CLAIMS

1. An oil-in-water emulsion comprising particles that are dispersed in an aqueous continuous phase, wherein the average diameter of said particles is from about 80 nm to 180 nm ; the emulsion comprises an oil and a cationic lipid, and

wherein:

(i) the ratio of oil:lipid (mole:mole) is at least about 8:1 (mole:mole);

(ii) the concentration of cationic lipid in said emulsion is at least about 2.5 mM; and

(iii) the cationic lipid is not DC-Cholesterol.

2. The oil-in-water emulsion of claim 1, wherein the average diameter of said particles is from about 80 nm to about 150 nm.

3. The oil-in-water emulsion of claim 1 or 2, wherein the average diameter of said particles is from about 80 nm to about 130 nm.

4. The oil-in-water emulsion of any one of claims 1-3, wherein the oil-in-water emulsion is stable.

5. The oil-in-water emulsion of any one of claims 1-4, wherein the ratio of oil:lipid (mole:mole) is from about 10:1 (mole:mole) to about 43:1 (mole:mole).

6. The oil-in-water emulsion of any one of claims 1-5, wherein said oil-in-water emulsion comprises from about 0.2% to about 8% (w/v) oil.

7. The oil-in-water emulsion of any one of claims 1-6, wherein said oil is squalene or squalane.

8. The oil-in-water emulsion of any one of claims 1-7, wherein said particles further comprise a surfactant.

9. The oil-in-water emulsion of claim 8, wherein said surfactant is a nonionic surfactant.

10. The oil-in-water emulsion of claim 8 or 9, wherein said surfactant is not a Polyethylene Glycol (PEG)-lipid.

11. The oil-in-water emulsion of any one of claims 8-10, wherein the cationic oil-in-water emulsion comprises from about 0.01% to about 2.5% (v/v) surfactant.

12. The oil-in-water emulsion of any one of claims 8-11, wherein said surfactant is SPAN85 (Sorbtian Trioleate), Tween 80 (polysorbate 80), or a combination thereof.

13. The oil-in-water emulsion of claim 12, wherein the cationic oil-in-water emulsion comprises about 0.5% (v/v) Tween 80 and about 0.5% (v/v) SPAN85.

14. The oil-in-water emulsion of any one of claims 1-13, wherein the head group of the cationic lipid comprises a quaternary amine.

15. The oil-in-water emulsion of any one of claims 1-13, wherein the cationic lipid is selected from the group consisting of: 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (DOEPC), N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), and N-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA).

16. The oil-in-water emulsion of claim 15, wherein said cationic lipid is DOTAP.

17. The oil-in-water emulsion of claim 16, wherein the concentration of DOTAP in said emulsion is at least about 2.58 mM (1.8 mg/mL).

18. The oil-in-water emulsion of claim 16, wherein the concentration of DOTAP in said emulsion is from about 2.58 mM (1.8 mg/mL) to about 7.16 mM (5 mg/mL).

19. A method for preparing the oil-in-water emulsion of any one of claims 1-18, comprising: (a) directly dissolving the cationic lipid in the oil to form an oil phase; (b) providing an aqueous phase of the emulsion; and (c) dispersing the oil phase in the aqueous phase by homogenization.

20. The method of claim 19, wherein step (a) further comprises heating the oil to a temperature between about 30°C to about 65°C.

21. A composition comprising an RNA molecule complexed with a particle of a cationic oil-in-water emulsion, wherein the particle comprises an oil that is in liquid phase at 25°C, and a cationic lipid; wherein:

(i) the ratio of oil:lipid (mole:mole) is at least about 8:1 (mole:mole);

(ii) the concentration of cationic lipid in said composition is at least about 1.25 mM; and

(iii) the cationic lipid is not DC-Cholesterol.

