The invention relates to compositions and methods for the preparation, manufacture and therapeutic use of modified polynucleotides comprising at least one intrabody construct.
RELATED APPLICATIONS


FIELD OF THE INVENTION

[0002] The invention relates to compositions, methods, processes, kits and devices for the design, preparation, manufacture and/or formulation of polynucleotides comprising intrabody constructs.

BACKGROUND OF THE INVENTION

[0003] Antibodies, also known as immunoglobulins, are glycoproteins produced by B cells. Using a unique and highly evolved system of recognition, antibodies can recognize a target and tag a target epitope, foreign entity or invading microbe for attack by the immune system thereby neutralizing its effect. The production of antibodies is the main function of the humoral immune system. Antibodies are secreted by a plasma cell which is a type of white blood cell.

[0004] Currently, the majority of antibodies are generated using recombinant or cloning strategies and product heterogeneity is common to monoclonal antibody and other recombinant biological production. Such heterogeneity is typically introduced either upstream during expression or downstream during manufacturing. Recombinant antibody engineering involves the use of viruses or yeast to create antibodies, rather than mice which are used in cloning strategies. All of these however, suffer from drawbacks associated with the systems used for generation including degree of purity, speed of development, cross reactivity, low affinity and variable specificity.

[0005] Production of antibodies in vivo whether via direct translation of an encoding polynucleotide that elicits antibody production by the body can address most, if not all, of the problems associated with traditional antibody technology. In vivo production within the body, allowing the expression of the antibody within the target cell, also allows for the production of antibodies that are not secreted but instead bind and regulate an
intracellular target, within the cell they are expressed. These so-called "intrabodies" open up the range of potential therapeutic targets to include intracellular proteins, including nuclear proteins, such as transcription factors, which are often dysregulated in cancer and other proliferative diseases.

[0006] To this end, the present invention provides polynucleotide compositions comprising at least one intrabody construct for the treatment of diseases and disorders.

SUMMARY OF THE INVENTION

[0007] Described herein are compositions, methods, processes, kits and devices for the design, preparation, manufacture and/or formulation of polynucleotides comprising intrabody constructs.

[0008] In one aspect, the invention features a polynucleotide comprising:

(a) a first region of linked nucleosides, the first region encoding a polypeptide of interest;

(b) a first flanking region located 5' relative to the first region comprising at least one 5'terminal cap;

(c) a second flanking region located 3' relative to the first region comprising a 3' tailing sequence of linked nucleosides,

wherein the polypeptide of interest comprises a polypeptide which is expressed intracellularly and comprises a domain which binds to an intracellular target,

and wherein the polynucleotide comprises at least one chemically modified nucleoside.

In one embodiment, the polypeptide is an antibody or antigen-binding fragment thereof. In one embodiment, the polypeptide is a single domain antibody. In one embodiment, the polypeptide comprises a non-antibody scaffold protein which binds to an intracellular target. In one embodiment, the polypeptide is a fusion protein comprising an intracellular polypeptide and a fibronectin domain. In another embodiment, the polypeptide is a fusion protein comprising an intracellular polypeptide and a Kunitz domain. In another embodiment, the polypeptide is a fusion protein comprising an intracellular polypeptide and a Stefin A mutant scaffold. In another embodiment, the
polypeptide is a fusion protein comprising an intracellular polypeptide and a transferrin domain.

In one embodiment, the polypeptide of interest prevents or disrupts a protein-protein interaction between the intracellular target and at least one other protein. In one embodiment, the intracellular target is MYC and the at least one other protein is MAX. In another embodiment, the intracellular target is STAT3 and the at least one other protein is STAT3.

In one embodiment, the polypeptide of interest prevents recruitment of the intracellular target to a regulatory element. In one embodiment, the intracellular target is MYC and/or MAX. In another embodiment, the intracellular target is STAT3.

In certain embodiments, the polynucleotide comprises a microRNA (miRNA) binding site. In one embodiment, the microRNA binding site regulates mRNA and protein expression. In various embodiments, the microRNA binding site is selected miR-122, miR-133, miR-206, miR-208, miR-17-92, miR-126, miR-142-3p, miR-142-5p, miR-16, miR-21, miR-223, miR-24, miR-27, let-7, miR-30c, miR-ld, miR-149, miR-192, miR-194, miR-204, let-7, miR-133, miR-126 and miR-132.

In another aspect, the invention features a lipid nanoparticle comprising a polynucleotide as described herein. The lipid nanoparticle can further comprise a targeting moiety conjugated to the surface of the lipid nanoparticle.

In another aspect, the invention features a composition comprising a lipid nanoparticle as described herein or the polynucleotide as described herein, and a pharmaceutically acceptable excipient.

In another aspect, the invention features a method of treating a disease in a subject comprising administering to a subject a lipid nanoparticle as described herein or a polynucleotide as described herein.

In another aspect, the invention features a polynucleotide comprising

(a) a first region of linked nucleosides, the first region encoding a polypeptide of interest;

(b) a first flanking region located 5' relative to the first region comprising at least one 5' terminal cap;
(c) a second flanking region located 3’ relative to the first region comprising a 3’ tailing sequence of linked nucleosides,

wherein the polynucleotide comprises at least one chemically modified nucleoside,

wherein the polypeptide of interest comprises a polypeptide which is expressed intracellularly and comprises a domain which binds to an intracellular target, and

wherein the polypeptide reaches a maximum intracellular concentration within 2 to 8 hours after the polynucleotide is introduced into a mammalian cell.

In one embodiment, the polypeptide reaches a maximum intracellular concentration within 4 to 6 hours after the polynucleotide is introduced into a mammalian cell. In one embodiment, the polypeptide is an antibody or antigen-binding fragment thereof. In one embodiment, the polypeptide is a single domain antibody. In one embodiment, the polypeptide comprises a non-antibody scaffold protein which binds to an intracellular target.

In another aspect, the invention features a polynucleotide comprising

(a) a first region of linked nucleosides, the first region encoding a single domain antibody;

(b) a first flanking region located 5’ relative to the first region comprising at least one 5’ terminal cap;

(c) a second flanking region located 3’ relative to the first region comprising a 3’ tailing sequence of linked nucleosides,

wherein the polynucleotide comprises at least one chemically modified nucleoside,

wherein the single domain antibody is expressed intracellularly and binds to an intracellular target, and

wherein the single domain antibody reaches a maximum intracellular concentration at about 6 hours after the polynucleotide is introduced into a mammalian cell.

In one embodiment, the single domain antibody is detectable within about 1 hour after the polynucleotide is introduced into a mammalian cell. In one embodiment, the single domain antibody is no longer detectable about 24 hours after the polynucleotide is
introduced into a mammalian cell. In one embodiment, the single domain antibody has a half-life of about 4-6 hours.

In another aspect, the invention features a method of reaching a maximum intracellular concentration of a polypeptide of interest in a minimum time following administration of a polynucleotide encoding the polypeptide of interest to a subject, comprising administering to the subject a polynucleotide comprising

(a) a first region of linked nucleosides, the first region encoding a polypeptide of interest;

(b) a first flanking region located 5' relative to the first region comprising at least one 5' terminal cap;

(c) a second flanking region located 3' relative to the first region comprising a 3' tailing sequence of linked nucleosides,

wherein the polynucleotide comprises at least one chemically modified nucleoside,

wherein the polypeptide of interest comprises a polypeptide which is expressed intracellularly and comprises a domain which binds to an intracellular target, and

wherein the polypeptide reaches a maximum intracellular concentration within 2 to 8 hours after the polynucleotide is administered to the subject.

In one embodiment, the polypeptide reaches a maximum intracellular concentration within 4 to 6 hours after the polynucleotide is introduced into the subject.

In another aspect, the invention features a method of reaching a maximum intracellular concentration of a single domain antibody in a minimum time following administration of a polynucleotide encoding the single domain antibody to a subject comprising administering to the subject a polynucleotide comprising

(a) a first region of linked nucleosides, the first region encoding a single domain antibody;
(b) a first flanking region located 5' relative to the first region comprising at least one 5' terminal cap;
(c) a second flanking region located 3' relative to the first region comprising a 3' tailing sequence of linked nucleosides, wherein the polynucleotide is administered intravenously encapsulated in an LNP and comprises at least one chemically modified nucleoside, wherein the single domain antibody is expressed intracellularly and binds to an intracellular target, and wherein the single domain polypeptide reaches a maximum liver accumulation at about 6 hours after the polynucleotide is administered to the subject.

In one embodiment, the single domain antibody is detectable within about 1 hour after the polynucleotide is administered to the subject. In one embodiment, the single domain antibody is no longer detectable about 24 hours after the polynucleotide is administered to the subject. In one embodiment, the single domain antibody has a half-life of about 4-6 hours.

In another aspect, the invention features a method for transiently expressing an intracellular polypeptide of interest comprising administering to the subject a polynucleotide comprising
(a) a first region of linked nucleosides, the first region encoding a polypeptide of interest;
(b) a first flanking region located 5' relative to the first region comprising at least one 5' terminal cap;
(c) a second flanking region located 3' relative to the first region comprising a 3' tailing sequence of linked nucleosides, wherein the polynucleotide comprises at least one chemically modified nucleoside, wherein the polypeptide of interest comprises a polypeptide which is expressed intracellularly and comprises a domain which binds to an intracellular target, and
wherein the polypeptide of interest is detectable 1-3 hours after the polynucleotide is administered to the subject and is no longer detectable 24-48 hours after the polynucleotide is administered to the subject.

In one embodiment, the polypeptide of interest has a half-life of 4-6 hours.

In another aspect, the invention features a method for providing a single domain antibody which is expressed intracellularly to a subject, comprising

administering to the subject intravenously a first dose of a polynucleotide encapsulated in an LNP,

wherein the polynucleotide comprises

(a) a first region of linked nucleosides, the first region encoding a single domain antibody;

(b) a first flanking region located 5' relative to the first region comprising at least one 5' terminal cap;

(c) a second flanking region located 3' relative to the first region comprising a 3' tailing sequence of linked nucleosides,

wherein the polynucleotide comprises at least one chemically modified nucleoside, and

wherein the single domain antibody is expressed intracellularly and binds to an intracellular target; and

administering to the subject intravenously a second dose of the polynucleotide encapsulated in an LNP at 12-15 hours following administration of the first dose.

In one embodiment, the single domain antibody has a half-life of about 4-6 hours.

[0009] 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

[0010] 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.

[00011] FIG. 1 is a schematic comparing (A) the human IgG antibody structure to (B) a bicistronic antibody of the invention.

[00012] FIG. 2 is a schematic of two types of antibodies of the present invention. (A) an scFv antibody and (B) a single domain IgG antibody.

[00013] FIG. 3 is a schematic of two types of antibodies of the present invention. (A) a bispecific antibody and (B) a single domain IgA antibody.

[00014] FIG. 4 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.

[00015] FIG. 5 is a graph showing intracellular expression of an anti-HBV Core Antigen (HBcAg) single domain antibody (VHH) in liver cells in vivo, as determined by LC-MS.

[00016] FIG. 6 is a graph showing the intracellular expression of an anti-HBV Core Antigen (HBcAg) single domain antibody (VHH) in vitro for half-life determination.

[00017] FIG 7. is a graph showing intracellular expression of an anti-HBV Core Antigen (HBcAg) single domain antibody (VHH) in liver cells in vivo after two successive doses of lipid nanoparticles carrying a modified mRNA (mmRNA) encoding the construct.

[00018] FIG 8. is a graph showing intracellular expression of an anti-HBV Core Antigen (HBcAg) single domain antibody (VHH) in liver cells in vivo within the first 20 hours post-injection of lipid nanoparticles carrying an mmRNA encoding the construct.

DETAILED DESCRIPTION

[00019] 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

8
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.

[00020] Described herein are compositions (including pharmaceutical compositions) and methods for the design, preparation, manufacture and/or formulation of antibodies, antibody fragments, and antibody variants including non-antibody derived scaffold proteins or domains which function as antibody mimetics where at least one component of the antibody, antibody fragment or antibody variant is encoded by a polynucleotide. As such the present invention is directed, in part, to polynucleotides, specifically IVT polynucleotides, chimeric polynucleotides and/or circular polynucleotides encoding one or more antibodies and/or components thereof.

[00021] Also provided are systems, processes, devices and kits for the selection, design and/or utilization of the antibodies described herein.

[00022] According to the present invention, the polynucleotides are preferably modified in a manner as to avoid the deficiencies of or provide improvements over other antibody molecules of the art.

[00023] Provided herein, therefore, are antibodies, antibody fragments and antibody variants including non-antibody derived scaffold proteins or domains which function as antibody mimetics encoded by polynucleotide(s) and compositions comprising at least one polynucleotide which have been designed to produce a therapeutic outcome and optionally 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, protein production capacity, secretion efficiency (when applicable), accessibility to circulation, protein half-life and/or modulation of a cell's status, antibody target affinity and/or specificity, reduction of antibody cross reactivity, increase of antibody purity, increase or alteration of antibody effector function and/or antibody activity.

[00024] The methods of the present invention are and can be utilized to engineer novel polynucleotides for the in vivo production of antibodies, antibody fragments and antibody variants in such a manner as to provide improvements over standard antibody technology.
In some embodiments, the polynucleotides are designed to produce one or more antibodies, or combinations of antibodies selected from the group consisting of IgA, IgG, IgM, IgE, and IgD.

The resultant antibodies expressed in a cell, tissue or organism from the polynucleotides of the present invention may have the following properties which mirror those of the natural isotype. They may also exhibit improved properties over the native or natural isotype.

Table 1. Features of Antibody Isotypes

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Presence</th>
<th>Function</th>
<th>Complement Fixation</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>Appears as major Ig in serum; may appear greater than 75%</td>
<td>Binding to macrophages, monocytes; acts as opsonin; Cross placental transport</td>
<td>Fixation of complement</td>
<td>Exists as monomer of two heavy and two light chains</td>
</tr>
<tr>
<td>IgA</td>
<td>Second most common Ig in serum</td>
<td>Binds PMNs and some lymphocytes; major class in secretions such as tears, saliva, colostrum, mucus</td>
<td>Does not fix complement unless aggregated</td>
<td>Serum form is monomer; secreted form is dimer with J chain; secretory piece made in epithelial cells</td>
</tr>
<tr>
<td>IgM</td>
<td>Third most common Ig in serum</td>
<td>Made by fetus and virgin B cells; B cell surface Ig lacks J chain</td>
<td>Fixation of complement; destruction of microorganisms by agglutination and/or clumping</td>
<td>Exist as pentamer or monomer; all heavy chains identical; all light chains identical; valence of 10Extra domain on CH4 chain; additional protein bound via disulfide known as J chain for polymerization; cells via Fc receptors</td>
</tr>
<tr>
<td>IgE</td>
<td>Least abundant in serum</td>
<td>Involved in allergic reactions and release of mediators of allergic symptoms; role in parasitic helminth disease</td>
<td>Does not fix complement</td>
<td>Exists as monomer with extra domain in constant region; binds to Fc receptors on basophils and mast cells before antigen interaction</td>
</tr>
<tr>
<td>IgD</td>
<td>Low levels in</td>
<td>Found on B cell</td>
<td>Does not bind</td>
<td>Only exists as</td>
</tr>
</tbody>
</table>
I. Compositions of the Invention

Polynucleotides as components of compositions

[00027] The compositions of the present invention comprise polynucleotides which encode antibodies, fragments of antibodies or variants of antibodies including non-antibody derived scaffold proteins or domains which function as antibody mimetics and are collectively referred to as "polynucleotides" or "constructs." Compositions of the invention which comprise at least one polynucleotide are referred to as "compositions."

The polypeptides encoded by the polynucleotides are collectively referred to as polypeptides, whether the polypeptides are variants, fragments or the entire antibodies.

Polynucleotides

[00028] The present invention provides nucleic acid molecules, specifically polynucleotides which, in some embodiments, encode one or more peptides or polypeptides of interest. Such peptides or polypeptides, according to the invention are those derived from the antibodies. 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.

[00029] 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, a-LNA having an a-L-ribo configuration (a diastereomer of LNA), 2'-amino-LNA having a 2'-amino functionalization, and 2'-amino- a-LNA having a 2'-amino functionalization), ethylene...
nucleic acids (ENA), cyclohexenyl nucleic acids (CeNA) or hybrids or combinations thereof.

[00030] In one embodiment, linear polynucleotides encoding one or more constructs of the present invention encoding antibodies, fragments or variants thereof which are made using only in vitro transcription (IVT) enzymatic synthesis methods are referred to as "IVT polynucleotides." Methods of making IVT polynucleotides are known in the art and are described in International Application WO2013/151666, published October 10, 2013, the content of which is incorporated herein by reference in its entirety.

[00031] 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. Such constructs are taught in, for example, International Application WO20 15/034928, published March 12, 2015, the content of which is incorporated herein by reference in its entirety.

[00032] 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. Such constructs are taught in for example International Application WO20 15/034925, published March 12, 2015, the content of which is incorporated herein by reference in its entirety.

[00033] In some embodiments, the polynucleotides include 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).

[00034] In one embodiment, the polynucleotides of the present invention may encode at least one peptide or polypeptide of interest.

[00035] According to the present invention, the polypeptides of interest comprise the antibodies, or fragments or variants thereof.

[00036] In another aspect, the invention features a polynucleotide having a sequence including Formula II:

$$[A_n]-[B_0]$$

**Formula II**

wherein each A and B is independently any nucleoside;

[00037] n and o are, independently 15 to 1000; and

[00038] L has the structure of Formula III:

$$\frac{1}{2} R^1 \frac{1}{2} (R^2) \frac{1}{2} R^3 \frac{1}{2} R^4 \frac{1}{2} (R^5) \frac{1}{2} R^6 \frac{1}{2} (R^7) \frac{1}{2}$$

**Formula III**

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

[00040] each of R1, R3, R5, and R7, is, independently, selected from optionally substituted C1-C6 alkylene, optionally substituted C1-C6 heteroalkylene, O, S, and NR8;

[00041] R2 and R6 are each, independently, selected from carbonyl, thiocarbonyl, sulfonyl, or phosphoryl;


13
heterocyclylene, optionally substituted C_6-C_{12} arylene, optionally substituted C_2-C_{10}polyethylene glycolene, or optionally substituted C_1-C_10 heteroalkylene, or a bond linking (R^1)_c-(R^2)_b-(R^3)_c to (R^5)_d-(R^6)_c-(R^7)_d, wherein if c, d, e, f, g, and h are 0, R^4 is not a bond; and

[00044] R^8 is hydrogen, optionally substituted Ci-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 Ci-C_7 heteroalkyl;

[00045] wherein L^1 is attached to [A_n] and [B_0] 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 of a six membered sugar ring of a nucleoside of [B_0] or at the 5' position of a five-membered sugar ring or 6' position of 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_0]).

[00046] In some embodiments, at least one of [A_n] and [B_0] includes the structure of Formula IV:

![Formula IV](image)

**Formula IV**

[00047] wherein each of N^1 and N^2 is independently a nucleobase;

[00048] 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;
each of g and h is, independently, 0 or 1;

each $X^1$ and $X^4$ is, independently, O, NH, or S; and

each $X^2$ is independently O or S; and

each $X^3$ is OH or SH, or a salt thereof.

In another aspect, the invention features a polynucleotide having a sequence including Formula II:

$$[A_n]^{-I}_{-1} - [B_0]$$

**Formula II**

wherein each A and B is independently any nucleoside;

n and o are, independently 15 to 1000; and

$L^1$ is a bond or has the structure of Formula III:

$$\frac{5}{3} - (R^1)_{a} - (R^2)_{c} - (R^3)_{c} - (R^4)_{c} - (R^5)_{c} - (R^6)_{c} - (R^7)_{c} - (R^8)_{c}$$

**Formula III**

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

each of $R^1$, $R^3$, $R^5$, and $R^7$, is, independently, selected from optionally substituted $CrC_6$ alkylene, optionally substituted $CrC_6$ heteroalkylene, O, S, and $NR^8$;

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

$R^4$ is optionally substituted $C_1$-$C_10$ alkylene, optionally substituted $C_2$-$C_10$ alkenylene, optionally substituted $C_2$-$C_10$ alkynylene, optionally substituted $C_2$-$C_9$ heterocyclene, optionally substituted $C_6$-$C_{12}$ aryne, optionally substituted $C_2$-$C_{10}$ polyethylene glycolene, or optionally substituted $C_1$-$C_{10}$ heteroalkylene, or a bond linking $(R^1)_{a} - (R^2)_{c} - (R^3)_{c}$ to $(R^5)_{c} - (R^6)_{c} - (R^7)_{c}$; and

$R^8$ 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 $C_2$-$C_6$ heterocyclen, optionally substituted $C_6$-$C_{12}$ aryl, or optionally substituted $C_1$-$C_7$ heteroalkyl;

wherein $L^1$ is attached to $[A_n]$ and $[B_0]$ 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 of a six membered sugar ring of a nucleoside of $[B_0]$ 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 \([B_0]\)).

[00063] wherein at least one of \([A_n]\) or \([B_0]\) includes the structure of Formula IV:

![Formula IV](image)

**Formula IV**

[00064] wherein each of \(N^1\) and \(N^2\) is independently a nucleobase;

[00065] 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 Q-Cealkyl, 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;

[00066] each of \(g\) and \(h\) is, independently, 0 or 1;

[00067] each \(X^1\) and \(X^4\) is, independently, O, NH, or S; and

[00068] each \(X^2\) is independently O or S; and

[00069] each \(X^3\) is OH or SH, or a salt thereof;

[00070] wherein at least one of \(X^1, X^2,\) or \(X^4\) is NH or S.

[00071] In some embodiments, \(X^1\) is NH. In other embodiments, \(X^4\) is NH. In certain embodiments, \(X^2\) is S.

[00072] 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.

[00073] 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 \([A_n]\)-\(L^1\)-\([B_0]\).
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 \([B_0]\).

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

\[
\begin{align*}
\text{or} \\
\end{align*}
\]

In certain embodiments, \(L^1\) is attached to \([A_n]\) at the 3' or 4' position of the sugar of one of the nucleosides and to \([B_0]\) at the 5' or 6' position of the sugar of one of the nucleosides.

In another aspect, the invention features a method of producing a composition including a polynucleotide, wherein the polynucleotide includes the structure of Formula V:

This method includes reacting a compound having the structure of Formula VI:
with a compound having the structure of Formula VII:

Formulas VI and VII

[00080] wherein each of N\(^1\) and N\(^2\) is, independently, a nucleobase;

[00081] 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 Q-Cealkyl, optionally substituted Ci-C\(_6\) heteroalkyl, optionally substituted C\(_2\)-C\(_6\) heteroalkynyl, optionally substituted C\(_2\)-C\(_6\) heteroalkenyl, optionally substituted C\(_2\)-C\(_6\) heteroalkenyl, optionally substituted amino, azido, or optionally substituted C\(_6\)-C\(_{10}\) aryl;

[00082] each of g and h is, independently, 0 or 1;

[00083] each X\(^1\) and X\(^4\) is, independently, O, NH, or S; and

[00084] each X\(^3\) is independently OH or SH, or a salt thereof;

[00085] each of R\(^{17}\) and R\(^{19}\) is, independently, a region of linked nucleosides; and

[00086] R\(^{18}\) is a halogen.

[00087] In another aspect, the invention features a method of producing a composition including a polynucleotide encoding a polypeptide, wherein the polynucleotide includes the structure of Formula VIII:
This method includes reacting a compound having the structure of Formula IX:

wherein each of N₁ and N₂ is, independently, a nucleobase;

each of R⁹, R¹⁰, R¹¹, R¹², R¹³, R¹⁴, R¹⁵, and R¹⁶ is, independently, H, halo, hydroxy, thiol, optionally substituted Q-Cealkyl, optionally substituted CrC₆ heteroalkyl, optionally substituted C₂-C₆ heteroalkenyl, optionally substituted C₂-C₆ heteroalkynyl, optionally substituted amino, azido, or optionally substituted C₆-C₆₀ aryl;

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

each X⁴ is, independently, O, NH, or S; and

each X² is independently O or S;
[00095] each $X^3$ is independently OH, SH, or a salt thereof;
[00096] each of $R^{20}$ and $R^{23}$ is, independently, a region of linked nucleosides; and
[00097] each of $R^{21}$ and $R^{22}$ is, independently, optionally substituted $C_1-C_6$ alkoxy.
[00098] In another aspect, the invention features a method of producing a composition including a polynucleotide encoding a polypeptide, wherein the polynucleotide includes the structure of Formula XI:

\[
\begin{align*}
\text{Formula XI}
\end{align*}
\]

[00099] This method includes reacting a compound having the structure of Formula XII:

\[
\begin{align*}
\text{Formula XII}
\end{align*}
\]

with a compound having the structure of Formula XIII:
wherein each of $N_1$ and $N_2$ is, independently, a nucleobase;

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 Q-Cealkyl, optionally substituted $\text{CrC}_6$ heteroalkyl, optionally substituted $\text{C}_2\text{-C}_6$ heteroalkenyl, optionally substituted $\text{C}_2\text{-C}_6$ heteroalkynyl, optionally substituted amino, azido, or optionally substituted $\text{C}_6\text{-C}_{10}$ aryl;

each of $g$ and $h$ is, independently, 0 or 1;

each $X^4$ is, independently, O, NH, or S; and

each $X^2$ is independently O or S;

each $X^3$ is independently OH, SH, or a salt thereof;

each of $R_{24}$ and $R_{26}$ is, independently, a region of linked nucleosides; and

$R^{25}$ is optionally substituted $\text{CrC}_6$ alkyne or optionally substituted $\text{CrC}_6$ heteroalkylene or $R^{25}$ and the alkynyl group together form optionally substituted cycloalkynyl.

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

$$[A_n]^{-1} [B_0].$$

**Formula II**

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

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

**Formula XIV**

with a compound having the structure of Formula XV:

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

**Formula XV**

wherein each A and B is independently any nucleoside;

n and o are, independently 15 to 1000; and

L has the structure of Formula III:

$$2^{-} (R^1)_a^{-}(R^2)_b^{-}(R^3)_c-R^{4^{-}}(R^5)_d^{-}(R^6)_e^{-}(RV^5).$$

**Formula III**
wherein a, b, c, d, e, and f are each, independently, 0 or 1;

wherein each A and B is independently any nucleoside;

n and o are, independently 15 to 1000;

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

$R^2$ and $R^6$ are each, independently, selected from carbonyl, thiocarbonyl, sulphonyl, or phosphoryl;

$R^4$ is an optionally substituted triazolene; and

$R^8$ 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 $C_7$-$C_6$ heterocycl, optionally substituted $C_6$-$C_{12}$ aryl, or optionally substituted $C_1$-$C_7$ heteroalkyl; and

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

wherein $L^1$ is attached to $[A_n]$ and $[B_0]$ at the sugar of one of the nucleosides.

In some embodiments, the optionally substituted triazolene has the structure:

For example, polynucleotides of the invention having a sequence comprising Formula II:

$$[A_n]I_2^1B_0].$$

Formula II

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

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

Formula XIV
with a compound having the structure of Formula XV:

$$R^{27}_c-(R^5)_d-(R^6)_e-(R^7)_f[B_0]$$

**Formula XV**

wherein each A and B is independently any nucleoside;

n and o are, independently 15 to 1000; and

L has the structure of Formula III:

$$L^1$$

**Formula III**

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

n and o are, independently 15 to 1000;

R, R, R, and R each, independently, is selected from optionally substituted C-C alkylene, optionally substituted Ci-C heteroalkylene, O, S, and NR;

R and R are each, independently, selected from carbonyl, thiocarbonyl, sulphonyl, or phosphoryl;

R is an optionally substituted triazolene; and

R is hydrogen, optionally substituted Ci-C alkyl, optionally substituted C-C alkynyl, optionally substituted C-C alkynyl, optionally substituted C-C heterocycl, optionally substituted C-C aryl, or optionally substituted C-C heteroalkyl; and

R is an optionally substituted C-C alkynyl or an optionally substituted C-C cycloalkynyl,

wherein L is attached to [A] and [B] at the sugar of one of the nucleosides.

Polynucleotides of the invention including the structure of Formula XI:
[000140] may be synthesized by reacting a compound having the structure of Formula XII:

with a compound having the structure of Formula XIII:

[000141] wherein each of \(N^1\) and \(N^2\) is independently a nucleobase;

[000142] 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 Q-Cealkyl, optionally substituted Ci-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;
each of g and h is, independently, 0 or 1;

each $X^4$ is, independently, $O$, NH, or S; and

each $X^2$ and $X^3$ is independently $O$ or S;

each of $R_{24}$ and $R_{26}$ is, independently, a region of linked nucleosides; and

$R_{25}$ is optionally substituted $C_1$-$C_6$ alkylene, optionally substituted $C_1$-$C_6$ heteroalkylene or $R_{25}$ and the alkynyl group together form optionally substituted cycloalkynyl.

For example, the circular polynucleotides of the invention may be synthesized as shown below:

$$[A_n]_N^3 + \text{Enzymatic capping} \rightarrow [A_m]_N^3 [B_0]_N^3$$

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

A 5’ cap structure may be included in a polynucleotide of the invention as shown below:

A poly-A tail may be included in a polynucleotide of the invention as shown below:
For example, polynucleotides of the invention including the structure of Formula V:
[000153] This method includes reacting a compound having the structure of Formula VI:

![Formula VI](image)

[000154] with a compound having the structure of Formula VII:

![Formula VII](image)

[000155] wherein each of $N^1$ and $N^2$ is, independently, a nucleobase;

[000156] 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 Q-Cealkyl, 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;

[000157] each of $g$ and $h$ is, independently, 0 or 1;

[000158] each $X^1$ and $X^4$ is, independently, O, NH, or S; and

[000159] each $X^3$ is independently OH or SH, or a salt thereof;

[000160] each of $R^{17}$ and $R^{19}$ is, independently, a region of linked nucleosides; and

[000161] $R^{18}$ is a halogen.

[000162] Polynucleotides of the invention including the structure of Formula VIII:
This method includes reacting a compound having the structure of Formula IX:

with a compound having the structure of Formula X:

wherein each of $N^1$ and $N^2$ is, independently, a nucleobase;

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 Q-Cealkyl, 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;

each of $g$ and $h$ is, independently, 0 or 1;

each $X^4$ is, independently, O, NH, or S; and

each $X^2$ is independently O or S;
each $X^3$ is independently OH, SH, or a salt thereof;

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

each of $R^{21}$ and $R^{22}$ is, independently, optionally substituted C$_1$-C$_6$ alkoxy.

Polynucleotides of the invention including the structure of Formula XI:

![Formula XI](image)

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

![Formula XII](image)

with a compound having the structure of Formula XIII:

![Formula XIII](image)

wherein each of $N^1$ and $N^2$ is, independently, a nucleobase;

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 Q-C$_{al}a$kyl, optionally substituted CrC$_6$
heteroalkyl, optionally substituted C₂-C₆ heteroalkenyl, optionally substituted C₂-C₆ heteroalkynyl, optionally substituted amino, azido, or optionally substituted C₆-C₁₀ aryl;

[000177] each of g and h is, independently, 0 or 1;
[000178] each X⁴ is, independently, O, NH, or S; and
[000179] each X² is independently O or S;
[000180] each X³ is independently OH, SH, or a salt thereof;
[000181] each of R₂⁴ and R₂⁶ is, independently, a region of linked nucleosides; and
[000182] R₂⁵ is optionally substituted CrC₆ alkylene or optionally substituted CrC₆ heteroalkylene or R₂⁵ and the alkynyl group together form optionally substituted cycloalkynylene.

[000183] Polynucleotides of the invention may be synthesized as shown below:

[000184] Other methods for the synthesis of the polynucleotides of the invention are shown below:

[000185] a)
where CEO is 2-cyanoethoxy, and X is O or S.

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).

Intrabody Constructs

According to the present invention, an intrabody construct is a polynucleotide which has been modified for expression inside a target cell and where the expression product binds an intracellular protein. Such constructs may have sub picomolar binding affinities and may be formulated for targeting to particular sites or tissues. For example, intrabody constructs may be formulated in any of the lipid nanoparticle formulations disclosed herein.

In one embodiment, the polynucleotides described herein may comprise an intrabody construct. The intrabody construct may encode a polypeptide which binds to an intracellular target to disrupt the protein-protein-interaction (PPI) between proteins or
an intracellular target to disrupt the protein-DNA-interaction (PDI) between proteins and DNA.

[000192] In one embodiment, the polynucleotides described herein may comprise an intrabody construct which may encode a polypeptide which may prevent the activation of transcription complexes that up-regulate gene expression of cancer related genes.

[000193] In one embodiment, the polynucleotides described herein may comprise an intrabody construct which may encode a polypeptide which may prevent recruitment of transactivation repressors that inhibit expression of tumor suppressors.

[000194] In one embodiment, the polynucleotides described herein may comprise an intrabody construct which may encode a polypeptide comprising a target binding scaffold, protein or polypeptide and an effector domain. As a non-limiting example, the effector domain may be a transcriptional repressor or transcriptional activator.

[000195] In one embodiment, the polynucleotides described herein may comprise an intrabody construct which may encode a polypeptide which comprises a nuclear localization signal (NLS) for efficient translocation to the nucleus.

[000196] In one embodiment, the polynucleotides described herein may comprise an intrabody construct which may encode a polypeptide which binds to the DNA binding domain of at least one transcription factor, preventing its interaction with its regulatory element within the regulatory region or promoter of a gene. In one embodiment, the transcription factor is a transcriptional activator. In one embodiment, the complex may be a dimer that may further interact with other proteins to up-regulate gene transcription. In another embodiment, the transcription factor is a transcriptional repressor.

[000197] In one embodiment, the polypeptide may bind to the DNA binding domain of V-myc myelocytomatosis viral oncogene homolog (MYC) to prevent its association with MYC regulatory elements within the regulatory or promoter region of a target gene, thereby reducing gene expression controlled by MYC. In one embodiment, the polypeptide may bind to the DNA binding domain of MYC and/or myc-associated factor X (MAX) to prevent the association of a MYC homodimer or MYC/MAX heterodimer with its regulatory element within the regulatory region or promoter of a target gene, thereby reducing gene expression controlled by MYC. In one embodiment, the polypeptide may bind to the DNA binding domain of Signal transducer and activator of
transcription 3 (STAT3) to prevent its association to its DNA regulatory element, termed gamma-activated sites (GAS), within a regulatory or promoter region of a target gene, thereby reducing gene expression controlled by STAT3.

[000198] In one embodiment the polypeptide may bind to the dimerization domain of at least one transcription factor, preventing dimerization with a partner to form either a homo- or a heterodimer. In one embodiment, the transcription factor is a transcriptional activator. In one embodiment, the transcription factor is a transcriptional repressor. In one embodiment the polypeptides may bind to the dimerization domain of MYC to prevent MYC homo-dimerization. In one embodiment, the polypeptides may bind to the dimerization domains of MYC and/or MAX, preventing MYC/MAX heterodimer formation. In one embodiment the polypeptides may bind to the dimerization domain of STAT3 to prevent homo-dimerization. Without wishing to be bound by theory, the intrabody construct - through its ability to prevent homo- and hetero-dimerization - may prevent subsequent DNA binding and transcriptional activation of MYC or STAT3 target genes.

[000199] In one embodiment, the polypeptide may bind an oncogene protein such as RAS, which is involved in signal transduction. In one embodiment, the polypeptide binds an activated RAS protein. The ability of anti-RAS intrabodies to inhibit RAS activity has been demonstrated (Biocca, S. et al. (1993) Biochem. Biophys. Res. Commun. 197:422-427; Biocca, S. et al. (1994) Biotechnology 12:396-399). Furthermore, anti-RAS intrabodies have been shown to induce apoptosis in human cancer cell lines and to elicit sustained tumor regression in nude mice (Cochet, O. et al. (1998) Cancer Res. 58:1170-1176).

[000200] In one embodiment, the polypeptide encoded by the intrabody construct binds a viral protein, such as an HBV core antigen (HBcAg). For example, single domain antibody (VHH) constructs that bind HBcAg, such as those described in the Examples, can be used.

[000201] In one embodiment, the polypeptide encoded by the intrabody construct comprises a fusion protein comprising a target binding portion or domain and an effector portion or domain connected by a linker. In one embodiment, the effector portion is a
transactivation domain. In another embodiment, the effector portion is a repressor domain.

[000202] In one embodiment, the fusion protein comprises a target binding domain which is able to bind to a transcriptional activator, wherein the target binding domain is connected by a linker to a trans-repression domain. In one embodiment, the target binding domain binds to the transactivation domain of the transcriptional activator. In one embodiment, the target binding domain does not bind the DNA binding domain of the transcriptional activator, allowing DNA binding to occur.

[000203] Without wishing to be bound by theory, the fusion protein of the invention may inhibit transactivation and instead repress transcription of the target genes. In one embodiment, such target genes may include proto-oncogenes and oncogenes.

[000204] In one embodiment, the target binding domain of the fusion protein is a MYC binding domain. In one embodiment, the target binding domain of the fusion protein is a STAT3 binding domain. The Krippel associated box (KRAB) domain is a non-limiting example of a trans-repression domain encoded by the intrabody constructs of the invention. Such fusion proteins can bind to endogenous MYC or STAT3 and recruit the fused trans-repressive domain to MYC or STAT3 regulatory elements within the regulatory or promoter regions of target genes, resulting in transcriptional repression of MYC or STAT3 target genes.

[000205] In one embodiment, the fusion protein may comprise a target binding portion or domain that can bind a transcriptional repressor connected by a linker to a transactivation portion or domain. A non-limiting example of a transactivation domain is VP64, which is composed of four tandem copies of VP16 (Herpes Simplex Viral Protein 16). Without wishing to be bound by theory, such fusion proteins may bind to regulatory elements within the regulatory or promoter region of target genes, thereby recruiting the transactivation domain so it can activate transcription of tumor suppressor genes. Tumor suppressors include, but are not limited to, PTEN, NM23, KAI1, and p53.

[000206] In one embodiment, the polynucleotides described herein may comprise an intrabody construct which may encode a polypeptide derived from non-antibody scaffold proteins or domains which function as antibody mimetics. Non-limiting examples of non-
antibody scaffold proteins of the invention include, but are not limited to, fibronectin domains, Kunitz domains, Stefin A triple mutant scaffolds, or transferrin domains.

[000207] In one embodiment, the polynucleotides described herein may comprise an intrabody construct, which may comprise a fibronectin type III domain.

[000208] In one embodiment the non-antibody scaffold may be based on the tenth fibronectin type III domain from human fibronectin. The tenth fibronectin type III domain has a structure containing seven beta strands forming a barrel and three exposed loops on each side, the loops being analogous to the three complementarity determining regions of an antibody.

[000209] In a non-limiting example, the scaffold may comprise a monobody. Monobodies are genetically engineered proteins that are able to bind other proteins or antigens and are based on the tenth fibronectin type III (10FN3) domain from human fibronectin.

[000210] Monobodies with specificity for different proteins can be created and tailored, modifying the loops, for example, BC (between the second and third beta strands), CD (between the third and fourth beta strands), DE (between the fourth and fifth beta strands) and FG (between the sixth and seventh strands). Certain loop modifications are, for example, described in Koide A. et al, High-affinity single-domain binding proteins with a binary-code interface. Proc. Natl. Acad. Sci. (2007) 104(16):6632-7, the contents of which is herein incorporated by reference in its entirety.

[000211] In some embodiments of the current invention, the intrabody contract encodes a FN3 domain in which the loops may form the interactions with the target protein. In some embodiments, the interactions with the target proteins are formed by the beta strands. In some embodiments, the intrabody construct encodes a FN3 domain, in which the binding interface comprises both loop and non-loop residues.

[000212] In one embodiment, the intrabody construct encodes a polypeptide comprising a protein scaffold from the protein Tenascin. In one embodiment, the protein scaffold is derived from the fibronectin type III domain of Tenascin (Tencon). In one embodiment of the invention the polynucleotides of the invention comprise and intrabody construct which encodes a "Centyrin", as described in Diem et al, Selection of high-affinity Centyrin FN3 domains from a simple library diversified at a combination of strand and
loop positions, Protein Eng Des Sel. 2014, the contents of which is herein incorporated by reference in its entirety.

[000213] In one embodiment, the intrabody construct encodes a Centyrin, in which portions of the C-strand, F-strand, CD-loop and FG-loop are randomized to provide a binding surface.

[000214] In one embodiment, the polynucleotides of the invention comprise an intrabody construct which encodes a fibronectin type III domain isolated from a library of fibronectin type III module (FN3) domains having a diversified C-CD-F-FG alternative surface, described in or made by the methods described in International Patent Publication WO2013049275, the contents of which is herein incorporated by reference in its entirety.

[000215] In one embodiment of the invention, any of the non-antibody scaffolds described in the embodiments above and encoded by the intrabody construct may bind c-MYC. In one example, the non-antibody scaffold may bind to the DNA binding domain of c-MYC, preventing c-MYC from binding to its DNA regulatory element. In another example, the dimerization domain of c-MYC is the target domain of the non-antibody scaffold, preventing c-MYC homo- or hetero-dimerization. In one embodiment, the target protein of interest may be MAX. In a non-limiting example, the non-antibody scaffold may bind to the dimerization domain of MAX, preventing hetero-dimerization with c-MYC.

