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Special emphasis is given in this review to cationic adjuvant liposome vaccine formulations. Examples of vaccines made with CAF01, an adjuvant composed of the synthetic immune stimulating mycobacterial cordfactor glycolipid trehalose-dibehenate (TDB) as immunomodulator and the cationic membrane forming molecule dimethyl-dioctadecylammonium DDA are presented. Other vaccines such as cationic liposome-DNA complexes (CLDC) and other adjuvants like muramyl dipeptide, monophosphoryl lipid A and listeriolysin O are mentioned as well.

The field of liposomes and liposome-based vaccines is vast. Therefore, this review concentrates on recent and relevant studies emphasizing current reports dealing with the most studied antigens and adjuvants and pertinent
examples of vaccines. Studies on liposome-based veterinary vaccines and experimental therapeutic cancer vaccines are also summarized.
Liposomes as vaccine delivery systems. A review of the recent advances

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Abstract

Liposomes and liposome-derived nanovesicles such as archaeosomes and virosomes have become important carriers systems in vaccine development and the interest for liposome-based vaccines has markedly increased. A key advantage of liposomes, archaeosomes and virosomes in general, and liposome-based vaccine delivery systems in particular, is their versatility and plasticity. Liposome composition and preparation can be chosen to achieve desired features such as selection of lipid, charge, size, size distribution, entrapment and location of antigens or adjuvants. Depending on the chemical properties, water soluble antigens (proteins, peptides, nucleic acids, carbohydrates, haptens) are entrapped within the aqueous inner space of liposomes, whereas lipophilic compounds (lipopeptides, antigens, adjuvants, linker molecules) are intercalated into the lipid bilayer and antigens or adjuvants can be attached to the liposome surface either by adsorption or stable chemical linking. Co-formulations containing different types of antigens and/or adjuvants can be combined with the parameters mentioned to tailor liposomal vaccines for individual applications. Special emphasis is given in this review to cationic adjuvant liposome vaccine formulations. Examples of vaccines made with CAF01, an adjuvant composed of the synthetic immune stimulating mycobacterial cordfactor glycolipid trehalose-dibehenate (TDB) as immunomodulator and the cationic membrane forming molecule dimethyl-dioctadecylammonium DDA are presented. Other vaccines such as cationic liposome-DNA complexes (CLDC) and other adjuvants like muramyl dipeptide, monophosphoryl lipid A and listeriolysin O are mentioned as well.

The field of liposomes and liposome-based vaccines is vast. Therefore, this review concentrates on recent and relevant studies emphasizing current reports dealing with the most studied antigens and adjuvants and pertinent examples of vaccines. Studies on
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**Keywords:** Liposomes, archaeosomes, virosomes, vaccines, antigens, adjuvants, veterinary vaccines, therapeutic cancer vaccines
**Introduction (7011 words)**

Classical vaccines rely on the use of whole killed or attenuated pathogens. Today, research is focused on the development of subunit vaccines because they are better defined, easier to produce and safer. Vaccines are manufactured on the basis of well characterized antigens, such as recombinant proteins and peptides. However, due to their synthetic nature, their immune response is often weak which is largely related to the inability of the antigens to induce maturation of dendritic cells (DC), the primary antigen presenting cells (APC) that react to foreign pathogens and trigger the immune response [Moser et al., 2010, Reed et al., 2013].

The immune system is composed of the innate and the adaptive systems. The first is responsible for first line host defense, rapidly recognizing and responding to foreign pathogens. The complement system and phagocytic cells belong to this defense system which depends on pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). Toll like receptors (TLR) present on APCs are the receptors for pathogens containing PAMPs. TLR activation is the hallmark of innate immune response. The second defense line, the adaptive immune system, mounts specific responses against molecular determinants on pathogenic agents. These responses are initiated by antigen-mediated triggering of T cells, the CD4+ T helper (T\textsubscript{H}1) cells, the CD8+ cytotoxic T lymphocytes (CTL) and B lymphocytes carrying antigen-specific surface receptors. T\textsubscript{H} cells have subpopulations, of which T\textsubscript{H}1 and T\textsubscript{H}2 are the most important [Nordly et al., 2009, Kawai et al., 2010].

The diverse mechanisms by which nanoparticles induce immune responses are summarized in Figure 1. Activation of PRRs triggers the initiation of the innate immune response. Activated CTLs recognize peptides bound to the major histocompatibility complex class I and II molecules (MHC-I, MHC-II) which express antigenic peptides on APCs and bind to T cells via the T cell receptor. A co-stimulatory signal is needed for full CTL and T\textsubscript{H} cell activation which differentiate into T\textsubscript{H}1 or T\textsubscript{H}2 and other T-helper lineages that produce cytokines. T\textsubscript{H} cells provide help to antigen-specific B cells, resulting in antibody production [Lin et al., 2010, Chen et al., 2013]. Each invasion of a foreign antigen requires activation of a specific type of adaptive immune response for efficient control and elimination. Thus, vaccine formulations should be designed rationally to induce specific protective responses. This includes the choice of antigen and adjuvant(s) and their pharmaceutical formulation.
Adjuvants
The ability to enhance the immune response of vaccines by certain compounds was first demonstrated with aluminium salts, - termed “adjuvants” - added to killed or attenuated pathogens. Their functions were related to the ability to form a depot which prolonged antigen exposure to APCs. However, efficient adjuvants also stimulate the immune system by direct interaction with APCs. The nature of immune adjuvants is large and heterogeneous. Adjuvants are divided into immunostimulants and delivery systems. Immunostimulants interact with specific receptors, like TLRs and others, while delivery systems increase the immune response by multiple mechanisms, depending on their particular characteristics [Leroux-Roels, 2010, Alving et al., 2012]. Thus, modern vaccines comprise adjuvants such as pathogen-derived subcellular components, recombinant proteins, peptides and nucleic acid sequences [Zepp, 2010, Perez et al., 2013, Reed et al., 2013]. In addition, due to better knowledge of the immune system and improvements in formulation technology, effective therapeutic cancer vaccines are developed [Joshi et al., 2012]. Today’s challenges in vaccine development are linked to complex pathogens (e.g. malaria, tuberculosis, HIV) and to antigens susceptible to genetic mutations (e.g. influenza) as well as to subjects with a compromised or dysfunctional immune system [Leroux-Roels, 2010].

Nanoparticulate carriers provide adjuvant activity by enhancing antigen delivery or by activating innate immune responses. Strength and mechanisms of immunostimulation induced by nanocarrier vaccines depend on various factors, such as chemical composition, particle size and homogeneity, charge, nature and location of antigens and/or adjuvants within the carrier and, last but not least, the site of administration (see Fig. 2) [Watson et al., 2012, Brito et al., 2013, Gregory et al., 2013, Smith et al., 2013, Zaman et al., 2013].

Liposomes: ideal carriers for antigens and/or adjuvants
The ability of liposomes to induce immune responses to incorporated or associated antigens was first reported by Gregoriadis and Allison [Allison et al., 1974, Allison et al., 1976]. Since then, liposomes and liposome-derived nanovesicles such as archaeosomes and virosomes have become important carrier systems and the interest for liposome-based vaccines has markedly increased. The field of liposomes and liposome-based vaccines is vast. Therefore, this review concentrates on recent reports highlighting the most studied antigens and
adjuvants in pertinent examples of vaccines, including summaries of veterinary and experimental therapeutic cancer vaccines. Other nanoparticulate vaccines based on lipoplexes, niosomes, virus-like particles, solid lipid nanoparticles and nanoemulsions are not covered in this review.

A key advantage of liposomes, archaeosomes and virosomes in general, and liposome-based delivery systems in particular, is their versatility and plasticity (see Table 1). Liposome composition and preparation can be chosen to achieve desired features such as lipid composition, charge, size, size distribution, entrapment and location of antigens or adjuvants. Depending on the chemical properties, water soluble compounds (proteins, peptides, nucleic acids, carbohydrates, haptens) are entrapped within the aqueous inner space, whereas lipophilic compounds (lipopeptides, antigens, adjuvants, linker molecules) are intercalated into the lipid bilayer and antigens can be attached to the liposome surface either by adsorption or stable chemical linking [Torchilin, 2005, Watson et al., 2012]. Co-formulations containing different types of antigens and/or adjuvants can be combined to tailor liposomal vaccines for individual applications (see Figure 2).

