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(54) Title: P-ETHOXY NUCLEIC ACIDS FOR LIPOSOMAL FORMULATION

(57) Abstract: Provided herein are therapeutic oligonucleotides that comprise at least one p-ethoxy backbone linkage but no more than 80% p-ethoxy backbone linkages. Provided herein are improved delivery systems for therapeutic oligonucleotides comprising a liposome that comprises neutral phospholipids and a p-ethoxy oligonucleotide that is entrapped in the liposome.

# DESCRIPTION

# P-ETHOXY NUCLEIC ACIDS FOR LIPOSOMAL FORMULATION

**[0001]** The present application claims the priority benefit of United States provisional application number 62/241,503, filed October 14, 2015, the entire contents of which is incorporated herein by reference.

## BACKGROUND OF THE INVENTION

### 1. Field of the Invention

**[0002]** The present invention relates generally to the field of medicine. More particularly, it concerns liposomal formulations of p-ethoxy oligonucleotides and methods of making and using such formulations in medicine.

### 2. Description of Related Art

**[0003]** Antisense oligonucleotides (oligos) complementary to specific regions of a target mRNA have been used to inhibit the expression of endogenous genes. When the antisense oligonucleotides bind to a target mRNA, a DNA-RNA hybrid is formed. This hybrid formation inhibits the translation of the mRNA and, thus, the expression of the encoded protein. If the protein is essential for the survival of the cell, the inhibition of its expression may lead to cell death. Therefore, antisense oligonucleotides can be useful tools in anticancer and antiviral therapies.

**[0004]** The main obstacles in using antisense oligonucleotides to inhibit gene expression are cellular instability, low cellular uptake, and poor intercellular delivery. Natural phosphodiesters are not resistant to nuclease hydrolysis; thus high concentrations of antisense oligonucleotides are needed before any inhibitory effect is observed. Modified phosphodiester analogs, such as p-ethoxy, have been made to overcome this nuclease hydrolysis problem, but they have not provided a satisfactory solution to the problem.

**[0005]** The cellular uptake of antisense oligonucleotides is low. To solve this problem, physical techniques, such as calcium-phosphate precipitation, DEAE-dextran mediation, or electroporation, have been used to increase the cellular uptake of oligonucleotides. These techniques are difficult to reproduce and are inapplicable *in vivo*. Cationic lipids, such as Lipofectin, have also been used to deliver oligonucleotides. An

electrostatic interaction is formed between the cationic lipids and the negatively charged oligonucleotides, which results in a complex that is then taken up by the target cells. Since these cationic lipids do not protect the oligonucleotides from nuclease digestion, are harmful to the cell membrane, and they are only useful in delivering the nuclease-resistant phosphorothioates, but not the nuclease-cleavable phosphodiesters.

**[0006]** Another modified phosphodiester (PD) analog that has been prepared is p-ethoxy (pE) oligos. The modifications of pE oligos are made in the phosphate backbone so that the modification will not interfere with the binding of these oligos to a target mRNA. pE oligos are made by adding an ethyl group to the non-bridging oxygen atom of the phosphate backbone, thus rendering these oligos uncharged compounds. In spite of their resistance to nucleases, the cellular uptake and intracellular delivery of pE oligos is poor because upon internalization, these oligos remain sequestered inside the endosomal/lysosomal vacuoles, impeding their access to target mRNA.

**[0007]** There is a need for improved antisense compositions for use in treatment of disease, and also a need for processes for making such improved compositions.

## SUMMARY OF THE INVENTION

**[0008]** In one embodiment, compositions are provided comprising a population of oligonucleotides. In some aspects, the oligonucleotides of the population are composed of nucleoside molecules linked together through phosphate backbone linkages, wherein at least one of the phosphate backbone linkages in each oligonucleotide is a p-ethoxy backbone linkage, and wherein no more than 80% of the phosphate backbone linkages in each oligonucleotide are p-ethoxy backbone linkages. In some aspects, at least one of the phosphate backbone linkages in each oligonucleotide is a phosphodiester backbone linkage. In some aspects, 10% to 80% of the phosphate backbone linkages are p-ethoxy backbone linkages; 20% to 80% of the phosphate backbone linkages are p-ethoxy backbone linkages; 30% to 80% of the phosphate backbone linkages are p-ethoxy backbone linkages; 40% to 80% of the phosphate backbone linkages are p-ethoxy backbone linkages; 50% to 80% of the phosphate backbone linkages are p-ethoxy backbone linkages; or 60% to 70% of the phosphate backbone linkages are p-ethoxy backbone linkages, or any range derivable therein. In some aspects, 20% to 90% of the phosphate backbone linkages are phosphodiester backbone linkages; 20% to 80% of the phosphate backbone linkages are phosphodiester

backbone linkages; 20% to 70% of the phosphate backbone linkages are phosphodiester backbone linkages; 20% to 60% of the phosphate backbone linkages are phosphodiester backbone linkages; 20% to 50% of the phosphate backbone linkages are phosphodiester backbone linkages; or 30% to 40% of the phosphate backbone linkages are phosphodiester backbone linkages, or any range derivable therein. In various aspects, at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or any value therein, of the phosphate backbone linkages are p-ethoxy backbone linkages. In various aspects, at most 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or any value therein, of the phosphate backbone linkages are phosphodiester backbone linkages. In some aspects, the composition is lyophilized.

**[0009]** In some aspects, the oligonucleotides of the population have a size ranging from 7 to 30 nucleotides. In certain aspects, the oligonucleotides of the population have a size ranging from 12 to 25 nucleotides. In various aspects, the oligonucleotides of the population have a size of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides. The size range may be an average size of the oligonucleotides in the population.

**[0010]** In some aspects, the oligonucleotides of the population have an average size of 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides, wherein no more than 5, 6, 7, 8, 8, 9, 10, 11, 11, 12, 13, 14, 15, 15, 16, 17, 18, 19, 20, 20, 21, 22, 23, or 24, respectively, of the phosphate backbone linkages in each oligonucleotide is a p-ethoxy backbone linkage. In some aspects, the oligonucleotides of the population have an average size of 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides and at least 2, 2, 2, 2, 3, 3, 3, 3, 4, 4, 4, 4, 4, 5, 5, 5, 5, 5, 5, 6, 6, 6, 6, or 6, respectively, of the phosphate backbone linkages in each oligonucleotide is a phosphodiester backbone linkage.

**[0011]** In some aspects, the population of oligonucleotides comprises a single species of oligonucleotides. In other aspects, the population of oligonucleotides comprises at least two species of oligonucleotides. A single species of oligonucleotide may have the same nucleotide sequence but either have or lack p-ethoxy linkages in different places within the molecule. In some aspects, the population of oligonucleotides comprises antisense

oligonucleotides, short interfering RNAs (siRNAs), microRNAs (miRNAs), or piwiRNAs (piRNAs).

**[0012]** In certain aspects, the oligonucleotides of the population inhibit the expression of at least one oncogenic protein, infectious agent protein, or self-antigen. In some aspects, the oligonucleotides of the population hybridize with at least one oncogenic oligonucleotide, infectious agent oligonucleotide, or self-antigen oligonucleotide.

**[0013]** In various aspects, the composition further comprises phospholipids. In some aspects, the phospholipids are uncharged or have a neutral charge at physiologic pH. In some aspects, the phospholipids are neutral phospholipids. In certain aspects, the neutral phospholipids are phosphatidylcholines. In certain aspects, the neutral phospholipids are dioleoylphosphatidyl choline. In some aspects, the phospholipids are essentially free of cholesterol.

**[0014]** In some aspects, the phospholipids and oligonucleotides are present at a molar ratio of from about 5:1 to about 100:1, or any ratio derivable therein. In various aspects, the phospholipids and oligonucleotides are present at a molar ratio of about 5:1, 10:1, 15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1, 55:1, 60:1, 65:1, 70:1, 75:1, 80:1, 85:1, 90:1 95:1, or 100:1. In some aspects, the oligonucleotides and phospholipids form an oligonucleotide–lipid complex, such as, for example, a liposome complex. In some aspects, at least 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the liposomes are less than 5 microns in diameter. In various aspects, the composition further comprises at least one surfactant, such as, for example, polysorbate 20. In some aspects, at least about 5% of the total liposomal p-Ethoxy antisense drug product consists of surfactant and at least about 90% of the liposomes are less than 5 microns in diameter. In some aspects, at least about 15% of the total liposomal p-Ethoxy antisense drug product consists of surfactant and at least about 90% of the liposomes are less than 3 microns in diameter. In some aspects, the population of oligonucleotides are incorporated in the population of liposomes.

**[0015]** In one aspect, the oligonucleotides of a population each comprise about 21 nucleotides in length and have about 30% phosphodiester backbone linkages. In one aspect, the population of oligonucleotides maybe further incorporated into a liposome composition

comprising at least about 5% surfactant, wherein at least about 90% of said liposomes have a diameter of less than about 5 microns.

**[0016]** In one embodiment, pharmaceutical compositions are provided comprising a composition of oligonucleotides and phospholipids of the present embodiments and a pharmaceutically acceptable carrier. In some aspects, the composition further comprises a chemotherapeutic agent.

**[0017]** In one embodiment, methods are provided for delivering a therapeutically effective amount of an oligonucleotide to a cell comprising contacting the cell with a pharmaceutical composition of the present embodiments. In some aspects, the method is a method of treating hyperplasia, cancer, an autoimmune disease, or an infectious disease.

**[0018]** In one embodiment, methods are provided for treating a subject with cancer, an autoimmune disease, or an infectious disease comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition of the present embodiments. In some aspects, the subject is a human. In some aspects, the cancer is a bladder, blood, pancreas, bone, bone marrow, brain, breast, colon, esophagus, stomach, head and neck, kidney, liver, lung, prostate, skin, testis, tongue, ovary, or uterine cancer. In some aspects, the autoimmune disease is Lupus erythematosis, Sjogren's disease, Crohn's disease, diabetes mellitus, multiple sclerosis, or rheumatoid arthritis. In some aspects, the infectious disease is a bacterial infection, fungal infection, viral infection, or parasitic infection. In some aspects, the composition is administered subcutaneously, intravenously, or intraperitoneally. In some aspects, the method further comprises administering at least a second anticancer therapy to the subject. In some aspects, the second anticancer therapy is a surgical therapy, chemotherapy, radiation therapy, cryotherapy, hormone therapy, immunotherapy, or cytokine therapy.

**[0019]** An oligonucleotide includes an antisense nucleic acid molecule that specifically hybridizes to a nucleic acid molecule encoding a target protein or regulating the expression of the target protein. "Specific hybridization" means that the antisense nucleic acid molecule hybridizes to the targeted nucleic acid molecule and regulates its expression. Preferably, "specific hybridization" also means that no other genes or transcripts are affected. An oligonucleotide can be a single-stranded nucleic acid and may comprise 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more nucleobases.

In particular aspects the oligonucleotide can comprise 15 to 30, 19 to 25, 20 to 23, or 21 contiguous nucleobases. In certain embodiments, the oligonucleotide inhibits the translation of a gene that promotes growth of a cancerous or pre-cancerous or hyperplastic mammalian cell (*e.g.*, a human cell). An oligonucleotide may induce apoptosis in the cell, and/or inhibit the translation of an oncogene or other target gene. In certain embodiments, the oligonucleotide component comprises a single species of oligonucleotide. In other embodiments, the oligonucleotide component comprises a 2, 3, 4 or more species of oligonucleotide that target 1, 2, 3, 4, or more genes. The composition may further comprise a chemotherapeutic or other anti-cancer agent, which may or may not be incorporated in a lipid component or liposome of the invention. In further embodiments, the oligonucleotide component is incorporated within the liposome or lipid component.

**[0020]** "Entrap," "encapsulate," and "incorporate" refer to the lipid or liposome forming an impediment to free diffusion into solution by an association with or around an agent of interest, e.g., a liposome may encapsulate an agent within a lipid layer or within an aqueous compartment inside or between lipid layers. In certain embodiments, the composition is comprised in a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier may be formulated for administration to a human subject or patient.

**[0021]** In certain embodiments, the lipid component has an essentially neutral charge because it comprises a neutral phospholipid or a net neutral charge. In certain aspects a neutral phospholipid may be a phosphatidylcholine, such as DOPC, egg phosphatidylcholine ("EPC"), dilauryloylphosphatidylcholine ("DLPC"), dimyristoylphosphatidylcholine ("DMPC"), dipalmitoylphosphatidylcholine ("DPPC"), distearoylphosphatidylcholine ("DSPC"), 1-myristoyl-2-palmitoyl phosphatidylcholine ("MPPC"), 1-palmitoyl-2-myristoyl phosphatidylcholine ("PMPC"), 1-palmitoyl-2-stearoyl phosphatidylcholine ("PSPC"), 1-stearoyl-2-palmitoyl phosphatidylcholine ("SPPC"), dimyristyl phosphatidylcholine ("DMPC"), 1,2-distearoyl-sn-glycero-3-phosphocholine ("DAPC"), 1,2-diarachidoyl-sn-glycero-3-phosphocholine ("DBPC"), 1,2-dieicosenoyl-sn-glycero-3-phosphocholine ("DEPC"), palmitoyloeoyl phosphatidylcholine ("POPC"), lysophosphatidylcholine, or dilinoleoylphosphatidylcholine. In other aspects the neutral phospholipid can be a phosphatidylethanolamine, such as dioleoylphosphatidylethanolamine ("DOPE"), distearoylphophatidylethanolamine ("DSPE"), dimyristoyl phosphatidylethanolamine ("DMPE"), dipalmitoyl phosphatidylethanolamine ("DPPE"), palmitoyloeoyl

phosphatidylethanolamine ("POPE"), or lysophosphatidylethanolamine. In certain embodiments, the phospholipid component can comprise 1, 2, 3, 4, 5, 6, 7, 8, or more kinds or types of neutral phospholipid. In other embodiments, a phospholipid component can comprise 2, 3, 4, 5, 6 or more kinds or type of neutral phospholipids.

**[0022]** In certain embodiments, a lipid component can have an essentially neutral charge because it comprises a positively charged lipid and a negatively charged lipid. The lipid component may further comprise a neutrally charged lipid(s) or phospholipid(s). The positively charged lipid may be a positively charged phospholipid. The negatively charged lipid may be a negatively charged phospholipid. The negatively charged phospholipid may be a phosphatidylserine, such as dimyristoyl phosphatidylserine ("DMPS"), dipalmitoyl phosphatidylserine ("DPPS"), or brain phosphatidylserine ("BPS"). The negatively charged phospholipid may be a phosphatidylglycerol, such as dilauryloylphosphatidylglycerol ("DLPG"), dimyristoylphosphatidylglycerol ("DMPG"), dipalmitoylphosphatidylglycerol ("DPPG"), distearoylphosphatidylglycerol ("DSPG"), or dioleoylphosphatidylglycerol ("DOPG"). In certain embodiments, the composition further comprises cholesterol or polyethyleneglycol (PEG). In other embodiments, the composition is essentially free of cholesterol. In certain embodiments, a phospholipid is a naturally-occurring phospholipid. In other embodiments, a phospholipid is a synthetic phospholipid.

**[0023]** Liposomes can be made of one or more phospholipids, as long as the lipid material is substantially uncharged. It is important that the composition be substantially free of anionic and cationic phospholipids and cholesterol. Suitable phospholipids include phosphatidyl cholines and others that are well known to persons that are skilled in this field.

**[0024]** Another aspect of the present invention involves methods for delivering oligonucleotide to a cell comprising contacting the cell with a neutral lipid composition of the invention. The methods will provide an inventive composition in an effective amount. An effective amount is an amount of therapeutic component that attenuates, slows, reduces or eliminates a cell, condition, or disease state in a subject. The cell may be comprised in a subject or patient, such as a human. The method may further comprise a method of treating cancer or other hyperplastic condition. The cancer may have originated in the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestine, gum, head, kidney, liver, lung, nasopharynx, neck, prostate, skin, stomach, testis, tongue, or uterus. In certain embodiments, the method further comprises a method of treating a non-cancerous disease or

hyperplastic condition. The cell may be a pre-cancerous or a cancerous cell. In certain embodiments, the compositions and methods inhibit the growth of the cell, induce apoptosis in the cell, and/or inhibit the translation of an oncogene. The oligonucleotide may inhibit the translation of a gene that is overexpressed in the cancerous cell.

**[0025]** In certain embodiments, the methods of the invention further comprise administering an additional therapy to the subject. The additional therapy may comprise administering a chemotherapeutic (*e.g.*, paclitaxel or docetaxel), a surgery, a radiation therapy, and/or a gene therapy. In certain aspects the chemotherapy is docetaxel, paclitaxel, cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, busulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, raloxifene, estrogen receptor binding agents, taxol, gemcitabien, navelbine, farnesyl-protein tansferase inhibitors, transplatinum, 5-fluorouracil, vincristin, vinblastin, methotrexate, or combinations thereof. In certain embodiments the chemotherapy is a taxane such as docetaxal or paclitaxel. The chemotherapy can be delivered before, during, after, or combinations thereof relative to a neutral lipid composition of the invention. A chemotherapy can be delivered within 0, 1, 5, 10, 12, 20, 24, 30, 48, or 72 hours or more of the neutral lipid composition. The neutral lipid composition, the second anti-cancer therapy, or both the neutral lipid composition and the anti-cancer therapy can be administered intratumorally, intravenously, intraperitoneally, subcutaneously, orally or by various combinations thereof.

**[0026]** It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method or composition of the invention, and vice versa. Furthermore, compositions of the invention can be used to achieve the methods of the invention.

**[0027]** As used herein, "essentially free," in terms of a specified component, is used herein to mean that none of the specified component has been purposefully formulated into a composition and/or is present only as a contaminant or in trace amounts. The total amount of the specified component resulting from any unintended contamination of a composition is therefore well below 0.05%, preferably below 0.01%. Most preferred is a composition in which no amount of the specified component can be detected with standard analytical methods.

**[0028]** As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one.

**[0029]** The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." As used herein "another" may mean at least a second or more.

**[0030]** Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

**[0031]** Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

## DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

**[0032]** The present invention provides compositions and methods for delivery of an oligonucleotide (*e.g.*, an inhibitor of gene expression) to a cell *via* a lipid composition, in certain aspects a lipid composition with a net charge of about zero, *i.e.*, a neutral lipid composition. In certain embodiments the lipid composition is a non-charged liposome. These methods may be effectively used to treat a cancer.

### I. Lipids and Liposomes

**[0033]** "Liposomes" is used herein to mean lipid-containing vesicles having a lipid bilayer, as well as other lipid carrier particles that can entrap or incorporate antisense oligonucleotides. As such, liposome is a generic term encompassing a variety of unilamellar, multilamellar, and multivesicular lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. In addition, liposomes may have an undefined lamellar structure. Liposomes may be characterized as having vesicular structures with a phospholipid bilayer

membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). However, the present invention also encompasses compositions that have different structures in solution than the normal vesicular structure. For example, the lipids may assume a micellar structure or merely exist as non-uniform aggregates of lipid molecules.

**[0034]** Liposomes are a form of nanoparticles that are carriers for delivering a variety of drugs into a diseased tissue. Optimal liposome size depends on the target tissue. In tumor tissue, the vasculature is discontinuous, and pore sizes vary from 100 to 780 nm (Siwak *et al.*, 2002). By comparison, pore size in normal vascular endothelium is <2 nm in most tissues, and 6 nm in post-capillary venules. Negatively charged liposomes are thought to be more rapidly removed from circulation than neutral or positively charged liposomes; however, recent studies have indicated that the type of negatively charged lipid affects the rate of liposome uptake by the reticulo-endothelial system (RES). For example, liposomes containing negatively charged lipids that are not sterically shielded (phosphatidylserine, phosphatidic acid, and phosphatidylglycerol) are cleared more rapidly than neutral liposomes. Interestingly, cationic liposomes (1,2-dioleoyl-3-trimethylammonium-propane [DOTAP]) and cationic-liposome-DNA complexes are more avidly bound and internalized by endothelial cells of angiogenic blood vessels via endocytosis than anionic, neutral, or sterically stabilized neutral liposomes (Thurston *et al.*, 1998; Krasnici *et al.*, 2003). Cationic liposomes may not be ideal delivery vehicles for tumor cells because surface interactions with the tumor cells create an electrostatically derived binding-site barrier effect, inhibiting further association of the delivery systems with tumor spheroids (Kostarelos *et al.*, 2004). However, neutral liposomes appear to have better intratumoral penetration. Toxicity with specific liposomal preparations has also been a concern. Cationic liposomes elicit dose-dependent toxicity and pulmonary inflammation by promoting release of reactive oxygen intermediates, and this effect is more pronounced with multivalent cationic liposomes than monovalent cationic liposomes, such as DOTAP (Dokka *et al.*, 2000). Neutral and negative liposomes do not appear to exhibit lung toxicity (Guitierrez-Puente *et al.*, 1999). Cationic liposomes, while efficiently taking up nucleic acids, have had limited success for *in vivo* gene down-regulation, perhaps because of their stable intracellular nature and resultant failure to

release nucleic acid contents. Lipids with neutral charge or lipid compositions with a neutralized charge, *e.g.*, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), are used herein because of the neutral properties and success in delivering antisense oligonucleotides *in vivo*.

**[0035]** The present invention provides methods and compositions for associating an oligonucleotide, such as an antisense oligonucleotide, with a lipid and/or liposome. The oligonucleotide may be incorporated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome *via* a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. The liposome or liposome/oligonucleotide-associated compositions provided herein are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming aggregates that are not uniform in either size or shape.

### A. Lipids

**[0036]** Lipids are fatty substances that may be naturally occurring or synthetic. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds that are well known to those of skill in the art that contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes. An example is the lipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC).

**[0037]** Lipid compositions of the present invention may comprise phospholipids. In certain embodiments, a single kind or type of phospholipid may be used in the creation of lipid compositions, such as liposomes. In other embodiments, more than one kind or type of phospholipid may be used.

**[0038]** Phospholipids include glycerophospholipids and certain sphingolipids. Phospholipids include, but are not limited to, dioleoylphosphatidylycholine ("DOPC"), egg phosphatidylcholine ("EPC"), dilauryloylphosphatidylcholine ("DLPC"), dimyristoylphosphatidylcholine ("DMPC"), dipalmitoylphosphatidylcholine ("DPPC"), distearoylphosphatidylcholine ("DSPC"), 1-myristoyl-2-palmitoyl phosphatidylcholine

("MPPC"), 1-palmitoyl-2-myristoyl phosphatidylcholine ("PMPC"), 1-palmitoyl-2-stearoyl phosphatidylcholine ("PSPC"), 1-stearoyl-2-palmitoyl phosphatidylcholine ("SPPC"), dilauryloylphosphatidylglycerol ("DLPG"), dimyristoylphosphatidylglycerol ("DMPG"), dipalmitoylphosphatidylglycerol ("DPPG"), distearoylphosphatidylglycerol ("DSPG"), distearoyl sphingomyelin ("DSSP"), distearoylphophatidylethanolamine ("DSPE"), dioleoylphosphatidylglycerol ("DOPG"), dimyristoyl phosphatidic acid ("DMPA"), dipalmitoyl phosphatidic acid ("DPPA"), dimyristoyl phosphatidylethanolamine ("DMPE"), dipalmitoyl phosphatidylethanolamine ("DPPE"), dimyristoyl phosphatidylserine ("DMPS"), dipalmitoyl phosphatidylserine ("DPPS"), brain phosphatidylserine ("BPS"), brain sphingomyelin ("BSP"), dipalmitoyl sphingomyelin ("DPSP"), dimyristyl phosphatidylcholine ("DMPC"), 1,2-distearoyl-sn-glycero-3-phosphocholine ("DAPC"), 1,2-diarachidoyl-sn-glycero-3-phosphocholine ("DBPC"), 1,2-dieicosenoyl-sn-glycero-3-phosphocholine ("DEPC"), dioleoylphosphatidylethanolamine ("DOPE"), palmitoyloeoyl phosphatidylcholine ("POPC"), palmitoyloeoyl phosphatidyletlianolamine ("POPE"), lysophosphatidylcholine, lysophosphatidylethanolamine, and dilinoleoylphosphatidylcholine.

**[0039]** Phospholipids include, for example, phosphatidylcholines, phosphatidylglycerols, and phosphatidylethanolamines; because phosphatidylethanolamines and phosphatidylcholines are non-charged under physiological conditions (*i.e.*, at about pH 7), these compounds may be particularly useful for generating neutral liposomes. In certain embodiments, the phospholipid DOPC is used to produce non-charged liposomes or lipid compositions. In certain embodiments, a lipid that is not a phospholipid (*e.g.*, a cholesterol) can also be used

**[0040]** Phospholipids may be from natural or synthetic sources. However, phospholipids from natural sources, such as egg or soybean phosphatidylcholine, brain phosphatidic acid, brain or plant phosphatidylinositol, heart cardiolipin, and plant or bacterial phosphatidylethanolamine, are not used in certain embodiments as the primary phosphatide (*i.e.*, constituting 50% or more of the total phosphatide composition) because this may result in instability and leakiness of the resulting liposomes.

