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(54) Title: TRANSFECTION REAGENTS

(57) Abstract: The present invention provides optimized transfection reagents comprising mixtures of cationiclipoids. In particular, the present invention provides DNA delivery vehicles based on identifying the optimal hydrophobicity of novel cationic phospholipid derivatives that, alone or in combination, form complexes with DNA (lipoplexes) and exhibit enhanced transfection activity.

TRANSFECTION REAGENTS

FIELD OF THE INVENTION

The present invention provides optimized transfection reagents comprising mixtures of cationic lipoids. In particular, the present invention provides DNA delivery vehicles based on identifying optimal hydrophobicity of novel cationic phospholipid derivatives that, alone or in combination, form complexes with DNA (lipoplexes) and exhibit enhanced transfection activity.

BACKGROUND

There are approximately four thousand different genetic diseases, many highly debilitating and frequently resulting in death at an early age. Because almost all of these diseases involve a defective protein, conventional treatment is difficult. A direct approach to treating such diseases involves providing a competent gene in the proper cells to compensate for the mutation. This requires some form of effective transfection process. Transfection is a process whereby a nucleic acid, primarily DNA, is transferred to a target cell and codes for an expressed protein. Transfection is implemented typically to modify the gene complement of the recipient cell for controlled expression of a particular gene. The means by which "foreign" DNA can be packaged and delivered to a host cell are many and varied. The most efficient of these make use of viruses, but viral vectors have shortcomings, not the least of which is the potential for immune response or disease transmission. It has become apparent that lipid-like compounds (e.g., lipoids) can be used to deliver DNA to cells. The lipoids that are most efficient in delivering DNA to cells are positively charged. Cationic lipoids are naturally attracted to and spontaneously form complexes with

polyanionic DNA. Such complexes, or "lipoplexes," are useful as transfection

vehicles both *in vitro* and *in vivo*. Lipoplexes offer several advantages in that they

provide a high DNA packing density, lower immunogeneicity, and are likely to be

able to transport DNA of considerably larger size than the viral vectors. The

5   possibility of targeting lipidic carriers to specific cell types also makes them

attractive candidates for gene therapy. However, the delivery of whole genes is

not the only form of gene therapy. Previous research has demonstrated that

antisense gene therapy may be useful to inhibit expression of genes that cause

disease. Additionally, recent research on the RNAi effect has shown that

10   administration of particular RNA oligonucleotides could be an especially effective

way of silencing genes that are deleterious. Similarly, it has become newly

appreciated that DNA oligonucleotides engineered for high affinity binding to

particular gene sequences may be useful in gene therapy given the proper delivery

system. These kinds of developments make it clear that gene therapy is likely to

15   evolve in a variety of different ways and that different modes will be effective

with different diseases.

        To date, the primary approach to improving the transfection properties of

cationic lipids has been the synthesis of new kinds of cationic amphipaths or the

inclusion of non-cationic helper lipids. While such approaches have met with

20   some success, improved transfection reagents that provide efficient transfection

(e.g., efficient nucleic acid uptake, low toxicity) are needed.

DESCRIPTION OF THE DRAWINGS

        Figure 1 shows data demonstrating that the extent of transfection of

25   HUAEC varies with the ratio of medium chain to long chain lipids. The cells

were seeded in 96-well plates at 24 hours before transfection at densities to give

about 80% confluence at the time of transfection. Chloroform solutions of

EDLPC with EDOPC and EDLPC with EDMPC were mixed at the different ratios

and then chloroform was removed under N_2 stream and vacuum. The lipid

30   mixtures were hydrated in HBSS at 1 mg/ml to form liposomes. Liposomes and

plasmid DNA were diluted in OptiMEM to 80 µg/ml for lipid and to 20 µg/ml for

DNA, and liposomes were pipetted into an equal volume of plasmid DNA

solution at a 4:1 weight ratio and mixed gently. The resultant DNA (plasmid with

β-galactosidase marker gene) – lipid complexes were incubated at room
temperature for 15 min and then added to the cells that were either in medium
lacking serum (black bars) or medium containing 5% serum (gray bars). The
lipoplexes had 35% (lipoid) excess positive over negative (DNA) charge. Cells

5   were assayed for β-galactosidase expression 24 hours after transfection. Data
represent the mean ±S.D. of a representative experiment performed in
quadruplicate. ~20,000 fluorescence units corresponded approximately to 0.1
milliunit of β-galactosidase.

       Figure 2 shows data demonstrating the change of transfection with the

10   ratio of EDLPC to EDOPC and the ratio of total lipids to DNA. Cells were
treated with DNA-lipid complex for 2 h in the absence ("no serum") or presence
of serum ("serum") and then washed with HBSS and supplemented with fresh
culture medium. Data represent the mean ±S.D. of a representative experiment
performed in quadruplicate.

15   Figure 3 shows a graph of the membrane fusion of fluorescence-labeled,
cationic lipoplexes with anionic liposomes. The lipids were labeled with 0.5
mol% each of NBD-PE and Rh-PE and hydrated at 1 mg/ml in PBS. Lipoplexes
were then prepared as for transfection. Two hundred microliters of the resulting
lipoplexes were titrated with 3-fold mol unlabeled egg PC liposomes containing

20   20% DOPG. The experiments were done at 37 °C. Ex=320nm, Em=535nm. %
membrane fusion=$(Fn-F_0)/(F_{100}-F_0) \times 100\%$, where Fn is the fluorescence after the
addition of anionic lipid, F_0 is the initial fluorescence of lipoplexes, and F_{100} is the
fluorescence when anionic lipid was mixed directly with cationic lipids in
chloroform and then lipoplexes were prepared as above.

25   Figure 4 shows oligonucleotide distribution of EDOPC and
EDLPC/EDOPC/DNA (60/40/16.7) lipoplexes in HUAECs. Lipoplexes were
labeled with a fluorescein derivative of a double-stranded dodecameric
oligonucleotide. Cells were incubated with the resulting lipoplexes in the
presence of serum for 2 h and imaged under a fluorescence microscope at 2 h later

30   after being washed with HBSS.

       Figure 5 shows graphs showing the effect of serum on the composition of
lipoplexes. Lipoplexes were treated with 5% serum at 37°C for different times,
and centrifuged at 14,000 rpm (4°C) for 1 h. The pellets were collected and

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extracted with chloroform. The chloroform phase was applied to a TLC plate, which was then developed in chloroform/methanol/H_2O (65:25:4). The separated EDLPC and EDOPC were obtained by extracting SiO_2 of the relevant regions of the TLC plate with chloroform/methanol/H_2O (10:10:1). EDLPC and EDOPC
5   were quantified with a phosphate assay.

Figure 6 shows a graph showing the effect of serum on the transfection of HUAEC. Cells were treated with DNA-lipid complex for 2 h in the absence ("no serum") or presence of 5% normal serum ("normal serum") or 5% delipidated serum ("delipidated serum") and then washed with HBSS and supplemented with
10   fresh regular culture medium. Data represent the mean ±S.D. of a representative experiment performed in quadruplicate.

Figure 7 shows a mixed lipoid effect in transfection with tetraalkylammonium compounds. Conditions were identical with those described in Figure 1 except that different lipoid mixtures were used.
15   Ditetradecyldimethyammonium (di C14 substituted quaternary ammonium) was mixed with dioctadecylammonium (di C18 quaternary ammonium).

Figure 8 shows the results of transfecting human dermal fibroblast cells with EDOPC /EPOPC (one oleoyl chain, which is 18C's with one double bond, and one palmitoyl, which is 16C's without double bond) mixtures.
20   Figure 9 shows the results of transfecting human dermal fibroblast cells with EDOPC / EDiphytanoyl PC (two phytanoyl chains, 16 carbon chains with 4 methyl branches) mixtures.

Figure 10 shows the results of transfecting HUAECs with EDOPC/POEPC, EDOPC/ EDiphytanoylPC, and EDOPC/SDOPC (DOPC with
25   an 18 carbon chain instead of an ethyl group on the phosphate oxygen) mixtures.

SUMMARY OF THE INVENTION

The present invention provides optimized transfection reagents comprising mixtures of, for example, cationic lipoids. In some embodiments, the mixture
30   comprises first and second lipoids having different fatty acid chains. In some embodiments, the different fatty acid chains comprise fatty acid chains that differ in length (e.g., a first chain having 18 carbons and a second chain having 10, 12 or 14 carbons). The present invention is not limited by the particular lengths or number of fatty chains in the lipoids. In some embodiments, the fatty acid chains

differ in mean cross-sectional area (i.e., width). The present invention is not

limited by the nature of the fatty acid chain constituents that provide difference in

cross-sectional area. An example of such a constituent includes, but is not limited

to, branches (e.g., methyl branches on the fatty acid chain). In some

5   embodiments, cross-sectional area is altered by mixing a first lipoid with a second

lipoid that differs in that it has a substitution of a long chain fatty acid in a

location where such a chain does not normally exist (e.g., substitution of an ethyl

group of 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (EDOPC) to a long chain

fatty acid (e.g., stearyl) to provide a mixture of EDOPC and SDOPC).

