Liposome-based vaccines for minimally or non-invasive administration: an update on current advancements

Matteo Aroffu[1,2], Maria Letizia Manca[1], José Luis Pedraz[2,3,4] & Maria Manconi[1]

[1]Department of Scienze della Vita e dell'Ambiente, University of Cagliari, via Ospedale 72, 09124, Cagliari, Italy
[2]NanoBioCel Group, School of Pharmacy, University of the Basque Country (UPV/EHU), 01006, Vitoria-Gasteiz, Spain
[3]Biomedical Research Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), 01006, Vitoria-Gasteiz, Spain
[4]BioAraba, NanoBioCel research Group, 01009, Vitoria-Gasteiz, Spain.

Abstract

Introduction: vaccination requires innovation to provide effective protection. Traditional vaccines have several drawbacks, which can be overcome with advanced technologies and different administration routes. Over the past 10 years, a significant amount of research has focused on the delivery of antigens into liposomes due to their dual role as antigen-carrying systems and vaccine adjuvants able to increase the immunogenicity of the carried antigen.

Areas covered: this review encompasses the progress made over the last 10 years with liposome-based vaccines designed for minimally or non-invasive administration, filling the gaps in previous reviews and providing insights on composition, administration routes, results achieved and Technology Readiness Level of the most recent formulations.

Expert opinion: liposome-based vaccines administered through minimally or non-invasive routes are expected to improve efficacy and complacency of vaccination programs. However, the translation from lab-scale production to large-scale production and collaborations with hospitals, research centres and companies are needed to allow new products to enter the market and improve the vaccination programmes in the future.

Article highlights

- Most of vaccines are injected parentally, resulting in poor compliance, high costs and weak mucosal protection.
- Oral, buccal, sublingual, respiratory and cutaneous routes are valid options to achieve cellular and humoral immunity at both local and systemic level.
- The use of liposomes can boost the efficacy of vaccines due to their capability as delivery systems and adjuvant properties.
- Tailoring liposome composition due to the administration route is of primary importance to achieve optimal results.
- The combination of liposome-based vaccines with minimally or non-invasive administration routes and medical devices are expected to improve vaccination programs.

Keywords: phospholipid vesicles; needle-free administration; compliance; immune response; mucosal immunisation; systemic immunisation; vaccination; Technology Readiness Level

1. Introduction

The latest data from the World Health Organization database point out how the higher the vaccination coverage, the lower the number of reported cases for that disease [1]. Vaccination represents indeed the most effective and successful prophylactic intervention ever created to protect people from life-threatening diseases all over the globe [2,3]. Additionally, it plays a significant role in combating antimicrobial resistance and enhancing community resilience and adaptability [3,4]. The Ebola virus, in 2014, and the SARS CoV-2, more recently, are the most striking examples of how infectious diseases can severely afflict and overwhelm public health programmes and clinical services in a short time, highlighting the huge role of vaccination in todays' communities [5,6]. Regrettably, global vaccine coverage has plateaued over the last decade, leading to an increasing number of unvaccinated children, especially in low-income and lower-middle-income countries [7,8]. Different reasons contributed to this issue including 1) supply limitations, 2) restricted access to services, and 3) in some cases, the outbreak of new conflicts. In high-income countries, one of the top ten reasons is the hesitancy of patients, who often refuse vaccines, as recently happened with the COVID-19 pandemic [9–11]. Vaccine hesitancy is a complex and context-specific issue varying across time, space and vaccine type, that is also dependent on factors such as complacency, convenience and confidence [12]. Most of the vaccines

used worldwide are administered parenterally by intramuscular or subcutaneous injection, which entails several disadvantages as the onset of pain or local injury, easy contamination of products, need to healthcare facilities, professional medical staff and expensive formulations [13]. Since these problems are common and geographically widespread, they represent a target for intervention to comprehensively improve human health, especially, but not only, in low-income countries. Non-invasive vaccination, by oral, buccal, sublingual, intranasal, pulmonary and transcutaneous routes may permit to reduce these drawbacks and increase safety. Compared with the current immunization strategies, non-invasive vaccination holds promise for activating local cellular and humoral immunity in skin and mucosae, which are the entrances of pathogens into the human body and are typically not stimulated by parenteral vaccination [14]. Furthermore, it avoids systemic disadvantages, improves patient compliance, facilitates self-administration, eliminates the need for specialized personnel, and greatly reduces mass immunization costs. Altogether, these advantages provided by non-invasive or minimally invasive administration routes hold great promise and might find wide application in future vaccination programs. To date indeed, only a few vaccines are administered intranasally (FluMist/Fluenz® and Nasovac™) or orally (Vaxchora®, Dukoral®, Rotarix™, RotaTeq®, Vivotif ®, and oral polio vaccine) and only in the United States, Europe, Asia and Cuba [14,15]. Unfortunately, due to their composition, they lack long-lasting protection and might raise some concerns about safety. Therefore, the development of other types of vaccines is highly auspicious. In line with this, the review provides an analysis of the recent advancements in vaccine development, focusing on the use of liposomes as valuable and safe nanotechnology to increase patients' compliance and vaccine acceptance. To better understand the mechanisms beyond their effectiveness, an overview of the immune system is provided. All the most recent strategies involving liposomal vaccines to be administered by non-invasive or minimally invasive routes and/or devices are deeply discussed, evaluating their feasibility in a real-life context.

2. The immune system

The immune system is an intricate and communicative network composed by a variety of cells, humoral factors, cytokines and immune organs [16]. As well-known, it provides protection for the body against foreign microorganisms or molecules (antigens) due to its ability to discern between what is "self" and what is "non-self" [16,17]. To make this possible, the system relies on two different but interrelated types of immunity: the innate and the adaptative immunity. The first harnesses barriers such as epithelia, mucus and cilia, as well as cells such as dendritic cells, macrophages, granulocytes and mast cells to protect the host quickly and non-specifically. The latter utilizes T and B cells to originate a delayed but specific response to the antigen, which can also culminate with the development of an immunological memory of the event [18,19]. The two responses are however closely related and converge. Following the first encounter with the pathogen, the cells belonging to the innate immunity (macrophages and dendritic cells), thanks to their ability to sense invading pathogens through specialised receptors called "pattern recognition receptors", initiate the response [20]. So far, four types of these receptors have been identified: tool-like receptors, C-type lectin receptors, retinoic acid-inducible gene (RIG)-I-like receptors and NOD-like receptors. However, regardless of the receptor involved, they enable macrophages and dendritic cells not only to recognise pathogens but also to selectively bind at least one of the highly conserved microbial structures called "pathogen-associated molecular patterns" (i.e. lipids, proteins, lipoproteins or glycoproteins), leading to the phagocytosis of the pathogen. This way, the antigen is enzymatically dismantled and subsequently exposed on the immune cell's membrane surface bound to a receptor belonging to the class of the "major histocompatibility complex". This class is composed by two elements: "major histocompatibility complex class I", expressed in nucleated cells, and "major histocompatibility complex class II", expressed on antigen-presenting cells. Since macrophages and dendric cells are antigen-presenting cells, they show to lymphocytes the processed antigen on the major histocompatibility complex class II. The interaction between the antigen bound to major histocompatibility complex class II expressed on these cells and the T-cell receptor expressed on lymphocytes results in the activation of naïve lymphocytes. At this point, the convergence of the innate and adaptive systems has occurred and the further interaction with co-receptor CD4 or CD8 expressed on naïve T lymphocytes leads to their differentiation into helper T cells and cytotoxic T lymphocytes. Helper T cells (CD4 T cells) play an important role in both cellular and humoral responses. In fact, when activated in the simultaneous presence of IFN-γ and IL-12, they also secrete IFN-γ inducing inflammation and increasing the activity of macrophages and cytotoxic T lymphocytes (CD8 T cells) throughout the cellular response (Th1 response) with the aim of killing the pathogen [21]. Instead, when they are activated by IL-4, they support the so-called humoral response (Th2 response) enabling B cells to produce antibodies. In this case, once the B cells interact with the antigen through their B-cell receptor, they become plasma cells and begin to produce specific antibodies in order to neutralize that antigen. During the process that leads to the differentiation of B cells after the first encounter with the antigen, generally referred as

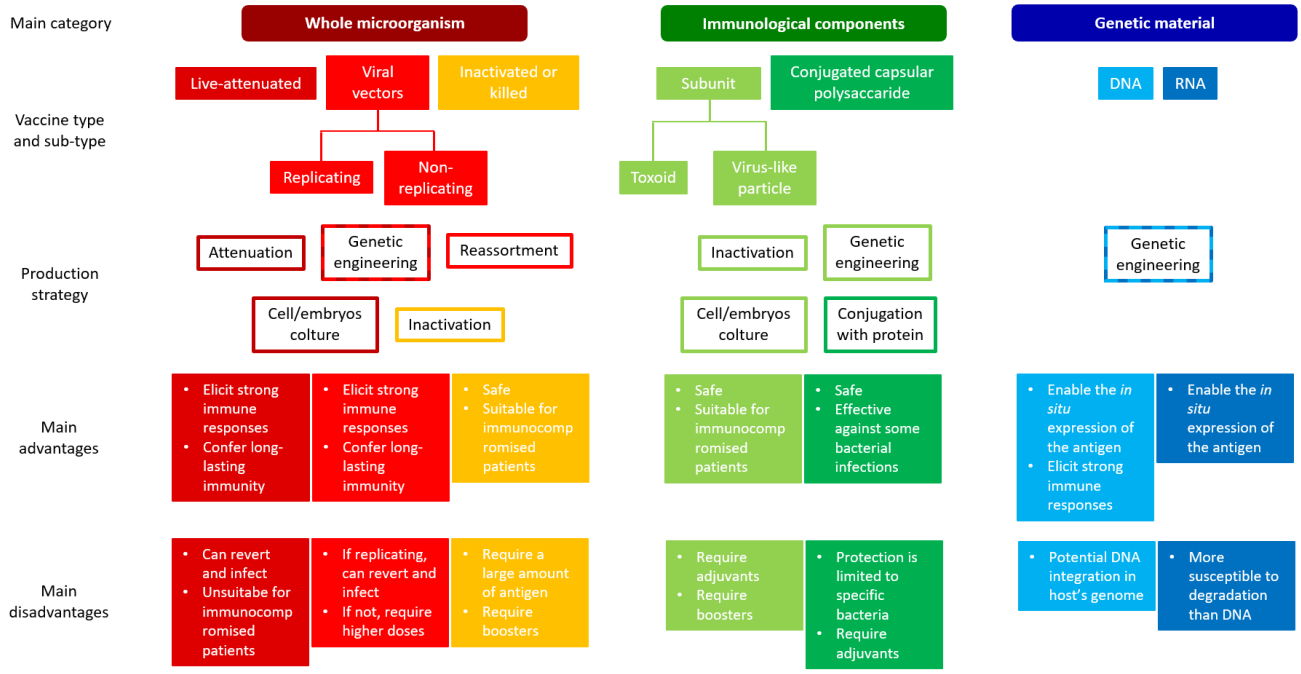
“primary response”, even a pool of memory B cells is produced, and this will be crucial to ensure the immunological memory. In fact, these memory B cells allow the host to counteract rapidly the antigen upon subsequent exposure, making the so-called “secondary response” even quicker than the primary due to their capacity to differentiate in plasma cells faster than naïve B cells [22].

3. Problems encountered in vaccine development and adopted strategies

Within the context of the achievement of an immunological memory, vaccines are invaluable resources because of their ability to stimulate the production of a clonally expanded population of antigen-specific lymphocytes, which ensure immunization by enabling the body to respond more rapidly and effectively to pathogens or their toxins that had been encountered previously in form of a “harmless version [17,23]. To ensure this stimulation, different strategies have been adopted throughout the century (**Table 1**) [24]. At first, attenuated vaccines were prepared reducing the virulence of pathogens through multiple passages in different tissues or hosts. Then, this method was modernised by growing pathogens in cell cultures, as in the case of the oral polio vaccine. Later, genetic reassortment, made it possible to manipulate segments of RNA virus genomes to safely handle viruses while maintaining their ability to stimulate the immune system. Unfortunately, their main problem was the possibility, for the living pathogen, to revert to a virulent state thus jeopardising the safety of the individual. Consequently, the next natural steps were 1) achieving the inactivation of pathogens through physical or chemical methods and 2) using only their capsular polysaccharides or proteins. Finally, genetic engineering emerged as an easier way to increase the amount of antigen needed for the vaccine, remove some genetic material from the microorganism to make it safer or provide micro-organism-derived vectors for antigen delivery. Unfortunately, when these technologies are applied to vaccine development, safety is not the only concern, as mode and ease of use, stability, cost-effectiveness, and ability to trigger an effective immune response are relevant as well. Conventional vaccines are commonly administered by parental route, which it is associated with pain, needle phobia and consequently low compliance for the patients [25]. Last but not least, it does not guarantee any immunization of skin and mucosae and can also seriously damage them, altering their protective function and favouring pathogens/other molecules to penetrate deep inside the organism. This effect is largely detrimental, especially when multiple administrations are needed [26].

Notwithstanding these important drawbacks, to date, parenteral vaccines have ensured tremendous achievements and have been extremely successful in preventing infections by pathogens expressing relatively conserved antigens through antibody-mediated effector mechanisms [27]. Nonetheless, advanced technologies and tools can offer new strategies to rationally design effective vaccines where conventional approaches have failed or may be improved. Accordingly, non-invasive or minimally invasive technologies such as nasal sprays, dry powder inhalers, metered dose inhalers, patches, powder/jets injectors and microneedles, have been tested as alternative approaches in vaccination [28–32]. Simultaneously, molecules/systems able to improve the magnitude of the immune response to the antigen (adjuvants), have been considered during vaccine design [33]. Currently, the credited mechanisms of adjuvanticity include the shipment of antigens to lymph nodes, the antigen safeguard, the increased reaction at the administration site, the induction on the release of cytokines and the engagement with pattern recognition receptors [34,35]. In this context, nanotechnologies are a valid approach, as they can display an adjuvant effect themselves while delivering the antigen and/or another adjuvant improving their stability and safety profiles [36–38].

Table 1. List of traditional vaccine types along with their production strategies and main advantages and disadvantages.