### B. Neutral Liposomes

**[0041]** "Neutral liposomes or lipid composition" or "non-charged liposomes or lipid composition," as used herein, are defined as liposomes or lipid compositions having one or more lipids that yield an essentially-neutral net charge (substantially non-charged). In certain

embodiments, neutral liposomes or lipid compositions may include mostly lipids and/or phospholipids that are themselves neutral. In certain embodiments, amphipathic lipids may be incorporated into or used to generate neutral liposomes or lipid compositions. For example, a neutral liposome may be generated by combining positively and negatively charged lipids so that those charges substantially cancel one another, thereby yielding an essentially-neutral net charge. By "essentially neutral" or "essentially non-charged," it is meant that few, if any, lipids within a given population (*e.g.*, a population of liposomes) include a charge that is not canceled by an opposite charge of another component (*e.g.*, fewer than 10% of components include a non-canceled charge, more preferably fewer than 5%, and most preferably fewer than 1%). In certain embodiments of the present invention, a composition may be prepared wherein the lipid component of the composition is essentially neutral but is not in the form of liposomes.

**[0042]** The size of the liposomes varies depending on the method of synthesis. A liposome suspended in an aqueous solution is generally in the shape of a spherical vesicle, and may have one or more concentric layers of lipid bilayer molecules. Each layer consists of a parallel array of molecules represented by the formula XY, wherein X is a hydrophilic moiety and Y is a hydrophobic moiety. In aqueous suspension, the concentric layers are arranged such that the hydrophilic moieties tend to remain in contact with an aqueous phase and the hydrophobic regions tend to self-associate. For example, when aqueous phases are present both within and without the liposome, the lipid molecules may form a bilayer, known as a lamella, of the arrangement XY-YX. Aggregates of lipids may form when the hydrophilic and hydrophobic parts of more than one lipid molecule become associated with each other. The size and shape of these aggregates will depend upon many different variables, such as the nature of the solvent and the presence of other compounds in the solution.

**[0043]** Liposomes within the scope of the present invention can be prepared in accordance with known laboratory techniques, such as, for example, the method of Bangham *et al.* (1965), the contents of which are incorporated herein by reference; the method of Gregoriadis (1979), the contents of which are incorporated herein by reference; the method of Deamer and Uster (1983), the contents of which are incorporated by reference; and the reverse-phase evaporation method as described by Szoka and Papahadjopoulos (1978). The aforementioned methods differ in their respective abilities to entrap aqueous material and their respective aqueous space-to-lipid ratios.

**[0044]** In certain embodiments, a neutral liposome may be used to deliver an oligonucleotide, such as an antisense oligonucleotide. The neutral liposome may contain a single species of oligonucleotide directed to the suppression of translation of a single gene, or the neutral liposome may contain multiple species of oligonucleotides that are directed to the suppression of translation of multiple genes. Further, the neutral liposome may also contain a chemotherapeutic in addition to the oligonucleotide; thus, in certain embodiments, a chemotherapeutic and an oligonucleotide may be delivered to a cell (*e.g.*, a cancerous cell in a human subject) in the same or separate compositions.

**[0045]** Dried lipids or lyophilized liposomes may be dehydrated and reconstituted at an appropriate concentration with a suitable solvent (*e.g.*, DPBS or Hepes buffer). The mixture may then be vigorously shaken in a vortex mixer. The liposomes may be resuspended at an appropriate total phospholipid concentration (*e.g.*, about 10-200 mM). Unencapsulated oligonucleotide may be removed by centrifugation at 29,000 g and the liposomal pellets washed. Alternatively, the unencapsulated oligonucleotides may be removed by dialyzing against an excess of solvent. The amount of oligonucleotide encapsulated can be determined in accordance with standard methods.

## II. Inhibition of Gene Expression

**[0046]** An inhibitory oligonucleotide can inhibit the transcription or translation of a gene in a cell. An oligonucleotide may be from 5 to 50 or more nucleotides long, and in certain embodiments from 7 to 30 nucleotides long. In certain embodiments, the oligonucleotide maybe 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides long. The oligonucleotide may comprise a nucleic acid and/or a nucleic acid analog. Typically, an inhibitory oligonucleotide will inhibit the translation of a single gene within a cell; however, in certain embodiments, an inhibitory oligonucleotide may inhibit the translation of more than one gene within a cell.

**[0047]** Within an oligonucleotide, the components of the oligonucleotide need not be of the same type or homogenous throughout (*e.g.*, an oligonucleotide may comprise a nucleotide and a nucleic acid or nucleotide analog). In certain embodiments of the present invention, the oligonucleotide may comprise only a single nucleic acid or nucleic acid analog. The inhibitory oligonucleotide may comprise 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18,

19, 20, 25, 30 or more contiguous nucleobases, including all ranges therebetween, that hybridize with a complementary nucleic acid to form a double-stranded structure.

## III. Nucleic Acids

**[0048]** The present invention provides methods and compositions for the delivery of an oligonucleotide *via* neutral liposomes. Because an oligonucleotide is composed of a nucleic acid, methods relating to nucleic acids (*e.g.*, production of a nucleic acid, modification of a nucleic acid, *etc.*) may also be used with regard to an oligonucleotide.

**[0049]** The term "nucleic acid" is well known in the art. A "nucleic acid" as used herein generally refers to a molecule (*i.e.*, a strand) of DNA, RNA, or a derivative or analog thereof, comprising a nucleobase. These definitions refer to a single-stranded or double-stranded nucleic acid. Double-stranded nucleic acids may be formed by fully complementary binding; however, in some embodiments, a double-stranded nucleic acid may be formed by partial or substantial complementary binding. As used herein, a single-stranded nucleic acid may be denoted by the prefix "ss" and a double-stranded nucleic acid by the prefix "ds."

### A. Nucleobases

**[0050]** As used herein a "nucleobase" refers to a heterocyclic base, such as, for example, a naturally occurring nucleobase (*i.e.*, an A, T, G, C or U) found in at least one naturally occurring nucleic acid (*i.e.*, DNA and RNA), and naturally or non-naturally occurring derivative(s) and analogs of such a nucleobase. A nucleobase generally can form one or more hydrogen bonds (*i.e.*, "anneal" or "hybridize") with at least one naturally occurring nucleobase in a manner that may substitute for naturally occurring nucleobase pairing (*e.g.*, the hydrogen bonding between A and T, G and C, and A and U). A nucleobase may be comprised in a nucleoside or nucleotide, using any chemical or natural synthesis method described herein or known to one of ordinary skill in the art.

**[0051]** "Purine" and/or "pyrimidine" nucleobase(s) encompass naturally occurring purine and/or pyrimidine nucleobases and also derivative(s) and analog(s) thereof, including but not limited to, a purine or pyrimidine substituted by one or more of an alkyl, carboxyalkyl, amino, hydroxyl, halogen (*i.e.*, fluoro, chloro, bromo, or iodo), thiol, or alkylthiol moiety. Preferred alkyl (*e.g.*, alkyl, caboxyalkyl, *etc.*) moieties comprise of from about 1, about 2, about 3, about 4, about 5, to about 6 carbon atoms. Other non-limiting

examples of a purine or pyrimidine include a deazapurine, a 2,6-diaminopurine, a 5-fluorouracil, a xanthine, a hypoxanthine, a 8-bromoguanine, a 8-chloroguanine, a bromothyline, a 8-aminoguanine, a 8-hydroxyguanine, a 8-methylguanine, a 8-thioguanine, an azaguanine, a 2-aminopurine, a 5-ethylcytosine, a 5-methylcyosine, a 5-bromouracil, a 5-ethyluracil, a 5-iodouracil, a 5-chlorouracil, a 5-propyluracil, a thiouracil, a 2-methyladenine, a methylthioadenine, a N,N-diemethyladenine, an azaadenines, a 8-bromoadenine, a 8-hydroxyadenine, a 6-hydroxyaminopurine, a 6-thiopurine, a 4-(6-aminohexyl/cytosine), and the like. Purine and pyrimidine derivatives or analogs include, but are not limited to (abbreviation/modified base description): ac4c/4-acetylcytidine, Mam5s2u/5-methoxyaminomethyl-2-thiouridine, Chm5u/5-(carboxyhydroxylmethyl) uridine, Man q/Beta, D-mannosylqueosine, Cm/2′-O-methylcytidine, Mcm5s2u/5-methoxycarbonylmethyl-2-thiouridine, Cmnm5s2u/5-carboxymethylamino-methyl-2-thioridine, Mcm5u/5-methoxycarbonylmethyluridine, Cmnm5u/5-carboxymethylaminomethyluridine, Mo5u/5-methoxyuridine, D/Dihydrouridine, Ms2i6a, 2-methylthio-N6-isopentenyladenosine, Fm/2′-O-methylpseudouridine, Ms2t6a/N-((9-beta-D-ribofuranosyl-2-methylthiopurine-6-yl)carbamoyl)threonine, Gal q/Beta,D-galactosylqueosine, Mt6a/N-((9-beta-D-ribofuranosylpurine-6-yl)N-methyl-carbamoyl)threonine, Gm/2′-O-methylguanosine, Mv/Uridine-5-oxyacetic acid methylester, I/Inosine, o5u/Uridine-5-oxyacetic acid (v), I6a/N6-isopentenyladenosine, Osyw/Wybutoxosine, m1a/1-methyladenosine, P/Pseudouridine, m1f/1-methylpseudouridine, Q/Queosine, m1g/1-methylguanosine, s2c/2-thiocytidine, m1I/1-methylinosine, s2t/5-methyl-2-thiouridine, m22g/2,2-dimethylguanosine, s2u/2-thiouridine, m2a/2-methyladenosine, s4u/4-thiouridine, m2g/2-methylguanosine, T/5-methyluridine, m3c/3-methylcytidine, t6a/N-((9-beta-D-ribofuranosylpurine-6-yl)carbamoyl)threonine, m5c/5-methylcytidine, Tm/2′-O-methyl-5-methyluridine, m6a/N6-methyladenosine, Um/2′-O-methyluridine, m7g/7-methylguanosine, Yw/Wybutosine, Mam5u/5-methylaminomethyluridine, or X/3-(3-amino-3-carboxypropyl)uridine, (acp3)u.

### B. Nucleosides

**[0052]** As used herein, a “nucleoside” refers to an individual chemical unit comprising a nucleobase covalently attached to a nucleobase linker moiety. A non-limiting example of a “nucleobase linker moiety” is a sugar comprising 5-carbon atoms (*i.e.*, a “5-carbon sugar”), including but not limited to a deoxyribose, a ribose, an arabinose, or a derivative or an analog of a 5-carbon sugar. Non-limiting examples of a derivative or an

analog of a 5-carbon sugar include a 2′-fluoro-2′-deoxyribose or a carbocyclic sugar where a carbon is substituted for an oxygen atom, in the sugar ring. As used herein, a "moiety" generally refers to a smaller chemical or molecular component of a larger chemical or molecular structure.

**[0053]** Different types of covalent attachment(s) of a nucleobase to a nucleobase linker moiety are known in the art. By way of non-limiting example, a nucleoside comprising a purine (*i.e.*, A or G) or a 7-deazapurine nucleobase typically comprises a covalent attachment of the 9 position of the purine or 7-deazapurine to a 1′-position of a 5-carbon sugar. In another non-limiting example, a nucleoside comprising a pyrimidine nucleobase (*i.e.*, C, T, or U) typically comprises a covalent attachment of the 1 position of the pyrimidine to a 1′-position of a 5-carbon sugar (Kornberg and Baker, 1992).

### C. Nucleotides

**[0054]** As used herein, a "nucleotide" refers to a nucleoside further comprising a "backbone linkage." A backbone linkage generally covalently attaches a nucleotide to another molecule comprising a nucleotide, or to another nucleotide to form a nucleic acid. The "backbone linkage" in naturally occurring nucleotides typically comprises a phosphate moiety (*e.g.*, a phosphodiester backbone linkage), which is covalently attached to a 5-carbon sugar. The attachment of the backbone moiety typically occurs at either the 3′- or 5′-position of the 5-carbon sugar. However, other types of attachments are known in the art, particularly when a nucleotide comprises derivatives or analogs of a naturally occurring 5-carbon sugar or phosphate moiety.

### D. Nucleic Acid Analogs

**[0055]** A nucleic acid may comprise, or be composed entirely of, a derivative or analog of a nucleobase, a nucleobase linker moiety, and/or backbone linkage that may be present in a naturally occurring nucleic acid. As used herein a "derivative" refers to a chemically modified or altered form of a naturally occurring molecule, while the terms "mimic" or "analog" refer to a molecule that may or may not structurally resemble a naturally occurring molecule or moiety, but possesses similar functions. Nucleobase, nucleoside, and nucleotide analogs or derivatives are well known in the art.

**[0056]** Non-limiting examples of nucleosides, nucleotides, or nucleic acids comprising 5-carbon sugar and/or backbone linkage derivatives or analogs, include those in

U.S. Pat. No. 5,681,947 which describes oligonucleotides comprising purine derivatives that form triple helixes with and/or prevent expression of dsDNA; U.S. Pat. Nos. 5,652,099 and 5,763,167 which describe nucleic acids incorporating fluorescent analogs of nucleosides found in DNA or RNA, particularly for use as fluorescent nucleic acids probes; U.S. Pat. No. 5,614,617 which describes oligonucleotide analogs with substitutions on pyrimidine rings that possess enhanced nuclease stability; U.S. Pat. Nos. 5,670,663, 5,872,232 and 5,859,221 which describe oligonucleotide analogs with modified 5-carbon sugars (*i.e.*, modified 2′-deoxyfuranosyl moieties) used in nucleic acid detection; U.S. Pat. No. 5,446,137 which describes oligonucleotides comprising at least one 5-carbon sugar moiety substituted at the 4′ position with a substituent other than hydrogen that can be used in hybridization assays; U.S. Pat. No. 5,886,165 which describes oligonucleotides with both deoxyribonucleotides with 3′-5′ backbone linkages and ribonucleotides with 2′-5′ backbone linkages; U.S. Pat. No. 5,714,606 which describes a modified backbone linkage wherein a 3′-position oxygen of the backbone linkage is replaced by a carbon to enhance the nuclease resistance of nucleic acids; U.S. Pat. No. 5,672,697 which describes oligonucleotides containing one or more 5′ methylene phosphonate backbone linkages that enhance nuclease resistance; U.S. Pat. Nos. 5,466,786 and 5,792,847 which describe the linkage of a substituent moiety that may comprise a drug or label to the 2′ carbon of an oligonucleotide to provide enhanced nuclease stability and ability to deliver drugs or detection moieties; U.S. Pat. No. 5,223,618 which describes oligonucleotide analogs with a 2 or 3 carbon backbone linkage attaching the 4′ position and 3′ position of adjacent 5-carbon sugar moiety to enhanced cellular uptake, resistance to nucleases, and hybridization to target RNA; U.S. Pat. No. 5,470,967 which describes oligonucleotides comprising at least one sulfamate or sulfamide backbone linkage that are useful as nucleic acid hybridization probes; U.S. Pat. Nos. 5,378,825, 5,777,092, 5,623,070, 5,610,289 and 5,602,240 which describe oligonucleotides with a three or four atom backbone linkage moiety replacing the phosphodiester backbone linkage used for improved nuclease resistance, cellular uptake, and regulating RNA expression; U.S. Pat. No. 5,858,988 which describes hydrophobic carrier agent attached to the 2′-O position of oligonucleotides to enhance their membrane permeability and stability; U.S. Pat. No. 5,214,136 which describes oligonucleotides conjugated to anthraquinone at the 5′ terminus that possess enhanced hybridization to DNA or RNA; enhanced stability to nucleases; U.S. Pat. No. 5,700,922 which describes PNA-DNA-PNA chimeras wherein the DNA comprises 2′-deoxy-erythro-pentofaranosyl nucleotides for enhanced nuclease resistance, binding affinity, and ability to activate RNase H; U.S. Pat. No. 5,708,154 which describes RNA

linked to a DNA to form a DNA-RNA hybrid; U.S. Pat. No. 5,908,845 which describes polyether nucleic acids wherein one or more nucleobases are linked to chiral carbon atoms in a polyether backbone; U.S. Pat. Nos. 5,786,461, 5,891,625, 5,786,461, 5,773,571, 5,766,855, 5,736,336, 5,719,262, 5,714,331, 5,539,082, and WO 92/20702 which describe peptide nucleic acids (PNA or peptide-based nucleic acid analog; or PENAM) that generally comprise one or more nucleotides or nucleosides that comprise a nucleobase moiety, a nucleobase linker moiety that is not a 5-carbon sugar (*e.g.*, aza nitrogen atoms, amido and/or ureido tethers), and/or a backbone linkage that is not a phosphate backbone linkage (*e.g.*, aminoethylglycine, polyamide, polyethyl, polythioamide, polysulfinamide, or polysulfonamide backbone linkage); and U.S. Pat. No. 5,855,911 which describes the hydrophobic, nuclease resistant p-ethoxy backbone linkage.

**[0057]** Other modifications and uses of nucleic acid analogs are known in the art, and it is anticipated that these techniques and types of nucleic acid analogs may be used with the present invention.

### E. Preparation of Nucleic Acids

**[0058]** A nucleic acid may be made by any technique known to one of ordinary skill in the art, such as chemical synthesis, enzymatic production or biological production. Non-limiting examples of a synthetic nucleic acid (*e.g.*, a synthetic oligonucleotide) include a nucleic acid made by *in vitro* chemical synthesis using phosphotriester, phosphite, or phosphoramidite chemistry and solid phase techniques, such as described in EP 266,032, incorporated herein by reference, or by deoxynucleoside H-phosphonate intermediates as described by Froehler *et al.* (1986) and U.S. Pat. No. 5,705,629, each incorporated herein by reference. In the methods of the present invention, one or more species of oligonucleotide may be used. Various mechanisms of oligonucleotide synthesis have been disclosed in, for example, U.S. Pat. Nos. 4,659,774, 4,816,571, 5,141,813, 5,264,566, 4,959,463, 5,428,148, 5,554,744, 5,574,146, 5,602,244, each of which is incorporated herein by reference.

### F. Purification of Nucleic Acids

**[0059]** A nucleic acid may be purified on polyacrylamide gels, cesium chloride centrifugation gradients, or by any other means known to one of ordinary skill in the art (see for example, Sambrook *et al.* (2001), incorporated herein by reference).

**[0060]** In certain embodiments, the present invention concerns a nucleic acid that is an isolated nucleic acid. As used herein, the term "isolated nucleic acid" refers to a nucleic acid molecule (*e.g.*, an RNA or DNA molecule) that has been isolated free of, or is otherwise free of, the bulk of the total genomic and transcribed nucleic acids of one or more cells. In certain embodiments, "isolated nucleic acid" refers to a nucleic acid that has been isolated free of, or is otherwise free of, the bulk of cellular components or *in vitro* reaction components, such as, for example, macromolecules, such as lipids or proteins, small biological molecules, and the like.

### G. Hybridization

**[0061]** As used herein, "hybridization," "hybridize(s)," or "capable of hybridizing" is understood to mean the forming of a double or triple stranded molecule or a molecule with partial double or triple stranded nature. The term "anneal" as used herein is synonymous with "hybridize."

**[0062]** As used herein "stringent condition(s)" or "high stringency" are those conditions that allow hybridization between or within one or more nucleic acid strand(s) containing complementary sequence(s), but precludes hybridization of random sequences. Stringent conditions tolerate little, if any, mismatch between a nucleic acid and a target strand. Such conditions are well known to those of ordinary skill in the art, and are preferred for applications requiring high selectivity.

**[0063]** Stringent conditions may comprise low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of about 50°C to about 70°C. It is understood that the temperature and ionic strength of a desired stringency are determined in part by the length of the particular nucleic acid(s), the length and nucleobase content of the target sequence(s), the charge composition of the nucleic acid(s), and to the presence or concentration of formamide, tetramethylammonium chloride, or other solvent(s) in a hybridization mixture.

**[0064]** It is also understood that these ranges, compositions and conditions for hybridization are mentioned by way of non-limiting examples only, and that the desired stringency for a particular hybridization reaction is often determined empirically by comparison to one or more positive or negative controls. Depending on the application envisioned it is preferred to employ varying conditions of hybridization to achieve varying

degrees of selectivity of a nucleic acid towards a target sequence. In a non-limiting example, identification or isolation of a related target nucleic acid that does not hybridize to a nucleic acid under stringent conditions may be achieved by hybridization at low temperature and/or high ionic strength. Such conditions are termed "low stringency" or "low stringency conditions," and non-limiting examples of low stringency include hybridization performed at about 0.15 M to about 0.9 M NaCl at a temperature range of about 20°C to about 50°C. Of course, it is within the skill of one in the art to further modify the low or high stringency conditions to suite a particular application.

## IV. Methods of Manufacturing Liposomal p-Ethoxy Antisense Drug Product

**[0065]** The liposomal p-ethoxy antisense drug product is composed of two cGMP products, both of which have a FDA-required Certificate of Analysis with FDA-approved release criteria. The raw materials, solvents, and final drug product are described herein. When manufactured, the drug product is a lyophilized crystal or powder of amber or white color that comprises the following materials: oligonucleotide (*e.g.*, p-ethoxy antisense drug substance), neutral lipids (*e.g.*, DOPC), and surfactant (*e.g.*, polysorbate 20). In preparation for administration to a patient, normal saline is added to the vial, at which time liposomes are formed with the p-ethoxy antisense incorporated into the interior.

**[0066]** Specific physical properties (*e.g.*, solubility and hydrophobicity, which then affect drug product solubility in saline, incorporation of oligo into liposomes, and liposome particle size) of the finished product can be defined using a pre-determined p-ethoxy and phosphodiester amidite raw material mix during production of the p-ethoxy antisense drug substance. Increasing the number of p-ethoxy molecules in the backbone of the oligonucleotide causes the molecule to be more hydrophobic (which results in larger liposome particles), less polar, and less soluble. As the oligonucleotide becomes less soluble due to a greater number of p-ethoxy backbone linkages, the reconstituted solution becomes whiter until particulates form as hydrophobicity becomes too high.

**[0067]** The effect of the surfactant (polysorbate 20) on liposome particle size was determined by titrating the amount of surfactant. In the absence of polysorbate 20, only 2.8% of the particles had a diameter of 300 nm or less. In the presence of 1x polysorbate 20 (about 5% of the total liposomal p-ethoxy antisense drug product), 12.5% of the particles had a diameter of 300 nm or less. With the addition of 3x-10x polysorbate 20, around 20% of the

particles had a diameter of 300 nm or less. Thus an increase in surfactant from 1x to 3x results in a decrease in particle size.

## V. Methods of Testing Liposomal P-ethoxy Antisense Drug Product

**[0068]** *Visual Inspection of Manufactured Drug Product*: After manufacturing, a sample vial containing drug product is selected and visually inspected. The absence of liquid is mandatory, and then amber crystals at the bottom of the vial are acceptable, and increasing in acceptance to a white, flocculated powder or appearance, the best result. The white appearance indicates a better drying process, with a high surface area to mass ratio, which is very conducive to reconstitution for use.

**[0069]** *Visual Inspection of Reconstituted Drug Ready for Patient IV*: Normal saline is added to a vial containing the manufactured Liposomal P-ethoxy Antisense Drug Product and shaken to reconstitute into a solution with the drug crystal or powder completely dissolved. Three main observations are made: 1) that the crystal or powder is completely dissolved, 2) there are no white clumps of undissolved material, and 3) the appearance is a milky white or skim milk appearance. The bluer the appearance of the reconstituted liquid, the better, as this signals a smaller liposome particle size that reflects light in the blue spectrum.

**[0070]** *Mass Spectrometry*: Mass spectrometry (mass spec) is used to display the profile of the various masses in a sample. When p-ethoxy antisense material is produced, a mass spec is run on the sample. The result shows peaks of material present on a grid that has increasing mass on the "x" axis to the right, and relative mass abundance on the "y" axis increasing upward. The profile from a sample is analyzed to determine the relative quantity of p-ethoxy backbones in the p-ethoxy sample, recognizing that the profile of peaks represents (starting farthest to the right), full length material with all backbones comprised of the p-ethoxy linkage, the next peak moving left a full length with one backbone with a p-ethoxy deletion (and therefore, the ethyl being knocked off and the result being a normal phosphodiester backbone linkage), and continuing. The mass spec pattern shifted to the right represents a p-ethoxy sample having more p-ethoxy backbones, and therefore having the properties of being more hydrophobic and less soluble; and likewise, shifted to the left having the opposite effects. Inspection of the mass spec chart of a sample also can be used to

determine if filtration during manufacturing produces any adverse effects on oligonucleotide composition present in the filtered drug product.

**[0071]** *UV Testing*: Ultraviolent light testing is used to determine the mass of oligonucleotide present in a sample. Oligonucleotides absorb light in the 260 nanometer range. As a result, UV testing of the finished reconstituted drug product has come to be used as a method in determining the quantity of oligonucleotide drug substance in a vial of drug product. In terms of manufacturing development and innovations, UV testing was used to determine if there were problems experienced during filtration in manufacturing or poor solubility of the p-ethoxy antisense drug substance, resulting in less oligonucleotide in solution and therefore a lower UV reading. The method will be validated and likely become part of the final product release testing.

**[0072]** *Liposome Particle Size*: A vial of finished drug product is reconstituted and tested for liposome particle size. The result is often a roughly normal distribution, having a central point, tails and average values or a roughly normal distribution of the majority of the particles and smaller, secondary peaks of the smaller liposomes particles resulting from second-order particle formation effects. It is important that liposome particles not be too large, as they may create adverse effects in patients (for example, create blood flow problems in smaller blood vessels in the lungs). As a result, the drug product release criteria include that particle size testing show that 90% of liposomes be about 5 microns or less in size or about 3 micron or less in size. In addition, smaller liposomes are preferred because they will have better uptake into cells, and secondly, smaller liposomes can penetrate vascular pores, thereby allowing the liposomes to penetrate inside tumors, increasing treatment effectiveness of a Liposomal P-ethoxy Antisense Drug Product.

