Thus, there remains a need in the art for (i) compositions which comprise LNPs comprising ionizable lipids and RNA and which are stable and can be stored in a temperature range compliant to regular technologies in pharmaceutical practice, in particular at a temperature of about -25°C or even in liquid form at temperatures between +2 and +20°C; (ii) compositions which are ready to use; (iii) compositions which, preferably, can repeatably be frozen and thawed; and (iv) methods for preparing and storing such compositions. The present disclosure addresses these and other needs.

The inventors surprisingly found that the compositions and methods described herein fulfill the above-mentioned requirements. In particular, it is demonstrated that by using a specific buffer substance, in particular tris(hydroxymethyl)aminomethane (Tris) and its protonated form, in a low concentration (e.g., at most about 25 mM) and excluding inorganic phosphate anions as well as citrate anions and anions of EDTA, it is possible to prepare compositions which are stable and which can be stored at about -25°C or even in liquid form.

Summary

In one aspect, the present disclosure provides a composition comprising lipid nanoparticles (LNPs) dispersed in an aqueous phase, wherein the LNPs comprise a cationically ionizable lipid and RNA; the aqueous phase comprises a buffer system comprising a buffer substance and a monovalent anion, the buffer substance being selected from the group consisting of tris(hydroxymethyl)aminomethane (Tris) and its protonated form, bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (Bis-Tris-methane) and its protonated form, and triethanolamine (TEA) and its protonated form, and the monovalent anion being selected from the group consisting of chloride, acetate, glycolate, and lactate; the concentration of the buffer substance in the composition is at most about 25 mM; and the aqueous phase is substantially free of inorganic phosphate anions, substantially free of citrate anions, and substantially free of anions of ethylenediaminetetraacetic acid (EDTA), wherein the concentration of the monovalent anion in the composition is at most equal to the concentration of the buffer substance in the composition.

In another aspect, the present disclosure provides a method of preparing a composition comprising LNPs dispersed in a final aqueous phase, wherein the LNPs comprise a cationically ionizable lipid and RNA; the final aqueous phase comprises a final buffer system comprising a final buffer substance and a final monovalent anion, the final buffer substance being selected from the group consisting of Tris and its protonated form, Bis-Tris-methane and its protonated form, and TEA and its protonated form, and the final monovalent anion being selected from the group consisting of chloride, acetate, glycolate, and lactate; the concentration of the final buffer substance in the composition is at most about 25 mM; and the final aqueous phase is

substantially free of inorganic phosphate anions, substantially free of citrate anions, and substantially free of anions of EDTA; wherein the method comprises: (I) preparing a formulation comprising LNPs dispersed in the final aqueous phase, wherein the LNPs comprise the cationically ionizable lipid and RNA; and (II) optionally freezing the formulation to about -10°C or below, thereby obtaining the composition, wherein step (I) comprises: (a) preparing an RNA solution containing water and a first buffer system; (b) preparing an ethanolic solution comprising the cationically ionizable lipid and, if present, one or more additional lipids; (c) mixing the RNA solution prepared under (a) with the ethanolic solution prepared under (b), thereby preparing a first intermediate formulation comprising the LNPs dispersed in a first aqueous phase comprising the first buffer system; and (d) filtrating the first intermediate formulation prepared under (c) using a final aqueous buffer solution comprising the final buffer system, thereby preparing the formulation comprising the LNPs dispersed in the final aqueous phase, wherein the concentration of the final monovalent anion in the composition is at most equal to the concentration of the final buffer substance in the composition.

In yet another aspect, the present disclosure provides a composition preparable by the method as described herein, which is in frozen form or in liquid form. In yet another aspect, the present disclosure provides a ready-to-use pharmaceutical composition preparable by a method, the method comprising the steps of providing a frozen composition prepared by the method as described herein, and thawing the frozen composition thereby obtaining the ready-to-use pharmaceutical composition; or the method comprising the step of providing a liquid composition prepared by the method as described herein, thereby obtaining the ready-to-use pharmaceutical composition.

In a further aspect, the present disclosure provides a composition as described herein for use in therapy. In a further aspect, the present disclosure provides a use of the composition as described herein in the manufacture of a medicament for inducing an immune response in a subject.

In a first example, the present disclosure provides a composition comprising lipid nanoparticles (LNPs) dispersed in an aqueous phase, wherein the LNPs comprise a cationically ionizable lipid and RNA; the aqueous phase comprises a buffer system comprising a buffer substance selected from the group consisting of Tris and its protonated form, bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (Bis-Tris-methane) and its protonated form, and triethanolamine (TEA) and its protonated form, and the monovalent anion being selected from the group consisting of chloride, acetate, glycolate, lactate, the anion of morpholinoethanesulfonic acid (MES), the anion of 3-(N-morpholino)propanesulfonic acid (MOPS), and the anion of 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES); the concentration of the buffer substance in the composition is at most about 25 mM; and the aqueous phase

is substantially free of inorganic phosphate anions, substantially free of citrate anions, and substantially free of anions of ethylenediaminetetraacetic acid (EDTA).

As demonstrated in the present application, using a buffer system based on the particular buffer substances specified above, in particular Tris and its protonated form, instead of PBS in a composition comprising LNPs inhibits the formation of a very stable folded form of RNA. Furthermore, the present application demonstrates that, surprisingly, by simply lowering the concentration of the buffer substance in a composition comprising LNPs and a buffer system, wherein the LNPs comprise a cationically ionizable lipid and RNA, it is possible to obtain an RNA LNP composition having improved RNA integrity after a freeze-thaw-cycle compared to a composition comprising the same buffer substance in a concentration of 50 mM. Thus, the claimed composition provides improved stability, can be stored in a temperature range compliant to regular technologies in pharmaceutical practice, and provides a ready-to-use composition.

In a preferred example of the first example, the buffer substance is Tris and its protonated form, i.e., a mixture of Tris and its protonated form.

In one example, the monovalent anion is selected from the group consisting of chloride, acetate, glycolate, lactate, morpholinoethanesulfonate, and 3-(N-morpholino)propanesulfonate, or from the group consisting of chloride, acetate, glycolate, lactate, morpholinoethanesulfonate, and 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonate, preferably from the group consisting of chloride, acetate, lactate, and morpholinoethanesulfonate, more preferably from the group consisting of chloride, acetate, and morpholinoethanesulfonate, or from the group consisting of chloride, acetate, and lactate, such as chloride or acetate.

In one example, the buffer substance is Tris and its protonated form and the monovalent anion is selected from the group consisting of chloride, acetate, glycolate, lactate, morpholinoethanesulfonate, and 3-(N-morpholino)propanesulfonate, or from the group consisting of chloride, acetate, glycolate, lactate, morpholinoethanesulfonate, and 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonate, preferably from the group consisting of chloride, acetate, lactate, and morpholinoethanesulfonate, more preferably from the group consisting of chloride, acetate, and morpholinoethanesulfonate, or from the group consisting of chloride, acetate, and lactate, such as chloride or acetate.

In one example of the first example, the concentration of the buffer substance, in particular the total concentration of Tris and its protonated form, in the composition is at most about 20 mM, such as at most about 19 mM, at most about 18 mM, at most about 17 mM, at most about 16 mM, at most about 15 mM, at most about 14 mM, at most about 13 mM, at most about 12 mM, at most about 11 mM, or at most about 10 mM. In one example, the lower limit of the buffer substance, in particular Tris and its protonated form, in the composition is at least about 1 mM, preferably at least about 2 mM, such as at least about 3 mM, at least about 4 mM, at least about 5 mM, at least about 6 mM, at least about 7 mM, at least about 8 mM, or at least about 9 mM. For example, the concentration of the buffer substance, in particular the total concentration of Tris and its protonated form, in the composition may be between about 1 mM and about 20 mM, such as between about 2 mM and about 15 mM, between about 5 mM and about 14 mM, between about 7 mM and about 13 mM, between about 8 mM and about 12 mM, between about 9 mM and about 11 mM, such as about 10 mM.

In one example of the first example, the aqueous phase is substantially free of inorganic sulfate anions and/or carbonate anions and/or dibasic organic acid anions and/or polybasic organic acid anions. In a first subgroup, at least one of these criteria applies. For example, in one example of this first subgroup, the aqueous phase is substantially free of inorganic sulfate anions. In a further example

of this first subgroup, the aqueous phase is substantially free of carbonate anions. In a further example of this first subgroup, the aqueous phase is substantially free of dibasic organic acid anions. In a further example of this first subgroup, the aqueous phase is substantially free of polybasic organic acid anions.

In a second subgroup of the first example, at least two of the above criteria apply. For example, in one example of this second subgroup, the aqueous phase is substantially free of inorganic sulfate anions and substantially free of carbonate anions. In a further example of this second subgroup, the aqueous phase is substantially free of inorganic sulfate anions and substantially free of dibasic organic acid anions. In a further example of this second subgroup, the aqueous phase is substantially free of inorganic sulfate anions and substantially free of polybasic organic acid anions. In a further example of this second subgroup, the aqueous phase is substantially free of carbonate anions and substantially free of dibasic organic acid anions. In a further example of this second subgroup, the aqueous phase is substantially free of carbonate anions and substantially free of polybasic organic acid anions. In a further example of this second subgroup, the aqueous phase is substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions.

In a third subgroup of the first example, at least three of the above criteria apply. For example, in one example of this third subgroup, the aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions and substantially free of dibasic organic acid anions. In a further example of this third subgroup, the aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions and substantially free of polybasic organic acid anions. In a further example of this third subgroup, the aqueous phase is substantially free of inorganic sulfate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions. In a further example of this third subgroup, the aqueous phase is substantially free of carbonate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions.

In a fourth subgroup of the first example, at least four of the above criteria apply. *I.e.*, in this fourth subgroup, the aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example), the composition comprises a cryoprotectant. In an alternative example of the first example (in particular in one example of the above first, second, third, or

fourth subgroup of the first example), the composition is substantially free of a cryoprotectant. Thus, particular examples of these examples are the following:

(1) the aqueous phase is substantially free of inorganic sulfate anions, and the composition comprises a cryoprotectant;

(2) the aqueous phase is substantially free of carbonate anions, and the composition comprises a cryoprotectant;

(3) the aqueous phase is substantially free of dibasic organic acid anions, and the composition comprises a cryoprotectant;

(4) the aqueous phase is substantially free of polybasic organic acid anions, and the composition comprises a cryoprotectant;

(5) the aqueous phase is substantially free of inorganic sulfate anions and substantially free of carbonate anions, and the composition comprises a cryoprotectant;

(6) the aqueous phase is substantially free of inorganic sulfate anions and substantially free of dibasic organic acid anions, and the composition comprises a cryoprotectant;

(7) the aqueous phase is substantially free of inorganic sulfate anions and substantially free of polybasic organic acid anions, and the composition comprises a cryoprotectant;

(8) the aqueous phase is substantially free of carbonate anions and substantially free of dibasic organic acid anions, and the composition comprises a cryoprotectant;

(9) the aqueous phase is substantially free of carbonate anions and substantially free of polybasic organic acid anions, and the composition comprises a cryoprotectant;

(10) the aqueous phase is substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the composition comprises a cryoprotectant;

(11) the aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions and substantially free of dibasic organic acid anions, and the composition comprises a cryoprotectant;

(12) the aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions and substantially free of polybasic organic acid anions, and the composition comprises a cryoprotectant;

(13) the aqueous phase is substantially free of inorganic sulfate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the composition comprises a cryoprotectant;

(14) the aqueous phase is substantially free of carbonate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the composition comprises a cryoprotectant;

(15) the aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the composition comprises a cryoprotectant;

(16) the aqueous phase is substantially free of inorganic sulfate anions, and the composition is substantially free of a cryoprotectant;

(17) the aqueous phase is substantially free of carbonate anions, and the composition is substantially free of a cryoprotectant;

(18) the aqueous phase is substantially free of dibasic organic acid anions, and the composition is substantially free of a cryoprotectant;

(19) the aqueous phase is substantially free of polybasic organic acid anions, and the composition is substantially free of a cryoprotectant;

(20) the aqueous phase is substantially free of inorganic sulfate anions and substantially free of carbonate anions, and the composition is substantially free of a cryoprotectant;

(21) the aqueous phase is substantially free of inorganic sulfate anions and substantially free of dibasic organic acid anions, and the composition is substantially free of a cryoprotectant;

(22) the aqueous phase is substantially free of inorganic sulfate anions and substantially free of polybasic organic acid anions, and the composition is substantially free of a cryoprotectant;

(23) the aqueous phase is substantially free of carbonate anions and substantially free of dibasic organic acid anions, and the composition is substantially free of a cryoprotectant;

(24) the aqueous phase is substantially free of carbonate anions and substantially free of polybasic organic acid anions, and the composition is substantially free of a cryoprotectant;

(25) the aqueous phase is substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the composition is substantially free of a cryoprotectant;

(26) the aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions and substantially free of dibasic organic acid anions, and the composition is substantially free of a cryoprotectant;

(27) the aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions and substantially free of polybasic organic acid anions, and the composition is substantially free of a cryoprotectant;

(28) the aqueous phase is substantially free of inorganic sulfate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the composition is substantially free of a cryoprotectant;

(29) the aqueous phase is substantially free of carbonate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the composition is substantially free of a cryoprotectant;

(30) the aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the composition is substantially free of a cryoprotectant.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), wherein the composition comprises a cryoprotectant, said cryoprotectant comprises one or more compounds selected from the group consisting of carbohydrates and sugar alcohols. For example, the cryoprotectant may be selected from the group consisting of sucrose, glucose, glycerol, sorbitol, and a combination thereof. In a preferred example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the composition comprises sucrose and/or glycerol as cryoprotectant.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), wherein the composition comprises a cryoprotectant, the concentration of the cryoprotectant in the composition is at least 1% w/v, such as at least 2% w/v, at least 3% w/v, at least 4% w/v, at least 5% w/v, at least 6% w/v, at least 7% w/v, at least 8% w/v, or at least 9% w/v. In one example, the concentration of the cryoprotectant in the composition is up to 25% w/v, such as up to 20% w/v, up to 19% w/v, up to 18% w/v, up to 17% w/v, up to 16% w/v, up to 15% w/v, up to 14% w/v, up to 13% w/v, up to 12% w/v, or up to 11% w/v. In one example, the concentration of the cryoprotectant in the composition is 1% w/v to 20% w/v, such as 2% w/v to 19% w/v, 3% w/v to 18% w/v, 4% w/v to 17% w/v, 5% w/v to 16% w/v, 5% w/v to 15% w/v, 6% w/v to 14% w/v, 7% w/v to 13% w/v, 8% w/v to 12% w/v, 9% w/v to 11% w/v, or about 10% w/v. In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the composition comprises a cryoprotectant (in particular, sucrose and/or glycerol) in a concentration of from 5% w/v to 15% w/v, such as from 6% w/v to 14% w/v, from 7% w/v to 13% w/v, from 8% w/v to 12% w/v, or from 9% w/v to 11% w/v, or in a concentration of about 10% w/v.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), wherein the composition comprises a cryoprotectant, the cryoprotectant is present in a concentration resulting in an osmolality of the composition in the range of from about 50 x 10^{-3} osmol/kg to about 400 x 10^{-3} osmol/kg (such as from about 50 x 10^{-3} osmol/kg to about 390 x 10^{-3} osmol/kg, from about 60 x 10^{-3} osmol/kg to about 380 x 10^{-3} osmol/kg, from about 70 x 10^{-3} osmol/kg to about 370 x 10^{-3} osmol/kg, from about 80 x 10^{-3} osmol/kg to about 360 x 10^{-3} osmol/kg, from about 90 x 10^{-3} osmol/kg to about 350 x 10^{-3} osmol/kg, from about 100 x 10^{-3} osmol/kg to about 340 x 10^{-3} osmol/kg, from about 120 x 10^{-3} osmol/kg to about 330 x 10^{-3} osmol/kg, from about 140 x 10^{-3} osmol/kg to about 320 x 10^{-3} osmol/kg, from about 160 x 10^{-3} osmol/kg to about 310 x 10^{-3} osmol/kg, from about 180 x 10^{-3} osmol/kg

