

# Expert Opinion

1. Introduction
2. Vesicular systems
3. Conclusion
4. Expert opinion

## Vesicular carrier constructs for topical immunisation

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Topical immunisation represents a convenient and novel approach to vaccination. Skin is exploited as a route of immunisation because it shows both specific and non-specific immune responses against foreign invaders, and these responses are a result of the presence of immunocompetent cells within the skin layers. These skin-resident antigen-presenting cells are highly efficient for the initiation of humoral and cellular immune responses. Vesicular carrier systems, particularly liposomes, vesosomes, niosomes and transfersomes, have been advocated for the topical delivery of biomacromolecules. This review describes the potential and feasibility of vesicular carrier-based vaccine delivery for topical immunisation.

**Keywords:** liposomes, niosomes, topical immunisation, transfersomes, vesosomes

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### 1. Introduction

Newer vaccines that are stable, economical, require fewer doses and can be administered using needle-free systems are a worldwide priority. An ideal theoretical vaccine may not be cogent unless formulated and delivered aptly. The most exciting development in terms of the non-invasive delivery of vaccines is topical immunisation [1]. Topical immunisation (i.e., non-invasive vaccination onto the skin) provides a convenient and novel approach of vaccination, and classical vaccination methods that use needles or require multiple doses using an invasive route suffer from the problems of administration, expense and patient compliance. The skin is exploited as a route of immunisation because it shows specific (immunity) and non-specific (inflammation) responses against foreign substances, and these responses are the result of the presence of immunocompetent cells within the skin layers (e.g., Langerhan's cells, dendritic cells and epidermotropic lymphocytes). Other cells present are mast cells in the dermis, resident antigen-presenting cells (APCs) and transient inflammatory lymphocytes. Apart from epidermal APCs and migratory T lymphocytes, all cells are collectively known as skin-associated lymphoid tissue and constitute the skin immune system. These cells altogether function in association with lymph nodes and are responsible for the generation of both cellular and humoral immune responses [2,3]. It has been reported that the nature of APCs that present the antigen to specific T helper cells will favour the isotype and magnitude of the B cell antibody response [4]. Dendritic cells are considered as major APCs and are widely distributed on the epithelial barriers at different sites of the body. They are capable of processing the antigen by MHC I and MHC II pathways, depending upon the nature of the recognised antigen. Antigen processed by MHC I molecules is presented to Th1 cells to elicit a cellular immune response, and that processed via MHC II molecules is presented to Th2 cells and elicits a humoral response. IgG1 is an antibody associated with inflammatory and mast cell or eosinophil responses, and IgG2a is able to act as an opsonin, activates the complement and binds to macrophages and enhances the phagocytic response [5]. Studies have revealed that switching to IgG2a is induced by

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IL-12 and IFN- $\gamma$  (Th1-dependent cytokines) and inhibited by IL-4, IL-5 and IL-10 (Th2-dependent cytokines).

Transcutaneous immunisation (TCI) appears to be particularly promising in gaining access to skin-resident APCs, which are highly efficient for the initiation of humoral and/or cellular immune responses. Consisting of an adjuvant as a stimulus in combination with an antigen, which defines the target, TCI offers the most attractive immunisation strategy and mounts highly specific full-blown adaptive immune responses. Topical immunisation with protein antigens was first demonstrated by Glenn *et al.* [1], who used the term 'transcutaneous immunisation'. TCI was found to be aided in terms of immunogenicity by the use of adjuvant(s), such as the *Vibrio cholera* toxin, *Escherichia coli* heat-labile enterotoxin and their mutants, or co-administrated with a protein antigen to induce potent immune responses [1,6,7]. The use of cholera toxin on the skin resulted in classic priming and secondary antibody responses to co-administered antigens. Mice first immunised with cholera toxin or heat-labile *E. coli* enterotoxin alone and subsequently boosted, demonstrate a typical secondary antibody response to the toxins [8]. TCI using ligands of Toll-like receptors (TLRs) and cytotoxic T-lymphocyte epitopes lead to the induction of potent T cell responses [9,10]. TLRs are a family of receptors involved in the recognition of a wide range of microbial molecules, for example lipopolysaccharide (LPS) from Gram-negative bacteria and peptidoglycan from Gram-positive bacteria. The prototypic receptor Toll was first identified in the fruit fly *Drosophila*, but several TLRs are found in mammals, particularly on mononuclear phagocytes. The binding of TLR leads to the production of inflammatory cytokines, including TNF- $\alpha$  and IL-12, and enhances a cell's antimicrobial killing mechanisms and antigen-presenting capacity.

