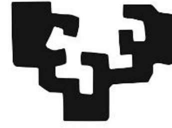
UNICA
UNIVERSITÀ DEGLI STUDI DI CAGLIARI



Universidad del País Vasco
Euskal Herriko Unibertsitatea

IN VITRO EVALUATION OF LIPOSOMES TAILORED FOR NON-INVASIVE DELIVERY OF THE MODEL ANTIGEN OVALBUMIN TO SKIN, NOSE, AND LUNGS

Matteo Aroffu

Prof. Maria Manconi & Prof. José Luis Pedraz

2024

(cc) 2024 Matteo Aroffu (cc by-nc-sa 4.0)

Acknowledgments

To research groups:

The authors thank the PhytoNanoGroup (PNG) at University of Cagliari.

The authors thank the ICTS “NANBIOSIS” and the Drug Formulation Unit (U10) of the CIBER in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), and consolidated groups (IT1448-22) at the University of Basque Country (UPV/EHU).

To the editorials:

The authors thank the editorials for granting permission to reuse the published articles. The published versions can be accessed at the following links:

Aroffu M et al., Expert Opinion on Drug Delivery, 2023, volume 20, issue 11
https://doi.org/10.1080/17425247.2023.2288856 (Appendix 1)

Contents

Chapter 1 – Introduction **1**
1. **State of the art** 2
2. **Hypothesis and objectives** 7
3. **Methodology** 8
3.1 Liposome preparation and characterization 8
3.2 Hydrogel disks preparation and characterization 12
3.3 Nasal spray characterization 12
3.4 Dry powder characterisation 13
3.5 Biological evaluation 14
3.6 Statistical analysis 17
4. **Results** 18
4.1. Enriched transfersomes for the cutaneous delivery of ovalbumin 18
4.2 Anionic and cationic liposomes for the nasal administration of ovalbumin 26
4.3 Freeze-dried anionic liposomes for the pulmonary administration of ovalbumin 33
5. **Discussion** 44
5.1 Enriched transfersomes for the cutaneous delivery of ovalbumin 44
5.2 Anionic and cationic liposomes for the nasal delivery of ovalbumin 46
5.3 Freeze-dried anionic liposomes for the pulmonary delivery of ovalbumin 48
6. **Bibliography** 52
Chapter 2 – Conclusions **70**
Chapter 3 – Appendix **73**
Liposome-based vaccines for minimally or noninvasive administration: an update on current advancements 74
Chapter 4 – Summary **127**
4.1 English summary 128
4.2 Italian summary 133
4.3 Spanish summary 139

Chapter 1 – Introduction

1. State of the art

Vaccination stands out as the most potent and successful preventive measure ever devised to safeguard individuals worldwide from life-threatening diseases [1,2]. It contributes not only to individual protection but also to community immunity and has a crucial role in preventing and controlling the outbreaks of various diseases, contributing significantly to public health efforts [2,3].

Vaccination works by leveraging the body's natural immune response to safeguard against infectious diseases. The ultimate goal is to establish immunity without causing the disease itself. Th process typically involves administering a vaccine, which contains weakened or inactivated forms of pathogens or specific components (antigens) that trigger an immune response. The immune system recognizes these antigens as foreign invaders and initiates a defence mechanism to neutralize or eliminate them. Upon vaccination, the immune system's primary components, such as white blood cells and antibodies, are activated. Memory cells are also produced, which "remember" the encountered antigens. If the vaccinated individual later encounters the actual pathogen, the immune system can mount a rapid and effective response, preventing or mitigating the infection. This adaptive immune response includes the production of antibodies that can neutralize the pathogen, as well as the activation of killer T cells that can destroy infected cells.

Unfortunately, despite its significance, global vaccine coverage has reached a plateau in the past decade, resulting in a growing number of unvaccinated children, particularly in low-income and lower-middle-income countries [4]. Several factors contribute to this issue, including supply constraints, limited access to services, and, in some instances, the emergence of new conflicts. Notably, in high-income countries, vaccine hesitancy ranks among the top ten reasons, as observed during the recent COVID-19 pandemic.

Vaccine hesitancy is a multifaceted and context-specific challenge. It is influenced by factors such as complacency, convenience, and confidence [5,6]. The majority of globally used vaccines are administered through parenteral methods, involving intramuscular or subcutaneous injections, presenting various disadvantages such as pain, local injury, product contamination risks, reliance on healthcare facilities, professional medical staff, and expensive formulations [7,8]. As these issues are widespread and affect diverse regions, addressing them becomes a focal point for comprehensive health improvement efforts, especially in low-income countries. Non-invasive vaccination methods, including oral, buccal, sublingual, intranasal, pulmonary, and transcutaneous routes, offer promising avenues to mitigate these challenges and enhance safety [9].

The oral route is widely accepted for its accessibility and high patient compliance, although challenges like the stomach's acidic environment and liver metabolism can limit bioavailability. Despite these hurdles, strategies exist to make oral administration effective. In vaccine formulations, oral administration allows self-administration, improves compliance, and stimulates the gastrointestinal immune system [10]. However, only a limited number of oral vaccines are licensed and used clinically [11]. To address challenges, alternative strategies like buccal and sublingual immunization have been explored [12]. The potential benefits of different dosage forms and the high patient compliance of oral, buccal, and sublingual routes encourage

scientists to develop new vaccines for enhanced immune responses at both systemic and mucosal levels.

The respiratory route emerges as a practical choice for mass vaccination due to its needleless, painless, and highly accessible nature [13,14]. Immunologically, its appeal lies in key components between the upper and lower respiratory tracts, offering protection against airborne diseases [14]. The ability to counter pathogens at the entry site and achieve systemic immunization enhances its strategic importance. Existing vaccines like Fluenz®, Flumist®, and Nasovac® target influenza through intranasal administration.

The skin, as the largest and most accessible route for therapeutics, has a historical use for immunization [15,16]. However, despite its potential, most of currently licensed vaccines are delivered through intramuscular and subcutaneous injections using hypodermic needles, leading to issues such as needle phobia, pain, injuries, infection risks, and high overall costs. Despite the skin's complex immune cell network, traditional methods do not achieve skin immunization [17–19]. To address this challanges, nanocarriers like liposomes, often coupled with devices or physical techniques, are explored as minimally invasive or non-invasive alternatives for immunization, offering potential solutions to current limitations.

On the whole, in contrast to existing immunization methods, non-invasive vaccination has the potential to stimulate local cellular and humoral immunity in the skin and mucosae, which are primary entry points for pathogens into the human body and are typically not adequately or entirely stimulated by parenteral vaccination [14]. Furthermore, non-invasive vaccination methods offer advantages such as avoiding systemic drawbacks, improving patient compliance, enabling self-administration, eliminating the need for specialized personnel, and significantly reducing costs associated with mass immunization. These benefits associated with non-invasive or minimally invasive administration routes hold substantial promise and could be widely adopted in future vaccination programs. However, at present, only a limited number of vaccines, including FluMist/Fluenz® and Nasovac™ for intranasal administration, and Vaxchora®, Dukoral®, Rotarix™, RotaTeq®, Vivotif®, and oral polio vaccine for oral administration, are employed, and this is limited to specific countries.

In the present era, it is imperative to expand exploration beyond alternative administration routes exclusively. It is evident that relying solely on these approaches is insufficient for achieving a notable enhancement in vaccine efficacy and safety. Consequently, there is a concurrent consideration for the integration of molecules or systems known as adjuvants, aimed at boosting the immune response to antigens [20–22]. Adjuvants enhance vaccine effectiveness through mechanisms like aiding antigen transport to lymph nodes, safeguarding the antigen, amplifying reactions at the administration site, inducing cytokine release, and engaging with pattern recognition receptors [23,24]. Within this context, nanotechnologies present a promising approach, serving a dual purpose by delivering antigens and/or additional adjuvants while also manifesting inherent adjuvant effects. This utilization of nanotechnologies contributes to improving the stability and safety profiles of the delivered components [25,26].

Nanotechnologies, arising from the study of phenomena at nanoscale dimensions, have become integral in medical applications [27]. These nanosystems, with unique properties like size, shape, surface area, and charge, play a crucial role in overcoming biological barriers,

enabling controlled drug release, and minimizing toxicity in medical treatments. These adaptable nanocarriers, now clinically approved, are instrumental in treating various conditions, including infections, chronic diseases, pain management, autoimmune diseases, mental disorders, and cancer [28]. A noteworthy exploration is in "nanovaccinology," leveraging non-viral vectors to deliver antigens and adjuvants, addressing challenges in conventional vaccines. This approach aims to enhance immunogenic responses, improve safety, and overcome logistical hurdles related to storage and administration [25,29].

Liposomes have gained recognition as highly effective carriers in the realm of vaccine development [30]. Comprising nanosized vesicles crafted from phospholipids, these structures create closed bilayers in water, enveloping an aqueous core and one or more interlamellar spaces [31]. This unique configuration not only allows liposomes to encapsulate hydrophilic molecules but also to entrap hydrophobic ones, showcasing their versatility [32,33]. The cell-like membrane structure, coupled with attributes such as high biocompatibility, low immunogenicity, and the capability to safeguard payloads, makes liposomes ideal candidates for enhancing antigen presentation. Beyond these advantages, liposomes play a pivotal role in modifying the biodistribution of payloads, reducing their toxicity, and extending their half-life in the body. This multifaceted functionality positions liposomes as promising tools to overcome the challenges encountered with conventional vaccines.

It's worth noting that ongoing research in liposomal vaccine development aims to further optimize their features, including refining the encapsulation process and exploring tailored modifications for specific medical applications. These advancements underscore the dynamic nature of liposomal technology in the continuous quest for more effective and targeted vaccine delivery systems.

In recent studies, novel approaches to oral vaccination have been explored using liposomal formulations. Liu and colleagues, in 2014, developed an oral vaccine employing DNA-loaded cationic liposomes targeting the influenza A virus M1 gene. The formulation induced robust humoral and cellular immune responses, providing respiratory immunity in mice [34]. Harde et al. in 2015 focused on oral vaccination using liposomes and layersomes encapsulating tetanus toxoid [35]. Layersomes, exhibiting enhanced stability in biological fluids, proved more effective in inducing immune responses in comparison to liposomes, emphasizing the importance of thoughtful formulation design. Wilkhu et al. successfully employed bilosomes for oral delivery of recombinant influenza hemagglutinin, demonstrating antigen retention, effective uptake in the small intestine, and protection against fever and lung inflammation in ferrets [36]. Jain and colleagues chemically modified bilosomes with glucomannan for oral delivery of bovine serum albumin, showcasing stability in simulated gastrointestinal fluids, sustained antigen release, and improved uptake [37,38].

Wang and colleagues introduced an innovative approach to oral mucosal vaccination by designing dry powder dually decorated liposomes, combining the advantages of oral mucosal administration with a cold chain-free, adjuvanted delivery system [38]. Subsequent studies coupling these liposomes with microneedles demonstrated improved stability and increased in vivo response [39–41]. Mašek and colleagues explored multi-layered nanofibrous

mucoadhesive films containing liposomes for buccal and sublingual vaccination and successful results were achieved in ex vivo and in vivo pig models [42]. Garcia-del Rio and his team introduced a mucoadhesive thermogelling hydrogel containing liposomes for sublingual application against Chlamydia trachomatis enhancing the immune responses in vivo [43]. Oberoi and collaborators explored the co-delivery of influenza antigens with traditional and methylglycol chitosan modified liposomes, emphasizing the significance of mucoadhesiveness for sublingual vaccines. Despite the interesting results, no sublingual vaccines have been commercialized to date [44].

Tasaniyananda et al. explored an inhalatory vaccine using liposomes to encapsulate cat allergens, reducing allergic manifestations in a mouse model of cat allergic rhinitis [45]. Yang et al. conjugated lipopeptide-based liposomes with cell-penetrating peptides to enhance membrane permeability and extend protection against group A Streptococcus [46]. Azuar et al. utilized instead cholic acid for conjugation, generating strong humoral immune responses in mice [47]. Senchi et al. investigated oligomannose-coated liposomes against human parainfluenza virus type 3, revealing significant viral-specific immunity at a reduced antigen dose [48]. Dhakal et al. enhanced immune responses against influenza using liposomes adjuvanted with monosodium urate crystals [49]. The vaccine reduced flu clinical signs and achieved broad protection but remained ineffective against different influenza virus subtypes. Wang et al. addressed this challenge by using negatively charged liposomes with 2',3'-cyclic guanosine monophosphate-adenosine monophosphate as an adjuvant [50]. During the COVID-19 outbreak, An et al. developed a single-dose intranasal vaccine encapsulating the same adjuvant in negatively charged liposomes with the trimeric S-protein of SARS-CoV-2 adsorbed on the surface [51]. The vaccine demonstrated safety and comprehensive immunity at nasal and lung levels.

Studies by Tada et al. showed that co-administration of antigens with cationic liposomes boosted uptake by dendritic cells, and liposomes carrying immunostimulatory CpG motifs increased mucosal immunity while reducing side effects [52,53]. Wenjing et al. found that certain cationic liposomes outperformed others in internalization by dendritic cells and efficacy in boosting mucosal and systemic antibody titres [54]. Yusuf et al. explored cationic liposomes with positive charge inducers as alternatives to address cost and toxicity concerns, demonstrating improved internalization and humoral responses [55]. Marasini et al. developed a trimethyl chitosan-coated liposome vaccine for Group A Streptococcus, achieving durable immunization and specific antibody titres [56]. Intranasal vaccines extend beyond local protection were obtained by Leroux-Roels et al. integrating the HIV-1 Gp41 P1 peptide into liposomes [57]. Wang, Jiang et al. created galactose-modified liposomes loading ovalbumin, stimulating mucosal and systemic immune responses [58]. Kakhi et al. developed liposomal vaccines against lung tumors, showcasing a strong immune response with lower vaccine doses [59,60]. While these liposomal vaccines exhibit promise, further studies are essential before clinical application.

Zhang et al. compared the skin delivery capabilities of liposomes, transfersomes, and ethosomes, carrying ovalbumin and saponin [61]. Archaeosomes, which lamellar vesicles containing lipids from Archaea, were formulated by Jia et al. demonstrating their superiority over liposomes [62]. Caimi and colleagues enriched archaeosomes with sodium cholate,

creating ultradeformable archaeosomes for delivering imiquimod, a topical adjuvant [63]. Higher stability against various conditions (thermal stress, sterilization, freeze-drying) and consistent immune responses were found with ultradeformable archaeosomes from Halorubrum tebenquichense [63]. Higa et al. explored ultradeformable archaeosomes against leishmaniasis [64]. Bernardi and colleagues formulated ovalbumin-loaded liposomes with silver nanoparticles to enhance iontophoresis efficiency and antigen delivery achieving a 92-fold improvement and higher responses in vivo compared to free ovalbumin [65]. Yuan-Chuan Chen explored microneedles to deliver a liposomal vaccine against plague to the skin, inducing higher antibody titres and survival rates compared to control groups administered topically [66]. Du and co-workers took advantage of hollow microneedle to deliver liposomes, co-loaded with ovalbumin and polyriboinosinic-polyribocytidylic acid [67,68]. Wu and colleagues explored the combination of ovalbumin-loaded transfersomes with self-dissolving microneedles, presenting a promising method for cutaneous vaccination [69]. Qiu et al. investigated cationic liposomes coupled with dissolving microneedles for transcutaneous immunization against hepatitis B [70]. Guo and co-workers developed polyvinylpyrrolidone dissolving microneedles combined with cationic liposomes, demonstrating rapid dissolution, balanced immune responses, and enhanced antibody levels [71]. Zhao and Zhang achieved similar success with polyvinylpyrrolidone-K17/K30 dissolving microneedles [72]. Zhang et al. incorporated ovalbumin-loaded ethosomes with a saponin into carbomer hydrogels for easier vaccine administration [73]. Yang et al. explored the immunization potential of ovalbumin-loaded ethosomes modified with hyaluronic acid and galactosylated chitosan, incorporated into nanofibrous mats [74].

Despite promising global research outcomes, to date, only 6 out 25 liposomal formulations in the market are vaccines, indicating persistent challenges in commercializing new products [75].

2. Hypothesis and objectives

Currently, immunisation can be achieved through different types of vaccines. Among them, subunit vaccines, which are made of proteins or sugars obtained from pathogens, shine for their safety and are therefore suitable for high-risk individuals. Regrettably, they often induce a weaker immune response than less safe types of vaccines (i.e., live attenuated or inactivated vaccines), may undergo to aggregation and require particular storage conditions.

Hypothesis 1: liposomes might be used to fill two needs with one deed. Having delivery and protecting shield capabilities, liposomes can prevent antigen aggregation and degradation. In addition, they can be used to load adjuvants or display intrinsic adjuvanticity themselves, producing stronger responses.

However, as stated in section 1, today's vaccines cannot be investigated without taking into careful consideration the administration route. This parameter is indeed a key element, affecting not only the perception of the vaccine but also the whereabouts of the response. For instance, vaccines administered via injection may elicit a more systemic response, whereas those administered through mucosal surfaces could trigger both local and distal responses.

Hypothesis 2: alternative routes to injection can improve adherence to vaccination and provide a first shield against pathogens infections right at the entry sites of the body, offering the local protection that is given up by injection.

On these premises, the peptide model antigen ovalbumin, which has been widely studied, was encapsulated in liposomes, and cutaneous, nasal, and pulmonary delivery were exploited as administration routes for liposomes.

Objectives: with that in mind, in this thesis we aimed at:

1. Producing liposomes with an eco-friendlier and safer method than the ones found in literature.

2. Tailoring the composition of the antigen-containing liposomes depending on the administration route investigating new or more cost-effective compositions.

3. Evaluating whether the composition affected the antigen presentation or not.

4. Testing and characterising semi-solid (imbibed hydrogels), liquid (nasal spray) or solid (dry powders) systems.

5. Selecting the most suitable formulation depending on the foreseen use.

6. Pursuing non-invasive administration to promote local protection.

3. Methodology

3.1 Liposome preparation and characterization

3.1.1 Liposome preparation

In all the studies, ovalbumin-encapsulated vesicles were prepared eco-friendly by direct sonication avoiding the use of organic solvents. When labelling was required, vesicles were prepared replacing ovalbumin with the ovalbumin–fluorescein isothiocyanate conjugate (referred as labelled ovalbumin, excitation: 495 nm; emission: 520 nm).

In the first study, ovalbumin-encapsulated transfersomes were prepared to promote antigen deposition in the skin. Lipoid S75 (60 mg/mL), sodium deoxycholate (5 mg/mL) and ovalbumin (5 mg/mL) were dispersed in freshly prepared 0.9% saline (2 mL) containing glycerol (10% w/v) or sodium hyaluronate (0.1% w/v) or their combination at the same concentrations (**Table 1**). The dispersions were sonicated 4 times (4 sec. on, 2 sec. off, 5 cycles, 14 µm amplitude) with a 5-minute pause at each repetition to avoid overheating phenomena. All the formulations were sterilized by filtration through Corning® syringe filters (pore size 0.2 µm) in autoclaved vials and stored at 4 °C under vacuum.

Table 1. Composition of ovalbumin-encapsulated transfersomes.

	Lipoid S75 (mg/mL)	**Sodium deoxicholate** (mg/mL)	**Ovalbumin** (mg/mL)	**Sodium hyaluronate** (% w/v)	**Glycerol** (% w/v)	**Saline solution** (mL)
Glycerol-transfersomes	60	5	5	-	10	1
Hyaluronan-transfersomes	60	5	5	0.1	-	1
Glycerohyaluronan-transfersomes	60	5	5	0.1	10	1

In the second study, ovalbumin-encapsulated anionic and cationic liposome were prepared for nasal administration. Anionic liposomes were prepared dispersing Phospholipon® 90G (69 mg/mL), cholesterol (1 mg/mL) and ovalbumin (5 mg/mL) in sterile bidistilled water. Cationic DOTAP-liposomes were prepared instead replacing 9 mg/mL of Phospholipon® 90G with 9 mg/mL of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), thus keeping constant the total lipidic weight (70 mg/mL). Both dispersions were then directly sonicated twice (4 sec. on, 2 sec. off, 5 cycles, 14 µm amplitude) using a Soniprep 150 sonicator (MSE Crowley, London, UK), allowing each sample to cool down 5 minutes between the two sonication sessions. All the formulations were sterilized by filtration through Corning® syringe filters (pore size 0.2 µm) in autoclaved vials and stored at 4 °C under vacuum.

In the third study, ovalbumin-encapsulated anionic liposomes were prepared and freeze-dried to obtain a suitable dry powder for lung delivery. P90H, SPC-3, DPPC or DSPC (14 mg) were dispersed in Milli-Q water (3 mL) along with 1 mg of cholesterol and 10 mg of ovalbumin (**Table 2**). This mixture was sonicated using a probe cell disruptor (Branson Sonifier 250, USA) three times (three cycles) for 90 seconds [76]. Pulsed mode was used (duty-cycle 70%, output control 5) and a 90-second cool down between cycles was set.

Table 2. Qualitative and quantitative composition of ovalbumin-encapsulated liposomes.

	P90H (mg)	**SPC-3 (mg)**	**DPPC (mg)**	**DSPC (mg)**	**Cholesterol (mg)**	**Ovalbumin (mg)**	**Water (mL)**
P90H liposomes	14	-	-	-	1	10	3
SPC-3 liposomes	-	14	-	-	1	10	3
DPPC liposomes	-	-	14	-	1	10	3
DSPC liposomes	-	-	-	14	1	10	3

All the formulations were freeze-dried after the addition of increasing amounts of Respitose, trehalose or inulin (15, 30 or 45 mg, respectively). Freeze-drying was carried out in a Lyobeta freeze-dryer (Telstar, Spain) for 42 hours [77]. Being the re-dispersibility index a measure of the effectiveness of the freeze-drying process, it was calculated for the ovalbumin-encapsulated liposomes, prepared without (0 mg) or in combination with trehalose or Respitose, or inulin (15, 30 and 45 mg), as a ratio between the mean diameter measured after freeze-drying and rehydration (final size) and the mean diameter measured before freeze-drying (initial size) [78].

3.1.2 Morphological analysis

In all the studies, cryogenic transmission electron microscopy was used to assess the formation of vesicles as well as their morphology. 5 μL of each colloidal dispersion were loaded on a glow-discharged holey carbon grid and blotted with filter paper to obtain a thin film. It was vitrified with a Vitrobot (FEI Company, Eindhoven, The Netherlands) submerging the grid (kept at 100% humidity and room temperature) into ethane maintained at its melting point with liquid nitrogen. Finally, it was transferred using a Gatan cryo-transfer (Gatan, Pleasanton, CA, US) to a Tecnai F20 TEM (FEI Company) and images were acquired at 200 kV, -175 °C and in low-dose imaging mode with a 4096 × 4096 pixel CCD Eagle camera (FEI Company). In the third study, the analysis was conducted before freeze-drying and after freeze-drying and rehydration.

3.1.3 Mean diameter, polydispersity index and zeta potential

The measurement of mean diameter, polydispersity index and zeta potential of vesicles was performed using a Zetasizer Ultra (Malvern Instruments, Worcestershire, UK). Mean diameter and polydispersity index were determined by means of the dynamic light scattering technique, which measures the Brownian motion of the particles in the sample. Zeta potential was instead detected through the mixed-mode measurement-phase analysis, which measures the electrophoretic mobility of the particles in dispersion. All the samples were properly diluted with water prior to analysis.

3.1.4 Stability studies

Colloidal dispersions were stored at 4 °C and kept under vacuum before and after every measurement. Mean diameter, polydispersity index and zeta potential were assessed with the Zetasizer Ultra (Malvern Instruments, Worcestershire, UK) at regular intervals to detect any variation.

3.1.5 Encapsulation efficiency

The encapsulation efficiency of liposomes was determined indirectly. Vesicle dispersions were properly diluted with distilled water and placed into Vivaspin® 2 centrifugal separators. The samples were centrifuged at 1000 rpm for 2 hours, at 25 °C, through a centrifuge equipped with swing bucket rotor. The amount of free ovalbumin collected in the purification filtrates was determined with the bicinchoninic acid protein assay kit, reading the absorbance at 562 nm with a UV spectrophotometer (Lambda 25, Perkin Elmer, Milan, Italy). All experiments were performed in triplicate. In the third study, all the experiments were performed before and after freeze-drying and rehydration of the vesicle dispersions.

3.1.6 Penetration studies

In the first study, labelled ovalbumin was encapsulated in the enriched transfersomes and *in vitro* deposition studies were performed using vertical Franz cells with an effective diffusion area of 0.785 cm^2, thermostated at 37 ± 1 °C, and maintained under continuous stirring (300 rpm). Excised dorsal skin of one-day-old pigs, dead from natural causes, were stripped to remove stratum corneum by adhesive tape Tesa® AG (Hamburg, Germany) (n = 10 strips) and sandwiched between the donor and receptor compartments of the cells. The receptor compartment was filled with saline solution and the skin specimens (n = 3 per formulation) were left to equilibrate at 37 ± 1 °C in this solution overnight. Labelled ovalbumin, in solution or encapsulated in the enriched transfersomes, and saline solution (100 µL) were applied onto the skin specimen in non-occlusive or occlusive conditions, the latter achieved with cling film. Alternatively, the hydrogel disks (section 2.2) previously imbibed with the same samples were applied to the skin as well. The Franz cells were covered with tin foil to avoid direct exposure to light. Every 2 hours, saline solution was withdrawn and replaced by new medium to mimic the sink conditions. Experiments were conducted for 4 and 8 hours and, at the end, epidermis and dermis were severed and sliced using a surgical scalpel, placed in amber, glass vials with 2 mL of a mixture of ethanol and methanol (1:1 v/v) and sonicated in an ice bath to extract the free labelled ovalbumin. The resulted solution was filtered with syringe filters (Corning®, pore size 0.2 µm) and the ovalbumin was fluorescently quantified with a plate reader (Lambda 25, Perkin Elmer, Milan, Italy).