to about 300 x 10^{-3} osmol/kg, or from about 200 x 10^{-3} osmol/kg to about 300 x 10^{-3} osmol/kg), based on the total weight of the composition.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), in particular in those examples of the first example, where the buffer substance is Tris and its protonated form, the monovalent anion is selected from the group consisting of chloride, acetate, glycolate, and lactate, and the concentration of the monovalent anion (in particular the total concentration of all monovalent anions) in the composition is at most equal to the concentration of the buffer substance in the composition. For example, the concentration of the monovalent anion (in particular the total concentration of all monovalent anions) in the composition may be less than the concentration of the buffer substance in the composition. Thus, in those examples of the first example, where the concentration of the buffer substance, in particular Tris and its protonated form, in the composition is at most about 20 mM, the concentration of the monovalent anion (in particular the total concentration of all monovalent anions) in the composition is at most equal to about 20 mM, *e.g.*, less than 20 mM. Generally, the concentration of the monovalent anion, such as chloride and/or acetate (in particular the total concentration of all monovalent anions) in the composition may be less than about 15 mM, such as less than about 14 mM, less than about 13 mM, less than about 12 mM, less than about 11 mM, less than about 10 mM, less than about 9 mM, less than about 8 mM, less than about 7 mM, less than about 6 mM, or less than about 5 mM. In one example, the chloride concentration in the composition is as defined above (e.g., less than about 15 mM, etc.) and the composition does not comprise acetate. In an alternative example, the acetate concentration in the composition is as defined above (e.g., less than about 15 mM, etc.) and the composition does not comprise chloride.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), in particular in those examples of the first example, where the buffer substance is Tris and its protonated form, the sodium concentration in the aqueous phase and/or composition is less than 20 mM, such as less than about 15 mM, e.g., less than about 14 mM, less than about 13 mM, less than about 12 mM, less than about 11 mM, less than about 10 mM, less than about 9 mM, less than about 8 mM, less than about 7 mM, less than about 6 mM, or less than about 5 mM.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), in particular in those examples of the first example, where the buffer substance is Tris and its protonated form, the monovalent anion is selected from the group consisting of the anions of MES, MOPS and HEPES, and the concentration of the monovalent anion (in particular the total concentration of all

monovalent anions) in the composition is at least equal to the concentration of the buffer substance in the composition. For example, the concentration of the monovalent anion (in particular the total concentration of all monovalent anions) in the composition may be higher than the concentration of the buffer substance in the composition. Thus, in those examples of the first example, where the concentration of the buffer substance, in particular Tris and its protonated form, in the composition is at most about 20 mM, the concentration of the monovalent anion (in particular the total concentration of all monovalent anions) in the composition is at least equal to about 20 mM, *e.g.*, higher than 20 mM. Generally, the concentration of the monovalent anion (in particular the total concentration of all monovalent anions) in the composition may be higher than about 20 mM, such as higher than about 21 mM, higher than about 22 mM, higher than about 23 mM, higher than about 24 mM, higher than about 25 mM, higher than about 26 mM, higher than about 27 mM, higher than about 28 mM, higher than about 29 mM, or higher than about 30 mM, and preferably at most 50 mM, such as at most 45 mM, at most 40 mM or at most 35 mM.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the pH of the composition is between about 6.5 and about 8.0. For example, the pH of the composition may be between about 6.9 and about 7.9, such as between about 7.0 and about 7.9, between about 7.1 and about 7.8, between about 7.2 and about 7.7, between about 7.3 and about 7.6, between about 7.4 and about 7.6, or about 7.5.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the composition comprises water as the main component and/or the total amount of solvent(s) other than water contained in the composition is less than about 1.0% (v/v). For example, the amount of water contained in the composition may be at least 50% (w/w), such as at least at least 55% (w/w), at least 60% (w/w), at least 65% (w/w), at least 70% (w/w), at least 75% (w/w), at least 80% (w/w), at least 85% (w/w), at least 90% (w/w), or at least 95% (w/w). In particular, if the composition comprises a cryoprotectant, the amount of water contained in the composition may be at least 50% (w/w), such as at least at least 55% (w/w), at least 60% (w/w), at least 65% (w/w), at least 70% (w/w), at least 75% (w/w), at least 80% (w/w), at least 85% (w/w), or at least 90% (w/w). If the composition is substantially free of a cryoprotectant, the amount of water contained in the composition may be at least 95% (w/w). Additionally or alternatively, the total amount of solvent(s) other than water contained in the composition may be less than about 1.0% (v/v), such as less than about 0.9% (v/v), less than about 0.8% (v/v), less than about 0.7% (v/v), less than about 0.6% (v/v), less than about 0.5% (v/v), less than about 0.4% (v/v), less than about 0.3% (v/v), less than about 0.2% (v/v), less than about 0.1% (v/v), less than about 0.05% (v/v), or less than about 0.01% (v/v). In this respect, a cryoprotectant which is liquid under

normal conditions will not be considered as a solvent other than water but as cryoprotectant. In other words, the above optional limitation that the total amount of solvent(s) other than water contained in the composition may be less than about 1.0% (v/v) does not apply to cryoprotectants which are liquids under normal conditions.

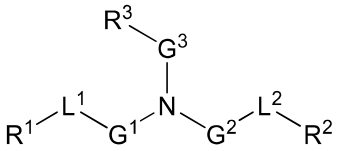
In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the osmolality of the composition is at most about 400 x 10^{-3} osmol/kg, such as at most about 390 x 10^{-3} osmol/kg, at most about 380 x 10^{-3} osmol/kg, at most about 370 x 10^{-3} osmol/kg, at most about 360 x 10^{-3} osmol/kg, at most about 350 x 10^{-3} osmol/kg, at most about 340 x 10^{-3} osmol/kg, at most about 330 x 10^{-3} osmol/kg, at most about 320 x 10^{-3} osmol/kg, at most about 310 x 10^{-3} osmol/kg, or at most about 300 x 10^{-3} osmol/kg. If the composition does not comprise a cryoprotectant, the osmolality of the composition may be below 300 x 10^{-3} osmol/kg, such as at most about 250 x 10^{-3} osmol/kg, at most about 200 x 10^{-3} osmol/kg, at most about 150 x 10^{-3} osmol/kg, at most about 100 x 10^{-3} osmol/kg, at most about 50 x 10^{-3} osmol/kg, at most about 40 x 10^{-3} osmol/kg, or at most about 30 x 10^{-3} osmol/kg. If the composition comprises a cryoprotectant, it is preferred that the main part of the osmolality of the composition is provided by the cryoprotectant. For example, the cryoprotectant may provide at least 50%, such as at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90%, of the osmolality of the composition.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the concentration of the RNA in the composition is about 5 mg/l to about 150 mg/l. For example, the concentration of the RNA in the composition may be about 10 mg/l to about 140 mg/l, such as about 20 mg/l to about 130 mg/l, about 25 mg/l to about 125 mg/l, about 30 mg/l to about 120 mg/l, about 35 mg/l to about 115 mg/l, about 40 mg/l to about 110 mg/l, about 45 mg/l to about 105 mg/l, or about 50 mg/l to about 100 mg/l.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the buffer substance is Tris and its protonated form, the pH of the composition is between about 6.5 and about 8.0, and the concentration of the RNA in the composition is about 5 mg/l to about 150 mg/l. In this example, it is preferred that the pH of the composition is between about 6.9 and about 7.9 and the concentration of the RNA in the composition is about 25 mg/l to about 125 mg/l, such as about 30 mg/l to about 120 mg/l. In particularly preferred example of the claimed composition, the buffer substance is Tris and its protonated form; the pH of the composition is between about 6.9 and about 7.9; the concentration of the RNA in the composition is about 30 mg/l to about 120 mg/l; the aqueous phase

is substantially free of inorganic sulfate anions, substantially free of dibasic organic acids and substantially free of polybasic organic acids; and the composition comprises a cryoprotectant. In an alternative particularly preferred example of the claimed composition, the buffer substance is Tris and its protonated form; the pH of the composition is between about 6.9 and about 7.9; the concentration of the RNA in the composition is about 30 mg/l to about 120 mg/l; the aqueous phase is substantially free of inorganic sulfate anions, substantially free of dibasic organic acids and substantially free of polybasic organic acids; and the composition is substantially free of a cryoprotectant.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the cationically ionizable lipid comprises a head group which includes at least one nitrogen atom which is capable of being protonated under physiological conditions. For example, the cationically ionizable lipid may have the structure of Formula (I):

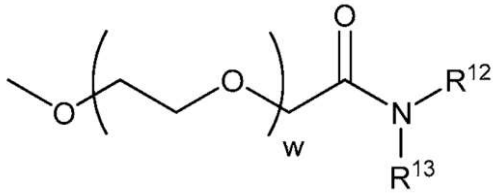


(I)

or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein L^1, L^2, G^1, G^2, G^3, R^1, R^2, and R^3 are as defined herein. Preferably, the cationically ionizable lipid is selected from the following: structures I-1 to I-36 (shown herein); and/or structures A to F (shown herein); and/or N,N-dimethyl-2,3-dioleyloxypropylamine (DODMA), 1,2-dioleoyl-3-dimethylammonium-propane (DODAP), heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino)butanoate (DLin-MC3-DMA), and 4-((di((9Z,12Z)-octadeca-9,12-dien-1-yl)amino)oxy)-N,N-dimethyl-4-oxobutan-1-amine (DPL-14). In a particularly preferred example, the cationically ionizable lipid is the lipid having the structure I-3.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the LNPs further comprise one or more additional lipids. Preferably, the one or more additional lipids are selected from the group consisting of polymer conjugated lipids, neutral lipids, steroids, and combinations thereof. In a preferred example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the LNPs comprise the cationically ionizable lipid as described herein, a polymer conjugated lipid (*e.g.*, a pegylated lipid or a polysarcosine-lipid conjugate or a conjugate of polysarcosine and a lipid-like material), a neutral lipid (*e.g.*, DSPC), and a steroid (*e.g.*, cholesterol).

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), wherein the LNPs further comprise a polymer conjugated lipid as one of the one or more additional lipids, the polymer conjugated lipid is a pegylated lipid. For example, the pegylated lipid may have the following structure:



or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein R^{12}, R^{13}, and w are as defined herein.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), wherein the LNPs further comprise a polymer conjugated lipid as one of the one or more additional lipids, the polymer conjugated lipid is a polysarcosine-lipid conjugate or a conjugate of polysarcosine and a lipid-like material. For example, the polysarcosine-lipid conjugate or conjugate of polysarcosine and a lipid-like material may be a member selected from the group consisting of a polysarcosine-diacylglycerol conjugate, a polysarcosine-dialkyloxypropyl conjugate, a polysarcosine-phospholipid conjugate, a polysarcosine-ceramide conjugate, and a mixture thereof.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), wherein the LNPs further comprise a neutral lipid as one of the one or more additional lipids, the neutral lipid is a phospholipid. Such phospholipid is preferably selected from the group consisting of phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidic acids, phosphatidylserines and sphingomyelins. Particular examples of phospholipids include distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dimyristoylphosphatidylcholine (DMPC), dipentadecanoylphosphatidylcholine, dilauroylphosphatidylcholine, dipalmitoylphosphatidylcholine (DPPC), diarachidoylphosphatidylcholine (DAPC), dibehenoylphosphatidylcholine (DBPC), ditricosanoylphosphatidylcholine (DTPC), dilignoceroylphatidylcholine (DLPC), palmitoyloleoyl-phosphatidylcholine (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 (C16 Lyso PC), dioleoylphosphatidylethanolamine (DOPE), distearoyl-phosphatidylethanolamine (DSPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-

phosphatidylethanolamine (DMPE), dilauroyl-phosphatidylethanolamine (DLPE), and diphytanoyl-phosphatidylethanolamine (DpyPE). In a particularly preferred example, the neutral lipid is DSPC.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), wherein the LNPs further comprise a steroid as one of the one or more additional lipids, the steroid is a sterol such as cholesterol.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the aqueous phase does not comprise a chelating agent.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the LNPs comprise at least about 75% of the RNA comprised in the composition. For example, the LNPs may comprise at least about 76%, such as at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, or at least about 95% of the RNA comprised in the composition.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the RNA (such as mRNA) is encapsulated within or associated with the LNPs.

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the RNA (such as mRNA) comprises a modified nucleoside in place of uridine. For example, the modified nucleoside may be selected from pseudouridine (ψ), N1-methyl-pseudouridine (m1ψ), and 5-methyl-uridine (m5U).

In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the RNA (such as mRNA) comprises one or more of the following (a) a 5’ cap, such as a cap1 or cap2 structure; (b) a 5’ UTR; (c) a 3’ UTR; and (d) a poly-A sequence, such as a poly-A sequence comprising at least 100 nucleotides, wherein the poly-A sequence preferably is an interrupted sequence of A nucleotides.

In one preferred example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the RNA (such as mRNA) encodes one or more polypeptides. For example, the one or more polypeptides may comprise an epitope for inducing an immune response against an antigen in a subject. In a preferred example, the RNA (such as mRNA) comprises an open reading frame (ORF) encoding an amino acid sequence comprising a SARS-CoV-2 S protein, an immunogenic variant thereof, or an immunogenic fragment of the SARS-CoV-2 S protein or the immunogenic variant thereof. In one example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the RNA (such as mRNA) comprises an ORF encoding a full-length SARS-CoV2 S protein variant with proline residue substitutions at positions 986 and 987 of SEQ ID NO: 1. For example, the SARS-CoV2 S protein variant may have at least 80% identity to SEQ ID NO: 7.

In one preferred example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the composition is in frozen form. Preferably, the RNA integrity after thawing the frozen composition is at least 50%, such as at least 52%, at least 54%, at least 55%, at least 56%, at least 58%, or at least 60%, *e.g.*, after thawing the frozen composition which has been stored at -20°C. Additionally or alternatively, the size ($Z_{average}$) (and/or size distribution and/or polydispersity index (PDI)) of the LNPs after thawing the frozen composition is equal to the size ($Z_{average}$) (and/or size distribution and/or PDI) of the LNPs before the composition has been frozen. In one example, the size ($Z_{average}$) of the LNPs after thawing the frozen composition is between about 50 nm and about 500 nm, preferably between about 40 nm and about 200 nm, more preferably between about 40 nm and about 120 nm. In one example, the PDI of the LNPs after thawing the frozen composition is less than 0.3, preferably less than 0.2, more preferably less than 0.1. In one example, the size ($Z_{average}$) of the LNPs after thawing the frozen composition is between about 50 nm and about 500 nm, preferably between about 40 nm and about 200 nm, more preferably between about 40 nm and about 120 nm, and the size ($Z_{average}$) (and/or size distribution and/or PDI) of the LNPs after thawing the frozen composition is equal to the size ($Z_{average}$) (and/or size distribution and/or PDI) of the LNPs before freezing. In one example, the size ($Z_{average}$) of the LNPs after thawing the frozen composition is between about 50 nm and about 500 nm, preferably between about 40 nm and about 200 nm, more preferably between about 40 nm and about 120 nm, and the PDI of the LNPs after thawing the frozen composition is less than 0.3 (preferably less than 0.2, more preferably less than 0.1).

