{2b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

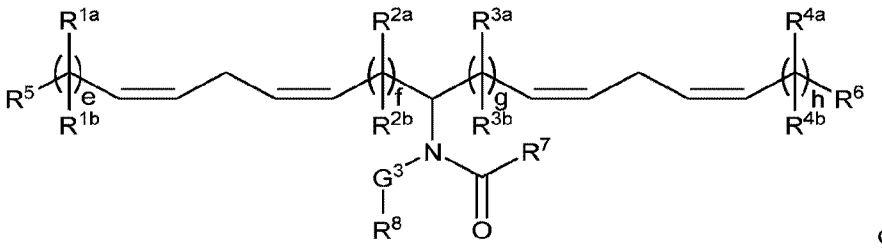
In other different embodiments of any of the foregoing, for at least one occurrence of R^{3a} and R^{3b}, R^{3a} is H or C_1-C_{12} alkyl, and R^{3b} together with the carbon atom to which it is bound is taken together with an adjacent R^{3b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

It is understood that "carbon-carbon" double bond refers to one of the following structures:



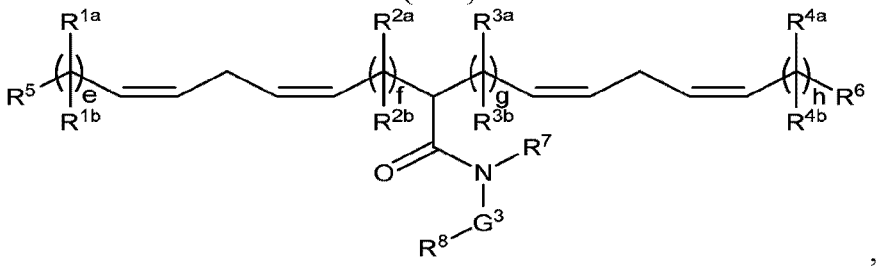
wherein R^c and R^d are, at each occurrence, independently H or a substituent. For example, in some embodiments R^c and R^d are, at each occurrence, independently H, C_1-C_{12} alkyl or cycloalkyl, for example H or C_1-C_{12} alkyl.

In various other embodiments, the compound has one of the following structures (VIC) or (VID):



or

(VIC)



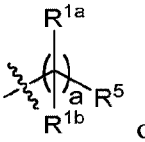
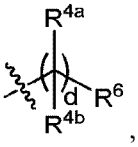
,

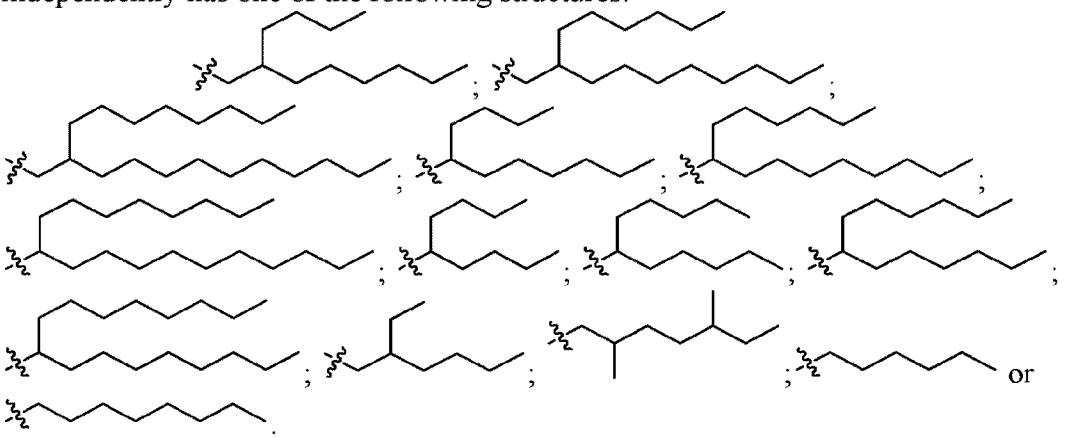
(VID)

wherein e, f, g and h are each independently an integer from 1 to 12.

In some embodiments, the compound has structure (VIC). In other embodiments, the compound has structure (VID).

In various embodiments of the compounds of structures (VIC) or (VID), e, f, g and h are each independently an integer from 4 to 10.

In other different embodiments,  or  , or both, independently has one of the following structures:



or

.

In certain embodiments of the foregoing, a, b, c and d are each independently an integer from 2 to 12 or an integer from 4 to 12. In other embodiments, a, b, c and d are each independently an integer from 8 to 12 or 5 to 9. In some certain embodiments, a is 0. In some embodiments, a is 1. In other embodiments, a is 2. In more embodiments, a is 3. In yet other embodiments, a is 4. In some embodiments, a is 5. In other embodiments, a is 6. In more embodiments, a is 7. In yet other embodiments, a is 8. In some embodiments, a is 9. In other embodiments, a is 10. In more embodiments, a is 11. In yet other embodiments, a is 12. In some embodiments, a is 13. In other embodiments, a is 14. In more embodiments, a is 15. In yet other embodiments, a is 16.

In some embodiments of structure (VI), b is 1. In other embodiments, b is 2. In more embodiments, b is 3. In yet other embodiments, b is 4. In some embodiments, b is 5. In other embodiments, b is 6. In more embodiments, b is 7. In yet other embodiments, b is 8. In some embodiments, b is 9. In other embodiments, b is 10. In more embodiments, b is 11. In yet other embodiments, b is 12. In some embodiments, b is 13. In other embodiments, b is 14. In more embodiments, b is 15. In yet other embodiments, b is 16.

In some embodiments of structure (VI), c is 1. In other embodiments, c is 2. In more embodiments, c is 3. In yet other embodiments, c is 4. In some embodiments, c is 5. In other embodiments, c is 6. In more embodiments, c is 7. In yet other embodiments, c is 8. In some embodiments, c is 9. In other embodiments, c is 10. In more embodiments, c is 11. In yet other embodiments, c is 12. In some embodiments, c is 13. In other embodiments, c is 14. In more embodiments, c is 15. In yet other embodiments, c is 16.

In some certain embodiments of structure (VI), d is 0. In some embodiments, d is 1. In other embodiments, d is 2. In more embodiments, d is 3. In yet other embodiments, d is 4. In some embodiments, d is 5. In other embodiments, d is 6. In more embodiments, d is 7. In yet other embodiments, d is 8. In some embodiments, d is 9. In other embodiments, d is 10. In more embodiments, d is 11. In

yet other embodiments, d is 12. In some embodiments, d is 13. In other embodiments, d is 14. In more embodiments, d is 15. In yet other embodiments, d is 16.

In some embodiments of structure (VI), e is 1. In other embodiments, e is 2. In more embodiments, e is 3. In yet other embodiments, e is 4. In some embodiments, e is 5. In other embodiments, e is 6. In more embodiments, e is 7. In yet other embodiments, e is 8. In some embodiments, e is 9. In other embodiments, e is 10. In more embodiments, e is 11. In yet other embodiments, e is 12.

In some embodiments of structure (VI), f is 1. In other embodiments, f is 2. In more embodiments, f is 3. In yet other embodiments, f is 4. In some embodiments, f is 5. In other embodiments, f is 6. In more embodiments, f is 7. In yet other embodiments, f is 8. In some embodiments, f is 9. In other embodiments, f is 10. In more embodiments, f is 11. In yet other embodiments, f is 12.

In some embodiments of structure (VI), g is 1. In other embodiments, g is 2. In more embodiments, g is 3. In yet other embodiments, g is 4. In some embodiments, g is 5. In other embodiments, g is 6. In more embodiments, g is 7. In yet other embodiments, g is 8. In some embodiments, g is 9. In other embodiments, g is 10. In more embodiments, g is 11. In yet other embodiments, g is 12.

In some embodiments of structure (VI), h is 1. In other embodiments, e is 2. In more embodiments, h is 3. In yet other embodiments, h is 4. In some embodiments, e is 5. In other embodiments, h is 6. In more embodiments, h is 7. In yet other embodiments, h is 8. In some embodiments, h is 9. In other embodiments, h is 10. In more embodiments, h is 11. In yet other embodiments, h is 12.

In some other various embodiments of structure (VI), a and d are the same. In some other embodiments, b and c are the same. In some other specific embodiments a and d are the same and b and c are the same.

The sum of a and b and the sum of c and d are factors which may be varied to obtain a lipid having the desired properties. In one embodiment, a and b are chosen such that their sum is an integer ranging from 14 to 24. In other embodiments, c and d are chosen such that their sum is an integer ranging from 14 to 24. In further embodiment, the sum of a and b and the sum of c and d are the same. For example, in

some embodiments the sum of a and b and the sum of c and d are both the same integer which may range from 14 to 24. In still more embodiments, a. b, c and d are selected such that the sum of a and b and the sum of c and d is 12 or greater.

The substituents at R^{1a}, R^{2a}, R^{3a} and R^{4a} are not particularly limited. In some embodiments, at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is H. In certain embodiments R^{1a}, R^{2a}, R^{3a} and R^{4a} are H at each occurrence. In certain other embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_{12} alkyl. In certain other embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_8 alkyl. In certain other embodiments at least one of R^{1a}, R^{2a}, R^{3a} and R^{4a} is C_1-C_6 alkyl. In some of the foregoing embodiments, the C_1-C_8 alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

In certain embodiments of the foregoing, R^{1a}, R^{1b}, R^{4a} and R^{4b} are C_1-C_{12} alkyl at each occurrence.

In further embodiments of the foregoing, at least one of R^{1b}, R^{2b}, R^{3b} and R^{4b} is H or R^{1b}, R^{2b}, R^{3b} and R^{4b} are H at each occurrence.

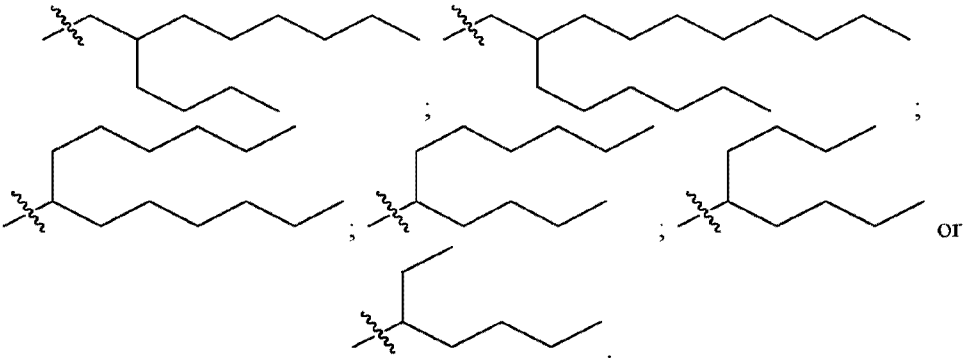
In certain embodiments of the foregoing, R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond. In other embodiments of the foregoing R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

The substituents at R^5 and R^6 are not particularly limited in the foregoing embodiments. In certain embodiments one of R^5 or R^6 is methyl. In other embodiments each of R^5 or R^6 is methyl.

The substituents at R^7 are not particularly limited in the foregoing embodiments. In certain embodiments R^7 is C_6-C_{16} alkyl. In some other embodiments, R^7 is C_6-C_9 alkyl. In some of these embodiments, R^7 is substituted with $-(C=O)OR^b$, $-O(C=O)R^b$, $-C(=O)R^b$, $-OR^b$, $-S(O)_xR^b$, $-S-SR^b$, $-C(=O)SR^b$, $-SC(=O)R^b$, $-NR^aR^b$, $-NR^aC(=O)R^b$, $-C(=O)NR^aR^b$, $-NR^aC(=O)NR^aR^b$, $-OC(=O)NR^aR^b$, $-NR^aC(=O)OR^b$,

$-NR^aS(O)_xNR^aR^b$, $-NR^aS(O)_xR^b$ or $-S(O)_xNR^aR^b$, wherein: R^a is H or C_1-C_{12} alkyl; R^b is C_1-C_{15} alkyl; and x is 0, 1 or 2. For example, in some embodiments R^7 is substituted with $-(C=O)OR^b$ or $-O(C=O)R^b$.

In various of the foregoing embodiments of structure (VI), R^b is branched C_3-C_{15} alkyl. For example, in some embodiments R^b has one of the following structures:

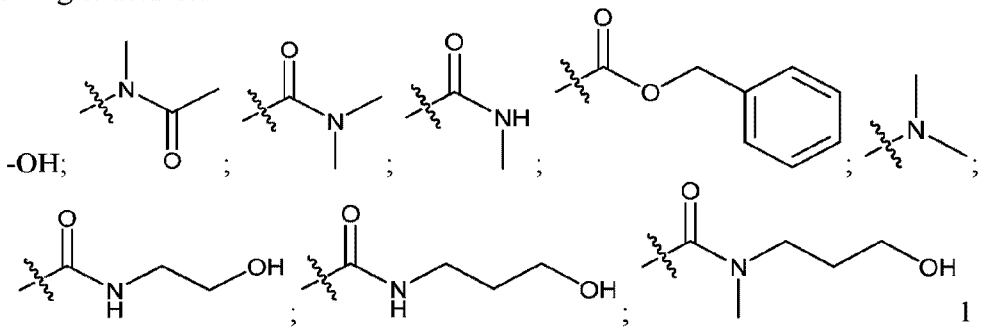


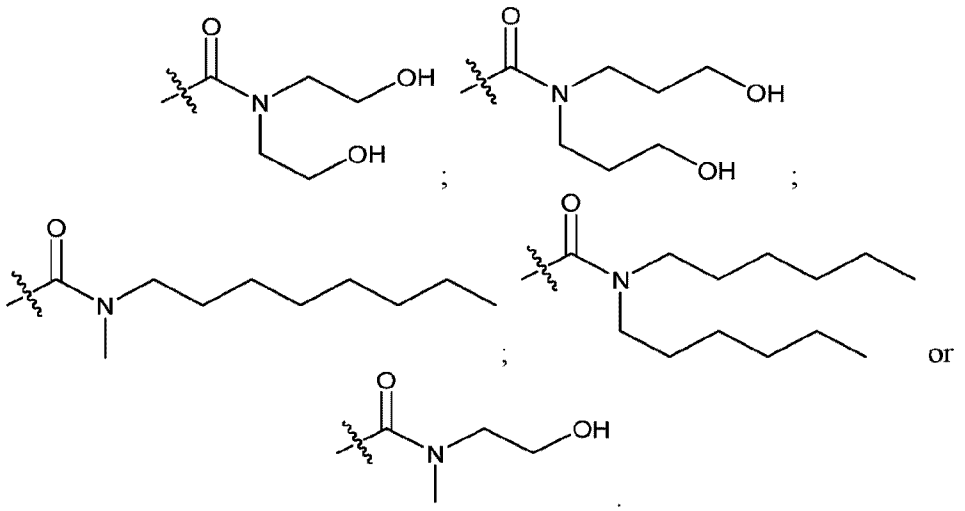
In certain embodiments, R^8 is OH.

In other embodiments of structure (VI), R^8 is $-N(R^9)(C=O)R^{10}$. In some other embodiments, R^8 is $-(C=O)NR^9R^{10}$. In still more embodiments, R^8 is $-NR^9R^{10}$. In some of the foregoing embodiments, R^9 and R^{10} are each independently H or C_1-C_8 alkyl, for example H or C_1-C_3 alkyl. In more specific of these embodiments, the C_1-C_8 alkyl or C_1-C_3 alkyl is unsubstituted or substituted with hydroxyl. In other of these embodiments, R^9 and R^{10} are each methyl.

In yet more embodiments of structure (VI), R^8 is $-(C=O)OR^{11}$. In some of these embodiments R^{11} is benzyl.

In yet more specific embodiments of structure (VI), R^8 has one of the following structures:

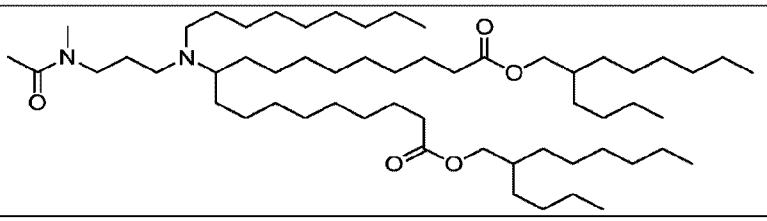
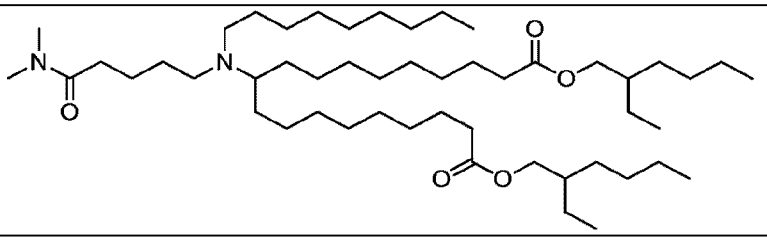


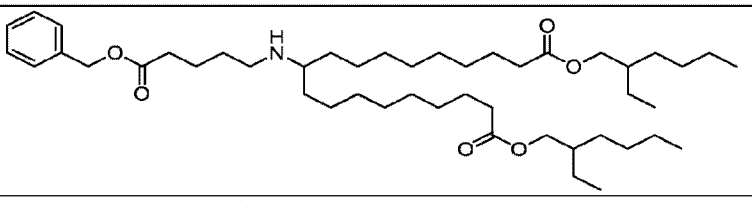
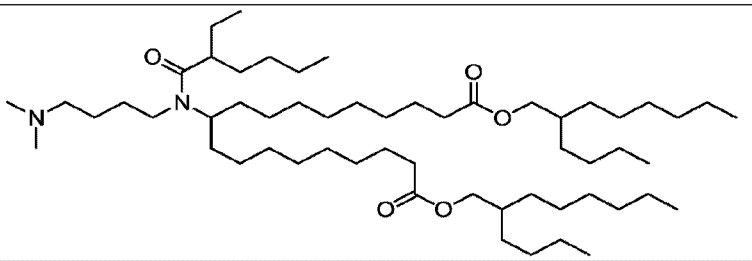
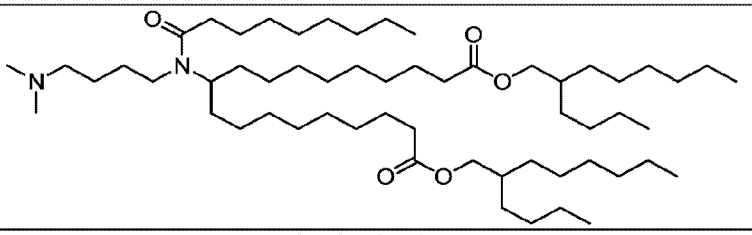
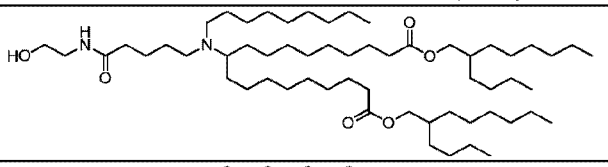
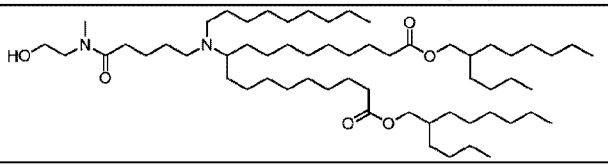
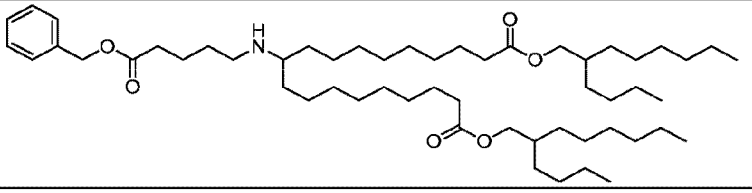
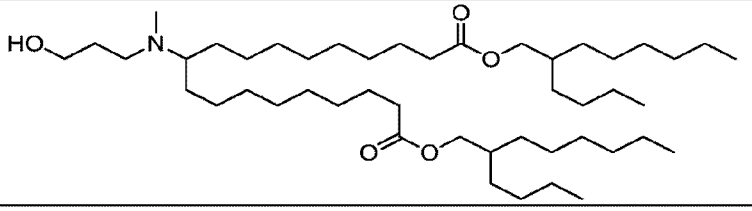


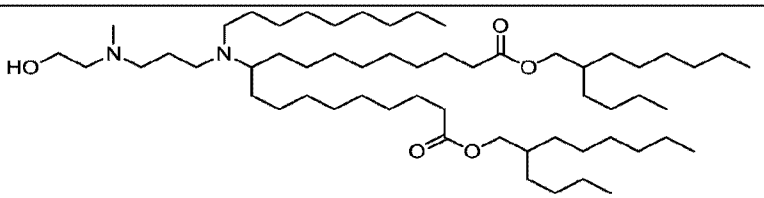
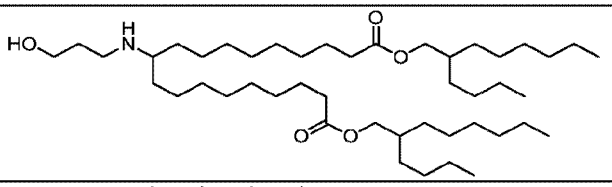
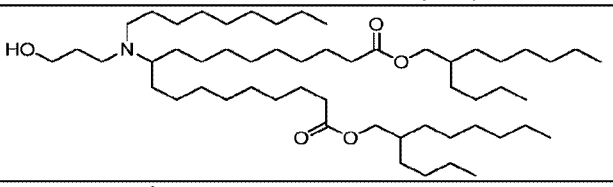
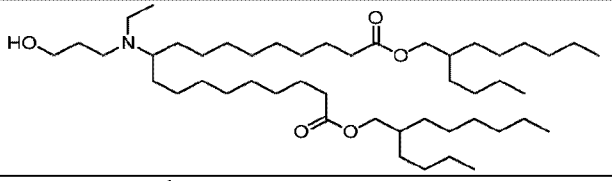
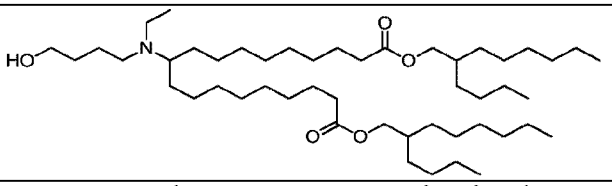
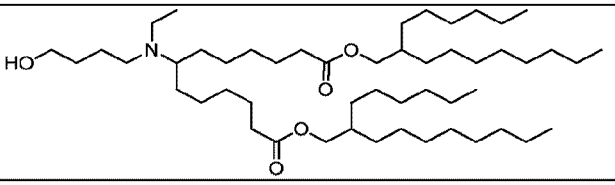
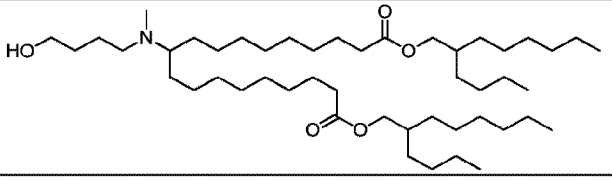
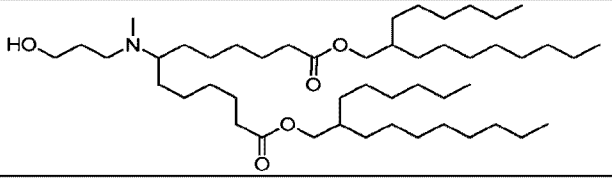
In still other embodiments of the foregoing compounds, G^3 is C_2-C_5 alkylene, for example C_2-C_4 alkylene, C_3 alkylene or C_4 alkylene. In some of these embodiments, R^8 is OH. In other embodiments, G^2 is absent and R^7 is C_1-C_2 alkylene, such as methyl.

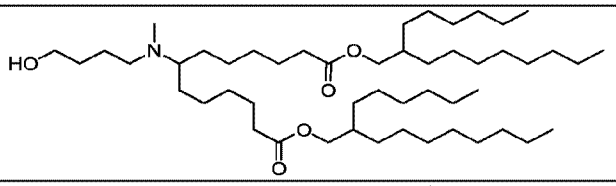
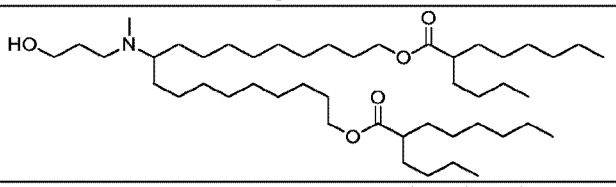
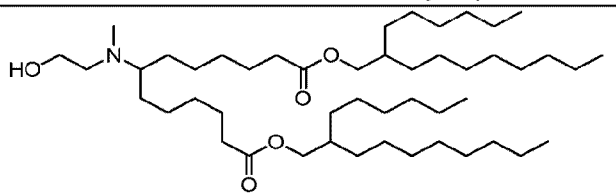
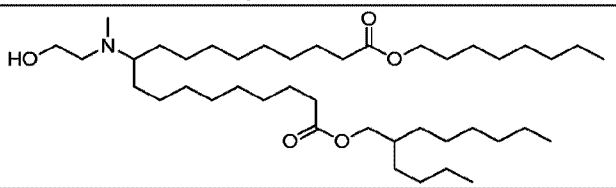
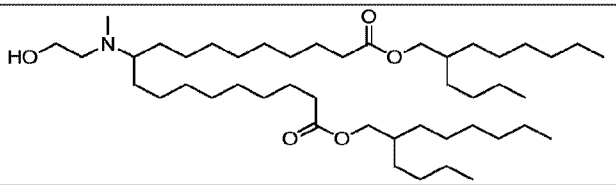
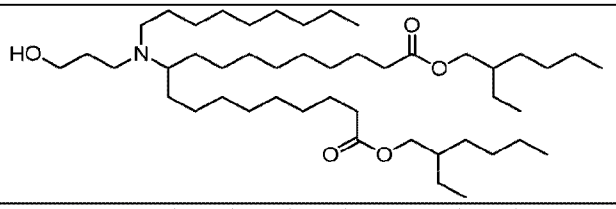
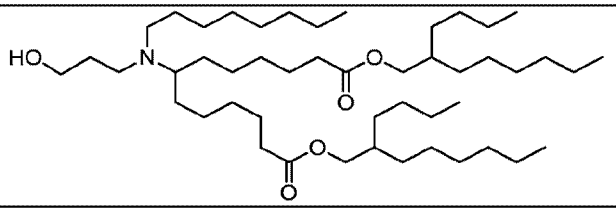
In various different embodiments, the compound has one of the structures set forth in Table 5 below.

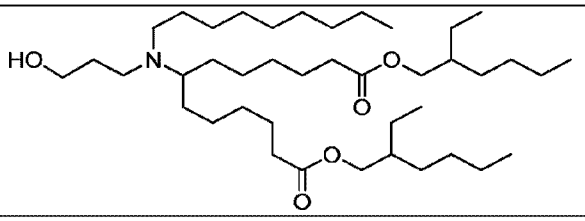
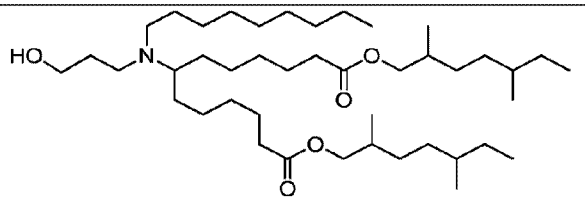
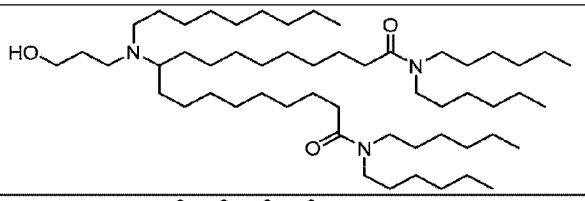
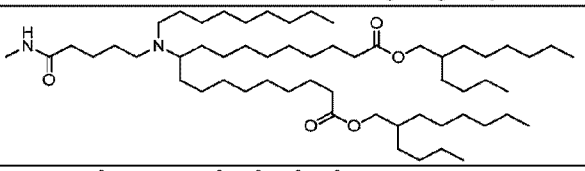
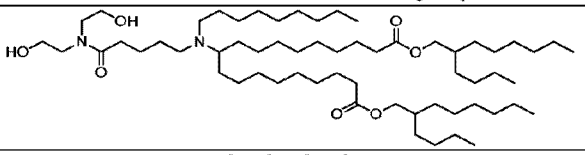
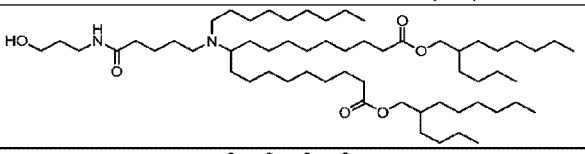
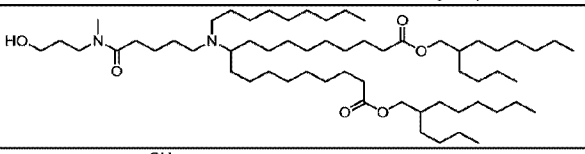
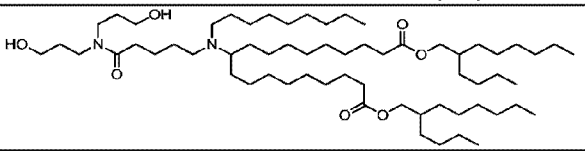
Table 5. Representative cationic lipids of structure (VI)

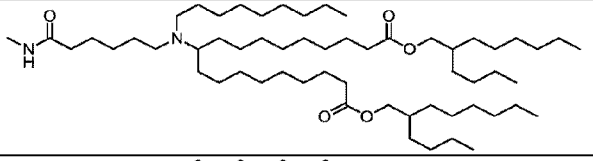
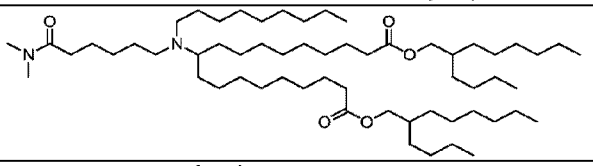
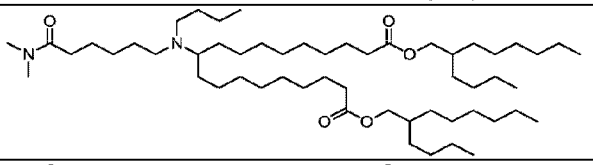
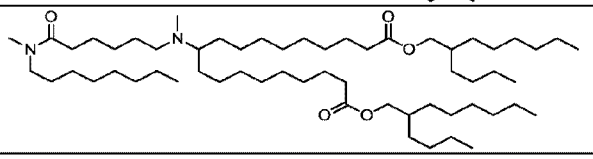
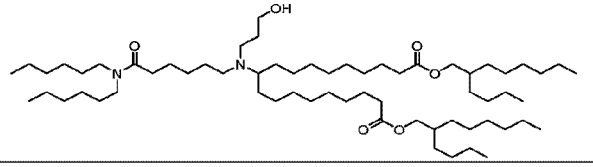
No.	Structure
VI-1	
VI-2	

No.	Structure
VI-3	
VI-4	
VI-5	
VI-6	
VI-7	
VI-8	
VI-9	

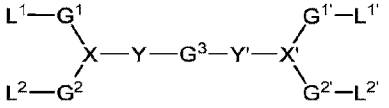
No.	Structure
VI-10	
VI-11	
VI-12	
VI-13	
VI-14	
VI-15	
VI-16	
VI-17	

No.	Structure
VI-18	
VI-19	
VI-20	
VI-21	
VI-22	
VI-23	
VI-24	

No.	Structure
VI-25	
VI-26	
VI-27	
VI-28	
VI-29	
VI-30	
VI-31	
VI-32	

No.	Structure
VI-33	
VI-34	
VI-35	
VI-36	
VI-37	

In one embodiment, the cationic lipid is a compound having the following structure (VII):



(VII)

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

X and X' are each independently N or CR;

Y and Y' are each independently absent, -O(C=O)-, -(C=O)O- or NR, provided that:

a)Y is absent when X is N;

b) Y' is absent when X' is N;

c) Y is -O(C=O)-, -(C=O)O- or NR when X is CR; and

d) Y' is -O(C=O)-, -(C=O)O- or NR when X' is CR,

L^1 and $L^{1'}$ are each independently $-O(C=O)R^1$, $-(C=O)OR^1$, $-C(=O)R^1$, $-OR^1$, $-S(O)_zR^1$, $-S-SR^1$, $-C(=O)SR^1$, $-SC(=O)R^1$, $-NR^aC(=O)R^1$, $-C(=O)NR^bR^c$, $-NR^aC(=O)NR^bR^c$, $-OC(=O)NR^bR^c$ or $-NR^aC(=O)OR^1$;

L^2 and $L^{2'}$ are each independently $-O(C=O)R^2$, $-(C=O)OR^2$, $-C(=O)R^2$, $-OR^2$, $-S(O)_zR^2$, $-S-SR^2$, $-C(=O)SR^2$, $-SC(=O)R^2$, $-NR^dC(=O)R^2$, $-C(=O)NR^eR^f$, $-NR^dC(=O)NR^eR^f$, $-OC(=O)NR^eR^f$;$-NR^dC(=O)OR^2$ or a direct bond to R^2;

G^1, $G^{1'}$, G^2 and $G^{2'}$ are each independently C_2-C_{12} alkylene or C_2-C_{12} alkenylene;

G^3 is C_2-C_{24} heteroalkylene or C_2-C_{24} heteroalkenylene;

R^a, R^b, R^d and R^e are, at each occurrence, independently H, C_1-C_{12} alkyl or C_2-C_{12} alkenyl;

R^c and R^f are, at each occurrence, independently C_1-C_{12} alkyl or C_2-C_{12} alkenyl;

R is, at each occurrence, independently H or C_1-C_{12} alkyl;

R^1 and R^2 are, at each occurrence, independently branched C_6-C_{24} alkyl or branched C_6-C_{24} alkenyl;

z is 0, 1 or 2, and

wherein each alkyl, alkenyl, alkylene, alkenylene, heteroalkylene and heteroalkenylene is independently substituted or unsubstituted unless otherwise specified.

In other different embodiments of structure (VII):

X and X' are each independently N or CR;

Y and Y' are each independently absent or NR, provided that:

a)Y is absent when X is N;

b) Y' is absent when X' is N;

c) Y is NR when X is CR; and

d) Y' is NR when X' is CR,

L^1 and $L^{1'}$ are each independently $-O(C=O)R^1$, $-(C=O)OR^1$, $-C(=O)R^1$, $-OR^1$, $-S(O)_zR^1$, $-S-SR^1$, $-C(=O)SR^1$, $-SC(=O)R^1$, $-NR^aC(=O)R^1$, $-C(=O)NR^bR^c$, $-NR^aC(=O)NR^bR^c$, $-OC(=O)NR^bR^c$ or $-NR^aC(=O)OR^1$;

L^2 and $L^{2'}$ are each independently $-O(C=O)R^2$, $-(C=O)OR^2$, $-C(=O)R^2$,

$-OR^2$, $-S(O)_zR^2$, $-S-SR^2$, $-C(=O)SR^2$, $-SC(=O)R^2$, $-NR^dC(=O)R^2$, $-C(=O)NR^eR^f$, $-NR^dC(=O)NR^eR^f$, $-OC(=O)NR^eR^f$;$-NR^dC(=O)OR^2$ or a direct bond to R^2;

G^1, $G^{1'}$, G^2 and $G^{2'}$ are each independently C_2-C_{12} alkylene or C_2-C_{12} alkenylene;

G^3 is C_2-C_{24} alkyleneoxide or C_2-C_{24} alkenyleneoxide;

R^a, R^b, R^d and R^e are, at each occurrence, independently H, C_1-C_{12} alkyl or C_2-C_{12} alkenyl;

R^c and R^f are, at each occurrence, independently C_1-C_{12} alkyl or C_2-C_{12} alkenyl;

R is, at each occurrence, independently H or C_1-C_{12} alkyl;

R^1 and R^2 are, at each occurrence, independently branched C_6-C_{24} alkyl or branched C_6-C_{24} alkenyl;

z is 0, 1 or 2, and

wherein each alkyl, alkenyl, alkylene, alkenylene, alkyleneoxide and alkenyleneoxide is independently substituted or unsubstituted unless otherwise specified.

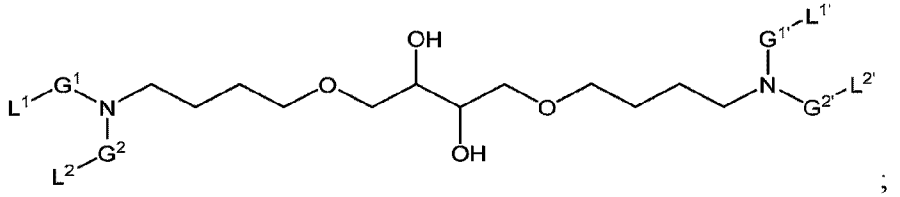
In some embodiments of structure (VII), G^3 is C_2-C_{24} alkyleneoxide or C_2-C_{24} alkenyleneoxide. In certain embodiments, G^3 is unsubstituted. In other embodiments, G^3 is substituted, for example substituted with hydroxyl. In more specific embodiments G^3 is C_2-C_{12} alkyleneoxide, for example, in some embodiments G^3 is C_3-C_7 alkyleneoxide or in other embodiments G^3 is C_3-C_{12} alkyleneoxide.

In other embodiments of structure (VII), G^3 is C_2-C_{24} alkyleneaminyl or C_2-C_{24} alkenyleneaminyl, for example C_6-C_{12} alkyleneaminyl. In some of these embodiments, G^3 is unsubstituted. In other of these embodiments, G^3 is substituted with C_1-C_6 alkyl.

In some embodiments of structure (VII), X and X' are each N, and Y and Y' are each absent. In other embodiments, X and X' are each CR, and Y and Y' are each NR. In some of these embodiments, R is H.

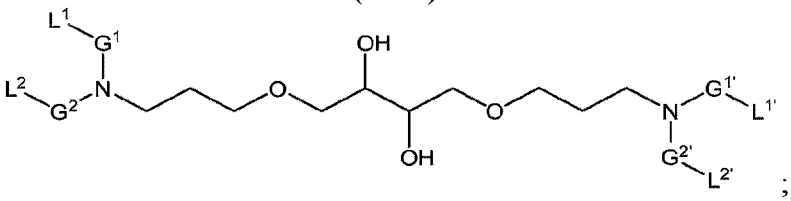
In certain embodiments of structure (VII), X and X' are each CR, and Y and Y' are each independently -O(C=O)- or -(C=O)O-.

In some of the foregoing embodiments of structure (VII), the compound has one of the following structures (VIIA), (VIIB), (VIIC), (VIID), (VIIE), (VIIF), (VIIG) or (VIIH):



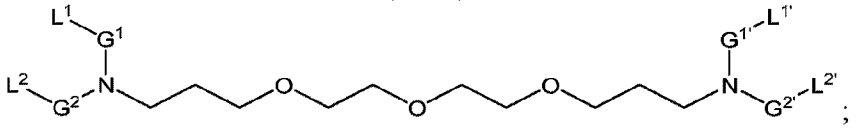
;

(VIIA)



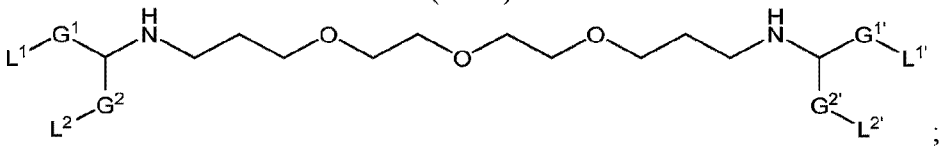
;

(VIIB)



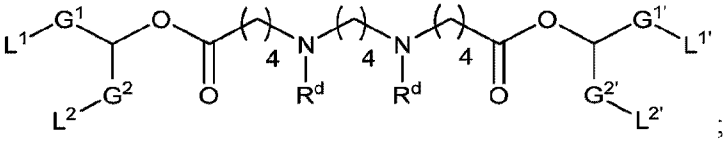
;

(VIIC)



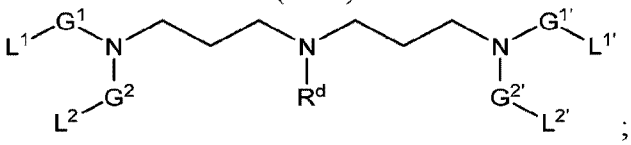
;

(VIID)



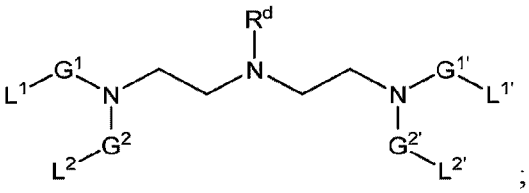
;

(VIIE)



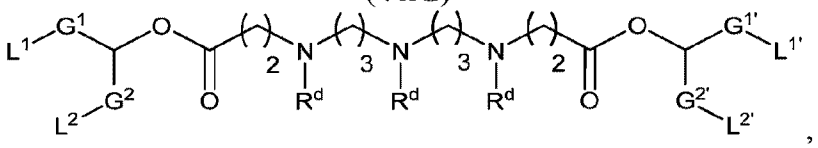
;

(VIIF)



; or

(VIIG)



,

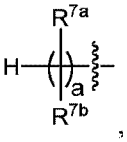
(VIIH)

wherein R^d is, at each occurrence, independently H or optionally substituted C_1-C_6 alkyl. For example, in some embodiments R^d is H. In other embodiments, R^d is C_1-C_6 alkyl, such as methyl. In other embodiments, R^d is substituted C_1-C_6 alkyl, such as C_1-C_6 alkyl substituted with -O(C=O)R, -(C=O)OR, -NRC(=O)R or -C(=O)N(R)$_2$, wherein R is, at each occurrence, independently H or C_1-C_{12} alkyl.

In some of the foregoing embodiments of structure (VII), L^1 and $L^{1'}$ are each independently -O(C=O)R^1, -(C=O)OR^1 or -C(=O)NR^bR^c, and L^2 and $L^{2'}$ are each independently -O(C=O)R^2, -(C=O)OR^2 or -C(=O)NR^eR^f. For example, in some embodiments L^1 and $L^{1'}$ are each -(C=O)OR^1, and L^2 and $L^{2'}$ are each -(C=O)OR^2.. In other embodiments L^1 and $L^{1'}$ are each -(C=O)OR^1, and L^2 and $L^{2'}$ are each -C(=O)NR^eR^f. In other embodiments L^1 and $L^{1'}$ are each -C(=O)NR^bR^c, and L^2 and $L^{2'}$ are each -C(=O)NR^eR^f.

In some embodiments of the foregoing, G^1, $G^{1'}$, G^2 and $G^{2'}$ are each independently C_2-C_8 alkylene, for example C_4-C_8 alkylene.

In some of the foregoing embodiments of structure (VII), R^1 or R^2, are each, at each occurrence, independently branched C_6-C_{24} alkyl. For example, in some embodiments, R^1 and R^2 at each occurrence, independently have the following structure:



,

wherein:

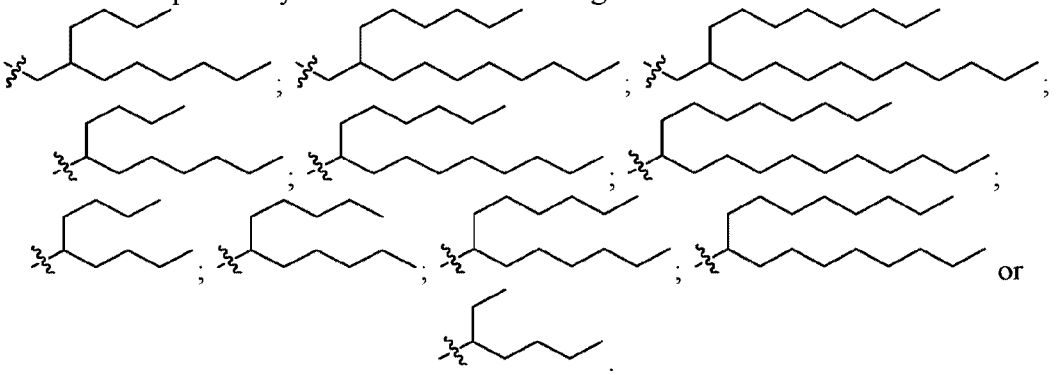
R^{7a} and R^{7b} are, at each occurrence, independently H or C_1-C_{12} alkyl; and

a is an integer from 2 to 12,

wherein R^{7a}, R^{7b} and a are each selected such that R^1 and R^2 each independently comprise from 6 to 20 carbon atoms. For example, in some embodiments a is an integer ranging from 5 to 9 or from 8 to 12.

In some of the foregoing embodiments of structure (VII), at least one occurrence of R^{7a} is H. For example, in some embodiments, R^{7a} is H at each occurrence. In other different embodiments of the foregoing, at least one occurrence of R^{7b} is C_1-C_8 alkyl. For example, in some embodiments, C_1-C_8 alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

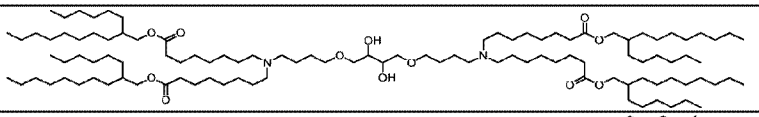
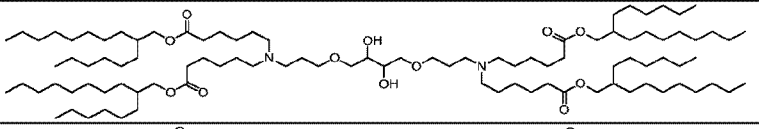
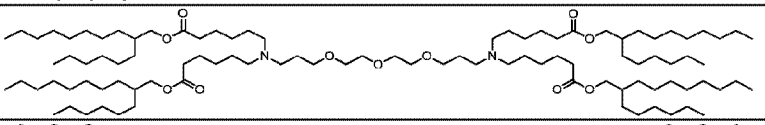
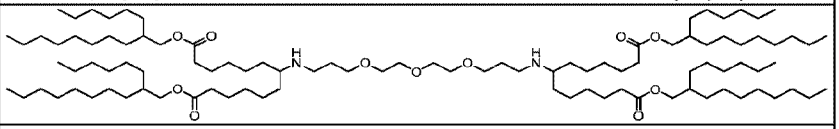
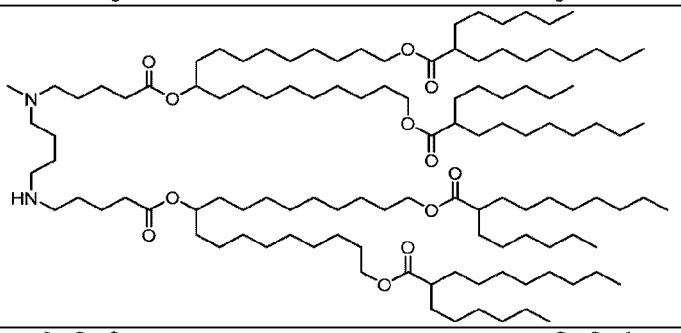
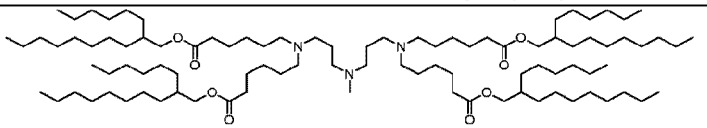
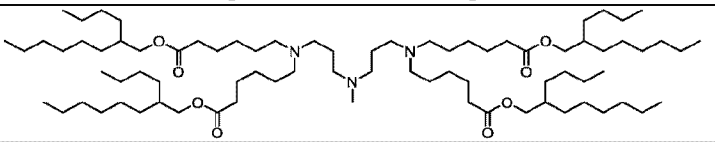
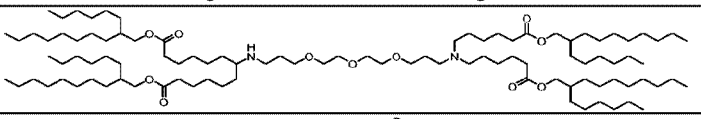
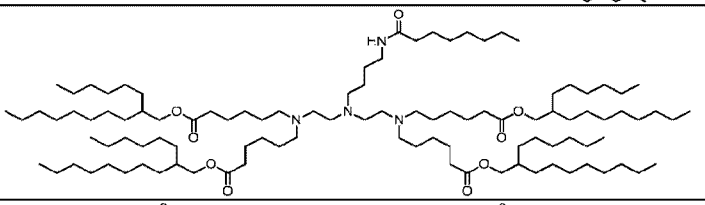
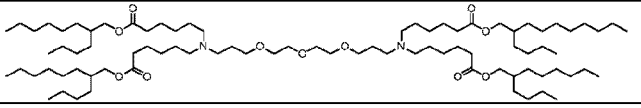
In different embodiments of structure (VII), R^1 or R^2, or both, at each occurrence independently has one of the following structures:

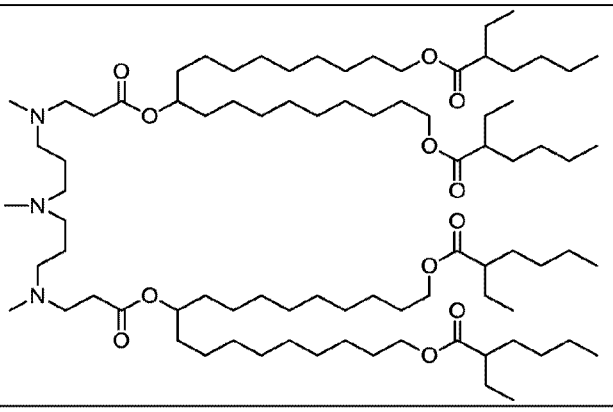


In some of the foregoing embodiments of structure (VII), R^b, R^c, R^e and R^f, when present, are each independently C_3-C_{12} alkyl. For example, in some embodiments R^b, R^c, R^e and R^f, when present, are n-hexyl and in other embodiments R^b, R^c, R^e and R^f, when present, are n-octyl.

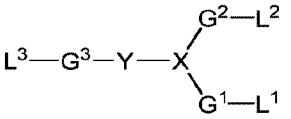
In various different embodiments of structure (VII), the cationic lipid has one of the structures set forth in Table 6 below.

Table 6. Representative cationic lipids of structure (VII)

No.	Structure
VII-1	
VII-2	
VII-3	
VII-4	
VII-5	
VII-6	
VII-7	
VII-8	
VII-9	
VII-10	

No.	Structure
VII-11	

In one embodiment, the cationic lipid is a compound having the following structure (VIII):



(VIII)

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

X is N, and Y is absent; or X is CR, and Y is NR;

L^1 is $-O(C=O)R^1$, $-(C=O)OR^1$, $-C(=O)R^1$, $-OR^1$, $-S(O)_xR^1$, $-S-SR^1$, $-C(=O)SR^1$, $-SC(=O)R^1$, $-NR^aC(=O)R^1$, $-C(=O)NR^bR^c$, $-NR^aC(=O)NR^bR^c$, $-OC(=O)NR^bR^c$ or $-NR^aC(=O)OR^1$;

L^2 is $-O(C=O)R^2$, $-(C=O)OR^2$, $-C(=O)R^2$, $-OR^2$, $-S(O)_xR^2$, $-S-SR^2$, $-C(=O)SR^2$, $-SC(=O)R^2$, $-NR^dC(=O)R^2$, $-C(=O)NR^eR^f$, $-NR^dC(=O)NR^eR^f$, $-OC(=O)NR^eR^f$; $-NR^dC(=O)OR^2$ or a direct bond to R^2;

L^3 is $-O(C=O)R^3$ or $-(C=O)OR^3$;

G^1 and G^2 are each independently C_2-C_{12} alkylene or C_2-C_{12} alkenylene;

G^3 is C_1-C_{24} alkylene, C_2-C_{24} alkenylene, C_1-C_{24} heteroalkylene or C_2-C_{24} heteroalkenylene;

R^a, R^b, R^d and R^e are each independently H or C_1-C_{12} alkyl or C_1-C_{12} alkenyl;

R^c and R^f are each independently C_1-C_{12} alkyl or C_2-C_{12} alkenyl;

each R is independently H or C_1-C_{12} alkyl;

R^1, R^2 and R^3 are each independently C_1-C_{24} alkyl or C_2-C_{24} alkenyl; and

x is 0, 1 or 2, and

wherein each alkyl, alkenyl, alkylene, alkenylene, heteroalkylene and heteroalkenylene is independently substituted or unsubstituted unless otherwise specified.

In more embodiments of structure (I):

X is N, and Y is absent; or X is CR, and Y is NR;

L^1 is $-O(C=O)R^1$, $-(C=O)OR^1$, $-C(=O)R^1$, $-OR^1$, $-S(O)_xR^1$, $-S-SR^1$, $-C(=O)SR^1$, $-SC(=O)R^1$, $-NR^aC(=O)R^1$, $-C(=O)NR^bR^c$, $-NR^aC(=O)NR^bR^c$, $-OC(=O)NR^bR^c$ or $-NR^aC(=O)OR^1$;

L^2 is $-O(C=O)R^2$, $-(C=O)OR^2$, $-C(=O)R^2$, $-OR^2$, $-S(O)_xR^2$, $-S-SR^2$, $-C(=O)SR^2$, $-SC(=O)R^2$, $-NR^dC(=O)R^2$, $-C(=O)NR^eR^f$, $-NR^dC(=O)NR^eR^f$, $-OC(=O)NR^eR^f$; $-NR^dC(=O)OR^2$ or a direct bond to R^2;

L^3 is $-O(C=O)R^3$ or $-(C=O)OR^3$;

G^1 and G^2 are each independently C_2-C_{12} alkylene or C_2-C_{12} alkenylene;

G^3 is C_1-C_{24} alkylene, C_2-C_{24} alkenylene, C_1-C_{24} heteroalkylene or C_2-C_{24} heteroalkenylene when X is CR, and Y is NR; and G^3 is C_1-C_{24} heteroalkylene or C_2-C_{24} heteroalkenylene when X is N, and Y is absent;

R^a, R^b, R^d and R^e are each independently H or C_1-C_{12} alkyl or C_1-C_{12} alkenyl;

R^c and R^f are each independently C_1-C_{12} alkyl or C_2-C_{12} alkenyl;

each R is independently H or C_1-C_{12} alkyl;

R^1, R^2 and R^3 are each independently C_1-C_{24} alkyl or C_2-C_{24} alkenyl; and

x is 0, 1 or 2, and

wherein each alkyl, alkenyl, alkylene, alkenylene, heteroalkylene and heteroalkenylene is independently substituted or unsubstituted unless otherwise specified.

In other embodiments of structure (I):

X is N and Y is absent, or X is CR and Y is NR;

L^1 is $-O(C=O)R^1$, $-(C=O)OR^1$, $-C(=O)R^1$, $-OR^1$, $-S(O)_xR^1$, $-S-SR^1$,

$-C(=O)SR^1$, $-SC(=O)R^1$, $-NR^aC(=O)R^1$, $-C(=O)NR^bR^c$, $-NR^aC(=O)NR^bR^c$, $-OC(=O)NR^bR^c$ or $-NR^aC(=O)OR^1$;

L^2 is $-O(C=O)R^2$, $-(C=O)OR^2$, $-C(=O)R^2$, $-OR^2$, $-S(O)_xR^2$, $-S-SR^2$, $-C(=O)SR^2$, $-SC(=O)R^2$, $-NR^dC(=O)R^2$, $-C(=O)NR^eR^f$, $-NR^dC(=O)NR^eR^f$, $-OC(=O)NR^eR^f$; $-NR^dC(=O)OR^2$ or a direct bond to R^2;

L^3 is $-O(C=O)R^3$ or $-(C=O)OR^3$;

G^1 and G^2 are each independently C_2-C_{12} alkylene or C_2-C_{12} alkenylene;

G^3 is C_1-C_{24} alkylene, C_2-C_{24} alkenylene, C_1-C_{24} heteroalkylene or C_2-C_{24} heteroalkenylene;

R^a, R^b, R^d and R^e are each independently H or C_1-C_{12} alkyl or C_1-C_{12} alkenyl;

R^c and R^f are each independently C_1-C_{12} alkyl or C_2-C_{12} alkenyl;

each R is independently H or C_1-C_{12} alkyl;

R^1, R^2 and R^3 are each independently branched C_6-C_{24} alkyl or branched C_6-C_{24} alkenyl; and

x is 0, 1 or 2, and

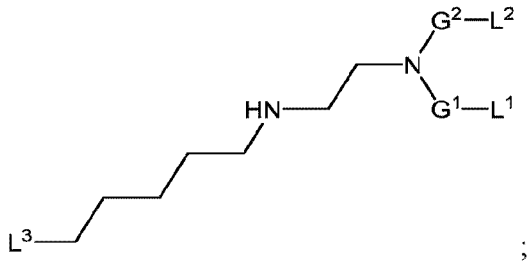
wherein each alkyl, alkenyl, alkylene, alkenylene, heteroalkylene and heteroalkenylene is independently substituted or unsubstituted unless otherwise specified.

In certain embodiments of structure (VIII), G^3 is unsubstituted. In more specific embodiments G^3 is C_2-C_{12} alkylene, for example, in some embodiments G^3 is C_3-C_7 alkylene or in other embodiments G^3 is C_3-C_{12} alkylene. In some embodiments, G^3 is C_2 or C_3 alkylene.

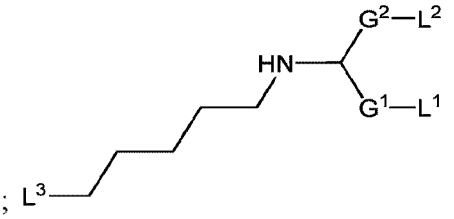
In other embodiments of structure (VIII), G^3 is C_1-C_{12} heteroalkylene, for example C_1-C_{12} aminylalkylene.

In certain embodiments of structure (VIII), X is N and Y is absent. In other embodiments, X is CR and Y is NR, for example in some of these embodiments R is H.

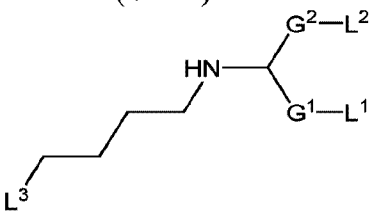
In some of the foregoing embodiments of structure (VIII), the compound has one of the following structures (VIIIA), (VIIIB), (VIIIC) or (VIIID):



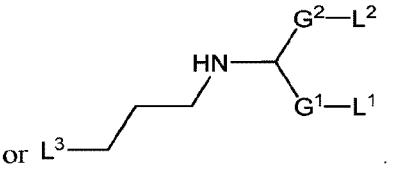
(VIIIA)



(VIIIB)



(VIIIC)

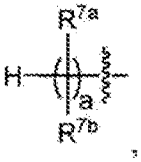


(VIIID)

In some of the foregoing embodiments of structure (VIII), L^1 is -$O(C=O)R^1$, -$(C=O)OR^1$ or -$C(=O)NR^bR^c$, and L^2 is -$O(C=O)R^2$, -$(C=O)OR^2$ or -$C(=O)NR^eR^f$. In other specific embodiments, L^1 is -$(C=O)OR^1$ and L^2 is -$(C=O)OR^2$. In any of the foregoing embodiments, L^3 is -$(C=O)OR^3$.

In some of the foregoing embodiments of structure (VIII), G^1 and G^2 are each independently C_2-C_{12} alkylene, for example C_4-C_{10} alkylene.

In some of the foregoing embodiments of structure (VIII), R^1, R^2 and R^3 are each, independently branched C_6-C_{24} alkyl. For example, in some embodiments, R^1, R^2 and R^3 each, independently have the following structure:



wherein:

R^{7a} and R^{7b} are, at each occurrence, independently H or C_1-C_{12} alkyl; and

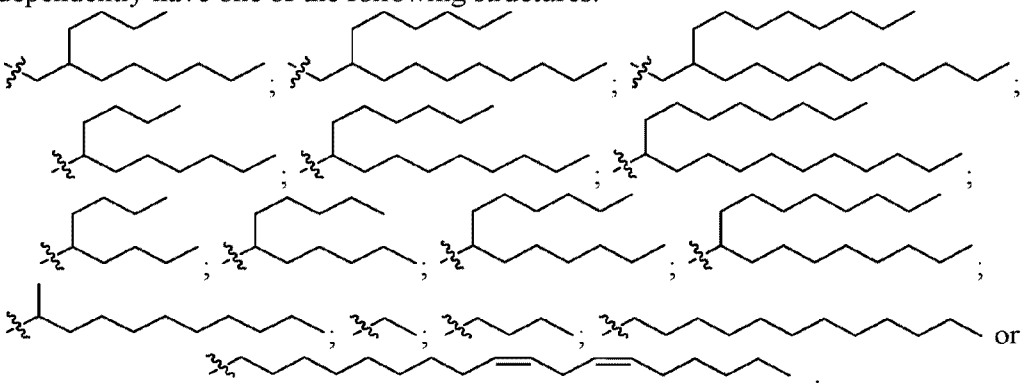
a is an integer from 2 to 12,

wherein R^{7a}, R^{7b} and a are each selected such that R^1 and R^2 each independently comprise from 6 to 20 carbon atoms. For example, in some embodiments a is an integer ranging from 5 to 9 or from 8 to 12.

In some of the foregoing embodiments of structure (VIII), at least one occurrence of R^{7a} is H. For example, in some embodiments, R^{7a} is H at each occurrence. In other different embodiments of the foregoing, at least one occurrence of R^{7b} is C_1-C_8 alkyl. For example, in some embodiments, C_1-C_8 alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

In some of the foregoing embodiments of structure (VIII), X is CR, Y is NR and R^3 is C_1-C_{12} alkyl, such as ethyl, propyl or butyl. In some of these embodiments, , R^1 and R^2 are each independently branched C_6-C_{24} alkyl.

In different embodiments of structure (VIII), R^1, R^2 and R^3 each, independently have one of the following structures:

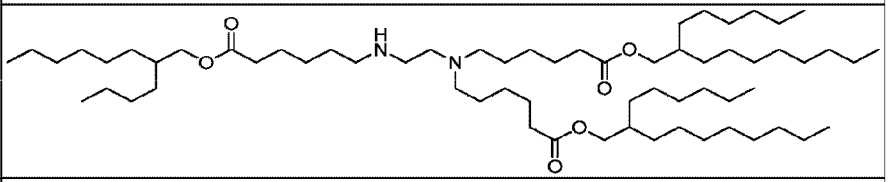
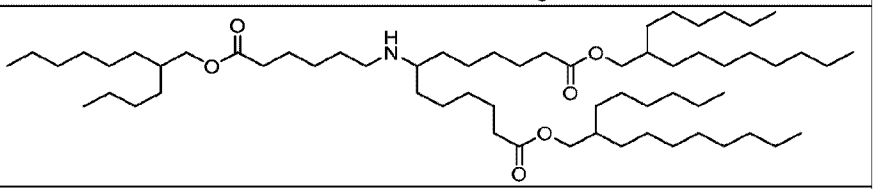
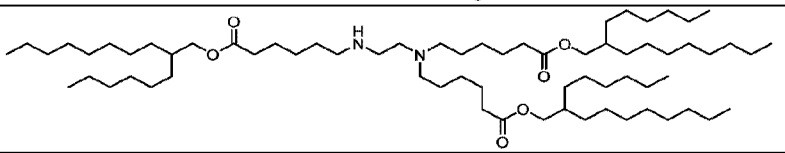
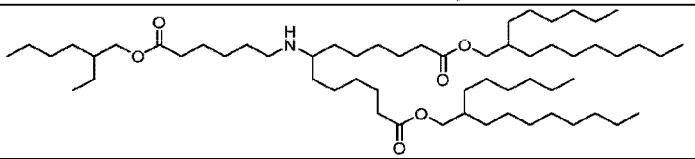
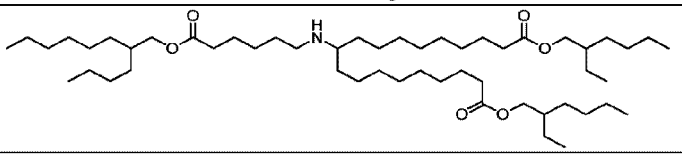
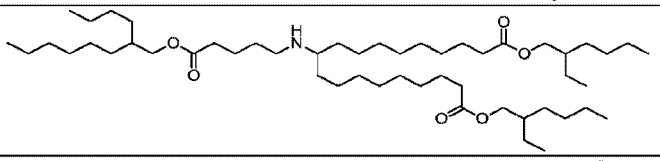
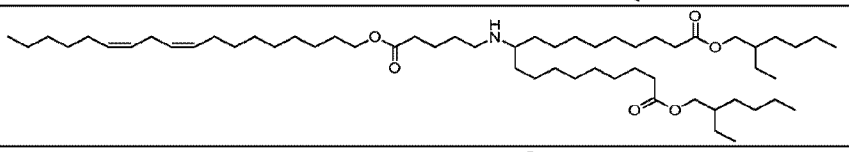
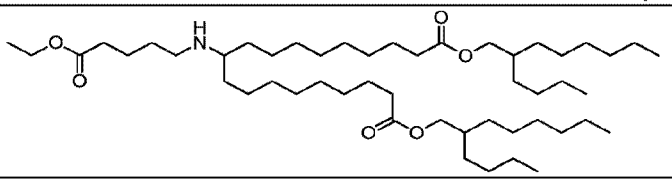


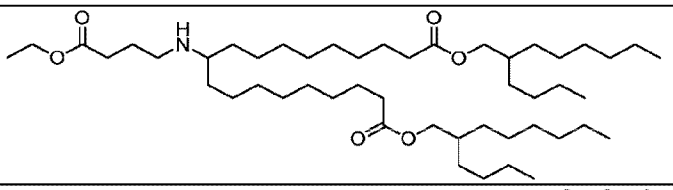
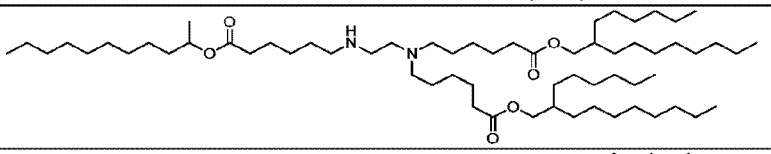
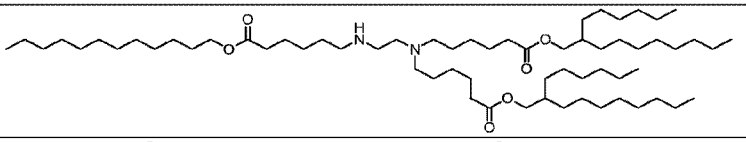
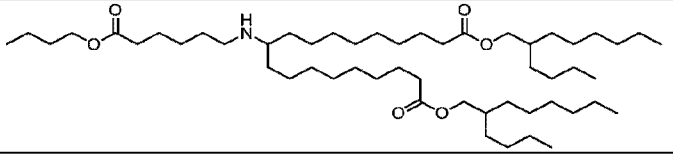
In certain embodiments of structure (VIII), R^1 and R^2 and R^3 are each, independently, branched C_6-C_{24} alkyl and R^3 is C_1-C_{24} alkyl or C_2-C_{24} alkenyl.

In some of the foregoing embodiments of structure (VIII), R^b, R^c, R^e and R^f are each independently C_3-C_{12} alkyl. For example, in some embodiments R^b, R^c, R^e and R^f are n-hexyl and in other embodiments R^b, R^c, R^e and R^f are n-octyl.

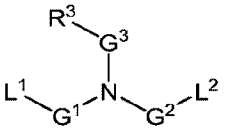
In various different embodiments of structure (VIII), the compound has one of the structures set forth in Table 7 below.

Table 7. Representative cationic lipids of structure (VIII)

No.	Structure
VIII-1	
VIII-2	
VIII-3	
VIII-4	
VIII-5	
VIII-6	
VIII-7	
VIII-8	

No.	Structure
VIII-9	
VIII-10	
VIII-11	
VIII-12	

In one embodiment, the cationic lipid is a compound having the following structure (IX):



(IX)

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

L^1 is $-O(C=O)R^1$, $-(C=O)OR^1$, $-C(=O)R^1$, $-OR^1$, $-S(O)_xR^1$, $-S-SR^1$, $-C(=O)SR^1$, $-SC(=O)R^1$, $-NR^aC(=O)R^1$, $-C(=O)NR^bR^c$, $-NR^aC(=O)NR^bR^c$, $-OC(=O)NR^bR^c$ or $-NR^aC(=O)OR^1$;

L^2 is $-O(C=O)R^2$, $-(C=O)OR^2$, $-C(=O)R^2$, $-OR^2$, $-S(O)_xR^2$, $-S-SR^2$, $-C(=O)SR^2$, $-SC(=O)R^2$, $-NR^dC(=O)R^2$, $-C(=O)NR^eR^f$, $-NR^dC(=O)NR^eR^f$, $-OC(=O)NR^eR^f$, $-NR^dC(=O)OR^2$ or a direct bond to R^2;

G^1 and G^2 are each independently C_2-C_{12} alkylene or C_2-C_{12} alkenylene;

G^3 is C_1-C_{24} alkylene, C_2-C_{24} alkenylene, C_3-C_8 cycloalkylene or C_3-C_8 cycloalkenylene;

R^a, R^b, R^d and R^e are each independently H or C_1-C_{12} alkyl or C_1-C_{12} alkenyl;

R^c and R^f are each independently C_1-C_{12} alkyl or C_2-C_{12} alkenyl;

R^1 and R^2 are each independently branched C_6-C_{24} alkyl or branched C_6-C_{24} alkenyl;

R^3 is $-N(R^4)R^5$;

R^4 is C_1-C_{12} alkyl;

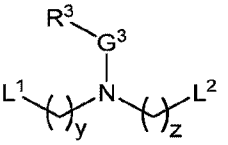
R^5 is substituted C_1-C_{12} alkyl; and

x is 0, 1 or 2, and

wherein each alkyl, alkenyl, alkylene, alkenylene, cycloalkylene, cycloalkenylene, aryl and aralkyl is independently substituted or unsubstituted unless otherwise specified.

In certain embodiments of structure (XI), G^3 is unsubstituted. In more specific embodiments G^3 is C_2-C_{12} alkylene, for example, in some embodiments G^3 is C_3-C_7 alkylene or in other embodiments G^3 is C_3-C_{12} alkylene. In some embodiments, G^3 is C_2 or C_3 alkylene.

In some of the foregoing embodiments of structure (IX), the compound has the following structure (IX A):

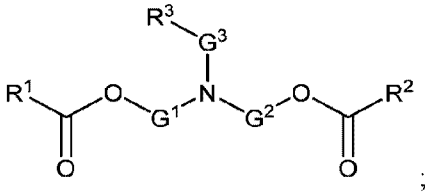


(IXA)

wherein y and z are each independently integers ranging from 2 to 12, for example an integer from 2 to 6, from 4 to 10, or for example 4 or 5. In certain embodiments, y and z are each the same and selected from 4, 5, 6, 7, 8 and 9.

In some of the foregoing embodiments of structure (IX), L^1 is $-O(C=O)R^1$, $-(C=O)OR^1$ or $-C(=O)NR^bR^c$, and L^2 is $-O(C=O)R^2$, $-(C=O)OR^2$ or $-C(=O)NR^eR^f$. For example, in some embodiments L^1 and L^2 are $-(C=O)OR^1$ and $-(C=O)OR^2$, respectively. In other embodiments L^1 is $-(C=O)OR^1$ and L^2 is $-C(=O)NR^eR^f$. In other embodiments L^1 is $-C(=O)NR^bR^c$ and L^2 is $-C(=O)NR^eR^f$.

In other embodiments of the foregoing, the compound has one of the following structures (IXB), (IXC), (IXD) or (IXE):



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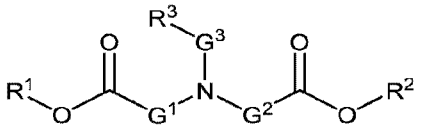
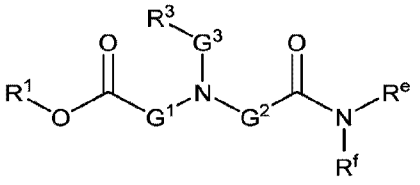
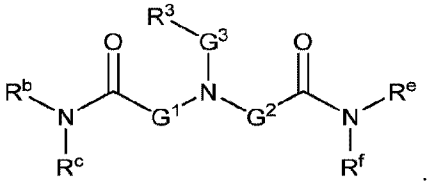
(IXB)



(IXC)



or



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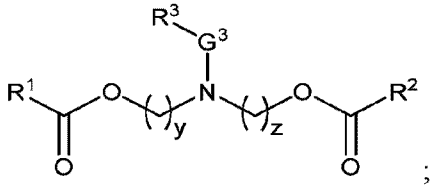
(IXD) (IXE)

In some of the foregoing embodiments, the compound has structure (IXB), in other embodiments, the compound has structure (IXC) and in still other embodiments the compound has the structure (IXD). In other embodiments, the compound has structure (IXE).

In some different embodiments of the foregoing, the compound has one of the following structures (IXF), (IXG), (IXH) or (IXJ):



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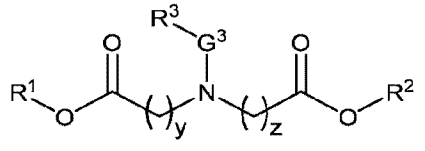
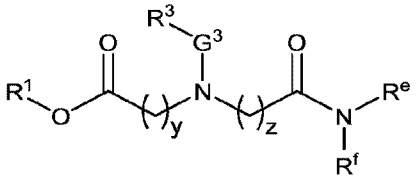
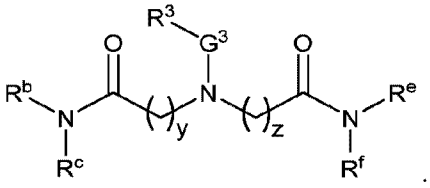
(IXF)



(IXG)



or



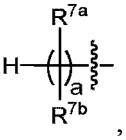
.

(IXH) (IXJ)

wherein y and z are each independently integers ranging from 2 to 12, for example an integer from 2 to 6, for example 4.

In some of the foregoing embodiments of structure (IX), y and z are each independently an integer ranging from 2 to 10, 2 to 8, from 4 to 10 or from 4 to 7. For example, in some embodiments, y is 4, 5, 6, 7, 8, 9, 10, 11 or 12. In some embodiments, z is 4, 5, 6, 7, 8, 9, 10, 11 or 12. In some embodiments, y and z are the same, while in other embodiments y and z are different.

In some of the foregoing embodiments of structure (IX), R^1 or R^2, or both is branched C_6-C_{24} alkyl. For example, in some embodiments, R^1 and R^2 each, independently have the following structure:



,

wherein:

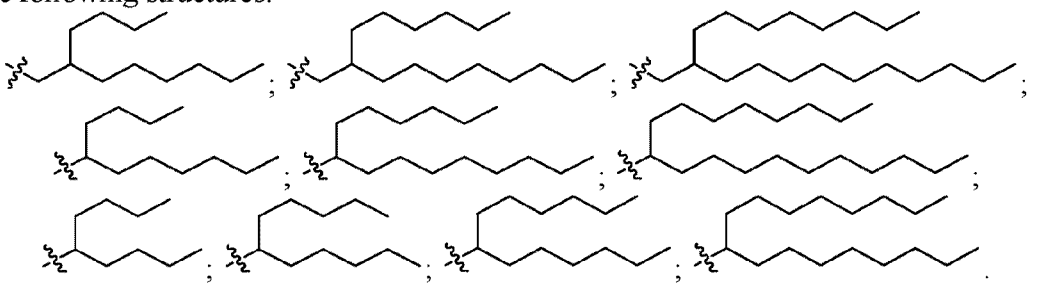
R^{7a} and R^{7b} are, at each occurrence, independently H or C_1-C_{12} alkyl; and

a is an integer from 2 to 12,

wherein R^{7a}, R^{7b} and a are each selected such that R^1 and R^2 each independently comprise from 6 to 20 carbon atoms. For example, in some embodiments a is an integer ranging from 5 to 9 or from 8 to 12.

In some of the foregoing embodiments of structure (IX), at least one occurrence of R^{7a} is H. For example, in some embodiments, R^{7a} is H at each occurrence. In other different embodiments of the foregoing, at least one occurrence of R^{7b} is C_1-C_8 alkyl. For example, in some embodiments, C_1-C_8 alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

In different embodiments of structure (IX), R^1 or R^2, or both, has one of the following structures:


;
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In some of the foregoing embodiments of structure (IX), R^b, R^c, R^e and R^f are each independently C_3-C_{12} alkyl. For example, in some embodiments R^b, R^c, R^e and R^f are n-hexyl and in other embodiments R^b, R^c, R^e and R^f are n-octyl.

In any of the foregoing embodiments of structure (IX), R^4 is substituted or unsubstituted: methyl, ethyl, propyl, n-butyl, n-hexyl, n-octyl or n-nonyl. For example, in some embodiments R^4 is unsubstituted. In other R^4 is substituted with one or more substituents selected from the group consisting of $-OR^g$, $-NR^gC(=O)R^h$, $-C(=O)NR^gR^h$, $-C(=O)R^h$, $-OC(=O)R^h$, $-C(=O)OR^h$ and $-OR^iOH$, wherein:

R^g is, at each occurrence independently H or C_1-C_6 alkyl;

R^h is at each occurrence independently C_1-C_6 alkyl; and

R^i is, at each occurrence independently C_1-C_6 alkylene.

In other of the foregoing embodiments of structure (IX), R^5 is substituted: methyl, ethyl, propyl, n-butyl, n-hexyl, n-octyl or n-nonyl. In some embodiments, R^5 is substituted ethyl or substituted propyl. In other different embodiments, R^5 is substituted with hydroxyl. In still more embodiments, R^5 is substituted with one or more substituents selected from the group consisting of $-OR^g$, $-NR^gC(=O)R^h$, $-C(=O)NR^gR^h$, $-C(=O)R^h$, $-OC(=O)R^h$, $-C(=O)OR^h$ and $-OR^iOH$, wherein:

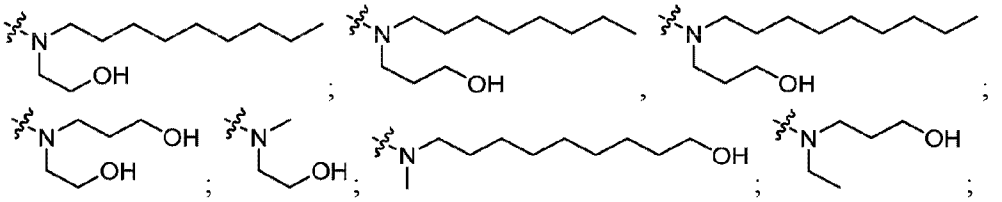
R^g is, at each occurrence independently H or C_1-C_6 alkyl;

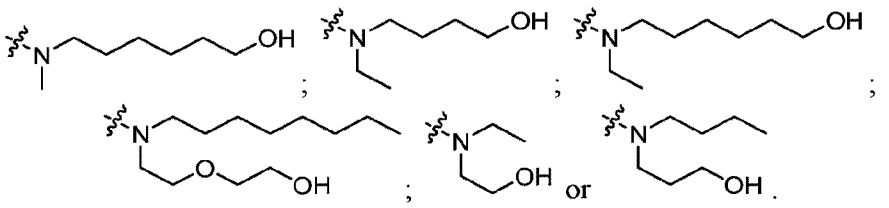
R^h is at each occurrence independently C_1-C_6 alkyl; and

R^i is, at each occurrence independently C_1-C_6 alkylene.

In other embodiments of structure (IX), R^4 is unsubstituted methyl, and R^5 is substituted: methyl, ethyl, propyl, n-butyl, n-hexyl, n-octyl or n-nonyl. In some of these embodiments, R^5 is substituted with hydroxyl.

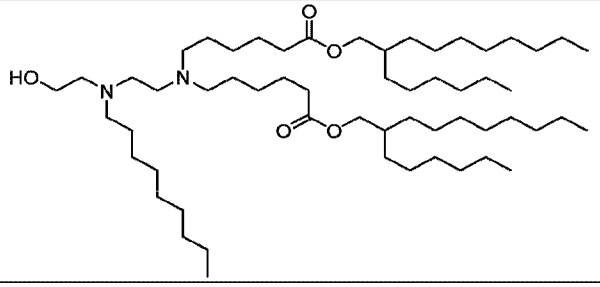
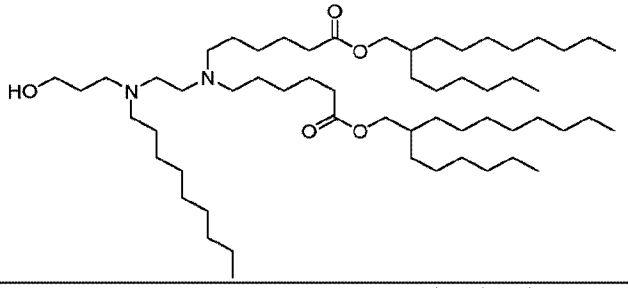
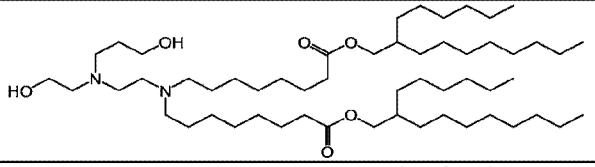
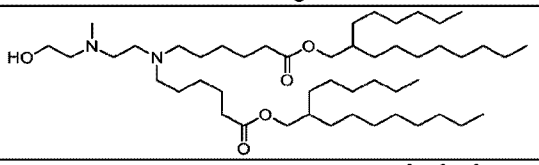
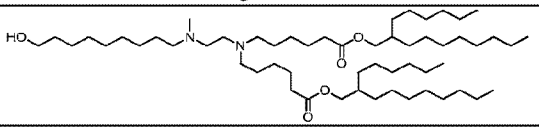
In some other specific embodiments of structure (IX), R^3 has one of the following structures:

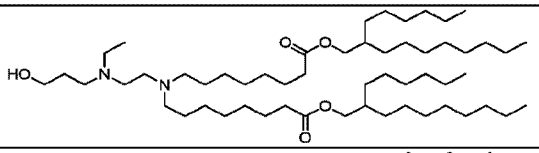
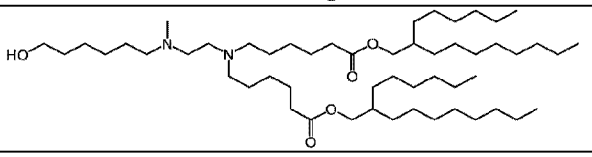
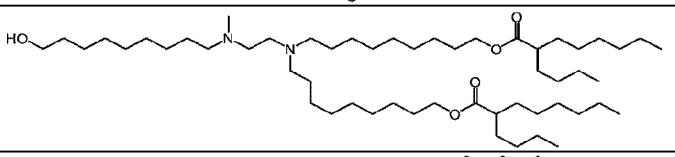
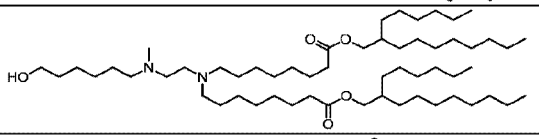
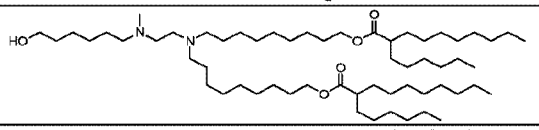
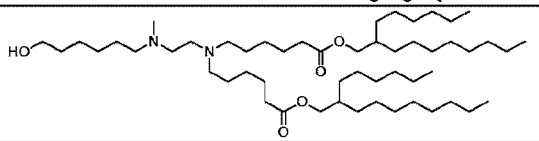
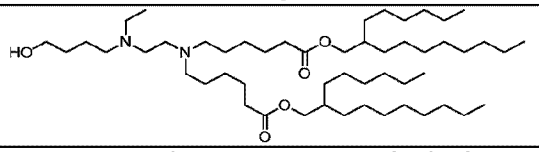
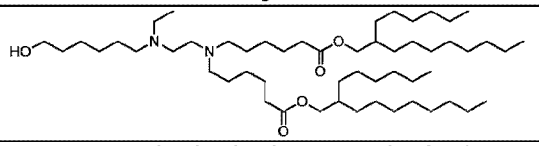
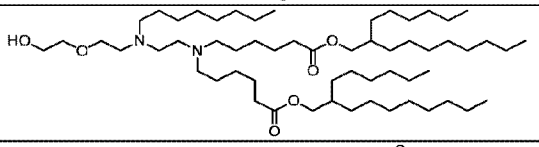
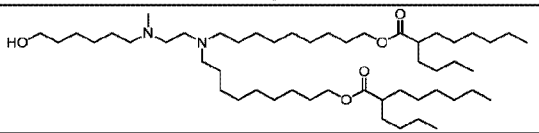
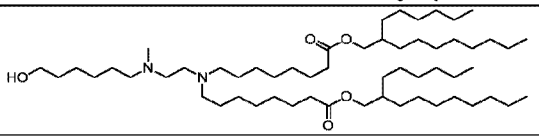


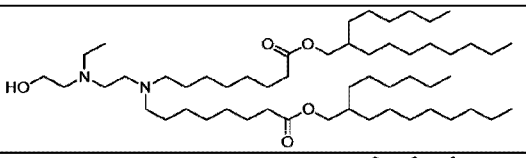
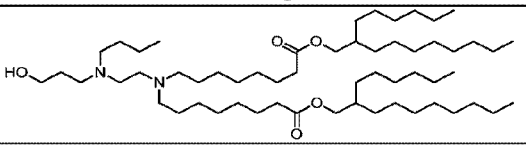


In various different embodiments of structure (IX), the cationic lipid has one of the structures set forth in Table 8 below.

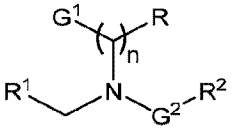
Table 8. Representative cationic lipids of structure (IX)

No.	Structure
IX-1	
IX-2	
IX-3	
IX-4	
IX-5	

No.	Structure
IX-6	
IX-7	
IX-8	
IX-9	
IX-10	
IX-11	
IX-12	
IX-13	
IX-14	
IX-15	
IX-16	

No.	Structure
IX-17	
IX-18	

In one embodiment, the cationic lipid is a compound having the following structure (X):



(X)

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

G^1 is –OH, $-NR^3R^4$, $-(C=O)NR^5$ or $-NR^3(C=O)R^5$;

G^2 is $-CH_2-$ or $-(C=O)-$;

R is, at each occurrence, independently H or OH;

R^1 and R^2 are each independently branched, saturated or unsaturated C_{12}-C_{36} alkyl;

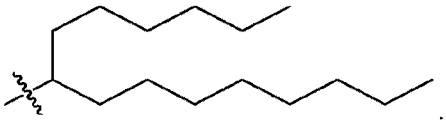
R^3 and R^4 are each independently H or straight or branched, saturated or unsaturated C_1-C_6 alkyl;

R^5 is straight or branched, saturated or unsaturated C_1-C_6 alkyl; and

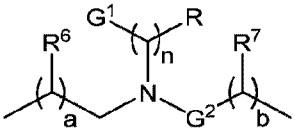
n is an integer from 2 to 6.

In some embodiments, R^1 and R^2 are each independently branched, saturated or unsaturated C_{12}-C_{30} alkyl, C_{12}-C_{20} alkyl, or C_{15}-C_{20} alkyl. In some specific embodiments, R^1 and R^2 are each saturated. In certain embodiments, at least one of R^1 and R^2 is unsaturated.

In some of the foregoing embodiments of structure (X), R^1 and R^2 have the following structure:



In some of the foregoing embodiments of structure (X), the compound has the following structure (XA):



(XA)

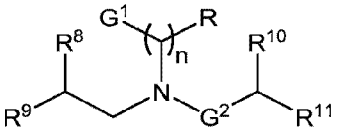
wherein:

R^6 and R^7 are, at each occurrence, independently H or straight or branched, saturated or unsaturated C_1-C_{14} alkyl;

a and b are each independently an integer ranging from 1 to 15,

provided that R^6 and a, and R^7 and b, are each independently selected such that R^1 and R^2, respectively, are each independently branched, saturated or unsaturated C_{12}-C_{36} alkyl.

In some of the foregoing embodiments, the compound has the following structure (XB):



(XB)

wherein:

R^8, R^9, R^{10} and R^{11} are each independently straight or branched, saturated or unsaturated C_4-C_{12} alkyl, provided that R^8 and R^9, and R^{10} and R^{11}, are each independently selected such that R^1 and R^2, respectively, are each independently branched, saturated or unsaturated C_{12}-C_{36} alkyl. In some embodiments of (XB), R^8, R^9, R^{10} and R^{11} are each independently straight or branched, saturated or unsaturated C_6-C_{10} alkyl. In certain embodiments of (XB), at least one of R^8, R^9, R^{10} and R^{11} is unsaturated. In other certain specific embodiments of (XB), each of R^8, R^9, R^{10} and R^{11} is saturated.

In some of the foregoing embodiments, the compound has structure (XA), and in other embodiments, the compound has structure (XB).

In some of the foregoing embodiments, G^1 is –OH, and in some embodiments G^1 is $–NR^3R^4$. For example, in some embodiments, G^1 is $–NH_2$, $-NHCH_3$ or $–N(CH_3)_2$. In certain embodiments, G^1 is $–(C=O)NR^5$. In certain other embodiments, G^1 is $–NR^3(C=O)R^5$. For example, in some embodiments G^1 is $–NH(C=O)CH_3$ or $–NH(C=O)CH_2CH_2CH_3$.

In some of the foregoing embodiments of structure (X), G^2 is $–CH_2–$. In some different embodiments, G^2 is $–(C=O)–$.

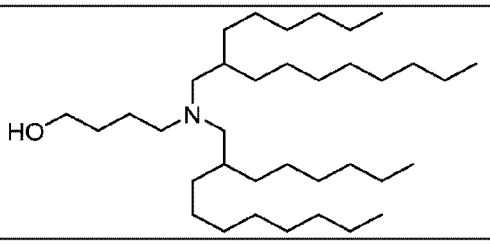
In some of the foregoing embodiments of structure (X), n is an integer ranging from 2 to 6, for example, in some embodiments n is 2, 3, 4, 5 or 6. In some embodiments, n is 2. In some embodiments, n is 3. In some embodiments, n is 4.

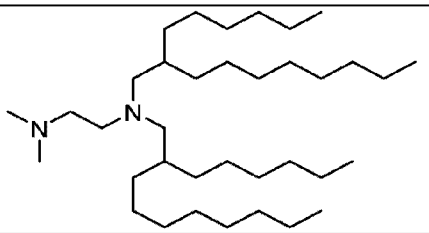
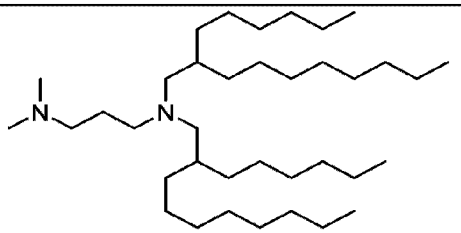
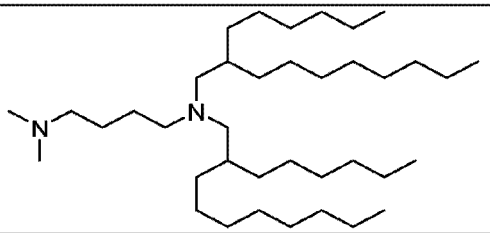
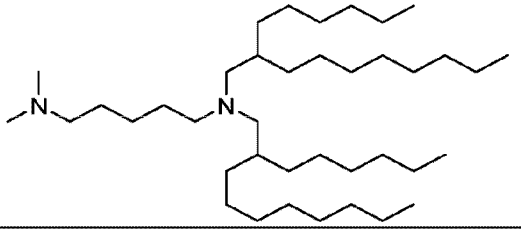
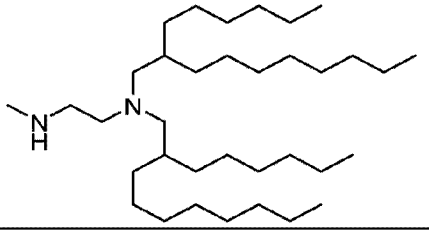
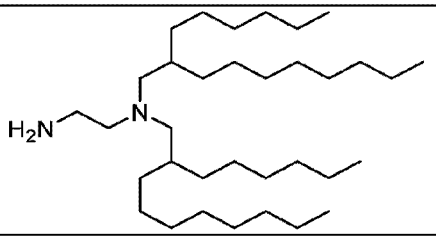
In certain of the foregoing embodiments of structure (X), at least one of R^1, R^2, R^3, R^4 and R^5 is unsubstituted. For example, in some embodiments, R^1, R^2, R^3, R^4 and R^5 are each unsubstituted. In some embodiments, R^3 is substituted. In other embodiments R^4 is substituted. In still more embodiments, R^5 is substituted. In certain specific embodiments, each of R^3 and R^4 are substituted. In some embodiments, a substituent on R^3, R^4 or R^5 is hydroxyl. In certain embodiments, R^3 and R^4 are each substituted with hydroxyl.

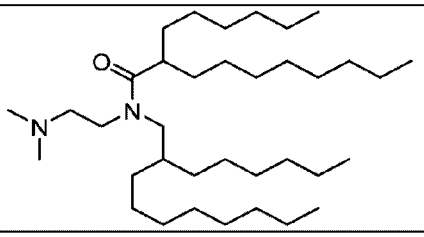
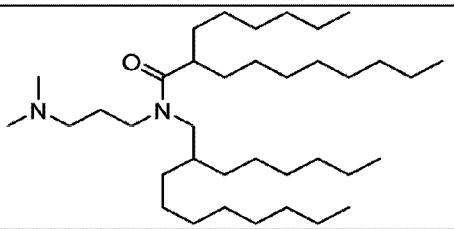
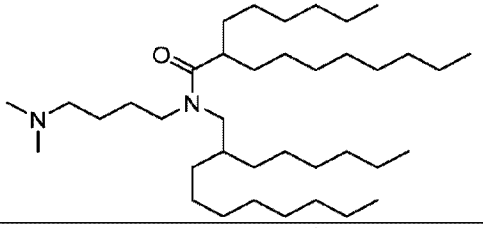
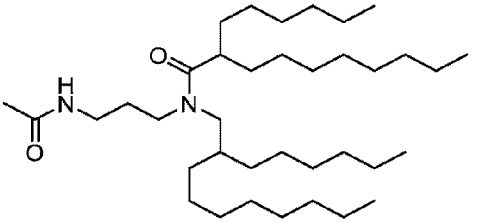
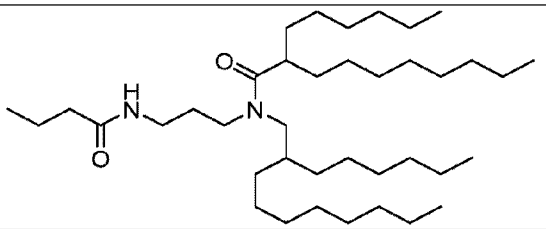
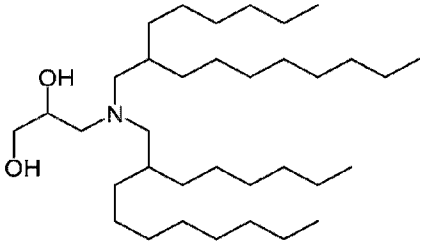
In some of the foregoing embodiments of structure (X), at least one R is OH. In other embodiments, each R is H.

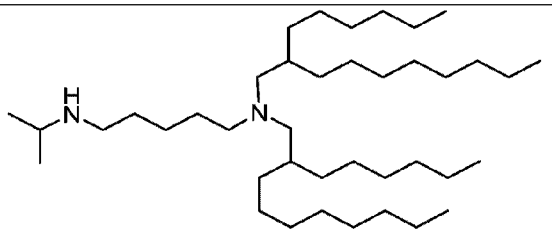
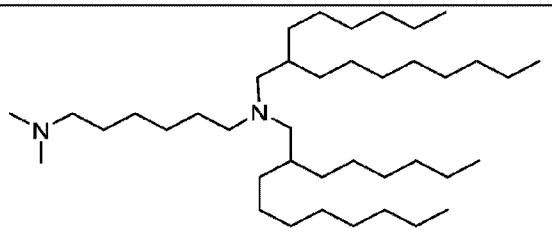
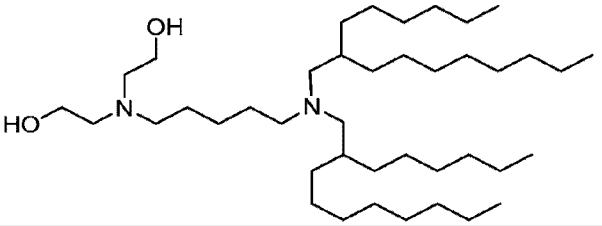
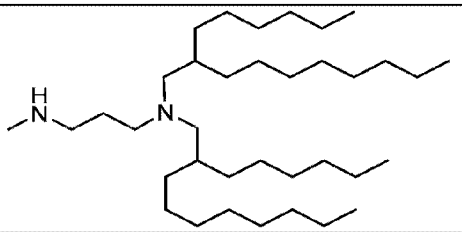
In various different embodiments of structure (X), the compound has one of the structures set forth in Table 9 below.

Table 9. Representative cationic lipids of structure (X)

No.	Structure
X-1	

No.	Structure
X-2	
X-3	
X-4	
X-5	
X-6	
X-7	

No.	Structure
X-8	
X-9	
X-10	
X-11	
X-12	
X-13	

No.	Structure
X-14	
X-15	
X-16	
X-17	

In any of Embodiments 1, 2, 3, 4 or 5, the LNPs further comprise a neutral lipid. In various 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 neutral lipid is present in any of the foregoing LNPs in a concentration ranging from 5 to 10 mol percent, from 5 to 15 mol percent, 7 to 13 mol percent, or 9 to 11 mol percent. In certain specific embodiments, the neutral lipid is present in a concentration of about 9.5, 10 or 10.5 mol percent. In some embodiments, the molar ratio of cationic lipid to the neutral lipid ranges from about 4.1:1.0 to about 4.9:1.0, from about 4.5:1.0 to about 4.8:1.0, or from about 4.7:1.0 to 4.8:1.0. In some embodiments, the molar ratio of total

cationic lipid to the neutral lipid ranges from about 4.1:1.0 to about 4.9:1.0, from about 4.5:1.0 to about 4.8:1.0, or from about 4.7:1.0 to 4.8:1.0.

Exemplary neutral lipids for use in any of Embodiments 1, 2, 3, 4 or 5 include, for example, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE) and dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1-trans PE, 1-stearioyl-2-oleoylphosphatidyethanol amine (SOPE), and 1,2-dielaidoyl-sn-glycero-3-phophoethanolamine (transDOPE). In one embodiment, the neutral lipid is 1,2-distearoyl-sn-glycero-3phosphocholine (DSPC). In some embodiments, the neutral lipid is selected from DSPC, DPPC, DMPC, DOPC, POPC, DOPE and SM. In some embodiments, the neutral lipid is DSPC.

In various embodiments of Embodiments 1, 2, 3, 4 or 5, any of the disclosed lipid nanoparticles comprise a steroid or steroid analogue. In certain embodiments, the steroid or steroid analogue is cholesterol. In some embodiments, the steroid is present in a concentration ranging from 39 to 49 molar percent, 40 to 46 molar percent, from 40 to 44 molar percent, from 40 to 42 molar percent, from 42 to 44 molar percent, or from 44 to 46 molar percent. In certain specific embodiments, the steroid is present in a concentration of 40, 41, 42, 43, 44, 45, or 46 molar percent.