3.1.7 Mucoadhesiveness

In the second study, the zeta potential variations and mucin adsorption onto vesicle surfaces were measured to assess their mucoadhesiveness [79]. Vesicles were diluted (1:80) with a mucin solution (0.5 mg/mL), vortexed 10 seconds and incubated in a humidified atmosphere at 5% CO_2 and 37 °C for 20 minutes, which corresponds to the mucociliary clearance time in the nose [80,81]. Zeta potential was measured at 37 °C, every 5 minutes during the 20-minute incubation period (0, 5, 10, 15 and 20 minutes), and compared with the zeta potential of the corresponding vesicles diluted with water.
To assay the mucin adsorption instead, the method developed by Salade and co-workers was employed with some modifications [81]. Once the incubation time had elapsed, the vesicle dispersions diluted with mucin were centrifuged at 4000 rpm for 10 minutes and 25 µL of supernatant were collected to quantify the amount of free mucin by the bicinchoninic acid protein assay kit. Vesicles diluted with water, either loading ovalbumin or not, were used as references. The percentage of mucin adsorbed onto the vesicles was indirectly calculated, according to the following formula:

Adsorbed mucin (%) = (total mucin – free mucin)/total mucin x 100

3.1.8 Mucus permeation of ovalbumin

In the third study, the capability to cross the mucus over time of labelled ovalbumin, in solution or encapsulated in liposomes, was assessed using a Transwell® artificial mucus model [82,83]. Artificial mucus was prepared mixing 250 mg of mucin, 500 mg of DNA, 250 mg of sodium chloride, 110 mg of potassium chloride, 250 µL of sterile egg yolk emulsion, 300 µL of a solution of diethylenetriaminepenta-acetic (1 mg/mL) and 1 mL of RPMI in 50 mL of Milli-Q water. 200 µL of artificial mucus were transferred into each donor chamber of the Transwell® (6.5 mm well, 5.0 µm pore), and 300 µL of PBS (10 mM, pH 7.4) were added to each acceptor chamber. The Transwell® was incubated at 37 °C for 1 hour prior to start the experiment and kept at this temperature for its entire duration (24 hours). 50 µL of labelled ovalbumin in solution or encapsulated in liposomes was added to the mucus, in the donor chamber, with 50 µL of PBS (liposomes alone) or in association with a mucolytic agent, BromAc®. BromAc® (50 µL) was added at two different concentrations corresponding to 2% (w/v) N-acetyl cysteine + 100 µg/mL Bromelain (lower dose) or 2% N-acetyl cysteine (w/v) + 250 µg/mL Bromelain (higher dose). PBS alone (100 µL) was used as control. At 1, 2, 4, 10 and 24 hours, 50 µL of the medium in the acceptor chamber were withdrawn, replaced with fresh PBS (10 mM, pH 7.4) and dissolved in 250 µL of absolute ethanol in order to quantify the quantity of labelled ovalbumin able to cross the mucus. At each time point, the permeated ovalbumin (%) was calculated as follows:

$$\text{Ovalbumin permeated (\%)} = \frac{[\text{Labelled ovalbumin}]_{\text{acceptor chamber}}}{[\text{Labelled ovalbumin}]_{\text{donor chamber}}} \cdot 100$$

3.2 Hydrogel disks preparation and characterization

3.2.1 Preparation of hydrogel disks

In the first study, a hydrogel was prepared dispersing polyvinyl alcohol (10% w/v) in Milli-Q water and sonicating the system (4 sec. on, 2 sec. off, 90 cycles, 14 µm amplitude) to obtain a clear dispersion and avoid bubble formation. Aliquots (300 µL) of dispersions were then poured into each well of a 24-well plate, frozen at -20 °C for 16 hours and subsequently thawed for 8 hours (freeze-thawed). After two cycles of freeze-thaw, samples were freeze-dried at 0.5 mBar for 8 hours after freezing samples (-20 °C) overnight with no addition of secondary drying. Finally, all hydrogels were cut round with a chisel (hydrogel disks).

3.2.2 Swelling ratio and imbibition time of hydrogel disks

In the first study, the ability of the hydrogel disks to capture water (swelling) overtime was measured as a function of weight changes. Therefore, the freeze-dried hydrogel disks (n = 3) were weighted on analytical balance, submerged in an excess of water (9 mL) and, at each time point (0.5, 1, 2, 3, 4, 6 and 8 hours), they were gently blotted with paper, reweighted, and submerged in water again, noting the weights. The hydrogel disk swelling (%) was calculated on the basis of the weight of the freeze-dried hydrogel whereas the equilibrium swelling and the time needed to reach it (minimum imbibition time) were determined once a constant hydrogel disk weight was achieved after repeated submersion in water [84].

3.2.3 Loading of labelled ovalbumin dispersions into hydrogel disks and analysis of their contents

In the first study, freeze-dried hydrogel disks (n = 3) were hydrated with 300 µL of labelled ovalbumin, in solution or encapsulated in transfersomes, and incubated for 3 and 12 hours within a 24-well plate. At each time point, each hydrogel disk was taken, placed in an amber vial (10 mL) with 2.7 mL of water and sonicated (4 sec. on, 2 sec. off, 40 cycles, 14 µ amplitude) to release vesicles. Subsequently, 10 µL were withdrawn, diluted with 2.49 mL of a mixture of ethanol and methanol (1:1 v/v) and sonicated to release labelled ovalbumin from the enriched transfersomes. A centrifugation cycle (4000 rpm, 10 minutes, 25 °C) was performed in order to deposit any debris before reading the fluorescence of supernatant (190 µL) with a plate reader (Lambda 25, Perkin Elmer, Milan, Italy).

3.3 Nasal spray characterization

3.3.1 Droplet size distribution by laser diffraction

In the second study, the droplet size distribution was evaluated by laser diffraction using the Spraytec® (Malvern Panalytical Ltd., Malvern, United Kingdon). Tests were carried out at nozzle tip-to-laser distances of 4 and 7 cm, complying with both the measurement range (3-7 cm) and the distance between measurements (3 cm) set by the Food and Drug Administration. 6 mL of each formulation were used to fill a commercial pump device (20 mL) kindly provided by FAES laboratories. All the measurements were performed in triplicate, at 25 °C, positioning the pump device at a 45° angle to the reading laser beam. After every actuation, the values D10, D50 and D90, which represent the respective percentage (10%, 50% and 90%) of all the particles below the registered size, along with the Span, which expresses the uniformity of the droplet size distribution, were collected.

3.3.2 Droplet deposition in the Alberta Idealized Nasal Inlet

In the second study, fluorescein isothiocyanate-dextran replaced ovalbumin as a fluorescent probe in anionic liposomes and cationic DOTAP-liposomes during their preparation. The Alberta Idealized Nasal Inlet (Copley Scientific, Nottingham, United Kingdom) was coated with the mucin solution (0.5 mg/mL) in section 2.1.6 and connected to the Next Generation Impactor (Copley Scientific, Nottingham, United Kingdom). The commercial Nasonex device was filled with 6 mL of dispersions of either negative or positive liposomes, oriented to 45° with respect to the Alberta Idealized Nasal Inlet and manually actuated three times for each formulation. A Petri dish was placed under the device to recover the sample in case of dripping. To simulate slow inhalation through a single nostril, a steady flow rate of 7.5 L/min was set. Experiments were performed in triplicate. After each experiment, the Alberta Idealized Nasal Inlet was disassembled and each region was washed with appropriate volumes of methanol to release and detect the fluorescein isothiocyanate-dextran (λ excitation: 495 nm; λ emission: 525 nm) spectrophotometrically (Lambda 25, Perkin Elmer, Milan, Italy).

To qualitatively assess the deposition, anionic liposomes and cationic DOTAP-liposomes were mixed with 20 µL of a solution (2 mg/mL) of blue patent dye and red ponceau dye, respectively. Pictures were taken after actuating the device in the same conditions reported above.

3.4 Dry powder characterisation

3.4.1 Dilution of freeze-dried liposomes and capsule filling

In the third study, just before testing with Spraytec® or Next Generation Impactor, the freeze-dried formulations were diluted to 1) finely regulate the dose of ovalbumin and 2) decrease the adherence of the powder particles. Respitose (1:10 w/w) was used as diluent and the resulting powders were homogenised for 10 seconds using a CapMixTM (3 M-ESPE, Diegem, Belgium) before filling size 3 hydroxypropyl methylcellulose Quali-V®-I capsules (Qualicaps, Madrid, Spain). 14 mg of powder (Respitose-diluted liposomes), containing 200 µg of ovalbumin, were used per capsule.

3.4.2 Particle size distribution by laser diffraction

The effect of the dilution with Respitose on the dry powders was assessed with the Spraytec® (Malvern Instruments, United Kingdom), which exploits laser diffraction to determine the size distribution of the dry powder particles generated by the Aerolizer® after actuation. Studies were performed in triplicate, coupling the Spraytec® with the inhalation cell and the US Pharmacopeia induction port. Being the Aerolizer® a low-resistance device, with the aim of complying with the Pharmacopeia requirements for pulmonary testing, the flow rate was set to 100 L/min and the duration of experiment was set to 2.4 seconds (Ph.Eur.10, 2.9.18 Preparation for inhalation). Size distributions of freeze-dried liposomes diluted 1:10 (w/v) with Respitose (Respitose-diluted liposomes), as well as freeze-dried undiluted liposomes and Respitose alone, were determined and compared. 1 capsule per formulation was discharged (1 actuation) by the Aerolizer® for each Spraytec® determination. The experiments were performed in triplicate (3 determinations per formulation). To quantify the width of particle-size distribution, the span was calculated as follows:

$$span = \frac{D_{90} - D_{10}}{D_{50}}$$

3.4.2. *In vitro* aerodynamic performance

In the third study, the aerodynamic performances of the freeze-dried ovalbumin-encapsulated liposomes were assessed using the Next Generation Impactor (Copley Scientific, Nottingham, United Kingdom) equipped with the adult Alberta Idealised Throat and the mixed inlet, and further connected to the BRS 3000 breath simulator (Copley Scientific, Nottingham, United Kingdom). The healthy, asthmatic and chronic obstructive pulmonary disease patient profiles were were created according to the equations developed by Delvadia and co-workers and literature data and studied [85,86]. The pre-separator was kept between the mixed inlet and the Next Generation Impactor due to the nature of the samples. The Alberta Idealised Throat and plates were coated with a mixture of isopropanol and propylene glycol (50% v/v) and allowed to dry before the experiments [87]. 5 capsules were discharged (5 actuations) by the Aerolizer® for each assessment. Appropriate volumes of a sodium dodecyl sulfate solution (2% w/v) were applied in each stage to release the antigen from the vesicles and the bicinchoninic acid protein assay kit was used for quantification by measuring the absorbance according to section 2.1.5. The experiments were performed in triplicate (15 capsules in total for each diluted formulation).

CITDAS V3.10 software (Copley Scientific, United Kingdom) was used to determine mass median aerodynamic diameter, geometric standard deviation, fine particle dose, extrafine particle dose and delivered dose. Mass median aerodynamic diameter is the average size of the particles under a flow (aerodynamic diameter) that actually reaches the stages of the impactor [88]. The geometric standard deviation is a dimensionless number that express the width of homogeneity in size distribution, and its value can be 1 only when particles have exactly the same mass median aerodynamic diameter [89]. Fine particle dose is the mass of the drug particles with an aerodynamic diameter < 5.0 μm (thus reaching the lungs) whereas extrafine particle dose is the mass of the particles with an aerodynamic diameter < 2.0 μm (thus reaching the deep lungs) [90]. The delivered dose, also known as emitted dose, corresponds to the mass of drug emitted per actuation that is actually available to the user at the mouthpiece. In addition, delivered fraction, fine particle fraction and extrafine particle fraction were calculated as a function of the total dose metered:

$$\text{Delivered fraction (\%)} = \frac{\text{Delivered dose}}{\text{Total dose}} \cdot 100$$

$$\text{Fine particle fraction (\%)} = \frac{\text{Fine particle dose}}{\text{Delivered dose}} \cdot 100$$

$$\text{extrafineparticle fraction (\%)} = \frac{\text{Extrafine particle dose}}{\text{Delivered dose}} \cdot 100$$

3.5 Biological evaluation

3.5.1 Biocompatibility assay

In the first study, fibroblasts (L929 cells) were grown in monolayers under standard conditions (37 °C, humidified, 5% CO_2), using Dulbecco's Modified Eagle Medium (DMEM) supplemented with foetal bovine serum (10% v/v), penicillin (100 U/mL), and streptomycin (100 μg/mL). Biocompatibility assay was carried out according to the *in vitro* methods for cytotoxicity assessment included in ISO 10993-

5 (https://nhiso.com/wp-content/uploads/2018/05/ISO-10993-5-2009.pdf). Briefly, fibroblasts L929 cells were seeded at a density of 1 × 10^5 cells/mL in 96-well plates (100 µl/well) and incubated under standard conditions for 24 hours. The complete medium was then replaced with medium-diluted formulations (glycerol-transfersomes, hyaluronan-transfersomes and glycerohyaluronan-transfersomes) or ovalbumin solution at different ovalbumin concentrations (0.05, 0.5, 50, and 500 µg/mL). After an additional incubation period (24 hours), the supernatant was removed, 100 µL of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium (MTT, 0.5 mg/mL) were added and the formazan formation, dissolved in DMSO, was determined for each concentration tested and compared to that determined in culture controls. The absorbance was read at 570 nm with a microplate reader (Lambda 25, Perkin Elmer, Milan, Italy).

In the third study, alveolar macrophages (RAW 264.7 cells) were seeded at a density of 1 × 105 cells/mL in 96-well plates (100 µl/well) and incubated under standard conditions for 24 hours. After, the complete medium was withdrawn and cells were incubated for 24 hours with ovalbumin in solution or encapsulated in liposomes, properly diluted with medium to reach 300, 60, 12, 2.4 and 0.48 µg/mL of ovalbumin (final concentration). Medium alone was used as positive control. Cell viability was evaluated by adding cell Counting Kit - 8 to each well and reading the absorbance at 450 nm by using a microplate reader (Thermo Fisher Scientific) after 4 hours of incubation, according to manufacturer instructions.

3.5.2 Culturing of bone marrow-derived dendritic cells and B3Z OT-I hybridoma cell line

In the first study, bone marrow derived dendritic cells were obtained from precursors isolated from the tibiae of euthanized eight-week-old, female, C57BL/6 mice (Charles River, Lecco, Italy) that had been housed in pathogen-free conditions, in accordance with institutional guidelines. Tibiae were deprived of their extremities and the contained marrow was washed with ice-cold RPMI 1640 medium. Cells were resuspended by pipetting, washed twice with medium, seeded and cultured with recombinant murine granulocyte/macrophage colony-stimulating factor (200 U/mL) in RPMI 1640 medium supplemented with 10% foetal calf serum, 60 µg/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate and 50 µM 2-mercaptoethanol. After 8 days, immature dendritic cells were collected and used in the antigen presentation assay.

In the first study, B3Z cells (OT-I hybridoma line) were used as they specifically recognize the ovalbumin octapeptide (257–264) SIINFEKL exposed on the major histocompatibility complex I (MHC I) of the surface of dendritic cells allowing the production of interleukin-2 in response. These cells were grown in complete RPMI 1640 medium (10% foetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 1% glutamine, 1% N-ethylmaleimide, 1% sodium pyruvate and 50 µM 2-mercaptoethanol) and co-cultured with bone marrow derived dendritic cells for the antigen presentation assay.

3.5.3. Antigen presentation assay and detection of interleukin-2

In the first and second study, bone marrow derived dendritic cells (1×10^6/mL) were incubated overnight with different concentrations of ovalbumin in solution or loaded in liposomes (1-5 µg/mL). A blank control was made incubating cells with only medium. Subsequently, the bone marrow derived

dendritic cells were co-cultured with B3Z cells (OT-I hybridoma line, 5×10^5/well) for 40 hours and the amount of interleukin-2 released into supernatants was assessed as a measure of uptake and processing of ovalbumin (ovalbumin-peptide 257-264). From each well, 100 µL of supernatant were analysed in duplicate using mouse IL-2 ELISA MAX Standard kit in two independent experiments, according to the manufacturer's instructions.

3.5.4 Human monocyte isolation and culturing of monocyte-derived macrophages

In the first study, discarded buffy coats of healthy blood donors were used, upon informed consent, to obtain monocytes. Briefly, Ficoll-Paque gradient density separation was adopted to separate human peripheral blood mononuclear cells while magnetic microbeads allowed for isolation of monocytes by CD14 positive selection. Only > 95% viable and pure monocytes (determined by trypan blue exclusion and cytosmears) were used in the experiments. Monocytes were grown in RPMI 1640 + Glutamax-I medium supplemented with 50 µg/mL of gentamicin sulphate, 5% of heat-inactivated human AB serum and 10 ng/mL of M-CSF. Differentiation in macrophages was achieved incubating cells at 37 °C in 5 % CO_2 for 7 days and replacing the medium every 3 days.

3.5.5 Biocorona formation on trasfersome surface

Before the antigen presentation assay, vesicles were admixed 1:1 (v/v) with heat-inactivated serum and incubated for 1 hour at 37 °C in an orbital shaker at 300 g to allow the formation of the serum protein biocorona on vesicles' surface and ensure their stability in culture. The serum pre-coated transfersomal vesicles were added directly to the culture plates adjusting their concentration to the desired values for each treatment.

3.5.6 Stimulation of monocyte-derived macrophages and detection of tumor necrosis factor-α

In the first and second study, monocyte-derived macrophages were exposed for 24 h to culture medium alone (negative control), medium containing 5 ng/mL of lipopolysaccharide from E. coli O55:B5 (positive control), medium containing ovalbumin or medium containing pre-coated vesicles diluted up to 200 ng/mL, 1 µg/mL, 5 µg/mL of ovalbumin. After exposure all supernatants were collected, centrifuged and frozen at −80 °C for subsequent cytokine analysis. Production of tumor necrosis factor-α was measured in the culture supernatants by ELISA using a MultiScan FC reader (ThermoScientific) according to the manufacturers' protocol. Results are reported as mean ± standard deviation of values from 2 replicate samples from the same donor.

3.5.7 Stimulation of alveolar macrophages and detection of tumor necrosis factor-α and interleukin-6

In the third study, RAW 264.7 cells were seeded at a density of 5 × 105 cells/well in 24-well plates, incubated for 24 hours with ovalbumin in solution or encapsulated in liposomes (5 and 1 µg/mL, ovalbumin final concentration), medium alone (negative control) or lipopolysaccharide (positive control, 5 ng/mL). Cell culture supernatants were collected, and the enzyme linked immunosorbent assays (ELISA) and MultiScan FC reader (ThermoScientific) were used to evaluate the expression levels of cytokines tumor necrosis factor-α and interleukin-6 according to the manufacturer's protocols.

3.5.8 Cellular uptake by flow cytometry

In the third study, RAW 264.7 cells were seeded at a density of 1 × 10^5 cells/mL in 24-well plates and incubated at 37 °C and 5% CO_2 for 24 hours. The medium was then replaced with labelled ovalbumin in solution or encapsulated in the vesicles properly diluted to reach 60 µg/mL of ovalbumin (final concentration). Medium alone was used as control. After 0.5, 1, 2, 4, 8 or 24 hours of incubation, the medium was removed, the cells were detached with Accutase®, the plates were centrifuged (5 minutes, 350 rfc) and the supernants were discarded. Cell pellets were resuspended in running buffer (200 µL) prior the analysis of live, single-stained cells with MACSQuant® Analyzer 10 Flow Cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). The obtained data were analysed with FlowJo V10 software (TreeStar, Ashland, USA).

3.5.9 Cellular uptake by flow cytometry

In the third study, RAW 264.7 cells were seeded at a density of 1 × 10^4 cells/well in a chamber slide system (Nunc™ Lab-Tek™ Chambered Coverglass) and incubated at 37 °C and 5% CO_2 for 24 hours. The medium was then replaced with labelled ovalbuminin solution or encapsulated in the vesicles properly diluted to reach 60 µg/mL of ovalbumin (final concentration). Medium alone was used as control. At specific time points (0.5, 24 and 48 hours), the samples and controls were removed, the wells were rinsed off three times with PBS and the cells were stained and subsequently incubated at 37 °C for 30 minutes. CellTrackerTM red was used to stain the cell membrane and NucBlue™ Live the nucleus. Live cells were observed with the Nikon Eclipse Ti2-E fluorescence imaging system after three additional washings with PBS to evaluate labelled ovalbumin internalization. Nikon's advanced Artificial Intelligence (AI) and software were used to make real-time analysis and optimize the measurements.

3.6 Statistical analysis

Results are expressed as the means ± standard deviations. Multiple comparisons of means (ANOVA) were used to substantiate statistical differences between groups, while Student's t-test was used to compare two samples. Significance was tested at the 0.05 level of probability (p). Data analysis was carried out with the software package XLStatistic for Excel.

4. Results

4.1. Enriched transfersomes for the cutaneous delivery of ovalbumin

4.1.1 Enriched transfersome characterisation

Mean diameter, polydispersity index, zeta potential and entrapment efficiency of ovalbumin-encapsulated enriched transfersomes were measured (**Table 3**). All the vesicles were very small < 60 nm and homogeneously dispersed, as the polydispersity index was ≈ 0.2. Hyaluronan-transfersomes had a slightly larger mean diameter (~57 nm) when compared with glycerol-transfersomes and glycerohyaluronan-transfersomes, and these differences were confirmed by cryogenic electron microscopy (**Fig. 1**). Along with size, morphology was affected by the composition of the hydrating medium: glycerol, sodium hyaluronate or their combination. Specifically: glycerol-transfersomes were small and had irregular and elongated shapes; hyaluronan-transfersomes were larger and more spherical; glycerohyaluronan-transfersomes were smaller, completely spherical and uniformly sized. Neither the zeta potential strongly negative (~-32 mV, $p > 0.05$ among the 3 values) nor the encapsulation efficiencies (~63%, $p > 0.05$ among the 3 values) were significantly affected by the hydrating phase.

Table 3. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency (EE) of ovalbumin-encapsulated enriched transfersomes. Same symbols (*, °, ^, #) indicate values that are not statistically different ($p > 0.05$). Mean values ± standard deviations, obtained from at least 3 measurements, are reported.

	MD (nm)	PI	ZP (mV)	EE (%)
Glycerol-transfersomes	50±1*	0.21±0.01°	-29±2^	65±7#
Hyaluronan-transfersomes	57±2	0.22±0.01°	-34±9^	61±6#
Glycerohyaluronan-transfersomes	49±3*	0.20±0.01°	-30±6^	64±5#

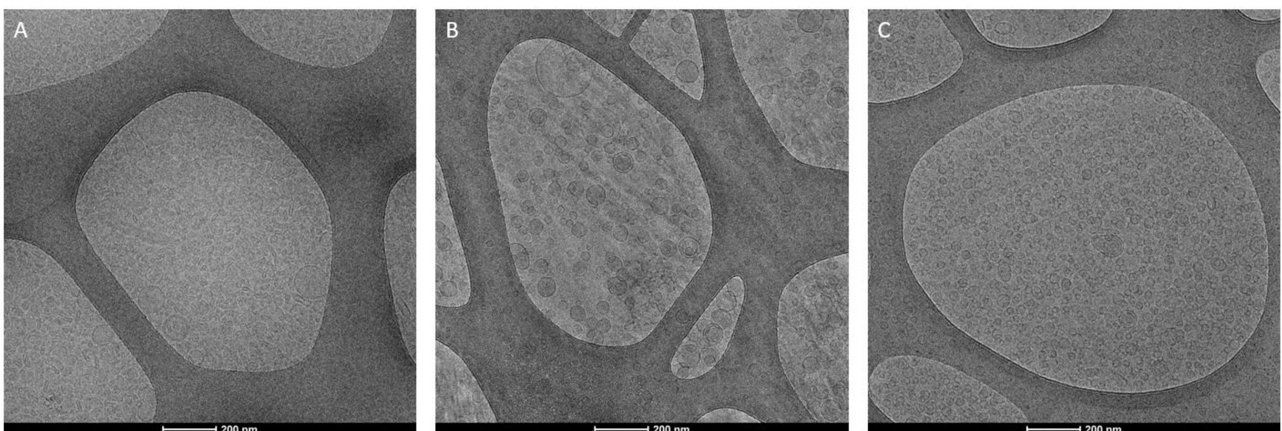


Figure 1. Representative cryogenic transmission electron microscopy images of glycerol-transfersomes (A), hyaluronan-transfersomes (B) and glycerohyaluronan-transfersomes (C).

Variations of mean diameter, polydispersity index and zeta potential were monitored overtime each 3 months for 9 months to evaluate the stability of the ovalbumin-encapsulated transfersomes once stored at 4 °C and under vacuum. All the vesicle dispersions were stable for 9 months of study, with no need of adding any preservative (**Fig. 2**). Comparing the mean diameters of fresh formulations with their respective mean diameters after 1, 3, 6 and 9 months, no significant differences were detected as glycerol-transfersomes, hyaluronan-transfersomes and glycerohyaluronan-transfersomes retained their initial hydrodynamic diameter < 60 nm ($p > 0.05$ among the values of each sample). Similarly, zeta potential did not undergo through significant variations overtime, remaining constant (~-32 mV, $p > 0.05$ among the values of each sample).

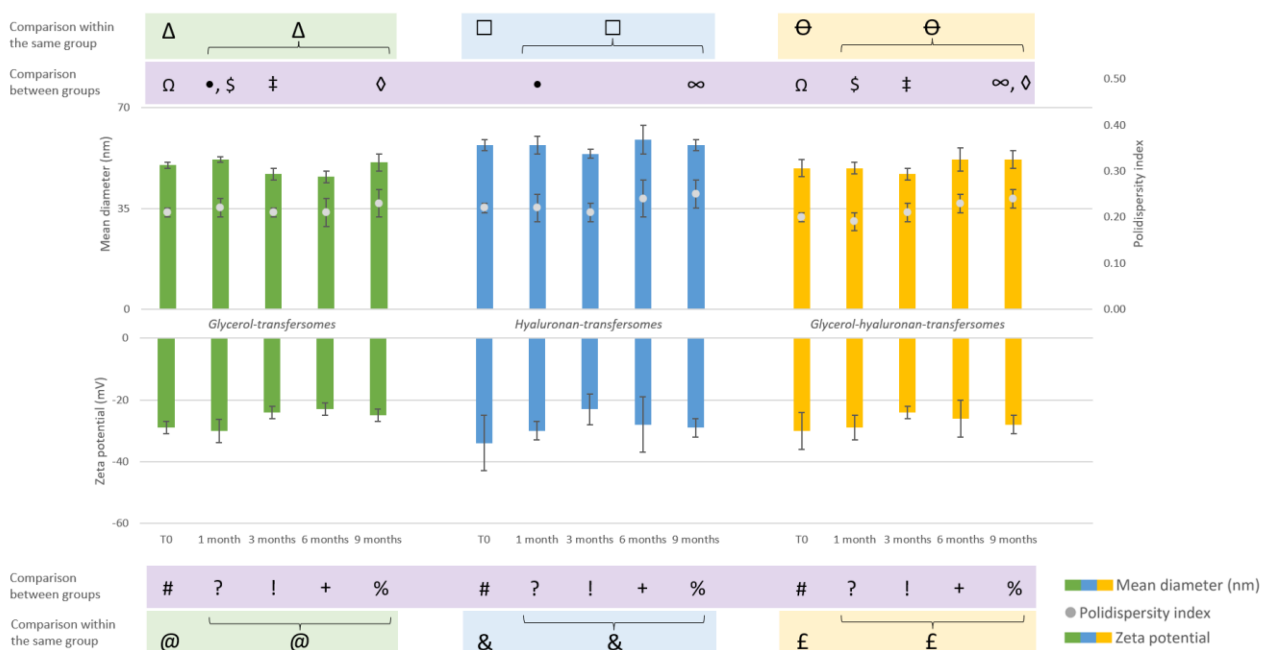


Figure 2. Stability overtime evaluated measuring mean diameter (upper panel, bars), polydispersity index (upper panel, dots) and zeta potential (lower panel, bars) of glycerol-transfersomes (green), hyaluronan-transfersomes (blue) and glycerohyaluronan-transfersomes (orange). Same symbols (Δ, □, ϴ, Ω, •, $, ‡, ◊, ∞, #, ?, !, +, %, @, & and £) indicate values that are not statistically different from the values at initial time (light green, blue and orange) or among the different groups (cross-comparison) at the same time point (violet). Mean values ± standard deviations, obtained from at least 3 measurements, are reported.

Since the enriched transfersomes were intended to be loaded into hydrogel disks, their stability was evaluated after freeze-thawing and freeze-driyng as they were both steps required to prepare the hydrogel disks. Thus, after these processes, the mean diameter and polydispersity index of the enriched transfersomes were measured again (**Fig. 3**). A significant increase of the size was found for all the formulations since the first cycle of freeze-thaw, as the mean diameter of hyaluronan-transfersomes changed from ~57 to ~92 nm ($p << 0.05$), and that of glycerohyaluronan-transfersomes

from 49 to ~100 nm ($p << 0.05$). This increase in size was remarkably higher if compared with that of glycerol-transfersomes, whose mean diameter increased only from ~50 to ~70 nm. Interestingly, the second cycle did not further significantly affect the mean diameters suggesting that, after a possible initial rearrangement of the double bilayer, all these vesicles are unaffected by subsequent freeze-thaw cycles. After freeze-drying mean diameter changed again, especially that of glycerol-transfersomes (from ~70 to ~370 nm) while glycerohyaluronan-transfersomes underwent the less variation (from ~98 to ~192 nm). The polydispersity index of glycerol-transfersomes and glycerohyaluronan-transfersomes changed drastically from initial values ~0.20 to final values ~0.38 whereas that of hyaluronan-transfersomes remained unchanged (~0.23) indicating that the new arranged vesicles were homogeneously dispersed.

