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(71) Applicant *(for all designated States except US)*: **PROTIVA BIOTHERAPEUTICS, INC.** [CA/CA]; 100 - 3480 Gilmore Way, Burnaby, British Columbia V5G 4Y1 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **MACLACHLAN, Ian** [CA/CA]; 1812 W. 7th Avenue, Suite 302, Vancouver, British Columbia V6J 1S8 (CA). **AMBEGIA, Ellen, Grace** [CA/CA]; 3150 W. 4th Avenue, #302, Vancouver, British Columbia V6K 1R7 (CA). **HEYES, James** [GB/CA]; 315 - 651 Moberly Road, Vancouver, British Columbia V5Z 4B2 (CA).

(74) Agents: **FETHERSTONHAUGH & CO.** et al.; Suite 2200, 650 West Georgia Street, Box 11560, Vancouver Centre, Vancouver, British Columbia V6B 4N8 (CA).

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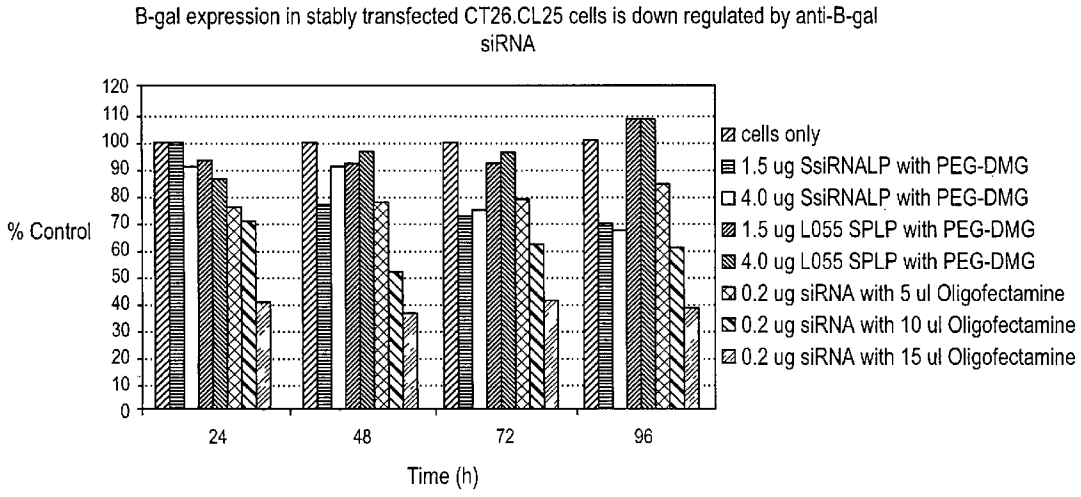
(54) Title: LIPID ENCAPSULATED INTERFERING RNA




(57) Abstract: The present invention provides compositions and methods for silencing gene expression by delivering nucleic acid-lipid particles comprising a siRNA molecule to a cell.

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# LIPID ENCAPSULATED INTERFERING RNA

## CROSS-REFERENCES TO RELATED APPLICATIONS

**[0001]** This application claims the benefit of U.S. Provisional Patent Application Nos. 60/529,406, filed December 11, 2003; 60/503,279, filed September 15, 2003, and 60/488,144, filed July 16, 2003, the disclosures of each of which are hereby incorporated by reference in their entirety for all purposes.

## FIELD OF THE INVENTION

**[0002]** The present invention relates to compositions and methods for the therapeutic delivery of a nucleic acid by delivering a serum-stable lipid delivery vehicle encapsulating the nucleic acid to provide efficient RNA interference (RNAi) in a cell or mammal. More particularly, the present invention is directed to using a small interfering RNA (siRNA) encapsulated in a serum-stable lipid particle having a small diameter suitable for systemic delivery.

## BACKGROUND OF THE INVENTION

**[0003]** RNA interference (RNAi) is an evolutionarily conserved, sequence specific mechanism triggered by double stranded RNA (dsRNA) that induces degradation of complementary target single stranded mRNA and "silencing" of the corresponding translated sequences (McManus and Sharp, *Nature Rev. Genet.* 3:737 (2002)). RNAi functions by enzymatic cleavage of longer dsRNA strands into biologically active "short-interfering RNA" (siRNA) sequences of about 21-23 nucleotides in length (Elbashir, *et al.*, Genes Dev. 15:188 (2001)). siRNA can be used downregulate or silence the translation of a gene product of interest. For example, it is desirable to downregulate genes associated with various diseases and disorders.