In one alternative preferred example of the first example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30)

listed above), the composition is in liquid form. Preferably, the RNA integrity of the liquid composition, when stored, e.g., at 0°C or higher for at least one week, is sufficient to produce the desired effect, e.g., to induce an immune response. For example, the RNA integrity of the liquid composition, when stored, e.g., at 0°C or higher for at least one week, may be at least 50%, such as at least 52%, at least 54%, at least 55%, at least 56%, at least 58%, or at least 60%. Additionally or alternatively, the size ($Z_{average}$) (and/or size distribution and/or polydispersity index (PDI)) of the LNPs of the liquid composition, when stored, e.g., at 0°C or higher for at least one week, is sufficient to produce the desired effect, e.g., to induce an immune response. For example, the size ($Z_{average}$) (and/or size distribution and/or polydispersity index (PDI)) of the LNPs of the liquid composition, when stored, e.g., at 0°C or higher for at least one week, is equal to the size ($Z_{average}$) (and/or size distribution and/or PDI) of the LNPs of the initial composition, i.e., before storage. In one example, the size ($Z_{average}$) of the LNPs after storage of the liquid composition e.g., at 0°C or higher for at least one week is between about 50 nm and about 500 nm, preferably between about 40 nm and about 200 nm, more preferably between about 40 nm and about 120 nm. In one example, the PDI of the LNPs after storage of the liquid composition e.g., at 0°C or higher for at least one week is less than 0.3, preferably less than 0.2, more preferably less than 0.1. In one example, the size ($Z_{average}$) of the LNPs after storage of the liquid composition e.g., at 0°C or higher for at least one week is between about 50 nm and about 500 nm, preferably between about 40 nm and about 200 nm, more preferably between about 40 nm and about 120 nm, and the size ($Z_{average}$) (and/or size distribution and/or PDI) of the LNPs after storage of the liquid composition e.g., at 0°C or higher for at least one week is equal to the size ($Z_{average}$) (and/or size distribution and/or PDI) of the LNPs before storage. In one example, the size ($Z_{average}$) of the LNPs after storage of the liquid composition e.g., at 0°C or higher for at least one week is between about 50 nm and about 500 nm, preferably between about 40 nm and about 200 nm, more preferably between about 40 nm and about 120 nm, and the PDI of the LNPs after storage of the liquid composition e.g., at 0°C or higher for at least one week is less than 0.3 (preferably less than 0.2, more preferably less than 0.1).

In a second example, the present disclosure provides a method of preparing a composition comprising LNPs dispersed in a final aqueous phase, wherein the LNPs comprise a cationically ionizable lipid and RNA; the final aqueous phase comprises a buffer system comprising a final buffer substance and a final monovalent anion, the final buffer substance being selected from the group consisting of Tris and its protonated form, Bis-Tris-methane and its protonated form, and TEA and its protonated form, and the final monovalent anion being selected from the group consisting of chloride, acetate, glycolate, lactate, the anion of MES, the anion of MOPS, and the anion of HEPES; the concentration of the final buffer substance in the composition is at most about 25 mM; and the final aqueous phase is substantially free of inorganic phosphate anions, substantially free of citrate anions, and substantially free of anions of EDTA; wherein the method comprises:

(I) preparing a formulation comprising LNPs dispersed in the final aqueous phase, wherein the LNPs comprise the cationically ionizable lipid and RNA; and
(II) optionally freezing the formulation to about -10°C or below,
thereby obtaining the composition,
wherein step (I) comprises:
(a) preparing an RNA solution containing water and a first buffer system;
(b) preparing an ethanolic solution comprising the cationically ionizable lipid and, if present, one or more additional lipids;
(c) mixing the RNA solution prepared under (a) with the ethanolic solution prepared under (b), thereby preparing an intermediate formulation comprising the LNPs dispersed in an intermediate aqueous phase comprising the first buffer system; and
(d) filtrating the first intermediate formulation prepared under (c) using a final aqueous buffer solution comprising the final buffer system,
thereby preparing the formulation comprising the LNPs dispersed in the final aqueous phase.

As demonstrated in the present application, using a particular buffer system based on the above specified buffer substances, in particular Tris and its protonated form, instead of PBS in a composition comprising LNPs inhibits the formation of a very stable folded form of RNA. Furthermore, the present application demonstrates that, surprisingly, by simply lowering the concentration of the buffer substance in a composition comprising LNPs and a buffer system, wherein the LNPs comprise a cationically ionizable lipid and RNA, it is possible to obtain an LNP RNA composition having improved RNA integrity after a freeze/thaw cycle compared to a composition comprising the same buffer substance in a concentration of 50 mM. Thus, the composition prepared by the claimed method provides improved stability, can be stored in a temperature range compliant to regular technologies in pharmaceutical practice, and provides a ready-to-use preparation.

In a particularly preferred example of the second example, the final buffer substance is Tris and its protonated form, i.e., a mixture of Tris and its protonated form.

In one example of the second example, the final monovalent anion is selected from the group consisting of chloride, acetate, glycolate, lactate, morpholinoethanesulfonate, and 3-(N-morpholino)propanesulfonate, or from the group consisting of chloride, acetate, glycolate, lactate, morpholinoethanesulfonate, and 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonate, preferably from the group consisting of chloride, acetate, lactate, and morpholinoethanesulfonate, more preferably from the group consisting of chloride, acetate, and morpholinoethanesulfonate, or from the group consisting of chloride, acetate, and lactate, such as chloride or acetate.

In one example of the second example, the final buffer substance is Tris and its protonated form and the final monovalent anion is selected from the group consisting of chloride, acetate, glycolate, lactate, morpholinoethanesulfonate, and 3-(N-morpholino)propanesulfonate, or from the group consisting of chloride, acetate, glycolate, lactate, morpholinoethanesulfonate, and 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonate, preferably from the group consisting of chloride, acetate, lactate, and morpholinoethanesulfonate, more preferably from the group consisting of chloride, acetate, lactate, and morpholinoethanesulfonate, more preferably from the group consisting of chloride, acetate, and morpholinoethanesulfonate, such as chloride or acetate

In one example, in particular if it is desired to prepare a composition in frozen form, the method of the second example comprises (II) freezing the formulation to about -10°C or below. Thus, in this example, conducting the method of the second example results in a composition in frozen form.

In an alternative example, in particular if it is desired to prepare a composition in liquid form, the method of the second example does not comprises step (II). Thus, in this example, conducting the method of the second example results in a composition in liquid form.

In one example of the second example, step (I) further comprises one or more steps selected from diluting and filtrating, such as tangential flow filtrating and diafiltrating, after step (c). For example, a diluting step may comprise adding a dilution solution to an intermediate formulation. Such dilution solution may comprise one or more additional compounds and optionally the final buffer system, wherein the one or more additional compounds may comprise a cryoprotectant. The one or more filtrating steps (including steps (d), (f'), (g'), and (h')) may be used to remove unwanted compounds (e.g., ethanol and/or one or more di- and/or polybasic organic acids) from the intermediate formulation and/or for increasing the RNA concentration of the intermediate formulation and/or for changing the pH and/or the buffer system of the intermediate formulation. To this end, an aqueous buffer solution can be used, which does not contain the unwanted compounds (such that the unwanted compounds are washed out from the intermediate formulation and into the aqueous buffer solution) and/or which is hypertonic compared to the aqueous buffer solution (such that water flows from the intermediate formulation to the aqueous buffer solution) and/or which has a pH and/or buffer system other than the pH and/or buffer system of the intermediate formulation.

In a preferred example of the second example, step (I) comprises:
(a') providing an aqueous RNA solution;
(b') providing a first aqueous buffer solution comprising a first buffer system;
(c') mixing the aqueous RNA solution provided under (a') with the first aqueous buffer solution provided under (b') thereby preparing an RNA solution containing water and the first buffer system;

(d') preparing an ethanolic solution comprising the cationically ionizable lipid and, if present, one or more additional lipids;

(e') mixing the RNA solution prepared under (c') with the ethanolic solution prepared under (d'), thereby preparing a first intermediate formulation comprising LNPs dispersed in a first aqueous phase comprising the first buffer system;

(f') optionally filtrating the first intermediate formulation prepared under (e') using a further aqueous buffer solution comprising a further buffer system, thereby preparing a further intermediate formulation comprising the LNPs dispersed in a further aqueous phase comprising the further buffer system, wherein the further aqueous buffer solution may be identical to or different from the first aqueous buffer solution;

(g') optionally repeating step (f') once or two or more times, wherein the further intermediate formulation comprising the LNPs dispersed in the further aqueous phase comprising the further buffer system obtained after step (f') of one cycle is used as the first intermediate formulation of the next cycle, wherein in each cycle the further aqueous buffer solution may be identical to or different from the first aqueous buffer solution;

(h') filtrating the first intermediate formulation obtained in step (e'), if step (f') is absent, or the further intermediate formulation obtained in step (f'), if step (f') is present and step (g') is not present, or the further intermediate formulation obtained after step (g'), if steps (f') and (g') are present, using a final aqueous buffer solution comprising the final buffer system and having a pH of at least 6.0; and

(i') optionally diluting the formulation obtained in step (h') with a dilution solution;

thereby preparing the formulation comprising the LNPs dispersed in the final aqueous phase.

In one example of the second example, the concentration of the final buffer substance, in particular the total concentration of Tris and its protonated form, in the composition is at most about 20 mM, such as at most about 19 mM, at most about 18 mM, at most about 17 mM, at most about 16 mM, at most about 15 mM, at most about 14 mM, at most about 13 mM, at most about 12 mM, at most about 11 mM, or at most about 10 mM. In one example, the lower limit of the final buffer substance, in particular Tris and its protonated form, in the composition is at least about 1 mM, preferably at least about 2 mM, such as at least about 3 mM, at least about 4 mM, at least about 5 mM, at least about 6 mM, at least about 7 mM, at least about 8 mM, or at least about 9 mM. For example, the concentration of the final buffer substance, in particular the total concentration of Tris and its protonated form, in the composition may be between about 1 mM and about 20 mM, such as between about 2 mM and about 15 mM, between about 5 mM and about 14 mM, between about 7 mM and about 13 mM, between about 8 mM and about 12 mM, between about 9 mM and about 11 mM, such as about 10 mM.

In one example of the second example, the final aqueous phase is substantially free of inorganic sulfate anions and/or carbonate anions and/or dibasic organic acid anions and/or polybasic organic acid anions. In a first subgroup of the second example, at least one of these criteria applies. For example, in one

example of this first subgroup of the second example, the final aqueous phase is substantially free of inorganic sulfate anions. In a further example of this first subgroup of the second example, the final aqueous phase is substantially free of carbonate anions. In a further example of this first subgroup of the second example, the final aqueous phase is substantially free of dibasic organic acid anions. In a further example of this first subgroup of the second example, the final aqueous phase is substantially free of polybasic organic acid anions.

In a second subgroup of the second example, at least two of the above criteria apply. For example, in one example of this second subgroup of the second example, the final aqueous phase is substantially free of inorganic sulfate anions and substantially free of carbonate anions. In a further example of this second subgroup of the second example, the final aqueous phase is substantially free of inorganic sulfate anions and substantially free of dibasic organic acid anions. In a further example of this second subgroup of the second example, the final aqueous phase is substantially free of inorganic sulfate anions and substantially free of polybasic organic acid anions. In a further example of this second subgroup of the second example, the final aqueous phase is substantially free of carbonate anions and substantially free of dibasic organic acid anions. In a further example of this second subgroup of the second example, the final aqueous phase is substantially free of carbonate anions and substantially free of polybasic organic acid anions. In a further example of this second subgroup of the second example, the final aqueous phase is substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions.

In a third subgroup of the second example, at least three of the above criteria apply. For example, in one example of this third subgroup of the second example, the final aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions and substantially free of dibasic organic acid anions. In a further example of this third subgroup of the second example, the final aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions and substantially free of polybasic organic acid anions. In a further example of this third subgroup of the second example, the final aqueous phase is substantially free of inorganic sulfate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions. In a further example of this third subgroup of the second example, the final aqueous phase is substantially free of carbonate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions.

In a fourth subgroup of the second example, at least four of the above criteria apply. *I.e.*, in this fourth subgroup of the second example, the final aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example), the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant. In an alternative example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example), the formulation obtained in step (I) and/or the composition is substantially free of a cryoprotectant. Thus, particular examples of these examples are the following:

(1) the final aqueous phase is substantially free of inorganic sulfate anions, and the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant;

(2) the final aqueous phase is substantially free of carbonate anions, and the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant;

(3) the final aqueous phase is substantially free of dibasic organic acid anions, the formulation obtained in step (I) and/or the composition and comprise(s) a cryoprotectant;

(4) the final aqueous phase is substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant;

(5) the final aqueous phase is substantially free of inorganic sulfate anions and substantially free of carbonate anions, and the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant;

(6) the final aqueous phase is substantially free of inorganic sulfate anions and substantially free of dibasic organic acid anions, and the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant;

(7) the final aqueous phase is substantially free of inorganic sulfate anions and substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant;

(8) the final aqueous phase is substantially free of carbonate anions and substantially free of dibasic organic acid anions, and the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant;

(9) the final aqueous phase is substantially free of carbonate anions and substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant;

(10) the final aqueous phase is substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant;

(11) the final aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions and substantially free of dibasic organic acid anions, and the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant;

(12) the final aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions and substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant;

(13) the final aqueous phase is substantially free of inorganic sulfate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant;

(14) the final aqueous phase is substantially free of carbonate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant;

(15) the final aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant;

(16) the final aqueous phase is substantially free of inorganic sulfate anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant;

(17) the final aqueous phase is substantially free of carbonate anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant;

(18) the final aqueous phase is substantially free of dibasic organic acid anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant;

(19) the final aqueous phase is substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant;

(20) the final aqueous phase is substantially free of inorganic sulfate anions and substantially free of carbonate anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant;

(21) the final aqueous phase is substantially free of inorganic sulfate anions and substantially free of dibasic organic acid anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant;

(22) the final aqueous phase is substantially free of inorganic sulfate anions and substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant;

(23) the final aqueous phase is substantially free of carbonate anions and substantially free of dibasic organic acid anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant;

(24) the final aqueous phase is substantially free of carbonate anions and substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant;

(25) the final aqueous phase is substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant;

(26) the final aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions and substantially free of dibasic organic acid anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant;

(27) the final aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions and substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant;

(28) the final aqueous phase is substantially free of inorganic sulfate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant;

(29) the final aqueous phase is substantially free of carbonate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant;

(30) the final aqueous phase is substantially free of inorganic sulfate anions, substantially free of carbonate anions, substantially free of dibasic organic acid anions and substantially free of polybasic organic acid anions, and the formulation obtained in step (I) and/or the composition is/are substantially free of a cryoprotectant.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), wherein formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant, said cryoprotectant comprises one or more compounds selected from the group consisting of carbohydrates and sugar alcohols. For example, the cryoprotectant may be selected from the group consisting of sucrose, glucose, glycerol, sorbitol, and a combination thereof. In a preferred example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the formulation obtained in step (I) and/or the composition comprise(s) sucrose and/or glycerol as cryoprotectant.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), wherein the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant, the concentration of the cryoprotectant in the formulation and/or composition is at least 1% w/v, such as at least 2% w/v, at least 3% w/v, at least 4% w/v, at least 5% w/v, at least 6% w/v, at least 7% w/v, at least 8% w/v or at least 9% w/v. In one example, the concentration of the cryoprotectant in the formulation and/or composition is up to 25% w/v, such as up to 20% w/v, up to

