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(54) Title: NICKED POLYNUCLEOTIDE COMPLEXES FOR MULTIVALENT RNA INTERFERENCE

(57) Abstract: The present invention includes multivalent nucleic acid complexes of nucleic acid molecules having two or more target-specific regions, in which the target-specific regions are complementary to a single target gene at more than one distinct nucleotide site, and/or in which the target regions are complementary to more than one target gene or target sequence. One oligonucleotide strand of the complex is nicked and thereby inactivated. Also included are methods of using the complexes for treatment of a variety of diseases and infections.

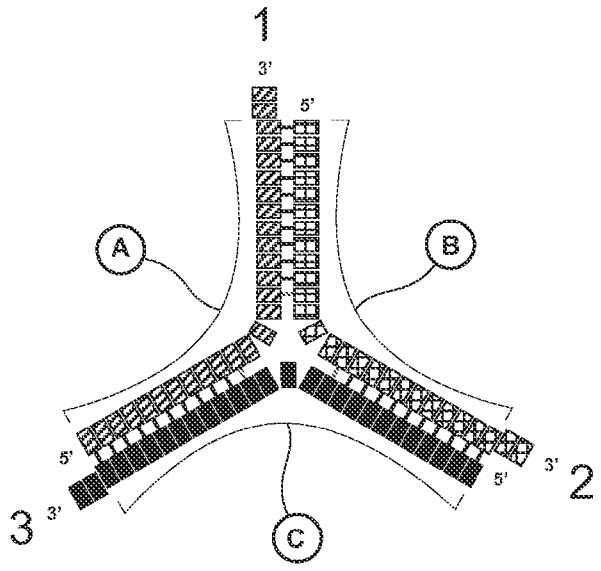


FIG. 1

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NICKED POLYNUCLEOTIDE COMPLEXES FOR MULTIVALENT RNA INTERFERENCE

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. provisional application 61/583,035 filed January 4, 2012, which is incorporated herein by reference in its entirety.

BACKGROUND

Technical Field

The present invention relates generally to precisely structured polynucleotide molecules, and methods of using the same for multivalent RNA interference and the treatment of disease.

Description of the Related Art

The phenomenon of gene silencing, or inhibiting the expression of a gene, holds significant promise for therapeutic and diagnostic purposes, as well as for the study of gene function itself. Examples of this phenomenon include antisense technology and dsRNA forms of posttranscriptional gene silencing (PTGS) which has become popular in the form of RNA interference (RNAi).

Antisense strategies for gene silencing have attracted much attention in recent years. The underlying concept is simple yet, in principle, effective: antisense nucleic acids (NA) base pair with a target RNA resulting in inactivation of the targeted RNA. Target RNA recognition by antisense RNA or DNA can be considered a hybridization reaction. Since the target is bound through sequence complementarily, this implies that an appropriate choice of antisense NA should ensure high specificity. Inactivation of the targeted RNA can occur via different pathways, dependent on the nature of the antisense NA (either modified or unmodified DNA or RNA, or a hybrid thereof) and on the properties of the biological system in which inhibition is to occur.

RNAi based gene suppression is a widely accepted method in which a sense and an antisense RNA form double-stranded RNA (dsRNA), *e.g.,* as a long RNA duplex, a 19-24 nucleotide duplex, or as a short-hairpin dsRNA duplex (shRNA), which is involved in gene modulation

by involving enzyme and/or protein complex machinery. The long RNA duplex and the shRNA duplex are pre-cursors that are processed into small interfering RNA (siRNA) by the endoribonuclease described as Dicer. The processed siRNA or directly introduced siRNA is believed to join the protein complex RISC for guidance to a complementary gene, which is cleaved by the RISC/siRNA complex.

However, many problems persist in the development of effective antisense and RNAi technologies. For example, DNA antisense oligonucleotides exhibit only short-term effectiveness and are usually toxic at the doses required; similarly, the use of antisense RNAs has also proved ineffective due to stability problems. Also, the siRNA used in RNAi has proven to result in significant off-target suppression due to either strand guiding cleaving complexes potential involvement in endogenous regulatory pathways. Various methods have been employed in attempts to improve antisense stability by reducing nuclease sensitivity and chemical modifications to siRNA have been utilized. These include modifying the normal phosphodiester backbone, *e.g.*, using phosphorothioates or methyl phosphonates, incorporating 2'-OMe-nucleotides, using peptide nucleic acids (PNAs) and using 3'-terminal caps, such as 3'-aminopropyl modifications or 3'-3' terminal linkages. However, these methods can be expensive and require additional steps. In addition, the use of non-naturally occurring nucleotides and modifications precludes the ability to express the antisense or siRNA sequences *in vivo*, thereby requiring them to be synthesized and administered afterwards. Additionally, the siRNA duplex exhibits primary efficacy to a single gene and off-target to a secondary gene. This unintended effect is negative and is not a reliable RNAi multivalence.

Consequently, there remains a need for effective and sustained methods and compositions for the targeted, direct inhibition of gene function *in vitro* and *in vivo*, particularly in cells of higher vertebrates, including a single-molecule complex capable of multivalent gene inhibition.

BRIEF SUMMARY

The present invention provides novel compositions and methods, which include precisely structured oligonucleotides that are useful in specifically regulating gene expression of one or more genes simultaneously when the nucleotide target site sequence of each is not identical to the other. In particular, the present invention provides isolated, three-stranded,

polynucleotide complexes, capable of silencing gene expression by RNA interference, wherein one of the three strands includes a nick that divides the nicked strand into two shorter portions. The nick inactivates the nicked strand which is thereby prevented from interacting with an off-target RNA molecule.

In certain embodiments, the present invention includes an isolated precisely structured three-stranded polynucleotide complex comprising a region or regions having a sequence complementary to a target gene or sequence at multiple sites or complementary to multiple genes at single sites. In related embodiments, the isolated precisely structured three-stranded polynucleotide complex comprises one or more regions having a sequence complementary to one or more regions within a target gene or sequence. In particular embodiments, the isolated precisely structured three-stranded polynucleotide complex comprises two or more, *e.g.,* three, regions having sequences complementary to the same region within a target gene or sequence.

Certain embodiments relate to polynucleotide complexes of at least three separate polynucleotides, comprising (a) a first polynucleotide comprising a target-specific region that is complementary to a first target sequence, a 5' region, and a 3' region; (b) a second polynucleotide comprising a target-specific region that is complementary to a second target sequence, a 5' region, and a 3' region; and (c) a third polynucleotide comprising a null region or a target-specific region that is complementary to a third target specific, a 5' region, and a 3' region, wherein each of the target-specific regions of the first, second, and third polynucleotides are complementary to a different target sequence or wherein two or more of the target-specific regions of the first, second, and third polynucleotides are complementary to the same target sequence, wherein the 5' region of the first polynucleotide is complementary to the 3' region of the third polynucleotide, wherein the 3' region of the first polynucleotide is complementary to the 5' region of the second polynucleotide, and wherein the 3' region of the second polynucleotide is complementary to the 5' region of the third polynucleotide, and wherein the three separate polynucleotides hybridize via their complementary 3' and 5' regions to form a polynucleotide complex with a first, second, and third single-stranded region, and a first, second, and third self-complementary region. The first polynucleotide, or the second polynucleotide or the third polynucleotide includes a nick that divides the nicked polynucleotide into two shorter polynucleotide portions. Typically, each of the shorter polynucleotide portions is from 5 to 12 nucleotides in length. Typically,

the nick is located within a target-specific region, although the nick can be located outside a target specific region.

In certain embodiments, the first, second, and/or third polynucleotide comprises about 8-30 or about 15-30 nucleotides. In certain embodiments, the first, second, and/or third polynucleotide comprises about 8-25 or about 17-25 nucleotides. In certain embodiments, one or more of the self-complementary regions comprises about 5-10 nucleotide pairs. In certain embodiments, one or more of the self-complementary regions comprises about 7-8 nucleotide pairs.

In certain embodiments, each of said first, second, and third target sequences are present in the same gene, cDNA, mRNA, or microRNA. In certain embodiments, at least two of said first, second, and third target sequences are present in different genes, cDNAs, mRNAs, or microRNAs.

In certain embodiments, all or a portion of the 5' and/or 3' region of each polynucleotide is also complementary to the target sequence for that polynucleotide. In certain embodiments, one or more of the self-complementary regions comprises a 3' overhang.

The present invention further includes a composition comprising a physiologically acceptable carrier and a polynucleotide of the present invention.

The present invention further includes a lipid-nucleic acid particle comprising a polynucleotide complex of the present invention encapsulated within a lipid particle.

The present invention further includes a pharmaceutical composition comprising a lipid-nucleic acid particle of the present invention and a pharmaceutically acceptable carrier or excipient.

In other embodiments, the present invention provides a method for reducing the expression of a gene, comprising introducing a polynucleotide complex of the present invention into a cell. In various embodiments, the cell is plant, animal, protozoan, viral, bacterial, or fungal. In one embodiment, the cell is mammalian.

In some embodiments, the polynucleotide complex is introduced directly into the cell, while in other embodiments, the polynucleotide complex is introduced extracellularly by a means sufficient to deliver the isolated polynucleotide into the cell.

In another embodiment, the present invention includes a method for treating a disease, comprising introducing a polynucleotide complex of the present invention into a cell, wherein overexpression of the targeted gene is associated with the disease. In one embodiment, the disease is a cancer.

The present invention further provides a method of treating an infection in a patient, comprising introducing into the patient a polynucleotide complex of the present invention, wherein the isolated polynucleotide mediates entry, replication, integration, transmission, or maintenance of an infective agent.

In yet another related embodiment, the present invention provides a method for identifying a function of a gene, comprising introducing into a cell a polynucleotide complex of the present invention, wherein the polynucleotide complex inhibits expression of the gene, and determining the effect of the introduction of the polynucleotide complex on a characteristic of the cell, thereby determining the function of the targeted gene. In one embodiment, the method is performed using high throughput screening.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 shows a polynucleotide complex of three separate polynucleotide molecules. (A) indicates the region comprising sequence complementary to a site on a target gene (hatched); (B) indicates the region comprising sequence complementary to a second site on the target gene or a site on a different gene (cross-hatched); (C) indicates the region comprising sequence complementary to a third site on the target gene or a site on a different gene (filled in black). The numbers 1, 2, and 3 indicate the 3' end of each oligonucleotide that guides gene silencing; (A) loads in the direction of 1, (B) in the direction 2, and (C) in the direction 3. The 3' and 5' regions of each molecule, which hybridize to each other to form their respective self-complementary or double-stranded regions, are indicated by connecting bars. Each polynucleotide comprises a two nucleotide 3' overhang.

Figure 2 shows a polynucleotide complex of three separate polynucleotide molecules wherein one of the polynucleotide molecules is nicked. (A) indicates the region comprising sequence complementary to a site on a target gene (hatched); (B) indicates the region comprising sequence complementary to a second site on the target gene or a site on a different gene (cross-hatched); (C) indicates the region comprising sequence complementary to a third site on the target gene or a site on a different gene (filled in black). The numbers 1, 2, and 3

indicate the 3' end of each oligonucleotide that guides gene silencing; (A) loads in the direction of 1, (B) in the direction 2, and (C) in the direction 3. The 3' and 5' regions of each molecule, which hybridize to each other to form their respective self-complementary or double-stranded regions, are indicated by connecting bars. Each polynucleotide comprises a two nucleotide 3' overhang. The polynucleotide that includes region (C) includes a nick (R) that divides region (C) into a first portion (PI) and a second portion (P2).

Figure 3 depicts a polynucleotide complex of the present invention having modified RNA bases. (L), (M), and (N) illustrate regions (defined by hashed lines) in which the Tm can be incrementally increased by the use of modified RNA *(e.g.,* 2'-0-methyl RNA or 2'-fluoro RNA instead of 2'-OH RNA) to preference the annealing and/or the silencing order of ends 1, 2 or 3. The polynucleotide complex includes a nick (R).

Figure 4 depicts a polynucleotide complex of the present invention wherein (O) illustrates a blunt-ended DNA strand that deactivates the silencing function of this strand. The polynucleotide complex includes a nick (R).

Figure 5 depicts a polynucleotide complex of the present invention wherein (P) illustrates an end that can be utilized for conjugation of a delivery chemistry, ligand, antibody, or other payload or targeting molecule. The oligonucleotide complex includes a nick (R).

DETAILED DESCRIPTION

The present invention provides novel compositions and methods for inhibiting the expression of a gene at two target sites, or for inhibiting the expression of multiple genes at one or two target sites, which sites are not of equivalent nucleotide sequences, in eukaryotes *in vivo* and *in* vitro. In particular, the present invention provides polynucleotide complexes comprising two, three, or more regions having sequences complementary to regions of one or more target genes, which are capable of targeting and reducing expression of the target genes. In various embodiments, the compositions and methods of the present invention may be used to inhibit the expression of a single target gene by targeting one site or multiple sites within the target gene or its expressed RNA. Alternatively, they may be used to target two or more different genes by targeting sites within two or more different genes or their expressed RNAs.

In certain embodiments, the present invention offers significant advantages over traditional siRNA molecules. First, when polynucleotide complexes of the present invention target two

regions within a single target gene, they are capable of achieving greater inhibition of gene expression from the target gene, as compared to an RNAi agent that targets only one region within a target gene. In addition, polynucleotide complexes of the present invention that target two different target genes may be used to inhibit the expression of multiple target genes associated with a disease or disorder using a single polynucleotide complex. Furthermore, polynucleotide complexes of the present invention do not require the additional non-targeting strand present in conventional double-stranded RNAi agents, so they do not have off-target effects caused by the non-targeting strand. Further, the presence of a nick in one of the polynucleotide strands inactivates the nicked strand and prevents it from silencing an off-target gene. Accordingly, the polynucleotide complexes of the present invention offer surprising advantages over polynucleotide inhibitors of the prior art, including antisense RNA and RNA interference molecules, including increased potency and increased effectiveness against one or more target genes.

The present invention is also based upon the recognition of the polynucleotide structure guiding a protein complex for cleavage using only one, or two of the guide strands, which are complementary to one or two distinct nucleic sequences of the target genes. This multivalent function results in a markedly broader and potent inhibition of a target gene or group of target genes than that of dsRNA, while utilizing many of the same endogenous mechanisms.

Certain embodiments of the present invention are also based upon the recognition of the polynucleotide structure directionally by presentation of the 3' overhangs and 5' phosphate resulting in a sense strand free complex, which contributes to greater specificity than that of dsRNA-based siRNA.

Given their effectiveness, the compositions of the present invention may be delivered to a cell or subject with an accompanying guarantee of specificity predicted by the single guide strand complementary to the target gene or multiple target genes.

