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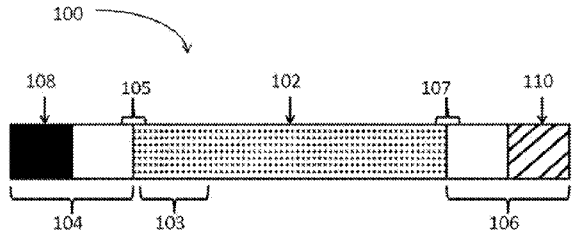
(54) Title: COMPOSITIONS AND METHODS FOR THE TREATMENT OF OPHTHALMIC DISEASES AND CONDITIONS

(57) Abstract: The present invention relates to compositions and methods for the preparation, manufacture and therapeutic use of polynucleotides in the treatment, prevention and/or amelioration of ophthalmic disease or conditions.

A.

FIG. 1



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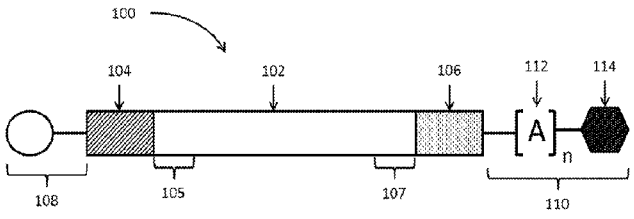

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COMPOSITIONS AND METHODS FOR THE TREATMENT OF OPHTHALMIC DISEASES AND CONDITIONS

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 62/034,883 filed August 8, 2014, entitled Compositions and Methods for the Treatment of Ophthalmic Diseases and Conditions, the contents of each of which are herein incorporated by reference in its entirety.

REFERENCE TO THE SEQUENCE LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled M124PCTSL.txt created on August 7, 2015 which is 151,281 bytes in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] The invention provides polynucleotides (e.g., mRNA) and formulations, methods, processes, kits and devices of using the same in the treatment, prophylaxis and/or therapy in the field of ophthalmology.

BACKGROUND OF THE INVENTION

[0004] Despite advances in the field of ophthalmology, there remains a long felt need for effective therapeutic modalities which offer improved profiles and broader applicability. For example, there are currently no or insufficient treaments for dry age-related macular degeneration, geographic atrophy (GA), or non-infectious uveitis, to name a few. Consequently, back-of-the eye indications in ophthalmology constitute a clear area with a critical and significant unmet medical need.

[0005] Intervention strategies for ophthalmic disorders are also lacking in the art. For example, there are no or insufficient available therapeutics for the prevention of dry AMD (dAMD), for the prevention of the transition from dAMD to wet AMD (wAMD), the prevention of apoptosis in wAMD, the prevention of vessel induction in wAMD or the prevention of vessel maturation in wAMD.

[0006] The present invention provides nucleic acid based compounds or polynucleotides (both coding and non-coding and combinations thereof) and formulations thereof for the

treatment, prevention, diagnosis of ophthalmic disorders and conditions including both back of the eye and front of the eye disorders.

SUMMARY OF THE INVENTION

[0007] Described herein are polynucleotides and formulations, methods, processes, kits and devices of using the same in the treatment, prophylaxis and/or therapy in the field of ophthalmology.

[0008] Described herein are methods of treating an ophthalmic disease, disorder or condition comprising administering to a subject a pharmaceutical composition comprising a polynucleotide encoding at least one ophthalmic polypeptide and wherein said polynucleotide is formulated in a pharmaceutically acceptable carrier or excipient. Provided herein are methods, wherein the polynucleotide is an mRNA. In a non-limiting example, the polynucleotide encodes two polypeptides. In one aspect the polynucleotide encodes more than two open reading frames.

[0009] In one embodiment, provided herein are methods, wherein the polynucleotide comprises at least one chemical modification.

[00010] In one non-limiting example, a method is provided herein, wherein the polynucleotide comprises a purified IVT transcript. In another non-limiting example, the polynucleotide comprises a chimeric transcript.

[00011] In one embodiment a method is provided, wherein the polynucleotide formulation comprises an osmolarity neutral buffer or solution.

[00012] In one embodiment a method is provided, wherein the ophthalmic polypeptide is an intracellular, nuclear or membrane bound polypeptide.

[00013] In one embodiment a method is provided herein, wherein the polynucleotide encodes one or more microRNA (miR) or microRNA binding sites (miRBS).

[00014] In one embodiment a method is provided herein, wherein the ophthalmic polypeptide is involved in a pathway selected from the group consisting of neovascularization, the complement cascade, vascular cell infiltration, NLRP3 mediated cell death, reducing HIF-1 transcription of VEGF and/or PDGF, modulating the antioxidant system, reducing apoptotic pathway signaling, modulating angiogenesis and those associated with retinopathies.

[00015] In one embodiment a method is provided herein, wherein the pathway is modulating neovascularization and the ophthalmic polynucleotide encodes a polypeptide which functions as

a dual inhibitor of VEGF and PDGF induced neovascularization. In one aspect of the invention, the pathway is the complement cascade and the ophthalmic polynucleotide encodes a polypeptide which inhibits the MASP complex. In another aspect, the pathway is vascular cell infiltration and the ophthalmic polynucleotide encodes a polypeptide which blocks early vascular cell infiltration. In another non-limiting example, the pathway is NLRP3 mediated cell death and the ophthalmic polynucleotide encodes a polypeptide which restores DICER1 expression in order to reduce NLRP3 mediated cell death in geographic atrophy. In another non-limiting example, the pathway is transcription and the ophthalmic polynucleotide encodes a polypeptide which functions to reduce HIF1 transcription of VEGF and PDGF.

[00016] In one embodiment of the invention, methods are provided, wherein the pathway is the antioxidant system and the ophthalmic polynucleotide encodes a polypeptide which functions to alleviate, prevent or treat drusen, choroidal neovascularization, and/or retinal pigment epithelium dysfunction. In one non-limiting example, the pathway is apoptosis and the ophthalmic polynucleotide encodes a polypeptide which functions to reduce apoptosis in the retinal pigment epithelium.

[00017] In another non-limiting example, the pathway is angiogenesis and the ophthalmic polynucleotide encodes a polypeptide which functions to alter the Notch signaling pathway or the semaphorin-plexin pathway. In one embodiment, the pathway is associated with a retinopathy and said retinopathy pathway is selected from the group consisting of mitochondrial oxidation, cell adhesion/tissue remodeling, the visual cycle/rod-cone homeostasis, inflammation (the complement system), Drusen formation involving the extracellular matrix, inflammation (immune homing), and neovascularization (involving either growth factors or blood components).

[00018] In one embodiment of the invention, methods are provided, wherein the retinopathy pathway is mitochondrial oxidation and the polynucleotide encodes TOMM40Lm Mitofusin 2, OPA1, SOD2, NADH dehydrogenase (1, 2, 4-6), Cytochromes (b,c) and or ATP synthase. In one aspect, the retinopathy pathway is cell adhesion/tissue remodeling and the polynucleotide encodes cadherin 5, vascular endothelial, Cadherin-related family member 1, Peripherin 2, ADAM metallopeptidase domain 9, Thrombospondin receptor and/or Integrin A5.

[00019] In one embodiment, a method is provided, wherein the retinopathy pathway is visual cycle/rod-cone homeostasis and the polynucleotide encodes retinal pigment epithelium-specific

protein, guanylate cyclase activator 1A, guanylate cyclase 2D, membrane, voltage dependent calcium channels (A2, LA1F), bestrophin 1, and/or ciliary neurotrophic factor. In one embodiment the retinopathy pathway is inflammation and the polynucleotide encodes complement component 3, complement component 5, complement component 5 receptor 1, complement component 2, complement factor D, complement factor H, complement factor B, and/or complement factor I.

[00020] In one embodiment, methods are provided wherein the retinopathy pathway is extracellular matrix homeostasis and the polynucleotide encodes amyloid beta (A4) precursor protein, tenascin XB, collagen type X, alpha 1, myelin basic protein, and/or collagen type VIII, alpha 1. In one embodiment, the retinopathy pathway is inflammation and the polynucleotide encodes chemokine receptor 3, chemokine receptor 4, carbohydrate (GlcNAc) sulfotrans 6 (lymphocyte ligand metabolism), and/or TNF receptor 10A. In one aspect, the retinopathy pathway is neovascularization and the polynucleotide encodes VEGF-A, PDGF, HtrA serine peptidase 1, insulin like GF binding protein 7, and/or placental growth factor. In another aspect, the retinopathy pathway is c neovascularization and the polynucleotide encodes plasminogen, Factor III, sphingosine -1 –phosphate receptor, hepatic lipase and/or cholesteryl ester transfer protein.

[00021] In one embodiment, methods are provided, wherein the disease or disorder is a retinopathy attendant to an orphan indication selected from the group consisting of Stargardt disease, Leber hereditary optic neuropathy, Cone rod dystrophy, Leber congenital amaurosis, Best vitelliform macular dystrophy, Choroideremia, Central areolar choroidal dystrophy, Macular corneal dystrophy, Autosomal dominant optic atrophy plus syndrome, North Carolina macular dystrophy, Hereditary vascular retinopathy and/or Autosomal dominant vitreoretinochoroidopathy. In one aspect, the disease or disorder is a glaucoma attendant to Axenfeld-Rieger syndrome.

[00022] In one embodiment, methods are provided, wherein the disease or disorder is cataracts attendant to an orphan indication, wherein the cataract is selected from the group consisting of anterior polar cataract, Hutterite type Cataract, coralliform cataract, cerulean cataract, pulverulent cataract, congenital cataract, Volkmann type, Coppock-like cataract, cataract with Y-shaped suture opacities, zonular cataract, partial congenital cataract, nuclear

cataract, total congenital cataract, and/or posterior polar cataract. In one non-limiting example, the disease or disorder is a dry eye attendant benign essential blepharospasm.

[00023] In one embodiment, methods are provided herein, wherein the formulation comprises a lipid nanoparticle and wherein said lipid nanoparticle comprises at least one lipid and/or at least one polymer.

[00024] In one embodiment, methods are provided herein, wherein the polynucleotide is encapsulated in the lipid nanoparticle. In some aspects, the lipid is selected from the group consisting of DLin-DMA, DLin-K-DMA, 98N12-5, C12-200, ckk, E12, DLin-MC3-DMA, DLin-KC2-DMA, DODMA, DOPE, DSPC, PLGA, PEG-DMG, PEG-DSG, PEG-DSPE, PEG-DOMG, PEGylated lipids, polyethylenimine (PEI) and chitosan. In one aspect, the lipid is an ionizable amino lipid.

[00025] In one embodiment, methods are provided wherein the ionizable amino lipid is selected from the group consisting of DLin-MC3-DMA and DLin-KC2-DMA.

[00026] In one embodiment, methods are provided herein, wherein contacting said mammalian cells or tissues occurs via a route of administration selected from the group consisting of intraocular, subconjunctival, subcutaneous, intravitreal, or intramuscular.

[00027] In one embodiment, methods are provided, wherein the mRNA comprises at least one 5′ terminal cap selected from the group consisting of Cap0, Cap1, ARCA, inosine, N1-methyl-guanosine, 2′fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine.

[00028] In one embodiment, methods are provided herein, wherein the 5' terminal cap is Cap1.

[00029] In one embodiment, methods are provided wherein the polynucleotide comprises at least two chemical modifications.

[00030] The details of various embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and the drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[00031] The foregoing and other objects, features and advantages will be apparent from the following description of particular embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the

different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of various embodiments of the invention.

[00032] FIG. 1 is a schematic of an IVT polynucleotide construct. A is a schematic of a polynucleotide construct taught in commonly owned co-pending US Patent Application 13/791,922 filed March 9, 2013, the contents of which are incorporated herein by reference. B is a schematic of a linear polynucleotide construct.

[00033] FIG. 2 is a schematic of a series of chimeric polynucleotides of the present invention.

[00034] FIG. 3 is a schematic of a series of chimeric polynucleotides illustrating various patterns of positional modifications and showing regions analogous to those regions of an mRNA polynucleotide.

[00035] FIG. 4 is a schematic of a series of chimeric polynucleotides illustrating various patterns of positional modifications based on Formula I.

[00036] FIG. 5 is a is a schematic of a series of chimeric polynucleotides illustrating various patterns of positional modifications based on Formula I and further illustrating a blocked or structured 3' terminus.

[00037] FIG. 6 comprises A-G which are schematics of a circular polynucleotide construct of the present invention. A and B are circular polynucleotides with and without a non-nucleic acid moiety. C is a circular polynucleotide with at least one spacer region. D is a circular polynucleotide with at least one sensor region. E is a circular polynucleotide with at least one spacer and sensor region. F and G are non-coding circular polynucleotides.

[00038] FIG. 7 is a PRIOR ART schematic of the five classes of known antibodies. The figure was taken from the URL www2.estrellamountain.edu/faculty/farabee/biobk/biobookimmun.html.

[00039] FIG. 8 is a schematic comparing (A) the human IgG antibody structure to (B) a bicistronic antibody of the invention.

[00040] FIG. 9 is a schematic of two types of antibodies of the present invention. (A) an scFv antibody and (B) a single domain IgG antibody.

[00041] FIG. 10 is a schematic of two types of antibodies of the present invention. (A) a bispecific antibody and (B) a single domain IgA antibody.

[00042] FIG. 11 is a schematic of certain polynucleotide constructs of the present invention illustrating (A) the modular design of the encoding polynucleotides and (B) the domains or regions of a standard antibody unit.

DETAILED DESCRIPTION

[00043] It is of great interest in the fields of therapeutics, diagnostics, reagents and for biological assays to be able design, synthesize and deliver a nucleic acid, e.g., a ribonucleic acid (RNA) inside a cell, whether *in vitro*, *in vivo, in situ* or *ex vivo*, such as to effect physiologic outcomes which are beneficial to the cell, tissue or organ and ultimately to an organism. One beneficial outcome is to cause intracellular translation of the nucleic acid and production of at least one encoded peptide or polypeptide of interest. In like manner, non-coding RNA has become a focus of much study; and utilization of non-coding polynucleotides, alone and in conjunction with coding polynucleotides, could provide beneficial outcomes in therapeutic scenarios.

[00044] Described herein are compositions (including pharmaceutical compositions) and methods for the design, preparation, manufacture and/or formulation of polynucleotides, specifically IVT polynucleotides, chimeric polynucleotides and/or circular polynucleotides for use in the field of ophthalmology.

[00045] The compositions of the present invention may be useful, among others, in (1) the inhibition of angiogenesis and vessel maturation (2) inhibition of inflammation-induced immune cell recruitment and associated cellular damage, (3) reduction of hypoxia-induced damage and gene induction, (4) inhibition of cell death and/or (5) management of retinal homeostasis.

[00046] Disorders which may be treated or prevented using the compositions of the invention include, but are not limited to, either front of the eye or back of the eye conditions such as diabetic retinopathy, dry eye, cataracts, retinal vein occlusion, macular edema, macular degeneration (wet & dry), refraction and accommodation disorders, keratoconus, amblyopia, glaucoma, Sstargardt disease, endophthalmitis, conjunctivitis, uveitis, retinal detachment, corneal ulcers, dacryocystitis, Duane retraction syndrome, and optic neuritis, eyelid disease, infections e.g. conjunctivitis, allergic problems, eye lash problems, watery eyes, corneal problems, or foreign bodies.

[00047] Also provided are systems, processes, devices and kits for the selection, design and/or utilization of the polynucleotides described herein.

[00048] Provided herein are polynucleotides useful in the ophthalmology field which have been designed to improve one or more of the stability and/or clearance in tissues, receptor uptake and/or kinetics, cellular access, engagement with translational machinery, mRNA half-life,

translation efficiency, immune evasion, immune induction (where necessary), protein production capacity, secretion efficiency (when applicable), accessibility to circulation, protein half-life and/or modulation of a cell's status, function and/or activity.

I. Compositions of the Invention

Ophthalmic Polynucleotides

[00049] The present invention provides nucleic acid molecules, specifically polynucleotides which, in some embodiments, encode one or more peptides or polypeptides of interest. The term "nucleic acid," in its broadest sense, includes any compound and/or substance that comprise a polymer of nucleotides. These polymers are often referred to as polynucleotides.

[00050] Exemplary nucleic acids or polynucleotides of the invention include, but are not limited to, ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs, including LNA having a β- D-ribo configuration, α-LNA having an α-L-ribo configuration (a diastereomer of LNA), 2′-amino-LNA having a 2′-amino functionalization, and 2′-amino- α-LNA having a 2′-amino functionalization), ethylene nucleic acids (ENA), cyclohexenyl nucleic acids (CeNA) or hybrids or combinations thereof.

[00051] In one embodiment, linear polynucleotides of the present invention which are made using only *in vitro* transcription (IVT) enzymatic synthesis methods are referred to as "IVT polynucleotides."

[00052] In one embodiment, polynucleotides of the present invention are fully synthetic such as the chimeric polynucleotides taught herein.

[00053] In another embodiment, the polynucleotides of the present invention which have portions or regions which differ in size and/or chemical modification pattern, chemical modification position, chemical modification percent or chemical modification population and combinations of the foregoing are known as "chimeric polynucleotides." A "chimera" according to the present invention is an entity having two or more incongruous or heterogeneous parts or regions. As used herein a "part" or "region" of a polynucleotide is defined as any portion of the polynucleotide which is less than the entire length of the polynucleotide.

[00054] In one embodiment, the chimeric polynucleotides may take the form or function as modified mRNA molecules which encode at least one polypeptide of interest.

[00055] In one embodiment, such chimeric polynucleotides are substantially non-coding.

[00056] In one aspect the chimeric polynucleotides has a sequence or structure comprising Formula I,

$$5' [A_n]_x\text{-}L1\text{-}[B_o]_y\text{-}L2\text{-}[C_p]_z\text{-}L3\ 3'$$

Formula I

[00057] wherein:

[00058] each of A and B independently comprise a region of linked nucleosides;

[00059] C is an optional region of linked nucleosides;

[00060] at least one of regions A, B, or C is positionally modified, wherein said positionally modified region comprises at least two chemically modified nucleosides of one or more of the same nucleoside type of adenosine, thymidine, guanosine, cytidine, or uridine, and wherein at least two of the chemical modifications of nucleosides of the same type are different chemical modifications;

[00061] n, o and p are independently an integer between 15-1000;

[00062] x and y are independently 1-20;

[00063] z is 0-5;

[00064] L1 and L2 are independently optional linker moieties, said linker moieties being either nucleic acid based or non-nucleic acid based; and

[00065] L3 is an optional conjugate or an optional linker moiety, said linker moiety being either nucleic acid based or non-nucleic acid based.

[00066] In another aspect, the invention features a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide has a sequence including Formula II:

$$[A_n]\text{-}L^1\text{-}[B_o]$$

Formula II

[00067] wherein each A and B is independently any nucleoside;

[00068] n and o are, independently 15 to 1000; and

[00069] L^1 is a bond or has the structure of Formula III:

$$\S\text{—}(R^1)_a\text{—}(R^2)_b\text{—}(R^3)_c\text{—}R^4\text{-}(R^5)_d\text{-}(R^6)_e\text{-}(R^7)_f\text{—}\S$$

Formula III

[00070] wherein a, b, c, d, e, and f are each, independently, 0 or 1;

[00071] each of R^1, R^3, R^5, and R^7, is, independently, selected from optionally substituted C_1-C_6 alkylene, optionally substituted C_1-C_6 heteroalkylene, O, S, and NR^8;

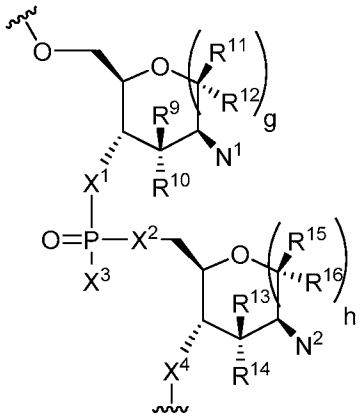
[00072] R^2 and R^6 are each, independently, selected from carbonyl, thiocarbonyl, sulfonyl, or phosphoryl;

[00073] R^4 is optionally substituted C_1–C_{10} alkylene, optionally substituted C_2–C_{10} alkenylene, optionally substituted C_2–C_{10} alkynylene, optionally substituted C_2–C_9 heterocyclylene, optionally substituted C_6–C_{12} arylene, optionally substituted C_2-C_{100} polyethylene glycolene, or optionally substituted C_1–C_{10} heteroalkylene, or a bond linking $(R^1)_a$-$(R^2)_b$-$(R^3)_c$ to $(R^5)_d$-$(R^6)_e$-$(R^7)_f$; and

[00074] R^8 is hydrogen, optionally substituted C_1–C_4 alkyl, optionally substituted C_2–C_4 alkenyl, optionally substituted C_2–C_4 alkynyl, optionally substituted C_2–C_6 heterocyclyl, optionally substituted C_6–C_{12} aryl, or optionally substituted C_1–C_7 heteroalkyl;

[00075] wherein L^1 is attached to $[A_n]$ and $[B_o]$ at the sugar of one of the nucleosides (e.g., at the 3' position of a five-membered sugar ring or 4' position of a six membered sugar ring of a nucleoside of $[A_n]$ and the 5' position of a five-membered sugar ring or 6' position of a six membered sugar ring of a nucleoside of $[B_o]$ or at the 5' position of a five-membered sugar ring or 6' position of a six membered sugar ring of a nucleoside of $[A_n]$ and the 3' position of a five-membered sugar ring or 4' position of a six membered sugar ring of a nucleoside of $[B_o]$).

[00076] wherein at least one of $[A_n]$ or $[B_o]$ includes the structure of Formula IV:



Formula IV

[00077] wherein each of N^1 and N^2 is independently a nucleobase;

[00078] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted C_1-C_6 alkyl, optionally substituted C_1-C_6 heteroalkyl, optionally substituted C_2-C_6 heteroalkenyl, optionally substituted C_2-C_6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-C_{10} aryl;

[00079] each of g and h is, independently, 0 or 1;

[00080] each X^1 and X^4 is, independently, O, NH, or S; and

[00081] each X^2 is independently O or S; and

[00082] each X^3 is OH or SH, or a salt thereof;

[00083] wherein at least one of X^1, X^2, or X^4 is NH or S.

[00084] In some embodiments, X^1 is NH. In other embodiments, X^4 is NH. In certain embodiments, X^2 is S.

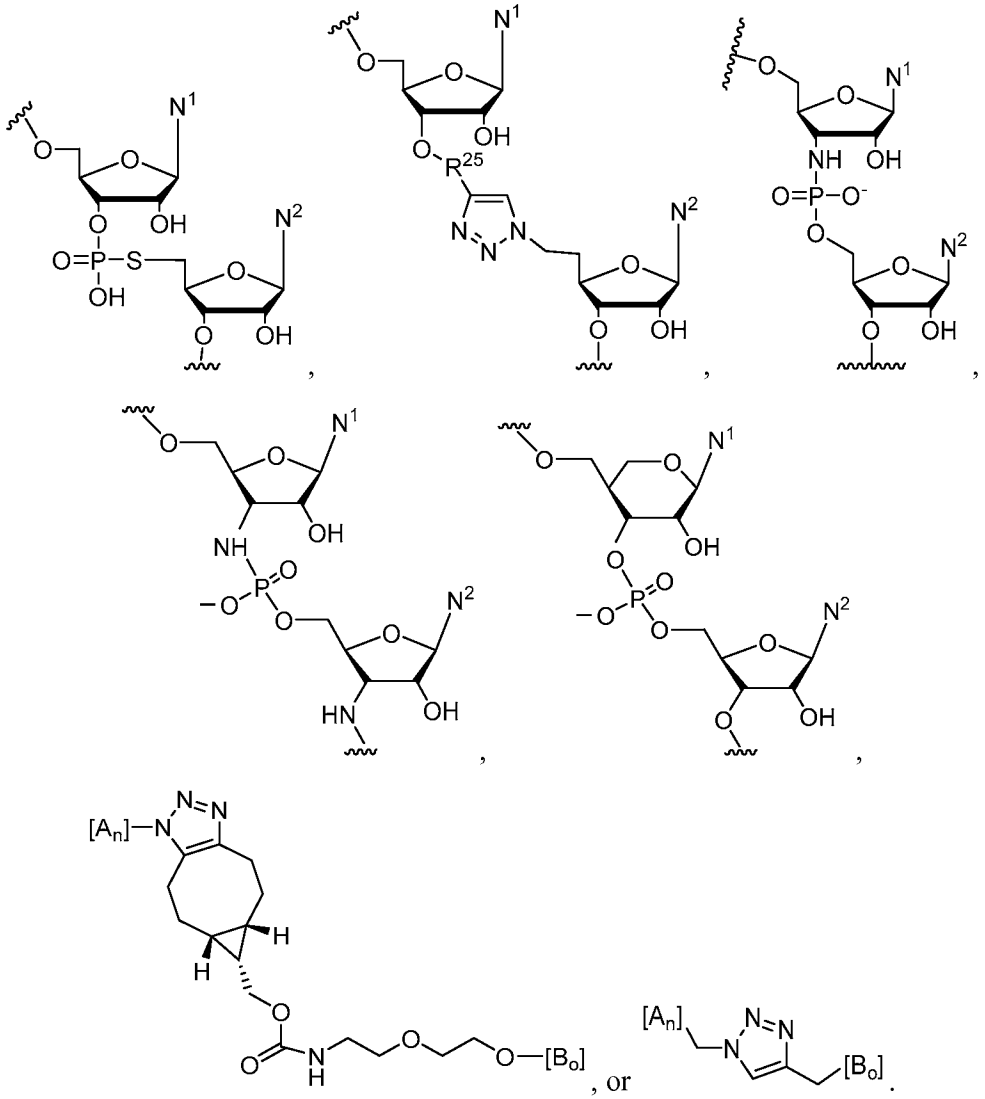
[00085] In some embodiments, the polynucleotide includes: (a) a coding region; (b) a 5' UTR including at least one Kozak sequence; (c) a 3' UTR; and (d) at least one 5' cap structure. In other embodiments, the polynucleotide further includes (e) a poly-A tail.

[00086] In some embodiments, one of the coding region, the 5' UTR including at least one Kozak sequence, the 3' UTR, the 5' cap structure, or the poly-A tail includes $[A_n]$-L^1-$[B_o]$.

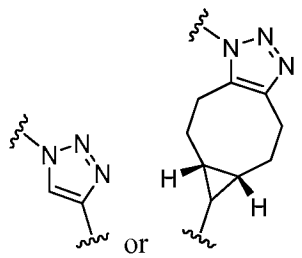
[00087] In other embodiments, one of the coding region, the 5' UTR including at least one Kozak sequence, the 3' UTR, the 5' cap structure, or the poly-A tail includes $[A_n]$ and another of the coding region, the 5' UTR including at least one Kozak sequence, the 3' UTR, the 5' cap structure, or the poly-A tail includes $[B_o]$.

[00088] In certain embodiments, the polynucleotide includes at least one modified nucleoside.

[00089] For example, in some embodiments, the chimeric polynucleotides of the invention include the structure:



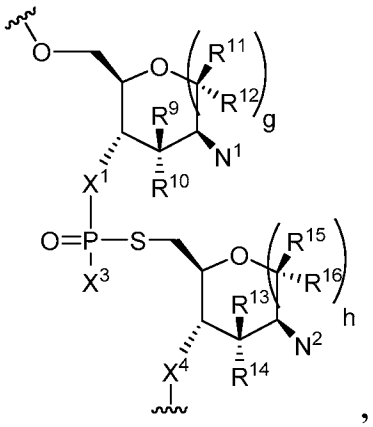
[00090] In some embodiments, R^4 is optionally substituted C_{2-9} heterocyclylene, for example, the heterocycle may have the structure:



[00091] In certain embodiments, L^1 is attached to $[A_n]$ at the 3' position of a five-membered sugar ring or 4' position of a six membered sugar ring of one of the nucleosides and to $[B_o]$ at the 5' position of a five-membered sugar ring or 6' position of a six membered sugar ring of one of the nucleosides.

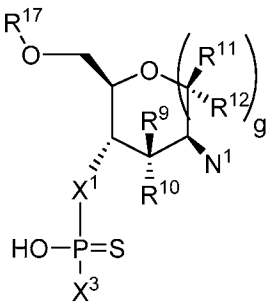
[00092] In some embodiments, the polynucleotide is circular.

[00093] In another aspect, the invention features a method of producing a composition including a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide includes the structure of Formula V:



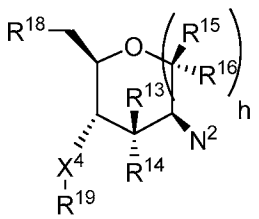
Formula V

[00094] This method includes reacting a compound having the structure of Formula VI:



Formula VI

with a compound having the structure of Formula VII:



Formula VII

[00095] wherein each of N^1 and N^2 is, independently, a nucleobase;

[00096] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted C_1-C_6 alkyl, optionally substituted C_1-C_6 heteroalkyl, optionally substituted C_2-C_6 heteroalkenyl, optionally substituted C_2-C_6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-C_{10} aryl;

[00097] each of g and h is, independently, 0 or 1;

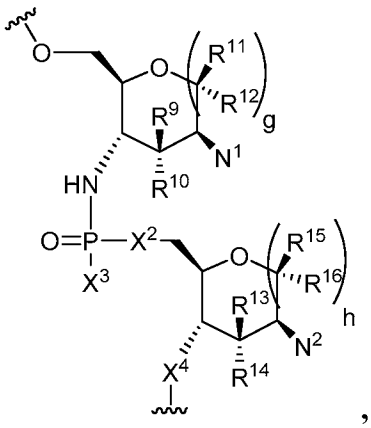
[00098] each X^1 and X^4 is, independently, O, NH, or S; and

[00099] each X^3 is independently OH or SH, or a salt thereof;

[000100] each of R^{17} and R^{19} is, independently, a region of linked nucleosides; and

[000101] R^{18} is a halogen.

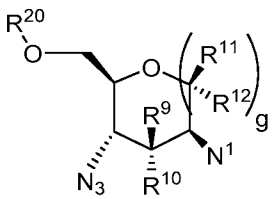
[000102] In another aspect, the invention features a method of producing a composition including a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide includes the structure of Formula VIII:



,

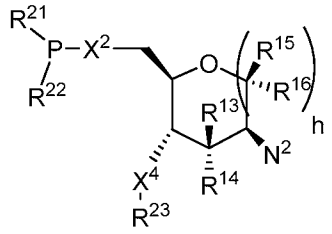
Formula VIII

[000103] This method includes reacting a compound having the structure of Formula IX:



Formula IX

with a compound having the structure of Formula X:



Formula X

[000104] wherein each of N^1 and N^2 is, independently, a nucleobase;

[000105] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted C_1-C_6 alkyl, optionally substituted C_1-C_6 heteroalkyl, optionally substituted C_2-C_6 heteroalkenyl, optionally substituted C_2-C_6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-C_{10} aryl;

[000106] each of g and h is, independently, 0 or 1;

[000107] each X^4 is, independently, O, NH, or S; and

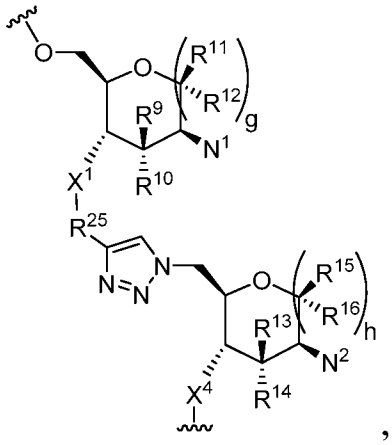
[000108] each X^2 is independently O or S;

[000109] each X^3 is independently OH, SH, or a salt thereof;

[000110] each of R^{20} and R^{23} is, independently, a region of linked nucleosides; and

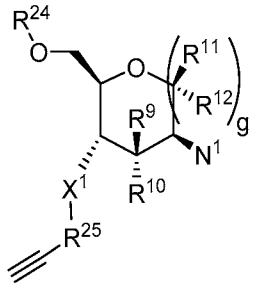
[000111] each of R^{21} and R^{22} is, independently, optionally substituted C_1-C_6 alkoxy.

[000112] In another aspect, the invention features a method of producing a composition including a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide includes the structure of Formula XI:



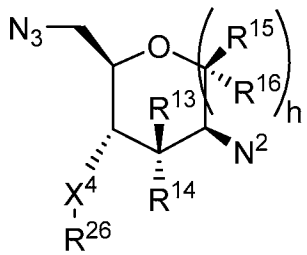
Formula XI

[000113] This method includes reacting a compound having the structure of Formula XII:



Formula XII

with a compound having the structure of Formula XIII:



Formula XIII

[000114] wherein each of N^1 and N^2 is, independently, a nucleobase;

[000115] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted C_1-C_6 alkyl, optionally substituted C_1-C_6 heteroalkyl, optionally substituted C_2-C_6 heteroalkenyl, optionally substituted C_2-C_6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-C_{10} aryl;

[000116] each of g and h is, independently, 0 or 1;

[000117] each X^4 is, independently, O, NH, or S; and

[000118] each X^2 is independently O or S;

[000119] each X^3 is independently OH, SH, or a salt thereof;

[000120] each of R^{24} and R^{26} is, independently, a region of linked nucleosides; and

[000121] R^{25} is optionally substituted C_1-C_6 alkylene or optionally substituted C_1-C_6 heteroalkylene or R^{25} and the alkynyl group together form optionally substituted cycloalkynyl.

[000122] In another aspect, the invention features a method of producing a composition including a chimeric polynucleotide encoding a polypeptide, wherein the polynucleotide has a sequence including Formula II:

$$[A_n]\text{-}L^1\text{-}[B_o],$$

Formula II

[000123] This method includes reacting a compound having the structure of Formula XIV

$$[A_n]\text{-}(R^1)_a\text{-}(R^2)_b\text{-}(R^3)_c\text{-}N_3$$

Formula XIV

with a compound having the structure of Formula XV:

$$R^{27}\text{-}(R^5)_d\text{-}(R^6)_e\text{-}(R^7)_f\text{-}[B_o]$$

Formula XV

[000124] wherein each A and B is independently any nucleoside;

[000125] n and o are, independently 15 to 1000; and

[000126] L^1 has the structure of Formula III:

$$\S\text{—}(R^1)_a\text{—}(R^2)_b\text{—}(R^3)_c\text{—}R^4\text{-}(R^5)_d\text{-}(R^6)_e\text{-}(R^7)_f\text{—}\S$$

Formula III

[000127] wherein a, b, c, d, e, and f are each, independently, 0 or 1;

[000128] wherein each A and B is independently any nucleoside;

[000129] n and o are, independently 15 to 1000;

[000130] R^1, R^3, R^5, and R^7 each, independently, is selected from optionally substituted C_1-C_6 alkylene, optionally substituted C_1-C_6 heteroalkylene, O, S, and NR^8;

[000131] R^2 and R^6 are each, independently, selected from carbonyl, thiocarbonyl, sulfonyl, or phosphoryl;

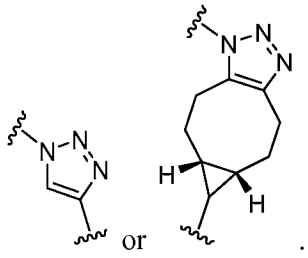
[000132] R^4 is an optionally substituted triazolene; and

[000133] R^8 is hydrogen, optionally substituted C_1–C_4 alkyl, optionally substituted C_3–C_4 alkenyl, optionally substituted C_2–C_4 alkynyl, optionally substituted C_2–C_6 heterocyclyl, optionally substituted C_6–C_{12} aryl, or optionally substituted C_1–C_7 heteroalkyl; and

[000134] R^{27} is an optionally substituted C_2-C_3 alkynyl or an optionally substituted C_8-C_{12} cycloalkynyl,

[000135] wherein L^1 is attached to $[A_n]$ and $[B_o]$ at the sugar of one of the nucleosides.

[000136] In some embodiments, the optionally substituted triazolene has the structure:



[000137] In one aspect, the chimeric polynucleotide may comprise at least one modification such as a modified nucleoside. As a non-limiting example, the at least one modification may be 1-methylpseudouridine.

[000138] In one aspect, at least one region A, B, or C may be codon optimized.

[000139] In another aspect, the chimeric polynucleotide may comprise at least two modifications. As a non-limiting example, the at least two modifications may be 1-methylpseudouridine and 5-methycytidine.

[000140] Provided herein are pharmaceutical compositions comprising at least one polynucleotide such as, but not limited to, an IVT polynucleotide or a chimeric polynucleotide.

[000141] The IVT polynucleotide may comprise a polyA tail which may be at least 100 nucleotides in length (SEQ ID NO: 95). The IVT polynucleotide may also comprise a 5'cap structure such as, but not limited to, Cap0, Cap1, ARCA, inosine, N1-methyl-guanosine, 2'fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine. The IVT polynucleotide may also be purified.

[000142] In one embodiment, the IVT polynucleotide may take the form or function as modified mRNA molecules which encode at least one polypeptide of interest.

[000143] In one aspect, the IVT polynucleotide may comprise at least one modification such as a modified nucleoside. The at least one modification may be located on one or more nucleosides such as, but not limited to the sugar and/or the nucleobase. As a non-limiting example, the at least one modification may be 1-methylpseudouridine.

[000144] In another aspect, the IVT polynucleotide may comprise at least two modifications. The at least two modifications may be located on one or more of a nucleoside and/or a backbone linkage between nucleosides, both a nucleoside and a backbone linkage. The backbone linkage may be modified by the replacement of one or more oxygen atoms or with a phosphorothioate

linkage. As a non-limiting example, the at least two modifications may be 1-methylpseudouridine and 5-methycytidine.

[000145] In one aspect, at least the coding region of the IVT polynucleotide is codon optimized.

[000146] In yet another embodiment, the polynucleotides of the present invention that are circular are known as "circular polynucleotides" or "circP." As used herein, "circular polynucleotides" or "circP" means a single stranded circular polynucleotide which acts substantially like, and has the properties of, an RNA. The term "circular" is also meant to encompass any secondary or tertiary configuration of the circP.

[000147] In some embodiments, the polynucleotide includes from about 30 to about 100,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 1,000, from 30 to 1,500, from 30 to 3,000, from 30 to 5,000, from 30 to 7,000, from 30 to 10,000, from 30 to 25,000, from 30 to 50,000, from 30 to 70,000, from 100 to 250, from 100 to 500, from 100 to 1,000, from 100 to 1,500, from 100 to 3,000, from 100 to 5,000, from 100 to 7,000, from 100 to 10,000, from 100 to 25,000, from 100 to 50,000, from 100 to 70,000, from 100 to 100,000, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 3,000, from 500 to 5,000, from 500 to 7,000, from 500 to 10,000, from 500 to 25,000, from 500 to 50,000, from 500 to 70,000, from 500 to 100,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 3,000, from 1,000 to 5,000, from 1,000 to 7,000, from 1,000 to 10,000, from 1,000 to 25,000, from 1,000 to 50,000, from 1,000 to 70,000, from 1,000 to 100,000, from 1,500 to 3,000, from 1,500 to 5,000, from 1,500 to 7,000, from 1,500 to 10,000, from 1,500 to 25,000, from 1,500 to 50,000, from 1,500 to 70,000, from 1,500 to 100,000, from 2,000 to 3,000, from 2,000 to 5,000, from 2,000 to 7,000, from 2,000 to 10,000, from 2,000 to 25,000, from 2,000 to 50,000, from 2,000 to 70,000, and from 2,000 to 100,000).

[000148] In one embodiment, the polynucleotides of the present invention may encode at least one peptide or polypeptide of interest. In another embodiment, the polynucleotides of the present invention may be non-coding.

[000149] In one embodiment, the length of a region encoding at least one peptide polypeptide of interest of the polynucleotides present invention is greater than about 30 nucleotides in length (e.g., at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500,

1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000 or up to and including 100,000 nucleotides). As used herein, such a region may be referred to as a "coding region" or "region encoding."

[000150] In one embodiment, the polynucleotides of the present invention is or functions as a messenger RNA (mRNA). As used herein, the term "messenger RNA" (mRNA) refers to any polynucleotide which encodes at least one peptide or polypeptide of interest and which is capable of being translated to produce the encoded peptide polypeptide of interest *in vitro, in vivo, in situ* or *ex vivo*.

[000151] In one embodiment, the polynucleotides of the present invention may be structurally modified or chemically modified. As used herein, a "structural" modification is one in which two or more linked nucleosides are inserted, deleted, duplicated, inverted or randomized in a polynucleotide without significant chemical modification to the nucleotides themselves. Because chemical bonds will necessarily be broken and reformed to effect a structural modification, structural modifications are of a chemical nature and hence are chemical modifications. However, structural modifications will result in a different sequence of nucleotides. For example, the polynucleotide "ATCG" may be chemically modified to "AT-5meC-G". The same polynucleotide may be structurally modified from "ATCG" to "ATCCCG". Here, the dinucleotide "CC" has been inserted, resulting in a structural modification to the polynucleotide.

[000152] In one embodiment, the polynucleotides of the present invention, such as IVT polynucleotides or circular polynucleotides, may have a uniform chemical modification of all or any of the same nucleoside type or a population of modifications produced by mere downward titration of the same starting modification in all or any of the same nucleoside type, or a measured percent of a chemical modification of all any of the same nucleoside type but with random incorporation, such as where all uridines are replaced by a uridine analog, e.g., pseudouridine. In another embodiment, the polynucleotides may have a uniform chemical modification of two, three, or four of the same nucleoside type throughout the entire polynucleotide (such as all uridines and all cytosines, etc. are modified in the same way).

[000153] When the polynucleotides of the present invention are chemically and/or structurally modified the polynucleotides may be referred to as "modified polynucleotides."

[000154] In one embodiment, the polynucleotides of the present invention may include a sequence encoding a self-cleaving peptide. The self-cleaving peptide may be, but is not limited to, a 2A peptide. As a non-limiting example, the 2A peptide may have the protein sequence: GSGATNFSLLKQAGDVEENPGP (SEQ ID NO: 1), fragments or variants thereof. In one embodiment, the 2A peptide cleaves between the last glycine and last proline. As another non-limiting example, the polynucleotides of the present invention may include a polynucleotide sequence encoding the 2A peptide having the protein sequence GSGATNFSLLKQAGDVEENPGP (SEQ ID NO: 1) fragments or variants thereof.

[000155] One such polynucleotide sequence encoding the 2A peptide is GGAAGCGGAGCTACTAACTTCAGCCTGCTGAAGCAGGCTGGAGACGTGGAGGAGAACCCTGGACCT (SEQ ID NO: 2). The polynucleotide sequence of the 2A peptide may be modified or codon optimized by the methods described herein and/or are known in the art.

[000156] In one embodiment, this sequence may be used to separate the coding region of two or more polypeptides of interest. As a non-limiting example, the sequence encoding the 2A peptide may be between a first coding region A and a second coding region B (A-2Apep-B). The presence of the 2A peptide would result in the cleavage of one long protein into protein A, protein B and the 2A peptide. Protein A and protein B may be the same or different peptides or polypeptides of interest. In another embodiment, the 2A peptide may be used in the polynucleotides of the present invention to produce two, three, four, five, six, seven, eight, nine, ten or more proteins.

IVT Polynucleotide Architecture

[000157] Traditionally, the basic components of an mRNA molecule include at least a coding region, a 5′UTR, a 3′UTR, a 5′ cap and a poly-A tail. The IVT polynucleotides of the present invention may function as mRNA but are distinguished from wild-type mRNA in their functional and/or structural design features which serve to overcome existing problems of effective polypeptide production using nucleic-acid based therapeutics.

[000158] Figure 1 shows a primary construct **100** of an IVT polynucleotide of the present invention. As used herein, "primary construct" refers to a polynucleotide of the present invention which encodes one or more polypeptides of interest and which retains sufficient structural and/or chemical features to allow the polypeptide of interest encoded therein to be translated.

[000159] According to FIG. **1A** and **1B**, the primary construct **100** of an IVT polynucleotide here contains a first region of linked nucleotides **102** that is flanked by a first flanking region **104** and a second flaking region **106**. The first flanking region **104** may include a sequence of linked nucleosides which function as a 5' untranslated region (UTR) such as the 5' UTR of any of the nucleic acids encoding the native 5'UTR of the polypeptide or a non-native 5'UTR such as, but not limited to, a heterologous 5'UTR or a synthetic 5'UTR. The polypeptide of interest may comprise at its 5' terminus one or more signal sequences encoded by the signal sequence region **103** of the polynucleotide. The flanking region **104** may comprise a region of linked nucleotides comprising one or more complete or incomplete 5′ UTRs sequences which may be completely codon optimized or partially codon optimized. The flanking region **104** may include at least one nucleic acid sequence including, but not limited to, miR sequences, TERZAK™ sequences and translation control sequences. The flanking region **104** may also comprise a 5′ terminal cap **108**. The 5′ terminal capping region **108** may include a naturally occurring cap, a synthetic cap or an optimized cap. Non-limiting examples of optimized caps include the caps taught by Rhoads in US Patent No. US7074596 and International Patent Publication No. WO2008157668, WO2009149253 and WO2013103659, the contents of each of which are herein incorporated by reference in its entirety. The second flanking region **106** may comprise a region of linked nucleotides comprising one or more complete or incomplete 3′ UTRs which may encode the native 3' UTR of the polypeptide or a non-native 3'UTR such as, but not limited to, a heterologous 3'UTR or a synthetic 3' UTR. The flanking region **106** may also comprise a 3′ tailing sequence **110**. The second flanking region **106** may be completely codon optimized or partially codon optimized. The flanking region **106** may include at least one nucleic acid sequence including, but not limited to, miR sequences and translation control sequences. The 3' tailing sequence **110** may be, but is not limited to, a polyA tail, a polyC tail, a polyA-G quartet and/or a stem loop sequence.

[000160] Bridging the 5′ terminus of the first region **102** and the first flanking region **104** is a first operational region **105**. Traditionally this operational region comprises a Start codon. The operational region may alternatively comprise any translation initiation sequence or signal including a Start codon.

[000161] Bridging the 3′ terminus of the first region **102** and the second flanking region **106** is a second operational region **107**. Traditionally this operational region comprises a Stop codon.

The operational region may alternatively comprise any translation initiation sequence or signal including a Stop codon. Multiple serial stop codons may also be used in the IVT polynucleotide. In one embodiment, the operation region of the present invention may comprise two stop codons. The first stop codon may be "TGA" or "UGA" and the second stop codon may be selected from the group consisting of "TAA," "TGA," "TAG," "UAA," "UGA" or "UAG."

[000162] Figure 1 shows a representative IVT polynucleotide primary construct **100** of the present invention. IVT polynucleotide primary construct refers to a polynucleotide transcript which encodes one or more polypeptides of interest and which retains sufficient structural and/or chemical features to allow the polypeptide of interest encoded therein to be translated.

[000163] Non-limiting examples of polypeptides of interest and polynucleotides encoding polypeptide of interest are described in Table 6 of International Publication Nos. WO2013151666, WO2013151668, WO2013151663, WO2013151669, WO2013151670, WO2013151664, WO2013151665, WO2013151736; Tables 6 and 7 International Publication No. WO2013151672; Tables 6, 178 and 179 of International Publication No. WO2013151671; Tables 6, 185 and 186 of International Publication No WO2013151667, the contents of each of which are incorporated herein by reference in their entirety.

[000164] Returning to FIG. **1**, the IVT polynucleotide primary construct **130** here contains a first region of linked nucleotides **132** that is flanked by a first flanking region **134** and a second flaking region **136**. As used herein, the "first region" may be referred to as a "coding region" or "region encoding" or simply the "first region." This first region may include, but is not limited to, the encoded polypeptide of interest. In one aspect, the first region **132** may include, but is not limited to, the open reading frame encoding at least one polypeptide of interest. The open reading frame may be codon optimized in whole or in part. The flanking region **134** may comprise a region of linked nucleotides comprising one or more complete or incomplete 5′ UTRs sequences which may be completely codon optimized or partially codon optimized. The flanking region **134** may include at least one nucleic acid sequence including, but not limited to, miR sequences, TERZAK™ sequences and translation control sequences. The flanking region **134** may also comprise a 5′ terminal cap **138**. The 5′ terminal capping region **138** may include a naturally occurring cap, a synthetic cap or an optimized cap. Non-limiting examples of optimized caps include the caps taught by Rhoads in US Patent No. US7074596 and International Patent Publication No. WO2008157668, WO2009149253 and WO2013103659.

The second flanking region **106** may comprise a region of linked nucleotides comprising one or more complete or incomplete 3′ UTRs. The second flanking region **136** may be completely codon optimized or partially codon optimized. The flanking region **134** may include at least one nucleic acid sequence including, but not limited to, miR sequences and translation control sequences. After the second flanking region **136** the IVT polynucleotide primary construct may comprise a 3′ tailing sequence **140**. The 3' tailing sequence **140** may include a synthetic tailing region **142** and/or a chain terminating nucleoside **144**. Non-liming examples of a synthetic tailing region include a polyA sequence, a polyC sequence, and a polyA-G quartet. Non-limiting examples of chain terminating nucleosides include 2'-O methyl, F and locked nucleic acids (LNA).

[000165] Bridging the 5′ terminus of the first region **132** and the first flanking region **134** is a first operational region **144**. Traditionally this operational region comprises a Start codon. The operational region may alternatively comprise any translation initiation sequence or signal including a Start codon.

[000166] Bridging the 3′ terminus of the first region **132** and the second flanking region **136** is a second operational region **146**. Traditionally this operational region comprises a Stop codon. The operational region may alternatively comprise any translation initiation sequence or signal including a Stop codon. According to the present invention, multiple serial stop codons may also be used.

[000167] The shortest length of the first region of the primary construct of the IVT polynucleotide of the present invention can be the length of a nucleic acid sequence that is sufficient to encode for a dipeptide, a tripeptide, a tetrapeptide, a pentapeptide, a hexapeptide, a heptapeptide, an octapeptide, a nonapeptide, or a decapeptide. In another embodiment, the length may be sufficient to encode a peptide of 2-30 amino acids, e.g. 5-30, 10-30, 2-25, 5-25, 10-25, or 10-20 amino acids. The length may be sufficient to encode for a peptide of at least 11, 12, 13, 14, 15, 17, 20, 25 or 30 amino acids, or a peptide that is no longer than 40 amino acids, e.g. no longer than 35, 30, 25, 20, 17, 15, 14, 13, 12, 11 or 10 amino acids. Examples of dipeptides that the polynucleotide sequences can encode or include, but are not limited to, carnosine and anserine.

[000168] The length of the first region of the primary construct of the IVT polynucleotide encoding the polypeptide of interest of the present invention is greater than about 30 nucleotides

in length (e.g., at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000 or up to and including 100,000 nucleotides).

[000169] In some embodiments, the IVT polynucleotide includes from about 30 to about 100,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 1,000, from 30 to 1,500, from 30 to 3,000, from 30 to 5,000, from 30 to 7,000, from 30 to 10,000, from 30 to 25,000, from 30 to 50,000, from 30 to 70,000, from 100 to 250, from 100 to 500, from 100 to 1,000, from 100 to 1,500, from 100 to 3,000, from 100 to 5,000, from 100 to 7,000, from 100 to 10,000, from 100 to 25,000, from 100 to 50,000, from 100 to 70,000, from 100 to 100,000, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 3,000, from 500 to 5,000, from 500 to 7,000, from 500 to 10,000, from 500 to 25,000, from 500 to 50,000, from 500 to 70,000, from 500 to 100,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 3,000, from 1,000 to 5,000, from 1,000 to 7,000, from 1,000 to 10,000, from 1,000 to 25,000, from 1,000 to 50,000, from 1,000 to 70,000, from 1,000 to 100,000, from 1,500 to 3,000, from 1,500 to 5,000, from 1,500 to 7,000, from 1,500 to 10,000, from 1,500 to 25,000, from 1,500 to 50,000, from 1,500 to 70,000, from 1,500 to 100,000, from 2,000 to 3,000, from 2,000 to 5,000, from 2,000 to 7,000, from 2,000 to 10,000, from 2,000 to 25,000, from 2,000 to 50,000, from 2,000 to 70,000, and from 2,000 to 100,000).

[000170] According to the present invention, the first and second flanking regions of the IVT polynucleotide may range independently from 15-1,000 nucleotides in length (e.g., greater than 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, and 900 nucleotides or at least 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, and 1,000 nucleotides).

[000171] According to the present invention, the tailing sequence of the IVT polynucleotide may range from absent to 500 nucleotides in length (e.g., at least 60, 70, 80, 90, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, or 500 nucleotides). Where the tailing region is a polyA tail, the length may be determined in units of or as a function of polyA Binding Protein binding. In this embodiment, the polyA tail is long enough to bind at least 4 monomers of PolyA Binding Protein. PolyA Binding Protein monomers bind to stretches of approximately 38 nucleotides. As

such, it has been observed that polyA tails of about 80 nucleotides (SEQ ID NO: 96) and 160 nucleotides (SEQ ID NO: 97) are functional.

[000172] According to the present invention, the capping region of the IVT polynucleotide may comprise a single cap or a series of nucleotides forming the cap. In this embodiment the capping region may be from 1 to 10, e.g. 2-9, 3-8, 4-7, 1-5, 5-10, or at least 2, or 10 or fewer nucleotides in length. In some embodiments, the cap is absent.

[000173] According to the present invention, the first and second operational regions of the IVT polynucleotide may range from 3 to 40, e.g., 5-30, 10-20, 15, or at least 4, or 30 or fewer nucleotides in length and may comprise, in addition to a Start and/or Stop codon, one or more signal and/or restriction sequences.

[000174] In one embodiment, the IVT polynucleotides of the present invention may be structurally modified or chemically modified. When the IVT polynucleotides of the present invention are chemically and/or structurally modified the polynucleotides may be referred to as "modified IVT polynucleotides."

[000175] In one embodiment, if the IVT polynucleotides of the present invention are chemically modified they may have a uniform chemical modification of all or any of the same nucleoside type or a population of modifications produced by mere downward titration of the same starting modification in all or any of the same nucleoside type, or a measured percent of a chemical modification of all any of the same nucleoside type but with random incorporation, such as where all uridines are replaced by a uridine analog, e.g., pseudouridine. In another embodiment, the IVT polynucleotides may have a uniform chemical modification of two, three, or four of the same nucleoside type throughout the entire polynucleotide (such as all uridines and all cytosines, etc. are modified in the same way).

[000176] In one embodiment, the IVT polynucleotides of the present invention may include a sequence encoding a self-cleaving peptide, described herein, such as but not limited to the 2A peptide. The polynucleotide sequence of the 2A peptide in the IVT polynucleotide may be modified or codon optimized by the methods described herein and/or are known in the art.

[000177] In one embodiment, this sequence may be used to separate the coding region of two or more polypeptides of interest in the IVT polynucleotide.

[000178] In one embodiment, the IVT polynucleotide of the present invention may be structurally and/or chemically modified. When chemically modified and/or structurally modified the IVT polynucleotide may be referred to as a "modified IVT polynucleotide."

[000179] In one embodiment, the IVT polynucleotide may encode at least one peptide or polypeptide of interest. In another embodiment, the IVT polynucleotide may encode two or more peptides or polypeptides of interest. Non-limiting examples of peptides or polypeptides of interest include heavy and light chains of antibodies, an enzyme and its substrate, a label and its binding molecule, a second messenger and its enzyme or the components of multimeric proteins or complexes.

[000180] In one embodiment, the IVT polynucleotide may include modified nucleosides such as, but not limited to, the modified nucleosides described in US Patent Publication No. US20130115272 including pseudouridine, 1-methylpseudouridine, 5-methoxyuridine and 5-methylcytosine. As a non-limiting example, the IVT polynucleotide may include 1-methylpseudouridine and 5-methylcytosine. As another non-limiting example, the IVT polynucleotide may include 1-methylpseudouridine. As yet another non-limiting example, the IVT polynucleotide may include 5-methoxyuridine and 5-methylcytosine. As a non-limiting example, the IVT polynucleotide may include 5-methoxyuridine.

Chimeric Polynucleotide Architecture

[000181] The chimeric polynucleotides or RNA constructs of the present invention maintain a modular organization similar to IVT polynucleotides, but the chimeric polynucleotides comprise one or more structural and/or chemical modifications or alterations which impart useful properties to the polynucleotide. As such, the chimeric polynucleotides which are modified mRNA molecules of the present invention are termed "chimeric modified mRNA" or "chimeric mRNA."

[000182] Chimeric polynucleotides have portions or regions which differ in size and/or chemical modification pattern, chemical modification position, chemical modification percent or chemical modification population and combinations of the foregoing.

[000183] Examples of parts or regions, where the chimeric polynucleotide functions as an mRNA and encodes a polypeptide of interest include, but are not limited to, untranslated regions (UTRs, such as the 5' UTR or 3' UTR), coding regions, cap regions, polyA tail regions, start regions, stop regions, signal sequence regions, and combinations thereof. Figure 2 illustrates

certain embodiments of the chimeric polynucleotides of the invention which may be used as mRNA. Figure 3 illustrates a schematic of a series of chimeric polynucleotides identifying various patterns of positional modifications and showing regions analogous to those regions of an mRNA polynucleotide. Regions or parts that join or lie between other regions may also be designed to have subregions. These are shown in the figure.

[000184] In some embodiments, the chimeric polynucleotides of the invention have a structure comprising Formula I.

$$5' [A_n]_x\text{-}L1\text{-}[B_o]_y\text{-}L2\text{-}[C_p]_z\text{-}L3\ 3'$$

Formula I

[000185] wherein:

[000186] each of A and B independently comprise a region of linked nucleosides;

[000187] C is an optional region of linked nucleosides;

[000188] at least one of regions A, B, or C is positionally modified, wherein the positionally modified region comprises at least two chemically modified nucleosides of one or more of the same nucleoside type of adenosine, thymidine, guanosine, cytidine, or uridine, and wherein at least two of the chemical modifications of nucleosides of the same type are different chemical modifications;

[000189] n, o and p are independently an integer between 15-1000;

[000190] x and y are independently 1-20;

[000191] z is 0-5;

[000192] L1 and L2 are independently optional linker moieties, the linker moieties being either nucleic acid based or non-nucleic acid based; and

[000193] L3 is an optional conjugate or an optional linker moiety, the linker moiety being either nucleic acid based or non-nucleic acid based.

[000194] In some embodiments the chimeric polynucleotide of Formula I encodes one or more peptides or polypeptides of interest. Such encoded molecules may be encoded across two or more regions.

[000195] In one embodiment, at least one of the regions of linked nucleosides of A may comprise a sequence of linked nucleosides which can function as a 5' untranslated region (UTR). The sequence of linked nucleosides may be a natural or synthetic 5' UTR. As a non-limiting example, the chimeric polynucleotide may encode a polypeptide of interest and the sequence of

linked nucleosides of A may encode the native 5' UTR of a polypeptide encoded by the chimeric polynucleotide or the sequence of linked nucleosides may be a non-heterologous 5' UTR such as, but not limited to a synthetic UTR.

[000196] In another embodiment, at least one of the regions of linked nucleosides of A may be a cap region. The cap region may be located 5' to a region of linked nucleosides of A functioning as a 5'UTR. The cap region may comprise at least one cap such as, but not limited to, Cap0, Cap1, ARCA, inosine, N1-methyl-guanosine, 2'fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, 2-azido-guanosine, Cap2 and Cap4.

[000197] In one embodiment, at least one of the regions of linked nucleosides of B may comprise at least one open reading frame of a nucleic acid sequence. The nucleic acid sequence may be codon optimized and/or comprise at least one modification.

[000198] In one embodiment, at least one of the regions of linked nucleosides of C may comprise a sequence of linked nucleosides which can function as a 3' UTR. The sequence of linked nucleosides may be a natural or synthetic 3' UTR. As a non-limiting example, the chimeric polynucleotide may encode a polypeptide of interest and the sequence of linked nucleosides of C may encode the native 3' UTR of a polypeptide encoded by the chimeric polynucleotide or the sequence of linked nucleosides may be a non-heterologous 3' UTR such as, but not limited to a synthetic UTR.

[000199] In one embodiment, at least one of the regions of linked nucleosides of A comprises a sequence of linked nucleosides which functions as a 5' UTR and at least one of the regions of linked nucleosides of C comprises a sequence of linked nucleosides which functions as a 3' UTR. In one embodiment, the 5' UTR and the 3' UTR may be from the same or different species. In another embodiment, the 5' UTR and the 3' UTR may encode the native untranslated regions from different proteins from the same or different species.

[000200] Figures 4 and 5 provide schematics of a series of chimeric polynucleotides illustrating various patterns of positional modifications based on Formula I as well as those having a blocked or structured 3' terminus.

[000201] Chimeric polynucleotides, including the parts or regions thereof, of the present invention may be classified as hemimers, gapmers, wingmers, or blockmers.

[000202] As used herein, a "hemimer" is chimeric polynucleotide comprising a region or part which comprises half of one pattern, percent, position or population of a chemical

modification(s) and half of a second pattern, percent, position or population of a chemical modification(s). Chimeric polynucleotides of the present invention may also comprise hemimer subregions. In one embodiment, a part or region is 50% of one and 50% of another.

[000203] In one embodiment the entire chimeric polynucleotide can be 50% of one and 50% of the other. Any region or part of any chimeric polynucleotide of the invention may be a hemimer. Types of hemimers include pattern hemimers, population hemimers or position hemimers. By definition, hemimers are 50:50 percent hemimers.

[000204] As used herein, a "gapmer" is a chimeric polynucleotide having at least three parts or regions with a gap between the parts or regions. The "gap" can comprise a region of linked nucleosides or a single nucleoside which differs from the chimeric nature of the two parts or regions flanking it. The two parts or regions of a gapmer may be the same or different from each other.

[000205] As used herein, a "wingmer" is a chimeric polynucleotide having at least three parts or regions with a gap between the parts or regions. Unlike a gapmer, the two flanking parts or regions surrounding the gap in a wingmer are the same in degree or kind. Such similarity may be in the length of number of units of different modifications or in the number of modifications. The wings of a wingmer may be longer or shorter than the gap. The wing parts or regions may be 20, 30, 40, 50, 60 70, 80, 90 or 95% greater or shorter in length than the region which comprises the gap.

[000206] As used herein, a "blockmer" is a patterned polynucleotide where parts or regions are of equivalent size or number and type of modifications. Regions or subregions in a blockmer may be 50, 51, 52, 53, 54, 55, 56, 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, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251,

252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490 or 500, nucleosides long.

[000207] Chimeric polynucleotides, including the parts or regions thereof, of the present invention having a chemical modification pattern are referred to as "pattern chimeras." Pattern chimeras may also be referred to as blockmers. Pattern chimeras are those polynucleotides having a pattern of modifications within, across or among regions or parts.

[000208] Patterns of modifications within a part or region are those which start and stop within a defined region. Patterns of modifications across a part or region are those patterns which start in on part or region and end in another adjacent part or region. Patterns of modifications among parts or regions are those which begin and end in one part or region and are repeated in a different part or region, which is not necessarily adjacent to the first region or part.

[000209] The regions or subregions of pattern chimeras or blockmers may have simple alternating patterns such as ABAB[AB]n where each "A" and each "B" represent different chemical modifications (at least one of the base, sugar or backbone linker), different types of chemical modifications (e.g., naturally occurring and non-naturally occurring), different percentages of modifications or different populations of modifications. The pattern may repeat n number of times where n=3-300. Further, each A or B can represent from 1-2500 units (e.g., nucleosides) in the pattern. Patterns may also be alternating multiples such as AABBAABB[AABB]n (an alternating double multiple) or AAABBBAAABBB[AAABBB]n (an alternating triple multiple) pattern. The pattern may repeat n number of times where n=3-300.

[000210] Different patterns may also be mixed together to form a second order pattern. For example, a single alternating pattern may be combined with a triple alternating pattern to form a second order alternating pattern A'B'. One example would be [ABABAB][AAABBBAAABBB] [ABABAB][AAABBBAAABBB] [ABABAB][AAABBBAAABBB], where [ABABAB] is A' and [AAABBBAAABBB] is B'. In like fashion, these patterns may be repeated n number of times, where n=3-300.

[000211] Patterns may include three or more different modifications to form an ABCABC[ABC]n pattern. These three component patterns may also be multiples, such as

AABBCCAABBCC[AABBCC]n and may be designed as combinations with other patterns such as ABCABCAABBCCABCABCAABBCC, and may be higher order patterns.

[000212] Regions or subregions of position, percent, and population modifications need not reflect an equal contribution from each modification type. They may form series such as "1-2-3-4", "1-2-4-8", where each integer represents the number of units of a particular modification type. Alternatively, they may be odd only, such as '1-3-3-1-3-1-5" or even only "2-4-2-4-6-4-8" or a mixture of both odd and even number of units such as "1-3-4-2-5-7-3-3-4".

[000213] Pattern chimeras may vary in their chemical modification by degree (such as those described above) or by kind (e.g., different modifications).

[000214] Chimeric polynucleotides, including the parts or regions thereof, of the present invention having at least one region with two or more different chemical modifications of two or more nucleoside members of the same nucleoside type (A, C, G, T, or U) are referred to as "positionally modified" chimeras. Positionally modified chimeras are also referred to herein as "selective placement" chimeras or "selective placement polynucleotides". As the name implies, selective placement refers to the design of polynucleotides which, unlike polynucleotides in the art where the modification to any A, C, G, T or U is the same by virtue of the method of synthesis, can have different modifications to the individual As, Cs, Gs, Ts or Us in a polynucleotide or region thereof. For example, in a positionally modified chimeric polynucleotide, there may be two or more different chemical modifications to any of the nucleoside types of As, Cs, Gs, Ts, or Us. There may also be combinations of two or more to any two or more of the same nucleoside type. For example, a positionally modified or selective placement chimeric polynucleotide may comprise 3 different modifications to the population of adenines in the molecule and also have 3 different modifications to the population of cytosines in the construct—all of which may have a unique, non-random, placement.

[000215] Chimeric polynucleotides, including the parts or regions thereof, of the present invention having a chemical modification percent are referred to as "percent chimeras." Percent chimeras may have regions or parts which comprise at least 1%, at least 2%, at least 5%, at least 8%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% positional, pattern or population of modifications. Alternatively, the percent chimera may be completely modified as to modification

position, pattern, or population. The percent of modification of a percent chimera may be split between naturally occurring and non-naturally occurring modifications.

[000216] Chimeric polynucleotides, including the parts or regions thereof, of the present invention having a chemical modification population are referred to as "population chimeras." A population chimera may comprise a region or part where nucleosides (their base, sugar or backbone linkage, or combination thereof) have a select population of modifications. Such modifications may be selected from functional populations such as modifications which induce, alter or modulate a phenotypic outcome. For example, a functional population may be a population or selection of chemical modifications which increase the level of a cytokine. Other functional populations may individually or collectively function to decrease the level of one or more cytokines. Use of a selection of these like-function modifications in a chimeric polynucleotide would therefore constitute a "functional population chimera." As used herein, a "functional population chimera" may be one whose unique functional feature is defined by the population of modifications as described above or the term may apply to the overall function of the chimeric polynucleotide itself. For example, as a whole the chimeric polynucleotide may function in a different or superior way as compared to an unmodified or non-chimeric polynucleotide.

[000217] It should be noted that polynucleotides which have a uniform chemical modification of all of any of the same nucleoside type or a population of modifications produced by mere downward titration of the same starting modification in all of any of the same nucleoside type, or a measured percent of a chemical modification of all any of the same nucleoside type but with random incorporation, such as where all uridines are replaced by a uridine analog, e.g., pseudouridine, are not considered chimeric. Likewise, polynucleotides having a uniform chemical modification of two, three, or four of the same nucleoside type throughout the entire polynucleotide (such as all uridines and all cytosines, etc. are modified in the same way) are not considered chimeric polynucleotides. One example of a polynucleotide which is not chimeric is the canonical pseudouridine/5-methyl cytosine modified polynucleotide of the prior art. These uniform polynucleotides are arrived at entirely via *in vitro* transcription (IVT) enzymatic synthesis; and due to the limitations of the synthesizing enzymes, they contain only one kind of modification at the occurrence of each of the same nucleoside type, i.e., adenosine (A),

thymidine (T), guanosine (G), cytidine (C) or uradine (U), found in the polynucleotide. Such polynucleotides may be characterized as IVT polynucleotides.

[000218] The chimeric polynucleotides of the present invention may be structurally modified or chemically modified. When the chimeric polynucleotides of the present invention are chemically and/or structurally modified the polynucleotides may be referred to as "modified chimeric polynucleotides."

[000219] In some embodiments of the invention, the chimeric polynucleotides may encode two or more peptides or polypeptides of interest. Such peptides or polypeptides of interest include the heavy and light chains of antibodies, an enzyme and its substrate, a label and its binding molecule, a second messenger and its enzyme or the components of multimeric proteins or complexes.

[000220] The regions or parts of the chimeric polynucleotides of the present invention may be separated by a linker or spacer moiety. Such linkers or spaces may be nucleic acid based or non-nucleosidic.

[000221] In one embodiment, the chimeric polynucleotides of the present invention may include a sequence encoding a self-cleaving peptide described herein, such as, but not limited to, a 2A peptide. The polynucleotide sequence of the 2A peptide in the chimeric polynucleotide may be modified or codon optimized by the methods described herein and/or are known in the art.

[000222] Notwithstanding the foregoing, the chimeric polynucleotides of the present invention may comprise a region or part which is not positionally modified or not chimeric as defined herein.

[000223] For example, a region or part of a chimeric polynucleotide may be uniformly modified at one or more A, T, C, G, or U but according to the invention, the polynucleotides will not be uniformly modified throughout the entire region or part.

[000224] Regions or parts of chimeric polynucleotides may be from 15-1000 nucleosides in length and a polynucleotide may have from 2-100 different regions or patterns of regions as described herein.

[000225] In one embodiment, chimeric polynucleotides encode one or more polypeptides of interest. In another embodiment, the chimeric polynucleotides are substantially non-coding. In

another embodiment, the chimeric polynucleotides have both coding and non-coding regions and parts.

[000226] Figure 4 illustrates the design of certain chimeric polynucleotides of the present invention when based on the scaffold of the polynucleotide of Figure 1. Shown in the figure are the regions or parts of the chimeric polynucleotides where patterned regions represent those regions which are positionally modified and open regions illustrate regions which may or may not be modified but which are, when modified, uniformly modified. Chimeric polynucleotides of the present invention may be completely positionally modified or partially positionally modified. They may also have subregions which may be of any pattern or design. Shown in Figure 2 are a chimeric subregion and a hemimer subregion.

[000227] In one embodiment, the shortest length of a region of the chimeric polynucleotide of the present invention encoding a peptide can be the length that is sufficient to encode for a dipeptide, a tripeptide, a tetrapeptide, a pentapeptide, a hexapeptide, a heptapeptide, an octapeptide, a nonapeptide, or a decapeptide. In another embodiment, the length may be sufficient to encode a peptide of 2-30 amino acids, e.g. 5-30, 10-30, 2-25, 5-25, 10-25, or 10-20 amino acids. The length may be sufficient to encode for a peptide of at least 11, 12, 13, 14, 15, 17, 20, 25 or 30 amino acids, or a peptide that is no longer than 40 amino acids, e.g. no longer than 35, 30, 25, 20, 17, 15, 14, 13, 12, 11 or 10 amino acids. Examples of dipeptides that the polynucleotide sequences can encode or include, but are not limited to, carnosine and anserine.

[000228] In one embodiment, the length of a region of the chimeric polynucleotide of the present invention encoding the peptide or polypeptide of interest is greater than about 30 nucleotides in length (e.g., at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000 or up to and including 100,000 nucleotides). As used herein, such a region may be referred to as a "coding region" or "region encoding."

[000229] In some embodiments, the chimeric polynucleotide includes from about 30 to about 100,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 1,000, from 30 to 1,500, from 30 to 3,000, from 30 to 5,000, from 30 to 7,000, from 30 to 10,000, from 30 to 25,000, from 30 to 50,000, from 30 to 70,000, from 100 to 250, from 100 to

500, from 100 to 1,000, from 100 to 1,500, from 100 to 3,000, from 100 to 5,000, from 100 to 7,000, from 100 to 10,000, from 100 to 25,000, from 100 to 50,000, from 100 to 70,000, from 100 to 100,000, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 3,000, from 500 to 5,000, from 500 to 7,000, from 500 to 10,000, from 500 to 25,000, from 500 to 50,000, from 500 to 70,000, from 500 to 100,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 3,000, from 1,000 to 5,000, from 1,000 to 7,000, from 1,000 to 10,000, from 1,000 to 25,000, from 1,000 to 50,000, from 1,000 to 70,000, from 1,000 to 100,000, from 1,500 to 3,000, from 1,500 to 5,000, from 1,500 to 7,000, from 1,500 to 10,000, from 1,500 to 25,000, from 1,500 to 50,000, from 1,500 to 70,000, from 1,500 to 100,000, from 2,000 to 3,000, from 2,000 to 5,000, from 2,000 to 7,000, from 2,000 to 10,000, from 2,000 to 25,000, from 2,000 to 50,000, from 2,000 to 70,000, and from 2,000 to 100,000).

[000230] According to the present invention, regions or subregions of the chimeric polynucleotides may also range independently from 15-1,000 nucleotides in length (e.g., greater than 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900 and 950 nucleotides or at least 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 and 1,000 nucleotides).

[000231] According to the present invention, regions or subregions of chimeric polynucleotides may range from absent to 500 nucleotides in length (e.g., at least 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, or 500 nucleotides). Where the region is a polyA tail, the length may be determined in units of or as a function of polyA Binding Protein binding. In this embodiment, the polyA tail is long enough to bind at least 4 monomers of PolyA Binding Protein. PolyA Binding Protein monomers bind to stretches of approximately 38 nucleotides. As such, it has been observed that polyA tails of about 80 nucleotides (SEQ ID NO: 96) to about 160 nucleotides (SEQ ID NO: 97) are functional. The chimeric polynucleotides of the present invention which function as an mRNA need not comprise a polyA tail.

[000232] According to the present invention, chimeric polynucleotides which function as an mRNA may have a capping region. The capping region may comprise a single cap or a series of nucleotides forming the cap. In this embodiment the capping region may be from 1 to 10, e.g. 2-

9, 3-8, 4-7, 1-5, 5-10, or at least 2, or 10 or fewer nucleotides in length. In some embodiments, the cap is absent.

[000233] The present invention contemplates chimeric polynucleotides which are circular or cyclic. As the name implies circular polynucleotides are circular in nature meaning that the termini are joined in some fashion, whether by ligation, covalent bond, common association with the same protein or other molecule or complex or by hybridization. Any of the circular polynucleotides as taught in for example in International Publication No. WO2015034925, (Attorney Docket No. M51.20) the contents of which are incorporated herein by reference in their entirety, may be made chimeric according to the present invention.

[000234] Chimeric polynucleotides, formulations and compositions comprising chimeric polynucleotides, and methods of making, using and administering chimeric polynucleotides are also described in co-pending International Publication No. WO2015034928 (Attorney Docket No. M57.20); the contents of which is incorporated by reference in its entirety.

Circular Polynucleotide Architecture

[000235] The present invention contemplates polynucleotides which are circular or cyclic. As the name implies circular polynucleotides are circular in nature meaning that the termini are joined in some fashion, whether by ligation, covalent bond, common association with the same protein or other molecule or complex or by hybridization. Any of the circular polynucleotides as taught in for example in International Publication No. WO2015034925, (Attorney Docket No. M51.20) the contents of which are incorporated herein by reference in their entirety.

[000236] Circular polynucleotides of the present invention may be designed according to the circular RNA construct scaffolds shown in Figure 6A-6G. These figures are also described in International Publication No. WO2015034925, (Attorney Docket No. M51.20), the contents of which are incorporated herein by reference in their entirety. Such polynucleotides are circular polynucleotides or circular constructs.

[000237] The circular polynucleotides or circPs of the present invention which encode at least one peptide or polypeptide of interest are known as circular RNAs or circRNA. As used herein, "circular RNA" or "circRNA" means a circular polynucleotide that can encode at least one peptide or polypeptide of interest. The circPs of the present invention which comprise at least one sensor sequence and do not encode a peptide or polypeptide of interest are known as circular sponges or circSP. As used herein, "circular sponges," "circular polynucleotide sponges" or

"circSP" means a circular polynucleotide which comprises at least one sensor sequence and does not encode a polypeptide of interest. As used herein, "sensor sequence" means a receptor or pseudo-receptor for endogenous nucleic acid binding molecules. Non-limiting examples of sensor sequences include, microRNA binding sites, microRNA seed sequences, microRNA binding sites without the seed sequence, transcription factor binding sites and artificial binding sites engineered to act as pseudo-receptors and portions and fragments thereof.

[000238] The circPs of the present invention which comprise at least one sensor sequence and encode at least one peptide or polypeptide of interest are known as circular RNA sponges or circRNA-SP. As used herein, "circular RNA sponges" or "circRNA-SP" means a circular polynucleotide which comprises at least one sensor sequence and at least one region encoding at least one peptide or polypeptide of interest.

[000239] Figure 6 shows a representative circular construct **200** of the circular polynucleotides of the present invention. As used herein, the term "circular construct" refers to a circular polynucleotide transcript which may act substantially similar to and have properties of a RNA molecule. In one embodiment the circular construct acts as an mRNA. If the circular construct encodes one or more peptides or polypeptides of interest (e.g., a circRNA or circRNA-SP) then the polynucleotide transcript retains sufficient structural and/or chemical features to allow the polypeptide of interest encoded therein to be translated. Circular constructs may be polynucleotides of the invention. When structurally or chemically modified, the construct may be referred to as a modified circP, modified circSP, modified circRNA or modified circRNA-SP.

[000240] Turning to FIG. 6A, the circular construct **200** here contains a first region of linked nucleotides **202** that is flanked by a first flanking region **204** and a second flanking region **206**. As used herein, the "first region" may be referred to as a "coding region," a "non-coding region" or "region encoding" or simply the "first region." In one embodiment, this first region may comprise nucleotides such as, but is not limited to, encoding at least one peptide or polypeptide of interest and/or nucleotides encoding a sensor region. The peptide or polypeptide of interest may comprise at its 5' terminus one or more signal peptide sequences encoded by a signal peptide sequence region **203**. The first flanking region **204** may comprise a region of linked nucleosides or portion thereof which may act similarly to an untranslated region (UTR) in an mRNA and/or DNA sequence. The first flanking region may also comprise a region of polarity **208**. The region of polarity **208** may include an IRES sequence or portion thereof. As a non-

limiting example, when linearized this region may be split to have a first portion be on the 5' terminus of the first region **202** and second portion be on the 3' terminus of the first region **202.** The second flanking region **206** may comprise a tailing sequence region **210** and may comprise a region of linked nucleotides or portion thereof **212** which may act similarly to a UTR in an mRNA and/or DNA.

[000241] Bridging the 5′ terminus of the first region **202** and the first flanking region **104** is a first operational region **205**. In one embodiment, this operational region may comprise a start codon. The operational region may alternatively comprise any translation initiation sequence or signal including a start codon.

[000242] Bridging the 3′ terminus of the first region **202** and the second flanking region **106** is a second operational region **207**. Traditionally this operational region comprises a stop codon. The operational region may alternatively comprise any translation initiation sequence or signal including a stop codon. According to the present invention, multiple serial stop codons may also be used. In one embodiment, the operation region of the present invention may comprise two stop codons. The first stop codon may be "TGA" or "UGA" and the second stop codon may be selected from the group consisting of "TAA," "TGA," "TAG," "UAA," "UGA" or "UAG."

[000243] Turning to Figure 6B, at least one non-nucleic acid moiety **201** may be used to prepare a circular construct **200** where the non-nucleic acid moiety **201** is used to bring the first flanking region **204** near the second flanking region **206**. Non-limiting examples of non-nucleic acid moieties which may be used in the present invention are described herein. The circular construct **200** may comprise more than one non-nucleic acid moiety wherein the additional non-nucleic acid moieties may be heterologous or homologous to the first non-nucleic acid moiety.

[000244] Turning to Figure 6C, the first region of linked nucleosides **202** may comprise a spacer region **214**. This spacer region **214** may be used to separate the first region of linked nucleosides **202** so that the circular construct can include more than one open reading frame, non-coding region or an open reading frame and a non-coding region.

[000245] Turning to Figure 6D, the second flanking region **206** may comprise one or more sensor regions **216** in the 3'UTR **212**. These sensor sequences as discussed herein operate as pseudo-receptors (or binding sites) for ligands of the local microenvironment of the circular construct. For example, microRNA binding sites or miRNA seeds may be used as sensors such that they function as pseudoreceptors for any microRNAs present in the environment of the

circular polynucleotide. As shown in Figure 9, the one or more sensor regions **216** may be separated by a spacer region **214**.

[000246] As shown in Figure 6E, a circular construct **200,** which includes one or more sensor regions **216,** may also include a spacer region **214** in the first region of linked nucleosides **202**. As discussed above for Figure 6B, this spacer region **214** may be used to separate the first region of linked nucleosides **202** so that the circular construct can include more than one open reading frame and/or more than one non-coding region.

[000247] Turning to Figure 6F, a circular construct **200** may be a non-coding construct known as a circSP comprising at least one non-coding region such as, but not limited to, a sensor region **216**. Each of the sensor regions **216** may include, but are not limited to, a miR sequence, a miR seed, a miR binding site and/or a miR sequence without the seed.

[000248] Turning to Figure 6G, at least one non-nucleic acid moiety **201** may be used to prepare a circular construct **200** which is a non-coding construct. The circular construct **200** which is a non-coding construct may comprise more than one non-nucleic acid moiety wherein the additional non-nucleic acid moieties may be heterologous or homologous to the first non-nucleic acid moiety.

[000249] Circular polynucleotides, formulations and compositions comprising circular polynucleotides, and methods of making, using and administering circular polynucleotides are also described in co-pending in International Publication No. WO2015034925, (Attorney Docket No. M51.20); each of which is incorporated by reference in its entirety.

Multimers of Polynucleotides

[000250] According to the present invention, multiple distinct chimeric polynucleotides and/or IVT polynucleotides may be linked together through the 3′-end using nucleotides which are modified at the 3′-terminus. Chemical conjugation may be used to control the stoichiometry of delivery into cells. For example, the glyoxylate cycle enzymes, isocitrate lyase and malate synthase, may be supplied into cells at a 1:1 ratio to alter cellular fatty acid metabolism. This ratio may be controlled by chemically linking chimeric polynucleotides and/or IVT polynucleotides using a 3′-azido terminated nucleotide on one polynucleotides species and a C5-ethynyl or alkynyl-containing nucleotide on the opposite polynucleotide species. The modified nucleotide is added post-transcriptionally using terminal transferase (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. After the addition of the 3′-modified

nucleotide, the two polynucleotides species may be combined in an aqueous solution, in the presence or absence of copper, to form a new covalent linkage via a click chemistry mechanism as described in the literature.

[000251] In another example, more than two chimeric polynucleotides and/or IVT polynucleotides may be linked together using a functionalized linker molecule. For example, a functionalized saccharide molecule may be chemically modified to contain multiple chemical reactive groups (SH-, NH_2-, N_3, etc…) to react with the cognate moiety on a 3′-functionalized mRNA molecule (i.e., a 3′-maleimide ester, 3′-NHS-ester, alkynyl). The number of reactive groups on the modified saccharide can be controlled in a stoichiometric fashion to directly control the stoichiometric ratio of conjugated chimeric polynucleotides and/or IVT polynucleotides.

[000252] In one embodiment, the chimeric polynucleotides and/or IVT polynucleotides may be linked together in a pattern. The pattern may be a simple alternating pattern such as $CD[CD]_x$ where each "C" and each "D" represent a chimeric polynucleotide, IVT polynucleotide, different chimeric polynucleotides or different IVT polynucleotides. The pattern may repeat x number of times, where x= 1-300. Patterns may also be alternating multiples such as $CCDD[CCDD]_x$ (an alternating double multiple) or $CCCDDD[CCCDDD]_x$ (an alternating triple multiple) pattern. The alternating double multiple or alternating triple multiple may repeat x number of times, where x= 1-300.

Conjugates and Combinations of Polynucleotides

[000253] In order to further enhance protein production, polynucleotides of the present invention can be designed to be conjugated to other polynucleotides, dyes, intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases (*e.g.* EDTA), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, $[MPEG]_2$, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.* biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases, proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies *e.g.*, an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell, hormones and hormone receptors, non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, or a drug.

[000254] Conjugation may result in increased stability and/or half life and may be particularly useful in targeting the polynucleotides to specific sites in the cell, tissue or organism.

[000255] According to the present invention, the polynucleotides may be administered with, conjugated to or further encode one or more of RNAi agents, siRNAs, shRNAs, miRNAs, miRNA binding sites, antisense RNAs, ribozymes, catalytic DNA, tRNA, RNAs that induce triple helix formation, aptamers or vectors, and the like.

Bifunctional Polynucleotides

[000256] In one embodiment of the invention are bifunctional polynucleotides (e.g., bifunctional IVT polynucleotides, bifunctional chimeric polynucleotides or bifunctional circular polynucleotides). As the name implies, bifunctional polynucleotides are those having or capable of at least two functions. These molecules may also by convention be referred to as multi-functional.

[000257] The multiple functionalities of bifunctional polynucleotides may be encoded by the RNA (the function may not manifest until the encoded product is translated) or may be a property of the polynucleotide itself. It may be structural or chemical. Bifunctional modified polynucleotides may comprise a function that is covalently or electrostatically associated with the polynucleotides. Further, the two functions may be provided in the context of a complex of a chimeric polynucleotide and another molecule.

[000258] Bifunctional polynucleotides may encode peptides which are anti-proliferative. These peptides may be linear, cyclic, constrained or random coil. They may function as aptamers, signaling molecules, ligands or mimics or mimetics thereof. Anti-proliferative peptides may, as translated, be from 3 to 50 amino acids in length. They may be 5-40, 10-30, or approximately 15 amino acids long. They may be single chain, multichain or branched and may form complexes, aggregates or any multi-unit structure once translated.

Noncoding Polynucleotides

[000259] As described herein, provided are polynucleotides having sequences that are partially or substantially not translatable, e.g., having a noncoding region. As one non-limiting example, the noncoding region may be the first region of the IVT polynucleotide or the circular polynucleotide. Alternatively, the noncoding region may be a region other than the first region. As another non-limiting example, the noncoding region may be the A, B and/or C region of the chimeric polynucleotide.

[000260] Such molecules are generally not translated, but can exert an effect on protein production by one or more of binding to and sequestering one or more translational machinery components such as a ribosomal protein or a transfer RNA (tRNA), thereby effectively reducing protein expression in the cell or modulating one or more pathways or cascades in a cell which in turn alters protein levels. The polynucleotide may contain or encode one or more long noncoding RNA (lncRNA, or lincRNA) or portion thereof, a small nucleolar RNA (sno-RNA), micro RNA (miRNA), small interfering RNA (siRNA) or Piwi-interacting RNA (piRNA). Examples of such lncRNA molecules and RNAi constructs designed to target such lncRNA any of which may be encoded in the polynucleotides are taught in International Publication, WO2012/018881 A2, the contents of which are incorporated herein by reference in their entirety.

Polypeptides of Interest

[000261] Polynucleotides of the present invention may encode one or more ophthalmic peptides or polypeptides of interest. They may also affect the levels, signaling or function of one or more peptides or polypeptides.

[000262] Polypeptides of interest, according to the present invention include any ophthalmology target as taught herein including but not limited to, retinal pigment epithelium-specific protein, adrenoceptor alpha 2A, amyloid beta (A4) precursor protein, complement component 3, complement component 5, complement factor D (adipsin), thrombospondin receptor, complement component 5 receptor 1, HIF1A , nerve growth factor receptor, STAT3, VEGFA, PDGFR, VEGFR1/2, plasminogen, tyrosine kinase, mTOR, Factor III, cadherin, chemokine receptor (3/4), integrin A5, placental growth factor, protein tyrosine phosphatase, S1PR1, vRaf, TGF-beta, HtrA serine peptidase 1, TNF receptor 10A , NOTCH4, insulin-like growth factor-binding protein 7, Ras responsive element binding protein 1, component factor H, component factor B, complement component 3, complement component 2, complement factor I, hepatic lipase, cholesteryl ester transfer protein, translocase of outer mitochondrial membrane 40, superoxide dismutase 2, mitochondrial, tenascin XB, collagen type X, alpha 1, myelin basic protein, collagen type VIII, alpha 1, bestrophin 1, carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6, retinitis pimentosa GTPases, guanylate cyclase system (2D, A1A), calcium channels (A2, LA1F), peripherin 2, cadherin 1, choroideremia (Rab escort protein 1), guanylate cyclase 2D, peripherin 2, mitochondrial encoded ATP synthase, mitochondrial encoded cytochromes, mitochondrial encoded NADH dehydrogenase, mitofusin 2, optic atrophy 1, three

prime repair exonuclease 1, three prime repair exonuclease 1. DICER1, HIF-PHD, Hey 1, dominant negative CCR3, anti-Eotaxin mAb, Dcr1, Sema3E, VEGF-trap, PDGF-trap Nitrin1R, aA, aB Crystallin, Hey 2, any of the siruins including SIRT1, DR4-Fc, DR5-Fc, PD1R, RhoJ, sFLT-1, IGFR I-Fc, IGFBP7, PEDF, NPPB, CD59, PLEKHA1, RPE65, ABCA4, and/or PDE.

[000263] According to the present invention, the polynucleotide may be designed to encode one or more polypeptides of interest or fragments thereof. Such polypeptide of interest may include, but is not limited to, whole polypeptides, a plurality of polypeptides or fragments of polypeptides, which independently may be encoded by one or more regions or parts or the whole of a polynucleotide. As used herein, the term "polypeptides of interest" refer to any polypeptide which is selected to be encoded within, or whose function is affected by, the polnucleotides of the present invention.

[000264] As used herein, "polypeptide" means a polymer of amino acid residues (natural or unnatural) linked together most often by peptide bonds. The term, as used herein, refers to proteins, polypeptides, and peptides of any size, structure, or function. In some instances the polypeptide encoded is smaller than about 50 amino acids and the polypeptide is then termed a peptide. If the polypeptide is a peptide, it will be at least about 2, 3, 4, or at least 5 amino acid residues long. Thus, polypeptides include gene products, naturally occurring polypeptides, synthetic polypeptides, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing. A polypeptide may be a single molecule or may be a multi-molecular complex such as a dimer, trimer or tetramer. They may also comprise single chain or multichain polypeptides such as antibodies or insulin and may be associated or linked. Most commonly disulfide linkages are found in multichain polypeptides. The term polypeptide may also apply to amino acid polymers in which one or more amino acid residues are an artificial chemical analogue of a corresponding naturally occurring amino acid.

[000265] The term "polypeptide variant" refers to molecules which differ in their amino acid sequence from a native or reference sequence. The amino acid sequence variants may possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence, as compared to a native or reference sequence. Ordinarily, variants will possess at least about 50% identity (homology) to a native or reference sequence, and preferably, they will be at least about 80%, more preferably at least about 90% identical (homologous) to a native or reference sequence.

[000266] In some embodiments "variant mimics" are provided. As used herein, the term "variant mimic" is one which contains one or more amino acids which would mimic an activated sequence. For example, glutamate may serve as a mimic for phosphoro-threonine and/or phosphoro-serine. Alternatively, variant mimics may result in deactivation or in an inactivated product containing the mimic, e.g., phenylalanine may act as an inactivating substitution for tyrosine; or alanine may act as an inactivating substitution for serine.

[000267] "Homology" as it applies to amino acid sequences is defined as the percentage of residues in the candidate amino acid sequence that are identical with the residues in the amino acid sequence of a second sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. It is understood that homology depends on a calculation of percent identity but may differ in value due to gaps and penalties introduced in the calculation.

[000268] By "homologs" as it applies to polypeptide sequences means the corresponding sequence of other species having substantial identity to a second sequence of a second species.

[000269] "Analogs" is meant to include polypeptide variants which differ by one or more amino acid alterations, e.g., substitutions, additions or deletions of amino acid residues that still maintain one or more of the properties of the parent or starting polypeptide.

[000270] The present invention contemplates several types of compositions which are polypeptide based including variants and derivatives. These include substitutional, insertional, deletion and covalent variants and derivatives. The term "derivative" is used synonymously with the term "variant" but generally refers to a molecule that has been modified and/or changed in any way relative to a reference molecule or starting molecule.

[000271] As such, polynucleotides encoding peptides or polypeptides containing substitutions, insertions and/or additions, deletions and covalent modifications with respect to reference sequences, in particular the polypeptide sequences disclosed herein, are included within the scope of this invention. For example, sequence tags or amino acids, such as one or more lysines, can be added to the peptide sequences of the invention (e.g., at the N-terminal or C-terminal ends). Sequence tags can be used for peptide purification or localization. Lysines can be used to increase peptide solubility or to allow for biotinylation. Alternatively, amino acid residues located at the carboxy and amino terminal regions of the amino acid sequence of a peptide or protein may optionally be deleted providing for truncated sequences. Certain amino acids (e.g.,

C-terminal or N-terminal residues) may alternatively be deleted depending on the use of the sequence, as for example, expression of the sequence as part of a larger sequence which is soluble, or linked to a solid support.

[000272] "Substitutional variants" when referring to polypeptides are those that have at least one amino acid residue in a native or starting sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

[000273] As used herein the term "conservative amino acid substitution" refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine and leucine for another non-polar residue. Likewise, examples of conservative substitutions include the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, and between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine, methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue.