On the whole, freeze-thawing and freeze-drying strongly affected the physico-chemical properties of all the enriched transfersomes. As a consequence, hydrogels disks were imbibed with the enriched tranfersomes only after the preparation by freeze-thawing and freeze-drying.

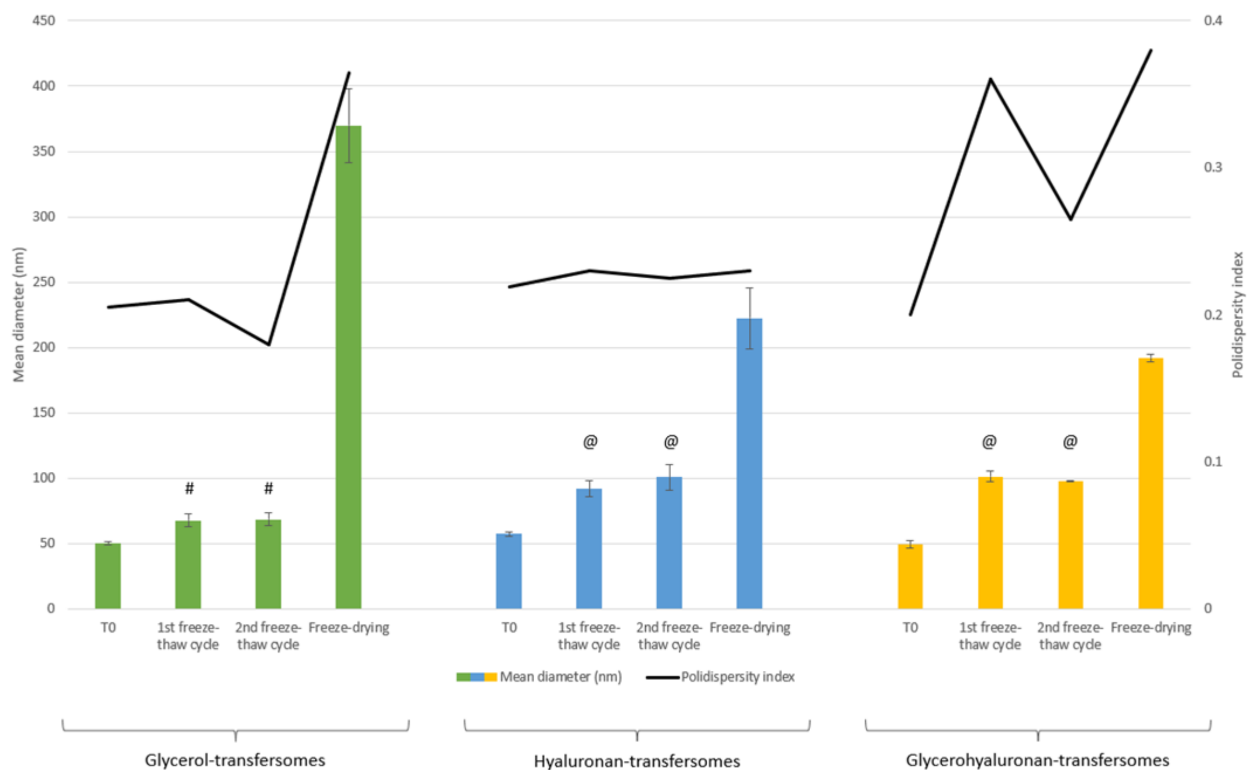


Figure 3. Mean diameter and polydispersity index of glycerol-transfersomes (green), hyaluronan-transfersomes (blue) and glycerohyaluronan-transfersomes (orange) after the freeze-thawing (2 cycles) and freeze-drying processes. Mean values along with the standard deviations are reported for each formulation obtained from at least 3 measurements.

4.1.2 Imbibed hydrogel disks characterization

Since the preparation steps of the hydrogels (freeze-thaw and freeze-dry) had a strong negative impact on the physico-chemical properties of the enriched transfersomes (**Fig. 3**), empty freeze-dried hydrogels disks were firstly prepared and after imbibed with formulations. To find the imbibition time, their ability to capture water was evaluated and the swelling ratio at different time points (0.5, 1, 2, 3,

4, 6 and 8 hours) were calculated (**table 4**). The time needed to reach the equilibrium was found allowing to select the minimum incubation time needed to completely imbibe the hydrogel disks (minimum imbibition time). After submerging the freeze-dried hydrogel disks in water for 0.5 hours, their swelling ratio hugely increased up to ~391% compared to the initial value (**Table 4**). Subsequent increases were detected at 1, 2 and 3 hours. From 3 hours onwards, there were no statistical differences among the swelling ratios (≈418%, $p > 0.05$ between the values at 3, 4, 6 and 8 hours). As a consequence, 3 hours was identified as the minimum imbibition time to completely imbibe the hydrogel disks with ovalbumin solution or ovalbumin-encapsulated enriched transfersomes in the next studies.

Table 4. Swelling ratio (%) and minimum imbibition time of empty hydrogel disks left swelling in water at different time points (0.5, 1, 2, 3, 4, 6 and 8 hours). Same symbols (○, □) indicate values that are not statistically different ($p > 0.05$). Mean values ± standard deviations, obtained from at least 3 measurements, are reported.

Time (h)	**0.5**	**1**	**2**	**3***	**4**	**6**	**8**
Swelling ratio (%)	391 ± 2	405 ± 2○	403 ± 2○	420 ± 4□	418 ± 2□	417 ± 2□	421 ± 4□
				***Attainment of the equilibrium of swelling and the minimum imbibition time**			

To confirm the effectiveness of the minimum imbibition time, empty freeze-dried hydrogels were allowed to imbibe with FITC-ovalbumin in solution or encapsulated in transfersomes for 3 or 12 hours. The amount of ovalbumin was quantified (**Table 5**). No statistical differences were found in the ovalbumin content among the hydrogel disks with respect to the time points (3 or 12 hours) or the formulations tested (solution, glycerol-transfersomes, hyaluronan-transfersomes or glycerohyaluronan-transfersomes), as it was always ~22% ($p > 0.05$ among the different values, correspondent to ~330 µg of ovalbumin per disk). Thus, according to the swelling study, 3 hours were enough to achieve the complete imbibition of the hydrogel disks also with the labelled ovalbumin in solution or encapsulated in the enriched transfersomes.

Table 5. Ovalbumin content (%) of imbibed hydrogel disks after 3 and 12 hours of swelling in presence of ovalbumin in solution or encapsulated in enriched transfersomes. Same symbols (○) indicate values that are not statistically different ($p > 0.05$). Mean values ± standard deviations, obtained from at least 3 measurements, are reported.

	Ovalbumin content in imbibed hydrogel disks (%)	
	3 h	12 h
Solution	18 ± 2○	19 ± 2○
Glycerol-transfersomes	20 ± 2○	21 ± 2○
Hyaluronan-transfersomes	21 ± 3○	24 ± 4○
Glycerohyaluronan-transfersomes	24 ± 4○	22 ± 2○

4.1.3 *In vitro* penetration studies

The capability of labelled ovalbumin (500 μg) in solution or encapsulated in enriched transfersomes to penetrate in the tape-stripped skin was evaluated *in vitro* using vertical Franz cells and calculating the amount of antigen accumulated in the epidermis and dermis at 4 and 8 hours after non-occlusive or occlusive (cling film) application (**Fig. 4**).

After the non-occlusive application of the solution, ovalbumin accumulation was ~6 μg/cm^2 in the epidermis ($p > 0.05$ between the values at 4 and 8 hours), and sensibly lower, ~0.5 μg/cm^2 in the dermis ($p > 0.05$ between values at 4 and 8 hours). After application of glycerol-transfersomes in the same condition, the deposition was comparable, especially at 4 hours. Using hyaluronan-transfersomes and glycerohyaluronan-transfersomes, it was instead higher, ≈ 10 μg/cm^2 in the epidermis ($p > 0.05$ among the values at 4 and 8 hours using the 2 formulations), and ≈ 1 μg/cm^2 in the dermis ($p > 0.05$ among the values at 4 and 8 hours using the 2 formulations), thus indicating that the accumulation of ovalbumin in these skin layers was not influenced by time but only by the used formulation.

By contrast, when ovalbumin formulations were applied under occlusive conditions (cling film), the accumulation was time and formulation dependent. In the epidermidis, the lowest accumulation, ≈7 μg/cm^2 ($p > 0.05$ between the values at 4 and 8 hours), was achieved by the solution. Higher accumulations were achieved by tranfersomes, with the highest value, ≈22 μg/cm^2 ($p > 0.05$ versus the values obtained solution and glycerol-transfersomes) provided by hyaluronan-transfersomes at 8 hours and glycerohyaluronan-transfersomes at 4 and 8 hours. In the dermis, the benefits associated with the occlusive application were significantly visible at 4 and 8 hours for all the formulations with respect to the non-occlusive application. Therefore, the occlusive approach significantly enhanced the antigen delivery in this deeper skin layer as the ovalbumin accumulated at 8 hours was ~2 μg/cm^2 using the solution, ~3 μg/cm^2 using glycerol-transfersomes, ~5 μg/cm^2 using hyaluronan-transfersomes and ~7 μg/cm^2 using glycerohyaluronan-transfersomes.

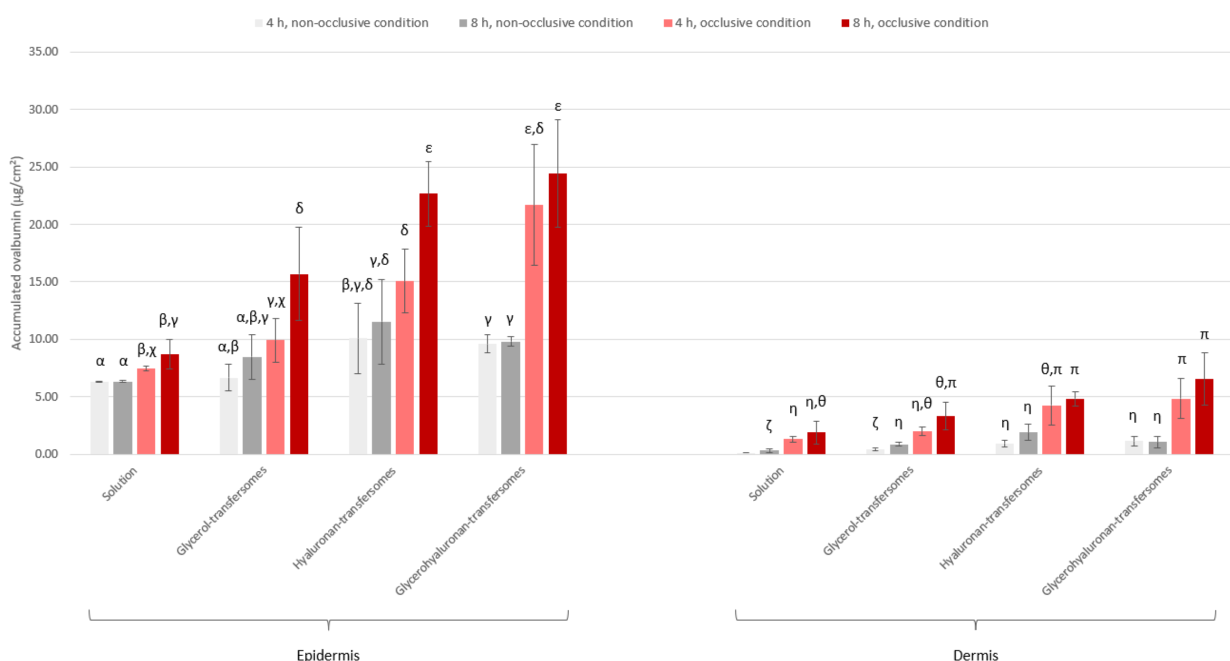


Figure 4. Amount of ovalbumin (μg/cm^2) accumulated in epidermis (left) and dermis (right) after application of FITC-ovalbumin, in solution or encapsulated in enriched transfersomes, with cling film (red) or not (grey) at 4 (light colours) and 8 hours (dark colours). Same symbols (α, β, γ, χ, δ, ε, ζ, η, θ, π) indicate values that are not statistically different ($p > 0.05$). Mean values ± standard deviations, obtained from at least 3 measurements, are reported.

Considering the promise of the occlusive condition (cling film), the labelled ovalbumin (~330 μg) in solution or encapsulated in enriched trasfersomes was loaded into polyvinyl alcohol hydrogel disks (imbibed hydrogel disks) to enable a comparison with the occlusive condition after application of the disks for 4 and 8 hours on tape-stripped skin (**Fig. 5**). With respect to the occlusive conditions (cling film), the antigen deposition was greatly improved, at 8 hours, by using the imbibed disks likely due to the better adhesion they provide to the skin. The accumulation achieved by the FITC-ovalbumin solution imbibed disks was ~9 μg/cm^2 ($p < 0.05$ versus values obtained using the ovalbumin solution with cling film) at 4 hours and it doubled (~18 μg/cm^2) at 8 hours, confirming the ability of the hydrogel disks to improve the payload delivery. Combining the hydrogel disks and enriched transfersomes, the antigen deposition was even further improved. In particular, transfersome-imbibed disks ensured the highest ovalbumin accumulation at 8 hours in the epidermis, ≈ 35 μg/cm^2 ($p > 0.05$ among the values obtained at 8 hours using the three transfersome-imbibed disks and $p < 0.05$ versus values obtained at 4 and 8 hours applying the solution-imbibed disks). At 4 hours instead, the highest deposition was achieved only using glycerohyaluronan-transfersome imbibed disk, and it was ≈ 23 μg/cm^2 ($p > 0.05$ between the values obtained under occlusive condition and $p < 0.05$ versus the values obtained at 4 hours applying the disks imbibed with glycerol-transfersomes and hyaluronan-transfersomes). In the dermis, the highest deposition at 8 hours, ≈ 10 μg/cm^2, was found when using disks imbibed with hyaluronan-transfersomes and glycerohyaluronan-transfersomes ($p > 0.05$ between the 2 values and $p < 0.05$ versus all the other values). By contrast, disks imbibed with either glycerol-transfersomes or ovalbumin solution led to a lower deposition, ≈ 5 μg/cm^2 ($p < 0.05$ between the 2 values), and were therefore less effective than hyaluronan-transfersomes and glycerohyaluronan-transfersomes.

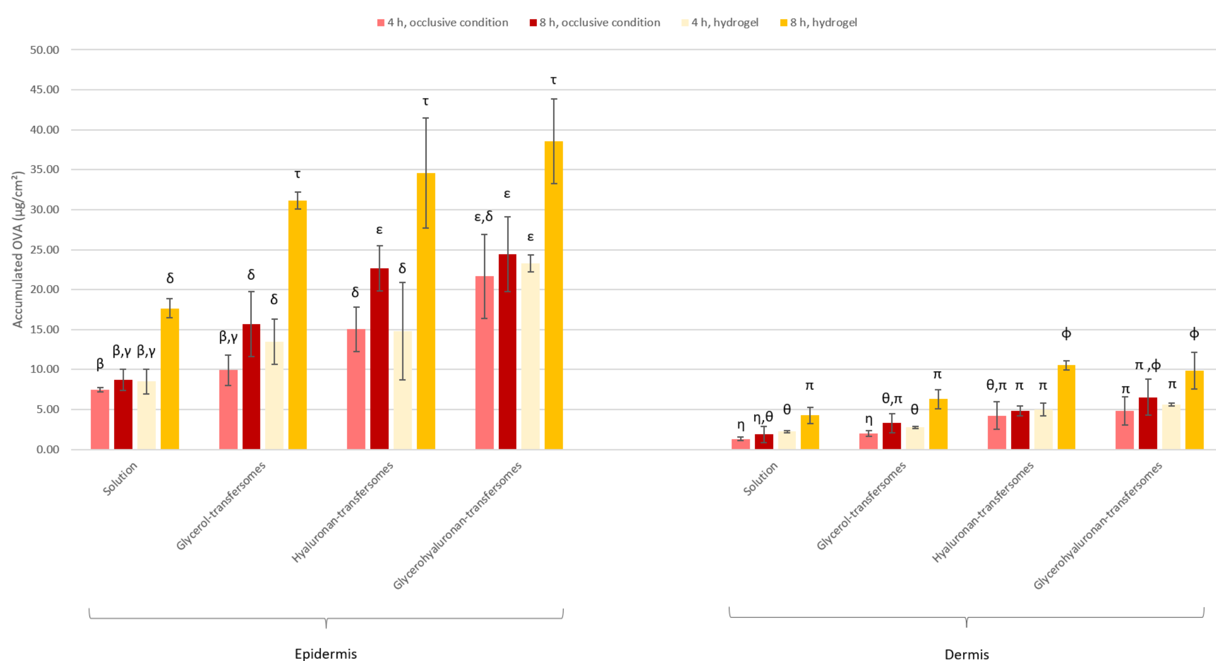


Figure 5. Amount of ovalbumin (μg/cm²) accumulated in epidermis (left) and dermis (right) after application of FITC-ovalbumin, in solution or encapsulated in enriched transfersomes, with cling film (red) or hydrogel disks (yellow) at 4 (light colours) and 8 hours (dark colours). Same symbols (α, β, γ, δ, τ, η, θ, π, ϕ) indicate values that are not statistically different ($p > 0.05$). Mean values ± standard deviations, obtained from at least 3 measurements, are reported.

4.1.4 Biological evaluation

The biocompatibility of ovalbumin in solution or encapsulated in enriched transfersomes was tested on the L929 fibroblast cell line (**Fig. 6**). After 24 hours of incubation, the cell viability of all the samples was > 60% and < 90% only using the highest concentration (500 μg/mL of ovalbumin in solution or encapsulated in transfersomes). Using ovalbumin concentrations ≤ 50 μg/mL the viability was > 90% and increased in a composition dependant manner (glycerohyaluronan-transfersomes > hyaluronan-transfersomes > glycerol-transfersomes > solution), denoting in any case the low toxicity of ovalbumin itself and the beneficial effect provided by its encapsulation into enriched transfersomes.

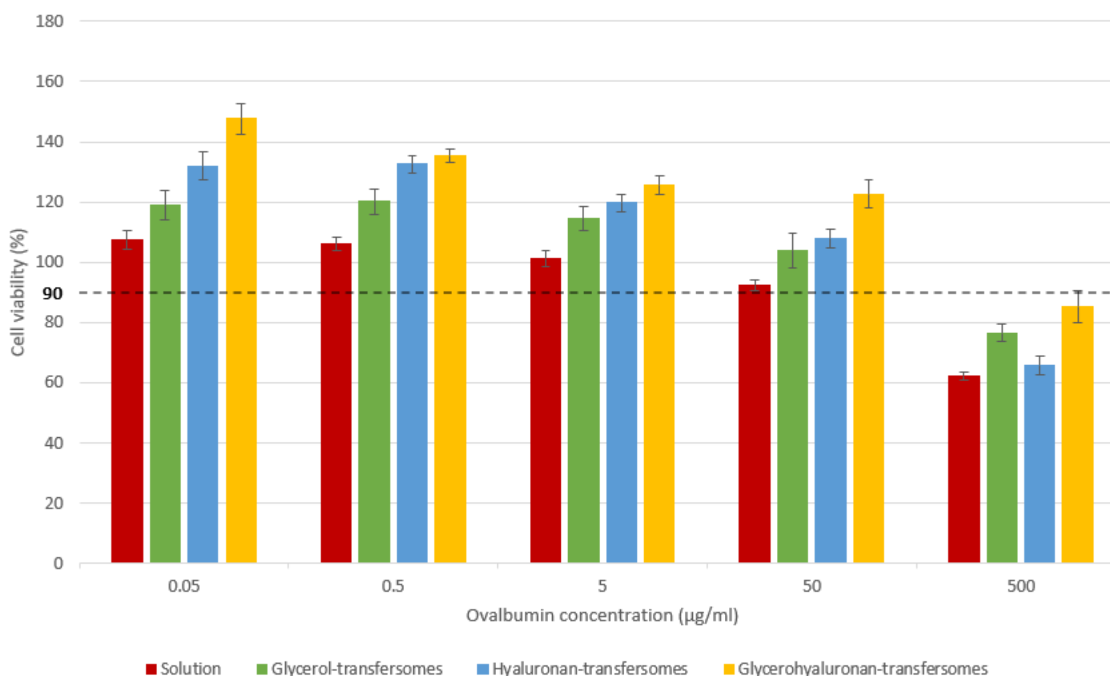


Figure 6. Cell viability of L929 fibroblasts incubated for 24 hours with ovalbumin solution (red), glycerol-transfersomes (green), hyaluronan-transfersomes (blue) and glycerohyaluronan-transfersomes (yellow) diluted with medium to reach 0.05, 0.5, 5, 50 and 500 μg/mL of ovalbumin. Mean values ± standard deviations, obtained from at least 3 measurements, are reported.

The ability of ovalbumin (5 or 1 μg/mL), in solution or encapsulated in enriched transfersomes, to stimulate antigen-presenting cells was evaluated measuring, after incubation, the production of interleukin-2 by B3Z cells co-incubated with dendritic cells and the expression of tumor necrosis factor-α by monocyte-derived macrophages (**Fig. 7**). The interleukin-2 produced by dendritic cells incubated with medium (negative control) was ~10 pg/mL, irrespective to the dose used, indicating the basal production from these cells. When the cells were incubated with ovalbumin (5 or 1 μg/mL),

in solution or encapsulated in enriched transfersomes, the value slightly increased up to ~14 pg/mL ($p > 0.05$ versus the control), indicating a minimum but not significant stimulating effect irrespective to the used formulation. Similarly, while the treatment of macrophages with lipopolysaccharide (positive control), a strong pro-inflammatory agent, stimulated the expression of high amount of tumor necrosis factor-α (~4000 pg/mL), the treatment with ovalbumin in solution or encapsulated in enriched transfersomes did not stimulate its production as the measured values were ~50 pg/mL and thus comparable to those found incubating the cells only with medium ($p > 0.05$ among the values obtained with all ovalbumin formulations and negative control, $p < 0.05$ versus positive control). On the whole, despite the good skin penetration performance and high compatibility of the enriched vesicles, no ability to stimulate skin-resident antigen-presenting cells was detected. However, no differences were revealed for either ovalbumin free or encapsulated, so that transfersomes displayed no adjuvanticity.

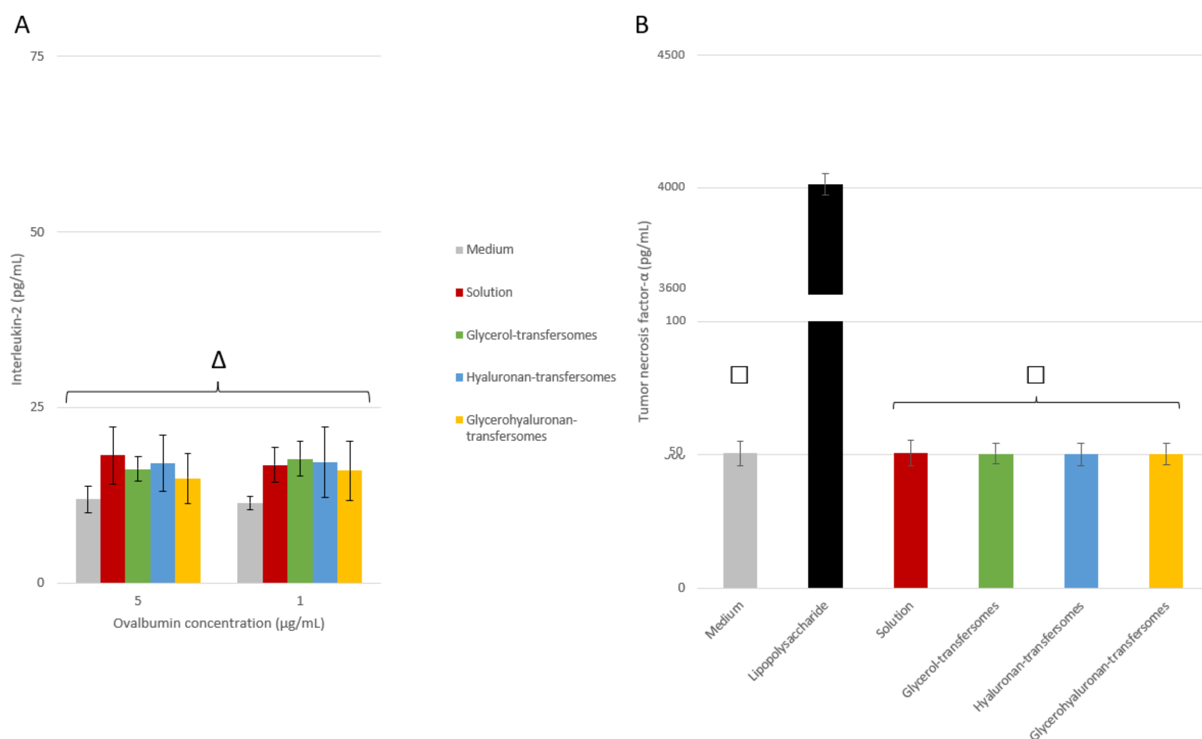


Figure 7. Production of interleukin-2 by B3Z cells co-cultured with dendritic cells (A) and tumor necrosis factor-α by macrophages (B) after incubation with ovalbumin (5 and 1 µg/mL) in solution (red) or encapsulated in glycerol-transfersomes (green), hyaluronan-transfersomes (blue) and glycerohyaluronan-transfersomes (orange). Medium alone was used as negative control while lipopolysaccharide was used a positive control during the tumor necrosis factor-α detection. Mean values ± standard deviations are reported. Results are representative of two independent experiments.

4.2 Anionic and cationic liposomes for the nasal administration of ovalbumin

4.2.1 Anionic and cationic liposome characterisation

The actual formation of liposomes, along with their morphology and lamellarity, was confirmed by cryogenic transmission electron microscopy (**Fig. 8**). Anionic liposomes were overall smaller than cationic DOTAP-liposomes, slightly irregular shaped and mostly unilamellar. By contrast, cationic DOTAP-liposomes had an overall more regular and spherical shape and a uni or oligolamellar structure.