10   In some embodiments, the different fatty acid chains are contained in a

single molecule (e.g., ethylphosphatidylcholine having an oleoyl and a decanoyl

chain). In other embodiments, the different fatty acid chains are present in

different molecules in the mixture (e.g., a mixture having 1,2-dioleoyl-*sn*-glycero-

3-ethylphosphocholine (EDOPC) and 1,2-dilauroyl-*sn*-glycero-3-

15   ethylphosphocholine (EDLPC)).

The present invention is not limited to any particular mechanism of action

and an understanding of the mechanism of action is not necessary to practice the

present invention. However, it is contemplated that improved transfection is

achieved where two or more (e.g., three, four, . . .) different fatty acid chains are

20   provided in the lipoid mixture.

In some embodiments, the mixture comprises first and second cationic

lipoids. The present invention provides optimized transfection reagents

comprising mixtures of cationic lipoids. In particular, the present invention

provides DNA delivery vehicles based on identifying the optimal hydrophobicity

25   of novel cationic phospholipid derivatives that, alone or in combination, form

complexes with DNA (lipoplexes) and exhibit enhanced transfection activity.

In some embodiments, the first cationic lipoid is a standard lipoid found in

transfection reagents. Such standard lipoids are those known in the art that are

used, for example, to form liposomes (although the present invention is not

30   limited to the use of liposome transfection reagents). Liposomes comprise spheres

of lipid bilayers that enclose an aqueous medium. Liposomes can generally be

formed by sonicating a lipid in an aqueous medium, by resuspension of dried lipid

layers in a buffer or by dialysis of lipids dissolved in an organic solvent or in an

aqueous solution against a buffer of choice. Phospholipids form closed, fluid-

filled spheres when they are mixed with water, in part because the molecules are
amphipathic: they have a hydrophobic (water-insoluble) tail and a hydrophilic
(water-soluble), or "polar," head. Two fatty acid chains containing from about 16
up to about 24 carbon atoms generally make up the hydrophobic tail of most

5   naturally occurring phospholipid molecules. Equivalent structures may be
employed in synthetic lipids. Phosphoric acid bound to any of several water-
soluble molecules composes the hydrophilic head. When a high enough
concentration of phospholipids is mixed with water, the hydrophobic tails
spontaneously herd together to exclude water, whereas the hydrophilic heads bind

10   to water. In most instances, the result is a bilayer in which the fatty acid tails
point into the membrane's interior and the polar head groups point outward. The
polar groups at one surface of the membrane point toward the liposome's interior
and those at the other surface point toward the external environment. As a
liposome forms, any water-soluble molecules that have been added to the water

15   are incorporated into the aqueous spaces in the interior of the spheres, whereas
any lipid-soluble molecules added to the solvent during vesicle formation are
incorporated into the lipid bilayer. Phospholipid-related materials that are found
in typical liposomes include, lecithin, lysolecithin, lysophosphatidylethanolamine,
phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, sphinogomyelin,

20   cephalin, cardiolipin, phosphatidic acid, cerebrosides, and ceramide. Some
specific examples of phospholipids include, but are not limited to,
dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC),
dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG),
dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine

25   (POPC), palmitoyloleoylphosphatidylethanolamine (POPE),
dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl) cyclohexane-1-
carboxylate (DOPE-MAL), diheptadecanoyl phosphatidylethanolamine,
dilauroylphosphatilylethanolamine, dimyristoylphosphatidylethanolamine,
distearoyl phosphatidylethanolamine, beta-linoleoyl-gammapalmitoyl

30   phosphatidylethanolamine and beta-oleoyl-gammapalmitoyl
phosphatidylethanolamine). Common cationic lipoids found in liposomes include
1,2-diolelyloxy-3-(trimethylamino) propane (DOTAP); N-1-(2,3,-
ditetradecyloxy)propyl-N,N-dimethyl-N-hydroxyethylammonium bromide
(DMRIE); N-1-(2,3,-dioleyloxy)propyl-N,N-dimethyl-N-hydroxy

ethylammonium bromide (DORIE); N-1-(2,3-dioleyloxy) propyl-N,N,N-
trimethylammonium chloride (DOTMA); 3α N-(N',N'-dimethylaminoethane)
carbamoly cholesterol (DC-Chol); and dimethyldioctadecylammonium (DDAB).
Numerous other cationic lipoids are known in the art.

5   In some embodiments, the second cationic lipoid is a lipoid with a
hydrophobic structure that is significantly different from that of the first lipoid..
The present invention is not limited to a particular mechanism. Indeed, an
understanding of the mechanism is not necessary to practice the present invention.
Nonetheless, the mechanism of action of such a hydrophobic structure may disrupt

10   the packing of the bilayer of a liposome or other structure so that the lipid
organization of the array may be different in the mixture, thereby enhancing
transfection. Differences in physical organization of lipids of transfection agents
may translate into effects on transfection, as measured by detecting transfection
efficiency with and without the second cationic lipoid or by comparing the

15   transfection reagents having the second cationic lipoid to other transfection
reagents (see e.g., Example 1 for such a method). Optimization to determine the
optimal composition for liposomes or other lipoid arrays can be achieved by a
variety of methods. For example, in some embodiments, the second cationic
lipoid has a smaller hydrophobic mass (e.g., shorter hydrophobic tail or net shorter

20   hydrophobic tails in a molecule with multiple tails). In some embodiments, the
second cationic lipoid is functionalized to add hydrophilicity (e.g., canceling some
of the hydrophobic mass). For example, in some embodiments, a lipid head group
is made larger or more hydrophilic. In some embodiments, a lipid tail is
functionalized to reduce hydrophobicity. For example, in some embodiments, the

25   functionalization comprises addition of one or more polar groups. In some
embodiments, a polar fluorophore is added (e.g., NBD), providing the added
feature of fluorescent detectability. In some embodiments, the second cationic
lipoid comprises a head group and a second component with sufficient
hydrophobicity to allow it to form a bilayer with the first cationic lipoid, but

30   otherwise with low hydrophobicity.

In some embodiments, the first or second lipoids are not cationic. In some
embodiments, the second lipoid is configured to have shorter acyl chains than a
first lipoid and/or that alters the packing of the bilayer of a liposome or other

structure) as compared to the same structure in the absence of a second cationic lipoid (see, e.g., Example 1).

In some embodiments, the present invention provides a composition comprising lipoid transfection reagents, wherein the reagents comprise a first

5   cationic lipoid (e.g., having a head group and a lipid tail), and a second cationic lipoid (e.g., having the same or a different head group and a different, second lipoid tail), wherein the second cationic lipoid, when combined with said first cationic lipoid in said reagents, decreases the hydrophobicity of the reagents compared to said reagents in the absence of the second cationic lipoid, and

10   wherein said decrease increases the ability of the reagents to transfect cells. In some preferred embodiments, the second cationic lipoid comprises either a short, medium, or long chain fatty acid and the first cationic lipoid comprises either a short, medium or long chain fatty acid. A short chain fatty acid is a fatty acid chain having 7 or less carbons. A medium chain fatty acid is a fatty acid chain

15   having between 8 and 15 carbons (e.g., laurate, myristate, etc.). A long chain fatty acid is a fatty acid chain having 16 or more carbons (e.g., palmitate, stearate, oleate, etc.). The lipid tails may be saturated or unsaturated.

In other embodiments, the second lipoid has lipid tails that may be the same length as those of the first lipoid, but are of a different shape. Such different

20   shapes arise by incorporating different carbon chain branches along the lipid tails (e.g., as when methyl branches are incorporated along the lipid tail, the result of which is to increase the cross-sectional area (in the plane of the bilayer) of the lipid). Generally, the chains may be either shorter and/or fatter than the chains of the first lipoid.

25   The cationic lipoids of the present invention may be of any form, including, but not limited to, natural or synthetic lipoids having head groups with one or more (e.g., two) fatty acyl or alkyl chains attached. Where more than one fatty chain is provided on a lipoid molecule, the chains may be the same or different. In some embodiments, the modified bilayer structure is achieved by

30   lengthening one of the two tails and shortening the other. In some embodiments, the shortened chain is reduced in size more than the lengthened chain is increased. In some embodiments, the two chains are of similar length, but of different cross sectional area. In some embodiments, the lipoid does not comprise a traditional head group/tail structure. For example, in some embodiments, cationic

cholesterol derivatives or similar structures may be used. In some embodiments, the cationic lipoids comprise any type of head group, including, but not limited to, chemically modified phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, and

5   sphingomyelin, and derivative thereof. In some preferred embodiments, the first cationic lipid comprises a dioleoyl O-ethylphosphatidylcholine. In some preferred embodiments, the second cationic lipid comprises a dilauroyl O-ethylphosphatidylcholine. The present invention is not limited by the ratio of the first and second cationic lipids within the composition. In some embodiments, the

10   composition further comprises one or more additional components such as cholesterol or other lipids. In some embodiments, the composition further comprises a nucleic acid molecule (e.g., a vector, naked DNA, antisense oligonucleotides, siRNA, etc.), a protein, a small molecule drug, or other desired agents.

15   The present invention also provides methods for transfecting cells. In some embodiments, the method comprises exposing a cell to the composition described above, wherein the composition comprises a nucleic acid molecule. The cell may reside *in vitro* (e.g., in culture), *ex vivo*, or *in vivo*. The cell may be isolated or may be associated with other cells (e.g., in a tissue).