[000216] In one embodiment, the target protein of interest may be STAT3. In one non-limiting example, the non-antibody scaffold may bind to the DNA binding domain of STAT3, preventing STAT3 from binding to its DNA regulatory element. In another non-limiting example, the non-antibody scaffold may bind to the dimerization domain of STAT3, preventing STAT3 homo-dimerization.

Bicistronic and/or Pseudo-bicistronic Constructs

[000217] According to the present invention, a bicistronic construct is a polynucleotide encoding a two-protein chain antibody on a single polynucleotide strand. (Fig. IB) 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**

According to the present invention, a single domain construct comprises one or two polynucleotides encoding a single monomeric variable antibody domain. See Figs 2B and 3B for examples. Typically single domain antibodies comprise one variable domain of a heavy-chain antibody (VH) or one variable domain of a light-chain antibody (VL). Single domain antibodies consisting solely of the heavy chain variable domain are referred to herein as VHH constructs and have been described in the art (for reviews see e.g., A.S.-Y. et al. Therapeutic Handbook of Experimental Pathology 181, Springer Verlag (Y. Chernajovsky and A. Nissim, eds.), 2008, pp 344-373; Smolarek, D. et al. (2012) Postepy Hig Med Dosw (online), 66:348-358).

**Single chain Fv Constructs**

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. 2A 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**

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. 3A for an example. Polynucleotides of the present invention may also encode trispecific antibodies having an affinity for three antigens.

**Linkers**
Examples of linkers which may be used in the polynucleotides of the present invention include those in Tables 1 and 2.

**Table 1. Nucleic Acid Sequences of Exemplary Linkers**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence in polynucleotide</th>
<th>SEQ ID NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLrigid</td>
<td>GAAGCTGCTGCAAGAGAGCTGC AGCTAGGAGGAGCTAGCTAGG AGGCTGCTGCAAGA</td>
<td>1</td>
</tr>
<tr>
<td>PLrigid is a 20 a.a. peptide that is based on an alpha-helix motif (EAAAR) (Merutka et al., 1991; Sommese et al., 2010)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2aa GS linker</td>
<td>GCAGC</td>
<td>2</td>
</tr>
<tr>
<td>Highly flexibly glycine linker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6aa (GS)x linker</td>
<td>GGTAGCGGCAGCGGTAGC</td>
<td>3</td>
</tr>
<tr>
<td>Highly flexible 6 amino acid linker. Translates to gsgsgs. Codon-optimize for E. coli, yeast, mammalian</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 aa flexible protein domain linker</td>
<td>GGTGAAAAATTTGTATTTTCAATCT GGTGGT</td>
<td>4</td>
</tr>
<tr>
<td>8 aa protein domain linker</td>
<td>TCCGCTTTGTACTGTGAGCTTCCC</td>
<td>5</td>
</tr>
<tr>
<td>15 aa flexible glycine-serine protein domain linker; Freiburg standard</td>
<td>GGTGGAGGAGGTCTGGAGGCGG TGGGAAGTGGTGCGAGGCTAGC</td>
<td>6</td>
</tr>
<tr>
<td>Short Linker (Gly-Gly-Ser-Gly)</td>
<td>GGTGTTCTGTTGGTGGTCTGGT CTGGTTCTGCTGGT</td>
<td>7</td>
</tr>
<tr>
<td>Middle Linker (Gly-Gly-Ser-Gly)x2</td>
<td>GGTGTTCTGTTGGTGGTCTGGT CTGGTTCTGCTGGT</td>
<td>8</td>
</tr>
<tr>
<td>Long Linker (Gly-Gly-Ser-Gly)x3</td>
<td>GGTGTTCTGTTGGTGGTCTGGT CTGGTTCTGCTGGT</td>
<td>9</td>
</tr>
</tbody>
</table>
| GSAT Linker                      | GGTGTTCTGGGCTAGGGTGGTGGAGCT GTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT GG

**Table 2. Protein Sequences of Exemplary Linkers**

<table>
<thead>
<tr>
<th>Name</th>
<th>Protein Sequence</th>
<th>SEQ ID NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>G9S linker</td>
<td>GGGGGGGGGS</td>
<td>12</td>
</tr>
</tbody>
</table>

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."

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.

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.

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).

[000227] When the polynucleotides of the present invention are chemically and/or structurally modified the polynucleotides may be referred to as "modified polynucleotides."

[000228] 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: GSGATNFSLKQAGDVEENPGP (SEQ ID NO: 13), 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 GSGATNFSLKQAGDVEENPGP (SEQ ID NO: 14) fragments or variants thereof.

[000229] One such polynucleotide sequence encoding the 2A peptide is GGAAGCGGAGCTACTAACTTACGGCTGAGCTGGAGACGGCTGGAG GAGAACCCTGGACCT (SEQ ID NO: 15). 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.

[000230] 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**

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.

IVT constructs and their features may be those of the primary constructs as described in copending application WO2015/085318, the contents of which are incorporated by reference in their entirety.

**Chimeric Polynucleotide Architecture**

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."

It is to be understood that the polynucleotides of the present invention may be encoded by a chimeric polynucleotide, RNA construct, chimeric modified mRNA or chimeric mRNA. Chimeric polynucleotides, formulations and compositions comprising chimeric polynucleotides, and methods of making, using and administering chimeric polynucleotides are also described in International Application WO2015/034928, published March 12, 2015, entitled Chimeric Polynucleotides, which is incorporated by reference in its entirety.

**Circular Polynucleotide Architecture**

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 International Application WO2015/034925, published March 12, 2015, the content of which is incorporated herein by reference in its entirety, may be made chimeric according to the present invention.

**Multimers of Polynucleotides**

**[000237]** 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.

**[000238]** 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-, NH₂-, N₃, 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.

**[000239]** 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 CCCDD[CCCDD] 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

[000240] 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.

[000241] 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.

[000242] 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

[000243] In one embodiment of the invention the compositions may comprise 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.
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.

Noncoding Polynucleotides

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.

Such molecules are generally not translated, but can exert an effect on the immune response or 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 (IncRNA, 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 IncRNA molecules and RNAi constructs designed to target such IncRNA 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

According to the present invention, the polynucleotides 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 polynucleotides of the present invention.

[000248] 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 one embodiment, the polypeptides of interest are antibodies encoded by the polynucleotides as described herein.

[000249] 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.

[000250] 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.

[000251] 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.

[000252] "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.

[000253] 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.

[000254] "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.

[000255] 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.

[000256] 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.

[000257] "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.

[000258] 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.

[000259] "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.

[000260] "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.
"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.

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.

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)).

"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.

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.
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.

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.

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.

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 dibromopyrrol agents used herein.

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 residues 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+7-0.5 being 3 or 4).
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).

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 residues 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).

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.

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.

[000275] 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.

[000276] Modifications and manipulations can be accomplished by methods known in the art such as, but not limited to, site directed mutagenesis or apriori 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.

[000277] 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.

[000278] 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.

[000279] 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 the polypeptides disclosed herein.

[000280] 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).

[000281] 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. Schaffer, 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."

[000282] 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.

*Cell-Penetrating Polypeptides*

[000283] The polynucleotides disclosed herein, may also 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.

[000284] 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.
Polypeptide Libraries

In one embodiment, the polynucleotides may be used to produce polypeptide libraries. These libraries may arise from the production of a population of polynucleotides, each containing various structural or chemical modification designs. In this embodiment, a population of polynucleotides may comprise a plurality of encoded polypeptides, including but not limited to, an antibody or antibody fragment, protein binding partner, scaffold protein, and other polypeptides taught herein or known in the art. In one embodiment, the polynucleotides may be suitable for direct introduction into a target cell or culture which in turn may synthesize the encoded polypeptides.

In certain embodiments, multiple variants of a protein, each with different amino acid modification(s), may be produced and tested to determine the best variant in terms of pharmacokinetics, stability, biocompatibility, and/or biological activity, or a biophysical property such as expression level. Such a library may contain 10, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, or over 10^9 possible variants (including, but not limited to, substitutions, deletions of one or more residues, and insertion of one or more residues).

Cytotoxic Nucleosides

In one embodiment, the polynucleotides of the present invention may incorporate one or more cytotoxic nucleosides. For example, cytotoxic nucleosides may be incorporated into polynucleotides such as bifunctional modified RNAs or mRNAs. Cytotoxic nucleoside anti-cancer agents include, but are not limited to, adenosine arabinoside, cytarabine, cytosine arabinoside, 5-fluorouracil, fludarabine, floxuridine, FTORAFUR® (a combination of tegafur and uracil), tegafur ((RS)-5-fluoro-1-(tetrahydrofuran-2-yl)pyrimidine-2,4(IH,3H)-dione), and 6-mercaptopurine.

A number of cytotoxic nucleoside analogues are in clinical use, or have been the subject of clinical trials, as anticancer agents. Examples of these and other cytotoxic nucleosides may be found in copending application US 61/912,635 filed December 6, 2013 (Attorney Docket Number M073.60), the contents of which are incorporated herein by reference in their entirety and may be used in the present invention.

Polynucleotides having Untranslated Regions (UTRs)

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.

[000290] 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.

[000291] Tables 1 and 2 of WO20 15/051169, the content of which is incorporated by reference in its entirety, provides a listing of exemplary UTRs which may be utilized in the polynucleotides of the present invention.

5’UTR and Translation Initiation

[000292] 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.

[000293] 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.

[000294] Untranslated regions useful in the design and manufacture of polynucleotides include, but are not limited, to those disclosed in co- owned WO2014/164253, the content of which is incorporated herein by reference in its entirety.

[000295] 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 polynucleotide levels.

[000296] 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.

[000297] Co-owned WO2014/164253 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.

[000298] 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.

[000299] 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.

3' UTR and the A U Rich Elements
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.

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

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.

[000303] 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).

[000304] 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.
Examples of tissues where microRNA are known to regulate mRNA, and thereby protein expression, include, but are not limited to, liver (miR-122), muscle (miR-133, miR-206, miR-208), endothelial cells (miR-17-92, miR-126), myeloid cells (miR-142-3p, miR-142-5p, miR-16, miR-21, miR-223, miR-24, miR-27), adipose tissue (let-7, miR-30c), heart (miR-ld, miR-149), kidney (miR-192, miR-194, miR-204), and lung epithelial cells (let-7, miR-133, miR-126). MicroRNA can also regulate complex biological processes such as angiogenesis (miR-132) (Anand and Cheresh Curr Opin Hematol 2011 18:171-176; herein incorporated by reference in its entirety).

Expression profiles, microRNA and cell lines useful in the present invention include those taught in for example, WO2014/13089 and WO2014/081507, the contents of which are incorporated by reference in their entirety.

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. A listing of microRNA, miR sequences and miR binding sites is listed in Table 9 of International Application WO2014/13089, and in Table 11 of WO2014/081507, each of which is herein incorporated by reference in their entireties.

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.

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.

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.

*Regions having a 5’*Cap

[000311] 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.

[000312] 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 antiterminal transcribed nucleotides of the 5’ end of the mRNA may optionally also be 2’-0-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.

[000313] 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 a-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 a-methyl-phosphonate and seleno-phosphate nucleotides.
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.

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'-0-methyl group (i.e., N7,3'-0-dimethyl-guanosine-5'-triphosphate-5'-guanosine (m7G-3'mppp-G; which may equivalently be designated 3' O-Me-m7G(5')ppp(5')G). The 3'-0 atom of the other, unmodified, guanine becomes linked to the 5'-terminal nucleotide of the capped polynucleotide. The N7- and 3'-0-methyated guanine provides the terminal moiety of the capped polynucleotide.

Another exemplary cap is mCAP, which is similar to ARCA but has a 2'-0-methyl group on guanosine (i.e., N7,2'-0-dimethyl-guanosine-5'-triphosphate-5'-guanosine, m7Gm-ppp-G).

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 phosphoroselenoate 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.

In another embodiment, the cap is a cap analog is a N7-(4-chlorophenoxyethyl) substituted dinucleotide form of a cap analog known in the art and/or described herein. Non-limiting examples of a N7-(4-chlorophenoxyethyl) substituted dinucleotide form of a cap analog include a N7-(4-chlorophenoxyethyl)-G(5')ppp(5')G and a N7-(4-chlorophenoxyethyl)-m 3'-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.

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.

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'-0-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'-0-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).

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.

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, Nl-methyl-guanosine, 2'fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine.

Viral Sequences

[000323] 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. WO20 12 129648; 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

[000324] 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

[000325] 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, including approximately 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240 or 250 residues long.

[000326] 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).

[000327] 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.

[000328] Unique poly-A tail lengths provide certain advantages to the polynucleotides of the present invention.

[000329] 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).

[000330] 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.

[000331] 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.

[000332] 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.

[000333] 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.

**Start Codon Region**

[000334] In some embodiments, the polynucleotides of the present invention may have regions that are analogous to or function like a start codon region.

[000335] 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.

[000336] 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.

[000337] 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).
[000338] 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.

[000339] 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.

[000340] 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.

[000341] 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
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**

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.

Additional signal sequences which may be utilized in the present invention include those taught in, for example, databases such as those found at http://www.signalpeptide.de/ or http://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.

**Protein Cleavage Signals and Sites**

In one embodiment, the polynucleotides may encode or 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.

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 a 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.

[000347] 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.

[000348] 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 and such sites are useful in the polynucleotides of the present invention.

Insertions and Substitutions

[000349] 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.

[000350] 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.

[000351] 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.

[000352] 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.

[000353] 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.

[000354] 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

[000355] 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.

[000356] 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, the contents of which are
herein incorporated by reference in its entirety. Methods identifying RNA regulatory
sequences involved in translational control are described in International Publication No.
WO2004067728, the contents of which are 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, the
contents of which are herein incorporated by reference in its entirety.

[000357] 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.
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 Nos. WO2004010106, WO2006044456, WO2006044682, WO2006044503 and WO2006044505, the contents of 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).

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. WO2005 118857, WO2006065480, WO2006065479 and WO2006058088, the contents of each of which is herein incorporated by reference in its entirety.

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

**Codon Optimization**

The polynucleotides of the invention, 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>
<thead>
<tr>
<th>Amino Acid</th>
<th>Single Letter Code</th>
<th>Codon Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>I</td>
<td>ATT, ATC, ATA</td>
</tr>
<tr>
<td>Leucine</td>
<td>L</td>
<td>CTT, CTC, CTA, CTG, TTA, TTG</td>
</tr>
<tr>
<td>Valine</td>
<td>V</td>
<td>GTT, GTC, GTA, GTG</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>F</td>
<td>TTT, TTC</td>
</tr>
<tr>
<td>Methionine</td>
<td>M</td>
<td>ATG</td>
</tr>
<tr>
<td>Cysteine</td>
<td>C</td>
<td>TGT, TGC</td>
</tr>
<tr>
<td>Alanine</td>
<td>A</td>
<td>GCT, GCC, GCA, GCG</td>
</tr>
<tr>
<td>Glycine</td>
<td>G</td>
<td>GGT, GGC, GGA, GGG</td>
</tr>
<tr>
<td>Proline</td>
<td>P</td>
<td>CCT, CCC, CCA, CCG</td>
</tr>
<tr>
<td>Threonine</td>
<td>T</td>
<td>ACT, ACC, ACA, ACG</td>
</tr>
<tr>
<td>Serine</td>
<td>S</td>
<td>TCT, TCC, TCA, TCG, AGT, AGC</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Y</td>
<td>TAT, TAC</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>W</td>
<td>TGG</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Q</td>
<td>CAA, CAG</td>
</tr>
<tr>
<td>Asparagine</td>
<td>N</td>
<td>AAT, AAC</td>
</tr>
<tr>
<td>Histidine</td>
<td>H</td>
<td>CAT, CAC</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>E</td>
<td>GAA, GAG</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>D</td>
<td>GAT, GAC</td>
</tr>
<tr>
<td>Lysine</td>
<td>K</td>
<td>AAA, AAG</td>
</tr>
<tr>
<td>Arginine</td>
<td>R</td>
<td>CGT, CGC, CCA, CGG, AGA, AGG</td>
</tr>
<tr>
<td>Selenocysteine</td>
<td>Sec</td>
<td>UGA in mRNA in presence of Selenocysteine insertion element (SECIS)</td>
</tr>
<tr>
<td>Stop codons</td>
<td>Stop</td>
<td>TAA, TAG, TGA</td>
</tr>
</tbody>
</table>

Table 3. Codon Options

[000362] 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 Xbal recognition.

Synthesis

[000363] Enzymatic (IVT), solid-phase, liquid-phase, combined synthetic methods,
small region synthesis, and ligation methods are taught in for example copending
application WO2015/085318, the contents of which are incorporated herein by reference in
their entirety, and may be utilized to manufacture the polynucleotides of the present
invention.

Modified and Conjugated Polynucleotides

[000364] 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.
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, 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.

[000365] 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.

Quantification

[000366] 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, bronchoalveolar 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.

[000367] Quantification may be 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.

[000368] It is often 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.

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.

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**

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.

[000372] 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.

[000373] In another embodiment, the polynucleotides may be sequenced by methods including, but not limited to reverse-transcriptase-PCR.

III. Modifications

[000374] 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 deoxyribonucleosides 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.

[000375] In a polypeptide, the term "modification" refers to a modification as compared to the canonical set of 20 amino acids.

[000376] 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.

Modifications of the polynucleotides of the compositions which are useful in the present invention include, but are not limited to, 2-methylthio-N6-(cis-hydroxyisopentenyl)adenosine, 2-methylthio-N6-methyladenosine, 2-methylthio-N6-threonyl carbamoyladenosine, N6-glycinylcarbamoyladenosine, N6-isopentenyladenosine, N6-methyladenosine, N6-threonylcarbamoyladenosine, 1,2'-0-dimethyladenosine, 1-methyladenosine, 2'-0-methyladenosine, 2'-0-ribosyladenosine (phosphate), 2-methyladenosine, 2-methylthio-N6 isopentenyladenosine, 2-methylthio-
N6-hydroxynorvalyl carbamoyladienosine, 2'-0-methyladenosine, 2'-0-ribosyladenosine (phosphate), isopentenyladenosine, N6- (cis-hydroxyisopentenyl)adenosine, N6,2'-0- dimethyladenosine, N6,2'-0-dimethyladenosine, N6,N6,2'-0-trimethyladenosine, N6,N6- dimethyladenosine, N6-acetyl adenosine, N6-hydroxynorvalyl carbamoyl adenosine, N6- methyl-N6-threonyl carbamoyl adenosine, 2-methyladenosine, 2-methylthio-N6- isopentenyladenosine, 7-deaza-adenosine, N1-methyl-adenosine, N6, N6 (dimethyl) adenine, N6-cis-hydroxy-isopentenyl-adenosine, a-thio-adenosine, 2 (amino)adenine, 2 (aminopropyl)adenine, 2 (methylthio) N6 (isopentenyl)adenine, 2- (alkyl)adenine, 2-(aminoalkyl)adenine, 2-(aminopropyl)adenine, 2-(halo)adenine, 2- (halo) adenine, 2-(propyl)adenine, 2'-Amino-2'-deoxy-ATP, 2'-Azido-2'-deoxy-ATP, 2'- Deoxy-2'-a-aminoadenosine TP, 2'-Deoxy-2'-a-azidoadenosine TP, 6 (alkyl)adenine, 6 (methyl)adenine, 6-(alkyl)adenine, 6-(methyl)adenine, 6-(alkynyl)adenine, 6-(alkynyl)adenine, 8 (alkenyl)adenine, 8 (alkynyl) adenine, 8 (amino) adenine, 8 (thioalkyl) adenine, 8-(alkenyl)adenine, 8-(alkynyl) adenine, 8-(amino)adenine, 8-(alkenyl)adenine, 8-(alkynyl)adenine, 8-(hydroxyl) adenine, 8-(thiol) adenine, 8-azido-adenosine,aza adenine, deaza adenine, N6 (methyl) adenine, N6-(isopentyl) adenine, 7-deaza-8-aza-adenosine, 7-methyladenine, 1-Deazaadenosine TP, 2'Fluoro-N6-Bz- deoxyadenosine TP, 2'-OMe-2'-Amino-ATP, 2'-0-methyl-N6-Bz-deoxyadenosine TP, 2'- a-Ethynyladenosine TP, 2-aminoadenine, 2-Amino adenosine TP, 2-Amino-ATP, 2'-a- Trifluoromethyladenosine TP, 2-Azidoadenosine TP, 2'-b-Ethynyladenosine TP, 2- Bromoadenosine TP, 2'-b-Trifluoromethyladenosine TP, 2-Chloroadenosine TP, 2'- Deoxy-2',2'-difluoroadenosine TP, 2'-Deoxy-2'-a-mercaptopadenosine TP, 2'-Deoxy-2'-a-thiometyloxyadenosine TP, 2'-Deoxy-2'-b-aminoadenosine TP, 2'-Deoxy-2'-b- azidoadenosine TP, 2'-Deoxy-2'-b-bromoadenosine TP, 2'-Deoxy-2'-b-chloroadenosine TP, 2'-Deoxy-2'-b-fluoroadenosine TP, 2'-Deoxy-2'-b-iodoadenosine TP, 2'-Deoxy-2'-b- mercaptopadenosine TP, 2'-Deoxy-2'-b-thiometyloxyadenosine TP, 2-Fluoroadenosine TP, 2-Iodoadenosine TP, 2-Mercaptopadenosine TP, 2-methoxy-adenine, 2-methylthio- adenine, 2-Trifluoromethyladenosine TP, 3-Deaza-3-bromoadenosine TP, 3-Deaza-3- chloroadenosine TP, 3-Deaza-3-fluoroadenosine TP, 3-Deaza-3-iodoadenosine TP, 3- Deazaadenosine TP, 4'-Azidoadenosine TP, 4'-Carbocyclic adenosine TP, 4'- Ethynyladenosine TP, 5'-Homo-adenosine TP, 8-Aza-ATP, 8-bromo-adenosine TP, 8-
Trifluoromethyladenosine TP, 9-Deazaadenosine TP, 2-aminopurine, 7-deaza-2,6-diaminopurine, 7-deaza-8-aza-2,6-diaminopurine, 7-deaza-8-aza-2-aminopurine, 2,6-diaminopurine, 7-deaza-8-aza-adenine, 7-deaza-2-aminopurine, 2-thiocytidine, 3-methylcytidine, 5-formylcytidine, 5-hydroxymethylcytidine, 5-methylcytidine, N4-acetylcystidine, 2′-0-methylcytidine, 2′-0-methylcytidine, 5,2′-0-dimethylcytidine, 5-formyl-2′-0-methylcytidine, lysidine, N4,2′-0-dimethylcytidine, N4-acetyl-2′-0-methylcytidine, N4,N4-Dimethyl-2′-OMe-Cytidine TP, 4-methylcytidine, 5-aza-cytidine, Pseudo-iso-cytidine, pyrrolo-cytidine, a-thio-cytidine, 2-(thio)cytosine, 2′-Amino-2′-deoxy-CTP, 2′-Azido-2′-deoxy-CTP, 2′-Deoxy-2′-a-aminocytidine TP, 2′-Deoxy-2′-a-azidocytidine TP, 3 (deaza) 5 (aza)cytosine, 3 (methyl)cytosine, 3-(alkyl)cytosine, 3-(deaza) 5 (aza)cytosine, 3-(methyl)cytidine, 4,2′-O-dimethylcytidine, 5 (halo)cytosine, 5 (methyl)cytosine, 5 (propynyl)cytosine, 5 (trifluoromethyl)cytosine, 5-(alkyl)cytosine, 5-(alkynyl)cytosine, 5-(halo)cytosine, 5-(propynyl)cytosine, 5-(trifluoromethyl)cytosine, 5-bromo-cytidine, 5-iodo-cytidine, 5-propynyl cytosine, 6-(azo)cytosine, 6-aza-cytidine, aza cytosine, deaza cytosine, N4 (acetyl)cytosine, 1-methyl-1-deaza-pseudoisocytidine, 1-methyl-pseudoisocytidine, 2-methoxy-5-methyl-cytidine, 2-methoxy-cytidine, 2-thio-5-methyl-cytidine, 4-methoxy-1-methyl-pseudoisocytidine, 4-methoxy-pseudoisocytidine, 4-thio-1-methyl-1-deaza-pseudoisocytidine, 4-thio-1-methyl-pseudoisocytidine, 4-thio-1-methyl-pseudoisocytidine, 4-thio-1-methyl-pseudoisocytidine, 4-thio-1-methyl-pseudoisocytidine, 5-aza-zebularine, 5-methyl-zebularine, pyrrolo-pseudoisocytidine, zebularine, (E)-5-(2-Bromovinyl)cytidine TP, 2,2′-anhydro-cytidine TP hydrochloride, 2′Fluor-N4-Bz-cytidine TP, 2′Fluoro-N4-Acetyl-cytidine TP, 2′-0-Methyl-N4-Acetyl-cytidine TP, 20′-methyl-N4-Bz-cytidine TP, 2′-a-Ethynylcytidine TP, 2′-a-Trifluoromethylcytidine TP, 2′-b-Ethynlycytidine TP, 2′-b-Trifluoromethylcytidine TP, 2′-Deoxy-2′-2′-difluorocytidine TP, 2′-Deoxy-2′-a-mercaptopcytidine TP, 2′-Deoxy-2′-a-thiomethoxycytidine TP, 2′-Deoxy-2′-b-aminocytidine TP, 2′-Deoxy-2′-b-azidocytidine TP, 2′-Deoxy-2′-b-bromocytidine TP, 2′-Deoxy-2′-b-chlorocytidine TP, 2′-Deoxy-2′-b-fluorocytidine TP, 2′-Deoxy-2′-b-iodocytidine TP, 2′-Deoxy-2′-b-mercaptopcytidine TP, 2′-Deoxy-2′-b-thiomethoxycytidine TP, 2′-0-Methyl-5-(1-propynyl)cytidine TP, 3′-Ethynlycytidine TP, 4′-Azidocytidine TP, 4′-Carbocyclic cytidine TP, 4′-Ethynlycytidine TP, 5-(1-Propynyl)ara-cytidine TP, 5-(2-Chloro-phenyl)-2-thiocytidine TP, 5-(4-Amino-phenyl)-2-thiocytidine TP, 5-Aminoallyl-
CTP, 5-Cyanocytidine TP, 5-Ethynylara-cytidine TP, 5-Ethynylcytidine TP, 5'-Homo-cytidine TP, 5-Methoxycytidine TP, 5-Trifluoromethyl-Cytidine TP, N4-Amino-cytidine TP, N4-Benzoyl-cytidine TP, pseudoisocytidine, 7-methylguanosine, N2,2'-0-dimethylguanosine, N2-methylguanosine, wyosine, 1,2'-0-dimethylguanosine, 1-methylguanosine, 2'-0-methylguanosine, 2'-0-ribosylguanosine (phosphate), 2'-0-methylguanosine, 2'-0-ribosylguanosine (phosphate), 7-aminomethyl-7-deazaguanosine, 7-cyano-7-deazaguanosine, archaeosine, methylwyosine, N2,7-dimethylguanosine, N2,N2,2'-0-trimethylguanosine, N2,N2,7,2'-0-trimethylguanosine, 6-thio-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, N1-methyl-guanosine, a-thio-guanosine, 2-(propyl)guanine, 2-(alkyl)guanine, 2'-Amino-2'-deoxy-GTP, 2'-Azido-2'-deoxy-GTP, 2'-Deoxy-2'-a-aminoguanosine TP, 2'-Deoxy-2'-a-azidoguanosine TP, 6 (methyl)guanine, 6-(alkyl)guanine, 6-(methyl)guanine, 6-methyl-guanosine, 7 (alkyl)guanine, 7 (deaza)guanine, 7 (methyl)guanine, 7-(alkyl)guanine, 7-(deaza)guanine, 7-(methyl)guanine, 8 (alkyl)guanine, 8 (alkynyl)guanine, 8 (halo)guanine, 8 (thioalkyl)guanine, 8-(alkenyl)guanine, 8-(alkyl)guanine, 8-(alkynyl)guanine, 8-(amino)guanine, 8-(halo)guanine, 8-(hydroxy)guanine, 8-(thioalkyl)guanine, 8-(thiol)guanine, aza guanine, deaza guanine, N (methyl)guanine, N-(methyl)guanine, 1-methyl-6-thio-guanosine, 6-thio-guanosine, 6-thio-7-deaza-8-aza-guanosine, 6-thio-7-deaza-guanosine, 6-thio-7-methyl-guanosine, 7-deaza-8-aza-guanosine, 7-methyl-8-oxo-guanosine, N2,N2-dimethyl-6-thio-guanosine, N2-methyl-6-thio-guanosine, 1-Me-GTP, 2'Fluoro-N2-isobutyl-guanosine TP, 2'0-methyl-N2-isobutyl-guanosine TP, 2'-a-Ethynylguanosine TP, 2'-a-Trifluoromethylguanosine TP, 2'-b-Ethynylguanosine TP, 2'-b-Trifluoromethylguanosine TP, 2'-Deoxy-2',2'-difluoroguanosine TP, 2'-Deoxy-2'-a-mercaptopoguanosine TP, 2'-Deoxy-2'-a-thiometelhoxyguanosine TP, 2'-Deoxy-2'-b-aminoguanosine TP, 2'-Deoxy-2'-b-azidoguanosine TP, 2'-Deoxy-2'-b-bromoguanosine TP, 2'-Deoxy-2'-b-chloroguanosine TP, 2'-Deoxy-2'-b-fluoroguanosine TP, 2'-Deoxy-2'-b-iodoguanosine TP, 2'-Deoxy-2'-b-mercaptopoguanosine TP, 2'-Deoxy-2'-b-thiomethoxyguanosine TP, 4'-Azidoguanosine TP, 4'-Carbocyclic guanosine TP, 4'-Ethynylguanosine TP, 5'-Homo-guanosine TP, 8-bromo-guanosine TP, 9-Deazaguanosine TP, N2-isobutyl-guanosine TP, 1-methylinosine, inosine, 1,2'-0-
dimethylinosine, 2′-0-methylinosine, 7-methylinosine, 2′-0-methylinosine, 
epoxyqueuosine, galactosyl-queuosine, mannosylqueuosine, queuosine, allyamino-
thymidine, aza thymidine, deaza thymidine, deoxy-thymidine, 2′-0-methyluridine, 2-
thiouridine, 3-methyluridine, 5-carboxymethyluridine, 5-hydroxyuridine, 5-
methyluridine, 5-taurinomethyl-2-thiouridine, 5-taurinomethyluridine, dihydrouridine, 
pseudouridine, (3-(3-amino-3-carboxypropyl)uridine, 1-methyl-3-(3-amino-5-
carboxypropyl)pseudouridine, 1-methylpseduouridine, 1-methyl-pseudouridine, 2′-0-
methyluridine, 2′-0-methylpseudouridine, 2′-0-methyluridine, 2-thio-2′-0-methyluridine, 
3-(3-amino-3-carboxypropyl)uridine, 3,2′-0-dimethyluridine, 3-Methyl-pseudo-Uridine 
TP, 4-thiouridine, 5-(carboxyhydroxymethyl)uridine, 5-(carboxyhydroxymethyl)uridine 
methyl ester, 5,2′-0-dimethyluridine, 5,6-dihydro-uridine, 5-aminomethyl-2-thiouridine, 
5-carbamoylmethyl-2′-0-methyluridine, 5-carbamoylmethyluridine, 5-
carboxyhydroxymethyluridine, 5-carboxyhydroxymethyluridine methyl ester, 5-
carboxymethylaminomethyl-2′-0-methyluridine, 5-carboxymethylaminomethyl-2-
thiouridine, 5-carboxymethylaminomethyl-2-thiouridine, 5-
carboxymethylaminomethyluridine, 5-carboxymethylaminomethyluridine, 5-
Carbamoylmethyluridine TP, 5-methoxycarbonylmethyl-2′-0-methyluridine, 5-
methoxycarbonylmethyl-2-thiouridine, 5-methoxycarbonylmethyluridine, 5-
methoxyuridine, 5-methyl-2-thiouridine, 5-methylaminomethyl-2-selenouridine, 5-
methylaminomethyl-2-thiouridine, 5-methylaminomethyluridine, 5-
Methyldihydrouridine, 5-Oxyacetic acid- Uridine TP, 5-Oxyacetic acid-methyl ester-
Uridine TP, N1-methyl-pseudo-uridine, uridine 5-oxyacetic acid, uridine 5-oxyacetic acid 
methyl ester, 3-(3-Amino-3-carboxypropyl)-Uridine TP, 5-(iso-Pentenylaminomethyl)-
2-thiouridine TP, 5-(iso-Pentenylaminomethyl)-2′-0-methyluridine TP, 5-(iso-
Pentenylaminomethyl)uridine TP, 5-propynyl uracil, a-thio-uridine, 1 (aminoalkylamino-
carbonylethylenyl)-2(thio)-pseudouracil, 1 (aminoalkylaminocarbonylethylenyl)-2,4-
(dithio)pseudouracil, 1 (aminoalkylaminocarbonylethylenyl)-4 (thio)pseudouracil, 1 
(aminoalkylaminocarbonylethylenyl)-pseudouracil, 1 (aminocarboxylethylenyl)-2(thio)-
pseudouracil, 1 (aminocarboxylethylenyl)-2,4-(dithio)pseudouracil, 1 
(aminocarboxylethylenyl)-4 (thio)pseudouracil, 1 (aminocarboxylethylenyl)-
pseudouracil, 1 substituted 2(thio)-pseudouracil, 1 substituted 2,4-(dithio)pseudouracil, 1
substituted 4 (thio)pseudouracil, 1 substituted pseudouracil, 1-(aminoalkylamino-carbonylethyl)-2-(thio)-pseudouracil, 1-Methyl-3-(3-amino-3-carboxypropyl) pseudouridine TP, 1-Methyl-3-(3-amino-3-carboxypropyl)pseudo-UTP, 1-Methyl-pseudo-UTP, 2 (thio)pseudouracil, 2' deoxy uridine, 2' fluorouridine, 2-(thio)uracil, 2,4-(dithio)pseudouracil, 2' methyl, 2'amino, 2'azido, 2'fluro-guanosine, 2'-Amino-2'-deoxy-UTP, 2'-Azido-2'-deoxy-UTP, 2'-Azido-deoxyuridine TP, 2'-0-methylpseudouridine, 2' deoxy uridine, 2' fluorouridine, 2'-Deoxy-2'-a-aminouridine TP, 2'-Deoxy-2'-a-azidouridine TP, 2'-methylpseudouridine, 3 (3 amino-3 carboxypropyl)uracil, 4 (thio)pseudouracil, 4-(thio )pseudouracil, 4-(thio)uracil, 4-thiouracil, 5 (1,3-diazone-l-alkyl)uracil, 5 (2-amino propyl)uracil, 5 (aminoalkyl)uracil, 5 (dimethylaminoalkyl)uracil, 5 (guanidiniumalkyl)uracil, 5 (methoxycarbonylmethyl)-2-(thio)uracil, 5 (methoxycarbonyl-methyl)uracil, 5 (methyl) 2 (thio)uracil, 5 (methyl) 2,4 (dithio)uracil, 5 (methyl) 4 (thio)uracil, 5 (methylaminomethyl)-2 (thio)uracil, 5 (methylaminomethyl)-2,4 (dithio)uracil, 5 (methylaminomethyl)-4 (thio)uracil, 5 (propynyl)uracil, 5 (trifluoromethyl)uracil, 5-(2-aminopropyl)uracil, 5-(alkyl)-2 (thio)pseudouracil, 5-(alkyl)-2,4 (dithio)pseudouracil, 5-(alkyl)-4 (thio)pseudouracil, 5-(alkyl)pseudouracil, 5-(alkyl)uracil, 5-(alkynyl)uracil, 5-(allylamo)uracil, 5-(cyanoalkyl)uracil, 5-(dialkylaminoalkyl)uracil, 5-(dimethylaminoalkyl)uracil, 5-(guanidiniumalkyl)uracil, 5-(halo)uracil, 5-(1,3-diazone-l-alkyl)uracil, 5-(methoxy)uracil, 5-(methoxycarbonylmethyl)-2-(thio)uracil, 5-(methoxycarbonyl-methyl)uracil, 5-(methyl) 2(thio)uracil, 5-(methyl) 2,4 (dithio) uracil, 5-(methyl) 4 (thio)uracil, 5-(methyl)-2-(thio)pseudouracil, 5-(methyl)-2,4 (dithio)pseudouracil, 5-(methyl)4 (thio)pseudouracil, 5-(methyl)pseudouracil, 5-(methyl) pseudouracil, 5-(methylaminomethyl)-2 (thio)uracil, 5-(methylaminomethyl)-2,4(dithio )uracil, 5-(methylaminomethyl)-4-(thio)uracil, 5-(propynyl)uracil, 5-(trifluoromethyl)uracil, 5-aminalkyl-uridine, 5-bromo-uridine, 5-iodo-uridine, 5-uracil, 6 (azo)uracil, 6-(azo)uracil, 6-aza-uridine, allyamino-uracil, aza uracil, deaza uracil, N3 (methyl)uracil, Pseudo-UTP-1-2-ethanoic acid, pseudouracil, 4-Thio-pseudo-UTP, 1-carboxymethyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 1-propynyl-uridine, 1-taurinomethyl-1-methyl-uridine, 1-taurinomethyl-4-thio-uridine, 1-taurinomethyl-pseudouridine, 2-methoxy-4-thio-pseudouridine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-
dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-
pseudouridine, 4-methoxy-pseudouridine, 4-thio-l-methyl-pseudouridine, 4-thio-
pseudouridine, 5-aza-uridine, dihydropseudouridine, (+)-l-(2-
Hydroxypropyl)pseudouridine TP, (2R)-l-(2-Hydroxypropyl)pseudouridine TP, (2S)-l-
(2-Hydroxypropyl)pseudouridine TP, (E)-5-(2-Bromo-vinyl)ara-uridine TP, (E)-5-(2-
Bromo-vinyl)uridine TP, (Z)-5-(2-Bromo-vinyl)ara-uridine TP, (Z)-5-(2-Bromo-
viny1)uridine TP, l-(2,2,2-Trifluoroethyl)-pseudo-UTP , l-(2,2,3,3,3-
Pentafluoropropyl)pseudouridine TP, l-(2,2-Diethoxyethyl)pseudouridine TP, l-(2,4,6-
Trimethylbenzyl)pseudouridine TP, l-(2,4,6-Trimethyl-phenyl)pseudo-UTP , l-(2-Amino-2-carboxyethyl)pseudo-UTP, l-(2-Amino-
ethyl)pseudo-UTP, l-(2-Hydroxyethyl)pseudouridine TP, l-(2-
Methoxyethyl)pseudouridine TP, l-(3,4-Bis-trifluoromethoxybenzyl)pseudouridine TP, l-(3,4-Dimethoxybenzyl)pseudouridine TP, l-(3-Amino-3-carboxypropyl)pseudo-UTP , l-(3-Amino-propyl)pseudo-UTP, l-(3-Cyclopropyl-prop-2-ynyl)pseudouridine TP, l-(4-
Amino-4-carboxybutyl)pseudo-UTP, l-(4-Amino-benzyl)pseudo-UTP, l-(4-Amino-
butyl)pseudo-UTP, l-(4-Amino-phenyl)pseudo-UTP , l-(4-Azidobenzyl)pseudouridine TP, l-(4-Bromobenzyl)pseudouridine TP, l-(4-Chlorobenzyl)pseudouridine TP, l-(4-
Fluorobenzyl)pseudouridine TP, l-(4-Iodobenzyl)pseudouridine TP, l-(4-
Methanesulfonylbenzyl)pseudouridine TP, l-(4-Methoxybenzyl)pseudouridine TP, l-(4-
Methoxy-benzyl)pseudo-UTP, l-(4-Methoxy-phenyl)pseudo-UTP , l-(4-
Methylbenzyl)pseudouridine TP, l-(4-Methyl-benzyl)pseudo-UTP , l-(4-
Nitrobenzyl)pseudouridine TP, l-(4-Nitro-benzyl)pseudo-UTP, l-(4-Nitro-
phenyl)pseudo-UTP , l-(4-Thiomethoxybenzyl)pseudouridine TP, l-(4-
Trifluoromethoxybenzyl)pseudouridine TP, l-(4-Trifluoromethylbenzyl)pseudouridine TP, l-(5-Amino-pentyl)pseudo-UTP, l-(6-Amino-hexyl)pseudo-UTP, 1,6-Dimethyl-
pseudo-UTP, l-[3-2-[2-(2-Aminoethoxy)-ethoxy]-ethoxy]-ethoxy)-
propionyl)pseudouridine TP, l-[3-2-(2-Aminoethoxy)-ethoxy]-propionyl} 
pseudouridine TP, l-(Acetyl)pseudouridine TP, l-(Alkyl-6-(l-propynyl))-pseudo-UTP, 1-
Alkyl-6-(2-propynyl)-pseudo-UTP, l-(Alkyl-6-allyl)-pseudo-UTP, l-(Alkyl-6-ethyl-
pseudo-UTP, l-(Alkyl-6-homoallyl)-pseudo-UTP, l-(Alkyl-6-vinyl)-pseudo-UTP, 1-
Allylpseudouridine TP, l-(Aminomethyl)-pseudo-UTP , l-Bensoylpseudouridine TP, l-
Deoxy-2'-a-mercaptouridine TP, 2'-Deoxy-2'-a-thiomethoxyuridine TP, 2'-Deoxy-2'-b-aminouridine TP, 2'-Deoxy-2'-b-azidouridine TP, 2'-Deoxy-2'-b-bromouridine TP, 2'-Deoxy-2'-b-chlorouridine TP, 2'-Deoxy-2'-b-fluorouridine TP, 2'-Deoxy-2'-b-iodouridine TP, 2'-Deoxy-2'-b-mercaptouridine TP, 2'-Deoxy-2'-b-thiomethoxyuridine TP, 2-methoxy-4-thio-uridine, 2-methoxyuridine, 2'-0-Methyl-5-(l-propynyl)uridine TP, 3-Alkyl-pseudo-UTP, 4'-Azidouridine TP, 4'-Carbocyclic uridine TP, 4'-Ethynyluridine TP, 5-(l-Propynyl)ara-uridine TP, 5-(2-Furanyl)uridine TP, 5-Cyanouridine TP, 5-Dimethylaminouridine TP, 5'-Homo-uridine TP, 5-Iodo-2'-fluoro-deoxyuridine TP, 5-Phenylethynyluridine TP, 5-Trideuteromethyl-6-deuterouridine TP, 5-Trinuoromethyl-Uridine TP, 5-Vinylarauridine TP, 6-(2,2,2-Trifluoroethyl)-pseudo-UTP, 6-(4-Morpholino)-pseudo-UTP, 6-(4-Thiomorpholino)-pseudo-UTP, 6-(Substituted-Phenyl)-pseudo-UTP, 6-Amino-pseudo-UTP, 6-Azido-pseudo-UTP, 6-Bromo-pseudo-UTP, 6-Butyl-pseudo-UTP, 6-Chloro-pseudo-UTP, 6-Cyano-pseudo-UTP, 6-Dimethylaminopseudo-UTP, 6-Ethoxy-pseudo-UTP, 6-Ethylcarboxylate-pseudo-UTP, 6-Ethyl-pseudo-UTP, 6-Fluoro-pseudo-UTP, 6-Formyl-pseudo-UTP, 6-Hydroxyamino-pseudo-UTP, 6-Hydroxy-pseudo-UTP, 6-Iodo-pseudo-UTP, 6-iso-Propyl-pseudo-UTP, 6-Methoxy-pseudo-UTP, 6-Methylamino-pseudo-UTP, 6-Methyl-pseudo-UTP, 6-Methyl-pseudo-UTP, 6-Phenyl-pseudo-UTP, 6-Phenyl-pseudo-UTP, 6-Propyl-pseudo-UTP, 6-tert-Butyl-pseudo-UTP, 6-Trifluoromethoxy-pseudo-UTP, 6-Trifluoromethyl-pseudo-UTP, Alpha-thio-pseudo-UTP, Pseudouridine l-(4-methylbenzenesulfonic acid) TP, Pseudouridine l-(4-methylbenzoic acid) TP, Pseudouridine TP l-[3-(2-ethoxy)]propionic acid, Pseudouridine TP l-[3-{2-[2-{2-(2-ethoxy)-ethoxy}]-ethoxy}-ethoxy]propionic acid, Pseudouridine TP l-[3-{2-[2-{2-(2-ethoxy)-ethoxy}-ethoxy]-ethoxy}]-ethoxy]propionic acid, Pseudouridine TP l-[3-{2-[2-ethoxy]-ethoxy}]-ethoxy]propionic acid, Pseudouridine TP l-[3-{2-[2-ethoxy]-ethoxy}] propionic acid, Pseudouridine TP 1-methylphosphonic acid, Pseudouridine TP 1-methylphosphonic acid diethyl ester, Pseudo-UTP-NI-3-propionic acid, Pseudo-UTP-NI-4-butanoic acid, Pseudo-UTP-NI-5-pentanoic acid, Pseudo-UTP-NI-6-hexanoic acid, Pseudo-UTP-NI-7-heptanoic acid, Pseudo-UTP-NI-methyl-p-benzoic acid, Pseudo-UTP-NI-p-benzoic acid, wybutosine, hydroxywybutosine, isowyosine, peroxywybutosine, undermodified hydroxywybutosine, and/or 4-demethylwyosine.
In some embodiments, an mRNA of the invention includes a combination of one or more of the aforementioned modified nucleobases (e.g., a combination of 2, 3 or 4 of the aforementioned modified nucleobases.)