22. The composition of claim 21, wherein the average diameter of said particles is from about 80 nm to 180 nm.

23. The composition of claim 21, wherein the average diameter of said particles is from about 80 nm to 150 nm.

24. The composition of claim 21, wherein the average diameter of said particles is from about 80 nm to about 130 nm.

25. The composition of any one of claims 21-24, wherein the N/P ratio of the composition is at least about 4:1.

26. The composition of any one of claims 21-24, wherein the N/P ratio of the composition is from about 4:1 to about 20:1.

27. The composition of any one of claims 21-24, wherein the N/P ratio of the composition is from about 4:1 to about 15:1.

28. The composition of any one of claims 21-27, wherein the ratio of oil:lipid (mole:mole) is from about 10:1 (mole:mole) to about 43:1 (mole:mole).

29. The composition of any one of claims 21-28, wherein said oil-in-water emulsion comprises from about 0.1% to about 5% (w/v) oil.

30. The composition of claim 29, wherein said oil is squalene or squalane.

31. The composition of any one of claims 21-30, wherein said particles further comprise a surfactant.

32. The composition of claim 31, wherein said surfactant is a nonionic surfactant.

33. The composition of claim 31 or 32, wherein said surfactant is not a Polyethylene Glycol (PEG)-lipid.

34. The composition of any one of claims 31-33, wherein the cationic oil-in-water emulsion comprises from about 0.005% to about 1.25% (v/v) surfactant.

35. The composition of any one of claims 31-33, wherein said surfactant is SPAN85 (Sorbtian Trioleate), Tween 80 (polysorbate 80), or a combination thereof.

36. The composition of claim 35, wherein the cationic oil-in-water emulsion comprises about 0.25% (v/v) Tween 80 and about 0.25% (v/v) SPAN85.

37. The composition of any one of claims 21-36, wherein the head group of the cationic lipid comprises a quaternary amine.

38. The composition of any one of claims 21-36, wherein said cationic lipid is selected from the group consisting of: 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (DOEPC), N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), and N-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA).

39. The composition of claim 38, wherein said cationic lipid is DOTAP.

40. The composition of claim 39, wherein the concentration of DOTAP in said composition is at least about 1.29 mM (0.9 mg/mL).

41. The composition of claim 39, wherein the concentration of DOTAP in said composition is from about 1.29 mM (0.9 mg/mL) to about 3.58 mM (2.5 mg/mL).

42. The composition of any one of claims 21-41, wherein the RNA molecule is a self-replicating RNA molecule that encodes an antigen.

43. The composition of claim 42, wherein the self-replicating RNA is an alphavirus-derived RNA replicon.

44. A method for preparing a composition comprising an RNA molecule complexed with a particle of a cationic oil-in-water emulsion, comprising:

(i) providing an oil-in-water emulsion of any one of claims 1-18;

(ii) providing an aqueous solution comprising the RNA molecule; and

(iii) combining the oil-in-water emulsion of (i) and the aqueous solution of (ii), thereby preparing the composition.

45. The method of claim 44, wherein the cationic oil-in-water emulsion of (i) and RNA solution of (ii) are combined at about 1:1 (v/v) ratio.

46. The method of claim 44 or 45, wherein the aqueous solution comprising the RNA molecule comprises a salt.

47. The method of claim 46, wherein the salt is NaCl.

48. The method of claim 47, wherein the aqueous solution comprises about 20 mM NaCl.

49. The method of any one of claims 44-48, wherein the aqueous solution comprising the RNA molecule is a buffer.

50. The method of claim 49, wherein the buffer is a citrate buffer.

51. The method of claim 50, wherein the buffer comprises about 2 mM citrate.

52. The method of any one of claims 44-51, wherein the aqueous solution comprising the RNA molecule comprises a nonionic tonicifying agent.

53. The method of claim 52, wherein the nonionic tonicifying agent is a sugar or sugar alcohol.

54. The method of claim 53, wherein the nonionic tonicifying agent is selected from the group consisting of sucrose, trehalose, sorbitol, dextrose and combination thereof.