## VI. Methods of Treatment

**[0073]** Certain aspects of the present invention provide an oligonucleotide–lipid complex *(e.g.*, an oligonucleotide incorporated into a non-charged liposome) for treating diseases, such as cancer, autoimmune disease, or infectious disease. Particularly, the oligonucleotide may have a sequence that allows for base pairing with a human nucleotide sequence and thus may inhibit the expression of a protein encoded by the human nucleotide sequence.

**[0074]** "Treatment" and "treating" refer to administration or application of a therapeutic agent to a subject or performance of a procedure or modality on a subject for the purpose of obtaining a therapeutic benefit of a disease or health-related condition. For example, a treatment may include administration of a pharmaceutically effective amount of an oligonucleotide–lipid complex.

**[0075]** "Subject" and "patient" refer to either a human or non-human, such as primates, mammals, and vertebrates. In particular embodiments, the subject is a human.

**[0076]** The term "therapeutic benefit" or "therapeutically effective" as used throughout this application refers to anything that promotes or enhances the well-being of the subject with respect to the medical treatment of this condition. This includes, but is not limited to, a reduction in the frequency or severity of the signs or symptoms of a disease. For example, treatment of cancer may involve, for example, a reduction in the size of a tumor, a reduction in the invasiveness of a tumor, reduction in the growth rate of the cancer, or prevention of metastasis. Treatment of cancer may also refer to prolonging survival of a subject with cancer. Treatment of an autoimmune disease may involve, for example, reducing the expression of a self-antigen against which there is an undesired immune response, inducing tolerance of a self-antigen against which there is an undesired immune response, or inhibiting the immune response towards the self-antigen. Treatment of an infectious disease may involve, for example, eliminate the infectious agent, reduce the level of the infectious agent, or maintain the level of the infectious agent at a certain level.

**[0077]** Tumors for which the present treatment methods are useful include any malignant cell type, such as those found in a solid tumor, a hematological tumor, metastatic cancer, or non-metastatic cancer. Exemplary solid tumors can include, but are not limited to, a tumor of an organ selected from the group consisting of pancreas, colon, cecum, esophagus, gastrointestine, gum, liver, skin, stomach, testis, tongue, uterus, stomach, brain, head, neck, ovary, kidney, larynx, sarcoma, bone, lung, bladder, melanoma, prostate, and breast. Exemplary hematological tumors include tumors of the bone marrow, T or B cell malignancies, leukemias, lymphomas, blastomas, myelomas, and the like. Further examples of cancers that may be treated using the methods provided herein include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, leukemia, squamous cell cancer, lung cancer (including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung), cancer of the peritoneum, hepatocellular cancer,

gastric or stomach cancer (including gastrointestinal cancer and gastrointestinal stromal cancer), pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, various types of head and neck cancer, melanoma, superficial spreading melanoma, lentigo malignant melanoma, acral lentiginous melanomas, nodular melanomas, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's macroglobulinemia), chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), Hairy cell leukemia, multiple myeloma, acute myeloid leukemia (AML) and chronic myeloblastic leukemia.

**[0078]** The cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; androblastoma,

malignant; sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malignant melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; hodgkin's disease; hodgkin's; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

**[0079]** Autoimmune diseases for which the present treatment methods are useful include, without limitation, spondyloarthropathy, ankylosing spondylitis, psoriatic arthritis, reactive arthritis, enteropathic arthritis, diabetes mellitus, celiac disease, autoimmune thyroid disease, autoimmune liver disease, Addison’s disease, transplant rejection, graft vs. host disease, host vs. graft disase, ulcerative colitis, Crohn’s disease, irritable bowel disease,

inflammatory bowel disease, rheumatoid arthritis, juvenile rheumatoid arthritis, familial Mediterranean fever, amyotrophic lateral sclerosis, Sjogren's syndrome, early arthritis, viral arthritis, multiple sclerosis, or psoriasis. The diagnosis and treatment of these diseases are well documented in the literature.

**[0080]** Infectious diseases for which the present treatment methods are useful include, without limitation, bacterial infections, viral infections, fungal infections, and parasitic infections. Exemplary viral infections include hepatitis B virus, hepatitis C virus, human immunodeficiency virus 1, human immunodeficiency virus 2, human papilloma virus, herpes simplex virus 1, herpes simplex virus 2, herpes zoster, varicella zoster, coxsackievirus A16, cytomegalovirus, ebola virus, enterovirus, Epstein-Barr virus, hanta virus, hendra virus, viral meningitis, respiratory syncytial virus, rotavirus, west nile virus, adenovirus, and influenza virus infections. Exemplary bacterial infections include *Chlamydia trachomatis*, *Listeria monocytogenes*, *Helicobacter pylori*, *Escherichia coli*, *Borelia burgdorferi*, *Legionella pneumophilia*, *Mycobacteria* sps (e.g., *M. tuberculosis*, *M. avium*, *M. intraceliuiar e*, *M. kansaii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitides*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic sps.), *Streptococcus pneumoniae*, pathogenic *Campylobacter* sp., *Enterococcus* sp., *Haemophilus influenzae*, *Bacillus anthracis*, *Corynebacterium diphtheriae*, *corynebacterium* sp., *Erysipelothrix rhusiopathiae*, *Clostridium perfringers*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides* sp., *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidium*, *Treponema pertenue*, *Leptospira*, *Rickettsia*, *Actinomyces israelli*, *Shigella* sps (e.g., *S.flexneri*, *S. sonnei*, *S. dysenteriae*), and *Salmonella* spp infections. Exemplary fungal infections include *Candida albicans*, *Candida glabrata*, *Aspergillus fumigatus*, *Aspergillus terreus*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, and *Chlamydia irachomatis* infections.

**[0081]** The oligonucleotide–lipid complex may be used herein as an antitumor, antiviral, antibacterial, antifungal, antiparasite, or anti-autoimmune agent in a variety of modalities. In a particular embodiment, the invention contemplates methods of using an oligonucleotide–lipid complex comprises contacting a population of diseased cells with a

therapeutically effective amount of an oligonucleotide–lipid complex for a time period sufficient to inhibit or reverse disease.

**[0082]** In one embodiment, the contacting *in vivo* is accomplished by administering, by intravenous, intraperitoneal, subcutaneous, or intratumoral injection, a therapeutically effective amount of a physiologically tolerable composition comprising an oligonucleotide–lipid complex of this invention to a patient. The oligonucleotide–lipid complex can be administered parenterally by injection or by gradual infusion over time.

**[0083]** Therapeutic compositions comprising oligonucleotide–lipid complex are conventionally administered intravenously or subcutaneously, such as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent, *i.e.*, carrier, or vehicle.

**[0084]** The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the route of administration. Suitable regimes for initial and booster administration are also contemplated and are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Exemplary multiple administrations are described herein and are particularly preferred to maintain continuously high serum and tissue levels of polypeptide. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for *in vivo* therapies are contemplated.

**[0085]** It is contemplated that an oligonucleotide of the invention can be administered systemically or locally to treat disease, such as to inhibit tumor cell growth or to kill cancer cells in cancer patients with locally advanced or metastatic cancers. They can be administered intravenously, intrathecally, subcutaneously, and/or intraperitoneally. They can be administered alone or in combination with anti-proliferative drugs. In one embodiment,

they are administered to reduce the cancer load in the patient prior to surgery or other procedures. Alternatively, they can be administered after surgery to ensure that any remaining cancer (*e.g.*, cancer that the surgery failed to eliminate) does not survive.

**[0086]** A therapeutically effective amount of an oligonucleotide is a predetermined amount calculated to achieve the desired effect, *i.e.*, to inhibit the expression of a target protein. Thus, the dosage ranges for the administration of oligonucleotides of the invention are those large enough to produce the desired effect. The dosage should not be so large as to cause adverse side effects, such as hyperviscosity syndromes, pulmonary edema, congestive heart failure, neurological effects, and the like. Generally, the dosage will vary with age of, condition of, sex of, and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any complication.

**[0087]** A composition of the present invention is preferably administered to a patient parenterally, for example by intravenous, intraarterial, intramuscular, intralymphatic, intraperitoneal, subcutaneous, intrapleural, or intrathecal injection, or may be used ex vivo. Preferred dosages are between 5-25 mg/kg. The administration is preferably repeated on a timed schedule until the cancer disappears or regresses, and may be in conjunction with other forms of therapy.

## VII. Pharmaceutical Preparations

**[0088]** A pharmaceutical composition comprising the liposomes will usually include a sterile, pharmaceutically acceptable carrier or diluent, such as water or saline solution.

**[0089]** Where clinical application of non-charged lipid component (*e.g.*, in the form of a liposome) containing an oligonucleotide is undertaken, it will generally be beneficial to prepare the lipid complex as a pharmaceutical composition appropriate for the intended application. This will typically entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One may also employ appropriate buffers to render the complex stable and allow for uptake by target cells.

**[0090]** The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other

untoward reaction when administered to an animal, such as a human, as appropriate. The preparation of a pharmaceutical composition that contains at least one non-charged lipid component comprising an oligonucleotide or additional active ingredient will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington: The Science and Practice of Pharmacy, 21st, 2005, incorporated herein by reference. Moreover, for animal (*e.g.*, human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

**[0091]** As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (*e.g.*, antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art. A pharmaceutically acceptable carrier is preferably formulated for administration to a human, although in certain embodiments it may be desirable to use a pharmaceutically acceptable carrier that is formulated for administration to a non-human animal but which would not be acceptable (*e.g.*, due to governmental regulations) for administration to a human. Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the therapeutic or pharmaceutical compositions is contemplated.

**[0092]** The actual dosage amount of a composition of the present invention administered to a patient or subject can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

**[0093]** In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1% of an active compound. In other embodiments, the an active compound may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about

5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 µg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, etc., can be administered.

**[0094]** An oligonucleotide of the present embodiments may be administered in a dose of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100 or more µg of nucleic acid per dose. Each dose may be in a volume of 1, 10, 50, 100, 200, 500, 1000 or more µl or ml.

**[0095]** Solutions of therapeutic compositions can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

**[0096]** The therapeutic compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain 10 mg, 25 mg, 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like.

**[0097]** Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium

chloride, Ringer's dextrose, etc. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to well-known parameters.

**[0098]** The therapeutic compositions of the present invention may include classic pharmaceutical preparations. Administration of therapeutic compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Topical administration may be particularly advantageous for the treatment of skin cancers, to prevent chemotherapy-induced alopecia or other dermal hyperproliferative disorder. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients. For treatment of conditions of the lungs, aerosol delivery can be used. Volume of the aerosol is between about 0.01 ml and 0.5 ml.

**[0099]** An effective amount of the therapeutic composition is determined based on the intended goal. The term “unit dose” or “dosage” refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses discussed above in association with its administration, i.e., the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the protection or effect desired.

**[00100]** Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual. Factors affecting the dose include the physical and clinical state of the patient, the route of administration, the intended goal of treatment (*e.g.*, alleviation of symptoms versus cure) and the potency, stability and toxicity of the particular therapeutic substance.

## VIII. Combination Treatments

**[00101]** In certain embodiments, the compositions and methods of the present invention involve an inhibitory oligonucleotide, or oligonucleotide capable of expressing an inhibitor of gene expression, in combination with a second or additional therapy. The

methods and compositions including combination therapies enhance the therapeutic or protective effect, and/or increase the therapeutic effect of another anti-cancer or anti-hyperproliferative therapy. Therapeutic and prophylactic methods and compositions can be provided in a combined amount effective to achieve the desired effect, such as the killing of a cancer cell and/or the inhibition of cellular hyperproliferation. This process may involve contacting the cells with both an inhibitor of gene expression and a second therapy. A tissue, tumor, or cell can be contacted with one or more compositions or pharmacological formulation(s) including one or more of the agents (i.e., inhibitor of gene expression or an anti-cancer agent), or by contacting the tissue, tumor, and/or cell with two or more distinct compositions or formulations, wherein one composition provides 1) an inhibitory oligonucleotide; 2) an anti-cancer agent, or 3) both an inhibitory oligonucleotide and an anti-cancer agent. Also, it is contemplated that such a combination therapy can be used in conjunction with a chemotherapy, radiotherapy, surgical therapy, or immunotherapy.

**[00102]** An inhibitory oligonucleotide may be administered before, during, after or in various combinations relative to an anti-cancer treatment. The administrations may be in intervals ranging from concurrently to minutes to days to weeks. In embodiments where the inhibitory oligonucleotide is provided to a patient separately from an anti-cancer agent, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the two compounds would still be able to exert an advantageously combined effect on the patient. In such instances, it is contemplated that one may provide a patient with the inhibitory oligonucleotide therapy and the anti-cancer therapy within about 12 to 24 or 72 h of each other and, more preferably, within about 6-12 h of each other. In some situations it may be desirable to extend the time period for treatment significantly where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between respective administrations.

**[00103]** In certain embodiments, a course of treatment will last 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90 days or more. It is contemplated that one agent may be given on day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52,

53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, and/or 90, any combination thereof, and another agent is given on day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, and/or 90, or any combination thereof. Within a single day (24-hour period), the patient may be given one or multiple administrations of the agent(s). Moreover, after a course of treatment, it is contemplated that there is a period of time at which no anti-cancer treatment is administered. This time period may last 1, 2, 3, 4, 5, 6, 7 days, and/or 1, 2, 3, 4, 5 weeks, and/or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 months or more, depending on the condition of the patient, such as their prognosis, strength, health, etc.

**[00104]** Various combinations may be employed. For the example below an inhibitory oligonucleotide therapy is "A" and an anti-cancer therapy is "B":

A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B

B/A/B/B B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A

B/B/A/A B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A

A/A/B/A

**[00105]** Administration of any compound or therapy of the present invention to a patient will follow general protocols for the administration of such compounds, taking into account the toxicity, if any, of the agents. Therefore, in some embodiments there is a step of monitoring toxicity that is attributable to combination therapy. It is expected that the treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies, as well as surgical intervention, may be applied in combination with the described therapy.

**[00106]** In specific aspects, it is contemplated that a standard therapy will include chemotherapy, radiotherapy, immunotherapy, surgical therapy or gene therapy and may be employed in combination with the inhibitor of gene expression therapy, anticancer therapy, or both the inhibitor of gene expression therapy and the anti-cancer therapy, as described herein.

### A. Chemotherapy

**[00107]** A wide variety of chemotherapeutic agents may be used in accordance with the present embodiments. The term "chemotherapy" refers to the use of drugs to treat cancer. A "chemotherapeutic agent" is used to connote a compound or composition that is administered in the treatment of cancer. These agents or drugs are categorized by their mode of activity within a cell, for example, whether and at what stage they affect the cell cycle. Alternatively, an agent may be characterized based on its ability to directly cross-link DNA, to intercalate into DNA, or to induce chromosomal and mitotic aberrations by affecting nucleic acid synthesis.

**[00108]** Examples of chemotherapeutic agents include alkylating agents, such as thiotepa and cyclosphosphamide; alkyl sulfonates, such as busulfan, improsulfan, and piposulfan; aziridines, such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines, including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide, and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards, such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, and uracil mustard; nitrosureas, such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics, such as the enediyne antibiotics (*e.g.,* calicheamicin, especially calicheamicin gammaI and calicheamicin omegaI1); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores, aclacinomysins, actinomycin, authrarnycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, such as mitomycin C, mycophenolic acid, nogalarnycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin,

tubercidin, ubenimex, zinostatin, and zorubicin; anti-metabolites, such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues, such as denopterin, pteropterin, and trimetrexate; purine analogs, such as fludarabine, 6-mercaptopurine, thiamiprine, and thioguanine; pyrimidine analogs, such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, and floxuridine; androgens, such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, and testolactone; anti-adrenals, such as mitotane and trilostane; folic acid replenisher, such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids, such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSKpolysaccharide complex; razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2”-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside (“Ara-C”); cyclophosphamide; taxoids, *e.g.*, paclitaxel and docetaxel gemcitabine; 6-thioguanine; mercaptopurine; platinum coordination complexes, such as cisplatin, oxaliplatin, and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (*e.g.*, CPT-11); topoisomerase inhibitor RFS 2000; difluorometlhylornithine (DMFO); retinoids, such as retinoic acid; capecitabine; carboplatin, procarbazine,plicomycin, gemcitabien, navelbine, farnesyl-protein tansferase inhibitors, transplatinum, and pharmaceutically acceptable salts, acids, or derivatives of any of the above.

### B. Radiotherapy

**[00109]** Other factors that cause DNA damage and have been used extensively include what are commonly known as γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves, proton beam irradiation (U.S. Pat. Nos. 5,760,395 and 4,870,287) and UV-irradiation. It is most likely that all of these factors affect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to

200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

**[00110]** The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic construct and a chemotherapeutic or radiotherapeutic agent are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing, for example, both agents are delivered to a cell in a combined amount effective to kill the cell or prevent it from dividing.

### C. Immunotherapy

**[00111]** In the context of cancer treatment, immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. Trastuzumab (Herceptin™) is such an example. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually affect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, etc.) and serve merely as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells. The combination of therapeutic modalities, i.e., direct cytotoxic activity and inhibition or reduction of ErbB2 would provide therapeutic benefit in the treatment of ErbB2 overexpressing cancers.

**[00112]** Another immunotherapy could also be used as part of a combined therapy with gen silencing therapy discussed above. In one aspect of immunotherapy, the tumor cell must bear some marker that is amenable to targeting, i.e., is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present invention. Common tumor markers include carcinoembryonic antigen, prostate specific antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, estrogen receptor, laminin receptor, erb B and p155. An alternative aspect of immunotherapy is to combine anticancer effects with immune stimulatory effects. Immune stimulating molecules also exist including: cytokines such as IL-2, IL-4, IL-12, GM-CSF, gamma-IFN, chemokines such as MIP-1, MCP-1, IL-8 and growth factors such as FLT3

ligand. Combining immune stimulating molecules, either as proteins or using gene delivery in combination with a tumor suppressor has been shown to enhance anti-tumor effects. Moreover, antibodies against any of these compounds can be used to target the anti-cancer agents discussed herein.

**[00113]** Examples of immunotherapies currently under investigation or in use are immune adjuvants e.g., Mycobacterium bovis, Plasmodium falciparum, dinitrochlorobenzene and aromatic compounds (U.S. Pat. Nos. 5,801,005 and 5,739,169; Hui and Hashimoto, 1998; Christodoulides et al., 1998), cytokine therapy, e.g., interferons α, β and γ; IL-1, GM-CSF and TNF (Bukowski et al., 1998; Davidson et al., 1998; Hellstrand et al., 1998) gene therapy, e.g., TNF, IL-1, IL-2, p53 (Qin et al., 1998; Austin-Ward and Villaseca, 1998; U.S. Pat. Nos. 5,830,880 and 5,846,945) and monoclonal antibodies, e.g., anti-ganglioside GM2, anti-HER-2, anti-p185 (Pietras et al., 1998; Hanibuchi et al., 1998; U.S. Pat. No. 5,824,311). It is contemplated that one or more anti-cancer therapies may be employed with the gene silencing therapies described herein.

**[00114]** In active immunotherapy, an antigenic peptide, polypeptide or protein, or an autologous or allogenic tumor cell composition or "vaccine" is administered, generally with a distinct bacterial adjuvant (Ravindranath and Morton, 1991; Morton et al., 1992; Mitchell et al., 1990; Mitchell et al., 1993).

**[00115]** In adoptive immunotherapy, the patient's circulating lymphocytes, or tumor infiltrated lymphocytes, are isolated in vitro, activated by lymphokines such as IL-2 or transduced with genes for tumor necrosis, and readministered (Rosenberg et al., 1988; 1989).

### D. Surgery

**[00116]** Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative, and palliative surgery. Curative surgery is a cancer treatment that may be used in conjunction with other therapies, such as the treatment of the present invention, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy and/or alternative therapies.

**[00117]** Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery

includes laser surgery, cryosurgery, electrosurgery, and microscopically controlled surgery (Mohs' surgery). It is further contemplated that the present invention may be used in conjunction with removal of superficial cancers, precancers, or incidental amounts of normal tissue.

**[00118]** Upon excision of part or all of cancerous cells, tissue, or tumor, a cavity may be formed in the body. Treatment may be accomplished by perfusion, direct injection or local application of the area with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well.

### E. Other Agents

**[00119]** It is contemplated that other agents may be used in combination with certain aspects of the present embodiments to improve the therapeutic efficacy of treatment. These additional agents include agents that affect the upregulation of cell surface receptors and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adhesion, agents that increase the sensitivity of the hyperproliferative cells to apoptotic inducers, or other biological agents. Increases in intercellular signaling by elevating the number of GAP junctions would increase the anti-hyperproliferative effects on the neighboring hyperproliferative cell population. In other embodiments, cytostatic or differentiation agents can be used in combination with certain aspects of the present embodiments to improve the anti-hyperproliferative efficacy of the treatments. Inhibitors of cell adhesion are contemplated to improve the efficacy of the present embodiments. Examples of cell adhesion inhibitors are focal adhesion kinase (FAKs) inhibitors and Lovastatin. It is further contemplated that other agents that increase the sensitivity of a hyperproliferative cell to apoptosis, such as the antibody c225, could be used in combination with certain aspects of the present embodiments to improve the treatment efficacy.

## IX. Kits and Diagnostics

**[00120]** In various aspects of the invention, a kit is envisioned containing therapeutic agents and/or other therapeutic and delivery agents. In some embodiments, the present invention contemplates a kit for preparing and/or administering a therapy of the invention. The kit may comprise reagents capable of use in administering an active or

effective agent(s) of the invention. Reagents of the kit may include at least one inhibitor of gene expression, one or more lipid component, one or more anti-cancer component of a combination therapy, as well as reagents to prepare, formulate, and/or administer the components of the invention or perform one or more steps of the inventive methods.

**[00121]** In some embodiments, the kit may also comprise a suitable container means, which is a container that will not react with components of the kit, such as an eppendorf tube, an assay plate, a syringe, a bottle, or a tube. The container may be made from sterilizable materials such as plastic or glass.

**[00122]** The kit may further include an instruction sheet that outlines the procedural steps of the methods, and will follow substantially the same procedures as described herein or are known to those of ordinary skill.

## X. Examples

**[00123]** The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

### Example 1 - Method of Manufacturing Liposomal p-Ethoxy Antisense Drug Product

**[00124]** The liposomal p-ethoxy antisense drug product is composed of two cGMP products, both of which have a FDA-required Certificate of Analysis with FDA-approved release criteria. The raw materials, solvents, and final drug product are described herein. When manufactured, the drug product is a lyophilized crystal or powder of amber or white color that comprises the following materials: oligonucleotide (*e.g.*, p-ethoxy antisense drug substance), neutral lipids (*e.g.*, DOPC), and surfactant (*e.g.*, polysorbate 20). In preparation for administration to a patient, normal saline is added to the vial, at which time liposomes are formed with the p-ethoxy antisense incorporated into the interior.

**[00125]** *P-ethoxy antisense drug substance.* Specific physical properties (*e.g.*, solubility and hydrophobicity, which then affect drug product solubility in saline, incorporation of oligo into liposomes, and liposome particle size) of the finished product can be defined using a pre-determined p-ethoxy and phosphodiester amidite raw material mix during production of the p-ethoxy antisense drug substance. While loss of the p-ethoxy backbone group randomly occurs during oligonucleotide manufacturing resulting in phosphodiester bonds at those linkages, that loss may not generate the preferred ratio of p-ethoxy : phosphodiester backbone linkage within the oligonucleotide. In this case, the mix of p-ethoxy and phosphodiester amidite raw material supplements the expected value of p-ethoxy backbone deletions, thus generating an oligonucleotide with the desired ratio. Increasing the number of p-ethoxy molecules in the backbone of the oligonucleotide causes the molecule to be more hydrophobic (which results in larger liposome particles; Table 1), less polar, and less soluble (Table 2). Methods of testing the charge-neutral, hydrophobic p-ethoxy drug substance include mass spectrometry to determine the distribution of oligonucleotide lengths and assays to determine the solubility of drug substance, which for practical purposes for solubility is a visual inspection of the drug product reconstituted in saline. As the oligonucleotide becomes less soluble due to a greater number of p-ethoxy backbone linkages the reconstituted solution becomes whiter until particulates form as hydrophobicity becomes too high.