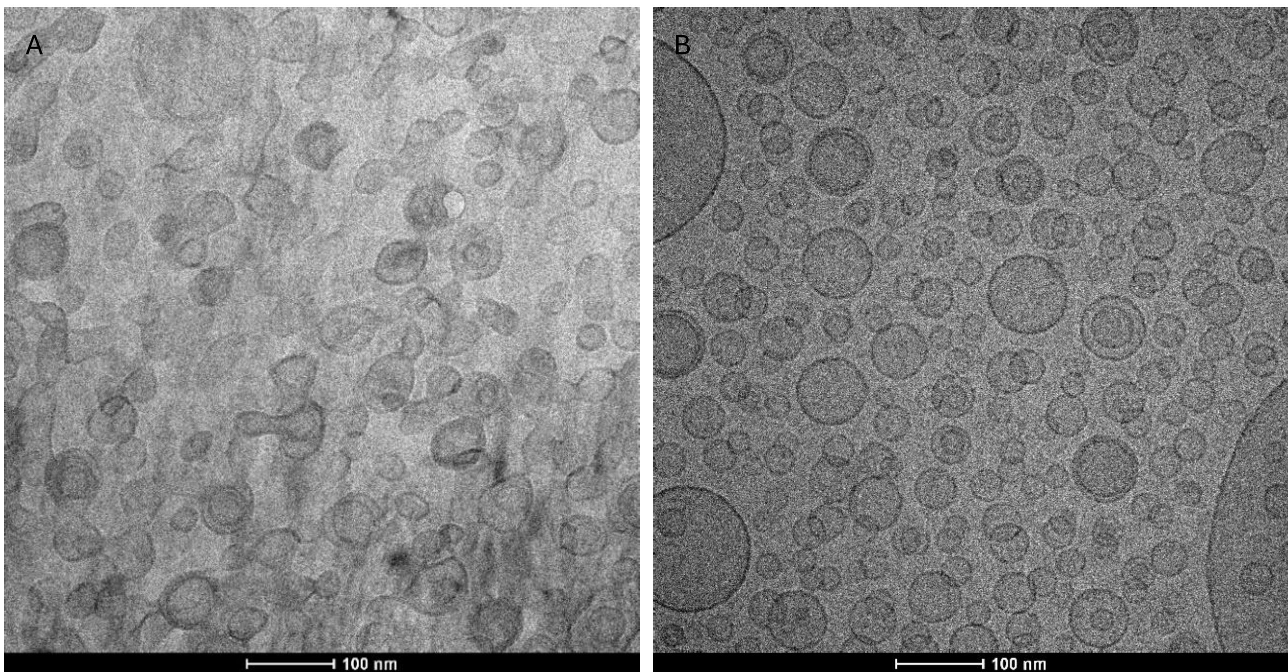


Figure 8. Representative images of anionic liposomes (A) and cationic DOTAP-liposomes (B).

The mean diameter, polydispersity index, zeta potential and entrapment efficiency of the vesicles were assessed (**Table 6**). The anionic liposomes were smaller and more homogeneously dispersed than the cationic DOTAP-liposomes, thus corroborating the observations made through cryogenic transmission electron microscopy. The addition of 1,2-dioleoyl-3-trimethylammonium-propane strongly affected the shape and surface charge of the vesicles, as the cationic DOTAP-liposomes had a zeta potential around +50 mV compared to the -8 mV zeta potential of the anionic liposomes prepared without it. Despite both the anionic and cationic DOTAP-liposomes were able to encapsulate high amounts of antigen (> 80%), the cationic DOTAP-liposomes led to higher encapsulation, thus suggesting an important role of the 1,2-dioleoyl-3-trimethylammonium-propane not only in the superficial charge but also in the vesicle assembling and antigen loading.

Table 6. Mean diameter, polydispersity index, zeta potential and encapsulation efficiency of ovalbumin loaded liposomes. Mean values ± standard deviations, obtained from at least 3 measurements, are reported.

	MD (nm)	**PI**	**ZP (mV)**	**EE (%)**
Liposomes	108±1	0.22±0.01	-8±1	83±1
DOTAP-liposomes	127±2	0.25±0.01	+53±2	99±1

The long-term stability of the vesicle dispersions was evaluated considering all the physico-chemical variations involving mean diameter, polydispersity index and zeta potential during a period of 3 months (**Table 7**). While the mean diameter and the polydispersity index of the anionic liposomes were basically unaffected over time, a slight increase in these parameters was observed for the cationic DOTAP-liposomes. This increase could be related to a rearrangement of vesicles, as confirmed by the slight decrease in zeta potential. After 6 months, it was not possible to measure these parameters again by virtue of the precipitation phenomena that underwent in the two dispersions.

Table 7. Mean diameter, polydispersity index and zeta potential of ovalbumin-encapsulated liposomes after 30 and 90 days. Mean values ± standard deviations, obtained from at least 3 measurements, are reported.

	MD (nm)			**PI**			**ZP (mV)**		
Days	**t0**	**30**	**90**	**t0**	**30**	**90**	**t0**	**30**	**90**
Liposomes	108±1	106±1	108±3	0.22±0.01	0.23±0.01	0.21±0.02	-8±1	-13±1	-12±2
DOTAP-liposomes	127±2	138±2	140±8	0.25±0.01	0.26±0.01	0.27±0.01	+53±2	+50±2	+46±6

The mucoadhesiveness of liposomes was evaluated diluting the dispersions with a mucin solution (0.5 mg/mL) and measuring the resulting zeta potential and the amount of mucin adsorbed on their surface (**Fig. 9**). The zeta potential of anionic liposomes did not undergo significant changes and became only slightly less negative. On the contrary, the zeta potential of cationic DOTAP-liposomes underwent a substantial change, from a strong positive value to a significant negative value. Looking at these changes of zeta potential, it can be concluded that greater interactions between mucus and vesicles occurred especially with cationic DOTAP-liposomes rather than anionic liposomes. The intensity of such interactions was evaluated measuring the amount of mucin adsorbed on vesicle surface after incubation with the same mucin solution (0.5 mg/mL) for 20 minutes at 37 °C (**Fig. 10**). The cationic DOTAP-liposomes adsorbed an 11-fold higher amount of mucin than the anionic liposomes, reaching a mucin adsorption, ~88%, compared to ~8% of anionic liposomes.

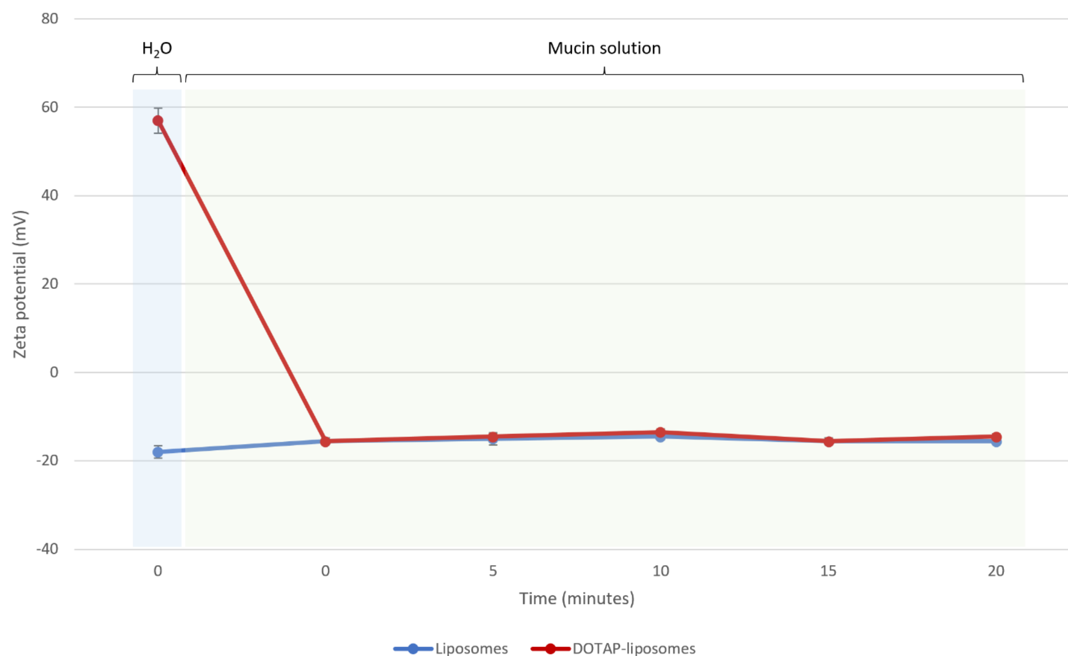


Figure 9. Zeta potential of anionic liposomes (in blue) and cationic DOTAP-liposomes (in red), before (highlighted in blue) and after (highlighted in green) being mixed with a mucin solution (0.5 mg/mL) and incubated for 20 minutes at 37 °C. Mean values ± standard deviations, obtained from at least 3 measurements, are reported.

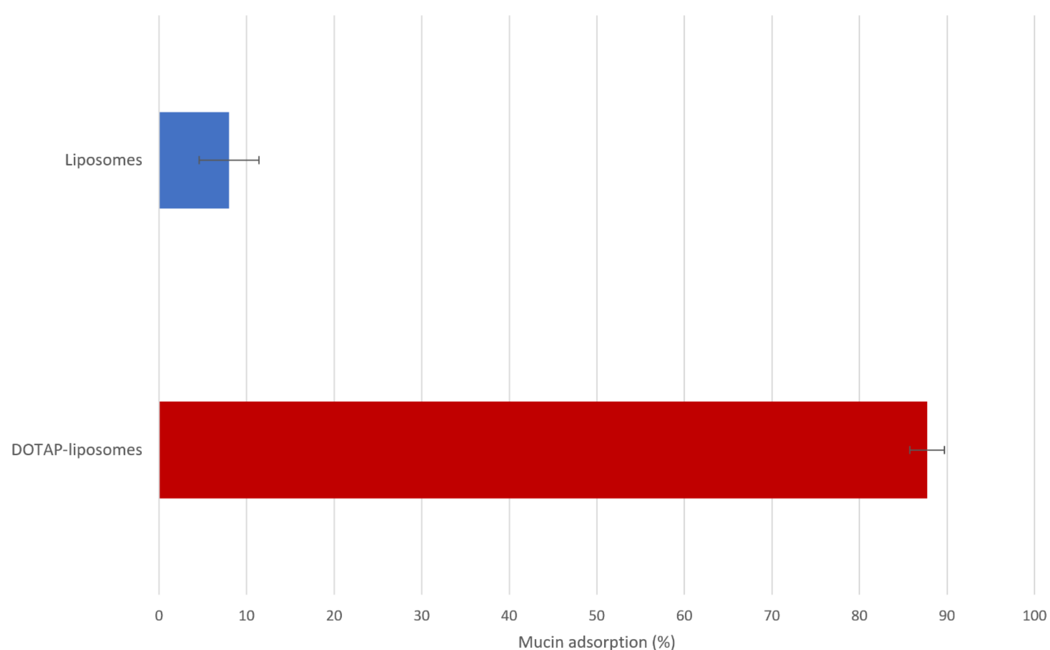


Figure 10. Percentage of mucin adsorbed on the surface of anionic liposomes (in blue) and cationic DOTAP-liposomes (in red), after their incubation with a mucin solution (0.5 mg/mL) for 20 minutes at 37 °C. Mean values ± standard deviations, obtained from at least 3 measurements, are reported.

4.2.2 Nasal spray characterisation

The adequacy of these formulations to be sprayed, and therefore used as nasal spray vaccines, was evaluated measuring the size of the droplets generated by the chosen device. To meet the requirements of European Medicines Agency and Food and Drug Administration, the nozzle tip of the device was positioned at 4 and 7 cm from the laser beam. The cumulative volumes of undersized droplets, expressed as D10, D50 and D90, were calculated along with the width of their size distribution, expressed as Span (**Fig. 11**). Overall, the narrow Span values (< 1.5) indicated a high homogeneity of size distribution among the generated droplets. When sprayed at 4 cm from the laser beam, the behaviour of both the anionic and cationic DOTAP-liposome dispersions was similar. The D90 and the D10 were ~90 mm and ~22 mm, respectively, indicating that the 90% and the 10% of the generated droplets had a physical diameter ≤ 90 mm and ≤ 22 mm. Instead, when the dispersions were sprayed at 7 cm from the laser beam, the D90 decreased to ~78 mm while the D10 increased to ~30 mm. The D50 was the only value unaffected by the laser beam distance or the dispersion used, remaining constant at ~50 mm. However, regardless of the tested distance or formulation, the generated droplets were always larger than 10 mm and therefore suitable to be deposited in the nasal cavity [123].

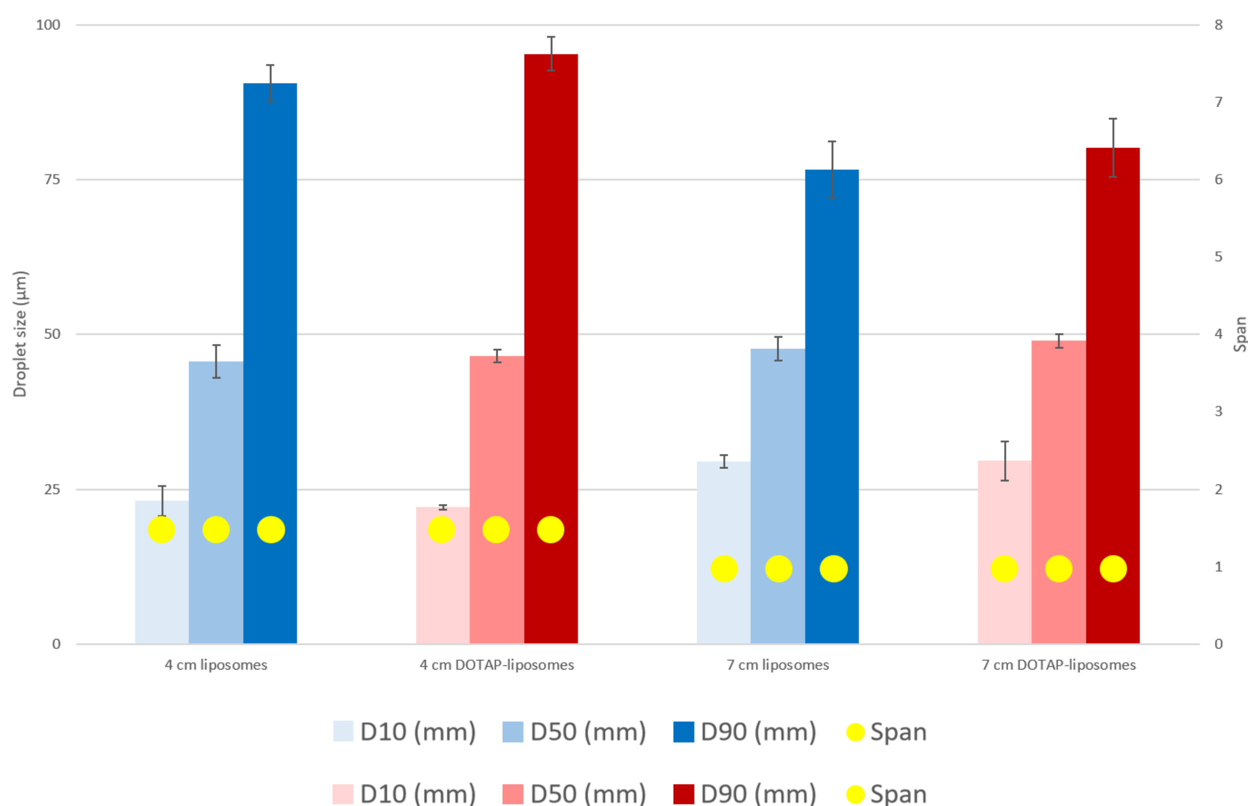


Figure 11. Droplet size analysis of liposomes (in blue) and DOTAP-liposomes (in red) sprayed at 4 cm (left bars) and 7 cm (right bars) from the laser beam. The cumulative volumes of undersized droplets (D10, D50 and D90) and the width of their size distribution (Span) are reported as mean values ± standard deviations of at least three measurements.

The specific regional deposition of both anionic liposomes and cationic DOTAP-liposomes was then assessed using a realistic nasal replica, the Alberta Idealized Nasal Inlet, which possesses a four-

region resolution (vestibule, olfactory region, turbinates and nasopharynx) to allow the recovery of the sample, connected to the Next Generation impactor, which simulates the lungs (**Fig. 12**) (https://www.copleyscientific.com/inhaler-testing/realistic-throat-and-nasal-models/alberta-idealised-nasal-inlet-aini/). To facilitate the quantification, liposome dispersions were fluorescently labelled with fluorescein isothiocyanate-dextran. The total recovery of labelled vesicles was ~95%, irrespective of the formulation. The deposition of anionic liposomes and cationic DOTAP-liposomes in the vestibule was ~45%, with no statistical differences between the values obtained with the two formulations ($p > 0.05$). Similarly, no statistical differences ($p > 0.05$) were detected in the olfactory region (values < 0.5%) or the lungs (0%). By contrast, in the turbinates, the deposition provided by cationic DOTAP-liposomes (~52%) was significantly higher than the one provided by anionic liposomes (~43%). In the nasopharynx instead, the deposition of anionic liposomes was slightly higher (~3%) than that of cationic DOTAP-liposomes (~0.8%). It is likely that the negative surface charge of anionic liposomes facilitates the deposition in the deeper stages because their negative charge is the same of mucin and generates repulsive forces. The quantitative recovery was confirmed qualitatively after mixing the anionic liposomes with blue patent dye and the cationic DOTAP-liposomes with red ponceau dye, as showed by the pictures at both sides of **Fig. 12**.

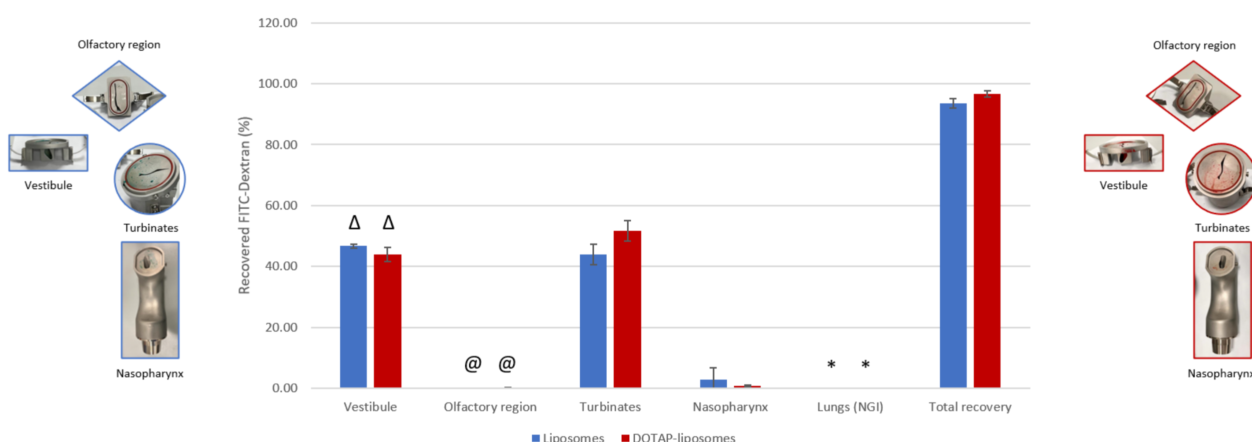


Figure 12. Fluorescein isothiocyanate-dextran recovered in the deposition regions of the Alberta Idealised Nasal Inlet (vestibule, olfactory region, turbinates and nasopharynx) and the Next Generation Impactor (lungs) after nebulisation of labelled anionic liposomes (in blue) and cationic DOTAP-liposomes (in red). Mean values ± standard deviations, obtained from at least 3 measurements, are reported. The same symbol (Δ, @, *) indicates values not statistically different ($p > 0.05$).

4.3.3 Biological evaluation

The efficacy of anionic liposomes and cationic DOTAP-liposomes as antigen delivery carriers was evaluated by measuring the interleukin-2 produced in a co-culture of dendritic cells exposed to liposome dispersions or culture medium (negative control) and B3Z cells (**Fig. 13**). A significant production of interleukin-2 was detected when the ovalbumin was delivered in anionic liposomes and cationic DOTAP-liposomes in comparison to the unencapsulated protein (ovalbumin solution, $p > 0.05$). Cationic DOTAP-liposomes led to higher production of interleukin-2 than anionic liposomes when used at the highest dose ($p > 0.05$). By contrast, no significative differences ($p > 0.05$) were

detected for the anionic liposomes with regard to the dose tested (5 or 1 µg/mL). Nonetheless, both formulations mediated significant antigen uptake, processing and presentation as they induced interleukin-2 production at levels significantly higher than those provided by the ovalbumin solution ($p > 0.05$).

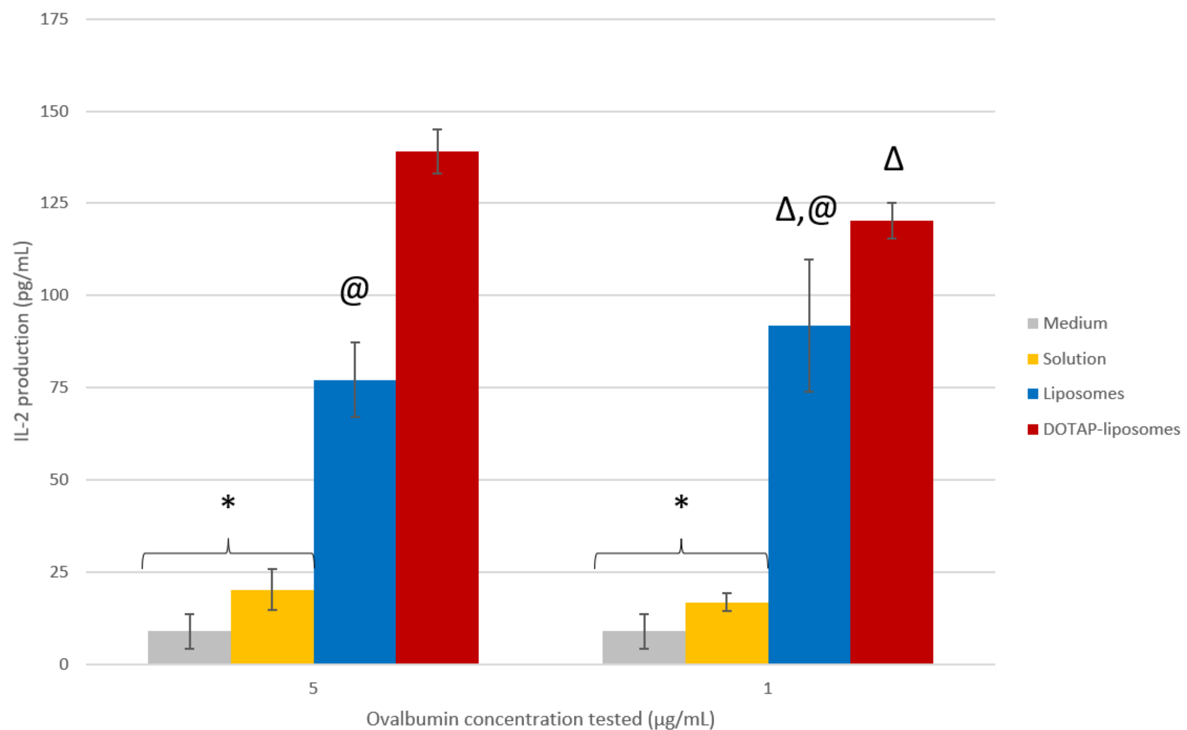


Figure 13. Interleukin-2 measured in the medium of dendritic cells pre-exposed to culture medium (negative control) or ovalbumin (5 and 1 µg/mL), in solution or encapsulated in anionic or cationic DOTAP-liposomes and co-cultured with B3Z cells. Mean values ± standard deviations, calculated from experimental replicates, are reported. Results are representative of two independent experiments. The same symbol (Δ, @, *) indicates values not statistically different ($p > 0.05$).

The innate immune primary response was evaluated after exposing human monocyte-derived macrophages for 24 h to ovalbumin in solution or encapsulated in anionic liposomes or cationic DOTAP-liposomes. Three different concentrations of ovalbumin were tested: 5 µg/mL (**Fig. 14**), 1 µg/mL and 200 ng/mL (data not shown). Macrophages exposed only to culture medium were used as negative control whereas macrophages exposed to lipopolysaccharide, a prototypical inflammatory stimulus, were used a positive control. Macrophage activation was evaluated measuring the production of the inflammatory cytokine tumor necrosis factor-α. Comparably to the ovalbumin solution, the ovalbumin-encapsulated vesicles did not induce a measurable reactivity in human macrophages, being unable to promote tumor necrosis factor-α production at any tested dose ($p < 0.05$).

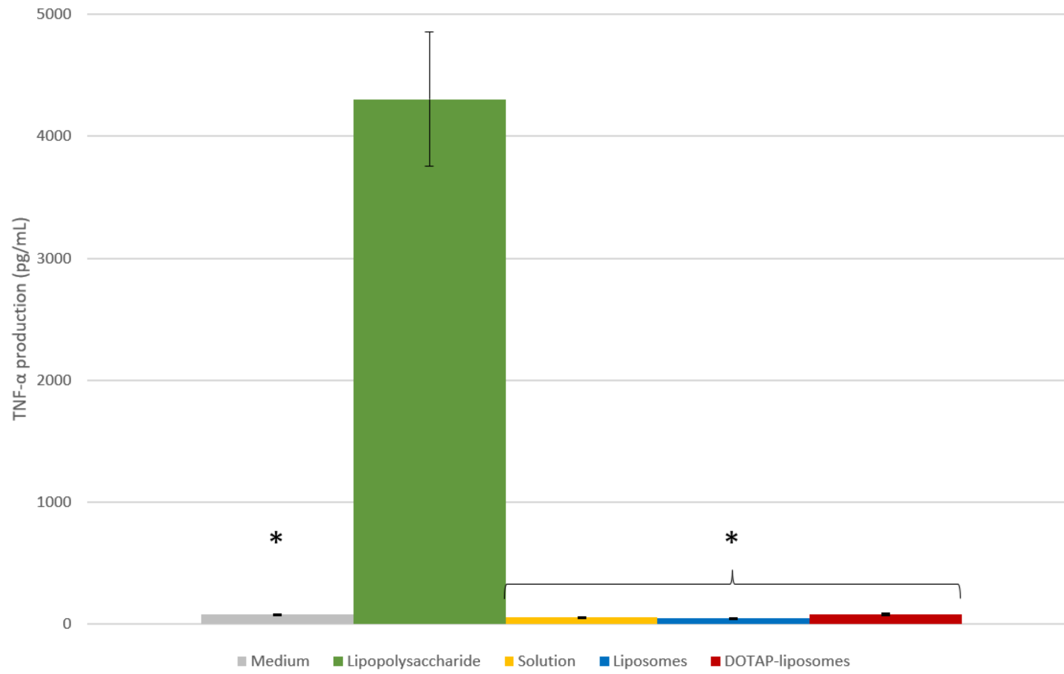


Figure 14. Tumor necrosis factor-α produced by human monocyte-derived macrophages upon exposure to culture medium (in grey, negative control), ovalbumin (5 µg/mL) in solution (in yellow) or encapsulated in anionic liposomes (in blue) and DOTAP-liposomes (in red) or 5 ng/mL of lipopolysaccharide (in green, positive control). Mean of two replicate experiments ± standard deviations are reported. The same symbol (*) indicates values not statistically different ($p > 0.05$).

4.3 Freeze-dried anionic liposomes for the pulmonary administration of ovalbumin

4.3.1 Anionic liposome characterisation

Since transforming vesicles in solid state was of primary importance for improving their stability and allowing their delivery to the lungs, the re-dispersibility index, an index of freeze-drying effectiveness, was calculated and used to enable a fast preliminary screening of the formulations (**Fig. 15**). Formulations having a re-dispersibility index < 1.3 are recognised to be effectively freeze-dried and can be easily and rapidly rehydrated [149,150]. Therefore, among the prepared formulations, the ones freeze-dried using the lowest amount of cryoprotectant (15 mg of trehalose or Respitose or inulin) or without it, were discarded as their re-dispersibility indexes were ~4 irrespective of the cryoprotectant or lipid used. By contrast, adding the medium and high amounts (30 and 45 mg) of trehalose and Respitose, the re-dispersibility index was < 1.3. Using 30 mg of inulin the index was > 1.3, while using the higher dose (45 mg) the index was < 1.3. A further selection was performed among the formulations having a re-dispersibility index < 1.3, since, 45 mg of trehalose, Respitose and inulin enabled a better reformation of SPC-3 and DPPC liposomes as their index was 1.1. The same dose (45 mg) of the three cryoprotectants was less effective in facilitating the reformation of P90H and DSPC liposomes as the re-dispersibility index was 1.2. Since the effectiveness of freeze-drying was in this case affected by both the cryoprotectant and the lipid used, SPC-3 and DPPC liposomes were selected over P90H and DSPC liposomes. Additionally, being inulin more cost-effective than trehalose, only SPC-3 and DPPC liposomes containing Respitose and inulin were further characterized as these specific combinations of lipids and cryoprotectants led to the best re-dispersibility index (1.1) and the best affinity to the pulmonary surfactant composition [151,152].