Multivalent RNAs

The present invention includes polynucleotide complexes that comprise two or more targeting regions complementary to regions of one or more target genes (inclusive of coding and non-coding sequences), mRNAs, or microRNAs. The polynucleotide complexes of the present invention may be referred to as multivalent RNAs (mv-RNAs), since they comprise at least two targeting regions complementary to one or more regions of one or more target

genes. Accordingly, the compositions and methods of the present invention may be used to inhibit or reduce expression of one or more target genes, either by targeting , one, two or more regions within a single target gene, or by targeting one or more regions within two or more target genes.

The polynucleotide complexes of the present invention include a polynucleotide strand that includes a nick, although it is also within the scope of the present invention for a polynucleotide strand to include more than one nick *(e.g.,* 2, 3, 4, 5, 6, 7 or 8 nicks). In this context, a nick refers to the absence of a covalent bond *(e.g.,* a phosphodiester bond) linking adjacent subunits *(e.g.,* nucleotides) within a polynucleotide strand. Thus, a strand containing a single nick is composed of two adjacent portions, referred to as a first portion and a second portion, that are not directly, covalently, linked to each other, and that are held within the polynucleotide complex by non-covalent bonding *(e.g.,* hydrogen bonding) to at least one other polynucleotide strand as described more fully herein. The first and second portions each typically have a length of from 5 to 12 subunits *(e.g.,* where the subunits are nucleotides). The nick(s) inactivates the nicked strand so that the nicked strand cannot cause the RNAi-mediated suppression of a target gene. The nick(s) thereby permits construction and use of polynucleotide complexes having two functional strands, that target different sequences for RNAi-mediated suppression of gene expression, and a third non-functional strand that does not cause unwanted, off-target, suppression of gene expression.

In certain embodiments, polynucleotide complexes of the present invention comprise three or more separate oligonucleotides, each having a 5' and 3' end, with two or more of the oligonucleotides comprising a targeting region, which oligonucleotides hybridize to each other as described herein to form a complex. Each of the strands is referred to herein as a "guide strand." Each guide strand comprises regions complementary to other guide strands. Polynucleotide molecules of this aspect of the invention also include at least one nick.

In certain embodiments, the present invention provides polynucleotide complexes that comprise at least two guide strands which comprise regions that are complementary to different sequences within one or more target genes. In various embodiments, the polynucleotide complexes of the present invention comprise two, three or more separate polynucleotides each comprising one or more guide strands, which can hybridize to each other to form a complex.

Certain embodiments of the present invention are directed to polynucleotide complexes having at least three guide strands, two or more of which are partially or fully complementary to one or more target genes; and each having about 4 to about 12, about 5 to about 10, or preferably about 7 to about 8, nucleotides on either end that are complementary to each other *(i.e.,* complementary to a region of another guide strand), allowing the formation of a polynucleotide complex *(see, e.g.,* Figure 2). For example, each end of a guide strand may comprise nucleotides that are complementary to nucleotides at one end of another of the guide strands of the polynucleotide complex or molecule. Certain embodiments may include polynucleotide complexes that comprise 4, 5, 6 or more individual polynucleotide molecules or guide strands.

In certain embodiments, a polynucleotide complex of the present invention comprises at least three separate polynucleotides, which include: (1) a first polynucleotide comprising a target-specific region that is complementary to a first target sequence, a 5' region, and a 3' region; (2) a second polynucleotide comprising a target-specific region that is complementary to a second target sequence, a 5' region, and a 3' region; and (3) a third polynucleotide comprising either a null region or a target-specific region that is complementary to a third target specific, a 5' region, and a 3' region, wherein each of the target-specific regions of the first, second, and third polynucleotides are complementary to a different target sequence or wherein two or more of the target-specific regions of the first, second, and third polynucleotides are complementary to the same target sequence, wherein the 5' region of the first polynucleotide is complementary to the 3' region of the third polynucleotide, wherein the 3' region of the first polynucleotide is complementary to the 5' region of the second polynucleotide, and wherein the 3' region of the second polynucleotide is complementary to the 5' region of the third polynucleotide, and wherein the three separate polynucleotides hybridize via their complementary 3' and 5' regions to form a polynucleotide complex with a first, second, and third single-stranded region, and a first, second, and third self-complementary region. In the polynucleotide complexes of this aspect of the invention, the first polynucleotide, the second polynucleotide or the third polynucleotide includes at least one nick (typically a single nick) that divides the nicked polynucleotide into at least two shorter polynucleotides *(e.g.,* a first polynucleotide portion and a second polynucleotide portion).

As described above, in particular embodiments, a polynucleotide complex of the present invention comprises at least three separate oligonucleotides, each having a 5' end and a 3' end wherein one of the oligonucleotides is nicked. To facilitate understanding of the present invention, Figure 1 herein depicts a polynucleotide complex that lacks a nick in one of the constituent oligonucleotides. Thus, a region at the 5' end of the first oligonucleotide anneals to a region at the 3' end of the third oligonucleotide; a region at the 5' end of the third oligonucleotide anneals to a region at the 3' end of the second oligonucleotide; and a region at the 5' end of the second oligonucleotide anneals to a region at the 3' end of the first oligonucleotide. Each of the three oligonucleotides can target the same or different genes. Although the ability to target up to three separate sequences on three separate genes is often a desirable property of trivalent mv-RNA complexes, such as the complex shown in Figure 1, in some circumstances it is desirable to use a trimeric mv-RNA complex in which one of the strands is inactivated. Such partially inactivated complexes are desirable, for example, where two functional targeting strands are sufficient to target one or two genes, and designing a third, inactive, strand presents significant problems, such as the difficulty of ensuring that the third strand does not interact with an off-target sequence thereby causing an undesirable inactivation of a gene that is essential for normal cellular function.

Accordingly, Figure 2 herein depicts the same complex that is depicted in Figure 1, except that the complex depicted in Figure 2 includes a nick (R) in region (C) of one strand, thereby dividing region (C) into a first portion (PI) and a second portion (P2). The nick functionally inactivates the nicked strand which is no longer useful for targeting a gene or other nucleic acid molecule. Moreover, the nicked strand is highly unlikely to cause off-target gene silencing.

If additional oligonucleotides are present in the complex shown in Figure 2, then they anneal to other oligonucleotides of the complex in a similar manner. The regions at the ends of the oligonucleotides that anneal to each other may include the ultimate nucleotides at either or both the 5' and/or 3' ends. Where the regions of both the hybridizing 3' and 5' ends include the ultimate nucleotides of the oligonucleotides, the resulting double-stranded region is blunt-ended. In particular embodiments, the region at the 3' end that anneals does not include the ultimate and/or penultimate nucleotides, resulting in a double-stranded region having a one or two nucleotide 3' overhang.

Figure 3 depicts a polynucleotide complex of the present invention having modified RNA bases. (L), (M), and (N) illustrate regions (defined by hashed lines) in which the Tm can be incrementally increased by the use of modified RNA *(e.g.,* 2'-0-methyl RNA or 2'-fluoro RNA instead of 2'-OH RNA) to favor the annealing and/or the silencing order of ends 1, 2 or 3. The polynucleotide complex includes a nick (R).

Figure 4 depicts a polynucleotide complex of the present invention wherein (O) illustrates a blunt-ended DNA strand that deactivates the silencing function of this strand. The polynucleotide complex includes a nick (R).

Figure 5 depicts a polynucleotide complex of the present invention wherein (P) illustrates an end that can be utilized for conjugation of a delivery chemistry, ligand, antibody, or other payload or targeting molecule. The oligonucleotide complex includes a nick (R).

It will be understood that, in Figures 2-5 herein, the location of a nick between subunits of a polynucleotide is indicated by the end of a line extending from the circled letter R. Gaps may be shown between subunits of a polynucleotide, but such gaps do not represent nicks unless expressly identified as a nick by a circled letter R.

As used herein, polynucleotides complexes of the present invention include isolated polynucleotides comprising three single-stranded regions, at least two of which are complementary to two or more target sequences, each target sequence located within one or more target genes, and comprising at least two or three self-complementary regions interconnecting the 5' or 3' ends of the single-stranded regions, by forming a double-stranded region, such as a stem-loop structure. The polynucleotides may also be referred to herein as the oligonucleotides.

In certain embodiments, the polynucleotide complexes of the present invention comprise two or more regions of sequence complementary to a target gene. In particular embodiments, these regions are complementary to the same target genes or genes, while in other embodiments, they are complementary to two or more different target genes or genes.

The term "complementary" refers to nucleotide sequences that are fully or partially complementary to each other, according to standard base pairing rules. The term "partially complementary" refers to sequences that have less than full complementarity, but still have a

sufficient number of complementary nucleotide pairs to support binding or hybridization within the stretch of nucleotides under physiological conditions.

In particular embodiments, the region of a guide strand complementary to a target gene *(i.e.,* the targeting region) may comprise one or more nucleotide mismatches as compared to the target gene. Optionally, the mismatched nucleotide(s) in the guide strand may be substituted with an unlocked (UNA) nucleic acid or a phosphoramidite nucleic acid *(e.g.*, rSpacer, Glen Research , Sterling, VA, USA), to allow base-pairing, *e.g.,* Watson-Crick base pairing, of the mismatched nucleotide(s) to the target gene.

As used herein, the term "self-complementary" or "self-complementary region" may refer to a region of a first nucleotide molecule that binds to a region of a second or third nucleotide molecule to form a polynucleotide complex of the invention *(i.e.,* an RNAi polynucleotide complex), wherein the complex is capable of RNAi interference activity against two or more target sites.

In certain embodiments, a "self complementary region" comprises a "3' region" of a first polynucleotide molecule that is bound or hybridized to a "5' region" of a separate polynucleotide molecule, to form a polynucleotide complex. These 3' and '5 regions are typically defined in relation to their respective target-specific region, in that the 5' regions are on the 5' end of the target-specific region and the 3' regions are on the 3' end of the target specific region. In certain embodiments, one or both of these 3' and 5' regions not only hybridize to their corresponding 3' or 5' regions to form a self-complementary region, but may be designed to also contain full or partial complementarity their respective target sequence, thereby forming part of the target-specific region. In these embodiments, the target-specific region contains both a single-stranded region and self-complementary *(i.e.,* double-stranded) region.

In certain embodiments, these "self-complementary regions" comprise about 5-12 nucleotide pairs, preferably 5-10 or 7-8 nucleotide pairs, including all integers in between. Likewise, in certain embodiments, each 3' region or 5' region comprises about 5-12 nucleotides, preferably 5-10 or 7-8 nucleotides, including all integers in between.

The term "non-complementary" indicates that in a particular stretch of nucleotides, there are no nucleotides within that align with a target to form A-T(U) or G-C hybridizations. The term "semi-complementary" indicates that in a stretch of nucleotides, there is at least one

nucleotide pair that aligns with a target to form an A-T(U) or G-C hybridizations, but there are not a sufficient number of complementary nucleotide pairs to support binding within the stretch of nucleotides under physiological conditions.

The term "isolated" refers to a material that is at least partially free from components that normally accompany the material in the material's native state. Isolation connotes a degree of separation from an original source or surroundings. Isolated, as used herein, *e.g.,* related to DNA, refers to a polynucleotide that is substantially away from other coding or non-coding sequences, and that the DNA molecule can contain large portions of unrelated coding DNA, such as large chromosomal fragments or other functional genes or polypeptide coding regions. Of course, this refers to the DNA molecule as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

In various embodiments, a polynucleotide complex of the present invention comprises RNA, DNA, or peptide nucleic acids, or a combination of any or all of these types of molecules. In addition, a polynucleotide may comprise modified nucleic acids, or derivatives or analogs of nucleic acids. General examples of nucleic acid modifications include, but are not limited to, biotin labeling, fluorescent labeling, amino modifiers introducing a primary amine into the polynucleotide, phosphate groups, deoxyuridine, halogenated nucleosides, phosphorothioates, 2'-0-Methyl RNA analogs, chimeric RNA analogs, wobble groups, universal bases, and deoxyinosine.

A "subunit" of a polynucleotide or oligonucleotide refers to one nucleotide (or nucleotide analog) unit. The term may refer to the nucleotide unit with or without the attached intersubunit linkage, although, when referring to a "charged subunit", the charge typically resides within the intersubunit linkage *{e.g.,* a phosphate or phosphorothioate linkage or a cationic linkage). A given synthetic MV-RNA may utilize one or more different types of subunits and/or intersubunit linkages, mainly to alter its stability, Tm, RNase sensitivity, or other characteristics, as desired. For instance, certain embodiments may employ RNA subunits with one or more 2'-0-methyl RNA subunits.

The cyclic subunits of a polynucleotide or an oligonucleotide may be based on ribose or another pentose sugar or, in certain embodiments, alternate or modified groups. Examples of modified oligonucleotide backbones include, without limitation, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters,

methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, phosphorodiamidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Also contemplated are peptide nucleic acids (PNAs), locked nucleic acids (LNAs), 2'-0-methyl oligonucleotides (2'-OMe), 2'-methoxyethoxy oligonucleotides (MOE), among other oligonucleotides known in the art.

The purine or pyrimidine base pairing moiety is typically adenine, cytosine, guanine, uracil, thymine or inosine. Also included are bases such as pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2,4,6-trimel 15thoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines *(e.g.,* 5-methylcytidine), 5-alkyluridines *(e.g.,* ribothymidine), 5-halouridine *(e.g.,* 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines *(e.g.* 6-methyluridine), propyne, quesosine, 2-thiouridine, 4-thiouridine, wybutosine, wybutoxosine, 4-acetyltidine, 5-(carboxyhydroxymethyl)uridine, 5'-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluridine, β-D-galactosylqueosine, 1-methyladenosine, 1-methylinosine, 2,2-dimethylguanosine, 3-methylcytidine, 2-methyladenosine, 2-methylguanosine, N6-methyladenosine, 7-methylguanosine, 5-methoxyaminomethyl-2-thiouridine, 5-methylaminomethyluridine, 5-methylcarbonyhnethyluridine, 5-methyloxyuridine, 5-methyl-2-thiouridine, 2-methylthio-N6-isopentenyladenosine, β-D-mannosylqueosine, uridine-5-oxyacetic acid, 2-thiocytidine, threonine derivatives and others (Burgin *et al.,* 1996, Biochemistry, 35, 14090; Uhlman & Peyman, supra). By "modified bases" in this aspect is meant nucleotide bases other than adenine (A), guanine (G), cytosine (C), thymine (T), and uracil (U), as illustrated above; such bases can be used at any position in the antisense molecule. Persons skilled in the art will appreciate that depending on the uses or chemistries of the oligomers, Ts and Us are interchangeable. For instance, with other antisense chemistries such as 2'-0-methyl antisense oligonucleotides that are more RNA-like, the T bases may be shown as U.

