

A LIPID DRUG DELIVERY SYSTEM FOR INCREASED ENDOSOMAL ESCAPE

Specification

The present invention relates to a drug delivery liposome formulation to increase escape from endosomes within cells and to improve drug delivery to cells and tissues as well as enhance the subsequent release of the delivered drug within such cells and tissues. The lipid bilayer of the liposome comprises an encapsulating agent, a fusogenic agent, and an acid-cleavable PEGylated lipid. The liposome encapsulates a therapeutic agent, such as a nucleic acid (RNA; DNA), a peptide, a protein, a small molecule, or a non-lipinski molecule. The lipid bilayer may comprise further helper lipids for enhancing endosomal escape or increasing system stability, such as cholesterol, solasodine, ceramide, or diosgenin.

Background of the invention

The biggest challenge for the delivery of macromolecular therapeutics "cargos" in general, such as mRNA, is to target them to the correct cells and, once endocytosed, let them cross the endosomal membrane in a process called "endosomal escape". Indeed, only a small fraction of exogenous macromolecules can escape from endosomes into the cytosol where they can exert their activity. As a consequence, the efficiency of cargo delivery and importantly the desired cargo effect is usually quite low.

In recent years, several types of RNA have emerged as potentially powerful therapeutics to inhibit gene expression, e.g. siRNA, to express proteins, e.g. mRNA, or for gene editing (e.g. CRISPR/Cas9 system).

The plasma membrane of cells is a biological barrier that prevents access of macromolecules, especially RNAs, into the cell's interior as cell defense mechanism. Consequently, numerous delivery systems have focused on facilitating the transport of molecular cargos across this membrane.

In particular, eukaryotic cells internalize extracellularly administered cargos by endocytosis. This can be mediated by specific cell surface interactions, or simply involve non-specific engulfment of molecules present in the extracellular fluid. Upon internalization, cargos are sequestered within membrane-bound endosomes. Through fusion and fission events, endosomes exchange some of their luminal content, and cargos can subsequently distribute throughout a complex endosomal pathway that includes early endosomes, recycling endosomes, multivesicular bodies, late endosomes, and lysosomes. Cargos trapped in this pathway may either be recycled to the cell surface or undergo degradation by exposure to lysosomal enzymes. Notably, while conceptually in the cell, endosomally-trapped cargos do not have access to the cytosol, i.e. the space between cytoplasmic organelles such as the nucleus. Thus, delivery systems that simply promote endocytosis of cargos but not the crossing of the endosomal membranes are not sufficient to permit successful cytosolic penetration. Instead, cargos have to cross

the membrane of endosomes. Therefore, delivery remains a membrane translocation challenge and some of the issues previously described in the context of crossing the plasma membrane apply. Yet, the endosomal pathway includes membrane systems that are different from the plasma membrane. Consequently, the mechanisms by which translocation is mediated, the efficiencies with which this is achieved, and the cellular responses that accompany delivery may all be distinct. The idea of promoting endosomal escape so as to increase cytosolic delivery has been pursued for several decades.

The ability to cross the endosomal membrane for successful delivery into the cytosol of target cells is a major challenge also for RNA-based therapeutics. It has been shown that only 1 % or less of endocytosed RNA therapeutics escape the endosome. Therefore, it is very difficult to experimentally investigate where and how this small quantity of RNA escapes from endosomes. Previous research tried to solve the problem posed by low endosomal escape by administering therapeutic RNA at higher doses, in order to achieve the desired effect; however, this causes side effects such as cytotoxicity and innate immune system activation.

A major step forward was the development of lipid nanoparticles (LNP) to deliver siRNA and mRNA. LNPs are not liposomes because they do not have a lipid bilayer only but have an amorphous lipid core matrix, and thus are not designed to fuse with cellular membranes as membrane vesicles and organelles can fuse. However, also this delivery vehicle suffers from the problem of low escape efficacy and cytotoxicity.

Therefore, there is still a need for new and efficient delivery systems to specifically increase crossing of endosomal membranes by macromolecule drugs.

It is the objective of the present invention to provide a liposomal drug delivery system for therapeutic agents inducing an increased endosomal escape, and thus an increased delivery of the therapeutic agent in the cell cytosol.

The objective of the present invention is solved by the teaching of the independent claims. Further advantageous features, aspects and details of the invention are evident from the dependent claims, the description, the figures, and the examples of the present application.

Summary of the invention

The present invention relates to a drug delivery liposome formulation to increase escape from endosomes within cells and to improve drug delivery to cells and tissues as well as enhance the subsequent release of the delivered drug within such cells and tissues. The lipid bilayer of the liposome comprises an encapsulating agent, a fusogenic agent, and an acid-cleavable PEGylated lipid. The liposome encapsulates a therapeutic agent, such as a nucleic acid (RNA; DNA), a peptide, a protein, a small

molecule, a non-lipinski molecule or another type of molecule. The lipid bilayer may further comprise helper lipids enhancing endosomal escape or increasing system stability, such as cholesterol, solasodine, ceramide, or diosgenin.

The inventors have surprisingly found that, when transfecting Hela cells with siRNA against eGFP delivered by a liposome composition, the combination of a fusogenic agent such as LBPA with an acid-cleavable PEG-lipid in said composition shows an unexpected synergetic effect on eGFP downregulation (Figure 3c). Indeed, the eGFP downregulation reached by liposomes comprising both components amount to much more than the sum of the effects reached by the liposomes comprising only one of the two components.

This effect is even more surprising as introduction of LBPA as fusogenic agent in MC3-based lipid nanoparticles (LNP) does not show a comparable improved effect on drug delivery and activity, such as siRNA mediated GFP downregulation. It is well known that lipid nanoparticles differ from liposomes for having an amorphous core structure made up of lipids, whereas liposomes in contrast have an aqueous core surrounded by one or multiple lipid bilayers.

Moreover, this effect even more surprisingly when considering that prior art (Matsuo et al., 2004, *Role of LBPA and Alix in multivesicular liposome formation and endosome organization*. Science, New Series, 303, 5657, 531-534) shows that LBPA drives formation of membrane invaginations within acidic liposomes, thus stimulating MVL formation, and controls inward fission process by transient interaction between LBPA membranes and Alix.

Remarkably, a similar effect to using LBPA as fusogenic agent can be obtained by using the compounds MGDG, PLPE (**Figure 12 f-j**), IsostA, GMO, POPE (eGFP- knock down efficiency by siRNA-liposomes, **Figure 9b**), NPPE, MO, OIA, PalmA (Table 11), and with the isoforms Hemi-LBPA, 3,3'-R,R, 2,2'-S,S-C18:0, 2,2'-S,S-C12:0, 3,3'-S,S, and 3,3'-R,S (liposome-siRNA-mediated GFP downregulation **Figure 7d**).

Therefore, the present invention in one embodiment provides a drug delivery system comprising a liposome having

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of at least one encapsulating agent;
 - ii) between 1 and 20 mol percent of an acid-cleavable polyethylene glycol conjugated lipid;
 - iii) between 15 and 45 mol percent of at least one fusogenic agent, and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume,

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm as determined by dynamic light scattering.

In an aspect the invention provides a composition comprising (a) a therapeutic agent or a pharmaceutically acceptable salt thereof; (b) a PEG-monoorthoester-lipid; (c) an amphiphilic lipid; (d) a cationic lipid and/or a beta-alanyl-prolyl-cysteine methyl ester; and optionally (e) a steroid and/or a ceramide and/or DOPE.

In another aspect the invention provides a pharmaceutical composition comprising the composition of the invention.

In another aspect the invention provides a method of producing the composition according to the invention, or the pharmaceutical composition according to the invention, comprising the steps of: (i) combining and mixing a composition comprising the components (b)-(d) and optionally (e) as defined herein; and (ii) mixing the composition obtained in step (i) with the therapeutic agent or with the pharmaceutically acceptable salt thereof (a).

In another aspect the invention relates to the composition of the invention or the pharmaceutical composition of the invention for use in the treatment or prophylaxis of a disease.

In another aspect the invention provides method of treatment or prophylaxis in a subject, comprising administering to the subject the composition according to the invention or the pharmaceutical composition according to the invention in a therapeutically effective amount.

In another aspect the invention relates to the composition according to the invention or the pharmaceutical composition according to the invention for use in the manufacture of a medicament.

In another aspect the invention provides a kit of parts comprising (i) the composition according to the invention or the pharmaceutical composition according to the invention; and (ii) an instruction manual.

In another aspect the invention provides a composition comprising an artificial nucleic acid molecule, wherein the artificial nucleic acid molecule is an mRNA molecule having at least one open reading frame encoding an antigen derived from a pathogen, a therapeutic protein, or a CRISPR/CAS9 ribonucleoprotein, for use as a medicament, for use as a vaccine or for use in gene therapy, wherein the artificial nucleic acid molecule is associated with or complexed with components (b)-(d) and optionally (e) as defined herein, and wherein the composition is administered intramuscularly, intravenously, subcutaneously, intratumorally, orally, by inhalation or transdermally/topical.

In another aspect the invention provides a method of delivering an siRNA, an mRNA and/or protein into a target cell, comprising the step of (i) contacting the target cell with a composition as defined herein, wherein component (a) is said siRNA, mRNA and/or protein.

In another aspect the invention provides a method of transfecting a cell, wherein the cell is selected from the group consisting of epithelial cells (preferably epithelial cells from cervical cancer), macrophages, a hepatocytes and cardiomyocytes, the method comprising the step of contacting the cell with the composition of the invention.

Another preferred embodiment of the invention is directed to the drug delivery system as disclosed above, wherein the encapsulating agent is a cationic lipid selected from 1,2-dialkanyloxy-3-trialkylammonium propane halide, 1,2-dialkenyloxy-3-trialkylammonium propane halide, 1,2-dialkanoyloxy-3-trialkylammonium propane halide, 1,2-dialkenoyloxy-3-trialkylammonium propane halide, tetraalkylammonium halide, 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine, and/or a lipidated polypeptide PD740.

Another preferred embodiment of the invention is directed to the drug delivery system as disclosed above, wherein the encapsulating agent is a cationic lipid selected from 1,2-di-C₁₂-C₂₀ alkanyloxy-3-tri-C₁-C₆ alkylammonium propane halide, 1,2-di-C₁₂-C₂₀ alkenyloxy-3-tri-C₁-C₆ alkylammonium propane halide, 1,2-di-C₁₂-C₂₀ alkenoyloxy-3-tri-C₁-C₆ alkylammonium propane halide, di-C₁₂-C₂₀ alkyl di-C₁-C₆ alkyl ammonium halide, and/or a lipidated polypeptide PD740.

Another more preferred embodiment of the invention is directed to the drug delivery system as disclosed above, wherein the encapsulating agent is a cationic lipid selected from the group consisting of 1,2-didodecyloxy-3-trimethylammonium propane chloride, 1,2-ditridecyloxy-3-trimethylammonium propane chloride, 1,2-ditetradecyloxy-3-trimethylammonium propane chloride, 1,2-dipentadecyloxy-3-trimethylammonium propane chloride, 1,2-dihexadecyloxy-3-trimethylammonium propane chloride, 1,2-diheptadecyloxy-3-trimethylammonium propane chloride, 1,2-dioctadecyloxy-3-trimethylammonium propane chloride, 1,2-dinonadecyloxy-3-trimethylammonium propane chloride, 1,2-diicosyloxy-3-trimethylammonium propane chloride, 1,2-didodecenyloxy-3-trimethylammonium propane chloride, 1,2-ditridecenyloxy-3-trimethylammonium propane chloride, 1,2-ditetradecenyloxy-3-trimethylammonium propane chloride, 1,2-dipentadecenyloxy-3-trimethylammonium propane chloride, 1,2-dihexadecenyloxy-3-trimethylammonium propane chloride, 1,2-diheptadecenyloxy-3-trimethylammonium propane chloride, 1,2-dioctadecenyloxy-3-trimethylammonium propane chloride, 1,2-dinonadecenyloxy-3-trimethylammonium propane chloride, 1,2-diicosenyloxy-3-trimethylammonium propane chloride, 1,2-dilauroleyloxy-3-trimethylammonium propane chloride, 1,2-dimyristoleoyloxy-3-trimethylammonium propane chloride, 1,2-dipalmitoleoyloxy-3-trimethylammonium propane chloride, 1,2-dipetroseloyloxy-3-trimethylammonium propane chloride, 1,2-dipetroselaidoyloxy-3-trimethylammonium propane chloride, 1,2-dioleoyloxy-3-trimethylammonium propane chloride, 1,2-dielaidoyloxy-3-trimethylammonium propane chloride, 1,2-divaccenoyloxy-3-trimethylammonium propane chloride, 1,2-digadoleoyloxy-3-trimethylammonium propane chloride, dimethyldidodecylammonium bromide, dimethylditridecylammonium bromide, dimethylditetradecylammonium bromide, dimethyldipentadecylammonium bromide, dimethyldihexadecylammonium bromide, dimethyldiheptadecylammonium bromide, dimethyldioctadecylammonium bromide, dimethyldinonadecylammonium bromide, dimethyldiicosylammonium bromide, and/or a lipidated polypeptide PD740.

Another still more preferred embodiment of the invention is directed to the drug delivery system as disclosed above, wherein the encapsulating agent is a cationic lipid selected from the group consisting of 1,2-di-O-octadecenyl-3-trimethylammonium propane chloride, dimethyldioctadecylammonium bromide, 1,2-

dioleoyl-3-trimethylammonium propane chloride, and/or a lipidated polypeptide PD740. A further more preferred embodiment of the invention is directed to the drug delivery system as disclosed above, wherein the encapsulating agent is a cationic lipid 1,2-dioleoyl-3-trimethylammonium propane chloride and/or a lipidated polypeptide PD740.

A preferred embodiment of the invention is further directed to the drug delivery system as disclosed above, wherein the acid-cleavable polyethylene glycol conjugated lipid is an acid-cleavable polyethylene glycol conjugated with a C₁₂-C₂₀ alkyl, or with a C₁₂-C₂₀ acyl, or with a mono-C₁₂-C₂₀ acylglycerol, or with a di-C₁₂-C₂₀ acylglycerol.

A further preferred embodiment of the invention is directed to the drug delivery system as disclosed above, wherein the acid-cleavable polyethylene glycol conjugated lipid comprises an acid-cleavable linkage selected from the group comprising an orthoester linkage, a hydrazone linkage, an acetal linkage, a vinyl ether linkage, and an imine linkage, and preferably a 2-methyl-2-alkoxy-1,3-dioxane or a 2-alkoxy-1,3-dioxolane, or an hydrazone. A further more preferred embodiment of the invention is directed to the drug delivery system as disclosed above, wherein the acid-cleavable polyethylene glycol conjugated lipid is selected from the group consisting of: *N*-(2-methyl-2-dodecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-tridecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-tetradecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-pentadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-hexadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-heptadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-nonadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, and *N*-(2-methyl-2-icosyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol.

A further still more preferred embodiment of the invention is directed to the drug delivery system as disclosed above, wherein the acid-cleavable polyethylene glycol conjugated lipid comprises polyethylene glycol having a number average molar mass M_n comprised between 400 and 5000 Da (Dalton).

A further still more preferred embodiment of the invention is directed to the drug delivery system as disclosed above, wherein the acid-cleavable polyethylene glycol conjugated lipid is α -methoxy- ω -{*N*-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅.

A preferred embodiment of the invention provides a drug delivery system as disclosed above, wherein the fusogenic agent is a glycolipid, a phosphatidylethanolamine, a phosphatidylglycerol, a fatty acid, or a fatty acid ester.

A further preferred embodiment of the invention provides a drug delivery system as disclosed above, wherein the fusogenic agent is a galactolipid, preferably an unsaturated monogalactosyldiacylglycerol.

A more preferred embodiment of the invention provides a drug delivery system as disclosed above, wherein the fusogenic agent is a 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine or an *N*-acyl-1,2-diacyl-*sn*-glycero-3-phosphoethanolamine.

A still more preferred embodiment of the invention provides a drug delivery system as disclosed above, wherein the fusogenic agent is a phosphatidylethanolamine selected from the group consisting of 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-

dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dimargaroyl-*sn*-glycero-3-phosphoethanolamine, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-didocosaheptaenoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dierucoyl-*sn*-glycero-3-phosphoethanolamine, 1-pentadecanoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-docosaheptaenoyl-*sn*-glycero-3-phosphoethanolamine, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine, 1-stearoyl-2-docosaheptaenoyl-*sn*-glycero-3-phosphoethanolamine, and *N*-palmitoyl-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine.

A further more preferred embodiment of the invention provides a drug delivery system as disclosed above, wherein the fusogenic agent is a phosphatidylglycerol selected from the group consisting of bis(monoacylglycerol)phosphate, 3-acylglycero-1-phospho-3'-(1',2'-diacyl)-glycerol, 3-acylglycero-1-phospho-glycerol, and 1,2-diacylglycero-3-phospho-glycerol.

A particular more preferred embodiment of the invention provides a drug delivery system as disclosed above, wherein the fusogenic agent is a bis(monoacylglycerol)phosphate selected from the group consisting of 2,2'-*S,S*-bis(monoacylglycerol)phosphate, 3,3'-*S,S*-bis(monoacylglycerol)phosphate, 3,3'-*R,R*-bis(monoacylglycerol)phosphate, and 3,3'-*R,S*-bis(monoacylglycerol)phosphate.

A bis(monoacylglycerol)phosphate may also be selected from 2,2'-*R,R*-bis(monoacylglycerol)phosphate and 2,2'-*R,S*-bis(monoacylglycerol)phosphate.

A particular still more preferred embodiment of the invention provides a drug delivery system as disclosed above, wherein the fusogenic agent is a bis(monoacylglycerol)phosphate selected from the group consisting of bis(monododecylglycerol)phosphate, bis(monotridecylglycerol)phosphate, bis(monotetradecylglycerol)phosphate, bis(monopentadecylglycerol)phosphate, bis(monohexadecylglycerol)phosphate, bis(monooctadecylglycerol)phosphate, bis(monononadecylglycerol)phosphate, bis(monoicosylglycerol)phosphate, bis(monolauroleoylglycerol)phosphate, bis(monomyristoleoylglycerol)phosphate, bis(monopalmitoleoylglycerol)phosphate, bis(monopetroseloylglycerol)phosphate, bis(monopetroselaidoylglycerol)phosphate, bis(monooleoylglycerol)phosphate, bis(monoelaidoylglycerol)phosphate, bis(monovaccenoylglycerol)phosphate, and bis(monogadoleoylglycerol)phosphate.

A further embodiment of the invention provides a drug delivery system as disclosed above, wherein the fusogenic agent is a 3-acylglycero-1-phospho-3'-(1',2'-diacyl)-glycerol selected from the group consisting of 3-dodecylglycero-1-phospho-3'-(1',2'-didodecyl)-glycerol, 3-tridecylglycero-1-phospho-3'-(1',2'-ditridecyl)-glycerol, 3-tetradecylglycero-1-phospho-3'-(1',2'-ditetradecyl)-glycerol, 3-pentadecylglycero-1-phospho-3'-(1',2'-dipentadecyl)-glycerol, 3-hexadecylglycero-1-phospho-3'-(1',2'-

dihexadecyl)-glycerol, 3-heptadecylglycero-1-phospho-3'-(1',2'-diheptadecyl)-glycerol, 3-octadecylglycero-1-phospho-3'-(1',2'-dioctadecyl)-glycerol, 3-nonadecylglycero-1-phospho-3'-(1',2'-dinonadecyl)-glycerol, 3-icosylglycero-1-phospho-3'-(1',2'-diicosyl)-glycerol, 3-lauroleoylglycero-1-phospho-3'-(1',2'-dilauroleoyl)-glycerol, 3-myristoleoylglycero-1-phospho-3'-(1',2'-dimyristoleoyl)-glycerol, 3-palmitoleoylglycero-1-phospho-3'-(1',2'-dpalmitoleoyl)-glycerol, 3-petroseloylglycero-1-phospho-3'-(1',2'-dipetroseloyl)-glycerol, 3-petroselaidoylglycero-1-phospho-3'-(1',2'-dipetroselaidoyl)-glycerol, 3-oleoylglycero-1-phospho-3'-(1',2'-dioleoyl)-glycerol, 3-elaidoylglycero-1-phospho-3'-(1',2'-dielaidoyl)-glycerol, 3-vaccenoylglycero-1-phospho-3'-(1',2'-divaccenoyl)-glycerol, and 3-gadoleoylglycero-1-phospho-3'-(1',2'-digadoleoyl)-glycerol. A further preferred

embodiment of the invention provides a drug delivery system as disclosed above, wherein the fusogenic agent is a 3-acylglycero-1-phospho-glycerol selected from the group consisting of 3-dodecylglycero-1-phospho-glycerol, 3-tridecylglycero-1-phospho-glycerol, 3-tetradecylglycero-1-phospho-glycerol, 3-pentadecylglycero-1-phospho-glycerol, 3-hexadecylglycero-1-phospho-glycerol, 3-heptadecylglycero-1-phospho-glycerol, 3-octadecylglycero-1-phospho-glycerol, 3-nonadecylglycero-1-phospho-glycerol, 3-icosylglycero-1-phospho-glycerol, 3-lauroleoylglycero-1-phospho-glycerol, 3-myristoleoylglycero-1-phospho-glycerol, 3-palmitoleoylglycero-1-phospho-glycerol, 3-petroseloylglycero-1-phospho-glycerol, 3-petroselaidoylglycero-1-phospho-glycerol, 3-oleoylglycero-1-phospho-glycerol, 3-elaidoylglycero-1-phospho-glycerol, 3-vaccenoylglycero-1-phospho-glycerol, and 3-gadoleoylglycero-1-phospho-glycerol. A further more preferred embodiment of the

invention provides a drug delivery system as disclosed above, wherein the fusogenic agent is a 1,2-diacylglycero-3-phospho-glycerol selected from the group consisting of 1,2-didodecylglycero-3-phospho-glycerol, 1,2-ditridecylglycero-3-phospho-glycerol, 1,2-ditetradecylglycero-3-phospho-glycerol, 1,2-dipentadecylglycero-3-phospho-glycerol, 1,2-dihexadecylglycero-3-phospho-glycerol, 1,2-diheptadecylglycero-3-phospho-glycerol, 1,2-dioctadecylglycero-3-phospho-glycerol, 1,2-dinonadecylglycero-3-phospho-glycerol, 1,2-diicosylglycero-3-phospho-glycerol, 1,2-dilauroleoylglycero-3-phospho-glycerol, 1,2-dimyristoleoylglycero-3-phospho-glycerol, 1,2-dipalmitoleoylglycero-3-phospho-glycerol, 1,2-dipetroseloylglycero-3-phospho-glycerol, 1,2-dipetroselaidoylglycero-3-phospho-glycerol, 1,2-dioleoylglycero-3-phospho-glycerol, 1,2-dielaidoylglycero-3-phospho-glycerol, 1,2-divaccenoylglycero-3-phospho-glycerol, and 1,2-digadoleoylglycero-3-phospho-glycerol. A further still more preferred embodiment of the

invention provides a drug delivery system as disclosed above, wherein the fusogenic agent is a fatty acid selected from the group consisting of myristic acid, pentadecanoic acid, palmitic acid, margaric acid, stearic acid, isostearic acid, nonadecylic acid, arachidic acid, myristoleic acid, palmitoleic acid, sapienic acid, oleic acid, elaidic acid, vaccenic acid, and linoleic acid; or a methyl ester or a glyceryl ester of one of the aforementioned fatty acids. A further still more preferred embodiment of the invention

provides a drug delivery system as disclosed above, wherein the fusogenic agent is selected from the

group consisting of monogalactosyldilinolenoylglycerol, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, *N*-palmitoyl-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, 2,2'-S,S-bis(monododecylglycerol)phosphate, 3,3'-S,S-bis(monododecylglycerol)phosphate, 3,3'-R,R-bis(monododecylglycerol)phosphate, 3,3'-R,S-bis(monododecylglycerol)phosphate, 2,2'-S,S-bis(monooctadecylglycerol)phosphate, 3,3'-S,S-bis(monooctadecylglycerol)phosphate, 3,3'-R,R-bis(monooctadecylglycerol)phosphate, 3,3'-R,S-bis(monooctadecylglycerol)phosphate, 2,2'-S,S-bis(monooleoylglycerol)phosphate, 3,3'-S,S-bis(monooleoylglycerol)phosphate, 3,3'-R,R-bis(monooleoylglycerol)phosphate, 3,3'-R,S-bis(monooleoylglycerol)phosphate, 3-dodecylglycero-1-phospho-3'-(1',2'-didodecyl)-glycerol, 3-octadecylglycero-1-phospho-3'-(1',2'-dioctadecyl)-glycerol, 3-oleoylglycero-1-phospho-3'-(1',2'-dioleoyl)-glycerol, glyceryl monoleate, methyl oleate, isostearic acid, palmitoleic acid, and oleic acid.

In another aspect, the present invention provides a drug delivery system as disclosed above, wherein the lipid bilayer comprises:

iv) between 2.5 and 35 mol percent of at least one neutral lipid.

In another preferred aspect, the present invention provides a drug delivery system as disclosed above, wherein the lipid bilayer comprises:

iv) between 2.5 and 35 mol percent of at least one neutral lipid selected from the group consisting of cholesterol, diosgenin, solasodine, and ceramide.

It is also herein provided a drug delivery system as disclosed above, wherein the therapeutic agent is a peptide, protein, an antibody, a nanobody, a nucleic acid, a ribonucleoprotein, a small molecule, a non-lipinski molecule, a biomimetic, or a natural compound. It is further herein provided a drug delivery system as disclosed above, wherein the therapeutic agent is a CRISPR/Cas9 ribonucleoprotein, a CRISPR guide RNA, or a Cas protein. A particular embodiment of the invention provides a drug delivery system as disclosed above, wherein the therapeutic agent is a nucleic acid selected from the group comprising ssDNA, dsDNA, ssRNA, dsRNA, aiRNA, miRNA, siRNA, piRNA, sdRNA, snRNA, snoRNA, PAR, tsRNA, endo-siRNA, MSY-RNA, tel-sRNA, crasiRNA, moRNA, xiRNA, lncRNA, ncRNA, circRNA, mRNA, sgRNA, crRNA, tracr RNA, guide RNA mix, self-amplifying RNA, ribozymes, and antisense oligonucleotides.

In a preferred embodiment, the composition disclosed herein comprises

as component (b) α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;

as component (c) 2,2'-S,S-bis(monooleoylglycerol)phosphate or 3,3'-S,S-bis(monooleoylglycerol)phosphate;

- as component (d) 1,2-dioleoyl-3-trimethylammonium propane chloride and/or PD740; and
- as component (e) cholesterol and/or solasodine.

In a preferred embodiment, the composition disclosed herein comprises

- as component (b) α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- as component (c) 2,2'-S,S-bis(monooleoylglycerol)phosphate or 3,3'-S,S-bis(monooleoylglycerol)phosphate;
- as component (d) 1,2-dioleoyl-3-trimethylammonium propane chloride; and
- as component (e) cholesterol.

In a preferred embodiment, the composition disclosed herein comprises

- as component (b) α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- as component (c) 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate; and as component (d) 1,2-dioleoyl-3-trimethylammonium propane chloride.

In a preferred embodiment, the composition disclosed herein comprises

- as component (b) between 1 and 20 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅, wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

A preferred embodiment of the invention provides a drug delivery system as disclosed above, wherein the lipid bilayer comprises

- i) between 30 and 75 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride and/or PD740,
- ii) between 1 and 20 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
- iii) between 15 and 45 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate, and
- iv) between 13 and 35 mol percent of cholesterol.

In one embodiment the composition of the invention comprises

- (b) between 1 and 20 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- (c) between 15 and 45 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate;
- (d) between 30 and 75 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride and/or PD740; and
- (e) between 13 and 35 mol percent of cholesterol;

wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

Another embodiment of the invention provides a drug delivery system as disclosed above, wherein the lipid bilayer comprises

- i) 50 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride,
- ii) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
- iii) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate, and
- iv) 25 mol percent of cholesterol.

In a preferred embodiment the composition of the invention comprises

- (b) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- (c) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate;
- (d) 50 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride; and
- (e) 25 mol percent of cholesterol,

wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

In a preferred embodiment the composition of the invention comprises

- (b) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- (c) 45 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate; and
- (d) 50 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride,

wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

Another preferred embodiment of the invention provides a drug delivery system as disclosed above, wherein the lipid bilayer comprises

- i) 50 mol percent of PD740,
- ii) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
- iii) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate, and
- iv) 25 mol percent of cholesterol.

In one embodiment the composition of the invention comprises

- (b) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- (c) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate;
- (d) 50 mol percent of PD740; and

- (e) 25 mol percent of cholesterol;

wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

A further preferred embodiment of the invention provides a drug delivery system as disclosed above, wherein the lipid bilayer comprises

- ia) 25 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride,
- ib) 25 mol percent of PD740,
- ii) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
- iii) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate, and
- iv) 25 mol percent of cholesterol.

In one embodiment the composition of the invention comprises

- (b) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- (c) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate;
- (d1) 25 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride;
- (d2) 25 mol percent of PD740; and
- (e) 25 mol percent of cholesterol;

wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

A further more preferred embodiment of the invention provides a drug delivery system as disclosed above, wherein the lipid bilayer comprises

- i) 33 mol percent of PD740,
- ii) 7 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
- iii) 27 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate, and
- iv) 33 mol percent of cholesterol.

In one embodiment the composition of the invention comprises

- (b) 7 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- (c) 27 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate;
- (d) 33 mol percent of PD740; and
- (e) 33 mol percent of cholesterol;

wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

An alternative embodiment of the invention provides a drug delivery system as disclosed above, wherein the lipid bilayer comprises

- i) 42 to 50 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride,
- ii) 4 to 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
- iii) 4 to 15 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate,
- iva) 13 to 15 mol percent of cholesterol, and
- ivb) 15 to 36 mol percent of solasodine.

In one embodiment the composition of the invention comprises

- (b) between 4 and 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- (c) between 4 and 15 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate;
- (d) between 42 and 50 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride;
- (e1) between 13 and 15 mol percent of cholesterol; and
- (e2) between 15 and 36 mol percent of solasodine;

wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

In one embodiment the composition of the invention comprises

- (b) α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- (c) monogalactosyldiacylglycerol or 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine;
- (d) 1,2-dioleoyl-3-trimethylammonium propane chloride and PD740; and
- (e) cholesterol.

A further alternative embodiment of the invention provides a drug delivery system as disclosed above, wherein the lipid bilayer comprises

- ia) 25 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride,
- ib) 25 mol percent of PD740,
- ii) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
- iii) 20 mol percent of monogalactosyldiacylglycerol or 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine, and
- iv) 25 mol percent of cholesterol.

In one embodiment the composition of the invention comprises

(b) 5 mol percent of α -methoxy- ω -[N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido]-polyethylene glycol₄₅;

(c) 20 mol percent of monogalactosyldiacylglycerol or 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine;

(d1) 25 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride;

(d2) 25 mol percent of PD740; and

(e) 25 mol percent of cholesterol,

wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

A particular embodiment of the present invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 1:1 to about 50:1. Another embodiment of the present invention relates to the composition as disclosed herein, wherein the N:P ratio is from about 1:1 to about 50:1.

Finally, another particular embodiment of the present invention provides a drug delivery system as disclosed above, wherein the liposome is a unilamellar and/or univesicular liposome.

Description of the figures:

Figure 1 Shows (a) schematic representation of endosomal escape induced by the drug delivery system disclosed herein; (b) Structure of labile, acid-cleavable PEG-lipid; (c) Structure of fusogenic lipid 2,2'-LBPA and isomerization to 3,3' isoform.

Figure 2 Shows in vitro characterization of the delivery system. (a) Liposomes of composition LPI visualized by cryo transmission electron microscopy (scale bar 100 nm). The image shows unilamellar, univesicular individual spherical vesicles composed of a single membrane surrounding an aqueous core with a diameter of 100 nm and less. Gold nanoparticles (10 nm) used for image alignment can be seen as electron-dense dots attached to some of the vesicles. (b) Extruded liposomes embedded in methyl cellulose and stained with sodium tungstate and uranyl acetate visualized by transmission electron microscopy (scale bar 100 nm). The image shows vesicles that are folded over or with a donut shape, as the spherical vesicles collapse during the embedding due to their aqueous core. Most of the vesicles are individual, while some are in contact with each other. Their size is between 50 and 300 nm along their longest axis. (c) Size distribution of extruded liposomes by dynamic light scattering (n = 6, mean $\pm$ SEM). (d) siRNA encapsulation in extruded liposomes, analyzed by Ribogreen assay (n = 12, mean $\pm$ SEM). (e) siRNA content before and after extrusion, analyzed by Ribogreen assay (n = 8 (without extrusion), 12 (with extrusion), mean $\pm$ SEM). (f,g) Agarose gel images and quantification of RNase A treatment kinetics of non-encapsulated (naked) siRNA (dark grey in (g)) and siRNA encapsulated in liposomes (light grey in (g)) (n = 3, mean $\pm$ SD). The image shows siRNA bands on the agarose gel for each timepoint. For siRNA in

liposomes the siRNA band is present for each timepoint, while for non-encapsulated siRNA the band gets fainter over time and is becoming not visible after 8 min. This shows that siRNA in liposomes is protected from RNase degradation. (h) Comparison of size distribution of extruded liposomes analyzed by dynamic light scattering directly after preparation and after 9 months, 1.6 years and 2.4 years storage at 4°C (n = 16 (fresh), n = 12 (9 months), n = 15 (1.6 years), n = 14 (2.4 years), mean ± SEM). (i) Comparison of siRNA encapsulation analyzed by Ribogreen assay directly after preparation and after 9 months, 1.6 and 2.4 years of storage at 4°C (n = 12 (fresh), n = 10 (9 months), n = 10 (1.6 years), n = 14 (2.4 years), mean ± SEM). (j) Comparison of siRNA amount after 2.4 years of storage at 4°C, expressed as percentage of the siRNA amount measured directly after liposome formation (n = 8, mean ± SEM). (k) Comparison of siRNA encapsulation analyzed by Ribogreen assay between liposomes made with siRNA at N/P ratio 20:1 and 5:1 (n=1, mean± SD). This example shows that the delivery system of the composition of this invention can be formulated as liposomal, unilamellar, nanoscale sized, encapsulates RNA to a high degree and protects the RNA from RNase degradation and the example shows that the in vitro parameters of the formulation are stable over time.

Figure 3 Shows anti eGFP siRNA uptake and eGFP downregulation in HeLa eGFP cells. (a) HeLa cells control (left column) and after 4 h uptake of liposomes with 10 nM Alexa 647-labeled siRNA (right) (scale bar 25 µm). A vesicular pattern of Alexa 647 labeled siRNA can be detected in the treated cells, but not in the untreated ones (bottom row). The vesicular pattern shows that liposomal siRNA is taken up by endocytosis. Position of cells is visualized by Hoechst/Cell Mask Blue staining (top row). (b) Downregulation of eGFP and toxicity (nuclei number) induced by siRNA encapsulated in liposomes compared to naked, non-encapsulated siRNA, both at 10 nM, in HeLa cells. Mean eGFP expression intensity and number of nuclei are expressed as percentage of untreated control (n = 6, mean ± SEM). (c) Comparison of eGFP downregulation by siRNA liposomes containing the two active components, LBPA and labile PEG lipid, to that by liposomes containing one active component and one non-active replacing component (n = 6, mean ± SEM, siRNA conc. = 10 nM). (d) eGFP downregulation in HeLa eGFP cells treated with different concentrations of siRNA in extruded liposomes for 5 h in the presence of serum (72 h after transfection, scale bar 100 µm). Decreased amount of GFP fluorescent signal can be detected with increasing amount of siRNA concentration (left column). The untreated cells are uniformly positive for GFP, while only a few positive cells can be detected in cells treated with 2.5 nM and most of the cells show no GFP signal when treated with 10 nM. This dose dependent reduction of protein levels corresponds to downregulation of GFP. The position of cells is indicated by DAPI staining (right column). Scalebar 100 µm. (e) Single cell distribution of eGFP intensity

normalized to median of untreated control after treatment with different concentrations of siRNA in extruded liposomes ($n = 1$, mean $\pm$ SD). (f) Comparison of eGFP downregulation mediated by siRNA-liposomes containing different LBPA isomers ($n = 36-53$ (2,2'-LBPA), 11-17 (3,3'-LBPA), mean $\pm$ SEM). (g) Comparison of toxicity by siRNA-liposomes containing different LBPA isomers ($n = 36-53$ (2,2'-LBPA), 11-17 (3,3'-LBPA), mean $\pm$ SEM). (h) Dose response curve of eGFP downregulation and toxicity ($n = 4$, line: fit with four-parameter log-logistic model, shading: 95% confidence interval). (i) Reproducibility of eGFP downregulation liposomes comprising 2,2'-LBPA ($n = 36-53$). (j) Reproducibility of toxicity by siRNA-liposomes comprising 2,2'-LBPA ($n = 36-53$). (k) Comparison of eGFP downregulation by siRNA-liposomes directly after their preparation and after 9 months, 1.6 years and 2.4 years of storage at 4°C ($n = 10$ (fresh), 11 (9 months), 14 (1.6 years), and 14 (2.4 years), mean $\pm$ SD); (l) Dose-response curve for eGFP downregulation and toxicity (nuclei number) in Hela cells by siRNA-liposomes used fresh directly after preparation or after 5 h of serum pre-incubation ($n = 3$, mean $\pm$ SD). This example shows that this exemplary composition of the invention is effective for siRNA delivery at nanomolar concentrations in presence of serum with low toxicity. The knockdown efficiency is reproducible and stable over time.

Figure 4 Shows uptake and eGFP downregulation in primary bone marrow-derived macrophages and hepatocytes from LifeAct-GFP mice. (a) Primary bone marrow-derived mouse macrophages after 6 h uptake of Alexa 647-labeled siRNA-liposomes (left column) and untreated control (right column) (scale bar 25 μ m, $c = 5$ nM). A vesicular pattern of Alexa 647 labeled siRNA can be detected in the treated cells, but not in the untreated ones (bottom row), indicating uptake by endocytosis. Presence of cells is indicated by DAPI staining (top row). (b) eGFP downregulation in primary macrophages treated with different concentrations of anti eGFP siRNA-liposomes for 5 h in the presence of serum (72 h after transfection, scale bar 100 μ m). Decrease of GFP signal can be seen with increased concentrations of siRNA (left column). Untreated cells are uniformly positive for GFP, while less cells are positive for GFP when treated with increasing siRNA concentration and most cells are negative when treated with 20 nM. This demonstrates dose dependent eGFP downregulation. The position of cells is indicated by DAPI staining (right column). (c) Quantification of eGFP downregulation and toxicity in primary macrophages ($n = 9$, mean $\pm$ SEM). (d) Dose response curve of eGFP downregulation and toxicity in primary macrophages ($n = 4$, line: fit with four-parameter log-logistic model, shading: 95% confidence interval). (e) Reproducibility of eGFP downregulation in primary macrophages ($n = 37-49$). (f) Reproducibility of toxicity in primary macrophages ($n = 37-49$). (g) eGFP downregulation in primary hepatocytes treated with different concentrations of anti eGFP siRNA-liposomes for 5 h in the presence of serum (72 h after transfection, scale bar 100 μ m). Decrease of eGFP

signal can be detected with increased concentrations of siRNA (left column). Cells are uniformly positive for eGFP in the untreated condition, while with increasing siRNA concentration less cells are positive. At 5 nM and above most treated cells are negative for eGFP. This corresponds to dose dependent eGFP downregulation. The position of cells is indicated by DAPI staining (right column). (h) Quantification of eGFP downregulation and toxicity in primary hepatocytes ($n = 2$, mean $\pm$ SEM) treated with different concentrations of siRNA-liposomes. This example shows that the aforementioned exemplary composition of the invention is effective for the delivery of siRNA into primary cells at nanomolar concentrations.

Figure 5 Shows in vitro characterization of liposomes with mRNA and delivery of mRNA in HeLa cells, primary macrophages and hepatocytes. (a) Encapsulation efficiency of mRNA in liposomes at charge ratio 6:1, analyzed by Ribogreen assay ($n = 9$, mean $\pm$ SEM). (b) Conventional transmission electron microscopy of liposomes with mRNA (scale bar 100 nm). The image shows spherical vesicles with a diameter of 100 nm and below. (c) Size distribution of liposomes analyzed by dynamic light scattering before and after addition of mRNA at charge ratio 6:1 ($n = 2$, mean $\pm$ SD). (d) HeLa cells treated with different concentrations of eGFP mRNA-liposomes at charge ratio 6:1 in the presence of serum for 24 h (scale bar 100 μ m). Number of cells expressing GFP and GFP fluorescence intensity increases with increasing mRNA dose treatment. Untreated cells show no GFP fluorescence, few cells are positive when treated with a 0.16 μ g/ml dose with low GFP intensity, more cells are positive and the fluorescence intensity is increased when treated with 0.31 μ g/ml and most cells are positive for GFP with high signal intensity for a treatment with 0.63 μ g/ml, showing dose dependent GFP expression. GFP fluorescence (left column) and cellular location by DAPI staining (right column). (e) Quantification of eGFP expression and toxicity in the HeLa cells treated with eGFP mRNA-liposomes at charge ratio 6:1, shown in (d) ($n = 3$, mean $\pm$ SEM). (f) Quantification of eGFP expression and toxicity in HeLa cells at charge ratio 3:1 ($n = 2$, mean $\pm$ SEM). (g) Quantification of eGFP expression by intensity in HeLa cells after treatment with liposomes of composition LP1 and mRNA mixed at different N/P ratios ($n = 1$, mean $\pm$ SD). (h) Quantification of percentage of HeLa cells positive for eGFP expression after treatment with liposomes of composition LP1 and mRNA mixed at different N/P ratios ($n = 1$, mean $\pm$ SD). (i) Quantification of toxicity by analyzing total cell number normalized to untreated control in HeLa cells after treatment with liposomes of composition LP1 and mRNA mixed at different N/P ratios ($n = 1$, mean $\pm$ SD). (j) Primary bone marrow-derived mouse macrophages treated with different concentrations of eGFP mRNA-liposomes at charge ratio 6:1 in the presence of serum for 24 h (scale bar 100 μ m). Both number of cells expressing eGFP and eGFP fluorescence intensity increases with increasing mRNA concentration (left column).

Untreated cells show no eGFP signal, treatment with 0.31 µg/ml results in few eGFP positive cells, a treatment with 0.63 µg/ml results in many cells positive with a higher signal intensity and for a treatment with 1.25 µg/ml most cells are positive with high signal intensity, showing dose dependent eGFP expression. Cellular location is visualized by DAPI staining (right column). (k) Quantification of eGFP expression and toxicity in primary mouse macrophages treated with eGFP mRNA-liposomes at charge ratio 6:1, shown in (j) ($n = 3$, mean $\pm$ SEM). (l) Quantification of eGFP expression and toxicity in primary mouse macrophages at charge ratio 3:1 ($n = 3$, mean $\pm$ SEM). (m) Primary hepatocytes treated with different concentrations of eGFP mRNA-liposomes at charge ratio 3:1 in the presence of serum for 24 h (scale bar 50 µm). Both number of cells expressing eGFP and eGFP fluorescence intensity increase with increasing mRNA dose treatment (left column). Untreated cells are negative for eGFP, when treated with 0.63 µg/ml a few cells show eGFP fluorescence, when treated with 1.25 µg/ml about half of the cells are positive for eGFP with intermediate and high intensity and for a treatment with 2.50 µg/ml about $\frac{3}{4}$ of the cells are positive for eGFP, mostly with high intensity. Cellular location is visualized by nuclear staining with DAPI (right column). (n) Quantification of eGFP expression in primary hepatocytes shown in (m) ($n = 1$, mean $\pm$ SEM). This example shows that the tested exemplary composition of the invention is effective for the delivery of mRNA into various cell types, including primary cells, inducing high protein expression in the majority of cells present. Different charge ratios N/P can be used. The example further shows that liposomes with mRNA are in a nanoscale size range and encapsulate RNA to a high degree.

Figure 6 Shows delivery of CRISPR Cas9 ribonucleoprotein complex (Cas9 RNP) and guide RNA in HeLa eGFP cells. (a) Downregulation of eGFP and toxicity of Cas9 RNP-liposomes in HeLa eGFP cells ($n = 3$, mean $\pm$ SEM). b) Comparison of downregulation efficiency of eGFP with Cas9 RNP in liposomes at charge ratio 9.10:1 (as in panel a) and 5.46:1 in HeLa eGFP cells ($n = 3$, mean $\pm$ SEM). (c) Comparison of toxicity by analyzing the number of nuclei compared to untreated control in HeLa eGFP cells treated with Cas9 RNP in liposomes at charge ratio 9.10:1 (as in panel a) and 5.46:1 ($n = 3$, mean $\pm$ SEM). (d) Comparison of different liposome compositions for the transfection of guide RNA mix against eGFP into HeLa cells previously transfected with BacMam Cas9; GFP intensity is normalized to untreated control. ($n=5$ (3,3'- and 2,2,'-LBPA), 3 (LP4), 1 (EPC), mean $\pm$ SEM). (e) Toxicity data for treatment of panel (b). ($n=5$ (3,3'- and 2,2,'-LBPA), 3 (LP4), 1 (EPC), mean $\pm$ SEM). (f) Comparison of downregulation efficiency of eGFP with guide RNA mix in liposomes at charge ratio 8.45:1 (as in panel c,d) and 5.07:1 in HeLa cells transfected with BacMan Cas9 ($n = 3$, mean $\pm$ SEM). (c) Comparison of toxicity by analyzing the number of nuclei compared to untreated control in HeLa cells expressing Cas9 after BacMam transfection treated

with guide RNA in liposomes at charge ratio 8.45:1 (as in panel c,d) and 5.07:1 ($n = 3$, mean $\pm$ SEM). This example shows that the tested composition of the invention is effective for delivery of ribonucleoprotein complexes, specifically CRISPR/Cas9 ribonucleoprotein complexes with guideRNA as well as for the delivery of guideRNA for CRISPR/Cas9 mediated knockout. Different charge ratios N/P and compositions of the invention can be used.

Figure 7 Shows exploration of suitable variations from the identified liposomal composition LP1. (a) Overview of different variations screened to optimize liposome formulation with anti eGFP siRNA as cargo in HeLa eGFP cells. (b) Identification of effective percentage ranges of the indicated components of the initial composition LP1 (black) with 10 nM siRNA in HeLa eGFP cells ($n=3$, mean $\pm$ SD). (c) Helper lipid variation in the basis formulation LP1 ($n=12$ (Solasodine), 1 (Ceramide), 2 (Diosgenin), 10 (LP1), mean $\pm$ SD). (d) Several isoforms of LBPA show a similar activity when replacing LBPA in LP1 formulation ($n=3$, mean $\pm$ SD). (e) Encapsulating agent variation in LP1 formulation. PD740 (LP2) shows increased activity compared to DOTAP (LP1) ($n=6$ (LP1), 3 (others), mean $\pm$ SD). (f) Test of formulations LP2, LP3, and LP4 containing PD740 to find optimal composition for eGFP knock down efficiency ($n= 17$ (LP1), 8 (LP2), 2 (LP3), 5 (LP4), mean $\pm$ SD). (g) Toxicity profiles of formulations from (f). (h, i) Dose response curves for eGFP intensity and nuclei number obtained using LP1-LP4 liposome formulations at 10 concentrations to extract EC50 and TC50 ($n = 6$ (LP1), 2 (LP2), 7 (LP3), 2 (LP4), mean $\pm$ SD). (j) Difference in eGFP intensity normalized to untreated control for HeLa eGFP cells treated with anti eGFP siRNA in liposomes at different N/P ratios compared to N/P ratio 20:1 ($n= 2$ (for N/P = 40:1, 20:1, 10:1), 1 (for N/P = 5:1), mean $\pm$ SD). (k) Difference in number of nuclei normalized to untreated control for HeLa eGFP cells treated with anti eGFP siRNA in liposomes at different N/P ratios compared to N/P ratio 20:1 ($n= 2$ (for N/P = 40:1, 20:1, 10:1), 1 (for N/P = 5:1), mean $\pm$ SD). This example shows different tested compositions and variations of compositions of the invention that are effective for siRNA delivery. Different compounds can be used as components (c), (d) and (e) of the composition, showing a comparable or increased effect for siRNA delivery in target cells. Different charge ratios N/P are effective for siRNA delivery using the compositions of the invention.

Figure 8 Shows screening of potential fusogenic agents. (a) Similar eGFP knock down in HeLa eGFP cells reached by replacing LBPA in formulation LP3 with MGDG or PLPE ($n= 7$ (LP3), 2 (PLPE), 3 (MGDG), mean $\pm$ SD, fit: 4 parameter logistic). (b) Formulation containing MGDG shows a lower toxicity profile than LP3 ($n= 7$ (LP3), 2 (PLPE), 3 (MGDG), mean $\pm$ SD, fit: 4 parameter logistic). (c) Effect of substituting LBPA with GMO, POPE or IsostA in LP3 composition on eGFP downregulation efficiency by anti eGFP siRNA-liposomes, comparison with formulation LP1 ($n= 6$ (LP1), 2 (GMO,

POPE), 3 (IsostA), mean $\pm$ SD, fit: 4 parameter logistic); (d) Toxicity profile for compositions from (c) (n= 6 (LP1), 2 (GMO, POPE), 3 (IsostA), mean $\pm$ SD, fit: 4 parameter logistic); (e, f) Compositions combining LBPA and fusogenic agents reach a similar knockdown efficiency and toxicity profile (n= 7 (LP3), 1 (all others), mean $\pm$ SD, fit: 4 parameter logistic). This example demonstrates that various compounds can serve as fusogenic agents or components (c) of the formulation of the invention, yielding similar or enhanced siRNA-mediated downregulation in cells compared to composition LP1. It also highlights that different compounds acting as cationic agents or components (d) are effective for siRNA-mediated downregulation when combined with various components (c).

Figure 9 Shows changed downregulation profiles in primary mouse macrophages in response to different liposome compositions. (a) Comparison of eGFP knock down efficiency of LP1 (classic) with compositions containing varying amount of PD740 (n= 5 (LP3), 4 (LP1), 1 (LP2, LP4), mean $\pm$ SD, fit: 4 parameter logistic). (b) Comparison of eGFP knock down efficiency of anti eGFP siRNA-liposomes with LP3 composition and compositions where LBPA has been replaced with other fusogenic agents (n= 5 (LP3), 2 (MGDG, IsostA), 1 (GMO, POPE), mean $\pm$ SD, fit: 4 parameter logistic). (c) Comparison of eGFP knockdown efficiency of siRNA-liposomes with LP3 compositions containing both LBPA and another fusogenic agent (n= 5 (LP3), 1 (others), mean $\pm$ SD, fit: 4 parameter logistic). This example shows that different compositions of the invention containing different compounds as fusogenic agents or component (c) and/or different compounds as cationic agents or component (d) of the composition of the invention are effective for siRNA mediated downregulation in primary mouse macrophages with the same or increased effect compared to composition LP1.

Figure 10 Shows (a) phenotypic comparison of siRNA-liposome treatment with treatment with the endosomal disruptive agent L-leucine methyl ester (LLOMe). HeLa cells were transfected with GFP-tagged galectin-8 by BacMam virus. Endosomal disruption is characterized by appearance of aggregated galectin-8 foci as seen for LLOMe treatment, which are not visible upon liposome treatment (scale bar 20 μ m). Untreated cells show a low background level of GFP fluorescence, cells treated with liposomes LP1 show the same background level as the untreated cells with a few cells exhibiting diffuse GFP fluorescence in cytosol and nuclei above background level (top row). Cell location is visualized by staining with DAPI and CMB (bottom row). (b) Phenotypic comparison of siRNA-liposome treatment with treatment with siRNA lipoplexes. HeLa cells were treated with A647-labeled siRNA complexed with lipofectamine 2000, resulting in endosomal bursting events (cells with release events are marked with arrows) (top row). Cells treated with A647-labeled siRNA in liposomes show a

vesicular pattern on the same timescale (bottom row), indicating that endosomes are not ruptured by this more gentle treatment. Background GFP fluorescence was used to visualize cellular position (scale bar 10 μ m), shown in the left panel of both rows. A647-siRNA was imaged at 6.4 s intervals for 5 min in the same position, images at 13, 45 and 77 s timepoint are shown in middle and right panels. This example shows that treatment of cells with the compositions of the invention does not disrupt endosomes and is milder than treatment with lipofectamine-based siRNA lipoplexes.

Figure 11 (a) Screening in HeLa eGFP cells of structural analogues of E18 to identify potential endosomal escape enhancers. Cells were treated with both chol-siRNA (200 nM) and the indicated compounds (6 μ M (treatments 1-24) and 7 μ M (treatments 25-32)). Downregulation of eGFP and nuclei number were quantified as percentage of untreated control. (n=2 (treatments 1-24), 1 (treatments 25-32), mean $\pm$ SEM). (b) Dose response curves of hits from screening showed in (a). HeLa cells were treated with varying concentrations of the indicated compounds in the presence of chol-siRNA (150 nM). (n=3, mean $\pm$ SEM). This example shows the identification of enhancers of siRNA delivery.

Figure 12 Shows comparison of different liposomal compositions for mRNA transfection in HeLa cells. HeLa cells were treated for 24 h with liposomes of varying composition containing eGFP mRNA in the presence of serum: (a,b) Quantification of eGFP expression (percentage of positive cells (a) and fluorescence intensity (b)) for treatment with mRNA-liposomes containing endosomal escape enhancer solasodine at two different concentrations. (n = 3, mean $\pm$ SEM). (c,d) Quantification of eGFP expression (percentage of positive cells (c) and fluorescence intensity (d)) for treatment with mRNA-liposomes with formulations LP3 and LP4 containing PD740 compared to LP1. (n = 2, mean $\pm$ SEM). (e) Quantification of toxicity of mRNA-liposome formulations shown in (c) and (d). (n = 2, mean $\pm$ SEM). (f, g, i, j) Quantification of eGFP expression (percentage of positive cells (f, i) and fluorescence intensity (g, j)) for the treatment with mRNA-liposomes LP-MGDG or LP-PLPE that comprise both PD740 as additional encapsulating agent and the alternative fusogenic agents MGDG or PLPE, respectively (n = 2 (f, g), mean $\pm$ SEM, n = 1 (i, j), mean $\pm$ SD), in comparison to LP1 and LP3. (h) Quantification of toxicity of mRNA-liposome formulations showed in (f, g). (n = 2, mean $\pm$ SEM). This example shows that different compositions of the invention containing different compounds as fusogenic agents or compounds (c) of the composition of the invention are effective for mRNA delivery with the same or increased effect compared to composition LP1.

Figure 13 Shows semi-automated production of liposomes with NanoAssemblr microfluidic system. (a) Liposomes of composition LP1 produced by NanoAssemblr showed same transfection efficiency for mRNA in HeLa cells in terms of percentage of cells

expressing eGFP as liposomes produced by extrusion ($n = 1$, mean $\pm$ SD). (b) Liposomes of composition LP1 produced by NanoAssemblr showed similar level of eGFP expression with mRNA in HeLa cells in terms of GFP intensity as liposomes produced by extrusion. ($n = 1$, mean $\pm$ SD). (c) Size distribution by DLS of liposomes of composition LP1 produced by NanoAssemblr compared to liposomes produced by extrusion. ($n = 1$, mean). This example shows that liposomes of the composition of the invention can be produced with microfluidic processes, which makes it possible to reach smaller average size without impacting mRNA delivery efficiency.

Figure 14 Shows interchangeability of HeLa eGFP and HeLa dsGFP cell line for siRNA downregulation experiments and stability of liposomes towards freezing at -80°C . (a) Comparison of eGFP downregulation with extruded liposomes of composition LP1 in HeLa eGFP and HeLa dsGFP cells ($n = 7$ (HeLa dsGFP), 4 (HeLa eGFP), mean $\pm$ SD). (b) Comparison of toxicity of treatment with extruded liposomes of composition LP1 in HeLa eGFP and HeLa dsGFP cells ($n = 7$ (HeLa dsGFP), 4 (HeLa eGFP), mean $\pm$ SD). (c) Size distribution measured by DLS for liposomes stored in different buffers at -80°C and 4°C . ($n=1$, mean). (d) Comparison of siRNA mediated knock down efficiency in HeLa dsGFP cells in terms of GFP intensity normalized to untreated control using liposomes stored at -80°C and 4°C . ($n=1$, mean $\pm$ SD). (e) Comparison of toxicity of siRNA containing liposomes stored at -80°C and 4°C for treatment of HeLa dsGFP cells. ($n=1$, mean $\pm$ SD). This example shows that liposomes of the composition of the invention containing siRNA can be stored at -80°C without impacting their size or downregulation efficiency.

