Abstract:

Title: LIPOSOMES WITH LIPIDS HAVING AN ADVANTAGEOUS pKa VALUE FOR RNA DELIVERY

Abstract: RNA encoding an immunogen is delivered in a liposome for the purposes of immunisation. The liposome includes lipids which have a pKa in the range of 5.0 to 7.6 and, preferably, a tertiary amine. These liposomes can have essentially neutral surface charge at physiological pH and are effective for immunisation.
LIPOSOMES WITH LIPIDS HAVING AN ADVANTAGEOUS PKA-VALUE FOR RNA DELIVERY

This application claims the benefit of US provisional applications 61/361,830 (filed July 6, 2010) and 61/378,837 (filed August 31, 2010), the complete contents of both of which are hereby incorporated herein by reference for all purposes.

TECHNICAL FIELD
This invention is in the field of non-viral delivery of RNA for immunisation.

BACKGROUND ART
The delivery of nucleic acids for immunising animals has been a goal for several years. Various approaches have been tested, including the use of DNA or RNA, of viral or non-viral delivery vehicles (or even no delivery vehicle, in a "naked" vaccine), of replicating or non-replicating vectors, or of viral or non-viral vectors.

There remains a need for further and improved nucleic acid vaccines.

DISCLOSURE OF THE INVENTION
According to the invention, RNA encoding an immunogen is delivered in a liposome for the purposes of immunisation. The liposome includes lipids which have a pKa in the range of 5.0 to 7.6. Ideally the lipid with a pKa in this range has a tertiary amine; such lipids behave differently from lipids such as DOTAP or DC-Choi, which have a quaternary amine group. At physiological pH amines with a pKa in the range of 5.0 to 7.6 have neutral or reduced surface charge, whereas a lipid such as DOTAP is strongly cationic. The inventors have found that liposomes formed from quaternary amine lipids (e.g. DOTAP) are less suitable for delivery of immunogen-encoding RNA than liposomes formed from tertiary amine lipids (e.g. DLinDMA).

Thus the invention provides a liposome having a lipid bilayer encapsulating an aqueous core, wherein: (i) the lipid bilayer comprises a lipid having a pKa in the range of 5.0 to 7.6, and preferably having a tertiary amine; and (ii) the aqueous core includes a RNA which encodes an immunogen. These liposomes are suitable for in vivo delivery of the RNA to a vertebrate cell and so they are useful as components in pharmaceutical compositions for immunising subjects against various diseases.

The invention also provides a process for preparing a RNA-containing liposome, comprising steps of: (a) mixing RNA with a lipid at a pH which is below the lipid's pKa but is above 4.5, to form a liposome in which the RNA is encapsulated; and (b) increasing the pH of the resulting liposome-containing mixture to be above the lipid's pKa.

The liposome
The invention utilises liposomes in which immunogen-encoding RNA is encapsulated. Thus the RNA is (as in a natural virus) separated from any external medium by the liposome's lipid bilayer.
and encapsulation in this way has been found to protect RNA from RNase digestion. The liposomes can include some external RNA (e.g. on their surface), but at least half of the RNA (and ideally all of it) is encapsulated in the liposome's core. Encapsulation within liposomes is distinct from, for instance, the lipid/RNA complexes disclosed in reference 1.

Various amphiphilic lipids can form bilayers in an aqueous environment to encapsulate a RNA-containing aqueous core as a liposome. These lipids can have an anionic, cationic or zwitterionic hydrophilic head group. Liposomes of the invention comprise a lipid having a pKa in the range of 5.0 to 7.6, and preferred lipids with a pKa in this range have a tertiary amine. For example, they may comprise 1,2-dilinolexyloxy-N,N-dimethyl-3-aminopropane (DLinDMA; pKa 5.8) and/or 1,2-dilinolenyloxy-N,N-dimethyl-3-aminopropane (DLenDMA). Another suitable lipid having a tertiary amine is 1,2-dioleloxy-N,N-dimethyl-3-aminopropane (DODMA). See Figure 3 & ref. 2. Some of the amino acid lipids of reference 3 may also be used, as can certain of the amino lipids of reference 4. Further useful lipids with tertiary amines in their headgroups are disclosed in reference 5, the complete contents of which are incorporated herein by reference.

Liposomes of the invention can be formed from a single lipid or from a mixture of lipids, provided that at least one of the lipids has a pKa in the range of 5.0 to 7.6 (and, preferably, a tertiary amine). Within this pKa range, preferred lipids have a pKa of 5.5 to 6.7 e.g. between 5.6 and 6.8, between 5.6 and 6.3, between 5.6 and 6.0, between 5.5 and 6.2, or between 5.7 and 5.9. The pKa is the pH at which 50% of the lipids are charged, lying halfway between the point where the lipids are completely charged and the point where the lipids are completely uncharged. It can be measured in various ways, but is preferably measured using the method disclosed below in the section entitled "pKa measurement". The pKa typically should be measured for the lipid alone rather than for the lipid in the context of a mixture which also includes other lipids (e.g. not as performed in reference 6, which looks at the pKa of a SNALP rather than of the individual lipids).

Where a liposome of the invention is formed from a mixture of lipids, it is preferred that the proportion of those lipids which have a pKa within the desired range should be between 20-80% of the total amount of lipids e.g. between 30-70%, or between 40-60%. For instance, useful liposomes are shown below in which 40% or 60% of the total lipid is a lipid with a pKa in the desired range. The remainder can be made of e.g. cholesterol (e.g. 35-50% cholesterol) and/or DMG (optionally PEGylated) and/or DSPC. Such mixtures are used below. These % values are mole percentages.

A liposome may include an amphiphilic lipid whose hydrophilic portion is PEGylated (i.e. modified by covalent attachment of a polyethylene glycol). This modification can increase stability and prevent non-specific adsorption of the liposomes. For instance, lipids can be conjugated to PEG using techniques such as those disclosed in reference 6 and 7. PEG provides the liposomes with a coat which can confer favourable pharmacokinetic characteristics. The combination of efficient encapsulation of a RNA (particularly a self-replicating RNA), a cationic lipid having a pKa in the
range 5.0-7.6, and a PEGylated surface, allows for efficient delivery to multiple cell types (including both immune and non-immune cells), thereby eliciting a stronger and better immune response than when using quaternary amines without PEGylation. Various lengths of PEG can be used e.g. between 0.5-8kDa.

Lipids used with the invention can be saturated or unsaturated. The use of at least one unsaturated lipid for preparing liposomes is preferred. Figure 3 shows three useful unsaturated lipids. If an unsaturated lipid has two tails, both tails can be unsaturated, or it can have one saturated tail and one unsaturated tail.

A mixture of DSPC, DLinDMA, PEG-DMG and cholesterol is used in the examples. An independent aspect of the invention is a liposome comprising DSPC, DLinDMA, PEG-DMG & cholesterol. This liposome preferably encapsulates RNA, such as a self-replicating RNA e.g. encoding an immunogen.

Liposomal particles are usually divided into three groups: multilamellar vesicles (MLV); small unilamellar vesicles (SUV); and large unilamellar vesicles (LUV). MLVs have multiple bilayers in each vesicle, forming several separate aqueous compartments. SUVs and LUVs have a single bilayer encapsulating an aqueous core; SUVs typically have a diameter <50nm, and LUVs have a diameter >50nm. Liposomal particles of the invention are ideally LUVs with a diameter in the range of 50-220nm. For a composition comprising a population of LUVs with different diameters: (i) at least 80% by number should have diameters in the range of 20-220nm, (ii) the average diameter (Zav, by intensity) of the population is ideally in the range of 40-200nm, and/or (iii) the diameters should have a polydispersity index <0.2. The liposome/RNA complexes of reference 1 are expected to have a diameter in the range of 600-800nm and to have a high polydispersity. The liposome can be substantially spherical.

Techniques for preparing suitable liposomes are well known in the art e.g. see references 8 to 10. One useful method is described in reference 11 and involves mixing (i) an ethanolic solution of the lipids (ii) an aqueous solution of the nucleic acid and (iii) buffer, followed by mixing, equilibration, dilution and purification. Preferred liposomes of the invention are obtainable by this mixing process.

**Mixing process**

As mentioned above, the invention provides a process for preparing a RNA-containing liposome, comprising steps of: (a) mixing RNA with a lipid at a pH which is below the lipid's pKa but is above 4.5; then (b) increasing the pH to be above the lipid's pKa.

Thus a cationic lipid is positively charged during liposome formation in step (a), but the pH change thereafter means that the majority (or all) of the positively charged groups become neutral. This process is advantageous for preparing liposomes of the invention, and by avoiding a pH below 4.5 during step (a) the stability of the encapsulated RNA is improved.
The pH in step (a) is above 4.5, and is ideally above 4.8. Using a pH in the range of 5.0 to 6.0, or in the range of 5.0 to 5.5, can provide suitable liposomes.

The increased pH in step (b) is above the lipid's pKa. The pH is ideally increased to a pH less than 9, and preferably less than 8. Depending on the lipid's pKa, the pH in step (b) may thus be increased to be within the range of 6 to 8 e.g. to pH 6.5±0.3. The pH increase of step (b) can be achieved by transferring the liposomes into a suitable buffer e.g. into phosphate-buffered saline. The pH increase of step (b) is ideally performed after liposome formation has taken place.

RNA used in step (a) can be in aqueous solution, for mixing with an organic solution of the lipid (e.g. an ethanolic solution, as in ref. 11). The mixture can then be diluted to form liposomes, after which the pH can be increased in step (b).

**The RNA**

The invention is useful for *in vivo* delivery of RNA which encodes an immunogen. The RNA is translated by non-immune cells at the delivery site, leading to expression of the immunogen, and it also causes immune cells to secrete type I interferons and/or pro-inflammatory cytokines which provide a local adjuvant effect. The non-immune cells may also secrete type I interferons and/or pro-inflammatory cytokines in response to the RNA.

The RNA is +-stranded, and so it can be translated by the non-immune cells without needing any intervening replication steps such as reverse transcription. It can also bind to TLR7 receptors expressed by immune cells, thereby initiating an adjuvant effect.

Preferred +-stranded RNAs are self-replicating. A self-replicating RNA molecule (replicon) can, when delivered to a vertebrate cell even without any proteins, lead to the production of multiple daughter RNAs by transcription from itself (via an antisense copy which it generates from itself). A self-replicating RNA molecule is thus typically a + -strand molecule which can be directly translated after delivery to a cell, and this translation provides a RNA-dependent RNA polymerase which then produces both antisense and sense transcripts from the delivered RNA. Thus the delivered RNA leads to the production of multiple daughter RNAs. These daughter RNAs, as well as collinear subgenomic transcripts, may be translated themselves to provide *in situ* expression of an encoded immunogen, or may be transcribed to provide further transcripts with the same sense as the delivered RNA which are translated to provide *in situ* expression of the immunogen. The overall results of this sequence of transcriptions is a huge amplification in the number of the introduced replicon RNAs and so the encoded immunogen becomes a major polypeptide product of the cells.

As shown below, a self-replicating activity is not required for a RNA to provide an adjuvant effect, although it can enhance post-transfection secretion of cytokines. The self-replicating activity is particularly useful for achieving high level expression of the immunogen by non-immune cells. It can also enhance apoptosis of the non-immune cells.
One suitable system for achieving self-replication is to use an alphavirus-based RNA replicon. These + -stranded replicons are translated after delivery to a cell to give of a replicase (or replicase-transcriptase). The replicase is translated as a polyprotein which auto-cleaves to provide a replication complex which creates genomic — strand copies of the + -strand delivered RNA. These — strand transcripts can themselves be transcribed to give further copies of the + -stranded parent RNA and also to give a subgenomic transcript which encodes the immunogen. Translation of the subgenomic transcript thus leads to in situ expression of the immunogen by the infected cell. Suitable alphavirus replicons can use a replicase from a sindbis virus, a semliki forest virus, an eastern equine encephalitis virus, a Venezuelan equine encephalitis virus, etc. Mutant or wild-type virus sequences can be used e.g. the attenuated TC83 mutant of VEEV has been used in replicons [12].

A preferred self-replicating RNA molecule thus encodes (i) a RNA-dependent RNA polymerase which can transcribe RNA from the self-replicating RNA molecule and (ii) an immunogen. The polymerase can be an alphavirus replicase e.g. comprising one or more of alphavirus proteins nsP1, nsP2, nsP3 and nsP4.

Whereas natural alphavirus genomes encode structural virion proteins in addition to the non-structural replicase polyprotein, it is preferred that a self-replicating RNA molecule of the invention does not encode alphavirus structural proteins. Thus a preferred self-replicating RNA can lead to the production of genomic RNA copies of itself in a cell, but not to the production of RNA-containing virions. The inability to produce these virions means that, unlike a wild-type alphavirus, the self-replicating RNA molecule cannot perpetuate itself in infectious form. The alphavirus structural proteins which are necessary for perpetuation in wild-type viruses are absent from self-replicating RNAs of the invention and their place is taken by gene(s) encoding the immunogen of interest, such that the subgenomic transcript encodes the immunogen rather than the structural alphavirus virion proteins.

Thus a self-replicating RNA molecule useful with the invention may have two open reading frames. The first (5') open reading frame encodes a replicase; the second (3') open reading frame encodes an immunogen. In some embodiments the RNA may have additional (e.g. downstream) open reading frames e.g. to encode further immunogens (see below) or to encode accessory polypeptides.

A self-replicating RNA molecule can have a 5' sequence which is compatible with the encoded replicase.