As noted above, certain polynucleotides or oligonucleotides provided herein include one or more peptide nucleic acid (PNAs) subunits. Peptide nucleic acids (PNAs) are analogs of DNA in which the backbone is structurally homomorphous with a deoxyribose backbone,

consisting of N-(2-aminoethyl) glycine units to which pyrimidine or purine bases are attached. PNAs containing natural pyrimidine and purine bases hybridize to complementary oligonucleotides obeying Watson-Crick base-pairing rules, and mimic DNA in terms of base pair recognition (Egholm, Buchardt *et al.* 1993). The backbone of PNAs is formed by peptide bonds rather than phosphodiester bonds, making them well-suited for antisense applications (see structure below). A backbone made entirely of PNAs is uncharged, resulting in PNA/DNA or PNA/RNA duplexes that exhibit greater than normal thermal stability. PNAs are not recognized by nucleases or proteases.

PNAs may be produced synthetically using any technique known in the art. PNA is a DNA analog in which a polyamide backbone replaces the traditional phosphate ribose ring of DNA. Despite a radical structural change to the natural structure, PNA is capable of sequence-specific binding in a helix form to DNA or RNA. Characteristics of PNA include a high binding affinity to complementary DNA or RNA, a destabilizing effect caused by single-base mismatch, resistance to nucleases and proteases, hybridization with DNA or RNA independent of salt concentration and triplex formation with homopurine DNA. Panagene™ has developed its proprietary Bts PNA monomers (Bts; benzothiazole-2-sulfonyl group) and proprietary oligomerisation process. The PNA oligomerisation using Bts PNA monomers is composed of repetitive cycles of deprotection, coupling and capping. Panagene's patents to this technology include US 6969766, US 721 1668, US 7022851, US 7125994, US 7145006 and US 7179896. Representative United States 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 in Nielsen *et al.,* Science, 1991, 254, 1497.

Also included are "locked nucleic acid" subunits (LNAs). The structures of LNAs are known in the art: for example, Wengel, *et al.,* Chemical Communications (1998) 455; Tetrahedron (1998) 54, 3607, and Accounts of Chem. Research (1999) 32, 301); Obika, *et al,* Tetrahedron Letters (1997) 38, 8735; (1998) 39, 5401, and Bioorganic Medicinal Chemistry (2008)16, 9230.

Polynucleotides and oligonucleotides may incorporate one or more LNAs; in some cases, the compounds may be entirely composed of LNAs. Methods for the synthesis of individual LNA nucleoside subunits and their incorporation into oligonucleotides are known in the art: U.S. Patents 7,572,582; 7,569,575; 7,084,125; 7,060,809; 7,053,207; 7,034,133; 6,794,499;

and 6,670,461. Typical intersubunit linkers include phosphodiester and phosphorothioate moieties; alternatively, non-phosphorous containing linkers may be employed. One embodiment includes an LNA containing compound where each LNA subunit is separated by a RNA or a DNA subunit *(i.e.,* a deoxyribose nucleotide). Further exemplary compounds may be composed of alternating LNA and RNA or DNA subunits where the intersubunit linker is phosphorothioate.

Certain polynucleotides or oligonucleotides may comprise morpholino-based subunits bearing base-pairing moieties, joined by uncharged or substantially uncharged linkages. The terms "morpholino oligomer" or "PMO" (phosphoramidate- or phosphorodiamidate morpholino oligomer) refer to an oligonucleotide analog composed of morpholino subunit structures, where (i) the structures are linked together by phosphorus-containing linkages, one to three atoms long, preferably two atoms long, and preferably uncharged or cationic, joining the morpholino nitrogen of one subunit to a 5' exocyclic carbon of an adjacent subunit, and (ii) each morpholino ring bears a purine or pyrimidine or an equivalent base-pairing moiety effective to bind, by base specific hydrogen bonding, to a base in a polynucleotide.

Variations can be made to this linkage as long as they do not interfere with binding or activity. For example, the oxygen attached to phosphorus may be substituted with sulfur (thiophosphorodiamidate). The 5' oxygen may be substituted with amino or lower alkyl substituted amino. The pendant nitrogen attached to phosphorus may be unsubstituted, monosubstituted, or disubstituted with (optionally substituted) lower alkyl. The purine or pyrimidine base pairing moiety is typically adenine, cytosine, guanine, uracil, thymine or inosine. The synthesis, structures, and binding characteristics of morpholino oligomers are detailed in U.S. Patent Nos. 5,698,685, 5,217,866, 5,142,047, 5,034,506, 5,166,315, 5,521,063, and 5,506,337, and PCT Appn. Nos. PCT/US07/1 1435 (cationic linkages) and US08/012804 (improved synthesis), all of which are incorporated herein by reference.

In one aspect of the invention, MV-RNA comprise at least one ligand tethered to an altered or non-natural nucleobase. Included are payload molecules and targeting molecules. A large number of compounds can function as the altered base. The structure of the altered base is important to the extent that the altered base should not substantially prevent binding of the oligonucleotide to its target, *e.g.,* mRNA. In certain embodiments, the altered base is difluorotolyl, nitropyrrolyl, nitroimidazolyl, nitroindolyl, napthalenyl, anthrancenyl, pyridinyl, quinolinyl, pyrenyl, or the divalent radical of any one of the non-natural

nucleobases described herein. In certain embodiments, the non-natural nucleobase is difluorotolyl, nitropyrrolyl, or nitroimidazolyl. In certain embodiments, the non-natural nucleobase is difluorotolyl.

A wide variety of ligands are known in the art and are amenable to the present invention. For example, the ligand can be a steroid, bile acid, lipid, folic acid, pyridoxal, B12, riboflavin, biotin, aromatic compound, polycyclic compound, crown ether, intercalator, cleaver molecule, protein-binding agent, or carbohydrate. In certain embodiments, the ligand is a steroid or aromatic compound. In certain instances, the ligand is cholesteryl.

In other embodiments, the polynucleotide or oligonucleotide is tethered to a ligand for the purposes of improving cellular targeting and uptake. For example, an MV-RNA agent may be tethered to an antibody, or antigen binding fragment thereof. As an additional example, an MV-RNA agent may be tethered to a specific ligand binding molecule, such as a polypeptide or polypeptide fragment that specifically binds a particular cell-surface receptor, or that more generally enhances cellular uptake, such as an arginine-rich peptide.

The term "analog" as used herein refers to a molecule, compound, or composition that retains the same structure and/or function *(e.g.,* binding to a target) as a polynucleotide herein. Examples of analogs include peptidomimetic and small and large organic or inorganic compounds.

The term "derivative" or "variant" as used herein refers to a polynucleotide that differs from a naturally occurring polynucleotide *(e.g.,* target gene sequence) by one or more nucleic acid deletions, additions, substitutions or side-chain modifications. In certain embodiments, variants have at least 70%, at least 80%> at least 90%>, at least 95%>, or at least 99%> sequence identity to a region of a target gene sequence. Thus, for example, in certain embodiments, an oligonucleotide of the present invention comprises a region that is complementary to a variant of a target gene sequence.

Polynucleotide complexes of the present invention comprise a sequence region, or two or more sequence regions, each of which is complementary, and in particular embodiments completely complementary, to a region of a target gene or polynucleotide sequences (or a variant thereof). In particular embodiments, a target gene is a mammalian gene, *e.g.,* a human gene, or a gene of a microorganism infecting a mammal, such as a virus. In certain embodiments, a target gene is a therapeutic target. For example, a target gene may be a gene

whose expression or overexpression is associated with a human disease or disorder. This may be a mutant gene or a wild type or normal gene. A variety of therapeutic target genes have been identified, and any of these may be targeted by polynucleotide complexes and molecules of the present invention. Therapeutic target genes include, but are not limited to, oncogenes, growth factor genes, translocations associated with disease such as leukemias, inflammatory protein genes, transcription factor genes, growth factor receptor genes, anti-apoptotic genes, interleukins, sodium channel genes, potassium channel genes, such as, but not limited to the following genes or genes encoding the following proteins: apolipoprotein B (ApoB), apolipoprotein B-100 (ApoB-100), bcl family members, including bcl-2 and bcl-x, MLL-AF4, Huntington gene, AML-MT68 fusion gene, IKK-B, Ahal, PCSK9, Eg5, transforming growth factor beta (TGFbeta), Navl.8, RhoA, HIF-lalpha, Nogo-L, Nogo-R, toll-like receptor 9 (TLR9), vascular endothelial growth factor (VEGF), SNCA, beta-catenin, CCR5, c-myc, p53, interleukin-1, interleukin 2, interleukin-12, interleukin-6, interleukin-17a (IL-17a), interleukin- 17f (IL-17f), Osteopontin (OPN) gene, psoriasis gene, and tumor necrosis factor gene.

In particular embodiments, polynucleotide complexes of the present invention comprise guide strands or target-specific regions targeting two or more genes, *e.g.,* two or more genes associated with a particular disease or disorder. For example, they may include guide strands complementary to interleukin-1 gene or mRNA and tumor necrosis factor gene or mRNA; complementary to interleukin-1 gene or mRNA and interleukin-12 gene or mRNA; or complementary to interleukin-1 gene or mRNA, interleukin-12 gene or mRNA and tumor necrosis factor gene or mRNA, for treatment of rheumatoid arthritis. In one embodiment, they include guide strands complementary to osteopontin gene or mRNA and TNF gene or mRNA.

Other examples of therapeutic target genes include genes and mRNAs encoding viral proteins, such as human immunodeficiency virus (HIV) proteins, HTLV virus proteins, hepatitis C virus (HCV) proteins, Ebola virus proteins, JC virus proteins, herpes virus proteins, human polyoma virus proteins, influenza virus proteins, and Rous sarcoma virus proteins. In particular embodiments, polynucleotide complexes of the present invention include guide strands complementary to two or more genes or mRNAs expressed by a particular virus, *e.g.,* two or more HIV protein genes or two or more herpes virus protein genes. In other embodiments, they include guide strands having complementary to two or

more herpes simplex virus genes or mRNAs, *e.g.,* the UL29 gene or mRNA and the Nectin-1 gene or mRNA of HSV-2, to reduce HSV-2 expression, replication or activity. In one embodiment, the polynucleotide complexes having regions targeting two or more HSV-2 genes or mRNAs are present in a formulation for topical delivery.

In particular embodiments, polynucleotide complexes of the present invention comprise one, two, three or more guide strands or target-specific regions that target an apolipoprotein B (ApoB) gene or mRNA, *e.g.,* the human ApoB gene or mRNA or the mouse ApoB gene or mRNA.

In certain embodiments, polynucleotide complexes of the present invention comprise one, two, three or more guide strands or regions that target HIV genes. In particular embodiments, they target one, two, three or more HIV genes or mRNAs encoding one or more proteins selected from HIV gag, HIV tat, HIV env, HIV gag-pol, HIV vif, and HIV nef proteins.

In certain embodiments, selection of a sequence region complementary to a target gene (or gene) is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding energy, and relative stability and cell specificity. Such sequences may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce structural integrity of the polynucleotide or prohibit specific binding to the target gene in a host cell.

Preferred target regions of the target gene or mRNA may include those regions at or near the AUG translation initiation codon and those sequences that are substantially complementary to 5' regions of the gene or mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software and/or the BLASTN 2.0.5 algorithm software (Altschul *et al.,* Nucleic Acids Res. 1997, 25(17):3389-402) or Oligoengine Workstation 2.0.

In one embodiment, target sites are preferentially not located within the 5' and 3' untranslated regions (UTRs) or regions near the start codon (within approximately 75 bases), since proteins that bind regulatory regions may interfere with the binding of the polynucleotide. In addition, potential target sites may be compared to an appropriate genome database, such as BLASTN 2.0.5, available on the NCBI server at www.ncbi.nlm, and potential target sequences with significant homology to other coding sequences eliminated.

In another embodiment, the target sites are located within the 5' or 3' untranslated region (UTRs). In addition, the self-complementary region of the polynucleotide may be composed of a particular sequence found in the gene of the target.

The target gene may be of any species, including, for example, plant, animal *(e.g.* mammalian), protozoan, viral *(e.g.,* HIV), bacterial or fungal.

As noted above, the target gene sequence and the complementary region of the polynucleotide may be complete complements of each other, or they may be less than completely complementary, as long as the strands hybridize to each other under physiological conditions.

The polynucleotide complexes of the present invention comprise at least one, two, or three regions complementary to one or more target genes. Typically, the region complementary to a target gene is 15 to 17 to 24 nucleotides in length, including integer values within these ranges. This region may be at least 16 nucleotides in length, at least 17 nucleotides in length, at least 20 nucleotides in length, at least 24 nucleotides in length, between 15 and 24 nucleotides in length, between 16 and 24 nucleotides in length, or between 17 and 24 nucleotides in length, inclusive of the end values, including any integer value within these ranges.

In one embodiment of the invention, the level of inhibition of target gene expression *(i.e.,* gene expression) is at least 90%, at least 95%, at least 98%>, and at least 99% or is almost 100%, and hence the cell or organism will in effect have the phenotype equivalent to a so-called "knock out" of a gene. However, in some embodiments, it may be preferred to achieve only partial inhibition so that the phenotype is equivalent to a so-called "knockdown" of the gene. This method of knocking down gene expression can be used therapeutically or for research *(e.g.,* to generate models of disease states, to examine the function of a gene, to assess whether an agent acts on a gene, to validate targets for drug discovery).

The polynucleotide complexes of the invention can be used to target and reduce or inhibit expression of genes (inclusive of coding and non-coding sequences), cDNAs, mRNAs, or microRNAs. In particular embodiments, their guide strands or targeting regions bind to mRNAs or microRNAs. Targeted sequences may be present in genes, cDNAs, mRNAs, or microRNAs.

The invention further provides arrays of the polynucleotide of the invention, including microarrays. Microarrays are miniaturized devices typically with dimensions in the micrometer to millimeter range for performing chemical and biochemical reactions and are particularly suited for embodiments of the invention. Arrays may be constructed via microelectronic and/or microfabrication using essentially any and all techniques known and available in the semiconductor industry and/or in the biochemistry industry, provided that such techniques are amenable to and compatible with the deposition and/or screening of polynucleotide sequences.

