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Liposomes or archaeosomes: a new vaccine delivery system?

What effects do the physiochemical differences between liposomes and archaeosomes have on the immunogenicity and what are the advances in altering their physiochemical characteristics on the immune response?

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Abstract

Vaccination is one of the most important medical interventions with a major impact on global health. In the past attenuated or inactivated pathogens were used in order to prevent bacterial or viral infections. Nowadays subunit vaccines are used because of their safety profile. However, due to their poor immunogenicity, these kinds of vaccines need adjuvants and particulate vaccines are a very promising option. Liposomes and archaeosomes belong to this class and they are optimal candidates for particulate vaccines due to their high safety profile and versatility. In this study the physiochemical properties of liposomes and archaeosomes are analysed and compared on the basis of their effect on the immunogenicity, moreover the possible advances in the modification of the physiochemical characteristics of the vesicles and how these changes affect the immune response are discussed. The archaeal lipids are well known to be more stable than conventional ester lipids due to their unsaturated isoprenoid chains ether linked to a *sn*-glycerol-1-phosphate backbone and are inherently immunogenic. Despite these features, the liposomes have overcome these problems by incorporating co-adjuvants and by the use of synthetic lipids. For each pathogen/disease a particular immune response is required to efficiently battle it, therefore the adjuvant formulation is designed depending on the kind of immune response that you want to trigger. In fact the different nature and the different physiochemical properties elicit different immune responses. The physiochemical properties that are considered in this review concern the choice of headgroup, which affects the recognition, binding and uptake of the vesicles, the membrane fluidity and the shape of the vesicles.

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List of abbreviations

DC, dendritic cell; **PRR**, pattern recognition receptors; **PAMP**, pathogen-associated molecular pattern; **TLR**, Toll-like receptors; **NLR**, Nod-like receptors; **CLR**, C-type lectin receptor; **APC**, antigen presenting; **MHC**, major histocompatibility complex; **Th**, CD4+ T cells; **IL**, interleukin; **INF**, interferon; **TNF**, tumor necrosis factor; **CTL**, CD8+ cytotoxic T lymphocytes; **G-1-P**, *sn*-glycerol-1-phosphate; **G-3-P**, *sn*-glycerol-3-phosphate; **DOPE**, 1,2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine; **DOTMA**, N-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride; **DOGS**, dioctadecylamido-glycylspermine; **DC-Chol**, 3b-[N-(N',N'-dimethylaminoethyl)carbamoyl)cholesterol; **ODA**, cis-9,10-octadecenamide; **TPL**, total polar lipid; **PEG**, Polyethylene glycol; **DPPC**, Dipalmitoylphosphatidylcholine; **DMPC**, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; **DMPG**, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; **CHOL**, cholesterol; **BSA**, bovine serum albumin; **DCP**, dihexadecyl phosphate; **HEL**, hen egg lysozyme; **OVA**, ovalbumin; **Pam**, Palmitoyl; **LPS**, Lipopolysaccharides; **MPLA**, monophosphoryllipid A; **HA** hemagglutinin; **NA**, neuraminidase; **PA**, Phosphatidic acid; **PC**, Phosphatidylcholine; **SA**, stearylamine; **DDAB**, Didecyldimethylammonium bromide; **DPPG**, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; **PGP**, phosphatidylglycerol phosphate; **DOPC**, 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine; **PS**, phosphatidylserine; **AS**, achaetidylserine; **AE**, archaetidylethanolamine; **PC**, phosphatidylcholine; **PG**, phophatidylglycerol; **DPPE**, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; $\mathbf{T_m}$, transition temperature; **DDA**, dimethyldioctadecylammonium; **TDB**, trehalose 6,6'-dibehenate ; **DODA**, dioctadecylamine; **DSPC**, 1,2-Distearoyl-sn-glycero-3-phosphocholine; **AMVAD**, Archaeal Lipid Mucosal Vaccine Adjuvant and Delivery; **LVS**, *Francisella tularensis*

Introduction

Vaccination is one of the most important medical interventions and has had a major impact on global health. Since the first vaccine designed in 1796 by Edward Jenner, who is often called "the father of immunology"[1] , many diseases such as mumps, measles, polio and diphtheria have been reduced in occurrence, and even the complete eradication of smallpox has been achieved. Many developed countries now employ national immunisation programmes which prevent childhood infection leading to a decrease in childhood mortality.[2-4]

Despite these great achievements there is an ongoing research to optimise existing vaccines (e.g. tuberculosis) and discover new vaccines against major infectious diseases such as HIV and malaria.[2]

The first used vaccines were based on attenuated or inactivated pathogens,[4] where the antigen and immune-stimulating agents come directly from the pathogen itself.[5] Of course this led to immunosafety issues contrasting with the high reactogenicity and a good safety profile required for the new generation of vaccines.[6] Most vaccines are administrated to normal, healthy people,[5] and due to the strict safety requirements, the attenuated or inactivated pathogens are no longer used. The focus moved instead on subunit vaccines which are mostly composed of peptide or proteins from the infectious agent against which the vaccine is formulated. However the high purity of the antigenic subunits makes them poorly immunogenic and therefore adjuvants are required. [2,3]

By definition an adjuvant is any compound that enhances the immune response against a vaccine antigen.[7] It comes from the Latin word 'adjuvare', which means indeed 'help' or 'to enhance'. [7,8] Adjuvants can be subdivided into two general classes: delivery systems (e.g. emulsions, liposomes and mineral salts) and immunostimulators (e.g. Toll-like receptor (TLR) agonists)[6] , although some systems can elicit both effects (e.g. archaeosomes). Adjuvants are used for several purposes: (1) immunomodulation (direct the type of immune response), (2) antigen's protection against degradation, (3) Targeting of specific cells and facilitate uptake by antigen presenting cells, (4) depot generation (accumulation of antigen at the site of injection) and (5) cytotoxic T-lymphocyte (CTL) induction. [4,7]

Until 2009 the only approved adjuvants in the United States were the aluminium salts, known as *alums*, which were suitable for vaccines because of their simplicity, tolerability, safety record and minimal cost.[5,7,9] However the alum salts showed some downsides which are mainly their sub-optimal Th1 response and the low cell mediated immunity. For vaccines against cancer or HIV, cell mediated immunity is essential. Therefore other particulate vaccines are investigated such as: gels, emulsions, polymeric particles, liposomes and archaeosomes.[10]