In certain embodiments, the molar ratio of cationic lipid to the steroid ranges from 1.0:0.9 to 1.0:1.2, or from 1.0:1.0 to 1.0:1.2. In some of these embodiments, the molar ratio of cationic lipid to cholesterol ranges from about 5:1 to 1:1. In certain embodiments, the steroid is present in a concentration ranging from 32 to 40 mol percent of the steroid.

In certain embodiments, the molar ratio of total cationic to the steroid ranges from 1.0:0.9 to 1.0:1.2, or from 1.0:1.0 to 1.0:1.2. In some of these

embodiments, the molar ratio of total cationic lipid to cholesterol ranges from about 5:1 to 1:1. In certain embodiments, the steroid is present in a concentration ranging from 32 to 40 mol percent of the steroid.

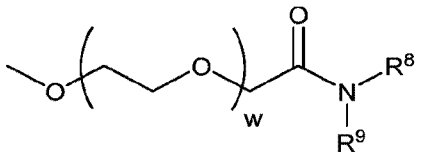
In some embodiments of Embodiments 1, 2, 3 4 or 5, the LNPs further comprise a polymer conjugated lipid. In various other embodiments of Embodiments 1, 2, 3 4 or 5, the polymer conjugated lipid is a pegylated lipid. For example, some embodiments include a pegylated diacylglycerol (PEG-DAG) such as 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (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 various embodiments, the polymer conjugated lipid is present in a concentration ranging from 1.0 to 2.5 molar percent. In certain specific embodiments, the polymer conjugated lipid is present in a concentration of about 1.7 molar percent. In some embodiments, the polymer conjugated lipid is present in a concentration of about 1.5 molar percent.

In certain embodiments, the molar ratio of cationic lipid to the polymer conjugated lipid ranges from about 35:1 to about 25:1. In some embodiments, the molar ratio of cationic lipid to polymer conjugated lipid ranges from about 100:1 to about 20:1.

In certain embodiments, the molar ratio of total cationic lipid (*i.e.*, the sum of the first and second cationic lipid) to the polymer conjugated lipid ranges from about 35:1 to about 25:1. In some embodiments, the molar ratio of total cationic lipid to polymer conjugated lipid ranges from about 100:1 to about 20:1.

In some embodiments of Embodiments 1, 2, 3 4 or 5, the pegylated lipid, when present, has the following Formula (XI):



(XI)

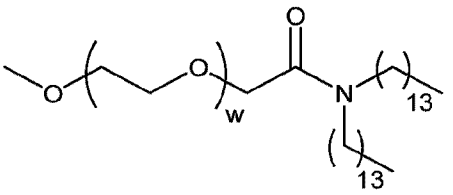
or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein:

R^{12} and R^{13} are each independently a straight or branched, saturated or unsaturated alkyl chain containing from 10 to 30 carbon atoms, wherein the alkyl chain is optionally interrupted by one or more ester bonds; and

w has a mean value ranging from 30 to 60.

In some embodiments, R^{12} and R^{13} are each independently straight, saturated alkyl chains containing from 12 to 16 carbon atoms. In other embodiments, the average w ranges from 42 to 55, for example, the average w is 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55. In some specific embodiments, the average w is about 49.

In some embodiments, the pegylated lipid has the following Formula (XIa):



(XIa)

wherein the average w is about 49.

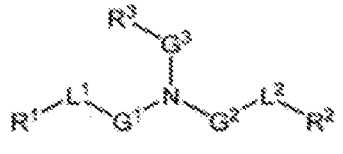
In some embodiments of Embodiments 1, 2, 3 4 or 5, the nucleic acid is selected from antisense and messenger RNA. For example, messenger RNA may be used to induce an immune response (*e.g.*, as a vaccine), for example by translation of immunogenic proteins.

In other embodiments of Embodiments 1, 2, 3 4 or 5, the nucleic acid is mRNA, and the mRNA to lipid ratio in the LNP (*i.e.*, N/P, were N represents the moles of cationic lipid and P represents the moles of phosphate present as part of the nucleic

[0426] In an embodiment, the transfer vehicle comprises a lipid or an ionizable lipid described in US patent publication number 20190314524.

[0427] Some embodiments of the present invention provide nucleic acid-lipid nanoparticle compositions comprising one or more of the novel cationic lipids described herein as structures listed in Table 10, that provide increased activity of the nucleic acid and improved tolerability of the compositions *in vivo*.

[0428] In one embodiment, an ionizable lipid has the following structure (XII):


(XII),

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

one of L^1 or L^2 is —O(C=O)—, —(C=O)O—, —C(=O)—, —O—, —S(O)$_x$—, —S—S—, —C(=O)S—, SC(=O)—, —NR^{a}C(=O)—, —C(=O)NR^a—, NR^{a}C(=)NR^a—, —OC(=O)NR^a— or —NR^{a}C(=O)O—, and the other of L^1 or L^2 is —O(C=O)—, —(C=O)O—, —C(=O)—, —O—, —S(O)$_x$—, —S—S—, —C(=O)S—, SC(=O)—, —NR^{a}C(=O)—, —C(=O)NR^a—, NR^{a}C(=O)NR^a—, —OC(=O)NR^a— or —NR^{a}C(=O)O— or a direct bond;

G^1 and G^2 are each independently unsubstituted C_1-C_{12} alkylene or C_1-C_{12} alkenylene;

G^3 is C_1-C_{24} alkylene, C_1-C_{24} alkenylene, C_3-C_8 cycloalkylene, C_3-C_8 cycloalkenylene;

R^a is H or C_1-C_{12} alkyl;

R^1 and R^2 are each independently C_6-C_{24} alkyl or C_6-C_{24} alkenyl;

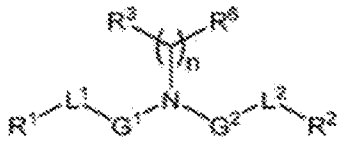
R^3 is H, OR^5, CN, —C(=O)OR^4, —OC(=O)R^4 or —NR^{5}C(=O)R^4;

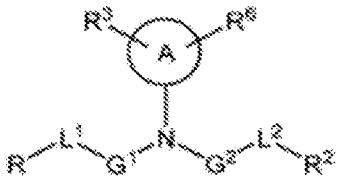
R^4 is C_1-C_{12} alkyl;

R^5 is H or C_1-C_6 alkyl; and

x is 0, 1 or 2.

[0429] In some embodiments, an ionizable lipid has one of the following structures (XIIA) or (XIIB):


(XIIA)


(XIIB)

wherein:

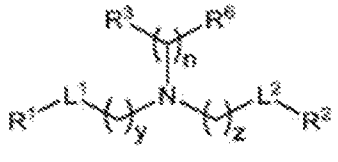
A is a 3 to 8-membered cycloalkyl or cycloalkylene ring;

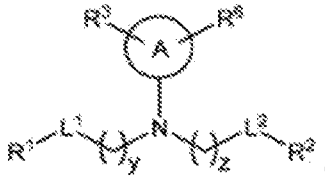
R^6 is, at each occurrence, independently H, OH or C_1-C_{24} alkyl; and

n is an integer ranging from 1 to 15.

[0430] In some embodiments, the ionizable lipid has structure (XIIA), and in other embodiments, the ionizable lipid has structure (XIIB).

[0431] In other embodiments, an ionizable lipid has one of the following structures (XIIC) or (XIID):

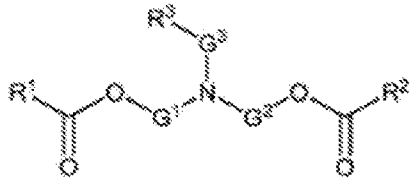

(XIIC)

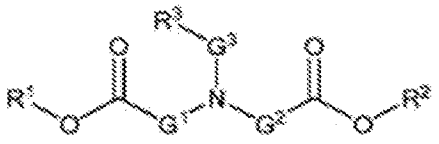

(XIID)

wherein y and z are each independently integers ranging from 1 to 12.

[0432] In some embodiments, one of L^1 or L^2 is —O(C=O)—. For example, in some embodiments each of L^1 and L^2 are —O(C=O)—. In some different embodiments of any of the foregoing, L^1 and L^2 are each independently —(C=O)O— or —O(C=O)—. For example, in some embodiments each of L^1 and L^2 is —(C=O)O—.

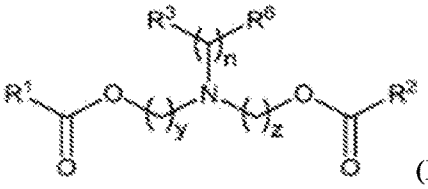
[0433] In some embodiments, an ionizable lipid has one of the following structures (XIIE) or (XIIF):


(XIIE)

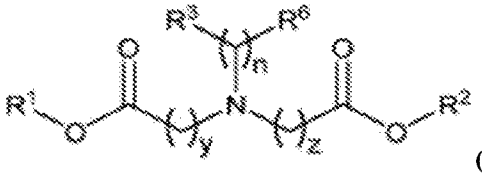


(XIIF)

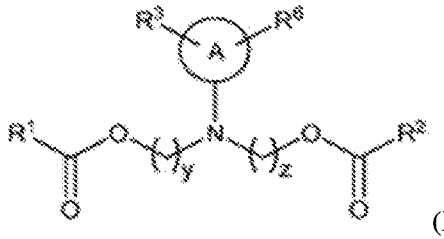
[0434] In some embodiments, an ionizable lipid has one of the following structures (XIIG), (XIIH), (XIII), or (XIIJ):



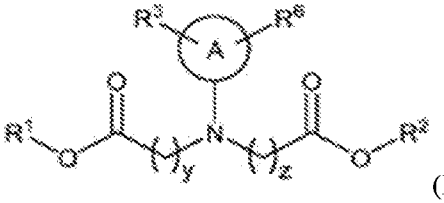
(XIIG)



(XIIH)



(XIII)



(XIIJ)

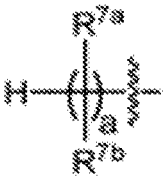
[0435] In some embodiments, n is an integer ranging from 2 to 12, for example from 2 to 8 or from 2 to 4. For example, in some embodiments, n is 3, 4, 5 or 6. In some embodiments, n is 3. In some embodiments, n is 4. In some embodiments, n is 5. In some embodiments, n is 6.

[0436] In some embodiments, y and z are each independently an integer ranging from 2 to 10. For example, in some embodiments, y and z are each independently an integer ranging from 4 to 9 or from 4 to 6.

[0437] In some embodiments, R^6 is H. In other embodiments, R^6 is C_1-C_{24} alkyl. In other embodiments, R^6 is OH.

[0438] In some embodiments, G^3 is unsubstituted. In other embodiments, G3 is substituted. In various different embodiments, G^3 is linear C_1-C_{24} alkylene or linear C_1-C_{24} alkenylene.

[0439] In some embodiments, R^1 or R^2, or both, is C_6-C_{24} alkenyl. For example, in some embodiments, R^1 and R^2 each, independently have the following structure:



wherein:

R^{7a} and R^{7b} are, at each occurrence, independently H or C_1-C_{12} alkyl; and

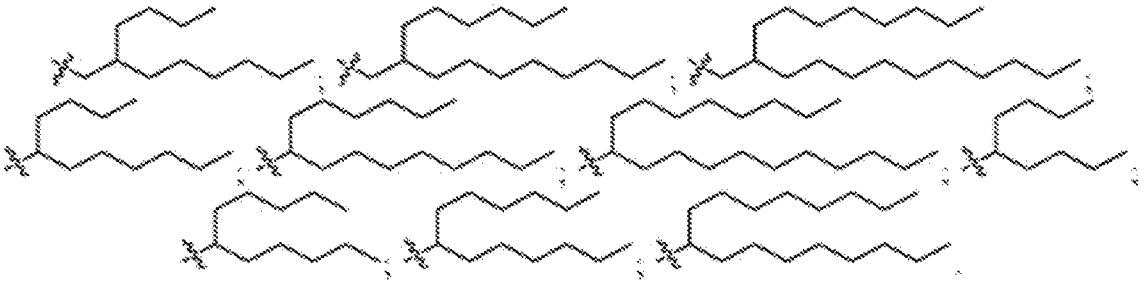
a is an integer from 2 to 12,

wherein R^{7a}, R^{7b} and a are each selected such that R^1 and R^2 each independently comprise from 6 to 20 carbon atoms.

[0440] In some embodiments, a is an integer ranging from 5 to 9 or from 8 to 12.

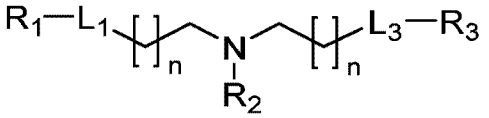
[0441] In some embodiments, at least one occurrence of R^{7a} is H. For example, in some embodiments, R^{7a} is H at each occurrence. In other different embodiments, at least one occurrence of R^{7b} is C_1-C_8 alkyl. For example, in some embodiments, C_1-C_8 alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

[0442] In different embodiments, R^1 or R^2, or both, has one of the following structures:



[0443] In some embodiments, R^3 is —OH, —CN, —C(=O)OR^4, —OC(=O)R^4 or —NHC(=O)R^4. In some embodiments, R^4 is methyl or ethyl.

[0444] In some embodiments, an ionizable lipid is a compound of Formula (1):



Formula (1),

wherein:

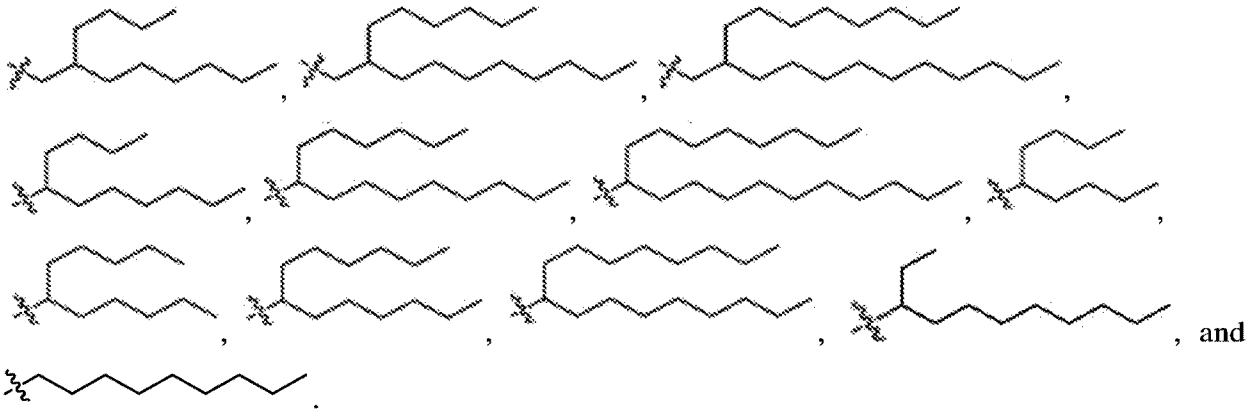
each n is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15; and

L_1 and L_3 are each independently –OC(O)–* or –C(O)O–*, wherein "*" indicates the attachment point to R_1 or R_3;

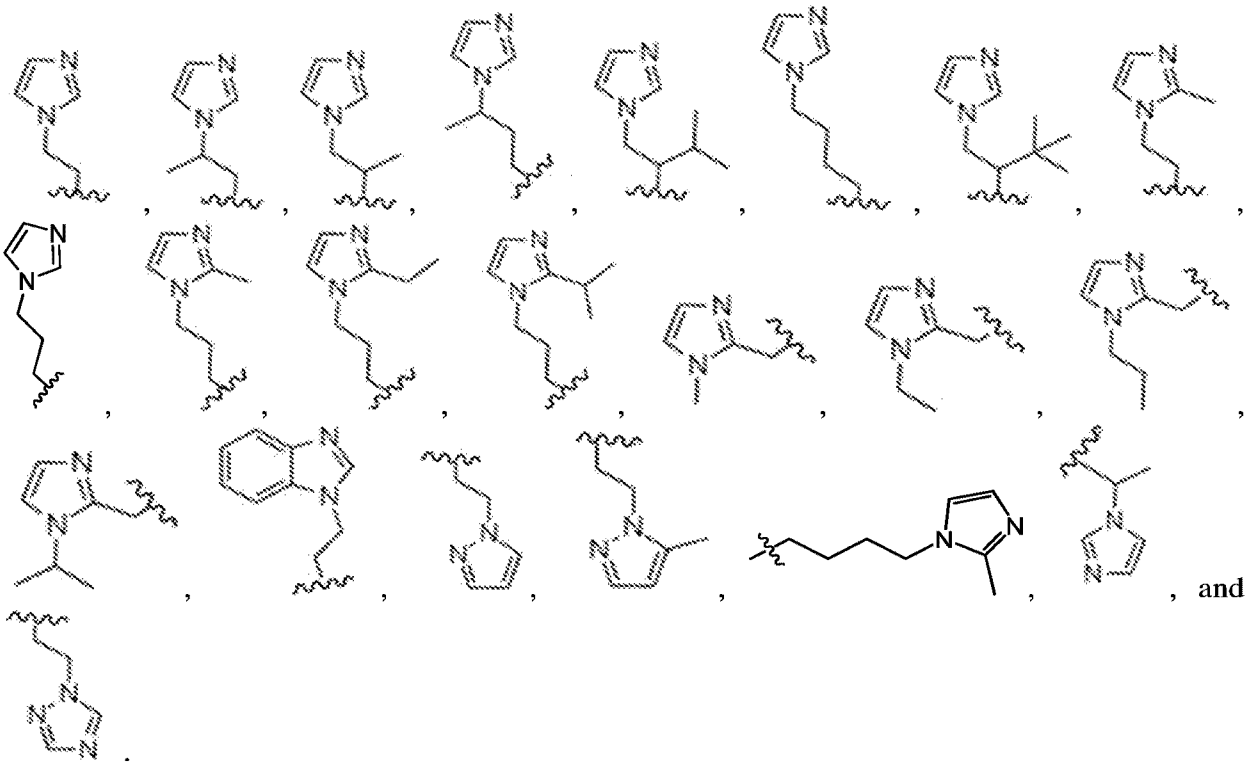
R_1 and R_3 are each independently a linear or branched C_9-C_{20} alkyl or C_9-C_{20} alkenyl, optionally substituted by one or more substituents selected from oxo, halo, hydroxy, cyano, alkyl, alkenyl, aldehyde, heterocyclylalkyl, hydroxyalkyl, dihydroxyalkyl, hydroxyalkylaminoalkyl, aminoalkyl, alkylaminoalkyl, dialkylaminoalkyl, (heterocyclyl)(alkyl)aminoalkyl, heterocyclyl, heteroaryl, alkylheteroaryl, alkynyl, alkoxy, amino, dialkylamino, aminoalkylcarbonylamino, aminocarbonylalkylamino, (aminocarbonylalkyl)(alkyl)amino, alkenylcarbonylamino, hydroxycarbonyl, alkyloxycarbonyl, aminocarbonyl, aminoalkylaminocarbonyl, alkylaminoalkylaminocarbonyl, dialkylaminoalkylaminocarbonyl, heterocyclylalkylaminocarbonyl, (alkylaminoalkyl)(alkyl)aminocarbonyl, alkylaminoalkylcarbonyl, dialkylaminoalkylcarbonyl, heterocyclylcarbonyl, alkenylcarbonyl, alkynylcarbonyl, alkylsulfoxide, alkylsulfoxidealkyl, alkylsulfonyl, and alkylsulfonealkyl.

[0445] In some embodiments, R_1 and R_3 are the same. In some embodiments, R_1 and R_3 are different.

[0446] In some embodiments, R_1 and R_3 are each independently a branched saturated C_9-C_{20} alkyl. In some embodiments, one of R_1 and R_3 is a branched saturated C_9-C_{20} alkyl, and the other is an unbranched saturated C_9-C_{20} alkyl. In some embodiments, R_1 and R_3 are each independently selected from a group consisting of:

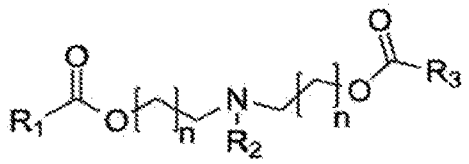


[0447] In various embodiments, R_2 is selected from a group consisting of:

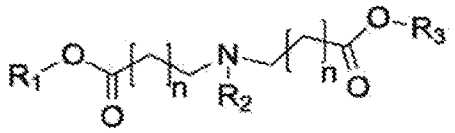


[0448] In some embodiments, R_2 may be as described in International Pat. Pub. No. WO2019/152848 A1, which is incorporated herein by reference in its entirety.

[0449] In some embodiments, an ionizable lipid is a compound of Formula (1-1) or Formula (1-2):



Formula (1-1)



Formula (1-2)

wherein n, R_1, R_2, and R_3 are as defined in Formula (1).

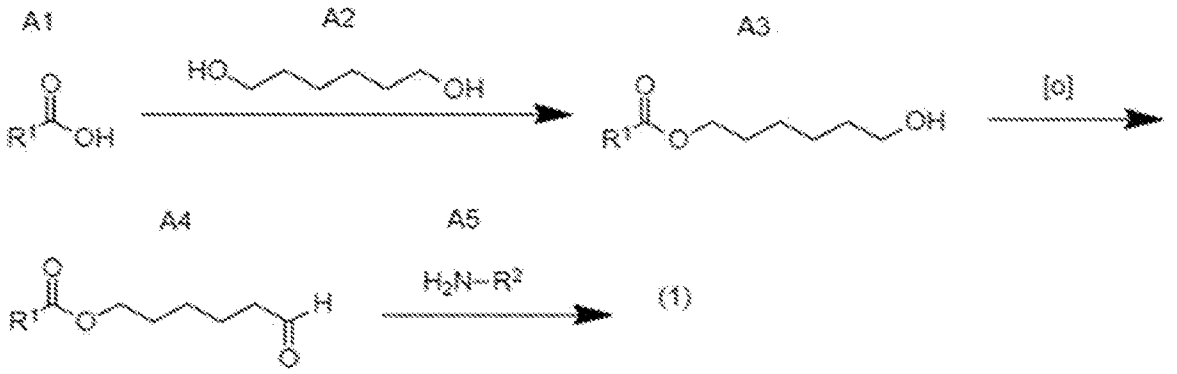
[0450] Preparation methods for the above compounds and compositions are described herein below and/or known in the art.

[0451] 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, *e.g.*, hydroxyl, amino, mercapto, and carboxylic acid. Suitable protecting groups for hydroxyl include, *e.g.*, 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, *e.g.*, t-butoxycarbonyl, benzyloxycarbonyl, and the like. Suitable protecting groups for mercapto include, *e.g.*, -C(O)-R'' (where R'' is alkyl, aryl, or arylalkyl), p-methoxybenzyl, trityl, and the like. Suitable protecting groups for carboxylic acid include, *e.g.*, 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, *e.g.*, 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.

[0452] It will also be appreciated by those skilled in the art, although such protected derivatives of compounds of this invention 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 invention which are pharmacologically active. Such derivatives may therefore be described as prodrugs. All prodrugs of compounds of this invention are included within the scope of the invention.

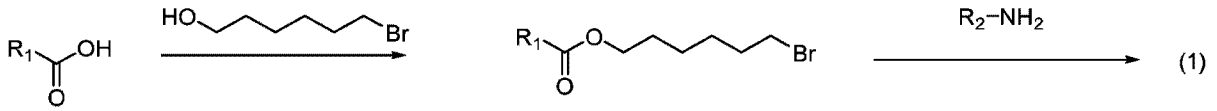
[0453] Furthermore, all compounds of the invention which exist in free base or acid form can be converted to their pharmaceutically acceptable salts by treatment with the appropriate inorganic or organic base or acid by methods known to one skilled in the art. Salts of the compounds of the invention can also be converted to their free base or acid form by standard techniques.

[0454] The following reaction scheme illustrates an exemplary method to make compounds of Formula (1):



[0455] A1 are purchased or prepared according to methods known in the art. Reaction of A1 with diol A2 under appropriate condensation conditions (*e.g.*, DCC) yields ester/alcohol A3, which can then be oxidized (*e.g.*, with PCC) to aldehyde A4. Reaction of A4 with amine A5 under reductive amination conditions yields a compound of Formula (1).

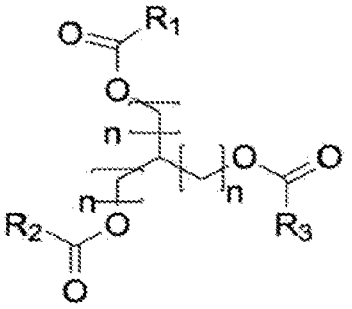
[0456] The following reaction scheme illustrates a second exemplary method to make compounds of Formula (1), wherein R_1 and R_3 are the same:



[0457] Modifications to the above reaction scheme, such as using protecting groups, may yield compounds wherein R_1 and R_3 are different. The use of protecting groups, as well as other modification methods, to the above reaction scheme will be readily apparent to one of ordinary skill in the art.

[0458] It is understood that one skilled in the art may be able to make these compounds by similar methods or by combining other methods known to one skilled in the art. It is also understood that one skilled in the art would be able to make other compounds of Formula (1) not specifically illustrated herein by using the appropriate starting materials and modifying the parameters of the synthesis. In general, starting materials 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 invention.

[0459] In some embodiments, an ionizable lipid is a compound of Formula (2):

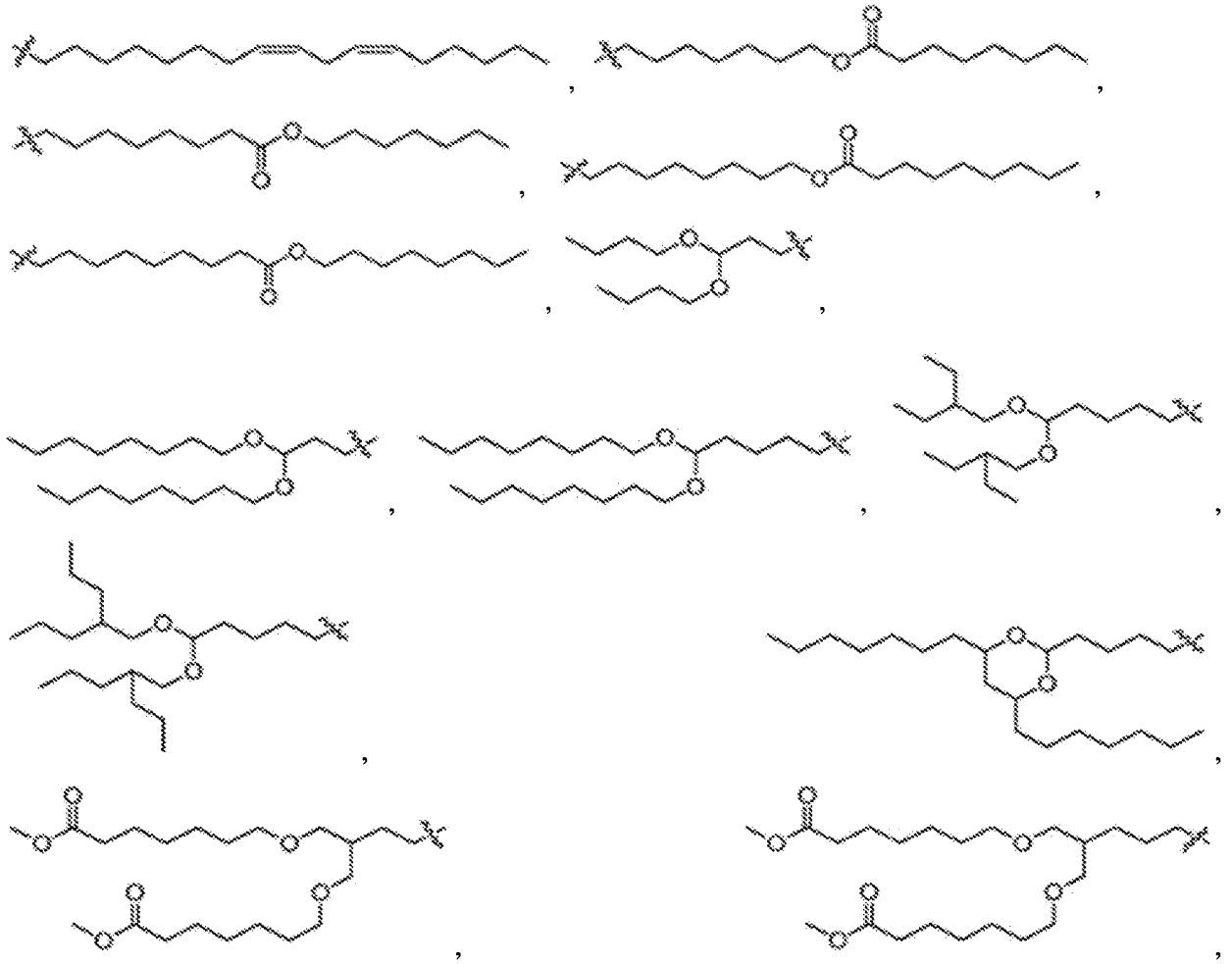


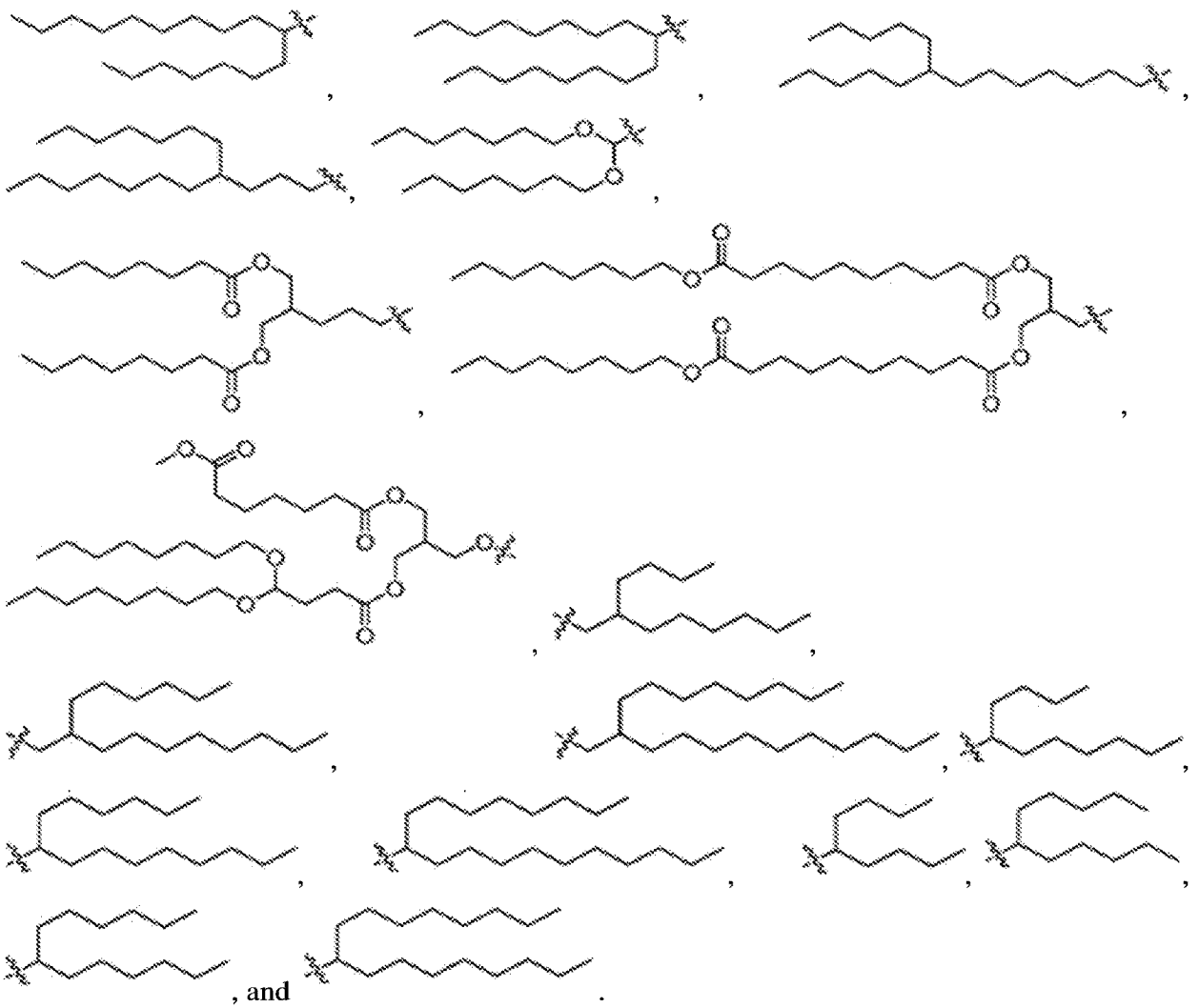
Formula (2),

wherein each n is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15.

[0460] In some embodiments, as used in Formula (2), R_1 and R_2 are as defined in Formula (1).

[0461] In some embodiments, as used in Formula (2), R_1 and R_2 are each independently selected from a group consisting of:

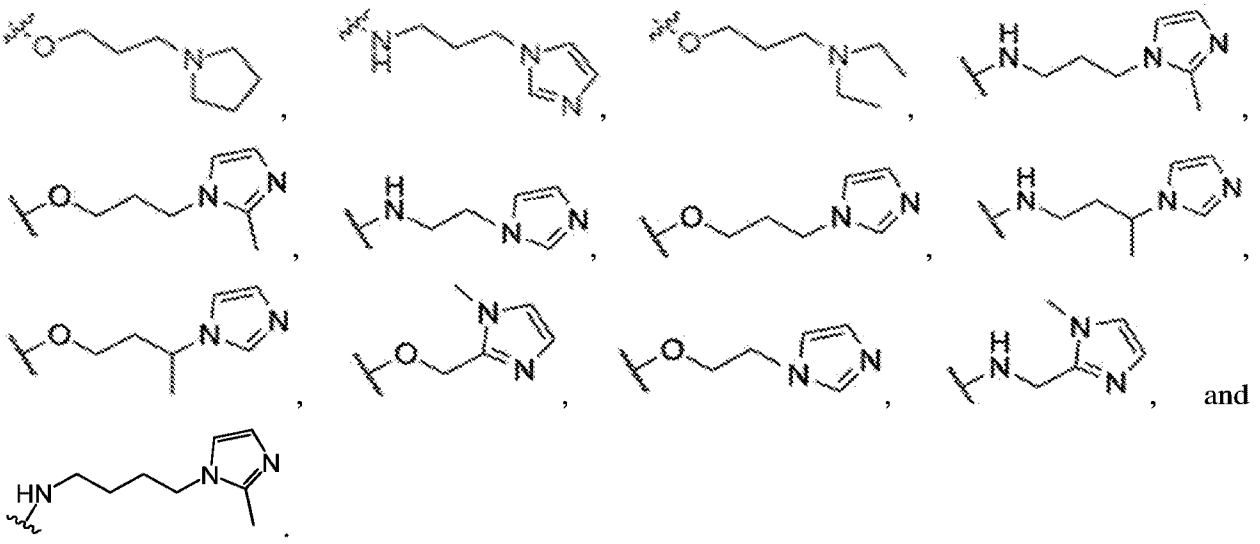




, and .

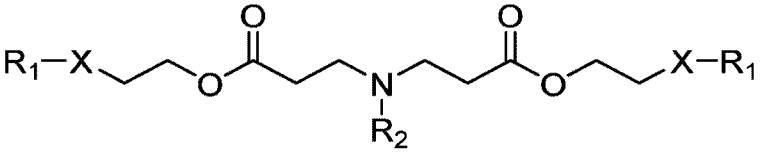
[0462] In some embodiments, R_1 and/or R_2 as used in Formula (2) may be as described in International Pat. Pub. No. WO2015/095340 A1, which is incorporated herein by reference in its entirety. In some embodiments, R_1 as used in Formula (2) may be as described in International Pat. Pub. No. WO2019/152557 A1, which is incorporated herein by reference in its entirety.

[0463] In some embodiments, as used in Formula (2), R_3 is selected from a group consisting of:



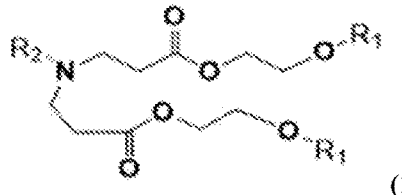
and

[0464] In some embodiments, an ionizable lipid is a compound of Formula (3)



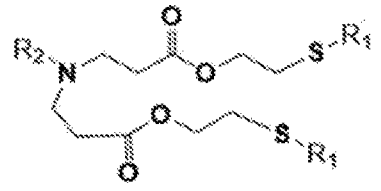
wherein X is selected from –O–, –S–, or –OC(O)–*, wherein * indicates the attachment point to R_1.

[0465] In some embodiments, an ionizable lipid is a compound of Formula (3-1):



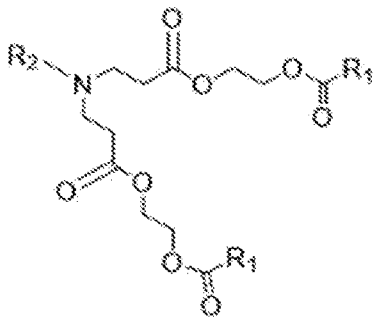
(3-1).

[0466] In some embodiments, an ionizable lipid is a compound of Formula (3-2):



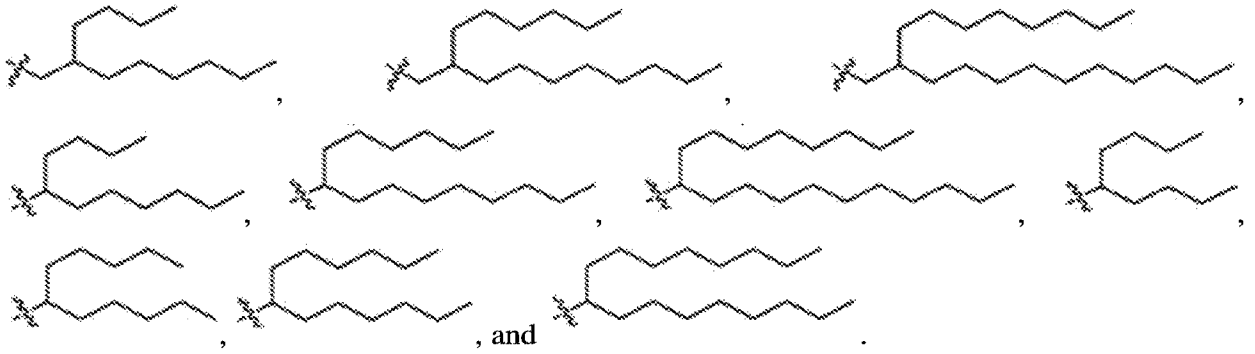
(3-2).

[0467] In some embodiments, an ionizable lipid is a compound of Formula (3-3):



(3-3).

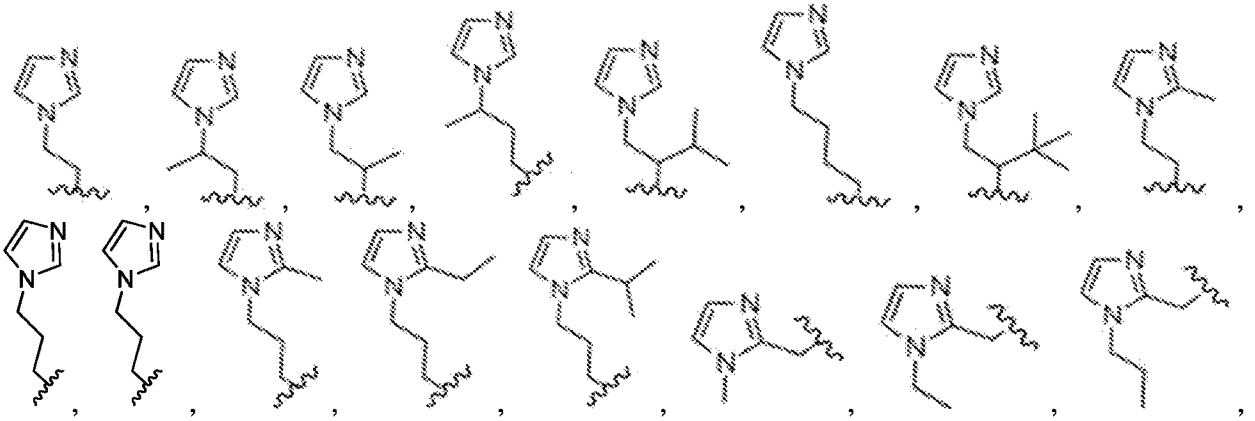
[0468] In some embodiments, as used in Formula (3-1), (3-2), or (3-3), each R_1 is independently a branched saturated C_9-C_{20} alkyl. In some embodiments, each R_1 is independently selected from a group consisting of:



, and .

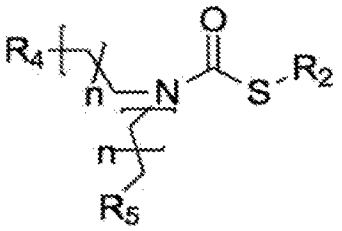
[0469] In some embodiments, each R_1 in Formula (3-1), (3-2), or (3-3) are the same.

[0470] In some embodiments, as used in Formula (3-1), (3-2), or (3-3), R_2 is selectd from a group consisting of:



[0471] In some embodiments, R_2 as used in Formula (3-1), (3-2), or (3-3) may be as described in International Pat. Pub. No. WO2019/152848A1, which is incorporated herein by reference in its entirety.

[0472] In some embodiments, an ionizable lipid is a compound of Formula (5):



(5),

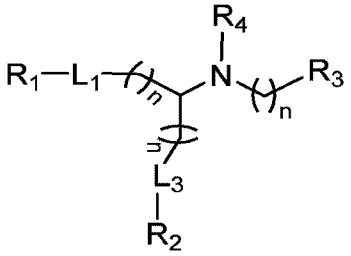
wherein:

each n is independently 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15; and

R_2 is as defined in Formula (1).

[0473] In some embodiments, as used in Formula (5), R_4 and R_5 are defined as R_1 and R_3, respectively, in Formula (1). In some embodiments, as used in Formula (5), R_4 and R_5 may be as described in International Pat. Pub. No. WO2019/191780 A1, which is incorporated herein by reference in its entirety.

[0474] In some embodiments, an ionizable lipid is a compound of Formula (6):



Formula (6)

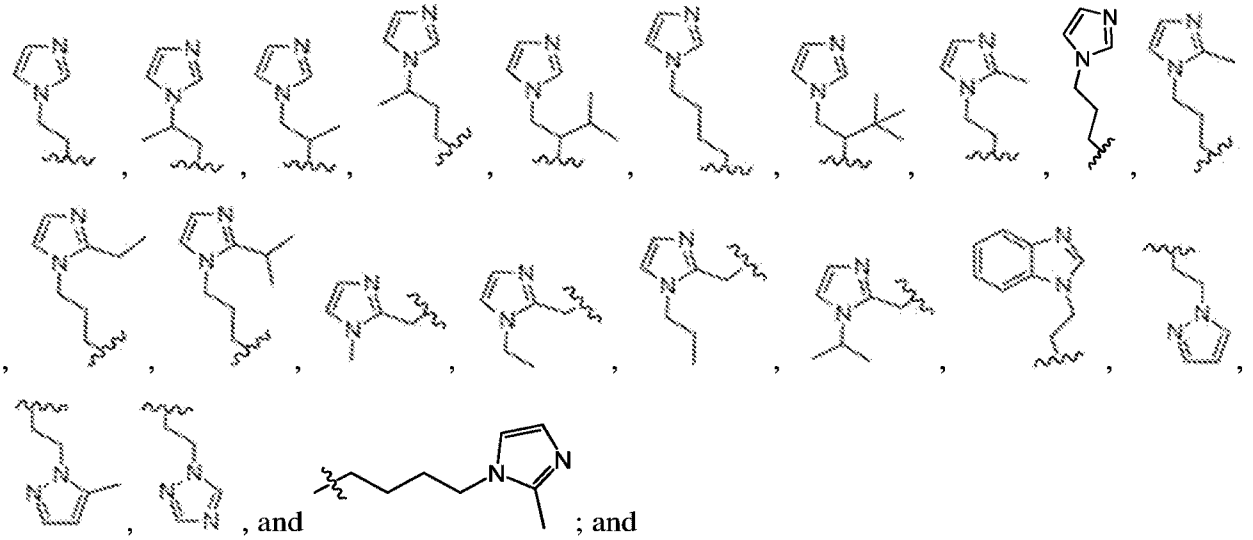
wherein:

each n is independently an integer from 0-15;

L_1 and L_3 are each independently –OC(O)–* or –C(O)O–*, wherein "*" indicates the attachment point to R_1 or R_3;

R_1 and R_2 are each independently a linear or branched C_9-C_{20} alkyl or C_9-C_{20} alkenyl, optionally substituted by one or more substituents selected from a group consisting of oxo, halo, hydroxy, cyano, alkyl, alkenyl, aldehyde, heterocyclylalkyl, hydroxyalkyl, dihydroxyalkyl, hydroxyalkylaminoalkyl, aminoalkyl, alkylaminoalkyl, dialkylaminoalkyl, (heterocyclyl)(alkyl)aminoalkyl, heterocyclyl, heteroaryl, alkylheteroaryl, alkynyl, alkoxy, amino, dialkylamino, aminoalkylcarbonylamino, aminocarbonylalkylamino, (aminocarbonylalkyl)(alkyl)amino, alkenylcarbonylamino, hydroxycarbonyl, alkyloxycarbonyl, aminocarbonyl, aminoalkylaminocarbonyl, alkylaminoalkylaminocarbonyl, dialkylaminoalkylaminocarbonyl, heterocyclylalkylaminocarbonyl, (alkylaminoalkyl)(alkyl)aminocarbonyl, alkylaminoalkylcarbonyl, dialkylaminoalkylcarbonyl, heterocyclylcarbonyl, alkenylcarbonyl, alkynylcarbonyl, alkylsulfoxide, alkylsulfoxidealkyl, alkylsulfonyl, and alkylsulfonealkyl;

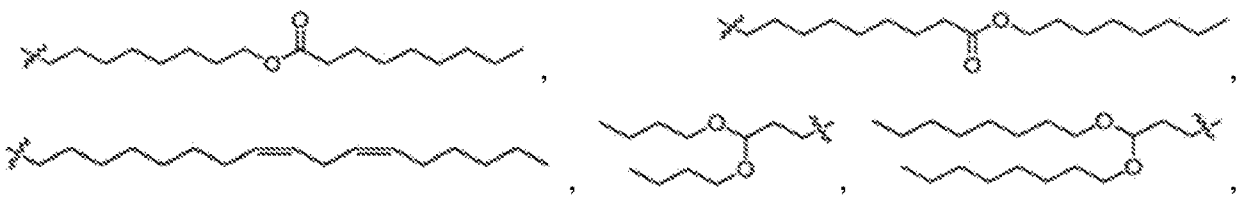
R_3 is selected from a group consisting of:

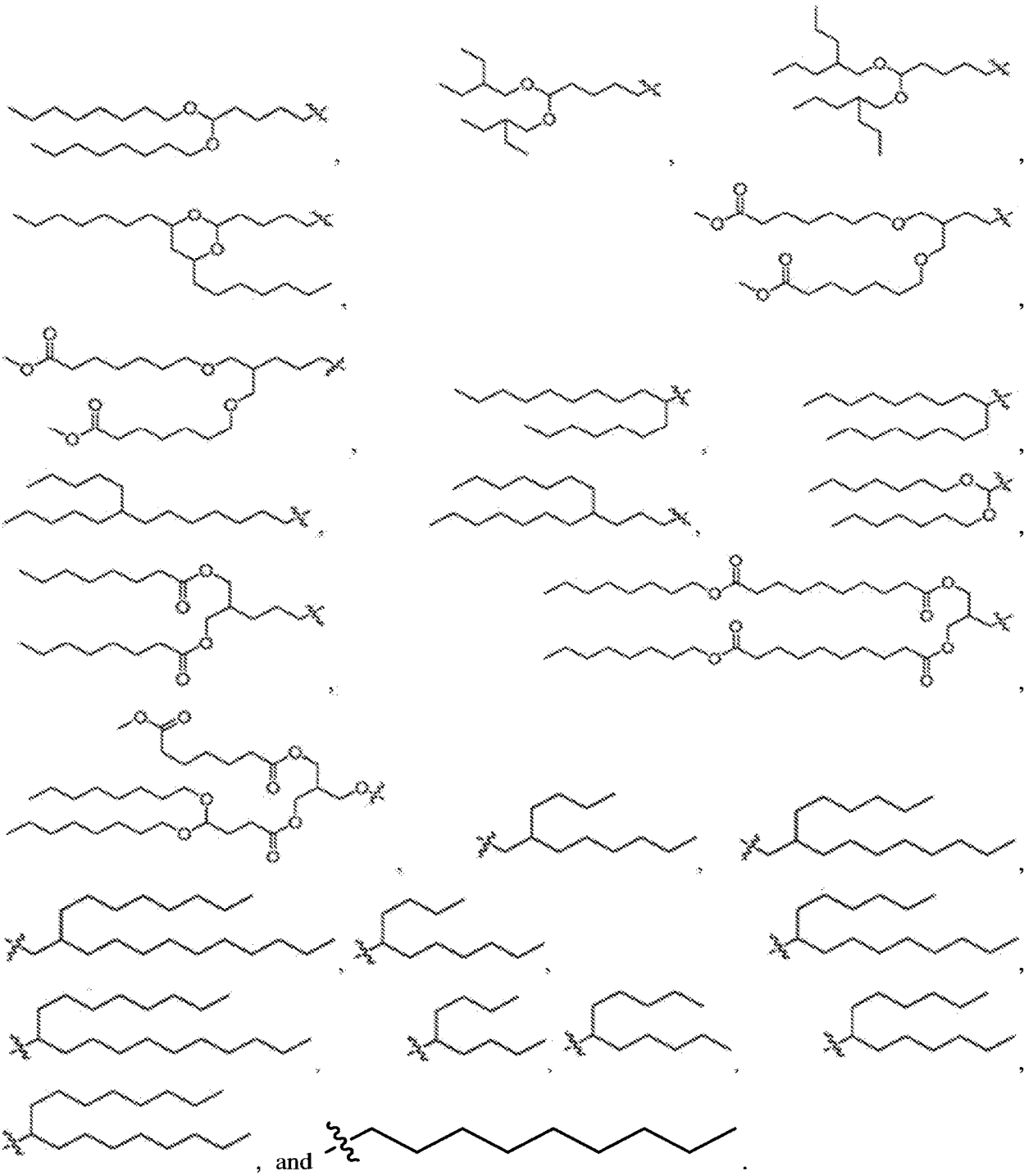


; and

R_4 is a linear or branched C_1-C_{15} alkyl or C_1-C_{15} alkenyl.

[0475] In some embodiments, R_1 and R_2 are each independently selected from a group consisting of:

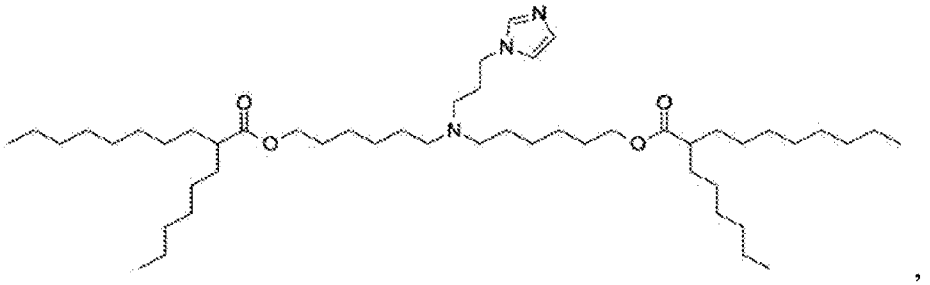




[0476] In some embodiments, R_1 and R_2 are the same. In some embodiments, R_1 and R_2 are different.

[0477] In some embodiments, an ionizable lipid of the disclosure is selected from Table 10a. In some embodiments, the ionizable lipid is Lipid 26 in Table 10a. In some embodiments, the ionizable lipid is Lipid 27 in Table 10a. In some embodiments, the ionizable lipid is Lipid 53 in Table 10a. In some embodiments, the ionizable lipid is Lipid 54 in Table 10a. In some embodiments, the ionizable lipid is Lipid 45 in Table 10a. In some embodiments, the ionizable lipid is Lipid 46 in Table 10a. In some embodiments, the ionizable lipid is Lipid 137 in Table 10a. In some embodiments, the ionizable lipid is Lipid 138 in Table 10a. In some embodiments, the ionizable lipid is Lipid 139 in Table 10a. In some embodiments, the ionizable lipid is Lipid 128 in Table 10a. In some embodiments, the ionizable lipid is Lipid 130 in Table 10a.

[0478] In some embodiments, an ionizable lipid of the disclosure is selected from the group consisting of:



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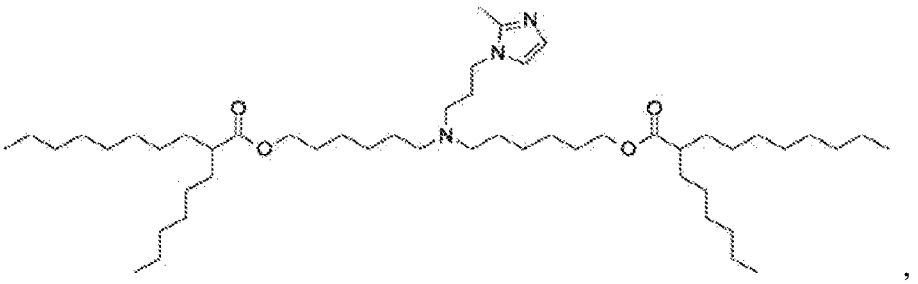

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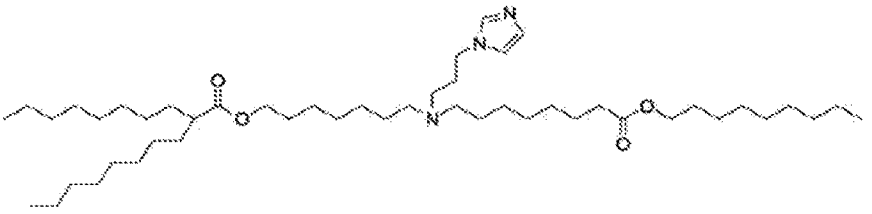

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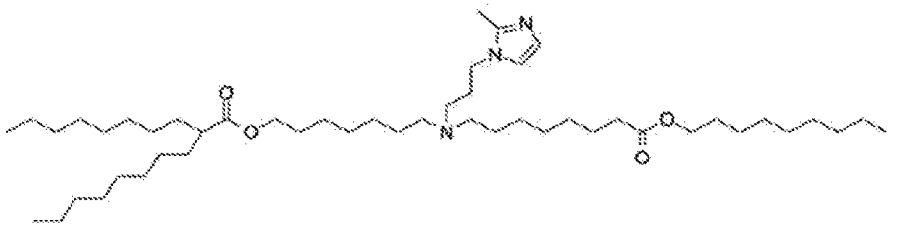

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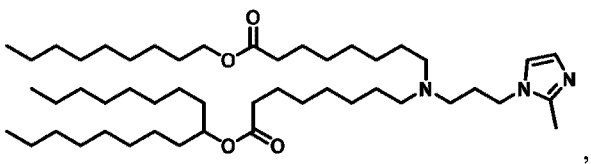

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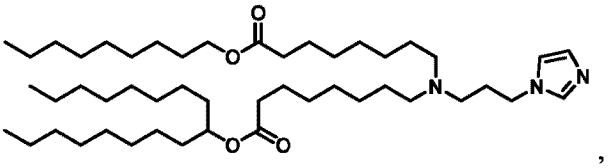

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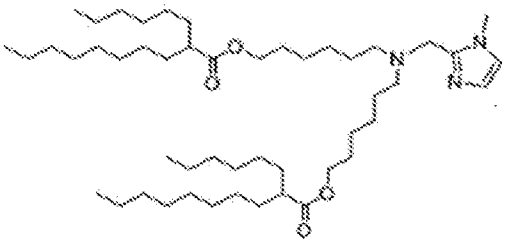

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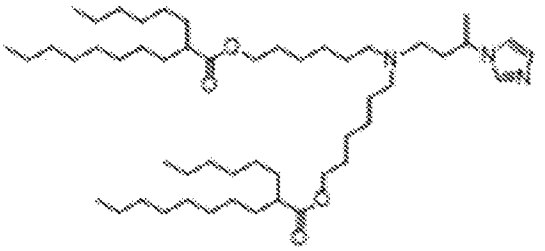

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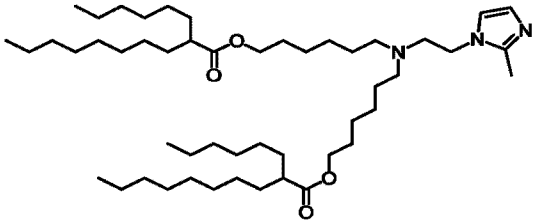

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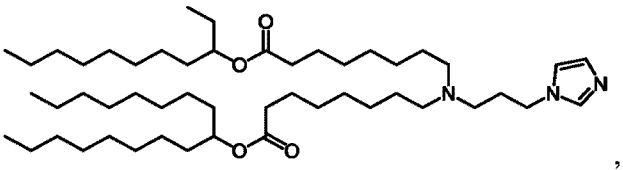

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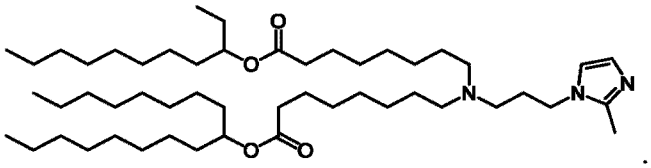
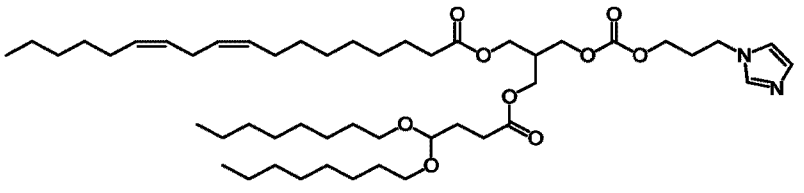
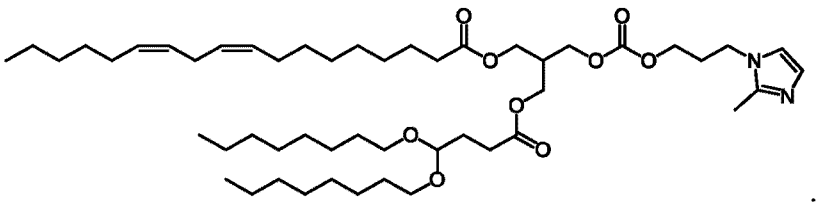


.

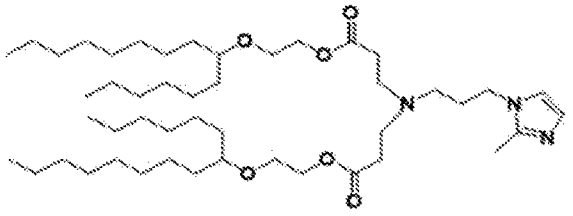
[0479] In some embodiments, an ionizable lipid of the disclosure is selected from the group consisting of:



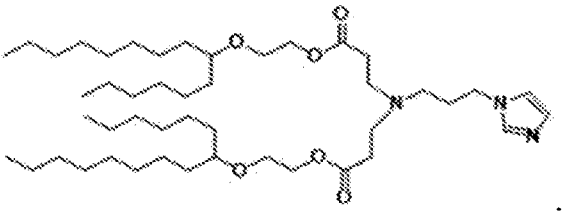
and



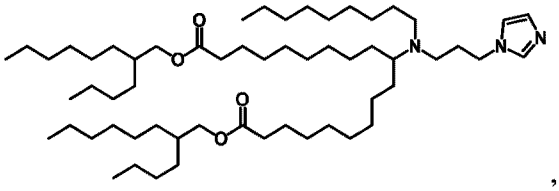
[0480] In some embodiments, an ionizable lipid of the disclosure is selected from the group consisting of:



and



[0481] In some embodiments, an ionizable lipid of the disclosure is selected from the group consisting of:



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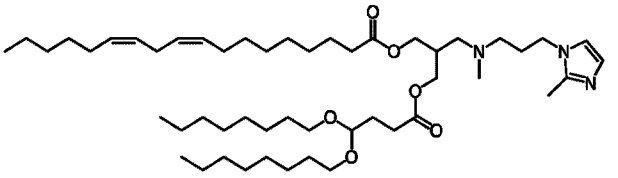

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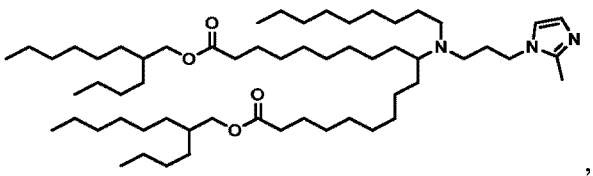

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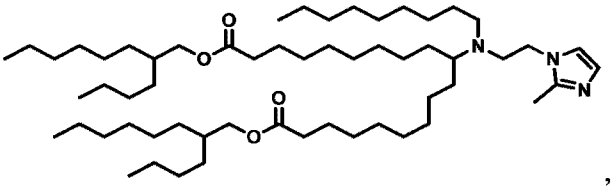

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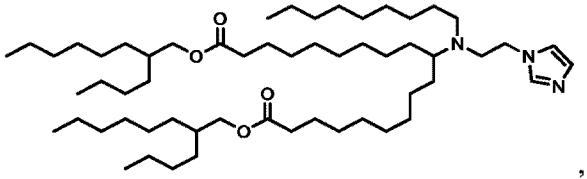

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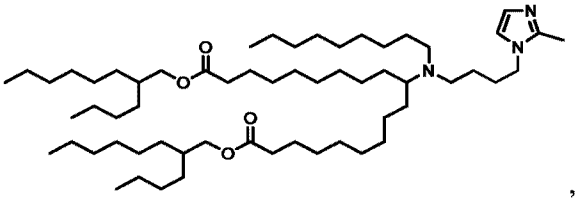

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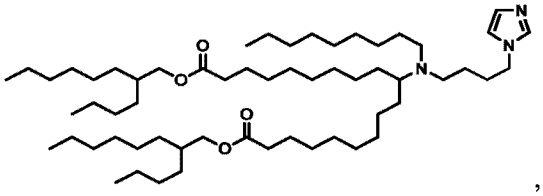

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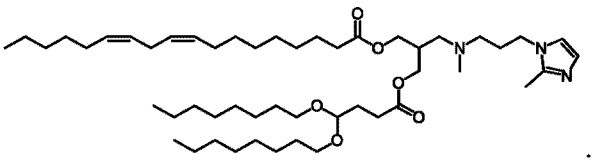

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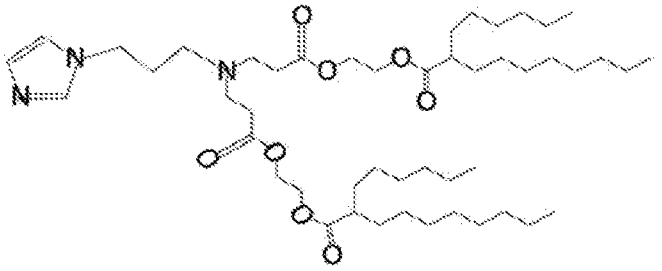
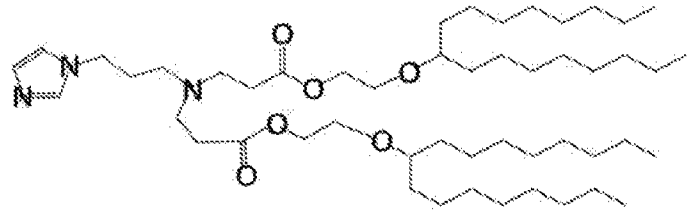
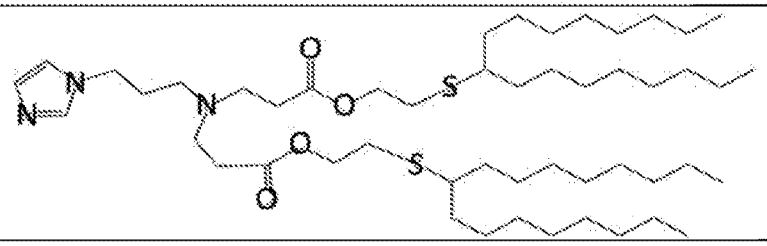
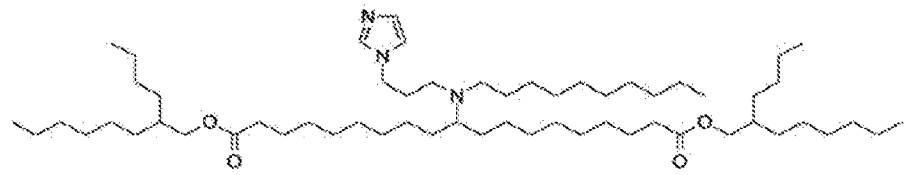
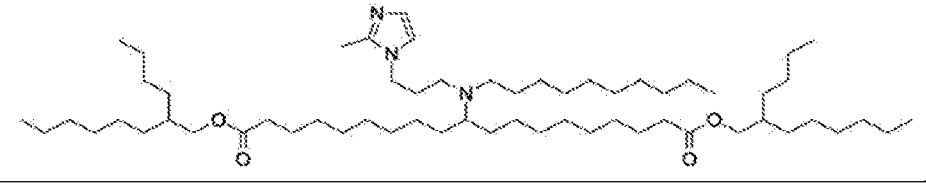
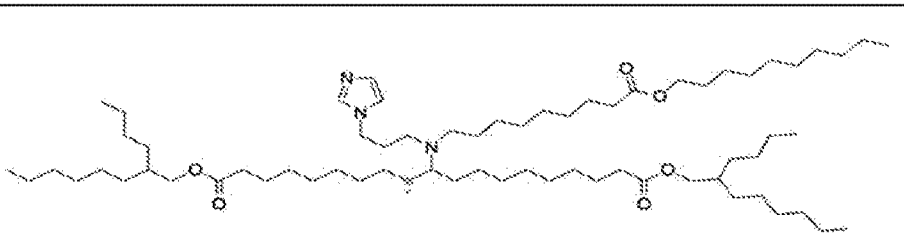
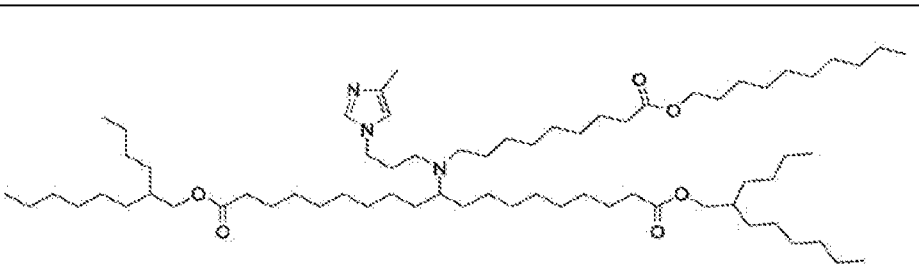
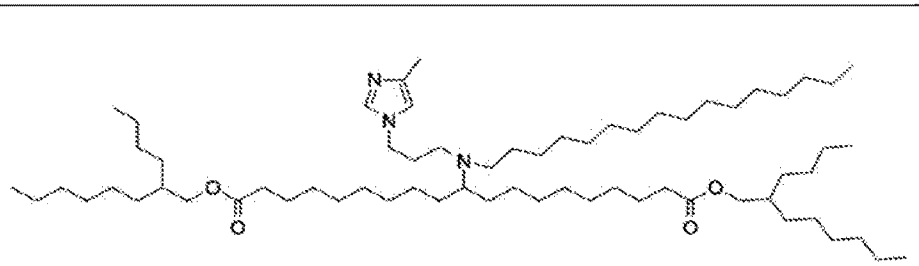
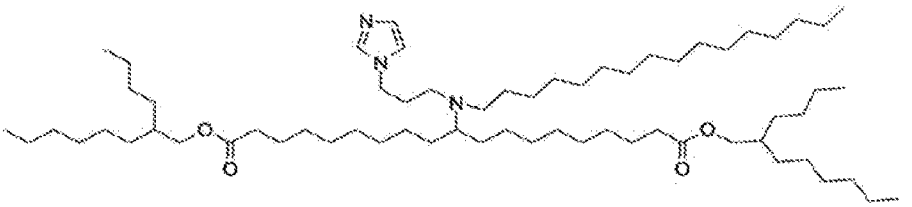
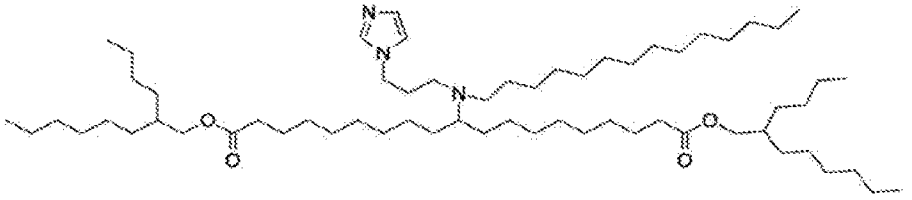
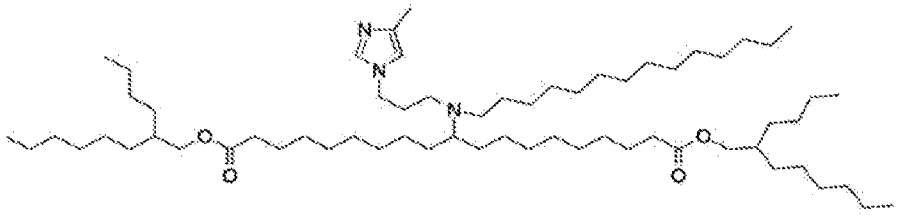
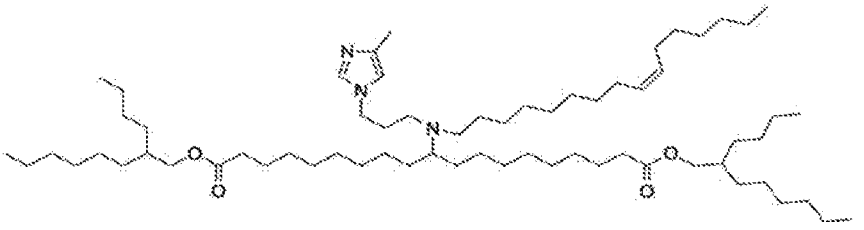
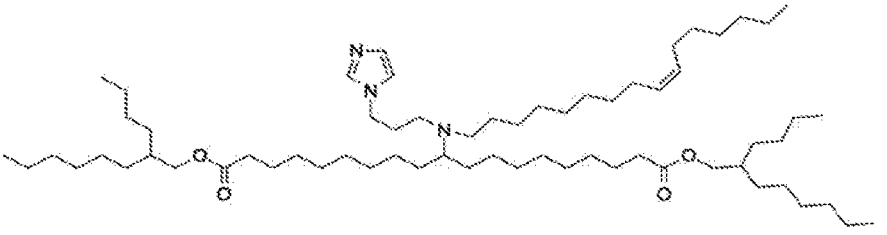
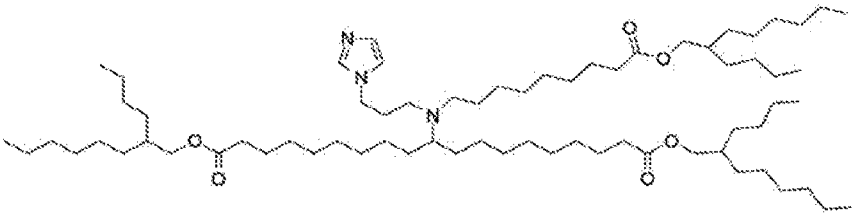
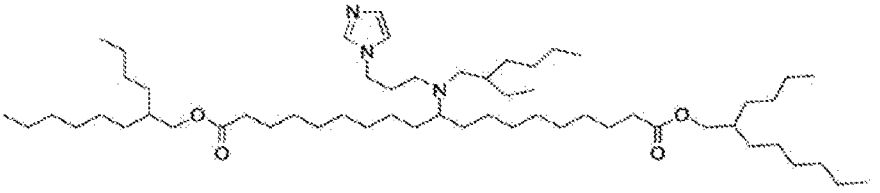
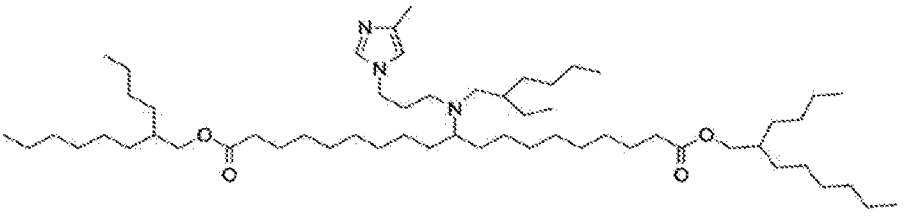
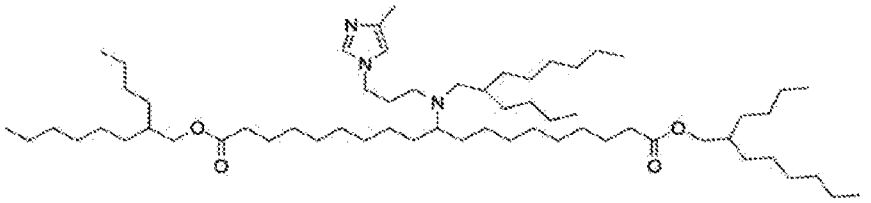
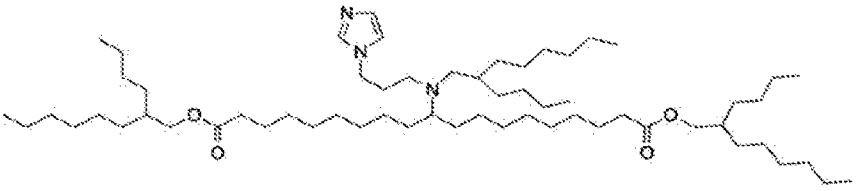
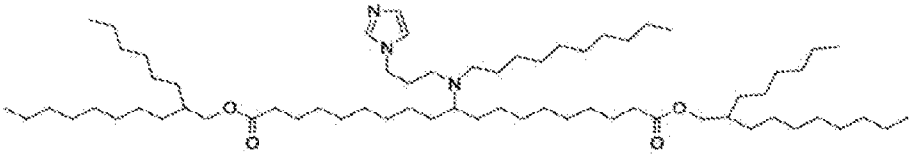
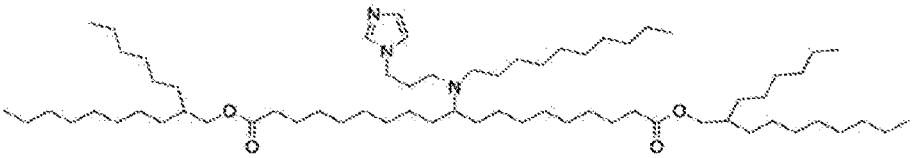
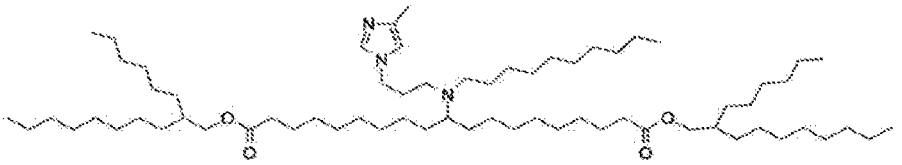
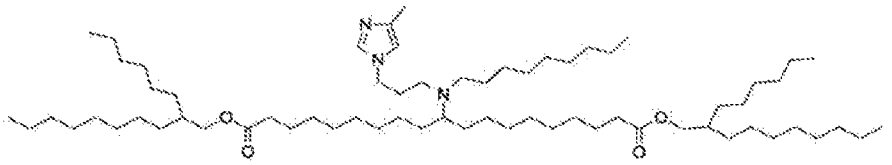
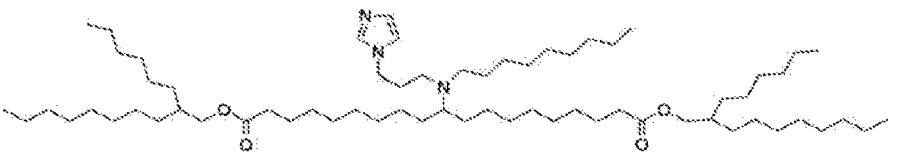
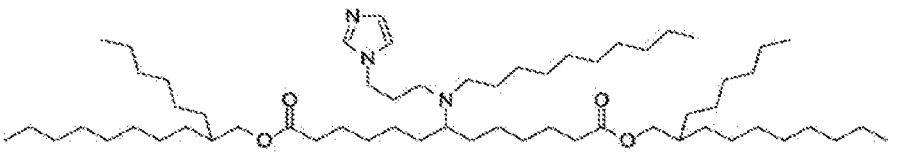
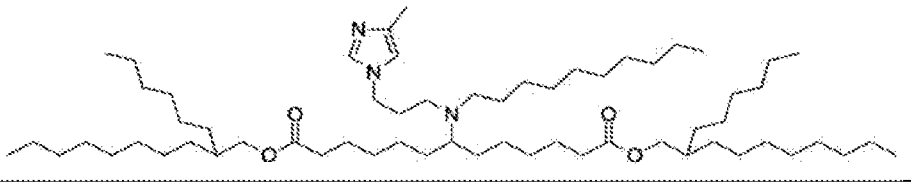
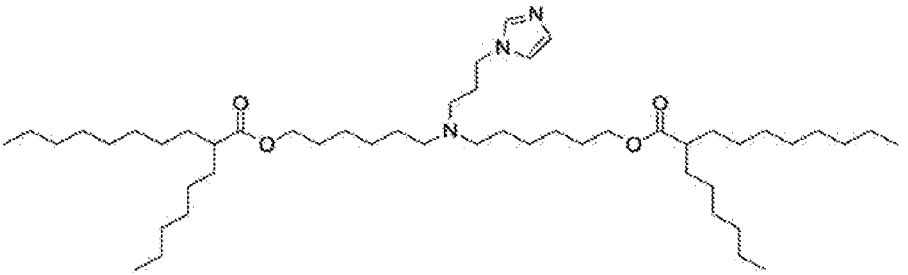
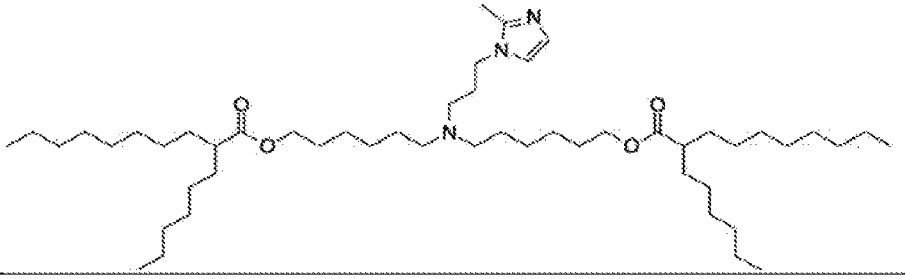
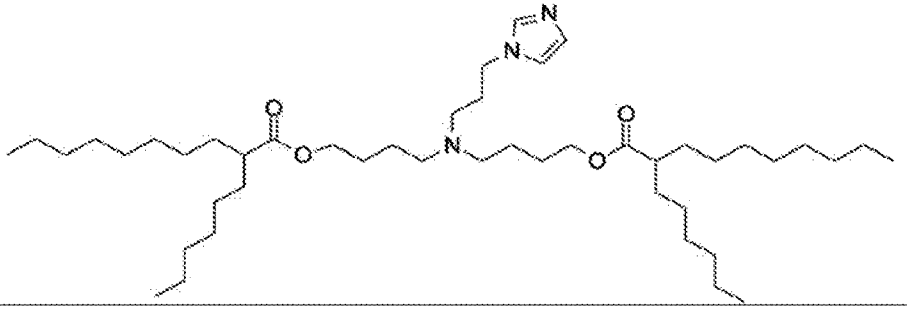
Table 10a

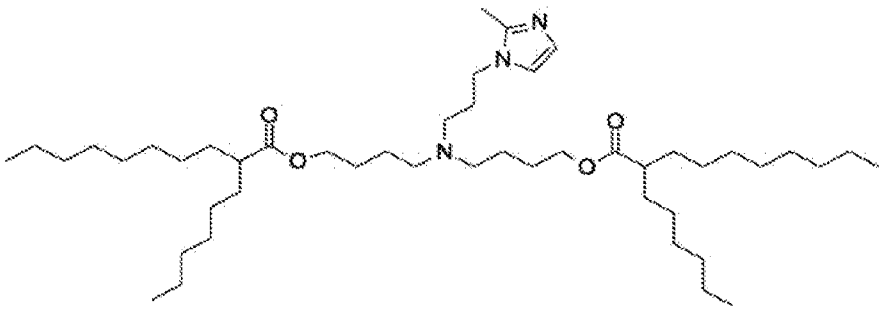
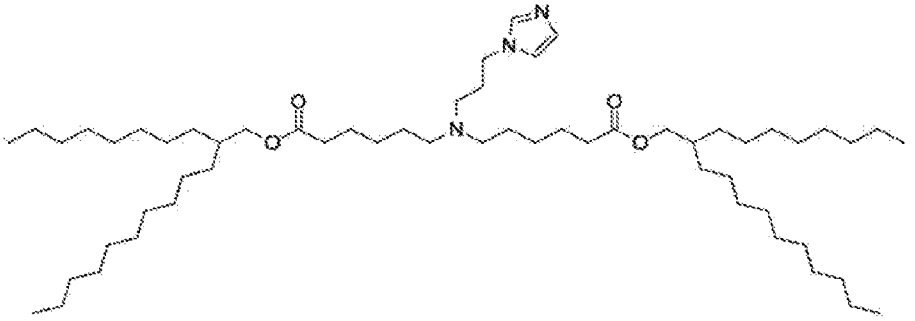
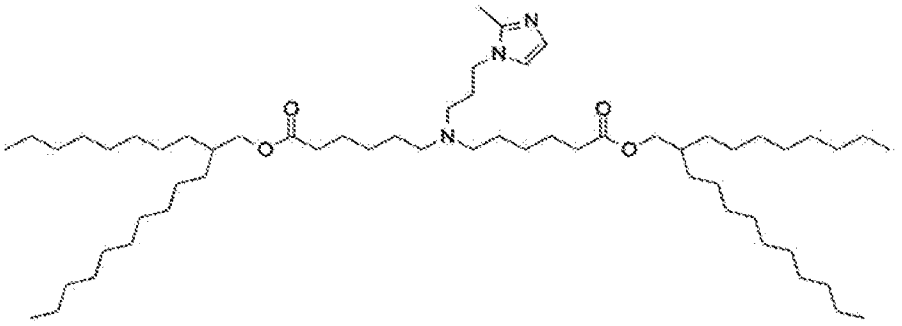
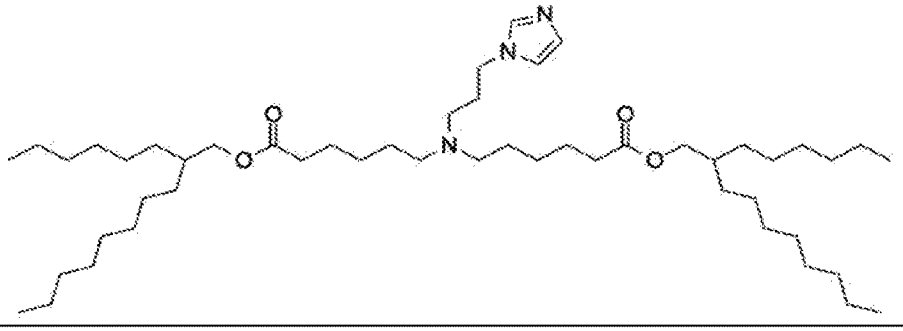
Ionizable lipid number	Structure
1	
2	
3	
4	
5	

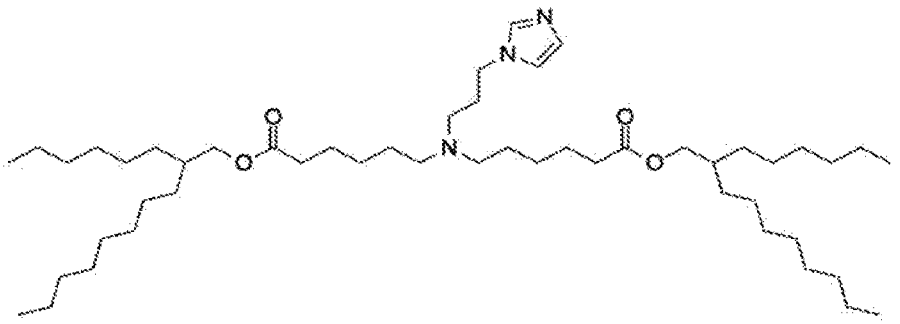
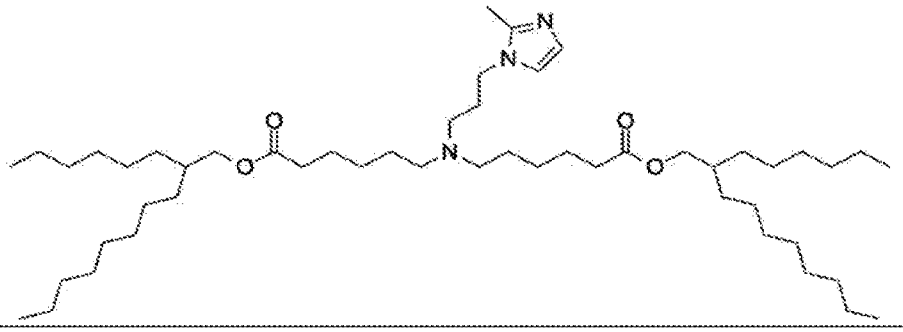
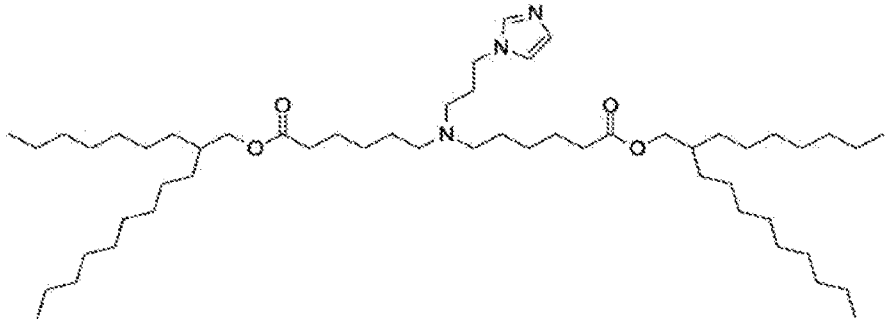
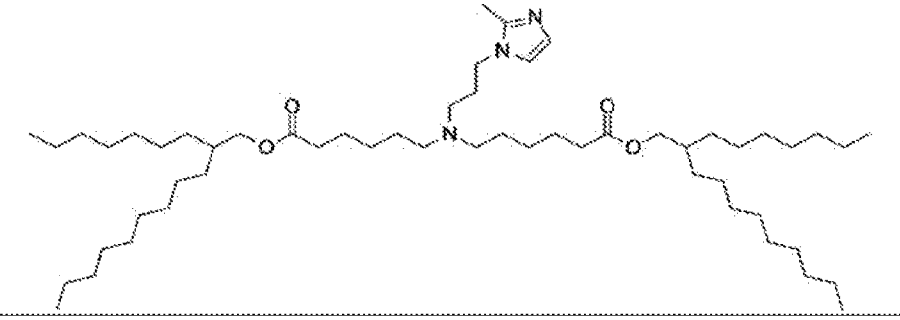
6	
7	
8	
9	
10	
11	

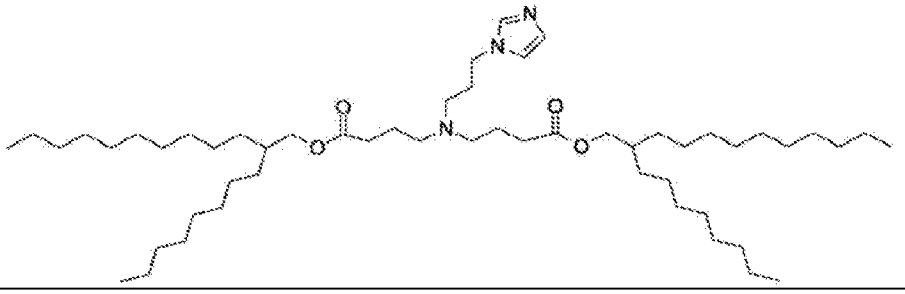
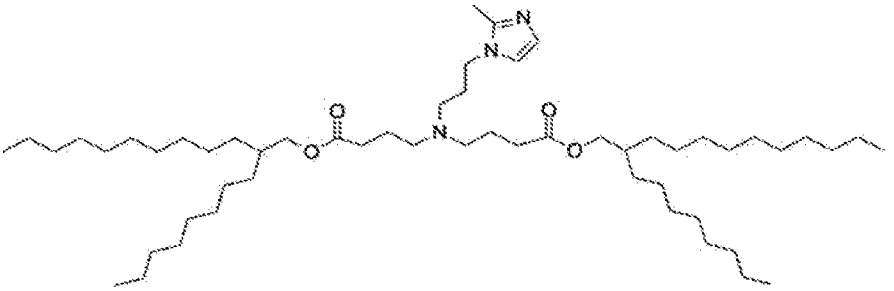
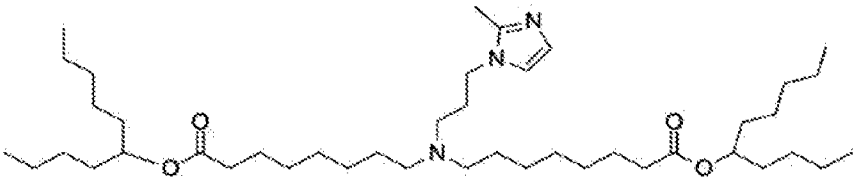
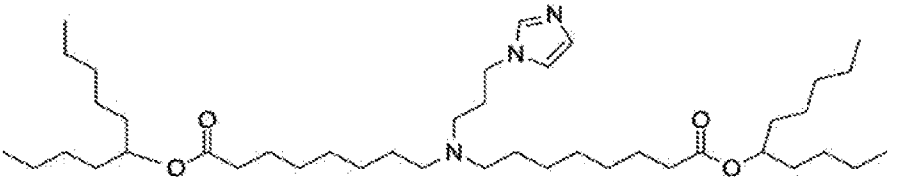
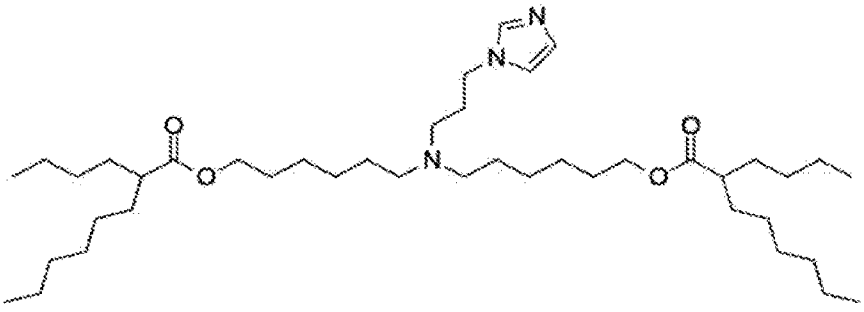
12	
13	
14	
15	
16	

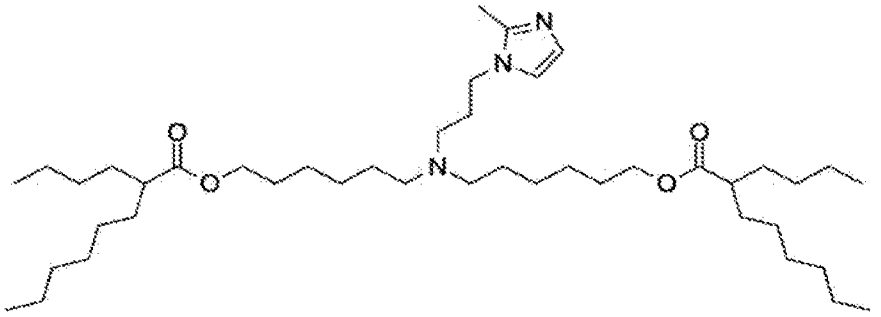
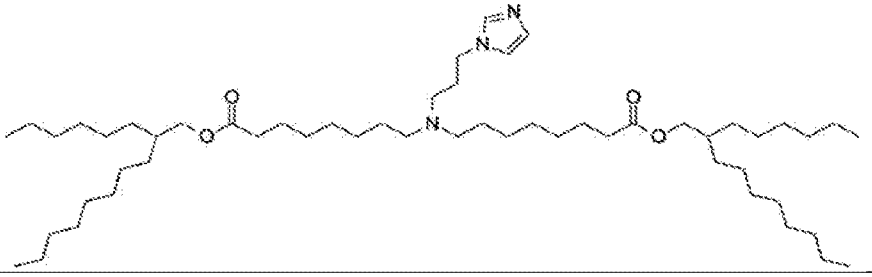
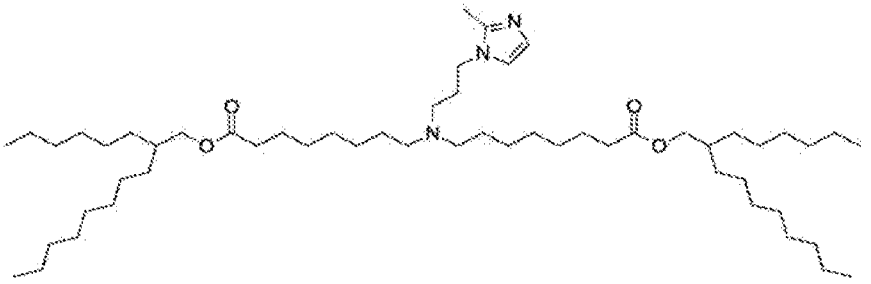
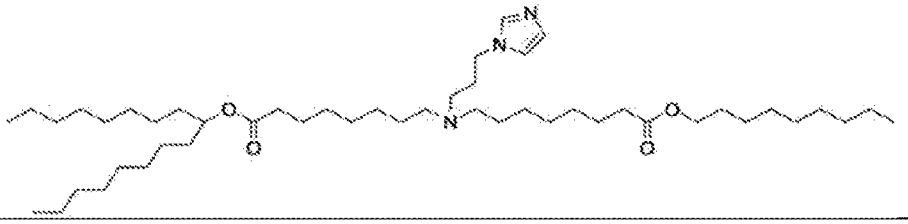
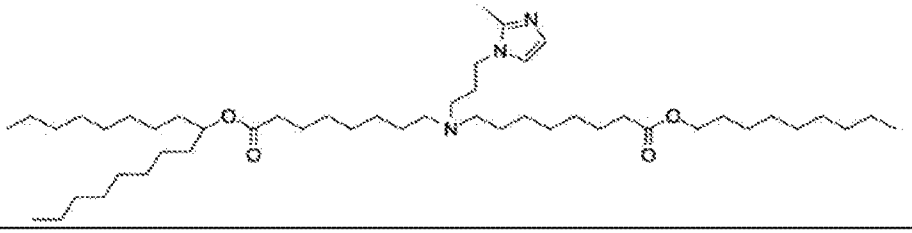
17	
18	
19	
20	
21	
22	
23	

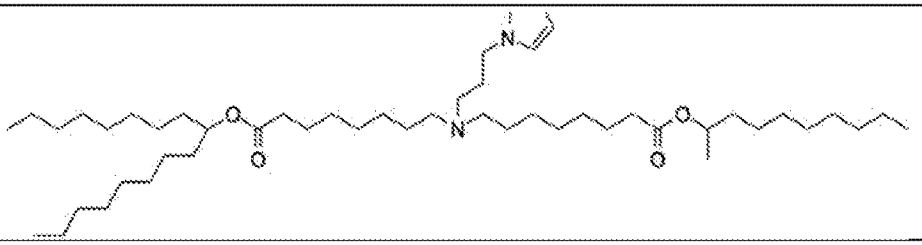
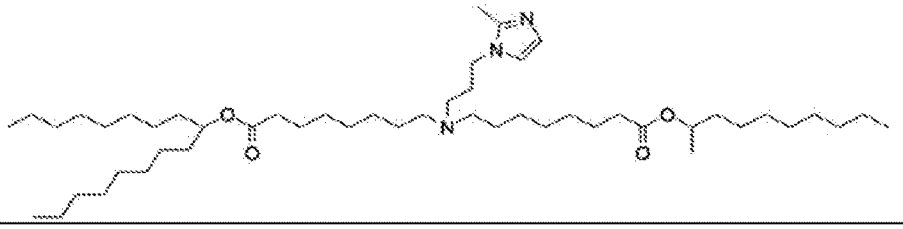
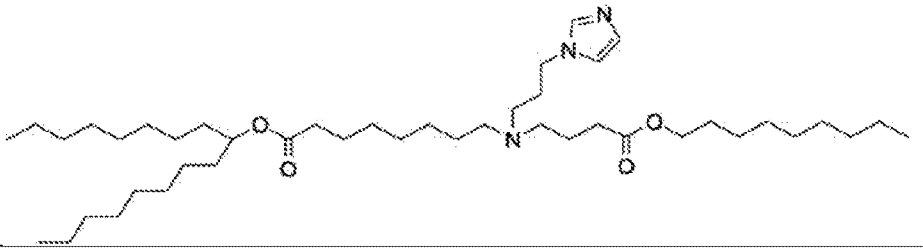
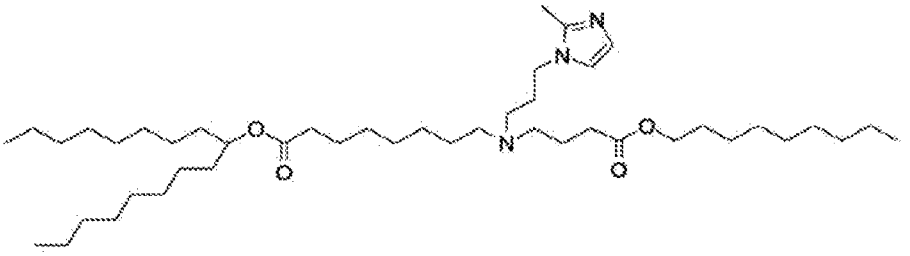
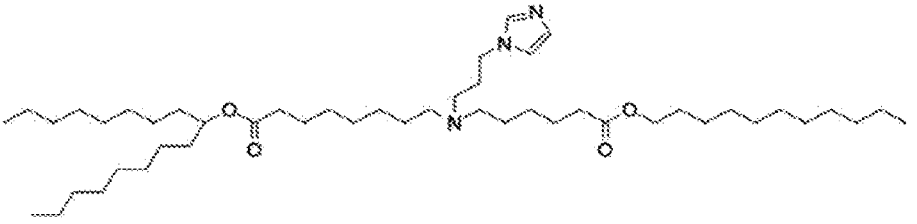
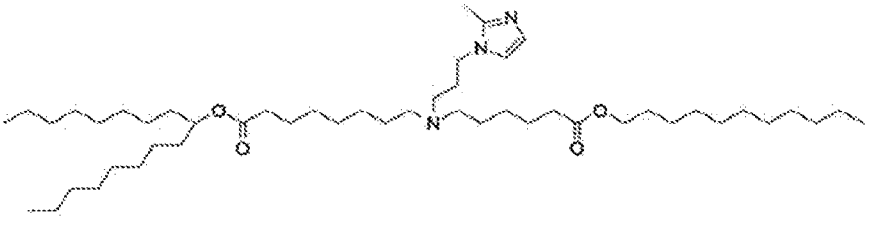
24	
25	
26	
27	
28	