Liposome-based antigens

Torchilin, 2005, Watson et al., 2012

As the majority of vaccines are administered by intramuscular or subcutaneous injection, liposome properties play a major role in local tissue distribution, retention, trafficking, uptake and processing by APCs. Earlier studies showed clear size dependent, but not unambiguous charge or lipid composition dependent effects at the injection site [Oussoren et al., 1997]. Newer studies with the cationic liposome formulation dimethyl-dioctadecylammonium (DDA) plus trehalose-dibehenate (TDB) (DDA/TDB, CAF01) showed no differences in liposome draining or antigen release from the injection site. However, differences in movement to regional lymph nodes (LN) were noted [Henriksen-Lacey et al., 2010, Henriksen-Lacey et al., 2011]. A cationic liposome pDNA vaccine of 500 nm and 140 nm size with encapsulated ovalbumin (OVA) encoding pDNA as antigen showed strongest retention at large vesicle size. Addition of poly(ethyleneglycol) (peg) coating resulted in enhanced lymphatic drainage, without improved immune response [Carstens et al., 2011]. Other pegylated DDA/TDB-liposomes reduced the depot effect and altered the immune response confirming these results [Kaur et al., 2012]. Badiee et al. evaluated liposomes of
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6 different sizes containing the surface glycoprotein of *Leishmania* (rgp63). Immunization with small liposomes induced a T\(_H\)2 response, whereas large liposomes induced a T\(_H\)1 response, higher IFN-\(\gamma\) levels and IgG2a/IgG1 ratios [Badiee *et al.*, 2012]. Adjuvant effects of neutral, positive or negative liposomes were evaluated when admixed with OVA, cationized OVA (cOVA) or *Bacillus anthracis* antigen by Yanasarn *et al.* Immunization with OVA admixed with different liposomes generated different antibody responses. Interestingly, OVA admixed with negative 1,2-dioleyl-sn-glycero-3-phosphatidic acid (DOPA) liposomes was as immunogenic as OVA admixed with positive 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP) liposomes. The cOVA antigen showed comparable adjuvant activities in all liposomes [Yanasarn *et al.*, 2011]. Neutral phosphatidylcholine (PC)/cholesterol small unilamellar vesicles (SUV) proved also to be effective vaccine carriers. We evaluated a vaccine with peptides derived from the glycoprotein of the lymphocytic choriomeningitis virus (LCMV). Liposome-encapsulated peptides were highly immunogenic and elicited protective antiviral immunity by *in vivo* antigen loading of DCs. Encapsulated cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpGs) further enhanced immune activation [Ludewig *et al.*, 2000]. We also used the vaccine to prime a CD8\(^{+}\) T cell response against 10 different hepatitis C virus (HCV) epitopes resulting in strong CTL responses. Challenge experiments with *Vaccinia* virus expressing HCV epitopes emphasized the utility of neutral liposomes as HCV vaccine [Engler *et al.*, 2004, Schwendener *et al.*, 2010]. Moon *et al.* describe novel interbilayer-crosslinked multilamellar vesicles (MLV) formed by crosslinking adjacent lipid bilayers within MLVs. These vesicles entrapped protein antigens in their core and lipid-based immunostimulatory molecules in the bilayers, forming a potent vaccine, eliciting strong T-cell and antibody responses [Moon *et al.*, 2011].

Investigation of hemagglutinin (HA) adsorption versus encapsulation and co-encapsulation of CpGs in 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-chol) liposomes showed that adsorbed HA was more immunogenic than encapsulated HA. Cholesterol enhanced the adjuvant effect and CpG-loaded liposomes were highly efficient at enhancing HA-specific humoral responses [Barnier Quer *et al.*, 2012, Barnier-Quer *et al.*, 2013]. Covalent attachment of protein antigens to nanocarriers can disrupt protein structure and mask epitopes, altering the antibody response. Watson *et al.* used metal chelation via nitrilotriacetic acid (NTA) to attach antigens to liposomes. OVA and a HIV-1 gp41 (N-MPR) peptide were attached via NTA or covalent linkage. Attachment of N-MPR, but not OVA,
elicited stronger antibody responses than antigen admixed with liposomes and covalent attachment was superior to NTA-anchored antigens [Watson et al., 2011]. Mannose receptors (MR) expressed on macrophages and APCs mediate endocytosis and cooperate in antigen capture and presentation. MR recognize carbohydrate moieties of many pathogens. Thus, targeting of glycosylated antigens or carrier systems to MR is a method to develop vaccines [Irache et al., 2008]. To establish a human papilloma virus 16 (HPV16) cancer vaccine, Mizuuchi et al. generated oligomannose-liposomes containing HPV16-E6 plasmid antigens (OML-HPV). HPV16-E6-specific CTLs were generated from HPV16-positive cervical carcinoma patients with OML-HPV, but not with standard liposomes [Mizuuchi et al., 2012]. OMLs in combination with entrapped dsRNA to induce anti-human parainfluenza virus-3 (HPIV3) immunity were studied by Senchi et al. [Senchi et al., 2013]. Hemagglutinin-neuraminidase (HN) antigen was co-encapsulated with adjuvant poly(I:C) into OMLs. Systemic and mucosal immune responses were generated and immune sera suppressed viral infection in vitro. Finally, Li et al. constructed a mannosylated liposome/protamine/DNA (Man-LPD) vaccine. Man-LPD exhibited higher intracellular uptake and transfection in vitro and induction of co-stimulatory molecules on BMDCs [Li et al., 2013].

**Peptides and proteins as antigens**

The antigen location in liposomes influences immunogenicity. Both, entrapped or surface-attached antigens induce T cell responses, the latter having advantages of availability for antibody or B cell recognition, whereas encapsulated antigens require vesicle disruption to be accessible. The necessity of CD4\(^+\) T cells to induce memory CD8\(^+\) T cells was investigated in mice immunized with liposome surface-coupled OVA peptides.CTL responses were induced and confirmed in mice lacking CD4\(^+\) T cells, suggesting that CD4\(^+\) T cells were not required for memory CD8\(^+\) T cell generation [Taneichi et al., 2010]. Phosphatidylserine (PS)-liposome conjugated antigens were efficiently captured by APCs resulting in T\(_H\) cell stimulation, validating PS as adjuvant for peptide vaccines [Ichihashi et al., 2013]. Takagi et al. coupled several HCV peptides to liposomes. One D\(^{b}\)-restricted and three HLA-A(*)0201-restricted peptides conferred complete protection to immunized mice and long-term memory [Takagi et al., 2013].
Liposome-encapsulated protein antigens have been used frequently in earlier work. More recently, Nagill et al. compared encapsulated 78kDa antigen of *Leishmania donovani* with antigen plus monophosphoryl lipid A (MPLA) resulting in decreased parasite burden after challenge [Nagill et al., 2010]. In another study Bal et al. co-encapsulated OVA and the TLR ligand Pam$_3$CysSK$_4$ or CpGs in DOTAP-liposomes. Encapsulation of both ligands did not obstruct activation of TLR-transfected cells and OVA/CpG-liposomes shifted the IgG1/IgG2a balance to IgG2a, whereas Pam$_3$CysSK$_4$ was less efficient [Bal et al., 2011]. Hepatitis B surface antigen (HBsAg) encapsulated liposomes coupled with *Ulex europaeus* agglutinin-1 were developed by Gupta et al. Lectinized liposomes were predominantly targeted to M-cells on intestinal Peyer’s patches after oral immunization, yielding high antibody titers in mucosal secretions [Gupta et al., 2011]. Another mucosal vaccine was described by Figueiredo et al. who encapsulated *Streptococcus equi* antigens in PC/cholesterol/stearylamine-liposomes or chitosan nanoparticles. Intranasal immunization of mice elicited mucosal, humoral and cellular responses with higher sIgA levels of the chitosan nanoparticles, due to enhanced mucoadhesive properties [Figueiredo et al., 2012]. Liposomes modified with pH-sensitive 3-methyl-glutarylated hyperbranched poly(glycidol) (MGlu-HPG) were used to encapsulate OVA. MGlu-HPG-liposomes induced a strong immune response which was suppressed with anti-MHC-I/MHC-II antibodies [Hebishima et al., 2012]. Ding et al. developed so called RAFTsomes by isolating membrane microdomains containing MHC-I and I-A$_b$ restricted epitopes from OVA primed DCs and reconstituted them on liposome surfaces. RAFTsome immunization gave high anti-OVA IgG1 levels and protection against OVA expressing EG.7 tumor challenge [Ding et al., 2013].

