

LIPID NANOPARTICLES

FIELD OF THE INVENTION

The present invention relates to the field of lipid nanoparticles (LNP); more specifically comprising an ionizable lipid, a phospholipid, a sterol, a PEG lipid and one or more nucleic acids. The LNP's of the present invention are characterized in having an ionizable lipid content of above 55 mol% and a ratio of cholesterol to phospholipid of 1 : 1 or less, such as about 1 : 2 or less. The present invention provides use of the LNP's for extrahepatic delivery of nucleic acid molecules, specifically mRNA; thereby making them highly suitable for use in the treatment of cancer, autoimmune diseases, genetic disorders, hematologic disorders, aging, fibrosis, neurological disorders, cardiovascular diseases or infectious diseases.

BACKGROUND TO THE INVENTION

One of the major challenges in the field of targeted delivery of biologically active substances is often their instability and low cell penetrating potential. This is specifically the case for the delivery of nucleic acid molecules, in particular (m)RNA molecules. Therefore, proper packaging is crucial for adequate protection and delivery. Hence, there is a continuous need for methods and compositions for packaging biologically active substances, such as nucleic acids.

In that respect, lipid-based nanoparticle compositions such as lipoplexes and liposomes have been used as packaging vehicles for biologically active substances to allow transport into cells and/or intracellular compartments. These lipid-based nanoparticle compositions typically comprise a mixture of different lipids such as cationic lipids, ionizable lipids, phospholipids, structural lipids (such as sterols or cholesterol), PEG (polyethylene glycol) lipids,... (as reviewed in Reichmuth et al., 2016).

Lipid based nanoparticles composed of a mixture of 4 lipids – a cationic or ionizable lipid, a phospholipid, a sterol and a PEGylated lipid – have been developed for the non-immunogenic delivery of siRNA and mRNA to the liver after systemic administration. While many of such lipid compositions are known in the art, the ones used in mRNA delivery *in vivo*, typically comprise an excess of cholesterol to phospholipid, such as the Onpattro-type formulation of ionizable lipid/phospholipid/cholesterol/PEG-lipid having a mol/mol ratio of 50/10/38.5/1.5, thus having a mol/mol ratio of cholesterol to phospholipid of about 4 : 1. It is further known that LNPs containing this ratio of lipids largely accumulates in liver (hepatic) tissues. The ability of LNPs to accumulate in organs and tissues beyond the liver, such as bone marrow and/or spleen, would greatly expand the clinical utility of these delivery systems. Accordingly, it was an object of the present invention to provide improved LNPs having increased spleen and bone marrow targeting over liver targeting.

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Although research in the LNP field is currently very active, only a few research groups have been investigating the impact of inverting the ratio of cholesterol to phospholipid, i.e. providing LNPs having an excess of phospholipid to cholesterol. For example in WO2023184038, LNPs are provided with ratio's of cholesterol to phospholipid of about 1 : 1 and about 1 : 2 in combination with rather low levels of ionizable lipid (max. 45 mol%) and a fixed amount of PEG lipid of 1.5 mol%. However, herein the researchers specifically looked at the effects of particular molar percentages of the different lipids, rather than to investigate the effect of the ratio of lipids. They concluded that extrahepatic delivery is increased when providing LNPs comprising a phospholipid at high molar concentrations (30 – 70 mol%) in combination with a low ionizable lipid content of 5 mol% to 50 mol%. Preferred combinations disclosed herein contain a phospholipid content of between 45 mol% and 55 mol% and an ionizable lipid content of less than 40 mol%. While various variants were prepared, only one low ionizable lipid containing LNP was tested (IcLNP) having a ratio of ionizable lipid/phospholipid/cholesterol/PEG-lipid having a mol/mol ratio of 27.4/50/21.1/1.5.

Also, Kawaguchi et al., 2023 (Journal of Pharmaceutical Sciences 112; p. 1401-1410), assessed liver targeting of various LNPs and concluded that after intramuscular or subcutaneous administration of mRNA-LNPs, protein expression in liver decreased if the cholesterol molar percentage decreased from 40 mol% to 20 mol% and 10 mol%. The various conditions tested used ratios of cholesterol to phospholipid ranging from about 1 : 4 to about 4 : 1 in combination with rather low levels of ionizable lipid (max. 52.5 mol%) and a fixed amount of PEG lipid of 1.5 mol%. In this study, again no specific analysis of the effect of the ratio of cholesterol to phospholipid was made.

WO2022251953 relates to LNPs having increased expression in target tissue such as spleen, bone marrow and/or liver, showing preferred liver over spleen and/or bone marrow targeting. Contrary to the aim of the present invention, WO2022251953 does not teach or suggest LNPs having preferred spleen and/or bone marrow targeting over liver targeting.

We have now surprisingly found that LNPs having rather high levels of ionizable lipids (i.e. at least 55 mol%) and a ratio of cholesterol to phospholipid of 1 : 1 or less results in increased targeting of LNPs to both spleen and bone marrow. Even further increased bone marrow targeting is obtained when one or more of the following conditions are complied with: 1) the ratio of cholesterol to phospholipid of about 1 : 2 or less, 2) the mol% of ionizable lipid is at least 60 mol% and/or 3) the mol% of PEG-lipid is above 1.5 (e.g. 3.0 mol%).

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a lipid nanoparticle (LNP) comprising an ionizable lipid, a phospholipid, a sterol, a stabilizing lipid; and one or more nucleic acid molecules;

5 characterized in that:

- said LNP comprises at least 55 mol% of said ionizable lipid; and
- the ratio of sterol to phospholipid is 1 : 1 or less.

Specifically, the present invention provides a lipid nanoparticle (LNP) comprising an ionizable lipid, a phospholipid, a sterol, a PEG lipid; and one or more nucleic acid molecules;

10 characterized in that:

- said LNP comprises at least 55 mol% of said ionizable lipid; and
- the ratio of sterol to phospholipid is 1 : 1 or less.

15 In particular, the present invention provides a lipid nanoparticle (LNP) comprising an ionizable lipid, a phospholipid, cholesterol, a PEG lipid; and one or more nucleic acid molecules;

characterized in that:

- said LNP comprises at least 55 mol% of said ionizable lipid; and
- the ratio of cholesterol to phospholipid is 1 : 1 or less.

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In a specific embodiment, the lipid nanoparticle (LNP) as defined herein comprises a ratio of sterol to phospholipid of 1 : 2 or less, preferably a cholesterol to phospholipid of 1 : 2 or less.

In a further embodiment, the LNP of the invention comprises at least 0.1 mol% of said stabilizing lipid, in particular at least 0.5 mol% of said stabilizing lipid, at least 1.0 mol% of said stabilizing lipid, 1.5 mol% of said stabilizing lipid, in particular at least 2.0 mol% of stabilizing lipid, more in particular at least 2.5 mol% of said stabilizing lipid, at least 3.0 mol% of said stabilizing lipid, at least 4.0 mol% of said stabilizing lipid, at least 5.0 mol% of said stabilizing lipid, at least 6.0 mol% of said stabilizing lipid, at least 7.0 mol% of said stabilizing lipid, at least 8.0 mol% of said stabilizing lipid, at least 9.0 mol% of said stabilizing lipid, at least 10.0 mol% of said stabilizing lipid.

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In a further embodiment, the LNP of the invention comprises at least 0.1 mol% of said PEG lipid, in particular at least 0.5 mol% of said PEG lipid, at least 1.0 mol% of said PEG lipid, at least 1.5 mol% of said PEG lipid, in particular at least 2.0 mol% of PEG lipid, more in particular at least 2.5 mol% of said PEG lipid, at least 3.0 mol% of said PEG lipid, at least 4.0 mol% of said PEG lipid, at least 5.0 mol% of said PEG lipid, at least 6.0 mol% of said PEG lipid, at least 7.0 mol% of said PEG lipid, at least 8.0 mol% of said PEG lipid, at least 9.0 mol% of said PEG lipid, at least 10.0 mol% of said PEG lipid.

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In another particular embodiment, the molar percentage of the ionizable lipid in the LNPs of the invention is at least 60 mol%, such as at least 65 mol%, at least 70 mol%, at least 75 mol%, at least 80 mol% or at least 85 mol%.

5 In yet a further embodiment, the molar percentage of the phospholipid in the LNPs of the invention is above 1 mol%, in particular between 2 mol% and 30 mol%, more in particular between 5 mol% and 28 mol%; more in particular between 10 mol% and 27 mol%; even more in particular between 20 mol% and 26 mol%.

10 In a further embodiment, the molar percentage of the sterol, preferably cholesterol, in the LNPs of the invention is below 22 mol%, in particular between 10 mol% and 15 mol%, more in particular between 12 mol% and 13 mol%.

In a further embodiment, the PEG lipid in the LNPs of the invention is selected from the list
15 comprising DMG-PEG and DSG-PEG; in particular DMG-PEG.

In yet a further embodiment, the present invention provides a lipid nanoparticle as defined herein, wherein said phospholipid is selected from the list comprising: DOPE, DOPC, DSPC, ESM and mixtures thereof; in particular DSPC.

20 In a further embodiment, the present invention provides a lipid nanoparticle as defined herein, wherein said sterol is selected from the list comprising cholesterol, ergosterol, campesterol, oxysterol, antrosterol, cholecalciferol, desmosterol, nicasterol, sitosterol and stigmasterol; preferably cholesterol.

25 In a very specific embodiment, the present invention provides an LNP comprising:

- about 60 mol% of said ionizable lipid;
- about 25.67 mol% of DSPC;
- about 12.83 mol% of cholesterol; and
- 30 - about 1.5 mol% of DMG-PEG.

In yet a further specific embodiment, the present invention provides an LNP comprising:

- about 60 mol% of said ionizable lipid;
- about 24.17 mol% of DSPC;
- 35 - about 12.83 mol% of cholesterol; and
- about 3.0 mol% of DMG-PEG.

In yet a further aspect, the present invention provides a pharmaceutical composition or a vaccine comprising one or more lipid nanoparticles as defined herein and an acceptable pharmaceutical

carrier.

The present invention also provides the lipid nanoparticles, pharmaceutical compositions or vaccines as defined herein for use in human or veterinary medicine; in particular for use in the treatment of cancer, autoimmune diseases, genetic disorders, hematological disorders, aging, fibrosis, neurological disorders, cardiovascular diseases or infectious diseases.

The invention further provides a pharmaceutical composition or a vaccine comprising one or more lipid nanoparticles as defined herein for use in increasing gene expression of the nucleic acid molecules *in vivo* in spleen and/or bone marrow.

Finally, the present invention provides a method for *in vivo* delivery of nucleic acid molecules to a subject in need thereof, the method comprising: administering to the subject a lipid nanoparticle or pharmaceutical composition as defined herein.

BRIEF DESCRIPTION OF THE DRAWINGS

With specific reference now to the figures, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the different embodiments of the present invention only. They are presented in the cause of providing what is believed to be the most useful and readily description of the principles and conceptual aspects of the invention. In this regard no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention. The description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

Fig. 1. Spleen/Liver ratio of mRNA expression upon intravenous injection of Fluc mRNA LNPs with different molar ratios of ionizable lipid, phospholipid, sterol and PEG-lipid. The condition with a cholesterol to phospholipid ratio of 3.9 : 1 represents a LNP that has a standard lipid molar ratio as used in the Onpattro formulation (INL1 / DSPC / Chol / DMG-PEG2000 = 50 / 10 / 38.5 / 1.5).

Fig. 2. Luciferase activity in bone marrow upon intravenous injection of Fluc mRNA LNPs with different molar ratios of ionizable lipid, phospholipid, sterol and PEG-lipid. The condition with a cholesterol to phospholipid ratio of 3.9 : 1 represents a LNP that has a standard lipid molar ratio as used in the Onpattro formulation.