Another promising technology that may be used for transdermal delivery is immunisation with microneedles. Microneedles are pointed projections fabricated into arrays that can create vaccine delivery pathways through the skin. These microneedles can be made of varying dimensions, as short as 25  $\mu\text{m}$  and as long as 1000  $\mu\text{m}$  in length. Recently, it has been suggested that microneedles, used in conjunction with transdermal patches, could increase skin permeability, allowing for transdermal delivery of large molecules [11,12]. Although microneedles have not yet been used clinically in immunisation, several companies, such as Biovalve Technologies, Inc., which makes MicroTrans<sup>TM</sup>, are presently working on developing this technology and are seeking FDA approval for their products.

However, the general consensus has been that penetration of the skin by any entity is largely restricted to molecules of a relatively small size (up to 500 Da). Cholera toxin is a relatively large molecule (86 kDa) that would not be expected to penetrate the skin. However, it has been found that a variety of vaccine antigens and adjuvants are able to induce potent immune responses following hydration and topical vaccine application, and, thus, it is hypothesised that

hydration is sufficient to allow large molecules access to the superficial layer of the skin, where they may encounter Langerhan's cells. Other methods to facilitate penetration of the epithelium barrier include abrasion, and electroporation. Once an antigen has moved through the stratum corneum, epidermal Langerhan's cells can take it up, migrate to a draining lymph node, present the antigen, and induce systemic immune responses. Non-invasive vaccination through application onto the skin can also be achieved by means of suitable carriers, such as liposomes, niosomes, transfersome and vesosomes (Figure 1), or through the application of naked DNA in solution. DNA vaccines are capable of eliciting both humoral and cellular immune responses. The feasibility of topical immunisation using DNA vaccines was first demonstrated by Tang and co-workers [13,14]. The topical application of both naked and liposome-entrapped plasmid vector for hepatitis B surface antigen (HBsAg) resulted in an antigen specific immune response [15]. Vesicular carrier systems (liposomes and niosomes) have reportedly been advocated for the topical delivery of bioactives [16-18].