**[0004]** Delivery of siRNA remains problematic (*see*, *e.g.*, Novina and Sharp, *Nature* 430::161-163 (2004); and Garber, *J. Natl. Cancer Inst.* 95(7):500-2 (2003)). An effective and safe nucleic acid delivery system is required for siRNA to be therapeutically useful. Naked dsRNA administered to most subjects will: (1) be degraded by endogenous nucleases; and (2) will not be able to cross cell membranes to contact and silence their target gene sequences.

**[0005]** Viral vectors are relatively efficient gene delivery systems, but suffer from a variety of safety concerns, such as potential for undesired immune responses. Furthermore, viral systems are rapidly cleared from the circulation, limiting transfection to "first-pass" organs such as the lungs, liver, and spleen. In addition, these systems induce immune responses that compromise delivery with subsequent injections. As a result, nonviral gene delivery systems are receiving increasing attention (Worgall, *et al.*, *Human Gene Therapy* 8:37 (1997); Peeters, *et al.*, Human Gene Therapy 7:1693 (1996); Yei, *et al.*, Gene *Therapy* 1: 192 (1994); Hope, *et al.*, *Molecular Membrane Biology* 15:1 (1998)).

**[0006]** Plasmid DNA-cationic liposome complexes are currently the most commonly employed nonviral gene delivery vehicles (Felgner, Scientific American 276:102 (1997); Chonn, *et al.*, *Current Opinion in Biotechnology* 6:698 (1995)). For instance, cationic liposome complexes made of an amphipathic compound, a neutral lipid, and a detergent for transfecting insect cells are disclosed in U.S. Patent No. 6,458,382. Cationic liposome complexes are also disclosed in U.S. Patent Application Publication No. 2003/0073640. Cationic liposome complexes, however, are large, poorly defined systems that are not suited for systemic applications and can elicit considerable toxic side effects (Harrison, *et al.*, *Biotechniques* 19:816 (1995); Li, *et al.*, *The Gene* 4:891 (1997); Tam, *et al*, *Gene Ther.* 7:1867 (2000)). As large, positively charged aggregates, lipoplexes are rapidly cleared when administered *in vivo*, with highest expression levels observed in first-pass organs, particularly the lungs (Huang, *et al.*, *Nature Biotechnology* 15:620 (1997); Templeton, *et al.*, *Nature Biotechnology* 15:647 (1997); Hofland, *et al.*, *Pharmaceutical Research* 14:742 (1997)).

**[0007]** Other liposomal delivery systems include, for example, the use of reverse micelles, anionic and polymer liposomes as disclosed in, *e.g.*, U.S. Patent No. 6,429,200; U.S. Patent Application No. 2003/0026831; and U.S. Patent Application Nos. 2002/0081736 and 2003/0082103, respectively.

**[0008]** Recent work has shown that nucleic acids can be encapsulated in small (about 70 nm diameter) "stabilized plasmid-lipid particles" (SPLP) that consist of a single plasmid encapsulated within a bilayer lipid vesicle (Wheeler, *et al.*, *Gene Therapy* 6:271 (1999)). These SPLPs typically contain the "fusogenic" lipid dioleoylphosphatidylethanolamine (DOPE), low levels of cationic lipid (*i.e.*, 10% or less), and are stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. SPLP have systemic application as they exhibit extended circulation lifetimes following intravenous (i.v.) injection, accumulate preferentially at distal tumor sites due to the enhanced vascular permeability in such regions,

and can mediate transgene expression at these tumor sites. The levels of transgene expression observed at the tumor site following i.v. injection of SPLP containing the luciferase marker gene are superior to the levels that can be achieved employing plasmid DNA-cationic liposome complexes (lipoplexes) or naked DNA.

**[0009]** However, there remains a strong need in the art for novel and more efficient methods and compositions for introducing nucleic acids, such as siRNA, into cells. The present invention addresses this and other needs.