Fig. 3. Spleen/Liver ratio of mRNA expression upon intravenous injection of Fluc mRNA LNPs with different molar ratios of ionizable lipid, phospholipid, sterol and PEG-lipid. LNP 5 represents a LNP with a cholesterol to phospholipid ratio of 3.9 : 1 and has a standard lipid molar ratio as used in the Onpattro formulation.

Fig. 4. Luciferase activity in bone marrow upon intravenous injection of Fluc mRNA LNPs with different molar ratios of ionizable lipid, phospholipid, sterol and PEG-lipid. LNP 5 represents a LNP with a cholesterol to phospholipid ratio of 3.9 : 1 and has a standard lipid molar ratio as used in the Onpattro formulation.

Fig. 5. Luciferase activity in bone marrow upon intravenous injection of Fluc mRNA LNPs with different molar ratios of ionizable lipid, phospholipid, sterol and PEG-lipid. LNP 5 represents a LNP with a cholesterol-to-phospholipid ratio of 3.9 : 1 and has a standard lipid molar ratio as used in the Onpattro formulation.

Fig. 6. displays the spleen/liver ratio of mRNA expression upon intravenous injection of Fluc mRNA LNPs with molar ratios of ionizable lipid, phospholipid, sterol and PEG-lipid as displayed in Table 4. A total of 5 different ionizable lipids have been used and formulations with a cholesterol-to-phospholipid ratio of 3.9 : 1 have a standard lipid molar ratio as used in the Onpattro formulation.

Fig. 7. displays the spleen/liver ratio of mRNA expression upon intravenous injection of Fluc mRNA LNPs with different molar ratios of ionizable lipid, phospholipid, sterol and PEG-lipid as displayed in Table 5. Two different ionizable lipids have been used in the formulation, and formulations displaying a cholesterol-to-phospholipid ratio of 3.9 : 1 have a standard lipid molar ratio as used in the Onpattro formulation.

Fig. 8. displays the amount of Thy1.1 positive cells in a certain liver cell type 16h after IV injection as determined via flow cytometry, exemplified for INL 2 containing formulations. The formulation with a cholesterol-to-phospholipid ratio of 3.9 : 1 has a standard lipid molar ratio as used in the Onpattro formulation.

Fig. 9. shows the ratio of the number of Thy1.1 positive splenic CD4+ T cells / CD8+ T cells / macrophages to the number of Thy1.1 positive hepatocytes following an intravenous injection of Thy1.1 mRNA LNPs with different molar ratios of ionizable lipid, phospholipid, sterol and PEG-lipid. Two different ionizable lipids have been used in the formulation, and formulations displaying a cholesterol-to-phospholipid ratio of 3.9 : 1 have a standard lipid molar ratio as used in the Onpattro formulation.

DETAILED DESCRIPTION OF THE INVENTION

The description and drawings merely illustrate the principles of the present invention. It will thus be appreciated that those skilled in the art will be able to devise various arrangements that, although not explicitly described or shown herein, embody the principles of the present invention and are included within its scope. Furthermore, all examples recited herein are principally intended expressly to be only for pedagogical purposes to aid the reader in understanding the principles of the present invention and the concepts contributed by the inventor(s) to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the present invention, as well as specific examples thereof, are intended to encompass equivalents thereof.

As used in the specification and the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. By way of example, "a, RNA polynucleotide" means one RNA polynucleotide or more than one RNA polynucleotide. The terms "comprising", "comprises" and "comprised of" as used herein are synonymous with "including", "includes" or "containing", "contains", and are inclusive or open-ended and do not exclude additional, non-recited members, elements, or method steps. The recitation of numerical ranges by endpoints includes all numbers and fractions subsumed within the respective ranges, as well as the recited endpoints.

The term "about" or "approximately" as used herein when referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, is meant to encompass variations of +/-10% or less, such as +/-5% or less from the specified value, insofar such variations are appropriate to perform the disclosed invention. It is to be understood that the value to which the modifier "about" or "approximately" refers is itself also specifically, and preferably, disclosed. Accordingly, where a ratio of 1 : 2 is specified, this also includes slight variations of such ratio, with a 10% variation meaning between 1 : 1.8 and 1 : 2.2 or with a 5 % variation meaning between 1 : 1.9 and 1 : 2.1. Similarly, a ratio of 1 : 1 is meant to include a 10% variation of between 1 : 0.9 and 1 : 1.1 or with a 5 % variation meaning between 1 : 0.95 and 1 : 1.05.

Whereas the term "one or more", such as one or more members of a group of members, is clear per se, by means of further exemplification, the term encompasses inter alia a reference to any one of said members, or to any two or more of said members, such as, e.g., any ≥ 3 , ≥ 4 , ≥ 5 , ≥ 6 or ≥ 7 etc. of said members, and up to all said members.

Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment, but may. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to a person skilled in the art from this disclosure, in one or more embodiments. Furthermore, while some embodiments described herein include some but not other features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the invention, and form different embodiments, as would be understood by those in the art. For example, any of the disclosed embodiments and/or claims can be used in any combination.

All documents cited in the present specification are hereby incorporated by reference in their entirety.

Unless otherwise specified, all terms used in disclosing the invention, including technical and scientific terms, have the meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. By means of further guidance, term definitions may be included to better appreciate the teaching of the present invention.

As already detailed herein above, the present invention provides a lipid nanoparticle (LNP) comprising an ionizable lipid, a phospholipid, a sterol, a stabilizing lipid; and one or more nucleic acid molecules;

characterized in that:

- said LNP comprises at least 55 mol% of said ionizable lipid; and
- the ratio of sterol to phospholipid is 1 : 1 or less.

As already detailed herein above, the present invention provides a lipid nanoparticle (LNP) comprising an ionizable lipid, a phospholipid, a sterol, a PEG lipid; and one or more nucleic acid molecules;

characterized in that:

- said LNP comprises at least 55 mol% of said ionizable lipid; and
- the ratio of sterol to phospholipid is 1 : 1 or less.

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In particular, the present invention provides a lipid nanoparticle (LNP) comprising an ionizable lipid, a phospholipid, a cholesterol, a PEG lipid; and one or more nucleic acid molecules; characterized in that:

- said LNP comprises at least 55 mol% of said ionizable lipid; and

- the ratio of cholesterol to phospholipid is 1 : 1 or less.

For these LNPs specifically, as evidenced in the examples part, it was found that a significantly increased delivery of the cargo to extrahepatic tissues, such as spleen and/or bone marrow is achieved.

The lipid nanoparticle described herein comprises a cargo that is in particular a nucleic acid. A "nucleic acid" in the context of the invention is a deoxyribonucleic acid (DNA) or preferably a ribonucleic acid (RNA), more preferably mRNA. Nucleic acids include according to the invention genomic DNA, cDNA, mRNA, recombinantly produced and chemically synthesized molecules. A nucleic acid may according to the invention be in the form of a molecule which is single stranded or double stranded and linear or closed covalently to form a circle. A nucleic acid can be employed for introduction into, i.e. transfection of cells, for example, in the form of RNA which can be prepared by in vitro transcription from a DNA template. The RNA can moreover be modified before application by stabilizing sequences, capping, and/or polyadenylation.

In the context of the present invention, the term "RNA" relates to a molecule which comprises ribonucleotide residues and preferably being entirely or substantially composed of ribonucleotide residues. "Ribonucleotide" relates to a nucleotide with a hydroxyl group at the 2'-position of a β -D-ribofuranosyl group. The term includes double stranded RNA, single stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as modified RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of a RNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in RNA molecules can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs. Nucleic acids may be comprised in a vector. The term "vector" as used herein includes any vectors known to the skilled person including plasmid vectors, cosmid vectors, phage vectors such as lambda phage, viral vectors such as adenoviral or baculoviral vectors, or artificial chromosome vectors such as bacterial artificial chromosomes (BAC), yeast artificial or analogs of naturally-occurring RNA. The term RNA is also meant to include, but is not limited to, small activating RNA (saRNA) and trans-amplifying RNA (taRNA), but also includes other types of RNA such as circularRNA (circRNA), siRNA (small interfering RNA) gRNA (guide RNA), base editor RNA, and self-amplifying RNA.

According to the present invention, the term "RNA" includes and preferably relates to "mRNA" which means "messenger RNA" and relates to a "transcript" which may be produced using DNA as template and encodes a peptide or protein. mRNA typically comprises a 5' untranslated

region (5' -UTR), a protein or peptide coding region and a 3' untranslated region (3'-UTR). mRNA has a limited half-life in cells and in vitro. Preferably, mRNA is produced by in vitro transcription using a DNA template. In one embodiment of the invention, the RNA is obtained by in vitro transcription or chemical synthesis. The in vitro transcription methodology is known to the skilled person. For example, there is a variety of in vitro transcription kits commercially available.

In a specific embodiment of the present invention, said mRNA molecules are mRNA molecules encoding immune-modulating proteins. Such proteins may be immune-stimulating proteins, immune-tolerizing proteins or immune-suppressive proteins, depending on the specific application domain. In that aspect, the invention is in particular suitable for use in the treatment of cancer, autoimmune diseases, genetic disorders, hematological disorders, aging, fibrosis, neurological disorders, cardiovascular diseases or infectious diseases.

In the context of the present invention, the term "mRNA molecules encoding immune-modulating proteins" is meant to be mRNA molecules encoding proteins that regulate or modify the activity of the immune system, helping to maintain a balanced and appropriately responsive immune response. Immune-modulating proteins can have diverse effects on the immune system, including dampening excessive responses or promoting a more robust defense when needed. These proteins play a crucial role in ensuring that the immune system responds appropriately to various challenges, avoiding excessive inflammation or immune reactions that could harm the body's own tissues.

In the context of the present invention, the term "mRNA molecules encoding immune-stimulating proteins" is meant to be mRNA molecules encoding proteins that have the ability to enhance or activate the immune system, which is the body's defense mechanism against pathogens such as bacteria, viruses, and other harmful entities. These proteins can stimulate various components of the immune system, including antigen-presenting cells (e.g. dendritic cells) and other immune cells, leading to an increased and more effective immune response. Immune-stimulating proteins may be naturally occurring or can be engineered for therapeutic purposes, such as in the development of vaccines or immunotherapies. They play a crucial role in supporting the body's ability to recognize and eliminate foreign invaders, contributing to overall immune system health and functionality.

In the context of the present invention, the term "mRNA molecules encoding immune-tolerizing proteins" is meant to be mRNA molecules encoding proteins that induce or promote immune tolerance within the body. Immune tolerance is a crucial aspect of the immune system's function, as it involves the ability to recognize and tolerate the body's own cells and tissues while effectively responding to foreign invaders, such as pathogens. Proteins with immune-tolerizing properties help regulate the immune response to prevent the immune system from attacking its

own healthy cells, a phenomenon known as autoimmunity. These proteins can modulate immune cells and signaling pathways to foster a state of tolerance, preventing the development of inappropriate or exaggerated immune reactions against self-antigens. Immune-tolerizing proteins are of particular interest in the context of autoimmune diseases, where the immune system mistakenly targets and damages the body's own tissues.

In the context of the present invention, the term "mRNA molecules encoding immune-suppressive proteins" is meant to be mRNA molecules encoding proteins that suppress or inhibit the activity of the immune system. These proteins play a crucial role in modulating the immune response to prevent excessive reactions that can lead to tissue damage or autoimmune disorders. Immunosuppressive proteins are often used in medical settings to intentionally dampen immune activity, such as in the context of organ transplantation or the treatment of autoimmune diseases. These proteins may act by inhibiting the function of immune cells, disrupting signaling pathways involved in immune activation, or promoting a state of immune tolerance.