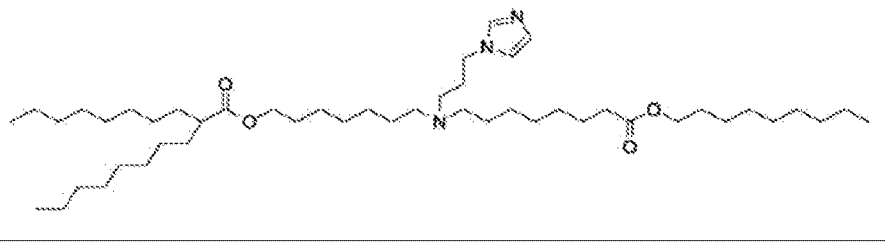
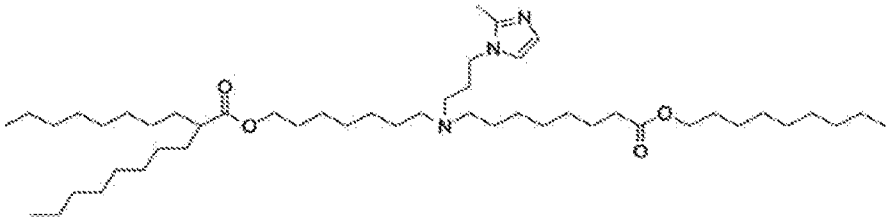
29	
30	
31	
32	

33	
34	
35	
36	

37	
38	
39	
40	
41	

42	
43	
44	
45	
46	

47	
48	
49	
50	
51	
52	

53	
54	
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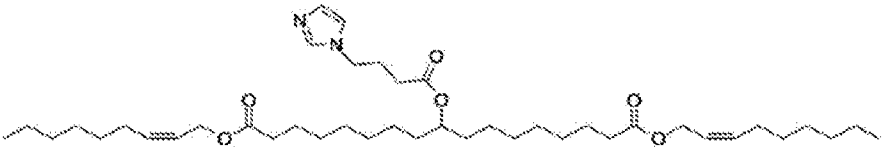
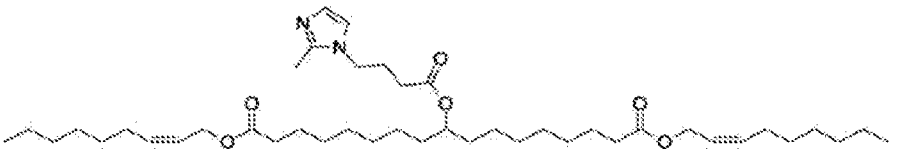
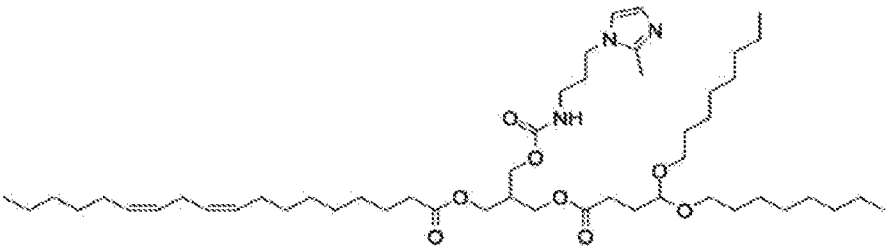
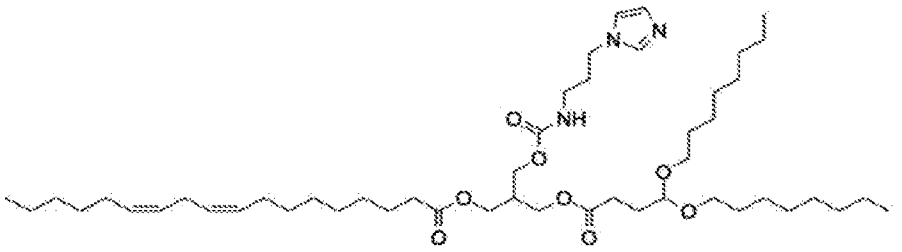
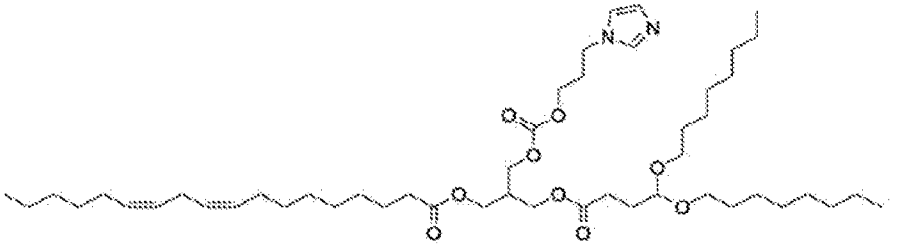
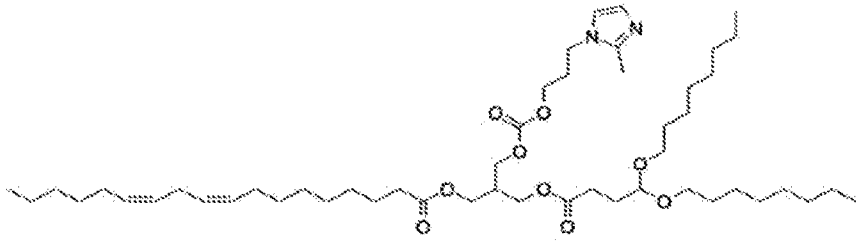
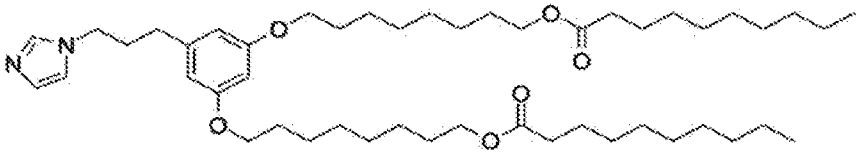
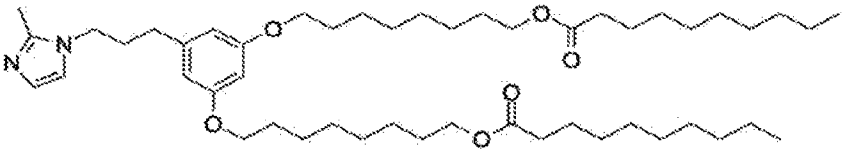
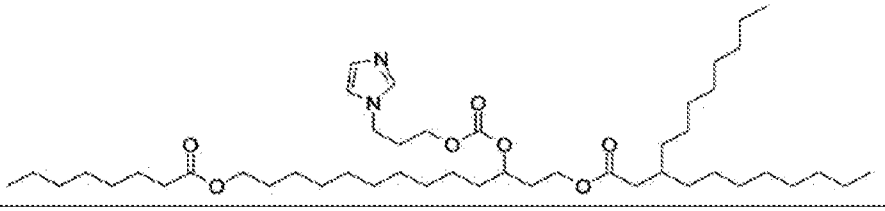
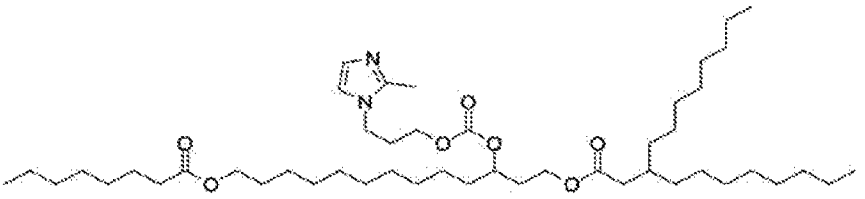
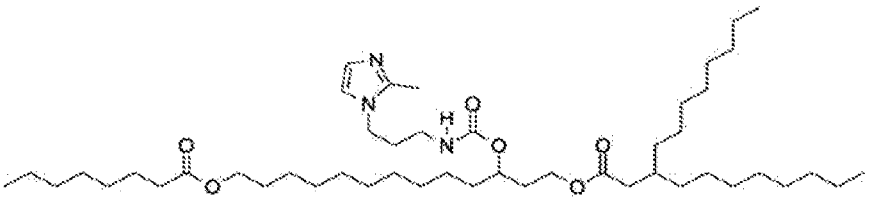
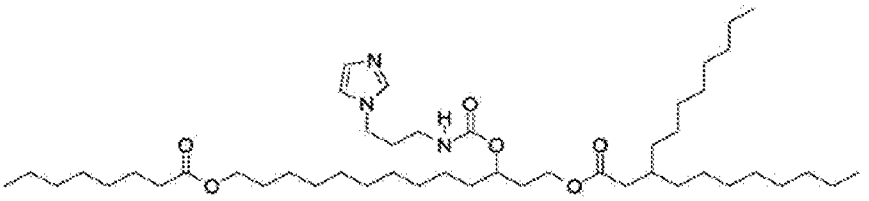
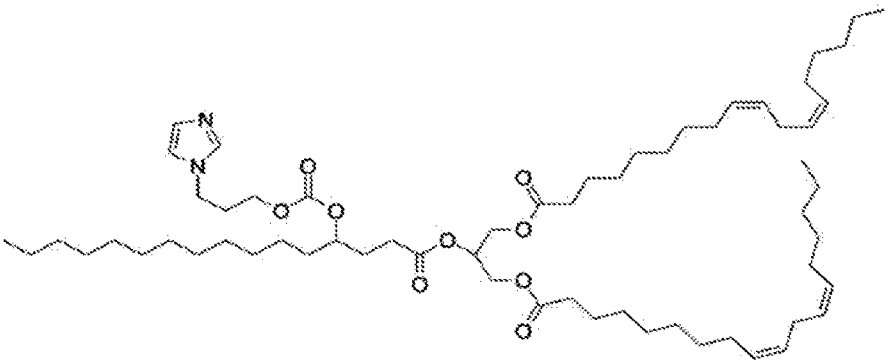
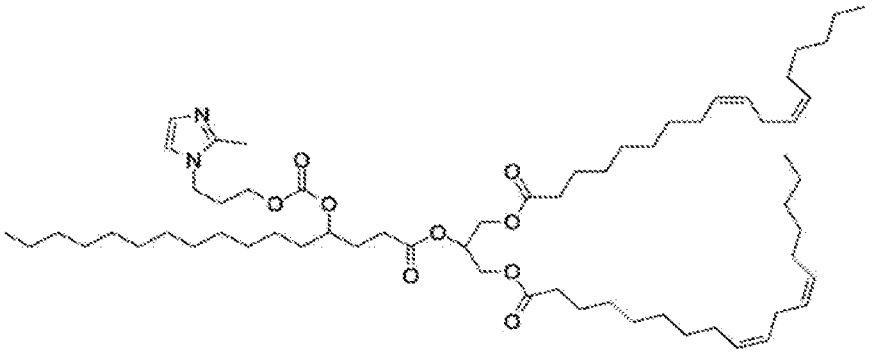
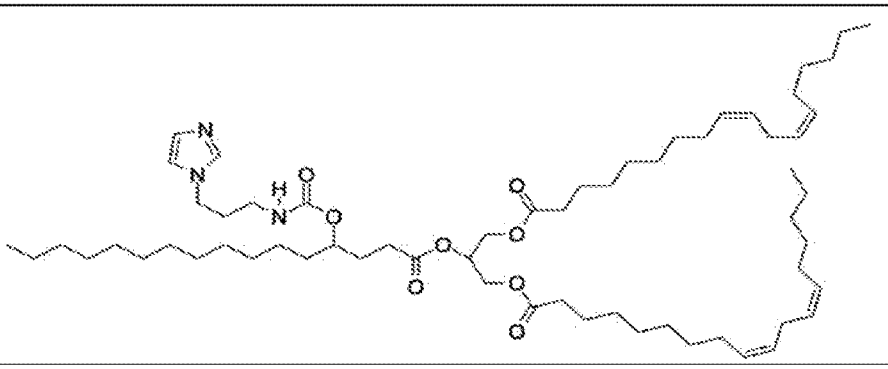
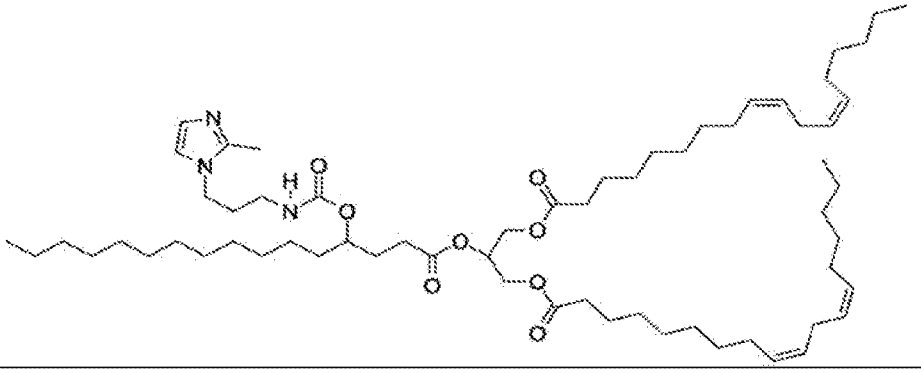
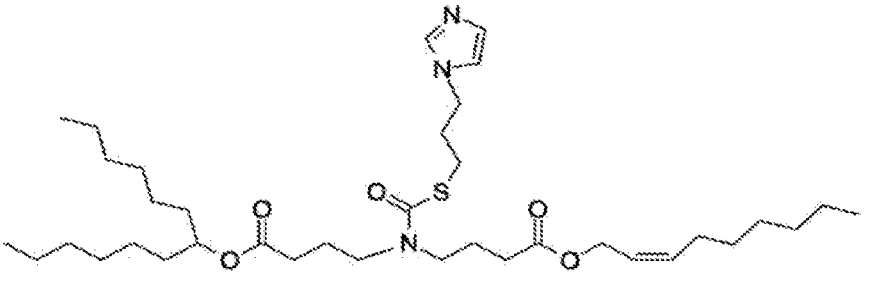
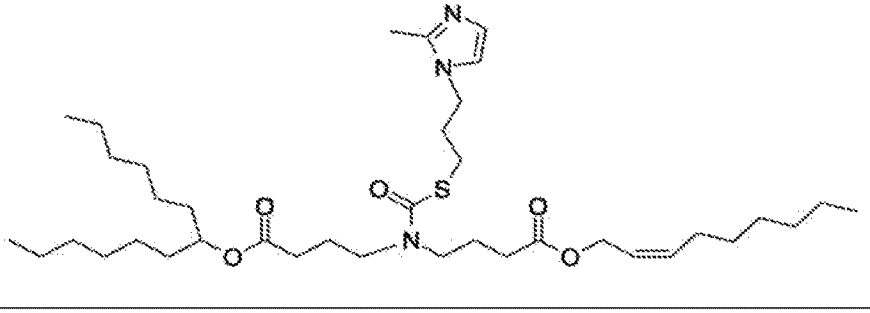
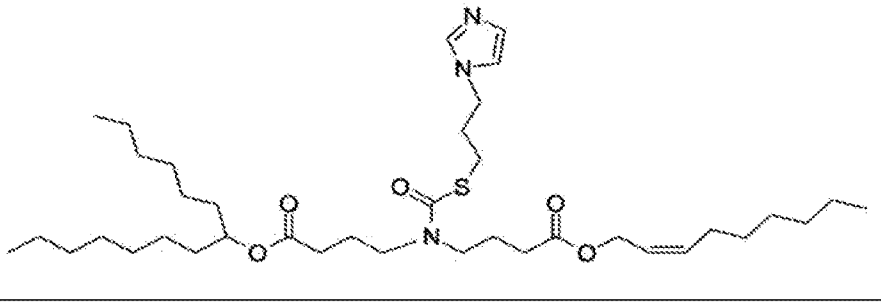
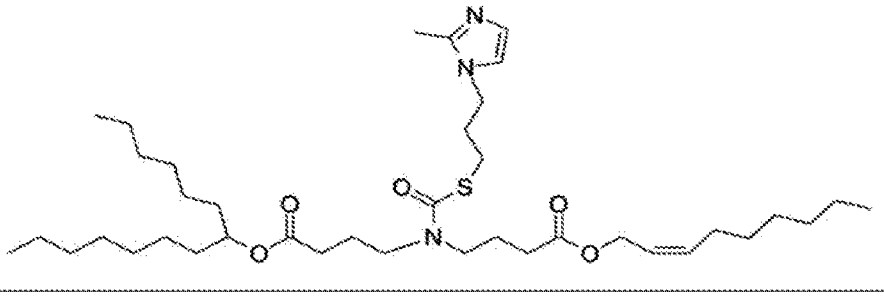
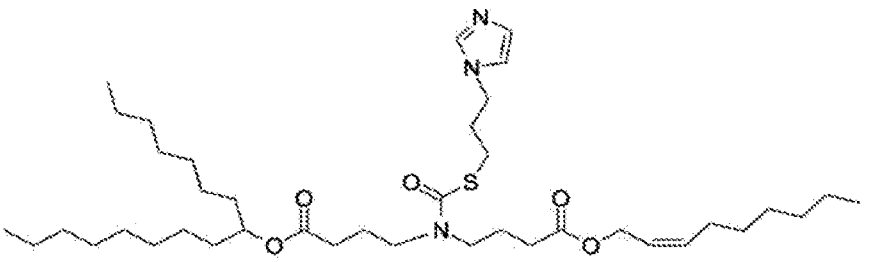
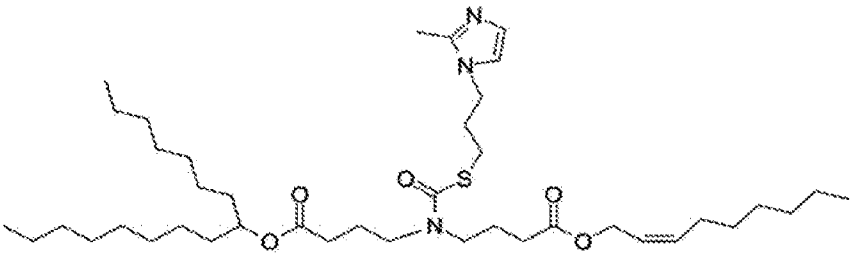
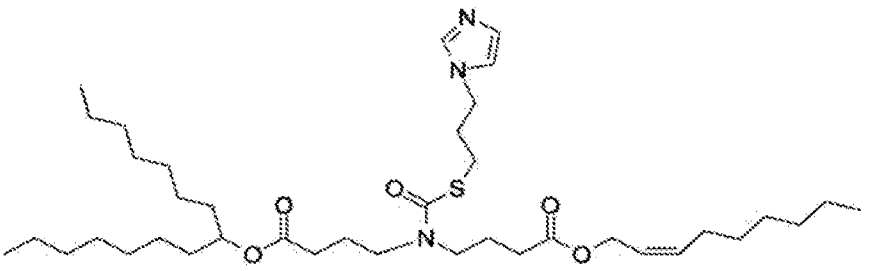
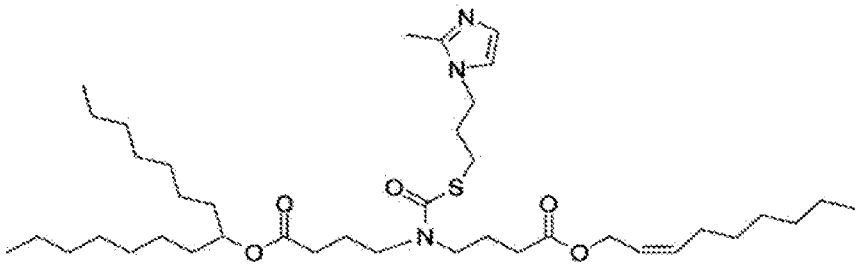
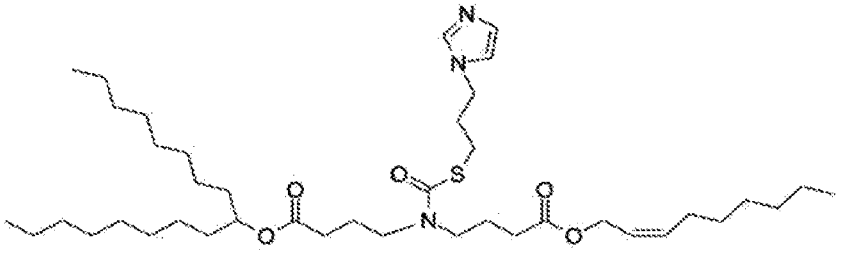
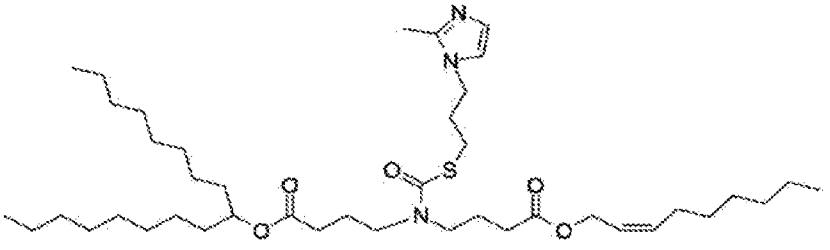
57	
58	
59	

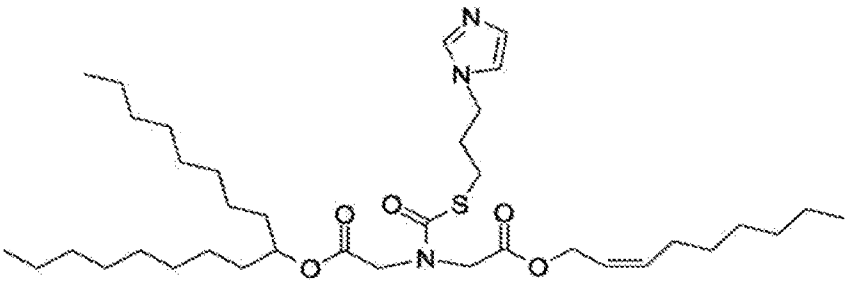
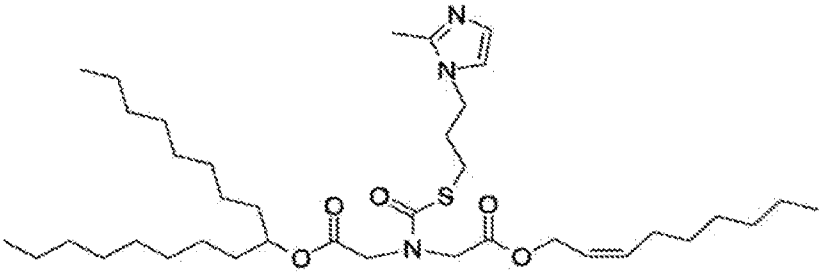
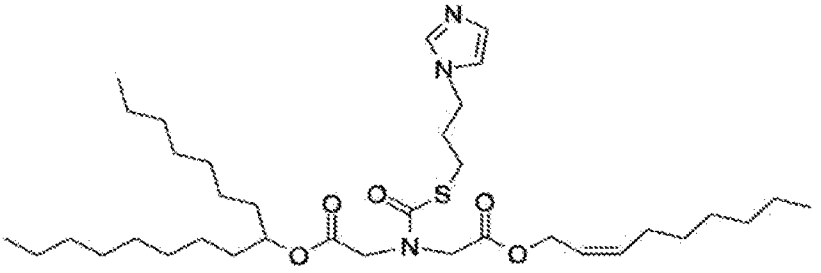
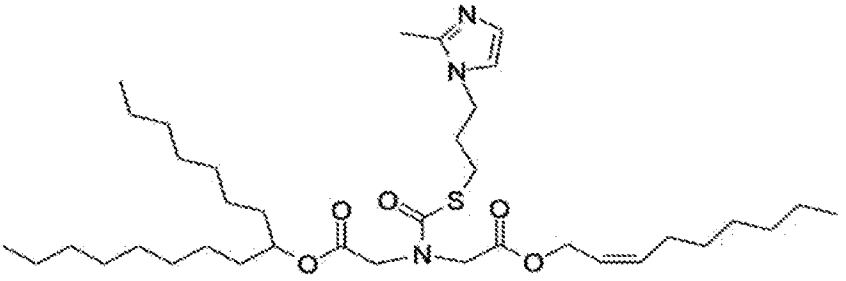
60	
61	
62	
63	
64	
65	

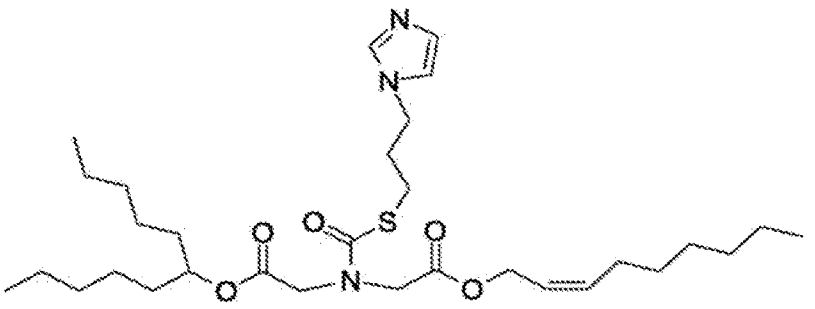
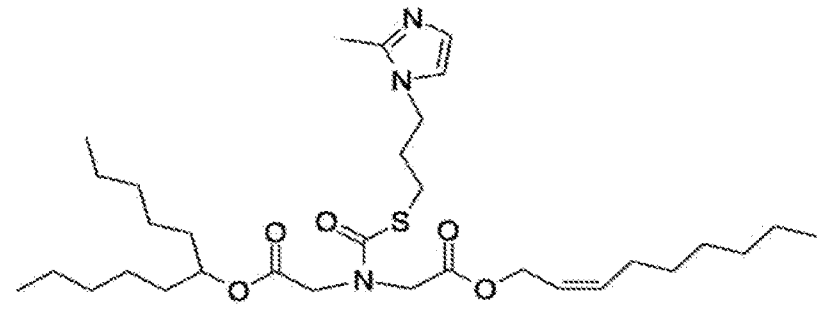
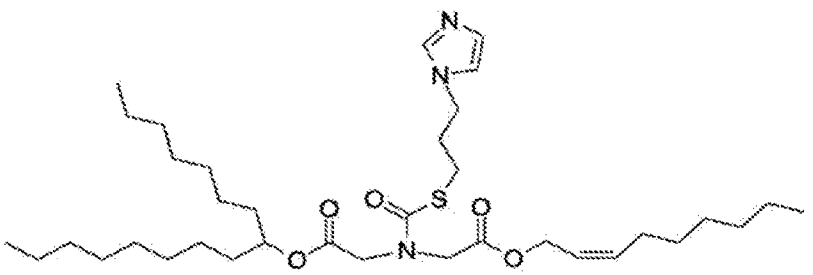
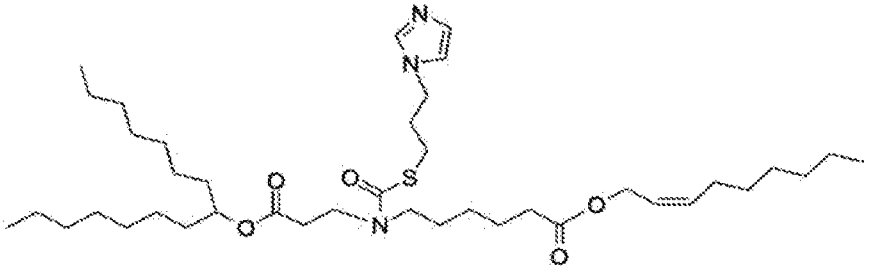
66	
67	
68	
69	
70	

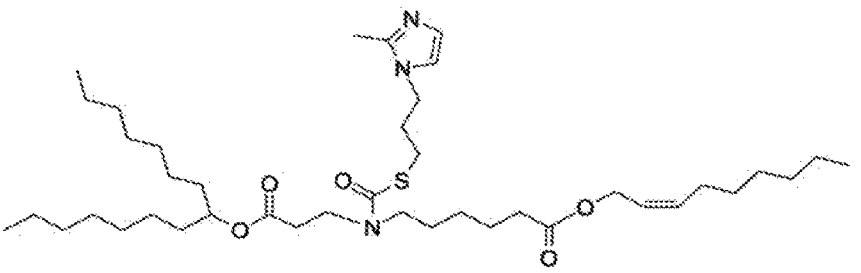
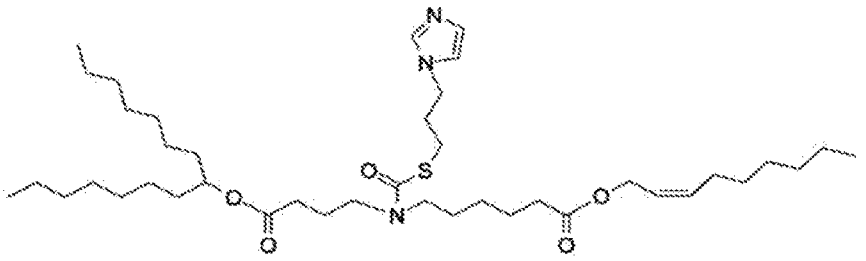
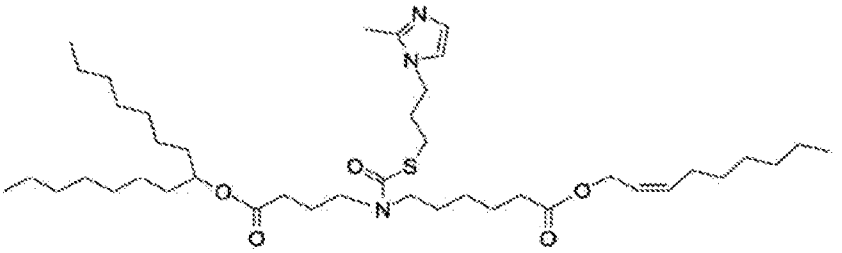
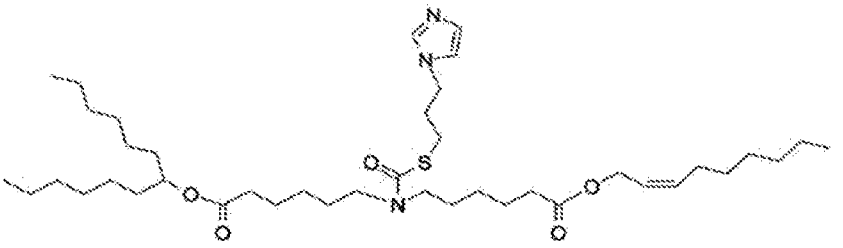
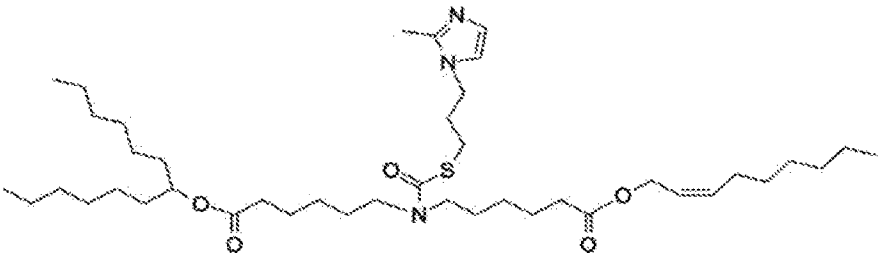
71	
72	
73	
74	

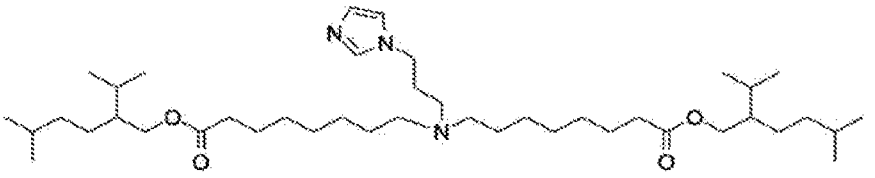
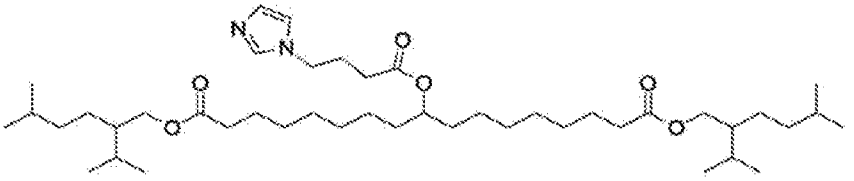
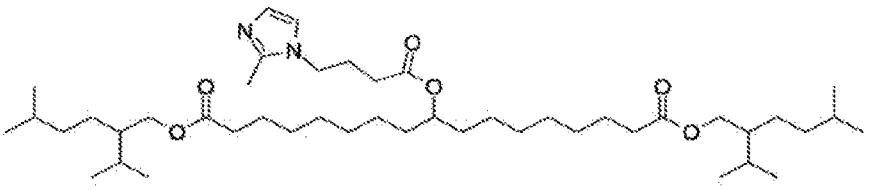
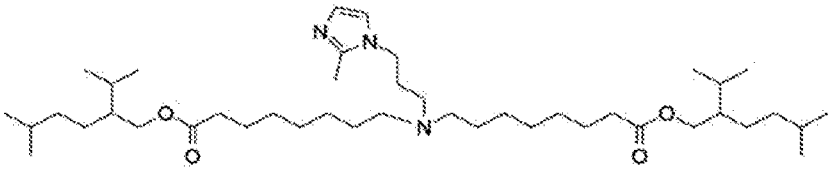
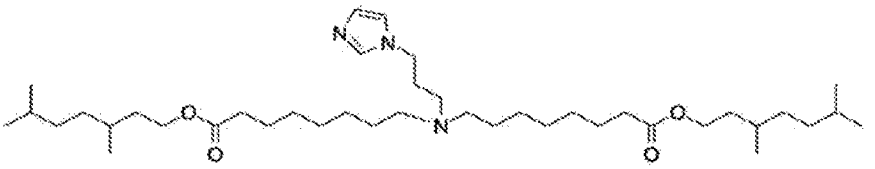
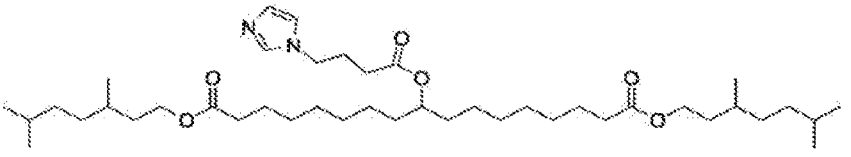
75	
76	
77	
78	

79	
80	
81	
82	

83	
84	
85	
86	

87	
88	
89	
90	

91	
92	
93	
94	
95	

96	
97	
98	
99	
100	
101	
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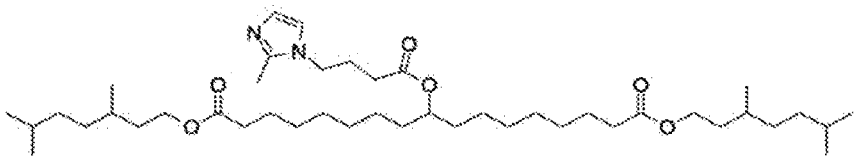
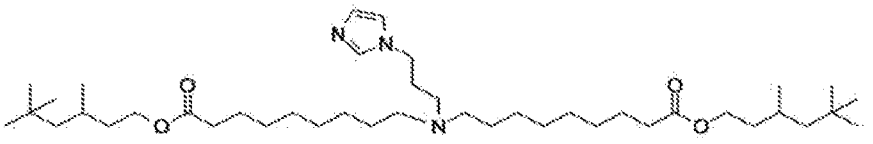
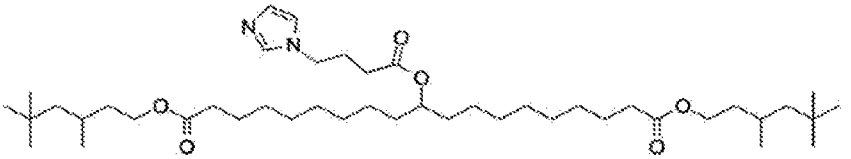
103	
104	
105	
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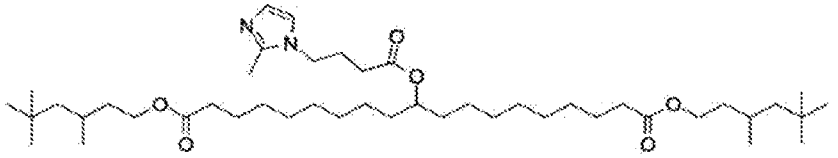
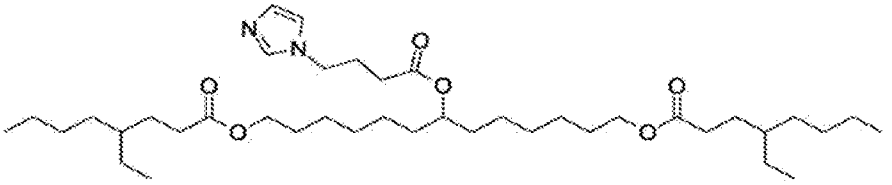
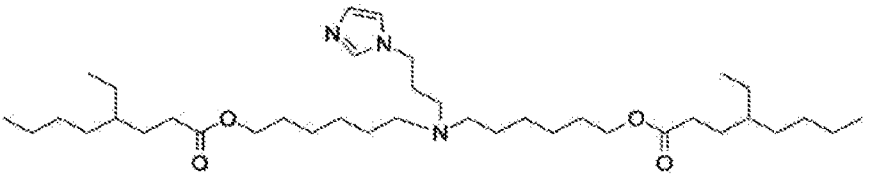
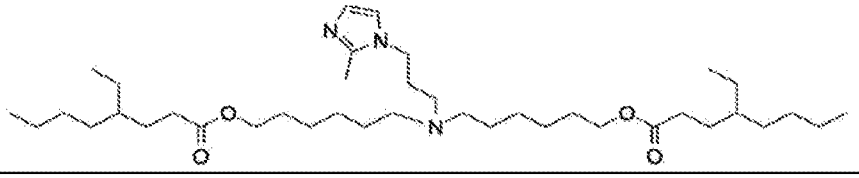
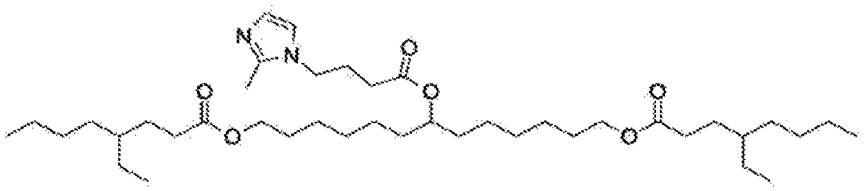
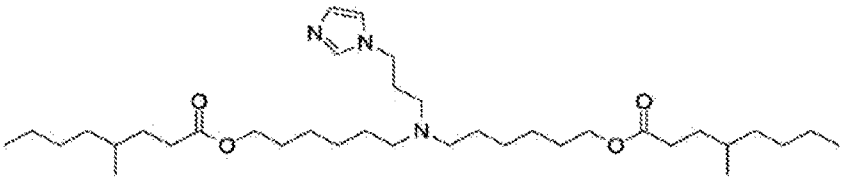
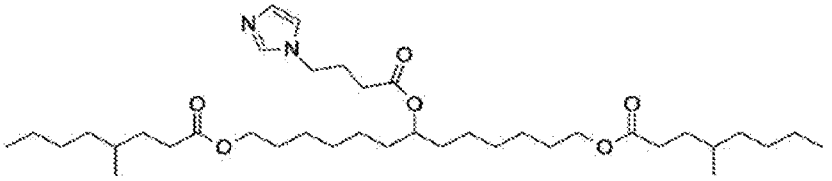
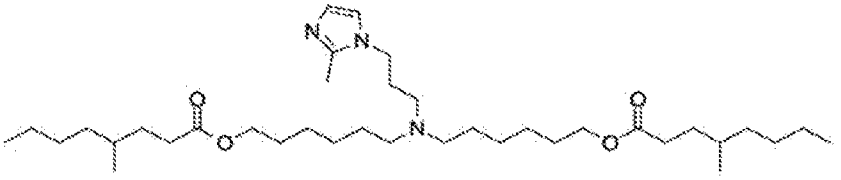
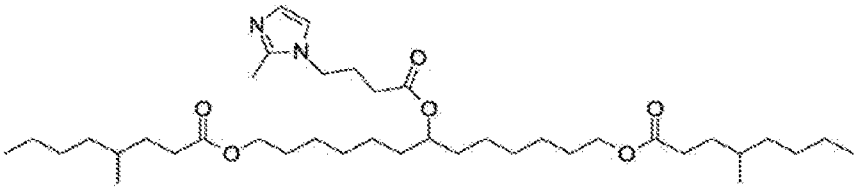
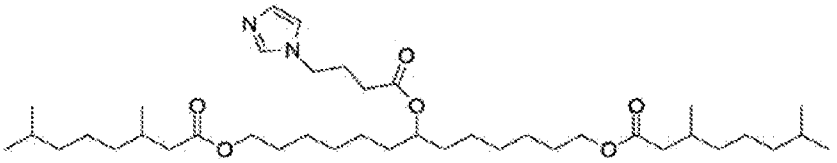
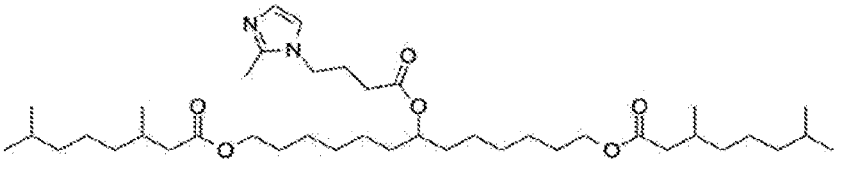
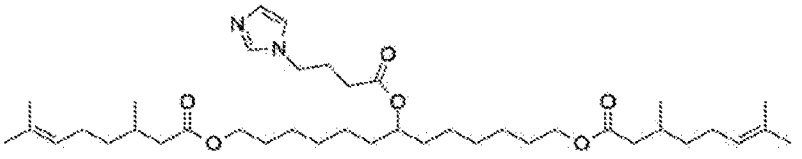
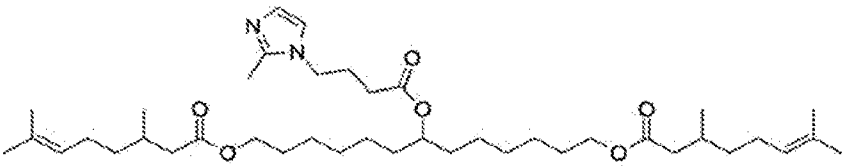
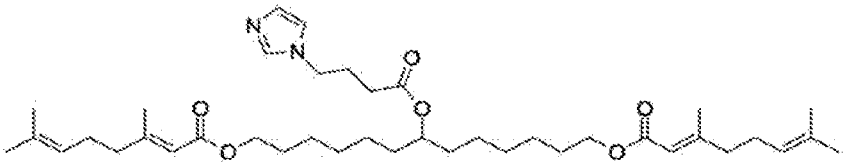
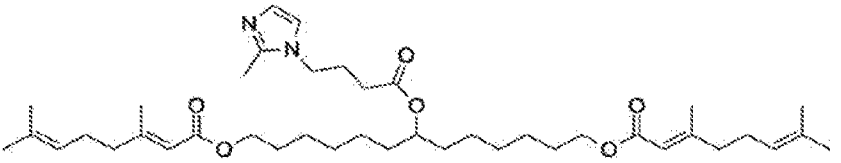
107	
108	
109	
110	

111	
112	
113	
114	
115	
116	
117	

118	
119	
120	
121	
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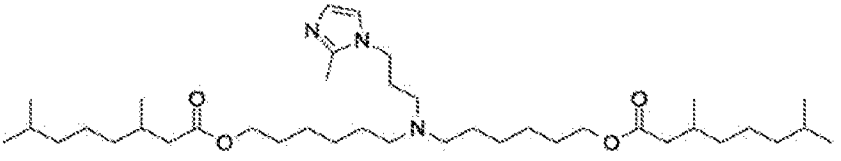
123	
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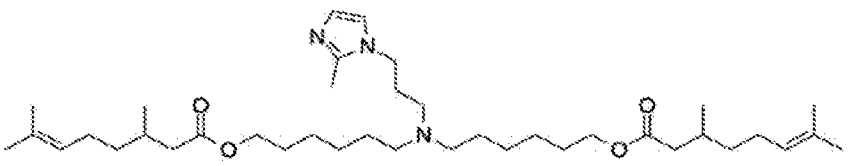
125	
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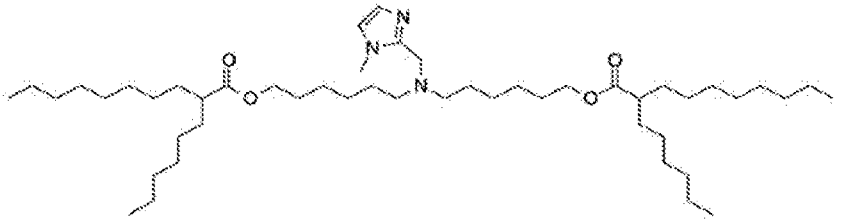
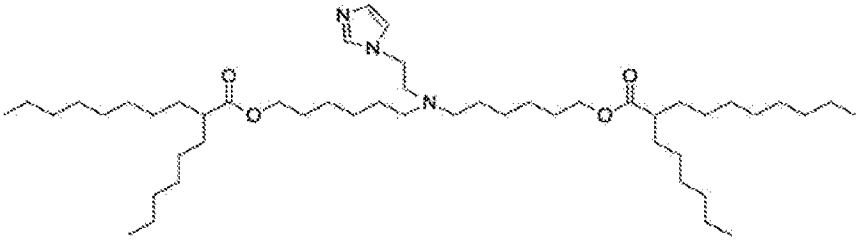
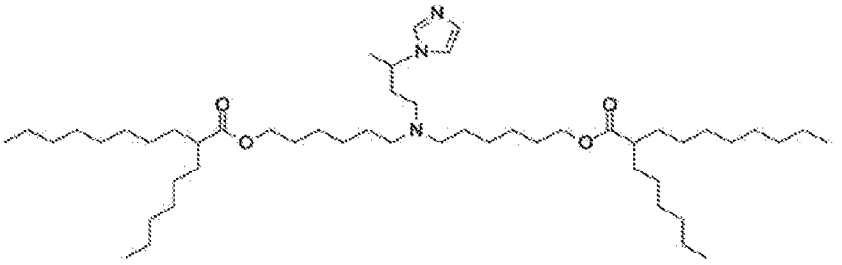
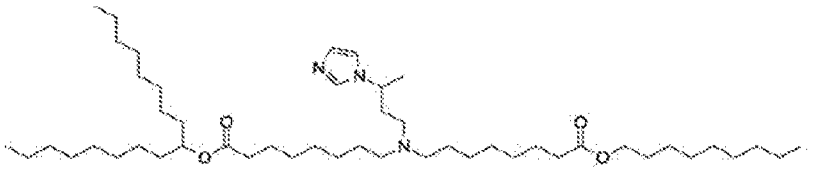
128	
129	
130	

131	
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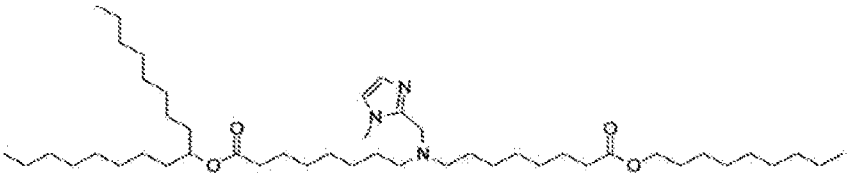
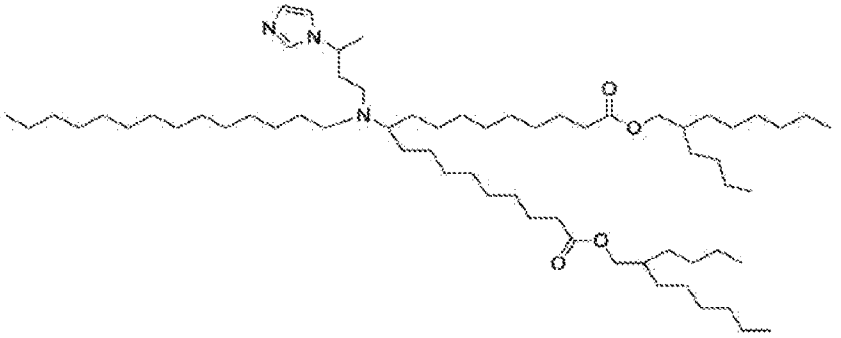
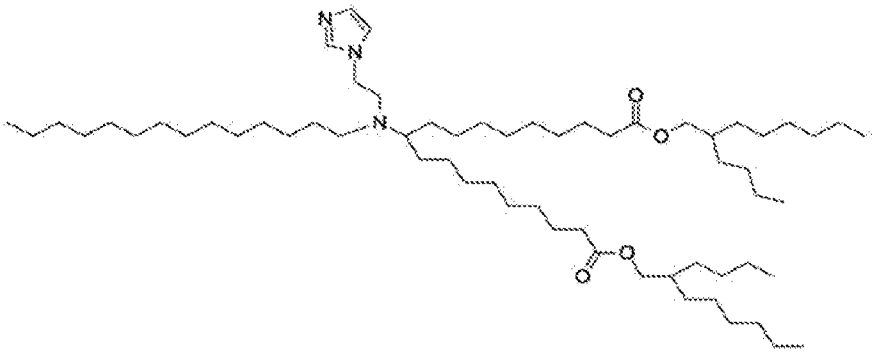
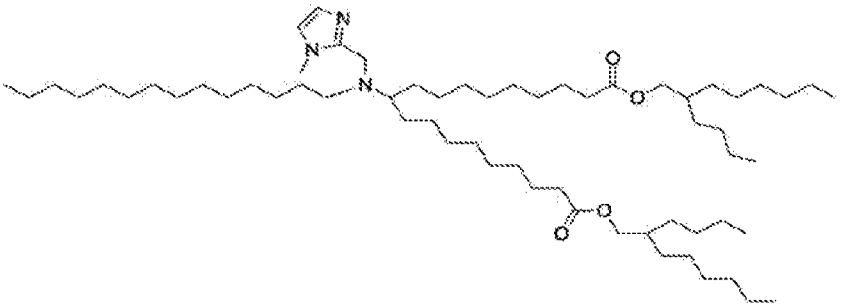
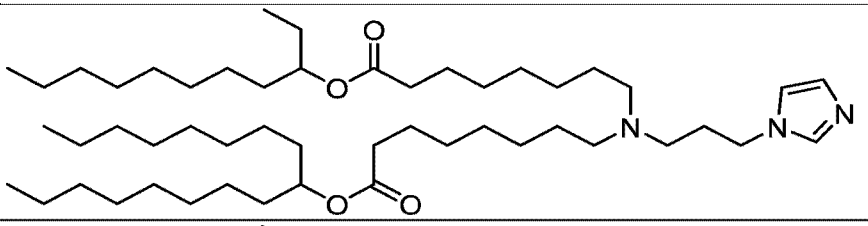
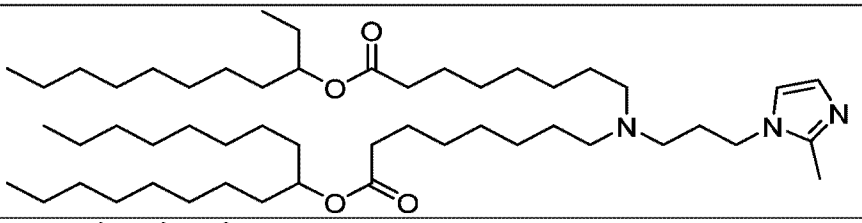
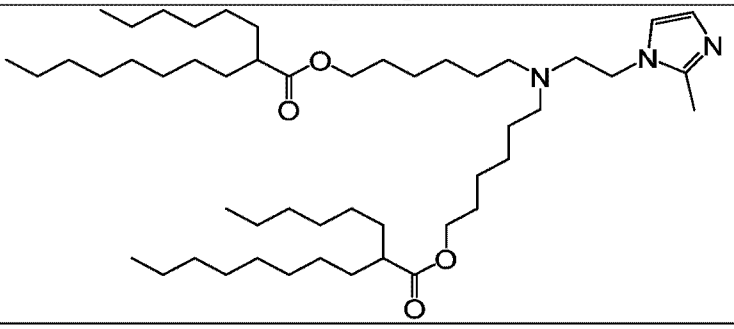
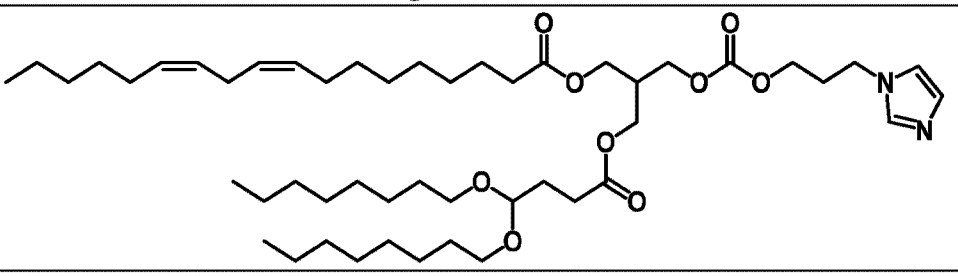
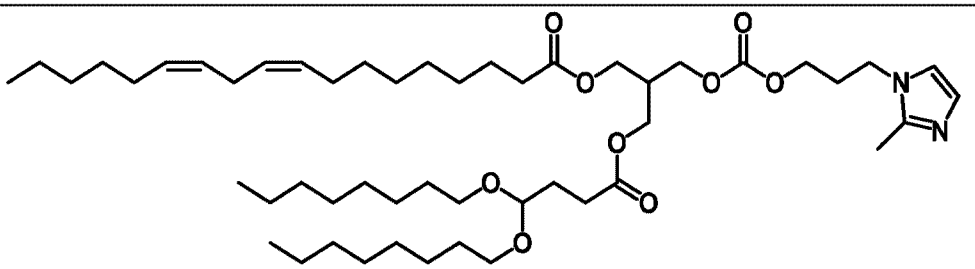
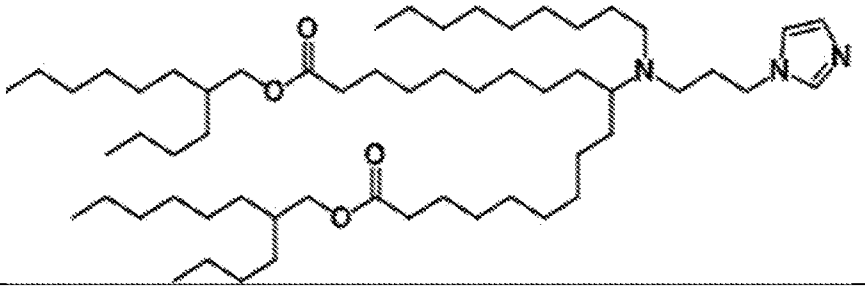
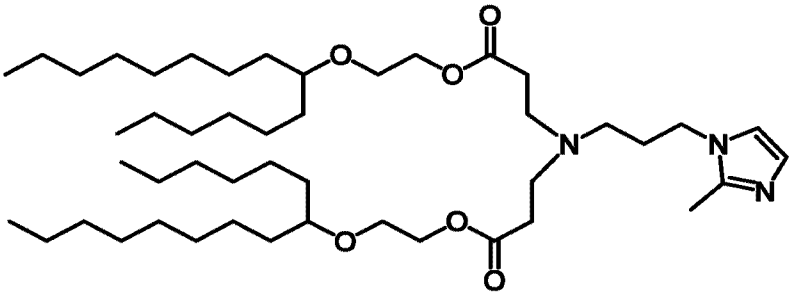
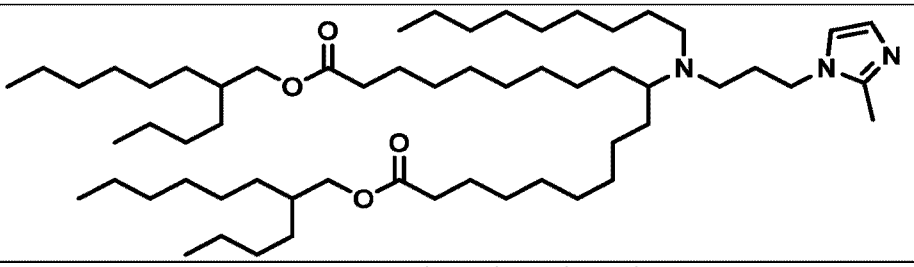
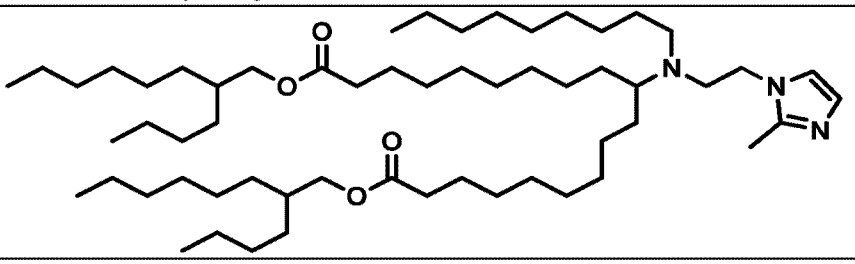
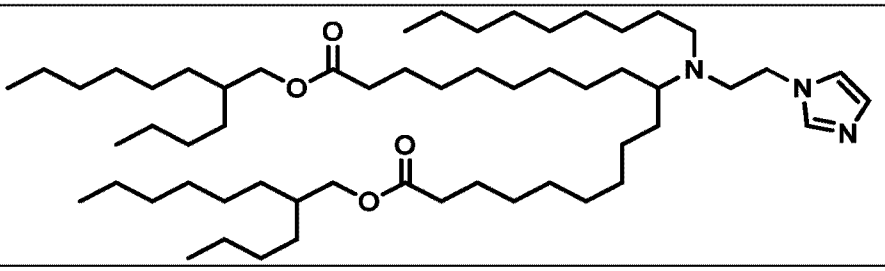
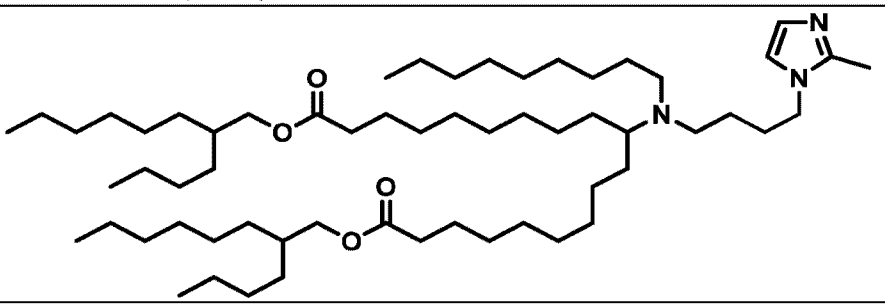
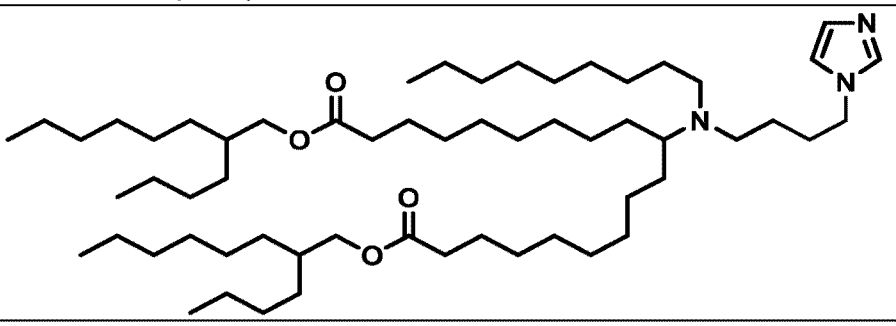
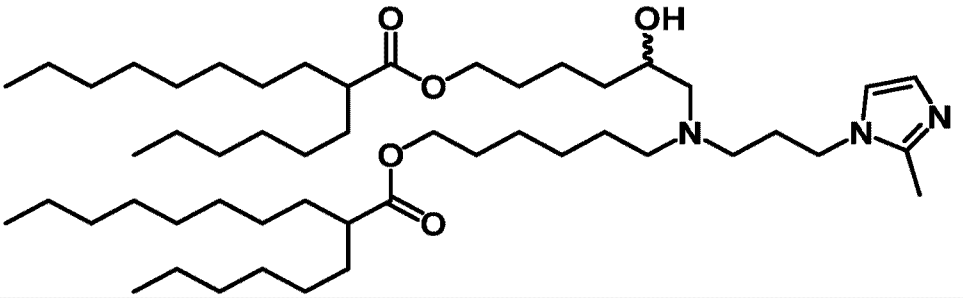
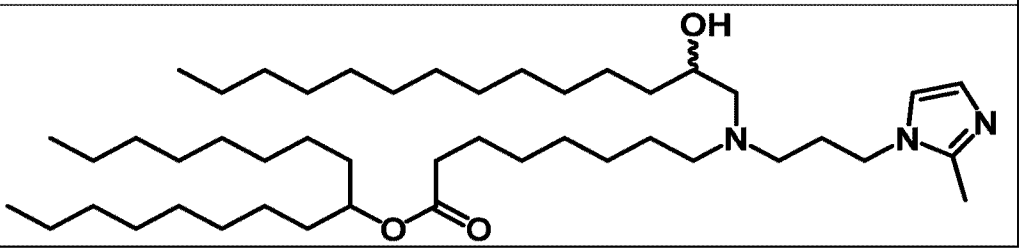
133	
134	
135	

136	
137	
138	
139	
140	

141	
142	
143	
144	
145	

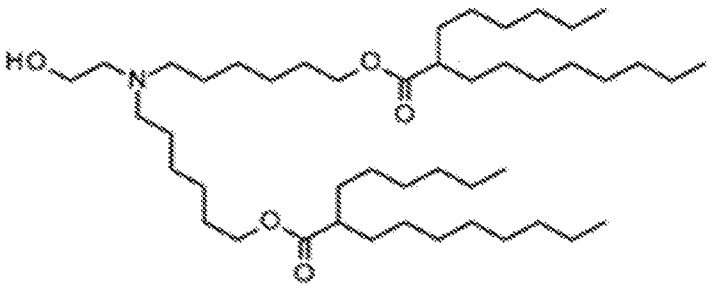
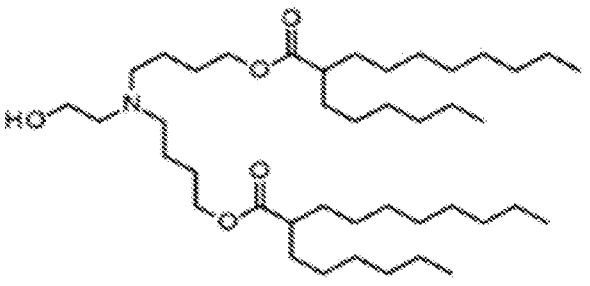
146	
147	
148	
149	
150	

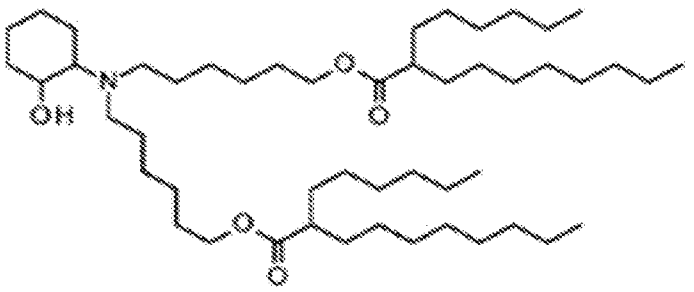
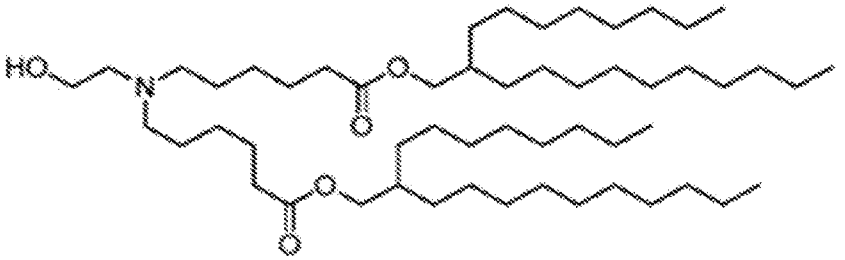
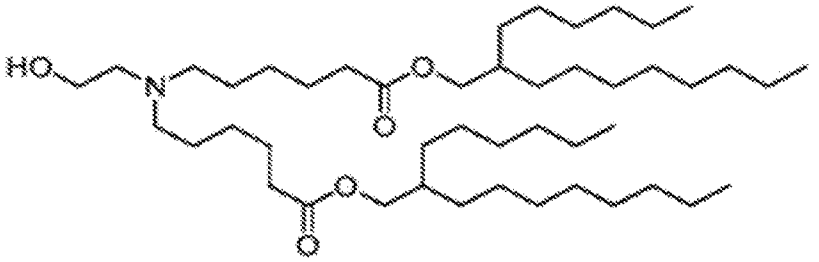
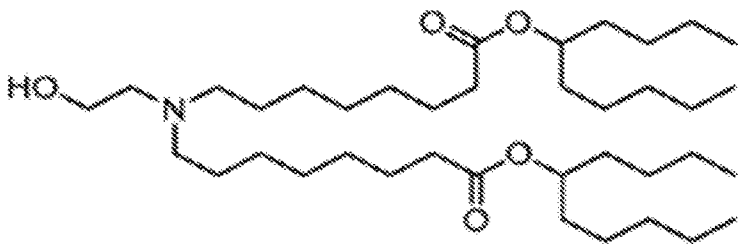
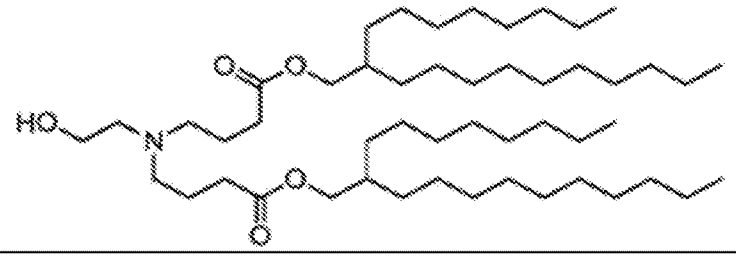
151	

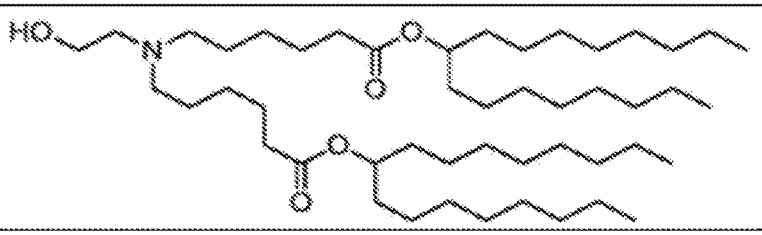
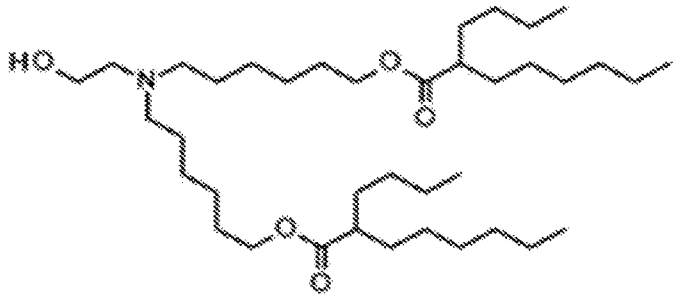
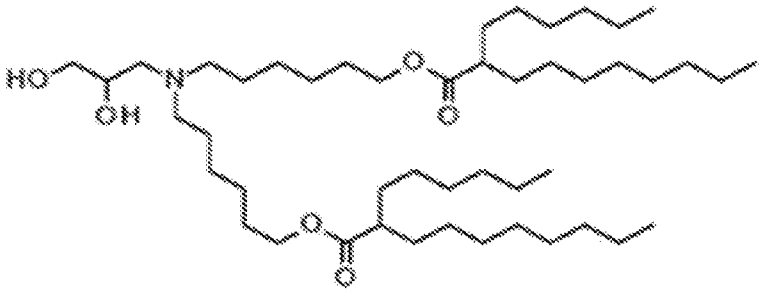
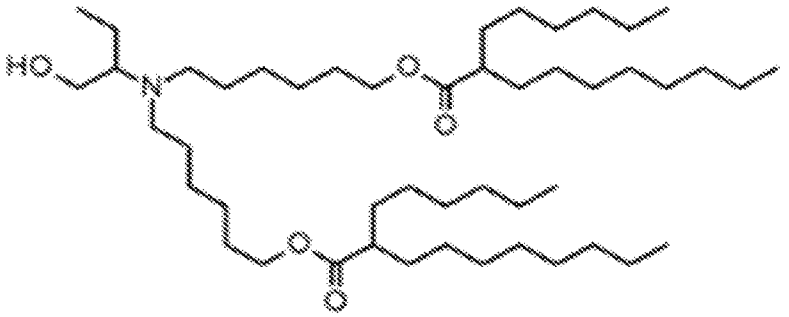
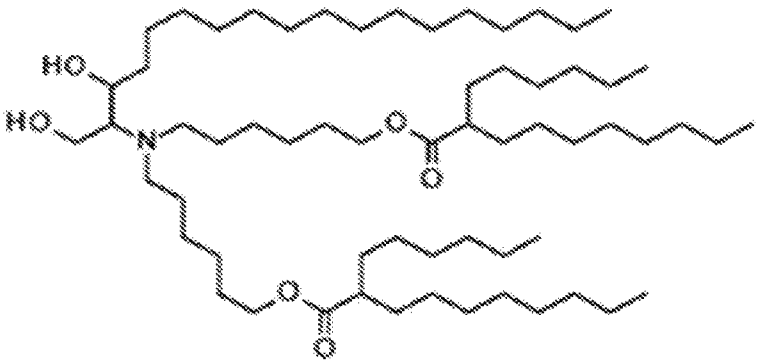
[0482] In some embodiments, the ionizable lipid has a beta-hydroxyl amine head group. In some embodiments, the ionizable lipid has a gamma-hydroxyl amine head group.

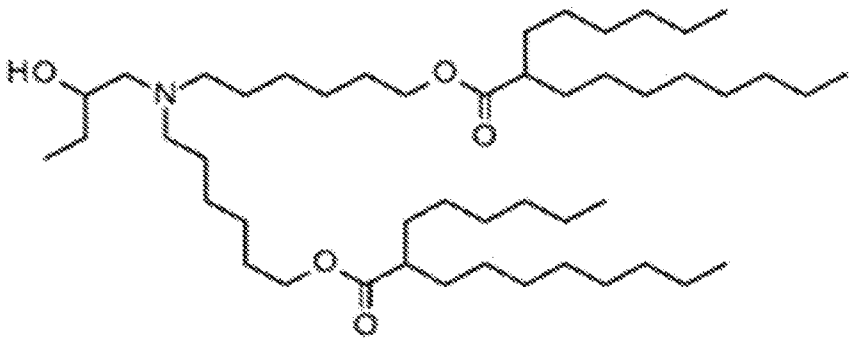
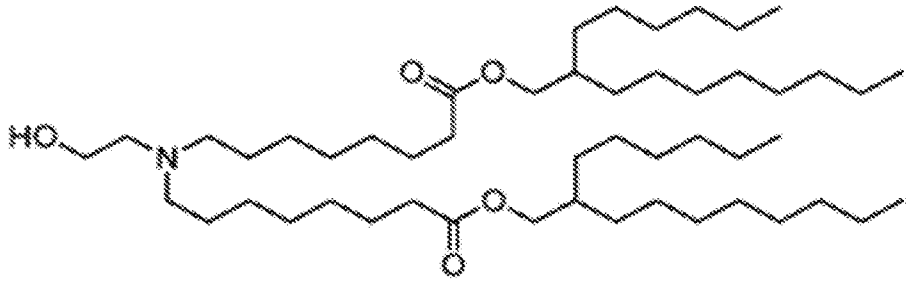
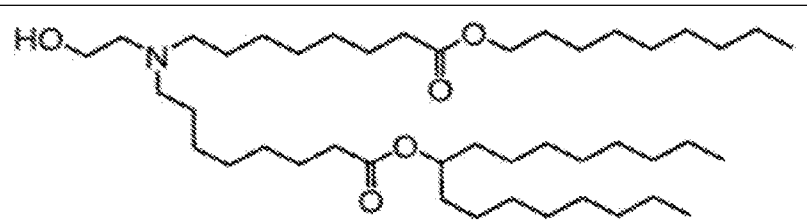
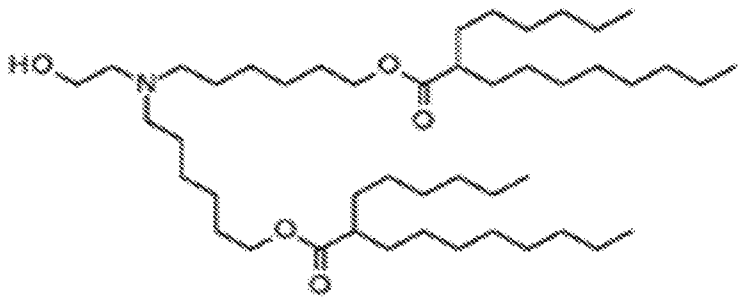
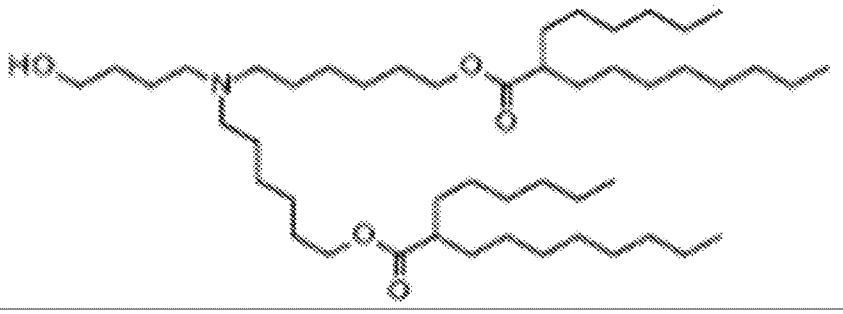
[0483] In some embodiments, an ionizable lipid of the disclosure is a lipid selected from Table 10b. In some embodiments, an ionizable lipid of the disclosure is Lipid 15 from Table 10b. In an embodiment, the ionizable lipid is described in US patent publication number US20170210697A1. In an embodiment, the ionizable lipid is described in US patent publication number US20170119904A1.

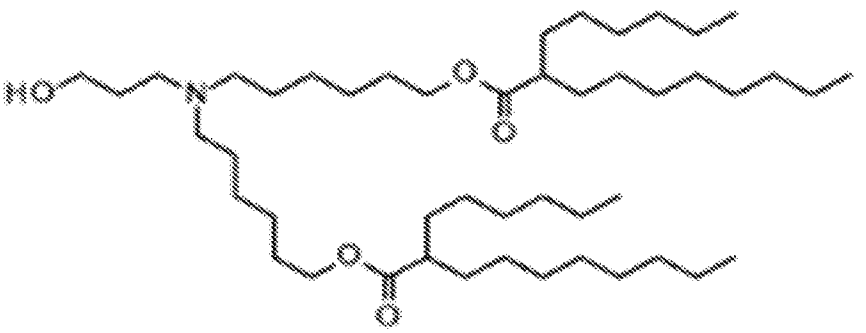
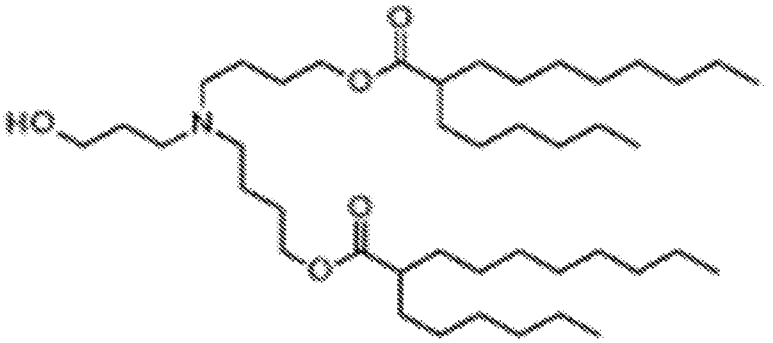
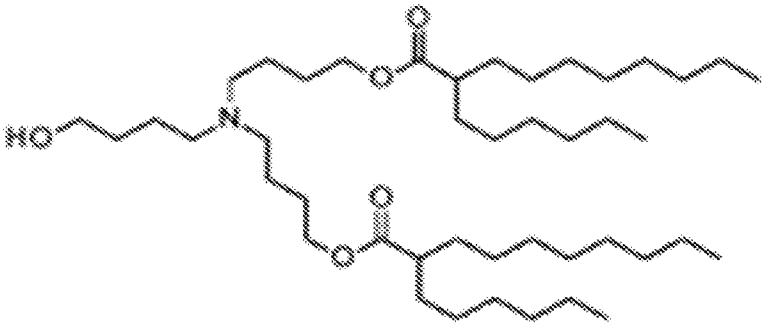
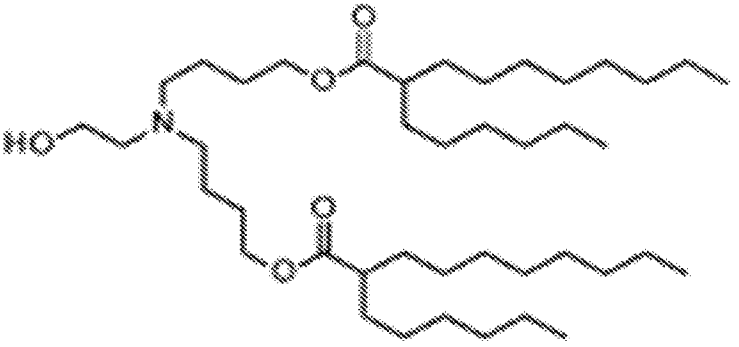
Table 10b

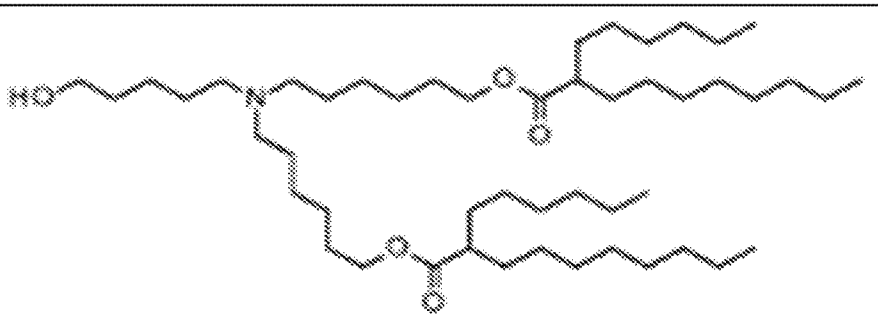
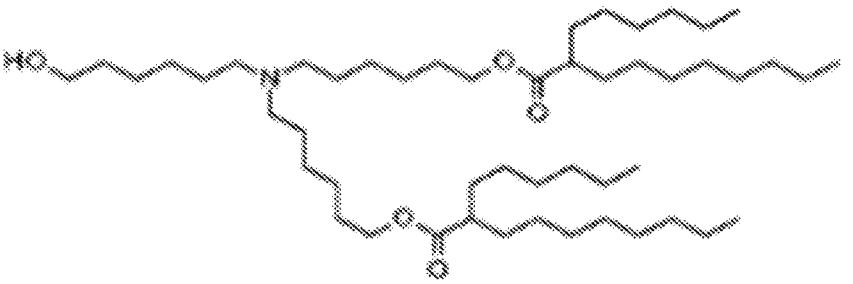
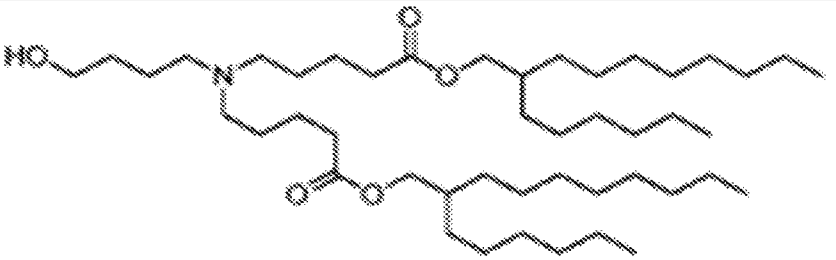
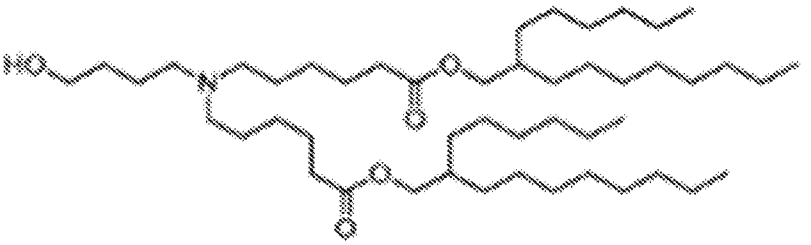
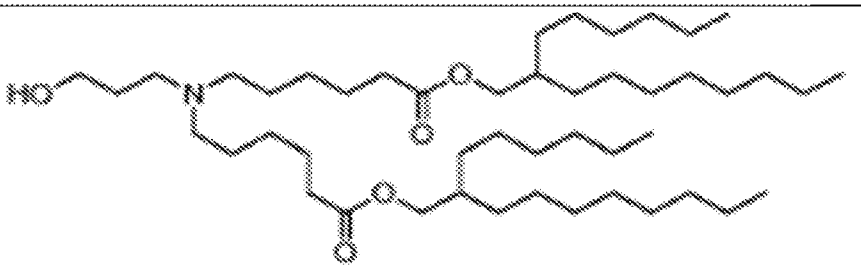
Ionizable lipid number	**Structure**
1	
2	

3	
4	
5	
6	
7	

8	
9	
10	
11	
12	

13	
14	
15	
16	
17	

18	
19	
20	
21	

22	
23	
24	
25	
26	

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 4
CONTENANT LES PAGES 1 À 268

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JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 4
CONTAINING PAGES 1 TO 268

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

WHAT IS CLAIMED IS:

1. A precursor RNA polynucleotide comprising, in the following order:
   a. a 5' enhanced intron element,
   b. a 5' enhanced exon element,
   c. a core functional element,
   d. a 3' enhanced exon element, and
   e. a 3' enhanced intron element,

   wherein the core functional element comprises, in the following order:
   i. a translation initiation element (TIE),
   ii. a coding element, and
   iii. optionally, a stop codon or a stop cassette.

2. A precursor RNA polynucleotide comprising, in the following order:
   a. a 5' enhanced intron element,
   b. a 5' enhanced exon element,
   c. a core functional element,
   d. a 3' enhanced exon element, and
   e. a 3' enhanced intron element

   wherein the core functional element comprises, in the following order:
   i. a coding region,
   ii. optionally, a stop codon or a stop cassette, and
   iii. a translation initiation element (TIE).

3. A precursor RNA polynucleotide comprising, in the following order:
   a. a 5' enhanced intron element,
   b. a 5' enhanced exon element,
   c. a core functional element,
   d. a 3' enhanced exon element, and
   e. a 3' enhanced intron element,

   wherein the core functional element comprises a noncoding element.

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4. The precursor RNA polynucleotide of claim 1 or 2, wherein the TIE comprises an untranslated region (UTR) or a fragment thereof, a aptamer complex or a fragment thereof, or a combination thereof.

5. The precursor RNA polynucleotide of claim 4, wherein the UTR or fragment thereof is derived from a viral or eukaryotic messenger RNA.

6. The precursor RNA polynucleotide of claim 5, wherein the UTR or fragment thereof comprises a viral internal ribosome entry site (IRES) or eukaryotic IRES.

7. The precursor RNA polynucleotide of claim 1 or 2, wherein the core functional element comprises two or more internal ribosome entry sites (IRESs).

8. The precursor RNA polynucleotide of claim 7, wherein the core functional element comprises a TIE, a coding element, a termination sequence, optionally a spacer, a TIE, a coding element, and a termination sequence, wherein the TIE comprises an IRES.

9. The precursor RNA polynucleotide of any one of claims 6-8, wherein the IRES comprises a sequence selected from SEQ ID NOs: 1-2983 and 3282-3287, or a fragment thereof.

10. The precursor RNA polynucleotide of any one of claims 6-9, wherein the IRES comprises a sequence selected from SEQ ID NOs: 75, 77, 137, 532, 566, 582, 648, 680, 693, 752, 785, 787, 791, 793, 820, 823, 839, 840, 843, 852, 857, 861, 862, 863, 864, 871, 874, 876, 922, 959, 983, 984, 1015, 1017, 1023, 1026, 1031, 1041, 1047, 1059, 1068, 1134, 1168, 1169, 1171, 1177, 1178, 1179, 1180, 1189, 1192, 1193, 1198, 1216, 1218, 1230, 1263, 1276, 1280, 1282, 1284, 1287, 1346, 1354, 1364, 1367, 1370, 1432, 1438, 1440, 2285, 2465, 2601, 2615, 2616, 2617, 2618, 2627, 2667, 2681, 2742, 2746, 2758, 2777, 2778, 3282, 3283, 3286, and 3287, or a fragment thereof.

11. The precursor RNA polynucleotide of any one of claims 6-8, wherein the IRES comprises one or more modified nucleotides compared to the wild-type viral IRES or eukaryotic IRES.

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12. The precursor RNA polynucleotide of any one of claims 6-11, wherein the IRES is capable of facilitating expression of a protein encoded by the precursor RNA in a cell.

13. The precursor RNA polynucleotide of any one of claims 12, wherein the IRES is capable of facilitating expression of the protein, such that the expression level of the protein is comparable to or higher than when a control IRES is used.

14. The precursor RNA polynucleotide of claim 13, wherein the control IRES comprises the sequence of SEQ ID NO: 3282.

15. The precursor RNA polynucleotide of any one of claims 12-14, wherein the IRES is derived from Enterovirus, Kobuvirus, Parechovirus, or Cardiovirus.

16. The precursor RNA polynucleotide of any one of claims 12-15, wherein the IRES is derived from Enterovirus or Kobuvirus.

17. The precursor RNA polynucleotide of any one of claims 12-14, wherein the cell is a myotube.

18. The precursor RNA polynucleotide of claim 17, wherein the IRES is derived from Bopivirus, Oscivirus, Hunnivirus, Passerivirus, Mischivirus, Kobuvirus, Enterovirus, Cardiovirus, Salivirus, Rabovirus, Parechovirus, Gallivirus, or Sicinivirus.

19. The precursor RNA polynucleotide of claim 17, wherein the IRES is derived from Hunnivirus, Passerivirus, Kobuvirus, Bopivirus, or Enterovirus.

20. The precursor RNA polynucleotide of claim 17, wherein the IRES is derived from Enterovirus I, Enterovirus F, Enterovirus E, Enterovirus J, Enterovirus C, Enterovirus A, Enterovirus B, Aichivirus B, Parechovirus A, Cardiovirus F, Cardiovirus B, or Cardiovirus E.

21. The precursor RNA polynucleotide of claim 17, wherein the IRES comprises a sequence selected from SEQ ID NOs: 137, 580, 785, 791, 820, 922, 1041, 1047, 1068, 1168, 1169, 1171, 1177, 1178, 1179, 1180, 1189, 1192, 1263, 1276, 1280, 1282, 1284, 1287, 1354, 1356, 1432, 1436, 1439, 1440, 2285, 2667, 2746, 2777, 2778, 3283, and 3284.

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22. The precursor RNA polynucleotide of any one of claims 12-14, wherein the cell is a hepatocyte.

23. The precursor RNA polynucleotide of claim 22, wherein the IRES is derived from Enterovirus, Bopivirus, Mischivirus, Gallivirus, Oscivirus, Cardiovirus, Kobuvirus, Rabovirus, Salivirus, Parechovirus, Hunnivirus, Tottorivirus, Passerivirus, Cosavirus, or Sicinivirus.

24. The precursor RNA polynucleotide of claim 23, wherein the IRES is derived from Enterovirus, Mischivirus, Kobuvirus, Bopivirus, or Gallivirus.

25. The precursor RNA polynucleotide of claim 23, wherein the IRES is derived from Enterovirus B, Enterovirus A, Enterovirus D, Enterovirus J, Enterovirus C, Rhinovirus B, Enterovirus H, Enterovirus I, Enterovirus E, Enterovirus F, Aichivirus B, Aichivirus A, Parechovirus A, Cardiovirus F, Cardiovirus E, or Cardiovirus B.

26. The precursor RNA polynucleotide of claim 22, wherein the IRES comprises a sequence selected from SEQ ID NOs: 137, 580, 648, 693, 752, 785, 791, 793, 820, 823, 839, 840, 861, 862, 863, 876, 922, 959, 983, 984, 1015, 1017, 1023, 1026, 1031, 1041, 1047, 1059, 1068, 1134, 1168, 1169, 1171, 1177, 1178, 1179, 1180, 1189, 1192, 1193, 1198, 1216, 1263, 1276, 1280, 1282, 1284, 1287, 1346, 1354, 1356, 1432, 1436, 1438, 1439, 1440, 2285, 2777, 2778, 3283, and 3284.

27. The precursor RNA polynucleotide of any one of claims 12-14, wherein the cell is a T cell.

28. The precursor RNA polynucleotide of claim 27, wherein the IRES is derived from Passerivirus, Bopivirus, Hunnivirus, Mischivirus, Enterovirus, Kobuvirus, Rabovirus, Tottorivirus, Salivirus, Cardiovirus, Parechovirus, Megrivirus, Allexivirus, Oscivirus, or Shanbavirus.

29. The precursor RNA polynucleotide of claim 28, wherein the IRES is derived from Passerivirus, Hunnivirus, Mischivirus, Enterovirus, or Kobuvirus.

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30. The precursor RNA polynucleotide of claim 28, wherein the IRES is derived from Enterovirus I, Enterovirus D, Enterovirus C, Enterovirus A, Enterovirus J, Enterovirus H, Aichivirus B, Parechovirus A, or Cardiovirus B.

31. The precursor RNA polynucleotide of claim 27, wherein IRES comprises a sequence selected from SEQ ID NOs: 77, 787, 793, 820, 839, 840, 843, 852, 857, 861, 862, 863, 864, 871, 874, 876, 959, 1193, 1216, 1284, 1287, 1346, 1356, 1364, 1432, 1438, 1440, 2667, 2681, 2742, 2746, 2758, 3283, and 3284.

32. The precursor RNA polynucleotide of claim 4, wherein the aptamer complex or a fragment thereof comprises a natural or synthetic aptamer sequence.

33. The precursor RNA polynucleotide of claim 4, wherein the aptamer complex or a fragment thereof comprises a sequence selected from SEQ ID NOs: 3266-3268.

34. The precursor RNA polynucleotide of claim 4, wherein the aptamer complex or a fragment thereof comprises more than one aptamer.

35. The precursor RNA polynucleotide of claim 1 or 2, wherein the TIE comprises an UTR and an aptamer complex.

36. The precursor RNA polynucleotide of claim 35, wherein the UTR is located upstream to the aptamer complex.

37. The precursor RNA polynucleotide of claim 1 or 2, wherein the TIE further comprises an accessory element.

38. The precursor RNA polynucleotide of claim 37, wherein the accessory element comprises a miRNA binding site or a fragment thereof, a restriction site or a fragment thereof, an RNA editing motif or a fragment thereof, a zip code element or a fragment thereof, an RNA trafficking element or a fragment thereof, or a combination thereof.

39. The precursor RNA polynucleotide of claim 37, wherein the accessory element comprises a binding domain to an IRES transacting factor (ITAF).

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40. The precursor RNA polynucleotide of claim 39, wherein the binding domain comprises a polyA region, a polyC region, a poly AC region, a polyprimidine tract, or a combination or variant thereof.

41. The precursor RNA polynucleotide of claim 39, wherein the ITAF comprises a poly(rC)-binding protein 1 (PCBP1), PCBP2, PCBP3, PCBP4, poly(A) -binding protein 1 (PABP1), polyprimidine-tract binding protein (PTB), Argonaute protein family member, HNRNPK (heterogeneous nuclear ribonucleoprotein K protein), or La protein, or a fragment or combination thereof.

42. The precursor RNA polynucleotide of claim 1 or 2, wherein the coding element comprises a sequence encoding for a therapeutic protein.

43. The precursor RNA polynucleotide of claim 42, wherein the therapeutic protein comprises a chimeric protein.

44. The precursor RNA polynucleotide of claim 43, wherein the chimeric protein comprises a chimeric antigen receptor (CAR), T-cell receptor (TCR), B-cell receptor (BCR), immune cell activation or inhibitory receptor, recombinant fusion protein, chimeric mutant protein, or fusion protein, or a combination thereof.

45. The precursor RNA polynucleotide of claim 42, wherein the therapeutic protein comprises an antibody, nanobody, non-antibody protein, immune modulatory ligand, receptor, structural protein, growth factor ligand or receptor, hormone or hormone receptor, transcription factor, checkpoint inhibitor or agonist, Fc fusion protein, anticoagulant, blood clotting factor, chaperone protein, antimicrobial protein, structural protein, biochemical enzyme, tight junction protein, mitochondrial stress response, cytoskeletal protein, metal-binding protein, or small molecule.

46. The precursor RNA polynucleotide of claim 45, wherein the immune modulatory ligand comprises an interferon, cytokine, chemokine, or interleukin.

47. The precursor RNA polynucleotide of claim 45, wherein the structural protein is a channel protein or nuclear pore protein.

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48. The precursor RNA polynucleotide of claim 3, wherein the noncoding element comprises more than one noncoding element.

49. The precursor RNA polynucleotide of claim 3 or 48, wherein the noncoding element comprises 50 to 15,000 nucleotides in length.

50. The precursor RNA polynucleotide of any one of claim 1 or 2, wherein the core functional element comprises a termination sequence.

51. The precursor RNA polynucleotide of claim 50, wherein the termination sequence is located at the 5' end of the 3' enhanced exon element.

52. The precursor RNA polynucleotide of claim 50, wherein the termination sequence is a stop codon.

53. The precursor RNA polynucleotide of claim 50, wherein the termination sequence is a stop cassette.

54. The precursor RNA polynucleotide of claim 53, wherein the stop cassette comprises one or more stop codons in one or more frames.

55. The precursor RNA polynucleotide of claim 54, wherein each frame comprises a stop codon.

56. The precursor RNA polynucleotide of claim 54, wherein each frame comprises two or more stop codons.

57. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 5' enhanced intron element comprises a 3' intron fragment.

58. The precursor RNA polynucleotide of claim 57, wherein the 3' intron fragment further comprises a first or a first and a second nucleotides of a 3' group 1 intron splice site dinucleotide.

59. The precursor RNA polynucleotide of claim 58, wherein the 3' intron fragment is located at the 3' end of the 5' enhanced intron element.

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60. The precursor RNA polynucleotide of claim 58, wherein the group I intron comprises is derived from a bacterial phage, viral vector, organelle genome, nuclear rDNA gene.

61. The precursor RNA polynucleotide of claim 60, wherein the nuclear rDNA gene comprises a nuclear rDNA gene derived from a fungi, plant, or algae, or a fragment thereof.

62. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 5' enhanced intron element comprises a leading untranslated sequence located at the 5' end.

63. The precursor RNA polynucleotide of claim 62, wherein the leading untranslated sequence comprises a spacer.

64. The precursor RNA polynucleotide of claim 62, wherein the leading untranslated sequence comprises the last nucleotide of a transcription start site.

65. The precursor RNA polynucleotide of claim 64, wherein the leading untranslated sequence comprises 1 to 100 additional nucleotides.

66. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 5' enhanced intron element comprises a 5' affinity sequence.

67. The precursor RNA polynucleotide of claim 66, wherein the 5' affinity sequence comprises a polyA, polyAC, or polypyrimidine sequence.

68. The precursor RNA polynucleotide of claim 67, wherein the 5' affinity sequence comprises 10 to 100 nucleotides.

69. The precursor RNA polynucleotide of any one of claims 1-3 or 57-68, wherein the 5' enhanced intron element comprises a 5' external spacer sequence.

70. The precursor RNA polynucleotide of claim 69, wherein the 5' external spacer sequence is located between the 5' affinity sequence and the 3' intron fragment.

71. The precursor RNA polynucleotide of claim 69, wherein the 5' external spacer sequence has a length of about 6 to 60 nucleotides.

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72. The precursor RNA polynucleotide of claim 69, wherein the 5' external spacer sequence comprises or consists of a sequence selected from SEQ ID NOs: 3094-3152.

73. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 5' enhanced intron element comprises, in the following order:

a. a leading untranslated sequence;

b. a 5' affinity sequence;

c. a 5' external spacer sequence; and

d. a 3' intron fragment including the first nucleotide of a 3' Group I intron splice site;

wherein the leading untranslated sequence comprises the last nucleotide of a transcription start site and 1 to 100 nucleotides.

74. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 5' enhanced intron element comprises, in the following order

a. a leading untranslated sequence;

b. a 5' external spacer sequence;

c. a 5' affinity sequence; and

d. a 3' intron fragment including the first nucleotide of a 3' group I splice site;

wherein the leading untranslated sequence comprises the last nucleotide of a transcription start site and 1 to 100 nucleotide.

75. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 5' enhanced intron element comprises, in the following order:

a. a leading untranslated sequence;

b. a 5' affinity sequence;

c. a 5' external spacer sequence; and

d. a 3' intron fragment including the first and second nucleotides of a 3' Group I intron splice site;

wherein the leading untranslated sequence comprises the last nucleotide of a transcription start site and 1 to 100 nucleotides; and wherein the 5' enhanced exon element comprises a 3' exon fragment lacking the second nucleotide of a 3' group I splice site dinucleotide.

76. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 5' enhanced intron element comprises, in the following order:

a. a leading untranslated sequence;

b. a 5' external spacer sequence;

c. a 5' affinity sequence; and

d. a 3' intron fragment including the first and second nucleotides of a 3' Group I splice site;

wherein the leading untranslated sequence comprises the last nucleotide of a transcription start site and 1 to 100 nucleotide; and wherein the 5' enhanced exon element comprises a 3' exon fragment lacking the second nucleotide of a 3' group I splice site dinucleotide.

77. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 5' enhanced exon element comprises a 3' exon fragment.

78. The precursor RNA polynucleotide of claim 78, wherein the 3' exon fragment further comprises the second nucleotide of a 3' group I intron splice site dinucleotide.

79. The precursor RNA polynucleotide of claim 78, wherein the 3' exon fragment comprises 1 to 100 natural nucleotides derived from a natural exon.

80. The precursor RNA polynucleotide of claim 79, wherein the natural exon derived from a Group I intron containing gene or a fragment thereof.

81. The precursor RNA polynucleotide of claim 79, wherein the natural exon derived from an anabaena bacterium, T4 phage virus, twort bacteriophage, tetrahymena, or azoarcus bacterium.

82. The precursor RNA polynucleotide of any of claims 1-3, wherein the 5' enhanced exon element comprises a 5' internal spacer sequence located downstream from the 3' exon fragment.

83. The precursor RNA polynucleotide of claim 82, wherein the 5' internal spacer sequence is about 6 to 60 nucleotides in length.

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84. The precursor RNA polynucleotide of claim 83, wherein the 5' internal spacer sequence comprises or consists of a sequence selected from SEQ ID NOs: 3094-3152.

85. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 5' enhanced exon element comprises in the following order:

a. a 3' exon fragment including the second nucleotide of a 3' group I intron splice site dinucleotide; and

b. a 5' internal spacer sequence,

wherein the 3' exon fragment comprises 1 to 100 natural nucleotides derived from a natural exon.

86. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 5' enhanced exon element comprises in the following order:

a. a 3' exon fragment; and

b. a 5' internal spacer sequence,

wherein the 3' exon fragment comprises 1 to 100 natural nucleotides derived from a natural exon; and wherein the 5' enhanced intron element comprises a 3' intron fragment comprising the first and second nucleotides of a 3' group I splice site dinucleotide.

87. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 3' enhanced exon element comprises a 5' exon fragment.

88. The precursor RNA polynucleotide of claim 87, wherein the 5' exon fragment comprises the first nucleotide of a 5' group I intron fragment.

89. The precursor RNA polynucleotide of claim 87, wherein the 5' exon fragment further comprises 1 to 100 nucleotides derived from a natural exon.

90. The precursor RNA polynucleotide of claim 87, wherein the natural exon is derived from a Group I intron containing gene or a fragment thereof.

91. The precursor RNA polynucleotide of any one of claims 1-3 or 87, wherein the 3' enhanced exon element comprises a 3' internal spacer sequence.

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92. The precursor RNA polynucleotide of claim 91, wherein the 3' internal spacer sequence is located between the termination sequence and the 5' exon fragment.

93. The precursor RNA polynucleotide of claim 91, wherein the 3' internal spacer is about 6 to 60 nucleotides in length.

94. The precursor RNA polynucleotide of any one of claims 91, wherein the 3' internal spacer comprises or consists of a sequence selected from SEQ ID NOs: 3094-3152.

95. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 3' enhanced exon element comprises:

a. a 3' internal spacer sequence; and

b. a 5' exon fragment including the first nucleotide of a 5' group I intron splice site dinucleotide,

wherein the 5' exon fragment comprises 1 to 100 nucleotides derived from a natural exon.

96. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 3' enhanced exon element comprises:

a. a 3' internal spacer sequence; and

b. a 5' exon fragment,

wherein the 5' exon fragment comprises 1 to 100 nucleotides derived from a natural exon; wherein the 3' enhanced intron element comprises a 5' intron fragment comprising the first and second nucleotide of a 5' group I intron splice site dinucleotide.

97. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 3' enhanced intron element comprises a 5' intron fragment.

98. The precursor RNA polynucleotide of claim 97, wherein the 5' intron fragment comprises a second nucleotide of a 5' group I intron splice site dinucleotide.

99. The precursor RNA polynucleotide of any one of claims 1-3 or 96-98, wherein the 3' enhanced intron element comprises a trailing untranslated sequence located at the 3' end of the 5' intron.

100. The precursor RNA polynucleotide of claim 99, wherein the trailing untranslated sequence comprises 3 to12 nucleotides.

101. The precursor RNA polynucleotide of any of claims 1-3 or 96-100, wherein the 3' enhanced intron fragment comprises a 3' external spacer sequence.

102. The precursor RNA polynucleotide of claim 101, wherein the 3' external spacer sequence is located between the 5' intron fragment and trailing untranslated sequence.

103. The precursor RNA polynucleotide of claim 101, wherein the 3' external spacer sequence has a length of 6 to 60 nucleotides in length.

104. The precursor RNA polynucleotide of any of claims 101, wherein the 3' external spacer sequence comprises or consists of a sequence selected SEQ ID NOs: 3094-3152.

105. The precursor RNA polynucleotide of any of claims 1-3, wherein the 3' enhanced intron element comprises a 3' affinity sequence.

106. The precursor RNA polynucleotide of claim 105, wherein the 3' affinity sequence is located between the 3' external spacer sequence and the trailing untranslated sequence.

107. The precursor RNA polynucleotide of claim 105, wherein the 3' affinity sequence comprises a polyA, poly AC, or polypyrimidine sequence.

108. The precursor RNA polynucleotide of claim 105, wherein the affinity sequence comprises 10 to 100 nucleotides.

109. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 5' enhanced intron element further comprises a 5' external duplex sequence; wherein the 3' enhanced intron element further comprises a 3' external duplex sequence.

110. The precursor RNA polynucleotide of claim 109, wherein the 5' external duplex sequence and 3' external duplex sequence are fully or partially complementary to each other.

111. The precursor RNA polynucleotide of claim 109, wherein the 5' external duplex sequence comprises fully synthetic or partially synthetic nucleotides.

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112. The precursor RNA polynucleotide of claim 109, wherein the 3' external duplex sequence comprises fully synthetic or partially synthetic nucleotides.

113. The precursor RNA polynucleotide of claim 109, wherein the 3' external duplex sequence is about 6 to about 50 nucleotides.

114. The precursor RNA polynucleotide of claim 109, wherein the 5' external duplex sequence is about 6 to about 50 nucleotides.

115. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 5' enhanced exon element further comprises a 5' internal duplex sequence; wherein the 3' enhanced exon element further comprises a 3' internal duplex sequence.

116. The precursor RNA polynucleotide of claim 115, wherein the 5' internal duplex sequence and 3' internal duplex sequence are fully or partially complementary to each other.

117. The precursor RNA polynucleotide of claim 115, wherein the 5' internal duplex sequence comprises fully synthetic or partially synthetic nucleotides.

118. The precursor RNA polynucleotide of claim 115, wherein the 3' internal duplex sequence comprises fully synthetic or partially synthetic nucleotides.

119. The precursor RNA polynucleotide of claim 115, wherein the 3' internal duplex sequence is about 6 to about 19 nucleotides.

120. The precursor RNA polynucleotide of claim 115, wherein the 5' internal duplex sequence is about 6 to about 19 nucleotides.

121. The precursor RNA polynucleotide of any one of claims 1-3, wherein the 3' enhanced intron fragment comprises in the following order:

a. a 5' intron fragment including the second nucleotide of a 5' group I intron splice site dinucleotide;
b. a 3' external spacer sequence; and
c. a 3' affinity sequence

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122. The precursor RNA polynucleotide of anyone of claims 1-3, wherein the 3' enhanced intron fragment comprises in the following order:

a. a 5' intron fragment including the first and second nucleotide of a 5' group I intron splice site dinucleotide;

b. a 3' external spacer sequence; and

c. a 3' affinity sequence

wherein the 3' enhanced exon element comprises a 5' exon fragment lacking the first nucleotide of a 5' group I intron splice site dinucleotide.

123. The precursor RNA polynucleotide of claim 1 or 2, comprising in the following order:

a. a leading untranslated sequence;

b. a 5' affinity sequence;

c. 5' external duplex sequence;

d. 5' spacer sequence;

e. 3' intron fragment;

f. 3' exon fragment;

g. 5' internal duplex sequence

h. 5' internal spacer sequence;

i. a translation initiation element;

j. a coding element;

k. a termination sequence;

l. a 3' internal spacer sequence;

m. a 3' internal duplex sequence;

n. a 5' exon fragment;

o. a 5' intron fragment;

p. a 3' external duplex sequence;

q. a 3' affinity sequence; and

r. a trailing untranslated sequence.

124. The precursor RNA polynucleotide of 3, comprising in the following order:

a. a leading untranslated sequence;

b. a 5' affinity sequence;

c. a 5' external spacer sequence;
d. a 3' intron fragment;
e. a 3' exon fragment;
f. a 5' internal duplex sequence;
g. a 5' internal spacer sequence;
h. a noncoding element;
i. a 3' internal spacer sequence;
j. a 3' internal duplex sequence;
k. a 5' exon fragment;
l. a 5' intron fragment;
m. a 3' external spacer sequence;
n. a 3' affinity sequence; and
o. a trailing untranslated sequence.

125. The precursor RNA polynucleotide of claim 1 or 2, comprising in the following order:
a. a leading untranslated sequence;
b. a 5' affinity sequence;
c. a 5' external spacer sequence;
d. a 3' intron fragment;
e. a 3' exon fragment;
f. a 5' internal duplex sequence;
g. a 5' internal spacer sequence;
h. a translation initiation element;
i. a coding element;
j. a termination sequence;
k. a 3' internal spacer sequence;
l. a 3' internal duplex sequence;
m. a 5' exon fragment;
n. a 5' intron fragment;
o. a 3' external spacer sequence; and
p. a 3' affinity sequence.

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126. The precursor RNA polynucleotide of claim 1 or 2, comprising in the following order:

a. a leading untranslated sequence;
b. a 5' affinity sequence;
c. a 5' external spacer sequence;
d. a 3' intron fragment;
e. a 3' exon fragment;
f. a 5' internal spacer sequence;
g. a translation initiation element;
h. a coding element;
i. a termination sequence;
j. a 3' internal spacer sequence;
k. a 5' exon fragment;
l. a 5' intron fragment;
m. a 3' external spacer sequence; and
n. a 3' affinity sequence.

127. The precursor RNA polynucleotide of 3, comprising in the following order:

a. a leading untranslated sequence;
b. a 5' affinity sequence;
c. a 5' external spacer sequence;
d. a 3' intron fragment;
e. a 3' exon fragment;
f. a 5' internal spacer sequence;
g. a noncoding element;
h. a 3' internal spacer sequence;
i. a 5' exon fragment;
j. a 5' intron fragment;
k. a 3' external spacer sequence;
l. a 3' affinity sequence; and
m. a trailing untranslated sequence.

128. The precursor RNA polynucleotide of claim 3, comprising the following order,:

a. a leading untranslated sequence;
b. a 5' affinity sequence;
c. 5' external duplex sequence;
d. 5' spacer sequence;
e. 3' intron fragment;
f. 3' exon fragment;
g. 5' internal duplex sequence
h. 5' internal spacer sequence;
i. a termination sequence;
j. a coding element;
k. a translation initiation element;
l. a 3' internal spacer sequence;
m. a 3' internal duplex sequence;
n. a 5' exon fragment;
o. a 5' intron fragment;
p. a 3' external duplex sequence;
q. a 3' affinity sequence; and
r. a trailing untranslated sequence.

129. The precursor RNA polynucleotide of claim 1 or 2, wherein the coding element comprises two or more protein coding regions.

130. The precursor RNA polynucleotide of claim 129, comprising a polynucleotide sequence encoding a proteolytic cleavage site or a ribosomal stuttering element between the first and second expression sequence.

131. The precursor RNA polynucleotide of claim 130, wherein the ribosomal stuttering element is a self-cleaving spacer.

132. The precursor RNA polynucleotide of claim 129, comprising a polynucleotide sequence encoding 2A ribosomal stuttering peptide.

133. A circular RNA polynucleotide produced from the precursor RNA polynucleotide of any one of claims 1-8.

134. The circular RNA polynucleotide of claim 133, consisting of natural nucleotides.

135. The circular RNA polynucleotide of any one of claims 134, wherein the protein coding or non-coding sequence is codon optimized.

136. The circular RNA polynucleotide of any one of claims 133-135, wherein the circular RNA polynucleotide is from about 0.1 to about 15 kilobases in length.

137. The circular RNA polynucleotide of any one of claims 133-136, optimized to lack at least one microRNA binding site present in an equivalent pre-optimized polynucleotide.

138. The circular RNA polynucleotide of any one of claims 133-137, optimized to lack at least one RNA-editing susceptible site present in an equivalent pre-optimized polynucleotide.

139. The circular RNA polynucleotide of any one of claims 133-138, having an *in vivo* duration of therapeutic effect in humans of at least 20 hours.

140. The circular RNA polynucleotide of any one of claims 133-139, having a functional half-life of at least 6 hours.

141. The circular RNA polynucleotide of claims 133-140, having a duration of therapeutic effect in a human cell greater than or equal to that of an equivalent linear RNA polynucleotide comprising the same expression sequence.

142. The circular RNA polynucleotide of claims 133-141, having an *in vivo* duration of therapeutic effect in human greater than that of an equivalent linear RNA polynucleotide having the same expression sequence.

143. The circular RNA polynucleotide of any one of claims 133-142, wherein the precursor RNA polynucleotide is transcribed from a vector or DNA comprising a PCR product, a linearized plasmid, non-linearized plasmid, linearized minicircle, a non-linearized minicircle, viral vector, cosmid, ceDNA, or an artificial chromosome.

144. A method of making a translation initiation element (TIE) comprising:
    a. obtaining a viral untranslated region (UTR);
    b. determining the functional unit of the UTR capable of binding to an initiation factor and/or initiating translation by progressively deleting sequence;
    c. removing non-functional units of the UTR; and
    d. optionally, modifying the ends of the UTR.

145. The method of claim 144, wherein the modification of the ends of the UTR is about 1 percent to 75% of the viral UTR.

146. The method of claim 144 or 145, wherein the functional unit of UTR is determined by deletion scanning from the 5' and 3' ends of the UTR or mutational scanning across the length of the UTR to identify important regions.

147. A pharmaceutical composition comprising a circular RNA polynucleotide of any one of claims 133-143, a nanoparticle, and optionally, a targeting moiety operably connected to the nanoparticle.

148. The pharmaceutical composition of claim 147, wherein the nanoparticle is a lipid nanoparticle, a core-shell nanoparticle, a biodegradable nanoparticle, a biodegradable lipid nanoparticle, a polymer nanoparticle, a polyplex or a biodegradable polymer nanoparticle.

149. The pharmaceutical composition of claim 147 or 148, comprising a targeting moiety, wherein the targeting moiety mediates receptor-mediated endocytosis, endosome fusion, or direct fusion into selected cells of a selected cell population or tissue in the absence of cell isolation or purification.

150. The pharmaceutical composition of any one of claims 147-149, comprising a targeting moiety operably connected to the nanoparticle.

151. The pharmaceutical composition of any one of claims 147-150, wherein the targeting moiety is a small molecule, scFv, nanobody, peptide, cyclic peptide, di or tri cyclic peptide, minibody, polynucleotide aptamer, engineered scaffold protein, heavy chain variable region, light chain variable region, or a fragment thereof.

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152. The pharmaceutical composition of any one of claims 147-151, wherein less than 1%, by weight, of the polynucleotides in the composition are double stranded RNA, DNA splints, DNA template, or triphosphorylated RNA.

153. The pharmaceutical composition of any one of claims 147-152, wherein less than 1%, by weight, of the polynucleotides and proteins in the pharmaceutical composition are double stranded RNA, DNA splints, DNA template, triphosphorylated RNA, phosphatase proteins, protein ligases, RNA polymerases, and capping enzymes.

154. A pharmaceutical composition comprising a circular RNA polynucleotide of any one of claims 133-143 and a liposome, dendrimer, carbohydrate carrier, glycan nanomaterial, fusome, exosome, or a combination thereof.

155. A pharmaceutical composition comprising a circular RNA polynucleotide of any one of claims 133-143 and a pharmaceutical salt, buffer, diluent or combination thereof.

156. A method of treating a subject in need thereof comprising administering a therapeutically effective amount of a composition comprising the circular RNA polynucleotide of any one of claims 147-155, a nanoparticle, and optionally, a targeting moiety operably connected to the nanoparticle.

157. The method of claim 156, wherein the targeting moiety is a small molecule, scFv, nanobody, peptide, cyclic peptide, di or tri cyclic peptide, minibody, heavy chain variable region, engineered scaffold protein, light chain variable region or fragment thereof.

158. The method of any one of claims 156-157, wherein the nanoparticle is a lipid nanoparticle, a core-shell nanoparticle, or a biodegradable nanoparticle.

159. The method of any one of claims 156-158, wherein the nanoparticle comprises one or more cationic lipids, ionizable lipids, or poly β-amino esters.

160. The method of any one of claims 156-159, wherein the nanoparticle comprises one or more non-cationic lipids.

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161. The method of any one of claims 156-160, wherein the nanoparticle comprises one or more PEG-modified lipids, polyglutamic acid lipids, or hyaluronic acid lipids.

162. The method of any one of claims 156-161, wherein the nanoparticle comprises cholesterol.

163. The method of any one of claims 156-162, wherein the nanoparticle comprises arachidonic acid, leukotriene, or oleic acid.

164. The method of any one of claims 156-163, wherein the composition comprises a targeting moiety, wherein the targeting moiety mediates receptor-mediated endocytosis selectively into cells of a selected cell population in the absence of cell selection or purification.

165. The method of any one of claims 156-164, wherein the nanoparticle comprises more than one circular RNA polynucleotide.

166. The method of any one of claims 156-165, wherein the subject has a cancer selected from the group consisting of: acute myeloid leukemia (AML); alveolar rhabdomyosarcoma; B cell malignancies; bladder cancer (e.g., bladder carcinoma); bone cancer; brain cancer (e.g., medulloblastoma and glioblastoma multiforme); breast cancer; cancer of the anus, anal canal, or anorectum; cancer of the eye; cancer of the intrahepatic bile duct; cancer of the joints; cancer of the neck; gallbladder cancer; cancer of the pleura; cancer of the nose, nasal cavity, or middle ear; cancer of the oral cavity; cancer of the vulva; chronic lymphocytic leukemia; chronic myeloid cancer; colon cancer; esophageal cancer, cervical cancer; fibrosarcoma; gastrointestinal carcinoid tumor; head and neck cancer (e.g., head and neck squamous cell carcinoma); Hodgkin lymphoma; hypopharynx cancer; kidney cancer; larynx cancer; leukemia; liquid tumors; lipoma; liver cancer; lung cancer (e.g., non-small cell lung carcinoma, lung adenocarcinoma, and small cell lung carcinoma); lymphoma; mesothelioma; mastocytoma; melanoma; multiple myeloma; nasopharynx cancer; non-Hodgkin lymphoma; B-chronic lymphocytic leukemia; hairy cell leukemia; Burkitt's lymphoma; ovarian cancer; pancreatic cancer; cancer of the peritoneum; cancer of the omentum; mesentery cancer; pharynx cancer; prostate cancer; rectal cancer; renal cancer; skin cancer; small intestine cancer; soft tissue cancer; solid tumors; synovial sarcoma; gastric cancer; teratoma; testicular cancer; thyroid cancer; and ureter cancer.

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167. The method of any one of claims 156-166, wherein the subject has an autoimmune disorder selected from scleroderma, Grave's disease, Crohn's disease, Sjogren's disease, multiple sclerosis, Hashimoto's disease, psoriasis, myasthenia gravis, autoimmune polyendocrinopathy syndromes, Type I diabetes mellitus (TIDM), autoimmune gastritis, autoimmune uveoretinitis, polymyositis, colitis, thyroiditis, and the generalized autoimmune diseases typified by human Lupus.

168. A eukaryotic cell comprising a circular RNA polynucleotide according to any of claims 133-143 or the pharmaceutical composition of any one of claims 147-155.

169. The eukaryotic cell of claim 168, wherein the eukaryotic cell is a human cell.

170. The eukaryotic cell of claim 169, wherein the eukaryotic cell is an immune cell.

171. The eukaryotic cell of claim 170, wherein the eukaryotic cell is a T cell, dendritic cell, macrophage, B cell, neutrophil, or basophil.

172. A prokaryotic cell comprising a circular RNA polynucleotide according to any of claims 133-143.

173. A method of purifying circular RNA, comprising hybridizing an oligonucleotide conjugated to a solid surface with an affinity sequence.

174. The method of claim 173, wherein one or more copies of the affinity sequence is present in a precursor RNA.

175. The method of claim 174, wherein the precursor RNA is the precursor RNA of any one of claims 44-54, 83-86, or 103-110.

176. The method of any one of claims 173-175, wherein the circular RNA is the circular RNA of any one of claims 117-127.

177. The method of any one of claims 173-176, wherein the affinity sequence is removed during formation of the circular RNA.

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178. The method of any one of claims 173-177, comprising separating the circular RNA from the precursor RNA.

179. The method of any one of claims 173-178, wherein the affinity sequence comprises a polyA sequence.

180. The method of claim 179, wherein the oligonucleotide that hybridizes to the affinity sequence is a deoxythymidine oligonucleotide.

181. The method of any one of claims 173-178, wherein the affinity sequence comprises a dedicated binding site (DBS).

182. The method of claim 181, wherein the DBS comprises the nucleotide sequence of: TATAATTCTACCCTATTGAGGCATTGACTA (SEQ ID NO: 3269).

183. The method of claim 181or 182, wherein the oligonucleotide that hybridizes to the affinity sequence comprises a sequence complementary to the DBS.

184. A method of purifying circular RNA comprising:
   a. contacting a composition comprising linear RNA and circular RNA with a binding agent that preferentially binds to the linear RNA over the circular RNA; and
   b. separating RNA bound to the binding agent from RNA that is not bound to the binding agent.

185. The method of claim 184, wherein the binding agent is conjugated to a solid support.

186. The method of claim 185, wherein the solid support comprises agarose, an agarose-derived resin, cellulose, a cellulose fiber, a magnetic bead, a high throughput microtiter plate, a non-agarose resin, a glass surface, a polymer surface, or a combination thereof.

187. The method of claim 185 or 186, wherein the solid support comprises agarose or cellulose.

188. The method of any one of claims 184-187, wherein the binding agent comprises an oligonucleotide that is complementary to a sequence present in the linear RNA and absent from the circular RNA.

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189. The method of any one of claims 184-188, wherein the binding agent comprises an oligonucleotide that is 100% complementary to a sequence present in the linear RNA and absent from the circular RNA.

190. The method of claim 188 or 189, wherein the sequence present in the linear RNA and absent from the circular RNA is an affinity sequence.

191. The method of any one of claims 188-190, wherein the sequence present in the linear RNA and absent from the circular RNA comprises a polyA sequence.

192. The method of any one of claims 184-191, wherein the binding agent comprises an oligonucleotide comprising a poly-deoxythymidine sequence.

193. The method of any one of claims 188-192, wherein the sequence present in the linear RNA and absent from the circular RNA comprises a DBS sequence.

194. The method of claim 193, wherein the DBS sequence comprises the nucleotide sequence of: TATAATTCTACCCTATTGAGGCATTGACTA (SEQ ID NO: 3269).

195. The method of any one of claims 188-194, wherein the sequence present in the linear RNA and absent from the circular RNA is 10-150 nucleotides in length.

196. The method of any one of claims 188-194, wherein the sequence present in the linear RNA and absent from the circular RNA is 10-70 nucleotides in length.

197. The method of any one of claims 188-194, wherein the sequence present in the linear RNA and absent from the circular RNA is 20-30 nucleotides in length.

198. The method of any one of claims 188-197, wherein the sequence present in the linear RNA and absent from the circular RNA is present at two locations in the linear RNA.

199. The method of any one of claims 188-198, wherein the sequence present in the linear RNA and absent from the circular RNA is encoded into the linear RNA during transcription of the linear RNA.

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200. The method of any one of claims 188-198, wherein the sequence present in the linear RNA and absent from the circular RNA is enzymatically added to the linear RNA.

201. The method of any one of claims 184-200, wherein the linear RNA does not comprise a methylguanylate cap.

202. The method of any one of claims 184-201, wherein the linear RNA comprises a precursor RNA or a fragment thereof.

203. The method of claim 202, wherein the precursor RNA is the precursor RNA of any one of claims 44-54, 83-86, or 103-110 or a fragment thereof.

204. The method of claim 202or 203, wherein the precursor RNA is produced using in vitro transcription (IVT).

205. The method of claim 202or 203, wherein the fragment comprises an intron.

206. The method of claim 204 or 205, wherein the linear RNA comprises a prematurely terminated RNA or RNA formed by abortive transcription.

207. The method of any one of claims 184-206, wherein the circular RNA comprises the circular RNA of any one of claims 117-127.

208. The method of any one of claims 184-207, wherein the circular RNA is produced using a method comprising splicing the precursor RNA.

209. The method of claim 208, wherein the sequence present in the linear RNA and absent from the circular RNA is excised during the splicing.

210. The method of any one of claims 184-209, wherein the circular RNA is less than 6 kilobases in size.

211. The method of any one of claims 184-210, wherein the separating comprises removing the unbound RNA from the solid support.

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212. The method of claim 211, wherein the removing comprises eluting the unbound RNA from the solid support.

213. The method of any one of claims 184-212, comprising heating the composition.

214. The method of any one of claims 184-213, comprising buffer exchange.

215. The method of claim 214, wherein buffer exchange is performed before the contacting.

216. The method of claim 214 or 215, wherein buffer exchange is performed after the separating.

217. The method of any one of claims 214-216, wherein buffer exchange is performed before the contacting, and the resulting buffer comprises greater than 1 mM monovalent salt.

218. The method of claim 217, wherein the monovalent salt is NaCl or KCl.

219. The method of claim 217 or 218, wherein the resulting buffer comprises Tris.

220. The method of any one of claims 217-219, wherein the resulting buffer comprises EDTA.

221. The method of any one of claims 214-220, wherein buffer exchange is performed after the separating into storage buffer, wherein the storage buffer comprises 1mM sodium citrate, pH 6.5.

222. The method of any one of claims 184-221, comprising filtering the circular RNA after the separating.

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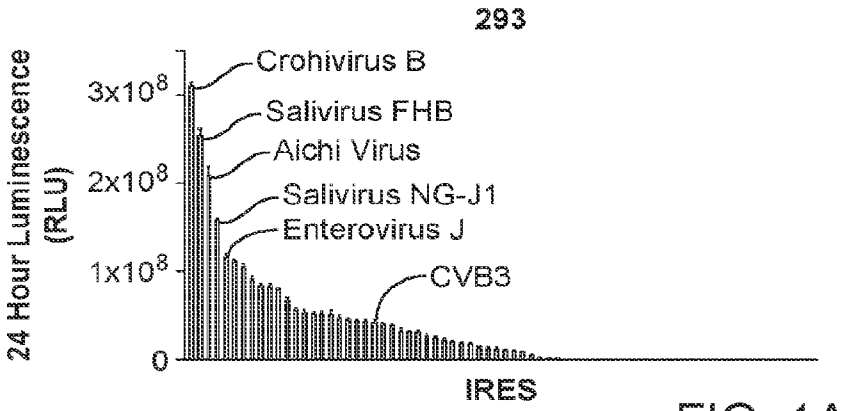

FIG. 1A

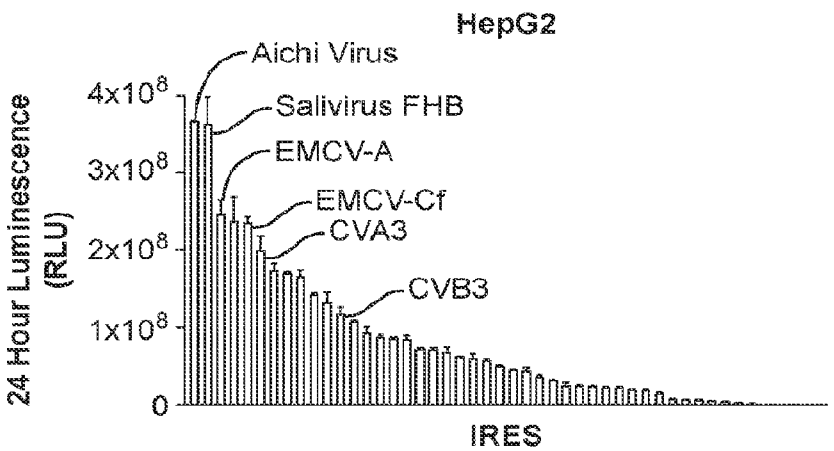

FIG. 1B

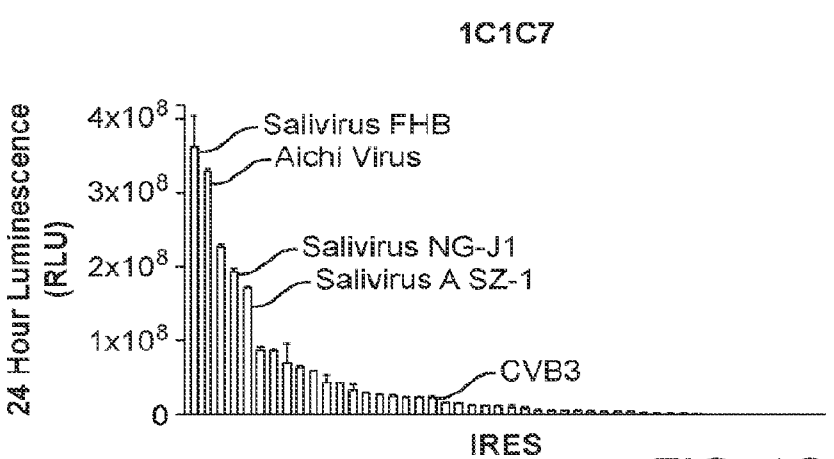

FIG. 1C

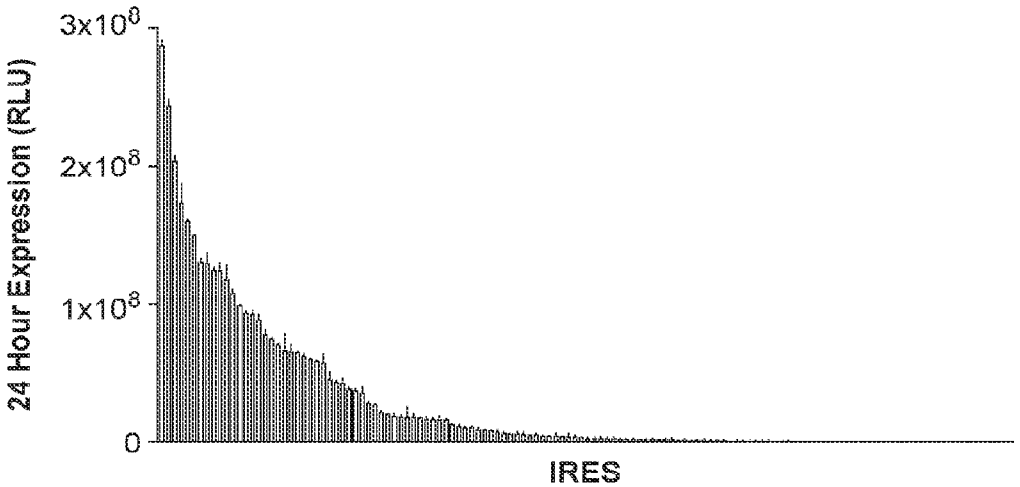
3x10^8
2x10^8
1x10^8
0
24 Hour Expression (RLU)
IRES

FIG. 1D

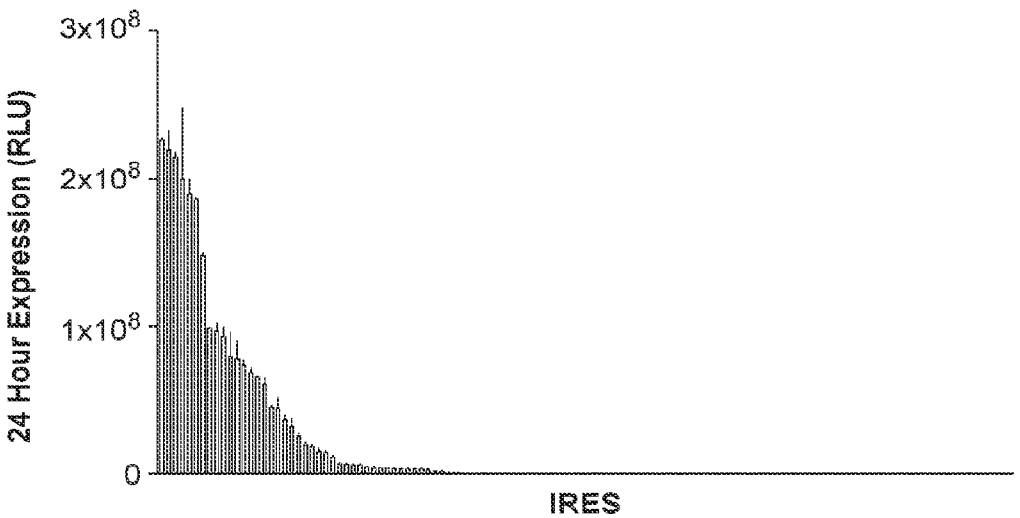
3x10^8
2x10^8
1x10^8
0
24 Hour Expression (RLU)
IRES

FIG. 1E

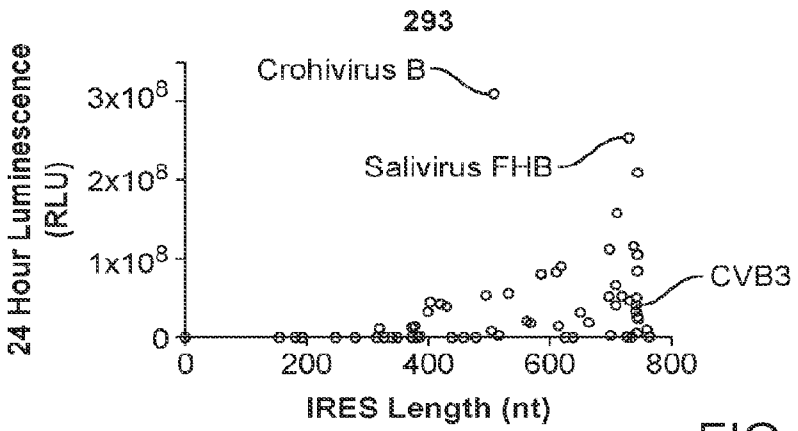

FIG. 2A

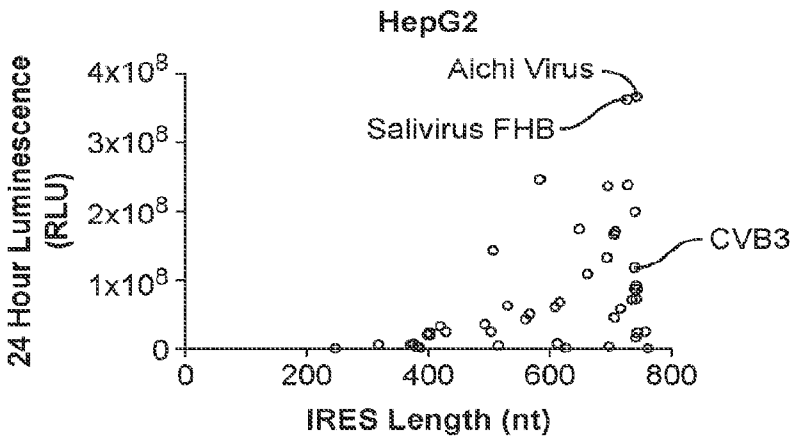

FIG. 2B

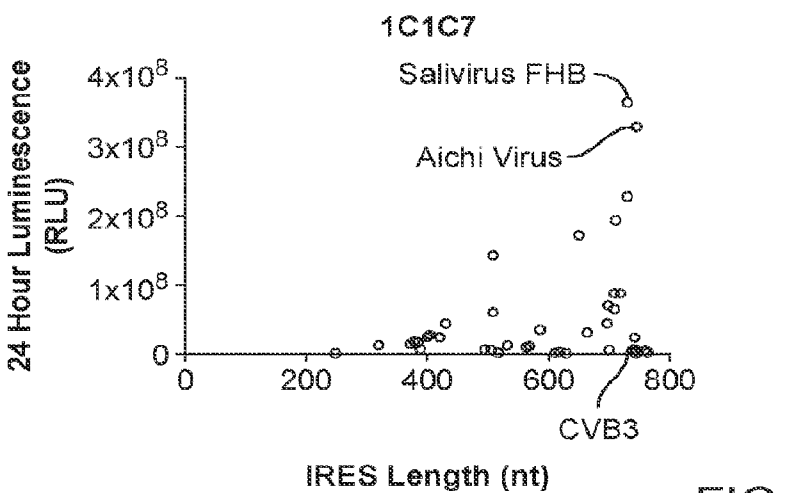

FIG. 2C

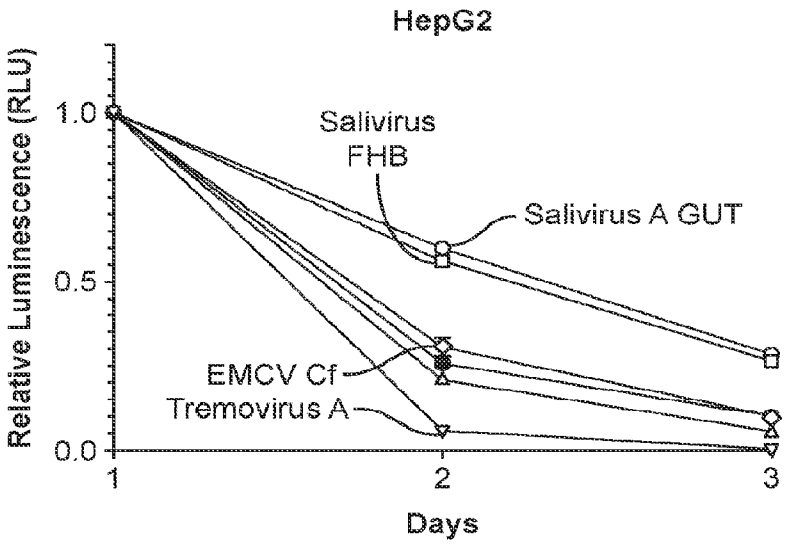
HepG2
Relative Luminescence (RLU)
1.0
0.5
0.0
Salivirus FHB
Salivirus A GUT
EMCV Cf
Tremovirus A
1
2
3
Days

FIG. 3A

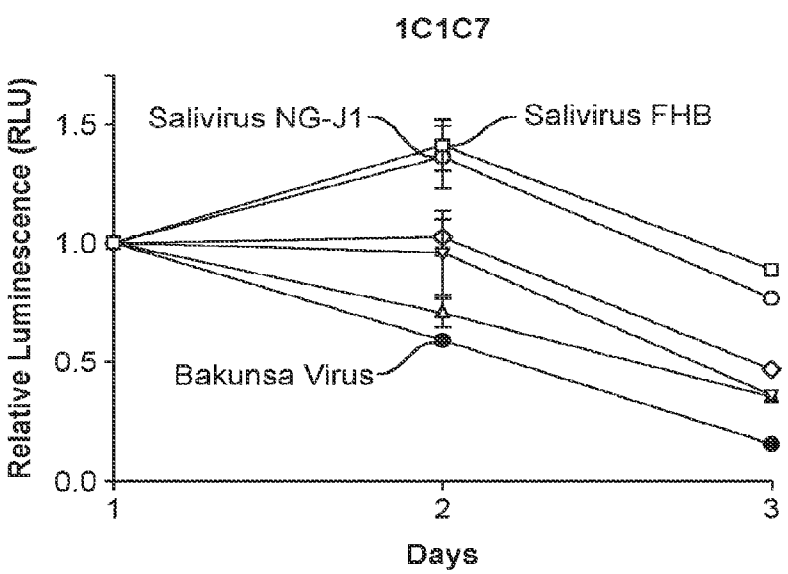
1C1C7
Relative Luminescence (RLU)
1.5
1.0
0.5
0.0
Salivirus NG-J1
Salivirus FHB
Bakunsa Virus
1
2
3
Days

FIG. 3B

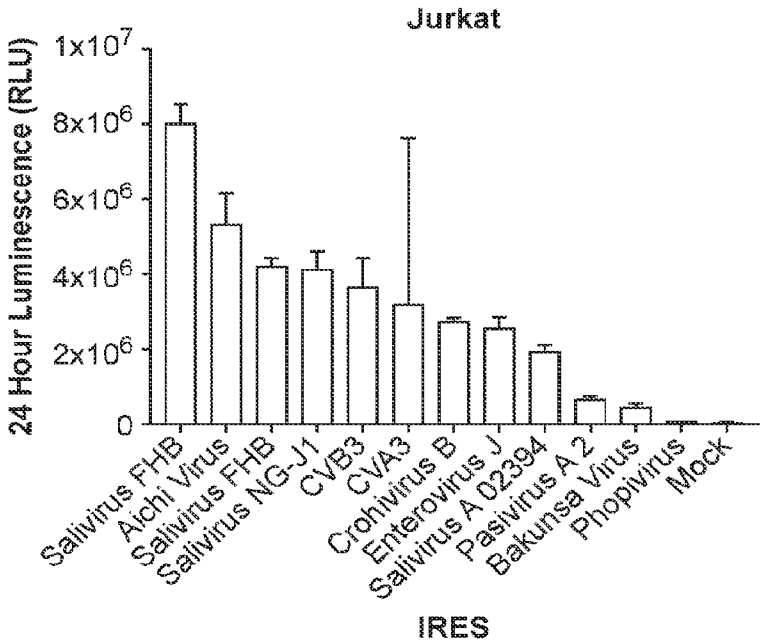
Jurkat
24 Hour Luminescence (RLU)
1x10^7
8x10^6
6x10^6
4x10^6
2x10^6
0
Salivirus FHB
Aichi Virus
Salivirus FHB
Salivirus NG-J1
CVB3
CVA3
Crohivirus B
Enterovirus J
Salivirus A 02394
Pasivirus A 2
Bakunsa Virus
Phopivirus
Mock
IRES

FIG. 4A

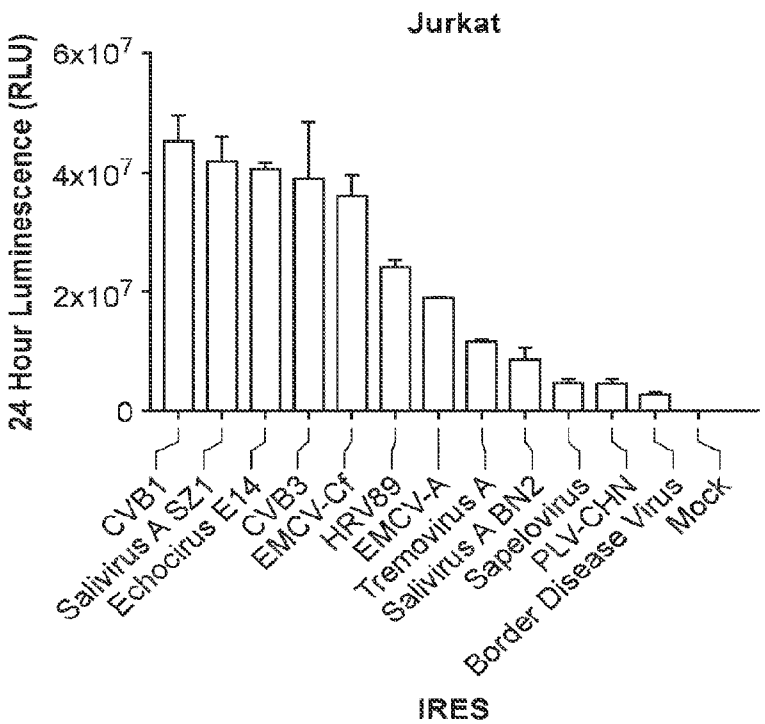
Jurkat
24 Hour Luminescence (RLU)
6x10^7
4x10^7
2x10^7
0
CVB1
Salivirus A SZ1
Echocirus E14
CVB3
EMCV-Cf
HRV89
EMCV-A
Tremovirus A
Salivirus A BN2
Sapelovirus
PLV-CHN
Border Disease Virus
Mock
IRES

FIG. 4B

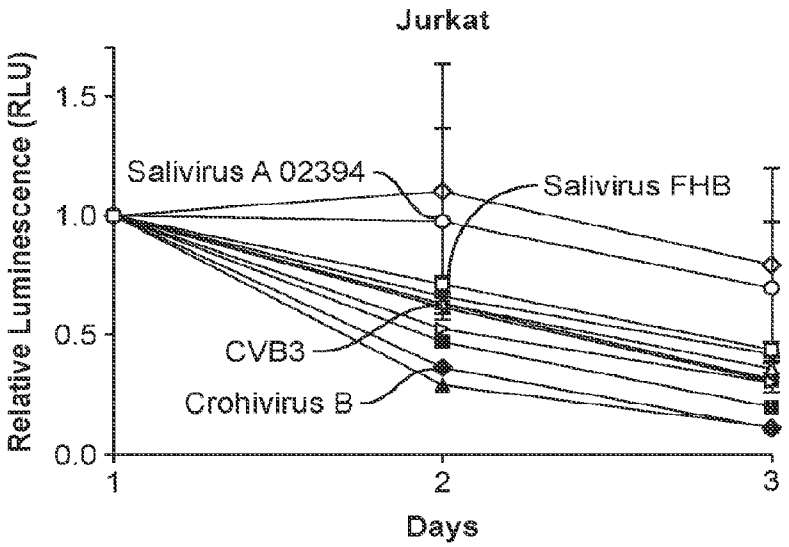
Jurkat
Relative Luminescence (RLU)
1.5
1.0
0.5
0.0
Salivirus A 02394
Salivirus FHB
CVB3
Crohivirus B
1
2
3
Days

FIG. 5A

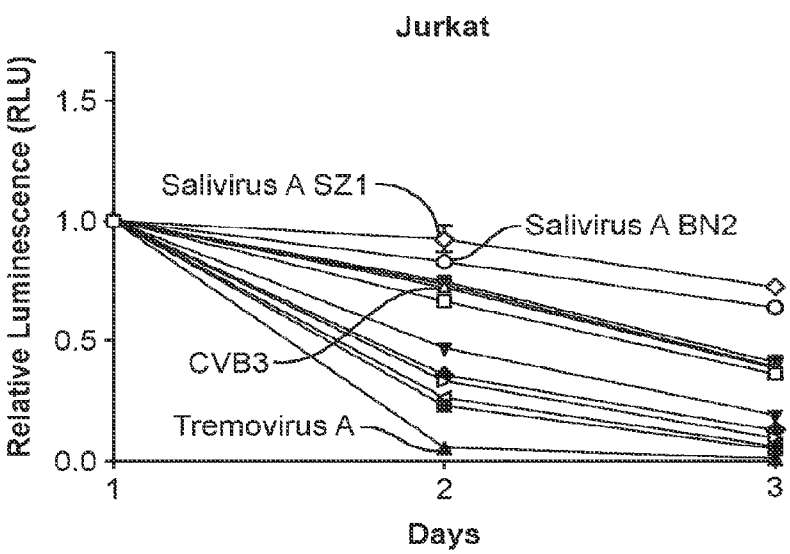
Jurkat
Relative Luminescence (RLU)
1.5
1.0
0.5
0.0
Salivirus A SZ1
Salivirus A BN2
CVB3
Tremovirus A
1
2
3
Days

FIG. 5B

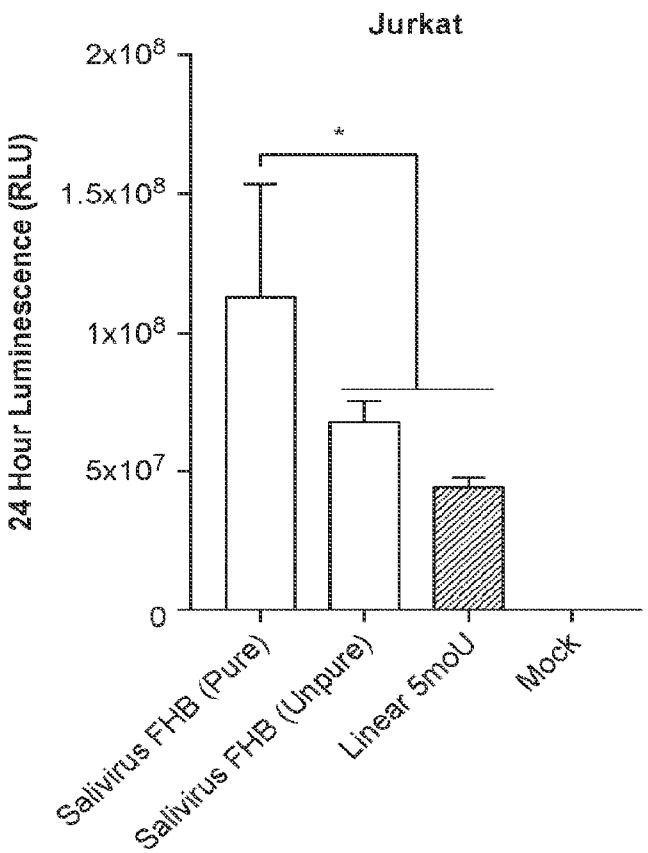
Jurkat
24 Hour Luminescence (RLU)
2x10^8
1.5x10^8
1x10^8
5x10^7
0
*
Salivirus FHB (Pure)
Salivirus FHB (Unpure)
Linear 5moU
Mock

FIG. 6A

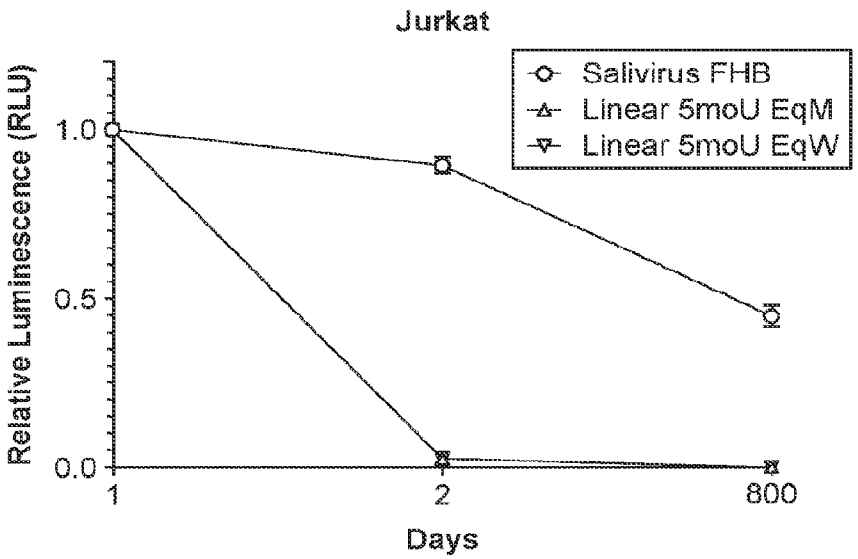
Jurkat
Salivirus FHB
Linear 5moU EqM
Linear 5moU EqW
Relative Luminescence (RLU)
1.0
0.5
0.0
1
2
800
Days

FIG. 6B

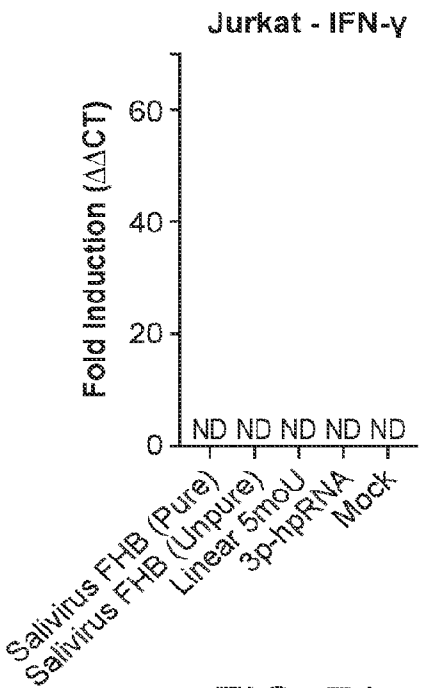
Jurkat - IFN-γ
Fold Induction (ΔΔCT)
60
40
20
0
ND ND ND ND ND
Salivirus FHB (Pure)
Salivirus FHB (Unpure)
Linear 5moU
3p-hpRNA
Mock

FIG. 7A

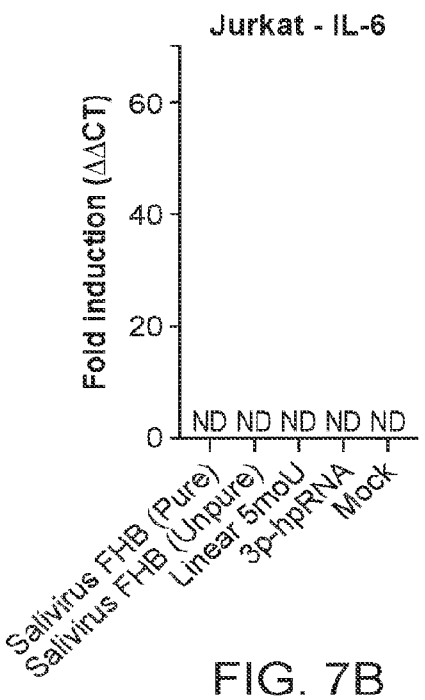
Jurkat - IL-6
Fold Induction (ΔΔCT)
60
40
20
0
ND ND ND ND ND
Salivirus FHB (Pure)
Salivirus FHB (Unpure)
Linear 5moU
3p-hpRNA
Mock

FIG. 7B

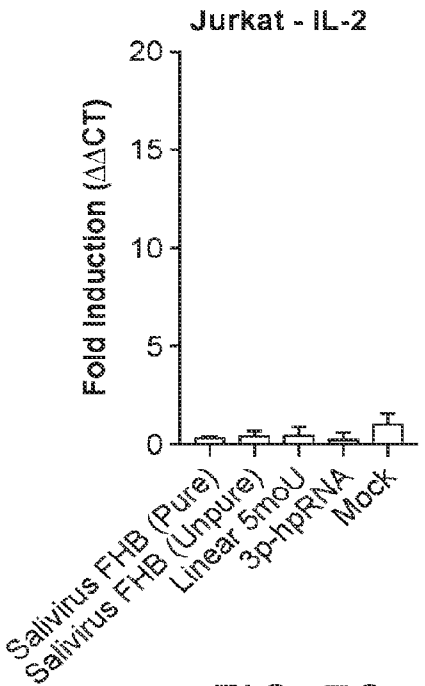
Jurkat - IL-2
Fold Induction (ΔΔCT)
20
15
10
5
0
Salivirus FHB (Pure)
Salivirus FHB (Unpure)
Linear 5moU
3p-hpRNA
Mock

FIG. 7C

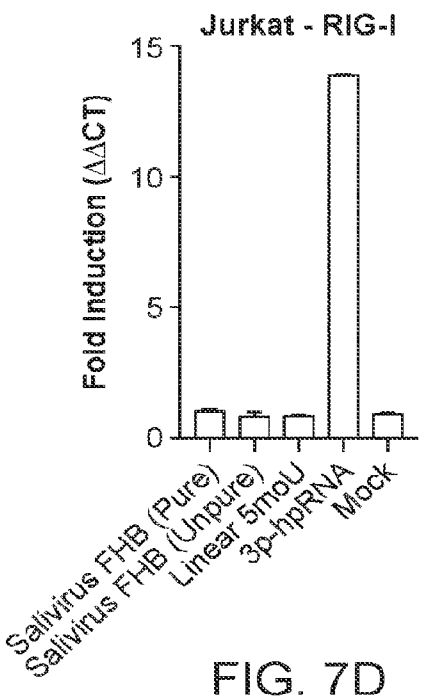
Jurkat - RIG-I
Fold Induction (ΔΔCT)
15
10
5
0
Salivirus FHB (Pure)
Salivirus FHB (Unpure)
Linear 5moU
3p-hpRNA
Mock

FIG. 7D

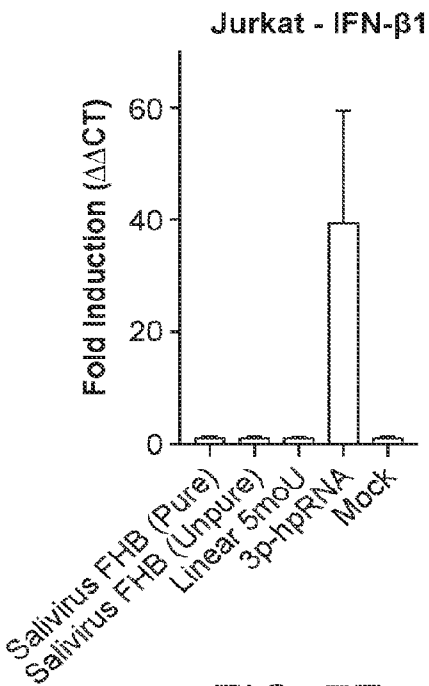
Jurkat - IFN-β1
Fold Induction (ΔΔCT)
60
40
20
0
Salivirus FHB (Pure)
Salivirus FHB (Unpure)
Linear 5moU
3p-hpRNA
Mock

FIG. 7E

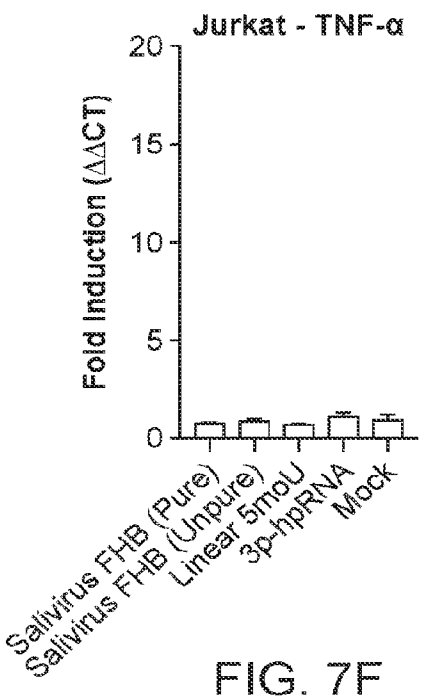
Jurkat - TNF-α
Fold Induction (ΔΔCT)
20
15
10
5
0
Salivirus FHB (Pure)
Salivirus FHB (Unpure)
Linear 5moU
3p-hpRNA
Mock

FIG. 7F

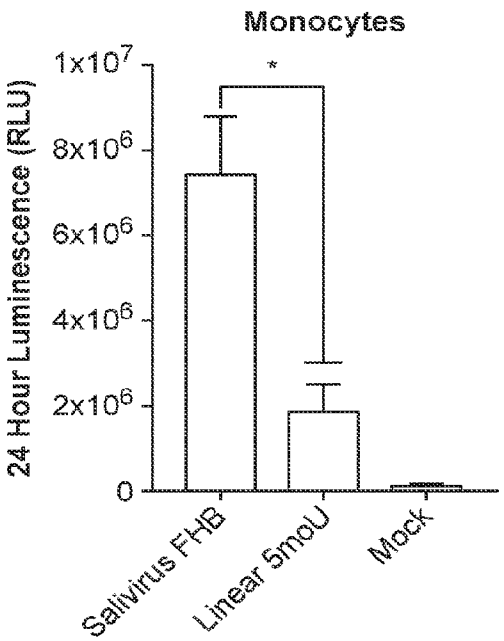
Monocytes
24 Hour Luminescence (RLU)
1x10^7
8x10^6
6x10^6
4x10^6
2x10^6
0
*
Salivirus FHB
Linear 5moU
Mock

FIG. 8A

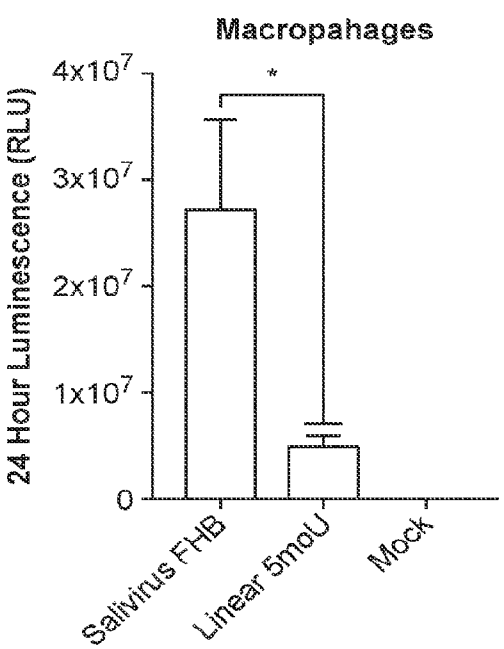
Macropahages
24 Hour Luminescence (RLU)
4x10^7
3x10^7
2x10^7
1x10^7
0
*
Salivirus FHB
Linear 5moU
Mock

FIG. 8B

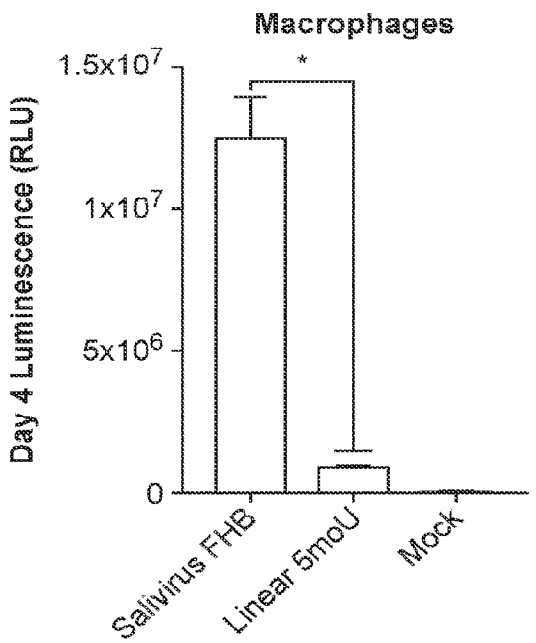
Macrophages
Day 4 Luminescence (RLU)
1.5x10^7
1x10^7
5x10^6
0
*
Salivirus FHB
Linear 5moU
Mock

FIG. 8C

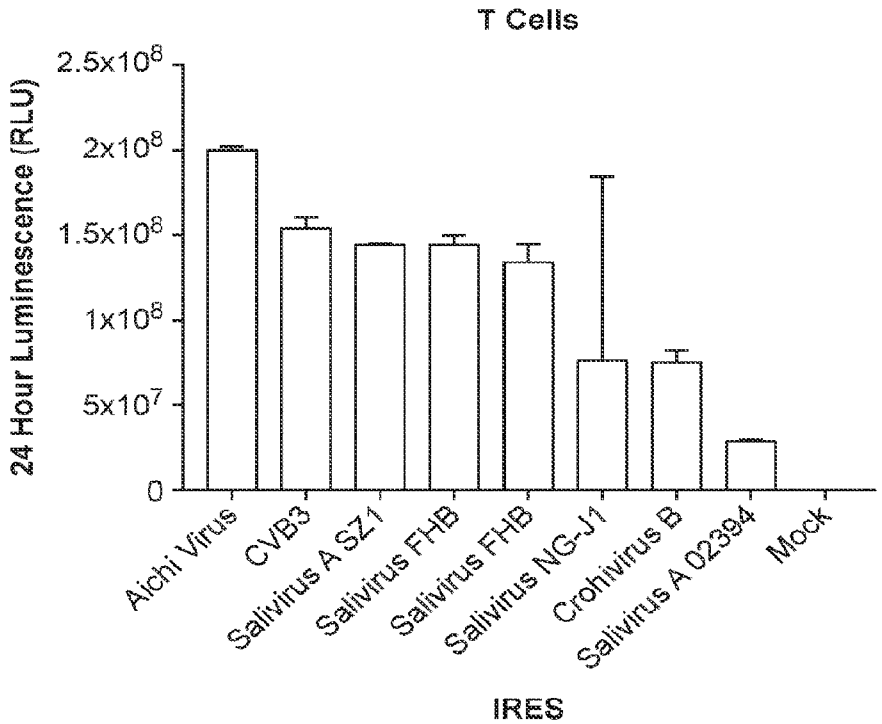
T Cells
24 Hour Luminescence (RLU)
2.5x10^8
2x10^8
1.5x10^8
1x10^8
5x10^7
0
Aichi Virus
CVB3
Salivirus A SZ1
Salivirus FHB
Salivirus FHB
Salivirus NG-J1
Crohivirus B
Salivirus A 02394
Mock
IRES

FIG. 9A

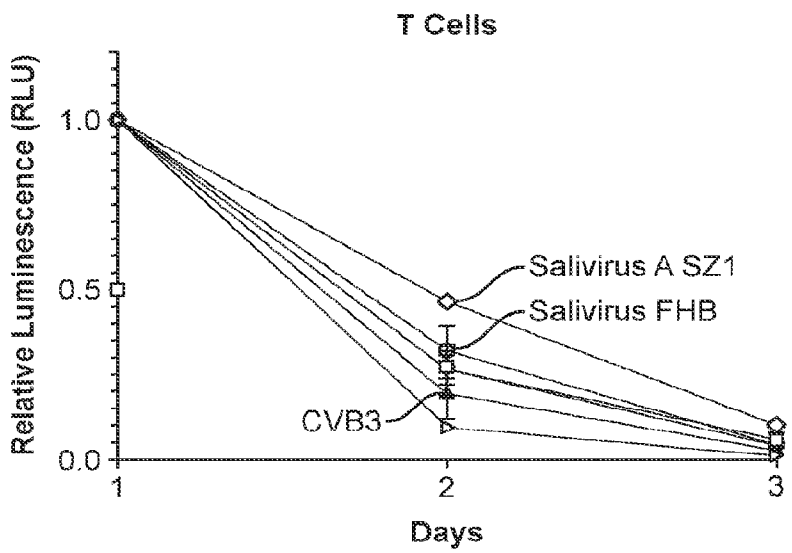
T Cells
Relative Luminescence (RLU)
1.0
0.5
0.0
Salivirus A SZ1
Salivirus FHB
CVB3
1
2
3
Days

FIG. 9B

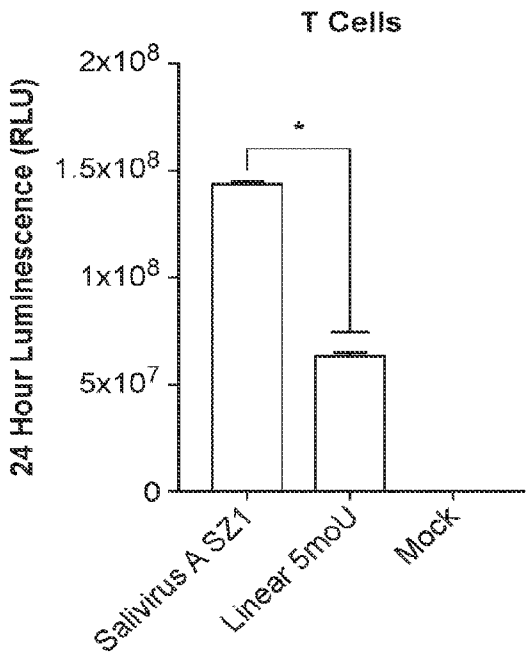

FIG. 10A

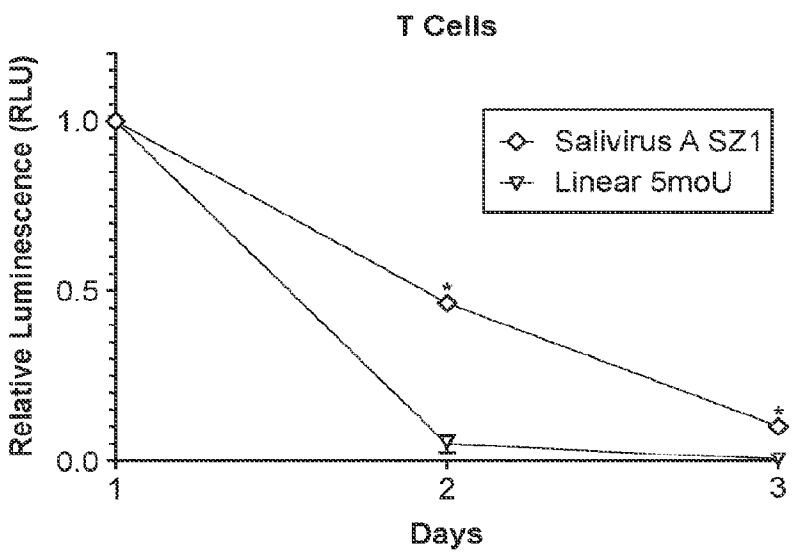

FIG. 10B

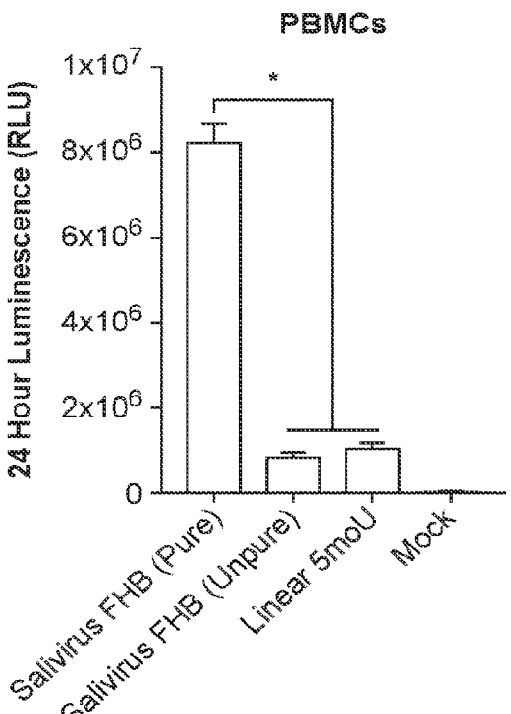
PBMCs
24 Hour Luminescence (RLU)
1x10^7
8x10^6
6x10^6
4x10^6
2x10^6
0
*
Salivirus FHB (Pure)
Salivirus FHB (Unpure)
Linear 5moU
Mock

FIG. 10C

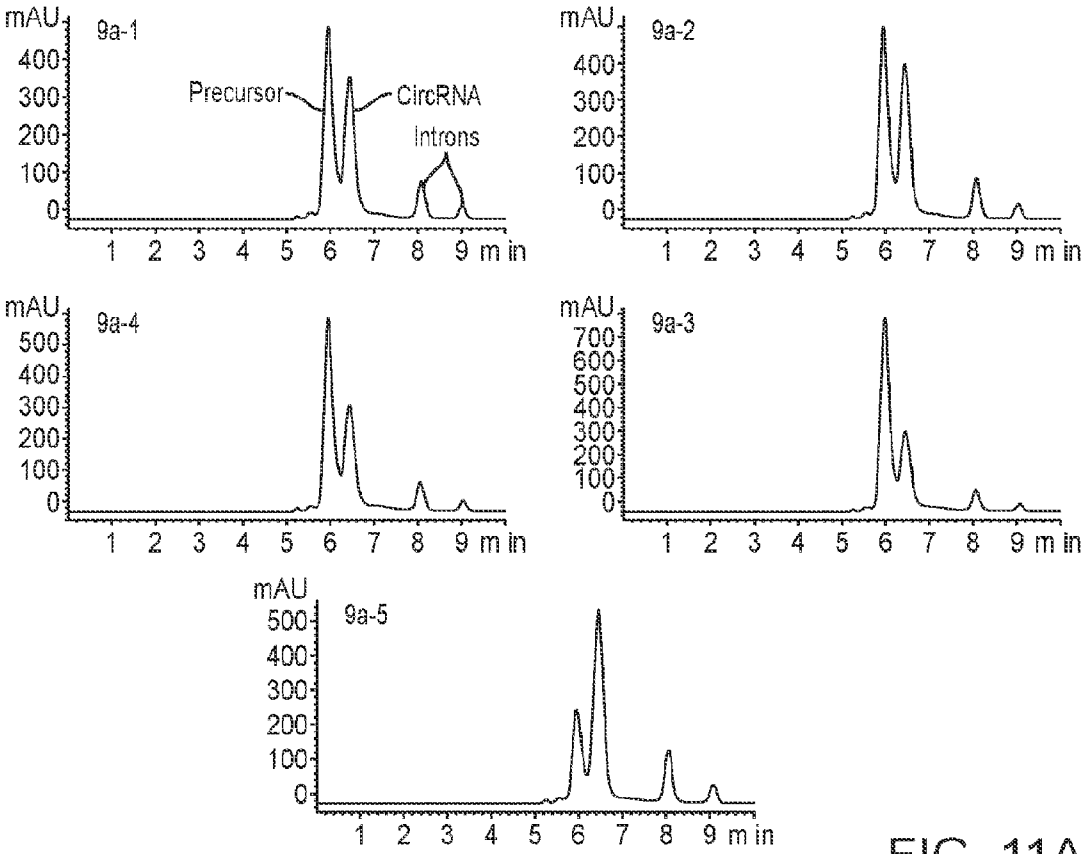

FIG. 11A

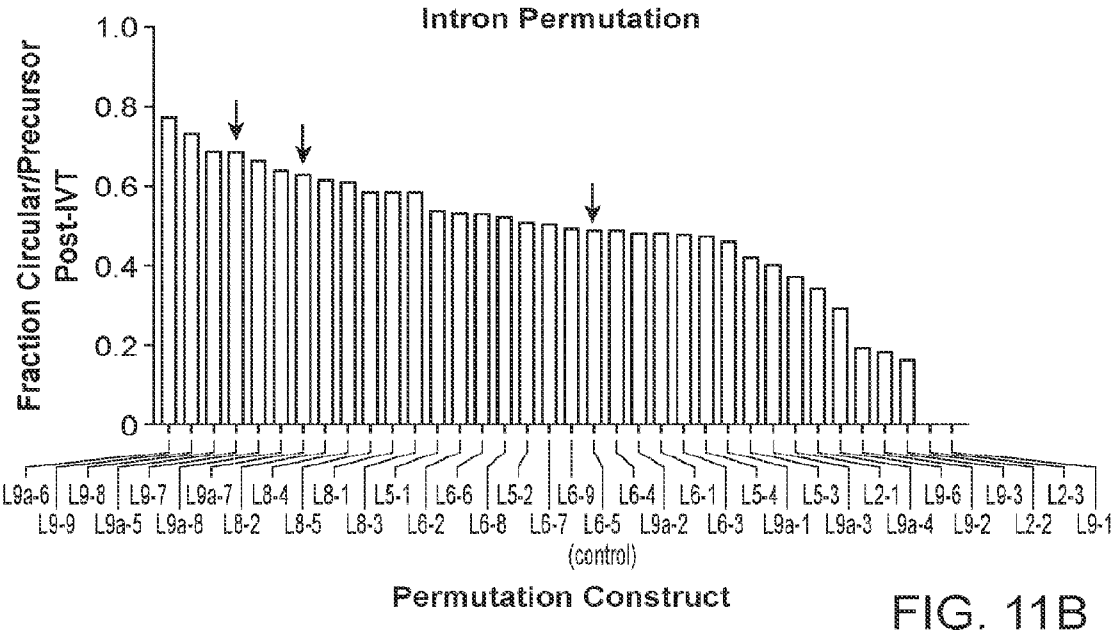

FIG. 11B

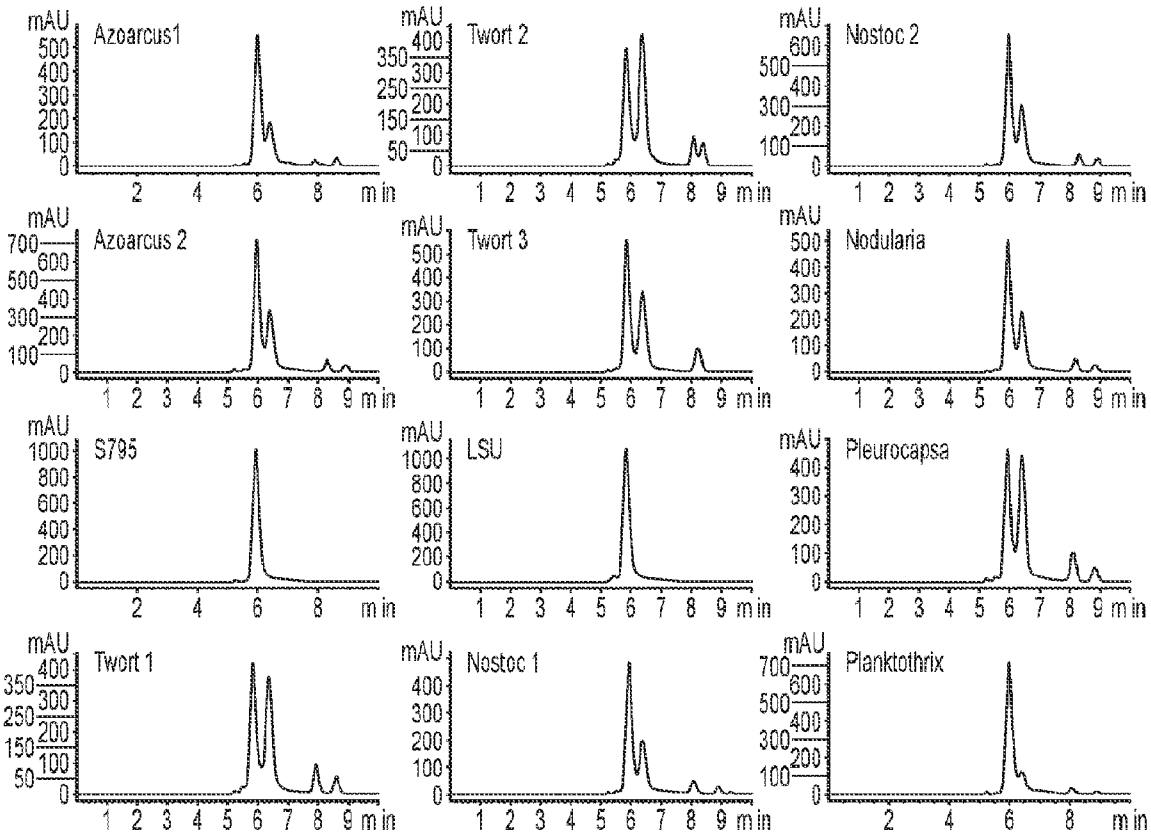

FIG. 12A

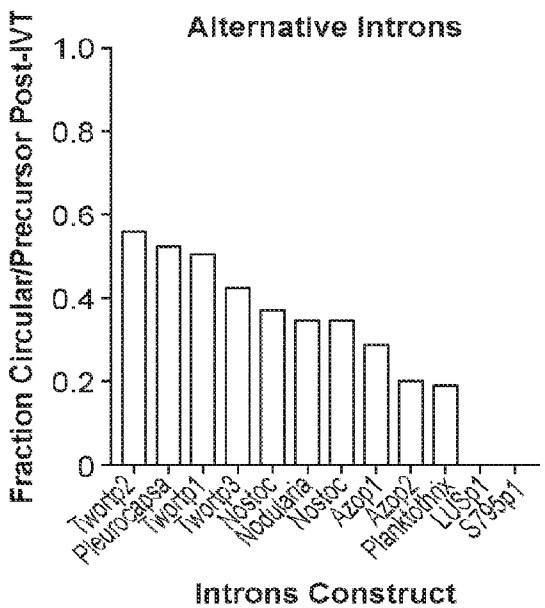

FIG. 12B

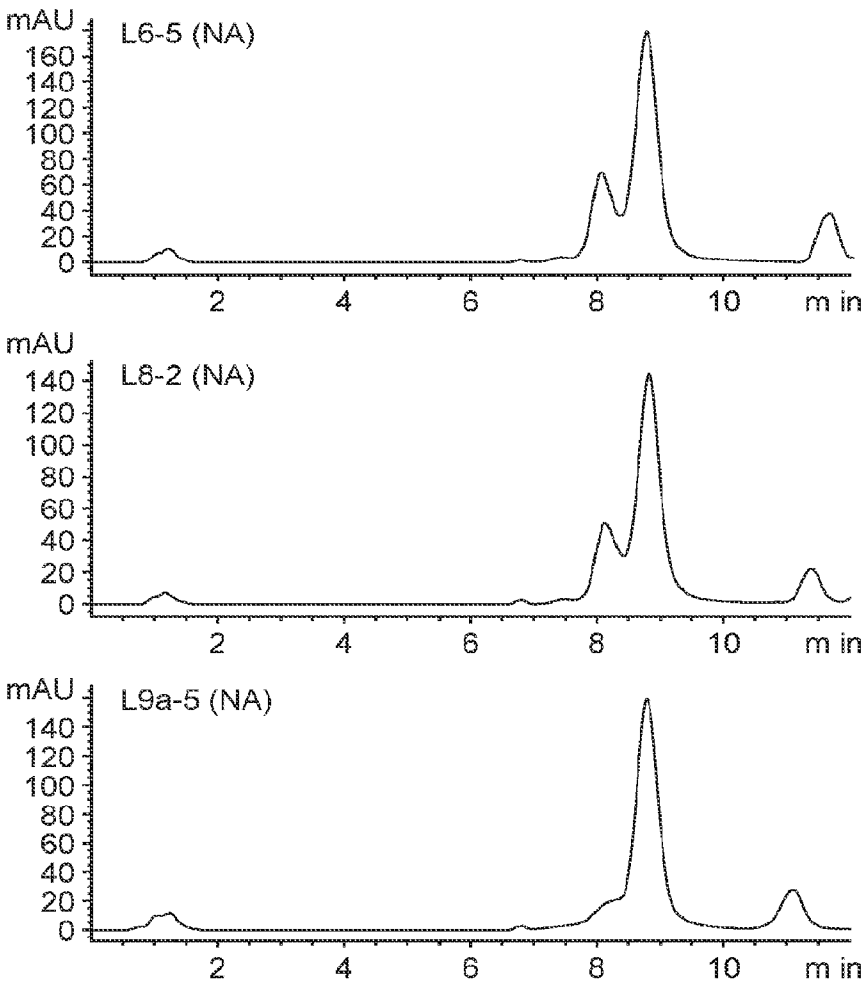
mAU
L6-5 (NA)
160
140
120
100
80
60
40
20
0
2
4
6
8
10
m in
mAU
L8-2 (NA)
140
120
100
80
60
40
20
0
2
4
6
8
10
m in
mAU
L9a-5 (NA)
140
120
100
80
60
40
20
0
2
4
6
8
10
m in

FIG. 13A

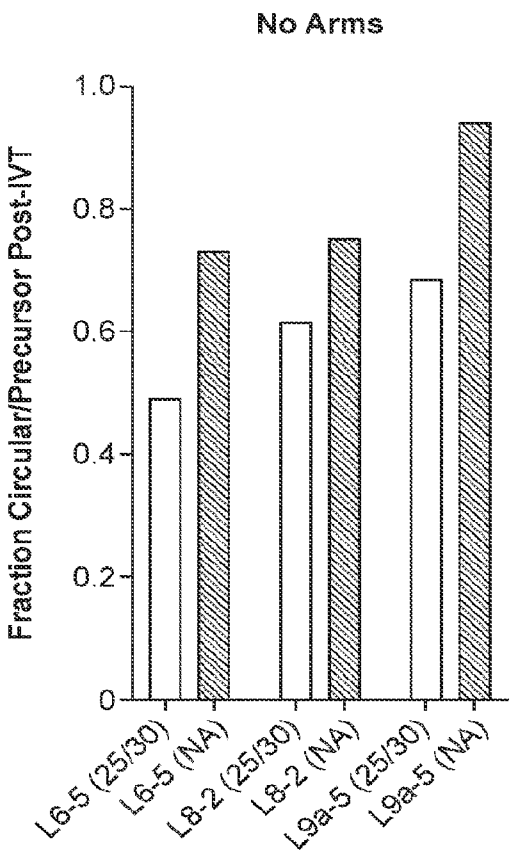
No Arms
Fraction Circular/Precursor Post-IVT
1.0
0.8
0.6
0.4
0.2
0
L6-5 (25/30)
L6-5 (NA)
L8-2 (25/30)
L8-2 (NA)
L9a-5 (25/30)
L9a-5 (NA)

FIG. 13B

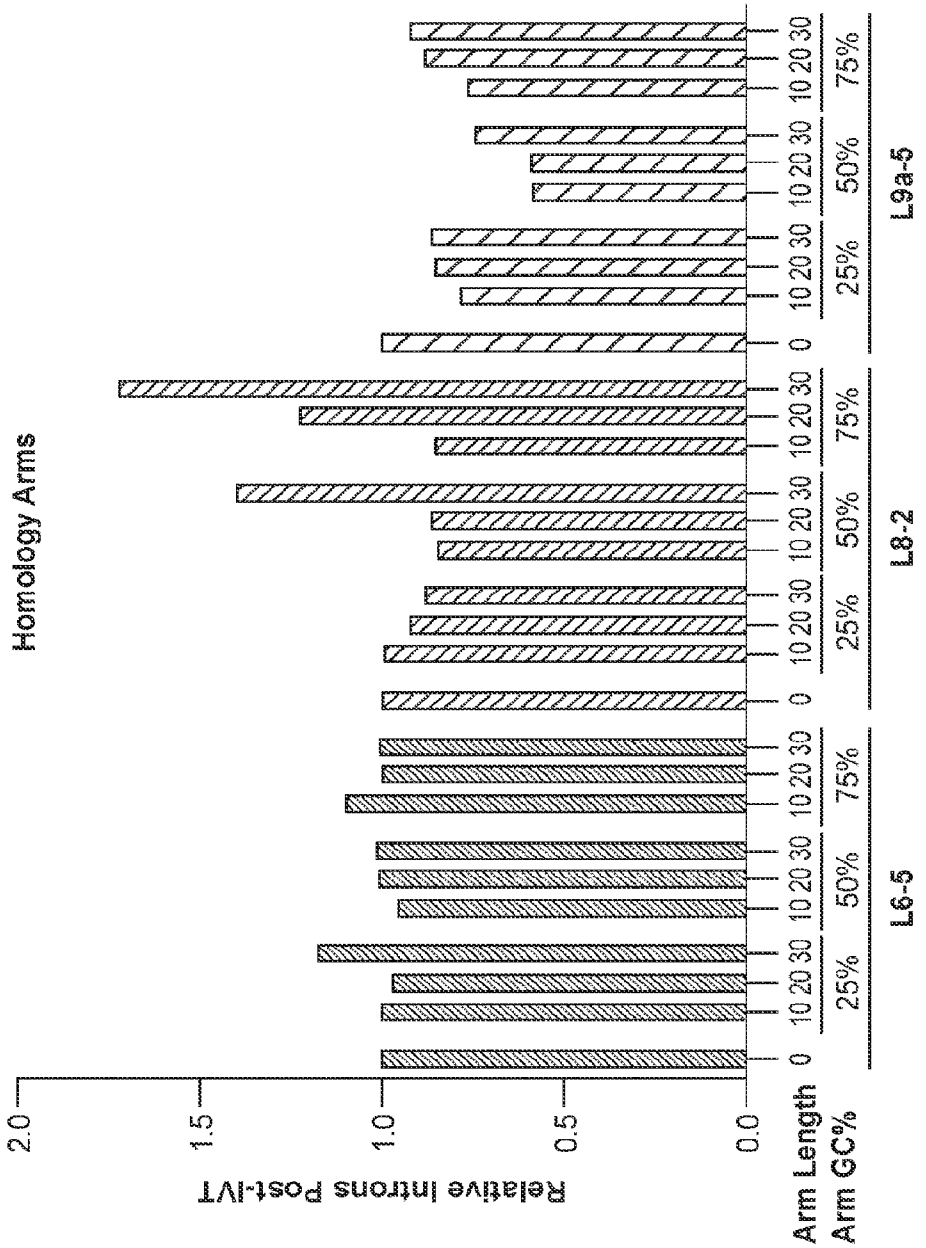
Homology Arms
Relative Introns Post-IVT
2.0
1.5
1.0
0.5
0.0
Arm Length
Arm GC%
0 10 20 30 10 20 30 10 20 30 0 10 20 30 10 20 30 10 20 30 0 10 20 30 10 20 30 10 20 30
25% 50% 75% 25% 50% 75% 25% 50% 75%
L6-5 L8-2 L9a-5
Permutation

FIG. 14

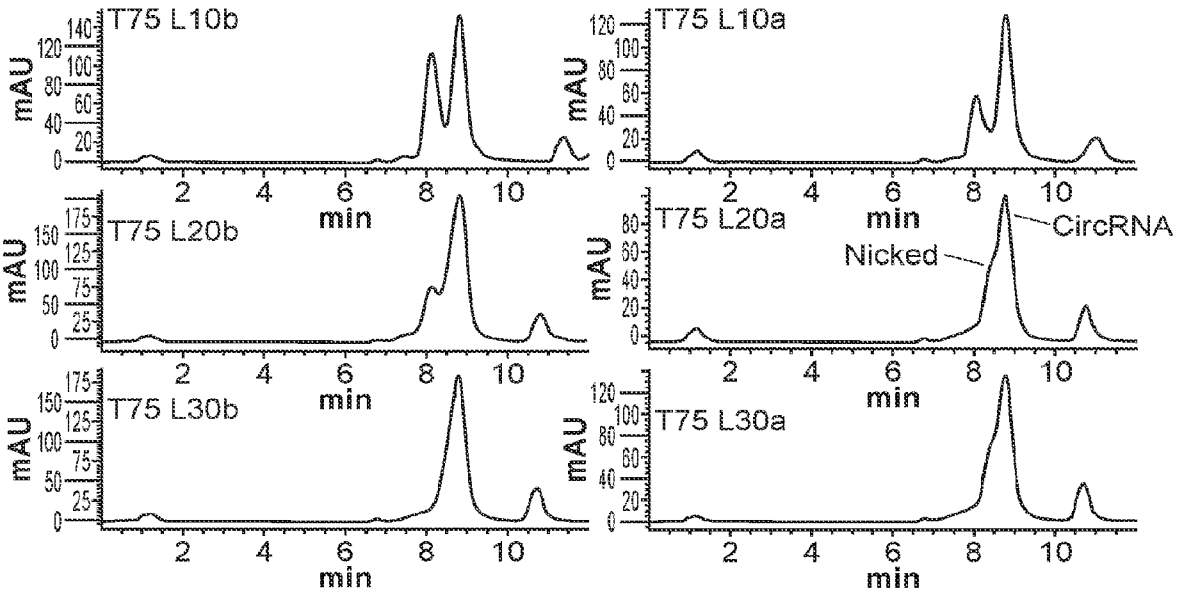

FIG. 15A

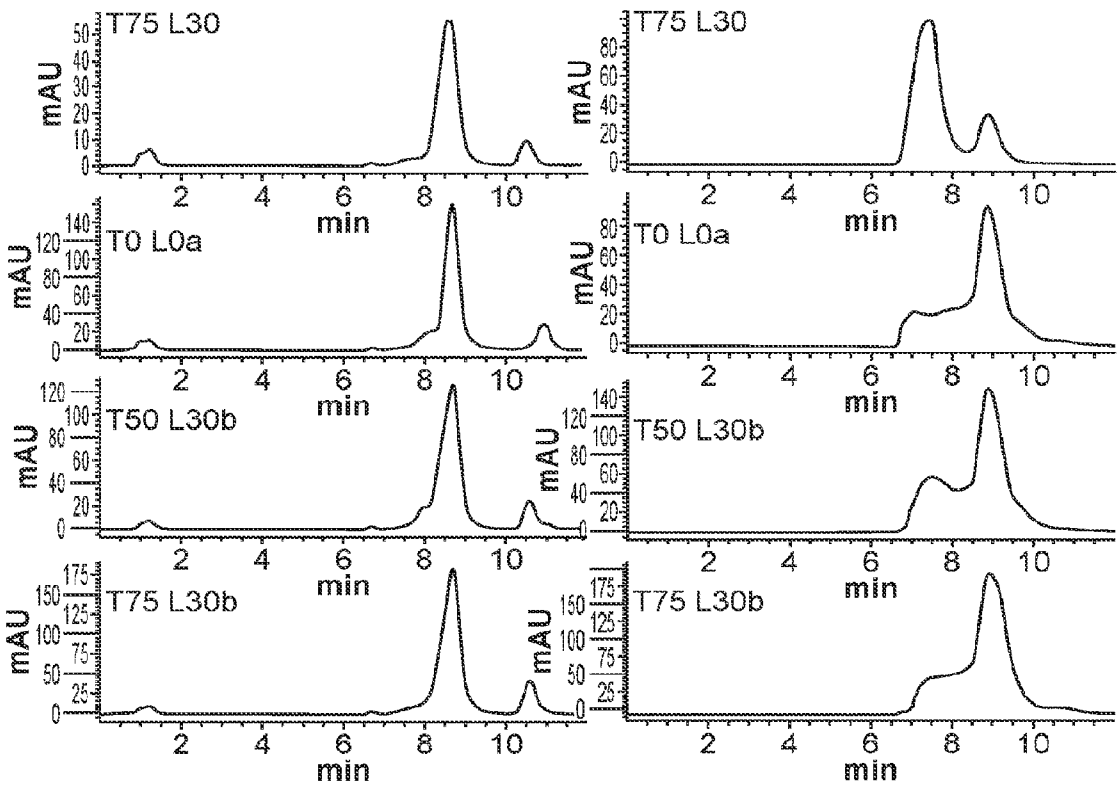

FIG. 15B

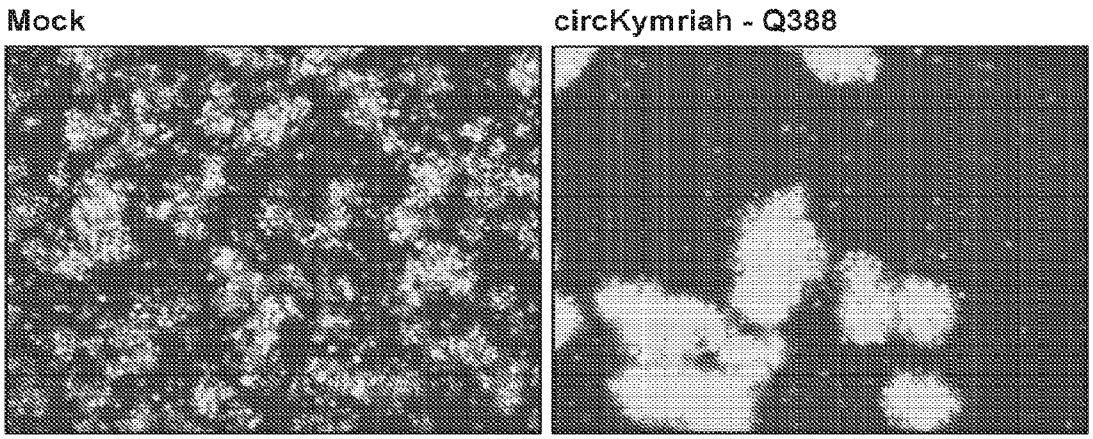

FIG. 16

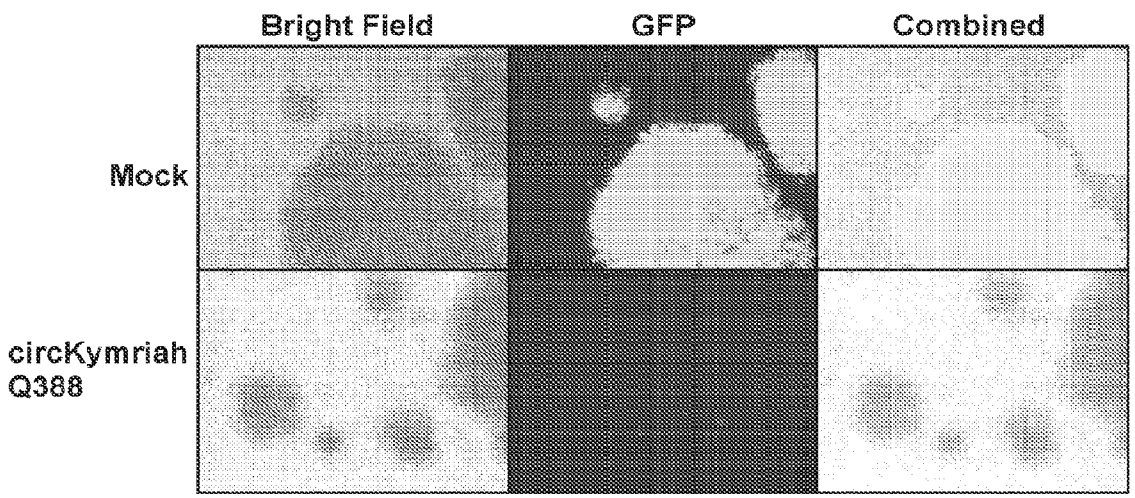

FIG. 17

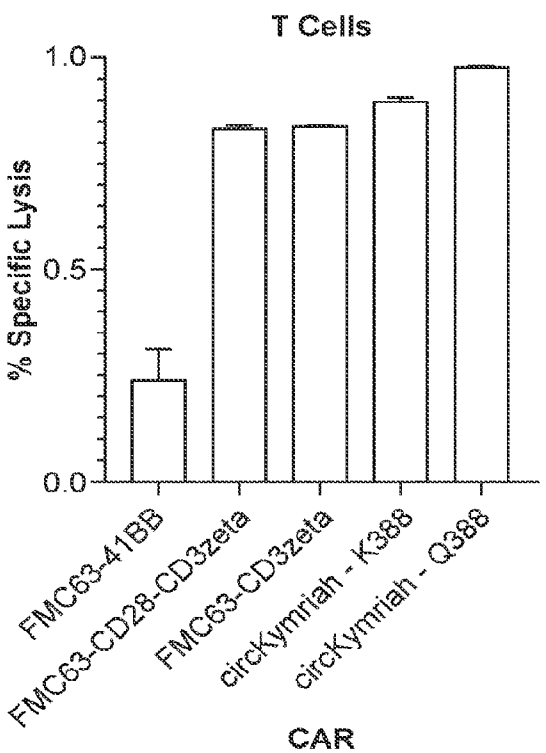
T Cells
% Specific Lysis
1.0
0.5
0.0
FMC63-41BB
FMC63-CD28-CD3zeta
FMC63-CD3zeta
circKymriah - K388
circKymriah - Q388
CAR