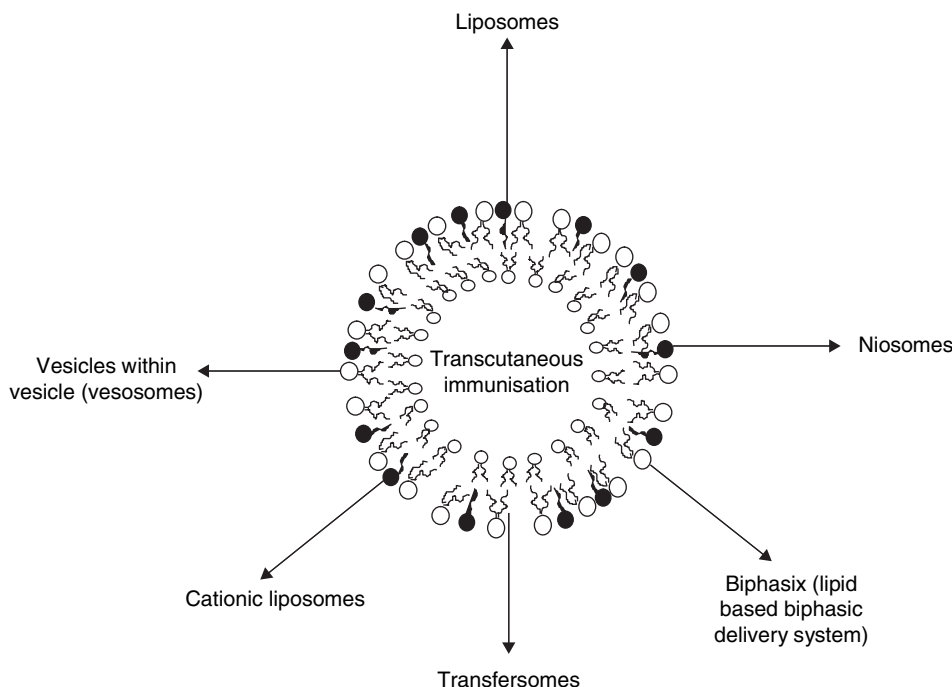
## 2. Vesicular systems

### 2.1 Liposomes

Liposomes are self-assembled phospholipids vesicles with a bilayer structure. Hydrophilic antigens can be incorporated into the internal space of the vesicle, and amphiphilic antigens can be inserted into the bilayer(s). Liposomes may be targeted to dendritic cells by incorporation or introduction of receptor-specific ligands into the bilayer [19]. This can be achieved by employing various physicochemical methods, including metal-chelating linkage of his-tagged antibodies to the bilayer [20], or by introduction of protein A into the bilayer, and attaching receptor-specific antibodies to the protein A [21]. Although liposomes in general show modest adjuvant effects, targeting liposomes containing antigenic peptides or DNA to professional APCs effectively enhances their capacity to induce humoral and cytotoxic T lymphocyte responses *in vivo* [19,22,23].

Microencapsulated liposome systems (MELs) have been investigated as a potential immunisation carrier for recombinant HBsAg [24]. MELs were prepared by first entrapping the HBsAg particles within liposomes composed of phosphatidylcholine:cholesterol (1:1 molar ratio), which were subsequently encapsulated in alginate-poly(L-lysine) hydrogel microspheres. The studies reported significant anti-HBsAg titres in mice administered with HBsAg in MELs, and indicated that MELs were relatively more efficient than conventional liposomes or alum in eliciting higher and prolonged antibody levels in mice. These observations suggest the immunoadjuvant potential of MELs to provide an HBsAg depot, as well as a sustained release of liposomal HBsAg.

Interestingly, vesosomes i.e. multicompartamental aggregates of tethered vesicles encapsulated within a large bilayer, are described for multicomponent or multifunctional drug



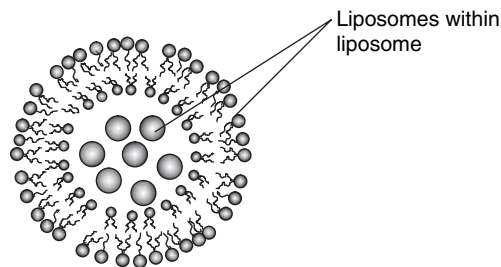
**Figure 1. Vesicular delivery systems for transcutaneous immunisation.**

delivery [25]. Recently, fusogenic vesosomes (liposomes within liposome) (Figure 2), were investigated for topical delivery of tetanus toxoid (TTx) antigen. The developed novel fusogenic vesosomes consisted of inner cationic liposomes encapsulated in an outer liposomal bilayer that was largely neutral with respect to electric potential (charge). The average vesicle size and zeta potential of the inner cationic liposomes was found to be  $104 \pm 4.2$  nm and  $32.4 \pm 4.2$ , respectively, whereas in the case of vesosomal systems, the average vesicle size and zeta potential was found to be  $3.7 \pm 0.8$   $\mu$ m and  $18.84 \pm 1.34$ , respectively. These fusogenic vesicular systems have the potential to microinject entrapped antigen directly into the cytoplasm of APCs, via a fusion event at the plasma membrane. The fusion-mediated delivery of encapsulated antigen to the cytosol of APCs is anticipated to involve, and hence cause the induction of, class-I-restricted antigen presentation in addition to presentation along with class-II-restricted MHC molecules, due to capture and endosomal processing of extracellular antigen encountered after the release or exocytic spill of antigen in the vicinity of APCs. The immune-stimulating activity of these vesicles has been studied by measuring the serum anti-TTx IgG titre and the isotype ratio of IgG2a/IgG1 following topical immunisation. The results were compared with alum-adsorbed TTx given intramuscularly, and topically administered plain TTx solution, plain liposomes and cationic fusogenic liposomes. Serum IgG titres after three consecutive topical administrations were significantly better ( $p < 0.05$ ) than a single administration of TTx antigen with vesosomal systems, suggesting an effective stimulation of a serum immune

response. Furthermore, notable serum anti-TTx antibody titres also occurred in animals primed with alum-adsorbed TTx that were subsequently boosted topically with vesosomal systems. In each immunisation study, the vesosomal systems were shown to elicit a combined Th1 and Th2 immune response following topical administration, suggesting the effectiveness of vesosomal systems for the topical delivery of vaccines [26].