20

DEFINITIONS

         To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

         As used herein the term "lipoid" refers to any natural or synthetic lipid

25   molecules, including non-natural compounds that are similar to lipids in structure and properties (e.g., they are amphipaths).

         As used herein the term "transfection efficiency" refers to, for example, the percentage of target cells, within a population of target cells, that contain an introduced exogenous nucleic acid molecule. Transfection efficiency can be

30   determined by transfecting a nucleic acid molecule encoding a reporter gene into a population of target cells and determining the percentage of cells having reporter activity. The term "transfection efficiency" also refers to the amount of gene product detected following transfection of the nucleic acid into the cell. This is determined, for example, by testing an entire cell population for the amount of

gene product produced after a given incubation period. Thus, the term

"transfection efficiency" involves assaying for the relative expression of the gene

product encoded by the introduced nucleic acid.

      As used herein, the term "liposome" refers to a vesicle bounded by a lipid

5   bilayer. A "cationic liposome" has a net positive charge.

      As used herein, the term "short chain fatty acid" refers to a fatty acid chain

having 7 or less carbons.

      As used herein, the term "medium chain fatty acid" refers to a fatty acid

chain having between 8 and 15 carbons (e.g., laurate, myristate, etc.).

10   As used herein, the term "long chain fatty acid" is a fatty acid chain having

16 or more carbons (e.g., palmitate, stearate, oleate, etc.).

      As used herein, the term "nucleic acid molecule" refers to any nucleic acid

containing molecule including, but not limited to DNA or RNA. The term

encompasses sequences that include any of the known base analogs of DNA and

15   RNA including, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-

methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-

(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-

carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil,

dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-

20   methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-

methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-

methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-

methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-

methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-

25   isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid,

oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-

thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester,

uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-

diaminopurine.

30   The term "gene" refers to a nucleic acid (e.g., DNA) sequence that

comprises coding sequences necessary for the production of a polypeptide or

precursor. The polypeptide can be encoded by a full length coding sequence or by

any portion of the coding sequence so long as the desired activity or functional

properties (e.g., enzymatic activity, ligand binding, signal transduction, etc.) of the

full-length or fragment are retained. The term also encompasses the coding region

of a structural gene and the including sequences located adjacent to the coding

region on both the 5' and 3' ends for a distance of about 1 kb or more on either end

such that the gene corresponds to the length of the full-length mRNA. The

5   sequences that are located 5' of the coding region and which are present on the

mRNA are referred to as 5' non-translated sequences. The sequences that are

located 3' or downstream of the coding region and which are present on the

mRNA are referred to as 3' non-translated sequences. The term "gene"

encompasses both cDNA and genomic forms of a gene. A genomic form or clone

10   of a gene contains the coding region interrupted with non-coding sequences

termed "introns" or "intervening regions" or "intervening sequences." Introns are

segments of a gene which are transcribed into nuclear RNA (hnRNA); introns

may contain regulatory elements such as enhancers. Introns are removed or

"spliced out" from the nuclear or primary transcript; introns therefore are absent in

15   the messenger RNA (mRNA) transcript. The mRNA functions during translation

to specify the sequence or order of amino acids in a nascent polypeptide.

As used herein, the term "gene expression" refers to the process of

converting genetic information encoded in a gene into RNA (e.g., mRNA, rRNA,

tRNA, or snRNA) through "transcription" of the gene (i.e., via the enzymatic

20   action of an RNA polymerase), and for protein encoding genes, into protein

through "translation" of mRNA. Gene expression can be regulated at many stages

in the process. "Up-regulation" or "activation" refers to regulation that increases

the production of gene expression products (i.e., RNA or protein), while "down-

regulation" or "repression" refers to regulation that decrease production.

25   Molecules (e.g., transcription factors) that are involved in up-regulation or down-

regulation are often called "activators" and "repressors," respectively.

Transcriptional control signals in eukaryotes comprise "promoter" and

"enhancer" elements. Promoters and enhancers consist of short arrays of DNA

sequences that interact specifically with cellular proteins involved in transcription

30   (T. Maniatis et al., Science 236:1237 [1987]). Promoter and enhancer elements

have been isolated from a variety of eukaryotic sources including genes in yeast,

insect and mammalian cells, and viruses (analogous control elements, i.e.,

promoters, are also found in prokaryotes). The selection of a particular promoter

and enhancer depends on what cell type is to be used to express the protein of

interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types (for review see, S. D. Voss et al., Trends Biochem. Sci., 11:287 [1986]; and T. Maniatis et al., supra). For example, the SV40 early gene enhancer is very active in a wide variety of cell

5   types from many mammalian species and has been widely used for the expression of proteins in mammalian cells (R. Dijkema et al., EMBO J. 4:761 [1985]). Two other examples of promoter/enhancer elements active in a broad range of mammalian cell types are those from the human elongation factor 1.alpha. gene (T. Uetsuki et al., J. Biol. Chem., 264:5791 [1989]; D. W. Kim et al., Gene 91:217

10   [1990]; and S. Mizushima and S. Nagata, Nuc. Acids. Res., 18:5322 [1990]) and the long terminal repeats of the Rous sarcoma virus (C. M. Gorman et al., Proc. Natl. Acad. Sci. USA 79:6777 [1982]) and the human cytomegalovirus (M. Boshart et al., Cell 41:521 [1985]). Some promoter elements serve to direct gene expression in a tissue-specific manner.

15   As used herein, the term "promoter/enhancer" denotes a segment of DNA which contains sequences capable of providing both promoter and enhancer functions (i.e., the functions provided by a promoter element and an enhancer element, see above for a discussion of these functions). For example, the long terminal repeats of retroviruses contain both promoter and enhancer functions.

20   The enhancer/promoter may be "endogenous" or "exogenous" or "heterologous." An "endogenous" enhancer/promoter is one which is naturally linked with a given gene in the genome. An "exogenous" or "heterologous" enhancer/promoter is one which is placed in juxtaposition to a gene by means of genetic manipulation (i.e., molecular biological techniques such as cloning and recombination) such that

25   transcription of that gene is directed by the linked enhancer/promoter.

The presence of "splicing signals" on an expression vector often results in higher levels of expression of the recombinant transcript. Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site (J. Sambrook et al., Molecular Cloning: A Laboratory

30   Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York [1989], pp. 16.7-16.8). A commonly used splice donor and acceptor site is the splice junction from the 16S RNA of SV40.

Efficient expression of recombinant DNA sequences in eukaryotic cells requires expression of signals directing the efficient termination and

polyadenylation of the resulting transcript. Transcription termination signals are generally found downstream of the polyadenylation signal and are a few hundred nucleotides in length. The term "poly A site" or "poly A sequence" as used herein denotes a DNA sequence that directs both the termination and polyadenylation of

5   the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. The poly A signal utilized in an expression vector may be "heterologous" or "endogenous." An endogenous poly A signal is one that is found naturally at the 3' end of the coding region of a given gene in the genome. A

10   heterologous poly A signal is one that is isolated from one gene and placed 3' of another gene. A commonly used heterologous poly A signal is the SV40 poly A signal. The SV40 poly A signal is contained on a 237 bp BamHI/BclI restriction fragment and directs both termination and polyadenylation (J. Sambrook, supra, at 16.6-16.7).

15   Eukaryotic expression vectors may also contain "viral replicons" or "viral origins of replication." Viral replicons are viral DNA sequences that allow for the extrachromosomal replication of a vector in a host cell expressing the appropriate replication factors. Vectors that contain either the SV40 or polyoma virus origin of replication replicate to high "copy number" (up to 10^4 copies/cell) in cells that

20   express the appropriate viral T antigen. Vectors that contain the replicons from bovine papillomavirus or Epstein-Barr virus replicate extrachromosomally at "low copy number" (~100 copies/cell).

            The term "transgene" as used herein refers to a foreign gene that is placed into an organism by, for example, introducing the foreign gene into newly

25   fertilized eggs or early embryos. The term "foreign gene" refers to any nucleic acid (e.g., gene sequence) that is introduced into the genome of an animal by experimental manipulations and may include gene sequences found in that animal so long as the introduced gene does not reside in the same location as does the naturally-occurring gene.

30   As used herein, the term "vector" is used in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The term "vehicle" is sometimes used interchangeably with "vector." Vectors are often derived from plasmids, bacteriophages, or plant or animal viruses.

The term "expression vector" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in

5   prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

The terms "overexpression" and "overexpressing" and grammatical equivalents, are used in reference to levels of mRNA to indicate a level of

10   expression approximately 3-fold higher than that typically observed in a given tissue in a control or non-transgenic animal. Levels of mRNA are measured using any of a number of techniques known to those skilled in the art including, but not limited to Northern blot analysis. Appropriate controls are included on the Northern blot to control for differences in the amount of RNA loaded from each

15   tissue analyzed (e.g., the amount of 28S rRNA, an abundant RNA transcript present at essentially the same amount in all tissues, present in each sample can be used as a means of normalizing or standardizing the mRNA-specific signal observed on Northern blots). The amount of mRNA present in the band corresponding in size to the correctly spliced transgene RNA is quantified; other

20   minor species of RNA which hybridize to the transgene probe are not considered in the quantification of the expression of the transgenic mRNA.