The latter two are spherical vesicles composed of lipids from *Bacteria/Eukarya* and *Archaea* respectively. Liposomes are constituted only by phospholipid bilayers vesicles [4] whereas archaeosomes can also consist of a lipid monolayer especially when they exclusively constitute of tetraether lipids. A combination of monolayer and bilayer has been observed in archaeosomes.[11] The use of liposomes and archaeosomes has some main advantages compared to other particulate systems especially that both systems have been shown to be safe and well tolerated. This is already well established for liposomes, due the presence of commercially approved liposome based drugs such as Doxil (Johnson & Johnson) and Ambisome (Gilead sciences) and numerous human trials of liposomal vaccines.[9,12] Archaeosome's safety has not yet been studied in humans since it is a relatively new system but all *in vitro* and *in vivo* safety evaluation showed no significant toxicity.[13] Moreover the safety of liposomes can be assessed, given the fact that many of the used lipids naturally occur in cell membranes such as phosphatidylcholine (PC) and cholesterol.[9] In the same way, in the case of archaeosomes, humans can have a high dietary intake of ether-linked lipids from meats and sea food. Furthermore the isoprenoid chains present in the archaeal lipid can also be found in fat-soluble vitamins and coenzyme Q10 present in the human body.[14] Beside the good safety profile of both particulate systems, they are also very versatile systems. Lipid formulations, vesicle preparation methods and addition of immunostimulatory molecules can be used to change

the physicochemical properties of the vesicles such that a specific response can be generated for a specific kind of antigen.[9]

In this study a comparison between liposomes and archaeosomes as adjuvants for vaccine delivery is highlighted. The focus lays on the effect of lipid composition and on their physicochemical properties on the immunogenicity.

The biological processes in the immune system upon vaccine administration will be briefly described, followed by a description of the liposomal and archaeosomal lipid content and the respective differences.

Immune responses upon vaccination

The first line of defence: the innate immune system

The purpose of vaccination is to produce immunity against a certain disease by activating the immune system. The immune system consists of two components: the innate and the adaptive immune systems. The innate immune system is the first line of defence and mainly consists of the complement system and phagocytic cells such as macrophages and dendritic cells (DCs).[4]
They are located in the periphery of the body and constantly sample their environment for the presence of foreign antigens, recognised by their pattern recognition receptors (PRRs) which bind to pathogen-associated molecular patterns (PAMPs) on the antigen. PRRs include: Toll-like receptors (TLRs), Nod-like receptors (NLRs) and C-type lectin receptor (CLRs) which recognise different antigenic substances.[15] The innate immune system is a non-specific response to all antigens, recognising and eliminating them from the body, but in vertebrates it can also activate the adaptive immune system through antigen presentation.[16]

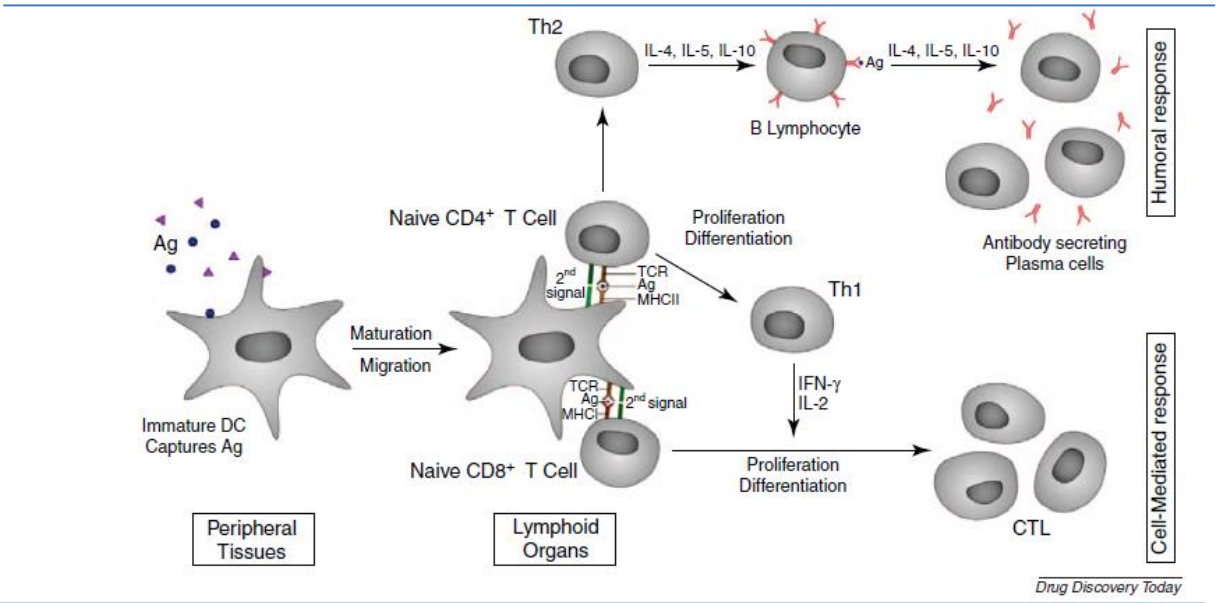

Fig. 1. Representation of the adaptive immune system where the humoral and cell-mediated response and the route thereto are highlighted [4]

The second line of defence: the adaptive immune system

DCs are regarded as the most powerful and versatile antigen presenting cells (APCs).[4] Immature DCs capture and internalise the antigen carrying them from the periphery of the body to the lymph nodes. Antigens, that are extracellularly captured, are loaded onto major histocompatibility complex (MHC) class II molecules (fig. 1). The complex of antigen and MHC molecule are transported to the surface of the cells and presented to $CD4^+$ T cells (Th). Th cells can be divided in several classes among which the most important are named Th1 and Th2 that can be distinguished by the type of secreted cytokines and the nature of induced response. Th1 cells produce a cell-mediated response by secreting interleukin-2 (IL-2), interferon-γ (INF-γ) cytokines and tumor necrosis factor-β (TNF-β) which stimulates the proliferation and differentiation of CD8+ cytotoxic T lymphocytes (CTLs). Th2 cells cause a humoral response by secreting IL-4, IL-5 and IL-10 which stimulate the production of circulating antibodies by maturation of B-lymphocytes into antibody secreting plasma cells, which are active in the defence against extracellular pathogens.[4,6]

The other major type of MHC molecules are MHC class I molecules which act in response to the presence of intracellular antigens. The antigen-MHC class I complex are presented to $CD8^+$ T cells and cause the cell-mediated response. However, through a path called cross presentation, also

extracellular antigens can elicit a MHC class I response. In this pathway antigens escape from an endosomal or lysosomal compartment after their uptake by the APC and thereby entering the cytosol, where they can associate with MHC class I molecules. [15]

The key to an effective vaccine is the choice of antigen and adjuvant. It is crucial to understand what kind of immune response is needed for a particular disease. Therefore the adjuvant formulation should be designed according to which immune response you want to elicit (e.g. Th1 versus Th2, $CD8^+$ versus $CD4^+$ t cells, etc.). The choice of formulation could also affect the longevity of the T-cell memory, affect the speed of the initial response (important in a fast pandemic outbreak) and alter the specificity or affinity of the response. [6,7]

As abovementioned the liposomes and archaeosomes are used adjuvants in vaccine formulations, therefore a further investigation of liposomal and archaeosomal formulations and their effect on the immunogenic response will be described in the following sections.