4. Formulative nanotechnologies

Nanotechnologies are the practical applications of a branch of science called nanoscience, which studies phenomena occurring at nanoscale dimensions (i.e. 0.1-1000 nm) to design, manufacture, characterise, and test materials, structures, systems and devices [39,40]. Due to their size, shape, surface area, charge, functionalization and safety, some nanosystems have found application in the medical field either for diagnostic or therapeutic purposes [41]. As a matter of fact, they have allowed not only to overcome biological barriers but also to control drug release, extend its blood circulation time and reduce its toxicity, thus proving to be outstanding tools in medicine [42]. For this reason, they have gained a relevance in the pre-clinical and clinical development of medicine, and several nanomaterials have been tested as carrier systems for different payloads (i.e. drugs, proteins, peptides and nucleic acids) [43]. Nanocarriers are colloidal systems usually formed by macro-molecules or supra-molecular aggregates, usually distinguished according to their structure into nanocapsules or nanospheres. The former consists of a homogenous spherical matrix in which the drugs are homogenously dispersed, the latter are core-systems surrounded by a spherical membrane in which drugs may be trapped in the membrane, encapsulated within the core or adsorbed onto the surfaces [44]. Over the years, they have been variously modified from unspecific composition and structure to a tailored one to achieve different goals (from passive to active targeting, from uncontrolled to controlled release etc.) [45]. All these modifications have been essential to develop therapeutic products now approved for clinical use [46]. To date, these nanoformulated products are available to treat infections, chronic diseases, pain, autoimmune diseases, mental disorders and even cancer [47]. Another growing area of interest is "nanovaccinology", in which antigens and/or adjuvants are delivered by non-viral vectors overcoming common issues of conventional vaccines (i.e. the poor immunogenic response, the not properly safe profile, the instability during their storage or distribution and/or after administration and the need for multiple administrations) [36,38,48,49].

5. Liposomes in vaccine development

Liposomes have been identified as one of the most effective carriers for vaccine development [50]. They are nanosized vesicles made of phospholipids, which in water form closed bilayers surrounding an aqueous core and one or more interlamellar spaces [51]. Due to this peculiar structure, liposomes can encapsulate hydrophilic molecules and entrap hydrophobic ones [52]. The cell-like membrane structure, along with the high biocompatibility, the low immunogenicity and the possibility of protecting the payloads, modifying their biodistribution, reducing their toxicity and even extending their half-life make them the perfect candidates to improve the antigen presentation and foster its uptake by professional antigen-presenting cells overcoming the problems of conventional vaccines [48].

Considering the promising role of liposomes in this field, the most recent (2013-2023) liposome-based vaccine tested through minimally or non-invasive administration routes are presented, discussed and summarised according to their administration routes (**Figure 1** and **Tables 2-5**).

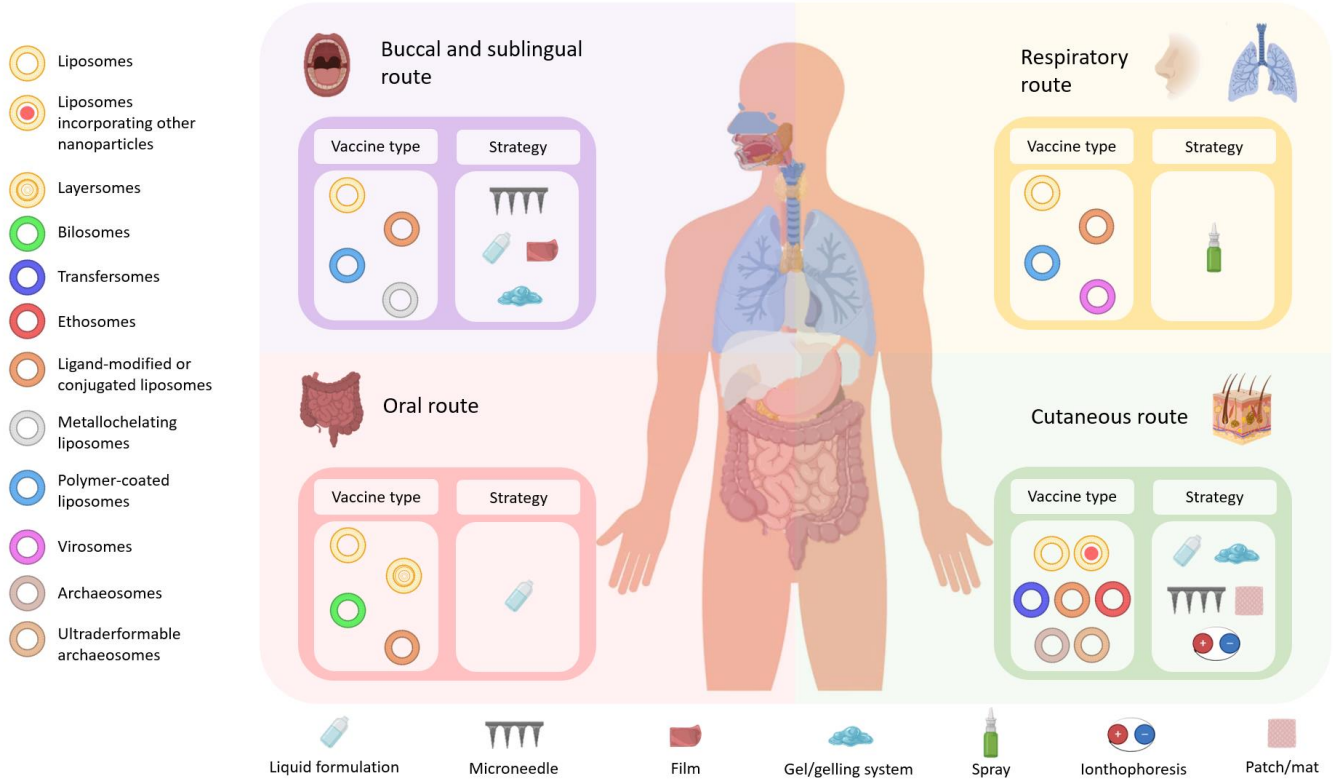


Figure 1. Liposome-based vaccines developed in the last decade (2013-2023) and tested trough minimally or non-invasive administration routes, alone or in combination with medical devices to pursue needle-free immunization.

6. Vaccination via oral, sublingual and buccal routes

The oral is the most accepted administration route because of its ease of access and high patient compliance [53]. However, not all molecules can be easily administered through this route as the strong acidic environment of the stomach, the presence of proteolytic enzymes in it, the inaccessibility of intestinal epithelial barrier due to tight junctions and mucus, as well as the strong metabolic activity that takes place in the liver, are factors that can seriously dampen the bioavailability of bioactives and thus their therapeutic effect [54]. Despite all these limitations, several strategies can make this route viable to achieve local or systemic effects [55].

Oral administration also offers important advantages in vaccine formulations, as they enable self-administration, improve compliance and ensure stimulation of the gastrointestinal immune system [56]. At this level, it is finely regulated by the gut-associated lymphoid tissue, which harbours the majority of immune cells in the whole body and can generate lasting immunity at both mucosal and systemic levels if stimulated. Accordingly, the oral vaccines available on the market act against acute enteric infections caused by pathogens that 1) remain in the gastrointestinal mucosa (e.g. enterotoxigenic *Escherichia coli* or *Vibrio cholerae*) or 2) spread from it causing systemic diseases (e.g. *Salmonella typhi*) [57]. However, although several vaccine dosage forms have been tested for years, only a small number is licensed and used clinically [58]. To address the limitations of a conventional oral administration, which requires swallowing of the antigen, two other valuable strategies are buccal and sublingual immunisation, which rely on local adsorption or the passage through the oral cavity allowing for enhanced local immunisation or to by-pass hepatic metabolism to achieve systemic immunisation [59].

Overall, beyond the challenges posed by oral administration, the possibility opened by different dosage forms, along with the advantages of liposomal administration and the high patience compliance provided by the oral, buccal and sublingual routes, have prompted scientists to formulate new vaccines to better elicit humoral and cellular immune responses at systemic and mucosal level (**Tables 2 and 3**) [60].

6.1. Liposome-based vaccines improved and tailored for oral administration

In 2014, Liu and colleagues designed an oral vaccine based on DNA-loaded cationic liposomes and aimed at stimulating the expression of the M1 gene of influenza A virus [61]. The resulting formulation successfully induced M1 gene expression *in vitro* in the tested cell line and *in vivo* in the intestine of orally treated mice, boosting both humoral and cellular immune responses. Interestingly, one week after the vaccination, no virus was found in the lungs of mice. The immunity achieved at respiratory level can be related to the migration of sensitized competent cells from the gastrointestinal mucosa to distant lymphoid tissues and mucosal sites [62].

In 2015, Harde and co-workers chose tetanus toxoid as model antigen for oral vaccination [63]. It was encapsulated in liposomes and layersomes, prepared from the formers by alternate layer-by-layer coating of polyacrylic acid and polyallylamine. Even though both vesicles retained the conformation and native 3D structure of the antigen, layersomes, which were more stable in simulated biological fluids, induced higher humoral, mucosal and cellular immune responses in mice. Overall, results confirmed their promising properties as oral delivery systems and emphasised as a proper design is crucial when developing formulations to counteract the gastrointestinal aggressive environment.

In this context, bilosomes are often used to pursue oral immunisation [64]. Indeed, they are basically lamellar vesicles (liposomes or niosomes) suitably modified with bile salts in order to achieve resistance to the metabolically active environment of the gastro-intestinal and facilitate the oral administration of antigenic payloads. Additionally, they act themselves as adjuvants capable of stimulating gastrointestinal immune responses [65].

Accordingly, these carriers were adopted by Wilkhu *et al.* for the oral delivery of recombinant influenza hemagglutinin [66]. No antigen loss was detected *in vitro* in the simulated gastric media when it was encapsulated in bilosomes. Additionally, biodistribution studies in mice demonstrated that bilosomes could both promote accumulation in the small intestine and antigen uptake within the Peyer's patch and mesentery lymph nodes. Lastly, ferrets orally immunised with antigen-containing bilosomes were effectively protected against fever and lung inflammation.

In another study, Jain and colleagues chemically functionalised bilosomes with glucomannan to deliver bovine serum albumin orally [67]. These novel carriers were stable in simulated gastro-intestinal fluids, sustained antigen release up to 24 h and significantly improve payload uptake *in vitro* and *in vivo* in comparison with unmodified bilosomes and free bovine serum albumin. Furthermore, this response was comparable to intramuscularly injected alum-adsorbed bovine serum albumin, thus confirming the suitability of these systems for easy mass vaccination.

Table 2. Liposome-based vaccines designed for oral administration.

Delivery system(s)	Composition	Antigen(s)	Additional adjuvant	Combination with other strategy or technology	Administration route	Reference
Cationic liposomes	N.R.	pcDNA3.1(+)/ M1 plasmid	-	-	Oral	[61]
Cationic liposomes; **Cationic layersomes**	Epikuron 200, cholesterol, and stearylamine; Cationic liposomes composition + polyacrylic acid sodium salt and polyallylamine hydrochloride	Tetanus toxoid	-	-	Oral	[63]
Bilosomes	Monopalmitoyl glycerol, cholesterol, dicetyl phosphate and sodium deoxycholate	Recombinant hemagglutinin (rHA)	-	-	Oral	[66]
Bilosomes; **Glucomannosylated bilosomes**	Span 80, cholesterol, sodium deoxycholate	Bovine serum albumin	-	-	Oral	[67]

	and stearyl amine; Bilosomes composition + glucomannan-O-Carboxymethyl-Distearyl Phosphatidyl Ethanolamine					

6.2. Liposome-based vaccines improved and tailored for buccal and sublingual administration

In 2014, Wang and collaborators combined the advantages of the oral mucosal administration with those of a cold chain-free, adjuvanted delivery system. To this end, they designed dually decorated liposomes in form of dry powder [68]. Bovine serum albumin was encapsulated as model antigen whereas monophosphoryl lipid A (a toll-like receptor 4 agonist) and a synthetic mannose conjugate (mannose-PEG-cholesterol conjugate, a C-type lectin receptor agonist) were used as adjuvants and to decorate liposome surface. Since the mannose receptor is widely expressed on antigen-presenting cells, the mannose dislocated on vesicles' surface was expected to serve as specific targeting molecule [69]. According to the results, vesicles effectively promoted antigen uptake by immunocytes mainly via these receptors. Additionally, the combination of antigen and adjuvants resulted in a mixed cell-mediated and humoral response following vesicle administration in mice. This multiple defence mechanism was confirmed one year later, when the same vesicles were coupled with microneedles [70]. They proved to be stable at room temperature and suitable devices for vaccination. Indeed, a greater response was observed *in vivo* for the formulation included within microneedles probably due to their ability to ensure better adherence to the mucosa [71]. On these premises, the same research group developed a vaccine against hepatitis B virus [72]. Microneedles were not only applicable in the controlled temperature chain but also allowed to boost immunization efficiency *in vivo* in comparison with the aqueous suspension since no antigen was either swallowed or trapped by mucus. Consequently, as highlighted by these studies, an approach that increases the residence time and the contact with the mucosa should be always considered when the goal is to achieve oral mucosa immunization. However, it should be noted that microneedles, albeit painlessly, partially disrupt epithelia and can cause local inflammation or allergies, so alternative approaches might also be considered [73,74]. Recently, Mašek and colleagues explored multi-layered nanofibrous mucoadhesive films containing liposomes for buccal and sublingual vaccination [75]. Promising results were achieved on an *ex vivo* and *in vivo* pig model as the films controlled the delivery of the vesicles trough the mucosa. Given the huge versatility of these systems, their use will be very likely in the future as the advent of 3D printing technologies will lead to faster, easier and more scalable manufacturing processes [76,77].

Garcia-del Rio and colleagues, developed a muco-adhesive thermogelling hydrogel containing liposomes to be used sublingually after parental prime against *Chlamydia trachomatis* [78]. Application under the tongue and liposome contact with the sublingual tissue were facilitated, avoiding antigen loss, promoting its absorption and increasing cellular and local immunoglobulin A immune responses during the *in vivo* studies.