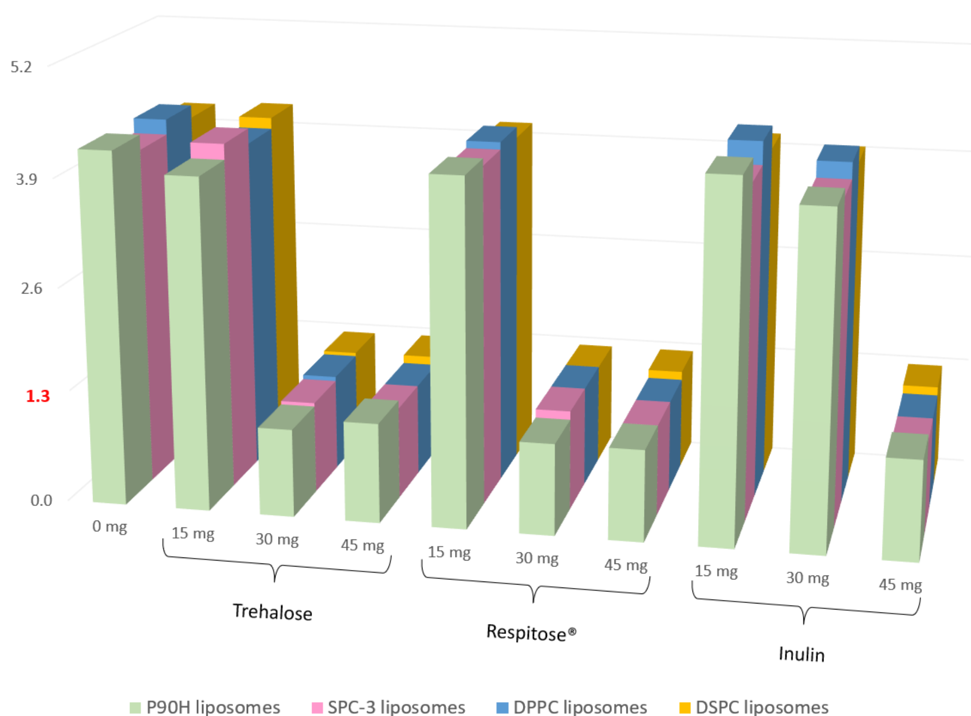


Figure 15. Re-dispersibility index of ovalbumin-encapsulated P90H (in green), SPC-3 (in pink), DPPC (in blue) and DSPC (in yellow) liposomes prepared with 0, 15, 30 or 45 mg of trehalose, Respitose and inulin.

Mean diameter, polydispersity index, zeta potential and encapsulation efficiency of the selected ovalbumin-encapsulated liposomes (Respitose and inulin SPC-3 and DPPC liposomes) were assessed before and after freeze-drying and rehydration to evaluate the effects of the process on these parameters (**Table 8**).

Before freeze-drying, all the liposomes manufactured by direct sonication were small (< 100 nm) and monodisperse (PI ≈ 0.2) (**Table 8**, **before freeze-drying**). Mean diameter and polydispersity index were not affected by the phospholipid nor the cryoprotectant and the encapsulation efficiency of all vesicles was always > 50%. Specifically, DPPC liposomes had significantly higher encapsulation efficiency, ~63 when prepared with Respitose and ~78% with inulin. On the contrary, that of SPC-3 liposomes was ~56% irrespective of the cryoprotectant used.

After freeze-drying and rehydration (**Table 8**, **after freeze-drying**), the high concentration of cryoprotectants selected (45 mg of either Respitose or inulin) led to the reforming of vesicles having similar mean diameter and polydispersity index to the former ones, in accordance with the narrow values of their re-dispersibility index (**Fig. 16**). Minor but not significant changes of zeta potential of vesicles were observed before and after the rehydration. Similarly, no statistical differences of encapsulation efficiencies were detected before and after the rehydration, thus highlighting an effective protection exerted by the cryoprotectants during freeze-drying.

Table 8. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and encapsulation efficiency (EE) of ovalbumin-encapsulated SPC-3 and DPPC liposomes prepared with 45 mg of Respitose or inulin before freeze-drying (on the left) and after freeze-drying and rehydration (on the right). Mean values ± standard deviations (n=3) are reported. The same symbol (Δ, ●, ◊, @, ○, ϟ, #) is used to indicate values not statistically different (p > 0.05).

		Before freeze-drying				**After freeze-drying**			
		MD (nm)	**PI**	**ZP (mV)**	**EE (%)**	**MD (nm)**	**PDI**	**ZP (mV)**	**EE (%)**
Respitose	*SPC-3 liposomes*	$86\pm2^{\#\Delta}$	0.19±0.01	$-15\pm1^{@}$	$54\pm7^{\circ}$	$97\pm4^{\Delta}$	0.23±0.01	$-14\pm1^{@}$	$53\pm4^{\circ}$
Inulin		$85\pm7^{\#\Delta}$	0.22±0.04	$-13\pm1^{@}$	$56\pm1^{\circ}$	$92\pm4^{\Delta}$	0.25±0.01	$-15\pm1^{@}$	$59\pm4^{\circ}$
Respitose	*DPPC liposomes*	$78\pm6^{\#}$	0.16±0.01	$-9\pm1^{\lozenge}$	$63\pm6^{\text{ϟ}}$	$84\pm3^{\#}$	0.18±0.02	$-11\pm3^{\lozenge}$	$62\pm3^{\text{ϟ}}$
Inulin		$82\pm7^{\#}$	0.21±0.03	$-9\pm2^{\lozenge}$	$78\pm1^{\bullet}$	$93\pm1^{\Delta}$	0.18±0.05	$-10\pm1^{\lozenge}$	$75\pm5^{\bullet}$

The structure and size of the selected formulations having re-dispersibility index = 1.1 (Respitose and inulin SPC-3 and DPPC liposomes) were observed by cryogenic transmission electron microscopy, before and after freeze-drying and rehydration (**Fig. 16**). Before freeze-drying, all the vesicles had a spherical, unilamellar morphology and small diameters, confirming that the selected cryoprotectants

do not affect the shape nor the size of the vesicles. After rehydration, lamellarity and dimensions were basically unchanged, but the morphology was slightly different as they had instead a polyhedral (faceted) shape. The existence of these peculiar assembling is reported elsewhere and, despite its origin has not been fully clarified, it seems associated to the minimization of elastic bending energy that in turn leads to a more energetically favourable form than spherical bilayers [153,154]. Thus, these findings confirm the effectiveness of the selected cryoprotectants in preserving the integrity of vesicles while favouring their reconstitution in a more stable assembling. Additionally, since reconstitution was easily achieved manually by a few seconds of shaking, a spontaneous and fast reconstitution can also be expected in the biological fluids lining the respiratory tract [155].

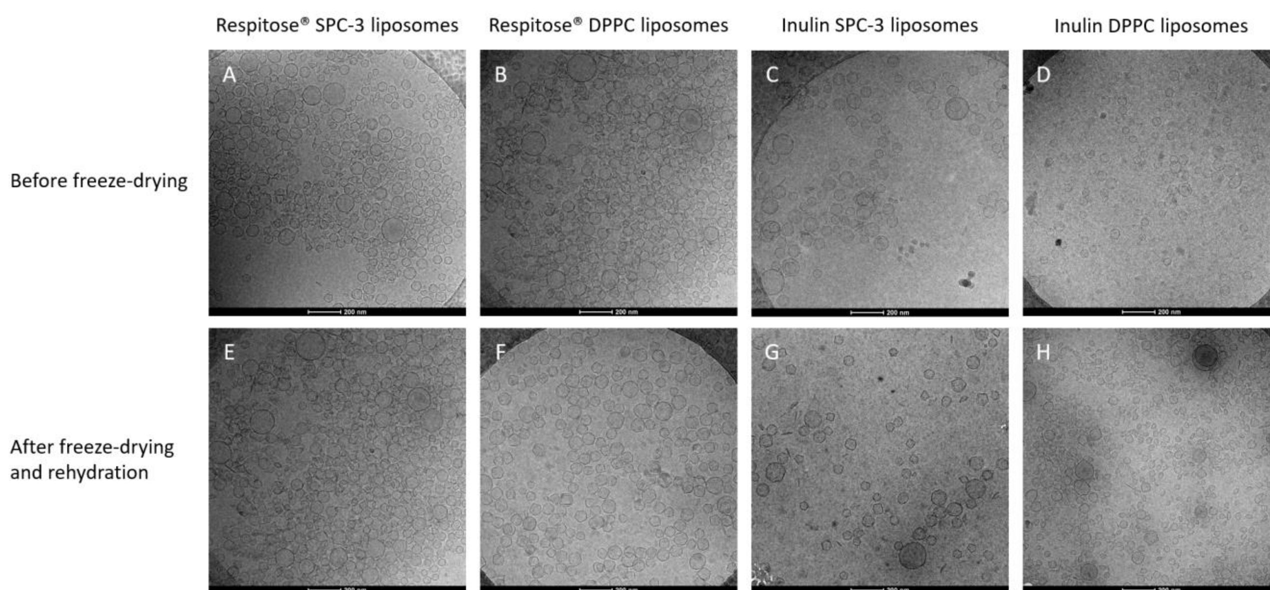


Figure 16. Representative cryogenic transmission electron microscopy images of SPC-3 and DPPC liposomes prepared with 45 mg of Respitose (1st and 2nd column) or inulin (3rd and 4th column), before freeze-drying (upper row) and after freeze-drying and rehydration (lower row).

Considering that mucus lining the respiratory mucosa is one of the main obstacles for inhaled formulations to reach the target tissues, the ability of ovalbumin in solution or encapsulated in liposomes to cross the mucus was evaluated using an artificial mucus model over 24 hours. A well-known mucolytic agent, BromAc®, was added to facilitate the ovalbumin permeation across the mucus, at two different doses (low and high) and results were compared with those obtained using liposomes alone (**Fig. 17**).

To allow for easier detection, labelled ovalbumin was used and at each time point the amount permeated in the receptor compartment was quantified. At 1, 2, 4 and 10 hours, the amount of ovalbumin permeated applying the solution was comparable to that found when it was encapsulated in liposomes as it was ≈ 0.5% ($p > 0.05$ among the values obtained using solution or liposomes) at 1 or 2 hours and ≈ 1% ($p > 0.05$ among the values obtained using solution or liposomes) at 4 or 10 hours. Only at 24 hours, the ovalbumin permeated through the mucus in the receptor compartment using the solution was lower (~3.8%, $p < 0.05$ versus values measured at other times) than that found using liposomes, irrespective to their composition (~5%, $p < 0.05$ versus value measured at 24 hours using

the solution). Despite being more effective than the ovalbumin solution, ovalbumin-encapsulated liposomes with no mucolytic agent had minimal capability of facilitating the passage of the payload through the mucus, being the amount recovered ~5% at 24 hours. The addition of BromAc® significantly increased mucus permeation of ovalbumin-encapsulated in liposomes in a concentration-dependent manner. Indeed, the same amount of labelled ovalbumin permeated that had been obtained with liposomes alone at 24 hours, was obtained, at only 2 hours, using the liposomes in association with the lower dose of BromAc® (2% N-acetyl cysteine and 100 µg/mL Bromelain). Using instead the higher dose of BromAc® (2% N-acetyl cysteine and 250 µg/mL Bromelain), the amount of ovalbumin penetrated in the mucus was significantly higher, as at 24 hours it was 7-fold higher than that found in mucus using liposomes alone. Therefore, BromAc® facilitated and accelerated mucus permeation of labelled ovalbumin, especially when it was delivered by liposomes.

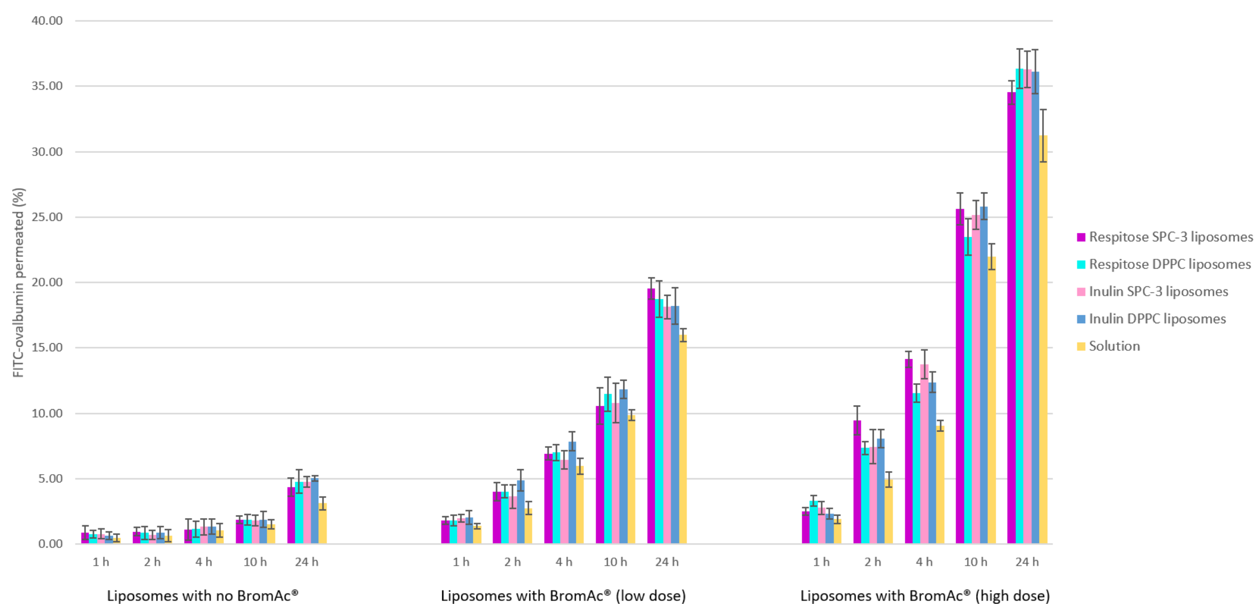


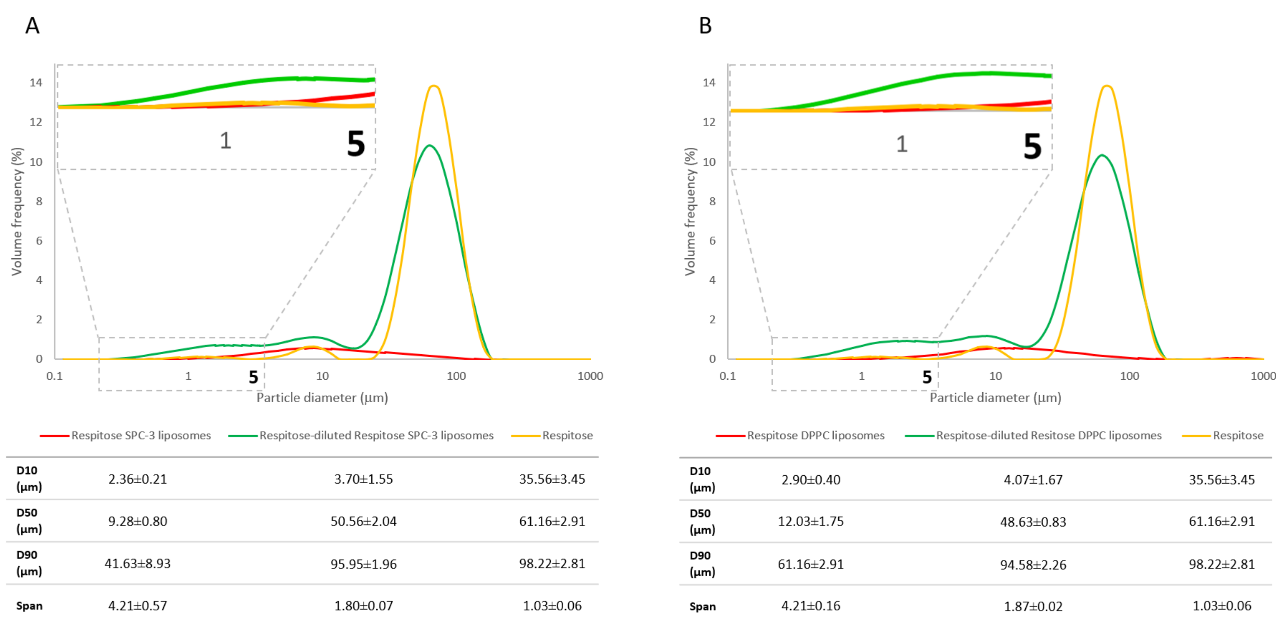
Figure 17. Labelled ovalbumin permeated (%) through the mucus at 1, 2, 4, 10 and 24 hours after its application in solution (yellow) or encapsulated in Respitose SPC-3 liposomes (violet), Respitose DPPC liposomes (clear blue), inulin SPC-3 liposomes (pink) and inulin DPPC liposomes (blue), with or without the low and high doses of BromAc®. Mean values ± standard deviations are reported.

4.3.2 Dry powders containing freeze-dried anionic liposomes characterization

Since the ovalbumin-encapsulated liposomes were supposed to be used as dry powders for inhalation, the physical diameter of the particles generated after the actuation of the Aerolizer® loaded with freeze-dried liposomes was measured under a flow of 100 L/min by laser diffraction. Size distribution, which is indicated by the coloured lines and represents the volume frequency of each particle size as well as specific percentiles of particles having a size lower than the measured value (D10, D50 and D90), and the span, an index of particle size distribution width, were reported (**Fig. 18**). Undiluted freeze-dried liposomes prepared with Respitose or inulin, freeze-dried liposomes prepared with Respitose or inulin and further diluted (1:10) with Respitose (Respitose-diluted liposomes) along

with Respitose alone were analysed to evaluate any effect of the inhalable-grade Respitose on the flowability of the resulting powders [156].

The particles generated after aerosolization of Respitose alone were highly homogeneous, as the span was ~1, with the 90% of the particle population lying < ~100 µm (D90 = 98.22±2.81 µm), the 50% < ~60 µm (D50 = 61.16±2.91 µm) and only the 10% < ~36 µm (D10 = 35.56±3.45 µm). The particles generated after aerosolization of undiluted freeze-dried liposomes were smaller, as the measured values were: D90 < ~60 µm, D50 < ~12 µm and D10 < ~3 µm regardless of the lipid or cryoprotectant used. The high values of measured span, ~3.11 and ~4.21, indicated the presence of multiple populations. The particles generated after aerosolization of freeze-dried liposomes, when diluted with Respitose, had a D90 ~94 µm, thus comparable to that of Respitose alone. This fraction of particles may indeed be ascribed to the diluent and not to the freeze-dried liposomes as a consequence of the magnitude of the performed dilution (1:10). Similarly, D50 increased from ~9-12 µm of undiluted freeze-dried liposomes to ~50 µm for the powders diluted with Respitose. D10 was ~2-4 µm, thus < 5 µm, which is the cut-off to achieve lung deposition [157]. Despite this, it must be highlighted that the dilution with Respitose increased, regardless of the formulation, the volume frequency of particles < 5 µm (**Fig. 18**), which can in turn increase the powder deposition in the lungs [158].

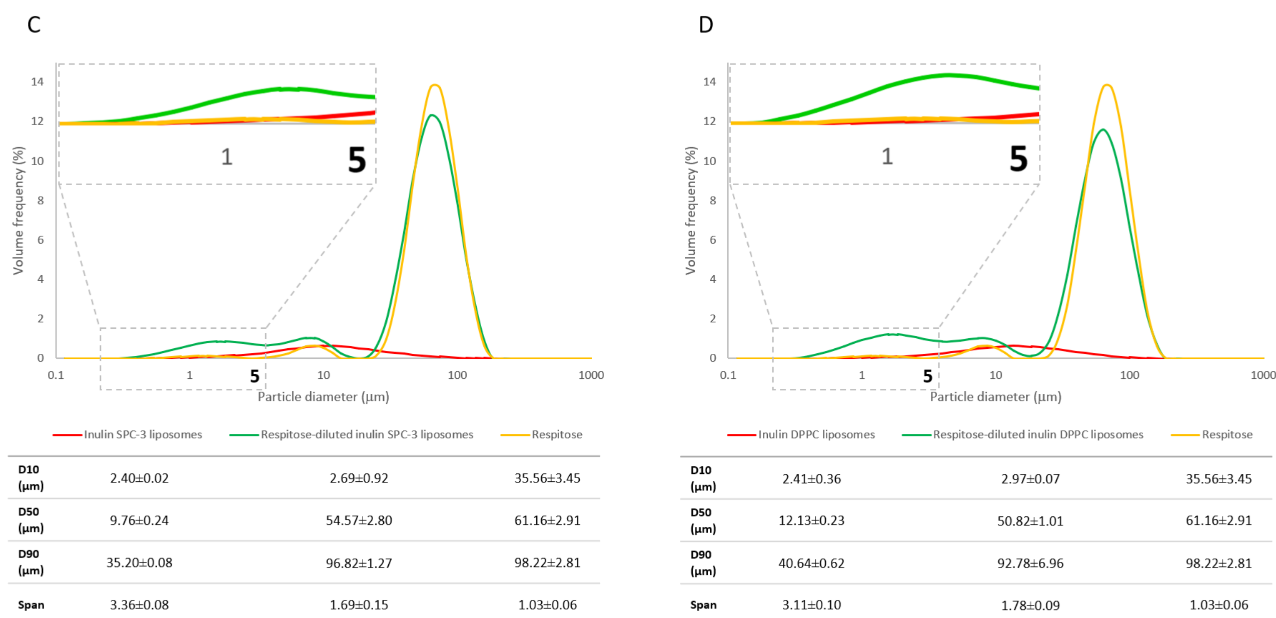


A

	Respitose SPC-3 liposomes	Respitose-diluted Respitose SPC-3 liposomes	Respitose
D10 (µm)	2.36±0.21	3.70±1.55	35.56±3.45
D50 (µm)	9.28±0.80	50.56±2.04	61.16±2.91
D90 (µm)	41.63±8.93	95.95±1.96	98.22±2.81
Span	4.21±0.57	1.80±0.07	1.03±0.06

B

	Respitose DPPC liposomes	Respitose-diluted Resitose DPPC liposomes	Respitose
D10 (µm)	2.90±0.40	4.07±1.67	35.56±3.45
D50 (µm)	12.03±1.75	48.63±0.83	61.16±2.91
D90 (µm)	61.16±2.91	94.58±2.26	98.22±2.81
Span	4.21±0.16	1.87±0.02	1.03±0.06



	Inulin SPC-3 liposomes	Respitose-diluted inulin SPC-3 liposomes	Respitose
D10 (μm)	2.40±0.02	2.69±0.92	35.56±3.45
D50 (μm)	9.76±0.24	54.57±2.80	61.16±2.91
D90 (μm)	35.20±0.08	96.82±1.27	98.22±2.81
Span	3.36±0.08	1.69±0.15	1.03±0.06

	Inulin DPPC liposomes	Respitose-diluted inulin DPPC liposomes	Respitose
D10 (μm)	2.41±0.36	2.97±0.07	35.56±3.45
D50 (μm)	12.13±0.23	50.82±1.01	61.16±2.91
D90 (μm)	40.64±0.62	92.78±6.96	98.22±2.81
Span	3.11±0.10	1.78±0.09	1.03±0.06

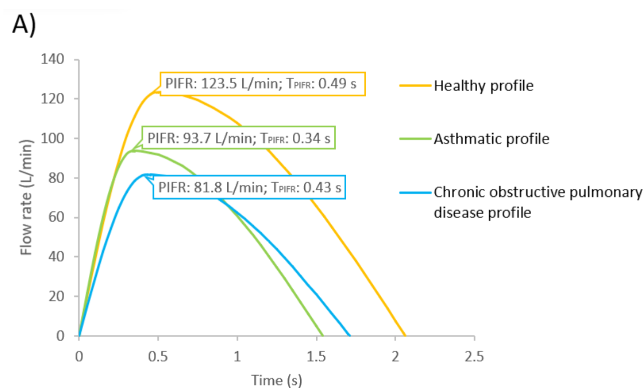
Figure 18. Particle size distribution of particles generated by the aerosolization of freeze-dried SPC-3 (A) and DPPC (B) liposomes prepared with Respitose, SPC-3 (C) and DPPC (D) liposomes prepared with inulin, undiluted (red line) or diluted 1:10 with Respitose (green line). Respitose alone (orange line) was used as control. The percentiles of undersized particles (D10, D50 and D90) along with the width of the particle size distribution (span) were reported (tables). Mean values ± standard deviations (n=3) are reported.

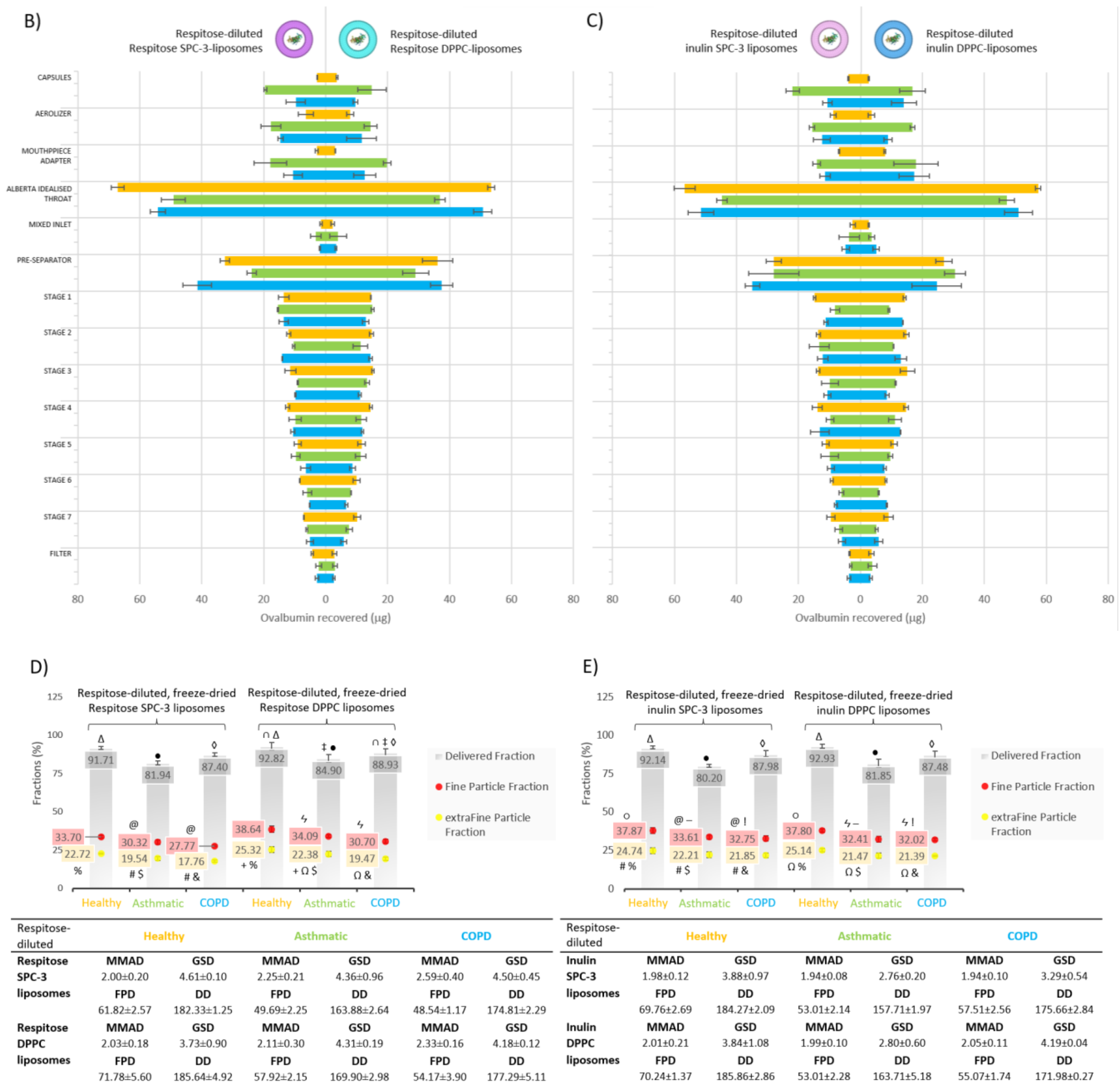
The performances of the dry powders obtained by the freeze-drying of liposomes were evaluated using the Next Generation Impactor simulating inhalation profiles of healthy, asthmatic and chronic obstructive pulmonary disease patients with the BRS 3000 breath simulator. The *in vitro-in vivo* correlation was improved using the Alberta idealised throat, which has an internal structure more anatomically accurate than the induction port. The parameters of inhalation profiles, the amount of ovalbumin deposited in the different stages of the impactor and the aerodynamic parameters were calculated (**Fig. 19**). The peak of inspiratory flow rate of healthy patients (123.51 L/min) was higher than those of asthmatic (93.70 L/min) or chronic obstructive pulmonary disease (> 81.80 L/min), implying a better ability of healthy patients to generate an appropriate flow though the device (Aerolizer®) (**Fig. 19A**). On the contrary, the time needed to reach this peak in simulated asthmatic (0.34 seconds) and chronic obstructive pulmonary disease (0.43 seconds) profiles was smaller, probably due to the smaller inhalation volumes proper of these pathological conditions, which in turn led also to reduced inhalation times (1.54 seconds and 1.71 seconds) in comparison with the healthy profile (2.06 seconds).