Microarrays of the invention are particularly desirable for high throughput analysis of multiple polynucleotides. A microarray typically is constructed with discrete region or spots that comprise the polynucleotide of the present invention, each spot comprising one or more the polynucleotide, preferably at positionally addressable locations on the array surface. Arrays of the invention may be prepared by any method available in the art. For example, the light-directed chemical synthesis process developed by Affymetrix (see, U.S. Pat. Nos. 5,445,934 and 5,856,174) may be used to synthesize biomolecules on chip surfaces by combining solid-phase photochemical synthesis with photolithographic fabrication techniques. The chemical deposition approach developed by Incyte Pharmaceutical uses pre-synthesized cDNA probes for directed deposition onto chip surfaces (see, *e.g.,* U.S. Pat. No. 5,874,554).

The three or more guide strands of a polynucleotide complex of the present invention may be individually chemically synthesized and annealed to produce the polynucleotide complex.

Methods of Regulating Gene Expression

The polynucleotides of the invention may be used for a variety of purposes, all generally related to their ability to inhibit or reduce expression of one or more target genes. Accordingly, the invention provides methods of reducing expression of one or more target genes comprising introducing a polynucleotide complex of the present invention into a cell comprising said one or more target genes. In particular embodiments, the polynucleotide complex comprises one or more guide strands that collectively target the one or more target genes. In one embodiment, a polynucleotide of the invention is introduced into a cell that contains a target gene or a homolog, variant or ortholog thereof, targeted by either one or two of the guide strands or targeting regions.

In addition, the polynucleotides of the present invention may be used to reduce expression indirectly. For example, a polynucleotide complex of the present invention may be used to reduce expression of a transactivator that drives expression of a second gene *(i.e.,* the target gene), thereby reducing expression of the second gene. Similarly, a polynucleotide may be used to increase expression indirectly. For example, a polynucleotide complex of the present invention may be used to reduce expression of a transcriptional repressor that inhibits expression of a second gene, thereby increasing expression of the second gene.

In various embodiments, a target gene is a gene derived from the cell into which a polynucleotide is to be introduced, an endogenous gene, an exogenous gene, a transgene, or a gene of a pathogen that is present in the cell after transfection thereof. Depending on the particular target gene and the amount of the polynucleotide delivered into the cell, the method of this invention may cause partial or complete inhibition of the expression of the target gene. The cell containing the target gene may be derived from or contained in any organism *(e.g.,* plant, animal, protozoan, virus, bacterium, or fungus). As used herein, "target genes" include genes, mRNAs, and microRNAs.

Inhibition of the expression of the target gene can be verified by means including, but not limited to, observing or detecting an absence or observable decrease in the level of protein encoded by a target gene, an absence or observable decrease in the level of a gene product expressed from a target gene *(e.g.,* mRNAO, and/or a phenotype associated with expression of the gene, using techniques known to a person skilled in the field of the present invention.

Examples of cell characteristics that may be examined to determine the effect caused by introduction of a polynucleotide complex of the present invention include, cell growth, apoptosis, cell cycle characteristics, cellular differentiation, and morphology.

A polynucleotide complex of the present invention may be directly introduced to the cell *(i.e.,* intracellularly), or introduced extracellularly into a cavity or interstitial space of an organism, *e.g.,* a mammal, into the circulation of an organism, introduced orally, introduced by bathing an organism in a solution containing the polynucleotide, or by some other means sufficient to deliver the polynucleotide into the cell.

In addition, a vector engineered to express a polynucleotide may be introduced into a cell, wherein the vector expresses the polynucleotide, thereby introducing it into the cell. Methods of transferring an expression vector into a cell are widely known and available in the art,

including, *e.g.,* transfection, lipofection, scrape-loading, electroporation, microinjection, infection, gene gun, and retrotransposition. Generally, a suitable method of introducing a vector into a cell is readily determined by one of skill in the art based upon the type of vector and the type of cell, and teachings widely available in the art. Infective agents may be introduced by a variety of means readily available in the art, including, *e.g.,* nasal inhalation.

Methods of inhibiting gene expression using the oligonucleotides of the invention may be combined with other knockdown and knockout methods, *e.g.,* gene targeting, antisense RNA, ribozymes, double-stranded RNA *(e.g.,* shRNA and siRNA) to further reduce expression of a target gene.

In different embodiments, target cells of the invention are primary cells, cell lines, immortalized cells, or transformed cells. A target cell may be a somatic cell or a germ cell. The target cell may be a non-dividing cell, such as a neuron, or it may be capable of proliferating *in vitro* in suitable cell culture conditions. Target cells may be normal cells, or they may be diseased cells, including those containing a known genetic mutation. Eukaryotic target cells of the invention include mammalian cells, such as, for example, a human cell, a murine cell, a rodent cell, and a primate cell. In one embodiment, a target cell of the invention is a stem cell, which includes, for example, an embryonic stem cell, such as a murine embryonic stem cell.

The polynucleotide complexes and methods of the present invention may be used to treat any of a wide variety of diseases or disorders, including, but not limited to, inflammatory diseases, cardiovascular diseases, nervous system diseases, tumors, demyelinating diseases, digestive system diseases, endocrine system diseases, reproductive system diseases, hemic and lymphatic diseases, immunological diseases, mental disorders, musculoskeletal diseases, neurological diseases, neuromuscular diseases, metabolic diseases, sexually transmitted diseases, skin and connective tissue diseases, urological diseases, and infections.

In certain embodiments, the methods are practiced on an animal, in particular embodiments, a mammal, and in certain embodiments, a human.

Accordingly, in one embodiment, the present invention includes methods of using a polynucleotide complex of the present invention for the treatment or prevention of a disease associated with gene deregulation, overexpression, or mutation. For example, a polynucleotide complex of the present invention may be introduced into a cancerous cell or

tumor and thereby inhibit expression of a gene required for or associated with maintenance of the carcinogenic/tumorigenic phenotype. To prevent a disease or other pathology, a target gene may be selected that is, *e.g.,* required for initiation or maintenance of a disease/pathology. Treatment may include amelioration of any symptom associated with the disease or clinical indication associated with the pathology.

In addition, the polynucleotides of the present invention are used to treat diseases or disorders associated with gene mutation. In one embodiment, a polynucleotide is used to modulate expression of a mutated gene or allele. In such embodiments, the mutated gene is a target of the polynucleotide complex, which will comprise a region complementary to a region of the mutated gene. This region may include the mutation, but it is not required, as another region of the gene may also be targeted, resulting in decreased expression of the mutant gene or gene. In certain embodiments, this region comprises the mutation, and, in related embodiments, the polynucleotide complex specifically inhibits expression of the mutant gene or gene but not the wild type gene or gene. Such a polynucleotide is particularly useful in situations, *e.g.,* where one allele is mutated but another is not. However, in other embodiments, this sequence would not necessarily comprise the mutation and may, therefore, comprise only wild-type sequence. Such a polynucleotide is particularly useful in situations, *e.g.,* where all alleles are mutated. A variety of diseases and disorders are known in the art to be associated with or caused by gene mutation, and the invention encompasses the treatment of any such disease or disorder with a the polynucleotide.

In certain embodiments, a gene of a pathogen is targeted for inhibition. For example, the gene could cause immunosuppression of the host directly or be essential for replication of the pathogen, transmission of the pathogen, or maintenance of the infection. In addition, the target gene may be a pathogen gene or host gene responsible for entry of a pathogen into its host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of an infection in the host, or assembly of the next generation of pathogen. Methods of prophylaxis *{i.e.,* prevention or decreased risk of infection), as well as reduction in the frequency or severity of symptoms associated with infection, are included in the present invention. For example, cells at risk for infection by a pathogen or already infected cells, particularly human immunodeficiency virus (HIV) infections, may be targeted for treatment by introduction of a the polynucleotide according to the invention. Thus, in one embodiment, polynucleotide complexes of the present invention

that target one or more HIV proteins are used to treat or inhibit HIV infection or acquired immune deficiency syndrome (AIDS).

In other specific embodiments, the present invention is used for the treatment or development of treatments for cancers of any type. Examples of tumors that can be treated using the methods described herein include, but are not limited to, neuroblastomas, myelomas, prostate cancers, small cell lung cancer, colon cancer, ovarian cancer, non-small cell lung cancer, brain tumors, breast cancer, leukemias, lymphomas, and others.

In one embodiment, polynucleotide complexes of the present invention that target apolipoprotein B (apoB) are used to treat, reduce, or inhibit atherosclerosis or heart disease. ApoB is the primary apolipoprotein of low-density lipoproteins (LDLs), which is responsible for carrying cholesterol to tissues. ApoB on the LDL particle acts as a ligand for LDL receptors, and high levels of ApoB can lead to plaques that cause vascular disease (atherosclerosis), leading to heart disease.

The polynucleotide complexes may be introduced into cells *in vitro* or *ex vivo* and then subsequently placed into an animal to affect therapy, or they may be directly introduced to a patient by *in vivo* administration. Thus, the invention provides methods of gene therapy, in certain embodiments. Compositions of the invention may be administered to a patient in any of a number of ways, including parenteral, intravenous, systemic, local, topical, oral, intratumoral, intramuscular, subcutaneous, intraperitoneal, inhalation, or any such method of delivery. In one embodiment, the compositions are administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. In a specific embodiment, the liposomal compositions are administered by intravenous infusion or intraperitoneally by a bolus injection.

Lipid Particles

The polynucleotide complexes of the invention can, for example, be delivered to a living subject *{e.g.,* a human being) using lipid particles wherein the polynucleotide complexes are enclosed within the lipid particles. The lipid particles thereby protect the polynucleotide complexes from chemical or enzymatic degradation, and also facilitate transport of the polynucleotide complexes to a tissue or organ of interest.

The lipid particles typically include one or more of the cationic (amino) lipids or salts thereof. In some embodiments, the lipid particles of the invention further comprise one or more non-cationic lipids. In other embodiments, the lipid particles further comprise one or more conjugated lipids capable of reducing or inhibiting particle aggregation.

Lipid particles include, but are not limited to, lipid vesicles such as liposomes. As used herein, a lipid vesicle includes a structure having lipid-containing membranes enclosing an aqueous interior. In particular embodiments, lipid vesicles are used to encapsulate nucleic acids within the lipid vesicles. In other embodiments, lipid vesicles are complexed with nucleic acids to form lipoplexes.

The lipid particles used in the practice of the present invention typically comprise a polynucleotide complex of the present invention, a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles. In some embodiments, the polynucleotide complex is fully encapsulated within the lipid portion of the lipid particle such that the polynucleotide complex in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.,* by a nuclease or protease. In other embodiments, the lipid particles described herein are substantially non-toxic to mammals such as humans. The lipid particles typically have a mean diameter of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm. The lipid particles of the invention also typically have a lipid:polynucleotide complex ratio (mass/mass ratio) of from about 1:1 to about 100:1, from about 1:1 to about 50:1, from about 2:1 to about 25:1, from about 3:1 to about 20:1, from about 5:1 to about 15:1, or from about 5:1 to about 10:1.

In preferred embodiments, the lipid particles of the invention are serum-stable nucleic acid-lipid particles (LNP) which comprise a polynucleotide complex of the present invention, a cationic lipid, a non-cationic lipid *{e.g.,* mixtures of one or more phospholipids and cholesterol), and a conjugated lipid that inhibits aggregation of the particles *{e.g.,* one or more PEG-lipid and/or POZ-lipid conjugates). The LNP may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more unmodified and/or modified polynucleotide complex. Nucleic acid-lipid particles and their method of preparation are described in, *e.g.,* U.S. Patent Nos. 5,753,613; 5,785,992; 5,705,385; 5,976,567; 5,981,501; 6,1 10,745; and 6,320,017; and PCT Publication No. WO 96/40964, the disclosures of which are each herein incorporated by reference in their entirety for all purposes.

In the nucleic acid-lipid particles of the invention, the nucleic acid may be fully encapsulated within the lipid portion of the particle, thereby protecting the nucleic acid from nuclease degradation. In preferred embodiments, a LNP comprising a polynucleotide complex is fully encapsulated within the lipid portion of the particle, thereby protecting the nucleic acid from nuclease degradation. In certain instances, the polynucleotide complex in the LNP is not substantially degraded after exposure of the particle to a nuclease at 37°C for at least about 20, 30, 45, or 60 minutes. In certain other instances, the polynucleotide complex in the LNP is not substantially degraded after incubation of the particle in serum at 37°C for at least about 30, 45, or 60 minutes or at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, or 36 hours. In other embodiments, the polynucleotide complex is complexed with the lipid portion of the particle. One of the benefits of the formulations of the present invention is that the nucleic acid-lipid particle compositions are substantially non-toxic to mammals such as humans.

The term "fully encapsulated" indicates that the nucleic acid *(e.g.,* polynucleotide complex) in the nucleic acid-lipid particle is not significantly degraded after exposure to serum or a nuclease assay that would significantly degrade free DNA or RNA. In a fully encapsulated system, preferably less than about 25% of the nucleic acid in the particle is degraded in a treatment that would normally degrade 100% of free nucleic acid, more preferably less than about 10%, and most preferably less than about 5% of the nucleic acid in the particle is degraded. "Fully encapsulated" also indicates that the nucleic acid-lipid particles are serum-stable, that is, that they do not rapidly decompose into their component parts upon *in vivo* administration.

In the context of nucleic acids, full encapsulation may be determined by performing a membrane-impermeable fluorescent dye exclusion assay, which uses a dye that has enhanced fluorescence when associated with nucleic acid. Specific dyes such as OliGreen® and RiboGreen® (Invitrogen Corp.; Carlsbad, CA) are available for the quantitative determination of plasmid DNA, single-stranded deoxyribonucleotides, and/or single- or double-stranded ribonucleotides. Encapsulation is determined by adding the dye to a liposomal formulation, measuring the resulting fluorescence, and comparing it to the fluorescence observed upon addition of a small amount of nonionic detergent. Detergent-mediated disruption of the liposomal bilayer releases the encapsulated nucleic acid, allowing it to interact with the membrane-impermeable dye. Nucleic acid encapsulation may be calculated as $E = (I_0 - I)/I_0$,

where I and I_0 refer to the fluorescence intensities before and after the addition of detergent *(see,* Wheeler *et al, Gene Ther.*, 6:271-281 (1999)).

In other embodiments, the present invention provides a nucleic acid-lipid particle *(e.g.*, LNP) composition comprising a plurality of nucleic acid-lipid particles.

In some instances, the LNP composition comprises nucleic acid *(e.g.,* polynucleotide complex of the invention) that is fully encapsulated within the lipid portion of the particles, such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% (or any fraction thereof or range therein) of the particles have the nucleic acid encapsulated therein.

In other instances, the LNP composition comprises nucleic acid *(e.g.,* polynucleotide complex) that is fully encapsulated within the lipid portion of the particles, such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% (or any fraction thereof or range therein) of the input nucleic acid is encapsulated in the particles.