19% w/v, up to 18% w/v, up to 17% w/v, up to 16% w/v, up to 15% w/v, up to 14% w/v, up to 13% w/v, up to 12% w/v, or up to 11% w/v. In one example, the concentration of the cryoprotectant in the formulation and/or composition is 1% w/v to 20% w/v, such as 2% w/v to 19% w/v, 3% w/v to 18% w/v, 4% w/v to 17% w/v, 5% w/v to 16% w/v, 5% w/v to 15% w/v, 6% w/v to 14% w/v, 7% w/v to 13% w/v, 8% w/v to 12% w/v, 9% w/v to 11% w/v, or about 10% w/v. In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the formulation and/or composition comprise(s) a cryoprotectant (in particular, sucrose and/or glycerol) in a concentration of from 5% w/v to 15% w/v, such as from 6% w/v to 14% w/v, from 7% w/v to 13% w/v, from 8% w/v to 12% w/v, or from 9% w/v to 11% w/v, or in a concentration of about 10% w/v. For example, the method of the second example may comprise a diluting step using a dilution solution, wherein the dilution solution comprises a sufficient amount of a cryoprotectant in order to achieve the above concentrations of cryoprotectant in the formulation obtained in step (I) and/or the composition.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), wherein the formulation obtained in step (I) and/or the composition comprise(s) a cryoprotectant, the cryoprotectant is present in a concentration resulting in an osmolality of the composition in the range of from about 50 x 10^{-3} osmol/kg to about 400 x 10^{-3} osmol/kg (such as from about 50 x 10^{-3} osmol/kg to about 390 x 10^{-3} osmol/kg, from about 60 x 10^{-3} osmol/kg to about 380 x 10^{-3} osmol/kg, from about 70 x 10^{-3} osmol/kg to about 370 x 10^{-3} osmol/kg, from about 80 x 10^{-3} osmol/kg to about 360 x 10^{-3} osmol/kg, from about 90 x 10^{-3} osmol/kg to about 350 x 10^{-3} osmol/kg, from about 100 x 10^{-3} osmol/kg to about 340 x 10^{-3} osmol/kg, from about 120 x 10^{-3} osmol/kg to about 330 x 10^{-3} osmol/kg, from about 140 x 10^{-3} osmol/kg to about 320 x 10^{-3} osmol/kg, from about 160 x 10^{-3} osmol/kg to about 310 x 10^{-3} osmol/kg, from about 180 x 10^{-3} osmol/kg to about 300 x 10^{-3} osmol/kg, or from about 200 x 10^{-3} osmol/kg to about 300 x 10^{-3} osmol/kg), based on the total weight of the formulation/composition. For example, the method of the second example may comprise a diluting step using a dilution solution, wherein the dilution solution comprises a sufficient amount of a cryoprotectant in order to achieve the above osmolality values in the formulation obtained in step (I) and/or the composition.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), in particular in those examples of the second example, where the final buffer substance is Tris and its protonated form, the final monovalent anion is selected from the group consisting of chloride, acetate, glycolate, and lactate, and the concentration of the final monovalent anion (in particular the total concentration of all final monovalent anions) in the composition is at most equal to the concentration of

the final buffer substance in the composition. For example, the concentration of the final monovalent anion (in particular the total concentration of all final monovalent anions) in the composition may be less than the concentration of the final buffer substance in the composition. Thus, in those examples of the second example, where the concentration of the final buffer substance, in particular Tris and its protonated form, in the composition is at most about 20 mM, the concentration of the final monovalent anion (in particular the total concentration of all final monovalent anions) in the composition is at most equal to about 20 mM, *e.g.*, less than 20 mM. Generally, the concentration of the monovalent anion, such as chloride and/or acetate (in particular the total concentration of all monovalent anions) in the composition may be less than about 15 mM, such as less than about 14 mM, less than about 13 mM, less than about 12 mM, less than about 11 mM, less than about 10 mM, less than about 9 mM, less than about 8 mM, less than about 7 mM, less than about 6 mM, or less than about 5 mM. In one example, the chloride concentration in the composition is as defined above (e.g., less than about 15 mM, etc.) and the composition does not comprise acetate. In an alternative example, the acetate concentration in the composition is as defined above (e.g., less than about 15 mM, etc.) and the composition does not comprise chloride.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), in particular in those examples of the second example, where the final buffer substance is Tris and its protonated form, the sodium concentration in the aqueous phase and/or composition is less than 20 mM, such as less than about 15 mM, e.g., less than about 14 mM, less than about 13 mM, less than about 12 mM, less than about 11 mM, less than about 10 mM, less than about 9 mM, less than about 8 mM, less than about 7 mM, less than about 6 mM, or less than about 5 mM.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), in particular in those examples of the second example, where the final buffer substance is Tris and its protonated form, the final monovalent anion is selected from the group consisting of the anions of MES, MOPS and HEPES, and the concentration of the final monovalent anion (in particular the total concentration of all final monovalent anions) in the composition is at least equal to the concentration of the final buffer substance in the composition. For example, the concentration of the final monovalent anion (in particular the total concentration of all final monovalent anions) in the composition may be higher than the concentration of the final buffer substance in the composition. Thus, in those examples of the second example, where the concentration of the final buffer substance, in particular Tris and its protonated form, in the composition is at most about 20 mM, the concentration of the final monovalent anion (in particular the total concentration of all final monovalent anions) in the composition is at least equal to about 20 mM, *e.g.*, higher than 20 mM. Generally, the concentration of the final

monovalent anion (in particular the total concentration of all final monovalent anions) in the composition may be higher than about 20 mM, such as higher than about 21 mM, higher than about 22 mM, higher than about 23 mM, higher than about 24 mM, higher than about 25 mM, higher than about 26 mM, higher than about 27 mM, higher than about 28 mM, higher than about 29 mM, or higher than about 30 mM, and preferably at most 50 mM, such as at most 45 mM, at most 40 mM or at most 35 mM.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the pH of the final buffer system (and the pH of the composition) is between about 6.5 and about 8.0. For example, the pH of the composition may be between about 6.9 and about 7.9, such as between about 7.0 and about 7.9, between about 7.1 and about 7.8, between about 7.2 and about 7.7, between about 7.3 and about 7.6, between about 7.4 and about 7.6, or about 7.5.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the first buffer system (and the pH of the RNA solution obtained in step (a)) has a pH of below 6.0, preferably at most about 5.5, such as at most about 5.0, at most about 4.9, at most about 4.8, at most about 4.7, at most about 4.6, or at most about 4.5. For example, the pH of first buffer system (and the pH of the RNA solution obtained in step (a)) may be between about 3.5 and about 5.9, such as between about 4.0 and about 5.5, or between about 4.5 and about 5.0. To this end, the RNA solution obtained in step (a) may further comprises one or more di- and/or polybasic organic acids (e.g., citrate anions and/or anions of EDTA). In this example, it is preferred that step (d) is conducted under conditions which remove the one or more di- and/or polybasic organic acids resulting in the formulation comprising the LNPs dispersed in final aqueous phase with the final aqueous phase being substantially free of the one or more di- and/or polybasic organic acids. For example, such conditions can include subjecting the intermediate formulation comprising the LNPs dispersed in the intermediate aqueous phase obtained in step (c) to at least one step of filtrating, such as tangential flow filtrating or diafiltrating, using a final buffer solution comprising the final buffer system (i.e., the final buffer substance and the final monovalent anion), wherein the final buffer solution does not contain the one or more di- and/or polybasic organic acids (and preferably does not contain ethanol). Alternatively, such conditions can include (i) subjecting the intermediate formulation comprising the LNPs dispersed in the intermediate aqueous phase obtained in step (c) (*i.e.*, a first intermediate formulation) to at least one step of filtrating, such as tangential flow filtrating or diafiltrating, using a further aqueous buffer solution comprising a further buffer system, thereby preparing a further intermediate formulation comprising the LNPs dispersed in a further aqueous phase comprising the further buffer system, wherein the further buffer system of the further aqueous buffer solution may be identical to or different from the buffer system used in step (a); (ii) optionally repeating step (i) once or two or more times, wherein the further

intermediate formulation comprising the LNPs dispersed in the further aqueous phase obtained after step (i) of one cycle is used as the first intermediate formulation of the next cycle, wherein in each cycle the further buffer system of the further aqueous buffer solution may be identical to or different from the first buffer system used in step (a); and (iii) subjecting the intermediate formulation obtained in step (i) (if step (ii) is not present), or the intermediate formulation obtained in step (ii) (if step (ii) is present) to at least one step of filtrating, such as tangential flow filtrating or diafiltrating, using the final aqueous buffer solution, wherein at least one of the intermediate and final aqueous buffer solutions (preferably all intermediate and final aqueous buffer solutions) does not contain the one or more di- and/or polybasic organic acids (and preferably does not contain ethanol.

Similarly, in one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), where step (I) comprises steps (a') to (e') and (h') (and optionally one or more of steps (f'), (g') and (i')), the first aqueous buffer solution (and the pH of the RNA solution obtained under step (c')) has a pH of below 6.0, preferably at most about 5.5, such as at most about 5.0, at most about 4.9, at most about 4.8, at most about 4.7, at most about 4.6, or at most about 4.5. For example, the pH of the first aqueous buffer solution (and the pH of the RNA solution obtained under step (c')) may be between about 3.5 and about 5.9, such as between about 4.0 and about 5.5, or between about 4.5 and about 5.0. To this end, the first aqueous buffer solution provided under (b') (and the first aqueous phase) may further comprises one or more di- and/or polybasic organic acids (e.g., citrate anions and/or anions of EDTA). In this example, it is preferred that least one of steps (f') to (h') is conducted under conditions which remove the one or more di- and/or polybasic organic acids from the first intermediate formulation and/or from the further intermediate formulation resulting in a further inter formulation comprising the LNPs dispersed in a further aqueous phase or in the final aqueous phase with the further and/or final aqueous phase being substantially free of the one or more di- and/or polybasic organic acids. For example, such conditions can include using a further aqueous buffer solution and/or a final buffer solution, wherein at least one of the further aqueous buffer solution(s) and the final buffer solution (preferably all of the further aqueous buffer solution(s) and the final buffer solution) does not contain the one or more di- and/or polybasic organic acids (and preferably does not contain ethanol). In one example, the filtrating steps can be tangential flow filtrating or diafiltrating, preferably tangential flow filtrating.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the first buffer system used in step (a) comprises the final buffer substance and the final monovalent anion used in step (d), preferably the buffer system and pH of the first buffer system used in step (a) are identical to the buffer system and pH of the final aqueous buffer solution used in step (d). For example, only one aqueous buffer solution is used in this example of the second example.

Similarly, in one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), where step (I) comprises steps (a') to (e') and (h') (and optionally one or more of steps (f'), (g') and (i')), each of the first buffer system and every further buffer system used in steps (b'), (f') and (g') comprises the final buffer substance and the final monovalent anion used in step (h'), preferably the buffer system and pH of each of the first aqueous buffer solution and of every further aqueous buffer solution used in steps (b'), (f') and (g') are identical to the buffer system and pH of the final aqueous buffer solution. For example, the aqueous buffer solutions used in steps (b'), (f'), if present, (g'), if present, and (h') of this example of the second example are identical.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the formulation and/or composition comprise(s) water as the main component and/or the total amount of solvent(s) other than water contained in the composition is less than about 1.0% (v/v). For example, the amount of water contained in the formulation and/or composition may be at least 50% (w/w), such as at least at least 55% (w/w), at least 60% (w/w), at least 65% (w/w), at least 70% (w/w), at least 75% (w/w), at least 80% (w/w), at least 85% (w/w), at least 90% (w/w), or at least 95% (w/w). In particular, if the formulation and/or composition comprise(s) a cryoprotectant, the amount of water contained in the formulation and/or composition comprise(s) may be at least 50% (w/w), such as at least at least 55% (w/w), at least 60% (w/w), at least 65% (w/w), at least 70% (w/w), at least 75% (w/w), at least 80% (w/w), at least 85% (w/w), or at least 90% (w/w). If the formulation and/or composition is/are substantially free of a cryoprotectant, the amount of water contained in the formulation and/or composition may be at least 95% (w/w). Additionally or alternatively, the total amount of solvent(s) other than water contained in the composition may be less than about 1.0% (v/v), such as less than about 0.9% (v/v), less than about 0.8% (v/v), less than about 0.7% (v/v), less than about 0.6% (v/v), less than about 0.5% (v/v), less than about 0.4% (v/v), less than about 0.3% (v/v), less than about 0.2% (v/v), less than about 0.1% (v/v), less than about 0.05% (v/v), or less than about 0.01% (v/v). In this respect, a cryoprotectant which is liquid under normal conditions will not be considered as a solvent other than water but as cryoprotectant. In other words, the above optional limitation that the total amount of solvent(s) other than water contained in the composition may be less than about 1.0% (v/v) does not apply to cryoprotectants which are liquids under normal conditions.

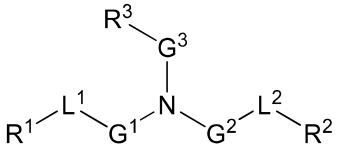
In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the osmolality of the composition is at most about 400 x 10^{-3} osmol/kg, such as at most about 390 x 10^{-3} osmol/kg, at most about 380 x 10^{-3} osmol/kg, at most about 370 x 10^{-3} osmol/kg, at most

about 360 x 10^{-3} osmol/kg, at most about 350 x 10^{-3} osmol/kg, at most about 340 x 10^{-3} osmol/kg, at most about 330 x 10^{-3} osmol/kg, at most about 320 x 10^{-3} osmol/kg, at most about 310 x 10^{-3} osmol/kg, or at most about 300 x 10^{-3} osmol/kg. If the composition does not comprise a cryoprotectant, the osmolality of the composition may be below 300 x 10^{-3} osmol/kg, such as at most about 250 x 10^{-3} osmol/kg, at most about 200 x 10^{-3} osmol/kg, at most about 150 x 10^{-3} osmol/kg, at most about 100 x 10^{-3} osmol/kg, at most about 50 x 10^{-3} osmol/kg, at most about 40 x 10^{-3} osmol/kg, or at most about 30 x 10^{-3} osmol/kg. If the composition comprises a cryoprotectant, it is preferred that the main part of the osmolality of the composition is provided by the cryoprotectant. For example, the cryoprotectant may provide at least 50%, such as at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90%, of the osmolality of the composition.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the concentration of the RNA in the composition is about 5 mg/l to about 150 mg/l. For example, the concentration of the RNA in the composition may be about 10 mg/l to about 140 mg/l, such as about 20 mg/l to about 130 mg/l, about 25 mg/l to about 125 mg/l, about 30 mg/l to about 120 mg/l, about 35 mg/l to about 115 mg/l, about 40 mg/l to about 110 mg/l, about 45 mg/l to about 105 mg/l, or about 50 mg/l to about 100 mg/l.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the final buffer substance is Tris and its protonated form, the pH of the composition is between about 6.5 and about 8.0, and the concentration of the RNA in the composition is about 5 mg/l to about 150 mg/l. In this example, it is preferred that the pH of the composition is between about 6.9 and about 7.9 and the concentration of the RNA in the composition is about 25 mg/l to about 125 mg/l, such as about 30 mg/l to about 120 mg/l. In particularly preferred example of the second example, the buffer substance is Tris and its protonated form; the pH of the composition is between about 6.9 and about 7.9; the concentration of the RNA in the composition is about 25 mg/l to about 125 mg/l, such as about 30 mg/l to about 120 mg/l; the final aqueous phase is substantially free of sulfate anions, substantially free of dibasic organic acids and substantially free of polybasic organic acids; and the composition comprises a cryoprotectant. In an alternative particularly preferred example of the second example, the buffer substance is Tris and its protonated form; the pH of the composition is between about 6.9 and about 7.9; the concentration of the RNA in the composition is about 25 mg/l to about 125 mg/l, such as about 30 mg/l to about 120 mg/l; the final aqueous phase is substantially free of sulfate anions, substantially free of dibasic organic acids and substantially free of polybasic organic acids; and the composition is substantially free of a cryoprotectant.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the cationically ionizable lipid comprises a head group which includes at least one nitrogen atom which is capable of being protonated under physiological conditions. For example, the cationically ionizable lipid may have the structure of Formula (I):

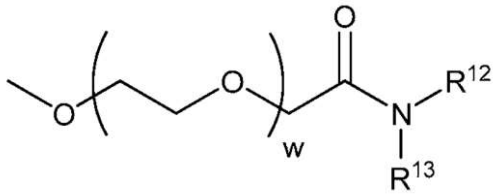


(I)

or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein L^1, L^2, G^1, G^2, G^3, R^1, R^2, and R^3 are as defined herein. Preferably, the cationically ionizable lipid is selected from the following: structures I-1 to I-36 (shown herein); and/or structures A to F (shown herein) ; and/or N,N-dimethyl-2,3-dioleyloxypropylamine (DODMA), 1,2-dioleoyl-3-dimethylammonium-propane (DODAP), heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino)butanoate (DLin-MC3-DMA), and 4-((di((9Z,12Z)-octadeca-9,12-dien-1-yl)amino)oxy)-N,N-dimethyl-4-oxobutan-1-amine (DPL-14). In a particularly preferred example, the cationically ionizable lipid is the lipid having the structure I-3.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the ethanolic solution prepared in step (b) or (d') further comprises one or more additional lipids and the LNPs further comprise the one or more additional lipids. Preferably, the one or more additional lipids are selected from the group consisting of polymer conjugated lipids, neutral lipids, steroids, and combinations thereof. In a preferred example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the one or more additional lipids comprise a polymer conjugated lipid (*e.g.*, a pegylated lipid or a polysarcosine-lipid conjugate or a conjugate of polysarcosine and a lipid-like material), a neutral lipid (*e.g.*, DSPC), and a steroid (*e.g.*, cholesterol), such that the LNPs comprise the cationically ionizable lipid as described herein, a polymer conjugated lipid (*e.g.*, a pegylated lipid or a polysarcosine-lipid conjugate or a conjugate of polysarcosine and a lipid-like material), a neutral lipid (*e.g.*, DSPC), and a steroid (*e.g.*, cholesterol).

