Advanced Review

Topical and mucosal liposomes for vaccine delivery

Eder Lilia Romero* and Maria Jose Morilla

Mucosal (and in minor extent transcutanous) stimulation can induce local or distant mucosa secretory IgA. Liposomes and other vesicles as mucosal and transcutaneous adjuvants are attractive alternatives to parenteral vaccination. Liposomes can be massively produced under good manufacturing practices and stored for long periods, at high antigen/vesicle mass ratios. However, their uptake by antigen-presenting cells (APC) at the inductive sites remains as a major challenge. As neurotoxicity is a major concern in intranasal delivery, complexes between archaeosomes and calcium as well as cationic liposomes complexed with plasmids encoding for antigenic proteins could safely elicit secretory and systemic antigen-specific immune responses. Oral bilosomes generate intense immune responses that remain to be tested against challenge, but the admixing with toxins or derivatives is mandatory to reduce the amount of antigen. Most of the current experimental designs, however, underestimate the mucus blanket 100- to 1000-fold thicker than a 100-nm diameter liposome, which has first to be penetrated to access the underlying M cells. Overall, designing mucoadhesive chemoenzymatic resistant liposomes, or selectively targeted to M cells, has produced less relevant results than tailoring the liposomes to make them mucus penetrating. Opposing, the nearly 10 μm thickness stratum corneum interposed between liposomes and underlying APC can be surpassed by ultradeformable liposomes (UDL), with lipid matrices that penetrate up to the limit with the viable epidermis. UDL made of phospholipids and detergents, proved to be better transfection agents than conventional liposomes and niosomes, without the toxicity of ethosomes, in the absence of classical immunomodulators.
 WIREs Nanomed Nanobiotechnol 2011 DOI: 10.1002/wnan.131

INTRODUCTION

In first place, a brief overview on the anatomical and phenomenological constraints for mucosal and transdermal delivery of particulate material to antigen-presenting cells (APC) will be presented. Later selected results upon administration of intranasal, oral, and transdermal liposomes and other vesicles as adjuvants will be critically discussed. On those basis, a relationship between structure and function of liposomes and immune response will be elaborated.

Intramuscular (i.m.) and subcutaneous (s.c.) administration are the two most widely used vaccination routes.[1] Briefly, immune responses are triggered after delivered antigens (Ag) are captured by APC and processed through different intracellular pathways. Ag processed via cytoplasm are expressed via MHC-I molecules, while those processed via the endo-lysosomal system are expressed via MHC-II molecules. The interaction between MHC-I and co-stimulatory molecules from APC with CD8+ T lymphocytes generates cytotoxic T lymphocytes (CTL) and memory cells, whereas the interaction between MHC-II and co-stimulatory molecules from APC with CD4+ T lymphocytes generates T helper lymphocytes and antibodies producing B cells. The adaptive immune responses are favored under inflammatory contexts induced by cells of the innate immunity. The i.m. route, however, is not optimal for Ag delivery to APC. The muscle content of APC is poor and the expression of MHC class II and co-stimulatory molecules is absent in myocytes which cannot directly prime T cells. Mucosal and transcutaneous are alternatives to parenteral administration that present a series of advantages. In first place, the problems

*Correspondence to: elromero@unq.edu.ar

Nanomedicine Research Program (NRP), Departamento de Ciencia y Tecnologia, Universidad Nacional de Quilmes (UNQ), Bernal, Argentina

DOI: 10.1002/wnan.131

associated with the use of injectables are avoided[2,3] and self-administration is feasible bringing higher patient compliance.

Other unique properties of nonparenteral vias are better understood after a closer look to the organization of the immune system, which is divided into inductive and effector sites. In the former areas, the Ag sampling leads to initial activation of immune cells, whereas in the latter the antibodies and cells of the immune system perform their specific function upon activation.[4] The inductive sites in mucosa are region specific lymphoid tissues and their surrounding regional lymph nodes, composed of B-cell follicles, APC, and T lymphocytes, known as mucosa-associated lymphoid tissue.[5] The two main inductive sites in mucosa- are the gut-associated lymphoid tissue (GALT) in the gastrointestinal tract (GIT) and the nasopharynx-associated lymphoid tissue (NALT) in the nose.[6] Both are similarly organized, with follicle-associated epithelium containing cells (M cell in GALT and M-like cells in NALT), specialized in take up and transport particulate Ag and organisms from the lumen to the underlining APC in the follicles, across the epithelial barrier (transcytosis).[7–10] The inductive sites in the skin (2 m^2 surface area in humans) lie between 10 and 200 μm below the stratum corneum (SC).[11] Keratinocytes, Langerhans cells, dermal dendritic cells, subsets of T lymphocytes, and lymph nodes constitute the skin-associated lymphoid tissue (SALT).[12–14]

Mucosal immunization is the only way to induce an effective B cells class switching and production of secretory IgA (sIgA),[15] both in local and distal mucosa.[16] It also induces Ag-specific serum IgA and IgG antibodies and favors cell-mediated immune response.[17,18] Mucosal immunity may be less affected by aging than that induced by parenteral route.[19] Immunization by the intranasal (i.n.) route (160 cm^2 surface area in humans)[20] offers a series of advantages over other mucosal routes: (1) it requires less amount of Ag than by oral route, (2) it is optimal to generate secretory immunity against pathogens transmitted via aerosols in the respiratory mucosa,[21–23] (3) it induces sIgA in saliva, urine, and rectum,[24] (4) it induces sIgA and CTL in female genital tract,[25] in a more predictable way and with major compliance than by vaginal route,[26] (5) it produces greater systemic antibody responses than immunization by other mucosal routes,[27] and (6) it can be used for vaccination of large populations within a short period of time in case of a sudden epidemic. On the other hand, the oral route is of major relevance in pediatric vaccination.[28,29]

The high density of APC, laying several micrometers under the body surface or beneath the GIT and nose mucosa, however, is only available for Ag delivery if a series of physical and phenomenological constraints can be surpassed. Soluble Ag, for instance, are sensitive to degradation in GIT. In general, soluble Ag are unsuitable to generate protective immunity upon oral or i.n. administration and in some cases can induce systemic tolerance.[30] Large doses of oral Ag can lead to a short-lived secretion of Ag-specific IgA, without the induction of a serum antibody response. It was observed that nearly 100-fold higher Ag amounts have to be given orally to induce a 100-fold lower response as compared to s.c. administration.[31–33] To surpass this problem, oral vaccination requires the coadministration of high doses of powerful adjuvants. For instance, ADP-ribosylating enterotoxins from *Escherichia coli* (thermolabile enterotoxin, LT) and from *Vibrio cholerae* (CT), responsible for fulminant gastrointestinal losses of water and electrolytes which accompany clinical infection, bind to ganglioside GM1 receptors present on enterocytes, dendritic cells, macrophages as well as B and T lymphocytes.[34] When coadministered with soluble Ag, CTL, Ag-specific sIgA, serum IgG, and long-term memory are induced.[35–37] The adjuvant activity of these toxins may result from improved Ag uptake as well as the induction of secretion of various cytokines.[34] Other powerful mucosal adjuvants are CpG-DNA (synthetic unmethylated CpG dinucleotide, which is a ligand of toll-like receptor 9 on cells of the innate immunity) and monophosphoryl lipid A (a ligand of the toll-like receptor 4), a nontoxic derivative of the lipopolysaccharides from *Salmonella minnesota*. CpG-DNA and monophosphoryl lipid A lead to the release of various pro-inflammatory cytokines that can influence adaptive immune responses and B-cell activation. Mucosal adjuvants, however, have to be given orally to mice in high doses in the presence of bicarbonate to prevent intragastric degradation. In humans, oral and i.n. administration of CT and LT is associated with diarrhea at doses as low as 5 μg[38,39] and neurotoxicity.[40,41] On the other hand, transcutaneous (topical) immunization requires the physical/chemical disruption of the SC[42,43] and also the use of immunomodulators such as LT,[44] its mutants (LTK63 and LTR72[45]), or CT.[35,36,46] Upon applied on hydrated skin, these immunomodulators induce strong systemic and mucosal responses,[47] posing risk of autoimmune or inflammatory reactions.[48] Transcutaneous application of mucosal immunomodulators such as CpG-DNA, lipopolysaccharides, muramyl dipeptide, alum, IL-2, and IL-12 generate weaker and more transient responses than CT or LT.[49] Currently no toxins or derivatives have been approved for human use.[50–53]

The induction of an immune response by nonparenteral vias is extraordinarily depending on the aggregation state of the Ag. Immature Langerhans cells and dermal dendritic cells, as well as dendritic cells in the basolateral pocket of the M cells readily take up particulate material.[16,54,55] As particles are more efficiently taken up by APC than soluble molecules, particulate Ag induces stronger immune responses than soluble Ag, independently of the route of administration.[8,56] However, the architecture of inductive sites in mucosa and skin interposes higher physical constrains for delivery of particles to APC than for soluble Ag. The structure of the SC (10- to 15-μm thick apposed layers of dead keratinized cells embedded in a lipid matrix lacking phospholipids) impairs the diffusion/penetration of transcutaneous soluble or particulate Ag.[11,44] In oral administration, apart from dilution in a degradative chemoenzymatic environment, the alimentary status and the tight epithelial junctions, the delivery of particulate Ag is mainly impaired by the mucus blanket.[57] The mucus (a viscous colloid containing antiseptic enzymes such as lysozyme, proteins such as lactoferrin and anionic glycoproteins known as mucins) from GIT, respiratory and urogenital mucosa is specialized in wrapping up particles, impairing their contact with the epithelium.[58] A nearly 10-μm thick mucus layer is secreted by enterocytes at 1–100 μm/s consisting of a luminal layer (rapidly cleared, within minutes to hours) on the top on an adherent layer (slowly cleared, within hours to days). A bed of 500-μm thick glycocalyx covering the apical side of the enterocytes that becomes considerably thinner (nearly 30 μm) on the surface of M cells is an additional physical barrier.[59] The penetration of particles is effectively impaired by the bulk viscosity of healthy human mucus which is typically 1000–10,000 times higher than the viscosity of water (at low shear rates).[60] Theoretically, no particulate material could diffuse across the mucus, neither virus nor small hydrophilic molecules. In the less destructive environment of the nose, the upper gel mucus layer is cleansed and replaced within 10 min, whereas the underlying sol mucus layer is slowly cleared. Particles trapped in the upper mucus layer are removed from respiratory epithelium to the nasopharynx and stomach by the mucociliary escalator at 10–100 μm/s.[61] Because of the physical barrier of the mucus, only mucus-penetrating infective agents such as wild or attenuated microorganisms reach the underlying epithelia and stimulate the mucosa.[52] Live or attenuated microorganisms, however, lead to risks of reactivation especially in immunocompromised people.[53] Hence, in order to avoid rapid mucus clearance and to reach the underlying epithelia, particles should quickly traverse at least the outermost layer of the mucus barrier (which are cleared most rapidly).[59]

It was recently demonstrated that properly engineered particles up to 500 nm diameter can rapidly enter and cross the human mucus, with diffusivities as high as only fourfold reduced compared to their rates in pure water.[62] One of the reasons that allow displacement of nanoparticles is the opening of low viscosity aqueous channels (mesh spaces) caused by the aggregation of mucin fibers into cables.[63] As the mucine concentration is increased, the probability of formation of cables and of the number of freely diffusive pathways (100- up to 500-nm mesh spacing) becomes higher. Taking this fact into consideration, it can be proposed that to reach the surface of M cells, particles need to be mucus penetrating instead of mucoadhesive.[63,64] Determined structural characteristics enable the particles displacement through the channels. For instance, particles between 35 and 75 nm have the optimal diameter to fit into the channels. The role of Z potential is more complex. The mucoadhesivity of cationic particles such as polyethylenimine, chitosan, and polylysine is caused by the multiple contact points established with the negatively charged mucins. Therefore, particles with low cationic charge density surfaces stick to the luminal layer of mucus and are rapidly removed. Hence, multiple ionic interactions are useless to improve the mucus penetration of particles. However, particles with high cationic charge density surfaces or wrapped by highly concentrated chitosan can induce the collapse of the mucin cables, increasing the chances of penetration of the particles. Indeed, a high number of particles can saturate the binding points of mucus, which no longer retain material into the mesh.[65,66] Anionic particles, on the other hand, are repelled in the surface of the anionic mucus, having lower chance of penetration across the low viscosity channels.[67] Particles with equal number of cationic/anionic charges and relatively hidden hydrophobic sites have maximal chances to penetrate. Finally, highly hydrophobic particles [poly(lactide-co-glycolide and polyanhydrides)] also establish multiple contact points with the mucin core, and their penetration velocity is decreased. Hydrophobic particles establishing minimal contact points have higher chances to penetrate.