FIG. 18

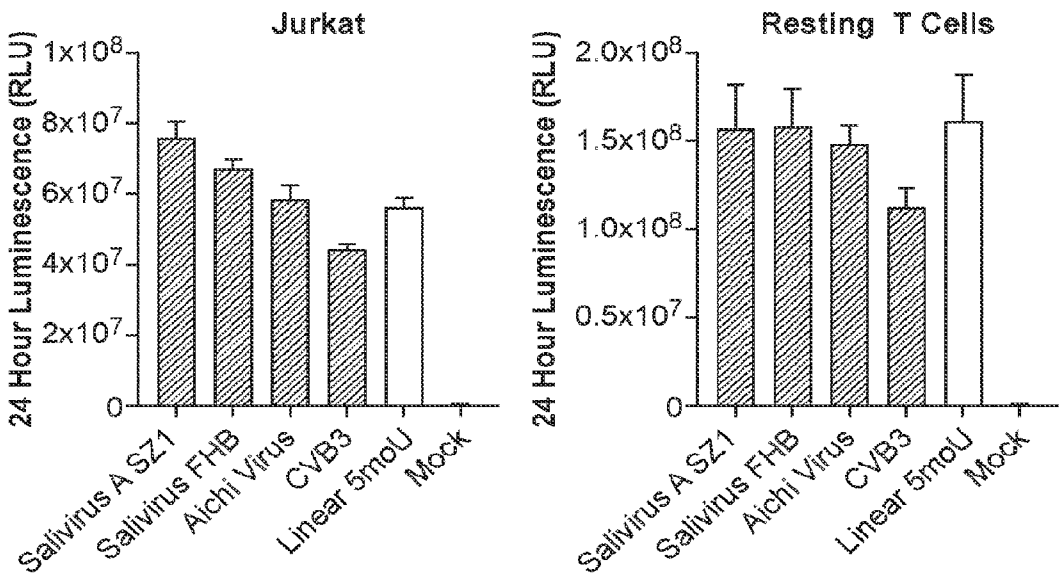

FIG. 19A

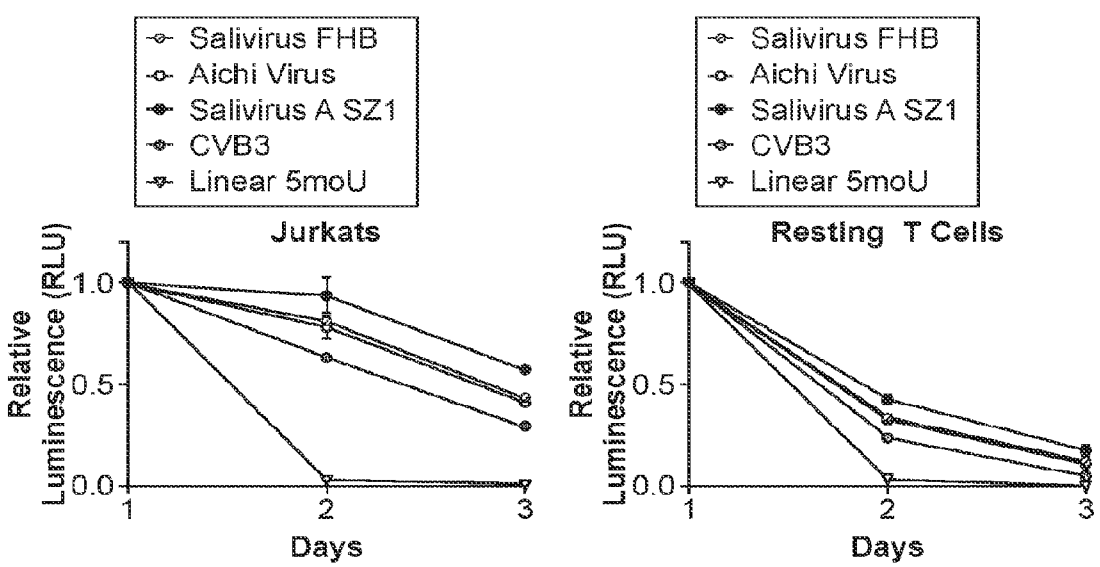

FIG. 19B

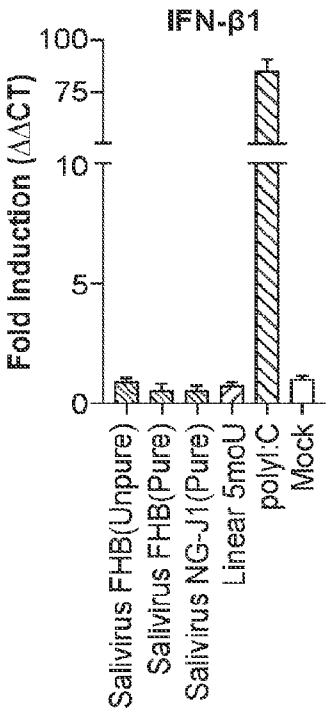

FIG. 20A

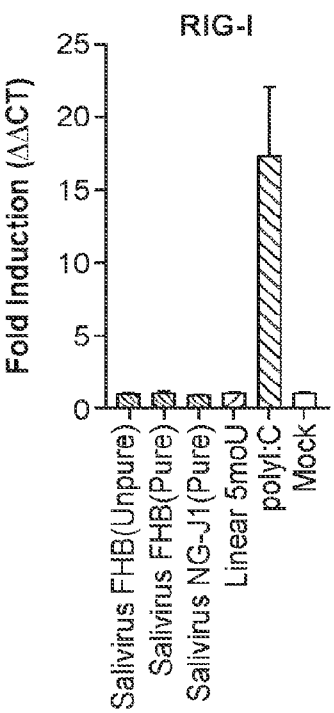

FIG. 20B

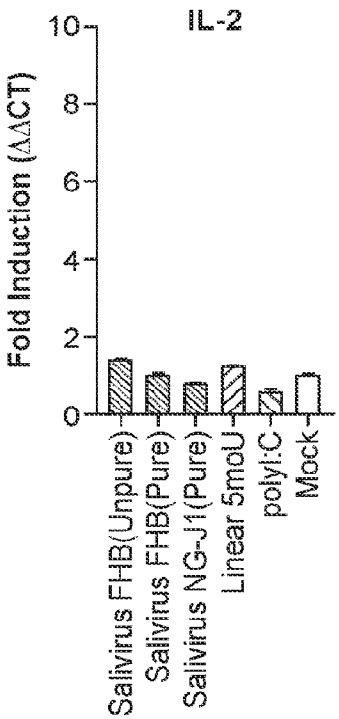

FIG. 20C

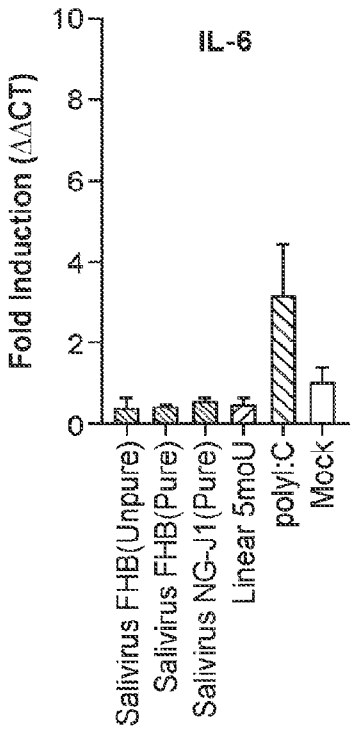

FIG. 20D

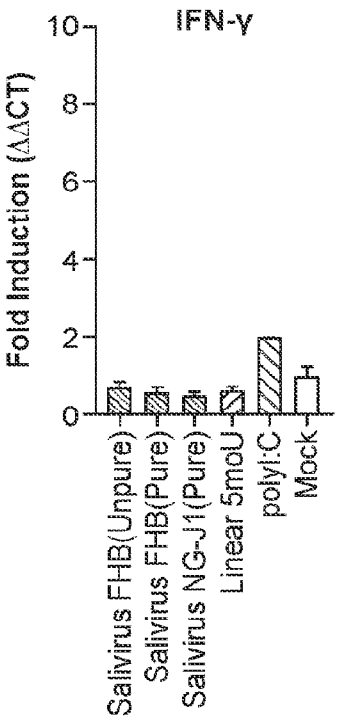

FIG. 20E

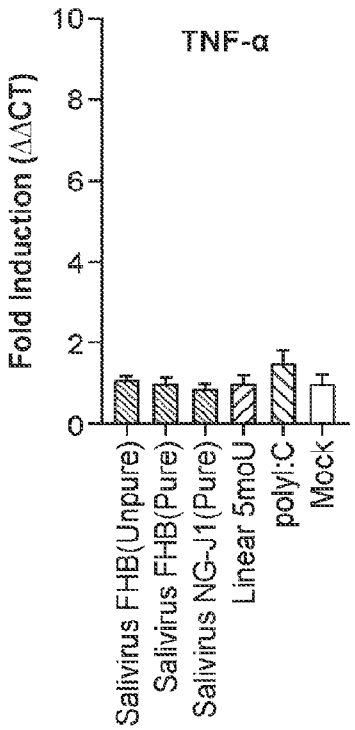

FIG. 20F

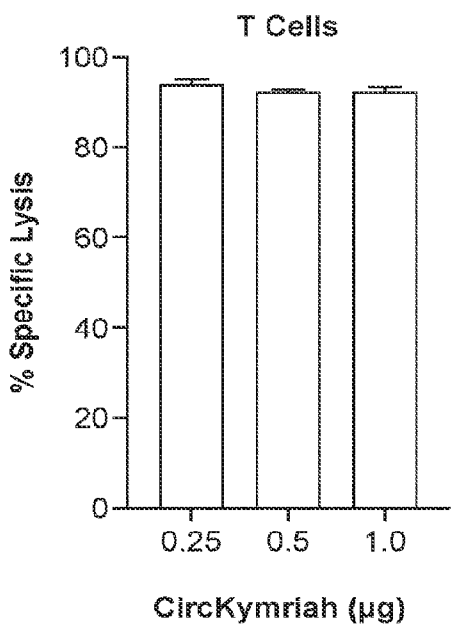
T Cells
100
80
60
40
20
0
% Specific Lysis
0.25
0.5
1.0
CircKymriah (µg)

FIG. 21A

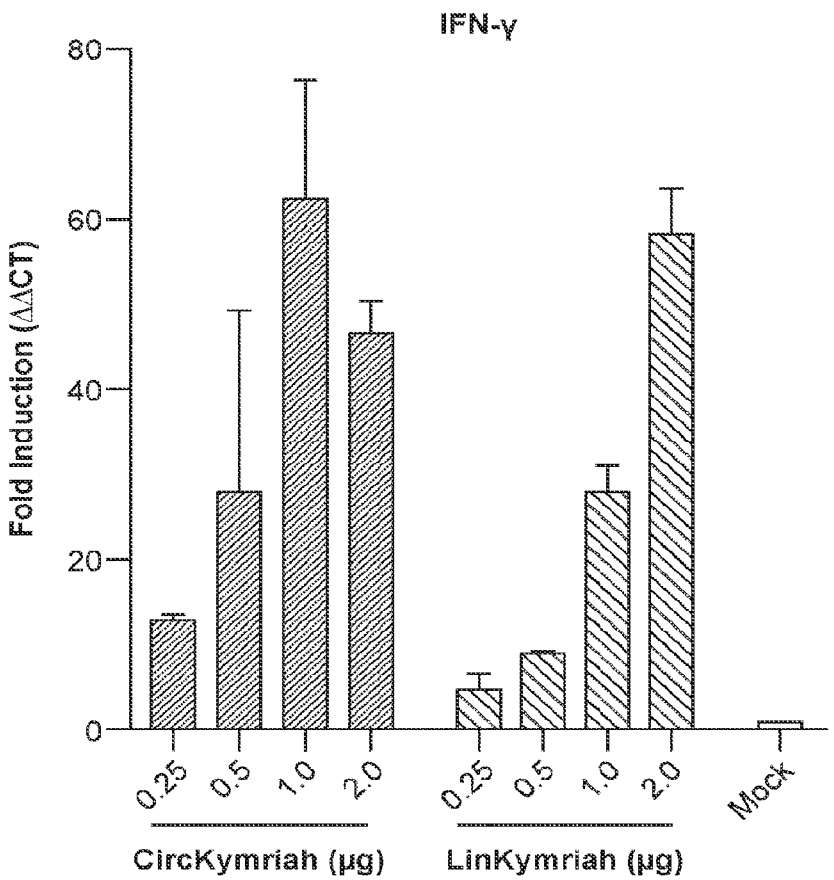
IFN-γ
Fold Induction (ΔΔCT)
80
60
40
20
0
0.25
0.5
1.0
2.0
0.25
0.5
1.0
2.0
Mock
CircKymriah (µg)
LinKymriah (µg)

FIG. 21B

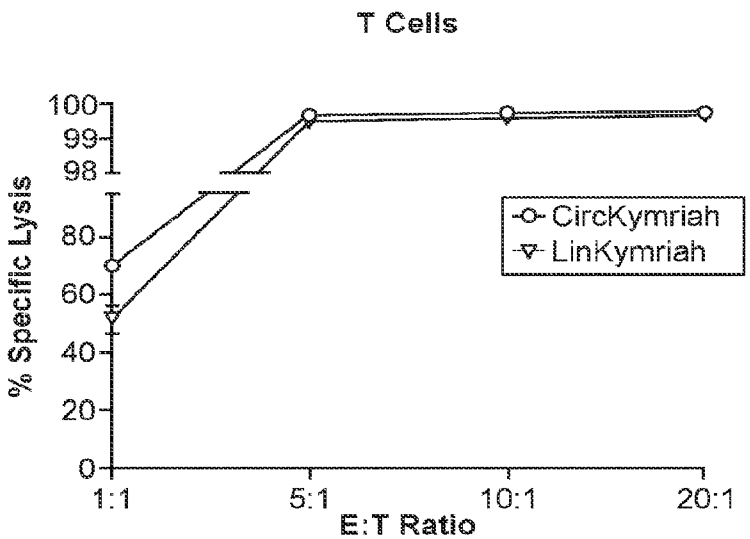

FIG. 22A

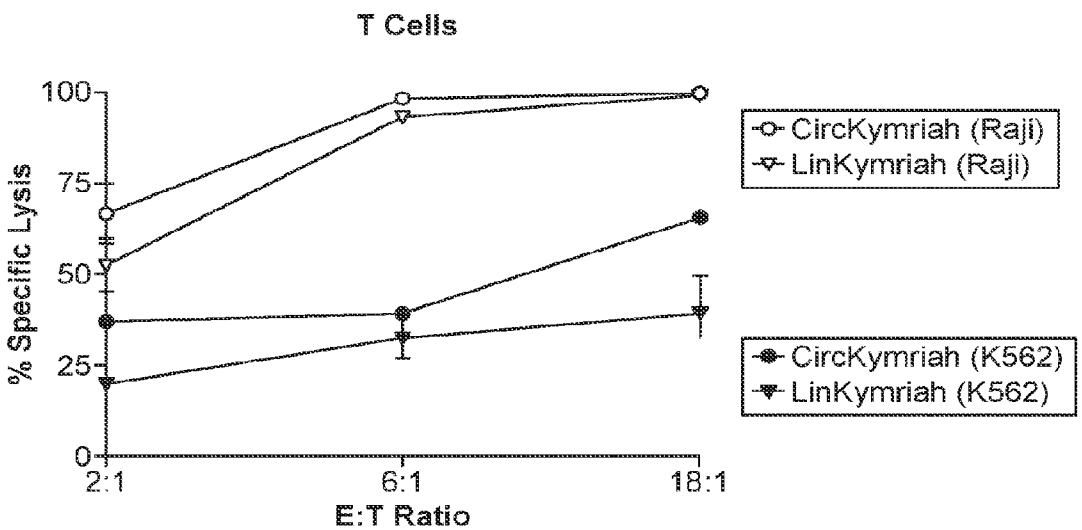

FIG. 22B

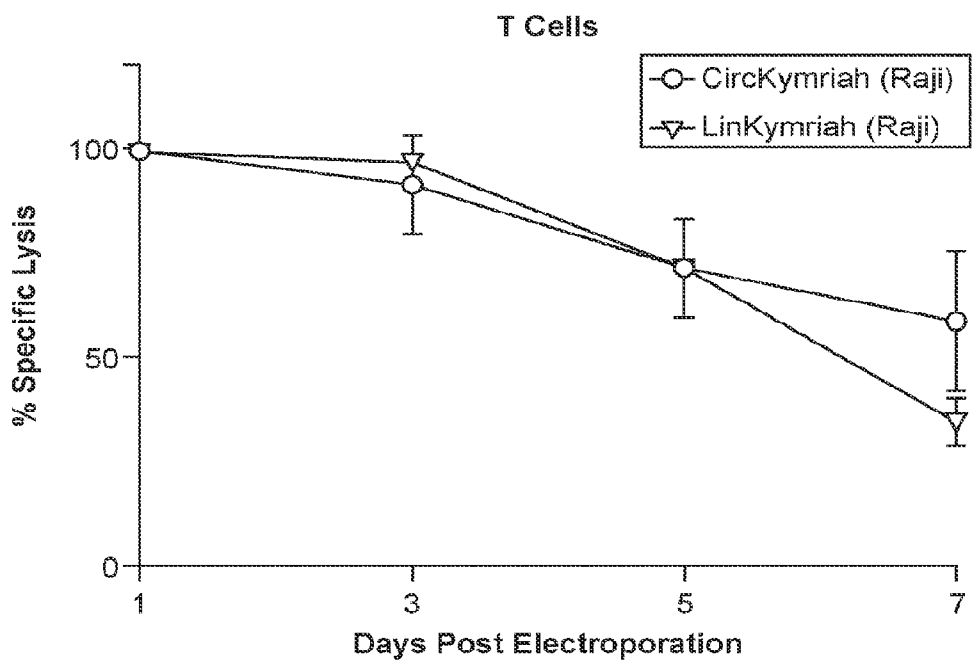
T Cells
CircKymriah (Raji)
LinKymriah (Raji)
% Specific Lysis
100
50
0
1
3
5
7
Days Post Electroporation

FIG. 23

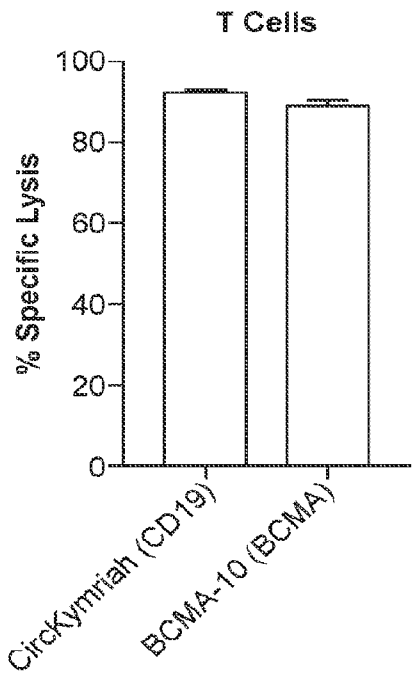
T Cells
% Specific Lysis
100
80
60
40
20
0
CircKymriah (CD19)
BCMA-10 (BCMA)

FIG. 24

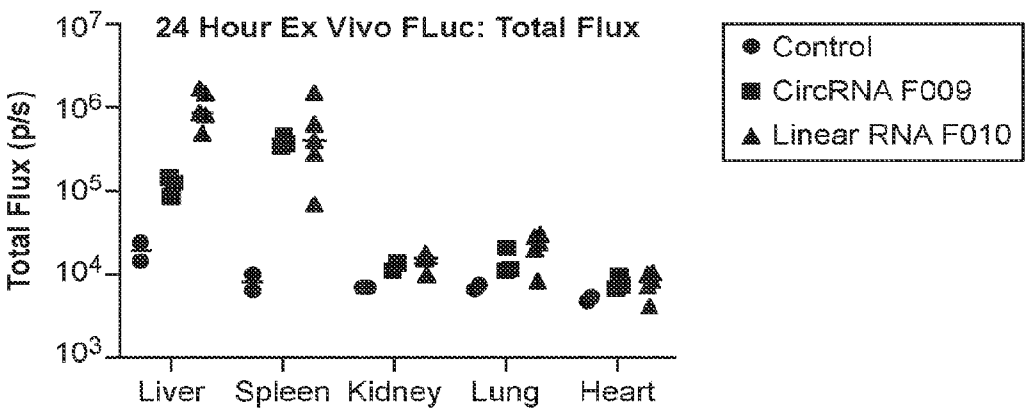
24 Hour Ex Vivo FLuc: Total Flux
Total Flux (p/s)
10^7
10^6
10^5
10^4
10^3
Liver
Spleen
Kidney
Lung
Heart
Control
CircRNA F009
Linear RNA F010

FIG. 25

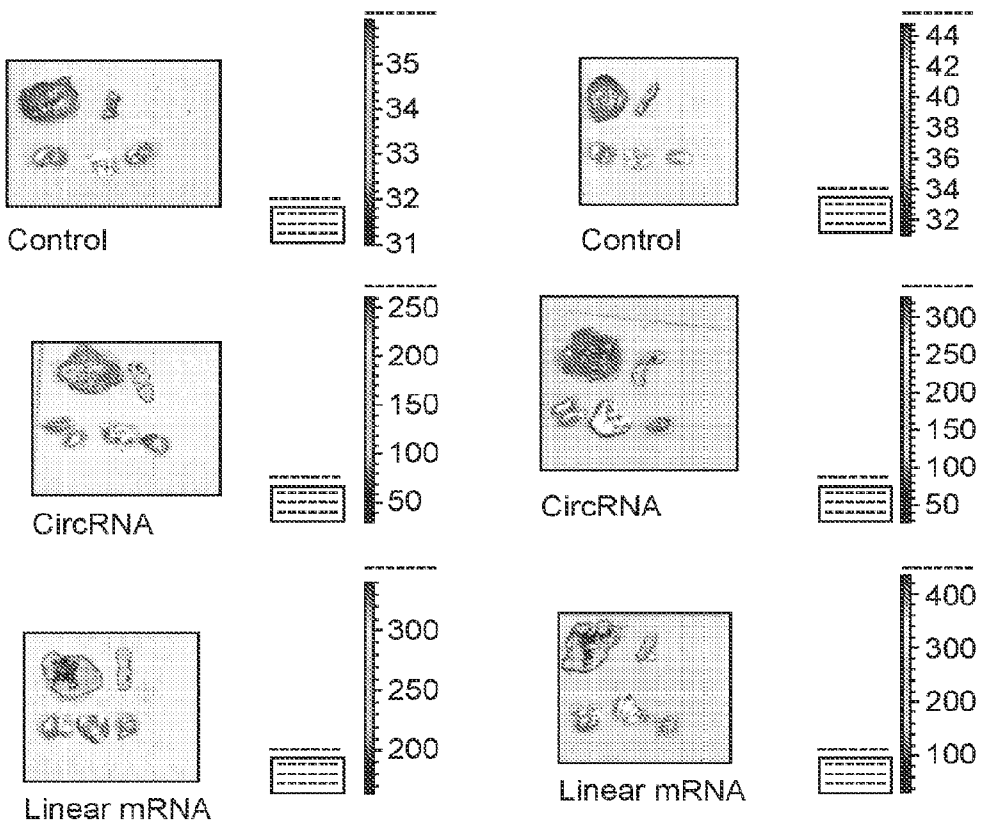
Control
35
34
33
32
31
Control
44
42
40
38
36
34
32
CircRNA
250
200
150
100
50
CircRNA
300
250
200
150
100
50
Linear mRNA
300
250
200
Linear mRNA
400
300
200
100

FIG. 26

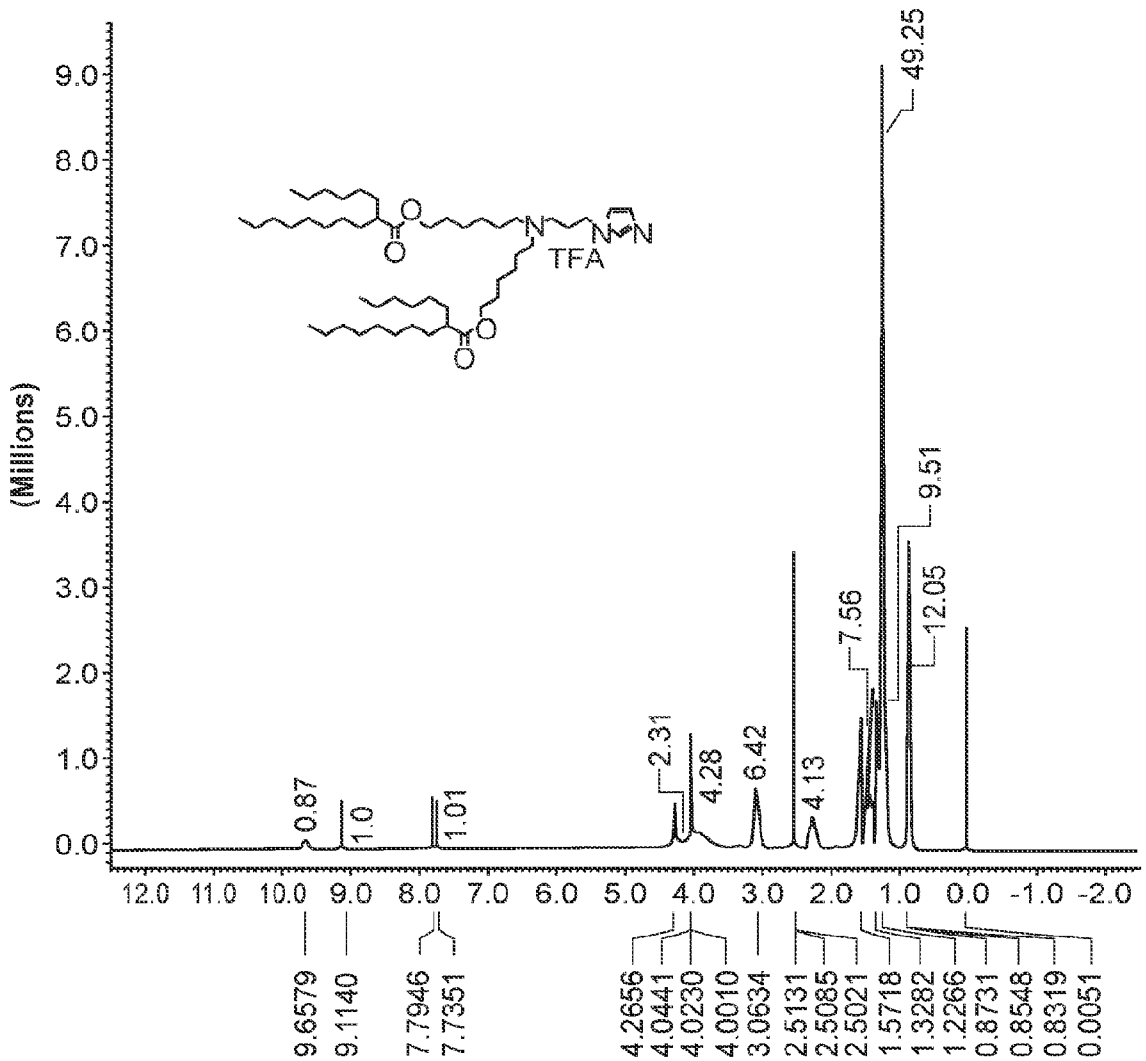
(Millions)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
TFA
49.25
9.51
12.05
7.56
2.31
4.28
6.42
4.13
0.87
1.0
1.01
12.0 11.0 10.0 9.0 8.0 7.0 6.0 5.0 4.0 3.0 2.0 1.0 0.0 -1.0 -2.0
9.6579
9.1140
7.7946
7.7351
4.2656
4.0441
4.0230
4.0010
3.0634
2.5131
2.5085
2.5021
1.5718
1.3282
1.2266
0.8731
0.8548
0.8319
0.0051

FIG. 27A

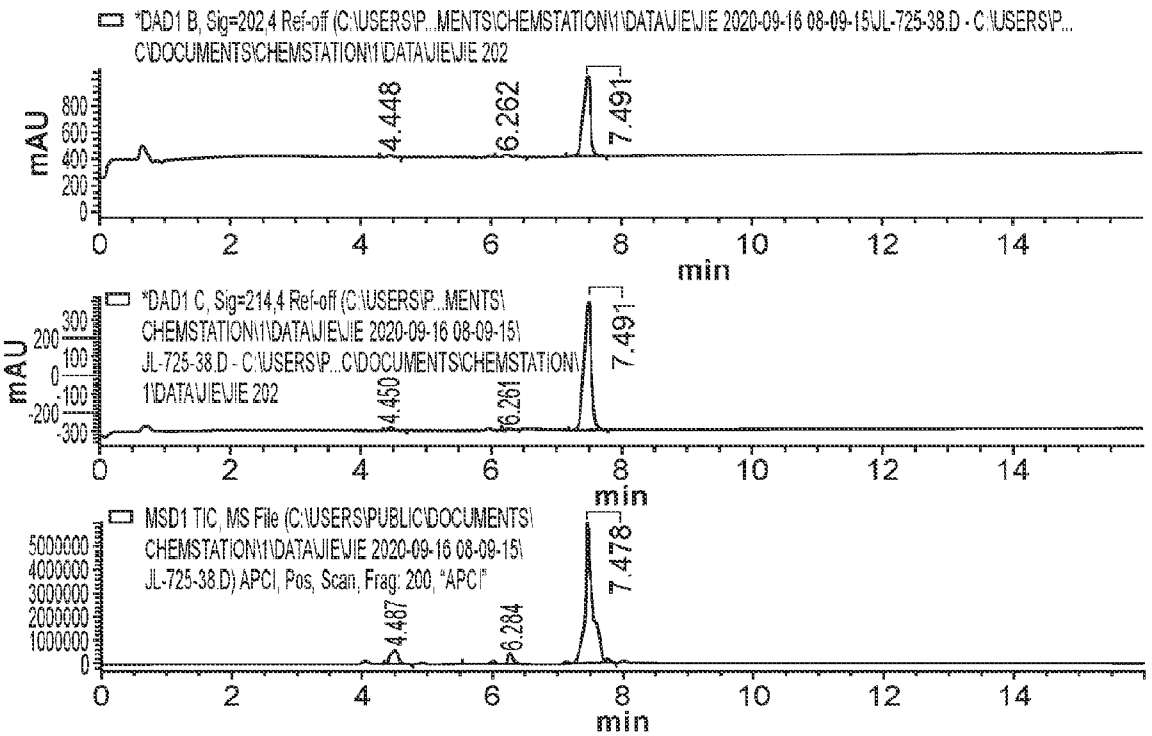

FIG. 27B

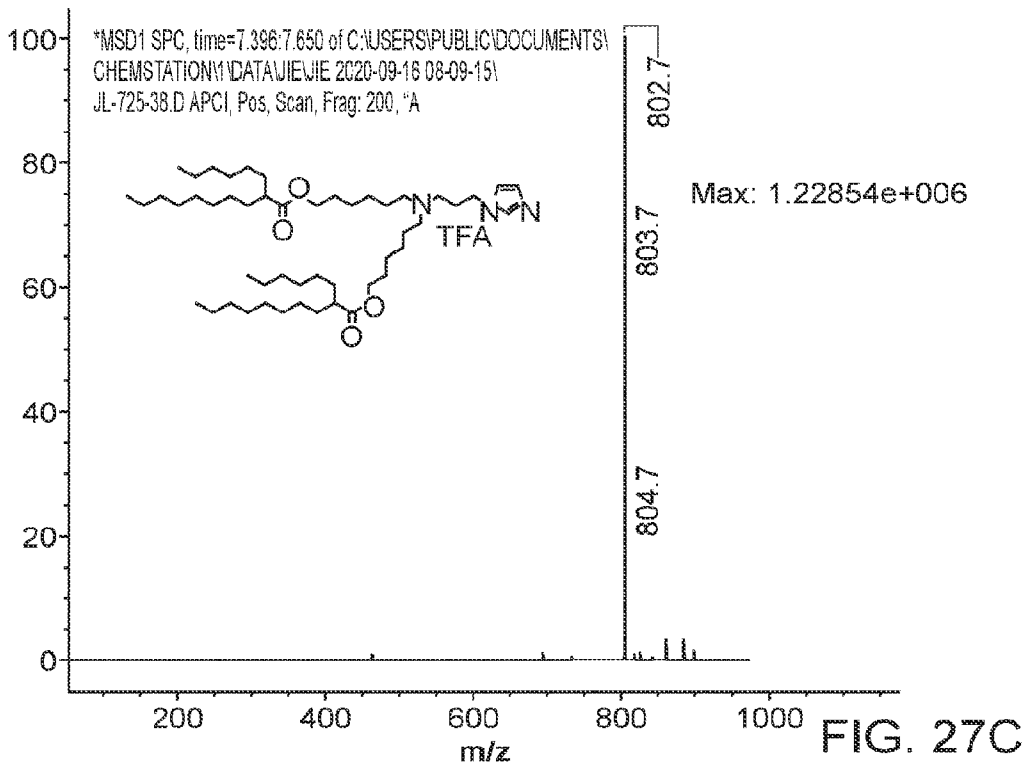

FIG. 27C

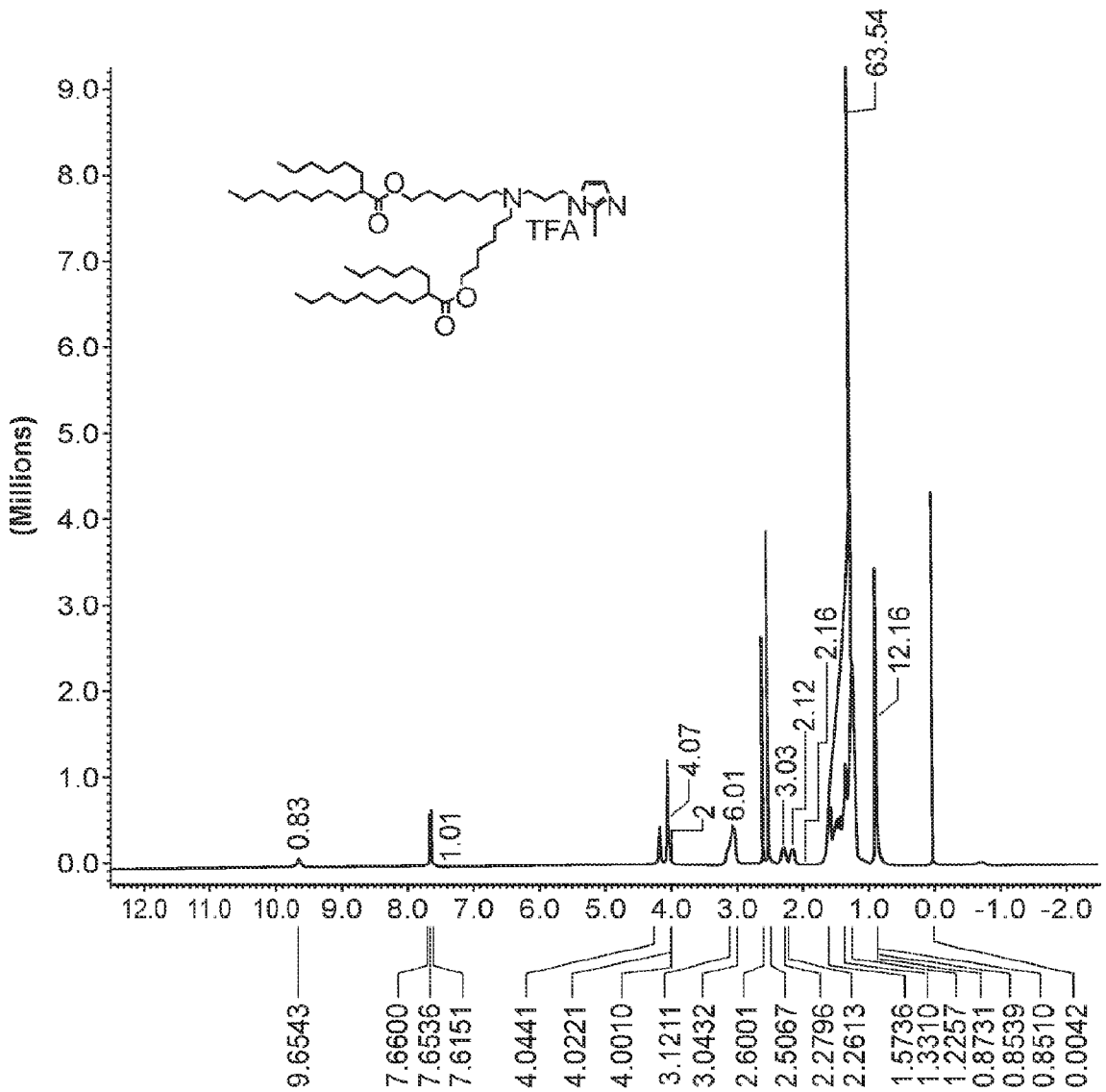
(Millions)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
TFA
63.54
12.16
2.16
2.12
4.07
3.03
6.01
2
1.01
0.83
12.0 11.0 10.0 9.0 8.0 7.0 6.0 5.0 4.0 3.0 2.0 1.0 0.0 -1.0 -2.0
9.6543
7.6600
7.6536
7.6151
4.0441
4.0221
4.0010
3.1211
3.0432
2.6001
2.5067
2.2796
2.2613
1.5736
1.3310
1.2257
0.8731
0.8539
0.8510
0.0042

FIG. 27D

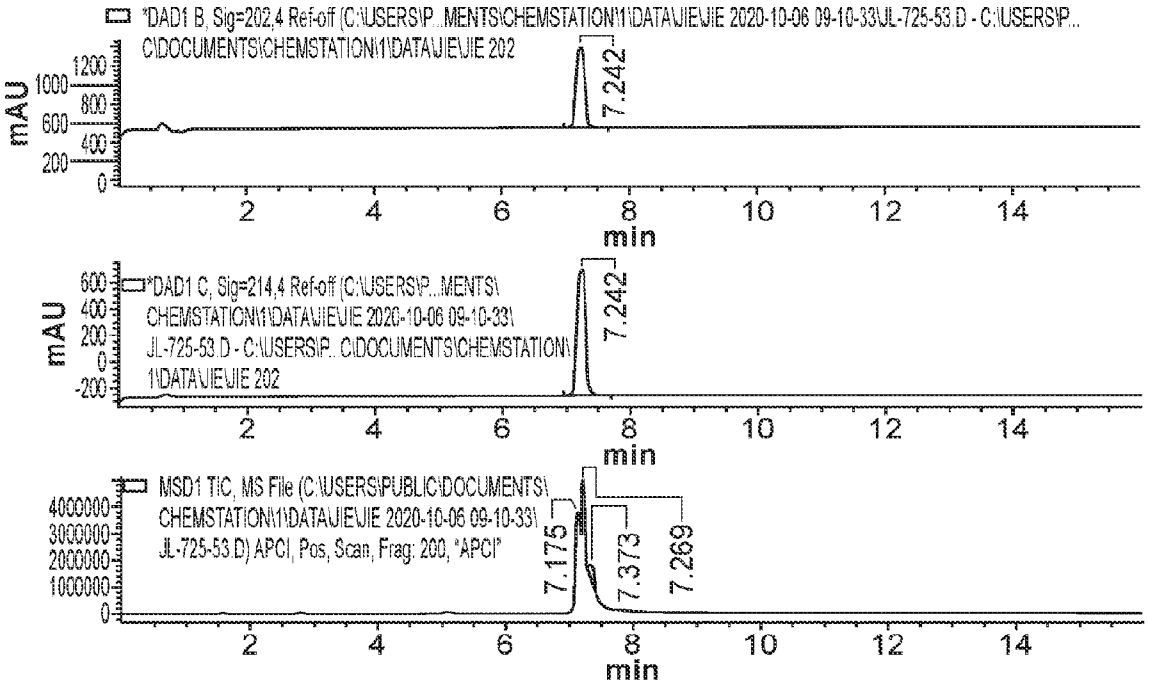

FIG. 27E

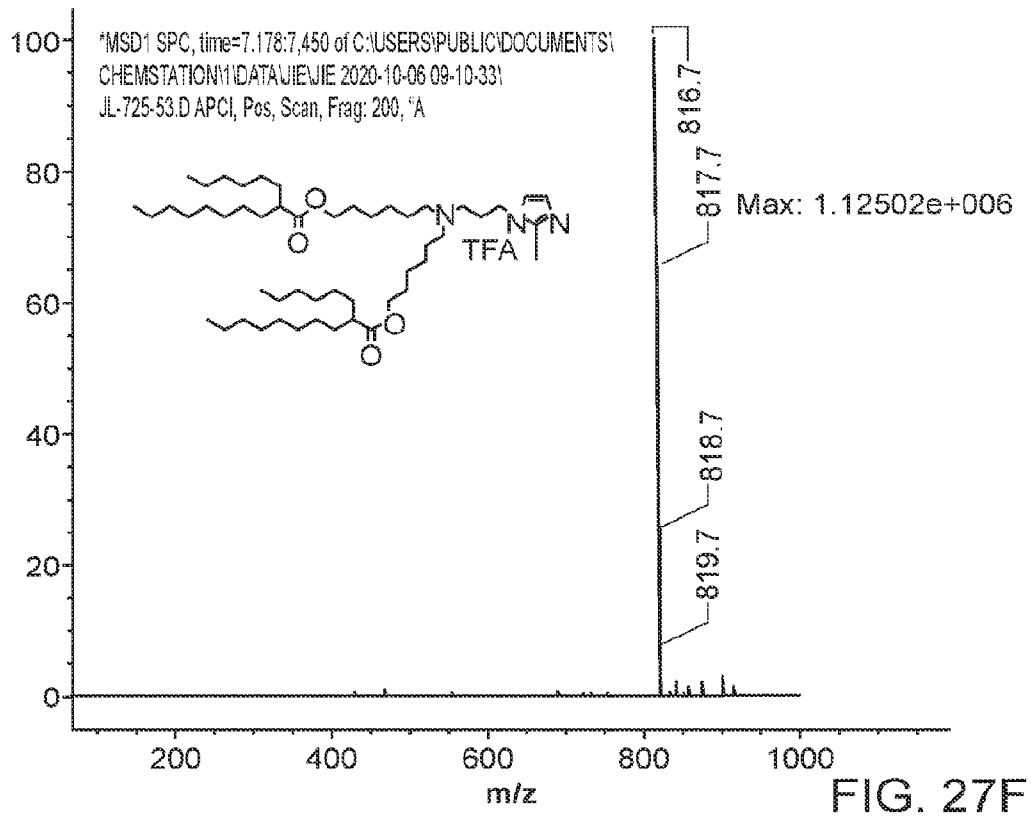

FIG. 27F

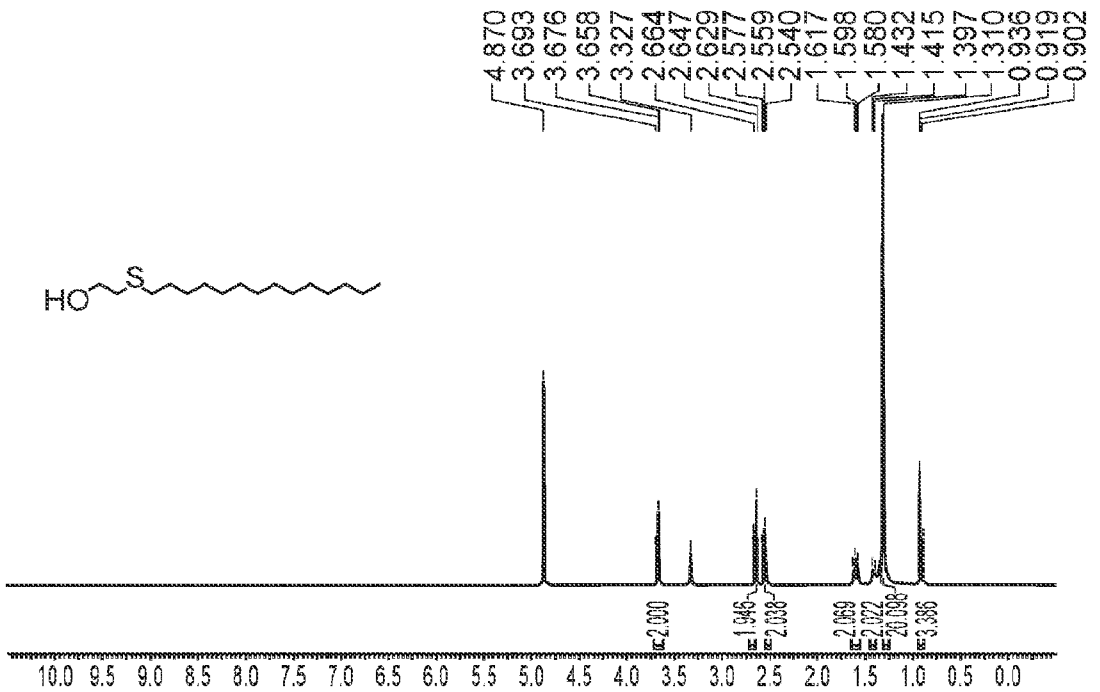

FIG. 28A

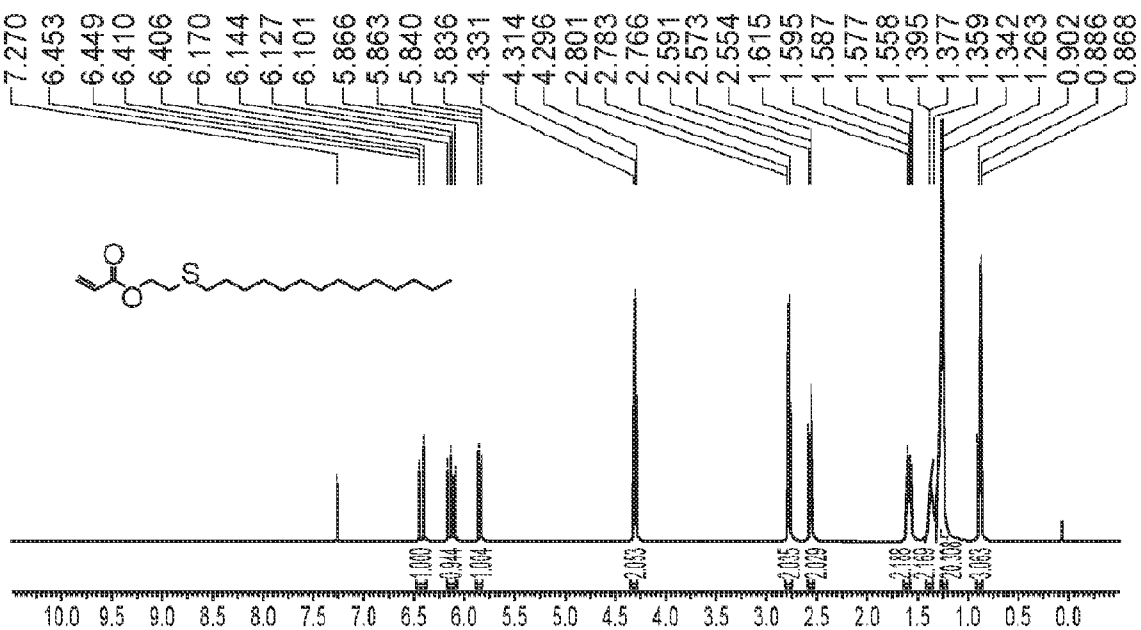

FIG. 28B

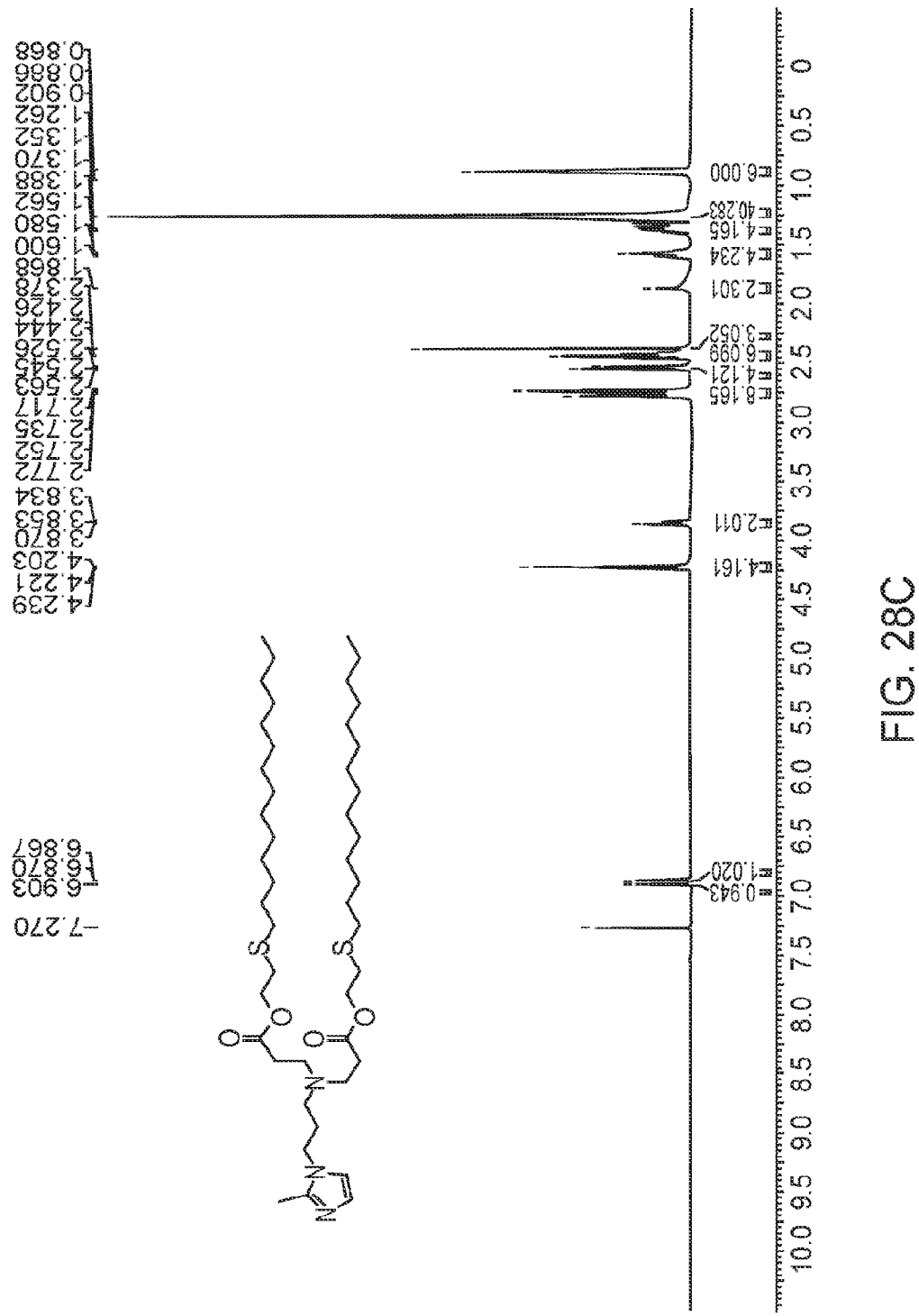

FIG. 28C

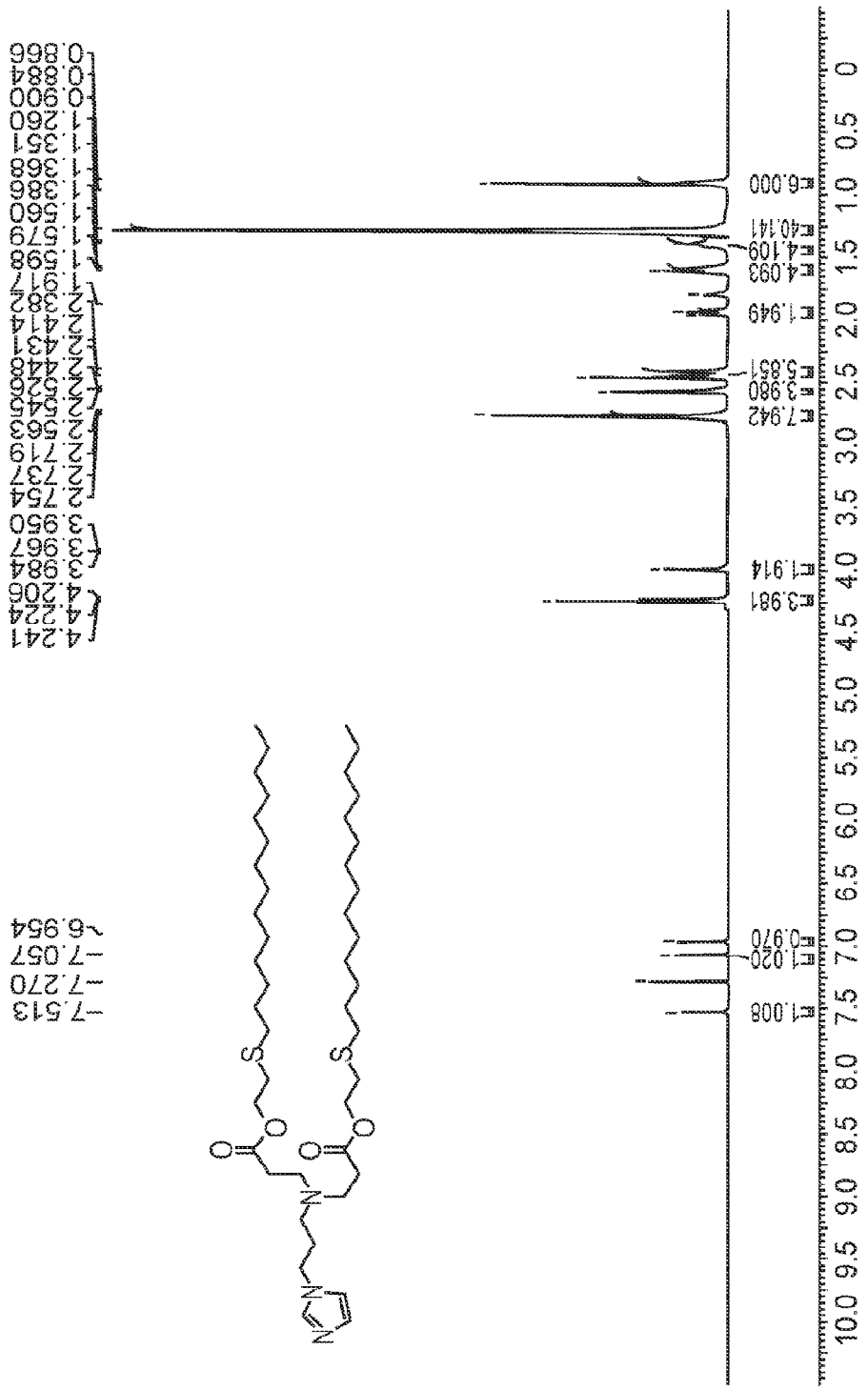

FIG. 29

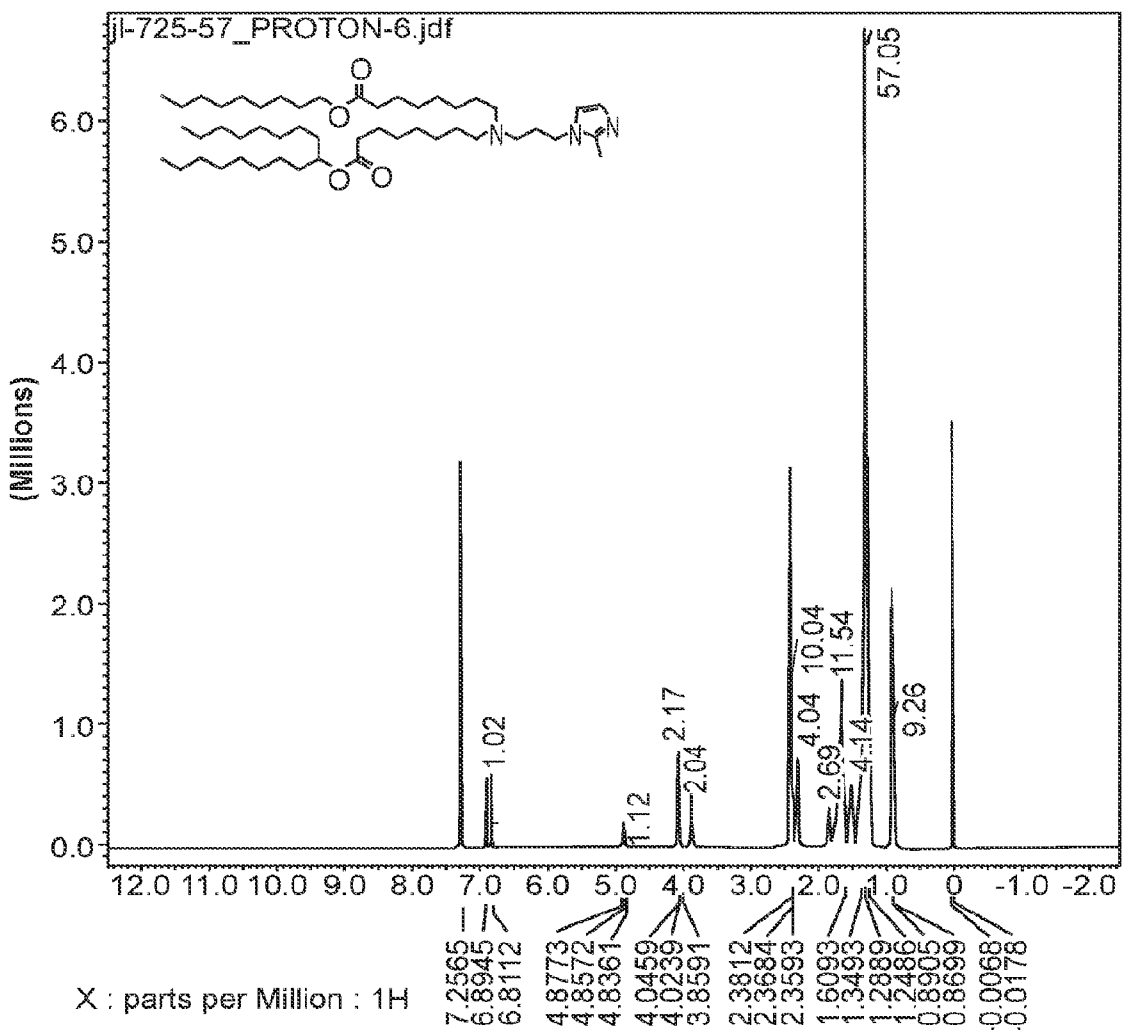
jl-725-57_PROTON-6.jdf
(Millions)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
57.05
10.04
11.54
4.04
2.69
4.14
9.26
1.02
2.17
2.04
1.12
12.0 11.0 10.0 9.0 8.0 7.0 6.0 5.0 4.0 3.0 2.0 1.0 0 -1.0 -2.0
X : parts per Million : 1H
7.2565
6.8945
6.8112
4.8773
4.8572
4.8361
4.0459
4.0239
3.8591
2.3812
2.3684
2.3593
1.6093
1.3493
1.2889
1.2486
0.8905
0.8699
-0.0068
-0.0178

FIG. 30A

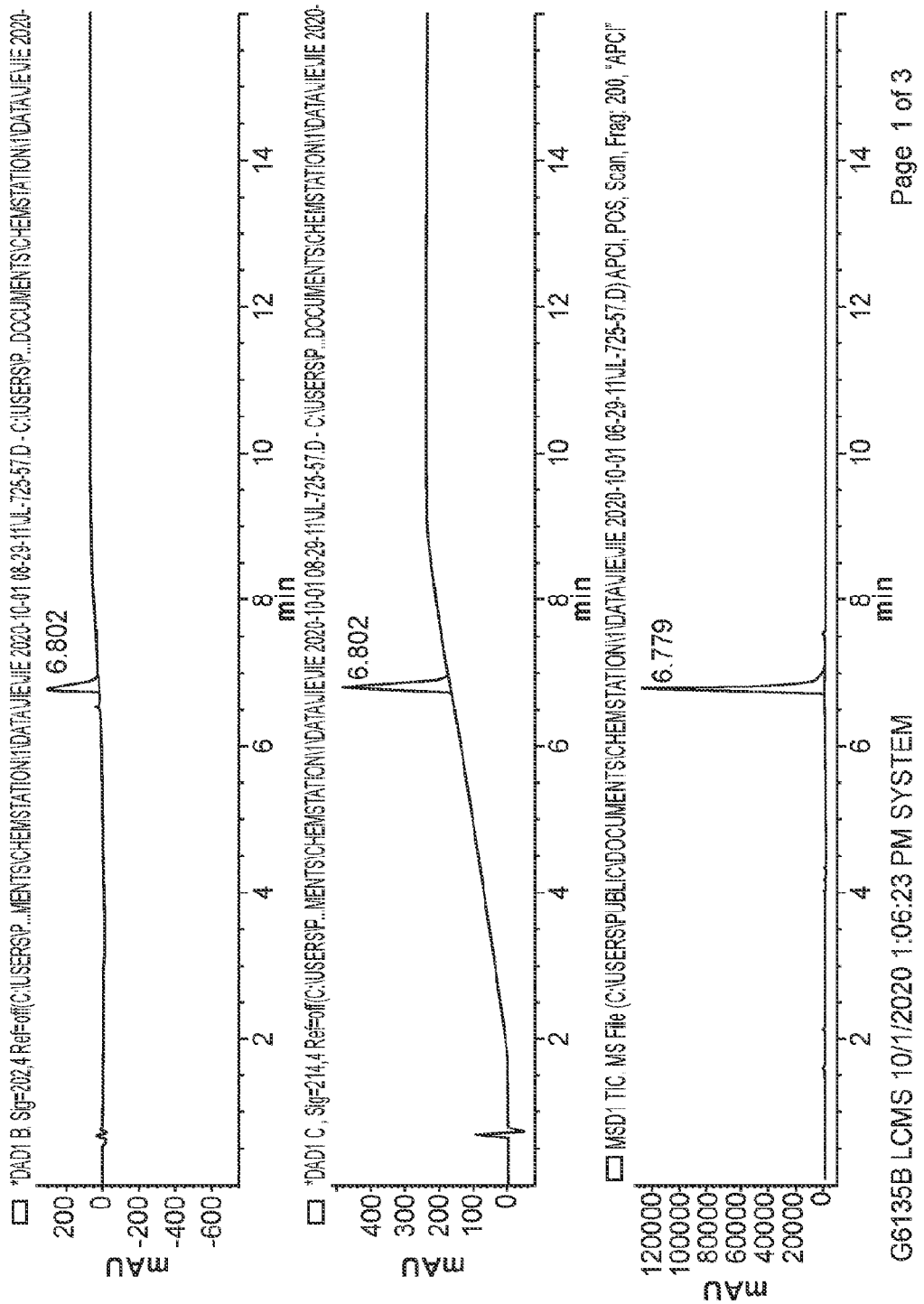
*DAD1 B, Sig=202,4 Ref=off (C:\USERSP...MENTS\CHEMSTATION\1\DATA\JIE\JIE 2020-10-01 08-29-11\JL-725-57.D - C:\USERSP...DOCUMENTS\CHEMSTATION\1\DATA\JIE\JIE 2020-
6.802
mAU
min
*DAD1 C, Sig=214,4 Ref=off (C:\USERSP...MENTS\CHEMSTATION\1\DATA\JIE\JIE 2020-10-01 08-29-11\JL-725-57.D - C:\USERSP...DOCUMENTS\CHEMSTATION\1\DATA\JIE\JIE 2020-
6.802
mAU
min
MSD1 TIC, MS File (C:\USERSP\PUBLIC\DOCUMENTS\CHEMSTATION\1\DATA\JIE\JIE 2020-10-01 08-29-11\JL-725-57.D) APCI, POS, Scan, Frag: 200, "APCI"
6.779
mAU
min
G6135B LCMS 10/1/2020 1:06:23 PM SYSTEM
Page 1 of 3

FIG. 30B

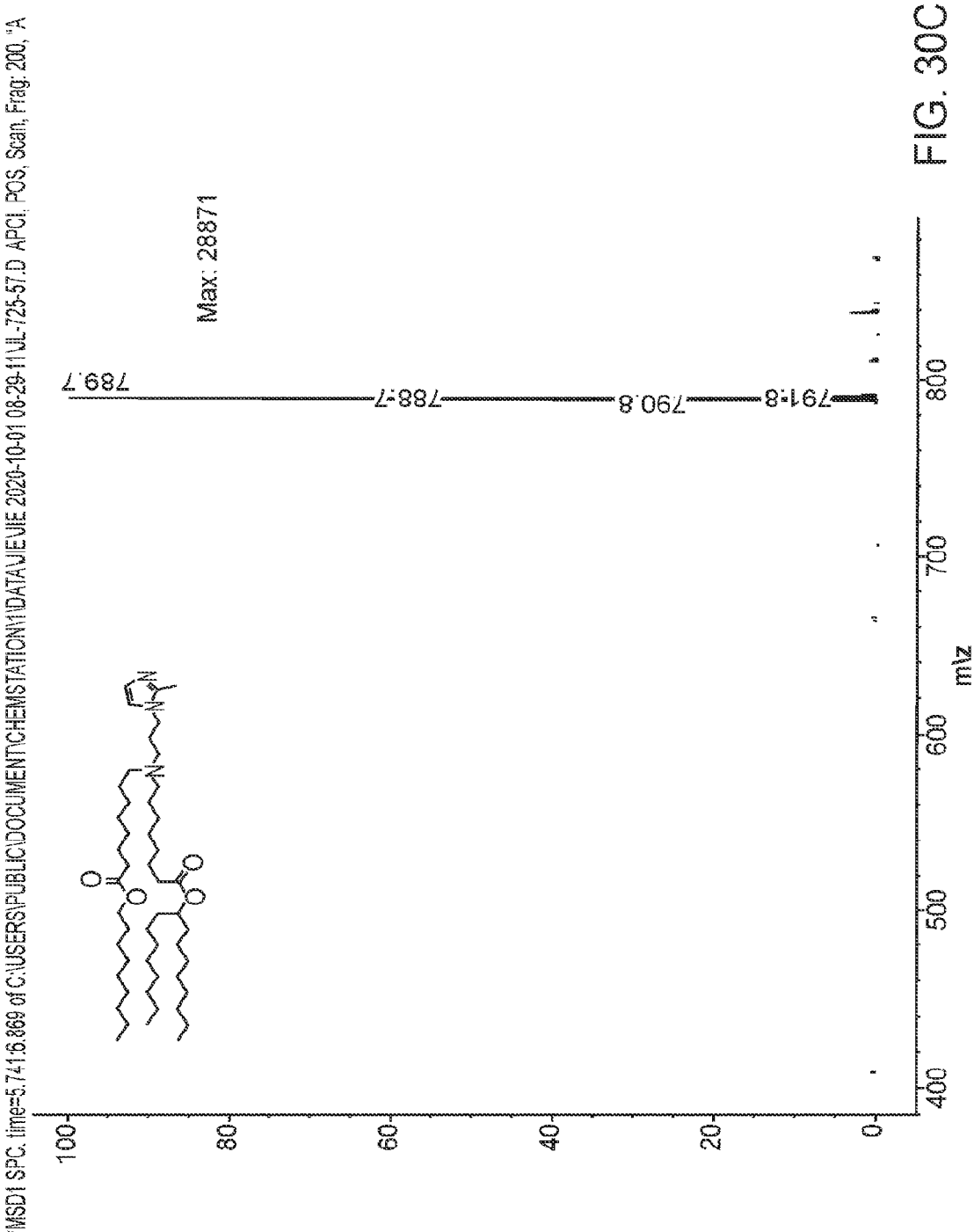
*MSD1 SPC, time=5.741:6.869 of C:\USERS\PUBLIC\DOCUMENTS\HEMSTATION\1\DATA\JEUE 2020-10-01 08-29-11\JL-725-57.D APCI, POS, Scan, Frag: 200, "A
Max: 28871
789.7
788.7
790.8
791.8
100
80
60
40
20
0
400
500
600
700
800
m/z

FIG. 30C

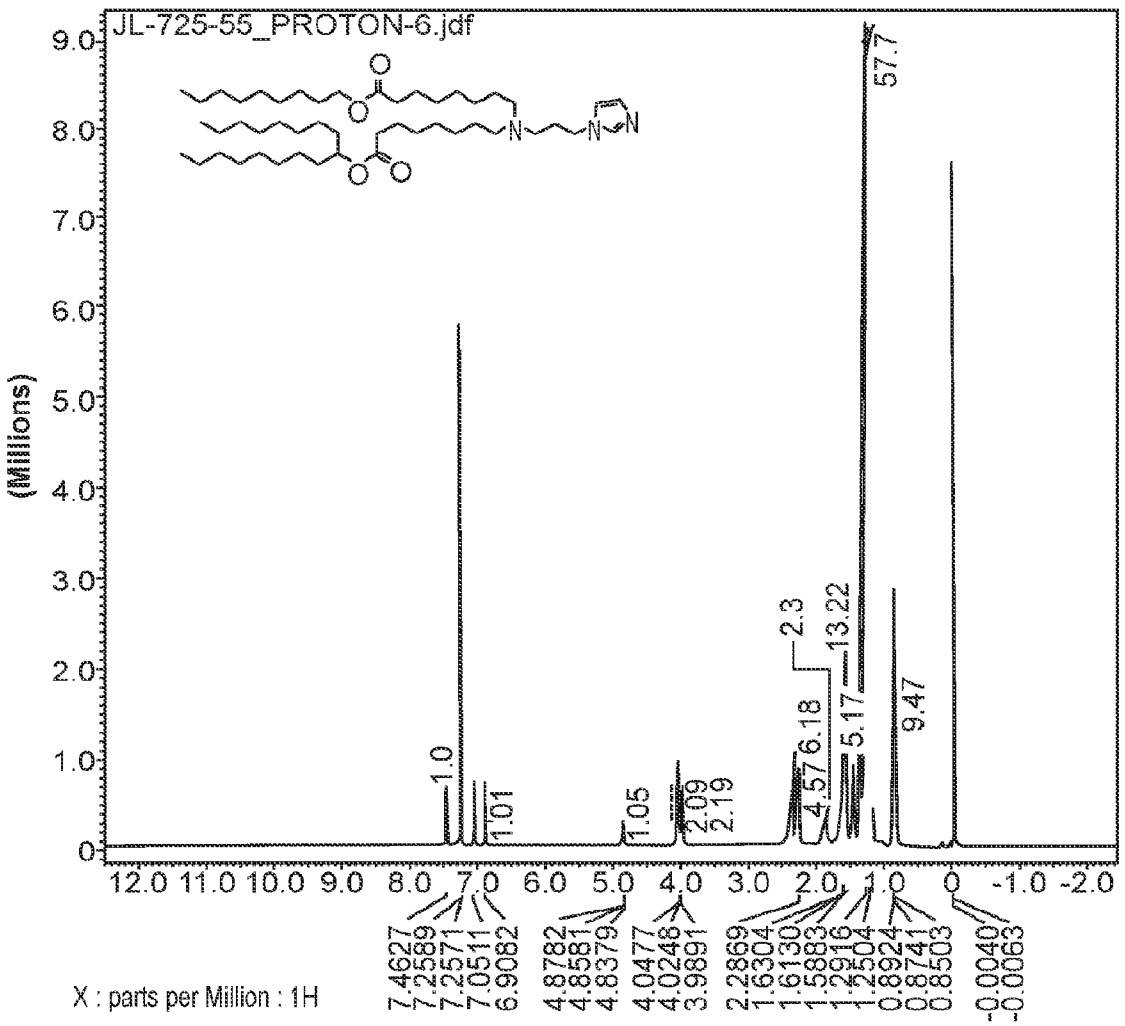
JL-725-55_PROTON-6.jdf
(Millions)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
12.0 11.0 10.0 9.0 8.0 7.0 6.0 5.0 4.0 3.0 2.0 1.0 0 -1.0 -2.0
1.0
1.01
1.05
2.09
2.19
4.57
6.18
2.3
13.22
5.17
57.7
9.47
7.4627
7.2589
7.2571
7.0511
6.9082
4.8782
4.8581
4.8379
4.0477
4.0248
3.9891
2.2869
1.6304
1.6130
1.5883
1.2916
1.2504
0.8924
0.8741
0.8503
-0.0040
-0.0063
X : parts per Million : 1H

FIG. 31A

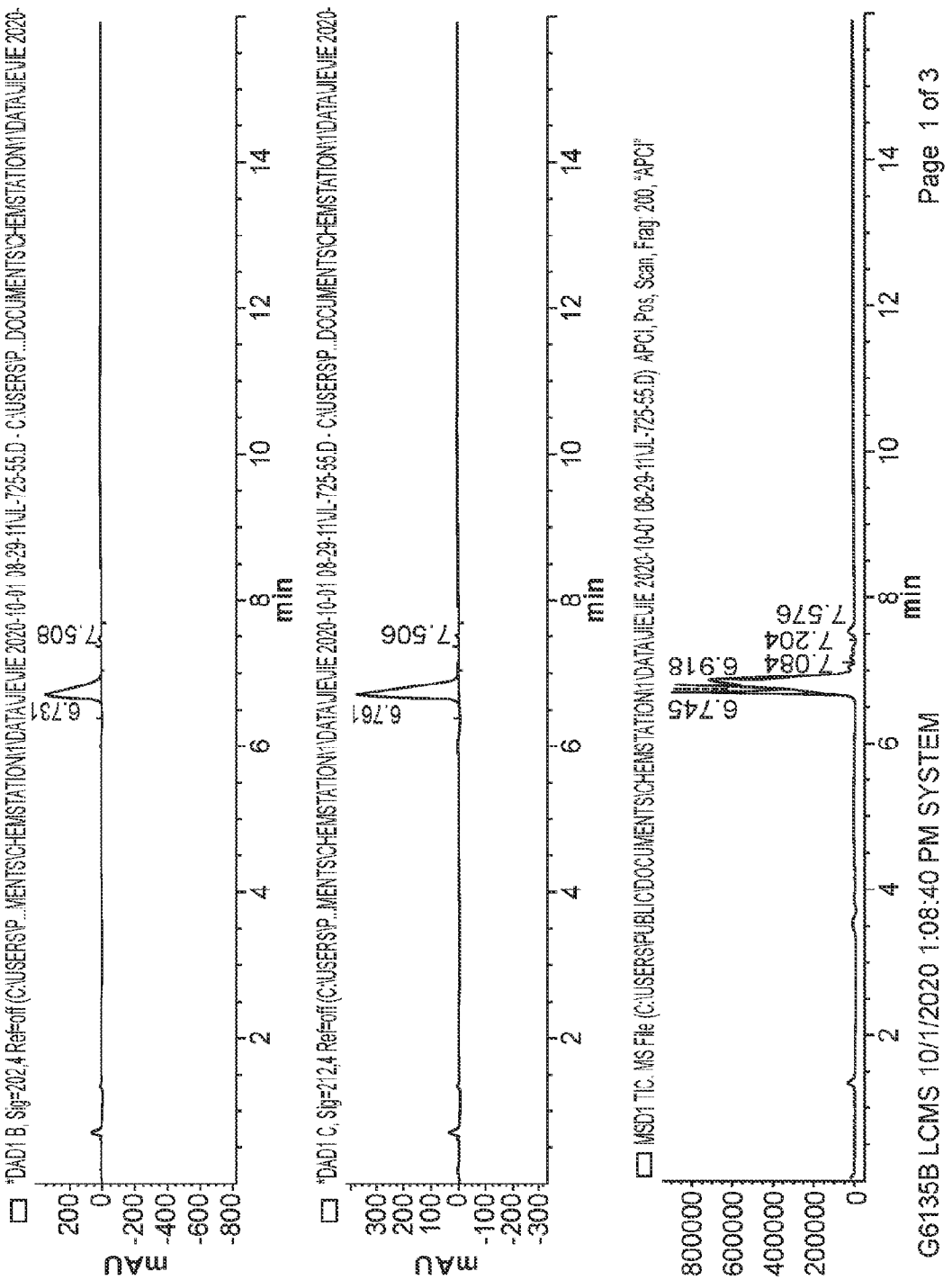
*DAD1 B, Sig=202,4 Ref=off (C:\USERSIP...MENTSICHEMSTATION\1\DATA\JIEWJIE 2020-10-01 08-29-11\WL-725-55.D - C:\USERSIP...DOCUMENTSICHEMSTATION\1\DATA\JIEWJIE 2020-
mAU
min
6.731
7.508
*DAD1 C, Sig=212,4 Ref=off (C:\USERSIP...MENTSICHEMSTATION\1\DATA\JIEWJIE 2020-10-01 08-29-11\WL-725-55.D - C:\USERSIP...DOCUMENTSICHEMSTATION\1\DATA\JIEWJIE 2020-
mAU
min
6.761
7.506
MSD1 TIC, MS File (C:\USERSIPUBLIC\DOCUMENTSICHEMSTATION\1\DATA\JIEWJIE 2020-10-01 08-29-11\WL-725-55.D) APCI, Pos, Scan, Frag: 200, "APCI"
6.745
6.918
7.084
7.204
7.576
min
G6135B LCMS 10/1/2020 1:08:40 PM SYSTEM
Page 1 of 3

FIG. 31B

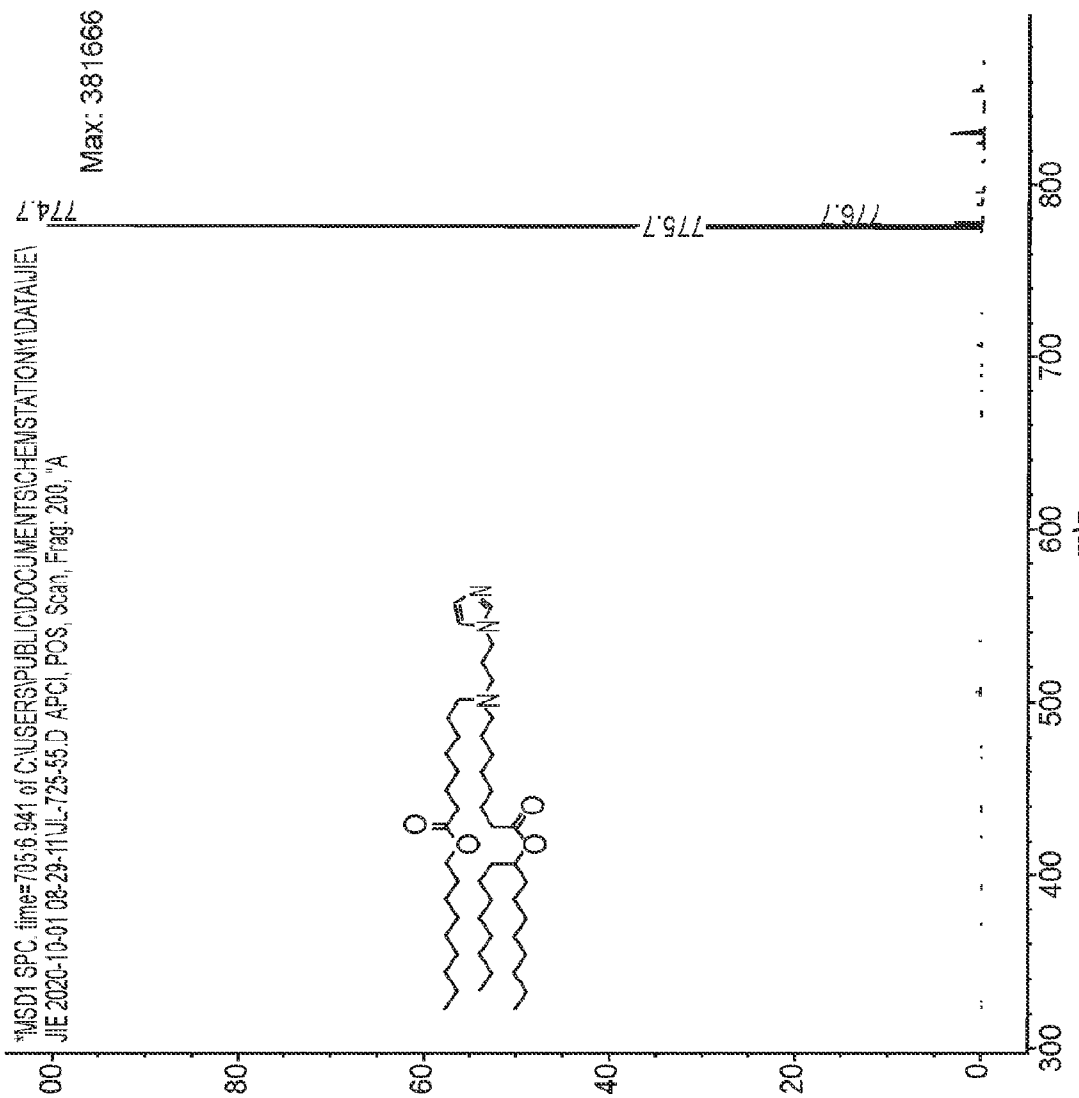
*MSD1 SPC, time=705.6.941 of C:\USERS\PUBLIC\DOCUMENTS\CHEMSTATION\1\DATA\JIE\JIE 2020-10-01 08-29-11\JL-725-55.D APCI, POS, Scan, Frag: 200, "A
Max: 381666
774.7
775.7
776.7
100
80
60
40
20
0
300
400
500
600
700
800
m/z

FIG. 31C

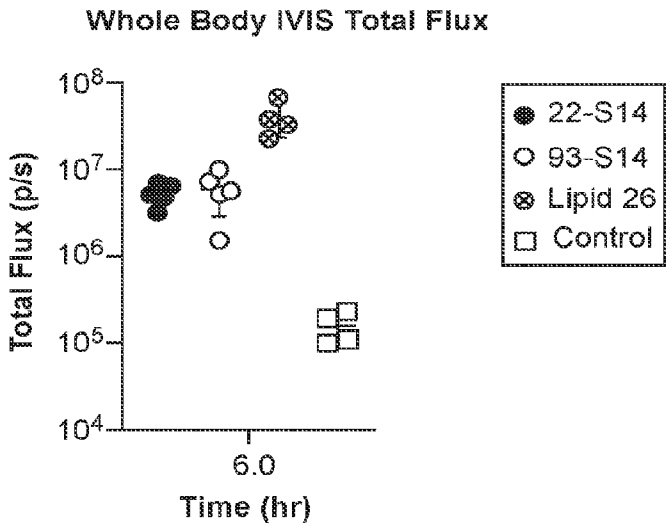

FIG. 32A

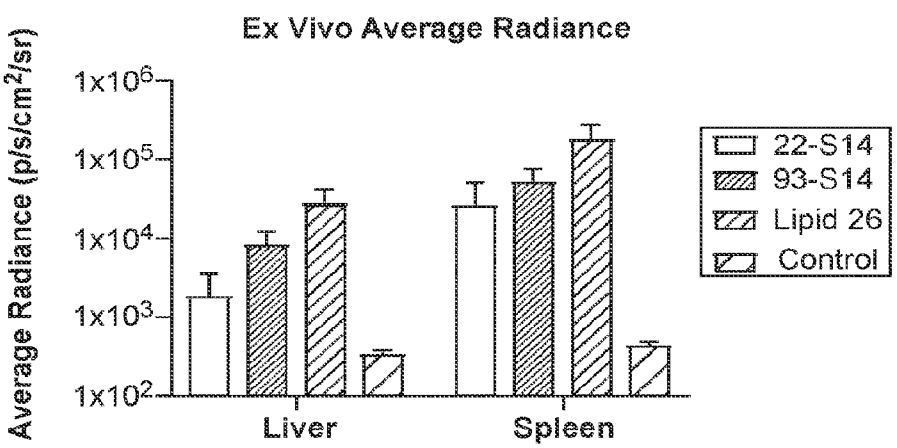

FIG. 32B

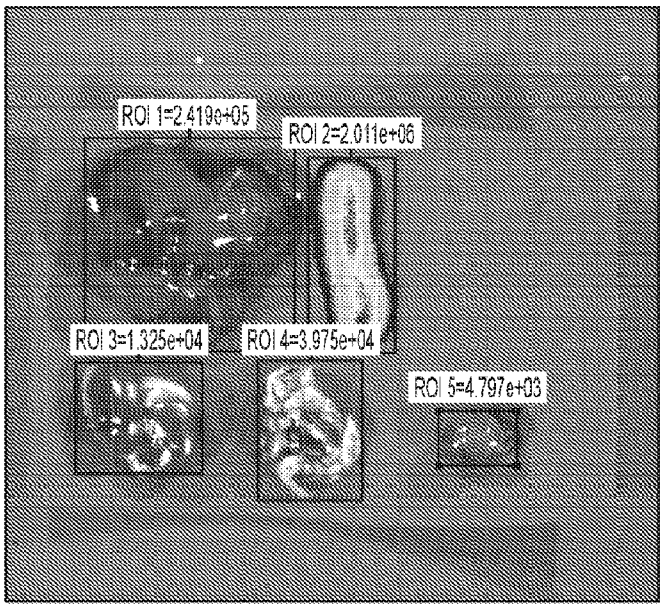

FIG. 33A

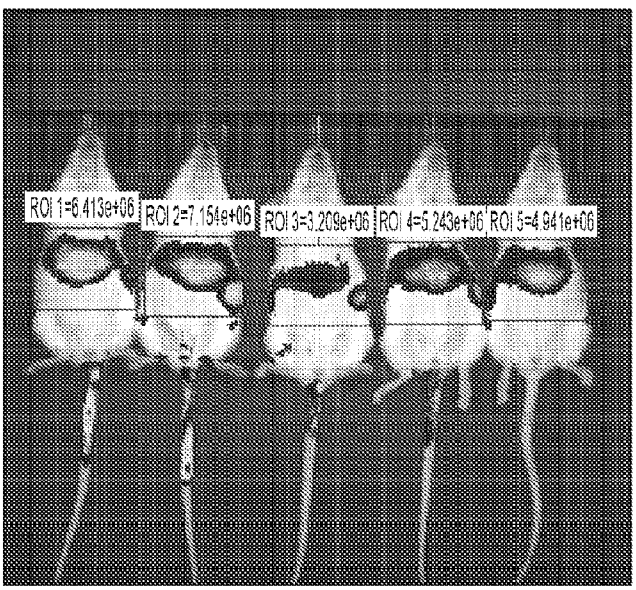

FIG. 33B

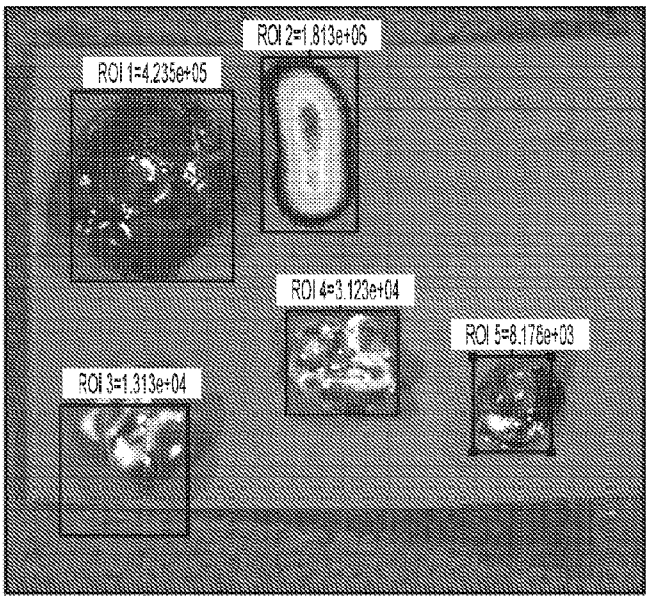

FIG. 34A

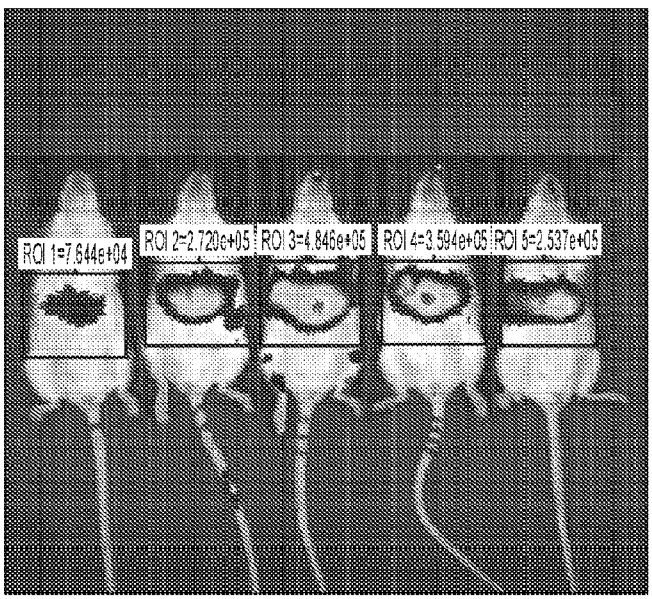

FIG. 34B

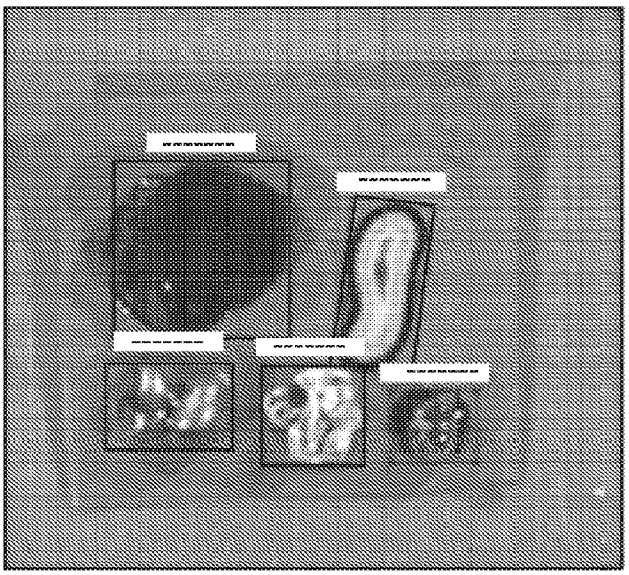

FIG. 35A

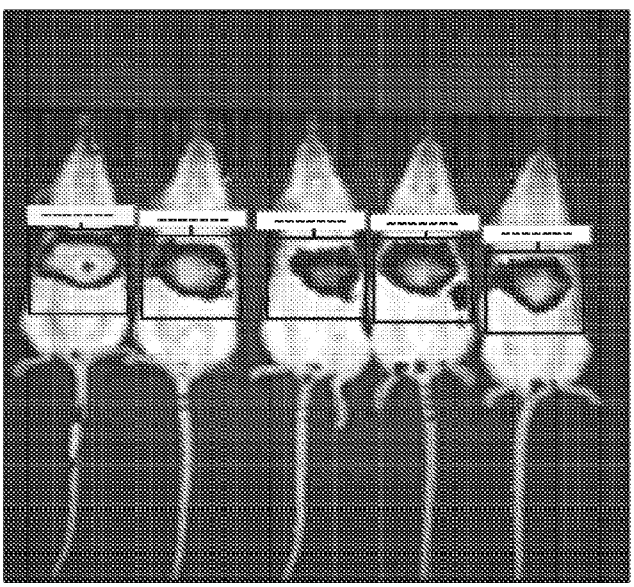

FIG. 35B

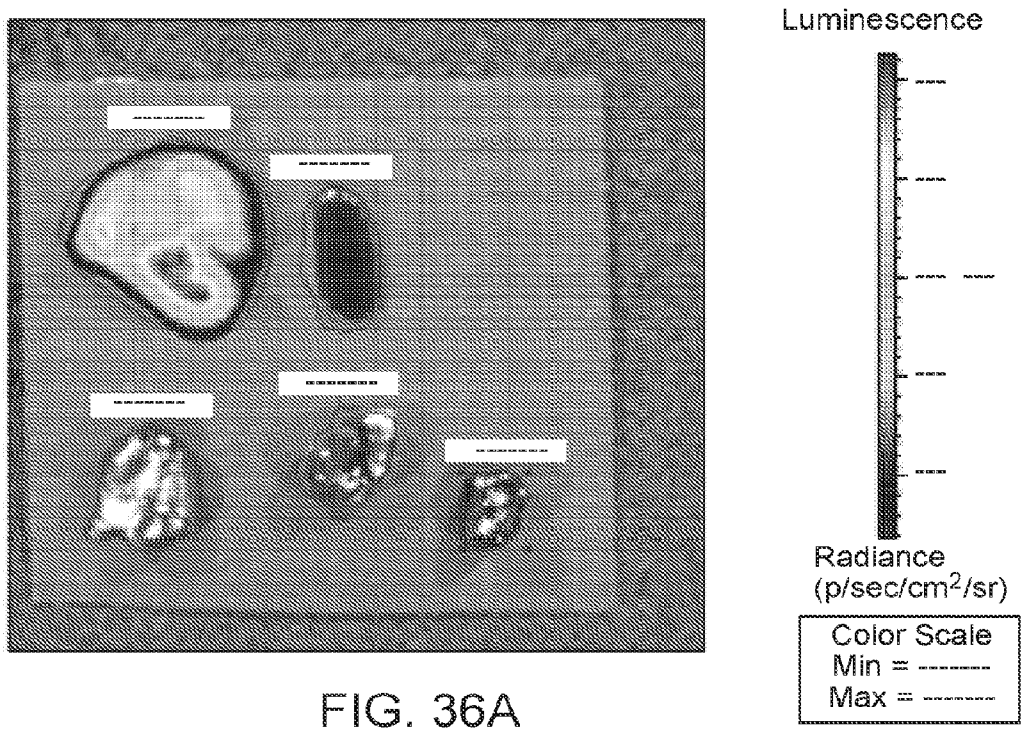

FIG. 36A

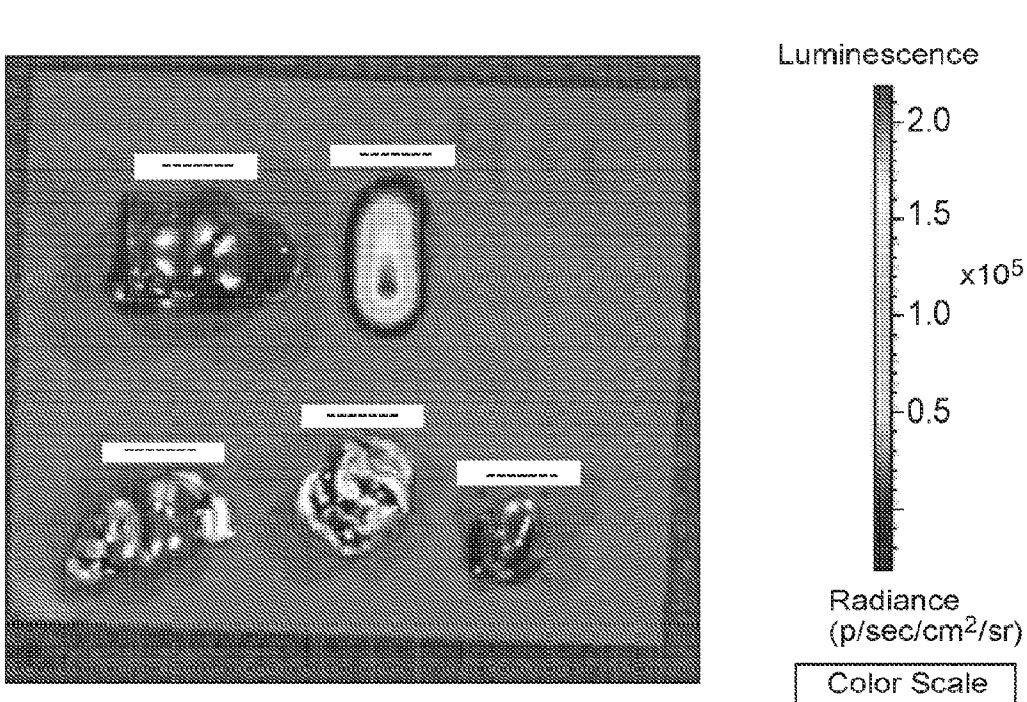

FIG. 36B

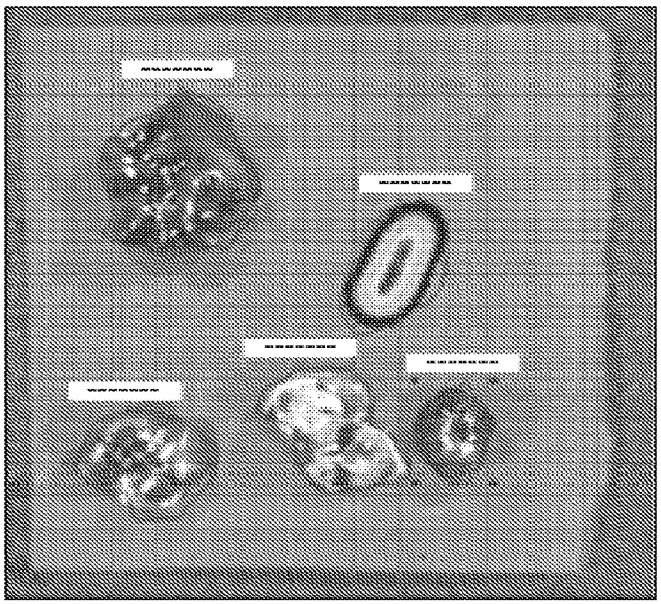

FIG. 36C

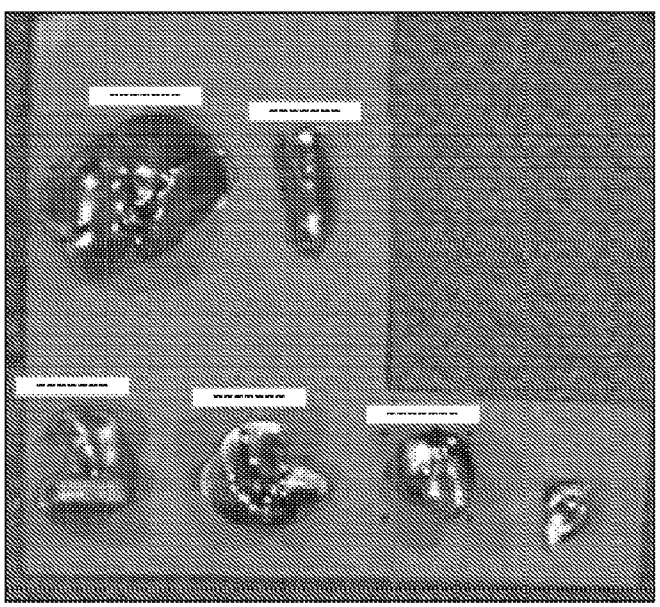

FIG. 36D

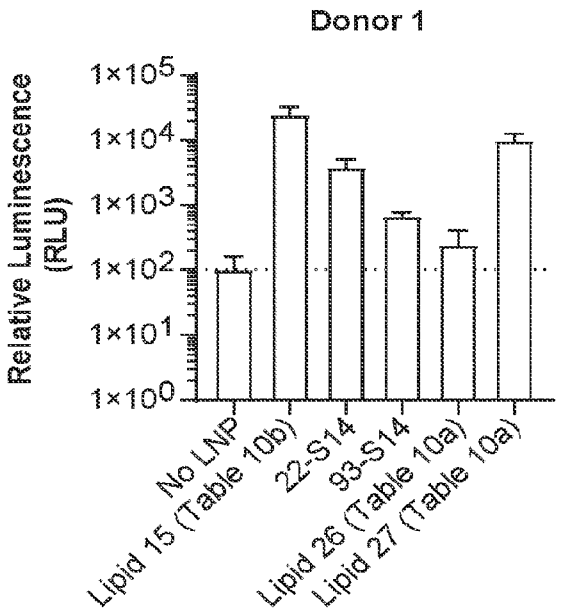

FIG. 37A

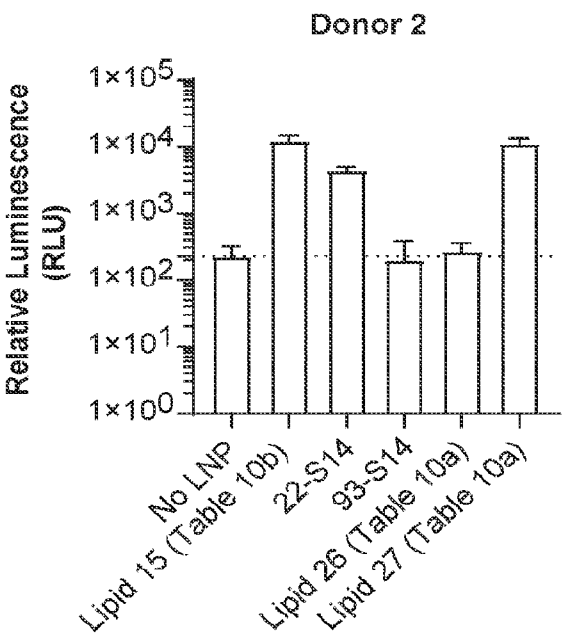

FIG. 37B

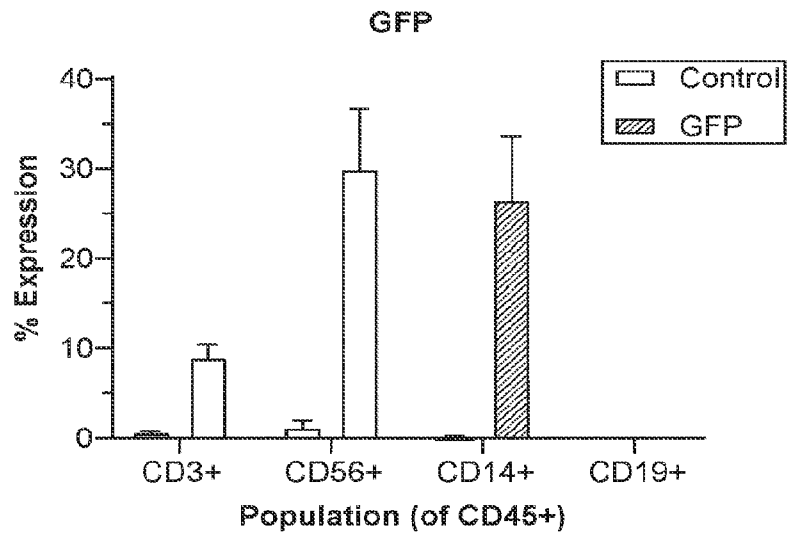

FIG. 38A

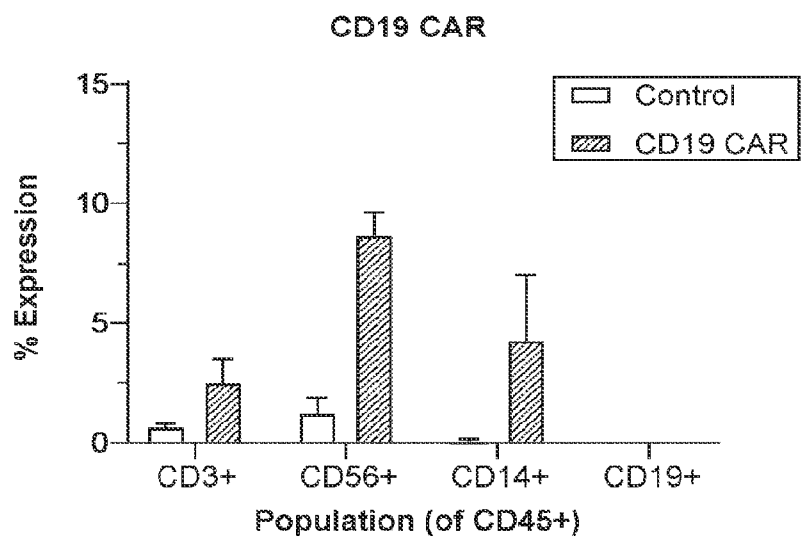

FIG. 38B

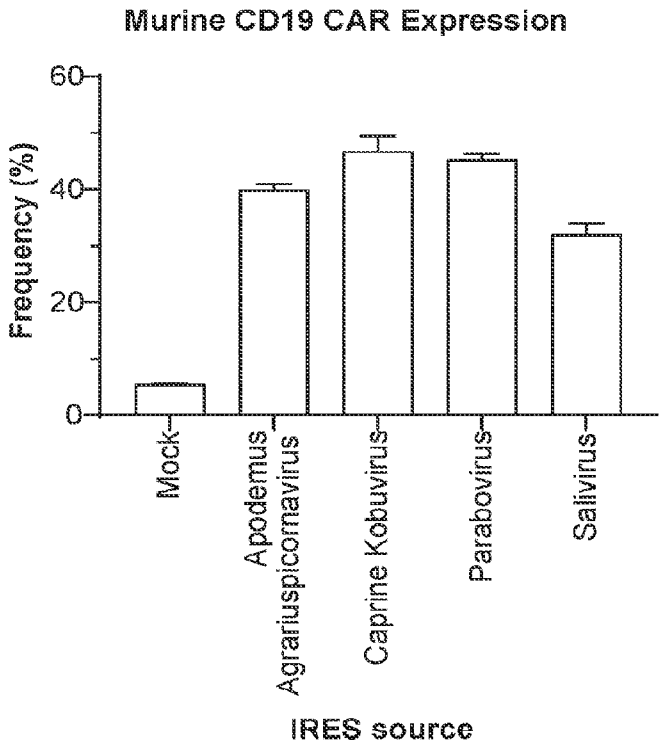
Murine CD19 CAR Expression
Frequency (%)
60
40
20
0
Mock
Apodemus Agrariuspicornavirus
Caprine Kobuvirus
Parabovirus
Salivirus
IRES source