Lipid structures of liposomes and archaeosomes

Woese et al.[17] proposed, on the basis of comparative rRNA studies, the existence of three domains of life: *Eukarya, Bacteria and Archaea*. [18,19]

Archaea are known by their ability to survive at extreme conditions such as high temperatures, high salt concentration and extreme pH environments (for instance acidic hot springs) therefore their physiological characteristics are adapted to these conditions. Due to these peculiar environments they possess membrane lipids that are unique and distinct from the other two domains of life.[20]

There are three main differences between archaeal membrane lipids and bacterial ones (fig. 2). First, in the *Archaea* the hydrocarbon chains are linked to the glycerolphosphate backbone by ether linkages while in *Bacteria* ester linkages are present. Second, the presence of the enantiomeric *sn*-glycerol-1-phosphate (G-1-P) in *Archaea* as glycerophosphate backbone compared to *Bacteria* which have *sn*-glycerol-3-phosphate (G-3-P). Lastly, the hydrocarbon chains of *Bacteria* are made of fatty acids while in *Archaea*, isoprenoidic chains are found.[20-22]

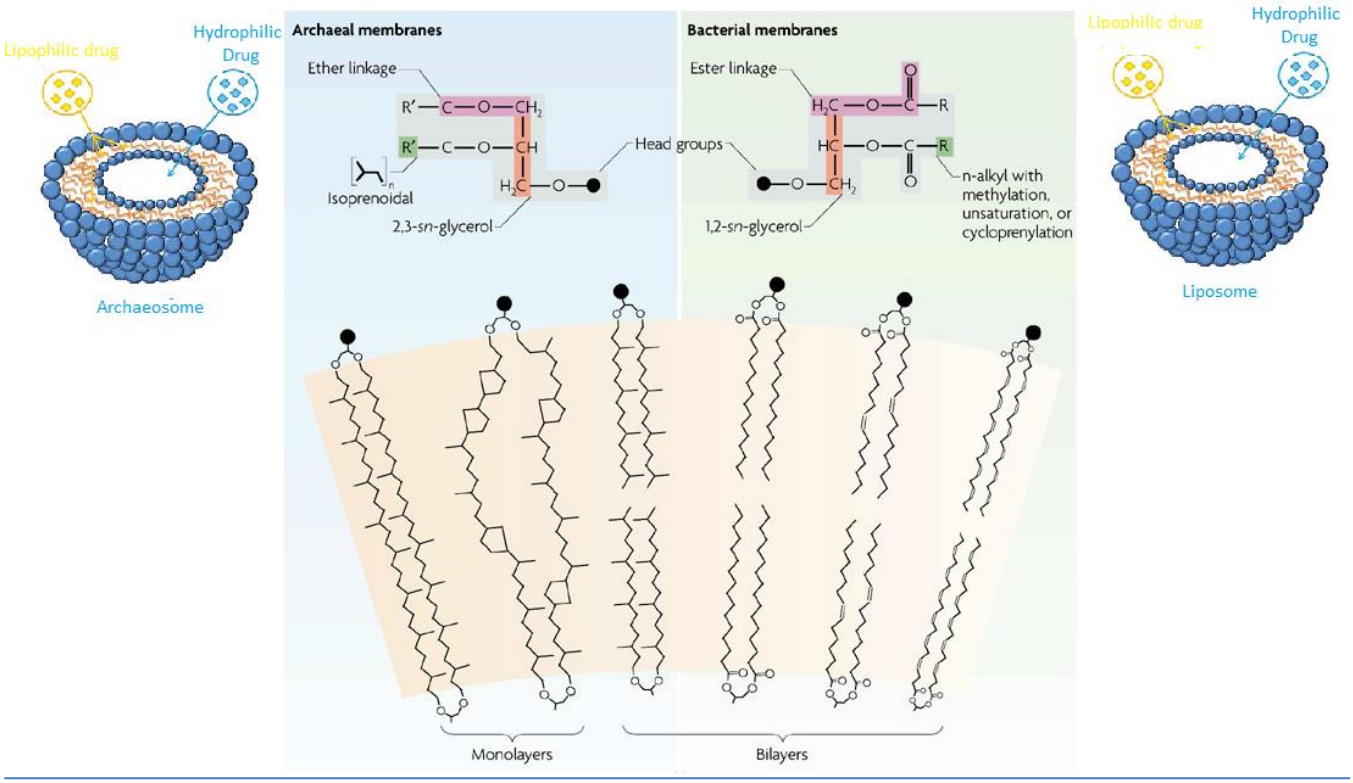

Fig. 2. Differences in archaeal and bacterial lipids (top); membranes that the respective lipids can form (bottom) and an illustration of archaeosomal and liposomal vesicles. [67]

Both liposomes and archaeosomes are microscopic vesicles consisting of a central aqueous compartment surrounded by one or more concentric phospholipid lamellas.[23] In the case of archaeosomes these lamellas are bilayers or monolayers depending on the type of lipids used [11] (fig. 2.). They can both incorporate either hydrophilic, hydrophobic and amphiphilic substances. Despite these similarities both systems were developed in an independent way with their own lipids compositions.