**Liposomal DNA vaccines**

Nucleic acid vaccines are an alternative to attenuated bacterial antigens or protein or peptide vaccines. MLVs as inexpensive carriers were used by Rodriguez et al. to deliver DNA to mice with plasmids encoding bovine herpesvirus type-1. Vaccinated mice developed specific IgG responses [Rodriguez et al., 2013]. The M1 gene of influenza A virus was used by Liu et al. to construct a cationic liposome/DNA vaccine with a M1-encoding plasmid for oral vaccination, resulting in M1 gene expression in intestines of vaccinated mice and strong immune responses and protection against challenge infection [Liu et al., 2013]. Liposomes were also used to deliver plasmid DNA encoding hsp65 to treat the pulmonary fungal...
infection *Paracoccidiomycosis* resulting in protective immune response and reduced fungal burden [Ribeiro *et al.*, 2013]. Amidi *et al.* proposed liposomes as artificial microbes that can be programmed to produce specific antigens for vaccination. A bacterial transcription and translation system together with a gene construct encoding β-galactosidase or a luciferase-NP fusion epitope as antigens were entrapped in liposomes. Vaccination of mice showed that such antigen-producing liposomes elicited higher specific immune responses against the produced antigen than control vaccines [Amidi *et al.*, 2011, Amidi *et al.*, 2012].

**Liposomal messenger RNA vaccines**

The immune system is naturally activated by foreign nucleic acids by inducing specific immune responses. Lack of persistence, genome integration and auto-antibody induction are advantages of mRNA and siRNA vaccines. Currently, mRNA vaccines are developed to treat various diseases including cancers. Pichon *et al.* loaded mannosylated nanoparticles with mRNA encoding a melanoma antigen [Pichon *et al.*, 2013]. The mRNA was formulated with histidylated liposomes promoting endosome destabilization, allowing cytosolic nucleic acid delivery which enhanced anti-B16F10 melanoma vaccination in mice. A liposome encapsulated double-stranded RNA (LE-PolyICLC) was tested in the influenza (H5N1-HPIV) model by Li *et al.* Intranasal LE-PolyICLC inhibited virus replication, reduced viral titers, increased survival of infected mice and attenuated pulmonary fibrosis [Li *et al.*, 2011].

**The MUC-1 (BLP25) antigen**

The MUC1 glycoprotein is often overexpressed and hypoglycosylated in tumor cells of cancers making it an attractive target for immunotherapy (for other examples see Tab. 2) [Acres *et al.*, 2005, Roulois *et al.*, 2013]. MUC1 variable number tandem repeats (VNTRs) conjugated to tumor-associated carbohydrate antigens (TACAs) break self-tolerance in humanized MUC1 transgenic mice. Sarkar *et al.* formulated an anti-cancer vaccine composed of a MUC1 glycopeptide containing a GalNAc-O-Thr (Tn) TACA conjugated to a TLR ligand. Additional surface-displayed l-rhamnose (Rha) epitopes were included in 1,2-dipalmitoyl-sn-glycero-3-phosphatidyl-choline (DPPC) liposomes. Mice were immunized with a Rha-Ficoll conjugate, followed by the vaccine resulting in a >8-fold increase in anti-MUC1-Tn, anti-Tn antibody titers and increased T cell proliferation [Sarkar *et al.*, 2013]. Another liposome
vaccine containing the immunoadjuvant Pam₃CysSK₄, a Th peptide epitope and a glycosylated MUC1 peptide was reported by Lakshminarayanan et al. Covalent surface-linkage of all three components was essential for maximum efficacy [Lakshminarayanan et al., 2012]. The BLP25 liposome (L-BLP25) vaccine which targets MUC1 extended survival of patients with non-small cell lung cancer (NSCLC) and showed promise in prostate cancer [North et al., 2005, North et al., 2006]. Butts et al. conducted phase II/IIB studies to evaluate L-BLP25 in patients with stage IIIA/IIIB NSCLC. Patients received either L-BLP25 plus best supportive care (BSC) or BSC alone. Survival time and rates were longer in patients receiving the combination compared to BSC alone [Butts et al., 2010, Butts et al., 2011]. Wu et al. conduct an ongoing L-BLP25 study (INSPIRE) in NSCLC patients of East-Asian ethnicity which is the first large therapeutic cancer vaccine study in an East-Asian population [Wu et al., 2011]. Accordingly, a L-BLP25 study was conducted in Japanese NSCLC patients showing consistency with studies of Caucasian patients [Ohyanagi et al., 2011].

**Liposomes as carriers for adjuvants**

*Liposomal DNA as adjuvant*

CpGs are adjuvants composed of un-methylated CpG dinucleotide sequences similar to those found in bacterial DNA. They trigger TLR9, activate DC maturation, increase antigen expression and induce Th1 immune responses [Shirota et al., 2014]. Antigens and CpGs must be co-localized in one APC to generate optimal immune responses [Krishnamachari et al., 2009]. CpG-encapsulation in liposomes of different properties altered antigen encapsulation efficiency, release and delivery rates, thus influencing the immune response. OVA/CpG co-encapsulation augmented Th1 and cell mediated immune response [Erikci et al., 2011]. Co-encapsulation of OVA and Pam₃CysSK₄ and/or CpGs in cationic liposomes shifted the IgG1/IgG2a balance to IgG2a, showing that antigen/adjuvant co-encapsulation shapes the type of immune response [Bal et al., 2011]. Nuclease-resistant phosphorothioate CpGs (PS-CpGs) or sensitive phosphodiester CpGs (PO-CpGs) were used by Shargh et al. in a *Leishmaniasis* model. PO-CpGs or PS-CpGs were encapsulated in DOTAP-liposomes for protection against nuclease degradation. Mice immunized with liposomal soluble *Leishmania* antigens (SLA) co-incorporated with PO-CpGs or PS-CpGs showed no significant difference in immune response. Thus, nuclease-sensitive PO-CpGs can be used as adjuvants [Shargh et al.,
Finally, CpGs incorporated in cationic DOTAP-liposomes but not in neutral 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) liposomes provided complete protection against challenge with *Burkholderia pseudomallei* in a mouse model [Puangpetch et al., 2012].

**Cationic liposome adjuvant vaccines**

The introduction of positively charged compounds is a common method used to alter liposome properties. Cationic liposomes are frequently used as cell transfection reagents and vaccine adjuvants. Most cationic lipids form bilayer liposomes but often additional lipids are needed. The high surface density of positive charges increases liposome adsorption on negatively charged cell surfaces. Cationic liposomes penetrate into cells through specific mechanisms and activate different cellular pathways depending on cell type, cationic lipid nature, but also on formulation types and liposome size [Korsholm et al., 2012, Lonez et al., 2012].