Oberoi and collaborators co-delivered influenza antigens with traditional and modified liposomes containing the synthetic toll-like-4 receptor agonist CRX-601, either coated or not with the muco-adhesive agent methylglycol chitosan [79]. Liposomes provided only a modest improvement in the immune response over the traditional ones whereas their combination with methylglycol chitosan led to the most consistent response highlighting the importance of mucoadhesiveness for sublingual vaccines.

Since the results obtained with liposome vaccination by this route have turned out to be very promising, some examples of its application in the so-called sublingual immunotherapy can already be found in literature [80–82]. Nonetheless, no sublingual vaccines against infectious diseases are available on the market [79].

Table 3. Liposome-based vaccines designed for buccal and sublingual administration.

Delivery system(s)	Composition	Antigen(s)	Additional adjuvant	Combination with other	Administration route	Reference

				strategy or technology		
PEG-mannosylated cationic liposomes	Mannose-PEG-cholesterol, soy phosphatidylcholine (SPC) and stearyl amine (SA)	Bovine serum albumin (BSA)	Monophosphoryl lipid A	-	Buccal	[68]
PEG-mannosylated cationic liposomes	Mannose-PEG-cholesterol, soy phosphatidylcholine (SPC) and stearyl amine (SA)	Bovine serum albumin (BSA)	Monophosphoryl lipid A	Dis[70]solving microneedles	Buccal	[70]
PEG-mannosylated cationic liposomes	Mannose-PEG-cholesterol, soy phosphatidylcholine (SPC) and stearyl amine (SA)	Hepatitis B virus (HBV) surface antigen	Monophosphoryl lipid A	Dissolving microneedles	Buccal	[72]
Liposomes; **Metallochelating liposomes**	Distearoyl phosphatidylethanolamine-polyethylene glycol, egg phosphatidylcholine (EPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl); DOGS-NTA-Ni-1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt), egg phosphatidylcholine (EPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-	-	-	Mucoadhesive film	Buccal/Sublingual	[75]

	phospho-(1'-rac-glycerol) (sodium salt)					
Cationic liposomes	Trehalose 6,6´dibehenate (TDB), dimethyldioctadecylammonium bromide (DDAB)	CTH522	CAF01	Mucoadhesive hydrogel	Sublingual	[78]
Liposomes; **Pluronic liposomes;** **PEGylated liposomes;** **Chitosan-coated liposomes;**	1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), cholesterol; 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) + Pluronic L64/F68/F127; Liposomes composition + [N-(carbonyl-methoxypolyethylenglycol-2000)-distearoyl-phosphoethanolamine (DSPE-PEG2K)/ N-(carbonyl-methoxypolyethylenglycol-5000)-dipalmitoyl-phosphoethanolamine (DPPE-PEG5K); Liposomes/Pluronic liposomes/PEGylated liposomes composition + methylglycol chitosan (MGC)/glycol chitosan (GC)/chitosan	Hemagglutinin (HA, detergent split)	CRX-601	-	Sublingual	[79]

	oligosaccharide lactate (CO)

7. Vaccination via respiratory route

The respiratory route is a solid option to use for mass vaccination being it needleless, painless, highly accessible and free of sterility requirements [83]. From an immunology perspective, what makes it appealing are the components located between the upper and lower respiratory tracts. In particular, epithelial compartments filled with immunocompetent cells, lymphoid tissues such as nose-, larynx- and bronchus-associated lymphoid tissue, and draining lymph nodes have revealed to be key elements in the protection against air-borne diseases [83]. In addition to the possibility of rapidly and massively thwarting the pathogen right at the site of entry, systemic immunisation can also be achieved through this route making it even more strategic. Currently, Fluenz®, Flumist® and Nasovac® are human vaccines against influenza already available on the market for intranasal administration [84]. Unfortunately, all of them are live attenuated vaccines, so many carriers have been proposed by scientists as alternatives to get safer profiles. Among them, liposomes are recognised as reliable and efficient systems and have been exploited for nasalor pulmonary immunization many times over (**Table 4**).

7.1 Liposome-based vaccines improved and tailored for nasal and pulmonary administration

Considering the high incidence of rhinitis, in 2016 Tasaniyananda and colleagues exploited a mouse model of cat allergic rhinitis to evaluate the therapeutic efficacy *in vivo* of an intranasal vaccine formulated with liposomes encapsulating the major cat allergen, Fel d 1, or the entire crude cat hair extract [85]. Both vaccines mediated the reduction of the mucus production and the allergic manifestations in mice. Additionally, they caused a shift of the pathogenic humoral immune response towards the non-pathogenic cell-mediated and regulatory T-cell responses. The liposomes loading the cat allergen were the most effective, but further tests are required before clinical application.

Yang et al., with the aim of eliciting protection against group A *Streptococcus*, conjugated the lipopeptide-based liposomes with cell-penetrating peptides to overcome membrane permeability issues [86]. Their efficacy was demonstrated *in vivo*, as the vesicles were able to boost the humoral response and provide an immune stimulation even greater than the cholera toxin-based adjuvant. Similar results had been obtained also by Azuar and colleagues, exploiting instead cholic acid as anchoring moiety and relying on the fact that bile salts possess immunomodulatory activity [87]. The conjugation between the bile salt and the antigenic peptide derived from Group A *Streptococcus* was easily achieved and, once encapsulated in liposomes, triggered strong humoral immune responses following intranasal administration in mice. It is likely that uptake by the same antigen-presenting cell of both adjuvant and antigen as single entity can induce stronger immune responses. Therefore, conjugation can be a valid strategy to induce high antibody titres.

Senchi et al. investigated the effectiveness of oligomannose-coated liposomes against the human parainfluenza virus type 3, an etiologic agent responsible for pneumonia and respiratory infections [88]. Full-length hemagglutinin-neuraminidase was used as antigen whereas oligomannose was used to coat liposomes and target antigen-presenting cells. While liposomes themselves did not promote a significant immune response, their intranasal coadministration with the adjuvant polyriboinosinic-polyribocytidylic acid led to significative viral-specific immunity *in vitro* and *in vivo*. Additionally, the combination allowed to reduce the dose of antigen needed to stimulate the immune response showing enormous promise in the immunisation against respiratory viruses. In another study, Dhakal and colleagues aimed at improving specific cellular and mucosal humoral immune responses against influenza virus using liposomes adjuvanted with monosodium urate crystals, an activator of the innate immune response [89,90]. The vaccine, administered as intranasal mist in an *in vivo* pig model, reduced the clinical signs of flu, virus load and pneumonic lesions. It was also confirmed that broad protection was achieved through both mucosal and systemic immune responses. Even though results were promising, it must be underlined that all the current influenza vaccines, including this one, fail to demonstrate efficacy against different subtypes of the virus because of the constant drift/shift of the surface antigens commonly used (i.e., hemagglutinin and neuraminidase). An attempt to overcome this problem was recently made by Wang and co-workers [91]. Since interferons are known to provide wide protection against viral infections, 2',3'-cyclic guanosine monophosphate-adenosine monophosphate was used as adjuvant and loaded in novel pulmonary surfactant-biomimetic liposomes. Vesicles were prepared with different phospholipids to obtain negative, neutral or positive surfaces and intranasally co-administered with the whole inactivated A/Vietnam/1203/2004(VN04) H5N1 vaccine. Among the formulations, the negatively charged vesicles were able to

stimulate the production of immunoglobulin G and immunoglobulin A, successfully generating prolonged immunity in two *in vivo* models, finally confirming their potential towards the development of a universal influenza vaccine. Recently, due to the outbreak of COVID-19, research has found itself in urgent need for a SARS-CoV-2 vaccine [92]. An and colleagues developed a single-dose intranasal vaccine encapsulating 2',3'-cyclic guanosine monophosphate–adenosine monophosphate in negatively charged liposomes on which surface the trimeric S-protein of the virus was adsorbed [93]. *In vivo* results indicated that the vaccine was safe and elicited comprehensive immunity at nasal and lung level, confirming its suitability for fast, mass vaccination.

A different trend in terms of superficial charge has instead been observed when the target is the nose immunization. Indeed, it must be considered that the residence time in the nasal mucosa is a critical parameter for antigen adsorption. Therefore, negative surface charged liposomes could be repulsed by negatively charged mucus and antigen-presenting cells located in the nasal cavity affecting the response [94]. On the contrary, cationic liposomes may allow to fulfill two needs with one deed providing 1) increased residence time in the mucus and 2) greater adjuvanticity [95]. These two aspects were investigated and confirmed in several comparative studies carried out by Tada and colleagues, who observed a boosted uptake of different antigens by dendritic cells after their co-administration, *in vivo*, with cationic liposomes made of 1,2-dioleoyl-3-trimethylammonium-propane and cholesteryl 3β-N-(dimethylaminoethyl)-carbamate [96–98]. In addition, they demonstrated that these carriers, when harbouring oligodeoxynucleotides containing immunostimulatory CpG motifs and co-administered intranasally *in vivo* with ovalbumin, were able to increase the mucosal levels of immunoglobulin A and reduce the side effects of these motifs [99]. By the comparison made by Wenjing et al. instead, the cationic liposomes prepared with dimethyldioctadecylammonium/trehalose 6,6,9-dibehenate and bearing the influenza antigen A achieved even significantly better results than the liposomes prepared with 1,2-dioleoyl-3-trimethylammonium-propane and cholesteryl 3β-N-(dimethylaminoethyl)-carbamate. Indeed, they observed that, these vesicles 1) were better internalized by dendritic cells *in vitro* and 2) were more efficient at boosting mucosal immunoglobulin A and systemic immunoglobulin G antibody titres *in vivo* [100]. However, it is difficult to address these results only to the vesicle composition. Indeed, the differences in size among the vaccines must be considered while looking at these results as the modulation of the immune response is also influenced by this parameter [101]. A different formulation of cationic liposomes was investigated by Yusuf and colleagues, this time prepared with dimethyldioctadecylammonium either alone or in combination with D-alpha-tocopheryl polyethylene glycol 1000 succinate. Clear evidence was provided on its ability to improve *in vitro* internalisation, *ex vivo* permeability into nasal bovine tissue and humoral response *in vivo* following the nasal administration of vesicles in mice [102]. On these basis, Zhuang and Qi delivered mRNA encoding hemagglutinin in cationic liposomes comprised of 1,2-dioleoyl-3-trimethylammonium-propane, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine and, alternatively, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol)-2000) or 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol)-mannose [103]. Both carriers were effective but the ones exploiting the combination of PEGylated and mannose ligands were more efficient in the gene delivery both *in vitro* and *in vivo* in mice. Overall, results suggest the use of ligands as a tool to further improve vaccination.

Despite the several advantages provided by cationic lipids, their high costs and toxicity remain their major drawbacks. Therefore, alternatives have been found in positive charge inducers such as stearylamine, chitosan and its derivatives [104,105]. *Ex vivo* studies have indeed confirmed their ability to improve the mucoadhesiveness and the ability of the so-modified liposomes to deliver protein cargos through the nasal mucosa [106,107]. Marasini et al. developed a vaccine using trimethyl chitosan-coated liposomes and tested it *in vivo* to evaluate its protective effects against Group A *Streptococcus* [108]. When the vaccine was intranasally administered to mice, durable immunization was achieved for over 4 months and specific mucosal and systemic antibody titres were stimulated after a single boost.

If most intranasal vaccines are destined to improve protection locally, some vaccines have been developed for other purposes. Leroux-Roels and colleagues, in a phase I study, evaluated the effect of a vaccine obtained by the integration of the HIV-1 Gp41 P1 peptide in liposomes. When administered intranasally, it was able to elicit distal mucosal responses even at vaginal level, where it may help in reducing sexually transmitted HIV-1 [109].

In 2012, with the aim of fulfilling the ideal mucosal vaccine requirements of both stimulating mucosal and systemic responses, Wang, Jiang et al. manufactured galactose-modified liposomes loading ovalbumin. Their capability to enhance the levels of mucosal immunoglobulin A and systemic immunoglobulin G against free ovalbumin was demonstrated after intranasal administration in mice [110]. Two years later, the same group also demonstrated their capability to foster the antigen uptake by dendritic cells compared to the corresponding unmodified liposomes both *in vitro* and *in vivo* [111]. Kakhi and colleagues developed a liposomal vaccine able to exert a strong immune response

against lungs tumour, increasing the INF-γ levels up to 155 times while using a vaccine dose 4 times lower than the respective subcutaneous vaccine [112]. Lastly, in another study they investigated the activity of di-epitopic liposomal constructs containing the ErbB2 T-cytotoxic epitope, the influenza-derived hemagglutinin T-helper epitope and the lipopeptide adjuvant Pam2CAG against lung tumour [113]. Different size, structure and compositions were tested but none of them impacted on vaccine immunity and antitumoral efficiency, in contrast to total dose of vaccine or dose of adjuvant. Despite the great promise showcased by these anti-tumour vaccines candidates, further studies are needed before clinical applications in cancer prophylaxis.

Table 4. Liposome-based vaccines designed for intranasal administration.