The term "transfection" as used herein refers to the introduction of foreign DNA into eukaryotic cells.

The term "stable transfection" or "stably transfected" refers to the

25   introduction and integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell which has stably integrated foreign DNA into the genomic DNA.

The term "transient transfection" or "transiently transfected" refers to the introduction of foreign DNA into a cell where the foreign DNA fails to integrate

30   into the genome of the transfected cell. The foreign DNA persists in the nucleus of the transfected cell for several days. During this time the foreign DNA is subject to the regulatory controls that govern the expression of endogenous genes in the chromosomes. The term "transient transfectant" refers to cells which have taken up foreign DNA but have failed to integrate this DNA.

As used herein, the term "cell culture" refers to any in vitro culture of cells. Included within this term are continuous cell lines (e.g., with an immortal phenotype), primary cell cultures, finite cell lines (e.g., non-transformed cells), and any other cell population maintained in vitro.

5   As used, the term "eukaryote" refers to organisms distinguishable from "prokaryotes." It is intended that the term encompass all organisms with cells that exhibit the usual characteristics of eukaryotes, such as the presence of a true nucleus bounded by a nuclear membrane, within which lie the chromosomes, the presence of membrane-bound organelles, and other characteristics commonly

10   observed in eukaryotic organisms. Thus, the term includes, but is not limited to such organisms as fungi, protozoa, and animals (e.g., humans).

As used herein, the term "in vitro" refers to an artificial environment and to processes or reactions that occur within an artificial environment. In vitro environments can consist of, but are not limited to, test tubes and cell culture. The

15   term "in vivo" refers to the natural environment (e.g., an animal or a cell) and to processes or reaction that occur within a natural environment.

The term "test compound" refers to any chemical entity, pharmaceutical, drug, and the like that can be used to treat or prevent a disease, illness, sickness, or disorder of bodily function. Test compounds comprise both known and potential

20   therapeutic compounds. A test compound can be determined to be therapeutic by screening using the screening methods of the present invention. A "known therapeutic compound" refers to a therapeutic compound that has been shown (e.g., through animal trials or prior experience with administration to humans) to be effective in such treatment or prevention.

25   The term "sample" as used herein is used in its broadest sense and includes environmental and biological samples. Environmental samples include material from the environment such as soil and water. Biological samples may be animal, including, human, fluid (e.g., blood, plasma and serum), solid (e.g., stool), tissue, liquid foods (e.g., milk), and solid foods (e.g., vegetables).

30

DESCRIPTION OF THE INVENTION

The present invention provides an alternative approach to improving transfection reagents. The compositions and methods of the present invention were shown to be unusually effective. In preferred embodiments, the present

invention provides the combination of two or more cationic lipoids to provide improved transfection reagents. In some embodiments, the first cationic lipoid is a standard cationic lipoid used in transfection reagents and the second cationic lipoid is of the nature where, when combined with the first cationic lipoid in

5   transfection reagents, alters the hydrophobicity of the hydrophobic mass either in the extent of hydrophobicity of the lipoids or in the organization of the lipoids compared to the reagents in the absence of the second cationic lipoid, and wherein such change in hydrophobicity increases the ability of the transfection reagents to transfect cells.

10   In some embodiments, the second cationic lipoid is a lipoid that has higher water solubility than the first lipoid and/or that increases the exposure of the hydrophobic core of the lipoid structure to an aqueous environment (e.g., disrupt the bilayer of a liposome) as compared to the same structure in the absence of the second cationic lipoid. The degree of increase in water solubility and/or increased

15   exposure of the hybrophobic core that finds use in the present invention can readily be measured by detecting transfection efficiency with and without the second cationic lipoid or by comparing the transfection reagents having the second cationic lipoid to other transfection reagents (see e.g., Example 1 for such a method). Optimization to increase water solubility and/or increased exposure of

20   the hydrophobic core can be achieved by a variety a methods. For example, in some embodiments, the second cationic lipoid has a smaller hydrophobic mass (e.g., shorter hydrophobic tail). In some embodiments, the second cationic lipoid is functionalized to add hydrophilicity (e.g., canceling some of the hydrophobic mass). In some embodiments, the functionalization comprises addition of one or

25   more polar groups. In some embodiments, a polar fluorophore is added (e.g., NBD), providing the added feature of fluorescent detectability.

        For example, particularly efficient transfection reagents were produced by the combination of dilauroyl (12 carbon chain) and dioleoyl (18 carbon chain) homologues of O-ethylphosphatidylcholine. This mixture transfected DNA into

30   human umbilical artery endothelial cells (HUAECs) more than 30-fold more efficiently than either compound separately. A unique advantage of this kind of combination agent is that transfection can be optimized either in the presence or absence of serum by adjusting the component ratio.

In some embodiments, the second lipoid has chains that are not significantly different in length from those of the first lipoid, but the second lipoid has chains that have a larger cross-sectional area.

In some embodiments, the second lipoid has chains that are differently
5   shaped from those of the first lipoid, so as to occupy space in the bilayer in a different way than those of the first lipoid.

Cationic lipids have been widely used for the delivery of plasmid and antisense DNA into eukaryotic cells; however, inefficiency of transfection is a major problem confronting their use in gene therapy. Vascular endothelial cells
10   act as an interface between circulating blood and various tissues and organs of the body, and are known to be involved in inflammatory processes such as leukocyte recruitment, cytokine production (see, e.g., Koning GA, et al., *Endothelium* 2002, 9:161-171; Neuhaus T *et al., Clinical Science* 2000; 98: 461-470; Stier S *et al.,* *FEBS Letters* 2000; 467: 299-304; each herein incorporated by reference in their
15   entireties), and to play a major role in the pathogenesis of atherosclerosis (see, e.g., Behrendt D, and Ganz P., *Am J Cardiol* 2002; 90: 40L-48L; Ulrich-Merzenich G, et al., *European Journal of Nutrition* 2002; 41: 27-34; each herein incorporated by reference in their entireties), as well as angiogenesis (see, e.g., Ellis LM. *Am Surg* 2003; 69: 3-10; Nam NH, Parang K. *Curr Drug Targets* 2003;
20   4: 159-179; Ranieri G, and Gasparini G., *Curr Drug Targets Immune Endocr* *Metabol Disord* 2001; 1: 241-253; Sylven C. *Drugs Today (Barc)* 2002; 38: 819-827; each herein incorporated by reference in their entireties), on which the growth and spread of tumors are dependent. Hence, they are of considerable interest as a gene therapy target (see, e.g., Baker AH., *J Card Surg* 2002; 17: 543-
25   548; Morishita R., *Circ J* 2002; 66: 1077-1086; each herein incorporated by reference in their entireties). Even though they are readily accessible, gene therapy with nonviral vectors of endothelial tissue has been seriously hampered by the fact that endothelial cells are very difficult to transfect. According to Struck et al., Biochemistry 1981, 20:4093-4099, the transfection efficiency of vascular
30   endothelial cells with cationic lipids was only 2%. It is known that the cytotoxicity of cationic lipids increases with the shortening of acyl groups and so cationic lipids used in transfection invariably have alkyl chains that are 14 or more carbon long. The present invention provides solutions to such problems.

For example, in one embodiment, a short chain cationic phosphocholine (1,2-dilauroyl-*sn*-glycero-3-ethylphosphocholine, EDLPC), when combined with longer chain compounds (1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine, EDOPC, or 1,2-dimyristol-*sn*-glycero-3-ethylphosphocholine, EDMPC)

5   dramatically enhances (up to 30-fold) the transfection efficiency of human umbilical artery endothelial cells (HUAECs) even though, individually, EDLPC, EDOPC or EDMPC are quite weak transfection reagents. Moreover, transfection efficiency can be adjusted to be optimal either in the presence or absence of serum by changing the EDLPC/EDOPC ratio and the ratio of total lipids to DNA. Under

10   optimal conditions, transfection efficiency can be achieved up to 15% both in the presence and absence of serum. Thus, these formulations constitute a novel form of cellular transfection reagent and offer entirely new formulations for optimizing *in vivo* gene delivery. At present, only phosphatidylethanolamine and cholesterol are used as the helper lipids to improve the transfection properties of cationic

15   lipids. Unlike these prior methods (although they may be used in conjunction with the present invention), the present invention employs compounds with different hydrophobicity-hydrophilicity balance to improve the gene delivery properties of lipoplexes. The properties of lipoplexes can be tuned by changing the ratio of the different lipoids (e.g., the ratio of medium chain to long chain

20   cationic lipoids and the ratio of lipoid to DNA).

In some preferred embodiments, one or more agents may be added to the cationic lipoid mixtures so as to further increase transfection efficiency. Examples of agents include, but are not limited to, cholesterol, polyamidoamine dendrons, histidylated lipids, octylglucoside, phycoerythrin, and non-cationic

25   lipids. In some preferred embodiments, the cationic lipoid mixtures may be transfected with additional transfection reagent sytems so as to further increase transfection efficiency. Examples of transfection reagent systems include, but are not limited to, LIPOFECTAMINE (Invitrogen), OPTIFECT (Invitrogen), 293FECTIN (Invitrogen), OLIGOFECTAMINE (Invitrogen), CELLFECTIN

30   (Invitrogen), LIPOFECTIN (Invitrogen), DMRIE-C (Invitrogen), EXGEN 500 (Euromedex), octylglucoside, FUGENE (Roche), EFFECTGENE (Qiagen), and SUPERFECT (Qiagen).