Depending on the intended use of the lipid particles of the invention, the proportions of the components can be varied and the delivery efficiency of a particular formulation can be measured using, *e.g.,* an endosomal release parameter (ERP) assay.

In particular embodiments, the present invention provides a lipid particle *(e.g.,* LNP) composition comprising a plurality of lipid particles described herein and an antioxidant. In certain instances, the antioxidant in the lipid particle composition reduces, prevents, and/or inhibits the degradation of a cationic lipid present in the lipid particle. The antioxidant in the lipid particle composition reduces, prevents, and/or inhibits the degradation of the nucleic acid payload, *e.g.,* by reducing, preventing, and/or inhibiting the formation of adducts between the nucleic acid and the cationic lipid. Non-limiting examples of antioxidants include hydrophilic antioxidants such as chelating agents *(e.g.,* metal chelators such as ethylenediaminetetraacetic acid (EDTA), citrate, and the like), lipophilic antioxidants *(e.g.,* vitamin E isomers, polyphenols, and the like), salts thereof; and mixtures thereof. If needed, the antioxidant is typically present in an amount sufficient to prevent, inhibit, and/or reduce the degradation of the cationic lipid and/or active agent present in the particle, *e.g.,* at least about 20 mM EDTA or a salt thereof, or at least about 100 mM citrate or a salt thereof. An antioxidant such as EDTA and/or citrate may be included at any step or at multiple steps in the lipid particle formation process described in Section VI *(e.g.,* prior to, during, and/or after lipid particle formation).

Additional embodiments related to methods of preventing the degradation of cationic lipids and/or active agents *(e.g.,* therapeutic nucleic acids) present in lipid particles are described in International Patent Application No. PCT/CA2010/001919, entitled "SNALP Formulations Containing Antioxidants," filed December 1, 2010, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

Cationic Lipids

Examples of cationic lipids or salts thereof which may also be included in the lipid particles of the present invention include, but are not limited to, 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), l,2-di-Y-linolenyloxy-*N,N* -dimethylaminopropane (γ-DLenDMA), 1,2-dilinoleyloxy-(N,N-dimethyl)-butyl-4-amine (C2-DLinDMA), 1,2-dilinoleoyloxy-(N,N-dimethyl)-butyl-4-amine (C2-DLinDAP), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[l ,3]-dioxolane (DLin-K-C2-DMA; also known as "XTC2" or "C2K"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[l,3]-dioxolane (DLin-K-C3-DMA; "C3K"), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[l,3]-dioxolane (DLin-K-C4-DMA; "C4K"), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-

[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 2,2-dioleoyl-4-dimethylaminomethyl-[l,3]-dioxolane (DO-K-DMA), 2,2-distearoyl-4-dimethylaminomethyl-[1,3]-dioxolane (DS-K-DMA), 2,2-dilinoleyl-4-N-morpholino-[l,3]-dioxolane (DLin-K-MA), 2,2-Dilinoleyl-4-trimethylamino-[l,3]-dioxolane chloride (DLin-K-TMA.Cl), 2,2-dilinoleyl-4,5-bis(dimethylaminomethyl)-[l,3]-dioxolane (DLin-K^2-DMA), 2,2-dilinoleyl-4-methylpiperzine-[1,3]-dioxolane (D-Lin-K-N-methylpiperzine), (6Z,9Z,28Z,3 lZ)-heptatriaconta-6,9,28,3 l-tetraen-19-yl 4-(dimethylamino) butanoate (DLin-M-C3-DMA; "MC3"), dilinoleylmethyl-3-dimethylaminopropionate (DLin-M-C2-DMA; also known as DLin-M-K-DMA or DLin-M-DMA), 1,2-dioeylcarbamoyloxy-3-dimethylaminopropane (DO-C-DAP), l,2-dimyristoleoyl-3-dimethylaminopropane (DMDAP), l,2-dioleoyl-3-trimethylaminopropane chloride (DOTAP.C1), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), l,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), l,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), l-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), l,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), l,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), l,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)- 1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-l,2-propanedio (DOAP), l,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-l-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy- 1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), l,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(l-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(l-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 -(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Choi), N-(l,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl- 1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), analogs thereof, and mixtures thereof.

Additional cationic lipids or salts thereof which may be present in the lipid particles described herein include novel cationic lipids such as CP-LenMC3, CP-y-LenMC3, CP-MC3, CP-DLen-C2K-DMA, CP-yDLen-C2K-DMA, CP-C2K-DMA, CP-DODMA, CP-DPetroDMA, CP-DLinDMA, CP-DLenDMA, CP-yDLenDMA, analogs thereof, and combinations thereof. Additional cationic lipids or salts thereof which may be present in the lipid particles described herein include MC3 analogs such as LenMC3, y-LenMC3, MC3MC, MC2C, MC2MC, MC3 Thioester, MC3 Ether, MC4 Ether, MC3 Alkyne, MC3 Amide, Pan-MC3, Pan-MC4, Pan-MC5, and combinations thereof. Additional cationic lipids or salts thereof which may be present in the lipid particles described herein include the novel cationic lipids described in International Patent Application No. PCT/CA20 10/00 1029, entitled "Improved Cationic Lipids and Methods for the Delivery of Nucleic Acids," filed June 30, 2010. Additional cationic lipids or salts thereof which may be present in the lipid particles described herein include the cationic lipids described in U.S. Patent Publication No. 20090023673. The disclosures of each of these patent documents are herein incorporated by reference in their entirety for all purposes.

In some embodiments, the additional cationic lipid forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the additional cationic lipid is the oxalate *(e.g.,* hemioxalate) salt thereof, which is preferably a crystalline salt.

The synthesis of cationic lipids such as DLinDMA and DLenDMA, as well as additional cationic lipids, is described in U.S. Patent Publication No. 20060083780, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

The synthesis of cationic lipids such as γ-DLenDMA, C2-DLinDMA and C2-DLinDAP, as well as additional cationic lipids, is described in International Patent Application No. PCT/CA20 10/00 1029, entitled "Improved Cationic Lipids and Methods for the Delivery of Nucleic Acids," filed June 30, 2010, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

The synthesis of cationic lipids such as DLin-K-DMA, as well as additional cationic lipids, is described in PCT Publication No. WO 09/086558, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

The synthesis of cationic lipids such as DLin-K-C2-DMA, DLin-K-C3-DMA, DLin-K-C4-DMA, DLin-K6-DMA, DLin-K-MPZ, DO-K-DMA, DS-K-DMA, DLin-K-MA, DLin-K-

TMA.C1, DLin-K^2-DMA, D-Lin-K-N-methylpiperzine, DLin-M-C2-DMA, DO-C-DAP, DMDAP, and DOTAP.C1, as well as additional cationic lipids, is described in PCT Publication No. WO 2010/042877, entitled "Improved Amino Lipids and Methods for the Delivery of Nucleic Acids," filed October 9, 2009, the disclosure of which is incorporated herein by reference in its entirety for all purposes.

The synthesis of cationic lipids such as DLin-C-DAP, DLinDAC, DLinMA, DLinDAP, DLin-S-DMA, DLin-2-DMAP, DLinTMA.Cl, DLinTAP.Cl, DLinMPZ, DLinAP, DOAP, and DLin-EG-DMA, as well as additional cationic lipids, is described in PCT Publication No. WO 09/086558, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

The synthesis of cationic lipids such as CLinDMA, as well as additional cationic lipids, is described in U.S. Patent Publication No. 20060240554, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

The synthesis of a number of other cationic lipids and related analogs has been described in U.S. Patent Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390, the disclosures of which are each herein incorporated by reference in their entirety for all purposes. Additionally, a number of commercial preparations of cationic lipids can be used, such as, *e.g.,* LIPOFECTIN® (including DOTMA and DOPE, available from GIBCO/BRL); LIPOFECTAMINE® (including DOSPA and DOPE, available from GIBCO/BRL); and TRANSFECTAM® (including DOGS, available from Promega Corp.).

In some embodiments, the cationic lipid comprises from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, from about 50 mol % to about 60 mol %, from about 55 mol % to about 65 mol %, or from about 55 mol % to about 70 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In particular embodiments, the cationic lipid comprises about 50 mol %, 51 mol %, 52 mol %, 53 mol %, 54 mol %, 55 mol %, 56 mol %, 57 mol %, 58 mol %, 59 mol %, 60 mol %, 61 mol %, 62 mol %, 63 mol %, 64 mol %, or 65 mol % (or any fraction thereof) of the total lipid present in the particle.

In other embodiments, the cationic lipid comprises from about 2 mol % to about 60 mol %, from about 5 mol % to about 50 mol %, from about 10 mol % to about 50 mol %, from about 20 mol % to about 50 mol %, from about 20 mol % to about 40 mol %, from about 30 mol % to about 40 mol %, or about 40 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

Additional percentages and ranges of cationic lipids suitable for use in the lipid particles of the present invention are described, for example, in PCT Publication No. WO 09/127060, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

It should be understood that the percentage of cationic lipid present in the lipid particles of the invention is a target amount, and that the actual amount of cationic lipid present in the formulation may vary, for example, by ± 5 mol %.

Non-Cationic Lipids

The non-cationic lipids used in the lipid particles of the invention *(e.g.,* LNP) can be any of a variety of neutral uncharged, zwitterionic, or anionic lipids capable of producing a stable complex.

Non-limiting examples of non-cationic lipids include phospholipids such as lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane- 1-carboxylate (DOPE-mal), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof. Other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids can also be

used. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having C10-C24 carbon chains, *e.g.,* lauroyl, myristoyl, palmitoyl, stearoyl, or oleoyl.

Additional examples of non-cationic lipids include sterols such as cholesterol and derivatives thereof. Non-limiting examples of cholesterol derivatives include polar analogues such as 5a-cholestanol, 5P-coprostanol, cholesteryl-(2'-hydroxy)-ethyl ether, cholesteryl-(4'-hydroxy)-butyl ether, and 6-ketocholestanol; non-polar analogues such as 5a-cholestane, cholestenone, 5a-cholestanone, 5P-cholestanone, and cholesteryl decanoate; and mixtures thereof. In preferred embodiments, the cholesterol derivative is a polar analogue such as cholesteryl-(4'-hydroxy)-butyl ether. The synthesis of cholesteryl-(2'-hydroxy)-ethyl ether is described in PCT Publication No. WO 09/127060, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

In some embodiments, the non-cationic lipid present in the lipid particles *{e.g.,* LNP) comprises or consists of a mixture of one or more phospholipids and cholesterol or a derivative thereof. In other embodiments, the non-cationic lipid present in the lipid particles *{e.g.,* LNP) comprises or consists of one or more phospholipids, *e.g.,* a cholesterol-free lipid particle formulation. In yet other embodiments, the non-cationic lipid present in the lipid particles *{e.g.,* LNP) comprises or consists of cholesterol or a derivative thereof, *e.g.,* a phospholipid-free lipid particle formulation.

Other examples of non-cationic lipids suitable for use in the present invention include nonphosphorous containing lipids such as, *e.g.,* stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerolricinoleate, hexadecyl stereate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyldimethyl ammonium bromide, ceramide, sphingomyelin, and the like.

In some embodiments, the non-cationic lipid comprises from about 10 mol % to about 60 mol %, from about 20 mol % to about 55 mol %, from about 20 mol % to about 45 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 50 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 50 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 35 mol % to about 45 mol %, from about 37 mol % to about 42 mol %, or about 35 mol %, 36 mol %, 37 mol %, 38

mol %, 39 mol %, 40 mol %, 41 mol %, 42 mol %, 43 mol %, 44 mol %, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

In embodiments where the lipid particles contain a mixture of phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40 mol %, 45 mol %, 50 mol %, 55 mol %, or 60 mol % of the total lipid present in the particle.

In some embodiments, the phospholipid component in the mixture may comprise from about 2 mol % to about 20 mol %, from about 2 mol % to about 15 mol %, from about 2 mol % to about 12 mol %, from about 4 mol % to about 15 mol %, or from about 4 mol % to about 10 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In certain preferred embodiments, the phospholipid component in the mixture comprises from about 5 mol % to about 10 mol %, from about 5 mol % to about 9 mol %, from about 5 mol % to about 8 mol %, from about 6 mol % to about 9 mol %, from about 6 mol % to about 8 mol %, or about 5 mol %, 6 mol %, 7 mol %, 8 mol %, 9 mol %, or 10 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

In other embodiments, the cholesterol component in the mixture may comprise from about 25 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 27 mol % to about 37 mol %, from about 25 mol % to about 30 mol %, or from about 35 mol % to about 40 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In certain preferred embodiments, the cholesterol component in the mixture comprises from about 25 mol % to about 35 mol %, from about 27 mol % to about 35 mol %, from about 29 mol % to about 35 mol %, from about 30 mol % to about 35 mol %, from about 30 mol % to about 34 mol %, from about 31 mol % to about 33 mol %, or about 30 mol %, 31 mol %, 32 mol %, 33 mol %, 34 mol %, or 35 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

In embodiments where the lipid particles are phospholipid-free, the cholesterol or derivative thereof may comprise up to about 25 mol %, 30 mol %, 35 mol %, 40 mol %, 45 mol %, 50 mol %, 55 mol %, or 60 mol % of the total lipid present in the particle.

In some embodiments, the cholesterol or derivative thereof in the phospholipid-free lipid particle formulation may comprise from about 25 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to

about 40 mol %, from about 31 mol % to about 39 mol %, from about 32 mol % to about 38 mol %, from about 33 mol % to about 37 mol %, from about 35 mol % to about 45 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 40 mol %, or about 30 mol %, 31 mol %, 32 mol %, 33 mol %, 34 mol %, 35 mol %, 36 mol %, 37 mol %, 38 mol %, 39 mol %, or 40 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

In other embodiments, the non-cationic lipid comprises from about 5 mol % to about 90 mol %, from about 10 mol % to about 85 mol %, from about 20 mol % to about 80 mol %, about 10 mol % *(e.g.,* phospholipid only), or about 60 mol % *(e.g.,* phospholipid and cholesterol or derivative thereof) (or any fraction thereof or range therein) of the total lipid present in the particle.

Additional percentages and ranges of non-cationic lipids suitable for use in the lipid particles of the present invention are described, for example, in PCT Publication No. WO 09/127060, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

It should be understood that the percentage of non-cationic lipid present in the lipid particles of the invention is a target amount, and that the actual amount of non-cationic lipid present in the formulation may vary, for example, by ± 5 mol %.

Lipid Conjugates

In addition to cationic and non-cationic lipids, the lipid particles of the invention *(e.g.,* LNP) may further comprise a lipid conjugate. The conjugated lipid is useful in that it prevents the aggregation of particles. Suitable conjugated lipids include, but are not limited to, PEG-lipid conjugates, POZ-lipid conjugates, ATTA-lipid conjugates, cationic-polymer-lipid conjugates (CPLs), and mixtures thereof. In certain embodiments, the particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate together with a CPL.