Delivery system(s)	Composition	Antigen(s)	Additional adjuvant	Combination with other strategy or technology	Administration route	Reference
Liposomes	Didodecyldimethylammonium bromide (DDAB), soy phosphatidylcholine (SPC) and cholesterol	Fel d 1/ crude cat hair extract (cCE)	-	-	Intranasal	[85]
Anionic liposomes	Dipalmitoylphosphatidylcholine (DPPC), cholesterol and cell-penetrating peptides (CPPs)	Synthetic lipopeptide-based antigen (LCP-1)	-	-	Intranasal	[86]
Cationic liposomes	Dipalmitoylphosphatidylcholine (DPPC), cholesterol and Didodecyldimethylammonium bromide (DDAB)	Peptide derived from Group A *Streptococcus*	Cholic acid	-	Intranasal	[87]
Liposomes	Dipalmitoylphosphatidylcholine (DPPC), cholesterol and oligomannose-dipalmitoyl-phosphoethanolamine (Man3-DPPE)	Hemagglutinin-neuraminidase (HN)	Polyriboinosinic-polyribocytidylic acid [poly(I:C)]	-	Intranasal	[88]
Liposomes	Soy lecithin, cholesterol and alpha tocopherol	Pooled influenza A virus peptides	Monosodium urate (MSU) crystals	-	Intranasal	[89]
Anionic liposomes	1,2-dipalmitoyl-sn-glycero-3-	Inactivated H1N1 vaccine	2′,3′-cyclic guanosin	Freeze-drying	Intranasal	[91]

	phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE-PEG2000) and cholesterol		e monophosphate-adenosine monophosphate (cGAMP)			
Anionic liposomes	1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE-PEG2000) and cholesterol	SARS-CoV-2 spike protein (S-protein)	2',3'-cyclic guanosine monophosphate-adenosine monophosphate (cGAMP)	-	Intranasal	[93]
Cationic liposomes	1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-chol)	Ovalbumin (OVA)	-	-	Intranasal	[96]
Cationic liposomes	1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and	Ovalbumin (OVA)	-	-	Intranasal	[97]

	3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-chol)					
Cationic liposomes	1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-chol)	Pneumococcal surface protein A (PspA)	-	-	Intranasal	[98]
Cationic liposomes	1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-chol)	Ovalbumin (OVA)	Oligodeoxynucleotides containing immunostimulatory CpG motifs (CpG ODNs)	-	Intranasal	[99]
Cationic liposomes (1) **Cationic liposomes (2)** **Neutral liposomes**	Dimethyldioctadecylammonium (DDA) and trehalose 6,6,9-dibehenate (TDB); 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-chol); 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol	Influenza antigen A (H3N2)	-	-	Intranasal	[100]

Anionic liposomes; **Cationic liposomes;** **PEGylated cationic liposomes**	Soy phosphatidylcholine (SPC); Anionic liposomes composition + Dimethyldioctadecylammonium (DDA); Cationic liposomes composition + D-alpha-Tocopheryl polyethylene glycol 1000 succinate (TPGS)	Ovalbumin (OVA)	-	Freeze-drying	Intranasal	[102]
PEGylated cationic liposomes; **PEG-mannosylated cationic liposomes;**	1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy (polyethylene glycol)-2000) (DSPE-$mPEG_{2000}$); 1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(polyethylene glycol)-	mRNA encoding hemagglutinin (HA)	-	-	Intranasal	[103]

	Mannose (DSPE-PEG-Mannose)					
Alginate/chitosan /trimethyl chitosan (TMC)-coated liposomes	Soy phosphatidylcholine (SPC), phospholipid dimyristoyl phosphatidylglycerol (DMPG) and cholesterol + alginate/chitosan/ trimethyl chitosan (TMC)	Bovine serum albumin (BSA)	-	Spray drying	Intranasal	[106]
Chitosan/Carbopol® 974P NF lipogel	Egg phosphatidylcholine (EPC) and cholesterol + Chitosan/Carbopol® 974P NF	Ovalbumin (OVA)	-	Gel	Intranasal	[107]
Trimethyl chitosan (TMC)-coated liposomes	Dipalmitoylphosphatidylcholine (DPPC), cholesterol and 1-α-phosphophatidyl-DL-glycerol sodium (PG)	Peptide derived from Group A *Streptococcus*	-	-	Intranasal	[108]
Virosomes	Hemagglutinin (HA) and neuramidase (NA) glicoproteins mixed with egg phosphatidylcholine (EPC) and phosphatidylethanolamine (PE)	HIV-1 Gp41 P1 peptide	-	-	Intranasal	[109]
Galactosylated liposomes	Phosphatidylcholine (PC), cholesterol and galactosyl-1,2-didodecanoyl-sn-glycero-3-phosphoethanolamine	Ovalbumin (OVA)	-	-	Intranasal	[110]

	(galactosyl-DLPE)					
Galactosylated liposomes	Phosphatidylcholine (PC), cholesterol and galactosyl-1,2-didodecanoyl-sn-glycero-3-phosphoethanolamine (galactosyl-DLPE)	Ovalbumin (OVA)	-	-	Intranasal	[111]

8. Vaccination via cutaneous route

Skin represents the largest and most accessible route for the administration of therapeutics and it has long been used to induce immunization. Unfortunately, apart from a few vaccines not administered by the skin route, almost all the currently licensed vaccines are delivered via intramuscular and subcutaneous injection through hypodermic needles [114]. Consequently, the potential of this route is hindered by needle phobia, pain, puncture injuries, the risk of infection by blood-borne pathogens or death and the overall high costs of transport, storage and disposal [115]. In addition to this, no skin immunization is achieved using these routes, even though it harbours a complex network of immune cells, comprising antigen-presenting cells such as Langerhans cells in the viable epidermis and dendritic cells and macrophages in the dermis, which are valuable targets for vaccination [116–118]. Therefore, nanocarriers such as liposomes and derivatives, alone or in combination with different devices (e.g. microneedles, jet/powder injectors and transdermal patches) as well as physical techniques (e.g. iontophoresis, sonophoresis and thermal ablation), that rely instead on epidermal and transcutaneous/transdermal routes, have been widely exploited as valid minimally-invasive or non-invasive approaches to pursue immunization, even at local level [119–124].

8.1. Liposome-based vaccines improved and tailored for cutaneous administration

Zhang and colleagues formulated three different types of phospholipid vesicles (liposomes, transfersomes and ethosomes) carrying ovalbumin and saponin, either modified with cholesterol and/or stearylamine or not, and tested their efficacy for transdermal immunization in mice [125]. Despite all the vesicles improved the skin permeation of the antigen and the antibody titres with respect to the free antigen, cationic ethosomes were the most effective. The authors hypothesised a synergistic effect between the ability of ethanol to induce a disorder in the lipid structure of the stratum corneum, thus increasing skin permeability, and the ability of stearylamine to induce a cationic charge on vesicle surface, thus favouring the recognition by immune cells. Tyagi and Garg prepared transfersomes to deliver the malaria antigen MSP-1_{19} from *Plasmodium falciparum* to immunocompetent Langerhans cells in the epidermis [126,127]. Due to the elasticity and deformability that span 80 provided to these carriers, transdermal immunization was achieved *in vivo* and comparable specific immunoglobulin G antibody responses were observed against both plain antigen alum-adsorbed and intramuscularly injected liposomes.

As an alternative to phospholipid vesicles, archaeosomes, which are basically lamellar vesicles formulated with lipids extract from *Archaea*, have aroused considerable attention in vaccinology [128,129]. An *ex vivo* study carried out by Jia et al. demonstrated their superiority, when applied onto the skin surface, in ensuring better distribution and higher ovalbumin accumulation in the viable skin than liposomes [130]. Caimi and co-workers enriched archaeosomes with sodium cholate and obtained ultradeformable archaeosomes for the delivery of imiquimod, a topical adjuvant [131,132]. In the comparison with the liposomal counterpart, they induced higher imiquimod accumulation in human skin explants. Consequently, upon topical application in mice, they led to higher systemic response while using only a 13-fold lower dose. However, as stated by Carrer et al., attention must be paid on the composition of the total polar archaeolipids extracted as high levels of phosphatidylglycerophosphate methyl ether seem to reduce their penetration by ∼1.5 folds [133]. Consequently, a certain variability on the outcome can be expected depending on the microorganism used. In another comparative study carried out by Caimi and co-workers, ultradeformable archaeosomes were obtained from *Halorubrum tebenquichense* and used to manufacture a topical vaccine by loading ovalbumin [134]. To produce a vaccine not only effective but also marketable, the stability of these vesicles was

evaluated under stress conditions (thermal stress, sterilisation and freeze-drying) along with their ability to elicit a systemic antigen-specific immune response. Ultradeformable archeosomes demonstrated higher stability than the respective transfersomes under both a wide range of temperatures (4, 40 and 80°) and sterilisation. Additionally, they proved to be the only formulation able to elicit the same immune response, irrespective of freeze-drying. Ultradeformable archaeosomes from the same *Archaea* were also exploited by Higa and colleagues in the development of a vaccine against leishmaniasis [135]. When applied onto mice's skin, they penetrated the stratum corneum down to the viable epidermis transporting the antigens, thus increasing the levels of the pro-inflammatory cytokine IL-1β, which is involved in the protection against *Leishmania spp*. However, further insights are needed to confirm if this secretion by macrophages may contribute to an *in vivo* lethal response to the *Leishmania* parasites.

Overall, the promising results obtained testing all these vaccine candidates can be addressed to the peculiar structures achieved by modifying conventional liposomes. Ethanol and edge activators such as span 80 and sodium cholate have indeed led to improved skin penetration while the polar lipids from *Archea* have provided greater thermal and pH stability as well as enhanced immunostimulatory effects [136,137]. However, since modified structures alone might not be enough to ensure proper immunization through the skin, recently research has also investigated new ways to facilitate topical application of vaccines and improve performances [119,138]. To avoid the damages from the high electrical voltage of electroporation, iontophoresis, which uses instead a weak electrical current, was investigated for the first time to achieve transcutaneous immunization by Bernardi and colleagues [139]. Ovalbumin-loaded liposomes were formulated incorporating silver nanoparticles to improve iontophoresis efficiency and thus antigen delivery. The application of the liposomal vaccine to the skin through iontophoresis 1) *ex vivo,* improved the delivery of the antigen to the viable epidermis by 92-fold in comparison to its passive delivery and 2) *in vivo*, elicited higher humoral and cellular responses in comparison to the subcutaneous injection of ovalbumin. Although results are noteworthy, it must be acknowledged that such method requires specific equipment and the capability to correctly set iontophoretic parameters. Therefore, other strategies might be more easily applied to commercial vaccines. In the regime of a painless, self-administration, liposomes have been combined with microneedles multiple times in drug delivery as well as in vaccination [140,141]. Yuan-Chuan Chen adopted these devices for the formulation of a vaccine against plague loading the F1 antigen of *Yersinia pestis* into liposomes [142]. The vaccine, applied to the skin through microneedles, induced adaptive immunity in mice increasing Immunoglobulin G antibody titres and survival rates with respect to the control groups (PBS and F1-Alugel) administered topically. Du and co-workers investigated the effect on the immune response provided by four different nanoparticles (polylactic-co-glycolic acid nanoparticles, liposomes, mesoporous silica nanoparticles and gelatin nanoparticles) co-loading ovalbumin, as antigen, and polyriboinosinic-polyribocytidylic acid, as adjuvant [143]. All the formulations were topically administered using hollow microneedles and the effect on the immune response was evaluated in mice. If on the one hand the co-encapsulation of antigen and adjuvant did not increase the total immunoglobulin G response with respect to the unloaded antigen or the adjuvant, on the other hand it was crucial in promoting a cell-mediated response in a nanoparticle-dependent manner. Specifically, cationic liposomes made of egg phosphatidyl choline, dioleoyl-3-trimethylammonium-propane chloride salt and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, due to both their composition and size, offered the highest immunization. These findings were confirmed in a subsequent study by the same group using the same combination of adjuvanted cationic liposomes and hollow microneedles but to deliver instead the diphtheria toxoid as a model antigen [144]. Part of these valuable results clearly lies in the ability of microneedles to grant liposomal vaccines a preferential pathway to antigen-presenting cells in the skin. However, this specific type of microneedles can suffer of microchannel blockage and, in addition, must be removed after use for disposal. A good alternative is therefore represented by dissolving microneedles, whose composition and structure allow to easily overcome these problems [145]. Wu and colleagues exploited these devices in combination with ovalbumin-loaded transfersomes [146]. Transfersomes with opposite surface charges were prepared to investigate charge influence on the immune response. Despite the anionic ovalbumin-loaded vesicles (prepared with sodium cholate) were more biocompatible and better internalised by dendritic cells, the cationic counterparts (prepared instead with polyquaternium-7 and stearylamine) were more efficient in the induction of a cell-mediated immune response. Consequently, their combination with hyaluronic acid, self-dissolving microneedles seems to be a suitable method for cutaneous vaccination. Furthermore, in a more general context, due to their dissolvable nature, they can also help in reducing the spread of blood-borne diseases. This dual prevention potential was studied by Qiu et al. by coupling cationic liposomes with dissolving microneedles in an attempt to induce transcutaneous immunisation against hepatitis B [147]. Polyvinylpyrrolidone-K17 and K30 were selected to prepare the microneedles whereas the plasmid DNA vector VR2012 encoding envelope proteins of hepatitis B virus was co-encapsulated with the toll-like receptor 9 adjuvant cytosine-phosphate-guanine

oligodeoxynucleotide in cationic liposomes consisting of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, cholesterol and dimethyldioctadecylammonium. *In vivo* studies confirmed the efficacy and suitability of this combination as the immune response outcomes were comparable to those of conventional needle administration of the liposomes while avoiding hazardous wastes. Very different results were achieved instead by Lanza and colleagues, who proposed cationic liposomes enclosed within dissolvable microneedle patches as next generation vaccine against leishmaniasis [148]. In this study, the recombinant antigen LiHyp1 was either co-encapsulated with the adjuvant cytosine-phosphate-guanine oligodeoxynucleotide or not. The liposome-based vaccine was immunogenic when injected but unfortunately its protective effect decreased significantly when inserted in the microneedle patch. The explanation of this undesirable result might lie in the high polydispersity index of the liposomal formulation and in the presence of antigen aggregates outside or attached to the vesicle's membrane, the magnitude of which further increased after incorporation in the microneedles. Therefore, composition and homogeneity of the liposome dispersion, outside and inside these devices, as well as its compatibility with them, must be verified when using microneedles. With that in mind, Guo and co-workers prepared polyvinylpyrrolidone dissolving microneedles and combined them with ovalbumin-loaded cationic liposomes adjuvanted by cytosine-phosphate-guanine oligodeoxynucleotide [149]. Liposomes were stable within the microneedles, which dissolved completely within 3 minutes, allowing them to generate balanced cellular and humoral immune response and higher levels of anti-ovalbumin immunoglobulin G antibodies than intramuscularly injected ovalbumin. Similar results were also achieved *in vivo* by Zhao and Zhang using polyvinylpyrrolidone-K17/polyvinylpyrrolidone-K30 dissolving microneedles to deliver through the skin cationic liposomes co-loading ovalbumin and the saponin adjuvant platycodin [150]. These microneedles dissolved within 1 minute generating minimal irritation in rabbit skin and facilitating the transition of vesicles through the epidermis. In addition, liposomes decreased the saponin-induced haemolysis while allowing to exploit its adjuvanticity. Consequently, the proposed system enhanced the immune response while being well tolerated.