In some embodiments, the first or second lipoids are not cationic but are configured to have a structural impact on the bilayer into which they are

incorporated (e.g., to impact the water solubility than a cationic lipoid used alone, to increase the exposure of the hydrophobic core of the lipoid structure to an acqueous environment, or to disrupt the packing of the bilayer of a liposome or other structure as compared to the same structure in the absence of a second

5   cationic lipoid).

Transgenes

The transfection system described herein is useful to express any polypeptide of interest or to transfect any nucleic acid of interest (e.g., siRNAs,

10   antisense oligonucleotides, expression vectors, etc.).

The transgene will generally encode a native or recombinant protein, although the expression of other polypeptides, such as epitopes or other immunologically active polypeptides, are contemplated within the scope of this invention. Examples of proteins that can be expressed using the method of the

15   present invention are hormones; cytokines, such as growth factors; enzymes; receptors; oncogenes; polypeptide vaccines, viral proteins, and structural and secretory proteins.

The transgene employed in the constructs of the invention can be cloned sequences that retain intronic regions. If the exonic structure of the gene is

20   known, the coding exons can be inserted in the constructs.

Expression of the polypeptide of interest can be directed by a promoter homologous to the polypeptide coding sequences (for example, human glucose-6-phosphate dehydrogenase under the control of its own transcription promoter sequences). Further, other homologous or heterologous expression control

25   elements (e.g., affecting transcription, translation, or post-translational events) may be used.

It should be understood that expression of the transgene in the mammalian cells of the invention can be stable or transient. Even transient expression, at a higher than normal level, is useful for functional studies in the cells or for the

30   production and recovery of proteins of interest.

Regulatory Sequences

In addition to selectable markers and transgenes, the constructs described herein may contain suitable regulatory elements. Regulatory elements (or control

elements) are selected for use in the host cell of interest; for example, selectable

markers may be included to allow propagation in microorganisms, (e.g., f1 origin

of replication and ampicillin resistance encoding sequences). Such regulatory

elements include, but are not limited to, transcription promoters, transcription

5   enhancer elements, transcription termination signals, polyadenylation sequences

(located 3' to the translation stop codon), sequences for optimization of initiation

of translation (located 5' to the coding sequence), translation termination

sequences, secretion signal sequences, and sequences that direct post-translational

modification (e.g., glycosylation sites). Transcription promoters can include

10   inducible promoters (where expression of a polynucleotide sequence operably

linked to the promoter is induced by an analyte, cofactor, regulatory protein, etc.),

repressible promoters (where expression of a polynucleotide sequence operably

linked to the promoter is induced by an analyte, cofactor, regulatory protein, etc.),

and constitutive promoters.

15

Cells

The cells (e.g., host cells) employed in this invention include all eukaryotic

cells including mammalian cells (*in vivo* or *in vitro*), cell lines, and cell cultures.

The cells can be derived from mammals, such as mice, rats, or other rodents, or

20   from primates, such as humans or monkeys. Mammalian germ cells or somatic

cells can be employed for this purpose. It will be understood that primary cell

cultures or immortalized cells can be employed in carrying out the techniques of

the present invention. The cells may also reside *in vivo*. Examples of cells used

in the present invention include, but are not limited to, HUAEC cells, human

25   dermal fibroblast cells, cancer cells (e.g., myeloma cells).

The transformed cells obtained by some embodiments of the present

invention can be employed for the preparation of continuous cell lines in which

the cells are essentially immortal, or for the preparation of established cell lines

that have the potential to be subcultured *in vitro*. Continuous cell lines and

30   established cell lines can be obtained from a variety of organisms and organs, such

as rodent embryos; primate kidneys; rodent and human tumors; and fibroblast,

epithelial, or lymphoid cells. Cells exhibiting the highest levels of expression can

be cloned, if desired.

20

EXAMPLES

The following examples are provided to demonstrate and further illustrate certain preferred embodiments of the present invention and are not to be construed as limiting the scope thereof.

5

Example 1:   EDLPC/EDOPC Transfection Reagents

Experiments conducted during the development of the present invention found that attention to the hydrophobic portions of medium and long-chain cationic lipids synergistically enhance transfection. It was found that a

10   combination of two cationic lipid derivatives with the same head group but tails of different chain lengths behave considerably differently as transfection agents than the separate molecules. For example, the combination of the dilauroyl (12 carbon chain) and the dioleoyl (18 carbon chain) homologues of O-ethylphosphatidylcholine transfected DNA into primary human umbilical artery

15   endothelial cells (HUAECS) more than 30-fold more efficiently than either compound separately. The present invention is not limited to a particular mechanism. Indeed, an understanding of the mechanism is not necessary to practice the present invention. Nonetheless, these results suggest that the hydrophobic portions of medium and long-chain cationic lipids is far more

20   important than previously assumed.   An advantage of this kind of combination agent is that transfection is optimized either in the presence or absence of serum by adjusting the component ratio.

Considering that there are more opportunities to modify and combine the hydrophobic moieties on cationic lipoids than there are for variation of the head

25   groups, a study of the transfection efficiency of lipids with different kinds of tails and different kinds of combinations of those tails leads to new and improved nonviral vectors was conducted. A unique advantage of the cationic phospholipoids for hydrophobic structure modification is that they allow use of specific enzymes in their synthesis, a feature not exhibited by the other cationic

30   lipoids described in the literature because those compounds are not based on a natural product.

Figure1 shows that combining EDLPC with EDOPC enhances by ~30-fold of the extent of transfection of HUAECS, compared to EDLPC or EDOPC alone. The ratio of EDLPC to EDOPC affected performance, with different ratios

21

optimal, depending upon whether serum is present or absent. The
EDLPC/EDMPC mixture exhibits the similar pattern to that of EDLPC/EDOPC,
but the extent of transfection is lower than that of EDLPC/EDOPC.

On the basis of the results presented above, EDLPC/EDOPC (80/20) and

5   EDLPC/EDOPC (60/40) were chosen to further optimize transfection; the ratio of
lipids to DNA and the amount of DNA were used as optimization parameters.
Figure 2 depicts the change of transfection with the ratio of EDLPC to EDOPC
and the ratio of total lipids to DNA. For some formulations, transfection without
serum was better than that in serum; but for others, transfection in serum was

10   better than that in the absence of serum. The highest transfection in the absence of
serum was obtained when EDLPC/EDOPC =80/20 and lipid/DNA=4/1, with 0.5
µg DNA/well; under these conditions the extent of expression was 8× higher than
that in the presence of serum. In contrast, the most efficient transfection in the
presence of serum was when EDLPC/EDOPC=60/40 and lipid/DNA=6/1, with

15   1.0 µg DNA/well, under which condition the expression was 20× that in the
absence of serum. According to X-gal staining, 15% of the cells treated under
both of these conditions were positive. This efficiency of transfection is more than
an order of magnitude higher than has been previously reported for transfection of
these primary cells. These two formulations were thus used in the subsequent

20   studies. Such assays can be used to readily determine optimal ratios and optimal
components of the transfection reagents of the present invention.

The cell viability and the percentage of cells transfected for the two
formulations were determined using the MTT method and X-gal staining,
respectively (Table 1). Those data revealed that the low transfection efficiency in

25   the absence of serum for EDLPC/EDOPC=60/40 and lipid/DNA=6/1 was due to
high cytotoxicity.

While the present invention is not limited to any particular mechanism of
action and an understanding of the mechanism of action is not necessary to
practice the present invention, it is contemplated that the medium chain lipid

30   facilitates mixing of the lipoplex lipid with cellular lipid, which could lead to the
neutralizing of the positive charge of the cationic lipid and facilitate release of
DNA from the complex. Under such circumstances, EDLPC could facilitate
fusion (or at least lipid mixing) of cationic liposomes with anionic liposomes.

The fusion of EDLPC/EDOPC (80/20), EDLPC/EDOPC (60/40) and pure

EDOPC lipoplexes were compared to phosphatidylglycerol-containing (anionic)

liposomes. Membrane fusion was measured using a FRET assay (see, e.g., Struck

DK, et al., *Biochemistry* 1981; 20: 4093-4099; herein incorporated by reference in

5   its entirety) that measures reduction of energy transfer between NBD-PE and Rh-

PE in cationic lipids of the lipoplexes as they fuse with egg-PC liposomes

containing 20% DOPG. From Figure 3, it is seen that the extent of fusion of

EDLPC/EDOPC (80/20) and EDLPC/EDOPC (60/40) lipoplexes is significantly

higher than that of pure EDOPC. The present invention is not limited to a

10   particular mechanism. Indeed, an understanding of the mechanism is not

necessary to practice the present invention. Nonetheless, these results indicate

that increased transfection efficiency is associated with membrane fusion

characteristics.