In a preferred embodiment, the lipid conjugate is a PEG-lipid. Examples of PEG-lipids include, but are not limited to, PEG coupled to dialkyloxypropyls (PEG-DAA) as described in, *e.g.,* PCT Publication No. WO 05/026372, PEG coupled to diacylglycerol (PEG-DAG) as described in, *e.g.,* U.S. Patent Publication Nos. 20030077829 and 2005008689, PEG coupled to phospholipids such as phosphatidylethanolamine (PEG-PE), PEG conjugated to ceramides as described in, *e.g.,* U.S. Patent No. 5,885,613, PEG conjugated to cholesterol or a derivative

thereof, and mixtures thereof. The disclosures of these patent documents are herein incorporated by reference in their entirety for all purposes. Additional PEG-lipids suitable for use in the invention include, without limitation, mPEG2000-l,2-di-O-alkyl-s/?3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Publication No. WO 09/086558, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Yet additional suitable PEG-lipid conjugates include, without limitation, 1-[8'-(1,2-dimyristoyl-3-propanoxy)-carboxamido-3',6'-dioxaoctanyl]carbamoyl-o>methyl-poly(ethylene glycol) (2KPEG-DMG). The synthesis of 2KPEG-DMG is described in U.S. Patent No. 7,404,969, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

PEG is a linear, water-soluble polymer of ethylene PEG repeating units with two terminal hydroxyl groups. PEGs are classified by their molecular weights; for example, PEG 2000 has an average molecular weight of about 2,000 daltons, and PEG 5000 has an average molecular weight of about 5,000 daltons. PEGs are commercially available from Sigma Chemical Co. and other companies and include, but are not limited to, the following: monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S-NHS), monomethoxypolyethylene glycol-amine (MePEG-NH_2), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM), as well as such compounds containing a terminal hydroxyl group instead of a terminal methoxy group *(e.g.,* HO-PEG-S, HO-PEG-S-NHS, HO-PEG-NH_2, *etc.).* Other PEGs such as those described in U.S. Patent Nos. 6,774,180 and 7,053,150 *(e.g.,* mPEG (20 KDa) amine) are also useful for preparing the PEG-lipid conjugates of the present invention. The disclosures of these patents are herein incorporated by reference in their entirety for all purposes. In addition, monomethoxypolyethylenegly col-acetic acid (MePEG-CH_2COOH) is particularly useful for preparing PEG-lipid conjugates including, *e.g.,* PEG-DAA conjugates.

The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons *(e.g.,* from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about

750 daltons to about 2,000 daltons, *etc.).* In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

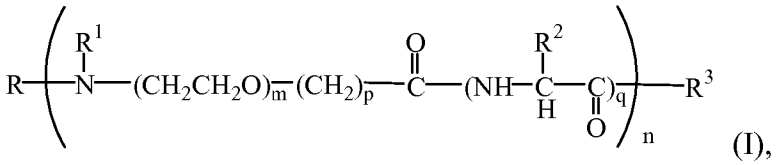
In certain instances, the PEG can be optionally substituted by an alkyl, alkoxy, acyl, or aryl group. The PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.,* non-ester containing linker moieties and ester-containing linker moieties. In a preferred embodiment, the linker moiety is a non-ester containing linker moiety. As used herein, the term "non-ester containing linker moiety" refers to a linker moiety that does not contain a carboxylic ester bond (-OC(O)-). Suitable non-ester containing linker moieties include, but are not limited to, amido (-C(O)NH-), amino (-NR-), carbonyl (-C(O)-), carbamate (-NHC(O)O-), urea (-NHC(O)NH-), disulphide (-S-S-), ether (-0-), succinyl (-(0)CCH $_2$CH$_2$C(0)-), succinamidyl (-NHC(0)CH $_2$CH$_2$C(0)NH-), ether, disulphide, as well as combinations thereof (such as a linker containing both a carbamate linker moiety and an amido linker moiety). In a preferred embodiment, a carbamate linker is used to couple the PEG to the lipid.

In other embodiments, an ester containing linker moiety is used to couple the PEG to the lipid. Suitable ester containing linker moieties include, *e.g.,* carbonate (-OC(O)O-), succinoyl, phosphate esters (-O-(O)POH-O-), sulfonate esters, and combinations thereof.

Phosphatidylethanolamines having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be conjugated to PEG to form the lipid conjugate. Such phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skilled in the art. Phosphatidyl-ethanolamines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of C_{10} to C_2o are preferred. Phosphatidylethanolamines with mono- or diunsaturated fatty acids and mixtures of saturated and unsaturated fatty acids can also be used. Suitable phosphatidylethanolamines include, but are not limited to, dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE), and distearoyl-phosphatidylethanolamine (DSPE).

The term "ATTA" or "polyamide" includes, without limitation, compounds described in U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated by

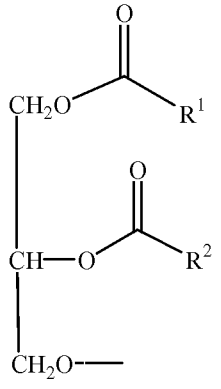
reference in their entirety for all purposes. These compounds include a compound having the formula:



(I),

wherein R is a member selected from the group consisting of hydrogen, alkyl and acyl; R^1 is a member selected from the group consisting of hydrogen and alkyl; or optionally, R and R^1 and the nitrogen to which they are bound form an azido moiety; R^2 is a member of the group selected from hydrogen, optionally substituted alkyl, optionally substituted aryl and a side chain of an amino acid; R^3 is a member selected from the group consisting of hydrogen, halogen, hydroxy, alkoxy, mercapto, hydrazino, amino and NR^4R^5, wherein R^4 and R^5 are independently hydrogen or alkyl; n is 4 to 80; m is 2 to 6; p is 1 to 4; and q is 0 or 1. It will be apparent to those of skill in the art that other polyamides can be used in the compounds of the present invention.

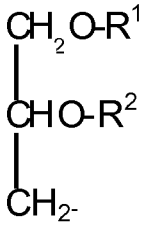
The term "diacylglycerol" or "DAG" includes a compound having 2 fatty acyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups include, but are not limited to, lauroyl (C_{12}), myristoyl (C14), palmitoyl (Ci_6), stearoyl (Cig), and icosoyl (C20). In preferred embodiments, R^1 and R^2 are the same, *i.e.*, R^1 and R^2 are both myristoyl *{i.e.*, dimyristoyl), R^1 and R^2 are both stearoyl *{i.e.*, distearoyl), *etc.* Diacylglycerols have the following general formula:



(II).

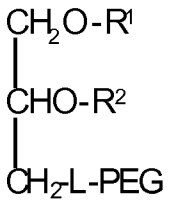
The term "dialkyloxypropyl" or "DAA" includes a compound having 2 alkyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons. The alkyl groups can be

saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:



(III).

In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:



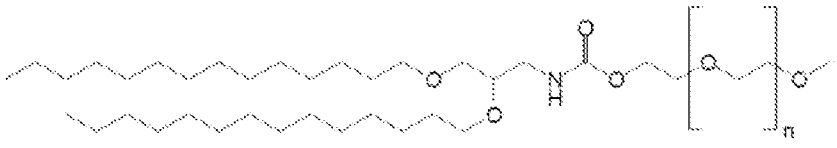
(IV),

wherein R^1 and R^2 are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester containing linker moiety or an ester containing linker moiety as described above. The long-chain alkyl groups can be saturated or unsaturated. Suitable alkyl groups include, but are not limited to, decyl (Cio), lauryl (C12), myristyl (C14), palmityl (Ci_6), stearyl (Cig), and icosyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.,* R^1 and R^2 are both myristyl *{i.e.,* dimyristyl), R^1 and R^2 are both stearyl *(i.e.,* distearyl), *etc.*

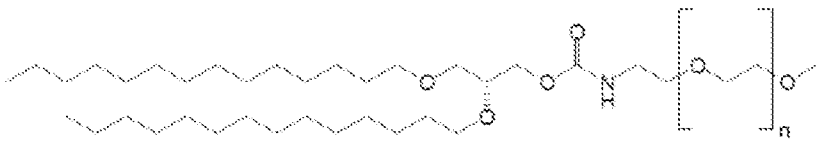
In Formula IV above, the PEG has an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG has an average molecular weight of from about 750 daltons to about 5,000 daltons *{e.g.,* from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.).* In preferred embodiments, the PEG has an average molecular weight of about 2,000 daltons or about 750 daltons. The PEG can be optionally substituted with alkyl, alkoxy, acyl, or aryl groups. In certain embodiments, the terminal hydroxyl group is substituted with a methoxy or methyl group.

In a preferred embodiment, "L" is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide linker moiety, a succinamidyl linker moiety, and combinations thereof. In a preferred embodiment, the non-ester containing linker moiety is a carbamate linker moiety *(i.e.,* a PEG-C-DAA conjugate). In another preferred embodiment, the non-ester containing linker moiety is an amido linker moiety *(i.e.,* a PEG-^-DAA conjugate). In yet another preferred embodiment, the non-ester containing linker moiety is a succinamidyl linker moiety *(i.e.,* a PEG-S-DAA conjugate).

In particular embodiments, the PEG-lipid conjugate is selected from:



(PEG-C-DMA); and



(PEG-C-DOMG).

The PEG-DAA conjugates are synthesized using standard techniques and reagents known to those of skill in the art. It will be recognized that the PEG-DAA conjugates will contain various amide, amine, ether, thio, carbamate, and urea linkages. Those of skill in the art will recognize that methods and reagents for forming these bonds are well known and readily available. *See, e.g.,* March, ADVANCED ORGANIC CHEMISTRY (Wiley 1992); Larock, COMPREHENSIVE ORGANIC TRANSFORMATIONS (VCH 1989); and Furniss, VOGEL'S TEXTBOOK OF PRACTICAL ORGANIC CHEMISTRY, 5th ed. (Longman 1989). It will also be appreciated that any functional groups present may require protection and deprotection at different points in the synthesis of the PEG-DAA conjugates. Those of skill in the art will recognize that such techniques are well known. *See, e.g.,* Green and Wuts, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS (Wiley 1991).

Preferably, the PEG-DAA conjugate is a PEG-didecyloxypropyl (C_{10}) conjugate, a PEG-dilauryloxypropyl (C_{12}) conjugate, a PEG-dimyristyloxypropyl (C_{14}) conjugate, a PEG-dipalmityloxypropyl (C_{16}) conjugate, or a PEG-distearyloxypropyl (C_{18}) conjugate. In these embodiments, the PEG preferably has an average molecular weight of about 750 or about 2,000 daltons. In one particularly preferred embodiment, the PEG-lipid conjugate comprises PEG2000-C-DMA, wherein the "2000" denotes the average molecular weight of the PEG, the "C" denotes a carbamate linker moiety, and the "DMA" denotes dimyristyloxypropyl. In another particularly preferred embodiment, the PEG-lipid conjugate comprises PEG750-C-DMA, wherein the "750" denotes the average molecular weight of the PEG, the "C" denotes a carbamate linker moiety, and the "DMA" denotes dimyristyloxypropyl. In particular embodiments, the terminal hydroxyl group of the PEG is substituted with a methyl group. Those of skill in the art will readily appreciate that other dialkyloxypropyls can be used in the PEG-DAA conjugates of the present invention.

In addition to the foregoing, it will be readily apparent to those of skill in the art that other hydrophilic polymers can be used in place of PEG. Examples of suitable polymers that can be used in place of PEG include, but are not limited to, polyvinylpyrrolidone, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide and polydimethylacrylamide, polylactic acid, polyglycolic acid, and derivatized celluloses such as hydroxymethylcellulose or hydroxyethylcellulose.

In addition to the foregoing components, the lipid particles *(e.g.,* LNP) of the present invention can further comprise cationic poly(ethylene glycol) (PEG) lipids or CPLs *(see, e.g.,* Chen *et al, Bioconj. Chem.,* 11:433-437 (2000); U.S. Patent No. 6,852,334; PCT Publication No. WO 00/62813, the disclosures of which are herein incorporated by reference in their entirety for all purposes).

In some embodiments, the lipid conjugate *(e.g.,* PEG-lipid) comprises from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 0.9 mol % to about 1.6 mol %, from about 0.9 mol % to about 1.8 mol %, from about 1 mol % to about 1.8 mol %, from about 1 mol % to about 1.7 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, or from about 1.4 mol % to

about 1.5 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

In other embodiments, the lipid conjugate *(e.g.,* PEG-lipid) comprises from about 0 mol % to about 20 mol %, from about 0.5 mol % to about 20 mol %, from about 2 mol % to about 20 mol %, from about 1.5 mol % to about 18 mol %, from about 2 mol % to about 15 mol %, from about 4 mol % to about 15 mol %, from about 2 mol % to about 12 mol %, from about 5 mol % to about 12 mol %, or about 2 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

In further embodiments, the lipid conjugate *(e.g.,* PEG-lipid) comprises from about 4 mol % to about 10 mol %, from about 5 mol % to about 10 mol %, from about 5 mol % to about 9 mol %, from about 5 mol % to about 8 mol %, from about 6 mol % to about 9 mol %, from about 6 mol % to about 8 mol %, or about 5 mol %, 6 mol %, 7 mol %, 8 mol %, 9 mol %, or 10 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

Additional examples, percentages, and/or ranges of lipid conjugates suitable for use in the lipid particles of the present invention are described in, *e.g.,* PCT Publication No. WO 09/127060, and PCT Publication No. WO 2010/006282, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

It should be understood that the percentage of lipid conjugate *(e.g.,* PEG-lipid) present in the lipid particles of the invention is a target amount, and that the actual amount of lipid conjugate present in the formulation may vary, for example, by ± 2 mol %.

One of ordinary skill in the art will appreciate that the concentration of the lipid conjugate can be varied depending on the lipid conjugate employed and the rate at which the lipid particle is to become fusogenic.

By controlling the composition and concentration of the lipid conjugate, one can control the rate at which the lipid conjugate exchanges out of the lipid particle and, in turn, the rate at which the lipid particle becomes fusogenic. For instance, when a PEG-DAA conjugate is used as the lipid conjugate, the rate at which the lipid particle becomes fusogenic can be varied, for example, by varying the concentration of the lipid conjugate, by varying the molecular weight of the PEG, or by varying the chain length and degree of saturation of the alkyl groups on the PEG-DAA conjugate. In addition, other variables including, for example,

pH, temperature, ionic strength, *etc.* can be used to vary and/or control the rate at which the lipid particle becomes fusogenic. Other methods which can be used to control the rate at which the lipid particle becomes fusogenic will become apparent to those of skill in the art upon reading this disclosure. Also, by controlling the composition and concentration of the lipid conjugate, one can control the lipid particle *{e.g.,* LNP) size.