Since high patient acceptance is required to ensure a compliant therapy, alternative strategies have been investigated as well. Zhang and colleagues included their ovalbumin-loaded ethosomes adjuvanted by a saponin in two different carbomer hydrogels to facilitate vaccine administration [151]. The ethosomes-containing gel prepared with PBS and ethanol was more stable than the respective gel prepared with water and more effective *in vivo* in boosting serum antibody titres than the same gel containing unencapsulated antigen and saponin. Yang et al. explored instead the immunization potential of ovalbumin-loaded ethosomes, modified with hyaluronic acid and galactosylated chitosan, and included in nanofibrous mats fabricated through a green electrospinning process [152]. The novel mats facilitated the application of the vaccine, which in turn effectively targeted the dendritic cells stimulating their maturation and ensuring skin and systemic anti-tumour immunity in mice.

Despite the great results obtained with these strategies, from a clinical translation point of view there might be a few issues that need to be solved, such as sterility requirements for medical devices such as microneedles, patches and mats, as well as the need for applicators to ensure proper dose delivery or device placement.

Table 5. Liposome-based vaccines designed for cutaneous administration.

Delivery system(s)	Composition	Antigen(s)	Additional adjuvant	Combination with other strategy or technology	Administration route	Reference
Anionic and cationic liposomes; **Anionic and cationic ethosomes;** **Anionic and cationic transfersomes;**	Soy phosphatidylcholine (SPC), cholesterol and stearylamine (if cationic); Liposomes composition + ethanol + stearylamine (if cationic);	Ovalbumin (OVA)	Saponin	-	Transcutaneous, transdermal	[125]

	Liposomes composition + sodium cholate + stearylamine (if cationic);					
Transfersomes	Soy phosphatidylcholine (SPC) and Span 80	MSP-1_{19}	-	-	Transcutaneous, transdermal	[126,127]
Anionic liposomes; **Archaeosomes;** **Semi-synthetic archaeosomes;** **Semi-synthetic archaeosomes;** **Semi-synthetic archaeosomes;** **Semi-synthetic archaeosomes;**	1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG) Total polar lipids (TPL) from *M. smithii*/*H. salinarum*/*H. volcanii;* Lactosylarchaeol (LA) and sulfated lactosylarchaeol (SLA); β-gentiotriosyl-A (Glc_3), α-mannotriosyl-A (Man_3), lactosylarchaeol (LA) and archaetidylglycerol-phosphate-O-CH3 (PGP); β-gentiotriosyl-A (Glc_3), α-mannotriosyl-A (Man_3), archaetidlyserine (AS) and archaetidylglycerol-	Ovalbumin (OVA)	-	-	Transcutaneous, transdermal	[130]

	phosphate-O-CH3 (PGP); Mannotriosyl-A (Man_3) and archaetidylglycerol-phosphate-O-CH3 (PGP);					
Anionic transfersomes; **Ultradeformable archaeosomes;**	Soy phosphatidylcholine (SPC) and sodium cholate; Total polar archaeolipids (TPA) from *H. tebenquichense*, soy phosphatidylcholine (SPC) and sodium cholate	Ovalbumin (OVA)	Imiquod	-	Transcutaneous, transdermal	[131]
Anionic liposomes; **Anionic transfersomes;** **Archaeosomes;** **Ultradeformable archaeosomes;**	Soy phosphatidylcholine (SPC); Liposomes composition + sodium cholate; Total polar archaeolipids (TPA) from *H. tebenquichense*; Archaeosomes composition + soy phosphatidylcholine (SPC) and sodium cholate;	Ovalbumin (OVA)	-	-	Transcutaneous, transdermal	[133]
Anionic transfersomes; **Ultradeformable archaeosomes;**	Soy phosphatidylcholine (SPC) and sodium cholate; Total polar archaeolipids (TPA) from *H. tebenquichense*, soy phosphatidylcholine (SPC)	Ovalbumin (OVA)	-	Freeze-drying	Transcutaneous, transdermal	[134]

	and sodium cholate					
Anionic liposomes; **Anionic transfersomes;** **Ultradeformable archaeosomes;**	Soy phosphatidylcholine (SPC); Liposomes composition + sodium cholate; Total polar archaeolipids (TPA) from *H. tebenquichense,* soy phosphatidylcholine (SPC) and sodium cholate;	*L. braziliensis* proteins (Detergent-solubilized)	-	-	Transcutaneous, transdermal	[135]
Anionic liposomes incorporating silver nanoparticles	Soy phosphatidylcholine (SPC) and 1,2-dioleoylphosphatidylethanolamine (DOPE)	Ovalbumin (OVA)	-	Iontophoresis	Transcutaneous, transdermal	[139]
PEGylated liposomes	1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, distearoyl-phosphoethanolamine-PEG_{2000} (DSPE-PEG_{2000}) and docosahexaenoic acid	*Y. pestis* F1 antigen	-	Solid microneedles	Transcutaneous, transdermal	[142]
Cationic liposomes	Egg phosphatidylcholine (EPC), 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)	Ovalbumin (OVA)	Polyriboinosinic-polyribocytidylic acid [poly(I:C)]	Hollow microneedles	Transcutaneous, transdermal	[143]

Cationic liposomes	Egg phosphatidylcholine (EPC), 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)	Diphtheria toxoid	Polyriboinosinic-polyribocytidylic acid [poly(I:C)]	Hollow microneedles	Transcutaneous, transdermal	[144]
Anionic transfersomes; **Cationic transfersomes;**	Egg phosphatidylcholine (EPC), sodium cholate (SC) and hyaluronic acid-monostearin (HA-GMS); Egg phosphatidylcholine (EPC), hyaluronic acid-monostearin (HA-GMS), polyquaternium-7 (PQ-7) and stearylamine (SA);	Ovalbumin (OVA)	-	Dissolving microneedles	Transcutaneous, transdermal	[146]
Cationic liposomes	Dimethyldioctadecylammonium (DDA), 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol	Plasmid vector VR2012 encoding the middle envelope proteins of Hepatitis B virus (HBV)	Cytosine-phosphate-guanine oligodeoxynucleotide (CpG ODN)	Dissolving microneedles	Transcutaneous, transdermal	[147]
Cationic liposomes	1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dipalmitoyl-sn-glycero-3-phosphocholi	Leishmania recombinant antigen LiHyp1	Cytosine-phosphate-guanine oligodeoxynucleotide (CpG ODN)	Dissolving microneedles, freeze-drying	Transcutaneous, transdermal	[148]

	ne (DPPC) and cholesterol					
Cationic liposomes	Dimethyldioctadecylammonium (DDA), 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol	Ovalbumin (OVA)	Cytosine-phosphate-guanine oligodeoxynucleotide (CpG ODN)	Dissolving microneedles	Transcutaneous, transdermal	[149]
Cationic liposomes	Hydrogenated egg phosphatidylcholine (HEPC), cholesterol and octadecylamine	Ovalbumin (OVA)	Platycodin (PD)	Dissolving microneedles	Transcutaneous, transdermal	[150]
Anionic/cationic ethosomes	Soy phosphocholine (SPC), cholesterol, ethanol and stearylamine (SA) (if cationic)	Ovalbumin (OVA)	Saponin	Hydrogel	Transcutaneous, transdermal	[151]
Galactosylated chitosan-modified ethosomes	Egg phosphatidylcholine (EPC), cholesterol, octadecylamine, hyaluronic acid and ethanol	Ovalbumin (OVA)	-	Mat	Transcutaneous, transdermal	[152]

9. Clinical trials involving liposome-based nanovaccines/adjuvants

From our screening in the literature, all the clinical trials concerning liposome-based vaccines in the decade 2013-2023 involve parental administration [153]. Firstly, 27 clinical trials were found, 11 of which were completed, 3 still active but not recruiting, 2 not recruiting, 9 recruiting, 1 terminated and 1 had an unknown status (**Table 6**). Only 9 out 27 trials involved universities, with the remaining involving either companies, research institutes or hospitals. In these studies, liposomes were used either as vaccines (10) or as adjuvants (17). When used as adjuvants, they were formulated as monophosphoryl lipid A liposomal adjuvant (MPLA liposomes), liposome-based adjuvant containing 3-O-desacyl-4'-monophosphoryl lipid A and the saponin Quillaja saponaria-21 (AS01), glucopyranosyl lipid adjuvant-liposome-Quillaja saponaria-21 formulations (GLA-LSQ and AP10-602), army liposome formulation containing the 43% of cholesterol (ALF43) or army liposome formulation containing a synthetic monophosphoryl lipid A with Quillaja saponaria-21 (ALFQ). On the whole, they were tested against acquired immunodeficiency syndrome (AIDS, 10 vaccines), malaria (4 vaccines), tuberculosis (1), herpes (1 vaccine), Coronavirus disease (COVID-19, 1 disease), Campylobacter infections, solid tumors (2 vaccines), Papillomavirus-associated oropharynx cancer (1 vaccine), cervical cancer (1 vaccine), ovarian or breast cancer (2 vaccines), melanoma (1 vaccine), glioma and glioblastoma (1 vaccine) and leukaemia (1 vaccine). Most of the trials (22) are on phase I, with only 4 trials on phase II and 1 on phase IV. Regardless of the clinical trial, no minimally or non-invasive route was explored. All the vaccines were indeed administered parenterally as follows: 66.7% intramuscularly, 18.5% subcutaneously and 14.8% intravenously. The only exception is represented by the aforementioned intranasal formulation of Leroux-Roels and colleagues, who published their paper in 2013 but reporting data from a phase I clinical trial completed in 2010.

Table 6. Clinical trials involving liposome-based vaccines/adjuvants.

Status	Title (*ClinicalTrials.gov ID*)	Liposome-based vaccine/adjuvant type	Administration route	Investigator and collaborator	Phase
Completed	Evaluating the Safety and Immunogenicity of an HIV-1 gp41 MPER-656 Liposome Vaccine in Healthy, HIV-uninfected Adult Participants (*NCT03934541*)	HIV-1 gp41 MPER-656 liposome vaccine	Parenteral (intramuscular)	Brigham and Women's Hospital and University of Alabama at Birmingham	**I**
	Amsterdam UMC Clinical Trial With a Native-like HIV-1 Envelope Vaccine (ACTHIVE-001) (*NCT03961438*)	ConM SOSIP.v7 gp140 adjuvanted with MPLA liposomes	Parenteral (intramuscular)	Academisch Medisch Centrum - Universiteit van Amsterdam (AMC-UvA)	**I**
	Phase 1 Study of ONT-10 in Patients With Solid Tumors (*NCT01556789*)	Liposomal MUC1 Cancer Vaccine	Parenteral (subcutaneous)	Cascadian Therapeutics Inc.	**I**
	Phase 1 Clinical Trial With Controlled Human Malaria Infection (CHMI) to Evaluate the Safety and Efficacy of the Plasmodium Falciparum Vaccine Candidate FMP012 Administered Intramuscularly With AS01B Adjuvant System in Healthy Malaria-Naïve Adults (*NCT02174978*)	Plasmodium Falciparum Malaria Protein FMP012 with liposomal AS01B	Parenteral (intramuscular)	Walter Reed Army Institute of Research (WRAIR), United States Agency for International Development (USAID), Military Infectious Diseases Research Program (MIDRP) and GlaxoSmithKline (GSK)	**I**
	Safety and Immunogenicity of Pfs25M-EPA/AS01 and Pfs230D1M-EPA/AS01 Vaccines, Transmission Blocking Vaccines Against Plasmodium Falciparum, at Full and Fractional Dosing in Adults in Mali (*NCT02942277*)	*P. aeruginosa* ExoProtein A (EPA)-conjugated Pfs25 and Pfs230 surface antigens adjuvanted with liposomal AS01	Parenteral (intramuscular)	National Institute of Allergy and Infectious Diseases (NIAID)	**I**
	rCSP/AP10-602 [GLA-LSQ] Vaccine Trial (*NCT03589794*)	Recombinant circumsporozoite protein (rCSP) antigen malaria vaccine adjuvanted with GLA-LSQ	Parenteral (intramuscular)	National Institute of Allergy and Infectious Diseases (NIAID)	**I**
	Safety and Immunogenicity of the Placental Malaria Vaccine Candidate PAMVAC Variously Adjuvanted (PAMVAC)	PAMVAC vaccine antigen adjuvanted with GLA-LSQ	Parenteral (intramuscular)	University Hospital Tuebingen	**I**

	(*NCT02647489*)				
	Clinical Trial of HIV Vaccine Combinations in Healthy Men and Women (Ad4HIV) (*NCT03408262*)	Recombinant glycoprotein of HIV-1 isolate 97CN54 adjuvanted with MPLA liposomes	Parenteral (intramuscular)	Imperial College London	**I**
	A Challenge Study to Assess the Safety, Immunogenicity and Efficacy of a Malaria Vaccine Candidate (*NCT02927145*)	Malaria vaccine RH5.1 with liposomal AS01	Parenteral (intramuscular)	University of Oxford	**II**
	Safety, Tolerability, and Immunogenicity of the Vaccine Candidates ID93 + AP10-602 and ID93 + GLA-SE Administered Intramuscularly in Healthy Adult Subjects (*NCT02508376*)	ID93 recombinant mycobacterium protein antigen adjuvanted with AP10-602	Parenteral (intramuscular)	National Institute of Allergy and Infectious Diseases (NIAID)	**I**
	Study of ONT-10 and Varlilumab to Treat Advanced Ovarian or Breast Cancer (*NCT02270372*)	Varlilumab with ONT-10 (liposomal synthetic glycopolypeptide MUC1 targeted antigen)	Parenteral (subcutaneous)	Cascadian Therapeutics Inc. and Celldex Therapeutics	**I**
Active not recruiting	Vaccine Therapy for Treating Patients With Previously Untreated Chronic Lymphocytic Leukemia (CLL) (*NCT01976520*)	Liposome-based vaccines containing an extract of a person's cancer cells and the immunostimulant IL-2	Parenteral (subcutaneous)	XEME Biopharma Inc.	**I**
	A Phase I, Randomized, Double-Blind, Placebo-Controlled Safety, Tolerability and Immunogenicity Study of Candidate HIV-1 Vaccines ChAdOx1.HTI and MVA.HTI With Recombinant HIV-1 Envelope Protein ConM SOSIP.v7 gp140 Vaccine, Adjuvanted With MPLA Liposomes in ART-Suppressed HIV-1 Positive Individuals (*NCT05208125*)	HIV envelope protein ConM SOSIP.v7 gp140 vaccine adjuvanted with MPLA liposomes	Parenteral (intramuscular)	IrsiCaixa	**I**
	A Study to Assess the Safety and Immune Response to Env-C DNA and Protein Vaccines in Kenya	HIV Env-C DNA and protein vaccines adjuvanted with ALF43	Parenteral (intramuscular)	National Institute of Allergy and Infectious Diseases (NIAID)	**I**