Liposomes can be composed of naturally derived phospholipids or pure surfactant components such as DOPE.[24] Naturally occurring phospholipids are phosphoglycerides and sphingomylin, moreover membranes can also contain glycolipids. Phosphoglycerides are made from phosphophatidate by formation of an ester bond between the phosphate group of the

phosphatidate and the hydroxyl group of an alcohol. The most commonly used alcohol moieties are: serine, ethanolamine, choline, glycerol and inositol. These all result in negatively charged or neutral phosphoglycerides.[25] To extent the number of applications of liposomes by altering their structure, several research groups made synthetic lipids, for example cationic lipids such as DOTMA, introduced for the first time by Felgner *et. al.*[26] which consists of a quaternary amine connected to two unsaturated aliphatic hydrocarbon chains via ether groups. Other synthetic cationic lipids are DOGS, DC-Chol and ODA which do not always retain the characteristics of a classical lipid with hydrocarbon tail ester linked to a glycerol head. [27]

Liposomes are classified in terms of composition and mechanism of intracellular delivery into five types, moreover they are often classified according to their size and number of bilayers (table 1.).[23,28]

Composition an mechanism of intracellular delivery	Size and number of bilayers
Conventional liposomes	Small unilamellar vesicles (SUV): 20–100 nm
pH-sensitive liposomes	Large unilamellar vesicles (LUV): >100 nm
Cationic liposomes	Giant unilamellar vesicles (GUV): >1000 nm
Immunoliposomes	Oligolamellar vesicle (OLV): 100–500 nm
Long circulating liposomes	Multilamellar vesicles (MLV): >500 nm

Table. 1. Two different ways of characterising liposomes

Up to now, no characterisation on composition or intracellular delivery for archaeosomes can be found in literature. However the size classification and number of bilayers of liposomes could be also applied to archaeaomes. Benvegnu *et al.*[11] divided the archaeosomes into 3 main categories: natural polar structures, hemi-synthetic lipids and totally synthetic lipid analogues.

Natural polar lipids can be obtained by solvent extraction methods from the total lipids of archaeal cells and can be separated into neutral and total polar lipid (TPL) fractions.[29] The two main polar lipid structures are: archaeol (2,3-di-O-diphytanyl-*sn*-glycerol, monopolar diether) and caldarchaeol (2,2',3, 3'-tetra-O-dibiphytanyl-*sn*-diglycerol, bipolar tetraether). Variation in lipids can be seen in the number of five carbon units or in the presence of cyclopentane rings in the caldarchaeol lipid chains (fig. 2).[29] The predominant polar headgroups of diethers are negatively charged molecules among which the most abundant are phosphoethanolamine and phosphoglycerol. Tetraethers can have neutral (sugar), negative (phosphoinositol) or zwitterionic headgroups. TPL fractions have been obtained from many different *Archaea* including mathanogens, halophiles and thermoacidophiles, and new organisms are continued to be probed on interesting lipid compositions.[11]

Hemi-synthetic lipids are made by the hydrolysis of the polar head group of natural polar lipids and chemical modification of the resulting dihydroxyl archaeal lipid core. Head groups that can be attached include: amines, hydrophilic polymers (PEG), phosphogroups, biotin, cell recognition ligands and sugars.[11]

Fully synthetic lipids are synthetized due to the difficulty in obtaining sufficient amounts of natural archaeal lipids. Several research groups have attempted the synthetic manufacture of lipids but this technique is still in its infancy and not all desirable structures can be obtained in good yield.[11]

Comparison in physiochemical characteristics and the impact on immunogenicity

A comparison between conventional liposomes and natural archaeosomes

The differences in immune response and adjuvanticity between conventional liposomes (made of natural ester lipids) and natural archaeosomes (made from the TPL of different archaeal species) have been extensively reviewed in previous studies[11,29] and therefore only the main outcomes will be discussed here.

There are five main important structural differences, already mentioned in the previous section, which make archaeosomes more stable vaccine delivery systems than liposomes although not all of these properties may actually produce a greater immunogenic response. (1) Ether linkages are more pH stable than ester linkages and the branching methyl groups in the lipids of *Archaea* reduce the crystallization and the membrane permeability; (2) saturated alkyl chains present in archaeosomes allow a better resistance to oxidative degradation; (3)The G-1-P backbone confers resistance against many phospholipase enzymes; (4) the caldarchaeol lipids enhance stability of the membrane; (5)enhanced membrane packing and reduced membrane fluidity can be achieved through addition of cyclic structure in the transmembrane portion of the lipids.[11] Some of these structural differences will be further discussed in relation with their impact on the immune response.

It was determined that archaeosomes made of lipids from various *Archaea* where internalised by APCs in much higher amount (3 to 53 times more) than conventional liposomes made of DPPC or DMPC:DMPG:CHOL in 1.8:0.2:1.5 molar ratio, leading to the conclusion that archaeosomes are more easily recognised and incorporated. In another study[30] , mice were immunised with BSA encapsulated into archaeaosomes made of the TPL of various archaeal species (*T. acidophilum, M. mazei, M. smithii, M. voltae, M.hungatei or M. concilii*) and compared to conventional liposomes (DMPC:DMPG, 1.8:0.2; DMPC:DMPG:CHOL, 1.8:0.2:1.5 and DMPC: DCP/CHOL, 7:1:2). The archaeaomes induced superior bovine serum albumin (BSA)-specific serum antibody responses, even comparable to Freund's adjuvant (very potent adjuvant). Moreover adjuvant effects were enhanced when increased amount of archaeal lipid was added to a conventional liposome formulation, namely DMPC:DMPG. Archaeosomes elicited both Th1 (IgG2a) and Th2 (IgG1) responses. Sprott *et al.*[31] showed that unlike most liposomal formulations, archaeosomes induce a strong and lasting CTL response to encapsulated protein or peptide without needing additional immune modulating adjuvants and that archaeosomes elicit both MHC-I and MHC-II responses. Furthermore archaeosomes from various species encapsulated with BSA, hen egg lysozyme (HEL) and ovalbumin (OVA) all elicited antigen specific dose dependent proliferation of spleen cells while conventional liposomes and alums are not able to induce such response. The fact that archaeosomes by themselves can alert the immune system is proven by the fact that empty *M. smithii* archaeosomes cause enhanced expression of MHC II costimulatory markers CD80 and CD86 in macrophages, while this phenomenon is not observed in conventional liposomes. [11,29]

All these studies show that natural archaeosomes can fulfil the role of delivery system as well as an immunostimulating adjuvant compared to conventional liposomes which can only be used as delivery system. However these systems have been developed and physical and structural modifications of the natural lipids are used to alter the immune response as required.

Immunostimulators used in liposomes

As described above archaeosomes are able to induce the immunoresponse by their only presence in the system, while conventional liposomes cannot. However addition of immunostimulators to liposomes can greatly improve the immunogenic response. The immunostimulators used include: lipoprotein, characterized by a hydrophobic lipid chain that anchors an immunostimmulatory protein in the liposomal membrane. Other examples of these molecules used in liposomes are Palmitoyl chain (Pam) diacylated and triacylated lipopeptides (Pam2CAG and Pam3CAG,respectively) recognised by TLR receptors; Poly(I:C) which promotes DC maturation and $CD8^+$ T cell activation; CpG DNA incorporated in liposomes also to create a cell mediated immune response.; LPS to elicit high

antibody responses as TLR4 agonist; MPLA, a derivative of lipid A from *S. Minnesota* which led to the MPLA-liposomal vaccine formulations Stimuvax (phase III clinical trials) for non-small cell lung carcinoma and AS01 (phase I/II/III) for malaria.[15] Virosomes are a special type of liposomes that possess two glycoproteins from influenza virus, hemagglutinin (HA) and neuraminidase (NA), and it is considered as a vaccine system on its own.[3] These are just a few examples, for a more complete list, Demento *et. al.*[15] has provided an extensive review.