Recent reviews have addressed the use of particulate Ag, specifically nanosized material.[68–70] Ideally, the structure of mucosal and transcutaneous particulate adjuvants must be suitable to cross the mucus and SC barriers. By overcoming the physical impairments (which are absent in parenteral administration) to gain the APC, nonparenteral particle delivery should

diminish the Ag dose as well as the need for potentially toxic immunomodulators coadministration.

Liposomes are nonimmunogenic vesicles made of 1,2 *sn*-glycerophospholipids with or without cholesterol.[71] Virosomes, archaeosomes, niosomes, bilosomes are also vesicles but their composition is based on amphiphilic molecules other than 1,2 *sn*-glycerophospholipids. For biological applications, any vesicle under 200–300 nm is a nanoparticle.[72] Liposomes and other vesicles have a number of competitive advantages over other nanoparticles. Liposomes, for instance, can be prepared in a wide range of sizes and compositions at high efficiency of Ag encapsulation and with high stability of Ag association. Liposomes between 20 and 100 nm diameter offer 0.2–2 μL/μmol lipid encapsulation volume, with an encapsulation efficiency of hydrosoluble active between 15 and 50%.[73] Techniques for massive production under good manufacturing practices[74–77] to afford long periods of storage[78] are available, and different to polymeric nanoparticles such as those made of polylactide-co-glycolide no major concerns on Ag stability are presented during storage.[79] As the European guidelines on adjuvants destined for human vaccines recommend completing distribution studies,[80] it is important to stress that methods of double radioactive labeling to follow Ag and lipids biodistribution of liposomes are already available[81] as well as to identify optimal high-throughput industrial production conditions for compositions of interest.[82] Liposomes are biodegradable and exhibit a huge record of safety when repeatedly administered by parenteral routes.

INTRANASAL ROUTE

Currently, an annual i.m. injection of a trivalent vaccine is generally used for protection against influenza virus.[83] However, the vaccine does not induce respiratory mucosal immune response, which is important in the first line of defense against influenza. The only licensed commercial mucosal influenza vaccine (FluMist) is live attenuated and quite effective, but remains too expensive for poor societies. Production of a nonliving vaccine would be cheaper.[84]

Until now, i.n. virosomes (that reached the market) and liposomes (still in preclinic developments), have been unable to replace conventional vaccination. Virosomes are 100–200 nm mean diameter vesicles[85] containing immunogenic viral fusion proteins in their membranes. In particular, influenza virosomes contain viral membrane lipids, hemagglutinin, and neuraminidase proteins.[86] Neuraminidase cleave *N*-acetylneuraminic acid from bound sugar residues. The decreased viscosity of mucus allows an easier access of virosomes to epithelial cells (note that virosomes were the first mucus-penetrating vesicles). Hemagglutinin has high affinity for *N*-acetylneuraminic acid on APC surface, and has got pH-sensitive fusogenic activity. As for the native influenza virus, binding of virosomes to the sialic acid residues on the surface of APC, will initiate uptake of the virosomes through receptor-mediated endocytosis. On the other hand, the immunostimulating reconstituted influenza virosomes (IRIVs) are vesicles of size similar to virosomes, which contain nearly 70 mol% of external lipids.[87] The addition of external lipids reduces the fusogenic activity of IRIVs that becomes negligible at 75 mol%.[86] Ag loaded in virosomes or IRIVs induces an immune response that depends on their intracellular processing pathway. Ag loaded in fusogenic virosomes or IRIVs are delivered to the cytoplasm, upon an acid-triggered fusion with the membrane of endosomes, and presented by MHC class I to induce CTL-mediated responses, which are optimal to attack virus and cancer cells. On the other hand, Ag in virosomes that do not fuse with endosomal membranes, are processed via an endo-lysosomal pathway and presented by MHC class II to induce either a B- or T-cell response.[88–90] Although lipids in virosomes served as an inert matrix to include the enzymes, the role of the optimal protein/lipid ratio is still unclear.

Preclinical studies showed that i.n. influenza virosomes or IRIVs require the coadministration of immunomodulators like LT[91] or lipopeptide (*N*-palmitoyl-S-2,3(bispalmitoyloxy)-propyl-cysteinyl -seryl-(lysil)3-lysine[92] in two successive immunizations to enhance sIgA levels and hemagglutination-inhibiting antibody response, which protected against challenge. Effectively, upon twice i.n. administration of NasalFlu®, a trivalent IRIV vaccine containing LT, an almost total prevention of virus shedding in ferret model of influenza was achieved,[93] without systemic or neurological adverse effects.[94] Promising clinical trials showed humoral- and cell-mediated responses, with high mucosal sIgA neutralizing antibodies.[94,95] NasalFlu® was marketed in Switzerland by Berna in 2001. However, after an increased occurrence of Bell's palsy observed in people who had recently received the vaccine, NasalFlu® had to be removed from the market.[41] IRIVs-based vaccine was the only i.n. vesicular adjuvant achieving the market, and its withdrawal underscores the dangers associated to the administration of exogenous material by i.n. route. Intranasal administration enables the direct contact with primary olfactory neurons that communicate through their axons with the olfactory bulb in the

brain.[96] This pathway leads to a direct nose-to-brain delivery.[97,98] For instance, accumulation of CT in the olfactory nerve and bulb was observed after its i.n. administration. This should occur upon its neuronal uptake via GM1, abundantly expressed in the central nervous system.[36,99] On the other hand, recently two cases of Bell's palsy temporally associated with i.n. administration of genetically detoxified LTK63 were reported. This was possibly owed to a transient interference with peripheral nerve function, caused by the accumulation of LTK63 molecules. Alternatively, it could result from inflammation arising from immune response to LTK63, after GM1 binding and retrograde neuronal transport.[100] Clearly the induction of immune responses in the absence of neurotoxicity is the bottle neck for i.n. delivery.

Depending on their size and lamellarity liposomes are classified as small unilamellar vesicles (SUV, $\leq$50 nm), large unilamellar vesicles (LUV, 200–400 nm), and multilamellar vesicles (MLV, several micrometers).[101] Especially upon s.c., i.m., or intraperitoneal (i.p.) administration, liposomes may act as a depot for slow release of the Ag over extended periods of time, to favor the Ag uptake by APC such as dendritic cells and macrophages.[102] In mucosal administration, liposomes protect the entrapped Ag against degradation or neutralization. An analysis of the structure of liposomes employed as i.n. adjuvants against influenza virus (Table 1) clearly indicated that the lipid matrix was not responsible for the elicited immune response. For instance, i.n. but not s.c. liposomes bearing T cell epitopes admixed with the immunomodulator anti-CD40 antibody protected mice against challenge. Remarkably, the sole activity of the anti-CD40 monoclonal antibody was not sufficient to replace the function of CD4+ T cells, required for the induction of CTL activity to clear the virus from the mucosal sites. In this case, liposomes were used as an inert lipid matrix for ligand attachment.[103] In another work, the protective efficacy elicited after i.n. liposomal CpG co-encapsulated with hemagglutinin and neuraminidase influenza proteins was higher as compared to free CpG, and similar to that of animals immunized with CT. Again liposomes were used as inert lipid matrices to load Ag and an immunomodulator.[104] In the following approach, i.n. cationic liposomes complexed with a plasmid encoding influenza hemagglutinin withstand a lethal challenge in mice, with a shift toward a Th1 response and involvement of B memory cell. Intramuscular naked or liposomal plasmid was also protective against a lethal challenge, with increased serum IgG but in the absence of sIgA. The intensity of the cell-mediated response after i.n. and i.m. administration was slightly lower for the liposomal formulation.[105] This was an example of liposomes as cationic lipid matrix to form a lipoplex with a plasmid.

The two followings works are examples of liposomes used as inert lipid matrix carrying lipid immunomodulators. In the first one, polycationic sphingolipid (ceramide carbamoyl-spermine) containing liposomes were highly efficacious following a single or repeated (2×) i.n. administrations. Serum and mucosal elicited antibodies were equivalent or superior to those obtained with commercial split virion trivalent vaccine coadministered with CT. No systemic adverse effects, only a mild local inflammatory response, were observed in mice and rabbits intranasally vaccinated with these liposomes. The ceramide carbamoyl-spermine should be recognized as a danger signal. A role for the size of the vesicles could not be identified.[106] In the second one, cationic cholesterol derivative (a special lipid immunomodulator) containing liposomes elicited similar serum responses but higher mucosal hemagglutination-inhibiting activity to that induced after s.c. immunization with the vaccine alone.[107]

Only one different approach relayed on the particular composition of a lipid matrix. Vesicles made of the immunomodulator trehalose 6,6′dibehenate and the cationic surfactant dimethyldioctadecylammonium bromide elicited both significantly higher IFN-γ and serum IgG levels than i.n. immunization with the commercially available influenza vaccine. Surprisingly, the induction of sIgA production was not reported. *In vitro* assays demonstrated that these liposomes enhanced Ag transport through the mucus layer on polarized Calu-3 cells and did not produce changes in epithelial integrity and viability.[108]

As exogenous Ag are mainly presented by B cells through the MHC class II pathway to Th2 cells, the current recombinant vaccine against hepatitis B provides humoral but no cellular immunity. This is not suitable as therapeutic vaccine in the treatment of chronic hepatitis B.[138] Moreover, the number of hepatitis B virus, hepatitis C virus, and human immunodeficiency virus infections among health care professionals attributable to sharps injuries are estimated to 83,000 incidences per year,[139] a good reason to search for alternatives to parenteral vaccination. Only two preclinical studies using liposomes as proofs of principle were carried out to date, and again liposomes were used as inert lipid matrices. In the first of them, i.n. glycol chitosan modified liposomes containing a plasmid encoding hepatitis B surface small protein (HBsAg), generated serum anti-HBsAg titers that were lower than those elicited by i.m. naked plasmid and alum adsorbed HBsAg (alum-HBsAg)