[000274] "Insertional variants" when referring to polypeptides are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native or starting sequence. "Immediately adjacent" to an amino acid means connected to either the alpha-carboxy or alpha-amino functional group of the amino acid.

[000275] "Deletional variants" when referring to polypeptides are those with one or more amino acids in the native or starting amino acid sequence removed. Ordinarily, deletional variants will have one or more amino acids deleted in a particular region of the molecule.

[000276] "Covalent derivatives" when referring to polypeptides include modifications of a native or starting protein with an organic proteinaceous or non-proteinaceous derivatizing agent, and/or post-translational modifications. Covalent modifications are traditionally introduced by

reacting targeted amino acid residues of the protein with an organic derivatizing agent that is capable of reacting with selected side-chains or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. The resultant covalent derivatives are useful in programs directed at identifying residues important for biological activity, for immunoassays, or for the preparation of anti-protein antibodies for immunoaffinity purification of the recombinant glycoprotein. Such modifications are within the ordinary skill in the art and are performed without undue experimentation.

[000277] Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues may be present in the polypeptides produced in accordance with the present invention.

[000278] Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)).

[000279] "Features" when referring to polypeptides are defined as distinct amino acid sequence-based components of a molecule. Features of the polypeptides encoded by the polynucleotides of the present invention include surface manifestations, local conformational shape, folds, loops, half-loops, domains, half-domains, sites, termini or any combination thereof.

[000280] As used herein when referring to polypeptides the term "surface manifestation" refers to a polypeptide based component of a protein appearing on an outermost surface.

[000281] As used herein when referring to polypeptides the term "local conformational shape" means a polypeptide based structural manifestation of a protein which is located within a definable space of the protein.

[000282] As used herein when referring to polypeptides the term "fold" refers to the resultant conformation of an amino acid sequence upon energy minimization. A fold may occur at the secondary or tertiary level of the folding process. Examples of secondary level folds include beta sheets and alpha helices. Examples of tertiary folds include domains and regions formed due to aggregation or separation of energetic forces. Regions formed in this way include hydrophobic and hydrophilic pockets, and the like.

[000283] As used herein the term "turn" as it relates to protein conformation means a bend which alters the direction of the backbone of a peptide or polypeptide and may involve one, two, three or more amino acid residues.

[000284] As used herein when referring to polypeptides the term "loop" refers to a structural feature of a polypeptide which may serve to reverse the direction of the backbone of a peptide or polypeptide. Where the loop is found in a polypeptide and only alters the direction of the backbone, it may comprise four or more amino acid residues. Oliva et al. have identified at least 5 classes of protein loops (J. Mol Biol 266 (4): 814-830; 1997). Loops may be open or closed. Closed loops or "cyclic" loops may comprise 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acids between the bridging moieties. Such bridging moieties may comprise a cysteine-cysteine bridge (Cys-Cys) typical in polypeptides having disulfide bridges or alternatively bridging moieties may be non-protein based such as the dibromozylyl agents used herein.

[000285] As used herein when referring to polypeptides the term "half-loop" refers to a portion of an identified loop having at least half the number of amino acid resides as the loop from which it is derived. It is understood that loops may not always contain an even number of amino acid residues. Therefore, in those cases where a loop contains or is identified to comprise an odd number of amino acids, a half-loop of the odd-numbered loop will comprise the whole number portion or next whole number portion of the loop (number of amino acids of the loop/2+/-0.5 amino acids). For example, a loop identified as a 7 amino acid loop could produce half-loops of 3 amino acids or 4 amino acids (7/2=3.5+/-0.5 being 3 or 4).

[000286] As used herein when referring to polypeptides the term "domain" refers to a motif of a polypeptide having one or more identifiable structural or functional characteristics or properties (e.g., binding capacity, serving as a site for protein-protein interactions).

[000287] As used herein when referring to polypeptides the term "half-domain" means a portion of an identified domain having at least half the number of amino acid resides as the domain from which it is derived. It is understood that domains may not always contain an even number of amino acid residues. Therefore, in those cases where a domain contains or is identified to comprise an odd number of amino acids, a half-domain of the odd-numbered domain will comprise the whole number portion or next whole number portion of the domain (number of amino acids of the domain/2+/-0.5 amino acids). For example, a domain identified as a 7 amino acid domain could produce half-domains of 3 amino acids or 4 amino acids

(7/2=3.5+/-0.5 being 3 or 4). It is also understood that sub-domains may be identified within domains or half-domains, these subdomains possessing less than all of the structural or functional properties identified in the domains or half domains from which they were derived. It is also understood that the amino acids that comprise any of the domain types herein need not be contiguous along the backbone of the polypeptide (i.e., nonadjacent amino acids may fold structurally to produce a domain, half-domain or subdomain).

[000288] As used herein when referring to polypeptides the terms "site" as it pertains to amino acid based embodiments is used synonymously with "amino acid residue" and "amino acid side chain." A site represents a position within a peptide or polypeptide that may be modified, manipulated, altered, derivatized or varied within the polypeptide based molecules of the present invention.

[000289] As used herein the terms "termini" or "terminus" when referring to polypeptides refers to an extremity of a peptide or polypeptide. Such extremity is not limited only to the first or final site of the peptide or polypeptide but may include additional amino acids in the terminal regions. The polypeptide based molecules of the present invention may be characterized as having both an N-terminus (terminated by an amino acid with a free amino group (NH2)) and a C-terminus (terminated by an amino acid with a free carboxyl group (COOH)). Proteins of the invention are in some cases made up of multiple polypeptide chains brought together by disulfide bonds or by non-covalent forces (multimers, oligomers). These sorts of proteins will have multiple N- and C-termini. Alternatively, the termini of the polypeptides may be modified such that they begin or end, as the case may be, with a non-polypeptide based moiety such as an organic conjugate.

[000290] Once any of the features have been identified or defined as a desired component of a polypeptide to be encoded by the polynucleotide of the invention, any of several manipulations and/or modifications of these features may be performed by moving, swapping, inverting, deleting, randomizing or duplicating. Furthermore, it is understood that manipulation of features may result in the same outcome as a modification to the molecules of the invention. For example, a manipulation which involved deleting a domain would result in the alteration of the length of a molecule just as modification of a nucleic acid to encode less than a full length molecule would.

[000291] Modifications and manipulations can be accomplished by methods known in the art such as, but not limited to, site directed mutagenesis or *a priori* incorporation during chemical synthesis. The resulting modified molecules may then be tested for activity using *in vitro* or *in vivo* assays such as those described herein or any other suitable screening assay known in the art.

[000292] According to the present invention, the polypeptides may comprise a consensus sequence which is discovered through rounds of experimentation. As used herein a "consensus" sequence is a single sequence which represents a collective population of sequences allowing for variability at one or more sites.

[000293] As recognized by those skilled in the art, protein fragments, functional protein domains, and homologous proteins are also considered to be within the scope of polypeptides of interest of this invention. For example, provided herein is any protein fragment (meaning a polypeptide sequence at least one amino acid residue shorter than a reference polypeptide sequence but otherwise identical) of a reference protein 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or greater than 100 amino acids in length. In another example, any protein that includes a stretch of about 20, about 30, about 40, about 50, or about 100 amino acids which are about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, or about 100% identical to any of the sequences described herein can be utilized in accordance with the invention. In certain embodiments, a polypeptide to be utilized in accordance with the invention includes 2, 3, 4, 5, 6, 7, 8, 9, 10, or more mutations as shown in any of the sequences provided or referenced herein.

Types of Polypeptides of Interest

[000294] The polynucleotides of the present invention may be designed to encode polypeptides of interest selected from any of several target categories including, but not limited to, biologics, antibodies, vaccines, therapeutic proteins or peptides, cell penetrating peptides, secreted proteins, plasma membrane proteins, cytoplasmic or cytoskeletal proteins, intracellular membrane bound proteins, nuclear proteins, proteins associated with human disease, targeting moieties or those proteins encoded by the human genome for which no therapeutic indication has been identified but which nonetheless have utility in areas of research and discovery.

[000295] In one embodiment, polynucleotides may encode variant polypeptides which have a certain identity with a reference polypeptide sequence. As used herein, a "reference polypeptide sequence" refers to a starting polypeptide sequence. Reference sequences may be wild type sequences or any sequence to which reference is made in the design of another sequence. A

"reference polypeptide sequence" may, e.g., be any one of those polypeptides taught herein or associated with an ophthalmic disease, disorder, or natural signaling pathway.

[000296] Reference molecules (polypeptides or polynucleotides) may share a certain identity with the designed molecules (polypeptides or polynucleotides). The term "identity" as known in the art, refers to a relationship between the sequences of two or more peptides, polypeptides or polynucleotides, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between them as determined by the number of matches between strings of two or more amino acid residues or nucleosides. Identity measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (i.e., "algorithms"). Identity of related peptides can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo et al., SIAM J. Applied Math. 48, 1073 (1988).

[000297] In some embodiments, the encoded polypeptide variant may have the same or a similar activity as the reference polypeptide. Alternatively, the variant may have an altered activity (e.g., increased or decreased) relative to a reference polypeptide. Generally, variants of a particular polynucleotide or polypeptide of the invention will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% but less than 100% sequence identity to that particular reference polynucleotide or polypeptide as determined by sequence alignment programs and parameters described herein and known to those skilled in the art. Such tools for alignment include those of the BLAST suite (Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.) Other tools are described herein, specifically in the definition of "Identity."

[000298] Default parameters in the BLAST algorithm include, for example, an expect threshold of 10, Word size of 28, Match/Mismatch Scores 1, -2, Gap costs Linear. Any filter can be applied as well as a selection for species specific repeats, e.g., Homo sapiens.

Antibodies

[000299] The polynucleotides disclosed herein, may encode one or more antibodies or fragments thereof. The term "antibody" includes monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region), antibody compositions with polyepitopic specificity, multispecific antibodies (e.g., bispecific antibodies, diabodies, and single-chain molecules), as well as antibody fragments. The term "immunoglobulin" (Ig) is used interchangeably with "antibody" herein. As used herein, the term "monoclonal antibody" refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations and/or post-translation modifications (e.g., isomerizations, amidations) that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site.

[000300] The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is(are) identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity. Chimeric antibodies of interest herein include, but are not limited to, "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g., Old World Monkey, Ape etc.) and human constant region sequences.

[000301] An "antibody fragment" comprises a portion of an intact antibody, preferably the antigen binding and/or the variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$ and Fv fragments; diabodies; linear antibodies; nanobodies; single-chain antibody molecules and multispecific antibodies formed from antibody fragments.

[000302] Any of the five classes of immunoglobulins, IgA, IgD, IgE, IgG and IgM, shown in Figure 7, may be encoded by the polynucleotides of the invention, including the heavy chains

designated alpha, delta, epsilon, gamma and mu, respectively. Also included are polynucleotide sequences encoding the subclasses, gamma and mu. Hence any of the subclasses of antibodies may be encoded in part or in whole and include the following subclasses: IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2.

[000303] In one embodiment, the polynucleotides described herein may encode a human IgG construct as described in Figure 8A.

[000304] In one embodiment, the polynucleotides have a modular design to encode at least one of the antibodies, fragments or variants thereof described herein. As a non-limiting example, as shown in Figure 5A, the polynucleotide construct may encode any of the following designs: (1) the heavy chain of an antibody, (2) the light chain of an antibody, (3) the heavy and light chain of the antibody, (4) the heavy chain and light chain separated by a linker, (5) the VH1, CH1, CH2, CH3 domains, a linker and the light chain and (6) the VH1, CH1, CH2, CH3 domains, VL region, and the light chain. Any of these designs may also comprise optional linkers between any domain and/or region.

[000305] In one embodiment, the polynucleotides have a modular design and encode a polypeptide of interest such as, but not limited to, an antibody, fragment or variant thereof described herein. Shown in Figure 5B are the domains or regions of a standard antibody unit.

[000306] According to the present invention, one or more antibodies or fragments currently being marketed or in development may be encoded by the polynucleotides of the present invention. While not wishing to be bound by theory, it is believed that incorporation into the polynucleotides of the invention will result in improved therapeutic efficacy due at least in part to the specificity, purity and selectivity of the polynucleotide designs.

[000307] In one embodiment, polynucleotides disclosed herein may encode monoclonal antibodies and/or variants thereof. Variants of antibodies may also include, but are not limited to, substitutional variants, conservative amino acid substitution, insertional variants, deletional variants and/or covalent derivatives. In one embodiment, the polynucleotide or regions thereof disclosed herein may encode an immunoglobulin Fc region. In another embodiment, the polynucleotide may encode a variant immunoglobulin Fc region. As a non-limiting example, the polynucleotide may encode an antibody having a variant immunoglobulin Fc region as described in U.S. Pat. No. 8,217,147 herein incorporated by reference in its entirety.

Cell-Penetrating Polypeptides

[000308] The polynucleotides disclosed herein, may encode one or more cell-penetrating polypeptides. As used herein, "cell-penetrating polypeptide" or CPP refers to a polypeptide which may facilitate the cellular uptake of molecules. A cell-penetrating polypeptide of the present invention may contain one or more detectable labels. The polypeptides may be partially labeled or completely labeled throughout. The polynucleotides may encode the detectable label completely, partially or not at all. The cell-penetrating peptide may also include a signal sequence. As used herein, a "signal sequence" refers to a sequence of amino acid residues bound at the amino terminus of a nascent protein during protein translation. The signal sequence may be used to signal the secretion of the cell-penetrating polypeptide.

[000309] In one embodiment, the polynucleotides may also encode a fusion protein. The fusion protein may be created by operably linking a charged protein to a therapeutic protein. As used herein, "operably linked" refers to the therapeutic protein and the charged protein being connected in such a way to permit the expression of the complex when introduced into the cell. As used herein, "charged protein" refers to a protein that carries a positive, negative or overall neutral electrical charge. Preferably, the therapeutic protein may be covalently linked to the charged protein in the formation of the fusion protein. The ratio of surface charge to total or surface amino acids may be approximately 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 or 0.9.

[000310] The cell-penetrating polypeptide encoded by the polynucleotides may form a complex after being translated. The complex may comprise a charged protein linked, e.g. covalently linked, to the cell-penetrating polypeptide. "Therapeutic protein" refers to a protein that, when administered to a cell has a therapeutic, diagnostic, and/or prophylactic effect and/or elicits a desired biological and/or pharmacological effect.

[000311] In one embodiment, the cell-penetrating polypeptide may comprise a first domain and a second domain. The first domain may comprise a supercharged polypeptide. The second domain may comprise a protein-binding partner. As used herein, "protein-binding partner" includes, but is not limited to, antibodies and functional fragments thereof, scaffold proteins, or peptides. The cell-penetrating polypeptide may further comprise an intracellular binding partner for the protein-binding partner. The cell-penetrating polypeptide may be capable of being secreted from a cell where the polynucleotides may be introduced. The cell-penetrating polypeptide may also be capable of penetrating the first cell.

[000312] In a further embodiment, the cell-penetrating polypeptide is capable of penetrating a second cell. The second cell may be from the same area as the first cell, or it may be from a different area. The area may include, but is not limited to, tissues and organs. The second cell may also be proximal or distal to the first cell.

[000313] In one embodiment, the polynucleotides may encode a cell-penetrating polypeptide which may comprise a protein-binding partner. The protein binding partner may include, but is not limited to, an antibody, a supercharged antibody or a functional fragment. The polynucleotides may be introduced into the cell where a cell-penetrating polypeptide comprising the protein-binding partner is introduced.

S*ecreted proteins*

[000314] Human and other eukaryotic cells are subdivided by membranes into many functionally distinct compartments. Each membrane-bounded compartment, or organelle, contains different proteins essential for the function of the organelle. The cell uses "sorting signals," which are amino acid motifs located within the protein, to target proteins to particular cellular organelles.

[000315] One type of sorting signal, called a signal sequence, a signal peptide, or a leader sequence, directs a class of proteins to an organelle called the endoplasmic reticulum (ER).

[000316] Proteins targeted to the ER by a signal sequence can be released into the extracellular space as a secreted protein. Similarly, proteins residing on the cell membrane can also be secreted into the extracellular space by proteolytic cleavage of a "linker" holding the protein to the membrane. While not wishing to be bound by theory, the molecules of the present invention may be used to exploit the cellular trafficking described above. As such, in some embodiments of the invention, polynucleotides are provided to express a secreted protein. The secreted proteins may be selected from those described herein or those in US Patent Publication, 20100255574, the contents of which are incorporated herein by reference in their entirety.

[000317] In one embodiment, these may be used in the manufacture of large quantities of human gene products.

Plasma membrane proteins

[000318] In some embodiments of the invention, the polynucleotides are provided to express a protein of the plasma membrane.

Cytoplasmic or cytoskeletal proteins

[000319] In some embodiments of the invention, the polynucleotides are provided to express a cytoplasmic or cytoskeletal protein.

Intracellular membrane bound proteins

[000320] In some embodiments of the invention, the polynucleotides are provided to express an intracellular membrane bound protein.

Nuclear proteins

[000321] In some embodiments of the invention, the polynucleotides are provided to express a nuclear protein.

Targeting Moieties

[000322] In some embodiments of the invention, the polynucleotides are provided to express a targeting moiety. These include a protein-binding partner or a receptor on the surface of the cell, which functions to target the cell to a specific tissue space or to interact with a specific moiety, either *in vivo* or *in vitro*. Suitable protein-binding partners include, but are not limited to, antibodies and functional fragments thereof, scaffold proteins, or peptides. Additionally, polynucleotides can be employed to direct the synthesis and extracellular localization of lipids, carbohydrates, or other biological moieties or biomolecules.

Polynucleotide Regions

[000323] In some embodiments, polynucleotides may be designed to comprise regions, subregions or parts which function in a similar manner as known regions or parts of other nucleic acid based molecules. Such regions include those mRNA regions discussed herein as well as noncoding regions. Noncoding regions may be at the level of a single nucleoside such as the case when the region is or incorporates one or more cytotoxic nucleosides.

Polynucleotides having Untranslated Regions (UTRs)

[000324] The polynucleotides of the present invention may comprise one or more regions or parts which act or function as an untranslated region. Where polynucleotides are designed to encode at least one polypeptide of interest, the polynucleotides may comprise one or more of these untranslated regions.

[000325] By definition, wild type untranslated regions (UTRs) of a gene are transcribed but not translated. In mRNA, the 5′UTR starts at the transcription start site and continues to the start codon but does not include the start codon; whereas, the 3′UTR starts immediately following the stop codon and continues until the transcriptional termination signal. There is growing body of

evidence about the regulatory roles played by the UTRs in terms of stability of the nucleic acid molecule and translation. The regulatory features of a UTR can be incorporated into the polynucleotides of the present invention to, among other things, enhance the stability of the molecule. The specific features can also be incorporated to ensure controlled down-regulation of the transcript in case they are misdirected to undesired organs sites.

[000326] Tables 1 and 2 provide a listing of exemplary UTRs which may be utilized in the polynucleotides of the present invention. Shown in Table 1 is a listing of a 5′-untranslated region of the invention. Variants of 5' UTRs may be utilized wherein one or more nucleotides are added or removed to the termini, including A, T, C or G.

Table 1. 5′-Untranslated Regions

5′ UTR Identifier	Name/ Description	Sequence	SEQ ID NO.
5UTR-001	Upstream UTR	GGGAAATAAGAGAGAAAAGAAGAGTAAGAAG AAATATAAGAGCCACC	3
5UTR-002	Upstream UTR	GGGAGATCAGAGAGAAAAGAAGAGTAAGAAG AAATATAAGAGCCACC	4
5UTR-003	Upstream UTR	GGAATAAAAGTCTCAACACAACATATACAAAA CAAACGAATCTCAAGCAATCAAGCATTCTACT TCTATTGCAGCAATTTAAATCATTTCTTTTAAA GCAAAAGCAATTTTCTGAAAATTTTCACCATTT ACGAACGATAGCAAC	5
5UTR-004	Upstream UTR	GGGAGACAAGCUUGGCAUUCCGGUACUGUUG GUAAAGCCACC	6
5UTR-005	Upstream UTR	GGGAGATCAGAGAGAAAAGAAGAGTAAGAAG AAATATAAGAGCCACC	7
5UTR-006	Upstream UTR	GGAATAAAAGTCTCAACACAACATATACAAAA CAAACGAATCTCAAGCAATCAAGCATTCTACT TCTATTGCAGCAATTTAAATCATTTCTTTTAAA GCAAAAGCAATTTTCTGAAAATTTTCACCATTT ACGAACGATAGCAAC	8
5UTR-007	Upstream UTR	GGGAGACAAGCUUGGCAUUCCGGUACUGUUG GUAAAGCCACC	9
5UTR-008	Upstream UTR	GGGAATTAACAGAGAAAAGAAGAGTAAGAAG AAATATAAGAGCCACC	10
5UTR-009	Upstream UTR	GGGAAATTAGACAGAAAAGAAGAGTAAGAAG AAATATAAGAGCCACC	11
5UTR-010	Upstream UTR	GGGAAATAAGAGAGTAAAGAACAGTAAGAAG AAATATAAGAGCCACC	12
5UTR-011	Upstream UTR	GGGAAAAAAGAGAGAAAAGAAGACTAAGAAG AAATATAAGAGCCACC	13
5UTR-012	Upstream UTR	GGGAAATAAGAGAGAAAAGAAGAGTAAGAAG ATATATAAGAGCCACC	14
5UTR-013	Upstream UTR	GGGAAATAAGAGACAAAACAAGAGTAAGAAG AAATATAAGAGCCACC	15
5UTR-014	Upstream UTR	GGGAAATTAGAGAGTAAAGAACAGTAAGTAG AATTAAAAGAGCCACC	16

5UTR-015	Upstream UTR	GGGAAATAAGAGAGAATAGAAGAGTAAGAAG AAATATAAGAGCCACC	17
5UTR-016	Upstream UTR	GGGAAATAAGAGAGAAAAGAAGAGTAAGAAG AAAATTAAGAGCCACC	18
5UTR-017	Upstream UTR	GGGAAATAAGAGAGAAAAGAAGAGTAAGAAG AAATTTAAGAGCCACC	19

[000327] Shown in Table 2 is a listing of 3′-untranslated regions of the invention. Variants of 3' UTRs may be utilized wherein one or more nucleotides are added or removed to the termini, including A, T, C or G.

Table 2. 3′-Untranslated Regions

3′ UTR Identifier	Name/ Description	Sequence	SEQ ID NO.
3UTR-001	Creatine Kinase	GCGCCTGCCCACCTGCCACCGACTGCTGGAACCCAGC CAGTGGGAGGGCCTGGCCCACCAGAGTCCTGCTCCCT CACTCCTCGCCCCGCCCCCTGTCCCAGAGTCCCACCTG GGGGCTCTCTCCACCCTTCTCAGAGTTCCAGTTTCAAC CAGAGTTCCAACCAATGGGCTCCATCCTCTGGATTCTG GCCAATGAAATATCTCCCTGGCAGGGTCCTCTTCTTTT CCCAGAGCTCCACCCCAACCAGGAGCTCTAGTTAATG GAGAGCTCCCAGCACACTCGGAGCTTGTGCTTTGTCTC CACGCAAAGCGATAAATAAAAGCATTGGTGGCCTTTG GTCTTTGAATAAAGCCTGAGTAGGAAGTCTAGA	20
3UTR-002	Myoglobin	GCCCCTGCCGCTCCCACCCCCACCCATCTGGGCCCCGG GTTCAAGAGAGAGCGGGGTCTGATCTCGTGTAGCCAT ATAGAGTTTGCTTCTGAGTGTCTGCTTTGTTTAGTAGA GGTGGGCAGGAGGAGCTGAGGGGCTGGGGCTGGGGT GTTGAAGTTGGCTTTGCATGCCCAGCGATGCGCCTCCC TGTGGGATGTCATCACCCTGGGAACCGGGAGTGGCCC TTGGCTCACTGTGTTCTGCATGGTTTGGATCTGAATTA ATTGTCCTTTCTTCTAAATCCCAACCGAACTTCTTCCA ACCTCCAAACTGGCTGTAACCCCAAATCCAAGCCATT AACTACACCTGACAGTAGCAATTGTCTGATTAATCACT GGCCCCTTGAAGACAGCAGAATGTCCCTTTGCAATGA GGAGGAGATCTGGGCTGGGCGGGCCAGCTGGGGAAG CATTTGACTATCTGGAACTTGTGTGTGCCTCCTCAGGT ATGGCAGTGACTCACCTGGTTTTAATAAAACAACCTG CAACATCTCATGGTCTTTGAATAAAGCCTGAGTAGGA AGTCTAGA	21
3UTR-003	α-actin	ACACACTCCACCTCCAGCACGCGACTTCTCAGGACGA CGAATCTTCTCAATGGGGGGGCGGCTGAGCTCCAGCC ACCCCGCAGTCACTTTCTTTGTAACAACTTCCGTTGCT GCCATCGTAAACTGACACAGTGTTTATAACGTGTACAT ACATTAACTTATTACCTCATTTTGTTATTTTTCGAAACA AAGCCCTGTGGAAGAAAATGGAAAACTTGAAGAAGC ATTAAAGTCATTCTGTTAAGCTGCGTAAATGGTCTTTG AATAAAGCCTGAGTAGGAAGTCTAGA	22
3UTR-004	Albumin	CATCACATTTAAAAGCATCTCAGCCTACCATGAGAAT AAGAGAAAGAAAATGAAGATCAAAAGCTTATTCATCT	23

		GTTTTTCTTTTTCGTTGGTGTAAAGCCAACACCCTGTCT AAAAAACATAAATTTCTTTAATCATTTTGCCTCTTTTCT CTGTGCTTCAATTAATAAAAAATGGAAAGAATCTAAT AGAGTGGTACAGCACTGTTATTTTTCAAAGATGTGTTG CTATCCTGAAAATTCTGTAGGTTCTGTGGAAGTTCCAG TGTTCTCTCTTATTCCACTTCGGTAGAGGATTTCTAGTT TCTTGTGGGCTAATTAAATAAATCATTAATACTCTTCT AATGGTCTTTGAATAAAGCCTGAGTAGGAAGTCTAGA	
3UTR-005	α-globin	GCTGCCTTCTGCGGGGCTTGCCTTCTGGCCATGCCCTT CTTCTCTCCCTTGCACCTGTACCTCTTGGTCTTTGAATA AAGCCTGAGTAGGAAGGCGGCCGCTCGAGCATGCATC TAGA	24
3UTR-006	G-CSF	GCCAAGCCCTCCCCATCCCATGTATTTATCTCTATTTA ATATTTATGTCTATTTAAGCCTCATATTTAAAGACAGG GAAGAGCAGAACGGAGCCCCAGGCCTCTGTGTCCTTC CCTGCATTTCTGAGTTTCATTCTCCTGCCTGTAGCAGT GAGAAAAAGCTCCTGTCCTCCCATCCCCTGGACTGGG AGGTAGATAGGTAAATACCAAGTATTTATTACTATGA CTGCTCCCCAGCCCTGGCTCTGCAATGGGCACTGGGAT GAGCCGCTGTGAGCCCCTGGTCCTGAGGGTCCCCACC TGGGACCCTTGAGAGTATCAGGTCTCCCACGTGGGAG ACAAGAAATCCCTGTTTAATATTTAAACAGCAGTGTTC CCCATCTGGGTCCTTGCACCCCTCACTCTGGCCTCAGC CGACTGCACAGCGGCCCCTGCATCCCCTTGGCTGTGA GGCCCCTGGACAAGCAGAGGTGGCCAGAGCTGGGAG GCATGGCCCTGGGGTCCCACGAATTTGCTGGGGAATC TCGTTTTTCTTCTTAAGACTTTTGGGACATGGTTTGACT CCCGAACATCACCGACGCGTCTCCTGTTTTTCTGGGTG GCCTCGGGACACCTGCCCTGCCCCCACGAGGGTCAGG ACTGTGACTCTTTTTAGGGCCAGGCAGGTGCCTGGAC ATTTGCCTTGCTGGACGGGGACTGGGGATGTGGGAGG GAGCAGACAGGAGGAATCATGTCAGGCCTGTGTGTGA AAGGAAGCTCCACTGTCACCCTCCACCTCTTCACCCCC CACTCACCAGTGTCCCCTCCACTGTCACATTGTAACTG AACTTCAGGATAATAAAGTGTTTGCCTCCATGGTCTTT GAATAAAGCCTGAGTAGGAAGGCGGCCGCTCGAGCAT GCATCTAGA	25
3UTR-007	Col1a2; collagen, type I, alpha 2	ACTCAATCTAAATTAAAAAAGAAAGAAATTTGAAAAA ACTTTCTCTTTGCCATTTCTTCTTCTTCTTTTTTAACTGA AAGCTGAATCCTTCCATTTCTTCTGCACATCTACTTGC TTAAATTGTGGGCAAAAGAGAAAAAGAAGGATTGATC AGAGCATTGTGCAATACAGTTTCATTAACTCCTTCCCC CGCTCCCCCAAAAATTTGAATTTTTTTTTCAACACTCTT ACACCTGTTATGGAAAATGTCAACCTTTGTAAGAAAA CCAAAATAAAAATTGAAAAATAAAAACCATAAACATT TGCACCACTTGTGGCTTTTGAATATCTTCCACAGAGGG AAGTTTAAAACCCAAACTTCCAAAGGTTTAAACTACC TCAAAACACTTTCCCATGAGTGTGATCCACATTGTTAG GTGCTGACCTAGACAGAGATGAACTGAGGTCCTTGTT TTGTTTTGTTCATAATACAAAGGTGCTAATTAATAGTA TTTCAGATACTTGAAGAATGTTGATGGTGCTAGAAGA ATTTGAGAAGAAATACTCCTGTATTGAGTTGTATCGTG TGGTGTATTTTTTAAAAAATTTGATTTAGCATTCATAT TTTCCATCTTATTCCCAATTAAAAGTATGCAGATTATT TGCCCAAATCTTCTTCAGATTCAGCATTTGTTCTTTGCC	26

		AGTCTCATTTTCATCTTCTTCCATGGTTCCACAGAAGC TTTGTTTCTTGGGCAAGCAGAAAAATTAAATTGTACCT ATTTTGTATATGTGAGATGTTTAAATAAATTGTGAAAA AAATGAAATAAAGCATGTTTGGTTTTCCAAAAGAACA TAT	
3UTR-008	Col6a2; collagen, type VI, alpha 2	CGCCGCCGCCCGGGCCCCGCAGTCGAGGGTCGTGAGC CCACCCCGTCCATGGTGCTAAGCGGGCCCGGGTCCCA CACGGCCAGCACCGCTGCTCACTCGGACGACGCCCTG GGCCTGCACCTCTCCAGCTCCTCCCACGGGGTCCCCGT AGCCCCGGCCCCCGCCCAGCCCCAGGTCTCCCCAGGC CCTCCGCAGGCTGCCCGGCCTCCCTCCCCCTGCAGCCA TCCCAAGGCTCCTGACCTACCTGGCCCCTGAGCTCTGG AGCAAGCCCTGACCCAATAAAGGCTTTGAACCCAT	27
3UTR-009	RPN1; ribophorin I	GGGGCTAGAGCCCTCTCCGCACAGCGTGGAGACGGGG CAAGGAGGGGGGTTATTAGGATTGGTGGTTTTGTTTTG CTTTGTTTAAAGCCGTGGGAAAATGGCACAACTTTACC TCTGTGGGAGATGCAACACTGAGAGCCAAGGGGTGGG AGTTGGGATAATTTTTATATAAAAGAAGTTTTTCCACT TTGAATTGCTAAAAGTGGCATTTTTCCTATGTGCAGTC ACTCCTCTCATTTCTAAAATAGGGACGTGGCCAGGCA CGGTGGCTCATGCCTGTAATCCCAGCACTTTGGGAGG CCGAGGCAGGCGGCTCACGAGGTCAGGAGATCGAGA CTATCCTGGCTAACACGGTAAAACCCTGTCTCTACTAA AAGTACAAAAAATTAGCTGGGCGTGGTGGTGGGCACC TGTAGTCCCAGCTACTCGGGAGGCTGAGGCAGGAGAA AGGCATGAATCCAAGAGGCAGAGCTTGCAGTGAGCTG AGATCACGCCATTGCACTCCAGCCTGGGCAACAGTGT TAAGACTCTGTCTCAAATATAAATAAATAAATAAATA AATAAATAAATAAATAAAAATAAAGCGAGATGTTGCC CTCAAA	28
3UTR-010	LRP1; low density lipoprotein receptor-related protein 1	GGCCCTGCCCCGTCGGACTGCCCCCAGAAAGCCTCCT GCCCCCTGCCAGTGAAGTCCTTCAGTGAGCCCCTCCCC AGCCAGCCCTTCCCTGGCCCCGCCGGATGTATAAATGT AAAAATGAAGGAATTACATTTTATATGTGAGCGAGCA AGCCGGCAAGCGAGCACAGTATTATTTCTCCATCCCCT CCCTGCCTGCTCCTTGGCACCCCCATGCTGCCTTCAGG GAGACAGGCAGGGAGGGCTTGGGGCTGCACCTCCTAC CCTCCCACCAGAACGCACCCCACTGGGAGAGCTGGTG GTGCAGCCTTCCCCTCCCTGTATAAGACACTTTGCCAA GGCTCTCCCCTCTCGCCCCATCCCTGCTTGCCCGCTCC CACAGCTTCCTGAGGGCTAATTCTGGGAAGGGAGAGT TCTTTGCTGCCCCTGTCTGGAAGACGTGGCTCTGGGTG AGGTAGGCGGGAAAGGATGGAGTGTTTTAGTTCTTGG GGGAGGCCACCCCAAACCCCAGCCCCAACTCCAGGGG CACCTATGAGATGGCCATGCTCAACCCCCCTCCCAGA CAGGCCCTCCCTGTCTCCAGGGCCCCCACCGAGGTTCC CAGGGCTGGAGACTTCCTCTGGTAAACATTCCTCCAGC CTCCCCTCCCCTGGGGACGCCAAGGAGGTGGGCCACA CCCAGGAAGGGAAAGCGGGCAGCCCCGTTTTGGGGAC GTGAACGTTTTAATAATTTTTGCTGAATTCCTTTACAA CTAAATAACACAGATATTGTTATAAATAAAATTGT	29
3UTR-011	Nnt1; cardiotrophin-like	ATATTAAGGATCAAGCTGTTAGCTAATAATGCCACCTC TGCAGTTTTGGGAACAGGCAAATAAAGTATCAGTATA CATGGTGATGTACATCTGTAGCAAAGCTCTTGGAGAA AATGAAGACTGAAGAAAGCAAAGCAAAAACTGTATA	30

	cytokine factor 1	GAGAGATTTTTCAAAAGCAGTAATCCCTCAATTTTAAA AAAGGATTGAAAATTCTAAATGTCTTTCTGTGCATATT TTTTGTGTTAGGAATCAAAAGTATTTTATAAAAGGAG AAAGAACAGCCTCATTTTAGATGTAGTCCTGTTGGATT TTTTATGCCTCCTCAGTAACCAGAAATGTTTTAAAAAA CTAAGTGTTTAGGATTTCAAGACAACATTATACATGGC TCTGAAATATCTGACACAATGTAAACATTGCAGGCAC CTGCATTTTATGTTTTTTTTTTCAACAAATGTGACTAAT TTGAAACTTTTATGAACTTCTGAGCTGTCCCCTTGCAA TTCAACCGCAGTTTGAATTAATCATATCAAATCAGTTT TAATTTTTTAAATTGTACTTCAGAGTCTATATTTCAAG GGCACATTTTCTCACTACTATTTTAATACATTAAAGGA CTAAATAATCTTTCAGAGATGCTGGAAACAAATCATTT GCTTTATATGTTTCATTAGAATACCAATGAAACATACA ACTTGAAAATTAGTAATAGTATTTTTGAAGATCCCATT TCTAATTGGAGATCTCTTTAATTTCGATCAACTTATAA TGTGTAGTACTATATTAAGTGCACTTGAGTGGAATTCA ACATTTGACTAATAAAATGAGTTCATCATGTTGGCAA GTGATGTGGCAATTATCTCTGGTGACAAAAGAGTAAA ATCAAATATTTCTGCCTGTTACAAATATCAAGGAAGA CCTGCTACTATGAAATAGATGACATTAATCTGTCTTCA CTGTTTATAATACGGATGGATTTTTTTTCAAATCAGTG TGTGTTTTGAGGTCTTATGTAATTGATGACATTTGAGA GAAATGGTGGCTTTTTTTAGCTACCTCTTTGTTCATTTA AGCACCAGTAAAGATCATGTCTTTTTATAGAAGTGTA GATTTTCTTTGTGACTTTGCTATCGTGCCTAAAGCTCT AAATATAGGTGAATGTGTGATGAATACTCAGATTATTT GTCTCTCTATATAATTAGTTTGGTACTAAGTTTCTCAA AAAATTATTAACACATGAAAGACAATCTCTAAACCAG AAAAAGAAGTAGTACAAATTTTGTTACTGTAATGCTC GCGTTTAGTGAGTTTAAAACACACAGTATCTTTTGGTT TTATAATCAGTTTCTATTTTGCTGTGCCTGAGATTAAG ATCTGTGTATGTGTGTGTGTGTGTGTGTGCGTTTGTGT GTTAAAGCAGAAAAGACTTTTTTAAAAGTTTTAAGTG ATAAATGCAATTTGTTAATTGATCTTAGATCACTAGTA AACTCAGGGCTGAATTATACCATGTATATTCTATTAGA AGAAAGTAAACACCATCTTTATTCCTGCCCTTTTTCTT CTCTCAAAGTAGTTGTAGTTATATCTAGAAAGAAGCA ATTTTGATTTCTTGAAAAGGTAGTTCCTGCACTCAGTT TAAACTAAAAATAATCATACTTGGATTTTATTTATTTT TGTCATAGTAAAAATTTTAATTTATATATATTTTTATTT AGTATTATCTTATTCTTTGCTATTTGCCAATCCTTTGTC ATCAATTGTGTTAAATGAATTGAAAATTCATGCCCTGT TCATTTTATTTTACTTTATTGGTTAGGATATTTAAAGG ATTTTTGTATATATAATTTCTTAAATTAATATTCCAAA AGGTTAGTGGACTTAGATTATAAATTATGGCAAAAAT CTAAAAACAACAAAAATGATTTTTATACATTCTATTTC ATTATTCCTCTTTTTCCAATAAGTCATACAATTGGTAG ATATGACTTATTTTATTTTTGTATTATTCACTATATCTT TATGATATTTAAGTATAAATAATTAAAAAAATTTATTG TACCTTATAGTCTGTCACCAAAAAAAAAAAATTATCT GTAGGTAGTGAAATGCTAATGTTGATTTGTCTTTAAGG GCTTGTTAACTATCCTTTATTTTCTCATTTGTCTTAAAT TAGGAGTTTGTGTTTAAATTACTCATCTAAGCAAAAAA TGTATATAAATCCCATTACTGGGTATATACCCAAAGG	

		ATTATAAATCATGCTGCTATAAAGACACATGCACACG TATGTTTATTGCAGCACTATTCACAATAGCAAAGACTT GGAACCAACCCAAATGTCCATCAATGATAGACTTGAT TAAGAAAATGTGCACATATACACCATGGAATACTATG CAGCCATAAAAAAGGATGAGTTCATGTCCTTTGTAGG GACATGGATAAAGCTGGAAACCATCATTCTGAGCAAA CTATTGCAAGGACAGAAAACCAAACACTGCATGTTCT CACTCATAGGTGGGAATTGAACAATGAGAACACTTGG ACACAAGGTGGGGAACACCACACACCAGGGCCTGTCA TGGGGTGGGGGGAGTGGGGAGGGATAGCATTAGGAG ATATACCTAATGTAAATGATGAGTTAATGGGTGCAGC ACACCAACATGGCACATGTATACATATGTAGCAAACC TGCACGTTGTGCACATGTACCCTAGAACTTAAAGTATA ATTAAAAAAAAAAAGAAAACAGAAGCTATTTATAAA GAAGTTATTTGCTGAAATAAATGTGATCTTTCCCATTA AAAAAATAAAGAAATTTTGGGGTAAAAAAACACAAT ATATTGTATTCTTGAAAAATTCTAAGAGAGTGGATGTG AAGTGTTCTCACCACAAAAGTGATAACTAATTGAGGT AATGCACATATTAATTAGAAAGATTTTGTCATTCCACA ATGTATATATACTTAAAAATATGTTATACACAATAAAT ACATACATTAAAAAATAAGTAAATGTA	
3UTR-012	Col6a1; collagen, type VI, alpha 1	CCCACCCTGCACGCCGGCACCAAACCCTGTCCTCCCAC CCCTCCCCACTCATCACTAAACAGAGTAAAATGTGAT GCGAATTTTCCCGACCAACCTGATTCGCTAGATTTTTT TTAAGGAAAAGCTTGGAAAGCCAGGACACAACGCTGC TGCCTGCTTTGTGCAGGGTCCTCCGGGGCTCAGCCCTG AGTTGGCATCACCTGCGCAGGGCCCTCTGGGGCTCAG CCCTGAGCTAGTGTCACCTGCACAGGGCCCTCTGAGG CTCAGCCCTGAGCTGGCGTCACCTGTGCAGGGCCCTCT GGGGCTCAGCCCTGAGCTGGCCTCACCTGGGTTCCCC ACCCCGGGCTCTCCTGCCCTGCCCTCCTGCCCGCCCTC CCTCCTGCCTGCGCAGCTCCTTCCCTAGGCACCTCTGT GCTGCATCCCACCAGCCTGAGCAAGACGCCCTCTCGG GGCCTGTGCCGCACTAGCCTCCCTCTCCTCTGTCCCCA TAGCTGGTTTTTCCCACCAATCCTCACCTAACAGTTAC TTTACAATTAAACTCAAAGCAAGCTCTTCTCCTCAGCT TGGGGCAGCCATTGGCCTCTGTCTCGTTTTGGGAAACC AAGGTCAGGAGGCCGTTGCAGACATAAATCTCGGCGA CTCGGCCCCGTCTCCTGAGGGTCCTGCTGGTGACCGGC CTGGACCTTGGCCCTACAGCCCTGGAGGCCGCTGCTG ACCAGCACTGACCCCGACCTCAGAGAGTACTCGCAGG GGCGCTGGCTGCACTCAAGACCCTCGAGATTAACGGT GCTAACCCCGTCTGCTCCTCCCTCCCGCAGAGACTGGG GCCTGGACTGGACATGAGAGCCCCTTGGTGCCACAGA GGGCTGTGTCTTACTAGAAACAACGCAAACCTCTCCTT CCTCAGAATAGTGATGTGTTCGACGTTTTATCAAAGGC CCCCTTTCTATGTTCATGTTAGTTTTGCTCCTTCTGTGT TTTTTTCTGAACCATATCCATGTTGCTGACTTTTCCAAA TAAAGGTTTTCACTCCTCTC	31
3UTR-013	Calr; calreticulin	AGAGGCCTGCCTCCAGGGCTGGACTGAGGCCTGAGCG CTCCTGCCGCAGAGCTGGCCGCGCCAAATAATGTCTCT GTGAGACTCGAGAACTTTCATTTTTTTCCAGGCTGGTT CGGATTTGGGGTGGATTTTGGTTTTGTTCCCCTCCTCC ACTCTCCCCCACCCCCTCCCCGCCCTTTTTTTTTTTTTT TTTTAAACTGGTATTTTATCTTTGATTCTCCTTCAGCCC	32

		TCACCCCTGGTTCTCATCTTTCTTGATCAACATCTTTTC TTGCCTCTGTCCCCTTCTCTCATCTCTTAGCTCCCCTCC AACCTGGGGGGCAGTGGTGTGGAGAAGCCACAGGCCT GAGATTTCATCTGCTCTCCTTCCTGGAGCCCAGAGGAG GGCAGCAGAAGGGGGTGGTGTCTCCAACCCCCCAGCA CTGAGGAAGAACGGGGCTCTTCTCATTTCACCCCTCCC TTTCTCCCCTGCCCCCAGGACTGGGCCACTTCTGGGTG GGGCAGTGGGTCCCAGATTGGCTCACACTGAGAATGT AAGAACTACAAACAAAATTTCTATTAAATTAAATTTTG TGTCTCC	
3UTR-014	Col1a1; collagen, type I, alpha 1	CTCCCTCCATCCCAACCTGGCTCCCTCCCACCCAACCA ACTTTCCCCCCAACCCGGAAACAGACAAGCAACCCAA ACTGAACCCCCTCAAAAGCCAAAAAATGGGAGACAAT TTCACATGGACTTTGGAAAATATTTTTTTCCTTTGCATT CATCTCTCAAACTTAGTTTTTATCTTTGACCAACCGAA CATGACCAAAAACCAAAAGTGCATTCAACCTTACCAA AAAAAAAAAAAAAAAAAAGAATAAATAAATAACTTTTT AAAAAAGGAAGCTTGGTCCACTTGCTTGAAGACCCAT GCGGGGGTAAGTCCCTTTCTGCCCGTTGGGCTTATGAA ACCCCAATGCTGCCCTTTCTGCTCCTTTCTCCACACCC CCCTTGGGGCCTCCCCTCCACTCCTTCCCAAATCTGTC TCCCCAGAAGACACAGGAAACAATGTATTGTCTGCCC AGCAATCAAAGGCAATGCTCAAACACCCAAGTGGCCC CCACCCTCAGCCCGCTCCTGCCCGCCCAGCACCCCCAG GCCCTGGGGGACCTGGGGTTCTCAGACTGCCAAAGAA GCCTTGCCATCTGGCGCTCCCATGGCTCTTGCAACATC TCCCCTTCGTTTTTGAGGGGGTCATGCCGGGGGAGCCA CCAGCCCCTCACTGGGTTCGGAGGAGAGTCAGGAAGG GCCACGACAAAGCAGAAACATCGGATTTGGGGAACGC GTGTCAATCCCTTGTGCCGCAGGGCTGGGCGGGAGAG ACTGTTCTGTTCCTTGTGTAACTGTGTTGCTGAAAGAC TACCTCGTTCTTGTCTTGATGTGTCACCGGGGCAACTG CCTGGGGGCGGGGATGGGGGCAGGGTGGAAGCGGCT CCCCATTTTATACCAAAGGTGCTACATCTATGTGATGG GTGGGGTGGGGAGGGAATCACTGGTGCTATAGAAATT GAGATGCCCCCCCAGGCCAGCAAATGTTCCTTTTTGTT CAAAGTCTATTTTTATTCCTTGATATTTTTCTTTTTTTTT TTTTTTTTTTGTGGATGGGGACTTGTGAATTTTTCTAAA GGTGCTATTTAACATGGGAGGAGAGCGTGTGCGGCTC CAGCCCAGCCCGCTGCTCACTTTCCACCCTCTCTCCAC CTGCCTCTGGCTTCTCAGGCCTCTGCTCTCCGACCTCT CTCCTCTGAAACCCTCCTCCACAGCTGCAGCCCATCCT CCCGGCTCCCTCCTAGTCTGTCCTGCGTCCTCTGTCCC CGGGTTTCAGAGACAACTTCCCAAAGCACAAAGCAGT TTTTCCCCCTAGGGGTGGGAGGAAGCAAAAGACTCTG TACCTATTTTGTATGTGTATAATAATTTGAGATGTTTTT AATTATTTTGATTGCTGGAATAAAGCATGTGGAAATG ACCCAAACATAATCCGCAGTGGCCTCCTAATTTCCTTC TTTGGAGTTGGGGGAGGGGTAGACATGGGGAAGGGG CTTTGGGGTGATGGGCTTGCCTTCCATTCCTGCCCTTT CCCTCCCCACTATTCTCTTCTAGATCCCTCCATAACCC CACTCCCCTTTCTCTCACCCTTCTTATACCGCAAACCTT TCTACTTCCTCTTTCATTTTCTATTCTTGCAATTTCCTT GCACCTTTTCCAAATCCTCTTCTCCCCTGCAATACCAT ACAGGCAATCCACGTGCACAACACACACACACACTCT	33

		TCACATCTGGGGTTGTCCAAACCTCATACCCACTCCCC TTCAAGCCCATCCACTCTCCACCCCCTGGATGCCCTGC ACTTGGTGGCGGTGGGATGCTCATGGATACTGGGAGG GTGAGGGGAGTGGAACCCGTGAGGAGGACCTGGGGG CCTCTCCTTGAACTGACATGAAGGGTCATCTGGCCTCT GCTCCCTTCTCACCCACGCTGACCTCCTGCCGAAGGAG CAACGCAACAGGAGAGGGGTCTGCTGAGCCTGGCGAG GGTCTGGGAGGGACCAGGAGGAAGGCGTGCTCCCTGC TCGCTGTCCTGGCCCTGGGGGAGTGAGGGAGACAGAC ACCTGGGAGAGCTGTGGGGAAGGCACTCGCACCGTGC TCTTGGGAAGGAAGGAGACCTGGCCCTGCTCACCACG GACTGGGTGCCTCGACCTCCTGAATCCCCAGAACACA ACCCCCCTGGGCTGGGGTGGTCTGGGGAACCATCGTG CCCCCGCCTCCCGCCTACTCCTTTTTAAGCTT	
3UTR-015	Plod1; procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1	TTGGCCAGGCCTGACCCTCTTGGACCTTTCTTCTTTGC CGACAACCACTGCCCAGCAGCCTCTGGGACCTCGGGG TCCCAGGGAACCCAGTCCAGCCTCCTGGCTGTTGACTT CCCATTGCTCTTGGAGCCACCAATCAAAGAGATTCAA AGAGATTCCTGCAGGCCAGAGGCGGAACACACCTTTA TGGCTGGGGCTCTCCGTGGTGTTCTGGACCCAGCCCCT GGAGACACCATTCACTTTTACTGCTTTGTAGTGACTCG TGCTCTCCAACCTGTCTTCCTGAAAAACCAAGGCCCCC TTCCCCCACCTCTTCCATGGGGTGAGACTTGAGCAGAA CAGGGGCTTCCCCAAGTTGCCCAGAAAGACTGTCTGG GTGAGAAGCCATGGCCAGAGCTTCTCCCAGGCACAGG TGTTGCACCAGGGACTTCTGCTTCAAGTTTTGGGGTAA AGACACCTGGATCAGACTCCAAGGGCTGCCCTGAGTC TGGGACTTCTGCCTCCATGGCTGGTCATGAGAGCAAA CCGTAGTCCCCTGGAGACAGCGACTCCAGAGAACCTC TTGGGAGACAGAAGAGGCATCTGTGCACAGCTCGATC TTCTACTTGCCTGTGGGGAGGGGAGTGACAGGTCCAC ACACCACACTGGGTCACCCTGTCCTGGATGCCTCTGAA GAGAGGGACAGACCGTCAGAAACTGGAGAGTTTCTAT TAAAGGTCATTTAAACCA	34
3UTR-016	Nucb1; nucleobindin 1	TCCTCCGGGACCCCAGCCCTCAGGATTCCTGATGCTCC AAGGCGACTGATGGGCGCTGGATGAAGTGGCACAGTC AGCTTCCCTGGGGGCTGGTGTCATGTTGGGCTCCTGGG GCGGGGGCACGGCCTGGCATTTCACGCATTGCTGCCA CCCCAGGTCCACCTGTCTCCACTTTCACAGCCTCCAAG TCTGTGGCTCTTCCCTTCTGTCCTCCGAGGGGCTTGCC TTCTCTCGTGTCCAGTGAGGTGCTCAGTGATCGGCTTA ACTTAGAGAAGCCCGCCCCCTCCCCTTCTCCGTCTGTC CCAAGAGGGTCTGCTCTGAGCCTGCGTTCCTAGGTGG CTCGGCCTCAGCTGCCTGGGTTGTGGCCGCCCTAGCAT CCTGTATGCCCACAGCTACTGGAATCCCCGCTGCTGCT CCGGGCCAAGCTTCTGGTTGATTAATGAGGGCATGGG GTGGTCCCTCAAGACCTTCCCCTACCTTTTGTGGAACC AGTGATGCCTCAAAGACAGTGTCCCCTCCACAGCTGG GTGCCAGGGGCAGGGGATCCTCAGTATAGCCGGTGAA CCCTGATACCAGGAGCCTGGGCCTCCCTGAACCCCTG GCTTCCAGCCATCTCATCGCCAGCCTCCTCCTGGACCT CTTGGCCCCCAGCCCCTTCCCCACACAGCCCCAGAAG GGTCCCAGAGCTGACCCCACTCCAGGACCTAGGCCCA GCCCCTCAGCCTCATCTGGAGCCCCTGAAGACCAGTC CCACCCACCTTTCTGGCCTCATCTGACACTGCTCCGCA	35

		TCCTGCTGTGTGTCCTGTTCCATGTTCCGGTTCCATCCA AATACACTTTCTGGAACAAA	
3UTR-017	α-globin	GCTGGAGCCTCGGTGGCCATGCTTCTTGCCCCTTGGGC CTCCCCCCAGCCCCTCCTCCCCTTCCTGCACCCGTACC CCCGTGGTCTTTGAATAAAGTCTGAGTGGGCGGC	36

5′ UTR and Translation Initiation

[000328] Natural 5′UTRs bear features which play roles in translation initiation. They harbor signatures like Kozak sequences which are commonly known to be involved in the process by which the ribosome initiates translation of many genes. Kozak sequences have the consensus CCR(A/G)CCAUGG, where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG), which is followed by another 'G'. 5′UTR also have been known to form secondary structures which are involved in elongation factor binding.

[000329] By engineering the features typically found in abundantly expressed genes of specific target organs, one can enhance the stability and protein production of the polynucleotides of the invention. For example, introduction of 5′ UTR of liver-expressed mRNA, such as albumin, serum amyloid A, Apolipoprotein A/B/E, transferrin, alpha fetoprotein, erythropoietin, or Factor VIII, could be used to enhance expression of a nucleic acid molecule, such as a polynucleotides, in hepatic cell lines or liver. Likewise, use of 5′ UTR from other tissue-specific mRNA to improve expression in that tissue is possible for muscle (MyoD, Myosin, Myoglobin, Myogenin, Herculin), for endothelial cells (Tie-1, CD36), for myeloid cells (C/EBP, AML1, G-CSF, GM-CSF, CD11b, MSR, Fr-1, i-NOS), for leukocytes (CD45, CD18), for adipose tissue (CD36, GLUT4, ACRP30, adiponectin) and for lung epithelial cells (SP-A/B/C/D). Untranslated regions useful in the design and manufacture of polynucleotides include, but are not limited, to those disclosed in co-pending, co-owned International Patent Publication No. WO2014164253 (Attorney Docket Number M42.20), the contents of each of which are incorporated herein by reference in its entirety.

[000330] Other non-UTR sequences may also be used as regions or subregions within the polynucleotides. For example, introns or portions of introns sequences may be incorporated into regions of the polynucleotides of the invention. Incorporation of intronic sequences may increase protein production as well as polynucletoide levels.

[000331] Combinations of features may be included in flanking regions and may be contained within other features. For example, the ORF may be flanked by a 5' UTR which may contain a

strong Kozak translational initiation signal and/or a 3' UTR which may include an oligo(dT) sequence for templated addition of a poly-A tail. 5'UTR may comprise a first polynucleotide fragment and a second polynucleotide fragment from the same and/or different genes such as the 5'UTRs described in US Patent Application Publication No. 20100293625, herein incorporated by reference in its entirety.

[000332] Co-pending, co-owned International Patent Publication No. WO2014164253 (Attorney Docket Number M42.20) provides a listing of exemplary UTRs which may be utilized in the polynucleotide of the present invention as flanking regions. Variants of 5' or 3' UTRs may be utilized wherein one or more nucleotides are added or removed to the termini, including A, T, C or G.

[000333] It should be understood that any UTR from any gene may be incorporated into the regions of the polynucleotide. Furthermore, multiple wild-type UTRs of any known gene may be utilized. It is also within the scope of the present invention to provide artificial UTRs which are not variants of wild type regions. These UTRs or portions thereof may be placed in the same orientation as in the transcript from which they were selected or may be altered in orientation or location. Hence a 5′ or 3′ UTR may be inverted, shortened, lengthened, made with one or more other 5′ UTRs or 3′ UTRs. As used herein, the term "altered" as it relates to a UTR sequence, means that the UTR has been changed in some way in relation to a reference sequence. For example, a 3′ or 5′ UTR may be altered relative to a wild type or native UTR by the change in orientation or location as taught above or may be altered by the inclusion of additional nucleotides, deletion of nucleotides, swapping or transposition of nucleotides. Any of these changes producing an "altered" UTR (whether 3′ or 5′) comprise a variant UTR.

[000334] In one embodiment, a double, triple or quadruple UTR such as a 5′ or 3′ UTR may be used. As used herein, a "double" UTR is one in which two copies of the same UTR are encoded either in series or substantially in series. For example, a double beta-globin 3′ UTR may be used as described in US Patent publication 20100129877, the contents of which are incorporated herein by reference in its entirety.

[000335] It is also within the scope of the present invention to have patterned UTRs. As used herein "patterned UTRs" are those UTRs which reflect a repeating or alternating pattern, such as ABABAB or AABBAABBAABB or ABCABCABC or variants thereof repeated once, twice, or

more than 3 times. In these patterns, each letter, A, B, or C represent a different UTR at the nucleotide level.

[000336] In one embodiment, flanking regions are selected from a family of transcripts whose proteins share a common function, structure, feature of property. For example, polypeptides of interest may belong to a family of proteins which are expressed in a particular cell, tissue or at some time during development. The UTRs from any of these genes may be swapped for any other UTR of the same or different family of proteins to create a new polynucleotide. As used herein, a "family of proteins" is used in the broadest sense to refer to a group of two or more polypeptides of interest which share at least one function, structure, feature, localization, origin, or expression pattern.

[000337] In one embodiment, flanking regions may be heterologous.

[000338] In one embodiment, the 5' untranslated region may be derived from a different species than the 3' untranslated region.

[000339] The untranslated region may also include translation enhancer elements (TEE). As a non-limiting example, the TEE may include those described in US Application No. 20090226470, herein incorporated by reference in its entirety, and those known in the art.

5'UTR and Histone Stem Loops

[000340] In one embodiment, the polynucleotides may include a nucleic acid sequence which is derived from the 5'UTR of a 5'-terminal oligopyrimidine (TOP) gene and at least one histone stem loop. Non-limiting examples of nucleic acid sequences which are derived from the 5'UTR of a TOP gene are taught in International Patent Publication No. WO2013143699, the contents of which are herein incorporated by reference in its entirety.

3' UTR and the AU Rich Elements

[000341] Natural or wild type 3' UTRs are known to have stretches of Adenosines and Uridines embedded in them. These AU rich signatures are particularly prevalent in genes with high rates of turnover. Based on their sequence features and functional properties, the AU rich elements (AREs) can be separated into three classes (Chen et al, 1995): Class I AREs contain several dispersed copies of an AUUUA motif within U-rich regions. C-Myc and MyoD contain class I AREs. Class II AREs possess two or more overlapping UUAUUUA(U/A)(U/A) nonamers. Molecules containing this type of AREs include GM-CSF and TNF-a. Class III ARES are less well defined. These U rich regions do not contain an AUUUA motif. c-Jun and Myogenin are

two well-studied examples of this class. Most proteins binding to the AREs are known to destabilize the messenger, whereas members of the ELAV family, most notably HuR, have been documented to increase the stability of mRNA. HuR binds to AREs of all the three classes. Engineering the HuR specific binding sites into the 3′ UTR of nucleic acid molecules will lead to HuR binding and thus, stabilization of the message *in vivo*.

[000342] Introduction, removal or modification of 3′ UTR AU rich elements (AREs) can be used to modulate the stability of polynucleotides of the invention. When engineering specific polynucleotides, one or more copies of an ARE can be introduced to make polynucleotides of the invention less stable and thereby curtail translation and decrease production of the resultant protein. Likewise, AREs can be identified and removed or mutated to increase the intracellular stability and thus increase translation and production of the resultant protein. Transfection experiments can be conducted in relevant cell lines, using polynucleotides of the invention and protein production can be assayed at various time points post-transfection. For example, cells can be transfected with different ARE-engineering molecules and by using an ELISA kit to the relevant protein and assaying protein produced at 6 hour, 12 hour, 24 hour, 48 hour, and 7 days post-transfection.

microRNA Binding Sites

[000343] microRNAs (or miRNA) are 19-25 nucleotide long noncoding RNAs that bind to the 3′UTR of nucleic acid molecules and down-regulate gene expression either by reducing nucleic acid molecule stability or by inhibiting translation. The polynucleotides of the invention may comprise one or more microRNA target sequences, microRNA sequences, or microRNA seeds. Such sequences may correspond to any known microRNA such as those taught in US Publication US2005/0261218 and US Publication US2005/0059005, the contents of which are incorporated herein by reference in their entirety.

[000344] A microRNA sequence comprises a “seed” region, i.e., a sequence in the region of positions 2-8 of the mature microRNA, which sequence has perfect Watson-Crick complementarity to the miRNA target sequence. A microRNA seed may comprise positions 2-8 or 2-7 of the mature microRNA. In some embodiments, a microRNA seed may comprise 7 nucleotides (e.g., nucleotides 2-8 of the mature microRNA), wherein the seed-complementary site in the corresponding miRNA target is flanked by an adenine (A) opposed to microRNA position 1. In some embodiments, a microRNA seed may comprise 6 nucleotides (e.g.,

nucleotides 2-7 of the mature microRNA), wherein the seed-complementary site in the corresponding miRNA target is flanked by an adenine (A) opposed to microRNA position 1. See for example, Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP; Mol Cell. 2007 Jul 6;27(1):91-105; each of which is herein incorporated by reference in their entirety. The bases of the microRNA seed have complete complementarity with the target sequence. By engineering microRNA target sequences into the polynucleotides (e.g., in a 3'UTR like region or other region) of the invention one can target the molecule for degradation or reduced translation, provided the microRNA in question is available. This process will reduce the hazard of off target effects upon nucleic acid molecule delivery. Identification of microRNA, microRNA target regions, and their expression patterns and role in biology have been reported (Bonauer et al., Curr Drug Targets 2010 11:943-949; Anand and Cheresh Curr Opin Hematol 2011 18:171-176; Contreras and Rao Leukemia 2012 26:404-413 (2011 Dec 20. doi: 10.1038/leu.2011.356); Bartel Cell 2009 136:215-233; Landgraf et al, Cell, 2007 129:1401-1414; each of which is herein incorporated by reference in its entirety).

[000345] For example, if the nucleic acid molecule is an mRNA and is not intended to be delivered to the liver but ends up there, then miR-122, a microRNA abundant in liver, can inhibit the expression of the gene of interest if one or multiple target sites of miR-122 are engineered into the 3′ UTR region of the polynucleotides. Introduction of one or multiple binding sites for different microRNA can be engineered to further decrease the longevity, stability, and protein translation of polynucleotides.

[000346] As used herein, the term "microRNA site" refers to a microRNA target site or a microRNA recognition site, or any nucleotide sequence to which a microRNA binds or associates. It should be understood that "binding" may follow traditional Watson-Crick hybridization rules or may reflect any stable association of the microRNA with the target sequence at or adjacent to the microRNA site.

[000347] Conversely, for the purposes of the polynucleotides of the present invention, microRNA binding sites can be engineered out of (i.e. removed from) sequences in which they occur, e.g., in order to increase protein expression in specific tissues. For example, miR-122 binding sites may be removed to improve protein expression in the liver. Regulation of expression in multiple tissues can be accomplished through introduction or removal or one or several microRNA binding sites.

[000348] Expression profiles, microRNA and cell lines useful in the present invention include those taught in for example, International Patent Publication Nos. WO2014081507 (Attorney Docket Number M39) and WO2014113089 (Attorney Docket Number M37), the contents of which are incorporated by reference in their entirety.

[000349] In the polynucleotides of the present invention, binding sites for microRNAs that are involved in such processes may be removed or introduced, in order to tailor the expression of the polynucleotides expression to biologically relevant cell types or to the context of relevant biological processes.

[000350] A listing of microRNA, and their binding sites are listed in Table 9 of U.S. Provisional Application No. 61/753,661 filed January 17, 2013, in Table 9 of U.S. Provisional Application No. 61/754,159 filed January 18, 2013, and in Table 7 of U.S. Provisional Application No. 61/758,921 filed January 31, 2013, each of which are herein incorporated by reference in their entireties.

[000351] Examples of use of microRNA to drive tissue or disease-specific gene expression are listed (Getner and Naldini, Tissue Antigens. 2012, 80:393-403; herein incorporated by reference in its entirety). In addition, microRNA seed sites can be incorporated into mRNA to decrease expression in certain cells which results in a biological improvement.

[000352] Lastly, through an understanding of the expression patterns of microRNA in different cell types, polynucleotides can be engineered for more targeted expression in specific cell types or only under specific biological conditions. Through introduction of tissue-specific microRNA binding sites, polynucleotides could be designed that would be optimal for protein expression in a tissue or in the context of a biological condition.

[000353] Transfection experiments can be conducted in relevant cell lines, using engineered polynucleotides and protein production can be assayed at various time points post-transfection. For example, cells can be transfected with different microRNA binding site-engineering polynucleotides and by using an ELISA kit to the relevant protein and assaying protein produced at 6 hour, 12 hour, 24 hour, 48 hour, 72 hour and 7 days post-transfection. *In vivo* experiments can also be conducted using microRNA-binding site-engineered molecules to examine changes in tissue-specific expression of formulated polynucleotides.

3'UTR and Albumin Variants

[000354] 3' UTRs of the polynucleotides described herein may comprise a nucleic acid sequence which is derived from the 3' UTR of an albumin gene or from a variant of the 3'UTR of the albumin gene. 3'UTRs and albumin variants are described in paragraphs [000256] – [000257] in International Publication No. WO2015038892, the contents of which are herein incorporated by reference in its entirety.

Regions having a 5′ Cap

[000355] The 5′ cap structure of a natural mRNA is involved in nuclear export, increasing mRNA stability and binds the mRNA Cap Binding Protein (CBP), which is responsible for mRNA stability in the cell and translation competency through the association of CBP with poly(A) binding protein to form the mature cyclic mRNA species. The cap further assists the removal of 5′ proximal introns removal during mRNA splicing.

[000356] Endogenous mRNA molecules may be 5′-end capped generating a 5′-ppp-5′-triphosphate linkage between a terminal guanosine cap residue and the 5′-terminal transcribed sense nucleotide of the mRNA molecule. This 5′-guanylate cap may then be methylated to generate an N7-methyl-guanylate residue. The ribose sugars of the terminal and/or anteterminal transcribed nucleotides of the 5′ end of the mRNA may optionally also be 2′-O-methylated. 5′-decapping through hydrolysis and cleavage of the guanylate cap structure may target a nucleic acid molecule, such as an mRNA molecule, for degradation.

[000357] In some embodiments, polynucleotides may be designed to incorporate a cap moiety. Modifications to the polynucleotides of the present invention may generate a non-hydrolyzable cap structure preventing decapping and thus increasing mRNA half-life. Because cap structure hydrolysis requires cleavage of 5′-ppp-5′ phosphorodiester linkages, modified nucleotides may be used during the capping reaction. For example, a Vaccinia Capping Enzyme from New England Biolabs (Ipswich, MA) may be used with α-thio-guanosine nucleotides according to the manufacturer's instructions to create a phosphorothioate linkage in the 5′-ppp-5′ cap. Additional modified guanosine nucleotides may be used such as α-methyl-phosphonate and seleno-phosphate nucleotides.

[000358] Additional modifications include, but are not limited to, 2′-O-methylation of the ribose sugars of 5′-terminal and/or 5′-anteterminal nucleotides of the polynucleotide (as mentioned above) on the 2′-hydroxyl group of the sugar ring. Multiple distinct 5′-cap structures

can be used to generate the 5′-cap of a nucleic acid molecule, such as a polynucleotide which functions as an mRNA molecule.

[000359] Cap analogs, which herein are also referred to as synthetic cap analogs, chemical caps, chemical cap analogs, or structural or functional cap analogs, differ from natural (i.e. endogenous, wild-type or physiological) 5′-caps in their chemical structure, while retaining cap function. Cap analogs may be chemically (i.e. non-enzymatically) or enzymatically synthesized and/or linked to the polynucleotides of the invention.

[000360] For example, the Anti-Reverse Cap Analog (ARCA) cap contains two guanines linked by a 5′-5′-triphosphate group, wherein one guanine contains an N7 methyl group as well as a 3′-O-methyl group (i.e., N7,3′-O-dimethyl-guanosine-5′-triphosphate-5′-guanosine (m^7G-3′mppp-G; which may equivalently be designated 3′ O-Me-m7G(5')ppp(5')G). The 3′-O atom of the other, unmodified, guanine becomes linked to the 5′-terminal nucleotide of the capped polynucleotide. The N7- and 3′-O-methlyated guanine provides the terminal moiety of the capped polynucleotide.

[000361] Another exemplary cap is mCAP, which is similar to ARCA but has a 2′-O-methyl group on guanosine (i.e., N7,2′-O-dimethyl-guanosine-5′-triphosphate-5′-guanosine, m^7Gm-ppp-G).

[000362] In one embodiment, the cap is a dinucleotide cap analog. As a non-limiting example, the dinucleotide cap analog may be modified at different phosphate positions with a boranophosphate group or a phophoroselenoate group such as the dinucleotide cap analogs described in US Patent No. US 8,519,110, the contents of which are herein incorporated by reference in its entirety.

[000363] In another embodiment, the cap is a cap analog is a N7-(4-chlorophenoxyethyl) substituted dicucleotide form of a cap analog known in the art and/or described herein. Non-limiting examples of a N7-(4-chlorophenoxyethyl) substituted dicucleotide form of a cap analog include a N7-(4-chlorophenoxyethyl)-G(5')ppp(5')G and a N7-(4-chlorophenoxyethyl)-$m^{3'-O}$G(5')ppp(5')G cap analog (See e.g., the various cap analogs and the methods of synthesizing cap analogs described in Kore et al. Bioorganic & Medicinal Chemistry 2013 21:4570-4574; the contents of which are herein incorporated by reference in its entirety). In another embodiment, a cap analog of the present invention is a 4-chloro/bromophenoxyethyl analog.

[000364] While cap analogs allow for the concomitant capping of a polynucleotide or a region thereof, in an in vitro transcription reaction, up to 20% of transcripts can remain uncapped. This, as well as the structural differences of a cap analog from an endogenous 5′-cap structures of nucleic acids produced by the endogenous, cellular transcription machinery, may lead to reduced translational competency and reduced cellular stability.

[000365] Polynucleotides of the invention may also be capped post-manufacture (whether IVT or chemical synthesis), using enzymes, in order to generate more authentic 5′-cap structures. As used herein, the phrase "more authentic" refers to a feature that closely mirrors or mimics, either structurally or functionally, an endogenous or wild type feature. That is, a "more authentic" feature is better representative of an endogenous, wild-type, natural or physiological cellular function and/or structure as compared to synthetic features or analogs, etc., of the prior art, or which outperforms the corresponding endogenous, wild-type, natural or physiological feature in one or more respects. Non-limiting examples of more authentic 5′cap structures of the present invention are those which, among other things, have enhanced binding of cap binding proteins, increased half-life, reduced susceptibility to 5′ endonucleases and/or reduced 5′decapping, as compared to synthetic 5′cap structures known in the art (or to a wild-type, natural or physiological 5′cap structure). For example, recombinant Vaccinia Virus Capping Enzyme and recombinant 2′-O-methyltransferase enzyme can create a canonical 5′-5′-triphosphate linkage between the 5′-terminal nucleotide of a polynucleotide and a guanine cap nucleotide wherein the cap guanine contains an N7 methylation and the 5′-terminal nucleotide of the mRNA contains a 2′-O-methyl. Such a structure is termed the Cap1 structure. This cap results in a higher translational-competency and cellular stability and a reduced activation of cellular pro-inflammatory cytokines, as compared, e.g., to other 5′cap analog structures known in the art. Cap structures include, but are not limited to, 7mG(5')ppp(5')N,pN2p (cap 0), 7mG(5')ppp(5')NlmpNp (cap 1), and 7mG(5')-ppp(5')NlmpN2mp (cap 2).

[000366] As a non-limiting example, capping chimeric polynucleotides post-manufacture may be more efficient as nearly 100% of the chimeric polynucleotides may be capped. This is in contrast to ~80% when a cap analog is linked to a chimeric polynucleotide in the course of an in vitro transcription reaction.

[000367] According to the present invention, 5′ terminal caps may include endogenous caps or cap analogs. According to the present invention, a 5′ terminal cap may comprise a guanine

analog. Useful guanine analogs include, but are not limited to, inosine, N1-methyl-guanosine, 2′fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine.

Viral Sequences

[000368] Additional viral sequences such as, but not limited to, the translation enhancer sequence of the barley yellow dwarf virus (BYDV-PAV), the Jaagsiekte sheep retrovirus (JSRV) and/or the Enzootic nasal tumor virus (See e.g., International Pub. No. WO2012129648; herein incorporated by reference in its entirety) can be engineered and inserted in the polynucleotides of the invention and can stimulate the translation of the construct *in vitro* and *in vivo*. Transfection experiments can be conducted in relevant cell lines at and protein production can be assayed by ELISA at 12hr, 24hr, 48hr, 72 hr and day 7 post-transfection.

IRES Sequences

[000369] Further, provided are polynucleotides which may contain an internal ribosome entry site (IRES). First identified as a feature Picorna virus RNA, IRES plays an important role in initiating protein synthesis in absence of the 5′ cap structure. An IRES may act as the sole ribosome binding site, or may serve as one of multiple ribosome binding sites of an mRNA. Polynucleotides containing more than one functional ribosome binding site may encode several peptides or polypeptides that are translated independently by the ribosomes ("multicistronic nucleic acid molecules"). When polynucleotides are provided with an IRES, further optionally provided is a second translatable region. Examples of IRES sequences that can be used according to the invention include without limitation, those from picornaviruses (e.g. FMDV), pest viruses (CFFV), polio viruses (PV), encephalomyocarditis viruses (ECMV), foot-and-mouth disease viruses (FMDV), hepatitis C viruses (HCV), classical swine fever viruses (CSFV), murine leukemia virus (MLV), simian immune deficiency viruses (SIV) or cricket paralysis viruses (CrPV).

Poly-A tails

[000370] During RNA processing, a long chain of adenine nucleotides (poly-A tail) may be added to a polynucleotide such as an mRNA molecule in order to increase stability. Immediately after transcription, the 3' end of the transcript may be cleaved to free a 3' hydroxyl. Then poly-A polymerase adds a chain of adenine nucleotides to the RNA. The process, called polyadenylation, adds a poly-A tail that can be between, for example, approximately 80 to

approximately 250 residues long (SEQ ID NO: 98), including approximately 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240 or 250 residues long.

[000371] PolyA tails may also be added after the construct is exported from the nucleus.

[000372] According to the present invention, terminal groups on the poly A tail may be incorporated for stabilization. Polynucleotides of the present invention may include des-3' hydroxyl tails. They may also include structural moieties or 2'-Omethyl modifications as taught by Junjie Li, et al. (Current Biology, Vol. 15, 1501–1507, August 23, 2005, the contents of which are incorporated herein by reference in its entirety).

[000373] The polynucleotides of the present invention may be designed to encode transcripts with alternative polyA tail structures including histone mRNA. According to Norbury, "Terminal uridylation has also been detected on human replication-dependent histone mRNAs. The turnover of these mRNAs is thought to be important for the prevention of potentially toxic histone accumulation following the completion or inhibition of chromosomal DNA replication. These mRNAs are distinguished by their lack of a 3′ poly(A) tail, the function of which is instead assumed by a stable stem–loop structure and its cognate stem–loop binding protein (SLBP); the latter carries out the same functions as those of PABP on polyadenylated mRNAs" (Norbury, "Cytoplasmic RNA: a case of the tail wagging the dog," Nature Reviews Molecular Cell Biology; AOP, published online 29 August 2013; doi:10.1038/nrm3645) the contents of which are incorporated herein by reference in its entirety.

[000374] Unique poly-A tail lengths provide certain advantages to the polynucleotides of the present invention.

[000375] Generally, the length of a poly-A tail, when present, is greater than 30 nucleotides in length. In another embodiment, the poly-A tail is greater than 35 nucleotides in length (e.g., at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000 nucleotides). In some embodiments, the polynucleotide or region thereof includes from about 30 to about 3,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 750, from 30 to 1,000, from 30 to 1,500, from 30 to 2,000, from 30 to 2,500, from 50 to 100, from 50 to 250, from 50 to 500, from 50 to 750, from 50 to 1,000, from 50 to 1,500, from 50 to 2,000, from 50 to 2,500, from 50 to 3,000, from 100 to 500, from 100 to 750, from 100 to 1,000, from 100 to 1,500, from 100 to

2,000, from 100 to 2,500, from 100 to 3,000, from 500 to 750, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 2,500, from 500 to 3,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 2,500, from 1,000 to 3,000, from 1,500 to 2,000, from 1,500 to 2,500, from 1,500 to 3,000, from 2,000 to 3,000, from 2,000 to 2,500, and from 2,500 to 3,000) (SEQ ID NO: 99).

[000376] In one embodiment, the poly-A tail is designed relative to the length of the overall polynucleotide or the length of a particular region of the polynucleotide. This design may be based on the length of a coding region, the length of a particular feature or region or based on the length of the ultimate product expressed from the polynucleotides.

[000377] In this context the poly-A tail may be 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100% greater in length than the polynucleotide or feature thereof. The poly-A tail may also be designed as a fraction of the polynucleotides to which it belongs. In this context, the poly-A tail may be 10, 20, 30, 40, 50, 60, 70, 80, or 90% or more of the total length of the construct, a construct region or the total length of the construct minus the poly-A tail. Further, engineered binding sites and conjugation of polynucleotides for Poly-A binding protein may enhance expression.

[000378] Additionally, multiple distinct polynucleotides may be linked together via the PABP (Poly-A binding protein) through the 3′-end using modified nucleotides at the 3′-terminus of the poly-A tail. Transfection experiments can be conducted in relevant cell lines at and protein production can be assayed by ELISA at 12hr, 24hr, 48hr, 72 hr and day 7 post-transfection.

[000379] In one embodiment, the polynucleotides of the present invention are designed to include a polyA-G Quartet region. The G-quartet is a cyclic hydrogen bonded array of four guanine nucleotides that can be formed by G-rich sequences in both DNA and RNA. In this embodiment, the G-quartet is incorporated at the end of the poly-A tail. The resultant polynucleotide is assayed for stability, protein production and other parameters including half-life at various time points. It has been discovered that the polyA-G quartet results in protein production from an mRNA equivalent to at least 75% of that seen using a poly-A tail of 120 nucleotides alone (SEQ ID NO: 100).

Start codon region

[000380] In some embodiments, the polynucleotides of the present invention may have regions that are analogous to or function like a start codon region.

[000381] In one embodiment, the translation of a polynucleotide may initiate on a codon which is not the start codon AUG. Translation of the polynucleotide may initiate on an alternative start codon such as, but not limited to, ACG, AGG, AAG, CTG/CUG, GTG/GUG, ATA/AUA, ATT/AUU, TTG/UUG (see Touriol et al. Biology of the Cell 95 (2003) 169-178 and Matsuda and Mauro PLoS ONE, 2010 5:11; the contents of each of which are herein incorporated by reference in its entirety). As a non-limiting example, the translation of a polynucleotide begins on the alternative start codon ACG. As another non-limiting example, polynucleotide translation begins on the alternative start codon CTG or CUG. As yet another non-limiting example, the translation of a polynucleotide begins on the alternative start codon GTG or GUG.

[000382] Nucleotides flanking a codon that initiates translation such as, but not limited to, a start codon or an alternative start codon, are known to affect the translation efficiency, the length and/or the structure of the polynucleotide. (See e.g., Matsuda and Mauro PLoS ONE, 2010 5:11; the contents of which are herein incorporated by reference in its entirety). Masking any of the nucleotides flanking a codon that initiates translation may be used to alter the position of translation initiation, translation efficiency, length and/or structure of a polynucleotide.

[000383] In one embodiment, a masking agent may be used near the start codon or alternative start codon in order to mask or hide the codon to reduce the probability of translation initiation at the masked start codon or alternative start codon. Non-limiting examples of masking agents include antisense locked nucleic acids (LNA) polynucleotides and exon-junction complexes (EJCs) (See e.g., Matsuda and Mauro describing masking agents LNA polynucleotides and EJCs (PLoS ONE, 2010 5:11); the contents of which are herein incorporated by reference in its entirety).

[000384] In another embodiment, a masking agent may be used to mask a start codon of a polynucleotide in order to increase the likelihood that translation will initiate on an alternative start codon.

[000385] In one embodiment, a masking agent may be used to mask a first start codon or alternative start codon in order to increase the chance that translation will initiate on a start codon or alternative start codon downstream to the masked start codon or alternative start codon.

[000386] In one embodiment, a start codon or alternative start codon may be located within a perfect complement for a miR binding site. The perfect complement of a miR binding site may help control the translation, length and/or structure of the polynucleotide similar to a masking

agent. As a non-limiting example, the start codon or alternative start codon may be located in the middle of a perfect complement for a miR-122 binding site. The start codon or alternative start codon may be located after the first nucleotide, second nucleotide, third nucleotide, fourth nucleotide, fifth nucleotide, sixth nucleotide, seventh nucleotide, eighth nucleotide, ninth nucleotide, tenth nucleotide, eleventh nucleotide, twelfth nucleotide, thirteenth nucleotide, fourteenth nucleotide, fifteenth nucleotide, sixteenth nucleotide, seventeenth nucleotide, eighteenth nucleotide, nineteenth nucleotide, twentieth nucleotide or twenty-first nucleotide.

[000387] In another embodiment, the start codon of a polynucleotide may be removed from the polynucleotide sequence in order to have the translation of the polynucleotide begin on a codon which is not the start codon. Translation of the polynucleotide may begin on the codon following the removed start codon or on a downstream start codon or an alternative start codon. In a non-limiting example, the start codon ATG or AUG is removed as the first 3 nucleotides of the polynucleotide sequence in order to have translation initiate on a downstream start codon or alternative start codon. The polynucleotide sequence where the start codon was removed may further comprise at least one masking agent for the downstream start codon and/or alternative start codons in order to control or attempt to control the initiation of translation, the length of the polynucleotide and/or the structure of the polynucleotide.

Stop Codon Region

[000388] In one embodiment, the polynucleotides of the present invention may include at least two stop codons before the 3' untranslated region (UTR). The stop codon may be selected from TGA, TAA and TAG. In one embodiment, the polynucleotides of the present invention include the stop codon TGA and one additional stop codon. In a further embodiment the addition stop codon may be TAA. In another embodiment, the polynucleotides of the present invention include three stop codons.

Signal Sequences

[000389] The polynucleotides may also encode additional features which facilitate trafficking of the polypeptides to therapeutically relevant sites. One such feature which aids in protein trafficking is the signal sequence. As used herein, a "signal sequence" or "signal peptide" is a polynucleotide or polypeptide, respectively, which is from about 9 to 200 nucleotides (3-60 amino acids) in length which is incorporated at the 5′ (or N-terminus) of the coding region or polypeptide encoded, respectively. Addition of these sequences result in trafficking of the

encoded polypeptide to the endoplasmic reticulum through one or more secretory pathways. Some signal peptides are cleaved from the protein by signal peptidase after the proteins are transported.

[000390] Additional signal sequences which may be utilized in the present invention include those taught in, for example, databases such as those found at www.signalpeptide.de/ or proline.bic.nus.edu.sg/spdb/. Those described in US Patents 8,124,379; 7,413,875 and 7,385,034 are also within the scope of the invention and the contents of each are incorporated herein by reference in their entirety.

Ophthalmic Target Selection

[000391] According to the present invention, the polynucleotides may comprise at least a first region of linked nucleosides encoding at least one polypeptide of interest. Non limiting examples of polypeptides of interest or "Targets" of the present invention include but are not limited to, retinal pigment epithelium-specific protein, adrenoceptor alpha 2A, amyloid beta (A4) precursor protein, complement component 3, complement component 5, complement factor D (adipsin), thrombospondin receptor, complement component 5 receptor 1, HIF1A , nerve growth factor receptor, STAT3, VEGFA, PDGFR, VEGFR1/2, plasminogen, tyrosine kinase, mTOR, Factor III, cadherin, chemokine receptor (3/4), integrin A5, placental growth factor, protein tyrosine phosphatase, S1PR1, vRaf, TGF-beta, HtrA serine peptidase 1, TNF receptor 10A , NOTCH4, insulin-like growth factor-binding protein 7, Ras responsive element binding protein 1, component factor H, component factor B, complement component 3, complement component 2, complement factor I, hepatic lipase, cholesteryl ester transfer protein, translocase of outer mitochondrial membrane 40, superoxide dismutase 2, mitochondrial, tenascin XB, collagen type X, alpha 1, myelin basic protein, collagen type VIII, alpha 1, bestrophin 1, carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6, retinitis pimentosa GTPases, guanylate cyclase system (2D, A1A), calcium channels (A2, LA1F), peripherin 2, cadherin 1, choroideremia (Rab escort protein 1), guanylate cyclase 2D, peripherin 2, mitochondrial encoded ATP synthase, mitochondrial encoded cytochromes, mitochondrial encoded NADH dehydrogenase, mitofusin 2, optic atrophy 1, three prime repair exonuclease 1, three prime repair exonuclease 1. DICER1, HIF-PHD, Hey 1, dominant negative CCR3, anti-Eotaxin mAb, Dcr1, Sema3E, VEGF-trap, PDGF-trap Nitrin1R, aA, aB Crystallin, Hey 2, any of the siruins including SIRT1, DR4-Fc,

DR5-Fc, PD1R, RhoJ, sFLT-1, IGFR I-Fc, IGFBP7, PEDF, NPPB, CD59, PLEKHA1, RPE65, ABCA4, and/or PDE.

[000392] Polynucleotides encoding these targets are useful in the treatment of ophthalmic diseases or disorders including, but not limited to diabetic retinopathy, dry eye, cataracts, retinal vein occlusion, macular edema, macular degeneration (wet & dry), refraction and accommodation disorders, keratoconus, amblyopia, glaucoma, Sstargardt disease, endophthalmitis, conjunctivitis, uveitis, retinal detachment, corneal ulcers, dacryocystitis, Duane retraction syndrome, and optic neuritis.

Protein Cleavage Signals and Sites

[000393] In one embodiment, the polypeptides of the present invention may include at least one protein cleavage signal containing at least one protein cleavage site. The protein cleavage site may be located at the N-terminus, the C-terminus, at any space between the N- and the C-termini such as, but not limited to, half-way between the N- and C-termini, between the N-terminus and the half way point, between the half way point and the C-terminus, and combinations thereof.

[000394] The polypeptides of the present invention may include, but is not limited to, a proprotein convertase (or prohormone convertase), thrombin or Factor Xa protein cleavage signal. Proprotein convertases are a family of nine proteinases, comprising seven basic amino acid-specific subtilisin-like serine proteinases related to yeast kexin, known as prohormone convertase 1/3 (PC1/3), PC2, furin, PC4, PC5/6, paired basic amino-acid cleaving enzyme 4 (PACE4) and PC7, and two other subtilases that cleave at non-basic residues, called subtilisin kexin isozyme 1 (SKI-1) and proprotein convertase subtilisin kexin 9 (PCSK9).

[000395] In one embodiment, the polynucleotides of the present invention may be engineered such that the polynucleotide contains at least one encoded protein cleavage signal. The encoded protein cleavage signal may be located in any region including but not limited to before the start codon, after the start codon, before the coding region, within the coding region such as, but not limited to, half way in the coding region, between the start codon and the half way point, between the half way point and the stop codon, after the coding region, before the stop codon, between two stop codons, after the stop codon and combinations thereof.

[000396] In one embodiment, the polynucleotides of the present invention may include at least one encoded protein cleavage signal containing at least one protein cleavage site. The encoded

protein cleavage signal may include, but is not limited to, a proprotein convertase (or prohormone convertase), thrombin and/or Factor Xa protein cleavage signal.

[000397] As a non-limiting example, U.S. Pat. No. 7,374,930 and U.S. Pub. No. 20090227660, herein incorporated by reference in their entireties, use a furin cleavage site to cleave the N-terminal methionine of GLP-1 in the expression product from the Golgi apparatus of the cells. In one embodiment, the polypeptides of the present invention include at least one protein cleavage signal and/or site with the proviso that the polypeptide is not GLP-1.

Insertions and Substitutions

[000398] In one embodiment, the 5'UTR of the polynucleotide may be replaced by the insertion of at least one region and/or string of nucleosides of the same base. The region and/or string of nucleotides may include, but is not limited to, at least 3, at least 4, at least 5, at least 6, at least 7 or at least 8 nucleotides and the nucleotides may be natural and/or unnatural. As a non-limiting example, the group of nucleotides may include 5-8 adenine, cytosine, thymine, a string of any of the other nucleotides disclosed herein and/or combinations thereof.

[000399] In one embodiment, the 5'UTR of the polynucleotide may be replaced by the insertion of at least two regions and/or strings of nucleotides of two different bases such as, but not limited to, adenine, cytosine, thymine, any of the other nucleotides disclosed herein and/or combinations thereof. For example, the 5'UTR may be replaced by inserting 5-8 adenine bases followed by the insertion of 5-8 cytosine bases. In another example, the 5'UTR may be replaced by inserting 5-8 cytosine bases followed by the insertion of 5-8 adenine bases.

[000400] In one embodiment, the polynucleotide may include at least one substitution and/or insertion downstream of the transcription start site which may be recognized by an RNA polymerase. As a non-limiting example, at least one substitution and/or insertion may occur downstream the transcription start site by substituting at least one nucleic acid in the region just downstream of the transcription start site (such as, but not limited to, +1 to +6). Changes to region of nucleotides just downstream of the transcription start site may affect initiation rates, increase apparent nucleotide triphosphate (NTP) reaction constant values, and increase the dissociation of short transcripts from the transcription complex curing initial transcription (Brieba et al, Biochemistry (2002) 41: 5144-5149; herein incorporated by reference in its entirety). The modification, substitution and/or insertion of at least one nucleoside may cause a silent mutation of the sequence or may cause a mutation in the amino acid sequence.

[000401] In one embodiment, the polynucleotide may include the substitution of 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 or at least 13 guanine bases downstream of the transcription start site.

[000402] In one embodiment, the polynucleotide may include the substitution of at least 1, at least 2, at least 3, at least 4, at least 5 or at least 6 guanine bases in the region just downstream of the transcription start site. As a non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases may be substituted by at least 1, at least 2, at least 3 or at least 4 adenine nucleotides. In another non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases may be substituted by at least 1, at least 2, at least 3 or at least 4 cytosine bases. In another non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases may be substituted by at least 1, at least 2, at least 3 or at least 4 thymine, and/or any of the nucleotides described herein.

[000403] In one embodiment, the polynucleotide may include at least one substitution and/or insertion upstream of the start codon. For the purpose of clarity, one of skill in the art would appreciate that the start codon is the first codon of the protein coding region whereas the transcription start site is the site where transcription begins. The polynucleotide may include, but is not limited to, at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7 or at least 8 substitutions and/or insertions of nucleotide bases. The nucleotide bases may be inserted or substituted at 1, at least 1, at least 2, at least 3, at least 4 or at least 5 locations upstream of the start codon. The nucleotides inserted and/or substituted may be the same base (e.g., all A or all C or all T or all G), two different bases (e.g., A and C, A and T, or C and T), three different bases (e.g., A, C and T or A, C and T) or at least four different bases. As a non-limiting example, the guanine base upstream of the coding region in the polynucleotide may be substituted with adenine, cytosine, thymine, or any of the nucleotides described herein. In another non-limiting example the substitution of guanine bases in the polynucleotide may be designed so as to leave one guanine base in the region downstream of the transcription start site and before the start codon (see Esvelt *et al.* Nature (2011) 472(7344):499-503; the contents of which is herein incorporated by reference in its entirety). As a non-limiting example, at least 5 nucleotides may be inserted at 1 location downstream of the transcription start site but upstream of the start codon and the at least 5 nucleotides may be the same base type.

Incorporating Post Transcriptional Control Modulators

[000404] In one embodiment, the polynucleotides of the present invention may include at least one post transcriptional control modulator. These post transcriptional control modulators may be, but are not limited to, small molecules, compounds and regulatory sequences. As a non-limiting example, post transcriptional control may be achieved using small molecules identified by PTC Therapeutics Inc. (South Plainfield, NJ) using their GEMS™ (Gene Expression Modulation by Small-Molecules) screening technology.

[000405] The post transcriptional control modulator may be a gene expression modulator which is screened by the method detailed in or a gene expression modulator described in International Publication No. WO2006022712, herein incorporated by reference in its entirety. Methods identifying RNA regulatory sequences involved in translational control are described in International Publication No. WO2004067728, herein incorporated by reference in its entirety; methods identifying compounds that modulate untranslated region dependent expression of a gene are described in International Publication No. WO2004065561, herein incorporated by reference in its entirety.

[000406] In one embodiment, the polynucleotides of the present invention may include at least one post transcriptional control modulator is located in the 5' and/or the 3' untranslated region of the polynucleotides of the present invention.