In another specific embodiment, said mRNA molecules are mRNA molecules encoding antigen-and/or disease-specific proteins. Alternatively, the mRNA molecules may encode proteins suitable for use in gene therapy, such as protein replacement therapy, enzyme replacement therapy, RNAi-based therapy, CRISPR-Cas based therapy, addition of transcription factors, generation of certain chemokines, immunomodulators and/or antisense oligonucleotide therapy.

According to the present invention, the term "antigen" comprises any molecule, preferably a peptide or protein, which comprises at least one epitope that will elicit an immune response and/or against which an immune response is directed; accordingly, the term antigen is also meant to encompass minimal epitopes from antigens. A "minimal epitope" as defined herein is meant to be the smallest structure which is capable of eliciting an immune response. Preferably, an antigen in the context of the present invention is a molecule which, optionally after processing, induces an immune response, which is preferably specific for the antigen or cells expressing the antigen. In particular, an "antigen" relates to a molecule which, optionally after processing, is presented by MHC molecules and reacts specifically with T lymphocytes (T cells).

In a specific embodiment, the antigen is a target-specific antigen which can be a tumor antigen, an auto-antigen, or a bacterial, viral or fungal antigen. Said target-specific antigen can be derived from either one of: total mRNA isolated from (a) target cell(s), one or more specific target mRNA molecules, protein lysates of (a) target cell(s), specific proteins from (a) target cell(s), or a synthetic target-specific peptide or protein and synthetic mRNA or DNA encoding a target-specific antigen or its derived peptides.

To avoid any misunderstanding, the LNP's of the present invention may comprise a single mRNA molecule, or they may comprise multiple mRNA molecules, such as a combination of one or more mRNA molecules encoding immune-modulating proteins and/or one or more mRNA molecules encoding antigen- and/or disease-specific proteins or proteins for use in gene therapy.

In a very specific embodiment, said mRNA molecules encoding immune-modulating proteins may be combined with one or more mRNA molecules encoding antigen- and/or disease-specific proteins or proteins for use in gene therapy.

As used herein, the term "nanoparticle" refers to any particle having a diameter making the particle suitable for systemic, in particular intravenous administration, of, in particular, nucleic acids, typically having a diameter of less than 1000 nanometers (nm), preferably less than 500 nm, even more preferably less than 200 nm, such as for example between 40 and 200 nm; preferably between 40 and 160 nm, preferably between 40 and 100 nm.

In a very specific embodiment, the nanoparticle of the present invention may be in the form of a lipid nanoparticle or lipid nanoparticle composition comprising a combination of lipids as defined herein. A lipid nanoparticle (LNP) is generally known as a nanosized particle composed of a combination of different lipids. While many different types of lipids may be included in such LNP, the LNP's of the present invention are typically composed of a combination of an ionizable lipid, a phospholipid, a sterol and a PEG lipid.

In the context of the present invention, the term lipid nanoparticle (LNP), also termed solid lipid nanoparticles (SLNP), is meant to be a nanoparticle comprising lipids. They are often used as a pharmaceutical drug delivery system or pharmaceutical formulation. LNPs as drug delivery vehicle were first approved in 2018 and are currently used in several candidate RNA based vaccines. A lipid nanoparticle is typically spherical with an average diameter between 10 and 1000 nanometers and possesses a lipid core matrix that can solubilize lipophilic molecules. The term lipid is used here in a broader sense and includes triglycerides, diglycerides, monoglycerides, fatty acids, steroids (e.g. cholesterol) and waxes. Biological membrane lipids such as phospholipids, sphingomyelins, bile acids and sterols are typically used as stabilizers in LNPs.

Accordingly, in the context of the present invention, the nanoparticles as disclosed herein further comprise one or more additional lipids either or not acting as stabilizers, such as helper lipids (e.g. a phospholipid), a sterol and/or a stabilizer lipids (such as PEGylated lipids and variants

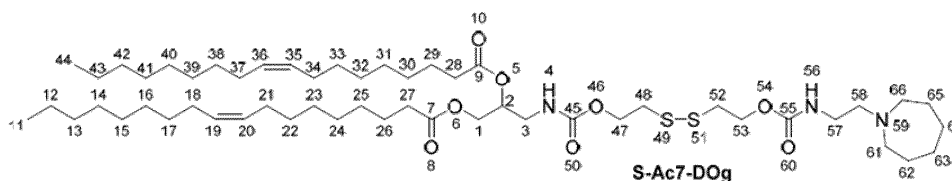
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thereof).

In the context of the present invention, the term lipid is meant to be a chemically defined substance that is insoluble in water but soluble in amongst others alcohol, ether and chloroform.

5 Ionizable or cationic lipids are lipids that are typically composed of three sections: an amine head group, a linker moiety and a hydrophobic tail. The term "ionizable" (or alternatively cationic) in the context of a compound or lipid means the presence of any uncharged group in said compound or lipid which is capable of becoming positively charged by receiving an ion (usually an H⁺ ion). Alternatively, any uncharged group in said compound or lipid may receive an electron
10 and thus becoming negatively charged.

In the context of the present invention any type of ionizable lipid can suitably be used. In a very specific embodiment, suitable ionizable lipids are as defined in any one of WO2022136641, PCT/EP2024/069656 or WO2024084056. In a very specific embodiment, the ionizable lipid is
15 S-Ac7-DOg:



Hence, in a specific embodiment, the present invention provides a lipid nanoparticle comprising:

- an ionizable lipid being S-Ac7-Dog
 - a phospholipid;
 - 20 - a sterol;
 - a PEG lipid; and
 - one or more nucleic acid molecules; in particular nucleic acid molecules;
- characterized in that:
- said LNP comprises at least 55 mol% of said ionizable lipid; and
 - 25 - the ratio of cholesterol to phospholipid is 1:1 or less.

In the context of the present invention, the term "phospholipid" is meant to be a helper lipid molecule consisting of two hydrophobic fatty acid "tails" and a hydrophilic "head" consisting of a phosphate groups. The two components are most often joined together by a glycerol molecule,
30 hence, the phospholipid of the present invention is preferably a glycerol-phospholipid. Furthermore, the phosphate group is often modified with simple organic molecules such as choline (i.e. rendering a phosphocholine) or ethanolamine (i.e. rendering a phosphoethanolamine).

Suitable helper lipids or phospholipids within the context of the invention can be selected from the list comprising: 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-*sn*-glycero-phosphocholine (DMPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-*sn*-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-di-O-octadecenyl-*sn*-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-*sn*-glycero-3-phosphocholine (OchemsPC), 1-hexadecyl-*sn*-glycero-3-phosphocholine (C 16 Lyso PC), 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine, 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphocholine, 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) sodium salt (DOPG), egg sphingomyelin (ESM), and mixtures thereof. The phospholipid may also be an anionic phospholipid, such as but not limited to *sn*-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-*sn*-1'-(3'-oleoyl-2'-hydroxy)-glycerol (BMP), 1,2-distearoyl-*sn*-glycero-3-phosphate; or 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), or salts thereof.

In a more specific embodiment, said helper lipid or phospholipid is selected from the list comprising: 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), egg sphingomyelin (ESM) and mixtures thereof; in particular DSPC.

In a specific embodiment, the present invention provides a lipid nanoparticle comprising:

- an ionizable lipid;
 - a phospholipid being DSPC;
 - a sterol;
 - a PEG lipid; and
 - one or more nucleic acid molecules; in particular nucleic molecules;
- characterized in that:
- said LNP comprises at least 55 mol% of said ionizable lipid; and
 - the ratio of cholesterol to phospholipid is 1 : 1 or less.

Hence, in a specific embodiment, the present invention provides a lipid nanoparticle comprising:

- an ionizable lipid being S-Ac7-Dog

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- a phospholipid being DSPC;
- a sterol;
- a PEG lipid; and
- one or more nucleic acid molecules; in particular nucleic molecules;

5 characterized in that:

- said LNP comprises at least 55 mol% of said ionizable lipid; and
- the ratio of cholesterol to phospholipid is 1 : 1 or less.

10 In the context of the present invention, the term "sterol", also known as steroid alcohol, is a subgroup of steroids that occur naturally in plants, animal and fungi, or can be produced by some bacteria. In the context of the present invention, any suitable sterol may be used, such as selected from the list comprising cholesterol, ergosterol, campesterol, oxysterol, antrosterol, cholecalciferol, desmosterol, nicasterol, sitosterol and stigmasterol; preferably cholesterol.

15 In another specific embodiment, the present invention provides a lipid nanoparticle comprising:

- an ionizable lipid;
- a phospholipid being DSPC;
- a sterol being cholesterol;
- a PEG lipid; and
- 20 - one or more nucleic acid molecules; in particular mRNA molecules;

characterized in that:

- said LNP comprises at least 55 mol% of said ionizable lipid; and
- the ratio of cholesterol to phospholipid is 1 : 1 or less.

25 Hence, in a specific embodiment, the present invention provides a lipid nanoparticle comprising:

- an ionizable lipid being S-Ac7-Dog
- a phospholipid being DSPC;
- a sterol being cholesterol;
- a PEG lipid; and
- 30 - one or more nucleic acid molecules; in particular mRNA molecules;

characterized in that:

- said LNP comprises at least 55 mol% of said ionizable lipid; and
- the ratio of cholesterol to phospholipid is 1 : 1 or less.

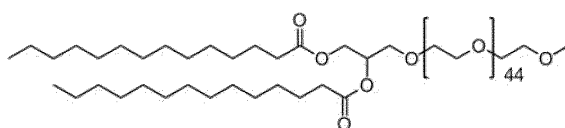
35 In the context of the present invention, the term "stabilizing lipid" is meant to be a component that helps maintain the structural integrity and stability of the lipid nanoparticles. Stabilizing lipids in the context of the invention, may for example be PEGylated lipids and variants thereof, sphingolipids and glycerolipids.

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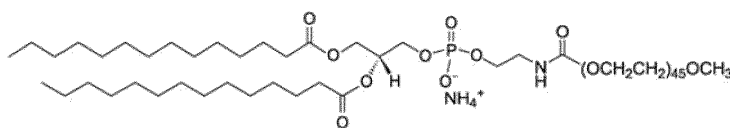
In the context of the present invention, the term “PEG lipid” or alternatively “PEGylated lipid” is meant to be any suitable lipid modified with a PEG (polyethylene glycol) group. The PEG lipids of the present invention may be C14-PEG lipids, C16-PEG lipids or C18-PEG lipids.

C14-PEG lipids contain a polyethylene glycol moiety, which defines the molecular weight of the lipids, as well as a fatty acid tail comprising 14 C-atoms. In a particular embodiment, said C14-PEG lipid is a diC14-PEG lipid, i.e. lipids having 2 C14 lipid tails. In a further embodiment, said diC14-PEG lipid is based on dimyristoyl, i.e. having 2 C14 tails, such as selected from the list comprising: a (dimyristoyl-based)-PEG2000 lipid such as DMG-PEG2000 lipid (1,2-dimyristoyl-*rac*-glycero-3-methoxypolyethylene glycol-2000) or 2-Dimyristoyl-*sn*-Glycero-3-Phosphoethanolamine glycol-2000 (DMPE-PEG2000).