TABLE 1 | Liposomes and Vesicles Used for i.n., Oral and Topical Immunization

Vesicle Type	Description	Particle Size, *Z* Potential	Route	Antigen	Effect	Reference
MLV	PS/PC (1:2.2) admixed with anti-CD40 monoclonal antibody	>1 μm	i.n.	CTL epitope for influenza A virus nucleoprotein	Protection against challenge in mice	103
MLV	DMPC/DMPG (9:1)	1.5 μm	i.n.	Influenza hemagglutinin and neuraminidase proteins, co-encapsulated with CpG	Induced high levels of lung IgG1 and IgA, nasal IgG1, IgG2a and IgA, and splenocyte IFNγ in mice and protection against challenge	104
Cationic LUV	DODAC/DOPE/ PEG-C8 (7:78:15, % w/w)	100 nm	i.n.	Plasmid encoding influenza hemagglutinin protein	Induced humoral IgG, IgA, and hemagglutinin-specific sIgA titers in bronchoalveolar lavage in mice, and protection against challenge	105
Cationic MLV	*N*-palmitoyl D-erythro-sphingosyl carbamoyl-spermine/ chol (3:2)	0.05–10 μm	i.n.	Monovalent subunit Ag preparation from influenza virus (80–90 wt% hemagglutinin, 5–10 wt% neuraminidase and trace amounts of nucleoprotein and matrix proteins)	Induced serum and mucosal (lung, nasal) strain cross-reactive hemagglutination-inhibiting antibody, IgG1, IgG2a, IgA, and splenocyte IFNγ. Protection against challenge persisted 9 months on young and old mice	106
Cationic LUV	3β[*N*-(*N*′,*N*′-Dimethylaminoethane)-carbamoylcholesterol/DOPE (2:3)	200 nm	i.n.	Split inactivated influenza vaccine (containing equal amounts of hemagglutinin, and nucleoprotein, and trace amounts of neuraminidase and matrix proteins)	Induced serum IgG1 and IgG2a, weak IgA responses in serum and in the naso-pharyngeal washes of mice	107
MLV	Dimethyldioctadecylammonium bromide/trehalose 6,6′-dibehenate (5:1, w/w)	450 nm, 60 mV	i.n.	Admixed with commercially available influenza split vaccine	Induced high IFN-γ and serum IgG levels in mice	108
MLV	PC/DOPE/chol (2:1:0.5) coated with glycol chitosan	700 nm, 9.8 mV	i.n.	Plasmid encoding HBsAg	Induced low serum IgG, but high sIgA in nasal, salivary, and vaginal secretion; IL-2; and IFNγ in spleen homogenates of mice	109
Liposomes *in situ* gelling system	LUV (PC/chol, 7:3) suspended in 0.3% w/v polyacrylic acid at pH 4.2, further suspended in polyacrylic acid solution containing HBsAg	720 nm, −36 mV	i.n.	HBsAg	Induced serum IgG and sIgA in salivary, intestinal, vaginal and nasal secretion of mice	110

TABLE 1 | continued

Vesicle Type	Description	Particle Size, *Z* Potential	Route	Antigen	Effect	Reference
MLV	DPPC/chol/DPPE-covalently conjugated with mannotriose (1:1:0.1)	~1 μm	i.n.	Ovoalbumin	Induced IgG and IgA in serum sIgA in nasal washes and IFNγ by secreting cells of NALT, in mice	111
MLV	PC/dicethylphosphate/Chol (7:1:7) covered with chitosan	2.4 μm	i.n.	Tetanus toxoid	Induced low sIgA in nasal lavage and serum IgG in rabbits	112
Proteoliposomes	Derived from *N. meningitides B*	70 nm	i.n.	Glycoprotein D2 from Herpes Simplex Virus type 2	Induced IgG and IgA in saliva and vaginal washes. Partially protected against vaginal challenge in mice	113
AMVAD	Complexes of archaeosomes made of lipids from *M. smithii* with $CaCl_2$	40 × 20 μm	i.n.	Ovoalbumin	Induced serum IgG and sIgA in fecal, vaginal wash, and nasal lavage of mice. High IgG1 versus IgG2a ratio in serum and CTL response	114
LUV	eggPC/DOPE (1:1) with 30% w/w 3-methylglutarylated poly(glycidol)	100 nm, −11 mV	i.n.	Ovoalbumin	Activate cellular immunity in mice	115
MLV, LUV mixture	eggPC/chol (1:1) mixed with aluminum hydroxide gel	0.1–0.5 μm	Oral	Diphtheria and tetanus toxoid	Induced specific antibodies against both toxoids in monkeys	116
MLV	Sphingomyelin bovine brain/chol (10:4 w/w)	>1 μm	Oral	Mixture of *Vibrio cholerae* Ag (crude fimbrial extract, LPS and heat-treated high molecular weight CT)	Induced intestinal IgM and IgA, but not IgG in human volunteers	117
LUV	DSPC/POPC/chol/DPPE-covalently conjugated with CTB	100 nm	Oral	Saliva-binding region of the AgI/II adhesin from *Streptococcus mutans*	Induced mucosal IgA and serum IgG, that persisted at high levels for at least 6 months in mice	118
LUV	PC/chol (1:1)	100–500 nm	Oral	Fusion peptide of CTB and *H. pylori* urease B subunit epitope	Induced serum IgG and mucosal sIgA in mice	119
MLV	eggPC/Chol/PE (7:2:1), coated with *O*-palmitoyl Pullulan (polysaccharide:lipids 4:6 w/w)	2.5–3.3 μm, ~0 mV	Oral	Bovine serum albumin	Induced serum IgG and IgA in mice	120
MLV	DSPC/chol/DSPE-PEG 2000 (10:5:1)	>1 μm	Oral	Ovoalbumin	Induced low serum IgG in mice induction of sIgA was not reported	121
Niosomes	Span 60/chol/stearylamine (6:3:1) coated with *O*-palmitoyl mannan	2.5 μm	Oral	HBsAg	Induced sIgA, combined serum IgG2a/IgG1 and IL-2 and IFNγ in spleen homogenates of mice	122

TABLE 1 | continued

Vesicle Type	Description	Particle Size, *Z* Potential	Route	Antigen	Effect	Reference
Bilosomes	1-Monopalmitoylglycerol/chol/dicetylphosphate (5:4:1), 20 mg/mL sodium deoxycholate	3 μm	Oral	Influenza subunit or tetanus toxoid	Induced serum IgG1, strong IgG2a response and rise in the numbers of IgA positive plasma cells in small intestine of mice	123, 124
Bilosomes	Sorbitan tris-tearate/chol/dicetylphosphate (7:3:1), 28 mg/mL sodium deoxycholate	200 nm	Oral	HBsAg	Induced serum IgG and sIgA in mucosal secretions of mice	125
Bilosomes	Sorbitan tristearate/chol/DPPE-covalently conjugated with CTB (7:3:1), 20 mg/mL sodium deoxycholate	100 nm	Oral	Bovine serum albumin	Induced serum IgG and sIgA in intestinal, salivary, and vaginal secretions of mice	126
Bilosomes	Sorbitan tristearate/chol/DPPE-covalently conjugated with CTB (7:3:1), 28 mg/mL sodium deoxycholate	200 nm	Oral	HBsAg	Induced serum IgG and sIgA in mucosal secretions of mice	127
Bilosomes	1-Monopalmitoylglycerol/chol/dicetylphosphate (5:4:1), 20 mg/mL sodium deoxycholate	250 and 980 nm	Oral	Influenza A vaccine	Both induced serum IgG1, larger bilosomes induced higher IgG2a levels and increased levels of IFN-γ in spleen supernatants of mice	128
UDL	SoyPC/(mixture of sodium cholate and sodium dodecyl sulfate 6/1) (9:2)	140–190 nm	Topical	Gap junction proteins, with or without monophosphoryl lipid A	Induced serum IgG and IgA in mice	129
Niosomes	(Span 60/Span 85)/chol/stearylamine coated with *O*-palmitoyl mannan	2.5 μm	Topical	Bovine serum albumin	Induced serum IgG, combined IgG2a/IgG1 response in mice	130
Liposomesniosomes UDL	SoyPC/chol (7:3 w:w); Span 85/chol (1:1 w:w); soyPC/Sodium deoxycholate (85:15 w:w)	200 nm	Topical	Tetanus toxoid	Induced serum IgG in different degrees in rats	131
Vesosomes	PC/DOPE/DOTAP (2:1:0.5) encapsulated within large bilayer (DPPC/chol, 97.5/2.5)	3.7 μm	Topical	Tetanus toxoid	Induced serum IgG in rats	132

TABLE 1 | continued

Vesicle Type	Description	Particle Size, Z Potential	Route	Antigen	Effect	Reference
Liposomes, niosomes	SoyPC/chol (7:3); Span 85/chol (7:3)	2.5 μm	Topical	Plasmid encoding HBsAg	Induced serum IgG, IFN-γ, and IL-2 in spleen homogenates of mice	133
Cationic UDL	SoyPC/octadecylamine (4:1 w/w)	200 nm	Topical	Plasmid encoding HBsAg	Induced serum IgG, IL-4 and IFN-γ in mice	134
UDL	SoyPC/span 80 (86:14 w/w)	~100 nm	Topical	HBsAg	Induced serum IgG, systemic and mucosal IgA in mice	135
Ethosomes	SoyPC/ethanol (2% w/v:25% v/v)	~150 nm	Topical	HBsAg	Induced serum IgG, systemic and mucosal IgA in mice	136
UDL	SoyPC/Cholate (6:1 w/w)	~100 nm	Topical	Plasmid encoding syncytial virus surface glycoproteins	Induced mucosal antibody response and IFN-γ producing cells of mice	137

Composition expressed as molar ratios, unless mass ratios is stated.
PS, phosphatidylserine; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DODAC, dioleoyldimethylammonium chloride; DOPE, dioleoylphosphatidylethanolamine; PEG, polyethylene glycol; Chol, cholesterol; DOPC, dioleoyl phosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; DSPC, distearoylphosphatidylcholine; POPC, palmitoyloleoyl phosphatidylcholine; DSPE, distearoylphosphatidylethanolamine; eggPC, PC from egg yolk; SoyPC, PC from Soybean; DOTAP, dioleoyltrimethylammonium propane; CTL, cytotoxic T lymphocytes; NALT, nasopharynx-associated lymphoid tissue; sIgA, secretory IgA; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; UDL, ultradeformable liposomes; AMVAD, archaeal lipid mucosal vaccine adjuvant and delivery.

but higher than those obtained with i.n. naked plasmid. Cytokine levels were lower than those produced by naked plasmid but higher than the induced by alum-HBsAg. The mice were seroprotective within 2 weeks. Glycol chitosan liposomes also elicited sIgA but the i.m. administrations did not.[109] However, though the authors stated the formulation was pH-sensitive because of the presence of DOPE, the composition of the lipid matrix did not correspond to a pH-sensitive vesicle.[140] In the second work from the same group, i.n. liposomes called *in situ* gelling system elicited serum anti-HBsAg titers comparable to those achieved upon i.m. alum-HBsAg. Additionally, these liposomes produced sIgA and cellular immune responses (measured by cytokine level) meanwhile alum-HBsAg did not.[110] Authors claim that this system possesses a 100% efficiency of Ag entrapment. At the pH of nasal mucosa (7.4–6.8), the polyacrylic acid solution becomes gel and a depot is created at the site of administration. The entrapped Ag is said to be released in three steps: (1) release of Ag from polyacrylic acid hydrogel, (2) release of liposomes from polyacrylic acid hydrogel, and (3) release of Ag entrapped in gel core liposomes. Hepatitis B has not been convincingly shown to be transmitted via feco-oral route. Therefore, the relevance of the observed mucosal immune response is debatable. But such a response may be useful in other forms of viral hepatitis (A, E, etc.) which are predominantly transmitted feco-orally.[141]