**The cationic adjuvant CAF01**

CAF01 is a novel adjuvant composed of the synthetic immunostimulating mycobacterial cordfactor glycolipid TDB and the cationic membrane forming molecule DDA. TDB induces strong Th1 and Th17 immune responses and the C-type lectin Mincle is the receptor for APC activation. The adjuvant effect also requires MyD88 and Schweneker et al. identified the Nlrp3 inflammasome as mediator for TDB triggered induction of immune response [Werninghaus et al., 2009, Desel et al., 2013, Schweneker et al., 2013]. Properties of cationic liposome forming lipids were studied with rigid DDA- or fluid dimethyldioleoylammonium (DODA) liposomes. When the antigen Ag85B-ESAT-6 was mixed with DDA/TDB or DODA/TDB liposomes, DDA-liposomes formed a depot resulting in continuous activation of APCs, whereas DODA-liposomes were rapidly cleared [Christensen et al., 2012]. Milicic et al. explored modifications of DDA/TDB-liposomes such as size, antigen association and addition of TLR agonists to assess their activity using OVA as antigen. SUV without TLR agonists showed higher antigen-specific antibody responses than MLVs. Addition of TLR3 and TLR9 agonists increased the adjuvant effects of MLVs but not of SUVs. The ability of DDA/TDB-SUVs to induce CD8+ CTL responses without immunostimulators could avoid safety risks.
associated with TLR agonists [Milicic et al., 2012]. Yu et al. tested several adjuvants, including DDA-monophosphoryl lipid A (DDA-MPLA), DDA-TDB (CAF01) and DDA-monomycyl glycerol (DDA-MMG, CAF04). *Chlamydia* antigens were used in a mouse genital tract infection model. DDA-MPLA and DDA-TDB elicited the best protective immune responses, characterized by CD4+ T cells co-expressing IFN-γ and TNF-α and by significantly reduced infection [Yu et al., 2012]. Ingvarsson et al. studied the parameters of CAF01 spray dried powder formulations using lactose, mannitol or trehalose as stabilizers. Immunization of mice with the tuberculosis antigen H56 demonstrated that spray drying with trehalose resulted in best preservation of adjuvant activity [Ingvarsson et al., 2011, Ingvarsson et al., 2013]. Lindenstrom et al. showed that CAF01 vaccination in mice led to establishment of T\(_H\)17 memory cells by retaining phenotypic and functional properties for 2 years. Challenge with *Mycobacterium tuberculosis* 2 years later induced T\(_H\)17 memory cells at levels comparable to T\(_H\)1 memory cells [Lindenstrom et al., 2012]. A trivalent influenza vaccine (TIV) with CAF01 enhanced the immune response determined by HA inhibition and antibody titers, promoting strong T\(_H\)1 responses. Maintenance of the T\(_H\)1/T\(_H\)17 cytokine profile over 20 weeks resulted in complete survival of H1N1 challenged mice [Rosenkrands et al., 2011]. A commercially available TIV was compared with the same vaccine mixed with CAF01 in ferrets. CAF01 induced increased influenza-specific IgA and IgG levels and promoted immunity and protection against challenge with H1N1. [Martel et al., 2011]. The combination of cationic liposomes and immunopotentiators such as MPL with DDA/TDB-liposomes was tested in mice using OVA as antigen. DDA/TDB/MPL-liposomes induced antigen-specific CD8+ T-cell and humoral responses [Nordly et al., 2011]. CAF01 was also used in a phase I trial with a therapeutic HIV-1 peptide vaccine. Safety and immunogenicity were assessed in untreated HIV-1-infected individuals. Vaccine-specific T cell responses were induced in 6/14 individuals, showing that therapeutic immunization with CAF01-adjuvanted HIV-1 peptide in humans is feasible [Roman et al., 2013]. In another clinical trial the potential of inducing T-cell immunity during chronic HIV-1 infection was investigated. Treatment-naive HIV-1-infected individuals were immunized with peptides/CAF01. Specific CD4+ and CD8+ T-cell responses were induced in all individuals [Karlsson et al., 2013]. Kamath et al. reported that physical linkage between antigens and immunomodulators is required to elicit T\(_H\)1/T\(_H\)17 responses. Separate same-site administration of a mycobacterial fusion antigen and CAF01 failed to elicit T\(_H\)1/T\(_H\)17 responses. Tracking experiments showed that
separate same-site administration elicited an early antigen-positive/adjuvant-negative DC population. Antigen targeting of LN DCs prior to their activation generated non-activated antigen-pulsed DCs that recruited antigen-specific T cells and triggered proliferation, but interfered with TH1 induction in a dose-dependent manner. Thus, synchronization of DC targeting and activation is a critical determinant for TH1/TH17 adjuvanticity [Kamath et al., 2012].

In summary, CAF01 adjuvant liposomes prove to be a valuable vaccine formulation for different antigens.

**CLDC adjuvant liposomes**

Another widely studied cationic liposome complex contains the cationic lipid 1-[2-(oleoyloxy)-ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium-chloride (DOTIM) and cholesterol. Cationic liposome-DNA complexes (CLDC) are prepared by mixing liposomes with DNA. CLDC (JVRS-100, Juvaris BioTherapeutics, Burlingame, CA) is a lyophilized powder composed of selected plasmid DNA complexed with liposomes. CLDCs facilitate APC uptake, activate TLRs and IFN production and stimulate the adaptive immune response. Several CLDC vaccines have been tested in various models. Gowen et al. analyzed liposomal delivery and CpG content of plasmid DNA with CLDCs. CpG-free or CpG-containing plasmids with and without liposomes, as well as poly(I:C) were evaluated to elicit protection against lethal Punta Toro virus (PTV) challenge in hamsters. CLDC-containing CpG-plasmid significantly improved survival, decreased viral loads and reduced liver damage. [Gowen et al., 2009]. CLDC enhanced anti-SIV immune responses induced by SIV vaccines. CLDC immunized rhesus macaques developed stronger SIV-specific T and B cell responses compared to controls, resulting in persistence and better memory responses [Fairman et al., 2009]. As no vaccines are available for common herpes simplex virus (HSV) infections CLDCs were evaluated for a HSV gD2 vaccine in a genital herpes guinea pig model. The CLDC/gD2 vaccine significantly decreased duration of acute and recurrent disease compared to gD2 alone. However, when evaluated as therapeutic vaccines they were ineffective, suggesting that such HSV-2 vaccines need improvement [Bernstein et al., 2010, Bernstein et al., 2011]. The protective effects of CLDCs against encephalitic arboviral infection were investigated in a Western equine encephalitis virus (WEEV) model. CLDC vaccinated mice were challenged with virulent WEEV. CLDC pre-treatment provided increased survival and higher cytokine levels, strong TH1
activation and protective immunity against lethal WEEF [Logue et al., 2010]. An influenza A virus vaccine adjuvanted with CLDC or alum was tested by Hong et al. CLDC induced more robust adaptive immune responses with higher levels of virus-specific IgG2a/c and CD4+ and CD8+ T cells plus cross-protection from lethal viral challenges. [Hong et al., 2010]. In another influenza A vaccine study, Dong et al. showed that addition of CLDC (JVRS-100) to a H5N1 split vaccine induced higher virus-specific responses than adjuvant-free formulations. CLDC vaccinated mice challenged with H5N1 had mild illness, very low viral titers, 100% survival and long lasting protective immunity [Dong et al., 2012]. Strategies to improve influenza vaccine efficacy in elderly individuals are needed. Thus, Carroll et al. determined whether CLDC (JVRS-100) could improve the efficacy of the influenza vaccine Fluzone in elderly rhesus macaques. Vaccination with Fluzone with or without CLDC and challenge with human H1N1 influenza virus showed that only the Fluzone/CLDC-vaccinated animals had lower virus replication. Thus, CLDC enhances immunogenicity and efficacy of a licensed vaccine in immunosenescent monkeys [Lay et al., 2009, Carroll et al., 2014]. CLDC (JVRS-100) was also evaluated as adjuvant for HBsAg in mice expressing hepatitis B virus (HBV). HBsAg+JVRS-100 elicited T and B cell responses, whereas HBsAg elicited only a B cell response. However, the response by HBsAg+JVRS-100 was not sufficient to cause destruction of infected liver cells, but it suppressed HBV DNA non-cytolytically [Morrey et al., 2011]. Similar results were obtained using the woodchuck model of HBV. HBV infection induced T cell responses to WHsAg and selected WHs peptides and expression of CD8+ CTL and T H1 cytokines. WHsAg plus CLDC elicited antibodies earlier, in more woodchucks and with higher titers than WHsAg and alum. [Cote et al., 2009]. There is a need for mucosal vaccines for pulmonary Yersinia pestis infections. The ability of an oral CLDC adjuvanted vaccine against lethal pneumonic plague was investigated by Jones et al. Oral immunization with Y. pestis F1 antigen combined with CLDC produced high titers of anti-F1 antibodies and long lasting CD4+ T cell dependent protection from lethal pulmonary challenge with Y. pestis [Jones et al., 2010].