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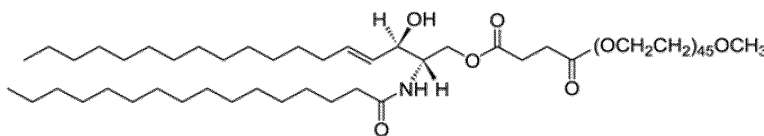
DMG-PEG2000



DMPE-PEG2000

C16-PEG lipids contain a polyethylene glycol moiety, which defines the molecular weight of the lipids, as well as a fatty acid tail comprising 16 C-atoms. In a particular embodiment, said C16-PEG lipid is a diC16-PEG lipid, i.e. lipids having 2 C16 lipid tails. In a particular embodiment, said diC16-PEG lipid is for example: N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]} (C16 PEG2000 Ceramide)

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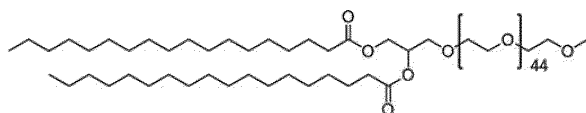


C16 PEG 2000 Ceramide

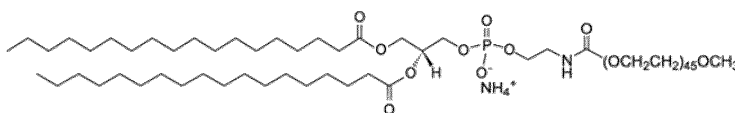
C18-PEG lipids contain a polyethylene glycol moiety, which defines the molecular weight of the lipids, as well as a fatty acid tail comprising 18 C-atoms. In a particular embodiment, said C18-PEG lipid is a diC18-PEG lipid, i.e. lipids having 2 C18 lipid tails. In a particular embodiment,

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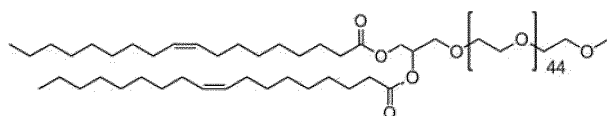
said diC18-PEG lipid is selected from the list comprising: a (distearoyl-based)-PEG lipid such as DSG-PEG2000 lipid (2-distearoyl-*rac*-glycero-3-methoxypolyethylene glycol-2000) or DSPE-PEG2000 lipid (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]); or a (dioleoyl-based)-PEG2000 lipid such as DOG-PEG2000 lipid (1,2-Dioleoyl-*rac*-glycerol) or DOPE-PEG2000 lipid (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000])



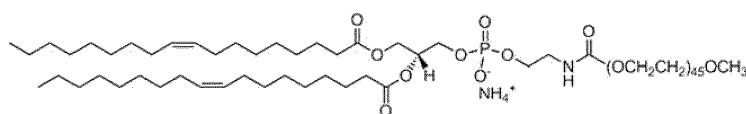
DSG-PEG2000



DSPE-PEG2000



DOG-PEG2000



DOPE-PEG2000

In a very specific embodiment, the PEG-lipid is DMG-PEG.

20

Hence, in a specific embodiment, the present invention provides a lipid nanoparticle comprising:

- an ionizable lipid;
- a phospholipid being DSPC;
- a sterol being cholesterol;
- a PEG lipid being DMG-PEG; and
- one or more nucleic acid molecules; in particular mRNA molecules;

25

characterized in that:

- said LNP comprises at least 55 mol% of said ionizable lipid; and
- the ratio of cholesterol to phospholipid is 1 : 1 or less.

30

Hence, in a specific embodiment, the present invention provides a lipid nanoparticle comprising:

- an ionizable lipid being S-Ac7-Dog
- a phospholipid being DSPC;
- a sterol being cholesterol;

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- a PEG lipid being DMG-PEG; and
- one or more nucleic acid molecules; in particular mRNA molecules;

characterized in that:

- said LNP comprises at least 55 mol% of said ionizable lipid; and
- 5 - the ratio of cholesterol to phospholipid is 1 : 1 or less.

We have moreover found that the extra hepatic delivery of LNPs of the invention may be further increased if one or more of the following conditions are complied with: 1) the ratio of cholesterol to phospholipid of 1 : 2 or less, 2) the mol% of ionizable lipid is at least 60 mol% and/or 3) the
10 mol% of PEG-lipid is above 1.5 (e.g. 3.0 mol%). Especially, these conditions result in increased bone marrow delivery as evidenced in the examples part.

Accordingly, in a specific embodiment, one or more of the following conditions applies:

- 15 - the lipid nanoparticle (LNP) as defined herein comprises a ratio of cholesterol to phospholipid of 1 : 1 or less, in particular 1:2 or less,
- the LNP of the invention comprises at least 1.5 mol% of PEG lipid, in particular at least 2.0 mol% of PEG lipid, more in particular at least 2.5 mol% of said PEG lipid, such as about 3.0 mol% of said PEG lipid, and/or
- 20 - the molar percentage of the ionizable lipid in the LNPs of the invention is at least 55 mol%; in particular at least 60 mol%,
- the molar percentage of the phospholipid in the LNPs of the invention is above 1 mol%, in particular between 2 mol% and 30 mol%, more in particular between 5 mol% and 28 mol%; more in particular between 10 mol% and 27 mol%; even more in particular between 20 mol% and 26 mol%.
- 25 - the molar percentage of the cholesterol in the LNPs of the invention is below 22 mol%, in particular between 10 mol% and 15 mol%, more in particular between 12 mol% and 13 mol%.

Accordingly, the present invention provides an LNP comprising a ratio of cholesterol to
30 phospholipid of 1 : 1 or less, such as equal to or below 1 : 1.1, 1 : 1.2, 1 : 1.3, 1 : 1.4, 1 : 1.5, 1 : 1.6, 1 : 1.7, 1 : 1.8, 1 : 1.9, 1 : 2, 1 : 2.1, 1 : 2.2, 1 : 2.3, 1 : 2.4, 1 : 2.5, 1 : 2.6, 1 : 2.7, 1 : 2.8, 1 : 2.9, 1 : 3, 1 : 3.1, 1 : 3.2, 1 : 3.3, 1 : 3.4, 1 : 3.5, 1 : 3.6, 1 : 3.7, 1 : 3.8, 1 : 3.9, 1 : 4, 1 : 4.1, 1 : 4.2, 1 : 4.3, 1 : 4.4, 1 : 4.5, 1 : 4.6, 1 : 4.7, 1 : 4.8, 1 : 4.9, 1 : 5, 1 : 5.1, 1 : 5.2, 1 : 5.3, 1 : 5.4, 1 : 5.5, 1 : 5.6, 1 : 5.7, 1 : 5.8, 1 : 5.9, 1 : 6, 1 : 6.1, 1 : 6.2, 1 : 6.3, 1 : 6.4, 1 : 6.5, 1 : 6.6, 1 : 6.7,
35 1 : 6.8, 1 : 6.9, 1 : 7, 1 : 7.1, 1 : 7.2, 1 : 7.3, 1 : 7.4, 1 : 7.5, 1 : 7.6, 1 : 7.7, 1 : 7.8, 1 : 7.9, 1 : 8, 1 : 8.1, 1 : 8.2, 1 : 8.3, 1 : 8.4, 1 : 8.5, 1 : 8.6, 1 : 8.7, 1 : 8.8, 1 : 8.9, 1 : 9, 1 : 9.1, 1 : 9.2, 1 : 9.3, 1 : 9.4, 1 : 9.5, 1 : 9.6, 1 : 9.7, 1 : 9.8, 1 : 9.9, 1 : 10.

In a further embodiment, the LNP of the invention comprises at least 0.1 mol% of said stabilizing

lipid, in particular at least 0.5 mol% of said stabilizing lipid, at least 1.0 mol% of said stabilizing lipid, 1.5 mol% of said stabilizing lipid, in particular at least 2.0 mol% of stabilizing lipid, more in particular at least 2.5 mol% of said stabilizing lipid, at least 3.0 mol% of said stabilizing lipid, at least 4.0 mol% of said stabilizing lipid, at least 5.0 mol% of said stabilizing lipid, at least 6.0 mol% of said stabilizing lipid, at least 7.0 mol% of said stabilizing lipid, at least 8.0 mol% of said stabilizing lipid, at least 9.0 mol% of said stabilizing lipid, at least 10.0 mol% of said stabilizing lipid.

The present invention further provides an LNP comprising at least 0.1 mol% of PEG lipid, at least 0.2 mol% of PEG lipid, at least 0.3 mol% of PEG lipid, at least 0.4 mol% of PEG lipid, at least 0.5 mol% of PEG lipid, at least 0.6 mol% of PEG lipid, at least 0.7 mol% of PEG lipid, at least 0.8 mol% of PEG lipid, at least 0.9 mol% of PEG lipid, at least 1.0 mol% of PEG lipid, at least 1.1 mol% of PEG lipid, at least 1.2 mol% of PEG lipid, at least 1.3 mol% of PEG lipid, at least 1.4 mol% of PEG lipid, at least 1.5 mol% of PEG lipid, such as about or above 1.6 mol%, 1.7 mol%, 1.8 mol%, 1.9 mol%, 2.0 mol%, 2.1 mol%, 2.2 mol%, 2.3 mol%, 2.4 mol%, 2.5 mol%, 2.6 mol%, 2.7 mol%, 2.8 mol%, 2.9 mol%, 3.0 mol%, 3.1 mol%, 3.2 mol%, 3.3 mol%, 3.4 mol%, 3.5 mol%, 3.6 mol%, 3.7 mol%, 3.8 mol%, 3.9 mol%, 4.0 mol%, 4.1 mol%, 4.2 mol%, 4.3 mol%, 4.4 mol%, 4.5 mol%, 4.6 mol%, 4.7 mol%, 4.8 mol%, 4.9 mol%, 5.0 mol%, 5.1 mol%, 5.2 mol%, 5.3 mol%, 5.4 mol%, 5.5 mol%, 5.6 mol%, 5.7 mol%, 5.8 mol%, 5.9 mol%, 6.0 mol%, 6.1 mol%, 6.2 mol%, 6.3 mol%, 6.4 mol%, 6.5 mol%, 6.6 mol%, 6.7 mol%, 6.8 mol%, 6.9 mol%, 7.0 mol%, 7.1 mol%, 7.2 mol%, 7.3 mol%, 7.4 mol%, 7.5 mol%, 7.6 mol%, 7.7 mol%, 7.8 mol%, 7.9 mol%, 8.0 mol%, 8.1 mol%, 8.2 mol%, 8.3 mol%, 8.4 mol%, 8.5 mol%, 8.6 mol%, 8.7 mol%, 8.8 mol%, 8.9 mol%, 9.0 mol%, 9.1 mol%, 9.2 mol%, 9.3 mol%, 9.4 mol%, 9.5 mol%, 9.6 mol%, 9.7 mol%, 9.8 mol%, 9.9 mol%, 10.0 mol%.

The present invention also provides an LNP comprising a molar percentage of the ionizable lipid of at least 55 mol%, such as equal to or above 55 mol%, 56 mol%, 57 mol%, 58 mol%, 59 mol%, 60 mol%, 61 mol%, 62 mol%, 63 mol%, 64 mol%, 65 mol%, 66 mol%, 67 mol%, 68 mol%, 69 mol%, 70 mol%, 71 mol%, 72 mol%, 73 mol%, 74 mol%, 75 mol%, 76 mol%, 77 mol%, 78 mol%, 79 mol%, 80 mol%, 81 mol%, 82 mol%, 83 mol%, 84 mol%, 85 mol%.

The present invention further provides an LNP comprising a molar percentage of the phospholipid of above 1 mol%, such as equal to or above 1 mol%, 2 mol%, 3 mol%, 4 mol%, 5 mol%, 6 mol%, 7 mol%, 8 mol%, 9 mol%, 10 mol%, 11 mol%, 12 mol%, 13 mol%, 14 mol%, 15 mol%, 16 mol%, 17 mol%, 18 mol%, 20 mol%, 21 mol%, 22 mol%, 23 mol%, 24 mol%, 25 mol%, 26 mol%, 27 mol%, 28 mol%, 29 mol%, 30 mol%, 31 mol%, 32 mol%, 33 mol%, 34 mol%, 35 mol%, 36 mol%, 37 mol%, 38 mol%, 39 mol%, 40 mol%.