Self-replicating RNA molecules can have various lengths but they are typically 5000-25000 nucleotides long e.g. 8000-15000 nucleotides, or 9000-12000 nucleotides. Thus the RNA is longer than seen in siRNA delivery.

A RNA molecule useful with the invention may have a 5' cap (e.g. a 7-methylguanosine). This cap can enhance in vivo translation of the RNA.
The 5' nucleotide of a RNA molecule useful with the invention may have a 5' triphosphate group. In a capped RNA this may be linked to a 7-methylguanosine via a 5'-to-5' bridge. A 5' triphosphate can enhance RIG-I binding and thus promote adjuvant effects.

A RNA molecule may have a 3' poly-A tail. It may also include a poly-A polymerase recognition sequence (e.g. AAUAAA) near its 3' end.

A RNA molecule useful with the invention will typically be single-stranded. Single-stranded RNAs can generally initiate an adjuvant effect by binding to TLR7, TLR8, RNA helicases and/or PKR. RNA delivered in double-stranded form (dsRNA) can bind to TLR3, and this receptor can also be triggered by dsRNA which is formed either during replication of a single-stranded RNA or within the secondary structure of a single-stranded RNA.

A RNA molecule useful with the invention can conveniently be prepared by in vitro transcription (IVT). IVT can use a (cDNA) template created and propagated in plasmid form in bacteria, or created synthetically (for example by gene synthesis and/or polymerase chain-reaction (PCR) engineering methods). For instance, a DNA-dependent RNA polymerase (such as the bacteriophage T7, T3 or SP6 RNA polymerases) can be used to transcribe the RNA from a DNA template. Appropriate capping and poly-A addition reactions can be used as required (although the replicon's poly-A is usually encoded within the DNA template). These RNA polymerases can have stringent requirements for the transcribed 5' nucleotide(s) and in some embodiments these requirements must be matched with the requirements of the encoded replicase, to ensure that the IVT-transcribed RNA can function efficiently as a substrate for its self-encoded replicase.

As discussed in reference 13, the self-replicating RNA can include (in addition to any 5' cap structure) one or more nucleotides having a modified nucleobase. Thus the RNA can comprise m5C (5-methylcytidine), m5U (5-methyluridine), m6A (N6-methyladenosine), s2U (2-thiouridine), Um (2'-0-methyluridine), mlA (1-methyladenosine); m2A (2-methyladenosine); Am (2'-0-methyladenosine); ms2m6A (2-methylthio-N6-methyladenosine); i6A (N6-isopentenyladenosine); ms2i6A (2-methylthio-N6-isopentenyladenosine); io6A (N6-(cis-hydroxyisopentenyl)adenosine); ms2io6A (2-methylthio-N6-(cis-hydroxyisopentenyl) adenosine); g6A (N6-glycinylcarbamoyladenosine); i6A (N6-threonyl carbamoyladenosine); ms2i6A (2-methylthio-N6-threonyl carbamoyladenosine); m6t6A (N6-methyl-N6-threonylcarbamoyladenosine); hn6A(N6-hydroxynorvalylcarbamoyl adenosine); ms2hn6A (2-methylthio-N6-hydroxynorvalyl carbamoyladenosine); Ar(p) (2'-0-ribosyladenosine (phosphate)); I (inosine); m1l (1-methylinosine); m'l'm (1,2'-0-dimethylinosine); m3C (3-methylcytidine); Cm (2T-0-methylcytidine); s2C (2-thiocytidine); ac4C (N4-acetylcytidine); f5C (5-fonnylcytidine); m5Cm (5,2'-0-dimethylcytidine); ac4Cm (N4acetyl2TOMethylcytidine); k2C (lysidine); mlG (1-methylguanosine); m2G (N2-methylguanosine); m7G (7-methylguanosine); Gm (2'-0-methylguanosine); m22G (N2,N2-dimethylguanosine); m2Gm (N2,2'-0-dimethylguanosine); m22Gm (N2,N2,2'-0-
trimethylguanosine); Gr(p) (2'-0-ribosylguanosine (phosphate)); yW (wybutosine); o2yW (peroxywybutosine); OHyW (hydroxywybutosine); OHyW* (undermodified hydroxywybutosine); imG (wyosine); mimG (methylguanosine); Q (queuosine); oQ (epoxyqueuosine); galQ (galtactosyl-queuosine); manQ (mannosyl-queuosine); preQ (7-cyano-7-deazaguanosine); preQ (7-aminomethyl-7-deazaguanosine); G (archaeosine); D (dihydouridine); m5Um (5,2'-0-dimethyluridine); s4U (4-thiouridine); m5s2U (5-methyl-2-thiouridine); s2Um (2-thio-2',0-methyluridine); acp3U (3-(3-amino-3-carboxypropyl)uridine); ho5U (5-hydroxyuridine); mo5U (5-methoxyuridine); cmo5U (uridine 5-oxyacetic acid); cmcm5U (uridine 5-oxyacetic acid methyl ester); chm5U (5-(carboxyhydroxymethyl)uridine); mcm5U (5-methoxycarbonylmethyl uridine methyl ester); cmcm5U (5-methoxycarbonylmethyl-2'-0-methyluridine); cmcm5s2U (5-methoxycarbonylmethyl-2-thiouridine); mm5s2U (5-aminomethyl-2-thiouridine); mmn5s2U (5-methylaminomethyl-2-thiouridine); mmn5se2U (5-methylaminomethyl-2-selenouridine); nc5Um (5-carbamoylmethyl uridine); nc5Um (5-carbamoylmethyl-2',0-methyluridine); cnm5m5U (5-carboxymethylaminomethyluridine); cmmn5Um (5-carboxymethylaminomethyl-2'-0-methyluridine); cmmn5s2U (5-carboxymethylaminomethyl-2-thiouridine); mm62A (N6,N6-dimethyladenosine); Tm (2',0-methylinosine); m4C (N4-methylcytidine); m4Cm (N4,2',0-dimethylcytidine); hm5C (5-hydroxymethylcytidine); m3U (3-methyluridine); cm5U (5-carboxymethyluridine); m6Am (N6,T-0-dimethyladenosine); nn62Am (N6,N6,0,2-trimethyladenosine); m2s7G (N2,7-dimethylguanosine); m2s27G (N2,N2,7-trimethylguanosine); m3Um (3,2T-0-dimethyluridine); m5D (5-methyldehydrouridine); £5Cm (5-formyl-2',0-methylcytidine); m1Gm (1,2',0-dimethylguanosine); m′Am (1,2-O-dimethyl adenosine) irinomethyluridine; tm5s2U (5-taurinomethyl-2-thiouridine)); imG-14 (4-demethyl guanosine); imG2 (isoguanosine); or ac6A (N6-acetyladenosine), hypoxanthine, inosine, 8-oxo-adenine, 7-substituted derivatives thereof, dihydouracil, pseudouracil, 2-thiouracil, 4-thiouracil, 5-aminouracil, 5-(Cl-C6)-alkyluracil, 5-methyluracil, 5-(C2-C6)-alkenyluracil, 5-(C2-C6)-alkynyluracil, 5-(hydroxymethyl)uracil, 5-chlorouracil, 5-fluorouracil, 5-bromouracil, 5-hydroxyuracil, 5-(Cl-C6)-alkylcytosine, 5-methylcytosine, 5-(C2-C6)-alkenylcytosine, 5-(C2-C6)-alkynylcytosine, 5-chlorocytosine, 5-fluorocytosine, 5-bromocytosine, N2-dimethylguanine, 7-deazaguanine, 8-azaguanine, 7-deaza-7-substituted guanine, 7-deaza-7-(C2-C6)alkynylguanine, 7-deaza-8-substituted guanine, 8-hydroxyguanine, 6-thioguanine, 8-oxoguanine, 2-aminopurine, 2-amin-6-chloropurine, 2,4-diaminopurine, 2,6-diaminopurine, 8-azapurine, substituted 7-deazapurine, 7-deaza-7-substituted purine, 7-deaza-8-substituted purine, or an abasic nucleotide. For instance, a self-replicating RNA can include one or more modified pyrimidine nucleobases, such as pseudouridine and/or 5-methylcytosine residues. In some embodiments, however, the RNA includes no modified nucleobases, and may include no modified nucleotides i.e. all of the nucleotides in the RNA are standard A, C, G and U ribonucleotides (except for any 5' cap structure, which may include a 7'-methylguanosine). In other embodiments, the RNA may include a 5' cap comprising a
7'-methylguanosine, and the first 1, 2 or 3 5' ribonucleotides may be methylated at the 2' position of
the ribose.
A RNA used with the invention ideally includes only phosphodiester linkages between nucleosides,
but in some embodiments it can contain phosphoramidate, phosphorothioate, and/or
methylphosphonate linkages.

Ideally, a liposome includes fewer than 10 different species of RNA e.g. 5, 4, 3, or 2 different
species; most preferably, a liposome includes a single RNA species i.e. all RNA molecules in the
liposome have the same sequence and same length.

The amount of RNA per liposome can vary. The number of individual self-replicating RNA
molecules per liposome is typically ≤50 e.g. <20, <10, <5, or 1-4 per liposome.

The immunogen
RNA molecules used with the invention encode a polypeptide immunogen. After administration of
the liposomes the RNA is translated in vivo and the immunogen can elicit an immune response in the
recipient. The immunogen may elicit an immune response against a bacterium, a virus, a fungus or a
parasite (or, in some embodiments, against an allergen; and in other embodiments, against a tumor
antigen). The immune response may comprise an antibody response (usually including IgG) and/or a
cell-mediated immune response. The polypeptide immunogen will typically elicit an immune
response which recognises the corresponding bacterial, viral, fungal or parasite (or allergen or
tumour) polypeptide, but in some embodiments the polypeptide may act as a mimotope to elicit an
immune response which recognises a bacterial, viral, fungal or parasite saccharide. The immunogen
will typically be a surface polypeptide e.g. an adhesin, a hemagglutinin, an envelope glycoprotein, a
spike glycoprotein, etc.

Self-replicating RNA molecules can encode a single polypeptide immunogen or multiple
polypeptides. Multiple immunogens can be presented as a single polypeptide immunogen (fusion
polypeptide) or as separate polypeptides. If immunogens are expressed as separate polypeptides then
one or more of these may be provided with an upstream IRES or an additional viral promoter
element. Alternatively, multiple immunogens may be expressed from a polyprotein that encodes
individual immunogens fused to a short autocatalytic protease (e.g. foot-and-mouth disease virus 2A
protein), or as inteins.

Unlike references 1 and 14, the RNA encodes an immunogen. For the avoidance of doubt, the
invention does not encompass RNA which encodes a firefly luciferase or which encodes a fusion
protein of E.coli β-galactosidase or which encodes a green fluorescent protein (GFP). Also, the RNA
is not total mouse thymus RNA.

In some embodiments the immunogen elicits an immune response against one of these bacteria:
Neisseria meningitidis: useful immunogens include, but are not limited to, membrane proteins such as adhesins, autotransporters, toxins, iron acquisition proteins, and factor H binding protein. A combination of three useful polypeptides is disclosed in reference 15.

Streptococcus pneumoniae: useful polypeptide immunogens are disclosed in reference 16. These include, but are not limited to, the RrgB pilus subunit, the beta-N-acetyl-hexosaminidase precursor (spr0057), spr0096, General stress protein GSP-781 (spr2021, SP2216), serine/threonine kinase StkP (SP1732), and pneumococcal surface adhesin PsaA.

Streptococcus pyogenes: useful immunogens include, but are not limited to, the polypeptides disclosed in references 17 and 18.

Moraxella catarrhalis.

Bordetella pertussis: Useful pertussis immunogens include, but are not limited to, pertussis toxin or toxoid (PT), filamentous haemagglutinin (FHA), pertactin, and agglutinogens 2 and 3.

Staphylococcus aureus: Useful immunogens include, but are not limited to, the polypeptides disclosed in reference 19, such as a hemolysin, esxA, esxB, ferrichrome-binding protein (sta006) and/or the staOl lipoprotein.

Clostridium tetani: the typical immunogen is tetanus toxoid.

Corynebacterium diphtheriae: the typical immunogen is diphtheria toxoid.

Haemophilus influenzae: Useful immunogens include, but are not limited to, the polypeptides disclosed in references 20 and 21.

Pseudomonas aeruginosa

Streptococcus agalactiae: useful immunogens include, but are not limited to, the polypeptides disclosed in reference 17.

Chlamydia trachomatis: Useful immunogens include, but are not limited to, PepA, LcrE, ArtJ, DnaK, CT398, OmpH-like, L7/L12, OmcA, AtoS, CT547, Eno, HtrA and MurG [e.g. as disclosed in reference 22. LcrE [23] and HtrA [24] are two preferred immunogens.

Chlamydia pneumoniae: Useful immunogens include, but are not limited to, the polypeptides disclosed in reference 25.

Helicobacter pylori: Useful immunogens include, but are not limited to, CagA, VacA, NAP, and/or urease [26].
Escherichia coli: Useful immunogens include, but are not limited to, immunogens derived from enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adhering *E. coli* (DAEC), enteropathogenic *E. coli* (EPEC), extraintestinal pathogenic *E. coli* (ExPEC) and/or enterohemorrhagic *E. coli* (EHEC). ExPEC strains include uropathogenic *E.coli* (UPEC) and meningitis/sepsis-associated *E.coli* (MNEC). Useful UPEC polypeptide immunogens are disclosed in references 27 and 28. Useful MNEC immunogens are disclosed in reference 29. A useful immunogen for several *E.coli* types is AcfD [30].