Figure 15 Shows mRNA delivery in THP1 cells. (a) Transfection of THP1 cells with eGFP encoding mRNA using liposomes of composition LP1. THP1 cells were treated with different concentrations of liposomes containing eGFP mRNA at charge ratio 6:1 for 24 h. Number of cells expressing eGFP and fluorescence intensity of eGFP increase with dosage. Scalebar 50 μm . Untreated cells show no GFP fluorescence, when treated with 0.625 $\mu\text{g/ml}$ a few positive cells with low GFP intensity can be seen, when treated with 1.25 $\mu\text{g/ml}$ many cells are positive with intermediate GFP signal intensity and when treated with 2.5 $\mu\text{g/ml}$ most cells are positive for GFP with high GFP signal intensity. (b) Quantification of eGFP expression by fluorescence intensity and percentage of eGFP positive cells and quantification of toxicity by total cell number in response to treatment. This example shows that the composition of the invention is effective for delivery of mRNA into THP-1 macrophages, a human primary macrophage model.

Figure 16 Shows GFP protein delivery in HeLa cells. (a) HeLa cells were treated for 24 h with 125 nM GFP protein alone or mixed with in liposomes. Both a standard, wild-type GFP ((-7)GFP) and a negatively supercharged GFP ((-30)GFP) were used. Both proteins

contain a nuclear location sequence (NLS). Top row shows DAPI stained nuclei and bottom row shows GFP stained with an Alexa647-labeled GFP antibody. No GFP signal can be detected in untreated cells and in cells that were treated with protein only, not mixed with liposomes. Cells treated with GFP protein-containing liposomes show GFP fluorescence. Cells treated with liposomes containing (-30)GFP protein show a nuclear stain and a vesicular pattern, while cells treated with liposomes containing (-7)GFP protein show nuclear and cytosolic signal. Scalebar 20 μ m. These results demonstrate that the liposomal delivery system can be used for (pure) protein delivery without the need of any nucleic acid and that it does not depend on the protein being highly negatively charged. (b) Quantification of Alexa647-labeled antiGFP antibody fluorescence intensity ($n = 1$, mean $\pm$ SD). (c) Quantification of toxicity via count of total cell number normalized to untreated control ($n = 1$, mean $\pm$ SD) (d) Quantification of percentage of nuclei that are GFP positive ($n = 1$, mean $\pm$ SD). This example shows that the composition of the invention is effective for delivery of proteins without the presence of nucleic acid or a high negative charge on the protein to be required.

Figure 17 Shows comparison with prior art delivery compositions for siRNA delivery. (a, b) Comparison of eGFP downregulation in HeLa dsGFP cells treated with liposomes of composition LP1 and DOTAP/Chol liposomes containing anti eGFP siRNA. (a) Quantification of toxicity by total nuclei number and (b) quantification of downregulation by GFP intensity normalized to untreated control in response to siRNA concentration ($n = 2$, depicted error is the standard deviation). (c) Comparison of eGFP downregulation in HeLa dsGFP cells with liposomes of composition LP1 and lipid nanoparticles "MC3" of composition MC3:DSPC:Chol:PEG-DMG=50:38.5:10:1.5 both containing siRNA. Quantification of downregulation efficacy by measuring eGFP intensity normalized to untreated control in response to siRNA concentration ($n=1$, depicted error is the standard deviation). This example shows that the composition of the invention is more effective for siRNA delivery compared to prior art liposome compositions and similarly effective to prior art lipid nanoparticle compositions.

Figure 18 Shows comparison with prior art delivery compositions for mRNA delivery. (a-c) Comparison of mRNA delivery in HeLa cells with liposomes of composition LP1 and DOTAP/Chol liposomes. Quantification of (b) eGFP expression, (c) percentage of eGFP positive cells and (a) total nuclei number in response to treatment ($n = 1$, depicted error is the standard deviation). This example shows that the composition of the invention is more effective for mRNA delivery compared with prior art compositions.

Figure 19 Shows mRNA delivery in cardiomyocytes. (a) Cardiomyocytes were treated with different concentrations of eGFP mRNA in liposomes of composition LP1 at N/P ratio 6:1. Number of cells expressing eGFP and fluorescence intensity of eGFP increase with dosage. Scalebar 100 μ m. Untreated cells show no eGFP fluorescence. When treated

with 0.08 $\mu\text{g/ml}$ a few positive cells with low eGFP intensity can be seen. Positive cell number and intensity increase with increased dosage, wherein at 0.625 $\mu\text{g/ml}$ many cells are positive with intermediate eGFP signal intensity and wherein for cells treated with 2.5 $\mu\text{g/ml}$ most cells are positive for eGFP with high eGFP signal intensity. (b) Quantification of eGFP expression by fluorescence intensity and percentage of eGFP positive cells and quantification of toxicity by total cell number in response to treatment ($n = 1$, mean $\pm$ SD). This example shows that the composition of this invention is effective for mRNA delivery into muscle cells, specifically cardiomyocytes.

Figure 20 Shows comparison of different labile PEG lipid PEG chain lengths and impact on siRNA delivery in HeLa dsGFP cells. (a-c) Comparison of anti eGFP siRNA treatment of HeLa dsGFP cells with variations of composition LP1 with varying PEG lipid length. (a) eGFP knockdown efficiency in response to concentrations tested (x-axis), based on average eGFP intensity per nucleus, normalized to untreated condition ($n = 1$, mean $\pm$ SD). (b) Number of nuclei in response to dose tested as a percentage of number of nuclei present in the untreated condition ($n = 1$, mean $\pm$ SD). (c) Number of nuclei positive for eGFP, normalized to total nuclei number of the untreated condition in response to concentrations tested ($n = 1$, mean $\pm$ SD). (d-f) Comparison of anti eGFP siRNA treatment of HeLa dsGFP cells with variations of composition DOTAP:LBPA:labile PEG lipid = 50:45:5 with varying PEG lipid length. (d) GFP knockdown efficiency based on average eGFP intensity per nucleus, normalized to untreated condition in response to concentrations tested ($n = 1$, mean $\pm$ SD). (e) Number of nuclei in response to concentrations tested as a percentage of number of nuclei present in the untreated condition ($n = 1$, mean $\pm$ SD). (f) Number of nuclei positive for eGFP, normalized to total nuclei number of the untreated condition in response to dose tested ($n = 1$, mean $\pm$ SD). (g-i) Comparison of anti eGFP siRNA treatment of HeLa dsGFP cells with variations of composition DOTAP:DOPE:labile PEG lipid = 45:45:10 with varying PEG lipid length. (g) GFP knockdown efficiency based on average eGFP intensity per nucleus, normalized to untreated condition in response to concentrations tested ($n = 1$, mean $\pm$ SD). (h) Number of nuclei in response to concentrations tested as a percentage of number of nuclei present in the untreated condition ($n = 1$, mean $\pm$ SD). (i) Number of nuclei positive for eGFP, normalized to total nuclei number of the untreated condition in response to concentrations tested ($n = 1$, mean $\pm$ SD). This example shows that compositions of the invention are effective for siRNA delivery irrespective of PEG chain length.

Figure 21 Shows comparison of different labile PEG lipid PEG chain lengths on mRNA delivery in HeLa cells. (a-c) Comparison of eGFP mRNA treatment of HeLa cells with variations of composition LP1 with varying PEG lipid length. (a) Mean eGFP intensity per nucleus in response to concentrations tested (x-axis) ($n = 1$, mean $\pm$ SD). (b) Number

of nuclei in response to dose tested as a percentage of number of nuclei present in the untreated condition ($n=1$, mean $\pm$ SD). (c) Number of nuclei positive for eGFP, normalized to total nuclei number of the untreated condition in response to concentrations tested ($n=1$, mean $\pm$ SD). (d-f) Comparison of eGFP mRNA treatment of HeLa cells with variations of composition DOTAP:LBPA:labile PEG lipid = 50:45:5 with varying PEG lipid length. (d) Mean eGFP intensity per nucleus in response to concentrations tested ($n=1$, mean $\pm$ SD). (e) Number of nuclei in response to concentrations tested as a percentage of number of nuclei present in the untreated condition ($n=1$, mean $\pm$ SD). (f) Number of nuclei positive for eGFP, normalized to total nuclei number of the untreated condition in response to dose tested ($n=1$, mean $\pm$ SD). (g-i) Comparison of eGFP mRNA treatment of HeLa cells with variations of composition DOTAP:DOPE:labile PEG lipid = 45:45:10 with varying PEG lipid length. (g) Mean eGFP intensity per nucleus in response to concentrations tested ($n=1$, mean $\pm$ SD). (h) Number of nuclei as a percentage of number of nuclei present in the untreated condition in response to concentrations tested ($n=1$, mean $\pm$ SD). (i) Number of nuclei positive for eGFP, normalized to total nuclei number of the untreated condition in response to concentrations tested ($n=1$, mean $\pm$ SD). This example shows that compositions of the invention can be used to deliver mRNA irrespective of the PEG chain length. The eGFP mRNA mediated expression tested was highest for PEG lipid PEG5000-orthoester-C18 in composition LP1 and for PEG2000-orthoester-C18 in the composition of this invention without cholesterol. The composition containing DOPE (DOTAP:DOPE:labile PEG lipid = 45:45:10) is not very effective for mRNA delivery at any PEG chain length. Thus, it is preferred that a composition of the invention that comprises mRNA preferably does not contain the combination DOTAP (as component d) and DOPE (as component e) but does contain cholesterol as component e.

Detailed description of the invention

The present invention relates to a drug delivery liposome formulation to increase escape from endosomes within cells and to improve drug delivery to cells and tissues as well as enhance the subsequent release of the delivered drug within such cells and tissues. The lipid bilayer of the liposome comprises an encapsulating agent, a fusogenic lipid agent for endosomal escape, and an acid-labile PEG lipid for specific activation inside the endosomal system.

The liposome-based drug delivery system disclosed herein is structurally and compositionally different from current LNP-based systems.

The liposome according to the present invention allows a combination of functions using a modular and bioinspired approach, and can fuse with endosomal membranes.

The encapsulating lipid or agent has the function of interacting with the negatively charged endosomal membrane as well as binding negatively charged macromolecules such as oligonucleotides.

The liposome-based delivery system disclosed herein allows specific activation by acidification: the PEG coat / PEG-lipid conjugate around liposomes is in a non-active state at pH 7. Under these conditions, the PEG coat also acts as a shield prior to acidification. The PEG is attached to a lipid anchor by an acid-labile linker that breaks between pH 5 and 6. The acid-cleavable PEGylated lipid increases the hydrophilicity of the liposome and provides a steric barrier against opsonisation (shield effect), whereas the adverse steric hindrance of PEG in regard to cellular uptake and subsequent endosomal escape is circumvented by cleavage of the PEG under acidic conditions within the endosomes.

Thus, the present invention provides in one embodiment a drug delivery system comprising a liposome having

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of at least one encapsulating agent;
 - ii) between 1 and 20 mol percent of an acid-cleavable polyethylene glycol conjugated lipid;
 - iii) between 15 and 45 mol percent of at least one fusogenic agent, and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume,

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm as determined by dynamic light scattering.

As used herein the term “about” in the context of a numerical value refers to said value $\pm 5\%$ of said value, most preferably it refers to exactly said value.

As used herein, the term “**liposome**” is meant to refer to lipid molecules assembled in a spherical configuration encapsulating an interior aqueous volume that is segregated from an aqueous exterior. Liposomes are vesicles that possess at least one lipid bilayer. Liposomes are typically used as carriers for drug/ therapeutic delivery in the context of pharmaceutical development. The liposomes described in the invention are designed using a bio-inspired approach, i.e. work by fusing the lipid bilayer with a cellular membrane and repositioning its lipid structure to deliver a therapeutic agent, drug or active pharmaceutical ingredient. As such, the liposomes are structurally different from the LNPs that contain an amorphous lipid-containing core and lack lipids compatible with functionally interacting with the cellular (endosomal) membranes.

An "aqueous volume" is used herein interchangeably with "aqueous core" or with "aqueous phase" or with "aqueous compartment" or with "aqueous lumen" or with "aqueous pocket".

As used herein, the term "**lipid**" is meant to refer to a group of organic compounds characterized by being insoluble in water, but soluble in many organic solvents. Lipid molecules are amphiphilic meaning that they have a hydrophilic or polar end and a hydrophobic or nonpolar end. The shape and amphipathic nature of the lipid molecules cause them to organize spontaneously into structures such as bilayers, and micelles in aqueous environments. Lipid molecules are usually divided into at least three classes: (1) "simple lipids," which include fats and oils as well as waxes; (2) "compound lipids," which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids.

In an aspect, the invention provides a composition comprising (a) a therapeutic agent or a pharmaceutically acceptable salt thereof; (b) a PEG-monoorthoester-lipid; (c) an amphiphilic lipid; (d) a cationic lipid and/or a beta-alanyl-prolyl-cysteine methyl ester; and optionally (e) a steroid and/or a ceramide and/or DOPE.

In one embodiment the therapeutic agent or pharmaceutically acceptable salt thereof is encapsulated by the composition.

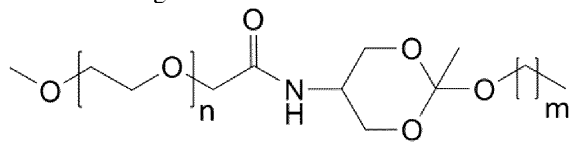
In one embodiment the invention provides a composition as described herein comprising (a) a therapeutic agent or a pharmaceutically acceptable salt thereof; (b) a PEG-monoorthoester-lipid; (c) an amphiphilic lipid; (d) a cationic lipid or a beta-alanyl-prolyl-cysteine methyl ester; and (e) a steroid, and preferably cholesterol.

In one embodiment the invention provides a composition as described herein comprising (a) a therapeutic agent or a pharmaceutically acceptable salt thereof; (b) a PEG-monoorthoester-lipid; (c) an amphiphilic lipid; (d) a cationic lipid and a beta-alanyl-prolyl-cysteine methyl ester; and (e) a steroid, and preferably cholesterol.

In one embodiment the invention provides a composition as described herein comprising (a) a therapeutic agent or a pharmaceutically acceptable salt thereof; (b) a PEG-monoorthoester-lipid; (c) an amphiphilic lipid; (d) a cationic lipid and/or beta-alanyl-prolyl-cysteine methyl ester; and (e) a steroid and a ceramide.

In one embodiment the invention provides a composition as described herein comprising (a) a therapeutic agent or a pharmaceutically acceptable salt thereof, wherein the therapeutic agent is mRNA; (b) a PEG-monoorthoester-lipid; (c) an amphiphilic lipid; (d) a cationic lipid and/or a beta-alanyl-prolyl-cysteine methyl ester; and (e) cholesterol.

In one embodiment the invention provides a composition comprising (a) a therapeutic agent or a pharmaceutically acceptable salt thereof; (b) a PEG-monoorthoester-lipid, wherein said PEG-monoorthoester lipid has the following structure:



wherein n is an integer between 35 and 55 and wherein m is an integer between 10 and 27; and preferably wherein n is an integer between 40 and 50 and m is an integer of between 15 and 23; (c) an amphiphilic lipid; (d) a cationic lipid and/or a beta-alanyl-prolyl-cysteine methyl ester; and optionally (e) a steroid and/or a ceramide; wherein components (a)-(d) and optionally (e) are formulated as a liposome.

In one embodiment, a composition as described herein does not comprise a steroid. In one embodiment, a composition as described herein does not comprise cholesterol.

"Liposome component ratios"

"Liposome component ratio" also abbreviated as "compound ratio" or "liposome agent ratio" or "agent ratio" or "component ratio" is defined as the molar ratio of that "Liposome component" or "compound" and is calculated as:

$$[n(\text{agent}) / n(\text{total})] * 100$$

wherein "n" is the molar amount or the number of moles used to prepare the liposomes.

The molar ratios of each liposome component of the optimized liposome compositions disclosed herein are listed in Table 1. The molar ratios of each liposome component of the tested liposome compositions in the Examples disclosed herein are listed in Tables 2 - 12.

The compound ratio of an encapsulating agent in the drug delivery system disclosed herein is not specifically restricted, but it is preferable that the liposome of the drug delivery system disclosed herein has 12.5, 15, 25, 30, 33, 35, 37.5, 40, 45, 47.5, 50, 52.5, 54, 55, 60 or 70 mol percent of at least one encapsulating agent. Preferably the liposome of the drug delivery system disclosed herein has between 10 and 90 mol percent, preferably between 12 and 90 mol percent, preferably between 15 and 90 mol percent, preferably between 20 and 90 mol percent, preferably between 25 and 90 mol percent, preferably between 30 and 90 mol percent, preferably between 40 and 90 mol percent, preferably between 10 and 80 mol percent, preferably between 12 and 80 mol percent, preferably between 15 and 80 mol percent, preferably between 20 and 80 mol percent, preferably between 25 and 80 mol percent, preferably between 30 and 80 mol percent, preferably between 40 and 80 mol percent, preferably between 10 and 75 mol percent, preferably between 12 and 75 mol percent, preferably between 15 and 75 mol percent, preferably between 20 and 75 mol percent, preferably between 25 and 75 mol percent, preferably between 30 and 75 mol percent, preferably between 40

and 75 mol percent, preferably between 10 and 70 mol percent, preferably between 12 and 70 mol percent, preferably between 15 and 70 mol percent, preferably between 20 and 70 mol percent, preferably between 25 and 70 mol percent, preferably between 30 and 70 mol percent, preferably between 40 and 70 mol percent, preferably between 10 and 60 mol percent, preferably between 12 and 60 mol percent, preferably between 15 and 60 mol percent, preferably between 20 and 60 mol percent, preferably between 25 and 60 mol percent, preferably between 30 and 60 mol percent, preferably between 40 and 60 mol percent, preferably between 10 and 55 mol percent, preferably between 12 and 55 mol percent, preferably between 15 and 5 mol percent, preferably between 20 and 55 mol percent, preferably between 25 and 5 mol percent, preferably between 30 and 5 mol percent, preferably between 40 and 55 mol percent, more preferred between 30 and 75 mol percent of at least one encapsulating agent.

The compound ratio of an acid-cleavable polyethylene glycol conjugated lipid in the drug delivery system disclosed herein is not specifically restricted, but it is preferable that the liposome of the drug delivery system disclosed herein has 1, 2, 2.5, 3, 4, 5, 6, 7, 7.5, 8, 9, 10, 11, or 20 mol percent of an acid-cleavable polyethylene glycol conjugated lipid.

Preferably the liposome of the drug delivery system disclosed herein has between 1 and 20 mol percent, preferably between 1 and 11 mol percent, preferably between 1 and 10 mol percent, preferably between 1 and 8 mol percent, preferably between 1 and 7 mol percent, preferably between 1 and 6 mol percent, preferably between 1 and 5 mol percent, preferably between 2 and 20 mol percent, preferably between 2 and 11 mol percent, preferably between 2 and 10 mol percent, preferably between 2 and 8 mol percent, preferably between 2 and 7 mol percent, preferably between 2 and 6 mol percent, preferably between 2 and 5 mol percent, preferably between 3 and 20 mol percent, preferably between 3 and 11 mol percent, preferably between 3 and 10 mol percent, preferably between 3 and 8 mol percent, preferably between 3 and 7 mol percent, preferably between 3 and 6 mol percent, preferably between 3 and 5 mol percent, preferably between 4 and 20 mol percent, preferably between 4 and 11 mol percent, preferably between 4 and 10 mol percent, preferably between 4 and 8 mol percent, preferably between 4 and 7 mol percent, preferably between 4 and 6 mol percent of an acid-cleavable polyethylene glycol conjugated lipid.

The compound ratio of a fusogenic agent in the drug delivery system disclosed herein is not specifically restricted, but it is preferable that the liposome of the drug delivery system disclosed herein has 10, 15, 20, 25, 27, 30, 35, 40, 45, 50, 55, 60 mol percent of at least one fusogenic agent.

Preferably the liposome of the drug delivery system disclosed herein has between 10 and 60 mol percent, preferably between 15 and 60 mol percent, preferably between 20 and 60 mol percent, preferably between 25 and 60 mol percent, preferably between 27 and 60 mol percent, preferably

between 30 and 60 mol percent, preferably between 35 and 60 mol percent, preferably between 40 and 60 mol percent, between 10 and 55 mol percent, preferably between 15 and 55 mol percent, preferably between 20 and 55 mol percent, preferably between 25 and 55 mol percent, preferably between 27 and 55 mol percent, preferably between 30 and 55 mol percent, preferably between 35 and 55 mol percent, preferably between 40 and 55 mol percent, between 10 and 50 mol percent, preferably between 15 and 50 mol percent, preferably between 20 and 50 mol percent, preferably between 25 and 50 mol percent, preferably between 27 and 50 mol percent, preferably between 30 and 50 mol percent, preferably between 35 and 50 mol percent, between 10 and 45 mol percent, preferably between 20 and 45 mol percent, preferably between 25 and 45 mol percent, preferably between 27 and 45 mol percent, preferably between 30 and 45 mol percent, preferably between 35 and 45 mol percent, between 10 and 35 mol percent, preferably between 20 and 35 mol percent, preferably between 25 and 35 mol percent, more preferred between 15 and 45 mol percent of at least one fusogenic agent

The compound ratio of a neutral lipid in the drug delivery system disclosed herein is not specifically restricted, but it is preferable that the liposome of the drug delivery system disclosed herein has 1, 2.5, 5, 10, 13, 20, 22.5, 25, 33, 36, 37.5, 40 mol percent of at least one neutral lipid, or is free of neutral lipid.

Preferably the liposome of the drug delivery system disclosed herein has preferably between 1 and 40 mol percent, preferably between 2.5 and 40 mol percent, preferably between 5 and 40 mol percent, preferably between 10 and 40 mol percent, preferably between 13 and 40 mol percent, preferably between 20 and 40 mol percent, preferably between 22.5 and 40 mol percent, preferably between 25 and 40 mol percent, between 1 and 37.5 mol percent, preferably between 2.5 and 37.5 mol percent, preferably between 5 and 37.5 mol percent, preferably between 10 and 37.5 mol percent, preferably between 13 and 37.5 mol percent, preferably between 20 and 37.5 mol percent, preferably between 22.5 and 37.5 mol percent, preferably between 25 and 37.5 mol percent, preferably between 2.5 and 36 mol percent, preferably between 5 and 36 mol percent, preferably between 10 and 36 mol percent, preferably between 13 and 36 mol percent, preferably between 20 and 36 mol percent, preferably between 22.5 and 36 mol percent, preferably between 25 and 36 mol percent, preferably between 2.5 and 33 mol percent, preferably between 5 and 33 mol percent, preferably between 10 and 33 mol percent, preferably between 13 and 33 mol percent, preferably between 20 and 33 mol percent, preferably between 22.5 and 33 mol percent, preferably between 25 and 33 mol percent, preferably between 2.5 and 25 mol percent, preferably between 5 and 25 mol percent, preferably between 10 and 25 mol percent, preferably between 13 and 25 mol, preferably between 2.5 and 20 mol percent, preferably between 5 and 20 mol percent, preferably between 10 and 20 mol percent, more preferred between 13 and 36 mol percent of at least one neutral lipid.

"Liposome size"

As used herein, "size" or "mean size" in the context of liposome compositions refers to the mean diameter measured as Z-average by DLS of a liposome.

The **Z-average particle diameter** of a liposome is a value measured by a dynamic light scattering method. "**Z average**" is defined as the intensity weighted mean hydrodynamic size of the ensemble collection of particles measured by **dynamic light scattering** (DLS). As defined by Malvern Panalytical Ltd., the Z average is derived from a Cumulants analysis of the measured correlation curve, wherein a single particle size is assumed and a single exponential fit is applied to the autocorrelation function.

The sizes of the liposomes in the liposome delivery system of the present invention are not specifically restricted, but it is preferable that the Z-Average diameter size range is comprised between 20 nm and 200 nm as determined by dynamic light scattering. In some embodiments, the Z-Average diameter size range is comprised between 30 nm and 200 nm as determined by dynamic light scattering. In some embodiments, the Z-Average diameter size range is comprised between 45 nm and 200 nm as determined by dynamic light scattering. In some embodiments, the Z-Average diameter size range is comprised between 60 nm and 200 nm as determined by dynamic light scattering. In some embodiments, the Z-Average diameter size range is comprised between 70 nm and 200 nm as determined by dynamic light scattering. In some embodiments, the Z-Average diameter size range is comprised between 80 nm and 200 nm as determined by dynamic light scattering. In other embodiments, the Z-Average diameter size range is comprised between 20 nm and 180 nm as determined by dynamic light scattering. In other embodiments, the Z-Average diameter size range is comprised between 30 nm and 180 nm as determined by dynamic light scattering. In other embodiments, the Z-Average diameter size range is comprised between 45 nm and 180 nm as determined by dynamic light scattering. In other embodiments, the Z-Average diameter size range is comprised between 60 nm and 180 nm as determined by dynamic light scattering. In other embodiments, the Z-Average diameter size range is comprised between 70 nm and 180 nm as determined by dynamic light scattering. In other embodiments, the Z-Average diameter size range is comprised between 80 nm and 180 nm as determined by dynamic light scattering. In further embodiments, the Z-Average diameter size range is comprised between 20 nm and 160 nm, or between 30 nm and 160 nm, or between 45 nm and 160 nm as determined by dynamic light scattering. In further embodiments, the Z-Average diameter size range is comprised between 60 nm and 160 nm as determined by dynamic light scattering. In further embodiments, the Z-Average diameter size range is comprised between 70 nm and 160 nm as determined by dynamic light scattering. In further embodiments, the Z-Average diameter size range is comprised between 80 nm and 160 nm as determined by dynamic light scattering. In alternative embodiments, the Z-Average diameter size range is comprised between 20 nm and 150 nm, or between 30 nm and 150 nm, between 45 nm and 150 nm as determined by dynamic light scattering. In alternative embodiments, the Z-Average diameter size range is comprised between 60 nm and 150 nm as

determined by dynamic light scattering. In alternative embodiments, the Z-Average diameter size range is comprised between 70 nm and 150 nm as determined by dynamic light scattering. In alternative embodiments, the Z-Average diameter size range is comprised between 80 nm and 150 nm as determined by dynamic light scattering. In other alternative embodiments, the Z-Average diameter size range is comprised between 20 nm and 140 nm, or between 30 nm and 140 nm, or between 45 nm and 140 nm as determined by dynamic light scattering. In other alternative embodiments, the Z-Average diameter size range is comprised between 60 nm and 140 nm as determined by dynamic light scattering. In other alternative embodiments, the Z-Average diameter size range is comprised between 70 nm and 140 nm as determined by dynamic light scattering. In other alternative embodiments, the Z-Average diameter size range is comprised between 80 nm and 140 nm as determined by dynamic light scattering. In further alternative embodiments, the Z-Average diameter size range is comprised between 20 nm and 130 nm, or between 30 nm and 130 nm, or between 45 nm and 130 nm as determined by dynamic light scattering. In further alternative embodiments, the Z-Average diameter size range is comprised between 60 nm and 130 nm as determined by dynamic light scattering. In further alternative embodiments, the Z-Average diameter size range is comprised between 70 nm and 130 nm as determined by dynamic light scattering. In further alternative embodiments, the Z-Average diameter size range is comprised between 80 nm and 130 nm as determined by dynamic light scattering. In preferred embodiments, the Z-Average diameter size is greater than 20 nm, 25 nm, 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, or 100 nm. In preferred embodiments, the Z-Average diameter size is also lower than 220 nm, 215 nm, 210 nm, 205 nm, 200 nm, 195 nm, 190 nm, 185 nm, 180 nm, 175 nm, 170 nm, 165 nm, 160 nm, 155 nm, 150 nm, 145 nm, 140 nm, 135 nm, 130 nm, 125 nm, 120 nm, 115 nm, or 100 nm.

As used herein, the "**polydispersity index**" is a ratio that describes the homogeneity of the particle size distribution of a system, which is derived from the dynamic light scattering correlation data. It can assume values between 0 and 1. A small value, e.g., less than 0.3, indicates a narrow particle size distribution.

As used herein, the "**zeta potential**" is the electrokinetic potential of a lipid, e.g., in a liposome composition.

As used herein, a "**lipid component**" is that component of a "**liposome composition**" that includes one or more lipids. For example, the lipid component may include one or more cationic/ionizable, PEGylated, structural, or other lipids, such as phospholipids. The lipid components of the inventive liposome delivery system compositions developed and tested in the present invention are listed in **Tables 1 - 12**. The lipid components of the inventive liposome delivery system are a fusogenic agent, an encapsulating agent or therapeutic encapsulating agent, a labile PEG lipid, optionally, a helper or enhancer lipid.

"Encapsulating agent"

The wording "**encapsulating agent**" refers to a compound able to interact and bind a therapeutic agent of interest, so that when the **encapsulating agent** is present in the lipid bilayer enclosing an aqueous volume of a liposome composition, the therapeutic agent is encapsulated within the aqueous volume of said liposome.

However, the therapeutic agent need not necessarily be encapsulated in the composition of the invention, such as in a liposome composition of the invention, but the therapeutic agent may also be associated with the composition of the invention and/or its components in another way. This includes the therapeutic agent being complexed or associated with or bound or attached or adsorbed to or integrated into the composition of the invention.

An encapsulating agent also refers to a molecule acting as trapping agent forming a complex with the drug (ie therapeutic agent)

PD740 (C₄₅H₈₂N₄O₆S₂) is a lipidated tripeptide analogue. PD740 is able to bind the therapeutic agent, such as RNA, probably by an electrostatic interaction due to the pKa of PD740.

The inventive liposome composition may also include one or more **cationic** and/or **ionizable lipids** (e.g., lipids that may have a positive or partial positive charge at physiological pH) as "encapsulating agent" in addition to the other liposome components listed elsewhere. As used herein, the term "**cationic lipid**" or "**cationic agent**" is meant to refer to a lipid, having a positively charged head group and one or two hydrocarbon chains.

Liposome compositions with the encapsulating agents PD740, DOTAP, DOTMA, DOMA showed highest siRNA mediated downregulation efficiency in HeLa cells. Liposome compositions with the cationic lipid EPC 18:1 as encapsulating agent showed intermediate siRNA mediated downregulation efficiency in HeLa cells. Liposome compositions with the encapsulating agents TTAB, CTAB, 16-BAC, SA, PD739, Gl67, MVL5, TAP 18:0 showed no siRNA mediated downregulation in HeLa cells.

As used herein, the term "**ionizable lipid**" is meant to refer to a lipid, e.g., cationic lipid, having at least one protonatable or deprotonatable group, such that the lipid is positively charged at a pH at or below physiological pH (e.g., pH 7.4), and neutral at a second pH, preferably at or above physiological pH. It will be understood by one of ordinary skill in the art that the addition or removal of protons as a function of pH is an equilibrium process, and that the reference to a charged or a neutral lipid refers to the nature of the predominant species and does not require that all of the lipid be present in the charged or neutral form. Generally, ionizable lipids have a pKa of the protonatable group in the range of about 4 to about 7. Preferred ionizable lipids are 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride, N-(4-carboxybenzyl)-N,N-dimethyl-

2,3-bis(oleoyloxy)propan-1-aminium, 1,2-distearoyl-3-dimethylammonium-propane, 1,2-dipalmitoyl-3-dimethylammonium-propane, 1,2-dimyristoyl-3-dimethylammonium-propane, 1,2-dioleoyl-3-dimethylammonium-propane (DODAP), 1,2-dioleyloxy-3-dimethylaminopropane (DODMA), 6-((2-hexyldecanoyl)oxy)-N-(6-((2-hexyldecanoyl)oxy)hexyl)-N-(4-hydroxybutyl)hexan-1-aminium, YSK05, Dlin-DMA, DLin-KC2-DMA, DLin-MC3-DMA (MC3), C12-200, SM-102, ALC-0315, Lipid 5. Further ionizable lipids can be found at ionizable cationic lipid <https://broadpharm.com/product-categories/lipid/ionizable-lipid>. In some embodiments, a ionizable lipid may include “cleavable lipid” or “SS- cleavable lipid”.

In one embodiment an encapsulating agent for the drug delivery system disclosed herein is a cationic lipid selected from 1,2-dialkanyloxy-3-trialkylammonium propane halide, 1,2-dialkenyloxy-3-trialkylammonium propane halide, 1,2-dialkanoyloxy-3-trialkylammonium propane halide, 1,2-dialkenoyloxy-3-trialkylammonium propane halide, tetraalkylammonium halide, and/or a lipidated polypeptide PD740. Preferentially, an encapsulating agent for the drug delivery system disclosed herein is a cationic lipid selected from 1,2-di-C₁₂-C₂₀ alkanyloxy-3-tri-C₁-C₆ alkylammonium propane halide, 1,2-di-C₁₂-C₂₀ alkenyloxy-3-tri-C₁-C₆ alkylammonium propane halide, 1,2-di-C₁₂-C₂₀ alkenoyloxy-3-tri-C₁-C₆ alkylammonium propane halide, di-C₁₂-C₂₀ alkyl di-C₁-C₆ alkyl ammonium halide, and/or a lipidated polypeptide PD740. More preferentially, an encapsulating agent for the drug delivery system disclosed herein is a cationic lipid selected from the group consisting of 1,2-didodecyloxy-3-trimethylammonium propane chloride, 1,2-ditridecyloxy-3-trimethylammonium propane chloride, 1,2-ditetradecyloxy-3-trimethylammonium propane chloride, 1,2-dipentadecyloxy-3-trimethylammonium propane chloride, 1,2-dihexadecyloxy-3-trimethylammonium propane chloride, 1,2-diheptadecyloxy-3-trimethylammonium propane chloride, 1,2-dioctadecyloxy-3-trimethylammonium propane chloride, 1,2-dinonadecyloxy-3-trimethylammonium propane chloride, 1,2-diicosyloxy-3-trimethylammonium propane chloride, 1,2-didodecenyloxy-3-trimethylammonium propane chloride, 1,2-ditridecenyloxy-3-trimethylammonium propane chloride, 1,2-ditetradecenyloxy-3-trimethylammonium propane chloride, 1,2-dipentadecenyloxy-3-trimethylammonium propane chloride, 1,2-dihexadecenyloxy-3-trimethylammonium propane chloride, 1,2-diheptadecenyloxy-3-trimethylammonium propane chloride, 1,2-dioctadecenyloxy-3-trimethylammonium propane chloride, 1,2-dinonadecenyloxy-3-trimethylammonium propane chloride, 1,2-diicosenyloxy-3-trimethylammonium propane chloride, 1,2-dilauroleyloxy-3-trimethylammonium propane chloride, 1,2-dimyristoleoyloxy-3-trimethylammonium propane chloride, 1,2-dipalmitoleoyloxy-3-trimethylammonium propane chloride, 1,2-dipetroseloyloxy-3-trimethylammonium propane chloride, 1,2-dipetroselaidoyloxy-3-trimethylammonium propane chloride, 1,2-dioleyloxy-3-trimethylammonium propane chloride, 1,2-dilaidoyloxy-3-trimethylammonium propane chloride, 1,2-divaccenoyloxy-3-trimethylammonium propane chloride, 1,2-digadoleoyloxy-3-trimethylammonium propane chloride, dimethyldidodecylammonium bromide, dimethylditridecylammonium bromide, dimethylditetradecylammonium bromide, dimethyldipentadecylammonium bromide,

dimethyldihexadecylammonium bromide, dimethyldiheptadecylammonium bromide, dimethyldioctadecylammonium bromide, dimethyldinonadecylammonium bromide, and dimethyldiicosylammonium bromide, and/or a lipidated polypeptide PD740. Still more preferentially, an encapsulating agent for the drug delivery system disclosed herein is a cationic lipid selected from the group consisting of 1,2-di-*O*-octadecenyl-3-trimethylammonium propane chloride, dimethyldioctadecylammonium bromide, and 1,2-dioleoyl-3-trimethylammonium propane chloride, and/or a lipidated polypeptide PD740. Particularly preferred, an encapsulating agent for the drug delivery system disclosed herein is a cationic lipid 1,2-dioleoyl-3-trimethylammonium propane chloride and/or a lipidated polypeptide PD740.

In one embodiment, the invention provides a composition as described herein, comprising (a) a therapeutic agent or a pharmaceutically acceptable salt thereof; (b) a PEG-monoorthoester-lipid; (c) an amphiphilic lipid; (d) a cationic lipid and/or a beta-alanyl-prolyl-cysteine methyl ester; and optionally (e) a steroid and/or a ceramide, wherein the cationic lipid in (d) is selected from the group consisting of 1,2-dialkanyloxy-3-trialkylammonium propane halide, 1,2-dialkenyloxy-3-trialkylammonium propane halide, 1,2-dialkanoyloxy-3-trialkylammonium propane halide, 1,2-dialkenoyloxy-3-trialkylammonium propane halide, tetraalkylammonium halide, 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine.

In one embodiment the cationic lipid in (d) is selected from the group consisting of 1,2-di-C12-C20 alkanyloxy-3-tri-C1-C6 alkylammonium propane halide, 1,2-di-C12-C20 alkenyloxy-3-tri-C1-C6 alkylammonium propane halide, 1,2-di-C12-C20 alkenoyloxy-3-tri-C1-C6 alkylammonium propane halide, di-C12-C20 alkyl di-C1-C6 alkyl ammonium halide.

In one embodiment the cationic lipid in (d) is selected from the group consisting of 1,2-didodecyloxy-3-trimethylammonium propane chloride, 1,2-ditridecyloxy-3-trimethylammonium propane chloride, 1,2-ditetradecyloxy-3-trimethylammonium propane chloride, 1,2-dipentadecyloxy-3-trimethylammonium propane chloride, 1,2-dihexadecyloxy-3-trimethylammonium propane chloride, 1,2-diheptadecyloxy-3-trimethylammonium propane chloride, 1,2-dioctadecyloxy-3-trimethylammonium propane chloride, 1,2-dinonadecyloxy-3-trimethylammonium propane chloride, 1,2-diicosyloxy-3-trimethylammonium propane chloride, 1,2-didodecenyloxy-3-trimethylammonium propane chloride, 1,2-ditridecenyloxy-3-trimethylammonium propane chloride, 1,2-ditetradecenyloxy-3-trimethylammonium propane chloride, 1,2-dipentadecenyloxy-3-trimethylammonium propane chloride, 1,2-dihexadecenyloxy-3-trimethylammonium propane chloride, 1,2-diheptadecenyloxy-3-trimethylammonium propane chloride, 1,2-dioctadecenyloxy-3-trimethylammonium propane chloride, 1,2-dinonadecenyloxy-3-trimethylammonium propane chloride, 1,2-diicosenyloxy-3-trimethylammonium propane chloride, 1,2-dilauroleoyloxy-3-trimethylammonium propane chloride, 1,2-dimyristoleoyloxy-3-trimethylammonium propane chloride, 1,2-dipalmitoleoyloxy-3-trimethylammonium propane chloride, 1,2-dipetroseloyloxy-3-trimethylammonium propane chloride, 1,2-dipetroselaidoyloxy-3-trimethylammonium propane chloride, 1,2-dioleoyloxy-3-

trimethylammonium propane chloride, 1,2-di-elaidoyloxy-3-trimethylammonium propane chloride, 1,2-divaccenoyloxy-3-trimethylammonium propane chloride, 1,2-digadoleoyloxy-3-trimethylammonium propane chloride, dimethyldidodecylammonium bromide, dimethylditridecylammonium bromide, dimethylditetradecylammonium bromide, dimethyldipentadecylammonium bromide, dimethyldihexadecylammonium bromide, dimethyldiheptadecylammonium bromide, dimethyldioctadecylammonium bromide, dimethyldinonadecylammonium bromide, and dimethyldiicosylammonium bromide.

In one embodiment the cationic lipid selected from the group consisting of 1,2-di-O-octadecenyl-3-trimethylammonium propane chloride, dimethyldioctadecylammonium bromide, and 1,2-dioleoyl-3-trimethylammonium propane chloride and preferably wherein the cationic lipid is 1,2-dioleoyl-3-trimethylammonium propane chloride.

In one embodiment the beta-alanyl-prolyl-cysteine methyl ester is a lipidated polypeptide. In a preferred embodiment the beta-alanyl-prolyl-cysteine methyl ester is or comprises N-[3-(Hexadecan-1-sulfonylamino)propionyl]-4-(R)-(aminoethyl)-L-prolyl-S-farnesyl-L-cysteine methyl ester (PD740).

In one embodiment the compound in (d) is selected from the group consisting of N-[3-(Hexadecan-1-sulfonylamino)propionyl]-4-(R)-(aminoethyl)-L-prolyl-S-farnesyl-L-cysteine methyl ester (PD740), 2,3-bis[[(Z)-octadec-9-enoyl]oxy]propyl-trimethylazanium (DOTAP), dioctadecyldimethylammonium bromide (DOMA), 1,2-di-O-octadecenyl-3-trimethylammonium propane (chloride salt) (DOTMA), 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (EPC 18:1), and mixtures thereof.

In one embodiment the compound in (d) is 2,3-bis[[(Z)-octadec-9-enoyl]oxy]propyl-trimethylazanium (DOTAP) and/or beta-alanyl-prolyl-cysteine methyl ester N-[3-(Hexadecan-1-sulfonylamino)propionyl]-4-(R)-(aminoethyl)-L-prolyl-S-farnesyl-L-cysteine methyl ester (PD740).

"Labile PEG-lipids"

The lipid component of a liposome composition as disclosed herein may include one or more labile PEG or labile PEG-modified lipids. A "pH-labile PEG-lipid" or "acid-labile PEG-lipid" refers to an "acid-cleavable PEG-lipid", i.e. a lipid attached to a PEG by a pH-sensitive acid-labile linkage, such as an orthoester or an hydrazone, (Fang Y et al., Cleavable PEGylation: a strategy for overcoming the "PEG dilemma" in efficient drug delivery. Drug Deliv. 2017;24(sup1):22-3), that breaks at pH between 5 and 6.

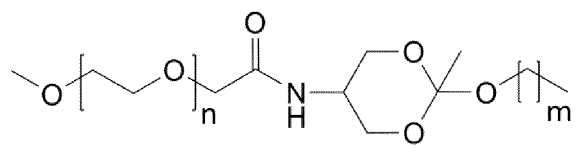
A "labile linkage" refers to a covalent bond that is capable of being selectively broken. Consequently, the labile bond may be broken in the presence of other covalent bonds in the liposome without the breakage of other covalent bonds eventually present. "pH-labile linkage" or "pH-sensitive linkage" refers to a linkage or bond that can be selectively broken under acidic conditions (pH < 7). This means that a pH-labile bond may be broken under acidic conditions in the presence of other covalent bonds without their breakage.

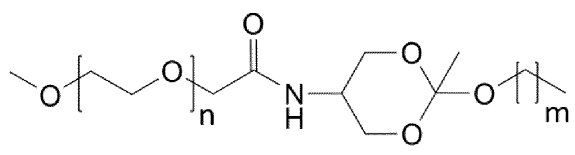
A labile PEG-lipid forms a PEG coat around the liposomes acting as a shield at neutral pH, i.e. pH around 7. As used herein, a "PEG lipid" or "PEGylated lipid" refers to a lipid comprising a polyethylene glycol component. In other words, the acid-cleavable PEGylated lipid increases the hydrophilicity of the liposome and provides a steric barrier against opsonisation (shield effect), whereas the endosomal escape after cellular uptake is allowed by the activating cleavage of the PEG in the acidic conditions within endosomes.

Particularly suitable acid-labile linkages have been described in the prior art and include orthoester linkages, hydrazone linkages, acetal linkages, vinyl ether linkages, and imine linkages (Fang Y et al., 2017). pH-sensitive PEG lipids containing orthoester linkers have been described by Masson C. et al., 2004, *pH-sensitive PEG lipids containing orthoester linkers: new potential tools for nonviral gene delivery*, J. Controlled Release, (99)3, 423-434. PEG lipids containing orthoester linkers provide a useful mechanism for pH dependent PEG modification, e.g. shedding of PEG. Thus, the composition of the invention suitably comprises a PEG-monoorthoester-lipid. In one embodiment an ester bond of the PEG-monoorthoester-lipid hydrolyses at a pH of 6 or less, preferably wherein said orthoester bond breaks at a pH of from pH 5 to pH 6.

An embodiment of the present invention discloses a drug delivery system as described herein, wherein the acid-cleavable polyethylene glycol conjugated lipid is an acid-cleavable polyethylene glycol conjugated with a C₁₂-C₂₀ alkyl, or with a C₁₂-C₂₀ acyl, or with a mono-C₁₂-C₂₀ acylglycerol, or with a di-C₁₂-C₂₀ acylglycerol, preferably an acid-cleavable polyethylene glycol conjugated with a mono-C₁₂-C₂₀ alkyl or a mono-C₁₂-C₂₀ acyl.

In one embodiment, the PEG-monoorthoester-lipid of the composition described herein comprises a polyethylene glycol conjugated with a C₁₂-C₂₀ alkyl, or with a C₁₂-C₂₀ acyl, or with a mono-C₁₂-C₂₀ acylglycerol, or with a di-C₁₂-C₂₀ acylglycerol, or wherein said PEG-monoorthoester lipid has the following structure:





wherein n is an integer between 20 and 200 and wherein m is an integer between 10 and 27; and preferably wherein n is an integer between 40 and 140.

The following integers for n are particularly preferred: n = 22, n = 45, n = 110.

It was surprisingly found that compositions with a PEG-monoorthoester-lipid having the formula of the above structure, wherein n is an integer of from 40 to 120 and preferably wherein n is an integer of from 100 to 120 and more preferably of 110, provided a particularly effective transfection of mRNA molecules into target cells. A similar effect was also confirmed for other therapeutic agents, such as for siRNA.

A more preferred embodiment of the present invention discloses a drug delivery system as described herein, wherein the acid-cleavable polyethylene glycol conjugated lipid comprises an acid-cleavable linkage selected from the group comprising an orthoester linkage, a hydrazone linkage, an acetal linkage, a vinyl ether linkage, and an imine linkage, preferably a 2-methyl-2-alkoxy-1,3-dioxane or a 2-alkoxy-1,3-dioxolane. A still more preferred embodiment of the present invention discloses a drug delivery system as described herein, wherein the acid-cleavable polyethylene glycol conjugated lipid is selected from the group consisting of:

N-(2-methyl-2-dodecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol,
 N-(2-methyl-2-tridecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol,
 N-(2-methyl-2-tetradecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol,
 N-(2-methyl-2-pentadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol,
 N-(2-methyl-2-hexadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol,
 N-(2-methyl-2-heptadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol,
 N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol,
 N-(2-methyl-2-nonadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, and
 N-(2-methyl-2-icosyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol.

In one embodiment, the PEG-monoorthoester-lipid of the composition described herein is selected from the group consisting of:

N-(2-methyl-2-dodecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, N-(2-methyl-2-tridecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, N-(2-methyl-2-tetradecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, N-(2-methyl-2-pentadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, N-(2-methyl-2-hexadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, N-(2-methyl-2-heptadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol (PEGOEC18), N-(2-

methyl-2-nonadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, and N-(2-methyl-2-icosyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol.

A further more preferred embodiment of the present invention discloses a drug delivery system as described herein, wherein the acid-cleavable polyethylene glycol conjugated lipid is α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅ (PEG2000-orthoester-C18, PEGOEC18). A further still more preferred embodiment of the present invention discloses a drug delivery system as described herein, wherein the acid-cleavable polyethylene glycol conjugated lipid comprises polyethylene glycol having a number average molar mass M_n comprised between 400 and 5000 Da. In one embodiment, the PEG-monoorthoester-lipid of the composition described herein comprises polyethylene glycol having a number average molar mass M_n comprised between 400 and 5000 Da.

In one embodiment, the PEG-monoorthoester-lipid of the composition described herein is N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol (PEGOEC18). In a preferred embodiment the PEG-monoorthoester-lipid of the composition described herein is α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅.

In a further still more preferred embodiment of the present invention, the acid-cleavable polyethylene glycol conjugated lipid or the PEG-monoorthoester-lipid comprises polyethylene glycol having a number average molar mass M_n comprised between 500 and 5000 Da, or between 600 and 5000 Da, or between 700 and 5000 Da, or between 800 and 5000 Da, or between 900 and 5000 Da, or between 1000 and 5000 Da, or between 2000 and 5000 Da, or between 500 and 4000 Da, or between 600 and 4000 Da, or between 700 and 4000 Da, or between 800 and 4000 Da, or between 900 and 4000 Da, or between 1000 and 4000 Da, or between 2000 and 4000 Da, or between 500 and 3000 Da, or between 600 and 3000 Da, or between 700 and 3000 Da, or between 800 and 3000 Da, or between 900 and 3000 Da, or between 1000 and 3000 Da, or between 2000 and 3000 Da, or between 500 and 2000 Da, or between 600 and 2000 Da, or between 700 and 2000 Da, or between 800 and 2000 Da, or between 900 and 2000 Da, or between 1000 and 2000 Da.

"Fusogenic agent"

A "fusogenic agent" or "fusogenic lipid" are used herein interchangeably to indicate a lipid that is recognized by the cellular machinery, either by cellular proteins or endosomal lipids and sugar moieties, and facilitates the fusion of the lipid bilayer of the delivery system with the limiting membrane of the endosome. However, fusogenic lipids can also be lipids that prefer non-lamellar phase behavior.

Preferably, a "fusogenic agent" or "fusogenic lipid" is selected from the group comprising a glycolipid, a phosphatidylethanolamine, a phosphatidylglycerol, a fatty acid, or a fatty acid ester. A particularly preferred **fusogenic agent** according to this invention is acylated diglycerolphosphate. Liposome compositions with MGDG, PLPE; S,S-3,3'- or S,S-2,2'-LBPA; as fusogenic agent showed highest activity for siRNA mediated downregulation in HeLa cells. Liposome compositions with

POPE, GMO, NPPE, MO or IsostA as fusogenic agent showed intermediate activity for siRNA mediated downregulation in HeLa cells. Liposome compositions with OIA or Palma as fusogenic agent showed low activity for siRNA mediated downregulation in HeLa cells. Liposome compositions with NAPE as fusogenic agent showed no activity for siRNA mediated downregulation in HeLa cells. Liposome compositions containing POPE, S,S-3,3'- or S,S-2,2'-LBPA, OIA, GMO or Palma showed high siRNA mediated downregulation in mouse macrophages. Liposome compositions with MO, IsostA and NAPE showed intermediate activity for siRNA downregulation in mouse macrophages, while NPPE showed low activity. Liposomes containing S,S-3,3'- or S,S-2,2'-LBPA, PLPE or MGDG as fusogenic agents showed high expression of eGFP by mRNA delivery in HeLa cells.

LBPA isomers S,S-2,2'-LBPA, R,R-3,3'-LBPA, S,S-3,3'-LBPA, R,S-3,3'-LBPA and isoforms S,R-Hemi, S,S-2,2'-C18:0-ether-LBPA and S,S-2,2'-C12:0-ether-LBPA showed a similar effect as LBPA in siRNA liposome compositions in terms of GFP downregulation in HeLa cells. LBPA isoforms S,S-BDP, DOPG, lysoPG showed a lower effect as LBPA in siRNA liposome compositions in terms of GFP downregulation in HeLa cells. LBPA isoforms R,S-C16:0-LBPA and R,S-C14:0-LBPA showed no activity in siRNA liposome compositions in terms of GFP downregulation in HeLa cells.

In some embodiments of the drug delivery system disclosed herein, the fusogenic agent is a glycolipid, a phosphatidylethanolamine, a phosphatidylglycerol, a fatty acid, or a fatty acid ester. In preferred embodiments of the drug delivery system disclosed herein, the fusogenic agent is a galactolipid, preferably an unsaturated monogalactosyldiacylglycerol. In further preferred embodiments of the drug delivery system disclosed herein, the fusogenic agent is a 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine or an *N*-acyl-1,2-diacyl-*sn*-glycero-3-phosphoethanolamine. In further more preferred embodiments of the drug delivery system disclosed herein, the fusogenic agent is a phosphatidylethanolamine selected from the group consisting of 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine,

1,2-dimargaroyl-*sn*-glycero-3-phosphoethanolamine, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dilinoyleoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dierucoyl-*sn*-glycero-3-phosphoethanolamine, 1-pentadecanoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine, 1-

stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine, 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine, and *N*-palmitoyl-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine. In still more preferred embodiments of the drug delivery system disclosed herein, the fusogenic agent is a phosphatidylglycerol selected from the group consisting of bis(monoacylglycerol)phosphate, 3-acylglycero-1-phospho-3'-(1',2'-diacyl)-glycerol, 3-acylglycero-1-phospho-glycerol, and 1,2-diacylglycero-3-phospho-glycerol.

An aspect of the present invention relates to the drug delivery system as disclosed herein, wherein the fusogenic agent is a bis(monoacylglycerol)phosphate selected from the group consisting of 2,2'-S,S-bis(monoacylglycerol)phosphate, 3,3'-S,S-bis(monoacylglycerol)phosphate, 3,3'-R,R-bis(monoacylglycerol)phosphate, and 3,3'-R,S-bis(monoacylglycerol)phosphate. A further aspect of the present invention relates to the drug delivery system as disclosed herein, wherein the fusogenic agent is a bis(monoacylglycerol)phosphate selected from the group consisting of bis(monododecylglycerol)phosphate, bis(monotridecylglycerol)phosphate, bis(monotetradecylglycerol)phosphate, bis(monopentadecylglycerol)phosphate, bis(monohexadecylglycerol)phosphate, bis(monoseptadecylglycerol)phosphate, bis(monooctadecylglycerol)phosphate, bis(monononadecylglycerol)phosphate, bis(monooicostylglycerol)phosphate, bis(monolauroleoylglycerol)phosphate, bis(monomyristoleoylglycerol)phosphate, bis(monopalmitoleoylglycerol)phosphate, bis(monopetroseloylglycerol)phosphate, bis(monopetroselaidoylglycerol)phosphate, bis(monooleoylglycerol)phosphate, bis(monolaidoylglycerol)phosphate, bis(monovaccenoylglycerol)phosphate, and bis(monogadoleoylglycerol)phosphate.

A further preferred aspect of the present invention relates to the drug delivery system as disclosed herein, wherein the fusogenic agent is a 3-acylglycero-1-phospho-3'-(1',2'-diacyl)-glycerol selected from the group consisting of 3-dodecylglycero-1-phospho-3'-(1',2'-didodecyl)-glycerol, 3-tridecylglycero-1-phospho-3'-(1',2'-ditridecyl)-glycerol, 3-tetradecylglycero-1-phospho-3'-(1',2'-ditetradecyl)-glycerol, 3-pentadecylglycero-1-phospho-3'-(1',2'-dipentadecyl)-glycerol, 3-hexadecylglycero-1-phospho-3'-(1',2'-dihexadecyl)-glycerol, 3-heptadecylglycero-1-phospho-3'-(1',2'-diheptadecyl)-glycerol, 3-octadecylglycero-1-phospho-3'-(1',2'-dioctadecyl)-glycerol, 3-nonadecylglycero-1-phospho-3'-(1',2'-dinonadecyl)-glycerol, 3-icosylglycero-1-phospho-3'-(1',2'-diicosyl)-glycerol, 3-lauroleoylglycero-1-phospho-3'-(1',2'-dilauroleoyl)-glycerol, 3-myristoleoylglycero-1-phospho-3'-(1',2'-dimyristoleoyl)-glycerol, 3-palmitoleoylglycero-1-phospho-3'-(1',2'-dipalmitoleoyl)-glycerol, 3-petroseloylglycero-1-phospho-3'-(1',2'-dipetroseloyl)-glycerol, 3-petroselaidoylglycero-1-phospho-3'-(1',2'-dipetroselaidoyl)-glycerol, 3-oleoylglycero-1-phospho-3'-(1',2'-dioleoyl)-glycerol, 3-elaidoylglycero-1-phospho-3'-(1',2'-dielaidoyl)-glycerol, 3-vaccenoylglycero-1-phospho-3'-(1',2'-divaccenoyl)-glycerol, and 3-gadoleoylglycero-1-phospho-3'-(1',2'-digadoleoyl)-glycerol.