The present invention further provides an LNP comprising a molar percentage of the cholesterol

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of below 22 mol%, such as equal to or below 22 mol%, 21 mol%, 20 mol%, 19 mol%, 18 mol%, 17 mol%, 16 mol%, 15 mol%, 14 mol%, 13 mol%, 12 mol%, 11 mol%, 10 mol%, 9 mol%, 8 mol%, 7 mol%, 6 mol%, 5 mol%, 3 mol%, 2 mol%, 1 mol%, 0.4 mol%.

- 5 The present invention thus also provides a lipid nanoparticle comprising:
- at least 55 mol% of an ionizable lipid; in particular at least 60 mol%
 - above 1 mol% of phospholipid, in particular between 2 mol% and 40 mol%, more in particular between 10 mol% and 30 mol%, more in particular between 20 mol% and 26 mol%.
 - 10 - below 22 mol% sterol, preferably cholesterol; in particular between 0.4 mol% and 20 mol%, more in particular between 2 mol% and 18 mol%; more in particular between 10 mol% and 15 mol%, more in particular between 12 mol% and 13 mol%;
 - at least 1.5 mol% of PEG lipid, in particular at least 2.0 mol% of PEG lipid, more in particular at least 2.5 mol% of said PEG lipid, such as at least 3.0 mol% of said PEG lipid,
 - 15 at least 4.0 mol% of said PEG lipid, at least 5.0 mol% of said PEG lipid, at least 6.0 mol% of said PEG lipid, at least 7.0 mol% of said PEG lipid, at least 8.0 mol% of said PEG lipid, at least 9.0 mol% of said PEG lipid, at least 10.0 mol% of said PEG lipid; and
 - one or more nucleic acid molecules; in particular mRNA molecules;
- characterized in that the ratio of cholesterol to phospholipid is 1 : 1 or less; in particular 1 : 2 or less.
- 20

In a specific embodiment, the present invention provides a lipid nanoparticle comprising:

- at least 55 mol% of S-Ac7-dOg; in particular at least 60 mol%
 - above 1 mol% of DSPC; in particular between 10 mol% and 30 mol%, more in particular
 - 25 between 20 mol% and 26 mol%;
 - below 22 mol% cholesterol in particular between 10 mol% and 15 mol%, more in particular between 12 mol% and 13 mol%;
 - at least 1.5 mol% of DMG-PEG lipid, in particular at least 2.0 mol% of DMG-PEG lipid, more in particular at least 2.5 mol%, such as about 3.0 mol% of DMG-PEG lipid, and
 - 30 - one or more nucleic acid molecules; in particular mRNA molecules;
- characterized in that the ratio of cholesterol to phospholipid is 1 : 1 or less; in particular 1 : 2 or less.

In another specific embodiment, the present invention provides a lipid nanoparticle comprising:

- 35 - at least 55 mol% of S-Ac7-dOg; in particular at least 60 mol%
- above 1 mol% of DSPC; in particular between 10 mol% and 30 mol%, more in particular between 20 mol% and 26 mol%;
- below 22 mol% cholesterol in particular between 10 mol% and 15 mol%, more in particular between 12 mol% and 13 mol%;

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- at least 1.5 mol% of DSG-PEG lipid, in particular at least 2.0 mol% of DSG-PEG lipid, more in particular at least 2.5 mol%, such as about 3.0 mol% of DSG-PEG lipid, and
- one or more nucleic acid molecules; in particular mRNA molecules;
characterized in that the ratio of cholesterol to phospholipid is 1 : 1 or less; in particular 1 : 2 or
5 less.

In a very specific embodiment, the present invention provides an LNP comprising:

- about 60 mol% of said ionizable lipid;
- about 25.67 mol% of phospholipid;
- 10 - about 12.83 mol% of cholesterol; and
- about 1.5 mol% of PEG lipid.

In another very specific embodiment, the present invention provides an LNP comprising:

- about 60 mol% of S-Ac7-Dog;
- 15 - about 25.67 mol% of DSPC;
- about 12.83 mol% of cholesterol; and
- about 1.5 mol% of DMG-PEG lipid.

This LNP is specifically characterized in that it has a very high spleen-centric and high bone
20 marrow cargo delivery.

In another very specific embodiment, the present invention provides an LNP comprising:

- about 60 mol% of said ionizable lipid;
- about 19.25 mol% of phospholipid;
- 25 - about 19.25 mol% of cholesterol; and
- about 1.5 mol% of PEG lipid.

In yet a further specific embodiment, the present invention provides an LNP comprising:

- about 60 mol% of S-Ac7-dOg;
- 30 - about 19.25 mol% of DSPC;
- about 19.25 mol% of cholesterol; and
- about 1.5 mol% of DMG-PEG.

In a very specific embodiment, the present invention provides an LNP comprising:

- 35 - about 60 mol% of said ionizable lipid;
- about 24.17 mol% of phospholipid;
- about 12.83 mol% of cholesterol; and
- about 3.0 mol% of PEG lipid.

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In yet a further specific embodiment, the present invention provides an LNP comprising:

- about 60 mol% of S-Ac7-dOg;
- 5 - about 24.17 mol% of DSPC;
- about 12.83 mol% of cholesterol; and
- about 3.0 mol% of DMG-PEG.

10 This LNP is specifically characterized in that it has a high spleen-centric and very high bone marrow cargo delivery.

Specifically interesting LNPs may have the following ionizable lipid/phospholipid/cholesterol/PEG lipid ratio:

- 60 / 25.67 / 12.83 / 1.5
- 15 - 60 / 19.25 / 19.25 / 1.5
- 60 / 24.17 / 12.83 / 3.0

Accordingly, the present invention provides LNPs which have the following ionizable lipid/DSPC/cholesterol/PEG lipid ratio:

- 20 - 60 / 25.67 / 12.83 / 1.5, wherein PEG lipid = DMG-PEG
- 60 / 25.67 / 12.83 / 1.5, wherein PEG lipid = DSG-PEG
- 60 / 19.25 / 19.25 / 1.5, wherein PEG lipid = DMG-PEG
- 60 / 19.25 / 19.25 / 1.5, wherein PEG lipid = DSG-PEG
- 60 / 24.17 / 12.83 / 3.0, wherein PEG lipid = DMG-PEG
- 25 - 60 / 24.17 / 12.83 / 3.0, wherein PEG lipid = DSG-PEG

Further, the present invention provides LNPs which have the following S-Ac7-dOg/DSPC/cholesterol/PEG lipid ratio:

- 60 / 25.67 / 12.83 / 1.5, wherein PEG lipid = DMG-PEG
- 30 - 60 / 25.67 / 12.83 / 1.5, wherein PEG lipid = DSG-PEG
- 60 / 19.25 / 19.25 / 1.5, wherein PEG lipid = DMG-PEG
- 60 / 19.25 / 19.25 / 1.5, wherein PEG lipid = DSG-PEG
- 60 / 24.17 / 12.83 / 3.0, wherein PEG lipid = DMG-PEG
- 60 / 24.17 / 12.83 / 3.0, wherein PEG lipid = DSG-PEG

35

Where in the context of the present invention mol% is used, it is meant to be the mol% of the specified component with respect to the empty nanoparticle, i.e. without nucleic acids. This means that the mol% of a component is calculated with respect to the total amount of ionizable lipids, phospholipids, sterols and PEG lipids, present in said LNP.

The inventors have found that the LNP's of the present invention are particularly suitable for the extrahepatic delivery of nucleic acids, be it immunogenic or non-immunogenic. Hence the present invention provides LNP's comprising one or more nucleic acid molecules, such as DNA
5 or RNA, more specifically mRNA.

In the context of the present invention, "immunogenic delivery of nucleic acid molecules" means delivery of nucleic acid molecules to cells whereby contact with cells, internalization and/or
10 expression inside the cells of said nucleic acids molecules results in induction of an immune response.

Given their predominant extrahepatic tissue targeting after IV administration, the LNPs of the present invention are specifically suitable for non-immunogenic approaches, including but not
15 limited to gene editing, protein replacement, gene editing of HSCs, the addition of transcription factors, the addition of chemokines, the addition of immunomodulators and in situ CAR formation of immune cells.

Protein replacement therapy involves the administration of specific proteins or nucleic acids encoding such proteins to patients who have a deficiency or dysfunction of that protein due to
20 genetic disorders or other conditions. The goal is to restore normal function by supplementing the missing or defective protein. This approach is commonly used in the treatment of diseases where a specific protein is absent or not functioning properly.

Gene editing allows scientists to modify an organism's DNA with precision, such as in HSCs (hematopoietic stem cells) and T -cells. This technology enables the addition, removal, or alteration of genetic material at specific locations in the genome. The most widely known and used gene editing technology is CRISPR-Cas9, or others such as TALENs (Transcription
25 Activator-Like Effector Nucleases) and ZFNs (Zinc Finger Nucleases). Using HSCs in this approach is particularly suitable for treating genetic blood disorders and certain immune deficiencies because HSCs have the ability to self-renew and differentiate into various blood cell lineages.
30

In situ CAR formation of immune cells by administration of nucleic acids, involves the direct modification of immune cells within the patient's body to express chimeric antigen receptors (CARs). This method aims to overcome some of the limitations associated with traditional CAR
35 T-cell therapy, which typically involves extracting T cells from the patient, genetically modifying them in the laboratory to express CARs, and then reinfusing them back into the patient.

The amount of nucleic acid in said LNP's is typically represented by the molar ratio, i.e. the ratio of cationic lipid (ionizable lipid) to RNA phosphates. In the context of the present invention, the molar ratio of the LNP's is about and between 3/1; in particular about and between 4/1 and 16/1. The amount of nucleic acid in said LNP's can alternatively be represented by the N/P ratio, i.e. the ratio of nitrogen atoms in ionizable lipids to phosphate groups in the nucleic acids. In the context of the present invention, the N/P ratio of the LNP's is about and between 3/1; in particular about and between 4/1 and 16/1.

10

In a further aspect, the present invention provides a pharmaceutical composition comprising one or more LNP's as defined herein. Such pharmaceutical compositions are particularly suitable as a vaccine. Thus, the invention also provides a vaccine comprising one or more LNP's according to the present invention.

15

In the context of the present invention, the term "vaccine" as used herein is meant to be any preparation intended to provide adaptive immunity (antibodies and/or T cell responses) against a disease. To that end, a vaccine as meant herein contains at least one nucleic acid molecule encoding an antigen to which an adaptive immune response is mounted. This antigen can be present in the format of a weakened or killed form of a microbe, a protein or peptide, or an antigen encoding a nucleic acid. An antigen in the context of this invention is meant to be a protein or peptide recognized by the immune system of a host as being foreign, thereby stimulating the production of antibodies against is, with the purpose of combating such antigens. Vaccines can be prophylactic (example: to prevent or ameliorate the effects of a future infection by any natural or wild-type pathogen), or therapeutic (example, to actively treat or reduce the symptoms of an ongoing disease). The administration of vaccines is called vaccination.

The vaccine of the invention may be used for inducing an immune response, in particular an immune response against a disease-associated antigen or cells expressing a disease-associated antigen, such as an immune response against cancer. Accordingly, the vaccine may be used for prophylactic and/or therapeutic treatment of a disease involving a disease-associated antigen or cells expressing a disease-associated antigen, such as cancer. Preferably said immune response is a T cell response. In one embodiment, the disease-associated antigen is a tumor antigen. The antigen encoded by the RNA comprised in the nanoparticles described herein preferably is a disease-associated antigen or elicits an immune response against a disease-associated antigen or cells expressing a disease-associated antigen.