In some embodiments, the modified nucleobase is pseudouridine (ψ), N1-methylpseudouridine (m1ψ), 2-thiouridine, 4′-thiouridine, 5-methylcytosine, 2-thio-l-methyl-l-deaza-pseudouridine, 2-thio-l-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-5-aza-uridine, 2-thio-2-thio-pseudouridine, 2-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methoxyuridine, or 2′-0-methyl uridine. In some embodiments, an mRNA of the invention includes a combination of one or more of the aforementioned modified nucleobases (e.g., a combination of 2, 3 or 4 of the aforementioned modified nucleobases.)

In some embodiments, the modified nucleobase is 1-methyl-pseudouridine (m1ψ), 5-methoxy-uridine (mo5U), 5-methyl-cytidine (m5C), pseudouridine (ψ), α-thio-guanosine, or α-thio-adenosine. In some embodiments, an mRNA of the invention includes a combination of one or more of the aforementioned modified nucleobases (e.g., a combination of 2, 3 or 4 of the aforementioned modified nucleobases.)

In some embodiments, the mRNA comprises pseudouridine (ψ). In some embodiments, the mRNA comprises pseudouridine (ψ) and 5-methyl-cytidine (m5C). In some embodiments, the mRNA comprises 1-methyl-pseudouridine (mψ)- In some embodiments, the mRNA comprises 1-methyl-pseudouridine (mψ) and 5-methyl-cytidine (m5C). In some embodiments, the mRNA comprises 2-thiouridine (s2U). In some embodiments, the mRNA comprises 2-thiouridine and 5-methyl-cytidine (m5C). In some embodiments, the mRNA comprises 5-methoxy-uridine (mo5U). In some embodiments, the mRNA comprises 5-methoxy-uridine (mo5U) and 5-methyl-cytidine (m5C). In some embodiments, the mRNA comprises 2′-0-methyl uridine. In some embodiments, the mRNA comprises 2′-0-methyl uridine and 5-methyl-cytidine (m5C). In some embodiments, the mRNA comprises comprises N6-methyl-adenosine (m6A). In some embodiments, the mRNA comprises N6-methyl-adenosine (m6A) and 5-methyl-cytidine (m5C).
In some embodiments, the modified nucleobase is a modified cytosine. Exemplary nucleobases and nucleosides having a modified cytosine include N4-acetylcytidine (ac4C), 5-methyl-cytidine (m5C), 5-halo-cytidine (e.g., 5-iodo-cytidine), 5-hydroxymethyl-cytidine (hm5C), 1-methyl-pseudoisocytidine, 2-thio-cytidine (s2C), 2-thio-5-methyl-cytidine. In some embodiments, an mRNA of the invention includes a combination of one or more of the aforementioned modified nucleobases (e.g., a combination of 2, 3 or 4 of the aforementioned modified nucleobases.)

In some embodiments, the modified nucleobase is a modified adenine. Exemplary nucleobases and nucleosides having a modified adenine include 7-deazaadenine, 1-methyl-adenosine (m1A), 2-methyl-adenine (m2A), N6-methyl-adenosine (m6A). In some embodiments, an mRNA of the invention includes a combination of one or more of the aforementioned modified nucleobases (e.g., a combination of 2, 3 or 4 of the aforementioned modified nucleobases.)

In some embodiments, the modified nucleobase is a modified guanine. Exemplary nucleobases and nucleosides having a modified guanine include inosine (I), 1-methyl-inosine (iVI), wytosine (imG), methylwyosine (mimG), 7-deaza-guanosine, 7-cyano-7-deaza-guanosine (preQ0), 7-aminoethyl-7-deaza-guanosine (preQO), 7-methylguanosine (m7G), 1-methyl-guanosine (m1G), 8-oxo-guanosine, 7-methyl-8-oxo-guanosine. In some embodiments, an mRNA of the invention includes a combination of one or more of the aforementioned modified nucleobases (e.g., a combination of 2, 3 or 4 of the aforementioned modified nucleobases.)

In certain embodiments, an mRNA of the invention is uniformly modified (i.e., fully modified, modified through-out the entire sequence) for a particular modification. For example, an mRNA can be uniformly modified with 5-methyl-cytidine (m5C), meaning that all cytosine residues in the mRNA sequence are replaced with 5-methyl-cytidine (m5C). Similarly, mRNAs of the invention can be uniformly modified for any type of nucleoside residue present in the sequence by replacement with a modified residue such as those set forth above.

Further modifications of the polynucleotides of the compositions which are useful in the present invention include, but are not limited to, 2,6-(diamino)purine, 1-
(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 1,3-(diaza)-2-(oxo)-phenthiazin-1-yl, 1,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 1,3,5-(triaza)-2,6-(dioxa)-naphthalene, 2-(amino)purine, 2,4,5-(trimethyl)phenyl, 2'-methyl, 2'amino, 2'azido, 2'fluoro-cytidine, 2' methyl, 2'amino, 2'azido, 2'fluoro-adenine, 2'methyl, 2'amino, 2'azido, 2'fluoro-uridine, 2'-amino-2'-deoxyribose, 2-amino-6-Chloro-purine, 2-aza-inosinyl, 2'-azido-2'-deoxyribose, 2'fluoro-2'-deoxyribose, 2'-fluoro-modified bases, 2'-0-methyl-ribose, 2-oxo-7-aminopyridopyrimidin-3-yl, 2-oxo-pyridopyrimidine-3-yl, 2-pyridinone, 3 nitropyrrrole, 3-(methyl)-7-(propynyl)isocarbostyrilyl, 3-(methyl)isocarbostyrilyl, 4-(fluro)-6-(methyl)benzimidazole, 4-(methyl)benzimidazole, 4-(methyl)indolyl, 4,6-(dimethyl)indolyl, 5 nitroindole, 5 substituted pyrimidines, 5-(methyl)isocarbostyrilyl, 5-nitroindole, 6-(aza)pyrimidine, 6-(azo)thymine, 6-(methyl)-7-(aza)indolyl, 6-chloropurine, 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, 7-(aminoalkylhydroxy)-l-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-(aminoalkylhydroxy)-l,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 7-(aminoalkylhydroxy)-l,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 7-(guanidiniumalkylhydroxy)-l-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-(guanidiniumalkylhydroxy)-l-(aza)-3-(diaza)-2-(oxo)-phenoxazin-1-yl, 7-(guanidiniumalkylhydroxy)-l,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 7-(propynyl)isocarbostyrilyl, 7-(propynyl)isocarbostyrilyl, 7-deaza-inosinyl, 7-substituted 1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-substituted 1,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 9-(methyl)-imidizopyridinyl, aminoinodolyl, anthracenyl, bis-ortho-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, bis-ortho-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, difluorotolyl, hypoxanthine, imidizopyridinyl, inosinyl, isocarbostyrilyl, isoguanisine, N2-substituted purines, N6-methyl-2-aminopurine, N6-substituted purines, N-alkylated derivative, napthalenyl, nitrobenzimidazolyl, nitroimidazolyl, nitroindazolyl, nitropyrazolyl, nubularine, 06-substituted purines, O-alkylated derivative, ortho-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl,
ortho-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, Oxoformycin TP, para-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, para-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, pentacetyl, phenanthracenyl, phenyl, propynyl-7-(aza)indolyl, pyrenyl, pyridopyrimidin-3-yl, pyridopyrimidin-3-yl, 2-oxo-7-amino-pyridopyrimidin-3-yl, pyrrolo-pyrimidin-2-on-3-yl, pyrrolopyrimidinyl, pyrrolopyrizinyl, substituted 1,2,4-triazoles, tetracenyl, tubercidine, xanthine, Xanthosine-5'-TP, 2-thio-zebularine, 5-aza-2-thio-zebularine, 7-deaza-2-amino-purine, pyridin-4-one ribonucleoside, 2-Amino-riboside-TP, Foraiycin A TP, Foraiycin B TP, Pyrrolosine TP, 2′-OH-ara-adenosine TP, 2′-OH-ara-cytidine TP, 2′-OH-ara-uridine TP, 2′-OH-ara-guanosine TP, 5′-(2-carbomethoxyvinyl)uridine TP, and/or N6-(19-Amino-pentaoxanonadecyl)adenosine TP.

Linkers which are useful in the present invention include, but are not limited to, 3′-alkylene phosphonates, 3′-amino phosphoramidate, alkene containing backbones, aminoalkylphosphoramidates, aminooalkylphosphotriesters, boranophosphates, -CH2-0-N(CH3)-CH2-, -CH2-N(CH3)-N(CH3)-CH2-, -CH2-NH-CH2-, chiral phosphonates, chiral phosphorothioates, formacetyl and thioformacetyl backbones, methylene (methylimino), methylene formacetyl and thioformacetyl backbones, methylenemimino and methylenehydratino backbones, morpholino linkages, -N(CH3)-CH2-CH2-, oligonucleosides with heteroatom internucleoside linkage, phosphinates, phosphoramidates, phosphorodithioates, phosphorothioate internucleoside linkages, phosphorothioates, phosphotriesters, PNA, siloxane backbones, sulfamate backbones, sulfide sulfoxide and sulfone backbones, sulfonate and sulfonamide backbones, thionoalkylphosphonates, thionoalkylphosphotriesters, and/or thionophosphoramidates, or any of the modifications, both naturally occurring and non-naturally occurring) described in International Applications WO20 13/052523, WO20 14/093924, WO2015/051173, WO20 15/05 1169, and PCT/US2015/036773, the contents of each of which are incorporated herein by reference in their entireties.

[000377] 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).

[000378] Additional modifications are described herein. For example, in certain embodiments, the at least one chemically modified nucleoside comprises a 1-methylpseudouridine or a 5-methyl-cytosine. In one embodiment, the polynucleotide is fully substituted with 1-methylpseudouridine (i.e., all uridine residues are replaced with 1-methylpseudouridine). In one embodiment, the polynucleotide is fully substituted with 5-methyl-cytosine (i.e., all cytosine residues are replaced with 5-methyl cytosine). In one embodiment, the polynucleotide is fully substituted with both 1-methylpseudouridine and with 5-methyl-cytosine.

Modified Polynucleotide Molecules

[000379] 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 Applications WO2013/052523, WO2014/093924, WO2015/051173, WO20 15/051169, and PCT/US2015/036773, the the contents of each of which are incorporated herein by reference in its entirety.

Combinations of Modified Sugars, Nucleobases, and Internucleoside Linkages

[000380] The chimeric 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.

[000381] Examples of modified nucleotides and modified nucleotide combinations are provided below in Table 4 and Table 5. These combinations of modified nucleotides can be used to form the chimeric polynucleotides of the invention. Unless otherwise noted,
the modified nucleotides may be completely substituted for the natural nucleotides of the chimeric 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.

[000382] Any combination of base/sugar or linker may be incorporated into the chimeric polynucleotides of the invention and such modifications are taught in International Applications WO20 13/052523, WO20 14/093924, WO2015/051173, WO2015/051169, and PCT/US2015/036773, the contents of each of which are incorporated herein by reference in its entirety.

<table>
<thead>
<tr>
<th>Modified Nucleotide</th>
<th>Modified Nucleotide Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-thio-ctydine</td>
<td>α-thio-ctydine/5-iodo-ctydine</td>
</tr>
<tr>
<td></td>
<td>α-thio-ctydine/N1-methyl-ctydine</td>
</tr>
<tr>
<td></td>
<td>α-thio-ctydine/α-thio-ctydine</td>
</tr>
<tr>
<td></td>
<td>α-thio-ctydine/5-methyl-ctydine</td>
</tr>
<tr>
<td></td>
<td>α-thio-ctydine/pseudo-ctydine</td>
</tr>
<tr>
<td></td>
<td>about 50% of the cytosines are α-thio-ctydine</td>
</tr>
<tr>
<td>pseudoisocytidine</td>
<td>pseudoisocytidine/5-iodo-ctydine</td>
</tr>
<tr>
<td></td>
<td>pseudoisocytidine/N1-methyl-ctydine</td>
</tr>
<tr>
<td></td>
<td>pseudoisocytidine/α-thio-ctydine</td>
</tr>
<tr>
<td></td>
<td>pseudoisocytidine/5-methyl-ctydine</td>
</tr>
<tr>
<td></td>
<td>pseudoisocytidine/pseudo-ctydine</td>
</tr>
<tr>
<td></td>
<td>about 25% of cytosines are pseudoisocytidine</td>
</tr>
<tr>
<td></td>
<td>pseudoisocytidine/about 50% of uridines are N1-methyl-ctydine and</td>
</tr>
<tr>
<td></td>
<td>about 50% of uridines are pseudo-ctydine</td>
</tr>
<tr>
<td></td>
<td>pseudoisocytidine/about 25% of uridines are N1-methyl-ctydine and</td>
</tr>
<tr>
<td></td>
<td>about 25% of uridines are pseudo-ctydine</td>
</tr>
<tr>
<td>pyrrolo-ctydine</td>
<td>pyrrolo-ctydine/5-iodo-ctydine</td>
</tr>
<tr>
<td></td>
<td>pyrrolo-ctydine/N1-methyl-ctydine</td>
</tr>
<tr>
<td></td>
<td>pyrrolo-ctydine/α-thio-ctydine</td>
</tr>
<tr>
<td></td>
<td>pyrrolo-ctydine/5-methyl-ctydine</td>
</tr>
<tr>
<td></td>
<td>pyrrolo-ctydine/pseudo-ctydine</td>
</tr>
<tr>
<td></td>
<td>about 50% of the cytosines are pyrrolo-ctydine</td>
</tr>
<tr>
<td>5-methyl-ctydine</td>
<td>5-methyl-ctydine/5-iodo-ctydine</td>
</tr>
<tr>
<td></td>
<td>5-methyl-ctydine/N1-methyl-ctydine</td>
</tr>
<tr>
<td></td>
<td>5-methyl-ctydine/α-thio-ctydine</td>
</tr>
</tbody>
</table>
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
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 5. Combinations

<table>
<thead>
<tr>
<th>1-(2,2,2-Trifluoroethyl)pseudo-UTP</th>
<th>25 % 5-Aminoallyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Ethyl-pseudo-UTP</td>
<td>25 % 5-Aminoallyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP</td>
</tr>
<tr>
<td>1-Methyl-pseudo-U-alpha-thio-TP</td>
<td>25 % 5-Bromo-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP</td>
</tr>
<tr>
<td>1-methyl-pseudouridine TP, ATP, GTP, CTP</td>
<td>25 % 5-Bromo-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP</td>
</tr>
<tr>
<td>1-methyl-pseudo-UTP/5-methyl-CTP/ATP/GTP</td>
<td>25 % 5-Bromo-CTP + 75 % CTP/1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>1-Propyl-pseudo-UTP</td>
<td>25 % 5-Carboxy-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP</td>
</tr>
<tr>
<td></td>
<td>25 % 5-Carboxy-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP</td>
</tr>
<tr>
<td></td>
<td>25 % 5-Ethyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP</td>
</tr>
<tr>
<td></td>
<td>25 % 5-Ethyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP</td>
</tr>
<tr>
<td>Percentage</td>
<td>Nucleic Acid Composition</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>25%</td>
<td>5-Ethynyl-CTP + 75% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Ethynyl-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Fluoro-CTP + 75% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Fluoro-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Formyl-CTP + 75% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Formyl-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Hydroxymethyl-CTP + 75% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Hydroxymethyl-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Iodo-CTP + 75% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Iodo-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Methoxy-CTP + 75% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Methoxy-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Methyl-CTP + 75% CTP/25% 5-Methoxy-UTP + 75% 1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Methyl-CTP + 75% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Methyl-CTP + 75% CTP/50% 5-Methoxy-UTP + 50% 1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Methyl-CTP + 75% CTP/50% 5-Methoxy-UTP + 50% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Methyl-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Methyl-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% 1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Methyl-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Phenyl-CTP + 75% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Phenyl-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Trifluoromethyl-CTP + 75% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Trifluoromethyl-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Trifluoromethyl-CTP + 75% CTP/1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>25%</td>
<td>N4-Ac-CTP + 75% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>N4-Ac-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>N4-Bz-CTP + 75% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>N4-Bz-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>N4-Methyl-CTP + 75% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>N4-Methyl-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>Pseudo-iso-CTP + 75% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>Pseudo-iso-CTP + 75% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-Bromo-CTP/75% CTP/Pseudo-UTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-methoxy-UTP/25% 5-methyl-CTP/ATP/GTP</td>
</tr>
<tr>
<td>Percentage</td>
<td>Nucleotide Combinations</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------------------------------------------------------</td>
</tr>
<tr>
<td>25%</td>
<td>5-methoxy-UTP/5-methyl-CTP/ATP/GTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-methoxy-UTP/CTP/ATP/GTP</td>
</tr>
<tr>
<td>25%</td>
<td>5-methoxy-UTP/50% 5-methyl-CTP/ATP/GTP</td>
</tr>
<tr>
<td>2-Amino-ATP</td>
<td></td>
</tr>
<tr>
<td>2-Thio-CTP</td>
<td></td>
</tr>
<tr>
<td>2-thio-pseudouridine TP, ATP, GTP, CTP</td>
<td></td>
</tr>
<tr>
<td>2-Thio-pseudo-UTP</td>
<td></td>
</tr>
<tr>
<td>2-Thio-UTP</td>
<td></td>
</tr>
<tr>
<td>3-Methyl-CTP</td>
<td></td>
</tr>
<tr>
<td>3-Methyl-pseudo-UTP</td>
<td></td>
</tr>
<tr>
<td>4-Thio-UTP</td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>5-Bromo-CTP + 50% CTP/1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-Hydroxymethyl-CTP + 50% CTP/1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-methoxy-UTP/5-methyl-CTP/ATP/GTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-Methyl-CTP + 50% CTP/25% 5-Methoxy-UTP + 75% 1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-Methyl-CTP + 50% CTP/25% 5-Methoxy-UTP + 75% 1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-Methyl-CTP + 50% CTP/50% 5-Methoxy-UTP + 50% 1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-Methyl-CTP + 50% CTP/50% 5-Methoxy-UTP + 50% 1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-Methyl-CTP + 50% CTP/5-Methoxy-UTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-Methyl-CTP + 50% CTP/75% 5-Methoxy-UTP + 25% 1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-Methyl-CTP + 50% CTP/75% 5-Methoxy-UTP + 25% 1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-Trifluoromethyl-CTP + 50% CTP/1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-Bromo-CTP/50% CTP/Pseudo-UTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-methoxy-UTP/25% 5-methyl-CTP/ATP/GTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-methoxy-UTP/50% 5-methyl-CTP/ATP/GTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP</td>
</tr>
<tr>
<td>50%</td>
<td>5-methoxy-UTP/CTP/ATP/GTP</td>
</tr>
<tr>
<td>5-Aminoallyl-CTP</td>
<td></td>
</tr>
<tr>
<td>5-Aminoallyl-CTP/ 5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Aminoallyl-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Bromo-CTP</td>
<td></td>
</tr>
<tr>
<td>5-Bromo-CTP/ 5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Bromo-CTP/l-Methyl-pseudo-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Bromo-CTP/Pseudo-UTP</td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td></td>
</tr>
<tr>
<td>5-bromocytidine TP, ATP, GTP, UTP</td>
<td></td>
</tr>
<tr>
<td>5-Bromo-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Carboxy-CTP/5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Ethyl-CTP/5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Ethynyl-CTP/5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Fluoro-CTP/5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Formyl-CTP/5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Hydroxymethyl-CTP</td>
<td></td>
</tr>
<tr>
<td>5-Hydroxymethyl-CTP/l-Methyl-pseudo-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Hydroxymethyl-CTP/5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-hydroxymethyl-cytidine TP, ATP, GTP, UTP</td>
<td></td>
</tr>
<tr>
<td>5-Iodo-CTP/5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Me-CTP/5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Methoxy carbonyl methyl-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Methoxy-CTP/5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-methoxy-uridine TP, ATP, GTP, UTP</td>
<td></td>
</tr>
<tr>
<td>5-methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Methoxy-UTP/N6-Isopentenyl-ATP</td>
<td></td>
</tr>
<tr>
<td>5-methoxy-UTP/25%5-methyl-CTP/ATP/GTP</td>
<td></td>
</tr>
<tr>
<td>5-methoxy-UTP/5-methyl-CTP/ATP/GTP</td>
<td></td>
</tr>
<tr>
<td>5-methoxy-UTP/75%5-methyl-CTP/ATP/GTP</td>
<td></td>
</tr>
<tr>
<td>5-methoxy-UTP/CTP/ATP/GTP</td>
<td></td>
</tr>
<tr>
<td>5-Methyl-2-thio-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Methylaminomethyl-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Methyl-CTP/5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Methyl-CTP/5-Methoxy-UTP(cap 0)</td>
<td></td>
</tr>
<tr>
<td>5-Methyl-CTP/5-Methoxy-UTP(No cap)</td>
<td></td>
</tr>
<tr>
<td>5-Methyl-CTP/25%5-Methoxy-UTP + 75%1-Methyl-pseudo-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Methyl-CTP/25%5-Methoxy-UTP + 75%UTP</td>
<td></td>
</tr>
<tr>
<td>5-Methyl-CTP/50%5-Methoxy-UTP + 50%1-Methyl-pseudo-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Methyl-CTP/50%5-Methoxy-UTP + 50%UTP</td>
<td></td>
</tr>
<tr>
<td>5-Methyl-CTP/5-Methoxy-UTP/N6-Me-ATP</td>
<td></td>
</tr>
<tr>
<td>Formulation</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>5-Methyl-CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Methyl-CTP/75 % 5-Methoxy-UTP + 25 % UTP</td>
<td></td>
</tr>
<tr>
<td>5-Phenyl-CTP/ 5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Trilluoro- methyl-CTP/ 5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Trilluoromethyl-CTP</td>
<td></td>
</tr>
<tr>
<td>5-Trilluoromethyl-CTP/ 5-Methoxy-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Trilluoromethyl-CTP/l-Methyl-pseudo-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Trilluoromethyl-CTP/Pseudo-UTP</td>
<td></td>
</tr>
<tr>
<td>5-Trilluoromethyl-UTP</td>
<td></td>
</tr>
</tbody>
</table>

5-trilluromethylcytidine 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/ 75 % 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
<table>
<thead>
<tr>
<th>Percentage</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>75%</td>
<td>5-Methyl-CTP + 25% CTP/50% 5-Methoxy-UTP + 50% UTP</td>
</tr>
<tr>
<td>75%</td>
<td>5-Methyl-CTP + 25% CTP/5-Methoxy-UTP</td>
</tr>
<tr>
<td>75%</td>
<td>5-Methyl-CTP + 25% CTP/75% 5-Methoxy-UTP + 25% 1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>75%</td>
<td>5-Methyl-CTP + 25% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>75%</td>
<td>5-Phenyl-CTP + 25% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>75%</td>
<td>5-Phenyl-CTP + 25% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>75%</td>
<td>5-Trifluoromethyl-CTP + 25% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>75%</td>
<td>5-Trifluoromethyl-CTP + 25% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>75%</td>
<td>5-Trifluoromethyl-CTP + 25% CTP/1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>75%</td>
<td>N4-Ac-CTP + 25% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>75%</td>
<td>N4-Ac-CTP + 25% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>75%</td>
<td>N4-Bz-CTP + 25% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>75%</td>
<td>N4-Bz-CTP + 25% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>75%</td>
<td>N4-Methyl-CTP + 25% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>75%</td>
<td>N4-Methyl-CTP + 25% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>75%</td>
<td>Pseudo-iso-CTP + 25% CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td>75%</td>
<td>Pseudo-iso-CTP + 25% CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
<tr>
<td>75%</td>
<td>5-Bromo-CTP/25% CTP/1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td>75%</td>
<td>5-Bromo-CTP/25% CTP/Pseudo-UTP</td>
</tr>
<tr>
<td>75%</td>
<td>5-methoxy-UTP/5% 5-methyl-CTP/ATP/GTP</td>
</tr>
<tr>
<td>75%</td>
<td>5-methoxy-UTP/50% 5-methyl-CTP/ATP/GTP</td>
</tr>
<tr>
<td>75%</td>
<td>5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP</td>
</tr>
<tr>
<td>75%</td>
<td>5-methoxy-UTP/CTP/ATP/GTP</td>
</tr>
<tr>
<td></td>
<td>8-Aza-ATP</td>
</tr>
<tr>
<td></td>
<td>Alpha-thio-CTP</td>
</tr>
<tr>
<td></td>
<td>CTP/25% 5-Methoxy-UTP + 75% 1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td></td>
<td>CTP/25% 5-Methoxy-UTP + 75% UTP</td>
</tr>
<tr>
<td></td>
<td>CTP/50% 5-Methoxy-UTP + 50% 1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td></td>
<td>CTP/50% 5-Methoxy-UTP + 50% UTP</td>
</tr>
<tr>
<td></td>
<td>CTP/5-Methoxy-UTP</td>
</tr>
<tr>
<td></td>
<td>CTP/5-Methoxy-UTP (cap 0)</td>
</tr>
<tr>
<td></td>
<td>CTP/5-Methoxy-UTP (No cap)</td>
</tr>
<tr>
<td></td>
<td>CTP/75% 5-Methoxy-UTP + 25% 1-Methyl-pseudo-UTP</td>
</tr>
<tr>
<td></td>
<td>CTP/75% 5-Methoxy-UTP + 25% UTP</td>
</tr>
</tbody>
</table>
According to the invention, polynucleotides of the invention may be synthesized to comprise the combinations or single modifications of Table 5.

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
[000385] The present invention provides polynucleotides and compositions and complexes optionally 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).

[000386] In some embodiments, compositions are administered to humans, human patients or subjects. For the purposes of the present disclosure, the phrase "active ingredient" generally refers to the composition or the polynucleotides contained therein to be delivered as described herein.

[000387] 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.

[000388] 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.

[000389] 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

[000390] The compositions 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); (4) alter the biodistribution (e.g., target 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 (antibody) 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, cells transfected with compositions (e.g., for transplantation into a subject), hyaluronidase, nanoparticle mimics and combinations thereof. Each of the foregoing methods is described in copending International Applications WO2015/085318, WO2013/090648 and WO2014/152211, the contents of each of which are incorporated herein by reference in their entireties.

[000391] Accordingly, the formulations of the invention can include one or more excipients, each in an amount that may increases the stability of the composition, increases cell transfection by the composition, increases the expression of polynucleotides encoded protein, and/or alters the release profile of polynucleotide encoded proteins. Further, the polynucleotides of the present invention may be formulated using self-assembled nucleic acid nanoparticles.
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.

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.

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.

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 or 5 polynucleotides.

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.

In one embodiment, the formulation contains at least three polynucleotides encoding proteins. In one embodiment, the formulation contains at least five polynucleotide encoding proteins.
[000398] 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.

[000399] 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

[000400] The synthesis of lipidoids has been extensively described and formulations containing these compounds are particularly suited for delivery of polynucleotides (see Mahon et al., Bioconj 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 entitieys).

[000401] 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, intramuscular, or subcutaneous routes.

[000402] *In vivo* delivery of nucleic acids may be affected by many parameters, including, but not limited to, the formulation composition, nature of particle PEGylation, degree of loading, polynucleotide to lipid ratio, and biophysical parameters such as, but not limited to, particle size (Akinc et al., Mol Ther. 2009 17:872-879; herein incorporated by reference in its entirety). As an example, small changes in the anchor chain length of poly(ethylene glycol) (PEG) lipids may result in significant effects on in vivo efficacy. Formulations with the different lipidoids, including, but not limited to pentapeptide laurylaminopropionyl]-triethylenetetramine hydrochloride (TETA-5LAP; aka 98N12-5, see Murugaiah et al., Analytical Biochemistry, 401:61 (2010); herein incorporated by reference in its entirety), C12-200 (including derivatives and variants), and MD1, can be tested for *in vivo* activity.

[000403] The lipidoid referred to herein as "98N12-5" is disclosed by Akinc et al., Mol Ther. 2009 17:872-879 and is incorporated by reference in its entirety.

[000404] The lipidoid referred to herein as "C12-200" is disclosed by Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869 and Liu and Huang, Molecular Therapy. 2010 669-670; both of which are herein incorporated by reference in their entirety. The lipidoid formulations can include particles comprising either 3 or 4 or more components in addition to polynucleotides. As an example, formulations with certain lipidoids, include, but are not limited to, 98N12-5 and may contain 42% lipidoid, 48% cholesterol and 10% PEG (CI4 alkyl chain length). As another example, formulations with certain lipidoids, include, but are not limited to, C12-200 and may contain 50% lipidoid, 10% disterylophosphatidyl choline, 38.5% cholesterol, and 1.5% PEG-DMG.

[000405] In one embodiment, a polynucleotide formulated with a lipidoid for systemic intravenous administration can target the liver. For example, a final optimized intravenous formulation using polynucleotides, and comprising a lipid molar composition of 42% 98N12-5, 48% cholesterol, and 10% PEG-lipid with a final weight ratio of about 7.5 to 1 total lipid to polynucleotides, and a C14 alkyl chain length on the PEG lipid, with a mean particle size of roughly 50-60 nm, can result in the distribution of the formulation to be greater than 90% to the liver. (see, Akinc et al., Mol Ther. 2009 17:872-879; herein
incorporated by reference in its entirety). In another example, an intravenous formulation using a C12-200 (see US provisional application 61/175,770 and published international application WO2010129709, each of which is herein incorporated by reference in their entirety) lipidoid may have a molar ratio of 50/10/38.5/1.5 of C12-200/disteroylphosphatidyl choline/cholesterol/PEG-DMG, with a weight ratio of 7 to 1 total lipid to polynucleotides, and a mean particle size of 80 nm may be effective to deliver polynucleotides to hepatocytes (see, Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869 herein incorporated by reference in its entirety). In another embodiment, an MD1 lipidoid-containing formulation may be used to effectively deliver polynucleotides to hepatocytes in vivo.

[000406] The characteristics of optimized lipidoid formulations for intramuscular or subcutaneous routes may vary significantly depending on the target cell type and the ability of formulations to diffuse through the extracellular matrix into the blood stream. While a particle size of less than 150 nm may be desired for effective hepatocyte delivery due to the size of the endothelial fenestrae (see, Akinc et al., Mol Ther. 2009 17:872-879 herein incorporated by reference in its entirety), use of a lipidoid-formulated compositions to deliver the formulation to other cells types including, but not limited to, endothelial cells, myeloid cells, and muscle cells may not be similarly size-limited.

[000407] Use of lipidoid formulations to deliver siRNA in vivo to other non-hepatocyte cells such as myeloid cells and endothelium has been reported (see Akinc et al., Nat Biotechnol. 2008 26:561-569; Leuschner et al., Nat Biotechnol. 2011 29:1005-1010; Cho et al. Adv. Funct. Mater. 2009 19:3112-3118; 8th International Judah Folkman Conference, Cambridge, MA October 8-9, 2010; each of which is herein incorporated by reference in its entirety). Effective delivery to myeloid cells, such as monocytes, lipidoid formulations may have a similar component molar ratio. Different ratios of lipidoids and other components including, but not limited to, disteroylphosphatidyl choline, cholesterol and PEG-DMG, may be used to optimize the formulation of the compositions for delivery to different cell types including, but not limited to, hepatocytes, myeloid cells, muscle cells, etc. For example, the component molar ratio may include, but is not limited to, 50% C12-200, 10% disteroylphosphatidyl choline, 38.5% cholesterol, and %1.5 PEG-DMG (see Leuschner et al., Nat Biotechnol 2011 29:1005-1010; herein incorporated by
reference in its entirety). The use of lipidoid formulations for the localized delivery of nucleic acids to cells (such as, but not limited to, adipose cells and muscle cells) via either subcutaneous or intramuscular delivery, may not require all of the formulation components desired for systemic delivery, and as such may comprise only the lipidoid and the composition.

[000408] Combinations of different lipidoids may be used to improve the efficacy of polynucleotides directed protein production as the lipidoids may be able to increase cell transfection by the composition; and/or increase the translation of encoded protein (see Whitehead et al., Mol. Ther. 2011, 19:1688-1694, herein incorporated by reference in its entirety).

Liposomes, Lipoplexes, and Lipid Nanoparticles

[000409] The compositions of the invention can be formulated using one or more liposomes, lipoplexes, or lipid nanoparticles. In one embodiment, pharmaceutical compositions of compositions 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 unilamellar 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.

[000410] The formation of liposomes may depend on the physicochemical characteristics such as, but not limited to, the pharmaceutical formulation entrapped and the liposomal ingredients, the nature of the medium in which the lipid vesicles are dispersed, the effective concentration of the entrapped substance and its potential toxicity, any additional processes involved during the application and/or delivery of the vesicles, the optimization size, polydispersity and the shelf-life of the vesicles for the
intended application, and the batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products.


[000412] In one embodiment, pharmaceutical compositions described herein may include, without limitation, liposomes such as those formed from 1,2-dioleyloxy-N,N-dimethylaminopropano (DODMA) liposomes, DiLa2 liposomes from Marina Biotech (Bothell, WA), 1,2-dilinoleoyloxy-3-dimethylaminopropano (DLin-DMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), and MC3 (US20100324120; 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).

[000413] 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; all 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.

In some embodiments, liposome formulations may comprise from about 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.

In one embodiment, pharmaceutical compositions may include liposomes which may be formed to deliver polynucleotides which may encode at least one antibody or any other polypeptide of interest. The polynucleotides or compositions may be encapsulated by the liposome and/or it may be contained in an aqueous core which may then be encapsulated by the liposome (see International Pub. Nos. WO2012031046, WO2012031043, WO2012030901 and WO2012006378 and US Patent Publication No. US20130189351, US20130195969 and US20130202684; the contents of each of which are herein incorporated by reference in their entirety).

In another embodiment, liposomes may be formulated for targeted delivery. As a non-limiting example, the liposome may be formulated for targeted delivery to the liver. 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 another embodiment, the polynucleotide which may encode protein may be formulated in a cationic oil-in-water emulsion where the emulsion particle comprises an oil core and a cationic lipid which can interact with the polynucleotide anchoring the
molecule to the emulsion particle (see International Pub. No. WO2012006380; herein incorporated by reference in its entirety).

[000418] In one embodiment, the compositions may be formulated in a water-in-oil emulsion comprising a continuous hydrophobic phase in which the hydrophilic phase is dispersed. As a non-limiting example, the emulsion may be made by the methods described in International Publication No. WO201087791, the contents of which are herein incorporated by reference in its entirety.

[000419] 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).

[000420] 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 compositions may be encapsulated in a liposome using reverse pH gradients and/or optimized internal buffer compositions as described in International Patent Publication No. WO2013086526, the contents of which are herein incorporated by reference in its entirety.

[000421] In one embodiment, the compositions may be formulated in liposomes such as, but not limited to, DiLa2 liposomes (Marina Biotech, Bothell, WA), SMARTICLES® (Marina Biotech, Bothell, WA), neutral DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) based liposomes (e.g., siRNA delivery for ovarian cancer (Landen et al. Cancer Biology & Therapy 2006 5(12)1708-1713); herein incorporated by reference in its entirety) and hyaluronan-coated liposomes (Quiet Therapeutics, Israel).

[000422] In one embodiment, the cationic lipid may be a low molecular weight cationic lipid such as those described in US Patent Application No. 20130090372, the contents of which are herein incorporated by reference in its entirety.
In one embodiment, the compositions may be formulated in a lipid vesicle which may have crosslinks between functionalized lipid bilayers.

In one embodiment, the compositions may be formulated in a liposome (e.g., LNP) comprising a cationic lipid. The liposome may have a molar ratio of nitrogen atoms in the cationic lipid to the phosphates in the RNA (N:P ratio) of between 1:1 and 20:1 as described in International Publication No. WO2013006825, herein incorporated by reference in its entirety. In another embodiment, the liposome may have a N:P ratio of greater than 20:1 or less than 1:1. In exemplary embodiments, the liposome has a N:P ratio of between 4:1 and 7:1.

In one embodiment, the compositions may be formulated in a lipid-polycation complex. The formation of the lipid-polycation complex may be accomplished by methods known in the art and/or as described in U.S. Pub. No. 20120178702, herein incorporated by reference in its entirety. As a non-limiting example, the polycation may include a cationic peptide or a polypeptide such as, but not limited to, polylysine, polyornithine and/or polyarginine and the cationic peptides described in International Pub. No. WO2012013326 or US Patent Pub. No. US20130142818; each of which is herein incorporated by reference in its entirety. In another embodiment, the compositions may be formulated in a lipid-polycation complex which may further include a neutral lipid such as, but not limited to, cholesterol or dioleoyl phosphatidylethanolamine (DOPE).