Bacillus anthracis

*Yersinia pestis*: Useful immunogens include, but are not limited to, those disclosed in references 31 and 32.

Staphylococcus epidermis

*Clostridium perfringens* or *Clostridium botulinum*

Legionella pneumophila

Coxiella burnetii

*Brucella*, such as *B.abortus*, *B.canis*, *B.melitensis*, *B.neotomae*, *B.ovis*, *B.suis*, *B.pinnipediae*.

Francisella, such as *F.novicida*, *F.philomiragia*, *F.tularensis*.

Neisseria gonorrhoeae

Treponemapallidum

Haemophilus ducreyi

*Enterococcus faecalis* or *Enterococcus faecium*

Staphylococcus saprophyticus

*Yersinia enterocolitica*

Mycobacterium tuberculosis

Rickettsia

Listeria monocytogenes

Vibrio cholerae

Salmonella typhi
Borrelia burgdorferi

Porphyromonas gingivalis

Klebsiella

In some embodiments the immunogen elicits an immune response against one of these viruses:

Orthomyxovirus: Useful immunogens can be from an influenza A, B or C virus, such as the hemagglutinin, neuraminidase or matrix M2 proteins. Where the immunogen is an influenza A virus hemagglutinin it may be from any subtype e.g. H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16.

Paramyxoviridae viruses: Viral immunogens include, but are not limited to, those derived from Pneumoviruses (e.g. respiratory syncytial virus, RSV), Rubulaviruses (e.g. mumps virus), Paramyxoviruses (e.g. parainfluenza virus), Metapneumoviruses and Morbilliviruses (e.g. measles).

Poxviridae: Viral immunogens include, but are not limited to, those derived from Orthopoxvirus such as Variola vera, including but not limited to, Variola major and Variola minor.

Picornavirus: Viral immunogens include, but are not limited to, those derived from Picornaviruses, such as Enteroviruses, Rhinoviruses, Heparnavirus, Cardioviruses and Aphthoviruses. In one embodiment, the enterovirus is a poliovirus e.g. a type 1, type 2 and/or type 3 poliovirus. In another embodiment, the enterovirus is an EV71 enterovirus. In another embodiment, the enterovirus is a coxsackie A or B virus.

Bunyavirus: Viral immunogens include, but are not limited to, those derived from an Orthobunyavirus, such as California encephalitis virus, a Phlebovirus, such as Rift Valley Fever virus, or a Nairovirus, such as Crimean-Congo hemorrhagic fever virus.

Heparnavirus: Viral immunogens include, but are not limited to, those derived from a Heparnavirus, such as hepatitis A virus (HAV).

Filovirus: Viral immunogens include, but are not limited to, those derived from a filovirus, such as an Ebola virus (including a Zaire, Ivory Coast, Reston or Sudan ebolavirus) or a Marburg virus.

Togavirus: Viral immunogens include, but are not limited to, those derived from a Togavirus, such as a Rubivirus, an Alphavirus, or an Arterivirus. This includes rubella virus.
Flavivirus: Viral immunogens include, but are not limited to, those derived from a Flavivirus, such as Tick-borne encephalitis (TBE) virus, Dengue (types 1, 2, 3 or 4) virus, Yellow Fever virus, Japanese encephalitis virus, Kyasanur Forest Virus, West Nile encephalitis virus, St. Louis encephalitis virus, Russian spring-summer encephalitis virus, Powassan encephalitis virus.

Pestivirus: Viral immunogens include, but are not limited to, those derived from a Pestivirus, such as Bovine viral diarrhea (BVDV), Classical swine fever (CSFV) or Border disease (BDV).

Hepadnavirus: Viral immunogens include, but are not limited to, those derived from a Hepadnavirus, such as Hepatitis B virus. A composition can include hepatitis B virus surface antigen (HBsAg).

Other hepatitis viruses: A composition can include an immunogen from a hepatitis C virus, delta hepatitis virus, hepatitis E virus, or hepatitis G virus.

Rhabdovirus: Viral immunogens include, but are not limited to, those derived from a Rhabdovirus, such as a Lyssavirus (e.g. a Rabies virus) and Vesiculovirus (VSV).

Caliciviridae: Viral immunogens include, but are not limited to, those derived from Calciviridae, such as Norwalk virus (Norovirus), and Norwalk-like Viruses, such as Hawaii Virus and Snow Mountain Virus.

Coronavirus: Viral immunogens include, but are not limited to, those derived from a SARS coronavirus, avian infectious bronchitis (IBV), Mouse hepatitis virus (MHV), and Porcine transmissible gastroenteritis virus (TGEV). The coronavirus immunogen may be a spike polypeptide.

Retrovirus: Viral immunogens include, but are not limited to, those derived from an Oncovirus, a Lentivirus (e.g. HIV-1 or HIV-2) or a Spumavirus.

Reovirus: Viral immunogens include, but are not limited to, those derived from an Orthoreovirus, a Rotavirus, an Orbivirus, or a Coltivirus.

Parvovirus: Viral immunogens include, but are not limited to, those derived from Parvovirus B19.

Herpesvirus: Viral immunogens include, but are not limited to, those derived from a human herpesvirus, such as, by way of example only, Herpes Simplex Viruses (HSV) (e.g. HSV types 1 and 2), Varicella-zoster virus (VZV), Epstein-Barr virus (EBV), Cytomegalovirus
(CMV), Human Herpesvirus 6 (HHV6), Human Herpesvirus 7 (HHV7), and Human Herpesvirus 8 (HHV8).

Papovaviruses: Viral immunogens include, but are not limited to, those derived from Papillomaviruses and Polyomaviruses. The (human) papillomavirus may be of serotype 1, 2, 4, 5, 6, 8, 11, 13, 16, 18, 31, 33, 35, 39, 41, 42, 47, 51, 57, 58, 63 or 65 e.g. from one or more of serotypes 6, 11, 16 and/or 18.

Adenovirus: Viral immunogens include those derived from adenovirus serotype 36 (Ad-36).

In some embodiments, the immunogen elicits an immune response against a virus which infects fish, such as: infectious salmon anemia virus (ISAV), salmon pancreatic disease virus (SPDV), infectious pancreatic necrosis virus (IPNV), channel catfish virus (CCV), fish lymphocystis disease virus (FLDV), infectious hematopoietic necrosis virus (IHNV), koi herpesvirus, salmon picorna-like virus (also known as picorna-like virus of Atlantic salmon), landlocked salmon virus (LSV), Atlantic salmon rotavirus (ASR), trout strawberry disease virus (TSD), coho salmon tumor virus (CSTV), or viral hemorrhagic septicemia virus (VHSV).

Helminthosporium spp, Fusarium spp, Aspergillus spp, Penicillium spp, Monolinia spp, Rhizoctonia spp, Paecilomyces spp, Pithomyces spp, and Cladosporium spp.

In some embodiments the immunogen elicits an immune response against a parasite from the Plasmodium genus, such as P.falciparum, P.vivax, P.malariae or P.ovale. Thus the invention may be used for immunising against malaria. In some embodiments the immunogen elicits an immune response against a parasite from the Caligidae family, particularly those from the Lepeophtheirus and Caligus genera e.g. sea lice such as Lepeophtheirus salmonis or Caligus rogercresseyi.

In some embodiments the immunogen elicits an immune response against: pollen allergens (tree-, herb, weed-, and grass pollen allergens); insect or arachnid allergens (inhalant, saliva and venom allergens, e.g. mite allergens, cockroach and mites allergens, hymenoptera venom allergens); animal hair and dandruff allergens (from e.g. dog, cat, horse, rat, mouse, etc.); and food allergens (e.g. a gliadin). Important pollen allergens from trees, grasses and herbs are such originating from the taxonomic orders of Fagales, Oleales, Pinales and platanaceae including, but not limited to, birch (Betula), alder (Alnus), hazel (Corylus), hornbeam (Carpinus) and olive (Olea), cedar (Cryptomeria and Juniperus), plane tree (Platanus), the order of Poales including grasses of the genera Lolium, Phleum, Poa, Cynodon, Dactylis, Holcus, Phalaris, Secale, and Sorghum, the orders of Asterales and Urticales including herbs of the genera Ambrosia, Artemisia, and Parietaria. Other important inhalation allergens are those from house dust mites of the genus Dermatophagoides and Euroglyphus, storage mite e.g. Lepidoglyphys, Glycyphagus and Tyrophagus, those from cockroaches, mides and fleas e.g. Blatella, Periplaneta, Chironomus and Ctenocephalides, and those from mammals such as cat, dog and horse, venom allergens including such originating from stinging or biting insects such as those from the taxonomic order of Hymenoptera including bees (Apidae), wasps (Vespidea), and ants (Formicoidae).

In some embodiments the immunogen is a tumor antigen selected from: (a) cancer-testis antigens such as NY-ESO-1, SSX2, SCP1 as well as RAGE, GAGE, GAGE and MAGE family polypeptides, for example, GAGE-1, GAGE-2, MAGE-1, MAGE-2, MAGE-3, MAGE-4, MAGE-5, MAGE-6, and MAGE-12 (which can be used, for example, to address melanoma, lung, head and neck, NSCLC, breast, gastrointestinal, and bladder tumors; (b) mutated antigens, for example, p53 (associated with various solid tumors, e.g., colorectal, lung, head and neck cancer), p21/Ras (associated with, e.g., melanoma, pancreatic cancer and colorectal cancer), CDK4 (associated with, e.g., melanoma), MUM1 (associated with, e.g., melanoma), caspase-8 (associated with, e.g., head and neck cancer), CIA 0205 (associated with, e.g., bladder cancer), HLA-A2-R1701, beta catenin (associated with, e.g., melanoma), TCR (associated with, e.g., T-cell non-Hodgkins lymphoma), BCR-abl (associated with, e.g., chronic myelogenous leukemia), triosephosphate isomerase, KIA 0205, CDC-27, and LDLR-FUT; (c) over-expressed antigens, for example, Galectin 4 (associated with, e.g., colorectal cancer), Galectin 9 (associated with, e.g., Hodgkin's disease), proteinase 3 (associated with, e.g., chronic myelogenous leukemia), WT 1 (associated with, e.g., various leukemias), carbonic anhydrase
(associated with, e.g., renal cancer), aldolase A (associated with, e.g., lung cancer), PRAME
(associated with, e.g., melanoma), HER-2/neu (associated with, e.g., breast, colon, lung and ovarian
cancer), mammaglobin, alpha-fetoprotein (associated with, e.g., hepatoma), KSA (associated with,
e.g., colorectal cancer), gastrin (associated with, e.g., pancreatic and gastric cancer), telomerase
catalytic protein, MUC-1 (associated with, e.g., breast and ovarian cancer), G-250 (associated with,
e.g., renal cell carcinoma), p53 (associated with, e.g., breast, colon cancer), and carcinoembryonic
antigen (associated with, e.g., breast cancer, lung cancer, and cancers of the gastrointestinal tract
such as colorectal cancer); (d) shared antigens, for example, melanoma-melanocyte differentiation
antigens such as MART-1/Melan A, gp100, MC1R, melanocyte-stimulating hormone receptor,
tyrosinase, tyrosinase related protein-1/TRP1 and tyrosinase related protein-2/TRP2 (associated with,
e.g., melanoma); (e) prostate associated antigens such as PAP, PSA, PSMA, PSH-P1, PSM-P1,
PSM-P2, associated with e.g., prostate cancer; (f) immunoglobulin idiotypes (associated with
myeloma and B cell lymphomas, for example). In certain embodiments, tumor immunogens include,
but are not limited to, p15, Hom/Mel-40, H-Ras, E2A-PRL, H-4-RET, IGH-IGK, MYL-RAR, Epstein
Barr virus antigens, EBNA, human papillomavirus (HPV) antigens, including E6 and E7, hepatitis B
and C virus antigens, human T-cell lymphotropic virus antigens, TSP-180, pl85erB2, pl80erB-3,
c-met, mm-23H1, TAG-72-4, CA 19-9, CA 72-4, CAM 17.1, NuMa, K-ras, pl6, TAGE, PSCA, CT7,
43-9F, 5T4, 791 Tgp72, beta-HCG, BCA225, BTAA, CA 125, CA 15-3 (CA 27.29YBCAA), CA 195,
CA 242, CA-50, CAM43, CD68\KP1, CO-029, FGF-5, Ga733 (EpCAM), HTgp-175, M344, MA-
50, MG7-Ag, MOV18, NB/70K, NY-CO-1, RCAS1, SDCCAG16, TA-90 (Mac-2 binding
protein/cyclophilin C-associated protein), TAAL6, TAG72, TLP, TPS, and the like.

Pharmaceutical compositions

Liposomes of the invention are useful as components in pharmaceutical compositions for
immunising subjects against various diseases. These compositions will typically include a
pharmaceutically acceptable carrier in addition to the liposomes. A thorough discussion of
pharmaceutically acceptable carriers is available in reference 33.