The LNP's and vaccines of the present invention are specifically intended for intravenous

administration, i.e. the infusion of liquid substance directly into a vein. The intravenous route is the fastest way to deliver fluids and medications throughout the body, i.e. systemically. The present invention thus provides intravenous vaccines, as well as the use of the disclosed vaccines and LNP's for intravenous administration. The vaccines and LNP's of the present invention can thus be administered intravenously. The present invention also provides the use of the vaccines and LNP's according to the present invention; wherein the vaccine is administered intravenously.

It was particularly found that the immunogenicity of the LNPs of the present invention increases upon multiple immunizations. Therefore, in a particular embodiment, the LNPs as defined herein are for use in vaccination purposes, wherein the LNPs are administered at least twice, preferably at least 3 times within a particular interval.

The present invention also provides the LNP's, pharmaceutical compositions and vaccines according to this invention for use in human or veterinary medicine. The use of the LNP's, pharmaceutical compositions and vaccines according to this invention for human or veterinary medicine is also intended. Finally, the invention provides a method for the prophylaxis and treatment of human and veterinary disorders, by administering the LNP's, pharmaceutical compositions and vaccines according to this invention to a subject in need thereof.

Such pharmaceutical compositions are particularly suitable in various fields such as prophylactic vaccines, therapeutic vaccines, protein replacement therapies, gene editing, gene silencing, small molecule delivery, and the like.

The present invention further provides the use of an LNP, a pharmaceutical composition or a vaccine according to the present invention for the immunogenic delivery of said one or more nucleic acid molecules. As such the LNP's, pharmaceutical compositions and vaccine of the present invention are highly useful in the treatment several human and veterinary disorders. Thus, the present invention provides the LNP's, pharmaceutical compositions and vaccines of the present invention for use in the treatment of cancer, autoimmune diseases, infectious diseases, hematologic disorders, aging, fibrosis, neurological disorders, or cardiovascular diseases.

In a specific aspect, the nanoparticles and pharmaceutical compositions as defined herein may be used in the induction of an immune response in a subject by providing to the subject a pharmaceutical composition wherein the active agent is an immunostimulatory oligonucleotide.

For example, the invention provides a vaccine comprising one or more nanoparticles according to the present invention. To that end, a vaccine as meant herein contains at least one active

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agent, such as a nucleic acid molecule, e.g. mRNA molecule encoding an antigen to which an adaptive immune response is mounted. This antigen can be present in the format of a weakened or killed form of a microbe, a protein or peptide, or an antigen encoding a nucleic acid, such as a disease-associated antigen for example a tumor antigen. Vaccines can be prophylactic (example: to prevent or ameliorate the effects of a future infection by any natural or "wild-type" pathogen), or therapeutic (example, to actively treat or reduce the symptoms of an ongoing disease).

In another aspect, the nanoparticles and pharmaceutical compositions as defined herein may be used in the treatment of a disease or disorder characterized by underexpression of a polypeptide in a subject by providing to the subject a pharmaceutical composition of the present invention, wherein the active agent is a plasmid that encodes the polypeptide or a functional variant or fragment thereof, such as in the context of protein replacement therapy and/or in the treatment of genetic disorders.

In yet a further aspect, the nanoparticles and compositions as defined herein may be used as a transfection agent that includes the compositions or nanoparticles described herein, wherein the composition or nanoparticles include a nucleic acid. The agent, when contacted with cells, can efficiently deliver nucleic acids to the cells. Yet another aspect is a method of delivering a nucleic acid to the interior of a cell, by obtaining or forming a composition or nanoparticles described herein, and contacting the composition or lipid particles with a cell.

The lipid nanoparticles of the present invention may be prepared in accordance with the protocols as specified in the Examples part. More generally, the LNP's may be prepared using a method comprising:

- preparing a first alcoholic composition comprising said ionizable lipid, said phospholipid, said sterol, said PEG lipid, and a suitable alcoholic solvent;
- preparing a second aqueous composition comprising said one or more nucleic acids and an aqueous solvent;
- mixing said first and second composition in a microfluidic mixing device.

In further detail, the lipid components are combined in suitable concentrations in an alcoholic vehicle such as ethanol. Thereto, an aqueous composition comprising the nucleic acid is added, and subsequently loaded in a microfluidic mixing device.

The aim of microfluidic mixing is to achieve thorough and rapid mixing of multiple samples (i.e. lipid phase and nucleic acid phase) in a microscale device. Such sample mixing is typically achieved by enhancing the diffusion effect between the different species flows. Thereto several microfluidic mixing devices can be used, such as for example reviewed in Lee et al., 2011. A

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particularly suitable microfluidic mixing device according to the present invention is the NanoAssemblr from Precision Nanosystems. Alternatively, LNPs can be prepared using T-mixing, utilizing a T-joint where 2 Harvard apparatus syringe pumps have been connected to each other.

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Other technologies suitable for preparing the LNP's of the present invention include dispersing the components in a suitable dispersing medium, for example, aqueous solvent and alcoholic solvent, and applying one or more of the following methods: ethanol dilution method, a simple hydration method, sonication, heating, vortex, an ether injecting method, a French press method, a cholic acid method, a Ca^{2+} fusion method, a freeze-thaw method, a reversed-phase evaporation method, T-junction mixing, Microfluidic Hydrodynamic Focusing, Staggered Herringbone Mixing, and the like.

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EXAMPLES

EXAMPLE 1: IN VIVO EXPERIMENTS

Bioluminescence imaging after IV administration of Fluc mRNA containing LNPs

20 **Materials and methods**

Animals

Mice were housed in IVC under specific pathogen-free conditions. All animal experiments were performed with approval from the Ethical Committee and animal care was according to established guidelines. Female Balb/C 6-weeks old (20-23g) were obtained from Charles River International Laboratories, Inc. (France) and housed (max 5 per cage) with free access to water and standard laboratory animal chow.

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Intravenous injection

For intravenous injection, mice are either placed in a warming chamber for vein dilation for no more than 10 min or the cage was placed under a red lamp for 10-20 min. When appropriately warm, mice were restrained individually. The tail was swabbed with gauze dampened in 70% ethanol. The needle of a BD microfine syringe (20-25G) with LNP solution was carefully inserted into one of the side tail veins and applying slow pressure to the plunger a maximum volume of 200 μL (for mice of 20-25g) was injected. The needle was removed from the vein and the local bleeding stopped by applying slight pressure to the puncture site with dry gauze. Animals were subsequently observed for at least 10 minutes in their cage. Each injection was equivalent to 10 μg Fluc mRNA and each treatment group contained 5 mice.

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Luminescence imaging

Imaging was performed 24h after IV injection of LNP formulations. Each mouse was injected i.p. with 100 µl D-luciferin (30 mg/mL.). Mice were then placed in an anesthesia induction chamber with oxygen supply (0.4-0.8 L/min) and isoflurane (5%) until they undergo narcosis. The flow of isoflurane was then reduced (3%) to maintain narcosis until mice are ready to be imaged. Mice are placed in a maximum of 3 per group in a stage inside the IVIS Lumina II (PerkinElmer) using the same anesthesia flow (3-4% Isoflurane). Imaging is performed with the parameters set to Luminescence, auto exposure with background overlay and medium binning (4), using field of view D. Imaging of the animals was performed no longer than 15 min after luciferin injection (peak of the signal). Once in vivo images were taken, the mice were sacrificed by means of cervical dislocation, dissected and imaging of respectively the liver, spleen and femur bone under similar imaging conditions.

mRNA synthesis

FireFly luciferase (Fluc) mRNA was produced from a linearized peTheRNA vector (see WO2015071295) using eTheRNA-optimized in vitro transcription (IVT) reaction conditions and purified via cellulose. Uridine was fully substituted by N1-Methylpseudouridine (N1ψ) to generate N1ψ-modified mRNA.

Example 1a: cholesterol to phospholipid ratio influences biodistribution of LNPs**LNP production**

Lipid based nanoparticles are produced by microfluidic mixing of an mRNA solution in sodium acetate buffer (100 mM, pH 4) and lipid solution in a 2 : 1 volume ratio at a speed of 9 mL/min or 16 ml/min using the NanoAssemblr Benchtop (Precision Nanosystems). The lipid solution contained a mixture of an ionizable lipid (S-Ac7-DOg), DSPC (Avanti), Cholesterol (Sigma) and DMG-PEG2000 (Avanti).

LNPs were produced at a different molar ratio ionizable lipid/helper lipid/cholesterol/DMG-PEG2000, with focus on cholesterol to phospholipid ratio. Fluc mRNA was encapsulated in all LNPs as reporter mRNA, at a mRNA/ionizable lipid molar ratio of 1/10.

A list of relevant physico-chemical properties for different LNP compositions based on INL 1 (ionizable lipid = S-Ac7-DOg), where an assessment of the ratio cholesterol to phospholipid is screened, is shown below in Table 1.

Table 1. Physico-chemical characteristics of LNPs

LNP #	Chol / HL Ratio ¹	Lipid Ratio (INL 1 / DSPC / Chol / DMG- PEG2000) [mol %]	Size [nm]	PDI	EE%
1	1 : 2	60 / 25.67 / 12.83 / 1.5	85.75	0.147	92
2	1 : 1	60 / 19.25 / 19.25 / 1.5	96.77	0.222	95
3	4 : 1	60 / 7.7 / 30.8 / 1.5	97.00	0.222	97
4	3.9 : 1	50 / 10 / 38.5 / 1.5	89.21	0.157	96

¹Chol / HL: cholesterol to phospholipid ratio

Figure 1 displays the spleen to liver ratio of mRNA expression upon intravenous injection of Fluc mRNA LNPs with molar ratios as displayed in Table 1. The spleen to liver ratio has been calculated as the ratio obtained by dividing the *ex vivo* average radiance of the spleen (normalized in function of photons/second/cm²/steradian) by the *ex vivo* average radiance of the liver, 24h post injection as captured by the IVIS. There, LNPs that contain a cholesterol to phospholipid ratio of 1 : 1 or lower display a shift in biodistribution in favor of the spleen as compared to LNPs that contain a cholesterol-to-helper lipid ratio that is higher. The condition with a cholesterol to phospholipid ratio of 3.9 : 1 represents a LNP that has a standard lipid molar ratio as used in the Onpattro formulation (INL 1 / DSPC / Chol / DMG-PEG2000 = 50 / 10 / 38.5 / 1.5).

Figure 2 depicts the luciferase activity in the bone marrow of the excised femur bone 24h post-injection. The femur bone was broken in order to expose the bone marrow to oxygen to allow for visualization with the IVIS. The luciferase activity is displayed as an *ex vivo* average radiance (normalized in function of photons/second/cm²/steradian). Here a cholesterol-to-helper lipid ratio of 1 : 2 shows a higher signal in the bone marrow compared to other LNPs, including the LNP with a cholesterol to phospholipid ratio of 3.9 : 1 that has a standard lipid molar ratio as used in the Onpattro formulation.

Example 1b: type and amount of PEG-lipid of LNPs containing a low cholesterol to phospholipid ratio has an impact on the in vivo biodistribution of LNPs.

LNP production

Lipid based nanoparticles are produced by microfluidic mixing of an mRNA solution in sodium acetate buffer (100 mM, pH 4) and lipid solution in a 2 : 1 volume ratio at a speed of 9 mL/min or 16 mL/min using the NanoAssemblr Benchtop (Precision Nanosystems). The lipid solution contained a mixture of an ionizable lipid (S-Ac7-Dog), DSPC (Avanti), Cholesterol (Sigma) and a PEG lipid selected from DMG-PEG2000 or DSG-PEG2000 (Avanti), as indicated in Tables 2

and 3.