Choice of polar headgroup

Charge

The charge of a liposomal membrane can be selected by the choice of lipid formulation that is used. Neutral and anionic liposomes can be made from natural or synthetic lipids, cationic liposomes always include synthetic cationic lipids. The charge's influence on the immunogenic response has been extensively studied for liposomes but not in such an extensive way for archaeosomes.

In a general study investigating phagocytic uptake of particulate formulation by macrophages and monocyte-derived DCs it was shown that large (4,5 µm) and/or positively charged vesicles where more efficiently assimilated than small (1 µm) and/or negatively charged vesicles.[32,33]

In an early review article[28] it was suggested that negatively charged liposomes are internalized by coated-pit endocytosis while cationic liposomes fuse with the cell membrane and release their contents directly into the cytosol. Moreover they claim that negative surface charge increases the intracellular uptake and hence shows a faster plasma clearance.[28]

In a study of Kraaijeveld[34] , UV-inactivated encephalomyocarditis and Semliki Forest viruses were admixed with positively charged (DPPC:ODA:Chol), negatively charged (DPPC:PA:Chol), or neutral(DPPC:Chol) liposomes and administered intraperitoneally to mice. Charged vesicles gave a better neutralising antibody response than neutral vesicles but with minimal difference between the two charges.[34] In a similar study[35] positively charged (PC:Chol:SA), negatively charged (PC:Chol:PA) and neutral (PC:Chol) liposomes were filled with OVA, diphtheria toxin or β-galactosidase and antibody and CTL responses in the spleen of mice were measured. Only positively charged liposomes elicited a CTL response and also gave the greatest anti-OVA serum IgG1 response.[35,36] These studies led to the hypothesis that there occurred a greater cytoplasmic release of the proteins encapsulated in positively charged vesicles. Supremacy of cationic formulation was also confirmed by Badiee *et al.*[37] who compared neutral (DPPC:Chol), negatively charged (DPPC:Chol:DCP), and positively charged (DPPC:Chol:DDAB) large multilamellar vesicles with *L. major* rgp63 protein on antibody response in mice and again the cationic vesicles elicited the greatest antigen-specific IgG1 and IgG2 response. Furthermore, Joseph *et al.*[38] showed that two out of five tried cationic liposomes with influenza antigen induced a systemic and local Th1 and Th2 response while all vaccine formulated of neutral or anionic lipids failed.[9] Moreover it has been shown in *in vitro* studies that while anionic liposomes interact with a limited fraction of dendritic cells, cationic liposomes interact with a high percentage of cells. This has been attributed to the fact that the positive charge of the liposome electrostatically interacts with negatively charged heparane sulfate proteoglycans. Furthermore most antigens are negatively charged and are therefore better absorbed by positively charged vesicles. [6]

The influence of charge on immunogenicity in archaeosomes has not been well studied probably due to the fact that the hemi-synthetic and fully synthetic production of cationic archaeal lipids has only recently been developed. Benvegnu *et al.*[39] studied cationic archaeosomes as gene delivery system, where there is electrostatic interaction with the negatively charged DNA. When cationic tetraethers were used no efficient transfection was achieved, while the addition of neutral DOPE (5%) lipid increased the transfection efficiency leading to the conclusion that the archaeosomes were too stable to release DNA to the cytosol. When neutral tetraether diol and cationic diester lipids were used the transfection efficiency was much higher. No other studies directly comparing positively, negatively and neutrally charged archaeosomes have been conducted, but it can be speculated that they give a similar effect on the immune response as the differently charged liposomes.

Carbohydrate head group

In the case of archaeosomes, the effect of a sugar as head group on the immunogenicity has been investigated in several studies.

In a first study using synthetic archaeal lipids, Sprott *et al.* [40] synthesised a series of several semi-synthetic disaccharide archaeols from *Halobacterium salinarum* lipids to see the effects of carbohydrate headgroup changes on the immune response. Archaeosomes were prepared by the addition of diglycosylarchaeol to DPPG/cholesterol and the CTL response on encapsulated OVA was measured. Among the dissarcharides assessed, β-gentiobiosylarchaeol gave the best CTL response, higher than for example α-tetraMannosylarchaeol which could be hypothesised to interact with mannose receptors on APCs. Moreover it was concluded that diglucosylarchaeols with 1,6 linkages gave a better response than either an α- or β-1,4 linkages. For gentiobiose linkages were preferred in the β rather than the α configuration. Later, Sprott *et al.*[41] fabricated series of semi-synthetic archaeal lipids from *H. salinarum* by addition of several sugar headgroups. Stable archaesomes were created by mixing the glycolipids with phosphatidylglycerol phosphate (PGP), an achaeaol-based anionic lipid. Four glycolipids (one biosyl and three triosyl headgroups) were assessed on $CD8^+$ T cell responses in mice. Gentiotriosylarchaeol appeared to be a better adjuvant than gentiobiosylarchaeol and among the triosyls, gentiotriosylarchaeol and mannotriosylarchaeol elicited a significantly greater effect than maltotriosylarchaeol. This suggest that the first two sugars head groups have positive interaction with receptors on DCs confirmed by previous studies where it was also shown that these sugars gave increased activation of DCs. Most interestingly however was that the incorporation of all three triglycosylarchaeols into the same archaeosome preparation, gave an immune response almost equal to that of the TPL of *M. smithii*, which is an archaeosome that gives a very potent immune response, possibly due to multiple interactions with receptors simultaneously. In both studies the exact receptors on the APC to which the sugar unit binds to produce an immune response was unknown.