A pharmaceutical composition of the invention may include one or more small molecule
immunopotentiators. For example, the composition may include a TLR2 agonist (e.g., Pam3CSK4), a
TLR4 agonist (e.g., an aminoalkyl glucosaminide phosphate, such as E6020), a TLR7 agonist (e.g.,
imiquimod), a TLR8 agonist (e.g. resiquimod) and/or a TLR9 agonist (e.g. IC31). Any such agonist
ideally has a molecular weight of <2000Da. Where a RNA is encapsulated, in some embodiments
such agonist(s) are also encapsulated with the RNA, but in other embodiments they are
unencapsulated. Where a RNA is adsorbed to a particle, in some embodiments such agonist(s) are
also adsorbed with the RNA, but in other embodiments they are unadsorbed.
Pharmaceutical compositions of the invention may include the liposomes in plain water (e.g. w.f.i.) or in a buffer e.g. a phosphate buffer, a Tris buffer, a borate buffer, a succinate buffer, a histidine buffer, or a citrate buffer. Buffer salts will typically be included in the 5-20mM range.

Pharmaceutical compositions of the invention may have a pH between 5.0 and 9.5 e.g. between 6.0 and 8.0.

Compositions of the invention may include sodium salts (e.g. sodium chloride) to give tonicity. A concentration of 10±2 mg/ml NaCl is typical e.g. about 9 mg/ml.

Compositions of the invention may include metal ion chelators. These can prolong RNA stability by removing ions which can accelerate phosphodiester hydrolysis. Thus a composition may include one or more of EDTA, EGTA, BAPTA, pentetic acid, etc.. Such chelators are typically present at between 10-500µM e.g. 0.1mM. A citrate salt, such as sodium citrate, can also act as a chelator, while advantageously also providing buffering activity.

Pharmaceutical compositions of the invention may have an osmolality of between 200 mOsm/kg and 400 mOsm/kg, e.g. between 240-360 mOsm/kg, or between 290-310 mOsm/kg.

Pharmaceutical compositions of the invention may include one or more preservatives, such as thiomersal or 2-phenoxyethanol. Mercury-free compositions are preferred, and preservative-free vaccines can be prepared.

Pharmaceutical compositions of the invention are preferably sterile.

Pharmaceutical compositions of the invention are preferably non-pyrogenic e.g. containing < 1 EU (endotoxin unit, a standard measure) per dose, and preferably <0.1 EU per dose.

Pharmaceutical compositions of the invention are preferably gluten free.

Pharmaceutical compositions of the invention may be prepared in unit dose form. In some embodiments a unit dose may have a volume of between 0.1-1.0ml e.g. about 0.5ml.

The compositions may be prepared as injectables, either as solutions or suspensions. The composition may be prepared for pulmonary administration e.g. by an inhaler, using a fine spray. The composition may be prepared for nasal, aural or ocular administration e.g. as spray or drops. Injectables for intramuscular administration are typical.

Compositions comprise an immunologically effective amount of liposomes, as well as any other components, as needed. By 'immunologically effective amount', it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (e.g. non-human primate, primate, etc.), the capacity of the individual's immune system to synthesise antibodies, the degree of

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protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. The liposome and RNA content of compositions of the invention will generally be expressed in terms of the amount of RNA per dose. A preferred dose has

<10(^g) RNA (e.g. from 10-10(^g), such as about 10µg, 25µg, 50µg, 75µg or 10(^g)), but expression can be seen at much lower levels e.g. <^g/dose, <100ng/dose, <10ng/dose, <1ng/dose, etc.

The invention also provides a delivery device (e.g. syringe, nebuliser, sprayer, inhaler, dermal patch, etc.) containing a pharmaceutical composition of the invention. This device can be used to administer the composition to a vertebrate subject.

Liposomes of the invention do not include ribosomes.

Methods of treatment and medical uses

In contrast to the particles disclosed in reference 14, liposomes and pharmaceutical compositions of the invention are for in vivo use for eliciting an immune response against an immunogen of interest.

The invention provides a method for raising an immune response in a vertebrate comprising the step of administering an effective amount of a liposome or pharmaceutical composition of the invention. The immune response is preferably protective and preferably involves antibodies and/or cell-mediated immunity. The method may raise a booster response.

The invention also provides a liposome or pharmaceutical composition of the invention for use in a method for raising an immune response in a vertebrate.

The invention also provides the use of a liposome of the invention in the manufacture of a medicament for raising an immune response in a vertebrate.

By raising an immune response in the vertebrate by these uses and methods, the vertebrate can be protected against various diseases and/or infections e.g. against bacterial and/or viral diseases as discussed above. The liposomes and compositions are immunogenic, and are more preferably vaccine compositions. Vaccines according to the invention may either be prophylactic (i.e. to prevent infection) or therapeutic (i.e. to treat infection), but will typically be prophylactic.

The vertebrate is preferably a mammal, such as a human or a large veterinary mammal (e.g. horses, cattle, deer, goats, pigs). Where the vaccine is for prophylactic use, the human is preferably a child (e.g. a toddler or infant) or a teenager; where the vaccine is for therapeutic use, the human is preferably a teenager or an adult. A vaccine intended for children may also be administered to adults e.g. to assess safety, dosage, immunogenicity, etc.

Vaccines prepared according to the invention may be used to treat both children and adults. Thus a human patient may be less than 1 year old, less than 5 years old, 1-5 years old, 5-15 years old, 15-55
years old, or at least 55 years old. Preferred patients for receiving the vaccines are the elderly \((e.g. \geq 50\) years old, \(\geq 60\) years old, and preferably \(\geq 65\) years), the young \((e.g. \leq 5\) years old), hospitalised patients, healthcare workers, armed service and military personnel, pregnant women, the chronically ill, or immunodeficient patients. The vaccines are not suitable solely for these groups, however, and may be used more generally in a population.

Compositions of the invention will generally be administered directly to a patient. Direct delivery may be accomplished by parenteral injection \((e.g.\) subcutaneously, intraperitoneally, intravenously, intramuscularly, intradermally, or to the interstitial space of a tissue; unlike reference 1, intraglottal injection is not typically used with the present invention). Alternative delivery routes include rectal, oral \((e.g.\) tablet, spray), buccal, sublingual, vaginal, topical, transdermal or transcutaneous, intranasal, ocular, aural, pulmonary or other mucosal administration. Intradermal and intramuscular administration are two preferred routes. Injection may be via a needle \((e.g.\) a hypodermic needle), but needle-free injection may alternatively be used. A typical intramuscular dose is 0.5 ml.

The invention may be used to elicit systemic and/or mucosal immunity, preferably to elicit an enhanced systemic and/or mucosal immunity.

Dosage can be by a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunisation schedule and/or in a booster immunisation schedule. In a multiple dose schedule the various doses may be given by the same or different routes \(e.g.\) a parenteral prime and mucosal boost, a mucosal prime and parenteral boost, \(etc.\) Multiple doses will typically be administered at least 1 week apart \((e.g.\) about 2 weeks, about 3 weeks, about 4 weeks, about 6 weeks, about 8 weeks, about 10 weeks, about 12 weeks, about 16 weeks, \(etc.\)). In one embodiment, multiple doses may be administered approximately 6 weeks, 10 weeks and 14 weeks after birth, \(e.g.\) at an age of 6 weeks, 10 weeks and 14 weeks, as often used in the World Health Organisation’s Expanded Program on Immunisation (“EPI”). In an alternative embodiment, two primary doses are administered about two months apart, \(e.g.\) about 7, 8 or 9 weeks apart, followed by one or more booster doses about 6 months to 1 year after the second primary dose, \(e.g.\) about 6, 8, 10 or 12 months after the second primary dose. In a further embodiment, three primary doses are administered about two months apart, \(e.g.\) about 7, 8 or 9 weeks apart, followed by one or more booster doses about 6 months to 1 year after the third primary dose, \(e.g.\) about 6, 8, 10, or 12 months after the third primary dose.

**General**

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, \(e.g.\), references 34-40, \(etc.\).
The term "comprising" encompasses "including" as well as "consisting" e.g. a composition "comprising" X may consist exclusively of X or may include something additional e.g. X + Y.

The term "about" in relation to a numerical value x is optional and means, for example, x±10%.

The word "substantially" does not exclude "completely" e.g. a composition which is "substantially free" from Y may be completely free from Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

References to charge, to cations, to anions, to zwitterions, etc., are taken at pH 7.

TLR3 is the Toll-like receptor 3. It is a single membrane-spanning receptor which plays a key role in the innate immune system. Known TLR3 agonists include poly(LC). "TLR3" is the approved HGNC name for the gene encoding this receptor, and its unique HGNC ID is HGNC: 11849. The RefSeq sequence for the human TLR3 gene is GL2459625.

TLR7 is the Toll-like receptor 7. It is a single membrane-spanning receptor which plays a key role in the innate immune system. Known TLR7 agonists include e.g. imiquimod. "TLR7" is the approved HGNC name for the gene encoding this receptor, and its unique HGNC ID is HGNC: 15631. The RefSeq sequence for the human TLR7 gene is GL: 67944638.

TLR8 is the Toll-like receptor 8. It is a single membrane-spanning receptor which plays a key role in the innate immune system. Known TLR8 agonists include e.g. resiquimod. "TLR8" is the approved HGNC name for the gene encoding this receptor, and its unique HGNC ID is HGNC: 15632. The RefSeq sequence for the human TLR8 gene is GL20302165.

The RIG-I-like receptor ("RLR") family includes various RNA helicases which play key roles in the innate immune system [41]. RLR-1 (also known as RIG-I or retinoic acid inducible gene I) has two caspase recruitment domains near its N-terminus. The approved HGNC name for the gene encoding the RLR-1 helicase is "DDX58" (for DEAD (Asp-Glu-Ala-Asp) box polypeptide 58) and the unique HGNC ID is HGNC: 19102. The RefSeq sequence for the human RLR-1 gene is GL77732514. RLR-2 (also known as MDA5 or melanoma differentiation-associated gene 5) also has two caspase recruitment domains near its N-terminus. The approved HGNC name for the gene encoding the RLR-2 helicase is "IFIH1" (for interferon induced with helicase C domain 1) and the unique HGNC ID is HGNC: 18873. The RefSeq sequence for the human RLR-2 gene is GL: 27886567. RLR-3 (also known as LGP2 or laboratory of genetics and physiology 2) has no caspase recruitment domains. The approved HGNC name for the gene encoding the RLR-3 helicase is "DHX58" (for DEXH (Asp-Glu-X-His) box polypeptide 58) and the unique HGNC ID is HGNC: 29517. The RefSeq sequence for the human RLR-3 gene is GL: 149408121.

PKR is a double-stranded RNA-dependent protein kinase. It plays a key role in the innate immune system. "EIF2AK2" (for eukaryotic translation initiation factor 2-alpha kinase 2) is the approved
HGNC name for the gene encoding this enzyme, and its unique HGNC ID is HGNC:9437. The RefSeq sequence for the human PKR gene is GL208431825.

**BRIEF DESCRIPTION OF DRAWINGS**

Figure 1 shows a gel with stained RNA. Lanes show (1) markers (2) naked replicon (3) replicon after RNase treatment (4) replicon encapsulated in liposome (5) liposome after RNase treatment (6) liposome treated with RNase then subjected to phenol/chloroform extraction.

Figure 2 is an electron micrograph of liposomes.

Figure 3 shows the structures of DLinDMA, DLenDMA and DODMA.

Figure 4 shows a gel with stained RNA. Lanes show (1) markers (2) naked replicon (3) replicon encapsulated in liposome (4) liposome treated with RNase then subjected to phenol/chloroform extraction.

Figure 5 shows protein expression at days 1, 3 and 6 after delivery of RNA as a virion-packaged replicon (squares), as naked RNA (diamonds), or in liposomes (+ = O.^g, x = ^g).

Figure 6 shows protein expression at days 1, 3 and 6 after delivery of four different doses of liposome-encapsulated RNA.

Figure 7 shows anti-F IgG titers in animals receiving virion-packaged replicon (VRP or VSRP), 1µg naked RNA, and 1µg liposome-encapsulated RNA.

Figure 8 shows anti-F IgG titers in animals receiving VRP, 1µg naked RNA, and O.lg or 1µg liposome-encapsulated RNA.

Figure 9 shows neutralising antibody titers in animals receiving VRP or either O.lg or 1µg liposome-encapsulated RNA.

Figure 10 shows expression levels after delivery of a replicon as naked RNA (circles), liposome-encapsulated RNA (triangle & square), or as a lipoplex (inverted triangle).

Figure 11 shows F-specific IgG titers (2 weeks after second dose) after delivery of a replicon as naked RNA (0.01-1µg), liposome-encapsulated RNA (0.01-10µg), or packaged as a virion (VRP, 10^6 infectious units or IU).

Figure 12 shows F-specific IgG titers (circles) and PRNT titers (squares) after delivery of a replicon as naked RNA (^g), liposome-encapsulated RNA (0.1 or ^g), or packaged as a virion (VRP, 10^6 IU). Titers in naive mice are also shown. Solid lines show geometric means.

Figure 13 shows intracellular cytokine production after restimulation with synthetic peptides representing the major epitopes in the F protein, 4 weeks after a second dose. The y-axis shows the % cytokine+ of CD8+CD4-.
Figure 14 shows F-specific IgG titers (mean logio titers ± std dev) over 63 days (Figure 14A) and 210 days (Figure 14B) after immunisation of calves. The three lines are easily distinguished at day 63 and are, from bottom to top: PBS negative control; liposome-delivered RNA; and the “Triangle 4” product.

Figure 15 shows SEAP expression (relative intensity) at day 6 against pKa of lipids used in the liposomes. Circles show levels for liposomes with DSPC, and squares for liposomes without DSPC; sometimes a square and circle overlap, leaving only the square visible for a given pKa.