	(*NCT04826094*)				
Not recruiting	Novel RNA-nanoparticle Vaccine for the Treatment of Early Melanoma Recurrence Following Adjuvant Anti-PD-1 Antibody Therapy (*NCT05264974*)	Autologous total tumor mRNA-loaded DOTAP liposome vaccine	Parenteral (intravenous)	University of Florida	**I**
	Safety and Efficacy of Neutralizing Antibodies and Vaccination for Induction of HIV Remission (RV582) (*NCT05769569*)	Neutralizing antibodies (VRC07-523LS, PGDM1400LS and N-803) adjuvanted with ALFQ	Parenteral (intramuscular)	Henry M. Jackson Foundation for the Advancement of Military Medicine, US Military HIV Research Program and Janssen Vaccines & Prevention B.V.	**I**
Recruiting	Clinical Trial to Evaluate the Safety and Immunogenicity of Recombinant HIV-1 Envelope Protein SOSIP v8.2 763 Vaccine, Adjuvanted With MPLA Liposomes, in Healthy, HIV-Uninfected Adults (HIVAC-FOUND) (*NCT05772286*)	Recombinant HIV-1 Envelope Protein SOSIP v8.2 763 vaccine adjuvanted with MPLA liposomes	Parenteral (intramuscular)	Fundacion Clinic per a la Recerca Biomédica and Polymun Scientific GmbH	**I**
	HIV Vaccine in HIV-uninfected Adults (RV546) (*NCT04658667*)	Full-length single chain gp120-CD4 chimera subunit HIV-1 vaccine and A244 gp120 envelope glycoprotein HIV-1 adjuvanted with ALFQ	Parenteral (intramuscular)	U.S. Army Medical Research and Development Command, Armed Forces Research Institute of Medical Sciences, ThailandMahidol University, Duke University University of Maryland (Baltimore) and Case Western Reserve University	**I**
	Safety and Immunogenicity of CJCV2 With and Without ALFQ (*NCT05500417*)	*Campylobacter jejuni* conjugate vaccine adjuvanted with ALFQ	Parenteral (intramuscular)	National Institute of Allergy and Infectious Diseases (NIAID)	**I**
	A Vaccine (PDS0101) Alone or in Combination With Pembrolizumab for the Treatment of Locally Advanced Human Papillomavirus-Associated Oropharynx Cancer (*NCT05232851*)	Liposomal HPV-16 E6/E7 Multipeptide Vaccine PDS0101	Parenteral (subcutaneous)	Mayo Clinic	**II**
	A Vaccine (PDS0101) and Chemoradiation for the Treatment of Stage IB3-	Liposomal HPV-16 E6/E7	Parenteral (subcutaneous)	M.D. Anderson Cancer Center	**II**

	IVA Cervical Cancer, the IMMUNOCERV Trial (*NCT04580771*)	Multipeptide Vaccine PDS0101			
	A Study of RNA-lipid Particle (RNA-LP) Vaccines for Newly Diagnosed Pediatric High-Grade Gliomas (pHGG) and Adult Glioblastoma (GBM) (PNOC020) (*NCT04573140*)	Autologous total tumor mRNA and pp65 full length lysosomal associated membrane protein mRNA-loaded DOTAP liposome vaccine	Parenteral (intravenous)	University of Florid	**I**
	Safety, Tolerability, and Immunogenicity of ALFQ in a HIV Vaccine Containing A244 and B.65321 in Healthy Adults (RV575) (*NCT05423418*)	Vaccine A244/B.63521 adjuvanted with ALFQ	Parenteral (intramuscular)	U.S. Army Medical Research and Development Command	**I**
	Recombinant Herpes Zoster Vaccine in Patients With Autoimmune Rheumatic Diseases (RZVRheum) (*NCT05879419*)	Recombinant *Herpes zoster* vaccine adjuvanted with liposomal AS01B	Parenteral (intramuscular)	University of Sao Paulo General Hospital and GlaxoSmithKline (GSK)	**IV**
	A Trial to Evaluate the Safety and Efficacy of CLDN6 CAR-T +/- CLDN6 RNA-LPX (*NCT04503278*)	Unmodified and modified RNA liposomal formulations	Parenteral (intravenous)	BioNTech Cell & Gene Therapies GmbH	**II**
Terminated	Ovarian Cancer Treatment With a Liposome Formulated mRNA Vaccine in Combination With (Neo-) Adjuvant Chemotherapy (OLIVIA) (*NCT04163094*)	Liposome Formulated mRNA Vaccine in Combination With (Neo-) Adjuvant Chemotherapy	Parenteral (intravenous)	University Medical Center Groningen	**I**
Unknown	SARS-COV-2-Spike-Ferritin-Nanoparticle (SpFN) Vaccine With ALFQ Adjuvant for Prevention of COVID-19 in Healthy Adults (*NCT04784767*)	SARS-COV-2-Spike-Ferritin-nanoparticle vaccine adjuvanted with ALFQ	Parenteral (intramuscular)	U.S. Army Medical Research and Development Command, Walter Reed Army Institute of Research (WRAIR) and Henry M. Jackson Foundation for the Advancement of Military Medicine	**I**

MPLA liposomes: monophosphoryl lipid A liposomal adjuvant; **AS01**: liposome-based adjuvant containing 3-O-desacyl-4'-monophosphoryl lipid A and the saponin Quillaja saponaria-21; **GLA-LSQ** and **AP10-602**: glucopyranosyl lipid adjuvant-liposome-Quillaja saponaria-21 based formulations; **ALF43**: army liposome formulation enriched with cholesterol (43%); **ALFQ**: army liposome formulation containing a synthetic monophosphoryl lipid A (MPLA, 3D-PHAD®) with Quillaja saponaria-21.

10. Stability and toxicity of liposome-based vaccines

Despite the great potential showcased by liposome-based vaccines, they are still far from flawless. Limitations, especially in terms of stability and toxicity, may represent the major cause for the limited number of clinical trials currently available. As extensively discussed by Jyothi and colleagues, liposomal formulations can be affected by physical, chemical and biological instabilities [154]. Sterility and apyrogenicity must be granted right from the development stage not to obtain false-positive results, as cell from the immune systems are extremely responsive to endotoxin contamination [155]. The pH and the impact of the biological fluids should be tested in an administration route-dependent manner, as skin and mucosae do not share the same pH values or enzymes [156]. Size and carrier structure, as well as integrity of the encapsulated antigen, must be monitored as well, as they can be altered not only by the site of administration but also by the storage conditions [157]. The liposome-based vaccines reported in this review were prepared under aseptic conditions and, in most cases, their stability was explored with regard to the administration route (i.e. simulated pH, ionic strength and biological fluids). By contrast, only a few addressed the problem of the physical stability on a long-term storage [158]. Most of the liposome-based vaccines were liquid and colloidal formulations are known for their limited stability overtime. Additionally, the antigens often lack of thermostability and thus need low (- 4 °C) or ultra-low storage temperatures (from - 20 to - 80 °C), which raise costs and are undesirable especially in the warmer countries [159]. In this respect, a proper selection of the post-processing method (i.e. spray drying, freeze-drying, spray freeze-drying, vacuum, or air-drying) might help in solving this problem and should always be considered during vaccine development [160]. Careful attention must also be paid on the composition of the nanocarrier as it can both affect colloidal/thermostability and cytotoxicity [161]. Liposome-based vaccines are usually prepared using phospholipids, which are vital components of the cell membranes in eukaryotic cells either obtained from natural sources or by synthesis. Composition can then be tailored to extend liposome circulation in the blood (i.e. PEGylation) or liposome behaviour in the skin (i.e. edge activators), in the gastro-intestinal tract (i.e. bile salts), in the nose (i.e. mucoadhesive polymers) etc. In some studies, cationic lipids were also exploited due to their ability to provide liposomes with a better interaction with antigen-presenting cells. Unfortunately, cationic liposomes are usually more toxic than neutral or negative liposomes and therefore a dose adjustment might be needed [105]. In any case, it must be pointed out that almost every nanocarrier present some degree of toxicity but what makes their use interesting is the possibility of reducing the side effects of some antigens, which if not encapsulated could generate even worse effects [162]. To date, there is no predictive model to know about the *in vivo* toxicity of any nanocarrier in advance.

11. Conclusions

Vaccination has revolutionized the field of medicine improving the quality of life and reducing the number of deaths worldwide. Substantial technological advances, as well as a deeper understanding of some of the processes underlying immunisation itself, have enabled increased vaccination coverage rates to be achieved with less effort. Nonetheless, vaccination is still mainly reliant on needle administration and thus still fails in meeting patients' compliance and reducing vaccination general costs. By the time this review was written, a lot of non-invasive or minimally invasive approaches to achieve immunisation were found in the literature and a lot of them relied on nanocarriers. Among them, liposomes, if properly designed, have immeasurable potential in vaccine development as they cannot only allow a needle-free delivery but also protect the antigen, modify its release, transport it to the target and boost its immunogenicity while improving its safety profile. Besides, due to the advancements in the field, a number of devices and/or techniques can nowadays be associated with them to further improve their performance, stability and even enhancing their skin and mucosae immunization properties painlessly.

12. Expert opinion

The optimal liposome delivery performances are confirmed by the 25 formulations available on the market [163]. However, among them, only 6 are vaccines. This means that, despite the very promising results achieved by researchers worldwide, some challenges still need to be addressed to commercialize new liposome-based vaccine products [164]. From our research in the literature, it has emerged that the studies involving liposomes have some technological limitations. The Technology Readiness Level is a tool for the assessment of the readiness of products [165,166]. We observed that the recent studies (2013-2023) on liposome-based vaccines were mainly carried out at laboratory level, which correspond to low Technology Readiness Levels (from 1 to 5), whereas only one of them reached clinical trials, which correspond instead to higher levels (up to 6-7). This is a huge limitation as these promising formulations, especially in combination with non-invasive devices, may substantially impact global health and safety, favouring mass vaccination, increasing vaccine coverage and providing effective herd immunity. Unfortunately, the

clinical trial iter is long and winding. In addition, passing this stage do not ensure that the product will enter the market: if the procedure for a medical product formulated with nanocarriers is difficult because of the need to comply with very specific requirements set by the regulatory authorities, the process for nanocarrier-based vaccines can be even more complicated. Moreover, when a needle-free route of administration is chosen, it is also of paramount importance using the appropriate device not only to improve patient compliance and ensure proper immunisation but also to guarantee what the vaccine plan sets. So it goes without saying that the device must also be approved by the European Medicines Agency (EMA) and Food and Drug Administration (FDA). At present, commercialising a nano-based vaccine (Technology Readiness Level 9) is still an end-to-end venture, especially because university laboratories are not full-equipped to deal with large-scale production, prototyping, quality control and clinical research. It is thus expected that in the next 10 years technologies capable of high precision, reproducibility and possibly to meet sterility requirements, such as microfluidics and 3D printing, will be widely used in this field. Furthermore, collaboration opportunities with hospitals and/or companies are expected to arise, leading to customised designs during the first stages of development or providing access to proper structures during the final stages prior commercialisation. Despite we acknowledge that tremendous progress has been made in recent years regarding the immune response, some grey areas are still present, so that continued research on this topic will be of utmost importance in the future to develop better vaccines. Finally, dissemination events in simplified language for the population will be needed to further improve acceptance of the vaccines and provide greater adherence to vaccination programs.

References

[1] World Health Organization. Immunization dashboard [Internet]. [cited 2023 Oct 9]. Available from: https://immunizationdata.who.int/.

[2] Greenwood B. The contribution of vaccination to global health: Past, present and future. Philosophical Transactions of the Royal Society B: Biological Sciences. 2014.

[3] Mantel C, Cherian T. New immunization strategies: adapting to global challenges. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz. 2020.

[4] Jansen KU, Knirsch C, Anderson AS. The role of vaccines in preventing bacterial antimicrobial resistance. Nat Med. 2018.

[5] Qureshi AI. Economic and Political Impact of Ebola Virus Disease. Ebola Virus Disease. 2016.

[6] Shlomo M, Barzani E. The Global Economic Impact of Covid-19: A Summary of Research. Samuel Neaman Institute for National Policy Research. 2020;

[7] Babirye JN, Engebretsen IM, Rutebemberwa E, et al. Urban settings do not ensure access to services: Findings from the immunisation programme in Kampala Uganda. BMC Health Serv Res. 2014;14.

[8] WHO. WHO and UNICEF warn of a decline in vaccinations during COVID-19. Who. 2020.

[9] Dubé E, Vivion M, MacDonald NE. Vaccine hesitancy, vaccine refusal and the anti-vaccine movement: Influence, impact and implications. Expert Rev Vaccines. 2014.

[10] Dror AA, Eisenbach N, Taiber S, et al. Vaccine hesitancy: the next challenge in the fight against COVID-19. Eur J Epidemiol. 2020;35.

[11] Troiano G, Nardi A. Vaccine hesitancy in the era of COVID-19. Public Health. 2021.

[12] MacDonald NE, Eskola J, Liang X, et al. Vaccine hesitancy: Definition, scope and determinants. Vaccine. 2015;33.

[13] Taddio A, McMurtry CM, Logeman C, et al. Prevalence of pain and fear as barriers to vaccination in children – Systematic review and meta-analysis. Vaccine. 2022.

[14] Skwarczynski M, Toth I. Non-invasive mucosal vaccine delivery: advantages, challenges and the future. Expert Opin Drug Deliv. 2020.

[15] Kehagia E, Papakyriakopoulou P, Valsami G. Advances in intranasal vaccine delivery: A promising non-invasive route of immunization. Vaccine. 2023.

[16] McComb S, Thiriot A, Akache B, et al. Introduction to the Immune System. Methods in Molecular Biology. 2019.

[17] Delves PJ, Roitt IM. The Immune System. N Engl J Med. 2000;343:37–49.