Preparation of Lipid Particles

The lipid particles of the present invention, *e.g.,* LNP, in which an active agent or therapeutic agent such as an interfering RNA *{e.g.,* siRNA) is entrapped within the lipid portion of the particle and is protected from degradation, can be formed by any method known in the art including, but not limited to, a continuous mixing method, a direct dilution process, and an in-line dilution process.

In particular embodiments, the non-cationic lipids are egg sphingomyelin (ESM), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), l-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), dipalmitoyl-phosphatidylcholine (DPPC), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, 14:0 PE (1,2-dimyristoyl-phosphatidylethanolamine (DMPE)), 16:0 PE (1,2-dipalmitoyl-phosphatidylethanolamine (DPPE)), 18:0 PE (1,2-distearoyl-phosphatidylethanolamine (DSPE)), 18:1 PE (1,2-dioleoyl-phosphatidylethanolamine (DOPE)), 18:1 trans PE (1,2-dielaidoyl-phosphatidylethanolamine (DEPE)), 18:0-18:1 PE (l-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE)), 16:0-18:1 PE (l-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)), polyethylene glycol-based polymers *{e.g.,* PEG 2000, PEG 5000, PEG-modified diacylglycerols, or PEG-modified dialkyloxypropyls), cholesterol, derivatives thereof, or combinations thereof.

In certain embodiments, the present invention provides nucleic acid-lipid particles *{e.g.,* LNP) produced via a continuous mixing method, *e.g.,* a process that includes providing an aqueous solution comprising a nucleic acid *{e.g.,* interfering RNA) in a first reservoir, providing an organic lipid solution in a second reservoir (wherein the lipids present in the organic lipid solution are solubilized in an organic solvent, *e.g.,* a lower alkanol such as ethanol), and mixing the aqueous solution with the organic lipid solution such that the organic lipid solution mixes with the aqueous solution so as to substantially instantaneously produce a lipid vesicle *{e.g.,* liposome) encapsulating the nucleic acid within the lipid vesicle. This process and the apparatus for carrying out this process are described in detail in U.S.

Patent Publication No. 20040142025, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

The action of continuously introducing lipid and buffer solutions into a mixing environment, such as in a mixing chamber, causes a continuous dilution of the lipid solution with the buffer solution, thereby producing a lipid vesicle substantially instantaneously upon mixing. As used herein, the phrase "continuously diluting a lipid solution with a buffer solution" (and variations) generally means that the lipid solution is diluted sufficiently rapidly in a hydration process with sufficient force to effectuate vesicle generation. By mixing the aqueous solution comprising a nucleic acid with the organic lipid solution, the organic lipid solution undergoes a continuous stepwise dilution in the presence of the buffer solution *(i.e.,* aqueous solution) to produce a nucleic acid-lipid particle.

The nucleic acid-lipid particles formed using the continuous mixing method typically have a size of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm (or any fraction thereof or range therein). The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

In another embodiment, the present invention provides nucleic acid-lipid particles *(e.g.,* LNP) produced via a direct dilution process that includes forming a lipid vesicle *(e.g.,* liposome) solution and immediately and directly introducing the lipid vesicle solution into a collection vessel containing a controlled amount of dilution buffer. In preferred aspects, the collection vessel includes one or more elements configured to stir the contents of the collection vessel to facilitate dilution. In one aspect, the amount of dilution buffer present in the collection vessel is substantially equal to the volume of lipid vesicle solution introduced thereto. As a non-limiting example, a lipid vesicle solution in 45% ethanol when introduced into the collection vessel containing an equal volume of dilution buffer will advantageously yield smaller particles.

In yet another embodiment, the present invention provides nucleic acid-lipid particles *(e.g.,* LNP) produced via an in-line dilution process in which a third reservoir containing dilution buffer is fluidly coupled to a second mixing region. In this embodiment, the lipid vesicle *(e.g.,* liposome) solution formed in a first mixing region is immediately and directly mixed with dilution buffer in the second mixing region. In preferred aspects, the second mixing region includes a T-connector arranged so that the lipid vesicle solution and the dilution buffer flows meet as opposing 180° flows; however, connectors providing shallower angles can be used, *e.g.,* from about 27° to about 180° *(e.g.,* about 90°). A pump mechanism delivers a controllable flow of buffer to the second mixing region. In one aspect, the flow rate of dilution buffer provided to the second mixing region is controlled to be substantially equal to the flow rate of lipid vesicle solution introduced thereto from the first mixing region. This embodiment advantageously allows for more control of the flow of dilution buffer mixing with the lipid vesicle solution in the second mixing region, and therefore also the concentration of lipid vesicle solution in buffer throughout the second mixing process. Such control of the dilution buffer flow rate advantageously allows for small particle size formation at reduced concentrations.

These processes and the apparatuses for carrying out these direct dilution and in-line dilution processes are described in detail in U.S. Patent Publication No. 20070042031, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

The nucleic acid-lipid particles formed using the direct dilution and in-line dilution processes typically have a size of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm (or any fraction thereof or range therein). The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

If needed, the lipid particles of the invention *(e.g.,* LNP) can be sized by any of the methods available for sizing liposomes. The sizing may be conducted in order to achieve a desired size range and relatively narrow distribution of particle sizes.

Several techniques are available for sizing the particles to a desired size. One sizing method, used for liposomes and equally applicable to the present particles, is described in U.S. Patent No. 4,737,323, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Sonicating a particle suspension either by bath or probe sonication produces a progressive size reduction down to particles of less than about 50 nm in size. Homogenization is another method which relies on shearing energy to fragment larger particles into smaller ones. In a typical homogenization procedure, particles are recirculated through a standard emulsion homogenizer until selected particle sizes, typically between about 60 and about 80 nm, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination, or QELS.

Extrusion of the particles through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing particle sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired particle size distribution is achieved. The particles may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in size.

In some embodiments, the nucleic acids present in the particles are precondensed as described in, *e.g.,* U.S. Patent Application No. 09/744,103, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

In other embodiments, the methods may further comprise adding non-lipid polycations which are useful to effect the lipofection of cells using the present compositions. Examples of suitable non-lipid polycations include, hexadimethrine bromide (sold under the brand name POLYBRENE®, from Aldrich Chemical Co., Milwaukee, Wisconsin, USA) or other salts of hexadimethrine. Other suitable polycations include, for example, salts of poly-L-ornithine, poly-L-arginine, poly-L-lysine, poly-**D**-lysine, polyallylamine, and polyethyleneimine. Addition of these salts is preferably after the particles have been formed.

In some embodiments, the nucleic acid to lipid ratios (mass/mass ratios) in a formed nucleic acid-lipid particle *(e.g.,* LNP) will range from about 0.01 to about 0.2, from about 0.05 to

about 0.2, from about 0.02 to about 0.1, from about 0.03 to about 0.1, or from about 0.01 to about 0.08. The ratio of the starting materials (input) also falls within this range. In other embodiments, the particle preparation uses about 400 μg nucleic acid per 10 mg total lipid or a nucleic acid to lipid mass ratio of about 0.01 to about 0.08 and, more preferably, about 0.04, which corresponds to 1.25 mg of total lipid per 50 μg of nucleic acid. In other preferred embodiments, the particle has a nucleic acid:lipid mass ratio of about 0.08.

In other embodiments, the lipid to nucleic acid ratios (mass/mass ratios) in a formed nucleic acid-lipid particle *(e.g.,* LNP) will range from about 1 (1:1) to about 100 (100:1), from about 5 (5:1) to about 100 (100:1), from about 1 (1:1) to about 50 (50:1), from about 2 (2:1) to about 50 (50:1), from about 3 (3:1) to about 50 (50:1), from about 4 (4:1) to about 50 (50:1), from about 5 (5:1) to about 50 (50:1), from about 1 (1:1) to about 25 (25:1), from about 2 (2:1) to about 25 (25:1), from about 3 (3:1) to about 25 (25:1), from about 4 (4:1) to about 25 (25:1), from about 5 (5:1) to about 25 (25:1), from about 5 (5:1) to about 20 (20:1), from about 5 (5:1) to about 15 (15:1), from about 5 (5:1) to about 10 (10:1), or about 5 (5:1), 6 (6:1), 7 (7:1), 8 (8:1), 9 (9:1), 10 (10:1), 11 (11:1), 12 (12:1), 13 (13:1), 14 (14:1), 15 (15:1), 16 (16:1), 17 (17:1), 18 (18:1), 19 (19:1), 20 (20:1), 21 (21:1), 22 (22:1), 23 (23:1), 24 (24:1), or 25 (25:1), or any fraction thereof or range therein. The ratio of the starting materials (input) also falls within this range.

As previously discussed, the conjugated lipid may further include a CPL. A variety of general methods for making LNP-CPLs (CPL-containing LNP) are discussed herein. Two general techniques include the "post-insertion" technique, that is, insertion of a CPL into, for example, a pre-formed LNP, and the "standard" technique, wherein the CPL is included in the lipid mixture during, for example, the LNP formation steps. The post-insertion technique results in LNP having CPLs mainly in the external face of the LNP bilayer membrane, whereas standard techniques provide LNP having CPLs on both internal and external faces. The method is especially useful for vesicles made from phospholipids (which can contain cholesterol) and also for vesicles containing PEG-lipids (such as PEG-DAAs and PEG-DAGs). Methods of making LNP-CPLs are taught, for example, in U.S. Patent Nos. 5,705,385; 6,586,410; 5,981,501; 6,534,484; and 6,852,334; U.S. Patent Publication No. 20020072121; and PCT Publication No. WO 00/62813, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

Pharmaceutical Formulations

Compositions of the invention may be formulated as pharmaceutical compositions suitable for delivery to a subject. The pharmaceutical compositions of the invention will often further comprise one or more buffers *(e.g.,* neutral buffered saline or phosphate buffered saline), carbohydrates *(e.g.,* glucose, mannose, sucrose, dextrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents such as EDTA or glutathione, adjuvants *(e.g.,* aluminum hydroxide), solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood of a recipient, suspending agents, thickening agents and/or preservatives. Alternatively, compositions of the present invention may be formulated as a lyophilizate.

The amount of the oligonucleotides administered to a patient can be readily determined by a physician based upon a variety of factors, including, *e.g.,* the disease and the level of the oligonucleotides expressed from the vector being used (in cases where a vector is administered). The amount administered per dose is typically selected to be above the minimal therapeutic dose but below a toxic dose. The choice of amount per dose will depend on a number of factors, such as the medical history of the patient, the use of other therapies, and the nature of the disease. In addition, the amount administered may be adjusted throughout treatment, depending on the patient's response to treatment and the presence or severity of any treatment-associated side effects.

Methods of Determining Gene Function

The invention further includes a method of identifying gene function in an organism comprising the use of a polynucleotide complex of the present invention to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics envisions determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention may be used in determining potential targets for pharmaceutics, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for the yeast, *D. melanogaster,* and *C. elegans* genomes, can be coupled with the invention to

determine gene function in an organism *(e.g.,* nematode). The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

In one embodiment, a polynucleotide of the present invention is used to inhibit gene expression based upon a partial sequence available from an expressed sequence tag (EST), *e.g.,* in order to determine the gene's function or biological activity. Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The ease with which a polynucleotide can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, solutions containing the polynucleotide that are capable of inhibiting different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. In one embodiment, the polynucleotides of the invention are used for chemocogenomic screening, *i.e.,* testing compounds for their ability to reverse a disease modeled by the reduction of gene expression using a polynucleotide of the invention.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, a polynucleotide can be introduced to the organism, and whether an alteration in the characteristic is correlated with inhibition can be determined.

The present invention is also useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or cellular compartments. The functional equivalent of conditional mutations may be produced

by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of a the polynucleotide at specific times of development and locations in the organism without introducing permanent mutations into the target genome. Similarly, the invention contemplates the use of inducible or conditional vectors that express a the polynucleotide only when desired.

The present invention also relates to a method of validating whether a gene product is a target for drug discovery or development. A polynucleotide that targets the gene that corresponds to the gene for degradation is introduced into a cell or organism. The cell or organism is maintained under conditions in which degradation of the gene occurs, resulting in decreased expression of the gene. Whether decreased expression of the gene has an effect on the cell or organism is determined. If decreased expression of the gene has an effect, then the gene product is a target for drug discovery or development.

Methods of Designing and Producing Polynucleotide Complexes

The polynucleotide complexes of the present invention comprise a novel and unique set of functional sequences, arranged in a manner so as to adopt a secondary structure containing one or more double-stranded regions (sometimes adjoined by stem-loop or loop structures), which imparts the advantages of the polynucleotide. Accordingly, in certain embodiments, the present invention includes methods of designing the polynucleotide complexes of the present invention. Such methods typically involve appropriate selection of the various sequence components of the polynucleotide complexes. The terms "primary strand", "secondary strand", and "key strand" refer to the various guide strands present within a polynucleotide complex of the present invention.

In one embodiment, the basic design of the polynucleotide complex is as follows:

DESIGN MOTIFS:

(primary strand)(UU)(secondary strand)(UU)(key strand)(UU)

Accordingly, in a related embodiment, the polynucleotide is designed as follows:

II. (secondary strand)(UU)(UU)(key strand)(UU)(primary strand)

III. (secondary strand)(UU)(loop or stem-loop)(key strand)(UU)(loop or stem-loop)(primary strand)(UU)

SET PARAMETERS

Set seed size for self complementarity at approx 38-43%. For a 19 nucleotide targets, a range or 7 or 8 nucleotides is preferred as SEED_SIZE.

For each gene, define a PRIMARY and SECONDARY target gene.

DEFINE PRIMARY STRANDS

Start with one or more target gene sequences. For each gene, build a list of PRIMARY target sequences 17-24 nucleotide motifs that meet criteria of G/C content, specificity, and poly-A or poly-G free. For each, find also a SECONDARY and KEY strand.

FIND SECONDARY AND KEY STRANDS

d. For each target sequence on each gene, clustal align base 1 through SEED_SIZE the reverse of each sequence to the SECONDARY gene

Record sequence with a perfect alignment. The target sequence on the SECONDARY gene is the alignment start, minus the length of the motif, plus SEED_SIZE to alignment start, plus SEED_SIZE. The SECONDARY strand is the reverse compliment.