55. The method of claim 52, wherein the nonionic tonicifying agent is sucrose.

56. The method of claim 55, wherein the aqueous solution comprises about 560 mM sucrose.

57. The method of any one of claims 44-56, wherein the aqueous solution comprising the RNA molecule comprises a polymer.

58. The method of claim 57, wherein the polymer is Pluronic® F127.

59. The method of claim 57 or 58, wherein the aqueous solution comprises from about 0.05% to about 20% (w/v) polymer.

60. The method of any one of claims 57-59, wherein the aqueous solution comprises about 1% (w/v) Pluronic® F127.

61. An oil-in-water emulsion comprising particles that are dispersed in an aqueous continuous phase, wherein the emulsion comprises an oil and a cationic lipid, the average diameter of said particles is from about 80 nm to 180 nm, the oil is present from 0.6% to 4% (w/v); and the concentration of cationic lipid in said emulsion is at least about 1.25 mM.

62. The oil-in-water emulsion of claim 61, wherein the oil-in-water emulsion is stable.

63. The oil-in-water emulsion of claim 61 or 62, wherein the concentration of cationic lipid in said emulsion is at least about 2.5 mM.

64. The oil-in-water emulsion of any one of claims 61-63, wherein the oil is squalene or squalane.

65. The oil-in-water emulsion of any one of claims 61-64, wherein the particle further comprises a surfactant.

66. The oil-in-water emulsion of claim 65, wherein the surfactant is a nonionic surfactant.

67. The oil-in-water emulsion of claim 66, wherein said surfactant is not a Polyethylene Glycol (PEG)-lipid.

68. The oil-in-water emulsion of any one of claims 65-67, wherein the cationic oil-in-water emulsion comprises from about 0.01% to about 2.5% (v/v) surfactant.

69. The oil-in-water emulsion of any one of claims 65-68, wherein said surfactant is SPAN85 (Sorbtian Trioleate), Tween 80 (polysorbate 80), or a combination thereof.

70. The oil-in-water emulsion of any one of claims 61-69, wherein the head group of the cationic lipid comprises a quaternary amine.

71. The oil-in-water emulsion of any one of claims 61-69, wherein the cationic lipid is selected from the group consisting of: 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (DOEPC), N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), and N-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA).

72. The oil-in-water emulsion of claim 71, wherein said cationic lipid is DOTAP.

73. A composition comprising an RNA molecule complexed with a particle of the cationic oil-in-water emulsion of any one of claims 61-72.

74. The composition of claim 73, wherein the N/P ratio of the composition is at least about 4:1.

75. The composition of claim 73, wherein the N/P ratio of the composition is from about 4:1 to about 20:1.

76. The composition of claim 73, wherein the N/P ratio of the composition is from about 4:1 to about 15:1.

77. The composition of any one of claims 73-76, wherein the RNA molecule is a self-replicating RNA molecule that encodes an antigen.

78. The composition of claim 77, wherein the self-replicating RNA is an alphavirus-derived RNA replicon.

79. The composition of any one of claims 73-78, wherein the average diameter of the emulsion particles is from about 80 nm to about 180 nm.

80. The composition of any one of claims 21-43 and 73-79, wherein the composition is buffered and has a pH of about 6.0 to about 8.0.

81. The composition of any one of claims 21-43 and 73-80, wherein the composition further comprises an inorganic salt, and the concentration of inorganic salt is no greater than 30 mM.

82. The composition of claim claims 21-43 and 73-81, wherein the composition further comprises a nonionic tonicifying agent, and is isotonic.

83. The oil-in-water emulsion of any one of claims 1-20, wherein the oil-in-water emulsion is stable.

84. A composition comprising an RNA molecule complexed with a particle of a cationic oil-in-water emulsion, wherein the composition is prepared using a process that comprises:

(i) providing an oil-in-water emulsion of any one of claims 61-72;

(ii) providing an aqueous solution comprising the RNA molecule; and

(iii) combining the oil-in-water emulsion of (i) and of the aqueous solution (ii), thereby preparing the composition.