[18] Chaplin DD. Overview of the immune response. Journal of Allergy and Clinical Immunology. 2010;125.

[19] Kellie S, Al-Mansour Z. Overview of the Immune System. Micro- and Nanotechnology in Vaccine Development. 2017;357:63–81.

[20] Jouault T, Sarazin A, Martinez-esparza M, et al. Host responses to a versatile commensal: PAMPs and PRRs interplay leading to tolerance or infection by Candida albicans. Cell Microbiol. 2009.

[21] Butcher MJ, Zhu J. Recent advances in understanding the Th1/Th2 effector choice. Fac Rev. 2021;10.

[22] Seifert M, Küppers R. Human memory B cells. Leukemia. 2016;30:2283–2292.

[23] et al. Janeway CA Jr Travers P Walport M. Immunobiology: The Immune System in Health and Disease. 5th edition. T-cell receptor gene rearrangement. New York: Garland Science; 2001;

[24] Plotkin S. History of vaccination. Proc Natl Acad Sci U S A. 2014;111:12283–12287.

[25] Nir Y, Paz A, Sabo E, et al. Fear of injections in young adults: Prevalence and associations. American Journal of Tropical Medicine and Hygiene. 2003;68.

[26] Wallace AS, Mantel C, Mayers G, et al. Experiences with provider and parental attitudes and practices regarding the administration of multiple injections during infant vaccination visits: Lessons for vaccine introduction. Vaccine. 2014;32.

[27] De Gregorio E, Rappuoli R. Vaccines for the future: Learning from human immunology. Microb Biotechnol. 2012.

[28] Masjedi M, Montahaei T, Sharafi Z, et al. Pulmonary vaccine delivery: An emerging strategy for vaccination and immunotherapy. J Drug Deliv Sci Technol. 2022.

[29] Cheng JY, Huang HN, Tseng WC, et al. Transcutaneous immunization by lipoplex-patch based DNA vaccines is effective vaccination against Japanese encephalitis virus infection. Journal of Controlled Release. 2009;135.

[30] Zheng Y, Ye R, Gong X, et al. Iontophoresis-driven microneedle patch for the active transdermal delivery of vaccine macromolecules. Microsyst Nanoeng. 2023;9.

[31] Boopathy A V., Mandal A, Kulp DW, et al. Enhancing humoral immunity via sustained-release implantable microneedle patch vaccination. Proc Natl Acad Sci U S A. 2019;116:16473–16478.

[32] Hao Y, Li W, Zhou XL, et al. Microneedles-based transdermal drug delivery systems: A review. J Biomed Nanotechnol. 2017;13:1581–1597.

[33] Riese P, Schulze K, Ebensen T, et al. Vaccine Adjuvants: Key Tools for Innovative Vaccine Design. Curr Top Med Chem. 2013;13:2562–2580.

[34] Guimarães LE, Baker B, Perricone C, et al. Vaccines, adjuvants and autoimmunity. Pharmacol Res. 2015;100:190–209.

[35] Bashiri S, Koirala P, Toth I, et al. Carbohydrate immune adjuvants in subunit vaccines. Pharmaceutics. 2020.

[36] Chattopadhyay S, Chen JY, Chen HW, et al. Nanoparticle vaccines adopting virus-like features for enhanced immune potentiation. Nanotheranostics. 2017;1:244–260.

[37] Fadeel B. Hide and seek: Nanomaterial interactions with the immune system. Front Immunol. 2019;10:1–10.

[38] Dumpa N, Goel K, Guo Y, et al. Stability of Vaccines. AAPS PharmSciTech. 2019;20:1–11.

[39] Bayda S, Adeel M, Tuccinardi T, et al. The history of nanoscience and nanotechnology: From chemical-physical applications to nanomedicine. Molecules. 2020;25:1–15.

[40] Haleem A, Javaid M, Singh RP, et al. Applications of nanotechnology in medical field: a brief review. Global Health Journal. 2023.

[41] Kinnear C, Moore TL, Rodriguez-Lorenzo L, et al. Form Follows Function: Nanoparticle Shape and Its Implications for Nanomedicine. Chem Rev. 2017;117:11476–11521.

[42] Chan WCW. Nanomedicine 2.0. Acc Chem Res. 2017;50:627–632.

[43] Lehner R, Wang X, Wolf M, et al. Designing switchable nanosystems for medical application. Journal of Controlled Release. 2012.

[44] Mishra B, Patel BB, Tiwari S. Colloidal nanocarriers: a review on formulation technology, types and applications toward targeted drug delivery. Nanomedicine. 2010.

[45] Scicluna MC, Vella-Zarb L. Evolution of Nanocarrier Drug-Delivery Systems and Recent Advancements in Covalent Organic Framework-Drug Systems. ACS Appl Nano Mater. 2020;3.

[46] Etheridge ML, Campbell SA, Erdman AG, et al. The big picture on nanomedicine: The state of investigational and approved nanomedicine products. Nanomedicine. 2013.

[47] Bobo D, Robinson KJ, Islam J, et al. Nanoparticle-Based Medicines: A Review of FDA-Approved Materials and Clinical Trials to Date. Pharm Res [Internet]. 2016;33:2373–2387. Available from: http://dx.doi.org/10.1007/s11095-016-1958-5.

[48] Facciolà A, Visalli G, Laganà P, et al. The new era of vaccines: The "nanovaccinology." Eur Rev Med Pharmacol Sci. 2019;23:7163–7182.

[49] Sulczewski FB, Liszbinski RB, Romão PRT, et al. Nanoparticle vaccines against viral infections. Arch Virol [Internet]. 2018;163:2313–2325. Available from: https://doi.org/10.1007/s00705-018-3856-0.

[50] Duong VA, Nguyen TTL, Maeng HJ. Recent Advances in Intranasal Liposomes for Drug, Gene, and Vaccine Delivery. Pharmaceutics. 2023.

[51] Manconi M, Caddeo C, Manca ML, et al. Oral delivery of natural compounds by phospholipid vesicles. Nanomedicine. 2020;15.

[52] Li M, Du C, Guo N, et al. Composition design and medical application of liposomes. Eur J Med Chem. 2019;164:640–653.

[53] Alqahtani MS, Kazi M, Alsenaidy MA, et al. Advances in Oral Drug Delivery. Front Pharmacol. 2021.

[54] Homayun B, Lin X, Choi HJ. Challenges and recent progress in oral drug delivery systems for biopharmaceuticals. Pharmaceutics. 2019.

[55] Ibrahim YHEY, Regdon G, Hamedelniel EI, et al. Review of recently used techniques and materials to improve the efficiency of orally administered proteins/peptides. DARU, Journal of Pharmaceutical Sciences. 2020.

[56] Jazayeri SD, Lim HX, Shameli K, et al. Nano and Microparticles as Potential Oral Vaccine Carriers and Adjuvants Against Infectious Diseases. Front Pharmacol. 2021.

[57] Mörbe UM, Jørgensen PB, Fenton TM, et al. Human gut-associated lymphoid tissues (GALT); diversity, structure, and function. Mucosal Immunol. 2021.

[58] Vela Ramirez JE, Sharpe LA, Peppas NA. Current state and challenges in developing oral vaccines. Adv Drug Deliv Rev. 2017.

[59] Trincado V, Gala RP, Morales JO. Buccal and sublingual vaccines: A review on oral mucosal immunization and delivery systems. Vaccines (Basel). 2021.

[60] Ou B, Yang Y, Lv H, et al. Current Progress and Challenges in the Study of Adjuvants for Oral Vaccines. BioDrugs. 2023.

[61] Liu J, Wu J, Wang B, et al. Oral vaccination with a liposome-encapsulated influenza DNA vaccine protects mice against respiratory challenge infection. J Med Virol. 2014;86.

[62] Van Splunter M, Van Hoffen E, Floris-Vollenbroek EG, et al. Oral cholera vaccination promotes homing of IgA+ memory B cells to the large intestine and the respiratory tract article. Mucosal Immunol. 2018;11.

[63] Harde H, Agrawal AK, Jain S. Tetanus toxoid-loaded layer-by-layer nanoassemblies for efficient systemic, mucosal, and cellular immunostimulatory response following oral administration. Drug Deliv Transl Res. 2015;5.

[64] Shukla A, Mishra V, Kesharwani P. Bilosomes in the context of oral immunization: Development, challenges and opportunities. Drug Discov Today. 2016.

[65] Aburahma MH. Bile salts-containing vesicles: promising pharmaceutical carriers for oral delivery of poorly water-soluble drugs and peptide/protein-based therapeutics or vaccines. Drug Deliv. 2016.

[66] Wilkhu JS, McNeil SE, Anderson DE, et al. Characterization and optimization of bilosomes for oral vaccine delivery. J Drug Target. 2013;21.

[67] Jain S, Indulkar A, Harde H, et al. Oral mucosal immunization using glucomannosylated bilosomes. J Biomed Nanotechnol. 2014;10.

[68] Wang N, Wang T, Zhang M, et al. Mannose derivative and lipid A dually decorated cationic liposomes as an effective cold chain free oral mucosal vaccine adjuvant-delivery system. European Journal of Pharmaceutics and Biopharmaceutics. 2014;88.

[69] Jahagirdar P, Lokhande AS, Dandekar P, et al. Mannose Receptor and Targeting Strategies. AAPS Advances in the Pharmaceutical Sciences Series. 2019.

[70] Zhen Y, Wang N, Gao Z, et al. Multifunctional liposomes constituting microneedles induced robust systemic and mucosal immunoresponses against the loaded antigens via oral mucosal vaccination. Vaccine. 2015;33.

[71] Li XJ, Li Y, Meng Y, et al. Composite dissolvable microneedle patch for therapy of oral mucosal diseases. Biomaterials Advances. 2022;139.

[72] Wang T, Zhen Y, Ma X, et al. Mannosylated and lipid A-incorporating cationic liposomes constituting microneedle arrays as an effective oral mucosal HBV vaccine applicable in the controlled temperature chain. Colloids Surf B Biointerfaces. 2015;126.

[73] Coffey JW, Gaiha G Das, Traverso G. Oral Biologic Delivery: Advances Toward Oral Subunit, DNA, and mRNA Vaccines and the Potential for Mass Vaccination during Pandemics. Annu Rev Pharmacol Toxicol. 2021.

[74] Jung JH, Jin SG. Microneedle for transdermal drug delivery: current trends and fabrication. J Pharm Investig. 2021.

[75] Mašek J, Lubasová D, Lukáč R, et al. Multi-layered nanofibrous mucoadhesive films for buccal and sublingual administration of drug-delivery and vaccination nanoparticles - important step towards effective mucosal vaccines. Journal of Controlled Release. 2017;249.

[76] Jacob S, Nair AB, Boddu SHS, et al. An updated overview of the emerging role of patch and film-based buccal delivery systems. Pharmaceutics. 2021.

[77] Uddin M, Allon A, Roni MA, et al. Overview and future potential of fast dissolving buccal films as drug delivery system for vaccines. Journal of Pharmacy and Pharmaceutical Sciences. 2019;22.

[78] Garcia-del Rio L, Diaz-Rodriguez P, Pedersen GK, et al. Sublingual Boosting with a Novel Mucoadhesive Thermogelling Hydrogel Following Parenteral CAF01 Priming as a Strategy Against Chlamydia trachomatis. Adv Healthc Mater. 2022;11.

[79] Oberoi HS, Yorgensen YM, Morasse A, et al. PEG modified liposomes containing CRX-601 adjuvant in combination with methylglycol chitosan enhance the murine sublingual immune response to influenza vaccination. Journal of Controlled Release. 2016;223.

[80] Aliu H, Rask C, Brimnes J, et al. Enhanced efficacy of sublingual immunotherapy by liposome-mediated delivery of allergen. Int J Nanomedicine. 2017;12.

[81] Ramírez W, Bourg V, Torralba D, et al. Safety of a proteoliposome from neisseria meningitides as adjuvant for a house dust mite allergy vaccine. J Immunotoxicol. 2017;14.

[82] Suzuki S, Sakurai D, Sakurai T, et al. Sublingual administration of liposomes enclosing alpha-galactosylceramide as an effective adjuvant of allergen immunotherapy in a murine model of allergic rhinitis. Allergology International. 2019;68.

[83] Hellfritzsch M, Scherließ R. Mucosal vaccination via the respiratory tract. Pharmaceutics. 2019.

[84] Wang J, Peng Y, Xu H, et al. The COVID-19 Vaccine Race: Challenges and Opportunities in Vaccine Formulation. AAPS PharmSciTech. 2020.

[85] Tasaniyananda N, Chaisri U, Tungtrongchitr A, et al. Mouse model of cat allergic rhinitis and intranasal liposome-adjuvanted refined Fel d 1 vaccine. PLoS One. 2016;11.

[86] Yang J, Firdaus F, Azuar A, et al. Cell-penetrating peptides-based liposomal delivery system enhanced immunogenicity of peptide-based vaccine against group a streptococcus. Vaccines (Basel). 2021;9.

[87] Azuar A, Zhao L, Hei TT, et al. Cholic Acid-based Delivery System for Vaccine Candidates against Group A Streptococcus. ACS Med Chem Lett. 2019;10.

[88] Senchi K, Matsunaga S, Hasegawa H, et al. Development of oligomannose-coated liposome-based nasal vaccine against human parainfluenza virus type 3. Front Microbiol. 2013;4.

[89] Dhakal S, Cheng X, Salcido J, et al. Liposomal nanoparticle-based conserved peptide influenza vaccine and monosodium urate crystal adjuvant elicit protective immune response in pigs. Int J Nanomedicine. 2018;13.

[90] Taus F, Santucci MB, Greco E, et al. Monosodium urate crystals promote innate anti-mycobacterial immunity and improve BCG efficacy as a vaccine against tuberculosis. PLoS One. 2015;10.

[91] Wang J, Li P, Yu Y, et al. Pulmonary surfactant-biomimetic nanoparticles potentiate heterosubtypic influenza immunity. Science (1979). 2020;367.

[92] Barouch DH. Covid-19 Vaccines — Immunity, Variants, Boosters. New England Journal of Medicine. 2022;387.

[93] An X, Martinez-Paniagua M, Rezvan A, et al. Single-dose intranasal vaccination elicits systemic and mucosal immunity against SARS-CoV-2. iScience. 2021;24.