In one embodiment, the compositions may be formulated in an aminoalcohol lipidoid. Aminoalcohol lipidoids which may be used in the present invention may be prepared by the methods described in U.S. Patent No. 8,450,298, the contents of which are herein incorporated by reference in its entirety.

The liposome formulation may be influenced by, but not limited to, the selection of the cationic lipid component, the degree of cationic lipid saturation, the nature of the PEGylation, ratio of all components and biophysical parameters such as size. In one example by Semple et al. (Semple et al. Nature Biotech. 2010 28:172-176; herein incorporated by reference in its entirety), the liposome formulation was composed of 57.1 % cationic lipid, 7.1% dipalmitoylphosphatidylcholine, 34.3 % cholesterol, and 1.4% PEG-c-DMA. As another example, changing the composition of the cationic lipid
could more effectively deliver siRNA to various antigen presenting cells (Basha et al. Mol Ther. 2011 19:2186-2200; herein incorporated by reference in its entirety). In some embodiments, liposome formulations may comprise from about 35 to about 45% cationic lipid, from about 40% to about 50% cationic lipid, from about 50% to about 60% cationic lipid and/or from about 55% to about 65% cationic lipid. In some embodiments, the ratio of lipid to mRNA in liposomes may be from about 5:1 to about 20:1, from about 10:1 to about 25:1, from about 15:1 to about 30:1 and/or at least 30:1.

[000428] In some embodiments, the ratio of PEG in the lipid nanoparticle (LNP) formulations may be increased or decreased and/or the carbon chain length of the PEG lipid may be modified from C14 to C18 to alter the pharmacokinetics and/or biodistribution of the LNP formulations. As a non-limiting example, LNP formulations may contain from about 0.5% to about 3.0%, from about 1.0% to about 3.5%, from about 1.5% to about 4.0%, from about 2.0% to about 4.5%, from about 2.5% to about 5.0% and/or from about 3.0% to about 6.0% of the lipid molar ratio of PEG-c-DOMG as compared to the cationic lipid, DSPC and cholesterol. In another embodiment the PEG-c-DOMG may be replaced with a PEG lipid such as, but not limited to, PEG-DSG (1,2-Distearoyl-sn-glycerol, methoxypolyethylene glycol), PEG-DMG (1,2-Dimyristoyl-sn-glycerol) and/or PEG-DPG (1,2-Dipalmitoyl-sn-glycerol, methoxypolyethylene glycol). The cationic lipid may be selected from any lipid known in the art such as, but not limited to, DLin-MC3-DMA, DLin-DMA, C12-200 and DLin-KC2-DMA.

[000429] In one embodiment, the compositions may be formulated in a lipid nanoparticle such as those described in International Publication No. WO2012170930, herein incorporated by reference in its entirety.

[000430] In one embodiment, the compositions 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 such as, but not limited to, DLin-DMA, DLin-D-DMA, DLin-MC3-DMA, DLin-KC2-DMA, DODMA and amino alcohol lipids. 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. As a non-limiting example, the cationic lipid may be 2-amino-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-2-{{(9Z,2Z)-octadeca-9,12-dien-1-yloxy}methyl}propan-1-ol (Compound 1 in US20130150625); 2-amino-3-[(9Z)-octadec-9-en-1-yloxy]-2-{{(9Z)-octadec-9-en-1-yloxy}methyl}propan-1-ol (Compound 2 in US20130150625); 2-amino-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-2-{{octyloxy}methyl}propan-1-ol (Compound 3 in US20130150625); and 2-(dimethylamino)-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-2-{{(9Z,12Z)-octadeca-9,12-dien-1-yloxy}methyl}propan-1-ol (Compound 4 in US20130150625); or any pharmaceutically acceptable salt or stereoisomer thereof.


[000432] As a non-limiting example, the cationic lipid may be selected from (20Z,23Z)-N,N-dimethylnonacosa-20,23-dien-10-amine, (17Z,20Z)-N,N-dimemylhexacosa-17,20-dien-9-amine, (1Z,19Z)-N5N-dimethylpentacosa-16,19-dien-8-amine, (13Z,16Z)-N,N-
dimethyldocosa-13,16-dien-5-amine, (12Z,15Z)-N,N-dimethylhenicosa-12,15-dien-4-
10-amine, (15Z,18Z)-N,N-dimethyltetracona-15,18-dien-7-amine, (14Z,17Z)-N,N-dimethyltricosa-
14,17-dien-4-amine, (19Z,22Z)-N,N-dimeihyloctacosa-19,22-dien-9-amine, (18Z,21Z)-N,N-dimethylheptacosa-
18,21-dien-8-amine, (17Z,20Z)-N,N-dimethylhexacosa-17,20-dien-7-amine, (16Z,19Z)-N,N-dimethylpentacosa-16,19-dien-
[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-l-[(octyloxy)methyl]ethylpyrrolidine, (2S)-

[000433] In one embodiment, the lipid may be a cleavable lipid such as those described in International Publication No. WO2012170889, the contents of which are herein incorporated by reference in its entirety.

[000434] In another embodiment, the lipid may be a cationic lipid such as, but not limited to, Formula (I) of U.S. Patent Application No. US2013064894, the contents of which are herein incorporated by reference in its entirety.

[000435] In one embodiment, the cationic lipid may be synthesized by methods known in the art and/or as described in International Publication Nos. WO2012040184,

[000436] In another embodiment, the cationic lipid may be a trialkyl cationic lipid. Non-limiting examples of trialkyl cationic lipids and methods of making and using the trialkyl cationic lipids are described in International Patent Publication No. WO2013126803, the contents of which are herein incorporated by reference in its entirety.

[000437] In one embodiment, the LNP formulations of the compositions may contain PEG-c-DOMG at 3% lipid molar ratio. In another embodiment, the LNP formulations compositions may contain PEG-c-DOMG at 1.5% lipid molar ratio.

[000438] In one embodiment, the pharmaceutical compositions of the compositions may include at least one of the PEGylated lipids described in International Publication No. WO2012099755, herein incorporated by reference.

[000439] 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).

[000440] In one embodiment, the LNP formulation may be formulated by the methods described in International Publication Nos. WO201 1127255 or WO2008 103276, the contents of each of which is herein incorporated by reference in their entirety. As a non-limiting example, the compositions described herein may be encapsulated in LNP formulations as described in WO201 1127255 and/or WO2008103276; each of which is herein incorporated by reference in their entirety.
In one embodiment, the compositions described herein may be formulated in a nanoparticle to be delivered by a parenteral route as described in U.S. Pub. No. US20 1202075845; the contents of which are herein incorporated by reference in its entirety.

In another embodiment, the polycationic lipids present herein may comprise one or more active agents or therapeutic agents; one or more cationic lipids comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle. The nucleic acid in the nanoparticle may be the polynucleotides described herein and/or are known in the art.

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 are herein incorporated by reference in their entirety. As a non-limiting example, modified RNA described herein may be encapsulated in LNP formulations as described in WO2011127255 and/or WO2008103276; the contents of which are herein incorporated by reference in their entirety.

In one embodiment, LNP formulations described herein may comprise a polycationic composition. As a non-limiting example, the polycationic composition may be selected from formula 1-60 of US Patent Publication No. US20050222064; the content of which is herein incorporated by reference in its entirety. In another embodiment, the
LNP formulations comprising a polycationic composition may be used for the delivery of the modified RNA described herein in vivo and/or in vitro.

[000447] In one embodiment, the LNP formulations described herein may additionally comprise a permeability enhancer molecule. Non-limiting permeability enhancer molecules are described in US Patent Publication No. US20050222064; the content of which is herein incorporated by reference in its entirety.

[000448] In one embodiment, the compositions may be formulated in liposomes such as, but not limited to, DiLa2 liposomes (Marina Biotech, Bothell, WA), SMARTICLES® (Marina Biotech, Bothell, WA), neutral DOPC (1,2-dioleoyl-sn-glycero-3-phosphochoeline) based liposomes (e.g., siRNA delivery for ovarian cancer (Landen et al. Cancer Biology & Therapy 2006 5(12)1708-1713); herein incorporated by reference in its entirety) and hyaluronan-coated liposomes (Quiet Therapeutics, Israel).

[000449] In one embodiment, the compositions may be formulated in a lyophilized gel-phase liposomal composition as described in US Publication No. US2012060293, herein incorporated by reference in its entirety.

[000450] The nanoparticle formulations may comprise a phosphate conjugate. The phosphate conjugate may increase in vivo circulation times and/or increase the targeted delivery of the nanoparticle. Phosphate conjugates for use with the present invention may be made by the methods described in International Application No. WO2013033438 or US Patent Publication No. US20130196948, the contents of each of which are herein incorporated by reference in its entirety. As a non-limiting example, the phosphate conjugates may include a compound of any one of the formulas described in International Application No. WO2013033438, herein incorporated by reference in its entirety.

[000451] The nanoparticle formulation may comprise a polymer conjugate. The polymer conjugate may be a water soluble conjugate. The polymer conjugate may have a structure as described in U.S. Patent Application No. 20130059360, the contents of which are herein incorporated by reference in its entirety. In one aspect, polymer conjugates with the polynucleotides of the present invention may be made using the methods and/or segmented polymeric reagents described in U.S. Patent Application No. 20130072709, herein incorporated by reference in its entirety. In another aspect, the polymer conjugate may have pendant side groups comprising ring moieties such as, but not limited to, the
polymer conjugates described in US Patent Publication No. US20130196948, the contents of which is herein incorporated by reference in its entirety.

[000452] The nanoparticle formulations may comprise a conjugate to enhance the delivery of nanoparticles of the present invention in a subject. Further, the conjugate may inhibit phagocytic clearance of the nanoparticles in a subject. In one aspect, the conjugate may be a "self" peptide designed from the human membrane protein CD47 (e.g., the "self" particles described by Rodriguez et al (Science 2013 339, 971-975), herein incorporated by reference in its entirety). As shown by Rodriguez et al. the self peptides delayed macrophage-mediated clearance of nanoparticles which enhanced delivery of the nanoparticles. In another aspect, the conjugate may be the membrane protein CD47 (e.g., see Rodriguez et al. Science 2013 339, 971-975, herein incorporated by reference in its entirety). Rodriguez et al. showed that, similarly to "self" peptides, CD47 can increase the circulating particle ratio in a subject as compared to scrambled peptides and PEG coated nanoparticles.

[000453] In one embodiment, the compositions of the present invention are formulated in nanoparticles which comprise a conjugate to enhance the delivery of the nanoparticles of the present invention in a subject. The conjugate may be the CD47 membrane or the conjugate may be derived from the CD47 membrane protein, such as the "self" peptide described previously. In another aspect the nanoparticle may comprise PEG and a conjugate of CD47 or a derivative thereof. In yet another aspect, the nanoparticle may comprise both the "self" peptide described above and the membrane protein CD47.

[000454] In another aspect, a "self" peptide and/or CD47 protein may be conjugated to a virus-like particle or pseudovirion, as described herein for delivery of the compositions of the present invention.

[000455] In another embodiment, pharmaceutical compositions comprising the polynucleotides of the present invention and a conjugate which may have a degradable linkage. Non-limiting examples of conjugates include an aromatic moiety comprising an ionizable hydrogen atom, a spacer moiety, and a water-soluble polymer. As a non-limiting example, pharmaceutical compositions comprising a conjugate with a degradable linkage and methods for delivering such pharmaceutical compositions are described in
US Patent Publication No. US20130184443, the contents of which are herein incorporated by reference in its entirety.

[000456] The nanoparticle formulations may be a carbohydrate nanoparticle comprising a carbohydrate carrier and a composition. As a non-limiting example, the carbohydrate carrier may include, but is not limited to, an anhydride-modified phytoglycogen or glycogen-type material, phtoglycogen octenyl succinate, phytoglycogen beta-dextrin, anhydride-modified phytoglycogen beta-dextrin. (See e.g., International Publication No. WO2012109121; the contents of which are herein incorporated by reference in its entirety).

[000457] Nanoparticle formulations of the present invention may be coated with a surfactant or polymer in order to improve the delivery of the particle. In one embodiment, the nanoparticle may be coated with a hydrophilic coating such as, but not limited to, PEG coatings and/or coatings that have a neutral surface charge. The hydrophilic coatings may help to deliver nanoparticles with larger payloads such as, but not limited to, compositions within the central nervous system. As a non-limiting example nanoparticles comprising a hydrophilic coating and methods of making such nanoparticles are described in US Patent Publication No. US20130183244, the contents of which are herein incorporated by reference in its entirety.

[000458] In one embodiment, the lipid nanoparticles of the present invention may be hydrophilic polymer particles. Non-limiting examples of hydrophilic polymer particles and methods of making hydrophilic polymer particles are described in US Patent Publication No. US20130210991, the contents of which are herein incorporated by reference in its entirety.

[000459] In another embodiment, the lipid nanoparticles of the present invention may be hydrophobic polymer particles.

[000460] Lipid nanoparticle formulations may be improved by replacing the cationic lipid with a biodegradable cationic lipid which is known as a rapidly eliminated lipid nanoparticle (reLNP). Ionizable cationic lipids, such as, but 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.

[000461] In one embodiment, the internal ester linkage may be located on either side of the saturated carbon.

[000462] In one embodiment, an immune response may be elicited by delivering a lipid nanoparticle which may include a nanospecies, a polymer and an immunogen. (U.S. Publication No. 20120189700 and International Publication No. WO2012099805; each of which is herein incorporated by reference in their entirety). The polymer may encapsulate the nanospecies or partially encapsulate the nanospecies. The immunogen may be a recombinant protein, a modified RNA and/or a polynucleotide described herein. In one embodiment, the lipid nanoparticle may be formulated for use in a vaccine such as, but not limited to, against a pathogen.

[000463] Lipid nanoparticles may be engineered to alter the surface properties of particles so the lipid nanoparticles may penetrate the mucosal barrier. Mucus is located on mucosal tissue such as, but not limited to, oral (e.g., the buccal and esophageal membranes and tonsil tissue), ophthalmic, gastrointestinal (e.g., stomach, small intestine, large intestine, colon, rectum), nasal, respiratory (e.g., nasal, pharyngeal, tracheal and bronchial membranes), genital (e.g., vaginal, cervical and urethral membranes). Nanoparticles larger than 10-200 nm which are preferred for higher drug encapsulation efficiency and the ability to provide the sustained delivery of a wide array of drugs have been thought to be too large to rapidly diffuse through mucosal barriers. Mucus is continuously secreted, shed, discarded or digested and recycled so most of the trapped particles may be removed from the mucosla tissue within seconds or within a few hours. Large polymeric nanoparticles (200nm -500nm in diameter) which have been coated densely with a low molecular weight polyethylene glycol (PEG) diffused through mucus only 4 to 6-fold lower than the same particles diffusing in water (Lai et al. PNAS 2007 104(5): 1482-487; Lai et al. Adv Drug Deliv Rev. 2009 61(2): 158-171; each of which is
The transport of nanoparticles may be determined using rates of permeation and/or fluorescent microscopy techniques including, but not limited to, fluorescence recovery after photobleaching (FRAP) and high resolution multiple particle tracking (MPT). As a non-limiting example, compositions which can penetrate a mucosal barrier may be made as described in U.S. Pat. No. 8,241,670 or International Patent Publication No. WO201310028, the contents of each of which are herein incorporated by reference in its entirety.

[000464] The lipid nanoparticle engineered to penetrate mucus may comprise a polymeric material (i.e. a polymeric core) and/or a polymer-vitamin conjugate and/or a tri-block co-polymer. The polymeric material may include, but is not limited to, polyamines, polyethers, polyamides, polyesters, polycarbonates, poly(styrenes), polyimidates, polysulfones, polyurethanes, polyacetylenes, polyethylenes, polyethyeneimines, polyisocyanates, polyacrylates, polymethacrylates, polyacrylonitriles, and polyarylates. The polymeric material may be biodegradable and/or biocompatible. Non-limiting examples of biocompatible polymers are described in International Patent Publication No. WO201316804, the contents of which are herein incorporated by reference in its entirety. The polymeric material may additionally be irradiated. As a non-limiting example, the polymeric material may be gamma irradiated (See e.g., International App. No. WO201282165, herein incorporated by reference in its entirety). Non-limiting examples of specific polymers include poly(caprolactone) (PCL), ethylene vinyl acetate polymer (EVA), poly(lactic acid) (PLA), poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA), poly(lactic acid-co-glycolic acid) (PLGA), poly(L-lactic acid-co-glycolic acid) (PLLGA), poly(D,L-lactide) (PDLA), poly(lactide) (PLLA), poly(D,L-lactide-co-caprolactone), poly(D,L-lactide-co-caprolactone-co-glycolide), poly(D,L-lactide-co-PEO-co-D,L-lactide), poly(D,L-lactide-co-PPO-co-D,L-lactide), polyalkyl cyanoacralate, polyurethane, poly-L-lysine (PLL), hydroxypropyl methacrylate (HPMA), polyethyleneglycol, poly-L-glutamic acid, poly(hydroxy acids), polyanhydrides, polyorthoesters, poly(ester amides), polyamides, poly(ester ethers), polycarbonates, polyalkylenes such as polyethylene and polypropylene, polyalkylene glycols such as poly(ethylene glycol) (PEG), polyalkylene oxides (PEO), polyalkylene terephthalates such as poly(ethylene terephthalate), polyvinyl alcohols (PVA), polyvinyl
ethers, polyvinyl esters such as poly(vinyl acetate), polyvinyl halides such as poly(vinyl chloride) (PVC), polyvinylpyrrolidone, polysiloxanes, polystyrene (PS), polyurethanes, derivatized celluloses such as alkyl celluloses, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, hydroxypropylcellulose, carboxymethylcellulose, polymers of acrylic acids, such as poly(methyl(meth)acrylate) (PMMA), poly(ethyl(meth)acrylate), poly(butyl(meth)acrylate), poly(isobutyl(meth)acrylate), poly(hexyl(meth)acrylate), poly(isodecyl(meth)acrylate), poly(lauryl(meth)acrylate), poly(phenyl(meth)acrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate) and copolymers and mixtures thereof, polydioxanone and its copolymers, polyhydroxyalkanoates, polypropylene fumarate, polyoxymethylene, poloxamers, poly(ortho)esters, poly(butric acid), poly(valeric acid), poly(lactide-co-caprolactone), PEG-PLGA-PEG and trimethylene carbonate, polyvinylpyrrolidone. The lipid nanoparticle may be coated or associated with a copolymer such as, but not limited to, a block co-polymer (such as a branched polyether-polyamide block copolymer described in International Publication No. WO2013012476, herein incorporated by reference in its entirety), and (poly(ethylene glycol))-(poly(propylene oxide))-(poly(ethylene glycol)) triblock copolymer (see e.g., US Publication 20120121718 and US Publication 20100003337 and U.S. Pat. No. 8,263,665; each of which is herein incorporated by reference in their entirety). The co-polymer may be a polymer that is generally regarded as safe (GRAS) and the formation of the lipid nanoparticle may be in such a way that no new chemical entities are created. For example, the lipid nanoparticle may comprise poloxamers coating PLGA nanoparticles without forming new chemical entities which are still able to rapidly penetrate human mucus (Yang et al. Angew. Chem. Int. Ed. 2011 50:2597-2600; the contents of which are herein incorporated by reference in its entirety). A non-limiting scalable method to produce nanoparticles which can penetrate human mucus is described by Xu et al. (See e.g., J Control Release 2013, 170(2):279-86; the contents of which are herein incorporated by reference in its entirety).

[000465] The vitamin of the polymer-vitamin conjugate may be vitamin E. The vitamin portion of the conjugate may be substituted with other suitable components such as, but not limited to, vitamin A, vitamin E, other vitamins, cholesterol, a hydrophobic moiety,
or a hydrophobic component of other surfactants (e.g., sterol chains, fatty acids, hydrocarbon chains and alkylene oxide chains).

[000466] The lipid nanoparticle engineered to penetrate mucus may include surface altering agents such as, but not limited to, polynucleotides, anionic proteins (e.g., bovine serum albumin), surfactants (e.g., cationic surfactants such as for example dimethyl dioctadecyl-ammonium bromide), sugars or sugar derivatives (e.g., cyclodextrin), nucleic acids, polymers (e.g., heparin, polyethylene glycol and poloxamer), mucolytic agents (e.g., N-acetylcysteine, mugwort, bromelain, papain, clerodendrum, acetylcysteine, bromhexine, carbocisteine, eprazinone, mesna, ambroxol, soberol, domiodol, letosteine, stepronin, tioprofin, gelsolin, thymosin β4 dornase alfa, neltenexine, erdosteine) and various DNases including rhDNase. The surface altering agent may be embedded or enmeshed in the particle's surface or disposed (e.g., by coating, adsorption, covalent linkage, or other process) on the surface of the lipid nanoparticle. (see e.g., US Publication 20100215580 and US Publication 20080166414 and US20130164343; each of which is herein incorporated by reference in their entirety).

[000467] In one embodiment, the mucus penetrating lipid nanoparticles may comprise at least one polynucleotide described herein. The polynucleotide may be encapsulated in the lipid nanoparticle and/or disposed on the surface of the particle. The polynucleotide may be covalently coupled to the lipid nanoparticle. Formulations of mucus penetrating lipid nanoparticles may comprise a plurality of nanoparticles. Further, the formulations may contain particles which may interact with the mucus and alter the structural and/or adhesive properties of the surrounding mucus to decrease mucoadhesion which may increase the delivery of the mucus penetrating lipid nanoparticles to the mucosal tissue.

[000468] In another embodiment, the mucus penetrating lipid nanoparticles may be a hypotonic formulation comprising a mucosal penetration enhancing coating. The formulation may be hypotonic for the epithelium to which it is being delivered. Non-limiting examples of hypotonic formulations may be found in International Patent Publication No. WO2013110028, the contents of which are herein incorporated by reference in its entirety.

[000469] In one embodiment, in order to enhance the delivery through the mucosal barrier the compositions may comprise or be a hypotonic solution. Hypotonic solutions
were found to increase the rate at which mucoinert particles such as, but not limited to, mucus-penetrating particles, were able to reach the vaginal epithelial surface (See e.g., Ensign et al. Biomaterials 2013 34(28):6922-9; the contents of which is herein incorporated by reference in its entirety).


[000471] 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

[000472] In one embodiment, the composition is formulated as a solid lipid nanoparticle. A solid lipid nanoparticle (SLN) may be spherical with an average diameter between 10 to 1000 nm. SLN possess a solid lipid core matrix that can solubilize lipophilic molecules and may be stabilized with surfactants and/or emulsifiers. In a further embodiment, the lipid nanoparticle may be a self-assembly lipid-polymer nanoparticle (see Zhang et al., ACS Nano, 2008, 2 (8), pp 1696-1702; the contents of which are herein incorporated by reference in its entirety). As a non-limiting example, the SLN may be the SLN described in International Patent Publication No. WO2013105101, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the SLN may be made by the methods or processes described in International Patent Publication No. WO2013105101, the contents of which are herein incorporated by reference in its entirety.

[000473] 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 composition; 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.

[000474] In one embodiment, the compositions 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 compositions 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.

[000475] In one embodiment, the controlled release formulation may include, but is not limited to, tri-block co-polymers. As a non-limiting example, the formulation may include two different types of tri-block co-polymers (International Pub. No. WO20 12131 104 and WO20 12131 106; each of which is herein incorporated by reference in its entirety).

[000476] In another embodiment, the compositions may be encapsulated into a lipid nanoparticle or a rapidly eliminated lipid nanoparticle and the lipid nanoparticles or a rapidly eliminated lipid nanoparticle may then be encapsulated into a polymer, hydrogel
and/or surgical sealant described herein and/or known in the art. As a non-limiting example, the polymer, hydrogel or surgical sealant may be PLGA, ethylene vinyl acetate (EVAc), poloxamer, GELSITE® (Nanotherapeutics, Inc. Alachua, FL), HYLENEX® (Halozyme Therapeutics, San Diego CA), surgical sealants such as fibrinogen polymers (Ethicon Inc. Cornelia, GA), TISSELL® (Baxter International, Inc Deerfield, IL), PEG-based sealants, and COSEAL® (Baxter International, Inc Deerfield, IL).

[000477] In another embodiment, the lipid nanoparticle may be encapsulated into any polymer known in the art which may form a gel when injected into a subject. As another non-limiting example, the lipid nanoparticle may be encapsulated into a polymer matrix which may be biodegradable.

[000478] In one embodiment, the composition for controlled release and/or targeted delivery may also include at least one controlled release coating. Controlled release coatings include, but are not limited to, OPADRY®, polyvinylpyrrolidone/vinyl acetate copolymer, polyvinylpyrrolidone, hydroxypropyl methylcellulose, hydroxypropyl cellulose, hydroxyethyl cellulose, EUDRAGIT RL®, EUDRAGIT RS® and cellulose derivatives such as ethylcellulose aqueous dispersions (AQUACOAT® and SURELEASE®).

[000479] In one embodiment, the composition controlled release and/or targeted delivery formulation may comprise at least one degradable polyester which may contain polycationic side chains. Degradeable polyesters include, but are not limited to, poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), and combinations thereof. In another embodiment, the degradable polyesters may include a PEG conjugation to form a PEGylated polymer.

[000480] In one embodiment, the composition controlled release and/or targeted delivery formulation comprising at least one polynucleotide may comprise at least one PEG and/or PEG related polymer derivatives as described in US Patent No. 8,404,222, herein incorporated by reference in its entirety.

[000481] In another embodiment, the composition controlled release delivery formulation comprising at least one polynucleotide may be the controlled release polymer system described in US20130130348, herein incorporated by reference in its entirety.
In one embodiment, the compositions of the present invention may be encapsulated in a therapeutic nanoparticle. Therapeutic nanoparticles may be formulated by methods described herein and known in the art such as, but not limited to, International Pub Nos. WO2010005740, WO2010030763, WO20 1005721, WO2010005723, WO2012054923, US Pub Nos. US201110262491, US20100104645, US20100087337, US20100068285, US201 10274759, US20100068286, US20120288541, US20130123351 and US20130230567 and US Pat No. 8,293,276, 8,293,276, 8,318,208 and 8,318,211; the contents of each of which are herein incorporated by reference in their entirety. In another embodiment, therapeutic polymer nanoparticles may be identified by the methods described in US Pub No. US20120140790, herein incorporated by reference in its entirety.

In one embodiment, the therapeutic nanoparticle compositions 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. As a non-limiting example, the sustained release nanoparticle may comprise a polymer and a therapeutic agent such as, but not limited to, the polynucleotides of the present invention (see International Pub No. 2010075072 and US Pub No. US20100216804, US20110217377 and US20120201859, each of which is herein incorporated by reference in their entirety). In another non-limiting example, the sustained release formulation may comprise agents which permit persistent bioavailability such as, but not limited to, crystals, macromolecular gels and/or particulate suspensions (see US Patent Publication No US20130150295, the contents of which is herein incorporated by reference in its entirety).

In one embodiment, the composition therapeutic nanoparticles may be formulated to be target specific. As a non-limiting example, the therapeutic nanoparticles may include a corticosteroid (see International Pub. No. WO2011084518; herein incorporated by reference in its entirety). In one embodiment, the therapeutic nanoparticles may be formulated to be cancer specific. As a non-limiting example, the therapeutic nanoparticles may be formulated in nanoparticles described in International Pub No. WO2008121949, WO2010005726, WO2010005725, WO2011084521 and US
In one embodiment, the nanoparticles of the present invention may comprise a polymeric matrix. As a non-limiting example, the nanoparticle may comprise two or more polymers such as, but not limited to, polyethylenes, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetalts, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polypeptides, polystyrenes, polyamines, polylysine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester) or combinations thereof.

In one embodiment, the therapeutic nanoparticle comprises a diblock copolymer. In one embodiment, the diblock copolymer may include PEG in combination with a polymer such as, but not limited to, polyethylenes, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetalts, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polypeptides, polystyrenes, polyamines, polylysine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester) or combinations thereof. In another embodiment, the diblock copolymer may comprise the diblock copolymers described in European Patent Publication No. the contents of which are herein incorporated by reference in its entirety. In yet another embodiment, the diblock copolymer may be a high-X diblock copolymer such as those described in International Patent Publication No. WO2013120052, the contents of which are herein incorporated by reference in its entirety.

As a non-limiting example the therapeutic nanoparticle comprises a PLGA-PEG block copolymer (see US Pub. No. US2012004293 and US Pat No. 8,236,330, each of which is herein incorporated by reference in their entirety). In another non-limiting example, the therapeutic nanoparticle is a stealth nanoparticle comprising a diblock copolymer of PEG and PLA or PEG and PLGA (see US Pat No 8,246,968 and International Publication No. WO2012166923, the contents of each of which are herein
incorporated by reference in its entirety). In yet another non-limiting example, the therapeutic nanoparticle is a stealth nanoparticle or a target-specific stealth nanoparticle as described in US Patent Publication No. US20130172406, the contents of which are herein incorporated by reference in its entirety.

[000488] In one embodiment, the therapeutic nanoparticle may comprise a multiblock copolymer (See e.g., U.S. Pat. No. 8,263,665 and 8,287,910 and US Patent Pub. No. US20130195987; the contents of each of which are herein incorporated by reference in its entirety).

[000489] In yet another non-limiting example, the lipid nanoparticle comprises the block copolymer PEG-PLGA-PEG (see e.g., the thermosensitive hydrogel (PEG-PLGA-PEG) was used as a TGF-betal gene delivery vehicle in Lee et al. Thermosensitive Hydrogel as a Tgf-β1 Gene Delivery Vehicle Enhances Diabetic Wound Healing. Pharmaceutical Research. 2003 20(12): 1995-2000; as a controlled gene delivery system in Li et al. Controlled Gene Delivery System Based on Thermosensitive Biodegradable Hydrogel. Pharmaceutical Research 2003 20(6):884-888; and Chang et al., Non-ionic amphiphilic biodegradable PEG-PLGA-PEG copolymer enhances gene delivery efficiency in rat skeletal muscle. J Controlled Release. 2007 118:245-253; each of which is herein incorporated by reference in its entirety). The compositions of the present invention may be formulated in lipid nanoparticles comprising the PEG-PLGA-PEG block copolymer.

[000490] In one embodiment, the therapeutic nanoparticle may comprise a multiblock copolymer (See e.g., U.S. Pat. No. 8,263,665 and 8,287,910 and US Patent Pub. No. US20130195987; the contents of each of which are herein incorporated by reference in its entirety).

[000491] In one embodiment, the block copolymers described herein may be included in a polyion complex comprising a non-polymeric micelle and the block copolymer. (See e.g., U.S. Pub. No. 20120076836; herein incorporated by reference in its entirety).

[000492] In one embodiment, the therapeutic nanoparticle may comprise 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.

[000493] In one embodiment, the therapeutic nanoparticles may comprise at least one poly(vinyl ester) polymer. The poly(vinyl ester) polymer may be a copolymer such as a random copolymer. As a non-limiting example, the random copolymer may have a structure such as those described in International Application No. WO2013032829 or US Patent Publication No US20130121954, the contents of which are herein incorporated by reference in its entirety. In one aspect, the poly(vinyl ester) polymers may be conjugated to the polynucleotides described herein. In another aspect, the poly(vinyl ester) polymer which may be used in the present invention may be those described in, herein incorporated by reference in its entirety.

[000494] In one embodiment, the therapeutic nanoparticle may comprise at least one diblock copolymer. The diblock copolymer may be, but it not limited to, a poly(lactic) acid-poly(ethylene)glycol copolymer (see e.g., International Patent Publication No. WO2013044219; herein incorporated by reference in its entirety). As a non-limiting example, the therapeutic nanoparticle may be used to treat cancer (see International publication No. WO2013044219; herein incorporated by reference in its entirety).

[000495] In one embodiment, the therapeutic nanoparticles may comprise at least one cationic polymer described herein and/or known in the art.

[000496] In one embodiment, the therapeutic nanoparticles may comprise at least one amine-containing polymer such as, but not limited to polylysine, polyethylene imine, poly(amidoamine) dendrimers, poly(beta-amino esters) (See e.g., U.S. Pat. No. 8,287,849; herein incorporated by reference in its entirety) and combinations thereof.

[000497] In another embodiment, the nanoparticles described herein may comprise an amine cationic lipid such as those described in International Patent Application No. WO2013059496, the contents of which are herein incorporated by reference in its entirety. In one aspect the cationic lipids may have a amino-amine or an amino-amide moiety.

[000498] In one embodiment, the therapeutic nanoparticles may comprise at least one degradable polyester which may contain polycationic side chains. Degradeable polyesters include, but are not limited to, poly(serine ester), poly(L-lactide-co-L-lysine),
poly(4-hydroxy-L-proline ester), and combinations thereof. In another embodiment, the degradable polyesters may include a PEG conjugation to form a PEGylated polymer. In another embodiment, the therapeutic nanoparticle may include a conjugation of at least one targeting ligand. The targeting ligand may be any ligand known in the art such as, but not limited to, a monoclonal antibody. (Kirpotin et al, Cancer Res. 2006 66:6732-6740; herein incorporated by reference in its entirety).

In one embodiment, the therapeutic nanoparticle may be formulated in an aqueous solution which may be used to target cancer (see International Pub No. WO201 1084513 and US Pub No. US20110294717, each of which is herein incorporated by reference in their entirety).

In one embodiment, the therapeutic nanoparticle comprising at least one composition may be formulated using the methods described by Podobinski et al in US Patent No. 8,404,799, the contents of which are herein incorporated by reference in its entirety.

In one embodiment, the compositions may be encapsulated in, linked to and/or associated with synthetic nanocarriers. Synthetic nanocarriers include, but are not limited to, those described in International Pub. Nos. WO2010005740, WO201005761, WO201213501, WO2012149252, WO2012149255, WO2012149259, WO2012149265, WO2012149268, WO2012149282, WO2012149301, WO2012149393, WO2012149405, WO2012149411, WO2012149454 and WO2013019669, and US Pub. Nos. US20110262491, US201000104645, US20100087337 and US20120244222, each of which is herein incorporated by reference in their entirety. The synthetic nanocarriers may be formulated using methods known in the art and/or described herein. As a non-limiting example, the synthetic nanocarriers may be formulated by the methods described in International Pub Nos. WO2010005740, WO2010030763 and WO201213501 and US Pub. Nos. US20110262491, US20100104645, US20100087337 and US20120244222, each of which is herein incorporated by reference in their entirety. In another embodiment, the synthetic nanocarrier formulations may be lyophilized by methods described in International Pub. No. WO201 1072218 and US Pat No. 8,211,473; the content of each of which is herein incorporated by reference in their entirety. In yet another embodiment, formulations of the present invention, including, but not limited to,
synthetic nanocarriers, may be lyophilized or reconstituted by the methods described in US Patent Publication No. US20130230568, the contents of which are herein incorporated by reference in its entirety.

[000503] In one embodiment, the synthetic nanocarriers may contain reactive groups to release the polynucleotides described herein (see International Pub. No. WO2010138192 and US Pub. No. US20120171229, each of which is herein incorporated by reference in their entirety).

[000504] In one embodiment, the synthetic nanocarriers may contain an immunostimulatory agent to enhance the immune response from delivery of the synthetic nanocarrier. As a non-limiting example, the synthetic nanocarrier may comprise a Th1 immunostimulatory agent which may enhance a Th1-based response of the immune system (see International Pub No. WO2010123569 and US Pub. No. US20110223201, each of which is herein incorporated by reference in its entirety).

[000505] 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. As a non-limiting example, the synthetic nanoparticle may be formulated to release the compositions after 24 hours and/or at a pH of 4.5 (see International Pub. Nos. WO2010138193 and WO2010138194 and US Pub Nos. US201100238 and US20110027217, each of which is herein incorporated by reference in their entireties).

[000506] In one embodiment, the synthetic nanocarriers may be formulated for controlled and/or sustained release of the polynucleotides described herein. As a non-limiting example, the synthetic nanocarriers for sustained release may be formulated by methods known in the art, described herein and/or as described in International Pub No. WO2010138192 and US Pub No. 20100303850, each of which is herein incorporated by reference in their entirety.

[000507] In one embodiment, the compositions may be formulated for controlled and/or sustained release wherein the formulation comprises at least one polymer that is a crystalline side chain (CYSC) polymer. CYSC polymers are described in U.S. Patent No. 8,399,007, herein incorporated by reference in its entirety.
In one embodiment, the synthetic nanocarrier may be formulated for use as a vaccine. In one embodiment, the synthetic nanocarrier may encapsulate at least one polynucleotide which encode at least one antibody.

In one embodiment, the synthetic nanocarrier may encapsulate at least one polynucleotide which encodes a peptide, fragment or region from a virus. As a non-limiting example, the synthetic nanocarrier may include, but is not limited to, the nanocarriers described in International Pub No. WO2012024621, WO201202629, WO2012024632 and US Pub No. US20120064110, US20120058153 and US20120058154, each of which is herein incorporated by reference in their entirety.

In one embodiment, the synthetic nanocarrier may be coupled to a polynucleotide which may be able to trigger a humoral and/or cytotoxic T lymphocyte (CTL) response (See e.g., International Publication No. WO2013019669, herein incorporated by reference in its entirety).

In one embodiment, the composition may be encapsulated in, linked to and/or associated with zwitterionic lipids. Non-limiting examples of zwitterionic lipids and methods of using zwitterionic lipids are described in US Patent Publication No. US20130216607, the contents of which are herein incorporated by reference in its entirety. In one aspect, the zwitterionic lipids may be used in the liposomes and lipid nanoparticles described herein.

In one embodiment, the compositions may be formulated in colloid nanocarriers as described in US Patent Publication No. US20130197100, the contents of which are herein incorporated by reference in its entirety.

In one embodiment, the nanoparticle may be optimized for oral administration. The nanoparticle may comprise at least one cationic biopolymer such as, but not limited to, chitosan or a derivative thereof. As a non-limiting example, the nanoparticle may be formulated by the methods described in U.S. Pub. No. 20120282343; herein incorporated by reference in its entirety.

In some embodiments, LNPs comprise the lipid KL52 (an amino-lipid disclosed in U.S. Application Publication No. 2012/0295832 expressly incorporated herein by reference in its entirety). Activity and/or safety (as measured by examining one or more of ALT/AST, white blood cell count and cytokine induction) of LNP
administration may be improved by incorporation of such lipids. LNPs comprising KL52 may be administered intravenously and/or in one or more doses. In some embodiments, administration of LNPs comprising KL52 results in equal or improved mRNA and/or protein expression as compared to LNPs comprising MC3.

[000515] In some embodiments, compositions may be delivered using smaller LNPs. Such particles may comprise a diameter from below 0.1 um up to 100 nm such as, but not limited to, less than 0.1 um, less than 1.0 um, less than 5 um, less than 10 um, less than 15 um, less than 20 um, less than 25 um, less than 30 um, less than 35 um, less than 40 um, less than 50 um, less than 55 um, less than 60 um, less than 65 um, less than 70 um, less than 75 um, less than 80 um, less than 85 um, less than 90 um, less than 95 um, less than 100 um, less than 125 um, less than 150 um, less than 175 um, less than 200 um, less than 225 um, less than 250 um, less than 275 um, less than 300 um, less than 325 um, less than 350 um, less than 375 um, less than 400 um, less than 425 um, less than 450 um, less than 475 um, less than 500 um, less than 525 um, less than 550 um, less than 575 um, less than 600 um, less than 625 um, less than 650 um, less than 675 um, less than 700 um, less than 725 um, less than 750 um, less than 775 um, less than 800 um, less than 825 um, less than 850 um, less than 875 um, less than 900 um, less than 925 um, less than 950 um, less than 975 um.

[000516] In another embodiment, the compositions may be delivered using smaller 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, about 10 to about 50 nM, from about 20 to about 50 nm, from about 30 to about 50 nm, from about 40 to about 50 nm, from about 20 to about 60 nm, from about 30 to about 60 nm, from about 40 to about 60 nm, from about 20 to about 70 nm, from about 30 to about 70 nm, from about 40 to about 70 nm, from about 50 to about 70 nm, from
about 60 to about 70 nm, from about 20 to about 80 nm, from about 30 to about 80 nm, from about 40 to about 80 nm, from about 50 to about 80 nm, from about 60 to about 80 nm, from about 20 to about 90 nm, from about 30 to about 90 nm, from about 40 to about 90 nm, from about 50 to about 90 nm, from about 60 to about 90 nm and/or from about 70 to about 90 nm.