Finally, three recent works employed liposomes as inert lipid matrix as proof of principles for different applications. In the first one, liposomes containing a mannotriose neoglycolipid (a ligand of the mannose receptor on APC) induced an immune response in mice that resulted in 5- to 10-fold increased serum IgG and IgA upon an i.n. boost with bare ovoalbumin.[111] The second one used anionic liposomes covered by low concentration of the mucoadhesive polymer chitosan. Coating of liposomes by chitosan failed to increase both the residence time of liposomes in nasal cavity and systemic responses. Conversely, coated liposomes could not induce the mucosal responses as efficiently as noncoated liposomes.[112] As predicted, the mucoadhesivity of coated liposomes was responsible for its removal and of its poor performance as adjuvant. The third one used proteoliposomes, vesicles containing major bacterial outer membrane proteins, lipopolysaccharide/lipooligosaccharide and phospholipids. Proteoliposomes contain multiple pathogen-associated molecular patterns as immunopotentiators as well as Th1 polarization activity.[142] The protection of i.n. *Neisseria meningitides* proteoliposomes containing glycoprotein D of herpes simplex virus type 2,

against challenge was only partial. Cochleates (large, continuous, solid, lipid bilayer sheet rolled up in a spiral, with no internal aqueous space) prepared by complex formation of proteoliposomes with Ca^{2+}, on the other hand, effectively protected against challenge with genital herpes.[113]

Different to the previous approaches, the following two works employed lipid matrices that played an active role on the induction of the immune response. In the first one, archaeal lipid mucosal vaccine adjuvant and delivery (AMVAD), complexes of archaeosomes made of lipids from *Methanobrevibacter smithii* with $CaCl_2$ were used as proof of principle. Archaeosomes are vesicles made of polar lipids extracted from the Archaea domain of life.[143] Archaeal polar lipids contain ether linked isoprenoid chains, mainly phytanyl (C20, archaeols) and bysphythanediyl (C40, caldarchaeols), in *sn*-2,3 enantiomeric configuration to glycerol moiety.[144] Mice i.p. or s.c. immunized with archaeosomes from *M. smithii* (40% archaeol, 60% caldarchaeol) containing soluble Ag, elicited Ag-specific systemic antibody and cellular immune response, including CD8+ CTL responses.[145,146] Intranasal AMVAD containing ovoalbumin induced serum IgG and mucosal sIgA responses similar to that obtained with CT, and mucosal and systemic immune memory responses in the absence of foreign immunomodulators.[114] A suitable explanation on the action mechanism of AMVAD, however, was not provided by the authors. It is especially intriguing the fact that i.n. archaeosomes induced inferior responses than those elicited with higher sized AMVAD. The *Z* potential of archaeosomes is strongly negative, and in consequence the archaeosomes should slip on the mucin layers. The screening of the negative charges by the Ca^{2+} should allow some vesicles to be mucus penetrating and that should be the reason, instead of the increased size, why these complexes functioned as better i.n. adjuvants. In the second one, pH-sensitive liposomes were also used as proof of principle. Similar to virosomes or IRIVs, the bilayer of pH-sensitive liposomes experiences an acid-triggered fusion with the membrane of endosomes once endocytosed. In consequence soluble Ag loaded in liposomes is released into the cytoplasm and it is presented by the MHC-I type pathway, responsible for cell-mediated responses.[147] Liposomes can be made pH-sensitive by wrapping inert lipid matrices with pH-sensitive polymers such as carboxylated polyglycidols. The hydrophobic/hydrophilic balance of the carboxylated polyglycidol polymer changes as the pH shifts from neutral (where the hydrophilic COO^- form predominates) to low (where the more hydrophobic COOH form predominates). Accordingly, the carboxylated polyglycidol polymer conformation changes from an extended form at neutral pH, to a collapsed form at low pH.[148] Especially, 3-methylglutarylated polyglycidol which has hydrophobic side chains, exhibited higher fusion ability than succinylated polyglycidol. Intranasal 3-methylglutarylated polyglycidol liposomes containing ovoalbumin induced cellular immunity in the absence of additional immunostimulating molecules, in a fashion comparable to that of Freund's complete adjuvant (which is a toxic adjuvant used only in veterinary).[115]

Due to their large size, neither MLV[103,104,106,108,109,111] nor AMVAD[114] are nano-sized vesicles. However, probably both are reduced to lower size/lamellarity structures during the transit across the mucosa. Otherwise the mucus penetration and/or the fitting into an endocytic pocket (two unavoidable steps previous to an efficient uptake/transcytosis by M or M-like cells) would be physically impossible. None of these formulations has been tested for neurotoxicity yet.

ORAL ROUTE

The destructive environment of the GIT is a challenge for the maintenance of colloidal and chemical stability of liposomes. One of the keys for oral, but not for i.n. immunization is to count on structurally stable liposomes against chemoenzymatic attack, in order to be taken up by M cells and transcitosed to APC of the GALT. Due to the weak structure of the lipid matrix, the first liposomes used to stimulate the GALT rendered lower antibodies titers than those induced by free Ag. Later, it was observed that saturated phospholipids of long acyl chain and high phase transition temperature (e.g. distearoylphosphatidylcholine) as well as inclusion of cholesterol, made the bilayers more resistant to hydrolysis. In this way liposomes were also protected against the action of bile salts, and their colloidal integrity was maintained.[149] The inclusion of phosphatidylglycerol in liposomes was observed to increase the liposomal capture by M cells, whereas the inclusion of phosphatidylserine was related to the induction of tolerance. In terms of size, SUV were observed to raise systemic responses, while LUV induced local response.[150] A selection of the most relevant results obtained with stable oral liposomes (Table 1), showed for instance that the adsorption of alum and admixed or included immunomodulators [CT and the recombinant B subunit of CT (CTB)] into the lipid matrix was required to generate mucosal and systemic responses.[116–118] Recently, oral liposomes containing a fusion peptide of CTB and *Helicobacter pylori* urease B subunit epitope elicited high protection

(in terms of gastritis score and histological evaluation) for both prophylactic and therapeutic vaccination protocols.[119] There is no further information on the outcome of these formulations. However, shortly after these works were published the induction of diarrhea was reported upon oral administration of toxin derivatives to humans.[38,39] On the other hand, the selective targeting to human M cells as a mean to increase immune responses, has generated inconclusive results. Orasomes™ are polymerized liposomes stable to detergent action due to the polymerization of the monomer 1,2-di(2,4-octadecadienoyl)-glycerol-3-phosphorylcholine (DODPC). Orasomes™ grafted to the *lectin Ulex europaeus 1* (UEA1) showed an increased uptake by mouse intestine (with M cells expressing receptors for UEA1) as compared to Orasomes grafted to the *lectin* wheat germ agglutinin.[151] Later, an *in situ* assay using a gut loop model showed that UEA1 grafted to Orasomes™ targeted the M cells in mouse Peyer Patches's, resulting in fourfold enhancement of liposomal binding.[152] However, the existence of specific ligands for M cells in humans remains to be demonstrated.[31] There was no further published information of Orasomes™. Recently, it was found that ligands of claudin 4, an β integrin that is over expressed in the surroundings of human M cells, increased the binding and capture by human M cells when attached to the surface of polymeric nanoparticles.[153]

Liposomes covered by polymers had increased structural stability but the immune responses were not improved. Pullulan is a natural polysaccharide that protects yeast plasma membrane against osmotic pressure and ionic strength. Liposomes covered with *O*-palmitoyl derivative from Pullulan exhibited an increased stability in simulated gastric fluid. However, serum IgG and IgA titers were comparable to those obtained by uncovered liposomes. IgG titers were lower and IgA similar to those obtained upon i.p. administration.[120] The same occurred with polyethylene glycol. Peguilated liposomes exhibited an increased stability in GIT but were less captured by M cells.[121] Niosomes, on the other hand, are nonionic vesicles made of 0–50 mol% mixtures of cholesterol with biodegradable and biocompatible nonionic surfactants (diacyl or monoacyl polyglycerol or poly(oxyethylene)). The high structural stability of niosomes is due to their high surface density of hydrated groups. Niosomes are more chemically stable and have lower cost than liposomes made of phospholipids.[154] Niosomes coated with *O*-palmitoyl mannan (a ligand of the mannose receptor in APC) containing HBsAg elicited Ag-specific antibodies (serum and mucosal) and cellular response. However, the serum titers were lower than those elicited by i.m. naked plasmid or HBsAg.[122] Finally, the advent of bilosomes allowed the induction of more intense immune responses than with other vesicles. Bilosomes are niosomes prepared by hydration of the nonionic surfactant film with buffer solution containing bile salts, in particular, deoxycholate.[155,156] Deoxycholate-containing vesicles are of remarkable stability against higher deoxycholate concentrations, being stable against effects of bile acids in the GIT.[155] Oral bilosomes induced Ag-specific antibodies in serum and mucosa.[123,124] The serum titers obtained by oral bilosomes loaded with tetanus toxoid were similar to those obtained upon s.c. administration.[124] In a further work, it was observed that bilosomes required five times higher dose of Ag (50 μg) to produce anti-HBsAg IgG titer similar to that elicited upon 10 μg i.m. alum-HBsAg.[125] To achieve an equivalent immune response to the same amount of Ag the addition of immunomodulators was needed. For instance oral bilosomes covalently conjugated to CTB and loaded with albumin induced similar response than parenteral administration of albumin with Freund's complete adjuvant, in the absence of adverse effects.[126] Mice immunized with 20 μg HBsAg loaded in bilosomes covalently conjugated with CTB, induced anti-HBsAg IgG antibody titers comparable to that elicited upon 10 μg i.m. alum-HBsAg. Also measurable sIgA was induced, which was negligible after i.m. administration.[127] Nano- and micrometer sized bilosomes induced systemic humoral and mucosal responses, but it was observed that size influenced the balance of the response. Large bilosomes entrapped influenza A antigens containing hemagluttinin (two population 60–350 and 400–2500 nm, *Z*-average 980 nm) induced higher IgG2a, significant IFNγ production and greater protection than small ones (single population range 10–100 nm, *Z*-average 250 nm) in ferret model of influenza. However, both oral bilosomes were superior to i.m. commercial vaccine, in terms of higher antibody production, lower temperatures, and reduced symptoms over time post-infection.[128] Authors attributed the differences to the fusogenic activity of the hemagluttinin. Large bilosomes are likely to have a greater entrapped hemagluttinin containing volume compared to small bilosomes, the amount of antigen capable of escaping the endosomes after phagocytosis, to be processed by the class I MHC pathway, and the induction of cytotoxic CD8+ T cells would be greater.