A further more preferred aspect of the present invention relates to the drug delivery system as disclosed herein, wherein the fusogenic agent is a 3-acylglycerol-1-phospho-glycerol selected from the group consisting of 3-dodecylglycerol-1-phospho-glycerol, 3-tridecylglycerol-1-phospho-glycerol, 3-tetradecylglycerol-1-phospho-glycerol,

3-pentadecylglycerol-1-phospho-glycerol, 3-hexadecylglycerol-1-phospho-glycerol, 3-heptadecylglycerol-1-phospho-glycerol, 3-octadecylglycerol-1-phospho-glycerol, 3-nonadecylglycerol-1-phospho-glycerol, 3-icosylglycerol-1-phospho-glycerol, 3-lauroleoylglycerol-1-phospho-glycerol, 3-myristoleoylglycerol-1-phospho-glycerol, 3-palmitoleoylglycerol-1-phospho-glycerol, 3-petroleoylglycerol-1-phospho-glycerol, 3-petroleaidoylglycerol-1-phospho-glycerol, 3-oleoylglycerol-1-phospho-glycerol, 3-elaidoylglycerol-1-phospho-glycerol, 3-vaccenoylglycerol-1-phospho-glycerol, and 3-gadoleoylglycerol-1-phospho-glycerol.

A still more preferred aspect of the present invention relates to the drug delivery system as disclosed herein, wherein the fusogenic agent is a 1,2-diacylglycerol-3-phospho-glycerol selected from the group consisting of 1,2-didodecylglycerol-3-phospho-glycerol, 1,2-ditridecylglycerol-3-phospho-glycerol, 1,2-ditetradecylglycerol-3-phospho-glycerol, 1,2-dipentadecylglycerol-3-phospho-glycerol, 1,2-dihexadecylglycerol-3-phospho-glycerol, 1,2-diheptadecylglycerol-3-phospho-glycerol, 1,2-dioctadecylglycerol-3-phospho-glycerol, 1,2-dinonadecylglycerol-3-phospho-glycerol, 1,2-diicosylglycerol-3-phospho-glycerol, 1,2-dilauroleoylglycerol-3-phospho-glycerol, 1,2-dimyristoleoylglycerol-3-phospho-glycerol, 1,2-dipalmitoleoylglycerol-3-phospho-glycerol, 1,2-dipetroleoylglycerol-3-phospho-glycerol, 1,2-dipetroleaidoylglycerol-3-phospho-glycerol, 1,2-dioleoylglycerol-3-phospho-glycerol, 1,2-di-elaidoylglycerol-3-phospho-glycerol, 1,2-divaccenoylglycerol-3-phospho-glycerol, and 1,2-digadoleoylglycerol-3-phospho-glycerol.

A particularly preferred aspect of the present invention relates to the drug delivery system as disclosed herein, wherein the fusogenic agent is a fatty acid selected from the group consisting of myristic acid, pentadecanoic acid, palmitic acid, margaric acid, stearic acid, isostearic acid, nonadecylic acid, arachidic acid, myristoleic acid, palmitoleic acid, sapienic acid, oleic acid, elaidic acid, vaccenic acid, and linoleic acid; or a methyl ester or a glyceryl ester of one of the aforementioned fatty acids.

A particularly preferred aspect of the present invention relates to the drug delivery system as disclosed herein, wherein the fusogenic agent is selected from the group consisting of monogalactosyl-dilinolenoylglycerol, 1-palmitoyl-2-linoleoyl-*sn*-glycerol-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphoethanolamine, *N*-palmitoyl-1,2-dipalmitoyl-*sn*-glycerol-3-phosphoethanolamine, 2,2'-S,S-bis(monododecylglycerol)phosphate, 3,3'-S,S-bis(monododecylglycerol)phosphate, 3,3'-R,R-bis(monododecylglycerol)phosphate, 3,3'-R,S-bis(monododecylglycerol)phosphate, 2,2'-S,S-bis(monooctadecylglycerol)phosphate, 3,3'-S,S-bis(monooctadecylglycerol)phosphate, 3,3'-R,R-bis(monooctadecylglycerol)phosphate, 3,3'-R,S-bis(monooctadecylglycerol)phosphate, 2,2'-S,S-

bis(monooleoylglycerol)phosphate, 3,3'-S,S-bis(monooleoylglycerol)phosphate, 3,3'-R,R-bis(monooleoylglycerol)phosphate, 3,3'-R,S-bis(monooleoylglycerol)phosphate, 3-dodecylglycero-1-phospho-3'-(1',2'-didodecyl)-glycerol, 3-octadecylglycero-1-phospho-3'-(1',2'-dioctadecyl)-glycerol, 3-oleoylglycero-1-phospho-3'-(1',2'-dioleoyl)-glycerol, glyceryl monoleate, methyl oleate, isotearic acid, palmitoleic acid, and oleic acid.

As used herein, the term “**non-cationic lipid**” is meant to refer to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

In some embodiments an amphiphilic lipid may have the fusogenic effect described herein. Thus, in one embodiment the present invention provides a drug delivery system comprising a composition comprising (c) an amphiphilic lipid as described herein, wherein the amphiphilic lipid is selected from the group consisting of glycolipids, phosphatidylethanolamines, phosphatidylglycerols, fatty acids, and fatty acid esters.

In one embodiment the amphiphilic lipid is a galactolipid, preferably an unsaturated monogalactosyldiacylglycerol.

In one embodiment the amphiphilic lipid is a 1,2-diacyl-sn-glycero-3-phosphoethanolamine or an N-acyl-1,2-diacyl-sn-glycero-3-phosphoethanolamine.

In one embodiment the amphiphilic lipid is a phosphatidylethanolamine selected from the group consisting of 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine, 1,2-dipentadecanoyl-sn-glycero-3-phosphoethanolamine, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, 1,2-dimargaroyl-sn-glycero-3-phosphoethanolamine, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dipalmitoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinenoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-dierucoyl-sn-glycero-3-phosphoethanolamine, 1-pentadecanoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine, 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine, 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphoethanolamine, and N-palmitoyl-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine.

In one embodiment the amphiphilic lipid is a phosphatidylglycerol selected from the group consisting of bis(monoacylglycerol)phosphate, 3-acylglycero-1-phospho-3'-(1',2'-diacyl)-glycerol, 3-acylglycero-1-phospho-glycerol, and 1,2-diacylglycero-3-phospho-glycerol.

In one embodiment the amphiphilic lipid is a bis(monoacylglycerol)phosphate selected from the group consisting of 2,2'-S,S-bis(monoacylglycerol)phosphate, 3,3'-S,S-bis(monoacylglycerol)phosphate, 3,3'-R,R-bis(monoacylglycerol)phosphate, and 3,3'-R,S-bis(monoacylglycerol)phosphate, S,R-Hemi, S,S-2,2'-C18:0-ether-LBPA and S,S-2,2'-C12:0-ether-LBPA. A bis(monoacylglycerol)phosphate may also be selected from 2,2'-R,R-bis(monoacylglycerol)phosphate and 2,2'-R,S-bis(monoacylglycerol)phosphate.

In a particularly preferred embodiment, the amphiphilic lipid is bis(monoacylglycerol)phosphates selected from 2,2'-S,S-bis(monoacylglycerol)phosphate and 3,3'-S,S-bis(monoacylglycerol)phosphate. Even more preferably the amphiphilic lipid is 3,3'-S,S-bis(monoacylglycerol)phosphate.

In one embodiment the amphiphilic lipid is a bis(monoacylglycerol)phosphate selected from the group consisting of bis(monododecylglycerol)phosphate, bis(monotridecylglycerol)phosphate, bis(monotetradecylglycerol)phosphate, bis(monopentadecylglycerol)phosphate, bis(monohexadecylglycerol)phosphate, bis(monooctadecylglycerol)phosphate, bis(monononadecylglycerol)phosphate, bis(monocosylglycerol)phosphate, bis(monolauryleoylglycerol)phosphate, bis(monomyristoleoylglycerol)phosphate, bis(monopalmitoleoylglycerol)phosphate, bis(monopetroseloylglycerol)phosphate, bis(monopetroselaidoylglycerol)phosphate, bis(monooleoylglycerol)phosphate, bis(monolaidoylglycerol)phosphate, bis(monovaccenoylglycerol)phosphate, and bis(monogadoleoylglycerol)phosphate.

In one embodiment the amphiphilic lipid is a 3-acylglycero-1-phospho-3'-(1',2'-diacyl)-glycerol selected from the group consisting of 3-dodecylglycero-1-phospho-3'-(1',2'-didodecyl)-glycerol, 3-tridecylglycero-1-phospho-3'-(1',2'-ditridecyl)-glycerol, 3-tetradecylglycero-1-phospho-3'-(1',2'-ditetradecyl)-glycerol, 3-pentadecylglycero-1-phospho-3'-(1',2'-dipentadecyl)-glycerol, 3-hexadecylglycero-1-phospho-3'-(1',2'-dihexadecyl)-glycerol, 3-heptadecylglycero-1-phospho-3'-(1',2'-diheptadecyl)-glycerol, 3-octadecylglycero-1-phospho-3'-(1',2'-dioctadecyl)-glycerol, 3-nonadecylglycero-1-phospho-3'-(1',2'-dinonadecyl)-glycerol, 3-icosylglycero-1-phospho-3'-(1',2'-diicosyl)-glycerol, 3-lauryleoylglycero-1-phospho-3'-(1',2'-dilauryleoyl)-glycerol, 3-myristoleoylglycero-1-phospho-3'-(1',2'-dimyristoleoyl)-glycerol, 3-palmitoleoylglycero-1-phospho-3'-(1',2'-dpalmitoleoyl)-glycerol, 3-petroseloylglycero-1-phospho-3'-(1',2'-dipetroseloyl)-glycerol, 3-petroselaidoylglycero-1-phospho-3'-(1',2'-dipetroselaidoyl)-glycerol, 3-oleoylglycero-1-phospho-3'-(1',2'-dioleoyl)-glycerol, 3-elaidoylglycero-1-phospho-3'-(1',2'-dielaidoyl)-glycerol, 3-vaccenoylglycero-1-phospho-3'-(1',2'-divaccenoyl)-glycerol, and 3-gadoleoylglycero-1-phospho-3'-(1',2'-digadoleoyl)-glycerol.

In one embodiment the amphiphilic lipid is a 3-acylglycero-1-phospho-glycerol selected from the group consisting of 3-dodecylglycero-1-phospho-glycerol, 3-tridecylglycero-1-phospho-glycerol, 3-tetradecylglycero-1-phospho-glycerol, 3-pentadecylglycero-1-phospho-glycerol, 3-hexadecylglycero-1-phospho-glycerol, 3-heptadecylglycero-1-phospho-glycerol, 3-octadecylglycero-1-phospho-glycerol, 3-nonadecylglycero-1-phospho-glycerol, 3-icosylglycero-1-

phospho-glycerol, 3-lauroleoylglycerol-1-phospho-glycerol, 3-myristoleoylglycerol-1-phospho-glycerol, 3-palmitoleoylglycerol-1-phospho-glycerol, 3-petroseloylglycerol-1-phospho-glycerol, 3-petroselaidoylglycerol-1-phospho-glycerol, 3-oleoylglycerol-1-phospho-glycerol, 3-elaidoylglycerol-1-phospho-glycerol, 3-vaccenoylglycerol-1-phospho-glycerol, and 3-gadoleoylglycerol-1-phospho-glycerol.

In one embodiment the amphiphilic lipid is a 1,2-diacylglycerol-3-phospho-glycerol selected from the group consisting of 1,2-didodecylglycerol-3-phospho-glycerol, 1,2-ditridecylglycerol-3-phospho-glycerol, 1,2-ditetradecylglycerol-3-phospho-glycerol, 1,2-dipentadecylglycerol-3-phospho-glycerol, 1,2-dihexadecylglycerol-3-phospho-glycerol, 1,2-diheptadecylglycerol-3-phospho-glycerol, 1,2-dioctadecylglycerol-3-phospho-glycerol, 1,2-dinonadecylglycerol-3-phospho-glycerol, 1,2-diicosylglycerol-3-phospho-glycerol, 1,2-dilauroleoylglycerol-3-phospho-glycerol, 1,2-dimyristoleoylglycerol-3-phospho-glycerol, 1,2-dipalmitoleoylglycerol-3-phospho-glycerol, 1,2-dipetroseloylglycerol-3-phospho-glycerol, 1,2-dipetroselaidoylglycerol-3-phospho-glycerol, 1,2-dioleoylglycerol-3-phospho-glycerol, 1,2-di-elaidoylglycerol-3-phospho-glycerol, 1,2-divaccenoylglycerol-3-phospho-glycerol, and 1,2-digadoleoylglycerol-3-phospho-glycerol.

In one embodiment the amphiphilic lipid is a fatty acid selected from the group consisting of myristic acid, pentadecanoic acid, palmitic acid, margaric acid, stearic acid, isostearic acid, nonadecylic acid, arachidic acid, myristoleic acid, palmitoleic acid, sapienic acid, oleic acid, elaidic acid, vaccenic acid, and linoleic acid; or a methyl ester or a glyceryl ester of one of the aforementioned fatty acids.

In one embodiment the amphiphilic lipid is selected from the group consisting of monogalactosyl-dilinolenoylglycerol, 1-palmitoyl-2-linoleoyl-sn-glycerol-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanolamine, N-palmitoyl-1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine, 2,2'-S,S-bis(monododecylglycerol)phosphate, 3,3'-S,S-bis(monododecylglycerol)phosphate, 3,3'-R,R-bis(monododecylglycerol)phosphate, 3,3'-R,S-bis(monododecylglycerol)phosphate, 2,2'-S,S-bis(monooctadecylglycerol)phosphate, 3,3'-S,S-bis(monooctadecylglycerol)phosphate, 3,3'-R,R-bis(monooctadecylglycerol)phosphate, 3,3'-R,S-bis(monooctadecylglycerol)phosphate, 2,2'-S,S-bis(monooleoylglycerol)phosphate, 3,3'-S,S-bis(monooleoylglycerol)phosphate, 3,3'-R,R-bis(monooleoylglycerol)phosphate, 3,3'-R,S-bis(monooleoylglycerol)phosphate, 3-dodecylglycerol-1-phospho-3'-(1',2'-didodecyl)-glycerol, 3-octadecylglycerol-1-phospho-3'-(1',2'-dioctadecyl)-glycerol, 3-oleoylglycerol-1-phospho-3'-(1',2'-dioleoyl)-glycerol, glyceryl monoleate, methyl oleate, isostearic acid, palmitoleic acid, and oleic acid.

In one embodiment the amphiphilic lipid is selected from the group consisting of (S,S) Bisoleoyl-lysobisphosphatidic acid (LBPA), monogalactosyldiacylglycerol (MGDG), 1-palmitoyl-2-linoleoyl-sn-glycerol-3-phosphoethanolamine (PLPE), isostearic acid (IsostA), glycerol mono oleate (GMO), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanolamine (POPE), N-Palmitoyl phosphatidylethanolamine 16:0 (NPPE), N-acylphosphatidylethanolamine 12:0 (NAPE), methyl

oleate (MO), palmitoleic acid (PalmA), oleic acid (OIA), or mixtures thereof, and even more preferably wherein the amphiphilic lipid is or comprises LBPA. In a preferred embodiment LBPA is selected from the group consisting of 3,3'-R,S-LBPA, 3,3'-R,R-LBPA, S,S-2,2'-C18:0-ether-LBPA, S,R-Hemi-LBPA, S,S-2,2'-C12:0-ether-LBPA, sn-[2,3-dioleoyl]-glycerol-1-phospho-sn-1'-[2',3'-dioleoyl]-glycerol (S,S-BDP), 18:1 (Δ^9 -Cis)PG 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG) and 1-oleoyl-2-hydroxy-sn-glycero-3-phospho-(1'-rac-glycerol) (lysoPG).

In another embodiment the amphiphilic lipid is selected from the group consisting of one of the following mixtures: (S,S) Bisoleoyl-lysobisphosphatidic acid (LBPA) and monogalactosyldiacylglycerol (MGDG), (S,S) Bisoleoyl-lysobisphosphatidic acid (LBPA) and isostearic acid (IsostA), (S,S) Bisoleoyl-lysobisphosphatidic acid (LBPA) and glycerol mono oleate (GMO), or (S,S) Bisoleoyl-lysobisphosphatidic acid (LBPA) and 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine (PLPE).

"Helper Lipids"

A liposome composition disclosed herein may include one or more components in addition to those described in the preceding sections. For example, a liposome composition may include one or more "**enhancer lipids**" or "**helper lipids**" that enhance endosomal escape, and, consequently, delivery of the cargo by the liposome composition. Preferred enhancers are neutral lipids selected from the group consisting of sterol-based compound E18, compounds 6-10,12-21, 23, 24, 27-32 of Figure 11a being analogs of E18, solasodine, solasodine acetate, diosgenin, ceramide, cholesterol. Further preferred enhancers are neutral lipids selected from the group consisting of cholesterol, diosgenin, solasodine, and ceramide.

Thus, in one embodiment the composition of the invention comprises (e) a steroid and/or a ceramide selected from the group consisting of cholesterol, solasodine, ceramide, diosgenin, DOPE, sphingomyelin, and mixtures thereof. In a preferred embodiment, said steroid and/or a ceramide is selected from the group consisting of cholesterol, diosgenin, solasodine, ceramide, and mixtures thereof. In a more preferred embodiment, the lipid is selected from the group consisting of cholesterol, solasodine and a mixture thereof.

In one embodiment the composition of the invention comprises (e) a steroid, wherein the steroid is cholesterol. In one embodiment said composition, comprising (e) cholesterol, is used to deliver a therapeutic agent to a cell selected from epithelial cells (preferably epithelial cells from cervical cancer), macrophages, hepatocytes and cardiomyocytes. In a preferred embodiment in this context, the therapeutic agent is an RNA, preferably siRNA or mRNA. In a more preferred embodiment the therapeutic agent is an RNA, preferably mRNA, and the cell to which the therapeutic agent is delivered is a hepatocyte.

In one embodiment the composition described herein comprises (e) between 2.5 and 35 mol percent of at least one neutral lipid, wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

In one embodiment the composition described herein comprises (e) between 2.5 and 35 mol percent of at least one neutral lipid selected from the group consisting of cholesterol, diosgenin, solasodine, and ceramide, wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

One embodiment of the present invention provides a drug delivery system comprising a liposome having:

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of at least one encapsulating agent;
 - ii) an acid-cleavable polyethylene glycol conjugated lipid;
 - iii) between 15 and 45 mol percent of at least one fusogenic agent,
 - iv) between 2.5 and 35 mol percent of at least one neutral lipid, and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume;

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering.

One embodiment of the present invention provides a drug delivery system comprising a liposome having:

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of at least one encapsulating agent;
 - ii) between 1 and 20 mol percent of an acid-cleavable polyethylene glycol conjugated lipid;
 - iii) between 15 and 45 mol percent of at least one fusogenic agent,
 - iv) between 2.5 and 35 mol percent of at least one neutral lipid selected from the group consisting of cholesterol, diosgenin, solasodine, and ceramide, and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume,

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering.

Endosomal escape is the process of cytosolic entry of macromolecules such as small interfering RNAs (siRNAs) from a vesicular compartment, following initial endocytosis of the macromolecule and delivery vehicle into the target cell.

A **"helper lipid"** is usually a lipid that contributes either to 1) the formation of the lipid bilayer, or 2) the recognition of the delivery system by the limiting membrane of the endosome and 3) the membrane fusion of the delivery system with the limiting membrane of the endosome.

Therefore, a **"helper lipid"** can be used to improve efficiency or physical characteristics such as stability, but is not essential for the function of the liposomal delivery system.

The preferred lipids Cholesterol, Diosgenin, Solasodine, and ceramide showed high **"helper"** activity for siRNA transfection in HeLa cells. The lipid DOPE showed intermediate **"helper"** activity for siRNA delivery in HeLa cells, whereas DOPC and sphingomyelin showed no **"helper"** activity for siRNA transfection in HeLa cells.

In other words, a particular aspect of the present invention provides a drug delivery system comprising a liposome having:

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of at least one encapsulating agent;
 - ii) between 1 and 20 mol percent of an acid-cleavable polyethylene glycol conjugated lipid;
 - iii) between 15 and 45 mol percent of at least one fusogenic agent,
 - iv) between 13 and 35 mol percent of at least one helper lipid, and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume,

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering.

A more particular aspect of the present invention provides a drug delivery system comprising a liposome having:

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of at least one encapsulating agent;
 - ii) between 1 and 20 mol percent of an acid-cleavable polyethylene glycol conjugated lipid;
 - iii) between 15 and 45 mol percent of at least one fusogenic agent,
 - iva) between 13 and 15 mol percent of cholesterol, and

- ivb) between 15 and 36 mol percent of solasodine, and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume,

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and
wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering.

A still more particular aspect of the present invention provides a drug delivery system comprising a liposome having:

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of at least one encapsulating agent;
 - ii) an acid-cleavable polyethylene glycol conjugated lipid;
 - iii) between 15 and 45 mol percent of at least one fusogenic agent,
 - iva) 13 mol percent of cholesterol, and
 - ivb) 36 mol percent of solasodine; and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume,

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and
wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering.

A further more particular aspect of the present invention provides a drug delivery system comprising a liposome having:

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of at least one encapsulating agent;
 - ii) an acid-cleavable polyethylene glycol conjugated lipid;
 - iii) between 15 and 45 mol percent of at least one fusogenic agent,
 - iva) 15 mol percent of cholesterol, and
 - ivb) 15 mol percent of solasodine; and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume,

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and
wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering.

As used herein, the term “**neutral lipid**” is meant to refer to any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such

lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebrosides, and diacylglycerols.

Therapeutic agents

The liposome compositions disclosed herein may include one or more therapeutic agents and/or prophylactic agents (or abbreviated as just therapeutic/s or prophylactic/s).

The term "**therapeutic agent**" or "**prophylactic agent**" as used herein refers to any agent that, when administered to a subject, has a therapeutic, diagnostic, and/or prophylactic effect and/or elicits a desired biological and/or pharmacological effect. Therapeutic agents are also referred to as "**actives**" or "**active agents**".

Moreover, the term "**therapeutic agent**" is meant to refer to a drug, a protein, a peptide, a gene, an oligonucleotide, a compound, an antibody, a nanobody, a nucleic acid, a ribonucleoprotein, a small molecule, a non-Lipinski molecule, a biomimetic, or a natural compound, or another pharmaceutically active ingredient.

In one embodiment of the composition disclosed herein the therapeutic agent is selected from the group consisting of a drug, a protein, a peptide, a gene, an oligonucleotide, a compound, an antibody, a nanobody, a nucleic acid, a ribonucleoprotein, a small molecule, a non-lipinski molecule, a biomimetic, or a natural compound.

In a preferred embodiment, a therapeutic agent such as a therapeutic siRNA is encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation.

As used herein, the term "**non-Lipinski molecule**" refers to molecules that do not conform to Lipinski's rule of five, which is a rule to predict high probability of success or failure due to drug likeness for molecules complying with 2 or more of the following rules: 1) Molecular mass less than 500 Dalton; 2) High lipophilicity (expressed as LogP less than 5); 3) Less than 5 hydrogen bond donors; 4) Less than 10 hydrogen bond acceptors; 5) Molar refractivity should be between 40-130. The rule was formulated by Christopher A. Lipinski in 1997, based on the observation that most orally administered drugs are relatively small and moderately lipophilic molecules. The rule is indicative of molecular properties such as absorption, distribution, metabolism, and excretion ("ADME"), which are important for drug's pharmacokinetics in the human body. Capecchi et al., described that seven million entries of the PubChem database are non-Lipinski molecules (non-Lipinski PubChem (NLP)), 183,185 of which are also found in the ChEMBL database (non-Lipinski ChEMBL (NLC)) (Capecchi et al., PubChem and ChEMBL beyond Lipinski. Mol. Inf. 2019, 38, 1900016).

The term "**biomimetics**" refers to a compound that mimics a biological compound in its structure or function.

The term "**natural products** (NPs)" refers to small molecules produced by living organisms with potential applications in pharmacology and other industries as many of them are bioactive. Exemplary "natural products (NPs)" can be found in COLleCtion of Open Natural prodUCts (COCONUT), <https://coconut.naturalproducts.net>.

In some embodiments, a **therapeutic agent** is a **polynucleotide** or **nucleic acid** (e.g., ribonucleic acid or deoxyribonucleic acid). The term "**polynucleotide**," in its broadest sense, includes any compound and/or substance that is or can be incorporated into an oligonucleotide chain. Exemplary polynucleotides for use in accordance with the present disclosure include, but are not limited to, one or more of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) including messenger mRNA (mRNA), hybrids thereof, RNAi-inducing agents, RNAi agents, siRNAs, shRNAs, miRNAs, antisense RNAs, ribozymes, catalytic DNA, RNAs that induce triple helix formation, aptamers, vectors, guide RNA, etc. In some embodiments, a therapeutic and/or prophylactic is an RNA as described above. As used herein, an "**RNA**" refers to a ribonucleic acid that may be naturally or non-naturally occurring. For example, an RNA may include modified and/or non-naturally occurring components such as one or more nucleobases, nucleosides, nucleotides, or linkers. An RNA may include a cap structure, a chain terminating nucleoside, a stem loop, a polyA sequence, and/or a polyadenylation signal. An RNA may have a nucleotide sequence encoding a polypeptide of interest. RNAs useful in the compositions and methods described herein can be selected from the group consisting of, but are not limited to, shortmers, antagomirs, antisense, ribozymes, small interfering RNA (siRNA), asymmetrical interfering RNA (aiRNA), microRNA (miRNA), piRNA, sdRNA, snRNA, snoRNA, PAR, tsRNA, endo-siRNA, MSY-RNA, tel-sRNA, crasiRNA, moRNA, xiRNA, lncRNA, circRNA, regulatory non-coding RNA (ncRNA), Dicer-substrate RNA (dsRNA), small hairpin RNA (shRNA), transfer RNA (tRNA), messenger RNA (mRNA) (e.g. Cas9 mRNA), single-guide RNA (sgRNA), guide RNA, CRISPR RNA (crRNA), tracr RNA, and mixtures thereof. In certain embodiments, the RNA is an mRNA.

In other embodiments, a therapeutic and/or prophylactic is a **siRNA**. A siRNA may be capable of selectively *knocking down* or *down regulating expression* of a gene of interest. For example, a siRNA could be selected to silence or downregulating a gene associated with a particular disease, disorder, or condition upon administration to a subject in need thereof of a liposome composition including the siRNA. A siRNA may comprise a sequence that is complementary to a mRNA sequence that encodes a gene or protein of interest. In some embodiments, the siRNA may be an immunomodulatory siRNA.

In some embodiments, a therapeutic agent is a **shRNA** or a vector or plasmid encoding the same. A shRNA may be produced inside a target cell upon delivery of an appropriate construct to the nucleus. Constructs and mechanisms relating to shRNA are well known in the relevant arts

In certain embodiments, a therapeutic and/or prophylactic is a "**CRISPR guide RNA**" such as a sgRNA, a crispr guide RNA (also referred to as cr guide RNA), or a tracr RNA, a guide RNA mix. SgRNA, crispr guide RNA, tracr RNA and/or a guide RNA mix can be used as gene editing tools. For example, a guide RNA-Cas9 complex can affect mRNA translation of cellular genes.

Thus, the term "**CRISPR guide RNA**" as used herein refer to a specific RNA sequence selected from the group comprising sgRNA, crRNA, tracr RNA, a guide RNA mix. A "**guide RNA**" (**gRNA**) or "**guide RNA mix**" refers to a specific RNA sequence that recognizes the target DNA region of interest and directs the Cas nuclease there for editing. The "**guide RNA mix**" is made up of two parts: crispr RNA ("**crRNA**"), a 17-20 nucleotide sequence complementary to the target DNA, and a "**tracr RNA**", which serves as a binding scaffold for the Cas nuclease.

A "**single guide RNA**" or "**sgRNA**" refers to a single RNA molecule that contains both a target DNA specific "**crRNA**" fused to the scaffold "**tracrRNA**".

Moreover, in some embodiments the therapeutic agent is a **ribonucleoprotein**. The term "ribonucleoprotein" refers to a protein present in the nucleus and cytoplasm that is complexed with, and influences the processing of different low molecular weight ribonucleic acids. An exemplary ribonucleoprotein is a ribonucleoprotein complex consisting of Cas9 protein and single guide RNA (sgRNA).

In some embodiments, a therapeutic agent for the liposome delivery system disclosed herein is a regulatory long non coding RNA. The term "**LncRNA**" refers to RNA species longer than 200 nt, lacking protein-coding ability, and involved in regulation of genes expression and of diverse cellular functions. In some embodiments, a therapeutic agent for the liposome delivery system disclosed herein is a regulatory circular noncoding RNAs (circRNAs).

In some embodiments, a therapeutic agent for the liposome delivery system disclosed herein is a regulatory small non coding RNA. The term "**small non coding RNA**" refers to RNA species 18–200 nts long, lacking protein-coding ability, and having diverse roles. Small non coding RNA comprise microRNAs (miRNA), Small interfering RNAs (siRNA), PIWI-interacting RNAs (piRNAs), Sno-derived RNAs (sdRNAs), small nuclear RNA (snRNA), Small nucleolar RNAs (snoRNAs), Promoter-associated RNAs (PARs), tRNA-derived small RNAs (tsRNAs), endo-siRNA, MSY2-associated RNAs (MSY-RNAs), Telomere small RNAs (tel-sRNAs), Centrosome-

associated RNAs (crasiRNAs), microRNA-offset RNAs (moRNAs), and X-inactivation RNAs (xiRNAs).

Thus, a preferred therapeutic agent for the liposome delivery system disclosed herein is a peptide, a protein, an antibody, a nanobody, a nucleic acid, a ribonucleoprotein, a small molecule, a non-lipinski molecule, a biomimetic, or a natural compound. A further preferred therapeutic agent for the liposome delivery system disclosed herein is a CRISPR/Cas9 ribonucleoprotein, a CRISPR guide RNA, or a CRISPR associated Cas protein. A further more preferred therapeutic agent for the liposome delivery system disclosed herein is a nucleic acid selected from the group comprising ssDNA, dsDNA, ssRNA, dsRNA, aiRNA, miRNA, siRNA, piRNA, sdRNA, snRNA, snoRNA, PAR, tsRNA, endo-siRNA, MSY-RNA, tel-sRNA, crasiRNA, moRNA, xiRNA, lncRNA, ncRNA, circRNA, mRNA, sgRNA, crRNA, tracr RNA, guide RNA mix, self-amplifying RNA, ribozymes, and antisense oligonucleotides.

The delivery system disclosed herein may not only be suitable for the delivery of cargo comprising nucleic acid molecules, but may also be used to deliver cargo not comprising nucleic acid molecules, such as proteins. It is preferred that such proteins are overall negatively charged.

In one embodiment the therapeutic agent is a CRISPR/CAS9 ribonucleoprotein, a CRISPR guide RNA or a CRISPR associated Cas protein.

In one embodiment the therapeutic agent is a nucleic acid selected from the group consisting of ssDNA, dsDNA, ssRNA, dsRNA, aiRNA, miRNA, siRNA, piRNA, sdRNA, snRNA, snoRNA, PAR, tsRNA, endo-siRNA, MSY-RNA, tel-sRNA, crasiRNA, moRNA, xiRNA, lncRNA, ncRNA, circRNA, mRNA, sgRNA, crRNA, tracr RNA, guide RNA mix, self-amplifying RNA, ribozymes, and antisense oligonucleotides.

In one embodiment the therapeutic agent is selected from the group consisting of a CRISPR/CAS9 ribonucleoprotein, a siRNA, a mRNA, a guide RNA mix, and a protein.

In a preferred embodiment the therapeutic agent is an anionic therapeutic agent. This can be particularly suitable, where the anionic therapeutic agent interacts with a cationic composition component of the composition of the invention, such as with a cationic lipid or a positively charged peptide component.

Thus, the present invention also discloses a drug delivery system comprising a liposome having

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of at least one encapsulating agent;
 - ii) between 1 and 20 mol percent of an acid-cleavable polyethylene glycol conjugated lipid;
 - iii) between 15 and 45 mol percent of at least one fusogenic agent, and

- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume;

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering, and

wherein the therapeutic agent is a drug, a protein, a peptide, a gene, an oligonucleotide, a compound, an antibody, a nanobody, a nucleic acid, a ribonucleoprotein, a small molecule, a non-lipinski molecule, a biomimetic, or a natural compound.

In particular, the present invention also discloses a drug delivery system comprising a liposome having

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of at least one encapsulating agent;
 - ii) between 1 and 20 mol percent of an acid-cleavable polyethylene glycol conjugated lipid;
 - iii) between 15 and 45 mol percent of at least one fusogenic agent, and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume;

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering, and

wherein the therapeutic agent is a CRISPR/Cas9 ribonucleoprotein, a CRISPR guide RNA, or a CRISPR associated Cas protein.

More in particular, the present invention also discloses a drug delivery system comprising a liposome having

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of at least one encapsulating agent;
 - ii) between 1 and 20 mol percent of an acid-cleavable polyethylene glycol conjugated lipid;
 - iii) between 15 and 45 mol percent of at least one fusogenic agent, and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume;

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering, and

wherein the therapeutic agent is a nucleic acid selected from the group comprising ssDNA, dsDNA, ssRNA, dsRNA, aiRNA, miRNA, siRNA, piRNA, sdRNA, snRNA, snoRNA, PAR, tsRNA, endo-siRNA, MSY-RNA, tel-sRNA, crasiRNA, moRNA, xiRNA, lncRNA, ncRNA, circRNA, mRNA,

sgRNA, crRNA, tracr RNA, guide RNA mix, self-amplifying RNA, ribozymes, and antisense oligonucleotides.

Therapeutic proteins useful to be delivered with the liposome delivery system of this disclosure include vaccine proteins, antibodies against intracellular targets/intrabodies, a nanobodies, gene editing enzymes, cytotoxic proteins (for cancer therapeutics). More in particular useful therapeutic proteins can be insulin, erythropoietin (EPO), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), luteinizing hormone-releasing hormone (LHRH) analogs, interferons, Hepatitis B surface antigen, a vaccine protein.

In one embodiment the composition as described herein is a vaccine formulation.

In one embodiment the composition as described herein is a drug delivery system.

In one aspect the present invention provides a pharmaceutical composition comprising the composition disclosed herein.

In another aspect the present invention relates to the composition disclosed herein or the pharmaceutical composition disclosed herein for use in the treatment or prophylaxis of a disease.

In one embodiment, the treatment or prophylaxis of a disease is a vaccination against said disease.

In one embodiment of the composition or the pharmaceutical composition for use described herein or of the method of treatment described elsewhere herein, the disease is selected from the group consisting of infectious diseases, cancer, and genetic defects and diseases.

In one embodiment the composition or pharmaceutical composition described herein comprises a therapeutic agent or a pharmaceutically acceptable salt thereof, wherein the therapeutic agent is mRNA, and wherein the treatment or prophylaxis of a diseases is a vaccination against an infectious diseases or against cancer and/or against cancer progression.

In one embodiment the composition or pharmaceutical composition described herein comprises a therapeutic agent or a pharmaceutically acceptable salt thereof, wherein the therapeutic agent is mRNA, and wherein the treatment of a diseases is in the form of introducing (partly or fully) missing protein activity. In a preferred embodiment the treatment of a diseases is in the form of introducing (partly or fully) missing protein activity into cells of a specific tissue and/or organ. Upon delivery of the mRNA the partly or fully missing protein can be produced from the encoding mRNA to compensate for the partly or fully missing protein activity.

Alternatively or additionally to delivering encoding mRNA, proteins may also be delivered directly. This is particularly useful, where proteins are more difficult to express intracellularly from delivered mRNA, for instance due to folding limitations. An example may be certain forms of antibodies. Direct protein delivery may be used for instance to inhibit the activity of key regulatory proteins involved in

disease progression, such as controlling cell cycle regulators in cancer. Direct protein delivery may be used for instance to inhibit intracellular protein aggregates and/or condensates that are associated with certain disease states. These include, but are not limited to, neurological diseases such as Alzheimer's and Huntington's diseases, certain cancers where aberrant phase separation of proteins involved in transcriptional regulation contributes to oncogenesis, and a variety of myopathies and cardiomyopathies caused by abnormal accumulation of ribonucleoprotein (RNP) granule components (such as RBM20) or mutations in BAG3 or similar scaffold proteins.

Direct protein delivery may further be used for instance to complement expression from delivered mRNA in situations where mRNAs can be intrinsically unstable. Direct protein delivery may further be used in situations where therapeutic applications, such as vaccines, need to be distributed to regions where cold chain logistics cannot be guaranteed. Proteins can be more stable therapeutic agent options under these conditions compared to more labile mRNA.

In one embodiment the composition or pharmaceutical composition described herein comprises a therapeutic agent or a pharmaceutically acceptable salt thereof, wherein the therapeutic agent is a CRISPR/CAS9 ribonucleoprotein, and wherein the disease is a genetic disease. In a preferred embodiment, the treatment is in the form of gene editing. In one embodiment the composition or pharmaceutical composition described herein comprises a therapeutic agent or a pharmaceutically acceptable salt thereof, wherein the therapeutic agent is siRNA, and wherein the treatment of a diseases is in the form of post-transcriptional gene silencing of a diseases-related gene via RNA interference (RNAi).

In another aspect the present invention relates to the composition as disclosed herein, or the pharmaceutical composition as disclosed herein for use in the manufacture of a medicament.

As used herein, the term "**polypeptide**" or "**polypeptide of interest**" refers to a polymer of amino acid residues typically joined by peptide bonds that can be produced naturally (e.g., isolated or purified) or synthetically.

As used herein, "**modified**" means non-natural. For example, an RNA may be a modified RNA that is an RNA including one or more nucleobases, nucleosides, nucleotides, or linkers that are non-naturally occurring. A "**modified**" species may also be referred to herein as an "**altered**" species. Species may be modified or altered chemically, structurally, or functionally. For example, a modified nucleobase species may include one or more substitutions that are not naturally occurring.

As used herein, "**naturally occurring**" means existing in nature without artificial aid.

As used herein, a "**linker**" is a moiety connecting two moieties, for example, the connection between two nucleosides of a cap species. A linker may include one or more groups including but not limited to phosphate groups (e.g., phosphates, boranophosphates, thiophosphates, selenophosphates, and phosphonates), alkyl groups, amidates, amides, esters, ethers, or glycerols.

Polynucleotides and nucleic acids may be naturally or non-naturally occurring. Polynucleotides and nucleic acids may include one or more modified (e.g., altered or alternative) nucleobases, nucleosides, nucleotides, or combinations thereof. The nucleic acids and polynucleotides useful in the drug delivery system disclosed herein can include any useful modification or alteration, such as to the nucleobase, the sugar, or the internucleoside linkage (e.g., to a linking phosphate / to a phosphodiester linkage / to the phosphodiester backbone). In certain embodiments, alterations are present in each of the nucleobase, the sugar, and the internucleoside linkage. Modifications according to the present disclosure may be alterations of ribonucleic acids (RNAs) to deoxyribonucleic acids (DNAs), e.g., the substitution of the 2'-OH of the ribofuranosyl ring to 2'-H, threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs), unlocked nucleic acid (UNA), or hybrids thereof. Modifications can concern modified nucleobases such as hypoxanthine, 6-methyladenine, 5-methyl pyrimidines, particularly 5-methylcytosine, (5-Me-C), 5-hydroxymethylcytosine (HMC), glycosyl HMC, gentobiosyl HMC, synthetic nucleobases, such as 2-aminoadenine, 2-(methylamino) adenine, 2-(imidazolylalkyl)adenine, 2-(aminoalkylamino)adenine or other heterosubstituted alkyladenines, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N6 (6-aminoethyl)adenine and 2,6-diaminopurine. Preferred RNA modifications are 2'-O-Methylation (2'-OMe), 2'-O-methoxyethyl (2'-MOE), 2'-Fluoro (2'-F), 5-methoxy-uridine (5moU), N1-methylpseudouridine, pseudouridine, phosphorothioate (s). The phrase "**pharmaceutically acceptable**" is used herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

As used herein, "**pharmaceutically acceptable salts**" refers to derivatives of the disclosed compounds wherein the parent compound is altered by converting an existing acid or base moiety to its salt form (e.g., by reacting a free base group with a suitable organic acid). Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. Representative acid addition salts include acetate, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium,

magnesium, and the like, as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. The pharmaceutically acceptable salts of the present disclosure include the conventional non-toxic salts of the parent compound formed, for example, from non-toxic inorganic or organic acids. The pharmaceutically acceptable salts of the present disclosure can be synthesized from the parent compound which contains a basic or acidic moiety by conventional chemical methods. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with a stoichiometric amount of the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, nonaqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred. Lists of suitable salts are known in the art. Examples of particularly suitable pharmaceutically acceptable salts of the therapeutic agent as disclosed herein, wherein the therapeutic agent is a nucleic acid molecule, such as RNA, include sodium salts.

The phrase "**pharmaceutically acceptable excipient**," as used herein, refers to any ingredient other than the compounds described herein (for example, a vehicle capable of suspending, complexing, or dissolving the active compound) and having the properties of being substantially nontoxic and non-inflammatory in a patient. Excipients may include, for example: anti-adherents, antioxidants, binders, coatings, compression aids, disintegrants, dyes (colors), emollients, emulsifiers, fillers (diluent), film formers or coatings, flavors, fragrances, glidants (flow enhancers), lubricants, preservatives, printing inks, sorbents, suspending or dispersing agents, sweeteners, and waters of hydration. Exemplary excipients include, but are not limited to: butylated hydroxytoluene (BHT), calcium carbonate, calcium phosphate (dibasic), calcium stearate, croscarmellose, cross-linked polyvinyl pyrrolidone, citric acid, crospovidone, cysteine, ethylcellulose, gelatin, hydroxypropyl cellulose, hydroxypropyl methylcellulose, lactose, magnesium stearate, maltitol, mannitol, methionine, methylcellulose, methyl paraben, microcrystalline cellulose, polyethylene glycol, polyvinyl pyrrolidone, povidone, pregelatinized starch, propyl paraben, retinyl palmitate, shellac, silicon dioxide, sodium carboxymethyl cellulose, sodium citrate, sodium starch glycolate, sorbitol, starch (corn), stearic acid, sucrose, talc, titanium dioxide, vitamin A, vitamin E (alpha-tocopherol), vitamin C, xylitol, and other species disclosed herein.

In one aspect the present invention provides a pharmaceutical composition comprising the drug delivery system disclosed herein and at least one pharmaceutically acceptable excipient.

It has been shown that the compositions of the invention are effective in transporting nucleic acid molecules into a variety of cells (e.g. hepatocytes, macrophages, HeLa cells, cardiomyocytes). Therefore, in a further aspect the invention provides a composition comprising an artificial nucleic acid molecule, wherein the artificial nucleic acid molecule is an mRNA molecule having at least one open reading frame encoding an antigen derived from a pathogen, a therapeutic protein, or a CRISPR/CAS9

ribonucleoprotein, for use as a medicament, for use as a vaccine or for use in gene therapy, wherein the artificial nucleic acid molecule is associated with or complexed with components (b)-(d) and optionally (e) as defined herein, and wherein the composition is administered intramuscularly, intravenously, subcutaneously, intratumorally, orally, by inhalation or transdermally/topical.

It is noted that intravenously administered drugs are typically transported to the liver. In view of the successful transfections of the cell types tested in the examples disclosed herein, an intravenous administration is a preferred mode of administration.

In yet another aspect, the invention provides a method of transfecting a cell, wherein the cell is selected from the group consisting of epithelial cells (preferably epithelial cells from cervical cancer), macrophages, hepatocytes and cardiomyocytes, the method comprising the step of contacting the cell with the composition according to the invention. In a preferred embodiment, the method of transfecting a cell is an in vitro method.

As used herein, the term "**therapeutically effective amount**" means an amount of an agent to be delivered (e.g., nucleic acid, drug, composition, therapeutic agent, diagnostic agent, prophylactic agent, etc.) that is sufficient, when administered to a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition.

In another aspect the present invention relates to a method of treatment or prophylaxis in a subject, comprising administering to the subject the composition of the invention or the pharmaceutical composition of the invention in a therapeutically effective amount.

The efficacy concentration "EC50" of a therapeutic agent, such as an mRNA or a siRNA, is the concentration associated with efficacy in approximately 50% of the population. For example, the EC50 of a particular mRNA or siRNA delivered by the inventive liposome composition is the concentration associated with expression or downregulation of the mRNA target, in approximately 50% of the population. The method for EC50 calculation is described in Example 12.

More in particular, the EC50 refers to a statistically or graphically estimated concentration that is expected to cause one or more specified effects in 50% of a group of organisms under specified conditions.

Another important parameter is the toxic concentration TC50 that refers to a dose of a substance administered to test organisms (here HeLa cells or primary cells) that produces toxic effects in 50 percent of test organisms. The method for TC50 calculation is described in Example 12.

On the basis of the EC50 and TC50 can be calculated the "Therapeutic Index"

of a particular agent. The therapeutic index is defined as the ratio between TC50 and EC50:

$$\text{Therapeutic index} = \text{TC50} / \text{EC50}$$

As used herein, "**transfection**" refers to the introduction of a species (e.g., an RNA) into a cell. Transfection may occur, for example, in vitro, ex vivo, or in vivo.

In another aspect the present invention provides a method of producing the composition according to any one of claims 1 to 52, or the pharmaceutical composition as disclosed herein, comprising the steps of (i) combining and mixing a composition comprising the components (b)-(d) and optionally (e) as defined elsewhere herein; and (ii) mixing the composition obtained in step (i) with the therapeutic agent or with the pharmaceutically acceptable salt thereof (a).

In another embodiment the invention provides a method of producing the composition according to the invention, or the pharmaceutical composition according to the invention, comprising the step of combining and mixing a composition comprising the components (a)-(d) and optionally (e) as defined herein. Thus, components (a)-(d) and optionally (e) as defined herein may also be combined and mixed in a single step.

In another aspect the present invention provides a kit of parts comprising (i) a composition as disclosed herein or a pharmaceutical composition disclosed herein; and (ii) an instruction manual.

In yet another aspect, the invention provides a method of delivering an siRNA, an mRNA and/or a protein into a target cell, comprising the step of (i) contacting the target cell with a composition as defined herein, wherein component (a) is said siRNA, mRNA and/or protein.

"N/P ratio"

As used herein, the "N/P ratio", also referred as "**amine to phosphate**" or "**positive to negative**" is the molar ratio of positively-chargeable amine (N = nitrogen) groups in a lipid to negatively-charged nucleic acid phosphate (P) groups in a nucleic acid molecule, such as an oligonucleotide, being encapsulated in the drug delivery system. Thus, the N/P ratio states the relative proportion of molar amount of positive to negative charges in the formulation. As a consequence, the "N/P ratio" is a measure to ensure encapsulation, and is used to determine how much encapsulating agent is necessary for a given amount of cargo or viceversa.

More in particular, the number of positive charges per molecule equals the number of amino groups present on each individual molecule (N) of encapsulating agent. For the cargo (in case of oligonucleotides) the number of negative charges per molecule is the number of phosphate groups present.

$$N/P \text{ ratio} = \frac{N}{P} = \frac{n(\text{positive charges})}{n(\text{negative charges})}$$

$n(\text{positive charges}) = n(\text{encapsulating agent}) * N_{\text{amino groups per molecule}}$

$n(\text{negative charges}) = n(\text{oligonucleotide}) * N_{\text{phosphate groups per oligonucleotide}}$

With $n(\text{encapsulating agent})$ and $n(\text{oligonucleotide})$ being the molar amount of respective entity, $N_{\text{amino groups per molecule}}$ the number of amino groups present on the encapsulating agent, $N_{\text{phosphate groups per oligonucleotide}}$ the number of phosphate groups on the oligonucleotide. For the latter, in case it is a double stranded oligonucleotide (e.g. siRNA) or several single oligonucleotides (e.g. guide RNA mix) the sum of phosphates of both strands is considered. More details about the calculation are given in Example 31.

Preferentially, in the drug delivery system of the invention the N/P ratio is from about 1:1 to about 50:1. Therefore, a preferred embodiment of the present invention provides a drug delivery system comprising a liposome having

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of at least one encapsulating agent;
 - ii) between 1 and 20 mol percent of an acid-cleavable polyethylene glycol conjugated lipid;
 - iii) between 15 and 45 mol percent of at least one fusogenic agent, and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume;

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering, and

wherein the N/P ratio is from about 1:1 to about 50:1.

A more preferred embodiment of the present invention provides a drug delivery system comprising a liposome having

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of at least one encapsulating agent;
 - ii) between 1 and 20 mol percent of an acid-cleavable polyethylene glycol conjugated lipid;
 - iii) between 15 and 45 mol percent of at least one fusogenic agent, and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume;

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering, and

wherein the therapeutic agent is a nucleic acid selected from the group comprising ssDNA, dsDNA, ssRNA, dsRNA, miRNA, siRNA, mRNA, sgRNA, crRNA, tracr RNA, guide RNA mix, self-amplifying RNA, ribozymes, and antisense oligonucleotides;
wherein the N/P ratio is from about 1:1 to about 50:1.

An embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 1:1 to about 6:1. A preferred embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 1:1 to about 10:1. A embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 1:1 to about 20:1. A embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 1:1 to about 30:1. A embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 1:1 to about 40:1. A embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 1:1 to about 50:1. An embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 2:1 to about 6:1. A embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 2:1 to about 10:1. A embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 2:1 to about 20:1. A embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 2:1 to about 30:1. A embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 2:1 to about 40:1. A embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 2:1 to about 50:1. An embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 3:1 to about 6:1. A embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 3:1 to about 10:1. A embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 3:1 to about 20:1. A embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 3:1 to about 30:1. A embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 3:1 to about 40:1. A embodiment of the invention relates to a drug delivery system as disclosed herein, wherein the N/P ratio is from about 3:1 to about 50:1.

A more particular aspect of the invention relates to a drug delivery system as disclosed herein, comprising

- (b) a siRNA as therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume; and

wherein the N/P ratio is from about 1:1 to about 40:1, and preferably from 5:1 to 40:1.

A further more particular aspect of the invention relates to a drug delivery system as disclosed herein, comprising

- (b) a mRNA as therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume; and

wherein the N/P ratio is from about 1:1 to about 20:1.

A further more particular aspect of the invention relates to a drug delivery system as disclosed herein, comprising

- (b) a guide RNA mix as therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume, said guide RNA mix comprising a crRNA and a tracr RNA; and

wherein the N/P ratio is from about 5:1 to about 10:1.

A further more particular aspect of the invention relates to a drug delivery system as disclosed herein, comprising

- (b) a guide RNA mix as therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume, said guide RNA mix comprising a crRNA and a tracr RNA; and

wherein the N/P ratio is 5.46:1, or 5.07:1, or 8.45:1, or 9.10:1 or any value comprised inbetween.

"Liposome compositions"

As used herein, a "**liposome composition**" refers to the composition of a liposome in term of lipids and therapeutic agents. More in particular, a "**liposome composition**" refers to the composition of a liposome as disclosed herein in terms of fusogenic agent, encapsulating agent, acid-cleavable PEG conjugated lipid, therapeutic agent, and optionally helper lipid.

In other word, a preferred liposome composition of the inventive drug delivery system disclosed herein comprises the following **liposome components** (also referred to as "**agents**" or "**compounds**"): an "**encapsulating agent**", an "**acid-cleavable agent or acid-cleavable PEG-lipid**", a "**fusogenic agent**", a "**therapeutic agent**", and optionally an "**helper lipid or agent**" (also referred to as "enhancer" or "enhancer lipid" or "enhancer agent")

The "cationic lipid and/or a beta-alanyl-prolyl-cysteine methyl ester" as recited as component (d) in the claims can preferably be any "encapsulating agent" as defined herein.

The "amphiphilic lipid" recited as component (c) in the claims can preferably be any "fusogenic agent" as defined herein.

The “PEG-monoorthoester-lipid” recited as component (b) in the claims can preferably be any PEG-monoorthoester-lipid as defined herein in the context of “acid-cleavable polyethylene glycol conjugated lipid”.

The “steroid and/or a ceramide” recited as component (e) in the claims can be any steroid and/or a ceramide as defined herein in the context of a “helper lipid” or a “helper agent”.

The wording **liposome component** is used within the meaning of the disclosed invention interchangeably with the terms **"agent"** and **"compound"**.

A **"liposome composition variation"** or **"variation"** refers to a liposome composition resulting from the replacement of a liposome component or from the variation of its ratio.

An embodiment of the present invention refers to a drug delivery system comprising a liposome having:

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride and/or PD740,
 - ii) between 1 and 11 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
 - iii) between 15 and 45 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate, and
 - iv) between 13 and 35 mol percent of cholesterol; and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume;

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering.

A preferred embodiment of the present invention refers to a drug delivery system comprising a liposome having:

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) 50 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride,
 - ii) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
 - iii) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate, and
 - iv) 25 mol percent of cholesterol; and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume;

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering.

A further preferred embodiment of the present invention refers to a drug delivery system comprising a liposome having:

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) 50 mol percent of PD740,
 - ii) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
 - iii) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate, and
 - iv) 25 mol percent of cholesterol; and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume;

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering.

A further more preferred embodiment of the present invention refers to a drug delivery system comprising a liposome having:

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - ia) 25 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride,
 - ib) 25 mol percent of PD740,
 - ii) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
 - iii) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate, and
 - iv) 25 mol percent of cholesterol; and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume;

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering.

A still more preferred embodiment of the present invention refers to a drug delivery system comprising a liposome having:

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) 33 mol percent of PD740,
 - ii) 7 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,

- iii) 27 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate, and
- iv) 33 mol percent of cholesterol; and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume;

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering.

An alternative embodiment of the present invention refers to a drug delivery system comprising a liposome having:

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 42 and 50 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride,
 - ii) between 4 and 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
 - iii) between 4 and 15 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate,
 - iva) between 13 and 15 mol percent of cholesterol, and
 - ivb) between 15 and 36 mol percent of solasodine.
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume;

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering.

A further alternative embodiment of the invention provides a drug delivery system comprising a liposome having:

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - ia) 25 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride,
 - ib) 25 mol percent of PD740,
 - ii) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
 - iii) 20 mol percent of monogalactosyldiacylglycerol or 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine, and
 - iv) 25 mol percent of cholesterol; and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume;

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering.

"Unilamellar liposomes"

In a preferred embodiment, the composition is a liposome composition. In a more preferred embodiment the liposome is a unilamellar and/or univesicular liposome.

Thus, a preferred embodiment of the present invention relates to a drug delivery system as disclosed herein, wherein the liposome is a unilamellar and/or univesicular liposome.

Unilamellar liposomes are characterized for having one aqueous compartment, delimited by a single bilayer membrane. Unilamellar liposomes are usually spherical vesicles. In contrast, multilamellar liposomes are characterized by a liposome structure having plural concentric circular membranes similar to "coats layers of onion" and having shell-like concentric circular aqueous compartments present between said membranes.

Moreover, the inventive drug delivery system comprises essentially only **"univesicular liposomes"**. However, the inventive drug delivery system can also comprise some **"multivesicular liposome"**.

In the present invention, **"univesicular liposome"** (ULV, the same meaning as that of **mononuclear liposome**) indicates a liposome structure having a single inner aqueous phase, and such liposomes have a Z-average particle diameter size of nanometer order, usually about 20 to 500 nm. On the other hand, the **"multivesicular liposome"** (MVL) indicates a liposome structure comprising a lipid membrane surrounding plural non-concentric circular inner aqueous phases. **Multivesicular liposomes** and the **multilamellar liposomes** have a Z-average particle diameter of micrometer order, usually about 0.2 to 25 μm .

As used herein, the term **"delivering"** means providing an entity to a destination. For example, delivering a therapeutic and/or prophylactic to a subject may involve administering a liposome composition including the **therapeutic and/or prophylactic** to the subject (e.g., by an intravenous, intramuscular, intradermal, or subcutaneous route). Administration of a liposome composition to a mammal or mammalian cell may involve contacting one or more cells with the liposome composition.

As used herein, the term **"enhanced delivery"** means delivery of more (e.g., at least 1.5 fold more, at least 2-fold more, at least 3-fold more, at least 4-fold more, at least 5-fold more, at least 6-fold more, at least 7-fold more, at least 8-fold more, at least 9-fold more, at least 10-fold more) of a **therapeutic and/or prophylactic** by a liposome to a target tissue or cell of interest compared to the level of delivery of a therapeutic and/or prophylactic without that liposome or by a previously known delivery system (e.g. lipofectamine) to a target tissue or cell of interest.

As used herein, the term "**isomer**" means any geometric isomer, tautomer, zwitterion, stereoisomer, enantiomer, or diastereomer of a compound. Compounds may include one or more chiral centers and/or double bonds and may thus exist as stereoisomers, such as double-bond isomers (i.e., geometric E/Z isomers) or diastereomers (*e.g.*, enantiomers (i.e., (+) or (-)) or *cis/trans* isomers). The present disclosure encompasses any and all isomers of the compounds described herein, including stereomerically pure forms (*e.g.*, geometrically pure, enantiomerically pure, or diastereomerically pure) and enantiomeric and stereoisomeric mixtures, *e.g.*, racemates. Enantiomeric and stereomeric mixtures of compounds and means of resolving them into their component enantiomers or stereoisomers are well-known.