**Table 1.** Liposome Particle Size Variability with Antisense Backbone Composition

| | | **Post-Manufacturing Backbone Ethyl Deletion** | | **Particle Size Characteristics: Cumulative Distribution Function** | | |
|---|---|---|---|---|---|---|
| **Experiment** | **Engineered Antisense Backbone** | **Principal Peak** | **Composite Deletion** | **90% Value (nm) **** | **50% Value (nm)** | **300 nm Value (%)** |
| 1 | 3 amidite substitution | -6 | -5.67 | 2130 | 911 | 15.30 |
| 2 | 3 amidite substitution | -6 | -5.67 | 2420 | 1004 | 15.50 |
| 3 | 3 amidite substitution | -6 | -6.12 | 3682 | 943 | 15.50 |
| 4 | 3 amidite substitution | -7 | -6.66 | 3805 | 978 | 14.60 |
| 5 | 100% p-ethoxy | -5 | -5.66 | 3924 | 976 | 16.00 |
| 6 | 2 amidite substitution | -5 | -5.32 | 4387 | 1888 | 11.60 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 7[a] | 100% p-ethoxy | -4 | -4.22 | 5057 | 1131 | 17.70 |
| 8 | 100% p-ethoxy | -4 | -4.52 | 5659 | 1359 | 10.00 |
| 9[b] | 100% p-ethoxy | -4 | -4.38 | 7571 | 1909 | 2.60 |
| 10[c] | 100% p-ethoxy | -4 | -4.38 | 7994 | 1653 | 14.40 |

** Drug product release criteria is for 90% of the liposome particles to be less than or equal to 5000 nm.

a. This lot was discarded due to poor solubility; specifically, antisense particles in the reconstituted solution.

b. This lot had lower DMSO and tBA volume with 2 mg antisense in a 20 mL vial, which added an additional component to liposome enlargement.

c. This lot was not released because it failed the particle size release spec.

**Table 2.** Liposome Particle Solubility with Antisense Backbone Composition

| | | **Post-Manufacturing Backbone Ethyl Deletion** | | **Drug Solubility** | |
|---|---|---|---|---|---|
| **Experiment** | **Engineered Antisense Backbone** | **Principal Peak** | **Composite Deletion** | **Visual Observation ****** | **Solubility Assessment** |
| 1 | 3 amidite substitution | -6 | -5.67 | skim milk solution | good |
| 2 | 3 amidite substitution | -6 | -5.67 | skim milk solution | good |
| 3 | 3 amidite substitution | -6 | -6.12 | skim milk solution | good |
| 4 | 3 amidite substitution | -7 | -6.66 | skim milk solution | good |
| 5 | 100% p-ethoxy | -5 | -5.66 | skim milk solution | good |
| 6 | 2 amidite substitution | -5 | -5.32 | skim milk solution | good |
| 7 | 100% p-ethoxy | -4 | -4.52 | white solution | pass |
| 8[b] | 100% p-ethoxy | -4 | -4.38 | white solution | pass |
| 9[c] | 100% p-ethoxy | -4 | -4.38 | white solution | pass |
| 10[a] | 100% p-ethoxy | -4 | -4.22 | white solution particles | fail |

** If the drug product sample has particles the lot will be rejected

a. This lot was discarded due to poor solubility; specifically, antisense particles in the reconstituted solution.

b. This lot had lower DMSO and tBA volume with 2 mg antisense in a 20 mL vial, which added an additional component to liposome enlargement.

c. This lot was not released because it failed the particle size release spec.

**[00126]** *Formulation, filtration, and lyophilization of liposomal p-ethoxy antisense drug product.* One gram (1 g) of pE oligos are dissolved in DMSO at a ratio of 10 mg oligonucleotide per 1 mL DMSO. Next, DOPC is added to tert-butyl alcohol at a ratio of 1 g DOPC per 1719 mL of tert-butyl alcohol. The oligo and DOPC are combined and mixed at a ratio of 1 g oligonucleotide per 2.67 g DOPC. Then, 20 mL of a 0.835% (v/v) solution of polysorbate 20 is added to the mixture resulting in a final concentration of 0.039 mg/mL. The solution is passed through a sterile filter prior to dispensing into glass vials for lyophilization.

**[00127]** The effect of the surfactant on liposome particle size was determined by titrating the amount of surfactant (Table 3). In the absence of polysorbate 20, only 2.8% of the particles had a diameter of 300 nm or less. In the presence of 1x polysorbate 20 (about 5% of the total liposomal p-ethoxy antisense drug product), 12.5% of the particles had a diameter of 300 nm or less. With the addition of 3x-10x polysorbate 20, around 20% of the particles had a diameter of 300 nm or less. Thus an increase in surfactant from 1x to 3x results in a decrease in particle size.

**Table 3.** Liposome Particle Size Variability with Surfactant

| | | **Particle Size Characteristics: Cumulative Distribution Function** | | |
|---|---|---|---|---|
| **Experiment** | **Amount of Surfactant** | **50% Value** | **90% Value **** | **300 nm Value** |
| 1 | 0x | 5301 nm | 10719 nm | 2.8% |
| 2 | 1x | 1053 nm | 4054 nm | 12.5% |
| 3 | 3x | 785 nm | 2926 nm | 19.1% |
| 4 | 5x | 721 nm | 2691 nm | 21.9% |
| 5 | 10x | 734 nm | 2937 nm | 21.4% |

** Drug product release criteria is for 90% of the liposome particles to be less than or equal to 5000 nm.

**[00128]** *Preparation of liposomal p-ethoxy antisense drug product for administration.* The lyophilized preparation was hydrated with normal saline (0.9%/10 mM NaCl) at a final oligo concentration of 10-5000 μM. The liposomal-p-ethoxy oligos were mixed by hand shaking.

## Example 2 - Methods of Testing Liposomal p-Ethoxy Antisense Drug Product

**[00129]** *Visual Inspection of Manufactured Drug Product*: After manufacturing, a sample vial containing drug product is selected and visually inspected. The absence of liquid is mandatory, and then amber crystals at the bottom of the vial are acceptable, and increasing in acceptance to a white, flocculated powder or appearance, the best result. The white appearance indicates a better drying process, with a high surface area to mass ratio, which is very conducive to reconstitution for use.

**[00130]** *Visual Inspection of Reconstituted Drug Ready for Patient IV*: Normal saline is added to a vial containing the manufactured Liposomal P-ethoxy Antisense Drug Product and shaken to reconstitute into a solution with the drug crystal or powder completely dissolved. Three main observations are made: 1) that the crystal or powder is completely dissolved, 2) there are no white clumps of undissolved material, and 3) the appearance is a milky white or skim milk appearance. The bluer the appearance of the reconstituted liquid, the better, as this signals a smaller liposome particle size that reflects light in the blue spectrum.

**[00131]** *Mass Spectrometry*: Mass spectrometry (mass spec) is used to display the profile of the various masses in a sample. When p-ethoxy antisense material is produced, a mass spec is run on the sample. The result shows peaks of material present on a grid that has increasing mass on the "x" axis to the right, and relative mass abundance on the "y" axis increasing upward. The profile from a sample is analyzed to determine the relative quantity of p-ethoxy backbones in the p-ethoxy sample, recognizing that the profile of peaks represents (starting farthest to the right), full length material with all backbones comprised of the p-ethoxy linkage, the next peak moving left a full length with one backbone with a p-ethoxy deletion (and therefore, the ethyl being knocked off and the result being a normal phosphodiester backbone linkage), and continuing. The mass spec pattern shifted to the right represents a p-ethoxy sample having more p-ethoxy backbones, and therefore having the properties of being more hydrophobic and less soluble; and likewise, shifted to the left having

the opposite effects. Inspection of the mass spec chart of a sample also can be used to determine if filtration during manufacturing produces any adverse effects on oligonucleotide composition present in the filtered drug product.

**[00132]** *UV Testing*: Ultraviolent light testing is used to determine the mass of oligonucleotide present in a sample. Oligonucleotides absorb light in the 260 nanometer range. As a result, UV testing of the finished reconstituted drug product has come to be used as a method in determining the quantity of oligonucleotide drug substance in a vial of drug product. In terms of manufacturing development and innovations, UV testing was used to determine if there were problems experienced during filtration in manufacturing or poor solubility of the p-ethoxy antisense drug substance, resulting in less oligonucleotide in solution and therefore a lower UV reading. The method will be validated and likely become part of the final product release testing.

**[00133]** *Liposome Particle Size*: A vial of finished drug product is reconstituted and tested for liposome particle size. The result is often a roughly normal distribution, having a central point, tails and average values or a roughly normal distribution of the majority of the particles and smaller, secondary peaks of the smaller liposomes particles resulting from second-order particle formation effects. It is important that liposome particles not be too large, as they may create adverse effects in patients (for example, create blood flow problems in smaller blood vessels in the lungs). As a result, the drug product release criteria include that particle size testing show that 90% of liposomes be about 5 microns or less in size. In addition, smaller liposomes are preferred because they will have better uptake into cells, and secondly, smaller liposomes can penetrate vascular pores, thereby allowing the liposomes to penetrate inside tumors, increasing treatment effectiveness of a Liposomal P-ethoxy Antisense Drug Product.

* * *

**[00134]** All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain

agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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**WHAT IS CLAIMED IS:**

1. A composition comprising a population of oligonucleotides, wherein oligonucleotides of the population are composed of nucleoside molecules linked together through phosphate backbone linkages, wherein at least one of the phosphate backbone linkages in each oligonucleotide is a p-ethoxy backbone linkage, and wherein no more than 80% of the phosphate backbone linkages in each oligonucleotide are p-ethoxy backbone linkages.

2. The composition of claim 1, wherein 10% to 80% of the phosphate backbone linkages are p-ethoxy backbone linkages.

3. The composition of claim 2, wherein 20% to 80% of the phosphate backbone linkages are p-ethoxy backbone linkages.

4. The composition of claim 3, wherein 30% to 80% of the phosphate backbone linkages are p-ethoxy backbone linkages.

5. The composition of claim 4, wherein 40% to 80% of the phosphate backbone linkages are p-ethoxy backbone linkages.

6. The composition of claim 5, wherein 50% to 80% of the phosphate backbone linkages are p-ethoxy backbone linkages.

7. The composition of claim 6, wherein 60% to 70% of the phosphate backbone linkages are p-ethoxy backbone linkages.

8. The composition of claim 1, wherein 20% to 90% of the phosphate backbone linkages are phosphodiester backbone linkages.

9. The composition of claim 8, wherein 20% to 80% of the phosphate backbone linkages are phosphodiester backbone linkages.

10. The composition of claim 9, wherein 20% to 70% of the phosphate backbone linkages are phosphodiester backbone linkages.

11. The composition of claim 10, wherein 20% to 60% of the phosphate backbone linkages are phosphodiester backbone linkages.

12. The composition of claim 11, wherein 20% to 50% of the phosphate backbone linkages are phosphodiester backbone linkages.

13. The composition of claim 12, wherein 30% to 40% of the phosphate backbone linkages are phosphodiester backbone linkages.

14. The composition of claim 1, wherein the oligonucleotides of the population have a size ranging from 7 to 30 nucleotides.

15. The composition of claim 14, wherein the oligonucleotides of the population have an average size of 7 nucleotides, wherein no more than 5 of the phosphate backbone linkages in each oligonucleotide is a p-ethoxy backbone linkage.

16. The composition of claim 14, wherein the oligonucleotides of the population have an average size of 10 nucleotides, wherein no more than 8 of the phosphate backbone linkages in each oligonucleotide is a p-ethoxy backbone linkage.

17. The composition of claim 14, wherein the oligonucleotides of the population have an average size of 30 nucleotides, wherein no more than 24 of the phosphate backbone linkages in each oligonucleotide is a p-ethoxy backbone linkage.

18. The composition of claim 14, wherein the oligonucleotides of the population have a size ranging from 12 to 25 nucleotides.

19. The composition of claim 18, wherein the oligonucleotides of the population have an average size of 15 nucleotides, wherein no more than 12 of the phosphate backbone linkages in each oligonucleotide is a p-ethoxy backbone linkage.

20. The composition of claim 18, wherein the oligonucleotides of the population have an average size of 18 nucleotides, wherein no more than 14 of the phosphate backbone linkages in each oligonucleotide is a p-ethoxy backbone linkage.

21. The composition of claim 18, wherein the oligonucleotides of the population have an average size of 20 nucleotides, wherein no more than 16 of the phosphate backbone linkages in each oligonucleotide is a p-ethoxy backbone linkage.

22. The composition of claim 18, wherein the oligonucleotides of the population have an average size of 25 nucleotides, wherein no more than 20 of the phosphate backbone linkages in each oligonucleotide is a p-ethoxy backbone linkage.

23. The composition of claim 1, wherein the population of oligonucleotides comprises a single species of oligonucleotides.

24. The composition of claim 1, wherein the population of oligonucleotides comprises at least two species of oligonucleotides.

25. The composition of claim 1, wherein the population of oligonucleotides comprises antisense oligonucleotides, short interfering RNAs, microRNAs, or piwiRNAs.

26. The composition of claim 1, wherein the oligonucleotides of the population inhibit the expression of at least one oncogenic protein, infectious agent protein, or self-antigen.

27. The composition of claim 1, wherein the oligonucleotides of the population hybridize with at least one oncogenic oligonucleotide, infectious agent oligonucleotide, or self-antigen oligonucleotide.

28. The composition of claim 1, further comprising phospholipids and wherein the oligonucleotides and phospholipids form an oligonucleotide–lipid complex.

29. The composition of claim 28, wherein the phospholipids are uncharged or have a neutral charge at physiologic pH.

30. The composition of claim 29, wherein the phospholipids are neutral phospholipids.

31. The composition of claim 30, wherein the neutral phospholipids are phosphatidylcholines.

32. The composition of claim 30, wherein the neutral phospholipids are dioleoylphosphatidyl choline.

33. The composition of claim 28, wherein the phospholipids are essentially free of cholesterol.

34. The composition of claim 28, wherein the phospholipids and oligonucleotides are present at a molar ratio of from about 5:1 to about 100:1.

35. The composition of claim 28, wherein the oligonucleotide–lipid complex is further defined as a population of liposomes.

36. The composition of claim 35, wherein at least 90% of the liposomes are less than 5 microns in diameter.

37. The composition of claim 35, wherein the population of oligonucleotides is incorporated in the population of liposomes.

38. The composition of claim 1, wherein the composition is lyophilized.

39. A pharmaceutical composition comprising a composition according to claim 28 and a pharmaceutically acceptable carrier.

40. The composition of claim 39, further comprising a chemotherapeutic agent.

41. A method for delivering a therapeutically effective amount of an oligonucleotide to a cell comprising contacting the cell with a pharmaceutical composition of claim 39.

42. The method of claim 41, wherein the method is a method of treating hyperplasia, cancer, autoimmune disease, or infectious disease.

43. A method of treating a subject with a cancer, an autoimmune disease, or an infectious disease comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition of claim 39.

44. The method of claim 43, wherein the subject is a human.

45. The method of claim 43, wherein the cancer is a bladder, blood, pancreas, bone, bone marrow, brain, breast, colon, esophagus, stomach, head and neck, kidney, liver, lung, prostate, skin, testis, tongue, ovary, or uterine cancer.

46. The method of claim 43, wherein the autoimmune disease is Lupus erythematosis, Sjogren's disease, Crohn's disease, diabetes mellitus, multiple sclerosis, or rheumatoid arthritis.

47. The method of claim 43, wherein the infectious disease is a bacterial infection, fungal infection, viral infection, or parasitic infection.

48. The method of claim 43, wherein the composition is administered subcutaneously, intravenously, or intraperitoneally.

49. The method of claim 43, further comprising administering at least a second anticancer therapy to the subject.

50. The method of claim 49, wherein the second anticancer therapy is a surgical therapy, chemotherapy, radiation therapy, cryotherapy, hormone therapy, immunotherapy, anti-viral therapy, immune suppression therapy, anti-bacterial therapy, anti-parasite therapy, anti-fungal therapy, or cytokine therapy.

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/057148

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | US 6 015 886 A (DALE; ARROW; SRIVASTAVA; RAZA; CHEMGENES CORPORATION [US]; OLIGOS ETC.) 18 January 2000 (2000-01-18) | 1-28,33, 35-50 |
| Y | column 21 - column 22; figure 14; example 2; table 3<br>the whole document | 29-32,34 |
| | ----- | |
| Y | WO 97/07784 A2 (BOARDS OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US])<br>6 March 1997 (1997-03-06)<br>page 2, line 25 - page 3, column 10 | 29-32,34 |
| | ----- | |
| Y | WO 01/60998 A2 (BOARDS OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US])<br>23 August 2001 (2001-08-23)<br>page 5 - page 6 | 29-32,34 |
| | ----- | |
| | -/-- | |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
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| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Macchia, Giovanni |

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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# INTERNATIONAL SEARCH REPORT

Information on patent family members

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| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| | | WO 0160998 A2 | 23-08-2001 |

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(54) Title: P-ETHOXY NUCLEIC ACIDS FOR LIPOSOMAL FORMULATION

(57) Abstract: Provided herein are therapeutic oligonucleotides that comprise at least one p-ethoxy backbone linkage but no more than 80% p-ethoxy backbone linkages. Provided herein are improved delivery systems for therapeutic oligonucleotides comprising a liposome that comprises neutral phospholipids and a p-ethoxy oligonucleotide that is entrapped in the liposome.

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(51)Int.Cl.
*C12N 15/113*(2006.01)

权利要求书3页 说明书30页

(54)发明名称

用于脂质体制剂的对乙氧基核酸

(57)摘要

本文提供包含至少一个对乙氧基主链键联但不超过80%对乙氧基主链键联的治疗性寡核苷酸。本文提供用于包含脂质体的治疗性寡核苷酸的改进的递送系统,所述脂质体包含中性磷脂和包埋在所述脂质体中的对乙氧基寡核苷酸。

CN 108291227 A

1.一种包含寡核苷酸群体的组合物,其中所述群体的寡核苷酸由通过磷酸酯主链键联连接在一起的核苷分子组成,其中每个寡核苷酸中的所述磷酸酯主链键联中的至少一个是对乙氧基主链键联,并且其中每个寡核苷酸中不超过80%的所述磷酸酯主链键联是对乙氧基主链键联。

2.如权利要求1所述的组合物,其中10%至80%的所述磷酸酯主链键联是对乙氧基主链键联。

3.如权利要求2所述的组合物,其中20%至80%的所述磷酸酯主链键联是对乙氧基主链键联。

4.如权利要求3所述的组合物,其中30%至80%的所述磷酸酯主链键联是对乙氧基主链键联。

5.如权利要求4所述的组合物,其中40%至80%的所述磷酸酯主链键联是对乙氧基主链键联。

6.如权利要求5所述的组合物,其中50%至80%的所述磷酸酯主链键联是对乙氧基主链键联。

7.如权利要求6所述的组合物,其中60%至70%的所述磷酸酯主链键联是对乙氧基主链键联。

8.如权利要求1所述的组合物,其中20%至90%的所述磷酸酯主链键联是磷酸二酯主链键联。

9.如权利要求8所述的组合物,其中20%至80%的所述磷酸酯主链键联是磷酸二酯主链键联。

10.如权利要求9所述的组合物,其中20%至70%的所述磷酸酯主链键联是磷酸二酯主链键联。

11.如权利要求10所述的组合物,其中20%至60%的所述磷酸酯主链键联是磷酸二酯主链键联。

12.如权利要求11所述的组合物,其中20%至50%的所述磷酸酯主链键联是磷酸二酯主链键联。

13.如权利要求12所述的组合物,其中30%至40%的所述磷酸酯主链键联是磷酸二酯主链键联。

14.如权利要求1所述的组合物,其中所述群体的所述寡核苷酸具有在7至30个核苷酸范围内的大小。

15.如权利要求14所述的组合物,其中所述群体的所述寡核苷酸具有7个核苷酸的平均大小,其中每个寡核苷酸中的所述磷酸酯主链键联中不超过5个是对乙氧基主链键联。

16.如权利要求14所述的组合物,其中所述群体的所述寡核苷酸具有10个核苷酸的平均大小,其中每个寡核苷酸中的所述磷酸酯主链键联中不超过8个是对乙氧基主链键联。

17.如权利要求14所述的组合物,其中所述群体的所述寡核苷酸具有30个核苷酸的平均大小,其中每个寡核苷酸中的所述磷酸酯主链键联中不超过24个是对乙氧基主链键联。

18.如权利要求14所述的组合物,其中所述群体的所述寡核苷酸具有在12至25个核苷酸范围内的大小。

19.如权利要求18所述的组合物,其中所述群体的所述寡核苷酸具有15个核苷酸的平

均大小，其中每个寡核苷酸中的所述磷酸酯主链键联中不超过12个是对乙氧基主链键联。

20.如权利要求18所述的组合物，其中所述群体的所述寡核苷酸具有18个核苷酸的平均大小，其中每个寡核苷酸中的所述磷酸酯主链键联中不超过14个是对乙氧基主链键联。

21.如权利要求18所述的组合物，其中所述群体的所述寡核苷酸具有20个核苷酸的平均大小，其中每个寡核苷酸中的所述磷酸酯主链键联中不超过16个是对乙氧基主链键联。

22.如权利要求18所述的组合物，其中所述群体的所述寡核苷酸具有25个核苷酸的平均大小，其中每个寡核苷酸中的所述磷酸酯主链键联中不超过20个是对乙氧基主链键联。

23.如权利要求1所述的组合物，其中所述寡核苷酸群体包含单一种类的寡核苷酸。

24.如权利要求1所述的组合物，其中所述寡核苷酸群体包含至少两种种类的寡核苷酸。

25.如权利要求1所述的组合物，其中所述寡核苷酸群体包含反义寡核苷酸、短干扰RNA、微小RNA或piwiRNA。

26.如权利要求1所述的组合物，其中所述群体的所述寡核苷酸抑制至少一种致癌蛋白、感染因子蛋白或自身抗原的表达。

27.如权利要求1所述的组合物，其中所述群体的所述寡核苷酸与至少一种致癌寡核苷酸、感染因子寡核苷酸或自身抗原寡核苷酸杂交。

28.如权利要求1所述的组合物，所述组合物还包含磷脂，并且其中所述寡核苷酸与磷脂形成寡核苷酸-脂质复合物。

29.如权利要求28所述的组合物，其中所述磷脂在生理pH下不带电或带有中性电荷。

30.如权利要求29所述的组合物，其中所述磷脂是中性磷脂。

31.如权利要求30所述的组合物，其中所述中性磷脂是磷脂酰胆碱。

32.如权利要求30所述的组合物，其中所述中性磷脂是二油酰基磷脂酰胆碱。

33.如权利要求28所述的组合物，其中所述磷脂基本上不含胆固醇。

34.如权利要求28所述的组合物，其中所述磷脂和寡核苷酸以约5:1至约100:1的摩尔比存在。

35.如权利要求28所述的组合物，其中所述寡核苷酸-脂质复合物进一步被定义为脂质体群体。

36.如权利要求35所述的组合物，其中至少90％的所述脂质体的直径小于5微米。

37.如权利要求35所述的组合物，其中所述寡核苷酸群体被并入所述脂质体群体中。

38.如权利要求1所述的组合物，其中所述组合物是冻干的。

39.一种药物组合物，所述药物组合物包含根据权利要求28所述的组合物和药学上可接受的载体。

40.如权利要求39所述的组合物，所述组合物还包含化学治疗剂。

41.一种将治疗有效量的寡核苷酸递送至细胞的方法，所述方法包括使所述细胞与如权利要求39所述的药物组合物接触。

42.如权利要求41所述的方法，其中所述方法是治疗增生、癌症、自身免疫性疾病或感染性疾病的方法。

43.一种治疗患有癌症、自身免疫性疾病或感染性疾病的受试者的方法，所述方法包括向所述受试者施用治疗有效量的如权利要求39所述的药物组合物。

44.如权利要求43所述的方法，其中所述受试者是人。

45.如权利要求43所述的方法，其中所述癌症是膀胱癌、血癌、胰腺癌、骨癌、骨髓癌、脑癌、乳腺癌、结肠癌、食道癌、胃癌、头颈部癌、肾癌、肝癌、肺癌、前列腺癌、皮肤癌、睾丸癌、舌癌、卵巢癌或子宫癌。

46.如权利要求43所述的方法，其中所述自身免疫性疾病是红斑狼疮、舍格伦病、克罗恩病、糖尿病、多发性硬化症或类风湿性关节炎。

47.如权利要求43所述的方法，其中所述感染性疾病是细菌感染、真菌感染、病毒感染或寄生虫感染。

48.如权利要求43所述的方法，其中所述组合物通过皮下、静脉内或腹膜内施用。

49.如权利要求43所述的方法，所述方法还包括向所述受试者施用至少第二种抗癌疗法。

50.如权利要求49所述的方法，其中所述第二种抗癌疗法是手术疗法、化学疗法、放射疗法、冷冻疗法、激素疗法、免疫疗法、抗病毒疗法、免疫抑制疗法、抗细菌疗法、抗寄生虫疗法、抗真菌疗法或细胞因子疗法。

# 用于脂质体制剂的对乙氧基核酸

[0001] 本申请要求2015年10月14日提交的美国临时申请号62/241,503的优先权权益,所述临时申请的全部内容以引用的方式并入本文。

[0002] 发明背景

1.发明领域

[0003] 本发明总体上涉及医学领域。更具体地说,它涉及对乙氧基寡核苷酸的脂质体制剂以及在医学中制备和使用此类制剂的方法。

[0004] 2.相关技术说明

[0005] 与靶mRNA的特定区域互补的反义寡核苷酸(寡核苷酸(oligos))已被用于抑制内源基因的表达。当反义寡核苷酸结合至靶mRNA时,形成DNA-RNA杂合体。所述杂合体形成抑制mRNA的翻译并因此抑制编码的蛋白质的表达。如果蛋白质对细胞的存活至关重要,则其表达的抑制可导致细胞死亡。因此,反义寡核苷酸可以是抗癌和抗病毒治疗中的有用工具。