## 2.2 Niosomes

Although liposomes have been widely studied and employed for DNA delivery through the topical route, they suffer from certain drawbacks, such as a higher cost and instability. The low cost, high purity, content uniformity, greater stability and ease of storage of non-ionic surfactants have presented niosomes as better possible alternatives to liposomes. These vesicles appear to be structurally similar, especially in terms of their physical properties to liposomes, being prepared by the same methods and methodologies and also under a variety of variable conditions that determine the formation of unilamellar or multilamellar structures [27,28]. Non-ionic surfactant-based vesicles (niosomes) have been investigated for the topical delivery of plasmid DNA encoding HBsAg. Niosomes composed of span 85 and cholesterol as constitutive lipids were prepared by a reverse-phase evaporation method. The study signifies the potential of niosomes as DNA vaccine carriers for effective topical immunisation. These niosomal systems are reportedly simple, stable and cost-effective compared with liposomes [29]. The immune-stimulating activity of these niosome-based systems was studied in terms of the



**Figure 2. A schematic representation of a vesosome (liposomes within a liposome).**

serum anti-HBsAg titre and also cytokine levels (IL-2 and IFN- $\gamma$ ) following the topical application of niosomes to Balb/c mice. The results were compared with naked DNA and liposome-encapsulated DNA applied topically, and also with naked DNA and pure recombinant HBsAg administered intramuscularly. These suggest that topical niosomes elicited a comparable serum antibody titre and endogenous cytokines levels when compared with intramuscular recombinant HBsAg and the topical application of liposomes.

Furthermore, the development of ligand-anchored niosomes, as a topical vaccine delivery carrier and adjuvant for the induction of both humoral and cellular immunity, opens the route for receptor-specific pilosebaceous vaccine delivery using vesicular systems. In one study, bovine serum albumin (BSA)-loaded niosomes consisting of sorbitan stearate/sorbitan trioleate (Span 60/Span 85), cholesterol and stearylamine as constitutive lipids were prepared by using a reverse-phase evaporation method and coated with a modified polysaccharide *O*-palmitoyl mannan to target them to Langerhan's cells – the major APCs found in abundance just below the stratum corneum. The immune-stimulating activity in terms of serum IgG titre and its subclasses (IgG2a/IgG1 ratio) following the topical application of niosomal formulations were compared with alum-adsorbed BSA following topical application and intramuscular injection. The study indicates that niosomal formulations elicited a significantly higher serum IgG titre upon topical application, compared with topically applied alum-adsorbed BSA ( $p < 0.05$ ) [30]. Furthermore, the significantly greater response of mannan-coated niosomes compared with plain niosomes could be attributed to the targeting of mannan to mannose receptors on dendritic cells (Langerhan's cells) and other APCs of skin-associated lymphoid tissue that are located at the basal layer of the epidermis and cover > 20% of the surface area [31]. In addition to this, mannan can elicit an intrinsic immunogenic effect that could considerably aggravate the immunogenic response of antigen through an adjuvant effect [32]. The results showed that Span 85-based formulations produced a significantly better immune response compared with Span 60-based formulations upon topical application ( $p < 0.05$ ). Span 85 is an unsaturated surfactant and the packing nature of unsaturated lipids/surfactants chains are reported to change the fluidity of the stratum corneum and

facilitate the skin permeation of bioactives to a higher extent compared with saturated lipids/surfactants [33,34].

A combined serum IgG2a/IgG1 response suggests that the niosomal formulations are capable of eliciting both humoral and cellular responses. The study conclusively suggests the potential clinical application of *O*-palmitoyl mannan-coated niosomes as a topical vaccine delivery carrier and adjuvant. These vesicular systems would be simple, stable, and cost-effective and might be clinically acceptable too [30].