FIG. 39

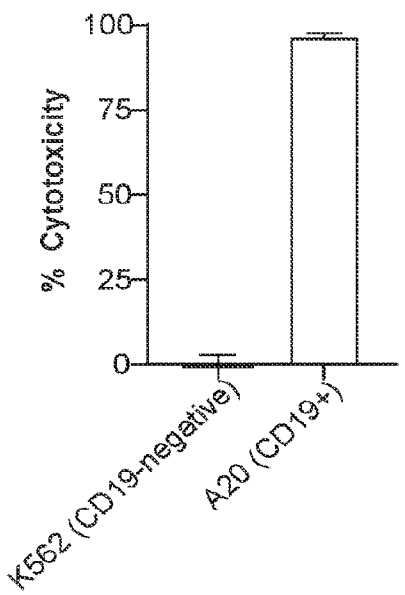
% Cytotoxicity
100
75
50
25
0
K562 (CD19-negative)
A20 (CD19+)

FIG. 40

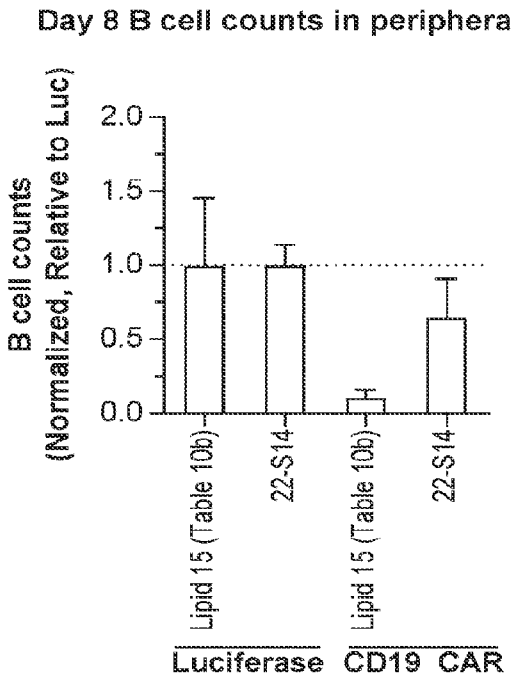

FIG. 41A

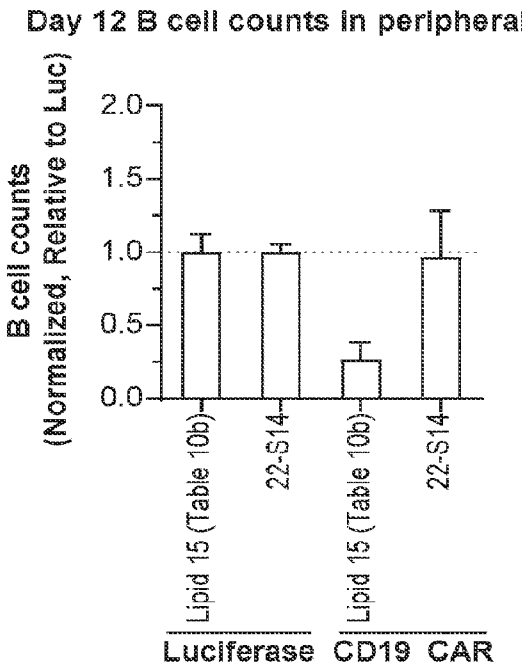

FIG. 41B

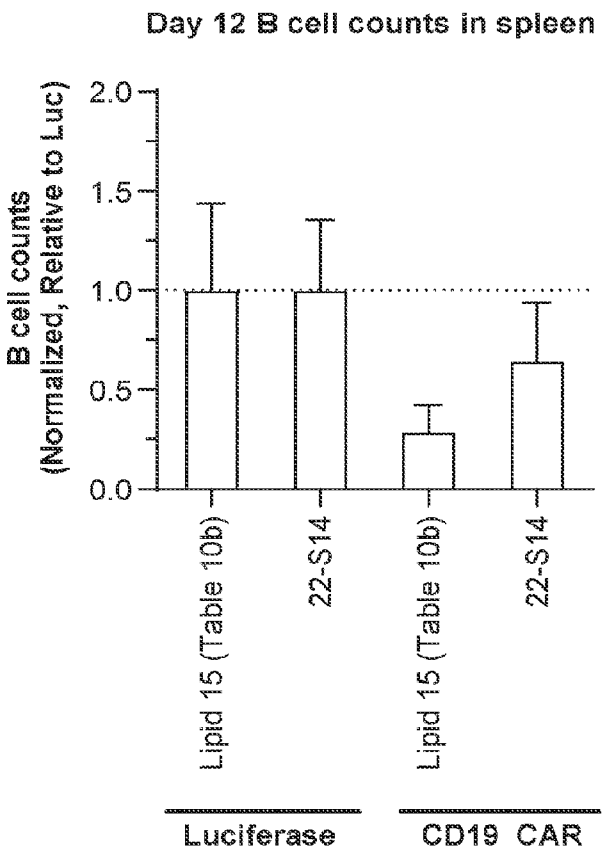
Day 12 B cell counts in spleen
B cell counts
(Normalized, Relative to Luc)
2.0
1.5
1.0
0.5
0.0
Lipid 15 (Table 10b)
22-S14
Lipid 15 (Table 10b)
22-S14
Luciferase
CD19 CAR

FIG. 41C

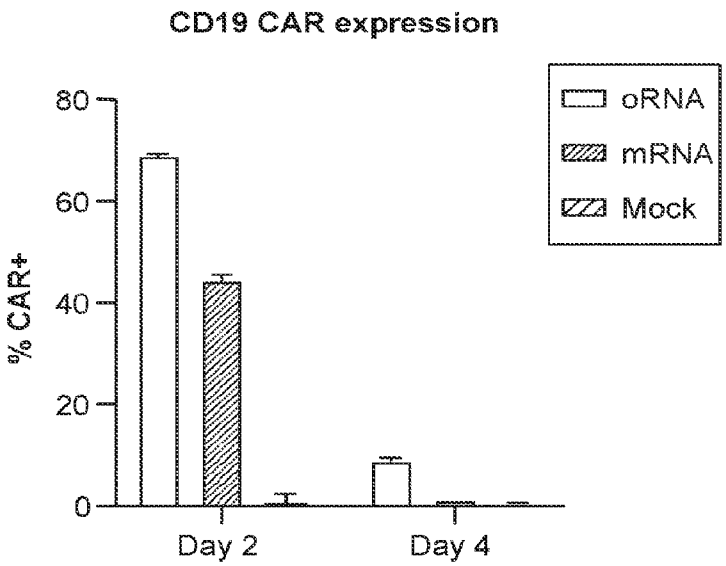

FIG. 42A

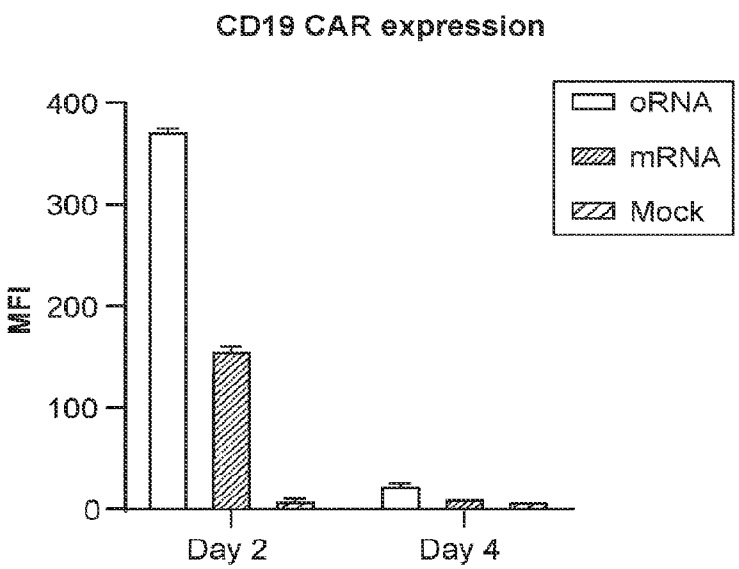

FIG. 42B

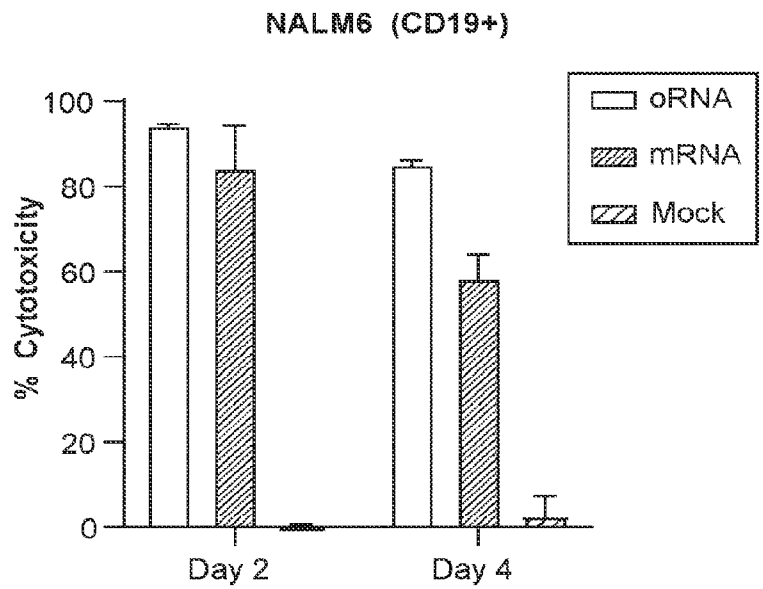

FIG. 43A

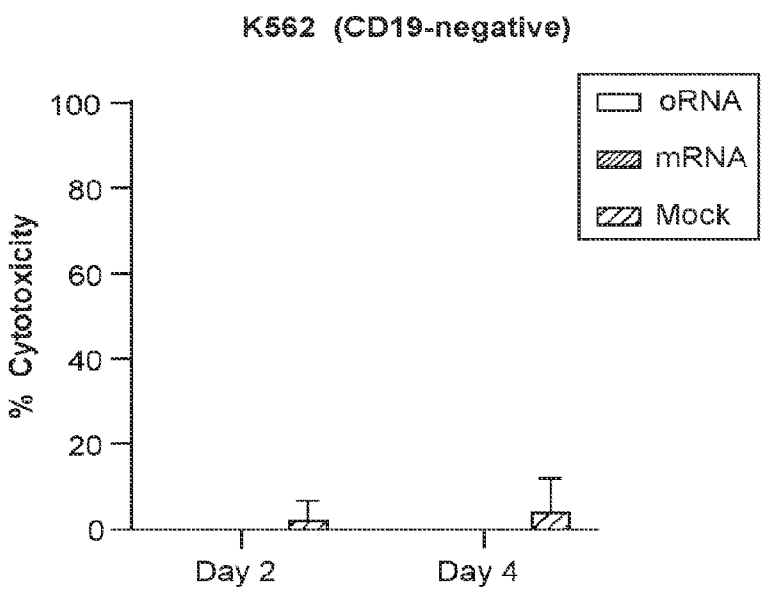

FIG. 43B

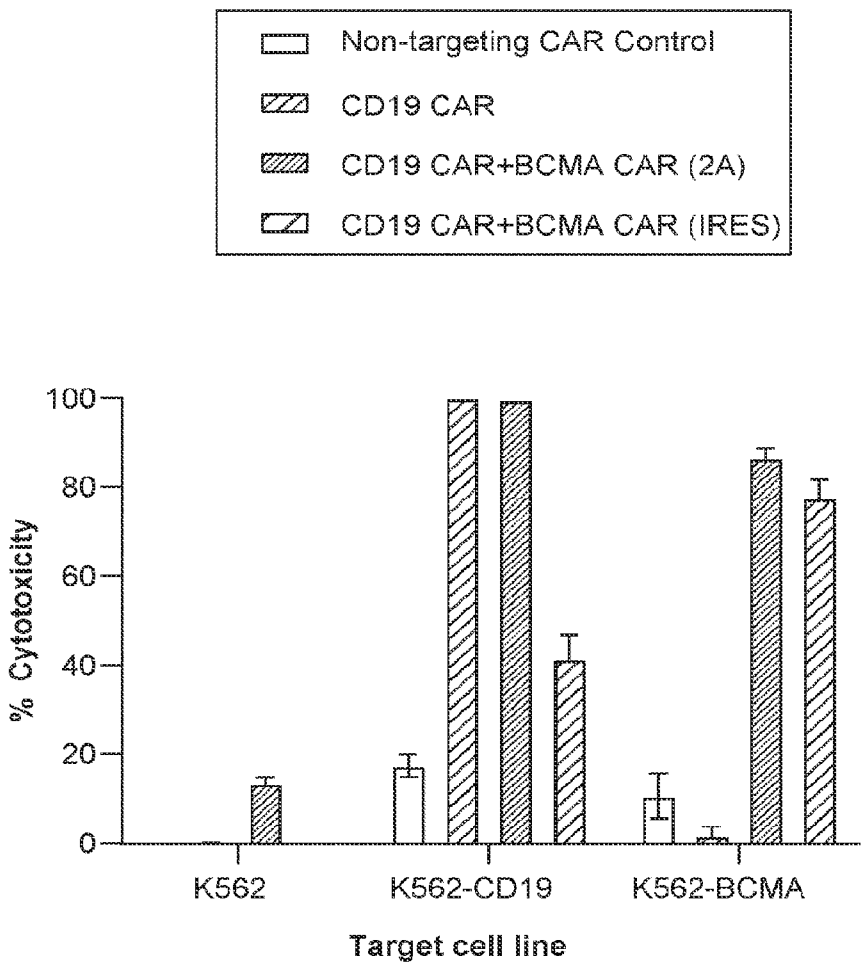
Non-targeting CAR Control
CD19 CAR
CD19 CAR+BCMA CAR (2A)
CD19 CAR+BCMA CAR (IRES)
% Cytotoxicity
100
80
60
40
20
0
K562
K562-CD19
K562-BCMA
Target cell line

FIG. 44

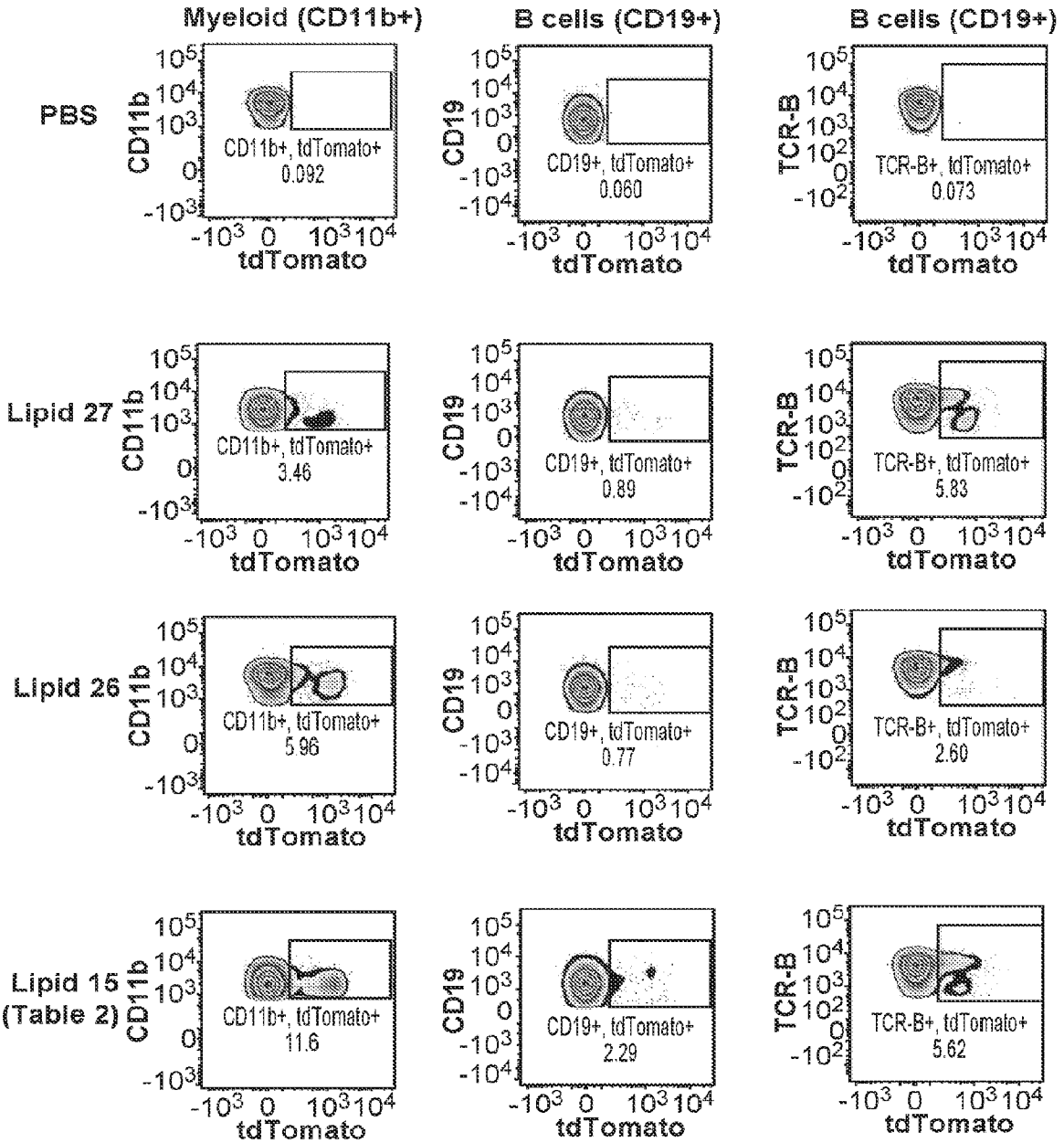
Myeloid (CD11b+)
B cells (CD19+)
B cells (CD19+)
PBS
Lipid 27
Lipid 26
Lipid 15 (Table 2)
CD11b
CD19
TCR-B
tdTomato
CD11b+, tdTomato+ 0.092
CD19+, tdTomato+ 0.060
TCR-B+, tdTomato+ 0.073
CD11b+, tdTomato+ 3.46
CD19+, tdTomato+ 0.89
TCR-B+, tdTomato+ 5.83
CD11b+, tdTomato+ 5.96
CD19+, tdTomato+ 0.77
TCR-B+, tdTomato+ 2.60
CD11b+, tdTomato+ 11.6
CD19+, tdTomato+ 2.29
TCR-B+, tdTomato+ 5.62

FIG. 45A

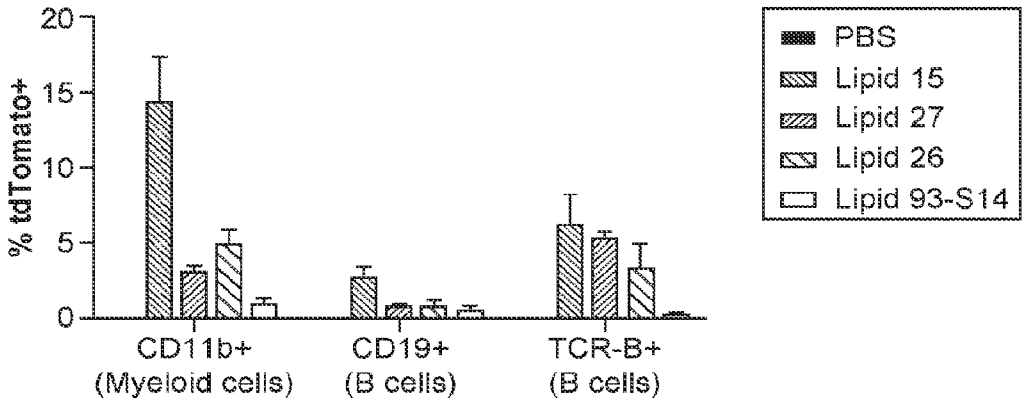

FIG. 45B

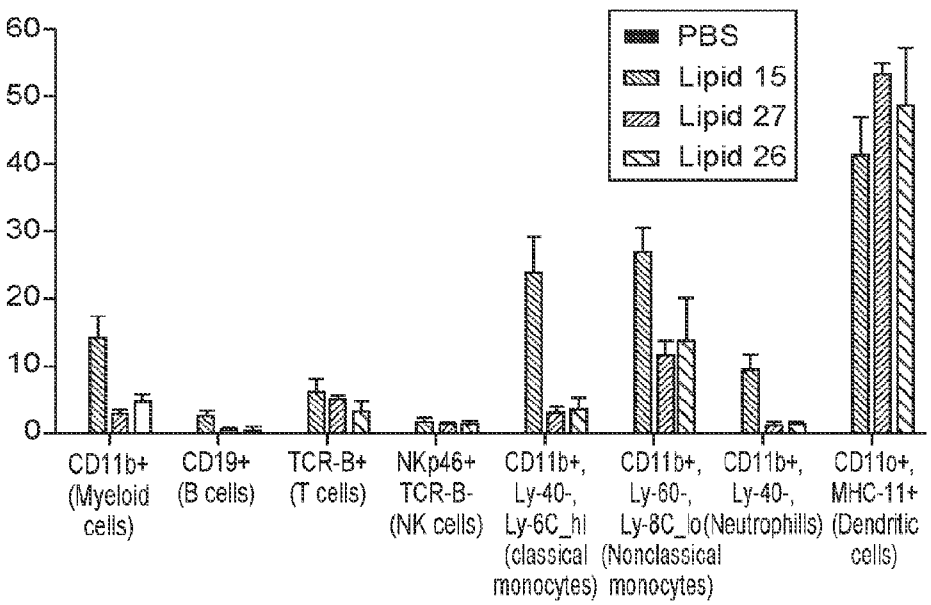

FIG. 45C

FIG. 46A

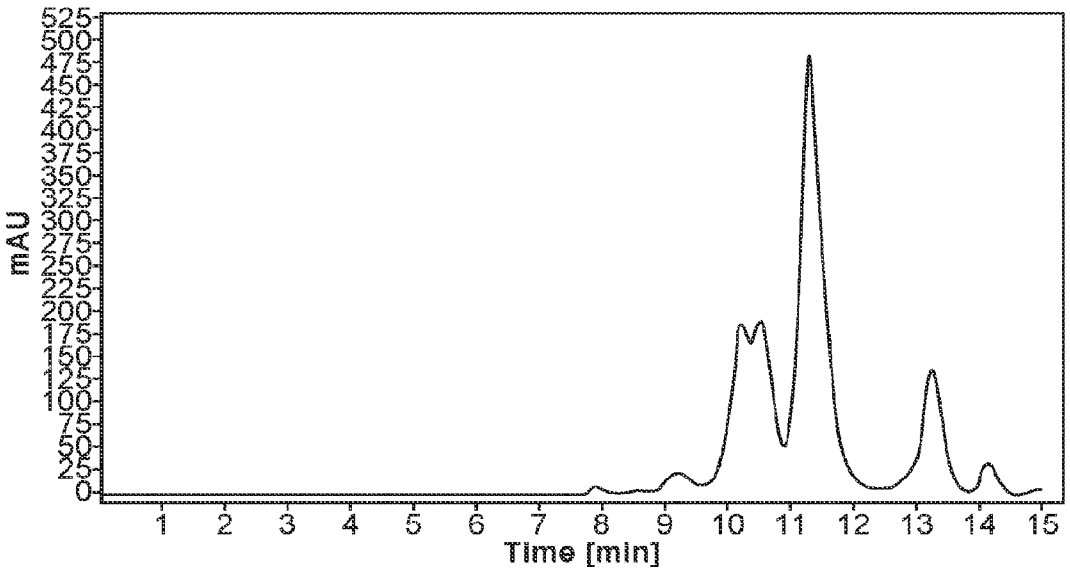

FIG. 46B

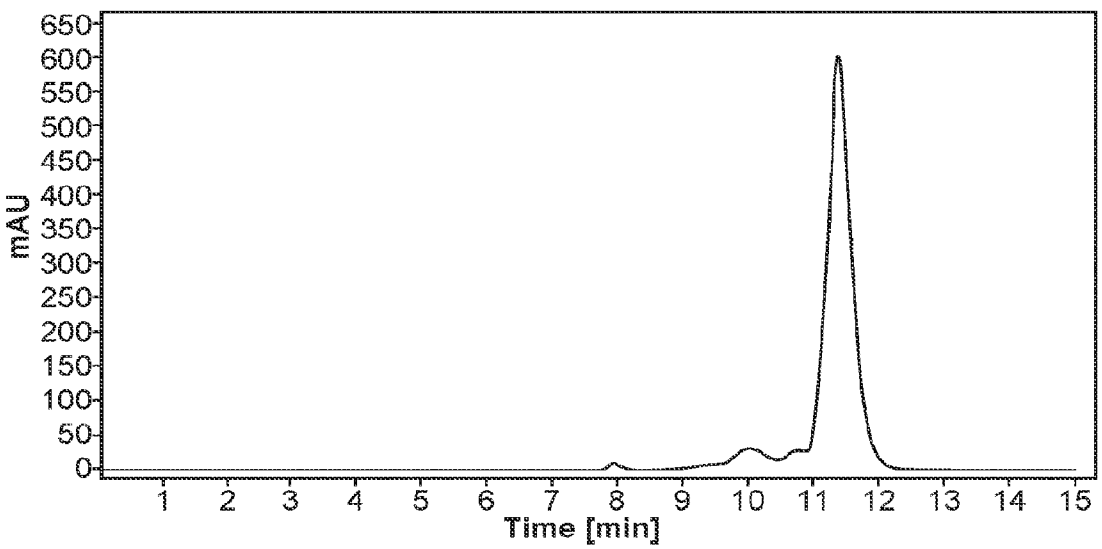

FIG. 46C

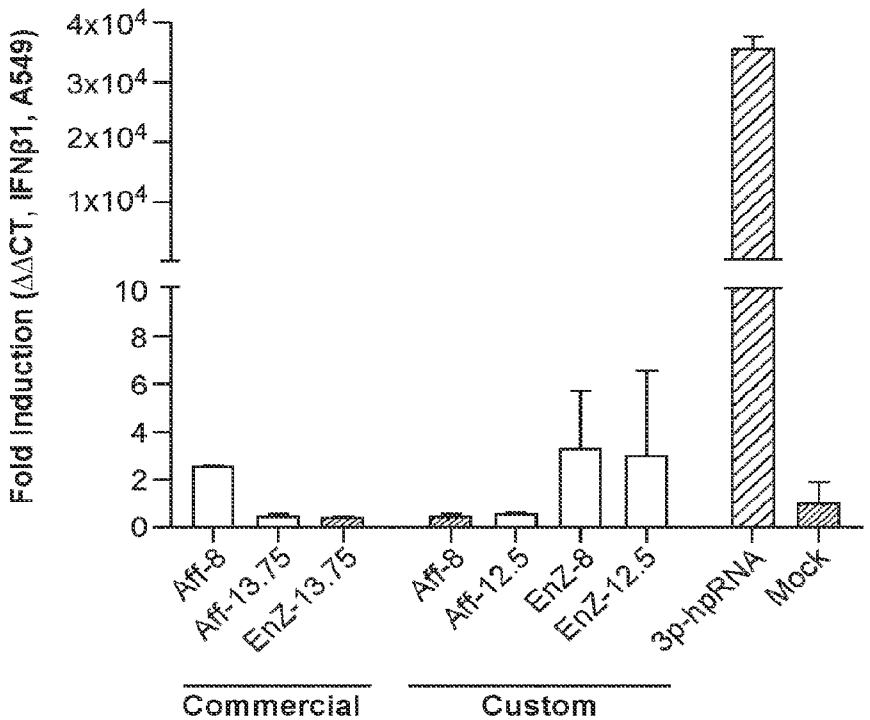
Fold Induction (ΔΔCT, IFNβ1, A549)
4x10^4
3x10^4
2x10^4
1x10^4
10
8
6
4
2
0
Aff-8
Aff-13.75
EnZ-13.75
Aff-8
Aff-12.5
EnZ-8
EnZ-12.5
3p-hpRNA
Mock
Commercial
Custom

FIG. 46D

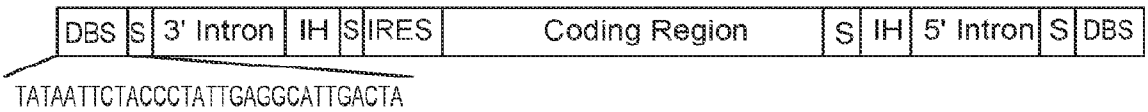

FIG. 47A

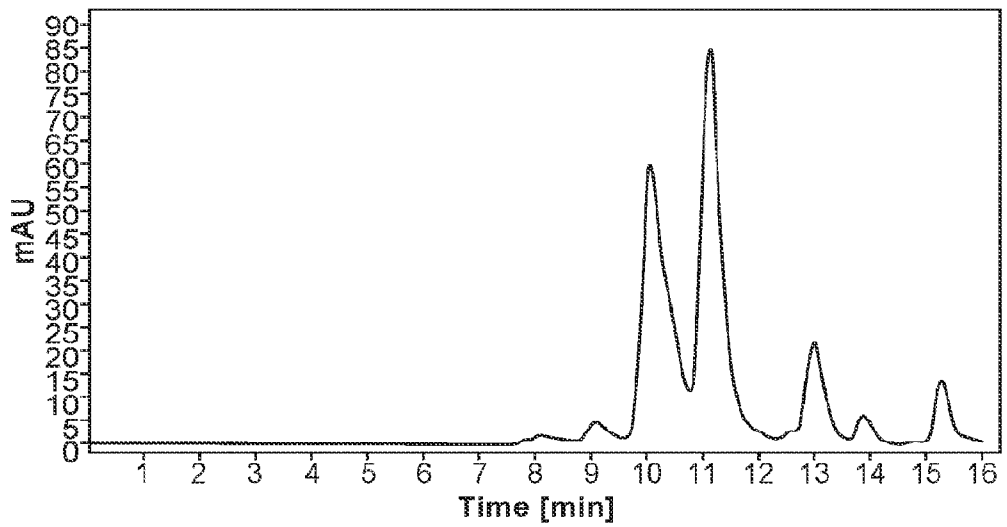

FIG. 47B

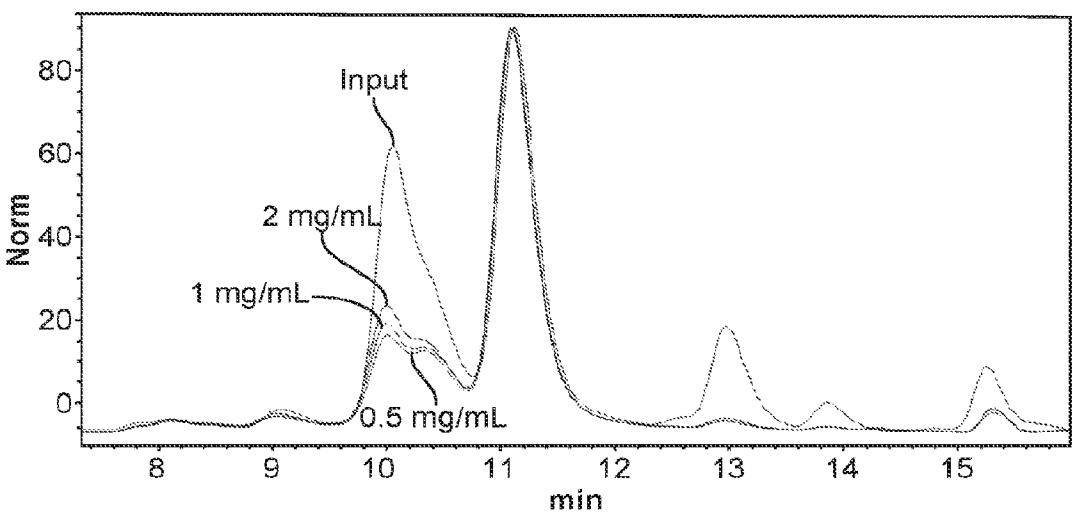

FIG. 47C

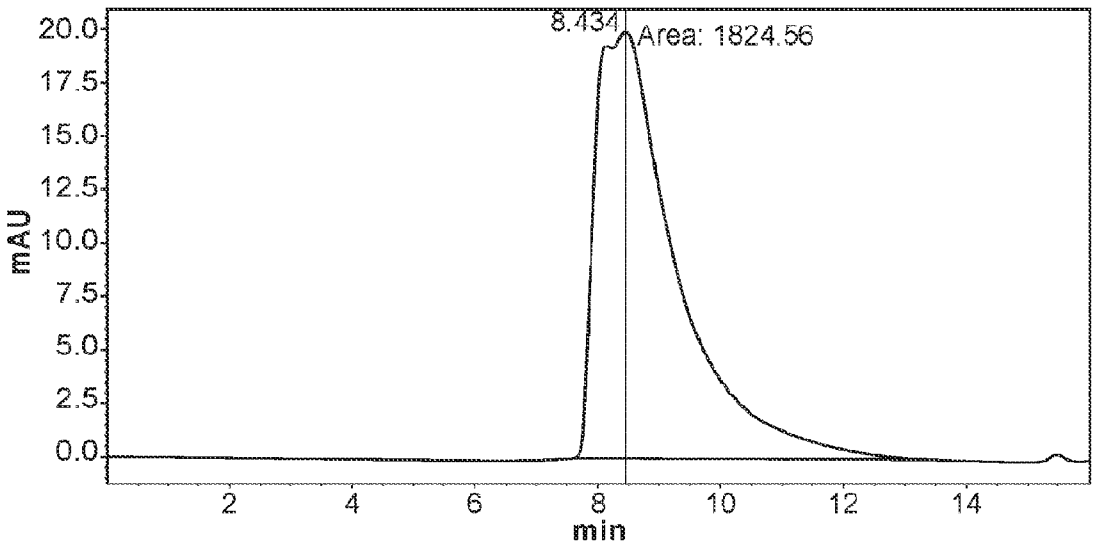

FIG. 48A

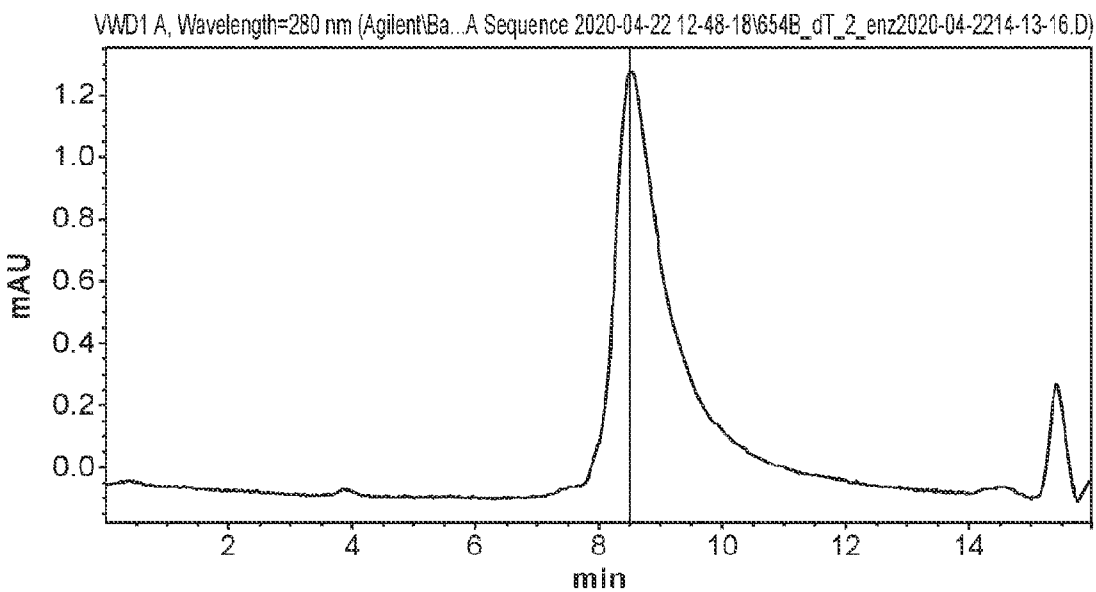

FIG. 48B

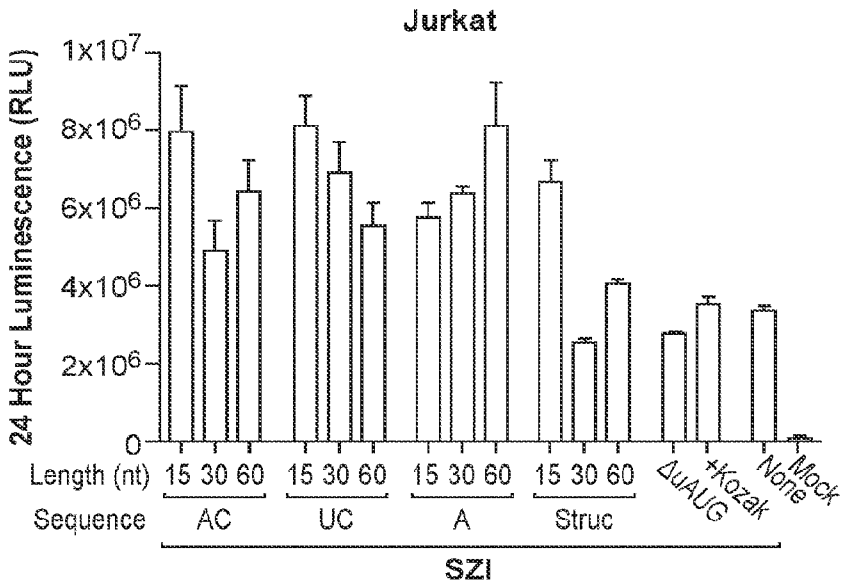

FIG. 49A

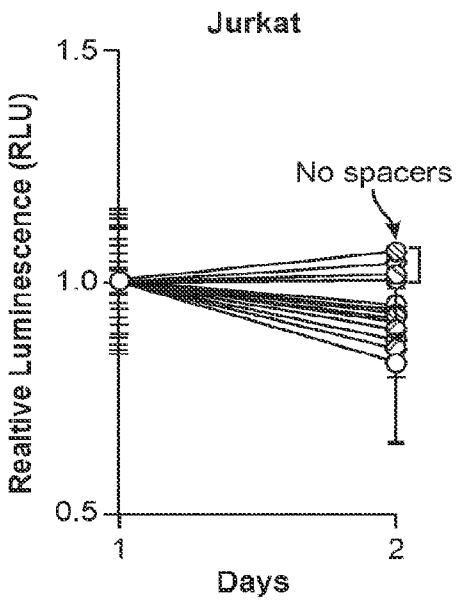

FIG. 49B

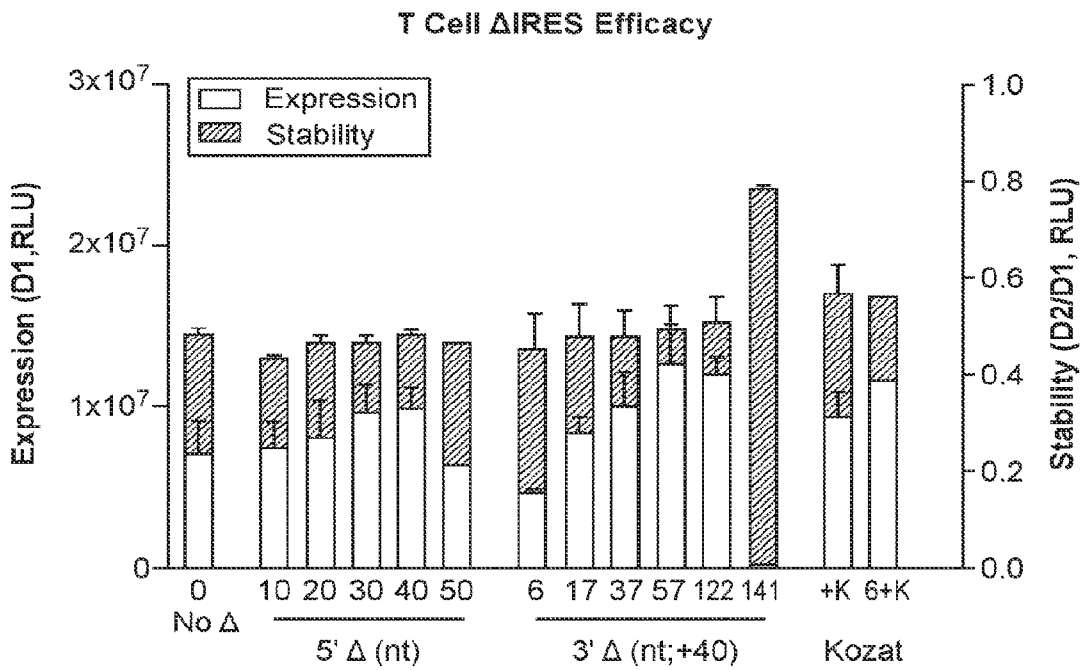
T Cell ΔIRES Efficacy
Expression
Stability
Expression (D1,RLU)
Stability (D2/D1, RLU)
3x10^7
2x10^7
1x10^7
0
1.0
0.8
0.6
0.4
0.2
0.0
0
No Δ
10 20 30 40 50
5' Δ (nt)
6 17 37 57 122 141
3' Δ (nt;+40)
+K 6+K
Kozat

FIG. 50

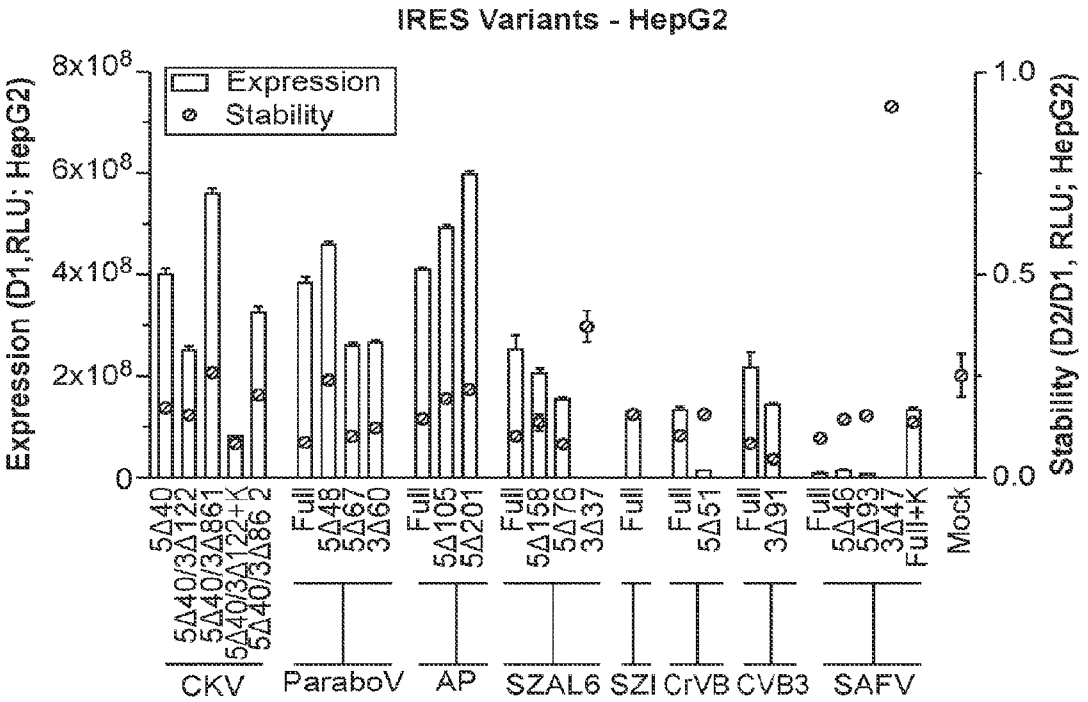
IRES Variants - HepG2
Expression
Stability
Expression (D1,RLU; HepG2)
Stability (D2/D1, RLU; HepG2)
8x10^8
6x10^8
4x10^8
2x10^8
0
1.0
0.5
0.0
5Δ40
5Δ40/3Δ122
5Δ40/3Δ861
5Δ40/3Δ122+K
5Δ40/3Δ862
Full
5Δ48
5Δ67
3Δ60
Full
5Δ105
5Δ201
Full
5Δ158
5Δ76
3Δ37
Full
Full
5Δ51
Full
3Δ91
Full
5Δ46
5Δ93
3Δ47
Full+K
Mock
CKV
ParaboV
AP
SZAL6
SZI
CrVB
CVB3
SAFV

FIG. 51

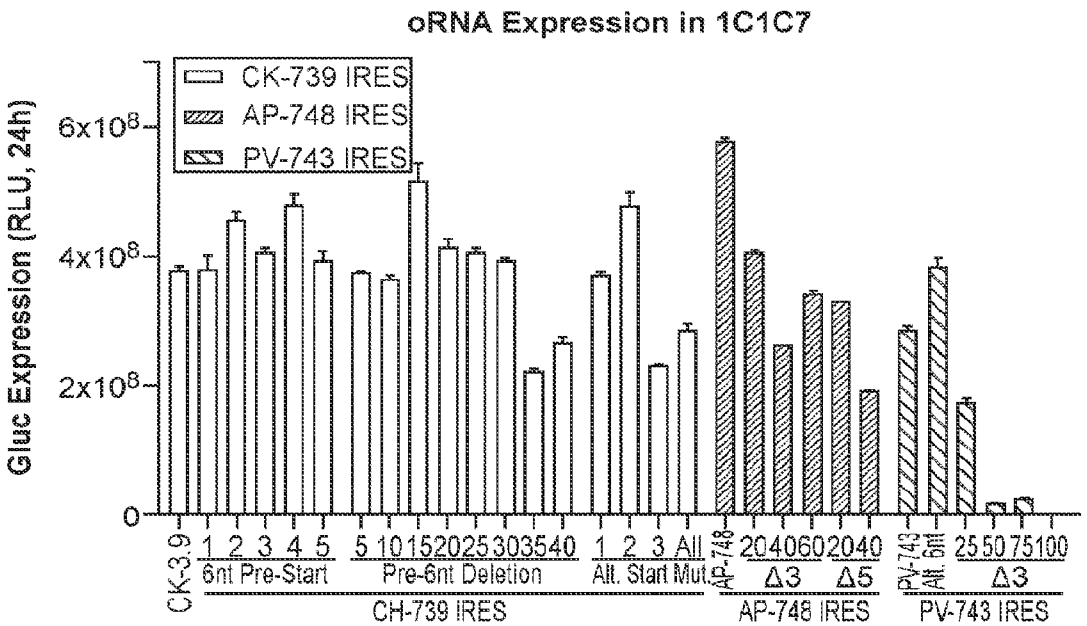
oRNA Expression in 1C1C7
CK-739 IRES
AP-748 IRES
PV-743 IRES
Gluc Expression (RLU, 24h)
6x10^8
4x10^8
2x10^8
0
CK-3.9
1 2 3 4 5
5 10 15 20 25 30 35 40
1 2 3 All
AP-748
20 40 60
20 40
PV-743 Alt. 6nt
25 50 75 100
6nt Pre-Start
Pre-6nt Deletion
Alt. Start Mut.
Δ3
Δ5
Δ3
CH-739 IRES
AP-748 IRES
PV-743 IRES

FIG. 52

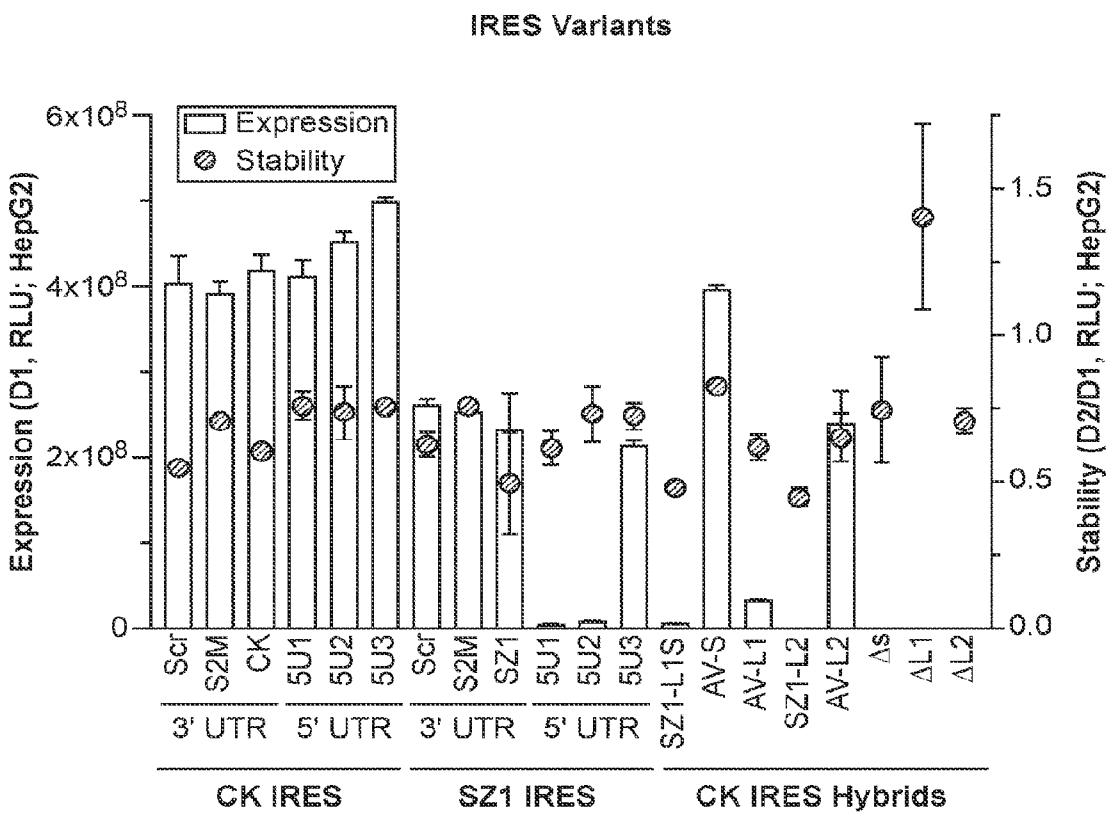
IRES Variants
Expression
Stability
Expression (D1, RLU; HepG2)
Stability (D2/D1, RLU; HepG2)
6x10^8
4x10^8
2x10^8
0
1.5
1.0
0.5
0.0
Scr
S2M
CK
5U1
5U2
5U3
Scr
S2M
SZ1
5U1
5U2
5U3
SZ1-L1S
AV-S
AV-L1
SZ1-L2
AV-L2
Δs
ΔL1
ΔL2
3' UTR
5' UTR
3' UTR
5' UTR
CK IRES
SZ1 IRES
CK IRES Hybrids

FIG. 53

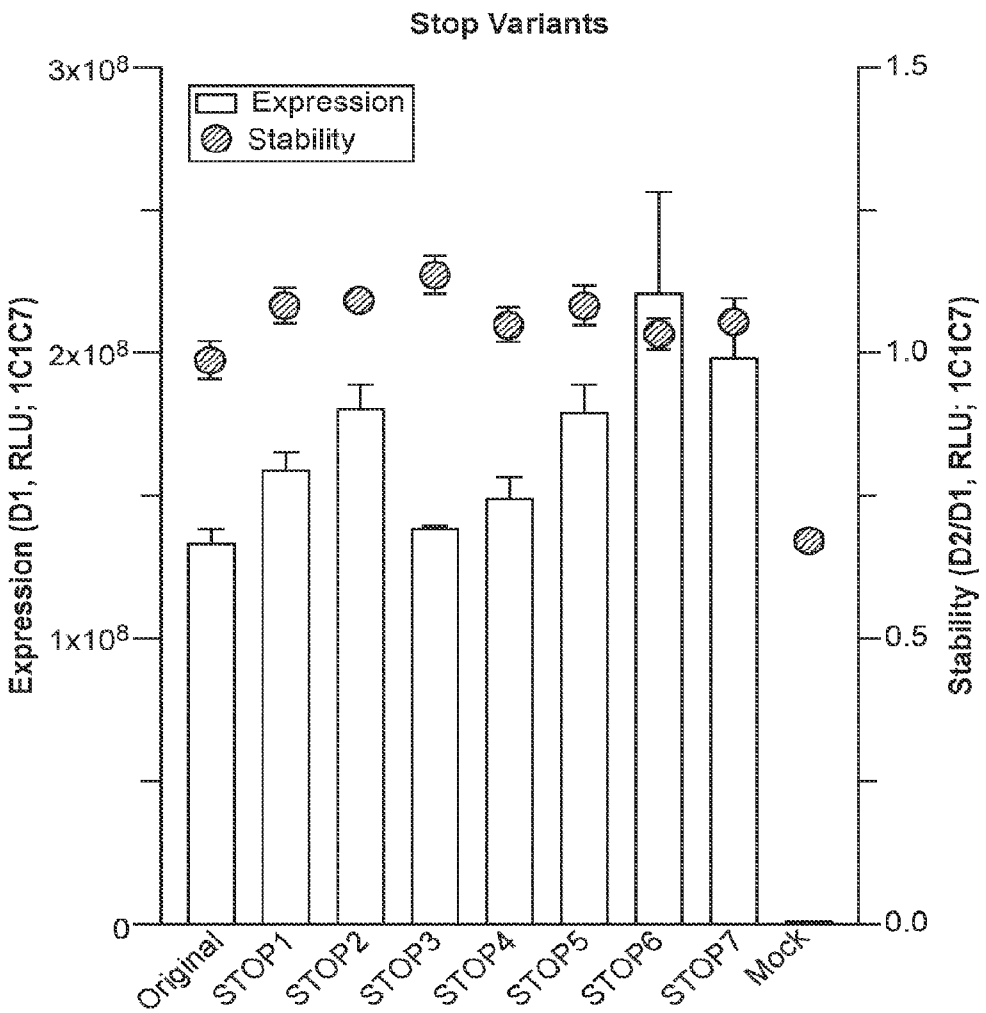
Stop Variants
Expression
Stability
Expression (D1, RLU; 1C1C7)
Stability (D2/D1, RLU; 1C1C7)
3x10^8
2x10^8
1x10^8
0
1.5
1.0
0.5
0.0
Original
STOP1
STOP2
STOP3
STOP4
STOP5
STOP6
STOP7
Mock

FIG. 54

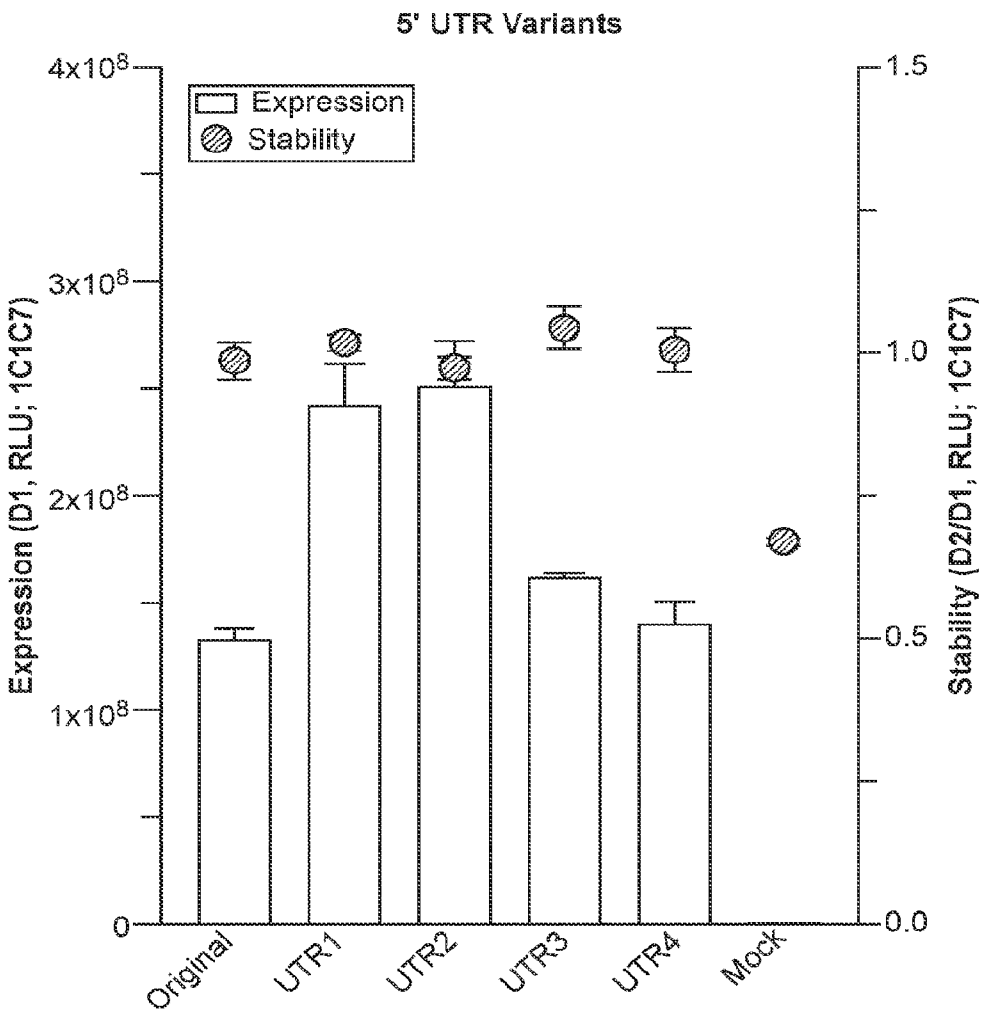
5' UTR Variants
Expression
Stability
Expression (D1, RLU; 1C1C7)
Stability (D2/D1, RLU; 1C1C7)
4x10^8
3x10^8
2x10^8
1x10^8
0
1.5
1.0
0.5
0.0
Original
UTR1
UTR2
UTR3
UTR4
Mock

FIG. 55

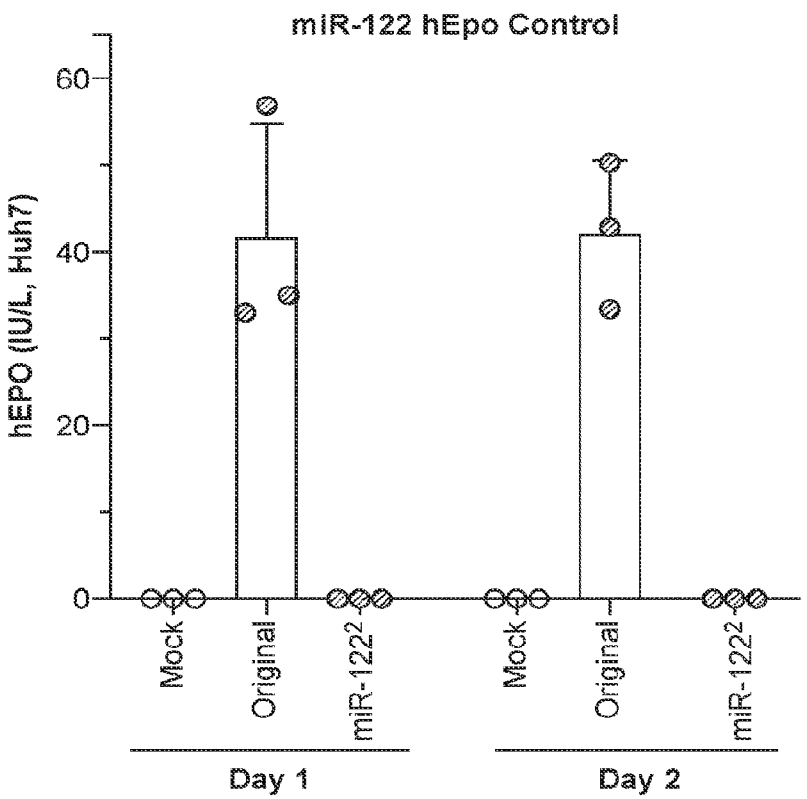
miR-122 hEpo Control
hEPO (IU/L, Huh7)
60
40
20
0
Mock
Original
miR-122
Mock
Original
miR-122
Day 1
Day 2

FIG. 56

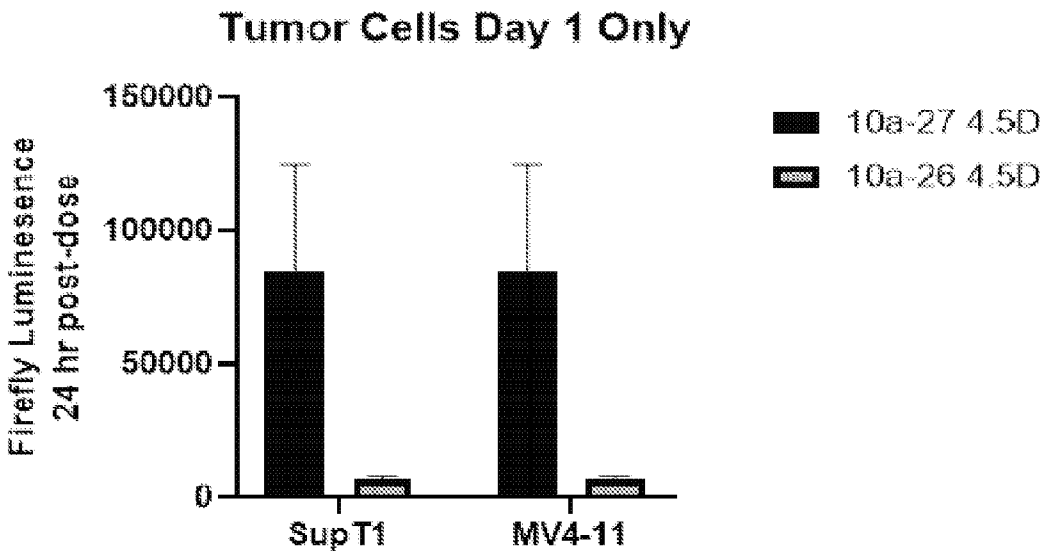
Tumor Cells Day 1 Only
Firefly Luminesence
24 hr post-dose
150000
100000
50000
0
SupT1
MV4-11
10a-27 4.5D
10a-26 4.5D

FIG. 57

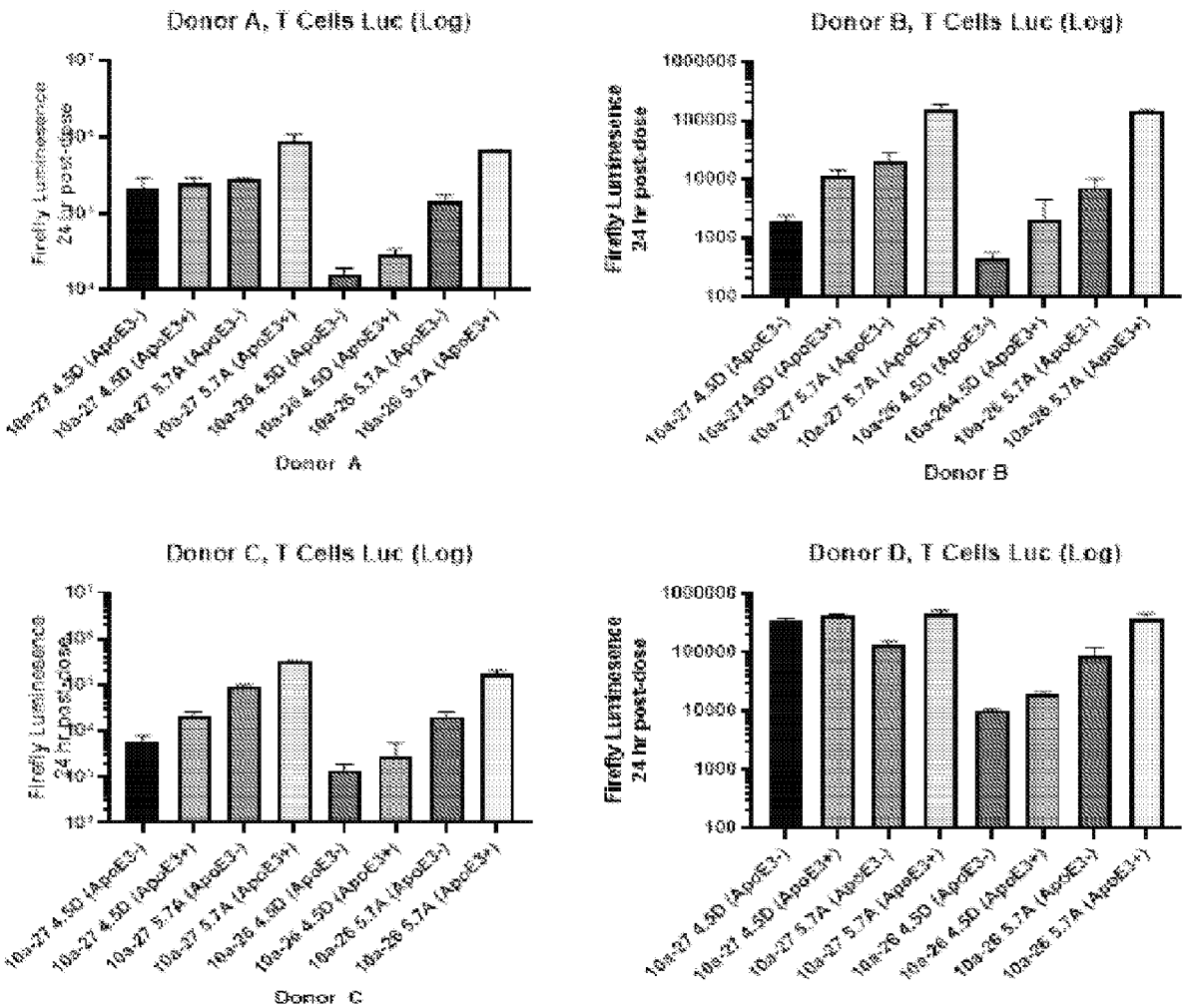
Donor A, T Cells Luc (Log)
Firefly Luminescence 24 hr post-dose
10a-27 4.5D (ApoE3-)
10a-27 4.5D (ApoE3+)
10a-27 5.7A (ApoE3-)
10a-27 5.7A (ApoE3+)
10a-26 4.5D (ApoE3-)
10a-26 4.5D (ApoE3+)
10a-26 5.7A (ApoE3-)
10a-26 5.7A (ApoE3+)
Donor A
Donor B, T Cells Luc (Log)
Firefly Luminescence 24 hr post-dose
Donor B
Donor C, T Cells Luc (Log)
Firefly Luminescence 24 hr post-dose
Donor C
Donor D, T Cells Luc (Log)
Firefly Luminescence 24 hr post-dose

FIG. 58

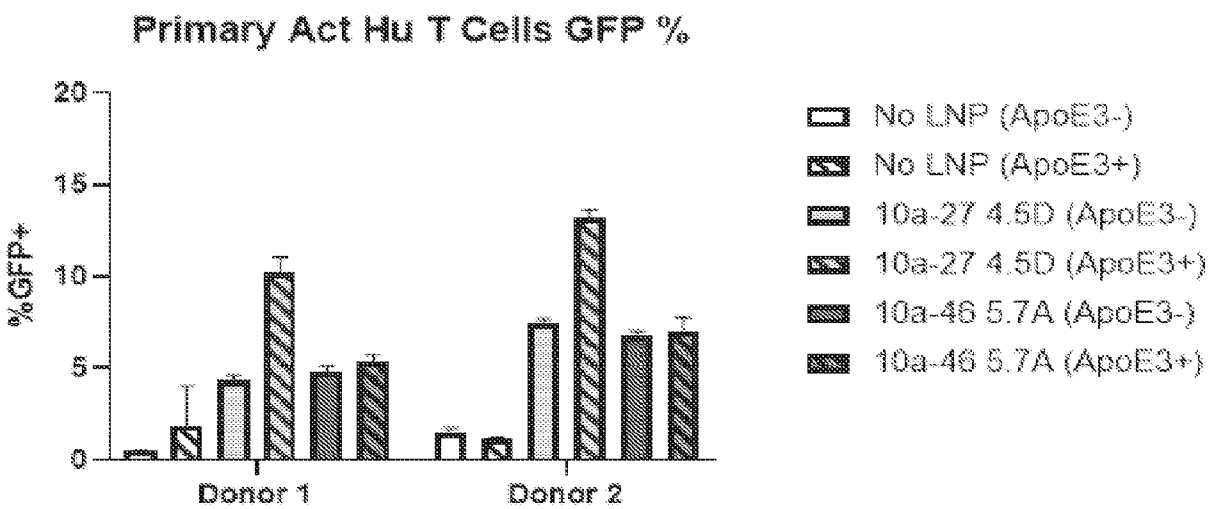
Primary Act Hu T Cells GFP %
%GFP+
20
15
10
5
0
Donor 1
Donor 2
No LNP (ApoE3-)
No LNP (ApoE3+)
10a-27 4.5D (ApoE3-)
10a-27 4.5D (ApoE3+)
10a-46 5.7A (ApoE3-)
10a-46 5.7A (ApoE3+)

FIG. 59

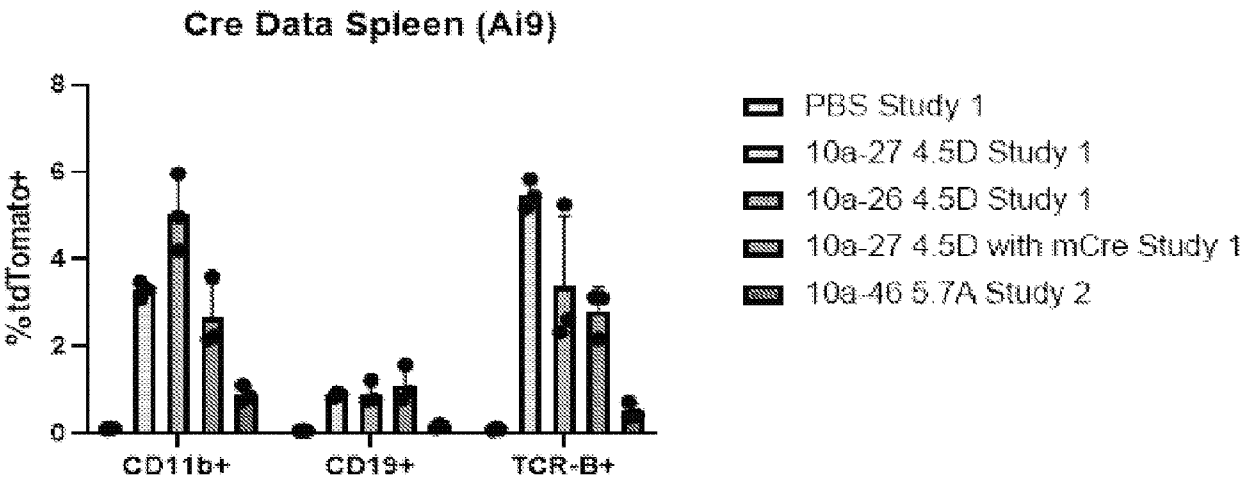
Cre Data Spleen (Ai9)
% tdTomato+
8
6
4
2
0
CD11b+
CD19+
TCR-B+
PBS Study 1
10a-27 4.5D Study 1
10a-26 4.5D Study 1
10a-27 4.5D with mCre Study 1
10a-46 5.7A Study 2

FIG. 60

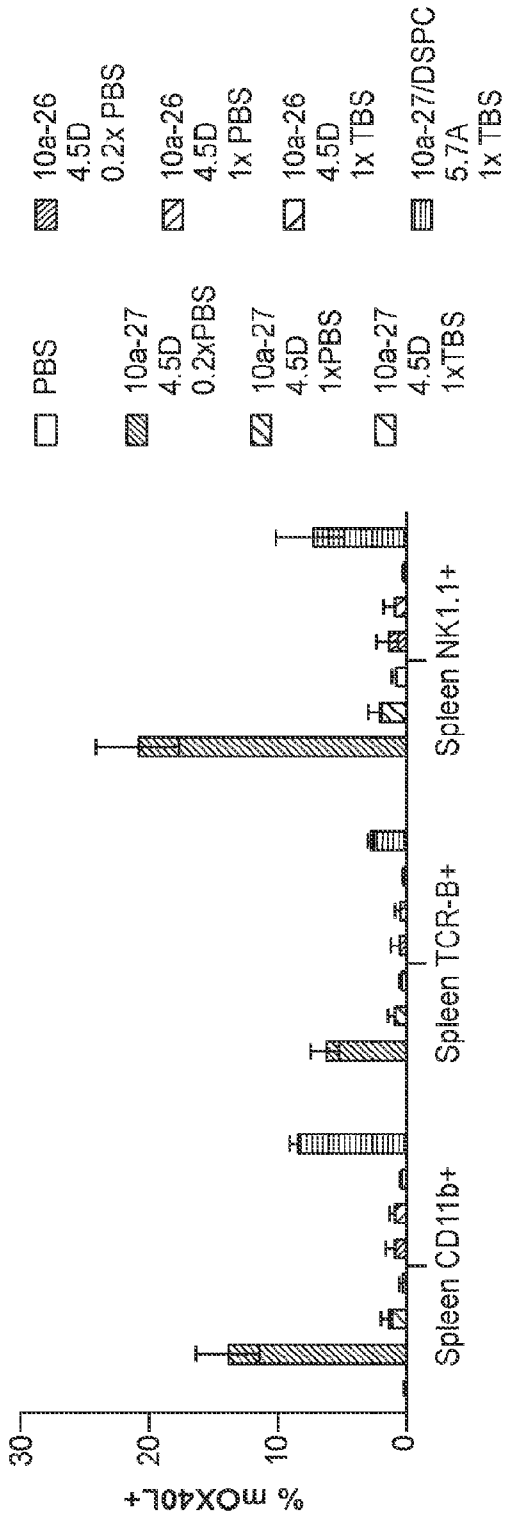
mOX40L
% mOX40L+
30
20
10
0
Spleen CD11b+
Spleen TCR-B+
Spleen NK1.1+
PBS
10a-27 4.5D 0.2xPBS
10a-27 4.5D 1xPBS
10a-27 4.5D 1xTBS
10a-26 4.5D 0.2x PBS
10a-26 4.5D 1x PBS
10a-26 4.5D 1x TBS
10a-27/DSPC 5.7A 1x TBS

FIG. 61

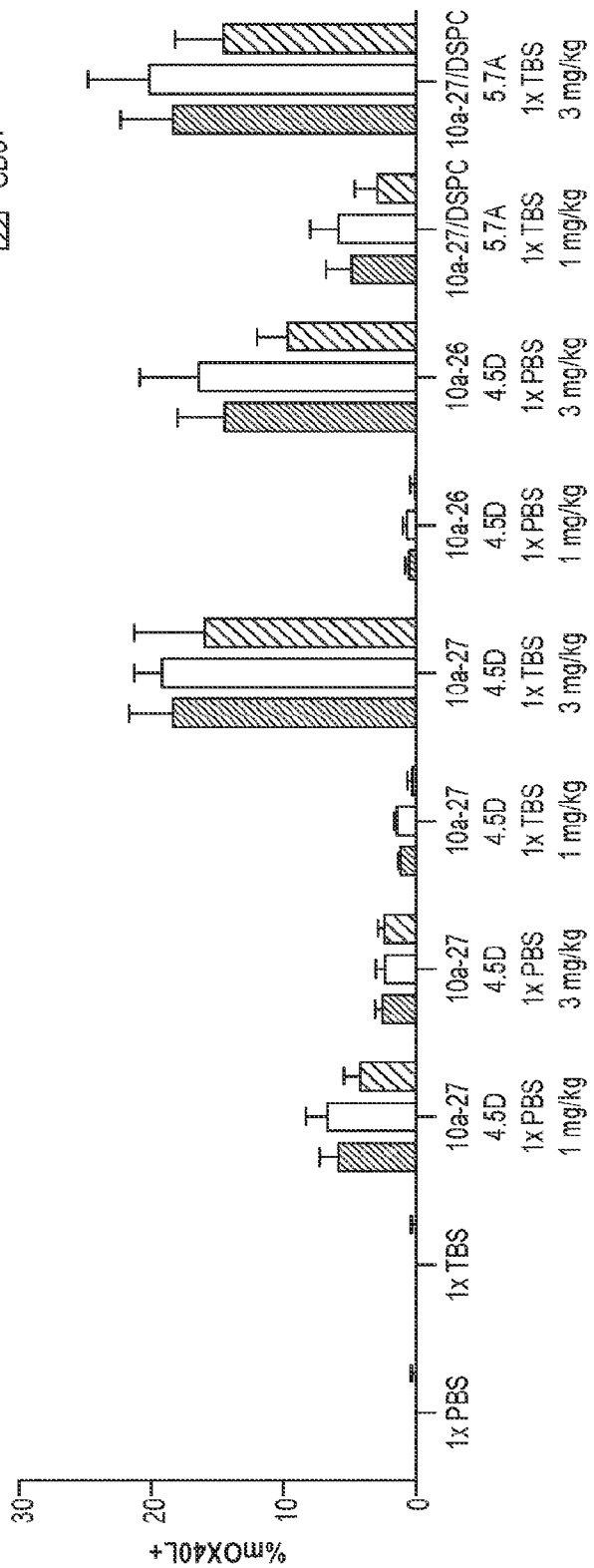
All TCR-B+
CD4+
CD8+
1 and 3 mg/kg, splenocytes (T cells)
%mOX40L+
0
10
20
30
1x PBS
1x TBS
10a-27 4.5D 1x PBS 1 mg/kg
10a-27 4.5D 1x PBS 3 mg/kg
10a-27 4.5D 1x TBS 1 mg/kg
10a-27 4.5D 1x TBS 3 mg/kg
10a-26 4.5D 1x PBS 1 mg/kg
10a-26 4.5D 1x PBS 3 mg/kg
10a-27/DSPC 5.7A 1x TBS 1 mg/kg
10a-27/DSPC 5.7A 1x TBS 3 mg/kg

FIG. 62A

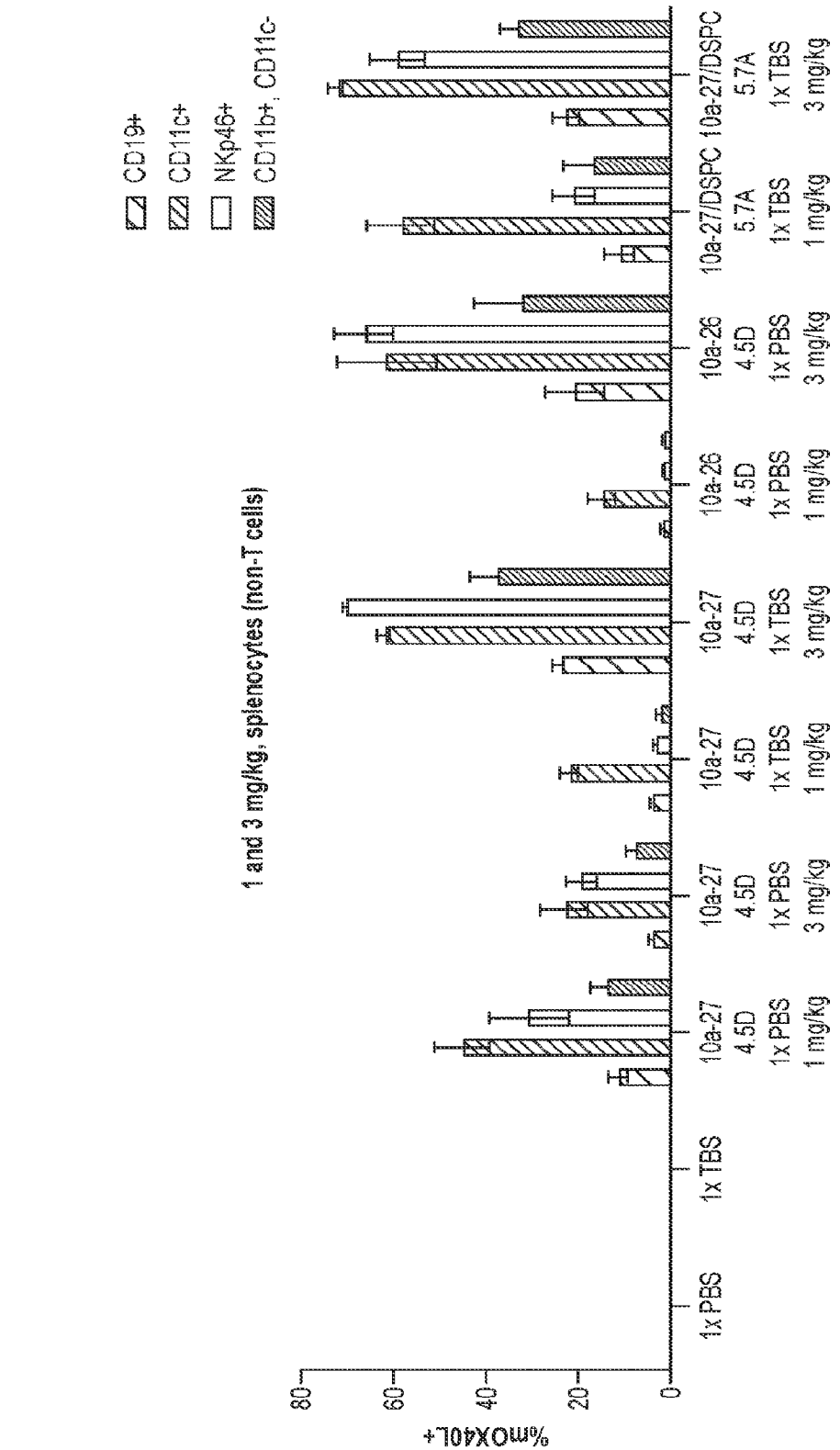
CD19+
CD11c+
NKp46+
CD11b+, CD11c-
1 and 3 mg/kg, splenocytes (non-T cells)
%mOX40L+
0
20
40
60
80
1x PBS
1x TBS
10a-27 4.5D 1x PBS 1 mg/kg
10a-27 4.5D 1x PBS 3 mg/kg
10a-27 4.5D 1x TBS 1 mg/kg
10a-27 4.5D 1x TBS 3 mg/kg
10a-26 4.5D 1x PBS 1 mg/kg
10a-26 4.5D 1x PBS 3 mg/kg
10a-27/DSPC 5.7A 1x TBS 1 mg/kg
10a-27/DSPC 5.7A 1x TBS 3 mg/kg

FIG. 62B

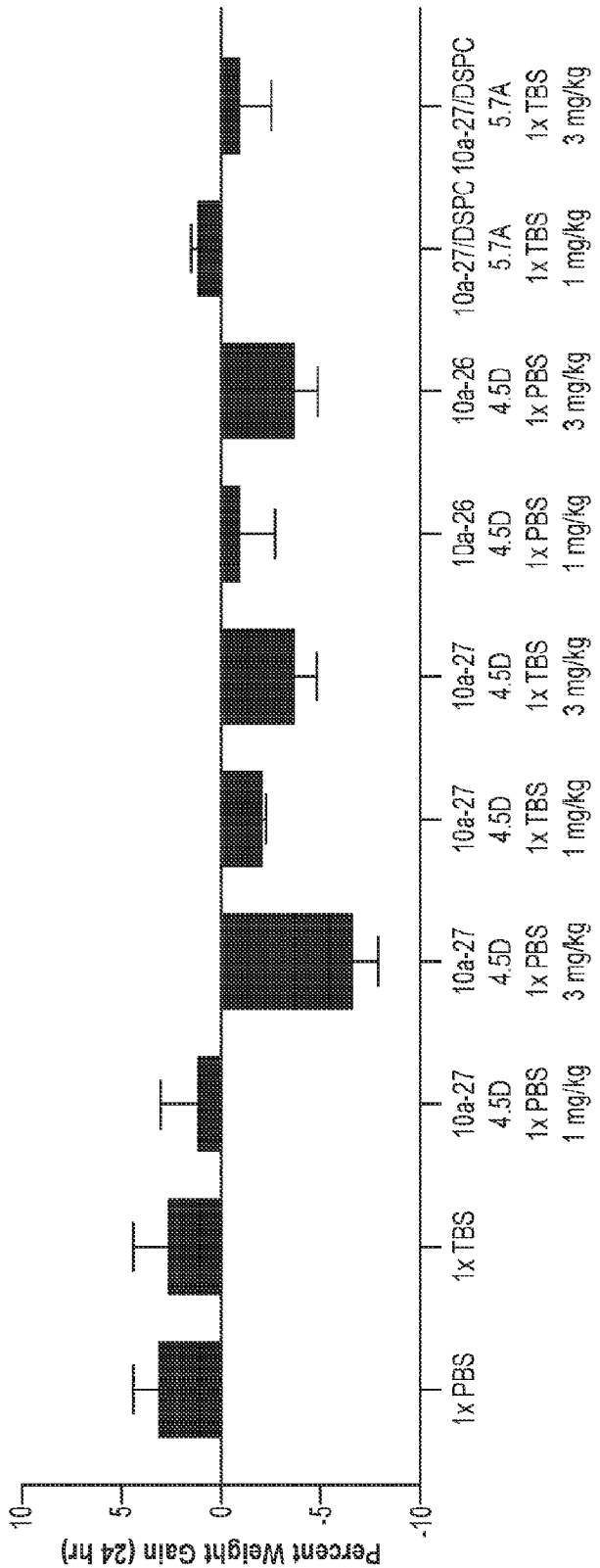
1 and 3 mg/kg, weight
Percent Weight Gain (24 hr)
10
5
0
-5
-10
1x PBS
1x TBS
10a-27 4.5D 1x PBS 1 mg/kg
10a-27 4.5D 1x PBS 3 mg/kg
10a-27 4.5D 1x TBS 1 mg/kg
10a-27 4.5D 1x TBS 3 mg/kg
10a-26 4.5D 1x PBS 1 mg/kg
10a-26 4.5D 1x PBS 3 mg/kg
10a-27/DSPC 5.7A 1x TBS 1 mg/kg
10a-27/DSPC 5.7A 1x TBS 3 mg/kg

FIG. 62C

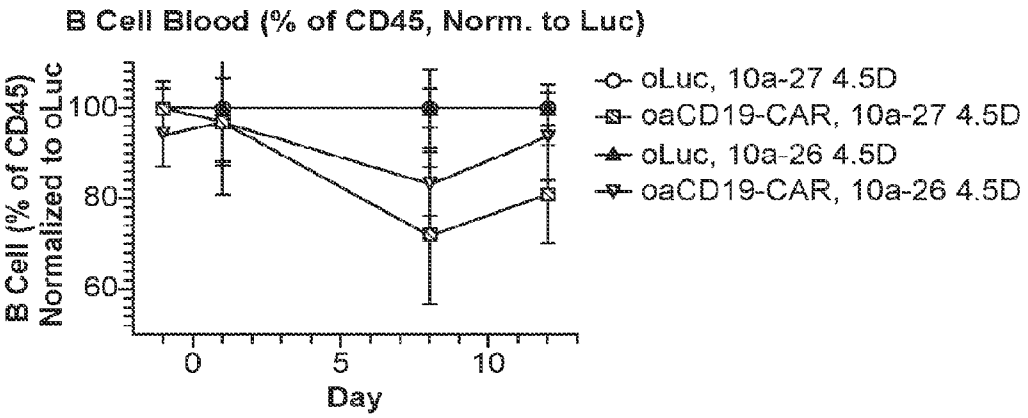

FIG. 63A

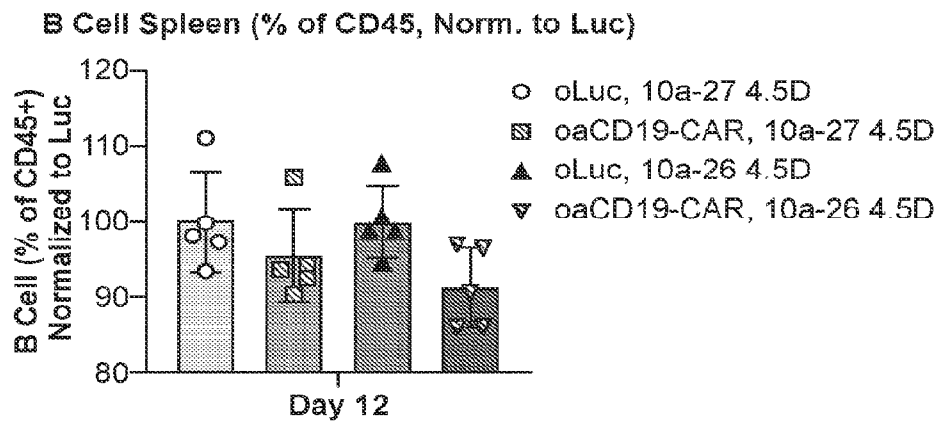

FIG. 63B

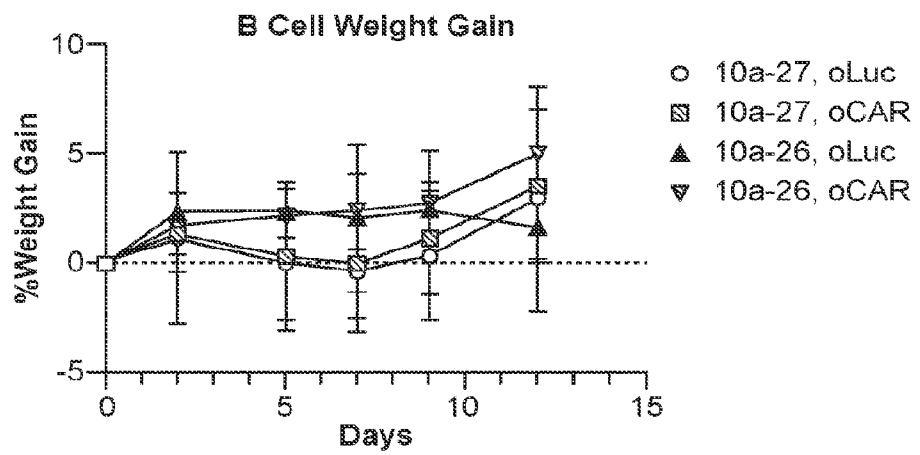

FIG. 63C

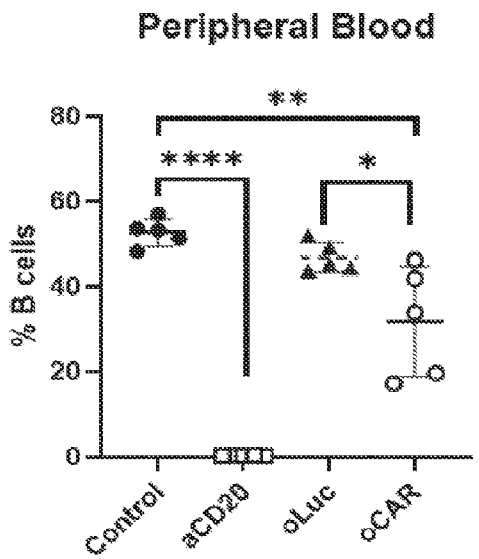

FIG. 64A

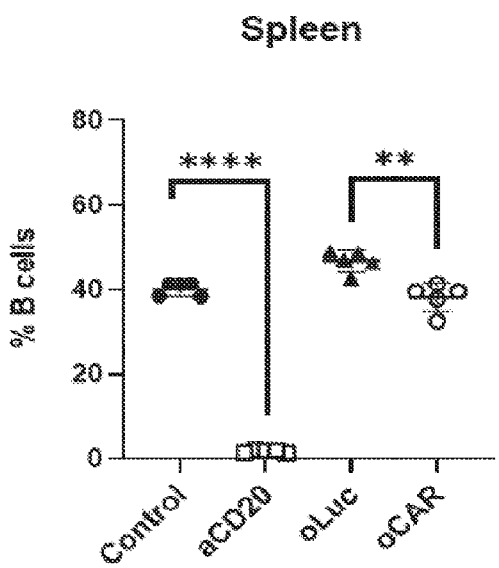

FIG. 64B

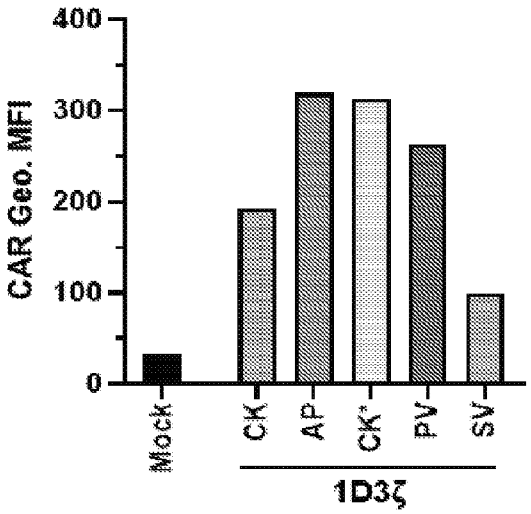

FIG. 65A

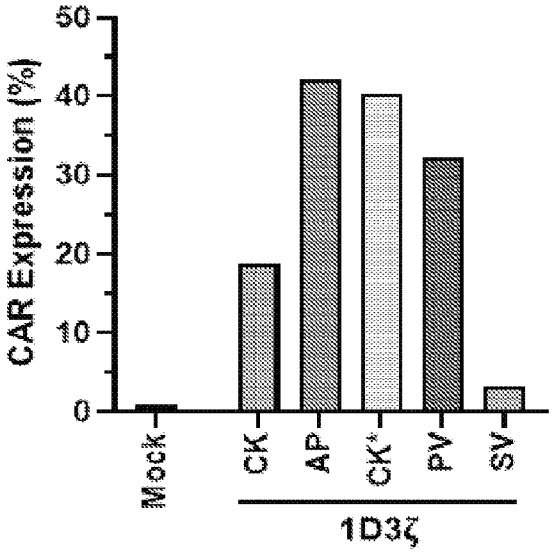

FIG. 65B

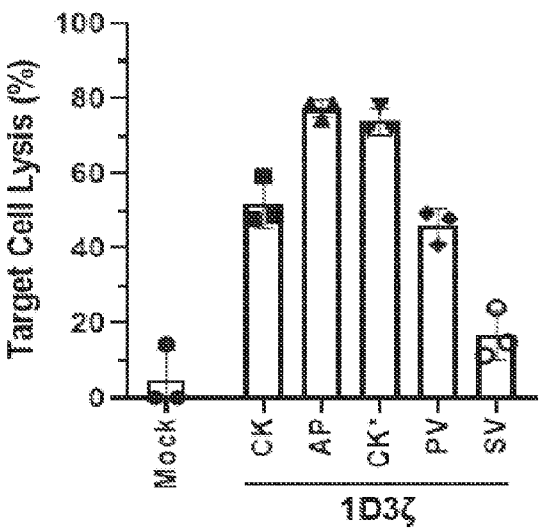

FIG. 65C

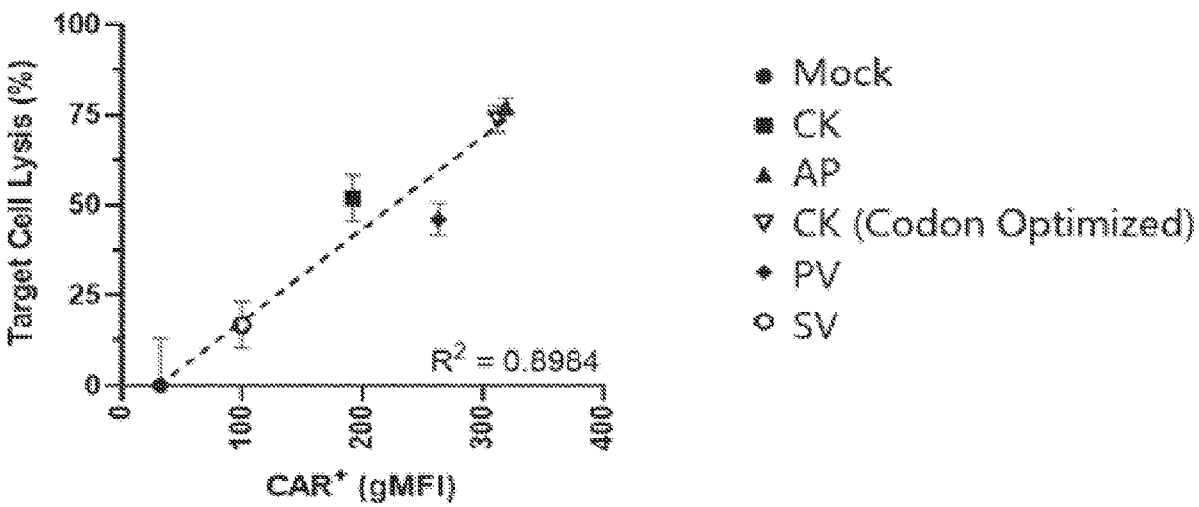
Target Cell Lysis (%)
100
75
50
25
0
0
100
200
300
400
CAR+ (gMFI)
R2 = 0.8984
Mock
CK
AP
CK (Codon Optimized)
PV
SV