As used herein, the term "**isoform**" refers to compounds that for example share the same backbone structure but include a different number of fatty acids attached, or include fatty acids of different chain length or with a different linkage.

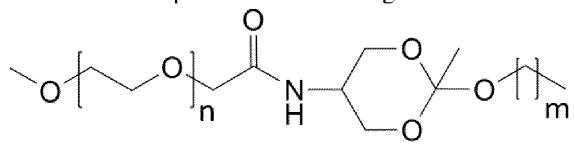
Certain embodiments of the invention

In one aspect, the invention provides a method of introducing an mRNA into a target cell comprising the steps of

(i) contacting said target cell with a composition comprising components a, b, c, d, e;

wherein (a) is said mRNA; and

wherein (b) is a PEG-monoorthoester lipid with the following structure:



wherein n is an integer between 35 and 120 and wherein m is an integer between 10 and 27; and preferably wherein n is an integer between 40 and 110 and m is an integer of between 15 and 23; and wherein (c) is an amphiphilic lipid; and

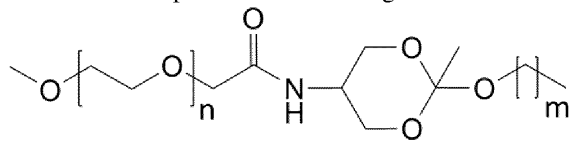
wherein (d) is a cationic lipid and/or a beta-alanyl-prolyl-cysteine methyl ester; and

wherein (e) is cholesterol.

Further provided is a composition comprising components a, b, c, d, e;

wherein (a) is said mRNA; and

wherein (b) is a PEG-monoorthoester lipid with the following structure:



wherein n is an integer between 35 and 120 and wherein m is an integer between 10 and 27; and preferably wherein n is an integer between 40 and 110 and m is an integer of between 15 and 23; and wherein (c) is an amphiphilic lipid; and wherein (d) is a cationic lipid and/or a beta-alanyl-prolyl-cysteine methyl ester; and wherein (e) is cholesterol; for use in the treatment of a disease or for use in the vaccination of a human subject.

Preferred embodiments in the above described method and composition for use are characterized by a combination of target cell, composition type and administration mode, as set out in the table below. The annotation as “any” indicates that any component falling under the definition disclosed herein of the categories above may be used in the respective combination.

Table 16

Embod.	Component (c)	Component (d)	Composition type	Component (a)	Administration mode
A1	LBPA	any	any	any	any
A2	LBPA	any	liposome	any	any
A3	LBPA	any	LNP	any	any
A4	LBPA	any	any	mRNA	any
A5	LBPA	any	liposome	mRNA	any
A6	LBPA	any	LNP	mRNA	any
A7	LBPA	any	any	siRNA	any
A8	LBPA	any	liposome	siRNA	any
A9	LBPA	any	LNP	siRNA	any
B1	LBPA	DOTAP	any	any	any
B2	LBPA	DOTAP	liposome	any	any
B3	LBPA	DOTAP	LNP	any	any
B4	LBPA	DOTAP	any	mRNA	any
B5	LBPA	DOTAP	liposome	mRNA	any
B6	LBPA	DOTAP	LNP	mRNA	any
B7	LBPA	DOTAP	any	mRNA	intravenously
B8	LBPA	DOTAP	liposome	mRNA	intravenously
B9	LBPA	DOTAP	LNP	mRNA	intravenously
B10	LBPA	DOTAP	any	mRNA	intramuscularly
B11	LBPA	DOTAP	liposome	mRNA	intramuscularly
B12	LBPA	DOTAP	LNP	mRNA	intramuscularly
B13	LBPA	DOTAP	any	mRNA	intratumorally
B14	LBPA	DOTAP	liposome	mRNA	intratumorally
B15	LBPA	DOTAP	LNP	mRNA	intratumorally
B16	LBPA	DOTAP	any	siRNA	any
B17	LBPA	DOTAP	liposome	siRNA	any
B18	LBPA	DOTAP	LNP	siRNA	any
B19	LBPA	DOTAP	any	siRNA	intravenously
B20	LBPA	DOTAP	liposome	siRNA	intravenously
B21	LBPA	DOTAP	LNP	siRNA	intravenously
B22	LBPA	DOTAP	any	siRNA	intramuscularly
B23	LBPA	DOTAP	liposome	siRNA	intramuscularly
B24	LBPA	DOTAP	LNP	siRNA	intramuscularly
C1	LBPA	PD740	any	any	any
C2	LBPA	PD740	Liposome	any	any
C3	LBPA	PD740	LNP	any	any
C4	LBPA	PD740	any	mRNA	any
C5	LBPA	PD740	Liposome	mRNA	any
C6	LBPA	PD740	LNP	mRNA	any
C7	LBPA	PD740	any	mRNA	intravenously

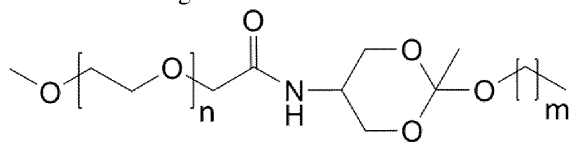
C8	LBPA	PD740	Liposome	mRNA	intravenously
C9	LBPA	PD740	LNP	mRNA	intravenously
C10	LBPA	PD740	any	mRNA	intramuscularly
C11	LBPA	PD740	Liposome	mRNA	intramuscularly
C12	LBPA	PD740	LNP	mRNA	intramuscularly
C13	LBPA	PD740	any	siRNA	any
C14	LBPA	PD740	Liposome	siRNA	any
C15	LBPA	PD740	LNP	siRNA	any
C16	LBPA	PD740	any	siRNA	intravenously
C17	LBPA	PD740	Liposome	siRNA	intravenously
C18	LBPA	PD740	LNP	siRNA	intravenously
C19	LBPA	PD740	any	siRNA	intramuscularly
C20	LBPA	PD740	Liposome	siRNA	intramuscularly
C21	LBPA	PD740	LNP	siRNA	intramuscularly
D1	LBPA	DOMA	any	any	any
D2	LBPA	DOMA	Liposome	any	any
D3	LBPA	DOMA	LNP	any	any
D4	LBPA	DOMA	any	mRNA	any
D5	LBPA	DOMA	Liposome	mRNA	any
D6	LBPA	DOMA	LNP	mRNA	any
D7	LBPA	DOMA	any	siRNA	any
D8	LBPA	DOMA	Liposome	siRNA	any
D9	LBPA	DOMA	LNP	siRNA	any
E1	LBPA	DOTMA	any	any	any
E2	LBPA	DOTMA	Liposome	any	any
E3	LBPA	DOTMA	LNP	any	any
E4	LBPA	DOTMA	any	mRNA	any
E5	LBPA	DOTMA	Liposome	mRNA	any
E6	LBPA	DOTMA	LNP	mRNA	any
E7	LBPA	DOTMA	any	siRNA	any
E8	LBPA	DOTMA	Liposome	siRNA	any
E9	LBPA	DOTMA	LNP	siRNA	any
F1	LBPA	EPC 18:1	any	any	any
F2	LBPA	EPC 18:1	Liposome	any	any
F3	LBPA	EPC 18:1	LNP	any	any
F4	LBPA	EPC 18:1	LNP	mRNA	any
F5	LBPA	EPC 18:1	LNP	mRNA	any
F6	LBPA	EPC 18:1	LNP	mRNA	any
F7	LBPA	EPC 18:1	LNP	siRNA	any
F8	LBPA	EPC 18:1	LNP	siRNA	any
F9	LBPA	EPC 18:1	LNP	siRNA	any
G1	any	DOTAP	any	any	any
G2	any	DOTAP	Liposome	any	any
G3	any	DOTAP	LNP	any	any
G4	any	DOTAP	any	mRNA	any
G5	any	DOTAP	Liposome	mRNA	any
G6	any	DOTAP	LNP	mRNA	any
G7	any	DOTAP	any	siRNA	any
G8	any	DOTAP	Liposome	siRNA	any
G9	any	DOTAP	LNP	siRNA	any
H1	LBPA	DOTAP	any	any	any
H2	LBPA	DOTAP	Liposome	any	any
H3	LBPA	DOTAP	LNP	any	any
H4	LBPA	DOTAP	any	mRNA	any
H5	LBPA	DOTAP	Liposome	mRNA	any
H6	LBPA	DOTAP	LNP	mRNA	any
H7	LBPA	DOTAP	any	siRNA	any
H8	LBPA	DOTAP	Liposome	siRNA	any
H9	LBPA	DOTAP	LNP	siRNA	any
I1	2,2'-S,S LBPA	DOTAP	any	any	any
I2	2,2'-S,S LBPA	DOTAP	Liposome	any	any
I3	2,2'-S,S LBPA	DOTAP	LNP	any	any
I4	2,2'-S,S LBPA	DOTAP	any	mRNA	any
I5	2,2'-S,S LBPA	DOTAP	Liposome	mRNA	any

I6	2,2'-S,S LBPA	DOTAP	LNP	mRNA	any
I7	2,2'-S,S LBPA	DOTAP	any	siRNA	any
I8	2,2'-S,S LBPA	DOTAP	Liposome	siRNA	any
I9	2,2'-S,S LBPA	DOTAP	LNP	siRNA	any
J1	3,3'-S,S LBPA	DOTAP	any	any	any
J2	3,3'-S,S LBPA	DOTAP	Liposome	any	any
J3	3,3'-S,S LBPA	DOTAP	LNP	any	any
J4	3,3'-S,S LBPA	DOTAP	any	mRNA	any
J5	3,3'-S,S LBPA	DOTAP	Liposome	mRNA	any
J6	3,3'-S,S LBPA	DOTAP	LNP	mRNA	any
J7	3,3'-S,S LBPA	DOTAP	any	siRNA	any
J8	3,3'-S,S LBPA	DOTAP	Liposome	siRNA	any
J9	3,3'-S,S LBPA	DOTAP	LNP	siRNA	any
K1	3,3'-R,S, LBPA	DOTAP	any	any	any
K2	3,3'-R,S, LBPA	DOTAP	Liposome	any	any
K3	3,3'-R,S, LBPA	DOTAP	LNP	any	any
K4	3,3'-R,S, LBPA	DOTAP	any	mRNA	any
K5	3,3'-R,S, LBPA	DOTAP	Liposome	mRNA	any
K6	3,3'-R,S, LBPA	DOTAP	LNP	mRNA	any
K7	3,3'-R,S, LBPA	DOTAP	any	siRNA	any
K8	3,3'-R,S, LBPA	DOTAP	Liposome	siRNA	any
K9	3,3'-R,S, LBPA	DOTAP	LNP	siRNA	any
L1	3,3'-R,R, LBPA	DOTAP	any	any	any
L2	3,3'-R,R, LBPA	DOTAP	Liposome	any	any
L3	3,3'-R,R, LBPA	DOTAP	LNP	any	any
L4	3,3'-R,R, LBPA	DOTAP	any	mRNA	any
L5	3,3'-R,R, LBPA	DOTAP	Liposome	mRNA	any
L6	3,3'-R,R, LBPA	DOTAP	LNP	mRNA	any
L7	3,3'-R,R, LBPA	DOTAP	any	siRNA	any
L8	3,3'-R,R, LBPA	DOTAP	Liposome	siRNA	any
L9	3,3'-R,R, LBPA	DOTAP	LNP	siRNA	any
M1	2,2'-S,S LBPA with 18:0 fatty acids	DOTAP	any	any	any
M2	2,2'-S,S LBPA with 18:0 fatty acids	DOTAP	Liposome	any	any
M3	2,2'-S,S LBPA with 18:0 fatty acids	DOTAP	LNP	any	any
M4	2,2'-S,S LBPA with 18:0 fatty acids	DOTAP	any	mRNA	any
M5	2,2'-S,S LBPA with 18:0 fatty acids	DOTAP	Liposome	mRNA	any
M6	2,2'-S,S LBPA with 18:0 fatty acids	DOTAP	LNP	mRNA	any
M7	2,2'-S,S LBPA with 18:0 fatty acids	DOTAP	any	siRNA	any
M8	2,2'-S,S LBPA with 18:0 fatty acids	DOTAP	Liposome	siRNA	any
M9	2,2'-S,S LBPA with 18:0 fatty acids	DOTAP	LNP	siRNA	any
N1	2,2'-S,S LBPA with 12:0 fatty acids	DOTAP	any	any	any
N2	2,2'-S,S LBPA with 12:0 fatty acids	DOTAP	Liposome	any	any
N3	2,2'-S,S LBPA with 12:0 fatty acids	DOTAP	LNP	any	any
N4	2,2'-S,S LBPA with 12:0 fatty acids	DOTAP	any	mRNA	any
N5	2,2'-S,S LBPA with 12:0 fatty acids	DOTAP	Liposome	mRNA	any
N6	2,2'-S,S LBPA with 12:0 fatty acids	DOTAP	LNP	mRNA	any
N7	2,2'-S,S LBPA with 12:0 fatty acids	DOTAP	any	siRNA	any
N8	2,2'-S,S LBPA with 12:0 fatty acids	DOTAP	Liposome	siRNA	any

N9	2,2'-S,S LBPA with 12:0 fatty acids	DOTAP	LNP	siRNA	any
O1	hemi-LBPA (3 fatty acids)	DOTAP	any	any	any
O2	hemi-LBPA (3 fatty acids)	DOTAP	Liposome	any	any
O3	hemi-LBPA (3 fatty acids)	DOTAP	LNP	any	any
O4	hemi-LBPA (3 fatty acids)	DOTAP	any	mRNA	any
O5	hemi-LBPA (3 fatty acids)	DOTAP	Liposome	mRNA	any
O6	hemi-LBPA (3 fatty acids)	DOTAP	LNP	mRNA	any
O7	hemi-LBPA (3 fatty acids)	DOTAP	any	siRNA	any
O8	hemi-LBPA (3 fatty acids)	DOTAP	Liposome	siRNA	any
O9	hemi-LBPA (3 fatty acids)	DOTAP	LNP	siRNA	any
P1	any	PD740, DOTAP	any	any	any
P2	any	PD740, DOTAP	Liposome	any	any
P3	any	PD740, DOTAP	LNP	any	any
P4	any	PD740, DOTAP	any	mRNA	any
P5	any	PD740, DOTAP	Liposome	mRNA	any
P6	any	PD740, DOTAP	LNP	mRNA	any
P7	any	PD740, DOTAP	any	siRNA	any
P8	any	PD740, DOTAP	Liposome	siRNA	any
P9	any	PD740, DOTAP	LNP	siRNA	any
Q1	LBPA	PD740, DOTAP	any	any	any
Q2	LBPA	PD740, DOTAP	Liposome	any	any
Q3	LBPA	PD740, DOTAP	LNP	any	any
Q4	LBPA	PD740, DOTAP	any	mRNA	any
Q5	LBPA	PD740, DOTAP	Liposome	mRNA	any
Q6	LBPA	PD740, DOTAP	LNP	mRNA	any
Q7	LBPA	PD740, DOTAP	any	mRNA	intravenously
Q8	LBPA	PD740, DOTAP	Liposome	mRNA	intravenously
Q9	LBPA	PD740, DOTAP	LNP	mRNA	intravenously
Q10	LBPA	PD740, DOTAP	any	mRNA	intramuscularly
Q11	LBPA	PD740, DOTAP	Liposome	mRNA	intramuscularly
Q12	LBPA	PD740, DOTAP	LNP	mRNA	intramuscularly
Q13	LBPA	PD740, DOTAP	any	siRNA	any
Q14	LBPA	PD740, DOTAP	Liposome	siRNA	any
Q15	LBPA	PD740, DOTAP	LNP	siRNA	any
Q16	LBPA	PD740, DOTAP	any	siRNA	intravenously
Q17	LBPA	PD740, DOTAP	Liposome	siRNA	intravenously
Q18	LBPA	PD740, DOTAP	LNP	siRNA	intravenously
Q19	LBPA	PD740, DOTAP	any	siRNA	intramuscularly
Q20	LBPA	PD740, DOTAP	Liposome	siRNA	intramuscularly
Q21	LBPA	PD740, DOTAP	LNP	siRNA	intramuscularly
R1	MGDG	PD740, DOTAP	any	any	any
R2	MGDG	PD740, DOTAP	Liposome	any	any
R3	MGDG	PD740, DOTAP	LNP	any	any
R4	MGDG	PD740, DOTAP	any	mRNA	any
R5	MGDG	PD740, DOTAP	Liposome	mRNA	any
R6	MGDG	PD740, DOTAP	LNP	mRNA	any
R7	MGDG	PD740, DOTAP	any	siRNA	any
R8	MGDG	PD740, DOTAP	Liposome	siRNA	any
R9	MGDG	PD740, DOTAP	LNP	siRNA	any
S1	PLPE	PD740, DOTAP	any	any	any
S2	PLPE	PD740, DOTAP	Liposome	any	any
S3	PLPE	PD740, DOTAP	LNP	any	any
S4	PLPE	PD740, DOTAP	any	mRNA	any
S5	PLPE	PD740, DOTAP	Liposome	mRNA	any

S6	PLPE	PD740, DOTAP	LNP	mRNA	any
S7	PLPE	PD740, DOTAP	any	siRNA	any
S8	PLPE	PD740, DOTAP	Liposome	siRNA	any
S9	PLPE	PD740, DOTAP	LNP	siRNA	any
T1	NAPE	PD740, DOTAP	any	any	any
T2	NAPE	PD740, DOTAP	liposome	any	any
T3	NAPE	PD740, DOTAP	LNP	any	any
T4	NAPE	PD740, DOTAP	any	mRNA	any
T5	NAPE	PD740, DOTAP	liposome	mRNA	any
T6	NAPE	PD740, DOTAP	LNP	mRNA	any
T7	NAPE	PD740, DOTAP	any	siRNA	any
T8	NAPE	PD740, DOTAP	liposome	siRNA	any
T9	NAPE	PD740, DOTAP	LNP	siRNA	any
U1	LBPA, MGDG	PD740, DOTAP	any	any	any
U2	LBPA, MGDG	PD740, DOTAP	Liposome	any	any
U3	LBPA, MGDG	PD740, DOTAP	LNP	any	any
U4	LBPA, MGDG	PD740, DOTAP	any	mRNA	any
U5	LBPA, MGDG	PD740, DOTAP	Liposome	mRNA	any
U6	LBPA, MGDG	PD740, DOTAP	LNP	mRNA	any
U7	LBPA, MGDG	PD740, DOTAP	any	siRNA	any
U8	LBPA, MGDG	PD740, DOTAP	Liposome	siRNA	any
U9	LBPA, MGDG	PD740, DOTAP	LNP	siRNA	any
V1	LBPA, PLPE	PD740, DOTAP	any	any	any
V2	LBPA, PLPE	PD740, DOTAP	Liposome	any	any
V3	LBPA, PLPE	PD740, DOTAP	LNP	any	any
V4	LBPA, PLPE	PD740, DOTAP	any	mRNA	any
V5	LBPA, PLPE	PD740, DOTAP	Liposome	mRNA	any
V6	LBPA, PLPE	PD740, DOTAP	LNP	mRNA	any
V7	LBPA, PLPE	PD740, DOTAP	any	siRNA	any
V8	LBPA, PLPE	PD740, DOTAP	Liposome	siRNA	any
V9	LBPA, PLPE	PD740, DOTAP	LNP	siRNA	any

In the context of any of the embodiments described herein above, wherein (b) is a PEG-monoorthoester lipid with the following structure:



it is most preferred that n is 22, 45 or 110. It is further most preferred that m is 17. Thus, it is preferred that the PEG-monoorthoester lipid is α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅ (PEG2000-orthoester-C18 or PEGOEC18).

Further provided herein is a composition comprising

- (a) a therapeutic agent or a pharmaceutically acceptable salt thereof;
- (b) a PEG-monoorthoester-lipid as disclosed herein;
- (c) an amphiphilic lipid;
- (d) a cationic lipid and/or a beta-alanyl-prolyl-cysteine methyl ester; and
- optionally (e) a steroid and/or a ceramide and/or DOPE; and

wherein the combination of the compounds (a), (c) and (d) is a combination of compounds (a), (c) and (d) selected from the group consisting of the embodiments (A1) through (V9) that are listed in Table 16 above.

Further provided herein is a composition comprising

- (a) a therapeutic agent or a pharmaceutically acceptable salt thereof;
- (b) a PEG-monoorthoester-lipid as disclosed herein;
- (c) an amphiphilic lipid;
- (d) a cationic lipid and/or a beta-alanyl-prolyl-cysteine methyl ester; and
- (e) cholesterol,

wherein the combination of the compounds (a), (c) and (d) is a combination of compounds (a), (c) and (d) selected from the group consisting of the embodiments (A1) through (V9) that are listed in Table 16 above.

Further provided herein is a composition comprising

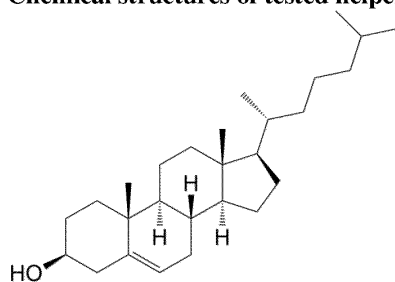
- (a) a therapeutic agent or a pharmaceutically acceptable salt thereof;
- (b) a PEG-monoorthoester-lipid as disclosed herein;
- (c) an amphiphilic lipid;
- (d) a cationic lipid and/or a beta-alanyl-prolyl-cysteine methyl ester; and
- (e) cholesterol,

wherein the combination of the compounds (a), (c) and (d) is a combination of compounds (a), (c) and (d) selected from the group consisting of the embodiments (A1) through (V9) that are listed in Table 16 above; and wherein the composition is of a type as set out in the same respective embodiment (A1) through (V9) that was selected from said table.

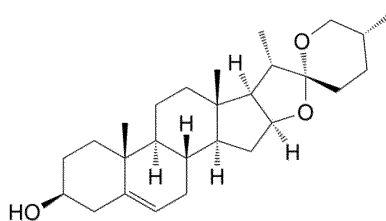
In the embodiments described herein above, wherein the administration mode is in vivo, in certain embodiments, these embodiments preferably relate to said composition as defined above for use in the treatment of a human subject by administering said composition to said subject.

Chemical structures

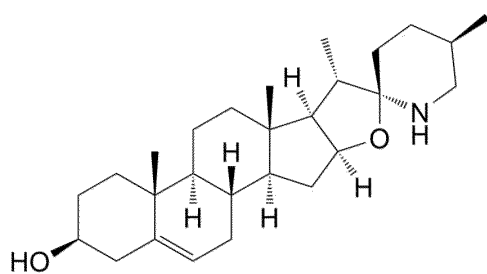
Chemical structures of tested helper lipids



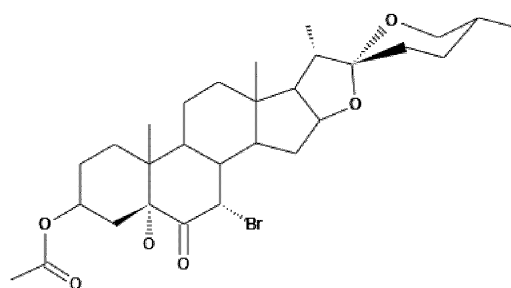
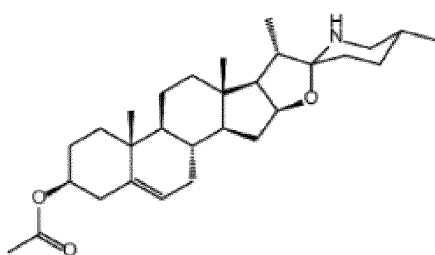
Cholesterol



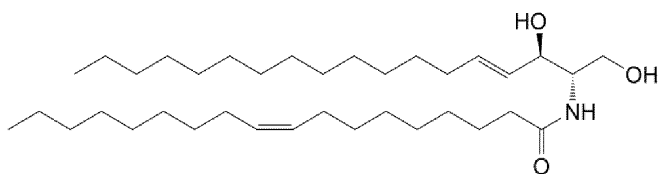
Diosgenin



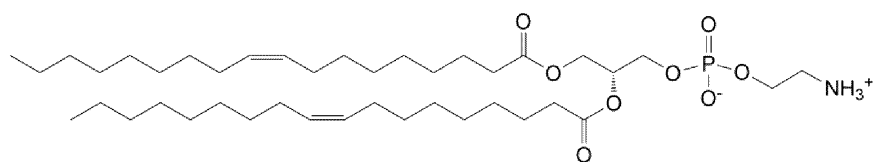
Solasodine

E18 ((5 α ,7 α)-7-bromo-5-hydroxy-6-oxospirostan - 3-yl acetate)

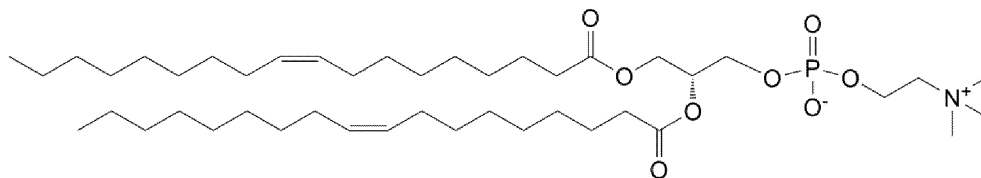
Solasodine acetate



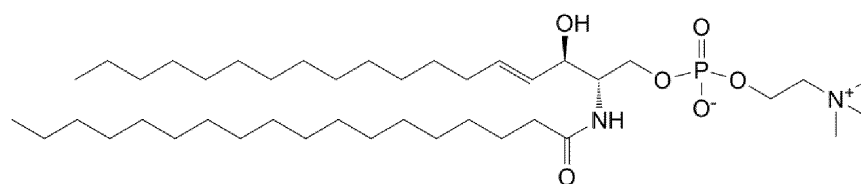
Ceramide



DOPE

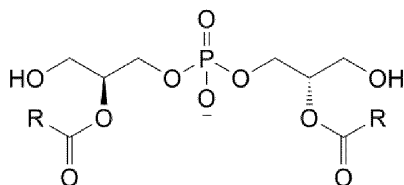


DOPC

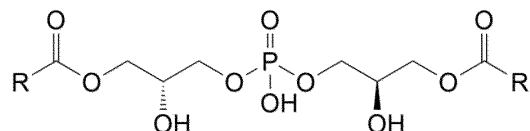


Sphingomyelin (porcine brain extract, mixture of different chain lengths)

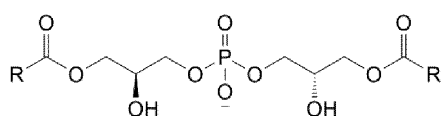
Chemical structures of tested LBPA isoforms



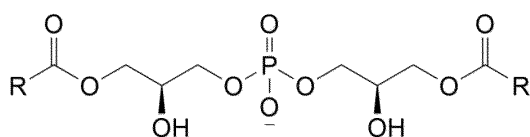
S,S-2,2'-LBPA



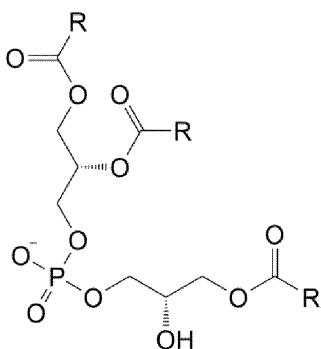
R,R-3,3'-LBPA



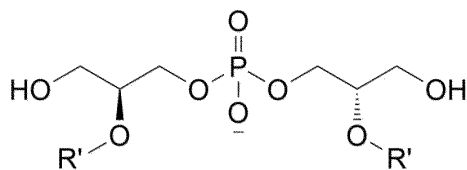
S,S-3,3'-LBPA



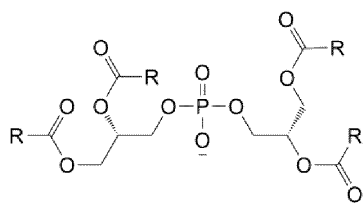
R,S-3,3'-LBPA



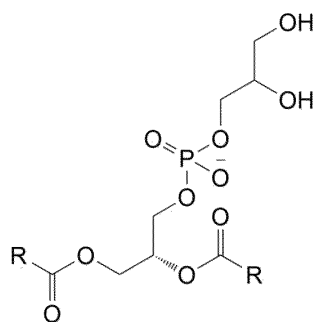
S,R - Hemi-LBPA



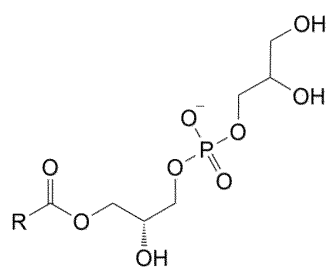
S,S-2,2'-C18:0-ether-LBPA
and
S,S-2,2'-C12:0-ether-LBPA



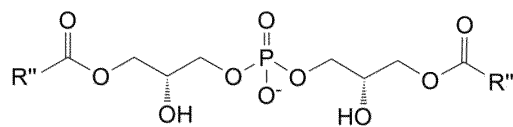
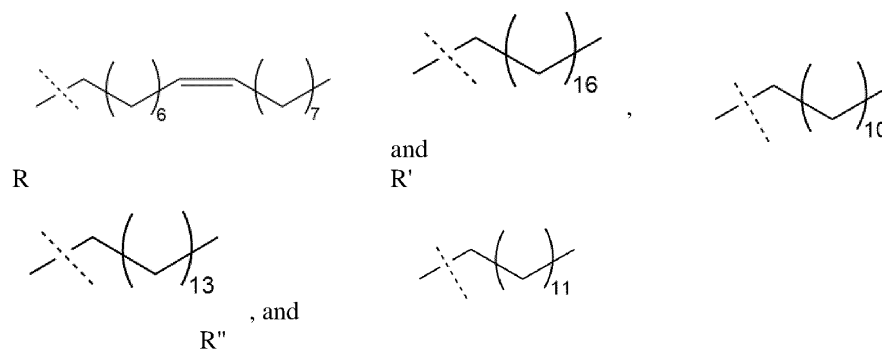
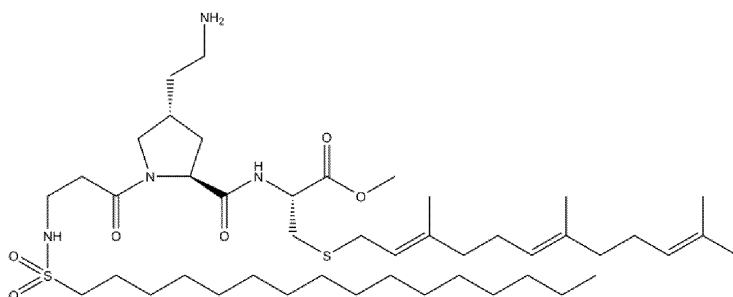
S,S-BDP



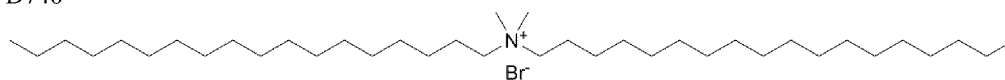
DOPG



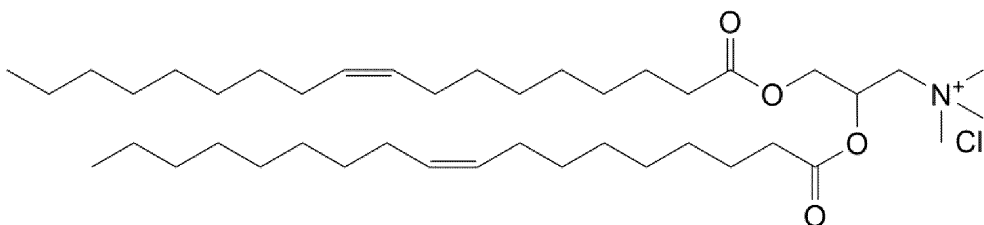
lysoPG

R,S-C16:0-LBPA
and
R,S-C14:0-LBPA**Chemical structures of encapsulating agents**

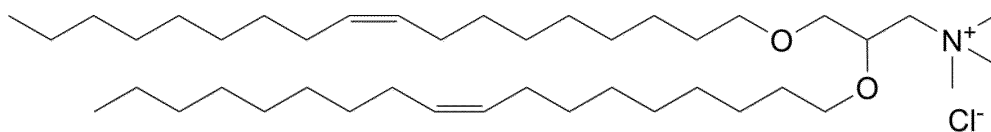
PD740



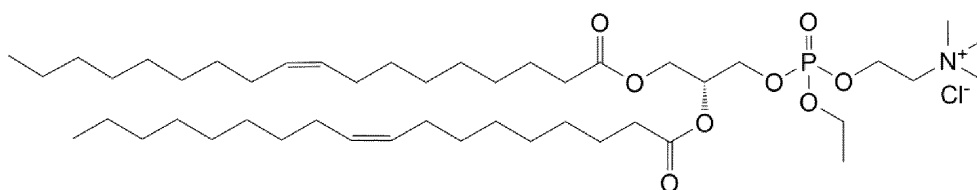
DOMA



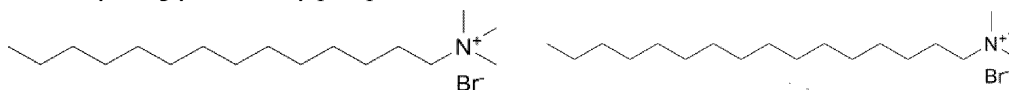
DOTAP



DOTMA

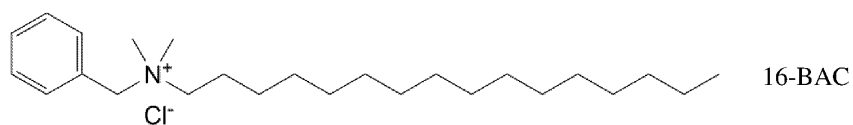


1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (EPC 18:1)

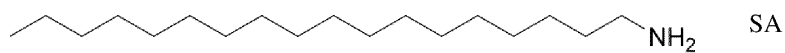


TTAB

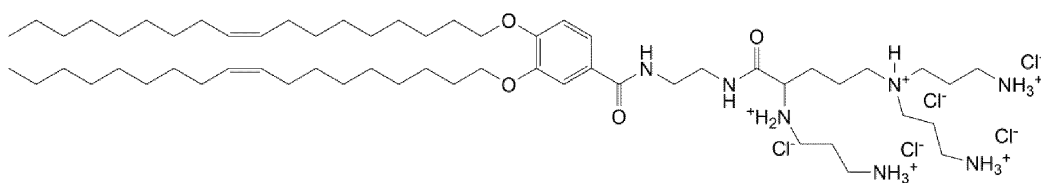
CTAB



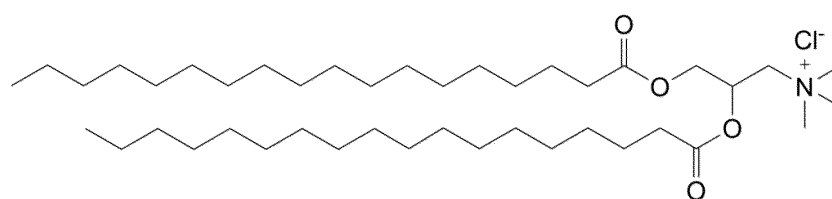
16-BAC



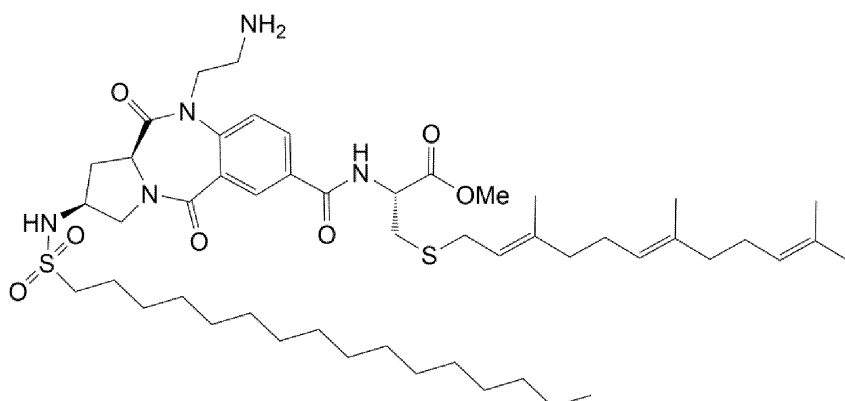
SA



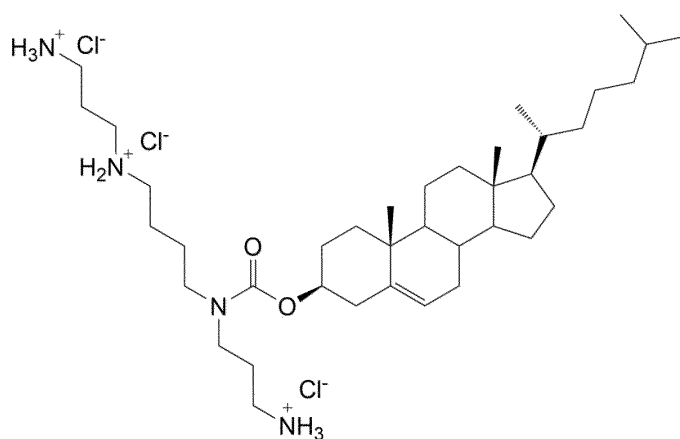
MVL5



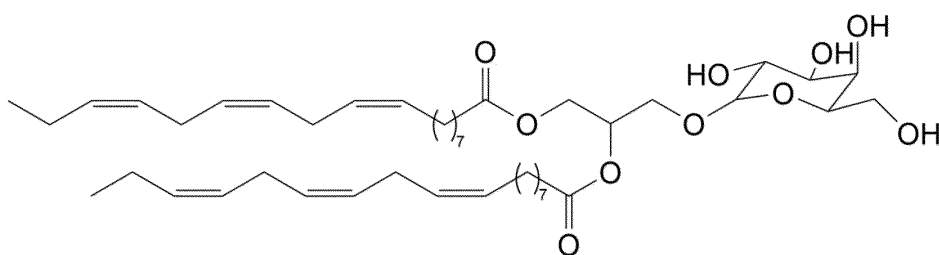
TAP 18:0



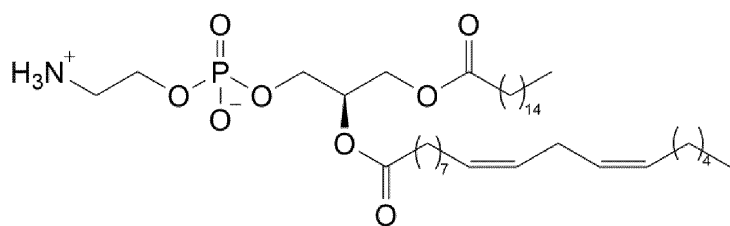
PD739



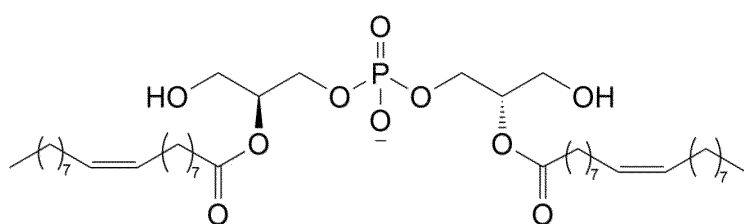
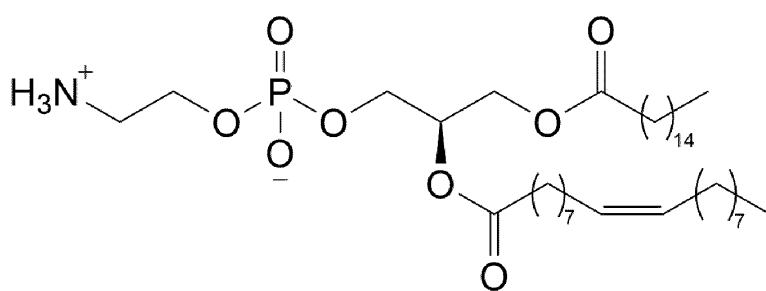
GL67

Fusogenic Agents

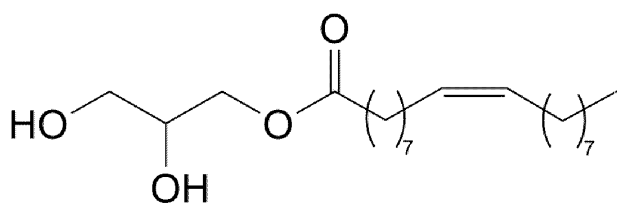
MGDG (plant extract, the chemical structure represents the main species of a mixture of different chain length)



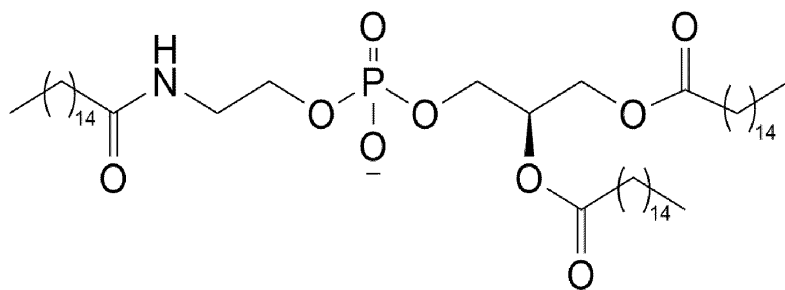
PLPE

*S,S*-2,2'-LBPA

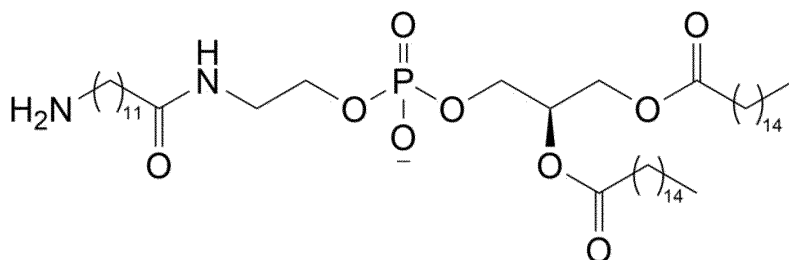
POPE



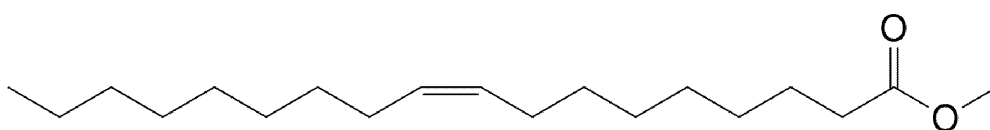
GMO



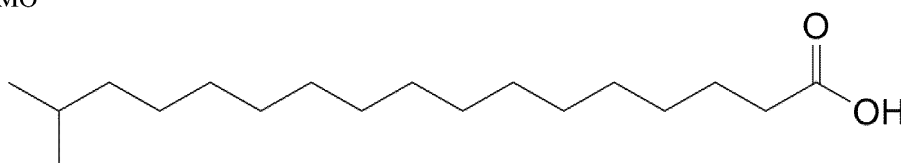
NPPE



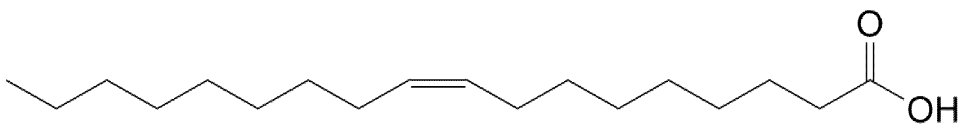
NAPE



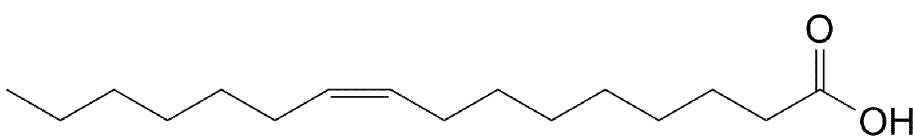
MO



Isost.A



OIA

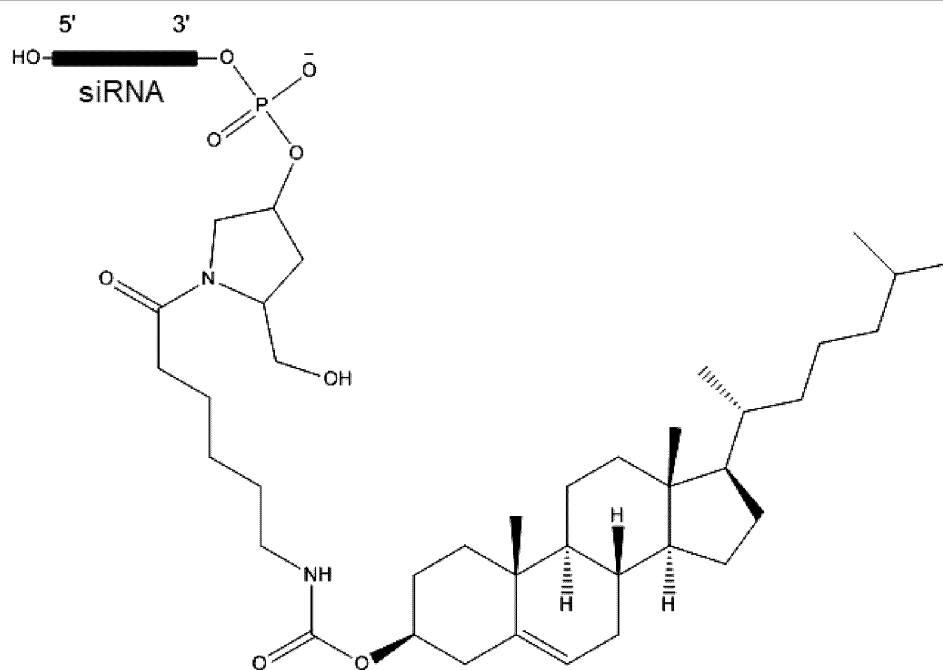


PalmA

[illegible]

α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol45
(PEG2000-orthoester-C18 or PEGOEC18)

Cholesterol conjugated siRNA sense strand (Chol-siRNA (SEQ ID NO 7))



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 scope of the invention.

Further modifications and alternative embodiments of various aspects of the invention will be apparent to those skilled in the art in view of this description. Accordingly, this description is to be construed as illustrative only and is for the purpose of teaching those skilled in the art the general manner of carrying out the invention. It is to be understood that the forms of the invention shown and described herein are to be taken as examples of embodiments. Elements and materials may be substituted for those illustrated and described herein, parts and processes may be reversed, and certain features of the invention may be utilized independently, all as would be apparent to one skilled in the art after having the benefit of this description of the invention. Changes may be made in the elements described herein without departing from the scope of the invention as described in the following claims.

Examples

Abbreviations list:

2'-OMe – 2'-O-methyl

2'-MOE – 2'-O-methoxyethyl

5-Me-C – 5-methylcytosine

5moU – 5-methoxyuridine

AcOH – acetic acid

aiRNA – asymmetrical interfering RNA

AllocCl – allyloxycarbonyl chloride

BACMAM – Baculovirus gene transfer into Mammalian cells

BHT – butylated hydroxytoluene (

BSA – bovine serum albumin

CAS – Chemical Abstracts Service

Cas – CRISPR associated protein

Cas9 – CRISPR associated protein 9

Chol – cholesterol

Chol-siRNA – cholesterol siRNA conjugate

CMB – Cell Mask Blue

conc. – concentration

COVID – coronavirus disease 2019
cr guide RNA – CRISPR guide RNA
crasiRNA – centrosome-associated RNA
CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats
cryoEM – cryogenic electron microscopy
DAPI – 4',6-Diamidino-2-phenylindol
DCM – dichlormethane
DDQ – 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone
DIPEA – diisopropylethyl amine
DMAP – 4-(Dimethylamino)pyridin
DMEM – Dulbecco's Modified Eagle Medium
DMF – dimethylformamide
DMSO – dimethylsulfoxid
DMTP – dimethylterephthalate
DNA – desoxyribonucleic acid
dsRNA – double stranded RNA
DLS – dynamic light scattering
dsGFP – destabilized eGFP
DSPE – 1,2-distearoyl-sn-glycero-3-phosphoethanolamine
E18 – (5 α ,7 α)-7-bromo-5-hydroxy-6-oxospirostan - 3-yl acetate
EC50 – half maximal effective concentration
EDTA – Ethylenediaminetetraacetic acid
EDC – N-Ethyl-N'-carbodiimide hydrochloride
eGFP/GFP – (enhanced) green fluorescent protein
EGTA – ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELSD – evaporative light scattering detector
EM – electron microscopy
EPO – Erythropoietin
EtOAc – ethyl acetate
EtOH – ethanol
eq. – equivalents
ESI – electrospray ionization
FACS – fluorescence-activated cell sorting
FBS – fetal bovine serum
FTMS – Fourier Transform Mass Spectrometry
G418 – geneticin
G-CSF – granulocyte-colony stimulating factor
eGFP/GFP – (enhanced) green fluorescent protein HEPES

GM-CSF – granulocyte-macrophage colony stimulating factor
GNA – glycol nucleic acid
guide RNA – mix of tracr RNA and cr guide RNA
HBTU – 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMC – 5-hydroxymethylcytosine
HOBt – hydroxybenzotriazole
HPLC – high performance liquid chromatography
KD – knock down
LBPA – lysobisphosphatidic acid
LHRH – luteinizing hormone-releasing hormone
LLOMe – L-leucine methyl ester
LMV – large multilamellar vesicle
LNA – locked nucleic acid
LNP – lipid nanoparticle
MCFS – macrophage colony-stimulating factor
MeCN – acetonitrile
MeOH – methanol
MM – mouse macrophages
MPI-CBG – Max Planck Institute of Molecular Cell Biology and Genetics
mRNA – messenger RNA
miRNA – micro RNA
moRNA – microRNA-offset RNA
MSY-RNA – MSY2-associated RNA
MVL – multivesicular liposome
ncRNA – regulatory non-coding RNA
NLS – nuclear location sequence
NMR – nuclear magnetic resonance spectroscopy
ORF – open reading frame
PAR – promoter-associated RNA
PBS – phosphate buffered saline
PDI – polydispersity index
PEG – polyethylene glycol
PEGOEC18 – PEG2000-orthoester-C18, α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅
PEPC – protein expression, purification and characterization facility
PEST amino acid sequence – proline-glutamate-serine-threonine-rich amino acid sequence (to target proteins for degradation)

piRNA – PIWI-interacting RNA
PNA – peptide nucleic acid
PMA - phorbol 12-myristate 13-acetate
PMBCl – para-methoxybenzyl chloride
PTFE – Polytetrafluoroethylene
pTosOH – p-toluene sulfonic acid
rcf – relative centrifugal force
Rf – retention factor
RNA – ribonucleic acid
RNP – ribonucleoprotein complex
rt – room temperature
SD – standard deviation
sdRNA – sno-derived RNA
SEM – standard error of the mean
shRNA – small hairpin RNA
siRNA – small interfering RNA
sgRNA – single guide RNA
snRNA – small nuclear RNA
snoRNA – small nucleolar RNA
ssRNA – single stranded RNA
TBAF – tetrabutylammonium fluoride
TBDMSCl – tert-butyldimethylsilyl chloride
TBE – tris-borate-EDTA buffer
tBuOOH – tert-Butyl hydroperoxide
TC50 – half maximal toxic concentration
TE – tris-EDTA buffer
tel-sRNA – telomere small RNA
TFA – trifluoroacetic acid
THF – tetrahydrofuran
TLC – thin layer chromatography
TNA – threose nucleic acid
tracr RNA – trans-activating crRNA
tRNA – transfer RNA
Tris – tris(hydroxymethyl)aminomethan
tsRNA – tRNA-derived small RNA
ULV – univesicular liposome
UNA – unlocked nucleic acid
UV – ultraviolet

xiRNA – X-inactivation RNA

General methods:

RNase free handling

All handling of liposome components to be used with RNA was done in an RNase free area using RNase free instruments. Area and instruments were cleaned with RNase zap (ThermoFisher Scientific, AM9780) repeatedly and regularly. RNase free plasticware was used (Eppendorf tubes, Falcon tubes, tips). All pipetting of compound solutions was done with RNase free filtered tips (ART tips, ThermoFisher Scientific) and Gilson Pipetman® pipettes. Glassware was made RNase free by heating to 220 °C for 2h. Lids for vials were made RNase free with RNase zap and subsequently washed with RNase free water and EtOH.

RNA quantification

RNA concentration was quantified by UV absorption at 260 nm using a NanoDrop instrument.

Compound aliquoting and solvent selection

Compounds were weighed into RNase-free glass vials (either 4 ml (Supelco 27138, screw top with solid green melamine cap with PTFE liner) or 1.5 ml (Certified CD™ Vial (Center Draining) Kit, 9 mm thread with cap/septa, Sigma Aldrich, 29307-U)) and dissolved in a suitable solvent as mentioned below.

Compound	Solvent
2,2'-LBPA	Chloroform, dried over K ₂ CO ₃ (see handling of 2,2'-LBPA)
labile PEG lipid	CD ₃ CN
16:0 BMP, 14:0 BMP	MeOH
NAPE	MeOH/CHCl ₃ (2.5:1)
all other compounds	Chloroform

Compound concentration was verified with quantitative NMR on a Bruker Avance III using ERETIC method (Akoka, Barantin and Trierweiler, 1999. Concentration Measurement by Proton NMR Using the ERETIC Method', Analytical Chemistry, 71(13), pp. 2554–2557) against a reference solution of DMTP (Standard for quantitative NMR, TraceCERT®, Merck, 07038).

Handling labile PEG lipid PEG2000-orthoester-C18

PEG2000-orthoester-C18 is not stable in chloroform. Deuterated acetonitrile was used as the solvent of choice to allow monitoring of the stability of aliquots over a longer time and quantification of compound concentration by quantitative NMR on a Bruker Avance III using ERETIC method (Akoka, Barantin and Trierweiler, 1999) against a reference solution of DMTP (Standard for quantitative NMR, TraceCERT®, Merck, 07038). Glassware and commonly used lab equipment used

for handling PEG2000-orthoester-C18 was thoroughly cleaned beforehand to avoid contamination with acids. The compound was handled at temperatures below 30 °C, including a lowered water bath temperature for solvent evaporation.

Handling 2,2'-LBPA

Isomerization of 2,2' - isomer to 3,3' is increased under acidic conditions. Therefore, chloroform used for aliquotation of 2,2'-LBPA was pretreated with K_2CO_3 (0.1 g/ml) for 1 h to remove residual HCl. Verification of correct isomer was performed using a phospholipase digestion assay and detection by quantitative TLC (Kobayashi et al., 2002. Separation and characterization of late endosomal membrane domains, The Journal of Biological Chemistry, 277(35), 32157–32164). Solvents used during purification were precooled on ice to avoid isomerization during the purification process. Evaporation of solvents was done at 35°C maximum.

Example 1 – Liposome formation with siRNA using extrusion or sonication

Liposomes (lipid vesicles) are formed when thin lipid films or lipid cakes are hydrated and stacks of liquid crystalline bilayers become fluid and swell. The hydrated lipid sheets detach during agitation and self-close to form large, multilamellar vesicles (LMV) which prevents interaction of water with the hydrocarbon core of the bilayer at the edges. Once these particles have formed, they are subjected to downsizing, i.e. size reduction, by sonication, wherein the energy input is in the form of sonic energy, or by extrusion, wherein the energy input is in the form of mechanical energy.

Individual lipid components were dissolved in suitable solvents as specified in Table 15. siRNA was dissolved in a suitable buffer and its concentration was determined by UV absorption at 260 nm using a NanoDrop instrument. The following protocol has actual amounts for preparation of 500 µl of liposome solution of composition LP1 (DOTAP:Chol:LBPA:labile PEG lipid= 50:25:20:5) at an siRNA concentration of 2 µM in 5% glucose solution (w/w) with anti eGFP siRNA (Sigma Aldrich, custom synthesis). Individual components such as buffer, concentrations, total volume as well as the drug binding agent, helper lipid, fusogenic agent, PEG lipid, siRNA and the stoichiometry of components can be varied. The preparation was prepared at a charge ratio N:P (amine to phosphate) of 20:1. The charge ratio N:P is calculated as described in example 31. The siRNA in this example has 42 charges per molecule (number of phosphate groups), while the liposomes have one charge per DOTAP molecule included (number of amino groups). This charge ratio can be varied. Amounts need to be adjusted depending on compounds and siRNA used.