TRANSCUTANEOUS ROUTE

The SC is the main impairment to transcutaneous penetration of molecules >500 Da[157] and

for transcutaneous delivery of vaccines.[158] Ultradeformable liposomes (UDL) are liposomes containing approximately 27mol% of border activators (small hydrophilic molecules of high mobility such as sodium cholate or detergents such as Span 80[159]). Border activators decrease the value of elastic energy (k) of UDL to that of the room thermal energy (kT) (~20 folds lower than k of conventional liposomes). The bilayer of UDL fluctuates at room temperature.[160] Border activators displace toward the zones under mechanical stress in the bilayer.[161,162] According to Cevc, the transepithelial humidity gradient provides a locomotive force (10^{11} to 10^{12} N/mol vesicles of 60 nm radii[161]), that impulse the locomotion of UDL across the inter-corneocyte nanochannels in the SC without coalesce nor collapse.[163,164] Under nonocclusive condition, UDL rapidly penetrate the SC (within 1 h) and can be found at several microns depth, close to the Langerhans cells.[165–167] In contrast, conventional liposomes dehydrate and fuse on the skin surface, and accumulate in the upper layers of the SC, not deeper than the first micron of the SC.[164]

Upon transcutaneous application on human skin explants, the lipid matrix of UDL penetrates between 7 and 10 folds deeper the SC than conventional liposomes. Besides, the hydrosoluble inner content of UDL is shuttled up to 50 μm within the viable epidermis.[168] UDL are capable of generating Ag-dependent systemic immune reactions more efficiently that conventional liposomes and niosomes. This could be owed to the higher penetration of the lipid matrix/inner content, to the neighborhood of skin APC. Up to date, however, a relation between amount and frequency of doses, penetration depth, and immune response (triggered by increased uptake by APC and/or release of pro-inflammatory cytokines) has not been surveyed.

An early report in 1998 showed that UDL raised Ag-specific IgG titers comparable to those obtained upon i.m. immunizations as well as serum IgA; the presence of the immunomodulator monophosphoryl lipid A did not improve the responses.[129] Seven years later, niosomes coated with *O*-palmitoyl mannan to target Langerhans cells elicited significantly higher serum IgG titers as compared with transcutaneous alum-bovine serum albumin and uncoated niosomes.[130] Then a comparative study showed that UDL produced higher serum IgG levels than niosomes and liposomes, with titers comparable to that elicited by i.m. alum-tetanus toxoid.[131] Fusogenic vesicles called vesosomes (multi-compartment structure consisting of drug-loaded liposomes encapsulated within another bilayer) elicited anti-tetanus toxoid serum IgG titers comparable to or higher than those obtained with i.m. alum-tetanus toxoid. Although vesosomes produced higher IgG2a/IgG1 ratio, a primary i.m. immunization with alum-tetanus toxoid was required.[132] Overall, the preparation method at laboratory scale of vesosomes is rather complex and the feasibility of their scaling up is uncertain.

Topical neutral liposomes and niosomes containing plasmid encoding for antigenic HBsAg elicited similar anti-HBsAg IgG titers, which were higher than topical but lower than i.m. naked plasmid. IFN-γ and IL-2 levels in spleen homogenates were similar to those raised by i.m. naked plasmid.[133] Transfection with topical UDL induced IgG titers comparable to i.m. naked plasmid or HBsAg, as well as serum IL-4 and IFN-γ.[134] Transfection with UDL required only one-third the amount of DNA carried by liposomes or niosomes.[133]

Ethosomes are liposomes that intercalate cosolvents such as ethanol into the polar head group of bilayers.[169] Ethanol increases the membrane permeability, making the vesicles soft flexible, which allow them to more easily penetrate into deeper layers of the skin. On the skin surface, ethanol causes a reduction in the phase transition temperature of the SC lipids, increasing their fluidity. If well regular ethosomes bear nearly 25% ethanol and most protein Ag are quite stable below 50%, ethosomes bear the risk of Ag degradation.[170]

Transcutaneous UDL[135] and ethosomes[136] containing HBsAg raised similar levels of anti-HBsAg serum IgG, those were in the order of that elicited by i.m. alum-HBsAg. However, only UDL and ethosomes generated significantly systemic and mucosal IgA levels. Finally, UDL and ethosomes were shown to be internalized fast by both murine[171] and human dendritic cells.[172] Human dendritic cells pulsed with UDL and ethosomes containing HBsAg stimulated T lymphocytes proliferation and induced IL-2 and IFNγ (Th1-type immune response). Ethosomes produced higher dendritic cells' toxicity than UDL. Finally, UDL containing plasmid encoding virus surface glycoproteins of respiratory syncytial virus induced both specific mucosal antibody response and IFNγ producing cells. Lungs from mice receiving topical vaccination also had fewer histopathologic anomalies after virus challenge than did mice receiving i.m. vaccination.[137]

CONCLUSION

Excepting the short period where the IRIVs achieved the market for nasal immunization, the last 15 years have witnessed the emergence of many types of vesicles for oral and nasal immunization. Up to date, none of those strategies evolved beyond preclinical studies. A reason for this could be the absence of a rational

design for the lipid matrix. Only in oral immunization a precise role for the matrix could be partly identified. The composition of oral vesicles was made to protect the chemical structure of the Ag during the transit by the GIT. To that purpose the Ag were included in MLV, or in liposomes in gel phase, or in niosomes/bilosomes (lacking of hydrolyzable ester bonds). Bilosomes, a milestone in the development of vesicles as oral adjuvants, are a simple matrix made of nonionic surfactants added with bile salts. Nasal liposomes were made mucoadhesive by wrapping them up with polymers. Mucoadhesive liposomes, however, were useless to increase the immune response. In that framework, a clear role for the size and the *Z* potential was not found. Most of the liposomal lipid matrices were simple inert platforms for ligand attachment. Only recently, matrices with special properties such as the pH-sensitivity were exploited to raise cell-mediated responses upon i.n. administration. In sum, most of the strategies for mucosal immunization focused not on lipid matrix design but on lab scale preparation of liposomes bearing the ligand best suited to increase (1) the binding to and subsequent uptake by M cells at the Peyer Patches's, (2) the binding to enterocytes, or (3) the interaction with APC. But the same as for polymeric particles, the feasibility of scaling up such adhesins/lectins/antibodies grafted vesicles as well as the absence of neurotoxicity upon i.n. administration remains to be demonstrated. Also antibodies could be unstable in the GIT, lost its adequate conformation on the liposomal surface. An inadequate target receptor expression in the FAE could occur. Lectins have disadvantages in that their receptors tend to be co-expressed in mucous and they have a tendency to be immunogenic, which could impede repeat immunization with the same or different antigens.[8]

Though a few intraepithelial APC in mucosa are directly accessible to Ag in the lumen, most of the APC lie under the mucosal epithelium. The M and M-like cells in intestine and nose are responsible for the transcytosis of particles straight to the follicular APC. An alternative and almost unexplored approach to increase the binding of adjuvants to APC is the design of liposomes/vesicles passively targeted to the transcytotic cells. A passive targeting does not depend on a ligand–receptor interaction but on the feasibility of a particle to remain close to a target cell. Passively targeted liposomes/vesicles do not require the attachment of ligands and their scaling up prospectives become more realistic. Designing mucus-penetrating liposome/vesicle is a way of passively targeting transcytotic cells. Note that targeted but not mucus-penetrating liposomes/vesicles can be trapped or wiped on the mucus blanket. In other words, the presence of ligands does not guarantee the binding of targeted liposomes/vesicles to transcytotic cells. Moreover, some of the structural features of mucus-penetrating particles are coincident with empirical data on particle features needed for an increased capture by M cells, surveyed among others by Florence in 2007[60]: size below 1 μm, with negative or neutral charge and relatively low hydrophobic surface. The success of the AMVAD could be owed to their special matrix made of glyco archaeolipids together with mucus penetration, a combination capable of inducing immune responses in the absence of targeting upon nasal administration. A similar explanation should account for the success of cochleates over the proteoliposomes. Newly designed liposomes/vesicles for oral adjuvancy should follow equivalent guidelines.

The rapid mucosal transport of large (200 and up to 500 nm) particles has important implications in therapeutics development, as larger liposomes/vesicles afford substantially higher drug encapsulation as well as reduced aggregation upon freeze drying. The endocytic uptake could be diminished, however, beyond an upper limit size of 200 nm. In the future, the engineering of liposomes/vesicles to make them mucus penetrating could help to increase the few mucosal vaccines currently on the market.[37,173]

On the other hand, the unusual penetration of UDL is responsible for its higher capacity to generate serum Ag-specific responses as compared to liposomes and niosomes, with no toxic or inflammatory reactions. If well the conservation by dehydration of UDL is more difficult than the usual procedures suitable for conventional liposomes (UDL cannot be submitted to lyophilization even in the presence of high sugar ratios[174]) their lipid matrices can be prepared with cheap raw material and their scaling up methods are the same as those used for conventional liposomes.

REFERENCES

1. Nicholas JF, Guy B. Intradermal, epidermal and transcutaneous vaccination: from immunology to clinical practice. *Expert Rev Vaccines* 2008, 7:1201–1214.
2. Kane A, Lloyd J, Zaffran M, Simonsen L, Kane M. Transmission of hepatitis B, hepatitis C and human immunodeficiency viruses through unsafe injections in

the developing world: model-based regional estimates. *Bull World Health Organ* 1999, 77:801–807.

3. Giudice EL, Campbell JD. Needle-free vaccine delivery. *Adv Drug Deliv Rev* 2006, 58:68–89.
4. Cesta MF. Normal structure, function, and histology of mucosa-associated lymphoid tissue. *Toxicol Pathol* 2006, 34:599–608.
5. Brandtzaeg P, Kiyono H, Pabst R, Russell MW. Terminology: nomenclature of mucosa-associated lymphoid tissue. *Mucosal Immunol* 2008, 1:31–37.
6. Dahl R, Mygind N. Anatomy, physiology and function of the nasal cavities in health and disease. *Adv Drug Deliv Rev* 1998, 29:3–12.
7. Neutra MR. Current concepts in mucosal immunity V. Role of M cells in transepithelial transport of antigens and pathogens to the mucosal immune system. *Am J Gastrointest Liver Physiol* 1998, 274:785–791.
8. Clark MA, Jepson MA, Hirst BH. Exploiting M cells for drug and vaccine delivery. *Adv Drug Deliv Rev* 2001, 50:81–106.
9. Gebert A, Steinmetz I, Fassbender S, Wendlandt K. Antigen transport into Peyer's patches: increased uptake by constant numbers of M Cells. *Am J Pathol* 2004, 164:65–72.
10. Jang MH, Kweon M, Iwatani K, Yamamoto M, Terahara K, Sasakawa C, Suzuki T, Nochi T, Yokota Y, Rennert PD, et al. Intestinal villous M cells: an antigen entry site in the mucosal epithelium. *Proc Natl Acad Sci U S A* 2004, 101:6110–6115.
11. Bos JD, Kapsenberg ML. The skin immune system: progress in cutaneous biology. *Immunol Today* 1993, 14:75–78.
12. Bos JD. Skin immune system. In: Bos JD, ed. *Skin Immune System: Cutaneous Immunology and Clinical Immunodermatology*. Boca Raton, FL: CRC Press; 2005, 3–17.
13. Streilein JW. Skin-associated lymphoid tissues (SALT): origins and functions. *J Invest Dermatol* 1983, 80:S12–S16.
14. Sallusto F. Origin and migratory properties of dendritic cells in the skin. *Curr Opin Allergy Clin Immunol* 2001, 1:441–448.
15. Lamm ME. Interaction of antigens and antibodies at mucosal surfaces. *Ann Rev Microbiol* 1997, 51:311–340.
16. Neutra MR. M cells in antigen sampling in mucosal tissues. *Curr Top Microbiol Immunol* 1999, 236:17–32.
17. MacPherson GG, Liu LM. Dendritic cells and Langerhans cells in the uptake of mucosal antigens. *Curr Top Microbiol Immunol* 1999, 236:33–53.
18. Kunkel EJ, Butcher EC. Plasma-cell homing. *Nat Rev Immunol* 2003, 3:822–829.
19. Hagiwara Y, McGhee JR, Fujihashi K, Kobayashi R, Yoshino N, Kataoka K, Etani Y, Kweon MN, Tamura S, Kurata T, et al. Protective mucosal immunity in aging is associated with functional CD4+ T cells in nasopharyngeal-associated lymphoreticular tissue. *J Immunol* 2003, 170:1754–1762.
20. Guyton AC. *Anatomy and Physiology*. Philadelphia, PA: *Saunders College Publishing*; 1985.
21. Davis SS. Nasal vaccines. *Adv Drug Deliv Rev* 2001, 51:21–24.
22. Ferro VA, Carter KC. Mucosal immunisation: successful approaches to targeting different tissues. *Methods* 2006, 38:61–64.
23. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. *Nat Rev Immunol* 2006, 6:148–158.
24. Staats HF, Montgomery SP, Palker TJ. Intranasal immunization is superior to vaginal, gastric, or rectal immunization for the induction of systemic and mucosal anti-HIV antibody responses. *AIDS Res Hum Retroviruses* 1997, 13:945–952.
25. Gallichan WS, Rosenthal KL. Long-term immunity and protection against herpes simplex virus type 2 in the murine female genital tract after mucosal but not systemic immunization. *J Infect Dis* 1998, 177:1155–1158.
26. Kozlowski PA, Williams SB, Lynch RM, Flanigan TP, Patterson RR, Cu-Uvin S, Neutra MR. Differential induction of mucosal and systemic antibody responses in women after nasal, rectal, or vaginal immunization: influence of the menstrual cycle. *J Immunol* 2002, 169:566–574.
27. Zinkernagel RM. Localization dose and time of antigens determine immune reactivity. *Semin Immunol* 2000, 12:163–171.
28. Jodar L, Duclos P, Milstien JB, Griffiths E, Aguado MT, Clements CJ. Ensuring vaccine safety in immunization programmes—a WHO perspective. *Vaccine* 2001, 19:1594–1605.
29. Mahmoud A. The global vaccination gap. *Science* 2004, 305:147.
30. Foster N, Hirst BH. Exploiting receptor biology for oral vaccination with biodegradable particulates. *Adv Drug Deliv Rev* 2005, 57:431–450.
31. Brayden DJ, Baird AW. Microparticle vaccine approaches to stimulate mucosal immunization. *Microbes Infect* 2001, 3:867–876.
32. Csaba N, Garcia-Fuentes M, Alonso MJ. The performance of nanocarriers for transmucosal drug delivery. *Expert Opin Drug Deliv* 2006, 3:463–478.
33. Mann JF, Acevedo R, Campo JD, Pérez O, Ferro VA. Delivery systems: a vaccine strategy for overcoming mucosal tolerance? *Expert Rev Vaccines* 2009, 8:103–112.
34. Cox E, Verdonck F, Vanrompay D, Goddeeris B. Adjuvants modulating mucosal immune responses or directing systemic responses towards the mucosa. *Vet Res* 2006, 37:511–539.