[0006] 使用反义寡核苷酸来抑制基因表达的主要障碍是细胞不稳定性、低细胞摄取和不良细胞间递送。天然磷酸二酯不耐核酸酶水解;因此在观察到任何抑制作用之前需要高浓度的反义寡核苷酸。已经制备了修饰的磷酸二酯类似物如对乙氧基来克服这种核酸酶水解问题,但是它们没有提供对所述问题的令人满意的解决方案。

[0007] 反义寡核苷酸的细胞摄取较低。为了解决这个问题,已经使用物理技术如磷酸钙沉淀、DEAE-葡聚糖介导或电穿孔来增加寡核苷酸的细胞摄取。这些技术难以再现并且不适用于体内。阳离子脂质如Lipofectin也已用于递送寡核苷酸。静电相互作用在阳离子脂质与带负电荷的寡核苷酸之间形成,这产生然后被靶细胞吸收的复合物。因为这些阳离子脂质不保护寡核苷酸免受核酸酶消化,所以对细胞膜有害,并且它们仅适用于递送耐核酸酶的硫代磷酸酯,而不适用于递送核酸酶可裂解的磷酸二酯。

[0008] 已经制备的另一种修饰的磷酸二酯(PD)类似物是对乙氧基(pE)寡核苷酸。pE寡核苷酸的修饰在磷酸酯主链中进行,以使得所述修饰不会干扰这些寡核苷酸与靶mRNA的结合。pE寡核苷酸通过向磷酸酯主链的非桥氧原子添加乙基来制备,从而使这些寡核苷酸成为不带电的化合物。尽管它们对核酸酶具有抗性,但pE寡核苷酸的细胞摄取和细胞内递送较差,因为在内化后,这些寡核苷酸保持在内体/溶酶体液泡内部螯合,从而阻碍它们接近靶mRNA。

[0009] 需要用于治疗疾病的改进的反义组合物,并且还需要用于制备此类改进的组合物的方法。

[0010] 发明概述

[0011] 在一个实施方案中,提供了包含寡核苷酸群体的组合物。在一些方面,所述群体的寡核苷酸由通过磷酸酯主链键联连接在一起的核苷分子组成,其中每个寡核苷酸中的磷酸酯主链键联中的至少一个是对乙氧基主链键联,并且其中每个寡核苷酸中不超过80%的磷酸酯主链键联是对乙氧基主链键联。在一些方面,每个寡核苷酸中的磷酸酯主链键联中的至少一个是磷酸二酯主链键联。在一些方面,10%至80%的所述磷酸酯主链键联是对乙氧

基主链键联;20％至80％的所述磷酸酯主链键联是对乙氧基主链键联;30％至80％的所述磷酸酯主链键联是对乙氧基主链键联;40％至80％的所述磷酸酯主链键联是对乙氧基主链键联;50％至80％的所述磷酸酯主链键联是对乙氧基主链键联;或60％至70％的所述磷酸酯主链键联是对乙氧基主链键联,或其中可推论出的任何范围。在一些方面,20％至90％的所述磷酸酯主链键联是磷酸二酯主链键联;20％至80％的所述磷酸酯主链键联是磷酸二酯主链键联;20％至70％的所述磷酸酯主链键联是磷酸二酯主链键联;20％至60％的所述磷酸酯主链键联是磷酸二酯主链键联;20％至50％的所述磷酸酯主链键联是磷酸二酯主链键联;或30％至40％的所述磷酸酯主链键联是磷酸二酯主链键联,或其中可推论出的任何范围。在各个方面,至少5％、10％、15％、20％、25％、30％、35％、40％、45％、50％、55％、60％、65％、60％、65％、70％、75％、80％、85％、90％或95％或其中任何值的所述磷酸酯主链键联是对乙氧基主链键联。在各个方面,至多5％、10％、15％、20％、25％、30％、35％、40％、45％、50％、55％、60％、65％、70％、75％、80％、85％、90％或95％或其中任何值的所述磷酸酯主链键联是磷酸二酯主链键联。在一些方面,所述组合物是冻干的。

[0012] 在一些方面,所述群体的寡核苷酸具有在7至30个核苷酸范围内的大小。在某些方面,所述群体的寡核苷酸具有在12至25个核苷酸范围内的大小。在各个方面,所述群体的寡核苷酸具有至少5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29或30个核苷酸的大小。所述大小范围可以是群体中的寡核苷酸的平均大小。

[0013] 在一些方面,所述群体的寡核苷酸具有7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29或30个核苷酸的平均大小,其中每个寡核苷酸中的磷酸酯主链键联中各自不超过5、6、7、8、8、9、10、11、11、12、13、14、15、15、16、17、18、19、20、20、21、22、23或24个是对乙氧基主链键联。在一些方面,所述群体的寡核苷酸具有7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29或30个核苷酸的平均大小,并且每个寡核苷酸中的磷酸酯主链键联中各自至少2、2、2、2、3、3、3、3、4、4、4、4、4、5、5、5、5、5、6、6、6、6或6个是磷酸二酯主链键联。

[0014] 在一些方面,所述寡核苷酸群体包含单一种类的寡核苷酸。在其他方面,所述寡核苷酸群体包含至少两种种类的寡核苷酸。单一种类的寡核苷酸可具有相同的核苷酸序列,但在分子内的不同位置中具有或缺乏对乙氧基键。在一些方面,所述寡核苷酸群体包含反义寡核苷酸、短干扰RNA(siRNA)、微小RNA(miRNA)或piwiRNA(piRNA)。

[0015] 在某些方面,所述群体的寡核苷酸抑制至少一种致癌蛋白、感染因子蛋白或自身抗原的表达。在一些方面,所述群体的寡核苷酸与至少一种致癌寡核苷酸、感染因子寡核苷酸或自身抗原寡核苷酸杂交。

[0016] 在各个方面,所述组合物还包含磷脂。在一些方面,所述磷脂在生理pH下不带电或带有中性电荷。在一些方面,所述磷脂是中性磷脂。在某些方面,所述中性磷脂是磷脂酰胆碱。在某些方面,所述中性磷脂是二油酰基磷脂酰胆碱。在一些方面,所述磷脂基本上不含胆固醇。

[0017] 在一些方面,所述磷脂和寡核苷酸以约5:1至约100:1或其中可推论出的任何比率的摩尔比存在。在各个方面,所述磷脂和寡核苷酸以约5:1、10:1、15:1、20:1、25:1、30:1、35:1、40:1、45:1、50:1、55:1、60:1、65:1、70:1、75:1、80:1、85:1、90:1、95:1或100:1的摩尔比存在。在一些方面,所述寡核苷酸与磷脂形成寡核苷酸-脂质复合物,例如像脂质体复合

物。在一些方面，至少75％、76％、77％、78％、79％、80％、81％、82％、83％、84％、85％、86％、87％、88％、89％、90％、91％、92％、93％、94％、95％、96％、97％、98％或99％的所述脂质体的直径小于5微米。在各个方面，所述组合物还包含至少一种表面活性剂，例如像聚山梨醇酯20。在一些方面，总脂质体对乙氧基反义药物产品的至少约5％由表面活性剂组成，并且至少约90％的所述脂质体的直径小于5微米。在一些方面，总脂质体对乙氧基反义药物产品的至少约15％由表面活性剂组成，并且至少约90％的所述脂质体的直径小于3微米。在一些方面，所述寡核苷酸群体被并入所述脂质体群体中。

[0018] 在一方面，所述群体的寡核苷酸各自包含约21个核苷酸的长度并且具有约30％磷酸二酯主链键联。在一方面，所述寡核苷酸群体可进一步并入包含至少约5％表面活性剂的脂质体组合物中，其中至少约90％的所述脂质体具有小于约5微米的直径。

[0019] 在一个实施方案中，提供了药物组合物，所述药物组合物包含本发明实施方案的寡核苷酸和磷脂的组合物以及药学上可接受的载体。在一些方面，所述组合物还包含化学治疗剂。

[0020] 在一个实施方案中，提供用于将治疗有效量的寡核苷酸递送至细胞的方法，所述方法包括使所述细胞与本发明实施方案的药物组合物接触。在一些方面，所述方法是一种治疗增生、癌症、自身免疫性疾病或感染性疾病的方法。

[0021] 在一个实施方案中，提供用于治疗患有癌症、自身免疫性疾病或感染性疾病的受试者的方法，所述方法包括向所述受试者施用治疗有效量的本发明实施方案的药物组合物。在一些方面，所述受试者是人。在一些方面，所述癌症是膀胱癌、血癌、胰腺癌、骨癌、骨髓癌、脑癌、乳腺癌、结肠癌、食道癌、胃癌、头颈部癌、肾癌、肝癌、肺癌、前列腺癌、皮肤癌、睾丸癌、舌癌、卵巢癌或子宫癌。在一些方面，所述自身免疫性疾病是红斑狼疮、舍格伦病、克罗恩病、糖尿病、多发性硬化症或类风湿性关节炎。在一些方面，所述感染性疾病是细菌感染、真菌感染、病毒感染或寄生虫感染。在一些方面，所述组合物通过皮下、静脉内或腹膜内施用。在一些方面，所述方法还包括向所述受试者施用至少第二种抗癌疗法。在一些方面，所述第二种抗癌疗法是手术疗法、化学疗法、放射疗法、冷冻疗法、激素疗法、免疫疗法或细胞因子疗法。

[0022] 寡核苷酸包含与编码靶蛋白或调控靶蛋白的表达的核酸分子特异性杂交的反义核酸分子。“特异性杂交”是指反义核酸分子与靶向核酸分子杂交并调控其表达。优选地，“特异性杂交”还指没有其他基因或转录物受影响。寡核苷酸可以是单链核酸，并且可包含7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30或更多个核碱基。在具体方面，所述寡核苷酸可包含15至30、19至25、20至23或21个连续核碱基。在某些实施方案中，所述寡核苷酸抑制促进癌性或癌前或增生性哺乳动物细胞(例如人细胞)的生长的基因的翻译。寡核苷酸可诱导细胞中的细胞凋亡，和/或抑制致癌基因或其他靶基因的翻译。在某些实施方案中，所述寡核苷酸组分包含单一种类的寡核苷酸。在其他实施方案中，所述寡核苷酸组分包含靶向1、2、3、4种或更多种基因的2、3、4种或更多种类的寡核苷酸。所述组合物还可包含化学治疗剂或其他抗癌剂，所述化学治疗剂或其他抗癌剂可以或可以不并入本发明的脂质组分或脂质体中。在其他实施方案中，所述寡核苷酸组分被并入脂质体或脂质组分内。

[0023] “包埋”、“封装”和“并入”是指脂质或脂质体通过与目标药剂缔合或在目标药剂周

围而形成对自由扩散到溶液中的障碍，例如，脂质体可将药剂封装在脂质层内或脂质层内部或之间的水性隔室内。在某些实施方案中，所述组合物包含在药学上可接受的载体中。所述药学上可接受的载体可被配制用于施用至人受试者或患者。

[0024] 在某些实施方案中，所述脂质组分具有基本上中性的电荷，因为它包含中性磷脂或净中性电荷。在某些方面，中性磷脂可以是磷脂酰胆碱，如DOPC、卵磷脂酰胆碱(“EPC”)、二月桂酰磷脂酰胆碱(“DLPC”)、二肉豆蔻酰磷脂酰胆碱(“DMPC”)、二棕榈酰磷脂酰胆碱(“DPPC”)、二硬脂酰磷脂酰胆碱(“DSPC”)、1-肉豆蔻酰基-2-棕榈酰磷脂酰胆碱(“MPPC”)、1-棕榈酰基-2-肉豆蔻酰磷脂酰胆碱(“PMPC”)、1-棕榈酰基-2-硬脂酰磷脂酰胆碱(“PSPC”)、1-硬脂酰基-2-棕榈酰磷脂酰胆碱(“SPPC”)、二肉豆蔻基磷脂酰胆碱(“DMPC”)、1,2-二硬脂酰基-sn-甘油基-3-磷酸胆碱(“DAPC”)、1,2-二花生酰基-sn-甘油基-3-磷酸胆碱(“DBPC”)、1,2-双二十碳烯酰基-sn-甘油基-3-磷酸胆碱(“DEPC”)、棕榈酰油酰基磷脂酰胆碱(“POPC”)、溶血磷脂酰胆碱或二亚油酰基磷脂酰胆碱。在其他方面，中性磷脂可以是磷脂酰乙醇胺，如二油酰磷脂酰乙醇胺(“DOPE”)、二硬脂酰磷脂酰乙醇胺(“DSPE”)、二肉豆蔻酰磷脂酰乙醇胺(“DMPE”)、二棕榈酰磷脂酰乙醇胺(“DPPE”)、棕榈酰油酰基磷脂酰乙醇胺(“POPE”)或溶血磷脂酰乙醇胺。在某些实施方案中，所述磷脂组分可包含1、2、3、4、5、6、7、8种或更多种类别或类型的中性磷脂。在其他实施方案中，磷脂组分可包含2、3、4、5、6种或更多种类别或类型的中性磷脂。

[0025] 在某些实施方案中，脂质组分可具有基本上中性的电荷，因为它包含带正电荷的脂质和带负电荷的脂质。所述脂质组分还可包含带中性电荷的脂质或磷脂。带正电荷的脂质可以是带正电荷的磷脂。带负电荷的脂质可以是带负电荷的磷脂。带负电荷的磷脂可以是磷脂酰丝氨酸，如二肉豆蔻酰磷脂酰丝氨酸(“DMPS”)、二棕榈酰磷脂酰丝氨酸(“DPPS”)或脑磷脂酰丝氨酸(“BPS”)。带负电荷的磷脂可以是磷脂酰甘油，如二月桂酰磷脂酰甘油(“DLPG”)、二肉豆蔻酰磷脂酰甘油(“DMPG”)、二棕榈酰磷脂酰甘油(“DPPG”)、二硬脂酰磷脂酰甘油(“DSPG”)或二油酰磷脂酰甘油(“DOPG”)。在某些实施方案中，所述组合物还包含胆固醇或聚乙二醇(PEG)。在其他实施方案中，所述组合物基本上不含胆固醇。在某些实施方案中，磷脂是天然存在的磷脂。在其他实施方案中，磷脂是合成磷脂。

[0026] 脂质体可由一种或多种磷脂制成，只要脂质材料基本上不带电。重要的是所述组合物基本上不含阴离子和阳离子磷脂和胆固醇。合适的磷脂包括磷脂酰胆碱和本领域技术人员熟知的其他磷脂。

[0027] 本发明的另一方面涉及用于将寡核苷酸递送至细胞的方法，所述方法包括使细胞与本发明的中性脂质组合物接触。所述方法将提供有效量的本发明组合物。有效量是减弱、减缓、减轻或消除受试者中的细胞、病状或疾病状态的治疗组分的量。所述细胞可包含在受试者或患者(如人)体内。所述方法还可包括治疗癌症或其他增生性病状的方法。所述癌症可起源于膀胱、血液、骨骼、骨髓、脑、乳房、结肠、食道、胃肠、牙龈、头部、肾、肝、肺、鼻咽、颈部、前列腺、皮肤、胃、睾丸、舌或子宫。在某些实施方案中，所述方法还包括治疗非癌性疾病或增生性病状的方法。所述细胞可以是癌前细胞或癌性细胞。在某些实施方案中，所述组合物和方法抑制细胞的生长，诱导细胞中的细胞凋亡和/或抑制致癌基因的翻译。所述寡核苷酸可抑制在癌性细胞中过量表达的基因的翻译。

[0028] 在某些实施方案中，本发明的方法还包括向所述受试者施用另外的治疗。另外的

疗法可包括施用化学治疗剂(例如紫杉醇或多西他赛)、手术疗法、放射疗法和/或基因疗法。在某些方面,化学疗法是多西他赛、紫杉醇、顺铂(CDDP)、卡铂、丙卡巴肼、氮芥、环磷酰胺、喜树碱、异环磷酰胺、美法仑、苯丁酸氮芥、白消安、亚硝基脲、更生霉素、柔红霉素、阿霉素、博来霉素、普卡霉素、丝裂霉素、依托泊苷(VP16)、它莫昔芬、雷洛昔芬、雌激素受体结合剂、紫杉酚、吉西他滨、诺维本、法尼基蛋白转移酶抑制剂、反铂、5-氟尿嘧啶、长春新碱、长春花碱、甲氨蝶呤或其组合。在某些实施方案中,化学疗法是紫杉烷,如多西他赛或紫杉醇。化学疗法可在相对于本发明的中性脂质组合物之前、期间、之后或其组合递送。化学疗法可在中性脂质组合物的0、1、5、10、12、20、24、30、48或72小时或更多小时内递送。所述中性脂质组合物、第二种抗癌疗法或中性脂质组合物和抗癌疗法两者可肿瘤内、静脉内、腹膜内、皮下、口服或通过其各种组合施用。

[0029] 预期本说明书中论述的任何实施例可关于本发明的任何方法或组合来实施,反之亦然。此外,本发明的组合物可用于实现本发明的方法。

[0030] 如本文所用,就指定组分而言“基本上不含”在本文中用于表示指定组分未被有目的地配制到组合物中和/或仅以污染物或痕量存在。因此,由组合物的任何意外污染产生的指定组分的总量远低于0.05%,优选低于0.01%。最优选的是用标准分析方法检测不到指定组分量的组合物。

[0031] 如本文在说明书中所用,“一个/种(a/an)”可指一个(种)或多个(种)。如本文在权利要求中所用,当与词语“包括/包含(comprising)”结合使用时,词语“一个/种”可指一个(种)或多于一个(种)。

[0032] 除非明确指明仅仅指代替代物,或替代物相互排斥,否则权利要求书中所用的术语“或”用于指“和/或”,尽管本公开支持仅仅指代替代物和“和/或”的定义。如本文所用,“另一”可指至少第二个(种)或更多个(种)。

[0033] 在整个本申请中,术语“约”用于指示值包括装置、用以测定所述值的方法的误差的固有变化,或研究受试者中存在的变化。

[0034] 通过以下详细描述,本发明的其他目的、特征和优点将变得显而易见。然而,应理解的是,尽管指示本发明的优选实施方案,但是详细描述和特定实施例仅通过说明的方式给出,因为从此详细描述中,本发明的精神和范围内的各种改变和修改对于本领域技术人员来说将变得显而易见。

[0035] 说明性实施方案的描述

[0036] 本发明提供用于经由脂质组合物,在某些方面具有约零的净电荷的脂质组合物(即中性脂质组合物)将寡核苷酸(例如,基因表达的抑制剂)递送至细胞的组合物和方法。在某些实施方案中,所述脂质组合物是不带电的脂质体。这些方法可有效地用于治疗癌症。

[0037] I.脂质和脂质体

[0038] “脂质体”在本文中用于表示具有脂质双层的含脂质囊泡,以及包埋或并入反义寡核苷酸的其他脂质载体颗粒。如此,脂质体是涵盖通过产生封闭的脂质双层或聚集体而形成的各种单层、多层和多囊脂质媒介物的通用术语。此外,脂质体可具有未定界的层状结构。脂质体可被表征为具有囊泡结构,所述囊泡结构具有磷脂双层膜和内部水介质。多层脂质体具有通过水介质分隔的多个脂质层。它们在磷脂悬浮于过量水溶液中时自发形成。脂质组分在形成封闭结构之前进行自我重排,并将水和溶解的溶质包埋在脂质双层之间

(Ghosh和Bachhawat,1991)。然而,与正常囊泡结构相比,本发明还涵盖在溶液中具有不同结构的组合物。例如,脂质可呈现胶束结构或仅仅以非均匀脂质分子聚集体形式存在。

[0039] 脂质体是一种形式的纳米颗粒,所述纳米颗粒是用于将各种药物递送到患病组织中的载体。最佳脂质体大小取决于靶组织。在肿瘤组织中,血管系统是不连续的,并且孔径从100至780nm变化(Siwak等人,2002)。相比之下,正常血管内皮中的孔径在大多数组织中是<2nm,并且在毛细血管后微静脉中是6nm。认为带负电荷的脂质体比中性或带正电荷的脂质体更快速地从循环中除去;然而,最近的研究已经表明,带负电荷的脂质的类型影响网状内皮系统(RES)摄取脂质体的速率。例如,含有未空间屏蔽的带负电荷的脂质(磷脂酰丝氨酸、磷脂酸和磷脂酰甘油)的脂质体比中性脂质体更快地清除。有趣的是,阳离子脂质体(1,2-二油酰基-3-三甲基铵-丙烷[DOTAP])和阳离子脂质体-DNA复合物比阴离子、中性或空间稳定的中性脂质体更易于结合并经由内吞作用被血管生成血管的内皮细胞内化(Thurston等人,1998;Krasnici等人,2003)。阳离子脂质体可能不是肿瘤细胞的理想递送媒介物,因为与肿瘤细胞的表面相互作用产生静电源性的结合位点屏障效应,从而抑制递送系统与肿瘤球体的进一步缔合(Kostarelos等人,2004)。然而,中性脂质体似乎具有更好的肿瘤内渗透。特定脂质体制剂的毒性也是一个问题。阳离子脂质体通过促进活性氧中间体的释放而引发剂量依赖性毒性和肺部炎症,并且这种效应在多价阳离子脂质体情况下比单价阳离子脂质体如DOTAP更显著(Dokka等人,2000)。中性脂质体和阴性脂质体似乎未表现出肺毒性(Guitierrez-Puente等人,1999)。阳离子脂质体虽然有效吸收核酸,但对于体内基因下调具有有限成功,这可能是由于其稳定的细胞内性质以及由此导致的释放核酸内容物失败。由于在体内递送反义寡核苷酸中的中性性质和成功,所以本文使用具有中性电荷的脂质或具有中和电荷的脂质组合物,例如1,2-二油酰基-sn-甘油基-3-磷酸胆碱(DOPC)。

[0040] 本发明提供用于将寡核苷酸(如反义寡核苷酸)与脂质和/或脂质体缔合的方法和组合物。所述寡核苷酸可并入脂质体的含水内部,散布在脂质体的脂质双层内,经由与脂质体和寡核苷酸两者缔合的连接分子连接至脂质体,包埋在脂质体中,与脂质体复合,分散在含有脂质的溶液中,与脂质混合,与脂质组合,以悬浮液形式含于脂质中,含于胶束中或与胶束复合,或以其他方式与脂质缔合。本文提供的脂质体或脂质体/寡核苷酸缔合的组合物不限于溶液中的任何特定结构。例如,它们可以存在于双层结构中,以胶束形式存在,或者具有“塌陷的”结构。它们也可以仅仅散布在溶液中,可能形成大小或形状不均匀的聚集体。

[0041] A.脂质

[0042] 脂质是可以为天然存在的或合成的脂肪物质。例如,脂质包括天然存在于细胞质中的脂肪微滴,以及含有长链脂肪烃及其衍生物的一类化合物,所述化合物是本领域的技术人员熟知的,如脂肪酸、醇、胺、氨基醇和醛。一个实例是脂质1,2-二油酰基-sn-甘油基-3-磷酸胆碱(DOPC)。

[0043] 本发明的脂质组合物可包含磷脂。在某些实施方案中,单一种类或类型的磷脂可用于产生脂质组合物,如脂质体。在其他实施方案中,可使用多于一种种类或类型的磷脂。

[0044] 磷脂包括甘油磷脂和某些鞘脂。磷脂包括但不限于二油酰磷脂酰胆碱(“DOPC”)、卵磷脂酰胆碱(“EPC”)、二月桂酰磷脂酰胆碱(“DLPC”)、二肉豆蔻酰磷脂酰胆碱(“DMPC”)、二棕榈酰磷脂酰胆碱(“DPPC”)、二硬脂酰磷脂酰胆碱(“DSPC”)、1-肉豆蔻酰基-2-棕榈酰磷脂酰胆碱(“MPPC”)、1-棕榈酰基-2-肉豆蔻酰磷脂酰胆碱(“PMPC”)、1-棕榈酰基-2-硬脂酰

磷脂酰胆碱(“PSPC”)、1-硬脂酰基-2-棕榈酰磷脂酰胆碱(“SPPC”)、二月桂酰磷脂酰甘油(“DLPG”)、二肉豆蔻酰磷脂酰甘油(“DMPG”)、二棕榈酰磷脂酰甘油(“DPPG”)、二硬脂酰磷脂酰甘油(“DSPG”)、二硬脂酰鞘磷脂(“DSSP”)、二硬脂酰磷脂酰乙醇胺(“DSPE”)、二油酰磷脂酰甘油(“DOPG”)、二肉豆蔻酰磷脂酸(“DMPA”)、二棕榈酰磷脂酸(“DPPA”)、二肉豆蔻酰磷脂酰乙醇胺(“DMPE”)、二棕榈酰磷脂酰乙醇胺(“DPPE”)、二肉豆蔻酰磷脂酰丝氨酸(“DMPS”)、二棕榈酰磷脂酰丝氨酸(“DPPS”)、脑磷脂酰丝氨酸(“BPS”)、脑鞘磷脂(“BSP”)、二棕榈酰鞘磷脂(“DPSP”)、二肉豆蔻酰磷脂酰胆碱(“DMPC”)、1,2-二硬脂酰基-sn-甘油基-3-磷酸胆碱(“DAPC”)、1,2-二花生酰基-sn-甘油基-3-磷酸胆碱(“DBPC”)、1,2-双二十碳烯酰基-sn-甘油基-3-磷酸胆碱(“DEPC”)、二油酰磷脂酰乙醇胺(“DOPE”)、棕榈酰油酰基磷脂酰胆碱(“POPC”)、棕榈酰油酰基磷脂酰乙醇胺(“POPE”)、溶血磷脂酰胆碱、溶血磷脂酰乙醇胺以及二亚油酰磷脂酰胆碱。