Lipid film generation:

Suitable volumes of DOTAP solution in $CHCl_3$ (0.59 mg, $8.40 \cdot 10^{-7}$ mol, 50 eq.) and cholesterol solution in $CHCl_3$ (0.16 mg, $4.20 \cdot 10^{-7}$ mol, 25 eq.) were added to an RNase-free glass vial (4 ml) (screw top vial with Thermoset cap with PTFE liner, Supelco 27138 (Merck)) and dried down under

nitrogen stream. The dried film was kept for 15 min at room temperature to ensure full evaporation. Suitable volume of a solution of LBPA in CHCl_3 (0.27 mg, 3.36×10^{-7} mol, 20 eq.) was added to the existing film and dried down under nitrogen stream. The dried film was kept for 15 min at room temperature to ensure full evaporation. Suitable volume of labile PEG lipid in deuterated acetonitrile (0.20 μg , 8.40×10^{-8} mol, 5 eq.) was added to the existing film and dried down under nitrogen stream. The dried film was kept for 15 min at room temperature or dried under vacuum for 2 h to ensure full evaporation.

Lipid film hydration:

A 2 μM solution of anti eGFP siRNA (1 nmol, charges: 42 nmol) in sterile 5% glucose solution (w/w) in RNase free water (500 μl) was added to the dried film in a sterile environment. The sample was vortexed for 5 min and left to mature at room temperature for 30 min. Total lipid concentration after hydration is 3.36 mM.

Downsizing can be achieved by 2 methods:

Downsizing by extrusion: In a sterile environment, the sample was extruded with a disposable NanoSizer MINI extruder (T&T Scientific) containing a 100 nm membrane with 22 passes and collected from the receiving syringe. Alternatively, an Avanti Mini Extruder and 100 nm polycarbonate membranes (Sigma Aldrich, 610005) can be used. The sample was incubated at room temperature for 30 min before storing it at 4 °C.

This method was used for final compositions.

Downsizing by sonication: The sample was sonicated in a water bath sonicator (Bandelin Sonorex Super RK 102 H) for 45 min. The sample was incubated at room temperature for 30 min before storing it at 4 °C.

This method is high throughput and was used for compositional screening.

Quality control:

Dynamic light scattering was used to check the presence of particles and analyse their size distribution (Example 6 for experimental details). The RiboGreen assay was used to assess siRNA concentration and siRNA encapsulation (Example 7 for experimental details).

Example 2 – Liposome formation without cargo using extrusion or sonication

Individual lipid components were dissolved in suitable solvents as specified in Table 15. The following protocol has actual amounts for preparation of 500 μl of liposome solution of composition LP1 (DOTAP:Chol:LBPA:labile PEG lipid= 50:25:20:5) in 5% glucose solution (w/w) at a total lipid concentration of 3.36 mM. Individual components such as buffer, concentrations, total volume as

well as encapsulating agent, helper lipid, fusogenic agent, PEG lipid and stoichiometry of components can be varied.

Lipid film generation was performed as described in Example 1 using the same components, amounts, and experimental conditions.

Lipid film hydration:

A sterile 5% glucose solution (w/w) in RNase free water (500 µl) was added to the dried film in a sterile environment. The sample was vortexed for 5 min and left to mature at room temperature for 30 min.

Downsizing was achieved by extrusion or sonication as described in Example 1 above.

Quality control:

Dynamic light scattering was used to check the presence of particles and analyse their size distribution (Example 6 for experimental details). The lipid concentration was quantified using a variation of Cholesterol/Cholesterol Ester-Glo™ Assay (Promega J3190) (Example 28 for experimental details).

Example 3 - Liposome formation with mRNA

Liposomes were prepared without cargo according to protocol from example 2.

Prior to transfection they were mixed with mRNA at an N/P ratio of 6:1. This ratio can be varied; and similar transfection data were obtained with a N/P ratio of 3:1.

The mRNA-containing liposomes were obtained mixing a liposome composition LP1 in 5% glucose solution (w/w) with CleanCap® EGFP mRNA (5moU) (Trilink, L-7201, 996 charges) at an N/P ratio of 6:1. To this aim, EGFP mRNA (10 µl, 100 µg/ml, PBS) was added to a solution of LP1 (10.92 µl, 3.36 mM total lipid) and mixed by pipetting up and down several times. The mixture was incubated for 15 min at room temperature and diluted to the concentration needed for transfection with 5% glucose solution, i.e. 12.5 µg/ml mRNA concentration.

Quality control:

Dynamic light scattering was used to analyse particle size distribution (Example 6 for experimental details). The RiboGreen assay was used to assess mRNA concentration and mRNA encapsulation (Example 7 for experimental details).

Example 4 – Liposome formation with guide RNA

Liposomes were prepared without cargo according to the protocol described in example 2. Prior to transfection they were mixed with guide RNA mix at an N/P ratio of 8.45:1. This ratio can be varied.

A liposome solution of composition LP1 in 5% glucose (w/w) at a total lipid concentration of 3.36 mM was prepared as described in example 2 and mixed with GFP cr guide and tracr RNA at an N/P ratio of 8.45:1. The guide RNA mix was prepared by mixing GFP cr guide 1 (0.84 μ l, 100 μ M, 50 charges) and tracr RNA (0.84 μ l, 100 μ M, 50 charges) and diluting it to 30 μ l total volume using 20 mM Hepes, pH 7.5, 150 mM KCl. This mix was incubated for 15 min at 37 °C. Afterwards, 3.55 μ l of this mix were mixed with LP1 solution (5 μ l) by pipetting up and down and then incubated at room temperature for 30 min. It was then diluted with 5% glucose solution to the concentration needed for transfection, i.e. 100 nM GFP cr guide RNA prior to transfection.

Example 5 – Liposome formation with Cas9 RNP

Liposomes were prepared without cargo according to the protocol described in example 2. Prior to transfection they were mixed with Cas9 protein/ guide RNA complex at an N/P ratio of 9.10:1. This ratio can be varied.

A liposome solution of composition LP1 in 5% glucose (w/w) at a total lipid concentration of 3.36 mM was mixed with Cas9 protein guide RNA ribonucleoprotein complex at an N/P ratio of 9.10:1. Cas9 protein guide RNA ribonucleoprotein complex was prepared by mixing GFP cr guide (0.84 μ l, 100 μ M, 50 negative charges), tracer RNA (0.84 μ l, 100 μ M, 50 negative charges) and Cas9 protein (4.29 μ l, 3 mg/ml, 22 positive charges) diluting it to 30 μ l total volume using 20 mM Hepes, pH 7.5, 150 mM KCl. This mix was incubated for 15 min at 37 °C. Afterwards, 3.55 μ l of this mix were mixed with LP1 solution (5 μ l) by pipetting up and down and then incubated at room temperature for 30 min. It was then diluted to the concentration needed for transfection, i.e. 100 nM GFP cr guide RNA prior to transfection.

Example 6 – Liposome size analysis by dynamic light scattering

Description of method:

The liposome sample (3.36 mM, 1 μ l) was diluted 1:150 in the initial "preparation" buffer (e.g. 5% Glucose (w/w) in RNase free water) to a total lipid concentration around 20 μ M and transferred to a micro UV cuvette (Brand®, BR759200). The sample was measured with the size program of a Malvern Zetasizer Nano ZS (laser 632.8 nm) at 25°C using 173° backscatter, automatic measurement settings and 120 s preequilibration time. 3 repeat measurements were conducted for each sample.

Data analysis:

The size Z-average, (Z-Ave), which corresponds to the liposome diameter in nm, and polydispersity index (PDI) were calculated on the basis of the light scattering data by the Malvern Zetasizer software and averaged over 3 measurements. The size distribution based on intensity, volume and number was exported as an average of 3 measurements. Distributions based on number of particles are shown in **Figures 2c, h, 5c, 12c and 13c**.

Results:Size distribution of extruded siRNA- liposomes

Dynamic light scattering analysis of extruded liposomes containing siRNA (prepared according to example 1) showed a narrow size distribution around 100 nm (**Fig. 2c**). The average diameter Z-Ave was 121.3 ± 2.8 nm and the average polydispersity index was 0.123 ± 0.007 (n=6, error: SEM).

siRNA-liposome size stability over time

After storage at 4 °C for 9 months, and 1.6 and 2.4 years the size distribution of extruded liposomes containing siRNA (prepared according to example 1) was unchanged (**Fig.2h**), demonstrating high stability of the liposomal system.

Size distribution of mRNA-liposomes

Dynamic light scattering several hours after mixing of empty extruded liposomes with mRNA (prepared according to example 3) showed a slight shift of the size distribution towards larger sizes for extruded liposomes with mRNA compared to the same liposomes without mRNA (**Fig. 5c**).

Example 7 – RNA quantification with RiboGreen assayDescription of method:

The concentration and encapsulation efficiency of RNA was determined using the Quant-it™ RiboGreen RNA Assay Kit from Invitrogen™ (ThermoFisher Scientific, R11490). The assay was performed in a Nunc™ F96 MicroWell™ Black Polystyrene Plate (ThermoFisher Scientific, 237105). Each sample was assayed in triplicate both in the absence and in the presence of 0.5% Triton X. The individual samples were compared to a four-point standard curve of the same RNA with and without 0.5% Triton X.

The standard curve for siRNA was recorded in the range between 10 and 50 nM, while the standard curve for mRNA was recorded in the range between 0.05 and 0.5 µg/ml.

Specific example for analysis of liposomes containing 2 µM siRNA (prepared according to example 1):

3 replicates of the sample (1.5 µl) were diluted to 50 µl using Tris-HCl buffer (10 mM, 1 mM EDTA, pH 7.5), another 3 replicates were diluted to 45 µl with Tris-HCl buffer, and addition of 5 µl of a 10% triton solution in RNase free water. The standard curve samples were prepared from an siRNA aliquot with fitting volumes with the same procedure. The Quant-it™ RiboGreen RNA Reagent was warmed to room temperature and diluted 200-fold in Tris-HCl buffer. 50 µl of this solution were added to each well. The fluorescence intensity was measured using a Perkin Elmer Envision plate reader using a 492 nm excitation and a 535 nm emission filter. The gain was adjusted for each measurement.

Data analysis:

The fluorescence intensity was plotted against the RNA concentration for the standard curves with and without triton and analyzed using the software R employing a weighted linear fit. The concentration (c(RNA)) and subsequently molar amount of RNA (n(RNA)) for each well was calculated based on the extracted slope and intercept of the relevant standard curve and averaged for each condition.

RNA content compared to theoretical RNA content in percentage was quantified as follows:

$$n(RNA)(\%) = \frac{n_{with\ triton}}{n_{theoretical}} \times 100\%$$

RNA loss compared to theoretical content is calculated as follows:

$$n(RNA\ remaining)(\%) = (1 - \frac{n_{with\ triton}}{n_{theoretical}}) \times 100\%$$

$$RNA\ loss(\%) = 100\% - n(RNA\ remaining)(\%)$$

$n_{with\ triton}$ is the molar amount detected in the triton treated sample, which equals the total RNA content of the sample, and $n_{theoretical}$ is the theoretical molar amount of the triton treated sample, based on the input.

Encapsulation efficiency is quantified as follows:

$$n(RNA_{outside})(\%) = \frac{n_{without\ triton}}{n_{with\ triton}} \times 100\%$$

$$\begin{aligned} Encapsulation\ efficiency(\%) &= n(RNA_{encapsulated})(\%) \\ &= \frac{n_{with\ triton} - n_{without\ triton}}{n_{with\ triton}} \times 100\% \end{aligned}$$

$n_{with\ triton}$ is the molar amount detected in the triton treated sample, which equals the total RNA content of the sample after liposome lysis, and $n_{without\ triton}$ is the molar amount detected in the untreated sample, which equals the RNA content outside the liposomes.

The molar concentration of the original sample (c(RNA)) was calculated by dividing the average molar amount per well of the triton treated sample by the original sample volume V_{Sample} (1.5 μ l).

$$c(RNA)(M) = \frac{n_{with\ triton}}{V_{Sample}}$$

This value was used as the concentration of the sample for subsequent transfections.

Change in concentration between two measurements (e.g. at a later timepoint) in percentage is calculated as follows:

$$Change\ in\ c(RNA)(\%) = \frac{c(RNA)_{Measurement\ 2}}{c(RNA)_{Measurement\ 1}} \times 100\%$$

"Measurement 1" is the original measurement, "measurement 2" is the one at a later timepoint.

Results:

siRNA encapsulation and content of extruded siRNA liposomes

Ribogreen assay shows successful binding of siRNA and average encapsulation efficiency of $97.0 \pm 0.83\%$ at charge ratio N/P 20:1 (positive/negative) for liposomes containing siRNA prepared by extrusion according to the protocol of example 1 (n=12, error: SEM) (**Fig. 2d**).

Average siRNA content loss due to extrusion for samples made according to the protocol in example 1 was 14.5 ± 2.21 % (n=12, error: SEM) (**Fig. 2e**).

siRNA encapsulation and content of extruded siRNA liposomes – stability over time

siRNA encapsulation efficiency of extruded liposomes made according to example 1 was unchanged after 9 months, 1.6 and 2.4 years of storage at 4°C (**Fig. 2i**). Also, siRNA concentration of these samples was not reduced after prolonged storage, even after 2.4 years at 4°C. (**Fig. 2j**).

siRNA encapsulation of liposomes at different N/P ratios

siRNA liposomes prepared at charge ratio N/P 5:1 showed an average encapsulation efficiency of 93%, which is close to full encapsulation that can be seen at N/P ratio 20:1 (**Fig. 2k**). By extension any N/P ratio in between or above should also lead to high encapsulation.

siRNA encapsulation and content of extruded mRNA liposomes

For mRNA-liposomes made according to protocol in example 3 at charge ratio 6:1 (positive/negative) around 40% of total mRNA was encapsulated while the rest was detected on the outside of the liposomes (**Fig. 5a**).

Example 8 – Cryogenic electron microscopy (EM)

Description of method:

Grids preparation and plunge-freezing

For cryo electron microscopy aqueous samples are cooled to cryogenic temperatures to preserve the sample in the most native state in vitreous ice by plunge freezing. Samples are spread into a thin film across an EM grid before rapidly submerging it in a cryogen, e.g. liquid ethane. A Lacey Carbon Supported Copper Grid (200 mesh) was glow-discharged for 12 s before insertion in the chamber of a plunge freezer EM GP2EM (Leica). Anti eGFP siRNA containing liposomes of composition LPI1 prepared according to the protocol detailed in example 1 were diluted to a total lipid concentration of 400 µM in 20 mM TRIS, 150 mM NaCl. 3 µl of this sample and 1 µl of 10 nm of gold nanoparticles suspension (BBI Solutions, EM.GC10) were added to the grid just before blotting. The plunge-freezing parameters were set to 30 °C, 99% humidity. Blotting was done on the sample-side of the grid for 1.4 s, and immediately followed by plunging in liquid ethane at -180°C. Frozen grids were preserved in liquid nitrogen.

Cryo-electron imaging

Transmission Electron Microscopy was done on a Titan Halo 300 kV FEG-TEM (FEI, Hillsboro, Oregon, USA) equipped with a field emission gun and an energy filter BioQuantum Model 967 from Gatan (Gatan, Pleasanton, California, USA). The images were zero loss filtered with a slit width of 20 eV and taken on a Gatan K2 summit direct detector in the counted mode. The program

DigitalMicrograph (Gatan) was used to control the energy filter and the K2 detector. Images were taken with the program SerialEM (Mastronarde, D.N., *Automated electron microscope tomography using robust prediction of specimen movements*. J. Struct. Biol. (2005) 152, 36–51) at a resolution of 3710 x 3838 pixels at a magnification resulting in a pixel size of 0.31 nm. The beam intensity was set between 6 and 10 electrons per pixel per second and the overall dose per image was adjusted to the range 50 - 100 electrons per Square-Ångstrom. Instead of a single image, twenty sub frames were taken with frame times between 0.1 s and 0.25 s and aligned using K2 align software, which is based on the MotionCorr algorithm, to compensate for any drift. The sample was in a Gatan 626 Cryoholder, cooled by liquid nitrogen without counter-heating so that the final sample temperature was constant and always below -165 °C.

Results:

CryoEM imaging shows that liposomal structure is unilamellar and univesicular (**Fig. 2a**). This means that the liposomes are composed of a single membrane layer around an aqueous core, making it possible for them to fuse with other membranes such as the endosomal membrane. They are in a diameter size range around 100 nm, in accordance with the data obtained by dynamic light scattering (**Fig. 2c**). Their morphology in solution is spherical.

Example 9 – conventional electron microscopy

Description of method:

For siRNA-liposomes, a sample (0.5 µl) of extruded liposomes of composition LP1 containing anti eGFP siRNA (prepared according to protocol detailed in example 1) was diluted 10x with water and 5 µl of the solution were incubated for 3 min on a non-carbon coated and not glow-discharged, copper, square 400-mesh grid (Plano G2400C), coated with formvar film.

For mRNA liposomes, a sample of liposomes of composition LP1 (prepared according to protocol detailed in example 2) (1 µl) was mixed mRNA solution (100 µg/ml, 1,83 µl) at charge ratio 3:1 and incubated for 15 min at room temperature (according to protocol detailed in example 3). The solution was diluted to 10 µl with 5% Glucose and incubated for 5 min at room temperature. 5 µl of the solution were incubated for 5 min on a non-carbon coated and not glow-discharged, copper, square 400-mesh grid (Plano G2400C), coated with formvar film.

The grid was washed on 3 drops of water for 5 s per drop. For the first staining step, the grid was incubated on a drop of 1% sodium tungstate (Electron Microscopy Sciences 21400) in water for 2 min, followed by washing on 3 drops of water, 1 s per drop. For the second staining step, the grid was incubated with 0.1% uranyl formate (Electron Microscopy Sciences 22450) in 1.8% methyl cellulose (pH 4.5, Sigma Aldrich M6385) on 3 consecutive drops, on ice, for a total of 2 min. The grid was lifted from the last drop using a nichrome wire loop, excess uranyl formate/methyl cellulose solution was removed using a filter paper, and the grid was air dried at room temperature. The protocol was adapted from Asadi et al., 2017 (Asadi, J. et al. *Enhanced imaging of lipid rich*

nanoparticles embedded in methylcellulose films for transmission electron microscopy using mixtures of heavy metals. Micron 99, 40–48 (2017)). The grid was imaged at 13,000x magnification in a Tecnai T12 transmission electron microscope (FEI, now Thermo Fisher Scientific), operated at 100 kV and equipped with an axial 2k CCD camera (TVIPS) and EM-MENU software (TVIPS).

Results:

Size and morphology of siRNA containing liposomes

Conventional electron microscopy (EM) imaging showed the size distribution and morphology of extruded liposomes containing siRNA in fixed state (**Fig. 2b**). During the fixation in methyl cellulose liposomes collapse onto themselves which results in a folded or donut shape. This is in accordance with literature examples of particles containing an aqueous core. Their size distribution is in accordance with data in solution obtained by dynamic light scattering (**Fig. 2c**).

Size and morphology of mRNA containing liposomes

Conventional EM imaging showed the size distribution and morphology of extruded liposomes containing mRNA at charge ratio 3:1 in fixed state (**Fig. 5b**). EM grids were prepared directly after mixing mRNA and liposomes for 15 min (preparation of mRNA containing liposomes according to protocol described in example 3). Extruded liposomes with mRNA appear to be smaller than liposomes with siRNA when embedded in methyl cellulose (Comparison Figs. 2b and 5b, Figs. 2c and 5c).

Example 10 – RNase degradation assay

Description of method:

A 4% (w/w) suspension of NuSieve GTG agarose (FMC Bioproducts (now Lonza), 50082) in Tris-borate-EDTA buffer (TBE, 89 mM TRIS base, 89 mM boric acid, 2 mM EDTA in RNase free water (ThermoFisher Scientific, AM9938)) was prepared. After soaking for 15 min, it was heated in a microwave at 360 W for 1 min, then kept for 15 min in a 60 °C water bath. Ethidium bromide (1 µl) was added to 27 ml of this molten solution and it was cast into a gel cast with a comb. The gel was left to set at room temperature and covered with TBE buffer afterwards.

Samples were prepared using anti eGFP siRNA (c= 1.85 µM, volume (V)= 7.5 µl) or siRNA-containing, extruded liposomes of composition LP1 made by the protocol detailed in example 1 (c (siRNA)= 1.73 uM, V= 7.5 µl). The siRNA or liposome solutions and RNase A (Sigma Aldrich, R6513) stock in TBE buffer (c=0.01 mg/ml) were incubated at 37 °C for 5 min. RNase A stock solution (V= 1 µl) was added to each sample to reach a final concentration 0.001 mg/ml and followed by addition of RNase inhibitor RNaseOUT™ (ThermoFisher Scientific, 10777019, 40 U/µl, V= 1.25 µl) after the indicated time of incubation (0-60 min, 9 time points) to stop the reaction. After treatment the samples were incubated for 2 min at 37 °C, then a 10% triton X solution (V= 1 µl) was added to each sample to lyse the liposomes. After 3 min incubation at room temperature, Orange G/bromophenol blue loading buffer (0.15% (w/v) Orange G, 0.03% (w/v) bromophenol blue in

glycerol/TBE buffer (3:2), V= 2 µl) was added and 10 µl of this solution was loaded into the gel pockets. The gel was run with 80 V for 20 min using an Amersham Pharmacia Biotech Electrophoresis Power Supply EPS 301.

Imaging and data analysis:

The gels were imaged using a Vilber Bio-Vision Gel Doc system with transillumination at 312 nm and an exposure of 440 ms. The images were analyzed with Fiji by creating an intensity plot for each lane with the gels submenu and calculating the area under the curve for the RNA band. The amount of RNA present in each sample relative to the starting point of the kinetic experiment was calculated by normalizing the area under the curve obtained to the one obtained for t= 0 min.

Results:

siRNA formulated in liposomes resulted in being protected from degradation by RNase A over time, in stark contrast to naked siRNA which was degraded within minutes at the same RNase concentration (**Fig. 2f, g**).

Example 11 – uptake of liposomes containing labeled siRNA in HeLa cells

Description of method:

HeLa cells stably expressing eGFP were obtained as described by Bramsen, J.B. et al. 2009 (Bramsen, J.B. et al., *A large-scale chemical modification screen identifies design rules to generate siRNAs with high activity, high stability and low toxicity*. Nucleic Acids Res. 37, 2867–2881 (2009)). Liposomes containing Alexa 647 labeled anti eGFP siRNA made by the protocol described in example 1 were transfected in HeLa eGFP cells to assess their uptake.

HeLa eGFP cells were cultured in DMEM media complemented with 10% FBS at 37 °C and 5% CO₂ and seeded the day before the transfection into a black 384 well cell culture microplate (Greiner 781092) in DMEM medium with 10% FBS and 50 µg/ml gentamicin. The transfection mixes were prepared in a 96 well master plate: the liposomes were diluted to 12x the final siRNA concentration (i.e. 120 nM) in 5% glucose. Untreated control samples were prepared using only buffer. Directly before transfection, equal volume of DMEM containing 20% FBS was added to each transfection mix to have a concentration of siRNA of 6x the final concentration of the transfection and 10% FBS. 10 µl of this transfection mix was added to each well of the 384 well plate containing 50 µl of medium, resulting in a final concentration of 10 nM. Each condition was tested in quadruplicate. The plate was incubated at 37 °C and 5% CO₂. After 5 h the plate was fixed with 3.7% formaldehyde in PBS for 15 min. After additional washing with PBS each well was stained with 6 µg/ml Hoechst 33342 and 0.5 µg/ml HCS CellMask™ Blue Stain (Invitrogen, H32720) in PBS containing 0.04% sodium azide. Every treatment was performed in quadruplicate.

Imaging and data analysis:

The plate was imaged at 40x magnification on a Yokogawa CellVoyager CV7000 automated laser spinning disk microscope. 4 fields per well were imaged. Images were adjusted and exported with MotionTracking (<http://motiontracking.mpi-cbg.de>).

Results:

Alexa 647-labeled siRNA in liposomes is taken up by HeLa cells and shows a specific vesicular pattern as expected for uptake by endocytosis (**Fig. 3a**). No Alexa 647 uptake can be seen in untreated cells.

Example 12 – transfection of HeLa eGFP cells with siRNA containing liposomes

Description of method:

Liposomes made by the protocol described in example 1 containing anti eGFP siRNA or AF647 labeled anti eGFP siRNA were transfected in HeLa eGFP cells to assess downregulation efficiency. The following description is for a dose response experiment with concentration points taken at 10, 5, 2.5 and 1 nM. This can be adjusted to use different concentrations, more or less points if necessary. HeLa eGFP cells (obtained as described in Bramsen, J. B. et al. (2009)) were cultured in DMEM media (ThermoFisher Scientific, 31966) complemented with 10% FBS (Merck, S0615) at 37°C and 5% CO₂ and seeded the day before the transfection into a black 384 well cell culture microplate (Greiner 781092) in DMEM medium with 10% FBS and 50 µg/ml gentamicin. The transfection mixes were prepared in a 96 well master plate: the liposomes were diluted to 10x the concentration of the highest concentration point (100 nM) in the "preparation" buffer (e.g. 5% glucose). Serial dilution was performed to obtain solutions at 10x concentration for the other concentration points. Untreated control samples were prepared using only buffer. Directly before transfection, equal volume of DMEM containing 20% FBS was added to each transfection mix to have a concentration of siRNA of 5x the final concentration of the transfection and 10% FBS. 10 µl of this transfection mix was added to each well of the 384 well plate containing 40 µl of medium. Each condition was tested in quadruplicate. The plate was incubated at 37°C and 5% CO₂. After 5 h, the transfection mix was washed away by replacing the medium three times with fresh DMEM and aspirating to 25 µl with a Biotek EL406 washer. Afterwards, 25 µl of DMEM containing 20% FBS and 100 µg/ml gentamicin was added. The plate was further incubated for 72 h and then fixed with 3.7% formaldehyde in PBS for 15 min. After additional washing with PBS each well was stained with 1 µg/ml DAPI (Sigma Aldrich, D9542-5MG) and 0.25 µg/ml HCS CellMask™ Blue Stain (Invitrogen, H32720) in PBS containing 0.04% sodium azide. Every treatment was performed in quadruplicate.

Imaging and data analysis:

The plate was imaged at 10x magnification on a Perkin Elmer Operetta high throughput microplate imager or a Yokogawa CellVoyager CV7000 automated laser spinning disk microscope. 9 fields per

well were imaged. The nuclei and cells were segmented based on the DAPI/CMB staining and the mean GFP intensity for each nucleus was recorded.

The nuclei number in percentage was calculated as follows:

The number of nuclei per image was summed up per well and then averaged per condition. This value was normalized to the value calculated in the same way from the untreated control wells.

The GFP intensity in percentage was calculated as follows:

Median of the mean GFP intensity per nucleus or per cell was calculated per image, averaged per well and averaged per condition. This value was normalized to the value calculated in the same way from the untreated control wells.

The analysis was conducted using Perkin Elmer Harmony software or CellProfiler for segmentation and parameter extraction and Knime Analytics platform for calculation, averaging and normalization.

The calculation for the single cell distribution plot of percentage GFP intensity was performed as follows:

The mean GFP intensity per nucleus was normalized to the median value of GFP intensity per nucleus of the untreated condition. The percentage was rounded up to the next integer number for each nucleus. For each condition (treatment, concentration) the occurrence of nuclei for each GFP intensity value was counted per well and then averaged over the quadruplicate repeats.

The difference in normalized GFP intensity and nuclei number for different N/P ratios was calculated as follows:

The GFP intensity or nuclei number in percentage for a specific N/P ratio at a specific concentration was calculated as above. Then the value of N/P ratio 20:1 at this concentration was subtracted the initial value.

EC50/TC50 calculation

EC50 and TC50 values for 4 and 10 concentration datasets were calculated by fitting a 4-parameter logistic equation to the GFP intensity and nuclei number normalized to untreated control in response to siRNA concentration respectively. Calculations were done using Knime Analytics platform. The lower limit was constrained to 100%, while the lower limit was left unconstrained for GFP intensity and set to 0 for Nuclei number. Fits were scored as non-applicable if the lower limit was estimated to be above 40% or below -10%. The therapeutic index is defined as the ratio between TC50 and EC50, as previously described in section "Therapeutic agents".

Scoring

Calculated based on result at dose 10 nM, if not stated otherwise.

Toxicity scoring:

Score	Nuclei number normalized to untreated
-	> 80%
†	60-80%
††	40-60%
†††	20-40%
††††	< 20%

Effect scoring to distinguish between non-working preparations (following toxicity or no effect) and low-working preparations (knock down (KD) effect visible, but fitting not possible):

Score	(Normalized GFP intensity)/(Normalized Nuclei number)
KD	< 0.8
no KD	> 0.8

Results:

siRNA downregulation with liposomes

The treatment of HeLa eGFP cells with 10 nM eGFP anti siRNA in LP1 liposomes caused 80% downregulation of eGFP intensity compared to untreated control, while treatment with the same concentration of naked siRNA showed no effect (**Fig 3b**).

Dependence of siRNA downregulation efficiency of liposomes on both active agents

To show that the functionality of the invention depends on the specific combination of the active agents as contained in the inventive liposome composition, i.e. on the combination of a fusogenic agent with an acid-cleavable PEG lipid, liposomes were prepared in which each of the active agents was replaced with a non-active form and compared with those comprising both active agents. The anti eGFP siRNA- liposomes were prepared according to the protocol in example 1. The non-fusogenic liposome sample was prepared by replacing the fusogenic agent LBPA in the composition LP1 with the non-fusogenic lipid DOPC. The non-acid cleavable sample was prepared by replacing the acid-cleavable PEG lipid in the composition LP1 with the non-acid cleavable PEG lipid DSPE-PEG2000. The downregulation of eGFP in HeLa eGFP cells transfected with formulations without active agents or comprising only one active agent was compared to the formulation containing both active agents (LP1). The downregulation effect was only successful if both active agents were present. Indeed compositions comprising only the non-fusogenic lipid or only the non-acid cleavable lipid as active agent did not show any eGFP downregulation (**Fig. 3c**). These results show that both active agents in the liposomal system have a surprising synergistic effect on inducing endosomal escape and efficient siRNA delivery.

Dose dependence of eGFP downregulation with siRNA-liposomes (four concentrations)

Testing the effect of liposomes containing siRNA against eGFP made by the protocol detailed in example 1 at different siRNA concentrations (10, 5, 2.5 and 0.5 nM) showed that the downregulation effect was dose dependent (**Fig. 3d**). A single cell analysis of distribution of mean GFP intensity per nucleus in the dose dependent experiment (**Fig 3e**) showed that the untreated control has a very wide distribution of mean eGFP intensities per nucleus. A clear, dose-dependent shift is visible with anti eGFP siRNA-liposome treatment, culminating in a narrow distribution around 20% of the median of the untreated condition at 10 nM dose. This indicates that GFP downregulation is achieved in the majority of treated cells.

Dose dependence of siRNA downregulation with liposomes containing different LBPA isomers

siRNA-LP1 liposomes containing one of the main isomer forms of LBPA, 2,2'-S,S and 3,3'-S,S, were prepared according to the protocol in Example 1. These liposome compositions showed same dose response in eGFP downregulation (**Fig. 3f**) and same low toxicity response (**Fig. 3g**). The EC50 for downregulation (determined from four point curve) was very similar: 2,2'-S,S-LBPA containing liposomes showed EC50 2.34 ± 0.18 nM, 3,3'-S,S-LBPA containing liposomes showed EC50 2.50 ± 0.35 nM. Effectiveness of delivery system does not depend on a specific LBPA isomer.

Dose dependency of GFP downregulation with siRNA liposomes (10 concentrations)

To explore the dose dependency of siRNA-mediated downregulation with liposomes of composition LP1 made by the protocol detailed in example 1 in greater detail, a dose response curve with 10 concentration points was explored. This allows for a more exact calculation of the effect (EC50) and toxic (TC50) concentration (**Fig. 3h**). By fitting a four-parameter log-logistics model, these values were calculated as being EC50: 0.93 nM, and TC50: 19.44 nM. Therefore, therapeutic index (TC50/EC50): 21.

Reproducibility of dose dependent siRNA mediated downregulation with liposomes

siRNA mediated downregulation at four concentrations using liposomes of composition LP1 containing 2,2'-LBPA as fusogenic agent prepared according to the protocol detailed in example 1 was shown to be reproducible over a large number of repeated experiments (**Fig. 3i**). The toxicity profile was reproducible as well (**Fig. 3j**).

Effect of liposome storage on siRNA mediated downregulation.

Extruded siRNA liposomes of composition LP1 prepared according to the protocol detailed in example 1 were tested for downregulation efficiency at four concentrations directly after formation as well as after 9 months, 1.6 and 2.4 years of storage at 4°C. The downregulation profile resulted in a similar curve showing that liposomes with siRNA are stable over years without losing efficiency of downregulation (**Fig. 3k**).

Stability of siRNA containing liposomes towards serum

Transfection is often done in serum free conditions to increase success rates. In contrast, all the experiments herein were performed within serum presence to be closer to *in vivo* conditions. Therefore, to check the stability of siRNA liposomes towards serum exposure, LP1 liposomes prepared according to the protocol detailed in example 1 were pretreated with serum for 5 h at room temperature. Their dose-dependent siRNA downregulation and toxicity was compared with a sample of the same liposome batch that was not pretreated with serum (**Fig. 3I**). No difference in downregulation efficiency (eGFP intensity) or toxicity profile (number of nuclei) could be observed indicating that downregulation efficiency of siRNA in liposomes is stable towards prolonged serum exposition (**Fig. 3I**).

Example 13 – Variation of chemical composition of the identified siRNA delivery system

Different compositional variations of anti eGFP siRNA-containing liposomes made by the protocol detailed in example 1 were prepared and tested for their downregulation efficiency and toxicity in HeLa eGFP cells at different siRNA concentrations according to experimental procedure described in example 12. In vitro parameters (size, stability, encapsulation and siRNA content) were analyzed for each liposome composition variation with the techniques detailed in examples 6 and 7. Results from the different compositions are detailed in the following paragraphs.

1. Stoichiometry variation

The percentage of each compound in the composition LP1 was varied resulting in 20 different liposome composition variations. Each of these liposome compositions was tested at 4 siRNA concentrations (Table 2, Fig. 7b, only 10 nM siRNA dose shown). Effective compositions were identified by the GFP downregulation assay in HeLa eGFP cells as having the following percentage ranges for each component:

DOTAP: 30-75%

Cholesterol: 0-35%

LBPA: 15-30% or 45% for cholesterol = 0%

Labile PEG lipid: 1-10%

2. Variation of helper lipid

The helper lipid cholesterol in formulation LP1 was replaced with similar compounds or delivery enhancers, which structures are shown in the section "Chemical Structures" (identified by the protocol detailed in example 25) generating 14 liposome composition variations. Each of these liposome variations was tested for knock down of eGFP in HeLa eGFP cells at 4 siRNA concentrations (table 3). The three compounds solasodine, ceramide, and diosgenin showed a similar effect as cholesterol in this composition (**Fig. 7c**).

3. LBPA isomer and isoform variation

6 LBPA isoforms and four structurally similar molecules were explored in the formulation LP1 (table 4). The resulting liposome composition variations were tested in HeLa eGFP cells at 4 siRNA concentrations each. Several LBPA isomers show a similar activity: in addition to 2,2'-S,S and 3,3'-S,S isomers (**Fig. 3f,g**) five more isomers or isoforms were showing a similar effect (3,3'-R,S, 3,3'-R,R, 2,2'-S,S with 18:0 or 12:0 fatty acids and hemi-LBPA (3 fatty acids)) (**Fig. 7d**).

4. Encapsulating agent variation

PD740 was tested as encapsulating agent. PD740 (N-[3-(Hexadecan-1-sulfonylamino)propionyl]-4-(R)-(aminoethyl)-L-prolyl-S-farnesyl-L-cysteine methyl ester) has been first synthesized by Biel et al. 2006 as a single isomer (Biel et al. Synthesis and Evaluation of Acyl Protein Thioesterase 1 (APT1) Inhibitors. Chem. Eur. J. 2006, 12, 4121 - 4143). It was tested by Gilleron et al. for siRNA transfection and identified as a delivery enhancer (Gilleron, J. et al. Identification of siRNA delivery enhancers by a chemical library screen. Nucleic Acids Res. 2015, 43, 7984–8001). PD740 was custom synthesized to prepare the inventive liposomes, obtaining PD740 as mixture of isomers of different ratios, which all had the same effect, so that the particular isomer ratio has not influence on the liposome drug delivery efficiency.

DOTAP in formulation LP1 was replaced with 12 different cationic agents or ionizable lipids, and the resulting liposome compositions were tested in HeLa cells at 4 siRNA concentrations (**Table 5**). One drug binding agent, PD740 showed increased activity at lower concentration (composition LP2 (see Table 1)) (**Fig. 7e**). Two other cationic lipids, DOMA and DOTMA, showed a similar activity profile as DOTAP (**Fig. 7e**). Another cationic lipid, EPC 18:1, shows downregulation activity, but at a lower EC50.

Variation of PD740 percentage

As liposomes with PD740 were identified as being more active than those with DOTAP as a encapsulating agent in the same composition, different variations of LP2 composition were explored using varying percentages of PD740 and mixtures of PD740 and DOTAP. 9 liposome composition variations were tested using HeLa eGFP knock down assay at 4 siRNA concentrations (**Table 6, Fig. 7f, 7g**). The composition LP3 containing equal amounts of PD740 and DOTAP showed an even higher effect than composition LP2 (**Fig. 7f**). The composition LP4 containing a lower amount of PD740 showed a similar efficiency profile as composition LP2, while exhibiting a decreased toxicity effect (**Fig. 7f,g**).

The most effective compositions LP1, LP2, LP3 and LP4 were further explored with a 10-concentration dose response experiment using lower siRNA concentrations to be able to extract both EC50 and TC50 values with higher accuracy (**Table 7**). Composition LP3 was confirmed as the most

effective, while composition LP4 was slightly less effective, but showed lower toxicity (Fig. 7h,i) confirming the results of Fig. 7f,g.

Variation of N/P ratio

All previous data were obtained with liposomes containing siRNA at an N/P ratio of 20:1. To explore the scope of possible N/P ratios for the delivery system, liposome formulations of composition LP1 containing siRNA against eGFP were also prepared at charge ratios 40:1, 10:1 and 5:1. The encapsulation of siRNA was 93% at N/P 5:1 (see example 7 and Fig. 2k), leading to the conclusion that any higher charge ratio will lead to an encapsulation above this value, close to 100%. eGFP downregulation with these liposomes was tested at 4 concentrations in HeLa eGFP cells. The GFP intensity and nuclei number were calculated as difference to the same formulation at N/P ratio 20:1 by subtracting the value for the N/P = 20:1 control formulation from the one obtained at the same concentration for the specific formulation in the same experiment. This leads to negative values if the percentage was lower and positive values if it was higher. At concentration 10 nM all N/P ratios showed a similar downregulation result. At lower concentrations the 5:1 formulation was less effective, while the 40:1 is more effective. At the lowest concentration tested (1 nM) the downregulation result was again close to within error for all ratios tested (Fig. 7j). The toxicity of all preparations is within error for all N/P ratios tested (Fig. 7k).

Screening of potential fusogenic agents

To show that other fusogenic agents than LBPA can be used to reach the same effect, LBPA in siRNA containing liposomes of composition LP3 was replaced with different fusogenic agents. The 14 resulting liposome variations were tested each at 10 siRNA concentrations for their GFP downregulation efficiency and toxicity in HeLa eGFP cells (**Table 8**). The compounds used were identified from literature as either being fusogenic or being lipids that prefer hexagonal or cubic phase packing. Liposome variations with MGDG and PLPE showed the same efficiency in downregulation as those with LBPA (**Fig. 8a**). Liposomes containing PLPE showed a similar toxicity profile as LP3 composition with LBPA, while liposomes containing MGDG were less toxic (**Fig. 8b**). Liposomes containing GMO, IsostA, and POPE showed an activity comparable to composition LP1 (**Fig. 8c**), with higher or comparable toxicity (**Fig. 8d**). These results show that other compounds can be used as fusogenic agents for the developed delivery system.

Combination of different fusogenic agents with LBPA in the same composition

Four liposome composition variations of LP3 containing both LBPA and an additional active fusogenic agent were tested at 10 siRNA concentrations in HeLa eGFP (**Table 9**). The downregulation efficiency of liposome compositions with MGDG and PLPE was comparable to LP3, while compositions with GMO and isostearic acid were lower in efficiency (**Fig. 8e**). All

compositions tested show a similar toxicity profile (**Fig. 8f**). These results suggest that the effect in HeLa cannot be boosted by a fusogenic agent combination, but is also not decreased.

Example 14 – uptake of liposomes containing labeled siRNA in primary bone marrow derived mouse macrophages

Description of method:

Murine Macrophage Purification from Bone Marrow

Homogeneous population of quiescent murine monocytes were derived from bone marrow protocol to seed them in 38 multi well plates (Greiner 781092). Briefly, femurs and tibia from C57Bl6 mouse strain were extracted from the mouse after euthanasia following cervical dislocation. The bones were mechanically cleaned from contaminating tissues and resuspended in cold PBS. Samples were transferred to a sterile laminar flow hood for extraction of bone marrow and purification of the relevant cell type.

Always working on ice, the cleaned bones were cut at both ends using a surgical scalpel. Using a 10 cc syringe with a 25 Gauge needle filled with 3 ml of cold PBS, the bone marrow was flushed in an empty 50 ml tube (Corning, 430829) until the cavity of the bone resulted empty. The process was repeated with each available bone, and at the end the cell suspension was disaggregated by continuous pipetting using a 5 ml pipette (Sarstedt, 86.1253.001). The cell suspension was passed through a wet 40 micron sieve (Falcon, 352340) to filter out big tissue clumps, and the filter was washed with cold PBS. The tube was centrifuged at 300xg for 10 minutes on a tabletop centrifuge (Beckman Coultr, Allegra X-22R), and after the centrifugation the supernatant was removed and the cellular pellet was resuspended in PBS, 0.5%BSA, 2mM EDTA.

The viable cell number of the complex mixture was determined using the Luna2 automated cell counter. A volume containing a total of 5×10^7 cells was then transferred to an empty 15 ml tube and the cell suspension was centrifuged at 300xg for 10 minutes, after which the supernatant was completely aspirated, being careful not to disrupt the cell pellet, which was then resuspended in 350 microliters of PBS, 0.5% BSA, 2mM EDTA. A Mouse Monocyte isolation kit (Miltenyi, 130-091-153) was used to purify the monocytes: 25 microliters of FCR Blocking reagent was added to the cell suspension, followed by addition of 50 microliters of Monocyte-Biotin antibody cocktail. The suspension was then incubated for 5 minutes on ice, after which 10 ml of cold PBS, 0.5%BSA, 2mM EDTA was added to the mix. Suspension was then centrifuged at 300xg for 10 minutes, supernatant was discarded and the pellet was again resuspended in 400 μ l of PBS, 0.5% BSA, 2mM EDTA. 100 μ l of AntiBiotin Cocktail solution was added to the suspension and incubated for 10 minutes on ice. During this incubation, LS columns (Miltenyi Biotec, 130-042-401) were assembled on a MACS magnetic stand (Miltenyi Biotec, 130-042-303) and rinsed with 3 ml of cold PBS, 0.5% BSA, 2 mM EDTA to drip in an empty beaker. Upon the end of the drip, the beaker was substituted with an empty 15 ml falcon tube on ice. Cell suspension was applied to the column, and the column was washed 3 times with 3 ml of PBS, 0.5% BSA, 2 mM EDTA collecting the flow through. This cell suspension

was again centrifuged at 300xg, supernatant discarded and pellet resuspended in 2 ml of PBS, 0.5% BSA, 2 mM EDTA. After evaluation of cell number using the LUNA2 automated cell counter, an appropriate number of cells were resuspended in DMEM/F12 (Gibco, 21331-020), 10% FBS (GE Healthcare, A15-102), 50 µg/ml of Gentamicin and 10 ng/ml of macrophage colony-stimulating factor MCSF (PeproTech, 315-02-100UG) to differentiate the monocytes into mouse macrophages (MM): typically MM cells were seeded and cultured at 37 °C and 5% CO₂ in 384 well format, using 15,000 cells per well, in 50 microliters of medium.

Assessment of uptake of liposomes

LP1-liposomes containing Alexa 647 labeled siRNA made by the protocol described in example 1 were transfected in MM in 384 well format to assess their uptake. Prior to transfection, MM were cultured for at least 5 days in DMEM/F12 10% FBS, 50 µg/ml of gentamicin, 10 ng/ml of MCSF into a black 384 well cell culture microplate (Greiner 781092). The transfection mixes were prepared in a 96 well master plate: the liposomes were diluted to 10x the siRNA concentration of the final concentration (5 nM) in 5% glucose. Untreated control samples were prepared using only buffer. Directly before transfection, equal volume of DMEM containing 20% FBS was added to each transfection mix to have a concentration of siRNA of 5x the final concentration for the transfection and 10% FBS. 10 µl of this transfection mix was added to each well of the 384 well plate containing 40 µl of medium, resulting in a final concentration of 5 nM. Each condition was typically tested in quadruplicate. The plate was cultured at 37 °C and 5% CO₂. After 5 h the plate was washed with PBS using the automated PowerWasher cell washer (TECAN) and fixed with 3.7% formaldehyde in PBS for 15 min. After additional washing with PBS each well was stained with 1 µg/ml DAPI (SIGMA, D9542-5MG) and 0.25 µg/ml HCS CellMask™ Blue Stain (Invitrogen, H32720) in PBS containing 0.02% sodium azide. Every treatment was performed in quadruplicate.

Imaging and data analysis:

The plate was imaged at 40x magnification on a Yokogawa CellVoyager CV7000 automated laser spinning disk microscope. 4 fields per well were imaged. Images were adjusted and exported with MotionTracking (<http://motiontracking.mpi-cbg.de>).

Results:

MM were incubated with Alexa 647-labeled siRNA formulated in LP1-liposomes. Internalization of labelled siRNA visualized by confocal microscopy yielded a specific vesicular pattern as expected for uptake by endocytosis (**Fig. 4a**). No aspecific signal could be seen in the 647 channel in untreated cells.

Example 15 – transfection of bone marrow derived macrophages from LifeAct eGFP mice with siRNA containing liposomes

Description of method

Isolation of primary cells

Primary mouse macrophages (MM) cells were isolated from a mouse strain expressing the LifeAct reporter: the procedure for isolations and differentiation is as described in example 14. The transfections were routinely made in 384 well formats, using 15,000 cells per well.

Transfection

Liposomes containing anti eGFP siRNA or A647 labeled anti eGFP siRNA made by the protocol described in example 1 were transfected in MM from LifeAct reporter mouse to assess eGFP downregulation efficiency.

The following description is for a dose response experiment with concentration points taken at 20, 10, 5, 1 and 0.1 nM. This can be adjusted to use different concentrations, more or less points if necessary.

The transfection mixes were prepared in a 96 well master plate: the liposomes were diluted to 10x the concentration of the highest concentration point (100 nM) in the same buffer used for liposome preparation (e.g. 5% glucose). A serial dilution was performed using automated multichannel pipettes (Eppendorf) to obtain solutions at 10x concentration for the other concentration points. Untreated control samples were prepared using only buffer. Directly before transfection, equal volume of DMEM/F12 containing 20% FBS and 20 ng/ml of MCSF was added to each transfection mix to have a concentration of siRNA of 5x the final concentration of the transfection and 10% FBS. 10 µl of this transfection mix was added to each well of the 384 well plate containing 40 µl of medium. Each condition was tested in quadruplicate. The plate was further cultured for 72 h and then fixed with 3.7% formaldehyde in PBS for 15 min. After additional washing with PBS each well was stained with 1 µg/ml DAPI and 0.25 µg/ml HCS CellMask™ Blue Stain in PBS containing 0.02% sodium azide. Every treatment was performed in quadruplicate.

Imaging and data analysis was performed as described in Example 12, except that 9 fields per well were recorded with the Perkin Elmer Operetta high throughput microplate imager and 4 fields per well, sufficient to cover >90% of the well, were recorded with the Yokogawa Cell Voyager CV7000 automated laser spinning disk microscope.

EC50/TC50 calculation

EC50 and TC50 values for 4 and 10 siRNA concentration datasets were calculated by fitting a 4-parameter logistic equation to the GFP intensity and nuclei number normalized to untreated control in response to siRNA concentration as described in example 12. For the 4 and 10 concentration datasets in macrophages only the GFP intensity data could be fitted, as mainly non-toxic concentrations were explored.

Scoring

Scoring of toxicity and effect was done as described in example 12.

Results:

siRNA downregulation with liposomes

MM isolated from a LifeAct-eGFP reporter mouse were incubated with various anti eGFP siRNA concentrations (1-20 nM) formulated in LP1 liposomes made according to protocol in example 1 for 5 hours. Downregulation of eGFP was determined by confocal microscopy imaging and quantitative image analysis as described above. Downregulation of eGFP intensity compared to untreated control increase in response to siRNA concentration, reaching value of 70% eGFP downregulation at of 20 nM, while no evident effect on cell viability as determined by the number of nuclei was detected (**Fig 4c**).

Dose dependency of siRNA downregulation with liposomes (10 concentrations)

To explore the dose dependency of siRNA downregulation with liposomes of composition LP1 made by the protocol detailed in example 1 in greater detail, a dose response curve with 10 concentration points was conducted. This allowed for a more exact calculation of the effect and toxic concentration (**Fig. 4d**). By fitting a four-parameter log-logistics model, the half maximal effective concentration (EC50) was calculated to be 6.37 nM. It was not possible to calculate the half maximal toxic concentration from this experiment as the fitting was not applicable. Toxicity would need to be explored at higher concentrations.

Reproducibility of dose dependent GFP downregulation and toxicity profile mediated by siRNA in liposomes

Downregulation by siRNA at four concentrations in liposomes of composition LP1 containing 3,3'-LBPA as the fusogenic agent and prepared according to the protocol detailed in example 1 was shown to be reproducible over a large number of repeated experiments (**Fig. 4e**). The toxicity profile was reproducible as well (**Fig. 4f**).

Example 16 – Variation of chemical composition of the identified siRNA delivery system in MM

20 liposome composition variations of initial compositions LP1 and LP3 previously tested on HeLa cells (datasets from tables 7-9, using PD740 and DOTAP as encapsulating agents, and other potential fusogenic compounds to replace LBPA or in combination with LBPA) were tested on bone-marrow derived macrophages from LifeAct eGFP mice at 10 concentrations to compare efficiency profiles (**Tables 10, 11, 12**). The different composition variations of anti eGFP siRNA-containing liposomes were made by the protocol detailed in example 1 and tested for their downregulation efficiency and toxicity in HeLa eGFP cells at different siRNA concentrations prior to testing on macrophages

(example 13). In vitro parameters (size, stability, encapsulation and siRNA content) were analyzed for each liposome variation with the techniques detailed in examples 6 and 7. Results obtained with the different liposome compositions are detailed in the following paragraphs.

Encapsulating agent variation

Similar as in HeLa cells, composition LP3 showed a higher downregulation (KD) efficiency and statistically significant lower EC50 than formulation LP1. The difference between the values is not as pronounced as in HeLa cells. Formulation LP4 is significantly less active in macrophages than in HeLa (**Fig. 9a, Table 10**).

Generalization of fusogenic agent concept

Replacement of LBPA in formulation LP3 with different fusogenic agents leads to a different downregulation efficiency in macrophages. Compositions with MGDG and PLPE that showed similar activity as LP3 with LBPA for HeLa transfection showed reduced activity compared to LP3 or no activity in mouse macrophages. Compositions with POPE, which in HeLa showed a reduced activity compared to LP3, showed a similar effect as LP3 in macrophages (**Fig. 9 b, Table 11**).

Combination of different fusogenic agents with LBPA in the same composition

In contrast to the result in HeLa, combinations of LBPA with an additional fusogenic agent within LP3 formulation had increased downregulation activity compared to LP3 (containing only LBPA) as showed by a significantly lower EC50 (**Fig. 9c, Table 12**).

Conclusion:

The efficiency of different compositions resulted to be cell type specific. Composition LP3 showed increased GFPdownregulation efficiency in response to siRNA compared to LP1 in macrophages, but different fusogenic agents showed a different effect in macrophages compared to HeLa cells. A combination of LBPA with an additional fusogenic agent can boost the efficiency of LP3 in macrophages, but not in HeLa cells.

Example 17 – transfection of primary hepatocytes from LifeAct eGFP mice with siRNA containing liposomes

Description of method

Hepatocyte isolation

Primary mouse hepatocytes were isolated from LifeAct eGFP mice via collagenase perfusion using a previously described protocol. Mice were anaesthetized using i.p. injection with Ketamin:Rompun (1.6:1) and fixed unto a sterile surface. The abdominal wall was opened to expose the organs and intestine and fat tissue are moved to expose the venae cavae and the hepatic portal vein. A needle (20G x 1 ½'', 0.9x40 mm, not sharpt) attached to a peristaltic pump was inserted into the venae

cavae, running with a flow rate of 10 ml/min, and the hepatic portal vein was cut to drain solutions. The liver was rinsed for 10 min with EGTA buffer (0.19 g/l EGTA, 0.04 M NaOH, 5.67 g/l glucose, 6.10 g/l NaCl, 0.18 g/l KCl, 0.16 g/l KH₂PO₄, 6.10 g/l HEPES, 0.07 g/l glutamine, 0.04 g/l L-alanine, 0.02 g/l L-aspartic acid, 0.06 g/l asparagine, 0.04 g/l citrulline, 0.02 g/l L-cysteine, 0.15 g/l L-histidine, 0.15 g/l L-glutamic acid, 0.15 g/l glycine, 0.06 g/l L-isoleucine, 0.12 g/l L-leucine, 0.20 g/l L-lysine, 0.08 g/l L-methionine, 0.10 g/l L-ornithine, 0.08 g/l L-phenylalanine, 0.08 g/l L-proline, 0.10 g/l L-serine, 0.20 g/l L-threonine, 0.10 g/l L-tryptophan, 0.08 g/l L-tyrosine, 0.12 g/l L-valine). The solution was switched to collagenase (0.35 g/l collagenase (1-2.5 U/ml) in collagenase buffer (5.46 g/l glucose, 5.87 g/l NaCl, 0.17 g/l KCl, 0.16 g/l KH₂PO₄, 5.87 g/l HEPES, 0.07 g/l glutamine, 0.74 g/l CaCl₂, 0.04 g/l L-alanine, 0.02 g/l L-aspartic acid, 0.06 g/l asparagine, 0.04 g/l citrulline, 0.02 g/l L-cysteine, 0.15 g/l L-histidine, 0.15 g/l L-glutamic acid, 0.15 g/l glycine, 0.06 g/l L-isoleucine, 0.12 g/l L-leucine, 0.20 g/l L-lysine, 0.08 g/l L-methionine, 0.10 g/l L-ornithine, 0.08 g/l L-phenylalanine, 0.08 g/l L-proline, 0.10 g/l L-serine, 0.20 g/l L-threonine, 0.10 g/l L-tryptophan, 0.08 g/l L-tyrosine, 0.12 g/l L-valine)) and perfused for 8 min. After the perfusion the liver was removed from the animal and put into 15 ml suspension buffer (5.63 g/l glucose, 6.05 g/l NaCl, 0.18 g/l KCl, 0.16 g/l KH₂PO₄, 6.05 g/l HEPES, 0.07 g/l glutamine, 0.15 g/l CaCl₂, 0.10 g/l MgSO₄, 0.04 g/l L-alanine, 0.02 g/l L-aspartic acid, 0.06 g/l asparagine, 0.04 g/l citrulline, 0.02 g/l L-cysteine, 0.15 g/l L-histidine, 0.15 g/l L-glutamic acid, 0.15 g/l glycine, 0.06 g/l L-isoleucine, 0.12 g/l L-leucine, 0.20 g/l L-lysine, 0.08 g/l L-methionine, 0.10 g/l L-ornithine, 0.08 g/l L-phenylalanine, 0.08 g/l L-proline, 0.10 g/l L-serine, 0.20 g/l L-threonine, 0.10 g/l L-tryptophan, 0.08 g/l L-tyrosine, 0.12 g/l L-valine). Hepatocytes were extracted from the liver tissue using forceps. The cell suspension was filtered through a cell strainer (100µm pore size) and diluted to 50 ml with suspension buffer. The cells were pelleted by centrifugation at 4 °C with 50xg for 5 ml and the pellet was washed with 50 ml suspension buffer and recentrifuged before being resuspended in 10 ml suspension buffer by gentle inversion.