Other cationic lipid complexes
Several other cationic lipid adjuvant complexes were evaluated in various vaccine models. Phillips et al. tested an alphavirus vaccine comprised of cationic lipid nucleic acid complexes (CLNCs) and the ectodomain (E1ecto) of WEEV. Interestingly, CLNC alone had therapeutic efficacy, as it increased survival of mice following lethal WEEV infection. Immunization with
the CLNC/WEEV/E1ecto mixture provided full protection after challenge. Passive serum transfer from immunized to naïve mice conferred protection to challenge, indicating that antibody is sufficient for protection. [Phillips et al., 2014]. Liposomes containing different cationic compounds and neutral DPPC were loaded with influenza HA by adsorption. DC-chol/DPPC-liposomes with a high amount of DC-chol had stronger immunogenicity compared to less DC-chol and elicited higher antibody titers compared to the other compounds and non-adjuvanted HA. Liposome-adsorbed HA was more immunogenic than encapsulated HA and incorporation of cholesterol in DC-chol-liposomes as well as CpGs enhanced adjuvancy [Barnier-Quer et al., 2013]. A similar study by Ma et al. showed that DOTAP/DOPC-liposome regulated immune responses relied on surface charge density and might occur through reactive oxygen species (ROS) signaling. High charge density liposomes potentlly enhanced DC maturation, ROS generation, antigen uptake and production of IgG2a and IFN-γ, whereas low-charge density liposomes failed to promote immune responses [Ma et al., 2011]. Lipid assemblies composed of a polycationic sphingolipid (ceramide carbamoylspermine, CCS) are effective adjuvants/carriers for several vaccines when complexed with cholesterol (CCS/C, VaxiSome). Ferrets immunized intranasally with CCS/C-influenza vaccine produced higher HI antibody titers compared to controls. Following viral challenge the vaccine reduced the severity of infection. Biodistribution studies showed that lipids and antigens are retained in nose and lung, increasing cytokine levels and expression of co-stimulatory molecules [Even-Or et al., 2011]. Chen et al. developed a cationic lipopolymer, the liposome-polyethyleneglycol-polyethyleneimine complex (LPPC) adjuvant for surface-adsorption of antigens or immunomodulators. LPPC enhanced presentation on APCs, surface marker expression, cytokine release and activated Th1-immunity. With LPS or CpGs, LPPC dramatically enhanced the IgA or IgG2A proportion of total Ig, demonstrating host immunity modulation [Chen et al., 2012]. Effects of pegylation of cationic DOTAP liposome vaccines on LN targeting and immunogenicity were studied by Zhuang et al. Peg-DOTAP-liposomes accelerated drainage into LNs, prolonged retention and APC uptake, increased anti-OVA antibody responses and modulated their biodistribution which improved vaccine efficiency [Zhuang et al., 2012]. The activity of cationic vaccines can be hampered by immobilization in the extracellular matrix caused by electrostatic interactions. Thus, Van den Berg et al. found that surface-shielding of DOTAP-liposomes by pegylation improved antigen expression drastically. Mice vaccinated with pegylated pVAX/Luc-NP antigen containing liposomes
elicited T cell responses comparable to naked DNA, suggesting that charge shielding improves dermally applied vaccines [Van Den Berg et al., 2010].

Other adjuvants

Muramyl dipeptide (MDP)

MDP originates from a bacterial peptidoglycan cell wall fragment and is responsible for the activity of Freund's complete adjuvant (FCA). After phagocytosis by APCs, MDP is detected by the NOD2 receptor that activates the immune response. Numerous MDP derivatives have been synthesized to evaluate their immunostimulatory effects and adjuvant activity [Traub et al., 2006, Ogawa et al., 2011]. It was early recognized that liposomes were ideal carriers for MDP and its derivatives [Alving, 1991]. For example, the lipophilic MDP analogs MDP-PE and MDP-glycerol-dipalmitate (MDP-GDP) were added to liposomal HBsAg formulations both of which induced higher antibody titers, TH1 response and IFN-γ levels [Jain et al., 2009]. Masek et al. used small Ni-chelating liposomes to attach His-tagged Candida albicans heat shock protein (hsp90-CA) as antigen and to co-incorporate the MDP derivative C18-O-6-norAbuMDP as adjuvant. The immune response was of TH1 and TH2 type, comparable to FCA, but without side effects [Masek et al., 2011]. Liposomes formulated as MDP with 1,2-dipalmitoyl-sn-glycero-3-phosphatidyl-ethanolamine (DPPE) are called Mifamurtide which is an adjuvant to standard chemotherapy for osteosarcoma. A study by Anderson et al. in patients with metastatic osteosarcoma showed that Mifamurtide had a manageable safety profile but that a randomized clinical trial is required to further determine its utility [Anderson et al., 2014].

Monophosphoryl lipid A (MPLA)

Monophosphoryl lipid A (MPLA) is the active moiety of the bacterial endotoxin lipopolysaccharide (LPS). TLR4 is the receptor for LPS forming a complex with MD2, representing the main LPS binding component. LPS supports the development of diverse TH cell types, depending on the tissue microenvironment. For instance, peripheral immunization with LPS drives TH1 priming in lymphoid tissue and TH17 priming in the intestinal mucosa [McAleer et al., 2010]. Several lipopeptides derived from microbial origin convey self-adjuvanting activity by TLR2 signaling recognizing many PAMPs [Kawai et al.,
MPLA-liposomes have considerable potency and safety with a variety of candidate vaccines, including malaria, HIV-1 and several types of cancer [Alving et al., 2012, Zaman et al., 2013]. Combination of MPLA with the saponin QS-21 and immunostimulants in various adjuvant formulations forms the basis of Adjuvant Systems (AS, GlaxoSmithKline-Biologicals) to promote protective immune responses. MPLA and aluminum salts are present in AS04, and both MPLA and QS-21 are present in AS01 and AS02 which are liposome- and emulsion-based formulations [Garcon et al., 2011]. Rizwan et al. analyzed the immunological activity of cubosomes containing MPLA and imiquimod. Cubosomes, a novel variation of MLVs, are composed of highly twisted lipid bilayers and non-intersecting water channels, providing increased encapsulation rates of lipophilic compounds. In MPLA-cubosomes sustained release of OVA was observed with induction of OVA-specific antibodies and CD8+ and CD4+ T cell proliferation at higher efficiency than liposomes plus adjuvants and alum [Rizwan et al., 2013]. Immune response and protection induced by liposomal soluble leishmanial antigen (SLA) formulated with lipid A-trehalose dicorynomycolate (MPLA-TDM) was evaluated by Ravindran et al. This vaccine induced strong and long lasting protection against experimental visceral Leishmaniasis [Ravindran et al., 2012]. An influenza vaccine with MPLA was developed by Adler-Moore et al. using the ectodomain of the M2e channel protein as a universal vaccine candidate. A liposomal M2e vaccine elicited anti-M2e antibodies that inhibited viral cell lysis and conferred complete protection to mice challenged with H1N1. Lymphocyte depletion markedly decreased protection, suggesting a primarily Th2 mediated immune response [Adler-Moore et al., 2011].

Listeriolysin

Cytolysins are virulence factors of various pathogenic bacteria. They form pores in target cell membranes, degrade membrane lipids or solubilize cell membranes. Bacteria use cytolysins to either inhibit functions of host immune cells or to gain access to intracellular niches. The bacterium Listeria monocytogenes can escape host immune defenses by lysis of the phagosomal membrane by use of listeriolysin O (LLO). LLO is used as vaccine adjuvant to provide cytosolic access for antigens in APCs [Dietrich et al., 2001]. LLO based vaccines were reported by Mandal et al. who prepared OVA/LLO-liposomes. OVA immunization resulted in higher CTL activity and high IFN-γ production. The vaccine also conferred protection to mice from lethal challenges with antigen-expressing tumor cells [Mandal et al., 2002]. LLO-
liposomes were also used to deliver the LCMV nucleoprotein (NP) to stimulate a NP-specific CTL response. Immunized mice generated high frequencies of NP-specific CD8\(^+\) T cells and full protection against a lethal intracerebral challenge with virulent LCMV [Mandal et al., 2004]. An anionic liposome-polycation-DNA complex combined with LLO was used as vaccine by Sun et al. to deliver OVA-cDNA. This formulation produced an enhanced CD8\(^+\) T cell response, higher CTL frequency and IFN-γ production after stimulation by an OVA-specific peptide [Sun et al., 2010]. Andrews et al. analyzed whether encapsulating CpGs in LLO-liposomes would enhance cell-mediated immune response and skew T\(_{H1}\)-type responses in a protein antigen-based vaccine utilizing LLO-liposomes. Co-encapsulation of CpGs in LLO-liposomes activated the NFκB pathway, maintaining cytosolic delivery of antigen mediated by co-encapsulated LLO. Immunization with OVA and CpG-LLO-liposomes showed enhanced T\(_{H1}\) immune responses. [Andrews et al., 2012].

Currently, 26 clinical trials are registered at clinicaltrials.org, a service of the U.S. NIH (see: clinicaltrials.org with the search terms liposome AND vaccine).