Sugars have been attached or incorporated in liposomal membranes for drug targeting to specific cells or tissues in drug delivery systems.[42] However, only a very recent article has investigated the attachment of a sugar headgroup to lipids in the liposomal membrane in order to alter the immune response. They covalently attached tetra-saccharide repeating unit from the polysaccharide of pneumococcal serotype 14 to diacylthiglycerol (PBS150). Glycosylceramide (PBS57) was previously developed as NKT cell antigen. A liposome was constructed of DOPC, cholesterol, PBS150 and PBS57 and gave high IgG titers after a single vaccination rather than multiple injections needed for the conventional *Streptococcus pneumoniae* vaccine Prevnar. A much weaker response was generated when PBS150 and PBS57 were not incorporated in a liposomal formulation. [43]

Phosphatidylserine and archaetidylserine

In the body, apoptotic cells are removed by macrophages and dendritic cells to prevent inflammation by toxic intracellular components. The apoptotic cells are recognised and internalised by the immune system through phosphatidylserine (PS) exposed on the surface of these cells.[44]
The possibility of increased uptake of archaeosomes and liposomes due the presence of serine in their lipids has been studied.

Total lipids analysis of the archaeon *M. smithii* showed a very high amount of achaetidylserine (AS) (30 mol% of the total lipids). Uptake studies revealed that *M. smithii* archaeosomes are incorporated by receptor mediated endocytosis through recognition of archaetidylserine by phophatidylserine receptors on the APC (e.g. CD36 on macrophages[45]).[14] In the study of Sprott *et al.*[41] discussed above the effect of incorporating AS and archaetidylethanolamine (AE) (another amine bearing group) into mannotriosylarchaeol/gentiotriosylarchaeol/PGP archaeosome on the CD8+ T cell response was tested. Addition of 30mol% AS resulted in a significantly higher response, rather similar to the response of archaeosomes made of the TPL of *M. smitthii*, as expected given the endogenous

presence of AS into the methanorganism. Response was highest with the highest mol% of AS added even though a higher mol% than 30 mol% could not be tested due to instability issues. Antibody responses were instead not significantly enhanced. Combination of AS and AE had no further effect on adjuvanticity. Moreover they hypothesised that AS, helps not only in the recognition of the archaeosome by APCs but also enhances membrane fusion promoted by calcium with the phagolysosome which helps release the antigen to the cytosol and MHC-I molecules.

Concerning the liposomes, Mori *et al.*[44] studied macrophage recognition, processing and antigen presentation of surface-coupled antigen in two different formulations, PS –liposome and phosphatidylcholine (PC)-liposome. Enhanced uptake of antigen (OVA) was observed for the PS-liposome in comparison with the PC-liposome. Moreover more OVA was processed by macrophages when coupled to the PS-liposome than when coupled to the PC-liposome and a threefold higher production of IL-2 cytokine was seen for the PS-liposome, together with a significantly higher specific anti-OVA IgG antibody production. Therefore PS (at least in comparison to PC) gives an enhanced recognition, processing and antigen presentation and thus enhances the adjuvant activity of the liposome.

Dicaire *et al.*[46] compared liposomes containing dipalmitoylphosphatidylserine and archaeosomes (*M. smithii)* containing an exact equivalent amount of archaetidylserine on the basis of their ability to promote short and long-term CTL activity in animals. It was shown that incorporation of PS into PC/cholesterol or phophatidylglycerol (PG)/cholesterol liposomes resulted in enhanced short term CTL responses, which correlated to the amount of surface head groups exposed. However, long term CTL responses were not observed. Archaeosomes showed higher amounts of surface head groups and thereby they induced higher short and long term CTL responses. Moreover it was shown that incorporation of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) (lipid of liposomes) and AE (same concentration) into the archaeosome enhanced rapidity of the CTL response although the incorporation of DPPE decreased long term CTL response. This lead to the hypothesis that polar head groups are important for short-term CTL responses while long term responses, beside head group effect, depends also on stability of the lipid core enhanced by the stable isoprenoid chains of archaeal lipids. Possibly the β-gentiobiosyl head groups of glycolipids found in *M. smithii* archaeosomes, could also explain the superior CTL response observed in the archaeosomes compared to the liposomes.

Membrane fluidity

Degree of saturation and chain length

A parameter that is really important for the immunogenicity in liposomes is the membrane fluidity. The fluidity of the membrane is determined by the level of saturation and the length of the hydrocarbon chains. Longer hydrocarbon chains and increased saturation lead to stronger van der Waals forces and thus a higher phase transition temperature (T_m) Below the T_m the membrane is in a solid gel like phase, while above the T_m it is in a liquid-crystalline ('fluid') phase. [28,32]

In a study liposomes with different PC lipid formulations, which resulted in different transition temperatures, were compared on their ability to elicit an anti-DNP antibody response. It was found that liposomes with higher T_m gave a higher antibody response.[9] Moreover it was found that neutral PC liposomes in their solid phase induce high cell mediated and humoral immune responses, while liposomes in fluid phase only induce a humoral immune response. Therefore it was hypothesised that humoral responses do not depend on fluidity changes. [32]

In one study investigating influenza vaccines based on polycationic lipid liposomes it was shown that immunogenicity did not really change when lipids with saturated lipid chains were replaced with monounsaturated analogues.[32] However in another study two liposome formulation one of DDA:TDB (rigid) and one of DODA:TDB (fluid) where compared. DODA is the monounsaturated analogue of DDA . The fully saturated liposomes showed higher levels of antigens at the site of injection (depot effect) and also induced a 100-fold higher Th1 response than its DODA:TDB

analogue, thereby contrasting the conclusion drawn above. [47] Furthermore it was found that increasing chain length of saturated PC increased serum antibody responses. [9]

Taken together these results indicated that there might be several explanations how membrane fluidity alters the immune response: (1) it could change the uptake mechanism by APCs leading to different pathways of antigen presentation, (2) it could affect the retention of the liposomes at the site of injection leading to a depot effect (lengthening the exposure to APCs). [32] Overall it is accepted that more rigid liposomes which contain longer and highly saturated chains and those that have higher T_m induce a higher antibody and cell mediated response.[9]

Membrane stability in archaeosomes is ensured by relatively long fully saturated isoprenoid chains, tetraether lipids and cyclopentane rings. Branching of the chains cause the membranes to be in a liquid crystalline state over a wide temperature range causing archaeal lipids to have a T_m (between -20 and -15°C) that is much lower than that of ester lipids (40-50°C).[20,48] No similar studies have been conducted for archaeosomes in this field since they are always in a liquid crystalline state at ambient temperature. Therefore whether this is a disadvantage of archaeosomes, since for liposomes a higher T_m meant a better immunogenic response or that archaeosomes still induce an immunogenic response due to the rigid nature of the lipids in the membranes is unknown.