[000517] In some embodiments, such LNPs are synthesized using methods comprising microfluidic mixers. Exemplary microfluidic mixers may include, but are not limited to a slit interdigital micromixer including, but not limited to those manufactured by Microinnova (Allerheiligen bei Wildon, Austria) and/or a staggered herringbone micromixer (SHM) (Zhigaltsev, I.V. et al., Bottom-up design and synthesis of limit size lipid nanoparticle systems with aqueous and triglyceride cores using millisecond microfluidic mixing have been published (Langmuir. 2012. 28:3633-40; Belliveau, N.M. et al., Microfluidic synthesis of highly potent limit-size lipid nanoparticles for in vivo delivery of siRNA. Molecular Therapy-Nucleic Acids. 2012. 1:e37; Chen, D. et al., Rapid discovery of potent siRNA-containing lipid nanoparticles enabled by controlled microfluidic formulation. J Am Chem Soc. 2012. 134(16):6948-51; each of which is herein incorporated by reference in its entirety). In some embodiments, methods of LNP generation comprising SHM, further comprise the mixing of at least two input streams wherein mixing occurs by microstructure-induced chaotic advection (MICA). According to this method, fluid streams flow through channels present in a herringbone pattern causing rotational flow and folding the fluids around each other. This method may also comprise a surface for fluid mixing wherein the surface changes orientations during fluid cycling. Methods of generating LNPs using SHM include those disclosed in U.S. Application Publication Nos. 2004/0262223 and 2012/0276209, each of which is expressly incorporated herein by reference in their entirety.

[000518] In one embodiment, the compositions of the present invention may be formulated in lipid nanoparticles created using a micromixer such as, but not limited to, a Slit Interdigital Microstructured Mixer (SEVIM-V2) or a Standard Slit Interdigital Micro Mixer (SSIMM) or Caterpillar (CPMM) or Impinging-jet (IJMM) from the Institut für Mikrotechnik Mainz GmbH, Mainz Germany).
In one embodiment, the compositions of the present invention may be formulated in lipid nanoparticles created using microfluidic technology (see Whitesides, George M. The Origins and the Future of Microfluidics. Nature, 2006 442: 368-373; and Abraham et al. Chaotic Mixer for Microchannels. Science, 2002 295: 647-651; each of which is herein incorporated by reference in its entirety). As a non-limiting example, controlled microfluidic formulation includes a passive method for mixing streams of steady pressure-driven flows in microchannels at a low Reynolds number (See e.g., Abraham et al. Chaotic Mixer for Microchannels. Science, 2002 295: 647-651; which is herein incorporated by reference in its entirety).

In one embodiment, the compositions of the present invention may be formulated in lipid nanoparticles created using a micromixer chip such as, but not limited to, those from Harvard Apparatus (Holliston, MA) or Dolomite Microfluidics (Royston, UK). A micromixer chip can be used for rapid mixing of two or more fluid streams with a split and recombine mechanism.

In one embodiment, the compositions of the invention may be formulated for delivery using the drug encapsulating microspheres described in International Patent Publication No. WO2013063468 or U.S. Patent No. 8,440,614, each of which is herein incorporated by reference in its entirety. The microspheres may comprise a compound of the formula (I), (II), (III), (IV), (V) or (VI) as described in International patent application No. WO2013063468, the contents of which are herein incorporated by reference in its entirety. In another aspect, the amino acid, peptide, polypeptide, lipids (APPL) are useful in delivering the compositions of the invention to cells (see International Patent Publication No. WO2013063468, herein incorporated by reference in its entirety).

In one embodiment, the compositions of the invention may be formulated in lipid nanoparticles having a diameter from about 10 to about 100 nm such as, but not limited to, about 10 to about 20 nm, about 10 to about 30 nm, about 10 to about 40 nm, about 10 to about 50 nm, about 10 to about 60 nm, about 10 to about 70 nm, about 10 to about 80 nm, about 10 to about 90 nm, about 20 to about 30 nm, about 20 to about 40 nm, about 20 to about 50 nm, about 20 to about 60 nm, about 20 to about 70 nm, about 20 to about 80 nm, about 20 to about 90 nm, about 20 to about 100 nm, about 30 to about 40 nm, about 30 to about 50 nm, about 30 to about 60 nm, about 30 to about 70 nm, about 30
to about 80 nm, about 30 to about 90 nm, about 30 to about 100 nm, about 40 to about 50 nm, about 40 to about 60 nm, about 40 to about 70 nm, about 40 to about 80 nm, about 40 to about 90 nm, about 40 to about 100 nm, about 50 to about 60 nm, about 50 to about 70 nm about 50 to about 80 nm, about 50 to about 90 nm, about 50 to about 100 nm, about 60 to about 70 nm, about 60 to about 80 nm, about 60 to about 90 nm, about 60 to about 100 nm, about 70 to about 80 nm, about 70 to about 90 nm, about 70 to about 100 nm, about 80 to about 90 nm, about 80 to about 100 nm and/or about 90 to about 100 nm.

[000523] In one embodiment, the lipid nanoparticles may have a diameter from about 10 to 500 nm.

[000524] 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.

[000525] In one aspect, the lipid nanoparticle may be a limit size lipid nanoparticle described in International Patent Publication No. WO2013059922, the contents of which are herein incorporated by reference in its entirety. The limit size lipid nanoparticle may comprise a lipid bilayer surrounding an aqueous core or a hydrophobic core; where the lipid bilayer may comprise a phospholipid such as, but not limited to, diacylphosphatidylcholine, a diacylphosphatidylethanolamine, a ceramide, a sphingomyelin, a dihydrospingomyelin, a cephalin, a cerebroside, a C8-C20 fatty acid diacylphosphatidylcholine, and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC). In another aspect the limit size lipid nanoparticle may comprise a polyethylene glycol-lipid such as, but not limited to, DLPE-PEG, DMPE-PEG, DPPC-PEG and DSPE-PEG.

[000526] In one embodiment, the compositions may be delivered, localized and/or concentrated in a specific location using the delivery methods described in International Patent Publication No. WO2013063530, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, a subject may be administered an empty polymeric particle prior to, simultaneously with or after delivering the compositions to the subject. The empty polymeric particle undergoes a change in volume
once in contact with the subject and becomes lodged, embedded, immobilized or entrapped at a specific location in the subject.

[000527] In one embodiment, the compositions may be formulated in an active substance release system (See e.g., US Patent Publication No. US20130102545, herein incorporated by reference in its entirety). The active substance release system may comprise 1) at least one nanoparticle bonded to an oligonucleotide inhibitor strand which is hybridized with a catalytically active nucleic acid and 2) a compound bonded to at least one substrate molecule bonded to a therapeutically active substance (e.g., polynucleotides described herein), where the therapeutically active substance is released by the cleavage of the substrate molecule by the catalytically active nucleic acid.

[000528] In one embodiment, the compositions may be formulated in a nanoparticle comprising an inner core comprising a non-cellular material and an outer surface comprising a cellular membrane. The cellular membrane may be derived from a cell or a membrane derived from a virus. As a non-limiting example, the nanoparticle may be made by the methods described in International Patent Publication No. WO2013052167, herein incorporated by reference in its entirety. As another non-limiting example, the nanoparticle described in International Patent Publication No. WO2013052167, herein incorporated by reference in its entirety, may be used to deliver the compositions described herein.

[000529] In one embodiment, the compositions may be formulated in porous nanoparticle-supported lipid bilayers (protocells). Protocells are described in International Patent Publication No. WO2013056132, the contents of which are herein incorporated by reference in its entirety.

[000530] In one embodiment, the compositions described herein may be formulated in polymeric nanoparticles as described in or made by the methods described in US Patent No. 8,420,123 and 8,518,963 and European Patent No. EP2073848B1, the contents of each of which are herein incorporated by reference in their entirety. As a non-limiting example, the polymeric nanoparticle may have a high glass transition temperature such as the nanoparticles described in or nanoparticles made by the methods described in US Patent No. 8,518,963, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the polymer nanoparticle for oral, parenteral
and topical formulations may be made by the methods described in European Patent No. EP2073848B1, the contents of which are herein incorporated by reference in its entirety. [000531] In another embodiment, the compositions described herein may be formulated in nanoparticles used in imaging. The nanoparticles may be liposome nanoparticles such as those described in US Patent Publication No US20130129636, herein incorporated by reference in its entirety. As a non-limiting example, the liposome may comprise gadolinium(III)2-{4,7-bis-carboxymethyl-10-[(N,N-distearylamidomethyl-N'-amido-methyl]-1,4,7,10-tetrazacyclododec-l-yl}-acetic acid and a neutral, fully saturated phospholipid component (see e.g., US Patent Publication No US20130129636, the contents of which is herein incorporated by reference in its entirety).

[000532] In one embodiment, the nanoparticles which may be used in the present invention are formed by the methods described in U.S. Patent Application No. US20130130348, the contents of which is herein incorporated by reference in its entirety.

[000533] The nanoparticles of the present invention may further include nutrients such as, but not limited to, those which deficiencies can lead to health hazards from anemia to neural tube defects (see e.g., the nanoparticles described in International Patent Publication NoWO2013072929, the contents of which is herein incorporated by reference in its entirety). As a non-limiting example, the nutrient may be iron in the form of ferrous, ferric salts or elemental iron, iodine, folic acid, vitamins or micronutrients.

[000534] In one embodiment, the compositions of the present invention may be formulated in a swellable nanoparticle. The swellable nanoparticle may be, but is not limited to, those described in U.S. Patent No. 8,440,231, the contents of which is herein incorporated by reference in its entirety. As a non-limiting embodiment, the swellable nanoparticle may be used for delivery of the compositions of the present invention to the pulmonary system (see e.g., U.S. Patent No. 8,440,231, the contents of which is herein incorporated by reference in its entirety).

[000535] The compositions of the present invention may be formulated in polyanhydride nanoparticles such as, but not limited to, those described in U.S. Patent No. 8,449,916, the contents of which is herein incorporated by reference in its entirety.

[000536] The nanoparticles and microparticles of the present invention may be geometrically engineered to modulate macrophage and/or the immune response. In one
aspect, the geometrically engineered particles may have varied shapes, sizes and/or surface charges in order to incorporated the polynucleotides of the present invention for targeted delivery such as, but not limited to, pulmonary delivery (see e.g., International Publication No WO2013082111, the contents of which is herein incorporated by reference in its entirety). Other physical features the geometrically engineering particles may have include, but are not limited to, fenestrations, angled arms, asymmetry and surface roughness, charge which can alter the interactions with cells and tissues. As a non-limiting example, nanoparticles of the present invention may be made by the methods described in International Publication No WO2013082111, the contents of which is herein incorporated by reference in its entirety.

[000537] In one embodiment, the nanoparticles of the present invention may be water soluble nanoparticles such as, but not limited to, those described in International Publication No. WO2013090601, the contents of which is herein incorporated by reference in its entirety. The nanoparticles may be inorganic nanoparticles which have a compact and zwitterionic ligand in order to exhibit good water solubility. The nanoparticles may also have small hydrodynamic diameters (HD), stability with respect to time, pH, and salinity and a low level of non-specific protein binding.

[000538] In one embodiment the nanoparticles of the present invention may be developed by the methods described in US Patent Publication No. US20130172406, the contents of which are herein incorporated by reference in its entirety.

[000539] In one embodiment, the nanoparticles of the present invention are stealth nanoparticles or target-specific stealth nanoparticles such as, but not limited to, those described in US Patent Publication No. US20130172406; the contents of which is herein incorporated by reference in its entirety. The nanoparticles of the present invention may be made by the methods described in US Patent Publication No. US20130172406, the contents of which are herein incorporated by reference in its entirety.

[000540] In another embodiment, the stealth or target-specific stealth nanoparticles may comprise a polymeric matrix. The polymeric matrix may comprise two or more polymers such as, but not limited to, polyethylenes, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols,
polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, polyamines, polyesters, polyanhydrides, polyethers, polyurethanes, polymethacrylates, polyacrylates, polycyanoacrylates or combinations thereof.

[000541] In one embodiment, the nanoparticle may be a nanoparticle-nucleic acid hybrid structure having a high density nucleic acid layer. As a non-limiting example, the nanoparticle-nucleic acid hybrid structure may be made by the methods described in US Patent Publication No. US20130171646, the contents of which are herein incorporated by reference in its entirety. The nanoparticle may comprise a nucleic acid such as, but not limited to, polynucleotides described herein and/or known in the art.

[000542] At least one of the nanoparticles of the present invention may be embedded in the core a nanostructure or coated with a low density porous 3-D structure or coating which is capable of carrying or associating with at least one payload within or on the surface of the nanostructure. Non-limiting examples of the nanostructures comprising at least one nanoparticle are described in International Patent Publication No. WO2013123523, the contents of which are herein incorporated by reference in its entirety.

Polymers, Biodegradable Nanoparticles, and Core-Shell Nanoparticles

[000543] The compositions of the invention can be formulated using natural and/or synthetic polymers. Non-limiting examples of polymers which may be used for delivery include, but are not limited to, DYNAMIC POLYCONJUGATE® (Arrowhead Research Corp., Pasadena, CA) formulations from MIRUS® Bio (Madison, WI) and Roche Madison (Madison, WI), PHASERX™ polymer formulations such as, without limitation, SMARTT POLYMER TECHNOLOGY™ (PHASERX®, Seattle, WA), DMRI/DOPE, poloxamer, VAXFECTIN® adjuvant from Vical (San Diego, CA), chitosan, cyclodextrin from Calando Pharmaceuticals (Pasadena, CA), dendrimers and poly(lactic-co-glycolic acid) (PLGA) polymers. RONDEL™ (RNAi/Oligonucleotide Nanoparticle Delivery) polymers (Arrowhead Research Corporation, Pasadena, CA) and pH responsive co-block polymers such as, but not limited to, PHASERX® (Seattle, WA).

[000544] A non-limiting example of chitosan formulation includes a core of positively charged chitosan and an outer portion of negatively charged substrate (U.S. Pub. No.
20120258176; herein incorporated by reference in its entirety). Chitosan includes, but is not limited to N-trimethyl chitosan, mono-N-carboxymethyl chitosan (MCC), N-palmitoyl chitosan (NPCS), EDTA-chitosan, low molecular weight chitosan, chitosan derivatives, or combinations thereof.

[000545] In one embodiment, the polymers used in the present invention have undergone processing to reduce and/or inhibit the attachment of unwanted substances such as, but not limited to, bacteria, to the surface of the polymer. The polymer may be processed by methods known and/or described in the art and/or described in International Pub. No. WO2012150467, herein incorporated by reference in its entirety.

[000546] A non-limiting example of PLGA formulations include, but are not limited to, PLGA injectable depots (e.g., ELIGARD® which is formed by dissolving PLGA in 66% N-methyl-2-pyrrolidone (NMP) and the remainder being aqueous solvent and leuprolide. Once injected, the PLGA and leuprolide peptide precipitates into the subcutaneous space).

[000547] Many of these polymer approaches have demonstrated efficacy in delivering oligonucleotides in vivo into the cell cytoplasm (reviewed in deFougerolles Hum Gene Ther. 2008 19:125-132; herein incorporated by reference in its entirety). Two polymer approaches that have yielded robust in vivo delivery of nucleic acids, in this case with small interfering RNA (siRNA), are dynamic polyconjugates and cyclodextrin-based nanoparticles (see e.g., US Patent Publication No. US20130156721, herein incorporated by reference in its entirety). The first of these delivery approaches uses dynamic polyconjugates and has been shown in vivo in mice to effectively deliver siRNA and silence endogenous target mRNA in hepatocytes (Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887; herein incorporated by reference in its entirety). This particular approach is a multicomponent polymer system whose key features include a membrane-active polymer to which nucleic acid, in this case siRNA, is covalently coupled via a disulfide bond and where both PEG (for charge masking) and N-acetylglactosamine (for hepatocyte targeting) groups are linked via pH-sensitive bonds (Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887; herein incorporated by reference in its entirety). On binding to the hepatocyte and entry into the endosome, the polymer complex disassembles in the low-pH environment, with the polymer
exposing its positive charge, leading to endosomal escape and cytoplasmic release of the siRNA from the polymer. Through replacement of the ^-acetylgalactosamine group with a mannose group, it was shown one could alter targeting from asialoglycoprotein receptor-expressing hepatocytes to sinusoidal endothelium and Kupffer cells. Another polymer approach involves using transferrin-targeted cyclodextrin-containing polycation nanoparticles. These nanoparticles have demonstrated targeted silencing of the EWS-FLII gene product in transferrin-receptor-expressing Ewing's sarcoma tumor cells (Hu-Lieskovavan et al., Cancer Res. 2005 65: 8984-8982; herein incorporated by reference in its entirety) and siRNA formulated in these nanoparticles was well tolerated in non-human primates (Heidel et al., Proc Natl Acad Sci USA 2007 104:5715-21; herein incorporated by reference in its entirety). Both of these delivery strategies incorporate rational approaches using both targeted delivery and endosomal escape mechanisms.

The polymer formulation can permit the sustained or delayed release of polynucleotides (e.g., following intramuscular or subcutaneous injection). The altered release profile for the polynucleotide can result in, for example, translation of an encoded protein over an extended period of time. The polymer formulation may also be used to increase the stability of the polynucleotide. Biodegradable polymers have been previously used to protect nucleic acids other than polynucleotide from degradation and been shown to result in sustained release of payloads in vivo (Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887; Sullivan et al., Expert Opin Drug Deliv. 2010 7:1433-1446; Convertine et al., Biomacromolecules. 2010 Oct 1; Chu et al., Acc Chem Res. 2012 Jan 13; Manganiello et al., Biomaterials. 2012 33:2301-2309; Benoit et al., Biomacromolecules. 2011 12:2708-2714; Singha et al., Nucleic Acid Ther. 2011 2:133-147; deFougerolles Hum Gene Ther. 2008 19:125-132; Schaffert and Wagner, Gene Ther. 2008 16:1131-1138; Chaturvedi et al., Expert Opin Drug Deliv. 2011 8:1455-1468; Davis, Mol Pharm. 2009 6:659-668; Davis, Nature 2010 464:1067-1070; each of which is herein incorporated by reference in its entirety).

In one embodiment, the compositions may be sustained release formulations. In a further embodiment, the sustained release formulations may be for subcutaneous delivery. Sustained release formulations may include, but are not limited to, PLGA microspheres, ethylene vinyl acetate (EVAc), poloxamer, GELSITE® (Nanotherapeutics,
Inc. Alachua, FL), HYLENEX® (Halozyme Therapeutics, San Diego CA), surgical
sealants such as fibrinogen polymers (Ethicon Inc. Cornelia, GA), TISSELL® (Baxter
International, Inc Deerfield, IL), PEG-based sealants, and COSEAL® (Baxter
International, Inc Deerfield, IL).

[000550] As a non-limiting example compositions may be formulated in PLGA
microspheres by preparing the PLGA microspheres with tunable release rates (e.g., days
and weeks) and encapsulating the modified mRNA in the PLGA microspheres while
maintaining the integrity of the modified mRNA during the encapsulation process.
EVAc are non-biodegradeable, biocompatible polymers which are used extensively in
pre-clinical sustained release implant applications (e.g., extended release products
Ocusert a pilocarpine ophthalmic insert for glaucoma or progestasert a sustained release
progesterone intrauterine devise; transdermal delivery systems Testoderm, Duragesic and
Selegiline; catheters). Poloxamer F-407 NF is a hydrophilic, non-ionic surfactant
triblock copolymer of polyoxyethylene-polyoxypropylene-polyoxyethylene having a low
viscosity at temperatures less than 5°C and forms a solid gel at temperatures greater than
15°C. PEG-based surgical sealants comprise two synthetic PEG components mixed in a
delivery device which can be prepared in one minute, seals in 3 minutes and is
reabsorbed within 30 days. GELSITE® and natural polymers are capable of in-situ
gelation at the site of administration. They have been shown to interact with protein and
peptide therapeutic candidates through ionic ineraction to provide a stabilizing effect.

[000551] Polymer formulations can also be selectively targeted through expression of
different ligands as exemplified by, but not limited by, folate, transferrin, and N-
acetylgalactosamine (GalNAc) (Benoit et al., Biomacromolecules. 2011 12:2708-2714;
2009 6:659-668; Davis, Nature 2010 464:1067-1070; each of which is herein
incorporated by reference in its entirety).

[000552] The compositions 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[a-(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, polylsine,
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 or combinations thereof.

[000553] As a non-limiting example, the compositions 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.

[000554] As another non-limiting example the compositions of the invention may be
formulated with a PLGA-PEG block copolymer (see US Pub. No. US2012004293 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 compositions 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).

[000555] 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 No. 8,460,696) 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 composition 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 compositions 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 dilykne unite comprising oligoamines (U.S. Pat. No. 8,236,280; herein incorporated by reference in its entirety).

[000556] The compositions 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), poly(cyanoacrylates and combinations thereof.

[000557] In one embodiment, the compositions of the present invention may be formulated with at least one polymer and/or derivatives thereof described in International Publication Nos. WO201115862, WO2012082574 and WO2012068187 and U.S. Pub. No. 20120283427, each of which are herein incorporated by reference in their entireties.

[000558] In another embodiment, the compositions of the present invention may be formulated with a polymer of formula Z as described in WO201115862, herein incorporated by reference in its entirety. In yet another embodiment, the compositions 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.

[000559] The compositions 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.

[000560] Formulations of compositions of the invention may include at least one amine-containing polymer such as, but not limited to polylysine, polyethylene imine, poly(amideamine) dendrimers, poly(amine-co-esters) or combinations thereof. As a non-limiting example, the poly(amine-co-esters) may be the polymers described in and/or made by the methods described in International Publication No WO2013082529, the contents of which are herein incorporated by reference in its entirety.

[000561] For example, the compositions of the invention may be formulated in a pharmaceutical compound including a poly(alkylene imine), a biodegradable cationic lipopolymer, a biodegradable block copolymer, a biodegradable polymer, or a biodegradable random copolymer, a biodegradable polyester block copolymer, a biodegradable polyester polymer, a biodegradable polyester random copolymer, a linear biodegradable copolymer, PAGA, a biodegradable cross-linked cationic multi-block copolymer or combinations thereof. The biodegradable cationic lipopolymer may be made by methods known in the art and/or described in U.S. Pat. No. 6,696,038, U.S. App. Nos. 20030073619 and 20040142474 each of which is herein incorporated by reference in their entireties. The poly(alkylene imine) may be made using methods known in the art and/or as described in U.S. Pub. No. 2010004315, herein incorporated by reference in its entirety. The biodegradable polymer, biodegradable block copolymer, the biodegradable random copolymer, biodegradable polyester block copolymer, biodegradable polyester polymer, or biodegradable polyester random copolymer may be made using methods known in the art and/or as described in U.S. Pat. Nos. 6,517,869 and 6,267,987, the contents of which are each incorporated herein by reference in their entirety. The linear biodegradable copolymer may be made using methods known in the art and/or as described in U.S. Pat. No. 6,652,886. The PAGA polymer may be made using methods known in the art and/or as described in U.S. Pat. No. 6,217,912 herein incorporated by reference in its entirety. The PAGA polymer may be copolymerized to form a copolymer or block copolymer with polymers such as but not limited to, poly-L-lysine, polyargin, polyornithine, histones, avidin, protamines, polylactides and poly(lactide-co-glycolides). The biodegradable cross-linked cationic
multi-block copolymers may be made by methods known in the art and/or as described in U.S. Pat. No. 8,057,821, 8,444,992 or U.S. Pub. No. 2012009145 each of which are herein incorporated by reference in their entireties. For example, the multi-block copolymers may be synthesized using linear polyethylenimine (LPEI) blocks which have distinct patterns as compared to branched polyethylenimines. Further, the composition or pharmaceutical composition may be made by the methods known in the art, described herein, or as described in U.S. Pub. No. 20100004315 or U.S. Pat. Nos. 6,267,987 and 6,217,912 each of which are herein incorporated by reference in their entireties.

[000562] The compositions of the invention may be formulated with at least one degradable polyester which may contain polycationic side chains. Degradable polyesters include, but are not limited to, poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), and combinations thereof. In another embodiment, the degradable polyesters may include a PEG conjugation to form a PEGylated polymer.

[000563] The compositions of the invention may be formulated with at least one crosslinkable polyester. Crosslinkable polyesters include those known in the art and described in US Pub. No. 20120269761, the contents of which is herein incorporated by reference in its entirety.

[000564] The compositions of the invention may be formulated in or with at least one cyclodextrin polymer. Cyclodextrin polymers and methods of making cyclodextrin polymers include those known in the art and described in US Pub. No. 20130184453, the contents of which are herein incorporated by reference in its entirety.

[000565] In one embodiment, the compositions of the invention may be formulated in or with at least one crosslinked cation-binding polymers. Crosslinked cation-binding polymers and methods of making crosslinked cation-binding polymers include those known in the art and described in International Patent Publication No. WO2013106072, WO2013106073 and WO2013106086, the contents of each of which are herein incorporated by reference in its entirety.

[000566] In one embodiment, the compositions of the invention may be formulated in or with at least one branched polymer. Branched polymers and methods of making branched polymers include those known in the art and described in International Patent
Publication No. WO20131 13071, the contents of each of which are herein incorporated by reference in its entirety.

[000567] In one embodiment, the compositions of the invention may be formulated in or with at least PEGylated albumin polymer. PEGylated albumin polymer and methods of making PEGylated albumin polymer include those known in the art and described in US Patent Publication No. US20130231287, the contents of each of which are herein incorporated by reference in its entirety. I n another embodiment, the compositions

[000568] In one embodiment, the polymers described herein may be conjugated to a lipid-terminating PEG. As a non-limiting example, PLGA may be conjugated to a lipid-terminating PEG forming PLGA-DSPE-PEG. As another non-limiting example, PEG conjugates for use with the present invention are described in International Publication No. WO2008 103276, herein incorporated by reference in its entirety. The polymers may be conjugated using a ligand conjugate such as, but not limited to, the conjugates described in U.S. Pat. No. 8,273,363, herein incorporated by reference in its entirety.

[000569] In one embodiment, the compositions disclosed herein may be mixed with the PEGs or the sodium phosphate/sodium carbonate solution prior to administration.

[000570] In another embodiment, a polynucleotides encoding a protein of interest may be mixed with the PEGs and also mixed with the sodium phosphate/sodium carbonate solution.

[000571] In yet another embodiment, polynucleotides encoding a protein of interest may be mixed with the PEGs and polynucleotides encoding a second protein of interest may be mixed with the sodium phosphate/sodium carbonate solution.

[000572] In one embodiment, the compositions described herein may be conjugated with another compound. Non-limiting examples of conjugates are described in US Patent Nos. 7,964,578 and 7,833,992, each of which are herein incorporated by reference in their entireties. In another embodiment, modified RNA of the present invention may be conjugated with conjugates of formula 1-122 as described in US Patent Nos. 7,964,578 and 7,833,992, each of which are herein incorporated by reference in their entireties. The compositions described herein may be conjugated with a metal such as, but not limited to, gold. (See e.g., Giljohann et al. Journ. Amer. Chem. Soc. 2009 131(6): 2072-2073; herein incorporated by reference in its entirety). In another embodiment, the compositions
described herein may be conjugated and/or encapsulated in gold-nanoparticles. (International Pub. No. WO201216269 and U.S. Pub. No. 20120302940 and US20130177523; the contents of each of which is herein incorporated by reference in its entirety).

[000573] As described in U.S. Pub. No. 20100004313, herein incorporated by reference in its entirety, a gene delivery composition may include a nucleotide sequence and a poloxamer. For example, the compositions of the present invention may be used in a gene delivery composition with the poloxamer described in U.S. Pub. No. 20100004313.

[000574] In one embodiment, the polymer formulation of the present invention may be stabilized by contacting the polymer formulation, which may include a cationic carrier, with a cationic lipopolymer which may be covalently linked to cholesterol and polyethylene glycol groups. The polymer formulation may be contacted with a cationic lipopolymer using the methods described in U.S. Pub. No. 20090042829 herein incorporated by reference in its entirety. The cationic carrier may include, but is not limited to, polyethylenimine, poly(trimethylenimine), poly(tetramethylenimine), polypropyleneimine, aminoglycoside-polyamine, dideoxy-diamino-b-cyclodextrin, spermine, spermidine, poly(2-dimethylamino)ethyl methacrylate, poly(lysine), poly(histidine), poly(arginine), cationized gelatin, dendrimers, chitosan, 1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP), N-[1-(2,3-dioleoyoxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolium chloride (DOTIM), 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 3B-[N—(N\N-Dimethylaminoethane)-carbamoyl]Cholesterol Hydrochloride (DC-Cholesterol HCl) diheptadecylamidoglycyl spermidine (DOGS), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1,2-dimyristoxypyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRJE), N,N-dioleyl,N,N-dimethylammonium chloride DODAC) and combinations thereof. As a non-limiting example, the compositions may be formulated with a cationic lipopolymer such as those described in U.S. Patent Application No. 20130065942, herein incorporated by reference in its entirety.
The compositions of the invention may be formulated in a polyplex of one or more polymers (See e.g., U.S. Pat. No. 8,501,478, U.S. Pub. No. 20120237565 and 20120270927 and 20130149783 and International Patent Pub. No. WO2013090861; the contents of each of which is herein incorporated by reference in its entirety). As a non-limiting example, the polyplex may be formed using the noval alpha-aminoamidine polymers described in International Publication No. WO2013090861, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the polyplex may be formed using the click polymers described in US Patent No. 8,501,478, the contents of which is herein incorporated by reference in its entirety.

In one embodiment, the polyplex comprises two or more cationic polymers. The cationic polymer may comprise a poly(ethylene imine) (PEI) such as linear PEL. In another embodiment, the polyplex comprises p(TETA/CBA) its PEGylated analog p(TETA/CBA)-g-PEG2k and mixtures thereof (see e.g., US Patent Publication No. US20130149783, the contents of which are herein incorporated by reference in its entirety.

The compositions of the invention can also be formulated as a nanoparticle using a combination of polymers, lipids, and/or other biodegradable agents, such as, but not limited to, calcium phosphate. Components may be combined in a core-shell, hybrid, and/or layer-by-layer architecture, to allow for fine-tuning of the nanoparticle so the delivery of the composition may be enhanced (Wang et al., Nat Mater. 2006 5:791-796; Fuller et al., Biomaterials. 2008 29:1526-1532; DeKoker et al., Adv Drug Deliv Rev. 2011 63:748-761; Endres et al., Biomaterials. 2011 32:7721-7731; Su et al., Mol Pharm. 2011 Jun 6;8(3):774-87; herein incorporated by reference in its entirety). As a non-limiting example, the nanoparticle may comprise a plurality of polymers such as, but not limited to hydrophilic-hydrophobic polymers (e.g., PEG-PLGA), hydrophobic polymers (e.g., PEG) and/or hydrophilic polymers (International Pub. No. WO20120225 129; the contents of which is herein incorporated by reference in its entirety).

As another non-limiting example the nanoparticle comprising hydrophilic polymers for the compositions may be those described in or made by the methods described in International Patent Publication No. WO20131 19936, the contents of which are herein incorporated by reference in its entirety.
In one embodiment, the biodegradable polymers which may be used in the present invention are poly(ether-anhydride) block copolymers. As a non-limiting example, the biodegradable polymers used herein may be a block copolymer as described in International Patent Publication No. WO2006063249, herein incorporated by reference in its entirety, or made by the methods described in International Patent Publication No. WO2006063249, herein incorporated by reference in its entirety.

In another embodiment, the biodegradable polymers which may be used in the present invention are alkyl and cycloalkyl terminated biodegradable lipids. As a non-limiting example, the alkyl and cycloalkyl terminated biodegradable lipids may be those described in International Publication No. WO2013086322 and/or made by the methods described in International Publication No. WO2013086322; the contents of which are herein incorporated by reference in its entirety.

In yet another embodiment, the biodegradable polymers which may be used in the present invention are cationic lipids having one or more biodegradable group located in a lipid moiety. As a non-limiting example, the biodegradable lipids may be those described in US Patent Publication No. US20130195920, the contents of which are herein incorporated by reference in its entirety.

Biodegradable calcium phosphate nanoparticles in combination with lipids and/or polymers have been shown to deliver polynucleotides in vivo. In one embodiment, a lipid coated calcium phosphate nanoparticle, which may also contain a targeting ligand such as anisamide, may be used to deliver the compositions of the present invention. For example, to effectively deliver siRNA in a mouse metastatic lung model a lipid coated calcium phosphate nanoparticle was used (Li et al., J Contr Rel. 2010 142: 416-421; Li et al., J Contr Rel. 2012 158:108-114; Yang et al., Mol Ther. 2012 20:609-615; herein incorporated by reference in its entirety). This delivery system combines both a targeted nanoparticle and a component to enhance the endosomal escape, calcium phosphate, in order to improve delivery of the siRNA.

In one embodiment, calcium phosphate with a PEG-polyanion block copolymer may be used to deliver the compositions (Kazikawa et al., J Contr Rel. 2004 97:345-356; Kazikawa et al., J Contr Rel. 2006 111:368-370; the contents of each of which are herein incorporated by reference in its entirety).
In one embodiment, a PEG-charge-conversional polymer (Pitella et al., Biomaterials. 2011 32:3106-3114; the contents of which are herein incorporated by reference in its entirety) may be used to form a nanoparticle to deliver the compositions of the present invention. The PEG-charge-conversional polymer may improve upon the PEG-polyanion block copolymers by being cleaved into a polycation at acidic pH, thus enhancing endosomal escape.

In one embodiment, a polymer used in the present invention may be a pentablock polymer such as, but not limited to, the pentablock polymers described in International Patent Publication No. WO2013055331, herein incorporated by reference in its entirety. As a non-limiting example, the pentablock polymer comprises PGA-PCL-PEG-PCL-PGA, wherein PEG is polyethylene glycol, PCL is poly(E-caprolactone), PGA is poly(glycolic acid), and PLA is poly(lactic acid). As another non-limiting example, the pentablock polymer comprises PEG-PCL-PLA-PCL-PEG, wherein PEG is polyethylene glycol, PCL is poly(E-caprolactone), PGA is poly(glycolic acid), and PLA is poly(lactic acid).

In one embodiment, a polymer which may be used in the present invention comprises at least one diepoxide and at least one aminoglycoside (See e.g., International Patent Publication No. WO2013055971, the contents of which are herein incorporated by reference in its entirety). The diepoxide may be selected from, but is not limited to, 1,4 butanediol diglycidyl ether (1,4 B), 1,4-cyclohexanediethanol diglycidyl ether (1,4 C), 4-vinylcyclohexene diepoxide (4VCD), ethleneglycol diglycidyl ether (EDGE), glycerol diglycidyl ether (GDE), neopentylglycol diglycidyl ether (NPDGE), poly(ethleneglycol) diglycidyl ether (PEGDE), poly(propylene glycol) diglycidyl ether (PPGDE) and resorcinol diglycidyl ether (RDE). The aminoglycoside may be selected from, but is not limited to, streptomycin, neomycin, framycetin, paromomycin, ribostamycin, kanamycin, amikacin, arbekacin, bekanamycin, dibekacin, tobramycin, spectinomycin, hygromycin, gentamicin, netilmicin, sisomicin, isepamicin, verdamicin, astromicin, and apramycin. As a non-limiting example, the polymers may be made by the methods described in International Patent Publication No. WO2013055971, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, compositions comprising any of the polymers comprising at least one
least one diepoxide and at least one aminoglycoside may be made by the methods described in International Patent Publication No. WO2013055971, the contents of which are herein incorporated by reference in its entirety.

[000587] In one embodiment, a polymer which may be used in the present invention may be a cross-linked polymer. As a non-limiting example, the cross-linked polymers may be used to form a particle as described in US Patent No. 8,414,927, the contents of which are herein incorporated by reference in its entirety. As another non-limiting example, the cross-linked polymer may be obtained by the methods described in US Patent Publication No. US20130172600, the contents of which are herein incorporated by reference in its entirety.

[000588] In another embodiment, a polymer which may be used in the present invention may be a cross-linked polymer such as those described in US Patent No. 8,461,132, the contents of which are herein incorporated by reference in its entirety. As a non-limiting example, the cross-linked polymer may be used in a therapeutic composition for the treatment of a body tissue. The therapeutic composition may be administered to damaged tissue using various methods known in the art and/or described herein such as injection or catheterization.

[000589] In one embodiment, a polymer which may be used in the present invention may be a di-alphatic substituted pegylated lipid such as, but not limited to, those described in International Patent Publication No. WO2013049328, the contents of which are herein incorporated by reference in its entirety.

[000590] In one embodiment, a block copolymer is PEG-PLGA-PEG (see e.g., the thermosensitive hydrogel (PEG-PLGA-PEG) was used as a TGF-beta1 gene delivery vehicle in Lee et al. Thermosensitive Hydrogel as a Tgf-β1 Gene Delivery Vehicle Enhances Diabetic Wound Healing. Pharmaceutical Research, 2003 20(12): 1995-2000; as a controlled gene delivery system in Li et al. Controlled Gene Delivery System Based on Thermosensitive Biodegradable Hydrogel. Pharmaceutical Research 2003 20(6):884-888; and Chang et al., Non-ionic amphiphilic biodegradable PEG-PLGA-PEG copolymer enhances gene delivery efficiency in rat skeletal muscle. J Controlled Release. 2007 118:245-253; each of which is herein incorporated by reference in its entirety) may be used in the present invention. The present invention may be formulated with PEG-
PLGA-PEG for administration such as, but not limited to, intramuscular and subcutaneous administration.

In another embodiment, the PEG-PLGA-PEG block copolymer is used in the present invention to develop a biodegradable sustained release system. In one aspect, the compositions of the present invention are mixed with the block copolymer prior to administration. In another aspect, the compositions of the present invention are co-administered with the block copolymer.

In one embodiment, the polymer used in the present invention may be a multi-functional polymer derivative such as, but not limited to, a multi-functional N-maleimidyl polymer derivatives as described in US Patent No US8454946, the contents of which are herein incorporated by reference in its entirety.

The use of core-shell nanoparticles has additionally focused on a high-throughput approach to synthesize cationic cross-linked nanogel cores and various shells (Siegwart et al., Proc Natl Acad Sci U S A. 2011 108:12996-13001; the contents of which are herein incorporated by reference in its entirety). The complexation, delivery, and internalization of the polymeric nanoparticles can be precisely controlled by altering the chemical composition in both the core and shell components of the nanoparticle. For example, the core-shell nanoparticles may efficiently deliver siRNA to mouse hepatocytes after they covalently attach cholesterol to the nanoparticle.

In one embodiment, a hollow lipid core comprising a middle PLGA layer and an outer neutral lipid layer containing PEG may be used for delivery of the compositions of the present invention. As a non-limiting example, in mice bearing a luciferase-expressing tumor, it was determined that the lipid-polymer-lipid hybrid nanoparticle significantly suppressed luciferase expression, as compared to a conventional lipoplex (Shi et al, Angew Chem Int Ed. 2011 50:7027-7031; herein incorporated by reference in its entirety).

In one embodiment, the lipid nanoparticles may comprise a core of the compositions 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.
Core-shell nanoparticles for use with the compositions of the present invention are described and may be formed by the methods described in U.S. Pat. No. 8,313,777 or International Patent Publication No. WO2013124867, the contents of each of which are herein incorporated by reference in their entirety.

In one embodiment, the polymer used with the formulations described herein may be a modified polymer (such as, but not limited to, a modified polyacetal) as described in International Publication No. WO201120053, the contents of which are herein incorporated by reference in its entirety.

In one embodiment, the formulation may be a polymeric carrier cargo complex comprising a polymeric carrier and at least one nucleic acid molecule. Non-limiting examples of polymeric carrier cargo complexes are described in International Patent Publications Nos. WO2013113326, WO2013113501, WO2013113325, WO2013113502 and WO2013113736 and European Patent Publication No. EP2623121, the contents of each of which are herein incorporated by reference in their entireties. In one aspect the polymeric carrier cargo complexes may comprise a negatively charged nucleic acid molecule such as, but not limited to, those described in International Patent Publication Nos. WO2013113325 and WO2013113502, the contents of each of which are herein incorporated by reference in its entirety.

As a non-limiting example, the core-shell nanoparticle may be used to treat an eye disease or disorder (See e.g. US Publication No. 20120321719, the contents of which are herein incorporated by reference in its entirety).

In one embodiment, the polymer used with the formulations described herein may be a modified polymer (such as, but not limited to, a modified polyacetal) as described in International Publication No. WO201120053, the contents of which are herein incorporated by reference in its entirety.

Peptides and Proteins

The compositions 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. The compositions 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 Biologies (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).

[000602] 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.

[000603] Formulations of the including peptides or proteins may be used to increase cell transfection by the composition, 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. WO20121 10636 and WO2013123298; the contents of which are herein incorporated by reference in its entirety).
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.

**Cells**

The compositions 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).

The compositions may be delivered in synthetic VLPs synthesized by the methods described in International Pub No. WO201 1085231 and WO20131 16656 and US Pub No. 201 10171248, the contents of each of which are herein incorporated by reference in their entireties.

Cell-based formulations of the compositions 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**

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 and any of these may be used to introduce the compositions of the present invention. 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.

[000609] 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.

[000610] 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 devices are sold by many companies worldwide including, but not limited to BTX® Instruments (HoUiston, 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). In one embodiment, compositions may be delivered by electroporation as described in Example 9.