85. The composition of any one of claims 21-43, 73-82 and 84, wherein the RNA molecule is a polycistronic RNA encoding two or more antigens.

86. The composition of claim 85, wherein the polycistronic RNA is a alphavirus replicon.

87. The composition of claim 86, wherein the alphavirus replicon contains a first nucleotide sequence encoding a first antigen and a second nucleotide sequence encoding a second antigen, wherein the first nucleotide sequence and the second nucleotide sequence are operably linked to control elements.

88. The composition of claim 87, wherein the first nucleotide sequence is operably linked to a first control element and the second nucleotide sequence is operably linked to a second control element.

89. The composition of claim 87 or 88, further comprising a third nucleotide sequence encoding a third protein or fragment thereof, wherein the third nucleotide sequences is operably linked to a control element.

90. The composition of claim 89, wherein the third nucleotide sequence is operably linked to a third control element.

91. The composition of claim 89 or 90, further comprising a fourth nucleotide sequence encoding a fourth protein or fragment thereof, wherein the fourth nucleotide sequences is operably linked to a control element.

92. The composition of claim 91, wherein the fourth nucleotide sequence is operably linked to a fourth control element.

93. The composition of claim 91 or 92, further comprising a fifth nucleotide sequence encoding a fifth protein or fragment thereof, wherein the fifth nucleotide sequences is operably linked to a control element.

94. The composition of claim 93, wherein the fifth nucleotide sequence is operably linked to a fifth control element.

95. The composition of any one of claims 87-94, wherein the control elements are independently selected from the group consisting of a subgenomic promoter, an IRES, and a viral 2A site.

96. A method of generating an immune response in a subject, comprising administering to a subject in need thereof the composition of any one of claims 21-43, 73-82 and 84-95.

97. The method of claim 96, wherein the total amount of the cationic lipid administered to the subject in a single administration is no more than about 30 mg.

98. The method of claim 96, wherein the total amount of DOTAP administered to the subject in a single administration is no more than about 24 mg.

99. The method of claim 96, wherein the total amount of DOTAP administered to the subject in a single administration is no more than about 4 mg.

100. An oil-in-water emulsion comprising particles that are dispersed in an aqueous continuous phase, wherein the average diameter of said particles is from about 80 nm to 180 nm ; the emulsion comprises an oil and a cationic lipid, and

wherein:

(i) the ratio of oil:lipid (mole:mole) is at least about 4:1 (mole:mole);

(ii) the concentration of cationic lipid in said emulsion is at least about 2.5 mM;

(iii) the oil is present from about 0.2% to about 8% (w/v); and

(iv) the cationic lipid is not DC-Cholesterol.

101. The oil-in-water emulsion of claim 100, wherein the oil is present from 0.6% to 4% (w/v).

102. The oil-in-water emulsion of claim 100 or 101, wherein the oil is present from about 1% to about 3.2% (w/v).

103. The oil-in-water emulsion of any one of claims 100-102, wherein the average diameter of said particles is from about 80 nm to about 150 nm.

104. The oil-in-water emulsion of any one of claims 100-103, wherein the average diameter of said particles is from about 80 nm to about 130 nm.

105. The oil-in-water emulsion of any one of claims 100-104, wherein the oil-in-water emulsion is stable.

106. The oil-in-water emulsion of any one of claims 100-105, wherein the ratio of oil:lipid (mole:mole) is from about 4:1 (mole:mole) to about 43:1 (mole:mole).

107. The oil-in-water emulsion of any one of claims 100-106, wherein said oil is squalene or squalane.

108. The oil-in-water emulsion of any one of claims 100-107, wherein said particles further comprise a surfactant.

109. The oil-in-water emulsion of claim 108, wherein said surfactant is a nonionic surfactant.

110. The oil-in-water emulsion of claim 108 or 109, wherein said surfactant is not a Polyethylene Glycol (PEG)-lipid.

111. The oil-in-water emulsion of any one of claims 108-110, wherein the cationic oil-in-water emulsion comprises from about 0.01% to about 2.5% (v/v) surfactant.