In order to determine if this pattern of fusion is also observed within cells

15   (e.g., the mixture is more prone to fuse with endosomal membranes facilitating

escape of DNA from endosomal degradation and nuclei entrance) the intracellular

distribution of fluorescent lipid and oligonucleotide in EDOPC and

EDLPC/EDOPC (60/40) lipoplexes was investigated. It was found that both lipid

and oligonucleotide in EDOPC lipoplexes remained in the cytoplasm for at least

20   20 hours, whereas a large amount of the oligonucleotide from EDLPC/EDOPC

(60/40) lipoplexes entered the nuclei, in particular at the early time point of 2 h,

although lipid in EDLPC/EDOPC (60/40) lipoplexes remained in the cytoplasm at

this and all other time points. Figure 4 shows oligonucleotide distribution of

EDOPC and EDLPC/EDOPC/DNA (60/40/16.7) lipoplexes in HUAECSs.

25   Lipoplexes were labeled with a fluorescein derivative of a double-stranded

dodecameric oligonucleotide. Cells were incubated with the resulting lipoplexes

in the presence of serum for 2 h and imaged under a fluorescence microscope after

being washed in HBSS. As shown in Figure 4, the results of these experiments

indicated that in the presense of the lipoid mixture there was an increase in the

30   nuclear distribution of highly fluorescent oligonucleotides. Similar images were

obtained with fluorescent plasmid DNA, although the fluorescence of the nucleus

was less intense.

Escape of lipoplexes from endosomes prior to their entry into lysosomes is

important for trangene efficient expression. It is contemplated that fusion of

lipoplexes with endosomal membranes facilitates DNA release from endosomes
into cytoplasm, and thus increase DNA expression. While the present invention is
not limited to any particular mechanism of action and an understanding of the
mechanism of action is not necessary to practice the present invention, it is

5   contemplated that this may be one reason that transfection by the mixtures of lipid
is much higher than that of pure EDOPC.

        The present invention is not limited to a particular mechanism. Indeed, an
understanding of the mechanism is not necessary to practice the present invention.
Nonetheless, it is contemplated that dissociation of DNA from the surface of a

10   lipoid is caused by neutralization of the lipoid by cellular anionic lipids. Such
neutralization implies fusion or transfer of lipids as a necessary prerequisite of
efficient transfection, and implies that the DNA must become sufficiently free of
the lipid-lipoid array to be transcribed in the nucleus. Unlike normal cellular
lipids, the combination of cationic lipoids and anionic lipids gives rise to a variety

15   of non-lamellar phases which may or may not be capable of retaining a molecule
as large as a typical plasmid. Generally, generation of lipid phases through
combination of cationic and anionic lipids is dependent upon lipids (see, e.g.,
Tarahovsky, Y.S., et al., 2004, Biophysical Journal 87:1054-1064; herein
incorporated by reference in its entirety). For example, mixtures such as EDOPC-

20   EDLPC, when reconstituted with anionic lipids such as phosphatidylglycerol, give
rise to a highly curved inverted micellar cubic phase. This phase is characterized
by a cubic array of balls (shells) in which amphipathic molecules are organized
with their polar portion facing a small aqueous core and their hydrophobic tails
facing those of other shells. These phases have aqueous spaces too small to entrap

25   either a plasmid or other DNA molecule. Separate experiments have revealed that
treatment of EDOPC-EDLPC lipoplexes with the anionic lipid,
phosphatidylserine, releases more DNA by far that does treatment of EDOPC
lipoplexes with phoshatidylserine. The present invention is not limited to a
particular mechanism. Indeed, an understanding of the mechanism is not

30   necessary to practice the present invention. Nonetheless, it is contemplated that
there are at least two important effects involved when certain kinds of lipoid
mixtures are used to prepare lipoplexes. First, the mixed lipoid lipoplex may
acquire anionic lipid from the cell (e.g., perhaps by membrane fusion or molecular
exchange) faster and/or to a larger extent than do lipoplexes composed of lipoids

of a single type. Second, the phase or 3-dimensional array assumed after the cellular anionic lipid and the lipoplex lipoid may have such a structure as to release faster and/or to a greater extent its cargo of DNA than do conventional lipoplexes.

5   Serum strongly influences properties of lipoplexes, so experiments were conducted to examine the effect of serum on the composition of these two formulations. In Figure 5, one sees that during 90 min incubation in serum, for EDLPC/EDOPC=60/40, 20% of the EDOPC and 10% of the EDLPC were extracted from the lipoplexes; in the case of EDLPC/EDOPC=80/20, 30% of the

10   EDOPC and 50% of the EDLPC are extracted. Furthermore, at early times (30min), which are contemplated to be more important for endocytosis, the extraction of EDLPC and EDOPC from EDLPC/EDOPC=80/20 was much larger than that from EDLPC/EDOPC=60/40.

           Gene expression in delipidated serum was tested (Figure 6). Transgene

15   expression of both two formulations decreased significantly in delipidated serum, in which ~80% lipids (including cholesterol, HDL cholesterol, LDL cholesterol and phospholipids) are absent relative to normal serum. While the present invention is not limited to any particular mechanism of action and an understanding of the mechanism of action is not necessary to practice the present

20   invention, it is contemplated that this indirectly confirms serum extraction of lipids, since delipidated serum, with a higher lipid binding capacity than normal serum, would also extract more lipids from the lipoplex.

           Human dermal fibroblasts are another medically important cell type through participation in wound healing. It was contemplated that human dermal

25   fibroblasts would be useful in gene therapy to accelerate wound healing. It was therefore of interest to determine if the "mixed lipid" effect also operates in these primary cells. It was found that the "mixed lipid" effect is more pronounced than with HUAECs. The response in serum was not as pronounced, but efforts were not made to optimize the conditions for this system.

30   A human multiple myeloma cell line that is extremely difficult to transfect was also investigated and the "mixing effect" was observed, although the transfection efficiency was very low (1-2%).

           The mixed lipoid effect is not limited to cationic phospholipoids. As shown in Figure 7, the effect is seen when a dimethylammonium with two C14

chains is mixed with EDOPC and when the C18 phospholipoid is replaced with a

dimethylammonium having two C18 chains. The TAP compounds, DOTAP and

DMTAP in various combinations, were investigated with each other and with

EDOPC. In all cases substantial increases at intermediate compositions was

5   observed. The present invention is not limited to a particular mechanism. Indeed,

an understanding of the mechanism is not necessary to practice the present

invention. Nonetheless, the mixed lipoid effect appears quite general, as would be

anticipated if some aspect of the hydrophobicity of the lipoplex needs to be

matched to the cell and the transfection conditions. Although other lipoids can be

10   synthesized to have chain length differences, it is unlikely that any other such

compounds offer the flexibility of structural variation as the cationic

phospholipoids. Thus, while the present invention is not limited to the use of

cationic lipoids, cationic lipoids are a preferred material. These compounds offer

enormous flexibility in constructing molecules with varied amount and

15   configuration of hydrophobic moieties.

**Example 2:   Transfection of Human Dermal Fibroblasts with
EDOPC/EPOPC and EDOPC/EDiphytanoyl PC Transfection
Reagents**

20

Figure 8 shows that combining EDOPC with EPOPC (one oleoyl chain,

which is 18C's with one double bond, and one palmitoyl chain, which is 16C's

without any double bond) shows little mixing effect in transfection of human

dermal fibroblast cells in the absence of serum.

25   Figure 9 shows that combining EDOPC with EDiphytanoyl PC (two

phytanoyl chains, 16 carbon chains with 4 methyl branches) shows marked mixing

effect in the transfection of human dermal fibroblast cells in the absence of serum.

**Example 3:   Transfection of HUAECs with EDOPC/EPOPC, EDOPC/
30   EDiphytanoylPC, EDOPC/SDOPC and EDOPC/EC18C10PC**

Figure 10 shows the results of transfecting HUAECs with

EDOPC/EPOPC, EDOPC/EDiphytanoylPC, EDOPC/SDOPC (DOPC with an 18

carbon chain instead of an ethyl group on the phosphate oxygen), and

35   EDOPC/EC18C10PC mixtures.

Example 4:   Synthesis of new cationic phospholipoids (derivatives of phosphatidylcholine) having hydrophobic moieties

New cationic phospholipids (derivatives of phosphatidylcholine) are

5   contemplated including, but not limited to, medium chain cationic PC's with

phosphate oxygen alkyl substituents ranging in length from 2 to 24 C's); lyso

cationic PC's with one long chain (C24) and a phosphate oxygen alkyl substituent

with 2 to 12 C's; cationic PC's with acyl groups having very much different chain

lengths; tetra-acyl cationic PC's with short acyl chains; and lipoids with very long

10   (>18 carbons) acyl chains. In some cases, acyl or alky substituents may be

branched so as to effectively increase the number of chains without increasing the

number of attachment points to the hydrophilic cationic head group.

All publications and patents mentioned are herein incorporated by

15   reference. Various modifications and variations of the described method and

system of the invention will be apparent to those skilled in the art without

departing from the scope and spirit of the invention. Although the invention has

been described in connection with specific preferred embodiments, it should be

understood that the invention as claimed should not be unduly limited to such

20   specific embodiments. Indeed, various modifications of the described modes for

carrying out the invention that are obvious to those skilled in the relevant fields

are intended to be within the scope of the following claims.