Micro-Organ

[000611] The compositions, e.g., 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., a polynucleotides of the present invention) encoding a polypeptide of interest, operably linked to one or more regulatory sequences. Micro-organs 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 [000672] - [000677].
Hyaluronidase

[000612] The intramuscular or subcutaneous localized injection of compositions 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

[000613] The compositions 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 compositions 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

[000614] The compositions 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 compositions may be bound to the nanotubes through forces such as, but not limited to, steric, ionic, covalent and/or other forces. Nanotubes 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 [000679] - [000684].

[000615] Conjugates
The compositions 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).

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-ethylacrylic acid), N-isopropylacrylamide polymers, or polyphosphazene.

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.

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.
In one embodiment, the conjugate of the present invention may function as a carrier for the compositions of the present invention. The conjugate may comprise a cationic polymer such as, but not limited to, polyamine, polylsine, 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.

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 composition of the present invention.

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-gulucosamine 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.

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-gulucosamine multivalent mannose, multivalent fucose, or aptamers. The ligand can be, for example, a lipopolysaccharide, or an activator of p38 MAP kinase.

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, aptamers, 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.

[000624] 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.

[000625] 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.

[000626] In another embodiment, the conjugate which may be used in the present invention may be an aptamer conjugate. Non-limiting examples of aptamer 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 compositions of the present invention.

[000627] 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 comprise releasable linkages to release the compositions upon and/or after delivery to a subject.

[000628] 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.

[000629] 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.

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.

Some embodiments featured in the invention include polynucleotides with phosphorothioate backbones and oligonucleosides with other modified backbones, and in particular -CH₂-NH-CH₂⁻, -CH₂-N(CH₃)-0-CH₂⁻ [known as a methylene (methylimino) or MMI backbone], -CH₂-O-N(CH₃)-CH₂⁻, -CH₂-N(CH₃)-N(CH₃)-CH₂—and —N(CH₃)—CH₂—CH₂—[wherein the native phosphodiester backbone is represented as --0—P(0)₂-0-CH₂⁻] 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.

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; 0-, S-, or N-alkyl; 0-, S-, or N-alkenyl; 0-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkaryl and alkynyl. Exemplary suitable modifications include 0[(CH₂)nO]mCH₃, 0(CH₂)nOCH₃, 0(CH₂)nNH₂, 0(CH₂)nC₃H₇, 0(CH₂)nONH₂, and 0(CH₂)nON[(CH₂)nCH₃]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: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, CI, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, 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'-0—CH₂CH₂OCH₃, also known as 2'-0-(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'-dimethylaminoxyethoxy, *i.e.*, a 0(CH₂)₂0N(CH₃)₂ group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-0-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e.*, 2'-0-CH₂0-CH₂N(CH₂)₂, also described in examples herein below. Other modifications include 2'-methoxy (2'-OCH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) 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.

**[000633]** In one embodiment, the compositions 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. WO201 1062965, 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.

**[000634]** In another embodiment, polynucleotides may be conjugated to SMARTT POLYMER TECHNOLOGY® (PHASERX®, Inc. Seattle, WA).

**[000635]** 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.

[000636] In yet another aspect, the conjugate may be a peptide that can assist in crossing the blood-brain barrier.

Self-Assembled Nanoparticles

Nucleic Acid Self-Assembled Nanoparticles

[000637] Self-assembled nanoparticles have a well-defined size which may be precisely controlled as the nucleic acid strands may be easily reprogrammable. For example, the optimal particle size for a cancer-targeting nanodelivery carrier is 20-100 nm as a diameter greater than 20 nm avoids renal clearance and enhances delivery to certain tumors through enhanced permeability and retention effect. Using self-assembled nucleic acid nanoparticles a single uniform population in size and shape having a precisely controlled spatial orientation and density of cancer-targeting ligands for enhanced delivery. As a non-limiting example, oligonucleotide nanoparticles were prepared using programmable self-assembly of short DNA fragments and therapeutic siRNAs. These nanoparticles are molecularly identical with controllable particle size and target ligand location and density. The DNA fragments and siRNAs self-assembled into a one-step reaction to generate DNA/siRNA tetrahedral nanoparticles for targeted in vivo delivery. (Lee et al., Nature Nanotechnology 2012 7:389-393; herein incorporated by reference in its entirety).

[000638] In one embodiment, the compositions disclosed herein, e.g., polynucleotide compositions, may be formulated as self-assembled nanoparticles. As a non-limiting example, nucleic acids may be used to make nanoparticles which may be used in a delivery system for the compositions of the present invention. Self-assembled nanoparticles 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 [00071 1] - [000715].

Polymer-Based Self-Assembled Nanoparticles

[000639] Polymers may be used to form sheets which self-assembled into nanoparticles. These nanoparticles may be used to deliver the compositions of the present invention. Polymer-based self-assembled nanoparticles 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 [000716] - [000720].

Self-Assembled Macromolecules

[000640] The compositions 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

[000641] The compositions 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).

[000642] In one embodiment, the inorganic nanoparticles may comprise a core of the compositions 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 compositions in the core.

Semi-conductive and Metallic Nanoparticles

[000643] The compositions 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.

[000644] In one embodiment, the semi-conductive and/or metallic nanoparticles may comprise a core of the compositions 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 compositions in the core.

Surgical Sealants: Gels and Hydrogels

[000645] In one embodiment, the compositions disclosed herein may be encapsulated into any hydrogel known in the art which may form a gel when injected into a subject. Hydrogels are a network of polymer chains that are hydrophilic, and are sometimes found as a colloidal gel in which water is the dispersion medium. Hydrogels are highly absorbent (they can contain over 99% water) natural or synthetic polymers. Hydrogels also possess a degree of flexibility very similar to natural tissue, due to their significant water content. The hydrogel described herein may used to encapsulate lipid nanoparticles which are biocompatible, biodegradable and/or porous. A hydrogel can be made in situ from solution injection or implanted.

[000646] Surgical sealants including gels and hydrogels 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 [000728] - [000773]. In one embodiment, the compositions disclosed herein may be formulated in a PEG-based surgical sealant or hydrogel.

[000647] In one embodiment, the surgical sealant or hydrogel may include at least one, at least two, at least three, at least four, at least five, at least six or more than six PEG lipids. The PEG lipids may be selected from, but are not limited to, pentaerythritol PEG ester tetra-succinimidyl and pentaerythritol PEG ether tetra-thiol, PEG-c-DOMG, PEG-DMG (1,2-Dimyristoyl-sn-glycerol, methoxypolyethylene Glycol), PEG-DSG (1,2-Distearoyl-sn-glycerol, methoxypolyethylene Glycol), PEG-DPG (1,2-Dipalmitoyl-sn-glycerol, methoxypolyethylene glycol), PEG-DSA (PEG coupled to 1,2-distearyloxypropyl-3-amine), PEG-DMA (PEG coupled to 1,2-dimyrystyloxypropyl-3-amine, PEG-c-DNA, PEG-c-DMA, PEG-S-DSG, PEG-c-DMA, PEG-DPG, PEG-DMG 2000 and those described herein and/or known in the art. The concentration and/or ratio of the PEG lipids in the surgical sealant or hydrogel may be varied in order to optimize the formulation for delivery and/or administration.

[000648] The amount of buffer and/or acid used in combination with the PEG lipids of the surgical sealant or hydrogel may also be varied. In one non-limiting example, the
ratio of buffer and/or acid with PEG lipids is 1:1. As a non-limiting example, the amount
of buffer and/or acid used with the PEG lipids may be increased to alter the ratio of
buffer/acid to PEG in order to optimize the surgical sealant or hydrogel. As another non-
limiting example, the amount of buffer and/or acid used with the PEG lipids may be
deincreased to alter the ratio of buffer/acid to PEG in order to optimize the surgical sealant
or hydrogel.

[000649] The amount of compositions loaded into the buffer, acid and/or PEG lipid
may be varied. The amount of compositions loaded into the buffer, acid and/or PEG lipid
may be, but is not limited to, at least 1 μL, at least 2 μL, at least 5 μL, at least 10 μL, at
least 15 μL, at least 20 μL, at least 25 μL, at least 30 μL, at least 35 μL, at least 40 μL, at
least 45 μL, at least 50 μL, at least 55 μL, at least 60 μL, at least 65 μL, at least 70 μL, at
least 75 μL, at least 80 μL, at least 85 μL, at least 90 μL, at least 100 μL, at least 125 μL,
at least 150 μL, at least 200 μL, at least 250 μL, at least 300 μL, at least 350 μL, at least
400 μL, at least 450 μL, at least 500 μL or more than 500 μL.

[000650] In one embodiment, the compositions of the present invention may be loaded
in PEGs and also in the buffer or the acid. The amount of compositions loaded in the
PEG may be the same, greater or less than the amount loaded in the buffer or acid. In
another embodiment, the compositions may be formulated, by the methods described
herein and/or known in the art, prior to loading in the PEGs, buffer or acid.

[000651] A non-limiting example of a PEG-based hydrogel which may be used in the
present invention is described in US Patent No. 8,524,215, the contents of which is herein
incorporated by reference in its entirety. The PEG-based hydrgel may be an absorbable
hydrogel prepared from a multi-arm PEG-vinylsulfone having about 3 to about 8 arms
and a multi-arm-PEG-R-sulfhydryl having about 3 to about 8 arms (See e.g., US Patent
No. 8,524,215). In one embodiment, the PEG-based hydrogel may be an absorbable
hydrogel. While not wishing to be bound by theory, an absorbable PEG-based hydrogel
may be beneficial to reduce the permanent chronic foreign body reaction since the
absorbable hydrogel can be absorbed and passed by the body.

[000652] In one embodiment, the hydrogel may be a thermosensitive hydrogel. In one
aspect the thermosensitive hydrogel may be, but is not limited to, a triblock polymer such
as those described herein and known in the art. As a non-limiting example, the tri-block
polymer may be PEG-PLGA-PEG (see e.g., the thermosensitive hydrogel (PEG-PLGA-PEG) was used as a TGF-beta gene delivery vehicle in Lee et al. Thermosensitive Hydrogel as a Tgf- β Gene Delivery Vehicle Enhances Diabetic Wound Healing, Pharmaceutical Research, 2003 20(12): 1995-2000; as a controlled gene delivery system in Li et al. Controlled Gene Delivery System Based on Thermosensitive Biodegradable Hydrogel. Pharmaceutical Research 2003 20(6):884-888; and Chang et al., Non-ionic amphiphilic biodegradable PEG-PLGA-PEG copolymer enhances gene delivery efficiency in rat skeletal muscle. J Controlled Release. 2007 118:245-253; each of which is herein incorporated by reference in its entirety). As a non-limiting example, the thermosensitive hydrogel may be used to make nanoparticles and liposomes by the methods described in International Publication No. WO20 13123407, the contents of which are herein incorporated by reference in its entirety.

[000653] In another embodiment, the hydrogel may be a biodegradable copolymer hydrogel (see e.g., the biodegradable hydrogels described by Nguyen and Lee (Injectable Biodegradable Hydrogels. Macromolecular Bioscience. 2010 10:563-579), herein incorporated by reference in its entirety). These hydrogels may exhibit a sol-gel phase transition that respond to external stimuli such as, but not limited to, temperature changes, pH alternations or both. Non-limiting examples of biodegradable copolymer hydrogels include triblock copolymers PEG-PLLA-PEG, PEG-PLA-PEG (see e.g., Chang et al., Non-ionic amphiphilic biodegradable PEG-PLGA-PEG copolymer enhances gene delivery efficiency in rat skeletal muscle. J Controlled Release. 2007 118:245-253, herein incorporated by reference in its entirety), PLGA-PEG-PLGA, PEG-PCL-PEG, PCL-PEG-PCL, polyesters such as poly [(R)-3-hydroxybutyrate] (PHB), polyphosphazenes such as L-sioleucine ethyl ester (IleOEt), D,L-leucine ethyl ester (LeuOEt), L-valine ethyl ester (ValOEt), or di-, tri- and oligo-peptides, polypeptides and chitosan. Temperature and pH sensitive polymers which may be used to form the biodegradable copolymer hydrogels include, but are not limited to, sulfamethazine-, poly (P-amino ester)-, poly(amoine urethane)-, and poly(amoine) based polymers. Formulations of the biodegradable copolymer hydrogels and compositions may be administered using site-specific control of release behavior.
[000654] In one embodiment, the hydrogel used in the present invention may be a PEG based hydrogel such as, but not limited to, those described in International Patent Publication No WO2013082590, herein incorporated by reference in its entirety. The PEG based hydrogel may have, but is not limited to, an overall polymer weight concentration of less than or equal to 50% at the time of curing. As a non-limiting example, the PEG based hydrogel may be made by the methods described in International Patent Publication No WO2013082590, the contents of which are herein incorporated by reference in its entirety.

[000655] In another embodiment, the compositions may be formulated in a nanostructured gel composition. The nanostructured gel may be capable of controlled release of the encapsulated compositions. Non-limiting examples of nanostructured gels or self-assembled gels are described in International Patent Publication No. WO2012040623, the contents of which are herein incorporated by reference in its entirety.

[000656] In one embodiment, the concentration of the compositions of the present invention in the surgical sealants, gels and/or hydrogels may be selected to provide a dosage within the range to have the desired therapeutic effect.

[000657] In one embodiment, the concentration of the polynucleotides of the composition of the present invention in the surgical sealants, gels and/or hydrogels may be at least 0.001 mg to at least 150 mg in at least 0.1 ml to at least 30 ml of the surgical sealant, gel or hydrogel. The concentration of the polynucleotides of the present invention may be at least 0.001 mg, at least 0.005 mg, at least 0.01 mg, at least 0.05 mg, at least 0.1 mg, at least 0.5 mg, at least 1 mg, at least 5 mg, at least 7 mg, at least 10 mg, at least 12, at least 15 mg, at least 17 mg, at least 20 mg, at least 22 mg, at least 25 mg, at least 27 mg, at least 30 mg, at least 32 mg, at least 35 mg, at least 40 mg, at least 45 mg, at least 50 mg, at least 55 mg, at least 60 mg, at least 65 mg, at least 70 mg, at least 75 mg, at least 80 mg, at least 85 mg, at least 90 mg, at least 95 mg, at least 100 mg, at least 105 mg, at least 110 mg, at least 115 mg, at least 120 mg, at least 125 mg, at least 130 mg, at least 135 mg, at least 140 mg, at least 145 mg or at least 150 mg in at least 0.1 ml, at least 0.2 ml, at least 0.3 ml, at least 0.4 ml, at least 0.5 ml, at least 0.6 ml, at least 0.7 ml, at least 0.8 ml, at least 0.9 ml, at least 1 ml, at least 2 ml, at least 3 ml, at least 4 ml,
at least 5 ml, at least 6 ml, at least 7 ml, at least 8 ml, at least 9 ml, at least 10 ml, at least 11 ml, at least 12 ml, at least 13 ml, at least 14 ml, at least 15 ml, at least 16 ml, at least 17 ml, at least 18 ml, at least 19 ml, at least 20 ml, at least 21 ml, at least 22 ml, at least 23 ml, at least 24 ml, at least 25 ml, at least 26 ml, at least 27 ml, at least 28 ml, at least 29 ml or at least 30 ml of the surgical sealant, gel or hydrogel.

[000658] In another embodiment, concentration of the polynucleotides of the composition of the present invention in the surgical sealants, gels and/or hydrogels may be at least 0.001 mg/ml at least 0.005 mg/ml, at least 0.01 mg/ml, at least 0.05 mg/ml, at least 0.1 mg/ml, at least 0.5 mg/ml, at least 1 mg/ml, at least 5 mg/ml, at least 7 mg/ml, at least 10 mg/ml, at least 12, at least 15 mg/ml, at least 17 mg/ml, at least 20 mg/ml, at least 22 mg/ml, at least 25 mg/ml, at least 27 mg/ml, at least 30 mg/ml, at least 32 mg/ml, at least 35 mg/ml, at least 40 mg/ml, at least 45 mg/ml or at least 50 mg/ml.

[000659] Technology allowing for large subcutaneous infusion volumes which are known in the art, such as, but not limited to, HYLENEX® (Halozyme Therapeutics, San Diego, CA) may also be used. The dispersion and/or adsorption of the modified mRNA described herein may be increased with the use of HYLENEX® as HYLENEX® temporarily breaks down hyaluronic acid causing a temporary degradation in the subcutaneous space (for about 24 hours) just beneath the outside surface of the skin opening microscopic channels and allowing fluid or drugs to be dispersed and absorbed in the body.

[000660] In one embodiment, the hydrogel is a PEG based hydrogel which may be used for a topical application (See e.g., US Patent Publication No. US20130149318, herein incorporated by reference in its entirety).

[000661] In another embodiment, the hydrogel is an absorbable hydrogel. The absorbably hydrogel may be a PEG-based hydrogel as described in and/or made by the methods described in International Publication No. WO2012018718, the contents of which are herein incorporated by reference in its entirety. The absorbable hydrogels may be used to form sustained release compositions for use with the present invention (see e.g., International Pub. No. WO2012018718, the contents of which are herein incorporated by reference in its entirety).
In one embodiment, the hydrogel may comprise a polymer described in International Publication No. WO2013091001, the contents of which are herein incorporated by reference in its entirety.

Suspensionformulations

In some embodiments, suspension formulations are provided comprising compositions, water immiscible oil depots, surfactants and/or co-surfactants and/or co-solvents. Combinations of oils and surfactants may enable suspension formulation with compositions. Delivery of compositions 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. Suspension formulations 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 [000775] - [000781].

Cations and Anions

Formulations of the compositions 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 composition 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).

In some embodiments, cationic nanoparticles comprising combinations of divalent and monovalent cations may be formulated with the compositions. Such nanoparticles may form spontaneously in solution over a give 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 compositions in cationic nanoparticles or in one or more depot comprising cationic nanoparticles may improve composition bioavailability by acting as a long-acting depot and/or reducing the rate of degradation by nucleases.

Molded Nanoparticles and Microparticles
The compositions disclosed herein may be formulated in nanoparticles and/or microparticles. These nanoparticles and/or microparticles may be molded into any size shape and chemistry. Molded nanoparticles and microparticles 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 [000784] - [000788].

**NanoJackets and NanoLiposomes**

The compositions disclosed herein may be formulated in NanoJackets and NanoLiposomes by Keystone Nano (State College, PA). NanoJackets are made of compounds that are naturally found in the body including calcium, phosphate and may also include a small amount of silicates. Nanojackets may range in size from 5 to 50 nm and may be used to deliver hydrophilic and hydrophobic compounds such as, but not limited to, the compositions of the present invention.

NanoLiposomes are made of lipids such as, but not limited to, lipids which naturally occur in the body. NanoLiposomes may range in size from 60-80 nm and may be used to deliver hydrophilic and hydrophobic compounds such as, but not limited to, the compositions. In one aspect, the compositions disclosed herein are formulated in a NanoLiposome such as, but not limited to, Ceramide NanoLiposomes.

**Pseudovirions**

In one embodiment, the compositions disclosed herein may be formulated in Pseudovirions (e.g., pseudo-virions). Pseudovirions 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 [000791] - [000796].

**Minicells**

In one aspect, the compositions may be formulated in bacterial minicells. As a non-limiting example, bacterial minicells may be those described in International Publication No. WO2013088250 or US Patent Publication No. US20130177499, the contents of each of which are herein incorporated by reference in its entirety. The bacterial minicells comprising therapeutic agents such as the compositions described herein may be used to deliver the therapeutic agents to brain tumors.
Semi-solid Compositions

[000672] In one embodiment, the compositions 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.

[000673] 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).

[000674] The semi-solid composition using the compositions 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-mm$^{-2}$, and/or a viscosity of at least 100 mPa-s).

Exosomes

[000675] In one embodiment, the compositions may be formulated in exosomes. The exosomes may be loaded with at least one composition and delivered to cells, tissues and/or organisms. As a non-limiting example, the compositions may be loaded in the exosomes described in International Publication No. WO2013084000, herein incorporated by reference in its entirety.

Silk-Based Delivery

[000676] In one embodiment, the compositions 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 compositions 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 Publication No. US2013017761 1, the contents of which are herein incorporated by reference in its entirety.
Microparticles

In one embodiment, formulations comprising the compositions may comprise microparticles. The microparticles may comprise a polymer described herein and/or known in the art such as, but not limited to, poly(a-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 the compositions. 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 and US20130195898 and US Patent No. 8,309,139 and 8,206,749, the contents of each of which are herein incorporated by reference in its entirety.

In another embodiment, the formulation may be a microemulsion comprising microparticles and the compositions of the present invention. 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 and 8,206,749, the contents of each of which are herein incorporated by reference in its entirety.

Amino Acid Lipids

In one embodiment, the compositions 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, the contents of which are herein incorporated by reference in its entirety.

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.

In one embodiment, the amino acid lipid formulations may be used to deliver the compositions to a subject.

In another embodiment, the amino acid lipid formulations may deliver a composition in releasable form which comprises an amino acid lipid that binds and releases the composition. As a non-limiting example, the release of the compositions 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.

**Microvesicles**

[000683] In one embodiment, the compositions 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.

[000684] 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.

**Interpolyelectrolyte Complexes**

[000685] In one embodiment, the compositions 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**

[000686] In one embodiment, the compositions 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.

**Excipients**

[000687] The 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.

[000688] 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.

[000689] 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.

[000690] Exemplary diluents, granulating and/or dispersing agents, surface active agents and/or emulsifiers, binding agents, preservatives, buffers, lubricating agents, oils, additives, cocoa butter and suppository waxes, coloring agents, coating agents, sweetening, flavoring, and/or perfuming agents 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 [000828] - [000838].

Cryoprotectants for mRNA

[000691] In some embodiments, the formulations may comprise cryoprotectants. 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 the compositions 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 the 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

[000692] In some embodiments, 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 formulations to yield a "pharmaceutically elegant" cake, stabilizing the lyophilized compositions 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 compositions during freezing and provide a bulking agent for lyophilization.

[000693] Non-limiting examples of formulations and methods for formulating the compositions 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

[000694] In some embodiments, the compositions may comprise at least one excipient which is an inactive ingredient. As used herein, the 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). A non-exhaustive list of inactive ingredients and the routes of administration the inactive ingredients may be formulated in are described in Table 9 of International Application WO2015/085318, the contents of which are incorporated herein by reference.

Delivery

[000695] The present disclosure encompasses the delivery of compositions 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

[000696] The compositions of the present invention may be delivered to a cell naked. As used herein in, "naked" refers to delivering compositions free from agents which promote transfection. For example, the compositions delivered to the cell may contain no modifications. The naked compositions may be delivered to the cell using routes of administration known in the art and described herein.

Formulated Delivery

[000697] The compositions 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 compositions may be delivered to the cell using routes of administration known in the art and described herein.

[000698] 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

[000699] The compositions of the present invention may be administered by any route which results in a therapeutically effective outcome. These include, but are not limited to enteral (into the intestine), gastroenteral, epidural (into the dura matter), oral (by way of the mouth), transdermal, peridural, intracerebral (into the cerebrum), intracerebroventricular (into the cerebral ventricles), epicutaneous (application onto the skin), intradermal, (into the skin itself), subcutaneous (under the skin), nasal administration (through the nose), intravenous (into a vein), intravenous bolus, intravenous drip, intraarterial (into an artery), intramuscular (into a muscle), intracardiac (into the heart), intraosseous infusion (into the bone marrow), intrathecal (into the spinal canal), intraperitoneal, (infusion or injection into the peritoneum), intravesical infusion, intravitreal, (through the eye), intracavernous injection (into a pathologic cavity) intracavitary (into the base of the penis), intravaginal administration, intrauterine, extra-amniotic administration, transdermal (diffusion through the intact skin for systemic distribution), transmucosal (diffusion through a mucous membrane), transvaginal, insufflation (snorting), sublingual, sublabial, enema, eye drops (onto the conjunctiva), in ear drops, auricular (in or by way of the ear), buccal (directed toward the cheek), conjunctival, cutaneous, dental (to a tooth or teeth), electro-osmosis, endocervical, endosinusial, endotracheal, extracorporeal, hemodialysis, infiltration, interstitial, intra-abdominal, intra-amniotic, intra-articular, intrabiliary, intrabursal, intracartilaginous (within a cartilage), intracaudal (within the cauda equine), intracistemal (within the cisterna magna cerebellomedularis), intracorneal (within the cornea), dental intracoronar, intracoronary (within the coronary arteries), intracorporal cavernosum (within the dilatable spaces of the corporal cavernosa of the penis), intradiscal (within a disc), intraductal (within a duct of a gland), intraduodenal (within the duodenum), intradural (within or beneath the dura), intraepidermal (to the epidermis), intraesophageal (to the esophagus), intragastric (within the stomach), intragingival (within the gingivae), intraileal (within the distal portion of the small intestine), intralesional (within or introduced directly to a localized lesion), intraluminal (within a lumen of a tube),
intralymphatic (within the lymph), intramedullary (within the marrow cavity of a bone),
intrameningeal (within the meninges), intraocular (within the eye), intraovarian (within the
ovary), intrapericardial (within the pericardium), intrapleural (within the pleura),
intraprostatic (within the prostate gland), intrapulmonary (within the lungs or its bronchi),
intrasal (within the nasal or periorbital sinuses), intraspinal (within the vertebral
column), intrasynovial (within the synovial cavity of a joint), intratendinous (within a
tendon), intratesticular (within the testicle), intrathecal (within the cerebrospinal fluid at
any level of the cerebrospinal axis), intrathoracic (within the thorax), intratubular (within the
tubules of an organ), intratumor (within a tumor), intratympanic (within the auras
media), intravascular (within a vessel or vessels), intraventricular (within a ventricle),
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), laryngeal
(directly upon the larynx), nasogastric (through the nose and into the stomach), occlusive
dressing technique (topical route administration which is then covered by a dressing
which occludes the area), ophthalmic (to the external eye), oropharyngeal (directly to the
mouth and pharynx), parenteral, percutaneous, periarticular, peridural, perineural,
periodontal, rectal, respiratory (within the respiratory tract by inhaling orally or nasally
for local or systemic effect), retrobulbar (behind the pons or behind the eyeball),
intramyocardial (entering the myocardium), soft tissue, subarachnoid, subconjunctival,
submucosal, topical, transplacental (through or across the placenta), transtracheal
(through the wall of the trachea), transtympanic (across or through the tympanic cavity),
ureteral (to the ureter), urethral (to the urethra), vaginal, caudal block, diagnostic, nerve
block, biliary perfusion, cardiac perfusion, photopheresis or spinal. In specific
embodiments, compositions may be administered in a way which allows them cross the
blood-brain barrier, vascular barrier, or other epithelial barrier. In one embodiment, a
formulaulation for a route of administration may include at least one inactive ingredient.

[000700] In one embodiment, the compositions are administered by intrasynovial
infusions.

[000701] 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 10 of copending application US 61/912,635 filed December 6, 2013 (Attorney Docket Number M073.60), the contents of which are incorporated herein by reference.

Non-limiting routes of administration for the compositions of the present invention are described below.

**Parenteral and Injectable Administration**

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-butylen 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.

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.

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.

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.

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).

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.

Rectal and Vaginal Administration

Rectal and vaginal administration and corresponding dosage forms 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 [000856] - [000859].

**Oral Administration**

[000710] Oral administration and corresponding dosage forms (e.g., liquid dosage forms) 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 [000860] - [000869].

**Topical or Transdermal Administration**

[000711] As described herein, compositions containing the compositions of the invention may be formulated for administration topically and/or transdermally. Topical, transdermal and transcutaneous administration and corresponding dosage forms 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 [000870] - [000888]

**Depot Administration**

[000712] As described herein, in some embodiments, the composition is formulated in depots for extended release. Generally, a specific organ or tissue (a "target tissue") is targeted for administration.

[000713] In some aspects of the invention, the compositions 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.

[000714] 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" composition (such as nucleic acids without a cell penetration agent or other agent) is also contemplated, and a pharmaceutically acceptable carrier.

[000715] 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.

[000716] 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.

[000717] In one embodiment, the invention provides for the compositions to be delivered in more than one injection or by split dose injections.

[000718] 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®).

Pulmonary Administration

[000719] A pharmaceutical composition may be prepared, packaged, and/or sold in a
formulation suitable for pulmonary administration via the buccal cavity. Pulmonary
administration and corresponding dosage forms 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 [000896] - [000901].

Intranasal, nasal and buccal Administration

[000720] Formulations described herein as being useful for pulmonary delivery are
useful for intranasal delivery of a pharmaceutical composition. Another formulation
suitable for intranasal administration is a coarse powder comprising the active ingredient
and having an average particle from about 0.2 µη to 500 µη. Such a formulation is
administered in the manner in which snuff is taken, i.e. by rapid inhalation through the
nasal passage from a container of the powder held close to the nose. Intranasal, nasal and
buccal administration and corresponding dosage forms 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
[000902] - [000905]

[000721]

Ophthalmic and Auricular (Otic) Administration

[000722] 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, conjunctival, intracorneal,
intraocular, intravitreal, ophthalmic and subconjunctiva. Ophthalmic and auricular
administration and corresponding dosage forms are described in co-pending International
Payload Administration: Detectable Agents and Therapeutic Agents

[000723] The compositions 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.

[000724] The compositions described herein can be used in intracellular targeting of a payload, e.g., detectable or therapeutic agent, to specific organelle. Exemplary intracellular targets can include, but are not limited to, the nuclear localization for advanced mRNA processing, or a nuclear localization sequence (NLS) linked to the mRNA containing an inhibitor.

[000725] In addition, the compositions described herein can be used to deliver therapeutic agents to cells or tissues, e.g., in living animals. For example, the compositions described herein can be used to deliver highly polar chemotherapeutics agents to kill cancer cells. The compositions attached to the therapeutic agent through a linker can facilitate member permeation allowing the therapeutic agent to travel into a cell to reach an intracellular target.

[000726] In some embodiments, the payload may be a therapeutic agent such as a cytotoxin, radioactive ion, chemotherapeutic, or other therapeutic agent. A cytotoxin or cytotoxic agent includes any agent that may be detrimental to cells. Examples include, but are not limited to, taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, teniposide, vincristine, vinblastine, colchicine, doxorubicin,
daunorubicin, dihydroxyanthracinedione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin, maytansinoids, *e.g.*, maytansinol (see U.S. Pat. No. 5,208,020 incorporated herein in its entirety), rachelmycin (CC-1065, see U.S. Pat. Nos. 5,475,092, 5,585,499, and 5,846,545, all of which are incorporated herein by reference), and analogs or homologs thereof. Radioactive ions include, but are not limited to iodine (*e.g.*, iodine 125 or iodine 131), strontium 89, phosphorous, palladium, cesium, iridium, phosphate, cobalt, yttrium 90, samarium 153, and praseodymium. Other therapeutic agents include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thiotepa chlorambucil, rachelmycin (CC-1065), melphalan, carmustine (BSNU), lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine, vinblastine, taxol and maytansinoids).

[000727] 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'-isothiocyanato stilbene-2,2'disulfonic acid; acid; acidine and derivatives (*e.g.*, acidine and acidine isothiocyanate); 5-(2'-aminoethyl)aminonaphthalene-l-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, dansyl chloride); 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-l,l-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-sulfopropyl)-IH-benz[e]indolium
hydroxide, inner salt, compound with n,n-diethylethanamine(l:l) (IR144); 5-chloro-2-[2-
[3-[(5-chloro-3-ethyl-2(3H)-benzoazolylidine)ethylidene]-2-(diphenylamino)-1-
cyclopenten-1-yl]ethenyl]-3-ethyl benzoazolium perchlorate (IR140); Malachite Green
isothiocyanate; 4-methylumbelliferone orthocresolphthalein; nitrotyrosine;
pararosaniline; Phenol Red; B-phycoerythrin; o-phthalaldialdehyde; 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 sulfonyle chloride rhodamine (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 naphthal cyanine.
In some embodiments, the detectable agent may be a non-detectable precursor 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

The polynucleotides or compositions 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.

Such combinations may include any of the agents identified in International Application WO2015/085318, the contents of which are incorporated herein by reference in their entirety.

The combinations referred to above can conveniently be presented for use in the form of a pharmaceutical formulation and thus pharmaceutical compositions comprising a combination as defined above together with a pharmaceutically acceptable diluent or carrier represent a further aspect of the invention.

The individual compounds of such combinations can be administered either sequentially or simultaneously in separate or combined pharmaceutical formulations. In one embodiment, the individual compounds will be administered simultaneously in a combined pharmaceutical formulation.
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

The present invention provides methods comprising administering compositions and in accordance with the invention to a subject in need thereof. 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.

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 0.005 mg/kg to about 0.05 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.

[000736] According to the present invention, the compositions may be administered in split-dose regimens. 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 administered 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 compositions of the present invention are administered to a subject in split doses. The compositions may be formulated in buffer only or in a formulation described herein.

Dosage Forms

[000737] 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

[000738] Liquid dosage forms for parenteral administration 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 paragraph [0001037].
Injectable

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-butanol. 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 monoo- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables.

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.

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 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 composition may be accomplished by dissolving or suspending the polynucleotide or composition in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the composition in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of polynucleotide 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.
Pulmonary

[000742] Pulmonary and intranasal formulations for delivery and administration are described in co-pending International Patent Publication No. WO2013151666, the contents of which is incorporated by reference in its entirety, such as, but not limited to, in paragraphs [000766] - [000781]

Coatings or Shells

[000743] 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

[000744] 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.

[000745] 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 a polynucleotide. 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

[000746] The pharmaceutical compositions described herein can be characterized by one or more of the following properties:

Bioavailability

[000747] The polynucleotides or compositions 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 or compositions administered to a mammal. Bioavailability can be assessed by measuring the area under the curve (AUC) or the maximum serum or plasma concentration \( C_{\text{max}} \) 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.

[000748] The \( C_{\text{max}} \) 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 \( C_{\text{max}} \) 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 polynucleotide or composition, 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 polynucleotide or composition 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%.

[000749] In some embodiments, liquid formulations of the polynucleotide or composition 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, the polynucleotide or composition formulations may be designed to improve bioavailability and/or therapeutic effect during repeat administrations. Such formulations may enable sustained release or degradation rates by nucleases.

Pharmacokinetics of intrabody compositions, including single domain antibodies and scaffold polypeptides, are described in further detail in Examples 19-21. The results demonstrate that transfection of the polynucleotide constructs into cells leads to expression of the encoded protein (e.g., single domain antibody or scaffold) very rapidly (e.g., within about 1 hour) such that maximum intracellular concentration for the protein can be reached, for example, within about 2-8 hours, or about 4-6 hours or about 6 hours. The half life of the encoded protein (e.g., single domain antibody or scaffold) can be, for example, about 4-6 hours, about 4-8 hours or about 4-10 hours. In another embodiment, the half life of the encoded protein (e.g., single domain antibody, such as a VHH antibody) is about 4 to about 5 hours. In another embodiment, the half life of the encoded protein (e.g., scaffold protein, such as an FN3 scaffold) is about 9 to about 10 hours. The protein (e.g., single domain antibody or scaffold) can be rapidly cleared as well, for example being no longer detectable about 24 hours after the polynucleotide construct is introduced into the cells. Alternatively, the minimum intracellular
concentration of the encoded protein (e.g., single domain antibody or scaffold) can be
reached within 24 hours after the polynucleotide construct is introduced into the cells.

Accordingly, in one aspect, the invention pertains to a polynucleotide
comprising
(a) a first region of linked nucleosides, the first region encoding a
polypeptide of interest;
(b) a first flanking region located 5' relative to the first region comprising
at least one 5' terminal cap;
(c) a second flanking region located 3' relative to the first region
comprising a 3' tailing sequence of linked nucleosides,

wherein the polynucleotide comprises at least one chemically modified
nucleoside,

wherein the polypeptide of interest comprises a polypeptide which is
expressed intracellularly and comprises a domain which binds to an intracellular target, and

wherein the polypeptide reaches a maximum intracellular concentration
within 2 to 8 hours after the polynucleotide is introduced into a mammalian cell.

In another embodiment, the polypeptide reaches a maximum intracellular
concentration within 4 to 6 hours after the polynucleotide is introduced into a mammalian
cell. In one embodiment, the polypeptide is an antibody or antigen-binding fragment
thereof. In one embodiment, the polypeptide is a single domain antibody. In one
embodiment, the polypeptide comprises a non-antibody scaffold protein which binds to
an intracellular target.

In another aspect, the invention pertains to a polynucleotide comprising
(a) a first region of linked nucleosides, the first region encoding a single
domain antibody;
(b) a first flanking region located 5' relative to the first region comprising
at least one 5' terminal cap;
(c) a second flanking region located 3' relative to the first region
comprising a 3' tailing sequence of linked nucleosides,
wherein the polynucleotide comprises at least one chemically modified nucleoside, wherein the single domain antibody is expressed intracellularly and binds to an intracellular target, and wherein the single domain antibody reaches a maximum intracellular concentration at about 6 hours after the polynucleotide is introduced into a mammalian cell.

In one embodiment, the single domain antibody is detectable within about 1 hour after the polynucleotide is introduced into a mammalian cell. In one embodiment, the single domain antibody is no longer detectable about 24 hours after the polynucleotide is introduced into a mammalian cell. In one embodiment, the minimum intracellular concentration of the single domain antibody is reached within 24 hours after the polynucleotide is introduced into the mammalian cell. In one embodiment, the single domain antibody has a half-life of about 4-6 hours.

In another aspect, the invention pertains to a method of reaching a maximum intracellular concentration of a polypeptide of interest in a minimum time following administration of a polynucleotide encoding the polypeptide of interest to a subject comprising administering to the subject a polynucleotide comprising

(a) a first region of linked nucleosides, the first region encoding a polypeptide of interest;

(b) a first flanking region located 5' relative to the first region comprising at least one 5' terminal cap;

(c) a second flanking region located 3' relative to the first region comprising a 3' tailing sequence of linked nucleosides,

wherein the polynucleotide comprises at least one chemically modified nucleoside,

wherein the polypeptide of interest comprises a polypeptide which is expressed intracellularly and comprises a domain which binds to an intracellular target, and

wherein the polypeptide reaches a maximum intracellular concentration within 2 to 8 hours after the polynucleotide is administered to the subject.
In one embodiment, the polypeptide reaches a maximum intracellular concentration within 4 to 6 hours after the polynucleotide is introduced into the subject. In one embodiment, the polypeptide is an antibody or antigen-binding fragment thereof. In one embodiment, the polypeptide is a single domain antibody. In one embodiment, the polypeptide comprises a non-antibody scaffold protein which binds to an intracellular target.

In another aspect, the invention provides a method of reaching a maximum intracellular concentration of a single domain antibody in a minimum time following administration of a polynucleotide encoding the single domain antibody to a subject comprising administering to the subject a polynucleotide comprising

(a) a first region of linked nucleosides, the first region encoding a single domain antibody;

(b) a first flanking region located 5' relative to the first region comprising at least one 5' terminal cap;

(c) a second flanking region located 3' relative to the first region comprising a 3' tailing sequence of linked nucleosides,

wherein the polynucleotide is administered intravenously encapsulated in an LNP and comprises at least one chemically modified nucleoside,

wherein the single domain antibody is expressed intracellularly and binds to an intracellular target, and

wherein the single domain polypeptide reaches a maximum liver accumulation at about 6 hours after the polynucleotide is administered to the subject.

In one embodiment, the single domain antibody is detectable within about 1 hour after the polynucleotide is administered to the subject. In one embodiment, the single domain antibody is no longer detectable about 24 hours after the polynucleotide is administered to the subject. In one embodiment, the minimum intracellular concentration of the single domain antibody is reached within 24 hours after the polynucleotide is introduced into the mammalian cell. In one embodiment, the single domain antibody has a half-life of about 4-6 hours.
In another aspect, the invention pertains to a method for transiently expressing an intracellular polypeptide of interest comprising administering to the subject a polynucleotide comprising

(a) a first region of linked nucleosides, the first region encoding a polypeptide of interest;

(b) a first flanking region located 5’ relative to the first region comprising at least one 5’ terminal cap;

(c) a second flanking region located 3’ relative to the first region comprising a 3’ tailing sequence of linked nucleosides,

wherein the polynucleotide comprises at least one chemically modified nucleoside,

wherein the polypeptide of interest comprises a polypeptide which is expressed intracellularly and comprises a domain which binds to an intracellular target, and

wherein the polypeptide of interest is detectable 1-3 hours after the polynucleotide is administered to the subject and is no longer detectable 24-48 hours after the polynucleotide is administered to the subject.

In one embodiment, the polypeptide of interest has a half-life of about 4-6 hours.

In another aspect, the invention pertains to a method for providing a single domain antibody which is expressed intracellularly to a subject, comprising

administering to the subject intravenously a first dose of a polynucleotide encapsulated in an LNP,

wherein the polynucleotide comprises

(a) a first region of linked nucleosides, the first region encoding a single domain antibody;

(b) a first flanking region located 5’ relative to the first region comprising at least one 5’ terminal cap;

(c) a second flanking region located 3’ relative to the first region comprising a 3’ tailing sequence of linked nucleosides,

wherein the polynucleotide comprises at least one chemically modified nucleoside, and

wherein the polynucleotide comprises at least one chemically modified nucleoside, and
wherein the single domain antibody is expressed intracellularly and binds to an intracellular target; and
administering to the subject intravenously a second dose of the polynucleotide encapsulated in an LNP at 12-15 hours following administration of the first dose.