[000407] In another embodiment, the polynucleotides of the present invention may include at least one post transcription control modulator to modulate premature translation termination. The post transcription control modulators may be compounds described in or a compound found by methods outlined in International Publication Nso. WO2004010106, WO2006044456, WO2006044682, WO2006044503 and WO2006044505, each of which is herein incorporated by reference in its entirety. As a non-limiting example, the compound may bind to a region of the 28S ribosomal RNA in order to modulate premature translation termination (See e.g., WO2004010106, herein incorporated by reference in its entirety).

[000408] In one embodiment, polynucleotides of the present invention may include at least one post transcription control modulator to alter protein expression. As a non-limiting example, the expression of VEGF may be regulated using the compounds described in or a compound found by the methods described in International Publication Nos. WO2005118857, WO2006065480, WO2006065479 and WO2006058088, each of which is herein incorporated by reference in its entirety.

[000409] The polynucleotides of the present invention may include at least one post transcription control modulator to control translation. In one embodiment, the post transcription control modulator may be a RNA regulatory sequence. As a non-limiting example, the RNA regulatory sequence may be identified by the methods described in International Publication No. WO2006071903, herein incorporated by reference in its entirety.

II. Design, Synthesis and Quantitation of Polynucleotides

Design-Codon Optimization

[000410] The polynucleotides, their regions or parts or subregions may be codon optimized. Codon optimization methods are known in the art and may be useful in efforts to achieve one or more of several goals. These goals include to match codon frequencies in target and host organisms to ensure proper folding, bias GC content to increase mRNA stability or reduce secondary structures, minimize tandem repeat codons or base runs that may impair gene construction or expression, customize transcriptional and translational control regions, insert or remove protein trafficking sequences, remove/add post translation modification sites in encoded protein (e.g. glycosylation sites), add, remove or shuffle protein domains, insert or delete restriction sites, modify ribosome binding sites and mRNA degradation sites, to adjust translational rates to allow the various domains of the protein to fold properly, or to reduce or eliminate problem secondary structures within the polynucleotide. Codon optimization tools, algorithms and services are known in the art, non-limiting examples include services from GeneArt (Life Technologies), DNA2.0 (Menlo Park CA) and/or proprietary methods. In one embodiment, the ORF sequence is optimized using optimization algorithms. Codon options for each amino acid are given in Table 3.

Table 3. Codon Options

Amino Acid	Single Letter Code	Codon Options
Isoleucine	I	ATT, ATC, ATA
Leucine	L	CTT, CTC, CTA, CTG, TTA, TTG
Valine	V	GTT, GTC, GTA, GTG
Phenylalanine	F	TTT, TTC
Methionine	M	ATG
Cysteine	C	TGT, TGC
Alanine	A	GCT, GCC, GCA, GCG
Glycine	G	GGT, GGC, GGA, GGG
Proline	P	CCT, CCC, CCA, CCG
Threonine	T	ACT, ACC, ACA, ACG
Serine	S	TCT, TCC, TCA, TCG, AGT, AGC

Tyrosine	Y	TAT, TAC
Tryptophan	W	TGG
Glutamine	Q	CAA, CAG
Asparagine	N	AAT, AAC
Histidine	H	CAT, CAC
Glutamic acid	E	GAA, GAG
Aspartic acid	D	GAT, GAC
Lysine	K	AAA, AAG
Arginine	R	CGT, CGC, CGA, CGG, AGA, AGG
Selenocysteine	Sec	UGA in mRNA in presence of Selenocystein insertion element (SECIS)
Stop codons	Stop	TAA, TAG, TGA

[000411] Features, which may be considered beneficial in some embodiments of the present invention, may be encoded by regions of the polynucleotide and such regions may be upstream (5') or downstream (3') to a region which encodes a polypeptide. These regions may be incorporated into the polynucleotide before and/or after codon optimization of the protein encoding region or open reading frame (ORF). It is not required that a polynucleotide contain both a 5′ and 3′ flanking region. Examples of such features include, but are not limited to, untranslated regions (UTRs), Kozak sequences, an oligo(dT) sequence, and detectable tags and may include multiple cloning sites which may have XbaI recognition.

[000412] In some embodiments, a 5′ UTR and/or a 3′ UTR region may be provided as flanking regions. Multiple 5′ or 3′ UTRs may be included in the flanking regions and may be the same or of different sequences. Any portion of the flanking regions, including none, may be codon optimized and any may independently contain one or more different structural or chemical modifications, before and/or after codon optimization.

[000413] After optimization (if desired), the polynucleotides components are reconstituted and transformed into a vector such as, but not limited to, plasmids, viruses, cosmids, and artificial chromosomes. For example, the optimized polynucleotide may be reconstituted and transformed into chemically competent *E. coli,* yeast, neurospora, maize, drosophila, etc. where high copy plasmid-like or chromosome structures occur by methods described herein.

[000414] Synthetic polynucleotides and their nucleic acid analogs play an important role in the research and studies of biochemical processes. Various enzyme-assisted and chemical-based methods have been developed to synthesize polynucleotides and nucleic acids.

Enzymatic Methods

In Vitro Transcription-enzymatic synthesis

[000415] cDNA encoding the polynucleotides described herein may be transcribed using an *in vitro* transcription (IVT) system. The system typically comprises a transcription buffer, nucleotide triphosphates (NTPs), an RNase inhibitor and a polymerase. The NTPs may be manufactured in house, may be selected from a supplier, or may be synthesized as described herein. The NTPs may be selected from, but are not limited to, those described herein including natural and unnatural (modified) NTPs. The polymerase may be selected from, but is not limited to, T7 RNA polymerase, T3 RNA polymerase and mutant polymerases such as, but not limited to, polymerases able to incorporate polynucleotides (e.g., modified nucleic acids).

RNA Polymerases useful for synthesis

[000416] Any number of RNA polymerases or variants may be used in the synthesis of the polynucleotides of the present invention.

[000417] RNA polymerases may be modified by inserting or deleting amino acids of the RNA polymerase sequence. As a non-limiting example, the RNA polymerase may be modified to exhibit an increased ability to incorporate a 2'-modified nucleotide triphosphate compared to an unmodified RNA polymerase (see International Publication WO2008078180 and U.S. Patent 8,101,385; herein incorporated by reference in their entireties).

[000418] Variants may be obtained by evolving an RNA polymerase, optimizing the RNA polymerase amino acid and/or nucleic acid sequence and/or by using other methods known in the art. As a non-limiting example, T7 RNA polymerase variants may be evolved using the continuous directed evolution system set out by Esvelt *et al.* (Nature (2011) 472(7344):499-503; herein incorporated by reference in its entirety) where clones of T7 RNA polymerase may encode at least one mutation such as, but not limited to, lysine at position 93 substituted for threonine (K93T), I4M, A7T, E63V, V64D, A65E, D66Y, T76N, C125R, S128R, A136T, N165S, G175R, H176L, Y178H, F182L, L196F, G198V, D208Y, E222K, S228A, Q239R, T243N, G259D, M267I, G280C, H300R, D351A, A354S, E356D, L360P, A383V, Y385C, D388Y, S397R, M401T, N410S, K450R, P451T, G452V, E484A, H523L, H524N, G542V, E565K, K577E, K577M, N601S, S684Y, L699I, K713E, N748D, Q754R, E775K, A827V, D851N or L864F. As another non-limiting example, T7 RNA polymerase variants may encode at least mutation as described in U.S. Pub. Nos. 20100120024 and 20070117112; herein incorporated by reference in their entireties. Variants of RNA polymerase may also include, but

are not limited to, substitutional variants, conservative amino acid substitution, insertional variants, deletional variants and/or covalent derivatives.

[000419] In one embodiment, the polynucleotide may be designed to be recognized by the wild type or variant RNA polymerases. In doing so, the polynucleotide may be modified to contain sites or regions of sequence changes from the wild type or parent polynucleotide.

[000420] Polynucleotide or nucleic acid synthesis reactions may be carried out by enzymatic methods utilizing polymerases. Polymerases catalyze the creation of phosphodiester bonds between nucleotides in a polynucleotide or nucleic acid chain. Currently known DNA polymerases can be divided into different families based on amino acid sequence comparison and crystal structure analysis. DNA polymerase I (pol I) or A polymerase family, including the Klenow fragments of *E. Coli*, *Bacillus* DNA polymerase I, *Thermus aquaticus* (Taq) DNA polymerases, and the T7 RNA and DNA polymerases, is among the best studied of these families. Another large family is DNA polymerase α (pol α) or B polymerase family, including all eukaryotic replicating DNA polymerases and polymerases from phages T4 and RB69. Although they employ similar catalytic mechanism, these families of polymerases differ in substrate specificity, substrate analog-incorporating efficiency, degree and rate for primer extension, mode of DNA synthesis, exonuclease activity, and sensitivity against inhibitors.

[000421] DNA polymerases are also selected based on the optimum reaction conditions they require, such as reaction temperature, pH, and template and primer concentrations. Sometimes a combination of more than one DNA polymerases is employed to achieve the desired DNA fragment size and synthesis efficiency. For example, Cheng et al. increase pH, add glycerol and dimethyl sulfoxide, decrease denaturation times, increase extension times, and utilize a secondary thermostable DNA polymerase that possesses a 3' to 5' exonuclease activity to effectively amplify long targets from cloned inserts and human genomic DNA (Cheng et al., *PNAS*, Vol. 91, 5695-5699 (1994), the contents of which are incorporated herein by reference in their entirety). RNA polymerases from bacteriophage T3, T7, and SP6 have been widely used to prepare RNAs for biochemical and biophysical studies. RNA polymerases, capping enzymes, and poly-A polymerases are disclosed in the copending Publication No. WO2014028429 (M032), the contents of which are incorporated herein by reference in their entirety.

[000422] In one embodiment, the RNA polymerase which may be used in the synthesis of the chimeric polynucleotides described herein is a Syn5 RNA polymerase (see Zhu et al. Nucleic

Acids Research 2013, the contents of which is herein incorporated by reference in its entirety). The Syn5 RNA polymerase is described in paragraphs [000283] – [000287] of International Patent Publication No. WO2015034928, the contents of which are herein incorporated by reference in its entirety.

[000423] Various tools in genetic engineering are based on the enzymatic amplification of a target gene which acts as a template. For the study of sequences of individual genes or specific regions of interest and other research needs, it is necessary to generate multiple copies of a target gene from a small sample of polynucleotides or nucleic acids. Such methods may be applied in the manufacture of the polynucleotides of the invention.

[000424] Polymerase chain reaction (PCR) has wide applications in rapid amplification of a target gene, as well as genome mapping and sequencing. The key components for synthesizing DNA comprise target DNA molecules as a template, primers complementary to the ends of target DNA strands, deoxynucleoside triphosphates (dNTPs) as building blocks, and a DNA polymerase. As PCR progresses through denaturation, annealing and extension steps, the newly produced DNA molecules can act as a template for the next circle of replication, achieving exponentially amplification of the target DNA. PCR requires a cycle of heating and cooling for denaturation and annealing. Variations of the basic PCR include, but are not limited to, asymmetric PCR (See e.g., Innis et al., *PNAS*, vol. 85, 9436-9440 (1988), the contents of which are incorporated herein by reference in their entirety), inverse PCR (see e.g., Ochman et al., *Genetics*, vol. 120(3), 621-623, (1988), the contents of which are incorporated herein by reference in their entirety), and reverse transcription PCR (RT-PCR) (see e.g., Freeman et al., *BioTechniques*, vol. 26(1), 112-22, 124-5 (1999), the contents of which are incorporated herein by reference in their entirety). In RT-PCR, a single stranded RNA is the desired target and is converted to a double stranded DNA first by reverse transcriptase.

[000425] A variety of isothermal *in vitro* nucleic acid amplification techniques have been developed as alternatives or complements of PCR. For example, strand displacement amplification (SDA) is based on the ability of a restriction enzyme to form a nick (Walker et al., *PNAS*, vol. 89, 392-396 (1992), the contents of which are incorporated herein by reference in their entirety). A restriction enzyme recognition sequence is inserted into an annealed primer sequence. Primers are extended by a DNA polymerase and dNTPs to form a duplex. Only one strand of the duplex is cleaved by the restriction enzyme. Each single strand chain is then

available as a template for subsequent synthesis. SDA does not require the complicated temperature control cycle of PCR.

[000426] Nucleic acid sequence-based amplification (NASBA), also called transcription mediated amplification (TMA), is also an isothermal amplification method that utilizes a combination of DNA polymerase, reverse transcriptase, RNAse H, and T7 RNA polymerase (Compton, *Nature*, vol. 350, 91-92 (1991), the contents of which are incorporated herein by reference in their entirety). A target RNA is used as a template and a reverse transcriptase synthesizes its complementary DNA strand. RNAse H hydrolyzes the RNA template, making space for a DNA polymerase to synthesize a DNA strand complementary to the first DNA strand which is complementary to the RNA target, forming a DNA duplex. T7 RNA polymerase continuously generates complementary RNA strands of this DNA duplex. These RNA strands act as templates for new cycles of DNA synthesis, resulting in amplification of the target gene.

[000427] Rolling-circle amplification (RCA) amplifies a single stranded circular polynucleotide and involves numerous rounds of isothermal enzymatic synthesis where Φ29 DNA polymerase extends a primer by continuously progressing around the polynucleotide circle to replicate its sequence over and over again. Therefore, a linear copy of the circular template is achieved. A primer can then be annealed to this linear copy and its complementary chain can be synthesized (Lizardi et al., *Nature Genetics*, vol. 19, 225-232 (1998), the contents of which are incorporated herein by reference in their entirety). A single stranded circular DNA can also serve as a template for RNA synthesis in the presence of an RNA polymerase (Daubendiek et al., *JACS*, vol. 117, 7818-7819 (1995), the contents of which are incorporated herein by reference in their entirety). An inverse rapid amplification of cDNA ends (RACE) RCA is described by Polidoros et al. (*BioTechniques,* vol. 41, 35-42 (2006), the contents of which are incorporated herein by reference in their entirety). A messenger RNA (mRNA) is reverse transcribed into cDNA, followed by RNAse H treatment to separate the cDNA. The cDNA is then circularized by CircLigase into a circular DNA. The amplification of the resulting circular DNA is achieved with RCA.

[000428] Any of the foregoing methods may be utilized in the manufacture of one or more regions of the polynucleotides of the present invention.

[000429] Assembling polynucleotides or nucleic acids by a ligase is also widely used. DNA or RNA ligases promote intermolecular ligation of the 5' and 3' ends of polynucleotide chains

through the formation of a phosphodiester bond. Ligase chain reaction (LCR) is a promising diagnosing technique based on the principle that two adjacent polynucleotide probes hybridize to one strand of a target gene and couple to each other by a ligase. If a target gene is not present, or if there is a mismatch at the target gene, such as a single-nucleotide polymorphism (SNP), the probes cannot ligase (Wiedmann et al., *PCR Methods and Application,* vol.3 (4), s51-s64 (1994), the contents of which are incorporated herein by reference in their entirety). LCR may be combined with various amplification techniques to increase sensitivity of detection or to increase the amount of products if it is used in synthesizing polynucleotides and nucleic acids.

[000430] Several library preparation kits for nucleic acids are now commercially available. They include enzymes and buffers to convert a small amount of nucleic acid samples into an indexed library for downstream applications. For example, DNA fragments may be placed in a NEBNEXT® ULTRA™ DNA Library Prep Kit by NEWENGLAND BIOLABS® for end preparation, ligation, size selection, clean-up, PCR amplification and final clean-up.

[000431] Continued development is going on to improvement the amplification techniques. For example, US Pat. 8,367,328 to Asada et al. the contents of which are incorporated herein by reference in their entirety, teaches utilizing a reaction enhancer to increase the efficiency of DNA synthesis reactions by DNA polymerases. The reaction enhancer comprises an acidic substance or cationic complexes of an acidic substance. US Pat. 7.384,739 to Kitabayashi et al. the contents of which are incorporated herein by reference in their entirety, teaches a carboxylate ion-supplying substance that promotes enzymatic DNA synthesis, wherein the carboxylate ioin-supplying substance is selected from oxalic acid, malonic acid, esters of oxalic acid, esters of malonic acid, salts of malonic acid, and esters of maleic acid. US Pat. 7,378,262 to Sobek et al. the contents of which are incorporated herein by reference in their entirety, discloses an enzyme composition to increase fidelity of DNA amplifications. The composition comprises one enzyme with 3' exonuclease activity but no polymerase activity and another enzyme that is a polymerase. Both of the enzymes are thermostable and are reversibly modified to be inactive at lower temperatures.

[000432] US Pat. No. 7,550,264 to Getts et al. teaches multiple round of synthesis of sense RNA molecules are performed by attaching oligodeoxynucleotides tails onto the 3' end of cDNA molecules and initiating RNA transcription using RNA polymerase, the contents of which are incorporated herein by reference in their entirety. US Pat. Publication No. 2013/0183718 to

Rohayem teaches RNA synthesis by RNA-dependent RNA polymerases (RdRp) displaying an RNA polymerase activity on single-stranded DNA templates, the contents of which are incorporated herein by reference in their entirety. Oligonucleotides with non-standard nucleotides may be synthesized with enzymatic polymerization by contacting a template comprising non-standard nucleotides with a mixture of nucleotides that are complementary to the nucleotides of the template as disclosed in US Pat. No. 6,617,106 to Benner, the contents of which are incorporated herein by reference in their entirety.

[000433] Solid-phase chemical synthesis may be used to manufacture the polynucleotides described herein or portions thereof. Solid-phase chemical synthesis manufacturing of the polynucleotides described herein are described in International Patent Application No. PCT/US2014/53907, the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000298]-[000307].

[000434] Liquid phase chemical synthesis may be used to manufacture the polynucleotides described herein or portions thereof. Liquid phase chemical synthesis manufacturing of the polynucleotides described herein are described in International Patent Application No. PCT/US2014/53907, the contents of which are herein incorporated by reference in its entirety, such as in paragraph [000308].

[000435] Combinations of different synthetic methods may be used to manufacture the polynucleotides described herein or portions thereof. These combinations are described in International Patent Application No. PCT/US2014/53907, the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000309] – [000312].

[000436] Small region synthesis which may be used for regions or subregions of the polynucleotides of the present invention. These synthesis methods are described in International Patent Application No. PCT/US2014/53907, the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000313] – [000314].

[000437] Ligation of polynucleotide regions or subregions may be used to prepare the polynucleotides described herein. These ligation methods are described in International Patent Application No. PCT/US2014/53907, the contents of which are herein incorporated by reference in its entirety, such as in paragraphs [000315] – [000322].

[000438] For example, polynucleotides of the invention having a sequence comprising Formula I:

$$[A_n]\text{-}L^1\text{-}[B_o],$$

Formula I

may be synthesized by reacting a compound having the structure of Formula XIV:

$$[A_n]\text{-}(R^1)_a\text{-}(R^2)_b\text{-}(R^3)_c\text{-}N_3$$

Formula XIV

with a compound having the structure of Formula XV:

$$R^{27}\text{-}(R^5)_d\text{-}(R^6)_e\text{-}(R^7)_f\text{-}[B_o]$$

Formula XV

[000439] wherein each A and B is independently any nucleoside;

[000440] n and o are, independently 15 to 1000; and

[000441] L^1 has the structure of Formula III:

$$\{\!\!-(R^1)_a-(R^2)_b-(R^3)_c-R^4-(R^5)_d-(R^6)_e-(R^7)_f-\!\!\}$$

Formula III

[000442] wherein a, b, c, d, e, and f are each, independently, 0 or 1;

[000443] wherein each A and B is independently any nucleoside;

[000444] n and o are, independently 15 to 1000;

[000445] R^1, R^3, R^5, and R^7 each, independently, is selected from optionally substituted C_1-C_6 alkylene, optionally substituted C_1-C_6 heteroalkylene, O, S, and NR^8;

[000446] R^2 and R^6 are each, independently, selected from carbonyl, thiocarbonyl, sulfonyl, or phosphoryl;

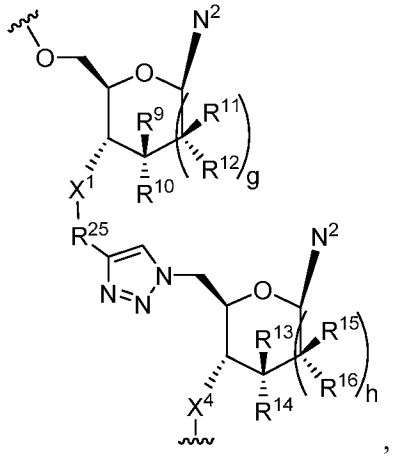
[000447] R^4 is an optionally substituted triazolene; and

[000448] R^8 is hydrogen, optionally substituted C_1–C_4 alkyl, optionally substituted C_3–C_4 alkenyl, optionally substituted C_2–C_4 alkynyl, optionally substituted C_2–C_6 heterocyclyl, optionally substituted C_6–C_{12} aryl, or optionally substituted C_1–C_7 heteroalkyl; and

[000449] R^{27} is an optionally substituted C_2-C_3 alkynyl or an optionally substituted C_8-C_{12} cycloalkynyl,

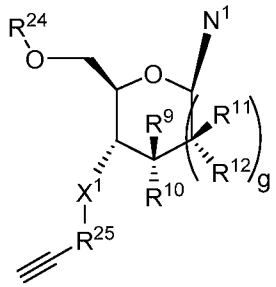
[000450] wherein L^1 is attached to $[A_n]$ and $[B_o]$ at the sugar of one of the nucleosides.

[000451] Chimeric polynucleotides of the invention including the structure of Formula XI:



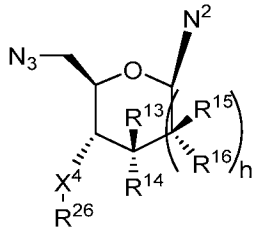
Formula XI

may be synthesized by reacting a compound having the structure of Formula XII:



Formula XII

with a compound having the structure of Formula XIII:



Formula XIII

[000452] wherein each of N^1 and N^2 is independently a nucleobase;

[000453] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted C_1-C_6 alkyl, optionally substituted C_1-C_6 heteroalkyl, optionally substituted C_2-C_6 heteroalkenyl, optionally substituted C_2-C_6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-C_{10} aryl;

[000454] each of g and h is, independently, 0 or 1;

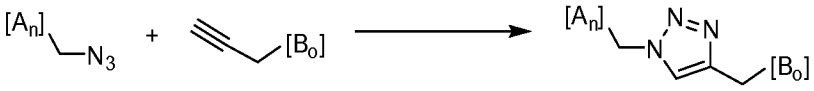
[000455] each X^4 is, independently, O, NH, or S; and

[000456] each X^2 and X^3 is independently O or S;

[000457] each of R^{24} and R^{26} is, independently, a region of linked nucleosides; and

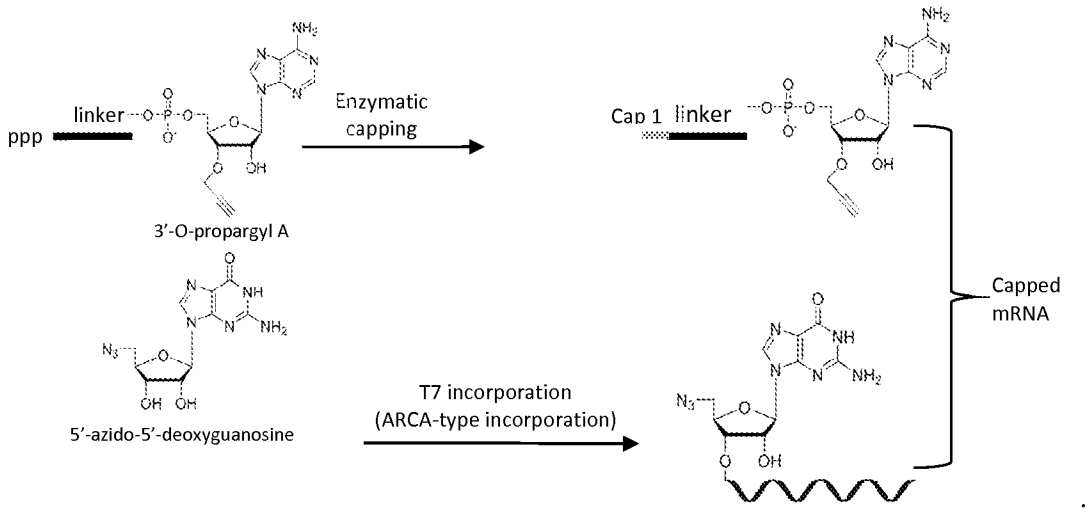
[000458] R^{25} is optionally substituted C_1-C_6 alkylene or optionally substituted C_1-C_6 heteroalkylene or R^{25} and the alkynyl group together form optionally substituted cycloalkynyl.

[000459] For example, the chimeric polynucleotides of the invention may be synthesized as shown below



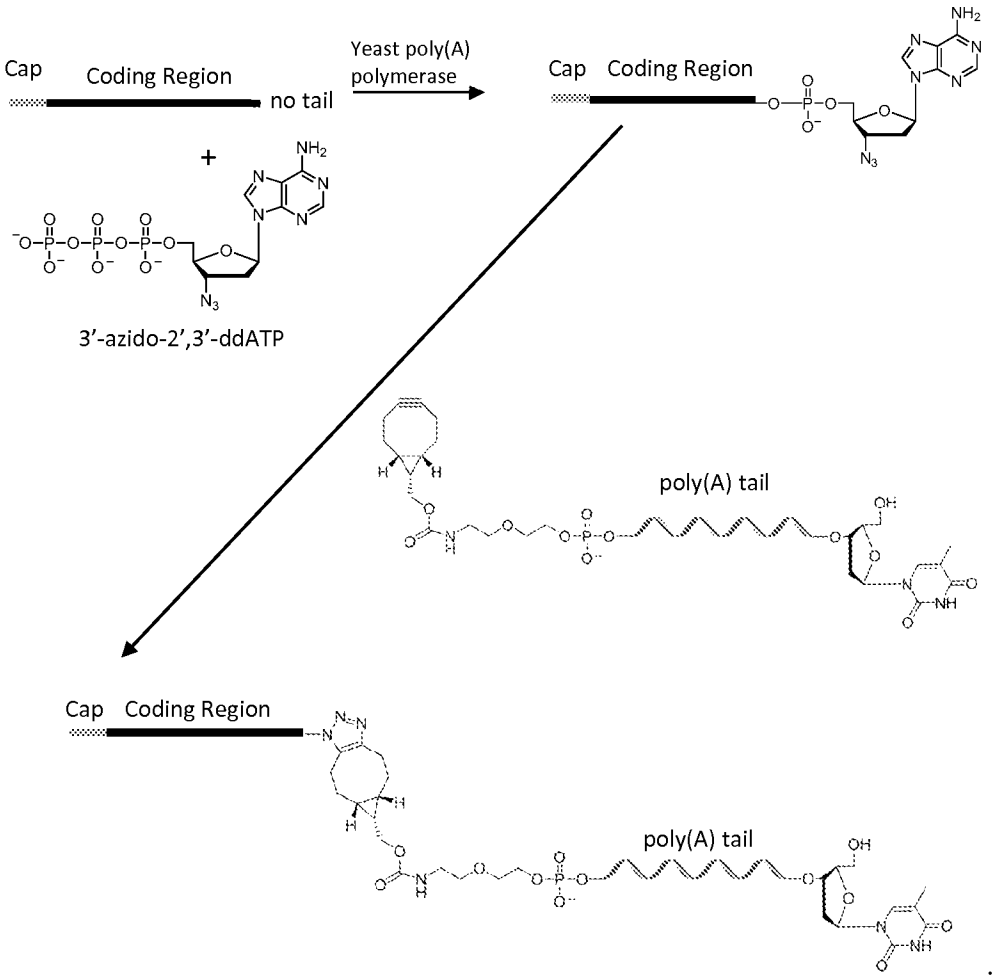
. In some embodiments, the 5' cap structure or poly-A tail may be attached to a chimeric polynucleotide of the invention with this method.

[000460] A 5' cap structure may be attached to a chimeric polynucleotide of the invention as shown below:



.

[000461] A poly-A tail may be attached to a chimeric polynucleotide of the invention as shown below:



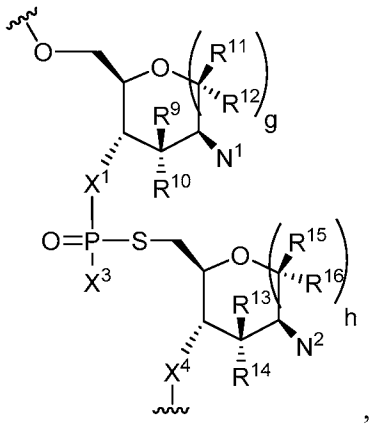
[000462] Sequential ligation can be performed on a solid substrate. For example, initial linker DNA molecules modified with biotin at the end are attached to streptavidin-coated beads. The 3'-ends of the linker DNA molecules are complimentary with the 5'-ends of the incoming DNA fragments. The beads are washed and collected after each ligation step and the final linear constructs are released by a meganuclease. This method allows rapid and efficient assembly of genes in an optimized order and orientation. (Takita, *DNA Research,* vol. 20(4), 1-10 (2013), the contents of which are incorporated herein by reference in their entirety). Labeled polynucleotides synthesized on solid-supports are disclosed in US Pat. Pub. No. 2001/0014753 to Soloveichik et al. and US Pat. Pub. No. 2003/0191303 to Vinayak et al., the contents of which are incorporated herein by reference for their entirety.

Modified and Conjugated Polynucleotides

[000463] Non-natural modified nucleotides may be introduced to polynucleotides or nucleic acids during synthesis or post-synthesis of the chains to achieve desired functions or properties. The modifications may be on internucleotide lineage, the purine or pyrimidine bases, or sugar. The modification may be introduced at the terminal of a chain or anywhere else in the chain; with chemical synthesis or with a polymerase enzyme. For example, hexitol nucleic acids (HNAs) are nuclease resistant and provide strong hybridization to RNA. Short messenger RNAs (mRNAs) with hexitol residues in two codons have been constructed (Lavrik et al., *Biochemistry,* 40, 11777-11784 (2001), the contents of which are incorporated herein by reference in their entirety). The antisense effects of a chimeric HNA gapmer oligonucleotide comprising a phosphorothioate central sequence flanked by 5' and 3' HNA sequences have also been studied (See e.g., Kang et al., *Nucleic Acids Research,* vol. 32(4), 4411-4419 (2004), the contents of which are incorporated herein by reference in their entirety). The preparation and uses of modified nucleotides comprising 6-member rings in RNA interference, antisense therapy or other applications are disclosed in US Pat. Application No. 2008/0261905, US Pat. Application No. 2010/0009865, and PCT Application No. WO97/30064 to Herdewijn et al.; the contents of each of which are herein incorporated by reference in their entireties). Modified nucleic acids and their synthesis are disclosed in copending PCT applications No. PCT/US2012/058519 (Attorney Docket Number M09), the contents of which are incorporated herein by reference for their entirety. The synthesis and strategy of modified polynucleotides is reviewed by Verma and Eckstein in *Annual Review of Biochemistry,* vol. 76, 99-134 (1998), the contents of which are incorporated herein by reference in their entirety.

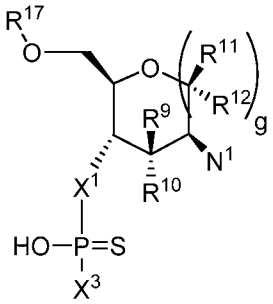
[000464] Either enzymatic or chemical ligation methods can be used to conjugate polynucleotides or their regions with different functional blocks, such as fluorescent labels, liquids, nanoparticles, delivery agents, etc. The conjugates of polynucleotides and modified polynucleotides are reviewed by Goodchild in *Bioconjugate Chemistry,* vol. 1(3), 165-187 (1990), the contents of which are incorporated herein by reference in their entirety. US Pat. No. 6,835,827 and US Pat. No. 6,525,183 to Vinayak et al. (the contents of each of which are herein incorporated by reference in their entireties) teach synthesis of labeled oligonucleotides using a labeled solid support.

[000465] For example, chimeric polynucleotides of the invention including the structure of Formula V:



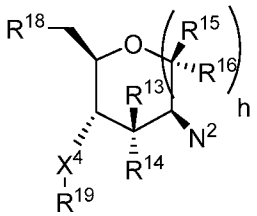
Formula V

[000466] This method includes reacting a compound having the structure of Formula VI:



Formula VI

with a compound having the structure of Formula VII:



Formula VII

[000467] wherein each of N^1 and N^2 is, independently, a nucleobase;

[000468] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted C_1-C_6 alkyl, optionally substituted C_1-C_6 heteroalkyl, optionally substituted C_2-C_6 heteroalkenyl, optionally substituted C_2-C_6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-C_{10} aryl;

[000469] each of g and h is, independently, 0 or 1;

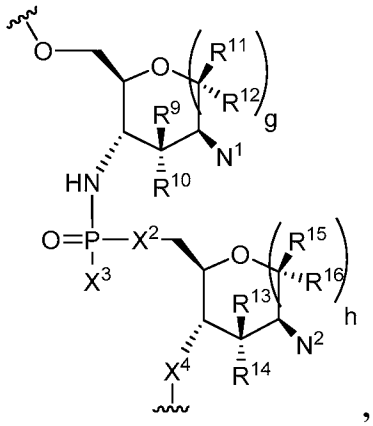
[000470] each X^1 and X^4 is, independently, O, NH, or S; and

[000471] each X^3 is independently OH or SH, or a salt thereof;

[000472] each of R^{17} and R^{19} is, independently, a region of linked nucleosides; and

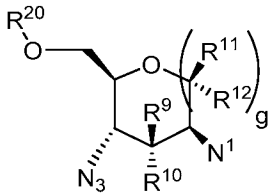
[000473] R^{18} is a halogen.

[000474] Chimeric polynucleotides of the invention including the structure of Formula VIII:



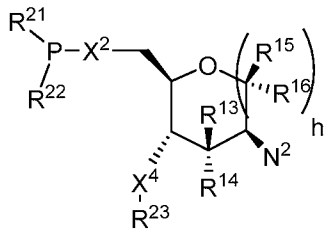
Formula VIII

[000475] This method includes reacting a compound having the structure of Formula IX:



Formula IX

with a compound having the structure of Formula X:



Formula X

[000476] wherein each of N^1 and N^2 is, independently, a nucleobase;

[000477] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted C_1-C_6 alkyl, optionally substituted C_1-C_6 heteroalkyl, optionally

substituted C_2-C_6 heteroalkenyl, optionally substituted C_2-C_6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-C_{10} aryl;

[000478] each of g and h is, independently, 0 or 1;

[000479] each X^4 is, independently, O, NH, or S; and

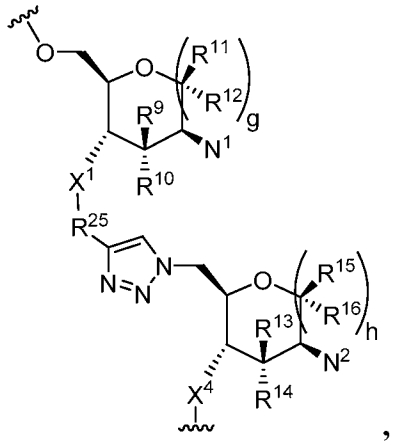
[000480] each X^2 is independently O or S;

[000481] each X^3 is independently OH, SH, or a salt thereof;

[000482] each of R^{20} and R^{23} is, independently, a region of linked nucleosides; and

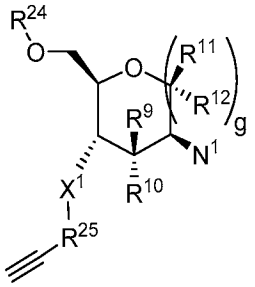
[000483] each of R^{21} and R^{22} is, independently, optionally substituted C_1-C_6 alkoxy.

[000484] Chimeric polynucleotides of the invention including the structure of Formula XI:



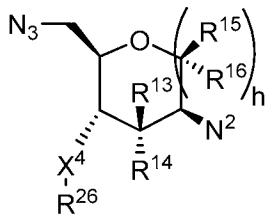
Formula XI

[000485] This method includes reacting a compound having the structure of Formula XII:



Formula XII

with a compound having the structure of Formula XIII:



Formula XIII

[000486] wherein each of N^1 and N^2 is, independently, a nucleobase;

[000487] each of R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} is, independently, H, halo, hydroxy, thiol, optionally substituted C_1-C_6 alkyl, optionally substituted C_1-C_6 heteroalkyl, optionally substituted C_2-C_6 heteroalkenyl, optionally substituted C_2-C_6 heteroalkynyl, optionally substituted amino, azido, or optionally substituted C_6-C_{10} aryl;

[000488] each of g and h is, independently, 0 or 1;

[000489] each X^4 is, independently, O, NH, or S; and

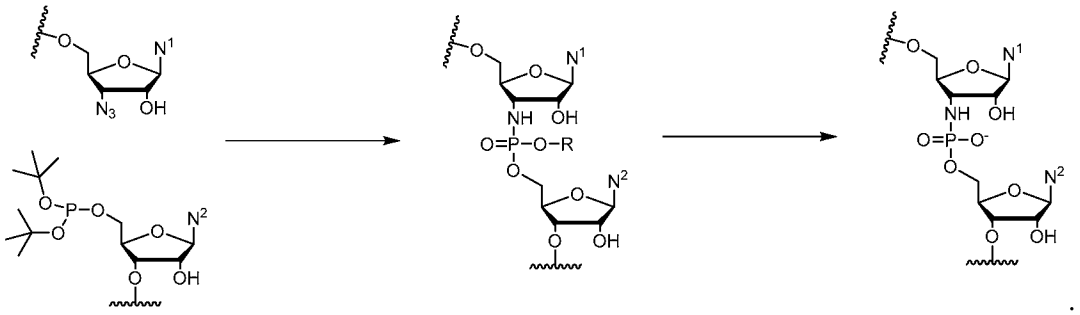
[000490] each X^2 is independently O or S;

[000491] each X^3 is independently OH, SH, or a salt thereof;

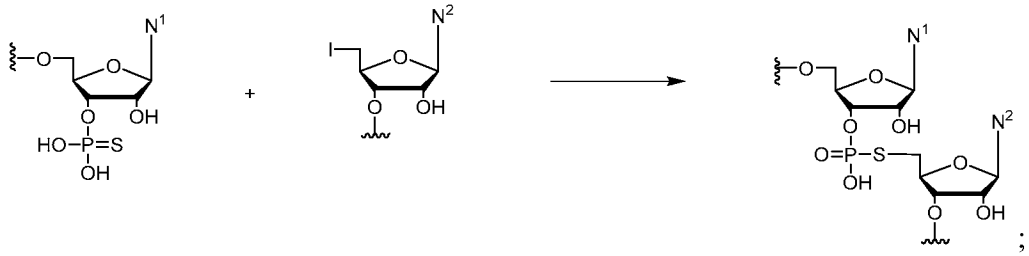
[000492] each of R^{24} and R^{26} is, independently, a region of linked nucleosides; and

[000493] R^{25} is optionally substituted C_1-C_6 alkylene or optionally substituted C_1-C_6 heteroalkylene or R^{25} and the alkynyl group together form optionally substituted cycloalkynylene.

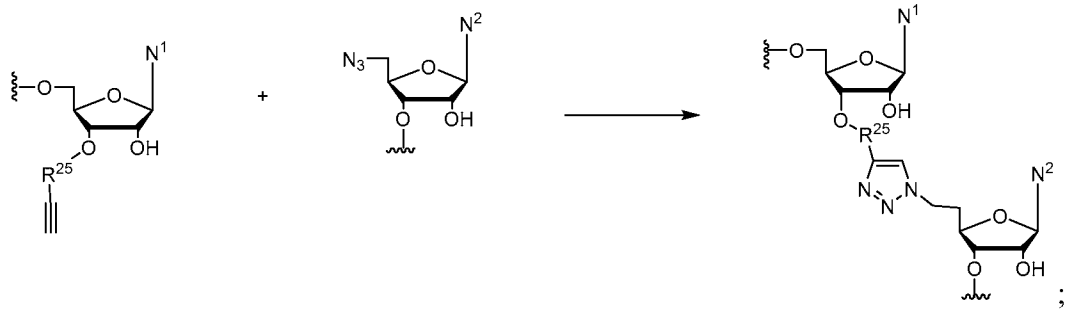
[000494] Chimeric polynucleotides of the invention may be synthesized as shown below:



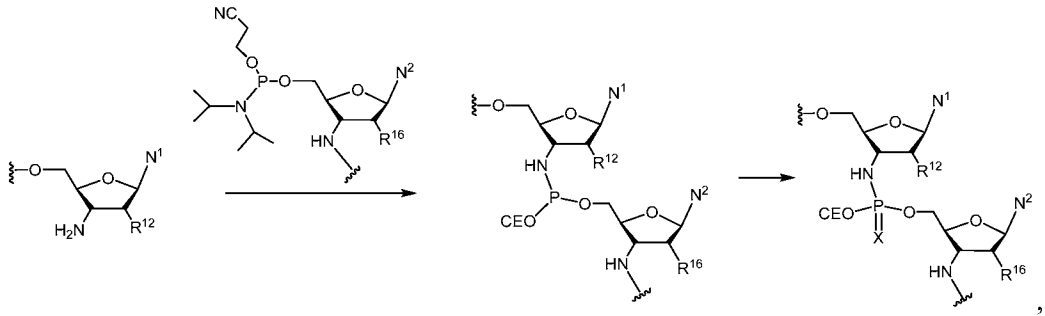
[000495] Other methods for the synthesis of the chimeric polynucleotides of the invention are shown below:



a) ;



b) ;



c) , where CEO is 2-cyanoethoxy, and X is O or S.

[000496] It will be understood that the reactive group shown at the 3' (or 4' position, when g or h is 1) and at the 5' (or 6' position, when g or h is 1) can be reversed. For example, the halogen, azido, or alkynyl group may be attached to the 5' position (or 6' position, when g or h is 1), and the thiophosphate, (thio)phosphoryl, or azido group may be attached to the 3' position (or 4' position, when g or h is 1).

Quantification

[000497] In one embodiment, the polynucleotides of the present invention may be quantified in exosomes or when derived from one or more bodily fluid. As used herein "bodily fluids" include peripheral blood, serum, plasma, ascites, urine, cerebrospinal fluid (CSF), sputum, saliva, bone marrow, synovial fluid, aqueous humor, amniotic fluid, cerumen, breast milk, broncheoalveolar lavage fluid, semen, prostatic fluid, cowper's fluid or pre-ejaculatory fluid, sweat, fecal matter, hair, tears, cyst fluid, pleural and peritoneal fluid, pericardial fluid, lymph, chyme, chyle, bile,

interstitial fluid, menses, pus, sebum, vomit, vaginal secretions, mucosal secretion, stool water, pancreatic juice, lavage fluids from sinus cavities, bronchopulmonary aspirates, blastocyl cavity fluid, and umbilical cord blood. Alternatively, exosomes may be retrieved from an organ selected from the group consisting of lung, heart, pancreas, stomach, intestine, bladder, kidney, ovary, testis, skin, colon, breast, prostate, brain, esophagus, liver, and placenta.

[000498] In the exosome quantification method, a sample of not more than 2mL is obtained from the subject and the exosomes isolated by size exclusion chromatography, density gradient centrifugation, differential centrifugation, nanomembrane ultrafiltration, immunoabsorbent capture, affinity purification, microfluidic separation, or combinations thereof. In the analysis, the level or concentration of a polynucleotide may be an expression level, presence, absence, truncation or alteration of the administered construct. It is advantageous to correlate the level with one or more clinical phenotypes or with an assay for a human disease biomarker. The assay may be performed using construct specific probes, cytometry, qRT-PCR, real-time PCR, PCR, flow cytometry, electrophoresis, mass spectrometry, or combinations thereof while the exosomes may be isolated using immunohistochemical methods such as enzyme linked immunosorbent assay (ELISA) methods. Exosomes may also be isolated by size exclusion chromatography, density gradient centrifugation, differential centrifugation, nanomembrane ultrafiltration, immunoabsorbent capture, affinity purification, microfluidic separation, or combinations thereof.

[000499] These methods afford the investigator the ability to monitor, in real time, the level of polynucleotides remaining or delivered. This is possible because the polynucleotides of the present invention differ from the endogenous forms due to the structural or chemical modifications.

[000500] In one embodiment, the polynucleotide may be quantified using methods such as, but not limited to, ultraviolet visible spectroscopy (UV/Vis). A non-limiting example of a UV/Vis spectrometer is a NANODROP® spectrometer (ThermoFisher, Waltham, MA). The quantified polynucleotide may be analyzed in order to determine if the polynucleotide may be of proper size, check that no degradation of the polynucleotide has occurred. Degradation of the polynucleotide may be checked by methods such as, but not limited to, agarose gel electrophoresis, HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and

hydrophobic interaction HPLC (HIC-HPLC), liquid chromatography-mass spectrometry (LCMS), capillary electrophoresis (CE) and capillary gel electrophoresis (CGE).

Purification

[000501] Purification of the polynucleotides described herein may include, but is not limited to, polynucleotide clean-up, quality assurance and quality control. Clean-up may be performed by methods known in the arts such as, but not limited to, AGENCOURT® beads (Beckman Coulter Genomics, Danvers, MA), poly-T beads, LNA™ oligo-T capture probes (EXIQON® Inc, Vedbaek, Denmark) or HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC-HPLC). The term "purified" when used in relation to a polynucleotide such as a "purified polynucleotide" refers to one that is separated from at least one contaminant. As used herein, a "contaminant" is any substance which makes another unfit, impure or inferior. Thus, a purified polynucleotide (e.g., DNA and RNA) is present in a form or setting different from that in which it is found in nature, or a form or setting different from that which existed prior to subjecting it to a treatment or purification method.

[000502] A quality assurance and/or quality control check may be conducted using methods such as, but not limited to, gel electrophoresis, UV absorbance, or analytical HPLC.

[000503] In another embodiment, the polynucleotides may be sequenced by methods including, but not limited to reverse-transcriptase-PCR.

III. Modifications

[000504] As used herein in a polynucleotide (such as a chimeric polynucleotide, IVT polynucleotide or a circular polynucleotide), the terms "chemical modification" or, as appropriate, "chemically modified" refer to modification with respect to adenosine (A), guanosine (G), uridine (U), thymidine (T) or cytidine (C) ribo- or deoxyribnucleosides in one or more of their position, pattern, percent or population. Generally, herein, these terms are not intended to refer to the ribonucleotide modifications in naturally occurring 5′-terminal mRNA cap moieties.

[000505] In a polypeptide, the term "modification" refers to a modification as compared to the canonical set of 20 amino acids.

[000506] The modifications may be various distinct modifications. In some embodiments, the regions may contain one, two, or more (optionally different) nucleoside or nucleotide

modifications. In some embodiments, a modified polynucleotide, introduced to a cell may exhibit reduced degradation in the cell, as compared to an unmodified polynucleotide.

[000507] Modifications which are useful in the present invention include, but are not limited to those in Table 4. Noted in the table are the symbol of the modification, the nucleobase type and whether the modification is naturally occurring or not.

Table 4. Modifications

Name	**Symbol**	**Base**	**Naturally Occurring**
2-methylthio-N6-(cis-hydroxyisopentenyl)adenosine	ms2i6A	A	YES
2-methylthio-N6-methyladenosine	ms2m6A	A	YES
2-methylthio-N6-threonyl carbamoyladenosine	ms2t6A	A	YES
N6-glycinylcarbamoyladenosine	g6A	A	YES
N6-isopentenyladenosine	i6A	A	YES
N6-methyladenosine	m6A	A	YES
N6-threonylcarbamoyladenosine	t6A	A	YES
1,2′-O-dimethyladenosine	m1Am	A	YES
1-methyladenosine	m1A	A	YES
2′-O-methyladenosine	Am	A	YES
2′-O-ribosyladenosine (phosphate)	Ar(p)	A	YES
2-methyladenosine	m2A	A	YES
2-methylthio-N6 isopentenyladenosine	ms2i6A	A	YES
2-methylthio-N6-hydroxynorvalyl carbamoyladenosine	ms2hn6A	A	YES
2'-O-methyladenosine	m6A	A	YES
2'-O-ribosyladenosine (phosphate)	Ar(p)	A	YES
isopentenyladenosine	Iga	A	YES
N6-(cis-hydroxyisopentenyl)adenosine	io6A	A	YES
N6,2′-O-dimethyladenosine	m6Am	A	YES
N^6,2'-O-dimethyladenosine	m^6Am	A	YES
N6,N6,2′-O-trimethyladenosine	m62Am	A	YES
N6,N6-dimethyladenosine	m62A	A	YES
N6-acetyladenosine	ac6A	A	YES
N6-hydroxynorvalylcarbamoyladenosine	hn6A	A	YES
N6-methyl-N6-threonylcarbamoyladenosine	m6t6A	A	YES
2-methyladenosine	m^2A	A	YES
2-methylthio-N^6-isopentenyladenosine	ms^2i^6A	A	YES
7-deaza-adenosine	--	A	NO
N1-methyl-adenosine	--	A	NO

N6, N6 (dimethyl)adenine	--	A	NO
N6-cis-hydroxy-isopentenyl-adenosine	--	A	NO
α-thio-adenosine	--	A	NO
2 (amino)adenine	--	A	NO
2 (aminopropyl)adenine	--	A	NO
2 (methylthio) N6 (isopentenyl)adenine	--	A	NO
2-(alkyl)adenine	--	A	NO
2-(aminoalkyl)adenine	--	A	NO
2-(aminopropyl)adenine	--	A	NO
2-(halo)adenine	--	A	NO
2-(halo)adenine	--	A	NO
2-(propyl)adenine	--	A	NO
2'-Amino-2'-deoxy-ATP	--	A	NO
2'-Azido-2'-deoxy-ATP	--	A	NO
2'-Deoxy-2'-a-aminoadenosine TP	--	A	NO
2'-Deoxy-2'-a-azidoadenosine TP	--	A	NO
6 (alkyl)adenine	--	A	NO
6 (methyl)adenine	--	A	NO
6-(alkyl)adenine	--	A	NO
6-(methyl)adenine	--	A	NO
7 (deaza)adenine	--	A	NO
8 (alkenyl)adenine	--	A	NO
8 (alkynyl)adenine	--	A	NO
8 (amino)adenine	--	A	NO
8 (thioalkyl)adenine	--	A	NO
8-(alkenyl)adenine	--	A	NO
8-(alkyl)adenine	--	A	NO
8-(alkynyl)adenine	--	A	NO
8-(amino)adenine	--	A	NO
8-(halo)adenine	--	A	NO
8-(hydroxyl)adenine	--	A	NO
8-(thioalkyl)adenine	--	A	NO

8-(thiol)adenine	--	A	NO
8-azido-adenosine	--	A	NO
aza adenine	--	A	NO
deaza adenine	--	A	NO
N6 (methyl)adenine	--	A	NO
N6-(isopentyl)adenine	--	A	NO
7-deaza-8-aza-adenosine	--	A	NO
7-methyladenine	--	A	NO
1-Deazaadenosine TP	--	A	NO
2'Fluoro-N6-Bz-deoxyadenosine TP	--	A	NO
2'-OMe-2-Amino-ATP	--	A	NO
2'O-methyl-N6-Bz-deoxyadenosine TP	--	A	NO
2'-a-Ethynyladenosine TP	--	A	NO
2-aminoadenine	--	A	NO
2-Aminoadenosine TP	--	A	NO
2-Amino-ATP	--	A	NO
2'-a-Trifluoromethyladenosine TP	--	A	NO
2-Azidoadenosine TP	--	A	NO
2'-b-Ethynyladenosine TP	--	A	NO
2-Bromoadenosine TP	--	A	NO
2'-b-Trifluoromethyladenosine TP	--	A	NO
2-Chloroadenosine TP	--	A	NO
2'-Deoxy-2',2'-difluoroadenosine TP	--	A	NO
2'-Deoxy-2'-a-mercaptoadenosine TP	--	A	NO
2'-Deoxy-2'-a-thiomethoxyadenosine TP	--	A	NO
2'-Deoxy-2'-b-aminoadenosine TP	--	A	NO
2'-Deoxy-2'-b-azidoadenosine TP	--	A	NO
2'-Deoxy-2'-b-bromoadenosine TP	--	A	NO
2'-Deoxy-2'-b-chloroadenosine TP	--	A	NO
2'-Deoxy-2'-b-fluoroadenosine TP	--	A	NO
2'-Deoxy-2'-b-iodoadenosine TP	--	A	NO
2'-Deoxy-2'-b-mercaptoadenosine TP	--	A	NO

2'-Deoxy-2'-b-thiomethoxyadenosine TP	--	A	NO
2-Fluoroadenosine TP	--	A	NO
2-Iodoadenosine TP	--	A	NO
2-Mercaptoadenosine TP	--	A	NO
2-methoxy-adenine	--	A	NO
2-methylthio-adenine	--	A	NO
2-Trifluoromethyladenosine TP	--	A	NO
3-Deaza-3-bromoadenosine TP	--	A	NO
3-Deaza-3-chloroadenosine TP	--	A	NO
3-Deaza-3-fluoroadenosine TP	--	A	NO
3-Deaza-3-iodoadenosine TP	--	A	NO
3-Deazaadenosine TP	--	A	NO
4'-Azidoadenosine TP	--	A	NO
4'-Carbocyclic adenosine TP	--	A	NO
4'-Ethynyladenosine TP	--	A	NO
5'-Homo-adenosine TP	--	A	NO
8-Aza-ATP	--	A	NO
8-bromo-adenosine TP	--	A	NO
8-Trifluoromethyladenosine TP	--	A	NO
9-Deazaadenosine TP	--	A	NO
2-aminopurine	--	A/G	NO
7-deaza-2,6-diaminopurine	--	A/G	NO
7-deaza-8-aza-2,6-diaminopurine	--	A/G	NO
7-deaza-8-aza-2-aminopurine	--	A/G	NO
2,6-diaminopurine	--	A/G	NO
7-deaza-8-aza-adenine, 7-deaza-2-aminopurine	--	A/G	NO
2-thiocytidine	s2C	C	YES
3-methylcytidine	m3C	C	YES
5-formylcytidine	f5C	C	YES
5-hydroxymethylcytidine	hm5C	C	YES
5-methylcytidine	m5C	C	YES
N4-acetylcytidine	ac4C	C	YES
2′-O-methylcytidine	Cm	C	YES
2'-O-methylcytidine	Cm	C	YES

5,2′-O-dimethylcytidine	m5 Cm	C	YES
5-formyl-2′-O-methylcytidine	f5Cm	C	YES
lysidine	k2C	C	YES
N4,2′-O-dimethylcytidine	m4Cm	C	YES
N4-acetyl-2′-O-methylcytidine	ac4Cm	C	YES
N4-methylcytidine	m4C	C	YES
N4,N4-Dimethyl-2'-OMe-Cytidine TP	--	C	YES
4-methylcytidine	--	C	NO
5-aza-cytidine	--	C	NO
Pseudo-iso-cytidine	--	C	NO
pyrrolo-cytidine	--	C	NO
α-thio-cytidine	--	C	NO
2-(thio)cytosine	--	C	NO
2'-Amino-2'-deoxy-CTP	--	C	NO
2'-Azido-2'-deoxy-CTP	--	C	NO
2'-Deoxy-2'-a-aminocytidine TP	--	C	NO
2'-Deoxy-2'-a-azidocytidine TP	--	C	NO
3 (deaza) 5 (aza)cytosine	--	C	NO
3 (methyl)cytosine	--	C	NO
3-(alkyl)cytosine	--	C	NO
3-(deaza) 5 (aza)cytosine	--	C	NO
3-(methyl)cytidine	--	C	NO
4,2'-O-dimethylcytidine	--	C	NO
5 (halo)cytosine	--	C	NO
5 (methyl)cytosine	--	C	NO
5 (propynyl)cytosine	--	C	NO
5 (trifluoromethyl)cytosine	--	C	NO
5-(alkyl)cytosine	--	C	NO
5-(alkynyl)cytosine	--	C	NO
5-(halo)cytosine	--	C	NO
5-(propynyl)cytosine	--	C	NO
5-(trifluoromethyl)cytosine	--	C	NO
5-bromo-cytidine	--	C	NO
5-iodo-cytidine	--	C	NO

5-propynyl cytosine	--	C	NO
6-(azo)cytosine	--	C	NO
6-aza-cytidine	--	C	NO
aza cytosine	--	C	NO
deaza cytosine	--	C	NO
N4 (acetyl)cytosine	--	C	NO
1-methyl-1-deaza-pseudoisocytidine	--	C	NO
1-methyl-pseudoisocytidine	--	C	NO
2-methoxy-5-methyl-cytidine	--	C	NO
2-methoxy-cytidine	--	C	NO
2-thio-5-methyl-cytidine	--	C	NO
4-methoxy-1-methyl-pseudoisocytidine	--	C	NO
4-methoxy-pseudoisocytidine	--	C	NO
4-thio-1-methyl-1-deaza-pseudoisocytidine	--	C	NO
4-thio-1-methyl-pseudoisocytidine	--	C	NO
4-thio-pseudoisocytidine	--	C	NO
5-aza-zebularine	--	C	NO
5-methyl-zebularine	--	C	NO
pyrrolo-pseudoisocytidine	--	C	NO
zebularine	--	C	NO
(E)-5-(2-Bromo-vinyl)cytidine TP	--	C	NO
2,2'-anhydro-cytidine TP hydrochloride	--	C	NO
2'Fluor-N4-Bz-cytidine TP	--	C	NO
2'Fluoro-N4-Acetyl-cytidine TP	--	C	NO
2'-O-Methyl-N4-Acetyl-cytidine TP	--	C	NO
2'O-methyl-N4-Bz-cytidine TP	--	C	NO
2'-a-Ethynylcytidine TP	--	C	NO
2'-a-Trifluoromethylcytidine TP	--	C	NO
2'-b-Ethynylcytidine TP	--	C	NO
2'-b-Trifluoromethylcytidine TP	--	C	NO
2'-Deoxy-2',2'-difluorocytidine TP	--	C	NO
2'-Deoxy-2'-a-mercaptocytidine TP	--	C	NO

2'-Deoxy-2'-a-thiomethoxycytidine TP	--	C	NO
2'-Deoxy-2'-b-aminocytidine TP	--	C	NO
2'-Deoxy-2'-b-azidocytidine TP	--	C	NO
2'-Deoxy-2'-b-bromocytidine TP	--	C	NO
2'-Deoxy-2'-b-chlorocytidine TP	--	C	NO
2'-Deoxy-2'-b-fluorocytidine TP	--	C	NO
2'-Deoxy-2'-b-iodocytidine TP	--	C	NO
2'-Deoxy-2'-b-mercaptocytidine TP	--	C	NO
2'-Deoxy-2'-b-thiomethoxycytidine TP	--	C	NO
2'-O-Methyl-5-(1-propynyl)cytidine TP	--	C	NO
3'-Ethynylcytidine TP	--	C	NO
4'-Azidocytidine TP	--	C	NO
4'-Carbocyclic cytidine TP	--	C	NO
4'-Ethynylcytidine TP	--	C	NO
5-(1-Propynyl)ara-cytidine TP	--	C	NO
5-(2-Chloro-phenyl)-2-thiocytidine TP	--	C	NO
5-(4-Amino-phenyl)-2-thiocytidine TP	--	C	NO
5-Aminoallyl-CTP	--	C	NO
5-Cyanocytidine TP	--	C	NO
5-Ethynylara-cytidine TP	--	C	NO
5-Ethynylcytidine TP	--	C	NO
5'-Homo-cytidine TP	--	C	NO
5-Methoxycytidine TP	--	C	NO
5-Trifluoromethyl-Cytidine TP	--	C	NO
N4-Amino-cytidine TP	--	C	NO
N4-Benzoyl-cytidine TP	--	C	NO
pseudoisocytidine	--	C	NO
7-methylguanosine	m7G	G	YES
N2,2′-O-dimethylguanosine	m2Gm	G	YES
N2-methylguanosine	m2G	G	YES
wyosine	imG	G	YES
1,2′-O-dimethylguanosine	m1Gm	G	YES
1-methylguanosine	m1G	G	YES
2′-O-methylguanosine	Gm	G	YES

2′-O-ribosylguanosine (phosphate)	Gr(p)	G	YES
2'-O-methylguanosine	Gm	G	YES
2'-O-ribosylguanosine (phosphate)	Gr(p)	G	YES
7-aminomethyl-7-deazaguanosine	preQ1	G	YES
7-cyano-7-deazaguanosine	preQ0	G	YES
archaeosine	G+	G	YES
methylwyosine	mimG	G	YES
N2,7-dimethylguanosine	m2,7G	G	YES
N2,N2,2′-O-trimethylguanosine	m22Gm	G	YES
N2,N2,7-trimethylguanosine	m2,2,7G	G	YES
N2,N2-dimethylguanosine	m22G	G	YES
N^2,7,2'-O-trimethylguanosine	$m^{2,7}$Gm	G	YES
6-thio-guanosine	--	G	NO
7-deaza-guanosine	--	G	NO
8-oxo-guanosine	--	G	NO
N1-methyl-guanosine	--	G	NO
α-thio-guanosine	--	G	NO
2 (propyl)guanine	--	G	NO
2-(alkyl)guanine	--	G	NO
2’-Amino-2’-deoxy-GTP	--	G	NO
2’-Azido-2’-deoxy-GTP	--	G	NO
2'-Deoxy-2'-a-aminoguanosine TP	--	G	NO
2'-Deoxy-2'-a-azidoguanosine TP	--	G	NO
6 (methyl)guanine	--	G	NO
6-(alkyl)guanine	--	G	NO
6-(methyl)guanine	--	G	NO
6-methyl-guanosine	--	G	NO
7 (alkyl)guanine	--	G	NO
7 (deaza)guanine	--	G	NO
7 (methyl)guanine	--	G	NO
7-(alkyl)guanine	--	G	NO
7-(deaza)guanine	--	G	NO
7-(methyl)guanine	--	G	NO
8 (alkyl)guanine	--	G	NO
8 (alkynyl)guanine	--	G	NO

8 (halo)guanine	--	G	NO
8 (thioalkyl)guanine	--	G	NO
8-(alkenyl)guanine	--	G	NO
8-(alkyl)guanine	--	G	NO
8-(alkynyl)guanine	--	G	NO
8-(amino)guanine	--	G	NO
8-(halo)guanine	--	G	NO
8-(hydroxyl)guanine	--	G	NO
8-(thioalkyl)guanine	--	G	NO
8-(thiol)guanine	--	G	NO
aza guanine	--	G	NO
deaza guanine	--	G	NO
N (methyl)guanine	--	G	NO
N-(methyl)guanine	--	G	NO
1-methyl-6-thio-guanosine	--	G	NO
6-methoxy-guanosine	--	G	NO
6-thio-7-deaza-8-aza-guanosine	--	G	NO
6-thio-7-deaza-guanosine	--	G	NO
6-thio-7-methyl-guanosine	--	G	NO
7-deaza-8-aza-guanosine	--	G	NO
7-methyl-8-oxo-guanosine	--	G	NO
N2,N2-dimethyl-6-thio-guanosine	--	G	NO
N2-methyl-6-thio-guanosine	--	G	NO
1-Me-GTP	--	G	NO
2'Fluoro-N2-isobutyl-guanosine TP	--	G	NO
2'O-methyl-N2-isobutyl-guanosine TP	--	G	NO
2'-a-Ethynylguanosine TP	--	G	NO
2'-a-Trifluoromethylguanosine TP	--	G	NO
2'-b-Ethynylguanosine TP	--	G	NO
2'-b-Trifluoromethylguanosine TP	--	G	NO
2'-Deoxy-2',2'-difluoroguanosine TP	--	G	NO
2'-Deoxy-2'-a-mercaptoguanosine TP	--	G	NO

2'-Deoxy-2'-a-thiomethoxyguanosine TP	--	G	NO
2'-Deoxy-2'-b-aminoguanosine TP	--	G	NO
2'-Deoxy-2'-b-azidoguanosine TP	--	G	NO
2'-Deoxy-2'-b-bromoguanosine TP	--	G	NO
2'-Deoxy-2'-b-chloroguanosine TP	--	G	NO
2'-Deoxy-2'-b-fluoroguanosine TP	--	G	NO
2'-Deoxy-2'-b-iodoguanosine TP	--	G	NO
2'-Deoxy-2'-b-mercaptoguanosine TP	--	G	NO
2'-Deoxy-2'-b-thiomethoxyguanosine TP	--	G	NO
4'-Azidoguanosine TP	--	G	NO
4'-Carbocyclic guanosine TP	--	G	NO
4'-Ethynylguanosine TP	--	G	NO
5'-Homo-guanosine TP	--	G	NO
8-bromo-guanosine TP	--	G	NO
9-Deazaguanosine TP	--	G	NO
N2-isobutyl-guanosine TP	--	G	NO
1-methylinosine	m1I	I	YES
inosine	I	I	YES
1,2′-O-dimethylinosine	m1Im	I	YES
2′-O-methylinosine	Im	I	YES
7-methylinosine		I	NO
2'-O-methylinosine	Im	I	YES
epoxyqueuosine	oQ	Q	YES
galactosyl-queuosine	galQ	Q	YES
mannosylqueuosine	manQ	Q	YES
queuosine	Q	Q	YES
allyamino-thymidine	--	T	NO
aza thymidine	--	T	NO
deaza thymidine	--	T	NO
deoxy-thymidine	--	T	NO
2'-O-methyluridine	--	U	YES
2-thiouridine	s2U	U	YES
3-methyluridine	m3U	U	YES
5-carboxymethyluridine	cm5U	U	YES
5-hydroxyuridine	ho5U	U	YES
5-methyluridine	m5U	U	YES

5-taurinomethyl-2-thiouridine	τm5s2U	U	YES
5-taurinomethyluridine	τm5U	U	YES
dihydrouridine	D	U	YES
pseudouridine	Ψ	U	YES
(3-(3-amino-3-carboxypropyl)uridine	acp3U	U	YES
1-methyl-3-(3-amino-5-carboxypropyl)pseudouridine	m1acp3Ψ	U	YES
1-methylpseduouridine	m1Ψ	U	YES
1-methyl-pseudouridine	--	U	YES
2′-O-methyluridine	Um	U	YES
2'-O-methylpseudouridine	Ψm	U	YES
2'-O-methyluridine	Um	U	YES
2-thio-2′-O-methyluridine	s2Um	U	YES
3-(3-amino-3-carboxypropyl)uridine	acp3U	U	YES
3,2′-O-dimethyluridine	m3Um	U	YES
3-Methyl-pseudo-Uridine TP	--	U	YES
4-thiouridine	s4U	U	YES
5-(carboxyhydroxymethyl)uridine	chm5U	U	YES
5-(carboxyhydroxymethyl)uridine methyl ester	mchm5U	U	YES
5,2′-O-dimethyluridine	m5Um	U	YES
5,6-dihydro-uridine	--	U	YES
5-aminomethyl-2-thiouridine	nm5s2U	U	YES
5-carbamoylmethyl-2′-O-methyluridine	ncm5Um	U	YES
5-carbamoylmethyluridine	ncm5U	U	YES
5-carboxyhydroxymethyluridine	--	U	YES
5-carboxyhydroxymethyluridine methyl ester	--	U	YES
5-carboxymethylaminomethyl-2′-O-methyluridine	cmnm5Um	U	YES
5-carboxymethylaminomethyl-2-thiouridine	cmnm5s2U	U	YES
5-carboxymethylaminomethyl-2-thiouridine	--	U	YES
5-carboxymethylaminomethyluridine	cmnm5U	U	YES
5-carboxymethylaminomethyluridine	--	U	YES
5-Carbamoylmethyluridine TP	--	U	YES
5-methoxycarbonylmethyl-2′-O-methyluridine	mcm5Um	U	YES
5-methoxycarbonylmethyl-2-thiouridine	mcm5s2U	U	YES
5-methoxycarbonylmethyluridine	mcm5U	U	YES
5-methoxyuridine	mo5U	U	YES
5-methyl-2-thiouridine	m5s2U	U	YES

5-methylaminomethyl-2-selenouridine	mnm5se2U	U	YES
5-methylaminomethyl-2-thiouridine	mnm5s2U	U	YES
5-methylaminomethyluridine	mnm5U	U	YES
5-Methyldihydrouridine	--	U	YES
5-Oxyacetic acid- Uridine TP	--	U	YES
5-Oxyacetic acid-methyl ester-Uridine TP	--	U	YES
N1-methyl-pseudo-uridine	--	U	YES
uridine 5-oxyacetic acid	cmo5U	U	YES
uridine 5-oxyacetic acid methyl ester	mcmo5U	U	YES
3-(3-Amino-3-carboxypropyl)-Uridine TP	--	U	YES
5-(iso-Pentenylaminomethyl)- 2-thiouridine TP	--	U	YES
5-(iso-Pentenylaminomethyl)-2'-O-methyluridine TP	--	U	YES
5-(iso-Pentenylaminomethyl)uridine TP	--	U	YES
5-propynyl uracil	--	U	NO
α-thio-uridine	--	U	NO
1 (aminoalkylamino-carbonylethylenyl)-2(thio)-pseudouracil	--	U	NO
1 (aminoalkylaminocarbonylethylenyl)-2,4-(dithio)pseudouracil	--	U	NO
1 (aminoalkylaminocarbonylethylenyl)-4 (thio)pseudouracil	--	U	NO
1 (aminoalkylaminocarbonylethylenyl)-pseudouracil	--	U	NO
1 (aminocarbonylethylenyl)-2(thio)-pseudouracil	--	U	NO
1 (aminocarbonylethylenyl)-2,4-(dithio)pseudouracil	--	U	NO
1 (aminocarbonylethylenyl)-4 (thio)pseudouracil	--	U	NO
1 (aminocarbonylethylenyl)-pseudouracil	--	U	NO
1 substituted 2(thio)-pseudouracil	--	U	NO
1 substituted 2,4-(dithio)pseudouracil	--	U	NO
1 substituted 4 (thio)pseudouracil	--	U	NO
1 substituted pseudouracil	--	U	NO
1-(aminoalkylamino-carbonylethylenyl)-2-(thio)-pseudouracil	--	U	NO
1-Methyl-3-(3-amino-3-carboxypropyl) pseudouridine TP	--	U	NO
1-Methyl-3-(3-amino-3-carboxypropyl)pseudo-UTP	--	U	NO

1-Methyl-pseudo-UTP	--	U	NO
2 (thio)pseudouracil	--	U	NO
2' deoxy uridine	--	U	NO
2' fluorouridine	--	U	NO
2-(thio)uracil	--	U	NO
2,4-(dithio)psuedouracil	--	U	NO
2' methyl, 2'amino, 2'azido, 2'fluro-guanosine	--	U	NO
2'-Amino-2'-deoxy-UTP	--	U	NO
2'-Azido-2'-deoxy-UTP	--	U	NO
2'-Azido-deoxyuridine TP	--	U	NO
2'-O-methylpseudouridine	--	U	NO
2′ deoxy uridine	2′ dU	U	NO
2′ fluorouridine	--	U	NO
2'-Deoxy-2'-a-aminouridine TP	--	U	NO
2'-Deoxy-2'-a-azidouridine TP	--	U	NO
2-methylpseudouridine	m3Ψ	U	NO
3 (3 amino-3 carboxypropyl)uracil	--	U	NO
4 (thio)pseudouracil	--	U	NO
4-(thio)pseudouracil	--	U	NO
4-(thio)uracil	--	U	NO
4-thiouracil	--	U	NO
5 (1,3-diazole-1-alkyl)uracil	--	U	NO
5 (2-aminopropyl)uracil	--	U	NO
5 (aminoalkyl)uracil	--	U	NO
5 (dimethylaminoalkyl)uracil	--	U	NO
5 (guanidiniumalkyl)uracil	--	U	NO
5 (methoxycarbonylmethyl)-2-(thio)uracil	--	U	NO
5 (methoxycarbonyl-methyl)uracil	--	U	NO
5 (methyl) 2 (thio)uracil	--	U	NO
5 (methyl) 2,4 (dithio)uracil	--	U	NO
5 (methyl) 4 (thio)uracil	--	U	NO
5 (methylaminomethyl)-2 (thio)uracil	--	U	NO

5 (methylaminomethyl)-2,4 (dithio)uracil	--	U	NO
5 (methylaminomethyl)-4 (thio)uracil	--	U	NO
5 (propynyl)uracil	--	U	NO
5 (trifluoromethyl)uracil	--	U	NO
5-(2-aminopropyl)uracil	--	U	NO
5-(alkyl)-2-(thio)pseudouracil	--	U	NO
5-(alkyl)-2,4 (dithio)pseudouracil	--	U	NO
5-(alkyl)-4 (thio)pseudouracil	--	U	NO
5-(alkyl)pseudouracil	--	U	NO
5-(alkyl)uracil	--	U	NO
5-(alkynyl)uracil	--	U	NO
5-(allylamino)uracil	--	U	NO
5-(cyanoalkyl)uracil	--	U	NO
5-(dialkylaminoalkyl)uracil	--	U	NO
5-(dimethylaminoalkyl)uracil	--	U	NO
5-(guanidiniumalkyl)uracil	--	U	NO
5-(halo)uracil	--	U	NO
5-(l,3-diazole-l-alkyl)uracil	--	U	NO
5-(methoxy)uracil	--	U	NO
5-(methoxycarbonylmethyl)-2-(thio)uracil	--	U	NO
5-(methoxycarbonyl-methyl)uracil	--	U	NO
5-(methyl) 2(thio)uracil	--	U	NO
5-(methyl) 2,4 (dithio)uracil	--	U	NO
5-(methyl) 4 (thio)uracil	--	U	NO
5-(methyl)-2-(thio)pseudouracil	--	U	NO
5-(methyl)-2,4 (dithio)pseudouracil	--	U	NO
5-(methyl)-4 (thio)pseudouracil	--	U	NO
5-(methyl)pseudouracil	--	U	NO
5-(methylaminomethyl)-2 (thio)uracil	--	U	NO
5-(methylaminomethyl)-2,4(dithio)uracil	--	U	NO
5-(methylaminomethyl)-4-(thio)uracil	--	U	NO
5-(propynyl)uracil	--	U	NO

5-(trifluoromethyl)uracil	--	U	NO
5-aminoallyl-uridine	--	U	NO
5-bromo-uridine	--	U	NO
5-iodo-uridine	--	U	NO
5-uracil	--	U	NO
6 (azo)uracil	--	U	NO
6-(azo)uracil	--	U	NO
6-aza-uridine	--	U	NO
allyamino-uracil	--	U	NO
aza uracil	--	U	NO
deaza uracil	--	U	NO
N3 (methyl)uracil	--	U	NO
P seudo-UTP-1-2-ethanoic acid	--	U	NO
pseudouracil	--	U	NO
4-Thio-pseudo-UTP	--	U	NO
1-carboxymethyl-pseudouridine	--	U	NO
1-methyl-1-deaza-pseudouridine	--	U	NO
1-propynyl-uridine	--	U	NO
1-taurinomethyl-1-methyl-uridine	--	U	NO
1-taurinomethyl-4-thio-uridine	--	U	NO
1-taurinomethyl-pseudouridine	--	U	NO
2-methoxy-4-thio-pseudouridine	--	U	NO
2-thio-1-methyl-1-deaza-pseudouridine	--	U	NO
2-thio-1-methyl-pseudouridine	--	U	NO
2-thio-5-aza-uridine	--	U	NO
2-thio-dihydropseudouridine	--	U	NO
2-thio-dihydrouridine	--	U	NO
2-thio-pseudouridine	--	U	NO
4-methoxy-2-thio-pseudouridine	--	U	NO
4-methoxy-pseudouridine	--	U	NO
4-thio-1-methyl-pseudouridine	--	U	NO
4-thio-pseudouridine	--	U	NO

5-aza-uridine	--	U	NO
dihydropseudouridine	--	U	NO
(±)1-(2-Hydroxypropyl)pseudouridine TP	--	U	NO
(2R)-1-(2-Hydroxypropyl)pseudouridine TP	--	U	NO
(2S)-1-(2-Hydroxypropyl)pseudouridine TP	--	U	NO
(E)-5-(2-Bromo-vinyl)ara-uridine TP	--	U	NO
(E)-5-(2-Bromo-vinyl)uridine TP	--	U	NO
(Z)-5-(2-Bromo-vinyl)ara-uridine TP	--	U	NO
(Z)-5-(2-Bromo-vinyl)uridine TP	--	U	NO
1-(2,2,2-Trifluoroethyl)-pseudo-UTP	--	U	NO
1-(2,2,3,3,3-Pentafluoropropyl)pseudouridine TP	--	U	NO
1-(2,2-Diethoxyethyl)pseudouridine TP	--	U	NO
1-(2,4,6-Trimethylbenzyl)pseudouridine TP	--	U	NO
1-(2,4,6-Trimethyl-benzyl)pseudo-UTP	--	U	NO
1-(2,4,6-Trimethyl-phenyl)pseudo-UTP	--	U	NO
1-(2-Amino-2-carboxyethyl)pseudo-UTP	--	U	NO
1-(2-Amino-ethyl)pseudo-UTP	--	U	NO
1-(2-Hydroxyethyl)pseudouridine TP	--	U	NO
1-(2-Methoxyethyl)pseudouridine TP	--	U	NO
1-(3,4-Bis-trifluoromethoxybenzyl)pseudouridine TP	--	U	NO
1-(3,4-Dimethoxybenzyl)pseudouridine TP	--	U	NO
1-(3-Amino-3-carboxypropyl)pseudo-UTP	--	U	NO
1-(3-Amino-propyl)pseudo-UTP	--	U	NO
1-(3-Cyclopropyl-prop-2-ynyl)pseudouridine TP	--	U	NO
1-(4-Amino-4-carboxybutyl)pseudo-UTP	--	U	NO
1-(4-Amino-benzyl)pseudo-UTP	--	U	NO
1-(4-Amino-butyl)pseudo-UTP	--	U	NO
1-(4-Amino-phenyl)pseudo-UTP	--	U	NO
1-(4-Azidobenzyl)pseudouridine TP	--	U	NO
1-(4-Bromobenzyl)pseudouridine TP	--	U	NO

1-(4-Chlorobenzyl)pseudouridine TP	--	U	NO
1-(4-Fluorobenzyl)pseudouridine TP	--	U	NO
1-(4-Iodobenzyl)pseudouridine TP	--	U	NO
1-(4-Methanesulfonylbenzyl)pseudouridine TP	--	U	NO
1-(4-Methoxybenzyl)pseudouridine TP	--	U	NO
1-(4-Methoxy-benzyl)pseudo-UTP	--	U	NO
1-(4-Methoxy-phenyl)pseudo-UTP	--	U	NO
1-(4-Methylbenzyl)pseudouridine TP	--	U	NO
1-(4-Methyl-benzyl)pseudo-UTP	--	U	NO
1-(4-Nitrobenzyl)pseudouridine TP	--	U	NO
1-(4-Nitro-benzyl)pseudo-UTP	--	U	NO
1(4-Nitro-phenyl)pseudo-UTP	--	U	NO
1-(4-Thiomethoxybenzyl)pseudouridine TP	--	U	NO
1-(4-Trifluoromethoxybenzyl)pseudouridine TP	--	U	NO
1-(4-Trifluoromethylbenzyl)pseudouridine TP	--	U	NO
1-(5-Amino-pentyl)pseudo-UTP	--	U	NO
1-(6-Amino-hexyl)pseudo-UTP	--	U	NO
1,6-Dimethyl-pseudo-UTP	--	U	NO
1-[3-(2-{2-[2-(2-Aminoethoxy)-ethoxy]-ethoxy}-ethoxy)-propionyl]pseudouridine TP	--	U	NO
1-{3-[2-(2-Aminoethoxy)-ethoxy]-propionyl } pseudouridine TP	--	U	NO
1-Acetylpseudouridine TP	--	U	NO
1-Alkyl-6-(1-propynyl)-pseudo-UTP	--	U	NO
1-Alkyl-6-(2-propynyl)-pseudo-UTP	--	U	NO
1-Alkyl-6-allyl-pseudo-UTP	--	U	NO
1-Alkyl-6-ethynyl-pseudo-UTP	--	U	NO
1-Alkyl-6-homoallyl-pseudo-UTP	--	U	NO
1-Alkyl-6-vinyl-pseudo-UTP	--	U	NO
1-Allylpseudouridine TP	--	U	NO
1-Aminomethyl-pseudo-UTP	--	U	NO
1-Benzoylpseudouridine TP	--	U	NO

1-Benzyloxymethylpseudouridine TP	--	U	NO
1-Benzyl-pseudo-UTP	--	U	NO
1-Biotinyl-PEG2-pseudouridine TP	--	U	NO
1-Biotinylpseudouridine TP	--	U	NO
1-Butyl-pseudo-UTP	--	U	NO
1-Cyanomethylpseudouridine TP	--	U	NO
1-Cyclobutylmethyl-pseudo-UTP	--	U	NO
1-Cyclobutyl-pseudo-UTP	--	U	NO
1-Cycloheptylmethyl-pseudo-UTP	--	U	NO
1-Cycloheptyl-pseudo-UTP	--	U	NO
1-Cyclohexylmethyl-pseudo-UTP	--	U	NO
1-Cyclohexyl-pseudo-UTP	--	U	NO
1-Cyclooctylmethyl-pseudo-UTP	--	U	NO
1-Cyclooctyl-pseudo-UTP	--	U	NO
1-Cyclopentylmethyl-pseudo-UTP	--	U	NO
1-Cyclopentyl-pseudo-UTP	--	U	NO
1-Cyclopropylmethyl-pseudo-UTP	--	U	NO
1-Cyclopropyl-pseudo-UTP	--	U	NO
1-Ethyl-pseudo-UTP	--	U	NO
1-Hexyl-pseudo-UTP	--	U	NO
1-Homoallylpseudouridine TP	--	U	NO
1-Hydroxymethylpseudouridine TP	--	U	NO
1-iso-propyl-pseudo-UTP	--	U	NO
1-Me-2-thio-pseudo-UTP	--	U	NO
1-Me-4-thio-pseudo-UTP	--	U	NO
1-Me-alpha-thio-pseudo-UTP	--	U	NO
1-Methanesulfonylmethylpseudouridine TP	--	U	NO
1-Methoxymethylpseudouridine TP	--	U	NO
1-Methyl-6-(2,2,2-Trifluoroethyl)pseudo-UTP	--	U	NO
1-Methyl-6-(4-morpholino)-pseudo-UTP	--	U	NO
1-Methyl-6-(4-thiomorpholino)-pseudo-UTP	--	U	NO

1-Methyl-6-(substituted phenyl)pseudo-UTP	--	U	NO
1-Methyl-6-amino-pseudo-UTP	--	U	NO
1-Methyl-6-azido-pseudo-UTP	--	U	NO
1-Methyl-6-bromo-pseudo-UTP	--	U	NO
1-Methyl-6-butyl-pseudo-UTP	--	U	NO
1-Methyl-6-chloro-pseudo-UTP	--	U	NO
1-Methyl-6-cyano-pseudo-UTP	--	U	NO
1-Methyl-6-dimethylamino-pseudo-UTP	--	U	NO
1-Methyl-6-ethoxy-pseudo-UTP	--	U	NO
1-Methyl-6-ethylcarboxylate-pseudo-UTP	--	U	NO
1-Methyl-6-ethyl-pseudo-UTP	--	U	NO
1-Methyl-6-fluoro-pseudo-UTP	--	U	NO
1-Methyl-6-formyl-pseudo-UTP	--	U	NO
1-Methyl-6-hydroxyamino-pseudo-UTP	--	U	NO
1-Methyl-6-hydroxy-pseudo-UTP	--	U	NO
1-Methyl-6-iodo-pseudo-UTP	--	U	NO
1-Methyl-6-iso-propyl-pseudo-UTP	--	U	NO
1-Methyl-6-methoxy-pseudo-UTP	--	U	NO
1-Methyl-6-methylamino-pseudo-UTP	--	U	NO
1-Methyl-6-phenyl-pseudo-UTP	--	U	NO
1-Methyl-6-propyl-pseudo-UTP	--	U	NO
1-Methyl-6-tert-butyl-pseudo-UTP	--	U	NO
1-Methyl-6-trifluoromethoxy-pseudo-UTP	--	U	NO
1-Methyl-6-trifluoromethyl-pseudo-UTP	--	U	NO
1-Morpholinomethylpseudouridine TP	--	U	NO
1-Pentyl-pseudo-UTP	--	U	NO
1-Phenyl-pseudo-UTP	--	U	NO
1-Pivaloylpseudouridine TP	--	U	NO
1-Propargylpseudouridine TP	--	U	NO
1-Propyl-pseudo-UTP	--	U	NO
1-propynyl-pseudouridine	--	U	NO
1-p-tolyl-pseudo-UTP	--	U	NO

1-tert-Butyl-pseudo-UTP	--	U	NO
1-Thiomethoxymethylpseudouridine TP	--	U	NO
1-Thiomorpholinomethylpseudouridine TP	--	U	NO
1-Trifluoroacetylpseudouridine TP	--	U	NO
1-Trifluoromethyl-pseudo-UTP	--	U	NO
1-Vinylpseudouridine TP	--	U	NO
2,2'-anhydro-uridine TP	--	U	NO
2'-bromo-deoxyuridine TP	--	U	NO
2'-F-5-Methyl-2'-deoxy-UTP	--	U	NO
2'-OMe-5-Me-UTP	--	U	NO
2'-OMe-pseudo-UTP	--	U	NO
2'-a-Ethynyluridine TP	--	U	NO
2'-a-Trifluoromethyluridine TP	--	U	NO
2'-b-Ethynyluridine TP	--	U	NO
2'-b-Trifluoromethyluridine TP	--	U	NO
2'-Deoxy-2',2'-difluorouridine TP	--	U	NO
2'-Deoxy-2'-a-mercaptouridine TP	--	U	NO
2'-Deoxy-2'-a-thiomethoxyuridine TP	--	U	NO
2'-Deoxy-2'-b-aminouridine TP	--	U	NO
2'-Deoxy-2'-b-azidouridine TP	--	U	NO
2'-Deoxy-2'-b-bromouridine TP	--	U	NO
2'-Deoxy-2'-b-chlorouridine TP	--	U	NO
2'-Deoxy-2'-b-fluorouridine TP	--	U	NO
2'-Deoxy-2'-b-iodouridine TP	--	U	NO
2'-Deoxy-2'-b-mercaptouridine TP	--	U	NO
2'-Deoxy-2'-b-thiomethoxyuridine TP	--	U	NO
2-methoxy-4-thio-uridine	--	U	NO
2-methoxyuridine	--	U	NO
2'-O-Methyl-5-(1-propynyl)uridine TP	--	U	NO
3-Alkyl-pseudo-UTP	--	U	NO
4'-Azidouridine TP	--	U	NO
4'-Carbocyclic uridine TP	--	U	NO

4'-Ethynyluridine TP	--	U	NO
5-(1-Propynyl)ara-uridine TP	--	U	NO
5-(2-Furanyl)uridine TP	--	U	NO
5-Cyanouridine TP	--	U	NO
5-Dimethylaminouridine TP	--	U	NO
5'-Homo-uridine TP	--	U	NO
5-iodo-2'-fluoro-deoxyuridine TP	--	U	NO
5-Phenylethynyluridine TP	--	U	NO
5-Trideuteromethyl-6-deuterouridine TP	--	U	NO
5-Trifluoromethyl-Uridine TP	--	U	NO
5-Vinylarauridine TP	--	U	NO
6-(2,2,2-Trifluoroethyl)-pseudo-UTP	--	U	NO
6-(4-Morpholino)-pseudo-UTP	--	U	NO
6-(4-Thiomorpholino)-pseudo-UTP	--	U	NO
6-(Substituted-Phenyl)-pseudo-UTP	--	U	NO
6-Amino-pseudo-UTP	--	U	NO
6-Azido-pseudo-UTP	--	U	NO
6-Bromo-pseudo-UTP	--	U	NO
6-Butyl-pseudo-UTP	--	U	NO
6-Chloro-pseudo-UTP	--	U	NO
6-Cyano-pseudo-UTP	--	U	NO
6-Dimethylamino-pseudo-UTP	--	U	NO
6-Ethoxy-pseudo-UTP	--	U	NO
6-Ethylcarboxylate-pseudo-UTP	--	U	NO
6-Ethyl-pseudo-UTP	--	U	NO
6-Fluoro-pseudo-UTP	--	U	NO
6-Formyl-pseudo-UTP	--	U	NO
6-Hydroxyamino-pseudo-UTP	--	U	NO
6-Hydroxy-pseudo-UTP	--	U	NO
6-Iodo-pseudo-UTP	--	U	NO
6-iso-Propyl-pseudo-UTP	--	U	NO
6-Methoxy-pseudo-UTP	--	U	NO

6-Methylamino-pseudo-UTP	--	U	NO
6-Methyl-pseudo-UTP	--	U	NO
6-Phenyl-pseudo-UTP	--	U	NO
6-Phenyl-pseudo-UTP	--	U	NO
6-Propyl-pseudo-UTP	--	U	NO
6-tert-Butyl-pseudo-UTP	--	U	NO
6-Trifluoromethoxy-pseudo-UTP	--	U	NO
6-Trifluoromethyl-pseudo-UTP	--	U	NO
Alpha-thio-pseudo-UTP	--	U	NO
Pseudouridine 1-(4-methylbenzenesulfonic acid) TP	--	U	NO
Pseudouridine 1-(4-methylbenzoic acid) TP	--	U	NO
Pseudouridine TP 1-[3-(2-ethoxy)]propionic acid	--	U	NO
Pseudouridine TP 1-[3-{2-(2-[2-(2-ethoxy)-ethoxy]-ethoxy)-ethoxy}]propionic acid	--	U	NO
Pseudouridine TP 1-[3-{2-(2-[2-{2(2-ethoxy)-ethoxy}-ethoxy]-ethoxy)-ethoxy}]propionic acid	--	U	NO
Pseudouridine TP 1-[3-{2-(2-[2-ethoxy]-ethoxy)-ethoxy}]propionic acid	--	U	NO
Pseudouridine TP 1-[3-{2-(2-ethoxy)-ethoxy}] propionic acid	--	U	NO
Pseudouridine TP 1-methylphosphonic acid	--	U	NO
Pseudouridine TP 1-methylphosphonic acid diethyl ester	--	U	NO
Pseudo-UTP-N1-3-propionic acid	--	U	NO
Pseudo-UTP-N1-4-butanoic acid	--	U	NO
Pseudo-UTP-N1-5-pentanoic acid	--	U	NO
Pseudo-UTP-N1-6-hexanoic acid	--	U	NO
Pseudo-UTP-N1-7-heptanoic acid	--	U	NO
Pseudo-UTP-N1-methyl-p-benzoic acid	--	U	NO
Pseudo-UTP-N1-p-benzoic acid	--	U	NO
wybutosine	yW	W	YES
hydroxywybutosine	OHyW	W	YES
isowyosine	imG2	W	YES
peroxywybutosine	o2yW	W	YES
undermodified hydroxywybutosine	OHyW*	W	YES

4-demethylwyosine	imG-14	W	YES

[000508] Other modifications which may be useful in the polynucleotides of the present invention are listed in Table 5.