LNPs were produced at a different molar ratio ionizable lipid/helper lipid/cholesterol/PEG lipid, with a low cholesterol to phospholipid ratio of 1 : 2. LNPs were compared to the standard formulation that contains a cholesterol to phospholipid ratio of 3.9 : 1. Fluc mRNA was encapsulated in all LNPs as reporter mRNA, at a mRNA/ionizable lipid molar ratio of 1/10.

Table 2 contains the list of physico-chemical properties of different LNP compositions based on INL 1 (S-Ac7-Dog) with a cholesterol to phospholipid ratio of about 1 : 2 and different amounts of DMG-PEG2000.

Table 2. Physico-chemical characteristics of LNPs

LNP #	Chol / HL Ratio ¹	Lipid Ratio (INL 1 / DSPC / Chol / DMG-PEG2000) Ratio [mol %]	Size [nm]	PDI	EE%
1	1 : 2	35 / 42.33 / 21.17 / 1.5	126.60	0.089	83
2	1 : 1.9	35 / 40.83 / 21.17 / 3.0	119.05	0.146	55
3	1 : 2	60 / 25.67 / 12.83 / 1.5	100.62	0.137	96
4	1 : 1.9	60 / 24.17 / 12.83 / 3.0	113.40	0.063	82
5	3.9 : 1	50 / 10 / 38.5 / 1.5	82.94	0.129	98

¹Chol / HL: cholesterol to phospholipid ratio

Figure 3 displays the relative spleen to liver ratio of mRNA expression upon intravenous injection of Fluc mRNA LNPs with molar ratios as displayed in Table 2. The spleen to liver ratio has been calculated as the ratio obtained by dividing the *ex vivo* average radiance of the spleen (normalized in function of photons/second/cm²/steradian) by the *ex vivo* average radiance of the liver, 24h post injection as captured by the IVIS. The data displayed below indicates that the combination of a molar percentage of 60% of ionizable lipid and a cholesterol to phospholipid ratio of 1 : 2 is beneficial compared to a condition containing a molar percentage of 35% ionizable lipid and a cholesterol to phospholipid ratio of 1 : 2, as well as compared to including an LNP with a cholesterol to phospholipid ratio of 3.9 : 1 that has a standard lipid molar ratio as used in the Onpattro formulation.

Figure 4 depicts the luciferase activity in the bone marrow of the excised femur bone 24h post-injection. The femur bone was broken in order to expose the bone marrow to oxygen to allow for visualization with the IVIS. The luciferase activity is displayed as an *ex vivo* average radiance (normalized in function of photons/second/cm²/steradian). LNP 4, containing a molar percentage of 3% DMG-PEG-2000 in combination with 60% ionizable lipid and a cholesterol to phospholipid

ratio of 1 : 2 shows a significantly higher signal in bone marrow compared to LNPs with lower mol% of ionizable lipid and/or lower mol% of DMG-PEG2000.

Table 3 contains the list of physico-chemical properties of different LNP compositions based on INL 1 (S-Ac7-Dog) with a cholesterol to phospholipid ratio of about 1 : 2 and different amounts of DSG-PEG2000.

Table 3. Physico-chemical characteristics of LNPs

LNP #	Chol / HL Ratio ¹	Lipid Ratio (INL 1 / DSPC / Chol / DSG- PEG2000) Ratio [mol %]	Size [nm]	PDI	EE%
6	1 : 2	35 / 42.33 / 21.17 / 1.5	206.35	0.222	66
7	1 : 1.9	35 / 40.83 / 31.17 / 3.0	151.05	0.190	83
8	1 : 2	60 / 25.67 / 12.83 / 1.5	125.90	0.173	92
9	1 : 1.9	60 / 24.17 / 12.83 / 3.0	75.80	0.041	100
5	3.9 : 1	50 / 10 / 38.5 / 1.5	82.94	0.129	98

¹Chol / HL: cholesterol to phospholipid ratio

Figure 5 depicts the luciferase activity in the bone marrow of the excised femur bone 24h post-injection. The femur bone was broken in order to expose the bone marrow to oxygen to allow for visualization with the IVIS. The luciferase activity is displayed as an ex vivo average radiance (normalized in function of photons/second/cm²/steradian). LNP 8 and LNP 9, containing a molar percentage of 60% ionizable lipid in combination with DSG-PEG2000 and a cholesterol to phospholipid ratio of 1 : 2, show a significantly higher signal in bone marrow compared to LNPs with lower mol% of ionizable lipid and LNP 5, which represents an LNP with a cholesterol to phospholipid ratio of 3.9 : 1 and a standard lipid molar ratio as used in the Onpattro formulation.

Example 1c: cholesterol to phospholipid ratio determines LNP biodistribution, irrespective of the ionizable lipid.

LNP production

Lipid based nanoparticles are produced mixing of an mRNA solution in sodium acetate buffer (100 mM, pH 4) and lipid solution in a 2 : 1 volume ratio (INL 1) or 3 : 1 volume ratio (INL 2 / INL 3 / INL 4 / INL 5) at a speed of 9 mL/min, 12 mL/min or 20 mL/min using a T-mixing set-up. The lipid solution contained a mixture of an ionizable lipid (INL 1 and INL 2 are both covered by patent application WO2022136641, INL 3 and INL 4 are both covered by patent application PCT/EP2024/069656 and INL 5 is covered by patent application WO2024084056), DSPC (Avanti), Cholesterol (Sigma) and DMG-PEG2000 (Avanti). LNPs were produced at different molar ratios of ionizable lipid/helper lipid/cholesterol/DMG-

PEG2000, with focus on cholesterol to phospholipid ratio. Fluc mRNA was encapsulated in all LNPs as reporter mRNA, at a mRNA/ionizable lipid molar ratio of 1/10 for INL 1 and 1/6 for INL 2, INL 3, INL 4 and INL 5.

5 A list of relevant physico-chemical properties for different LNP compositions is shown below in Table 4.

Table 4. Physico-chemical characteristics of LNPs

LNP #	INL	Chol / HL Ratio ¹	Lipid Ratio (INL / DSPC / Chol / DMG-PEG2000) [mol %]	Size [nm]	PDI	EE%
1	INL 1	3.9 : 1	50 / 10 / 38.5 / 1.5	76.4	0.093	99
2	INL 1	1 : 2	60 / 25.67 / 12.83 / 1.5	79.5	0.136	98
3	INL 2	3.9 : 1	50 / 10 / 38.5 / 1.5	75.4	0.090	97
4	INL 2	1 : 2	60 / 25.67 / 12.83 / 1.5	97.5	0.046	98
5	INL 3	3.9 : 1	50 / 10 / 38.5 / 1.5	76.0	0.106	98
6	INL 3	1 : 2	60 / 25.67 / 12.83 / 1.5	87.5	0.074	98
7	INL 4	3.9 : 1	50 / 10 / 38.5 / 1.5	78.9	0.047	98
8	INL 4	1 : 2	60 / 25.67 / 12.83 / 1.5	93.1	0.045	91
9	INL 5	3.9 : 1	50 / 10 / 38.5 / 1.5	98.8	0.126	94
10	INL 5	1 : 2	60 / 25.67 / 12.83 / 1.5	86.3	0.157	46

¹Chol / HL: cholesterol to phospholipid ratio

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Figure 6 displays the spleen to liver ratio of mRNA expression upon intravenous injection of Fluc mRNA LNPs with molar ratios as displayed in Table 4. The spleen to liver ratio has been calculated as the ratio obtained by dividing the *ex vivo* average radiance of the spleen (normalized in function of photons/second/cm²/steradian) by the *ex vivo* average radiance of the liver, 24h post injection as captured by the IVIS. There, LNPs that contain a cholesterol to phospholipid ratio of 1 : 2 display a shift in biodistribution in favor of the spleen as compared to LNPs that contain a cholesterol to phospholipid ratio that is higher. As can be seen from Figure 6, a similar impact on spleen to liver ratio can be observed irrespective of the ionizable lipid that has been used in the LNP formulation. The condition with a cholesterol to phospholipid ratio of 3.9 : 1 represents a LNP that has a standard lipid molar ratio as used in the Onpattro formulation (INL / DSPC / Chol / DMG-PEG2000 = 50 / 10 / 38.5 / 1.5).

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Example 1d: combination of cholesterol to phospholipid ratio together with the ionizable lipid percentage determines LNP biodistribution, irrespective of the ionizable lipid.

LNP production

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- Lipid based nanoparticles are produced by microfluidic mixing of an mRNA solution in sodium acetate buffer (100 mM, pH 4) and lipid solution in a 3 : 1 volume ratio at a speed of 12 mL/min or 20 mL/min using a T-mixing set-up. The lipid solution contained a mixture of an ionizable lipid (INL 2 is covered by patent application WO2022136641, INL 5 is covered by patent WO2024084056), DSPC (Avanti), Cholesterol (Sigma) and DMG-PEG2000 (Avanti).
- 10
- LNPs were produced at a different molar ratio ionizable lipid/helper lipid/cholesterol/DMG-PEG2000, with focus on cholesterol to phospholipid ratio. Fluc mRNA was encapsulated in all LNPs as reporter mRNA, at a mRNA/ionizable lipid molar ratio of 1/6 for all formulations.
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- A list of relevant physico-chemical properties for different LNP compositions based on INL 2 and INL 5 is shown below in Table 5.

Table 5. Physico-chemical characteristics of LNPs

LNP #	INL	Chol / HL Ratio ¹	Lipid (INL / DSPC / Chol / DMG-PEG2000) [mol %]	Size [nm]	PDI	EE%
1	INL 2	3.9 : 1	50 / 10 / 38.5 / 1.5	75.4	0.090	97
2	INL 2	1 : 2	35 / 42.33 / 21.17 / 1.5	100	0.061	95
3	INL 2	1 : 2	60 / 25.67 / 12.83 / 1.5	97.5	0.046	98
4	INL 5	3.9 : 1	50 / 10 / 38.5 / 1.5	98.8	0.126	94
5	INL 5	1 : 2	35 / 42.33 / 21.17 / 1.5	104.7	0.080	62
6	INL 5	1 : 2	60 / 25.67 / 12.83 / 1.5	86.3	0.157	46

¹Chol / HL: cholesterol to phospholipid ratio

- 20
- Figure 7 displays the spleen to liver ratio of mRNA expression upon intravenous injection of Fluc mRNA LNPs with molar ratios as displayed in Table 5. The spleen to liver ratio has been calculated as the ratio obtained by dividing the *ex vivo* average radiance of the spleen (normalized in function of photons/second/cm²/steradian) by the *ex vivo* average radiance of the liver, 24h post injection as captured by the IVIS. There, LNPs that contain a cholesterol to phospholipid ratio of 1 : 2 display a shift in biodistribution in favor of the spleen as compared to
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LNPs that contain a cholesterol-to-helper lipid ratio that is higher. The data also indicates that the combination of a molar percentage of 60% ionizable lipid and a cholesterol to phospholipid ratio of 1 : 2 is again beneficial compared to a condition with a similar cholesterol to phospholipid ratio and a 35% ionizable lipid percentage. This has already been shown earlier with an
5 alternative ionizable lipid, indicating that the effect is irrespective of the ionizable lipid structure.

Flow cytometry-based cellular tropism analysis after IV administration of Thy1.1 mRNA containing LNPs

10 **Materials and methods**

Animals

Mice were housed in IVC under specific pathogen-free conditions. All animal experiments were performed with approval from the Ethical Committee and animal care was according to established guidelines. Female 6-weeks old Balb/C mice (20-23g) were obtained from Charles
15 River International Laboratories, Inc. (France) and housed (max 5 per cage) with free access to water and standard laboratory animal chow.