In one embodiment, the single domain antibody has a half-life of about 4-6 hours.

**[000750]** In some embodiments, cationic nanoparticles comprising combinations of divalent and monovalent cations may be formulated with the polynucleotide or composition. 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 the polynucleotide or composition in cationic nanoparticles or in one or more depot comprising cationic nanoparticles may improve bioavailability by acting as a long-acting depot and/or reducing the rate of degradation by nucleases.

**Therapeutic Window**

**[000751]** The polynucleotide or composition, when formulated into a composition with a delivery agent as described herein, can exhibit an increase in the therapeutic window of the administered polynucleotide or composition composition as compared to the therapeutic window of the administered polynucleotide or composition 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 polynucleotide or composition 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**

202
[000752] The polynucleotide or composition, when formulated into a composition with a delivery agent as described herein, can exhibit an improved volume of distribution (V₁₀₀), 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 polynucleotide or composition 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

[000753] In one embodiment, the biological effect of the polynucleotide or composition 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 polynucleotide or composition of the present invention.

Detection of Polynucleotides by Mass Spectrometry

[000754] 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.
Mass spectrometry is performed using a mass spectrometer which includes an ion source for ionizing the fractionated sample and creating charged molecules for further analysis. For example ionization of the sample may be performed by electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), photoionization, electron ionization, fast atom bombardment (FAB)/liquid secondary ionization (LSIMS), matrix assisted laser desorption/ionization (MALDI), field ionization, field desorption, thermospray/plasmaspray ionization, and particle beam ionization. The skilled artisan will understand that the choice of ionization method can be determined based on the analyte to be measured, type of sample, the type of detector, the choice of positive versus negative mode, etc.

After the sample has been ionized, the positively charged or negatively charged ions thereby created may be analyzed to determine a mass-to-charge ratio (i.e., m/z). Suitable analyzers for determining mass-to-charge ratios include quadrupole analyzers, ion traps analyzers, and time-of-flight analyzers. The ions may be detected using several detection modes. For example, selected ions may be detected (i.e., using a selective ion monitoring mode (SIM)), or alternatively, ions may be detected using a scanning mode, e.g., multiple reaction monitoring (MRM) or selected reaction monitoring (SRM).

Liquid chromatography-multiple reaction monitoring (LC-MS/MRM) coupled with stable isotope labeled dilution of peptide standards has been shown to be an effective method for protein verification (e.g., Keshishian et al., Mol Cell Proteomics 2009 8:2339-2349; Kuhn et al., Clin Chem 2009 55:1108-1117; Lopez et al., Clin Chem 2010 56:281-290; each of which are herein incorporated by reference in its entirety).

In one embodiment, a biological sample which may contain protein encoded by the polynucleotides of the present invention may be analyzed for protein using electrospray ionization. Electrospray ionization (ESI) mass spectrometry (ESEVIS) uses electrical energy to aid in the transfer of ions from the solution to the gaseous phase before they are analyzed by mass spectrometry. Samples may be analyzed using methods known in the art (e.g., Ho et al., Clin Biochem Rev. 2003 24(1):3-12; herein incorporated by reference in its entirety).
In one embodiment, a biological sample which may contain protein encoded by the polynucleotides of the present invention may be analyzed for protein in a tandem ESI-MS system (e.g., MS/MS). As non-limiting examples, the droplets may be analyzed using a product scan (or daughter scan) a precursor scan (parent scan) a neutral loss or a multiple reaction monitoring.

In one embodiment, a biological sample which may contain protein encoded by the polynucleotides of the present invention may be analyzed using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MALDIMS). MALDI provides for the nondestructive vaporization and ionization of both large and small molecules, such as proteins.

In one embodiment, the analyte-matrix mixture may be formed using the dried-droplet method. A biologic sample is mixed with a matrix to create a saturated matrix solution where the matrix-to-sample ratio is approximately 5000:1. An aliquot (approximately 0.5-2.0 uL) of the saturated matrix solution is then allowed to dry to form the analyte-matrix mixture.

In one embodiment, the analyte-matrix mixture may be formed using the thin-layer method. A matrix homogeneous film is first formed and then the sample is then applied and may be absorbed by the matrix to form the analyte-matrix mixture.

In one embodiment, the analyte-matrix mixture may be formed using the thick-layer method. A matrix homogeneous film is formed with a nitro-cellulose matrix additive. Once the uniform nitro-cellulose matrix layer is obtained the sample is applied and absorbed into the matrix to form the analyte-matrix mixture.

In one embodiment, the analyte-matrix mixture may be formed using the sandwich method. A thin layer of matrix crystals is prepared as in the thin-layer method followed by the addition of droplets of aqueous trifluoroacetic acid, the sample and matrix. The sample is then absorbed into the matrix to form the analyte-matrix mixture.

V. Uses of Compositions of the Invention
Therapeutics

Production of Antibodies
In one embodiment of the invention, the polynucleotides of the compositions, particularly the immunomodulatory agents or moieties, encode antibodies and/or 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, IgE, IgD, 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.

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.

Antibodies used in 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.

**Therapeutic Agents**

The polynucleotides or compositions of the present invention can be used as therapeutic or prophylactic agents. They are provided for use in medicine. For example, a polynucleotide or composition 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 or
compositions, cells containing polynucleotides or compositions or polypeptides translated from the polynucleotides.

[000769] Provided herein are methods of inducing translation of a polypeptide (an antibody, variant or fragment thereof) in a cell, tissue or organism using the polynucleotides described herein. Such translation can be in vivo, ex vivo, in culture, or in vitro. The cell, tissue or organism is contacted with an effective amount of a composition containing a composition which contains a polynucleotide that has at least one a translatable region encoding the polypeptide of interest (antibody).

[000770] 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 polynucleotide (e.g., size, and extent of modified nucleosides) and other components of the polynucleotide, and other determinants.

[000771] Aspects of the invention are directed to methods of inducing in vivo translation of a polypeptide in a mammalian subject in need thereof. Therein, an effective amount of a composition containing a polynucleotide that has at least one structural or chemical modification and a translatable region encoding the polypeptide (antibody) is administered to the subject using the delivery methods described herein. The polynucleotide is provided in an amount and under other conditions such that the polynucleotide is localized into a cell of the subject and the polypeptide is translated in the cell from the polynucleotide. The cell in which the polynucleotide is localized, or the tissue in which the cell is present, may be targeted with one or more than one rounds of polynucleotide administration.

[000772] In certain embodiments, the administered compositions comprising polynucleotides directs production of one or more polypeptides that provide a functional immune system-related activity which is substantially absent in the cell, tissue or organism in which the 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 polypeptides that increases (e.g., synergistically) a functional activity related to the immune system which is present but substantially deficient in the cell in which the polypeptide is translated.
[000773] In other embodiments, the administered compositions comprising polynucleotides directs production of one or more polypeptides that replace an immune related polypeptide (or multiple polypeptides) that is substantially absent in the cell in which the polypeptide is translated. Such absence may be due to genetic mutation of the encoding gene or regulatory pathway thereof. In some embodiments, the polypeptide increases the level of an endogenous protein in the cell to a desirable level; such an increase may induce or boost an immune response by bringing the level of the endogenous protein from a subnormal level to a normal level or from a normal level to a super-normal level.

[000774] Alternatively, the 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 or the subject's immune system; for example, due to mutation of the endogenous protein resulting in altered activity or localization.

[000775] Additionally, the polypeptide antagonizes, directly or indirectly, the activity of a biological moiety present in, on the surface of, or secreted from the cell. Examples of antagonized biological moieties include lipids (e.g., cholesterol), a lipoprotein (e.g., low density lipoprotein), a nucleic acid, a carbohydrate, a protein toxin such as shiga and tetanus toxins, or a small molecule toxin such as botulinum, cholera, and diphtheria toxins. Additionally, the antagonized biological molecule may be an endogenous protein that exhibits an undesirable activity, such as a cytotoxic or cytostatic activity.

[000776] The 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.

[000777] In some embodiments, polynucleotides of the invention 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: autoimmune disorders (e.g., diabetes, lupus, multiple sclerosis, psoriasis, rheumatoid arthritis); inflammatory disorders (e.g., arthritis, pelvic inflammatory disease); infectious diseases (e.g., viral infections (e.g., HIV, HCV, RSV), bacterial infections, fungal infections, sepsis); neurological disorders (e.g., Alzheimer's...
disease, Huntington's disease; autism; Duchenne muscular dystrophy); cardiovascular disorders (e.g. atherosclerosis, hypercholesterolemia, thrombosis, clotting disorders, angiogenic disorders such as macular degeneration); proliferative disorders (e.g. cancer, benign neoplasms); respiratory disorders (e.g. chronic obstructive pulmonary disease); digestive disorders (e.g. inflammatory bowel disease, ulcers); musculoskeletal disorders (e.g. fibromyalgia, arthritis); endocrine, metabolic, and nutritional disorders (e.g. diabetes, osteoporosis); urological disorders (e.g. renal disease); psychological disorders (e.g. depression, schizophrenia); skin disorders (e.g. wounds, eczema); blood and lymphatic disorders (e.g. anemia, hemophilia); etc.

[000778] In another embodiment, the present invention provides a method for treating hematopoietic disorders, cardiovascular disease, oncology, diabetes, cystic fibrosis, neurological diseases, inborn errors of metabolism, skin and systemic disorders, and blindness. The identity of molecular targets to treat these specific diseases has been described (Templeton ed., Gene and Cell Therapy: Therapeutic Mechanisms and Strategies, 3rd Edition, Bota Raton, FL: CRC Press; herein incorporated by reference in its entirety).

[000779] In one embodiment, the present invention provides a method of treating cancer and proliferative diseases.

[000780] In certain embodiments, the administration may be local or systemic. In certain embodiments, the administration may be subcutaneous. In certain embodiments, the administration may be intravenous. In certain embodiments, the administration may be oral. In certain embodiments, the administration may be topical. In certain embodiments, the administration may be by inhalation. In certain embodiments, the administration may be rectal. In certain embodiments, the administration may be vaginal.

[000781] Other aspects of the present disclosure relate to transplantation of cells containing polynucleotides to a mammalian subject.

[000782] 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. Provided are methods of identifying, diagnosing, and classifying subjects on these bases, which may include clinical diagnosis, biomarker levels, genome-wide association studies (GWAS), and other methods known in the art.
VI. Kits and Devices

Kits

[000783] 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.

[000784] In one aspect, the present invention provides kits comprising the molecules (including any proteins or polynucleotides) of the invention. In one embodiment, the kit comprises one or more functional antibodies or function fragments thereof.

[000785] Said kits can be for protein production, comprising a first polynucleotides comprising a translatable region of an antibody. 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.

[000786] 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.

[000787] The components may also be varied in order to increase the stability of polynucleotides 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.

[000788] 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.

[000789] 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

[000790] The present invention provides for devices which may incorporate compositions comprising 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.

[000791] Devices for administration may be employed to deliver the compositions of the present invention according to single, multi- or split-dosing regimens taught herein. Such devices are taught in, for example, International Application WO2013151666, the contents of which are incorporated herein by reference in their entirety.

[000792] 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.

[000793] 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 WO2013151666, the contents of which are incorporated herein by reference in their entirety.

[000794] 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).

Methods and Devices utilizing catheters and/or lumens

[000795] Methods and devices using catheters and lumens may be employed to administer the compositions of the present invention on a single, multi- or split dosing schedule. Such methods and devices are described in International Application WO2013151666, the contents of which are incorporated herein by reference in their entirety.

Methods and Devices utilizing electrical current

[000796] Methods and devices utilizing electric current may be employed to deliver the compositions of the present invention according to the single, multi- or split dosing regimens taught herein. Such methods and devices are described in International Application WO2013151666, the contents of which are incorporated herein by reference in their entirety.

VII. Definitions

[000797] 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

[000798] About: As used herein, the term "about" means +/- 10% of the recited value.

[000799] 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.

[000800] Adjuvant: As used herein, the term "adjuvant" means a substance that enhances a subject's immune response to an antigen.

[000801] 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.

**[000802]** Antigen: As used herein, the term "antigen" refers to a substance or molecule that induces, elicits or triggers an immune response in a cell, tissue or organism. An antigen may originate either from the body, such as cancer antigen, or from the external environment, for instance, from infectious agents. Antigens may be, in whole or part, endogenous or exogenous peptides, proteins or polypeptides of interest or fragments thereof.

**[000803]** 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).

**[000804]** 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.

**[000805]** 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.

[000806] **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.

[000807] **Biodegradable:** As used herein, the term "biodegradable" means capable of being broken down into innocuous products by the action of living things.

[000808] **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.

[000809] **Chimera:** As used herein, "chimera" is an entity having two or more incongruous or heterogeneous parts or regions.

[000810] **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.

[000811] **Compound:** As used herein, the term "compound," is meant to include all stereoisomers, geometric isomers, tautomers, and isotopes of the structures depicted.

[000812] 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.

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.

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.

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.

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.

[000817] 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 a polynucleotide or polypeptide or may apply to a portion, region or feature thereof.

[000818] 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.

[000819] 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.

[000820] 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.
[000821] 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.

[000822] Delivery: As used herein, "delivery" refers to the act or manner of delivering a compound, substance, entity, moiety, cargo or payload.

[000823] 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.

[000824] 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.

[000825] 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.

[000826] 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.

[000827] 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.

[000828] 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.

[000829] Differentiate: As used herein, "differentiate" refers to the process where an uncommitted or less committed cell acquires the features of a committed cell.
Distal: As used herein, the term "distal" means situated away from the center or away from a point or region of interest.

Dosing regimen: As used herein, a "dosing regimen" is a schedule of administration or physician determined regimen of treatment, prophylaxis, or palliative care.

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.

Encapsulate: As used herein, the term "encapsulate" means to enclose, surround or encase.

Encoded protein cleavage signal: As used herein, "encoded protein cleavage signal" refers to the nucleotide sequence which encodes a protein cleavage signal.

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.

Effective Amount: As used herein, the term "effective amount" of an agent is 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.

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.

Feature: As used herein, a "feature" refers to a characteristic, a property, or a distinctive element.

Formulation: As used herein, a "formulation" includes at least a polynucleotide encoding the polypeptide of interest and a delivery agent.
[000840] **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.

[000841] **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.

[000842] **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.

[000843] **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)).
[000844] 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.

[000845] 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).

[000846] 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).

[000847] 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.
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.

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, alkylation, oxygen, sulfur, sulfoxide, sulfenyl, 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 polynucleotides) or polynucleotides conjugates, or to provide a cleavage site to separate two or more polypeptides after translation, 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.

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.
[000851] *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 polynucleotide 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.

[000852] *Mucus:* As used herein, "mucus" refers to the natural substance that is viscous and comprises mucin glycoproteins.

[000853] *Nanoparticle:* As used herein, "nanoparticle" refers to a particle having any one structural feature on a scale of less than about 1000 nm that exhibits novel properties as compared to a bulk sample of the same material. Routinely, nanoparticles have any one structural feature on a scale of less than about 500 nm, less than about 200 nm, or about 100 nm. Also routinely, nanoparticles have any one structural feature on a scale of from about 50 nm to about 500 nm, from about 50 nm to about 200 nm or from about 70 to about 120 nm. In exemplary embodiments, a nanoparticle is a particle having one or more dimensions of the order of about 1 - 1000 nm. In other exemplary embodiments, a nanoparticle is a particle having one or more dimensions of the order of about 10 - 500 nm. In other exemplary embodiments, a nanoparticle is a particle having one or more dimensions of the order of about 50 - 200 nm. A spherical nanoparticle would have a diameter, for example, of between about 50-100 or 70-120 nanometers. A nanoparticle most often behaves as a unit in terms of its transport and properties. It is noted that novel properties that differentiate nanoparticles from the corresponding bulk material typically develop at a size scale of under 1000 nm, or at a size of about 100 nm, but nanoparticles can be of a larger size, for example, for particles that are oblong, tubular, and the like. Although the size of most molecules would fit into the above outline, individual molecules are usually not referred to as nanoparticles.

[000854] *Naturally occurring:* As used herein, "naturally occurring" means existing in nature without artificial aid.
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.

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.

Off-target: As used herein, "off target" refers to any unintended effect on any one or more target, gene, or cellular transcript.

Open reading frame: As used herein, "open reading frame" or "ORE" refers to a sequence which does not contain a stop codon in a given reading frame.

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.

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 said alkyl is optionally substituted"). It is not intended to mean that the feature "X" (e.g. alkyl) per se is optional.

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.

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.

Paratope: As used herein, a "paratope" refers to the antigen-binding site of an antibody.

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.

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.

[000866] *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, suspending 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.

[000867] *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.

[000868] 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.

[000869] Physicochemical: As used herein, "physicochemical" means of or relating to a physical and/or chemical property.

[000870] 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.

[000871] 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.

[000872] 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

[000873] Proliferate: As used herein, the term "proliferate" means to grow, expand or increase or cause to grow, expand or increase rapidly. "Proliferative" means having the ability to proliferate. "Anti-proliferative" means having properties counter to or inapposite to proliferative properties.

[000874] Prophylactic: As used herein, "prophylactic" refers to a therapeutic or course of action used to prevent the spread of disease.

[000875] Prophylaxis: As used herein, a "prophylaxis" refers to a measure taken to maintain health and prevent the spread of disease. An "immune prophylaxis" refers to a measure to produce active or passive immunity to prevent the spread of disease.

[000876] 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.

[000877] Protein cleavage signal: As used herein "protein cleavage signal" refers to at least one amino acid that flags or marks a polypeptide for cleavage.

[000878] 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.

[000879] Proximal: As used herein, the term "proximal" means situated nearer to the center or to a point or region of interest.

[000880] 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 (m1Ψ), 1-methyl-4-thio-pseudouridine (m1s4Ψ), 4-thio-1 -methyl-
pseudouridine, 3-methyl-pseudouridine (m^3\psi), 2-thio-l-methyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-l-methyl-1-deaza-pseudouridine, dihydrospseudouridine, 2-thio-dihydrospseudouridine, 2-methoxyuridine, 2-methoxy-4-thio-uridine, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, Nl-methyl-pseudouridine, 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine (acp^3 \psi), and 2'-0-methyl-pseudouridine (\psi \eta).

**Purified:** As used herein, "purify," "purified," "purification" means to make substantially pure or clear from unwanted components, material defilement, admixture or imperfection.

**Repeated transfection or repeated dose:** As used herein, the term "repeated transfection" or "dose" 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.

**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.

**Signal Sequences:** As used herein, the phrase "signal sequences" refers to a sequence which can direct the transport or localization of a protein.

**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.
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.

Split dose: As used herein, a "split dose" is the division of single unit dose or total daily dose into two or more doses.

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.

Stabilized: As used herein, the term "stabilize", "stabilized," "stabilized region" means to make or become stable.

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.

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.

Substantially equal: As used herein as it relates to time differences between doses, the term means plus/minus 2%.

Substantially simultaneously: As used herein and as it relates to plurality of doses, the term means within 2 seconds.
[000894] **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.

[000895] **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.

[000896] **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.

[000897] **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.

[000898] **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.
[000899] **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.

[000900] **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.

[000901] **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.

[000902] **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.

[000903] **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.

[000904] **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.

[000905] **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**

[000906] 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.

[000907] 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.

[000908] 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.

[000909] 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.

[000910] 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.

[000911] 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.

[000912] Section and table headings are not intended to be limiting.
EXAMPLES

Example 1. Manufacture of Polynucleotides

[000913] According to the present invention, the manufacture of polynucleotides and or parts or regions thereof may be accomplished utilizing the methods taught in International Application WO2014/152027, the contents of which is incorporated herein by reference in its entirety.

[000914] Purification methods may include those taught in International Application WO2014/152030 and WO2014/152031, each of which is incorporated herein by reference in its entirety.

[000915] Detection and characterization methods of the polynucleotides may be performed as taught in WO2014/144039, which is incorporated herein by reference in its entirety.

[000916] Characterization of the 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, WO2014/144711 and WO2014/144767, the contents of each of which is incorporated herein by reference in its entirety.

Example 2. Synthesis of Polynucleotides

[000917] Enzymatic (IVT), solid-phase, liquid-phase, combined synthetic methods, small region synthesis, and ligation methods are taught in for example International Application WO2015/085308, the contents of which are incorporated herein by reference in their entirety, and may be utilized to manufacture the polynucleotides of the present invention.

Example 3. Enzymatic Capping

[000918] Capping of a polynucleotide is performed as follows where the mixture includes: IVT RNA 60 µg-180µg and dH₂O up to 72 µl. The mixture is incubated at 65°C for 5 minutes to denature RNA, and then is transferred immediately to ice.
The protocol then involves the mixing of 1Ox Capping Buffer (0.5 M Tris-HCl (pH 8.0), 60 mM KCl, 12.5 mM MgCl₂) (10.0 μl); 20 mM GTP (5.0 μl); 20 mM S-Adenosyl Methionine (2.5 μl); RNase Inhibitor (100 U); 2'-0-Methyltransferase (400U); Vaccinia capping enzyme (Guanylyl transferase) (40 U); dH₂O (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.

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 4. PolyA Tailing Reaction**

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); 1Ox Tailing Buffer (0.5 M Tris-HCl (pH 8.0), 2.5 M NaCl, 100 mM MgCl₂)(12.0 μl); 20 mM ATP (6.0 μl); Poly-A Polymerase (20 U); dH₂O 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.

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, 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 5. Natural 5’ Caps and 5’ Cap Analogues**

5’-capping of polynucleotides may be completed concomitantly during the in viYro-transcription reaction using the following chemical RNA cap analogs to generate the 5’-guanosine cap structure according to manufacturer protocols: 3’-0-Me-m7G(5’ppp(5’)) G [the ARCA cap];G(50ppp(5’))A; G(5’ppp(5’))G; m7G(5’ppp(50A;
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’-0 methyl-transferase to generate: m7G(50ppp(50G-2’-O-methyl. Cap 2 structure may be generated from the Cap 1 structure followed by the 2’-0-methylation of the 5’-antepenultimate nucleotide using a 2’-0 methyl-transferase. Cap 3 structure may be generated from the Cap 2 structure followed by the 2’-0-methylation of the 5’-preantepenultimate nucleotide using a 2’-0 methyl-transferase. Enzymes are preferably derived from a recombinant source.

[000924] 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 6. Capping Assays

A. Protein Expression Assay

[000925] 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

[000926] 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
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

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 7. Agarose Gel Electrophoresis of Modified RNA or RT PCR Products

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 8. Nanodrop Modified RNA Quantification and UV Spectral Data

Modified chimeric polynucleotides in TE buffer (1 µl) are used for Nanodrop UV absorbance readings to quantitate the yield of each chimeric polynucleotide from a chemical synthesis or in vitro transcription reaction.

Example 9. Formulation of Modified mRNA Using Lipidoids

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 10. Method of Screening for Protein Expression**

A. *Electrospray Ionization*

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.

B. *Matrix-Assisted Laser Desorption/Ionization*

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).

C. *Liquid Chromatography-Mass spectrometry-Mass spectrometry*

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.

**Example 11. Cyclization and/or concatemerization**
According to the present invention, a chimeric polynucleotide may be cyclized, or concatemerized, to generate a translation competent molecule to assist interactions between poly-A binding proteins and 5'-end binding proteins. The mechanism of cyclization or concatemerization may occur through at least 3 different routes: 1) chemical, 2) enzymatic, and 3) ribozyme catalyzed. The newly formed 5'73'-linkage may be intramolecular or intermolecular.

In the first route, the 5'-end and the 3'-end of the nucleic acid contain chemically reactive groups that, when close together, form a new covalent linkage between the 5'-end and the 3'-end of the molecule. The 5'-end may contain an NHS-ester reactive group and the 3'-end may contain a 3'-amino-terminated nucleotide such that in an organic solvent the 3'-amino-terminated nucleotide on the 3'-end of a synthetic mRNA molecule will undergo a nucleophilic attack on the 5'-NHS-ester moiety forming a new 5'73'-amide bond.

In the second route, T4 RNA ligase may be used to enzymatically link a 5'-phosphorylated nucleic acid molecule to the 3'-hydroxyl group of a nucleic acid forming a new phosphodiester linkage. In an example reaction, 1µg of a nucleic acid molecule is incubated at 37°C for 1 hour with 1-10 units of T4 RNA ligase (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. The ligation reaction may occur in the presence of a split polynucleotide capable of base-pairing with both the 5'- and 3'-region in juxtaposition to assist the enzymatic ligation reaction.

In the third route, either the 5'- or 3'-end of the cDNA template encodes a ligase ribozyme sequence such that during in vitro transcription, the resultant nucleic acid molecule can contain an active ribozyme sequence capable of ligating the 5'-end of a nucleic acid molecule to the 3'-end of a nucleic acid molecule. The ligase ribozyme may be derived from the Group I Intron, Group I Intron, Hepatitis Delta Virus, Hairpin ribozyme or may be selected by SELEX (systematic evolution of ligands by exponential enrichment). The ribozyme ligase reaction may take 1 to 24 hours at temperatures between 0 and 37°C.

**Example 12. Chimeric Synthesis of RNA**

**A. Capping**
The RNA (220 µL) eluted from the IVT step is denatured by heating to 65 °C for 15 minutes followed by cooling on ice for at least 2 min. The capping reaction is performed in 300 µL with the denatured RNA (220 µL), GTP (1 mM), SAM (0.5 mM), RNase Inhibitor (1 U/µL), IX Capping buffer, and Vaccinia capping complex (0.4 U/µL) (NEB). These reactions are incubated at 37 °C for 2 hr on the thermomixer. The reactions are purified using MEGAclear spin columns (Ambion) and eluted in 250 µL water. The eluted mRNA was analyzed by CE (Agilent 2100 Bioanalyzer) and quantified by UV absorbance.

B. Incorporation of 3''-azido-2''3''-dideoxyadenosine-5''-triphosphate (3''-azido-ddATP)

3''-azido-ddATP is incorporated into the 3'-end of tailless RNA using yeast poly(A) polymerase as depicted in the following scheme (Scheme 1), which illustrates the general synthesis of 3''-azido RNA by incorporation of 3''-azido ddATP onto the 3'-end of RNA using yeast poly(A) polymerase.

![Scheme 1](image)

In 100 µL reactions, RNA transcript (0.2 µM), 3''-azido-ddATP (500 µM), murine RNase inhibitor (NEB) (1 U/µL), 1x reaction buffer (20 mM Tris-HCl, pH 7.0, 0.6 mM MnCl₂, 20 µM EDTA, 0.2 mM DTT, 100 µg/mL acetylated BSA, 10% glycerol), and yeast poly(A) polymerase (2400 U, Affymetrix) are incubated at 37°C for 1 hr, followed by ethanol precipitation. The RNA is dissolved in 100 µL DEPC-treated H₂O and further purified by gel filtration using an illustra NICK column or illustra MicroSpin G-25 column (GE Healthcare).

The RNA is concentrated, if necessary, by ultrafiltration using an Amicon Ultra-0.5 centrifugal device (100K NMWL), quantified by UV absorbance, and analyzed by capillary electrophoresis (CE) (Agilent 2100 Bioanalyzer). The RNA obtained at this
point is a mixture of unmodified and 3'-azido RNA which cannot be distinguished by CE, and this mixture is used without further purification in subsequent reactions.

C. Synthesis of 5′-bicyclo[6.1.0]nonyne (BCN) poly(A) tails 1-6

[000946] 5′-bicyclo[6.1.0]nonyne (BCN) poly(A) tails 1-6 are synthesized for generating RNA-poly(A) tail conjugates using strain-promoted azide-alkyne cycloaddition (SPAAC) chemistry. The following scheme (Scheme 2) shows the structures of 5′-BCN poly(A) tails 1-6. Tails 1-3 are stabilized on the 3′ end with two 2′-OMe A’s and an inverted T. Tails 4-6 contain only A.

![Diagram of 5′-BCN poly(A) tails 1-6](image-url)
While tails 1 and 4 could be synthesized directly by solid phase phosphoramidite oligomerization technology, tails 2, 3, 5, and 6 are first synthesized as the 5'-amino derivatives (tails 2a, 3a, 5a, and 6a) which are then coupled to the reactive BCN group via NHS chemistry. The following scheme (Scheme 3) illustrates the synthesis of tails 2, 3, 5, and 6 by coupling the corresponding 5'-amino oligoribonucleotides to the BCN N-hydroxysuccinimide ester I.

[000948] Tails 1, 2a, 3a, 4, 5a, and 6a are assembled on an Expedite 8909 DNA/RNA synthesizer (Perseptive) employing solid phase phosphoramidite oligomerization technology. Syntheses are initiated on a solid support made of controlled pore glass (CPG, 1000 Å) with either immobilized 3'-0-dimethoxytrityl-thymidine at a loading of 31 μmol/g (obtained from Prime Synthesis, Aston, PA, USA) generating a 3'-3'-linkage at the 3'-end or immobilized 5'-0-dimethoxytrityl-adenosine loaded at 32 μmol/g (Chemgenes, Wilmington, MA; USA).

[000949] In order to introduce an amino-linker at the 5'-end either a trifluoracetyl (TFA)-protected aminohexyl phosphoramidite (SAFC Proligo, Hamburg, Germany) or the corresponding propyl derivative from Glen Research (Sterling, Virginia, USA) is employed. All amidites are dissolved in anhydrous acetonitrile (100 mM) and molecular sieves (3A) are added. 5-Ethyl thiotetrazole (ETT, 500 mM in acetonitrile) is used as activator solution. Coupling times are 5 minutes for the nucleoside phosphoramidites and
12 minutes for the linker amidites. Ancillary reagents for RNA synthesis are purchased from SAFC Proligo (Hamburg, Germany). After finalization of the solid phase synthesis, the dried solid support is transferred to a 15 mL polypropylene tube and the RNA is cleaved from the solid support and deprotected by methods known in the field (Wincott F., et al, Nucleic Acid Res., 1995, 23, 2677-84).

[000950] Crude oligomers are purified by RP HPLC using an XBridge C18 19x 50 mm column (Waters, Eschborn, Germany) on an AKTA Explorer system (GE Healthcare, Freiburg, Germany). Buffer A was 100 mM triethylammonium acetate (Biosolve, Valkenswaard, The Netherlands) and buffer B contained 95% acetonitrile in buffer A. A flow rate of 15 mL/min is employed. UV traces at 260 and 280 were recorded. A gradient of 5% B to 45% B within 57 column volumes was employed. Appropriate fractions are pooled and precipitated with 3M NaOAc, pH=5.2 and 70% ethanol. The pellet is isolated by centrifugation, dissolved in water and the concentration of the solution is determined by absorbance measurement at 260 nm in a UV photometer (Eppendorf, Germany).

[000951] For the coupling step to produce tails 2, 3, 5, and 6 by NHS chemistry as depicted in Scheme 3 above, the respective amine-modified oligoribonucleotide is dissolved in 100 mM sodium borate/KCl buffer (pH 8.5) to yield a concentration of 500 µM. Click-easy® BCN N-hydroxysuccinimide ester I (5 mg, Berry & Associates, Inc., Dexter; MI, USA) is dissolved in 50 µL DMSO. The reaction is initiated by addition of about 3 equivalents BCN derivative to the RNA solution. The progress of the reaction is monitored by the change of retention time on an anion exchange HPLC column (Dionex DNA Pac PA200, 4x250 mm, Dionex, Idstein, Germany). After completion of the reaction the oligoribonucleotide conjugate is precipitated using 3 M NaOAc (pH 5.2)/EtOH and purified on a C18 XBridge reversed phase HPLC column (Waters, Eschborn, Germany).

D. Poly(A) tail Conjugation Using Strain-promoted Azide-alkyne Cycloaddition (SPAAC)

[000952] RNA transcripts modified on the 3'-end with 3'-azido-ddATP are ligated to 80 nt 5'-BCN poly(A) tails using strain-promoted azide-alkyne cycloaddition (SPAAC) to give RNA-poly(A) tail conjugates of the general form shown in the following scheme.
The scheme shows the general synthesis of RNA-poly(A) tail conjugates by SPAAC with 3'-azido RNA and 5'-BCN poly(A) tail.

(Scheme 4).

[000953] 3'-azido RNA and tail 1 are mixed in at least a 1:50 molar ratio, respectively, in water and diluted with ethanol to a final concentration of 70% ethanol. Generally, the concentration of 3'-azido RNA is between 150 - 400 nM in the reaction mixture. The reactions are shaken at room temperature for 1 hr, diluted with water to 200 µl, if necessary, ethanol precipitated, and dissolved in DEPC-treated water.

[000954] Alternatively, the reactions are purified by MEGAclear kit (Ambion) and eluted in water. The RNA reaction mixture is analyzed by CE (Agilent 2100 Bioanalyzer).

[000955] Conjugates may also be made in this manner with RNA which already contain a poly(A) tail through transcription by T7 RNA polymerase, and tails 1 and 4.

[000956] In order to remove excess unreacted 5'-BCN tail, the reaction mixture is reacted with biotin azide (500 µM) in 10% DMSO by shaking for 1 hr at room temperature, followed by a MEGAclear purification.

[000957] The reaction mixture is then subjected to streptavidin capture with M-280 Streptavidin Dynabeads (Life Technologies). The beads (200 µL, 2 mg) are washed with a high salt buffer (10 mM Tris-HCl, pH 7.4, 0.5 M NaCl, and 1 mM EDTA) three times and resuspended in 200 µl high salt buffer. The reaction mixture, which contains

245
approximately 1.3 nmol 5'-BCN tail, is diluted to 200 µL for a final concentration of IX high salt buffer, and added to the beads. The sample and beads are mixed at room temperature for 15 min. This supernatant is saved and ethanol precipitated.

[000958] CE was used to confirm that the 5'-biotin-tails were removed from the reaction mixtures. The purity of the clicked constructs after this procedure are greater than 80%.

E. DNA Splint-templated poly(A) tail Conjugation Using SPAAC

[000959] A DNA splint complementary to the 3'-end of the RNA and to the poly(A) tail is used to template the SPAAC reaction. RNA-poly(A) tail conjugates are synthesized by mixing 3'-azido RNA, 5'-BCN poly(A) tail, and splint in a molar ratio of 1: 3: 3 with final concentrations of 100 nM: 300 nM: 300 nM, respectively, in a 100 µL reaction containing 1 M NaCl. The RNA and DNA splint mixture is heated to 70 °C for 5 min, cooled at 1 °C/min until reaching 25 °C, and maintained at 25 °C overnight. Salts are removed by ultrafiltration (Amicon Ultra-0.5 centrifugal device 100K NMWL).

[000960] The DNA splint is removed by digestion with TURBO DNase (Ambion) in 50 µL reactions containing no more than 200 ng/µL of the reaction mixture, 1x reaction buffer, and TURBO DNase (2 U). These reactions are incubated for 30 min at 37°C and terminated by the addition of 2 µL of 0.5 M EDTA. The buffer components are again removed by ultrafiltration. The RNA-poly(A) tail conjugates are purified from unmodified and unreacted 3'-azido RNA using oligo(T) Dynabeads (Ambion). The oligo(T) purification is performed as directed by the manufacturer's protocol, except the beads were washed and the RNA sample prepared in a high salt buffer containing 10 mM Tris-HCl, pH 7.4, 0.5 M NaCl, and 1 mM EDTA, the beads are washed after binding with a low salt buffer containing 10 mM Tris-HCl, pH 7.4, 0.1 M NaCl, and 1 mM EDTA, and the RNA-poly(A) tail conjugates are eluted in 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA.

[000961] All steps in the click reaction and purification are analyzed by CE (Agilent 2100 Bioanalyzer).

F. Analysis of 3''-azido-ddATP Incorporation

[000962] Since 3'-azido RNA and RNA - tail conjugates are blocked on the 3'-end for poly(A) extension by poly(A) polymerase, only the unmodified RNA is a substrate for
enzymatic tailing. The percentage of unmodified RNA, and therefore 3'-azido RNA, can be determined by calculating the % difference in the area of the peak corresponding to the unmodified RNA and 3'-azido RNA mixture after removal of the unmodified RNA and normalization to the area of the RNA - tail 1 conjugate peak.

[000963] In many cases, the click reaction goes to completion under the conditions described, allowing for a determination of azide incorporation simply by determining the % yield of the RNA - tail conjugate.

[000964] In 10 µL, the RNA mixture after the SPAAC reaction in 70% ethanol is treated with E. coli poly(A) polymerase (NEB) (5 U) in a reaction containing the RNA reaction mixture (300 - 400 ng/mL), ATP (1 mM), and 1x reaction buffer (50 mM Tris-HCl, pH 7.9, 250 mM NaCl, 10 mM MgCl₂). Reactions containing no enzyme are also used for comparative controls. Controls where unmodified RNA is mixed with tail and treated with poly(A) polymerase may also be performed to ensure that all unmodified RNA would become tailed. Salts are removed from the reactions by ultrafiltration, and the reactions are analyzed by CE.

[000965] In the control reactions, all unmodified RNA is lengthened by treatment with PAP. In all these cases, after the SPAAC reaction and treatment with PAP, no RNA is left in the peak representing the putative mixture of unmodified RNA and 3'-azido RNA, indicating the click reactions went to completion and azide incorporation could be determined from % yield of the RNA-tail conjugate.

G. Total Area under the Curve of mCherry Fluorescence

[000966] The mRNA (50 ng) generated are transfected using LIPOFECTAMINE2000TM into HeLa cells. The cells are placed in the Incucyte kinetic imaging system (Essen Bioscience) where mCherry fluorescence was measured every 2hrs for 142hrs. Each transfection is performed in triplicate. The total area under the curve is integrated using GraphPad Prism.

H. Activity in HeLa Cells

[000967] The mRNA (25 ng) generated are transfected in triplicate using LIPOFECTAMINE2000TM into HeLa cells. After incubation overnight, the cells are lysed in GLO lysis buffer (Promega). NanoGlo substrate is added and luminescent signal is quantified using Synergy MicroPlate Reader (BioTek).
1. Expression in HeLa cells

[000968] The mRNA (250 ng) generated are transfected in triplicate using LISPOFECTAMINE2000TM into HeLa cells. After incubation overnight, the supernatant is collected and used to measure the levels of protein (R&D Systems).

J. IFNP Levels in Supernatant of BJ Fibroblasts Transfected with mRNA

[000969] The mRNA generated (500 ng) is transfected in triplicate using LISPOFECTAMINE2000TM into BJ fibroblasts. After incubation for 48hrs, the supernatant is collected and used to measure the levels of human Interferon-β (R&D Systems).

Example 13. Inhibition of MYC-mediated Transactivation

[000970] MYC is a transcription factor known to be dysregulated in a majority of human cancers, and plays a role in many cellular processes involved in carcinogenesis, such as proliferation, anabolic cell growth and metabolism. MYC affects the transcriptional regulation of a variety of target genes through the dimerization with other partner proteins, such as MAX, and MYC-containing complexes then associate with DNA in at the regulatory regions of target genes to regulate gene expression. In order to activate transcription, MYC recruits other transcriptional factors through its transactivation domain.

A. PPT inhibition of MYC dimerization

[000971] Polypeptides that bind c-MYC and inhibit MYC dimerization with other proteins are screened and selected using methods known in the art. mRNA encoding the polypeptides with favorable screening results for binding c-MYC and/or inhibiting MYC dimerization are designed and synthesized. The mRNA comprises at least one chemical modification and may be formulated in any of the formulation described herein.

1) Screen and select anti-MYC intracellular binders that inhibit MYC dimerization with other proteins.

[000972] Selected mRNAs encoding the MYC dimerization inhibitor or a control mRNA are transfected into exponentially growing MYC expressing cancer cell lines together with a reporter construct controlled by an upstream promoter containing MYC regulatory elements. As another control, a mock transfection is performed in parallel. At a set number of days post-transfection, cells are harvested, lysed, and reporter activity is
measured. A reduction in measured reporter gene activity as compared to the samples transfected with the control mRNA and mock transfected samples demonstrates the ability of the polynucleotide to inhibit MYC-mediated transactivation.

2) Screen for anti-MYC binders that reduce cancer cell activity or intracellular MYC activity by transfecting cancer cell lines with encoding polynucleotides.

[000973] Selected mRNAs encoding the MYC dimerization inhibitor or a control RNA are transfected into MYC expressing cancer cell lines in exponential growth. At predetermined days post-transfection, the effect of mRNAs encoding the MYC dimerization inhibitor on proliferation, viability, and apoptosis is measured using the proliferation, viability and apoptosis assay and compared to samples transfected with control RNA. A further control includes mock-transfected cells. A reduction in proliferation, viability, and/or increase in apoptosis of the cancer cells demonstrates the ability of the polynucleotide to reduce cancer cell activity.

B. DNA binding inhibition of MYC

[000974] Polypeptides that bind c-MYC and inhibit MYC DNA binding are screened and selected using methods known in the art. mRNA encoding the polypeptides with favorable screening results for binding c-MYC and/or inhibiting MYC DNA binding are designed and synthesized. The mRNA comprises at least one chemical modification and may be formulated in any of the formulation described herein.