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), wherein the one or more additional lipids comprise a polymer conjugated lipid, the polymer conjugated lipid is a pegylated lipid. For example, the pegylated lipid may have the following structure:



or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein R^{12}, R^{13}, and w are as defined herein.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), wherein the one or more additional lipids comprise a polymer conjugated lipid, the polymer conjugated lipid is a polysarcosine-lipid conjugate or a conjugate of polysarcosine and a lipid-like material. For example, the polysarcosine-lipid conjugate or conjugate of polysarcosine and a lipid-like material may be a member selected from the group consisting of a polysarcosine-diacylglycerol conjugate, a polysarcosine-dialkyloxypropyl conjugate, a polysarcosine-phospholipid conjugate, a polysarcosine-ceramide conjugate, and a mixture thereof.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), wherein the one or more additional lipids comprise a neutral lipid , the neutral lipid is a phospholipid. Such phospholipid is preferably selected from the group consisting of phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidic acids, phosphatidylserines and sphingomyelins. Particular examples of phospholipids include distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dimyristoylphosphatidylcholine (DMPC), dipentadecanoylphosphatidylcholine, dilauroylphosphatidylcholine, dipalmitoylphosphatidylcholine (DPPC), diarachidoylphosphatidylcholine (DAPC), dibehenoylphosphatidylcholine (DBPC), ditricosanoylphosphatidylcholine (DTPC), dilignoceroylphatidylcholine (DLPC), palmitoyloleoyl-phosphatidylcholine (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 (C16 Lyso PC), dioleoylphosphatidylethanolamine (DOPE), distearoyl-phosphatidylethanolamine (DSPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), dilauroyl-phosphatidylethanolamine (DLPE), and diphytanoyl-phosphatidylethanolamine (DPyPE). In a particularly preferred example, the neutral lipid is DSPC.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed

above), wherein the one or more additional lipids comprise a steroid, the steroid is a sterol such as cholesterol.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the ethanolic solution comprises the cationically ionizable lipid, the polymer conjugated lipid, the neutral lipid, and the steroid in a molar ratio of 20% to 60% of the cationically ionizable lipid, 0.5% to 15% of the polymer conjugated lipid, 5% to 25% of the neutral lipid, and 25% to 55% of the steroid, based on the total molar amount of lipids in the ethanolic solution. For example, the molar ratio may be 40% to 55% of the cationically ionizable lipid, 1.0% to 10% of the polymer conjugated lipid, 5% to 15% of the neutral lipid, and 30% to 50% of the steroid, such as 45% to 55% of the cationically ionizable lipid, 1.0% to 5% of the polymer conjugated lipid, 8% to 12% of the neutral lipid, and 35% to 45% of the steroid, based on the total molar amount of lipids in the ethanolic solution.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the first example, such as in any of the examples (1) to (30) listed above), the final aqueous phase does not comprise a chelating agent.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the LNPs comprise at least about 75% of the RNA comprised in the composition. For example, the LNPs may comprise at least about 76%, such as at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, or at least about 95% of the RNA comprised in the composition.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the RNA (such as mRNA) is encapsulated within or associated with the LNPs.

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the RNA (such as mRNA) comprises a modified nucleoside in place of uridine. For example, the modified nucleoside may be selected from pseudouridine (ψ), N1-methyl-pseudouridine (m1ψ), and 5-methyl-uridine (m5U).

In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the RNA (such as mRNA) comprises one or more of the following (a) a 5' cap, such as a cap1 or cap2 structure; (b) a 5' UTR; (c) a 3' UTR; and (d) a poly-A sequence, such as a poly-A sequence comprising at least 100 nucleotides, wherein the poly-A sequence preferably is an interrupted sequence of A nucleotides.

In one preferred example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the RNA (such as mRNA) encodes one or more polypeptides. For example, the one or more polypeptides may comprise an epitope for inducing an immune response against an antigen in a subject. In a preferred example, the RNA (such as mRNA) comprises an open reading frame (ORF) encoding an amino acid sequence comprising a SARS-CoV-2 S protein, an immunogenic variant thereof, or an immunogenic fragment of the SARS-CoV-2 S protein or the immunogenic variant thereof. In one example of the second example (in particular in one example of the above first, second, third, or fourth subgroup of the second example, such as in any of the examples (1) to (30) listed above), the RNA (such as mRNA) comprises an ORF encoding a full-length SARS-CoV2 S protein variant with proline residue substitutions at positions 986 and 987 of SEQ ID NO:1. For example, the SARS-CoV2 S protein variant may have at least 80% identity to SEQ ID NO:7.

In a third example, the present disclosure provides a method of storing a composition, comprising preparing a composition according to the method of the second example and storing the composition at a temperature ranging from about -90°C to about -10°C, such as from about -90°C to about -40°C or from about -40°C to about -25°C or from about -25°C to about -10°C, or a temperature of about -20°C. In one example of the third example, storing the frozen composition is for at least 1 week, such as at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 1 month, at least 2 months, at least 3 months, at least 6 months, at least 12 months, at least 24 months, or at least 36 months, preferably at least 4 weeks. In one example of the third example, storing the frozen composition is for at least 4 weeks, preferably at least 1 month, more preferably at least 2 months, more preferably at least 3 months, more preferably at least 6 months at -20°C. In one example of the third example, the composition can be stored at -70°C.

In one example of the third example, the method of storing a composition comprises preparing a composition according to the method of the second example comprising step (II) (i.e., freezing the formulation to about -10°C or below); storing the frozen composition at a temperature ranging from about -90°C to about -10°C for a certain period of time (e.g., at least one week); and storing the frozen composition a temperature ranging from about 0°C to about 20°C for a certain period of time (e.g., at least one week).

It is understood that any example described herein in the context of the first or second example (in particular, any example of the above first, second, third, or fourth subgroup of the first example, such as any of the examples (1) to (30) of the first example listed above or any example of the above first, second, third, or fourth subgroup of the second example, such as any of the examples (1) to (30) of the second example listed above) may also apply to any example of the third example.

In a fourth example, the present disclosure provides a method of storing a composition, comprising preparing a liquid composition according to the method of the second example and storing the liquid composition at a temperature ranging from about 0°C to about 20°C, such as from about 1°C to about 15°C, from about 2°C to about 10°C, or from about 2°C to about 8°C, or at a temperature of about 5°C. In one example of the fourth example, storing the liquid composition is for at least 1 week, such as at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 1 month, at least 2 months, at least 3 months, or at least 6 months, preferably at least 4 weeks. In one example of the fourth example, storing the liquid composition is for at least 4 weeks, preferably at least 1 month, more preferably at least 2 months, more preferably at least 3 months, more preferably at least 6 months at 5°C.

In one example of the fourth example, the method of storing a composition comprises preparing a composition according to the method of the second example comprising step (II) (i.e., freezing the formulation to about -10°C or below); and storing the frozen composition at a temperature ranging from about 0°C to about 20°C for a certain period of time (e.g., at least one week).

It is understood that any example described herein in the context of the first, second or third example (in particular, any example of the above first, second, third, or fourth subgroup of the first example, such as any of the examples (1) to (30) of the first example listed above or any example of the above first, second, third, or fourth subgroup of the second example, such as any of the examples (1) to (30) of the second example listed above) may also apply to any example of the fourth example.

In a fifth example, the present disclosure provides a composition preparable by the method of the second, third or fourth example. In one example of the fifth example, the composition can be in frozen form which, preferably, can be stored at a temperature of about -90°C or higher, such as about -90°C to about -10°C. For example, the frozen composition of the fifth example can be stored at a temperature ranging from about -90°C to about -40°C or from about -40°C to about -25°C or from about -25°C to about -10°C, or a temperature to about -20°. In one example of the fifth example, the composition can be stored for at least 1 week, such as at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 1 month, at least 2 months, at least 3 months, at least 6 months, at least 12 months, at least 24 months, or at least 36 months, preferably at least 4 weeks. For example, the frozen composition can be stored for at least 4

weeks, preferably at least 1 month, more preferably at least 2 months, more preferably at least 3 months, more preferably at least 6 months at -20°C.

In one example of the fifth example, where the composition is in frozen form, the RNA integrity after thawing the frozen composition is at least 50%, such as at least 52%, at least 54%, at least 55%, at least 56%, at least 58%, or at least 60%, *e.g.*, after thawing the frozen composition which has been stored at -20°C.

Additionally or alternatively, in one example of the fifth example, where the composition is in frozen form, the size ($Z_{average}$) (and/or size distribution and/or polydispersity index (PDI)) of the LNPs after thawing the frozen composition is equal to the size ($Z_{average}$) (and/or size distribution and/or PDI) of the LNPs before the composition has been frozen. In one example, the size ($Z_{average}$) of the LNPs after thawing the frozen composition is between about 50 nm and about 500 nm, preferably between about 40 nm and about 200 nm, more preferably between about 40 nm and about 120 nm. In one example, the PDI of the LNPs after thawing the frozen composition is less than 0.3, preferably less than 0.2, more preferably less than 0.1. In one example, the size ($Z_{average}$) of the LNPs after thawing the frozen composition is between about 50 nm and about 500 nm, preferably between about 40 nm and about 200 nm, more preferably between about 40 nm and about 120 nm, and the size ($Z_{average}$) (and/or size distribution and/or PDI) of the LNPs after thawing the frozen composition is equal to the size ($Z_{average}$) (and/or size distribution and/or PDI) of the LNPs before freezing. In one example, the size ($Z_{average}$) of the LNPs after thawing the frozen composition is between about 50 nm and about 500 nm, preferably between about 40 nm and about 200 nm, more preferably between about 40 nm and about 120 nm, and the PDI of the LNPs after thawing the frozen composition is less than 0.3 (preferably less than 0.2, more preferably less than 0.1).

In an alternative example of the fifth example, the composition is in liquid form.

In one example of the fifth example, where the composition is in liquid form, the RNA integrity of the liquid composition, when stored, e.g., at 0°C or higher for at least one week, is sufficient to produce the desired effect, e.g., to induce an immune response. For example, the RNA integrity of the liquid composition, when stored, e.g., at 0°C or higher for at least one week, may be at least 50%, such as at least 52%, at least 54%, at least 55%, at least 56%, at least 58%, or at least 60%.

Additionally or alternatively, in one example of the fifth example, where the composition is in liquid form, the size ($Z_{average}$) (and/or size distribution and/or polydispersity index (PDI)) of the LNPs of the liquid composition, when stored, e.g., at 0°C or higher for at least one week, is sufficient to produce the desired effect, e.g., to induce an immune response. For example, the size ($Z_{average}$) (and/or size

distribution and/or polydispersity index (PDI)) of the LNPs of the liquid composition, when stored, e.g., at 0°C or higher for at least one week, is equal to the size ($Z_{average}$) (and/or size distribution and/or PDI) of the LNPs of the initial composition, i.e., before storage. In one example, the size ($Z_{average}$) of the LNPs after storage of the liquid composition e.g., at 0°C or higher for at least one week is between about 50 nm and about 500 nm, preferably between about 40 nm and about 200 nm, more preferably between about 40 nm and about 120 nm. In one example, the PDI of the LNPs after storage of the liquid composition e.g., at 0°C or higher for at least one week is less than 0.3, preferably less than 0.2, more preferably less than 0.1. In one example, the size ($Z_{average}$) of the LNPs after storage of the liquid composition e.g., at 0°C or higher for at least one week is between about 50 nm and about 500 nm, preferably between about 40 nm and about 200 nm, more preferably between about 40 nm and about 120 nm, and the size ($Z_{average}$) (and/or size distribution and/or PDI) of the LNPs after storage of the liquid composition e.g., at 0°C or higher for at least one week is equal to the size ($Z_{average}$) (and/or size distribution and/or PDI) of the LNPs before storage. In one example, the size ($Z_{average}$) of the LNPs after storage of the liquid composition e.g., at 0°C or higher for at least one week is between about 50 nm and about 500 nm, preferably between about 40 nm and about 200 nm, more preferably between about 40 nm and about 120 nm, and the PDI of the LNPs after storage of the liquid composition e.g., at 0°C or higher for at least one week is less than 0.3 (preferably less than 0.2, more preferably less than 0.1).

It is understood that any example described herein in the context of the first, second, third, or fourth example (in particular, any example of the above first, second, third, or fourth subgroup of the first example, such as any of the examples (1) to (30) of the first example listed above or any example of the above first, second, third, or fourth subgroup of the second example, such as any of the examples (1) to (30) of the second example listed above) may also apply to any example of the fifth example.

In a sixth example, the present disclosure provides a method for preparing a ready-to-use pharmaceutical composition, the method comprising the steps of providing a frozen composition prepared by the method of the second or third example and thawing the frozen composition thereby obtaining the ready-to-use pharmaceutical composition.

It is understood that any example described herein in the context of the first, second, third, fourth, or fifth example (in particular, any example of the above first, second, third, or fourth subgroup of the first example, such as any of the examples (1) to (30) of the first example listed above or any example of the above first, second, third, or fourth subgroup of the second example, such as any of the examples (1) to (30) of the second example listed above) may also apply to any example of the sixth example.

In a seventh example, the present disclosure provides a method for preparing a ready-to-use pharmaceutical composition, the method comprising the steps of providing a liquid composition prepared by the method of the second or fourth example thereby obtaining the ready-to-use pharmaceutical composition.

It is understood that any example described herein in the context of the first, second, third, fourth, fifth, or sixth example (in particular, any example of the above first, second, third, or fourth subgroup of the first example, such as any of the examples (1) to (30) of the first example listed above or any example of the above first, second, third, or fourth subgroup of the second example, such as any of the examples (1) to (30) of the second example listed above) may also apply to any example of the seventh example.

In an eighth example, the present disclosure provides a ready-to-use pharmaceutical composition preparable by the method of the sixth or seventh example.

It is understood that any example described herein in the context of the first, second, third, fourth, fifth, sixth, or seventh example (in particular, any example of the above first, second, third, or fourth subgroup of the first example, such as any of the examples (1) to (30) of the first example listed above or any example of the above first, second, third, or fourth subgroup of the second example, such as any of the examples (1) to (30) of the second example listed above) may also apply to any example of the eighth example.

In a ninth example, the present disclosure provides a composition of any one of the first, fifth, and eighth example for use in therapy.

It is understood that any example described herein in the context of the first, second, third, fourth, fifth, sixth, seventh, or eighth example may also apply to any example of the ninth example.

In a tenth example, the present disclosure provides a composition of any one of the first, fifth, and eighth example for use in inducing an immune response.

It is understood that any example described herein in the context of the first, second, third, fourth, fifth, sixth, seventh, eighth, or ninth example may also apply to any example of the tenth example.

Further itemised examples are as follows:

1. A composition comprising lipid nanoparticles (LNPs) dispersed in an aqueous phase, wherein the LNPs comprise a cationically ionizable lipid and RNA; the aqueous phase comprises a buffer system comprising a buffer substance and a monovalent anion, the buffer substance being selected from the group consisting of tris(hydroxymethyl)aminomethane (Tris) and its protonated form, bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (Bis-Tris-methane) and its protonated form, and triethanolamine (TEA) and its protonated form, and the monovalent anion being selected from the group consisting of chloride, acetate, glycolate, lactate, the anion of morpholinoethanesulfonic acid (MES), the anion of 3-(N-morpholino)propanesulfonic acid (MOPS), and the anion of 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES); the concentration of the buffer substance in the composition is at most about 25 mM; and the aqueous phase is substantially free of inorganic phosphate anions, substantially free of citrate anions, and substantially free of anions of ethylenediaminetetraacetic acid (EDTA).