To find each KEY strand, define SEED_A as base 1 through SEED_SIZE of the PRIMARY strand, define SEED_B as bases at motif length minus SEED_SIZE to motif length of the SECONDARY strand. Set a MID_SECTION as characters "|" repeated of length motif sequence length minus SEED_A length plus SEED_B length. Set key alignment sequence as SEED_A, MID_SECTION, SEED_B. Clustal align to the target gene for the key segment. Record KEY target sequence as bases at alignment hit on key target gene to bases alignment hit plus motif length. The KEY strand is the reverse compliment.

CONSTRUCT OPTIONAL POLYNUCLEOTIDE

g. Build candidate Stem A & B with (4-24) nucleotides that have melting temperature dominant to equal length region of target. Stem strands have A-T, G-C complementarity to

each other. Length and composition depend upon which endoribonuclease is chosen for pre-processing of the stem-loop structure.

h. Build candidate Stem C & D with (4-24) nucleotides that have melting temperature dominant to equal length region of target. Stem strands have A-T, G-C complementarity to each other, but no complementarity to Stem A & B. Length and composition depend upon which endoribonuclease is chosen for pre-processing of the stem-loop structure.

i. Build loop candidates with (4-12) A-T rich nucleotides into loop A & B. Length and composition depend upon which endoribonuclease is chosen for pre-processing of the stem-loop structure. Tetraloops as described are suggested for longer stems processed by RNase III or Pacl RNase III endoribonucleases as drawn in. Larger loops are suggested for preventing RNase III or Pacl processing and placed onto shorter stems.

j. Form a contiguous sequence for each motif candidate.

k. Fold candidate sequence using software with desired parameters.

1. From output, locate structures with single stranded target regions which are flanked at either one or both ends with a desired stem/loop structure.

The programs of the present invention may further use input regarding the genomic sequence of the organism containing the target gene, *e.g.,* public or private databases, as well as additional programs that predict secondary structure and/or hybridization characteristics of particular sequences, in order to ensure that the polynucleotide adopts the correct secondary structure and does not hybridize to non-target genes.

The present invention is based, in part, upon the surprising discovery that the polynucleotide complexes, as described herein, are extremely effective in reducing target gene expression of one or more genes. The polynucleotide offer significant advantages over previously described antisense RNAs, including increased potency, and increased effectiveness to multiple target genes. Furthermore, the polynucleotide of the invention offer additional advantages over traditional dsRNA molecules used for siRNA, since the use of the polynucleotide substantially eliminates the off-target suppression associated with dsRNA molecules and offers multivalent RNAi.

It is understood that the compositions and methods of the present invention may be used to target a variety of different target genes. The term "target gene" may refer to a gene, an mRNA, or a microRNA. Accordingly, target sequences provided herein may be depicted as either DNA sequences or RNA sequences. One of skill the art will appreciate that the compositions of the present invention may include regions complementary to either the DNA or RNA sequences provided herein. Thus, where either a DNA or RNA target sequence is provided, it is understood that the corresponding RNA or DNA target sequence, respectively, may also be targeted.

The practice of the present invention will employ a variety of conventional techniques of cell biology, molecular biology, microbiology, and recombinant DNA, which are within the skill of the art. Such techniques are fully described in the literature. See, for example, Molecular Cloning: A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch, and Maniatis (Cold Spring Harbor Laboratory Press, 1989); and DNA Cloning, Volumes I and II (D. N. Glover ed. 1985).

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference in their entirety. as if each one was individually incorporated by reference. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments.

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

EXAMPLES

EXAMPLE 1

This Example describes a method for making a polynucleotide complex of the present invention.

Step A: Anneal oligonucleotides A and B.

1) Combine 30μL of oligos A and B with10μL of 10 X Annealing Buffer *(e.g.,* lOOmM Tris-HCL, pH 7.5, 1M NaCl, lOmM EDTA).

2) Vortex gently.

3) Heat for 5 minutes at 90°C in heat block.

4) Step cool to 76°C at a rate of 1°C per minute.

5) Incubate at 76°C for 20 minutes.

Step B: Add oligonucleotide C.

1) Add 30μL of oligo C to annealed oligos A and B formed in Step A.

2) Incubate for 60 minutes at 76°C.

3) Cool to 48°C at a rate of 1°C per minute.

Step C: Reduce Scaffolding.

1) Cycle heat between 40-50°C for 10 minutes, then reduce cycling range by 1°C per cycle until range is 40°C.

2) Remove from heat block and let cool to room temperature.

EXAMPLE 2

This Example describes another method for making a polynucleotide complex of the present invention.

Step 1: mix oligonucleotide strands in correct ratios in the annealing buffer described in Example 1. The oligonucleotide strands are two un-nicked strands, and the first and second portions of the nicked oligonucleotide strand.

Step 2: heat the mixture of oligonucleotides prepared in Step 1 at 95°C for 2 minutes.

Step 3: cool the heated mixture prepared in Step 2 to ambient temperature within a 37°C chamber.

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CLAIMS:

1. A polynucleotide complex of at least three separate polynucleotides, comprising

(a) a first polynucleotide comprising a target-specific region that is complementary to a first target sequence, a 5' region, and a 3' region;

(b) a second polynucleotide comprising a target-specific region that is complementary to a second target sequence, a 5' region, and a 3' region; and

(c) a third polynucleotide comprising a null region or a target-specific region that is complementary to a third target specific, a 5' region, and a 3' region,

wherein each of the target-specific regions of the first, second, and third polynucleotides are complementary to a different target sequence,

wherein the 5' region of the first polynucleotide is complementary to the 3' region of the third polynucleotide, wherein the 3' region of the first polynucleotide is complementary to the 5' region of the second polynucleotide, and wherein the 3' region of the second polynucleotide is complementary to the 5' region of the third polynucleotide,

wherein the three separate polynucleotides hybridize via their complementary 3' and 5' regions to form a polynucleotide complex with a first, second, and third single-stranded region, and a first, second, and third self-complementary region,

and wherein one of the first polynucleotide, the second polynucleotide and the third polynucleotide comprises a nick to form a nicked polynucleotide comprising a first portion and a second portion.

2. The polynucleotide complex of claim 1, wherein the first, second, and/or third polynucleotide comprises about 15-30 nucleotides.

3. The polynucleotide complex of claim 1, wherein the first, second, and/or third polynucleotide comprises about 17-25 nucleotides.

4. The polynucleotide complex of claim 1, wherein one or more of the self-complementary regions comprises about 5-10 nucleotide pairs.

5. The polynucleotide complex of claim 1, wherein one or more of the self-complementary regions comprises about 7-8 nucleotide pairs.

6. The polynucleotide complex of claim 1, wherein each of said first, second, and third target sequences are present in the same gene, cDNA, mRNA, or microRNA.

7. The polynucleotide complex of claim 1, wherein at least two of said first, second, and third target sequences are present in different genes, cDNAs, mRNAs, or microRNAs.

8. The polynucleotide complex of claim 1, wherein all or a portion of the 5' and/or 3' region of each polynucleotide is also complementary to the target sequence for that polynucleotide.

9. The polynucleotide complex of claim 1, wherein one or more of the self-complementary regions comprises a 3' overhang.

10. The polynucleotide complex of claim 1, wherein each of the first portion and the second portion comprises from 5 to 12 nucleotides. Typically, the nick is located within a target-specific region, although the nick can be located outside a target specific region.

11. The polynucleotide complex of claim 1, wherein the nick is located within a target-specific region.

12. The polynucleotide complex of claim 1, wherein the nick is located outside a target-specific region.

13. A method of reducing expression of a gene, comprising introducing a polynucleotide complex of any one of claims 1-12 into a cell.

14. The method of claim 13, wherein said method is practiced *in vitro.*

15. The method of claim 13, wherein said method is practiced *in vivo.*

16. A method for making a trimeric polynucleotide complex of Claim 1, the method comprising the step of incubating a first oligonucleotide, a second oligonucleotide, a first portion of a third oligonucleotide and a second portion of the third oligonucleotide in an annealing buffer under conditions whereby a trimeric polynucleotide complex of Claim 1 is formed.

17. A lipid-nucleic acid particle comprising a polynucleotide complex of Claim 1 encapsulated within a lipid particle.

18. A pharmaceutical composition comprising a lipid-nucleic acid particle of Claim 17 and a pharmaceutically acceptable carrier or excipient.

5

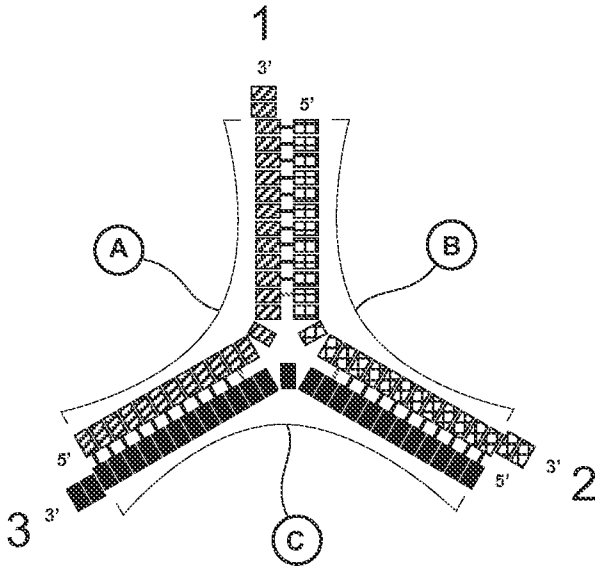


FIG. 1

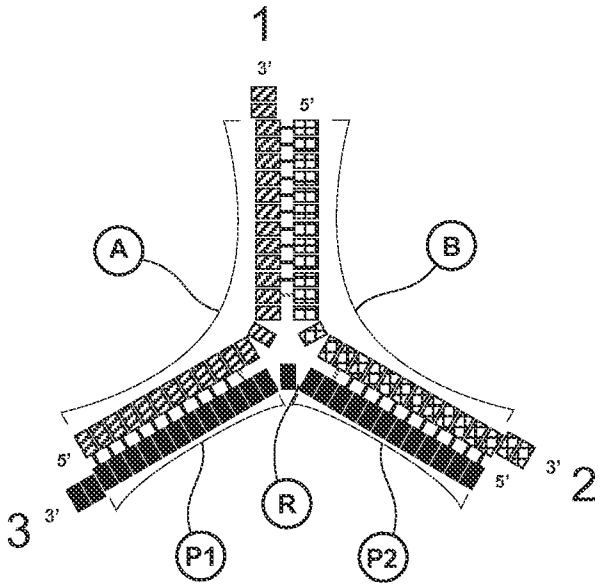


FIG. 2

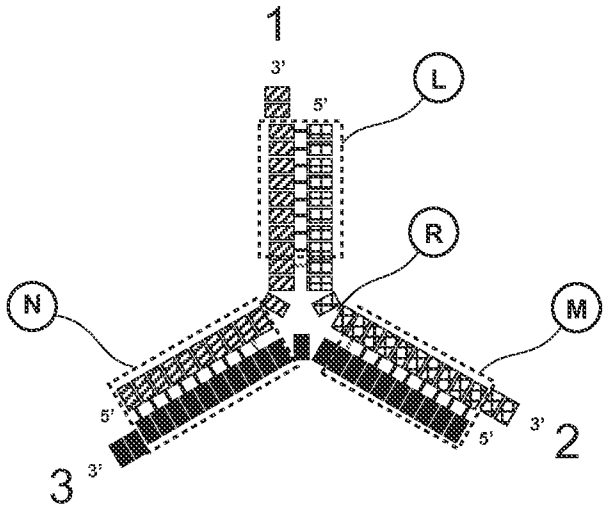


FIG. 3

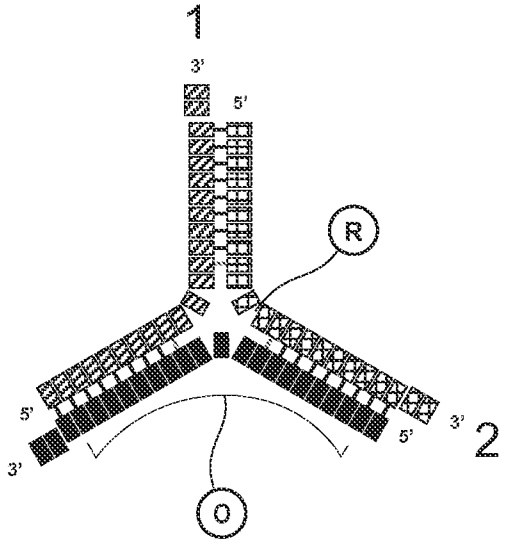


FIG. 4

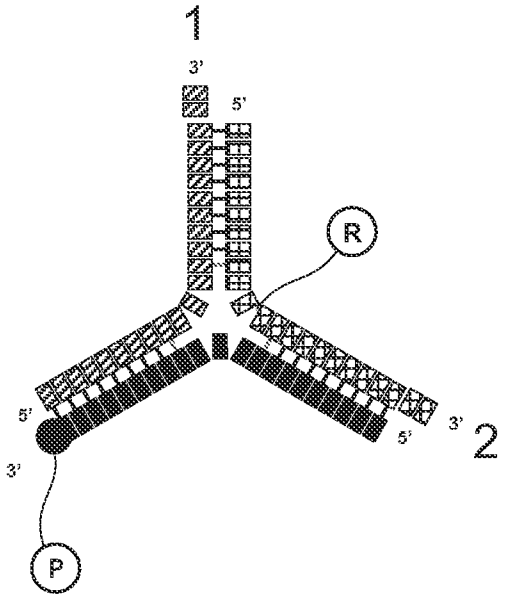


FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/20109

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07H 21/00, C12N 15/11 (2013.01)
USPC - 536/24.5, 514/44A
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C07H 21/00, C12N 15/11 (2013.01)
USPC - 536/24.5, 514/44A

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/490, 424/493, 536/22, 514/44R

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Pat Base; PubWEST (PGPB,USPT,USOC,EPAB,JPAB); USPTO Web Page; PCT Patentscope; Google Scholar
Search terms - multivalent complex, oligonucleotides, nick, fragments, self-complementary, target region, annealing buffer, pharmaceutical composition, in vitro, in vivo, liposome-encapsulation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2010/141511 A2 (HAUSER) 09 December 2010 (09.12.2010) pg 3, para 4; pg 3 para 6 - pg 4, para 4; pg 6, para 5; pg 11, para 1; pg 15, para 3 ~ pg 16, para 1; pg 38, para 3 ~ pg 39, para 1; pg 43, para 2; pg 52, para 1	1-18
Y	US 2010/0209487 A1 (QUAY et al.) 19 August 2010 (19.08.2010) para [0001], [0005]-[0007], [0030], [0046], [0123], [0140], [0146], [0147], [0182], [0203], [0208], [0211]	1-18

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 February 2013 (14.02.2013)	14 MAR 2013

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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