1) Screen and select anti-MYC intracellular binders that inhibit MYC heterodimers binding to DNA.

[000975] Selected mRNAs encoding the MYC DNA binding inhibitor or a control RNA are transfected into exponentially growing MYC expressing cancer cell lines together with a reporter construct controlled by an upstream promoter containing MYC regulatory elements. As another control, a mock transfection is performed in parallel. At set days post-transfection, cells are harvested, lysed, and reporter activity is measured. A reduction in measured reporter gene activity as compared to the samples transfected with the control mRNA and mock transfected samples demonstrates the ability of the polynucleotide to inhibit MYC-mediated transactivation.
2) Screen for anti-MYC binders that reduces cancer cell activity or intracellular MYC activity by transfecting cancer cell lines with encoding mRNAs.

[000976] Selected mRNAs encoding the MYC DNA binding inhibitor or a control RNA are transfected into MYC expressing cancer cell lines in exponential growth, in 96 well plates. At set days post-transfection, the effect of RNAs encoding the putative MYC dimerization inhibitor on proliferation, viability, and apoptosis is measured using proliferation, viability and apoptosis assays and compared to samples transfected with control RNA. A further control includes mock-transfected cells. A reduction in proliferation, viability, and/or increase in apoptosis of the cancer cells demonstrates the ability of the polynucleotide to reduce cancer cell activity.

c. Targeted Trans-repression of MYC-regulated genes

[000977] Polypeptides that bind c-MYC, for example in its transactivation domain, are screened for and selected, and an encoding mRNA is designed. The KRAB domain mRNA sequence is connected with an appropriate linker to the encoding RNA of the MYC binder to create a trans-repressor, and the mRNA is synthesized. The mRNA comprises at least one chemical modification and may be formulated in any of the formulation described herein.

[000978] Cancer cell lines known to express MYC are transfected with the mRNA in order to determine the ability of the mRNA to reduce cancer cell activity and MYC transactivation activity.

[000979] Selected mRNAs encoding the MYC transrepressor or a control mRNA are transfected into exponentially growing MYC expressing cancer cell lines together with a reporter construct controlled by an upstream promoter containing MYC regulatory elements. As another control, a mock transfection is performed in parallel. At set days post-transfection, cells are harvested, lysed, and reporter activity is measured. A reduction in measured reporter gene activity as compared to the samples transfected with the control mRNA and mock transfected samples demonstrates the ability of the polynucleotide to inhibit MYC-mediated transactivation through repression in trans.

[000980] Selected mRNAs encoding the MYC trans-repressor or a control mRNA are transfected into MYC expressing cancer cell lines in exponential growth, in 96 well plates. At set days post-transfection, the effect of mRNAs encoding the MYC
dimerization inhibitor on proliferation, viability, and apoptosis is measured using proliferation, viability and apoptosis assays and compared to samples transfected with control mRNA. A further control includes mock-transfected cells. A reduction in proliferation, viability, and/or increase in apoptosis of the cancer cells demonstrates the ability of the polynucleotide to reduce cancer cell activity.

**Example 14. Inhibition of STAT3-mediated Transactivation**

Activated STAT3 increases tumor cell proliferation, survival and invasion while suppressing anti-tumor immunity. STAT3 has this dual role in tumor inflammation and immunity by promoting pro-oncogenic inflammatory pathways. STAT3 is activated by phosphorylation, which enables homodimerization. The activated transcription factor reaches the nucleus and binds to consensus DNA-recognition motif called gamma-activated sites (GAS) in the promoter region of cytokine-inducible genes and activates transcription of these genes. The transactivation domain of STAT3 at the protein C-terminus interacts with other proteins, such as CBP/p300, to activate transcription of its target genes.

**A. PPT inhibition of STAT3 dimerization screen**

Polypeptides that bind STAT3 and inhibit STAT3 dimerization are screened for and selected using methods known in the art. mRNA encoding the polypeptides with favorable screening results for binding STAT3 and/or inhibiting STAT3 dimerization are designed and synthesized. The mRNA comprises at least one chemical modification and may be formulated in any of the formulation described herein.

1) **Screen and select anti-STAT3 intracellular binders that inhibit STAT3 dimerization.**

Selected mRNAs encoding the STAT3 dimerization inhibitor or a control mRNA are transfected into exponentially growing STAT3 expressing cancer cell lines together with a reporter construct controlled by an upstream promoter containing STAT3 regulatory elements. As another control, a mock transfection is performed in parallel. At set days post-transfection, cells are harvested, lysed, and reporter activity is measured. A reduction in measured reporter gene activity as compared to the samples transfected with the control mRNA and mock transfected samples demonstrates the ability of the polynucleotide to inhibit STAT3-mediated transactivation.
2) **Screen for anti-STAT3 binders that reduces cancer cell activity or intracellular STAT3 activity in cell lines transfected with encoding mRNAs.**

[000984] Selected mRNAs encoding the STAT3 dimerization inhibitor or a control RNA are transfected into MYC expressing cancer cell lines in exponential growth, in 96 well plates. At set days post-transfection, the effect of mRNAs encoding the STAT3 dimerization inhibitor on proliferation, viability, and apoptosis is measured using proliferation, viability and apoptosis assays and compared to samples transfected with control mRNA. A further control includes mock-transfected cells. A reduction in proliferation, viability, and/or increase in apoptosis of the cancer cells demonstrates the ability of the polynucleotide to reduce cancer cell activity.

**B. Experiment 2. DNA binding inhibition of STAT3**

[000985] Polypeptides that bind STAT3 and inhibit STAT3 DNA binding are screened for and selected using methods known in the art. mRNA encoding the polypeptides with favorable screening results for binding STAT3 and/or inhibiting STAT3 DNA binding are designed and synthesized. The mRNA comprises at least one chemical modification and may be formulated in any of the formulation described herein.

1) **Screen and select anti-STAT3 intracellular binders that inhibit STAT3 homodimers binding to DNA.**

[000986] Selected mRNAs encoding the STAT3 DNA binding inhibitor or a control RNA are transfected into exponentially growing STAT3 expressing cancer cell lines together with a reporter construct controlled by an upstream promoter containing STAT3 regulatory elements. As another control, a mock transfection is performed in parallel. At set days post-transfection, cells are harvested, lysed, and reporter activity is measured. A reduction in measured reporter gene activity as compared to the samples transfected with the control mRNA and mock transfected samples demonstrates the ability of the polynucleotide to inhibit STAT3-mediated transactivation.

2) **Screen for anti-STAT3 binders that reduce cancer cell activity or intracellular STAT3 activity in cell lines transfected with encoding mRNAs.**

[000987] Selected mRNAs encoding the STAT3 DNA binding inhibitor or a control RNA are transfected into MYC expressing cancer cell lines in exponential growth, in 96 well plates. At set days post-transfection, the effect of mRNAs encoding the putative
MYC dimerization inhibitor on proliferation, viability, and apoptosis is measured using proliferation, viability and apoptosis assays and compared to samples transfected with control mRNA. A further control includes mock-transfected cells. A reduction in proliferation, viability, and/or increase in apoptosis of the cancer cells demonstrates the ability of the polynucleotide to reduce cancer cell activity.

**c. Targeted Trans-repression of STAT3-regulated genes**

[000988] Polypeptides that bind STAT3, for example in its transactivation domain, are screened for and selected, and an encoding mRNA is designed. The KRAB domain mRNA sequence is connected with an appropriate linker to the encoding RNA of the MYC binder to create a trans-repressor, and the resulting mRNA is synthesized. The mRNA comprises at least one chemical modification and may be formulated in any of the formulation described herein.

[000989] Cancer cell lines known to express STAT3 are transfected with the mRNA in order to determine the ability of the mRNA to reduce cancer cell activity and STAT3 transactivation activity.

[000990] Selected mRNAs encoding the STAT3 trans-repressor or a control mRNA are transfected into exponentially growing STAT3 expressing cancer cell lines together with a reporter construct controlled by an upstream promoter containing STAT3 regulatory elements. As another control, a mock transfection is performed in parallel. At set days post-transfection, cells are harvested, lysed, and reporter activity is measured. A reduction in measured reporter gene activity as compared to the samples transfected with the control mRNA and mock transfected samples demonstrates the ability of the polynucleotide to inhibit STAT3-mediated transactivation through repression *in trans*.

[000991] Selected mRNAs encoding the STAT3 trans-repressor or a control RNA are transfected into STAT3 expressing cancer cell lines in exponential growth, in 96 well plates. At set days post-transfection, the effect of mRNAs encoding the STAT3 dimerization inhibitor on proliferation, viability, and apoptosis is measured using proliferation, viability and apoptosis assays and compared to samples transfected with control mRNA. A further control includes mock-transfected cells. A reduction in proliferation, viability, and/or increase in apoptosis of the cancer cells demonstrates the ability of the polynucleotide to reduce cancer cell activity.
Example 15. Generation of an Intrabody Construct Comprising a Non-antibody Scaffold

[000992] In one embodiment of the current invention, a non-antibody scaffold intrabody is generated and characterized according to the methods described in Diem et al. Selection of high-affinity Centyrin FN3 domains from a simple library diversified at a combination of strand and loop positions, Protein Eng Des Sel. 2014, the contents of which is herein incorporated by reference in its entirety.

[000993] In one embodiment, certain portions of strands and loops of the Tenascin FN3 framework (Tencon) are randomized to generate a FN3 domain library. In a non-limiting example, portions of the C-strand, F-strand, CD-loop and FG-loop are randomized to provide a binding surface, as for example described in Protein Eng Des Sel. 2014.

[000994] In one embodiment, the library of FN3 domains having a diversified C-CD-F-FG alternative surface formed by a C beta-strand, a CD loop, an F beta-strand, and an FG loop is constructed according to the methods described in International Patent Publication WO2013049275, herein incorporated by reference in its entirety. The method comprises providing a reference FN3 domain polypeptide having an amino acid sequence at least 80% identical to the amino acid sequence of SEQ ID NO: 27 of WO2013049275 and introducing diversity into the reference FN3 domain polypeptide by mutating at least one C beta-strand residue and at least one F beta-strand residue to form the FN3 domain library having the diversified C-CD-F-FG alternative surface.

[000995] Once the library of randomized FN3 domains is obtained, the library is then panned against the target protein of interest.

[000996] In one embodiment, the target protein of interest is c-MYC. In one example, the DNA binding domain of c-MYC is the target domain within the target protein of interest. In another example, the dimerization domain of c-MYC is the target domain within the target protein of interest. In one embodiment, protein of interest is MAX. In a non-limiting example, the dimerisation domain of Max is the target domain of interest. In one embodiment, the target protein of interest is STAT3. In one non-limiting example, the target domain of interest within the protein of interest is the DNA binding domain of STAT3. In another non-limiting example the dimerization domain is the domain of interest.
The library may be panned against the targets of interest using an in
vitro library selection system, such as CIS display, and binders may be screened for and
identified using ELISA, alone or in combination with biological activity assays. Higher
affinity is obtained by repeating the screening at higher stringency. Higher affinity is also
generated by creating further libraries, where some residues deemed critical for affinity
are held constant and others are further randomized.

ORF variants for each amino acid sequence may then be generated from the
binders identified, using the standard human codon usage frequency table.

Example 16. Expression of Anti-Ras Intrabodies and Interaction with
Ras

A series of modified mRNA (mmRNA) constructs encoding anti-Ras
intrabodies were prepared. The constructs had the sequence structures as shown below in

Table 6

<table>
<thead>
<tr>
<th>Name</th>
<th>5'UTR SEQ ID</th>
<th>mRNA SEQ ID</th>
<th>3'UTR SEQ ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.dAb.HRAS.VH(CC).cyto.nHis.eV5_HS3UPCRfree</td>
<td>20</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>i.dAb.HRAS.VH(AV).cyto.nHis.eV5_HS3UPCRfree</td>
<td>20</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>i.dAb.HRAS.VHH(AV).mem.nHis.eV5_HS3UPCRfree</td>
<td>20</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td>uAb.RAS.nHis.eV5_HS3U</td>
<td>20</td>
<td>25</td>
<td>21</td>
</tr>
</tbody>
</table>

Additionally, all constructs contained a Cap 1 5' Cap (described in Example
5), a poly A tail of 140 nucleotides and were fully modified with 1-methyl-pseudouridine
(ιηψ). The mRNA sequences used in the constructs shown in Table 6 include an epitope
tag coding sequence (V5) incorporated into the sequence. Sequences corresponding to
SEQ ID NOs: 22-25 but without the V5 tag are shown in SEQ ID NOs: 29-32.

The constructs were transfected into HT1080 cells and the cells were lysed 6
hours later. To determine whether the intrabodies were expressed and interacted with
endogenous Ras in the cells, the lysate was first immunoprecipitated with an antibody
(anti-V5 antibody) that bound an epitope tag (V5) included in all of the intrabody
constructs and then the immunoprecipitate was subjected to Western blot analysis using
an anti-Ras antibody. More specifically, approximately 700 μg of protein were
immunoprecipitated with an anti-V5 antibody bound to protein A/G sepharose beads.
Samples were thrice washed, eluted and resolved by SDS-PAGE. Western blot analysis of the gel was performed by standard methods using an anti-Ras antibody. The results showed that all constructs specific for Ras immunoprecipitated Ras to varying levels, whereas control constructs that did not contain an anti-Ras intrabody did not immunoprecipitate Ras.

To determine whether the anti-Ras intrabodies preferentially bound activated (i.e., GTP-bound) Ras, HT1080 cells were transfected with the constructs, cells were lysed 6 hours later and approximately 700 μg of protein were immunoprecipitated with anti-V5 bound to protein A/G sepharose as described above. Then, supernatants were removed and 250 ng of purified HRAS + GTPyS (a non-hydrolyzable form of GTP) or GDP was incubated with each sample. Samples were thrice washed, eluted, and resolved by SDS-PAGE, followed by standard Western blotting with anti-Ras antibody. The results showed that the constructs encoding anti-Ras intrabody preferentially bound GTPyS-Ras, while the non-specific controls did not, thus indicating that the anti-Ras intrabodies preferentially bound activated Ras. Binding to active (i.e., GTP-bound) Ras is a desirable property of the anti-Ras intrabodies because mutations that lead to oncogenic Ras are typically activating mutations.

Example 17. Expression of Anti-HBV Core Antigen Intrabodies and Interaction with HBV Core Antigen

A series of modified mRNA (mmRNA) constructs encoding anti-HBV core antigen intrabodies were prepared. The constructs had the structures as shown below in Table 7:

<table>
<thead>
<tr>
<th>Name</th>
<th>5'UTR SEQ ID</th>
<th>mRNA SEQ ID</th>
<th>3'UTR SEQ ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHH.C4.cyto.cV5</td>
<td>20</td>
<td>26</td>
<td>21</td>
</tr>
<tr>
<td>VHH.C2.cyto.cV5</td>
<td>20</td>
<td>27</td>
<td>21</td>
</tr>
<tr>
<td>VHH.C6.cyto.cV5</td>
<td>20</td>
<td>28</td>
<td>21</td>
</tr>
</tbody>
</table>

Additionally, all constructs contained a Cap 15' Cap (described in Example 5), a poly A tail of 140 nucleotides and were fully modified with 1-methyl-pseudouridine (ιηψ). The mRNA sequences used in the constructs shown in Table 7 include an epitope
tag coding sequence (V5) incorporated into the sequence. Sequences corresponding to SEQ ID NOs: 26-28 but without the V5 tag are shown in SEQ ID NOs: 33-35.

[0001007] Hep3B cells were transfected with either the VHH construct mmRNAs against HBV core Ag or a control construct. Cells were lysed and the extracts normalized using the Pierce BCA assay. Input samples were collected and then the extracts subjected to immunoprecipitation using 7.5uL of a 1:1 mix of protein A and protein G agarose beads and 0.5uL anti-V5 antibody (Life Tech) for 3h at 4°C in the presence of 500 ng of recombinant HBV core antigen (ABDserotec cat#OBT0909). After 3h, the beads were washed 3 times and then resuspended in IX LDS to elute the immune complexes. Samples were then run on SDS-PAGE and analyzed by western blotting for HBV Core antigen (ABT Serotec 4939-8520 in PBS-0.05% Tween-1% milk, overnight @ 4C with shaking). Membranes were stripped and reprobed with anti-V5 antibody. The results showed that the mmRNA encoded anti-HBV core Ag intrabodies were able to immunoprecipitate their target antigen (HBV core Ag), whereas the control construct did not.

[0001008] Example 18. Expression of Intrabody Constructs Comprising an FN3 Scaffold

[0001009] A series of modified mRNA (mmRNA) intrabody constructs encoding an FN3 scaffold displaying a peptide aptamer known to bind a target protein were prepared. The constructs also contained a V5 epitope tag. DLD1 cells were transfected with the mmRNA constructs using Lipofectamine 2000, incubated overnight, lysed, and immunoprecipitated with anti-V5 antibodies. The immunoprecipitates were then subjected to standard Western blot analysis using an antibody against the target protein to which the aptamer binds. The results showed that the peptide aptamer displayed by the FN3 scaffold was able to bind to its target protein.

[0001010] Another series of mmRNA intrabody constructs were prepared encoding an FN3 scaffold presenting peptides that bound to a protein domain. The constructs also included a V5 epitope tag. K562 cells were electroporated with the FN3 scaffold constructs presenting peptides specific for the protein domain. Immunoprecipitations were performed on the cell lysates using an anti-V5 antibody and then subjected to SDS-PAGE. The gel was transferred to nitrocellulose and subjected to standard Western blot
analysis using antibodies against the protein containing the domain to which the FN3-presented peptides bind. The results showed that the FN3 scaffold constructs were capable of interacting with the protein containing the domain to which presented peptides bind in K562 cells.

[0001011] Example 19. Intracellular Expression of Anti-HBV Core Antigen Intrabody in Mouse Livers in Vivo Following Injection of mmRNA Construct Encoding Intrabody as Determined by LC-MS

In order to determine the pharmacokinetic profile of an intracellular protein synthesized in vivo via mmRNA injection into mice, an anti-HBV Core Antigen (HBcAg) intrabody comprising a single domain antibody, antiHBcAg.VHH.C4.cyto.V5, (described further in Example 17) was formulated in MC3 lipid nanoparticles and injected intravenously mice. MC3 lipid nanoparticles included MC3 50%, DSPC 10%, Cholesterol 38.5%, PEG-DMG 1.5%, N:P -5.5. (Values are based on mol. %.) The mmRNA was administered at a dose of 0.5 mg/kg to eight groups of three mice each. At a designated time point after injection, the mice were sacrificed and the livers excised. Group 1 mice were a control at time point 0 hours. Groups 2-8 mice were treatment mice, with time points post-injection for liver harvesting of 1 hour, 2 hours, 4 hours, 6 hours, 15 hours, 24 hours and 48 hours, respectively.

[0001012] After excising the livers, approximately 1/3 of the tissue was homogenized using an Optima Max XP polytron in 8M urea/lO0mM ammonium bicarbonate. The homogenized cells were centrifuged at 100,000 x g for 20 minutes at 4 °C and protein concentration was assessed via a Pierce 660 protein assay. 100 µg of lysate was reduced with 15mM TCEP for 1 hour at 37°C in the presence of heavy isotopic peptides (2nM per peptide per reaction) and the samples were brought to a final volume of 100 µl in 8M urea/lO0mM ammonium bicarbonate.

[0001013] Heavily-labeled isotopic peptides identified as unique to the anti-HBcAg construct are as follows:

[0001014] (i) LMQAGGSLR (SEQ ID NO: 16);
[0001015] (ii) TFYAMGWFR (SEQ ID NO: 17);
[0001016] (iii) EFVAAINR (SEQ ID NO: 18);
GDGTTFYAESVK (SEQ ID NO: 19).

Following labeling of the lysate samples with the heavy-isotope labeled signature peptides, the reduced cysteines in the sample were alkylated with 25mM iodoacetamide for 1 hour at room temperature. Next, the proteins in the sample were digested with trypsin (protease:protein = 1:50). 2 µg of trypsin were used per 100 µg sample and the samples were incubated overnight at 37°C. The samples were then cleaned up by reducing the pH with formic acid to approximately pH 3 and the peptides were captured over a prepared C18 resin. The resin was washed once with 0.1% formic acid and the peptides were eluted with 80% acetonitrile/0.1% formic acid. The samples were dried using a speed vacuum and resuspended in 400 µl of 0.1% formic acid. Finally, 1 ml of each sample was injected for analysis by liquid chromatography-mass spectrometry (LC-MS).

Representative results of the intracellular expression of the anti-HBcAg intrabody encoded by the injected mmRNA in mouse liver are shown in Figure 5, which shows the LC-MS results for the LMQAGGSLR (SEQ ID NO: 16) signature peptide. The results show that the intrabody is expressed in the liver cells in vivo as early as 1 hour post-injection of the mmRNA construct, with expression increasing at 2 hours and 4 hours post-injection, and peaking at 6 hours post-injection. At 6 hours post-injection, expression levels were approximately 50-80 ng intrabody/mg mouse liver. Expression had significantly decreased by 15 hours post-injection and remained low at 24 and 48 hours post-injection. A similar expression pattern was observed for the three other signature peptides studied (SEQ ID NOs: 17-19), with expression starting at 1 hour post-injection, increasing at 2 and 4 hours post-injection, peaking at 6 hours post-injection, subsiding by 15 hours post-injection and remaining low at 24 and 48 hours post-injection.

These results demonstrate the intracellular expression in mouse liver cells in vivo of an mmRNA-encoded intrabody, with very rapid (within 1 hour) onset of expression and peak expression by 6 hours post-injection. Moreover, these results demonstrate that the expression had subsided by 15 hours post-injection. Thus, these results demonstrate the ability of the mmRNA constructs to allow both for rapid production of the intrabody intracellularly, as well as rapid clearance of the intrabody from the cells. Furthermore, following such clearance, intrabody expression can be re-
instated in the cells by injection of another dose of the mmRNA construct (see Example 21).

Example 20. Half-Life Determination for Protein Scaffolds

The half-life of mmRNA-encoded protein scaffolds in cells was determined by Bio-orthogonal non-canonical amino acid tagging (BONCAT). An mmRNA construct encoding a protein scaffold of interest that did not contain any presenting polypeptides (i.e., inert constructs not containing a binding polypeptide (s)) was transfected into cells, substituting an unnatural amino acid (azidohomoalanine; AHA) for a natural amino acid (methionine). AHA contains a reactive group (a terminal azide), which can be conjugated to a fluorophore via click chemistry. Newly synthesized proteins in the cells were separated by molecular weight (SDS-PAGE) at various time points post-transfection and visualized via in-gel fluorescence.

An mmRNA encoding an antiHBcAg VHH construct (single domain intrabody) was transfected into cells and protein production analyzed as described above to determine the half-life of the VHH scaffold. The results are shown in Figure 6. A half-life of 4.507 hours post-transfection was determined based on the data for the VHH scaffold.

Similar experiments were conducted using an mmRNA encoding a fibronectin III (FN3) scaffold transfected into cells, with protein production analyzed as described above to determine the half-life of the FN3 scaffold. A half-life of 9.467 hours post-transfection was determined based on the data for the FN3 scaffold.

Example 21. Additional Pharmacokinetic Analysis of Intracellular Expression of Anti- HBV Core Antigen Intrabody in Mouse Livers in Vivo

Following Multiple Injections of mmRNA Construct Encoding Intrabody

Additional studies were performed to assess the pharmacokinetics of intracellular expression of an anti-HBcAg VHH intrabody in mouse livers in vivo following multiple dosing. Mice were dosed on day 0 and on day 7 with an LNP carrying an mmRNA construct encoding the intrabody, which included a V5 epitope tag. Liver protein was recovered and analyzed for expression of the anti-HBcAg VHH intrabody using the V5 epitope tag for detection and quantification. Protein amounts were normalized to tubulin.
The results are shown in Figures 7 and 8. Figure 7 shows intracellular anti-HBcAg VHH expression over 250 hours, following administration of the mmRNA at day 0 and day 7 (168 hours). Figure 8 is a more detailed graph of the first 30 hours post-injection of the first dose at day 0. Consistent with the results reported in Example 19, the results in Figure 9 demonstrate that there was rapid onset of protein production, with protein detectable within 1 hour of mmRNA administration, followed by increased protein production, with peak production being reached within only several hours after administration of the first dose. Protein production then began to subside significantly and was essentially at baseline by 24 hours post-injection. The results in Figure 7 demonstrate that after the initial peak and subsiding of protein expression following administration of the first dose, intracellular intrabody production is re-instated in the cells following administration of a second dose on day 7. The time course of protein production for the second dose mimicked that of the first dose, with rapid onset of protein production and peak levels being reached within several hours post-injection, followed by rapid subsiding of protein expression.

These results demonstrate the ability of the mmRNA constructs to allow both for rapid production of the intrabody intracellularly, as well as rapid clearance of the intrabody from the cells. Furthermore, following such clearance, intrabody expression can be re-instated in the cells by injection of another dose of the mmRNA construct.
<table>
<thead>
<tr>
<th>SEQ ID NO:</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAAAGCTGCTGCAAGAGAAGCTGCAGCTAGGAGGGACTAGGGAGGTGGCTGCTGCAAGAGA</td>
</tr>
<tr>
<td>2</td>
<td>GGCAGC</td>
</tr>
<tr>
<td>3</td>
<td>GGTAGCGGCAAGCGGTAGC</td>
</tr>
<tr>
<td>4</td>
<td>GGTGAATAATATTGTATATTTATAATGTCCTGTTG</td>
</tr>
<tr>
<td>5</td>
<td>TCCGCTTGTACTGGATGCTTCC</td>
</tr>
<tr>
<td>6</td>
<td>GGTGGAGAAGTGTTCTGGAGGCGGTGGAGATGGTGCGGAGGAGCGTCAG</td>
</tr>
<tr>
<td>7</td>
<td>GGTGGTTCTGGTT</td>
</tr>
<tr>
<td>8</td>
<td>GGTGGTTCTGGTTGGTGGTTCTGGT</td>
</tr>
<tr>
<td>9</td>
<td>GGTGGTTCTGGTTGGTGGTTCTGGT</td>
</tr>
<tr>
<td>10</td>
<td>GGTGGTTCTGGTTGGTGGTTCTGGT</td>
</tr>
<tr>
<td>11</td>
<td>GGTGGTTCTGGTTGGTGGTTCTGGT</td>
</tr>
<tr>
<td>12</td>
<td>GGGGGGAGG</td>
</tr>
<tr>
<td>13</td>
<td>GSGATNSLLKQQAGDVEENPG</td>
</tr>
<tr>
<td>14</td>
<td>GSGATNSLLKQQAGDVEENPG</td>
</tr>
<tr>
<td>15</td>
<td>GGAAGCGGAGCTACTAATCTGCTGACTGAGCGACTGGGAAGAGATGGGAGGAGAA</td>
</tr>
<tr>
<td>16</td>
<td>CCCCCTGACCCT</td>
</tr>
<tr>
<td>17</td>
<td>LMQAGGSLR</td>
</tr>
<tr>
<td>18</td>
<td>TFYAMGWR</td>
</tr>
<tr>
<td>19</td>
<td>EFVAAINR</td>
</tr>
<tr>
<td>20</td>
<td>GDGTTFYAESVK</td>
</tr>
<tr>
<td>21</td>
<td>TCAAGCTTTGGAGCCATGACTAGAAGCTATGCTGACTGAGCGACTGGGAAGAGATGGGAGGAGAA</td>
</tr>
<tr>
<td>22</td>
<td>ACACTGCGCACCTCCAAAACTATTACTACCGGATGTCGTAAGGAGCGAGTCTGAGGGTCCCGG</td>
</tr>
</tbody>
</table>

262
| 33 | GAAAGAAGTGATCGACGCATTCATCTCGGAAAACGGTTGGGTGGAGGACTAC
ATGCAGGTGAAACTGGAGGAGAGCGGAGGTCGGCTCATGCAGGCTGGGGGCAGTCT
GCGGTTGTCATGCGTCGCCAGCGGACGCACGTTTTACGCCATGGGCTGGTTCCGCCA
GGCCCCGGGGAAAGAGGGAATTTGTAGCTGCAATTAACCGTGGCGACGGCACAA
CATTTTACGCGCGAATCTGTAAGGGGAGGAGTTTCATACTATCTACGTGGCAGACTAT
GCGGCTGCAGGAGGAGAGATCGCAATCACTCAATGGCGAGCTACTGAG
| 34 | ATGCAGGTTCAGCTGTGGAAGTCGGGATTATGCAGGAGGAGGATCTCTAGGATCTCT
CGGTTATCTTGTGCTCCCTCTGGGAGGACCTGGGCTCTGGGGCTGGGTTTT
CGACAGCCGCGCGAAAGAGGAAGGAATTCGTGGGCCGCAACTCAGCCGCGCT
CAATATTCTTTATGGGAGATGTTGAAGGCGAGATTTACGATATGTCGCGATAATGC
GTTGAAATACCGTTTTACTTGCCTCAAGGCCGGAACAGACCGGCTGCTCTA
CTACTGTGCGAAGCCTACTGGGTCTCGGGACATATGCTGATACAGTCATATGCTT
CGTCACCTGGGGCGAGGTTACACAAAAAGTTACGCTCAGCTCAGT
| 35 | ATGGAGATCCAAATTGGAGGATCTCTGGGGGGAATGGGGCTGGTCGACAGCCGCGGCTCT
AAGACTGTCATGGCGGCTTCGGGCCACCTCTCAAGAATGCGATGGGGCTGGTT
CAGACAAAGACCCAGGAGAAGAGCCGGAGGTCTGTCGCGGCGGATCGATGGGGTCGAG
GAAGCAGGTTTATTAGGACCGCTCGTGAAGGGTGTTTCACTATTCTGGAGAGAAATG
CAGAGAACCAGAATGTTGACCTAATTCAGATGAAGTCTGAAGGCAAGGAGACACTGCA
GTTAACATTGGGCGGCTCGGGACAGCAACTACAGTGCGACCGGCACTTTGCGCGGA
CGCTAGATCGAGTTATGGGGTCAGGTAACATGCTTGCTCCTTCT
|
Claims

1. A polynucleotide comprising:
   (a) a first region of linked nucleosides, the first region encoding a polypeptide of interest;
   (b) a first flanking region located 5' relative to the first region comprising at least one 5'terminal cap; and
   (c) a second flanking region located 3' relative to the first region comprising a 3'tailing sequence of linked nucleosides,

   wherein the polypeptide of interest comprises a polypeptide which is expressed intracellularly and comprises a domain which binds to an intracellular target, and wherein the polynucleotide comprises at least one chemically modified nucleoside.

2. The polynucleotide of claim 1, wherein the polypeptide is an antibody or antigen-binding fragment thereof.

3. The polynucleotide of claim 1, wherein the polypeptide is a single domain antibody.

4. The polynucleotide of claim 1, wherein the polypeptide comprises a non-antibody scaffold protein which binds to an intracellular target.

5. The polynucleotide of claim 1, wherein the polypeptide is a fusion protein comprising an intracellular polypeptide and a fibronectin domain.

6. The polynucleotide of claim 1, wherein the polypeptide is a fusion protein comprising an intracellular polypeptide and a Kunitz domain.

7. The polynucleotide of claim 1, wherein the polypeptide is a fusion protein comprising an intracellular polypeptide and a Stefin A mutant scaffold.
8. The polynucleotide of claim 1, wherein the polypeptide is a fusion protein comprising an intracellular polypeptide and a transferrin domain.

9. The polynucleotide of claim 1, wherein the polypeptide of interest prevents or disrupts a protein-protein interaction between the intracellular target and at least one other protein.

10. The polynucleotide of claim 9, wherein the intracellular target is MYC and the at least one other protein is MAX.

11. The polynucleotide of claim 9, wherein the intracellular target is STAT3 and the at least one other protein is STAT3.

12. The polynucleotide of claim 1, wherein the polypeptide of interest prevents recruitment of the intracellular target to a regulatory element.

13. The polynucleotide of claim 12, wherein the intracellular target is MYC and/or MAX.

14. The polynucleotide of claim 12, wherein the intracellular target is STAT3.

15. The polynucleotide of claim 1, wherein the at least one chemically modified nucleoside comprises a 1-methylpseudouridine or a 5-methyl-cytosine.

16. The polynucleotide of claim 1, comprising a microRNA (miRNA) binding site.

17. The polynucleotide of claim 15, wherein the miRNA binding site regulates mRNA and protein expression.
18. The polynucleotide of claim 17, wherein the miRNA binding site is selected
from miR-122, miR-133, miR-206, miR-208, miR-17-92, miR-126, miR-142-3p, miR-
142-5p, miR-16, miR-21, miR-223, miR-24, miR-27, let-7, miR-30c, miR-1d, miR-149,
miR-192, miR-194, miR-204, let-7, miR-133, miR-126 and miR-132.

19. A lipid nanoparticle comprising the polynucleotide of claim 1.

20. A lipid nanoparticle of claim 19, further comprising a targeting moiety
conjugated to the surface of the lipid nanoparticle.

21. A composition comprising the lipid nanoparticle of claim 19 or the
polynucleotide of claim 1, and a pharmaceutically acceptable excipient.

22. A method of treating a disease in a subject comprising administering to a
subject the lipid nanoparticle of claim 19 or the polynucleotide of claim 1.

23. A polynucleotide comprising

(a) a first region of linked nucleosides, the first region encoding a
polypeptide of interest;

(b) a first flanking region located 5’ relative to the first region comprising
at least one 5’ terminal cap; and

(c) a second flanking region located 3’ relative to the first region
comprising a 3’ tailing sequence of linked nucleosides,

wherein the polynucleotide comprises at least one chemically modified
nucleoside, wherein the polypeptide of interest comprises a polypeptide which is
expressed intracellularly and comprises a domain which binds to an intracellular target,
and wherein the polypeptide reaches a maximum intracellular concentration within 2 to 8
hours after the polynucleotide is introduced into a mammalian cell.
24. The polynucleotide of claim 23, wherein the polypeptide reaches a maximum intracellular concentration within 4 to 6 hours after the polynucleotide is introduced into a mammalian cell.

25. The polynucleotide of claim 23, wherein the polypeptide is an antibody or antigen-binding fragment thereof.

26. The polynucleotide of claim 23, wherein the polypeptide is a single domain antibody.

27. The polynucleotide of claim 23, wherein the polypeptide comprises a non-antibody scaffold protein which binds to an intracellular target.

28. A polynucleotide comprising

(a) a first region of linked nucleosides, the first region encoding a single domain antibody;

(b) a first flanking region located 5' relative to the first region comprising at least one 5' terminal cap; and

(c) a second flanking region located 3' relative to the first region comprising a 3' tailing sequence of linked nucleosides,

wherein the polynucleotide comprises at least one chemically modified nucleoside, wherein the single domain antibody is expressed intracellularly and binds to an intracellular target, and wherein the single domain antibody reaches a maximum intracellular concentration at about 6 hours after the polynucleotide is introduced into a mammalian cell.
29. The polynucleotide of claim 28, wherein the single domain antibody is detectable within about 1 hour after the polynucleotide is introduced into a mammalian cell.

30. The polynucleotide of claim 28, wherein the single domain antibody is no longer detectable about 24 hours after the polynucleotide is introduced into a mammalian cell.

31. The polynucleotide of claim 28, wherein the single domain antibody has a half-life of 4-6 hours.

32. A method of reaching a maximum intracellular concentration of a polypeptide of interest in a minimum time following administration of a polynucleotide encoding the polypeptide of interest to a subject comprising administering to the subject a polynucleotide comprising

   (a) a first region of linked nucleosides, the first region encoding a polypeptide of interest;

   (b) a first flanking region located 5' relative to the first region comprising at least one 5' terminal cap; and

   (c) a second flanking region located 3' relative to the first region comprising a 3' tailing sequence of linked nucleosides,

   wherein the polynucleotide comprises at least one chemically modified nucleoside, wherein the polypeptide of interest comprises a polypeptide which is expressed intracellularly and comprises a domain which binds to an intracellular target, and wherein the polypeptide reaches a maximum intracellular concentration within 2 to 8 hours after the polynucleotide is administered to the subject.
33. The method of claim 32, wherein the polypeptide reaches a maximum intracellular concentration within 4 to 6 hours after the polynucleotide is introduced into the subject.

34. The method of claim 32, wherein the polypeptide is an antibody or antigen-binding fragment thereof.

35. The method of claim 32, wherein the polypeptide is a single domain antibody.

36. The method of claim 32, wherein the polypeptide comprises a non-antibody scaffold protein which binds to an intracellular target.

37. A method of reaching a maximum intracellular concentration of a single domain antibody in a minimum time following administration of a polynucleotide encoding the single domain antibody to a subject comprising administering to the subject a polynucleotide comprising

(a) a first region of linked nucleosides, the first region encoding a single domain antibody;

(b) a first flanking region located 5' relative to the first region comprising at least one 5' terminal cap; and

(c) a second flanking region located 3' relative to the first region comprising a 3' tailing sequence of linked nucleosides,

wherein the polynucleotide is administered intravenously encapsulated in an LNP and comprises at least one chemically modified nucleoside, wherein the single domain antibody is expressed intracellularly and binds to an intracellular target, and wherein the single domain polypeptide reaches a maximum liver accumulation at about 6 hours after the polynucleotide is administered to the subject.
38. The method of claim 37, wherein the single domain antibody is detectable within about 1 hour after the polynucleotide is administered to the subject.

39. The method of claim 37, wherein the single domain antibody is no longer detectable about 24 hours after the polynucleotide is administered to the subject.

40. The method of claim 37, wherein the single domain antibody has a half-life of 4-6 hours.

41. A method for transiently expressing an intracellular polypeptide of interest comprising administering to the subject a polynucleotide comprising

   (a) a first region of linked nucleosides, the first region encoding a polypeptide of interest;

   (b) a first flanking region located 5' relative to the first region comprising at least one 5' terminal cap; and

   (c) a second flanking region located 3' relative to the first region comprising a 3' tailing sequence of linked nucleosides,

   wherein the polynucleotide comprises at least one chemically modified nucleoside, wherein the polypeptide of interest comprises a polypeptide which is expressed intracellularly and comprises a domain which binds to an intracellular target, and wherein the polypeptide of interest is detectable 1-3 hours after the polynucleotide is administered to the subject and is no longer detectable 24-48 hours after the polynucleotide is administered to the subject.

42. The method of claim 41, wherein the polypeptide of interest has a half-life of 4-6 hours.

43. A method for providing a single domain antibody which is expressed intracellularly to a subject, comprising
administering to the subject intravenously a first dose of a polynucleotide encapsulated in an LNP,

wherein the polynucleotide comprises

(a) a first region of linked nucleosides, the first region encoding a single domain antibody;

(b) a first flanking region located 5’ relative to the first region comprising at least one 5’ terminal cap; and

(c) a second flanking region located 3’ relative to the first region comprising a 3’ tailing sequence of linked nucleosides,

wherein the polynucleotide comprises at least one chemically modified nucleoside, and wherein the single domain antibody is expressed intracellularly and binds to an intracellular target; and

administering to the subject intravenously a second dose of the polynucleotide encapsulated in an LNP at 12-15 hours following administration of the first dose.

44. The method of claim 43, wherein the single domain antibody has a half-life of 4-6 hours.
Fig. 6

VHH Half-Life: Fluorescence Normalized to 0h
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K 48/00 CO7K16/00 C12N 15/13
ADD.

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)

A61K CO7K

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

Electronis database consulted during the international search (name of database and, where possible, search terms used)

EPO-Internal, WPI Data, EMBASE, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>WO 2013/151666 A2 (MODERNA THERAPEUTICS [US]) 10 October 2013 (2013-10-10) cited in the application on paragraph(s) [0005], [0095], [0151], [0158], [0181], [0187], [0508], [0509], [0934], [0940]</td>
<td>1-44</td>
</tr>
</tbody>
</table>

* 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 invention 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 obvious as it contains and/or involves 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

**A** document member of the same patent family

Date of the actual completion of the international search: 6 November 2015

Date of mailing of the international search report: 16/11/2015

Name and mailing address of the ISA/European Patent Office, P. B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax (+31-70) 340-3018

Authorized officer

Saame, Tina
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 13ter.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 13ter.1 (a)).
      - on paper or in the form of an image file (Rule 13ter.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:
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patent document cited in search report</td>
<td>Publication date</td>
<td>Patent family member(s)</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>wO 2013151666 A2</td>
<td>10-10-2013</td>
<td>AU 2013243949 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2868391 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 104411338 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2015518705 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wO 2013151666 A2</td>
</tr>
<tr>
<td>wO 2007024708 A2</td>
<td>01-03-2007</td>
<td>EP 1979364 A2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 2578685 A2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2009286852 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2013111615 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2013197068 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2013261172 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2015038558 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wO 2007024708 A2</td>
</tr>
<tr>
<td>wO 2015105926 A1</td>
<td>16-07-2015</td>
<td>NONE</td>
</tr>
</tbody>
</table>

Form PCT/ISA/210 (patent family annex) (April 2005)