2. The composition of item 1, wherein the buffer substance is Tris and its protonated form.

3. The composition of item 1 or 2, wherein the concentration of the buffer substance, in particular Tris and its protonated form, in the composition is at most about 20 mM, preferably at most about 15 mM, more preferably at most about 10 mM, such as about 10 mM.

4. The composition of any one of items 1 to 3, wherein the aqueous phase is substantially free of inorganic sulfate anions and/or carbonate anions and/or dibasic organic acid anions and/or polybasic organic acid anions, in particular substantially free of inorganic sulfate anions, carbonate anions, dibasic organic acid anions and polybasic organic acid anions.

5. The composition of any one of items 1 to 4, wherein the monovalent anion is selected from the group consisting of chloride, acetate, glycolate, and lactate, and the concentration of the monovalent anion in the composition is at most equal to, preferably less than the concentration of the buffer substance in the composition, such as less than about 9 mM.

6. The composition of any one of items 1 to 4, wherein the monovalent anion is selected from the group consisting of the anions of MES, MOPS, and HEPES, and the concentration of the monovalent anion in the composition is at least equal to, preferably higher than the concentration of the buffer substance in the composition.

7. The composition of any one of items 1 to 6, wherein the pH of the composition is between about 6.5 and about 8.0, preferably between about 6.9 and about 7.9, such as between about 7.0 and about 7.8.

8. The composition of any one of items 1 to 7, wherein water is the main component in the composition and/or the total amount of solvent(s) other than water contained in the composition is less than about 0.5% (v/v).

9. The composition of any one of items 1 to 8, wherein the osmolality of the composition is at most about 400 x 10^{-3} osmol/kg.

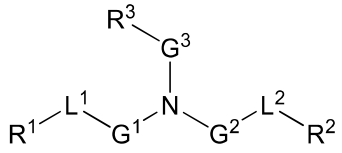
10. The composition of any one of items 1 to 9, wherein the concentration of the RNA in the composition is about 5 mg/l to about 150 mg/l, preferably about 10 mg/l to about 130 mg/l, more preferably about 30 mg/l to about 120 mg/l.

11. The composition of any one of items 1 to 10, wherein the composition comprises a cryoprotectant, preferably in a concentration of at least about 1% w/v, wherein the cryoprotectant preferably comprises one or more compounds selected from the group consisting of carbohydrates and sugar alcohols, more preferably the cryoprotectant is selected from the group consisting of sucrose, glucose, glycerol, sorbitol, and a combination thereof, more preferably the cryoprotectant comprises sucrose and/or glycerol.

12. The composition of any one of items 1 to 10, wherein the composition is substantially free of a cryoprotectant.

13. The composition of any one of items 1 to 12, wherein the cationically ionizable lipid comprises a head group which includes at least one nitrogen atom which is capable of being protonated under physiological conditions.

14. The composition of any one of items 1 to 13, wherein the cationically ionizable lipid has the structure of Formula (I):



(I)

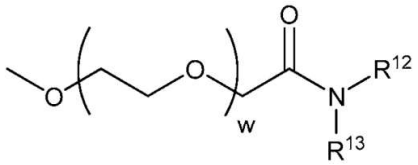
or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein:
one of L^1 or L^2 is –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -$S(O)_x$-, -S-S-, -C(=O)S-, SC(=O)-, -$NR^aC(=O)$-, -$C(=O)NR^a$-, $NR^aC(=O)NR^a$-, -$OC(=O)NR^a$- or -$NR^aC(=O)O$-, and the other of L^1 or L^2 is –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -$S(O)_x$-, -S-S-, -C(=O)S-, SC(=O)-, -$NR^aC(=O)$-, -$C(=O)NR^a$-, $NR^aC(=O)NR^a$-, -$OC(=O)NR^a$- or -$NR^aC(=O)O$- or a direct bond;

G^1 and G^2 are each independently unsubstituted C_1-C_{12} alkylene or C_2-C_{12} alkenylene;
G^3 is C_1-C_{24} alkylene, C_2-C_{24} alkenylene, C_3-C_8 cycloalkylene, C_3-C_8 cycloalkenylene;
R^a is H or C_1-C_{12} alkyl;
R^1 and R^2 are each independently C_6-C_{24} alkyl or C_6-C_{24} alkenyl;
R^3 is H, OR^5, CN, -C(=O)OR^4, -OC(=O)R^4 or –$NR^5C(=O)R^4$;
R^4 is C_1-C_{12} alkyl;
R^5 is H or C_1-C_6 alkyl; and
x is 0, 1 or 2.

15. The composition of any one of items 1 to 13, wherein:
(α) the cationically ionizable lipid is selected from the structures I-1 to I-36 shown herein; or
(β) the cationically ionizable lipid is selected from the structures A to F shown herein; or
(γ) the cationically ionizable lipid is the lipid having the structure I-3 shown herein.

16. The composition of any one of items 1 to 15, wherein the LNPs further comprise one or more additional lipids, preferably selected from the group consisting of polymer conjugated lipids, neutral lipids, steroids, and combinations thereof, more preferably the LNPs comprise the cationically ionizable lipid, a polymer conjugated lipid, a neutral lipid, and a steroid.

17. The composition of item 16, wherein the polymer conjugated lipid comprises a pegylated lipid, wherein the pegylated lipid preferably has the following structure:



or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein:
R^{12} and R^{13} are each independently a straight or branched, saturated or unsaturated alkyl chain containing from 10 to 30 carbon atoms, wherein the alkyl chain is optionally interrupted by one or more ester bonds; and w has a mean value ranging from 30 to 60.

18. The composition of item 16, wherein the polymer conjugated lipid comprises a polysarcosine-lipid conjugate or a conjugate of polysarcosine and a lipid-like material, wherein the polysarcosine-lipid conjugate or conjugate of polysarcosine and a lipid-like material preferably is a member selected from the group consisting of a polysarcosine-diacylglycerol conjugate, a polysarcosine-dialkyloxypropyl conjugate, a polysarcosine-phospholipid conjugate, a polysarcosine-ceramide conjugate, and a mixture thereof.

19. The composition of any one of items 16 to 18, wherein the neutral lipid is a phospholipid, preferably selected from the group consisting of phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidic acids, phosphatidylserines and sphingomyelins, more preferably selected from the group consisting of distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dimyristoylphosphatidylcholine (DMPC), dipentadecanoylphosphatidylcholine, dilauroylphosphatidylcholine, dipalmitoylphosphatidylcholine (DPPC), diarachidoylphosphatidylcholine (DAPC), dibehenoylphosphatidylcholine (DBPC), ditricosanoylphosphatidylcholine (DTPC), dilignoceroylphatidylcholine (DLPC), palmitoyloleoyl-phosphatidylcholine (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 (C16 Lyso PC), dioleoylphosphatidylethanolamine (DOPE), distearoyl-phosphatidylethanolamine (DSPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), dilauroyl-phosphatidylethanolamine (DLPE), and diphytanoyl-phosphatidylethanolamine (DPyPE).

20. The composition of any one of items 16 to 19, wherein the steroid comprises a sterol such as cholesterol.

21. The composition of any one of items 1 to 20, wherein the aqueous phase does not comprise a chelating agent.

22. The composition of any one of items 1 to 21, wherein the LNPs comprise at least about 75%, preferably at least about 80% of the RNA comprised in the composition.

23. The composition of any one of items 1 to 22, wherein the RNA is encapsulated within or associated with the LNPs.

24. The composition of any one of items 1 to 23, wherein the RNA comprises a modified nucleoside in place of uridine, wherein the modified nucleoside is preferably selected from pseudouridine (ψ), N1-methyl-pseudouridine (m1ψ), and 5-methyl-uridine (m5U).

25. The composition of any one of items 1 to 24, wherein the RNA comprises at least one of the following, preferably all of the following: a 5’ cap; a 5’ UTR; a 3’ UTR; and a poly-A sequence.

26. The composition of item 25, wherein the poly-A sequence comprises at least 100 A nucleotides, wherein the poly-A sequence preferably is an interrupted sequence of A nucleotides.

27. The composition of item 25 or 26, wherein the 5' cap is a cap1 or cap2 structure.

28. The composition of any one of items 1 to 27, wherein the RNA encodes one or more polypeptides, wherein the one or more polypeptides preferably comprise an epitope for inducing an immune response against an antigen in a subject.

29. The composition of item 28, wherein the RNA comprises an open reading frame (ORF) encoding an amino acid sequence comprising a SARS-CoV-2 S protein, an immunogenic variant thereof, or an immunogenic fragment of the SARS-CoV-2 S protein or the immunogenic variant thereof.

30. The composition of item 28 or 29, wherein the RNA comprises an ORF encoding a full-length SARS-CoV2 S protein variant with proline residue substitutions at positions 986 and 987 of SEQ ID NO: 1.

31. The composition of item 29 or 30, wherein the SARS-CoV2 S protein variant has at least 80% identity to SEQ ID NO: 7.

32. The composition of any one of items 1 to 31, wherein the composition is in frozen form.

33. The composition of item 32, wherein the RNA integrity after thawing the frozen composition is at least 50% compared to the RNA integrity before the composition has been frozen.

34. The composition of item 32 or 33, wherein the size ($Z_{average}$) and/or size distribution and/or polydispersity index (PDI) of the LNPs after thawing the frozen composition is equal to the size ($Z_{average}$) and/or size distribution and/or PDI of the LNPs before the composition has been frozen.

35. The composition of any one of items 1 to 31, wherein the composition is in liquid form.

36. A method of preparing a composition comprising LNPs dispersed in a final aqueous phase, wherein the LNPs comprise a cationically ionizable lipid and RNA; the final aqueous phase comprises a final buffer system comprising a final buffer substance and a final monovalent anion, the final buffer substance being selected from the group consisting of Tris and its protonated form, Bis-Tris-methane and its protonated form, and TEA and its protonated form,

and the final monovalent anion being selected from the group consisting of chloride, acetate, glycolate, lactate, the anion of MES, the anion of MOPS, and the anion of HEPES; the concentration of the final buffer substance in the composition is at most about 25 mM; and the final aqueous phase is substantially free of inorganic phosphate anions, substantially free of citrate anions, and substantially free of anions of EDTA;

wherein the method comprises:

(I) preparing a formulation comprising LNPs dispersed in the final aqueous phase, wherein the LNPs comprise the cationically ionizable lipid and RNA; and

(II) optionally freezing the formulation to about -10°C or below,

thereby obtaining the composition,

wherein step (I) comprises:

(a) preparing an RNA solution containing water and a first buffer system;

(b) preparing an ethanolic solution comprising the cationically ionizable lipid and, if present, one or more additional lipids;

(c) mixing the RNA solution prepared under (a) with the ethanolic solution prepared under (b), thereby preparing a first intermediate formulation comprising the LNPs dispersed in a first aqueous phase comprising the first buffer system; and

(d) filtrating the first intermediate formulation prepared under (c) using a final aqueous buffer solution comprising the final buffer system,

thereby preparing the formulation comprising the LNPs dispersed in the final aqueous phase.

37. The method of item 36, wherein step (I) further comprises one or more steps selected from diluting and filtrating.

38. The method of item 36 or 37, wherein step (I) comprises:

(a') providing an aqueous RNA solution;

(b') providing a first aqueous buffer solution comprising a first buffer system;

(c') mixing the aqueous RNA solution provided under (a') with the first aqueous buffer solution provided under (b') thereby preparing an RNA solution containing water and the first buffer system;

(d') preparing an ethanolic solution comprising the cationically ionizable lipid and, if present, one or more additional lipids;

(e') mixing the RNA solution prepared under (c') with the ethanolic solution prepared under (d'), thereby preparing a first intermediate formulation comprising LNPs dispersed in a first aqueous phase comprising the first buffer system;

(f') optionally filtrating the first intermediate formulation prepared under (e') using a further aqueous buffer solution comprising a further buffer system, thereby preparing a further

intermediate formulation comprising the LNPs dispersed in a further aqueous phase comprising the further buffer system, wherein the further aqueous buffer solution may be identical to or different from the first aqueous buffer solution;

(g') optionally repeating step (f') once or two or more times, wherein the further intermediate formulation comprising the LNPs dispersed in the further aqueous phase comprising the further buffer system obtained after step (f') of one cycle is used as the first intermediate formulation of the next cycle, wherein in each cycle the further aqueous buffer solution may be identical to or different from the first aqueous buffer solution;

(h') filtrating the first intermediate formulation obtained in step (e'), if step (f') is absent, or the further intermediate formulation obtained in step (f'), if step (f') is present and step (g') is not present, or the further intermediate formulation obtained after step (g'), if steps (f') and (g') are present, using a final aqueous buffer solution comprising the final buffer system and having a pH of at least 6.0; and

(i') optionally diluting the formulation obtained in step (h') with a dilution solution;

thereby preparing the formulation comprising the LNPs dispersed in the final aqueous phase.

39. The method of any one of items 36 to 38, wherein filtrating is tangential flow filtrating or diafiltrating, preferably tangential flow filtrating.

40. The method of any one of items 36 to 39, which comprises (II) freezing the formulation to about -10°C or below.

41. The method of any one of items 36 to 40, wherein the final buffer substance is Tris and its protonated form.

42. The method of any one of items 36 to 41, wherein the concentration of the final buffer substance, in particular Tris and its protonated form, in the composition is at most about 20 mM, preferably at most about 15 mM, more preferably at most about 10 mM, such as about 10 mM.

43. The method of any one of items 36 to 42, wherein the final aqueous phase is substantially free of inorganic sulfate anions and/or carbonate anions and/or dibasic organic acid anions and/or polybasic organic acid anions, in particular substantially free of inorganic sulfate anions, carbonate anions dibasic organic acid anions and polybasic organic acid anions.

44. The method of any one of items 36 to 43, wherein (i) the RNA solution prepared in step (a) further comprises one or more di- and/or polybasic organic acid anions, and step (d) is conducted under conditions which remove the one or more di- and/or polybasic organic acid anions

resulting in the formulation comprising the LNPs dispersed in the final aqueous phase with the final aqueous phase being substantially free of the one or more di- and/or polybasic organic acid anions present in the RNA solution prepared in step (a); or (ii) the first aqueous buffer solution and the first aqueous phase comprise one or more di- and/or polybasic organic acid anions and least one of steps (f') to (h') is conducted under conditions which remove the one or more di- and/or polybasic organic acid anions from the first intermediate formulation and/or from the further intermediate formulation.

45. The method of any one of items 36 to 44, wherein (i) the RNA solution obtained in step (a) has a pH of below 6.0, preferably at most about 5.0, more preferably at most about 4.5; or (ii) the first aqueous buffer solution has a pH of below 6.0, preferably at most about 5.0, more preferably at most about 4.5.

46. The method of item 44 or 45, wherein the one or more di- and/or polybasic organic acid anions comprise citrate anions and/or anions of EDTA.

47. The method of any one of items 36 to 43, wherein (i) the first buffer system used in step (a) comprises the final buffer substance and the final monovalent anion used in step (d), preferably the buffer system and pH of the first buffer system used in step (a) are identical to the buffer system and pH of the final aqueous buffer solution used in step (d); or (ii) each of the first buffer system and every further buffer system used in steps (b'), (f') and (g') comprises the final buffer substance and the final monovalent anion used in step (h'), preferably the buffer system and pH of each of the first aqueous buffer solution and of every further aqueous buffer solution used in steps (b'), (f') and (g') are identical to the buffer system and pH of the final aqueous buffer solution.

48. The method of any one of items 36 to 47, wherein the final monovalent anion is selected from the group consisting of chloride, acetate, glycolate, and lactate, and the concentration of the final monovalent anion in the composition is at most equal to, preferably less than the concentration of the final buffer substance in the composition, such as less than about 9 mM.

49. The method of any one of items 36 to 48, wherein the final monovalent anion is selected from the group consisting of the anions of MES, MOPS, and HEPES, and the concentration of the final monovalent anion in the composition is at least equal to, preferably higher than the concentration of the final buffer substance in the composition.