Hepatocyte plating

Cells were plated into 96 well plates (Greiner 655090) precoated with collagen using a previously published protocol. Rat tail collagen (10 mg, Roche 11179179001) was dissolved in sterile 0.2% acetic acid (10 ml) and incubated at 4 °C for at least 6 h, this solution was diluted to a concentration of 0.9 mg/ml in 10x DMEM and the pH was adjusted to 7.4. The collagen bottom layer was added to the wells by adding 25 µl of this solution and letting it polymerize at 37°C for 1h before adding 150 µl Williams E medium (Pan Biotech P04-29150), substituted with 10% FBS (Pan Biotech P40-37500), 100 nM dexamethasone (Sigma D4902-500MG) and penicillin/streptomycin (Gibco 15140-122). The medium was removed and replaced by cell solution containing 30,000 cells per well. Cells were incubated for 1,5 h at 37°C in an atmosphere with 5% CO₂ atmosphere for attachment, washed two times with PBS and 90 µl Williams E medium was added.

Hepatocyte transfection

siRNA liposomes of composition LP1 containing anti eGFP siRNA prepared to protocol described in example 1 were diluted to 10x the desired concentration for transfection in a master plate and 10 µl of this solution was added to each well. The plate was incubated for 5 h, washed with PBS and a second collagen layer was added on top by adding 50 µl of 0.6 mg/ml collagen solution. The plate was incubated for 1 h at 37 °C before 150 µl of Williams E medium was added on top. The plate was incubated for 3 d at 37 °C and the medium was replaced with fresh medium each day. For fixation medium is removed and 100 µl of 3.7% formaldehyde solution is added and incubated at room temperature for 15 min. The plate is washed three times with PBS and stained with 1 µg/ml DAPI (SIGMA, D9542-5MG) and 0.5 µg/ml HCS CellMask™ Blue Stain (Invitrogen, H32720) in PBS containing 0.02% sodium azide.

Imaging and data analysis:

The plate was imaged with 10x magnification on a Yokogawa CellVoyager CV7000 automated laser spinning disk microscope (6 fields per well). The cell area was masked based on the DAPI/CMB staining and the total GFP intensity normalized by masked area for each image was analyzed.

The GFP intensity in percentage was calculated as follows:

The mean GFP intensity per well was averaged per condition. This value was normalized to the value calculated the same way from the untreated control wells.

The analysis was conducted using MotionTracking software (<http://motiontracking.mpi-cbg.de>, Collinet, C. et al., Systems survey of endocytosis by multiparametric image analysis. Nature 464, 243–249 (2010)) for segmentation, parameter extraction, averaging and normalization.

Results:

Primary hepatocytes isolated from a LifeAct-eGFP reporter mouse were incubated with various concentrations (0.1-20 nM) of siRNA against eGFP formulated in liposomes of composition LP1 made according to protocol in example 1 for 5 hours. Downregulation of eGFP was determined by confocal microscopy imaging and quantitative image analysis as previously described (**Fig. 4g**). Increasing concentrations of liposome-siRNA caused up to 97% downregulation of eGFP intensity compared to untreated control, while it had limited effect on cell viability as determined by the number of nuclei (**Fig 4h**).

Example 18 – transfection of HeLa cells with mRNA containing liposomes

Description of method

Liposomes were made by the protocol described in example 3 containing an mRNA coding for eGFP fluorescent reporter (TriLink Biotechnologies, L-7201) and were used to transfect HeLa cells, (Accession: CVCL_1922, https://www.cellosaurus.org/CVCL_1922) (2000 cells/well in a 384 well

plate) to assess the ability to induce expression of the fluorescent reporter, as monitored by automated microscopy. The cells were plated and cultured as in example 12.

The following description is for a dose response experiment with concentration points ranging from 1.25 µg/ml to 0.039 µg/ml. This can be adjusted to use different concentrations, more or less points if necessary.

Liposomes containing mRNA were prepared (e.g. 5% glucose) at the highest concentration necessary for transfection as described in example 3 using a lipid to mRNA ratio of 6:1. Serial dilution was performed using automated multichannel pipettes (Eppendorf) to obtain solutions at 10x concentration for the other concentration points. Untreated control samples were prepared using only buffer. Directly before transfection, equal volume of DMEM containing 20% FBS was added to each transfection mix to have a concentration of mRNA of 5x the final concentration of the transfection and 10% FBS. 10 µl of this transfection mix was added to each well of the 384 well plate containing 40 µl of medium. Each condition was tested in quadruplicate. The plate was further cultured for 24 hours and then fixed with 3.7% formaldehyde for 15 min. After additional washing with PBS each well was stained with 1 µg/ml DAPI and 0.25 µg/ml HCS CellMask™ Blue Stain in PBS containing 0.02% sodium azide. Every treatment was performed in quadruplicate.

Imaging and data analysis:

The plate was imaged with 10x magnification on a Yokogawa CellVoyager CV7000 automated laser spinning disk microscope. 4 fields per well were imaged. The nuclei and cells were segmented based on the DAPI/CMB staining and the mean GFP intensity for each nucleus was recorded.

The total cell number in percentage was calculated as follows:

The number of nuclei per image was summed up per well and then averaged per condition. This value was normalized to the value calculated the same way from the untreated control wells and multiplied by 100.

The GFP intensity was calculated as follows:

Median of the mean GFP intensity per nucleus or per cell was calculated per image, averaged per well and averaged per condition.

Additionally, the number of cells expressing GFP was calculated from single cell data, utilizing a threshold evaluated on the comparison of the distribution of the value of the mean intensity of eGFP in the untreated cells and cells transfected with the eGFP mRNA. The threshold was chosen in order to exclude false positives due to cell autofluorescence: cells having values of mean eGFP fluorescence above that threshold were considered as GFP positive, while cells below were considered GFP negative. For each well, the number of cells that were evaluated as positives was counted and the percentage of GFP expressing cells was evaluated calculating the ratio of these positive cells and the total number of cells per condition, and multiplying it by 100.

The analysis was conducted using CellProfiler for segmentation and parameter extraction and Knime Analytics platform for calculation, averaging and normalization.

Results:

Liposomes of composition LP1 carrying eGFP mRNA can induce eGFP expression in HeLa cells at a low charge ratio of 6:1 (positive/negative) in a dose-dependent manner within 24 h. Over 90% of all cells were GFP positive by using liposomes with eGFP mRNA at a dose of 0.31 µg/ml without toxicity (Fig. 5d, e). A similar result can be obtained at a charge ratio of 3:1 (Fig. 5f). Comparing a wider range of N/P ratios ranging between 1:1 and 20:1 showed that similar number of cells can be transfected at the same concentration with any ratio above 3:1 (Fig. 5h), the highest eGFP expression at the same concentration can be reached with 3:1 ratio. Both the lower and higher ratios show lower expression (Fig. 5g). Toxicity for all N/P ratios above 3:1 was similar and is lower for the less effective N/P ratio 1:1 (Fig. 5i).

Exploration of chemical space of the identified delivery system for mRNA delivery

Different liposomal compositions were tested for their transfection efficiency of mRNA in HeLa cells and compared with the initial composition LP1.

Compositions containing varying amounts of solasodine showed a similar response in terms of total cell number expressing eGFP at a specific mRNA concentration (**Fig. 12a**). In terms of eGFP intensity, solasodine containing compositions show slightly increased expression levels in one out of three experiments (**Fig. 12b**).

Compositions LP3 and LP4 (the most effective compositions for siRNA delivery in HeLa cells and primary macrophages (see figure 7h, 7i and 9a; Tables 7 and 10)) did not result to be the most effective for mRNA delivery. mRNA containing LP3 showed a similar effect as mRNA containing LP1, while mRNA containing LP4 was much less effective (**Fig. 12c,d**). Both showed higher toxicity at concentrations 0.3125 and 0.625 µg/ml compared to LP1 (**Fig. 12e**).

Compositions with MGDG and PLPE (combining PD740, DOTAP and MGDG/PLPE as fusogenic agents) were among the most effective compositions for siRNA delivery and GFP downregulation in HeLa and primary macrophages (see **Fig. 8e,f** and **9b**, **Tables 8** and **11**). For mRNA delivery in HeLa cells they showed a response at much lower concentration than the original composition LP1, reaching most cells already at the lowest concentration tested (Fig. **12f**). GFP expression intensity was also increased at the lowest concentration (**Fig 12g**). Toxicity levels were within the error range of the treatment with LP1 and not as pronounced as for treatment with LP3 (same formulation except LBPA as fusogenic agent) (**Fig. 12h**). These compositions were also tested at lower concentrations to see full dose response curve. These experiments confirmed that by using LP-MGDG and LP-PLPE compositions for mRNA delivery more cells were reached and GFP expression was higher at lower concentrations of mRNA compared to composition LP1 (**Fig. 12i,j**).

Example 19 – transfection of primary bone marrow derived mouse macrophages with mRNA containing liposomesDescription of methodIsolation of primary cells

Primary MM cells were isolated from wild type C57Bl6 mouse strain. The procedure for isolations and differentiation is as described in example 14. The transfections were routinely made in 384 well formats, using 15,000 cells per well and 4 replicates for each condition

Transfection

Liposomes complexed with an mRNA coding for fluorescent reporter eGFP (as described in example 3) were transfected in MM to assess the ability to induce expression of the fluorescent reporter, which was monitored by automated microscopy.

Transfection of MM cells with mRNA containing liposomes was performed as described in Example 18 for Hela cells.

Imaging and data analysis was performed as described in Example 18.

Results:

eGFP expression in primary bone marrow-derived mouse macrophages after delivery of eGFP mRNA in liposomes at a charge ratio of 6:1 is dose-dependent with the majority of cells expressing eGFP at a dose of 1.25 µg/ml after 24 h without toxic effects (**Fig. 5j,k**). Liposome mRNA complexes at a charge ratio of 3:1 also show downregulation, albeit with lower efficiency, reaching 60% of cells at a dose of 1.25 µg/ml and showing lower fluorescence intensity (**Fig. 5l**).

Example 20 – transfection of primary mouse hepatocytes with mRNA containing liposomesDescription of methodHepatocyte isolation and plating

Primary mouse hepatocytes were isolated from wild type C57Bl6 mice via collagenase perfusion and plated as described in example 17.

Hepatocyte transfection

Liposomes prepared as in example 2 were complexed with an mRNA coding for eGFP (as described in example 3) at ratio N/P 3:1 were transfected into hepatocytes to assess the ability to induce expression of the fluorescent reporter. The following description is for a dose response experiment with concentrations ranging from 2,5 to 0,3125 µg/ml. Liposome mRNA complexes were diluted to 10x the desired concentration for transfection in a master plate and 10 µl of this solution was added to each well of a 96-well plate containing 90 µl of cells in Williams E medium. The plate was incubated for 5 h, washed with PBS and a second collagen layer was added on top by adding 50 µl

of 0.6 mg/ml collagen solution. The plate was incubated for 1 h at 37 °C before 150 µl of Williams E medium was added on top. The plate was incubated for 24 h at 37 °C before removing the medium and adding 100 µl of 3.7% formaldehyde solution for fixation. The plate was incubated at room temperature for 15 min. The plate was washed three times with PBS and stained with 1 µg/ml DAPI (SIGMA, D9542-5MG) and 0,5 µg/ml HCS CellMask™ Blue Stain (Invitrogen, H32720) in PBS containing 0.02% sodium azide.

Imaging and data analysis:

The plate was imaged with 40x magnification on a Yokogawa CellVoyager CV7000 automated laser spinning disk microscope (9 fields per well). The cell area was masked based on the DAPI/CMB staining and the total GFP intensity normalized by masked area for each image was analyzed.

The GFP intensity in percentage was calculated as follows:

The mean GFP intensity per well was averaged per condition. The value was subtracted with the mean GFP intensity calculated the same way for untreated conditions (background). The analysis was conducted using MotionTracking software (<http://motiontracking.mpi-cbg.de>) for segmentation, parameter extraction, averaging and normalization.

Results:

eGFP expression in primary hepatocytes could be generated using eGFP mRNA liposomes at N/P ratio 3:1 and was dose dependent (**Fig. 5m,n**).

Example 21 – transfection of HeLa eGFP cells with CRISPR Cas9 RNP containing liposomes

Description of method

Liposomes were complexed with CRISPR Cas9 ribonucleoprotein complex as described in example 5 and transfected in HeLa eGFP cells to assess their ability to induce sequence specific editing of eGFP: the functional result of the sequence specific gene editing would be the lack of eGFP expression, which is evaluated by automated microscopy.

Complex Cas9/guide formation

The first step in the process is the preparation of the complex between the guide/tracer and a recombinant CAS9 protein.

The recombinant Cas9 protein (<https://www.uniprot.org/uniprotkb/Q99ZW2/entry>) was obtained from the Protein Expression, Purification and Characterization (PEPC) facility of the MPI-CBG, where the protein is available in aliquots of 10µl, at the concentration of 5 mg/ml.

A custom designed guide sequence against eGFP (target sequence: GGAGCGCACCATTCTTCTTCA, SEQ ID NO 5) and tracr RNA were purchased from IDT (Table 14) and are available as a 100 µM stock.

A typical complexing reaction was performed in a total volume of 35 μL , mixing 3 μL of Cas9 protein, 32 μL of protein buffer (20 mM Hepes, pH 7.5, 150 mM KCl), 0.98 μL of GFP cr guide RNA and 0.98 μL of tracr RNA for 15 minutes at 37°C. The resulting concentration of the GFP cr guide RNA in the complexing solution is 2.8 μM .

Transfection of liposome-RNP complex

The ribonucleic protein complex (RNP) was then incubated with liposomes as in example 5. The transfection mixes were prepared in a 96 well master plate: the complexes were diluted to 10x the concentration of the highest concentration point (100 nM) of GFP cr guide RNA in the preparation buffer (e.g. 5% glucose).

The following description is for a dose response experiment with GFP cr guide RNA concentrations of 10, 5, 2.5, 1 and 0.1 nM.

HeLa eGFP cells were cultured in DMEM media complemented with 10% FBS at 37 °C and 5% CO₂ and seeded the day before the transfection into a black 384 well cell culture microplate (Greiner 781092) in DMEM medium with 10% FBS and 50 $\mu\text{g}/\text{ml}$ gentamicin. Serial dilution of liposome composition and cell transfection were performed as described in Example 12.

Imaging was performed using a Yokogawa CellVoyager CV7000 automated laser spinning disk microscope as described in Example 12, but imaging only 4 fields per well. Data analysis was performed using CellProfiler for segmentation and parameter extraction and Knime analytics platform for calculation, averaging and normalization as described in Example 12.

Results:

Using liposomes of composition LP1 loaded with Cas9 ribonucleoprotein with guide RNA mix containing GFP cr guide RNA and tracr RNA at charge ratio 9.10:1 downregulation of eGFP intensity to 20% of expression compared to untreated control is possible at 2.5 nM RNA concentration with low toxicity. Increased toxicity is observed at higher concentrations (Fig. 6a). Lowering the charge ratio to 5,46:1 shows a similar result both for downregulation and toxicity response (Fig. 6b,c).

Example 22 – transfection of HeLa eGFP cells with guide RNA containing liposomes

Description of method

Liposomes made by the protocol described in example 4 were transfected in HeLa eGFP cells to assess their ability to induce sequence specific editing of eGFP: the functional result of the gene editing would be the lack of eGFP expression evaluated with automated microscopy. Differently from Example 21 the Cas9 protein is not delivered by the liposomes, but is independently delivered by infection of the HeLa cells with a BACMAM virus containing an orf for it. The BACMAM virus was produced by the Protein Expression and Purification (PEPC) facility of the MPI-CBG.

Guide complex formation

The first step in the process is the preparation of the cr guide/tracr RNA complex as species of RNA to be transfected in cells infected with a BACMAM virus containing Cas9. A custom designed guide sequence against eGFP (GFP cr guide RNA, target sequence: GGAGCGCACCATCTTCTTCA) and tracr RNA were purchased from IDT and are available as a 100 μ M stock.

A typical complexing reaction was performed in a total volume of 30 μ L, using 28.32 μ L of 20 mM Hepes, pH 7.5, 150 mM KCl, 0.84 μ L of GFP cr guide RNA and 0.84 μ L of tracr RNA for 15 minutes at 37°C. The resulting concentration of the GFP cr guide RNA in the complexing solution is 2.8 μ M.

Transfection of guide RNA liposomes

The guide RNA complex was then incubated with liposomes prepared by the protocol detailed in example 2 as described in example 4. The transfection mixes were prepared in a 96 well master plate: the complexes were diluted to 10x the concentration of the highest concentration point (100 nM) in the buffer that they were prepared in (e.g. 5% glucose).

For Cas9 expression HeLa eGFP cells were mixed with 0.71 μ l of BACMAM virus solution per 1000 cells during seeding.

Seeding, culture and transfection of Hela eGFP cells as well as imaging and data analysis were performed in Example 21.

Results:

After testing the delivery of the Cas9 protein and guide RNA mix together in form of the Cas9-guide RNA ribonucleoprotein complex (Example 21), this Example aims to determine the delivery of guide RNA mix alone into HeLa eGFP cells that were expressing the Cas9 protein. Cas9 protein was expressed in these cells prior to transfection using a BacMam system.

Different liposome compositions were tested using a charge ratio of 8.45:1 which corresponds to the same amount of guide RNA mix as for the delivery of Cas9 RNP at charge ratio 9.10:1 (as used in Fig. 6a). Both isoforms of LBPA in composition LP1 showed similar activity for knock out of eGFP in response to RNA concentration. A similar level could also be reached using composition LP4, while replacing the encapsulating agent for EPC 18:1 resulted in a slightly lower efficiency (Fig. 6d). Toxicity was comparable for both LBPA isoforms and EPC 18:1, while it was increased for composition LP4 (Fig. 6e). Toxicity is decreased compared to transfection of Cas9 RNP at the same concentration (see Fig. 6 a).

Using a lower charge ratio of 5.07:1 for transfection with guide RNA mix and liposomes of composition LP1 results in a similar dose response both for eGFP reduction as well as for toxicity (Fig. 6f,g).

Example 23 – Galectin recruitment

Description of method

Following method is to investigate the question if the liposomal delivery system causes endosomal damage by disrupting endosomal membranes during the escape process using galectin recruitment assay similar to a previous report. HeLa eGFP cells (Bramsen, J. B. et al., 2009) were cultured DMEM media complemented with 10% FBS at 37 °C and 5% CO₂. The cells were seeded at the density of 600cells/40ul/well into a black 384 well cell culture microplate (Greiner 781092) in DMEM medium with 10% FBS and 50 µg/ml gentamicin. GFP-Galectin 8 plasmid (Galectin 8/LGALS8 cDNA ORF Clone, Human, C-GFPSpark® tag, SinoBiological HG10301-ACG) and lipofectamine 3000 were prepared in OptiMEM at 10x the final concentration and mixed in a 1:1 ratio via pipetting to reach 5x the final concentration. The transfection mix was incubated at room temperature for 15 min and 10 µl were added to each well of cells containing 40 µl total volume. The final concentrations were 10 ng/well for the Galectin plasmid and 0.1 µl/well for lipofectamine. The cells were incubated for 72h at 37 °C and 5% CO₂ before being washed with BioTek EL406 washer using serum medium for 2 cycles. In each cycle, the medium was aspirated to 20 µl and 90 µl of medium was added. For final aspiration, the medium was aspirated to 40 µl. Stock solutions of the lysosomal permeating agent L-leucyl-L-leucine methyl ester (LLOMe) (final concentration of 2 mM) at 5x the final concentration in medium, liposomes prepared according to protocol detailed in example 1 at 5x the final concentration (5 nM) in Glucose solution with 10% FBS and glucose with 10% FBS without liposomes as a control were prepared in 96 well plate format. 10 µl of these solutions were added to cells containing 40 µl medium. The plate was incubated at 37 °C and 5% CO₂ for 2h. Afterwards the plate was washed three times with PBS with a Tecan Microplate Power Washer PW384 and fixed with 3.7% formaldehyde in PBS for 10 min. After additional washing with PBS each well was stained with 1 µg/ml DAPI (SIGMA, D9542-5MG) and 0.25 µg/ml HCS CellMask™ Blue Stain (Invitrogen, H32720).

Imaging and data analysis:

The plates were imaged in Yokogawa CV7000 cell voyager automated laser spinning disk microscope at a magnification of 60x. 12 fields were imaged per well. The resulting images were adjusted and exported with MotionTracking (<http://motiontracking.mpi-cbg.de>, Collinet, C. et al, 2010).

Results:

Galectins (soluble, cytosolic carbohydrate-binding proteins) are recruited to galactoside sugars on the luminal membrane of endosomes after damage, resulting in a punctuate aggregation pattern. This pattern is visible for release events with lipoplexed siRNA (lipofectamine 2000) and siRNA in lipid nanoparticles.

GFP-tagged Gal-8 was expressed in HeLa cells and the galectin recruitment pattern was observed after treatment with the inventive liposomal delivery system compared to endosomal disruption agent L-Leucine methyl ester (LLOMe). No distinct pattern could be detected and liposome treated cells

looked like untreated control (**Fig. 10a**). This means that the escape of siRNA from endosomes with the liposomal system does not disturb the endosomal integrity and no part of the internal membrane is exposed to the cytosolic side.

Example 24 – endosome bursting visualization

Description of method

Lipoplexes and liposomes containing fluorescently labelled siRNAs were incubated in HeLa eGFP cells to investigate siRNA escape mechanism, adapting a protocol that has previously been used to study lipoplexes and lipid nanoparticles (Wittrup, A. et al. *Visualizing lipid-formulated siRNA release from endosomes and target gene knockdown*. Nat. Biotechnol. 33, 870–876 (2015)). The following description is an experimental procedure for live cell fluorescence microscopy imaging of siRNA escape.

HeLa eGFP cells (Bramsen, J. B. et al., 2009) were cultured DMEM media complemented with 10% FBS at 37 °C and 5% CO₂ and seeded the day before the transfection into a black 384 well cell culture microplate (Greiner 781092) in DMEM medium with 10% FBS and 50 µg/ml gentamicin. On the day of transfection, anti eGFP siRNA labeled with Alexa Fluor 647 (5 µl, 1000 nM) was mixed with lipofectamine 2000 (4 µl) as reported previously to form siRNA lipoplex transfection mix (Wittrup, A. et al., 2015). 5 µl of this transfection mix was added to the well containing cells in 45 µl of medium. Liposomes of composition LP1 containing A647 labeled anti eGFP siRNA were prepared as reported in example 1 and diluted to a working concentration of 250 nM (5x final concentration 50 nM). 10 µl of this transfection mix was added to the well containing cells in 40 µl of medium. Each transfection was done in triplicate.

Imaging and data analysis:

For live cell imaging, the plate was incubated at 37 °C and 5% CO₂ in CV7000 cell voyager microscope imaging chamber. Imaging at a magnification of 60x was started immediately after adding the transfection mix to the respective well. All wells were imaged in the GFP channel (525 nm) first to confirm the location of the cells, then a time course measurement in the Alexa647 channel (676 nm) was started. For each timepoint a Z-stack of 14 planes spaced 0.7µm apart was acquired first with short exposure (25 ms) to capture the siRNA uptake, and then one image was taken in one plane with long exposure (250 ms) at low laser power to visualize a faint cytoplasmic stain of escaped siRNA Alexa 647. Each field was imaged for 5 min with timepoints taken at 6.4 s intervals. 6 fields per well were imaged.

The resulting image datasets were analyzed with MotionTracking (<http://motiontracking.mpi-cbg.de>, Collinet, C. et al, 2010). For visualizing the cellular location a mask was put on top of the GFP image with a threshold of 120 to distinguish cells from background. The mask was exported. For each timepoint the short and long exposure images in the A647 channel were maximum projected. Individual images as well as videos were exported.

Results:

Lipoplexes such as lipofectamine 2000 show a typical release pattern of whole cell fluorescence when labeled cargo is used, while endosomal release by softer methods (which show effective gene downregulation using far lower concentrations) such as transfection with lipid nanoparticles does not show such pattern.

Treatment with liposomes loaded with siRNA labeled with Alexa647 showed a vesicular staining pattern which is similar to those observed for LNPs, and different from the whole cell fluorescence pattern induced by lipofectamine treatment (Fig. 10b).

Example 25 – enhancer screening by transfection of HeLa eGFP cells with cholesterol conjugated siRNA and small molecule enhancers

Description of method

HeLa eGFP cells were incubated with siRNAs conjugated with cholesterol and candidate enhancer compounds to assess their effect on downregulation efficiency mediated by cholesterol conjugated siRNAs.

HeLa eGFP cells were cultured as described for Example 12. On the day of transfection, the test compounds and Chol-siRNA mixes were prepared in a 96 well format master plate. The compounds were diluted to 6x of the final concentration 36 or 42 μM , i.e. 6 μM for compounds 1-24, and 7 μM for compounds 25-32 in reduced serum medium, OptiMEM (ThermoFisher 31985070). Chol-siRNAs against eGFP was custom synthesized by Axolabs. Cholesterol was conjugated to the 3'-end of sense strand of the siRNA molecule by a short L-prolinol-N-6-hexanoyl aminocarbonyl (Structure on page 61). Chol-siRNA against eGFP (Axolabs, custom synthesis) was prepared at 6X (1200 nM) of the final concentration 200 nM in OptiMEM. The cell plates were washed with OptiMEM using Biotek EL406 washer for 2 cycles. In each cycle, the medium was aspirated to 20 μl and 90 μL of OptiMEM was added. For final aspiration, the medium was aspirated to 50 μl . 10 μl of preprepared master mixes of compounds and Chol-siRNA were added to each well of the 384 well plate containing 50 μl of OptiMEM. Each condition was tested in quadruplicate. The plate was incubated at 37 °C and 5% CO₂. After 5 h, the compound and Chol-siRNA mixes were washed away from the cells by replacing the medium three times with fresh DMEM (80 μl) and aspirating to 25 μl with a Biotek EL406 washer. Afterwards, 25 μl of DMEM containing 20% FBS and 100 $\mu\text{g/ml}$ gentamicin was added. The plate was further cultured for 72 h and then fixed with 3.7% formaldehyde in PBS for 10 min. After additional washing with PBS each well was stained with 1 $\mu\text{g/ml}$ Hoechst 33324 and 0.5 $\mu\text{g/ml}$ HCS CellMask™ Blue Stain (Invitrogen, H32720).

Imaging and data analysis:

The plate was imaged with 10x magnification on a Perkin Elmer Operetta high throughput microplate imager. 9 fields per well were imaged. The nuclei and cells were segmented based on the Hoechst 33324/CMB staining and the mean GFP intensity for each nucleus was recorded.

The nuclei number in percentage and GFP intensity in percentage were calculated as described in Example 12.

Results:

In a screen of structural analogues of sterol-based compound E18 (Table 13), some sterols were identified to increase downregulation efficiency of Chol-siRNA at suboptimal concentration in HeLa eGFP cells (Fig. 11a).

Example 26 – enhancer dose response pattern with a fixed concentration of cholesterol-conjugated siRNA

Description of method

Compounds solasodine, solasodine acetate and diosgenin were identified as hits in the screening described in Example 25, and further characterized by determining their dose response in combination with 150 nM Chol-siRNA (Fig. 10d).

HeLa eGFP cells were cultured as described in the previous example 12. On the day of transfection, the test compounds and Chol-siRNA mixes were prepared in a glass vial in a 96 well format master plate. The test compounds were diluted to 160 nM (4x the highest concentration of the final 18 concentrations (40, 32, 25.6, 20.5, 16.4, 13.1, 10.5, 8.4, 6.7, 5.4, 4.3, 3.4, 2.7, 2.2, 1.8, 1.4, 1.1, 0.9 μ M) in reduced serum medium, OptiMEM. Chol-siRNA samples were prepared at 4x of the final concentration 150 nM in OptiMEM. The cell plates were washed with OptiMEM using Biotek EL406 washer for 2 cycles. In each cycle, the medium was aspirated till 20 μ L and 90 μ L of OptiMEM was added. For final aspiration, the medium was aspirated till 40 μ L. 10 μ L of preprepared master mixes of compounds and Chol-siRNA were added to each well of the 384 well plate containing 40 μ L of OptiMEM. Each condition was tested in quadruplicate. The plate was incubated at 37 °C and 5% CO₂. After 5 h, the compound and Chol-siRNA mixes were washed away from the cells by replacing the medium three times with fresh DMEM (80 μ L) and aspirating to 25 μ L with a Biotek EL406 washer. Afterwards, 25 μ L of DMEM containing 20% FBS and 100 μ g/ml gentamicin was added. The plate was further cultured for 72 h and then fixed with 3.7% formaldehyde in PBS for 10 min. After additional washing with PBS each well was stained with 1 μ g/ml DAPI (SIGMA, D9542-5MG) and 0.25 μ g/ml HCS CellMask™ Blue Stain (Invitrogen, H32720).

Imaging and data analysis were performed as described for Example 25, except that nuclei and cells were segmented based on the DAPI/CMB staining.

Results:

The dose response curve for eGFP downregulation by chol-siRNA combined with solasodine, or solasodine acetate or diosgenin is shown in Fig. 11b.

Compounds solasodine and diosgenin were tested as siRNA delivery enhancer in combination with the helper lipid cholesterol or as replacement of cholesterol in the inventive liposomal delivery system. Hela cells incubated with siRNA-liposomes comprising solasodine or diosgenin instead of cholesterol showed a similar downregulation pattern as those incubated with siRNA-liposomes comprising cholesterol (Fig. 7c, example 13).

Moreover, compositions LP-Solasodine2 and LP-Solasodine3 containing both solasodine and cholesterol showed a similar effect on expression pattern for mRNA containing liposomes (Fig. 12a,b, example 18).

Example 27 – liposome formation with NanoAssemblr

Description of method

Empty liposomes of composition LP1 were produced with a NanoAssemblr Ignite microfluidic system using NxGen cartridges. A lipid film of composition LP1 was produced according to the protocol detailed in example 1. The dried film was kept at vacuum for 1 h and stored at -20 °C before usage. The lipids were dissolved in ethanol at a final total lipid concentration of 12.5 mM. This solution was used as the organic phase in the NanoAssemblr setup. The aqueous phase was 10 mM TRIS, pH 7.4. These solutions were run at a flow rate ratio of 1:1 with a total flow rate of 10 ml/min. The total run volume was 0.95 ml, of which 0.3 ml were discarded as initial waste and 0.05 ml were discarded as final waste. The rest was collected as the sample and directly post-processed to remove residual ethanol.

Post processing

Dialysis

250 µl of the sample were diluted with 240 µl of TRIS buffer (10 mM, pH 7.4). They were dialyzed against 13.5 ml TRIS buffer (10 mM, pH 7.4) using a Slide-A-Lyzer™ MINI dialysis device (10K MWCO, 0.5 ml, ThermoFisher 88401) for 2 h with orbital shaking at room temperature. The buffer was exchanged and the dialysis was continued for another 2 h under the same conditions before collecting the sample.

Diafiltration

350 µl of the sample were diluted 40x with 13.65 ml TRIS buffer (10 mM, pH 7.4). The sample was sterile filtered through an Acrodisc® syringe filter (Supor® Membrane; 13mm, 0.2µm, PALL 4602). The solution was transferred into an Amicon® Ultra-15 centrifugal filter unit (10K MWCO, Merck UFC901024D) and centrifuged with 2000 rcf at 20 °C for 65 min using a Heraeus Megafuge 1.0 R. The sample was collected.

Quality control

To compare this liposome production techniques with previous methods for manual production (Examples 1-5) the liposomes were analyzed with the same techniques as the previous ones. Their size was checked with dynamic light scattering as detailed in example 6. For lipid concentration quantification, a Chol-Glo assay was used to estimated cholesterol content (example 28). The efficiency was analyzed by combining these empty liposomes produced with the NanoAssemblr with mRNA the same way as detailed in example 3 and transfecting HeLa cells with them using the protocol described in example 18. This was compared to liposomes produced with extrusion as detailed in example 2.

Results:

To increase production of liposomes to a higher throughput it is desirable to move from a manual manufacturing protocol to an automated one. The method of choice is microfluidics, as employed for LNP production.

Liposomes were produced by Precision NanoSystem's NanoAssemblr Ignite microfluidic system. The working principle is to combine lipids in ethanol with an aqueous solution under controlled flow and geometrical conditions. Due to high content of ethanol a post-production step is needed. Ethanol can be removed either by dialysis or by diafiltration. Both methods were tested.

Liposomes produced with microfluidics were mixed with eGFP mRNA as described in example 3 at an N/P ratio of 6:1. They were tested for dose response of eGFP expression in HeLa cells as described in example 18. They showed the same dose response for mRNA transfection in HeLa cells in terms of GFP positive cells independently from the post-production technique used as liposomes produced by extrusion (**Fig. 13a**). The overall expression of GFP seemed to be a bit lower for liposomes where ethanol was removed by diafiltration, while dialysis-treated liposomes showed the same expression levels as extruded liposomes (**Fig. 13b**).

Size of liposomes made with the different described techniques was assessed by dynamic light scattering as described in example 6. Before post-processing, liposomes made by NanoAssemblr reached much smaller sizes than those produced by extrusion. The size increased during post-processing, but both treatments still yielded liposomes with a smaller size distribution than those manually produced by extrusion (**Fig. 13c**).

Example 28 – Lipid concentration quantification with Cholesterol-Glo assay

Description of method

The lipid concentration of liposomes without cargo was analyzed using the Cholesterol/Cholesterol Ester-Glo™ assay from Promega (J3190), in which cholesterol from detergent lysed liposomes is metabolized enzymatically in combination with activation of luciferin production, which is then detected by luminescence of a luciferase reaction.

The assay was performed in a white polystyrene Corning® 96 Well Half-Area Microplate (Merck, CLS3696). Each sample was assayed in triplicate. The individual samples were compared to a four-point standard curve of pure cholesterol, in the range between 1 and 20 μ M. The samples (liposomes produced by either of examples 1-5, 24) were aimed to have a final concentration of 7.5 μ M.

For a simultaneous test of 5 samples cholesterol detection reagent was prepared by mixing cholesterol detection solution (1550 μ l) with reductase substrate solution (15 μ l). This solution was kept at room temperature in the dark until further usage. 3 replicates of fitting volume of the sample or the standard curve points were pipetted into each well and diluted to 40 μ l with Cholesterol lysis solution. The plate was briefly shaken by hand and incubated for 30 min at 37 °C. 40 μ l of cholesterol detection reagent prepared before were added to each well, the plate was briefly shaken by hand and incubated at room temperature in the dark for 1 h. The luminescence was read with a Tecan Spark® 20M multimode microplate reader.

Data analysis

The luminescence was plotted against the cholesterol concentration for the standard curve and analyzed using R employing a weighted linear fit. The cholesterol concentration for each well was calculated based on the extracted slope and intercept of the standard curve and averaged for each sample condition. The original cholesterol concentration of each sample was calculated using the input information (volumes). The concentration of the other lipids in the formulation was estimated based on the input ratio of compounds.

Example 29 – transfection of HeLa dsGFP cells with siRNA containing liposomes

Description of method

In addition to the HeLa eGFP cell line used, we generated an additional cell line derived from HeLa cells, expressing a destabilized form of eGFP (dsGFP) with an in vivo half-life of ~1 hour which allows for determination of protein downregulation within a reduced timeframe: 16 hours against the usual 72 hr for HeLa eGFP. dsGFP is a fusion protein of eGFP with mouse ornithine decarboxylase which contains a proline-glutamate-serine-threonine-rich (PEST) amino acid sequence that targets the protein for degradation and thus results in rapid protein turnover.¹⁷

Cell line generation

The cell line has been generated by stable transfection of the pOCC447-pd1GFP DNA construct in HeLa cells. The transfection was performed in 6 well format (ThermoFisher, 140675) using 1 μ g of DNA and FuGENE® 6 (Promega, E2691) as a transfection reagent using manufacturer recommendation to have the optimal ratio of volume of FuGENE® 6 to microgram of plasmid (3:1). Briefly, 100,000 cells were seeded in a well of 6 well plate in a volume of 2 ml of complete medium DMEM (ThermoFisher, 31966), 10% FBS (Merck, S0615) and incubated at 37°C overnight. Afterwards the medium was exchanged with 1900 microliters of complete medium.

The transfection reaction was prepared by combining 1 µg of plasmid DNA and 3 µl of FUGENE for 15 minutes in 100 µl of OptiMEM (ThermoFisher, 11058). At the end of the incubation, the transfection mix (100 µl) was added to cells to reach a total volume of 2000 µl.

After one day, the cell culture medium was changed to full medium containing 200 µg/ml of Geneticin (G418, ThermoFisher 10131035) and after another day G418 was raised to 400 µg/ml. The selection was kept until day 15, when mock transfected cells were completely dead, while the cells containing the plasmid were showing surviving clones, expressing dsGFP.

Transfected cells surviving the antibiotic selection, were treated with trypsin (ThermoFisher, 25300-54) for 5 minutes to produce a monodisperse solution of cells which was subjected to FACS analysis to sort single expressing clones in each well of a 384 well plate (Greiner, 781092). The growth of the single clones was followed by fluorescence microscopy to finally identify clones having homogeneous expression of dsGFP and a growth comparable to the parental cell line. 10 clones were identified to follow these criteria, and were subsequently tested in experiments using siRNA against eGFP to finally identify a clone showing a good downregulation of the protein in response to siRNA used for downregulation with the HeLa eGFP line.

Transfection with siRNA containing liposomes

The cells were cultured and transfected as described in example 12, with the only difference that they were fixed 24 h after transfection, not 72 h after transfection.

Imaging and Data Analysis

Imaging and data analysis were conducted as described in example 12.

Results:

Comparison of eGFP downregulation in HeLa dsGFP cells with HeLa eGFP cells

HeLa eGFP and HeLa dsGFP were transfected with the same batch of extruded liposomes of composition LP1 containing siRNA against eGFP. The downregulation response is the same at each concentration in both cell lines (Fig. 14a). The toxicity analyzed with the number of nuclei compared to untreated control is also within error and non-toxic over the whole concentration range (Fig. 14b). HeLa dsGFP can be used as a replacement of HeLa eGFP to increase experimental throughput due to shorter experiment time.

Example 30 – Liposome storage at -80°C

Description of method

The robustness of liposomes towards freezing at -80 °C was tested. To assess this question, liposomes containing siRNA against eGFP produced by extrusion according to the protocol detailed in example 1 were stored overnight at -80 °C and compared to samples that were kept at 4 °C overnight. Liposomes of composition LP1 (6 µl) were either used in 5% glucose solution without further

dilution or diluted with 2x cryoprotectant buffer (20 mM TRIS, 600 mM sucrose, pH 7.4) (6 µl). One sample of each was snap frozen in liquid nitrogen and transferred into a -80 °C freezer overnight. Another sample of each was kept at 4 °C overnight. Samples for size measurement by dynamic light scattering and transfection in HeLa cells expressing eGFP were taken. Samples for dynamic light scattering were prepared and measured as detailed in example 6. siRNA downregulation was tested by transfection of liposomes in HeLa dsGFP cells as described in example 29.

Results:

One of the main strategies to stabilize mRNA-containing particles and protect them from degradation for long-term storage is storage at low temperature (<-70°C). Extruded liposomes containing siRNA produced by the protocol described in example 1 were tested for both size and functionality after storage at -80°C in different buffers. The buffer used for most of the shown experiments was 5% glucose solution. This was compared to a cryoprotectant buffer used by Pfizer for their COVID vaccine Comirnaty (10 mM TRIS, 300 mM sucrose, pH 7.4).

The size of liposomes was assessed by dynamic light scattering as described in example 6 and compared after storage at 4°C and -80°C. Liposomes stored in cryoprotectant buffer at -80°C were slightly increased in size compared to overlapping size distributions of those stored in cryoprotectant buffer at 4°C and in 5% Glucose at both -80°C and 4°C (Fig.14c).

siRNA downregulation in HeLa dsGFP cells was assessed for liposome samples stored in different buffers at different temperatures as described in example 29. Both dose response and toxicity were unchanged after low temperature storage; they were also independent of buffer choice (Fig. 14d,e). Liposomes were stable towards freezing and storage at -80°C in the original buffer. Therefore, it can be concluded that inclusion of a cryoprotectant agent in the buffer is not needed.

Example 31 - Calculation of N/P ratio

Preparation of 1 ml of a liposome solution of composition LP1 (encapsulating agent DOTAP) containing 2 µM anti eGFP siRNA with an N/P ratio of 20:1.

From these parameters, the necessary amount of DOTAP is calculated as follows:

$$N = 20$$

$$P = 1$$

$$n(\text{positive charges}) = 20 * n(\text{negative charges})$$

$$n(\text{eGFP siRNA}) = V * c = 1 * 10^{-3} \text{ l} * 2 * 10^{-6} \frac{\text{mol}}{\text{l}} = 2 * 10^{-9} \text{ mol}$$

$$N_{\text{phosphate groups per oligonucleotide}} = 42 \text{ (21 on each strand)}$$

$$\begin{aligned} n(\text{negative charges}) &= n(\text{eGFP siRNA}) * N_{\text{phosphate groups per oligonucleotide}} \\ &= 2 * 10^{-9} \text{ mol} * 42 = 8.4 * 10^{-8} \text{ mol} \end{aligned}$$

$$n(\text{positive charges}) = 20 * n(\text{negative charges}) = 20 * 8.4 * 10^{-8} \text{ mol} = 1.68 * 10^{-6} \text{ mol}$$

$$N_{\text{amino groups per DOTAP molecule}} = 1$$

$$n(\text{DOTAP}) = \frac{n(\text{positive charges})}{N_{\text{amino groups per DOTAP molecule}}} = \frac{1.68 * 10^{-6} \text{ mol}}{1} = 1.68 * 10^{-6} \text{ mol}$$

The lipid film is prepared accordingly with the molar amount of DOTAP calculated. From the percentages of each individual other compound of the liposome composition, the molar amounts of each other component can be calculated.

In case of the Cas9 ribonucleoprotein complex the number of negative charges is determined by the sum of charges present on the protein and both oligonucleotides. As the protein has a net positive charge, the number of negative charges is lower than the total number of phosphates present on the oligonucleotides.

$$\begin{aligned} n(\text{negative charges}) \\ &= n(\text{phosphate groups on oligonucleotides}) \\ &- n(\text{net charge of Cas9 protein}) \end{aligned}$$

$$n(\text{net charge of Cas9 protein}) = n(\text{Cas9}) * N_{\text{net charge}}$$

$$\begin{aligned} n(\text{phosphate groups on oligonucleotides}) \\ &= n(\text{guide RNA}) * N_{\text{phosphate groups on guide RNA}} + \\ &n(\text{tracr RNA}) * N_{\text{phosphate groups on tracr RNA}} \end{aligned}$$

With $n(x)$ being the molar amount of respective entity x , $N_{\text{net charge}}$ the net charge of Cas9 protein being +22 as reported in literature (Zuris, J. A. et al. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nat. Biotechnol. 2015, 33, 73–80), $N_{\text{phosphate groups on } x}$ the number of phosphate groups present on the respective oligonucleotide

Example 32 – Synthesis of 2,2'-LBPA, PEG2000-orthoester-C18, PEG5000-orthoester-C18, PEG1000-orthoester-C18, PD740.

Description of method:

All reagents for the described syntheses were purchased from commercial sources (Sigma Aldrich/Merck, Roth, AppliChem, AcrosOrganics, Biozol, ChemImpex, AlfaAesar, TCI, Avanti Lipids (abbreviated as Avanti in the Tables)) and were used without further purification, except where noted. Dry solvents were purchased from Sigma Aldrich. All reactions were performed in oven-dried glassware, unless noted otherwise. Solvents for flash chromatography were obtained from

VWR. Performance under inert gas atmosphere is indicated. Analytical thin layer chromatography (TLC) was performed on precoated silica plates (Merck, 60 F254). The TLC plates were visualized with UV light (254 or 366 nm) and by staining either with 3% phosphomolybdic acid in EtOH, 1% KMnO₄ in water, 1.2% ninhydrin in EtOH containing 1.5% acetic acid or 2% formaldehyde in concentrated sulphuric acid. Preparative column chromatography was performed on 40-63 µm silica gel (230-400 mesh) from VWR with a pressure of 1-1.5 bar.

¹H and ¹³C NMR spectra were measured on a 400 MHz Bruker Ascend™ Nanobay spectrometer. NMR chemical shifts (δ) were recorded in ppm and coupling constants (*J*) were reported in Hz. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad. ¹³C-NMR-spectra were broadband hydrogen decoupled. Mass spectra (ESI) were recorded using a QExactive instrument (Thermo Fisher Scientific) equipped with a robotic nanoflow ion source. FTMS spectra were acquired within the range of *m/z* 200-1200 with the target mass resolution of *m/z* 200=140000. The spectra were evaluated using the Xcalibur Qual Browser software.

HPLC purification was performed on a Knauer HPLC (pump: AZURA P2.1S, UV/VIS detector: AZURA UVD 2.1S, Evaporative Light-Scattering Detector: SEDEX LT-ELSD Model 85LT).

Synthesis of 2,2'-LBPA

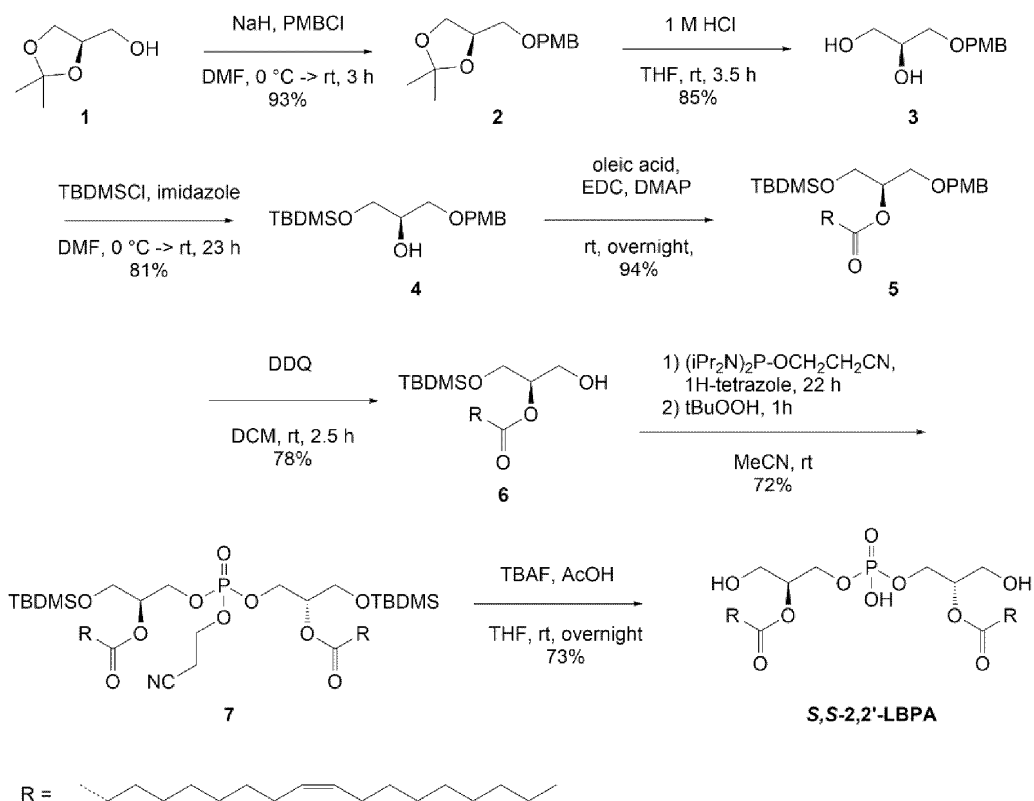
2,2'-LBPA was synthesized according to literature protocols with slight modifications. **2** was synthesized according to Chen, et al., 1996, Synthesis of Photoactivatable 1,2-O-Diacyl-sn-glycerol Derivatives of 1-L-Phosphatidyl-D-myo-inositol 4,5-Bisphosphate (PtdInsP 2) and 3,4,5-Trisphosphate (PtdInsP 3)', *J. Org. Chem.*, 61(18), 6305–6312.

Synthesis of compound **3** was conducted according Näser et al., 2005, Synthesis of ¹³C-labeled γ-hydroxybutyrates for EPR studies with 4-hydroxybutyryl-CoA dehydratase, *Bioorganic Chemistry*, 33(1), 53–66. The rest of the steps were conducted according to Jiang, G., et al., 2006, Practical Enantiospecific Syntheses of Lysobisphosphatidic Acid and Its Analogues, *The Journal of Organic Chemistry*, 71(3), 934–939. The final product was purified by gradient flash chromatography using precooled solvents to avoid isomerization. Ion exchanged was performed according to the protocol published in Jiang, G. *et al.* (2005) Concise synthesis of ether analogues of lysobisphosphatidic acid, *Organic letters*, 7(18), 3837–3840.

MS *m/z*: 792.577 [M+NH₄]⁺.

¹H-NMR (400 MHz, CDCl₃, 22 °C): δ = 0.88 (t, 6H, CH₃), 1.20 – 1.38 (m, 40H, CH₂), 1.51 – 1.64 (m, 4H, CH₂CH₂CO), 1.96 – 2.06 (m, 8H, CH₂-CH=CH), 2.31 (t, 4H, CH₂CO), 3.68 – 3.89 (m, 4H, 3,3'-CH₂), 3.96 – 4.20 (m, 4H, 1,1'-CH₂), 4.89 – 5.02 (m, 2H, 2,2'-CH), 5.30 – 5.40 (m, 4H, CH=CH).

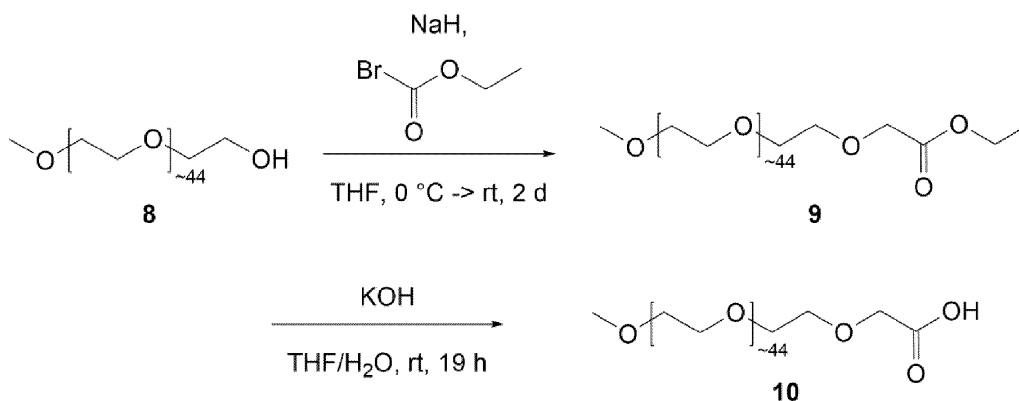
Synthesis scheme 2,2'-LBPA:



Synthesis of labile PEG lipid PEG2000-orthoester-C18

MeO-PEG2000-COOH

Synthesis scheme MeO-PEG2000-COOH:



Ethyl 2-(poly(ethylene glycol) methyl ether)acetate **9**

Poly(ethylene glycol) methyl ether **8** (2.06 g, 1.03 mmol, 1.00 eq., average molecular weight ~2,000) was dissolved in dry THF (40 ml) under argon atmosphere and cooled to 0°C. Sodium hydride (0.25 g, 10.30 mmol, 10.00 eq.) was added in portions. The mixture was stirred at 0°C for 5 min. Ethylbromoacetate (0.86 ml, 1.29 g, 7.73 mmol, 7.50 eq.) was added and the mixture was warmed up to room temperature and stirred at room temperature for 2 d. The reaction was quenched with saturated ammonium chloride solution (15 ml) before removing the solvent under reduced pressure. The solution was diluted with dichloromethane (200 mL) and washed with brine (2x100 ml). The solvent was evaporated under reduced pressure.

2-(Poly(ethylene glycol) methyl ether)acetate **10**

Compound **9** (0.46 g) was dissolved in THF (20 mL). Potassium hydroxide (0.31 g) in water (2 ml) was added. The mixture was stirred for 19 h at room temperature. THF was removed under reduced pressure. Water (40 ml) was added and the solution was acidified with hydrochloric acid (1 M in water, 7 ml). The product was extracted with DCM (3x40 ml). The organic solution was dried over sodium sulfate, filtered and the solvent was removed under reduced pressure. The crude product was purified by HPLC (Macherey-Nagel Nucleodur HTec, C18, VP 250/32, 20 µm, gradient: 0-52% B in 35 min, A= 10% acetonitrile in water, B= 90% acetonitrile in water, 20 ml/min).

MS *m/z*: 1850.072 (C₈₃H₁₆₅O₄₃, n=40), 1894.097 (C₈₅H₁₆₉O₄₄, n=41), 1938.123 (C₈₇H₁₇₃O₄₅, n=42), 1982.148 (C₈₉H₁₇₇O₄₆, n=43), 2026.177 (C₉₁H₁₈₁O₄₇, n=44), 2070.200 (C₉₃H₁₈₅O₄₈, n=45), 2114.225 (C₉₅H₁₈₉O₄₉, n=46), 2158.251 (C₉₇H₁₉₃O₅₀, n=47), 2202.277 (C₉₉H₁₉₇O₅₁, n=48), 2246.302 (C₁₀₁H₂₀₁O₅₂, n=49), 2290.327 (C₁₀₃H₂₀₅O₅₃, n=50) [M-H]⁻.

¹H-NMR (400 MHz, CDCl₃, 22 °C): δ = 3.38 (s, 3H, OMe), 3.44-3.86 (m, 190H, O-CH₂-CH₂-O), 4.16 (s, 2H, O-CH₂-COOMe).

PEG2000-orthoester-C18

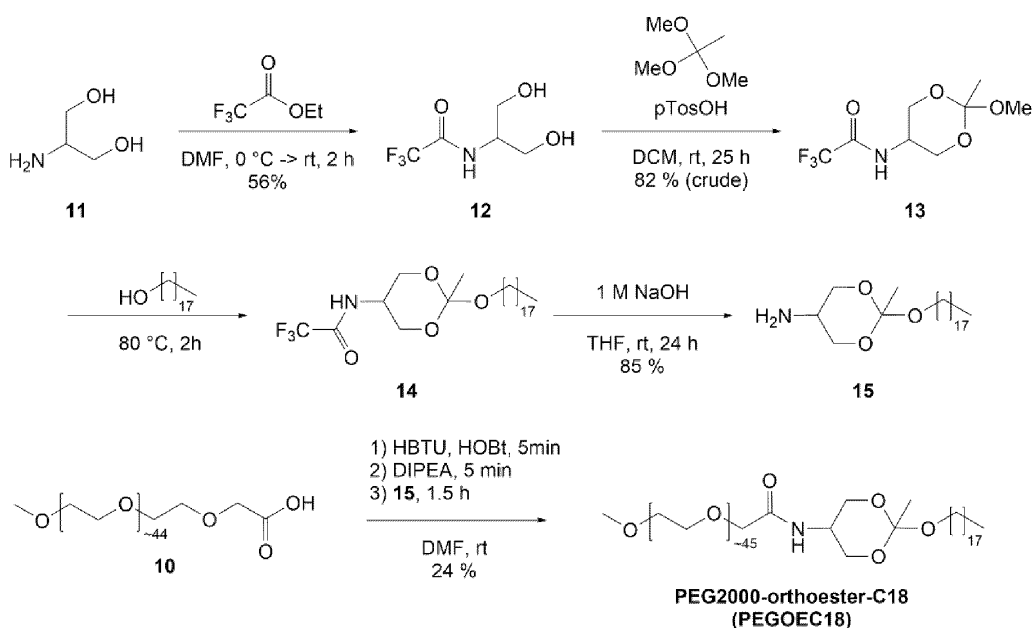
PEG2000-orthoester-C18 was synthesized according to a literature procedure (Masson et al., 2004) with a slight modification in the last step using different coupling reagents.

2-(Poly(ethylene glycol) methyl ether)acetate **10** (39.5 mg, 19.2 µmol, 1.00 eq.), HBTU (10.6 mg, 27.9 µmol, 1.45 eq.) and HOBt (3.9 mg, 28.8 µmol, 1.50 eq.) were dissolved in dry DMF (1 ml) under argon. DIPEA (pre-dried over molecular sieves (4°Å), 10 µl, 7.5 mg, 57.6 µmol, 3.00 eq.) was added and the solution was stirred for 5 min at room temperature. Compound **15** was dissolved in dry DMF (0.5 ml) and dry DCM (0.5 ml) under argon atmosphere in a second flask and the solution was added to the first solution. The mixture was stirred for 2 h at room temperature before being diluted with DCM (150 ml). The solution was washed K₂CO₃ solution (0.5 g/l, 40 ml), water (100 ml) and saturated NaCl solution (100 ml). The organic phase was quickly dried over Na₂SO₄,

filtrated and the solvent was removed under reduced pressure. The crude product was purified by precipitation in Et₂O. The precipitate was collected by centrifugation at 3240xg at 4 °C for 30 min.