35. Clements JD, Hartzog NM, Lyon FL. Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. *Vaccine* 1988, 6:269–277.
36. Elson CO. Cholera toxin and its subunits as potential oral adjuvants. *Immunol Today* 1989, 146:29–33.
37. van Ginkel FW, Nguyen HH, McGhee JR. Vaccines for mucosal immunity to combat emerging infectious diseases. *Emerg Infect Dis* 2000, 6:123–132.
38. Jackson RJ, Fujihashi K, Xu-Amano J, Kyoto H, Elson CO, McGhee J. Optimizing oral vaccines: induction of systemic and mucosal B cell and antibody responses to tetanus toxoid by use of cholera toxin as and adjuvant. *Infect Immun* 1993, 61:4272–4279.
39. Michetti P, Creéis C, Kotloff KL, Porta N, Blanco JL, Bachean D, Herranz M, Saldinger PF, Cortéis-Theulaz I, Losonsky G, et al. Oral immunization with urease and *Escherichia coli* heath-labile enterotoxin is safe and immunogenic in *Helicobacter pylori*-infected adults. *Gastroenterology* 1999, 116:804–812.
40. Couch RB. Nasal vaccination, *Escherichia coli* enterotoxin, and Bell's palsy. *N Engl J Med* 2004, 350: 860–861.
41. Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, Spyr C, Steffen R. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *N Engl J Med* 2004, 350:896–903.
42. Combadiere B, Mahe B. Particle-based vaccines for transcutaneous vaccination. *Comp Immunol Microb Infect Dis* 2008, 31:293–315.
43. Carstens MG. Opportunities and challenges in vaccine delivery. *Eur J Pharm Sci* 2009, 36:605–608.
44. Beignon A-S, Briand J-P, Muller S, Partidos CD. Immunization onto bare skin with heat-labile enterotoxin of *Escherichia coli* enhances immune responses to coadministered protein and peptide antigens and protects mice against lethal toxin challenge. *Immunology* 2001, 102:344–351.
45. Beignon A-S, Briand J-P, Rappuoli R, Muller S, Partidos CD. The LTR72 mutant of heat-labile enterotoxin of *Escherichia coli* enhances the ability of peptide antigens to elicit CD4+ T cells and secrete IFNγ after co-application onto bare skin. *Infect Immun* 2002, 70:3012–3019.
46. Glenn GM, Rao M, Matyas GR, Alving CR. Skin immunization made possible by cholera toxin. *Nature* 1998, 391:851.
47. Glenn GM, Scharton-Kersten T, Vassell R, Mallett CP, Hale TL, Alving CR. Transcutaneous immunization with cholera toxin protects mice against lethal mucosal toxin challenge. *J Immunol* 1998, 161:3211–3214.
48. Harandi AM, Davies G, Olesen OF. Vaccine adjuvants: scientific challenges and strategic initiatives. *Expert Rev Vaccines* 2009, 8:293–298.
49. Scharton-Kersten T, Yu J, Vassell R, O'Hagan DT, Alving CR, Glenn GM. Transcutaneous immunization with bacterial ADP-ribosylating exotoxins, subunits, and unrelated adjuvants. *Infect Immun* 2000, 68:5306–5313.
50. Glueck R. Pre-clinical and clinical investigation of the safety of a novel adjuvant for intranasal immunization. *Vaccine* 2001, 20:S42–S44.
51. Kenney RT, Edelman R. Survey of human-use adjuvants. *Expert Rev Vaccines* 2003, 2:167–188.
52. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. *Nat Med* 2005, 11S:S45–S53.
53. Hooke AM, Bellanti JA, Oeschger MP. Live attenuated bacterial vaccines: new approaches for safety and efficacy. *Lancet* 1985, 1:1472–1474.
54. Partidos CD, Beignon A-S, Mawas F, Belliard G, Briand J-P, Muller S. Immunity under the skin: potential application for topical delivery of vaccines. *Vaccine* 2003, 21:776–780.
55. Florence AT. Nanoparticles uptake by the oral route: fulfilling its potential? *Drug Discov Today* 2005, 2:75–81
56. Friede M, Aguado MT. Need for new vaccine formulations and potential of particulate antigen and DNA delivery systems. *Adv Drug Deliv Rev* 2005, 57:325–331.
57. Kaliner M, Shelhamer JH, Borson B, Nadel J, Patow C, Marom Z. Human respiratory mucus. *Am Rev Respir Dis* 1986, 134:612–621.
58. Lai SK, Wang YY, Wirtz D, Hanes J. Micro- and macro-rheology of mucus: implications for drug and gene delivery to mucosal tissues. *Adv Drug Deliv Rev* 2009, 61:86–100.
59. Cone R. Mucus. In: Michael WS, Lamm E, McGhee JR, Mayer L, Mestecky J, Bienenstock J, eds. *Mucosal Immunlogy*. San Diego, CA: Academic Press; 1999, 43–64.
60. Florence AT. The oral absorption of micro-and nanoparticulates: neither exceptional nor unusual. *Pharm Res* 2007, 14:259–266.
61. Karchev T, Kabakchiev P. M-cells in the epithelium of the nasopharyngeal tonsil. *Rhinology* 1984, 22:201–210.
62. Lai SK, O'Hanlon DE, Harrold S, Man ST, Wang YY, Cone R, Hanes J. Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus. *Proc Natl Acad Sci U S A* 2007, 104:1482–1487.
63. Cone RA. Barrier properties of mucus. *Adv Drug Deliv Rev* 2009, 61:75–85.
64. Yoncheva K, Gómez S, Campanero MA, Gamazo C, Irache JM. Bioadhesive properties of pegylated nanoparticles. *Expert Opin Drug Deliv* 2005, 2: 205–218.
65. Ponchel G, Montisci M-J, Dembri A, Durrer C, Duchene D. Mucoadhesion of colloidal particulate systems

in the gastro-intestinal tract. *Eur J Pharm Biopharm* 1997, 44:25–31.