[0045] 磷脂包括例如磷脂酰胆碱、磷脂酰甘油和磷脂酰乙醇胺;因为磷脂酰乙醇胺和磷脂酰胆碱在生理条件下(即在约pH 7下)不带电,所以这些化合物对于产生中性脂质体可能特别有用。在某些实施方案中,磷脂DOPC用于产生不带电的脂质体或脂质组合物。在某些实施方案中,也可以使用不是磷脂(例如胆固醇)的脂质。

[0046] 磷脂可来自天然或合成来源。然而,来自天然来源的磷脂,如卵或大豆磷脂酰胆碱、脑磷脂酸、脑或植物磷脂酰肌醇、心磷脂以及植物或细菌磷脂酰乙醇胺在某些实施方案中不被用作主要磷脂(即,构成总磷脂组合物的50%或更多),因为这可导致所得脂质体的不稳定性和泄漏。

[0047] B.中性脂质体

[0048] 如本文使用的“中性脂质体或脂质组合物”或“不带电的脂质体或脂质组合物”被定义为具有一种或多种产生基本上中性净电荷(基本上不带电)的脂质的脂质体或脂质组合物。在某些实施方案中,中性脂质体或脂质组合物可主要包含本身为中性的脂质和/或磷脂。在某些实施方案中,两亲性脂质可并入中性脂质体或脂质组合物中或用于产生中性脂质体或脂质组合物。例如,中性脂质体可通过组合带正电荷和带负电荷的脂质而产生,以使得那些电荷基本上彼此抵消,从而产生基本上中性的净电荷。“基本上中性的”或“基本上不带电的”是指在给定群体(例如脂质体群体)内很少(如果有的话)脂质包含未被另一种成分的相反电荷抵消的电荷(例如,少于10%的组分包含未抵消的电荷,更优选少于5%,并且最优选少于1%)。在本发明的某些实施方案中,可制备组合物,其中所述组合物的脂质组分基本上是中性的,但不呈脂质体的形式。

[0049] 脂质体的大小取决于合成方法而变化。悬浮于水溶液中的脂质体通常呈球形囊泡形状,并可具有一个或多个脂质双层分子的同心层。每层由通过式XY表示的分子平行阵列组成,其中X是亲水性部分,并且Y是疏水性部分。在水性悬浮液中,所述同心层被排列成使得亲水性部分倾向于保持与水相接触,并且疏水性区域倾向于自缔合。例如,当水相存在于脂质体内部和外部两者时,脂质分子可形成排列XY-YX的称为薄层的双层。当多于一种脂质分子的亲水性部分和疏水性部分彼此缔合时,可形成脂质的聚集体。这些聚集体的大小和形状将取决于许多不同的变量,如溶剂的性质和溶液中其他化合物的存在。

[0050] 本发明范围内的脂质体可根据已知的实验室技术来制备,例如像Bangham等人(1965)的方法,其内容以引用的方式并入本文;Gregoriadis(1979)的方法,其内容以引用

的方式并入本文;Deamer和Uster(1983)的方法,其内容以引用的方式并入本文;以及由Szoka和Papahadjopoulos(1978)描述的反相蒸发方法。上述方法在其各自包埋水性材料的能力及其各自的水性空间与脂质比率方面不同。

[0051] 在某些实施方案中,中性脂质体可用于递送寡核苷酸,如反义寡核苷酸。所述中性脂质体可含有单一种类的针对抑制单个基因的翻译的寡核苷酸,或者所述中性脂质体可含有多种种类的针对抑制多种基因的翻译的寡核苷酸。此外,除了寡核苷酸之外,所述中性脂质体还可含有化学治疗剂;因此,在某些实施方案中,化学治疗剂和寡核苷酸可在同一组合物或分开的组合物中递送至细胞(例如人受试者中的癌性细胞)。

[0052] 干燥的脂质或冻干的脂质体可脱水并用合适的溶剂(例如,DPBS或Hepes缓冲液)以适当的浓度重构。所述混合物然后可在涡旋混合器中剧烈振荡。所述脂质体可以适当的总磷脂浓度(例如,约10-200mM)重新悬浮。未封装的寡核苷酸可通过在29,000g下离心除去并洗涤脂质体球粒。或者,未封装的寡核苷酸可通过用过量溶剂进行透析来除去。封装的寡核苷酸的量可根据标准方法来测定。

[0053] II.基因表达的抑制

[0054] 抑制性寡核苷酸可抑制细胞中的基因的转录或翻译。寡核苷酸的长度可以是5至50个或更多个核苷酸,并且在某些实施方案中可以是7至30个核苷酸。在某些实施方案中,寡核苷酸的长度可以是7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29或30个核苷酸。寡核苷酸可包含核酸和/或核酸类似物。典型地,抑制性寡核苷酸将抑制细胞内的单一基因的翻译;然而,在某些实施方案中,抑制性寡核苷酸可抑制细胞内的多于一种基因的翻译。

[0055] 在寡核苷酸内,寡核苷酸的组分不需要全部具有相同类型或同源的(例如,寡核苷酸可包含核苷酸和核酸或核苷酸类似物)。在本发明的某些实施方案中,寡核苷酸可仅包含单一核酸或核酸类似物。抑制性寡核苷酸可包含5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30或更多个连续核碱基,包括其间的所有范围,所述核碱基与互补核酸杂交以形成双链结构。

[0056] III.核酸

[0057] 本发明提供用于经由中性脂质体递送寡核苷酸的方法和组合物。因为寡核苷酸由核酸组成,所以与核酸有关的方法(例如核酸的产生,核酸的修饰等)也可关于寡核苷酸使用。

[0058] 术语“核酸”是本领域中熟知的。如本文所用的“核酸”通常是指DNA、RNA或其衍生物或类似物的分子(即,一条链),其包含核碱基。这些定义是指单链或双链核酸。双链核酸可通过完全互补的结合形成;然而,在一些实施方案中,双链核酸可通过部分或实质性互补结合而形成。如本文所用,单链核酸可由前缀“ss”表示,并且双链核酸可由前缀“ds”表示。

[0059] A.核碱基

[0060] 如本文所用,“核碱基”是指在至少一种天然存在的核酸(即DNA和RNA)中发现的杂环碱基,例如像天然存在的核碱基(即,A、T、G、C或U),以及这种核碱基的天然或非天然存在的衍生物和类似物。核碱基通常可以可取代天然存在的核碱基配对的方式与至少一种天然存在的核碱基形成一个或多个氢键(即“退火”或“杂交”)(例如,A与T、G与C以及A与U之间的氢键合)。使用本文所述或本领域普通技术人员已知的任何化学或天然合成方法,核碱基可

包含于核苷或核苷酸中。

[0061] “嘌呤”和/或“嘧啶”核碱基涵盖天然存在的嘌呤和/或嘧啶核碱基以及还有其衍生物和类似物，包括但不限于被烷基、羧基烷基、氨基、羟基、卤素(即氟、氯、溴或碘)、硫醇或烷基硫醇部分中的一个或多个取代的嘌呤或嘧啶。优选的烷基(例如，烷基、羧基烷基等)部分包含约1个、约2个、约3个、约4个、约5个至约6个碳原子。嘌呤或嘧啶的其他非限制性实例包括脱氮嘌呤、2,6-二氨基嘌呤、5-氟尿嘧啶、黄嘌呤、次黄嘌呤、8-溴鸟嘌呤、8-氯鸟嘌呤、溴胸腺嘧啶、8-氨基鸟嘌呤、8-羟基鸟嘌呤、8-甲基鸟嘌呤、8-硫鸟嘌呤、氮鸟嘌呤、2-氨基嘌呤、5-乙基胞嘧啶、5-甲基胞嘧啶、5-溴尿嘧啶、5-乙基尿嘧啶、5-碘尿嘧啶、5-氯尿嘧啶、5-丙基尿嘧啶、硫尿嘧啶、2-甲基腺嘌呤、甲硫基腺嘌呤、N,N-二甲基腺嘌呤、氮杂腺嘌呤、8-溴腺嘌呤、8-羟基腺嘌呤、6-羟基氨基嘌呤、6-巯基嘌呤、4-(6-氨基己基/胞嘧啶)等。嘌呤和嘧啶衍生物或类似物包括但不限于(缩写/修饰的碱基描述)：ac4c/4-乙酰胞苷；Mam5s2u/5-甲氧基氨基甲基-2-硫代尿苷；Chm5u/5-(羧基羟基甲基)尿苷；Man q/β,D-甘露糖基辫苷；Cm/2′-O-甲基胞苷；Mcm5s2u/5-甲氧基羰基甲基-2-硫代尿苷；Cmnm5s2u/5-羧甲基氨基-甲基-2-硫代尿苷；Mcm5u/5-甲氧基羰基甲基尿苷；Cmnm5u/5-羧甲基氨基甲基尿苷；Mo5u/5-甲氧基尿苷；D/二氢尿苷；Ms2i6a,2-甲硫基-N6-异戊烯腺苷；Fm/2′-O-甲基假尿苷；Ms2t6a/N-((9-β-D-呋喃核糖基-2-甲硫基嘌呤-6-基)氨基甲酰基)苏氨酸；Gal q/β,D-半乳糖基辫苷；Mt6a/N-((9-β-D-呋喃核糖基嘌呤-6-基)N-甲基-氨基甲酰基)苏氨酸；Gm/2′-O-甲基鸟苷；Mv/尿苷-5-羟乙酸甲酯；I/肌苷；o5u/尿苷-5-羟乙酸(v)；I6a/N6-异戊烯腺苷；Osyw/怀丁氧苷；m1a/1-甲基腺苷；P/假尿苷；m1f/1-甲基假尿苷；Q/辫苷；m1g/1-甲基鸟苷；s2c/2-硫代胞苷；m1I/1-甲基肌苷；s2t/5-甲基-2-硫代尿苷；m22g/2,2-二甲基鸟苷；s2u/2-硫代尿苷；m2a/2-甲基腺苷；s4u/4-硫代尿苷；m2g/2-甲基鸟苷；T/5-甲基尿苷；m3c/3-甲基胞苷；t6a/N-((9-β-D-呋喃核糖基嘌呤-6-基)氨基甲酰基)苏氨酸；m5c/5-甲基胞苷；Tm/2′-O-甲基-5-甲基尿苷；m6a/N6-甲基腺苷；Um/2′-O-甲基尿苷；m7g/7-甲基鸟苷；Yw/怀丁苷；Mam5u/5-甲基氨基甲基尿苷；或X/3-(3-氨基-3-羧丙基)尿苷，(acp3)u。

[0062] B.核苷

[0063] 如本文所用，“核苷”是指包含共价连接至核碱基接头部分的核碱基的单独化学单位。“核碱基接头部分”的非限制性实例是包含5-碳原子的糖(即“5-碳糖”)，包括但不限于脱氧核糖、核糖、阿拉伯糖、或5-碳糖的衍生物或类似物。5-碳糖的衍生物或类似物的非限制性实例包括2'-氟-2'-脱氧核糖，或碳环糖，其中碳取代糖环中的氧原子。如本文所用，“部分”通常是指较大化学或分子结构的较小化学或分子组分。

[0064] 核碱基与核碱基接头部分的不同类型的共价连接是本领域中已知的。作为非限制性实例，包含嘌呤(即A或G)或7-脱氮嘌呤核碱基的核苷通常包含嘌呤或7-脱氮嘌呤的9位与5-碳糖的1'-位的共价连接。在另一个非限制性实例中，包含嘧啶核碱基(即C、T或U)的核苷通常包含嘧啶的1位与5-碳糖的1'位的共价连接(Kornberg和Baker，1992年)。

[0065] C.核苷酸

[0066] 如本文所用，“核苷酸”是指还包含“主链键联”的核苷。主链键联通常将核苷酸共价连接至包含核苷酸的另一分子或另一核苷酸以形成核酸。天然存在的核苷酸中的“主链键联”通常包含磷酸酯部分(例如，磷酸二酯主链键联)，其共价连接至5-碳糖。主链部分的连接通常发生在5-碳糖的3'位或5'位。但是，其他类型的连接是本领域中已知的，尤其当核

苷酸包含天然存在的5-碳糖或磷酸酯部分的衍生物或类似物时。

[0067] D.核酸类似物

[0068] 核酸可包含可存在于天然存在的核酸中的核碱基的衍生物或类似物、核碱基接头部分和/或主链键联，或者完全由其组成。如本文所用，“衍生物”是指天然存在的分子的化学修饰或改变的形式，而术语“模拟”或“类似物”是指可以或可以不与天然存在的分子或部分在结构上相似，但具有类似的功能。核碱基、核苷和核苷酸类似物或衍生物是本领域中熟知的。

[0069] 包含5-碳糖和/或主链键联衍生物或类似物的核苷、核苷酸或核酸的非限制性实例包括以下中的那些：美国专利号5,681,947，其描述了包含与dsDNA形成三股螺旋和/或阻止dsDNA表达的嘌呤衍生物的寡核苷酸；美国专利号5,652,099和5,763,167，其描述了并入在DNA或RNA中发现的核苷的荧光类似物的核酸，特别是用作荧光核酸探针；美国专利号5,614,617，其描述了具有增强的核酸酶稳定性的在嘧啶环上具有取代的寡核苷酸类似物；美国专利号5,670,663、5,872,232和5,859,221，其描述了用于核酸检测的具有修饰的5-碳糖(即，修饰的2’-脱氧呋喃糖基部分)的寡核苷酸类似物；美国专利号5,446,137，其描述了可用于杂交测定中的包含至少一个在4’位被除氢之外的取代基取代的5-碳糖部分的寡核苷酸；美国专利号5,886,165，其描述了含有具有3’-5’主链键联的脱氧核糖核苷酸和具有2’-5’主链键联的核糖核苷酸两者的寡核苷酸；美国专利号5,714,606，其描述了修饰的主链键联，其中主链键联的3’-位氧被碳取代以增强核酸的核酸酶抗性；美国专利号5,672,697，其描述了含有增强核酸酶抗性的一个或多个5’亚甲基膦酸酯主链键联的寡核苷酸；美国专利号5,466,786和5,792,847，其描述了可包含药物或标记的取代基部分与寡核苷酸的2’碳的键联以提供增强的核酸酶稳定性和递送药物或检测部分的能力；美国专利号5,223,618，其描述了具有连接相邻5-碳糖部分的4’位和3’位以增强细胞摄取、对核酸酶的抗性和与靶RNA的杂交的2或3碳主链键联的寡核苷酸类似物；美国专利号5,470,967，其描述了可用作核酸杂交探针的包含至少一个氨基磺酸酯或磺酰胺主链键联的寡核苷酸；美国专利号5,378,825、5,777,092、5,623,070、5,610,289和5,602,240，其描述了用于改进核酸酶抗性、细胞摄取和调控RNA表达的具有替代磷酸二酯主链键联的三或四个原子的主链键联部分的寡核苷酸；美国专利号5,858,988，其描述了连接至寡核苷酸的2’-O位以增强它们的膜渗透性和稳定性的疏水性载体剂；美国专利号5,214,136，其描述了在5’末端与蒽醌缀合的寡核苷酸，所述寡核苷酸具有增强的与DNA或RNA的杂交、对核酸酶的增强的稳定性；美国专利号5,700,922，其描述了PNA-DNA-PNA嵌合体，其中所述DNA包含2′-脱氧-赤-戊呋喃糖基核苷酸以获得增强的核酸酶抗性、结合亲和力以及活化RNA酶H的能力；美国专利号5,708,154，其描述了连接至DNA以形成DNA-RNA杂合体的RNA；美国专利号5,908,845，其描述了聚醚核酸，其中一个或多个核碱基连接至聚醚主链中的手性碳原子；美国专利号5,786,461、5,891,625、5,786,461、5,773,571、5,766,855、5,736,336、5,719,262、5,714,331、5,539,082以及WO 92/20702，其描述了通常包含一个或多个核苷酸或核苷的肽核酸(PNA或基于肽的核酸类似物；或PENAM)，所述核苷酸或核苷包含核碱基部分、不是5-碳糖的核碱基接头部分(例如，氮杂氮原子、酰胺基和/或脲基系链)和/或不是磷酸酯主链键联的主链键联(例如，氨基乙基甘氨酸、聚酰胺、聚乙基、聚硫代酰胺、聚亚磺酰胺或聚磺酰胺主链键联)；以及美国专利号5,855,911，其描述了疏水性、核酸酶抗性的对乙氧基主链键联。

[0070] 核酸类似物的其他修饰和用途在本领域中是已知的，并且预期这些技术和类型的核酸类似物可用于本发明。

[0071] E.核酸的制备

[0072] 可通过本领域普通技术人员已知的任何技术，如化学合成、酶法产生或生物性产生来制备核酸。合成核酸(例如，合成寡核苷酸)的非限制性实例包括使用磷酸三酯、亚磷酸酯或亚磷酰胺化学和固相技术通过体外化学合成制备的核酸，如以引用的方式并入本文的EP 266,032中所描述；或者通过脱氧核苷H-膦酸酯中间体制备的核酸，如Froehler等人(1986)和美国专利号5,705,629所描述，其各自以引用的方式并入本文。在本发明的方法中，可使用一种或多种种类的寡核苷酸。寡核苷酸合成的各种机制已经公开于例如美国专利号4,659,774、4,816,571、5,141,813、5,264,566、4,959,463、5,428,148、5,554,744、5,574,146、5,602,244中，其各自以引用的方式并入本文。

[0073] F.核酸的纯化

[0074] 核酸可在聚丙烯酰胺凝胶上、氯化铯离心梯度或通过本领域普通技术人员已知的任何其他方式(参见例如Sambrook等人(2001)，以引用的方式并入本文)进行纯化。

[0075] 在某些实施方案中，本发明涉及为分离的核酸的核酸。如本文所用，术语“分离的核酸”是指已经分离为不含或者以其他方式不含一种或多种细胞的大部分总基因组和转录核酸的核酸分子(例如，RNA或DNA分子)。在某些实施方案中，“分离的核酸”是指已经分离为不含或以其他方式不含大部分细胞组分或体外反应组分的核酸，例如像大分子如脂质或蛋白质、小生物分子等。

[0076] G.杂交

[0077] 如本文所用，“杂交(hybridization)”、“杂交(hybridize)”或“能够杂交”应理解为是指形成双链或三链分子或具有部分双链或三链性质的分子。如本文使用的术语“退火”与“杂交”同义。

[0078] 如本文所用，“严格条件”或“高严格性”是允许一条或多条含有互补序列的核酸链之间或之内的杂交，但阻止随机序列杂交的那些条件。严格条件容许核酸与靶链之间的很少(如果有的话)错配。此类条件是本领域普通技术人员熟知的，并且对于需要高选择性的应用是优选的。

[0079] 严格条件可包括低盐和/或高温条件，如在约50℃至约70℃的温度下由约0.02M至约0.15M NaCl提供。应理解，所需严格性的温度和离子强度部分地由特定核酸的长度、靶序列的长度和核碱基含量、核酸的电荷组成以及杂交混合物中甲酰胺、四甲基氯化铵或其他溶剂的存在或浓度决定。

[0080] 还应理解，用于杂交的这些范围、组成和条件仅作为非限制性实例提及，并且用于特定杂交反应的所需严格性通常通过与一种或多种阳性或阴性对照进行比较来凭经验确定。取决于所设想的应用，优选使用不同的杂交条件以实现核酸对靶序列的不同程度的选择性。在非限制性实例中，鉴定或分离在严格条件下不与核酸杂交的相关靶核酸可通过在低温和/或高离子强度下杂交来实现。此类条件被称为“低严格性”或“低严格性条件”，并且低严格性的非限制性实例包括在约0.15M至约0.9M NaCl在约20℃至约50℃的温度范围进行杂交。当然，本领域技术人员能够进一步修改低或高严格性条件以适应特定应用。

[0081] IV.制造脂质体对乙氧基反义药物产品的方法

[0082] 脂质体对乙氧基反义药物产品由两种cGMP产品组成，两种产品均具有FDA批准的分析证书和FDA批准的发布标准。本文描述了原材料、溶剂和最终药物产品。当制造时，所述药物产品是包含以下材料的琥珀色或白色冻干晶体或粉末：寡核苷酸(例如对乙氧基反义药品)、中性脂质(例如，DOPC)和表面活性剂(例如，聚山梨醇酯20)。在准备向患者施用时，将生理盐水加入小瓶中，此时形成脂质体，其中对乙氧基反义并入内部中。

[0083] 可在生产对乙氧基反义药品期间使用预先确定的对乙氧基和磷酸二酯亚酰胺原材料混合物来限定最终产品的特定物理性质(例如，溶解度和疏水性，其然后影响盐水中的药物产品溶解度，寡核苷酸并入脂质体中以及脂质体粒度)。增加寡核苷酸主链中对乙氧基分子的数量导致分子疏水性更高(其产生更大的脂质体颗粒)、极性更低、溶解性更低。当寡核苷酸由于更大量的对乙氧基主链键联而变得较不可溶时，重构溶液变得更白，直到微粒形成，因为疏水性变得太高。

[0084] 表面活性剂(聚山梨醇酯20)对脂质体粒度的影响通过滴定表面活性剂的量来确定。在不存在聚山梨醇酯20的情况下，仅2.8％的颗粒具有300nm或更小的直径。在1x聚山梨醇酯20(总脂质体对乙氧基反义药物产品的约5％)存在下，12.5％的颗粒具有300nm或更小的直径。通过加入3x-10x聚山梨醇酯20，约20％的颗粒具有300nm或更小的直径。因此表面活性剂从1x增加至3x导致粒度减小。

[0085] V.测试脂质体对乙氧基反义药物产品的方法

[0086] 制造的药物产品的目视检查：在制造后，选择含有药物产品的样品小瓶并且目视检查。液体不存在是强制性的，且然后小瓶底部的琥珀晶体是可接受的，并且接受性增加至白色絮凝状粉末或外观，这是最好的结果。白色外观表明更好的干燥过程，具有高表面积与质量比，这非常有利于重构使用。

[0087] 准备好用于患者IV的重构药物的视觉检查：将生理盐水加入到含有制造的脂质体对乙氧基反义药物产品的小瓶中，并且振荡以重新构成药物晶体或粉末完全溶解的溶液。得出三个主要观察结果：1)晶体或粉末完全溶解，2)不存在不溶性材料的白色团块，和3)外观是乳白色或脱脂乳外观。重构液体的外观越蓝越好，因为这表示反映蓝色光谱中的光的更小脂质体粒度。

[0088] 质谱法：质谱法(质谱)用于展示样品中各种质量的分布型。当产生对乙氧基反义物质时，在样品上运行质谱。结果显示栅格上存在的材料的峰在右侧的“x”轴上具有递增质量，并且“y”轴上的相对质量丰度向上递增。对来自样品的分布型进行分析以确定对乙氧基样品中对乙氧基主链的相对数量，从而认识到峰的分布型代表(从最右开始)所有主链均包含对乙氧基键联的全长材料，向左移动的下一个峰代表一个主链具有对乙氧基缺失(且因此，乙基被敲除，并且结果是正常磷酸二酯主链键联)的全长，并且继续。向右偏移的质谱图表示具有更多对乙氧基主链的对乙氧基样品，且因此具有更高疏水性和更低溶解性的性质；并且同样，向左偏移具有相反的效应。样品的质谱图表的检查也可用于确定制造期间的过滤是否对存在于过滤的药物产品中的寡核苷酸组合物产生任何不利影响。

[0089] UV测试：使用紫外光测试来确定样品中存在的寡核苷酸的质量。寡核苷酸吸收260纳米范围内的光。因此，最终重构的药物产品的UV测试已被用作确定一瓶药物产品中寡核苷酸药品的量的方法。就制造开发和创新而言，UV测试用于确定是否存在在制造中的过滤期间经历的问题或对乙氧基反义药品的较差溶解性，从而导致溶液中寡核苷酸较少且因此

UV读数较低。所述方法将被验证并可能成为最终产品发布测试的一部分。

[0090] 脂质体粒度:将一瓶成品药物产品重构并测试其脂质体粒度。结果通常是大致正态分布,具有中心点、尾部和平均值,或大部分颗粒的大致正态分布,以及由二阶颗粒形成效应产生的较小脂质体颗粒的次级峰。重要的是脂质体颗粒不要太大,因为它们可能会在患者中引起不良作用(例如,在肺部的较小血管中产生血流问题)。结果,药物产品发布标准包括粒度测试显示90%的脂质体的大小为约5微米或更小,或约3微米或更小。此外,更小的脂质体是优选的,因为它们将具有更好的细胞摄取,并且其次,更小的脂质体可穿透血管孔隙,从而允许脂质体穿透内部肿瘤,从而增加脂质体对乙氧基反义药物产品的治疗有效性。

[0091] VI.治疗方法

[0092] 本发明的某些方面提供一种用于治疗诸如癌症、自身免疫性疾病或感染性疾病的疾病的寡核苷酸-脂质复合物(例如并入不带电的脂质体中的寡核苷酸)。具体地说,所述寡核苷酸可具有允许与人核苷酸序列碱基配对的序列,且因此可抑制由人核苷酸序列编码的蛋白质的表达。