**Veterinary vaccines**

Knowledge of molecular details of immune mechanisms is relatively scarce for veterinary and pet animals and special concerns regarding the use of vaccine adjuvants must be considered. Demands like compatibility to human consumption, animal production, costs, challenges met by different species, vaccine administration for large numbers of animals and others must be evaluated [Heegaard et al., 2011, Underwood et al., 2012]. Table 2 summarizes some of the most recent experimental studies of liposome-based veterinary vaccines.

**Therapeutic cancer vaccines**

Although most cancers modify host proteins that can function as antigens the development of effective vaccines against such antigens is hampered by the weak immune response and the immunosuppressive effects induced by cancers. Tumor-associated antigens include viral proteins (e.g. HPV), chromosomal translocation products (e.g. bcr/abl), over-expressed
proteins like HER2/neu, telomerase, MUC-1 and others [Kozako et al., 2012]. In Table 3 some recent examples of experimental liposome-based cancer vaccines are listed.

**Archaeosomes**

Archaeabacteria (Archaea) were discovered and classified by Woese and Fox as new group of prokaryotes, besides the Eubacteria (Bacteria) [Woese et al., 1977]. Archaea contain DNA-dependent RNA polymerases and proteinaceous cell walls that lack peptidoglycan. Their cell membranes are composed of L-glycerol ether lipids with isoprenoid chains instead of D-glycerol ester lipids with fatty acid chains [Spang et al., 2013]. Archaeal lipids are uniquely constituted of ether-linked isoprenoid phytanyl-archaeol (diether) and/or caldarchaeol (tetraether) cores conferring high membrane stability. Archaeosomes are liposomes prepared with archaeal glycerolipids. The head groups displayed on the glycerol-lipid cores of archaeosomes interact with APCs and induce Th1, Th2 and CD8+ T cell responses to the entrapped antigen. The immune responses are persistent and subject to strong memory responses [Krishnan et al., 2008, Benvegnu et al., 2009]. The polar lipid from the archaeon, *Methanobrevibacter smithii* has been well characterized for its adjuvant potential. It contains archaetidyl-serine, promoting interaction with a PS receptor on APCs. These archaeosomes mediate MHC-I cross-priming and promote co-stimulation by APCs without inflammatory cytokine production. [Krishnan et al., 2000]. Patel et al. showed that archaeosomes prepared from *Methanobrevibacter smithii* lipids were suitable adjuvants for multivalent mucosal vaccines. Archaeosomes containing the encapsulated antigens OVA, bovine serum albumin and hen egg lysozyme conferred strong and sustained specific antibody responses to all three antigens [Patel et al., 2004]. Intranasal immunization of mice with the archaeal lipid mucosal vaccine adjuvant and delivery (AMVAD) system, obtained by interaction of archaeosomes/antigens with multivalent cations induced robust mucosal antigen-specific IgA responses. AMVAD formulations are stable, safe and show protective efficacy in murine models of infection/challenge [Patel et al., 2010]. Archaeosomes prepared from lipids of the non-pathogenic bacteria *Leptospira biflexa* (leptosomes) and *Mycobacterium smegmatis* (smegmosomes) were used as adjuvants. Both vesicles caused strong APC activation, cytokine release and expression of co-stimulatory signals which was significantly higher for smegmosomes compared to leptosomes. APC activation by both formulations induced immune responses in mice to entrapped OVA [Faisal et al., 2009, Faisal et al., 2011]. Borrero et al. studied immune response and cross reactivity against
Mycobacterium tuberculosis (MTB) with archaeosomes containing a mixture of cell wall glycolipids such as phosphatidylinositol mannosides of *M. smegmatis* (Ms) and DSPC/cholesterol. Ms containing liposomes induced a specific IgG response and recognition of MTB surface antigens, showing that immunogenic Ms-glycolipids could enhance subunit vaccines against tuberculosis [Borrero et al., 2013]. The relation between archaeal lipid structures and their activity was explored by synthesizing novel head groups linked to archaeol. Archaeosomes consisting of various combinations of synthesized lipids with entrapped OVA antigen were used to immunize mice. Addition of the glycolipids gentiotauroyl-archaeol, mannotriosyl-archaeol or maltotriosyl-archaeol to archaetidylglycerophosphate-O-methyl (AOM) archaeosomes significantly enhanced CD8+ T cell responses, but diminished antibody titers. All three triglycosyl archaeols combined with AOM resulted in additive CD8+ T cell responses [Sprott et al., 2012]. Ansari et al. showed that archaeosome-entrapped secretory antigens (SAgs) of *L. monocytogenes* resulted in upregulation of Th1 cytokines and boosted protective effects by reducing listerial burden in infected mice. Archaeosome-entrapped SAgs enhanced CTL response and increased survival of immunized animals [Ansari et al., 2012]. Finally, Singha et al. used *E. coli* lipid liposomes (escheriosomes) based DNA delivery to induce superoxide dismutase (SOD) and IL-18 specific immune responses in murine *Brucellosis*. Escheriosome-mediated delivery of SOD- and IL-18-encoding DNA induced specific immune responses in immunized mice. Co-expression of SOD+IL-18 resulted in stronger IgG2a type response compared free SOD-DNA [Singha et al., 2011].

Currently, no clinical trials with archaeosomal vaccines are registered at clinicaltrials.org, a service of the U.S. NIH (see: clinicaltrials.org, search terms archaeosome AND vaccine).

In summary, vaccines prepared with archaeal lipids, the archaeosomes, represent a new interesting and promising alternative to classical liposomes and virosomes.

**Virosomes**

Virosomes are liposomes prepared by combining natural or synthetic phospholipids with virus envelope phospholipids, viral spike glycoproteins and other viral proteins. First virosomes were prepared and characterized by Almeida et al. [Almeida et al., 1975], followed by Helenius et al. who incorporated Semliki Forest virus glycoproteins in liposomes...
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[Helenius et al., 1977, Balcarova et al., 1981]. Significant progress was made with virosomes termed “immunopotentiating reconstituted influenza virosomes” (IRIVs). IRIVs are SUVs with spike projections of the influenza surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). The fusogenic properties of HA are their primary features. IRIVs allow antigen presentation in the context of MHC-I and MHC-II and induce B- and T-cell responses [Gluck, 1992, Gluck et al., 2005]. The first virome-based influenza vaccine used in humans was Inflexal V™ which has an excellent tolerability profile and immunogenicity in healthy and immunocompromized persons [Herzog et al., 2009]. Another virome vaccine containing inactivated hepatitis A virus (HAV), Epaxal™, was developed as hepatitis A vaccine. It is exellently tolerable and highly immunogenic, conferring protection of at least 9-11 years in vaccinated individuals [Ambrosch et al., 1997, Gluck et al., 2000, Bovier et al., 2010]. Immunogenicity and safety of Epaxal™ was evaluated in Thai children with HIV infection. Prevalence of HAV protective antibodies was 100% after vaccination, showing that Epaxal™ is an effective HAV vaccine for HIV-infected children [Saksawad et al., 2011]. Another vaccine contains an aspartyl proteinase-2 (Sap-2) of Candida albicans incorporated into IRIVs. Following intravaginal administration anti-Sap2 antibodies were detected in vaginal fluids of rats inducing long-lasting protection [De Bernardis et al., 2012]. Walczak et al. demonstrated that a heterologous prime-boost with Semliki Forest virus (rSFV) encoding a fusion protein of E6 and E7 of HPV16 and virosomes containing the HPV16-E7 protein resulted in higher numbers of antigen-specific CTL in mice than homologous protocols [Walczak et al., 2011]. Today, a second generation of influenza virosomes has evolved for various preclinical and clinical stage vaccine candidates. Additional components are included to optimize particle assembly and stability and to enhance immunostimulatory effects [Moser et al., 2013]. GPI-0100, a saponin derivative, enhanced immunogenicity and protective efficacy of a viroosomal influenza vaccine, providing full protection of infected mice at extremely low antigen doses [Liu et al., 2013]. A combination of reconstituted respiratory syncytial virus (RSV) envelopes with incorporated MPLA (RSV-MPLA) virosomes was studied by Kamphuis et al. in enhanced respiratory disease (ERD)-prone rats. Vaccination with RSV-MPLA induced higher antibody levels and protection against infection [Kamphuis et al., 2013]. Jamali et al. developed a DNA vaccine using cationic influenza virosomes (CIV). CIV-delivered epitope-encoding DNA induced equal numbers of IFN-γ and granzyme B-producing T cells than a 10-fold higher dose of naked pDNA [Jamali et al., 2012]. Another DNA/virosome vaccine was reported by Kheiri
et al. who prepared a vaccine complex containing an influenza nucleoprotein encoding plasmid that induced much higher T cell responses and protection as plasmid alone [Kheiri et al., 2012]. In clinical trials, IRIVs have shown vast potential for delivery of peptides derived from *Plasmodium falciparum* antigens [Peduzzi et al., 2008]. An IRIV-formulated fusion protein composed of two malaria antigens was described by Tamborrini et al. Compared to other vaccines the adjuvant-free formulation elicited specific IgG1 antibody profiles in mice and cross-reactivity with blood-stage parasites [Tamborrini et al., 2011]. Virosomes containing surface HIV-1 gp41-derived P1 lipid conjugated peptides (MYM-V101) as prophylactic HIV-1 vaccine were prepared. MYM-V101 was safe and well-tolerated administered by intramuscular and intranasal routes in healthy women. P1-specific serum IgGs and IgAs were detected in all recipients but P1-specific Th1 responses were not found [Leroux-Roels et al., 2013].