Incorporation of cholesterol

Cholesterol rather than changing the T_m of the liposomes eliminates the solid ordered and fluid disordered phases by reorganisation of membrane lipids and forms an intermediate fluid ordered state. Hereby it improves stability, rigidity and decreases leakage of encapsulated antigen by reducing the permeability of water soluble molecules through the membrane.[23,32,49]

There is some ambiguity in the field of liposomes on whether or not incorporation of cholesterol increases the immunogenicity. Bakouche *et al.*[50] designed liposomes of 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) with varying amount of cholesterol and tested GCSA anti-body response. Immunogenicity was increased over a 10 fold range relative to DSPC alone with an optimum at 20 mol%. [9] Barnier-Quer *et al.*[51] also studied the effect of cholesterol in the lipid bilayer of cationic liposomes on the immunogenicity. They concluded that incorporation of cholesterol enhances the adjuvant effect of liposomes against influenza hemagglutinin by increased uptake by DCs. Moreover higher IgG1 and IgG2a/c where observed in eDPPC:Chol liposomes than in eDPPC:DPPC liposomes, however when DDA:Chol liposomes where compared to DDA:DPPC liposomes no significantly higher antibody titers where observed.[51] Nakano *et al.*[52] observed that the humoral immune response was reduced in PC liposomes with incorporation of cholesterol. Van Houte *et al.*[53] showed instead that incorporation of cholesterol in liposomes with low and high T_m increased the immunogenicity, whereas liposomes with intermediate T_m where by themselves are already immunogenic and cholesterol did not enhance this effect. [32]

Although for archaeosomes there are no direct comparison studies on the effect of cholesterol on the immunogenicity, there are some mentions in literature of the stabilising effect of cholesterol to archaeosomal vesicles. Moghimipour *et. al.* [49] studied physiochemical properties and drug encapsulation efficiency of archaeosomes made of lipids extracted of *S. acidocaldarius*. It was shown that encapsulation efficiency of methylene blue without cholesterol in the membrane was less than 7% but when cholesterol was added it increased to 62%. More encapsulated antigen could result in a higher immunogenic response. Cholesterol has also been used to increase membrane quality to prevent conversion of archaeosomes to needle-shaped crystals[40] , or to prevent aggregation and increase membrane stability (especially when synthetic archaeal lipids are used) .[11] In one study it was found that the optimum of β-D-Glc-(1,6)-β-D-Glc-archaeol to cholesterol was 15-45mol%/10-45 mol% and that forming a stable archaeosome by adding cholesterol was critical for good CD8+T cell immune responses. [54]

Importance of lipid formulation

Especially in archaeosomes there is one type of lipid which has drawn much attention to its possible immunogenic effect, namely caldarchaeol lipids (membrane spanning tetraether lipids). It has been speculated that caldarchaeol lipids confer stability to the vesicles and thereby create a depot effect at the site of injection.[14] Moreover it has been suggested that caldarchaeols can be used instead of cholesterol to confer stability to vesicle membranes since cholesterol can be oxidised.[11]

Researchers noted the TPL archaeosomes made of archaeal species with high content of caldarchaeol showed long-lasting $CD8^+$ T cell memory responses[41] , however compared to for example *H. salinarum* archaeosomes, *M. smithii* and *T. acidophilum* archaeosomes (high caldarchaeol content) show slightly lower antibody titres.[55] Therefore Krishnan *et. al.*[14] suggested that high caldarchaeol content lowers antigen processing by the phagosomal compartment for MHC class II presentation thereby favouring MHC class I presentation. *M. smithii* and *T. acidophilum* TPL archaeosomes, were the only archaeosomes in a study with multiple TPL archaeosomes tested, that produced a strong CTL recall response at >50 weeks, probably due to their high caldarchaeol content.[56] However, that long term CTL responses in archaeosomes can only be induced by caldarchaeols, has been proven false by Sprott *et al.*[41] who synthesised archaeosomes of archaeol lipids with sugar head groups that elicited long term CD8+ T cell responses.

For liposomes, it seems that there is not a particular kind of lipid besides cholesterol that through membrane stabilisation elicits one immunological route over another. However it has been noted that some cationic lipids (as discussed earlier) are inherently immunostimulatory, for example TDB in the liposomal formulation CAF01 has been noted for its immunostimulatory effect through the TLR-independent Syk/Card9-dependent pathway. [9,57]

Shape of the vesicles

Particle size

Although particle size has been extensively reviewed for liposomes, this review would not be complete without a mention of this critical physical property.

In terms of what sort of immunogenic response the vesicles induce, it was posed[6] that smaller sized particles (<500 nm) induce CTL responses through the Th1 route and larger particles (>500) induce a humoral response through the Th2 route. Possibly due to the fact that the small particles look more like a virus-like particles while the larger ones more like a bacterial cell.[6]
It was suggested[9] , based on two different studies[58,59] that Th1 response was elicited by vesicles of 250-750 nm. One of these studies[58] showed that immunised mice treated with larger liposomal vesicles (225 and 560 nm filled with OVA) produced higher amounts of IgG2a and IFN-γ (Th1) and the IgG1 response was similar for the different sizes (100 nm, 155nm). In another study[59] , in which vesicles of <200 nm, 700 nm and 1,5 µm in diameter were compared, the 700 nm vesicle produced the highest IFN-γ response.[9] Moreover, a recent review article suggested yet another point of view, that smaller liposomes (≈250 nm) showed Th2 response while larger liposomes (≈980 nm) produced high levels of IgG2 and IFN-γ characteristic of Th1 response.[32] Therefore all these results do not give a clear understanding about the effect of the particle size on the immunogenicity.

Particle size also has an effect on trafficking to the lymph nodes, antigen uptake and processing by APCs. It is the general understanding that larger particles remain at the site of injection and are phagocytised by APCs which are then transported in high quantities to the lymph nodes, while smaller particles drain to the lymph nodes by themselves.[9,32] However a recent study on the biodistribution of DDA:TDB liposomes showed no significant difference in drainage to the lymph nodes of the different sized vesicles (<200 nm, 500-600nm, 1500 nm) nor in the macrophage uptake.[47]

Oddly enough comparison studies on immunogenic response and uptake assays for differently sized archaeosomes have not been conducted. Whether this is because it has already been extensively studied for liposomes (although there seems to be some disagreement) and since

archaeosomes are sufficiently similar the liposomes, these conclusion can be used. Or it might be that the synthesis of differently sized archaeosomes is quite difficult

Lamellarity

Lamellarity is a characteristic which has not been properly and extensively evaluated for liposomes nor for archaeosomes although it is a very important physical feature which might have influence on immunogenicity.

This characteristic for liposomes was investigated only by Watson *et al.*[9] In this review, two studies[60,61] comparing lamellarity were taken into consideration. One of them[60] claimed that BSA specific antibody responses were higher in unilamellar vesicles than multilamellar vesicles (size and composition were kept the same), while in the other study[61] , size was not kept constant between the different lamellar vesicles and therefore conclusions are not trustworthy.