## BRIEF SUMMARY OF THE INVENTION

**[0010]** The present invention provides stable nucleic acid-lipid particles (SNALP) useful for encapsulating one or more siRNA molecules, methods of making SNALPs comprising siRNA, SNALPs comprising siRNA and methods of delivering and/or administering the SNALPs to a subject to silence expression of a target gene sequence.

**[0011]** In one embodiment, the invention provide nucleic acid-lipid particles comprising: a cationic lipid, a non-cationic lipid, a conjugated lipid that inhibits aggregation of particles and a siRNA. In some embodiments, the siRNA molecule is fully encapsulated within the lipid bilayer of the nucleic acid-lipid particle such that the nucleic acid in the nucleic acid-lipid particle is resistant in aqueous solution to degradation by a nuclease. The nucleic acid particle are substantially non-toxic to mammals. The siRNA molecule may comprise about 15 to about 60 nucleotides. The siRNA molecule may be derived from a double-stranded RNA greater than about 25 nucleotides in length. In some embodiments the siRNA is transcribed from a plasmid, in particular a plasmid comprising a DNA template of a target sequence.

**[0012]** The cationic lipid may be one or more of N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), and N,N-dimethyl-2,3-dioleyloxy)propylamine (DODMA), and a mixture thereof. The non-cationic lipid may be one or more of dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), egg phosphatidylcholine (EPC), distearoylphosphatidylcholine (DSPC), cholesterol, and combinations thereof.

**[0013]** The conjugated lipid that inhibits aggregation of particles may be one or more of a polyethyleneglycol (PEG)-lipid conjugate, a polyamide (ATTA)-lipid conjugate, and combinations thereof. The PEG-lipid conjugate may be one or more of a PEG-dialkyloxypropyl (DAA), a PEG-diacylglycerol (DAG), a PEG-phospholipid, a PEG-ceramide, and combinations thereof. The PEG-DAG conjugate may be one or more of a PEG-dilauroylglycerol ($C_{12}$), a PEG-dimyristoylglycerol ($C_{14}$), a PEG-dipalmitoylglycerol ($C_{16}$), and a PEG-distearoylglycerol ($C_{18}$), and combinations thereof. The PEG-DAA conjugate may be one or more of a PEG-dilauryloxypropyl ($C_{12}$), a PEG-dimyristyloxypropyl ($C_{14}$), a PEG-dipalmityloxypropyl ($C_{16}$), and a PEG-distearyloxypropyl ($C_{18}$), and combinations thereof. The nucleic acid-lipid particle may further comprise a cationic polymer lipid.

**[0014]** In some embodiments, the particles are made by providing an aqueous solution in a first reservoir and an organic lipid solution in a second reservoir and mixing the aqueous solution with the organic lipid solution so as to substantially instantaneously produce a liposome encapsulating an interfering RNA. In some embodiments, the particles are made by formation of hydrophobic intermediate complexes in either detergent-based or organic solvent-based systems, followed by removal of the detergent or organic solvent. Preferred embodiments are charge-neutralized.

**[0015]** In one embodiment, the interfering RNA is transcribed from a plasmid and the plasmid is combined with cationic lipids in a detergent solution to provide a coated nucleic acid-lipid complex. The complex is then contacted with non-cationic lipids to provide a solution of detergent, a nucleic acid-lipid complex and non-cationic lipids, and the detergent is then removed to provide a solution of serum-stable nucleic acid-lipid particles, in which the plasmid comprising an interfering RNA template is encapsulated in a lipid bilayer. The particles thus formed have a size of about 50-150 nm.

**[0016]** In another embodiment, serum-stable nucleic acid-lipid particles are formed by preparing a mixture of cationic lipids and non-cationic lipids in an organic solvent; contacting an aqueous solution of nucleic acids comprising interfering RNA with the mixture of cationic and non-cationic lipids to provide a clear single phase; and removing the organic solvent to provide a suspension of nucleic acid-lipid particles, in which the nucleic acid is encapsulated in a lipid bilayer, and the particles are stable in serum and have a size of about 50-150 nm.

**[0017]** The nucleic acid-lipid particles of the present invention are useful for the therapeutic delivery of nucleic acids comprising a siRNA sequence. In particular, it is an

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