The deposition of ovalbumin was affected by the inhalation profiles in a similar manner for Respitose-diluted freeze-dried liposomes, regardless of the lipid and the use of Respitose (**Fig. 19B**) or inulin (**Fig. 19C**) as cryoprotectants.

A higher deposition was detected in capsules, device and mouthpiece adapter when the pathological conditions were tested with regard to the healthy one. The deposition in the pre-separator was ~40 μg,

except in the case of liposomes freeze-dried with inulin (~30 µg), irrespective to the profile and can be related to the particles with larger diameter that were not aerosolized. Differences in the stages 1-7, whose cut-off was instead smaller than pre-separator (6.14-0.24 µm), were detected in a profile-dependent manner. In particular, in stages 4-7, a higher deposition of ovalbumin was observed under healthy conditions compared to pathological conditions. As a consequence, the fine particle fractions and the extrafine particle fractions were affected, to a certain extent, by the profile studied (**Fig. 19D and E**). Specifically, fine particle fractions were higher when testing the healthy profile (~38%) with respect to the pathological profiles (~32%) for all the samples except Respitose SPC-3 liposomes (~33% and 28%, respectively, $p < 0.05$ with the values of the other formulations). Extrafine particle fractions instead were comparable in case of liposomes freeze-dried with inulin but not in case of those freeze-dried with Respitose. This can be due to the way the extrafine particle fraction is calculated, since it expresses the fraction of particles having a median mass aerodynamic diameter < 2 µm. Indeed, while the median mass aerodynamic diameters remained constant for inulin SPC-3 and DPPC liposomes (~2 µm, table in **Fig. 19E**), they became progressively bigger for Respitose SPC-3 and DPPC liposomes while testing the pathological conditions (from ~2 to ~2.6 µm, table in **Fig. 19D**). As a consequence, extrafine particle fraction was constant (~23%) across the profiles studied for inulin SPC-3 and DPPC liposomes, which in turn might be able to deliver the dose to the alveoli more consistently. Nonetheless, the aerodynamic diameter, always below 5 µm, allowed a Fine Particle Fraction > ~28% and an extraFine Particle Fraction > ~21% to be delivered through every profile tested by all the formulatios, so that they might be exploited to pursue immunisation even outside the alveolar region.





Respitose-diluted	Healthy		Asthmatic		COPD	
Respitose SPC-3 liposomes	MMAD	GSD	MMAD	GSD	MMAD	GSD
	2.00±0.20	4.61±0.10	2.25±0.21	4.36±0.96	2.59±0.40	4.50±0.45
	FPD	DD	FPD	DD	FPD	DD
	61.82±2.57	182.33±1.25	49.69±2.25	163.88±2.64	48.54±1.17	174.81±2.29
Respitose DPPC liposomes	MMAD	GSD	MMAD	GSD	MMAD	GSD
	2.03±0.18	3.73±0.90	2.11±0.30	4.31±0.19	2.33±0.16	4.18±0.12
	FPD	DD	FPD	DD	FPD	DD
	71.78±5.60	185.64±4.92	57.92±2.15	169.90±2.98	54.17±3.90	177.29±5.11

Respitose-diluted	Healthy		Asthmatic		COPD	
Inulin SPC-3 liposomes	MMAD	GSD	MMAD	GSD	MMAD	GSD
	1.98±0.12	3.88±0.97	1.94±0.08	2.76±0.20	1.94±0.10	3.29±0.54
	FPD	DD	FPD	DD	FPD	DD
	69.76±2.69	184.27±2.09	53.01±2.14	157.71±1.97	57.51±2.56	175.66±2.84
Inulin DPPC liposomes	MMAD	GSD	MMAD	GSD	MMAD	GSD
	2.01±0.21	3.84±1.08	1.99±0.10	2.80±0.60	2.05±0.11	4.19±0.04
	FPD	DD	FPD	DD	FPD	DD
	70.24±1.37	185.86±2.86	53.01±2.28	163.71±5.18	55.07±1.74	171.98±0.27

Figure 19. A) Peak inspiratory flow rate (PIFR) and time needed to reach it (T_{PIFR}) of inhalation profiles simulating of healthy (orange), asthmatic (green) and chronic obstructive pulmonary disease (blue) condition. **B-C)** Ovalbumin recovered after nebulisation, with the Aerolizer®, of the freeze-dried SPC-3 and DPPC liposomes prepared with Respitose or inulin. **D-E)** Values of median mass aerodynamic diameter (MMAD), geometric standard deviation (GSD), fine particle dose (FPD) and delivered dose (DD) of aerolized powders. The same symbol (Δ, ●, ◊, @, ○, ¬, !, ϟ, #, %, $, &, Ω, ‡, ∩, +) is used to indicate values not statistically different ($p > 0.05$).

4.3.3 Biological evaluation

The biocompatibility of ovalbumin in solution or encapsulated in the vesicles was evaluated directly on the antigen target (the macrophage cell line RAW 264.7). A comparison was performed diluting the samples at five different concentrations (0.48, 2.4, 12, 60 and 300 µg/mL) and incubating the cells for 24 hours (**Fig. 20**). All the formulations were highly biocompatible regardless of the dilution, as the

cell viability was always ≥ 100%. The viability measured after incubation of cells with ovalbumin in solutions was ≥ 100% as well, indicating that the encapsulation and liposome composition did not influence cell viability.

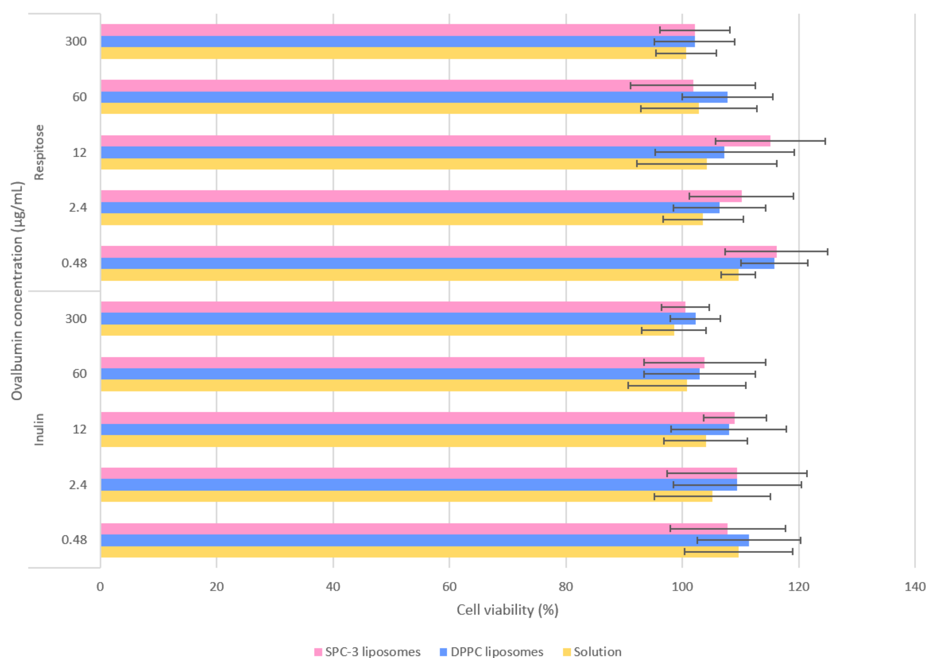


Figure 20. Viability of RAW 264.7 cells after exposure to ovalbumin (0.48-300 µg/mL) in solution mixed with Respitose (yellow, upper half) or inulin (yellow, lower half) or encapsulated in Respitose SPC-3 liposomes (pink, upper half), Respitose DPPC liposomes (blue, upper half), inulin SPC-3 liposomes (pink, lower half) or inulin DPPC liposomes (blue, lower half) for 24 hours.

Ovalbumin was taken up by macrophages regardless of the formulation. However, significant differences were found in terms of magnitude of the uptake (**Fig. 21**). The uptake of the solution was significantly lower for the solution (~0.04 arbitrary units) than the liposomes at both 0.5 and 24 hours. Similar values were detected for Respitose SPC-3 and DPPC liposomes as well as inulin SPC-3 liposomes (~0.08 arbitrary units, $p > 0.05$ between the formulations). The highest value was reached instead by inulin DPPC liposomes (~0.14 arbitrary units, $p < 0.05$ against the values obtained by the other liposomes or the solution). At 24 hours, Respitose SPC-3 and DPPC liposomes reached comparable values to those of inulin DPPC liposomes at 0.5 hours. Inulin SPC-3 and DPPC liposomes had the highest uptake at 24 hours (~0.23 arbitrary units, $p > 0.05$ between the formulations). By contrast, the uptake of FITC-ovalbumin in solution did not change overtime (~0.04 arbitrary units, $p < 0.05$ against the value at 0.5 hours), confirming the efficacy of these carriers in boosting the uptake of ovalbumin.

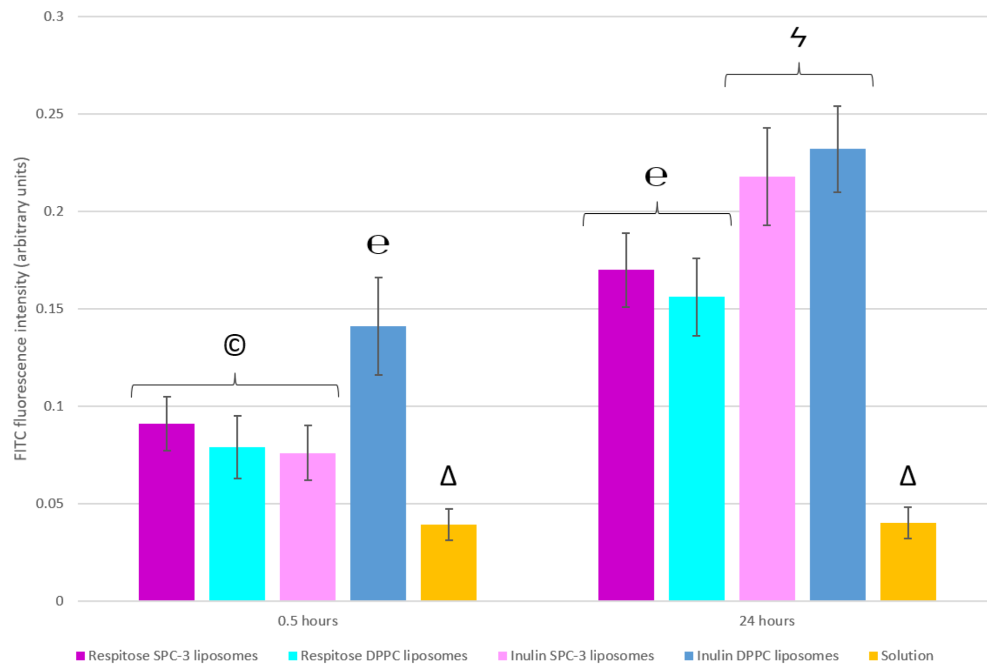
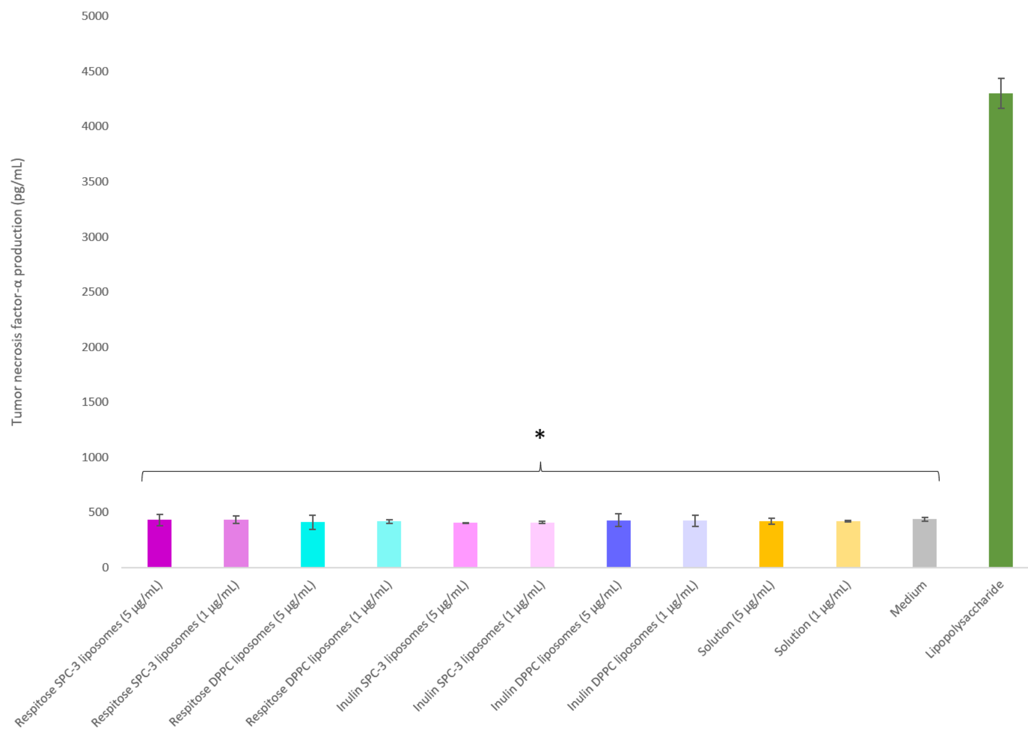


Figure 21. Fluorescence intensity detected in RAW 264.7 cells after 0.5 (left) or 24 hours (right) of exposure to FITC-ovalbumin in solution (yellow) or encapsulated in Respitose SPC-3 liposomes (violet), Respitose DPPC liposomes (clear blue), inulin SPC-3 liposomes (pink) and inulin DPPC liposomes (blue).

In order to assess the immunostimulatory effect of the formulations, RAW 264.7 cells were incubated for 24 hours with ovalbumin (1 and 5 µg/mL), in solution or encapsulated in liposomes (**Fig. 22**). The production of tumor necrosis factor-α by cells exposed to liposomes and ovalbumin solution, was ~ 500 pg/mL ($p < 0.05$ among the values), corresponding to the basal value measured in cells incubated with medium alone (**Fig. 22, A**), confirming that formulations did not cause any inflammatory effect. Differently, the production of interleukin-6, which mediate the immune response, was affected by the used formulation. Indeed, its expression in cells incubated with ovalbumin solution was ~ 50 pg/mL, a value slightly higher than that obtained incubating the cells with medium alone (basal value) ~45 pg/mL. When cells were incubated with liposomes prepared with inulin and DPPC liposomes prepared with Respitose, the amount of interleukin-6 expressed was ~ 50 pg/mL ($p > 0.05$ versus value obtained with ovalbumin solution), not statistically different than that expressed incubating the cells with ovalbumin solution. Only when ovalbumin was encapsulated in SPC-3 liposomes prepared with Respitose, at the two tested doses (1 and 5 µg/mL) the amount of interleukin-6 increased up to ~65 pg/mL ($p < 0.05$ versus others), underlining the improved effectiveness of these vesicles.

A



B

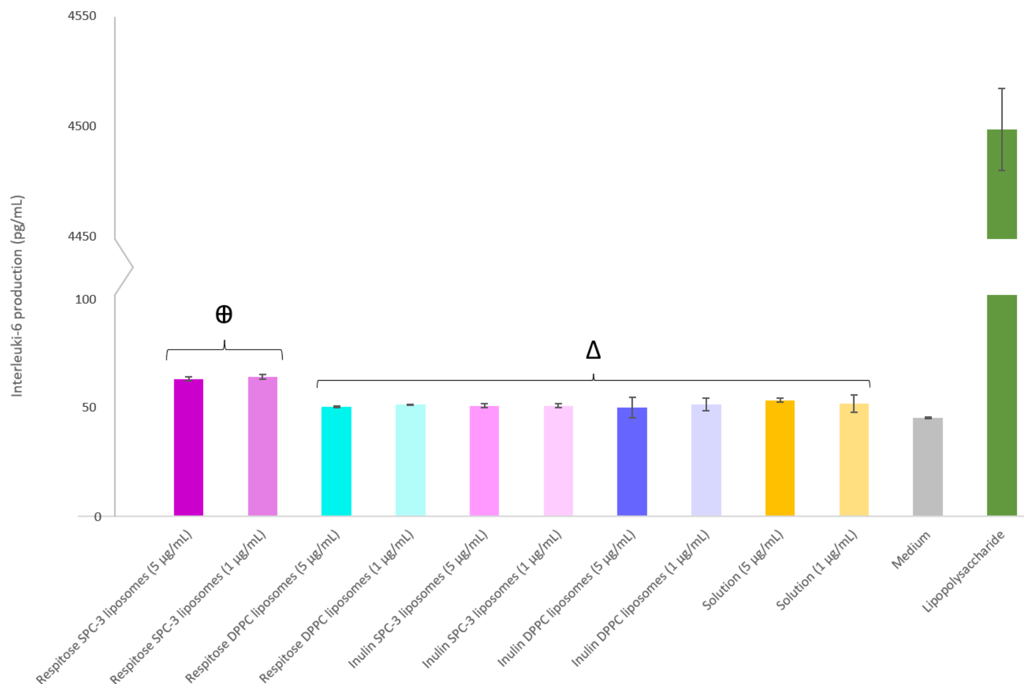


Figure 22. Production of tumor necrosis factor-α **(A)** and interleukin-6 **(B)** by RAW 264.7 cell after 24 hours of exposure to ovalbumin-encapsulated Respitose DPPC liposomes (clear blue), Respitose SPC-3 liposomes (violet), inulin DPPC liposomes (blue) and inulin SPC-3 liposomes (pink) or ovalbumin solution (yellow). Darker colours indicate the highest concentration of ovalbumin tested (5 µg/mL) whereas light colours indicate the lowest (1 µg/mL). Mean values ± standard deviations,

calculated from experimental replicates, are reported. Results are representative of two independent experiments. The same symbol (*, Δ, ⊕) is used to indicate values not statistically different ($p > 0.05$).

5. Discussion

5.1 Enriched transfersomes for the cutaneous delivery of ovalbumin

The aim of this first study was to develop and test advanced formulations tailored for antigen delivery in the skin combining formulative nanotechnology and physical strategies to overcome the serious hurdles related to the hypodermic injection of vaccines [91]. For this reason, transfersomes, which are ultra-deformable vesicles capable of carrying antigens and drugs across the skin and protecting them from environmental stress, were selected as carriers [92–94]. Lipoid S75, a commercial mixture of phosphatidylcholine, and sodium deoxycholate, a bile salt that acts as an edge-activating surfactant, were used in the preparation due to their skin penetration enhancing properties [95,96]. Furthermore, transfersomes were enriched with glycerol, sodium hyaluronate or their combination to develop glycerol-transfersomes, hyaluronan-transfersomes and glycerohyaluronan-transfersomes, as they are expected to be more effective than unenriched vesicles [97–100]. Ovalbumin was used as model antigen in high dose (5 mg/mL) and no organic solvents were involved during the preparation of vesicles, which was performed by direct sonication, an eco-friendly and one-step method currently used for drug-loaded phospholipid vesicles variously improved [101–103]. In this respect, this study substantially differs from those performed by other authors, that require instead solvent removal to prevent toxicity [104].

Vesicles prepared with this method were sized < 60 nm, thus suitable for skin delivery as a mean diameter < 100 nm is recommended to favour skin penetration [105,106]. Their high homogeneity (polydispersity index ~0.2) and negative superficial charge (~30 mV) ensured stability up to 9 months with no need for preservatives.

The ability of the enriched transfersomes to boost the antigen delivery in the skin was evaluated *in vitro*. Only the antigen retained in the skin was quantified as the carriers were meant to be used for local cutaneous immunization [107]. Upon non-occlusive application, the ovalbumin deposition in the epidermis and dermis was slightly improved with respect to the solution but only using hyaluronan-transfersomes and glycerohyaluronan-transfersomes. Using instead occlusive conditions (cling film), the delivery performances of enriched transfersomes were boosted, especially at 8 hours, and glycerohyaluronan-transfersomes proved to be the most effective carriers, having the best delivery capabilities. Results are in agreement with what reported by Ferderber and colleagues, who demonstrated that the amount of drug permeated and retained in the skin can be increased using phosphatidyl choline-based nanocarriers (including transfersomes) under occlusive conditions [108]. The improvement provided by the occlusive application of enriched transfersomes is probably related to the prevention of diffusional water loss from skin surface, which in turn can alter the stratum corneum function and synergize the moisturizer properties of glycerol and hyaluronic acid as well as the penetration enhancer properties of phosphatidylcholine and sodium deoxycholate, ultimately promoting skin penetration [109–112].

As the occlusive condition achieved by cling film was promising, polyvinyl alcohol hydrogel disks were exploited foreseeing an easier and faster application while keeping the advantages of occlusiveness. Therefore, ad hoc hydrogel disks were successfully prepared by freeze-thawing and freeze-drying cycles and loaded by imbibition with the ovalbumin-encapsulated enriched vesicles or in solution. Disks were imbibed as high as 22% of the ovalbumin left swelling (300 µL) irrespective of the formulation, thus reaching values commonly reported [113]. Despite the dose of ovalbumin applied using transfersomes imbibed disks was lower (~330 µg) than that used with transfersomes occlusively applied by cling film (500 µg) , it is important to highlight that yet at 4 hours disks imbibed with enriched transfersomes were as effective as occlusive conditions in favouring the delivery of ovalbumin and at 8 hours, they were even more effective, exalting the suitability of the combination of enriched transfersomes (especially those containing glycerol and hyaluronan together) and polyvinyl alcohol disks for antigen delivery and deposition in the epidermis and dermis. This effect may be related to the occlusive or semi-occlusive dressing created by the polyvinyl alcohol disks, which grant higher adherence to the skin thus synergizing the effect of the vesicle components [114–116]. Results are in fairly agreement with those obtained by other authors who exploited hydrogel formulations to promote the penetration of antigenic proteins into the skin or other medications [117–119].

To complete the *in vitro* evaluation of the prepared systems, the biocompatibility and immune response were assayed. Biocompatibility was tested using fibroblasts, as representative cells of the skin. According to previous studies, all the formulations, including ovalbumin solution, were highly biocompatible (cell viability > 100%) up to 50 µg/mL [120,121]. Considering that formulations were developed to be used for skin immunization, their ability to stimulate some of the most representative antigen-presenting cells resident in the skin (i.e. dendritic cells and macrophages) was evaluated *in vitro*. Interleukin-2 production was assessed as a measure of effective antigen presentation by ovalbumin-specific T cells in a model of response with dendritic cells while the production of tumor necrosis factor-α was assessed as a measure of inflammatory response mounted by macrophages. On the bright side, no inflammatory response was detected at any dose tested of ovalbumin in solution or encapsulated in enriched transfersomes, confirming the suitability of these carriers for a vaccine formulation. Unfortunately, no stimulation of the *in vitro* immune response was detected after incubation with ovalbumin, in solution or encapsulated in transfersomes, indicating that additional tests might be needed. By contrast, the prepared system, especially glycerohyaluronan-transfersomes imbibed disks seemed to be optimal carriers for skin delivery as the penetration studies have clearly disclosed their capability to modulate and boost the accumulation of ovalbumin. Consequently, an enhanced activation of the dendritic cells might be expected *in vivo* due to a facilitated exposure to the antigen. Furthermore, as stated by Wang and colleagues, vesicles might be further enriched with molecules having intrinsic or specific adjuvanticity, such as squalene, saponins or toll like receptor agonists (e.g., MPLA and CpG ODN), to boost or modulate immune responses providing the same phospholipid vesicles with adjuvanticity [122].

5.2 Anionic and cationic liposomes for the nasal delivery of ovalbumin

In this second study, with the aim of formulating liposomes tailored to be easily administered intranasally to stimulate immunity at nasal level and block the pathogens at the entry site, the model antigen ovalbumin was encapsulated in two kinds of phospholipid vesicles, having negative (liposomes) and positive (DOTAP-liposomes) superficial charge, respectively.

Primary importance was devoted to the selection of vesicle composition, which strongly affects vesicle performances and surface charge ultimately playing a key role in mucus adhesion, particle retainment in situ and cellular uptake by immune system's antigen presenting cells. Thus, a commercial mixture of phosphatidylcholines (Phospholipon® 90G) was selected and used as a main lipidic component by virtue of their uptake-enhancing properties, whereas dioleoyl-3-trimethylammonium propane, a cationic phospholipid featuring an unsaturated fatty acid, was chosen to both impart positive charge to the vesicles and induce the immune response [95,124]. Currently, cationic liposomes have gained attention as adjuvants for vaccine delivery as they are capable of boosting antigen delivery and promote antigenic protein-uptake by antigen presenting cells [125,126]. Although the mechanism of their adjuvanticity is not fully understood, what is well known is the ability of 1,2-dioleoyl-3-trimethylammonium-propane to promote cell surface contact by electrostatic interaction, foster uptake and antigen presentation by dendritic cells and even increase the humoral response [127]. During the preparation of the ovalbumin-encapsulated liposomes, cholesterol was also added to Phospholipon® 90G and 1,2-dioleoyl-3-trimethylammonium-propane because of its membrane-stabilising effects [128]. By contrast, positively charged cholesterol, which has also been used for the production of vaccines, was not considered for this study because of its higher cost and its toxicity in combination with 1,2-dioleoyl-3-trimethylammonium-propane, as reported by several authors [129–132].

Irrespective of the formulation, direct sonication was used as organic solvent-free and eco-friendly method to prepare the phospholipid vesicles [101]. The chosen method was consistent, allowing to obtain homogeneous systems (polydispersity index < 0.3) with high encapsulation efficiencies (> 80%) as much as other techniques such as thin film evaporation, ethanol injection and microfluidics, which however rely on organic solvents to dissolve lipids and are therefore not environmentally safe and require several dissipative preparation steps [104].