Currently, several clinical trials with virosome vaccines are registered at clinicaltrials.org, a service of the U.S. NIH (see: clinicaltrials.org, search terms virosome AND vaccine).

**Conclusions**

The enormous versatility of liposomes and the related archaeosomes and virosomes endows them as highly valuable carrier systems for vaccines. Besides improving antigen stability and presentation to immunocompetent cells, depending on their specific properties as composition, size and surface properties, these nanocarriers also possess the ability to overcome biological barriers such as skin and mucosa and provide controlled and slow release of antigens. Together with the ability to induce strong immune responses provided by co-formulated adjuvants, liposome-based vaccines provide properties that are fundamental for the development of modern vaccine formulations. It is predictable, that these delivery systems will be increasingly applied in the near future with success, leading to major improvements in vaccine development.

**Declaration of Conflicting Interests**

The author declares that there is no conflict of interest.

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References


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<table>
<thead>
<tr>
<th>Vesicle Type</th>
<th>Composition</th>
<th>Characteristics</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposome</td>
<td>Neutral or anionic lipids (PC, PG, PS, cholesterol) plus immunomodulators (MPLA, CpG, lipopeptides, glycolipids, etc.) and antigens (OVA, plasmids, mRNA, etc.)</td>
<td>Flexible compositions and antigen or adjuvant incorporation (encapsulation, adsorption, covalent surface attachment) Th1 and cell-mediated immune responses</td>
<td>[Torchilin, 2005, Watson et al., 2012]</td>
</tr>
<tr>
<td>Liposome</td>
<td>Cationic lipids (DDA, DC-chol, DOTAP, etc.) plus neutral phospholipids, cholesterol plus immunomodulators (TDB, MPLA, CAF01, etc.)</td>
<td>Long depot effect at site of injection. Strong electrostatic interactions with APCs and strong Th1 and Th17 mediated immunostimulatory effects.</td>
<td>[Christensen et al., 2011, Korsholm et al., 2012]</td>
</tr>
<tr>
<td>Archaeosome</td>
<td>Polar glycerolipids from Archaea and other bacteria plus phospholipids, cholesterol and antigens (OVA, plasmids)</td>
<td>Very stable formulations due to ether lipid bilayers. Archaeal glycerolipids are strong adjuvants mediating Th1 and cellular immune responses without need of TLR agonists.</td>
<td>[Krishnan et al., 2008, Patel et al., 2010]</td>
</tr>
<tr>
<td>Virosome</td>
<td>Vesicles reconstituted from virus membranes (Influenza, Semliki Forest, respiratory syncytial virus) and phospholipids. Hemaglutinin (HA), Neuraminidase (NA)</td>
<td>Strong binding to cells and high immunogenicity induced by HA and NA. Human influenza and hepatitis A vaccines (Inflexal, Epaxal)</td>
<td>[Gluck et al., 2005, Herzog et al., 2009, Kamphuis et al., 2013]</td>
</tr>
</tbody>
</table>

Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine.
Table 2: Examples of liposomal Veterinary vaccines

<table>
<thead>
<tr>
<th>Type of liposome</th>
<th>Antigen Vaccine</th>
<th>Adjuvant or Challenge</th>
<th>Animal Species Application</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC/cholesterol</td>
<td>Newcastle Disease vaccine</td>
<td>Glycosphingolipid (GSL) encapsulated in liposomes</td>
<td>Chickens, oral</td>
<td>Enhanced immune response with GSL-liposomes; T-, B-cell proliferation</td>
<td>[Yu et al., 2013]</td>
</tr>
<tr>
<td>PC/cholesterol, sonicated SUV</td>
<td>Newcastle Disease vaccine</td>
<td>Glycyrrhetic acid (GA) encapsulated in liposomes</td>
<td>Chickens</td>
<td>Enhanced immune response with GA-liposomes; Higher IgG, IgM antibody titers; T-, B-cell proliferation</td>
<td>[Zhao et al., 2011]</td>
</tr>
<tr>
<td>DPPC/cholesterol, MLV</td>
<td>Newcastle Disease (La Sota) strain</td>
<td>Newcastle Disease, Sato strain</td>
<td>Chickens, intranasal</td>
<td>High mucosal anti-NDV IgA, high serum IgG; high HA inhibition and survival rate; macrophage activation via ERK 1/2 and NfkB</td>
<td>[Lin et al., 2011]</td>
</tr>
<tr>
<td>DOTAP/cholesterol, cationic MLV</td>
<td>Newcastle Disease (La Sota) strain</td>
<td>Newcastle virus (Herts 33)</td>
<td>Chickens, oral</td>
<td>Higher antibody titers, 100% survival</td>
<td>[Onuigbo et al., 2012]</td>
</tr>
<tr>
<td>DPPC/cholesterol, DPPS/cholesterol, SA/cholesterol, MLV</td>
<td>Newcastle Disease (La Sota) strain</td>
<td>Newcastle Disease, Sato strain</td>
<td>Chickens, intranasal</td>
<td>DPPC/Chol-liposomes 320-fold higher HA-inhibition than SA/Chol liposomes; 90% survival with DPPC/Chol vaccine</td>
<td>[Tseng et al., 2010]</td>
</tr>
<tr>
<td>PC/cholesterol/α-Tocopherol SUV</td>
<td>Newcastle Disease vaccine</td>
<td>Epimedium polysaccharide-propolis flavone (EP), EP liposomes; Challenge: NDV F&lt;sub&gt;E&lt;/sub&gt;, strain</td>
<td>Chickens, intramuscular</td>
<td>High protection, high T-, B-cell proliferation; low mortality</td>
<td>[Fan et al., 2012]</td>
</tr>
<tr>
<td>PC/cholesterol/SA/cholesterol, MLV</td>
<td>Newcastle Disease vaccine</td>
<td>Non endotox LPS core types R1-R4</td>
<td>Chickens, intramuscular</td>
<td>Increased antibody response and lower lesion scores with MLV</td>
<td>[Dissanayake et al., 2010]</td>
</tr>
<tr>
<td>PC/cholesterol/DDA Cationic SUV</td>
<td>Inactivated avian pathogenic E. coli vaccine (APEC)</td>
<td>Inactivated APEC adsorbed to SUV; Challenge: APEC (PDI-386 strain O78)</td>
<td>Chickens, eye drops or coarse spraying</td>
<td>Higher mucosal and serum antibodies, reduced bacteria in blood</td>
<td>[Yaguchi et al., 2009]</td>
</tr>
<tr>
<td>Liposome-associated SEF14</td>
<td>S. enteritidis fimbrial protein, SEF14</td>
<td>Live S. enteritidis</td>
<td>Chickens, oral</td>
<td>High IgG, IgA in intestinal mucus and serum. Low bacterial and low excretion of S. enteritidis in feces</td>
<td>[Pang et al., 2013]</td>
</tr>
<tr>
<td>DOTA/DOPE Cationic MLV</td>
<td>n.a.</td>
<td>Plasmid DNA of microneme Toxoplasma gondii protein, MIC3 adsorbed to MLV</td>
<td>Sheep, intramuscular</td>
<td>High IgG2 and IgG1 serum levels, high anti-MIC3 antibodies.</td>
<td>[Hiszczynska-Sawicka et al., 2012]</td>
</tr>
<tr>
<td>DPPC/DOPE/DOTP Cationic SUV</td>
<td>Recombinant Foot-and-Mouth-disease protein vaccine (iGG-FMDV)</td>
<td>Porcine IFN-α DNA adsorbed to SUV; Challenge: FMDV isolate HKY 2002</td>
<td>Swine, intramuscular</td>
<td>Strong inflammatory cytokine production and T1,T2 response, high IFN-α, no viremia or lesions</td>
<td>[Cheng et al., 2007]</td>
</tr>
<tr>
<td>DPPC/cholesterol/曼多洛-[DPPE]-liposomes</td>
<td>M3-NcGRA7</td>
<td>Dense granule protein 7 of Neospora caninum (M3-NcGRA7) encapsulated in liposomes</td>
<td>Challenge: Tachyzoites of N. caninum</td>
<td>Cattle, subcutaneous</td>
<td>Decreased parasite load</td>
</tr>
<tr>
<td>DDA/TDB-liposomes (CAF01)</td>
<td>rec. M. avium paratuberculosis proteins</td>
<td>CAF01, rec. M. avium paratuberculosis proteins</td>
<td>Calves of different age, subcutaneous</td>
<td>Age dependent IFN-γ response, humoral response age independent</td>
<td>[Thakur et al., 2013]</td>
</tr>
</tbody>
</table>