Table 1 Cell viability and X-gal staining

EDLPC/EDOPC	Lipids/DNA	μg DNA /well	Serum	Cell viability %	X-gal staining %
80:20	4:1	0.5	-	77.5	~15
80:20	4:1	0.5	+	94.7	n.d.*
60:40	6:1	1.0	-	40.0	n.d.*
60:40	6:1	1.0	+	82.6	~15

*Not determined

5

CLAIMS

We claim:

5   1.   A composition comprising: lipoid transfection reagents comprising
a first cationic lipoid having a head group and a hydrophobic tail, and a second
cationic lipoid, wherein the second cationic lipoid comprises a hydrophobic tail
that is different than said hydrophobic tail of said first cationic lipid such that said
transfection reagents have a higher transfection efficiency compared to said
10   reagents that have said first cationic lipoid, but lack said second cationic lipoid.

        2.   The composition of Claim 1, wherein said hydrophobic tail of said
second cationic lipoid is shorter than said hydrophobic tail of said first cationic
lipoid.
15

        3.   The composition of Claim 1, wherein said hydrophobic tail of said
second cationic lipoid is longer than said hydrophobic tail of said first cationic
lipoid.

20   4.   The composition of Claim 1, wherein said hydrophobic tail of said
second cationic lipoid has a larger cross-sectional area than said hydrophobic tail
of said first cationic lipoid.

        5.   The composition of Claim 1, wherein said hydrophobic tail of said
25   second cationic lipoid has a smaller cross-sectional area than said hydrophobic tail
of said first cationic lipoid.

        6.   The composition of Claim 1, wherein said hydrophobic tail of said
second cationic lipoid has a different three dimensional shape than said
30   hydrophobic tail of said first cationic lipoid.

        7.   The composition of Claim 1, wherein said hydrophobic tail of said
second cationic lipoid has a different chemical composition than said hydrophobic
tail of said first cationic lipoid.

8.   The composition of claim 1, wherein said first and second cationic lipoids comprise different short chain fatty acids.

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9.   The composition of claim 1, wherein said first and second cationic lipoids comprise different medium chain fatty acids.

10.   The composition of claim 1, wherein said first and second cationic

10   lipoids comprise different long chain fatty acids.

11.   The composition of claim 1, wherein said second cationic lipoid is EDLPC.

15   12.   The composition of claim 1, wherein said first cationic lipoid is selected from the group consisting of EDOPC, EDMPC, EPOPC, EDiphytanoyl PC, SDOPC and EC18C10PC.

13.   The composition of claim 1, wherein said lipoid transfection

20   reagents further comprise a transfection enhancing agent.

14.   The composition of claim 13, wherein said transfection enhancing agent is selected from the group consisting of cholesterol, polyamidoamine dendrons, histidylated lipids, octylglucoside, and phycoerythrin.

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15.   A method of transfecting a cell comprising, exposing a cell to the composition of claim 1, wherein said composition further comprises a nucleic acid molecule.

30   16.   The method of claim 13, wherein said cell is in vivo.

17.   The method of claim 13, wherein said cell is in vitro.

18.   The method of claim 13, wherein said cell is ex vivo.

19.   The method of claim 13, wherein said cells are human dermal fibroblast cells.

5   20.   The method of claim 13, wherein said cell is a HUAEC cell.

21.   The method of claim 13, wherein said cell is a cancer cell.

22.   The method of claim 16, wherein said cancer cell is a myeloma
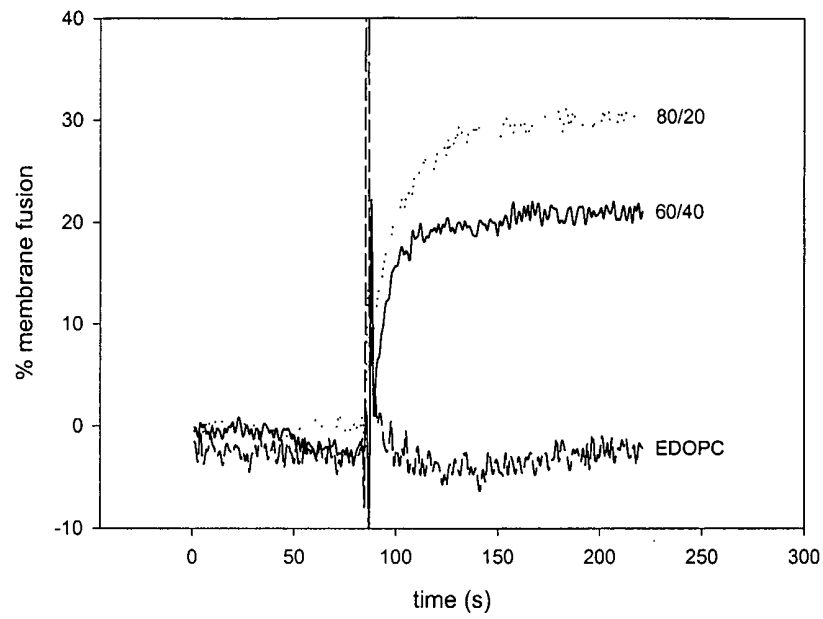
10   cell.

FIGURE 1

FIGURE 2

Transfection of HUAEC with EDLPC/EDOPC(80/20)

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Transfection of HUAEC with EDLPC/EDOPC (60/40)
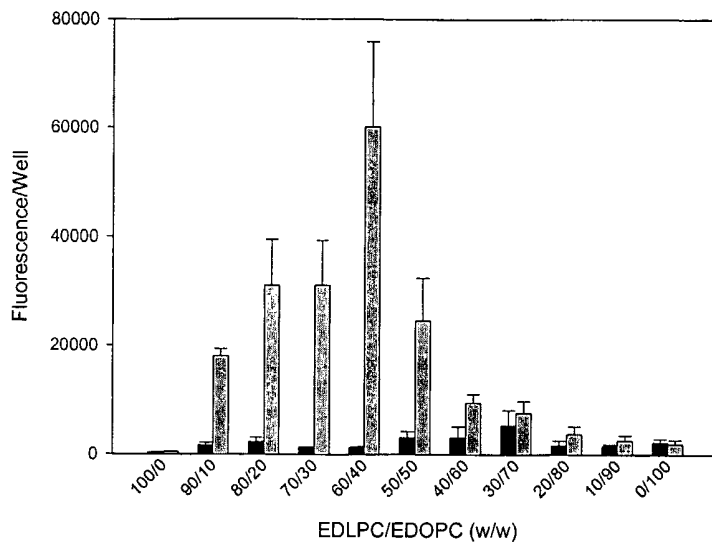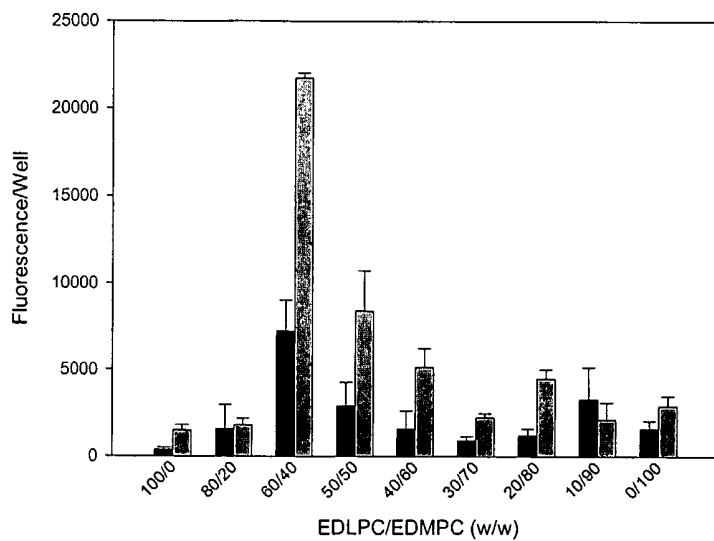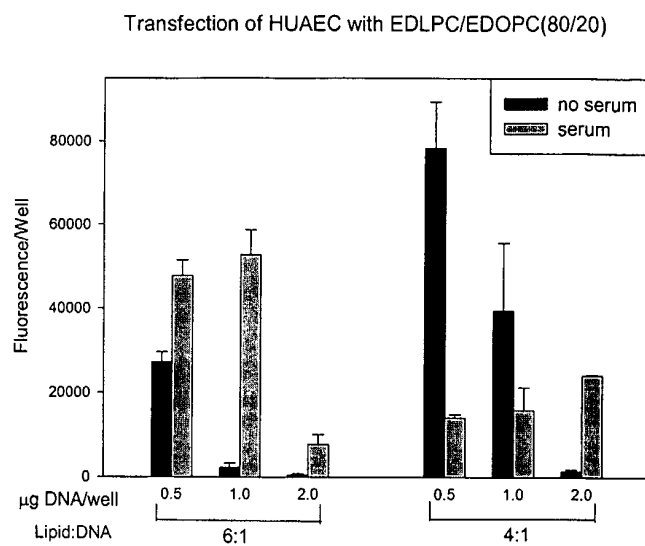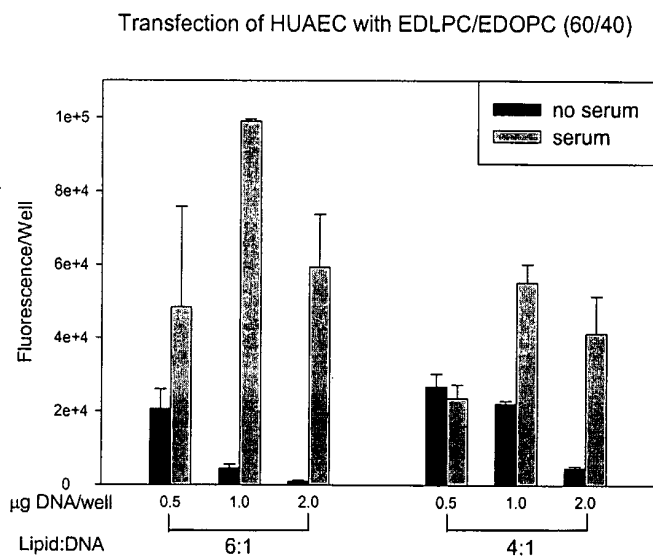
FIGURE 3