Figure 16 shows anti-F titers expression (relative to RVOI, 100%) two weeks after a first dose of replicon encoding F protein. The titers are plotted against pKa in the same way as in Figure 15. The star shows RV02, which used a cationic lipid having a higher pKa than the other lipids. Triangles show data for liposomes lacking DSPC; circles are for liposomes which included DSPC.

Figure 17 shows total IgG titers after replicon delivery in liposomes using, from left to right, RVOI, RV16, RV17, RV18 or RV19. Bars show means. The upper bar in each case is 2wp2 (i.e. 2 weeks after second dose), whereas the lower bar is 2wp1.

Figure 18 shows IgG titers in 13 groups of mice. Each circle is an individual mouse, and solid lines show geometric means. The dotted horizontal line is the assay’s detection limit. The 13 groups are, from left to right, A to M as described below.

Figure 19 shows (A) IL-6 and (B) IFNa (pg/ml) released by pDC. There are 4 pairs of bars, from left to right: control; immunised with RNA+DOTAP; immunised with RNA+lipofectamine; and immunised with RNA in liposomes. In each pair the black bar is wild-type mice, grey is rsq1 mutant.

**MODES FOR CARRYING OUT THE INVENTION**

**RNA replicons**

Various replicons are used below. In general these are based on a hybrid alphavirus genome with non-structural proteins from Venezuelan equine encephalitis virus (VEEV), a packaging signal from sindbis virus, and a 3’ UTR from Sindbis virus or a VEEV mutant. The replicon is about 10kb long and has a poly-A tail.

Plasmid DNA encoding alphavirus replicons (named: pT7-mVEEV-FL-RSVF or A317; pT7-mVEEV-SEAP or A306; pSP6-VCR-GFP or A50) served as a template for synthesis of RNA in vitro. The replicons contain the alphavirus genetic elements required for RNA replication but lack those encoding gene products necessary for particle assembly; the structural proteins are instead replaced by a protein of interest (either a reporter, such as SEAP or GFP, or an immunogen, such as full-length RSV F protein) and so the replicons are incapable of inducing the generation of infectious particles. A bacteriophage (T7 or SP6) promoter upstream of the alphavirus cDNA facilitates the
synthesis of the replicon RNA \textit{in vitro} and a hepatitis delta virus (HDV) ribozyme immediately downstream of the poly(A)-tail generates the correct 3'-end through its self-cleaving activity.

Following linearization of the plasmid DNA downstream of the HDV ribozyme with a suitable restriction endonuclease, run-off transcripts were synthesized in vitro using T7 or SP6 bacteriophage derived DNA-dependent RNA polymerase. Transcriptions were performed for 2 hours at 37°C in the presence of 7.5 mM (T7 RNA polymerase) or 5 mM (SP6 RNA polymerase) of each of the nucleoside triphosphates (ATP, CTP, GTP and UTP) following the instructions provided by the manufacturer (Ambion). Following transcription the template DNA was digested with TURBO DNase (Ambion). The replicon RNA was precipitated with LiCl and reconstituted in nuclease-free water. Uncapped RNA was capped post-transcriptionally with Vaccinia Capping Enzyme (VCE) using the ScriptCap m7G Capping System (Epigen Biotechnologies) as outlined in the user manual; replicons capped in this way are given the "v" prefix e.g. vA317 is the A317 replicon capped by VCE. Post-transcriptionally capped RNA was precipitated with LiCl and reconstituted in nuclease-free water. The concentration of the RNA samples was determined by measuring OD$_{260nm}$.

Integrity of the \textit{in vitro} transcripts was confirmed by denaturing agarose gel electrophoresis.

\textbf{Liposomal encapsulation}

RNA was encapsulated in liposomes made by the method of references 11 and 42. The liposomes were made of 10% DSPC (zwitterionic), 40% DLinDMA (cationic), 48% cholesterol and 2% PEG-conjugated DMG (2kDa PEG). These proportions refer to the \% moles in the total liposome.

DLinDMA (1,2-dilinoleoyloxy-N,N-dimethyl-3-aminopropane) was synthesized using the procedure of reference 6. DSPC (1,2-Diastearoyl-sn-glycero-3-phosphocholine) was purchased from Genzyme. Cholesterol was obtained from Sigma-Aldrich. PEG-conjugated DMG (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol), ammonium salt), DOTAP (1,2-dioleoyl-3-trimethylammonium-propane, chloride salt) and DC-chol (3P-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride) were from Avanti Polar Lipids.

Briefly, lipids were dissolved in ethanol (2ml), a RNA replicon was dissolved in buffer (2ml, 100mM sodium citrate, pH 6) and these were mixed with 2ml of buffer followed by 1 hour of equilibration. The mixture was diluted with 6ml buffer then filtered. The resulting product contained liposomes, with \~95% encapsulation efficiency.

For example, in one particular method, fresh lipid stock solutions were prepared in ethanol. 37 mg of DLinDMA, 11.8 mg of DSPC, 27.8 mg of cholesterol and 8.07 mg of PEG-DMG were weighed and dissolved in 7.55 mL of ethanol. The freshly prepared lipid stock solution was gently rocked at 37°C for about 15 min to form a homogenous mixture. Then, 755 µL of the stock was added to 1.245 mL ethanol to make a working lipid stock solution of 2 mL. This amount of lipids was used to form liposomes with 250 µg RNA. A 2 mL working solution of RNA was also prepared from a stock
solution of ~1µg/µL in 100 mM citrate buffer (pH 6). Three 20 mL glass vials (with stir bars) were rinsed with RNase Away solution (Molecular BioProducts) and washed with plenty of MilliQ water before use to decontaminate the vials of RNases. One of the vials was used for the RNA working solution and the others for collecting the lipid and RNA mixes (as described later). The working lipid and RNA solutions were heated at 37°C for 10 min before being loaded into 3 cc luer-lok syringes.

2 mL citrate buffer (pH 6) was loaded in another 3 cc syringe. Syringes containing RNA and the lipids were connected to a T mixer (PEEK™ 500 µm ID junction, Idex Health Science) using FEP tubing (fluorinated ethylene-propylene; all FEP tubing used has a 2mm internal diameter and a 3mm outer diameter). The outlet from the T mixer was also FEP tubing. The third syringe containing the citrate buffer was connected to a separate piece of FEP tubing. All syringes were then driven at a flow rate of 7 mL/min using a syringe pump. The tube outlets were positioned to collect the mixtures in a 20 mL glass vial (while stirring). The stir bar was taken out and the ethanol/aqueous solution was allowed to equilibrate to room temperature for 1 h. 4 mL of the mixture was loaded into a 5 cc syringe, which was connected to a piece of FEP tubing and in another 5 cc syringe connected to an equal length of FEP tubing, an equal amount of 100 mM citrate buffer (pH 6) was loaded. The two syringes were driven at 7 mL/min flow rate using the syringe pump and the final mixture collected in a 20 mL glass vial (while stirring). Next, the mixture collected from the second mixing step (liposomes) were passed through a Mustang Q membrane (an anion-exchange support that binds and removes anionic molecules, obtained from Pall Corporation). Before using this membrane for the liposomes, 4 mL of 1 M NaOH, 4 mL of 1 M NaCl and 10 mL of 100 mM citrate buffer (pH 6) were successively passed through it. Liposomes were warmed for 10 min at 37°C before passing through the membrane. Next, liposomes were concentrated to 2 mL and dialyzed against 10-15 volumes of 1X PBS using by tangential flow filtration before recovering the final product. The TFF system and hollow fiber filtration membranes were purchased from Spectrum Labs (Rancho Dominguez) and were used according to the manufacturer’s guidelines. Polysulfone hollow fiber filtration membranes with a 100 kD pore size cutoff and 8 cm² surface area were used. For in vitro and in vivo experiments formulations were diluted to the required RNA concentration with 1X PBS.

Figure 2 shows an example electron micrograph of liposomes prepared by these methods. These liposomes contain encapsulated RNA encoding full-length RSV F antigen. Dynamic light scattering of one batch showed an average diameter of 141 nm (by intensity) or 78 nm (by number).

The percentage of encapsulated RNA and RNA concentration were determined by Quant-iT RiboGreen RNA reagent kit (Invitrogen), following manufacturer’s instructions. The ribosomal RNA standard provided in the kit was used to generate a standard curve. Liposomes were diluted 10x or 100x in IX TE buffer (from kit) before addition of the dye. Separately, liposomes were diluted 10x or 100x in IX TE buffer containing 0.5% Triton X before addition of the dye (to disrupt the liposomes and thus to assay total RNA). Thereafter an equal amount of dye was added to each solution and then -180 µL of each solution after dye addition was loaded in duplicate into a 96 well
tissue culture plate. The fluorescence (Ex 485 nm, Em 528 nm) was read on a microplate reader. All
liposome formulations were dosed in vivo based on the encapsulated amount of RNA.

Encapsulation in liposomes was shown to protect RNA from RNase digestion. Experiments used
3.8mAU of RNase A per microgram of RNA, incubated for 30 minutes at room temperature. RNase
was inactivated with Proteinase K at 55°C for 10 minutes. A 1:1 v/v mixture of sample to 25:24:1
v/v/v, phenol:chloroform:isoamyl alcohol was then added to extract the RNA from the lipids into the
aqueous phase. Samples were mixed by vortexing for a few seconds and then placed on a centrifuge
for 15 minutes at 12k RPM. The aqueous phase (containing the RNA) was removed and used to
analyze the RNA. Prior to loading (400 ng RNA per well) all the samples were incubated with
formaldehyde loading dye, denatured for 10 minutes at 65°C and cooled to room temperature.
Ambion Millennium markers were used to approximate the molecular weight of the RNA construct.
The gel was run at 90 V. The gel was stained using 0.1% SYBR gold according to the manufacturer’s
guidelines in water by rocking at room temperature for 1 hour. Figure 1 shows that RNase
completely digests RNA in the absence of encapsulation (lane 3). RNA is undetectable after
encapsulation (lane 4), and no change is seen if these liposomes are treated with RNase (lane 4).
After RNase-treated liposomes are subjected to phenol extraction, undigested RNA is seen (lane 6).
Even after 1 week at 4°C the RNA could be seen without any fragmentation (Figure 4, arrow).
Protein expression in vivo was unchanged after 6 weeks at 4°C and one freeze-thaw cycle. Thus
liposome-encapsulated RNA is stable.

To assess in vivo expression of the RNA a reporter enzyme (SEAP; secreted alkaline phosphatase)
was encoded in the replicon, rather than an immunogen. Expression levels were measured in sera
diluted 1:4 in IX Phospha-Light dilution buffer using a chemiluminescent alkaline phosphate
substrate. 8-10 week old BALB/c mice (5/group) were injected intramuscularly on day 0, 50µl per
dose, or 1µg RNA dose. The same vector was also administered without the liposomes (in
RNase free IX PBS) at 1µg. Virion-packaged replicons were also tested. Virion-packaged replicons
used herein (referred to as "VRPs") were obtained by the methods of reference 43, where the
alphavirus replicon is derived from the mutant VEEV or a chimera derived from the genome of
VEEV engineered to contain the 3' UTR of Sindbis virus and a Sindbis virus packaging signal (PS),
packaged by co-electroporating them into BHK cells with defective helper RNAs encoding the
Sindbis virus capsid and glycoprotein genes.

As shown in Figure 5, encapsulation increased SEAP levels by about ½ log at the 1µg dose, and at
day 6 expression from a O.1µg encapsulated dose matched levels seen with 1µg unencapsulated dose.
By day 3 expression levels exceeded those achieved with VRPs (squares). Thus expressed increased
when the RNA was formulated in the liposomes relative to the naked RNA control, even at a 10x
lower dose. Expression was also higher relative to the VRP control, but the kinetics of expression
were very different (see Figure 5). Delivery of the RNA with electroporation resulted in increased
expression relative to the naked RNA control, but these levels were lower than with liposomes.
To assess whether the effect seen in the liposome groups was due merely to the liposome components, or was linked to the encapsulation, the replicon was administered in encapsulated form (with two different purification protocols, 0.1 μg RNA), or mixed with the liposomes after their formation (a non-encapsulated "lipoplex", 0.1 μg RNA), or as naked RNA (μg). Figure 10 shows that the lipoplex gave the lowest levels of expression, showing that shows encapsulation is essential for potent expression.

*In vivo* studies using liposomal delivery confirmed these findings. Mice received various combinations of (i) self-replicating RNA replicon encoding full-length RSV F protein (ii) self-replicating GFP-encoding RNA replicon (iii) GFP-encoding RNA replicon with a knockout in nsP4 which eliminates self-replication (iv) full-length RSV F-protein. 13 groups in total received:

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>0.1 μg of (i), naked</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>0.1 μg of (i), encapsulated in liposome</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>0.1 μg of (i), with separate liposomes</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>0.1 μg of (i), naked</td>
<td>10 μg of (ii), naked</td>
</tr>
<tr>
<td>F</td>
<td>0.1 μg of (i), naked</td>
<td>10 μg of (iii), naked</td>
</tr>
<tr>
<td>G</td>
<td>0.1 μg of (i), encapsulated in liposome</td>
<td>10 μg of (ii), naked</td>
</tr>
<tr>
<td>H</td>
<td>0.1 μg of (i), encapsulated in liposome</td>
<td>10 μg of (iii), naked</td>
</tr>
<tr>
<td>I</td>
<td>0.1 μg of (i), encapsulated in liposome</td>
<td>1 μg of (ii), encapsulated in liposome</td>
</tr>
<tr>
<td>J</td>
<td>0.1 μg of (i), encapsulated in liposome</td>
<td>1 μg of (iii), encapsulated in liposome</td>
</tr>
<tr>
<td>K</td>
<td>5 μg F protein</td>
<td>-</td>
</tr>
<tr>
<td>L</td>
<td>5 μg F protein</td>
<td>1 μg of (ii), encapsulated in liposome</td>
</tr>
<tr>
<td>M</td>
<td>5 μg F protein</td>
<td>1 μg of (iii), encapsulated in liposome</td>
</tr>
</tbody>
</table>

Results in Figure 18 show that F-specific IgG responses required encapsulation in the liposome rather than mere co-delivery (compare groups C & D). A comparison of groups K, L and M shows that the RNA provided an adjuvant effect against co-delivered protein, and this effect was seen with both replicating and non-replicating RNA.