Intravenous injection

For intravenous injection, mice were placed in a warming chamber for tail vein dilation for no
20 longer than 10 min. When the veins were sufficiently dilated, mice were restrained individually. The tail was wiped with gauze soaked in 70% ethanol. The needle of a BD microfine syringe (20-25G) filled with LNP suspension was carefully inserted into one of the tail veins and by applying slow pressure to the plunger a maximum volume of 200 µL was injected. The needle was removed from the vein and the local bleeding stopped by applying slight pressure to the
25 puncture site with dry gauze. Animals were subsequently observed for potential LNP-induced reactogenicity for at least 10 minutes in their cage. Each injection was equivalent to either 5 or 20 µg Thy1.1 mRNA and each treatment group contained 5 mice.

Flow cytometry

Euthanasia of all mice was performed 16h after IV injection of the LNP formulations. Both the
30 liver and the spleen were isolated and collected in MACS Tissue Storage Solution (Miltenyi Biotec). The organs were mechanically and enzymatically dissociated using a gentleMACS Tissue Dissociator and Liver or Spleen Dissociation Kit (Miltenyi) according to the manufacturer's guidelines. Subsequently, red blood cells were removed by means of RBC Lysis
35 Buffer (Thermo Fisher Scientific), the remaining cells were resuspended in FACS buffer (PBS with 1% BSA and 2.5 mM EDTA) and the hepatic single cell suspension was applied to a 40 µm strainer. Following cell counting using ViaStain AOPI Staining Solution and a Cellca MX 2FL (Revvity), for each staining condition, 2 million live cells were prestained with LIVE/DEAD Fixable Near IR (876) Viability Kit (Thermo Fisher Scientific) and Purified Rat Anti-Mouse CD16/CD32

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(BD Biosciences) and subsequently stained with one of the following antibody cocktails: antibody cocktail 1 (hepatic myeloid immune cell stain) containing CD90.1-BV786, TIM4-PE, CD11b-BV605 (BD Biosciences), Ly6G-PE-Cy5, Ly6C-AF700, CD31-PE-Cy7, CD146-APC (BioLegend), CD45-BUV496 and F4/80-eFluor450 (Thermo Fisher Scientific); antibody cocktail 2 (hepatic lymphoid immune cell stain) containing CD11b-BV605, CD8-FITC (BD Biosciences), CD4-BV421, CD19-BV785 (BioLegend), CD90.1-APC, CD45-BUV496, CD3-PerCP-eFluor710 and NK1.1-PE-eFluor610 (Thermo Fisher Scientific); antibody cocktail 3 (splenic lymphoid immune cell stain) containing CD3-BUV661, CD8-FITC (BD Biosciences), CD4-BV421, CD19-BV785 (BioLegend), CD90.1-APC, CD45-BUV496 and NK1.1-PE-eFluor610 (Thermo Fisher Scientific); antibody cocktail 4 (splenic myeloid immune cell stain) containing CD11b-BV605, CD11c-BV650 (BD Biosciences), Ly6C-AF700, CD3-biotin, CD19-biotin, NKp46-biotin (BioLegend), CD90.1-APC, CD45-BUV496, F4/80-eFluor450 and Ly6G-PE (Thermo Fisher Scientific). Cells stained with antibody cocktail 4 were subsequently stained with streptavidin-FITC (BD Biosciences). Flow cytometry analysis was performed using a CytoFLEX LX flow cytometer (Beckman Coulter) and FlowJo software (FlowJo LLC, BD Biosciences).

mRNA synthesis

Thymus cell antigen 1.1 (Thy1.1) mRNA was produced from a linearized peTheRNA vector (see WO2015071295) using eTheRNA-optimized in vitro transcription (IVT) reaction conditions and purified via cellulose. Uridine was fully substituted by N1-Methylpseudouridine (N1 ψ) to generate N1 ψ -modified mRNA.

Example 1e: impact of cholesterol to phospholipid ratio on cellular tropism.

LNP production

Lipid based nanoparticles are produced by T-junction mixing of an mRNA solution in sodium acetate buffer (100 mM, pH 4) and lipid solution in a 2 : 1 volume ratio (INL 1) or 3 : 1 volume ratio (INL 2) at a speed of 9 mL/min (INL 1) or 12 mL/min (INL 2). The lipid solution contained a mixture of an ionizable lipid (INL 1 and INL 2 are both covered by patent application WO2022136641, DSPC (Avanti), Cholesterol (Sigma) and DMG-PEG2000 (Avanti).

LNPs were produced at a different molar ratio ionizable lipid/helper lipid/cholesterol/DMG-PEG2000, with focus on cholesterol to phospholipid ratio. Thy1.1 mRNA was encapsulated in all LNPs as reporter mRNA, at a mRNA/ionizable lipid molar ratio of 1/10 for INL 1 and 1/6 for INL 2.

A list of relevant physico-chemical properties for different LNP compositions is shown below in Table 6.

Table 6. Physico-chemical characteristics of LNPs

LNP #	INL	Chol / HL Ratio ¹	Lipid (INL / DSPC / Chol / DMG-PEG2000) [mol %]	Size [nm]	PDI	EE%
1	INL 1	3.9 : 1	47.5 / 10.5 / 40.5 / 1.5	72.4	0.049	99
2	INL 1	1 : 2	60 / 25.67 / 12.83 / 1.5	78.7	0.047	100
3	INL 2	3.9 : 1	50 / 10 / 38.5 / 1.5	78.4	0.074	98
4	INL 2	1 : 2	60 / 25.67 / 12.83 / 1.5	86.5	0.015	98

¹Chol / HL: cholesterol to phospholipid ratio

Figure 8 displays the amount of Thy1.1 positive cells in a certain liver cell type 16h after IV injection as determined via flow cytometry, exemplified for INL 2 containing formulations. As can be seen from the graph, there is a clear drop in expression in most cell types, including hepatocytes, liver sinusoidal endothelial cells, neutrophils, monocytes, B cells, CD4+ and CD8+ T cells, NKT cells and NK cells when switching the cholesterol to phospholipid ratio from 3.9 : 1 to 1 : 2, while increasing the molar percentage of ionizable lipid to 60 mol%. These data confirm that the molar composition of the formulation is capable of de-targeting the liver.

Additionally, Figure 9 shows the ratio of the number of Thy1.1 positive splenic CD4+ T cells / CD8+ T cells / macrophages to the number of Thy1.1 positive hepatocytes. This again shows that by decreasing the cholesterol to phospholipid ratio, there is a shift in biodistribution in favor of the spleen, which is again irrespective of the chemical structure of the ionizable lipid in the formulation.

CLAIMS

1. A lipid nanoparticle (LNP) comprising an ionizable lipid, a phospholipid, a sterol, a stabilizing lipid; and one or more nucleic acid molecules;
- 5 characterized in that
- said LNP comprises at least 55 mol% of said ionizable lipid; and
 - the ratio of sterol to phospholipid is 1 : 1 or less.
2. A lipid nanoparticle (LNP) as defined in claim 1 wherein the ratio of sterol to phospholipid is 1 : 2 or less.
3. A lipid nanoparticle as defined in anyone of claims 1 or 2; wherein said LNP comprises at least 0.1 mol% of said stabilizing lipid, in particular, at least 0.5 mol% of said stabilizing lipid, in particular at least 1.0 mol% of said stabilizing lipid, in particular at least 1.5 mol% of said stabilizing lipid, in particular at least 2.0 mol% of stabilizing lipid, more in particular at least 2.5 mol% of said stabilizing lipid, at least 3.0 mol% of said stabilizing lipid, at least 4.0 mol% of said stabilizing lipid, at least 5.0 mol% of said stabilizing lipid, at least 6.0 mol% of said stabilizing lipid, at least 7.0 mol% of said stabilizing lipid, at least 8.0 mol% of said stabilizing lipid, at least 9.0 mol% of said stabilizing lipid, at least 10.0 mol% of said stabilizing lipid.
4. A lipid nanoparticle as defined in anyone of claims 1 to 3; wherein said stabilizing lipid is a PEG lipid or variant thereof.
5. A lipid nanoparticle as defined in claim 4; wherein said LNP comprises at least 0.1 mol% of said PEG lipid, in particular, at least 0.5 mol% of said PEG lipid, in particular at least 1.0 mol% of said PEG lipid, in particular at least 1.5 mol% of said PEG lipid, in particular at least 2.0 mol% of PEG lipid, more in particular at least 2.5 mol% of said PEG lipid, at least 3.0 mol% of said PEG lipid, at least 4.0 mol% of said PEG lipid, at least 5.0 mol% of said PEG lipid, at least 6.0 mol% of said PEG lipid, at least 7.0 mol% of said PEG lipid, at least 8.0 mol% of said PEG lipid, at least 9.0 mol% of said PEG lipid, at least 10.0 mol% of said PEG lipid.
6. A lipid nanoparticle as defined in anyone of claims 1 to 5; wherein the molar percentage of said phospholipid is above 1 mol%, in particular between 2 mol% and 40 mol%, more in particular between 2 mol% and 30 mol%, more in particular between 5 mol% and 28 mol%; more in particular between 10 mol% and 27 mol%; even more in particular between 20 mol% and 26 mol%.
7. A lipid nanoparticle as defined in anyone of claims 1 to 6; wherein the molar percentage of said sterol is below 22 mol%, in particular between 0.4 mol% and 20 mol%, more in particular

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between 2 mol% and 18 mol%; more in particular between 10 mol% and 15 mol%, more in particular between 12 mol% and 13 mol%.

8. A lipid nanoparticle as defined in anyone of claims 4 to 7; wherein said PEG lipid is selected from the list comprising DMG-PEG and DSG-PEG.

9. A lipid nanoparticle as defined in anyone of claims 1 to 8; wherein said phospholipid is selected from the list comprising: DOPE, DOPC, DSPC, ESM and mixtures thereof; in particular DSPC.

10. A lipid nanoparticle as defined in anyone of claims 1 to 9; wherein said sterol is selected from the list comprising cholesterol, ergosterol, campesterol, oxysterol, antrosterol, cholecalciferol, desmosterol, nicasterol, sitosterol and stigmasterol; preferably cholesterol.

11. A lipid nanoparticle as defined in anyone of claims 1 to 10; wherein said LNP comprises:

- about 60 mol% of said ionizable lipid;
- about 25.67 mol% of DSPC;
- about 12.83 mol% of cholesterol; and
- about 1.5 mol% of DMG-PEG.

12. A lipid nanoparticle as defined in anyone of claims 1 to 11; wherein said LNP comprises:

- about 60 mol% of said ionizable lipid;
- about 24.17 mol% of DSPC;
- about 12.83 mol% of cholesterol; and
- about 3.0 mol% of DMG-PEG.

13. A pharmaceutical composition or a vaccine comprising one or more lipid nanoparticles as defined in anyone of claims 1 to 12 and an acceptable pharmaceutical carrier.

14. A lipid nanoparticle as defined in anyone of claims 1 to 12 or a pharmaceutical composition or vaccine as defined in claim 13 for use in human or veterinary medicine.

15. A lipid nanoparticle as defined in anyone of claims 1 to 12 or a pharmaceutical composition or vaccine as defined in claim 13 for use in the treatment of cancer, autoimmune diseases, genetic disorders, hematological disorders, aging, fibrosis, neurological disorders, cardiovascular diseases or infectious diseases.

16. A lipid nanoparticle as defined in anyone of claims 1 to 12 or a pharmaceutical composition or vaccine as defined in claim 13 for use in increasing gene expression of the nucleic acid molecules *in vivo* in spleen and/or bone marrow.
- 5 17. A method for *in vivo* delivery of mRNA to a subject in need thereof, the method comprising: administering to the subject a lipid nanoparticle as defined in any one of claims 1 to 12 or the pharmaceutical composition or vaccine as defined in claim 13.