50. The method of any one of items 36 to 49, wherein the pH of the composition is between about 6.5 and about 8.0, preferably between about 6.9 and about 7.9, such as between about 7.0 and about 7.8.

51. The method of any one of items 36 to 50, wherein water is the main component in the formulation and/or composition and/or the total amount of solvent(s) other than water contained in the composition is less than about 0.5% (v/v).

52. The method of any one of items 36 to 51, wherein the osmolality of the composition is at most about 400 x 10^{-3} osmol/kg.

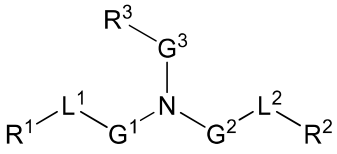
53. The method of any one of items 36 to 52, wherein the concentration of the RNA in the composition is about 5 mg/l to about 150 mg/l, preferably about 10 mg/l to about 130 mg/l, more preferably about 30 mg/l to about 120 mg/l.

54. The method of any one of items 36 to 53, wherein (i) step (I) further comprises diluting the formulation prepared under (d) with a dilution solution, or step (i') is present, wherein the dilution solution comprises a cryoprotectant; and/or (ii) the formulation obtained in step (I) and the composition comprise a cryoprotectant, preferably in a concentration of at least about 1% w/v, wherein the cryoprotectant preferably comprises one or more selected from the group consisting of carbohydrates and sugar alcohols, more preferably the cryoprotectant is selected from the group consisting of sucrose, glucose, glycerol, sorbitol, and a combination thereof, more preferably the cryoprotectant comprises sucrose and/or glycerol.

55. The method of any one of items 36 to 53, wherein the formulation obtained in step (I) and the composition is substantially free of a cryoprotectant.

56. The method of any one of items 36 to 55, wherein the cationically ionizable lipid comprises a head group which includes at least one nitrogen atom which is capable of being protonated under physiological conditions.

57. The method of any one of items 36 to 56, wherein the cationically ionizable lipid has the structure of Formula (I):



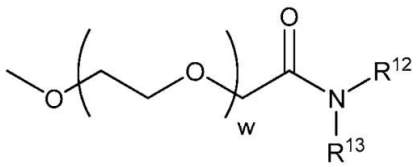
(I)

or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein:
one of L^1 or L^2 is –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -$S(O)_x$-, -S-S-, -C(=O)S-, SC(=O)-, -$NR^aC(=O)$-, -$C(=O)NR^a$-, $NR^aC(=O)NR^a$-, -$OC(=O)NR^a$- or -$NR^aC(=O)O$-, and the other of L^1 or L^2 is –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -$S(O)_x$-, -S-S-, -C(=O)S-, SC(=O)-, -$NR^aC(=O)$-, -$C(=O)NR^a$-, $NR^aC(=O)NR^a$-, -$OC(=O)NR^a$- or -$NR^aC(=O)O$- or a direct bond;
G^1 and G^2 are each independently unsubstituted C_1-C_{12} alkylene or C_2-C_{12} alkenylene;
G^3 is C_1-C_{24} alkylene, C_2-C_{24} alkenylene, C_3-C_8 cycloalkylene, C_3-C_8 cycloalkenylene;
R^a is H or C_1-C_{12} alkyl;
R^1 and R^2 are each independently C_6-C_{24} alkyl or C_6-C_{24} alkenyl;
R^3 is H, OR^5, CN, -C(=O)OR^4, -$OC(=O)R^4$ or –$NR^5C(=O)R^4$;
R^4 is C_1-C_{12} alkyl;
R^5 is H or C_1-C_6 alkyl; and
x is 0, 1 or 2.

58. The method of any one of items 36 to 56, wherein:
(α) the cationically ionizable lipid is selected from the structures I-1 to I-36 shown herein; or
(β) the cationically ionizable lipid is selected from the structures A to F shown herein; or
(γ) the cationically ionizable lipid is the lipid having the structure I-3 shown herein.

59. The method of any one of items 36 to 58, wherein the ethanolic solution prepared in step (b) or (d') further comprises one or more additional lipids and the LNPs further comprise the one or more additional lipids, wherein the one or more additional lipids are preferably selected from the group consisting of polymer conjugated lipids, neutral lipids, steroids, and combinations thereof, more preferably the one or more additional lipids comprise a polymer conjugated lipid, a neutral lipid, and a steroid.

60. The method of item 59, wherein the polymer conjugated lipid comprises a pegylated lipid, wherein the pegylated lipid preferably has the following structure:



or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein:
R^{12} and R^{13} are each independently a straight or branched, saturated or unsaturated alkyl chain containing from 10 to 30 carbon atoms, wherein the alkyl chain is optionally interrupted by one or more ester bonds; and w has a mean value ranging from 30 to 60.

61. The method of item 59, wherein the polymer conjugated lipid comprises a polysarcosine-lipid conjugate or a conjugate of polysarcosine and a lipid-like material, wherein the polysarcosine-lipid conjugate or conjugate of polysarcosine and a lipid-like material preferably is a member selected from the group consisting of a polysarcosine-diacylglycerol conjugate, a polysarcosine-dialkyloxypropyl conjugate, a polysarcosine-phospholipid conjugate, a polysarcosine-ceramide conjugate, and a mixture thereof.

62. The method of any one of items 59 to 61, wherein the neutral lipid is a phospholipid, preferably selected from the group consisting of phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidic acids, phosphatidylserines and sphingomyelins, more preferably selected from the group consisting of distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dimyristoylphosphatidylcholine (DMPC), dipentadecanoylphosphatidylcholine, dilauroylphosphatidylcholine, dipalmitoylphosphatidylcholine (DPPC), diarachidoylphosphatidylcholine (DAPC), dibehenoylphosphatidylcholine (DBPC), ditricosanoylphosphatidylcholine (DTPC), dilignoceroylphatidylcholine (DLPC), palmitoyloleoyl-phosphatidylcholine (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 (C16 Lyso PC), dioleoylphosphatidylethanolamine (DOPE), distearoyl-phosphatidylethanolamine (DSPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), dilauroyl-phosphatidylethanolamine (DLPE), and diphytanoyl-phosphatidylethanolamine (DPyPE).

63. The method of any one of items 59 to 62, wherein the steroid comprises a sterol such as cholesterol.

64. The method of any one of items 36 to 63, wherein the cationically ionizable lipid, the polymer conjugated lipid, the neutral lipid, and the steroid are present in the ethanolic solution in a molar ratio of 20% to 60% of the cationically ionizable lipid, 0.5% to 15% of the polymer conjugated lipid, 5% to 25% of the neutral lipid, and 25% to 55% of the steroid, preferably in a molar ratio of 45% to 55% of the cationically ionizable lipid, 1.0% to 5% of the polymer conjugated lipid, 8% to 12% of the neutral lipid, and 35% to 45% of the steroid.

65. The method of any one of items 36 to 64, wherein the final aqueous phase does not comprise a chelating agent.

66. The method of any one of items 36 to 65, wherein the LNPs comprise at least about 75%, preferably at least about 80% of the RNA comprised in the composition.

67. The method of any one of items 36 to 66, wherein the RNA is encapsulated within or associated with the LNPs.

68. The method of any one of items 36 to 67, wherein the RNA comprises a modified nucleoside in place of uridine, wherein the modified nucleoside is preferably selected from pseudouridine (ψ), N1-methyl-pseudouridine (m1ψ), and 5-methyl-uridine (m5U).

69. The method of any one of items 36 to 68, wherein the RNA comprises at least one of the following, preferably all of the following: a 5' cap; a 5' UTR; a 3' UTR; and a poly-A sequence.

70. The method of item 69, wherein the poly-A sequence comprises at least 100 A nucleotides, wherein the poly-A sequence preferably is an interrupted sequence of A nucleotides.

71. The method of item 69 or 70, wherein the 5' cap is a cap1 or cap2 structure.

72. The method of any one of items 36 to 71, wherein the RNA encodes one or more polypeptides, wherein the one or more polypeptides preferably comprise an epitope for inducing an immune response against an antigen in a subject.

73. The method of item 72, wherein the RNA comprises an open reading frame (ORF) encoding an amino acid sequence comprising a SARS-CoV-2 S protein, an immunogenic variant thereof, or an immunogenic fragment of the SARS-CoV-2 S protein or the immunogenic variant thereof.

74. The method of item 72 or 73, wherein the RNA comprises an ORF encoding a full-length SARS-CoV2 S protein variant with proline residue substitutions at positions 986 and 987 of SEQ ID NO: 1.

75. The method of item 73 or 74, wherein the SARS-CoV2 S protein variant has at least 80% identity to SEQ ID NO: 7.

76. The method of any one of items 36 to 39 and 41 to 75, which does not comprise step (II).

77. A method of storing a composition, comprising preparing a composition according to the method of any one of items 36 to 75 and storing the composition at a temperature ranging from

about -90°C to about -10°C, such as from about -90°C to about -40°C or from about -25°C to about -10°C.

78. The method of item 77, wherein storing the composition is for at least 1 week, such as at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 1 month, at least 2 months, at least 3 months, at least 6 months, at least 12 months, at least 24 months, or at least 36 months.

79. A method of storing a composition, comprising preparing a composition according to the method of any one of items 36 to 78 and storing the composition at a temperature ranging from about 0°C to about 20°C, such as from about 1°C to about 15°C, from about 2°C to about 10°C, or from about 2°C to about 8°C, or at a temperature of about 5°C.

80. The method of item 79, wherein storing the composition is for at least 1 week, such as at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 1 month, at least 2 months, at least 3 months, or at least 6 months.

81. A composition preparable by the method of any one of items 36 to 80.

82. The composition of item 81, which is in frozen form.

83. The composition of item 82, wherein the RNA integrity after thawing the frozen composition is at least 50% compared to the RNA integrity of the composition before the composition has been frozen.

84. The composition of item 82 or 83, wherein the size ($Z_{average}$) and/or size distribution and/or polydispersity index (PDI) of the LNPs after thawing the frozen composition is equal to the size ($Z_{average}$) and/or size distribution and/or PDI of the LNPs before the composition has been frozen.

85. The composition of item 81, which is in liquid form.

86. The composition of item 85, wherein the RNA integrity after storage of the composition for at least 1 week is at least 50% compared to the RNA integrity before storage.

87. The composition of item 85 or 86, wherein the size ($Z_{average}$) and/or size distribution and/or polydispersity index (PDI) of the LNPs after storage of the composition for at least one week is equal to the size ($Z_{average}$) and/or size distribution and/or PDI of the LNPs before storage.

88. A method for preparing a ready-to-use pharmaceutical composition, the method comprising the steps of providing a frozen composition prepared by the method of any one of items 36 to 75, 77, and 78, and thawing the frozen composition thereby obtaining the ready-to-use pharmaceutical composition.

89. A method for preparing a ready-to-use pharmaceutical composition, the method comprising the step of providing a liquid composition prepared by the method of any one of items 36 to 39, 41 to 76, 79, and 80, thereby obtaining the ready-to-use pharmaceutical composition.

90. A ready-to-use pharmaceutical composition preparable by the method of item 88 or 89.

91. A composition of any one of items 1 to 35, 81 to 87, and 90 for use in therapy.

92. A composition of any one of items 1 to 35, 81 to 87, and 90 for use in inducing an immune response in a subject.

Further examples of the present disclosure are disclosed herein.

CLAIMS

1. A composition comprising lipid nanoparticles (LNPs) dispersed in an aqueous phase, wherein the LNPs comprise a cationically ionizable lipid and RNA; the aqueous phase comprises a buffer system comprising a buffer substance and a monovalent anion, the buffer substance being selected from the group consisting of tris(hydroxymethyl)aminomethane (Tris) and its protonated form, bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (Bis-Tris-methane) and its protonated form, and triethanolamine (TEA) and its protonated form, and the monovalent anion being selected from the group consisting of chloride, acetate, glycolate, and lactate; the concentration of the buffer substance in the composition is at most about 25 mM; and the aqueous phase is substantially free of inorganic phosphate anions, substantially free of citrate anions, and substantially free of anions of ethylenediaminetetraacetic acid (EDTA),
wherein the concentration of the monovalent anion in the composition is at most equal to the concentration of the buffer substance in the composition.

2. The composition of claim 1, wherein
   (i) the buffer substance is Tris and its protonated form;
   (ii) the concentration of the buffer substance in the composition is at most about 20 mM, preferably at most about 15 mM, more preferably at most about 10 mM, such as about 10 mM;
   (iii) the aqueous phase is substantially free of inorganic sulfate anions and/or carbonate anions and/or dibasic organic acid anions and/or polybasic organic acid anions, in particular substantially free of inorganic sulfate anions, carbonate anions, dibasic organic acid anions and polybasic organic acid anions;
   (iv) the concentration of the monovalent anion in the composition is less than the concentration of the buffer substance in the composition or is less than about 9 mM;
   (v) the pH of the composition is between about 6.5 and about 8.0, preferably between about 6.9 and about 7.9, such as between about 7.0 and about 7.8;
   (vi) water is the main component in the composition and/or the total amount of solvent(s) other than water contained in the composition is less than about 0.5% (v/v);
   (vii) the osmolality of the composition is at most about 400 x 10^{-3} osmol/kg;
   (viii) the concentration of the RNA in the composition is about 5 mg/l to about 150 mg/l, preferably about 10 mg/l to about 130 mg/l, more preferably about 30 mg/l to about 120 mg/l;
   (ix) the composition comprises a cryoprotectant, preferably in a concentration of at least about 1% w/v, wherein the cryoprotectant preferably comprises one or more compounds selected from the group consisting of carbohydrates and sugar alcohols, more preferably

the cryoprotectant is selected from the group consisting of sucrose, glucose, glycerol, sorbitol, and a combination thereof, more preferably the cryoprotectant comprises sucrose and/or glycerol, or

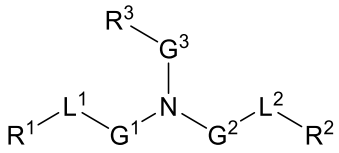
the composition is substantially free of a cryoprotectant; and/or

(x) the aqueous phase does not comprise a chelating agent.