As most of the vaccines nowadays struggle in ensuring a palatable route of administration, that is painless and non-invasive, the nasal route was then elected to administer ovalbumin-encapsulated liposomes to the nasal cavity and elicit a response in situ. This is currently regarded as a promising preventive strategy, especially against those disease affecting the respiratory system, which is often exposed to airborne pathogens [133]. To assess the nasal administration performance, both formulations were loaded in a commercial spray device (Nasonex®) and the droplet distribution was evaluated in accordance with European Medicines Agency and Food and Drug Administration recommendations [134,135]. The obtained results indicated that both formulations, once sprayed, generated droplets bigger than 10 mm, with up to the 90% of the droplets lying in a dimensional range of 80-90 mm. Being 5 mm the required aerodynamic diameter to ensure lung deposition, the data collected highlighted that a high percentage of droplets was deposited nasally with extreme precision,

avoiding any pulmonary deposition [123]. The characterisation of the specific deposition site was further carried out with the Alberta Idealised Nasal Inlet, whose geometry allows the assessment of deposited materials in four anatomical regions (vestibule, turbinates, olfactory region and nasopharynx) with high correlation *in vitro-in vivo* [136]. According to the results of a previous study performed by Chen and colleagues, the obtained data of droplet distribution indicated that the droplets generated by the device deposited in the nose but not in the lungs [136]. Specifically, the percentage of anionic liposomes and cationic DOTAP-liposomes detected in the vestibule was comparable (~45%) whereas that of cationic DOTAP-liposomes in the turbinates was higher (~52%). In a vaccine context, the deposition in the posterior nasal cavity is recognised as the site where the immune response is mainly produced [137,138]. Accordingly, Xu and colleagues achieved better efficacy *in vivo* for their vaccine candidate when it had demonstrated better tendency to deposit at the turbinates region during the *in vitro* studies on a nasal replica [139]. In this regard, the cationic DOTAP-liposomes are therefore expected to be more suitable than the anionic ones. As proved by the muchoadhesiveness test, the positive surface charge ensured a significantly better mucin adsorption than anionic liposomes (88% vs ~8%) and this is likely the reason why their deposition in the posterior nasal cavity was higher [140,141].

However, as deposition alone is not sufficient to ensure a proper immune response, the capability of cationic DOTAP-liposomes and anionic liposomes to deliver the antigen to the antigen-presenting cells, whose main activity is to process the antigen and present it to the lymphocytes involved in the immune response, was tested [142]. Bone marrow-derived dendritic cells were exposed to two different concentrations of ovalbumin (5 and 1 μg/mL), either in solution or encapsulated in phospholipid vesicles, and their activation was evaluated in terms of interleukin-2 production by the co-cultured T cells. Both formulations provided a significant boost in the interleukin-2 production compared to the ovalbumin solution, thus demonstrating their ability to be effectively sensed and engulfed by the antigen-presenting cells. Among the formulations, cationic DOTAP-liposomes provided the highest production of interleukin-2, in a concentration-independent manner. The differences in the efficiency of the response were probably mediated by the chemical and physical proprieties of the vesicles. Indeed, as reported elsewhere, positively charged liposomes display greater adjuvanticity than negatively charged or neutral liposomes due to their better ability to interact with the negatively charged membranes of antigen presenting cells [143,144].

Since the introduction of foreign material in the organism may be sensed by the innate immune system as a harmful stimulus, the capacity of human macrophages to trigger an inflammatory response upon exposure to the phospholipid vesicles was finally tested [145]. Macrophages were thus exposed to either cationic DOTAP-liposomes or anionic liposomes and the inflammatory cytokine tumor necrosis factor-α production was assessed after 24 hours. Although the measure of one inflammatory cytokine is not exhaustive, the lack of response at any concentration tested using ovalbumin solution or ovalbumin-encapsulated liposomes suggests that the carriers themselves have no capability of exerting proinflammatory effects following their administration and are perceived as safe by the immune system [146–148].

5.3 Freeze-dried anionic liposomes for the pulmonary delivery of ovalbumin

The main objective of this study was to formulate and freeze-dry ovalbumin-encapsulated liposomes to obtain suitable powders for lung delivery through the low-resistance device Aerolizer®. Liposomes were chosen as carriers thanks to their versatility and capability of carrying, protecting and delivering the model antigen ovalbumin. In the manufacturing process, 4 different phospholipids commonly employed in lung delivery (P90H, SPC3, DSPC and DPPC) were used along with cholesterol. Among them, P90H and SPC-3 are natural mixtures of lipids that differ in the content of phosphatidylcholine (90 and 98%, respectively) whereas DPPC and DSPC are synthetic lipids with different transition temperature (41 °C and 54 °C, respectively) [159–161]. DPPC is also the main component of the pulmonary surfactant and it had already been successfully used to develop a pulmonary surfactant-biomimetic vaccine against influenza virus [162,163]. To facilitate the freeze-drying of ovalbumin-encapsulated liposomes while preventing structural breakage, three different cryoprotectants (trehalose, Respitose or inulin) were added to the dispersions at three different lipid-to-cryoprotectant ratios (1:1, 1:2 and 1:3). According to the results obtained by Samad et al., the re-dispersibility index ≤ 1.3 was used as key value to select the most suitable vesicles, which did not break during drying and, when rehydrated, spontaneously reformed retaining their main physico-chemical properties (i.e. mean diameter) [149]. The spontaneous reformation of vesicles was affected by the cryoprotectant, so that only 20 formulations out of 40 complied with these values. Interestingly, also the lipid influenced the freeze-drying process. After rehydration, DPPC and SPC-3 allowed to better retain the original size of the vesicles (measured before freeze-drying) than DSPC and P90H, as indicated by their lower re-dispersibility index (1.1). This value was an effective, time-saving and reliable tool for freeze-drying assessment and permitted to select SPC-3 and DPPC liposomes prepared with 45 mg of trehalose, Respitose and inulin as the more suitable formulations to prepare dry powders. However, liposomes prepared with trehalose were discarded due to the high cost of this disaccharides in comparison with that of the other disaccharide (Respitose) and the oligosaccharide (inulin). Thus, only 4 formulations were selected for further studies [151,164]. By combining the results from the dimensional analysis and the morphological characterisation, shape, effective formation or reconstitution after rehydration and ovalbumin encapsulation were confirmed, thus highlighting the suitability of these systems to be exploited as carriers. Additionally, cryogenic transmission electron microscopy analysis disclosed that, after freeze-drying and rehydration, polyhedral faceted vesicles were formed as energetically favoured systems compared to spherical bilayer vesicles [154].

As these vesicles were designed to be administered to the lungs, their ability to promote the penetration of mucus by ovalbumin was evaluated and compared with that of the ovalbumin solution. Results were in agreement with those of Ingarvarsson and colleagues, which found that the permeation of the free ovalbumin across the mucus was lower with respect to that obtained upon its encapsulation into liposomes [165]. Accordingly, other authors have reported lower diffusion ratios for unencapsulated drugs than the correspondent drug-loaded liposomes [166]. The enhanced passage of the payloads through the mucus provided by liposomes is related to their physico-chemical properties that, if properly tuned, allow to avoid steric obstruction and association with mucins [167]. However, since the permeation (~5% at 24 hours) was not considered enough to ensure

sufficient antigen delivery to the macrophages, liposomes were subsequently applied in combination with BromAc®, a novel, patented mucolytic agent. Two different concentrations of BromAc® (low: 2% N-acetyl cysteine + 100 μg/mL of bromelain; high: 2% N-acetyl cysteine + 250 μg/mL of bromelain), successfully used to treat muco-obstructive respiratory diseases, were tested in this study [168–170]. The addition of BromAc® boosted the permeation of ovalbumin, in solution or encapsulated in liposomes, through mucus. This effect was particularly pronounced at the highest dose of BromAc®, probably due to the synergistic action of the mucolytic components in breaking glycosidic linkages and disulphide bonds [168,169]. However, at each time point, the mucus penetration in presence of BromAc® was significantly higher for the ovalbumin-encapsulated liposomes than the ovalbumin solution (i.e. ~36% and ~31% after 24 hours, respectively). Some authors reported that ovalbumin and N-acetyl cysteine can form complexes, undergoing to conformational changes in their structure and rearrangements of the hydrogen bonds, which likely affect the ability of ovalbumin to cross the mucus [171]. By contrast, encapsulation limits complexation and structure modifications thus favouring the antigen permeation through mucus.

The dry powders obtained from ovalbumin-encapsulated inulin SPC-3 and DPPC liposomes and Respitose SPC-3 and DPPC liposomes were diluted with Respitose (1:10), before being sprayed with the Aerolizer®, to ensure an appropriate filling of the capsules and thus regulate the dose of ovalbumin. The particle size analysis of the resultant powders was carried out by means of Spraytec®, confirming the anti-adhesive effect of Respitose on the freeze-dried antigen-encapsulated liposomal powders (whose volume distribution shifted indeed towards the 5 μm region) [172].

The Next Generation Impactor was used to deeply evaluate the aerodynamic performances of the particles and was coupled with the Alberta idealised throat and BRS 3000 breath simulator, which provide better match to the *in vivo* behaviour than the usual Next Generation Impactor setting [173]. Thanks to the breath simulator, the profiles of healthy, asthmatic and chronic obstructive pulmonary disease were set up, as differences in peak inspirator flow rate, time to reach the peak inspirator flow rate and inhalation volume can influence the deposition of the particles [174]. According to this, all the four ovalbumin-encapsulated liposomes generated higher deposition in capsule, dry powder inhaler and mouthpiece adapter when the pathological profiles were tested. This can probably be related to the lower peak inspiratory flow rate compared to the healthy profile, which is probably insufficient to allow particles to reach the lungs. A similar behaviour was reported by Saha et al. using a realistic mouth-throat model [175]. On the other hand, an overall higher deposition was detected in the Alberta Idealised Throat when the healthy profile was studied, probably as a consequence of the magnitude of the impaction that has been reported to be bigger when the flow rate increases [176]. By the combination of the previous phenomena (lower deposition in capsule, dry powder inhaler and mouthpiece adapter and higher deposition in the Alberta Idealised Throat), the healthy profile allowed also to achieve higher deposition in the stages of the impactor, which represent the lungs, thus providing higher fine particle fraction and fine particle dose than the pathological profiles. Such behaviour is known to be a consequence of the higher inhalation volume and flow rates generated by the healthy profiles with respect to the pathological ones and was also recently confirmed by Ahookhosh and co-workers in a realistic respiratory airway replica [177–179]. Despite the different depositions, the prepared formulations were able to generate particles with an aerodynamic diameter

< 5 μm, thus suitable for lung delivery, irrespective of the tested profile. Only using Respitose SPC-3 or DPPC liposomes, values of mass median aerodynamic diameter grew when switching from healthy to pathologic condition and the extra fine particle fraction significantly decreased ($p < 0.05$). Being extra fine particle fraction related to alveolar delivery, this may result in a reduction of antigen availability in the districts where alveolar macrophages reside, thus leading to therapeutic failure [180]. On the other hand, aerodynamic diameter of inulin SPC-3/DPPC liposomes did not change, possibly because of the presence of higher cohesive forces in the particles freeze-dried with Respitose than with inulin. As a consequence, extrafine particle fraction remained constant irrespective to the condition tested, thus providing reliability on the delivery capacity of these systems. To date, since this is an essential metric to evaluate the delivery of formulations in the small airways, awareness has raised on its importance in clinical practice. Consequently, several efforts have been made by many authors to optimize this parameter [90,181,182].

By comparing the data obtained from the Next Generation Impactor and Spraytec®, it can be noticed that Spraytec® revealed slightly bigger particles, probably because the Spraytec® setting involved the use of the United States Pharmacopeia induction port instead of the Alberta Idealised Throat. Some studies have already demonstrated that Alberta Idealised Throat, due to the more anatomically accurate structure, tend to retain more particles than the induction port [183]. As a result, it is reasonable to think that larger particles are stopped by the branches of Alberta Idealised Throat, in contrast to the Spraytec®'s United States Pharmacopeia induction port. In addition, the Alberta Idealised Throat is usually coated whereas the induction port cannot be coated, thus influencing even more particle deposition and changes in results.

As last step, all the ovalbumin-encapsulated liposomes were tested *in vitro* on alveolar macrophages (RAW 264.7), along with the ovalbumin solution, foreseeing a primary targeting to this lung resident cellular line. Both the ovalbumin solution and the liposomes were biocompatible over a wide range of ovalbumin concentrations (0.48-300 μg/mL), in agreement with previous studies in which ovalbumin was loaded in liposomes with a similar composition (phosphatidylcholine and cholesterol) and was tested in similar concentrations [120,184]. Flow cytometry and fluorescence microscopy were used to assess the uptake of ovalbumin, in solution or encapsulated in liposomes, by RAW 264.7 cells. Comparable percentages of cells were able to uptake ovalbumin regardless of the treatment or the time point [185]. However, fluorescence microscopy revealed significant, time-related differences among the formulations. Particularly, the ovalbumin-encapsulated liposomes were taken up by macrophages in a greater extent than ovalbumin in solution at both 0.5 and 24 hours. The highest uptake was achieved by inulin SPC-3 and DPPC liposomes at 24 hours, followed by Respitose SPC-3 and DPPC liposomes. When used as solution, the percentage of ovalbumin taken up was similar at 0.5 and 24 hours, confirming the efficacy of the liposomes in favouring the uptake of the antigen by macrophages. A similar result was achieved by Korsholm and colleagues [186].

Lastly, the ability of formulations to stimulate a response on RAW 264.7 cells was evaluated using the ovalbumin solution as reference. Since the volume of fluid in the lungs have been reported to be in the range of 10-35 mL, ovalbumin, in solution or encapsulated, was tested at the low concentrations, 5 and 1 μg/mL, considering the dilution that formulations may undergo after administration [187–189].

No production of tumor necrosis factor-α was detected after the incubation of macrophages with ovalbumin in solution or encapsulated in liposomes at any dose tested. Tumor necrosis factor-α stimulates the acute phase of the immune response but it is also an inducer of the inflammatory response, so that all the formulations were perceived as safe by the RAW 264.7 cells [190]. The production of interleukin-6 was improved, with respect to the ovalbumin solution, only when ovalbumin was encapsulated in SPC-3 liposomes prepared with Respitose. The absence of a comparable response by the ovalbumin-encapsulated Respitose DPPC liposomes might be related to the capability of DPPC to act as inhibitor of cytokine release from innate immune cells [191]. Similarly, the lack of adjuvanticity by inulin SPC 3 or DPPC liposomes might be due to the ability of inulin in reducing the expression of interleukin-6 mRNA in these cells [192,193]. Similar results involving the production of interleukin-6 and no expression of tumor necrosis factor-α were reported in a previous study by Ahmed et al., which also used liposomes [194].

6. Bibliography

[1] B. Greenwood, The contribution of vaccination to global health: Past, present and future, Philosophical Transactions of the Royal Society B: Biological Sciences 369 (2014). https://doi.org/10.1098/rstb.2013.0433.

[2] C. Mantel, T. Cherian, New immunization strategies: adapting to global challenges, Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz 63 (2020). https://doi.org/10.1007/s00103-019-03066-x.

[3] K.U. Jansen, C. Knirsch, A.S. Anderson, The role of vaccines in preventing bacterial antimicrobial resistance, Nat Med 24 (2018). https://doi.org/10.1038/nm.4465.

[4] WHO, WHO and UNICEF warn of a decline in vaccinations during COVID-19, Who 41 (2020).

[5] N.E. MacDonald, J. Eskola, X. Liang, M. Chaudhuri, E. Dube, B. Gellin, S. Goldstein, H. Larson, M.L. Manzo, A. Reingold, K. Tshering, Y. Zhou, P. Duclos, S. Guirguis, B. Hickler, M. Schuster, Vaccine hesitancy: Definition, scope and determinants, Vaccine 33 (2015). https://doi.org/10.1016/j.vaccine.2015.04.036.

[6] E. Dubé, M. Vivion, N.E. MacDonald, Vaccine hesitancy, vaccine refusal and the anti-vaccine movement: Influence, impact and implications, Expert Rev Vaccines 14 (2014). https://doi.org/10.1586/14760584.2015.964212.

[7] A. Taddio, C.M. McMurtry, C. Logeman, V. Gudzak, A. de Boer, K. Constantin, S. Lee, R. Moline, E. Uleryk, T. Chera, N.E. MacDonald, B. Pham, Prevalence of pain and fear as barriers to vaccination in children – Systematic review and meta-analysis, Vaccine 40 (2022). https://doi.org/10.1016/j.vaccine.2022.10.026.

[8] Y. Nir, A. Paz, E. Sabo, I. Potasman, Fear of injections in young adults: Prevalence and associations, American Journal of Tropical Medicine and Hygiene 68 (2003). https://doi.org/10.4269/ajtmh.2003.68.341.

[9] J.O. Morales, K.R. Fathe, A. Brunaugh, S. Ferrati, S. Li, M. Montenegro-Nicolini, Z. Mousavikhamene, J.T. McConville, M.R. Prausnitz, H.D.C. Smyth, Challenges and Future Prospects for the Delivery of Biologics: Oral Mucosal, Pulmonary, and Transdermal Routes, AAPS Journal 19 (2017). https://doi.org/10.1208/s12248-017-0054-z.

[10] J.W. Coffey, G. Das Gaiha, G. Traverso, Oral Biologic Delivery: Advances Toward Oral Subunit, DNA, and mRNA Vaccines and the Potential for Mass Vaccination during Pandemics, Annu Rev Pharmacol Toxicol 61 (2021). https://doi.org/10.1146/annurev-pharmtox-030320-092348.

[11] J.E. Vela Ramirez, L.A. Sharpe, N.A. Peppas, Current state and challenges in developing oral vaccines, Adv Drug Deliv Rev 114 (2017). https://doi.org/10.1016/j.addr.2017.04.008.

[12] V. Trincado, R.P. Gala, J.O. Morales, Buccal and sublingual vaccines: A review on oral mucosal immunization and delivery systems, Vaccines (Basel) 9 (2021). https://doi.org/10.3390/VACCINES9101177.

[13] J.A. Omlor, J. Nguyen, R. Bals, Q.T. Dinh, Nanotechnology in respiratory medicine, Respir Res 16 (2015) 1–9. https://doi.org/10.1186/s12931-015-0223-5.

[14] M. Hellfritzsch, R. Scherließ, Mucosal vaccination via the respiratory tract, Pharmaceutics 11 (2019). https://doi.org/10.3390/pharmaceutics11080375.

[15] D.R. De Araújo, D.C. Da Silva, R.M. Barbosa, M. Franz-Montan, C.M. Cereda, C. Padula, P. Santi, E. De Paula, Strategies for delivering local anesthetics to the skin: Focus on liposomes, solid lipid nanoparticles, hydrogels and patches, Expert Opin Drug Deliv 10 (2013). https://doi.org/10.1517/17425247.2013.828031.

[16] L. Engelke, G. Winter, S. Hook, J. Engert, Recent insights into cutaneous immunization: How to vaccinate via the skin, Vaccine 33 (2015). https://doi.org/10.1016/j.vaccine.2015.05.012.

[17] A. V. Nguyen, A.M. Soulika, The dynamics of the skin's immune system, Int J Mol Sci 20 (2019) 1–53. https://doi.org/10.3390/ijms20081811.

[18] C. Handfield, J. Kwock, A.S. MacLeod, Innate Antiviral Immunity in the Skin, Trends Immunol 39 (2018) 328–340. https://doi.org/10.1016/j.it.2018.02.003.

[19] F.O. Nestle, P. Di Meglio, J.Z. Qin, B.J. Nickoloff, Skin immune sentinels in health and disease, Nat Rev Immunol 9 (2009) 679–691. https://doi.org/10.1038/nri2622.

[20] M. Kaurav, J. Madan, M.S. Sudheesh, R.S. Pandey, Combined adjuvant-delivery system for new generation vaccine antigens: alliance has its own advantage, Artif Cells Nanomed Biotechnol 46 (2018) S818–S831. https://doi.org/10.1080/21691401.2018.1513941.

[21] P. Riese, K. Schulze, T. Ebensen, B. Prochnow, C. Guzmán, Vaccine Adjuvants: Key Tools for Innovative Vaccine Design, Curr Top Med Chem 13 (2013) 2562–2580. https://doi.org/10.2174/15680266113136660183.

[22] G. Del Giudice, R. Rappuoli, A.M. Didierlaurent, Correlates of adjuvanticity: A review on adjuvants in licensed vaccines, Semin Immunol 39 (2018) 14–21. https://doi.org/10.1016/j.smim.2018.05.001.

[23] L.E. Guimarães, B. Baker, C. Perricone, Y. Shoenfeld, Vaccines, adjuvants and autoimmunity, Pharmacol Res 100 (2015) 190–209. https://doi.org/10.1016/j.phrs.2015.08.003.

[24] S. Bashiri, P. Koirala, I. Toth, M. Skwarczynski, Carbohydrate immune adjuvants in subunit vaccines, Pharmaceutics 12 (2020). https://doi.org/10.3390/pharmaceutics12100965.

[25] S. Chattopadhyay, J.Y. Chen, H.W. Chen, C.M. Jack Hu, Nanoparticle vaccines adopting virus-like features for enhanced immune potentiation, Nanotheranostics 1 (2017) 244–260. https://doi.org/10.7150/ntno.19796.

[26] B. Fadeel, Hide and seek: Nanomaterial interactions with the immune system, Front Immunol 10 (2019) 1–10. https://doi.org/10.3389/fimmu.2019.00133.

[27] S. Mishra, Nanotechnology in medicine, Indian Heart J 68 (2016) 437–439. https://doi.org/10.1016/j.ihj.2016.05.003.

[28] S. Bayda, M. Adeel, T. Tuccinardi, M. Cordani, F. Rizzolio, The history of nanoscience and nanotechnology: From chemical-physical applications to nanomedicine, Molecules 25 (2020) 1–15. https://doi.org/10.3390/molecules25010112.

[29] A. Facciolà, G. Visalli, P. Laganà, V. La Fauci, R. Squeri, G.F. Pellicanò, G. Nunnari, M. Trovato, A. Di Pietro, The new era of vaccines: The "nanovaccinology," Eur Rev Med Pharmacol Sci 23 (2019) 7163–7182. https://doi.org/10.26355/eurrev_201908_18763.

[30] M. Aroffu, M.L. Manca, J.L. Pedraz, M. Manconi, Liposome-based vaccines for minimally or noninvasive administration: an update on current advancements, Expert Opin Drug Deliv 20 (2023) 1573–1593. https://doi.org/10.1080/17425247.2023.2288856.

[31] M. Manconi, C. Caddeo, M.L. Manca, A.M. Fadda, Oral delivery of natural compounds by phospholipid vesicles, Nanomedicine 15 (2020). https://doi.org/10.2217/nnm-2020-0085.

[32] M. Li, C. Du, N. Guo, Y. Teng, X. Meng, H. Sun, S. Li, P. Yu, H. Galons, Composition design and medical application of liposomes, Eur J Med Chem 164 (2019) 640–653. https://doi.org/10.1016/j.ejmech.2019.01.007.

[33] K.S. Ahmed, S.A. Hussein, A.H. Ali, S.A. Korma, Q. Lipeng, C. Jinghua, Liposome: composition, characterisation, preparation, and recent innovation in clinical applications, J Drug Target 27 (2019). https://doi.org/10.1080/1061186X.2018.1527337.

[34] J. Liu, J. Wu, B. Wang, S. Zeng, F. Qi, C. Lu, Y. Kimura, B. Liu, Oral vaccination with a liposome-encapsulated influenza DNA vaccine protects mice against respiratory challenge infection, J Med Virol 86 (2014). https://doi.org/10.1002/jmv.23768.

[35] H. Harde, A.K. Agrawal, S. Jain, Tetanus toxoid-loaded layer-by-layer nanoassemblies for efficient systemic, mucosal, and cellular immunostimulatory response following oral administration, Drug Deliv Transl Res 5 (2015). https://doi.org/10.1007/s13346-015-0247-x.

[36] J.S. Wilkhu, S.E. McNeil, D.E. Anderson, Y. Perrie, Characterization and optimization of bilosomes for oral vaccine delivery, J Drug Target 21 (2013). https://doi.org/10.3109/1061186X.2012.747528.

[37] S. Jain, A. Indulkar, H. Harde, A.K. Agrawal, Oral mucosal immunization using glucomannosylated bilosomes, J Biomed Nanotechnol 10 (2014). https://doi.org/10.1166/jbn.2014.1800.

[38] N. Wang, T. Wang, M. Zhang, R. Chen, R. Niu, Y. Deng, 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 88 (2014). https://doi.org/10.1016/j.ejpb.2014.04.007.

[39] Y. Zhen, N. Wang, Z. Gao, X. Ma, B. Wei, Y. Deng, T. Wang, Multifunctional liposomes constituting microneedles induced robust systemic and mucosal immunoresponses against the loaded antigens via oral mucosal vaccination, Vaccine 33 (2015). https://doi.org/10.1016/j.vaccine.2015.03.081.

[40] X.J. Li, Y. Li, Y. Meng, X.Q. Pu, J.W. Qin, R. Xie, W. Wang, Z. Liu, L. Jiang, X.J. Ju, L.Y. Chu, Composite dissolvable microneedle patch for therapy of oral mucosal diseases, Biomaterials Advances 139 (2022). https://doi.org/10.1016/j.bioadv.2022.213001.

[41] T. Wang, Y. Zhen, X. Ma, B. Wei, S. Li, N. Wang, 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 126 (2015). https://doi.org/10.1016/j.colsurfb.2015.01.005.

[42] J. Mašek, D. Lubasová, R. Lukáč, P. Turánek-Knotigová, P. Kulich, J. Plocková, E. Mašková, L. Procházka, Š. Koudelka, N. Sasithorn, J. Gombos, E. Bartheldyová, F. Hubatka, M. Raška, A.D. Miller, J. Turánek, 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 249 (2017). https://doi.org/10.1016/j.jconrel.2016.07.036.

[43] L. Garcia-del Rio, P. Diaz-Rodriguez, G.K. Pedersen, D. Christensen, M. Landin, Sublingual Boosting with a Novel Mucoadhesive Thermogelling Hydrogel Following Parenteral CAF01 Priming as a Strategy Against Chlamydia trachomatis, Adv Healthc Mater 11 (2022). https://doi.org/10.1002/adhm.202102508.

[44] H. Aliu, C. Rask, J. Brimnes, T.L. Andresen, Enhanced efficacy of sublingual immunotherapy by liposome-mediated delivery of allergen, Int J Nanomedicine 12 (2017). https://doi.org/10.2147/IJN.S137033.

[45] N. Tasaniyananda, U. Chaisri, A. Tungtrongchitr, W. Chaicumpa, N. Sookrung, Mouse model of cat allergic rhinitis and intranasal liposome-adjuvanted refined Fel d 1 vaccine, PLoS One 11 (2016). https://doi.org/10.1371/journal.pone.0150463.

[46] J. Yang, F. Firdaus, A. Azuar, Z.G. Khalil, N. Marasini, R.J. Capon, W.M. Hussein, I. Toth, M. Skwarczynski, Cell-penetrating peptides-based liposomal delivery system enhanced immunogenicity of peptide-based vaccine against group a streptococcus, Vaccines (Basel) 9 (2021). https://doi.org/10.3390/vaccines9050499.

[47] A. Azuar, L. Zhao, T.T. Hei, R.J. Nevagi, S. Bartlett, W.M. Hussein, Z.G. Khalil, R.J. Capon, I. Toth, M. Skwarczynski, Cholic Acid-based Delivery System for Vaccine Candidates against Group A Streptococcus, ACS Med Chem Lett 10 (2019). https://doi.org/10.1021/acsmedchemlett.9b00239.

[48] K. Senchi, S. Matsunaga, H. Hasegawa, H. Kimura, A. Ryo, Development of oligomannose-coated liposome-based nasal vaccine against human parainfluenza virus type 3, Front Microbiol 4 (2013). https://doi.org/10.3389/fmicb.2013.00346.