¹H-NMR (400 MHz, CD₃CN, 22 °C): δ = 0.92 (t, 3H, CH₃), 1.25 – 1.50 (m, 33H, 15x CH₂, 1x CH₃), 1.52 – 1.68 (m, 2H, CH₂), 3.34 (s, 3H, OCH₃), 3.38 – 3.83 (m, 193H, OCH₂CH₂O), 3.99 (s, 2H, OCH₂), 4.27 (m, 2H, OCH₂).

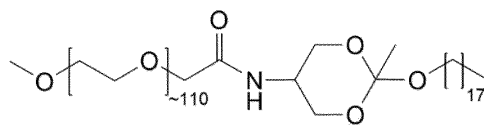
Synthesis scheme PEG2000-orthoester-C18:



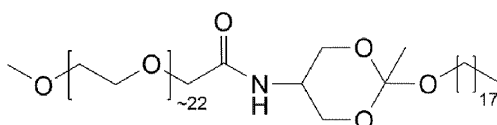
Synthesis of labile PEG lipids PEG5000-orthoester-C18 and PEG1000-orthoester-C18

Variations of labile PEG lipid PEG2000-orthoester-C18 (PEGOEC18) with different PEG chain lengths were synthesized according to the synthesis protocol for PEGOEC18. A longer variant with PEG of an average molecular weight of 5000, corresponding to an average of about 110 repeat units, and a shorter variant with an average molecular weight of 1000, corresponding to an average of about 22 repeat units, were chosen. The PEG carboxylic acid variant starting materials MeO-PEG5000-COOH and MeO-PEG1000-COOH were obtained from Broadpharm (PEG5000: BP-23257, PEG1000: BP-28714).

PEG5000-orthoester-C18

**PEG5000-orthoester-C18**

¹H-NMR (400 MHz, CD₃CN, 22 °C): δ = 0.91 (t, 3H, CH₃), 1.27 – 1.49 (m, 33H, 15x CH₂, 1x CH₃), 1.57 - 1.67 (m, 2H, CH₂), 3.32 (s, 3H, OCH₃), 3.37 – 3.83 (m, 305H, OCH₂CH₂O), 3.97 (s, 2H, OCH₂), 4.26 (m, 2H, OCH₂).

PEG1000-orthoester-C18**PEG1000-orthoester-C18**

¹H-NMR (400 MHz, CD₃CN, 22 °C): δ = 0.91 (t, 3H, CH₃), 1.24 – 1.49 (m, 33H, 15x CH₂, 1x CH₃), 1.56 - 1.67 (m, 2H, CH₂), 3.32 (s, 3H, OCH₃), 3.37 – 3.82 (m, 91H, OCH₂CH₂O), 3.98 (s, 2H, OCH₂), 4.26 (m, 2H, OCH₂).

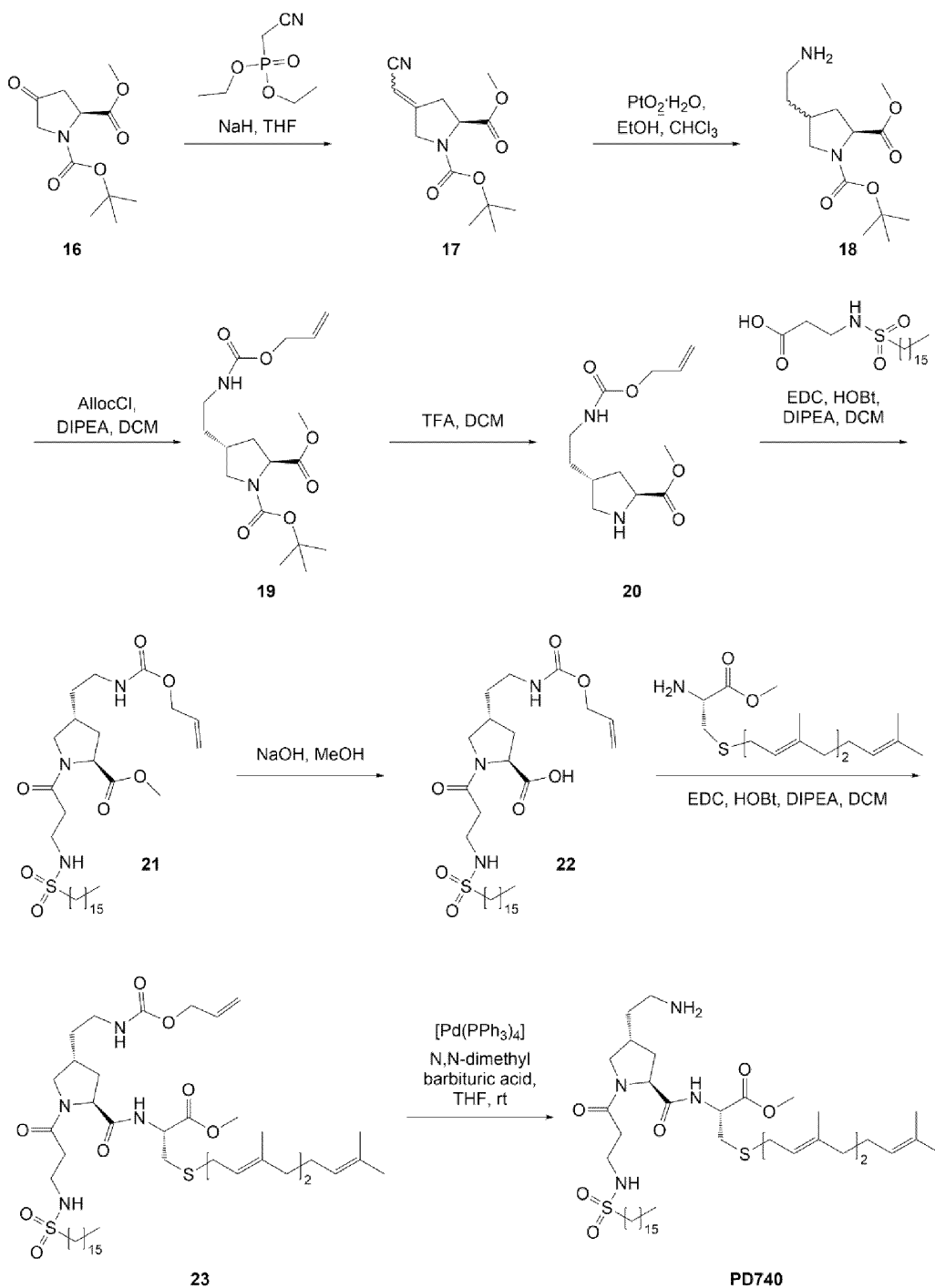
Synthesis of PD740

PD740 was obtained by custom synthesis in collaboration with Chemveda Life Science Pvt. Ltd. according to previously published protocols (Biel, M. *et al.* (2006) Synthesis and evaluation of acyl protein thioesterase 1 (APT1) inhibitors, *Chemistry - A European Journal*, 12(15), 4121–4143) which were slightly modified.

MS *m/z*: 839.574 [M+H]⁺.

¹H-NMR (400 MHz, CDCl₃, 22 °C): δ = 0.88 (t, 3H, SO₂(CH₂)₁₆CH₃), 1.25 (m, 24H, SO₂(CH₂)₃(CH₂)₁₂CH₃), 1.39 (m, 2H, SO₂(CH₂)₂CH₂(CH₂)₁₂CH₃), 1.60 (s, 6H, 2x terminal farnesyl CH₃), 1.67 (s, 6H, 2x internal farnesyl CH₃), 1.76 (m, 4H, CH₂CH₂NH₂ and SO₂CH₂CH₂(CH₂)₁₃CH₃), 1.94 – 2.15 (m, 8H, internal farnesyl CH₂), 2.22 – 2.55 (m, 3H, β -CH₂ and γ -CH Pro), 2.65 (m, 2H, α -CH₂ β -Ala), 2.73 – 3.30 (m, 9H, SO₂CH₂(CH₂)₁₄CH₃, CH₂NH₂, farnesyl SCH₂, β -CH₂ Cys, δ -CH_{2a} Pro), 3.39 (m, 2H, β -CH₂ β -Ala), 3.74 (s, 3H, OCH₃), 3.92 (m, 1H, β -CH_{2b} Pro), 4.48 (m, 1H, α -CH Pro), 4.66 (m, 1H, α -CH Cys), 5.10 (m, 2H, 2x farnesyl CH), 5.20 (m, 1H, farnesyl SCH₂CH=C).

Synthesis scheme PD740:



Example 33 - Transfection of THP-1 cells with eGFP mRNA using liposomes of composition LP1

Description of method:

To test whether macrophages of human origin can be transfected using the composition of the invention, the THP-1 leukemic monocyte cell line was differentiated into THP-1 macrophages, a model for human primary macrophages, and treated with different concentrations of eGFP mRNA containing liposomes of composition LP1.

THP-1 cells (DSMZ, #ACC 16) were cultured in RPMI medium (Life Technologies, 61870) complemented with 10% FBS superior (Merck #S0615), 1 mM sodium pyruvate (Thermo Fisher Scientific, #11360039) and 50 μ M mercaptoethanol (Sigma-Aldrich, M6250) at 37°C and 5% CO₂ and seeded three days before the transfection into a black 384 well cell culture microplate (Greiner, 781092) in RPMI medium with 10% FBS, 1 mM sodium pyruvate and 50 μ M mercaptoethanol, 50 μ g/ml gentamicin and 20 ng/ml PMA (Sigma-Aldrich, P 8139) for differentiation.

Liposomes containing eGFP mRNA (TriLink Biotechnologies, L-7201) were prepared at 10x the highest concentration necessary for transfection as described in example 3 using a lipid to mRNA charge ratio of 6:1. Serial dilution was performed using automated multichannel pipettes (Eppendorf) to obtain solutions at 10x concentration for all concentration points. Untreated control samples were prepared using only buffer. Directly before transfection, equal volume of RPMI containing 20% FBS was added to each transfection mix to have a concentration of mRNA of 5x the final concentration of the transfection and 10% FBS. 10 μ l of this transfection mix was added to each well of the 384 well plate containing 40 μ l of medium. Each condition was tested in quadruplicate. The plate was further cultured for 24 hours and then fixed with 3.7% formaldehyde for 15 min. After additional washing with PBS each well was stained with 1 μ g/ml DAPI and 0.25 μ g/ml HCS CellMask™ Blue Stain in PBS containing 0.02% sodium azide.

Imaging and data analysis:

Imaging and data analysis including the quantification of eGFP expression, percentage of eGFP positive cells and total cell number in response to treatment were conducted as described in example 18.

Results:

Liposomes of composition LP1 containing eGFP mRNA induce eGFP expression in THP-1 cells in a dose dependent manner. THP-1 cells show increasing amounts of eGFP expression in an increasing number of cells in response to dosage while untreated cells show no eGFP fluorescence (Fig. 15a,b). The quantification shows that the total cell number is within the range of the untreated control conditions even at the highest dosages tested. About 80% of the treated cells are positive for GFP expression after 24 h treatment at 2.5 μ g/ml dose (Fig. 15b).

Example 34 - Delivery of protein cargo into HeLa cells with liposome composition in LP1 composition liposomes

Description of method:

Besides delivery of protein in a complex with RNA (CRISPR Cas9 ribonucleoprotein complex, example 21), protein can also be delivered as sole cargo using a composition of the invention. Suitably the protein can be negatively charged and then interact with the positively charged liposome composition. A negatively supercharged version of GFP, (-30)GFP, was expressed, as well as a standard eGFP protein, (-7)GFP, as a control. Both protein versions contained a nuclear localization sequence (NLS) to allow nuclear accumulation and thus simplify delivery quantification.

Protein production

E. coli BL21 (DE3)-competent cells (ThermoFisher Scientific, EC0114) were transformed with g-30-GFP-NLS-6xHis and g-7-GFP-NLS-6xHis (Genscript, pET29a, custom) encoding supercharged GFP ((-30)GFP) and standard GFP ((-7)GFP) C-terminal NLS followed by His-tag. The sequence was based on previously published supercharged GFP proteins (Lawrence, M. S. et al, Supercharging Proteins Can Impart Unusual Resilience. JACS. 2007, 129, 10110-10112; Zuris, J. A. et al, Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nat. Biotechnol. 2015, 33, 73–80).

The resulting expression strain was inoculated in Luria-Bertani (LB) broth containing 50 mg/mL kanamycin (Sigma-Aldrich, K4000) at 37 °C overnight. The cells precultured at 37 °C with shaking at 200 rpm. Bacteria were added to start with an OD₆₀₀ of 0.05 into 100 ml culture. When OD₆₀₀ reached 0.6, the expression was induced with isopropyl b-d-1- thiogalactopyranoside (IPTG, Sigma Aldrich, R0392) (1 mM) and incubated at 18 °C and 180 rpm for ~16 h. The cells were harvested by centrifugation at 4,000 × g for 10 min and resuspended in lysis buffer [PBS, 10 mM imidazole, pH 7.4]. The cells were lysed by sonication (6x 30-second cycles, 30-second breaks, 30% amplitude), and the soluble lysate was obtained by centrifugation at 15,000 × g for 50 min.

The cell lysate was filtered through a 0.45 µm membrane filter and added to a His GraviTrap ml Ni-NTA Superflow™ gravity column (Cytiva, 11003399), preequilibrated with 10 ml binding buffer (PBS, 10 mM imidazole, pH 7.4). The column was washed with 10 ml binding buffer to remove nonspecifically bound proteins. The proteins were eluted with 3x3 ml of elution buffer (PBS, 250 mM imidazole, pH 7.4). The elution fractions were placed onto a PD10 desalting column (Cytiva, 17085101) and eluted with PBS. The eluted fractions were analysed with SDS-PAGE and protein concentration was determined by UV absorption at 280 nm using a NanoDrop instrument.

HeLa cell transfection

HeLa cells (Accession: CVCL_1922, https://www.cellosaurus.org/CVCL_1922) were plated and cultured as described in example 12. Liposomes of composition LP1 were made according to example 2 and mixed with (-30)GFP or (-7)GFP protein for 15 min at room temperature at a molar ratio of 180:1 (total lipid/protein), corresponding to a charge ratio N/P of 3:1 (positive to negative charges) for the negatively supercharged protein.

Serial dilution of liposomes complexed with protein and protein alone was performed using automated multichannel pipettes (Eppendorf) to obtain solutions at 10x concentration in PBS for all concentration points. Untreated control samples were prepared using only buffer. Directly before transfection, equal volume of DMEM containing 20% FBS was added to each transfection mix to have a protein concentration corresponding to 5x the final concentration of the transfection and 10% FBS. 10 µl of this transfection mix was added to each well of the 384 well plate containing 40 µl of medium with cells. Each condition was tested in quadruplicate. The plate was further cultured for 24 hours and then fixed with 3.7% formaldehyde for 20 min. After additional washing with PBS each well was stained with 1 µg/ml DAPI and 0.25 µg/ml HCS CellMask™ Blue Stain in PBS containing 0.02% sodium azide.

To increase GFP fluorescence signal to noise in view of a certain amount of autofluorescence, cells were stained with an antiGFP antibody and Alexa647 conjugated secondary antibody. Cells were permeabilized with 25 µl of 0.2% Triton X in PBS on top of 25 µl solution for 10 min at room temperature. After washing with PBS and aspiration to 12.5 µl volume, 12.5 µl of 6% BSA in PBS were added and incubated for 60 min at room temperature for blocking. After aspiration to 12.5 µl, the primary eGFP antibody (Goat anti eGFP, produced in antibody facility, MPI-CBG) was added in 12.5 µl of PBS containing 3% BSA at a final dilution of 1:10000. The solution was incubated for 1 h at room temperature, washed with PBS and aspirated to 12.5 µl. The labeled secondary antibody (Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647, Invitrogen, A-21447) was added in 12.5 µl of PBS containing 6% BSA at a final dilution of 1:2000 and incubated at room temperature for 60 minutes. The plate was washed with PBS.

Imaging and data analysis:

The plate was imaged with 40x magnification on a Yokogawa CellVoyager CV7000 automated laser spinning disk microscope. 3 fields per well were imaged. The nuclei and cells were segmented based on the DAPI/CMB staining and the mean GFP and Alexa647 intensity for each nucleus was recorded. The total cell number in percentage was calculated as follows:

The number of nuclei per image was summed up per well and then averaged per condition. This value was normalized to the value calculated the same way from the untreated control wells and multiplied by 100.

The Alexa647 GFP antibody intensity was calculated as follows:

Median of the mean Alexa647 intensity per nucleus was calculated per image, averaged per well and averaged per condition.

Additionally, the number of nuclei positive for eGFP was calculated from single cell data, utilizing a threshold evaluated on the comparison of the distribution of the value of the mean intensity of Alexa647 in the nuclei of untreated cells and cells transfected with the protein. The threshold was chosen in order to exclude false positives due to autofluorescence: nuclei having values of mean

Alexa647 fluorescence above that threshold were considered as GFP positive, while nuclei below were considered GFP negative. For each well, the number of nuclei that were evaluated as positives was counted and the percentage of GFP positive nuclei was evaluated calculating the ratio of these positive nuclei and the total number of nuclei per condition and multiplying it by 100. The percentage of positive nuclei was then averaged by treatment condition.

The analysis was conducted using CellProfiler for segmentation and parameter extraction and Knime Analytics platform for calculation, averaging and normalization.

Results:

HeLa cells treated with liposomes containing supercharged (-30)GFP or standard, wild-type (-7)GFP show both cytoplasmic and nuclear signal as revealed by anti-GFP antibody staining (visualized by secondary antibody staining labeled with Alexa647). Cells treated with liposomes containing (-30)GFP protein show a nuclear stain and a vesicular pattern, while cells treated with liposomes containing (-7)GFP protein show nuclear and cytosolic signal. Treatment with proteins alone (not mixed with liposomes) does not show any signal, akin to untreated cells (Fig. 16a). Quantification of the images shows that the mean nuclear fluorescence intensity in liposome treated conditions increases with protein concentration, while the conditions only treated with protein itself show only background level fluorescence intensity over the full concentration range. The fluorescence intensity is higher at lower concentrations for (-30)GFP than for (-7)GFP, but at the highest concentration tested (125 nM) the fluorescence intensity is the same (Fig. 16b). The total cell number is stable over all concentrations tested for protein treated conditions and liposomes with (-7)GFP protein (Fig. 16c). The number of nuclei positive for GFP increases with increased protein concentration for both liposomes containing GFP, with the (-30)GFP protein reaching higher percentages than the (-7)GFP protein at lower concentrations. At the highest concentration tested (125 nM) about 75% of the nuclei are positive for both (-30)GFP and (-7)GFP liposome treatment. Cells treated with only (-30)GFP or (-7)GFP protein show no GFP positive nuclei (Fig. 16d). The signal of Alexa647 colocalizes with the GFP fluorescence signal in all images taken, but has improved signal to noise (data now shown). The results show that not only the negatively supercharged (-30)GFP can successfully be delivered to HeLa cells with the composition of the invention but also the control, close to neutral (-7)GFP protein. This is surprising, as prior art cationic protein delivery systems generally require the protein to be negatively charged to be delivered. In contrast, protein delivery with the composition of the invention is expected to be independent of protein charge. Overall, these results demonstrate that the liposomal delivery system can be used for (pure) protein delivery without the need of any nucleic acid and that it does not depend on the protein being highly negatively charged.

Example 35 - LP1 composition compared to state of the art delivery compositions for siRNA delivery

Description of method:

Delivery of siRNA in liposomes of composition LP1 was compared to delivery in state of the art liposome compositions. Liposomes of composition LP1 containing anti eGFP siRNA were made as described in example 1. DOTAP/Chol (ratio 1:1, N/P ratio = 20:1) liposomes containing anti eGFP siRNA were made according to the same protocol. HeLa dsGFP cells were treated with different concentrations of liposomes containing siRNA for 24 h according to example 29.

Furthermore, delivery of siRNA in liposomes of composition LP1 was compared to delivery in state of the art LNP compositions. Liposomes of composition LP1 containing anti eGFP siRNA at N/P ratio 20:1 were made as described in example 1. Lipid nanoparticles MC3 of composition MC3:DSPC:Chol:PEG-DMG=50:38.5:10:1.5, containing anti eGFP siRNA at charge ratio N/P = 5:1, were prepared using NanoAssemblr Ignite, lipid concentration 12.5 mM, flow rate ratio 3:1, total flow rate 12 ml/min, as described in example 27. The aqueous buffer used for the microfluidic was 100 mM citrate pH 3.0. After production the sample was diluted with PBS and post-processed by diafiltration, as described in example 27. HeLa dsGFP cells were treated with different concentrations of liposomes and LNPs containing anti eGFP siRNA for 24 h according to example 29.

Imaging and data analysis:

Imaging and data analysis were conducted as described in example 12.

Results:

Composition LP1 is more effective than state of the art DOTAP/Chol liposomes for siRNA downregulation in HeLa dsGFP cells (Fig 17a). Full knockdown is reached at a lower concentration compared to state of the art liposome delivery and without detection of toxicity (Fig. 17a).

Furthermore, siRNA delivery using the composition formulation of the invention (e.g. using the formulation LP1) is unexpectedly just as efficient as delivery of siRNA using an LNP composition according to some of the best performing transfection compositions to date (MC3) (Fig. 17b).

Example 36 - LP1 composition compared to state of the art delivery compositions for mRNA delivery

Delivery of mRNA in liposomes of composition LP1 was compared to delivery in state of the art liposome compositions. Liposomes of composition LP1 containing mRNA were made as described in example 3 at N/P ratio 6:1. DOTAP/Chol (ratio 1:1) liposomes containing mRNA were made according to the same protocol at charge ratio N/P 6:1. HeLa cells were treated with different concentrations of mRNA in liposomes for 24 h according to the protocol in example 18.

Imaging and data analysis:

eGFP expression from mRNA was quantified as described in example 18 as percentage of eGFP positive cells and total nuclei number in response to treatment.

Results:

mRNA delivery into HeLa cells using composition LP1 of the invention is more efficient compared to mRNA delivery using a state of the art liposomal composition (DOTAP/Chol). The eGFP fluorescence in LP1 treated cells is higher than the eGFP fluorescence that can be reached with the state of the art composition, corresponding to a higher level of expression due to presence of an increased amount of mRNA in the cells. In addition, close to all cells can be transfected at the tested dosages, while the state of the art composition only achieves expression in around 40% of the cells. The toxicity profile (number of cells normalized to untreated control) is similar for both treatments (Fig. 18).

Example 37 – Transfection of cardiomyocytes with eGFP mRNA using liposomes of composition LP1Description of method:

To test whether yet a further cell type can be transfected using the composition of the invention, cardiomyocytes were treated with different concentrations of eGFP mRNA containing liposomes of composition LP1.

A 384-well cell culture microplate (Greiner, 781092) was pre-coated with 25 µl/well 0.1% Gelatin (Stemcell Technologies, 07903) for 1 hour at 37 °C. Cardiomyocytes (iCell Cardiomyocytes², Fujifilm Cellular Dynamics, C1016) were seeded in plating medium (Fujifilm Cellular Dynamics, M1001) into the precoated plate and kept at 37°C and 5% CO₂ three days before the transfection. The next day the plating medium was replaced with maintenance medium (Fujifilm Cellular Dynamics, M1003). The cells were kept at 37°C and 5% CO₂ for two more days.

Liposomes containing eGFP mRNA (TriLink Biotechnologies, L-7201) were prepared at 10x the highest concentration necessary for transfection as described in example 3 using a lipid to mRNA charge ratio of 3:1. Serial dilution was performed using automated multichannel pipettes (Eppendorf) to obtain solutions at 10x concentration for all concentration points. Untreated control samples were prepared using only buffer. Directly before transfection, equal volume of maintenance medium was added to each transfection mix to have a concentration of mRNA of 5x the final concentration of the transfection. 10 µl of this transfection mix were added to each well of the 384 well plate containing 40 µl of medium. Each condition was tested in quadruplicate. The plate was further cultured for 20 hours and then fixed with 3.7% formaldehyde for 20 min. After washing with PBS each well was stained with 1 µg/ml DAPI and 0.5 µg/ml HCS CellMask™ Blue Stain in PBS containing 0.02% sodium azide.

Imaging and data analysis:

Imaging and data analysis including the quantification of eGFP expression, percentage of eGFP positive cells and of total cell number in response to treatment were conducted as described in example 18.

Results:

Cardiomyocytes can be transfected with mRNA with the composition of the invention. The GFP fluorescence signal increases in an increasing number of cells with higher dosages and is detectable even at the lowest concentration tested (0.08 µg/ml) (Fig. 19a). The quantification shows that around 25 % of the cells show eGFP expression even at the lowest dose and about 90% of the cells are positive for eGFP at the highest dose tested (2.5 µg/ml). Transfection does not induce toxicity, as demonstrated by the constant number of total cells over the full range of concentrations tested (Fig. 19b).

Example 38 – Influence of PEG chain length of labile PEG lipid on siRNA delivery

To assess if PEG chain length of the labile PEG lipid has an influence on siRNA delivery efficiency, empty liposomes of three different compositions were produced as described in example 2 with labile PEG lipids of varying lengths, synthesized as described in example 32. The following 3 compositions were used: DOTAP:3,3'-S,S-LBPA:labile PEG lipid in a ratio of 50:45:5, DOTAP:3,3'-S,S-LBPA:Cholesterol:labile PEG lipid in a ratio of 50:20:25:5 and DOTAP:DOPE:labile PEG lipid in a ratio of 45:45:10. Labile PEG lipids of standard chain length 2000 (PEGOEC18), longer PEG chain length (PEG5000-orthoester-C18) and shorter PEG chain length (PEG1000-orthoester-C18), were each used.

The compositions were mixed with siRNA against eGFP directly before transfection analogous to example 3 at an N/P ratio of 20:1 and incubated for 15 min at room temperature. Transfection into HeLa dsGFP cells was conducted as described in example 29.

Imaging and Data Analysis

Imaging and data analysis were conducted as described in example 12.

Results:

Treatment of HeLa dsGFP cells with anti eGFP siRNA using liposomes of compositions LP1, incorporating three different labile PEG lipids with varying PEG chain lengths, demonstrates that PEG chain length does not affect overall knockdown efficiency. This is evidenced by the average GFP intensity normalized to untreated condition in response to dosage (Fig. 20a) and the percentage of nuclei retaining the eGFP signal post-treatment (Fig. 20c). Toxicity determined by the total number of cells normalized to untreated condition is similar over the range of treatments, only at the highest concentration tested the shorter PEG length liposomes seem to reduce the cell number slightly (Fig. 20b).

For the composition of the invention without cholesterol (DOTAP:3,3'-S,S-LBPA:labile PEG lipid = 50:45:5) the influence of PEG chain length on siRNA downregulation was minimal. At lower concentrations liposomes containing PEG lipid PEG1000-orthoester-C18 were slightly more

effective than liposomes with PEG lipid PEG5000-orthoester-C18, both in terms of average eGFP intensity normalized to untreated condition and number of cells positive for eGFP signal (Fig 20d,f). Liposomes with PEG lipid PEGOEC18 with a chain length of PEG2000 were slightly less effective than those with PEG5000-orthoester-C18, but mostly within error margin (Fig 20d,f). Overall all liposome compositions without cholesterol were less effective than the ones with composition LP1 ((Fig 20d,f). The toxicity response of the liposome formulation without cholesterol was the same for all PEG chain lengths (Fig. 20e).

In addition, a comparative composition comprising the PEG monoorthoester lipid was tested (DOTAP:DOPE:labile PEG lipid = 45:45:10). With this composition, siRNA mediated downregulation of eGFP and percentage of eGFP positive cells in response to dosage was the same for the two shorter PEG lipid chain lengths, and only slightly lower for liposomes comprising PEG lipid PEG5000-orthoester-C18 (Fig. 20g,i). The toxicity response was independent of PEG lipid chain length (Fig. 20h). However, eGFP downregulation with this composition was less effective in comparison to the compositions of this invention. While the divergence in terms of average GFP intensity is small (comparing Fig. 20a,d,g), approximately 50% of the cells treated with the comparative composition still exhibit eGFP signal at the highest concentration tested. In contrast, the compositions of this invention reduce the number of cells showing eGFP signal near background level (comparing Fig. 20 c,f,i).

Example 39– Influence of PEG chain length of labile PEG lipid on mRNA delivery

Liposomes of three different compositions with three different labile PEG lipids of differing PEG chain length were produced as described in example 38 and tested for mRNA transfection on HeLa cells. The compositions were mixed with eGFP mRNA as described in example 3 directly before transfection at N/P ratio 6:1 and incubated at room temperature for 15 min. Transfection of HeLa cells was conducted as described in example 18.

Imaging and Data Analysis:

Imaging and data analysis were conducted as described in example 18.

Results:

Transfection of HeLa cells with eGFP mRNA in liposomes of compositions LP1 with three different labile PEG lipids with varying PEG chain lengths shows that expression analyzed by average eGFP intensity is highest when using PEG5000-orthoester-C18, compared to intermediate level of expression with the shortest PEG lipid (PEG1000-orthoester-C18) and lower level of expression with the intermediate PEG lipid (PEG2000-orthoester-C18, PEGOEC18). The average eGFP intensity is reduced at the highest concentration tested (0.625 µg/ml) compared to 0.3125 µg/ml. (Fig. 21a). This correlates to the degree of toxicity at the highest concentration. The total number of nuclei normalized to untreated condition in response to dosage is comparable while being a little lower for liposome

composition LP1 with PEG5000-orthoester-C18 (Fig. 21b). The number of nuclei positive for eGFP increases in response to dosage, with the same trend for the PEG lipid chain length as for eGFP expression (described above), with the composition including PEG5000 lipid being most effective (Fig. 21c).

For the composition of the invention without cholesterol (DOTAP:3,3'-S,S-LBPA:labile PEG lipid = 50:45:5), the composition containing PEG2000-orthoester-C18 shows the highest expression, followed by the composition with longer PEG chain length (PEG5000-orthoester-C18). Overall the expression with the composition without cholesterol is lower than the expression with composition LP1 (Fig. 21d). In terms of nuclei positive for eGFP, the response to this composition is mostly independent from PEG chain length, with the liposomes including PEG2000-orthoester-C18 reaching a slightly higher percentage than the other two compositions at 0.31 µg/ml mRNA concentration (Fig. 21f). The total number of cells for cells treated with liposomes containing PEG1000-orthoester-C18 show the least toxicity. The toxicity response of cells to the compositions containing the two longer PEG chain lipids is about the same (Fig. 21e).

In addition, a comparative composition comprising the PEG monoorthoester lipid was tested (DOTAP:DOPE:labile PEG lipid = 45:45:10). eGFP mRNA delivery with liposomes containing PEG lipids of different chain lengths resulted in very low expression of eGFP for all of the PEG lipids tested (Fig 21g). As a result of the expression being only slightly above background level, the percentage of nuclei positive for eGFP in response to increased dosage displays a large variability and shows no clear dose response (Fig. 21i). The total number of cells normalized to untreated control stays constant in response to dosage, independent of PEG lipid chain length (Fig. 21h).

Tables

Table 1: Composition of most successful formulations

Composition Name	Component				Compound ratio*
	Encapsulating agent	Helper lipid	Fusogenic Agent	Labile lipid	
LP1	DOTAP	Chol	LBPA	PEGOEC18	50:25:20:5
LP2	PD740	Chol	LBPA	PEGOEC18	50:25:20:5
LP3	PD740,DOTAP	Chol	LBPA	PEGOEC18	25:25:25:20:5
LP4	PD740	Chol	LBPA	PEGOEC18	33:33:27:7
LP-Solasodine 2	DOTAP	Chol, Solasodine	LBPA	PEGOEC18	50:15:15:15:5
LP-Solasodine 3	DOTAP	Chol, Solasodine	LBPA	PEGOEC18	42:13:36:4:4
LP-MGDG	PD740, DOTAP	Chol	MGDG	PEGOEC18	25:25:25:20:5
LP-PLPE	PD740, DOTAP	Chol	PLPE	PEGOEC18	25:25:25:20:5

*The compound ratio is reported for each compound in the same order as shown in the columns "Component", (also referred to as "Composition agent").

The compound ratio is calculated as (n(agent)/ n(total) %), wherein "n" is the chemical amount in moles or molar concentration.

In all tables, the symbol "," between two compounds means that both compounds are present to exert the function of that particular component, e.g. as "encapsulating agent".

PEGOEC18 is an abbreviation for " α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅" or α -methyl- ω -[2-[[2-methyl-2-(octadecyloxy)-1,3-dioxan-5-yl]amino]-2-oxoethoxy]-poly(oxy-1,2-ethanediyl)₄₅

Table 2: Stoichiometric variation within composition LP1 - – compositions tested - HeLa

Compound varied	Component				Compound ratio
	Encapsulating agent	Helper lipid	Fusogenic Agent	Labile PEG lipid	
-	DOTAP	Chol	LBPA	PEGOEC18	50:25:20:5
DOTAP	DOTAP	Chol	LBPA	PEGOEC18	12.5:37.5:25:20:5
	DOTAP	Chol	LBPA	PEGOEC18	25:25:25:20:5
Cholesterol (Chol)	DOTAP	-	LBPA	PEGOEC18	70:25:5
	DOTAP	Chol	LBPA	PEGOEC18	60:15:20:5
	DOTAP	Chol	LBPA	PEGOEC18	40:35:20:5
LBPA	DOTAP	Chol	DOPC	PEGOEC18	50:25:20:5
	DOTAP	Chol	-	PEGOEC18	70:25:5
	DOTAP	Chol	LBPA	PEGOEC18	55:25:15:5
	DOTAP	DOPC/ Chol	LBPA	PEGOEC18	50:5:25:15:5
	DOTAP	Chol	LBPA	PEGOEC18	45:25:25:5
	DOTAP	Chol	LBPA	PEGOEC18	40:25:30:5
	DOTAP	Chol	LBPA	PEGOEC18	35:25:35:5
	DOTAP	-	LBPA	PEGOEC18	50:45:5
Labile PEG lipid	DOTAP	Chol	LBPA	PEGOEC18	25:25:50:5
	DOTAP	Chol	LBPA	PEGOEC18	54:25:20:1
	DOTAP	Chol	LBPA	PEGOEC18	52.5:25:20:2.5
	DOTAP	Chol	LBPA	PEGOEC18	47.5:25:20:7.5
	DOTAP	Chol	LBPA	PEGOEC18	45:25:20:10

Table 3: Helper lipid variation - – 4 concentration dose response - HeLa

Composition				
Encapsulating agent	PD740, DOTAP	PD740, DOTAP	DOTAP	DOTAP
Helper lipid	Chol, Solasodine	Chol, Ceramide	Diosgenin	Chol, Solasodine
Fusogenic Agent	LBPA	LBPA	LBPA	LBPA
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	25:25:15:15:15:5	25:25:15:15:15:5	50:25:20:5	50:22.5:2.5:20:5
EC50 Calculation				
EC50 (nM)	0.91	1.34	1.65	1.81
EC50 S.E.	0.23	0.2	0.24	0.82
EC50 model fitting	app.	app.	app.	app.
n	2	2	2	1

Toxicity score	††††	††††	††	-
Eff. score	no KD	no KD	KD	KD
Composition				
Encapsulating agent	DOTAP	DOTAP	DOTAP	DOTAP
Helper lipid	Solasodine	Ceramide	Chol, Solasodine	Chol, Solasodine
Fusogenic Agent	LBPA	LBPA	LBPA	LBPA
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	50:25:20:5	50:25:20:5	50:15:15: 15:5	50:20:5: 20:5
EC50 Calculation				
EC50 (nM)	1.95	1.96	2.11	2.32
EC50 S.E.	0.96	1.97	0.76	0.66
EC50 model fitting	app.	app.	app.	app.
n	1	1	12	3
Toxicity score	-	-	-	-
Eff. score	KD	KD	KD	KD
Composition				
Encapsulating agent	DOTAP	DOTAP	DOTAP	DOTAP
Helper lipid	Chol	Chol, Diosgenin, Solasodine	Chol, Solasodine	DOPE
Fusogenic Agent	LBPA	LBPA	LBPA	LBPA
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	50:25:20:5	50:10:10:5: 20:5	50:24:1: 20:5	50:25:20:5
EC50 Calculation				
EC50 (nM)	2.37	2.47	2.48	3.74
EC50 S.E.	0.9	0.7	2.89	0.78
EC50 model fitting	app.	app.	n.a.	n.a.
n	28	1	1	2
Toxicity score	-	-	-	-
Eff. score	KD	KD	KD	KD
Composition				
Encapsulating agent	DOTAP	DOTAP		
Helper lipid	DOPC	Spingomyelin		
Fusogenic Agent	LBPA	LBPA		
Labile PEG lipid	PEGOEC18	PEGOEC18		
Compound ratio	50:25:20:5	50:25:20:5		
EC50 Calculation				
EC50 (nM)	83.64	109.72		
EC50 S.E.	396.41	484.86		
EC50 model fitting	n.a.	n.a.		
n	1	1		
Toxicity score	-	-		
Eff. score	KD	KD		

Abbreviations: S.E.: Standard Error; app.: applicable; n.a.: not applicable; n = repetition number;
 KD: knock down; PEGOEC18: PEG2000- orthoester-C18; composition: Compos.; Eff.= effect.

Table 4: LBPA isoform variation – 4 concentration dose response – HeLa

Composition

Encapsulating agent	DOTAP	DOTAP	DOTAP	DOTAP	DOTAP	DOTAP
Helper lipid	Chol	Chol	Chol	Chol	Chol	Chol
Fusogenic Agent	3,3'-R,S-LBPA	LBPA	3,3'-R,R-LBPA	S,S-2,2'-C18:0-ether-LBPA	Hemi-LBPA	S,S-2,2'-C12:0-ether-LBPA
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	50:25:20:5	50:25:20:5	50:25:20:5	50:25:20:5	50:25:20:5	50:25:20:5
EC50 Calculation						
EC50 (nM)	1.36	1.56	1.64	1.76	1.78	1.95
EC50 S.E.	0.25	0.16	0.4	0.42	0.87	0.28
EC50 model fitting	app.	app.	app.	app.	app.	app.
n	3	9	3	3	3	3
Toxicity score	†	-	-	†	-	†
Effect score	KD	KD	KD	KD	KD	KD

Composition					
Encapsulating agent	DOTAP	DOTAP	DOTAP	DOTAP	DOTAP
Helper lipid	Chol	Chol	Chol	Chol	Chol
Fusogenic Agent	3,3'-C16:0-LBPA	BDP	DOPG	lysoPG	3,3'-C14:0-LBPA
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	50:25:20:5	50:25:20:5	50:25:20:5	50:25:20:5	50:25:20:5
EC50 Calculation					
EC50 (nM)	0.12	4.34	36.85	59.72	561.59
EC50 S.E.	234.34	5.59	300.81	395.38	
EC50 model fitting	n.a.	n.a.	n.a.	n.a.	n.a.
n	3	3	3	3	3
Toxicity score	-	-	-	-	-
Effect score	no KD	KD	KD	KD	no KD

Abbreviations: S.E.: Standard Error; app.: applicable; n.a.: not applicable; n = repetition number; KD: knock down.

Table 5: Cationic agent variation – 4 concentration dose response – HeLa

Composition					
Encapsulating agent	PD740	CTAB	DOTAP	TTAB	DOMA
Helper lipid	Chol	Chol	Chol	Chol	Chol
Fusogenic Agent	LBPA	LBPA	LBPA	LBPA	LBPA
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	50:25:20:5	50:25:20:5	50:25:20:5	50:25:20:5	50:25:20:5
EC50 calculation					
EC50 (nM)	0.48	0.48	1.47	1.57	2.24
EC50 S.E.	0.06	0.15	0.18	0.49	0.24
EC50 model fitting	app.	app.	app.	app.	app.

n	3	3	7	3	3
Toxicity score	††	††††	-	††††	†
Effect score	KD	no KD	KD	KD	KD
Composition					
Encapsulating agent	DOTMA	EPC 18:1	TAP 18:0	MVL5	GL67
Helper lipid	Chol	Chol	Chol	Chol	Chol
Fusogenic Agent	LBPA	LBPA	LBPA	LBPA	LBPA
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	50:25:20:5	50:25:20:5	50:25:20:5	50:25:20:5	50:25:20:5
EC50 calculation					
EC50 (nM)	2.8	6.97	61.74	0.79	1.12
EC50 S.E.	1.25	8.5	236.22	1	1.06
EC50 model fitting	app.	app.	app.	n.a.	n.a.
n	3	3	3	1	1
Toxicity score	†	-	-	-	-
Effect score	KD	KD	no KD	no KD	no KD
Composition					
Encapsulating agent	16-BAC	PD739	SA		
Helper lipid	Chol	Chol	Chol		
Fusogenic Agent	LBPA	LBPA	LBPA		
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18		
Compound ratio	50:25:20:5	50:25:20:5	50:25:20:5		
EC50 calculation					
EC50 (nM)	5.46	65.93	82.96		
EC50 S.E.	5.31	231.32	237.77		
EC50 model fitting	n.a.	n.a.	n.a.		
n	3	3	3		
Toxicity score	††††	-	†		
Effect score	no KD	no KD	no KD		

Abbreviations: S.E.: Standard Error; app.: applicable; n.a.: not applicable; n = repetition number; KD: knock down.

Table 6: PD740 % variation – 4 concentration dose response – HeLa

Composition					
Name	LP3	LP4	-	-	-
Encapsulating agent	PD740, DOTAP	PD740	PD740	PD740, DOTAP	PD740
Helper lipid	Chol	Chol	Chol	Chol	Chol, Solasodine
Fusogenic Agent	LBPA	LBPA	LBPA	LBPA	LBPA
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	25:25:25: 20:5	33:33:27:7	50:25:20:5, N/P=40:1	37.5:12.5: 25:20:5	50:15:15: 15:5
EC50 calculation					
EC50 (nM)	0.42	0.58	0.67	0.9	0.91
EC50 S.E.	0.02	0.06	0.02	0.05	0.23
EC50 model fitting	app.	app.	app.	app.	app.
n	2	5	2	2	2

siRNA 10 nM					
Toxicity score	††††	†††	†††	††††	††††
Effect score	KD	KD	KD	no KD	no KD
siRNA 5 nM					
Toxicity score	††	†	††	††	†††
Effect score	KD	KD	KD	KD	KD
Composition					
Name	LP2		LP1		
Encapsulating agent	PD740	PD740	DOTAP	PD740, DOTAP	
Helper lipid	Chol	Chol, Ceramide	Chol	Chol	
Fusogenic Agent	LBPA	LBPA	LBPA	LBPA	
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	
Compound ratio	50:25:20:5	50:15:15: 15:5	50:25:20:5	12.5:37.5: 25:20:5	
EC50 calculation					
EC50 (nM)	1	1.34	1.45	0.5	
EC50 S.E.	0.05	0.2	0.13	0.65	
EC50 model fitting	app.	app.	app.	n.a.	
n	8	2	17	2	
siRNA 10 nM					
Toxicity score	††††	††††	-	-	
Effect score	no KD	no KD	KD	no KD	
siRNA 5 nM					
Toxicity score	†	††	-	-	
Effect score	KD	KD	KD	no KD	

Abbreviations: S.E.: Standard Error; app.: applicable; n.a.: not applicable; n = repetition number; KD: knock down.

Table 7: PD740 % variation – 10 concentration dose response – HeLa

Composition					
Name	LP3	LP4	LP4	LP1	LP2
Encapsulating agent	DOTAP, PD740	PD740	DOTAP, PD740	DOTAP	PD740
Helper lipid	Chol	Chol	Chol	Chol	Chol
Fusogenic Agent	LBPA	LBPA	LBPA	LBPA	LBPA
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	25:25:20:15:5	33:33:27:7	37.5:12.5:20: 15:5	50:20:15:5	50:20:15:5
EC50 calculation					
EC50 (nM)	0.21	0.29	0.65	0.7	0.73
EC50 S.E.	0.01	0.01	0.05	0.03	0.05
EC50 model fitting	app.	app.	app.	app.	app.
n	7	2	2	6	2
TC50 (nM)	3.15	8.53	10.26	17.92	4.85
TC50 S.E.	0.14	0.57	0.48	0.57	0.33

TC50 fitting	model	app.	app.	app.	app.	app.
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Abbreviations: S.E.: Standard Error; app.: applicable; n.a.: not applicable; n = repetition number; KD: knock down.

Table 8: Fusogenic agent variation in combination with PD740 – 10 concentration dose response – HeLa

Composition						
Name	-	-	LP3	-	-	-
Encapsulating agent	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740
Helper lipid	Chol	Chol	Chol	Chol	Chol	Chol
Fusogenic Agent	PLPE	MGDG	LBPA	IsostA	GMO	POPE
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	25:25:20:15:5	25:25:20:15:5	25:25:20:15:5	25:25:20:15:5	25:25:20:15:5	25:25:20:15:5
EC50 calculation						
EC50 (nM)	0.14	0.2	0.21	0.54	0.65	0.7
EC50 S.E.	0.15	0.02	0.01	0.04	0.07	0.05
EC50 model fitting	app.	app.	app.	app.	app.	app.
n	2	3	7	3	2	2
TC50						
TC50 (nM)	3.34	6.66	3.15	9.3	5.42	5.64
TC50 S.E.	0.18	0.82	0.14	0.68	0.32	0.43
TC50 model fitting	app.	app.	app.	app.	app.	app.
Composition						
Name	LP1	-	-	-	-	-
Encapsulating agent	DOTAP	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740
Helper lipid	Chol	Chol	Chol	Chol	Chol	Chol
Fusogenic Agent	LBPA	NPPE	MO	PalmA	OIA	NAPE
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	50:20:15:5	25:25:20:15:5	25:25:20:15:5	25:25:20:15:5	25:25:20:15:5	25:25:20:15:5
EC50 calculation						
EC50 (nM)	0.7	0.73	0.77	0.97	0.97	10.05
EC50 S.E.	0.03	0.05	0.12	0.11	0.19	86
EC50 model fitting	app.	app.	app.	app.	app.	n.a.
n	6	2	2	2	2	2
TC50						
TC50 (nM)	17.92	8.11	5.89	12.43	9.96	8.86
TC50 S.E.	0.57	0.71	0.46	1.79	1.57	1.73
TC50 model fitting	app.	app.	app.	app.	app.	app.

Abbreviations: S.E.: Standard Error; app.: applicable; n.a.: not applicable; n = repetition number;
KD: knock down.

Table 9: Combination of fusogenic agents with LBPA – 10 concentration dose response - HeLa

Composition						
Name	-	-	LP3	-	-	LP1
Encapsulating agent	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740	DOTAP
Helper lipid	Chol	Chol	Chol	Chol	Chol	Chol
Fusogenic Agent	LBPA, MGDG	LBPA, PLPE	LBPA	LBPA, GMO	LBPA, Isost.A.	LBPA
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	25:25:15:15:15:5	25:25:15:15:15:5	25:25:20:15:5	25:25:15:15:15:5	25:25:15:15:15:5	50:20:15 :5
EC50						
EC50 (nM)	0.19	0.19	0.21	0.3	0.57	0.7
EC50 S.E.	0.01	0.01	0.01	0.02	0.06	0.03
EC50 model fitting	app.	app.	app.	app.	app.	app.
n	1	1	7	1	1	6
TC50						
TC50 (nM)	4.4	2.88	3.15	2.59	2.59	17.92
TC50 S.E.	0.43	0.38	0.14	0.16	0.16	0.57
TC50 model fitting	app.	app.	app.	app.	app.	app.

Abbreviations: S.E.: Standard Error; app.: applicable; n.a.: not applicable; n = repetition number;
KD: knock down.

Table 10: PD740 percentage variation – 10 concentration dose response – MMP

Composition						
Name	LP3	LP3	LP2	-	LP1	LP4
Encapsulating agent	DOTAP, PD740 (1 st batch)	DOTAP, PD740 (different 2 nd batch)	PD740	DOTAP, PD740	DOTAP	PD740
Helper lipid	Chol	Chol	Chol	Chol	Chol	Chol
Fusogenic Agent	LBPA	LBPA	LBPA	LBPA	LBPA	LBPA
Labile lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18

Compound ratio	25:25:20:15:5	25:25:20:15:5	50:20:15:5	37.5:12.5:20:15:5	50:20:15:5	33:33:27:7
EC50 calculation						
EC50 (nM)	4.05	4.35	5.1	6.11	6.21	23.02
EC50 S.E.	0.2	0.62	0.67	0.61	0.34	5.86
EC50 model fitting	app.	app.	app.	app.	app.	app.
n	5	1	1	1	4	1
TC50						
Toxicity score (12.5 nM)	-	-	-	-	-	-
Effect score (12.5 nM)	KD	KD	KD	KD	KD	no KD

Abbreviations: S.E.: Standard Error; app.: applicable; n.a.: not applicable; n = repetition number; KD: knock down.

Table 11: Fusogenic agent variation in combination with PD740 – 10 concentration dose response - MMP

Composition				
Name		LP3		
Encapsulating agent	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740
Helper lipid	Chol	Chol	Chol	Chol
Fusogenic Agent	POPE	LBPA	OIA	GMO
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	25:25:20:15:5	25:25:20:15:5	25:25:20:15:5	25:25:20:15:5
EC50 calculation				
EC50 (nM)	3.82	4.05	5.42	5.79
EC50 S.E.	0.32	0.2	0.57	0.21
EC50 model fitting	app.	app.	app.	app.
n	1	5	1	1
Toxicity score 12.5 nM	-	-	-	-
Effect score 12.5 nM	KD	KD	KD	KD
Composition				
Name	LP1			
Encapsulating agent	DOTAP	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740
Helper lipid	Chol	Chol	Chol	Chol
Fusogenic Agent	LBPA	PalmA	MO	IsostA
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	50:20:15:5	25:25:20:15:5	25:25:20:15:5	25:25:20:15:5
EC50 calculation				

EC50 (nM)	6.21	6.51	7.24	7.36
EC50 S.E.	0.34	0.37	0.41	0.46
EC50 model fitting	app.	app.	app.	app.
n	4	1	1	1
Toxicity score 12.5 nM	-	-	-	-
Effect score 12.5 nM	KD	KD	KD	KD
Composition				
Name				
Encapsulating agent	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740
Helper lipid	Chol	Chol	Chol	Chol
Fusogenic Agent	NAPE	NPPE	MGDG	PLPE
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	25:25:20:15:5	25:25:20:15:5	25:25:20:15:5	25:25:20:15:5
EC50 calculation				
EC50 (nM)	8.89	12.65	1.95	4.61
EC50 S.E.	1.3	1.35	0.7	0.82
EC50 model fitting	app.	app.	n.a.	n.a.
n	1	1	1	1
Toxicity score 12.5 nM	-	-	††	††
Effect score 12.5 nM	KD	KD	no KD	no KD

Abbreviations: S.E.: Standard Error; app.: applicable; n.a.: not applicable; n = repetition number; KD: knock down.

Table 12: Combination of fusogenic agents with LBPA – 10 concentration dose response - MMP

Composition						
Name					LP3	LP1
Cationic agent	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740	DOTAP, PD740	DOTAP
Helper lipid	Chol	Chol	Chol	Chol	Chol	Chol
Fusogenic Agent	LBPA, MGDG	LBPA, Isost.A.	LBPA, GMO	LBPA, PLPE	LBPA	LBPA
Labile PEG lipid	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18	PEGOEC18
Compound ratio	25:25:15:15:15:5	25:25:15:15:15:5	25:25:15:15:15:5	25:25:15:15:15:5	25:25:20:15:5	50:20:15:5
EC50 calculation						
EC50 (nM)	3	3	3.06	3.58	4.05	6.21
EC50 S.E.	0.55	0.22	0.27	2.19	0.2	0.34
EC50 model fitting	app.	app.	app.	app.	app.	app.
n	1	1	1	1	5	4

Toxicity score 12.5 nM						
Effect score 12.5 nM	KD	KD	KD	KD	KD	KD

Abbreviations: S.E.: Standard Error; app.: applicable; n.a.: not applicable; n = repetition number; KD: knock down.