66. Peppas NA, Carr DA. Impact of absorption and transport on intelligent therapeutics and nanoscale delivery of protein therapeutic agents. *Chem Eng Sci* 2009, 64:4553–4565.
67. Ugwoke MI, Agu RU, Verbeke N, Kinget R. Nasal mucoadhesive drug delivery: background, applications, trends and future perspectives. *Adv Drug Deliv Rev* 2005, 57:1640–1665.
68. Peek LJ, Russell MC, Berkland C. Nanotechnology in vaccine delivery. *Adv Drug Deliv Rev* 2008, 60:915–928.
69. Shahiwala A, Vyas T, Amiji MM. Nanocarriers for systemic and mucosal vaccine delivery. *Recent Pat Drug Deliv Formul* 2007, 1:1–9.
70. Chadwick S, Kriegel C, Amiji MM. Nanotechnology solutions for mucosal immunization. *Adv Drug Deliv Rev* 2010, 62:394–407.
71. Alving CR. Liposomes as carriers of antigens and adjuvants. *J Immunol Methods* 1991, 140:1–13.
72. ISO/TS 27687:2008 nanotechnologies—terminology and definitions for nano-objects—nanoparticle, nanofibre and nanoplate.
73. Yechiel E. Part V: Liposomes. In: Meyer RR, ed. *Delivery System Handbook for Personal Care and Cosmetic Products: Technology, Applications and Formulations*. New York: Willian Andrew Inc; 2005, 304–319.
74. Gregoriadis G, Gursel I, Gursel M, McCormack B. Liposomes as immunological adjuvants and vaccine carriers. *J Control Release* 1996, 41:49–56.
75. Alving CR. Liposomes as adjuvants for vaccines. In: Levine MM, Woodrow GC, Kaper JB, Cobon GS, eds. *New Generation Vaccines*. New York: Marcel Dekker; 1997, 207–213.
76. Gregoriadis G, McCormack B, Obrenovic M, Saffie R, Zadi B, Perrie Y. Vaccine entrapment in liposomes. *Methods* 1999, 19:156–162.
77. Kersten G, Hirschberg H. Antigen delivery systems. *Expert Rev Vaccines* 2004, 3:453–462.
78. Mohammed AR, Bramwell VW, Coombes AGA, Perrie I. Lyophilisation and sterilization of liposomal vaccines to produce stable and sterile products. *Methods* 2006, 40:30–38.
79. Jiang W, Gupta RK, Deshpande MC, Schwendeman SP. Biodegradable poly(lactic co-glycolic acid) microparticles for injectable delivery of vaccine antigens. *Adv Drug Deliv Rev* 2005, 57:391–410.
80. European Commission Enterprise and Industry. European Medicine Agency. *Guidelines on Adjuvants in Vaccines for Human Use*. London, UK: 2005, 18. Available at: http://www.ema.europa.eu/ema/pages/includes/document/open_document.jsp?webContentId=WC500003809. (Accessed April 20, 2010).
81. Henriksen-Lacey M, Bramwell V, Perrie Y. Radiolabelling of antigen and liposomes for vaccine biodistribution studies. *Pharmaceutics* 2010, 2: 91–104.
82. Wong A. Quantitative modeling of the high-throughput production and *in vivo* kinetics of (drug-encapsulating) liposomes. *PLoS ONE* 2010, 5:e10280.
83. Centers for disease control and prevention. Available at: www.cdc.gov/flu. (Accessed April 25, 2010).
84. Brandtzaeg P. Induction of secretory immunity and memory at mucosal surfaces. *Vaccine* 2007, 25:5467–5484.
85. Wilschut J. Influenza vaccines: the virosome concept. *Immunol Lett* 2009, 122:118–121.
86. Stegmann T, Morselt HW, Booy FP, van Breemen JF, Scherphof G, Wilschut J. Functional reconstitution of influenza virus envelopes. *EMBO J* 1987, 6: 2651–2659.
87. Zurbriggen R. Immunostimulating reconstituted influenza virosomes. *Vaccine* 2003, 21:921–924.
88. Daemen T, de Mare A, Bungener L, de Jonge J, Huckriede A, Wilschut J. Virosomes for antigen and DNA delivery. *Adv Drug Deliv Rev* 2005, 57:451–463.
89. Glück R, Moser C, Metcalfe IC. Influenza virosomes as an efficient system for adjuvanted vaccine delivery. *Expert Opin Biol Ther* 2004, 4:1139–1145.
90. Cusi MG. Applications of influenza virosomes as a delivery system. *Hum Vaccin* 2006, 2:1–7.
91. Durrer P, Glück U, Spyr C, Lang AB, Zurbriggen R, Herzog C, Glück R. Mucosal antibody response induced with a nasal virosome-based influenza vaccine. *Vaccine* 2003, 21:4328–4334.
92. Huckriede A, Bungener L, Stegmann T, Daemen T, Medema J, Palache AM, Wilschut J. The virosome concept for influenza vaccines. *Vaccine* 2005, 23:S26–S38.
93. Lambkin R, Oxford JS, Bossuyt S, Mann A, Metcalfe IC, Herzog C, Viret JF, Glück R. Strong local and systemic protective immunity induced in the ferret model by an intranasal virosome-formulated influenza subunit vaccine. *Vaccine* 2004, 22:4390–4396.
94. Glueck R. Pre-clinical and clinical investigation of the safety of a novel adjuvant for intranasal immunization. *Vaccine* 2001, 20:S42–S44.
95. Durrer P, Glück U, Spyr C, Lang AB, Zurbriggen R, Herzog C, Glück R. Mucosal antibody response induced with a nasal virosome-based influenza vaccine. *Vaccine* 2003, 21:4328–4334.
96. Mathison S, Nagilla R, Kompella UB. Nasal route for direct delivery of solutes to the central nervous system: fact or fiction? *J Drug Target* 1998, 5:415–441.
97. Illum L. Is nose-to-brain transport of drugs in man a reality? *J Pharm Pharmacol* 2004, 56:3–17.

98. Dhanda DS, Frey WH, Leopold D, Kompella U. Nose to brain delivery: approaches for drug deposition in human olfactory epithelium. *Drug Deliv Technol* 2005, 5:1–9.
99. Fujihashi K, Koga T, van Ginkel FW, Hagiwara Y, McGhee JR. A dilemma for mucosal vaccination: efficacy versus toxicity using enterotoxin-based adjuvants. *Vaccine* 2002, 20:2431–2438.
100. Lewis DJM, Huo Z, Barnett S, Kromann I, Giemza R, Galiza E, Woodrow M, Thierry-Carstensen B, Andersen P, Novicki D, et al. Transient facial nerve paralysis (Bell's Palsy) following intranasal delivery of a genetically detoxified mutant of *Escherichia coli* heat labile toxin. *PLoS ONE* 2009, 4:e6999.
101. New RR. Introduction. In: New RR, ed. *Liposomes A Practical Approach*. Oxford: Oxford University Press; 1990, 1–31.
102. Alving CR. Theorical basis for development of liposomes as carriers of vaccines. In: Lasic DD, Papahadjopoulos D, eds. *Medical Applications of Liposomes*. Amsterdam: Elsevier Science BV; 1998, 145–165.
103. Ninomiya A, Ogasawara K, Kajino K, Takada A, Kida H. Intranasal administration of a synthetic peptide vaccine encapsulated in liposome together with an anti-CD40 antibody induces protective immunity against influenza A virus in mice. *Vaccine* 2002, 20:3123–3129.
104. Joseph A, Louria-Hayon I, Plis-Finarov A, Zeira E, Zakay-Rones Z, Raz E, Hayashi T, Takabayashi K, Barenholz Y, Kedar E. Liposomal immunostimulatory DNA sequence (ISS-ODN): an efficient parenteral and mucosal adjuvant for influenza and hepatitis B vaccines. *Vaccine* 2002, 20:3342–3354.
105. Wang D, Christopher ME, Nagata LP, Li MA, Wong JP, Samuel J. Intranasal immunization with liposome-encapsulated plasmid DNA encoding influenza virus hemagglutinin elicits mucosal, cellular and humoral immune responses. *J Clin Virol* 2004, 31S:S99–S106.
106. Joseph A, Itskovitz-Cooper N, Samira S, Flasterstein O, Eliyahu H, Simberg D, Goldwaser I, Barenholz Y, Keda E. A new intranasal influenza vaccine based on a novel polycationic lipid-ceramide carbamoyl-spermine (CCS) I. Immunogenicity and efficacy studies in mice. *Vaccine* 2006, 24:3990–4006.
107. Guy B, Pascal N, Francon A, Bonnin A, Gimenez S, Lafay-Vialon E, Trannoy E, Haensler J. Design, characterization and preclinical efficacy of a cationic lipid adjuvant for influenza split vaccine. *Vaccine* 2001, 19:1794–805.
108. Christensen D, Foged C, Rosenkrands I, Lundberg CV, Andersen P, Agger EM, Nielsen HM. CAF01 liposomes as a mucosal vaccine adjuvant: *in vitro* and *in vivo* investigations. *Int J Pharm* 2010, 390:19–24.
109. Khatri K, Goyal AK, Gupta PN, Mishra N, Mehta A, Vyas SP. Surface modified liposomes for nasal delivery of DNA vaccine. *Vaccine* 2008, 26:2225–2233.
110. Tiwari S, Goyal AK, Mishra N, Vaidya B, Mehta A, Dube D, Vyas SP. Liposome *in situ* gelling system: novel carrier based vaccine adjuvant for intranasal delivery of recombinant protein vaccine. *Procedia Vaccinol* 2009, 1:148–163.
111. Ishii M, Kojima N. Mucosal adjuvant activity of oligomannose-coated liposomes for nasal immunization. *Glycoconj J* 2010, 2:115– 123.
112. Amin M, Jaafari MR, Tafaghodi M. Impact of chitosan coating of anionic liposomes on clearance rate, mucosal and systemic immune responses following nasal administration in rabbits. *Colloids Surf B Biointerfaces* 2009, 74:225–229.
113. del Campo J, Lindqvist M, Cuello M, Bäckström M, Cabrerra O, Persson J, Perez O, Harandi AM. Intranasal immunization with a proteoliposome-derived cochleate containing recombinant gD protein confers protective immunity against genital herpes in mice. *Vaccine* 2010, 28:1193– 1200.
114. Patel GB, Zhou H, Ponce A, Chen W. Mucosal and systemic immune responses by intranasal immunization using archaeal lipid-adjuvanted vaccines. *Vaccine* 2007, 25:8622–8636.
115. Yuba E, Kojima C, Harada A, Tana, Watarai S, Kono K. pH-Sensitive fusogenic polymer-modified liposomes as a carrier of antigenic proteins for activation of cellular immunity. *Biomaterials* 2010, 31:943–951.
116. Mirchamsy H, Manhouri H, Hamedi M, Ahourai P, Fateh G, Hamzeloo Z. Stimulating role of toxoids-laden liposomes in oral immunization against diphtheria and tetanus infections. *Biologicals* 1996, 24: 343–350.
117. Chaicumpa W, Chongsa-nguan M, Kalambaheti T, Wilairatana P, Srimanote P, Makakunkijcharoen Y, Looareesuwan S, Sakolvaree Y. Immunogenicity of liposome-associated and refined antigen oral cholera vaccines in Thai volunteers. *Vaccine* 1998, 16:678–684.
118. Harokopakis A, Hajishengallis EG, Michalek SM. Effectiveness of liposomes possessing surface-linked recombinant B subunit of cholera toxin as an oral antigen delivery system. *Infect Immun* 1998, 66:4299–4304.
119. Zhao W, Wu W, Xu X. Oral vaccination with liposome-encapsulated recombinant fusion peptide of urease B epitope and cholera toxin B subunit affords prophylactic and therapeutic effects against *H. pylori* infection in BALB/c mice. *Vaccine* 2007, 25:7664–7673.
120. Venkatesan N, Vyas SP. Polysaccharide coated liposomes for oral immunization—development and characterization. *Int J Pharm* 2000, 203:169–177.
121. Minato S, Iwanaga K, Kakemi M, Yamashita S, Oku N. Application of polyethyleneglycol (PEG)-modified liposomes for oral vaccine: effect of lipid dose on systemic and mucosal immunity. *J Control Release* 2003, 89:189–197.

122. Jain S, Singh P, Mishra V, Vyas SP. Mannosylated niosomes as adjuvant-carrier system for oral genetic immunization against hepatitis B. *Immunol Lett* 2005, 101:41–49.

123. Conacher M, Alexander J, Brewer JM. Oral immunisation with peptide and protein antigens by formulation in lipid vesicles incorporating bile salts (bilosomes). *Vaccine* 2001, 19:2965–2974.

124. Mann JFS, Scales HE, Shakir E, Alexander J, Carter KC, Mullen AB, Ferro VA. Oral delivery of tetanus toxoid using vesicles containing bile salts (bilosomes) induces significant systemic and mucosal immunity. *Methods* 2006, 38:90–95.

125. Shukla A, Khatri K, Gupta PN, Goyal AK, Mehta A, Vyas SP. Oral immunization against hepatitis B using bile salt stabilized vesicles (bilosomes). *J Pharm Pharmaceut Sci* 2008, 11:59–66.

126. Singh P, Prabakaran D, Jain S, Mishra V, Jaganathan KS, Vyas SP. Cholera toxin B subunit conjugated bile salt stabilized vesicles (bilosomes) for oral immunization *Int J Pharm* 2004, 278:379–390.

127. Shukla A, Katare OP, Singh B, Vyas SP. M-cell targeted delivery of recombinant hepatitis B surface antigen using cholera toxin B subunit conjugated bilosomes. *Int J Pharm* 2010, 385:47–52.

128. Mann JFS, Shakir E, Carter KC, Mullen AB, Alexander J, Ferro VA. Lipid vesicle size of an oral influenza vaccine delivery vehicle influences the Th1/Th2 bias in the immune response and protection against infection. *Vaccine* 2009, 27:3643–3649.

129. Paul A, Cevc G, Bachhawat BK. Transdermal immunisation with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, transfersomes. *Vaccine* 1998, 16: 188–195.

130. Jain S, Vyas SP. Mannosylated niosomes as carrier adjuvant system for topical immunization. *J Pharm Pharmacol* 2005, 57:1177–1184.