Further SEAP experiments showed a clear dose response *in vivo*, with expression seen after delivery of as little as 1 ng RNA (Figure 6). Further experiments comparing expression from encapsulated and naked replicons indicated that 0.01 μg encapsulated RNA was equivalent to 1 μg of naked RNA. At a 0.1 μg dose of RNA the encapsulated material gave a 12-fold higher expression at day 6; at a 0.1 μg dose levels were 24-fold higher at day 6.

Rather than looking at average levels in the group, individual animals were also studied. Whereas several animals were non-responders to naked replicons, encapsulation eliminated non-responders.
Further experiments replaced DLinDMA with DOTAP. Although the DOTAP liposomes gave better expression than naked replicon, they were inferior to the DLinDMA liposomes (2- to 3-fold difference at day 1). Whereas DOTAP has a quaternary amine, and so have a positive charge at the point of delivery, DLinDMA has a tertiary amine.

To assess in vivo immunogenicity a replicon was constructed to express full-length F protein from respiratory syncytial virus (RSV). This was delivered naked (\(^{\text{ag}}\)), encapsulated in liposomes (0.1 or \(^{\text{ag}}\)), or packaged in virions (10^6 IU; "VRP") at days 0 and 21. Figure 7 shows anti-F IgG titers 2 weeks after the second dose, and the liposomes clearly enhance immunogenicity. Figure 8 shows titers 2 weeks later, by which point there was no statistical difference between the encapsulated RNA at O.\(^{\text{ag}}\), the encapsulated RNA at \(^{\text{ag}}\), or the VRP group. Neutralisation titers (measured as 60% plaque reduction, "PRNT60") were not significantly different in these three groups 2 weeks after the second dose (Figure 9). Figure 12 shows both IgG and PRNT titers 4 weeks after the second dose.

Figure 13 confirms that the RNA elicits a robust CD8 T cell response.

Further experiments compared F-specific IgG titers in mice receiving VRP, O.\(^{\text{ag}}\) liposome-encapsulated RNA, or 1\(\mu\)g liposome-encapsulated RNA. Titer ratios (VRP:liposome) at various times after the second dose were as follows:

<table>
<thead>
<tr>
<th></th>
<th>2 weeks</th>
<th>4 weeks</th>
<th>8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1(\mu)g</td>
<td>2.9</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>1(\mu)g</td>
<td>2.3</td>
<td>0.9</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Thus the liposome-encapsulated RNA induces essentially the same magnitude of immune response as seen with virion delivery.

Further experiments showed superior F-specific IgG responses with a 10\(\mu\)g dose, equivalent responses for 1\(\mu\)g and O.\(^{\text{ag}}\) doses, and a lower response with a O.\(0^{\text{ag}}\) dose. Figure 11 shows IgG titers in mice receiving the replicon in naked form at 3 different doses, in liposomes at 4 different doses, or as VRP (10^6 IU). The response seen with 1\(\mu\)g liposome-encapsulated RNA was statistically insignificant (ANOVA) when compared to VRP, but the higher response seen with 10\(\mu\)g liposome-encapsulated RNA was statistically significant (p<0.05) when compared to both of these groups.

A further study confirmed that the O.\(^{\text{ag}}\) of liposome-encapsulated RNA gave much higher anti-F IgG responses (15 days post-second dose) than O.\(^{\text{ag}}\) of delivered DNA, and even was more immunogenic than 20\(\mu\)g plasmid DNA encoding the F antigen, delivered by electroporation (Elgen™ DNA Delivery System, Inovio).

Further study was performed in cotton rats (Sigmodon hispidis) instead of mice. At a 1\(\mu\)g dose liposome encapsulation increased F-specific IgG titers by 8.3-fold compared to naked RNA and increased PRNT titers by 9.5-fold. The magnitude of the antibody response was equivalent to that
induced by 5×10^6 IU VRP. Both naked and liposome-encapsulated RNA were able to protect the cotton rats from RSV challenge (1×10^5 plaque forming units), reducing lung viral load by at least 3.5 logs. Encapsulation increased the reduction by about 2-fold.

A large-animal study was performed in cattle. Cows were immunised with 66µg of replicon encoding full-length RSV F protein at days 0 and 21, formulated inside liposomes. PBS alone was used as a negative control, and a licensed vaccine was used as a positive control ("Triangle 4" from Fort Dodge, containing killed virus). Figure 14 shows F-specific IgG titers over a 63 day period starting from the first immunisation. The RNA replicon was immunogenic in the cows, although it gave lower titers than the licensed vaccine. All vaccinated cows showed F-specific antibodies after the second dose, and titers were very stable from the period of 2 to 6 weeks after the second dose (and were particularly stable for the RNA vaccine).

**Mechanism of action**

Bone marrow derived dendritic cells (pDC) were obtained from wild-type mice or the "Resq" (rsq) mutant strain. The mutant strain has a point mutation at the amino terminus of its TLR7 receptor which abolishes TLR7 signalling without affecting ligand binding [44]. The cells were stimulated with replicon RNA formulated with DOTAP, lipofectamine 2000 or inside a liposome. As shown in Figure 19, IL-6 and INFα were induced in WT cells but this response was almost completely abrogated in mutant mice. These results shows that TLR7 is required for RNA recognition in immune cells, and that liposome-encapsulated replicons can cause immune cells to secrete high levels of both interferons and pro-inflammatory cytokines.

**pKa measurement**

The pKa of a lipid is measured in water at standard temperature and pressure using the following technique:

- 2mM solution of lipid in ethanol is prepared by weighing the lipid and dissolving in ethanol.
- 0.3mM solution of fluorescent probe toluene nitrosulphonic acid (TNS) in ethanokmethanol 9:1 is prepared by first making 3mM solution of TNS in methanol and then diluting to 0.3mM with ethanol.
- An aqueous buffer containing sodium phosphate, sodium citrate sodium acetate and sodium chloride, at the concentrations 20mM, 25mM, 20mM and 150 mM, respectively, is prepared. The buffer is split into eight parts and the pH adjusted either with 12N HCl or 6N NaOH to 4.44-4.52, 5.27, 6.15-6.21, 6.57, 7.10-7.20, 7.72-7.80, 8.27-8.33 and 10.47-1 1.12. 400nL of 2mM lipid solution and 800µL of 0.3mM TNS solution are mixed.
- 1.5µL of probe/lipid mix are added to 242.5µL of buffer in a 1mL 96 well plate. This is done with all eight buffers. After mixing, 100µL of each probe/lipid/buffer mixture is transferred to a 250nL black with clear bottom 96 well plate (e.g. model COSTAR 3904, Corning). A
convenient way of performing this mixing is to use the Tecan Genesis RSP150 high throughput liquid handler and Gemini Software.

- Fluorescence of each probe/lipid/buffer mixture is measured (e.g. with a SpectraMax M5 spectrophotometer and SoftMax pro 5.2 software) with 322nm excitation, 431nm emission (auto cutoff at 420nm).

- After the measurement, the background fluorescence value of an empty well on the 96 well plate is subtracted from each probe/lipid/buffer mixture. The fluorescence intensity values are then normalized to the value at lowest pH. The normalized fluorescence intensity is then plotted against pH and a line of best fit is provided.

- The point on the line of best fit at which the normalized fluorescence intensity is equal to 0.5 is found. The pH corresponding to normalized fluorescence intensity equal to 0.5 is found and is considered the pKa of the lipid.

This method gives a pKa of 5.8 for DLinDMA. The pKa values measured by this method for cationic lipids of reference 5 are included below.

**Encapsulation in liposomes using alternative cationic lipids**

As an alternative to using DLinDMA, the cationic lipids of reference 5 are used. These lipids can be synthesised as disclosed in reference 5.

The liposomes formed above using DLinDMA are referred to hereafter as the "RV01" series. The DLinDMA was replaced with various cationic lipids in series "RV02" to "RV12" as described below.

Two different types of each liposome were formed, using 2% PEG2000-DMG with either (01) 40% of the cationic lipid, 10% DSPC, and 48% cholesterol, or (02) 60% of the cationic lipid and 38% cholesterol. Thus a comparison of the (01) and (02) liposomes shows the effect of the neutral zwitterionic lipid.

RV02 liposomes were made using the following cationic lipid (pKa >9, without a tertiary amine):

\[
\text{[Chemical structure of RV02 liposome cationic lipid]}
\]
RV03 liposomes were made using the following cationic lipid (pKa 6.4):

![Chemical Structure](image)

RV04 liposomes were made using the following cationic lipid (pKa 6.62):

![Chemical Structure](image)
RV05 liposomes were made using the following cationic lipid (pKa 5.85):

![RV05 lipid structure](image)

RV06 liposomes were made using the following cationic lipid (pKa 7.27):

![RV06 lipid structure](image)

RV07 liposomes were made using the following cationic lipid (pKa 6.8):

![RV07 lipid structure](image)
RV08 liposomes were made using the following cationic lipid (pKa 5.72):

RV09 liposomes were made using the following cationic lipid (pKa 6.07):

RV10 liposomes were made for comparison using the following cationic lipid (pKa 7.86):
RV1 liposomes were made using the following cationic lipid (pKa 6.41):

![Chemical structure of RV1 cationic lipid](image1)

RV12 liposomes were made using the following cationic lipid (pKa 7):

![Chemical structure of RV12 cationic lipid](image2)

RV16 liposomes were made using the following cationic lipid (pKa 6.1) [45]:

![Chemical structure of RV16 cationic lipid](image3)
RV17 liposomes were made using the following cationic lipid (pKa 6.1) [45]:

RV18 liposomes were made using DODMA. RV19 liposomes were made using DOTMA, and RV13 liposomes were made with DOTAP, both having a quaternary amine headgroup.

These liposomes were characterised and were tested with the SEAP reporter described above. The following table shows the size of the liposomes (Z average and polydispersity index), the % of RNA encapsulation in each liposome, together with the SEAP activity detected at days 1 and 6 after injection. SEAP activity is relative to "RV01(02)" liposomes made from DlinDMA, cholesterol and PEG-DMG:

<table>
<thead>
<tr>
<th>RV</th>
<th>Lipid pKa</th>
<th>Zav (pdl)</th>
<th>% encapsulation</th>
<th>SEAP day 1</th>
<th>SEAP day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV01 (01)</td>
<td>5.8</td>
<td>154.6 (0.131)</td>
<td>95.5</td>
<td>80.9</td>
<td>71.1</td>
</tr>
<tr>
<td>RV01 (02)</td>
<td>5.8</td>
<td>162.0 (0.134)</td>
<td>85.3</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>RV02 (01)</td>
<td>&gt;9</td>
<td>133.9 (0.185)</td>
<td>96.5</td>
<td>57</td>
<td>45.7</td>
</tr>
<tr>
<td>RV02 (02)</td>
<td>&gt;9</td>
<td>134.6 (0.082)</td>
<td>97.6</td>
<td>54.2</td>
<td>4.3</td>
</tr>
<tr>
<td>RV03 (01)</td>
<td>6.4</td>
<td>158.3 (0.212)</td>
<td>62.0</td>
<td>65.7</td>
<td>44.9</td>
</tr>
<tr>
<td>RV03 (02)</td>
<td>6.4</td>
<td>164.2 (0.145)</td>
<td>86</td>
<td>62.2</td>
<td>39.7</td>
</tr>
<tr>
<td>RV04 (01)</td>
<td>6.62</td>
<td>131.0 (0.145)</td>
<td>74.0</td>
<td>9.1</td>
<td>154.8</td>
</tr>
<tr>
<td>RV04 (02)</td>
<td>6.62</td>
<td>134.6 (0.117)</td>
<td>81.5</td>
<td>90.4</td>
<td>142.6</td>
</tr>
<tr>
<td>RV05 (01)</td>
<td>5.85</td>
<td>164.0 (0.162)</td>
<td>76.0</td>
<td>76.9</td>
<td>329.8</td>
</tr>
<tr>
<td>RV05 (02)</td>
<td>5.85</td>
<td>177.8 (0.117)</td>
<td>72.8</td>
<td>67.1</td>
<td>227.9</td>
</tr>
<tr>
<td>RV06 (01)</td>
<td>7.27</td>
<td>116.0 (0.180)</td>
<td>79.8</td>
<td>25.5</td>
<td>12.4</td>
</tr>
<tr>
<td>RV06 (02)</td>
<td>7.27</td>
<td>136.3 (0.164)</td>
<td>74.9</td>
<td>24.8</td>
<td>23.1</td>
</tr>
<tr>
<td>RV07 (01)</td>
<td>6.8</td>
<td>140.6 (0.184)</td>
<td>77</td>
<td>26.5</td>
<td>163.3</td>
</tr>
<tr>
<td>RV07 (02)</td>
<td>6.8</td>
<td>138.6 (0.122)</td>
<td>87</td>
<td>29.7</td>
<td>74.8</td>
</tr>
<tr>
<td>RV08 (01)</td>
<td>5.72</td>
<td>176.7 (0.185)</td>
<td>50</td>
<td>76.5</td>
<td>187</td>
</tr>
<tr>
<td>RV08 (02)</td>
<td>5.72</td>
<td>199.5 (0.191)</td>
<td>46.3</td>
<td>82.4</td>
<td>329.8</td>
</tr>
<tr>
<td>RV09 (01)</td>
<td>6.07</td>
<td>165.3 (0.169)</td>
<td>72.2</td>
<td>65.1</td>
<td>453.9</td>
</tr>
<tr>
<td>RV09 (02)</td>
<td>6.07</td>
<td>179.5 (0.157)</td>
<td>65</td>
<td>68.5</td>
<td>658.2</td>
</tr>
<tr>
<td>RV10 (01)</td>
<td>7.86</td>
<td>129.7 (0.184)</td>
<td>78.4</td>
<td>113.4</td>
<td>47.8</td>
</tr>
<tr>
<td>RV10 (02)</td>
<td>7.86</td>
<td>147.6 (0.131)</td>
<td>80.9</td>
<td>78.2</td>
<td>10.4</td>
</tr>
<tr>
<td>RV11 (01)</td>
<td>6.41</td>
<td>129.2 (0.186)</td>
<td>71</td>
<td>113.6</td>
<td>242.2</td>
</tr>
<tr>
<td>RV11 (02)</td>
<td>6.41</td>
<td>139 (0198)</td>
<td>75.2</td>
<td>71.8</td>
<td>187.2</td>
</tr>
<tr>
<td>RV12 (01)</td>
<td>7</td>
<td>135.7 (0.161)</td>
<td>78.8</td>
<td>65</td>
<td>10</td>
</tr>
<tr>
<td>RV12 (02)</td>
<td>7</td>
<td>158.3 (0.287)</td>
<td>69.4</td>
<td>78.8</td>
<td>8.2</td>
</tr>
</tbody>
</table>