Table 13: Treatment conditions with small molecule enhancers from figure 11a

Nr	Chol-siRNA	Compound	Supplier	Catalogue number	CAS number
1	no	EtOH	-	-	-
2	no	untreated	-	-	-
3	no	interferin	Polyplus-transfection SA	101000028	
4	yes	DMSO			
5	yes	interferin	Polyplus-transfection SA	101000028	
6	yes	Phar129414	MolPort	MolPort-000-795-045	1029083-66-6
7	yes	Hecogenin acetate	Santa Cruz Biotechnology	sc-295139	915-35-5
8	yes	Phar175861	MolPort	MolPort-000-836-434	1262296-77-4
9	yes	Peimine	LKT Laboratories Inc.	P1634	23496-41-5
10	yes	U18666A	Sigma Aldrich	U3633	3039-71-2
11	yes	EtOH			
12	yes	Stock1N-55313	MolPort	MolPort-002-527-957	1268849-74-6
13	yes	Stock1N-30551	MolPort	MolPort-002-515-494	1262295-27-1
14	yes	Stock1N-19417	MolPort	MolPort-002-512-728	897043-62-8
15	yes	Hecogenin	Santa Cruz Biotechnology	SC-295138	467-55-0
16	yes	Stock1N-55183	MolPort	MolPort-002-527-866	4952-51-6
17	yes	Tomatidine	MedChemExpress	HY-N2149	77-59-8
18	yes	Laxogenin	MolPort	000-713-417	1177-71-5
19	yes	Neoruscogenin	AvaChem scientific	3160	17676-33-4
20	yes	Sarasapogenin	Target Molecule Corp.	T2733	126-19-2
21	yes	Ruscogenin	BioZol	TRC-R701750-5mg	472-11-7
22	yes	E18	ChemDiv	N039-0029	1818436-99-5
23	yes	Diosgenin	Sigma Aldrich	D1634	512-04-9
24	yes	Solasidine	Santa Cruz Biotechnology	sc-296408	126-17-0
25	no	EtOH			
26	yes	EtOH			
27	yes	STOCK1N-41490	MolPort	MolPort-002-507-205	4860-15-5
28	yes	4185-0010	MolPort	MolPort-000-733-972	125443-26-7
29	yes	Solanidine	Carbosynth limited	FS765161801	80-78-4
30	yes	Spirostan 6 one	ChemDiv	1554-0030	1092561-88-0
31	yes	O-Acetylsolasodine	MolPort	MolPort-002-507-236	6159-99-5

32	yes	STOCK1N-52849	MolPort	MolPort-002-526-294	3669-17-8
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Table 14: Sequences used in this study

Abbreviation	Full name	Source	Sequence	Notes
Anti eGFP siRNA sense	siRNA against eGFP, 2'-OMe-modified	Sigma Aldrich, custom synthesis	SEQ ID NO 1	Purity: HPLC in vivo quality
Anti eGFP siRNA antisense	siRNA against eGFP, 2'-OMe-modified		SEQ ID NO 2	
AF647 anti eGFP siRNA sense	Alexa647-labeled siRNA against eGFP	Sigma Aldrich, custom synthesis	SEQ ID NO 3	purity: HPLC;
AF647 eGFP anti siRNA antisense	Alexa647-labeled siRNA against eGFP		SEQ ID NO 4	
GFP cr guide 1 , GFP_cr4	GFP cr guide RNA	IDT, custom synthesis	SEQ ID NO 5	Target sequence
tracr RNA	Alt-R® CRISPR-Cas9 tracrRNA	IDT, Cat. No: 1072532	commercial	Sequence not publicly available
GFP-Gal8 plasmid	pCMV3-LGALS8-GFPspark (Human LGALS8 transcript variant 1 Gene ORF cDNA clone expression plasmid, C-GFPspark tag)	SinoBiological , Cat.No: HG10301-ACG)	commercial	Sequence only contains ORF, linker and GFP
Cas9 protein	CRISPR-associated endonuclease Cas9/Csn1	PEPC facility, MPI-CBG	online db	Source of sequence https://www.uniprot.org/uniprotkb/Q99ZW2/entry
dsGFP plasmid	pOCC447-pd1GFP plasmid	PEPC facility, MPI-CBG	SEQ ID NO 6	
antiGFP Chol-siRNA sense	Cholesterol-conjugated siRNA against eGFP, cholesterol-bearing sense strand	Axolabs, custom synthesis	SEQ ID NO 7	
antiGFP Chol-siRNA antisense	Cholesterol-conjugated siRNA against eGFP, antisense strand	Axolabs, custom synthesis	SEQ ID NO 8	It forms a double stranded siRNA with SEQ ID NO 7
eGFP mRNA	CleanCap® EGFP mRNA (5moU)	Trilink, Cat. No: L-7201	online	Sequence: only open reading frame; full sequence not publicly available
guide RNA (mix)	Mix of GFP cr guide RNA (GFP_cr4) and tracr RNA (Alt-R® CRISPR-Cas9 tracrRNA)			
30-GFP-NLS-6xHis	Plasmid encoding (-30)GFP protein	Genscript, custom	SEQ ID No 9	
g-7-GFP-NLS-6xHis	Plasmid encoding (-7)GFP protein	Genscript, custom	SEQ ID No 10	

(-30)GFP protein			SEQ ID No 11	
(-7)GFP protein			SEQ ID No 12	

Table 15

Compound	CAS	Supplier, order number	Full name	Suitable Solvent
DOTAP	113669-21-9	Avanti, 890890	2,3-bis[[<i>(Z)</i> -octadec-9-enoyl]oxy]propyl-trimethylazanium	CHCl ₃
Chol	57-88-5	Avanti, 700000P	Cholesterol	CHCl ₃
DSPE-PEG	474922-77-5	Avanti, 880120C-200mg	18:0 PEG2000 PE	CHCl ₃
S,S-3,3'-LBPA	1246303-12-7	Avanti, 857135P	18:1 BMP (S,S)	CHCl ₃
DOPC	4235-95-4	Avanti, 850375P	18:1 (Δ 9-Cis) PC (DOPC)	CHCl ₃
R,S-3,3'-LBPA	326495-20-9	Avanti, 857133P	sn-(3--(9Z-octadecenoyl)-2-hydroxy)-glycerol-1-phospho-sn-3'-(1'--(9Z-octadecenoyl)-2'-hydroxy)-glycerol (ammonium salt)	CHCl ₃
Diosgenin	512-04-9	Sigma, D1634	(25R)-5-Spirosten-3 β -ol	CHCl ₃
Ceramide	5966-28-9	Avanti, 860519P	C18:1 Ceramide (d18:1/18:1(9Z))	CHCl ₃
DOPG	67254-28-8	Avanti, 840475	18:1 (Δ 9-Cis) PG, 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)	CHCl ₃
18:1 Lyso PG	326495-24-3	Avanti, 858125	1-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt), chloroform	CHCl ₃
R,S-C16:0-LBPA	97466-60-9	Avanti, 110728	Hexadecanoic acid, phosphinobis[oxo(2-hydroxy-3,1-propanediyl)] ester, (<i>R</i> *, <i>S</i> *)-(9CI)	MeOH
S,S-BDP	1246298-28-1	Avanti, 857137	sn-[2,3-dioleoyl]-glycerol-1-phospho-sn-1'-[2',3'-dioleoyl]-glycerol (ammonium salt)	CHCl ₃
R,R-3,3'-LBPA	1246303-13-8	Avanti, 857136	sn-(1-oleoyl-2-hydroxy)-glycerol-3-phospho-sn-3'-(1'-oleoyl-2'-hydroxy)-glycerol (ammonium salt)	CHCl ₃
R,S-C14:0-LBPA	325466-03-3	Avanti, 857131	sn-(3-tetradecanoyl-2-hydroxy)-glycerol-1-phospho-sn-3'-(1'-tetradecanoyl-2'-hydroxy)-glycerol (ammonium salt)	MeOH
S,R-Hemi-LBPA	474943-36-7	Avanti, 857134	sn-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-sn-3'-(1',2'-dioleoyl)-glycerol (ammonium salt)	CHCl ₃
16-BAC	122-18-9	Sigma, B4136-25G	cetalkonium chloride	CHCl ₃

DOMA	3700-67-2	Sigma Aldrich, D2779	dioctadecyldimethylammonium bromide	CHCl ₃
Stearyl amine (SA)	124-30-1	Sigma Aldrich, 74750	stearylamine	CHCl ₃
TTAB	1119-97-7	Sigma Aldrich, T4762	(1-Tetradecyl)trimethylammonium bromide; 98%	CHCl ₃
CTAB	57-09-0	ABCR, AB117004	(1-Hexadecyl)trimethylammonium bromide; 98%	CHCl ₃
S,S-2,2'-C18:0-ether-LBPA		Echelon, L-B180e		CHCl ₃
S,S-2,2'-C12:0-ether-LBPA		Echelon, L-B120e		CHCl ₃
DOPE	4004-05-1	Avanti, 850725	1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine	CHCl ₃
TAP 18:0	220609-41-6	Avanti, 890880P-25mg	2,3-di(octadecanoyloxy)propyl-trimethylazanium;chloride	CHCl ₃
DOTMA	104872-42-6	Avanti, 890898P	1,2-di-O-octadecenyl-3-trimethylammonium propane (chloride salt)	CHCl ₃
EPC 18:1	474945-24-9	Avanti, 890704	1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (chloride salt)	CHCl ₃
Solasodine (Sola)	126-17-0	Santa Cruz Biotechnology, sc-296408		CHCl ₃
GL67 (cholesteryl spermine)	179075-30-0	Avanti, 890893P-5mg	N ⁴ -Cholesteryl-Spermine	CHCl ₃ - only soluble with sonication
MVL5	464926-03-2	Avanti, 890000P-1mg		CHCl ₃
Sphingomyelin (mix from porcine brain)	383907-91-3	Avanti, 860062	Sphingomyelin (Brain, Porcine)	CHCl ₃
Solasodine-Oac (SolaOAc)	6159-99-5	MolPort, MolPort-002-507-236	(3beta,25R)-spiro[5-en-3-yl]acetate, IUPAC: (1S,2S,4S,5'R,7S,8R,9S,12S,13R,16S)-5',7,9,13-tetramethyl-5-oxaspiro[pentacyclo[10.8.0.0 ^{2,9} .0 ^{4,8} .0 ^{13,18}]icosane-6,2'-piperidin]-18-en-16-yl acetate	CHCl ₃
NPPE (16:0 NAPE (+2x16:0))	57984-41-5	Enzo, BML-LP108-0001	N-palmitoyl-1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine	CHCl ₃

NAPE (12:0 amine, 2X16:0)	474944-08-6	Avanti, 870140	N- <i>N</i> -(12-Amino Dodecanoyl)-1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphatidylethanolamine	MeOH , CHCl ₃ (around 2.5:1) - barely soluble
glycerol mono oleate (GMO)	111-03-5	sigma, M7765	1-Oleoyl- <i>rac</i> -glycerol	CHCl ₃
palmitoleic acid (Palma)	373-49-9	Sigma, P9417-100MG	<i>cis</i> -9-Hexadecenoic acid	CHCl ₃
methyl oleate (MO)	112-62-9	Sigma, 311111-5G	Oleic acid methyl ester	CHCl ₃
oleic acid (OLA)	112-80-1	Sigma, O1008		CHCl ₃
isostearic acid (IsostA)	2724-58-5	Sigma, M6281-25MG	16-Methylheptadecanoic acid	CHCl ₃
POPE	26662-94-2	Avanti, 850757P-25mg	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphoethanolamine	CHCl ₃
monogalactosyl diacylglycerol (MGDG)	426820-72-6	Avanti, 840523P-5mg	mixture of different chain lengths	CHCl ₃
PLPE (palmitoyl linoleoyl) (16:0, 18:2)	26662-95-3	Avanti, 850756C-25mg	1-palmitoyl-2-linoleoyl- <i>sn</i> -glycero-3-phosphoethanolamine	CHCl ₃
PD739	866888-46-2	donation by Waldmann laboratory, MPI of Molecular Physiology	methyl N-((2 <i>S</i> ,11 <i>aS</i>)-10-(2-aminoethyl)-2-(hexadecylsulfonamido)-5,11-dioxo-2,3,5,10,11,11 <i>a</i> -hexahydro-1 <i>H</i> -benzo[<i>e</i>]pyrrolo[1,2- <i>a</i>][1,4]diazepine-7-carbonyl)-S-((2 <i>E</i> ,6 <i>E</i>)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)-L-cysteinate	CHCl ₃
PD740	1818425-26-1	Originally donation by Waldmann laboratory, MPI of Molecular Physiology ; additional compound synthesized according to Example 32	N-[3-(Hexadecan-1-sulfonylamino)propionyl]-4-(<i>R</i>)-(aminoethyl)-L-prolyl-S-farnesyl-L-cysteine methyl ester	CHCl ₃

Certain patent items:

Also the following items are provided and are part of the invention:

Item 1: A drug delivery system comprising a liposome having

- (a) a lipid bilayer enclosing an aqueous volume, wherein the lipid bilayer comprises
 - i) between 30 and 75 mol percent of at least one encapsulating agent;
 - ii) between 1 and 20 mol percent of an acid-cleavable polyethylene glycol conjugated lipid;
 - iii) between 15 and 45 mol percent of at least one fusogenic agent, and
- (b) a therapeutic agent or a pharmaceutically acceptable salt thereof, encapsulated within the aqueous volume;

wherein the encapsulating agent is a cationic lipid and/or a lipidated polypeptide; and

wherein the liposome has a Z-Average diameter size range comprised between 20 nm and 200 nm, as determined by dynamic light scattering.

Item 2: The drug delivery system according to item 1, wherein the encapsulating agent is a cationic lipid selected from 1,2-dialkanyloxy-3-trialkylammonium propane halide, 1,2-dialkenyloxy-3-trialkylammonium propane halide, 1,2-dialkanoyloxy-3-trialkylammonium propane halide, 1,2-dialkenoyloxy-3-trialkylammonium propane halide, tetraalkylammonium halide, 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine, and/or a lipidated polypeptide PD740.

Item 3: The drug delivery system according to items 1 or 2, wherein the encapsulating agent is a cationic lipid selected from 1,2-di-C₁₂-C₂₀ alkanyloxy-3-tri-C₁-C₆ alkylammonium propane halide, 1,2-di-C₁₂-C₂₀ alkenyloxy-3-tri-C₁-C₆ alkylammonium propane halide, 1,2-di-C₁₂-C₂₀ alkenoyloxy-3-tri-C₁-C₆ alkylammonium propane halide, di-C₁₂-C₂₀ alkyl di-C₁-C₆ alkyl ammonium halide, and/or a lipidated polypeptide PD740.

Item 4: The drug delivery system according to any one of the items 1 to 3, wherein the encapsulating agent is a cationic lipid selected from the group consisting of 1,2-didodecyloxy-3-trimethylammonium propane chloride, 1,2-ditridecyloxy-3-trimethylammonium propane chloride, 1,2-ditetradecyloxy-3-trimethylammonium propane chloride, 1,2-dipentadecyloxy-3-trimethylammonium propane chloride, 1,2-dihexadecyloxy-3-trimethylammonium propane chloride, 1,2-diheptadecyloxy-3-trimethylammonium propane chloride, 1,2-dioctadecyloxy-3-trimethylammonium propane chloride, 1,2-dinonadecyloxy-3-trimethylammonium propane chloride, 1,2-diicosyloxy-3-trimethylammonium propane chloride, 1,2-didodecenyloxy-3-trimethylammonium propane chloride, 1,2-ditridecenyloxy-3-trimethylammonium propane chloride, 1,2-ditetradecenyloxy-3-trimethylammonium propane chloride, 1,2-dipentadecenyloxy-3-trimethylammonium propane chloride, 1,2-dihexadecenyloxy-3-

trimethylammonium propane chloride, 1,2-diheptadecenyloxy-3-trimethylammonium propane chloride, 1,2-dioctadecenyloxy-3-trimethylammonium propane chloride, 1,2-dinonadecenyloxy-3-trimethylammonium propane chloride, 1,2-diicosenyloxy-3-trimethylammonium propane chloride, 1,2-dilauroleoyloxy-3-trimethylammonium propane chloride, 1,2-dimyristoleoyloxy-3-trimethylammonium propane chloride, 1,2-dipalmitoleoyloxy-3-trimethylammonium propane chloride, 1,2-dipetroseloyloxy-3-trimethylammonium propane chloride, 1,2-dipetroselaidoyloxy-3-trimethylammonium propane chloride, 1,2-dioleoyloxy-3-trimethylammonium propane chloride, 1,2-di-laidoyloxy-3-trimethylammonium propane chloride, 1,2-divaccenoyloxy-3-trimethylammonium propane chloride, 1,2-digadoleoyloxy-3-trimethylammonium propane chloride, dimethyldidodecylammonium bromide, dimethylditridecylammonium bromide, dimethylditetracylammonium bromide, dimethyldipentadecylammonium bromide, dimethyldihexadecylammonium bromide, dimethyldiheptadecylammonium bromide, dimethyldioctadecylammonium bromide, dimethyldinonadecylammonium bromide, and dimethyldiicosylammonium bromide, and/or a lipidated polypeptide PD740.

Item 5: The drug delivery system according to any one of the items 1 to 4, wherein the encapsulating agent is a cationic lipid selected from the group consisting of 1,2-di-*O*-octadecenyl-3-trimethylammonium propane chloride, dimethyldioctadecylammonium bromide, and 1,2-dioleoyl-3-trimethylammonium propane chloride, and/or a lipidated polypeptide PD740.

Item 6: The drug delivery system according to any one of the items 1 to 5, wherein the encapsulating agent is a cationic lipid 1,2-dioleoyl-3-trimethylammonium propane chloride and/or a lipidated polypeptide PD740.

Item 7: The drug delivery system according to any one of the items 1 to 6, wherein the acid-cleavable polyethylene glycol conjugated lipid is an acid-cleavable polyethylene glycol conjugated with a C₁₂-C₂₀ alkyl, or with a C₁₂-C₂₀ acyl, or with a mono-C₁₂-C₂₀ acylglycerol, or with a di-C₁₂-C₂₀ acylglycerol.

Item 8: The drug delivery system according to any one of the items 1 to 7, wherein the acid-cleavable polyethylene glycol conjugated lipid comprises an acid-cleavable linkage selected from the group comprising an orthoester linkage, a hydrazone linkage, an acetal linkage, a vinyl ether linkage, and an imine linkage, and preferably 2-methyl-2-alkoxy-1,3-dioxane or 2-alkoxy-1,3-dioxolane.

Item 9: The drug delivery system according to any one of the items 1 to 8, wherein the acid-cleavable polyethylene glycol conjugated lipid is selected from the group consisting of: *N*-(2-methyl-2-dodecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-tridecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-tetradecyloxy-

[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-pentadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-hexadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-heptadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-nonadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, and *N*-(2-methyl-2-icosyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol.

Item 10: The drug delivery system according to any one of the items 1 to 9, wherein the acid-cleavable polyethylene glycol conjugated lipid comprises polyethylene glycol having a number average molar mass M_n comprised between 400 and 5000 Da.

Item 11: The drug delivery system according to any one of the items 1 to 10, wherein the acid-cleavable polyethylene glycol conjugated lipid is α -methoxy- ω -{*N*-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol.

Item 12: The drug delivery system according to any one of the items 1 to 11, wherein the fusogenic agent is a glycolipid, a phosphatidylethanolamine, a phosphatidylglycerol, a fatty acid, or a fatty acid ester.

Item 13: The drug delivery system according to item 12, wherein the fusogenic agent is a galactolipid, preferably an unsaturated monogalactosyldiacylglycerol.

Item 14: The drug delivery system according to item 12, wherein the fusogenic agent is a 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine or an *N*-acyl-1,2-diacyl-*sn*-glycero-3-phosphoethanolamine.

Item 15: The drug delivery system according to item 12, wherein the fusogenic agent is a phosphatidylethanolamine selected from the group consisting of 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dimargaroyl-*sn*-glycero-3-phosphoethanolamine, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dilinoyleoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dierucoyl-*sn*-glycero-3-phosphoethanolamine, 1-pentadecanoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine,

1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine, 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine, 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine, and *N*-palmitoyl-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine.

Item 16: The drug delivery system according to item 12, wherein the fusogenic agent is a phosphatidylglycerol selected from the group consisting of bis(monoacylglycerol)phosphate, 3-acylglycerol-1-phospho-3'-(1',2'-diacyl)-glycerol, 3-acylglycerol-1-phospho-glycerol, and 1,2-diacylglycerol-3-phospho-glycerol.

Item 17: The drug delivery system according to item 16, wherein the fusogenic agent is a bis(monoacylglycerol)phosphate selected from the group consisting of 2,2'-S,S-bis(monoacylglycerol)phosphate, 3,3'-S,S-bis(monoacylglycerol)phosphate, 3,3'-R,R-bis(monoacylglycerol)phosphate, and 3,3'-R,S-bis(monoacylglycerol)phosphate.

Item 18: The drug delivery system according to item 16, wherein the fusogenic agent is a bis(monoacylglycerol)phosphate selected from the group consisting of bis(monododecylglycerol)phosphate, bis(monotridecylglycerol)phosphate, bis(monotetradecylglycerol)phosphate, bis(monopentadecylglycerol)phosphate, bis(monohexadecylglycerol)phosphate, bis(monoheptadecylglycerol)phosphate, bis(monooctadecylglycerol)phosphate, bis(monononadecylglycerol)phosphate, bis(monocosylglycerol)phosphate, bis(monolauroleoylglycerol)phosphate, bis(monomyristoleoylglycerol)phosphate, bis(monopalmitoleoylglycerol)phosphate, bis(monopetroseloylglycerol)phosphate, bis(monopetroselaidoylglycerol)phosphate, bis(monooloylglycerol)phosphate, bis(monolaidoylglycerol)phosphate, bis(monovaccenoylglycerol)phosphate, and bis(monogadoleoylglycerol)phosphate.

Item 19: The drug delivery system according to item 16, wherein the fusogenic agent is a 3-acylglycerol-1-phospho-3'-(1',2'-diacyl)-glycerol selected from the group consisting of 3-dodecylglycero-1-phospho-3'-(1',2'-didodecyl)-glycerol, 3-tridecylglycero-1-phospho-3'-(1',2'-ditridecyl)-glycerol, 3-tetradecylglycero-1-phospho-3'-(1',2'-ditetradecyl)-glycerol, 3-pentadecylglycero-1-phospho-3'-(1',2'-dipentadecyl)-glycerol, 3-hexadecylglycero-1-phospho-3'-(1',2'-dihexadecyl)-glycerol, 3-heptadecylglycero-1-phospho-3'-(1',2'-diheptadecyl)-glycerol, 3-octadecylglycero-1-phospho-3'-(1',2'-dioctadecyl)-glycerol, 3-nonadecylglycero-1-phospho-3'-(1',2'-dinonadecyl)-glycerol, 3-icosylglycero-1-phospho-3'-(1',2'-diicosyl)-glycerol, 3-lauroleoylglycerol-1-phospho-3'-(1',2'-dilauroleoyl)-glycerol, 3-myristoleoylglycerol-1-phospho-3'-(1',2'-dimyristoleoyl)-glycerol, 3-palmitoleoylglycerol-1-phospho-3'-(1',2'-dpalmitoleoyl)-glycerol, 3-petroseloylglycerol-1-

phospho-3'-(1',2'-dipetroseloyl)-glycerol, 3-petroselaidoylglycero-1-phospho-3'-(1',2'-dipetroselaidoyl)-glycerol, 3-oleoylglycero-1-phospho-3'-(1',2'-dioleoyl)-glycerol, 3-elaidoylglycero-1-phospho-3'-(1',2'-dielaidoyl)-glycerol, 3-vaccenoylglycero-1-phospho-3'-(1',2'-divaccenoyl)-glycerol, and 3-gadoleoylglycero-1-phospho-3'-(1',2'-digadoleoyl)-glycerol.

Item 20: The drug delivery system according to item 16, wherein the fusogenic agent is a 3-acylglycero-1-phospho-glycerol selected from the group consisting of 3-dodecylglycero-1-phospho-glycerol, 3-tridecylglycero-1-phospho-glycerol, 3-tetradecylglycero-1-phospho-glycerol, 3-pentadecylglycero-1-phospho-glycerol, 3-hexadecylglycero-1-phospho-glycerol, 3-heptadecylglycero-1-phospho-glycerol, 3-octadecylglycero-1-phospho-glycerol, 3-nonadecylglycero-1-phospho-glycerol, 3-icosylglycero-1-phospho-glycerol, 3-lauroleoylglycero-1-phospho-glycerol, 3-myristoleoylglycero-1-phospho-glycerol, 3-palmitoleoylglycero-1-phospho-glycerol, 3-petroseloylglycero-1-phospho-glycerol, 3-petroselaidoylglycero-1-phospho-glycerol, 3-oleoylglycero-1-phospho-glycerol, 3-elaidoylglycero-1-phospho-glycerol, 3-vaccenoylglycero-1-phospho-glycerol, and 3-gadoleoylglycero-1-phospho-glycerol.

Item 21: The drug delivery system according to item 16, wherein the fusogenic agent is a 1,2-diacylglycero-3-phospho-glycerol selected from the group consisting of 1,2-didodecylglycero-3-phospho-glycerol, 1,2-ditridecylglycero-3-phospho-glycerol, 1,2-ditetradecylglycero-3-phospho-glycerol, 1,2-dipentadecylglycero-3-phospho-glycerol, 1,2-dihexadecylglycero-3-phospho-glycerol, 1,2-diheptadecylglycero-3-phospho-glycerol, 1,2-diiodadecylglycero-3-phospho-glycerol, 1,2-dinonadecylglycero-3-phospho-glycerol, 1,2-diicosylglycero-3-phospho-glycerol, 1,2-dilauroleoylglycero-3-phospho-glycerol, 1,2-dimyristoleoylglycero-3-phospho-glycerol, 1,2-dipalmitoleoylglycero-3-phospho-glycerol, 1,2-dipetroseloylglycero-3-phospho-glycerol, 1,2-dipetroselaidoylglycero-3-phospho-glycerol, 1,2-dioleoylglycero-3-phospho-glycerol, 1,2-dielaidoylglycero-3-phospho-glycerol, 1,2-divaccenoylglycero-3-phospho-glycerol, and 1,2-digadoleoylglycero-3-phospho-glycerol.

Item 22: The drug delivery system according to item 12, wherein the fusogenic agent is a fatty acid selected from the group consisting of myristic acid, pentadecanoic acid, palmitic acid, margaric acid, stearic acid, isostearic acid, nonadecylic acid, arachidic acid, myristoleic acid, palmitoleic acid, sapienic acid, oleic acid, elaidic acid, vaccenic acid, and linoleic acid; or a methyl ester or a glyceryl ester of one of the aforementioned fatty acids.

Item 23: The drug delivery system according to any one of the items 1 to 22, wherein the fusogenic agent is selected from the group consisting of monogalactosyldilinolenoylglycerol, 1-palmitoyl-2-

linoleoyl-*sn*-glycero-3-phosphoethanolamine,
 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, *N*-palmitoyl-1,2-dipalmitoyl-*sn*-
 glycero-3-phosphoethanolamine, 2,2'-S,S-bis(monododecylglycerol)phosphate, 3,3'-S,S-
 bis(monododecylglycerol)phosphate, 3,3'-R,R-bis(monododecylglycerol)phosphate, 3,3'-R,S-
 bis(monododecylglycerol)phosphate, 2,2'-S,S-bis(monooctadecylglycerol)phosphate, 3,3'-S,S-
 bis(monooctadecylglycerol)phosphate, 3,3'-R,R-bis(monooctadecylglycerol)phosphate, 3,3'-
 R,S-bis(monooctadecylglycerol)phosphate, 2,2'-S,S-bis(monooleoylglycerol)phosphate, 3,3'-
 S,S-bis(monooleoylglycerol)phosphate, 3,3'-R,R-bis(monooleoylglycerol)phosphate, 3,3'-R,S-
 bis(monooleoylglycerol)phosphate, 3-dodecylglycero-1-phospho-3'-(1',2'-didodecyl)-glycerol,
 3-octadecylglycero-1-phospho-3'-(1',2'-dioctadecyl)-glycerol, 3-oleoylglycero-1-phospho-3'-
 (1',2'-dioleoyl)-glycerol, glyceryl monoleate, methyl oleate, isotearic acid, palmitoleic acid, and
 oleic acid.

Item 24: The drug delivery system according to any one of the items 1 to 23, wherein the lipid bilayer further comprises:

iv) between 2.5 and 35 mol percent of at least one neutral lipid.

Item 25: The drug delivery system according to any one of the items 1 to 24, wherein the lipid bilayer further comprises:

iv) between 2.5 and 35 mol percent of at least one neutral lipid selected from the group consisting of cholesterol, diosgenin, solasodine, and ceramide.

Item 26: The drug delivery system according to any one of the items 1 to 25, wherein the therapeutic agent is a drug, a protein, a peptide, a gene, an oligonucleotide, a compound, an antibody, a nanobody, a nucleic acid, a ribonucleoprotein, a small molecule, a non-lipinski molecule, a biomimetic, or a natural compound.

Item 27: The drug delivery system according to any one of the items 1 to 26, wherein the therapeutic agent is a CRISPR/CAS9 ribonucleoprotein, a CRISPR guide RNA or a CRISPR associated Cas protein.

Item 28: The drug delivery system according to item 26, wherein the therapeutic agent is a nucleic acid selected from the group comprising ssDNA, dsDNA, ssRNA, dsRNA, aiRNA, miRNA, siRNA, piRNA, sdRNA, snRNA, snoRNA, PAR, tsRNA, endo-siRNA, MSY-RNA, tel-sRNA, crasiRNA, moRNA, xiRNA, lncRNA, ncRNA, circRNA, mRNA, sgRNA, crRNA, tracr RNA, guide RNA mix, self-amplifying RNA, ribozymes, and antisense oligonucleotides.

Item 29: The drug delivery system according to any one of the items 1 to 26, wherein the therapeutic agent is selected from the group comprising a CRISPR/CAS9 ribonucleoprotein, a siRNA, a mRNA, or a guide RNA mix.

Item 30: The drug delivery system according to any one of the items 1 to 29, wherein the lipid bilayer comprises

- i) between 30 and 75 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride and/or PD740,
- ii) between 1 and 20 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
- iii) between 15 and 45 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate, and
- iv) between 13 and 35 mol percent of cholesterol.

Item 31: The drug delivery system according to any one of the items 1 to 29, wherein the lipid bilayer comprises

- i) 50 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride,
- ii) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
- iii) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate, and
- iv) 25 mol percent of cholesterol.

Item 32: The drug delivery system according to any one of the items 1 to 29, wherein the lipid bilayer comprises

- i) 50 mol percent of PD740,
- ii) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
- iii) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate, and
- iv) 25 mol percent of cholesterol.

Item 33: The drug delivery system according to any one of the items 1 to 29, wherein the lipid bilayer comprises

- ia) 25 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride,
- ib) 25 mol percent of PD740,
- ii) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,

- iii) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate, and
- iv) 25 mol percent of cholesterol.

Item 34: The drug delivery system according to any one of the items 1 to 29, wherein the lipid bilayer comprises

- i) 33 mol percent of PD740,
- ii) 7 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
- iii) 27 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate, and
- iv) 33 mol percent of cholesterol.

Item 35: The drug delivery system according to any one of the items 1 to 29, wherein the lipid bilayer comprises

- i) between 42 and 50 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride,
- ii) between 4 and 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
- iii) between 4 and 15 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate,
- iva) between 13 and 15 mol percent of cholesterol, and
- ivb) between 15 and 36 mol percent of solasodine.

Item 36: The drug delivery system according to any one of the items 1 to 29, wherein the lipid bilayer comprises

- ia) 25 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride,
- ib) 25 mol percent of PD740,
- ii) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
- iii) 20 mol percent of monogalactosyldiacylglycerol or 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine, and
- iv) 25 mol percent of cholesterol.

Item 37: The drug delivery system according to any one of the items 1 to 37, wherein the N:P ratio is from about 1:1 to about 50:1.

Item 38: The drug delivery system according to any one of the items 1 to 37, wherein the liposome is a unilamellar and/or univesicular liposome.

Claims

1. A composition comprising
 - (a) a therapeutic agent or a pharmaceutically acceptable salt thereof;
 - (b) a PEG-monoorthoester-lipid;
 - (c) an amphiphilic lipid;
 - (d) a cationic lipid and/or a beta-alanyl-prolyl-cysteine methyl ester; and
 - optionally (e) a steroid and/or a ceramide and/or DOPE.
2. The composition according to claim 1, wherein the composition is a liposome composition, preferably wherein the liposome is a unilamellar and/or univesicular liposome.
3. The composition according to any one of claims 1 or 2, wherein the therapeutic agent or pharmaceutically acceptable salt thereof (a) is encapsulated by the mixture comprising of (b), (c), (d) and (e) if (e) is part of said composition.
4. The composition according to any one of claims 1 to 3, wherein the PEG-monoorthoester-lipid hydrolyses at a pH of 6 or less, preferably wherein said orthoester bond hydrolyses at a pH of between pH 5 and pH 6.
5. The composition according to any one of the preceding claims comprising
 - (a) a therapeutic agent or a pharmaceutically acceptable salt thereof;
 - (b) a PEG-monoorthoester-lipid;
 - (c) an amphiphilic lipid;
 - (d) a cationic lipid or a beta-alanyl-prolyl-cysteine methyl ester; and
 - (e) a steroid, and preferably cholesterol.
6. The composition according to any one of the claims 1 to 4 comprising
 - (a) a therapeutic agent or a pharmaceutically acceptable salt thereof;
 - (b) a PEG-monoorthoester-lipid;
 - (c) an amphiphilic lipid;
 - (d) a cationic lipid and a beta-alanyl-prolyl-cysteine methyl ester; and
 - (e) a steroid, and preferably cholesterol.
7. The composition according to any one of the claims 1 to 4 comprising
 - (a) a therapeutic agent or a pharmaceutically acceptable salt thereof;
 - (b) a PEG-monoorthoester-lipid;
 - (c) an amphiphilic lipid;
 - (d) a cationic lipid and/or beta-alanyl-prolyl-cysteine methyl ester; and

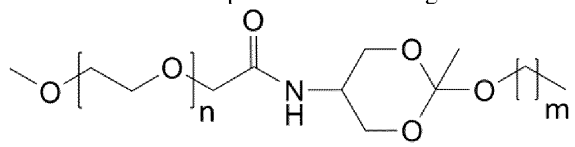
- (e) a steroid and a ceramide.
8. The composition according to any one of the claims 1 to 4 comprising
 - (a) a therapeutic agent or a pharmaceutically acceptable salt thereof, wherein the therapeutic agent is mRNA;
 - (b) a PEG-monoorthoester-lipid;
 - (c) an amphiphilic lipid;
 - (d) a cationic lipid and/or a beta-alanyl-prolyl-cysteine methyl ester; and
 - (e) cholesterol.
 9. The composition according to any one of claims 1 to 4, wherein said composition does not comprise a steroid, and preferably does not comprise cholesterol.
 10. The composition according to any of the preceding claims, wherein the compound (d) is selected from the group consisting of 1,2-dialkanyloxy-3-trialkylammonium propane halide, 1,2-dialkenyloxy-3-trialkylammonium propane halide, 1,2-dialkanoyloxy-3-trialkylammonium propane halide, 1,2-dialkenoyloxy-3-trialkylammonium propane halide, tetraalkylammonium halide, 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine, and/or is a lipidated polypeptide PD740.
 11. The composition according to any of the preceding claims, wherein the compound (d) is selected from the group consisting of 1,2-di-C12-C20 alkanyloxy-3-tri-C1-C6 alkylammonium propane halide, 1,2-di-C12-C20 alkenyloxy-3-tri-C1-C6 alkylammonium propane halide, 1,2-di-C12-C20 alkenoyloxy-3-tri-C1-C6 alkylammonium propane halide, di-C12-C20 alkyl di-C1-C6 alkyl ammonium halide, and/or is a lipidated polypeptide PD740.
 12. The composition according to any one of the preceding claims, wherein the compound (d) is selected from the group consisting of 1,2-didodecyloxy-3-trimethylammonium propane chloride, 1,2-ditridecyloxy-3-trimethylammonium propane chloride, 1,2-ditetradecyloxy-3-trimethylammonium propane chloride, 1,2-dipentadecyloxy-3-trimethylammonium propane chloride, 1,2-dihexadecyloxy-3-trimethylammonium propane chloride, 1,2-diheptadecyloxy-3-trimethylammonium propane chloride, 1,2-di-octadecyloxy-3-trimethylammonium propane chloride, 1,2-dinonadecyloxy-3-trimethylammonium propane chloride, 1,2-diicosyloxy-3-trimethylammonium propane chloride, 1,2-didodecenyloxy-3-trimethylammonium propane chloride, 1,2-ditridecenyloxy-3-trimethylammonium propane chloride, 1,2-ditetradecenyloxy-3-trimethylammonium propane chloride, 1,2-dipentadecenyloxy-3-trimethylammonium propane chloride, 1,2-dihexadecenyloxy-3-trimethylammonium propane chloride, 1,2-

diheptadecenyl-3-trimethylammonium propane chloride, 1,2-dioctadecenyl-3-trimethylammonium propane chloride, 1,2-dinonadecenyl-3-trimethylammonium propane chloride, 1,2-diicosenyl-3-trimethylammonium propane chloride, 1,2-dilauroyl-3-trimethylammonium propane chloride, 1,2-dimyristoyl-3-trimethylammonium propane chloride, 1,2-dipalmitoyl-3-trimethylammonium propane chloride, 1,2-dipetroselinoyl-3-trimethylammonium propane chloride, 1,2-dipetroselinoyl-3-trimethylammonium propane chloride, 1,2-dioleoyl-3-trimethylammonium propane chloride, 1,2-di-1,3-bis(sn-3'-phosphatidyl)-sn-glycero-3-ethylphosphocholine (EPC 18:1), 1,2-divaccenyl-3-trimethylammonium propane chloride, 1,2-digadoleoyl-3-trimethylammonium propane chloride, dimethyldidodecylammonium bromide, dimethylditridecylammonium bromide, dimethylditetradecylammonium bromide, dimethyldipentadecylammonium bromide, dimethyldihexadecylammonium bromide, dimethyldiheptadecylammonium bromide, dimethyldioctadecylammonium bromide, dimethyldinonadecylammonium bromide, and dimethyldiicosylammonium bromide, and/or is a lipidated polypeptide PD740.

13. The composition according to any one of the preceding claims, wherein the compound (d) is selected from the group consisting of 1,2-di-O-octadecenyl-3-trimethylammonium propane chloride, dimethyldioctadecylammonium bromide, and 1,2-dioleoyl-3-trimethylammonium propane chloride, and/or is a lipidated polypeptide PD740, and preferably wherein the compound (d) is 1,2-dioleoyl-3-trimethylammonium propane chloride and/or a lipidated polypeptide PD740.
14. The composition according to any one of the preceding claims, wherein the beta-alanyl-prolyl-cysteine methyl ester is a lipidated polypeptide, and preferably wherein the beta-alanyl-prolyl-cysteine methyl ester is or comprises N-[3-(Hexadecan-1-sulfonylamino)propionyl]-4-(R)-(aminoethyl)-L-prolyl-S-farnesyl-L-cysteine methyl ester (PD740).
15. The composition according to any one of the preceding claims, wherein the compound in (d) is selected from the group consisting of N-[3-(Hexadecan-1-sulfonylamino)propionyl]-4-(R)-(aminoethyl)-L-prolyl-S-farnesyl-L-cysteine methyl ester (PD740), 2,3-bis[(Z)-octadec-9-enoyl]oxypropyl-trimethylazanium (DOTAP), dioctadecyldimethylammonium bromide (DOMA), 1,2-di-O-octadecenyl-3-trimethylammonium propane (chloride salt) (DOTMA), 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (EPC 18:1), and mixtures thereof.
16. The composition according to any one of the preceding claims, wherein the compound in (d) is 2,3-bis[(Z)-octadec-9-enoyl]oxypropyl-trimethylazanium (DOTAP) and/or beta-alanyl-

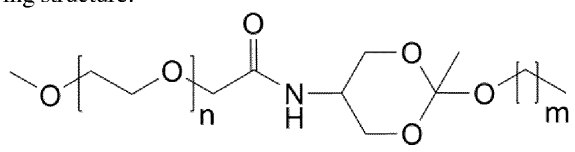
prolyl-cysteine methyl ester N-[3-(Hexadecan-1-sulfonylamino)propionyl]-4-(R)-(aminoethyl)-L-prolyl-S-farnesyl-L-cysteine methyl ester (PD740).

17. The composition according to any one of the preceding claims, wherein the PEG-monoorthoester-lipid comprises a polyethylene glycol conjugated with a C₁₂-C₂₀ alkyl, or with a C₁₂-C₂₀ acyl, or with a mono-C₁₂-C₂₀ acylglycerol, or with a di-C₁₂-C₂₀ acylglycerol, or wherein said PEG-monoorthoester lipid has the following structure:



wherein n is an integer between 35 and 120 and wherein m is an integer between 10 and 27; and preferably wherein n is an integer between 40 and 110 and m is an integer of between 15 and 23.

18. The composition according to any one of claims 1-16, wherein said PEG-monoorthoester lipid has the following structure:



wherein n is an integer between 20 and 200 and wherein m is an integer between 10 and 27; and preferably wherein n is an integer between 100 and 120; and preferably wherein the composition comprises as component (e) cholesterol.

19. The composition according to any one of the preceding claims, wherein the PEG-monoorthoester-lipid is selected from the group consisting of: *N*-(2-methyl-2-dodecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-tridecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-tetradecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-pentadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-hexadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-heptadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, *N*-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol (PEGOEC18), *N*-(2-methyl-2-nonadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol, and *N*-(2-methyl-2-icosyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol.
20. The composition according to any one of the preceding claims, wherein the PEG-monoorthoester-lipid comprises polyethylene glycol having a number average molar mass *M_n* comprised between 400 and 5000 Da.

21. The composition according to any one of the preceding claims, wherein the PEG-monoorthoester-lipid is N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido-polyethyleneglycol (PEGOEC18), and preferably α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅.
22. The composition according to any one of the preceding claims, wherein the amphiphilic lipid is selected from the group consisting of a glycolipid, a phosphatidylethanolamine, a phosphatidylglycerol, a fatty acid, and a fatty acid ester.
23. The composition according to claim 22, wherein the amphiphilic lipid is a galactolipid, preferably an unsaturated monogalactosyldiacylglycerol.
24. The composition according to claim 22, wherein the amphiphilic lipid is a 1,2-diacyl-sn-glycero-3-phosphoethanolamine or an N-acyl-1,2-diacyl-sn-glycero-3-phosphoethanolamine.
25. The composition according to claim 22, wherein the amphiphilic lipid is a phosphatidylethanolamine selected from the group consisting of 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine, 1,2-dipentadecanoyl-sn-glycero-3-phosphoethanolamine, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, 1,2-dimargaroyl-sn-glycero-3-phosphoethanolamine, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dipalmitoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoeoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-dierucoyl-sn-glycero-3-phosphoethanolamine, 1-pentadecanoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine, 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine, 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphoethanolamine, and N-palmitoyl-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine.
26. The composition according to claim 22, wherein the amphiphilic lipid is a phosphatidylglycerol selected from the group consisting of bis(monoacylglycerol)phosphate, 3-acylglycero-1-

phospho-3'-(1',2'-diacyl)-glycerol, 3-acylglycero-1-phospho-glycerol, and 1,2-diacylglycero-3-phospho-glycerol.

27. The composition according to claim 26, wherein the amphiphilic lipid is a bis(monoacylglycerol)phosphate selected from the group consisting of 2,2'-S,S-bis(monoacylglycerol)phosphate, 3,3'-S,S-bis(monoacylglycerol)phosphate, 3,3'-R,R-bis(monoacylglycerol)phosphate, 3,3'-R,S-bis(monoacylglycerol)phosphate, S,R-Hemi, S,S-2,2'-C18:0-ether-LBPA and S,S-2,2'-C12:0-ether-LBPA.
28. The composition according to claim 26, wherein the amphiphilic lipid is a bis(monoacylglycerol)phosphate selected from the group consisting of

bis(monododecylglycerol)phosphate,	bis(monotridecylglycerol)phosphate,
bis(monotetradecylglycerol)phosphate,	bis(monopentadecylglycerol)phosphate,
bis(monohexadecylglycerol)phosphate,	bis(monoheptadecylglycerol)phosphate,
bis(monooctadecylglycerol)phosphate,	bis(monononadecylglycerol)phosphate,
bis(monoicosylglycerol)phosphate,	bis(monolauroleoylglycerol)phosphate,
bis(monomyristoleoylglycerol)phosphate,	bis(monopalmitoleoylglycerol)phosphate,
bis(monopetroseloylglycerol)phosphate,	bis(monopetroselaidoylglycerol)phosphate,
bis(monooleoylglycerol)phosphate,	bis(monoelaidoylglycerol)phosphate,
bis(monovaccenoylglycerol)phosphate, and	bis(monogadoleoylglycerol)phosphate.
29. The composition according to claim 26, wherein the amphiphilic lipid is a 3-acylglycero-1-phospho-3'-(1',2'-diacyl)-glycerol selected from the group consisting of 3-dodecylglycero-1-phospho-3'-(1',2'-didodecyl)-glycerol, 3-tridecylglycero-1-phospho-3'-(1',2'-ditridecyl)-glycerol, 3-tetradecylglycero-1-phospho-3'-(1',2'-ditetradecyl)-glycerol, 3-pentadecylglycero-1-phospho-3'-(1',2'-dipentadecyl)-glycerol, 3-hexadecylglycero-1-phospho-3'-(1',2'-dihexadecyl)-glycerol, 3-heptadecylglycero-1-phospho-3'-(1',2'-diheptadecyl)-glycerol, 3-octadecylglycero-1-phospho-3'-(1',2'-dioctadecyl)-glycerol, 3-nonadecylglycero-1-phospho-3'-(1',2'-dinonadecyl)-glycerol, 3-icosylglycero-1-phospho-3'-(1',2'-diicosyl)-glycerol, 3-lauroleoylglycero-1-phospho-3'-(1',2'-dilauroleoyl)-glycerol, 3-myristoleoylglycero-1-phospho-3'-(1',2'-dimyristoleoyl)-glycerol, 3-palmitoleoylglycero-1-phospho-3'-(1',2'-dipalmitoleoyl)-glycerol, 3-petroseloylglycero-1-phospho-3'-(1',2'-dipetroseloyl)-glycerol, 3-petroselaidoylglycero-1-phospho-3'-(1',2'-dipetroselaidoyl)-glycerol, 3-oleoylglycero-1-phospho-3'-(1',2'-dioleoyl)-glycerol, 3-elaidoylglycero-1-phospho-3'-(1',2'-dielaidoyl)-glycerol, 3-vaccenoylglycero-1-phospho-3'-(1',2'-divaccenoyl)-glycerol, and 3-gadoleoylglycero-1-phospho-3'-(1',2'-digadoleoyl)-glycerol.

30. The composition according to claim 26, wherein the amphiphilic lipid is a 3-acylglycerol-1-phospho-glycerol selected from the group consisting of 3-dodecylglycero-1-phospho-glycerol, 3-tridecylglycero-1-phospho-glycerol, 3-tetradecylglycero-1-phospho-glycerol, 3-pentadecylglycero-1-phospho-glycerol, 3-hexadecylglycero-1-phospho-glycerol, 3-heptadecylglycero-1-phospho-glycerol, 3-octadecylglycero-1-phospho-glycerol, 3-nonadecylglycero-1-phospho-glycerol, 3-icosylglycero-1-phospho-glycerol, 3-lauroleoylglycerol-1-phospho-glycerol, 3-myristoleoylglycerol-1-phospho-glycerol, 3-palmitoleoylglycerol-1-phospho-glycerol, 3-petroseloylglycerol-1-phospho-glycerol, 3-petroselaidoylglycerol-1-phospho-glycerol, 3-oleoylglycerol-1-phospho-glycerol, 3-elaidoylglycerol-1-phospho-glycerol, 3-vaccenoylglycerol-1-phospho-glycerol, and 3-gadoleoylglycerol-1-phospho-glycerol.
31. The composition according to claim 26, wherein the amphiphilic lipid is a 1,2-diacylglycerol-3-phospho-glycerol selected from the group consisting of 1,2-didodecylglycero-3-phospho-glycerol, 1,2-ditridecylglycero-3-phospho-glycerol, 1,2-ditetradecylglycero-3-phospho-glycerol, 1,2-dipentadecylglycero-3-phospho-glycerol, 1,2-dihexadecylglycero-3-phospho-glycerol, 1,2-diheptadecylglycero-3-phospho-glycerol, 1,2-diiodadecylglycero-3-phospho-glycerol, 1,2-dinonadecylglycero-3-phospho-glycerol, 1,2-diicosylglycero-3-phospho-glycerol, 1,2-dilauroleoylglycerol-3-phospho-glycerol, 1,2-dimyristoleoylglycerol-3-phospho-glycerol, 1,2-dipalmitoleoylglycerol-3-phospho-glycerol, 1,2-dipetroseloylglycerol-3-phospho-glycerol, 1,2-dipetroselaidoylglycerol-3-phospho-glycerol, 1,2-dioleoylglycerol-3-phospho-glycerol, 1,2-di-elaidoylglycerol-3-phospho-glycerol, 1,2-divaccenoylglycerol-3-phospho-glycerol, and 1,2-digadoleoylglycerol-3-phospho-glycerol.
32. The composition according to claim 22, wherein the amphiphilic lipid is a fatty acid selected from the group consisting of myristic acid, pentadecanoic acid, palmitic acid, margaric acid, stearic acid, isostearic acid, nonadecylic acid, arachidic acid, myristoleic acid, palmitoleic acid, sapienic acid, oleic acid, elaidic acid, vaccenic acid, and linoleic acid; or a methyl ester or a glyceryl ester of one of the aforementioned fatty acids.
33. The composition according to any one of the claims 1 to 32, wherein the amphiphilic lipid is selected from the group consisting of monogalactosyldilinolenoylglycerol, 1-palmitoyl-2-linoleoyl-sn-glycerol-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanolamine, N-palmitoyl-1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine, 2,2'-S,S-bis(monododecylglycerol)phosphate, 3,3'-S,S-bis(monododecylglycerol)phosphate, 3,3'-R,R-bis(monododecylglycerol)phosphate, 3,3'-S,S-bis(monododecylglycerol)phosphate, 2,2'-S,S-bis(monooctadecylglycerol)phosphate, 3,3'-S,S-bis(monooctadecylglycerol)phosphate, 3,3'-R,R-bis(monooctadecylglycerol)phosphate, 3,3'-

R,S-bis(monooctadecylglycerol)phosphate, 2,2'-S,S-bis(monooleoylglycerol)phosphate, 3,3'-S,S-bis(monooleoylglycerol)phosphate, 3,3'-R,R-bis(monooleoylglycerol)phosphate, 3,3'-R,S-bis(monooleoylglycerol)phosphate, 3-dodecylglycero-1-phospho-3'-(1',2'-didodecyl)-glycerol, 3-octadecylglycero-1-phospho-3'-(1',2'-dioctadecyl)-glycerol, 3-oleoylglycero-1-phospho-3'-(1',2'-dioleoyl)-glycerol, glyceryl monoleate, methyl oleate, isotearic acid, palmitoleic acid, and oleic acid.

34. The composition according to claim 22, wherein the amphiphilic lipid is selected from the group consisting of (S,S) Bisoleoyl-lysobisphosphatidic acid (LBPA), monogalactosyldiacylglycerol (MGDG), 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine (PLPE), isostearic acid (IsostA), glycerol mono oleate (GMO), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), N-Palmitoyl phosphatidylethanolamine 16:0 (NPPE), N-acylphosphatidylethanolamine 12:0 (NAPE), methyl oleate (MO), palmitoleic acid (PalmA), oleic acid (OIA), or mixtures thereof, and even more preferably wherein the amphiphilic lipid is or comprises LBPA.
35. The composition according to claim 34, wherein LBPA is selected from the group consisting of 3,3'-R,S-LBPA, 3,3'-R,R-LBPA, S,S-2,2'-C18:0-ether-LBPA, S,R-Hemi-LBPA, S,S-2,2'-C12:0-ether-LBPA, 3,3'-C16:0-LBPA, sn-[2,3-dioleoyl]-glycerol-1-phospho-sn-1'-[2',3'-dioleoyl]-glycerol (S,S-BDP), 18:1 (Δ^9 -Cis)PG 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG), 1-oleoyl-2-hydroxy-sn-glycero-3-phospho-(1'-rac-glycerol) (lysoPG) and 3,3'-C14:0-LBPA.
36. The composition according to any of the preceding claims, wherein the composition comprises (e) a steroid and/or a ceramide selected from the group consisting of cholesterol, solasodine, ceramide, diosgenin, DOPE, sphingomyelin, and mixtures thereof, preferably selected from the group consisting of cholesterol, diosgenin, solasodine, ceramide, and mixtures thereof, more preferably selected from the group consisting of cholesterol, solasodine and a mixture thereof.
37. The composition according to any one of the preceding claims, wherein the composition comprises:
 - iv) between 2.5 and 35 mol percent of at least one neutral lipid, wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

38. The composition according to any one of the preceding claims, wherein the composition comprises:
- iv) between 2.5 and 35 mol percent of at least one neutral lipid selected from the group consisting of cholesterol, diosgenin, solasodine, and ceramide,
- wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.
39. The composition according to any one of the preceding claims, wherein the therapeutic agent is selected from the group consisting of a drug, a protein, a peptide, a gene, an oligonucleotide, a compound, an antibody, a nanobody, a nucleic acid, a ribonucleoprotein, a small molecule, a non-lipinski molecule, a biomimetic, or a natural compound.
40. The composition according to any one of the preceding claims, wherein the therapeutic agent is a CRISPR/CAS9 ribonucleoprotein, a CRISPR guide RNA or a CRISPR associated Cas protein.
41. The drug delivery system according to any one of the claims 1 to 39, wherein the therapeutic agent is a nucleic acid selected from the group consisting of ssDNA, dsDNA, ssRNA, dsRNA, aiRNA, miRNA, siRNA, piRNA, sdRNA, snRNA, snoRNA, PAR, tsRNA, endo-siRNA, MSY-RNA, tel-sRNA, crasiRNA, moRNA, xiRNA, lncRNA, ncRNA, circRNA, mRNA, sgRNA, crRNA, tracr RNA, guide RNA mix, self-amplifying RNA, ribozymes, and antisense oligonucleotides.
42. The composition according to any one of the claims 1 to 39, wherein the therapeutic agent is selected from the group consisting of a CRISPR/CAS9 ribonucleoprotein, a siRNA, a mRNA, a guide RNA mix or a protein.
43. The composition according to any one of the preceding claims, wherein the therapeutic agent is an anionic therapeutic agent.
44. The composition according to any one of the claims 1 to 43 comprising
- as component (b) α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- as component (c) 2,2'-S,S-bis(monooleoylglycerol)phosphate or 3,3'-S,S-bis(monooleoylglycerol)phosphate;
- as component (d) 1,2-dioleoyl-3-trimethylammonium propane chloride and/or PD740; and
- as component (e) cholesterol and/or solasodine.
45. The composition according to any one of the claims 1 to 43 comprising

- as component (b) α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- as component (c) 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate;
- as component (d) 1,2-dioleoyl-3-trimethylammonium propane chloride; and
- as component (e) cholesterol.
46. The composition according to any one of the claims 1-4, 9-35, 37 or 39-43 comprising
- as component (b) α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- as component (c) 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate; and
- as component (d) 1,2-dioleoyl-3-trimethylammonium propane chloride.
47. The composition according to any one of the claims 1 to 44 comprising
- as component (b) between 1 and 20 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅,
- wherein said mol percent is calculated taking the total moles of all components except component as component (a) that are comprised in said composition as 100%.
48. The composition according to any one of the claims 1 to 47 comprising
- as component (b) between 1 and 20 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- as component (c) between 15 and 45 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate;
- as component (d) between 30 and 75 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride and/or PD740; and
- as component (e) between 13 and 35 mol percent of cholesterol;
- wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.
49. The composition according to any one of the claims 1 to 43 comprising
- as component (b) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
- as component (c) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate;
- as component (d) 50 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride; and
- as component (e) 25 mol percent of cholesterol,

wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

50. The composition according to any one of the claims 1-4, 9-35, 37 or 39-43 comprising
 - as component (b) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
 - as component (c) 45 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate; and
 - as component (d) 50 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride,
 wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

51. The composition according to any one of the claims 1 to 43 comprising
 - as component (b) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
 - as component (c) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate;
 - as component (d) 50 mol percent of PD740; and
 - as component (e) 25 mol percent of cholesterol;
 wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

52. The composition according to any one of the claims 1 to 43 comprising
 - as component (b) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
 - as component (c) 20 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate;
 - as component (d1) 25 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride;
 - as component (d2) 25 mol percent of PD740; and
 - as component (e) 25 mol percent of cholesterol;
 wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

53. The composition according to any one of the claims 1 to 43 comprising
 - as component (b) 7 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;

as component (c) 27 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate;
 as component (d) 33 mol percent of PD740; and
 as component (e) 33 mol percent of cholesterol; wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

54. The composition according to any one of the claims 1 to 43 comprising
 as component (b) between 4 and 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
 as component (c) between 4 and 15 mol percent of 2,2'-S,S-bis(monooleoylglycerol)phosphate or of 3,3'-S,S-bis(monooleoylglycerol)phosphate;
 as component (d) between 42 and 50 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride;
 as component e: component (e1) between 13 and 15 mol percent of cholesterol; and
 component (e2) between 15 and 36 mol percent of solasodine;
 wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.
55. The composition according to any one of the claims 1 to 43 comprising
 as component (b) α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
 as component (c) monogalactosyldiacylglycerol or 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine;
 as component (d) 1,2-dioleoyl-3-trimethylammonium propane chloride and PD740; and
 as component (e) cholesterol.
56. The composition according to any one of the claims 1 to 43 comprising
 as component (b) 5 mol percent of α -methoxy- ω -{N-(2-methyl-2-octadecyloxy-[1,3]dioxan-5-yl)-amido}-polyethylene glycol₄₅;
 as component (c) 20 mol percent of monogalactosyldiacylglycerol or 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine;
 as component (d): component (d1) 25 mol percent of 1,2-dioleoyl-3-trimethylammonium propane chloride; and component (d2) 25 mol percent of PD740; and
 as component (e) 25 mol percent of cholesterol,
 wherein said mol percent is calculated taking the total moles of all components except component (a) that are comprised in said composition as 100%.

57. The composition according to any one of the preceding claims, wherein the N:P ratio is from about 1:1 to about 50:1.
58. The composition according to any one of claims 1 to 57, wherein the composition is a vaccine formulation.
59. The composition according to any one of claims 1 to 57, wherein the composition is a drug delivery system.
60. A pharmaceutical composition comprising the composition according to any one of claims 1 to 41.
61. A method of producing the composition according to any one of claims 1 to 59, or the pharmaceutical composition according to claim 60, comprising the steps of:
 - (i) combining and mixing a composition comprising the components (b)-(d) and optionally (e) as defined in any one of claims 1 to 36 or 42 to 59; and
 - (ii) mixing the composition obtained in step (i) with the therapeutic agent or with the pharmaceutically acceptable salt thereof (a).
62. The composition according to any one of claims 1 to 59 or the pharmaceutical composition according to claim 60 for use in the treatment or prophylaxis of a disease.
63. A method of treatment or prophylaxis in a subject, comprising administering to the subject the composition according to any one of claims 1 to 59 or the pharmaceutical composition according to claim 60 in a therapeutically effective amount.
64. The composition or the pharmaceutical composition for use according to claim 62 or the method of treatment according to claim 63, wherein the treatment or prophylaxis of a disease is a vaccination against said disease.
65. The composition or the pharmaceutical composition for use according to claim 62 or the method of treatment according to claim 63, wherein the disease is selected from the group consisting of infectious diseases, cancer, and genetic defects and diseases.
66. The composition according to any one of claims 1 to 59, or the pharmaceutical composition according to claim 60 for use in the manufacture of a medicament.
67. A kit of parts comprising

- (i) the composition according to any one of claims 1 to 59 or the pharmaceutical composition according to claim 60; and
 - (ii) an instruction manual.
65. A composition comprising an artificial nucleic acid molecule, wherein the artificial nucleic acid molecule is an mRNA molecule having at least one open reading frame encoding an antigen derived from a pathogen, a therapeutic protein, or a CRISPR/CAS9 ribonucleoprotein, for use as a medicament, for use as a vaccine or for use in gene therapy, wherein the artificial nucleic acid molecule is associated with or complexed with components (b)-(d) and optionally (e) as defined in any one of claims 1 to 38 or 44 to 59, and wherein the composition is administered intramuscularly, intravenously, subcutaneously, intratumorally, orally, by inhalation or transdermally/topical.
68. A method of delivering an siRNA, an mRNA and/or protein into a target cell, comprising the step of
- (i) contacting the target cell with a composition as defined in any one of claims 1 to 59, wherein component (a) is said siRNA, mRNA and/or protein.
69. A method of transfecting a cell, wherein the cell is selected from the group consisting of epithelial cells, macrophages, hepatocytes and cardiomyocytes, the method comprising the step of contacting the cell with the composition according to any one of claims 1 to 59.