112. The oil-in-water emulsion of any one of claims 108-111, wherein said surfactant is SPAN85 (Sorbtian Trioleate), Tween 80 (polysorbate 80), or a combination thereof.

113. The oil-in-water emulsion of claim 112, wherein the cationic oil-in-water emulsion comprises about 0.5% (v/v) Tween 80 and about 0.5% (v/v) SPAN85.

114. The oil-in-water emulsion of any one of claims 100-113, wherein the head group of the cationic lipid comprises a quaternary amine.

115. The oil-in-water emulsion of any one of claims 100-113, wherein the cationic lipid is selected from the group consisting of: 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (DOEPC), N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), and N-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA).

116. The oil-in-water emulsion of claim 115, wherein said cationic lipid is DOTAP.

117. The oil-in-water emulsion of claim 116, wherein the concentration of DOTAP in said emulsion is at least about 2.58 mM (1.8 mg/mL).

118. The oil-in-water emulsion of claim 116, wherein the concentration of DOTAP in said emulsion is from about 2.58 mM (1.8 mg/mL) to about 7.16 mM (5 mg/mL).

119. A method for preparing the oil-in-water emulsion of any one of claims 100-118, comprising: (a) directly dissolving the cationic lipid in the oil to form an oil phase; (b) providing an aqueous phase of the emulsion; and (c) dispersing the oil phase in the aqueous phase by homogenization.

120. The method of claim 119, wherein step (a) further comprises heating the oil to a temperature between about 30°C to about 65°C.

121. A composition comprising an RNA molecule complexed with a particle of a cationic oil-in-water emulsion, wherein the particle comprises an oil that is in liquid phase at 25°C, and a cationic lipid; wherein:

(i) the ratio of oil:lipid (mole:mole) is at least about 4:1 (mole:mole);

(ii) the concentration of cationic lipid in said composition is at least about 1.25 mM; and

(iii) the oil is present from about 0.1% to about 4% (w/v); and

(iv) the cationic lipid is not DC-Cholesterol.

122. The composition of claim 121, wherein the oil is present from 0.3% to 2% (w/v).

123. The oil-in-water emulsion of claim 121 or 122, wherein the oil is present from about 0.5% to about 1.6% (w/v).

124. The composition of any one of claims 121-123, wherein the average diameter of said particles is from about 80 nm to 180 nm.

125. The composition of any one of claims 121-124, wherein the average diameter of said particles is from about 80 nm to 150 nm.

126. The composition of any one of claims 121-125, wherein the average diameter of said particles is from about 80 nm to about 130 nm.

127. The composition of any one of claims 121-126, wherein the N/P ratio of the composition is at least about 4:1.

128. The composition of any one of claims 121-126, wherein the N/P ratio of the composition is from about 4:1 to about 20:1.

129. The composition of any one of claims 121-126, wherein the N/P ratio of the composition is from about 4:1 to about 15:1.

130. The composition of any one of claims 121-129, wherein the ratio of oil:lipid (mole:mole) is from about 4:1 (mole:mole) to about 43:1 (mole:mole).

131. The composition of any one of claims 121-130, wherein said oil is squalene or squalane.

132. The composition of any one of claims 121-131, wherein said particles further comprise a surfactant.

133. The composition of claim 132, wherein said surfactant is a nonionic surfactant.

134. The composition of claim 132 or 133, wherein said surfactant is not a Polyethylene Glycol (PEG)-lipid.

135. The composition of any one of claims 132-134, wherein the cationic oil-in-water emulsion comprises from about 0.005% to about 1.25% (v/v) surfactant.

136. The composition of any one of claims 132-135, wherein said surfactant is SPAN85 (Sorbtian Trioleate), Tween 80 (polysorbate 80), or a combination thereof.