Table 5. Additional Modification types

Name	Type
2,6-(diamino)purine	Other
1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl	Other
1,3-(diaza)-2-(oxo)-phenthiazin-l-yl	Other
1,3-(diaza)-2-(oxo)-phenoxazin-1-yl	Other
1,3,5-(triaza)-2,6-(dioxa)-naphthalene	Other
2 (amino)purine	Other
2,4,5-(trimethyl)phenyl	Other
2‘ methyl, 2’amino, 2’azido, 2’fluro-cytidine	Other
2’ methyl, 2’amino, 2’azido, 2’fluro-adenine	Other
2’methyl, 2’amino, 2’azido, 2’fluro-uridine	Other
2'-amino-2'-deoxyribose	Other
2-amino-6-Chloro-purine	Other
2-aza-inosinyl	Other
2'-azido-2'-deoxyribose	Other
2'fluoro-2'-deoxyribose	Other
2'-fluoro-modified bases	Other
2'-O-methyl-ribose	Other
2-oxo-7-aminopyridopyrimidin-3-yl	Other
2-oxo-pyridopyrimidine-3-yl	Other
2-pyridinone	Other
3 nitropyrrole	Other
3-(methyl)-7-(propynyl)isocarbostyrilyl	Other
3-(methyl)isocarbostyrilyl	Other
4-(fluoro)-6-(methyl)benzimidazole	Other
4-(methyl)benzimidazole	Other
4-(methyl)indolyl	Other
4,6-(dimethyl)indolyl	Other
5 nitroindole	Other
5 substituted pyrimidines	Other
5-(methyl)isocarbostyrilyl	Other

5-nitroindole	Other
6-(aza)pyrimidine	Other
6-(azo)thymine	Other
6-(methyl)-7-(aza)indolyl	Other
6-chloro-purine	Other
6-phenyl-pyrrolo-pyrimidin-2-on-3-yl	Other
7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-l-yl	Other
7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl	Other
7-(aminoalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenoxazin-1-yl	Other
7-(aminoalkylhydroxy)-l,3-(diaza)-2-(oxo)-phenthiazin-l-yl	Other
7-(aminoalkylhydroxy)-l,3-(diaza)-2-(oxo)-phenoxazin-l-yl	Other
7-(aza)indolyl	Other
7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazinl-yl	Other
7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-l-yl	Other
7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl	Other
7-(guanidiniumalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenoxazin-1-yl	Other
7-(guanidiniumalkyl-hydroxy)-l,3-(diaza)-2-(oxo)-phenthiazin-l-yl	Other
7-(guanidiniumalkylhydroxy)-l,3-(diaza)-2-(oxo)-phenoxazin-l-yl	Other
7-(propynyl)isocarbostyrilyl	Other
7-(propynyl)isocarbostyrilyl, propynyl-7-(aza)indolyl	Other
7-deaza-inosinyl	Other
7-substituted 1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl	Other
7-substituted 1,3-(diaza)-2-(oxo)-phenoxazin-1-yl	Other
9-(methyl)-imidizopyridinyl	Other
aminoindolyl	Other
anthracenyl	Other
bis-ortho-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl	Other
bis-ortho-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl	Other

difluorotolyl	Other
hypoxanthine	Other
imidizopyridinyl	Other
inosinyl	Other
isocarbostyrilyl	Other
isoguanisine	Other
N2-substituted purines	Other
N6-methyl-2-amino-purine	Other
N6-substituted purines	Other
N-alkylated derivative	Other
napthalenyl	Other
nitrobenzimidazolyl	Other
nitroimidazolyl	Other
nitroindazolyl	Other
nitropyrazolyl	Other
nubularine	Other
O6-substituted purines	Other
O-alkylated derivative	Other
ortho-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl	Other
ortho-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl	Other
Oxoformycin TP	Other
para-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl	Other
para-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl	Other
pentacenyl	Other
phenanthracenyl	Other
phenyl	Other
propynyl-7-(aza)indolyl	Other
pyrenyl	Other
pyridopyrimidin-3-yl	Other
pyridopyrimidin-3-yl, 2-oxo-7-amino-pyridopyrimidin-3-yl	Other

pyrrolo-pyrimidin-2-on-3-yl	Other
pyrrolopyrimidinyl	Other
pyrrolopyrizinyl	Other
stilbenzyl	Other
substituted 1,2,4-triazoles	Other
tetracenyl	Other
tubercidine	Other
xanthine	Other
Xanthosine-5'-TP	Other
2-thio-zebularine	Other
5-aza-2-thio-zebularine	Other
7-deaza-2-amino-purine	Other
pyridin-4-one ribonucleoside	Other
2-Amino-riboside-TP	Other
Formycin A TP	Other
Formycin B TP	Other
Pyrrolosine TP	Other
2'-OH-ara-adenosine TP	Other
2'-OH-ara-cytidine TP	Other
2'-OH-ara-uridine TP	Other
2'-OH-ara-guanosine TP	Other
5-(2-carbomethoxyvinyl)uridine TP	Other
N6-(19-Amino-pentaoxanonadecyl)adenosine TP	Other

[000509] The polynucleotides can include any useful linker between the nucleosides. Such linkers, including backbone modifications are given in Table 6.

Table 6. Linker modifications

Name	**TYPE**
3'-alkylene phosphonates	Linker
3'-amino phosphoramidate	Linker
alkene containing backbones	Linker

aminoalkylphosphoramidates	Linker
aminoalkylphosphotriesters	Linker
boranophosphates	Linker
-CH2-0-N(CH3)-CH2-	Linker
-CH2-N(CH3)-N(CH3)-CH2-	Linker
-CH2-NH-CH2-	Linker
chiral phosphonates	Linker
chiral phosphorothioates	Linker
formacetyl and thioformacetyl backbones	Linker
methylene (methylimino)	Linker
methylene formacetyl and thioformacetyl backbones	Linker
methyleneimino and methylenehydrazino backbones	Linker
morpholino linkages	Linker
-N(CH3)-CH2-CH2-	Linker
oligonucleosides with heteroatom internucleoside linkage	Linker
phosphinates	Linker
phosphoramidates	Linker
phosphorodithioates	Linker
phosphorothioate internucleoside linkages	Linker
phosphorothioates	Linker
phosphotriesters	Linker
PNA	Linker
siloxane backbones	Linker
sulfamate backbones	Linker
sulfide sulfoxide and sulfone backbones	Linker
sulfonate and sulfonamide backbones	Linker
thionoalkylphosphonates	Linker
thionoalkylphosphotriesters	Linker
thionophosphoramidates	Linker

[000510] The polynucleotides can include any useful modification, such as to the sugar, the nucleobase, or the internucleoside linkage (*e.g.* to a linking phosphate / to a phosphodiester linkage / to the phosphodiester backbone). One or more atoms of a pyrimidine nucleobase may be replaced or substituted with optionally substituted amino, optionally substituted thiol, optionally substituted alkyl (e.g., methyl or ethyl), or halo (e.g., chloro or fluoro). In certain embodiments, modifications (e.g., one or more modifications) are present in each of the sugar and the internucleoside linkage. Modifications according to the present invention may be

modifications of ribonucleic acids (RNAs) to deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs) or hybrids thereof). Additional modifications are described herein.

[000511] In some embodiments, the polynucleotides of the invention do not substantially induce an innate immune response of a cell into which the mRNA is introduced. Featues of an induced innate immune response include 1) increased expression of pro-inflammatory cytokines, 2) activation of intracellular PRRs (RIG-I, MDA5, etc, and/or 3) termination or reduction in protein translation.

[000512] In certain embodiments, it may desirable to intracellularly degrade a polynulcleotide introduced into the cell. For example, degradation of a polynulcleotide may be preferable if precise timing of protein production is desired. Thus, in some embodiments, the invention provides a polynulcleotide containing a degradation domain, which is capable of being acted on in a directed manner within a cell.

[000513] Traditionally, the basic components of an mRNA molecule include at least a coding region, a 5′UTR, a 3′UTR, a 5′ cap and a poly-A tail. Building on this wild type modular structure, the present invention expands the scope of functionality of traditional mRNA molecules by providing polynucleotides which maintain a modular organization, but which comprise one or more structural and/or chemical modifications or alterations which impart useful properties to the polynucleotide including, in some embodiments, the lack of a substantial induction of the innate immune response of a cell into which the polynucleotides are introduced. As used herein, a "structural" feature or modification is one in which two or more linked nucleotides are inserted, deleted, duplicated, inverted or randomized in a polynucleotide without significant chemical modification to the nucleotides themselves. Because chemical bonds will necessarily be broken and reformed to effect a structural modification, structural modifications are of a chemical nature and hence are chemical modifications. However, structural modifications will result in a different sequence of nucleotides. For example, the polynucleotide "ATCG" may be chemically modified to "AT-5meC-G". The same polynucleotide may be structurally modified from "ATCG" to "ATCCCG". Here, the dinucleotide "CC" has been inserted, resulting in a structural modification to the polynucleotide.

[000514] Any of the regions of the polynucleotides may be chemically modified as taught herein or as taught in International Publication Number WO2013052523 (Attorney Docket

Number M9) and International Publication Number WO2014093924 (Attorney Docket Number M36) the contents of each of which are incoroporated herein by reference in its entirety.

Modified Polynucleotide Molecules

[000515] The present invention also includes building blocks, e.g., modified ribonucleosides, and modified ribonucleotides, of polynucleotide molecules. For example, these building blocks can be useful for preparing the polynucleotides of the invention. Such building blocks are taught in International Publication Number WO2013052523 (Attorney Docket Number M9) and International Publication Number WO2014093924 (Attorney Docket Number M36) the contents of each of which are incoroporated herein by reference in its entirety.

Modifications on the Sugar

[000516] The modified nucleosides and nucleotides (e.g., building block molecules), which may be incorporated into a polynucleotide (e.g., RNA or mRNA, as described herein), can be modified on the sugar of the ribonucleic acid. For example, the 2′ hydroxyl group (OH) can be modified or replaced with a number of different substituents. Exemplary substitutions at the 2′-position include, but are not limited to, H, halo, optionally substituted C_{1-6} alkyl; optionally substituted C_{1-6} alkoxy; optionally substituted C_{6-10} aryloxy; optionally substituted C_{3-8} cycloalkyl; optionally substituted C_{3-8} cycloalkoxy; optionally substituted C_{6-10} aryloxy; optionally substituted C_{6-10} aryl-C_{1-6} alkoxy, optionally substituted C_{1-12} (heterocyclyl)oxy; a sugar (e.g., ribose, pentose, or any described herein); a polyethyleneglycol (PEG), $-O(CH_2CH_2O)_nCH_2CH_2OR$, where R is H or optionally substituted alkyl, and n is an integer from 0 to 20 (e.g., from 0 to 4, from 0 to 8, from 0 to 10, from 0 to 16, from 1 to 4, from 1 to 8, from 1 to 10, from 1 to 16, from 1 to 20, from 2 to 4, from 2 to 8, from 2 to 10, from 2 to 16, from 2 to 20, from 4 to 8, from 4 to 10, from 4 to 16, and from 4 to 20); "locked" nucleic acids (LNA) in which the 2′-hydroxyl is connected by a C_{1-6} alkylene or C_{1-6} heteroalkylene bridge to the 4'-carbon of the same ribose sugar, where exemplary bridges included methylene, propylene, ether, or amino bridges; aminoalkyl, as defined herein; aminoalkoxy, as defined herein; amino as defined herein; and amino acid, as defined herein

[000517] Generally, RNA includes the sugar group ribose, which is a 5-membered ring having an oxygen. Exemplary, non-limiting modified nucleotides include replacement of the oxygen in ribose (e.g., with S, Se, or alkylene, such as methylene or ethylene); addition of a double bond (e.g., to replace ribose with cyclopentenyl or cyclohexenyl); ring contraction of ribose (e.g., to

form a 4-membered ring of cyclobutane or oxetane); ring expansion of ribose (e.g., to form a 6- or 7-membered ring having an additional carbon or heteroatom, such as for anhydrohexitol, altritol, mannitol, cyclohexanyl, cyclohexenyl, and morpholino that also has a phosphoramidate backbone); multicyclic forms (e.g., tricyclo; and "unlocked" forms, such as glycol nucleic acid (GNA) (e.g., R-GNA or S-GNA, where ribose is replaced by glycol units attached to phosphodiester bonds), threose nucleic acid (TNA, where ribose is replace with α-L-threofuranosyl-(3′→2′)) , and peptide nucleic acid (PNA, where 2-amino-ethyl-glycine linkages replace the ribose and phosphodiester backbone). The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a polynucleotide molecule can include nucleotides containing, e.g., arabinose, as the sugar. Such sugar modifications are taught International Publication Number WO2013052523 (Attorney Docket Number M9) and International Publication Number WO2014093924 (Attorney Docket Number M36) the contents of each of which are incoroporated herein by reference in its entirety.

Modifications on the Nucleobase

[000518] The present disclosure provides for modified nucleosides and nucleotides. As described herein "nucleoside" is defined as a compound containing a sugar molecule (e.g., a pentose or ribose) or a derivative thereof in combination with an organic base (e.g., a purine or pyrimidine) or a derivative thereof (also referred to herein as "nucleobase"). As described herein, "nucleotide" is defined as a nucleoside including a phosphate group. The modified nucleotides may by synthesized by any useful method, as described herein (e.g., chemically, enzymatically, or recombinantly to include one or more modified or non-natural nucleosides). The polynucleotides may comprise a region or regions of linked nucleosides. Such regions may have variable backbone linkages. The linkages may be standard phosphoester linkages, in which case the polynucleotides would comprise regions of nucleotides.

[000519] The modified nucleotide base pairing encompasses not only the standard adenosine-thymine, adenosine-uracil, or guanosine-cytosine base pairs, but also base pairs formed between nucleotides and/or modified nucleotides comprising non-standard or modified bases, wherein the arrangement of hydrogen bond donors and hydrogen bond acceptors permits hydrogen bonding between a non-standard base and a standard base or between two complementary non-standard

base structures. One example of such non-standard base pairing is the base pairing between the modified nucleotide inosine and adenine, cytosine or uracil.

[000520] The modified nucleosides and nucleotides can include a modified nucleobase. Examples of nucleobases found in RNA include, but are not limited to, adenine, guanine, cytosine, and uracil. Examples of nucleobase found in DNA include, but are not limited to, adenine, guanine, cytosine, and thymine. Such modified nucleobases (including the distinctions between naturally occurring and non-naturally occurring) are taught in International Publication Number WO2013052523 (Attorney Docket Number M9) and International Publication Number WO2014093924 (Attorney Docket Number M36) the contents of each of which are incoroporated herein by reference in its entirety.

Combinations of Modified Sugars, Nucleobases, and Internucleoside Linkages

[000521] The polynucleotides of the invention can include a combination of modifications to the sugar, the nucleobase, and/or the internucleoside linkage. These combinations can include any one or more modifications described herein.

[000522] Examples of modified nucleotides and modified nucleotide combinations are provided below in Tables 7 and 8. These combinations of modified nucleotides can be used to form the polynucleotides of the invention. Unless otherwise noted, the modified nucleotides may be completely substituted for the natural nucleotides of the polynucleotides of the invention. As a non-limiting example, the natural nucleotide uridine may be substituted with a modified nucleoside described herein. In another non-limiting example, the natural nucleotide uridine may be partially substituted (e.g., about 0.1%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99.9%) with at least one of the modified nucleoside disclosed herein. Any combination of base/sugar or linker may be incorporated into the polynucleotides of the invention and such modifcations are taught in International Publication Number WO2013052523 (Attorney Docket Number M9) and International Publication Number WO2014093924 (Attorney Docket Number M36); International Publication Number WO2015051173 (Attorney Docket Number M71); International Publication Number WO2015051169 (Attorney Docket Number M72); International Publication Number WO2015089511 (Attorney Docket Number M79), the contents of each of which are incoroporated herein by reference in its entirety.

Table 7. Combinations

Modified Nucleotide	Modified Nucleotide Combination
α-thio-cytidine	α-thio-cytidine/5-iodo-uridine
	α-thio-cytidine/N1-methyl-pseudouridine
	α-thio-cytidine/α-thio-uridine
	α-thio-cytidine/5-methyl-uridine
	α-thio-cytidine/pseudo-uridine
	about 50% of the cytosines are α-thio-cytidine
pseudoisocytidine	pseudoisocytidine/5-iodo-uridine
	pseudoisocytidine/N1-methyl-pseudouridine
	pseudoisocytidine/α-thio-uridine
	pseudoisocytidine/5-methyl-uridine
	pseudoisocytidine/pseudouridine
	about 25% of cytosines are pseudoisocytidine
	pseudoisocytidine/about 50% of uridines are N1-methyl-pseudouridine and about 50% of uridines are pseudouridine
	pseudoisocytidine/about 25% of uridines are N1-methyl-pseudouridine and about 25% of uridines are pseudouridine
pyrrolo-cytidine	pyrrolo-cytidine/5-iodo-uridine
	pyrrolo-cytidine/N1-methyl-pseudouridine
	pyrrolo-cytidine/α-thio-uridine
	pyrrolo-cytidine/5-methyl-uridine
	pyrrolo-cytidine/pseudouridine
	about 50% of the cytosines are pyrrolo-cytidine
5-methyl-cytidine	5-methyl-cytidine/5-iodo-uridine
	5-methyl-cytidine/N1-methyl-pseudouridine
	5-methyl-cytidine/α-thio-uridine
	5-methyl-cytidine/5-methyl-uridine
	5-methyl-cytidine/pseudouridine
	about 25% of cytosines are 5-methyl-cytidine
	about 50% of cytosines are 5-methyl-cytidine
	5-methyl-cytidine/5-methoxy-uridine
	5-methyl-cytidine/5-bromo-uridine
	5-methyl-cytidine/2-thio-uridine
	5-methyl-cytidine/about 50% of uridines are 2-thio-uridine
	about 50% of uridines are 5-methyl-cytidine/ about 50% of uridines are 2-thio-uridine
N4-acetyl-cytidine	N4-acetyl-cytidine /5-iodo-uridine
	N4-acetyl-cytidine /N1-methyl-pseudouridine
	N4-acetyl-cytidine /α-thio-uridine
	N4-acetyl-cytidine /5-methyl-uridine
	N4-acetyl-cytidine /pseudouridine
	about 50% of cytosines are N4-acetyl-cytidine
	about 25% of cytosines are N4-acetyl-cytidine
	N4-acetyl-cytidine /5-methoxy-uridine
	N4-acetyl-cytidine /5-bromo-uridine

	N4-acetyl-cytidine /2-thio-uridine
	about 50% of cytosines are N4-acetyl-cytidine/ about 50% of uridines are 2-thio-uridine

Table 8. Combinations

1-(2,2,2-Trifluoroethyl)pseudo-UTP
1-Ethyl-pseudo-UTP
1-Methyl-pseudo-U-alpha-thio-TP
1-methyl-pseudouridine TP, ATP, GTP, CTP
1-methyl-pseudo-UTP/5-methyl-CTP/ATP/GTP
1-methyl-pseudo-UTP/CTP/ATP/GTP
1-Propyl-pseudo-UTP
25 % 5-Aminoallyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Aminoallyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Bromo-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Bromo-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Bromo-CTP + 75 % CTP/1-Methyl-pseudo-UTP
25 % 5-Carboxy-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Carboxy-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Ethyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Ethyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Ethynyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Ethynyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Fluoro-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Fluoro-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Formyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Formyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Hydroxymethyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Hydroxymethyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Iodo-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Iodo-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Methoxy-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Methoxy-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Methyl-CTP + 75 % CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP
25 % 5-Methyl-CTP + 75 % CTP/25 % 5-Methoxy-UTP + 75 % UTP

25 % 5-Methyl-CTP + 75 % CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP
25 % 5-Methyl-CTP + 75 % CTP/50 % 5-Methoxy-UTP + 50 % UTP
25 % 5-Methyl-CTP + 75 % CTP/5-Methoxy-UTP
25 % 5-Methyl-CTP + 75 % CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP
25 % 5-Methyl-CTP + 75 % CTP/75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Phenyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Phenyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Trifluoromethyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % 5-Trifluoromethyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % 5-Trifluoromethyl-CTP + 75 % CTP/1-Methyl-pseudo-UTP
25 % N4-Ac-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % N4-Ac-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % N4-Bz-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % N4-Bz-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % N4-Methyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % N4-Methyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25 % Pseudo-iso-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
25 % Pseudo-iso-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
25% 5-Bromo-CTP/75% CTP/ Pseudo-UTP
25% 5-methoxy-UTP/25% 5-methyl-CTP/ATP/GTP
25% 5-methoxy-UTP/5-methyl-CTP/ATP/GTP
25% 5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP
25% 5-methoxy-UTP/CTP/ATP/GTP
25% 5-metoxy-UTP/50% 5-methyl-CTP/ATP/GTP
2-Amino-ATP
2-Thio-CTP
2-thio-pseudouridine TP, ATP, GTP, CTP
2-Thio-pseudo-UTP
2-Thio-UTP
3-Methyl-CTP
3-Methyl-pseudo-UTP
4-Thio-UTP
50 % 5-Bromo-CTP + 50 % CTP/1-Methyl-pseudo-UTP
50 % 5-Hydroxymethyl-CTP + 50 % CTP/1-Methyl-pseudo-UTP

50 % 5-methoxy-UTP/5-methyl-CTP/ATP/GTP
50 % 5-Methyl-CTP + 50 % CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP
50 % 5-Methyl-CTP + 50 % CTP/25 % 5-Methoxy-UTP + 75 % UTP
50 % 5-Methyl-CTP + 50 % CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP
50 % 5-Methyl-CTP + 50 % CTP/50 % 5-Methoxy-UTP + 50 % UTP
50 % 5-Methyl-CTP + 50 % CTP/5-Methoxy-UTP
50 % 5-Methyl-CTP + 50 % CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP
50 % 5-Methyl-CTP + 50 % CTP/75 % 5-Methoxy-UTP + 25 % UTP
50 % 5-Trifluoromethyl-CTP + 50 % CTP/1-Methyl-pseudo-UTP
50% 5-Bromo-CTP/ 50% CTP/Pseudo-UTP
50% 5-methoxy-UTP/25% 5-methyl-CTP/ATP/GTP
50% 5-methoxy-UTP/50% 5-methyl-CTP/ATP/GTP
50% 5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP
50% 5-methoxy-UTP/CTP/ATP/GTP
5-Aminoallyl-CTP
5-Aminoallyl-CTP/ 5-Methoxy-UTP
5-Aminoallyl-UTP
5-Bromo-CTP
5-Bromo-CTP/ 5-Methoxy-UTP
5-Bromo-CTP/1-Methyl-pseudo-UTP
5-Bromo-CTP/Pseudo-UTP
5-bromocytidine TP, ATP, GTP, UTP
5-Bromo-UTP
5-Carboxy-CTP/ 5-Methoxy-UTP
5-Ethyl-CTP/5-Methoxy-UTP
5-Ethynyl-CTP/5-Methoxy-UTP
5-Fluoro-CTP/ 5-Methoxy-UTP
5-Formyl-CTP/ 5-Methoxy-UTP
5-Hydroxy- methyl-CTP/ 5-Methoxy-UTP
5-Hydroxymethyl-CTP
5-Hydroxymethyl-CTP/1-Methyl-pseudo-UTP
5-Hydroxymethyl-CTP/5-Methoxy-UTP
5-hydroxymethyl-cytidine TP, ATP, GTP, UTP
5-Iodo-CTP/ 5-Methoxy-UTP

5-Me-CTP/5-Methoxy-UTP
5-Methoxy carbonyl methyl-UTP
5-Methoxy-CTP/5-Methoxy-UTP
5-methoxy-uridine TP, ATP, GTP, UTP
5-methoxy-UTP
5-Methoxy-UTP
5-Methoxy-UTP/ N6-Isopentenyl-ATP
5-methoxy-UTP/25% 5-methyl-CTP/ATP/GTP
5-methoxy-UTP/5-methyl-CTP/ATP/GTP
5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP
5-methoxy-UTP/CTP/ATP/GTP
5-Methyl-2-thio-UTP
5-Methylaminomethyl-UTP
5-Methyl-CTP/ 5-Methoxy-UTP
5-Methyl-CTP/ 5-Methoxy-UTP(cap 0)
5-Methyl-CTP/ 5-Methoxy-UTP(No cap)
5-Methyl-CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP
5-Methyl-CTP/25 % 5-Methoxy-UTP + 75 % UTP
5-Methyl-CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP
5-Methyl-CTP/50 % 5-Methoxy-UTP + 50 % UTP
5-Methyl-CTP/5-Methoxy-UTP/N6-Me-ATP
5-Methyl-CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP
5-Methyl-CTP/75 % 5-Methoxy-UTP + 25 % UTP
5-Phenyl-CTP/ 5-Methoxy-UTP
5-Trifluoro- methyl-CTP/ 5-Methoxy-UTP
5-Trifluoromethyl-CTP
5-Trifluoromethyl-CTP/ 5-Methoxy-UTP
5-Trifluoromethyl-CTP/1-Methyl-pseudo-UTP
5-Trifluoromethyl-CTP/Pseudo-UTP
5-Trifluoromethyl-UTP
5-trifluromethylcytidine TP, ATP, GTP, UTP
75 % 5-Aminoallyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Aminoallyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Bromo-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP

75 % 5-Bromo-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Carboxy-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Carboxy-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Ethyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Ethyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Ethynyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Ethynyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Fluoro-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Fluoro-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Formyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Formyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Hydroxymethyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Hydroxymethyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Iodo-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Iodo-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Methoxy-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Methoxy-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-methoxy-UTP/5-methyl-CTP/ATP/GTP
75 % 5-Methyl-CTP + 25 % CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP
75 % 5-Methyl-CTP + 25 % CTP/25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Methyl-CTP + 25 % CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP
75 % 5-Methyl-CTP + 25 % CTP/50 % 5-Methoxy-UTP + 50 % UTP
75 % 5-Methyl-CTP + 25 % CTP/5-Methoxy-UTP
75 % 5-Methyl-CTP + 25 % CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP
75 % 5-Methyl-CTP + 25 % CTP/75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Phenyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Phenyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Trifluoromethyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % 5-Trifluoromethyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % 5-Trifluoromethyl-CTP + 25 % CTP/1-Methyl-pseudo-UTP
75 % N4-Ac-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % N4-Ac-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % N4-Bz-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % N4-Bz-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP

75 % N4-Methyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % N4-Methyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75 % Pseudo-iso-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP
75 % Pseudo-iso-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP
75% 5-Bromo-CTP/25% CTP/ 1-Methyl-pseudo-UTP
75% 5-Bromo-CTP/25% CTP/ Pseudo-UTP
75% 5-methoxy-UTP/25% 5-methyl-CTP/ATP/GTP
75% 5-methoxy-UTP/50% 5-methyl-CTP/ATP/GTP
75% 5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP
75% 5-methoxy-UTP/CTP/ATP/GTP
8-Aza-ATP
Alpha-thio-CTP
CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP
CTP/25 % 5-Methoxy-UTP + 75 % UTP
CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP
CTP/50 % 5-Methoxy-UTP + 50 % UTP
CTP/5-Methoxy-UTP
CTP/5-Methoxy-UTP (cap 0)
CTP/5-Methoxy-UTP(No cap)
CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP
CTP/75 % 5-Methoxy-UTP + 25 % UTP
CTP/UTP(No cap)
N1-Me-GTP
N4-Ac-CTP
N4Ac-CTP/1-Methyl-pseudo-UTP
N4Ac-CTP/5-Methoxy-UTP
N4-acetyl-cytidine TP, ATP, GTP, UTP
N4-Bz-CTP/ 5-Methoxy-UTP
N4-methyl CTP
N4-Methyl-CTP/ 5-Methoxy-UTP
Pseudo-iso-CTP/ 5-Methoxy-UTP
PseudoU-alpha-thio-TP
pseudouridine TP, ATP, GTP, CTP
pseudo-UTP/5-methyl-CTP/ATP/GTP

UTP-5-oxyacetic acid Me ester
Xanthosine

[000523] According to the invention, polynucleotides of the invention may be synthesized to comprise the combinations or single modifications of Table 8.

[000524] Where a single modification is listed, the listed nucleoside or nucleotide represents 100 percent of that A, U, G or C nucleotide or nucleoside having been modified. Where percentages are listed, these represent the percentage of that particular A, U, G or C nucleobase triphosphate of the total amount of A, U, G, or C triphosphate present. For example, the combination: 25 % 5-Aminoallyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP refers to a polynucleotide where 25% of the cytosine triphosphates are 5-Aminoallyl-CTP while 75% of the cytosines are CTP; whereas 25% of the uracils are 5-methoxy UTP while 75% of the uracils are UTP. Where no modified UTP is listed then the naturally occurring ATP, UTP, GTP and/or CTP is used at 100% of the sites of those nucleotides found in the polynucleotide. In this example all of the GTP and ATP nucleotides are left unmodified.

IV. Pharmaceutical Compositions

Formulation, Administration, Delivery and Dosing

[000525] The present invention provides polynucleotides, compositions and complexes thereof in combination with one or more pharmaceutically acceptable excipients. Pharmaceutical compositions may optionally comprise one or more additional active substances, e.g. therapeutically and/or prophylactically active substances. Pharmaceutical compositions of the present invention may be sterile and/or pyrogen-free. General considerations in the formulation and/or manufacture of pharmaceutical agents may be found, for example, in *Remington: The Science and Practice of Pharmacy* 21st ed., Lippincott Williams & Wilkins, 2005 (incorporated herein by reference in its entirety).

[000526] In some embodiments, compositions comprising at least one polynucleotide described herein are administered to humans, human patients or subjects. For the purposes of the present disclosure, the phrase "active ingredient" generally refers to the polynucleotides described herein.

[000527] Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for administration to

humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to any other animal, *e.g.,* to non-human animals, e.g. non-human mammals. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and/or perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions is contemplated include, but are not limited to, humans and/or other primates; mammals, including commercially relevant mammals such as cattle, pigs, horses, sheep, cats, dogs, mice, and/or rats; and/or birds, including commercially relevant birds such as poultry, chickens, ducks, geese, and/or turkeys.

[000528] Formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with an excipient and/or one or more other accessory ingredients, and then, if necessary and/or desirable, dividing, shaping and/or packaging the product into a desired single- or multi-dose unit.

[000529] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the invention will vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100%, e.g., between .5 and 50%, between 1-30%, between 5-80%, at least 80% (w/w) active ingredient.

Formulations

[000530] The polynucleotides of the invention can be formulated using one or more excipients to: (1) increase stability; (2) increase cell transfection; (3) permit the sustained or delayed release (e.g., from a depot formulation of the polynucleotide); (4) alter the biodistribution (e.g., target the polynucleotide to specific tissues or cell types); (5) increase the translation of encoded protein *in vivo*; and/or (6) alter the release profile of encoded protein in vivo. In addition to traditional excipients such as any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, excipients of the present invention can include, without limitation, lipidoids, liposomes, lipid nanoparticles, polymers, lipoplexes, core-shell

nanoparticles, peptides, proteins, carbohydrates, cells transfected with polynucleotides (e.g., for transplantation into a subject), hyaluronidase, nanoparticle mimics and combinations thereof.

[000531] Accordingly, the formulations of the invention can include one or more excipients, each in an amount that together increases the stability of the polynucleotide, increases cell transfection by the polynucleotide, increases the expression of polynucleotides encoded protein, and/or alters the release profile of polynucleotide encoded proteins. Formulations may be in simple buffers which may comprise any of the excipients, diluents, solvents or other components described herein. Buffers may be osmolarity neutral.

[000532] Formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of associating the active ingredient with an excipient and/or one or more other accessory ingredients.

[000533] A pharmaceutical composition in accordance with the present disclosure may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a "unit dose" refers to a discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

[000534] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the present disclosure may vary, depending upon the identity, size, and/or condition of the subject being treated and further depending upon the route by which the composition is to be administered. For example, the composition may comprise between 0.1% and 99% (w/w) of the active ingredient. By way of example, the composition may comprise between 0.1% and 100%, e.g., between .5 and 50%, between 1-30%, between 5-80%, at least 80% (w/w) active ingredient.

[000535] In some embodiments, the formulations described herein may contain at least one polynucleotide. As a non-limiting example, the formulations may contain 1, 2, 3, 4, 5 or more than 5 polynucleotides described herein. As a non-limiting example, the formulation may comprise more than one type of polynucleotide described herein such as an IVT polynucleotide, a chimeric polynucleotide and/or a circular polynucleotide.

[000536] In some embodiments, the formulations described herein may contain at least one polynucleotide encoding a polypeptide of interest and at least one nucleic acid sequence such as, but not limited to, siRNA, shRNA, snoRNA and miRNA.

[000537] In one embodiment, the formulations described herein may comprise more than one type of polynucleotide. In one embodiment, the formulation may comprise a chimeric polynucleotide in linear and circular form. In another embodiment, the formulation may comprise a circular polynucleotide and an IVT polynucleotide. In yet another embodiment, the formulation may comprise an IVT polynucleotide, a chimeric polynucleotide and a circular polynucleotide.

[000538] In one embodiment the formulation may contain polynucleotide encoding proteins selected from categories such as, but not limited to, human proteins, veterinary proteins, biological proteins, antibodies, immunogenic proteins, therapeutic peptides and proteins, secreted proteins, plasma membrane proteins, cytoplasmic and cytoskeletal proteins, intracellular membrane bound proteins, nuclear proteins, and proteins associated with ophtalmic. In one embodiment, the formulation contains at least three polynucleotides encoding proteins. In one embodiment, the formulation contains at least five polynucleotide encoding proteins.

[000539] Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes, but is not limited to, any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, and the like, as suited to the particular dosage form desired. Various excipients for formulating pharmaceutical compositions and techniques for preparing the composition are known in the art (see Remington: The Science and Practice of Pharmacy, 21st Edition, A. R. Gennaro, Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference in its entirety). The use of a conventional excipient medium may be contemplated within the scope of the present disclosure, except insofar as any conventional excipient medium may be incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition.

[000540] In one embodiment, the formulations of the polynucleotides described herein may also comprise a component such as, but not limited to, DLin-MC3-DMA lipid, cholesterol, PEG-DMG, DOPE, DSPC, Methoxy PEG-DSPC, Hydrogenated soy phospatidyl glycerol,

sphingomyelin, DOPC, DPPC, dierucoylphophadtidylcholine (DEPC), tricaprylin (C8:0), triolein (C18:1), soybean oil, methoxy-PEG-40-carbonyl-distearoylphosphatidylethanolamine, L-dimyristoylphosphatidylcholine, L-dimyristoylphosphatidylglycerol, egg phosphatidylglycerol, MPEG5000 DPPE, DPPA (dipalmitoyl phosphatide), phosphatidylcholine, DPPG, LECIVA-S90 Ipurified PC from soy), LECIVA-S70 (pure phospholipid from soy lecithin), LIPOVA-E120 (purified egg lecithin USP), Egg lecithin, propylene glycol, glycerol, polysorbate 80, glutathione (reduced), butylated hydroxytoluene (BHA), ascorbyl palmitate, alpha-tocopherol, sodium carbonate, TRIS, histidine, calcium chloride, sodium phosphate, sodium citrate, ammonium sulfate, mannitol, sucrose, lactose, trehalose, disodium succinate hexahydrate and nitrogen.

[000541] In one embodiment, the formulations of the polynucleotides described herein may comprise a lipid such as, but not limited to, DLin-DMA, DLin-K-DMA, 98N12-5, C12-200, ckk, E12, DLin-MC3-DMA, DLin-KC2-DMA, DODMA, DOPE, DSPC, PLGA,, PEG-DMG, PEG-DSG, PEG-DSPE, PEG-DOMG, PEGylated lipids, polyethylenimine (PEI) and chitosan. As a non-limiting example, the lipid may be cationic lipid such as, but not limited to, C12-200, DLin-DMA, DLin-K-DMA and DODMA. As another non-limiting example, the lipid may be an ionizable lipid such as, but not limited to, DLin-MC3-DMA and DLin-KC2-DMA.

[000542] In some embodiments, the particle size of the lipid nanoparticle may be increased and/or decreased. The change in particle size may be able to help counter biological reaction such as, but not limited to, inflammation or may increase the biological effect of the modified mRNA delivered to mammals.

[000543] Pharmaceutically acceptable excipients used in the manufacture of pharmaceutical compositions include, but are not limited to, inert diluents, surface active agents and/or emulsifiers, preservatives, buffering agents, lubricating agents, and/or oils. Such excipients may optionally be included in the pharmaceutical formulations of the invention.

Lipidoids

[000544] The synthesis of lipidoids has been extensively described and formulations containing these compounds are particularly suited for delivery of polynucleotides (see Mahon et al., Bioconjug Chem. 2010 21:1448-1454; Schroeder et al., J Intern Med. 2010 267:9-21; Akinc et al., Nat Biotechnol. 2008 26:561-569; Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-

1869; Siegwart et al., Proc Natl Acad Sci U S A. 2011 108:12996-3001; all of which are incorporated herein in their entireties).

[000545] While these lipidoids have been used to effectively deliver double stranded small interfering RNA molecules in rodents and non-human primates (see Akinc et al., Nat Biotechnol. 2008 26:561-569; Frank-Kamenetsky et al., Proc Natl Acad Sci U S A. 2008 105:11915-11920; Akinc et al., Mol Ther. 2009 17:872-879; Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869; Leuschner et al., Nat Biotechnol. 2011 29:1005-1010; all of which is incorporated herein in their entirety), the present disclosure describes their formulation and use in delivering polynucleotides.

[000546] Complexes, micelles, liposomes or particles can be prepared containing these lipidoids and therefore, can result in an effective delivery of the polynucleotide, as judged by the production of an encoded protein, following the injection of a lipidoid formulation via localized and/or systemic routes of administration. Lipidoid complexes of polynucleotides can be administered by various means including, but not limited to, intravenous, intravitreall, intramuscular, or subcutaneous routes.

Liposomes, Lipoplexes, and Lipid Nanoparticles

[000547] The polynucleotides of the invention can be formulated using one or more liposomes, lipoplexes, or lipid nanoparticles. In one embodiment, pharmaceutical compositions of polynucleotides include liposomes. Liposomes are artificially-prepared vesicles which may primarily be composed of a lipid bilayer and may be used as a delivery vehicle for the administration of nutrients and pharmaceutical formulations. Liposomes can be of different sizes such as, but not limited to, a multilamellar vesicle (MLV) which may be hundreds of nanometers in diameter and may contain a series of concentric bilayers separated by narrow aqueous compartments, a small unicellular vesicle (SUV) which may be smaller than 50 nm in diameter, and a large unilamellar vesicle (LUV) which may be between 50 and 500 nm in diameter. Liposome design may include, but is not limited to, opsonins or ligands in order to improve the attachment of liposomes to unhealthy tissue or to activate events such as, but not limited to, endocytosis. Liposomes may contain a low or a high pH in order to improve the delivery of the pharmaceutical formulations.

[000548] In one embodiment, pharmaceutical compositions described herein may include, without limitation, liposomes such as those formed from 1,2-dioleyloxy-*N,N*-

dimethylaminopropane (DODMA) liposomes, DiLa2 liposomes from Marina Biotech (Bothell, WA), 1,2-dilinoleyloxy-3-dimethylaminopropane (DLin-DMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), and MC3 (US20100324120; the contents of which is herein incorporated by reference in its entirety) and liposomes which may deliver small molecule drugs such as, but not limited to, DOXIL® from Janssen Biotech, Inc. (Horsham, PA).

[000549] In one embodiment, formulations of the polynucleotides described herein may comprise a reactive component attached to dienophile as a chemically cleavable group which may provoking the release in vitro of the formulation and/or the polynucleotide. As a non-limiting example, the chemically cleavable group may be any of the chemically cleavable groups described in International Patent Publication No. WO2014081303, the contents of which is herein incorporated by reference in its entirety.

[000550] In one embodiment, pharmaceutical compositions described herein may include, without limitation, liposomes such as those formed from the synthesis of stabilized plasmid-lipid particles (SPLP) or stabilized nucleic acid lipid particle (SNALP) that have been previously described and shown to be suitable for oligonucleotide delivery *in vitro* and *in vivo* (see Wheeler et al. Gene Therapy. 1999 6:271-281; Zhang et al. Gene Therapy. 1999 6:1438-1447; Jeffs et al. Pharm Res. 2005 22:362-372; Morrissey et al., Nat Biotechnol. 2005 2:1002-1007; Zimmermann et al., Nature. 2006 441:111-114; Heyes et al. J Contr Rel. 2005 107:276-287; Semple et al. Nature Biotech. 2010 28:172-176; Judge et al. J Clin Invest. 2009 119:661-673; deFougerolles *Hum Gene Ther.* 2008 19:125-132; U.S. Patent Publication No US20130122104 and US20130303587; the contents of each of which are incorporated herein in their entireties). The original manufacture method by Wheeler et al. was a detergent dialysis method, which was later improved by Jeffs et al. and is referred to as the spontaneous vesicle formation method. The liposome formulations are composed of 3 to 4 lipid components in addition to the polynucleotide. As an example a liposome can contain, but is not limited to, 55% cholesterol, 20% disteroylphosphatidyl choline (DSPC), 10% PEG-S-DSG, and 15% 1,2-dioleyloxy-*N,N*-dimethylaminopropane (DODMA), as described by Jeffs et al. As another example, certain liposome formulations may contain, but are not limited to, 48% cholesterol, 20% DSPC, 2% PEG-c-DMA, and 30% cationic lipid, where the cationic lipid can be 1,2-distearloxy-*N,N*-dimethylaminopropane (DSDMA), DODMA, DLin-DMA, or 1,2-dilinolenyloxy-3-

dimethylaminopropane (DLenDMA), as described by Heyes et al. the contents of which are herein incorporated by reference in its entirety.

[000551] In some embodiments, the polynucleotides of the invention may be formulated as components of SNALP particles described in and made by the methods of US Patent Publication No. 20140065228, the contents of which is herein incorporated by reference in its entirety, further comprising: a lipid (e.g., cationic lipid or an ionizable amino lipid) comprising from 50 mol % to 65 mol % of the total lipid present in the particle; a non-cationic lipid of neutral overall charge comprising up to 49.5 mol % of the total lipid present in the particle and comprising a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the particle; and a conjugated lipid that inhibits aggregation of particles comprising from 0.5 mol % to 2 mol % of the total lipid present in the particle.

[000552] In some embodiments, liposome formulations may comprise from about 25.0% cholesterol to about 40.0% cholesterol, from about 30.0% cholesterol to about 45.0% cholesterol, from about 35.0% cholesterol to about 50.0% cholesterol and/or from about 48.5% cholesterol to about 60% cholesterol. In a preferred embodiment, formulations may comprise a percentage of cholesterol selected from the group consisting of 28.5%, 31.5%, 33.5%, 36.5%, 37.0%, 38.5%, 39.0% and 43.5%. In some embodiments, formulations may comprise from about 5.0% to about 10.0% DSPC and/or from about 7.0% to about 15.0% DSPC.

[000553] In one embodiment, pharmaceutical compositions may include liposomes which may be formed to deliver polynucleotides which may encode at least one immunogen or any other polypeptide of interest.

[000554] In one embodiment, the composition of the invention may comprise a liposome peptide conjugate.

[000555] In one embodiment, the pharmaceutical compositions may include liposomes comprising liposomal shells consisting of distearoyl phosphocholine (DSPC) and distearoyl phosphatidylethanolamine-m-polyethylene glycol (DSPE-m-PEG) as described in International Patent Publication No. WO2014054026, the contents of which are incorporated herein by reference in its entirety.

[000556] In another embodiment, liposomes may be formulated for targeted delivery. The liposome used for targeted delivery may include, but is not limited to, the liposomes described in

and methods of making liposomes described in US Patent Publication No. US20130195967, the contents of which are herein incorporated by reference in its entirety. In a non-limiting example, according to US Patent Publication No. US20130195967, the polynucleotides may be formulated in a liposome that further comprises a polyamine; and a lipid component; wherein the lipid component comprises a neutral phospholipid and essentially no cationic lipid, and wherein the polynucleotide and the lipid component are present at certain ratios as described in US Patent Publication No. US20130195967, the contents of which are herein incorporated by reference in its entirety, and wherein the liposome is from 30 to 500 nanometers in diameter.

[000557] In one embodiment, pharmaceutical compositions described herein may include liposomes and lipoplexes that include a polyamine compound or a lipid moiety described in or are made by the methods as described in US Publication No. 20140121393, the contents of which is herein incorporated by reference in its entirety.

[000558] In one embodiment, the pharmaceutical compositions described herein may include a polyamine compound or a lipid moiety, wherein one or more compounds each individually have a structure defined by formula (I) of claim 1 of European Patent Publication No. EP2695608, the contents of which is herein incorporated by reference in its entirety.

[000559] In another embodiment, the lipid formulation may include at least cationic lipid, a lipid which may enhance transfection and a least one lipid which contains a hydrophilic head group linked to a lipid moiety (International Pub. No. WO2011076807 and U.S. Pub. No. 20110200582; the contents of each of which is herein incorporated by reference in their entirety). In another embodiment, the polynucleotides encoding an immunogen may be formulated in a lipid vesicle which may have crosslinks between functionalized lipid bilayers (see U.S. Pub. No. 20120177724, the contents of which is herein incorporated by reference in its entirety).

[000560] In one embodiment, the polynucleotides may be formulated in a liposome as described in International Patent Publication No. WO2013086526, herein incorporated by reference in its entirety. The polynucleotides may be encapsulated in a liposome using reverse pH gradients and/or optimized internal buffer compositions as described in International Patent Publication No. WO2013086526, herein incorporated by reference in its entirety.

[000561] In one embodiment, the polynucleotides may be delivered in a liposome comprising an ionizable lipid. As a non-limiting example, the ionizable lipid may be any of the formulas of ionizable lipids described in International Patent Publication No. WO2013149140 and US Patent

Publication No. US20130330401, the contents of each of which are herein incorporated by reference in their entirety.

Nanoparticles

[000562] In some embodiments, the ratio of lipid to mRNA in the lipid nanoparticles may be from about 5:1 to about 10:1, from about 5:1 to about 15:1, from about 5:1 to about 20:1, from about 5:1 to about 25:1, from about 5:1 to about 30:1, from about 5:1 to about 35:1, from about 5:1 to about 40:1, from about 5:1 to about 45:1, from about 5:1 to about 50:1, from about 5:1 to about 55:1, from about 5:1 to about 60:1, from about 5:1 to about 70:1, from about 10:1 to about 15:1, from about 10:1 to about 20:1, from about 10:1 to about 25:1, from about 10:1 to about 30:1, from about 10:1 to about 35:1, from about 10:1 to about 40:1, from about 10:1 to about 45:1, from about 10:1 to about 50:1, from about 10:1 to about 55:1, from about 10:1 to about 60:1, from about 10:1 to about 70:1, from about 15:1 to about 20:1, from about 15:1 to about 25:1,from about 15:1 to about 30:1, from about 15:1 to about 35:1, from about 15:1 to about 40:1, from about 15:1 to about 45:1, from about 15:1 to about 50:1, from about 15:1 to about 55:1, from about 15:1 to about 60:1 or from about 15:1 to about 70:1..

[000563] In one embodiment, the polydispersity index (PDI) of the lipid nanoparticle formulations comprising the polynucleotides described herein is between 0.03 and 0.2 such as, but not limited to, at least 0.03, at least 0.04, at least 0.05, at least 0.06, at least 0.07, at least 0.08, at least 0.09, at least 0.1, at least 0.11, at least 0.12, at least 0.13, at least 0.14, at least 0.15, at least 0.16, at least 0.17, at least 0.18, at least 0.19 or at least 0.2.

[000564] In one embodiment, the zeta potential of the lipid nanoparticle formulations comprising the polynucleotides described herein is from about -20 to about +20 at a pH in the range of 6-8.

[000565] In one embodiment, the polynucleotides may be formulated in a lipid nanoparticle such as those described in or made by the method of International Patent Publication No. WO2013177421, the contents of which are incorporated herein by reference in its entirety.

[000566] In one embodiment, the lipid nanoparticles described herein may comprise a lipid such as, but not limited to, a cationic lipid or an ionizable lipid, a non-cationic lipid of neutral overall charge (e.g., zwitterionic lipids and phospholipids including, but not limited to, DSPC and DOPE), cholesterol and a PEG lipid. The components of the lipid nanoparticle may be tailored for optimal delivery of the polynucleotides based on the delivery route and the desired

outcome. As a non-limiting example, the lipid nanoparticle may comprise 40-60% lipid (either cationic lipid or an ionizable lipid), 8-16% non-cationic lipid of neutral overall charge, 30-45% cholesterol and 1-5% PEG lipid. As another non limiting example, the lipid nanoparticle may comprise 50% lipid (either cationic lipid or an ionizable lipid), 10% non-cationic lipid of neutral overall charge, 38.5% cholesterol and 1.5% PEG lipid. As yet another non-limiting example, the 40-60%, lipid (either cationic lipid or an ionizable lipid) may be DODMA, DLin-KC2-DMA or DLin-MC3-DMA, the 8-15% non-cationic lipid of neutral overall charge may be DSPC or DOPE and the 1-5% PEG lipid may be PEG 2000-DMG or anionic mPEG-DSPC and the lipid nanoparticle may comprise 30-45% cholesterol. The lipid nanoparticle may further comprise a buffer such as, but not limited to, citrate or phosphate at a pH of 7, salt and/or sugar. Salt and/or sugar may be included in the formulations described herein for isotonicity.

[000567] In one embodiment, the pharmaceutical compositions of the invention may comprise a nucleic acid lipid particle comprising a lipid formulation comprising 45-65 mol % of a lipid (e.g., either cationic lipid or an ionizable lipid), 5 mol % to about 10 mol %, of a non-cationic lipid of overall neutral charge, 25-40 mol % of a sterol, and 0.5-5 mol % of a PEG or PEG-modified lipid. Non-limiting examples of nucleic acid particles are disclosed in US Patent Publication No 20140121263, the contents of which are herein incorporated by reference in its entirety.

[000568] In one embodiment, the lipid nanoparticles described herein may comprise polynucleotides (e.g., mRNA) in a lipid:mRNA weight ratio of 5:1, 10:1, 15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1, 55:1, 60:1 or 70:1, or a range or any of these ratios such as, but not limited to, 5:1 to about 10:1, from about 5:1 to about 15:1, from about 5:1 to about 20:1, from about 5:1 to about 25:1, from about 5:1 to about 30:1, from about 5:1 to about 35:1, from about 5:1 to about 40:1, from about 5:1 to about 45:1, from about 5:1 to about 50:1, from about 5:1 to about 55:1, from about 5:1 to about 60:1, from about 5:1 to about 70:1, from about 10:1 to about 15:1, from about 10:1 to about 20:1, from about 10:1 to about 25:1, from about 10:1 to about 30:1, from about 10:1 to about 35:1, from about 10:1 to about 40:1, from about 10:1 to about 45:1, from about 10:1 to about 50:1, from about 10:1 to about 55:1, from about 10:1 to about 60:1, from about 10:1 to about 70:1, from about 15:1 to about 20:1, from about 15:1 to about 25:1,from about 15:1 to about 30:1, from about 15:1 to about 35:1, from about 15:1 to about 40:1, from about 15:1 to about 45:1, from about 15:1 to about 50:1, from about 15:1 to about

55:1, from about 15:1 to about 60:1 or from about 15:1 to about 70:1.. As a non-limiting example, the lipid nanoparticle described herein may comprise mRNA in a lipid:mRNA weight ratio of 20:1. As another non-limiting example, the lipid nanoparticle comprises 40-60% lipid (e.g., DODMA, DLin-KC2-DMA or DLin-MC3-DMA), 8-15% non-cationic lipid of neutral overall charge (e.g., DSPC or DOPE), 30-45% cholesterol and 1-5% PEG lipid (e.g., PEG 2000-DMG or anionic mPEG-DSPC). As yet another non-limiting example, the lipid nanoparticle comprises 50% lipid (e.g., DODMA, DLin-KC2-DMA or DLin-MC3-DMA), 10% non-cationic lipid of neutral overall charge (e.g., DSPC or DOPE), 38.5% cholesterol and 1.5% PEG lipid (e.g., PEG 2000-DMG).

[000569] In one embodiment, formulations comprising the polynucleotides and lipid nanoparticles described herein may comprise 0.15 mg/ml to 2 mg/ml of the polynucleotide described herein (e.g., mRNA), 50% lipid (e.g., DLin-MC3-DMA), 38.5% Cholesterol, 10% non-cationic lipid of neutral overall charge (e.g., DSPC), 1.5% PEG lipid (e.g., PEG-2K-DMG), 10 mM of citrate buffer and the formulation may additionally comprise up to 10% w/w of sucrose (e.g., at least 1% w/w, at least 2% w/w/, at least 3% w/w, at least 4% w/w, at least 5% w/w, at least 6% w/w, at least 7% w/w, at least 8% w/w, at least 9% w/w or 10% w/w).

[000570] In one embodiment, the lipid nanoparticles described herein may comprise a PEG lipid which is a non-diffusible PEG. Non-limiting examples of non-diffusible PEGs include PEG-DSG and PEG-DSPE. As a non-limiting example, the lipid nanoparticle comprising the PEG lipid comprises 40-60% lipid (e.g., DODMA, DLin-KC2-DMA or DLin-MC3-DMA), 8-15% non-cationic lipid of neutral overall charge (e.g., DSPC or DOPE), 30-45% cholesterol and 0.5-10% PEG lipid (e.g., PEG-DSG or PEG-DSPE). As another non-limiting example, the lipid nanoparticle comprising the PEG lipid comprises 50% lipid (e.g., DODMA, DLin-KC2-DMA or DLin-MC3-DMA), 10% non-cationic lipid of neutral overall charge (e.g., DSPC or DOPE), 39.5%, 38.5%, 35% or 30% cholesterol and 0.5%, 1.5%, 5% or 10% PEG lipid (e.g., PEG-DSG, PEG-DMG, PEG-DOMG, or PEG-DSPE).

[000571] In one embodiment, the lipid nanoparticles described herein may comprise 50% DLin-KC2-DMA, 10% DSPC, 39.5% cholesterol and 0.5% PEG-DSG. In one embodiment, the lipid nanoparticles described herein may comprise 50% DLin-KC2-DMA, 10% DSPC, 39.5% cholesterol and 0.5% PEG-DSPE.

[000572] In one embodiment, the lipid nanoparticles described herein may comprise 50% DLin-KC2-DMA, 10% DSPC, 38.5% cholesterol and 1.5% PEG-DSG. In one embodiment, the lipid nanoparticles described herein may comprise 50% DLin-KC2-DMA, 10% DSPC, 38.5% cholesterol and 1.5% PEG-DSPE.

[000573] In one embodiment, the lipid nanoparticles described herein may comprise 50% DLin-KC2-DMA, 10% DSPC, 35% cholesterol and 5% PEG-DSG. In one embodiment, the lipid nanoparticles described herein may comprise 50% DLin-KC2-DMA, 10% DSPC, 35% cholesterol and 5% PEG-DSPE.

[000574] In one embodiment, the lipid nanoparticles described herein may comprise 50% DLin-KC2-DMA, 10% DSPC, 39.5% cholesterol and 0.5% PEG-DSG. In one embodiment, the lipid nanoparticles described herein may comprise 50% DLin-KC2-DMA, 10% DSPC, 30% cholesterol and 10% PEG-DSPE.

[000575] In one embodiment, the lipid nanoparticles described herein may comprise the polynucleotides described herein in a concentration from approximately 0.1 mg/ml to 2 mg/ml such as, but not limited to, 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml, 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml, 1.0 mg/ml, 1.1 mg/ml, 1.2 mg/ml, 1.3 mg/ml, 1.4 mg/ml, 1.5 mg/ml, 1.6 mg/ml, 1.7 mg/ml, 1.8 mg/ml, 1.9 mg/ml, 2.0 mg/ml or greater than 2.0 mg/ml.

[000576] In one embodiment, the lipid nanoparticles described herein may be lyophilized in order to improve storage stability of the formulation and/or polynucleotides.

[000577] In one embodiment, the lipid nanoparticles described herein may be spray dried in order to improve storage stability of the formulation and/or polynucleotides.

[000578] In one embodiment, the polynucleotides may be formulated in a lipid nanoparticle such as those described in International Publication No. WO2012170930, herein incorporated by reference in its entirety.

[000579] In one embodiment, nanoparticles formulations may comprise surface modified nanostructured lipid carrier nanoparticles which encapsulate active substances for topical application to a selected target skin tissue in the skin of a subject. In a non-limiting example, the surface of the nanoparticles is modified with a cell penetrating peptide, and the nanoparticles comprise a blend of solid lipids and liquid lipids, which is solid at body temperature, and the formulation releases the active substance in a controlled manner upon application to the skin so that it penetrates the skin and is delivered to the epidermis to a depth of from about 80 to about

120 microns, as described in US Patent No. 8,715,736 , the contents of which is herein incorporated by reference in its entirety.

[000580] In one embodiment, the formulation comprising the polynucleotide is a nanoparticle which may comprise at least one lipid. The lipid may be selected from, but is not limited to, DLin-DMA, DLin-K-DMA, 98N12-5, C12-200, DLin-MC3-DMA, DLin-KC2-DMA, DODMA, PLGA, PEG, PEG-DMG, PEGylated lipids and amino alcohol lipids. In another aspect, the lipid may be a cationic lipid or an ionizable amino lipid. Non-limiting examples of cationic lipids include C12-200, DLin-DMA, DLin-K-DMA and DODMA. Non-limiting examples of ionizable amino lipids include DLin-MC3-DMA and DLin-KC2-DMA. The amino alcohol cationic lipid may be the lipids described in and/or made by the methods described in US Patent Publication No. US20130150625, herein incorporated by reference in its entirety.

[000581] In one embodiment, the cationic lipid may be selected from, but not limited to, a cationic lipid described in International Publication Nos. WO2012040184, WO2011153120, WO2011149733, WO2011090965, WO2011043913, WO2011022460, WO2012061259, WO2012054365, WO2012044638, WO2010080724, WO201021865, WO2008103276, WO2013086373 and WO2013086354, US Patent Nos. 7,893,302, 7,404,969, 8,283,333, 8,466,122 and 8,569,256 and US Patent Publication No. US20100036115, US20120202871, US20130064894, US20130129785, US20130150625, US20130178541, US20130225836 and US20140039032; the contents of each of which are herein incorporated by reference in their entirety.

[000582] In one embodiment, the lipid may be a cleavable lipid such as those described in International Publication No. WO2012170889, herein incorporated by reference in its entirety.

[000583] In another embodiment, the lipid may be a cationic lipid such as, but not limited to, Formula (I) of U.S. Patent Application No. US20130064894, the contents of which are herein incorporated by reference in its entirety.

[000584] In one embodiment, the cationic lipid may be synthesized by methods known in the art and/or as described in International Publication Nos. WO2012040184, WO2011153120, WO2011149733, WO2011090965, WO2011043913, WO2011022460, WO2012061259, WO2012054365, WO2012044638, WO2010080724, WO201021865, WO2013086373 and WO2013086354; the contents of each of which are herein incorporated by reference in their entirety.

[000585] In one embodiment, the cationic lipid may have a positively charged hydrophilic head and a hydrophobic tail that are connected via a linker structure. As a non-limiting example, the hydrophilic head group may be primary, secondary, tertiary amines or quaternary ammonium salts. As another non-limiting example, the lipids may have guanidino, imidazole, pyridinium, phosphorus, and arsenic groups.

[000586] In one embodiment, the lipid or lipids which may be used in the formulation and/or delivery of polynucleotides described herein may be, but is not limited to, 1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), 1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), cholesterol, *N*-[1-(2,3-Dioleyloxy)propyl]*N,N,N*-trimethylammonium chloride (DOTMA), 1,2-Dioleoyloxy-3-trimethylammonium-propane (DOTAP), Dioctadecylamidoglycylspermine (DOGS), *N*-(3-Aminopropyl)-*N,N*-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide (GAP-DLRIE), cetyltrimethylammonium bromide (CTAB), 6-lauroxyhexyl ornithinate (LHON), 1-)2,3-Dioleoloxypropyl)2,4,6-trimethylpyridinium (2Oc), 2,3-Dioleyloxy-*N*-[2(sperminecarboxamido)-ehtyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 1,2-Dioleyl-3-trimethylammonium-propane (DOPA), *N*-(2-Hydroxyethyl)-*N,N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (MDRIE), Dimyristooxypropyl dimethyl hydroxyethyl ammonium bromide (DMRI), 3β-[*N*-(*N',N'*-Dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol), Bis-guanidium-tren-cholesterol (BGTC), 1,3-Dioleoxy-2-(6-carboxy-spermyl)-propylamide (DOSPER), Dimethyloctadecylammonium bromide (DDAB), Dioctadecylamidoglicylspermidin (DSL), rac-[(2,3-Dioctadecyloxypropyl)(2-hydroxyethyl)]-dimethylammonium chloride (CLIP-1), rac-[2(2,3-Dihexadecyloxypropyl-oxymethyloxy)ehtyl]trimethylammonium chloride (CLIP-6), Ethyldimyrisotylphosphatidylcholine (EDMPC), 1,2-Distearyloxy-*N,N*-dimethyl-3-aminopropane (DSDMA), 1,2-Dimyristoyl-trimethylammoniumpropane (DMTAP), *O,O'*-Dimyristyl-*N*-lysyl asparate (DMKE), 1,2-Distearoyl-sn-glycero-3-ethylphosphocholine (DSEPC), *N*-Palmitoyl-D-erythro-spingosyl carbamoyl-spermine (CCS), *N-t*-Butyl-*No*-tetradecyl-3-tetradecylaminopropionamidine (diC14-amidine), Octadecenolyoxy[ethyl-2-heptadecenyl-3 hydroxyethyl] imidazolinium chloride (DOTIM), *N*1-Cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN) and 2-(3-[Bis-(3-amino-propyl)-amino]propylamino)-N-ditetradecylcarbamoylme-ethyl-acetamide (RPR2091290).

[000587] In one embodiment, the polymers which may be used in the formulation and/or delivery of polynucleotides described herein may be, but is not limited to, poly(ethylene)glycol (PEG), polyethylenimine (PEI), dithiobis(succinimidylpropionate) (DSP), Dimethyl-3,3'-dithiobispropionimidate (DTBP), poly(ethylene imine) biscarbamate (PEIC), poly(L-lysine) (PLL), histidine modified PLL, poly(*N*-vinylpyrrolidone) (PVP), poly(propylenimine (PPI), poly(amidoamine) (PAMAM), poly(amido ethylenimine) (SS-PAEI), triehtylenetetramine (TETA), poly(β-aminoester), poly(4-hydroxy-L-proine ester) (PHP), poly(allylamine), poly(α-[4-aminobutyl]-L-glycolic acid (PAGA), Poly(D,L-lactic-co-glycolid acid (PLGA), Poly(*N*-ethyl-4-vinylpyridinium bromide), poly(phosphazene)s (PPZ), poly(phosphoester)s (PPE), poly(phosphoramidate)s (PPA), poly(*N*-2-hydroxypropylmethacrylamide) (pHPMA), poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA), poly(2-aminoethyl propylene phosphate) PPE_EA), Chitsoan, galactosylated chitosan, *N*-dodecylated chitosan, histone, collagen and dextran-spermine. In one embodiment, the polymer may be an inert polymer such as, but not limited to, PEG. In one embodiment, the polymer may be a cationic polymer such as, but not limited to, PEI, PLL, TETA, poly(allylamine), Poly(*N*-ethyl-4-vinylpyridinium bromide), pHPMA and pDMAEMA. In one embodiment, the polymer may be a biodegradable PEI such as, but not limited to, DSP, DTBP and PEIC. In one embodiment, the polymer may be biodegradable such as, but not limited to, histine modified PLL, SS-PAEI, poly(β-aminoester), PHP, PAGA, PLGA, PPZ, PPE, PPA and PPE-EA.

[000588] In one embodiment, the LNP formulation may contain PEG-DMG 2000 (1,2-dimyristoyl-sn-glycero-3-phophoethanolamine-N-[methoxy(polyethylene glycol)-2000). In one embodiment, the LNP formulation may contain PEG-DMG 2000, a cationic lipid known in the art and at least one other component. In another embodiment, the LNP formulation may contain PEG-DMG 2000, a cationic lipid known in the art, DSPC and cholesterol. As a non-limiting example, the LNP formulation may contain PEG-DMG 2000, DLin-DMA, DSPC and cholesterol. As another non-limiting example the LNP formulation may contain PEG-DMG 2000, DLin-DMA, DSPC and cholesterol in a molar ratio of 2:40:10:48 (see e.g., Geall et al., Nonviral delivery of self-amplifying RNA vaccines, PNAS 2012; PMID: 22908294; herein incorporated by reference in its entirety).

[000589] In one embodiment, the LNP formulation may be formulated by the methods described in International Publication Nos. WO2011127255 or WO2008103276, the contents of

each of which is herein incorporated by reference in their entirety. As a non-limiting example, the polynucleotides described herein may be encapsulated in LNP formulations as described in WO2011127255 and/or WO2008103276; each of which is herein incorporated by reference in their entirety. As another non-limiting example, polynucleotides described herein may be formulated in a nanoparticle to be delivered by a parenteral route as described in U.S. Pub. No. 20120207845 and International Publication No. WO2014008334; the contents of each of which are herein incorporated by reference in its entirety.

[000590] Lipid nanoparticle formulations may be improved by replacing the lipid which is either cationic or an ionizable amino lipid with a biodegradable lipid which is known as a rapidly eliminated lipid nanoparticle (reLNP). Lipids, which may be replaced with a biodegradable lipid include, but are not limited to, DLinDMA, DLin-KC2-DMA, and DLin-MC3-DMA, have been shown to accumulate in plasma and tissues over time and may be a potential source of toxicity. The rapid metabolism of the rapidly eliminated lipids can improve the tolerability and therapeutic index of the lipid nanoparticles by an order of magnitude from a 1 mg/kg dose to a 10 mg/kg dose in rat. Inclusion of an enzymatically degraded ester linkage can improve the degradation and metabolism profile of the cationic component, while still maintaining the activity of the reLNP formulation. The ester linkage can be internally located within the lipid chain or it may be terminally located at the terminal end of the lipid chain. The internal ester linkage may replace any carbon in the lipid chain.

[000591] In one embodiment, the internal ester linkage may be located on either side of the saturated carbon.

[000592] Lipid nanoparticles may be engineered to alter the surface properties of particles so the lipid nanoparticles may penetrate the mucosal barrier.

[000593] In one embodiment, the polynucleotides may be formulated in neurophilic nanoparticles. Neurophilic nanoparticles may be useful to deliver compounds (e.g., compounds suitable for therapeutic purposes) to cells found in the peripheral nervous system and/or endothelial cells that form the blood brain barrier. The neuropilic nanoparticles may comprise at least a phospholipid, a non-ionic surfactant and a cholesterol. As a non-limiting example, the neurophilic nanoparticles are the liposomal nanoparticles described in International Patent Publication No. WO2013151650, the contents of which are herein incorporated by reference in its entirety. These neurophilic nanoparticles may be advantageous for targeting neural cells,

endothelial cells of the blood vessels and epithelial cells of the choroid plexus that serve the brain.

[000594] In one embodiment such formulations may also be constructed or compositions altered such that they passively or actively are directed to different cell types *in vivo*, including but not limited to hepatocytes, immune cells, tumor cells, endothelial cells, antigen presenting cells, and leukocytes (Akinc et al. Mol Ther. 2010 18:1357-1364; Song et al., Nat Biotechnol. 2005 23:709-717; Judge et al., J Clin Invest. 2009 119:661-673; Kaufmann et al., Microvasc Res 2010 80:286-293; Santel et al., Gene Ther 2006 13:1222-1234; Santel et al., Gene Ther 2006 13:1360-1370; Gutbier et al., Pulm Pharmacol. Ther. 2010 23:334-344; Basha et al., Mol. Ther. 2011 19:2186-2200; Fenske and Cullis, Expert Opin Drug Deliv. 2008 5:25-44; Peer et al., Science. 2008 319:627-630; Peer and Lieberman, Gene Ther. 2011 18:1127-1133; all of which are incorporated herein by reference in its entirety). One example of passive targeting of formulations to liver cells includes the DLin-DMA, DLin-KC2-DMA and DLin-MC3-DMA-based lipid nanoparticle formulations which have been shown to bind to apolipoprotein E and promote binding and uptake of these formulations into hepatocytes in vivo (Akinc et al. Mol Ther. 2010 18:1357-1364; herein incorporated by reference in its entirety). Formulations can also be selectively targeted through expression of different ligands on their surface as exemplified by, but not limited by, folate, transferrin, N-acetylgalactosamine (GalNAc), and antibody targeted approaches (Kolhatkar et al., Curr Drug Discov Technol. 2011 8:197-206; Musacchio and Torchilin, *Front Biosci.* 2011 16:1388-1412; Yu et al., Mol Membr Biol. 2010 27:286-298; Patil et al., Crit Rev Ther Drug Carrier Syst. 2008 25:1-61; Benoit et al., Biomacromolecules. 2011 12:2708-2714; Zhao et al., Expert Opin Drug Deliv. 2008 5:309-319; Akinc et al., Mol Ther. 2010 18:1357-1364; Srinivasan et al., Methods Mol Biol. 2012 820:105-116; Ben-Arie et al., Methods Mol Biol. 2012 757:497-507; Peer 2010 J Control Release. 20:63-68; Peer et al., Proc Natl Acad Sci U S A. 2007 104:4095-4100; Kim et al., Methods Mol Biol. 2011 721:339-353; Subramanya et al., Mol Ther. 2010 18:2028-2037; Song et al., Nat Biotechnol. 2005 23:709-717; Peer et al., Science. 2008 319:627-630; Peer and Lieberman, Gene Ther. 2011 18:1127-1133; all of which are incorporated herein by reference in its entirety).

[000595] Liposomes, lipoplexes, or lipid nanoparticles may be used to improve the efficacy of polynucleotides directed protein production as these formulations may be able to increase cell transfection by the polynucleotide; and/or increase the translation of encoded protein. One such

example involves the use of lipid encapsulation to enable the effective systemic delivery of polyplex plasmid DNA (Heyes et al., Mol Ther. 2007 15:713-720; herein incorporated by reference in its entirety). The liposomes, lipoplexes, or lipid nanoparticles may also be used to increase the stability of the polynucleotide.

[000596] In one embodiment, the polynucleotides of the present invention can be formulated for controlled release and/or targeted delivery. As used herein, "controlled release" refers to a pharmaceutical composition or compound release profile that conforms to a particular pattern of release to effect a therapeutic outcome. In one embodiment, the polynucleotides may be encapsulated into a delivery agent described herein and/or known in the art for controlled release and/or targeted delivery. As used herein, the term "encapsulate" means to enclose, surround or encase. As it relates to the formulation of the compounds of the invention, encapsulation may be substantial, complete or partial. The term "substantially encapsulated" means that at least greater than 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.9 or greater than 99.999% of the pharmaceutical composition or compound of the invention may be enclosed, surrounded or encased within the delivery agent. "Partially encapsulation" means that less than 10, 10, 20, 30, 40 50 or less of the pharmaceutical composition or compound of the invention may be enclosed, surrounded or encased within the delivery agent. Advantageously, encapsulation may be determined by measuring the escape or the activity of the pharmaceutical composition or compound of the invention using fluorescence and/or electron micrograph. For example, at least 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the pharmaceutical composition or compound of the invention are encapsulated in the delivery agent.

[000597] In one embodiment, the therapeutic nanoparticle may be formulated for sustained release. As used herein, "sustained release" refers to a pharmaceutical composition or compound that conforms to a release rate over a specific period of time. The period of time may include, but is not limited to, hours, days, weeks, months and years.

[000598] In one embodiment, the synthetic nanocarriers may be formulated for targeted release. In one embodiment, the synthetic nanocarrier is formulated to release the polynucleotides at a specified pH and/or after a desired time interval.

[000599] In one embodiment, polynucleotides may be delivered using LNPs which may comprise a diameter from about 1 nm to about 100 nm, from about 1 nm to about 10 nm, about 1

nm to about 20 nm, from about 1 nm to about 30 nm, from about 1 nm to about 40 nm, from about 1 nm to about 50 nm, from about 1 nm to about 60 nm, from about 1 nm to about 70 nm, from about 1 nm to about 80 nm, from about 1 nm to about 90 nm, from about 5 nm to about from 100 nm, from about 5 nm to about 10 nm, about 5 nm to about 20 nm, from about 5 nm to about 30 nm, from about 5 nm to about 40 nm, from about 5 nm to about 50 nm, from about 5 nm to about 60 nm, from about 5 nm to about 70 nm, from about 5 nm to about 80 nm, from about 5 nm to about 90 nm, from about 10 nm to about from 100 nm, about 10 nm to about 20 nm, from about 10 nm to about 30 nm, from about 10 nm to about 40 nm, from about 10 nm to about 50 nm, from about 10 nm to about 60 nm, from about 10 nm to about 70 nm, from about 10 nm to about 80 nm, from about 10 nm to about 90 nm, from about 20 nm to about from 100 nm, from about 20 nm to about 30 nm, from about 20 nm to about 40 nm, from about 20 nm to about 50 nm, from about 20 nm to about 60 nm, from about 20 nm to about 70 nm, from about 20 nm to about 80 nm, from about 20 nm to about 90 nm, from about 30 nm to about from 100 nm, from about 30 nm to about 40 nm, from about 30 nm to about 50 nm, from about 30 nm to about 60 nm, from about 30 nm to about 70 nm, from about 30 nm to about 80 nm, from about 30 nm to about 90 nm, from about 40 nm to about from 100 nm, from about 40 nm to about 50 nm, from about 40 nm to about 60 nm, from about 40 nm to about 70 nm, from about 40 nm to about 80 nm, from about 40 nm to about 90 nm, from about 50 nm to about from 100 nm, from about 50 nm to about 60 nm, from about 50 nm to about 70 nm, from about 50 nm to about 80 nm, from about 50 nm to about 90 nm, from about 60 nm to about from 100 nm, from about 60 nm to about 70 nm, from about 60 nm to about 80 nm, from about 60 nm to about 90 nm, from about 70 nm to about from 100 nm, from about 70 nm to about 80 nm, from about 70 nm to about 90 nm, from about 80 nm to about from 100 nm, from about 80 nm to about 90 nm or from about 90 nm to about from 100 nm.

[000600] In one embodiment, the lipid nanoparticle may have a diameter greater than 100 nm, greater than 150 nm, greater than 200 nm, greater than 250 nm, greater than 300 nm, greater than 350 nm, greater than 400 nm, greater than 450 nm, greater than 500 nm, greater than 550 nm, greater than 600 nm, greater than 650 nm, greater than 700 nm, greater than 750 nm, greater than 800 nm, greater than 850 nm, greater than 900 nm, greater than 950 nm or greater than 1000 nm.

[000601] The polynucleotides of the invention may be formulated with or in a polymeric compound. The polymer may include at least one polymer such as, but not limited to,

polyethenes, polyethylene glycol (PEG), poly(l-lysine)(PLL), PEG grafted to PLL, cationic lipopolymer, biodegradable cationic lipopolymer, polyethyleneimine (PEI), cross-linked branched poly(alkylene imines), a polyamine derivative, a modified poloxamer, a biodegradable polymer, elastic biodegradable polymer, biodegradable block copolymer, biodegradable random copolymer, biodegradable polyester copolymer, biodegradable polyester block copolymer, biodegradable polyester block random copolymer, multiblock copolymers, linear biodegradable copolymer, poly[α-(4-aminobutyl)-L-glycolic acid) (PAGA), biodegradable cross-linked cationic multi-block copolymers, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, polyamines, polylysine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), acrylic polymers, amine-containing polymers, dextran polymers, dextran polymer derivatives or combinations thereof .

[000602] As a non-limiting example, the polynucleotides of the invention may be formulated with the polymeric compound of PEG grafted with PLL as described in U.S. Pat. No. 6,177,274; herein incorporated by reference in its entirety. The formulation may be used for transfecting cells *in vitro* or for *in vivo* delivery of polynucleotide. In another example, the polynucleotide may be suspended in a solution or medium with a cationic polymer, in a dry pharmaceutical composition or in a solution that is capable of being dried as described in U.S. Pub. Nos. 20090042829 and 20090042825; each of which are herein incorporated by reference in their entireties.

[000603] As another non-limiting example the polynucleotides of the invention may be formulated with a PLGA-PEG block copolymer (see US Pub. No. US20120004293 and US Pat No. 8,236,330, herein incorporated by reference in their entireties) or PLGA-PEG-PLGA block copolymers (See U.S. Pat. No. 6,004,573, herein incorporated by reference in its entirety). As a non-limiting example, the polynucleotides of the invention may be formulated with a diblock copolymer of PEG and PLA or PEG and PLGA (see US Pat No 8,246,968, herein incorporated by reference in its entirety).

[000604] In one embodiment, a polymer combination may be used for the formulation and/or delivery of the polynucleotides described herein. As a non-limiting example, the polymer

combination may be two polymers used at a ratio of 1:1, 1:2, 1:2.5, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:12.5, 1:15, 1:20, 1:25, 1:30, 1:40 or at least 1:50. In order to reduce the shear stress on the lipids during the delivery of the polynucleotides a polymer may be used to stabilize the polymers senstitive to degradation during delivery.

[000605] In one embodiment, a polymer combination of PLGA and PEG may be used for the formulation and/or delivery of the polynucleotides described herein. As a non-limiting example, PEG may be used with PLGA in the delivery and/or formulation of the polynucleotides to reduce the degradation of PLGA during delivery. As another non-limiting example, the PLGA and PEG lipids used in the formulation and/or delivery of the polynucleotides may be in a 50:50 ratio. As yet another non-limiting example, the PLGA has a size of approximately 15K and the PEG has a size of approximately 2K and used in the formulation and/or delivery of the polynucleotides in a 50:50 ratio.

[000606] A polyamine derivative may be used to deliver nucleic acids or to treat and/or prevent a disease or to be included in an implantable or injectable device (U.S. Pub. No. 20100260817 (now U.S. Patent Nos. 8,460,696) and 20140050775, are the contents of each of which is herein incorporated by reference in its entirety). As a non-limiting example, a pharmaceutical composition may include the polynucleotide and the polyamine derivative described in U.S. Pub. No. 20100260817 (now U.S. Patent No. 8,460,696; the contents of which are incorporated herein by reference in its entirety. As a non-limiting example the polynucleotides of the present invention may be delivered using a polyamine polymer such as, but not limited to, a polymer comprising a 1,3-dipolar addition polymer prepared by combining a carbohydrate diazide monomer with a dilkyne unite comprising oligoamines (U.S. Pat. No. 8,236,280; herein incorporated by reference in its entirety). As another non-limiting example, the polynucleotides of the present invention may be delivered using or formulated in compositions comprising polyamine derivatives such as those described in formulas I-VI described in US Patent Publication No. US20140050775, the contents of which are herein incorporated by reference in its entirety.

[000607] In one embodiment, the polynucleotides of the invention may be delivered in a formulation with branched polyamines, such carbamate functionalized branched polyethylenimines comprising hydrophobic carbamate end groups, as described in International

Patent Publication No. WO2014042920, the contents of which is herein incorporated by reference in its entirety.

[000608] The polynucleotides of the invention may be formulated with at least one acrylic polymer. Acrylic polymers include but are not limited to, acrylic acid, methacrylic acid, acrylic acid and methacrylic acid copolymers, methyl methacrylate copolymers, ethoxyethyl methacrylates, cyanoethyl methacrylate, amino alkyl methacrylate copolymer, poly(acrylic acid), poly(methacrylic acid), polycyanoacrylates and combinations thereof. As a non-limiting example, the polynucleotides may be formulated with at least one poly(acrylate) copolymer as described in US Patent Publication No. US20130317079, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the polynucleotides may be formulated with at least one poly(acrylate) polymer as described in International Patent Publication No. WO2013158141, the contents of which are herein incorporated by reference in its entirety.

[000609] In one embodiment, the polynucleotides of the present invention may be formulated with at least one polymer and/or derivatives thereof described in International Publication Nos. WO2011115862, WO2012082574 and WO2012068187 and U.S. Pub. No. 20120283427 and US Patent No. 8,735,570, each of which are herein incorporated by reference in their entireties. In another embodiment, the polynucleotides of the present invention may be formulated with a polymer of formula Z as described in WO2011115862, herein incorporated by reference in its entirety. In yet another embodiment, the polynucleotides may be formulated with a polymer of formula Z, Z' or Z'' as described in International Pub. Nos. WO2012082574 or WO2012068187 and U.S. Pub. No. 2012028342, each of which are herein incorporated by reference in their entireties. The polymers formulated with the modified RNA of the present invention may be synthesized by the methods described in International Pub. Nos. WO2012082574 or WO2012068187, each of which are herein incorporated by reference in their entireties.

[000610] The polynucleotides of the invention may be formulated with at least one acrylic polymer. Acrylic polymers include but are not limited to, acrylic acid, methacrylic acid, acrylic acid and methacrylic acid copolymers, methyl methacrylate copolymers, ethoxyethyl methacrylates, cyanoethyl methacrylate, amino alkyl methacrylate copolymer, poly(acrylic acid), poly(methacrylic acid), polycyanoacrylates and combinations thereof.

[000611] No. 8,715,741, the contents of which is herein incorporated by reference in its entirety.

Peptides and Proteins

[000612] The polynucleotides of the invention can be formulated with peptides and/or proteins in order to increase transfection of cells by the polynucleotide. In one embodiment, peptides such as, but not limited to, cell penetrating peptides and proteins and peptides that enable intracellular delivery may be used to deliver pharmaceutical formulations. A non-limiting example of a cell penetrating peptide which may be used with the pharmaceutical formulations of the present invention includes a cell-penetrating peptide sequence attached to polycations that facilitates delivery to the intracellular space, e.g., HIV-derived TAT peptide, penetratins, transportans, or hCT derived cell-penetrating peptides (see, e.g., Caron et al., Mol. Ther. 3(3):310-8 (2001); Langel, Cell-Penetrating Peptides: Processes and Applications (CRC Press, Boca Raton FL, 2002); El-Andaloussi et al., Curr. Pharm. Des. 11(28):3597-611 (2003); and Deshayes et al., Cell. Mol. Life Sci. 62(16):1839-49 (2005), all of which are incorporated herein by reference in their entirety). The compositions can also be formulated to include a cell penetrating agent, e.g., liposomes, which enhance delivery of the compositions to the intracellular space. Polynucleotides of the invention may be complexed to peptides and/or proteins such as, but not limited to, peptides and/or proteins from Aileron Therapeutics (Cambridge, MA) and Permeon Biologics (Cambridge, MA) in order to enable intracellular delivery (Cronican et al., ACS Chem. Biol. 2010 5:747-752; McNaughton et al., Proc. Natl. Acad. Sci. USA 2009 106:6111-6116; Sawyer, Chem Biol Drug Des. 2009 73:3-6; Verdine and Hilinski, Methods Enzymol. 2012;503:3-33; all of which are herein incorporated by reference in its entirety).

[000613] In one embodiment, the cell-penetrating polypeptide may comprise a first domain and a second domain. The first domain may comprise a supercharged polypeptide. The second domain may comprise a protein-binding partner. As used herein, "protein-binding partner" includes, but are not limited to, antibodies and functional fragments thereof, scaffold proteins, or peptides. The cell-penetrating polypeptide may further comprise an intracellular binding partner for the protein-binding partner. The cell-penetrating polypeptide may be capable of being secreted from a cell where the polynucleotide may be introduced.

[000614] Formulations of the including peptides or proteins may be used to increase cell transfection by the polynucleotide, alter the biodistribution of the polynucleotide (e.g., by

targeting specific tissues or cell types), and/or increase the translation of encoded protein. (See e.g., International Pub. No. WO2012110636 and WO2013123298; the contents of which are herein incorporated by reference in its entirety).

[000615] In one embodiment, the cell penetrating peptide may be, but is not limited to, those described in US Patent Publication No US20130129726, US20130137644 and US20130164219, each of which is herein incorporated by reference in its entirety.

[000616] In one embodiment, the polynucleotides may be complexed with oligopeptides as described in US Patent Publication No. US20140037660, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the oligopeptide may be between 8 and 15 amino acids in length and have formula I as described in US Patent Publication No. US20140037660, the contents of which are herein incorporated by reference in its entirety.

[000617] In one embodiment, the formulations may comprise a nanocomplex containing cationic peptide useful for biomolecule delivery across varied cell lines, which contains peptide sequences comprising 9 arginines, 7 histidines, and 2 cysteines, as described in International Patent Publication WO2014072999, the contents of which is incorporated herein by reference in its entirety.

[000618] In one embodiment, the formulations may comprise a nanocomplex comprising a amphipathic peptide sequence according to SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5 and SEQ ID No. 6 of International Patent Publication WO2014072997, the contents of which is incorporated herein by reference in its entirety.

Cells

[000619] The polynucleotides of the invention can be transfected *ex vivo* into cells, which are subsequently transplanted into a subject. As non-limiting examples, the pharmaceutical compositions may include red blood cells to deliver modified RNA to liver and myeloid cells, virosomes to deliver modified RNA in virus-like particles (VLPs), and electroporated cells such as, but not limited to, from MAXCYTE® (Gaithersburg, MD) and from ERYTECH® (Lyon, France) to deliver modified RNA. Examples of use of red blood cells, viral particles and electroporated cells to deliver payloads other than polynucleotides have been documented (Godfrin et al., Expert Opin Biol Ther. 2012 12:127-133; Fang et al., Expert Opin Biol Ther. 2012 12:385-389; Hu et al., Proc Natl Acad Sci U S A. 2011 108:10980-10985; Lund et al., Pharm Res. 2010 27:400-420; Huckriede et al., J Liposome Res. 2007;17:39-47; Cusi, Hum

Vaccin. 2006 2:1-7; de Jonge et al., Gene Ther. 2006 13:400-411; all of which are herein incorporated by reference in its entirety).

[000620] The polynucleotides may be delivered in synthetic VLPs synthesized by the methods described in International Pub No. WO2011085231 and WO2013116656 and US Pub No. 20110171248, the contents of each of which are herein incorporated by reference in their entireties.

[000621] Cell-based formulations of the polynucleotides of the invention may be used to ensure cell transfection (e.g., in the cellular carrier), alter the biodistribution of the polynucleotide (e.g., by targeting the cell carrier to specific tissues or cell types), and/or increase the translation of encoded protein.

Introduction Into Cells

[000622] A variety of methods are known in the art and suitable for introduction of nucleic acid into a cell, including viral and non-viral mediated techniques. Examples of typical non-viral mediated techniques include, but are not limited to, electroporation, calcium phosphate mediated transfer, nucleofection, sonoporation, heat shock, magnetofection, liposome mediated transfer, microinjection, microprojectile mediated transfer (nanoparticles), cationic polymer mediated transfer (DEAE-dextran, polyethylenimine, polyethylene glycol (PEG) and the like) or cell fusion.

[000623] The technique of sonoporation, or cellular sonication, is the use of sound (e.g., ultrasonic frequencies) for modifying the permeability of the cell plasma membrane. Sonoporation methods are known to those in the art and are used to deliver nucleic acids *in vivo* (Yoon and Park, Expert Opin Drug Deliv. 2010 7:321-330; Postema and Gilja, Curr Pharm Biotechnol. 2007 8:355-361; Newman and Bettinger, Gene Ther. 2007 14:465-475; all herein incorporated by reference in their entirety). Sonoporation methods are known in the art and are also taught for example as it relates to bacteria in US Patent Publication 20100196983 and as it relates to other cell types in, for example, US Patent Publication 20100009424, each of which are incorporated herein by reference in their entirety. Sonorporation may be combined with microbubbles (air-filled vesicles stabilized by surface active molecules such as albumin, polymers or phospholipids) to increase transdermal penetration of drugs. While not wishing to be bound by theory, upon absorption of the ultrasound waves, the microbubbles cavitate, oscillate, break up and release localized shock waves that can disrupt the nearby cell membranes

and promote penetration of drugs. The size of the microspheres may be optimized to ensure efficient transfection of the drug. As a non-limiting example, the microbubbles may be about 1 to about 6 um in diameter, e.g., about 1 um, about 2 um, about 3 um, about 4 um, about 5 um or about 6 um in diameter.

Electroporation

[000624] Electroporation techniques are also well known in the art and are used to deliver nucleic acids *in vivo* and clinically (Andre et al., Curr Gene Ther. 2010 10:267-280; Chiarella et al., Curr Gene Ther. 2010 10:281-286; Hojman, Curr Gene Ther. 2010 10:128-138; all herein incorporated by reference in their entirety). Electroporation parameters, when optimized, may produce a transfection efficiency which may be equal to the efficiency achieved by viral vectors. Electroporation devices are sold by many companies worldwide including, but not limited to BTX® Instruments (Holliston, MA) (e.g., the AgilePulse In Vivo System) and Inovio (Blue Bell, PA) (e.g., Inovio SP-5P intramuscular delivery device or the CELLECTRA® 3000 intradermal delivery device). Electroporation may be used after, before and/or during administration of the polynucleotides described herein. As a non-limiting example, electroporation may be used after local injection. As another non-limiting example, electroporation may be used after systemic injection. In one embodiment, polynucleotides may be delivered by electroporation as described in Example 9.

[000625] In one embodiment, the polynucleotides described herein may be administered using electroporation where the device is an integrated device where the injection and electrical pulse are coordinated. The integrated device ensures that the electrode position is consistent and the electrical field is consistent around the needle for each administration. As a non-limiting example, the polynucleotides described herein may be administered using TRIGRID™ technology such as the TRIGRID™ integrated device. The needle of the integrated device may be co-localized within the perimeter of the four electrodes.

[000626] In one embodiment, electroporation may be used to improve the generation of T and B cell responses from administration of a therapeutic agent (e.g., polynucleotides (see e.g., Cu et al. *Enhanced Delivery and Potency of Self-Amplifying mRNA Vaccines by Electroporation in Situ*. Vaccines 2013, 1, 367-383; the contents of which are herein incorporated by reference in its entirety)).

Micro-Organ

[000627] The polynucleotides may be contained in a micro-organ which can then express an encoded polypeptide of interest in a long-lasting therapeutic formulation. In one aspect, the micro-organ may comprise a vector comprising a nucleic acid sequence (e.g., the polynucleotides of the present invention) encoding a polypeptide of interest, operably linked to one or more regulatory sequences. As a non-limiting example, the long-lasting therapeutic micro-organ used with the present invention may be those described in US Patent No US845948, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the micro-organ may be used to maintain a desired level of a polypeptide of interest for a sustained period of time (e.g., maintaining physiological hemoglobin levels as described in US Patent No US845948, the contents of which are herein incorporated by reference in its entirety).

[000628] The micro-organ may be able to produce the polypeptide of interest for at least a day, at least two days, at least three days, at least four days, at least five days, at least six days, a least 7 days, at least 8 days, at least 9 days, at least 10 days, at least 11 days, at least 12 days, at least 13 days, at least 14 days, at least 3 weeks, at least 1 month and/or at least 2 months, at least 3 months, at least 4 months, at least 5 months, at least 6 months or greater than 6 months.

[000629] In one embodiment, the micro-organ may have a diameter of at least 0.5 mm to at least 20 mm such as, but not limited to, at least 0.5 mm, at least 1 mm, at least 1.5 mm, at least 2 mm, at least 2.5 mm, at least 3 mm, at least 3.5 mm, at least 4 mm, at least 4.5 mm, at least 5 mm, at least 5.5 mm, at least 6 mm, at least 6.5 mm, at least 7 mm, at least 7.5 mm, at least 8 mm, at least 8.5 mm, at least 9 mm, at least 9.5 mm, at least 10 mm, at least 10.5 mm, at least 11 mm, at least 11.5 mm, at least 12 mm, at least 12.5 mm, at least 13 mm, at least 13.5 mm, at least 14 mm, at least 14.5 mm, at least 15 mm, at least 15.5. mm, at least 16 mm, at least 16.5 mm, at least 17 mm, at least 17.5 mm, at least 18 mm, at least 18.5 mm, at least 19 mm, at least 19.5 mm or at least 20 mm. In another embodiment, the micro-organ may have a diameter of 0.5-2.5 mm, 1-2.5 mm, 1.5-2.5 mm, 0.5-3 mm, 1-3 mm, 1.5-3 mm, 0.5-3.5 mm, 1-3.5 mm, 1.5-3.5 mm, 0.5-4 mm, 1-4 mm, 1.5-4 mm, 2-4 mm, 0.5-5 mm, 1-5 mm, 1.5-5 mm, 2-5 mm, 2.5-5 mm, 3-5 mm, 0.5-6 mm, 1-6 mm, 1.5-6 mm, 2-6 mm, 2.5-6 mm, 3-6 mm, 3.5-6 mm, 4-6 mm, 0.5-7 mm, 1-7 mm, 1.5-7 mm, 2-7 mm, 2.5-7 mm, 3-7 mm, 3.5-7 mm, 4-7 mm, 4.5-7 mm, 5-7 mm, 0.5-8 mm, 1-8 mm, 1.5-8 mm, 2-8 mm, 2.5-8 mm, 3-8 mm, 3.5-8 mm, 4-8 mm, 4.5-8 mm, 5-8 mm, 5.5-8

mm, 6-8 mm, 0.5-9 mm, 1-9 mm, 1.5-9 mm, 2-9 mm, 2.5-9 mm, 3-9 mm, 3.5-9 mm, 4-9 mm, 4.5-9 mm, 5-9 mm, 5.5-9 mm, 6-9 mm, 6.5-9 mm, 7-9 mm, 0.5-10 mm, 1-10 mm, 1.5-10 mm, 2-10 mm, 2.5-10 mm, 3-10 mm, 3.5-10 mm, 4-10 mm, 4.5-10 mm, 5-10 mm, 5.5-10 mm, 6-10 mm, 6.5-10 mm, 7-10 mm, 7.5-10 nm or 8-10 nm.

[000630] In one embodiment, the micro-organ may have a length of at least 2 mm to at least 150 mm such as, but not limited to, at least 2 mm, at least 3 mm, at least 4 mm, at least 5 mm, at least 6 mm, at least 7 mm, at least 8 mm, at least 9 mm, at least 10 mm, at least 15 mm, at least 20 mm, at least 25 mm, at least 30 mm, at least 35 mm, at least 40 mm, at least 45 mm, at least 50 mm, at least 55 mm, at least 60 mm, at least 65 mm, at least 70 mm, at least 75 mm, at least 80 mm, at least 85 mm, at least 90 mm, at least 95 mm, at least 100 mm, at least 105 mm, at least 110 mm, at least 115 mm, at least 120 mm, at least 125 mm, at least 130 mm, at least 135 mm, at least 140 mm, at least 145 mm or at least 150 mm. In another embodiment, the micro-organ may have a length of 5-100 mm, 10-100 mm, 15-100 mm, 20-100 mm, 25-10 mm, 30-100 mm, 35-100 mm, 40-100 mm, 45-100 mm, 50-100 mm, 55-100 mm, 60-100 mm, 65-100 mm, 70-100 mm, 75-100 mm, 80-100 mm, 85-100 mm, 90-100 mm, 5-90 mm, 10-90 mm, 15-90 mm, 20-90 mm, 25-10 mm, 30-90 mm, 35-90 mm, 40-90 mm, 45-90 mm, 50-90 mm, 55-90 mm, 60-90 mm, 65-90 mm, 70-90 mm, 75-90 mm, 80-90 mm, 5-80 mm, 10-80 mm, 15-80 mm, 20-80 mm, 25-10 mm, 30-80 mm, 35-80 mm, 40-80 mm, 45-80 mm, 50-80 mm, 55-80 mm, 60-80 mm, 65-80 mm, 70-80 mm, 5-70 mm, 10-70 mm, 15-70 mm, 20-70 mm, 25-10 mm, 30-70 mm, 35-70 mm, 40-70 mm, 45-70 mm, 50-70 mm, 55-70 mm, 60-70 mm, 5-60 mm, 10-60 mm, 15-60 mm, 20-60 mm, 25-10 mm, 30-60 mm, 35-60 mm, 40-60 mm, 45-60 mm, 50-60 mm, 5-50 mm, 10-50 mm, 15-50 mm, 20-50 mm, 25-10 mm, 30-50 mm, 35-50 mm, 40-50 mm, 5-40 mm, 10-40 mm, 15-40 mm, 20-40 mm, 25-10 mm, 30-40 mm, 5-30 mm, 10-30 mm, 15-30 mm, 20-30 mm, 5-20 mm, 10-20 mm or 5-10 mm.