For archaeosomes the only mention of different lamellar vesicles, was in a study of Sprott *et. al.*[55] They assessed the incorporation of AS in the two different types of lamellar archaeosomes and concluded that unilammelar and multilamellar behaved the same in binding of phosphoserine. Assessment of the influence of lamellarity on immunogenicity was not made.

Cochleates and Archaeal Lipid Mucosal Vaccine Adjuvant and Delivery (AMVAD) systems

Cochleates and AMVADs are vaccine delivery systems on their own and the amount of information on these systems is too extensive to review here. However this study would not be complete without a mention of them, since they are well known for mucosal immune responses.

A cochleate is defined as “a multilayered, cylindrical structure comprised of a continuous, solid, lipid bilayer sheet rolled up in a spiral, uniquely devoid of any internal aqueous space(s)” and calcium ions hold the structure in its rolled form. The most important component of the mucosal defence system is IgA antibody production but also IgG antibodies play a role in mucosal defence. In one study, 3 out of 5 orally immunised mice showed good IgA titers in serum and saliva as well as high IgG titers. Antibody responses increased for up to 5 months following single immunization with influenza glycoprotein cochleates. [62] Since then successful cochleate formulations like AFCo1 have been designed.[63]

AMVADs are larger spherical structures that aggregate like a bunch of grapes, containing no aqueous compartment and containing at least one type of archaeal lipid. AMVADs have been shown to elicit strong immunogenic responses in mice against *Francisella tularensis* (LVS), when intranasally immunized with cell free extract of LVS linked to AMVAD (antibody response, splenocyte proliferation and IL-17 production) and protected against a lethal intranasal dose of LVS.[64] This is one of several examples that show the qualities of AMVAD as a mucosal vaccine.[65,66]

Discussion and conclusion

Vaccination is one of the most important techniques to prevent and cure diseases. Although the discipline has been developed and improved since the first vaccine made by Edward Jenner, a better understanding of the immune system and how it interacts with different types of vaccines would be helpful to develop new vaccines against major infectious diseases.

The future of vaccine should be tailor-made and not vaccines based on trial and error. To achieve this goal a profound understanding of the immune system is necessary, in order to understand what kind of immune response a certain pathogen elicits.[6] To each pathogen should be assigned a profile of the immune response that is necessary to prevent an infection (humoral v.s. cell mediated, Th1 v.s. Th2, etc.). Based on this profile a choice of antigen and adjuvant can be made. To achieve this goal the adjuvants systems therefore needs to be characterised on their physiochemical properties and on their effects on the immune response.

In this thesis a comparison of physiochemical differences of liposomes and archaeosomes and the effect on the immunogenicity was made, moreover the advances in physiochemical characteristics alteration and how this effect the immune response was considered. Although these systems are in concept very similar since both are vesicles made of lipids which can incorporate hydrophobic, hydrophilic and amphiphilic substances and deliver them to APCs; the disciplines have more or less developed themselves independently. Characteristics that have been extensively studied for one system are almost not considered for the other and vice versa.

Archaeal lipids, because they are derived from organisms that thrive at extreme condition are more stable than conventional lipids. They are more resistant against oxidative stress, high temperature, alkaline or acidic pH, action of phospholipases, bile salts and serum media and therefore can be fabricated and stored more easily than liposomes. Furthermore natural archaeosomes are inherently immunogenic, which conventional liposomes are not. Several studies, in which conventional liposomes and archaeosomes are compared, therefore claim that archaeosomes are a more promising delivery system then liposomes.[11,29] However these conclusions are based on conventional liposomes but the field of liposomes has improved itself since then. Nowadays, liposomes can incorporate co-adjuvants to improve immunogenicity and some synthetic cationic lipids have been shown to be inherently immunogenic. Moreover physical alterations in both particulate vaccine systems can alter the immune response.

In liposomes charge seems to greatly affect the immunogenicity and cationic liposomes are claimed to be the best, since they can form electrostatic interaction with the antigen, interact with high number of APCs, fuse with the membrane thereby releasing antigen directly in the cytosol and elicit both humoral and cell-mediated responses. This property has not really been studied in archaeosomes, possibly due to the fact that the synthesis of semi-synthetic cationic archaeal lipids it is a quite recent technique or maybe because of the endogenous characteristics of the natural archaeosomes mentioned above. However, comparative charge studies for archaeosomes are worth a try. Both in archaeosomes and liposomes (beside additional adjuvants) surface alterations, which represent the vesicle part that can interact with receptors on APCs, were studied to see if immunogenicity can be improved. In archaeosomes addition of certain sugar head groups improves the cell mediated response, in liposomes this technique has not been well studied until a recent article recorded higher antibody titers through addition of sugar headgroups. This field can be more extensively studied by characterising the receptors on the different APCs and possible sugar candidates which can interact with them. One receptor which is well characterised is the phosphatidylserine receptor present both in archaeosomes and liposomes which allow a better incorporation of either archeatidylserine or phosphatidylserine, which both seem to improve the immune response. Alteration in membrane fluidity has been well studied, especially in liposomes. In both systems it seems that, the more rigid the vesicle the better immune response is elicited, although this is not unanimously supported. In liposomes more rigid vesicles can be made through unsaturation of the fatty acid tails and incorporation of cholesterol. In archaeosomes the incorporation of cholesterol has also been used besides the more common method of incorporating

membrane spanning caldarchaeol. The increased immune responses observed upon the incorporation of caldarchaeol are due to better stability gained or some other unknown properties. Particle size has been extensively studied in liposomes but conflicting conclusion were drawn whether a certain size can elicit a specific response (humoral or cell-mediated). In archaeosomes this property has not been studied. Furthermore lamellarity is a property which should be more extensively studied in both systems, since there is a poor knowledge about how this property affects immunogenicity. Special aggregated types of vaccine delivery systems are cochleates and AMVADs which are both very promising mucosal vaccine delivery systems.

In conclusion, this review analysis underlines that the discussion should not focus on which delivery system (archaeosomes or liposomes) is better, but on how the choice of certain lipids, headgroups, stabilising agents, co-adjuvants, particle properties (e.g. size) affects the immune response and on what kind of response is elicited. Moreover there should be a wider understanding of how these properties affect each other (for example addition of cholesterol may be beneficial for liposomes with a high T_m but not for vesicles with an intermediate T_m). In this way it will be possible to create a powerful toolbox which might be useful in designing vesicles with a specific immune response against a specific disease.

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