[94] Walkowski W, Bassett J, Bhalla M, et al. Intranasal vaccine delivery technology for respiratory tract disease application with a special emphasis on pneumococcal disease. Vaccines (Basel). 2021;9.

[95] Christensen D, Korsholm KS, Andersen P, et al. Cationic liposomes as vaccine adjuvants. Expert Rev Vaccines. 2011.

[96] Tada R, Hidaka A, Iwase N, et al. Intranasal immunization with dotap cationic liposomes combined with DC-cholesterol induces potent antigen-specific mucosal and systemic immune responses in mice. PLoS One. 2015;10:1–21.

[97] Tada R, Hidaka A, Kiyono H, et al. Intranasal administration of cationic liposomes enhanced granulocyte-macrophage colony-stimulating factor expression and this expression is dispensable for mucosal adjuvant activity. BMC Res Notes. 2018;11.

[98] Tada R, Suzuki H, Takahashi S, et al. Nasal vaccination with pneumococcal surface protein A in combination with cationic liposomes consisting of DOTAP and DC-chol confers antigen-mediated protective immunity against Streptococcus pneumoniae infections in mice. Int Immunopharmacol. 2018;61.

[99] Tada R, Muto S, Iwata T, et al. Attachment of class B CpG ODN onto DOTAP/DC-chol liposome in nasal vaccine formulations augments antigen-specific immune responses in mice. BMC Res Notes. 2017;10.

[100] Qu W, Li N, Yu R, et al. Cationic DDA/TDB liposome as a mucosal vaccine adjuvant for uptake by dendritic cells in vitro induces potent humoural immunity. Artif Cells Nanomed Biotechnol. 2018;46.

[101] Baranov M V., Kumar M, Sacanna S, et al. Modulation of Immune Responses by Particle Size and Shape. Front Immunol. 2021.

[102] Yusuf H, Ali AA, Orr N, et al. Novel freeze-dried DDA and TPGS liposomes are suitable for nasal delivery of vaccine. Int J Pharm. 2017;533.

[103] Zhuang X, Qi Y, Wang M, et al. MRNA vaccines encoding the HA protein of influenza a H1N1 virus delivered by cationic lipid nanoparticles induce protective immune responses in mice. Vaccines (Basel). 2020;8.

[104] Yang K, Lu Y, Xie F, et al. Cationic liposomes induce cell necrosis through lysosomal dysfunction and late-stage autophagic flux inhibition. Nanomedicine. 2016;12.

[105] Knudsen KB, Northeved H, Pramod Kumar EK, et al. In vivo toxicity of cationic micelles and liposomes. Nanomedicine. 2015;11.

[106] Chen KH, Di Sabatino M, Albertini B, et al. The effect of polymer coatings on physicochemical properties of spray-dried liposomes for nasal delivery of BSA. European Journal of Pharmaceutical Sciences. 2013;50.

[107] Kaplan M, Tuğcu-Demiröz F, Vural İ, et al. Development and characterization of gels and liposomes containing ovalbumin for nasal delivery. J Drug Deliv Sci Technol. 2018;44.

[108] Marasini N, Ghaffar KA, Giddam AK, et al. Highly Immunogenic Trimethyl Chitosan-based Delivery System for Intranasal Lipopeptide Vaccines against Group A Streptococcus. Curr Drug Deliv. 2016;14.

[109] Leroux-Roels G, Maes C, Clement F, et al. Randomized Phase I: Safety, Immunogenicity and Mucosal Antiviral Activity in Young Healthy Women Vaccinated with HIV-1 Gp41 P1 Peptide on Virosomes. PLoS One. 2013;8.

[110] Wang HW, Jiang PL, Lin SF, et al. Application of galactose-modified liposomes as a potent antigen presenting cell targeted carrier for intranasal immunization. Acta Biomater [Internet]. 2013;9:5681–5688. Available from: http://dx.doi.org/10.1016/j.actbio.2012.11.007.

[111] Jiang PL, Lin HJ, Wang HW, et al. Galactosylated liposome as a dendritic cell-targeted mucosal vaccine for inducing protective anti-tumor immunity. Acta Biomater. 2015;11.

[112] Kakhi Z, Frisch B, Bourel-Bonnet L, et al. Airway administration of a highly versatile peptide-based liposomal construct for local and distant antitumoral vaccination. Int J Pharm. 2015;496.

[113] Kakhi Z, Frisch B, Heurtault B, et al. Liposomal constructs for antitumoral vaccination by the nasal route. Biochimie. 2016;130.

[114] Zhang L, Wang W, Wang S. Effect of vaccine administration modality on immunogenicity and efficacy. Expert Rev Vaccines. 2015.

[115] Wallis J, Shenton DP, Carlisle RC. Novel approaches for the design, delivery and administration of vaccine technologies. Clin Exp Immunol. 2019.

[116] Levin C, Perrin H, Combadiere B. Tailored immunity by skin antigen-presenting cells. Hum Vaccin Immunother. 2015.

[117] Nguyen A V., Soulika AM. The dynamics of the skin's immune system. Int J Mol Sci. 2019;20:1–53.

[118] Quaresma JAS. Organization of the skin immune system and compartmentalized immune responses in infectious diseases. Clin Microbiol Rev. 2019;32:1–35.

[119] Pielenhofer J, Sohl J, Windbergs M, et al. Current Progress in Particle-Based Systems for Transdermal Vaccine Delivery. Front Immunol. 2020.

[120] Sun X, Zeng L, Huang Y. Transcutaneous delivery of DNA/mRNA for cancer therapeutic vaccination. Journal of Gene Medicine. 2019.

[121] Hasan M, Khatun A, Fukuta T, et al. Noninvasive transdermal delivery of liposomes by weak electric current. Adv Drug Deliv Rev. 2020.

[122] Jeong WY, Kwon M, Choi HE, et al. Recent advances in transdermal drug delivery systems: a review. Biomater Res. 2021.

[123] Ita K. Transdermal delivery of vaccines – Recent progress and critical issues. Biomedicine and Pharmacotherapy. 2016.

[124] Badizadegan K, Goodson JL, Rota PA, et al. The potential role of using vaccine patches to induce immunity: platform and pathways to innovation and commercialization. Expert Rev Vaccines. 2020.

[125] Zhang Y, Ng W, Feng X, et al. Lipid vesicular nanocarrier: Quick encapsulation efficiency determination and transcutaneous application. Int J Pharm. 2017;516.

[126] Tyagi RK, Garg NK, Jadon R, et al. Elastic liposome-mediated transdermal immunization enhanced the immunogenicity of P. falciparum surface antigen, MSP-119. Vaccine. 2015;33.

[127] Tyagi RK, Garg NK, Dalai SK, et al. Transdermal immunization of P. falciparum surface antigen (MSP-119) via elastic liposomes confers robust immunogenicity. Hum Vaccin Immunother. 2016;12.

[128] Corthésy B, Bioley G. Lipid-based particles: Versatile delivery systems for mucosal vaccination against infection. Front Immunol. 2018.

[129] Romero EL, Morilla MJ. Ether lipids from archaeas in nano-drug delivery and vaccination. Int J Pharm. 2023;634.

[130] Jia Y, McCluskie MJ, Zhang D, et al. In vitro evaluation of archaeosome vehicles for transdermal vaccine delivery. J Liposome Res. 2018;28.

[131] Caimi AT, Altube MJ, de Farias MA, et al. Novel imiquimod nanovesicles for topical vaccination. Colloids Surf B Biointerfaces. 2019;174.

[132] Hanna E, Abadi R, Abbas O. Imiquimod in dermatology: an overview. Int J Dermatol. 2016.

[133] Carrer DC, Higa LH, Tesoriero MVD, et al. Structural features of ultradeformable archaeosomes for topical delivery of ovalbumin. Colloids Surf B Biointerfaces. 2014;121.

[134] Caimi AT, Parra F, de Farias MA, et al. Topical vaccination with super-stable ready to use nanovesicles. Colloids Surf B Biointerfaces. 2017;152.

[135] Higa LH, Arnal L, Vermeulen M, et al. Ultradeformable archaeosomes for needle free nanovaccination with leishmania braziliensis antigens. PLoS One. 2016;11.

[136] Nayak BS, Mohanty B, Mishra B, et al. Transethosomes: Cutting edge approach for drug permeation enhancement in transdermal drug delivery system. Chem Biol Drug Des. 2023.

[137] Agbayani G, Jia Y, Akache B, et al. Mechanistic insight into the induction of cellular immune responses by encapsulated and admixed archaeosome-based vaccine formulations. Hum Vaccin Immunother. 2020;16.

[138] Jamaledin R, Di Natale C, Onesto V, et al. Progress in microneedle-mediated protein delivery. J Clin Med. 2020.

[139] Bernardi DS, Bitencourt C, da Silveira DSC, et al. Effective transcutaneous immunization using a combination of iontophoresis and nanoparticles. Nanomedicine. 2016;12.

[140] Waghule T, Singhvi G, Dubey SK, et al. Microneedles: A smart approach and increasing potential for transdermal drug delivery system. Biomedicine and Pharmacotherapy. 2019.

[141] Menon I, Bagwe P, Gomes KB, et al. Microneedles: A new generation vaccine delivery system. Micromachines (Basel). 2021.

[142] Chen YC, Chen SJ, Cheng HF, et al. Development of Yersinia pestis F1 antigen-loaded liposome vaccine against plague using microneedles as a delivery system. J Drug Deliv Sci Technol. 2020;55.

[143] Du G, Hathout RM, Nasr M, et al. Intradermal vaccination with hollow microneedles: A comparative study of various protein antigen and adjuvant encapsulated nanoparticles. Journal of Controlled Release [Internet]. 2017;266:109–118. Available from: https://doi.org/10.1016/j.jconrel.2017.09.021.

[144] Du G, Leone M, Romeijn S, et al. Immunogenicity of diphtheria toxoid and poly(I:C) loaded cationic liposomes after hollow microneedle-mediated intradermal injection in mice. Int J Pharm. 2018;547.

[145] Sartawi Z, Blackshields C, Faisal W. Dissolving microneedles: Applications and growing therapeutic potential. Journal of Controlled Release. 2022.

[146] Wu X, Li Y, Chen X, et al. A surface charge dependent enhanced Th1 antigen-specific immune response in lymph nodes by transfersome-based nanovaccine-loaded dissolving microneedle-assisted transdermal immunization. J Mater Chem B. 2019;7.

[147] Qiu Y, Guo L, Zhang S, et al. DNA-based vaccination against hepatitis B virus using dissolving microneedle arrays adjuvanted by cationic liposomes and CpG ODN. Drug Deliv. 2016;23.

[148] Lanza JS, Vucen S, Flynn O, et al. A TLR9-adjuvanted vaccine formulated into dissolvable microneedle patches or cationic liposomes protects against leishmaniasis after skin or subcutaneous immunization. Int J Pharm. 2020;586.

[149] Guo L, Chen J, Qiu Y, et al. Enhanced transcutaneous immunization via dissolving microneedle array loaded with liposome encapsulated antigen and adjuvant. Int J Pharm. 2013;447.

[150] Zhao JH, Zhang QB, Liu B, et al. Enhanced immunization via dissolving microneedle array-based delivery system incorporating subunit vaccine and saponin adjuvant. Int J Nanomedicine. 2017;12:4763–4772.

[151] Zhang Y, Ng W, Hu J, et al. Formulation and in vitro stability evaluation of ethosomal carbomer hydrogel for transdermal vaccine delivery. Colloids Surf B Biointerfaces. 2018;163.

[152] Yang X, Wang X, Hong H, et al. Galactosylated chitosan-modified ethosomes combined with silk fibroin nanofibers is useful in transcutaneous immunization. Journal of Controlled Release. 2020;327:88–99.

[153] Clinical trials. Clinical trials involving liposome-based vaccines 2013-2023 [internet]. Available from: https://clinicaltrials.gov/search?cond=vaccine&term=liposomal%20vaccine&intr=vaccine&start=2013-01-01_.

[154] Sainaga Jyothi VGS, Bulusu R, Venkata Krishna Rao B, et al. Stability characterization for pharmaceutical liposome product development with focus on regulatory considerations: An update. Int J Pharm. 2022.

[155] Costa JP, Jesus S, Colaço M, et al. Endotoxin contamination of nanoparticle formulations: A concern in vaccine adjuvant mechanistic studies. Vaccine. 2023;41.

[156] Morales JO, Fathe KR, Brunaugh A, et al. Challenges and Future Prospects for the Delivery of Biologics: Oral Mucosal, Pulmonary, and Transdermal Routes. AAPS Journal. 2017;19.

[157] Guner S, Oztop MH. Food grade liposome systems: Effect of solvent, homogenization types and storage conditions on oxidative and physical stability. Colloids Surf A Physicochem Eng Asp. 2017;513.

[158] Crommelin DJA, Talsma H, Grit M, et al. Physical Stability on Long-Term Storage. Phospholipids Handbook. 2020.

[159] Chung YH, Church D, Koellhoffer EC, et al. Integrating plant molecular farming and materials research for next-generation vaccines. Nat Rev Mater. 2022.

[160] D'Amico C, Fontana F, Cheng R, et al. Development of vaccine formulations: past, present, and future. Drug Deliv Transl Res. 2021;11.

[161] Ahmed KS, Hussein SA, Ali AH, et al. Liposome: composition, characterisation, preparation, and recent innovation in clinical applications. J Drug Target. 2019.

[162] Hossen S, Hossain MK, Basher MK, et al. Smart nanocarrier-based drug delivery systems for cancer therapy and toxicity studies: A review. J Adv Res. 2019.

[163] Peng T, Xu W, Li Q, et al. Pharmaceutical liposomal delivery—specific considerations of innovation and challenges. Biomater Sci. 2022.

[164] Hua S, de Matos MBC, Metselaar JM, et al. Current trends and challenges in the clinical translation of nanoparticulate nanomedicines: Pathways for translational development and commercialization. Front Pharmacol. 2018.

[165] Kis Z, Shattock R, Shah N, et al. Emerging Technologies for Low-Cost, Rapid Vaccine Manufacture. Biotechnol J. 2019.

[166] Yousif M, Hewage C, Nawaf L. IOT technologies during and beyond COVID-19: A comprehensive review. Future Internet. 2021.