[000631] In one embodiment, the polynucleotides may be delivered to a subject using a genetically modified micro-organ such as, but not limited to, those described in US Patent Publication No. US20130251679, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the micro-organ may be provided to a subject in order to secrete a protein encoded by the polynucleotides described herein.

Hyaluronidase

[000632] The intramuscular or subcutaneous localized injection of polynucleotides of the invention can include hyaluronidase, which catalyzes the hydrolysis of hyaluronan. By catalyzing the hydrolysis of hyaluronan, a constituent of the interstitial barrier, hyaluronidase lowers the viscosity of hyaluronan, thereby increasing tissue permeability (Frost, Expert Opin. Drug Deliv. (2007) 4:427-440; herein incorporated by reference in its entirety). It is useful to speed their dispersion and systemic distribution of encoded proteins produced by transfected cells. Alternatively, the hyaluronidase can be used to increase the number of cells exposed to a polynucleotide of the invention administered intramuscularly or subcutaneously.

Nanoparticle Mimics

[000633] The polynucleotides of the invention may be encapsulated within and/or absorbed to a nanoparticle mimic. A nanoparticle mimic can mimic the delivery function organisms or particles such as, but not limited to, pathogens, viruses, bacteria, fungus, parasites, prions and cells. As a non-limiting example the polynucleotides of the invention may be encapsulated in a non-viron particle which can mimic the delivery function of a virus (see International Pub. No. WO2012006376 and US Patent Publication No. US20130171241 and US20130195968, the contents of each of which are herein incorporated by reference in its entirety).

Nanotubes

[000634] The polynucleotides of the invention can be attached or otherwise bound to at least one nanotube such as, but not limited to, rosette nanotubes, rosette nanotubes having twin bases with a linker, carbon nanotubes and/or single-walled carbon nanotubes, The polynucleotides may be bound to the nanotubes through forces such as, but not limited to, steric, ionic, covalent and/or other forces.

[000635] In one embodiment, the nanotube can release one or more polynucleotides into cells. The size and/or the surface structure of at least one nanotube may be altered so as to govern the interaction of the nanotubes within the body and/or to attach or bind to the polynucleotides disclosed herein. In one embodiment, the building block and/or the functional groups attached to the building block of the at least one nanotube may be altered to adjust the dimensions and/or properties of the nanotube. As a non-limiting example, the length of the nanotubes may be altered to hinder the nanotubes from passing through the holes in the walls of normal blood vessels but still small enough to pass through the larger holes in the blood vessels of tumor tissue.

[000636] In one embodiment, at least one nanotube may also be coated with delivery enhancing compounds including polymers, such as, but not limited to, polyethylene glycol. In another embodiment, at least one nanotube and/or the polynucleotides may be mixed with pharmaceutically acceptable excipients and/or delivery vehicles.

[000637] In one embodiment, the polynucleotides are attached and/or otherwise bound to at least one rosette nanotube. The rosette nanotubes may be formed by a process known in the art and/or by the process described in International Publication No. WO2012094304, herein incorporated by reference in its entirety. At least one polynucleotide may be attached and/or otherwise bound to at least one rosette nanotube by a process as described in International Publication No. WO2012094304, herein incorporated by reference in its entirety, where rosette nanotubes or modules forming rosette nanotubes are mixed in aqueous media with at least one polynucleotide under conditions which may cause at least one polynucleotides to attach or otherwise bind to the rosette nanotubes.

[000638] In one embodiment, the polynucleotides may be attached to and/or otherwise bound to at least one carbon nanotube. As a non-limiting example, the polynucleotides may be bound to a linking agent and the linked agent may be bound to the carbon nanotube (See e.g., U.S. Pat No. 8,246,995; herein incorporated by reference in its entirety). The carbon nanotube may be a single-walled nanotube (See e.g., U.S. Pat No. 8,246,995; herein incorporated by reference in its entirety).

[000639] In one embodiment, carbon nanotubes are filled with a therapeutic agent (e.g., polynucleotides) formulated in a temperature sensitive gel as described in International Patent Publication No. WO2014015334, the contents of which are herein incorporated by reference in its entirety. The release of the therapeutic agent may be triggered by inductive heating such as, but not limited to, from an alternating or pulsed magnetic field or an electrical field.

Conjugates

[000640] The polynucleotides of the invention include conjugates, such as a polynucleotide covalently linked to a carrier or targeting group, or including two encoding regions that together produce a fusion protein (e.g., bearing a targeting group and therapeutic protein or peptide).

[000641] The conjugates of the invention include a naturally occurring substance, such as a protein (e.g., human serum albumin (HSA), low-density lipoprotein (LDL), high-density lipoprotein (HDL), or globulin); an carbohydrate (e.g., a dextran, pullulan, chitin, chitosan,

inulin, cyclodextrin or hyaluronic acid); or a lipid. The ligand may also be a recombinant or synthetic molecule, such as a synthetic polymer, e.g., a synthetic polyamino acid, an oligonucleotide (e.g. an aptamer). Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

[000642] Representative U.S. patents that teach the preparation of polynucleotide conjugates, particularly to RNA, include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941; 6,294,664; 6,320,017; 6,576,752; 6,783,931; 6,900,297; 7,037,646; each of which is herein incorporated by reference in their entireties.

[000643] In one embodiment, the conjugate of the present invention may function as a carrier for the polynucleotides of the present invention. The conjugate may comprise a cationic polymer such as, but not limited to, polyamine, polylysine, polyalkylenimine, and polyethylenimine which may be grafted to with poly(ethylene glycol). As a non-limiting example, the conjugate may be similar to the polymeric conjugate and the method of synthesizing the polymeric conjugate described in U.S. Pat. No. 6,586,524 herein incorporated by reference in its entirety.

[000644] A non-limiting example of a method for conjugation to a substrate is described in US Patent Publication No. US20130211249, the contents of which are herein incorporated by

reference in its entirety. The method may be used to make a conjugated polymeric particle comprising a polynucleotide.

[000645] The conjugates can also include targeting groups, e.g., a cell or tissue targeting agent, e.g., a lectin, glycoprotein, lipid or protein, e.g., an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-D-glucosasamine, N-acetyl-glucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, an RGD peptide, an RGD peptide mimetic or an aptamer.

[000646] Targeting groups can be proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies e.g., an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Targeting groups may also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-D-glucosasamine, N-acetyl-glucosamine multivalent mannose, multivalent fucose, or aptamers. The ligand can be, for example, a lipopolysaccharide, or an activator of p38 MAP kinase.

[000647] The targeting group can be any ligand that is capable of targeting a specific receptor. Examples include, without limitation, folate, GalNAc, galactose, mannose, mannose-6P, apatamers, integrin receptor ligands, chemokine receptor ligands, transferrin, biotin, serotonin receptor ligands, PSMA, endothelin, GCPII, somatostatin, LDL, and HDL ligands. In particular embodiments, the targeting group is an aptamer. The aptamer can be unmodified or have any combination of modifications disclosed herein.

[000648] As a non-limiting example, the targeting group may be a glutathione receptor (GR)-binding conjugate for targeted delivery across the blood-central nervous system barrier (See e.g., US Patent Publication No. US2013021661012, the contents of which are herein incorporated by reference in its entirety.

[000649] In one embodiment, the conjugate of the present invention may be a synergistic biomolecule-polymer conjugate. The synergistic biomolecule-polymer conjugate may be long-acting continuous-release system to provide a greater therapeutic efficacy. The synergistic

biomolecule-polymer conjugate may be those described in US Patent Publication No. US20130195799, the contents of which are herein incorporated by reference in its entirety.

[000650] In one embodiment, the formulation may comprise a polymer conjugate which may be formulated into a nanoparticle, as described in US Patent No. 8,668,926, the contents of which is herein incorporated by reference in its entirety.

[000651] In another embodiment, the conjugate which may be used in the present invention may be an aptamer conjugate. Non-limiting examples of apatamer conjugates are described in International Patent Publication No. WO2012040524, the contents of which are herein incorporated by reference in its entirety. The aptamer conjugates may be used to provide targeted delivery of formulations comprising polynucleotides.

[000652] In one embodiment, the conjugate which may be used in the present invention may be an amine containing polymer conjugate. Non-limiting examples of amine containing polymer conjugate are described in US Patent No. US 8,507,653, the contents of which are herein incorporated by reference in its entirety. The factor IX moiety polymer conjugate may be comprise releasable linkages to release the polynucleotides upon and/or after delivery to a subject.

[000653] In one embodiment, the pharmaceutical compositions of the present invention may include polymeric backbone having attached a therapeutically active agent and a bone targeting moiety, for example to treat or monitor bone-related diseases or disorders, as described in International Patent Publication WO2012153297, the contents of which is herein incorporated by reference in its entirety.

[000654] In one embodiment, the pharmaceutical compositions of the present invention may include a targeted particle comprising a polymer conjugated to a surfactant, hydrophilic polymer or lipid, the particle having bound thereto a plurality of small molecule targeting moieties that specifically bind to the Zn2+ NAAG/PSMA binding pocket within prostate specific membrane antigen (PSMA), as described in US Patent No. 8,709,483 the contents of which is herein incorporated by reference in its entirety.

[000655] In some embodiments, the formulation may include polypeptide conjugates linked through a modified amino acid. In a non-limiting example, the conjugates may comprise the compound of claim 1 and dependent claims of International Patent Publication No. WO2014074218, the contents of which is incorporated herein by reference in its entirety.

[000656] In one embodiment, pharmaceutical compositions of the present invention may include chemical modifications such as, but not limited to, modifications similar to locked nucleic acids.

[000657] Representative U.S. Patents that teach the preparation of locked nucleic acid (LNA) such as those from Santaris, include, but are not limited to, the following: U.S. Pat. Nos. 6,268,490; 6,670,461; 6,794,499; 6,998,484; 7,053,207; 7,084,125; and 7,399,845, each of which is herein incorporated by reference in its entirety.

[000658] Representative U.S. patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found, for example, in Nielsen *et al.*, Science, 1991, 254, 1497-1500.

[000659] Some embodiments featured in the invention include polynucleotides with phosphorothioate backbones and oligonucleosides with other modified backbones, and in particular --CH_2--NH--CH_2--, --CH_2--N(CH_3)--O--CH_2--[known as a methylene (methylimino) or MMI backbone], --CH_2--O--N(CH_3)--CH_2--, --CH_2--N(CH_3)--N(CH_3)--CH_2-- and --N(CH_3)--CH_2--CH_2--[wherein the native phosphodiester backbone is represented as --O—P(O)$_2$--O--CH_2--] of the above-referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above-referenced U.S. Pat. No. 5,602,240. In some embodiments, the polynucleotides featured herein have morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

[000660] Modifications at the 2′ position may also aid in delivery. Preferably, modifications at the 2′ position are not located in a polypeptide-coding sequence, i.e., not in a translatable region. Modifications at the 2′ position may be located in a 5′UTR, a 3′UTR and/or a tailing region. Modifications at the 2′ position can include one of the following at the 2' position: H (i.e., 2′-deoxy); F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Exemplary suitable modifications include O[(CH_2)$_n$O] $_m$$CH_3$, O($CH_2$).$_nOCH_3$, O($CH_2$)$_n$$NH_2$, O($CH_2$) $_n$$CH_3$, O($CH_2$)$_nONH_2$, and O($CH_2$)$_n$ON[($CH_2$)$_n$$CH_3$)]$_2$, where n and m are from 1 to about 10. In other embodiments, the polynucleotides include one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted

silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties, or a group for improving the pharmacodynamic properties, and other substituents having similar properties. In some embodiments, the modification includes a 2'-methoxyethoxy (2'-O--$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, 1995, 78:486-504) *i.e.*, an alkoxy-alkoxy group. Another exemplary modification is 2'-dimethylaminooxyethoxy, *i.e.*, a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e.*, 2'-O--CH_2--O--CH_2--$N(CH_2)_2$, also described in examples herein below. Other modifications include 2'-methoxy (2'-OCH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked dsRNAs and the 5' position of 5' terminal nucleotide. Polynucleotides of the invention may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920; the contents of each of which is herein incorporated by reference in their entirety.

[000661] In still other embodiments, the polynucleotide is covalently conjugated to a cell penetrating polypeptide. The cell-penetrating peptide may also include a signal sequence. The conjugates of the invention can be designed to have increased stability; increased cell transfection; and/or altered the biodistribution (e.g., targeted to specific tissues or cell types).

[000662] In one embodiment, the polynucleotides may be conjugated to an agent to enhance delivery. As a non-limiting example, the agent may be a monomer or polymer such as a targeting monomer or a polymer having targeting blocks as described in International Publication No. WO2011062965, herein incorporated by reference in its entirety. In another non-limiting example, the agent may be a transport agent covalently coupled to the polynucleotides of the present invention (See e.g., U.S. Pat. Nos. 6,835.393 and 7,374,778, each of which is herein incorporated by reference in its entirety). In yet another non-limiting example, the agent may be a membrane barrier transport enhancing agent such as those described

in U.S. Pat. Nos. 7,737,108 and 8,003,129, each of which is herein incorporated by reference in its entirety.

[000663] In one embodiment, the pharmaceutical compositions of the invention comprise polymeric reagents that provide a conjugate, allowing a degradable linkage between a polymer and another moiety, as described in or synthesized and conjugated to active agents and other moieties by the methods of US Patent Publication 20140107349, the contents of which is incorporated herein by reference in its entirety.

[000664] In another embodiment, polynucleotides may be conjugated to SMARTT POLYMER TECHNOLOGY® (PHASERX®, Inc. Seattle, WA).

[000665] In another aspect, the conjugate may be a peptide that selectively directs the nanoparticle to neurons in a tissue or organism. As a non-limiting example, the peptide used may be, but is not limited to, the peptides described in US Patent Publication No US20130129627, herein incorporated by reference in its entirety.

[000666] In yet another aspect, the conjugate may be a peptide that can assist in crossing the blood-brain barrier.

[000667] In one embodiment, the conjugate may be an aptamer-mRNA conjugate which may be used for targeted expression. As a non-limiting example, the aptamer-mRNA conjugate may include any of the aptamers and/or conjugates described in US Patent Publication No. US20130022538, the contents of which is herein incorporated by reference in its entirety. The aptamer-mRNA conjugate may include an aptamer component that can bind to a membrane associated protein on a target cell.

[000668] In one embodiment, the conjugate may be a water-soluble polymer conjugate such as the conjugates described in US Patent No. US8636994, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the water-soluble polymer conjugate may comprise at least one residue of an antimicrobial agent (see e.g., the conjugates described in US Patent No. US8636994, the contents of which are herein incorporated by reference in its entirety).

[000669] In one embodiment, the conjugate may be a targeting amino acid chain bound to a biocompatiable polymer such as, but not limited to, the targeting amino acids and biocompatiable polymers described in International Patent Publication No. WO2014025890, the contents of which are herein incorporated by reference in its entirety. As a non-limiting

example, the targeting amino acid may be any of the targeting amino acid chains described in SEQ ID NO: 1-62 of International Patent Publication No. WO2014025890, the contents of which are herein incorporated by reference in its entirety. In one embodiment, the targeting amino acid chain is smaller than 50 amino acids in length.

[000670] In one embodiment, the conjugate may be a targeted poly amino-acid subunits which contains a targeting amino acid chain conjugated to a carboxylic acid such as, but not limited to, the targeting amino acids and carboxylic acids described in US Patent Publication No. US20140045950, the contents of which are herein incorporated by reference in its entirety. In one embodiment, the targeted drug delivery vehicle comprises 5 to 50 targeted amino acid subunits. As a non-limiting example, the targeting amino acid may be any of the targeting amino acid chains described in SEQ ID NO: 1-62 of US Patent Publication No. US20140045950, the contents of which are herein incorporated by reference in its entirety.

[000671] In one embodiment, the formulations may include small molecule conjugates according to the formula of claim 1 and dependent claims of US Patent Publication No. 20140135381, the contents of which is herein incorporated by reference in its entirety.

[000672] In one embodiment, the formulations may include a conjugate comprising at least one modified amino acid residue of formula (I) of claim 1 of US Patent Publication 20140141025, the contents of which is herein incorporated by reference in its entirety. In a non limiting example, the conjugate may be a polypeptide conjugate made according to a method using hydrazinyl-indole compounds, as described in US Patent Publication 20140141025, the contents of which is herein incorporated by reference in its entirety.

[000673] In one embodiment, the composition may include water-soluble polymer conjugates and/or polymer-based compositions. In a non-limiting example, the water soluble polymer may have the structure described in claim 1 of US Patent No. 8,728,493, the contents of which is herein incorporated by reference in its entirety.

[000674] In some embodiments, the water-soluble polymer is polyethylene glycol) or a derivative thereof. In a non-limiting example, the water-soluble polymer is described in US Patent Publication 20140141483, the contents of which is herein incorporated by reference in its entirety.

Self-Assembled Nanoparticles

[000675] In one embodiment, the polynucleotides disclosed herein may be formulated as self-assembled nanoparticles. These may be nucleic acid based or polymer based.

Self-Assembled Macromolecules

[000676] The polynucleotides may be formulated in amphiphilic macromolecules (AMs) for delivery. AMs comprise biocompatible amphiphilic polymers which have an alkylated sugar backbone covalently linked to poly(ethylene glycol). In aqueous solution, the AMs self-assemble to form micelles. Non-limiting examples of methods of forming AMs and AMs are described in US Patent Publication No. US20130217753, the contents of which are herein incorporated by reference in its entirety.

Inorganic Nanoparticles

[000677] The polynucleotides of the present invention may be formulated in inorganic nanoparticles (U.S. Pat. No. 8,257,745, herein incorporated by reference in its entirety). The inorganic nanoparticles may include, but are not limited to, clay substances that are water swellable. As a non-limiting example, the inorganic nanoparticle may include synthetic smectite clays which are made from simple silicates (See e.g., U.S. Pat. No. 5,585,108 and 8,257,745 each of which are herein incorporated by reference in their entirety).

Semi-conductive and Metallic Nanoparticles

[000678] The polynucleotides of the present invention may be formulated in water-dispersible nanoparticle comprising a semiconductive or metallic material (U.S. Pub. No. 20120228565; herein incorporated by reference in its entirety) or formed in a magnetic nanoparticle (U.S. Pub. No. 20120265001 and 20120283503; each of which is herein incorporated by reference in its entirety). The water-dispersible nanoparticles may be hydrophobic nanoparticles or hydrophilic nanoparticles.

High Density Lipoprotein (HDL) Particles

[000679] In one embodiment, the polynucleotides of the invention may be formulated in high density lipoprotein-nucleic acid particles. As a non-limiting example, the particles may comprise a nucleic acid component and a polypeptide comprising a positively charged region which associates with the nucleic acid component as described in US Patent No. 8,734,853, the contents of which is herein incorporated by reference in its entirety.

Micelles

[000680] In one embodiment, the polynucleotides may be formulated in a micelle or coated on a micelle for delivery.

Surgical Sealants: Gels and Hydrogels

[000681] In one embodiment, the polynucleotides disclosed herein may be encapsulated into any hydrogel known in the art which may form a gel when injected into a subject.

Polyethylene glycol

[000682] In one embodiment, the polynucleotides of the present invention may be loaded in PEGs as the buffer or the acid.

Nanolipogel

[000683] In one embodiment, the polynucleotides may be formulated in and/or delivered using a nanolipogel.

Suspension formulations

[000684] In some embodiments, suspension formulations are provided comprising polynucleotides, water immiscible oil depots, surfactants and/or co-surfactants and/or co-solvents. Combinations of oils and surfactants may enable suspension formulation with polynucleotides. Delivery of polynucleotides in a water immiscible depot may be used to improve bioavailability through sustained release of mRNA from the depot to the surrounding physiologic environment and prevent polynucleotides degradation by nucleases.

[000685] In some embodiments, suspension formulations of mRNA may be prepared using combinations of polynucleotides, oil-based solutions and surfactants. Such formulations may be prepared as a two-part system comprising an aqueous phase comprising polynucleotides and an oil-based phase comprising oil and surfactants. Exemplary oils for suspension formulations may include, but are not limited to sesame oil and Miglyol (comprising esters of saturated coconut and palmkernel oil-derived caprylic and capric fatty acids and glycerin or propylene glycol), corn oil, soybean oil, peanut oil, beeswax and/or palm seed oil. Exemplary surfactants may include, but are not limited to Cremophor, polysorbate 20, polysorbate 80, polyethylene glycol, transcutol, Capmul®, labrasol, isopropyl myristate, and/or Span 80. In some embodiments, suspensions may comprise co-solvents including, but not limited to ethanol, glycerol and/or propylene glycol.

[000686] Suspensions may be formed by first preparing polynucleotides formulation comprising an aqueous solution of polynucleotide and an oil-based phase comprising one or

more surfactants. Suspension formation occurs as a result of mixing the two phases (aqueous and oil-based). In some embodiments, such a suspension may be delivered to an aqueous phase to form an oil-in-water emulsion. In some embodiments, delivery of a suspension to an aqueous phase results in the formation of an oil-in-water emulsion in which the oil-based phase comprising polynucleotides forms droplets that may range in size from nanometer-sized droplets to micrometer-sized droplets. In some embodiments, specific combinations of oils, surfactants, cosurfactants and/or co-solvents may be utilized to suspend polynucleotides in the oil phase and/or to form oil-in-water emulsions upon delivery into an aqueous environment.

[000687] In some embodiments, suspensions may provide modulation of the release of polynucleotides into the surrounding environment. In such embodiments, polynucleotides release may be modulated by diffusion from a water immiscible depot followed by resolubilization into a surrounding environment (e.g. an aqueous environment).

[000688] In some embodiments, polynucleotides within a water immiscible depot (e.g. suspended within an oil phase) may result in altered polynucleotides stability (e.g. altered degradation by nucleases).

[000689] In some embodiments, polynucleotides may be formulated such that upon injection, an emulsion forms spontaneously (e.g. when delivered to an aqueous phase). Such particle formation may provide a high surface area to volume ratio for release of polynucleotides from an oil phase to an aqueous phase.

[000690] In one embodiment the polynucleotide formulations may comprise in oil-in-water emulsions based on fatty alcohols and mono-or diesters of glycerol for use as antifoams or deaerators for aqueous compositions, as described or made by the methods described in US Patent Publication No. 20140107229, the contents of which is incorporated herein by reference in its entirety.

[000691] In one embodiment, the polynucleotides may be formulated in a nanoemulsion such as, but not limited to, the nanoemulsions described in US Patent No. 8,496,945 and International Patent Publication Nos. WO2013130535 and WO2012129483, the contents of each of which are herein incorporated by reference in their entirety. The nanoemulsions may comprise nanoparticles described herein. As a non-limiting example, the nanoparticles may comprise a liquid hydrophobic core which may be surrounded or coated with a lipid or surfactant layer. The lipid or surfactant layer may comprise at least one membrane-integrating peptide and may also

comprise a targeting ligand (see e.g., US Patent No. 8,496,945, the contents of which are herein incorporated by reference in its entirety). As another non-limiting example, the nanoemulsion may be an oil-in-water emulsion comprising an aqueous phase, an oil phase, a surfactant and a phospholipid such as the nanoemulsions described in International Patent Publication No. WO2012129483, the contents of which are herein incorporated by reference in its entirety.

Cations and Anions

[000692] Formulations of polynucleotides disclosed herein may include cations or anions. In one embodiment, the formulations include metal cations such as, but not limited to, Zn2+, Ca2+, Cu2+, Mg+ and combinations thereof. As a non-limiting example, formulations may include polymers and a polynucleotides complexed with a metal cation (See e.g., U.S. Pat. Nos. 6,265,389 and 6,555,525, each of which is herein incorporated by reference in its entirety).

[000693] In some embodiments, cationic nanoparticles comprising combinations of divalent and monovalent cations may be formulated with polynucleotides. Such nanoparticles may form spontaneously in solution over a given period (e.g. hours, days, etc.). Such nanoparticles do not form in the presence of divalent cations alone or in the presence of monovalent cations alone. The delivery of polynucleotides in cationic nanoparticles or in one or more depot comprising cationic nanoparticles may improve polynucleotide bioavailability by acting as a long-acting depot and/or reducing the rate of degradation by nucleases.

Molded Nanoparticles and Microparticles

[000694] The polynucleotides disclosed herein may be formulated in nanoparticles and/or microparticles (also referred to as microspheres). These nanoparticles and/or microparticles may be molded into any size shape and chemistry. As an example, the nanoparticles and/or microparticles may be made using the PRINT® technology by LIQUIDA TECHNOLOGIES® (Morrisville, NC) (See e.g., International Pub. No. WO2007024323; the contents of which are herein incorporated by reference in its entirety).

[000695] In one embodiment, the molded nanoparticles may comprise a core of the polynucleotides disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the polynucleotides in the core.

[000696] In one embodiment, the polynucleotides of the present invention may be formulated in microparticles. The microparticles may contain a core of the polynucleotides and a cortext of

a biocompatible and/or biodegradable polymer. As a non-limiting example, the microparticles which may be used with the present invention may be those described in U.S. Patent No. 8,460,709, U.S. Patent Publication No. US20130129830 and International Patent Publication No WO2013075068, each of which is herein incorporated by reference in its entirety. As another non-limiting example, the microparticles may be designed to extend the release of the polynucleotides of the present invention over a desired period of time (see e.g., extended release of a therapeutic protein in U.S. Patent Publication No. US20130129830, herein incorporated by reference in its entirety).

[000697] In another non-limiting example, the microparticles may be polymer microparticles containing multi-block vinylic polymers, as described in European Patent Publication EP2038320, the contents of which is herein incorporated by reference in its entirety.

[000698] The microparticle for use with the present invention may have a diameter of at least 1 micron to at least 100 microns (e.g., at least 1 micron, at least 5 micron, at least 10 micron, at least 15 micron, at least 20 micron, at least 25 micron, at least 30 micron, at least 35 micron, at least 40 micron, at least 45 micron, at least 50 micron, at least 55 micron, at least 60 micron, at least 65 micron, at least 70 micron, at least 75 micron, at least 80 micron, at least 85 micron, at least 90 micron, at least 95 micron, at least 97 micron, at least 99 micron, and at least 100 micron).

[000699] The micorparticle may be a hydrogel microparticle. In one embodiment, the hydrogel microparticle may be made using the methods described in International Patent publication No. WO2014025312, the contents of which are herein incorporated by reference in its entirety. The hydrogel microparticles may include one or more species of living cells attached thereon and/or encapsulated therein such as the hydrogel microparticles described in International Patent publication No. WO2014025312, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the polynucleotides described herein may be formulated in or delivered using hydrogel microparticles

Semi-solid Compositions

[000700] In one embodiment, the polynucleotides may be formulated with a hydrophobic matrix to form a semi-solid composition. As a non-limiting example, the semi-solid composition or paste-like composition may be made by the methods described in International Patent Publication No WO201307604, herein incorporated by reference in its entirety. The semi-solid

composition may be a sustained release formulation as described in International Patent Publication No WO201307604, herein incorporated by reference in its entirety.

[000701] In another embodiment, the semi-solid composition may further have a micro-porous membrane or a biodegradable polymer formed around the composition (see e.g., International Patent Publication No WO201307604, herein incorporated by reference in its entirety).

[000702] The semi-solid composition using the polynucleotides of the present invention may have the characteristics of the semi-solid mixture as described in International Patent Publication No WO201307604, herein incorporated by reference in its entirety (e.g., a modulus of elasticity of at least 10^{-4} $N \cdot mm^{-2}$, and/or a viscosity of at least 100mPa·s).

Exosomes

[000703] In one embodiment, the polynucleotides may be formulated in exosomes. The exosomes may be loaded with at least one polynucleotide and delivered to cells, tissues and/or organisms. As a non-limiting example, the polynucleotides may be loaded in the exosomes described in International Publication No. WO2013084000, herein incorporated by reference in its entirety.

[000704] In one embodiment, the exosome are obtained from cells that have been induced to undergo oxidative stress such as, but not limited to, the exosomes described in International Patent Publication No. WO2014028763, the contents of which are herein incorporated by reference in its entirety.

Silk-Based Delivery

[000705] In one embodiment, the polynucleotides may be formulated in a sustained release silk-based delivery system. The silk-based delivery system may be formed by contacting a silk fibroin solution with a therapeutic agent such as, but not limited to, the polynucleotides described herein and/or known in the art. As a non-limiting example, the sustained release silk-based delivery system which may be used in the present invention and methods of making such system are described in US Patent No 8530625 and US Patent Publication No. US20130177611, the contents of each of which are herein incorporated by reference in their entirety.

Polymeric Microparticles

[000706] In one embodiment, formulations comprising polynucleotides may comprise microparticles. The microparticles may comprise a polymer described herein and/or known in the art such as, but not limited to, poly(α-hydroxy acid), a polyhydroxy butyric acid, a

polycaprolactone, a polyorthoester and a polyanhydride. The microparticle may have adsorbent surfaces to adsorb biologically active molecules such as polynucleotides. As a non-limiting example microparticles for use with the present invention and methods of making microparticles are described in US Patent Publication No. US2013195923, US20130195898 and US 20130236550 and US Patent No. 8,309,139, 8,206,749, and 8,734,832 the contents of each of which are herein incorporated by reference in its entirety. As a non-limiting example, the formulations comprising polynucleotides may comprise any of the microparticles described in or made by the methods described in US Patent Publication No. US20130236550, the contents of which are herein incorporated by reference in its entirety.

[000707] In another embodiment, the formulation may be a microemulsion comprising microparticles and polynucleotides. As a non-limiting example, microemulsions comprising microparticles are described in US Patent Publication No. US2013195923 and US20130195898 and US Patent No. 8,309,139, 8,206,749, and 8,734,832, the contents of each of which are herein incorporated by reference in its entirety.

[000708] In one embodiment, the formulation may include a reverse microemulsion comprising at least one hydrophilic, biologically-active agent solubilized by a hydrophobic reverse emulsion surfactant in a non-stinging, volatile, hydrophobic solvent, wherein said non-stinging, volatile, hydrophobic solvent is selected from the group consisting of volatile linear and cyclic siloxanes, volatile linear, branched and cyclic alkanes, volatile fluorocarbons and chlorofluorocarbons, liquid carbon dioxide under pressure, and combinations thereof, as described in International Patent Publication No. WO2014074289, the contents of which is incorporated herein by reference in its entirety.

Amino Acid Lipids

[000709] In one embodiment, the polynucleotides may be formulated in amino acid lipids. Amino acid lipids are lipophilic compounds comprising an amino acid residue and one or more lipophilic tails. Non-limiting examples of amino acid lipids and methods of making amino acid lipids are described in US Patent No. 8,501,824 and US Patent Publication No. US20140037714, the contents of each of which are herein incorporated by reference in their entirety.

[000710] In one embodiment, the amino acid lipids have a hydrophilic portion and a lipophilic portion. The hydrophilic portion may be an amino acid residue and a lipophilic portion may comprise at least one lipophilic tail.

[000711] In one embodiment, the amino acid lipid formulations may be used to deliver the polynucleotides to a subject.

[000712] In another embodiment, the amino acid lipid formulations may deliver a polynucleotide in releasable form which comprises an amino acid lipid that binds and releases the polynucleotides. As a non-limiting example, the release of the polynucleotides may be provided by an acid-labile linker such as, but not limited to, those described in U.S. Patent Nos. 7,098,032, 6,897,196, 6,426,086, 7,138,382, 5,563,250, and 5,505,931, the contents of each of which are herein incorporated by reference in its entirety.

[000713] In one embodiment, the amino acid lipid is a targeting amino acid lipid as described in International Publication No., WO2013135359, the contents of which are herein incorporated by reference in its entirety, such as but not limited to an amino acid lipid having formula I. As a non-limiting example, the targeting amino acid may target specific tissues and/or cells.

[000714] In another embodiment, the amino acid lipid is an ether-lipid having the general formula I as described in WO2013135360, the contents of which are herein incorporated by reference in its entirety.

[000715] In one embodiment, the amino acid lipid is an amino acid lipid of Formula I as described in US Patent Publication No., US20140037714, the contents of which are herein incorporated by reference in its entirety.

Microvesicles

[000716] In one embodiment, polynucleotides may be formulated in microvesicles. Non-limiting examples of microvesicles include those described in US Patent Publication No. US20130209544, the contents of which are herein incorporated by reference in its entirety.

[000717] In one embodiment, the microvesicle is an ARRDC1-mediated microvesicles (ARMMs). Non-limiting examples of ARMMs and methods of making ARMMs are described in International Patent Publication No. WO2013119602, the contents of which are herein incorporated by reference in its entirety.

[000718] In one embodiment, the microvesicles which may be used to formulate polynucleotides may be made by the methods described in International Publication No. WO2013138427, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, microvesicles comprising polynucleotides may be used to treat diseases

such as cancer as described in International Publication No. WO2013138427, the contents of which are herein incorporated by reference in its entirety.

[000719] In one embodiment, the microvesicles which may be used to formulate polynucleotides may be cell-derived microvesicles described in US Publication No. US20130195765, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, microvesicles comprising polynucleotides may be used to treat diseases such as cancer as described in US Publication No. US20130195765, the contents of which are herein incorporated by reference in its entirety.

Interpolyelectrolyte Complexes

[000720] In one embodiment, the polynucleotides may be formulated in an interpolyelectrolyte complex. Interpolyelectrolyte complexes are formed when charge-dynamic polymers are complexed with one or more anionic molecules. Non-limiting examples of charge-dynamic polymers and interpolyelectrolyte complexes and methods of making interpolyelectrolyte complexes are described in US Patent No. 8,524,368, the contents of which is herein incorporated by reference in its entirety.

Crystalline Polymeric Systems

[000721] In one embodiment, the polynucleotides may be formulated in crystalline polymeric systems. Crystalline polymeric systems are polymers with crystalline moieties and/or terminal units comprising crystalline moieties. Non-limiting examples of polymers with crystalline moieties and/or terminal units comprising crystalline moieties termed "CYC polymers," crystalline polymer systems and methods of making such polymers and systems are described in US Patent No. US 8,524,259, the contents of which are herein incorporated by reference in its entirety.

Polymer and Synthetic Scaffolds

[000722] In one embodiment, the polynucleotides may be formulated in, delivered by or associated with polymer scaffolds. In one embodiment, the polymer scaffold may be a polyester urethane polymer scaffold (PEUR).

[000723] In one embodiment, the polynucleotides may be formulated in, delivered by or associated with biodegradable, synthetic scaffolds such as, but not limited to, prefabricated ε-caprolactone and ethyl ethylene phosphate copolymer (PCLEEP) nanofibers, poly(lactic-*co*-glycolic acid) (PLGA) nanofibers, and porous polyester urethane (PEUR) scaffold design (see

e.g., Nelson et al. *Tunable Delivery of siRNA from a Biodegradable Scaffold to Promote Angiogenesis In Vivo.* Adv. Mater. 2013; the contents of which are herein incorporated by reference in its entirety).

Polymer Implant

[000724] In one embodiment, the polynucleotides may be formulated in or delivered using polymer implants. As a non-limiting example, the polymer implant is inserted into or onto damaged human tissue and the polynucleotides are released from the polymer implant. (See e.g., MariGen Omega3 from Kerecis for the treatment of damaged tissue).

[000725] In one embodiment, the polynucleotides may be formulated in or delivered using delivery devices comprising polymer implants.

Lipomers

[000726] In one embodiment, the polynucleotides may be formulated in or delivered using a conjugated lipomer. As a non-limiting example, the conjugated lipomer may be a conjugated polyethyleneimine (PEI) polymer or a conjugated aza-macrocycle which contains one or more groups of the formula (iii) as described in International Patent Publication No. WO2012135025, the contents of which are herein incorporated by reference in its entirety.

Poloxamer Delivery

[000727] In one embodiment, the polynucleotides may be formulated in or delivered using a pharmaceutical vehicle comprising at least one poloxamer. In one embodiment, the pharmaceutical vehicle may be suitable for the delivery of drugs to the mucosal surfaces such as, but not limited to, the pharmaceutical vehicles described in International Patent Publication No. WO2014027006, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example the poloxamers used in the pharmaceutical vehicles are Poloxamer 407 and Poloxamer 188.

Excipients

[000728] Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes, but are not limited to, any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants, flavoring agents, stabilizers, antioxidants, osmolality adjusting agents, pH adjusting agents and the like, as suited to the particular dosage form desired. Various excipients for formulating

pharmaceutical compositions and techniques for preparing the composition are known in the art (see *Remington*: *The Science and Practice of Pharmacy*, 21st Edition, A. R. Gennaro (Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference in its entirety). The use of a conventional excipient medium may be contemplated within the scope of the present disclosure, except insofar as any conventional excipient medium is incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition, its use is contemplated to be within the scope of this invention.

[000729] In some embodiments, a pharmaceutically acceptable excipient may be at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% pure. In some embodiments, an excipient is approved for use for humans and for veterinary use. In some embodiments, an excipient may be approved by United States Food and Drug Administration. In some embodiments, an excipient may be of pharmaceutical grade. In some embodiments, an excipient may meet the standards of the United States Pharmacopoeia (USP), the European Pharmacopoeia (EP), the British Pharmacopoeia, and/or the International Pharmacopoeia.

[000730] Pharmaceutically acceptable excipients used in the manufacture of pharmaceutical compositions include, but are not limited to, inert diluents, dispersing and/or granulating agents, surface active agents and/or emulsifiers, disintegrating agents, binding agents, preservatives, buffering agents, lubricating agents, and/or oils. Such excipients may optionally be included in pharmaceutical compositions. The composition may also include excipients such as cocoa butter and suppository waxes, coloring agents, coating agents, sweetening, flavoring, and/or perfuming agents.

[000731] Exemplary diluents include, but are not limited to, calcium carbonate, sodium carbonate, calcium phosphate, dicalcium phosphate, calcium sulfate, calcium hydrogen phosphate, sodium phosphate lactose, sucrose, cellulose, microcrystalline cellulose, kaolin, mannitol, sorbitol, inositol, sodium chloride, dry starch, cornstarch, powdered sugar, *etc.*, and/or combinations thereof.

[000732] Exemplary granulating and/or dispersing agents include, but are not limited to, potato starch, corn starch, tapioca starch, sodium starch glycolate, clays, alginic acid, guar gum, citrus pulp, agar, bentonite, cellulose and wood products, natural sponge, cation-exchange resins, calcium carbonate, silicates, sodium carbonate, cross-linked poly(vinyl-pyrrolidone)

(crospovidone), sodium carboxymethyl starch (sodium starch glycolate), carboxymethyl cellulose, cross-linked sodium carboxymethyl cellulose (croscarmellose), methylcellulose, pregelatinized starch (starch 1500), microcrystalline starch, water insoluble starch, calcium carboxymethyl cellulose, magnesium aluminum silicate (VEEGUM®), sodium lauryl sulfate, quaternary ammonium compounds, *etc.*, and/or combinations thereof.

[000733] Exemplary surface active agents and/or emulsifiers include, but are not limited to, natural emulsifiers (e.g. acacia, agar, alginic acid, sodium alginate, tragacanth, chondrux, cholesterol, xanthan, pectin, gelatin, egg yolk, casein, wool fat, cholesterol, wax, and lecithin), colloidal clays (e.g. bentonite [aluminum silicate] and VEEGUM® [magnesium aluminum silicate]), long chain amino acid derivatives, high molecular weight alcohols (e.g. stearyl alcohol, cetyl alcohol, oleyl alcohol, triacetin monostearate, ethylene glycol distearate, glyceryl monostearate, and propylene glycol monostearate, polyvinyl alcohol), carbomers (e.g. carboxy polymethylene, polyacrylic acid, acrylic acid polymer, and carboxyvinyl polymer), carrageenan, cellulosic derivatives (e.g. carboxymethylcellulose sodium, powdered cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose), sorbitan fatty acid esters (e.g. polyoxyethylene sorbitan monolaurate [TWEEN®20], polyoxyethylene sorbitan [TWEEN®60], polyoxyethylene sorbitan monooleate [TWEEN®80], sorbitan monopalmitate [SPAN®40], sorbitan monostearate [SPAN®60], sorbitan tristearate [SPAN®65], glyceryl monooleate, sorbitan monooleate [SPAN®80]), polyoxyethylene esters (e.g. polyoxyethylene monostearate [MYRJ®45], polyoxyethylene hydrogenated castor oil, polyethoxylated castor oil, polyoxymethylene stearate, and SOLUTOL®), sucrose fatty acid esters, polyethylene glycol fatty acid esters (e.g. CREMOPHOR®), polyoxyethylene ethers, (e.g. polyoxyethylene lauryl ether [BRIJ®30]), poly(vinyl-pyrrolidone), diethylene glycol monolaurate, triethanolamine oleate, sodium oleate, potassium oleate, ethyl oleate, oleic acid, ethyl laurate, sodium lauryl sulfate, PLUORINC®F 68, POLOXAMER®188, cetrimonium bromide, cetylpyridinium chloride, benzalkonium chloride, docusate sodium, etc. and/or combinations thereof.

[000734] Exemplary binding agents include, but are not limited to, starch (*e.g.* cornstarch and starch paste); gelatin; sugars (*e.g.* sucrose, glucose, dextrose, dextrin, molasses, lactose, lactitol, mannitol); amino acids (e.g., glycine); natural and synthetic gums (*e.g.* acacia, sodium alginate, extract of Irish moss, panwar gum, ghatti gum, mucilage of isapol husks,

carboxymethylcellulose, methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, microcrystalline cellulose, cellulose acetate, poly(vinyl-pyrrolidone), magnesium aluminum silicate (VEEGUM®), and larch arabogalactan); alginates; polyethylene oxide; polyethylene glycol; inorganic calcium salts; silicic acid; polymethacrylates; waxes; water; alcohol; *etc.*; and combinations thereof.

[000735] Exemplary preservatives may include, but are not limited to, antioxidants, chelating agents, antimicrobial preservatives, antifungal preservatives, alcohol preservatives, acidic preservatives, and/or other preservatives. Oxidation is a potential degradation pathway for mRNA, especially for liquid mRNA formulations. In order to prevent oxidation, antioxidants can be added to the formulation. Exemplary antioxidants include, but are not limited to, alpha tocopherol, ascorbic acid, acorbyl palmitate, benzyl alcohol, butylated hydroxyanisole, EDTA, m-cresol, methionine, butylated hydroxytoluene, monothioglycerol, potassium metabisulfite, propionic acid, propyl gallate, sodium ascorbate, sodium bisulfite, sodium metabisulfite, thioglycerol and/or sodium sulfite. Exemplary chelating agents include ethylenediaminetetraacetic acid (EDTA), citric acid monohydrate, disodium edetate, dipotassium edetate, edetic acid, fumaric acid, malic acid, phosphoric acid, sodium edetate, tartaric acid, and/or trisodium edetate. Exemplary antimicrobial preservatives include, but are not limited to, benzalkonium chloride, benzethonium chloride, benzyl alcohol, bronopol, cetrimide, cetylpyridinium chloride, chlorhexidine, chlorobutanol, chlorocresol, chloroxylenol, cresol, ethyl alcohol, glycerin, hexetidine, imidurea, phenol, phenoxyethanol, phenylethyl alcohol, phenylmercuric nitrate, propylene glycol, and/or thimerosal. Exemplary antifungal preservatives include, but are not limited to, butyl paraben, methyl paraben, ethyl paraben, propyl paraben, benzoic acid, hydroxybenzoic acid, potassium benzoate, potassium sorbate, sodium benzoate, sodium propionate, and/or sorbic acid. Exemplary alcohol preservatives include, but are not limited to, ethanol, polyethylene glycol, phenol, phenolic compounds, bisphenol, chlorobutanol, hydroxybenzoate, and/or phenylethyl alcohol. Exemplary acidic preservatives include, but are not limited to, vitamin A, vitamin C, vitamin E, beta-carotene, citric acid, acetic acid, dehydroacetic acid, ascorbic acid, sorbic acid, and/or phytic acid. Other preservatives include, but are not limited to, tocopherol, tocopherol acetate, deteroxime mesylate, cetrimide, butylated hydroxyanisol (BHA), butylated hydroxytoluened (BHT), ethylenediamine, sodium lauryl sulfate (SLS), sodium lauryl ether sulfate (SLES), sodium bisulfite, sodium metabisulfite, potassium

sulfite, potassium metabisulfite, GLYDANT PLUS®, PHENONIP®, methylparaben, GERMALL®115, GERMABEN®II, NEOLONE™, KATHON™, and/or EUXYL®.

[000736] In some embodiments, the pH of polynucleotide formulations and compositions described herein are maintained between pH 5 and pH 8 (e.g., between pH 5 and pH 7, between pH 6 and pH 8, between pH 6.5 – pH 7.5) for chemical stability. Exemplary buffers to control pH may include, but are not limited to sodium phosphate, sodium citrate, sodium succinate, histidine (or histidine-HCl), sodium carbonate, and/or sodium malate. In another embodiment, the exemplary buffers listed above may be used with additional monovalent counterions (including, but not limited to potassium). Divalent cations may also be used as buffer counterions; however, these are not preferred due to complex formation and/or mRNA degradation.

[000737] Exemplary buffering agents may also include, but are not limited to, citrate buffer solutions, acetate buffer solutions, phosphate buffer solutions, ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium glubionate, calcium gluceptate, calcium gluconate, D-gluconic acid, calcium glycerophosphate, calcium lactate, propanoic acid, calcium levulinate, pentanoic acid, dibasic calcium phosphate, phosphoric acid, tribasic calcium phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium chloride, sodium citrate, sodium lactate, dibasic sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, Ringer's solution, ethyl alcohol, *etc.*, and/or combinations thereof.

[000738] Exemplary lubricating agents include, but are not limited to, magnesium stearate, calcium stearate, stearic acid, silica, talc, malt, glyceryl behanate, hydrogenated vegetable oils, polyethylene glycol, sodium benzoate, sodium acetate, sodium chloride, leucine, magnesium lauryl sulfate, sodium lauryl sulfate, *etc.*, and combinations thereof.

[000739] Exemplary oils include, but are not limited to, almond, apricot kernel, avocado, babassu, bergamot, black current seed, borage, cade, camomile, canola, caraway, carnauba, castor, cinnamon, cocoa butter, coconut, cod liver, coffee, corn, cotton seed, emu, eucalyptus, evening primrose, fish, flaxseed, geraniol, gourd, grape seed, hazel nut, hyssop, isopropyl

myristate, jojoba, kukui nut, lavandin, lavender, lemon, litsea cubeba, macademia nut, mallow, mango seed, meadowfoam seed, mink, nutmeg, olive, orange, orange roughy, palm, palm kernel, peach kernel, peanut, poppy seed, pumpkin seed, rapeseed, rice bran, rosemary, safflower, sandalwood, sasquana, savoury, sea buckthorn, sesame, shea butter, silicone, soybean, sunflower, tea tree, thistle, tsubaki, vetiver, walnut, and wheat germ oils. Exemplary oils include, but are not limited to, butyl stearate, caprylic triglyceride, capric triglyceride, cyclomethicone, diethyl sebacate, dimethicone 360, isopropyl myristate, mineral oil, octyldodecanol, oleyl alcohol, silicone oil, and/or combinations thereof.

[000740] Excipients such as cocoa butter and suppository waxes, coloring agents, coating agents, sweetening, flavoring, and/or perfuming agents can be present in the composition, according to the judgment of the formulator.

[000741] Exemplary additives include physiologically biocompatible buffers (e.g., trimethylamine hydrochloride), addition of chelants (such as, for example, DTPA or DTPA-bisamide) or calcium chelate complexes (as for example calcium DTPA, CaNaDTPA-bisamide), or, optionally, additions of calcium or sodium salts (for example, calcium chloride, calcium ascorbate, calcium gluconate or calcium lactate). In addition, antioxidants and suspending agents can be used.

Cryoprotectants for mRNA

[000742] In some embodiments, polynucleotide formulations may comprise cyroprotectants. As used herein, there term "cryoprotectant" refers to one or more agent that when combined with a given substance, helps to reduce or eliminate damage to that substance that occurs upon freezing. In some embodiments, cryoprotectants are combined with polynucleotides in order to stabilize them during freezing. Frozen storage of mRNA between -20°C and -80°C may be advantageous for long term (e.g. 36 months) stability of polynucleotide. In some embodiments, cryoprotectants are included in polynucleotide formulations to stabilize polynucleotide through freeze/thaw cycles and under frozen storage conditions. Cryoprotectants of the present invention may include, but are not limited to sucrose, trehalose, lactose, glycerol, dextrose, raffinose and/or mannitol. Trehalose is listed by the Food and Drug Administration as being generally regarded as safe (GRAS) and is commonly used in commercial pharmaceutical formulations.

Bulking agents

[000743] In some embodiments, polynucleotide formulations may comprise bulking agents. As used herein, ther term "bulking agent" refers to one or more agents included in formulations to impart a desired consistency to the formulation and/or stabilization of formulation components. In some embodiments, bulking agents are included in lyophilized polynucleotide formulations to yield a "pharmaceutically elegant" cake, stabilizing the lyophilized polynucleotides during long term (e.g. 36 month) storage. Bulking agents of the present invention may include, but are not limited to sucrose, trehalose, mannitol, glycine, lactose and/or raffinose. In some embodiments, combinations of cryoprotectants and bulking agents (for example, sucrose/glycine or trehalose/mannitol) may be included to both stabilize polynucleotides during freezing and provide a bulking agent for lyophilization.

[000744] Non-limiting examples of formulations and methods for formulating the polynucleotides of the present invention are also provided in International Publication No WO2013090648 filed December 14, 2012, the contents of which are incorporated herein by reference in their entirety.

Inactive Ingredients

[000745] In some embodiments, polynucleotide formulations may comprise at least one excipient which is an inactive ingredient. As used herein, ther term "inactive ingredient" refers to one or more inactive agents included in formulations. In some embodiments, all, none or some of the inactive ingredients which may be used in the formulations of the present invention may be approved by the US Food and Drug Administration (FDA).

Delivery

[000746] The present disclosure encompasses the delivery of polynucleotides for any of therapeutic, pharmaceutical, diagnostic or imaging by any appropriate route taking into consideration likely advances in the sciences of drug delivery. Delivery may be naked or formulated.

Naked Delivery

[000747] The polynucleotides of the present invention may be delivered to a cell naked. As used herein in, "naked" refers to delivering polynucleotides free from complexing agents, for example, lipid agents and poloymer agents, etc.. For example, the polynucleotides delivered to the cell may contain no modifications. The naked polynucleotides may be delivered to the cell using routes of administration known in the art and described herein.

Formulated Delivery

[000748] The polynucleotides of the present invention may be formulated, using the methods described herein. The formulations may contain polynucleotides which may be modified and/or unmodified. The formulations may further include, but are not limited to, cell penetration agents, a pharmaceutically acceptable carrier, a delivery agent, a bioerodible or biocompatible polymer, a solvent, and a sustained-release delivery depot. The formulated polynucleotides may be delivered to the cell using routes of administration known in the art and described herein.

[000749] The compositions may also be formulated for direct delivery to an organ or tissue in any of several ways in the art including, but not limited to, direct soaking or bathing, via a catheter, by gels, powder, ointments, creams, gels, lotions, and/or drops, by using substrates such as fabric or biodegradable materials coated or impregnated with the compositions, and the like.

Administration

[000750] The polynucleotides of the present invention may be administered by any route which results in a therapeutically effective outcome. These include, but are not limited to intravenous (into a vein), intraarterial (into an artery), intramuscular (into a muscle), intravitreal, (through the eye), intracavernous injection (into a pathologic cavity), transmucosal (diffusion through a mucous membrane), eye drops (onto the conjunctiva), conjunctival, intracorneal (within the cornea), intraocular (within the eye), iontophoresis (by means of electric current where ions of soluble salts migrate into the tissues of the body), irrigation (to bathe or flush open wounds or body cavities), ophthalmic (to the external eye), retrobulbar (behind the pons or behind the eyeball), subconjunctival, submucosal, and/or topical,.

[000751] In one embodiment, a formulation for a route of administration may include at least one inactive ingredient. Non-limiting examples of routes of administration and inactive ingredients which may be included in formulations for the specific route of administration is shown in Table 9. In Table 9, "AN" means anesthetic, "CNBLK" means cervical nerve block, "NBLK" means nerve block, "IV" means intravenous, "IM" means intramuscular and "SC" means subcutaneous.

Table 9. Routes of Adminsitration and Inactive Ingredients

Route of Administration	Inactive Ingredient
Intraocular	Benzalkonium Chloride; Calcium Chloride; Citric Acid Monohydrate; Hydrochloric Acid; Magnesium Chloride; Polyvinyl Alcohol; Potassium Chloride; Sodium Acetate; Sodium Chloride; Sodium Citrate; Sodium Hydroxide

Intravitreal	Calcium Chloride; Carboxymethylcellulose Sodium; Cellulose, Microcrystalline; Hyaluronate Sodium; Hydrochloric Acid; Magnesium Chloride; Magnesium Stearate; Polysorbate 80; Polyvinyl Alcohol; Potassium Chloride; Sodium Acetate; Sodium Bicarbonate; Sodium Carbonate; Sodium Chloride; Sodium Hydroxide; Sodium Phosphate, Dibasic, Heptahydrate; Sodium Phosphate, Monobasic, Monohydrate; Trisodium Citrate Dihydrate
Irrigation	Acetic Acid; Activated Charcoal; Benzoic Acid; Hydrochloric Acid; Hypromelloses; Methylparaben; Nitrogen; Sodium Bisulfite; Sodium Citrate; Sodium Hydroxide; Sulfuric Acid
Any Delivery Route	Alcohol; Benzyl Alcohol; Citric Acid Monohydrate; Gelfoam Sponge; Hydrochloric Acid; Methylparaben; Poly(Dl-Lactic-Co-Glycolic Acid), (50:50; Poly(Dl-Lactic-Co-Glycolic Acid), Ethyl Ester Terminated, (50:50; Polyquaternium-7 (70/30 Acrylamide/Dadmac ; Propylene Glycol; Propylparaben; Sodium Chloride; Sodium Citrate ; Sodium Hydroxide; Sodium Lactate ; Sodium Phosphate, Monobasic, Monohydrate
Nerve Block	Acetic Acid; Acetone Sodium Bisulfite; Ascorbic Acid; Benzyl Alcohol; Calcium Chloride; Carbon Dioxide; Chlorobutanol; Citric Acid; Citric Acid Monohydrate; Edetate Calcium Disodium; Edetate Disodium; Hydrochloric Acid; Hydrochloric Acid, Diluted; Lactic Acid; Methylparaben; Monothioglycerol; Nitrogen; Potassium Chloride; Potassium Metabisulfite; Potassium Phosphate, Monobasic; Propylparaben; Sodium Bisulfite; Sodium Carbonate; Sodium Chlorate; Sodium Chloride; Sodium Citrate; Sodium Hydroxide; Sodium Lactate; Sodium Lactate, L-; Sodium Metabisulfite; Sodium Phosphate; Sodium Phosphate, Dibasic, Heptahydrate
Ophthalmic	Acetic Acid; Alcohol; Alcohol, Dehydrated; Alginic Acid; Amerchol-Cab; Ammonium Hydroxide; Anhydrous Trisodium Citrate; Antipyrine; Benzalkonium Chloride; Benzethonium Chloride; Benzododecinium Bromide; Boric Acid; Caffeine; Calcium Chloride; Carbomer 1342; Carbomer 934p; Carbomer 940; Carbomer Homopolymer Type B (Allyl Pentaerythritol Crosslinked); Carboxymethylcellulose Sodium; Castor Oil; Cetyl Alcohol; Chlorobutanol; Chlorobutanol, Anhydrous; Cholesterol; Citric Acid; Citric Acid Monohydrate; Creatinine; Diethanolamine; Diethylhexyl Phthalate **See Cder Guidance: Limiting The Use Of Certain Phthalates As Excipients In Cder-Regulated Products; Divinylbenzene Styrene Copolymer; Edetate Disodium; Edetate Disodium Anhydrous; Edetate Sodium; Ethylene Vinyl Acetate Copolymer; Gellan Gum (Low Acyl); Glycerin; Glyceryl Stearate; High Density Polyethylene; Hydrocarbon Gel, Plasticized; Hydrochloric Acid; Hydrochloric Acid, Diluted; Hydroxyethyl Cellulose; Hydroxypropyl Methylcellulose 2906; Hypromellose 2910 (15000 Mpa.S); Hypromelloses; Jelene; Lanolin; Lanolin Alcohols; Lanolin Anhydrous; Lanolin Nonionic Derivatives; Lauralkonium Chloride; Lauroyl Sarcosine; Light Mineral Oil; Magnesium Chloride; Mannitol; Methylcellulose (4000 Mpa.S); Methylcelluloses; Methylparaben; Mineral Oil; Nitric Acid; Nitrogen; Nonoxynol-9; Octoxynol-40; Octylphenol Polymethylene; Petrolatum; Petrolatum, White; Phenylethyl Alcohol; Phenylmercuric Acetate; Phenylmercuric Nitrate; Phosphoric Acid; Polidronium Chloride; Poloxamer 188; Poloxamer 407; Polycarbophil; Polyethylene Glycol 300; Polyethylene Glycol 400; Polyethylene Glycol 8000; Polyoxyethylene - Polyoxypropylene 1800; Polyoxyl 35 Castor Oil; Polyoxyl 40 Hydrogenated Castor Oil; Polyoxyl 40 Stearate; Polypropylene Glycol;

	Polysorbate 20; Polysorbate 60; Polysorbate 80; Polyvinyl Alcohol; Potassium Acetate; Potassium Chloride; Potassium Phosphate, Monobasic; Potassium Sorbate; Povidone K29/32; Povidone K30; Povidone K90; Povidones; Propylene Glycol; Propylparaben; Soda Ash; Sodium Acetate; Sodium Bisulfate; Sodium Bisulfite; Sodium Borate; Sodium Borate Decahydrate; Sodium Carbonate; Sodium Carbonate Monohydrate; Sodium Chloride; Sodium Citrate; Sodium Hydroxide; Sodium Metabisulfite; Sodium Nitrate; Sodium Phosphate; Sodium Phosphate Dihydrate; Sodium Phosphate, Dibasic; Sodium Phosphate, Dibasic, Anhydrous; Sodium Phosphate, Dibasic, Dihydrate; Sodium Phosphate, Dibasic, Heptahydrate; Sodium Phosphate, Monobasic; Sodium Phosphate, Monobasic, Anhydrous; Sodium Phosphate, Monobasic, Dihydrate; Sodium Phosphate, Monobasic, Monohydrate; Sodium Sulfate; Sodium Sulfate Anhydrous; Sodium Sulfate Decahydrate; Sodium Sulfite; Sodium Thiosulfate; Sorbic Acid; Sorbitan Monolaurate; Sorbitol; Sorbitol Solution; Stabilized Oxychloro Complex; Sulfuric Acid; Thimerosal; Titanium Dioxide; Tocophersolan; Trisodium Citrate Dihydrate; Triton 720; Tromethamine; Tyloxapol; Zinc Chloride
Soft Tissue	Acetic Acid; Anhydrous Trisodium Citrate; Benzyl Alcohol; Carboxymethylcellulose; Carboxymethylcellulose Sodium; Citric Acid; Creatinine; Edetate Disodium; Hydrochloric Acid; Methylcelluloses; Methylparaben; Myristyl-.Gamma.-Picolinium Chloride; Phenol; Phosphoric Acid; Polyethylene Glycol 3350; Polyethylene Glycol 4000; Polysorbate 80; Propylparaben; Sodium Acetate; Sodium Bisulfite; Sodium Chloride; Sodium Citrate; Sodium Hydroxide; Sodium Phosphate; Sodium Phosphate, Dibasic; Sodium Phosphate, Dibasic, Heptahydrate; Sodium Phosphate, Monobasic; Sodium Phosphate, Monobasic, Anhydrous; Sodium Sulfite
Subconjunctival	Benzyl Alcohol; Hydrochloric Acid; Sodium Hydroxide

[000752] Non-limiting routes of administration for the polynucleotides of the present invention are described below.

Parenteral and Injectable Administration

[000753] Liquid dosage forms for parenteral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, oral compositions can include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and/or perfuming agents. In certain

embodiments for parenteral administration, compositions are mixed with solubilizing agents such as CREMOPHOR®, alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof.

[000754] A pharmaceutical composition for parenteral administration may comprise at least one inactive ingredient. Any or none of the inactive ingredients used may have been approved by the US Food and Drug Administration (FDA). A non-exhaustive list of inactive ingredients for use in pharmaceutical compositions for parenteral administration includes hydrochloric acid, mannitol, nitrogen, sodium acetate, sodium chloride and sodium hydroxide.

[000755] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables. The sterile formulation may also comprise adjuvants such as local anesthetics, preservatives and buffering agents.

[000756] Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[000757] Injectable formulations may be for direct injection into a region of a tissue, organ and/or subject. As a non-limiting example, a tissue, organ and/or subject may be directly injected a formulation by intramyocardial injection into the ischemic region. (See e.g., Zangi et al. Nature Biotechnology 2013; the contents of which are herein incorporated by reference in its entirety).

[000758] In order to prolong the effect of an active ingredient, it is often desirable to slow the absorption of the active ingredient from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor

water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

[000759] In one embodiment, injectable formulations may comprise an excipient in addition to the polynucleotides described herein. As a non-limiting example the excipient may be N-acetyl-D-glucosasamine.

[000760] In one embodiment, formulations comprising the polynucleotides described herein may be formulated for intramuscular delivery may comprise an excipient. As a non-limiting example the excipient may be N-acetyl-D-glucosasamine.

[000761] In one embodiment, formulations comprising the polynucleotides described herein may be delivered with a microneedle device with an autodisable feature for intradermal delivery, as described in International Patent Publication No. WO2014064543, the contents of which is incorporated herein by reference in its entirety.

[000762] In another embodiment, the formulations of the invention may be delivered to the blood vessel lumen and wall. In some embodiments the formulations may include for example, antirestenotic, antithrombotic, antiplatelet, antiproliferative, antineoplastic, immunosuppressive, angiogenic, anti¬inflammatory, or antiangiogenic agents and/or vasodilators for delivery to a blood vessel. In a non-limiting example, the formulations may be delivered with a drug delivery device having an exterior surface and an interior surface; a plurality of openings in the device body; and a first therapeutic agent and a second therapeutic agent disposed in the openings and arranged to deliver the first therapeutic agent primarily to the exterior surface and to deliver the second therapeutic agent primarily to the interior surface, as described in European Patent No. EP1635893, the contents of which is herein incorporated by reference in its entirety.

Depot Administration

[000763] As described herein, in some embodiments, the composition is formulated in depots for extended release. Generally, a specific organ or tissue (a "target tissue", e.g., the eye) is targeted for administration.

[000764] In some aspects of the invention, the polynucleotides are spatially retained within or proximal to a target tissue. Provided are method of providing a composition to a target tissue of a mammalian subject by contacting the target tissue (which contains one or more target cells) with the composition under conditions such that the composition, in particular the nucleic acid component(s) of the composition, is substantially retained in the target tissue, meaning that at least 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the composition is retained in the target tissue. Advantageously, retention is determined by measuring the amount of the nucleic acid present in the composition that enters one or more target cells. For example, at least 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the nucleic acids administered to the subject are present intracellularly at a period of time following administration. For example, intramuscular injection to a mammalian subject is performed using an aqueous composition containing a ribonucleic acid and a transfection reagent, and retention of the composition is determined by measuring the amount of the ribonucleic acid present in the muscle cells.

[000765] Aspects of the invention are directed to methods of providing a composition to a target tissue of a mammalian subject, by contacting the target tissue (containing one or more target cells) with the composition under conditions such that the composition is substantially retained in the target tissue. The composition contains an effective amount of a polynucleotides such that the polypeptide of interest is produced in at least one target cell. The compositions generally contain a cell penetration agent, although "naked" nucleic acid (such as nucleic acids without a cell penetration agent or other agent) is also contemplated, and a pharmaceutically acceptable carrier.

[000766] In some circumstances, the amount of a protein produced by cells in a tissue is desirably increased. Preferably, this increase in protein production is spatially restricted to cells within the target tissue. Thus, provided are methods of increasing production of a protein of interest in a tissue of a mammalian subject. A composition is provided that contains polynucleotides characterized in that a unit quantity of composition has been determined to

produce the polypeptide of interest in a substantial percentage of cells contained within a predetermined volume of the target tissue.

[000767] In some embodiments, the composition includes a plurality of different polynucleotides, where one or more than one of the polynucleotides encodes a polypeptide of interest. Optionally, the composition also contains a cell penetration agent to assist in the intracellular delivery of the composition. A determination is made of the dose of the composition required to produce the polypeptide of interest in a substantial percentage of cells contained within the predetermined volume of the target tissue (generally, without inducing significant production of the polypeptide of interest in tissue adjacent to the predetermined volume, or distally to the target tissue). Subsequent to this determination, the determined dose is introduced directly into the tissue of the mammalian subject.

[000768] In one embodiment, the invention provides for the polynucleotides to be delivered in more than one injection or by split dose injections.

[000769] In one embodiment, the invention may be retained near target tissue using a small disposable drug reservoir, patch pump or osmotic pump. Non-limiting examples of patch pumps include those manufactured and/or sold by BD® (Franklin Lakes, NJ), Insulet Corporation (Bedford, MA), SteadyMed Therapeutics (San Francisco, CA), Medtronic (Minneapolis, MN) (e.g., MiniMed), UniLife (York, PA), Valeritas (Bridgewater, NJ), and SpringLeaf Therapeutics (Boston, MA). A non-limiting example of an osmotic pump include those manufactured by DURECT® (Cupertino, CA) (e.g., DUROS® and ALZET ®).

Ophthalmic Administration

[000770] A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for delivery to and/or around the eye and/or delivery to the ear (e.g., auricular (otic) administration). Non-limiting examples of route of administration for delivery to and/or around the eye include retrobulbar, conjuctival, intracorneal, intraocular, intravitreal, ophthlamic and subconjuctiva. Such formulations may, for example, be in the form of eye drops or ear drops including, for example, a 0.1/1.0% (w/w) solution and/or suspension of the active ingredient in an aqueous or oily liquid excipient. Such drops may further comprise buffering agents, salts, and/or one or more other of any additional ingredients described herein. Other ophthalmically-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form and/or in a liposomal preparation. Ear drops

and/or eye drops are contemplated as being within the scope of this invention. A multilayer thin film device may be prepared to contain a pharmaceutical composition for delivery to the eye and/or surrounding tissue.

[000771] A pharmaceutical composition for ophthalmic administration may comprise at least one inactive ingredient. Any or none of the inactive ingredients used may have been approved by the US Food and Drug Administration (FDA). A non-exhaustive list of inactive ingredients for use in pharmaceutical compositions for ophthalmic administration includes acetic acid, alcohol, alcohol, dehydrated, alginic acid, amerchol-cab, ammonium hydroxide, anhydrous trisodium citrate, antipyrine, benzalkonium chloride, benzethonium chloride, benzododecinium bromide, boric acid, caffeine, calcium chloride, carbomer 1342, carbomer 934p, carbomer 940, carbomer homopolymer type b (allyl pentaerythritol crosslinked), carboxymethylcellulose sodium, castor oil, cetyl alcohol, chlorobutanol, chlorobutanol, anhydrous, cholesterol, citric acid, citric acid monohydrate, creatinine, diethanolamine, diethylhexyl phthalate, divinylbenzene styrene copolymer, edetate disodium, edetate disodium anhydrous, edetate sodium, ethylene vinyl acetate copolymer, gellan gum (low acyl), glycerin, glyceryl stearate, high density polyethylene, hydrocarbon gel, plasticized, hydrochloric acid, hydrochloric acid, diluted, hydroxyethyl cellulose, hydroxypropyl methylcellulose 2906, hypromellose 2910 (15000 mpa.s), hypromelloses, jelene, lanolin, lanolin alcohols, lanolin anhydrous, lanolin nonionic derivatives, lauralkonium chloride, lauroyl sarcosine, light mineral oil, magnesium chloride, mannitol, methylcellulose (4000 mpa.s), methylcelluloses, methylparaben, mineral oil, nitric acid, nitrogen, nonoxynol-9, octoxynol-40, octylphenol polymethylene, petrolatum, petrolatum, white, phenylethyl alcohol, phenylmercuric acetate, phenylmercuric nitrate, phosphoric acid, polidronium chloride, poloxamer 188, poloxamer 407, polycarbophil, polyethylene glycol 300, polyethylene glycol 400, polyethylene glycol 8000, polyoxyethylene - polyoxypropylene 1800, polyoxyl 35 castor oil, polyoxyl 40 hydrogenated castor oil, polyoxyl 40 stearate, polypropylene glycol, polysorbate 20, polysorbate 60, polysorbate 80, polyvinyl alcohol, potassium acetate, potassium chloride, potassium phosphate, monobasic, potassium sorbate, povidone k29/32, povidone k30, povidone k90, povidones, propylene glycol, propylparaben, soda ash, sodium acetate, sodium bisulfate, sodium bisulfite, sodium borate, sodium borate decahydrate, sodium carbonate, sodium carbonate monohydrate, sodium chloride, sodium citrate, sodium hydroxide, sodium metabisulfite, sodium nitrate, sodium phosphate, sodium phosphate dihydrate, sodium

phosphate, dibasic, sodium phosphate, dibasic, anhydrous, sodium phosphate, dibasic, dihydrate, sodium phosphate, dibasic, heptahydrate, sodium phosphate, monobasic, sodium phosphate, monobasic, anhydrous, sodium phosphate, monobasic, dihydrate, sodium phosphate, monobasic, monohydrate, sodium sulfate, sodium sulfate anhydrous, sodium sulfate decahydrate, sodium sulfite, sodium thiosulfate, sorbic acid, sorbitan monolaurate, sorbitol, sorbitol solution, stabilized oxychloro complex, sulfuric acid, thimerosal, titanium dioxide, tocophersolan, trisodium citrate dihydrate, triton 720, tromethamine, tyloxapol and zinc chloride.

[000772] A pharmaceutical composition for retrobulbar administration may comprise at least one inactive ingredient. Any or none of the inactive ingredients used may have been approved by the US Food and Drug Administration (FDA). A non-exhaustive list of inactive ingredients for use in pharmaceutical compositions for retrobulbar administration includes hydrochloric acid and sodium hydroxide.

[000773] A pharmaceutical composition for intraocular administration may comprise at least one inactive ingredient. Any or none of the inactive ingredients used may have been approved by the US Food and Drug Administration (FDA). A non-exhaustive list of inactive ingredients for use in pharmaceutical compositions for intraocular administration includes benzalkonium chloride, calcium chloride, citric acid monohydrate, hydrochloric acid, magnesium chloride, polyvinyl alcohol, potassium chloride, sodium acetate, sodium chloride, sodium citrate and sodium hydroxide.

[000774] A pharmaceutical composition for intravitreal administration may comprise at least one inactive ingredient. Any or none of the inactive ingredients used may have been approved by the US Food and Drug Administration (FDA). A non-exhaustive list of inactive ingredients for use in pharmaceutical compositions for intravitreal administration includes calcium chloride, carboxymethylcellulose sodium, cellulose, microcrystalline, hyaluronate sodium, hydrochloric acid, magnesium chloride, magnesium stearate, polysorbate 80, polyvinyl alcohol, potassium chloride, sodium acetate, sodium bicarbonate, sodium carbonate, sodium chloride, sodium hydroxide, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate and trisodium citrate dehydrate.

[000775] A pharmaceutical composition for subconjunctival administration may comprise at least one inactive ingredient. Any or none of the inactive ingredients used may have been approved by the US Food and Drug Administration (FDA). A non-exhaustive list of inactive

ingredients for use in pharmaceutical compositions for subconjunctival administration includes benzyl alcohol, hydrochloric acid and sodium hydroxide.

[000776] A pharmaceutical composition may be administered by a sustained release drug delivery system as described in US Patent Publication No. 20140107594, the contents of which is incorporated herein by reference in its entirety, for example through implantation into limited anatomical spaces, such as the eye.

Payload Administration: Detectable Agents and Therapeutic Agents

[000777] The polynucleotides described herein can be used in a number of different scenarios in which delivery of a substance (the "payload") to a biological target is desired, for example delivery of detectable substances for detection of the target, or delivery of a therapeutic agent. Detection methods can include, but are not limited to, both imaging *in vitro* and *in vivo* imaging methods, *e.g.*, immunohistochemistry, bioluminescence imaging (BLI), Magnetic Resonance Imaging (MRI), positron emission tomography (PET), electron microscopy, X-ray computed tomography, Raman imaging, optical coherence tomography, absorption imaging, thermal imaging, fluorescence reflectance imaging, fluorescence microscopy, fluorescence molecular tomographic imaging, nuclear magnetic resonance imaging, X-ray imaging, ultrasound imaging, photoacoustic imaging, lab assays, or in any situation where tagging/staining/imaging is required.

[000778] The polynucleotides can be designed to include both a linker and a payload in any useful orientation. For example, a linker having two ends is used to attach one end to the payload and the other end to the nucleobase, such as at the C-7 or C-8 positions of the deaza-adenosine or deaza-guanosine or to the N-3 or C-5 positions of cytosine or uracil. The polynucleotide of the invention can include more than one payload (e.g., a label and a transcription inhibitor), as well as a cleavable linker. In one embodiment, the modified nucleotide is a modified 7-deaza-adenosine triphosphate, where one end of a cleavable linker is attached to the C7 position of 7-deaza-adenine, the other end of the linker is attached to an inhibitor (e.g., to the C5 position of the nucleobase on a cytidine), and a label (e.g., Cy5) is attached to the center of the linker (see, e.g., compound 1 of A*pCp C5 Parg Capless in Fig. 5 and columns 9 and 10 of U.S. Pat. No. 7,994,304, incorporated herein by reference). Upon incorporation of the modified 7-deaza-adenosine triphosphate to an encoding region, the resulting polynucleotide having a cleavable linker attached to a label and an inhibitor (e.g., a

polymerase inhibitor). Upon cleavage of the linker (e.g., with reductive conditions to reduce a linker having a cleavable disulfide moiety), the label and inhibitor are released. Additional linkers and payloads (e.g., therapeutic agents, detectable labels, and cell penetrating payloads) are described herein and in International Application PCT/US2013/30062 filed March 9, 2013 (Attorney Docket Number M300), the contents of which are incorporated herein by reference in their entirety.

[000779] In some embodiments, the payload may be a detectable agent, such as various organic small molecules, inorganic compounds, nanoparticles, enzymes or enzyme substrates, fluorescent materials, luminescent materials (e.g., luminol), bioluminescent materials (e.g., luciferase, luciferin, and aequorin), chemiluminescent materials, radioactive materials (e.g., ^{18}F, ^{67}Ga, ^{81m}Kr, ^{82}Rb, ^{111}In, ^{123}I, ^{133}Xe, ^{201}Tl, ^{125}I, ^{35}S, ^{14}C, ^{3}H, or ^{99m}Tc (e.g., as pertechnetate (technetate(VII), TcO_4^-)), and contrast agents (e.g., gold (*e.g.*, gold nanoparticles), gadolinium (e.g., chelated Gd), iron oxides (e.g., superparamagnetic iron oxide (SPIO), monocrystalline iron oxide nanoparticles (MIONs), and ultrasmall superparamagnetic iron oxide (USPIO)), manganese chelates (e.g., Mn-DPDP), barium sulfate, iodinated contrast media (iohexol), microbubbles, or perfluorocarbons). Such optically-detectable labels include for example, without limitation, 4-acetamido-4'-isothiocyanatostilbene-2,2′disulfonic acid; acridine and derivatives (e.g., acridine and acridine isothiocyanate); 5-(2′-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS); 4-amino-N-[3-vinylsulfonyl)phenyl]naphthalimide-3,5 disulfonate; N-(4-anilino-l-naphthyl)maleimide; anthranilamide; BODIPY; Brilliant Yellow; coumarin and derivatives (e.g., coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), and 7-amino-4-trifluoromethylcoumarin (Coumarin 151)); cyanine dyes; cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5′ 5"-dibromopyrogallol-sulfonaphthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2′-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2′-disulfonic acid; 5-[dimethylamino]-naphthalene-1-sulfonyl chloride (DNS, dansylchloride); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives (e.g., eosin and eosin isothiocyanate); erythrosin and derivatives (e.g., erythrosin B and erythrosin isothiocyanate); ethidium; fluorescein and derivatives (e.g., 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2′,7'-dimethoxy-4'5′-dichloro-6-carboxyfluorescein, fluorescein, fluorescein isothiocyanate, X-rhodamine-5-(and-6)-

isothiocyanate (QFITC or XRITC), and fluorescamine); 2-[2-[3-[[1,3-dihydro-1,1-dimethyl-3-(3-sulfopropyl)-2H-benz[e]indol-2-ylidene]ethylidene]-2-[4-(ethoxycarbonyl)-1-piperazinyl]-1-cyclopenten-1-yl]ethenyl]-1,1-dimethyl-3-(3-sulforpropyl)-1H-benz[e]indolium hydroxide, inner salt, compound with n,n-diethylethanamine(1:1) (IR144); 5-chloro-2-[2-[3-[(5-chloro-3-ethyl-2(3H)-benzothiazol- ylidene)ethylidene]-2-(diphenylamino)-1-cyclopenten-1-yl]ethenyl]-3-ethyl benzothiazolium perchlorate (IR140); Malachite Green isothiocyanate; 4-methylumbelliferone orthocresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives(e.g., pyrene, pyrene butyrate, and succinimidyl 1-pyrene); butyrate quantum dots; Reactive Red 4 (CIBACRON™ Brilliant Red 3B-A); rhodamine and derivatives (e.g., 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride rhodarnine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, sulforhodamine B, sulforhodamine 101, sulfonyl chloride derivative of sulforhodamine 101 (Texas Red), N,N,N',N'tetramethyl-6-carboxyrhodamine (TAMRA) tetramethyl rhodamine, and tetramethyl rhodamine isothiocyanate (TRITC)); riboflavin; rosolic acid; terbium chelate derivatives; Cyanine-3 (Cy3); Cyanine-5 (Cy5); cyanine-5.5 (Cy5.5), Cyanine-7 (Cy7); IRD 700; IRD 800; Alexa 647; La Jolta Blue; phthalo cyanine; and naphthalo cyanine.

[000780] In some embodiments, the detectable agent may be a non-detectable pre-cursor that becomes detectable upon activation (e.g., fluorogenic tetrazine-fluorophore constructs (e.g., tetrazine-BODIPY FL, tetrazine-Oregon Green 488, or tetrazine-BODIPY TMR-X) or enzyme activatable fluorogenic agents (e.g., PROSENSE® (VisEn Medical))). In vitro assays in which the enzyme labeled compositions can be used include, but are not limited to, enzyme linked immunosorbent assays (ELISAs), immunoprecipitation assays, immunofluorescence, enzyme immunoassays (EIA), radioimmunoassays (RIA), and Western blot analysis.

Combinations

[000781] The polynucleotides may be used in combination with one or more other therapeutic, prophylactic, diagnostic, or imaging agents. By "in combination with," it is not intended to imply that the agents must be administered at the same time and/or formulated for delivery together, although these methods of delivery are within the scope of the present disclosure. Compositions can be administered concurrently with, prior to, or subsequent to, one or more other desired therapeutics or medical procedures. In general, each agent will be administered at

a dose and/or on a time schedule determined for that agent. In some embodiments, the present disclosure encompasses the delivery of pharmaceutical, prophylactic, diagnostic, or imaging compositions in combination with agents that may improve their bioavailability, reduce and/or modify their metabolism, inhibit their excretion, and/or modify their distribution within the body.

[000782] In one embodiment, the polynucleotides described here may be used in combination with one or more other agents as described in International Patent Application No. PCT/US2014/027077, the contents of which are incorporated by reference in its entirety, such as in paragraphs [000978] – [001023].

[000783] In one embodiment, polynucleotides may be co-administered with at least one amino acid and/or at least one small molecule additive. As used herein, "co-administered" means the administration of two or more components. These components for co-administration include, but are not limited to active ingredients, polynucleotides, amino acids, inactive ingredients and excipients. Co-administration refers to the administration of two or more components simultaneously or with a time lapse between administration such as 1 second, 5 seconds, 10 seconds, 15 seconds, 30 seconds, 45 seconds, 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 11 minutes, 12 minutes, 13 minutes, 14 minutes, 15 minutes, 16 minutes, 17 minutes, 18 minutes, 19 minutes, 20 minutes, 21 minutes, 22 minutes, 23 minutes, 24 minutes, 25 minutes, 26 minutes, 27 minutes, 28 minutes, 29 minutes, 30 minutes, 31 minutes, 32 minutes, 33 minutes, 34 minutes, 35 minutes, 36 minutes, 37 minutes, 38 minutes, 39 minutes, 40 minutes, 41 minutes, 42 minutes, 43 minutes, 44 minutes, 45 minutes, 46 minutes, 47 minutes, 48 minutes, 49 minutes, 50 minutes, 51 minutes, 52 minutes, 53 minutes, 54 minutes, 55 minutes, 56 minutes, 57 minutes, 58 minutes, 59 minutes, 1 hour, 1.5 hours, 2 hours, 2.5 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 1 day, 1.5 days, 2 days, or more than 3 days.

[000784] In one embodiment, polynucleotides may be co-administered with at least one amino acid and/or at least one small molecule additive may be used to enhance cellular uptake, enhance intracellular release, increase translation, increase the duration of protein exposure, reduce the dosage requirement of the polynucleotide, reduce conformational diversity, improve chemical stability of the polynucleotide, increase the storage shelf life of formulations, conformational

stability of the polynucleotide (e.g., in formulation, storage, during transport *in vivo*), reduced variability, form predictable physical structures, increase the dosage options for polynucleotides and/or increase the half-life of polynucleotide formulations. As a non-limiting example, a formulation with polynucleotides and at least one amino acid may be formulated in nanoparticles greater than 100 nm or in micron aggregates. These larger dosage forms may be used in various delivery options such as depots.

[000785] In one embodiment, the co-administration of the polynucleotide may be prior to the amino acid. In another embodiment, the co-administration of the polynucleotide may be after to the amino acid.

[000786] In one embodiment, the amount of polynucleotide co-administered in any dosage form may be from about 0.0001 mg/kg to about 100 mg/kg, from about 0.001 mg/kg to about 0.05 mg/kg, from about 0.005 mg/kg to about 0.05 mg/kg, from about 0.001 mg/kg to about 0.005 mg/kg, from about 0.05 mg/kg to about 0.5 mg/kg, from about 0.01 mg/kg to about 50 mg/kg, from about 0.1 mg/kg to about 40 mg/kg, from about 0.5 mg/kg to about 30 mg/kg, from about 0.01 mg/kg to about 10 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, or from about 1 mg/kg to about 25 mg/kg, of subject body weight (see e.g., the range of unit doses described in International Publication No WO2013078199, herein incorporated by reference in its entirety). The polynucleotide may be administered once a day, more than once a day, every other day, weekly, monthly, bimonthly or by a dosage schedule outlined herein.

[000787] In one embodiment, the amount of polynucleotide co-administered in any dosage form may be from about 0.1 ug to about 50 mg, including 0.1 ug, 0.2 ug, 0.3 ug, 0.4 ug, 0.5 ug, 0.6 ug, 0.7 ug, 0.8 ug, 0.9 ug, 1.0 ug, 2 ug, 3 ug, 4 ug, 5 ug, 6 ug, 7 ug, 8 ug, 9 ug, 10 ug, 11 ug, 12 ug, 13 ug, 14 ug, 15 ug, 16 ug, 17 ug, 18 ug, 19 ug, 20 ug, 21 ug, 22 ug, 23 ug, 24 ug, 25 ug, 26 ug, 27 ug, 28 ug, 29 ug, 30 ug, 31 ug, 32 ug, 33 ug, 34 ug, 35 ug, 36 ug, 37 ug, 38 ug, 39 ug, 40 ug, 41 ug, 42 ug, 43 ug, 44 ug, 45 ug, 46 ug, 47 ug, 48 ug, 49 ug, 50 ug, 51 ug, 52 ug, 53 ug, 54 ug, 55 ug, 56 ug, 57 ug, 58 ug, 59 ug, 60 ug, 61 ug, 62 ug, 63 ug, 64 ug, 65 ug, 66 ug, 67 ug, 68 ug, 69 ug, 70 ug, 71 ug, 72 ug, 73 ug, 74 ug, 75 ug, 76 ug, 77 ug, 78 ug, 79 ug, 80 ug, 81 ug, 82 ug, 83 ug, 84 ug, 85 ug, 86 ug, 87 ug, 88 ug, 89 ug, 90 ug, 91 ug, 92 ug, 93 ug, 94 ug, 95 ug, 96 ug, 97 ug, 98 ug, 99 ug, 0.1 mg, 0.2 mg, 0.3 mg, 0.4 mg, 0.5 mg, 0.6 mg, 0.7 mg, 0.8 mg, 0.9 mg, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 11 mg, 12 mg, 13 mg, 14 mg, 15 mg, 16 mg, 17 mg, 18 mg, 19 mg, 20 mg, 21 mg, 22 mg, 23 mg, 24 mg, 25 mg, 26 mg,

27 mg, 28 mg, 29 mg, 30 mg, 31 mg, 32 mg, 33 mg, 34 mg, 35 mg, 36 mg, 37 mg, 38 mg, 39 mg, 40 mg, 41 mg, 42 mg, 43 mg, 44 mg, 45 mg, 46 mg, 47 mg, 48 mg, 49 mg and 50 mg. The polynucleotide may be administered once a day, more than once a day, every other day, weekly, monthly, bimonthly or by a dosage schedule outlined herein.

[000788] In one embodiment, the amount of amino acid co-administered is from about 0.0001 mg/kg to about 100 mg/kg, from about 0.001 mg/kg to about 0.005 mg/kg, from about 0.001 mg/kg to about 0.05 mg/kg, from about 0.001 mg/kg to about 0.5 mg/kg, from about 0.001 mg/kg to about 1 mg/kg, from about 0.001 mg/kg to about 5 mg/kg, from about 0.001 mg/kg to about 10 mg/kg, from about 0.001 mg/kg to about 15 mg/kg, from about 0.001 mg/kg to about 20 mg/kg, from about 0.001 mg/kg to about 25 mg/kg, from about 0.001 mg/kg to about 30 mg/kg, from about 0.001 mg/kg to about 35 mg/kg, from about 0.001 mg/kg to about 40 mg/kg, from about 0.001 mg/kg to about 45 mg/kg, from about 0.001 mg/kg to about 50 mg/kg, from about 0.005 mg/kg to about 0.05 mg/kg, from about 0.005 mg/kg to about 0.5 mg/kg, from about 0.005 mg/kg to about 1 mg/kg, from about 0.005 mg/kg to about 5 mg/kg, from about 0.005 mg/kg to about 10 mg/kg, from about 0.005 mg/kg to about 15 mg/kg, from about 0.005 mg/kg to about 20 mg/kg, from about 0.005 mg/kg to about 25 mg/kg, from about 0.005 mg/kg to about 30 mg/kg, from about 0.005 mg/kg to about 35 mg/kg, from about 0.005 mg/kg to about 40 mg/kg, from about 0.005 mg/kg to about 45 mg/kg, from about 0.005 mg/kg to about 50 mg/kg, from about 0.05 mg/kg to about 0.5 mg/kg, from about 0.05 mg/kg to about 1 mg/kg, from about 0.05 mg/kg to about 5 mg/kg, from about 0.05 mg/kg to about 10 mg/kg, from about 0.05 mg/kg to about 15 mg/kg, from about 0.05 mg/kg to about 20 mg/kg, from about 0.05 mg/kg to about 25 mg/kg, from about 0.05 mg/kg to about 30 mg/kg, from about 0.05 mg/kg to about 35 mg/kg, from about 0.05 mg/kg to about 40 mg/kg, from about 0.05 mg/kg to about 45 mg/kg, from about 0.05 mg/kg to about 50 mg/kg, from about 0.01 mg/kg to about 0.5 mg/kg, from about 0.01 mg/kg to about 1 mg/kg, from about 0.01 mg/kg to about 5 mg/kg, from about 0.01 mg/kg to about 10 mg/kg, from about 0.01 mg/kg to about 15 mg/kg, from about 0.01 mg/kg to about 20 mg/kg, from about 0.01 mg/kg to about 25 mg/kg, from about 0.01 mg/kg to about 30 mg/kg, from about 0.01 mg/kg to about 35 mg/kg, from about 0.01 mg/kg to about 40 mg/kg, from about 0.01 mg/kg to about 45 mg/kg, from about 0.01 mg/kg to about 50 mg/kg, from about 0.1 mg/kg to about 0.5 mg/kg, from about 0.1 mg/kg to about 1 mg/kg, from about 0.1 mg/kg to about 5 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, from about 0.1 mg/kg to

about 15 mg/kg, from about 0.1 mg/kg to about 20 mg/kg, from about 0.1 mg/kg to about 25 mg/kg, from about 0.1 mg/kg to about 30 mg/kg, from about 0.1 mg/kg to about 35 mg/kg, from about 0.1 mg/kg to about 40 mg/kg, from about 0.1 mg/kg to about 45 mg/kg, from about 0.1 mg/kg to about 50 mg/kg, from about 0.5 mg/kg to about 30 mg/kg, from about 0.5 mg/kg to about 1 mg/kg, from about 0.5 mg/kg to about 5 mg/kg, from about 0.5 mg/kg to about 10 mg/kg, from about 0.5 mg/kg to about 15 mg/kg, from about 0.5 mg/kg to about 20 mg/kg, from about 0.5 mg/kg to about 25 mg/kg, from about 0.5 mg/kg to about 30 mg/kg, from about 0.5 mg/kg to about 35 mg/kg, from about 0.5 mg/kg to about 40 mg/kg, from about 0.5 mg/kg to about 45 mg/kg, or from about 0.5 mg/kg to about 50 mg/kg, of subject body weight (see e.g., the range of unit doses described in International Publication No WO2013078199, herein incorporated by reference in its entirety). The amino acid may be administered once a day, more than once a day, every other day, weekly, monthly, bimonthly or by a dosage schedule outlined herein. As a non-limiting example, the amount of amino acid co-administered per dose is a maximum of 48 mg/kg.

[000789] In one embodiment, the ratio of polynucleotide to total amino acid (P:AA Ratio) may be 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18, 1:19, 1:20, 1:21, 1:22, 1:23, 1:24, 1:25, 1:26, 1:27, 1:28, 1:29, 1:30, 1:31, 1:32, 1:33, 1:34, 1:35, 1:36, 1:37, 1:38, 1:39, 1:40, 1:41, 1:42, 1:43, 1:44, 1:45, 1:46, 1:47, 1:48, 1:49, 1:50, 1:51, 1:52, 1:53, 1:54, 1:55, 1:56, 1:57, 1:58, 1:59, 1:60, 1:61, 1:62, 1:63, 1:64, 1:65, 1:66, 1:67, 1:68, 1:69, 1:70, 1:71, 1:72, 1:73, 1:74, 1:75, 1:76, 1:77, 1:78, 1:79, 1:80, 1:81, 1:82, 1:83, 1:84, 1:85, 1:86, 1:87, 1:88, 1:89, 1:90, 1:91, 1:92, 1:93, 1:94, 1:95, 1:96, 1:97, 1:98, 1:99, 1:100, 1:110, 1:120, 1:130, 1:140, 1:150, 1:160, 1:170, 1:180, 1:190, 1:200, 1:225, 1:250, 1:275, 1:300, 1:325, 1:350, 1:400, 1:450, 1:500, 1:550, 1:600 or greater than 1:600. The ratio may be molar, percent, weight, molar mass, nitrogen and phosphorus (N:P) ratio or any other ratio known or described herein.

[000790] In one embodiment, the ratio of total amino acid to polynucleotide may be 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18, 1:19, 1:20, 1:21, 1:22, 1:23, 1:24, 1:25, 1:26, 1:27, 1:28, 1:29, 1:30, 1:31, 1:32, 1:33, 1:34, 1:35, 1:36, 1:37, 1:38, 1:39, 1:40, 1:41, 1:42, 1:43, 1:44, 1:45, 1:46, 1:47, 1:48, 1:49, 1:50, 1:51, 1:52, 1:53, 1:54, 1:55, 1:56, 1:57, 1:58, 1:59, 1:60, 1:61, 1:62, 1:63, 1:64, 1:65, 1:66, 1:67, 1:68, 1:69, 1:70, 1:71, 1:72, 1:73, 1:74, 1:75, 1:76, 1:77, 1:78, 1:79, 1:80, 1:81, 1:82, 1:83, 1:84, 1:85, 1:86, 1:87, 1:88,

1:89, 1:90, 1:91, 1:92, 1:93, 1:94, 1:95, 1:96, 1:97, 1:98, 1:99, 1:100, 1:110, 1:120, 1:130, 1:140, 1:150, 1:160, 1:170, 1:180, 1:190, 1:200, 1:225, 1:250, 1:275, 1:300, 1:325, 1:350, 1:400, 1:450, 1:500, 1:550, 1:600 or greater than 1:600. The ratio may be molar, percent, weight, molar mass, nitrogen and phosphorus (N:P) ratio, length, or any other ratio known or described herein.