131. Gupta PN, Mishra V, Rawat A, Dubey P, Mahor S, Jain S, Chatterji DP, Vyas SP. Non-invasive vaccine delivery in transfersomes, niosomes and liposomes: a comparative study. *Int J Pharm* 2005, 293: 73–82.

132. Mishra V, Mahor S, Rawat A, Dubey P, Gupta PN, Singh P, Vyas SP. Development of novel fusogenic vesosomes for transcutaneous immunization. *Vaccine* 2006, 24:5559–5570.

133. Vyas SP, Singh RP, Jain S, Mishra V, Mahor S, Singh P, Gupta PN, Rawat A, Dubey P. Non-ionic surfactant based vesicles (niosomes) for non-invasive topical genetic immunization against hepatitis B. *Int J Pharm* 2005, 296:80–86.

134. Wang J, Hu J-H, Li F-Q, Liu G-Z, Zhu Q-G, Liu J-Y, Ma H-J, Peng C, Si F-G. Strong cellular and humoral immune responses induced by transcutaneous immunization with HBsAg DNA-cationic deformable liposomes complex. *Exp Dermatol* 2007, 16:724–729.

135. Mishra D, Dubey V, Asthana A, Saraf DK, Jain NK. Elastic liposomes mediated transcutaneous immunization against hepatitis B. *Vaccine* 2006, 24: 4847–4855.

136. Mishra D, Mishra PK, Dubey V, Nahar M, Dabadghao S, Jain NK. Systemic and mucosal immune response induced by transcutaneous immunization using hepatitis B surface antigen-loaded modified liposomes. *Eur J Pharm Biopharm* 2008, 33:424–433.

137. Xu J, Ding YZ, Yang Y. Enhancement of mucosal and cellular immune response in mice by vaccination with respiratory syncytial virus DNA encapsulated with transfersome. *Viral Immunol* 2008, 21:483–489.

138. Centers for disease control and prevention. Available at: www.cdc.gov/vaccines/vpd-vac/hepb. (Accessed April 27, 2010).

139. Prüss-Üstün A, Rapiti E, Hutin Y. *Sharps Injuries: Global Burden of Disease from Sharps Injuries to Health-Care Workers*. Geneva: World Health Organization; 2003. Available at: http://www.who.int/peh/burden/9241562463/sharptoc.htm. (Accessed April 27, 2010).

140. Drummond DC, Zignani M, Leroux JC. Current status of pH-sensitive liposomes in drug delivery. *Prog Lipid Res* 2000, 39:409–460.

141. Shaw-Stiffel TA. Chronic hepatitis. In: Mandell GL, Bennett JE, Dolin R, eds. *Principles and Practice of Infectious Diseases*. Philadelphia, PA: Churchill Livingstone; 2000, 1297–1332.

142. Lowell GH, Kaminski RW, Vancott TC, Slike B, Kersey K, Zawoznik E, Loomis-Price L, Smith G, Redfield RR, Amselem S, et al. Proteosomes, emulsomes, and cholera toxin B improve nasal immunogenicity of human immunodeficiency virus gp160 in mice: induction of serum, intestinal, vaginal, and lung IgA and IgG. *J Infect Dis* 1997, 75:292–301.

143. Sprott GD, Brisson J-R, Dicaire CJ, Pelletier AK, Deschatelets LA, Krishnan L, Patel GB. A structural comparison of the total polar lipids from the human archaea *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* and its relevance to the adjuvant activities of their liposomes. *Biochem Biophys Acta* 1999, 1440:275–288.

144. Kates M. The phytanyl ether-linked polar lipids and isoprenoid neutral lipids of extremely halophilic bacteria. *Prog Chem Fats Lipids* 1978, 15:301–342.

145. Krishnan L, Sad S, Patel GB, Sprott GD. Archaeosomes induces long-term CD8+ cytotoxic T cell response to entrapped soluble protein by the exogenous cytosolic pathway, in the absence of CD4+ T cell help. *J Immunol* 2000, 165:5177–5185.

146. Krishnan L, Sad S, Patel GB, Sprott GD. The potent adjuvant activity of archaeosomes correlates to the

recruitment and activation of macrophages and dendritic cells *in vivo*. *J Immunol* 2001, 166:1885–1893.

147. Torchillin VP. Recent advances with liposomes as pharmaceutical carriers. *Nat Rev Drug Discov* 2005, 4:145–160.
148. Sakaguchi N, Kojima C, Harada A, Kono K. Preparation of pH-sensitive poly(glycidol) derivatives with varying hydrophobicities: their ability to sensitize stable liposomes to pH. *Bioconj Chem* 2008, 19:1040–1048.
149. Han M, Watarai S, Kobayashi K, Yasuda T. Application of liposomes for development of oral vaccines: study of *in vitro* stability of liposomes and antibody response to antigen associated with liposomes after oral immunization. *J Vet Med Sci* 1997, 59:1109–1114.
150. Alves AC, Ramaldes GA, Oliveira MC, Cardoso VN, Mota-Santos TA, Faria AMC, Montijo CM. Ovoalbumin encapsulation into liposomes results in distinct degrees of oral immunization in mice. *Cell Immunol* 2008, 254:63–73.
151. Chen H, Torchilin V, Langer R. Lectin-bearing polymerized liposomes as potential oral vaccine carriers. *Pharm Res* 1996, 13:1378–1383.
152. Clark MA, Blair H, Liang L, Brey RN, Brayden D, Hirst BH. Targeting polymerized liposome vaccine carriers to intestinal M cells. *Vaccine* 2002, 20: 208–217.
153. Fievez V, Plapied L, des Rieux A, Pourcelle V, Freichels H, Wascotte V, Vanderhaeghen M-L, Jerôme C, Vanderplasschen A, Marchand-Brynaert J, et al. Targeting nanoparticles to M cells with non-peptidic ligands for oral vaccination. *Eur J Pharm Biopharm* 2009, 73:16–24.
154. Ribier A, Handjani-Vila RM, Bardez E, Valeur B. Bilayer fluidity of non ionic vesicles. An investigation by differential polarized phase fluorimetry. *Colloids Surf* 1984, 10:155–161.
155. Schubert R, Jaroni H, Schoelmerich J, Schmidt KH. Studies on the mechanism of bile salt-induced liposomal membrane damage. *Digestion* 1983, 28:181–90.
156. Conacher M, Alexander J, Brewer JM. Niosomes as immunological adjuvants. In: Uchegbu IF, ed. *Synthetic Surfactant Vesicles*. Singapore: International Publishers Distributors Ltd; 2000, 185–205.
157. Bouwstra JA, Ponec M. The skin barrier in healthy and diseased state. *Biochim Biophys Acta* 2006, 1758:2080–2020.
158. Lambert PH, Laurent PE. Intradermal vaccine delivery: will new delivery systems transform vaccine administration? *Vaccine* 2008, 26:3197–3208.
159. Cevc G, Blume G. Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. *Biochim Biophys Acta* 1992, 1104:226–232.
160. Cevc G. Material transport across permeability barriers by means of lipid vesicles. In: Lipowsky RSE, ed. *Handbook of Biological Physics*. Amsterdam: Elsevier; 1995, 465–490.
161. Cevc G. Transfersomes®-innovative transdermal drug carriers. In: Rathbone M, Roberts M, Hadgraft J, eds. *Modified Release Drug Delivery Technology*. New York: M. Dekker; 2002, 533–546.
162. Cevc G, Schatzlein A, Richardsen H. Ultradeformable lipid vesicles can penetrate the skin and other semipermeable barriers unfragmented. Evidence from double label CLSM experiments and direct size measurements. *Biochim Biophys Acta* 2002, 1564:21–30.
163. Cevc G, Gebauer D. Hydration-driven transport of deformable lipid vesicles through fine pores and the skin barrier. *Biophys J* 2003, 84:1010–1024.
164. Verma DD, Verma S, Blume G, Fahr A. Liposomes increase skin penetration of entrapped and non-entrapped hydrophilic substances into human skin: a skin penetration and confocal laser scanning microscopy study. *Eur J Pharm Biopharm* 2003, 55:271–277.
165. Honeywell-Nguyen PL, de Graaff AM, Groenink HW, Bouwstra JA. The *in vivo* and *in vitro* interactions of elastic and rigid vesicles with human skin. *Biochim Biophys Acta* 2002, 1573:130–140.
166. Honeywell-Nguyen PL, Gooris GS, Bouwstra JA. Quantitative assessment of the transport of elastic and rigid vesicle components and a model drug from these vesicle formulations into human skin *in vivo*. *J Invest Dermatol* 2004, 123:902–910.
167. Honeywell-Nguyen PL, Bouwstra JA. Vesicles as a tool for transdermal and dermal delivery. *Drug Discov Today* 2005, 2:67–74.
168. Montanari J, Maidana C, Esteva MI, Salomon C, Morilla MJ, Romero EL. Sunlight triggered photodynamic ultradeformable liposomes against *Leishmania braziliensis* are also leishmanicidal in the dark. *J Control Release* 2010, 147:368–376.
169. Touitou E, Dayan N, Bergelson L, Godin B, Eliaz M. Ethosomes-novel vesicular carriers: characterization and delivery properties. *J Control Release* 2000, 65:403–418.
170. Ito K, Kajiura T, Abe K. Effect of ethanol on antigenicity of hepatitis B virus envelope proteins. *Jpn J Infect Dis* 2002, 55:117–121.
171. Mishra D, Mishra PK, Dubey V, Dabadghao S, Jain NK. Evaluation of uptake and generation of immune response by murine dendritic cells pulsed with hepatitis B surface antigen-loaded elastic liposomes. *Vaccine* 2007, 25:6939–6944.
172. Mishra D, Mishra PK, Dabadghao S, Dubey V, Nahar M, Jain NK. Comparative evaluation of hepatitis B surface antigen-loaded elastic liposomes and ethosomes for human dendritic cell uptake and immune

response. *Nanomed: Nanotech Biol Med* 2010, 6:110–118.

173. Dietrich G, Griot-Wenk M, Metcalfe IC, Lang AB, Viret J-F. Experience with registered mucosal vaccines. *Vaccine* 2003, 21:678–683.

174. Montanari J, Roncaglia DI, Lado LA, Morilla MJ, Romero EL. Avoiding failed reconstitution of ultradeformable liposomes upon dehydration. *Int J Pharm* 2009, 372:184–190.

FURTHER READING

Brandtzaeg P. Induction of secretory immunity and memory at mucosal surfaces. *Vaccine* 2007, 25:5467–5484.

Chaplin DD. Overview of the immune response. *J Allergy Clin Immunol* 2010, S3–S23. doi:10.1016/j.jaci.2009.12.980.

El Maghraby GM, Barry BW, Williams AC. Liposomes and skin: from drug delivery to model membranes. *Eur J Pharm Sci* 2008, 34:203–222.

Groschwitz KR, Hogan SP. Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol* 2009, 3–20.

Rand RP, Fuller NL, Gruner SM, Parsegian VA. Membrane curvature, lipid segregation, and structural transitions for phospholipids under dual-solvent stress. *Biochemistry* 1990, 29:76–87.

Takahashi I, Nochi T, Yuki Y, Kiyono H. New horizon of mucosal immunity and vaccines. *Curr Opin Immunol* 2009, 21:352–358.

van Wijk F, Cheroutre H. Intestinal T cells: facing the mucosal immune dilemma with synergy and diversity. *Semin Immunol* 2009, 21:130–138.

Holl MM. Nanotoxicology: a personal perspective. *WIREs Nanomed Nanobiotechnol* 2009, 1:353–359.