[0093] “治疗(Treatment)”和“治疗(treating)”是指为了获得疾病或健康相关病状的治疗益处的目的向受试者施用或施加治疗剂或对受试者进行手术或模态。例如,治疗可包括施用药学有效量的寡核苷酸-脂质复合物。

[0094] “受试者”和“患者”是指人或非人,如灵长类动物、哺乳动物和脊椎动物。在具体实施方案中,受试者是人。

[0095] 如贯穿本申请中使用的术语“治疗益处”或“治疗有效的”是指促进或增强受试者关于此病状的医学治疗的福祉的任何物质。这包括但不限于疾病的体征或症状的频率或严重程度的降低。例如,癌症的治疗可涉及例如肿瘤大小减小、肿瘤的侵袭性降低、癌症的生长速率降低或预防转移。癌症的治疗也可指癌症受试者的存活期延长。治疗自身免疫性疾病可涉及例如减少针对其存在不期望的免疫应答的自身抗原的表达,诱导针对其存在不期望的免疫应答的自身抗原的耐受性,或抑制针对所述自身抗原的免疫应答。感染性疾病的治疗可涉及例如消除感染因子、降低感染因子的水平或将感染因子的水平维持在一定水平。

[0096] 本发明的治疗方法可用的肿瘤包括任何恶性细胞类型,如在实体瘤、血液肿瘤、转移性癌症或非转移性癌症中发现的那些。示例性实体瘤可包括但不限于选自由以下各项组成的组的器官的肿瘤:胰腺、结肠、盲肠、食道、胃肠、牙龈、肝、皮肤、胃、睾丸、舌、子宫、胃、脑、头部、颈部、卵巢、肾、喉、肉瘤、骨、肺、膀胱、黑色素瘤、前列腺以及乳房。示例性血液肿瘤包括骨髓肿瘤、T或B细胞恶性肿瘤、白血病、淋巴瘤、母细胞瘤、骨髓瘤等。可使用本文提供的方法治疗的癌症的其他实例包括但不限于癌、淋巴瘤、母细胞瘤、肉瘤、白血病、鳞状细胞癌、肺癌(包括小细胞肺癌、非小细胞肺癌癌症、肺腺癌以及肺鳞状癌)、腹膜癌、肝细胞癌、胃癌(gastric cancer)或胃癌(stomach cancer)(包括胃肠癌和胃肠道间质癌)、胰腺癌、成胶质细胞瘤、宫颈癌、卵巢癌、肝癌、膀胱癌、乳腺癌、结肠癌、结肠直肠癌、子宫内膜癌或子宫癌、唾液腺癌、肾癌(kidney cancer)或肾癌(renal cancer)、前列腺癌、外阴癌、甲状腺癌、各种类型的头颈癌、黑色素瘤、表面扩散型黑色素瘤、恶性小痣黑色素瘤、肢端雀斑样痣黑色素瘤、结节性黑色素瘤、以及B细胞淋巴瘤(包括低度/滤泡性非霍奇金淋巴瘤(NHL)、小淋巴细胞性(SL)NHL、中度/滤泡性NHL、中度弥漫性NHL、高度免疫母细胞性NHL、高

度成淋巴细胞性NHL、高度小非分裂细胞NHL、巨大肿块NHL、套细胞淋巴瘤、AIDS相关性淋巴瘤以及瓦尔登斯特伦氏巨球蛋白血症)、慢性淋巴细胞性白血病(CLL)、急性成淋巴细胞性白血病(ALL)、毛细胞白血病、多发性骨髓瘤、急性骨髓性白血病(AML)以及慢性成髓细胞性白血病。

[0097] 癌症可具体地具有以下组织学类型,但不限于这些:瘤,恶性;癌;癌,未分化;巨细胞和梭形细胞癌;小细胞癌;乳头状癌;鳞状细胞癌;淋巴上皮癌;基底细胞癌;毛基质癌;移行细胞癌;乳头状移行细胞癌;腺癌;胃泌素瘤,恶性;胆管癌;肝细胞癌;混合型肝细胞癌和胆管癌;小梁腺癌;腺样囊性癌;腺瘤性息肉腺癌;腺癌,家族性结肠息肉病;实体癌;类癌瘤,恶性;支气管-肺泡腺癌;乳头状腺癌;嫌色细胞癌;嗜酸细胞癌;嗜酸性腺癌;嗜碱性粒细胞癌;透明细胞腺癌;颗粒细胞癌;滤泡性腺癌;乳头状和滤泡性腺癌;非包囊硬化性癌;肾上腺皮质癌;子宫内膜样癌;皮肤附属器癌;大汗腺腺癌;皮脂腺癌;耵聍腺腺癌;粘液表皮样癌;囊腺癌;乳头状囊腺癌;乳头状浆液性囊腺癌;粘液性囊腺癌;粘液性腺癌;印戒细胞癌;浸润性导管癌;髓样癌;小叶癌;炎性癌;佩吉特病,乳房;腺泡细胞癌;腺鳞癌;伴随鳞状化生的腺癌;胸腺瘤,恶性;卵巢间质瘤,恶性;泡膜细胞瘤,恶性;粒层细胞瘤,恶性;男性细胞瘤,恶性;滋养细胞癌;莱迪希细胞瘤,恶性;脂质细胞瘤,恶性;副神经节瘤,恶性;乳房外副神经节瘤,恶性;嗜铬细胞瘤;皮肤丝球肉瘤;恶性黑色素瘤;无色素性黑色素瘤;表面扩散型黑色素瘤;巨大色素痣内恶性黑色素瘤;上皮样细胞黑色素瘤;蓝色痣,恶性;肉瘤;纤维肉瘤;纤维性组织细胞瘤,恶性;粘液肉瘤;脂肪肉瘤;平滑肌肉瘤;横纹肌肉瘤;胚胎性横纹肌肉瘤;腺泡型横纹肌肉瘤;间质肉瘤;混合瘤,恶性;苗勒管混合瘤;肾母细胞瘤;肝母细胞瘤;癌肉瘤;间质瘤,恶性;布伦纳瘤,恶性;叶状瘤,恶性;滑膜肉瘤;间皮瘤,恶性;无性细胞瘤;胚胎性癌;畸胎瘤,恶性;甲状腺肿样卵巢瘤,恶性;绒毛膜癌;中肾瘤,恶性;血管肉瘤;血管内皮瘤,恶性;卡波济氏肉瘤;血管外皮细胞瘤,恶性;淋巴管肉瘤;骨肉瘤;皮质旁成骨肉瘤;软骨肉瘤;成软骨细胞瘤,恶性;间叶性软骨肉瘤;骨巨细胞瘤;尤因氏肉瘤;牙源性肿瘤,恶性;成釉细胞性牙肉瘤;成釉细胞瘤,恶性;成釉细胞纤维肉瘤;松果体瘤,恶性;脊索瘤;神经胶质瘤,恶性;室管膜瘤;星形细胞瘤;原浆型星形细胞瘤;纤维性星形细胞瘤;成星形细胞瘤;成胶质细胞瘤;少突神经胶质瘤;成少突神经胶质细胞瘤;原始神经外胚层;小脑肉瘤;成神经节细胞瘤;成神经细胞瘤;成视网膜细胞瘤;嗅神经源性肿瘤;脑膜瘤,恶性;神经纤维肉瘤;神经鞘瘤,恶性;颗粒细胞瘤,恶性;恶性淋巴瘤;霍奇金病;霍奇金;副肉芽肿;恶性淋巴瘤,小淋巴细胞性;恶性淋巴瘤,大细胞,弥漫性;恶性淋巴瘤,滤泡性;蕈样霉菌病;其他特定非霍奇金淋巴瘤;恶性组织细胞增多症;多发性骨髓瘤;肥大细胞肉瘤;免疫增生性小肠病;白血病;淋巴细胞性白血病;浆细胞白血病;红白血病;淋巴肉瘤细胞白血病;骨髓性白血病;嗜碱细胞性白血病;嗜酸性粒细胞白血病;单核细胞白血病;肥大细胞白血病;巨核细胞白血病;髓样肉瘤;以及毛细胞白血病。

[0098] 本发明治疗方法可用的自身免疫性疾病包括但不限于脊椎关节病、强直性脊柱炎、银屑病性关节炎、反应性关节炎、肠病性关节炎、糖尿病、乳糜泻、自身免疫性甲状腺疾病、自身免疫性肝病、艾迪生氏病、移植排斥、移植物抗宿主病、宿主抗移植物病、溃疡性结肠炎、克罗恩氏病、肠易激综合征、炎性肠病、类风湿性关节炎、青少年类风湿性关节炎、家族性地中海热、肌萎缩性侧索硬化、舍格伦综合征、早期关节炎、病毒学关节炎、多发性硬化症或银屑病。文献中详细记载了这些疾病的诊断和治疗。

[0099] 本发明治疗方法可用的感染性疾病包括但不限于细菌感染、病毒感染、真菌感染和寄生虫感染。示例性病毒感染包括乙型肝炎病毒、丙型肝炎病毒、人免疫缺陷病毒1、人免疫缺陷病毒2、人乳头瘤病毒、单纯疱疹病毒1、单纯疱疹病毒2、带状疱疹、水痘带状疱疹、柯萨奇病毒A16、巨细胞病毒、埃博拉病毒、肠病毒、埃-巴二氏病毒、汉坦病毒、亨德拉病毒、病毒性脑膜炎、呼吸道合胞病毒、轮状病毒、西尼罗病毒、腺病毒以及流感病毒感染。示例性细菌感染包括沙眼衣原体、单核细胞增多性李斯特氏菌、幽门螺杆菌、大肠杆菌、伯氏疏螺旋体、嗜肺军团菌、分枝杆菌属(例如,结核分枝杆菌、鸟分枝杆菌、胞内分枝杆菌、堪萨斯分枝杆菌、戈登分枝杆菌)、金黄色葡萄球菌、淋病奈瑟菌、脑膜炎奈瑟菌、酿脓链球菌(甲类链球菌)、无乳链球菌(乙类链球菌)、链球菌(草绿色组)、粪链球菌、牛链球菌、肺炎链球菌、致病性弯曲杆菌属、肠球菌属、流感嗜血杆菌、炭疽芽孢杆菌、白喉棒状杆菌、棒状杆菌属、猪红斑丹毒丝菌、产气荚膜梭菌、破伤风梭菌、产气肠杆菌、肺炎克雷白氏杆菌、多杀巴斯德氏菌、拟杆菌属、具核梭杆菌、念珠状链杆菌、梅毒螺旋体、细弱密螺旋体、钩端螺旋体、立克次氏体、衣氏放线菌、志贺氏杆菌属(例如,弗氏志贺菌、宋内志贺菌、痢疾志贺菌)以及沙门氏菌属感染。示例性真菌感染包括白色念珠菌、光滑念珠菌、烟曲霉、土曲霉、新型隐球菌、荚膜组织胞浆菌、粗球孢子菌、皮炎芽生菌以及沙眼衣原体感染。

[0100] 寡核苷酸-脂质复合物在本文中可以各种形式用作抗肿瘤剂、抗病毒剂、抗细菌剂、抗真菌剂、抗寄生虫剂或抗自身免疫剂。在一个具体实施方案中,本发明考虑使用寡核苷酸-脂质复合物的方法包括使患病细胞群体与治疗有效量的寡核苷酸-脂质复合物接触足以抑制或逆转疾病的时间段。

[0101] 在一个实施方案中,体内接触通过经由静脉内、腹膜内、皮下或肿瘤内注射向患者施用治疗有效量的包含本发明的寡核苷酸-脂质复合物的生理上可耐受的组合物来实现。寡核苷酸-脂质复合物可通过注射或通过随时间推移逐渐输注而在肠胃外施用。

[0102] 例如,包含寡核苷酸-脂质复合物的治疗组合物通常静脉内或皮下施用,例如像通过注射单位剂量施用。当关于治疗组合物使用时,术语“单位剂量”是指适合作为单一剂量用于受试者的物理上分离的单位,每个单位含有与所需稀释剂(即载体或媒介物)缔合的经计算可产生所需治疗效果的预定量的活性物质。

[0103] 所述组合物以与剂量制剂相容的方式并以治疗有效量施用。待施用的量取决于待治疗的受试者、受试者的系统利用活性成分的能力以及所需的治疗效果的程度。施用所需要的活性成分的精确量取决于医师的判断并且是每一个个体所特有的。然而,用于全身施加的合适剂量范围在本文中公开并取决于施用途径。还考虑用于初始和加强施用的合适方案,并且通过初始施用、接着通过随后注射或其他施用在一个或多个小时间隔的重复剂量来代表。示例性多次施用在本文中描述并且特别优选用于持续维持多肽的高血清和组织水平。或者,考虑足以将血液中的浓度维持在体内治疗所规定的范围内的连续静脉内输注。

[0104] 考虑本发明的寡核苷酸可全身或局部施用以治疗疾病,如抑制肿瘤细胞生长或杀死患有局部晚期或转移癌症的癌症患者的癌细胞。它们可静脉内、鞘内、皮下和/或腹膜内施用。它们可单独施用或与抗增生药物组合施用。在一个实施方案中,在手术或其他程序之前施用它们以降低患者的癌症负荷。或者,可在手术后施用它们以确保任何剩余的癌症(例如,手术未能消除的癌症)不会存活。

[0105] 寡核苷酸的治疗有效量是经计算可实现所需效果(即抑制靶蛋白表达)的预定量。

因此，本发明的寡核苷酸的施用的剂量范围是足够大到产生所需效果的那些剂量范围。所述剂量不应大到导致不良的副作用，如高粘滞综合征、肺水肿、充血性心力衰竭等。一般来说，所述剂量将随着患者的年龄、病状、性别和疾病程度而变化且可由本领域的技术人员来确定。如果有任何并发症，则所述剂量可由单个医师调节。

[0106] 本发明的组合物优选肠胃外施用至患者，例如通过静脉内、动脉内、肌内、淋巴内、腹膜内、皮下、胸膜内或鞘内注射施用，或者可离体使用。优选的剂量是介于5-25mg/kg之间。优选按照时间表重复施用，直到癌症消失或消退，并且可与其他形式的治疗结合。

[0107] VII.药物制剂

[0108] 包含脂质体的药物组合物通常将包含无菌的药学上可接受的载体或稀释剂，如水或盐水溶液。

[0109] 当进行含有寡核苷酸的不带电的脂质组分(例如呈脂质体形式)的临床施加时，将脂质复合物制备成适合于预期施加的药物组合物通常将是有益的。这通常将需要制备基本上不含热原以及可能对人或动物有害的任何其他杂质的药物组合物。还可使用适当的缓冲液以使复合物稳定并允许被靶细胞摄取。

[0110] 短语“药物或药理学上可接受的”包括视情况而定当向动物如人施用时不产生副作用、过敏或其他不良反应的分子实体和组合物。含有至少一种包含寡核苷酸或另外的活性成分的不带电的脂质组分的药物组合物的制备鉴于本公开将是本领域技术人员已知的，如Remington:The Science and Practice of Pharmacy,第21版,2005所例示，其以引用的方式并入本文。此外，对于动物(例如人)施用，应该理解，制剂应满足FDA生物标准局要求的无菌性、致热原性、一般安全性和纯度标准。

[0111] 如本文所用，“药学上可接受的载体”包括任何和所有的溶剂、分散介质、包衣剂、表面活性剂、抗氧化剂、防腐剂(例如抗菌剂、抗真菌剂)、等渗剂、吸收延迟剂、盐、防腐剂、药物、药物稳定剂、凝胶、粘合剂、赋形剂、崩解剂、润滑剂、甜味剂、调味剂、染料等、类似物质以及其组合，如本领域的普通技术人员所已知的。药学上可接受的载体优选被配制用于施用至人，但是在某些实施方案中，可能希望使用药学上可接受的载体，所述载体被配制用于施用至非人动物但对于施用至人将是不可接受的(例如由于政府法规)。除非任何常规载体与活性成分不相容，否则考虑其在治疗或药物组合物中的用途。

[0112] 施用至患者或受试者的本发明组合物的实际剂量可通过身体和生理因素如体重、病状的严重程度、所治疗疾病的类型、先前或同时的治疗干预、患者的特发病和施用途径来确定。无论如何，负责施用的从业者都将确定组合物中活性成分的浓度和适合于个体受试者的剂量。

[0113] 在某些实施方案中，药物组合物可包含例如至少约0.1％的活性化合物。在其他实施方案中，活性化合物可占例如单位的重量的约2％至约75％之间，或约25％至约60％之间，以及其中可导出的任何范围。在其他非限制性实例中，剂量还可包括每次施用约1微克/千克/体重、约5微克/千克/体重、约10微克/千克/体重、约50微克/千克/体重、约100微克/千克/体重、约200微克/千克/体重、约350微克/千克/体重、约500微克/千克/体重、约1毫克/千克/体重、约5毫克/千克/体重、约10毫克/千克/体重、约50毫克/千克/体重、约100毫克/千克/体重、约200毫克/千克/体重、约350毫克/千克/体重、约500毫克/千克/体重至约1000毫克/千克/体重或更多，以及其中可导出的任何范围。在来自本文所列数字的可导范

围的非限制性实例中，约5μg/kg/体重至约100mg/kg/体重、约5微克/千克/体重至约500毫克/千克/体重等可被施用。

[0114] 本发明实施方案的寡核苷酸可以每剂量1、2、3、4、5、6、7、8、9、10、15、20、25、30、40、50、60、70、80、90、100或更多μg的核酸。每个剂量可以是1、10、50、100、200、500、1000或更多μl或ml的体积。

[0115] 治疗组合物的溶液可在适合与表面活性剂如羟丙基纤维素混合的水中制备。分散体也可在甘油、液体聚乙二醇、其混合物以及油中制备。在普通的储存和使用条件下，这些制剂含有防腐剂以防止微生物的生长。

[0116] 本发明的治疗组合物有利地以可注射组合物的形式作为液体溶液或悬浮液施用；也可制备适于在注射前溶解或悬浮于液体中的固体形式。这些制剂也可被乳化。用于这种目的的典型组合物包含药学上可接受的载体。例如，所述组合物可含有每毫升磷酸盐缓冲盐水10mg、25mg、50mg或达约100mg的人血清白蛋白。其他药学上可接受的载体包括水溶液、无毒赋形剂，包括盐、防腐剂、缓冲剂等。

[0117] 非水性溶剂的实例是丙二醇、聚乙二醇、植物油以及可注射的有机酯如油酸乙酯。水性载体包括水、醇/水溶液、盐水溶液、肠胃外媒介物如氯化钠、林格氏葡萄糖等。静脉内媒介物包括流体和营养补充剂。防腐剂包括抗微生物剂、抗氧化剂、螯合剂和惰性气体。药物组合物的各种组分的pH和精确浓度根据熟知的参数进行调节。

[0118] 本发明的治疗组合物可包括经典的药物制剂。根据本发明的治疗组合物的施用将经由任何常用途径进行，只要靶组织可经由所述途径可用。这包括口服、经鼻、经颊、直肠、阴道或局部。局部施用对于皮肤癌的治疗可特别有利，以预防化疗诱导的脱发或其他皮肤过度增生性疾病。或者，可通过原位、皮内、皮下、肌内、腹膜内或静脉内注射施用。此类组合物通常将作为药学上可接受的组合物施用，所述组合物包含生理学上可接受的载体、缓冲剂或其他赋形剂。为了治疗肺部的病状，可使用气雾剂递送。气雾剂的体积是介于约0.01ml与0.5ml之间。

[0119] 基于预期的目标来确定治疗组合物的有效量。术语“单位剂量”或“剂量”是指适用于受试者的物理上分离的单位，每个单位含有经计算可产生与其施用(即适当的途径和治疗方案)相关的上述讨论的所需应答的预定量的治疗组合物。根据治疗次数和单位剂量，待施用的量取决于所需的保护或效果。

[0120] 治疗组合物的精确量还取决于医师的判断并且是每个个体特有的。影响剂量的因素包括患者的身体和临床状态、施用途径、预期治疗目标(例如缓解症状与治愈)以及特定治疗物质的效力、稳定性和毒性。

[0121] VIII.组合治疗

[0122] 在某些实施方案中，本发明的组合物和方法涉及抑制性寡核苷酸或能够表达基因表达的抑制剂的寡核苷酸与第二种或另外的疗法的组合。包括组合疗法的方法和组合物增强治疗或预防效果，和/或增加另一种抗癌或抗增生疗法的治疗效果。可以有效实现所需效果，如杀死癌细胞和/或抑制细胞过度增殖的组合量来提供治疗性和预防性方法和组合物。此过程可涉及使细胞与基因表达抑制剂和第二种疗法接触。可使组织、肿瘤或细胞与包含一种或多种药剂(即，基因表达的抑制剂或抗癌剂)的一种或多种组合物或药物制剂接触，或通过使所述组织、肿瘤和/或细胞与两种或更多种不同的组合物或制剂接触，其中一种组

合物提供1)抑制性寡核苷酸;2)抗癌剂,或3)抑制性寡核苷酸和抗癌剂两者。此外,预期这种组合疗法可与化学疗法、放射疗法、手术疗法或免疫疗法结合使用。

[0123] 抑制性寡核苷酸可相对于抗癌治疗在之前、期间或之后或以各种组合施用。施用可以在从同时到数分钟至数天至数周范围内的时间间隔内。在与抗癌剂分开向患者提供抑制性寡核苷酸的实施方案中,通常确保显著时间段未在每次递送时间之间到期,以使得所述两种化合物仍能够对患者发挥有利的组合效果。在此类情况下,预期可在彼此的约12至24或72小时内且更优选在彼此的约6至12小时内向患者提供抑制性寡核苷酸疗法和抗癌疗法。在一些情形下,可能希望显著延长治疗的时间段,其中在各次施用之间经过数天(2、3、4、5、6或7天)至数周(1、2、3、4、5、6、7或8周)。

[0124] 在某些实施方案中,疗程将持续1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、50、51、52、53、54、55、56、57、58、59、60、61、62、63、64、65、66、67、68、69、70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、86、87、88、89、90天或更多天。考虑一种药剂可在第1天、第2天、第3天、第4天、第5天、第6天、第7天、第8天、第9天、第10天、第11天、第12天、第13天、第14天、第15天、第16天、第17天、第18天、第19天、第20天、第21天、第22天、第23天、第24天、第25天、第26天、第27天、第28天、第29天、第30天、第31天、第32天、第33天、第34天、第35天、第36天、第37天、第38天、第39天、第40天、第41天、第42天、第43天、第44天、第45天、第46天、第47天、第48天、第49天、第50天、第51天、第52天、第53天、第54天、第55天、第56天、第57天、第58天、第59天、第60天、第61天、第62天、第63天、第64天、第65天、第66天、第67天、第68天、第69天、第70天、第71天、第72天、第73天、第74天、第75天、第76天、第77天、第78天、第79天、第80天、第81天、第82天、第83天、第84天、第85天、第86天、第87天、第88天、第89天和/或第90天、其任何组合给予,并且另一种药剂在第1天、第2天、第3天、第4天、第5天、第6天、第7天、第8天、第9天、第10天、第11天、第12天、第13天、第14天、第15天、第16天、第17天、第18天、第19天、第20天、第21天、第22天、第23天、第24天、第25天、第26天、第27天、第28天、第29天、第30天、第31天、第32天、第33天、第34天、第35天、第36天、第37天、第38天、第39天、第40天、第41天、第42天、第43天、第44天、第45天、第46天、第47天、第48天、第49天、第50天、第51天、第52天、第53天、第54天、第55天、第56天、第57天、第58天、第59天、第60天、第61天、第62天、第63天、第64天、第65天、第66天、第67天、第68天、第69天、第70天、第71天、第72天、第73天、第74天、第75天、第76天、第77天、第78天、第79天、第80天、第81天、第82天、第83天、第84天、第85天、第86天、第87天、第88天、第89天和/或第90天或其任何组合给予。在一天内(24小时时间段),可向患者给予一次或多次药剂施用。此外,在一个疗程后,预期存在不施用抗癌治疗的时间段。此时间段可持续1、2、3、4、5、6、7天和/或1、2、3、4、5周和/或1、2、3、4、5、6、7、8、9、10、11、12个月或更长,这取决于患者的状况,如他们的预后、力量、健康等。

[0125] 可采用各种组合。对于以下实例,抑制性寡核苷酸疗法是“A”,并且抗癌疗法是“B”。

[0126] A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B

[0127] B/A/B/B B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A

[0128] B/B/A/A B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A

[0129] A/A/B/A

[0130] 考虑到药剂的毒性(如果有的话),向患者施用任何化合物或疗法将遵循用于施用此类化合物的通用方案。因此,在一些实施方案中,存在归因于组合疗法的监测毒性的步骤。预期治疗周期将根据需要重复。还考虑各种标准疗法以及手术干预可与所描述的疗法组合应用。

[0131] 在具体方面,预期标准疗法将包括化学疗法、放射疗法、免疫疗法、手术疗法或基因疗法,并且可与基因表达的抑制剂疗法、抗癌疗法或基因表达的抑制剂疗法和抗癌疗法两者组合使用,如本文所描述。

[0132] A.化学疗法

[0133] 可根据本发明的实施方案使用各种各样的化学治疗剂。术语“化学疗法”是指使用药物来治疗癌症。“化学治疗剂”用于暗示在癌症治疗中施用的化合物或组合物。这些药剂或药物按其在细胞内的活动模式(例如,它们是否影响细胞周期且在哪一阶段影响细胞周期)分类。或者,药剂可基于其直接交联DNA、嵌入DNA中或通过影响核酸合成诱导染色体和有丝分裂畸变的能力来进行表征。