[000791] In one embodiment, the ratio of total amino acid to the polynucleotide may be greater than the polynucleotide. In another embodiment, the ratio of the mass of the total amino acid may be greater than the mass of the polynucleotide. In yet another embodiment, the ratio of the molar composition of the total amino acid may be greater than the molar composition of the polynucleotide. The ratio of total amino acid may be 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x, 11x, 12x, 13x, 14x, 15x, 16x, 17x, 18x, 19x, 20x, 21x, 22x, 23x, 24x, 25x, 26x, 27x, 28x, 29x, 30x, 31x, 32x, 33x, 34x, 35x, 36x, 37x, 38x, 39x, 40x, 41x, 42x, 43x, 44x, 45x, 46x, 47x, 48x, 49x, 50x, 51x, 52x, 53x, 54x, 55x, 56x, 57x, 58x, 59x, 60x, 61x, 62x, 63x, 64x, 65x, 66x, 67x, 68x, 69x, 70x, 71x, 72x, 73x, 74x, 75x, 76x, 77x, 78x, 79x, 80x, 81x, 82x, 83x, 84x, 85x, 86x, 87x, 88x, 89x, 90x, 91x, 92x, 93x, 94x, 95x, 96x, 97x, 98x, 99x, 100x, 110x, 120x, 130x, 140x, 150x, 160x, 170x, 180x, 190x, 200x, 225x, 250x, 275x, 300x, 325x, 350x, 400x, 450x, 500x, 550x, 600x or greater than 650x to the polynucleotide.

[000792] It will further be appreciated that therapeutically, prophylactically, diagnostically, or imaging active agents utilized in combination may be administered together in a single composition or administered separately in different compositions. In general, it is expected that agents utilized in combination with be utilized at levels that do not exceed the levels at which they are utilized individually. In some embodiments, the levels utilized in combination will be lower than those utilized individually. In one embodiment, the combinations, each or together may be administered according to the split dosing regimens described herein.

Dosing

[000793] The present invention provides methods comprising administering modified mRNAs and their encoded proteins or complexes in accordance with the invention to a subject in need thereof. Nucleic acids, proteins or complexes, or pharmaceutical, imaging, diagnostic, or prophylactic compositions thereof, may be administered to a subject using any amount and any route of administration effective for preventing, treating, diagnosing, or imaging a disease, disorder, and/or condition (*e.g.*, a disease, disorder, and/or condition relating to working memory deficits). The exact amount required will vary from subject to subject, depending on the species,

age, and general condition of the subject, the severity of the disease, the particular composition, its mode of administration, its mode of activity, and the like. Compositions in accordance with the invention are typically formulated in dosage unit form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of the compositions of the present invention may be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective, prophylactically effective, or appropriate imaging dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts.

[000794] In certain embodiments, compositions in accordance with the present invention may be administered at dosage levels sufficient to deliver from about 0.0001 mg/kg to about 100 mg/kg, from about 0.001 mg/kg to about 0.05 mg/kg, from about 0.005 mg/kg to about 0.05 mg/kg, from about 0.001 mg/kg to about 0.005 mg/kg, from about 0.05 mg/kg to about 0.5 mg/kg, from about 0.01 mg/kg to about 50 mg/kg, from about 0.1 mg/kg to about 40 mg/kg, from about 0.5 mg/kg to about 30 mg/kg, from about 0.01 mg/kg to about 10 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, or from about 1 mg/kg to about 25 mg/kg, of subject body weight per day, one or more times a day, to obtain the desired therapeutic, diagnostic, prophylactic, or imaging effect (see e.g., the range of unit doses described in International Publication No WO2013078199, herein incorporated by reference in its entirety). The desired dosage may be delivered three times a day, two times a day, once a day, every other day, every third day, every week, every two weeks, every three weeks, or every four weeks. In certain embodiments, the desired dosage may be delivered using multiple administrations (*e.g.*, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or more administrations). When multiple administrations are employed, split dosing regimens such as those described herein may be used.

[000795] According to the present invention, it has been discovered that administration of polynucleotides in split-dose regimens produce higher levels of proteins in mammalian subjects.

As used herein, a "split dose" is the division of single unit dose or total daily dose into two or more doses, e.g, two or more administrations of the single unit dose. As used herein, a "single unit dose" is a dose of any therapeutic administed in one dose/at one time/single route/single point of contact, i.e., single administration event. As used herein, a "total daily dose" is an amount given or prescribed in 24 hr period. It may be administered as a single unit dose. In one embodiment, the polynucleotides of the present invention are administed to a subject in split doses. The polynucleotides may be formulated in buffer only or in a formulation described herein.

Dosage Forms

[000796] A pharmaceutical composition described herein can be formulated into a dosage form described herein, such as a topical, intranasal, intratracheal, or injectable (e.g., intravenous, intraocular, intravitreal, intramuscular, intracardiac, intraperitoneal, subcutaneous).

Liquid dosage forms

[000797] Liquid dosage forms for parenteral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art including, but not limited to, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. In certain embodiments for parenteral administration, compositions may be mixed with solubilizing agents such as CREMOPHOR®, alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof.

Injectable

[000798] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art and may include suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed include, but are not limited to, water, Ringer's solution, U.S.P.,

and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables.

[000799] Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[000800] In order to prolong the effect of an active ingredient, it may be desirable to slow the absorption of the active ingredient from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the polynucleotides then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered polynucleotides may be accomplished by dissolving or suspending the polynucleotides in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the polynucleotides in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of polynucleotides to polymer and the nature of the particular polymer employed, the rate of polynucleotides release can be controlled. Examples of other biodegradable polymers include, but are not limited to, poly(orthoesters) and poly(anhydrides). Depot injectable formulations may be prepared by entrapping the polynucleotides in liposomes or microemulsions which are compatible with body tissues.

[000801] Localized injection of naked DNA was demonstrated intramuscularly in 1990 and later was injected into several other tissues including liver, skin and brain. The uptake of the DNA was mostly localized in the area of the needle track. Different agents may be used to enhance overall gene expression. In one embodiment, the polynucleotides may be administered with an agent to enhance expression. Non-limiting examples of agents include transferrin, water-immiscible solvents, nonionic polymers, surfactants, and nuclease inhibitors.

[000802] A needle-free delivery method known as jet injection may be used to deliver a drug to a tissue. The jet injection method uses a high-speed ultrafine stream of solution driven by a pressurized gas. The penetration power of this method may be adjusted by altering the gas pressure and the mechanical properties of the target tissue. The fluid being administered travels

through the path of least resistance and may facilitate transport outside the traditional zone of delivery. As a non-limiting example, the solution may include the polynucleotides described herein. The solution (approximately 3-5 ul) may be loaded into the jet injection device and administered to a tissue at a pressure of approximately 1-3 bars. Commerical liquid jet injectors include, but are not limited to, Vitaject and bioject 2000 (Bioject), Advantagect (Activa systems), Injex 30 (Injex equidyne) and Mediject VISION (Antares Pharma).

[000803] Microneedles may be used to inject the polynucleotides and formulations thereof described herein. Microneedles are an array of microstructured projections which can be coated with a drug that can be administered to a subject to provide delivery of therapeutic agents (e.g., polynucleotides) within the epidermis. Microneedles can be approximately 1 um in diameter and from about 1 um to about 100 um (e.g., about 1 um, about 2 um, about 3 um, about 4 um, about 5 um, about 6 um, about 7 um, about 8 um, about 9 um, about 10 um, about 12 um, about 14 um, about 15 um, about 16 um, about 18 um, about 20 um, about 25 um, about 30 um, about 35 um, about 40 um, about 45 um, about 50 um, about 55 um, about 60 um, about 65 um, about 70 um about 75 um, about 80 um, about 85 um, about 90 um, about 95 um, or about 100 um) in length. The material used to make microneedles may be, but is not limited to, metals, silicon, silicon dioxide, polymers, glass and other materials and the material selected may depend on the type of agent to be delivered and the tissue contacted. In one embodiment, the miconeedles may be solid and may either be straight, bend or filtered. In one embodiment, the miconeedles may be hollow and may either be straight, bend or filtered.

[000804] In one embodiment, the polynucleotides and formulations thereof may be administered using a microneedle drug delivery system. The microneedles may be hollow, solid or a combination thereof. As a non-limiting example, the microneedle drug delivery system may be the 3M Hollow Microstructured Transdermal System (hMTS). As another non-limiting example, the microneedle drug delivery system may be a microneedle patch comprising solid microneedle technology from 3M (3M Drug Delivery Systems).

[000805] In one embodiment, the formulations described herein may be administered using a multi-prong needle device. As a non-limiting example, the device may administer more than one formulation in a single delivery. The formulations may be delivered at the same time or the formulations may have a pre-determined interval between each formulation delivery.

[000806] In one embodiment, the formulations described herein may be administered to more than one location to a tissue, organ or subject at the same time using a multi-prong needle device. The formulations may be administered at the same time or the formulations may have a pre-determined interval between each administration of a formulation.

[000807] In one embodiment, the amount of formulation comprising the polynucleotides administered may be varied depending on the type of injection and/or the cell, tissue or organ administered the formulation. As a non-limiting example, for intramuscular injection the formulation may be more concentrated to produce a polypeptide of interest as compared to a formulation for intravenous delivery.

Coatings or Shells

[000808] Solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally comprise opacifying agents and can be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. Solid compositions of a similar type may be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

Multi-dose and repeat-dose administration

[000809] In some embodiments, compounds and/or compositions of the present invention may be administered in two or more doses (referred to herein as "multi-dose administration"). Such doses may comprise the same components or may comprise components not included in a previous dose. Such doses may comprise the same mass and/or volume of components or an altered mass and/or volume of components in comparison to a previous dose. In some embodiments, multi-dose administration may comprise repeat-dose administration. As used herein, the term "repeat-dose administration" refers to two or more doses administered consecutively or within a regimen of repeat doses comprising substantially the same components provided at substantially the same mass and/or volume. In some embodiments, subjects may display a repeat-dose response. As used herein, the term "repeat-dose response" refers to a response in a subject to a repeat-dose that differs from that of another dose administered within a repeat-dose administration regimen. In some embodiments, such a response may be the

expression of a protein in response to a repeat-dose comprising mRNA. In such embodiments, protein expression may be elevated in comparison to another dose administered within a repeat-dose administration regimen or protein expression may be reduced in comparison to another dose administered within a repeat-dose administration regimen. Alteration of protein expression may be from about 1% to about 20%, from about 5% to about 50% from about 10% to about 60%, from about 25% to about 75%, from about 40% to about 100% and/or at least 100%. A reduction in expression of mRNA administered as part of a repeat-dose regimen, wherein the level of protein translated from the administered RNA is reduced by more than 40% in comparison to another dose within the repeat-dose regimen is referred to herein as "repeat-dose resistance."

Properties of the Pharmaceutical Compositions

[000810] The pharmaceutical compositions described herein can be characterized by one or more of the following properties:

Bioavailability

[000811] The polynucleotides, when formulated into a composition with a delivery agent as described herein, can exhibit an increase in bioavailability as compared to a composition lacking a delivery agent as described herein. As used herein, the term "bioavailability" refers to the systemic availability of a given amount of polynucleotides administered to a mammal. Bioavailability can be assessed by measuring the area under the curve (AUC) or the maximum serum or plasma concentration (C_{max}) of the unchanged form of a compound following administration of the compound to a mammal. AUC is a determination of the area under the curve plotting the serum or plasma concentration of a compound along the ordinate (Y-axis) against time along the abscissa (X-axis). Generally, the AUC for a particular compound can be calculated using methods known to those of ordinary skill in the art and as described in G. S. Banker, Modern Pharmaceutics, Drugs and the Pharmaceutical Sciences, v. 72, Marcel Dekker, New York, Inc., 1996, herein incorporated by reference in its entirety.

[000812] The C_{max} value is the maximum concentration of the compound achieved in the serum or plasma of a mammal following administration of the compound to the mammal. The C_{max} value of a particular compound can be measured using methods known to those of ordinary skill in the art. The phrases "increasing bioavailability" or "improving the pharmacokinetics," as used herein mean that the systemic availability of a first polynucleotides, measured as AUC, C_{max}, or C_{min} in a mammal is greater, when co-administered with a delivery agent as described herein,

than when such co-administration does not take place. In some embodiments, the bioavailability of the polynucleotides can increase by at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%.

[000813] In some embodiments, liquid formulations of polynucleotides may have varying in vivo half-life, requiring modulation of doses to yield a therapeutic effect. To address this, in some embodiments of the present invention, polynucleotides formulations may be designed to improve bioavailability and/or therapeutic effect during repeat administrations. Such formulations may enable sustained release of polynucleotides and/or reduce polynucleotide degradation rates by nucleases. In some embodiments, suspension formulations are provided comprising polynucleotides, water immiscible oil depots, surfactants and/or co-surfactants and/or co-solvents. Combinations of oils and surfactants may enable suspension formulation with polynucleotides. Delivery of polynucleotides in a water immiscible depot may be used to improve bioavailability through sustained release of polynucleotides from the depot to the surrounding physiologic environment and/or prevent polynucleotide degradation by nucleases.

[000814] In some embodiments, cationic nanoparticles comprising combinations of divalent and monovalent cations may be formulated with polynucleotides. Such nanoparticles may form spontaneously in solution over a given period (e.g. hours, days, etc). Such nanoparticles do not form in the presence of divalent cations alone or in the presence of monovalent cations alone. The delivery of polynucleotides in cationic nanoparticles or in one or more depot comprising cationic nanoparticles may improve polynucleotide bioavailability by acting as a long-acting depot and/or reducing the rate of degradation by nucleases.

Therapeutic Window

[000815] The polynucleotides, when formulated into a composition with a delivery agent as described herein, can exhibit an increase in the therapeutic window of the administered polynucleotides composition as compared to the therapeutic window of the administered polynucleotides composition lacking a delivery agent as described herein. As used herein "therapeutic window" refers to the range of plasma concentrations, or the range of levels of therapeutically active substance at the site of action, with a high probability of eliciting a

therapeutic effect. In some embodiments, the therapeutic window of the polynucleotides when co-administered with a delivery agent as described herein can increase by at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%.

Volume of Distribution

[000816] The polynucleotides, when formulated into a composition with a delivery agent as described herein, can exhibit an improved volume of distribution (V_{dist}), e.g., reduced or targeted, relative to a composition lacking a delivery agent as described herein. The volume of distribution (Vdist) relates the amount of the drug in the body to the concentration of the drug in the blood or plasma. As used herein, the term "volume of distribution" refers to the fluid volume that would be required to contain the total amount of the drug in the body at the same concentration as in the blood or plasma: Vdist equals the amount of drug in the body/concentration of drug in blood or plasma. For example, for a 10 mg dose and a plasma concentration of 10 mg/L, the volume of distribution would be 1 liter. The volume of distribution reflects the extent to which the drug is present in the extravascular tissue. A large volume of distribution reflects the tendency of a compound to bind to the tissue components compared with plasma protein binding. In a clinical setting, Vdist can be used to determine a loading dose to achieve a steady state concentration. In some embodiments, the volume of distribution of the polynucleotides when co-administered with a delivery agent as described herein can decrease at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%.

Biological Effect

[000817] In one embodiment, the biological effect of the modified mRNA delivered to the animals may be categorized by analyzing the protein expression in the animals. The protein expression may be determined from analyzing a biological sample collected from a mammal administered the modified mRNA of the present invention. In one embodiment, the expression

protein encoded by the modified mRNA administered to the mammal of at least 50 pg/ml may be preferred. For example, a protein expression of 50-200 pg/ml for the protein encoded by the modified mRNA delivered to the mammal may be seen as a therapeutically effective amount of protein in the mammal.

Detection of Polynucleotides Acids by Mass Spectrometry

[000818] Mass spectrometry (MS) is an analytical technique that can provide structural and molecular mass/concentration information on molecules after their conversion to ions. The molecules are first ionized to acquire positive or negative charges and then they travel through the mass analyzer to arrive at different areas of the detector according to their mass/charge (m/z) ratio. Methods of detecting polynucleotides are described in co-pending International Patent Publication No. WO2015038892, the contents of which is incorporated by reference in its entirety, such as, but not limited to, in paragraphs [0001055] – [0001067].

V. Uses of polynucleotides of the Invention

[000819] The polynucleotides of the present invention are designed, in preferred embodiments, to provide for avoidance or evasion of deleterious bio-responses such as the immune response and/or degradation pathways, overcoming the threshold of expression and/or improving protein production capacity, improved expression rates or translation efficiency, improved drug or protein half-life and/or protein concentrations, optimized protein localization, to improve one or more of the stability and/or clearance in tissues, receptor uptake and/or kinetics, cellular access by the compositions, engagement with translational machinery, secretion efficiency (when applicable), accessibility to circulation, and/or modulation of a cell's status, function and/or activity.

Therapeutics

[000820] The polynucleotides of the present invention, such as, but not limited to, IVT polynucleotides, chimeric polynucleotides, modified nucleic acids and modified RNAs, and the proteins translated from them described herein can be used as therapeutic or prophylactic agents. They are provided for use in medicine. For example, a polynucleotide described herein can be administered to a subject, wherein the polynucleotides is translated *in vivo* to produce a therapeutic or prophylactic polypeptide in the subject. Provided are compositions, methods, kits, and reagents for diagnosis, treatment or prevention of a disease or condition in humans and other

mammals. The active therapeutic agents of the invention include polynucleotides, cells containing polynucleotides or polypeptides translated from the polynucleotides.

[000821] Provided herein are methods of inducing translation of a recombinant polypeptide in a cell population using the polynucleotides described herein. Such translation can be *in vivo*, *ex vivo*, *in culture*, or *in vitro*. The cell population is contacted with an effective amount of a composition containing a nucleic acid that has at least one nucleoside modification, and a translatable region encoding the recombinant polypeptide. The population is contacted under conditions such that the nucleic acid is localized into one or more cells of the cell population and the recombinant polypeptide is translated in the cell from the nucleic acid.

[000822] An "effective amount" of the composition is provided based, at least in part, on the target tissue, target cell type, means of administration, physical characteristics of the nucleic acid (e.g., size, and extent of modified nucleosides), and other determinants. In general, an effective amount of the composition provides efficient protein production in the cell, preferably more efficient than a composition containing a corresponding unmodified nucleic acid. Increased efficiency may be demonstrated by increased cell transfection (i.e., the percentage of cells transfected with the nucleic acid), increased protein translation from the nucleic acid, decreased nucleic acid degradation (as demonstrated, e.g., by increased duration of protein translation from a modified nucleic acid), or reduced innate immune response of the host cell.

[000823] Aspects of the invention are directed to methods of inducing *in vivo* translation of a recombinant polypeptide in a mammalian subject in need thereof. Therein, an effective amount of a composition containing a nucleic acid that has at least one structural or chemical modification and a translatable region encoding the recombinant polypeptide is administered to the subject using the delivery methods described herein. The nucleic acid is provided in an amount and under other conditions such that the nucleic acid is localized into a cell of the subject and the recombinant polypeptide is translated in the cell from the nucleic acid. The cell in which the nucleic acid is localized, or the tissue in which the cell is present, may be targeted with one or more than one rounds of nucleic acid administration.

[000824] In certain embodiments, the administered polynucleotides directs production of one or more recombinant polypeptides that provide a functional activity which is substantially absent in the cell, tissue or organism in which the recombinant polypeptide is translated. For example, the missing functional activity may be enzymatic, structural, or gene regulatory in nature. In

related embodiments, the administered polynucleotides directs production of one or more recombinant polypeptides that increases (e.g., synergistically) a functional activity which is present but substantially deficient in the cell in which the recombinant polypeptide is translated.

[000825] In other embodiments, the administered polynucleotides directs production of one or more recombinant polypeptides that replace a polypeptide (or multiple polypeptides) that is substantially absent in the cell in which the recombinant polypeptide is translated. Such absence may be due to genetic mutation of the encoding gene or regulatory pathway thereof. In some embodiments, the recombinant polypeptide increases the level of an endogenous protein in the cell to a desirable level; such an increase may bring the level of the endogenous protein from a subnormal level to a normal level or from a normal level to a super-normal level.

[000826] Alternatively, the recombinant polypeptide functions to antagonize the activity of an endogenous protein present in, on the surface of, or secreted from the cell. Usually, the activity of the endogenous protein is deleterious to the subject; for example, due to mutation of the endogenous protein resulting in altered activity or localization. Additionally, the recombinant polypeptide antagonizes, directly or indirectly, the activity of a biological moiety present in, on the surface of, or secreted from the cell.

[000827] The recombinant proteins described herein may be engineered for localization within the cell, potentially within a specific compartment such as the nucleus, or are engineered for secretion from the cell or translocation to the plasma membrane of the cell.

[000828] In some embodiments, modified mRNAs and their encoded polypeptides in accordance with the present invention may be used for treatment of any of a variety of diseases, disorders, and/or conditions, including but not limited to one or more of the following: diabetic retinopathy, dry eye, cataracts, retinal vein occlusion, macular edema, macular degeneration (wet & dry), refraction and accommodation disorders, keratoconus, amblyopia, glaucoma, Sstargardt disease, endophthalmitis, conjunctivitis, uveitis, retinal detachment, corneal ulcers, dacryocystitis, Duane retraction syndrome, and optic neuritis.

[000829] The subject to whom the therapeutic agent may be administered suffers from or may be at risk of developing a disease, disorder, or deleterious condition.

Ophthalmic diseases

[000830] The compositions of the present invention may be useful, among others, in (1) the inhibition of angiogenesis and vessel maturation (2) inhibition of inflammation-induced immune

cell recruitment and associated cellular damage, (3) reduction of hypoxia-induced damage and gene induction, (4) inhibition of cell death and/or (5) management of retinal homeostasis. In some embodiments the polynucleotide compositions may be used for the treatment of ophthalmic disorder or disease which involve or implicate the inhibition of angiogenesis and vessel maturation. Such disorders or disease include wet AMD. Targets involved in such implicated pathways include VEGF and PDGF. In one embodiment, a polynucleotide of the invention encodes, as a bicistronic or polycistronic construct, VEGFR1/2-Fc and PDGFRbb-Fc. Hence both targets are delivered on a single mRNA construct. Without wishing to be bound by theory, associated formulation or osmolarity challenges seen by applying two and more than one protein formulation would not appear given that the polycistronic mRNA is delivered in one formulation with a defined osmolarity and characteristics.

[000831] In some embodiments the polynucleotide compositions may be used for the treatment of ophthalmic disorder or disease which involve or implicate the inhibition of inflammation induced cellular damage and recruitment of immune cell into the retinal space, an area that is normally held immune-privileged to avoid irreversible damage of the retina by evading activated immune cells. Such disorders or disease include wet AMD as well as early and late dry AMD. Targets involved in such implicated pathways include components of the complement system and the chemokine system such as C3, MAPs, Eotaxin-1 and/or CCR3. In one embodiment, a polynucleotide of the invention encodes one or more of the targets CFH, MAP-1, bertilimumab, and a dominant form of CCR3 or other chemokine receptor to block immune cell infiltration.

[000832] In some embodiments the polynucleotide compositions may be used for the treatment of ophthalmic disorder or disease which involve or implicate the reduction of hypoxia induced damage and gene induction. Hypoxia-induced gene induction is the triggering event for transition from a healthy retina to dry AMD. Targets involved in such implicated pathways include DICER1,Alu-RNA, SOD1 (when overexpressed) and HIF-1alpha. In one embodiment, a polynucleotide of the invention encodes one or more of the targets DICER1, SOD1, and/or HIF-PHD. Such a therapeutic would be administered during an early stage of the disease and could be in particular helpful for preventive treatment of patients with a genetic/allelic predisposition for dry and/or wet AMD.

[000833] In some embodiments the polynucleotide compositions may be used for the treatment of ophthalmic disorder or disease which involve or implicate the inhibition of cell death in retinal

pigment epithelial cells, endothelial cells and photoreceptor cells. Such disorders or disease include geographic atrophy (GA) and also wet AMD. Targets involved in such implicated pathways include TRAIL and/or Alu-RNA. In one embodiment, a polynucleotide of the invention encodes one or more of the targets DcR1 and/or DICER1. In another embodiment, a polynucleotide of the invention encodes DcR1 together with a miR142-3p site focusing expression to non-immune cells. Without wishing to be bound by theory, as TRAIL-induced apopotosis induction is active in infiltrating immune cells, this focuses the anti-apoptotic signal to all cell critical for retinal homeostasis and functional eye sight.

In some embodiments the polynucleotide compositions may be used for the treatment of ophthalmic disorder or disease which involve or implicate the management of retinal homeostasis. Such disorders or disease include late dry AMD. Targets involved in such implicated pathways include those involved in notch mediated angiogenesis and Plexin-D1. In one embodiment, a polynucleotide of the invention encodes one or more of the targets Notch4, Hey1, and/or furin-resistant Sema3E. Such a therapeutic might be expressed selectively in the choroidal endothelial cells.

Modulation of Biological Pathways

[000834] The rapid translation polynucleotides introduced into cells provides a desirable mechanism of modulating target biological pathways. Such modulation includes antagonism or agonism of a given pathway. In one embodiment, a method is provided for antagonizing a biological pathway in a cell by contacting the cell with an effective amount of a composition comprising a polynucleotide encoding a polypeptide of interest, under conditions such that the polynucleotides is localized into the cell and the polypeptide is capable of being translated in the cell from the polynucleotides, wherein the polypeptide inhibits the activity of a polypeptide functional in the biological pathway.

[000835] Exemplary biological pathways which may be altered or modulated using the ophthalmic polynucleotides of the present invention are those involving neovascularization, the complement cascade, vascular cell infiltration, NLRP3 mediated cell death, reducing HIF-1 transcription of VEGF and/or PDGF, modulating the antioxidant system, reducing apoptotic pathway signaling, and modulating angiogenesis.

Modulating neovascularization

[000836] In some embodiments, the ophthalmic polynucleotides of the present invention function for dual inhibition of VEGF and PDGF induced neovascularization. Published efficacy of VEGF/PDGF inhibition has been demonstrated in multiple studies of laser-induced CNV mice and in pre-clinical models of existing therapeutics

[000837] Without wishing to be bound by theory, associated formulation or osmolarity challenges seen by applying two and more than one protein formulation would not appear given that the polycistronic mRNA is delivered in one formulation with a defined osmolarity and characteristics.

Modulating the complement cascade

[000838] In some embodiments, the ophthalmic polynucleotides of the present invention function to interrupt the complement cascade by inhibiting the MASP complex and C3b. In an animal model, C3 inhibitor compastatin (POT-4 precursor) suppresses drusen formation in cynomolgus monkey model of macular degeneration (Chi, ZL. Adv Exp Med Biol. 2010, the contents of which are incorporated herein by reference in its entirety).

Controlling vascular cell infiltration

[000839] In some embodiments, the ophthalmic polynucleotides of the present invention function to block early vascular cell infiltration by inhibiting CCR3. It is known that CCR3 blockade is more effective at reducing CNV than VEGF-A neutralization in laser injury mouse model of AMD (Takeda, et al. Nature, 2009, the contents of which are incorporated herein by reference in its entirety). It is also known that CCR3 antagonist reduces CNV in alkali burn animal model (Zhou et al, Int J Ophthalmol. 2012, the contents of which are incorporated herein by reference in its entirety). However, to date no efficacy has been shown in man.

Modulating NLRP3 mediated cell death

[000840] In some embodiments, the ophthalmic polynucleotides of the present invention function to restore DICER1 expression in order to reduce NLRP3 mediated cell death in geographic atrophy. It is known that DICER1 is reduced in the RPE of humans with GA and that NLRP3 and IL18 mRNA are significantly increased in GA vs normal human eyes, localized to RPE. Further it is known that DICER knockdown induces RPE generation in wild-type mice due to MyD88 mediated apoptosis and that DICER overexpression rescues Alu-RNA induced RPE degeneration.

Reducing HIF-1 transcription of VEGF and/or PDGF

[000841] In some embodiments, the ophthalmic polynucleotides of the present invention function to reduce HIF1 transcription of VEGF and PDGF. It is known that HIF-1a knockout mice are protected against inflammation and vascular leakage after laser injury (Schipani and Clemens, IBMS BoneKEy, 2008, the contents of which are incorporated herein by reference in its entirety).

Modulating the antioxidant system

[000842] In some embodiments, the ophthalmic polynucleotides of the present invention function to modulate the antioxidant system. SOD2 polymorphisms increase the risk of AMD and mutations in phototransduction and mitochondrial oxidative metabolism lead to congenital disease. It is known that SOD1 deficient mice show drusen, choroidal neovascularization, and retinal pigment epithelium dysfunction and that SOD2 knockdown mice also mimic early AMD phenotype (Imamura et al, Proc Natl Acad Sci USA, 2006; Justilien et al, Invest Ophthalmol Vis Sci. 2007, the contents of which are incorporated herein by reference in its entirety).

Reducing apoptotic pathway signaling

[000843] In some embodiments, the ophthalmic polynucleotides of the present invention which inhibit TRAIL function to reduce apoptosis in the retinal pigment epithelium. It is known that polymorphism reducing expression of DR4 reduces risk of developing AMD and that DcR1 expression levels significantly lower in AMD patients vs. controls (Arakawa et al. Nature Genetics, 2011; Anand , et. al. Scientific Reports, 2014, the contents of which are incorporated herein by reference in its entirety).

[000844] Further, markers of authophagy (Atg5, Atg12) are found in drusen in AMD donor eyes and aged mice (Wang et al, PLoS One, 2009, the contents of which are incorporated herein by reference in its entirety).

Modulating angiogenesis

[000845] In some embodiments, the ophthalmic polynucleotides of the present invention function to modulate angiogenesis by either altering the Notch signaling pathway or the semaphorin-plexin pathways. Such polynucleotides may encode a polypeptide which may include one or more cleavage, e.g., furin, sites or have one or more cleavage sites removed to provide resistance to degradation.

Managing retinopathies

[000846] There are 8 major pathways involved across the natural history of the disease. These include mitochondrial oxidation, cell adhesion/tissue remodeling, the visual cycle/rod-cone homeostasis, inflammation (the complement system), Drusen formation involving the extracellular matrix, inflammation (immune homing), neovascularization (involving either growth factors or blood components).

[000847] In some embodiments ophthalmic polynucleotides encoding TOMM40Lm Mitofusin 2, OPA1, SOD2, NADH dehydrogenase (1, 2, 4-6), Cytochromes (b,c) and or ATP synthase may be used to modulate the mitochondrial oxidation pathway.

[000848] In some embodiments ophthalmic polynucleotides encoding Cadherin 5, vascular endothelial, Cadherin-related family member 1, Peripherin 2, ADAM metallopeptidase domain 9, Thrombospondin receptor and/or Integrin A5 may be used to modulate the cell adhesion/tissue remodeling pathway.

[000849] In some embodiments ophthalmic polynucleotides encoding Retinal pigment epithelium-specific protein, guanylate cyclase activator 1A, guanylate cyclase 2D, membrane, voltage dependent calcium channels (A2, LA1F), bestrophin 1, and/or ciliary neurotrophic factor may be used to modulate the visual cycle/rod-cone homeostasis pathway.

[000850] In some embodiments ophthalmic polynucleotides encoding complement component 3, complement component 5, complement component 5 receptor 1, complement component 2, complement factor D, complement factor H, complement factor B, and/or complement factor I may be used to modulate the inflammation (the complement system) pathway.

[000851] In some embodiments ophthalmic polynucleotides encoding amyloid beta (A4) precursor protein, tenascin XB, collagen type X, alpha 1, myelin basic protein, and/or collagen type VIII, alpha 1 may be used to modulate the Drusen formation involving the extracellular matrix pathway.

[000852] In some embodiments ophthalmic polynucleotides encoding chemokine receptor 3, chemokine receptor 4, carbohydrate (GlcNAc) sulfotrans 6 (lymphocyte ligand metabolism), and/or TNF receptor 10A may be used to modulate the inflammation (immune homing) pathway.

[000853] In some embodiments ophthalmic polynucleotides encoding VEGF-A,

[000854] PDGF, HtrA serine peptidase 1, insulin like GF binding protein 7, and/or placental growth factor may be used to modulate the neovascularization (involving growth factors) pathway.

[000855] In some embodiments ophthalmic polynucleotides encoding plasminogen, Factor III, sphingosine -1 –phosphate receptor, hepatic lipase and/or cholesteryl ester transfer protein may be used to modulate the neovascularization (involving blood components) pathway.

Orphan indications

[000856] In some embodiments, polynucleotide compositions may be used to treat retinopathies associated with orphan indications such as Stargardt disease, Leber hereditary optic neuropathy, Cone rod dystrophy, Leber congenital amaurosis, Best vitelliform macular dystrophy, Choroideremia, Central areolar choroidal dystrophy, Macular corneal dystrophy, Autosomal dominant optic atrophy plus syndrome, North Carolina macular dystrophy, Hereditary vascular retinopathy and/or Autosomal dominant vitreoretinochoroidopathy

[000857] In some embodiments, polynucleotide compositions may be used to treat glaucoma associated with orphan indications such as Axenfeld-Rieger syndrome.

[000858] In some embodiments, polynucleotide compositions may be used to treat cataracts associated with orphan indications such as anterior polar cataract, Hutterite type Cataract, coralliform cataract, cerulean cataract, pulverulent cataract, congenital cataract, Volkmann type, Coppock-like cataract, cataract with Y-shaped suture opacities, zonular cataract, partial congenital cataract, nuclear cataract, total congenital cataract, and/or posterior polar cataract.

[000859] In some embodiments, polynucleotide compositions may be used to treat dry-eye associated with orphan indications such as benign essential blepharospasm.

Modulation of the Immune Response

Avoidance of the immune response

[000860] As described herein, a useful feature of the polynucleotides of the invention is the capacity to reduce, evade or avoid the innate immune response of a cell. In one aspect, provided herein are polynucleotides encoding a polypeptide of interest which when delivered to cells, results in a reduced immune response from the host as compared to the response triggered by a reference compound, e.g. an unmodified polynucleotide corresponding to a polynucleotide of the invention, or a different polynucleotides of the invention. As used herein, a "reference compound" is any molecule or substance which when administered to a mammal, results in an innate immune response having a known degree, level or amount of immune stimulation. A reference compound need not be a nucleic acid molecule and it need not be any of the polynucleotides of the invention. Hence, the measure of a polynucleotides avoidance, evasion or

failure to trigger an immune response can be expressed in terms relative to any compound or substance which is known to trigger such a response.

[000861] The term "innate immune response" includes a cellular response to exogenous single stranded nucleic acids, generally of viral or bacterial origin, which involves the induction of cytokine expression and release, particularly the interferons, and cell death. As used herein, the innate immune response or interferon response operates at the single cell level causing cytokine expression, cytokine release, global inhibition of protein synthesis, global destruction of cellular RNA, up regulation of major histocompatibility molecules, and/or induction of apoptotic death, induction of gene transcription of genes involved in apoptosis, anti-growth, and innate and adaptive immune cell activation. Some of the genes induced by type I IFNs include PKR, ADAR (adenosine deaminase acting on RNA), OAS (2',5'-oligoadenylate synthetase), RNase L, and Mx proteins. PKR and ADAR lead to inhibition of translation initiation and RNA editing, respectively. OAS is a dsRNA-dependent synthetase that activates the endoribonuclease RNase L to degrade ssRNA.

[000862] In some embodiments, the innate immune response comprises expression of a Type I or Type II interferon, and the expression of the Type I or Type II interferon is not increased more than two-fold compared to a reference from a cell which has not been contacted with a polynucleotide of the invention.

[000863] In some embodiments, the innate immune response comprises expression of one or more IFN signature genes and where the expression of the one of more IFN signature genes is not increased more than three-fold compared to a reference from a cell which has not been contacted with the polynucleotides of the invention.

[000864] While in some circumstances, it might be advantageous to eliminate the innate immune response in a cell, the invention provides polynucleotides that upon administration result in a substantially reduced (significantly less) the immune response, including interferon signaling, without entirely eliminating such a response.

[000865] In some embodiments, the immune response is lower by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, 99.9%, or greater than 99.9% as compared to the immune response induced by a reference compound. The immune response itself may be measured by determining the expression or activity level of Type 1 interferons or the expression of interferon-regulated genes such as the toll-like receptors (e.g., TLR7 and TLR8). Reduction of innate

immune response can also be measured by measuring the level of decreased cell death following one or more administrations to a cell population; e.g., cell death is 10%, 25%, 50%, 75%, 85%, 90%, 95%, or over 95% less than the cell death frequency observed with a reference compound. Moreover, cell death may affect fewer than 50%, 40%, 30%, 20%, 10%, 5%, 1%, 0.1%, 0.01% or fewer than 0.01% of cells contacted with the polynucleotides.

[000866] In another embodiment, the polynucleotides of the present invention is significantly less immunogenic than an unmodified in vitro-synthesized polynucleotide with the same sequence or a reference compound. As used herein, "significantly less immunogenic" refers to a detectable decrease in immunogenicity. In another embodiment, the term refers to a fold decrease in immunogenicity. In another embodiment, the term refers to a decrease such that an effective amount of the polynucleotides can be administered without triggering a detectable immune response. In another embodiment, the term refers to a decrease such that the polynucleotides can be repeatedly administered without eliciting an immune response sufficient to detectably reduce expression of the recombinant protein. In another embodiment, the decrease is such that the polynucleotides can be repeatedly administered without eliciting an immune response sufficient to eliminate detectable expression of the recombinant protein.

[000867] In another embodiment, the polynucleotides is 2-fold less immunogenic than its unmodified counterpart or reference compound. In another embodiment, immunogenicity is reduced by a 3-fold factor. In another embodiment, immunogenicity is reduced by a 5-fold factor. In another embodiment, immunogenicity is reduced by a 7-fold factor. In another embodiment, immunogenicity is reduced by a 10-fold factor. In another embodiment, immunogenicity is reduced by a 15-fold factor. In another embodiment, immunogenicity is reduced by a fold factor. In another embodiment, immunogenicity is reduced by a 50-fold factor. In another embodiment, immunogenicity is reduced by a 100-fold factor. In another embodiment, immunogenicity is reduced by a 200-fold factor. In another embodiment, immunogenicity is reduced by a 500-fold factor. In another embodiment, immunogenicity is reduced by a 1000-fold factor. In another embodiment, immunogenicity is reduced by a 2000-fold factor. In another embodiment, immunogenicity is reduced by another fold difference.

[000868] Methods of determining immunogenicity are well known in the art, and include, e.g. measuring secretion of cytokines (e.g. IL-12, IFNalpha, TNF-alpha, RANTES, MIP-1alpha or beta, IL-6, IFN-beta, or IL-8), measuring expression of DC activation markers (e.g. CD83, HLA-

DR, CD80 and CD86), or measuring ability to act as an adjuvant for an adaptive immune response.

[000869] The polynucleotides of the invention, including the combination of modifications taught herein may have superior properties making them more suitable as therapeutic modalities.

[000870] It has been determined that the "all or none" model in the art is sorely insufficient to describe the biological phenomena associated with the therapeutic utility of modified mRNA. The present inventors have determined that to improve protein production, one may consider the nature of the modification, or combination of modifications, the percent modification and survey more than one cytokine or metric to determine the efficacy and risk profile of a particular modified mRNA.

[000871] In one aspect of the invention, methods of determining the effectiveness of a modified mRNA as compared to unmodified involves the measure and analysis of one or more cytokines whose expression is triggered by the administration of the exogenous nucleic acid of the invention. These values are compared to administration of an unmodified nucleic acid or to a standard metric such as cytokine response, PolyIC, R-848 or other standard known in the art.

[000872] One example of a standard metric developed herein is the measure of the ratio of the level or amount of encoded polypeptide (protein) produced in the cell, tissue or organism to the level or amount of one or more (or a panel) of cytokines whose expression is triggered in the cell, tissue or organism as a result of administration or contact with the modified nucleic acid. Such ratios are referred to herein as the Protein:Cytokine Ratio or "PC" Ratio. The higher the PC ratio, the more efficacious the modified nucleic acid (polynucleotide encoding the protein measured). Preferred PC Ratios, by cytokine, of the present invention may be greater than 1, greater than 10, greater than 100, greater than 1000, greater than 10,000 or more. Modified nucleic acids having higher PC Ratios than a modified nucleic acid of a different or unmodified construct are preferred.

[000873] The PC ratio may be further qualified by the percent modification present in the polynucleotide. For example, normalized to a 100% modified nucleic acid, the protein production as a function of cytokine (or risk) or cytokine profile can be determined.

[000874] In one embodiment, the present invention provides a method for determining, across chemistries, cytokines or percent modification, the relative efficacy of any particular modified the polynucleotides by comparing the PC Ratio of the modified nucleic acid (polynucleotides).

[000875] Polynucleotides containing varying levels of nucleobase substitutions could be produced that maintain increased protein production and decreased immunostimulatory potential. The relative percentage of any modified nucleotide to its naturally occurring nucleotide counterpart can be varied during the IVT reaction (for instance, 100, 50, 25, 10, 5, 2.5, 1, 0.1, 0.01% 5 methyl cytidine usage versus cytidine; 100, 50, 25, 10, 5, 2.5, 1, 0.1, 0.01% pseudouridine or N1-methyl-pseudouridine usage versus uridine). Polynucleotides can also be made that utilize different ratios using 2 or more different nucleotides to the same base (for instance, different ratios of pseudouridine and N1-methyl-pseudouridine). Polynucleotides can also be made with mixed ratios at more than 1 "base" position, such as ratios of 5 methyl cytidine/cytidine and pseudouridine/N1-methyl-pseudouridine/uridine at the same time. Use of modified mRNA with altered ratios of modified nucleotides can be beneficial in reducing potential exposure to chemically modified nucleotides. Lastly, positional introduction of modified nucleotides into the polynucleotides which modulate either protein production or immunostimulatory potential or both is also possible. The ability of such polynucleotides to demonstrate these improved properties can be assessed in vitro (using assays such as the PBMC assay described herein), and can also be assessed in vivo through measurement of both polynucleotides-encoded protein production and mediators of innate immune recognition such as cytokines.

[000876] In another embodiment, the relative immunogenicity of the polynucleotides and its unmodified counterpart are determined by determining the quantity of the polynucleotides required to elicit one of the above responses to the same degree as a given quantity of the unmodified nucleotide or reference compound. For example, if twice as much polynucleotides is required to elicit the same response, than the polynucleotides is two-fold less immunogenic than the unmodified nucleotide or the reference compound.

[000877] In another embodiment, the relative immunogenicity of the polynucleotides and its unmodified counterpart are determined by determining the quantity of cytokine (e.g. IL-12, IFNalpha, TNF-alpha, RANTES, MIP-1alpha or beta, IL-6, IFN-beta, or IL-8) secreted in response to administration of the polynucleotides, relative to the same quantity of the unmodified nucleotide or reference compound. For example, if one-half as much cytokine is secreted, than the polynucleotides is two-fold less immunogenic than the unmodified nucleotide. In another

embodiment, background levels of stimulation are subtracted before calculating the immunogenicity in the above methods.

[000878] Provided herein are also methods for performing the titration, reduction or elimination of the immune response in a cell or a population of cells. In some embodiments, the cell is contacted with varied doses of the same polynucleotides and dose response is evaluated. In some embodiments, a cell is contacted with a number of different polynucleotides at the same or different doses to determine the optimal composition for producing the desired effect. Regarding the immune response, the desired effect may be to avoid, evade or reduce the immune response of the cell. The desired effect may also be to alter the efficiency of protein production.

[000879] The polynucleotides of the present invention may be used to reduce the immune response using the method described in International Publication No. WO2013003475, herein incorporated by reference in its entirety.

[000880] In one embodiment, the polynucleotides may encode at least one antibody or a fragment or portion thereof. The antibodies may be broadly neutralizing antibodies which may inhibit and protect against a broad range of infectious agents. As a non-limiting example, the polynucleotides encoding at least one antibody or fragment or portion thereof are provided to protect a subject against an infection disease and/or treat the disease. As another non-limiting example, the polynucleotides encoding two or more antibodies or fragments or portions thereof which are able to neutralize a wide spectrum of infectious agents are provided to protect a subject against an infection disease and/or treat the disease.

[000881] In one embodiment, the polynucleotide may encode an antibody heavy chain or an antibody light chain. The optimal ratio of polynucleotide encoding antibody heavy chain and antibody light chain may be evaluated to determine the ratio that produces the maximal amount of a functional antibody and/or desired response. The polynucleotide may also encode a single svFv chain of an antibody.

[000882] According to the present invention, the polynucleotides which encode one or more broadly neutralizing antibodies.

[000883] In one embodiment, the effective amount of the polynucleotides provided to a cell, a tissue or a subject may be enough for immune prophylaxis.

[000884] In another instance, the present invention provides antibody therapeutics containing the polynucleotides which encode one or more antibodies, and/or other anti-infectious reagents.

[000885] In one embodiment, the polynucleotide compositions of the present invention may be administered with other prophylactic or therapeutic compounds. As a non-limiting example, the prophylactic or therapeutic compound may be an adjuvant or a booster. As used herein, when referring to a prophylactic composition, such as a vaccine, the term "booster" refers to an extra administration of the pr prophylactic ophalytic composition. A booster (or booster vaccine) may be given after an earlier administration of the prophylactic composition. The time of administration between the initial administration of the prophylactic composition and the booster may be, but is not limited to, 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 15 minutes, 20 minutes 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 1 day, 36 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 10 days, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 1 year, 18 months, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years, 10 years, 11 years, 12 years, 13 years, 14 years, 15 years, 16 years, 17 years, 18 years, 19 years, 20 years, 25 years, 30 years, 35 years, 40 years, 45 years, 50 years, 55 years, 60 years, 65 years, 70 years, 75 years, 80 years, 85 years, 90 years, 95 years or more than 99 years.

Production of Antibodies

[000886] In one embodiment of the invention, the polynucleotides may encode antibodies and fragments of such antibodies. These may be produced by any one of the methods described herein. The antibodies may be of any of the different subclasses or isotypes of immunoglobulin such as, but not limited to, IgA, IgG, or IgM, or any of the other subclasses. Exemplary antibody molecules and fragments that may be prepared according to the invention include, but are not limited to, immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that may contain the paratope. Such portion of antibodies that contain the paratope include, but are not limited to Fab, Fab', $F(ab')_2$, F(v) and those portions known in the art.

[000887] The polynucleotides of the invention may encode variant antibody polypeptides which may have a certain identity with a reference polypeptide sequence, or have a similar or dissimilar binding characteristic with the reference polypeptide sequence.

[000888] Antibodies obtained by the methods of the present invention may be antibodies comprising non-human antibody-derived variable region(s) sequences, derived from the immunized animals, and human antibody-derived constant region(s) sequences. In addition, they can also be humanized antibodies comprising complementary determining regions (CDRs) of non-human antibodies derived from the immunized animals and the framework regions (FRs) and constant regions derived from human antibodies. In another embodiment, the methods provided herein may be useful for enhancing antibody protein product yield in a cell culture process.

Bicistronic and/or Pseudo-bicistronic Constructs

[000889] According to the present invention, a bicistronic construct is a polynucleotide encoding a two-protein chain antibody on a single polynucleotide strand. (Fig. 8B) A pseudo-bicistronic construct is a polynucleotide encoding a single chain antibody discontinuously on a single polynucleotide strand. For bicistronic constructs, the encoded two strands or two portions/regions and/or domains (as is the case with pseudo-bicistronic) are separated by at least one nucleotide not encoding the strands or domains. More often the separation comprises a cleavage signal or site or a non-coding region of nucleotides. Such cleavage sites include, for example, furin cleavage sites encoded as an "RKR" site in the resultant polypeptide.

Single Domain Constructs

[000890] According to the present invention, a single domain construct comprises one or two polynucleotides encoding a single monomeric variable antibody domain. See Figs 9B and 10B for examples. Typically single domain antibodies comprise one variable domain (VH) of a heavy-chain antibody.

Single chain Fv Constructs

[000891] According to the present invention, a single chain Fv constructs is a polynucleotide encoding at least two coding regions and a linker region. The scFv construct may encode a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins, connected with a short linker peptide of ten to about 25 amino acids. See Fig. 9A for an example. The linker is usually rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the VH with the C-terminus of the VL, or vice versa. Other linkers include those known in the art and disclosed herein.

Bispecific Constructs

[000892] According to the present invention, a bispecific construct is a polynucleotide encoding portions or regions of two different antibodies. Bispecific constructs encode polypeptides which may bind two different antigens. See Fig. 10A for an example. Polynucleotides of the present invention may also encode trispecific antibodies having an affinity for three antigens.

Linkers

[000893] Examples of linkers which may be used in the polynucleotides of the present invention include those in Tables 11 and 12.

Table 11. Nucleic Acid Sequences of Exemplary Linkers

Name	**SEQ ID NO or Sequence**
PLrigid PLrigid is a 20 a.a. peptide that is based on an alpha-helix motif (EAAAR) (SEQ ID NO: 101) (Merutka et al., 1991; Sommese et al., 2010)	37
2aa GS linker Highly flexibly glycine linker	GGCAGC
6aa [GS]x linker Highly flexible 6 amino acid linker. Translates to gsgsgs (SEQ UD NIL 102). Codon-optimize for E. coli, yeast, mammalian	38
10 aa flexible protein domain linker	39
8 aa protein domain linker	40
15 aa flexible glycine-serine protein domain linker; Freiburg standard	41
Short Linker (Gly-Gly-Ser-Gly (SEQ ID NO: 103))	42
Middle Linker (Gly-Gly-Ser-Gly)x2 (SEQ ID NO: 104)	43
Long Linker (Gly-Gly-Ser-Gly)x3 (SEQ ID NO: 105)	44
GSAT Linker	45
SEG-Linker	46

Table 12. Protein Sequences of Exemplary Linkers

Name	**SEQ ID NO**
G9S linker	47

[000894] Table references: Merutka G, Shalongo W, Stellwagen E. (1991) A model peptide with enhanced helicity. Biochem. 30: 4245-4248 and Sommese RF, Sivaramakrishnan S, Baldwin RL, Spudich JA. (2010) Helicity of short E-R/K peptides. Protein Sci. 19: 2001-2005.

VI. Kits and Devices

Kits

[000895] The invention provides a variety of kits for conveniently and/or effectively carrying out methods of the present invention. Typically kits will comprise sufficient amounts and/or numbers of components to allow a user to perform multiple treatments of a subject(s) and/or to perform multiple experiments.

[000896] In one aspect, the present invention provides kits comprising the molecules (polynucleotides) of the invention. In one embodiment, the kit comprises one or more functional antibodies or function fragments thereof.

[000897] The kits can be for protein production, comprising a first polynucleotides comprising a translatable region. The kit may further comprise packaging and instructions and/or a delivery agent to form a formulation composition. The delivery agent may comprise a saline, a buffered solution, a lipidoid or any delivery agent disclosed herein.

[000898] In one embodiment, the buffer solution may include sodium chloride, calcium chloride, phosphate and/or EDTA. In another embodiment, the buffer solution may include, but is not limited to, saline, saline with 2mM calcium, 5% sucrose, 5% sucrose with 2mM calcium, 5% Mannitol, 5% Mannitol with 2mM calcium, Ringer's lactate, sodium chloride, sodium chloride with 2mM calcium and mannose (See e.g., U.S. Pub. No. 20120258046; herein incorporated by reference in its entirety). In a further embodiment, the buffer solutions may be precipitated or it may be lyophilized. The amount of each component may be varied to enable consistent, reproducible higher concentration saline or simple buffer formulations. The components may also be varied in order to increase the stability of modified RNA in the buffer solution over a period of time and/or under a variety of conditions. In one aspect, the present invention provides kits for protein production, comprising: a polynucleotide comprising a translatable region, provided in an amount effective to produce a desired amount of a protein encoded by the translatable region when introduced into a target cell; a second polynucleotide comprising an inhibitory nucleic acid, provided in an amount effective to substantially inhibit the innate immune response of the cell; and packaging and instructions.

[000899] In one aspect, the present invention provides kits for protein production, comprising a polynucleotide comprising a translatable region, wherein the polynucleotide exhibits reduced degradation by a cellular nuclease, and packaging and instructions.

[000900] In one aspect, the present invention provides kits for protein production, comprising a polynucleotide comprising a translatable region, wherein the polynucleotide exhibits reduced degradation by a cellular nuclease, and a mammalian cell suitable for translation of the translatable region of the first nucleic acid.

Devices

[000901] The present invention provides for devices which may incorporate polynucleotides that encode polypeptides of interest. These devices contain in a stable formulation the reagents to synthesize a polynucleotide in a formulation available to be immediately delivered to a subject in need thereof, such as a human patient

[000902] Devices for administration may be employed to deliver the polynucleotides of the present invention according to single, multi- or split-dosing regimens taught herein. Such devices are taught in, for example, International Application PCT/US2013/30062 filed March 9, 2013 (Attorney Docket Number M300), the contents of which are incorporated herein by reference in their entirety.

[000903] Method and devices known in the art for multi-administration to cells, organs and tissues are contemplated for use in conjunction with the methods and compositions disclosed herein as embodiments of the present invention. These include, for example, those methods and devices having multiple needles, hybrid devices employing for example lumens or catheters as well as devices utilizing heat, electric current or radiation driven mechanisms.

[000904] According to the present invention, these multi-administration devices may be utilized to deliver the single, multi- or split doses contemplated herein. Such devices are taught for example in, International Application PCT/US2013/30062 filed March 9, 2013 (Attorney Docket Number M300), the contents of which are incorporated herein by reference in their entirety.

[000905] In one embodiment, the polynucleotide is administered subcutaneously or intramuscularly via at least 3 needles to three different, optionally adjacent, sites simultaneously, or within a 60 minutes period (e.g., administration to 4 ,5, 6, 7, 8, 9, or 10 sites simultaneously or within a 60 minute period).

[000906] Methods of delivering therapeutic agents using solid biodegradable microneedles are described by O'hagan et al. in US Patent Publication No. US20130287832, the contents of which are herein incorporated by reference in its entirety. The microneedles are fabricated from the therapeutic agent (e.g., influenza vaccine) in combination with at least one solid excipient. After penetrating the skin, the microneedles dissolve in situ and release the therapeutic agent to the subject. As a non-limiting example, the therapeutic agents used in the fabrication of the microneedles are the polynucleotides described herein.

[000907] A microneedle assembly for transdermal drug delivery is described by Ross et al. in US Patent No. US8636696, the contents of which are herein incorporated by reference in its entirety. The assembly has a first surface and a second surface where the microneedles project outwardly from the second surface of the support. The assembly may include a channel and aperture to form a junction which allows fluids (e.g., therapeutic agents or drugs) to pass.

VII. Definitions

[000908] At various places in the present specification, substituents of compounds of the present disclosure are disclosed in groups or in ranges. It is specifically intended that the present disclosure include each and every individual subcombination of the members of such groups and ranges. For example, the term "C_{1-6} alkyl" is specifically intended to individually disclose methyl, ethyl, C_3 alkyl, C_4 alkyl, C_5 alkyl, and C_6 alkyl. Herein a phrase of the form "optionally substituted X" (*e.g.*, optionally substituted alkyl) is intended to be equivalent to "X, wherein X is optionally substituted" (*e.g.*, "alkyl, wherein said alkyl is optionally substituted"). It is not intended to mean that the feature "X" (*e.g.* alkyl) *per se* is optional.

[000909] *About:* As used herein, the term "about" means +/- 10% of the recited value.

[000910] *Administered in combination:* As used herein, the term "administered in combination" or "combined administration" means that two or more agents are administered to a subject at the same time or within an interval such that there may be an overlap of an effect of each agent on the patient. In some embodiments, they are administered within about 60, 30, 15, 10, 5, or 1 minute of one another. In some embodiments, the administrations of the agents are spaced sufficiently closely together such that a combinatorial (*e.g.*, a synergistic) effect is achieved.

[000911] *Adjuvant*: As used herein, the term "adjuvant" means a substance that enhances a subject's immune response to an antigen.

[000912] *Animal:* As used herein, the term "animal" refers to any member of the animal kingdom. In some embodiments, "animal" refers to humans at any stage of development. In some embodiments, "animal" refers to non-human animals at any stage of development. In certain embodiments, the non-human animal is a mammal (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, or a pig). In some embodiments, animals include, but are not limited to, mammals, birds, reptiles, amphibians, fish, and worms. In some embodiments, the animal is a transgenic animal, genetically-engineered animal, or a clone.

[000913] *Antigen:* As used herein, the term "antigen" refers to the substance that binds specifically to the respective antibody. An antigen may originate either from the body, such as cancer antigen used herein, or from the external environment, for instance, from infectious agents.

[000914] *Antigens of interest or desired antigens*: As used herein, the terms "antigens of interest" or "desired antigens" include those proteins and other biomolecules provided herein that are immunospecifically bound by the antibodies and fragments, mutants, variants, and alterations thereof described herein. Examples of antigens of interest include, but are not limited to, insulin, insulin-like growth factor, hGH, tPA, cytokines, such as interleukins (IL), e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, interferon (IFN) alpha, IFN beta, IFN gamma, IFN omega or IFN tau, tumor necrosis factor (TNF), such as TNF alpha and TNF beta, TNF gamma, TRAIL; G-CSF, GM-CSF, M-CSF, MCP-1 and VEGF.

[000915] *Approximately:* As used herein, the term "approximately" or "about," as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term "approximately" or "about" refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[000916] *Associated with:* As used herein, the terms "associated with," "conjugated," "linked," "attached," and "tethered," when used with respect to two or more moieties, means that the moieties are physically associated or connected with one another, either directly or via one or more additional moieties that serves as a linking agent, to form a structure that is sufficiently

stable so that the moieties remain physically associated under the conditions in which the structure is used, *e.g.*, physiological conditions. An "association" need not be strictly through direct covalent chemical bonding. It may also suggest ionic or hydrogen bonding or a hybridization based connectivity sufficiently stable such that the "associated" entities remain physically associated.

[000917] *Bicistronic or Multicistronic:*As used herein a bicistronic construct is one that encodes two protein coding regions. The proteins may be the same or different. A multicistronic construct or mRNA is one that encodes two or more protein coding regions.

[000918] *Bifunctional:* As used herein, the term "bifunctional" refers to any substance, molecule or moiety which is capable of or maintains at least two functions. The functions may effect the same outcome or a different outcome. The structure that produces the function may be the same or different. For example, bifunctional modified RNAs of the present invention may encode a cytotoxic peptide (a first function) while those nucleosides which comprise the encoding RNA are, in and of themselves, cytotoxic (second function). In this example, delivery of the bifunctional modified RNA to a cancer cell would produce not only a peptide or protein molecule which may ameliorate or treat the cancer but would also deliver a cytotoxic payload of nucleosides to the cell should degradation, instead of translation of the modified RNA, occur.

[000919] *Biocompatible*: As used herein, the term "biocompatible" means compatible with living cells, tissues, organs or systems posing little to no risk of injury, toxicity or rejection by the immune system.

[000920] *Biodegradable*: As used herein, the term "biodegradable" means capable of being broken down into innocuous products by the action of living things.

[000921] *Biologically active*: As used herein, the phrase "biologically active" refers to a characteristic of any substance that has activity in a biological system and/or organism. For instance, a substance that, when administered to an organism, has a biological effect on that organism, is considered to be biologically active. In particular embodiments, a polynucleotide of the present invention may be considered biologically active if even a portion of the polynucleotides is biologically active or mimics an activity considered biologically relevant.

[000922] *Chemical terms*: The following provides the definition of various chemical terms from "acyl" to "thiol."

[000923] The term "acyl," as used herein, represents a hydrogen or an alkyl group (e.g., a haloalkyl group), as defined herein, that is attached to the parent molecular group through a carbonyl group, as defined herein, and is exemplified by formyl (i.e., a carboxyaldehyde group), acetyl, trifluoroacetyl, propionyl, butanoyl and the like. Exemplary unsubstituted acyl groups include from 1 to 7, from 1 to 11, or from 1 to 21 carbons. In some embodiments, the alkyl group is further substituted with 1, 2, 3, or 4 substituents as described herein.

[000924] Non-limiting examples of optionally substituted acyl groups include, alkoxycarbonyl, alkoxycarbonylacyl, arylalkoxycarbonyl, aryloyl, carbamoyl, carboxyaldehyde, (heterocyclyl) imino, and (heterocyclyl)oyl:

[000925] The "alkoxycarbonyl" group, which as used herein, represents an alkoxy, as defined herein, attached to the parent molecular group through a carbonyl atom (e.g., -C(O)-OR, where R is H or an optionally substituted C_{1-6}, C_{1-10}, or C_{1-20} alkyl group). Exemplary unsubstituted alkoxycarbonyl include from 1 to 21 carbons (e.g., from 1 to 11 or from 1 to 7 carbons). In some embodiments, the alkoxy group is further substituted with 1, 2, 3, or 4 substituents as described herein.

[000926] The "alkoxycarbonylacyl" group, which as used herein, represents an acyl group, as defined herein, that is substituted with an alkoxycarbonyl group, as defined herein (e.g., -C(O) -alkyl-C(O)-OR, where R is an optionally substituted C_{1-6}, C_{1-10}, or C_{1-20} alkyl group). Exemplary unsubstituted alkoxycarbonylacyl include from 3 to 41 carbons (e.g., from 3 to 10, from 3 to 13, from 3 to 17, from 3 to 21, or from 3 to 31 carbons, such as C_{1-6} alkoxycarbonyl-C_{1-6} acyl, C_{1-10} alkoxycarbonyl-C_{1-10} acyl, or C_{1-20} alkoxycarbonyl-C_{1-20} acyl). In some embodiments, each alkoxy and alkyl group is further independently substituted with 1, 2, 3, or 4 substituents, as described herein (e.g., a hydroxy group) for each group.

[000927] The "arylalkoxycarbonyl" group, which as used herein, represents an arylalkoxy group, as defined herein, attached to the parent molecular group through a carbonyl (e.g., -C(O)-O-alkyl-aryl). Exemplary unsubstituted arylalkoxy groups include from 8 to 31 carbons (e.g., from 8 to 17 or from 8 to 21 carbons, such as C_{6-10} aryl-C_{1-6} alkoxy-carbonyl, C_{6-10} aryl-C_{1-10} alkoxy-carbonyl, or C_{6-10} aryl-C_{1-20} alkoxy-carbonyl). In some embodiments, the arylalkoxycarbonyl group can be substituted with 1, 2, 3, or 4 substituents as defined herein.

[000928] The "aryloyl" group, which as used herein, represents an aryl group, as defined herein, that is attached to the parent molecular group through a carbonyl group. Exemplary

unsubstituted aryloyl groups are of 7 to 11 carbons. In some embodiments, the aryl group can be substituted with 1, 2, 3, or 4 substituents as defined herein.

[000929] The "carbamoyl" group, which as used herein, represents $-C(O)-N(R^{N1})_2$, where the meaning of each R^{N1} is found in the definition of "amino" provided herein.

[000930] The "carboxyaldehyde" group, which as used herein, represents an acyl group having the structure –CHO.

[000931] The "(heterocyclyl) imino" group, which as used herein, represents a heterocyclyl group, as defined herein, attached to the parent molecular group through an imino group. In some embodiments, the heterocyclyl group can be substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[000932] The "(heterocyclyl)oyl" group, which as used herein, represents a heterocyclyl group, as defined herein, attached to the parent molecular group through a carbonyl group. In some embodiments, the heterocyclyl group can be substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[000933] The term "alkyl," as used herein, is inclusive of both straight chain and branched chain saturated groups from 1 to 20 carbons (e.g., from 1 to 10 or from 1 to 6), unless otherwise specified. Alkyl groups are exemplified by methyl, ethyl, n- and iso-propyl, n-, sec-, iso- and tert-butyl, neopentyl, and the like, and may be optionally substituted with one, two, three, or, in the case of alkyl groups of two carbons or more, four substituents independently selected from the group consisting of: (1) C_{1-6} alkoxy; (2) C_{1-6} alkylsulfinyl; (3) amino, as defined herein (e.g., unsubstituted amino (i.e., $-NH_2$) or a substituted amino (i.e., $-N(R^{N1})_2$, where R^{N1} is as defined for amino); (4) C_{6-10} aryl-C_{1-6} alkoxy; (5) azido; (6) halo; (7) (C_{2-9} heterocyclyl)oxy; (8) hydroxy, optionally substituted with an *O*-protecting group; (9) nitro; (10) oxo (e.g., carboxyaldehyde or acyl); (11) C_{1-7} spirocyclyl; (12) thioalkoxy; (13) thiol; (14) $-CO_2R^{A'}$, optionally substituted with an *O*-protecting group and where $R^{A'}$ is selected from the group consisting of (a) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c) C_{6-10} aryl, (d) hydrogen, (e) C_{1-6} alk-C_{6-10} aryl, (f) amino-C_{1-20} alkyl, (g) polyethylene glycol of $-(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h) amino-polyethylene glycol of $-NR^{N1}(CH_2)_{s2}(CH_2CH_2O)_{s1}(CH_2)_{s3}NR^{N1}$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6

or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; (15) $-C(O)NR^{B'}R^{C'}$, where each of $R^{B'}$ and $R^{C'}$ is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{6-10} aryl, and (d) C_{1-6} alk-C_{6-10} aryl; (16) $-SO_2R^{D'}$, where $R^{D'}$ is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) C_{1-6} alk-C_{6-10} aryl, and (d) hydroxy; (17) $-SO_2NR^{E'}R^{F'}$, where each of $R^{E'}$ and $R^{F'}$ is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{6-10} aryl and (d) C_{1-6} alk-C_{6-10} aryl; (18) $-C(O)R^{G'}$, where $R^{G'}$ is selected from the group consisting of (a) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c) C_{6-10} aryl, (d) hydrogen, (e) C_{1-6} alk-C_{6-10} aryl, (f) amino-C_{1-20} alkyl, (g) polyethylene glycol of $-(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h) amino-polyethylene glycol of $-NR^{N1}(CH_2)_{s2}(CH_2CH_2O)_{s1}(CH_2)_{s3}NR^{N1}$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; (19) $-NR^{H'}C(O)R^{I'}$, wherein $R^{H'}$ is selected from the group consisting of (a1) hydrogen and (b1) C_{1-6} alkyl, and $R^{I'}$ is selected from the group consisting of (a2) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b2) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c2) C_{6-10} aryl, (d2) hydrogen, (e2) C_{1-6} alk-C_{6-10} aryl, (f2) amino-C_{1-20} alkyl, (g2) polyethylene glycol of $-(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h2) amino-polyethylene glycol of $-NR^{N1}(CH_2)_{s2}(CH_2CH_2O)_{s1}(CH_2)_{s3}NR^{N1}$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; (20) $-NR^{J'}C(O)OR^{K'}$, wherein $R^{J'}$ is selected from the group consisting of (a1) hydrogen and (b1) C_{1-6} alkyl, and $R^{K'}$ is selected from the group consisting of (a2) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b2) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c2) C_{6-10} aryl, (d2) hydrogen, (e2) C_{1-6} alk-C_{6-10} aryl, (f2) amino-C_{1-20} alkyl, (g2) polyethylene glycol of $-(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to

6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h2) amino-polyethylene glycol of $-NR^{N1}(CH_2)_{s2}(CH_2CH_2O)_{s1}(CH_2)_{s3}NR^{N1}$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; and (21) amidine. In some embodiments, each of these groups can be further substituted as described herein. For example, the alkylene group of a C_1-alkaryl can be further substituted with an oxo group to afford the respective aryloyl substituent.

[000934] The term "alkylene," as used herein, represent a saturated divalent hydrocarbon group derived from a straight or branched chain saturated hydrocarbon by the removal of two hydrogen atoms, and is exemplified by methylene, ethylene, isopropylene, and the like. The term "C_{x-y} alkylene" and the prefix "C_{x-y} alk-" represent alkylene groups having between x and y carbons. Exemplary values for x are 1, 2, 3, 4, 5, and 6, and exemplary values for y are 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, or 20 (e.g., C_{1-6}, C_{1-10}, C_{2-20}, C_{2-6}, C_{2-10}, or C_{2-20} alkylene). In some embodiments, the alkylene can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for an alkyl group. Similarly, the suffix "-ene" appended to any group indicates that the group is a divalent group.

[000935] Non-limiting examples of optionally substituted alkyl and alkylene groups include acylaminoalkyl, acyloxyalkyl, alkoxyalkyl, alkoxycarbonylalkyl, alkylsulfinyl, alkylsulfinylalkyl, aminoalkyl, carbamoylalkyl, carboxyalkyl, carboxyaminoalkyl, haloalkyl, hydroxyalkyl, perfluoroalkyl, and sulfoalkyl:

[000936] The "acylaminoalkyl" group, which as used herein, represents an acyl group, as defined herein, attached to an amino group that is in turn attached to the parent molecular group through an alkylene group, as defined herein (i.e., –alkyl-$N(R^{N1})$-C(O)-R, where R is H or an optionally substituted C_{1-6}, C_{1-10}, or C_{1-20} alkyl group (e.g., haloalkyl) and R^{N1} is as defined herein). Exemplary unsubstituted acylaminoalkyl groups include from 1 to 41 carbons (e.g., from 1 to 7, from 1 to 13, from 1 to 21, from 2 to 7, from 2 to 13, from 2 to 21, or from 2 to 41 carbons). In some embodiments, the alkylene group is further substituted with 1, 2, 3, or 4 substituents as described herein, and/or the amino group is $–NH_2$ or $–NHR^{N1}$, wherein R^{N1} is, independently, OH, NO_2, NH_2, NR^{N2}_2, SO_2OR^{N2}, SO_2R^{N2}, SOR^{N2}, alkyl, aryl, acyl (e.g., acetyl,

trifluoroacetyl, or others described herein), or alkoxycarbonylalkyl, and each R^{N2} can be H, alkyl, or aryl.

[000937] The "acyloxyalkyl" group, which as used herein, represents an acyl group, as defined herein, attached to an oxygen atom that in turn is attached to the parent molecular group though an alkylene group (i.e., –alkyl-O-C(O)-R, where R is H or an optionally substituted C_{1-6}, C_{1-10}, or C_{1-20} alkyl group). Exemplary unsubstituted acyloxyalkyl groups include from 1 to 21 carbons (e.g., from 1 to 7 or from 1 to 11 carbons). In some embodiments, the alkylene group is, independently, further substituted with 1, 2, 3, or 4 substituents as described herein.

[000938] The "alkoxyalkyl" group, which as used herein, represents an alkyl group that is substituted with an alkoxy group. Exemplary unsubstituted alkoxyalkyl groups include between 2 to 40 carbons (e.g., from 2 to 12 or from 2 to 20 carbons, such as C_{1-6} alkoxy-C_{1-6} alkyl, C_{1-10} alkoxy-C_{1-10} alkyl, or C_{1-20} alkoxy-C_{1-20} alkyl). In some embodiments, the alkyl and the alkoxy each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective group.

[000939] The "alkoxycarbonylalkyl" group, which as used herein, represents an alkyl group, as defined herein, that is substituted with an alkoxycarbonyl group, as defined herein (e.g., -alkyl-C(O)-OR, where R is an optionally substituted C_{1-20}, C_{1-10}, or C_{1-6} alkyl group). Exemplary unsubstituted alkoxycarbonylalkyl include from 3 to 41 carbons (e.g., from 3 to 10, from 3 to 13, from 3 to 17, from 3 to 21, or from 3 to 31 carbons, such as C_{1-6} alkoxycarbonyl-C_{1-6} alkyl, C_{1-10} alkoxycarbonyl-C_{1-10} alkyl, or C_{1-20} alkoxycarbonyl-C_{1-20} alkyl). In some embodiments, each alkyl and alkoxy group is further independently substituted with 1, 2, 3, or 4 substituents as described herein (e.g., a hydroxy group).

[000940] The "alkylsulfinylalkyl" group, which as used herein, represents an alkyl group, as defined herein, substituted with an alkylsulfinyl group. Exemplary unsubstituted alkylsulfinylalkyl groups are from 2 to 12, from 2 to 20, or from 2 to 40 carbons. In some embodiments, each alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[000941] The "aminoalkyl" group, which as used herein, represents an alkyl group, as defined herein, substituted with an amino group, as defined herein. The alkyl and amino each can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the respective

group (e.g., $CO_2R^{A'}$, where $R^{A'}$ is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) hydrogen, and (d) C_{1-6} alk-C_{6-10} aryl, e.g., carboxy, and/or an *N*-protecting group).

[000942] The "carbamoylalkyl" group, which as used herein, represents an alkyl group, as defined herein, substituted with a carbamoyl group, as defined herein. The alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

[000943] The "carboxyalkyl" group, which as used herein, represents an alkyl group, as defined herein, substituted with a carboxy group, as defined herein. The alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein, and the carboxy group can be optionally substituted with one or more *O*-protecting groups.

[000944] The "carboxyaminoalkyl" group, which as used herein, represents an aminoalkyl group, as defined herein, substituted with a carboxy, as defined herein. The carboxy, alkyl, and amino each can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the respective group (e.g., $CO_2R^{A'}$, where $R^{A'}$ is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) hydrogen, and (d) C_{1-6} alk-C_{6-10} aryl, e.g., carboxy, and/or an *N*-protecting group, and/or an *O*-protecting group).

[000945] The "haloalkyl" group, which as used herein, represents an alkyl group, as defined herein, substituted with a halogen group (i.e., F, Cl, Br, or I). A haloalkyl may be substituted with one, two, three, or, in the case of alkyl groups of two carbons or more, four halogens. Haloalkyl groups include perfluoroalkyls (e.g., $-CF_3$), $-CHF_2$, $-CH_2F$, $-CCl_3$, $-CH_2CH_2Br$, $-CH_2CH(CH_2CH_2Br)CH_3$, and $-CHICH_3$. In some embodiments, the haloalkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for alkyl groups.

[000946] The "hydroxyalkyl" group, which as used herein, represents an alkyl group, as defined herein, substituted with one to three hydroxy groups, with the proviso that no more than one hydroxy group may be attached to a single carbon atom of the alkyl group, and is exemplified by hydroxymethyl, dihydroxypropyl, and the like. In some embodiments, the hydroxyalkyl group can be substituted with 1, 2, 3, or 4 substituent groups (e.g., *O*-protecting groups) as defined herein for an alkyl.

[000947] The "perfluoroalkyl" group, which as used herein, represents an alkyl group, as defined herein, where each hydrogen radical bound to the alkyl group has been replaced by a fluoride radical. Perfluoroalkyl groups are exemplified by trifluoromethyl, pentafluoroethyl, and the like.

[000948] The "sulfoalkyl" group, which as used herein, represents an alkyl group, as defined herein, substituted with a sulfo group of $-SO_3H$. In some embodiments, the alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein, and the sulfo group can be further substituted with one or more *O*-protecting groups (e.g., as described herein).

[000949] The term "alkenyl," as used herein, represents monovalent straight or branched chain groups of, unless otherwise specified, from 2 to 20 carbons (e.g., from 2 to 6 or from 2 to 10 carbons) containing one or more carbon-carbon double bonds and is exemplified by ethenyl, 1-propenyl, 2-propenyl, 2-methyl-1-propenyl, 1-butenyl, 2-butenyl, and the like. Alkenyls include both cis and trans isomers. Alkenyl groups may be optionally substituted with 1, 2, 3, or 4 substituent groups that are selected, independently, from amino, aryl, cycloalkyl, or heterocyclyl (e.g., heteroaryl), as defined herein, or any of the exemplary alkyl substituent groups described herein.

[000950] Non-limiting examples of optionally substituted alkenyl groups include, alkoxycarbonylalkenyl, aminoalkenyl, and hydroxyalkenyl:

[000951] The "alkoxycarbonylalkenyl" group, which as used herein, represents an alkenyl group, as defined herein, that is substituted with an alkoxycarbonyl group, as defined herein (e.g., -alkenyl-C(O)-OR, where R is an optionally substituted C_{1-20}, C_{1-10}, or C_{1-6} alkyl group). Exemplary unsubstituted alkoxycarbonylalkenyl include from 4 to 41 carbons (e.g., from 4 to 10, from 4 to 13, from 4 to 17, from 4 to 21, or from 4 to 31 carbons, such as C_{1-6} alkoxycarbonyl-C_{2-6} alkenyl, C_{1-10} alkoxycarbonyl-C_{2-10} alkenyl, or C_{1-20} alkoxycarbonyl-C_{2-20} alkenyl). In some embodiments, each alkyl, alkenyl, and alkoxy group is further independently substituted with 1, 2, 3, or 4 substituents as described herein (e.g., a hydroxy group).

[000952] The "aminoalkenyl" group, which as used herein, represents an alkenyl group, as defined herein, substituted with an amino group, as defined herein. The alkenyl and amino each can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the respective group (e.g., $CO_2R^{A'}$, where $R^{A'}$ is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) hydrogen, and (d) C_{1-6} alk-C_{6-10} aryl, e.g., carboxy, and/or an *N*-protecting group).

[000953] The "hydroxyalkenyl" group, which as used herein, represents an alkenyl group, as defined herein, substituted with one to three hydroxy groups, with the proviso that no more than one hydroxy group may be attached to a single carbon atom of the alkyl group, and is

exemplified by dihydroxypropenyl, hydroxyisopentenyl, and the like. In some embodiments, the hydroxyalkenyl group can be substituted with 1, 2, 3, or 4 substituent groups (e.g., *O*-protecting groups) as defined herein for an alkyl.

[000954] The term "alkynyl," as used herein, represents monovalent straight or branched chain groups from 2 to 20 carbon atoms (e.g., from 2 to 4, from 2 to 6, or from 2 to 10 carbons) containing a carbon-carbon triple bond and is exemplified by ethynyl, 1-propynyl, and the like. Alkynyl groups may be optionally substituted with 1, 2, 3, or 4 substituent groups that are selected, independently, from aryl, cycloalkyl, or heterocyclyl (e.g., heteroaryl), as defined herein, or any of the exemplary alkyl substituent groups described herein.

[000955] Non-limiting examples of optionally substituted alkynyl groups include alkoxycarbonylalkynyl, aminoalkynyl, and hydroxyalkynyl:

[000956] The "alkoxycarbonylalkynyl" group, which as used herein, represents an alkynyl group, as defined herein, that is substituted with an alkoxycarbonyl group, as defined herein (e.g., -alkynyl-C(O)-OR, where R is an optionally substituted C_{1-20}, C_{1-10}, or C_{1-6} alkyl group). Exemplary unsubstituted alkoxycarbonylalkynyl include from 4 to 41 carbons (e.g., from 4 to 10, from 4 to 13, from 4 to 17, from 4 to 21, or from 4 to 31 carbons, such as C_{1-6} alkoxycarbonyl-C_{2-6} alkynyl, C_{1-10} alkoxycarbonyl-C_{2-10} alkynyl, or C_{1-20} alkoxycarbonyl-C_{2-20} alkynyl). In some embodiments, each alkyl, alkynyl, and alkoxy group is further independently substituted with 1, 2, 3, or 4 substituents as described herein (e.g., a hydroxy group).

[000957] The "aminoalkynyl" group, which as used herein, represents an alkynyl group, as defined herein, substituted with an amino group, as defined herein. The alkynyl and amino each can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the respective group (e.g., $CO_2R^{A'}$, where $R^{A'}$ is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) hydrogen, and (d) C_{1-6} alk-C_{6-10} aryl, e.g., carboxy, and/or an *N*-protecting group).

[000958] The "hydroxyalkynyl" group, which as used herein, represents an alkynyl group, as defined herein, substituted with one to three hydroxy groups, with the proviso that no more than one hydroxy group may be attached to a single carbon atom of the alkyl group. In some embodiments, the hydroxyalkynyl group can be substituted with 1, 2, 3, or 4 substituent groups (e.g., *O*-protecting groups) as defined herein for an alkyl.

[000959] The term "amino," as used herein, represents $-N(R^{N1})_2$, wherein each R^{N1} is, independently, H, OH, NO_2, $N(R^{N2})_2$, SO_2OR^{N2}, SO_2R^{N2}, SOR^{N2}, an *N*-protecting group, alkyl, alkenyl, alkynyl, alkoxy, aryl, alkaryl, cycloalkyl, alkcycloalkyl, carboxyalkyl (e.g., optionally substituted with an *O*-protecting group, such as optionally substituted arylalkoxycarbonyl groups or any described herein), sulfoalkyl, acyl (e.g., acetyl, trifluoroacetyl, or others described herein), alkoxycarbonylalkyl (e.g., optionally substituted with an *O*-protecting group, such as optionally substituted arylalkoxycarbonyl groups or any described herein), heterocyclyl (e.g., heteroaryl), or alkheterocyclyl (e.g., alkheteroaryl), wherein each of these recited R^{N1} groups can be optionally substituted, as defined herein for each group; or two R^{N1} combine to form a heterocyclyl or an *N*-protecting group, and wherein each R^{N2} is, independently, H, alkyl, or aryl. The amino groups of the invention can be an unsubstituted amino (i.e., $-NH_2$) or a substituted amino (i.e., $-N(R^{N1})_2$). In a preferred embodiment, amino is $-NH_2$ or $-NHR^{N1}$, wherein R^{N1} is, independently, OH, NO_2, NH_2, $NR^{N2}{}_2$, SO_2OR^{N2}, SO_2R^{N2}, SOR^{N2}, alkyl, carboxyalkyl, sulfoalkyl, acyl (e.g., acetyl, trifluoroacetyl, or others described herein), alkoxycarbonylalkyl (e.g., t-butoxycarbonylalkyl) or aryl, and each R^{N2} can be H, $C_{1\text{-}20}$ alkyl (e.g., $C_{1\text{-}6}$ alkyl), or $C_{6\text{-}10}$ aryl.

[000960] Non-limiting examples of optionally substituted amino groups include acylamino and carbamyl:

[000961] The "acylamino" group, which as used herein, represents an acyl group, as defined herein, attached to the parent molecular group though an amino group, as defined herein (i.e., $-N(R^{N1})$-C(O)-R, where R is H or an optionally substituted $C_{1\text{-}6}$, $C_{1\text{-}10}$, or $C_{1\text{-}20}$ alkyl group (e.g., haloalkyl) and R^{N1} is as defined herein). Exemplary unsubstituted acylamino groups include from 1 to 41 carbons (e.g., from 1 to 7, from 1 to 13, from 1 to 21, from 2 to 7, from 2 to 13, from 2 to 21, or from 2 to 41 carbons). In some embodiments, the alkyl group is further substituted with 1, 2, 3, or 4 substituents as described herein, and/or the amino group is $-NH_2$ or $-NHR^{N1}$, wherein R^{N1} is, independently, OH, NO_2, NH_2, $NR^{N2}{}_2$, SO_2OR^{N2}, SO_2R^{N2}, SOR^{N2}, alkyl, aryl, acyl (e.g., acetyl, trifluoroacetyl, or others described herein), or alkoxycarbonylalkyl, and each R^{N2} can be H, alkyl, or aryl.

[000962] The "carbamyl" group, which as used herein, refers to a carbamate group having the structure $-NR^{N1}C(=O)OR$ or $-OC(=O)N(R^{N1})_2$, where the meaning of each R^{N1} is found in the definition of "amino" provided herein, and R is alkyl, cycloalkyl , alkcycloalkyl, aryl, alkaryl, heterocyclyl (e.g., heteroaryl), or alkheterocyclyl (e.g., alkheteroaryl), as defined herein.

[000963] The term "amino acid," as described herein, refers to a molecule having a side chain, an amino group, and an acid group (e.g., a carboxy group of $-CO_2H$ or a sulfo group of $-SO_3H$), wherein the amino acid is attached to the parent molecular group by the side chain, amino group, or acid group (e.g., the side chain). In some embodiments, the amino acid is attached to the parent molecular group by a carbonyl group, where the side chain or amino group is attached to the carbonyl group. Exemplary side chains include an optionally substituted alkyl, aryl, heterocyclyl, alkaryl, alkheterocyclyl, aminoalkyl, carbamoylalkyl, and carboxyalkyl. Exemplary amino acids include alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, hydroxynorvaline, isoleucine, leucine, lysine, methionine, norvaline, ornithine, phenylalanine, proline, pyrrolysine, selenocysteine, serine, taurine, threonine, tryptophan, tyrosine, and valine. Amino acid groups may be optionally substituted with one, two, three, or, in the case of amino acid groups of two carbons or more, four substituents independently selected from the group consisting of: (1) C_{1-6} alkoxy; (2) C_{1-6} alkylsulfinyl; (3) amino, as defined herein (e.g., unsubstituted amino (i.e., $-NH_2$) or a substituted amino (i.e., $-N(R^{N1})_2$, where R^{N1} is as defined for amino); (4) C_{6-10} aryl-C_{1-6} alkoxy; (5) azido; (6) halo; (7) (C_{2-9} heterocyclyl)oxy; (8) hydroxy; (9) nitro; (10) oxo (e.g., carboxyaldehyde or acyl); (11) C_{1-7} spirocyclyl; (12) thioalkoxy; (13) thiol; (14) $-CO_2R^{A'}$, where $R^{A'}$ is selected from the group consisting of (a) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c) C_{6-10} aryl, (d) hydrogen, (e) C_{1-6} alk-C_{6-10} aryl, (f) amino-C_{1-20} alkyl, (g) polyethylene glycol of $-(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h) amino-polyethylene glycol of $-NR^{N1}(CH_2)_{s2}(CH_2CH_2O)_{s1}(CH_2)_{s3}NR^{N1}$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; (15) $-C(O)NR^{B'}R^{C'}$, where each of $R^{B'}$ and $R^{C'}$ is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{6-10} aryl, and (d) C_{1-6} alk-C_{6-10} aryl; (16) $-SO_2R^{D'}$, where $R^{D'}$ is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) C_{1-6} alk-C_{6-10} aryl, and (d) hydroxy; (17) $-SO_2NR^{E'}R^{F'}$, where each of $R^{E'}$ and $R^{F'}$ is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{6-10} aryl and (d) C_{1-6} alk-C_{6-10} aryl; (18) $-C(O)R^{G'}$, where $R^{G'}$ is

selected from the group consisting of (a) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c) C_{6-10} aryl, (d) hydrogen, (e) C_{1-6} alk-C_{6-10} aryl, (f) amino-C_{1-20} alkyl, (g) polyethylene glycol of $-(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h) amino-polyethylene glycol of $-NR^{N1}(CH_2)_{s2}(CH_2CH_2O)_{s1}(CH_2)_{s3}NR^{N1}$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; (19) $-NR^{H'}C(O)R^{I'}$, wherein $R^{H'}$ is selected from the group consisting of (a1) hydrogen and (b1) C_{1-6} alkyl, and $R^{I'}$ is selected from the group consisting of (a2) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b2) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c2) C_{6-10} aryl, (d2) hydrogen, (e2) C_{1-6} alk-C_{6-10} aryl, (f2) amino-C_{1-20} alkyl, (g2) polyethylene glycol of $-(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h2) amino-polyethylene glycol of $-NR^{N1}(CH_2)_{s2}(CH_2CH_2O)_{s1}(CH_2)_{s3}NR^{N1}$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; (20) $-NR^{J'}C(O)OR^{K'}$, wherein $R^{J'}$ is selected from the group consisting of (a1) hydrogen and (b1) C_{1-6} alkyl, and $R^{K'}$ is selected from the group consisting of (a2) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b2) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c2) C_{6-10} aryl, (d2) hydrogen, (e2) C_{1-6} alk-C_{6-10} aryl, (f2) amino-C_{1-20} alkyl, (g2) polyethylene glycol of $-(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h2) amino-polyethylene glycol of $-NR^{N1}(CH_2)_{s2}(CH_2CH_2O)_{s1}(CH_2)_{s3}NR^{N1}$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; and (21) amidine. In some embodiments, each of these groups can be further substituted as described herein.

[000964] The term "aryl," as used herein, represents a mono-, bicyclic, or multicyclic carbocyclic ring system having one or two aromatic rings and is exemplified by phenyl, naphthyl, 1,2-dihydronaphthyl, 1,2,3,4-tetrahydronaphthyl, anthracenyl, phenanthrenyl, fluorenyl, indanyl, indenyl, and the like, and may be optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from the group consisting of: (1) C_{1-7} acyl (e.g., carboxyaldehyde); (2) C_{1-20} alkyl (e.g., C_{1-6} alkyl, C_{1-6} alkoxy-C_{1-6} alkyl, C_{1-6} alkylsulfinyl-C_{1-6} alkyl, amino-C_{1-6} alkyl, azido-C_{1-6} alkyl, (carboxyaldehyde)-C_{1-6} alkyl, halo-C_{1-6} alkyl (e.g., perfluoroalkyl), hydroxy-C_{1-6} alkyl, nitro-C_{1-6} alkyl, or C_{1-6} thioalkoxy-C_{1-6} alkyl); (3) C_{1-20} alkoxy (e.g., C_{1-6} alkoxy, such as perfluoroalkoxy); (4) C_{1-6} alkylsulfinyl; (5) C_{6-10} aryl; (6) amino; (7) C_{1-6} alk-C_{6-10} aryl; (8) azido; (9) C_{3-8} cycloalkyl; (10) C_{1-6} alk-C_{3-8} cycloalkyl; (11) halo; (12) C_{1-12} heterocyclyl (e.g., C_{1-12} heteroaryl); (13) (C_{1-12} heterocyclyl)oxy; (14) hydroxy; (15) nitro; (16) C_{1-20} thioalkoxy (e.g., C_{1-6} thioalkoxy); (17) $-(CH_2)_qCO_2R^{A'}$, where q is an integer from zero to four, and $R^{A'}$ is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) hydrogen, and (d) C_{1-6} alk-C_{6-10} aryl; (18) $-(CH_2)_qCONR^{B'}R^{C'}$, where q is an integer from zero to four and where $R^{B'}$ and $R^{C'}$ are independently selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{6-10} aryl, and (d) C_{1-6} alk-C_{6-10} aryl; (19) $-(CH_2)_qSO_2R^{D'}$, where q is an integer from zero to four and where $R^{D'}$ is selected from the group consisting of (a) alkyl, (b) C_{6-10} aryl, and (c) alk-C_{6-10} aryl; (20) $-(CH_2)_qSO_2NR^{E'}R^{F'}$, where q is an integer from zero to four and where each of $R^{E'}$ and $R^{F'}$ is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{6-10} aryl, and (d) C_{1-6} alk-C_{6-10} aryl; (21) thiol; (22) C_{6-10} aryloxy; (23) C_{3-8} cycloalkoxy; (24) C_{6-10} aryl-C_{1-6} alkoxy; (25) C_{1-6} alk-C_{1-12} heterocyclyl (e.g., C_{1-6} alk-C_{1-12} heteroaryl); (26) C_{2-20} alkenyl; and (27) C_{2-20} alkynyl. In some embodiments, each of these groups can be further substituted as described herein. For example, the alkylene group of a C_1-alkaryl or a C_1-alkheterocyclyl can be further substituted with an oxo group to afford the respective aryloyl and (heterocyclyl)oyl substituent group.

[000965] The "arylalkyl" group, which as used herein, represents an aryl group, as defined herein, attached to the parent molecular group through an alkylene group, as defined herein. Exemplary unsubstituted arylalkyl groups are from 7 to 30 carbons (e.g., from 7 to 16 or from 7 to 20 carbons, such as C_{1-6} alk-C_{6-10} aryl, C_{1-10} alk-C_{6-10} aryl, or C_{1-20} alk-C_{6-10} aryl). In some embodiments, the alkylene and the aryl each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective groups. Other groups preceded by the

prefix "alk-" are defined in the same manner, where "alk" refers to a C_{1-6} alkylene, unless otherwise noted, and the attached chemical structure is as defined herein.

[000966] The term "azido" represents an $-N_3$ group, which can also be represented as –N=N=N.

[000967] The term "bicyclic," as used herein, refer to a structure having two rings, which may be aromatic or non-aromatic. Bicyclic structures include spirocyclyl groups, as defined herein, and two rings that share one or more bridges, where such bridges can include one atom or a chain including two, three, or more atoms. Exemplary bicyclic groups include a bicyclic carbocyclyl group, where the first and second rings are carbocyclyl groups, as defined herein; a bicyclic aryl groups, where the first and second rings are aryl groups, as defined herein; bicyclic heterocyclyl groups, where the first ring is a heterocyclyl group and the second ring is a carbocyclyl (e.g., aryl) or heterocyclyl (e.g., heteroaryl) group; and bicyclic heteroaryl groups, where the first ring is a heteroaryl group and the second ring is a carbocyclyl (e.g., aryl) or heterocyclyl (e.g., heteroaryl) group. In some embodiments, the bicyclic group can be substituted with 1, 2, 3, or 4 substituents as defined herein for cycloalkyl, heterocyclyl, and aryl groups.

[000968] The term "boranyl," as used herein, represents $-B(R^{B1})_3$, where each R^{B1} is, independently, selected from the group consisting of H and optionally substituted alkyl. In some embodiments, the boranyl group can be substituted with 1, 2, 3, or 4 substituents as defined herein for alkyl.

[000969] The terms "carbocyclic" and "carbocyclyl," as used herein, refer to an optionally substituted C_{3-12} monocyclic, bicyclic, or tricyclic structure in which the rings, which may be aromatic or non-aromatic, are formed by carbon atoms. Carbocyclic structures include cycloalkyl, cycloalkenyl, cycloalkynyl, and aryl groups.

[000970] The term "carbonyl," as used herein, represents a C(O) group, which can also be represented as C=O.