Figure 15 plots the SEAP levels at day 6 against the pKa of the cationic lipids. The best results are seen where the lipid has a pKa between 5.6 and 6.8, and ideally between 5.6 and 6.3.

These liposomes were also used to deliver a replicon encoding full-length RSV F protein. Total IgG titers against F protein two weeks after the first dose (2wpl) are plotted against pKa in Figure 16. The best results are seen where the pKa is where the cationic lipid has a pKa between 5.7-5.9, but pKa alone is not enough to guarantee a high titer e.g. the lipid must still support liposome formation.

**RSI immunogenicity**

Further work was carried out with a self-replicating replicon (vA317) encoding RSV F protein. BALB/c mice, 4 or 8 animals per group, were given bilateral intramuscular vaccinations (50 µL per leg) on days 0 and 21 with the replicon (~g) alone or formulated as liposomes with the RV01 or RV05 lipids (see above; pKa of 5.8 or 5.85) or with RV13. The RV01 liposomes had 40% DlinDMA, 10% DSPC, 48% cholesterol and 2% PEG-DMG, but with differing amounts of RNA. The RV05(01) liposomes had 40% cationic lipid, 48% cholesterol, 10% DSPC, and 2% PEG-DMG; the RV05(02) liposomes had 60% cationic lipid, 38% cholesterol, and 2% PEG-DMG. The RV13 liposomes had 40% DOTAP, 10% DPE, 48% cholesterol and 2% PEG-DMG. For comparison, naked plasmid DNA (20 µg) expressing the same RSV-F antigen was delivered either using electroporation or with RV01(10) liposomes (O.µg DNA). Four mice were used as a naive control group.

Liposomes were prepared by method (A) or method (B). In method (A) fresh lipid stock solutions in ethanol were prepared. 37 mg of cationic lipid, 11.8 mg of DSPC, 27.8 mg of cholesterol and 8.07 mg of PEG-DMG were weighed and dissolved in 7.55 mL of ethanol. The freshly prepared lipid stock solution was gently rocked at 37°C for about 15 min to form a homogenous mixture. Then, 226.7 µL of the stock was added to 1.773 mL ethanol to make a working lipid stock solution of 2 mL. This amount of lipids was used to form liposomes with 75 µg RNA to give an 8:1 nitrogen to phosphate ratio (except that in RV01 (08) and RV01 (09) this ratio was modified to 4:1 or 16:1). A 2 mL working solution of RNA (or, for RV01(10), DNA) was also prepared from a stock solution of ~1µg/µL in 100 mM citrate buffer (pH 6). Three 20 mL glass vials (with stir bars) were rinsed with RNase Away solution (Molecular BioProducts) and washed with plenty of MilliQ water before use to decontaminate the vials of RNases. One of the vials was used for the RNA working solution and the
others for collecting the lipid and RNA mixes (as described later). The working lipid and RNA solutions were heated at 37°C for 10 min before being loaded into 3 cc syringes. 2 mL of citrate buffer (pH 6) was loaded in another 3 cc syringe. Syringes containing RNA and the lipids were connected to a T mixer (PEEK™ 500 μη ID junction) using FEP tubing. The outlet from the T mixer was also FEP tubing. The third syringe containing the citrate buffer was connected to a separate piece of FEP tubing. All syringes were then driven at a flow rate of 7 mL/min using a syringe pump. The tube outlets were positioned to collect the mixtures in a 20 mL glass vial (while stirring). The stir bar was taken out and the ethanol/aqueous solution was allowed to equilibrate to room temperature for 1 hour. Then the mixture was loaded in a 5 cc syringe, which was fitted to a piece of FEP tubing and in another 5 cc syringe with equal length of FEP tubing, an equal volume of 100 mM citrate buffer (pH 6) was loaded. The two syringes were driven at 7mL/min flow rate using a syringe pump and the final mixture collected in a 20 mL glass vial (while stirring). Next, liposomes were concentrated to 2 mL and dialyzed against 10-15 volumes of IX PBS using TFF before recovering the final product. The TFF system and hollow fiber filtration membranes were purchased from Spectrum Labs and were used according to the manufacturer's guidelines. Polyethersulfone (PES) hollow fiber filtration membranes (part number P-Cl-IOOE-100-OIN) with a 100 kD pore size cutoff and 20 cm² surface area were used. For in vitro and in vivo experiments, formulations were diluted to the required RNA concentration with IX PBS.

Preparation method (B) differed in two ways from method (A). Firstly, after collection in the 20 mL glass vial but before TFF concentration, the mixture was passed through a Mustang Q membrane (an anion-exchange support that binds and removes anionic molecules, obtained from Pall Corporation, Ann Arbor, MI, USA). This membrane was first washed with 4 mL of 1 M NaOH, 4 mL of 1 M NaCl and 10 mL of 100 mM citrate buffer (pH 6) in turn, and liposomes were warmed for 10 min at 37°C before being filtered. Secondly, the hollow fiber filtration membrane was Polysulfone (part number P/N: X1AB-100-20P).

The Z average particle diameter, polydispersity index and encapsulation efficiency of the liposomes were as follows:

<table>
<thead>
<tr>
<th>RV</th>
<th>Zav (nm)</th>
<th>pdl</th>
<th>% encapsulation</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV01 (10)</td>
<td>158.6</td>
<td>0.088</td>
<td>90.7</td>
<td>(A)</td>
</tr>
<tr>
<td>RV01 (08)</td>
<td>156.8</td>
<td>0.144</td>
<td>88.6</td>
<td>(A)</td>
</tr>
<tr>
<td>RV01 (05)</td>
<td>136.5</td>
<td>0.136</td>
<td>99</td>
<td>(B)</td>
</tr>
<tr>
<td>RV01 (09)</td>
<td>153.2</td>
<td>0.067</td>
<td>76.7</td>
<td>(A)</td>
</tr>
<tr>
<td>RV05 (01)</td>
<td>148</td>
<td>0.127</td>
<td>80.6</td>
<td>(A)</td>
</tr>
<tr>
<td>RV05 (02)</td>
<td>177.2</td>
<td>0.136</td>
<td>72.4</td>
<td>(A)</td>
</tr>
<tr>
<td>RV01 (10)</td>
<td>134.7</td>
<td>0.147</td>
<td>87.8 *</td>
<td>(A)</td>
</tr>
<tr>
<td>RV13 (02)</td>
<td>128.3</td>
<td>0.179</td>
<td>97</td>
<td>(A)</td>
</tr>
</tbody>
</table>
Serum was collected for antibody analysis on days 14, 36 and 49. Spleens were harvested from mice at day 49 for T cell analysis.

F-specific serum IgG titers (GMT) were as follows:

<table>
<thead>
<tr>
<th>RV</th>
<th>Day 14</th>
<th>Day 36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naked DNA plasmid</td>
<td>439</td>
<td>6712</td>
</tr>
<tr>
<td>Naked A317 RNA</td>
<td>78</td>
<td>2291</td>
</tr>
<tr>
<td>RV01 (10)</td>
<td>3020</td>
<td>26170</td>
</tr>
<tr>
<td>RV01 (08)</td>
<td>2326</td>
<td>9720</td>
</tr>
<tr>
<td>RV01 (05)</td>
<td>5352</td>
<td>54907</td>
</tr>
<tr>
<td>RV01 (09)</td>
<td>4428</td>
<td>51316</td>
</tr>
<tr>
<td>RV05 (01)</td>
<td>1356</td>
<td>5346</td>
</tr>
<tr>
<td>RV05 (02)</td>
<td>961</td>
<td>6915</td>
</tr>
<tr>
<td>RV01 (10) DNA</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>RV13 (02)</td>
<td>644</td>
<td>3616</td>
</tr>
</tbody>
</table>

The proportion of T cells which are cytokine-positive and specific for RSV F51-66 peptide are as follows, showing only figures which are statistically significantly above zero:

<table>
<thead>
<tr>
<th>RV</th>
<th>CD4+CD8-</th>
<th>CD4-CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFNγ</td>
<td>IL2</td>
</tr>
<tr>
<td>Naked DNA plasmid</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>Naked A317 RNA</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>RV01 (10)</td>
<td>0.07</td>
<td>0.10</td>
</tr>
<tr>
<td>RV01 (08)</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>RV01 (05)</td>
<td>0.08</td>
<td>0.12</td>
</tr>
<tr>
<td>RV01 (09)</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td>RV05 (01)</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>RV05 (02)</td>
<td>0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>RV01 (10) DNA</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>RV13 (02)</td>
<td>0.03</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Thus the liposome formulations significantly enhanced immunogenicity relative to the naked RNA controls, as determined by increased F-specific IgG titers and T cell frequencies. Plasmid DNA
formulated with liposomes, or delivered naked using electroporation, was significantly less
immunogenic than liposome-formulated self-replicating RNA.

The RV01 and RV05 RNA vaccines were more immunogenic than the RV13 (DOTAP) vaccine. These formulations had comparable physical characteristics and were formulated with the same self-replicating RNA, but they contain different cationic lipids. RV01 and RV05 both have a tertiary amine in the headgroup with a pKa of about 5.8, and also include unsaturated alkyl tails. RV13 has unsaturated alkyl tails but its headgroup has a quaternary amine and is very strongly cationic. These results suggest that lipids with tertiary amines with pKas in the range 5.0 to 7.6 are superior to lipids such as DOTAP, which are strongly cationic, when used in a liposome delivery system for RNA.

*Further alternatives to DLinDMA*

The cationic lipid in RV01 liposomes (DLinDMA) was replaced by RV16, RV17, RV18 or RV19. Total IgG titers are shown in Figure 17. The lowest results are seen with RV19 *i.e.* the DOTMA quaternary amine.

*BHK expression*

Liposomes with different lipids were incubated with BHK cells overnight and assessed for protein expression potency. From a baseline with RV05 lipid expression could be increased 18x by adding 10% 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (DPyPE) to the liposome, 10x by adding 10% 18:2 (cis) phosphatidylcholine, and 900x by instead using RV01.

*RSV immunogenicity in different mouse strains*

Replicon "vA142" encodes the full-length wild type surface fusion (F) glycoprotein of RSV but with the fusion peptide deleted, and the 3’ end is formed by ribozyme-mediated cleavage. It was tested in three different mouse strains.