FIG. 66

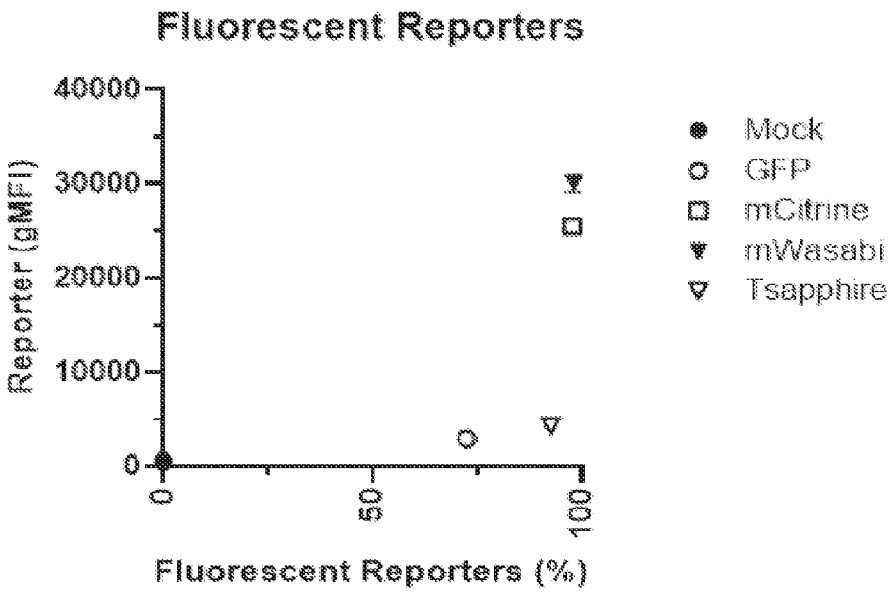

FIG. 67A

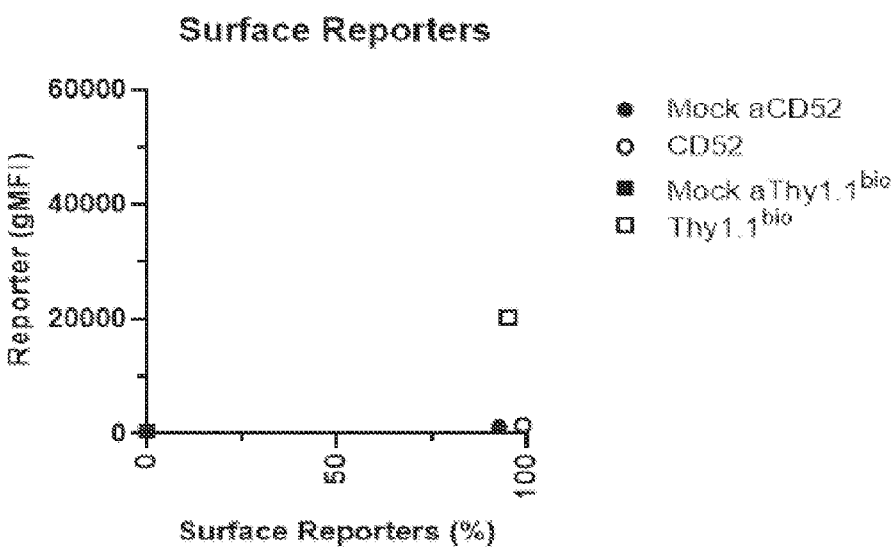

FIG. 67B

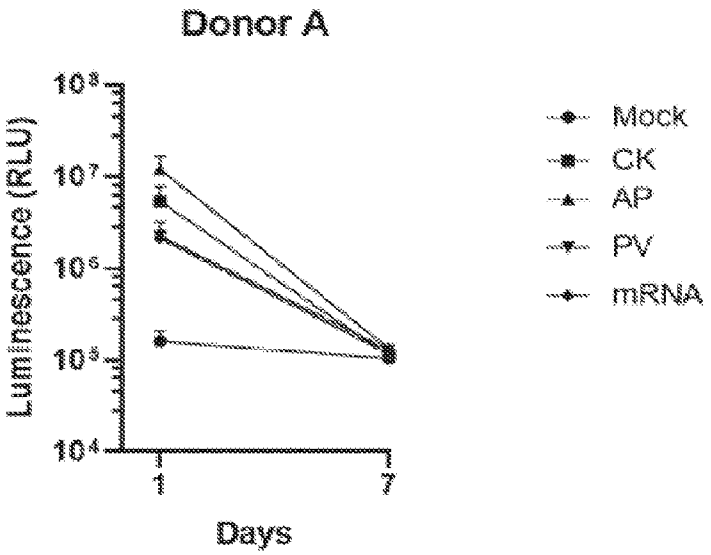

FIG. 68A

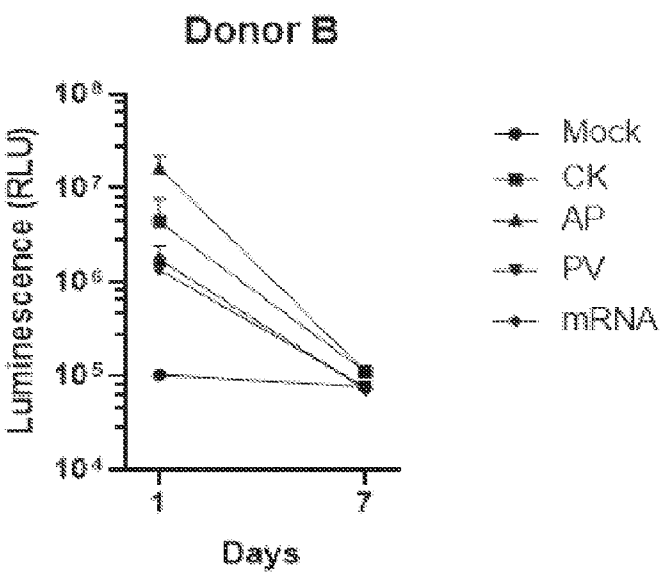

FIG. 68B

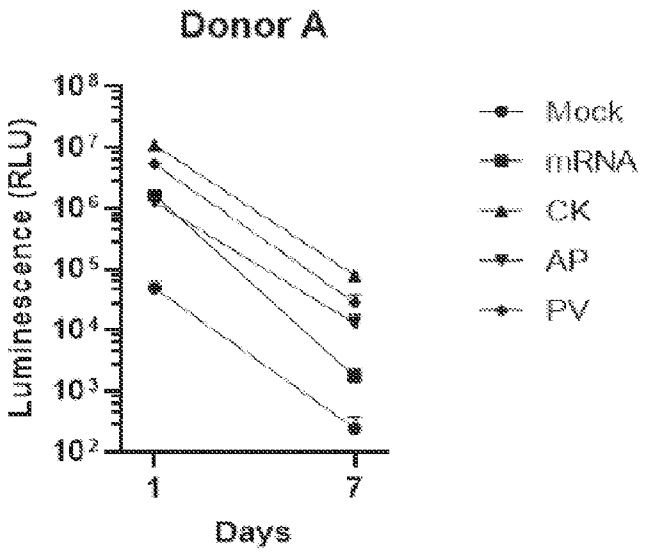

FIG. 68C

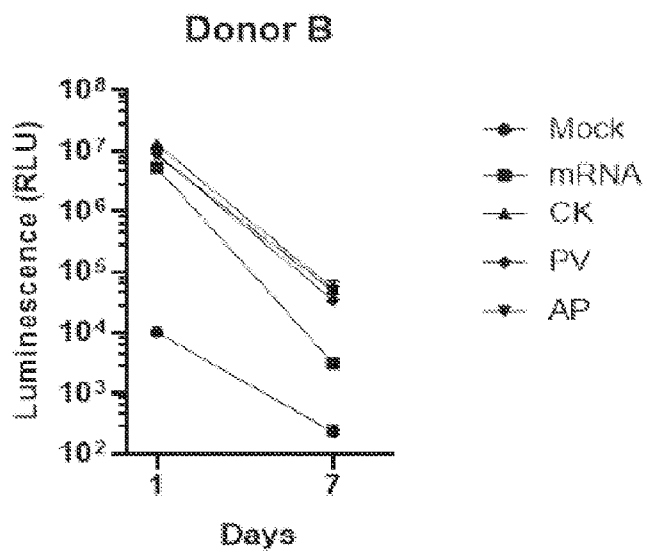

FIG. 68D

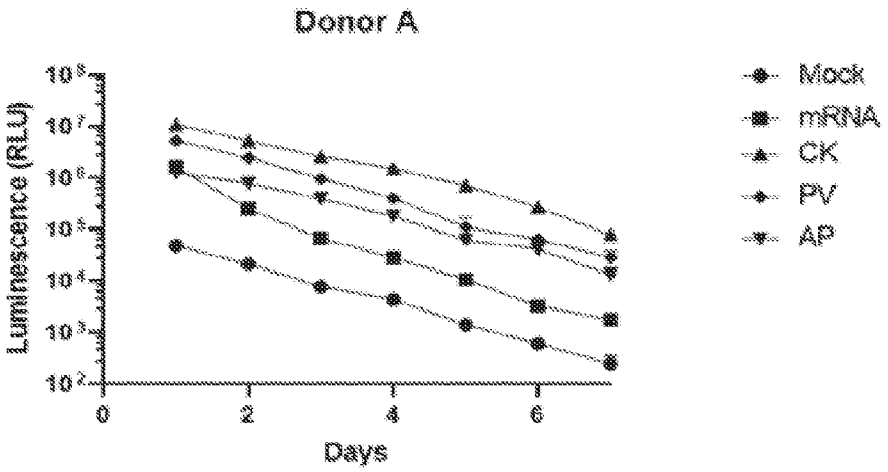

FIG. 68E

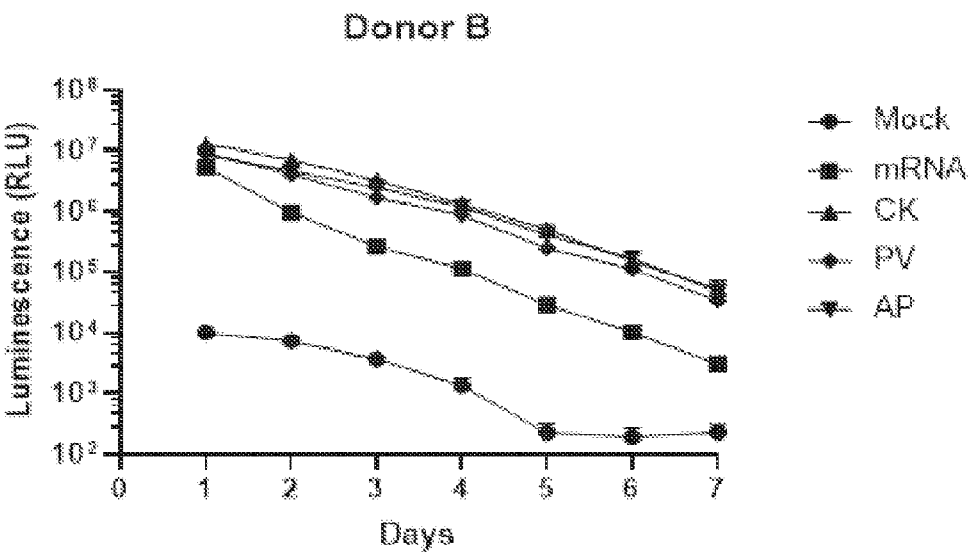

FIG. 68F

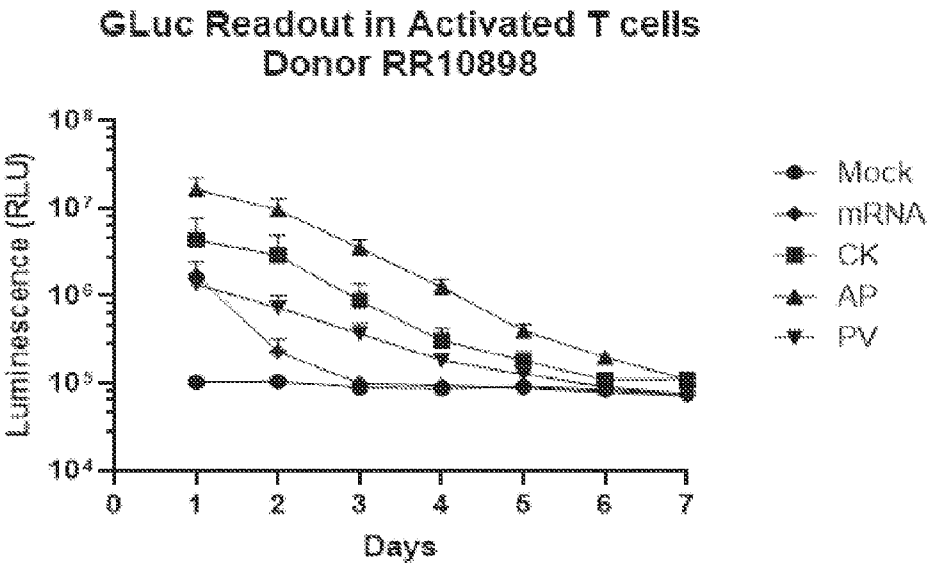
GLuc Readout in Activated T cells
Donor RR10898
Luminescence (RLU)
10^8
10^7
10^6
10^5
10^4
0
1
2
3
4
5
6
7
Days
Mock
mRNA
CK
AP
PV

FIG. 68G

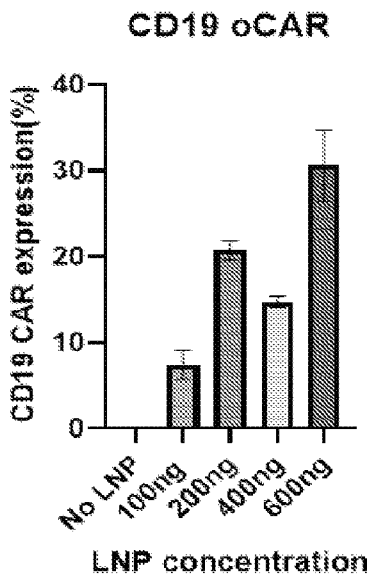

FIG. 69A

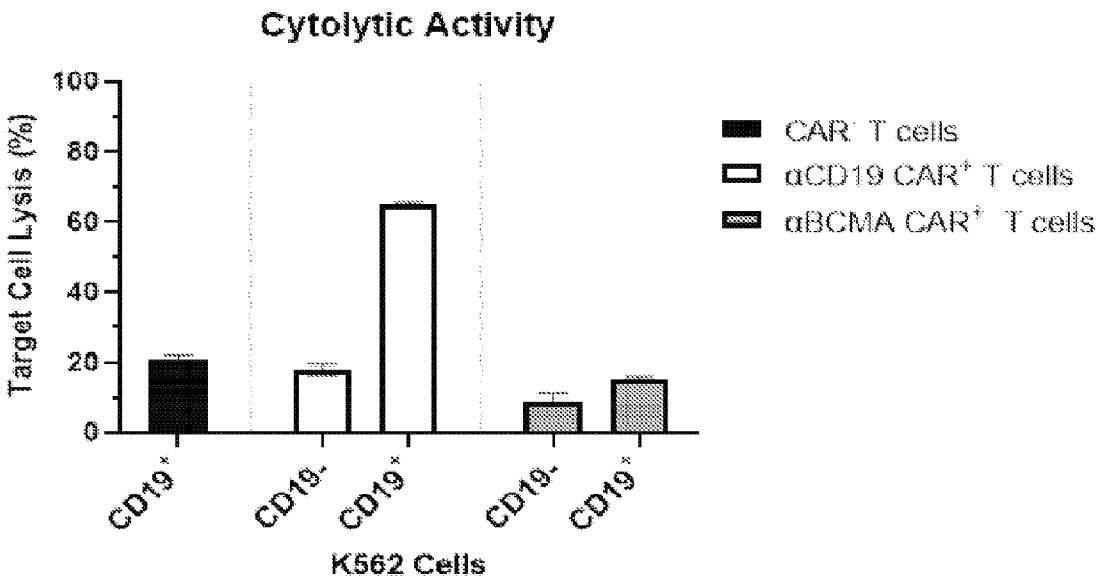

FIG. 69B

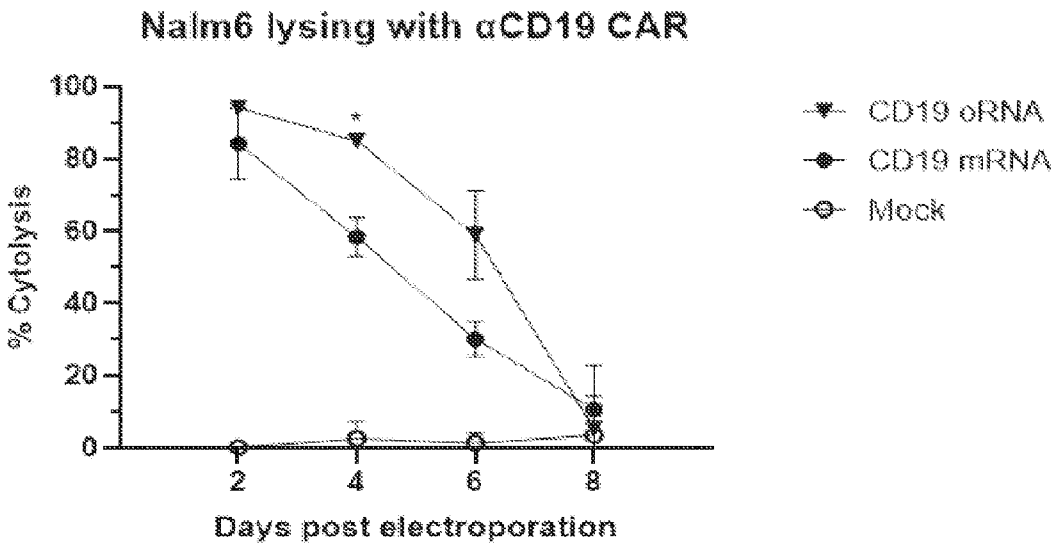

FIG. 70A

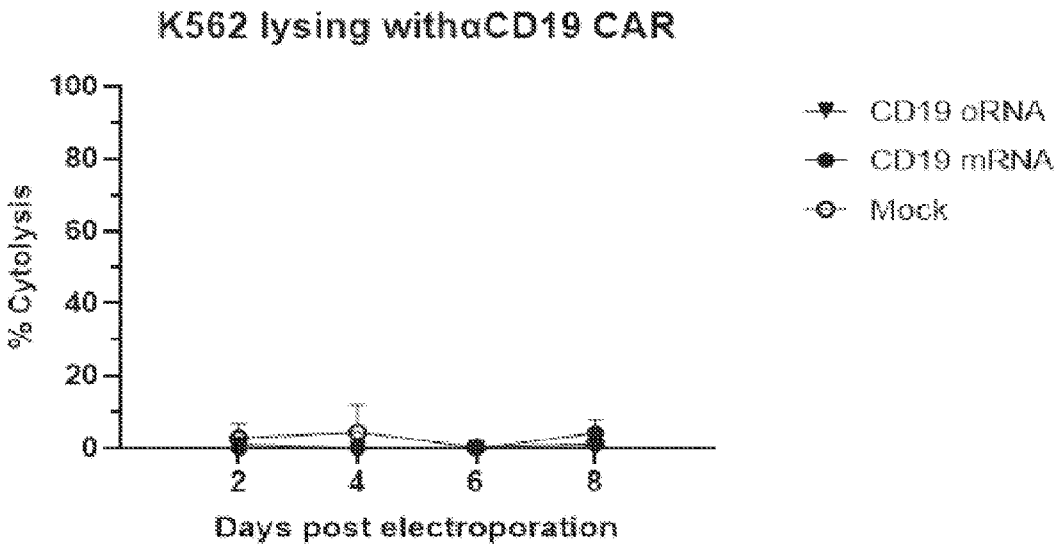

FIG. 70B

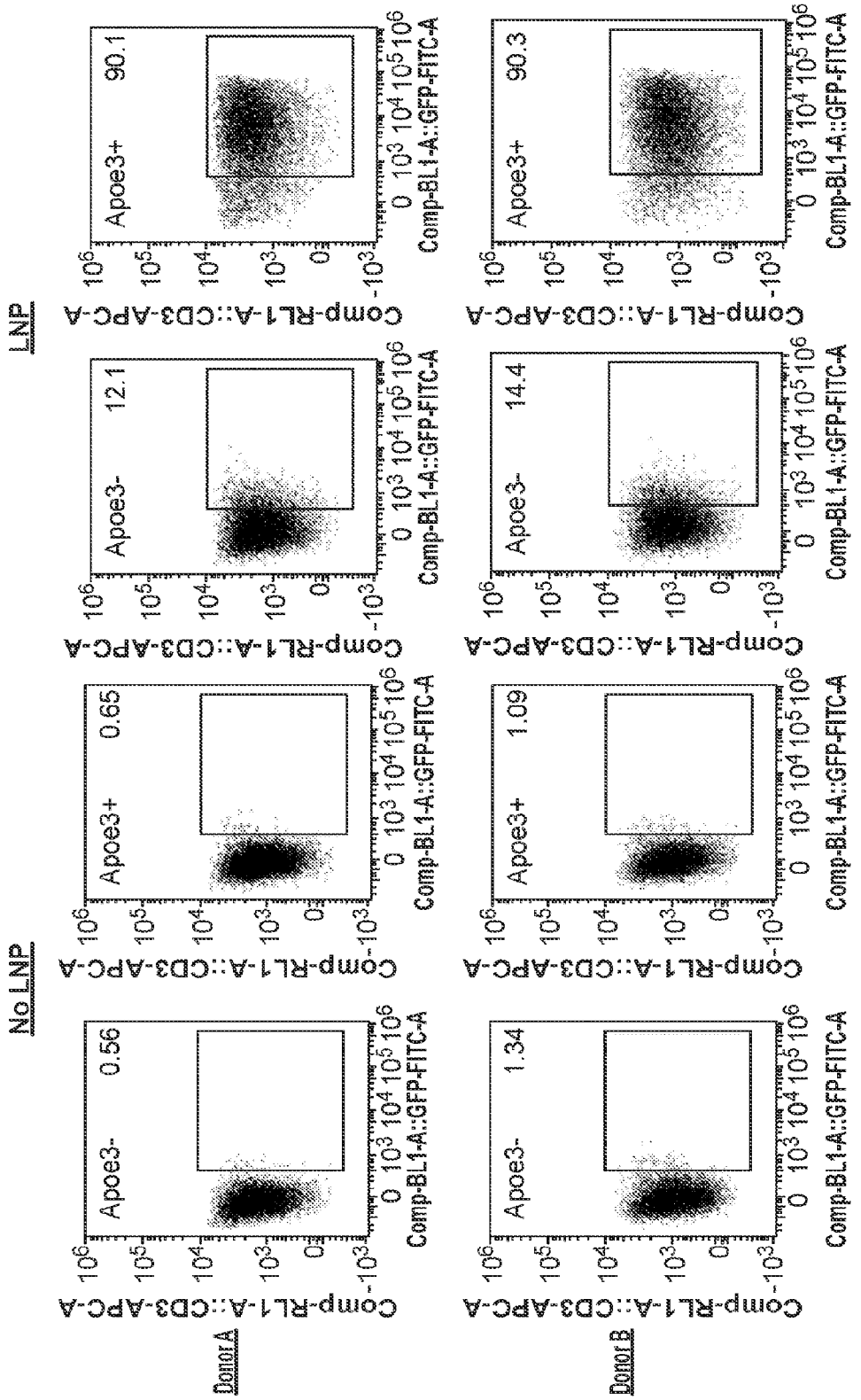
No LNP
LNP
Donor A
Donor B
Apoe3- 0.56
Apoe3+ 0.65
Apoe3- 12.1
Apoe3+ 14.4
Apoe3- 1.34
Apoe3+ 1.09
Apoe3- 90.1
Apoe3+ 90.3
Comp-RL1-A::CD3-APC-A
Comp-BL1-A::GFP-FITC-A

FIG. 71A

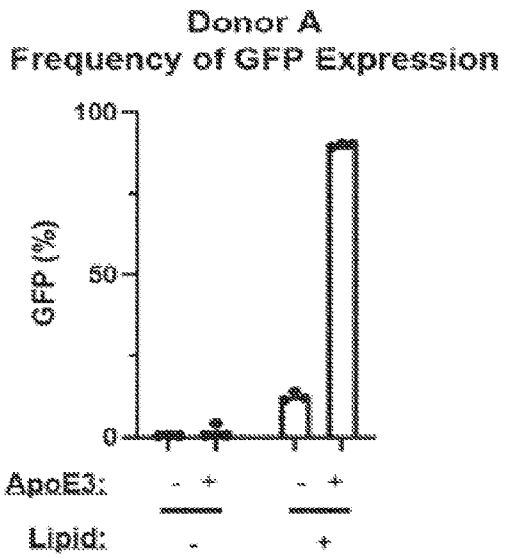

FIG. 71B

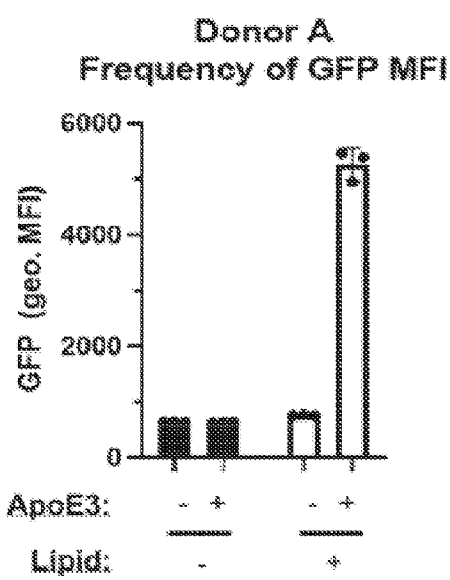

FIG. 71C

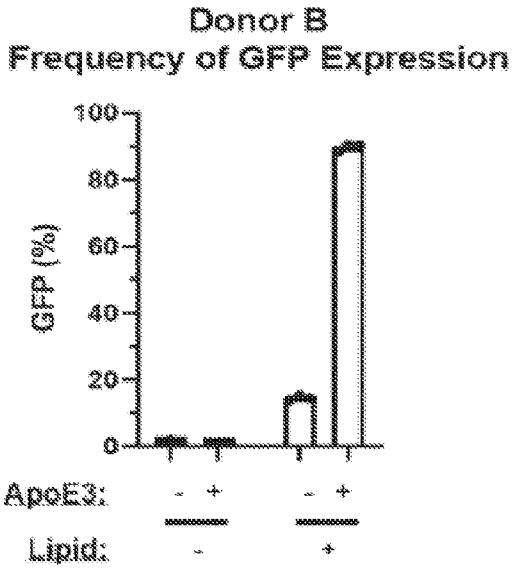

FIG. 71D

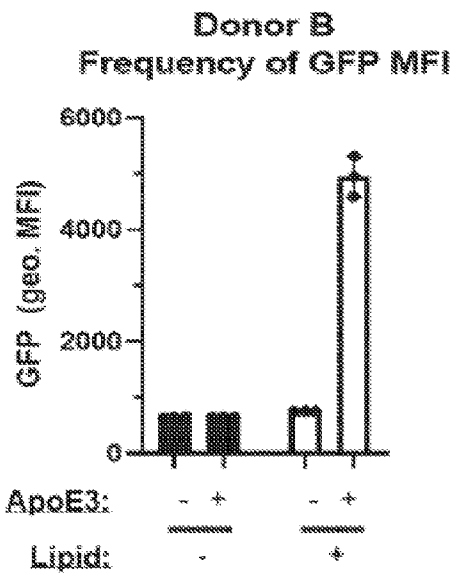

FIG. 71E

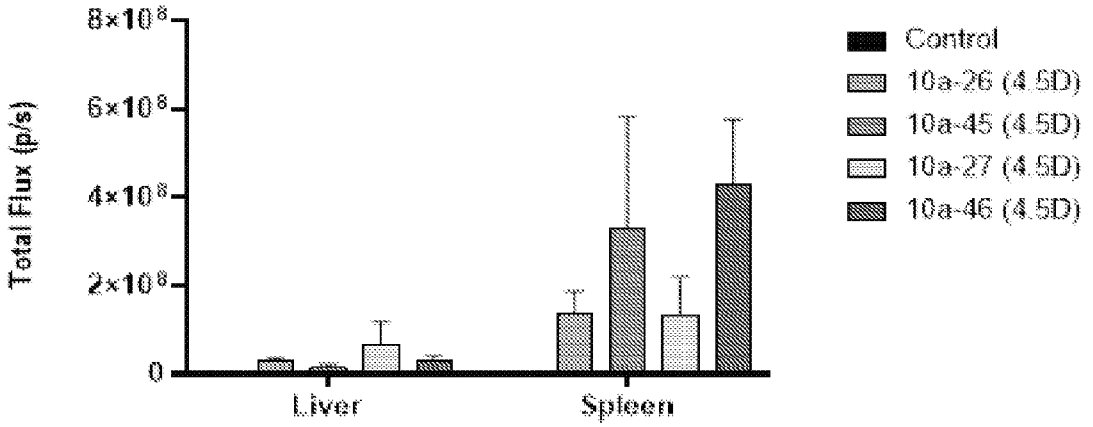

FIG. 72A

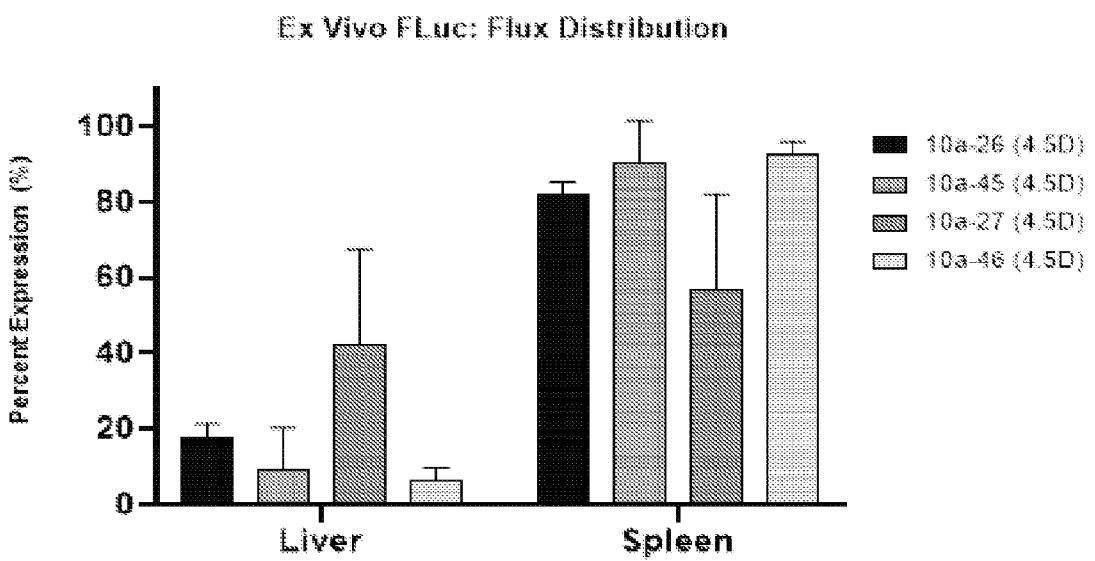

FIG. 72B

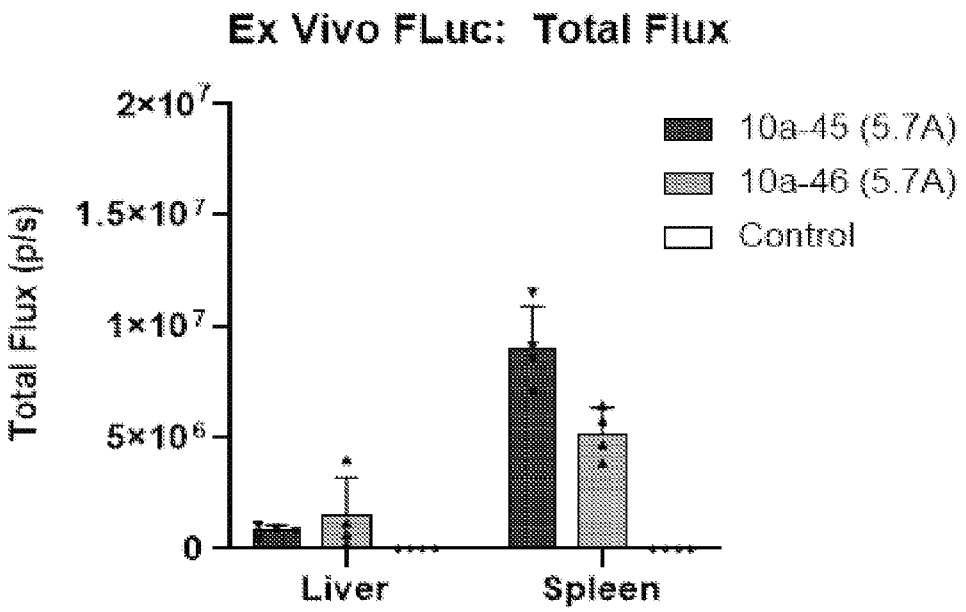

FIG. 72C

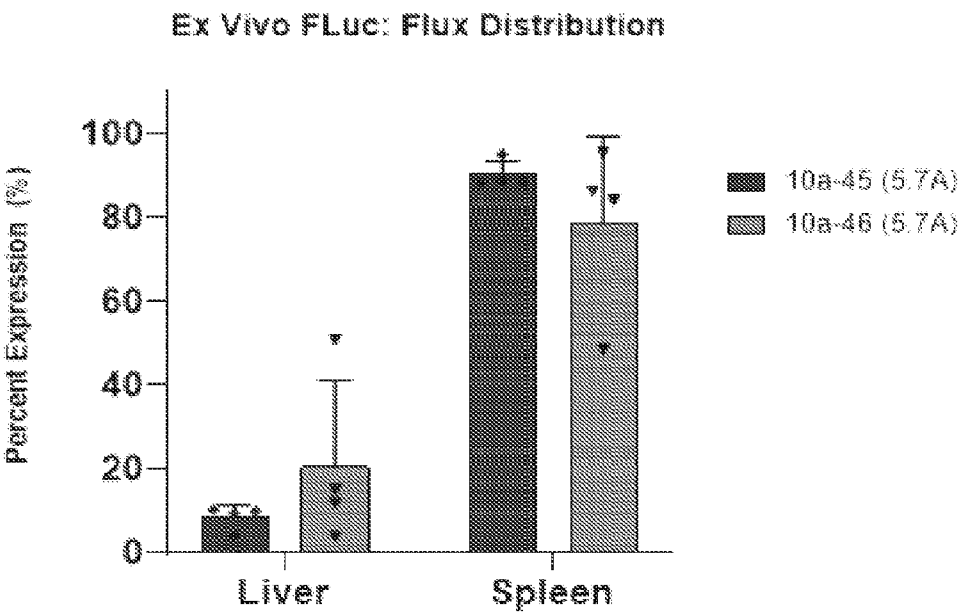

FIG. 72D

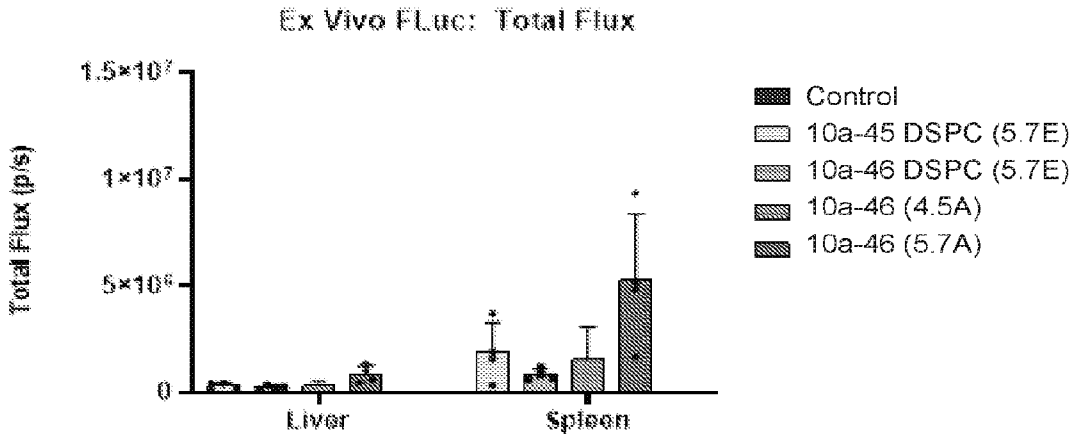

FIG. 72E

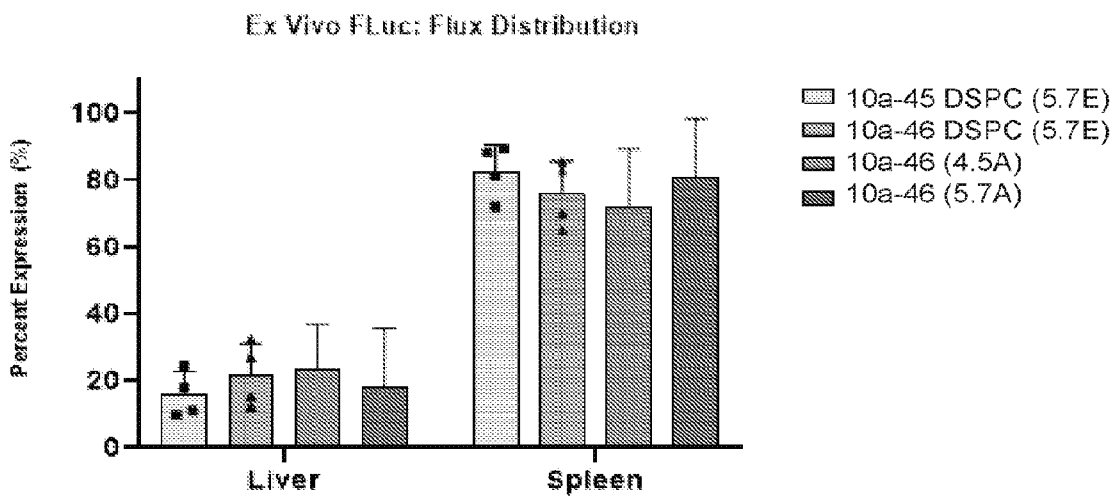

FIG. 72F

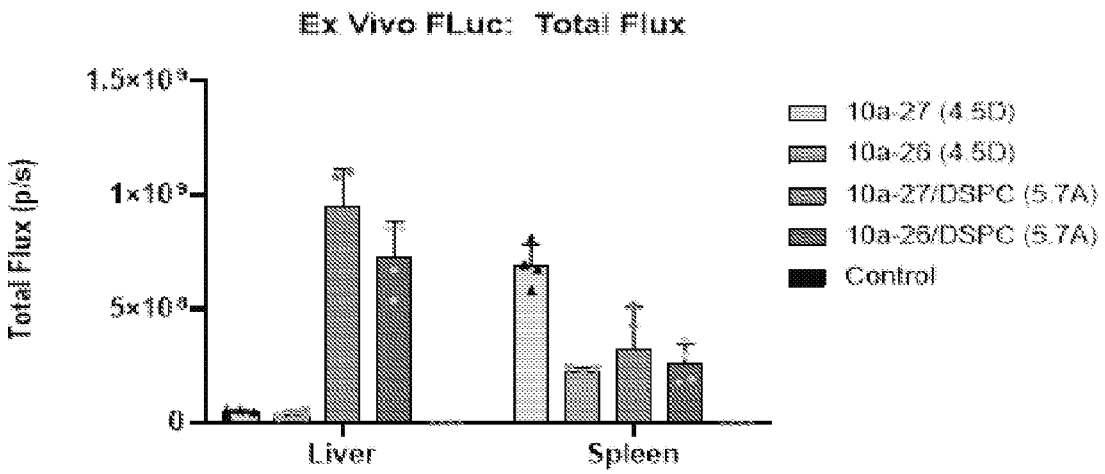

FIG. 72G

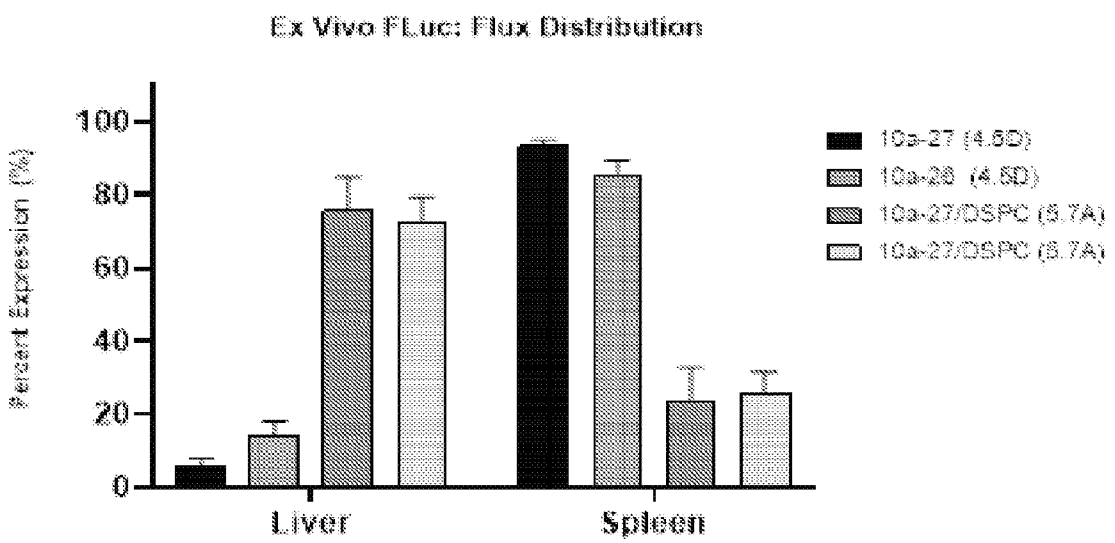

FIG. 72H

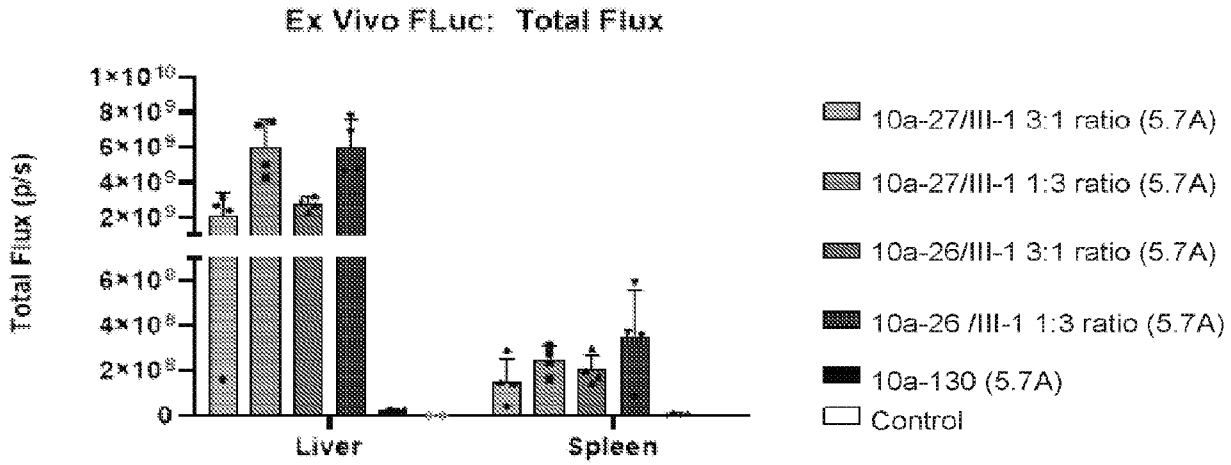

FIG. 72I

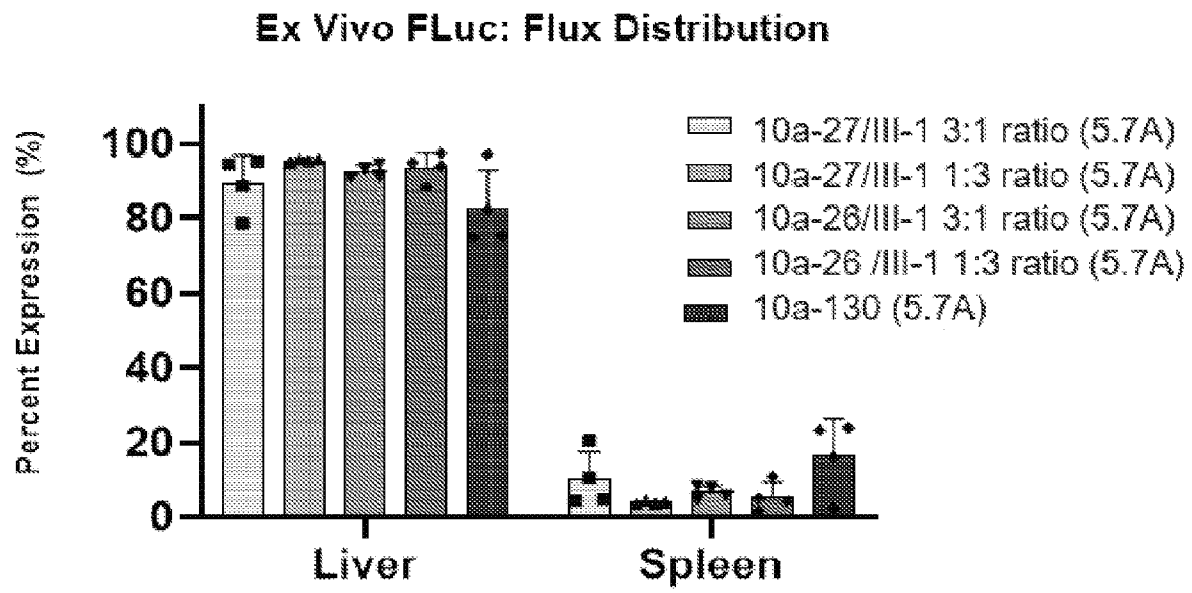

FIG. 72J

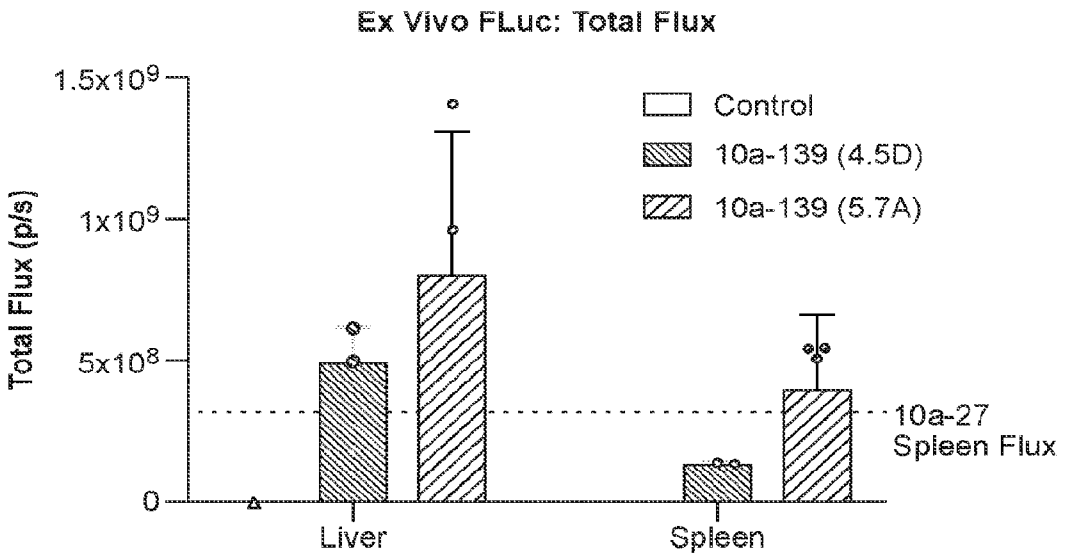

FIG. 72K

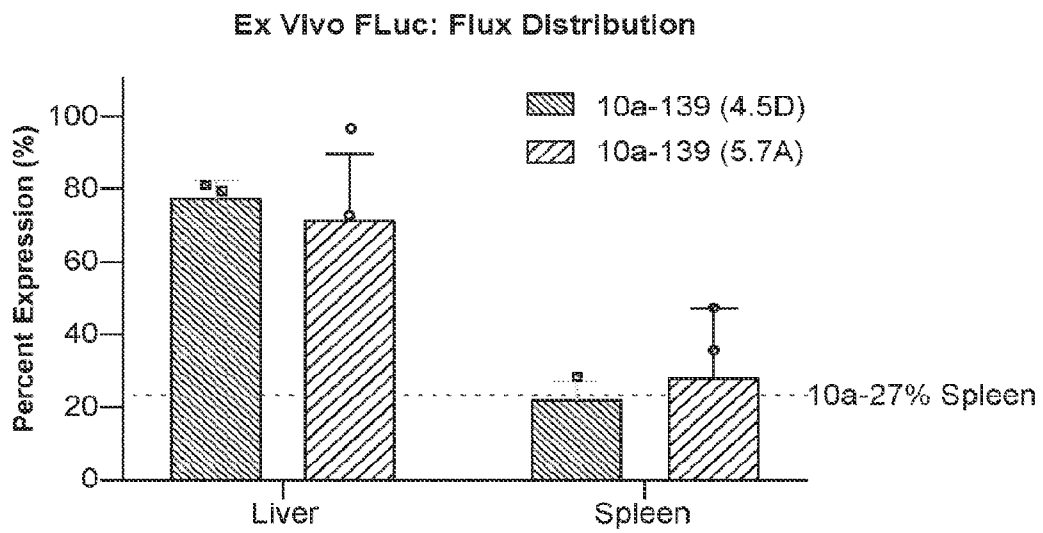

FIG. 72L

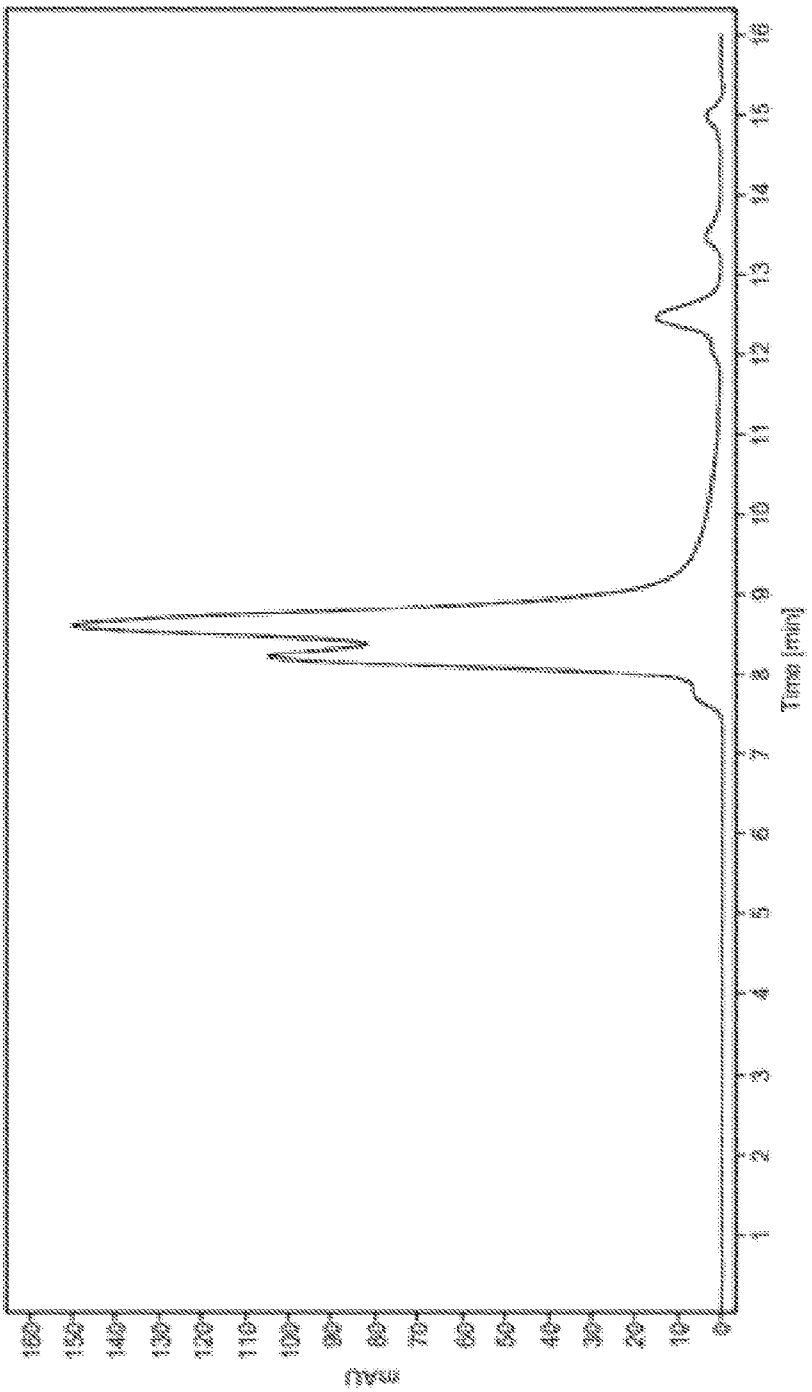

FIG. 73A

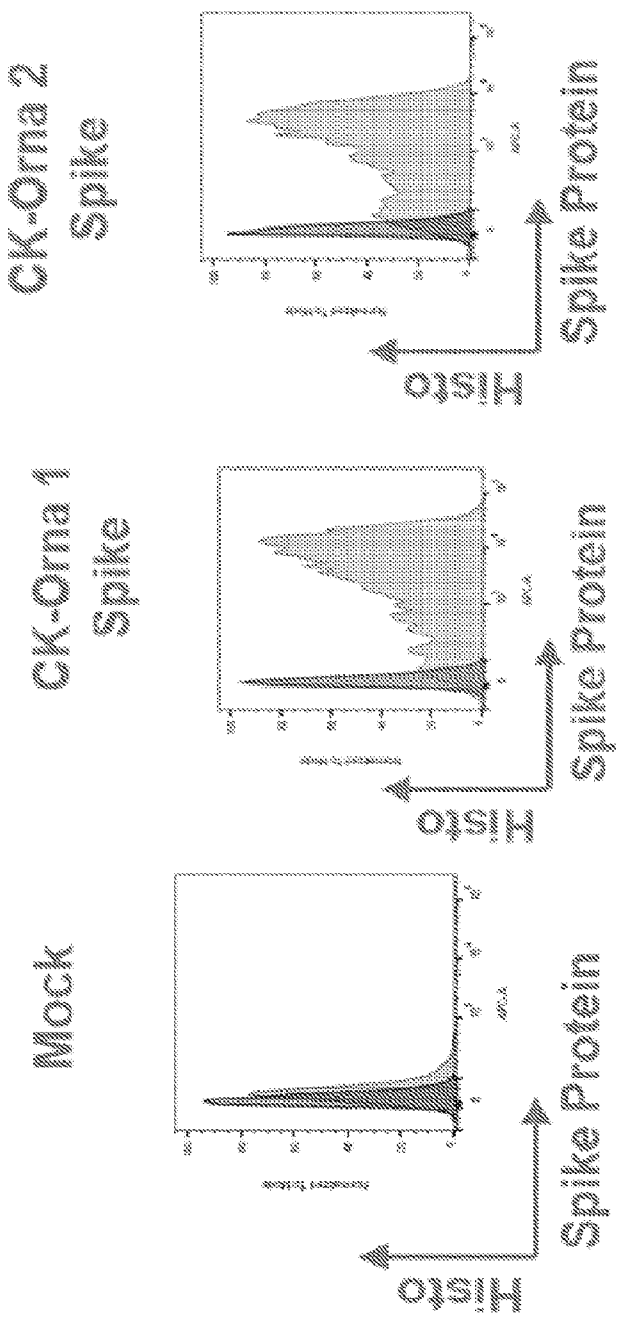
CK-Oma 2
Spike
Histo
Spike Protein
CK-Oma 1
Spike
Histo
Spike Protein
Mock
Histo
Spike Protein

FIG. 73B

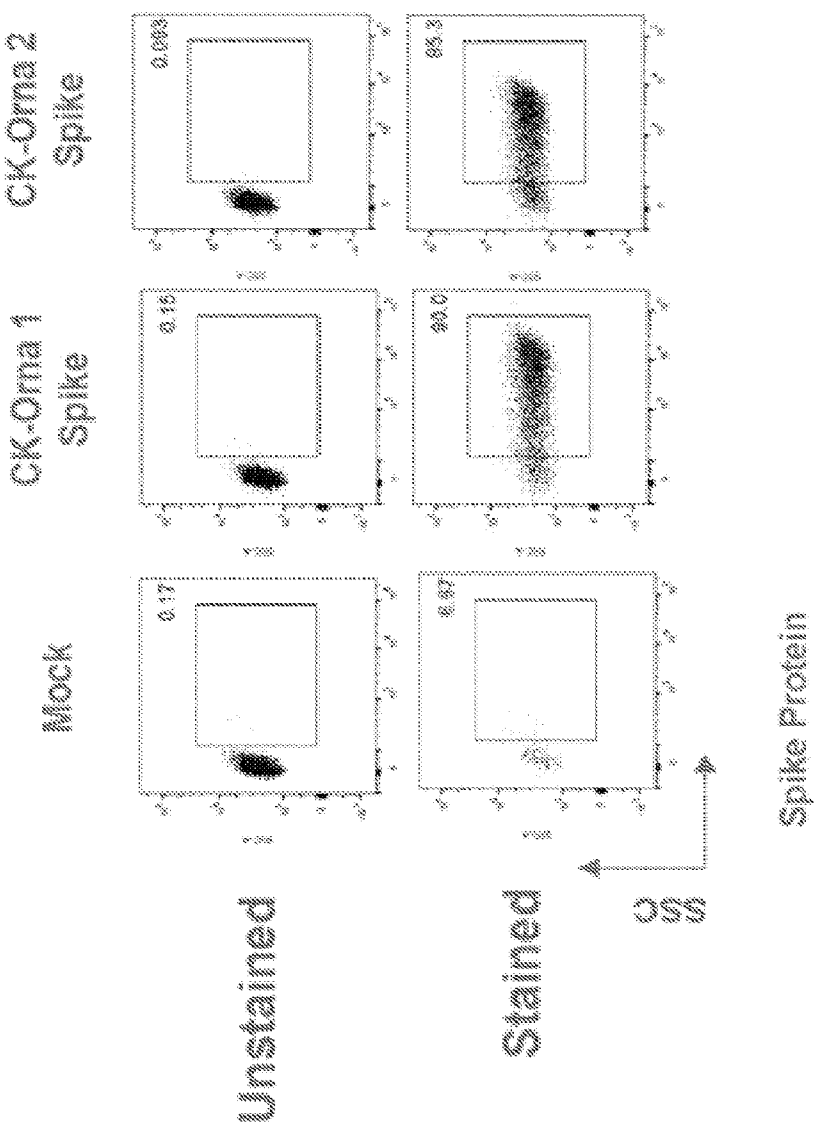
Mock
CK-Orna 1 Spike
CK-Orna 2 Spike
Unstained
Stained
SSC
Spike Protein

FIG. 73C

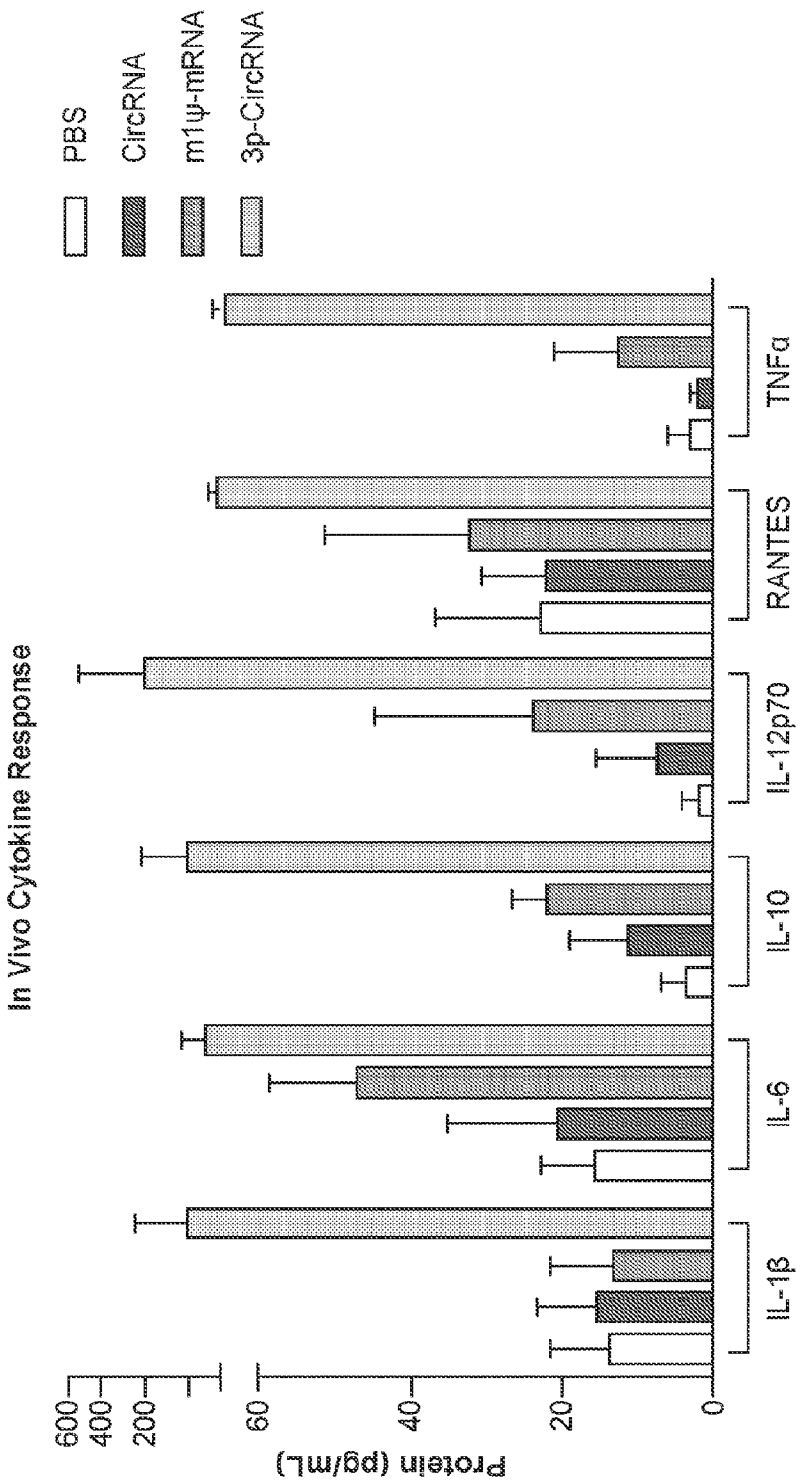
In Vivo Cytokine Response
PBS
CircRNA
m1ψ-mRNA
3p-CircRNA
Protein (pg/mL)
600
400
200
60
40
20
0
IL-1β
IL-6
IL-10
IL-12p70
RANTES
TNFα

FIG. 74

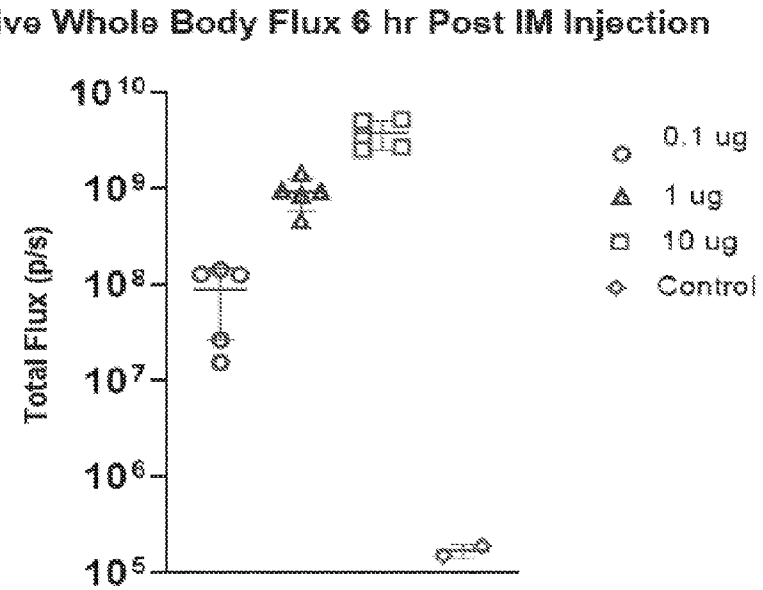

FIG. 75A

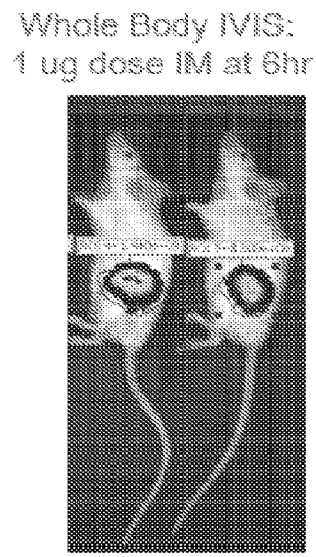

FIG. 75B

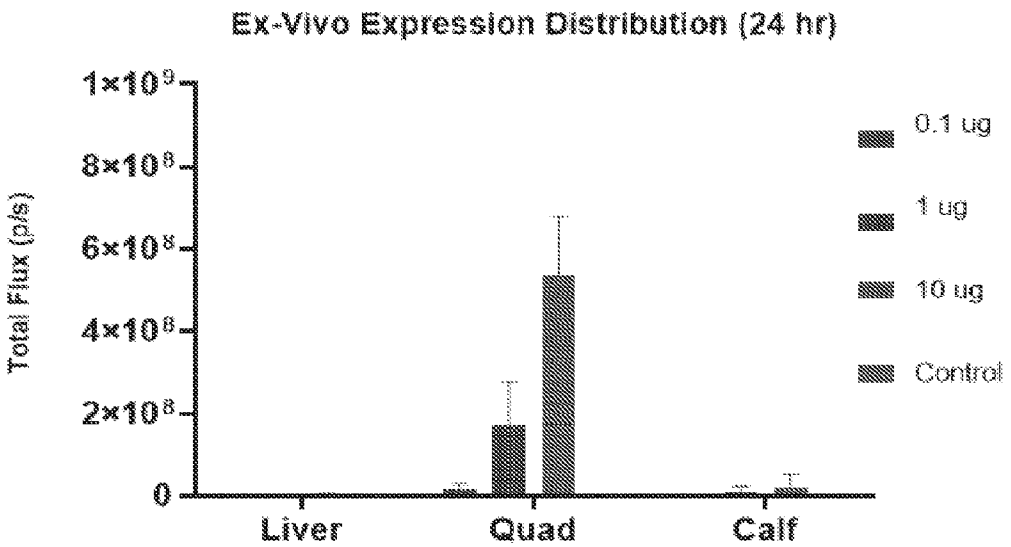
Ex-Vivo Expression Distribution (24 hr)
Total Flux (p/s)
1×10^9
8×10^8
6×10^8
4×10^8
2×10^8
0
Liver
Quad
Calf
0.1 ug
1 ug
10 ug
Control

FIG. 75C

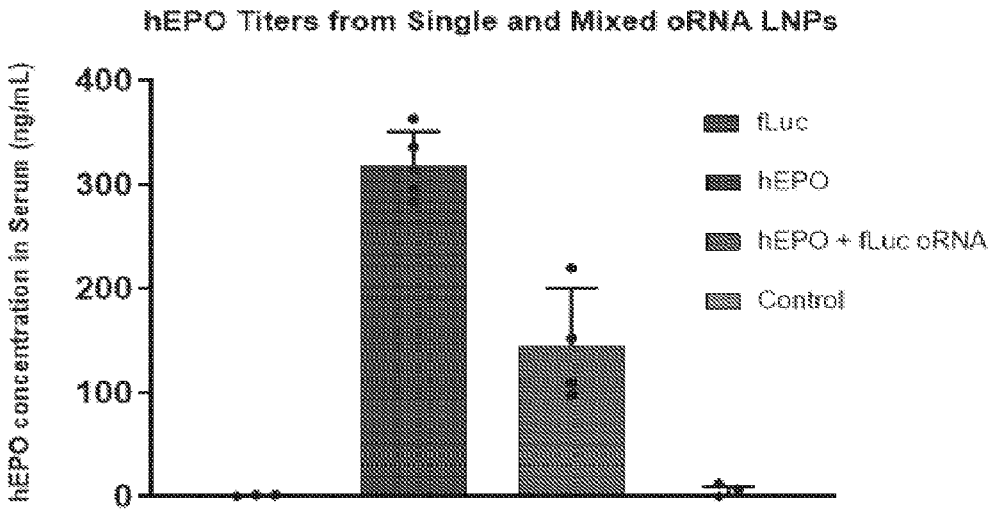

FIG. 76A

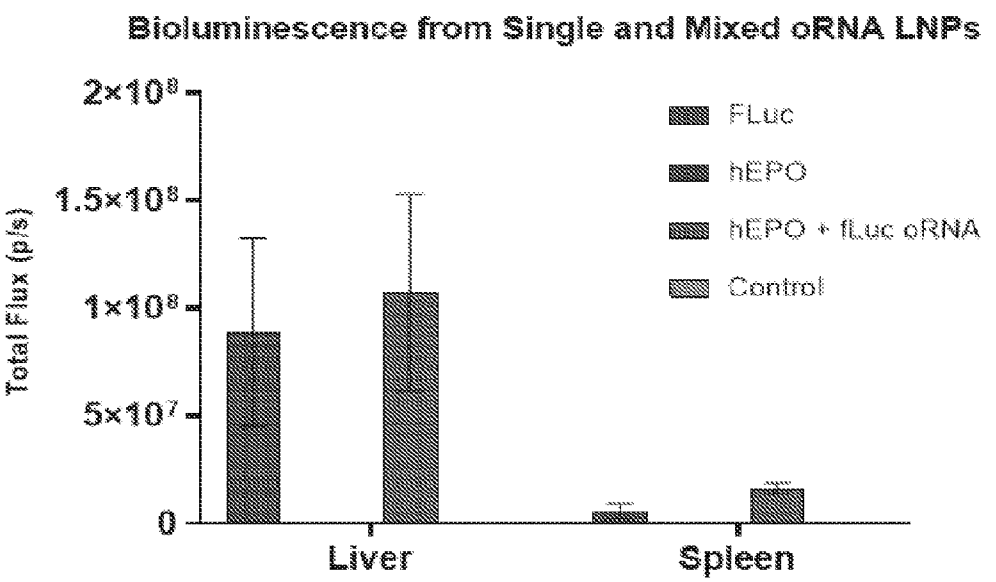

FIG. 76B

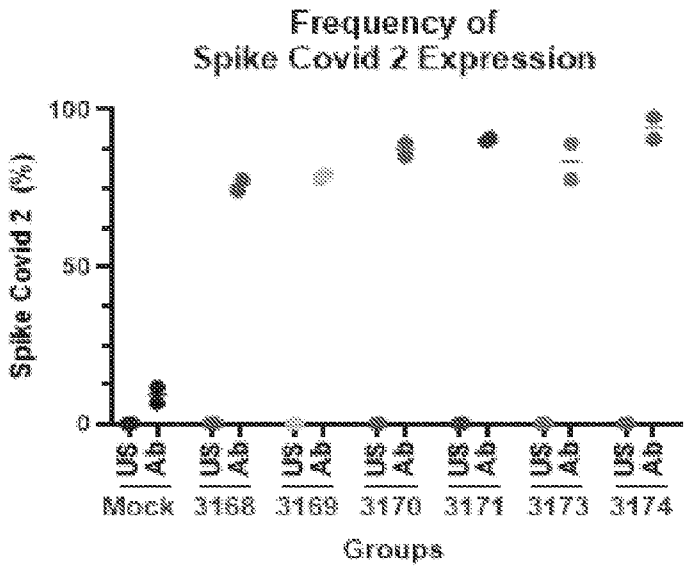

FIG. 77A

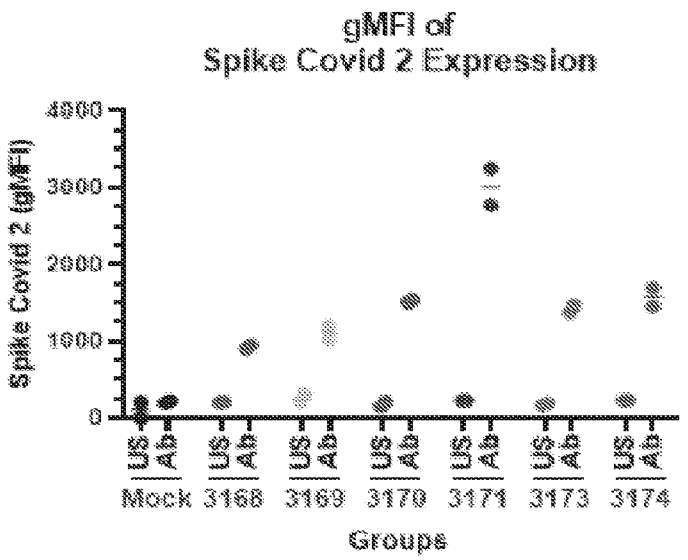

FIG. 77B

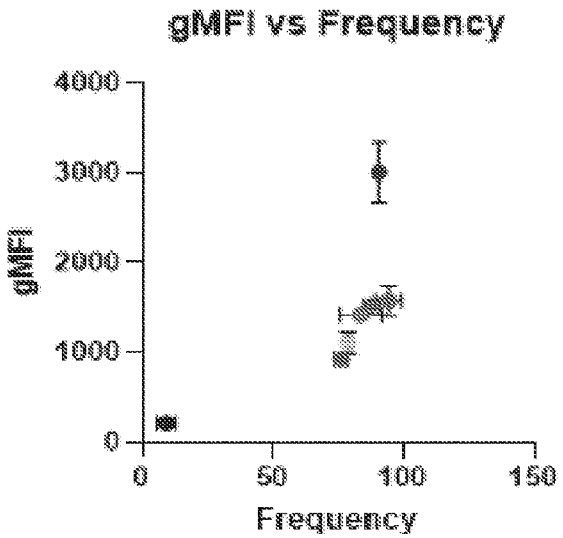
gMFI vs Frequency
4000
3000
2000
1000
0
gMFI
0
50
100
150
Frequency

FIG. 77C

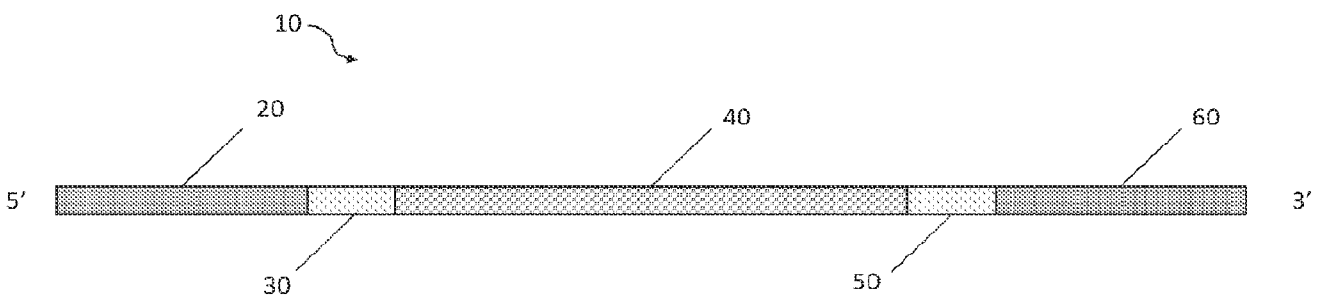

FIG. 78

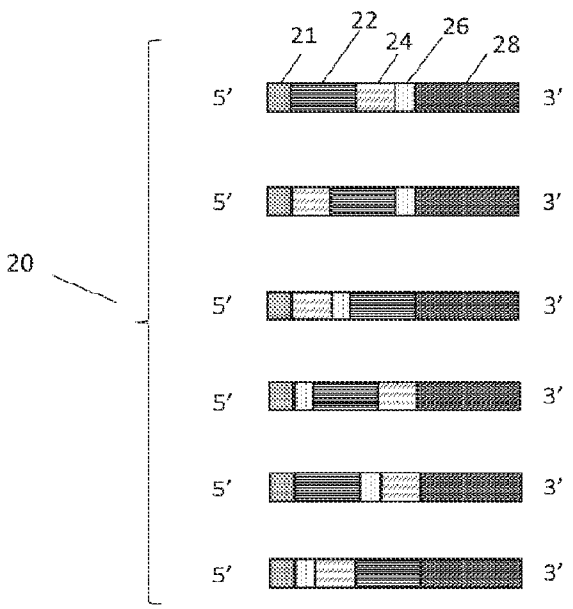

FIG. 79

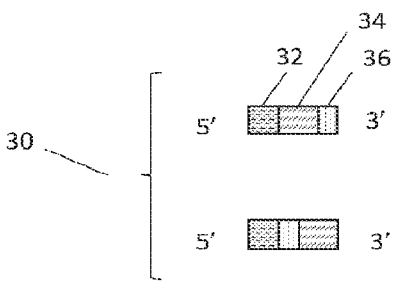

FIG. 80

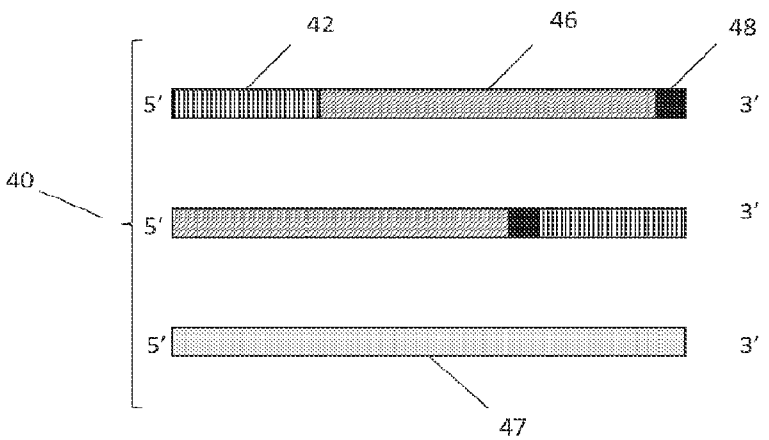

FIG. 81

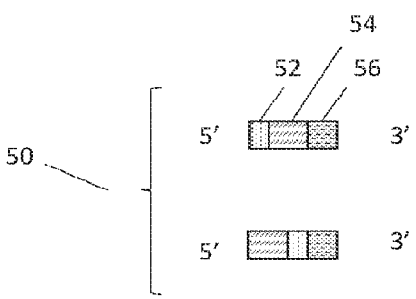

FIG. 82

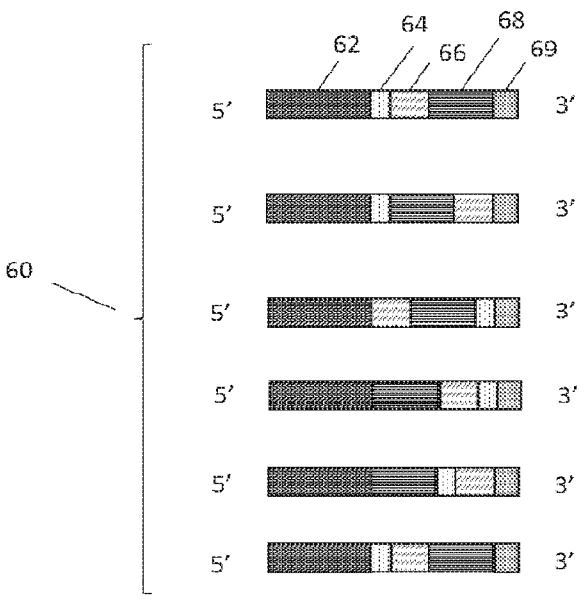

FIG. 83

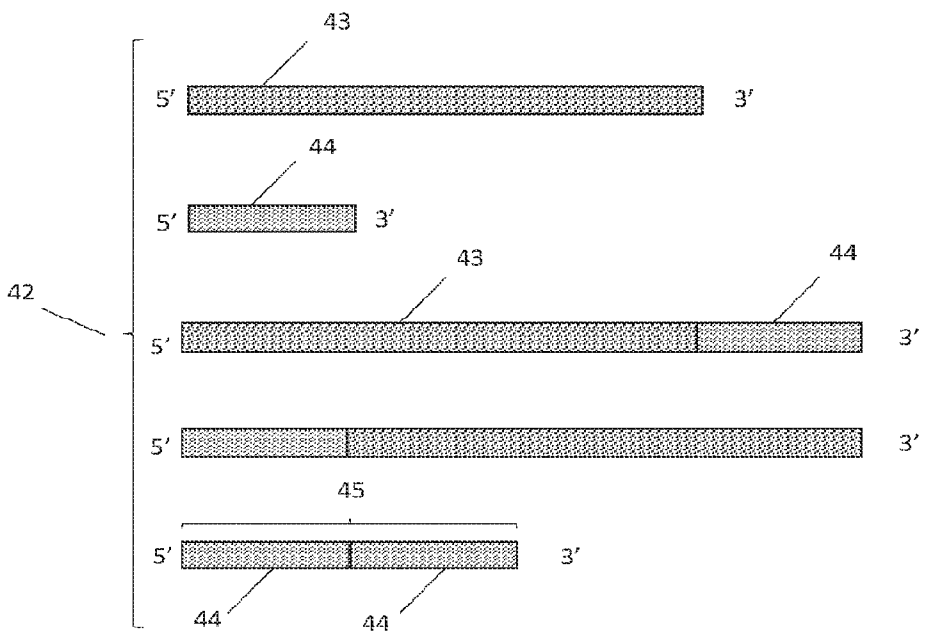

FIG. 84

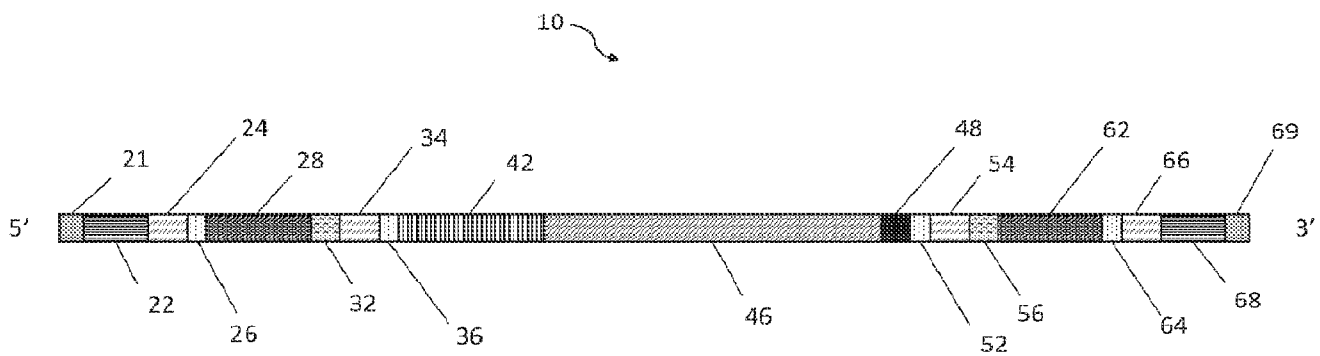

FIG. 85

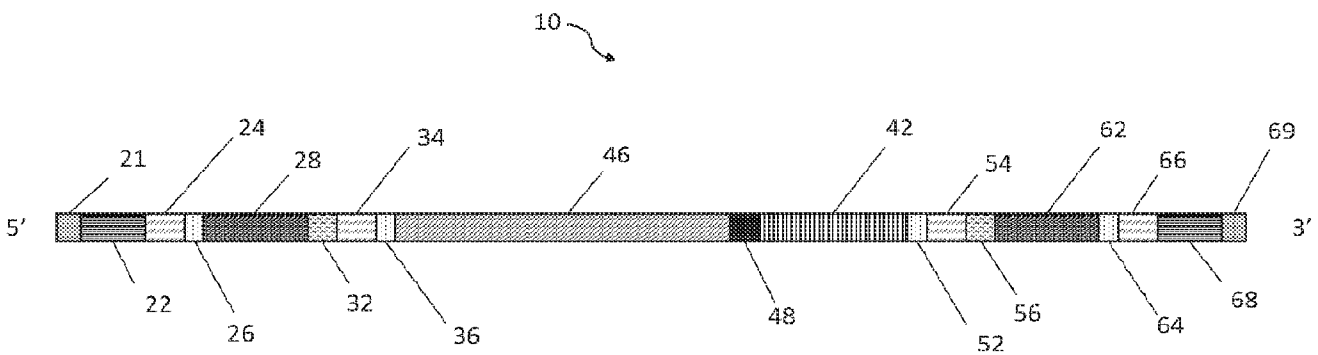

FIG. 86

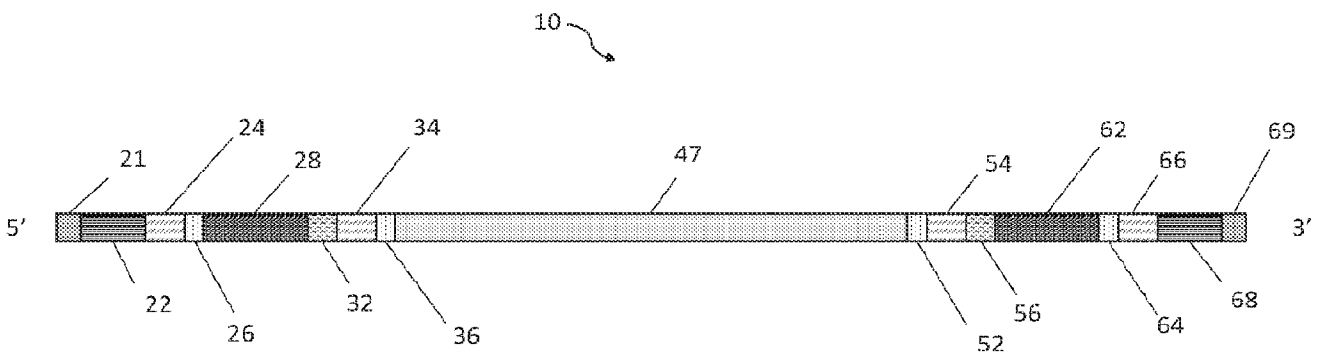

FIG. 87

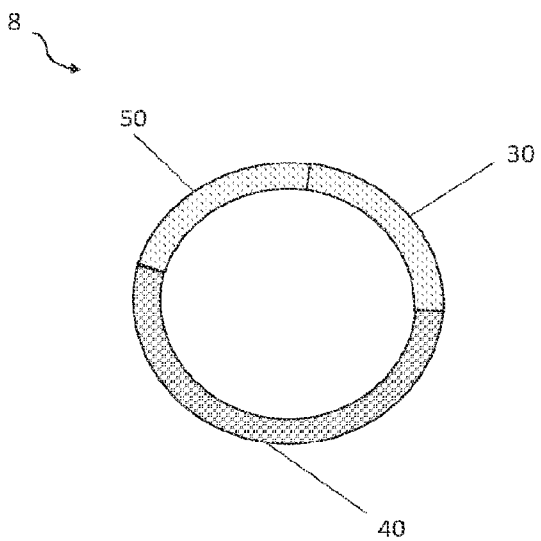

FIG. 88

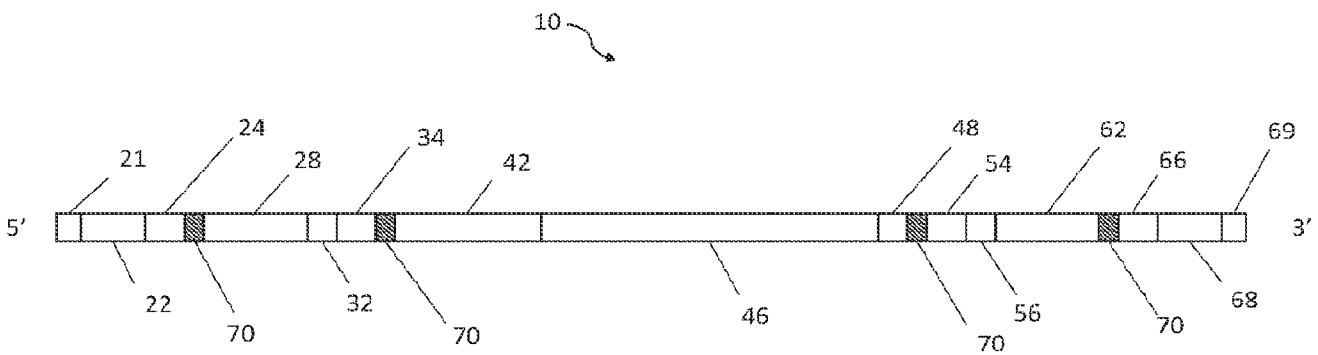

FIG. 89A

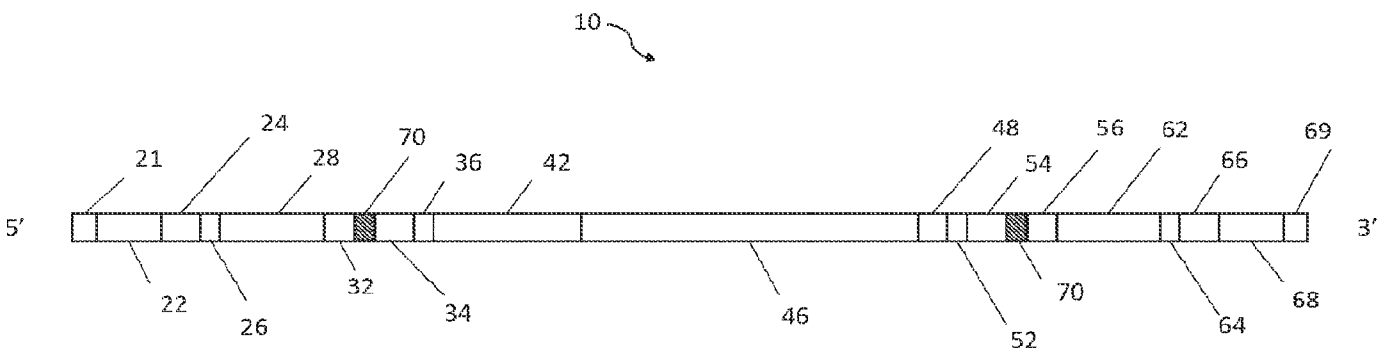

FIG. 89B

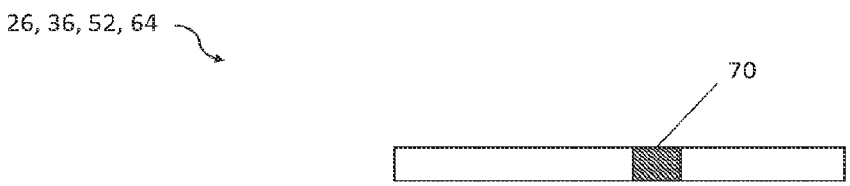

FIG. 89C

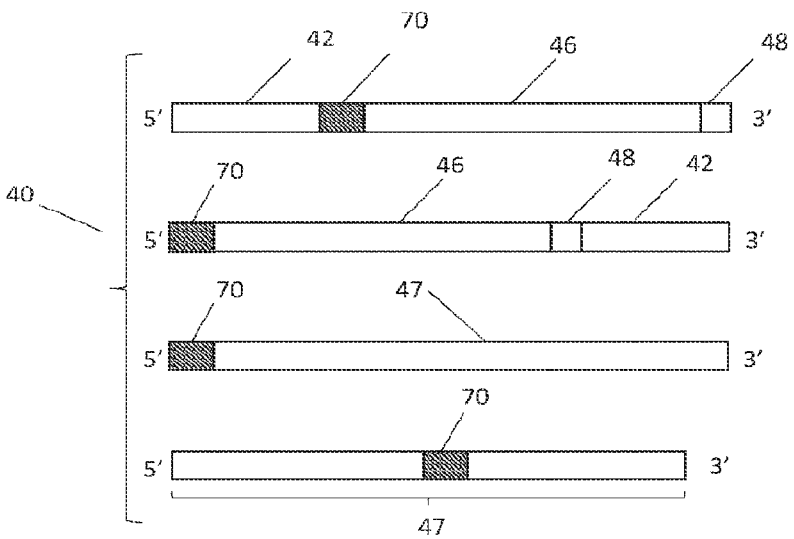

FIG. 89D

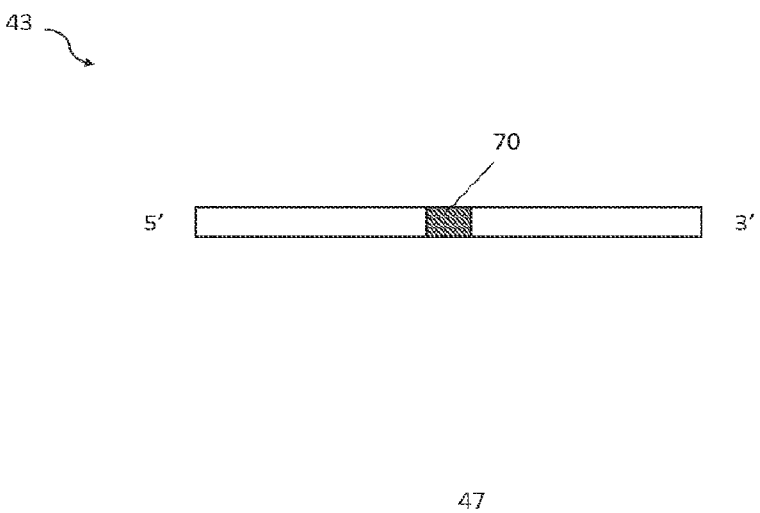

FIG. 89E

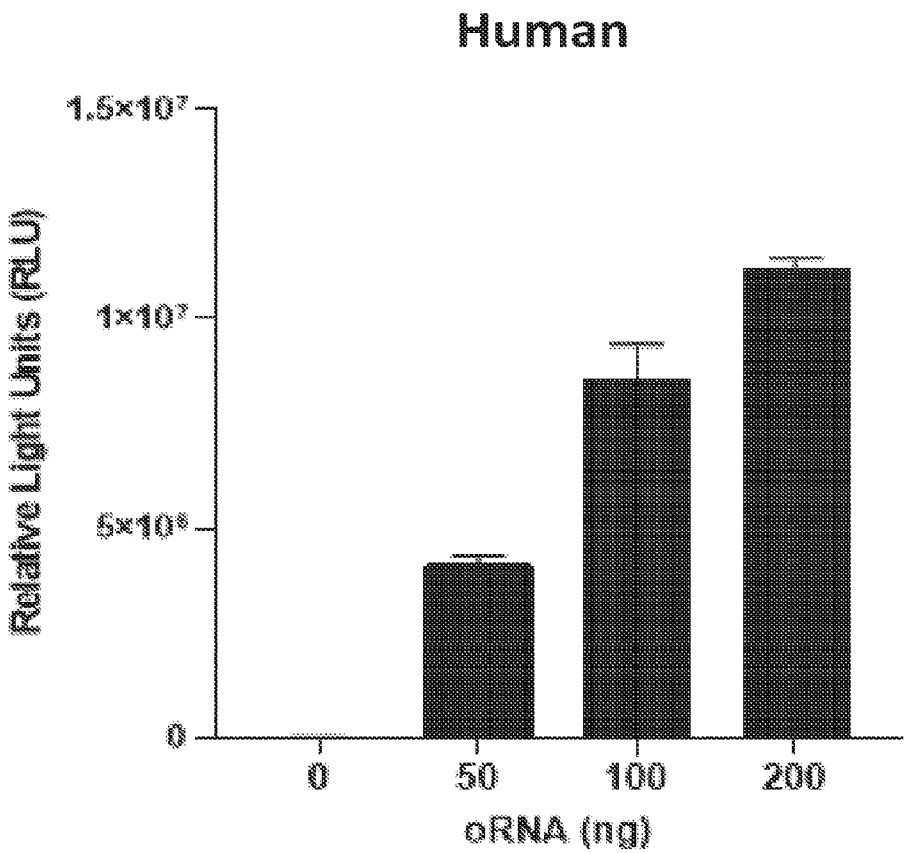
Human
1.5×10⁷
1×10⁷
5×10⁶
0
Relative Light Units (RLU)
0
50
100
200
oRNA (ng)

FIG. 90A

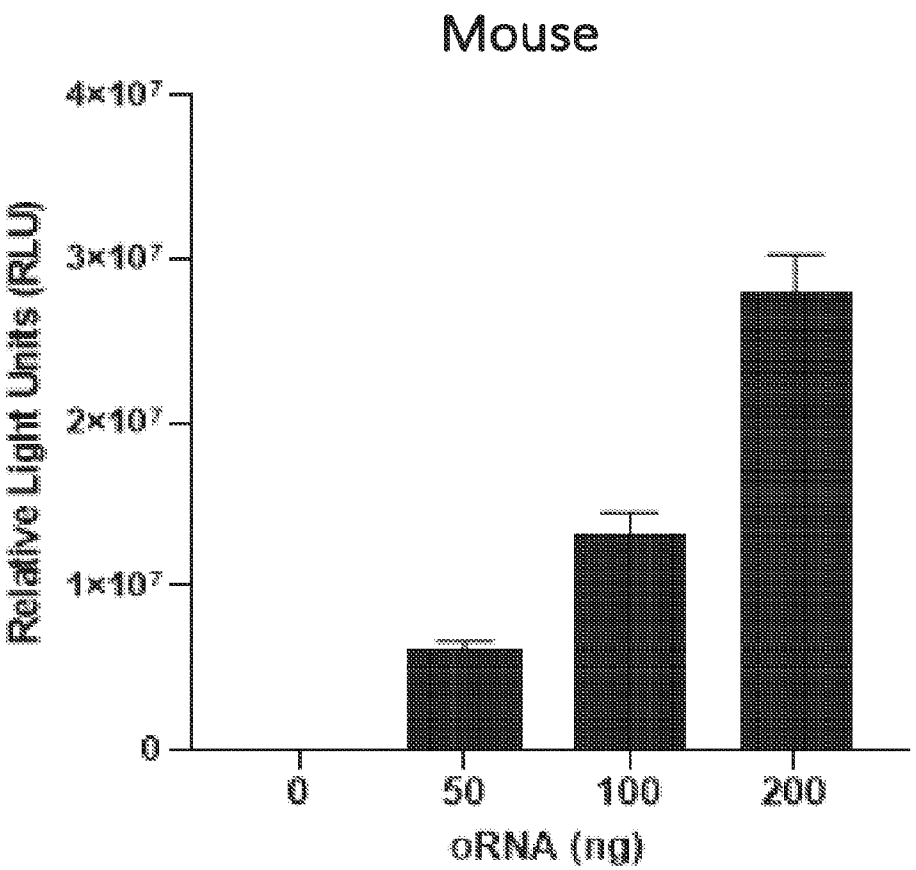
Mouse
Relative Light Units (RLU)
4×10⁷
3×10⁷
2×10⁷
1×10⁷
0
0
50
100
200
oRNA (ng)