[000971] The term "carboxy," as used herein, means $-CO_2H$.

[000972] The term "cyano," as used herein, represents an –CN group.

[000973] The term "cycloalkyl," as used herein represents a monovalent saturated or unsaturated non-aromatic cyclic hydrocarbon group from three to eight carbons, unless otherwise specified, and is exemplified by cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl,

bicycle heptyl, and the like. When what would otherwise be a cycloalkyl group includes one or more carbon-carbon double bonds, the group is referred to as a "cycloalkenyl" group. For the purposes of this invention, cycloalkenyl excludes aryl groups. When what would otherwise be a cycloalkyl group includes one or more carbon-carbon triple bonds, the group is referred to as a "cycloalkynyl" group. Exemplary cycloalkenyl groups include cyclopentenyl, cyclohexenyl, and the like. The cycloalkyl groups of this invention can be optionally substituted with: (1) C_{1-7} acyl (e.g., carboxyaldehyde); (2) C_{1-20} alkyl (e.g., C_{1-6} alkyl, C_{1-6} alkoxy-C_{1-6} alkyl, C_{1-6} alkylsulfinyl-C_{1-6} alkyl, amino-C_{1-6} alkyl, azido-C_{1-6} alkyl, (carboxyaldehyde)-C_{1-6} alkyl, halo-C_{1-6} alkyl (e.g., perfluoroalkyl), hydroxy-C_{1-6} alkyl, nitro-C_{1-6} alkyl, or C_{1-6} thioalkoxy-C_{1-6} alkyl); (3) C_{1-20} alkoxy (e.g., C_{1-6} alkoxy, such as perfluoroalkoxy); (4) C_{1-6} alkylsulfinyl; (5) C_{6-10} aryl; (6) amino; (7) C_{1-6} alk-C_{6-10} aryl; (8) azido; (9) C_{3-8} cycloalkyl; (10) C_{1-6} alk-C_{3-8} cycloalkyl; (11) halo; (12) C_{1-12} heterocyclyl (e.g., C_{1-12} heteroaryl); (13) (C_{1-12} heterocyclyl)oxy; (14) hydroxy; (15) nitro; (16) C_{1-20} thioalkoxy (e.g., C_{1-6} thioalkoxy); (17) $-(CH_2)_qCO_2R^{A'}$, where q is an integer from zero to four, and $R^{A'}$ is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) hydrogen, and (d) C_{1-6} alk-C_{6-10} aryl; (18) $-(CH_2)_qCONR^{B'}R^{C'}$, where q is an integer from zero to four and where $R^{B'}$ and $R^{C'}$ are independently selected from the group consisting of (a) hydrogen, (b) C_{6-10} alkyl, (c) C_{6-10} aryl, and (d) C_{1-6} alk-C_{6-10} aryl; (19) $-(CH_2)_qSO_2R^{D'}$, where q is an integer from zero to four and where $R^{D'}$ is selected from the group consisting of (a) C_{6-10} alkyl, (b) C_{6-10} aryl, and (c) C_{1-6} alk-C_{6-10} aryl; (20) $-(CH_2)_qSO_2NR^{E'}R^{F'}$, where q is an integer from zero to four and where each of $R^{E'}$ and $R^{F'}$ is, independently, selected from the group consisting of (a) hydrogen, (b) C_{6-10} alkyl, (c) C_{6-10} aryl, and (d) C_{1-6} alk-C_{6-10} aryl; (21) thiol; (22) C_{6-10} aryloxy; (23) C_{3-8} cycloalkoxy; (24) C_{6-10} aryl-C_{1-6} alkoxy; (25) C_{1-6} alk-C_{1-12} heterocyclyl (e.g., C_{1-6} alk-C_{1-12} heteroaryl); (26) oxo; (27) C_{2-20} alkenyl; and (28) C_{2-20} alkynyl. In some embodiments, each of these groups can be further substituted as described herein. For example, the alkylene group of a C_1-alkaryl or a C_1-alkheterocyclyl can be further substituted with an oxo group to afford the respective aryloyl and (heterocyclyl)oyl substituent group.

[000974] The "cycloalkylalkyl" group, which as used herein, represents a cycloalkyl group, as defined herein, attached to the parent molecular group through an alkylene group, as defined herein (e.g., an alkylene group of from 1 to 4, from 1 to 6, from 1 to 10, or form 1 to 20 carbons). In some embodiments, the alkylene and the cycloalkyl each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective group.

[000975] The term "diastereomer," as used herein means stereoisomers that are not mirror images of one another and are non-superimposable on one another.

[000976] The term "enantiomer," as used herein, means each individual optically active form of a compound of the invention, having an optical purity or enantiomeric excess (as determined by methods standard in the art) of at least 80% (i.e., at least 90% of one enantiomer and at most 10% of the other enantiomer), preferably at least 90% and more preferably at least 98%.

[000977] The term "halo," as used herein, represents a halogen selected from bromine, chlorine, iodine, or fluorine.

[000978] The term "heteroalkyl," as used herein, refers to an alkyl group, as defined herein, in which one or two of the constituent carbon atoms have each been replaced by nitrogen, oxygen, or sulfur. In some embodiments, the heteroalkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for alkyl groups. The terms "heteroalkenyl" and heteroalkynyl," as used herein refer to alkenyl and alkynyl groups, as defined herein, respectively, in which one or two of the constituent carbon atoms have each been replaced by nitrogen, oxygen, or sulfur. In some embodiments, the heteroalkenyl and heteroalkynyl groups can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for alkyl groups.

[000979] Non-limiting examples of optionally substituted heteroalkyl, heteroalkenyl, and heteroalkynyl groups include acyloxy, alkenyloxy, alkoxy, alkoxyalkoxy, alkoxycarbonylalkoxy, alkynyloxy, aminoalkoxy, arylalkoxy, carboxyalkoxy, cycloalkoxy, haloalkoxy, (heterocyclyl)oxy, perfluoroalkoxy, thioalkoxy, and thioheterocyclylalkyl:

[000980] The "acyloxy" group, which as used herein, represents an acyl group, as defined herein, attached to the parent molecular group though an oxygen atom (i.e., –O-C(O)-R, where R is H or an optionally substituted C_{1-6}, C_{1-10}, or C_{1-20} alkyl group). Exemplary unsubstituted acyloxy groups include from 1 to 21 carbons (e.g., from 1 to 7 or from 1 to 11 carbons). In some embodiments, the alkyl group is further substituted with 1, 2, 3, or 4 substituents as described herein.

[000981] The "alkenyloxy" group, which as used here, represents a chemical substituent of formula –OR, where R is a C_{2-20} alkenyl group (e.g., C_{2-6} or C_{2-10} alkenyl), unless otherwise specified. Exemplary alkenyloxy groups include ethenyloxy, propenyloxy, and the like. In some

embodiments, the alkenyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein (e.g., a hydroxy group).

[000982] The "alkoxy" group, which as used herein, represents a chemical substituent of formula –OR, where R is a C_{1-20} alkyl group (e.g., C_{1-6} or C_{1-10} alkyl), unless otherwise specified. Exemplary alkoxy groups include methoxy, ethoxy, propoxy (e.g., n-propoxy and isopropoxy), t-butoxy, and the like. In some embodiments, the alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein (e.g., hydroxy or alkoxy).

[000983] The "alkoxyalkoxy" group, which as used herein, represents an alkoxy group that is substituted with an alkoxy group. Exemplary unsubstituted alkoxyalkoxy groups include between 2 to 40 carbons (e.g., from 2 to 12 or from 2 to 20 carbons, such as C_{1-6} alkoxy-C_{1-6} alkoxy, C_{1-10} alkoxy-C_{1-10} alkoxy, or C_{1-20} alkoxy-C_{1-20} alkoxy). In some embodiments, the each alkoxy group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[000984] The "alkoxycarbonylalkoxy" group, which as used herein, represents an alkoxy group, as defined herein, that is substituted with an alkoxycarbonyl group, as defined herein (e.g., -O-alkyl-C(O)-OR, where R is an optionally substituted C_{1-6}, C_{1-10}, or C_{1-20} alkyl group). Exemplary unsubstituted alkoxycarbonylalkoxy include from 3 to 41 carbons (e.g., from 3 to 10, from 3 to 13, from 3 to 17, from 3 to 21, or from 3 to 31 carbons, such as C_{1-6} alkoxycarbonyl-C_{1-6} alkoxy, C_{1-10} alkoxycarbonyl-C_{1-10} alkoxy, or C_{1-20} alkoxycarbonyl-C_{1-20} alkoxy). In some embodiments, each alkoxy group is further independently substituted with 1, 2, 3, or 4 substituents, as described herein (e.g., a hydroxy group).

[000985] The "alkynyloxy" group, which as used herein, represents a chemical substituent of formula –OR, where R is a C_{2-20} alkynyl group (e.g., C_{2-6} or C_{2-10} alkynyl), unless otherwise specified. Exemplary alkynyloxy groups include ethynyloxy, propynyloxy, and the like. In some embodiments, the alkynyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein (e.g., a hydroxy group).

[000986] The "aminoalkoxy" group, which as used herein, represents an alkoxy group, as defined herein, substituted with an amino group, as defined herein. The alkyl and amino each can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the respective group (e.g., $CO_2R^{A'}$, where $R^{A'}$ is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) hydrogen, and (d) C_{1-6} alk-C_{6-10} aryl, e.g., carboxy).

[000987] The "arylalkoxy" group, which as used herein, represents an alkaryl group, as defined herein, attached to the parent molecular group through an oxygen atom. Exemplary unsubstituted arylalkoxy groups include from 7 to 30 carbons (e.g., from 7 to 16 or from 7 to 20 carbons, such as C_{6-10} aryl-C_{1-6} alkoxy, C_{6-10} aryl-C_{1-10} alkoxy, or C_{6-10} aryl-C_{1-20} alkoxy). In some embodiments, the arylalkoxy group can be substituted with 1, 2, 3, or 4 substituents as defined herein.

[000988] The "aryloxy" group, which as used herein, represents a chemical substituent of formula –OR′, where R′ is an aryl group of 6 to 18 carbons, unless otherwise specified. In some embodiments, the aryl group can be substituted with 1, 2, 3, or 4 substituents as defined herein.

[000989] The "carboxyalkoxy" group, which as used herein, represents an alkoxy group, as defined herein, substituted with a carboxy group, as defined herein. The alkoxy group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the alkyl group, and the carboxy group can be optionally substituted with one or more *O*-protecting groups.

[000990] The "cycloalkoxy" group, which as used herein, represents a chemical substituent of formula –OR, where R is a C_{3-8} cycloalkyl group, as defined herein, unless otherwise specified. The cycloalkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein. Exemplary unsubstituted cycloalkoxy groups are from 3 to 8 carbons. In some embodiment, the cycloalkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

[000991] The "haloalkoxy" group, which as used herein, represents an alkoxy group, as defined herein, substituted with a halogen group (i.e., F, Cl, Br, or I). A haloalkoxy may be substituted with one, two, three, or, in the case of alkyl groups of two carbons or more, four halogens. Haloalkoxy groups include perfluoroalkoxys (e.g., $-OCF_3$), $-OCHF_2$, $-OCH_2F$, $-OCCl_3$, $-OCH_2CH_2Br$, $-OCH_2CH(CH_2CH_2Br)CH_3$, and $-OCHICH_3$. In some embodiments, the haloalkoxy group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for alkyl groups.

[000992] The "(heterocyclyl)oxy" group, which as used herein, represents a heterocyclyl group, as defined herein, attached to the parent molecular group through an oxygen atom. In some embodiments, the heterocyclyl group can be substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[000993] The "perfluoroalkoxy" group, which as used herein, represents an alkoxy group, as defined herein, where each hydrogen radical bound to the alkoxy group has been replaced by a fluoride radical. Perfluoroalkoxy groups are exemplified by trifluoromethoxy, pentafluoroethoxy, and the like.

[000994] The "alkylsulfinyl" group, which as used herein, represents an alkyl group attached to the parent molecular group through an -S(O)- group. Exemplary unsubstituted alkylsulfinyl groups are from 1 to 6, from 1 to 10, or from 1 to 20 carbons. In some embodiments, the alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[000995] The "thioarylalkyl" group, which as used herein, represents a chemical substituent of formula –SR, where R is an arylalkyl group. In some embodiments, the arylalkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

[000996] The "thioalkoxy" group as used herein, represents a chemical substituent of formula –SR, where R is an alkyl group, as defined herein. In some embodiments, the alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

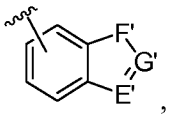
[000997] The "thioheterocyclylalkyl" group, which as used herein, represents a chemical substituent of formula –SR, where R is an heterocyclylalkyl group. In some embodiments, the heterocyclylalkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

[000998] The term "heteroaryl," as used herein, represents that subset of heterocyclyls, as defined herein, which are aromatic: i.e., they contain $4n+2$ pi electrons within the mono- or multicyclic ring system. Exemplary unsubstituted heteroaryl groups are of 1 to 12 (e.g., 1 to 11, 1 to 10, 1 to 9, 2 to 12, 2 to 11, 2 to 10, or 2 to 9) carbons. In some embodiment, the heteroaryl is substituted with 1, 2, 3, or 4 substituents groups as defined for a heterocyclyl group.

[000999] The term "heteroarylalkyl" refers to a heteroaryl group, as defined herein, attached to the parent molecular group through an alkylene group, as defined herein. Exemplary unsubstituted heteroarylalkyl groups are from 2 to 32 carbons (e.g., from 2 to 22, from 2 to 18, from 2 to 17, from 2 to 16, from 3 to 15, from 2 to 14, from 2 to 13, or from 2 to 12 carbons, such as C_{1-6} alk-C_{1-12} heteroaryl, C_{1-10} alk-C_{1-12} heteroaryl, or C_{1-20} alk-C_{1-12} heteroaryl). In some embodiments, the alkylene and the heteroaryl each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective group. Heteroarylalkyl groups are a subset of heterocyclylalkyl groups.

[0001000] The term "heterocyclyl," as used herein represents a 5-, 6- or 7-membered ring, unless otherwise specified, containing one, two, three, or four heteroatoms independently selected from the group consisting of nitrogen, oxygen, and sulfur. The 5-membered ring has zero to two double bonds, and the 6- and 7-membered rings have zero to three double bonds. Exemplary unsubstituted heterocyclyl groups are of 1 to 12 (e.g., 1 to 11, 1 to 10, 1 to 9, 2 to 12, 2 to 11, 2 to 10, or 2 to 9) carbons. The term "heterocyclyl" also represents a heterocyclic compound having a bridged multicyclic structure in which one or more carbons and/or heteroatoms bridges two non-adjacent members of a monocyclic ring, e.g., a quinuclidinyl group. The term "heterocyclyl" includes bicyclic, tricyclic, and tetracyclic groups in which any of the above heterocyclic rings is fused to one, two, or three carbocyclic rings, e.g., an aryl ring, a cyclohexane ring, a cyclohexene ring, a cyclopentane ring, a cyclopentene ring, or another monocyclic heterocyclic ring, such as indolyl, quinolyl, isoquinolyl, tetrahydroquinolyl, benzofuryl, benzothienyl and the like. Examples of fused heterocyclyls include tropanes and 1,2,3,5,8,8a-hexahydroindolizine. Heterocyclics include pyrrolyl, pyrrolinyl, pyrrolidinyl, pyrazolyl, pyrazolinyl, pyrazolidinyl, imidazolyl, imidazolinyl, imidazolidinyl, pyridyl, piperidinyl, homopiperidinyl, pyrazinyl, piperazinyl, pyrimidinyl, pyridazinyl, oxazolyl, oxazolidinyl, isoxazolyl, isoxazolidiniyl, morpholinyl, thiomorpholinyl, thiazolyl, thiazolidinyl, isothiazolyl, isothiazolidinyl, indolyl, indazolyl, quinolyl, isoquinolyl, quinoxalinyl, dihydroquinoxalinyl, quinazolinyl, cinnolinyl, phthalazinyl, benzimidazolyl, benzothiazolyl, benzoxazolyl, benzothiadiazolyl, furyl, thienyl, thiazolidinyl, isothiazolyl, triazolyl, tetrazolyl, oxadiazolyl (e.g., 1,2,3-oxadiazolyl), purinyl, thiadiazolyl (e.g., 1,2,3-thiadiazolyl), tetrahydrofuranyl, dihydrofuranyl, tetrahydrothienyl, dihydrothienyl, dihydroindolyl, dihydroquinolyl, tetrahydroquinolyl, tetrahydroisoquinolyl, dihydroisoquinolyl, pyranyl, dihydropyranyl, dithiazolyl, benzofuranyl, isobenzofuranyl, benzothienyl, and the like, including dihydro and tetrahydro forms thereof, where one or more double bonds are reduced and replaced with hydrogens. Still other exemplary heterocyclyls include: 2,3,4,5-tetrahydro-2-oxo-oxazolyl; 2,3-dihydro-2-oxo-1H-imidazolyl; 2,3,4,5-tetrahydro-5-oxo-1H-pyrazolyl (e.g., 2,3,4,5-tetrahydro-2-phenyl-5-oxo-1H-pyrazolyl); 2,3,4,5-tetrahydro-2,4-dioxo-1H-imidazolyl (e.g., 2,3,4,5-tetrahydro-2,4-dioxo-5-methyl-5-phenyl-1H-imidazolyl); 2,3-dihydro-2-thioxo-1,3,4-oxadiazolyl (e.g., 2,3-dihydro-2-thioxo-5-phenyl-1,3,4-oxadiazolyl); 4,5-dihydro-5-oxo-1*H*-triazolyl (e.g., 4,5-dihydro-3-methyl-4-amino 5-oxo-1*H*-triazolyl); 1,2,3,4-tetrahydro-2,4-

dioxopyridinyl (e.g., 1,2,3,4-tetrahydro-2,4-dioxo-3,3-diethylpyridinyl); 2,6-dioxo-piperidinyl (e.g., 2,6-dioxo-3-ethyl-3-phenylpiperidinyl); 1,6-dihydro-6-oxopyridiminyl; 1,6-dihydro-4-oxopyrimidinyl (e.g., 2-(methylthio)-1,6-dihydro-4-oxo-5-methylpyrimidin-1-yl); 1,2,3,4-tetrahydro-2,4-dioxopyrimidinyl (e.g., 1,2,3,4-tetrahydro-2,4-dioxo-3-ethylpyrimidinyl); 1,6-dihydro-6-oxo-pyridazinyl (e.g., 1,6-dihydro-6-oxo-3-ethylpyridazinyl); 1,6-dihydro-6-oxo-1,2,4-triazinyl (e.g., 1,6-dihydro-5-isopropyl-6-oxo-1,2,4-triazinyl); 2,3-dihydro-2-oxo-1*H*-indolyl (e.g., 3,3-dimethyl-2,3-dihydro-2-oxo-1*H*-indolyl and 2,3-dihydro-2-oxo-3,3′-spiropropane-1*H*-indol-1-yl); 1,3-dihydro-1-oxo-2*H*-iso-indolyl; 1,3-dihydro-1,3-dioxo-2*H*-iso-indolyl; 1*H*-benzopyrazolyl (e.g., 1-(ethoxycarbonyl)- 1*H*-benzopyrazolyl); 2,3-dihydro-2-oxo-1*H*-benzimidazolyl (e.g., 3-ethyl-2,3-dihydro-2-oxo-1*H*-benzimidazolyl); 2,3-dihydro-2-oxo-benzoxazolyl (e.g., 5-chloro-2,3-dihydro-2-oxo-benzoxazolyl); 2,3-dihydro-2-oxo-benzoxazolyl; 2-oxo-2H-benzopyranyl; 1,4-benzodioxanyl; 1,3-benzodioxanyl; 2,3-dihydro-3-oxo,4*H*-1,3-benzothiazinyl; 3,4-dihydro-4-oxo-3*H*-quinazolinyl (e.g., 2-methyl-3,4-dihydro-4-oxo-3*H*-quinazolinyl); 1,2,3,4-tetrahydro-2,4-dioxo-3*H*-quinazolyl (e.g., 1-ethyl-1,2,3,4-tetrahydro-2,4-dioxo-3*H*-quinazolyl); 1,2,3,6-tetrahydro-2,6-dioxo-7*H*-purinyl (e.g., 1,2,3,6-tetrahydro-1,3-dimethyl-2,6-dioxo-7 *H* -purinyl); 1,2,3,6-tetrahydro-2,6-dioxo-1 *H* –purinyl (e.g., 1,2,3,6-tetrahydro-3,7-dimethyl-2,6-dioxo-1 *H* -purinyl); 2-oxobenz[*c,d*]indolyl; 1,1-dioxo-2H-naphth[1,8-*c,d*]isothiazolyl; and 1,8-naphthylenedicarboxamido. Additional heterocyclics include 3,3a,4,5,6,6a-hexahydro-pyrrolo[3,4-b]pyrrol-(2H)-yl, and 2,5-diazabicyclo[2.2.1]heptan-2-yl, homopiperazinyl (or diazepanyl), tetrahydropyranyl, dithiazolyl, benzofuranyl, benzothienyl, oxepanyl, thiepanyl, azocanyl, oxecanyl, and thiocanyl. Heterocyclic groups also include groups of the formula



, where

[0001001] E′ is selected from the group consisting of -N- and -CH-; F′ is selected from the group consisting of -N=CH-, -NH-CH_2-, -NH-C(O)-, -NH-, -CH=N-, -CH_2-NH-, -C(O)-NH-, -CH=CH-, -CH_2-, -CH_2CH_2-, -CH_2O-, -OCH_2-, -O-, and -S-; and G′ is selected from the group consisting of -CH- and -N-. Any of the heterocyclyl groups mentioned herein may be optionally substituted with one, two, three, four or five substituents independently selected from the group consisting of: (1) C_{1-7} acyl (e.g., carboxyaldehyde); (2) C_{1-20} alkyl (e.g., C_{1-6} alkyl, C_{1-6} alkoxy-

C_{1-6} alkyl, C_{1-6} alkylsulfinyl-C_{1-6} alkyl, amino-C_{1-6} alkyl, azido-C_{1-6} alkyl, (carboxyaldehyde)-C_{1-6} alkyl, halo-C_{1-6} alkyl (e.g., perfluoroalkyl), hydroxy-C_{1-6} alkyl, nitro-C_{1-6} alkyl, or C_{1-6} thioalkoxy-C_{1-6} alkyl); (3) C_{1-20} alkoxy (e.g., C_{1-6} alkoxy, such as perfluoroalkoxy); (4) C_{1-6} alkylsulfinyl; (5) C_{6-10} aryl; (6) amino; (7) C_{1-6} alk-C_{6-10} aryl; (8) azido; (9) C_{3-8} cycloalkyl; (10) C_{1-6} alk-C_{3-8} cycloalkyl; (11) halo; (12) C_{1-12} heterocyclyl (e.g., C_{2-12} heteroaryl); (13) (C_{1-12} heterocyclyl)oxy; (14) hydroxy; (15) nitro; (16) C_{1-20} thioalkoxy (e.g., C_{1-6} thioalkoxy); (17) $-(CH_2)_qCO_2R^{A'}$, where q is an integer from zero to four, and $R^{A'}$ is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) hydrogen, and (d) C_{1-6} alk-C_{6-10} aryl; (18) $-(CH_2)_qCONR^{B'}R^{C'}$, where q is an integer from zero to four and where $R^{B'}$ and $R^{C'}$ are independently selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{6-10} aryl, and (d) C_{1-6} alk-C_{6-10} aryl; (19) $-(CH_2)_qSO_2R^{D'}$, where q is an integer from zero to four and where $R^{D'}$ is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, and (c) C_{1-6} alk-C_{6-10} aryl; (20) $-(CH_2)_qSO_2NR^{E'}R^{F'}$, where q is an integer from zero to four and where each of $R^{E'}$ and $R^{F'}$ is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{6-10} aryl, and (d) C_{1-6} alk-C_{6-10} aryl; (21) thiol; (22) C_{6-10} aryloxy; (23) C_{3-8} cycloalkoxy; (24) arylalkoxy; (25) C_{1-6} alk-C_{1-12} heterocyclyl (e.g., C_{1-6} alk-C_{1-12} heteroaryl); (26) oxo; (27) (C_{1-12} heterocyclyl)imino; (28) C_{2-20} alkenyl; and (29) C_{2-20} alkynyl. In some embodiments, each of these groups can be further substituted as described herein. For example, the alkylene group of a C_1-alkaryl or a C_1-alkheterocyclyl can be further substituted with an oxo group to afford the respective aryloyl and (heterocyclyl)oyl substituent group.

[0001002] The "heterocyclylalkyl" group, which as used herein, represents a heterocyclyl group, as defined herein, attached to the parent molecular group through an alkylene group, as defined herein. Exemplary unsubstituted heterocyclylalkyl groups are from 2 to 32 carbons (e.g., from 2 to 22, from 2 to 18, from 2 to 17, from 2 to 16, from 3 to 15, from 2 to 14, from 2 to 13, or from 2 to 12 carbons, such as C_{1-6} alk-C_{1-12} heterocyclyl, C_{1-10} alk-C_{1-12} heterocyclyl, or C_{1-20} alk-C_{1-12} heterocyclyl). In some embodiments, the alkylene and the heterocyclyl each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective group.

[0001003] The term "hydrocarbon," as used herein, represents a group consisting only of carbon and hydrogen atoms.

[0001004] The term "hydroxy," as used herein, represents an –OH group.

[0001005] The term "isomer," as used herein, means any tautomer, stereoisomer, enantiomer, or diastereomer of any compound of the invention. It is recognized that the compounds of the invention can have one or more chiral centers and/or double bonds and, therefore, exist as stereoisomers, such as double-bond isomers (i.e., geometric E/Z isomers) or diastereomers (e.g., enantiomers (i.e., (+) or (-)) or cis/trans isomers). According to the invention, the chemical structures depicted herein, and therefore the compounds of the invention, encompass all of the corresponding stereoisomers, that is, both the stereomerically pure form (e.g., geometrically pure, enantiomerically pure, or diastereomerically pure) and enantiomeric and stereoisomeric mixtures, e.g., racemates. Enantiomeric and stereoisomeric mixtures of compounds of the invention can typically be resolved into their component enantiomers or stereoisomers by well-known methods, such as chiral-phase gas chromatography, chiral-phase high performance liquid chromatography, crystallizing the compound as a chiral salt complex, or crystallizing the compound in a chiral solvent. Enantiomers and stereoisomers can also be obtained from stereomerically or enantiomerically pure intermediates, reagents, and catalysts by well-known asymmetric synthetic methods.

[0001006] The term "*N*-protected amino," as used herein, refers to an amino group, as defined herein, to which is attached one or two *N*-protecting groups, as defined herein.

[0001007] The term "*N*-protecting group," as used herein, represents those groups intended to protect an amino group against undesirable reactions during synthetic procedures. Commonly used *N*-protecting groups are disclosed in Greene, "Protective Groups in Organic Synthesis," 3rd Edition (John Wiley & Sons, New York, 1999), which is incorporated herein by reference. *N*-protecting groups include acyl, aryloyl, or carbamyl groups such as formyl, acetyl, propionyl, pivaloyl, t-butylacetyl, 2-chloroacetyl, 2-bromoacetyl, trifluoroacetyl, trichloroacetyl, phthalyl, o-nitrophenoxyacetyl, α-chlorobutyryl, benzoyl, 4-chlorobenzoyl, 4-bromobenzoyl, 4-nitrobenzoyl, and chiral auxiliaries such as protected or unprotected D, L or D, L-amino acids such as alanine, leucine, phenylalanine, and the like; sulfonyl-containing groups such as benzenesulfonyl, p-toluenesulfonyl, and the like; carbamate forming groups such as benzyloxycarbonyl, p-chlorobenzyloxycarbonyl, p-methoxybenzyloxycarbonyl, p-nitrobenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, p-bromobenzyloxycarbonyl, 3,4-dimethoxybenzyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 2,4-dimethoxybenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitro-4,5-

dimethoxybenzyloxycarbonyl, 3,4,5-trimethoxybenzyloxycarbonyl, 1-(p-biphenylyl)-1-methylethoxycarbonyl, α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl, benzhydryloxy carbonyl, t-butyloxycarbonyl, diisopropylmethoxycarbonyl, isopropyloxycarbonyl, ethoxycarbonyl, methoxycarbonyl, allyloxycarbonyl, 2,2,2,-trichloroethoxycarbonyl, phenoxycarbonyl, 4-nitrophenoxy carbonyl, fluorenyl-9-methoxycarbonyl, cyclopentyloxycarbonyl, adamantyloxycarbonyl, cyclohexyloxycarbonyl, phenylthiocarbonyl, and the like, alkaryl groups such as benzyl, triphenylmethyl, benzyloxymethyl, and the like and silyl groups, such as trimethylsilyl, and the like. Preferred *N*-protecting groups are formyl, acetyl, benzoyl, pivaloyl, t-butylacetyl, alanyl, phenylsulfonyl, benzyl, t-butyloxycarbonyl (Boc), and benzyloxycarbonyl (Cbz).

[0001008] The term "nitro," as used herein, represents an $-NO_2$ group.

[0001009] The term "*O*-protecting group," as used herein, represents those groups intended to protect an oxygen containing (e.g., phenol, hydroxyl, or carbonyl) group against undesirable reactions during synthetic procedures. Commonly used *O*-protecting groups are disclosed in Greene, "Protective Groups in Organic Synthesis," 3rd Edition (John Wiley & Sons, New York, 1999), which is incorporated herein by reference. Exemplary *O*-protecting groups include acyl, aryloyl, or carbamyl groups, such as formyl, acetyl, propionyl, pivaloyl, t-butylacetyl, 2-chloroacetyl, 2-bromoacetyl, trifluoroacetyl, trichloroacetyl, phthalyl, o-nitrophenoxyacetyl, α-chlorobutyryl, benzoyl, 4-chlorobenzoyl, 4-bromobenzoyl, *t*-butyldimethylsilyl, tri-*iso*-propylsilyloxymethyl, 4,4'-dimethoxytrityl, isobutyryl, phenoxyacetyl, 4-isopropylpehenoxyacetyl, dimethylformamidino, and 4-nitrobenzoyl; alkylcarbonyl groups, such as acyl, acetyl, propionyl, pivaloyl, and the like; optionally substituted arylcarbonyl groups, such as benzoyl; silyl groups, such as trimethylsilyl (TMS), tert-butyldimethylsilyl (TBDMS), tri-iso-propylsilyloxymethyl (TOM), triisopropylsilyl (TIPS), and the like; ether-forming groups with the hydroxyl, such methyl, methoxymethyl, tetrahydropyranyl, benzyl, p-methoxybenzyl, trityl, and the like; alkoxycarbonyls, such as methoxycarbonyl, ethoxycarbonyl, isopropoxycarbonyl, n-isopropoxycarbonyl, n-butyloxycarbonyl, isobutyloxycarbonyl, sec-butyloxycarbonyl, t-butyloxycarbonyl, 2-ethylhexyloxycarbonyl, cyclohexyloxycarbonyl, methyloxycarbonyl, and the like; alkoxyalkoxycarbonyl groups, such as methoxymethoxycarbonyl, ethoxymethoxycarbonyl, 2-methoxyethoxycarbonyl, 2-ethoxyethoxycarbonyl, 2-butoxyethoxycarbonyl, 2-methoxyethoxymethoxycarbonyl, allyloxycarbonyl,

propargyloxycarbonyl, 2-butenoxycarbonyl, 3-methyl-2-butenoxycarbonyl, and the like; haloalkoxycarbonyls, such as 2-chloroethoxycarbonyl, 2-chloroethoxycarbonyl, 2,2,2-trichloroethoxycarbonyl, and the like; optionally substituted arylalkoxycarbonyl groups, such as benzyloxycarbonyl, p-methylbenzyloxycarbonyl, p-methoxybenzyloxycarbonyl, p-nitrobenzyloxycarbonyl, 2,4-dinitrobenzyloxycarbonyl, 3,5-dimethylbenzyloxycarbonyl, p-chlorobenzyloxycarbonyl, p-bromobenzyloxy-carbonyl, fluorenylmethyloxycarbonyl, and the like; and optionally substituted aryloxycarbonyl groups, such as phenoxycarbonyl, p-nitrophenoxycarbonyl, o-nitrophenoxycarbonyl, 2,4-dinitrophenoxycarbonyl, p-methyl-phenoxycarbonyl, m-methylphenoxycarbonyl, o-bromophenoxycarbonyl, 3,5-dimethylphenoxycarbonyl, p-chlorophenoxycarbonyl, 2-chloro-4-nitrophenoxy-carbonyl, and the like); substituted alkyl, aryl, and alkaryl ethers (e.g., trityl; methylthiomethyl; methoxymethyl; benzyloxymethyl; siloxymethyl; 2,2,2,-trichloroethoxymethyl; tetrahydropyranyl; tetrahydrofuranyl; ethoxyethyl; 1-[2-(trimethylsilyl)ethoxy]ethyl; 2-trimethylsilylethyl; t-butyl ether; p-chlorophenyl, p-methoxyphenyl, p-nitrophenyl, benzyl, p-methoxybenzyl, and nitrobenzyl); silyl ethers (e.g., trimethylsilyl; triethylsilyl; triisopropylsilyl; dimethylisopropylsilyl; t-butyldimethylsilyl; t-butyldiphenylsilyl; tribenzylsilyl; triphenylsilyl; and diphenymethylsilyl); carbonates (e.g., methyl, methoxymethyl, 9-fluorenylmethyl; ethyl; 2,2,2-trichloroethyl; 2-(trimethylsilyl)ethyl; vinyl, allyl, nitrophenyl; benzyl; methoxybenzyl; 3,4-dimethoxybenzyl; and nitrobenzyl); carbonyl-protecting groups (e.g., acetal and ketal groups, such as dimethyl acetal, 1,3-dioxolane, and the like; acylal groups; and dithiane groups, such as 1,3-dithianes, 1,3-dithiolane, and the like); carboxylic acid-protecting groups (e.g., ester groups, such as methyl ester, benzyl ester, t-butyl ester, orthoesters, and the like; and oxazoline groups.

[0001010] The term "oxo" as used herein, represents =O.

[0001011] The prefix "perfluoro," as used herein, represents anyl group, as defined herein, where each hydrogen radical bound to the alkyl group has been replaced by a fluoride radical. For example, perfluoroalkyl groups are exemplified by trifluoromethyl, pentafluoroethyl, and the like.

[0001012] The term "protected hydroxyl," as used herein, refers to an oxygen atom bound to an *O*-protecting group.

[0001013] The term "spirocyclyl," as used herein, represents a C_{2-7} alkylene diradical, both ends of which are bonded to the same carbon atom of the parent group to form a spirocyclic group, and also a C_{1-6} heteroalkylene diradical, both ends of which are bonded to the same atom. The heteroalkylene radical forming the spirocyclyl group can containing one, two, three, or four heteroatoms independently selected from the group consisting of nitrogen, oxygen, and sulfur. In some embodiments, the spirocyclyl group includes one to seven carbons, excluding the carbon atom to which the diradical is attached. The spirocyclyl groups of the invention may be optionally substituted with 1, 2, 3, or 4 substituents provided herein as optional substituents for cycloalkyl and/or heterocyclyl groups.

[0001014] The term "stereoisomer," as used herein, refers to all possible different isomeric as well as conformational forms which a compound may possess (e.g., a compound of any formula described herein), in particular all possible stereochemically and conformationally isomeric forms, all diastereomers, enantiomers and/or conformers of the basic molecular structure. Some compounds of the present invention may exist in different tautomeric forms, all of the latter being included within the scope of the present invention.

[0001015] The term "sulfonyl," as used herein, represents an $-S(O)_2-$ group.

[0001016] The term "thiol," as used herein represents an –SH group.

[0001017] *Chimera*: As used herein, "chimera" is an entity having two or more incongruous or heterogeneous parts or regions.

[0001018] *Chimeric polynucleotide*: As used herein, "chimeric polynucleotides" are those nucleic acid polymers having portions or regions which differ in size and/or chemical modification pattern, chemical modification position, chemical modification percent or chemical modification population and combinations of the foregoing.

[0001019] *Compound:* As used herein, the term "compound," is meant to include all stereoisomers, geometric isomers, tautomers, and isotopes of the structures depicted.

[0001020] The compounds described herein can be asymmetric (*e.g.*, having one or more stereocenters). All stereoisomers, such as enantiomers and diastereomers, are intended unless otherwise indicated. Compounds of the present disclosure that contain asymmetrically substituted carbon atoms can be isolated in optically active or racemic forms. Methods on how to prepare optically active forms from optically active starting materials are known in the art, such as by resolution of racemic mixtures or by stereoselective synthesis. Many geometric

isomers of olefins, C=N double bonds, and the like can also be present in the compounds described herein, and all such stable isomers are contemplated in the present disclosure. Cis and trans geometric isomers of the compounds of the present disclosure are described and may be isolated as a mixture of isomers or as separated isomeric forms.

[0001021] Compounds of the present disclosure also include tautomeric forms. Tautomeric forms result from the swapping of a single bond with an adjacent double bond and the concomitant migration of a proton. Tautomeric forms include prototropic tautomers which are isomeric protonation states having the same empirical formula and total charge. Examples prototropic tautomers include ketone – enol pairs, amide – imidic acid pairs, lactam – lactim pairs, amide – imidic acid pairs, enamine – imine pairs, and annular forms where a proton can occupy two or more positions of a heterocyclic system, such as, 1H- and 3H-imidazole, 1H-, 2H- and 4H- 1,2,4-triazole, 1H- and 2H- isoindole, and 1H- and 2H-pyrazole. Tautomeric forms can be in equilibrium or sterically locked into one form by appropriate substitution.

[0001022] Compounds of the present disclosure also include all of the isotopes of the atoms occurring in the intermediate or final compounds. "Isotopes" refers to atoms having the same atomic number but different mass numbers resulting from a different number of neutrons in the nuclei. For example, isotopes of hydrogen include tritium and deuterium.

[0001023] The compounds and salts of the present disclosure can be prepared in combination with solvent or water molecules to form solvates and hydrates by routine methods.

[0001024] *Conserved*: As used herein, the term "conserved" refers to nucleotides or amino acid residues of a polynucleotide sequence or polypeptide sequence, respectively, that are those that occur unaltered in the same position of two or more sequences being compared. Nucleotides or amino acids that are relatively conserved are those that are conserved amongst more related sequences than nucleotides or amino acids appearing elsewhere in the sequences.

[0001025] In some embodiments, two or more sequences are said to be "completely conserved" if they are 100% identical to one another. In some embodiments, two or more sequences are said to be "highly conserved" if they are at least 70% identical, at least 80% identical, at least 90% identical, or at least 95% identical to one another. In some embodiments, two or more sequences are said to be "highly conserved" if they are about 70% identical, about 80% identical, about 90% identical, about 95%, about 98%, or about 99% identical to one another. In some embodiments, two or more sequences are said to be "conserved" if they are at least 30%

identical, at least 40% identical, at least 50% identical, at least 60% identical, at least 70% identical, at least 80% identical, at least 90% identical, or at least 95% identical to one another. In some embodiments, two or more sequences are said to be "conserved" if they are about 30% identical, about 40% identical, about 50% identical, about 60% identical, about 70% identical, about 80% identical, about 90% identical, about 95% identical, about 98% identical, or about 99% identical to one another. Conservation of sequence may apply to the entire length of an polynucleotide or polypeptide or may apply to a portion, region or feature thereof.

[0001026] *Controlled Release:* As used herein, the term "controlled release" refers to a pharmaceutical composition or compound release profile that conforms to a particular pattern of release to effect a therapeutic outcome.

[0001027] *Cyclic or Cyclized:* As used herein, the term "cyclic" refers to the presence of a continuous loop. Cyclic molecules need not be circular, only joined to form an unbroken chain of subunits. Cyclic molecules such as the engineered RNA or mRNA of the present invention may be single units or multimers or comprise one or more components of a complex or higher order structure.

[0001028] *Cytostatic*: As used herein, "cytostatic" refers to inhibiting, reducing, suppressing the growth, division, or multiplication of a cell (*e.g.*, a mammalian cell (*e.g.*, a human cell)), bacterium, virus, fungus, protozoan, parasite, prion, or a combination thereof.

[0001029] *Cytotoxic*: As used herein, "cytotoxic" refers to killing or causing injurious, toxic, or deadly effect on a cell (*e.g.*, a mammalian cell (*e.g.*, a human cell)), bacterium, virus, fungus, protozoan, parasite, prion, or a combination thereof.

[0001030] *Delivery:* As used herein, "delivery" refers to the act or manner of delivering a compound, substance, entity, moiety, cargo or payload.

[0001031] *Delivery Agent*: As used herein, "delivery agent" refers to any substance which facilitates, at least in part, the *in vivo* delivery of a polynucleotide to targeted cells.

[0001032] *Destabilized:* As used herein, the term "destable," "destabilize," or "destabilizing region" means a region or molecule that is less stable than a starting, wild-type or native form of the same region or molecule.

[0001033] *Detectable label:* As used herein, "detectable label" refers to one or more markers, signals, or moieties which are attached, incorporated or associated with another entity that is readily detected by methods known in the art including radiography, fluorescence,

chemiluminescence, enzymatic activity, absorbance and the like. Detectable labels include radioisotopes, fluorophores, chromophores, enzymes, dyes, metal ions, ligands such as biotin, avidin, streptavidin and haptens, quantum dots, and the like. Detectable labels may be located at any position in the peptides or proteins disclosed herein. They may be within the amino acids, the peptides, or proteins, or located at the N- or C- termini.

[0001034] *Diastereomer:* As used herein, the term "diastereomer," means stereoisomers that are not mirror images of one another and are non-superimposable on one another.

[0001035] *Digest*: As used herein, the term "digest" means to break apart into smaller pieces or components. When referring to polypeptides or proteins, digestion results in the production of peptides.

[0001036] *Differentiated cell*: As used herein, the term "differentiated cell" refers to any somatic cell that is not, in its native form, pluripotent. Differentiated cell also encompasses cells that are partially differentiated.

[0001037] *Differentiation*: As used herein, the term "differentiation factor" refers to a developmental potential altering factor such as a protein, RNA or small molecule that can induce a cell to differentiate to a desired cell-type.

[0001038] *Differentiate*: As used herein, "differentiate" refers to the process where an uncommitted or less committed cell acquires the features of a committed cell.

[0001039] *Distal:* As used herein, the term "distal" means situated away from the center or away from a point or region of interest.

[0001040] *Dosing regimen*: As used herein, a "dosing regimen" is a schedule of administration or physician determined regimen of treatment, prophylaxis, or palliative care.

[0001041] *Dose splitting factor (DSF)*-ratio of PUD of dose split treatment divided by PUD of total daily dose or single unit dose. The value is derived from comparison of dosing regimens groups.

[0001042] *Enantiomer:* As used herein, the term "enantiomer" means each individual optically active form of a compound of the invention, having an optical purity or enantiomeric excess (as determined by methods standard in the art) of at least 80% (i.e., at least 90% of one enantiomer and at most 10% of the other enantiomer), preferably at least 90% and more preferably at least 98%.

[0001043] *Encapsulate:* As used herein, the term "encapsulate" means to enclose, surround or encase.

[0001044] *Encoded protein cleavage signal*: As used herein, "encoded protein cleavage signal" refers to the nucleotide sequence which encodes a protein cleavage signal.

[0001045] *Engineered:* As used herein, embodiments of the invention are "engineered" when they are designed to have a feature or property, whether structural or chemical, that varies from a starting point, wild type or native molecule.

[0001046] *Effective Amount:* As used herein, the term "effective amount" of an agentis that amount sufficient to effect beneficial or desired results, for example, clinical results, and, as such, an "effective amount" depends upon the context in which it is being applied. For example, in the context of administering an agent that treats cancer, an effective amount of an agent is, for example, an amount sufficient to achieve treatment, as defined herein, of cancer, as compared to the response obtained without administration of the agent.

[0001047] *Expression*: As used herein, "expression" of a nucleic acid sequence refers to one or more of the following events: (1) production of an RNA template from a DNA sequence (*e.g.*, by transcription); (2) processing of an RNA transcript (*e.g.*, by splicing, editing, 5′ cap formation, and/or 3′ end processing); (3) translation of an RNA into a polypeptide or protein; and (4) post-translational modification of a polypeptide or protein.

[0001048] *Feature:* As used herein, a "feature" refers to a characteristic, a property, or a distinctive element.

[0001049] *Formulation*: As used herein, a "formulation" includes at least a polynucleotide and a delivery agent.

[0001050] *Fragment:* A "fragment," as used herein, refers to a portion. For example, fragments of proteins may comprise polypeptides obtained by digesting full-length protein isolated from cultured cells.

[0001051] *Functional*: As used herein, a "functional" biological molecule is a biological molecule in a form in which it exhibits a property and/or activity by which it is characterized.

[0001052] *Homology*: As used herein, the term "homology" refers to the overall relatedness between polymeric molecules, *e.g.* between nucleic acid molecules (*e.g.* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. In some embodiments, polymeric molecules are considered to be "homologous" to one another if their sequences are at least 25%,

30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical or similar. The term "homologous" necessarily refers to a comparison between at least two sequences (polynucleotide or polypeptide sequences). In accordance with the invention, two polynucleotide sequences are considered to be homologous if the polypeptides they encode are at least about 50%, 60%, 70%, 80%, 90%, 95%, or even 99% for at least one stretch of at least about 20 amino acids. In some embodiments, homologous polynucleotide sequences are characterized by the ability to encode a stretch of at least 4–5 uniquely specified amino acids. For polynucleotide sequences less than 60 nucleotides in length, homology is determined by the ability to encode a stretch of at least 4–5 uniquely specified amino acids. In accordance with the invention, two protein sequences are considered to be homologous if the proteins are at least about 50%, 60%, 70%, 80%, or 90% identical for at least one stretch of at least about 20 amino acids.

[0001053] *Identity*: As used herein, the term "identity" refers to the overall relatedness between polymeric molecules, *e.g.*, between polynucleotide molecules (*e.g.* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of the percent identity of two polynucleotide sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second nucleic acid sequences for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the percent identity between two nucleotide sequences can be determined using methods such as those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic

Press, New York, 1993; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; each of which is incorporated herein by reference. For example, the percent identity between two nucleotide sequences can be determined using the algorithm of Meyers and Miller (CABIOS, 1989, 4:11-17), which has been incorporated into the ALIGN program (version 2.0) using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The percent identity between two nucleotide sequences can, alternatively, be determined using the GAP program in the GCG software package using an NWSgapdna.CMP matrix. Methods commonly employed to determine percent identity between sequences include, but are not limited to those disclosed in Carillo, H., and Lipman, D., SIAM J Applied Math., 48:1073 (1988); incorporated herein by reference. Techniques for determining identity are codified in publicly available computer programs. Exemplary computer software to determine homology between two sequences include, but are not limited to, GCG program package, Devereux, J., *et al.*, *Nucleic Acids Research*, 12(1), 387 (1984)), BLASTP, BLASTN, and FASTA Altschul, S. F. *et al.*, *J. Molec. Biol.*, 215, 403 (1990)).

[0001054] *Inhibit expression of a gene:* As used herein, the phrase "inhibit expression of a gene" means to cause a reduction in the amount of an expression product of the gene. The expression product can be an RNA transcribed from the gene (*e.g.*, an mRNA) or a polypeptide translated from an mRNA transcribed from the gene. Typically a reduction in the level of an mRNA results in a reduction in the level of a polypeptide translated therefrom. The level of expression may be determined using standard techniques for measuring mRNA or protein.

[0001055] *Isomer:* As used herein, the term "isomer" means any tautomer, stereoisomer, enantiomer, or diastereomer of any compound of the invention. It is recognized that the compounds of the invention can have one or more chiral centers and/or double bonds and, therefore, exist as stereoisomers, such as double-bond isomers (i.e., geometric E/Z isomers) or diastereomers (e.g., enantiomers (i.e., (+) or (-)) or cis/trans isomers). According to the invention, the chemical structures depicted herein, and therefore the compounds of the invention, encompass all of the corresponding stereoisomers, that is, both the stereomerically pure form (e.g., geometrically pure, enantiomerically pure, or diastereomerically pure) and enantiomeric

and stereoisomeric mixtures, e.g., racemates. Enantiomeric and stereoisomeric mixtures of compounds of the invention can typically be resolved into their component enantiomers or stereoisomers by well-known methods, such as chiral-phase gas chromatography, chiral-phase high performance liquid chromatography, crystallizing the compound as a chiral salt complex, or crystallizing the compound in a chiral solvent. Enantiomers and stereoisomers can also be obtained from stereomerically or enantiomerically pure intermediates, reagents, and catalysts by well-known asymmetric synthetic methods.

[0001056] *In vitro*: As used herein, the term "*in vitro*" refers to events that occur in an artificial environment, *e.g.*, in a test tube or reaction vessel, in cell culture, in a Petri dish, *etc.*, rather than within an organism (*e.g.*, animal, plant, or microbe).

[0001057] *In vivo*: As used herein, the term "*in vivo*" refers to events that occur within an organism (*e.g.*, animal, plant, or microbe or cell or tissue thereof).

[0001058] *Isolated*: As used herein, the term "isolated" refers to a substance or entity that has been separated from at least some of the components with which it was associated (whether in nature or in an experimental setting). Isolated substances may have varying levels of purity in reference to the substances from which they have been associated. Isolated substances and/or entities may be separated from at least about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or more of the other components with which they were initially associated. In some embodiments, isolated agents are more than about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. As used herein, a substance is "pure" if it is substantially free of other components. *Substantially isolated*: By "substantially isolated" is meant that the compound is substantially separated from the environment in which it was formed or detected. Partial separation can include, for example, a composition enriched in the compound of the present disclosure. Substantial separation can include compositions containing at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, or at least about 99% by weight of the compound of the present disclosure, or salt thereof. Methods for isolating compounds and their salts are routine in the art.

[0001059] *IVT Polynucleotide*: As used herein, an "IVT polynucleotide" is a linear polynucleotide which may be made using only *in vitro* transcription (IVT) enzymatic synthesis methods.

[0001060] *Linker:* As used herein, a "linker" refers to a group of atoms, e.g., 10-1,000 atoms, and can be comprised of the atoms or groups such as, but not limited to, carbon, amino, alkylamino, oxygen, sulfur, sulfoxide, sulfonyl, carbonyl, and imine. The linker can be attached to a modified nucleoside or nucleotide on the nucleobase or sugar moiety at a first end, and to a payload, e.g., a detectable or therapeutic agent, at a second end. The linker may be of sufficient length as to not interfere with incorporation into a nucleic acid sequence. The linker can be used for any useful purpose, such as to form polynucleotide multimers (e.g., through linkage of two or more chimeric polynucleotides molecules or IVT polynucleoties) or polynucleotides conjugates, as well as to administer a payload, as described herein. Examples of chemical groups that can be incorporated into the linker include, but are not limited to, alkyl, alkenyl, alkynyl, amido, amino, ether, thioether, ester, alkylene, heteroalkylene, aryl, or heterocyclyl, each of which can be optionally substituted, as described herein. Examples of linkers include, but are not limited to, unsaturated alkanes, polyethylene glycols (e.g., ethylene or propylene glycol monomeric units, e.g., diethylene glycol, dipropylene glycol, triethylene glycol, tripropylene glycol, tetraethylene glycol, or tetraethylene glycol), and dextran polymers and derivatives thereof., Other examples include, but are not limited to, cleavable moieties within the linker, such as, for example, a disulfide bond (-S-S-) or an azo bond (-N=N-), which can be cleaved using a reducing agent or photolysis. Non-limiting examples of a selectively cleavable bond include an amido bond can be cleaved for example by the use of tris(2-carboxyethyl)phosphine (TCEP), or other reducing agents, and/or photolysis, as well as an ester bond can be cleaved for example by acidic or basic hydrolysis.

[0001061] *MicroRNA (miRNA) binding site:* As used herein, a microRNA (miRNA) binding site represents a nucleotide location or region of a nucleic acid transcript to which at least the "seed" region of a miRNA binds.

[0001062] *Modified:* As used herein "modified" refers to a changed state or structure of a molecule of the invention. Molecules may be modified in many ways including chemically, structurally, and functionally. In one embodiment, the mRNA molecules of the present invention are modified by the introduction of non-natural nucleosides and/or nucleotides, e.g., as

it relates to the natural ribonucleotides A, U, G, and C. Noncanonical nucleotides such as the cap structures are not considered "modified" although they differ from the chemical structure of the A, C, G, U ribonucleotides.

[0001063] *Naturally occurring:* As used herein, "naturally occurring" means existing in nature without artificial aid.

[0001064] *Neutralizing antibody*: As used herein, a "neutralizing antibody" refers to an antibody which binds to its antigen and defends a cell from an antigen or infectious agent by neutralizing or abolishing any biological activity it has.

[0001065] *Non-human vertebrate:* As used herein, a "non human vertebrate" includes all vertebrates except *Homo sapiens*, including wild and domesticated species. Examples of non-human vertebrates include, but are not limited to, mammals, such as alpaca, banteng, bison, camel, cat, cattle, deer, dog, donkey, gayal, goat, guinea pig, horse, llama, mule, pig, rabbit, reindeer, sheep water buffalo, and yak.

[0001066] *Off-target:* As used herein, "off target" refers to any unintended effect on any one or more target, gene, or cellular transcript.

[0001067] *Ophthalmic* As used herein, ther term "ophthalmic" means related to or associated with the eye or conditions of the eye or the sense of sight.

[0001068] *Ophthalmic Polypeptide:* As used herein, an "ophthalmic polypeptide" is a polypeptide that is involved in, associated with an ophthalmic disease or disorder or which expressed in a biological pathway associated with or involved in the etiology of any ophthalmology disease or disorder. Ophthalmic polypeptides may be encoded by an ophthalmic polynucleotide of the present invention.

[0001069] *Open reading frame:* As used herein, "open reading frame" or "ORF" refers to a sequence which does not contain a stop codon in a given reading frame.

[0001070] *Operably linked:* As used herein, the phrase "operably linked" refers to a functional connection between two or more molecules, constructs, transcripts, entities, moieties or the like.

[0001071] *Optionally substituted:* Herein a phrase of the form "optionally substituted X" (*e.g.*, optionally substituted alkyl) is intended to be equivalent to "X, wherein X is optionally substituted" (*e.g.*, "alkyl, wherein the alkyl is optionally substituted"). It is not intended to mean that the feature "X" (*e.g.* alkyl) *per se* is optional.

[0001072] *Osmolarity:* as used herein, "osmolarity" refers to the measure of solute concentration, defined as the number of osmoles of solute per liter of solution (Osm/L).

[0001073] *Osmolarity neutral buffer or solution:* as used herein, an "osmolarity neutral buffer" is one which can be applied without significant changes in normal osmolarity.

[0001074] *Part*: As used herein, a "part" or "region" of a polynucleotide is defined as any portion of the polynucleotide which is less than the entire length of the polynucleotide.

[0001075] *Peptide:* As used herein, "peptide" is less than or equal to 50 amino acids long, e.g., about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

[0001076] *Paratope:* As used herein, a "paratope" refers to the antigen-binding site of an antibody.

[0001077] *Patient:* As used herein, "patient" refers to a subject who may seek or be in need of treatment, requires treatment, is receiving treatment, will receive treatment, or a subject who is under care by a trained professional for a particular disease or condition.

[0001078] *Pharmaceutically acceptable*: The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[0001079] *Pharmaceutically acceptable excipients:* The phrase "pharmaceutically acceptable excipient," as used herein, refers any ingredient other than the compounds described herein (for example, a vehicle capable of suspending or dissolving the active compound) and having the properties of being substantially nontoxic and non-inflammatory in a patient. Excipients may include, for example: antiadherents, antioxidants, binders, coatings, compression aids, disintegrants, dyes (colors), emollients, emulsifiers, fillers (diluents), film formers or coatings, flavors, fragrances, glidants (flow enhancers), lubricants, preservatives, printing inks, sorbents, suspensing or dispersing agents, sweeteners, and waters of hydration. Exemplary excipients include, but are not limited to: butylated hydroxytoluene (BHT), calcium carbonate, calcium phosphate (dibasic), calcium stearate, croscarmellose, crosslinked polyvinyl pyrrolidone, citric acid, crospovidone, cysteine, ethylcellulose, gelatin, hydroxypropyl cellulose, hydroxypropyl methylcellulose, lactose, magnesium stearate, maltitol, mannitol, methionine, methylcellulose, methyl paraben, microcrystalline cellulose, polyethylene glycol, polyvinyl pyrrolidone,

povidone, pregelatinized starch, propyl paraben, retinyl palmitate, shellac, silicon dioxide, sodium carboxymethyl cellulose, sodium citrate, sodium starch glycolate, sorbitol, starch (corn), stearic acid, sucrose, talc, titanium dioxide, vitamin A, vitamin E, vitamin C, and xylitol.

[0001080] *Pharmaceutically acceptable salts*: The present disclosure also includes pharmaceutically acceptable salts of the compounds described herein. As used herein, "pharmaceutically acceptable salts" refers to derivatives of the disclosed compounds wherein the parent compound is modified by converting an existing acid or base moiety to its salt form (e.g., by reacting the free base group with a suitable organic acid). Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. Representative acid addition salts include acetate, acetic acid, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzene sulfonic acid, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, 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, toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like, as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. The pharmaceutically acceptable salts of the present disclosure include the conventional non-toxic salts of the parent compound formed, for example, from non-toxic inorganic or organic acids. The pharmaceutically acceptable salts of the present disclosure can be synthesized from the parent compound which contains a basic or acidic moiety by conventional chemical methods. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with a stoichiometric amount of the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, nonaqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred. Lists of suitable salts are found in *Remington's Pharmaceutical Sciences*, 17th ed.,

Mack Publishing Company, Easton, Pa., 1985, p. 1418, *Pharmaceutical Salts: Properties, Selection, and Use*, P.H. Stahl and C.G. Wermuth (eds.), Wiley-VCH, 2008, and Berge et al., *Journal of Pharmaceutical Science*, 66, 1-19 (1977), each of which is incorporated herein by reference in its entirety.

[0001081] *Pharmaceutically acceptable solvate*: The term "pharmaceutically acceptable solvate," as used herein, means a compound of the invention wherein molecules of a suitable solvent are incorporated in the crystal lattice. A suitable solvent is physiologically tolerable at the dosage administered. For example, solvates may be prepared by crystallization, recrystallization, or precipitation from a solution that includes organic solvents, water, or a mixture thereof. Examples of suitable solvents are ethanol, water (for example, mono-, di-, and tri-hydrates), *N*-methylpyrrolidinone (NMP), dimethyl sulfoxide (DMSO), *N,N'*-dimethylformamide (DMF), *N,N'*-dimethylacetamide (DMAC), 1,3-dimethyl-2-imidazolidinone (DMEU), 1,3-dimethyl-3,4,5,6-tetrahydro-2-(1H)-pyrimidinone (DMPU), acetonitrile (ACN), propylene glycol, ethyl acetate, benzyl alcohol, 2-pyrrolidone, benzyl benzoate, and the like. When water is the solvent, the solvate is referred to as a "hydrate."

[0001082] *Pharmacokinetic:* As used herein, "pharmacokinetic" refers to any one or more properties of a molecule or compound as it relates to the determination of the fate of substances administered to a living organism. Pharmacokinetics is divided into several areas including the extent and rate of absorption, distribution, metabolism and excretion. This is commonly referred to as ADME where: (A) Absorption is the process of a substance entering the blood circulation; (D) Distribution is the dispersion or dissemination of substances throughout the fluids and tissues of the body; (M) Metabolism (or Biotransformation) is the irreversible transformation of parent compounds into daughter metabolites; and (E) Excretion (or Elimination) refers to the elimination of the substances from the body. In rare cases, some drugs irreversibly accumulate in body tissue.

[0001083] *Physicochemical:* As used herein, "physicochemical" means of or relating to a physical and/or chemical property.

[0001084] *Polypeptide per unit drug (PUD)*: As used herein, a PUD or product per unit drug, is defined as a subdivided portion of total daily dose, usually 1 mg, pg, kg, etc., of a product (such as a polypeptide) as measured in body fluid or tissue, usually defined in concentration such as pmol/mL, mmol/mL, etc divided by the measure in the body fluid.

[0001085] *Preventing*: As used herein, the term "preventing" refers to partially or completely delaying onset of an infection, disease, disorder and/or condition; partially or completely delaying onset of one or more symptoms, features, or clinical manifestations of a particular infection, disease, disorder, and/or condition; partially or completely delaying onset of one or more symptoms, features, or manifestations of a particular infection, disease, disorder, and/or condition; partially or completely delaying progression from an infection, a particular disease, disorder and/or condition; and/or decreasing the risk of developing pathology associated with the infection, the disease, disorder, and/or condition.

[0001086] *Prodrug*: The present disclosure also includes prodrugs of the compounds described herein. As used herein, "prodrugs" refer to any substance, molecule or entity which is in a form predicate for that substance, molecule or entity to act as a therapeutic upon chemical or physical alteration. Prodrugs may by covalently bonded or sequestered in some way and which release or are converted into the active drug moiety prior to, upon or after administered to a mammalian subject. Prodrugs can be prepared by modifying functional groups present in the compounds in such a way that the modifications are cleaved, either in routine manipulation or *in vivo*, to the parent compounds. Prodrugs include compounds wherein hydroxyl, amino, sulfhydryl, or carboxyl groups are bonded to any group that, when administered to a mammalian subject, cleaves to form a free hydroxyl, amino, sulfhydryl, or carboxyl group respectively. Preparation and use of prodrugs is discussed in T. Higuchi and V. Stella, "Pro-drugs as Novel Delivery Systems," Vol. 14 of the A.C.S. Symposium Series, and in *Bioreversible Carriers in Drug Design*, ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987, both of which are hereby incorporated by reference in their entirety.

[0001087] *Prophylactic*: As used herein, "prophylactic" refers to a therapeutic or course of action used to prevent the spread of disease.

[0001088] *Prophylaxis:* As used herein, a "prophylaxis" refers to a measure taken to maintain health and prevent the spread of disease. An "immune phrophylaxis" refers to a measure to produce active or passive immunity to prevent the spread of disease.

[0001089] *Protein cleavage site*: As used herein, "protein cleavage site" refers to a site where controlled cleavage of the amino acid chain can be accomplished by chemical, enzymatic or photochemical means.

[0001090] *Protein cleavage signal:* As used herein "protein cleavage signal" refers to at least one amino acid that flags or marks a polypeptide for cleavage.

[0001091] *Protein of interest:* As used herein, the terms "proteins of interest" or "desired proteins" include those provided herein and fragments, mutants, variants, and alterations thereof.

[0001092] *Proximal:* As used herein, the term "proximal" means situated nearer to the center or to a point or region of interest.

[0001093] *Pseudouridine:* As used herein, pseudouridine refers to the C-glycoside isomer of the nucleoside uridine. A "pseudouridine analog" is any modification, variant, isoform or derivative of pseudouridine. For example, pseudouridine analogs include but are not limited to 1-carboxymethyl-pseudouridine, 1-propynyl-pseudouridine, 1-taurinomethyl-pseudouridine, 1-taurinomethyl-4-thio-pseudouridine, 1-methylpseudouridine ($m^1\psi$), 1-methyl-4-thio-pseudouridine ($m^1s^4\psi$), 4-thio-1-methyl-pseudouridine, 3-methyl-pseudouridine ($m^3\psi$), 2-thio-1-methyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-1-deaza-pseudouridine, dihydropseudouridine, 2-thio-dihydropseudouridine, 2-methoxyuridine, 2-methoxy-4-thio-uridine, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, N1-methyl-pseudouridine, 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine ($acp^3\ \psi$), and 2′-O-methyl-pseudouridine (ψm).

[0001094] *Purified:* As used herein, "purify," "purified," "purification" means to make substantially pure or clear from unwanted components, material defilement, admixture or imperfection.

[0001095] *Repeated transfection:* As used herein, the term "repeated transfection" refers to transfection of the same cell culture with a polynucleotide a plurality of times. The cell culture can be transfected at least twice, at least 3 times, at least 4 times, at least 5 times, at least 6 times, at least 7 times, at least 8 times, at least 9 times, at least 10 times, at least 11 times, at least 12 times, at least 13 times, at least 14 times, at least 15 times, at least 16 times, at least 17 times at least 18 times, at least 19 times, at least 20 times, at least 25 times, at least 30 times, at least 35 times, at least 40 times, at least 45 times, at least 50 times or more.

[0001096] *Sample:* As used herein, the term "sample" or "biological sample" refers to a subset of its tissues, cells or component parts (e.g. body fluids, including but not limited to blood, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid and semen). A sample further may include a homogenate, lysate or

extract prepared from a whole organism or a subset of its tissues, cells or component parts, or a fraction or portion thereof, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs. A sample further refers to a medium, such as a nutrient broth or gel, which may contain cellular components, such as proteins or nucleic acid molecule.

[0001097] *Signal Sequences:* As used herein, the phrase "signal sequences" refers to a sequence which can direct the transport or localization of a protein.

[0001098] *Single unit dose*: As used herein, a "single unit dose" is a dose of any therapeutic administered in one dose/at one time/single route/single point of contact, i.e., single administration event.

[0001099] *Similarity*: As used herein, the term "similarity" refers to the overall relatedness between polymeric molecules, *e.g.* between polynucleotide molecules (*e.g.* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of percent similarity of polymeric molecules to one another can be performed in the same manner as a calculation of percent identity, except that calculation of percent similarity takes into account conservative substitutions as is understood in the art.

[0001100] *Split dose*: As used herein, a "split dose" is the division of single unit dose or total daily dose into two or more doses.

[0001101] *Stable:* As used herein "stable" refers to a compound that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and preferably capable of formulation into an efficacious therapeutic agent.

[0001102] *Stabilized:* As used herein, the term "stabilize", "stabilized," "stabilized region" means to make or become stable.

[0001103] *Stereoisomer:* As used herein, the term "stereoisomer" refers to all possible different isomeric as well as conformational forms which a compound may possess (e.g., a compound of any formula described herein), in particular all possible stereochemically and conformationally isomeric forms, all diastereomers, enantiomers and/or conformers of the basic molecular structure. Some compounds of the present invention may exist in different tautomeric forms, all of the latter being included within the scope of the present invention.

[0001104] *Subject:* As used herein, the term "subject" or "patient" refers to any organism to which a composition in accordance with the invention may be administered, *e.g.*, for experimental, diagnostic, prophylactic, and/or therapeutic purposes. Typical subjects include animals (*e.g.*, mammals such as mice, rats, rabbits, non-human primates, and humans) and/or plants.

[0001105] *Substantially*: As used herein, the term "substantially" refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term "substantially" is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[0001106] *Substantially equal*: As used herein as it relates to time differences between doses, the term means plus/minus 2%.

[0001107] *Substantially simultaneously*: As used herein and as it relates to plurality of doses, the term means within 2 seconds.

[0001108] *Suffering from*: An individual who is "suffering from" a disease, disorder, and/or condition has been diagnosed with or displays one or more symptoms of a disease, disorder, and/or condition.

[0001109] *Susceptible to*: An individual who is "susceptible to" a disease, disorder, and/or condition has not been diagnosed with and/or may not exhibit symptoms of the disease, disorder, and/or condition but harbors a propensity to develop a disease or its symptoms. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition (for example, cancer) may be characterized by one or more of the following: (1) a genetic mutation associated with development of the disease, disorder, and/or condition; (2) a genetic polymorphism associated with development of the disease, disorder, and/or condition; (3) increased and/or decreased expression and/or activity of a protein and/or nucleic acid associated with the disease, disorder, and/or condition; (4) habits and/or lifestyles associated with development of the disease, disorder, and/or condition; (5) a family history of the disease, disorder, and/or condition; and (6) exposure to and/or infection with a microbe associated with development of the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will develop the disease, disorder,

and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will not develop the disease, disorder, and/or condition.

[0001110] *Sustained release:* As used herein, the term "sustained release" refers to a pharmaceutical composition or compound release profile that conforms to a release rate over a specific period of time.

[0001111] *Synthetic*: The term "synthetic" means produced, prepared, and/or manufactured by the hand of man. Synthesis of polynucleotides or polypeptides or other molecules of the present invention may be chemical or enzymatic.

[0001112] *Targeted Cells:* As used herein, "targeted cells" refers to any one or more cells of interest. The cells may be found *in vitro*, *in vivo*, *in situ* or in the tissue or organ of an organism. The organism may be an animal, preferably a mammal, more preferably a human and most preferably a patient.

[0001113] *Therapeutic Agent:* The term "therapeutic agent" refers to any agent that, when administered to a subject, has a therapeutic, diagnostic, and/or prophylactic effect and/or elicits a desired biological and/or pharmacological effect.

[0001114] *Therapeutically effective amount:* As used herein, the term "therapeutically effective amount" means an amount of an agent to be delivered (*e.g.*, nucleic acid, drug, therapeutic agent, diagnostic agent, prophylactic agent, *etc.*) that is sufficient, when administered to a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition.

[0001115] *Therapeutically effective outcome*: As used herein, the term "therapeutically effective outcome" means an outcome that is sufficient in a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition.

[0001116] *Total daily dose:* As used herein, a "total daily dose" is an amount given or prescribed in 24 hr period. It may be administered as a single unit dose.

[0001117] *Transcription factor:* As used herein, the term "transcription factor" refers to a DNA-binding protein that regulates transcription of DNA into RNA, for example, by activation or repression of transcription. Some transcription factors effect regulation of transcription alone, while others act in concert with other proteins. Some transcription factor can both activate and

repress transcription under certain conditions. In general, transcription factors bind a specific target sequence or sequences highly similar to a specific consensus sequence in a regulatory region of a target gene. Transcription factors may regulate transcription of a target gene alone or in a complex with other molecules.

[0001118] *Transcription*: As used herein, the term "transcription" refers to methods to introduce exogenous nucleic acids into a cell. Methods of transfection include, but are not limited to, chemical methods, physical treatments and cationic lipids or mixtures.

[0001119] *Treating*: As used herein, the term "treating" refers to partially or completely alleviating, ameliorating, improving, relieving, delaying onset of, inhibiting progression of, reducing severity of, and/or reducing incidence of one or more symptoms or features of a particular infection, disease, disorder, and/or condition. For example, "treating" cancer may refer to inhibiting survival, growth, and/or spread of a tumor. Treatment may be administered to a subject who does not exhibit signs of a disease, disorder, and/or condition and/or to a subject who exhibits only early signs of a disease, disorder, and/or condition for the purpose of decreasing the risk of developing pathology associated with the disease, disorder, and/or condition.

[0001120] *Unmodified*: As used herein, "unmodified" refers to any substance, compound or molecule prior to being changed in any way. Unmodified may, but does not always, refer to the wild type or native form of a biomolecule. Molecules may undergo a series of modifications whereby each modified molecule may serve as the "unmodified" starting molecule for a subsequent modification.

Equivalents and Scope

[0001121] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments in accordance with the invention described herein. The scope of the present invention is not intended to be limited to the above Description, but rather is as set forth in the appended claims.

[0001122] In the claims, articles such as "a," "an," and "the" may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context.

The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

[0001123] It is also noted that the term "comprising" is intended to be open and permits but does not require the inclusion of additional elements or steps. When the term "comprising" is used herein, the term "consisting of" is thus also encompassed and disclosed.

[0001124] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present disclosure; other, suitable methods and materials known in the art can also be used.

[0001125] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[0001126] In addition, it is to be understood that any particular embodiment of the present invention that falls within the prior art may be explicitly excluded from any one or more of the claims. Since such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the compositions of the invention (*e.g.*, any nucleic acid or protein encoded thereby; any method of production; any method of use; *etc.*) can be excluded from any one or more claims, for any reason, whether or not related to the existence of prior art.

[0001127] All cited sources, for example, references, publications, databases, database entries, and art cited herein, are incorporated into this application by reference, even if not expressly stated in the citation. In case of conflicting statements of a cited source and the instant application, the statement in the instant application shall control.

[0001128] Section and table headings are not intended to be limiting.

EXAMPLES

[0001129] Methods of cyclization and/or concatemerization are described in Example 13 of International Publication No. WO2015034928, the contents of which is herein incorporated by reference in its entirety.

[0001130] Methods of chimeric synthesis of RNA are described in Example 14 of US Provisional Application No. 62/034,883, filed August 8, 2014, entitled Compositions and Methods for the Treatment of Ophthalmic Diseases and Conditions, the contents of which are herein incorporated by reference in its entirety.

Example 1. Manufacture of chimeric polynucleotides

[0001131] According to the present invention, the manufacture of chimeric polynucleotides and or parts or regions thereof may be accomplished utilizing the methods taught in International Publication No. WO2014152027 (Attorney Docket number M500), the contents of which is incorporated herein by reference in its entirety.

[0001132] Purification methods may include those taught in International Publication No. WO2014152030 (Attorney Docket number M501); International Publication No. WO2014152031 (Attorney Docket number M502), each of which is incorporated herein by reference in its entirety.

[0001133] Characterization of the chimeric polynucleotides of the invention may be accomplished using a procedure selected from the group consisting of polynucleotide mapping, reverse transcriptase sequencing, charge distribution analysis, and detection of RNA impurities, wherein characterizing comprises determining the RNA transcript sequence, determining the purity of the RNA transcript, or determining the charge heterogeneity of the RNA transcript. Such methods are taught in, for example, International Publication No. WO2014144039 (Attorney Docket number M505); International Publication No. WO2014144711 (Attorney Docket number M506) and International Publication No. WO2014144767 (Attorney Docket number M507) the contents of each of which is incorporated herein by reference in its entirety.

Example 2. Chimeric polynucleotide synthesis: triphosphate route

Introduction

[0001134] According to the present invention, two regions or parts of a chimeric polynucleotide may be joined or ligated using triphosphate chemistry.

[0001135] According to this method, a first region or part of 100 nucleotides or less is chemically synthesized with a 5' monophosphate and terminal 3'desOH or blocked OH. If the region is longer than 80 nucleotides, it may be synthesized as two strands for ligation.

[0001136] If the first region or part is synthesized as a non-positionally modified region or part using in vitro transcription (IVT), conversion the 5'monophosphate with subsequent capping of the 3' terminus may follow.

[0001137] Monophosphate protecting groups may be selected from any of those known in the art.

[0001138] The second region or part of the chimeric polynucleotide may be synthesized using either chemical synthesis or IVT methods. IVT methods may include an RNA polymerase that can utilize a primer with a modified cap. Alternatively, a cap of up to 130 nucleotides may be chemically synthesized and coupled to the IVT region or part.

[0001139] It is noted that for ligation methods, ligation with DNA T4 ligase, followed by treatment with DNAse should readily avoid concatenation.

[0001140] The entire chimeric polynucleotide need not be manufactured with a phosphate-sugar backbone. If one of the regions or parts encodes a polypeptide, then it is preferable that such region or part comprise a phosphate-sugar backbone.

[0001141] Ligation is then performed using any known click chemistry, orthoclick chemistry, solulink, or other bioconjugate chemistries known to those in the art.

Synthetic route

[0001142] The chimeric polynucleotide is made using a series of starting segments. Such segments include:

[0001143] (a) Capped and protected 5' segment comprising a normal 3'OH (SEG. 1)

[0001144] (b) 5' triphosphate segment which may include the coding region of a polypeptide and comprising a normal 3'OH (SEG. 2)

[0001145] (c) 5' monophosphate segment for the 3' end of the chimeric polynucleotide (e.g., the tail) comprising cordycepin or no 3'OH (SEG. 3)

[0001146] After synthesis (chemical or IVT), segment 3 (SEG. 3) is treated with cordycepin and then with pyrophosphatase to create the 5'monophosphate.

[0001147] Segment 2 (SEG. 2) is then ligated to SEG. 3 using RNA ligase. The ligated polynucleotide is then purified and treated with pyrophosphatase to cleave the diphosphate. The

treated SEG.2-SEG. 3 construct is then purified and SEG. 1 is ligated to the 5' terminus. A further purification step of the chimeric polynucleotide may be performed.

[0001148] Where the chimeric polynucleotide encodes a polypeptide, the ligated or joined segments may be represented as: 5'UTR (SEG. 1), open reading frame or ORF (SEG. 2) and 3'UTR+PolyA (SEG. 3).

[0001149] The yields of each step may be as much as 90-95%.

Example 3: PCR for cDNA Production

[0001150] PCR procedures for the preparation of cDNA are performed using 2x KAPA HIFI™ HotStart ReadyMix by Kapa Biosystems (Woburn, MA). This system includes 2x KAPA ReadyMix12.5 µl; Forward Primer (10 uM) 0.75 µl; Reverse Primer (10 uM) 0.75 µl; Template cDNA -100 ng; and dH_20 diluted to 25.0 µl. The reaction conditions are at 95° C for 5 min. and 25 cycles of 98° C for 20 sec, then 58° C for 15 sec, then 72° C for 45 sec, then 72° C for 5 min. then 4° C to termination.

[0001151] The reverse primer of the instant invention incorporates a poly-T_{120} (SEQ ID NO: 106) for a poly-A_{120} (SEQ ID NO: 100) in the mRNA. Other reverse primers with longer or shorter poly(T) tracts can be used to adjust the length of the poly(A) tail in the polynucleotide mRNA.

[0001152] The reaction is cleaned up using Invitrogen's PURELINK™ PCR Micro Kit (Carlsbad, CA) per manufacturer's instructions (up to 5 µg). Larger reactions will require a cleanup using a product with a larger capacity. Following the cleanup, the cDNA is quantified using the NANODROP™ and analyzed by agarose gel electrophoresis to confirm the cDNA is the expected size. The cDNA is then submitted for sequencing analysis before proceeding to the *in vitro* transcription reaction.

Example 4. In vitro Transcription (IVT)

A. Synthesis of mRNA Constructs in Preparation for IVT

Restriction Digest of Plasmid DNA

[0001153] DNA plasmid is digested by incubation at 37°C for 2 hr in a 50 µL reaction containing DNA plasmid (50 ng/µL), BSA (1X), 1X NEBuffer 4 (50mM potassium acetate, 20mM Tris-acetate, 10mM magnesium acetate, 1mM DTT, pH 7.9), and XbaI (400 U/mL) (New England Biolabs). The restriction digest is analyzed by 1% agarose gel and used directly for PCR.

DNA Template Amplification

The desired DNA template is amplified by PCR in 100 uL reactions using linearized plasmid (20 ng), dNTPs (0.2 μM each), forward primer (0.2 μM), reverse primer (0.2 μM), 1X Q5 reaction buffer, and Q5 high-fidelity DNA polymerase (20 U/mL) (New England Biolabs). All components are kept on ice until added to the thermocycler. The reaction conditions are at 95° C for 4 min. and 30 cycles of 98° C for 15 sec, then 72° C for 45 sec, then 72° C for 20 sec per kb, then 72° C for 5 min. then 4° C to termination. The PCR product is analyzed by capillary electrophoresis (CE) (Agilent 2100 Bioanalyzer) and desalted by ultrafiltration (Amicon).

B. IVT Reaction

[0001154] In vitro transcription (IVT) reactions are performed in 50 uL containing template DNA (25 ng/μL), NTPs (7.6 mM each), 1X T7 IVT buffer, RNase Inhibitor (1 U/μL), Pyrophosphatase (1 U/μL), and T7 RNA polymerase (7 U/μL) (NEB). In general, 24 50uL reactions per construct are used. Modified mRNA may be generated using 5-methyl-CTP and 1-methyl-pseudoUTP or any chosen modified triphosphate. IVT reactions are incubated at 37 °C for 4 hr, after which 2.5 μL of DNase I (2000 U/mL) (NEB) is added and the reaction allowed to incubated for another 45 min. The reactions are combined and purified using MEGAclear spin columns (Ambion) and eluted in 250 μL water. The IVT product is analyzed by CE (Agilent 2100 Bioanalyzer).

Example 5. Enzymatic Capping

[0001155] Capping of a polynucleotide is performed as follows where the mixture includes: IVT RNA 60 μg-180μg and dH_20 up to 72 μl. The mixture is incubated at 65° C for 5 minutes to denature RNA, and then is transferred immediately to ice.

[0001156] The protocol then involves the mixing of 10x Capping Buffer (0.5 M Tris-HCl (pH 8.0), 60 mM KCl, 12.5 mM $MgCl_2$) (10.0 μl); 20 mM GTP (5.0 μl); 20 mM S-Adenosyl Methionine (2.5 μl); RNase Inhibitor (100 U); 2′-O-Methyltransferase (400U); Vaccinia capping enzyme (Guanylyl transferase) (40 U); dH_20 (Up to 28 μl); and incubation at 37° C for 30 minutes for 60 μg RNA or up to 2 hours for 180 μg of RNA.

[0001157] The polynucleotide is then purified using Ambion's MEGACLEAR™ Kit (Austin, TX) following the manufacturer's instructions. Following the cleanup, the RNA is quantified using the NANODROP™ (ThermoFisher, Waltham, MA) and analyzed by agarose gel electrophoresis to confirm the RNA is the proper size and that no degradation of the RNA has

occurred. The RNA product may also be sequenced by running a reverse-transcription-PCR to generate the cDNA for sequencing.

Example 6. PolyA Tailing Reaction

[0001158] Without a poly-T in the cDNA, a poly-A tailing reaction must be performed before cleaning the final product. This is done by mixing Capped IVT RNA (100 µl); RNase Inhibitor (20 U); 10x Tailing Buffer (0.5 M Tris-HCl (pH 8.0), 2.5 M NaCl, 100 mM $MgCl_2$)(12.0 µl); 20 mM ATP (6.0 µl); Poly-A Polymerase (20 U); dH_20 up to 123.5 µl and incubation at 37° C for 30 min. If the poly-A tail is already in the transcript, then the tailing reaction may be skipped and proceed directly to cleanup with Ambion's MEGACLEAR™ kit (Austin, TX) (up to 500 µg). Poly-A Polymerase is preferably a recombinant enzyme expressed in yeast.

[0001159] It should be understood that the processivity or integrity of the polyA tailing reaction may not always result in an exact size polyA tail. Hence polyA tails of approximately between 40-200 nucleotides (SEQ ID NO: 107), e.g., about 40, 50, 60, 70, 80, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 150-165, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164 or 165 are within the scope of the invention.

Example 7. Natural 5′ Caps and 5′ Cap Analogues

[0001160] 5′-capping of polynucleotides may be completed concomitantly during the *in vitro*-transcription reaction using the following chemical RNA cap analogs to generate the 5′-guanosine cap structure according to manufacturer protocols: 3′-O-Me-m7G(5')ppp(5') G [the ARCA cap];G(5')ppp(5')A; G(5')ppp(5')G; m7G(5')ppp(5')A; m7G(5')ppp(5')G (New England BioLabs, Ipswich, MA). 5′-capping of modified RNA may be completed post-transcriptionally using a Vaccinia Virus Capping Enzyme to generate the "Cap 0" structure: m7G(5')ppp(5')G (New England BioLabs, Ipswich, MA). Cap 1 structure may be generated using both Vaccinia Virus Capping Enzyme and a 2′-O methyl-transferase to generate: m7G(5')ppp(5')G-2′-O-methyl. Cap 2 structure may be generated from the Cap 1 structure followed by the 2′-O-methylation of the 5′-antepenultimate nucleotide using a 2′-O methyl-transferase. Cap 3 structure may be generated from the Cap 2 structure followed by the 2′-O-methylation of the 5′-preantepenultimate nucleotide using a 2′-O methyl-transferase. Enzymes are preferably derived from a recombinant source.

[0001161] When transfected into mammalian cells, the modified mRNAs have a stability of between 12-18 hours or more than 18 hours, e.g., 24, 36, 48, 60, 72 or greater than 72 hours.

Example 8. Capping Assays

A. Protein Expression Assay

[0001162] Chimeric polynucleotides encoding a polypeptide, containing any of the caps taught herein can be transfected into cells at equal concentrations. 6, 12, 24 and 36 hours post-transfection the amount of protein secreted into the culture medium can be assayed by ELISA. Synthetic chimeric polynucleotides that secrete higher levels of protein into the medium would correspond to a synthetic chimeric polynucleotide with a higher translationally-competent Cap structure.

B. Purity Analysis Synthesis

[0001163] Chimeric polynucleotides encoding a polypeptide, containing any of the caps taught herein can be compared for purity using denaturing Agarose-Urea gel electrophoresis or HPLC analysis. Chimeric polynucleotides with a single, consolidated band by electrophoresis correspond to the higher purity product compared to chimeric polynucleotides with multiple bands or streaking bands. Synthetic chimeric polynucleotides with a single HPLC peak would also correspond to a higher purity product. The capping reaction with a higher efficiency would provide a more pure chimeric polynucleotide population.

C. Cytokine Analysis

[0001164] Chimeric polynucleotides encoding a polypeptide, containing any of the caps taught herein can be transfected into cells at multiple concentrations. 6, 12, 24 and 36 hours post-transfection the amount of pro-inflammatory cytokines such as TNF-alpha and IFN-beta secreted into the culture medium can be assayed by ELISA. Chimeric polynucleotides resulting in the secretion of higher levels of pro-inflammatory cytokines into the medium would correspond to a chimeric polynucleotides containing an immune-activating cap structure.

D. Capping Reaction Efficiency

[0001165] Chimeric polynucleotides encoding a polypeptide, containing any of the caps taught herein can be analyzed for capping reaction efficiency by LC-MS after nuclease treatment. Nuclease treatment of capped chimeric polynucleotides would yield a mixture of free nucleotides and the capped 5′-5-triphosphate cap structure detectable by LC-MS. The amount of capped product on the LC-MS spectra can be expressed as a percent of total chimeric polynucleotide from the reaction and would correspond to capping reaction efficiency. The cap structure with higher capping reaction efficiency would have a higher amount of capped product by LC-MS.

Example 9. Agarose Gel Electrophoresis of Modified RNA or RT PCR Products

[0001166] Individual chimeric polynucleotides (200-400 ng in a 20 µl volume) or reverse transcribed PCR products (200-400 ng) are loaded into a well on a non-denaturing 1.2% Agarose E-Gel (Invitrogen, Carlsbad, CA) and run for 12-15 minutes according to the manufacturer protocol.

Example 10. Nanodrop Modified RNA Quantification and UV Spectral Data

[0001167] Modified chimeric polynucleotides in TE buffer (1 µl) are used for Nanodrop UV absorbance readings to quantitate the yield of each chimeric polynucleotide from an chemical synthesis or *in vitro* transcription reaction.

Example 11. Formulation of Modified mRNA Using Lipidoids

[0001168] Chimeric polynucleotides are formulated for *in vitro* experiments by mixing the chimeric polynucleotides with the lipidoid at a set ratio prior to addition to cells. *In vivo* formulation may require the addition of extra ingredients to facilitate circulation throughout the body. To test the ability of these lipidoids to form particles suitable for *in vivo* work, a standard formulation process used for siRNA-lipidoid formulations may be used as a starting point. After formation of the particle, chimeric polynucleotide is added and allowed to integrate with the complex. The encapsulation efficiency is determined using a standard dye exclusion assays.

Example 12. Method of Screening for Protein Expression

A. Electrospray Ionization

[0001169] A biological sample which may contain proteins encoded by a chimeric polynucleotide administered to the subject is prepared and analyzed according to the manufacturer protocol for electrospray ionization (ESI) using 1, 2, 3 or 4 mass analyzers. A biologic sample may also be analyzed using a tandem ESI mass spectrometry system.

[0001170] Patterns of protein fragments, or whole proteins, are compared to known controls for a given protein and identity is determined by comparison.

B. Matrix-Assisted Laser Desorption/Ionization

[0001171] A biological sample which may contain proteins encoded by one or more chimeric polynucleotides administered to the subject is prepared and analyzed according to the manufacturer protocol for matrix-assisted laser desorption/ionization (MALDI).
Patterns of protein fragments, or whole proteins, are compared to known controls for a given protein and identity is determined by comparison.

C. Liquid Chromatography-Mass spectrometry-Mass spectrometry

[0001172] A biological sample, which may contain proteins encoded by one or more chimeric polynucleotides, may be treated with a trypsin enzyme to digest the proteins contained within. The resulting peptides are analyzed by liquid chromatography-mass spectrometry-mass spectrometry (LC/MS/MS). The peptides are fragmented in the mass spectrometer to yield diagnostic patterns that can be matched to protein sequence databases via computer algorithms. The digested sample may be diluted to achieve 1 ng or less starting material for a given protein. Biological samples containing a simple buffer background (e.g. water or volatile salts) are amenable to direct in-solution digest; more complex backgrounds (e.g. detergent, non-volatile salts, glycerol) require an additional clean-up step to facilitate the sample analysis.

[0001173] Patterns of protein fragments, or whole proteins, are compared to known controls for a given protein and identity is determined by comparison.

Example 13. Detection of protein in retinal pigment epithelial cells (RPE)

Protocol

[0001174] RPE cells were cultured and passaged and then split into 96 well plates. 20,000 cells were grown in growth media with 10% fetal calf serum.

[0001175] Growth media was replaced with fresh media for the transfection assay. Cells were transfected with mRNAs and negative or positive controls. Cells were treated with 200 ng/well of mRNA and 0.4 uL Lipofection2000/well. Cell supernatant and/or lysate was collected 16 hours post transfect and frozen for further study.

[0001176] All mRNA were fully modified with N1-methylpseudouridine replacing each uridine

[0001177] Western blot analysis was performed to detect protein. Alternatively ELISA analysis was performed as noted. The mRNA constructs used in the experiment are listed in Table 13.

Table 13. mRNA Constructs

Gene ID	Construct Name	Encoded Protein	Function of Encoded Protein	SEQ ID NO
147905	RhoJ.nV5_Hs3U	RhoJ	Cytoskeleton and cell regulation. RhoJ is an endothelially expressed member of the Cdc42 (cell division cycle 42). It is expressed in both the developing mammalian vasculature and the vascular beds of a number of adult tissues. Rho proteins regulate the dynamic assembly of cytoskeletal components for	48

			several physiologic processes, such as cell proliferation and motility and the establishment of cell polarity. They are also involved in pathophysiologic process, such as cell transformation and metastasis	
147906	PEDF.cV5_Hs3U	PEDF (SERPINF1)	anti-angiogenic, anti-tumorigenic, and neurotrophic. In humans, pigment epithelium-derived factor is encoded by the SERPINF1 gene. A multifunctional secreted protein that has anti-angiogenic, anti-tumorigenic, and neurotrophic functions (50 kDa). A therapeutic candidate for treatment of such conditions as choroidal neovascularization, heart disease, and cancer.	49
147907	CD59.cV5_Hs3U	CD59	CD59 is a 128aa-long protein (18 -22 kDa). Expression CD59 is expressed in all organs and in almost all cell types. It is essential to protect cells from self-destruction by complement-mediated lysis. CD59 is expressed on the surface of cells. It is attached to the cellular membrane via a glycosylphosphatidylinositol tail (GPI-anchored molecule). In urine, CD59 can be found as soluble protein. CD59 was found recently to attenuate angiogenesis by preventing the complement-mediated lysis of newly grown blood vessels.	50
147908	Enbrel_Hs3U (FC)	Etanercept	TNF inhibitor. The mRNA encoded for TNF receptor 2 and IgG1 Fc protein. Etanercept (trade name Enbrel) is a biopharmaceutical that treats autoimmune diseases by interfering with tumor necrosis factor (TNF; a soluble inflammatory cytokine) by acting as a TNF inhibitor. The prototypic fusion protein was first synthesized and shown to be highly active and unusually stable as a modality for blockade of TNF in vivo. It is a large molecule, with a molecular weight of 150 kDa., that binds to TNFα and decreases its role in disorders	51

			involving excess inflammation in humans and other animals.	
147909	IL1RA.V5_Hs3U	IL-1R antagonist	inhibit IL-1 activities	52
147910	CD59_Hs3U	CD59	See above	53
147911	bestropin1_Hs3U	BEST1	Calcium-activated anion channels. Bestrophin-1 is a protein that in humans is encoded by the BEST1 gene and it belongs to the bestrophin family of calcium-activated anion channels. Bestrophins are transmembrane (TM) proteins. BEST1 has been shown by two independent studies to be regulated by Microphthalmia-associated transcription factor. It can be associated with Vitelliform macular dystrophy.	54
147912	RPE65_Hs3U	Retinal Pigment Epithelium-Specific Protein 65kDa	Plays important roles in the production of 11-cis retinal and in visual pigment regeneration. Located in the retinal pigment epithelium and is involved in the production of 11-cis retinal and in visual pigment regeneration. There are two forms, a soluble form called sRPE65, and a membrane-bound form known as mRPE65. mRPE65 serves as the palmitoyl donor for lecithin retinol acyl transferase (LRAT), the enzyme that catalyzes the vitamin A to all trans retinol step of the chromophore regeneration process and Both mRPE65 and sRPE65 also serve as regulatory proteins. Mutations in this gene have been associated with Leber congenital amaurosis type 2 and retinitis pigmentosa.	55
147913	myocilin_Hs3U	Myocilin (MYCO)	Myocilin have a role in cytoskeletal function and regulating intraocular pressure (glaucoma). Trabecular meshwork inducible glucocorticoid response, also known as MYOC. Mutations in MYOC are a major cause of glaucoma (Decreased secretion and increased accumulation appear to be the initial steps in myocilin-associated glaucoma).	56

			Myocilin have a role in cytoskeletal function. MYOC is expressed in many ocular tissues, including the trabecular meshwork, and was revealed to be the trabecular meshwork glucocorticoid-inducible response protein (TIGR). The trabecular meshwork is a specialized eye tissue essential in regulating intraocular pressure. The secreted recombinant human MYOC consists of 483 amino acids and has a predicted molecular mass of 54.7 kDa. Since most of the MYOC was cleaved at Glu 214, generating a C-terminal fragment of 32 kDa, the purified human MYOC migrates as an approximately 33 kDa band in SDS-PAGE under reducing conditions.	
147914	myocilin.cV5_Hs3U	Myocilin (MYCO)	See above	57
144875	CCR3_FLAG_Hs3U	C-C chemokine receptor type 3 (CCR3)	Accumulation and activation of eosinophils and other inflammatory cells in the allergic airway, and possibly at sites of parasitic infection. A protein that in humans is encoded by the CCR3 gene, has also recently been designated CD193 (cluster of differentiation 193). It belongs to family 1 of the G protein-coupled receptors. This receptor binds and responds to a variety of chemokines It is highly expressed in eosinophils and basophils, and is also detected in TH1 and TH2 cells, as well as in airway epithelial cells. This receptor may contribute to the accumulation and activation of eosinophils and other inflammatory cells in the allergic airway, and possibly at sites of parasitic infection. It is also known to be an entry co-receptor for HIV-1. 67 kDa including tags.	58

[0001178] In the table, V5 refers to an epitope tag used to identify the resultant proteins. The V5 tag is encoded at either the N or C terminus of the polypeptide. Proteins could then be detected using the V5 antibody for the presence of the V5 tag. The primary antibody was the Novex Life

Technology (cat#R96025)-Mouse anti-V5. The secondary antibody was the Licor Donkey-anti-mouse antibody. FLAG is a standard Flag tag.