137. The composition of claim 136, wherein the cationic oil-in-water emulsion comprises about 0.25% (v/v) Tween 80 and about 0.25% (v/v) SPAN85.

138. The composition of any one of claims 121-137, wherein the head group of the cationic lipid comprises a quaternary amine.

139. The composition of any one of claims 121-138, wherein said cationic lipid is selected from the group consisting of: 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (DOEPC), N,N-dioleoyl-

N,N-dimethylammonium chloride (DODAC), and N-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA).

140. The composition of claim 139, wherein said cationic lipid is DOTAP.

141. The composition of claim 140, wherein the concentration of DOTAP in said composition is at least about 1.29 mM (0.9 mg/mL).

142. The composition of claim 140, wherein the concentration of DOTAP in said composition is from about 1.29 mM (0.9 mg/mL) to about 3.58 mM (2.5 mg/mL).

143. The composition of any one of claims 121-142, wherein the RNA molecule is a self-replicating RNA molecule that encodes an antigen.

144. The composition of claim 143, wherein the self-replicating RNA is an alphavirus-derived RNA replicon.

145. A method for preparing a composition comprising an RNA molecule complexed with a particle of a cationic oil-in-water emulsion, comprising:

(i) providing an oil-in-water emulsion of any one of claims 100-118;

(ii) providing an aqueous solution comprising the RNA molecule; and

(iii) combining the oil-in-water emulsion of (i) and the aqueous solution of (ii), thereby preparing the composition.

146. The method of claim 145, wherein the cationic oil-in-water emulsion of (i) and RNA solution of (ii) are combined at about 1:1 (v/v) ratio.

147. The oil-in-water emulsion of claim 61, wherein the oil is present from about 1% to about 3.2% (w/v).

148. The composition of any one of claims 121-144, wherein the RNA molecule is a polycistronic RNA encoding two or more antigens.

149. The composition of claim 148, wherein the polycistronic RNA is an alphavirus replicon.

150. The composition of claim 149, wherein the alphavirus replicon contains a first nucleotide sequence encoding a first antigen and a second nucleotide sequence encoding a second antigen, wherein the first nucleotide sequence and the second nucleotide sequence are operably linked to control elements.

151. The composition of claim 150, wherein the first nucleotide sequence is operably linked to a first control element and the second nucleotide sequence is operably linked to a second control element.

152. The composition of claim 150 or 151, further comprising a third nucleotide sequence encoding a third protein or fragment thereof, wherein the third nucleotide sequences is operably linked to a control element.

153. The composition of claim 152, wherein the third nucleotide sequence is operably linked to a third control element.

154. The composition of claim 152 or 153, further comprising a fourth nucleotide sequence encoding a fourth protein or fragment thereof, wherein the fourth nucleotide sequences is operably linked to a control element.

155. The composition of claim 154, wherein the fourth nucleotide sequence is operably linked to a fourth control element.

156. The composition of claim 154 or 155, further comprising a fifth nucleotide sequence encoding a fifth protein or fragment thereof, wherein the fifth nucleotide sequences is operably linked to a control element.

157. The composition of claim 156, wherein the fifth nucleotide sequence is operably linked to a fifth control element.

158. The composition of any one of claims 150-157, wherein the control elements are independently selected from the group consisting of a subgenomic promoter, an IRES, and a viral 2A site.

159. A method of generating an immune response in a subject, comprising administering to a subject in need thereof the composition of any one of claims 121-144.

160. The method of claim 159, wherein the total amount of the cationic lipid administered to the subject in a single administration is no more than about 30 mg.

161. The method of claim 159, wherein the total amount of DOTAP administered to the subject in a single administration is no more than about 24 mg.

162. The method of claim 159, wherein the total amount of DOTAP administered to the subject in a single administration is no more than about 4 mg.

163. The oil-in-water emulsion or composition of any one of claims 1-18, 21-43, 61-83, 85-95, 100-118, 121-144 or 147-158 further comprising an antioxidant.

CLAIMS

1. An invention not claimed in any divisional application.