### 2.3 Transfersomes

Transfersomes have high deformable and flexible characteristics that allow them to penetrate intact skin if applied in a non-occlusive way *in vivo* [35,36]; these characteristics endow them with the ability of delivering bioactives across the skin barrier [37]. Hence, specially designed, deformable lipid vesicles, referred to as transfersomes, can be used for the non-invasive delivery of protein antigen(s), as well as DNA or for *in vivo* genetic transfection(s). Transfersomes can transport large macromolecules spontaneously through the skin in an immunologically active form. Gap junction proteins (GJP) incorporated into transfersomes and applied to the intact skin surface give rise to specific antibody titres that are marginally higher than those elicited by subcutaneous injections of GJP in transfersomes, mixed lipid micelles or liposomes [38]. It has been further suggested that topical protein delivery by means of transfersomes appears to increase the relative concentration of anti-GJP IgA in the serum.

The immune-stimulating activity of transfersomes has also been investigated for TTx by measuring the serum anti-TTx IgG titre following topical immunisation. The immune response was compared with the titre measured following the same dose of alum-adsorbed TTx (AATT) given intramuscularly, topically administered plain TTx solution, and a physical mixture of TTx and transfersomes. The results indicated that the transfersomal formulation, with a lipid composition of soya phosphatidylcholine and sodium deoxycholate in 85:15% w/w, with TTx as the antigen, having a size of  $188 \pm 9$  nm and a deformability index of  $121.5 \pm 4.21$ , when applied topically after secondary immunisation, elicited an immune response (anti-TT-IgG) that was comparable to one produced by intramuscular AATT. Fluorescence microscopic studies also indicated the penetration of transfersomes through/across the skin, which tend to deliver the antigen to the immunocompetent Langerhan's cells [39].

In another important study, elastic vesicle transfersomes, non-ionic surfactant vesicles (niosomes) and liposomes were evaluated for their relative potential in non-invasive delivery of TTx. The study compared the serum anti-TTx IgG titre following topical immunisation using these vesicles and the equivalent dose of antigen as alum-adsorbed AATT given intramuscularly. The results revealed that topically administered TTx containing transfersomes, after secondary immunisation, could elicit an immune response (anti-TTx-IgG) equivalent to one produced following intramuscularly



alum-adsorbed TTx-based immunisation. In comparison to transfersomes, niosomes and liposomes elicited a weaker immune response. It can be surmised that this difference in immune response elicited by transfersomes, niosomes and liposomes is due to the unequal capability and divergent tendency of these formulations, especially with respect to penetration across the intact skin barrier. A unique property associated with transfersomes is their deformability. This property, in combination with the sensitivity of transfersomes to the water gradient across the skin, makes this carrier a potential system for topical immunisation. The horny region of the skin is associated with sparsely distributed pores. These pores act as permeability shunt and locally lower the skin permeability barrier. Furthermore, these pores are potential sites for the application of deformable bodies, which are strongly driven under the effect of the *trans*-epidermal water gradient. Liposomes and niosomes cannot enter the intact skin spontaneously because of their low deformability. Transfersomes are unique carriers and adjuvants exploitable in transdermal immunisation. Thus, transfersomes hold promise for the effective non-invasive topical delivery of antigen(s) [40].

A novel, ultra-deformable transfersome formulation prepared using a cationic lipid (DOTAP) and sodium cholate has been found to be capable of transfecting several cell lines, as well as penetrating the intact skin of mice when applied transdermally. Sodium cholate, being an edge activator, is responsible for the flexibility of transfersomes. DOTAP, being positively charged, allowed the liposome to firmly complex with DNA. Thus, ultra-deformable transfersomes have both the ultraflexibility of transfersomes and the firm binding of DNA as the cationic liposomes [41]. In a different study, cationic transfersomes composed of cationic lipid DOTMA and sodium deoxycholate as constitutive lipids were prepared and plasmid DNA encoding HBsAg was incorporated in the cationic transfersomes using a charge neutralisation method. The immune-stimulating activity was studied by measuring the serum anti-HBsAg titre and cytokine level (IL-2 and IFN- $\gamma$ ) following topical application of the plasmid DNA-loaded cationic transfersomes in Balb/c mice. The results were compared with naked DNA applied topically, as well as naked DNA and pure recombinant HBsAg administered intramuscularly. The results revealed that DNA-loaded cationic transfersomes elicited a significantly ( $p < 0.05$ ) higher anti-HBsAg antibody titre and cytokine level compared with naked DNA. The study signifies the potential of cationic transfersomes as DNA vaccine carriers for effective topical immunisation [42].