[0001179] All proteins were detected as expressed from the mRNA transfected and ran on the gels at the correct size. RhoJ and CD59 were detected by Western Blot using anti-V5. IL-RA was detected by Western blot and found in both supernatant and lysate

[0001180] Reporter genes, mCherry and GFP were investigated and both reporter genes when expressed from an mRNA expressed protein of the correct size and function.

Example 14. Quantification of functional protein retinal pigment epithelial cells (RPE)

Using the protocol outlined in Example 15, mRNA expressing aflibercept (Eylea™) or bevacizumab (Avastin™) in RPE cells. Supernatants were assessed with a VEGF binding ELISA for the presence of the proteins. The mRNA used are listed in Table 14.

Table 14. Aflibercept and Bevacizumab Constructs

Gene ID	Construct Name	Encodes	SEQ ID NO
144864	aflibercept_HS3U	Aflibercept (VEGF-Trap)	59
144865	Bevacizumab.HC_HS3U	Bevacizumab heavy chain	60
144866	Bevacizumab.LC_HS3U	Bevacizumab light chain	61
144867	Bevacizumab.RKR_HS3U	Bevacizumab RKR site	62

[0001181] The data show that functional proteins are expressed in the assay using the mRNA of the invention. Aflibercept was expressed at 100,000 pg/mL and Bevacizumab at 90,000 pg/mL. The controls, a nonspecific mRNA and PBS showed no expression.

Example 15. Cell line studies

[0001182] Using the protocol of Example 15, different cell lines and varied formulations were tested. mRNA encoding either Aflibercept or Bevacizumab from Example 16 were utilized. Formulations included PBS buffer and lipid nanoparticle (LNP). Binding to recombinant human fusion protein was measured (pg/mL) and the data are given in Table 15. The controls, a nonspecific mRNA and PBS showed no expression. Shown in Table 16, are the values of protein detected (pg/mL) for Bevacizumab with and without the RKR signal in the supernatant and cell lysate of the specified cell lines.

[0001183] For detection of the IgG fusion proteins by Western blot, the antibody used was from Millipore CalBiochem (cat # 401954) goat anti-human IgG, heavy and light chain specific primary antibody and the Licor secondary antibody (cat # 926-32214).

Table 15. Formulation and Cell line (supernatant)

	HepB3 Cells	HeLa Cells	RPE Cells
Aflibercept-PBS	650	550	250
Aflibercept-LNP	610	600	100
Bevacizumab-PBS	210	190	80
Bevacizumab-LNP	300	250	100

Table 16. Supernatant vs. Lysate

	HepB3 Cells	HeLa Cells	RPE Cells
Bevacizumab-Supernatant	90,000	45,000	40,000
Bevacizumab-lysate	100,000	70,000	40,000
Bevacizumab-RKR-Supernatant	0	0	0
Bevacizumab-RKR-Lysate	30,000	60,000	35,000

Example 16. Combination mRNA treatment: In vitro study, VEGF-Trap/PDGF-Trap

[0001184] Using the protocol of Example 15, different combinations of modified mRNA were tested for expression of protein. In vitro transfection of modified mRNA encoding Aflibercept (VEGF trap) in a lipid nanoparticle, PDGF Trap in a lipid nanoparticle or a combination of both was performed. Also tested was the VEGF-Trap in PBS buffer and buffer alone as a control.

[0001185] Hep3B cells (15,000 cells/well) were transfected with 250 ng mRNA formulated in an MC3 lipid nanoparticle in a 96 well plate format. Supernatants which contain proteins expressed from the mRNA were collected 16 hours post transfection. Proteins were detected by Western blot with FC antibody or VEGFR1 and VEGFR2 antibodies for the presence of the VEGF-Trap.

[0001186] The same experiment was repeated in RPE cells (Seeded at 20,000 cells). The mRNA constructs are listed in Table 17 and the protein expression data for Hep3B cells and dose response data for RPE cells are shown in Tables 18 and 19.

Table 17. mRNA Constructs

Gene ID	Description	Protein Encoded	SEQ ID NO
144864	aflibercept_Hs3U	Aflibercept (VEGF-Trap)	59
148534	PDGFRbtrap.huIgG4Fc.V5_Hs3U	PDGFR trap; IgG4 with V5 tag	63
148535	PDGFRbtrap.huIgG1Fc.V5_Hs3U	PDGFR trap; IgG1 with V5 tag	64
148536	antiPDGF.B1.LC.V5_Hs3U	PDGFB1 light chain with V5 tag	65
148537	antiPDGF.B1.HC.V5_Hs3U	PDGFB1 heavy chain with V5 tag	66
148538	antiPDGF.E1.HC.V5_Hs3U	PDGFE1 heavy chain with V5 tag	67
148539	antiPDGF.E1.LC.V5_Hs3U	PDGFE1 light chain with V5 tag	68

Table 18. Protein Expression (ng/mL)

	VEGF Trap Protein Average Expression (ng/mL); n=2
VEGF-Trap-LNP	339.2
VEGF-Trap-LNP	429.95
VEGF-Trap-PBS	368.5
VEGF-Trap and PDGF-Trap-LNP	441.1

Table 19. VEGF-Trap Dose Response in RPE cells (ng/mL)

mRNA concentration (ng/well;96 well)	VEGF Trap Protein Average Expression (ng/mL); n=2
250	1813
125	3593.2
62.5	4047.1
31.3	3869.7
15.7	1825.7
7.8	330
3.9	211.8
1.9	57.7
0	0

Example 17. Expression in vivo: Intra-vitreal injection; Reporter proteins

[0001187] Evaluation of protein expression 18 hours following intravitreal injection of modified mRNA encoding luciferase or mCherry in the Sprague Dawley rat was conducted. Fourteen (14) male Sprague Dawley (SD) rats were selected for the study. Prior to dosing, the animals were approximately 6 week old with body weight that ranged between 120 to 210 g.

[0001188] Animals were dosed according to Table 20.

Table 20. Dosing

Group No.	Dose Level (µg Modified RNA/eye)	Dose Volume (µL/eye)	Treatment		No. of Males
			Right Eye (OD)	Left Eye (OS)	
1/ Vehicle Control	0	5	Delivery buffer only	Delivery buffer only	2
2/ Modified RNA in delivery buffer (2 mg/mL mCherry or Luciferase)	10	5	mCherry	Luciferase	5
3/ Modified RNA in Lipoplex formulation (2 mg/mL mCherry or Luciferase)	10	5	mCherry	Luciferase	5

[0001189] Dosing consisted of a 5 µL intravitreal injection of the 2 mg/mL solution per eye for Group 2.

[0001190] For Lipoplex-Luc modified RNA mRNA were formulated with Lipoplex, as follows. 22.5 µL RNAiMax was equilibrated in delivery buffer. After equilibration, RNAiMax (a total 75 µL volume) was added to mRNA (a total 75 µL volume) and incubated for a further 20 minutes at room temperature. Dosing consisted of a 5 µL intravitreal injection of the 2 mg/mL Lipoplexed solution per eye (Group 3.)

[0001191] Materials were administered to the left or right eyes of each animal on Day 1 as indicated in the Experimental Design table above. The animals were anesthetized (isoflurane) for the dose administration, which was performed by a board certified veterinary ophthalmologist. Topical antibiotics (gentamicin ophthalmic ointment or solution) were applied to both eyes twice on the day before treatment, immediately following the injection and at least once on the day following the injection. Prior to dosing, mydriatic drops (1% tropicamide and/or 2.5% phenylephrine) were applied to each eye. During dosing, animals were maintained under anesthesia with isoflurane/oxygen gas.

[0001192] The conjunctivae were flushed with 0.9% Sodium Chloride for Injection USP. A 10 µL Hamilton syringe 32-gauge, ½-inch needle was used for each intravitreal injection (one syringe/group/treatment). Test materials were administered by intravitreal injection into the eyes of each animal at a dose volume of 5 µL/eye. Both eyes were examined by slit-lamp biomicroscopy and/or indirect ophthalmoscopy immediately following each treatment to document any abnormalities (especially to the lens, vitreous and retina) caused by the administration procedure.

[0001193] Mortality/moribundity checks, detailed clinical signs, body weights, and food evaluation were monitored. All animals were considered suitable for study use. There were no unscheduled deaths during the study. There were no treatment or adverse procedure-related clinical signs and there were no effects on body weights, body weight gains or appetence.

[0001194] At termination, study animals underwent exsanguination from the abdominal aorta after isoflurane anesthesia. The animals were euthanized in group order.

[0001195] At approximately 18 hours post dose, all left eyes receiving luciferase modified RNA (with and without Lipoplex) and 2 eyes receiving buffer only were enucleated and placed separately (cornea facing the side) in a cryomold containing OCT, frozen in liquid nitrogen and placed on dry ice until storage in a freezer set to maintain -80°C for future tissue sectioning and immunofluorescent analysis.

[0001196] At approximately 18 hours post dose, all right eyes receiving mCherry modified RNA (with and without Lipoplex) and 2 eyes receiving buffer only were enucleated and flash frozen in liquid nitrogen and placed on dry ice until stored in a freezer set to maintain -80°C for further testing.

[0001197] The study revealed positive reporter (Luc and mCherry) protein expression in the rat eyes 18h post IVT administration of modified mRNA.

Example 18. Expression in vivo: Intra-vitreal injection; Aflibercept and DcR1.

[0001198] Following the protocol of Example 18, rats were treated with a single dose of 5ul/eye of an mRNA encoding Aflibercept in PBS (6ug), an mRNA encoding Aflibercept in a lipid nanoparticle (2ug), an mRNA encoding DcR1 in PBS (6ug), an mRNA encoding DcR1 in a lipid nanoparticle (2ug). The control for the study was the PBS buffer. The constructs are given in Table 21.

Table 21. Constructs

Gene ID	Construct Name	Encodes	SEQ ID NO
144864	aflibercept_Hs3U	Aflibercept a.k.a. Eylea	59
144865	Bevacizumab.HC_Hs3U	Bevacizumab heavy chain	60
144866	Bevacizumab.LC_Hs3U	Bevacizumab light chain	61
144867	Bevacizumab.RKR_Hs3U	Bevacizumab RKR site	62
144873	DcR1_Hs3U	GPI-linked receptor	69

[0001199] Serum and eye vitreous humor, and retina were collected 8 hours after injection. Protein was analyzed by Western blot or ELISA.

[0001200] Both proteins were detected in retina extract at 8 hours.

Example 19. Expression in vivo: Bispecific antibodies and intracellular proteins, Rabbit study

[0001201] The purpose of the study was to show ocular protein expression 24 and 48 hours following a single dose of mRNA via intra-vitreous injection in New Zealand White rabbits and the evaluation of four mRNAs encoding with different proteins.

[0001202] The treatment regimen is outlined in Table 22.

Table 22. Study outline

G #	Treatment	Treatment			N=		Conc	Vol	Time Point (sac) post dosing
		Right Eye (OD)	Dose Level (µg mRNA per eye)	Left Eye (OS)		Eyes	mg/ml	ul	
1	VEGF-Trap (mRNA1)-LNP	mRNA1-LNP	50ug	mRNA1-LNP	4	8	0.5	900	24h
								900	
2	VEGF-Trap-LNP	mRNA1-LNP	50ug	mRNA1-LNP	4	8			48h
3	PDGF Trap (mRNA2)-LNP	mRNA2-LNP	50ug	mRNA2-LNP	2	4	0.5	450	24h
4	VEGF Trap+ PDGF Trap-LNP	mRNA1+2-LNP	17.5 ug each	mRNA1+2-LNP	2	4	0.5	300+120= 420 ul	24h
5	VEGF Trap-PBS	Naked mRNA	200ug	Naked mRNA	4	8	2.0	900	24h
6	Vehicle – PBS	Vehicle – PBS	-	Vehicle – PBS	4	4	-	1000	48h

[0001203] Formulation: The mRNAs were formulated in an MC3-lipid nanoparticle or PBS buffer. All dose volumes were 100 ul per eye.

[0001204] Animal species: New Zealand White rabbits.

[0001205] Acclimation Period: Minimum of 5 days.

[0001206] Groups: 6 groups. Each rabbit received binocularly test article via a single intravitreal injection.

[0001207] Number of Animals/Sex/Age/Weight Range: Twenty (20) male or female rabbits, at least 8 weeks old (young adult) and weighing at least 1.75 kg.

[0001208] Duration of the study: 2 Days.

[0001209] Animal and Eye Preparation: Animals were sedated with appropriate anesthesia using 35-50 mg/kg ketamine and 5-10 mg/kg xylazine (intramuscular or intraperitoneal injection) / 1-3% isoflurane (inhalation) before procedures. Pupils were dilated with 1% tropicamide ophthalmic solution prior to procedures. The cornea was topically anaesthetized with 0.5% proparacaine HCl ophthalmic solution (or equivalent) prior to procedures. The eyes of sedated animals were kept moist and animals were kept warm until recovery. Following recovery from anesthesia, the animals were returned to their cages.

[0001210] Ocular Examinations: All eyes of the animals were examined pre-dose and only rabbits showing no signs of eye irritation, ocular defects or pre-existing corneal injury were used in the study.

[0001211] Body weight: All animals were weighed prior to dose administration.

[0001212] Administration: Animals were injected intravitreally with one article once on Day 1 and with buprenorphine 0.02-0.1 mg/kg SQ or IM once following administration. The data are shown in Table 23. The groups are numbered 1-6. VH stands for vitreous humor.

Table 23. Protein Expression

	Sample protein concentration	**total protein amount per 30ul well**	**VEGF trap concentration**	**Estimated VEGF trap per 30ul of well**	**Estimated VEGF trap per ug of total protein**
	ng/ul	ng	pg/ml	pg	pg/ug of protein
G1-Retina	642.15	19264.5	106	3.18	0.17
G2-Retina	399.83	11994.9	305	9.15	0.76
G3-Retina	596.2	17886		0	0.00
G4-Retina	626.79	18803.7	27	0.81	0.04

G5-Retina	617.14	18514.2	14	0.42	0.02
G6-Retina	437.02	13110.6		0	0.00
G1-VH	49.99	1499.7	40	1.2	0.80
G2-VH	72.33	2169.9	55	1.65	0.76
G3-VH	55.2	1656		0	0.00
G4-VH	74.87	2246.1	15	0.45	0.20
G5-VH	86.42	2592.6	15	0.45	0.17
G6-VH	19.4	582		0	0.00

[0001213] Higher expression of mRNA encoding VEGF-Trap protein was seen at 48 hours rather than 24 hours, (290 pg/mL vs. 90 pg/mL for retina lysates and 55 pg/mL vs. 40 pg/mL for vitreous humor samples).

Example 20. Ophthalmic Targets

[0001214] Ophthalmology targets which may be encoded by any of the polynucleotides of the invention may also include those in Table 24. These are useful in the treatment of wet age related macular degeneration (wAMD), dry age related macular degeneration (dAMD), diabetic retinopathy and macular edema (DME), and/or central retinal vein occlusion (CRVO). mRNA sequences encoding selected targets are given in Table 25.

Table 24. Ophthalmic Targets

Target	Target (full name)	Indication	Pathway/Mechanism of Action
Ranibizumab (Lucentis ™)	anti-VEGF Fab	wAMD, DME, CRVO, DME	Inhibitor of VEGF-induced angiogenesis
Aflibercept (Eylea ™) VEGF-Trap	VEGFR1/2-Fc	wAMD, DME, CRVO, DME	Inhibitor of VEGF-induced angiogenesis
IGFR I-Fc	Insulin-like growth factor 1 R-Fc	wAMD	Inhibitor of VEGF-induced angiogenesis (upstream of VEGF)
PDGF-trap	PDGFRbb-Fc	wAMD, DME, CRVO	Inhibition of PDGF-induced retinal pericyte survival, vessel remodeling
Sema3E	Semaphorin 3E	wAMD	VEGF-independent angiogenesis Inhibitor
PD1R	PlexinD1R (overexpression) (Sema3E pathway)	wAMD	VEGF-independent angiogenesis Inhibitor
RhoJ	RhoJ (overexpression) (Sema3E pathway)	wAMD	VEGF-independent angiogenesis Inhibitor

PEDF	Pigment epithelial derived factor	wAMD	VEGF/Sema3E-independent angiogenesis Inhibitor
Dcr1	Dcr1/TRAILR3 (GPI anchored), TRAIL decoy	wAMD, dAMD	Inhibition of TRAIL-induced apoptosis
DR4-Fc	DR4/TRAILR1-Fc, TRAIL decoy	wAMD, dAMD	Inhibition of TRAIL-induced apoptosis
DR5-Fc	DR5/TRAILR2-Fc, TRAIL decoy	wAMD, dAMD	Inhibition of TRAIL-induced apoptosis
aA Crystallin	aA Crystallin subunit	wAMD, dAMD	Strong disease association, unknown molecular function
aB Crystallin	aB Crystallin subunit	wAMD, dAMD	Strong disease association, unknown molecular function
aA Crystallin secreted	aA Crystallin subunit incl secretion signal	wAMD, dAMD	Strong disease association, unknown molecular function
aB Crystallin secreted	aB Crystallin subunit incl secretion signal	wAMD, dAMD	Strong disease association, unknown molecular function
NPPB	Natriuretic peptide precursor B	wAMD	Genetic association
CD59	MAC-inhibitory protein	wAMD	Genetic association
PLEKHA1	Pleckstrin homology domain containing, family A member w/t	wAMD, dAMD	Genetic association
SIRT1	Sirtuin 1	dAMD, Age-induced vision loss, cataract, optic neuritis	Hypoxia-induced, non-VEGF-mediated neovascularization
Unc5b-Fc	Netrin-1-Receptor-Fc	Oxygen/Ischemia-induced retinal disease (OIR)	Hypoxia-induced, non-VEGF-mediated neovascularization
RPE65	w/t RPE65 retinoid isomerohydrolase	Leber's Congenital Amaurosis (LCA)	Genetic association
ABCA4	ABCA4	Stargardt disease: ABCA4	Genetic association
PDE	w/t cGMP phosphodiesterase (PDE)	Retinitis Pigmentosa	Genetic association
TNFR-Fc	TNFR-Fc	Auto-immune uveitis	Inflammation
IL1RA	IL1-RA	Auto-immune uveitis	Inflammation

IL6R-Fc	IL6R-Fc or IL6-minibody	Auto-immune uveitis	Inflammation
TNFRFc/IL1RA/ IL6RFc	TNFR-Fc +/- IL1-RA +/- IL6R-Fc or IL6-minibody	Idiopathic uveitis	Inflammation
TNFRFc/ IL1RA	TNFR-Fc +/- IL1-RA	Behcet disease-related uveitis	Inflammation
IL6R-Fc	IL6R-Fc or IL6-minibody	Vogt-Koyanagi-Harada (VKH) disease	Inflammation
IL6R-Fc	IL6R-Fc or IL6-minibody	Ankylosing spondylitis	Inflammation
TGFb1	TGFb1	Fuchs' heterochromic iridocyclitis (FHC)	Inflammation

Table 25. Ophthalmic Targets

Gene ID	Description	Target	SEQ ID NO
144868	lucentis.LC_Hs3U	Ranibizumab	70
144869	lucentis.HC_Hs3U	Ranibizumab	71
144870	lucentis.RKR_Hs3U	Ranibizumab with RKR site	72
144871	AlphaCrystallinA_Hs3U	Alpha Crystallin A	73
144872	AlphaCrystallinB_Hs3U	Alpha crystalline B	74
144874	UNC5B.FLAG_Hs3U	Netrin-1-Receptor-Fc with Flag tag	75
146301	SIRT3.V5_Hs3U	Sirtuin 3 with V5 epitope	76
146302	SIRT6.V5_HS3U	Sirtuin 6 with V5 epitope	77
146303	SIRT1.V5_HS3U	Sirtuin 1 with V5 epitope	78
147585	SIRT2.V5_HS3U	Sirtuin 2 with V5 epitope	79
147586	SIRT4.V5_HS3U	Sirtuin 4 with V5 epitope	80
147587	SIRT5.V5_HS3U	Sirtuin 5 with V5 epitope	81
147588	SIRT7.V5_Hs3U	Sirtuin 7 with V5 epitope	82
147903	SEMA3E.cV5_Hs3U	Semaphorin 3E with C-terminus V5 epitope	83
147904	SEMA3E.nV5_Hs3U	Semaphorin 3E with N-terminus V5 epitope	84
147919	PLXND1.nV5_Hs3U	Plexin D1 with N-terminus V5 epitope	85
149385	DPPIV.Fc_HS3U	DPP IV FC fusion	86
149386	CD276v5_HS3U	CD27 with V5 epitope	87
153262	DICER1.v5_HS3U	Dicer 1 with V5 epitope	88

153263	SOD1.v5_HS3U	Superoxide dismutase with V5 epitope	89
153264	PHD2.v5_HS3U	PHD-2 with V5 epitope	90
153265	Notch4.v5_HS3U	Notch 4 with V5 epitope	91
153266	HEY1.v5_HS3U	Hey1 with V5 epitope	92
153267	anti-CCL11.HC.v5_HS3U	CCL11 heavy chain with V5 epitope; anti-eotaxin mAb	93
153268	anti-CCL11.LC.v5_HS3U	CCL11 light chain with V5 epitope; anti-eotaxin mAb	94

Example 21. In vitro Transfection of mRNA

[0001215] Modified mRNA was tested for expression of protein. In vitro transfection of modified mRNA encoding Myocilin, PEDF, Sema3E-cV5 and Sema3E-nV5 (i.e., Sema3E with a C or N terminal V5 tag) in a lipid nanoparticle was performed. PBS was transfected in parallel as a negative control.

[0001216] RPE cells (20,000 cells/well) and HeLa cells (15,000 cells/well) were transfected with 200 ng mRNA plus 0.4 ul of Lipofectamine2000/well in a 96 well plate format. Supernatants which contain proteins expressed from the mRNA were collected 16 hours post transfection. Proteins were detected by Western blot with anti-Myoclin, anti-PEDF, anti Sema3E-cV5 and anti Sema3E-nV5 antibodies.

[0001217] For extracts from both RPE and HeLa cells transfected with the mRNAs, bands of the appropriate molecular weight were observed (i.e. Myocilin 54.7 kDa, PEDF 50 kDa, and Sema 3E approx. 90 kDa). Extracts from RPE and HeLa cells transfected with PBS only did not show any bands. The mRNA constructs used are listed in Table 26.

Table 26 mRNA Constructs

Gene ID	Description	Protein Encoded	SEQ ID NO
147906	PEDF.cV5_Hs3U	PEDF	49
147913	myocilin_Hs3U	Myocilin	56
147914	myocilin.cV5_Hs3U	Myocilin with V5 tag	57
147903	SEMA3E.cV5_Hs3U	Sema3E with V5 tag	83
147904	SEMA3E.nV5_Hs3U	Sema3E with V5 tag	84

OTHER EMBODIMENTS

[0001218] It is to be understood that the words which have been used are words of description rather than limitation, and that changes may be made within the purview of the appended claims without departing from the true scope and spirit of the invention in its broader aspects.

[0001219] While the present invention has been described at some length and with some particularity with respect to the several described embodiments, it is not intended that it should be limited to any such particulars or embodiments or any particular embodiment, but it is to be construed with references to the appended claims so as to provide the broadest possible interpretation of such claims in view of the prior art and, therefore, to effectively encompass the intended scope of the invention.

[0001220] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, section headings, the materials, methods, and examples are illustrative only and not intended to be limiting.

Claims

1. A method of treating an ophthalmic disease, disorder or condition comprising administering to a subject a pharmaceutical composition comprising a polynucleotide encoding at least one ophthalmic polypeptide and wherein said polynucleotide is formulated in a pharmaceutically acceptable carrier or excipient.

2. The method of claim 1, wherein the polynucleotide is an mRNA.

3. The method of claim 2, wherein the polynucleotide encodes two polypeptides.

4. The method of claim 2, wherein the polynucleotide encodes more than two open reading frames.

5. The method of claims 1-4, wherein the polynucleotide comprises at least one chemical modification.

6. The method of claim 5, wherein the polynucleotide comprises a purified IVT transcript.

7. The method of claim 5, wherein the polynucleotide comprises a chimeric transcript.

8. The method of claim 6 or 7, wherein the polynucleotide formulation comprises an osmolarity neutral buffer or solution.

9. The method of claim 1, wherein the ophthalmic polypeptide is an intracellular, nuclear or membrane bound polypeptide.

10. The method of any of the preceding claims wherein the polynucleotide encodes one or more microRNA (miR) or microRNA binding sites (miRBS).

11. The method of claim 1, wherein the ophthalmic polypeptide is involved in a pathway selected from the group consisting of neovascularization, the complement cascade, vascular cell infiltration, NLRP3 mediated cell death, reducing HIF-1 transcription of VEGF and/or PDGF, modulating the antioxidant system, reducing apoptotic pathway signaling, modulating angiogenesis and those associated with retinopathies.

12. The method of claim 11, wherein the pathway is modulating neovascularization and the ophthalmic polynucleotide encodes a polypeptide which functions as a dual inhibitor of VEGF and PDGF induced neovascularization.

13. The method of claim 11, wherein the pathway is the complement cascade and the ophthalmic polynucleotide encodes a polypeptide which inhibits the MASP complex.

14. The method of claim 11, wherein the pathway is vascular cell infiltration and the ophthalmic polynucleotide encodes a polypeptide which blocks early vascular cell infiltration.

15. The method of claim 11, wherein the pathway is NLRP3 mediated cell death and the ophthalmic polynucleotide encodes a polypeptide which restores DICER1 expression in order to reduce NLRP3 mediated cell death in geographic atrophy.

16. The method of claim 11, wherein the pathway is transcription and the ophthalmic polynucleotide encodes a polypeptide which functions to reduce HIF1 transcription of VEGF and PDGF.

17. The method of claim 11, wherein the pathway is the antioxidant system and the ophthalmic polynucleotide encodes a polypeptide which functions to alleviate, prevent or treat drusen, choroidal neovascularization, and/or retinal pigment epithelium dysfunction.

18. The method of claim 11, wherein the pathway is apoptosis and the ophthalmic polynucleotide encodes a polypeptide which functions to reduce apoptosis in the retinal pigment epithelium.

19. The method of claim 11, wherein the pathway is angiogenesis and the ophthalmic polynucleotide encodes a polypeptide which functions to alter the Notch signaling pathway or the semaphorin-plexin pathway.

20. The method of claim 11, wherein the pathway is associated with a retinopathy and said retinopathy pathway is selected from the group consisting of mitochondrial oxidation, cell adhesion/tissue remodeling, the visual cycle/rod-cone homeostasis, inflammation (the complement system), Drusen formation involving the extracellular matrix, inflammation

(immune homing), and neovascularization (involving either growth factors or blood components).

21. The method of claim 20, wherein the retinopathy pathway is mitochondrial oxidation and the polynucleotide encodes TOMM40Lm Mitofusin 2, OPA1, SOD2, NADH dehydrogenase (1, 2, 4-6), Cytochromes (b,c) and or ATP synthase.

22. The method of claim 20, wherein the retinopathy pathway is cell adhesion/tissue remodeling and the polynucleotide encodes cadherin 5, vascular endothelial, Cadherin-related family member 1, Peripherin 2, ADAM metallopeptidase domain 9, Thrombospondin receptor and/or Integrin A5.

23. The method of claim 20, wherein the retinopathy pathway is visual cycle/rod-cone homeostasis and the polynucleotide encodes retinal pigment epithelium-specific protein, guanylate cyclase activator 1A, guanylate cyclase 2D, membrane, voltage dependent calcium channels (A2, LA1F), bestrophin 1, and/or ciliary neurotrophic factor

24. The method of claim 20, wherein the retinopathy pathway is inflammation and the polynucleotide encodes complement component 3, complement component 5, complement component 5 receptor 1, complement component 2, complement factor D, complement factor H, complement factor B, and/or complement factor I.

25. The method of claim 20, wherein the retinopathy pathway is extracellular matrix homeostasis and the polynucleotide encodes amyloid beta (A4) precursor protein, tenascin XB, collagen type X, alpha 1, myelin basic protein, and/or collagen type VIII, alpha 1.

26. The method of claim 20, wherein the retinopathy pathway is inflammation and the polynucleotide encodes chemokine receptor 3, chemokine receptor 4, carbohydrate (GlcNAc) sulfotrans 6 (lymphocyte ligand metabolism), and/or TNF receptor 10A.

27. The method of claim 20, wherein the retinopathy pathway is neovascularization and the polynucleotide encodes VEGF-A, PDGF, HtrA serine peptidase 1, insulin like GF binding protein 7, and/or placental growth factor.

28. The method of claim 20, wherein the retinopathy pathway is c neovascularization and the polynucleotide encodes plasminogen, Factor III, sphingosine -1 –phosphate receptor, hepatic lipase and/or cholesteryl ester transfer protein.

29. The method of claim 1, wherein the disease or disorder is a retinopathy attendant to an orphan indication selected from the group consisting of Stargardt disease, Leber hereditary optic neuropathy, Cone rod dystrophy, Leber congenital amaurosis, Best vitelliform macular dystrophy, Choroideremia, Central areolar choroidal dystrophy, Macular corneal dystrophy, Autosomal dominant optic atrophy plus syndrome, North Carolina macular dystrophy, Hereditary vascular retinopathy and/or Autosomal dominant vitreoretinochoroidopathy.

30. The method of claim 1, wherein the disease or disorder is a glaucoma attendant to Axenfeld-Rieger syndrome.

31. The method of claim 1, wherein the disease or disorder is cataracts attendant to an orphan indication, wherein the cataract is selected from the group consisting of anterior polar cataract, Hutterite type Cataract, coralliform cataract, cerulean cataract, pulverulent cataract, congenital cataract, Volkmann type, Coppock-like cataract, cataract with Y-shaped suture opacities, zonular cataract, partial congenital cataract, nuclear cataract, total congenital cataract, and/or posterior polar cataract.

32. The method of claim 1, wherein the disease or disorder is a dry eye attendant benign essential blepharospasm.

33. The method of claim 1, wherein the formulation comprises a lipid nanoparticle and wherein said lipid nanoparticle comprises at least one lipid and/or at least one polymer.

34. The method of claim 33, wherein the polynucleotide is encapsulated in the lipid nanoparticle.

35. The method of claim 33, wherein the lipid is selected from the group consisting of DLin-DMA, DLin-K-DMA, 98N12-5, C12-200, ckk, E12, DLin-MC3-DMA, DLin-KC2-DMA, DODMA, DOPE, DSPC, PLGA, PEG-DMG, PEG-DSG, PEG-DSPE, PEG-DOMG, PEGylated lipids, polyethylenimine (PEI) and chitosan.

36. The method of claim 33, wherein the lipid is an ionizable amino lipid.

37. The method of claim 36, wherein the ionizable amino lipid is selected from the group consisting of DLin-MC3-DMA and DLin-KC2-DMA.

38. The method of claim 1, wherein contacting said mammalian cells or tissues occurs via a route of administration selected from the group consisting of intraocular, subconjunctival, subcutaneous, intravitreal, or intramuscular.

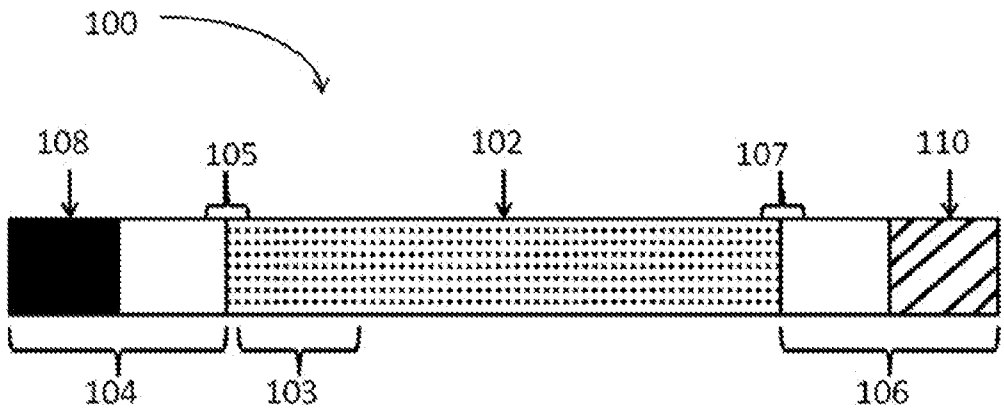
39. The method of claim 2, wherein the mRNA comprises at least one 5′ terminal cap selected from the group consisting of Cap0, Cap1, ARCA, inosine, N1-methyl-guanosine, 2′fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine.

40. The method of claim 39, wherein the 5' terminal cap is Cap1.

41. The method of claim 5, wherein the polynucleotide comprises at least two chemical modifications.

A.

FIG. 1


100
108
105
102
107
110
104
103
106

B.

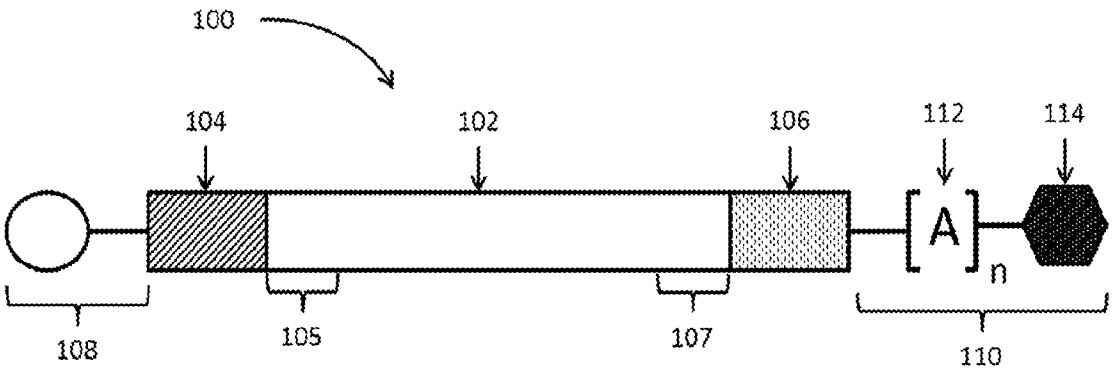

100
104
102
106
112
114
A
n
108
105
107
110

FIG. 2

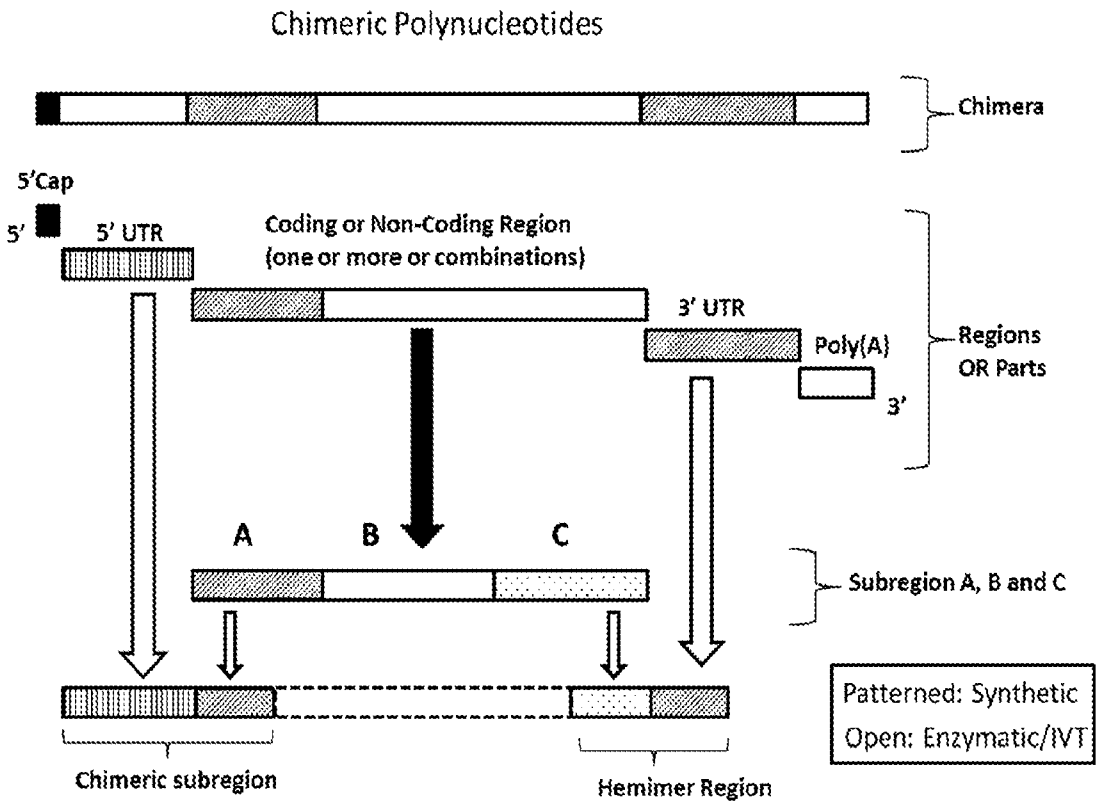

Chimeric Polynucleotides
Chimera
5'Cap
5'
5' UTR
Coding or Non-Coding Region
(one or more or combinations)
3' UTR
Poly(A)
3'
Regions
OR Parts
A
B
C
Subregion A, B and C
Patterned: Synthetic
Open: Enzymatic/IVT
Chimeric subregion
Hemimer Region

FIG. 3

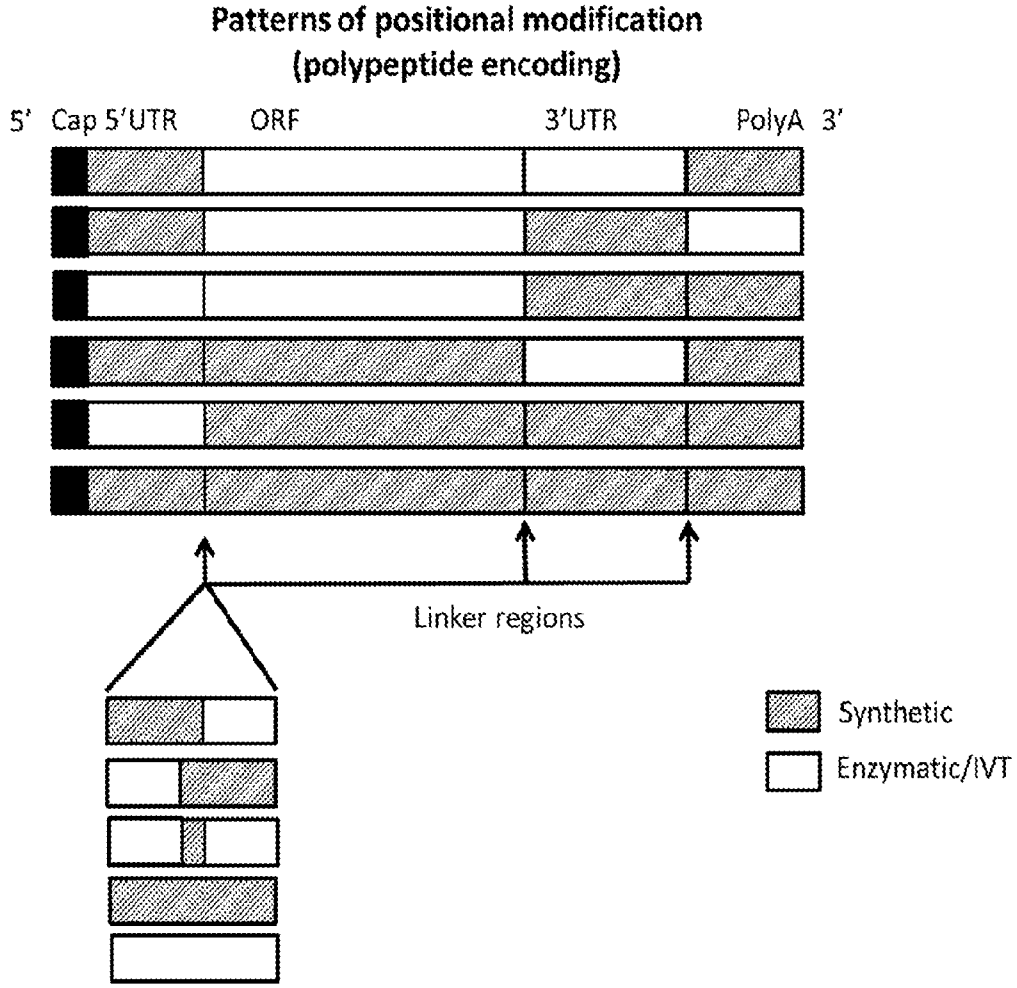

Patterns of positional modification
(polypeptide encoding)
5' Cap 5'UTR
ORF
3'UTR
PolyA 3'
Linker regions
Synthetic
Enzymatic/IVT

FIG. 4

Patterns of positional modification

A (L) B (L) C (L)
5'[NNNNN$_n$]$_x$ L1 [NNNNNNNNNNNN$_o$]$_y$ L2 [XXXXXXXXXXXX$_p$]$_z$ L3 3'
5'[NNNNN$_n$]$_x$ L1 [XXXXXXXXXXXXXXX$_o$]$_y$ L2 [XXXXXXXXXXXX$_p$]$_z$ L3 3'
5'[XXXXXX$_n$]$_x$ L1 [XXXXXXXXXXXXXXX$_o$]$_y$ L2 [XXXXXXXXXXXX$_p$]$_z$ L3 3'
5'[NNNNN$_n$]$_x$ L1 [NNNNNNNNNNNN$_o$]$_y$ L2 [XXXXXXXXXXXX$_p$]$_z$ L3 3'

A, B, C- Region or Part of chimeric polynucleotide
N-nucleoside
n, o, p-number of nucleosides
x, y, z-number of regions
X-selective placement nucleoside
L-linker (optional)

FIG. 5

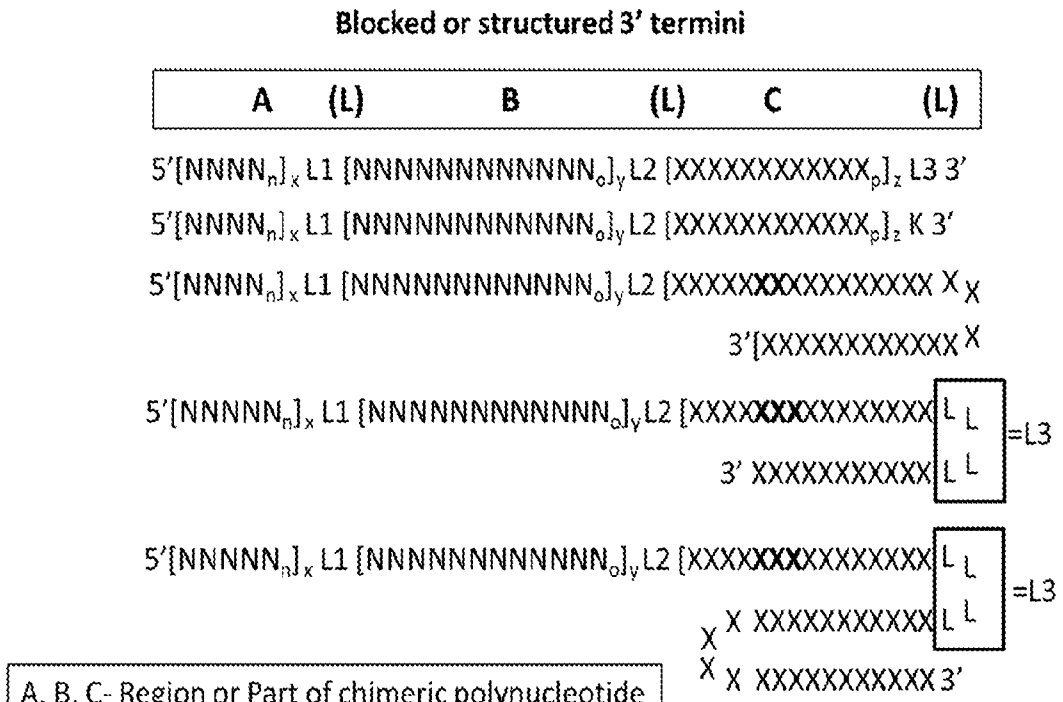

Blocked or structured 3' termini
A (L) B (L) C (L)
5'[NNNN$_n$]$_x$ L1 [NNNNNNNNNNNN$_o$]$_y$ L2 [XXXXXXXXXXXX$_p$]$_z$ L3 3'
5'[NNNN$_n$]$_x$ L1 [NNNNNNNNNNNN$_o$]$_y$ L2 [XXXXXXXXXXXX$_p$]$_z$ K 3'
5'[NNNN$_n$]$_x$ L1 [NNNNNNNNNNNN$_o$]$_y$ L2 [XXXXXXXXXXXXXXX X X
3'[XXXXXXXXXXXX X
5'[NNNN$_n$]$_x$ L1 [NNNNNNNNNNNN$_o$]$_y$ L2 [XXXXXXXXXXXXXXX L L =L3
3' XXXXXXXXXXX L L
5'[NNNN$_n$]$_x$ L1 [NNNNNNNNNNNN$_o$]$_y$ L2 [XXXXXXXXXXXXXXX L L =L3
X X XXXXXXXXXX L L
X
X X XXXXXXXXXX 3'
A, B, C- Region or Part of chimeric polynucleotide
N-nucleoside
n, o, p-number of nucleosides
x, y, z-number of regions
X-selective placement nucleoside
K-non-nucleosidic moiety or conjugate
L-linker (optional)

FIG. 6A

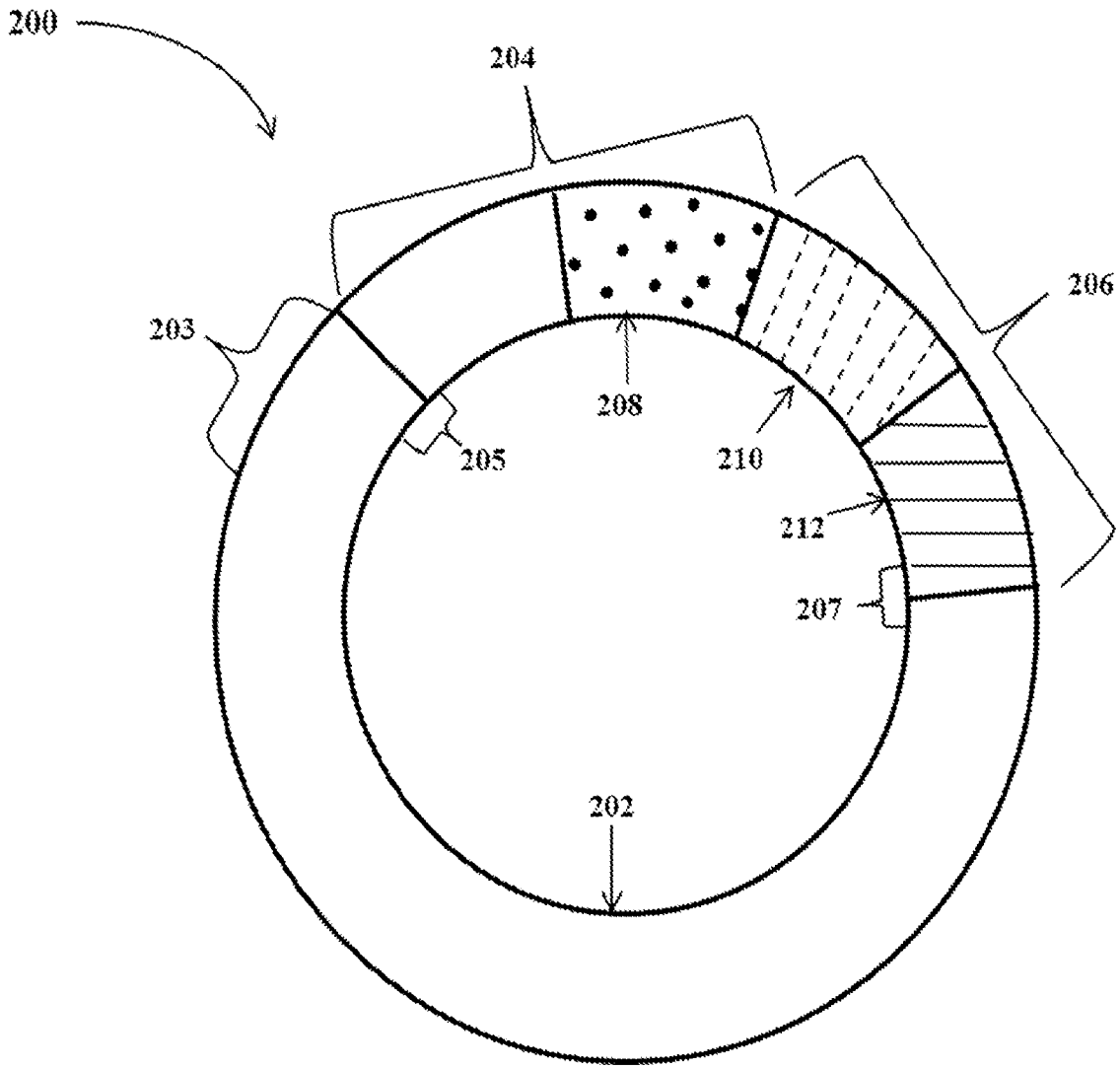


FIG. 6B

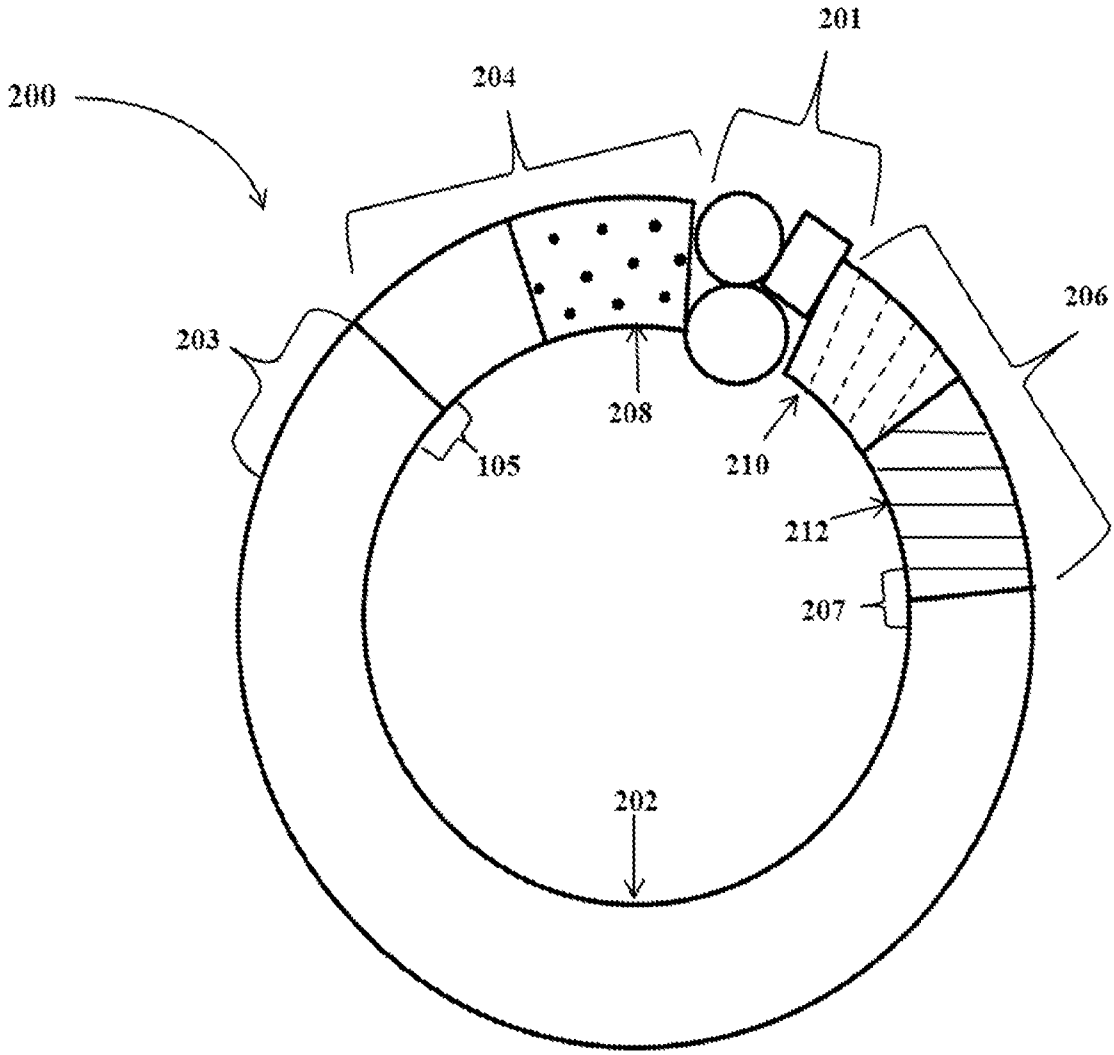


FIG. 6C

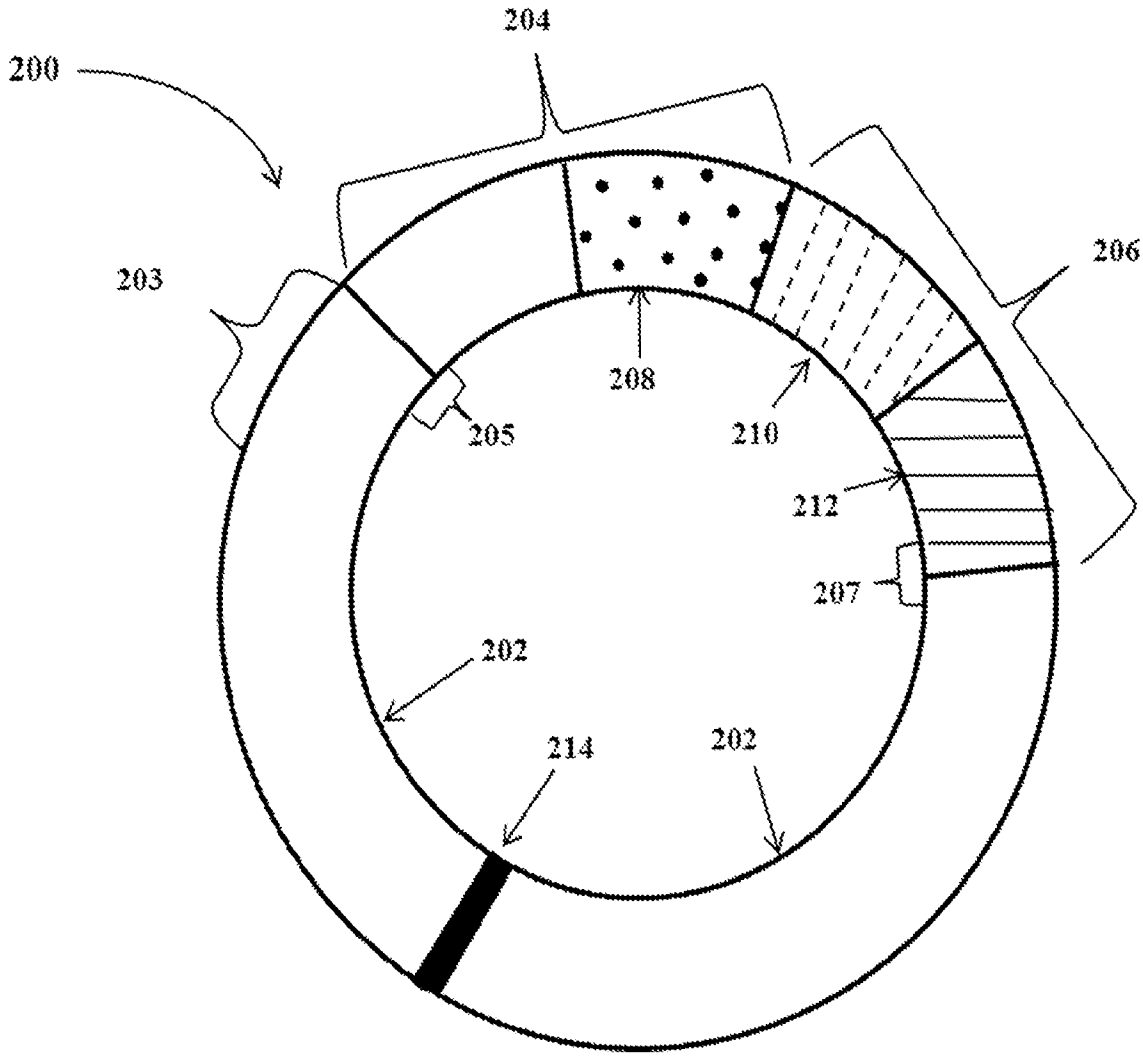


FIG. 6D

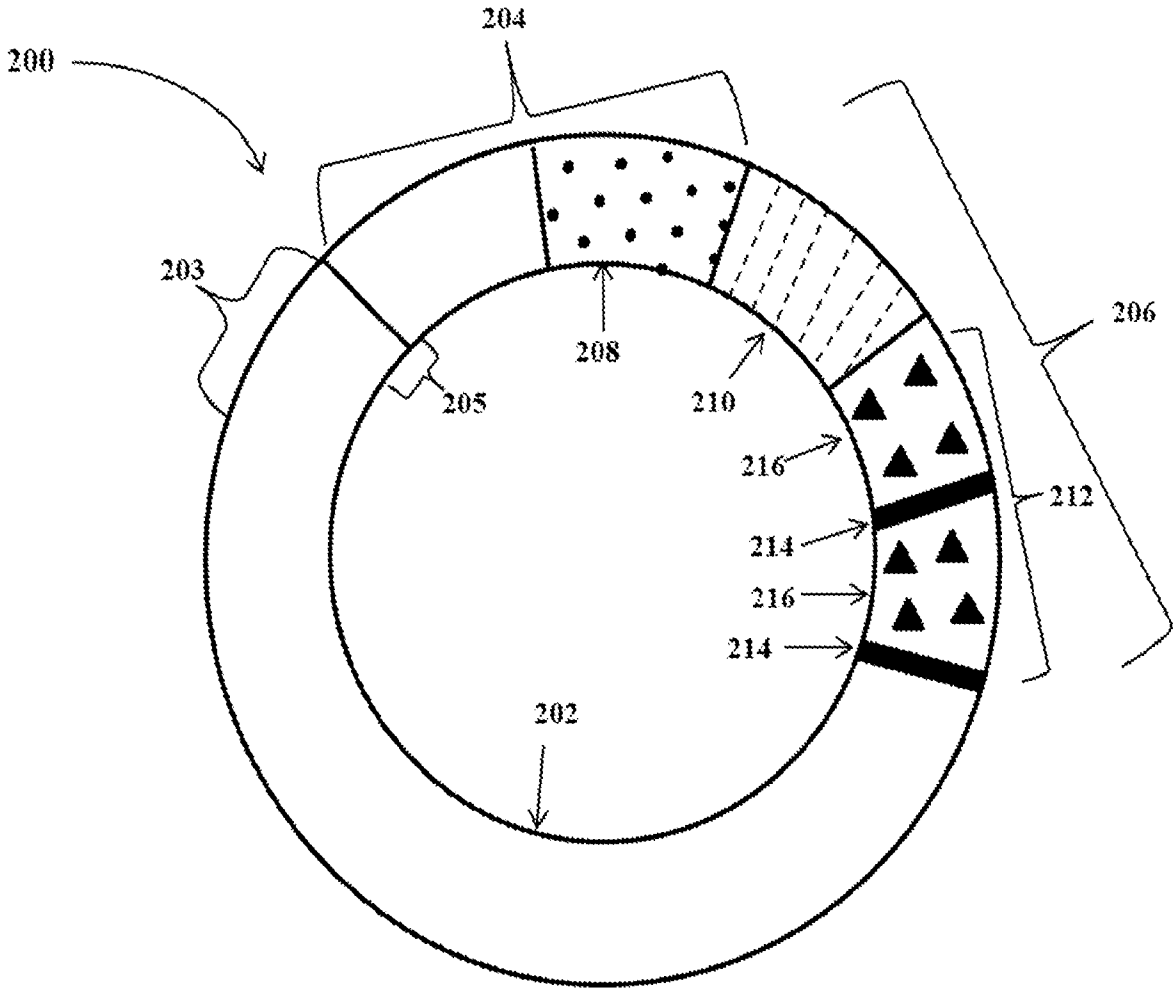


FIG. 6E

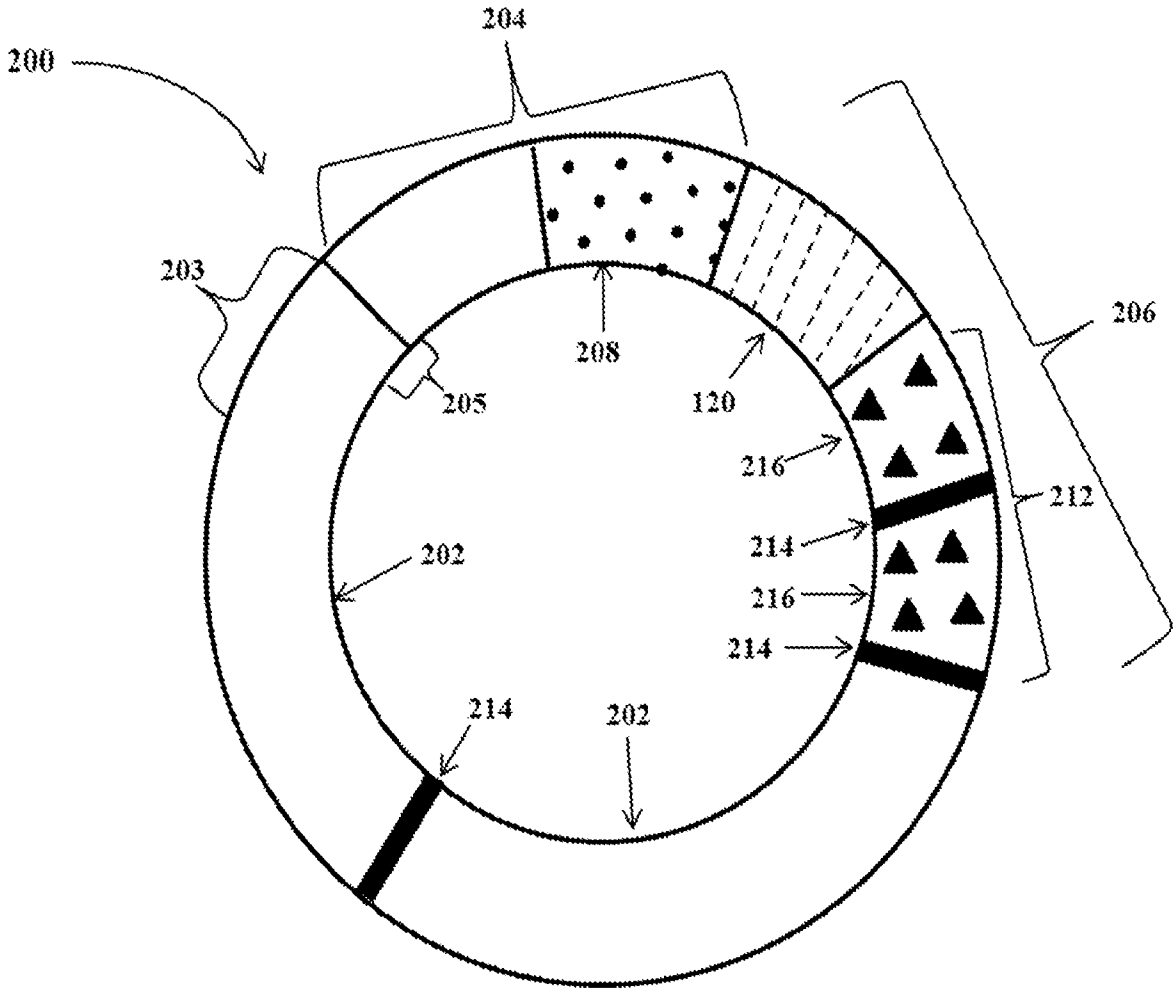


FIG. 6F

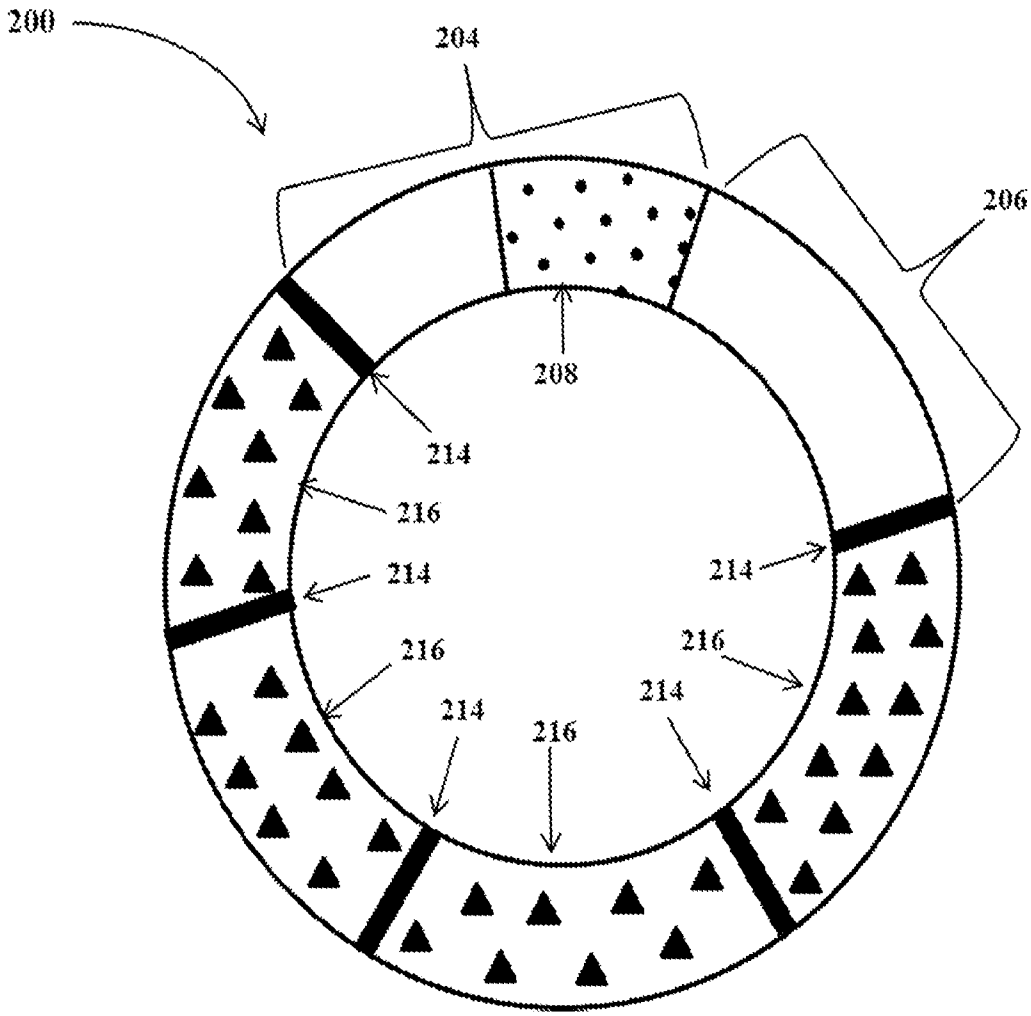


FIG. 6G

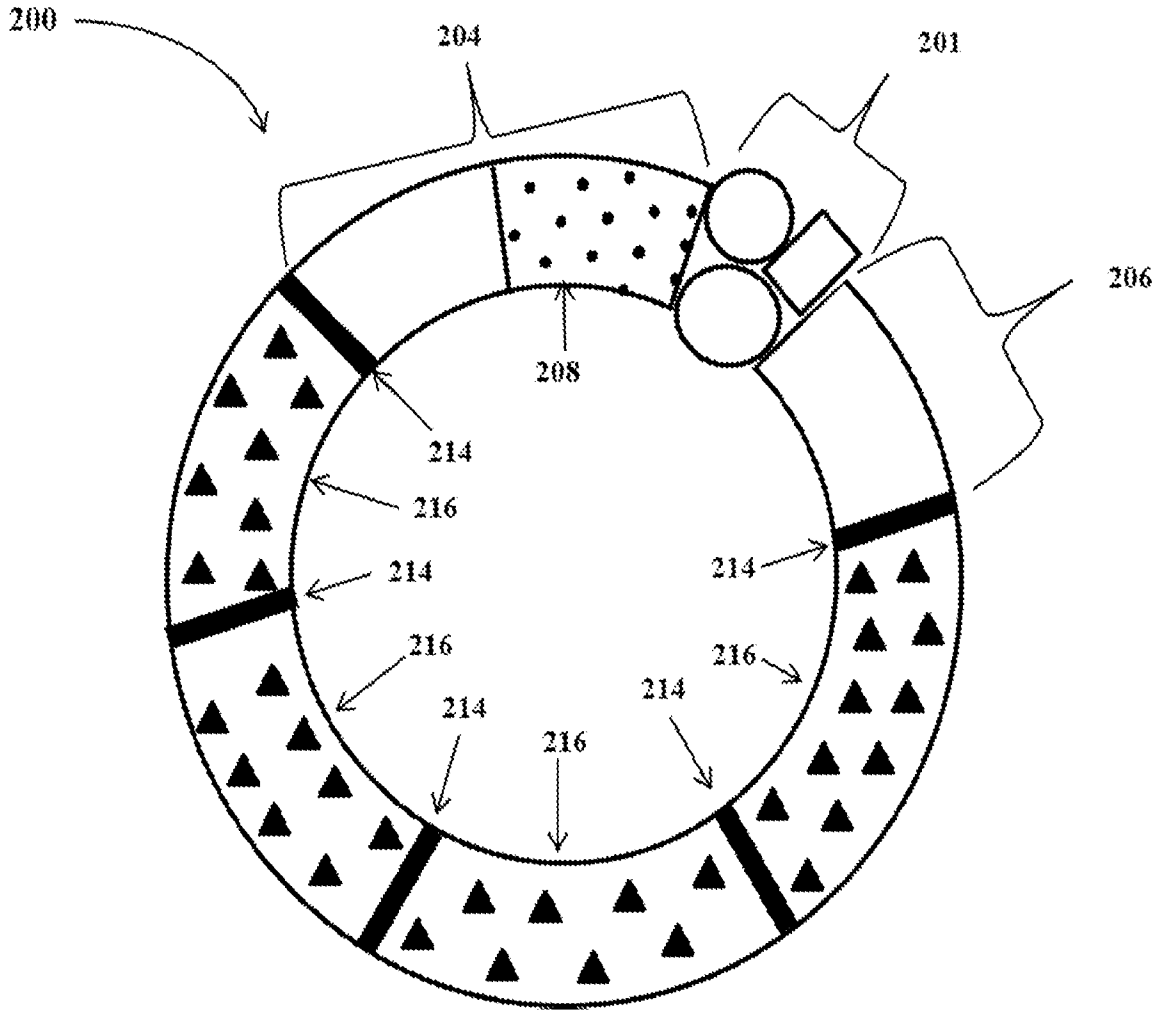
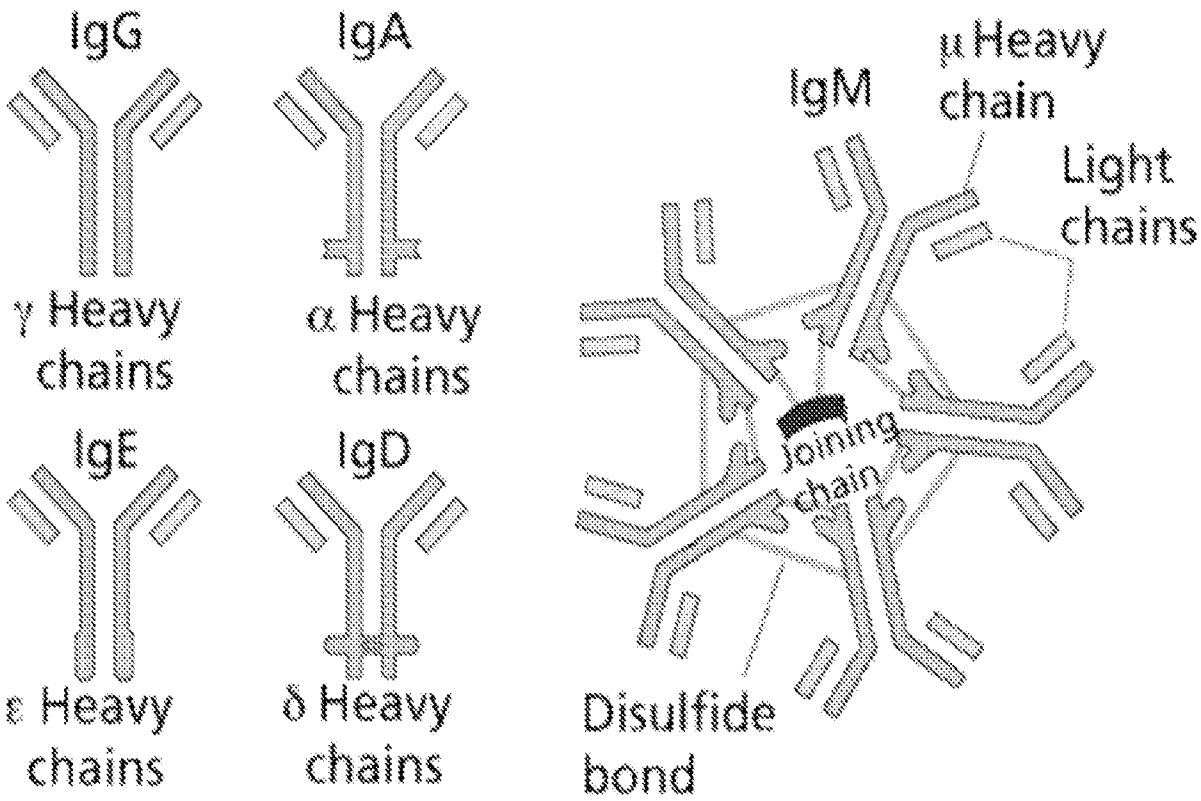


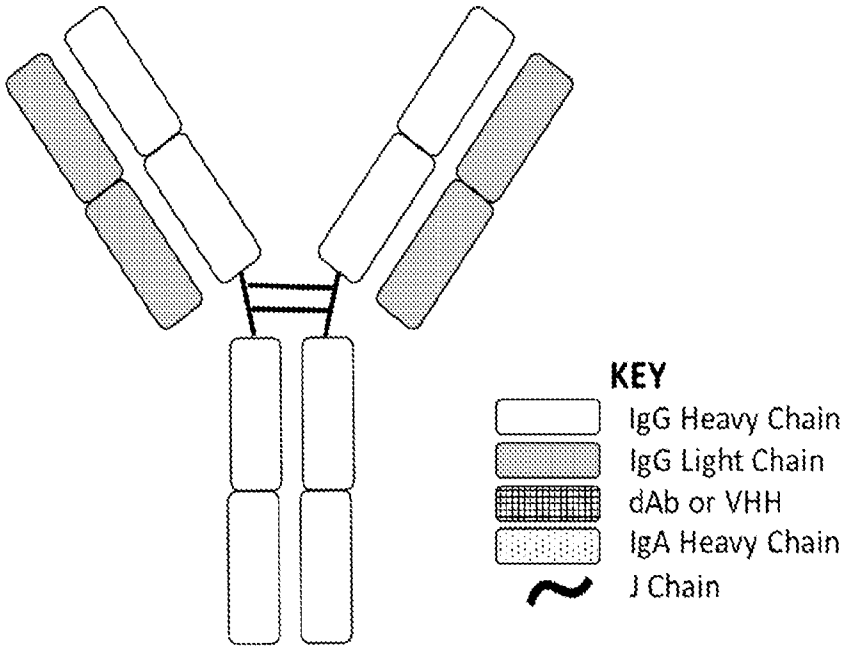
FIG. 7



The Five Classes of Immunoglobulins

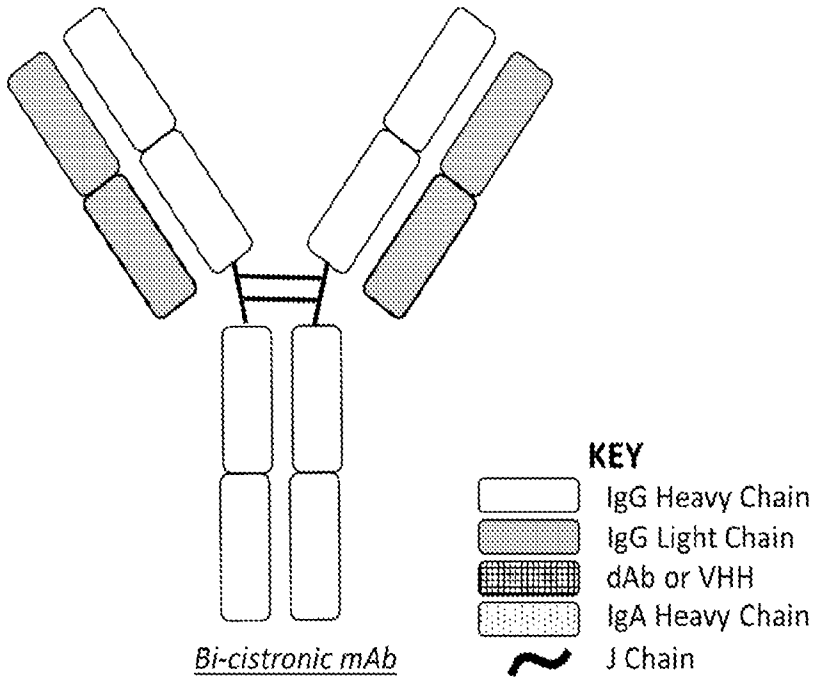
PRIOR ART

FIG. 8A



Human IgG

FIG. 8B



Bi-cistronic mAb

FIG. 9

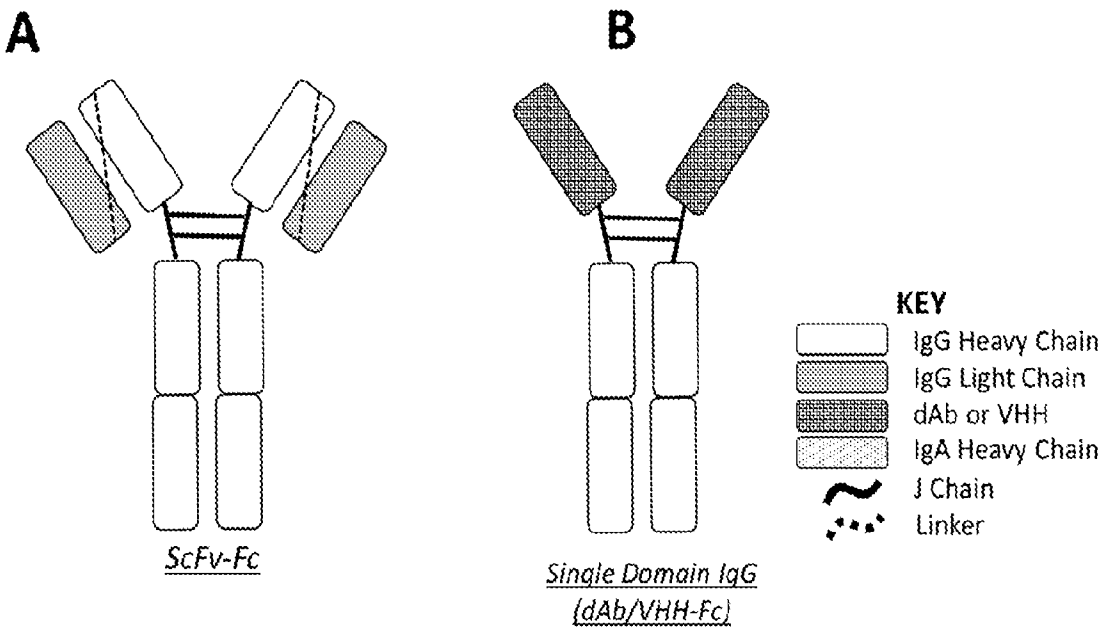

A
B
ScFv-Fc
Single Domain IgG
(dAb/VHH-Fc)
KEY
IgG Heavy Chain
IgG Light Chain
dAb or VHH
IgA Heavy Chain
J Chain
Linker

FIG. 10

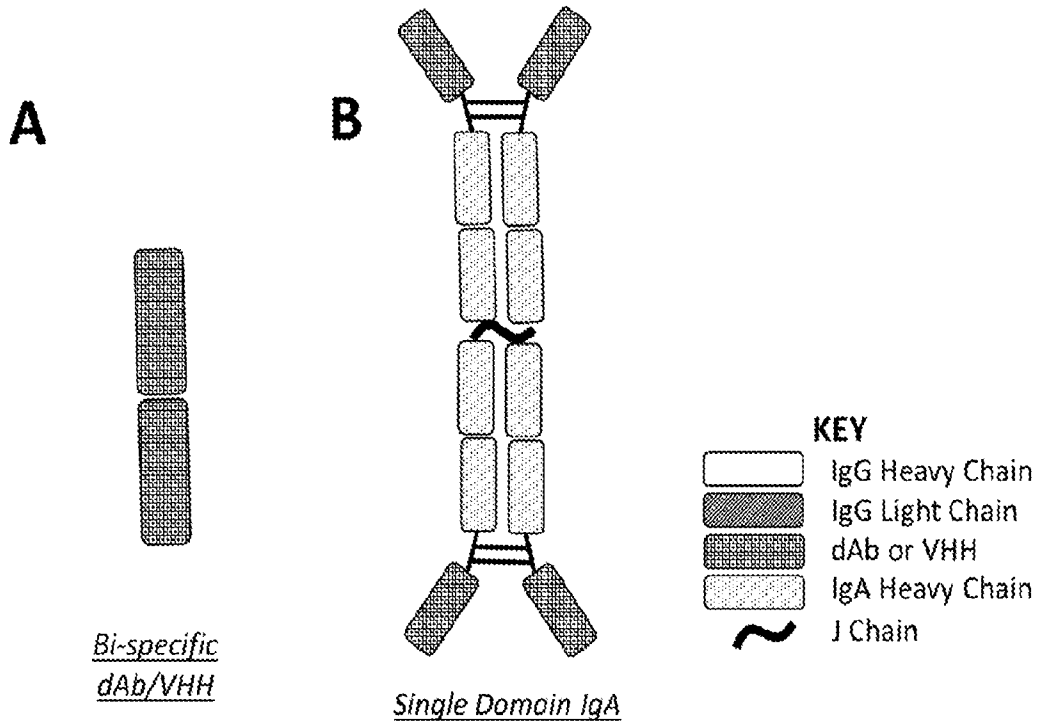

A
B
Bi-specific dAb/VHH
Single Domain IgA
KEY
IgG Heavy Chain
IgG Light Chain
dAb or VHH
IgA Heavy Chain
J Chain

FIG. 11A

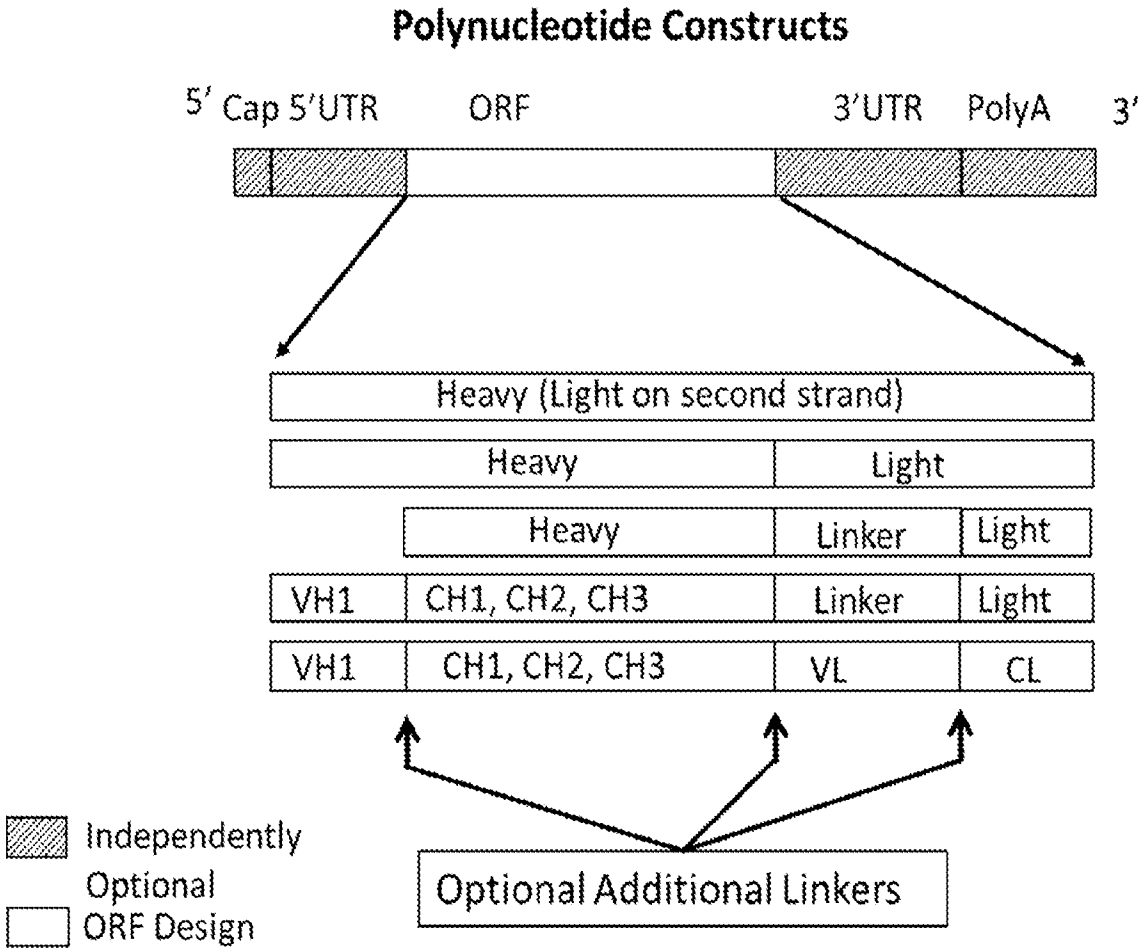
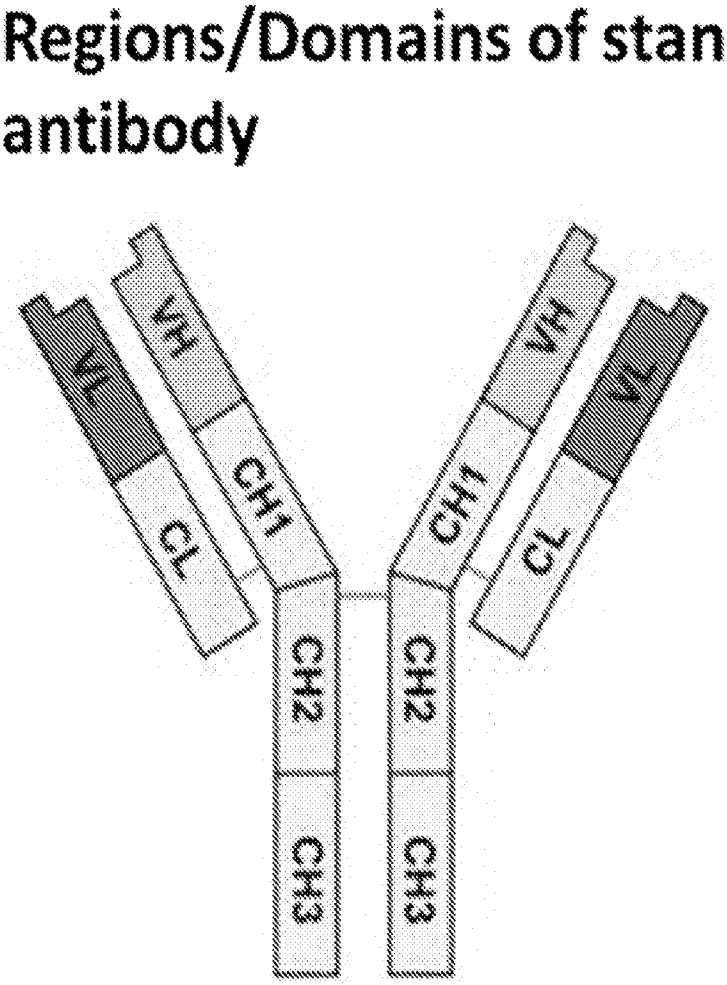

Polynucleotide Constructs
5′ Cap 5′UTR
ORF
3′UTR
PolyA
3′
Heavy (Light on second strand)
Heavy
Light
Heavy
Linker
Light
VH1
CH1, CH2, CH3
Linker
Light
VH1
CH1, CH2, CH3
VL
CL
Optional Additional Linkers
Independently Optional ORF Design

FIG. 11B


Regions/Domains of standard IgG antibody
VL
VH
CL
CH1
CH2
CH3
VH
VL
CH1
CL
CH2
CH3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/44201

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 19/34 (2015.01)
CPC - C12N 15/10; C12Q 1/686, 1/6869
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12P 19/34 (2015.01)
CPC: C12N 15/10; C12Q 1/686, 1/6869; 6869; USPC: 435/91.1, 89, 85, 84, 72, 41

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatSeer Google; Google Scholar; Dialog ProQuest; Entrez PubMed; Science Direct; polynucleotide, polypeptide, 'ophthalmic condition,' 'modified nucleotides,' liposomes, 'VEGF,' retinopathy, neovascualrization, glaucoma, cataract, complement, retinopathy

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2006/0193830 A1 (HAUSWIRTH, WW et al.) August 31, 2006; abstract; paragraphs [0004], [0010], [0013], [0023], [0027], [0028], [0033], [0039], [0053], [0055], [0057], [0061], [0064], [0072], [0076], [0090], [0094]	1, 2, 11, 20, 27, 28, 33, 34, 38 --- 3, 4, 9, 12-19, 21-26, 29-32, 35-37, 39, 40
Y	US 2014/0200261 A1 (HOGE, SG et al.) July 17, 2014; paragraphs [0010], [0013], [0015], [0018], 0019], [0028], [0038], [0069], [0229], [0267], [0296], [0394], [0445], [0458], [0976]	3, 4, 9, 21, 35-37, 39, 40
Y	US 2013/0131142 A1 (LIBERTINE, L et al.) May 23, 2013; abstract; paragraphs [0002], [0009]-[0011], [0554], [0630], [0832]	12, 16-18
Y	US 2007/0178068 A1 (REICH, SJ et al.) August 02, 2007; paragraphs [0010], [0028], [0035]	13, 24
Y	US 2013/0337557 A1 (COMBETTE, J-M et al.) December 19, 2013; paragraphs [0001], [0147], [0184], [0185]	14
Y	US 2014/0178309 A1 (AMBATI, J et al.) June 26, 2014; paragraphs [0009], [0105], [0172]	15
Y	WO 2003/011317 A1 (LORANTIS LIMITED) February 13, 2003; abstract; page 1, lines 4-5; page 10, lines 13-15	19
Y	US 2014/0056911 A1 (GENZYME CORPORATION et al.) February 27, 2014; paragraphs [0006], [0033]	22, 23, 25

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 2015 (16.11.2015)	21 DEC 2015
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/44201

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011/0160126 A1 (McDONALD, JR et al.) June 30, 2011; paragraphs [0049], [0053], [0108], [0113], [0227], [0406], [0441]	26
Y	US 2013/0143814 A1 (ROSA, R et al.) June 06, 2013; paragraphs [0124], [0126], [0166], [0169]	29, 32
Y	US 2006/0217332 A1 (VARGEESE, C et al.) September 28, 2006; paragraphs [0013], [0215], [0340], [0346], [0651]	30
Y	US 2010/0291097 A1 (PFEIFER, A et al.) November 18, 2010; paragraphs [0033], [0037], [0162], [0332]	31

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/44201

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/44201

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-8, 10, 41
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.