3. The composition of claim 1 or 2, wherein the cationically ionizable lipid

(i) comprises a head group which includes at least one nitrogen atom which is capable of being protonated under physiological conditions;

(ii) has the structure of Formula (I):



(I)

or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein:

one of L^1 or L^2 is –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -$S(O)_x$-, -S-S-, -C(=O)S-, SC(=O)-, -$NR^aC(=O)$-, -$C(=O)NR^a$-, $NR^aC(=O)NR^a$-, -$OC(=O)NR^a$- or -$NR^aC(=O)O$-, and the other of L^1 or L^2 is –O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -$S(O)_x$-, -S-S-, -C(=O)S-, SC(=O)-, -$NR^aC(=O)$-, -$C(=O)NR^a$-, $NR^aC(=O)NR^a$-, -$OC(=O)NR^a$- or -$NR^aC(=O)O$- or a direct bond;

G^1 and G^2 are each independently unsubstituted C_1-C_{12} alkylene or C_2-C_{12} alkenylene;

G^3 is C_1-C_{24} alkylene, C_2-C_{24} alkenylene, C_3-C_8 cycloalkylene, C_3-C_8 cycloalkenylene;

R^a is H or C_1-C_{12} alkyl;

R^1 and R^2 are each independently C_6-C_{24} alkyl or C_6-C_{24} alkenyl;

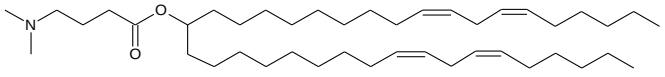
R^3 is H, OR^5, CN, -$C(=O)OR^4$, -$OC(=O)R^4$ or –$NR^5C(=O)R^4$;

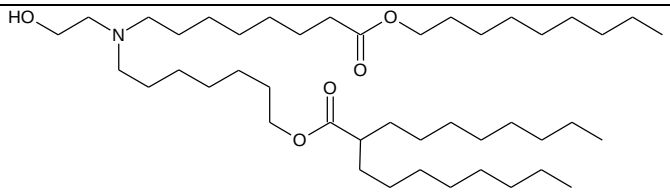
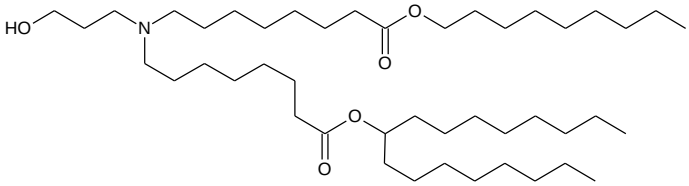
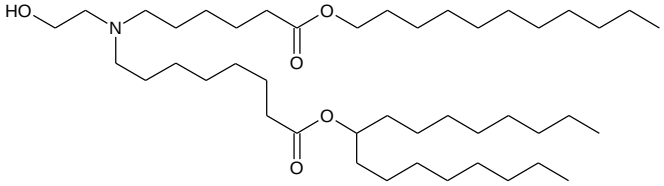
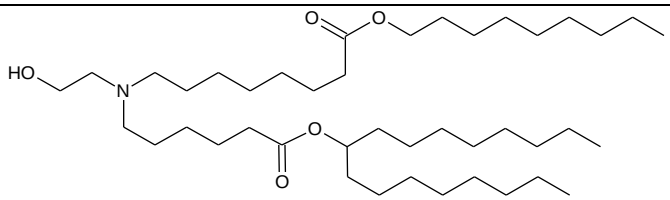
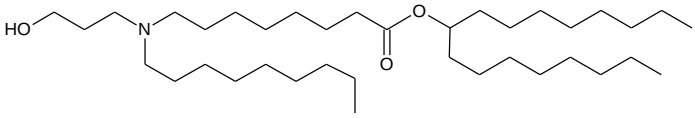
R^4 is C_1-C_{12} alkyl;

R^5 is H or C_1-C_6 alkyl; and

x is 0, 1 or 2;

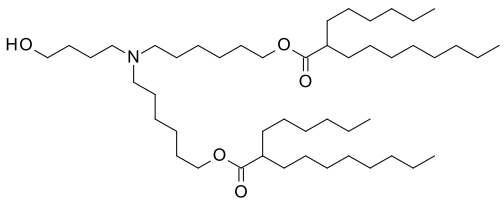
(iii) is selected from the following structures A to F:

No.	Structure
A	

No.	Structure
B	
C	
D	
E	
F	

or

(iv) is a lipid having the structure

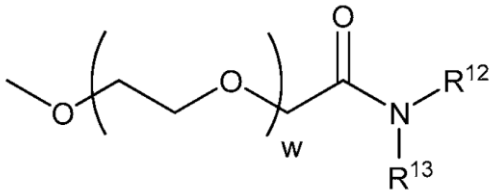
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4. The composition of any one of claims 1 to 3, wherein the LNPs further comprise one or more additional lipids, preferably selected from the group consisting of polymer conjugated lipids, neutral lipids, steroids, and combinations thereof, more preferably the LNPs comprise the cationically ionizable lipid, a polymer conjugated lipid, a neutral lipid, and a steroid;

wherein optionally:

(i) the polymer conjugated lipid comprises a pegylated lipid, wherein the pegylated lipid preferably has the following structure:



or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein:

R^{12} and R^{13} are each independently a straight or branched, saturated or unsaturated alkyl chain containing from 10 to 30 carbon atoms, wherein the alkyl chain is optionally interrupted by one or more ester bonds; and w has a mean value ranging from 30 to 60; or

the polymer conjugated lipid comprises a polysarcosine-lipid conjugate or a conjugate of polysarcosine and a lipid-like material, wherein the polysarcosine-lipid conjugate or conjugate of polysarcosine and a lipid-like material preferably is a member selected from the group consisting of a polysarcosine-diacylglycerol conjugate, a polysarcosine-dialkyloxypropyl conjugate, a polysarcosine-phospholipid conjugate, a polysarcosine-ceramide conjugate, and a mixture thereof;

(ii) the neutral lipid is a phospholipid, preferably selected from the group consisting of phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidic acids, phosphatidylserines and sphingomyelins, more preferably selected from the group consisting of distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dimyristoylphosphatidylcholine (DMPC), dipentadecanoylphosphatidylcholine, dilauroylphosphatidylcholine, dipalmitoylphosphatidylcholine (DPPC), diarachidoylphosphatidylcholine (DAPC), dibehenoylphosphatidylcholine (DBPC), ditricosanoylphosphatidylcholine (DTPC), dilignoceroylphatidylcholine (DLPC), palmitoyloleoyl-phosphatidylcholine (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 (C16 Lyso PC), dioleoylphosphatidylethanolamine (DOPE), distearoyl-phosphatidylethanolamine (DSPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), dilauroyl-phosphatidylethanolamine (DLPE), and diphytanoyl-phosphatidylethanolamine (DPyPE); and/or

(iii) the steroid comprises a sterol such as cholesterol.

5. The composition of any one of claims 1 to 4, wherein

(i) the LNPs comprise at least about 75%, preferably at least about 80% of the RNA comprised in the composition;

(ii) the RNA is encapsulated within or associated with the LNPs;

(iii) the RNA comprises a modified nucleoside in place of uridine, wherein the modified nucleoside is preferably selected from pseudouridine (ψ), N1-methyl-pseudouridine (m1ψ), and 5-methyl-uridine (m5U);

(iv) the RNA comprises at least one of the following, preferably all of the following: a 5' cap; a 5' UTR; a 3' UTR; and a poly-A sequence;
wherein optionally:
the poly-A sequence comprises at least 100 A nucleotides, wherein the poly-A sequence preferably is an interrupted sequence of A nucleotides; and/or
the 5' cap is a cap1 or cap2 structure;

(v) the RNA encodes one or more polypeptides, wherein the one or more polypeptides preferably comprise an epitope for inducing an immune response against an antigen in a subject,
wherein optionally:
the RNA comprises an open reading frame (ORF) encoding an amino acid sequence comprising a SARS-CoV-2 S protein, an immunogenic variant thereof, or an immunogenic fragment of the SARS-CoV-2 S protein or the immunogenic variant thereof; and/or
the RNA comprises an ORF encoding a full-length SARS-CoV2 S protein variant with proline residue substitutions at positions 986 and 987 of SEQ ID NO: 1; and/or
the RNA comprises an ORF encoding a full-length SARS-CoV2 S protein variant, wherein the SARS-CoV2 S protein variant has at least 80% identity to SEQ ID NO: 7.

6. The composition of any one of claims 1 to 5, wherein the composition is in frozen form,
wherein optionally
the RNA integrity after thawing the frozen composition is at least 50% compared to the RNA integrity before the composition has been frozen; and/or
the size ($Z_{average}$) and/or size distribution and/or polydispersity index (PDI) of the LNPs after thawing the frozen composition is equal to the size ($Z_{average}$) and/or size distribution and/or PDI of the LNPs before the composition has been frozen.

7. The composition of any one of claims 1 to 5, wherein the composition is in liquid form.

8. A method of preparing a composition comprising LNPs dispersed in a final aqueous phase, wherein the LNPs comprise a cationically ionizable lipid and RNA; the final aqueous phase comprises a final buffer system comprising a final buffer substance and a final monovalent

anion, the final buffer substance being selected from the group consisting of Tris and its protonated form, Bis-Tris-methane and its protonated form, and TEA and its protonated form, and the final monovalent anion being selected from the group consisting of chloride, acetate, glycolate, and lactate; the concentration of the final buffer substance in the composition is at most about 25 mM; and the final aqueous phase is substantially free of inorganic phosphate anions, substantially free of citrate anions, and substantially free of anions of EDTA;

wherein the method comprises:

(I) preparing a formulation comprising LNPs dispersed in the final aqueous phase, wherein the LNPs comprise the cationically ionizable lipid and RNA; and

(II) optionally freezing the formulation to about -10°C or below,

thereby obtaining the composition,

wherein step (I) comprises:

(a) preparing an RNA solution containing water and a first buffer system;

(b) preparing an ethanolic solution comprising the cationically ionizable lipid and, if present, one or more additional lipids;

(c) mixing the RNA solution prepared under (a) with the ethanolic solution prepared under (b), thereby preparing a first intermediate formulation comprising the LNPs dispersed in a first aqueous phase comprising the first buffer system; and

(d) filtrating the first intermediate formulation prepared under (c) using a final aqueous buffer solution comprising the final buffer system,

thereby preparing the formulation comprising the LNPs dispersed in the final aqueous phase,

wherein the concentration of the final monovalent anion in the composition is at most equal to the concentration of the final buffer substance in the composition.

9. The method of claim 8, wherein

(i) step (I) further comprises one or more steps selected from diluting and filtrating;

(ii) step (I) comprises:

(a') providing an aqueous RNA solution;

(b') providing a first aqueous buffer solution comprising a first buffer system;

(c') mixing the aqueous RNA solution provided under (a') with the first aqueous buffer solution provided under (b') thereby preparing an RNA solution containing water and the first buffer system;

(d') preparing an ethanolic solution comprising the cationically ionizable lipid and, if present, one or more additional lipids;

(e') mixing the RNA solution prepared under (c') with the ethanolic solution prepared under (d'), thereby preparing a first intermediate formulation comprising LNPs dispersed in a first aqueous phase comprising the first buffer system;

(f') optionally filtrating the first intermediate formulation prepared under (e') using a further aqueous buffer solution comprising a further buffer system, thereby preparing a further intermediate formulation comprising the LNPs dispersed in a further aqueous phase comprising the further buffer system, wherein the further aqueous buffer solution may be identical to or different from the first aqueous buffer solution;

(g') optionally repeating step (f') once or two or more times, wherein the further intermediate formulation comprising the LNPs dispersed in the further aqueous phase comprising the further buffer system obtained after step (f') of one cycle is used as the first intermediate formulation of the next cycle, wherein in each cycle the further aqueous buffer solution may be identical to or different from the first aqueous buffer solution;

(h') filtrating the first intermediate formulation obtained in step (e'), if step (f') is absent, or the further intermediate formulation obtained in step (f'), if step (f') is present and step (g') is not present, or the further intermediate formulation obtained after step (g'), if steps (f') and (g') are present, using a final aqueous buffer solution comprising the final buffer system and having a pH of at least 6.0; and

(i') optionally diluting the formulation obtained in step (h') with a dilution solution;

thereby preparing the formulation comprising the LNPs dispersed in the final aqueous phase;

(iii) filtrating is tangential flow filtrating or diafiltrating; and/or

(iv) the method comprises (II) freezing the formulation to about -10°C or below.

10. The method of claim 8 or 9, wherein

(i) the final buffer substance is Tris and its protonated form;

(ii) the concentration of the final buffer substance, in particular Tris and its protonated form, in the composition is at most about 20 mM, preferably at most about 15 mM, more preferably at most about 10 mM, such as about 10 mM; and/or

(iii) the final aqueous phase is substantially free of inorganic sulfate anions and/or carbonate anions and/or dibasic organic acid anions and/or polybasic organic acid anions, in particular substantially free of inorganic sulfate anions, carbonate anions dibasic organic acid anions and polybasic organic acid anions.

11. The method of any one of claims 8 to 10, wherein (i) the RNA solution prepared in step (a) further comprises one or more di- and/or polybasic organic acid anions, and step (d) is conducted under conditions which remove the one or more di- and/or polybasic organic acid anions resulting in the formulation comprising the LNPs dispersed in the final aqueous phase with the final aqueous phase being substantially free of the one or more di- and/or polybasic organic acid

anions present in the RNA solution prepared in step (a); or (ii) the first aqueous buffer solution and the first aqueous phase comprise one or more di- and/or polybasic organic acid anions and least one of steps (f') to (h') is conducted under conditions which remove the one or more di- and/or polybasic organic acid anions from the first intermediate formulation and/or from the further intermediate formulation, wherein the one or more di- and/or polybasic organic acid anions preferably comprise citrate anions and/or anions of EDTA, and/or

wherein (i) the RNA solution obtained in step (a) has a pH of below 6.0, preferably at most about 5.0, more preferably at most about 4.5; or (ii) the first aqueous buffer solution has a pH of below 6.0, preferably at most about 5.0, more preferably at most about 4.5.

12. The method of any one of claims 8 to 11, wherein

(a) (i) the first buffer system used in step (a) comprises the final buffer substance and the final monovalent anion used in step (d), preferably the buffer system and pH of the first buffer system used in step (a) are identical to the buffer system and pH of the final aqueous buffer solution used in step (d); or (ii) each of the first buffer system and every further buffer system used in steps (b'), (f') and (g') comprises the final buffer substance and the final monovalent anion used in step (h'), preferably the buffer system and pH of each of the first aqueous buffer solution and of every further aqueous buffer solution used in steps (b'), (f') and (g') are identical to the buffer system and pH of the final aqueous buffer solution;

(b) the concentration of the final monovalent anion in the composition is less than the concentration of the final buffer substance in the composition or is less than about 9 mM;

(c) the pH of the composition is between about 6.5 and about 8.0, preferably between about 6.9 and about 7.9, such as between about 7.0 and about 7.8;

(d) water is the main component in the formulation and/or composition and/or the total amount of solvent(s) other than water contained in the composition is less than about 0.5% (v/v);

(e) the osmolality of the composition is at most about 400 x 10^{-3} osmol/kg;

(f) the concentration of the RNA in the composition is about 5 mg/l to about 150 mg/l, preferably about 10 mg/l to about 130 mg/l, more preferably about 30 mg/l to about 120 mg/l; and/or

(g) (i) step (I) further comprises diluting the formulation prepared under (d) with a dilution solution, or step (i') is present, wherein the dilution solution comprises a cryoprotectant; and/or (ii) the formulation obtained in step (I) and the composition comprise a cryoprotectant, preferably in a concentration of at least about 1% w/v, wherein the cryoprotectant preferably comprises one or more selected from the group consisting of

carbohydrates and sugar alcohols, more preferably the cryoprotectant is selected from the group consisting of sucrose, glucose, glycerol, sorbitol, and a combination thereof, more preferably the cryoprotectant comprises sucrose and/or glycerol,

or

the formulation obtained in step (I) and the composition is substantially free of a cryoprotectant.

13. The method of any one of claims 8 to 12, wherein the cationically ionizable lipid is as specified in claim 3.

14. The method of any one of claims 8 to 13, wherein the ethanolic solution prepared in step (b) or (d') further comprises one or more additional lipids and the LNPs further comprise the one or more additional lipids, wherein the one or more additional lipids are preferably as specified in claim 4.

15. The method of any one of claims 8 to 14, wherein

(i) the cationically ionizable lipid, the polymer conjugated lipid, the neutral lipid, and the steroid are present in the ethanolic solution in a molar ratio of 20% to 60% of the cationically ionizable lipid, 0.5% to 15% of the polymer conjugated lipid, 5% to 25% of the neutral lipid, and 25% to 55% of the steroid, preferably in a molar ratio of 45% to 55% of the cationically ionizable lipid, 1.0% to 5% of the polymer conjugated lipid, 8% to 12% of the neutral lipid, and 35% to 45% of the steroid;

(ii) the final aqueous phase does not comprise a chelating agent;

(iii) the LNPs comprise at least about 75%, preferably at least about 80% of the RNA comprised in the composition;

(iv) the RNA is encapsulated within or associated with the LNPs; and/or

(v) the RNA is as specified in claim 5.

16. The method of any one of claims 8 to 15, which does not comprise step (II).

17. A composition preparable by the method of any one of claims 8 to 16, which is in frozen form or in liquid form.

18. A ready-to-use pharmaceutical composition preparable by a method, the method comprising

(i) the steps of providing a frozen composition prepared by the method of any one of claims 8 to 15, and thawing the frozen composition thereby obtaining the ready-to-use pharmaceutical composition; or

(ii) the method comprising the step of providing a liquid composition prepared by the method of any one of claims 8, 9 (i) to (iii), and 10 to 16, thereby obtaining the ready-to-use pharmaceutical composition.

19. A composition of any one of claims 1 to 7, 17, and 18 for use in therapy.

20. Use of the composition of any one of claims 1 to 7, 17, and 18 in the manufacture of a medicament for inducing an immune response in a subject.