BALB/c mice were given bilateral intramuscular vaccinations (50 µL per leg) on days 0 and 22. Animals were divided into 8 test groups (5 animals per group) and a naive control (2 animals):

- **Group 1** were given naked replicon (1 µg).
- **Group 2** were given 1 µg replicon delivered in liposomes "RV01(37)" with 40% DLinDMA, 10% DSPC, 48% Choi, 2% PEG-conjugated DMG.
- **Group 3** were the same as group 2, but at O^2g RNA.
- **Group 4** were given 1 µg replicon in "RV17(10)" liposomes (40% RV17 (see above), 10% DSPC, 49.5% cholesterol, 0.5% PEG-DMG).
- **Group 5** were 1 µg replicon in "RV05(11)" liposomes (40% RV07 lipid, 30% 18:2 PE (DLoPE, 28% cholesterol, 2% PEG-DMG).
- **Group 6** were given O^2g replicon in "RV17(10)" liposomes.
- **Group 7** were given 5 µg RSV-F subunit protein adjuvanted with aluminium hydroxide.
Group 8 were a naive control (2 animals)

Sera were collected for antibody analysis on days 14, 35 and 49. F-specific serum IgG GMTs were:

<table>
<thead>
<tr>
<th>Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>82</td>
<td>2463</td>
<td>1789</td>
<td>2496</td>
<td>1171</td>
<td>1295</td>
<td>1293</td>
<td>5</td>
</tr>
<tr>
<td>35</td>
<td>1538</td>
<td>34181</td>
<td>25605</td>
<td>23579</td>
<td>13718</td>
<td>8887</td>
<td>73809</td>
<td>5</td>
</tr>
</tbody>
</table>

At day 35 F-specific IgG1 and IgG2a titers (GMT) were as follows:

<table>
<thead>
<tr>
<th>IgG</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>94</td>
<td>6238</td>
<td>4836</td>
<td>7425</td>
<td>8288</td>
<td>1817</td>
<td>78604</td>
</tr>
<tr>
<td>IgG2a</td>
<td>5386</td>
<td>77064</td>
<td>59084</td>
<td>33749</td>
<td>14437</td>
<td>17624</td>
<td>24</td>
</tr>
</tbody>
</table>

RSV serum neutralizing antibody titers at days 35 and 49 were as follows (data are 60% plaque reduction neutralization titers of pools of 2-5 mice, 1 pool per group):

<table>
<thead>
<tr>
<th>Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>&lt;20</td>
<td>143</td>
<td>20</td>
<td>101</td>
<td>32</td>
<td>30</td>
<td>111</td>
<td>&lt;20</td>
</tr>
<tr>
<td>49</td>
<td>&lt;20</td>
<td>139</td>
<td>&lt;20</td>
<td>83</td>
<td>41</td>
<td>32</td>
<td>1009</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

Spleens were harvested at day 49 for T cell analysis. Average net F-specific cytokine-positive T cell frequencies (CD4+ or CD8+) were as follows, showing only figures which were statistically significantly above zero (specific for RSV peptides F51-66, F164-178, F309-323 for CD4+, or for peptides F85-93 and F249-258 for CD8+):

<table>
<thead>
<tr>
<th>Group</th>
<th>CD4+CD8-</th>
<th>CD4-CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFNγ</td>
<td>IL2</td>
</tr>
<tr>
<td>1</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>2</td>
<td>0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>3</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>4</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>5</td>
<td>0.03</td>
<td>0.08</td>
</tr>
<tr>
<td>6</td>
<td>0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C57BL/6 mice were immunised in the same way, but a 9th group received VRPs (lx1 0⁶ IU) expressing the full-length wild-type surface fusion glycoprotein of RSV (fusion peptide deletion).

Sera were collected for antibody analysis on days 14, 35 & 49. F-specific IgG titers (GMT) were:
At day 35 F-specific IgGl and IgG2a titers (GMT) were as follows:

<table>
<thead>
<tr>
<th>IgG</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>66</td>
<td>247</td>
<td>14</td>
<td>328</td>
<td>468</td>
<td>92</td>
<td>56258</td>
<td>79</td>
</tr>
<tr>
<td>IgG2a</td>
<td>2170</td>
<td>7685</td>
<td>5055</td>
<td>6161</td>
<td>1573</td>
<td>2944</td>
<td>35</td>
<td>14229</td>
</tr>
</tbody>
</table>

RSV serum neutralizing antibody titers at days 35 and 49 were as follows (data are 60% plaque reduction neutralization titers of pools of 2-5 mice, 1 pool per group):

<table>
<thead>
<tr>
<th>Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>&lt;20</td>
<td>27</td>
<td>29</td>
<td>36</td>
<td>&lt;20</td>
<td>28</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>49</td>
<td>&lt;20</td>
<td>44</td>
<td>30</td>
<td>23</td>
<td>36</td>
<td>&lt;20</td>
<td>33</td>
<td>&lt;20</td>
<td>37</td>
</tr>
</tbody>
</table>

Spleens were harvested at day 49 for T cell analysis. Average net F-specific cytokine-positive T cell frequencies (CD8+) were as follows, showing only figures which were statistically significantly above zero (specific for RSV peptides F85-93 and F249-258):

<table>
<thead>
<tr>
<th>Group</th>
<th>IFNγ</th>
<th>IL2</th>
<th>IL5</th>
<th>TNFα</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.42</td>
<td>0.13</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.21</td>
<td>0.37</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.01</td>
<td>0.26</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.26</td>
<td>0.23</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.13</td>
<td>0.70</td>
<td>1.77</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.59</td>
<td>0.19</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.10</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>2.83</td>
<td>0.72</td>
<td>2.26</td>
<td></td>
</tr>
</tbody>
</table>

Nine groups of C3H/HeN mice were immunised in the same way. F-specific IgG titers (GMT) were:

<table>
<thead>
<tr>
<th>Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>5</td>
<td>2049</td>
<td>1666</td>
<td>1102</td>
<td>298</td>
<td>984</td>
<td>3519</td>
<td>5</td>
<td>806</td>
</tr>
<tr>
<td>35</td>
<td>152</td>
<td>27754</td>
<td>19008</td>
<td>17693</td>
<td>3424</td>
<td>6100</td>
<td>62297</td>
<td>5</td>
<td>17249</td>
</tr>
</tbody>
</table>

At day 35 F-specific IgGl and IgG2a titers (GMT) were as follows:
RSV serum neutralizing antibody titers at days 35 and 49 were as follows:

<table>
<thead>
<tr>
<th>Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>&lt;20</td>
<td>539</td>
<td>260</td>
<td>65</td>
<td>101</td>
<td>95</td>
<td>443</td>
<td>&lt;20</td>
<td>595</td>
</tr>
<tr>
<td>49</td>
<td>&lt;20</td>
<td>456</td>
<td>296</td>
<td>35</td>
<td>82</td>
<td>125</td>
<td>1148</td>
<td>&lt;20</td>
<td>387</td>
</tr>
</tbody>
</table>

Thus three different lipids (RVOl, RV05, RV17; pKa 5.8, 5.85, 6.1) were tested in three different inbred mouse strains. For all 3 strains RVOl was more effective than RV17; for BALB/c and C3H strains RV05 was less effective than either RVOl or RV17, but it was more effective in B6 strain. In all cases, however, the liposomes were more effective than two cationic nanoemulsions which were tested in parallel.

**CMV immunogenicity**

RV01 liposomes with DLinDMA as the cationic lipid were used to deliver RNA replicons encoding cytomegalovirus (CMV) glycoproteins. The "vA160" replicon encodes full-length glycoproteins H and L (gH/gL), whereas the "vA322" replicon encodes a soluble form (gHsol/gL). The two proteins are under the control of separate subgenomic promoters in a single replicon; co-administration of two separate vectors, one encoding gH and one encoding gL, did not give good results.

BALB/c mice, 10 per group, were given bilateral intramuscular vaccinations (50 µL per leg) on days 0, 21 and 42 with VRPs expressing gH/gL (1x10^6 IU), VRPs expressing gHsol/gL (1x10^6 IU) and PBS as the controls. Two test groups received 1µg of the vA160 or vA322 replicon formulated in liposomes (40% DLinDMA, 10% DSPC, 48% Choi, 2% PEG-DMG; made using method (A) as discussed above, but with 150µg RNA batch size).

The vA160 liposomes had a Zav diameter of 168nm, a pdl of 0.144, and 87.4% encapsulation. The vA322 liposomes had a Zav diameter of 162nm, a pdl of 0.131, and 90% encapsulation.

The replicons were able to express two proteins from a single vector.

Sera were collected for immunological analysis on day 63 (3wp3). CMV neutralization titers (the reciprocal of the serum dilution producing a 50% reduction in number of positive virus foci per well, relative to controls) were as follows:

<table>
<thead>
<tr>
<th>gH/gL VRP</th>
<th>gHsol/gL VRP</th>
<th>gH/gL liposome</th>
<th>gHsol/gL liposome</th>
</tr>
</thead>
<tbody>
<tr>
<td>4576</td>
<td>2393</td>
<td>4240</td>
<td>10062</td>
</tr>
</tbody>
</table>
RNA expressing either a full-length or a soluble form of the CMV gH/gL complex thus elicited high titers of neutralizing antibodies, as assayed on epithelial cells. The average titers elicited by the liposome-encapsulated RNAs were at least as high as for the corresponding VRPs.

It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

REFERENCES
[26] WO03/0 18054.
[34] Methods In Enzymology (S. Colowick and N. Kaplan, eds., Academic Press, Inc.)
[40] PCR (Introduction to Biotechniques Series), 2nd ed. (Newton & Graham eds., 1997, Springer Verlag)
CLAIMS

1. A liposome having a lipid bilayer encapsulating an aqueous core, wherein: (i) the lipid bilayer comprises a lipid having a pKa in the range of 5.0 to 7.6; and (ii) the aqueous core includes a RNA which encodes an immunogen.

2. The liposome of claim 1, wherein the lipid having a pKa in the range of 5.0 to 7.6 has a tertiary amine.

3. The liposome of any preceding claim, wherein pKa in the range of 5.0 to 7.6 is between 5.7-5.9.

4. The liposome of claim 1, wherein the lipid having a pKa in the range of 5.0 to 7.6 has the formula shown herein for RV01, RV02, RV03, RV04, RV05, RV06, RV07, RV08, RV09, RV11, RV12, RV16 or RV17.

5. The liposome of any preceding claim, having a diameter in the range of 20-220nm.

6. The liposome of any preceding claim, wherein the RNA molecule encodes (i) a RNA-dependent RNA polymerase which can transcribe RNA from the RNA molecule and (ii) an immunogen.

7. The liposome of claim 5, wherein the RNA molecule has two open reading frames, the first of which encodes an alphavirus replicase and the second of which encodes the immunogen.

8. The liposome of any preceding claim, wherein the RNA molecule is 9000-12000 nucleotides long.

9. The liposome of any preceding claim, wherein the immunogen can elicit an immune response \textit{in vivo} against a bacterium, a virus, a fungus or a parasite.

10. The liposome of any preceding claim, wherein the immunogen can elicit an immune response \textit{in vivo} against respiratory syncytial virus glycoprotein F.

11. A pharmaceutical composition comprising the liposome of any preceding claim.

12. A method for raising a protective immune response in a vertebrate, comprising the step of administering to the vertebrate an effective amount of the liposome of claims 1-10, or the pharmaceutical composition of claim 11.

13. A process for preparing a RNA-containing liposome, comprising steps during liposome formation of: (a) mixing RNA with a lipid at a pH which is below the lipid's pKa but is above 4.5; then (b) increasing the pH to be above the lipid's pKa.

14. The process of claim 13, wherein: RNA used in step (a) is in aqueous solution, for mixing with an organic solution of the lipid to give a mixture which is then diluted to form liposomes; and the pH is increased in step (b) after liposome formation.
FIGURE 19A

WT
TLR7rsq1

FIGURE 19B

WT
TLR7rsq1
# INTERNATIONAL SEARCH REPORT

| A. CLASSIFICATION OF SUBJECT MATTER |
| INV. A61K9/127 A61K39/00 C12N15/86 |

According to International Patent Classification (IPC) or both national classification and IPC.

| B. FIELDS SEARCHED |
| Minimum documentation searched (classification system followed by classification symbols) |
| A61K C12N |

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched.

| C. DOCUMENTS CONSIDERED TO BE RELEVANT |
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| X | WO 2010/015098 A1 (UNIV DE CONCEPCION [CL]) | 1, 3, 6-9 |
| Y | Claims; examples 7, 11 |
| 18 August 2011 (2011-08-18) | 1-14 |
| | | -/- |

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another document or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is taken together with one or more other such documents, such combination being obvious to a person skilled in the art
- "A" document member of the same patent family

Date of the actual completion of the international search: 19 September 2011

Date of mailing of the international search report: 27/09/2011

Name and mailing address of the ISA:
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3018

Authorized officer: Somrner, Birgit
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<td>X</td>
<td>M MOCKEY ET AL: &quot;mRNA-based cancer vaccine: preventi on of B16 melanoma progressi on and metastasis by systemic injection of MARTI mRNA histi dylated 1i popolyply exes&quot;, CANCER GENE THERAPY, vol. 14, no. 9, 1 September 2007 (2007-09-01), pages 802-814, XP55007324, ISSN: 0929-1903, DOI: 10.1038/sjcgt.7701072</td>
<td>1, 3, 11, 12</td>
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<td>Y</td>
<td>abstract; material and methods</td>
<td>1-14</td>
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<tr>
<td>X</td>
<td>KITA H ET AL: &quot;Replication of Genetic Information with Self-Encoded Replicase in Liposomes&quot;, CHEMBIOCHEM, vol. 9, no. 15, 13 October 2008 (2008-10-13), pages 2403-2410, XP55007329, ISSN: 1439-4227, DOI: 10.1002/cbic.200800360</td>
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<td>Y</td>
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<td>w0 2011/076807 A2 (NOVARTIS AG [CH]; BARYZA JEREMY [US]; BOWMAN KEITH [US]; GEALL ANDREW) 30 June 2011 (2011-06-30) claims; examples 77</td>
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This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. □ Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. □ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. X As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos. :

4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos. :

Remark on Protest  □ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

□ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

□ No protest accompanied the payment of additional search fees.
This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-12

   a liposome comprising a lipid having a $pK_a$ in the range of 5.0-7.6 and including a RNA which encodes an immunogen as well as subject-matter related thereto;

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2. claims: 13, 14

   a process for preparing a RNA-containing liposome as well as subject-matter related thereto;

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