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FIGURE 4

BDOPC
FL-oligo

60/40/16.7
FL-oligo

FIGURE 5

EDLPC/EDOPC=60/40, Lipid/DNA=6/1

EDLPC/EDOPC=80/20, Lipid/DNA=4/1

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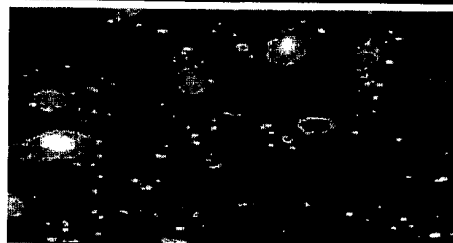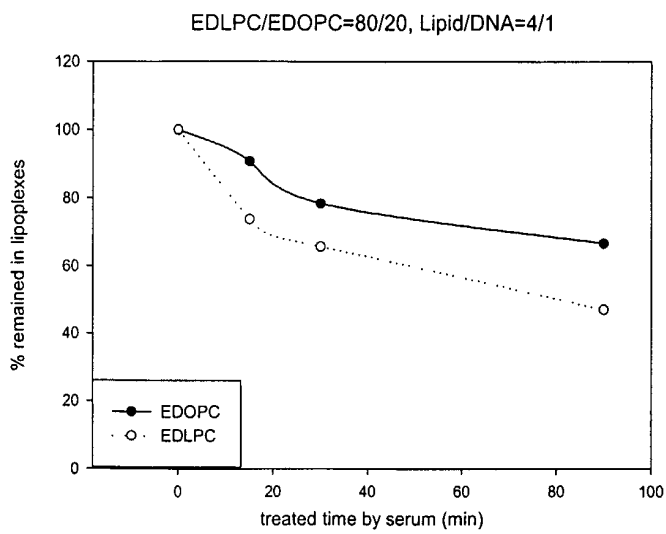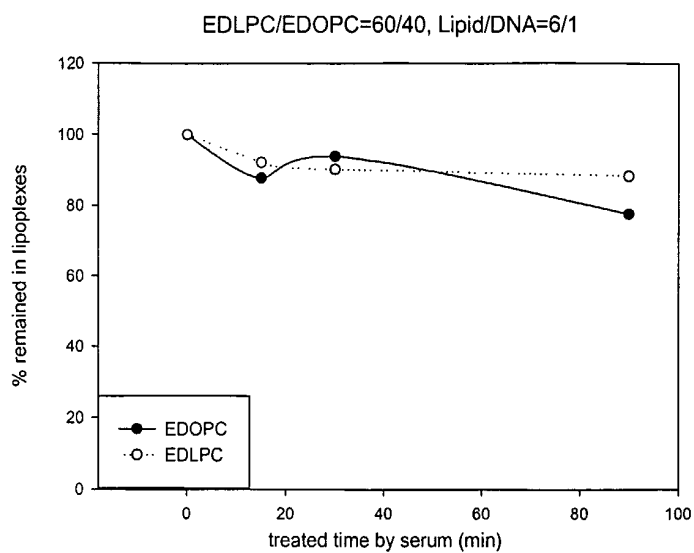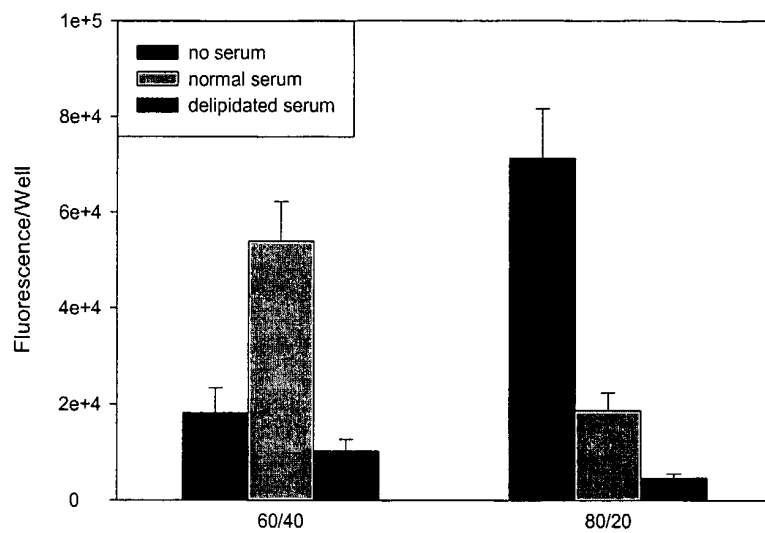
FIGURE 6

FIGURE 7

Transfection of HUAEC
with EDOPC/di-C14-dimethylammonium
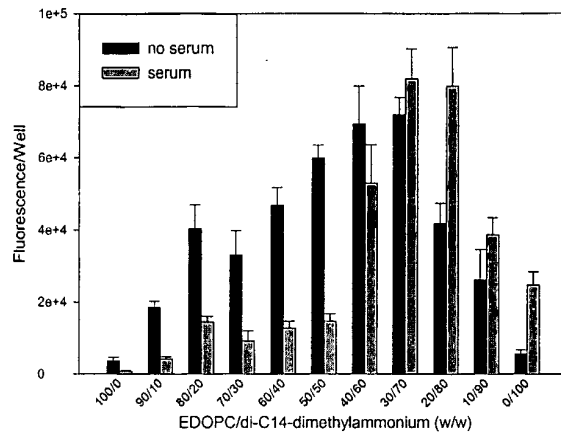
Transfection of HUAEC with
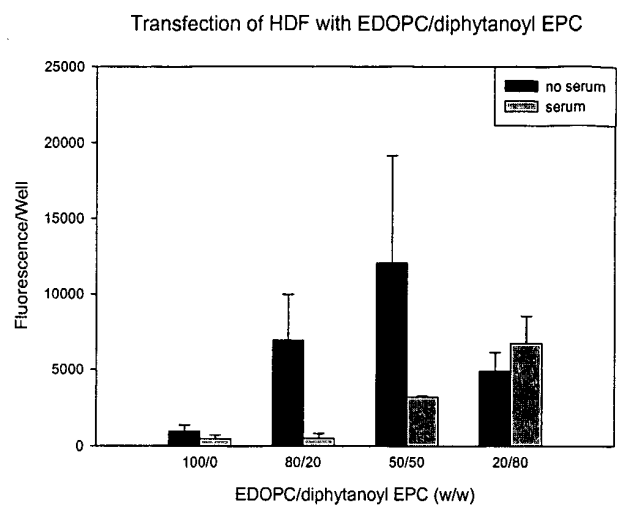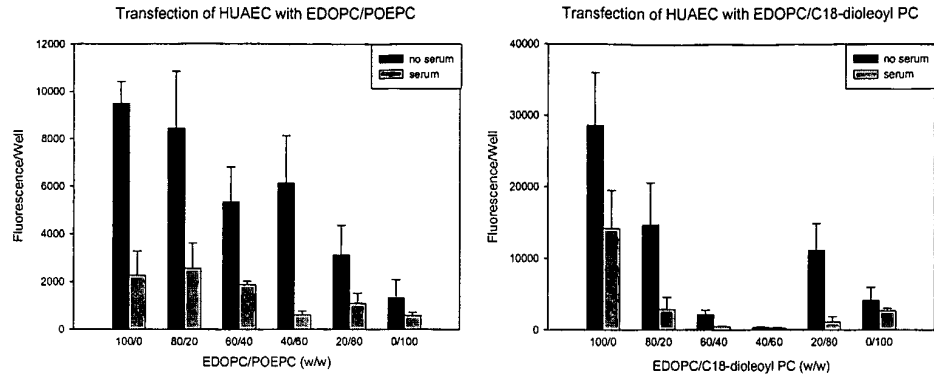di-C$_{18}$-dimethylammonium/di-C$_{14}$-dimethylammonium

FIGURE 8

Transfection of HDF with EDOPC/POEPC

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FIGURE 9

Transfection of HDF with EDOPC/diphytanoyl EPC

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FIGURE 10

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