[0134] 化学治疗剂的实例包括烷化剂,如噻替派和环磷酰胺;烷基磺酸酯,如白消安、英丙舒凡和哌泊舒凡;氮杂环丙烷,如苯佐多巴(benzodopa)、卡波醌、米特多巴(meturedopa)和尤利多巴(uredopa);乙烯亚胺和甲基蜜胺,包括六甲蜜胺、三亚乙基蜜胺、三亚乙基磷酰胺、三亚乙基硫代磷酰胺和三羟甲基蜜胺;乙酰精宁(特别是布拉他辛和布拉他辛酮);喜树碱(包括合成类似物拓扑替康);苔藓抑素;海绵他汀;CC-1065(包括其阿多来新、卡折来新和比折来新合成类似物);念珠藻素(cryptophycin)(特别是念珠藻素1和念珠藻素8);多拉司他汀;倍癌霉素(duocarmycin)(包括合成类似物、KW-2189和CB1-TM1);艾榴塞洛素(eleutherobin);水鬼蕉碱;匍枝珊瑚醇;海绵素(spongistatin);氮芥,诸如苯丁酸氮芥、萘氮芥、氯磷酰胺、雌莫司汀、异环磷酰胺、氮芥、氮芥氧化物盐酸盐、美法仑、新恩比兴、苯芥胆甾醇、泼尼莫司汀、曲磷胺以及尿嘧啶氮芥;亚硝基脲,诸如卡莫司汀、氯脲菌素、福莫司汀、洛莫司汀、尼莫司汀和雷莫司汀;抗生素,诸如烯二炔抗生素(例如卡奇霉素,尤其卡奇霉素γ1I和卡奇霉素ωI1;达内霉素,包括达内霉素A;二膦酸盐,如氯膦酸盐;埃斯培拉霉素;以及新制癌菌素生色团和相关色蛋白烯二炔抗生素生色团)、阿克拉霉素、放线菌素、安曲霉素、重氮丝氨酸、博莱霉素、放线菌素C、卡柔比星、洋红霉素、嗜癌菌素、色霉素、更生霉素、柔红霉素、地托比星、6-重氮基-5-氧代-L-正亮氨酸、阿霉素(包括吗啉基-阿霉素、氰基吗啉基-阿霉素、2-吡咯啉并-阿霉素和去氧阿霉素)、表柔比星、依索比星、伊达比星、麻西罗霉素、丝裂霉素(诸如丝裂霉素C)、霉酚酸、诺拉霉素、橄榄霉素、培洛霉素、泊非霉素、嘌呤霉素、三铁阿霉素、罗多比星、链黑菌素、链佐星、杀结核菌素、乌苯美司、净司他丁以及佐柔比星;抗代谢药,如甲氨蝶呤和5-氟尿嘧啶(5-FU);叶酸类似物,如二甲叶酸、蝶罗呤、三甲曲沙;嘌呤类似物,如氟达拉滨、6-巯基嘌呤、硫咪嘌呤和硫鸟嘌呤;嘧啶类似物,如安西他滨、阿扎胞苷、6-氮尿苷、卡莫氟、阿糖胞苷、双去氧尿苷、去氧氟尿苷、依诺他滨以及氟尿苷;雄激素,如卡普睾酮、屈他雄酮丙酸酯、环硫雄醇、美雄烷以及睾内酯;抗肾上腺素药,如米托坦和曲洛斯坦;叶酸补充剂,如亚叶酸;醋葡醛内酯;醛磷酰胺糖苷;氨基乙酰丙酸;恩尿嘧啶;安吖啶;倍曲布西;比生群;依达曲沙;德福法明;秋水仙胺;地吖醌;依氟鸟氨酸;依利醋铵;埃坡西龙;依托格鲁;硝酸镓;羟基脲;香菇多糖;氯尼达明;美登醇,如美登素和安丝菌素;米托胍腙;米托蒽醌;莫哌达醇;二胺硝吖啶(nitraerine);喷司他丁;蛋氨氮芥;

吡柔比星;洛索蒽醌;鬼臼酸;2-乙基酰肼;丙卡巴肼;PSK多糖复合物;雷佐生;根霉素(rhizoxin);西佐喃;锗螺胺;细交链孢菌酮酸;三亚胺醌;2,2',2″-三氯三乙胺;单端孢霉烯族毒素(特别是T-2毒素、疣孢菌素A、杆孢菌素A和蛇形菌素);乌拉坦;长春地辛;达卡巴嗪;甘露莫司汀;二溴甘露醇;二溴卫矛醇;哌泊溴烷;gacytosine;阿糖胞苷("Ara-C");环磷酰胺;紫杉烷类,例如紫杉醇和多西他赛吉西他滨;6-硫鸟嘌呤;巯基嘌呤;铂配位络合物,如顺铂、奥沙利铂和卡铂;长春花碱;铂;依托泊苷(VP-16);异环磷酰胺;米托蒽醌;长春新碱;长春瑞滨;诺安托;替尼泊苷;依达曲沙;柔红霉素;氨蝶呤;希罗达;伊班膦酸盐;伊立替康(例如CPT-11);拓扑异构酶抑制剂RFS 2000;二氟甲基鸟氨酸(DMFO);类视黄醇,如视黄酸;卡培他滨;卡铂;丙卡巴肼;普卡霉素;吉西他滨;诺维本;法尼基蛋白转移酶抑制剂;反铂;以及上述任一种的药学上可接受的盐、酸或衍生物。

[0135] B.放射疗法

[0136] 引起DNA损伤并一直广泛使用的其他因素包括通常称为γ射线、X射线和/或将放射性同位素定向递送至肿瘤细胞的因素。也考虑了其他形式的DNA损伤因素,如微波、质子束照射(美国专利号5,760,395和4,870,287)和UV照射。很可能所有这些因素都实现对DNA、DNA的前体、DNA的复制和修复以及染色体的组装和维持广泛范围的损伤。对于X射线,剂量范围在50至200伦琴的每日剂量持续延长的时间段(3至4周)至2000至6000伦琴的单次剂量的范围内。放射性同位素的剂量范围广泛变化,并且取决于同位素的半衰期、发射的辐射的强度和类型以及肿瘤细胞的摄取。

[0137] 当应用于细胞时,术语"接触"和"暴露"用于描述治疗性构建体和化学治疗剂和/或放射性治疗剂递送至靶细胞或直接与靶细胞并置的过程。为了实现细胞杀死,例如,两种药剂以有效杀死细胞或防止其分裂的组合量递送至细胞。

[0138] C.免疫疗法

[0139] 在癌症治疗的背景下,免疫疗法通常依赖于使用免疫效应细胞和分子来靶向并破坏癌细胞。曲妥珠单抗(Herceptin™)是这样一个实例。免疫效应物可以是例如,对肿瘤细胞表面上的一些标志物具有特异性的抗体。单独的抗体可充当治疗的效应物,或者它可募集其他细胞以实际地影响细胞杀伤。所述抗体也可缀合至药物或毒素(化学治疗剂、放射性核素、蓖麻毒蛋白A链、霍乱毒素、百日咳毒素等)并且仅充当靶向剂。或者,效应物可以是携带与肿瘤细胞靶标直接或间接相互作用的表面分子的淋巴细胞。各种效应细胞包括细胞毒性T细胞和NK细胞。治疗方式,即直接细胞毒性活性和抑制或降低ErbB2的组合将在治疗过量表达ErbB2的癌症中提供治疗益处。

[0140] 另一种免疫疗法也可用作上文论述的与基因沉默疗法的组合治疗的一部分。在免疫疗法的一个方面,肿瘤细胞必须携带一些适合靶向的标志物,即不存在于大部分其他细胞上。存在许多肿瘤标志物,并且这些标志物中的任一种都可能适合于在本发明的背景下靶向。常见肿瘤标志物包括癌胚抗原、前列腺特异性抗原、泌尿系统肿瘤相关抗原、胚胎抗原、酪氨酸酶(p97)、gp68、TAG-72、HMFG、唾液酸化的路易斯抗原、MucA、MucB、PLAP、雌激素受体、层粘连蛋白受体、erb B以及p155。免疫疗法的一个替代方面是将抗癌作用与免疫刺激作用组合。还存在免疫刺激分子,包括:细胞因子如IL-2、IL-4、IL-12、GM-CSF、γ-IFN,趋化因子如MIP-1、MCP-1、IL-8,以及生长因子如FLT3配体。已显示将免疫刺激分子(作为蛋白质或使用基因递送)与肿瘤抑制基因组合增强抗肿瘤作用。此外,针对任何这些化合物的抗

体可用于靶向本文论述的抗癌剂。

[0141] 目前正在研究或使用的免疫疗法的实例是免疫佐剂,例如牛型分枝杆菌、镰状疟原虫、二硝基氯苯和芳族化合物(美国专利号5,801,005和5,739,169;Hui和Hashimoto,1998;Christodoulides等人,1998)、细胞因子疗法(例如干扰素α、β和γ;IL-1、GM-CSF和TNF)(Bukowski等人,1998;Davidson等人,1998;Hellstrand等人,1998)、基因疗法(例如,TNF、IL-1、IL-2、p53)(Qin等人,1998;Austin-Ward和Villaseca,1998;美国专利号5,830,880和5,846,945)以及单克隆抗体(例如,抗神经节苷脂GM2、抗HER-2、抗p185)(Pietras等人,1998;Hanibuchi等人,1998;美国专利号5,824,311)。预期一种或多种抗癌疗法可与本文所述的基因沉默疗法一起使用。

[0142] 在主动免疫治疗中,抗原肽、多肽或蛋白质或自体或同种异体肿瘤细胞组合物或“疫苗”通常与不同的细菌佐剂一起施用(Ravindranath和Morton,1991;Morton等人,1992;Mitchell等人,1990;Mitchell等人,1993)。

[0143] 在过继免疫治疗中,在体外分离患者的循环淋巴细胞或肿瘤浸润淋巴细胞,通过诸如IL-2的淋巴因子活化或用基因转导用于肿瘤坏死,并且再次施用(Rosenberg等人,1988;1989)。

[0144] D.外科手术

[0145] 大约60%的患有癌症的人将经历一些类型的手术,所述手术包括预防性、诊断性或疾病分期、治愈性和姑息性手术。治愈性手术是可与其他疗法,如本发明的治疗、化学疗法、放射疗法、激素疗法、基因疗法、免疫疗法和/或替代疗法结合使用的癌症治疗。

[0146] 治愈性手术包括切除,其中全部或部分癌组织被物理地移除、切除和/或破坏。肿瘤切除是指物理除去至少一部分肿瘤。除肿瘤切除外,手术治疗包括激光手术、冷冻手术、电外科手术和显微镜控制的手术(莫氏手术)。进一步考虑本发明可与除去浅表癌症、初癌或偶发量的正常组织结合使用。

[0147] 在切除部分或全部癌细胞、组织或肿瘤时,可在体内形成空腔。治疗可通过灌注、直接注射或局部涂敷具有另外的抗癌疗法的区域来完成。这种治疗可例如每1、2、3、4、5、6或7天,或者每1、2、3、4或5周或每1、2、3、4、5、6、7、8、9、10、11或12个月重复。这些治疗也可具有不同的剂量。

[0148] E.其他药剂

[0149] 预期其他药剂可与本发明实施方案的某些方面组合使用以提高治疗的治疗功效。这些另外的药剂包括影响细胞表面受体的上调和GAP连接的那些药剂、细胞抑制剂和分化剂、细胞粘附的抑制剂、增加过度增殖细胞对凋亡诱导剂的敏感性的药剂或其他生物剂。通过提高GAP连接的数量增加细胞间信号传导将增加对邻近过度增殖细胞群体的抗过度增殖作用。在其他实施方案中,细胞抑制剂或分化剂可与本发明实施方案的某些方面组合使用以提高治疗的抗过度增殖功效。预期细胞粘附的抑制剂可提高本发明实施方案的功效。细胞粘附抑制剂的实例是粘着斑激酶(FAK)抑制剂和洛伐他汀。进一步考虑增加过度增殖细胞对凋亡的敏感性的其他药剂(如抗体c225)可与本发明实施方案的某些方面组合使用以提高治疗功效。

[0150] IX.试剂盒和诊断学

[0151] 在本发明的各个方面中,设想一种含有治疗剂和/或其他治疗剂和递送剂的试剂

盒。在一些实施方案中，本发明考虑一种用于制备和/或施用本发明的疗法的试剂盒。所述试剂盒可包括能够用于施用本发明的活性剂或有效剂的试剂。所述试剂盒的试剂可包括基因表达的至少一种抑制剂、一种或多种脂质组分、组合疗法的一种或多种抗癌组分，以及用于制备、配制和/或施用本发明的组分或执行本发明方法的一个或多个步骤的试剂。

[0152] 在一些实施方案中，所述试剂盒还可包括合适的容器装置，所述容器装置是不会与试剂盒的组分反应的容器，如埃彭道夫管、测定板、注射器、瓶或管。所述容器可由诸如塑料或玻璃的可灭菌材料制成。

[0153] 所述试剂盒还可包括概述所述方法的程序步骤的说明书，并且将遵循与本文所描述基本相同的程序或是本领域普通技术人员已知的。

[0154] X.实施例

[0155] 包括以下实施例以展示本发明的优选实施方案。本领域的普通技术人员应理解的是，在以下实施例中公开的技术代表由本发明人发现的在本发明的实践中起良好作用的技术，并且因此可被认为构成本发明实践的优选模式。然而，根据本公开，本领域的技术人员应理解，在不脱离本发明的精神和范围的情况下可在已公开并仍获得类似或相似结果的特定实施方案中做出许多改变。

[0156] 实施例1-制造脂质体对乙氧基反义药物产品的方法

[0157] 脂质体对乙氧基反义药物产品由两种cGMP产品组成，两种产品均具有FDA批准的分析证书和FDA批准的发布标准。本文描述了原材料、溶剂和最终药物产品。当制造时，所述药物产品是包含以下材料的琥珀色或白色冻干晶体或粉末：寡核苷酸(例如对乙氧基反义药品)、中性脂质(例如，DOPC)和表面活性剂(例如，聚山梨醇酯20)。在准备向患者施用时，将生理盐水加入小瓶中，此时形成脂质体，其中对乙氧基反义并入内部中。

[0158] 对乙氧基反义药品可在生产对乙氧基反义药品期间使用预先确定的对乙氧基和磷酸二酯亚酰胺原材料混合物来限定最终产品的特定物理性质(例如，溶解度和疏水性，其然后影响盐水中的药物产品溶解度，寡核苷酸并入脂质体中以及脂质体粒度)。虽然在寡核苷酸制造期间发生对乙氧基主链基团的损失，从而在那些键联处产生磷酸二酯键，但所述损失可能不在寡核苷酸内产生优选比例的对乙氧基:磷酸二酯主链键联。在这种情况下，对乙氧基和磷酸二酯亚酰胺原材料的混合物补充了对乙氧基主链缺失的期望值，从而产生具有所需比例的寡核苷酸。增加寡核苷酸主链中对乙氧基分子的数量导致分子疏水性更高(其产生更大的脂质体颗粒；表1)、极性更低以及溶解性更低(表2)。测试电荷中性疏水性对乙氧基药品的方法包括用于确定寡核苷酸长度的分布的质谱法和用于确定药品的溶解度的测定，出于实际考虑关于溶解度的所述测定是在盐水中重构的药物产品的目视检查。当寡核苷酸由于更大量的对乙氧基主链键联而变得较不可溶时，重构溶液变得更白，直到微粒形成，因为疏水性变得太高。

[0159] 表1.随反义主链组成的脂质体粒度可变性

[0160]

| | | 制造后主链乙基缺失 | | 粒度特征：累积分布函数 | | |
|---|---|---|---|---|---|---|
| 实验 | 工程化的反义主链 | 主峰 | 复合缺失 | 90%值 (nm) ** | 50%值 (nm) | 300 nm 值(%) |
| 1 | 3 亚酰胺取代 | -6 | -5.67 | 2130 | 911 | 15.30 |
| 2 | 3 亚酰胺取代 | -6 | -5.67 | 2420 | 1004 | 15.50 |
| 3 | 3 亚酰胺取代 | -6 | -6.12 | 3682 | 943 | 15.50 |
| 4 | 3 亚酰胺取代 | -7 | -6.66 | 3805 | 978 | 14.60 |
| 5 | 100%对乙氧基 | -5 | -5.66 | 3924 | 976 | 16.00 |
| 6 | 2 亚酰胺取代 | -5 | -5.32 | 4387 | 1888 | 11.60 |
| 7[a] | 100%对乙氧基 | -4 | -4.22 | 5057 | 1131 | 17.70 |
| 8 | 100%对乙氧基 | -4 | -4.52 | 5659 | 1359 | 10.00 |
| 9[b] | 100%对乙氧基 | -4 | -4.38 | 7571 | 1909 | 2.60 |
| 10[c] | 100%对乙氧基 | -4 | -4.38 | 7994 | 1653 | 14.40 |

[0161] **药物发布标准是90％的脂质体颗粒为小于或等于5000nm。

[0162] a.由于较差溶解性,此批次被丢弃;具体地说,重构溶液中的反义颗粒。

[0163] b.此批次在20mL小瓶中在2mg反义情况下具有较低DMSO和tBA体积,其向脂质体扩大添加了另外的组分。

[0164] c.此批次没有发布,因为它未通过粒度发布规范。

[0165] 表2.反义主链组成的脂质体颗粒溶解度

[0166]

| | | 制造后主链乙基缺失 | | 药物溶解度 | |
|---|---|---|---|---|---|
| 实验 | 工程化的反义主链 | 主峰 | 复合缺失 | 目视观察** | 溶解度评估 |
| 1 | 3 亚酰胺取代 | -6 | -5.67 | 脱脂乳溶液 | 良好 |
| 2 | 3 亚酰胺取代 | -6 | -5.67 | 脱脂乳溶液 | 良好 |
| 3 | 3 亚酰胺取代 | -6 | -6.12 | 脱脂乳溶液 | 良好 |
| 4 | 3 亚酰胺取代 | -7 | -6.66 | 脱脂乳溶液 | 良好 |
| 5 | 100%对乙氧基 | -5 | -5.66 | 脱脂乳溶液 | 良好 |
| 6 | 2 亚酰胺取代 | -5 | -5.32 | 脱脂乳溶液 | 良好 |
| 7 | 100%对乙氧基 | -4 | -4.52 | 白色溶液 | 通过 |
| 8[b] | 100%对乙氧基 | -4 | -4.38 | 白色溶液 | 通过 |
| 9[c] | 100%对乙氧基 | -4 | -4.38 | 白色溶液 | 通过 |
| 10[a] | 100%对乙氧基 | -4 | -4.22 | 白色溶液颗粒 | 未通过 |

[0167] **如果药品样品中具有颗粒，则所述批次被拒绝。

[0168] a.由于较差溶解性，此批次被丢弃；具体地说，重构溶液中的反义颗粒。

[0169] b.此批次在20mL小瓶中在2mg反义情况下具有较低DMSO和tBA体积，其向脂质体扩大添加了另外的组分。

[0170] c.此批次没有发布，因为它未通过粒度发布规范。

[0171] 脂质体对乙氧基反义药物产品的配制、过滤和冻干。将1克(1g)的pE寡核苷酸以10mg寡核苷酸/1mL DMSO的比例溶解于DMSO中。接着，将DOPC以1g DOPC/1719mL叔丁醇的比例加入到叔丁醇中。将寡核苷酸和DOPC以1g寡核苷酸/2.67g DOPC的比例组合并混合。然后，将20mL的0.835％(v/v)聚山梨醇酯20溶液加入混合物中，从而得到0.039mg/mL的最终浓度。在将溶液分配到玻璃瓶中进行冻干之前，使溶液通过无菌过滤器。

[0172] 表面活性剂对脂质体粒度的影响通过滴定表面活性剂的量来确定(表3)。在不存在聚山梨醇酯20的情况下，仅2.8％的颗粒具有300nm或更小的直径。在1x聚山梨醇酯20(总脂质体对乙氧基反义药物产品的约5％)存在下，12.5％的颗粒具有300nm或更小的直径。通过加入3x-10x聚山梨醇酯20，约20％的颗粒具有300nm或更小的直径。因此表面活性剂从1x增加至3x导致粒度减小。

[0173] 表3.随表面活性剂的脂质体粒度可变性

[0174]

| | | 粒度特征：累积分布函数 | | |
|---|---|---|---|---|
| 实验 | 表面活性剂的量 | 50%值 | 90%值** | 300 nm 值 |
| 1 | 0x | 5301 nm | 10719 nm | 2.8% |
| 2 | 1x | 1053 nm | 4054 nm | 12.5% |
| 3 | 3x | 785 nm | 2926 nm | 19.1% |
| 4 | 5x | 721 nm | 2691 nm | 21.9% |
| 5 | 10x | 734 nm | 2937 nm | 21.4% |

[0175] **药物发布标准是90％的脂质体颗粒为小于或等于5000nm。

[0176] 用于施用的脂质体对乙氧基反义药物产品的制备。将冻干制剂以10-5000μM的最终寡核苷酸浓度用生理盐水(0.9％/10mM NaCl)水合。通过手动振荡混合脂质体-对乙氧基寡核苷酸。

[0177] 实施例2-测试脂质体对乙氧基反义药物产品的方法

[0178] 制造的药物产品的目视检查：在制造后，选择含有药物产品的样品小瓶并且目视检查。液体不存在是强制性的，且然后小瓶底部的琥珀晶体是可接受的，并且接受性增加至白色絮凝状粉末或外观，这是最好的结果。白色外观表明更好的干燥过程，具有高表面积与质量比，这非常有利于重构使用。

[0179] 准备好用于患者IV的重构药物的视觉检查：将生理盐水加入到含有制造的脂质体对乙氧基反义药物产品的小瓶中，并且振荡以重新构成药物晶体或粉末完全溶解的溶液。得出三个主要观察结果：1)晶体或粉末完全溶解，2)不存在不溶性材料的白色团块，和3)外观是乳白色或脱脂乳外观。重构液体的外观越蓝越好，因为这表示反映蓝色光谱中的光的更小脂质体粒度。

[0180] 质谱法：质谱法(质谱)用于展示样品中各种质量的分布型。当产生对乙氧基反义物质时，在样品上运行质谱。结果显示栅格上存在的材料的峰在右侧的“x”轴上具有递增质量，并且“y”轴上的相对质量丰度向上递增。对来自样品的分布型进行分析以确定对乙氧基样品中对乙氧基主链的相对数量，从而认识到峰的分布型代表(从最右开始)所有主链均包含对乙氧基键联的全长材料，向左移动的下一个峰代表一个主链具有对乙氧基缺失(且因此，乙基被敲除，并且结果是正常磷酸二酯主链键联)的全长，并且继续。向右偏移的质谱图表示具有更多对乙氧基主链的对乙氧基样品，且因此具有更高疏水性和更低溶解性的性质；并且同样，向左偏移具有相反的效应。样品的质谱图表的检查也可用于确定制造期间的过滤是否对存在于过滤的药物产品中的寡核苷酸组合物产生任何不利影响。

[0181] UV测试：使用紫外光测试来确定样品中存在的寡核苷酸的质量。寡核苷酸吸收260纳米范围内的光。因此，最终重构的药物产品的UV测试已被用作确定一瓶药物产品中寡核苷酸药品的量的方法。就制造开发和创新而言，UV测试用于确定是否存在在制造中的过滤期间经历的问题或对乙氧基反义药品的较差溶解性，从而导致溶液中寡核苷酸较少且因此UV读数较低。所述方法将被验证并可能成为最终产品发布测试的一部分。

[0182] 脂质体粒度：将一瓶成品药物产品重构并测试其脂质体粒度。结果通常是大致正态分布，具有中心点、尾部和平均值，或大部分颗粒的大致正态分布，以及由二阶颗粒形成效应产生的较小脂质体颗粒的次级峰。重要的是脂质体颗粒不要太大，因为它们可能会在

患者中引起不良作用(例如,在肺部的较小血管中产生血流问题)。结果,药物产品发布标准包括粒度测试显示90%的脂质体的大小为约5微米或更小。此外,更小的脂质体是优选的,因为它们将具有更好的细胞摄取,并且其次,更小的脂质体可穿透血管孔隙,从而允许脂质体穿透内部肿瘤,从而增加脂质体对乙氧基反义药物产品的治疗有效性。

[0183] * * *

[0184] 根据本公开,本文公开的并且要求保护的所有方法可在无需过度实验的情况下进行和实施。尽管本发明的组合物和方法已经根据优选实施方案进行了描述,但对本领域技术人员显而易见的是可使本文所述的方法和本文所述方法的步骤或步骤的顺序发生变化,而不偏离本发明的概念、精神和范围。更具体地说,显而易见的是在化学上和生理学上相关的某些试剂可取代本文所述的试剂,同时达到相同或相似结果。对本领域技术人员来说显而易见的是所有此类相似的替代和修改被认为在如由随附权利要求限定的本发明的精神、范围和概念内。

[0185] 参考文献

[0186] 以下参考文献以引用方式特别并入本文,在某种程度上,它们提供示例性程序或对本文所阐述的那些进行补充的其他细节。

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