### 3. Conclusion

The pioneering work of Dr Glenn and the Iomai Company demonstrated the successful exploitation of TCI for the reliable treatment of several infectious diseases via induction of humoral responses following the simple application of

an immunisation patch. Topical immunisation appears to be a fundamental vaccine delivery route that enables the use of a variety of antigens and adjuvants. This review describes the potential and feasibility of vesicular system-based vaccine delivery for TCI, and a summary of work performed in the field so far is presented in Table 1. The immunological competence of the skin, the ease with which antigens can be targeted to defined sites on the skin, the rapid turn-over of skin cells, the efficacy of antigen/DNA-based vaccines and the various reports that animals can be immunised by antigen/DNA-based non-invasive vaccine delivery, may allow for the development of a unique method for vaccination. Skin-targeted non-invasive vaccines could be delivered by a patch containing a uniform dose of antigen/DNA with suitable carriers and adjuvants. As antigen/DNA-based topical immunisation is simple, economical, painless and potentially safe, it may be able to boost vaccine coverage in a wide variety of disease settings.

### 4. Expert opinion

Topical immunisation represents a novel combination using established knowledge relating to skin penetration, the potency of adjuvant-based immunostimulation, and data showing that Langerhan's cells are highly desirable targets because of their APC function. The near-term challenge will be to take this promising insight into successful product development. A wide variety of antigen delivery systems are being explored for antigen delivery into the cytosol of dendritic cells for the induction of class I presentation. Targeting systems carrying DNA-encoding protein antigens are internalised via receptor-mediated endocytosis or phagocytosis, resulting in expression of the antigens in the cytosol. Other delivery systems carry protein and peptide antigens into the cell via receptor-mediated endocytosis and phagocytosis. These antigens may gain access to the MHC class I loading machinery and become cross-presented. In contrast, antigen delivery systems based on toxins deliver protein and peptide antigens directly across the cell membrane into the cytosol without the need for endocytosis or phagocytosis.

The immunological competence of the skin as a site of vaccine delivery has been clearly demonstrated when antigen/DNA-based vaccines are delivered to the epidermis by the topical administration of antigen. Thus, the development of non-invasive immunisation methods using delivery systems capable of transporting protein molecules through intact skin will greatly facilitate the administration of human and veterinary vaccines. The development of transcutaneous vaccine formulations has a sound scientific and commercial basis. The take-up of the full course of injected vaccines in adult target populations, such as that available for hepatitis B, and topically applied vaccines can largely address this problem. Topically applied vaccines offer an opportunity to stimulate a 'useful' type of immunisation (mucosal) against biological agents, including aerosolised threats.

Table 1. A summary of the different vesicular systems used for topical immunisation.

Vesicular delivery system	Antigen/DNA	Ref.
Liposomes	HBsAg TTx pDNA encoding HBsAg pEGFP encoding green fluorescent protein	[24] [26,40] [29,43] [41]
Niosomes	pDNA encoding HBsAg TTx Bovine serum albumin	[29] [40] [30]
Vesosomes (liposomes within liposomes)	TTx	[26]
Transfersomes	Gap junction proteins TTx pDNA encoding HBsAg	[38] [39,40] [42]
Lipid-based biphasic delivery system – Biphaxix®	Recombinant <i>Pasteurella haemolytica</i> leukotoxin and hen egg lysozyme Luciferase as a reporter gene and bovine herpes virus type-1 glycoprotein D	[44] [45]

HBsAg: Hepatitis B surface antigen; pDNA: Plasmid DNA; TTx: Tetanus toxoid.

Vesicular carrier construct(s) could also be used in conjunction with microneedles to bring about a robust immune response following transdermal delivery. Finally, from a commercial perspective, a non-injectable vaccine delivery system may act

as a platform for delivering a variety of antigens or biomacromolecules. Many of the vaccine-related costs, especially those associated with sterile injections, would also be offset by a non-injected system.

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