FIG. 90B

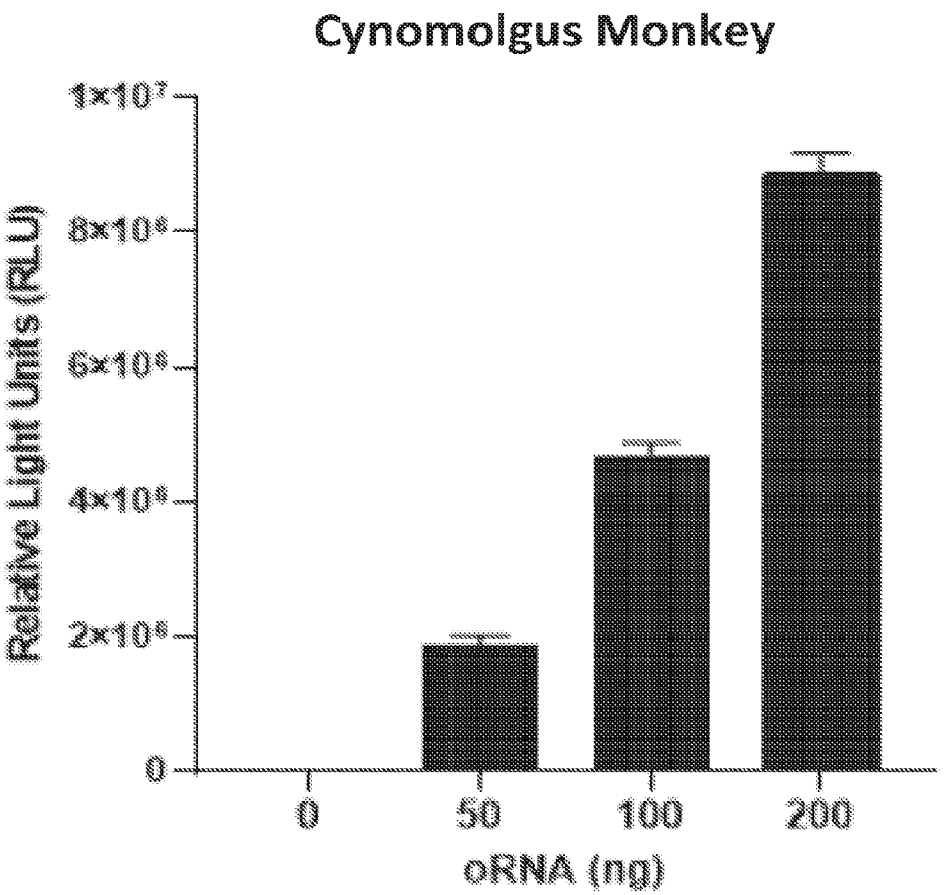
Cynomolgus Monkey
Relative Light Units (RLU)
1×10⁷
8×10⁶
6×10⁶
4×10⁶
2×10⁶
0
0
50
100
200
oRNA (ng)

FIG. 90C

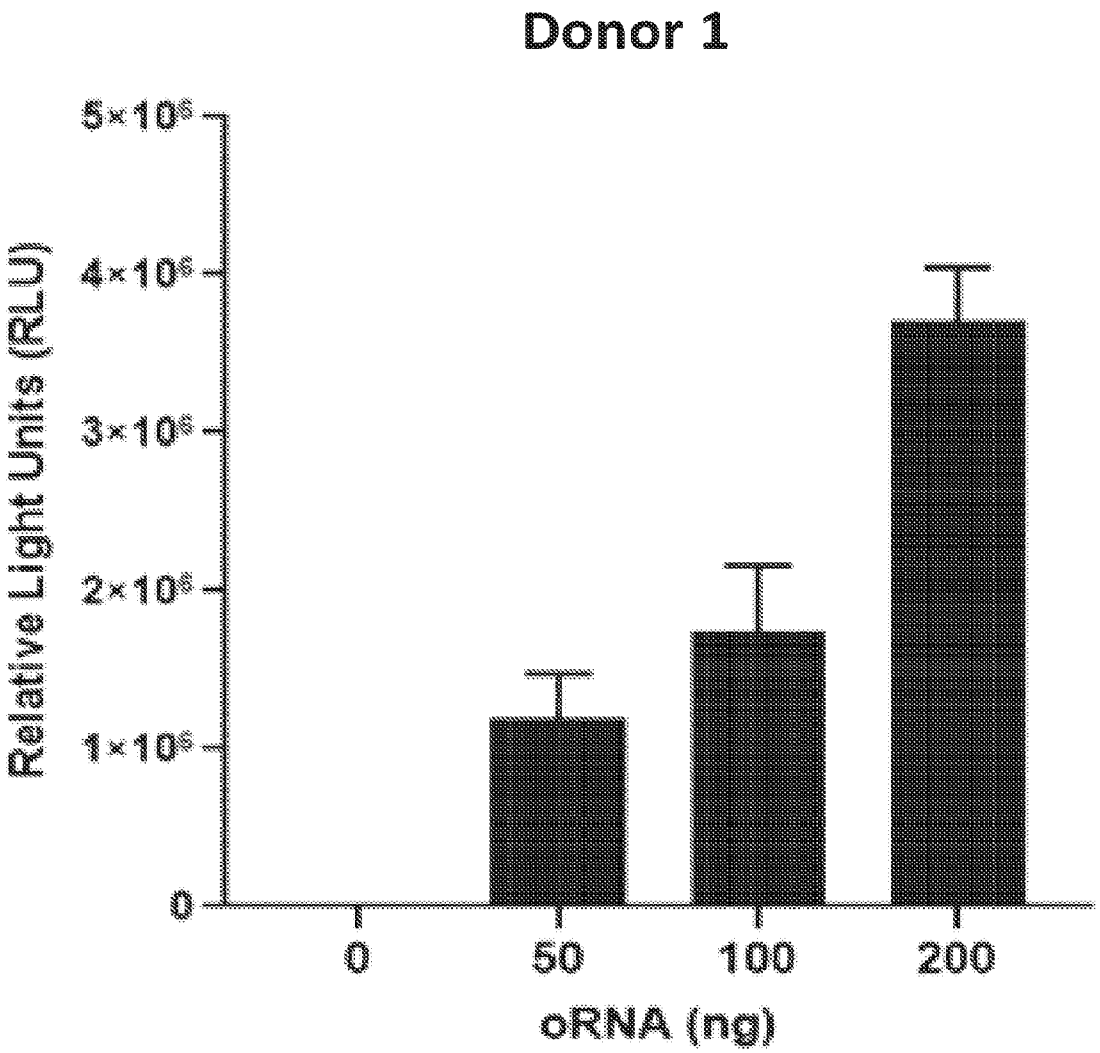
Donor 1
Relative Light Units (RLU)
5×10^6
4×10^6
3×10^6
2×10^6
1×10^6
0
0
50
100
200
oRNA (ng)

FIG. 91A

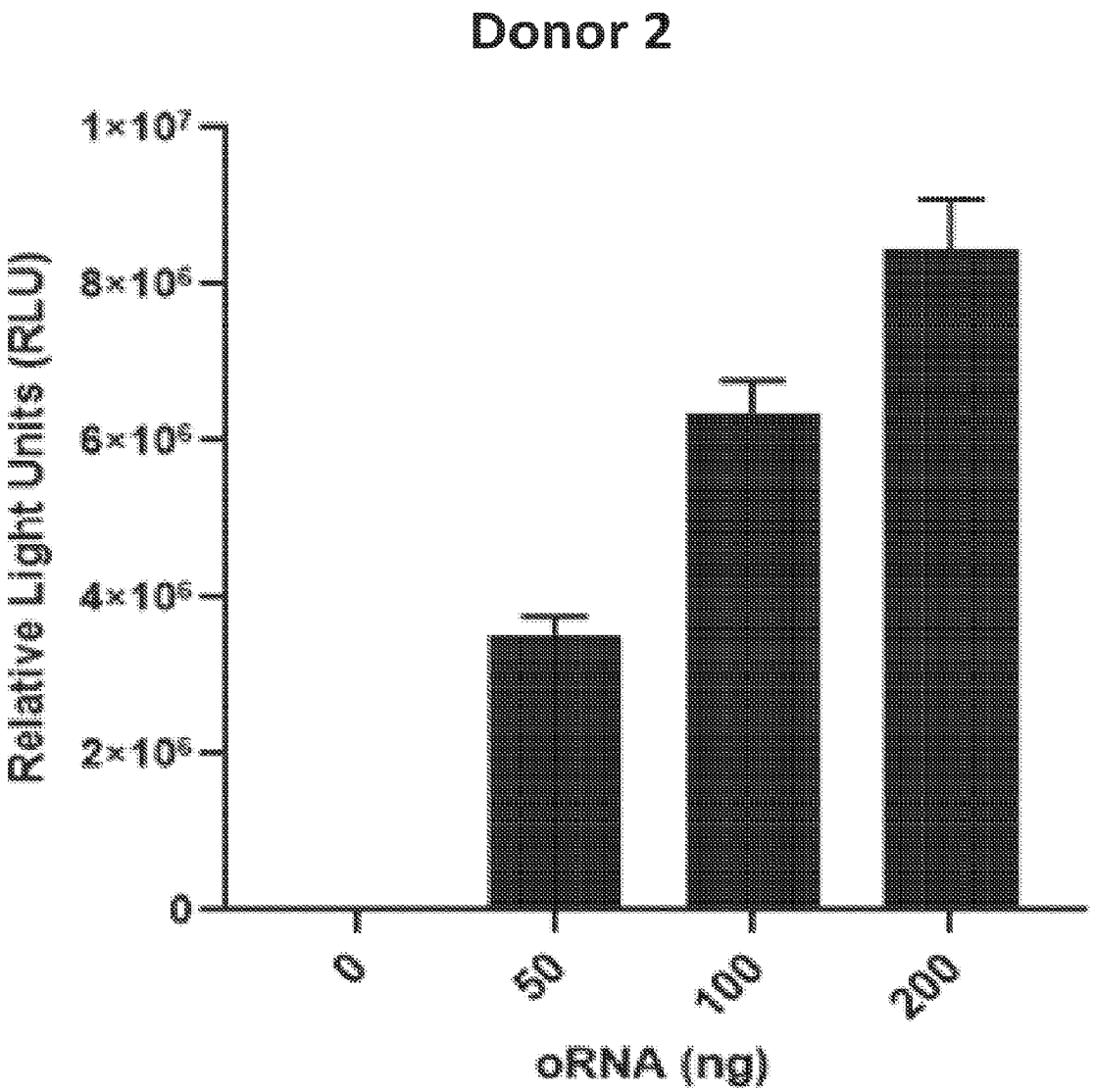
Donor 2
Relative Light Units (RLU)
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
0
50
100
200
oRNA (ng)

FIG. 91B

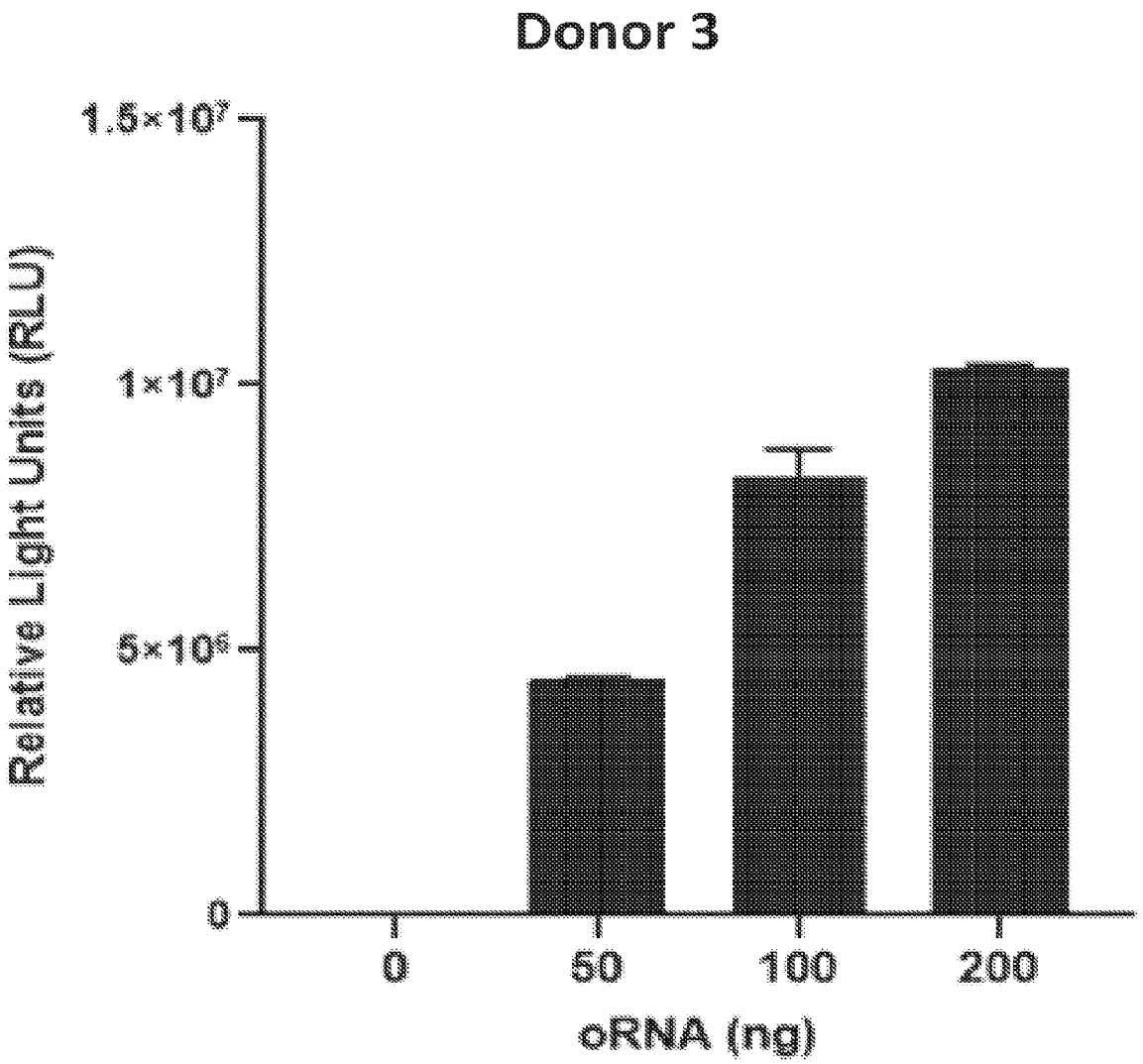
Donor 3
Relative Light Units (RLU)
1.5×10^7
1×10^7
5×10^6
0
0
50
100
200
oRNA (ng)

FIG. 91C

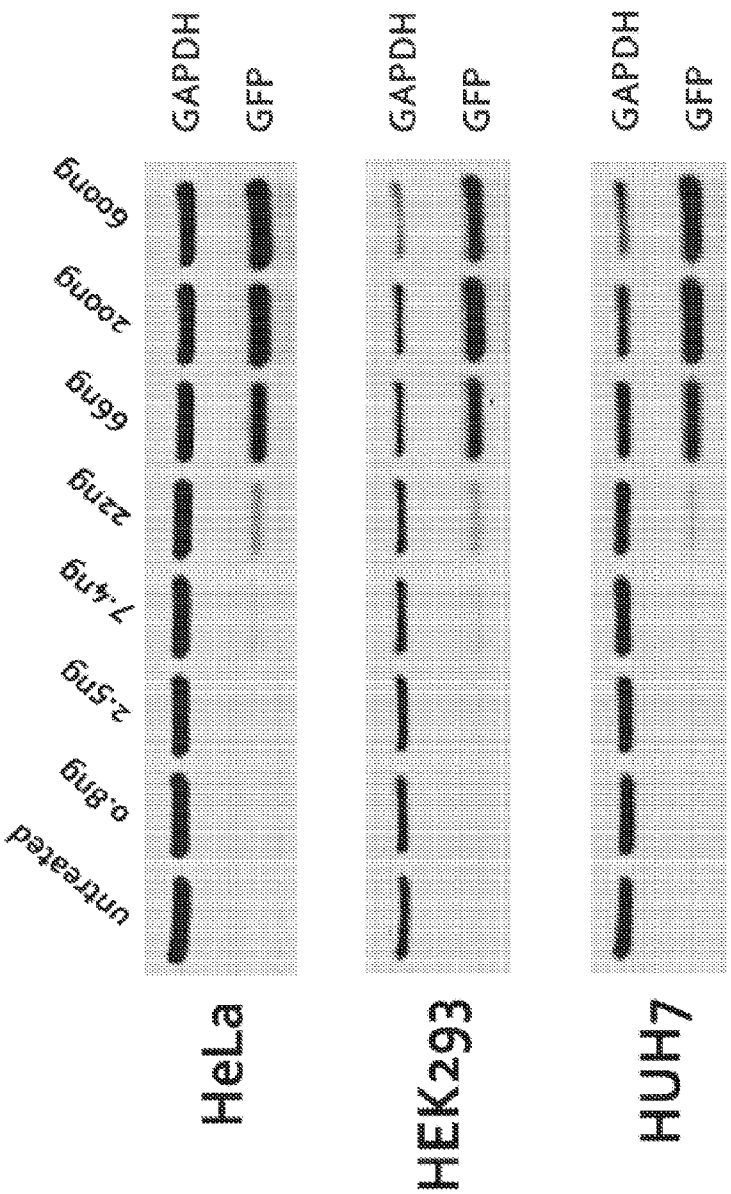
HeLa
HEK293
HUH7
GAPDH
GFP
untreated
0.8ng
2.5ng
7.4ng
22ng
66ng
200ng
600ng

FIG. 92

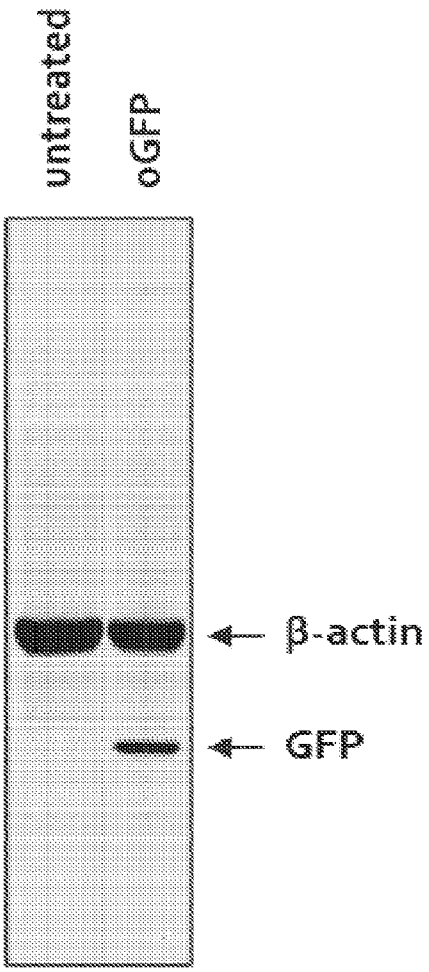

FIG. 93

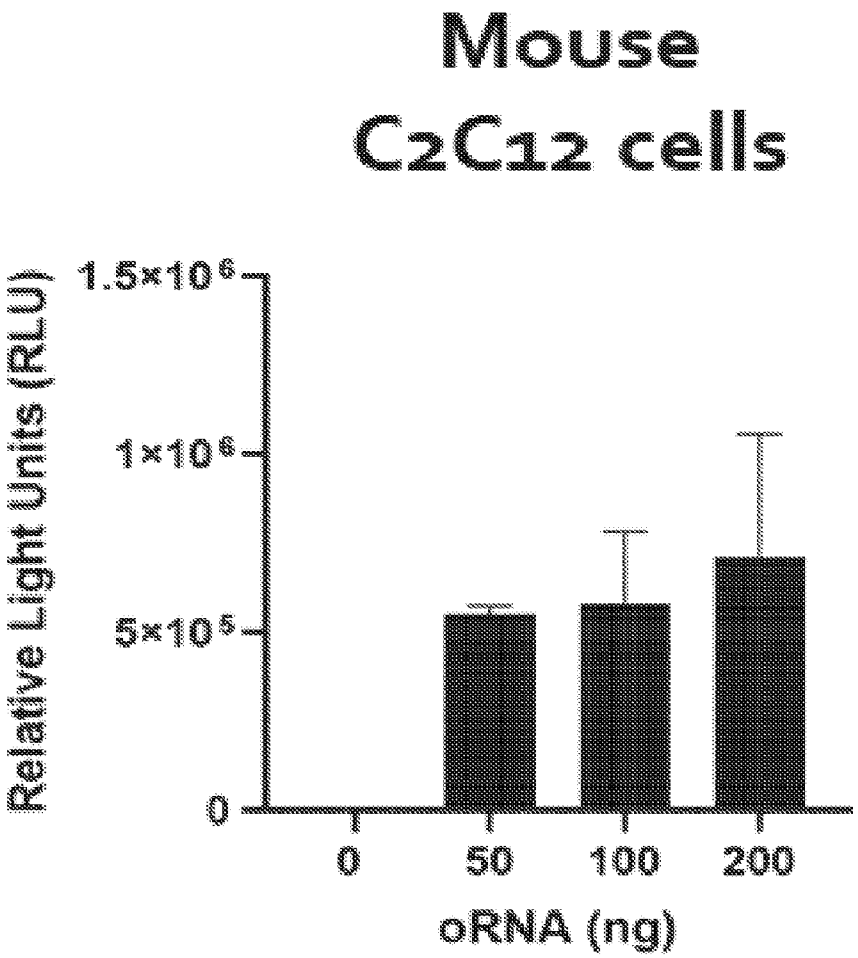
Mouse
C2C12 cells
Relative Light Units (RLU)
1.5×10^6
1×10^6
5×10^5
0
0
50
100
200
oRNA (ng)

FIG. 94A

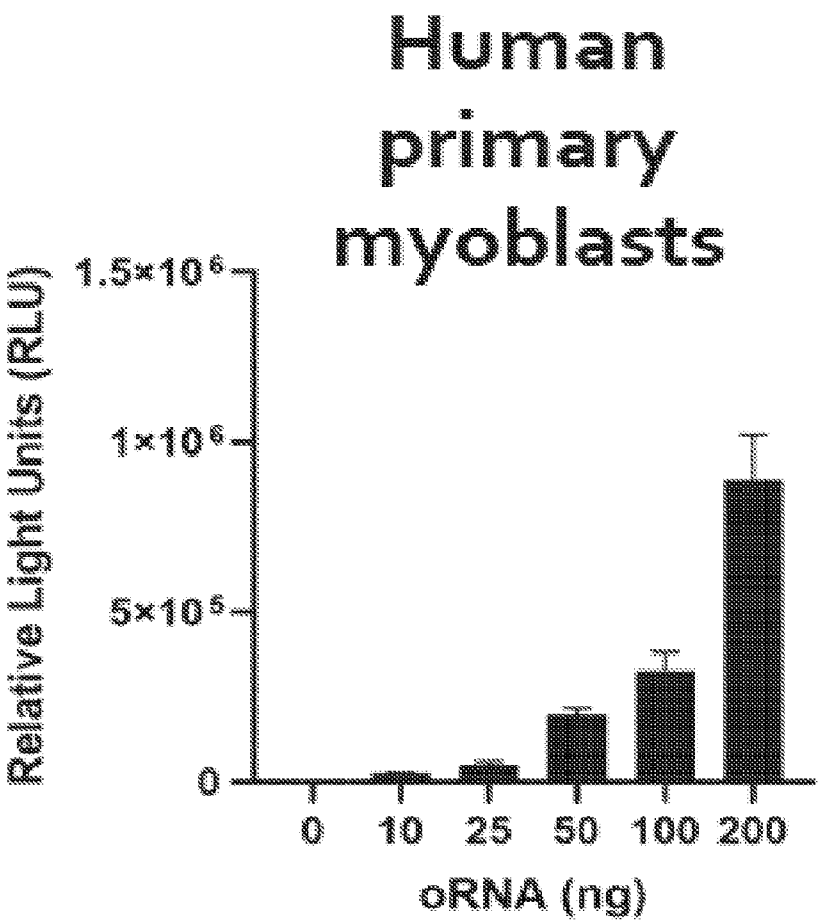
Human primary myoblasts
Relative Light Units (RLU)
1.5×10⁶
1×10⁶
5×10⁵
0
0
10
25
50
100
200
oRNA (ng)

FIG. 94B

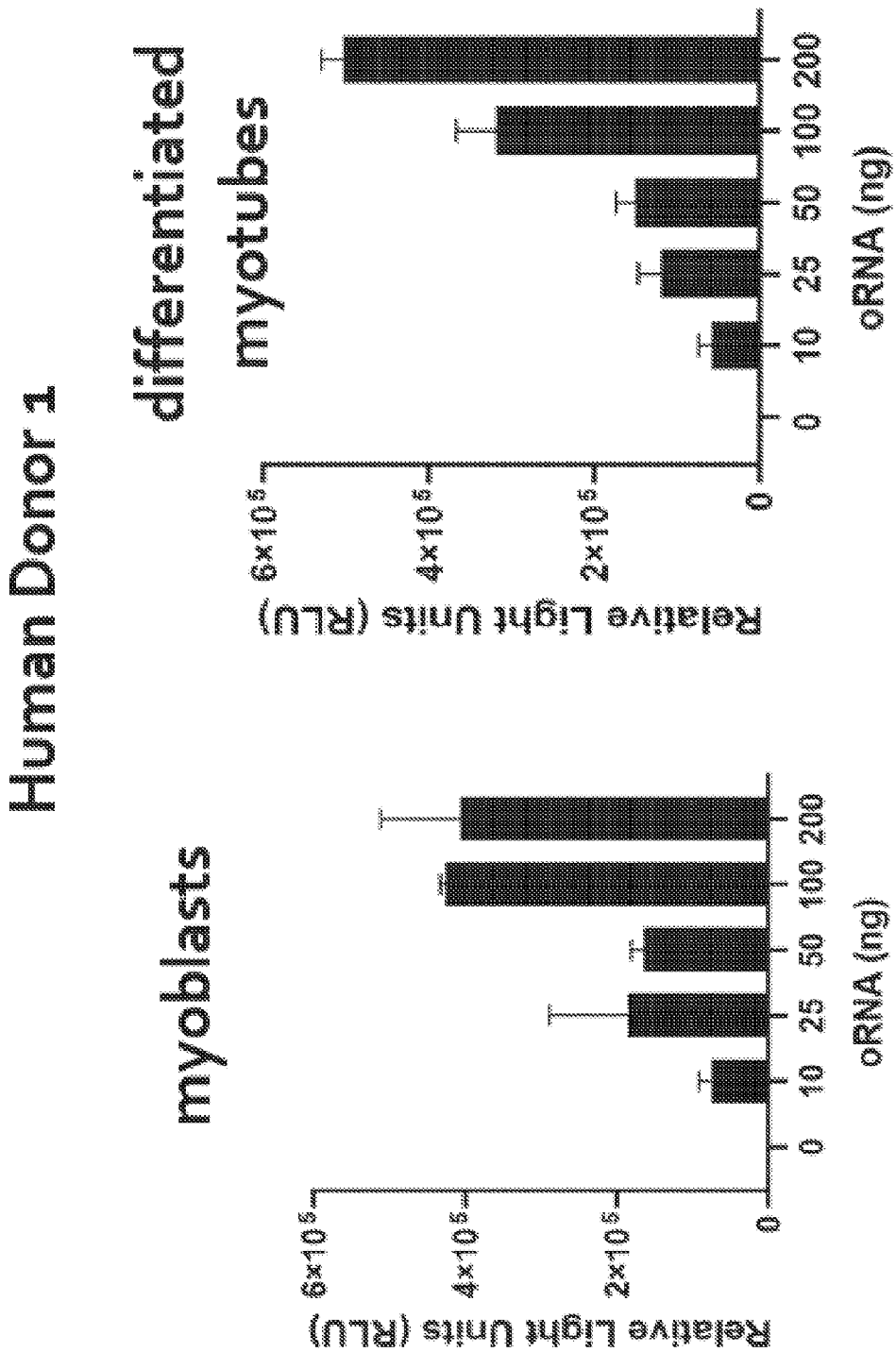
Human Donor 1
myoblasts
differentiated myotubes
Relative Light Units (RLU)
6x10^5
4x10^5
2x10^5
0
0 10 25 50 100 200
oRNA (ng)

FIG. 95A

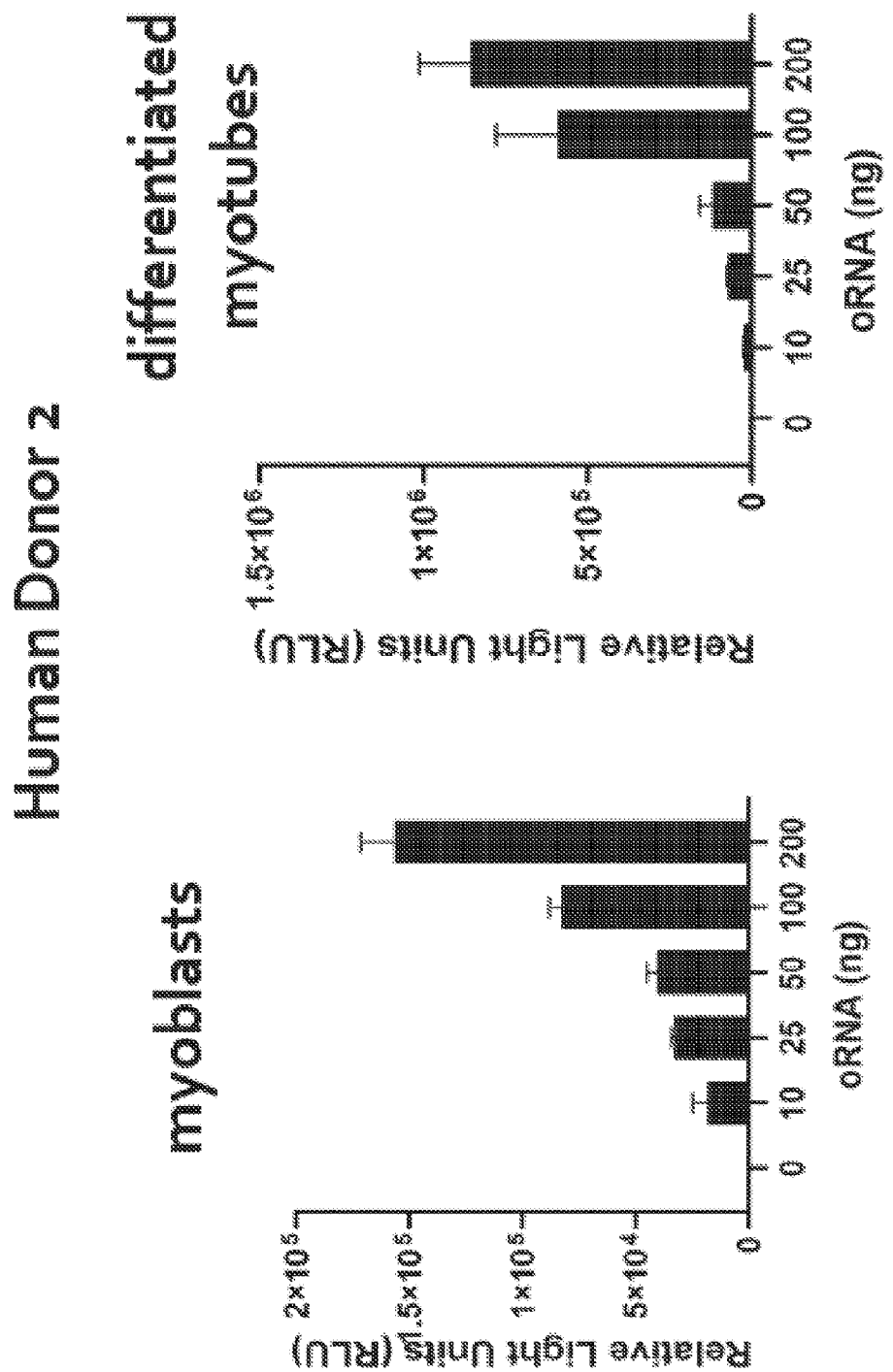
Human Donor 2
myoblasts
differentiated myotubes
Relative Light Units (RLU)
2×10^5
1.5×10^5
1×10^5
5×10^4
0
1.5×10^6
1×10^6
5×10^5
0 10 25 50 100 200
oRNA (ng)

FIG. 95B

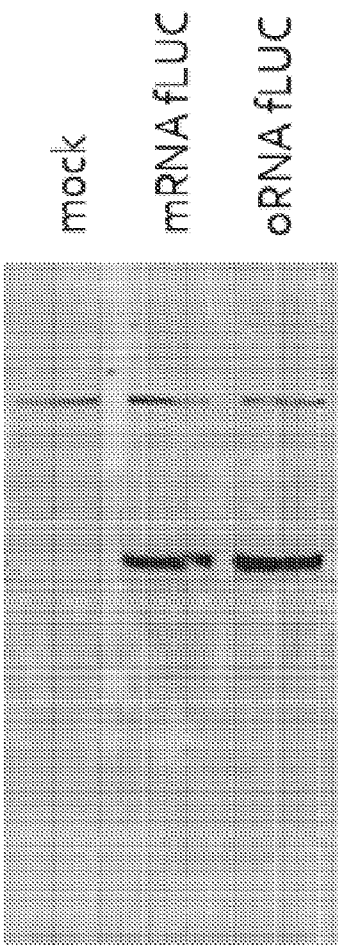

FIG. 96A

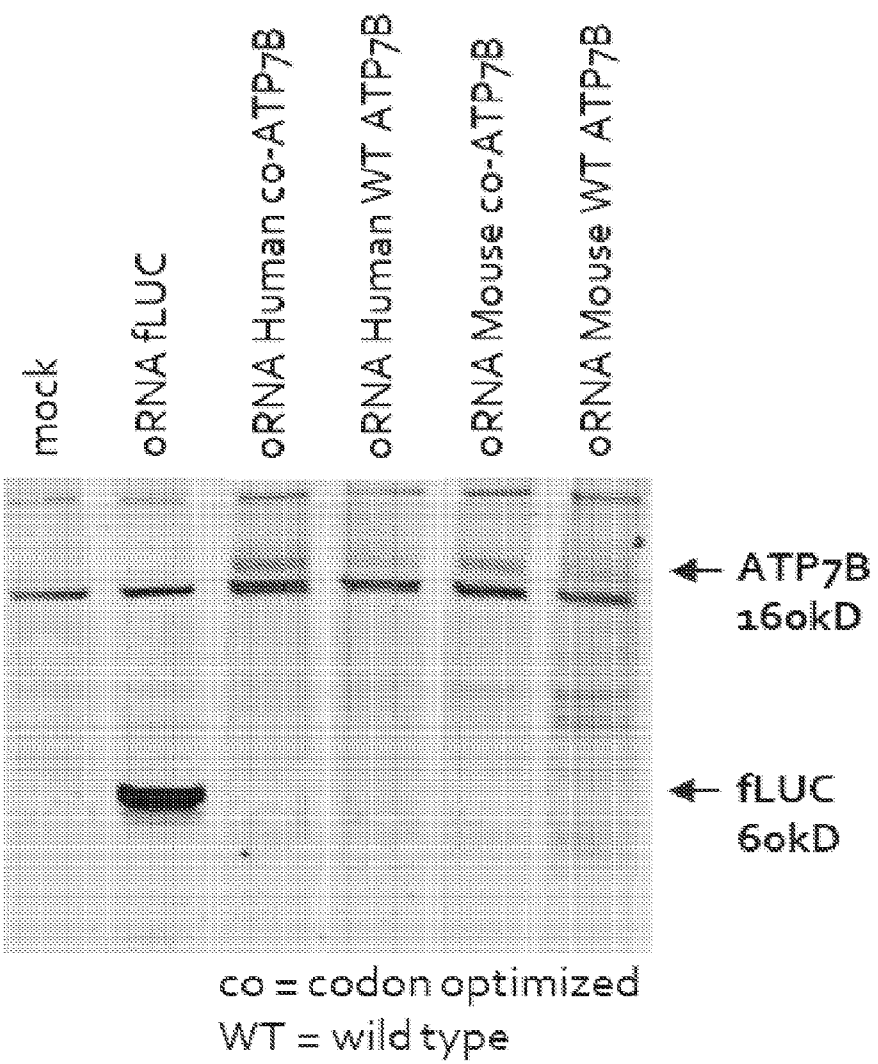
mock
oRNA fLUC
oRNA Human co-ATP7B
oRNA Human WT ATP7B
oRNA Mouse co-ATP7B
oRNA Mouse WT ATP7B
ATP7B
160kD
fLUC
60kD
co = codon optimized
WT = wild type

FIG. 96B

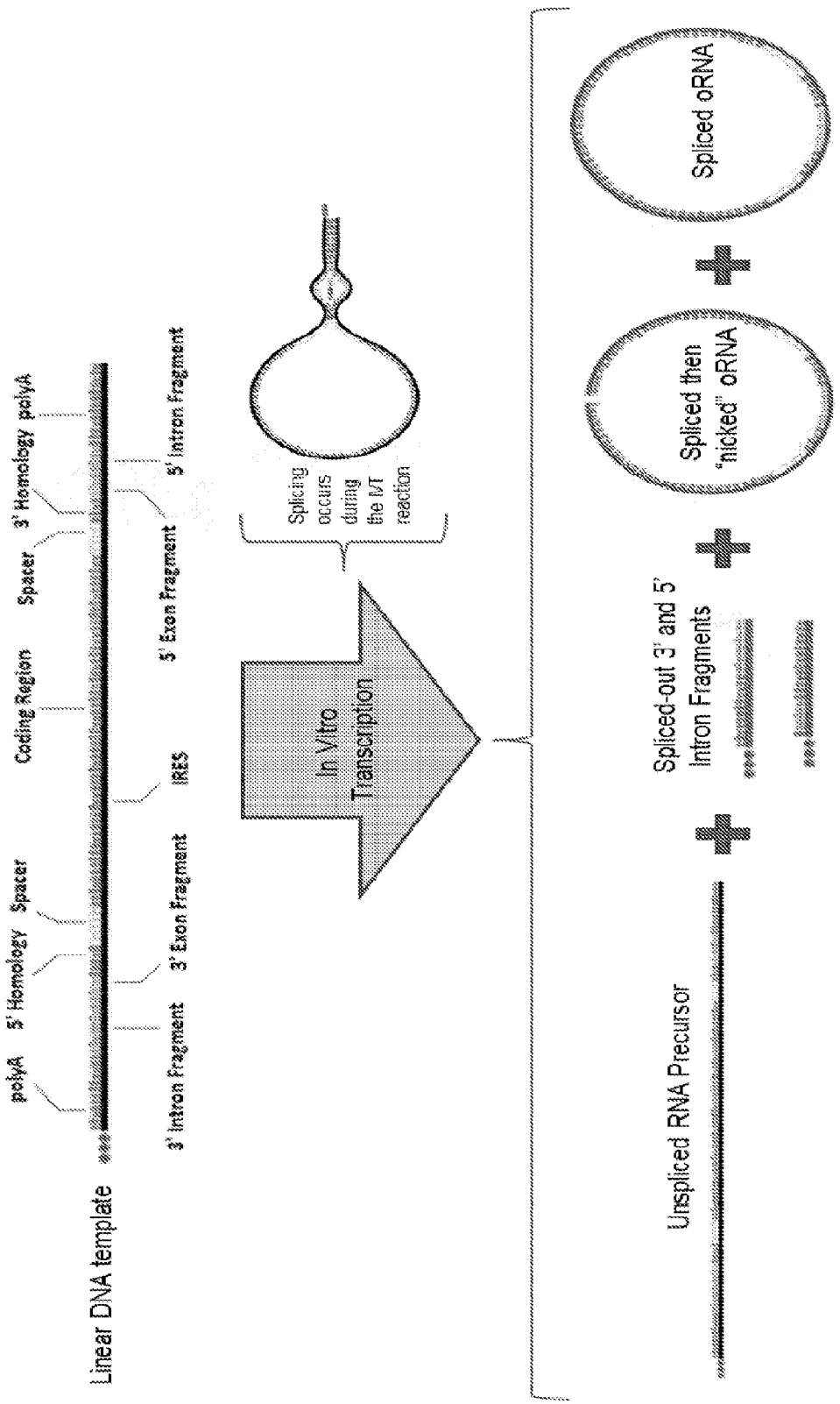
Linear DNA template
polyA
5' Homology
Spacer
3' Exon Fragment
3' Intron Fragment
IRES
Coding Region
Spacer
5' Exon Fragment
3' Homology
polyA
5' Intron Fragment
In Vitro Transcription
Splicing occurs during the IVT reaction
Unspliced RNA Precursor
Spliced-out 3' and 5' Intron Fragments
Spliced then "nicked" oRNA
Spliced oRNA

FIG. 97A

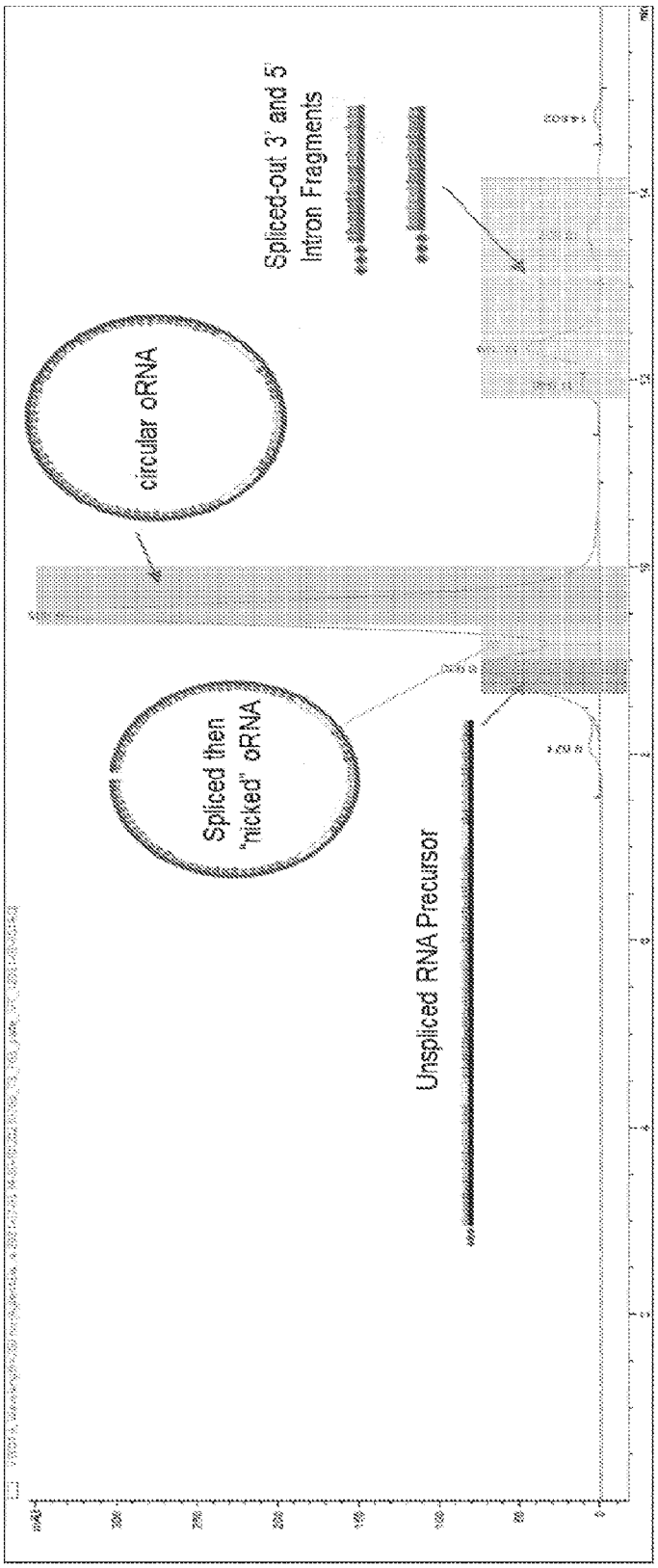
circular oRNA
Spliced-out 3' and 5' Intron Fragments
Spliced then "nicked" oRNA
Unspliced RNA Precursor

FIG. 97B

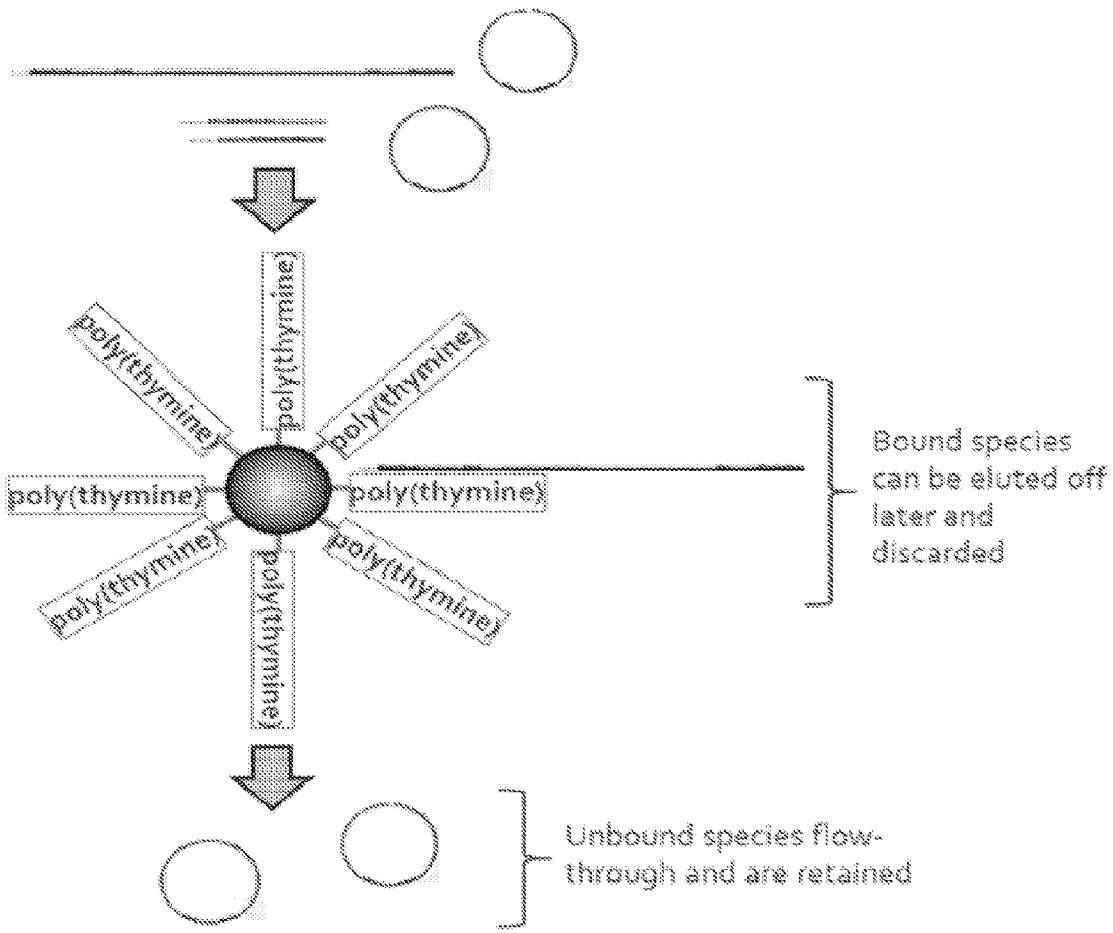
poly(thymine)
poly(thymine)
poly(thymine)
poly(thymine)
poly(thymine)
poly(thymine)
poly(thymine)
poly(thymine)
Bound species can be eluted off later and discarded
Unbound species flow-through and are retained

FIG. 98

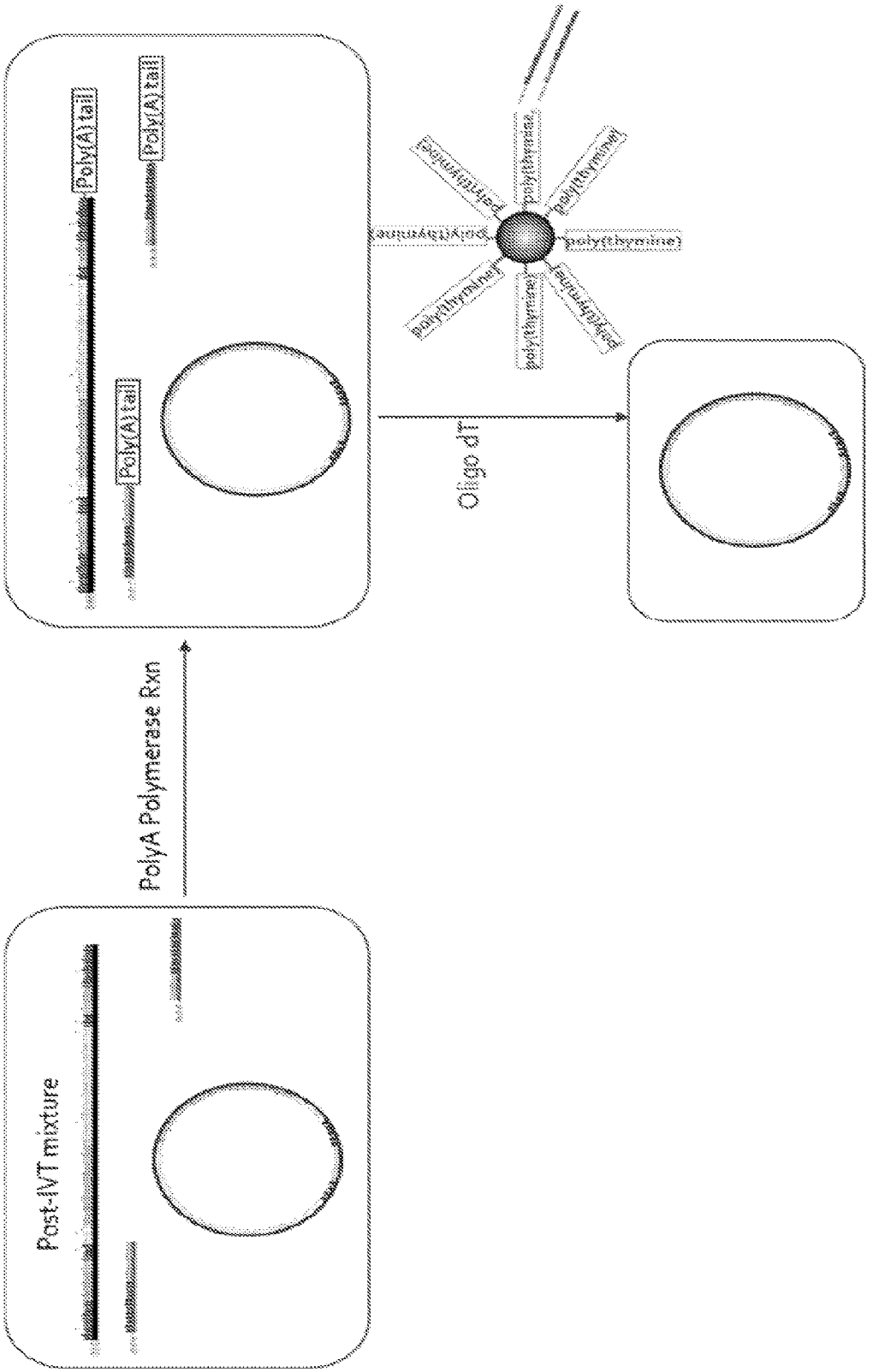
Post-IVT mixture
PolyA Polymerase Rxn
Poly(A) tail
Poly(A) tail
Poly(A) tail
Oligo dT
polythymine
polythymine
polythymine
polythymine
polythymine
polythymine
polythymine

FIG. 99A

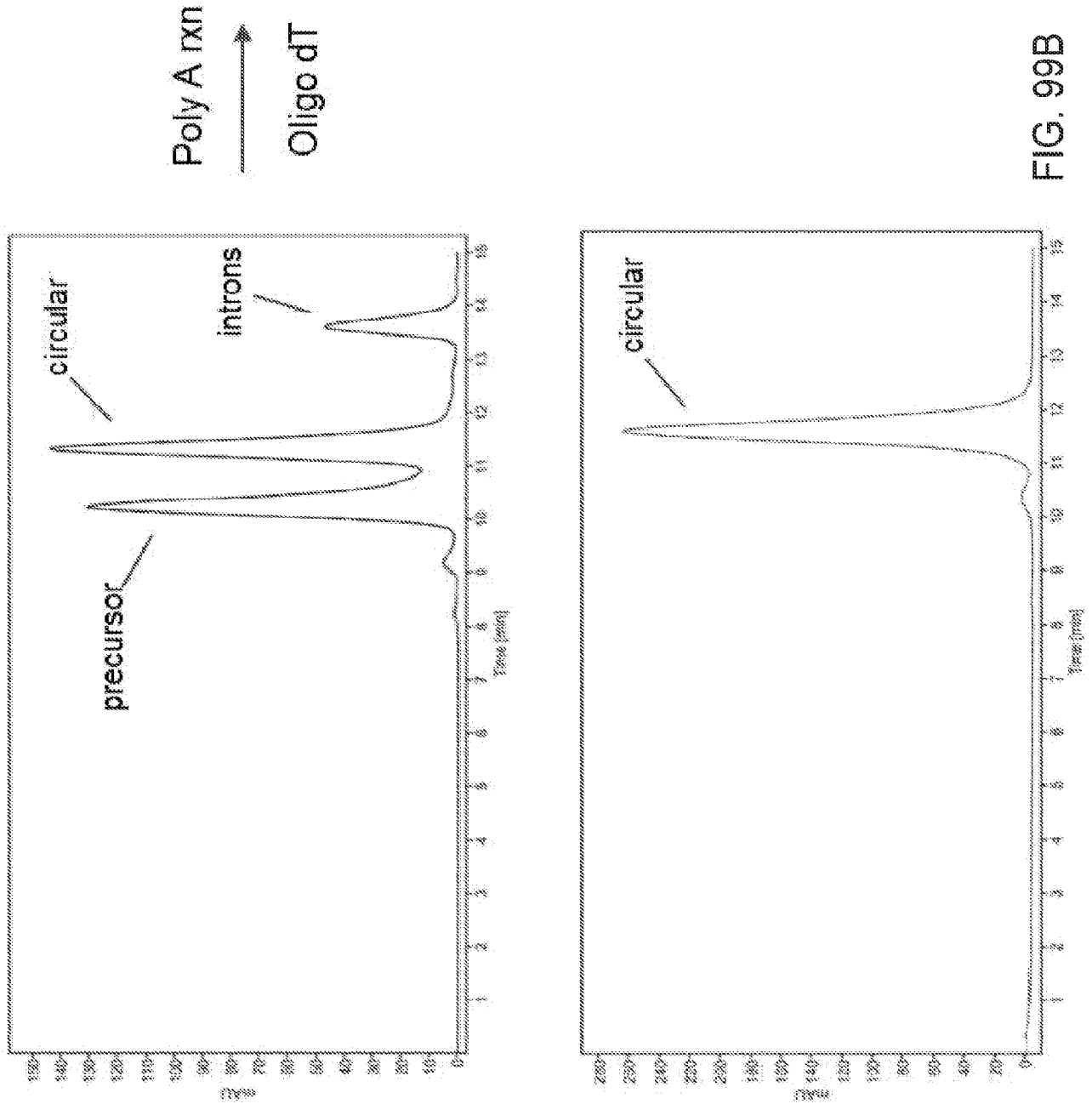

FIG. 99B

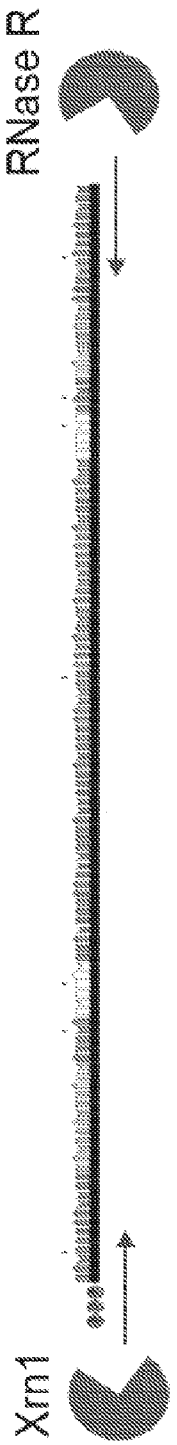

FIG. 100A

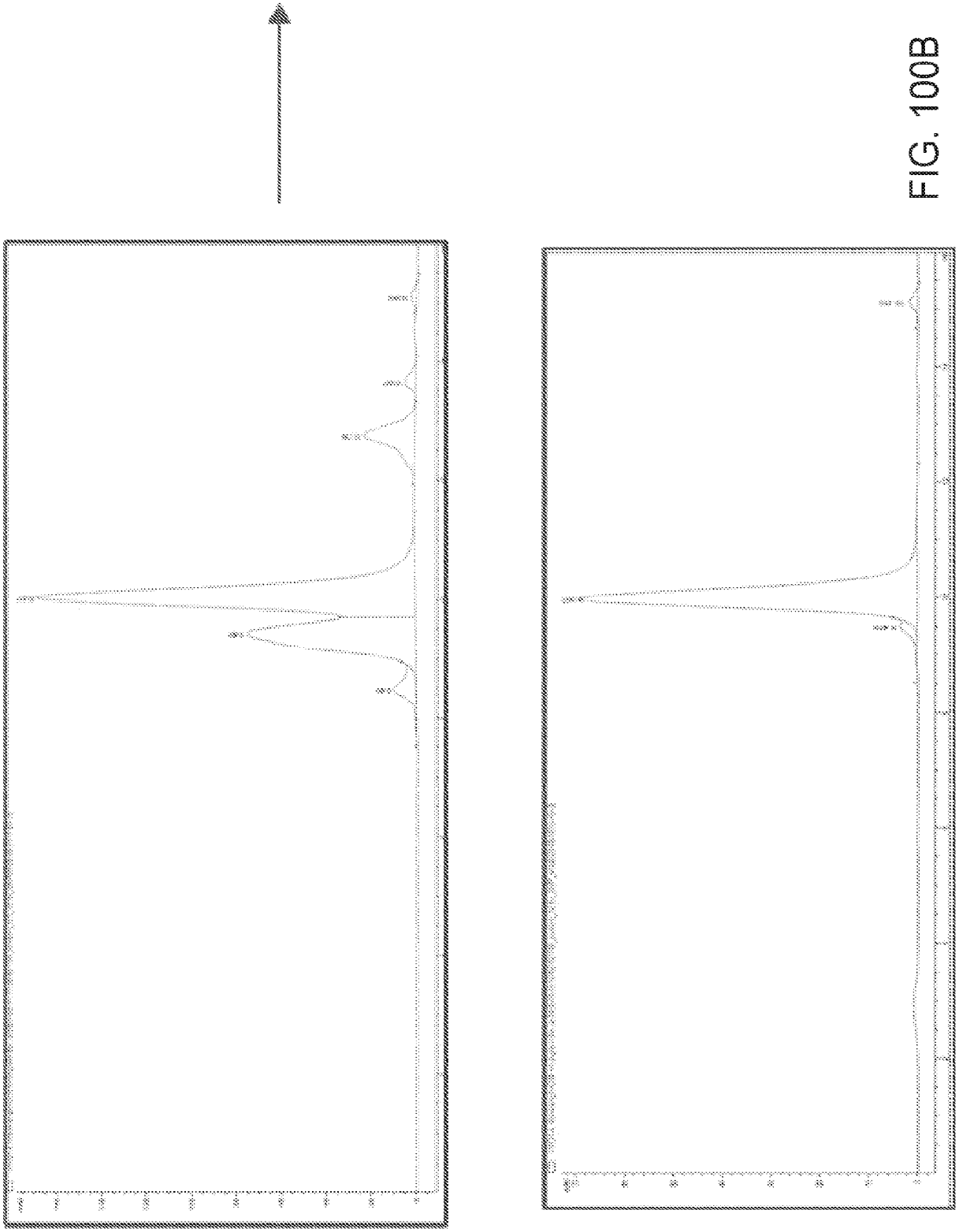

FIG. 100B

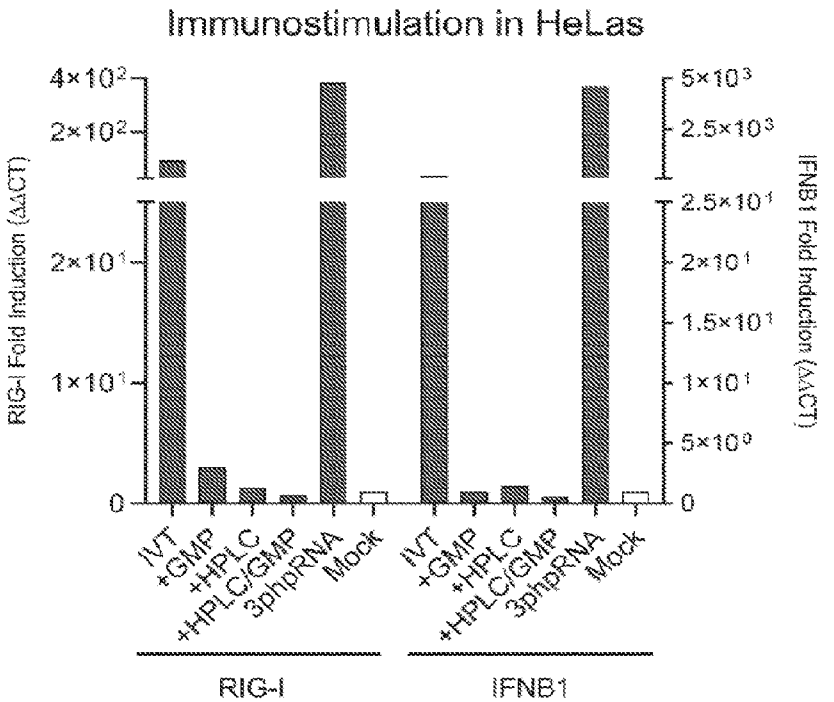

FIG. 101A

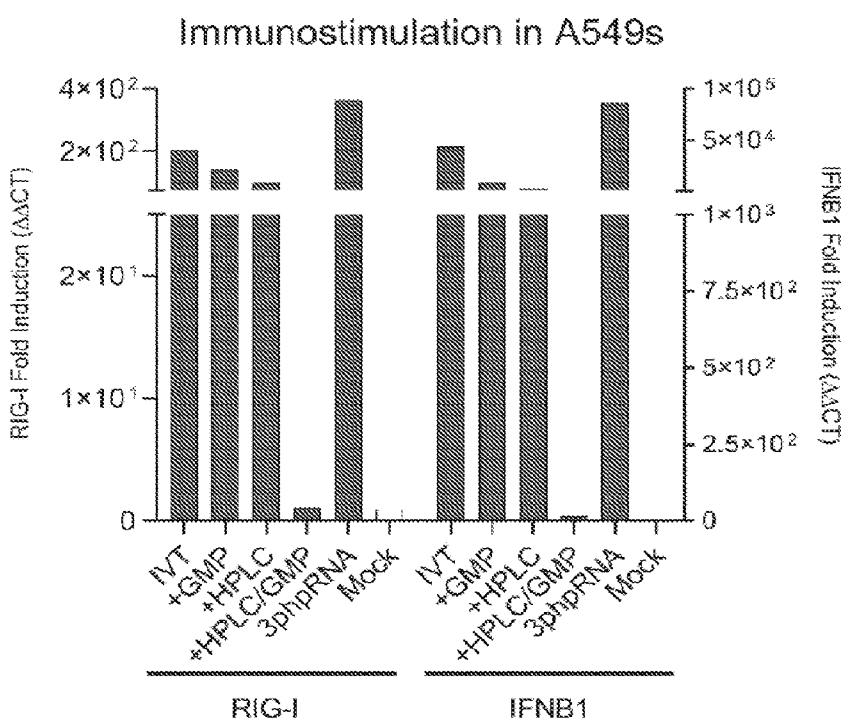

FIG. 101B

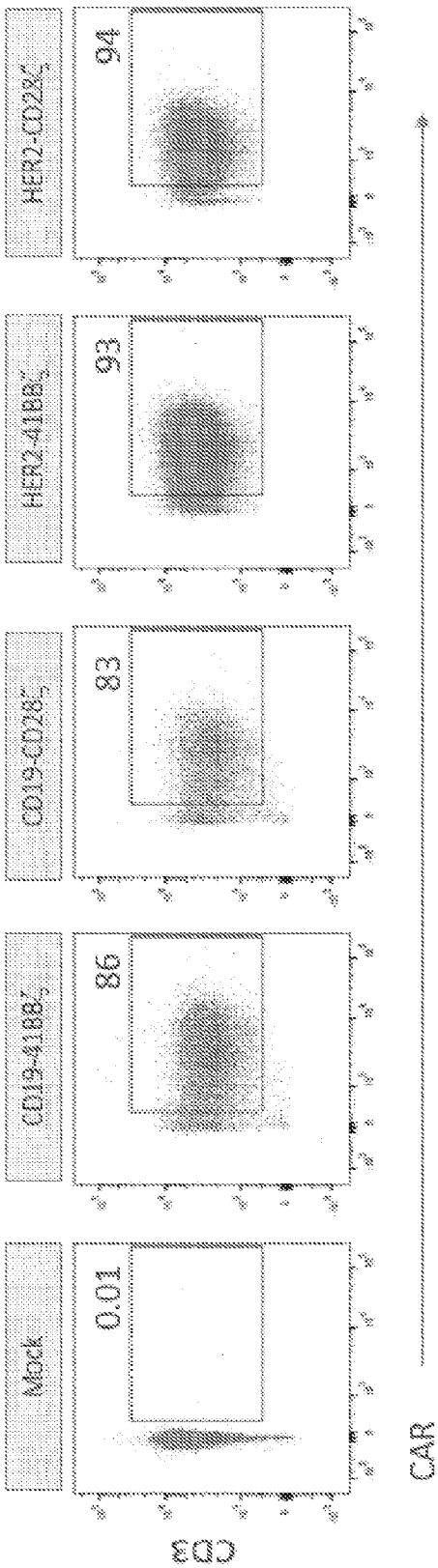
Mock
0.01
CD19-41BBζ
86
CD19-CD28ζ
83
HER2-41BBζ
93
HER2-CD28ζ
94
CD3
CAR

FIG. 102A

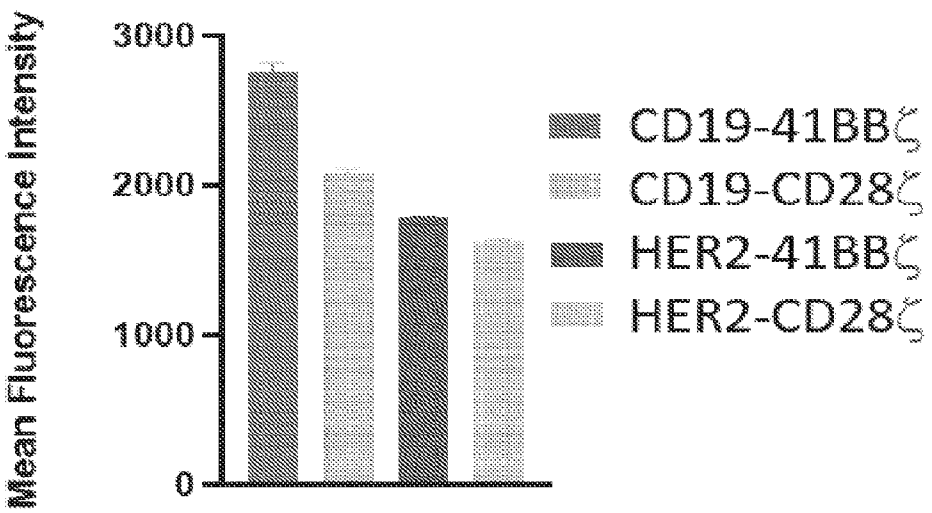
Mean Fluorescence Intensity
3000
2000
1000
0
CD19-41BBζ
CD19-CD28ζ
HER2-41BBζ
HER2-CD28ζ

FIG. 102B

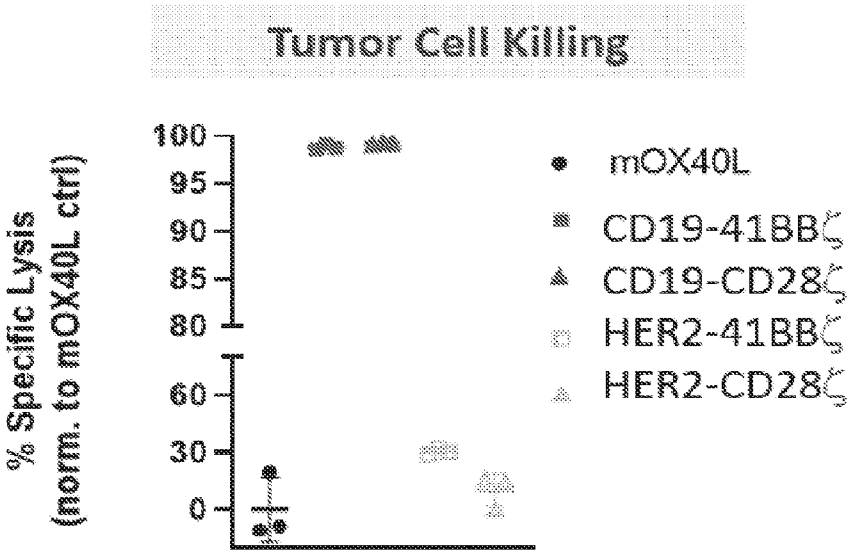

FIG. 103A

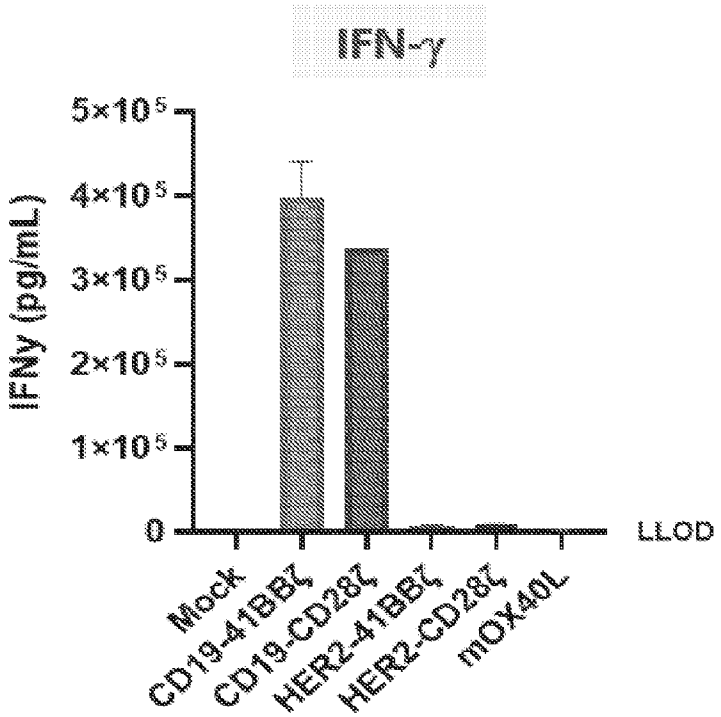

FIG. 103B

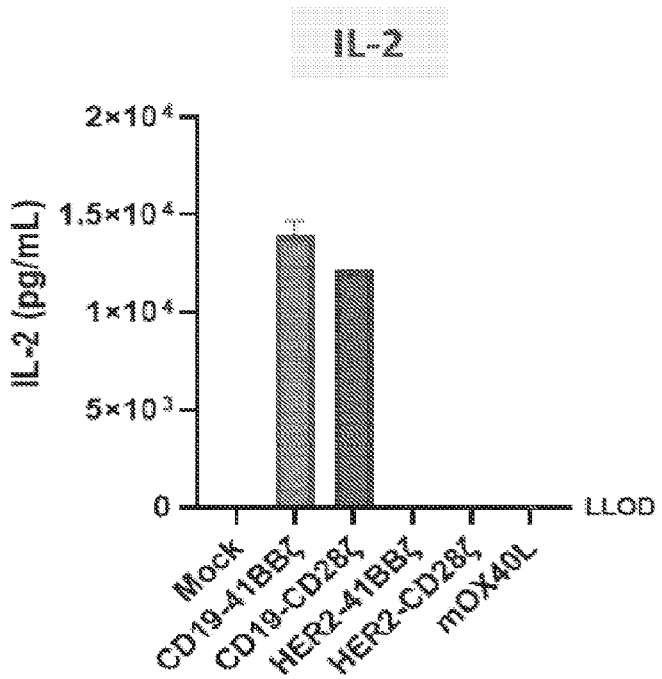
IL-2
IL-2 (pg/mL)
2×10⁴
1.5×10⁴
1×10⁴
5×10³
0
LLOD
Mock
CD19-41BBζ
CD19-CD28ζ
HER2-41BBζ
HER2-CD28ζ
mOX40L

FIG. 103C

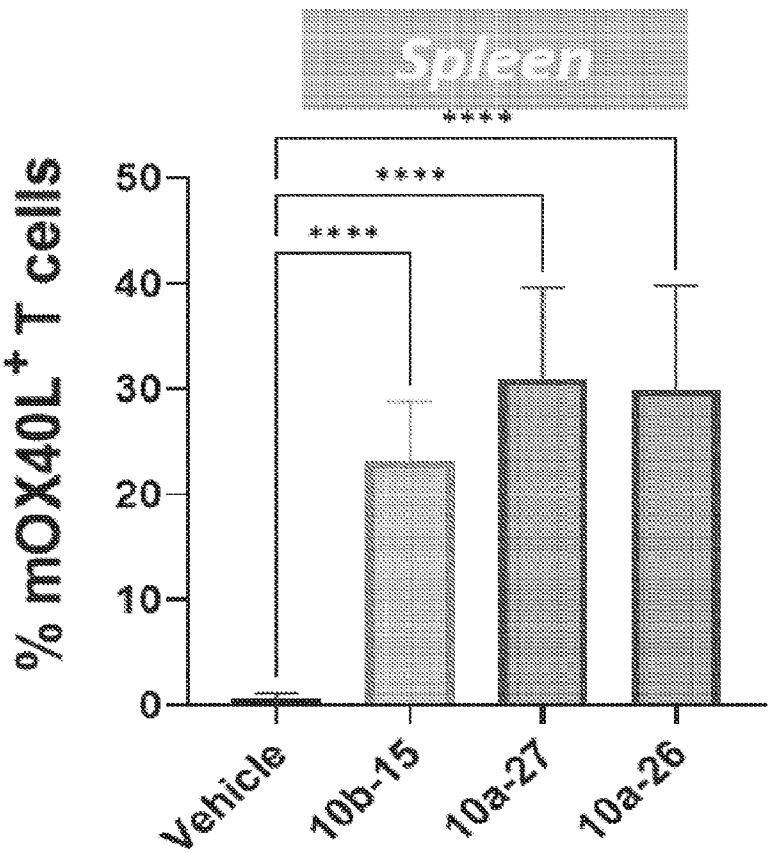
Spleen
% mOX40L+ T cells
50
40
30
20
10
0
****
****
****
Vehicle
10b-15
10a-27
10a-26

FIG. 104A

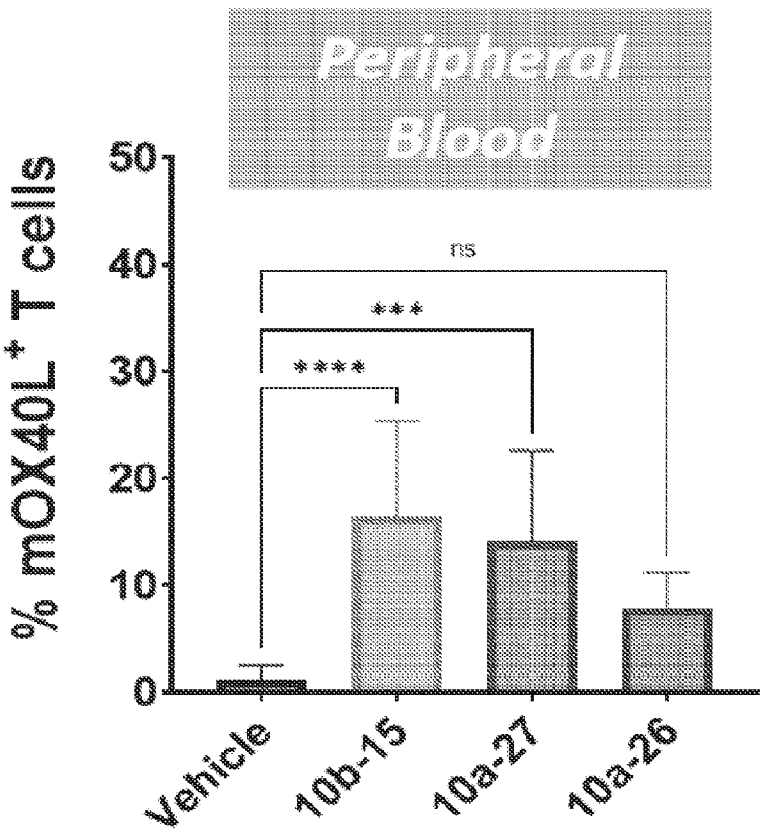
Peripheral Blood
% mOX40L+ T cells
50
40
30
20
10
0
ns
***
****
Vehicle
10b-15
10a-27
10a-26

FIG. 104B

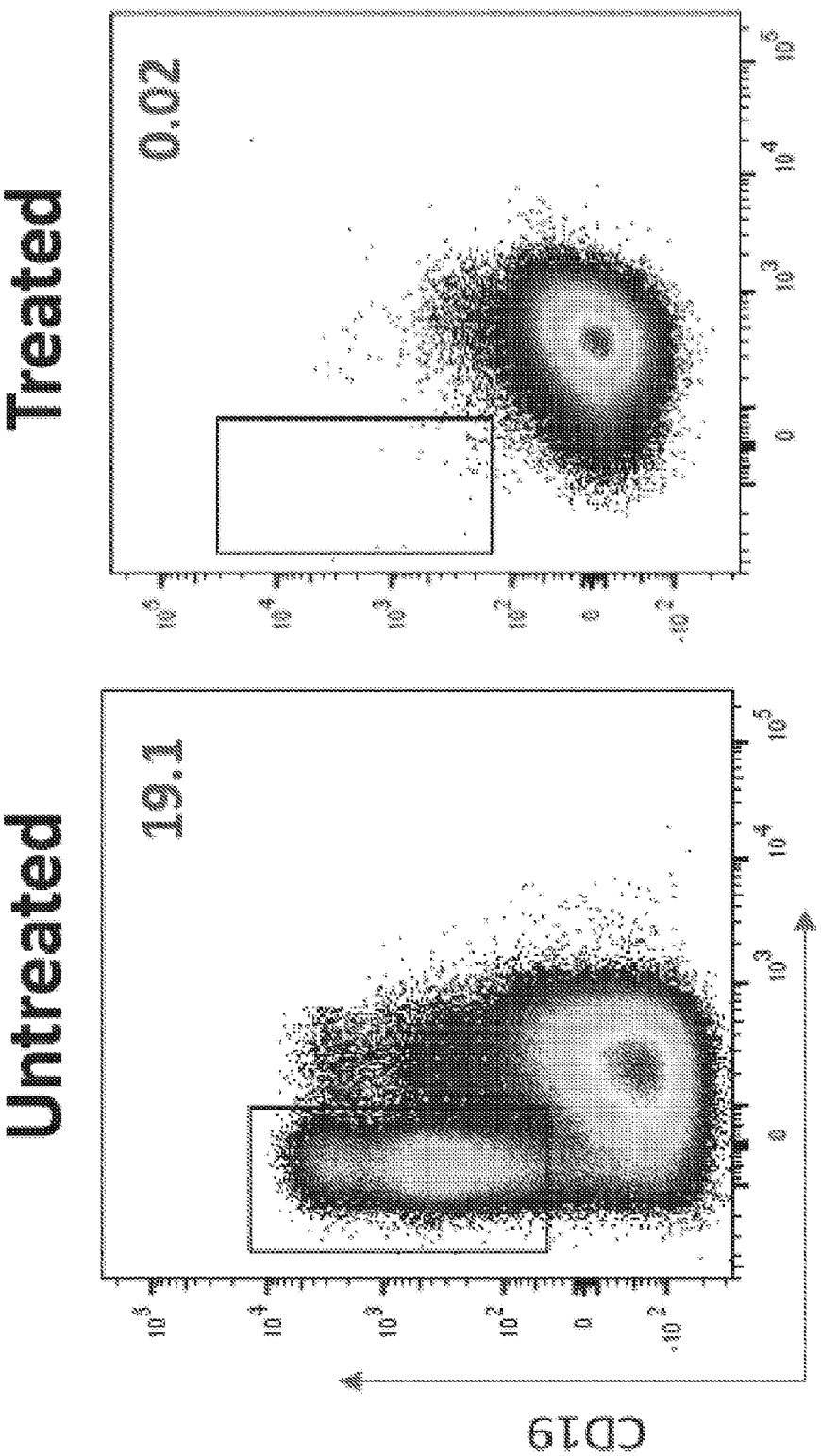

FIG. 105

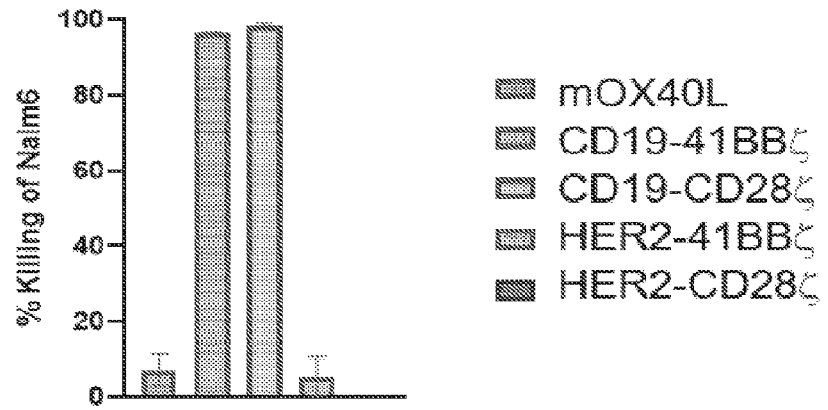

FIG. 106A

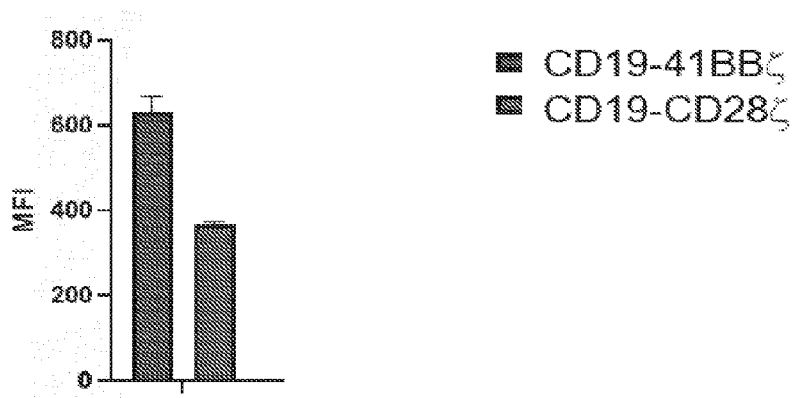

FIG. 106B

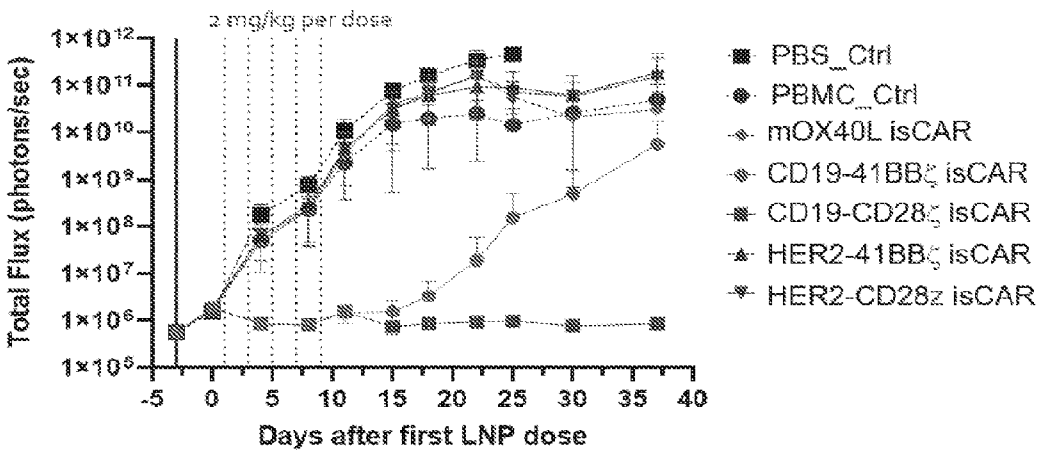
2 mg/kg per dose
Total Flux (photons/sec)
1×10^12
1×10^11
1×10^10
1×10^9
1×10^8
1×10^7
1×10^6
1×10^5
-5
0
5
10
15
20
25
30
35
40
Days after first LNP dose
PBS_Ctrl
PBMC_Ctrl
mOX40L isCAR
CD19-41BBζ isCAR
CD19-CD28ζ isCAR
HER2-41BBζ isCAR
HER2-CD28z isCAR

FIG. 107

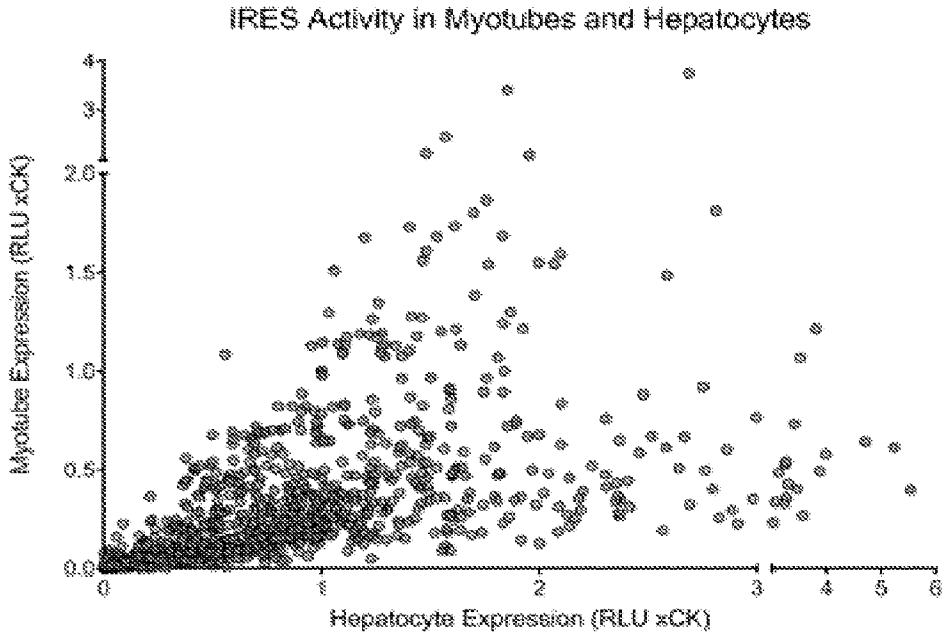

FIG. 108A

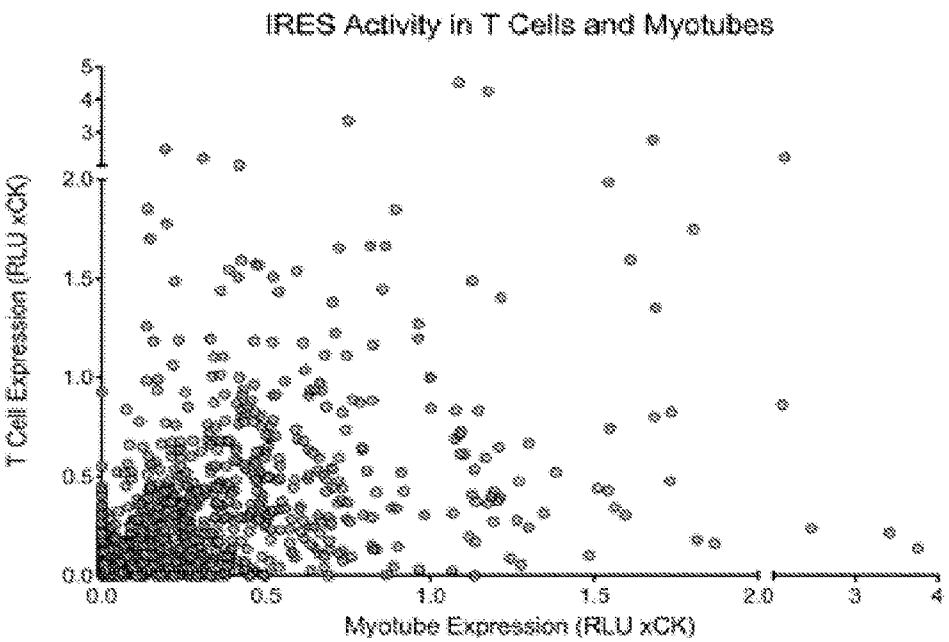

FIG. 108B

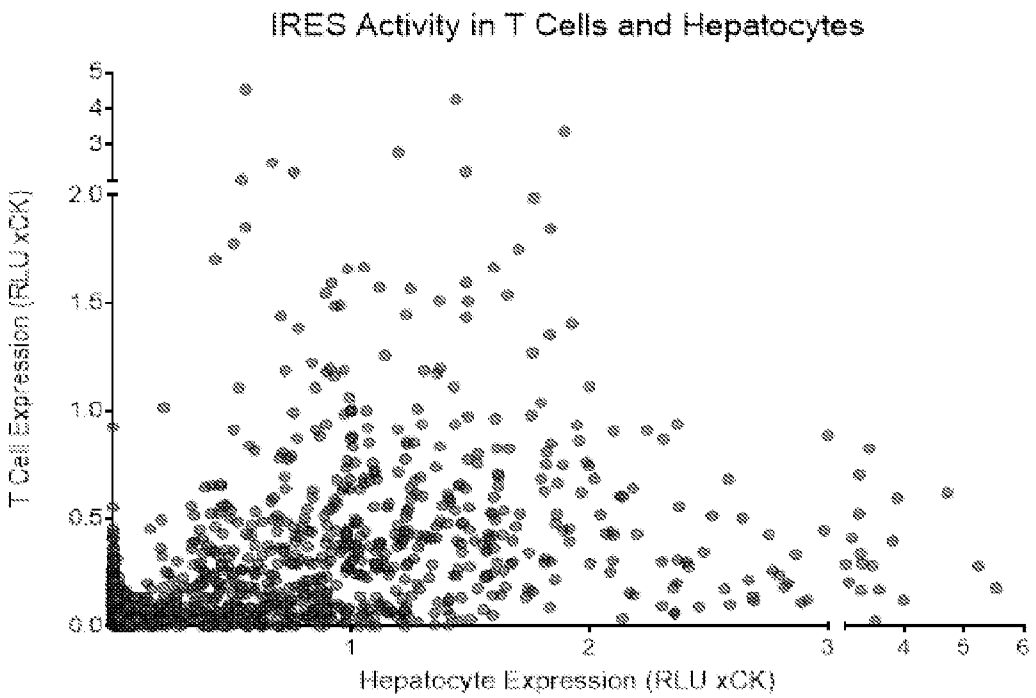
IRES Activity in T Cells and Hepatocytes
T Cell Expression (RLU xCK)
Hepatocyte Expression (RLU xCK)
0.0
0.5
1.0
1.5
2.0
3
4
5
1
2
3
4
5
6

FIG. 108C

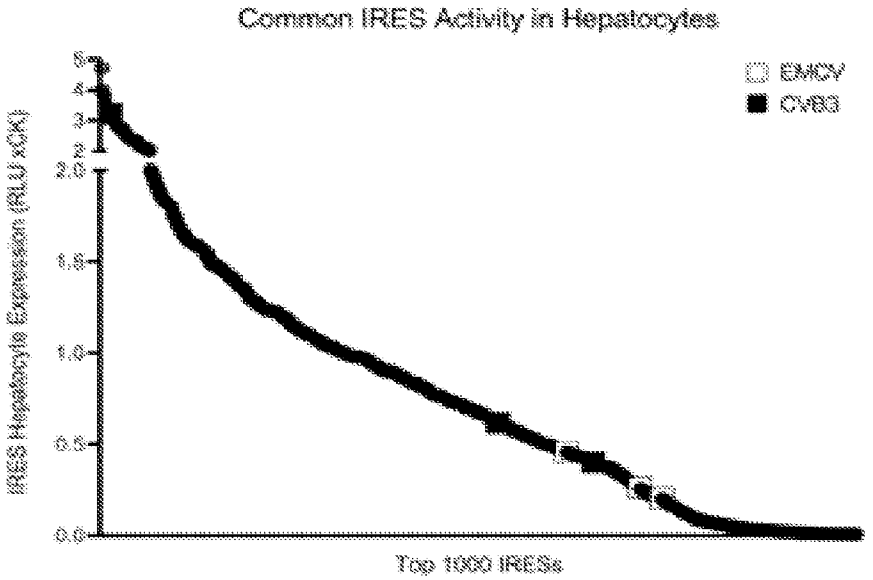

FIG. 109A

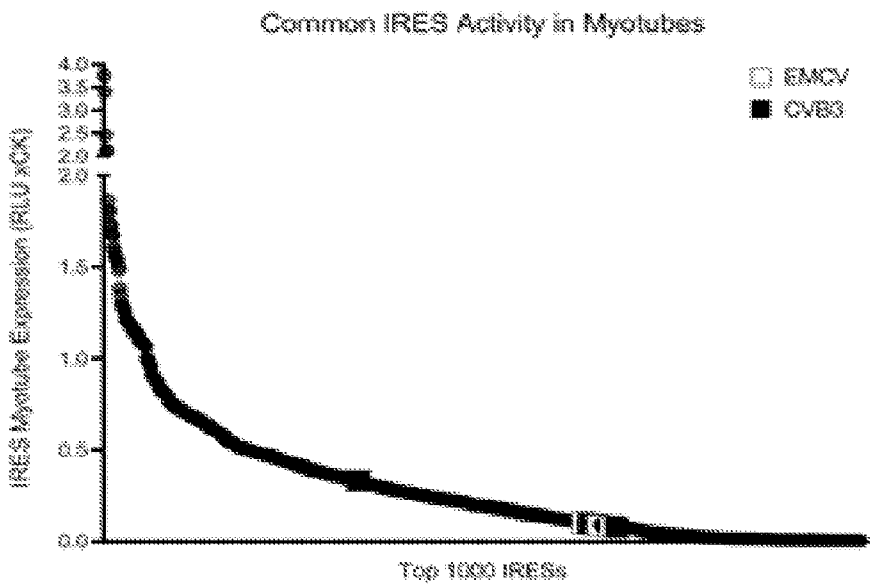

FIG. 109B

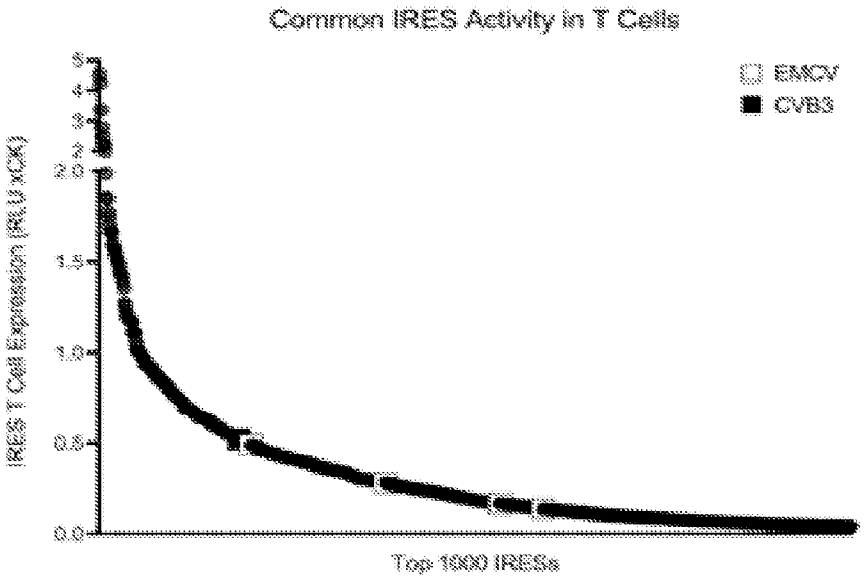
Common IRES Activity in T Cells
EMCV
CVB3
IRES T Cell Expression (RLU xCK)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Top 1000 IRESs

FIG. 109C

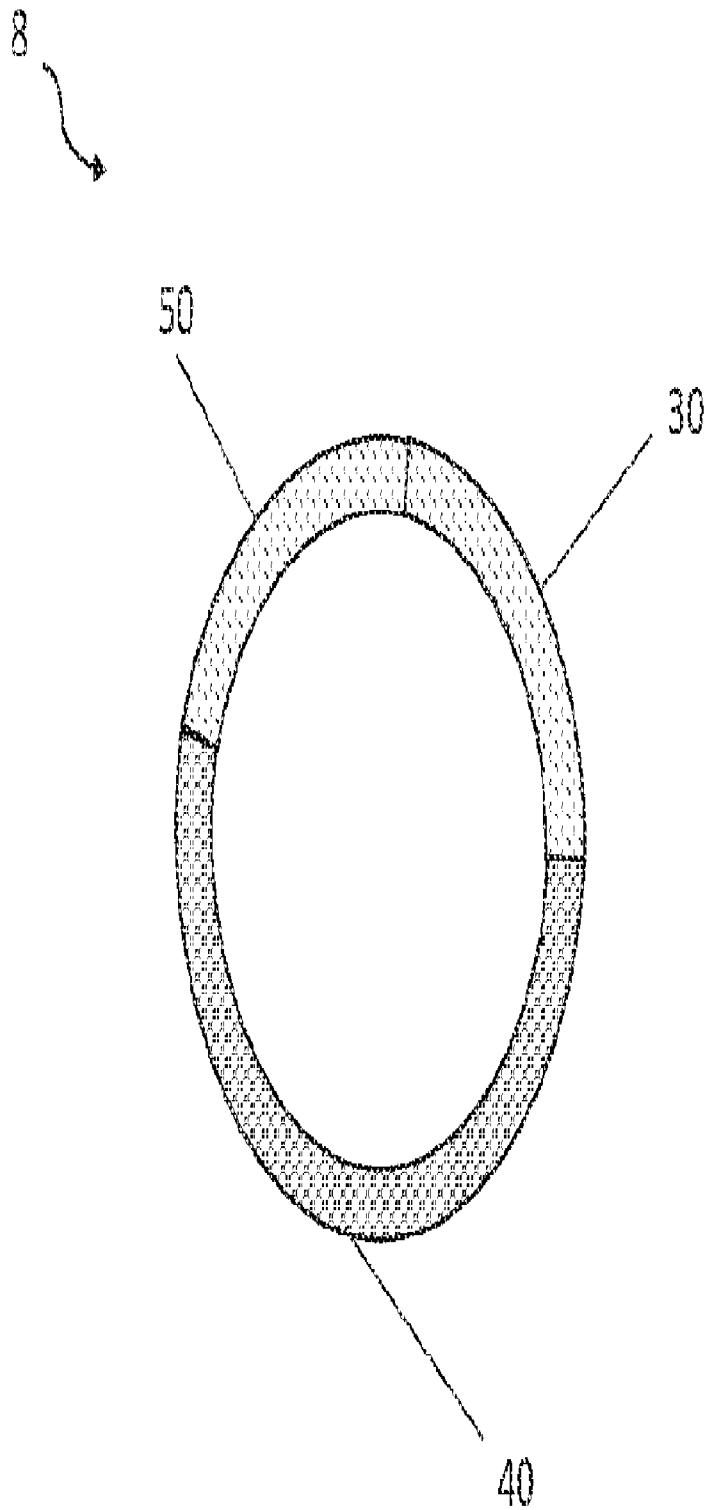

FIG. 88