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidyl glycerol; DPPS, 1,2-dipalmitoyl-phosphatidylserine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine; DSPC, 1,2-distearyl-sn-glycero-3-phosphocholine; pegDSPE, pegylated 1,2-distearyl-sn-glycero-3-phosphatidylethanolamine; DCPC, 1,2-dihexanoyl-sn-glycero-3-phosphocholine.
**Table 3: Examples of liposomal therapeutic cancer vaccines**

<table>
<thead>
<tr>
<th>Type of liposome</th>
<th>Antigen</th>
<th>Adjuvant/Treatment</th>
<th>Tumor model</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC chol/PC/peg-DSPE, surface-linked anti-CD20 (rituximab)</td>
<td>Encapsulated Bcl-2 antisense oligonucleotide (G3139)</td>
<td>Bcl-2-targeted antisense G3139 as antisense therapeutic</td>
<td>Selective B cell targeting in Raji B cell lymphoma in NOD-SCID mice</td>
<td>Reduced adverse immuno-stimulatory effects of G3139. 80% survival due to inhibition of TLR9-driven immuno-stimulation</td>
<td>[Yu et al., 2013]</td>
</tr>
<tr>
<td>Pegylated PC/cholesterol liposomes with octa-arginine-SA + α-GC</td>
<td>α-galactosylceramide (α-GC), lipid antigen</td>
<td>Activation of NK T cells by α-GC, spleen targeting</td>
<td>B16 melanoma lung metastases</td>
<td>65% inhibition of lung metastases</td>
<td>[Nakamura et al., 2013]</td>
</tr>
<tr>
<td>Nanoliposomes</td>
<td>Encapsulated tumor associated antigen ESO-1 + tetanus toxoid helper peptide</td>
<td>Co-administration of palm-I1 and MAP-IFN-γ as adjuvants</td>
<td>In vitro targeting to Fcy receptors on DCs</td>
<td>Highest immune response with nanoliposomes compared to soluble antigens</td>
<td>[Cruz et al., 2013]</td>
</tr>
<tr>
<td>DOTAP-PIC-liposome complex (200 -500 nm)</td>
<td>Hepa 1-6 cell lysates</td>
<td>polyriboinosinic: polyribocytidylic acid (poly I:C, PIC)</td>
<td>Hepa 1-6, subcutaneous</td>
<td>High tumor-specific CTL response and IFN-γ levels; significant tumor growth inhibition with DOTAP-PIC + antigen</td>
<td>[Wang et al., 2012]</td>
</tr>
<tr>
<td>Cationic TDB/DDA liposomes (CAF01)</td>
<td>Ovalbumin and HP16 E7 protein</td>
<td>Prophylactic and therapeutic vaccinations with CAF01 + poly I:C (= CAF05)</td>
<td>1) Lung B16-OVA 2) TC-1 expressing HP16 E7 protein, s.c.</td>
<td>Significant reduction of tumor growth with CAF05 vaccine by CD8 T cell induced target cell lysis</td>
<td>[Hansen et al., 2012]</td>
</tr>
<tr>
<td>PC/PG/cholesterol + DOG(Man), liposomes + mannosylated ligands</td>
<td>ErbB2 CTL and influenza virus HA T3-peptide epitopes</td>
<td>TLR2/1 (Pam3,CAG), TLR2/6 (Pam3,CAG, Pam3GD) agonists</td>
<td>Mice bearing RenCa-lacZ/ErbB2 tumors</td>
<td>100% cures after vaccination with mannosylated liposomes + TLR2 ligand Pam3CAG</td>
<td>[Thomann et al., 2011]</td>
</tr>
<tr>
<td>Mannosylated and histidylated lipopolyplexes with MART-1 mRNA (Man[11]-LPR100)</td>
<td>MART-1 (MelanA) mRNA</td>
<td>Immunization with Man[11]-LPR100 followed by challenge with B16F10 cells.</td>
<td>Delivery of mRNA to splenic DCs in vivo and anti-B16F10 melanoma vaccination in mice.</td>
<td>Immunization with Man[11]-LPR100 gave better transfection of DCs and increased survival</td>
<td>[Perche et al., 2011]</td>
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<td>DOTAP/DOPE (SUV) + MPLA and surface-adsorbed bFGF</td>
<td>bFGF as angiogenesis stimulator</td>
<td>Human bFGF plus monophosphoryl lipid A (MPLA)</td>
<td>C57 mice vaccinated with liposomal bFGF and challenged with Lewis lung carcinoma cells (LL-2)</td>
<td>Significant Inhibition of lung metastases in vaccinated mice</td>
<td>[Zhong et al., 2010]</td>
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<tr>
<td>Anti-GD2-targeted CpG encapsulated in stealth liposomes (TL-CpG)</td>
<td>Neuroblastoma (NB) growth inhibiting CpGs</td>
<td>TLR9 dependent growth inhibition of NB induced by TL-CpG</td>
<td>HTLA-230 neuroblastoma expressing high levels of GD2 and TLR9</td>
<td>TL-CpG inhibited proliferation of TLR9-expressing NB cells, induced apoptosis and prolonged survival of NB tumor xenografts.</td>
<td>[Brignole et al., 2010]</td>
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</tbody>
</table>

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; DPPS, 1,2-dipalmitoyl-phosphatidylserine; PG, phosphatidylglycerol; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine; DSPC, 1,2-distearyl-sn-glycero-3-phosphocholine; pegDSPE, pegylated 1,2-distearyloyl-sn-glycero-3-phosphatidylethanolamine; DCPC, 1,2-dihexanoyl-sn-glycero-3-phosphocholine.
Figure 1. Mechanisms by which nanoparticles alter the induction of immune responses. The immunostimulatory activity of nanocarriers such as liposomes, archaeosomes and virosomes depends on diverse mechanisms: antigen delivery, particle size-dependent tissue penetration and access to the lymphatics (a); depot effects, promoting persistence, stability, conformational integrity and gradual release of vaccine antigens (b); and antigen display facilitating B cell receptor (BCR) co-aggregation, triggering and activation (c). Additional mechanisms include TLR-dependent and TLR-independent signal transduction (not shown); cross-presentation into MHC-I pathways, caused by nanoparticle-mediated leakage of antigens into the cytosol after phagosome uptake (d); and release of cytokines, chemokines and immunomodulatory molecules that regulate the immune response (not shown). APC, antigen presenting cell; DC, dendritic cell; ER, endoplasmic reticulum; TCR, T cell receptor. Reproduced and modified by permission [Smith et al., 2013].

Figure 2. Schematic representation of a small unilamellar liposome showing the versatility of incorporation of various compounds either by encapsulation in the aqueous inner space or by integration in the bilayer or surface-attachment on the lipid bilayer membrane. Reproduced and modified by permission [Heegaard et al., 2011].
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