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 15 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 15 mM; and the final aqueous phase is

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 15 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 10 mM, or 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 6.5 and 8.0, preferably between 6.9 and 7.9, such as between 7.0 and 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 5 mg/l to 150 mg/l, preferably 10 mg/l to 130 mg/l, more preferably 30 mg/l to 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,

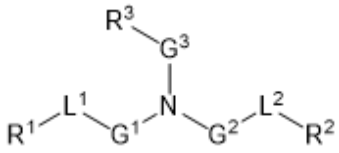
sorbitol, and a combination thereof, more preferably the cryoprotectant comprises sucrose and/or glycerol, or

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) 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;

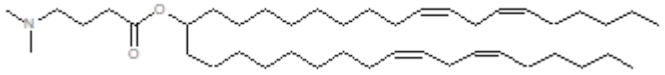
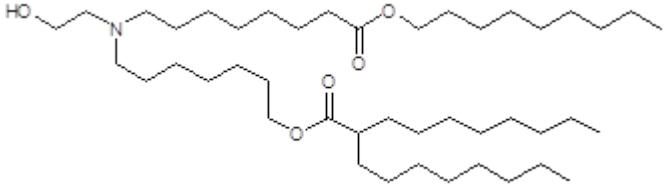
R^3 is H, OR^5, CN, -$C(=O)OR^4$, -$OC(=O)R^4$ or –$NR^5C(=O)R^4$;

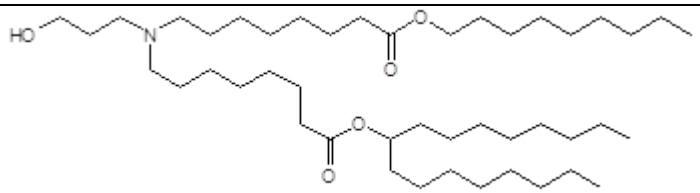
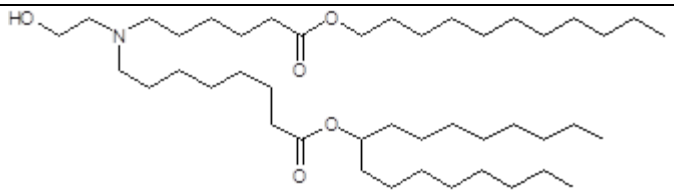
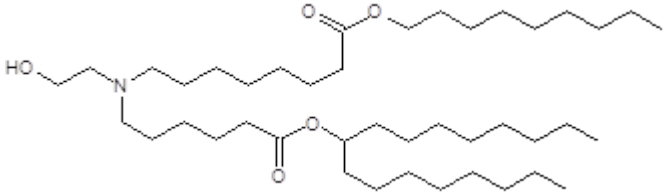
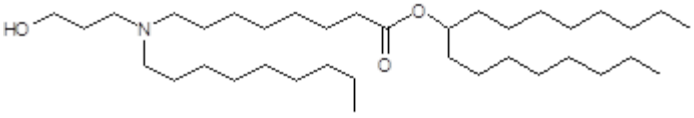
R^4 is C_1-C_{12} alkyl;

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

x is 0, 1 or 2;

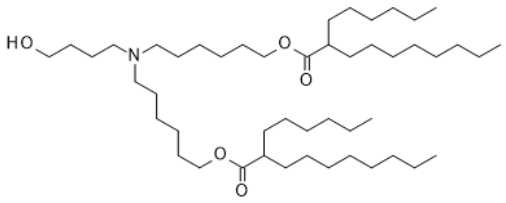
(ii) is selected from the following structures A to F:

No.	Structure
A	
B	

No.	Structure
C	
D	
E	
F	

or

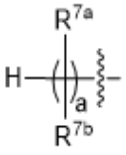
(iii) is a lipid having the structure

I-3	

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4. The composition of claim 3, wherein the cationically ionizable lipid has the structure of Formula (I), wherein

(i) R^1 and R^2 each, independently have the following structure:



,

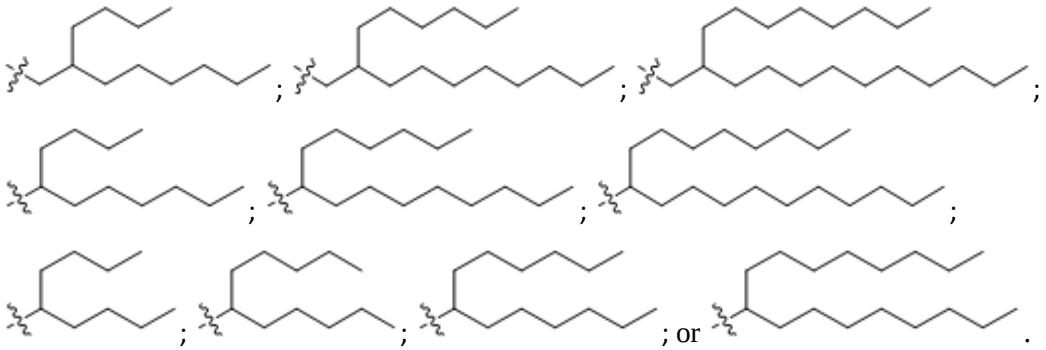
wherein:

R^{7a} and R^{7b} are, at each occurrence, independently C_1-C_{12} alkyl; and

a is an integer from 2 to 12,

wherein R^{7a}, R^{7b} and a are each selected such that R^1 and R^2 each independently comprise from 6 to 20 carbon atoms; or

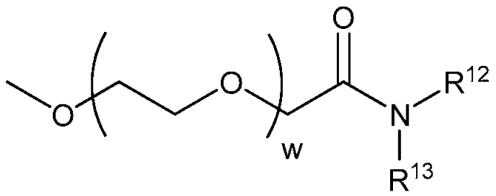
(ii) R^1 and R^2 both, independently have one of the following structures:



5. The composition of any one of claims 1 to 4, wherein the LNPs further comprise 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.

6. The composition of any one of claims 1 to 5, 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.

7. The composition of any one of claims 1 to 6, 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.

8. The composition of any one of claims 1 to 6, wherein the composition is in liquid form.

9. 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 15 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.

10. The method of claim 9, 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.

11. The method of claim 9 or 10, 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 10 mM, or 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.

12. The method of any one of claims 9 to 11, 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.

13. The method of any one of claims 9 to 12, 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 6.5 and 8.0, preferably between 6.9 and 7.9, such as between 7.0 and 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 5 mg/l to 150 mg/l, preferably 10 mg/l to 130 mg/l, more preferably 30 mg/l to 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.

14. The method of any one of claims 9 to 13, wherein the cationically ionizable lipid is as specified in claim 3 or 4.

15. The method of any one of claims 9 to 14, wherein the ethanolic solution prepared in step (b) or (d') further comprises a polymer conjugated lipid, a neutral lipid, and a steroid and the LNPs further comprise the polymer conjugated lipid, the neutral lipid, and the steroid, wherein the polymer conjugated lipid, the neutral lipid, and the steroid are preferably as specified in claim 5.

16. The method of any one of claims 9 to 15, 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.

17. The method of any one of claims 9 to 16, which does not comprise step (II).

18. A composition preparable by the method of any one of claims 9 to 17, which is in frozen form or in liquid form.

19. 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 9 to 16, 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 9, 10 (i) to (iii), and 11 to 17, thereby obtaining the ready-to-use pharmaceutical composition.

20. A composition of any one of claims 1 to 8, 18, and 19 for use in therapy.

21. Use of the composition of any one of claims 1 to 8, 18, and 19 in the manufacture of a medicament for inducing an immune response in a subject.