[49] S. Dhakal, X. Cheng, J. Salcido, S. Renu, K. Bondra, Y.S. Lakshmanappa, C. Misch, S. Ghimire, N. Feliciano-Ruiz, B. Hogshead, S. Krakowka, K. Carson, J. McDonough, C.W. Lee, G.J. Renukaradhya, Liposomal nanoparticle-based conserved peptide influenza vaccine and monosodium urate crystal adjuvant elicit protective immune response in pigs, Int J Nanomedicine 13 (2018). https://doi.org/10.2147/IJN.S178809.

[50] J. Wang, P. Li, Y. Yu, Y. Fu, H. Jiang, M. Lu, Z. Sun, S. Jiang, L. Lu, M.X. Wu, Pulmonary surfactant-biomimetic nanoparticles potentiate heterosubtypic influenza immunity, Science (1979) 367 (2020). https://doi.org/10.1126/science.aau0810.

[51] X. An, M. Martinez-Paniagua, A. Rezvan, S.R. Sefat, M. Fathi, S. Singh, S. Biswas, M. Pourpak, C. Yee, X. Liu, N. Varadarajan, Single-dose intranasal vaccination elicits systemic and mucosal immunity against SARS-CoV-2, IScience 24 (2021). https://doi.org/10.1016/j.isci.2021.103037.

[52] R. Tada, A. Hidaka, N. Iwase, S. Takahashi, Y. Yamakita, T. Iwata, S. Muto, E. Sato, N. Takayama, E. Honjo, H. Kiyono, J. Kunisawa, Y. Aramaki, P.N. Boyaka, Intranasal immunization with dotap cationic liposomes combined with DC-cholesterol induces potent antigen-specific mucosal and systemic immune responses in mice, PLoS One 10 (2015) 1–21. https://doi.org/10.1371/journal.pone.0139785.

[53] R. Tada, A. Hidaka, H. Kiyono, J. Kunisawa, Y. Aramaki, Intranasal administration of cationic liposomes enhanced granulocyte-macrophage colony-stimulating factor expression and this expression is dispensable for mucosal adjuvant activity, BMC Res Notes 11 (2018). https://doi.org/10.1186/s13104-018-3591-3.

[54] W. Qu, N. Li, R. Yu, W. Zuo, T. Fu, W. Fei, Y. Hou, Y. Liu, J. Yang, Cationic DDA/TDB liposome as a mucosal vaccine adjuvant for uptake by dendritic cells in vitro induces potent humoural immunity, Artif Cells Nanomed Biotechnol 46 (2018). https://doi.org/10.1080/21691401.2018.1438450.

[55] H. Yusuf, A.A. Ali, N. Orr, M.M. Tunney, H.O. McCarthy, V.L. Kett, Novel freeze-dried DDA and TPGS liposomes are suitable for nasal delivery of vaccine, Int J Pharm 533 (2017). https://doi.org/10.1016/j.ijpharm.2017.09.011.

[56] N. Marasini, K.A. Ghaffar, A.K. Giddam, M.R. Batzloff, M.F. Good, M. Skwarczynski, I. Toth, Highly Immunogenic Trimethyl Chitosan-based Delivery System for Intranasal Lipopeptide Vaccines against Group A Streptococcus, Curr Drug Deliv 14 (2016). https://doi.org/10.2174/1567201813666160721141322.

[57] G. Leroux-Roels, C. Maes, F. Clement, F. van Engelenburg, M. van den Dobbelsteen, M. Adler, M. Amacker, L. Lopalco, M. Bomsel, A. Chalifour, S. Fleury, Randomized Phase I: Safety, Immunogenicity and Mucosal Antiviral Activity in Young Healthy Women Vaccinated with HIV-1 Gp41 P1 Peptide on Virosomes, PLoS One 8 (2013). https://doi.org/10.1371/journal.pone.0055438.

[58] H.W. Wang, P.L. Jiang, S.F. Lin, H.J. Lin, K.L. Ou, W.P. Deng, L.W. Lee, Y.Y. Huang, P.H. Liang, D.Z. Liu, Application of galactose-modified liposomes as a potent antigen presenting cell targeted carrier for intranasal immunization, Acta Biomater 9 (2013) 5681–5688. https://doi.org/10.1016/j.actbio.2012.11.007.

[59] Z. Kakhi, B. Frisch, B. Heurtault, F. Pons, Liposomal constructs for antitumoral vaccination by the nasal route, Biochimie 130 (2016). https://doi.org/10.1016/j.biochi.2016.07.003.

[60] Z. Kakhi, B. Frisch, L. Bourel-Bonnet, J. Hemmerlé, F. Pons, B. Heurtault, Airway administration of a highly versatile peptide-based liposomal construct for local and distant antitumoral vaccination, Int J Pharm 496 (2015). https://doi.org/10.1016/j.ijpharm.2015.11.027.

[61] Y. Zhang, W. Ng, X. Feng, F. Cao, H. Xu, Lipid vesicular nanocarrier: Quick encapsulation efficiency determination and transcutaneous application, Int J Pharm 516 (2017). https://doi.org/10.1016/j.ijpharm.2016.11.011.

[62] Y. Jia, M.J. McCluskie, D. Zhang, R. Monette, U. Iqbal, M. Moreno, J. Sauvageau, D. Williams, L. Deschatelets, Z.J. Jakubek, L. Krishnan, In vitro evaluation of archaeosome vehicles for transdermal vaccine delivery, J Liposome Res 28 (2018). https://doi.org/10.1080/08982104.2017.1376683.

[63] A.T. Caimi, F. Parra, M.A. de Farias, R.V. Portugal, A.P. Perez, E.L. Romero, M.J. Morilla, Topical vaccination with super-stable ready to use nanovesicles, Colloids Surf B Biointerfaces 152 (2017). https://doi.org/10.1016/j.colsurfb.2016.12.039.

[64] L.H. Higa, L. Arnal, M. Vermeulen, A.P. Perez, P. Schilrreff, C. Mundiña-Weilenmann, O. Yantorno, M.E. Vela, M.J. Morilla, E.L. Romero, Ultradeformable archaeosomes for needle free nanovaccination with leishmania braziliensis antigens, PLoS One 11 (2016). https://doi.org/10.1371/journal.pone.0150185.

[65] D.S. Bernardi, C. Bitencourt, D.S.C. da Silveira, E.L.C.M. da Cruz, M.A. Pereira-da-Silva, L.H. Faccioli, R.F.V. Lopez, Effective transcutaneous immunization using a combination of iontophoresis and nanoparticles, Nanomedicine 12 (2016). https://doi.org/10.1016/j.nano.2016.07.001.

[66] Y.C. Chen, S.J. Chen, H.F. Cheng, M.K. Yeh, Development of Yersinia pestis F1 antigen-loaded liposome vaccine against plague using microneedles as a delivery system, J Drug Deliv Sci Technol 55 (2020). https://doi.org/10.1016/j.jddst.2019.101443.

[67] G. Du, R.M. Hathout, M. Nasr, M.R. Nejadnik, J. Tu, R.I. Koning, A.J. Koster, B. Slütter, A. Kros, W. Jiskoot, J.A. Bouwstra, J. Mönkäre, Intradermal vaccination with hollow microneedles: A comparative study of various protein antigen and adjuvant encapsulated nanoparticles, Journal of Controlled Release 266 (2017) 109–118. https://doi.org/10.1016/j.jconrel.2017.09.021.

[68] G. Du, M. Leone, S. Romeijn, G. Kersten, W. Jiskoot, J.A. Bouwstra, Immunogenicity of diphtheria toxoid and poly(I:C) loaded cationic liposomes after hollow microneedle-

mediated intradermal injection in mice, Int J Pharm 547 (2018). https://doi.org/10.1016/j.ijpharm.2018.06.001.

[69] X. Wu, Y. Li, X. Chen, Z. Zhou, J. Pang, X. Luo, M. Kong, 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 7 (2019). https://doi.org/10.1039/c9tb00448c.

[70] Y. Qiu, L. Guo, S. Zhang, B. Xu, Y. Gao, Y. Hu, J. Hou, B. Bai, H. Shen, P. Mao, DNA-based vaccination against hepatitis B virus using dissolving microneedle arrays adjuvanted by cationic liposomes and CpG ODN, Drug Deliv 23 (2016). https://doi.org/10.3109/10717544.2014.992497.

[71] L. Guo, J. Chen, Y. Qiu, S. Zhang, B. Xu, Y. Gao, Enhanced transcutaneous immunization via dissolving microneedle array loaded with liposome encapsulated antigen and adjuvant, Int J Pharm 447 (2013). https://doi.org/10.1016/j.ijpharm.2013.02.006.

[72] J.H. Zhao, Q.B. Zhang, B. Liu, X.H. Piao, Y.L. Yan, X.G. Hu, K. Zhou, Y.T. Zhang, N.P. Feng, Enhanced immunization via dissolving microneedle array-based delivery system incorporating subunit vaccine and saponin adjuvant, Int J Nanomedicine 12 (2017) 4763–4772. https://doi.org/10.2147/IJN.S132456.

[73] Y. Zhang, W. Ng, J. Hu, S.S. Mussa, Y. Ge, H. Xu, Formulation and in vitro stability evaluation of ethosomal carbomer hydrogel for transdermal vaccine delivery, Colloids Surf B Biointerfaces 163 (2018). https://doi.org/10.1016/j.colsurfb.2017.12.031.

[74] X. Yang, X. Wang, H. Hong, G. Elfawal, S. Lin, J. Wu, Y. Jiang, C. He, X. Mo, G. Kai, H. Wang, Galactosylated chitosan-modified ethosomes combined with silk fibroin nanofibers is useful in transcutaneous immunization, Journal of Controlled Release 327 (2020) 88–99. https://doi.org/10.1016/j.jconrel.2020.07.047.

[75] S. Hua, M.B.C. de Matos, J.M. Metselaar, G. Storm, Current trends and challenges in the clinical translation of nanoparticulate nanomedicines: Pathways for translational development and commercialization, Front Pharmacol 9 (2018). https://doi.org/10.3389/fphar.2018.00790.

[76] M.L. Manca, M. Manconi, A.M. Falchi, I. Castangia, D. Valenti, S. Lampis, A.M. Fadda, Close-packed vesicles for diclofenac skin delivery and fibroblast targeting, Colloids Surf B Biointerfaces 111 (2013) 609–617. https://doi.org/http://dx.doi.org/10.1016/j.colsurfb.2013.07.014.

[77] A. Huguet-Casquero, T.B. López-Méndez, E. Gainza, J.L. Pedraz, Development and validation of an eco-friendly HPLC-DAD method for the determination of oleuropein and its applicability to several matrices: olive oil, olive leaf extracts and nanostructured lipid carriers, Green Chemistry 22 (2020) 3495–3505. https://doi.org/10.1039/D0GC00965B.

[78] M.T. Mabrouk, W.-C. Huang, B. Deng, N. Li-Purcell, A. Seffouh, J. Ortega, G. Ekin Atilla-Gokcumen, C.A. Long, K. Miura, J.F. Lovell, Lyophilized, antigen-bound liposomes with

reduced MPLA and enhanced thermostability, Int J Pharm 589 (2020) 119843. https://doi.org/10.1016/j.ijpharm.2020.119843.

[79] E. Hagesaether, M.I. Adamczak, M. Hiorth, I. Tho, Characterization of Bioadhesion, Mucin-interactions and Mucosal Permeability of Pharmaceutical Nano- and Microsystems, in: Characterization of Pharmaceutical Nano and Microsystems, Wiley, 2021: pp. 171–205. https://doi.org/10.1002/9781119414018.ch5.

[80] S. Gänger, K. Schindowski, Tailoring Formulations for Intranasal Nose-to-Brain Delivery: A Review on Architecture, Physico-Chemical Characteristics and Mucociliary Clearance of the Nasal Olfactory Mucosa, Pharmaceutics 2018, Vol. 10, Page 116 10 (2018) 116. https://doi.org/10.3390/PHARMACEUTICS10030116.

[81] L. Salade, N. Wauthoz, M. Deleu, M. Vermeersch, C. De Vriese, K. Amighi, J. Goole, Development of coated liposomes loaded with ghrelin for nose-to-brain delivery for the treatment of cachexia, Int J Nanomedicine Volume 12 (2017) 8531–8543. https://doi.org/10.2147/IJN.S147650.

[82] J. Zhao, L. Qin, R. Song, J. Su, Y. Yuan, X. Zhang, S. Mao, Elucidating inhaled liposome surface charge on its interaction with biological barriers in the lung, European Journal of Pharmaceutics and Biopharmaceutics 172 (2022) 101–111. https://doi.org/10.1016/j.ejpb.2022.01.009.

[83] J. Zhao, J. Su, L. Qin, X. Zhang, S. Mao, Exploring the influence of inhaled liposome membrane fluidity on its interaction with pulmonary physiological barriers, Biomater Sci 8 (2020) 6786–6797. https://doi.org/10.1039/D0BM01529F.

[84] A. Ahsan, M.A. Farooq, Therapeutic potential of green synthesized silver nanoparticles loaded PVA hydrogel patches for wound healing, J Drug Deliv Sci Technol 54 (2019) 101308. https://doi.org/10.1016/j.jddst.2019.101308.

[85] W. Azouz, P. Chetcuti, H.S.R. Hosker, D. Saralaya, J. Stephenson, H. Chrystyn, The Inhalation Characteristics of Patients When They Use Different Dry Powder Inhalers, J Aerosol Med Pulm Drug Deliv 28 (2015) 35–42. https://doi.org/10.1089/jamp.2013.1119.

[86] R.R. Delvadia, X. Wei, P.W. Longest, J. Venitz, P.R. Byron, *In Vitro* Tests for Aerosol Deposition. IV: Simulating Variations in Human Breath Profiles for Realistic DPI Testing, J Aerosol Med Pulm Drug Deliv 29 (2016) 196–206. https://doi.org/10.1089/jamp.2015.1215.

[87] R. Scherließ, J. Janke, Preparation of Poly-Lactic-Co-Glycolic Acid Nanoparticles in a Dry Powder Formulation for Pulmonary Antigen Delivery, Pharmaceutics 13 (2021) 1196. https://doi.org/10.3390/pharmaceutics13081196.

[88] V. Melis, M.L. Manca, E. Bullita, E. Tamburini, I. Castangia, M.C. Cardia, D. Valenti, A.M. Fadda, J.E. Peris, M. Manconi, Inhalable polymer-glycerosomes as safe and effective carriers for rifampicin delivery to the lungs, Colloids Surf B Biointerfaces 143 (2016) 301–308. https://doi.org/10.1016/j.colsurfb.2016.03.044.

[89] N.A. Wiggins, The Development of a Mathematical Approximation Technique to Determine the Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation (GSD) of Drug Particles in an Inhalation Aerosol Sprat, Drug Dev Ind Pharm 17 (1991) 1971–1986. https://doi.org/10.3109/03639049109048062.

[90] J.Y. Tse, K. Kadota, T. Imakubo, H. Uchiyama, Y. Tozuka, Enhancement of the extra-fine particle fraction of levofloxacin embedded in excipient matrix formulations for dry powder inhaler using response surface methodology, European Journal of Pharmaceutical Sciences 156 (2021) 105600. https://doi.org/10.1016/j.ejps.2020.105600.

[91] M.J. Papania, D. Zehrung, C. Jarrahian, Technologies to Improve Immunization, in: Plotkin's Vaccines, Elsevier, 2018: pp. 1320-1353.e17. https://doi.org/10.1016/B978-0-323-35761-6.00068-7.

[92] Simrah, A. Hafeez, S.A. Usmani, M.P. Izhar, Transfersome, an ultra-deformable lipid-based drug nanocarrier: an updated review with therapeutic applications, Naunyn Schmiedebergs Arch Pharmacol 397 (2024) 639–673. https://doi.org/10.1007/s00210-023-02670-8.

[93] M.W. Akram, H. Jamshaid, F.U. Rehman, M. Zaeem, J. zeb Khan, A. Zeb, Transfersomes: a Revolutionary Nanosystem for Efficient Transdermal Drug Delivery, AAPS PharmSciTech 23 (2021) 7. https://doi.org/10.1208/s12249-021-02166-9.

[94] R. Gupta, A. Kumar, Transfersomes: The Ultra-Deformable Carrier System for Non-Invasive Delivery of Drug, Curr Drug Deliv 18 (2021) 408–420. https://doi.org/10.2174/1567201817666200804105416.

[95] M. Ghadiri, P.M. Young, D. Traini, Strategies to enhance drug absorption via nasal and pulmonary routes, Pharmaceutics 11 (2019). https://doi.org/10.3390/pharmaceutics11030113.

[96] G.M. El Zaafarany, G.A.S. Awad, S.M. Holayel, N.D. Mortada, Role of edge activators and surface charge in developing ultradeformable vesicles with enhanced skin delivery, Int J Pharm 397 (2010) 164–172. https://doi.org/10.1016/j.ijpharm.2010.06.034.

[97] R. Sklenarova, M. Allaw, M. Perra, I. Castangia, J. Frankova, J. Luis Pedraz, M. Letizia Manca, M. Manconi, Co-delivering of oleuropein and lentisk oil in phospholipid vesicles as an effective approach to modulate oxidative stress, cytokine secretion and promote skin regeneration, European Journal of Pharmaceutics and Biopharmaceutics 185 (2023) 126–136. https://doi.org/10.1016/j.ejpb.2023.02.018.

[98] M.L. Manca, M. Firoznezhad, C. Caddeo, F. Marongiu, E. Escribano-Ferrer, G. Sarais, J.E. Peris, I. Usach, M. Zaru, M. Manconi, A.M. Fadda, Phytocomplexes extracted from grape seeds and stalks delivered in phospholipid vesicles tailored for the treatment of skin damages, Ind Crops Prod 128 (2019). https://doi.org/10.1016/j.indcrop.2018.11.052.

[99] M. Firoznezhad, I. Castangia, C.I.G. Tuberoso, F. Cottiglia, F. Marongiu, M. Porceddu, I. Usach, E. Escribano-Ferrer, M.L. Manca, M. Manconi, Formulation and In Vitro Efficacy

Assessment of Teucrium marum Extract Loading Hyalurosomes Enriched with Tween 80 and Glycerol, Nanomaterials 12 (2022) 1096. https://doi.org/10.3390/nano12071096.

[100] I. Castangia, M.L. Manca, M. Allaw, J. Hellström, D. Granato, M. Manconi, Jabuticaba (Myrciaria jaboticaba) Peel as a Sustainable Source of Anthocyanins and Ellagitannins Delivered by Phospholipid Vesicles for Alleviating Oxidative Stress in Human Keratinocytes, Molecules 26 (2021) 6697. https://doi.org/10.3390/molecules26216697.

[101] M. Manconi, M. Rezvani, M.L. Manca, E. Escribano-Ferrer, S. Fais, G. Orrù, T. Lammers, F. Asunis, A. Muntoni, D. Spiga, G. De Gioannis, Bridging biotechnology and nanomedicine to produce biogreen whey-nanovesicles for intestinal health promotion, Int J Pharm 633 (2023) 122631. https://doi.org/10.1016/j.ijpharm.2023.122631.

[102] M. Perra, L. Fancello, I. Castangia, M. Allaw, E. Escribano-Ferrer, J.E. Peris, I. Usach, M.L. Manca, I.K. Koycheva, M.I. Georgiev, M. Manconi, Formulation and Testing of Antioxidant and Protective Effect of Hyalurosomes Loading Extract Rich in Rosmarinic Acid Biotechnologically Produced from Lavandula angustifolia Miller, Molecules 27 (2022) 2423. https://doi.org/10.3390/molecules27082423.

[103] F. Fulgheri, M. Aroffu, M. Ramírez, L. Román-Álamo, J.E. Peris, I. Usach, A. Nacher, M. Manconi, X. Fernàndez-Busquets, M.L. Manca, Curcumin or quercetin loaded nutriosomes as oral adjuvants for malaria infections, Int J Pharm 643 (2023) 123195. https://doi.org/10.1016/j.ijpharm.2023.123195.

[104] M. Aroffu, M.L. Manca, J.L. Pedraz, M. Manconi, Liposome-based vaccines for minimally or noninvasive administration: an update on current advancements, Expert Opin Drug Deliv 20 (2023) 1573–1593. https://doi.org/10.1080/17425247.2023.2288856.

[105] R. Touti, M. Noun, F. Guimberteau, S. Lecomte, C. Faure, What is the fate of multi-lamellar liposomes of controlled size, charge and elasticity in artificial and animal skin?, European Journal of Pharmaceutics and Biopharmaceutics 151 (2020) 18–31. https://doi.org/10.1016/j.ejpb.2020.03.017.

[106] A.C. Carita, J.O. Eloy, M. Chorilli, R.J. Lee, G.R. Leonardi, Recent Advances and Perspectives in Liposomes for Cutaneous Drug Delivery, Curr Med Chem 25 (2018) 606–635. https://doi.org/10.2174/0929867324666171009120154.

[107] Y. Zhang, W. Ng, X. Feng, F. Cao, H. Xu, Lipid vesicular nanocarrier: Quick encapsulation efficiency determination and transcutaneous application, Int J Pharm 516 (2017) 225–230. https://doi.org/10.1016/j.ijpharm.2016.11.011.

[108] K. Ferderber, S. Hook, T. Rades, Phosphatidyl choline-based colloidal systems for dermal and transdermal drug delivery, J Liposome Res 19 (2009) 267–277. https://doi.org/10.3109/08982100902814006.

[109] J.W. Fluhr, R. Darlenski, C. Surber, Glycerol and the skin: Holistic approach to its origin and functions, British Journal of Dermatology 159 (2008) 23–34. https://doi.org/10.1111/j.1365-2133.2008.08643.x.

[110] N. Hassan, A. Ahad, M. Ali, J. Ali, Chemical permeation enhancers for transbuccal drug delivery, Expert Opin Drug Deliv 7 (2010) 97–112. https://doi.org/10.1517/17425240903338758.

[111] C. Ni, Z. Zhang, Y. Wang, Z. Zhang, X. Guo, H. Lv, Hyaluronic acid and HA-modified cationic liposomes for promoting skin penetration and retention, Journal of Controlled Release 357 (2023) 432–443. https://doi.org/10.1016/j.jconrel.2023.03.049.

[112] H. Zhai, H.I. Maibach, Occlusion vs. skin barrier function, Skin Research and Technology 8 (2002) 1–6. https://doi.org/10.1046/j.0909-752x.2001.10311.x.

[113] S. Jacob, A.B. Nair, J. Shah, N. Sreeharsha, S. Gupta, P. Shinu, Emerging Role of Hydrogels in Drug Delivery Systems, Tissue Engineering and Wound Management, Pharmaceutics 13 (2021) 357. https://doi.org/10.3390/pharmaceutics13030357.

[114] T. de P. de L. Lima, M.F. Passos, Skin wounds, the healing process, and hydrogel-based wound dressings: a short review, J Biomater Sci Polym Ed 32 (2021) 1910–1925. https://doi.org/10.1080/09205063.2021.1946461.

[115] S. Napavichayanun, W. Bonani, Y. Yang, A. Motta, P. Aramwit, Fibroin and Polyvinyl Alcohol Hydrogel Wound Dressing Containing Silk Sericin Prepared Using High-Pressure Carbon Dioxide, Adv Wound Care (New Rochelle) 8 (2019) 452–462. https://doi.org/10.1089/wound.2018.0856.

[116] A. Ahsan, M.A. Farooq, Therapeutic potential of green synthesized silver nanoparticles loaded PVA hydrogel patches for wound healing, J Drug Deliv Sci Technol 54 (2019) 101308. https://doi.org/10.1016/j.jddst.2019.101308.

[117] D.R. de Araújo, D.C. da Silva, R.M. Barbosa, M. Franz-Montan, C.M. Cereda, C. Padula, P. Santi, E. de Paula, Strategies for delivering local anesthetics to the skin: focus on liposomes, solid lipid nanoparticles, hydrogels and patches, Expert Opin Drug Deliv 10 (2013) 1551–1563. https://doi.org/10.1517/17425247.2013.828031.

[118] P. Basu, N. Saha, T. Saha, P. Saha, Polymeric hydrogel based systems for vaccine delivery: A review, Polymer (Guildf) 230 (2021) 124088. https://doi.org/10.1016/j.polymer.2021.124088.

[119] Y. Ishii, T. Nakae, F. Sakamoto, K. Matsuo, K. Matsuo, Y.-S. Quan, F. Kamiyama, T. Fujita, A. Yamamoto, S. Nakagawa, N. Okada, A transcutaneous vaccination system using a hydrogel patch for viral and bacterial infection, Journal of Controlled Release 131 (2008) 113–120. https://doi.org/10.1016/j.jconrel.2008.07.025.

[120] M.T. Hussain, M. Tiboni, Y. Perrie, L. Casettari, Microfluidic production of protein loaded chimeric stealth liposomes, Int J Pharm 590 (2020) 119955. https://doi.org/10.1016/j.ijpharm.2020.119955.

[121] K. Taguchi, Y. Okamoto, K. Matsumoto, M. Otagiri, V. Chuang, When Albumin Meets Liposomes: A Feasible Drug Carrier for Biomedical Applications, Pharmaceuticals 14 (2021) 296. https://doi.org/10.3390/ph14040296.

[122] N. Wang, M. Chen, T. Wang, Liposomes used as a vaccine adjuvant-delivery system: From basics to clinical immunization, Journal of Controlled Release 303 (2019) 130–150. https://doi.org/10.1016/j.jconrel.2019.04.025.

[123] L. Salade, N. Wauthoz, J. Goole, K. Amighi, How to characterize a nasal product. The state of the art of in vitro and ex vivo specific methods, Int J Pharm 561 (2019) 47–65. https://doi.org/10.1016/j.ijpharm.2019.02.026.

[124] L. Huang, M. Cai, X. Xie, Y. Chen, X. Luo, Uptake enhancement of curcumin encapsulated into phosphatidylcholine-shielding micelles by cancer cells, J Biomater Sci Polym Ed 25 (2014) 1407–1424. https://doi.org/10.1080/09205063.2014.941261.

[125] R. Tada, A. Hidaka, N. Iwase, S. Takahashi, Y. Yamakita, T. Iwata, S. Muto, E. Sato, N. Takayama, E. Honjo, H. Kiyono, J. Kunisawa, Y. Aramaki, Intranasal Immunization with DOTAP Cationic Liposomes Combined with DC-Cholesterol Induces Potent Antigen-Specific Mucosal and Systemic Immune Responses in Mice, PLoS One 10 (2015) e0139785. https://doi.org/10.1371/journal.pone.0139785.

[126] P. Zamani, A.A. Momtazi-Borojeni, M.E. Nik, R.K. Oskuee, A. Sahebkar, Nanoliposomes as the adjuvant delivery systems in cancer immunotherapy, J Cell Physiol 233 (2018) 5189–5199. https://doi.org/10.1002/jcp.26361.

[127] Y. Mai, J. Guo, Y. Zhao, S. Ma, Y. Hou, J. Yang, Intranasal delivery of cationic liposome-protamine complex mRNA vaccine elicits effective anti-tumor immunity, Cell Immunol 354 (2020) 104143. https://doi.org/10.1016/j.cellimm.2020.104143.

[128] P. Nakhaei, R. Margiana, D.O. Bokov, W.K. Abdelbasset, M.A. Jadidi Kouhbanani, R.S. Varma, F. Marofi, M. Jarahian, N. Beheshtkhoo, Liposomes: Structure, Biomedical Applications, and Stability Parameters With Emphasis on Cholesterol, Front Bioeng Biotechnol 9 (2021). https://doi.org/10.3389/fbioe.2021.705886.

[129] R. Tada, A. Hidaka, H. Kiyono, J. Kunisawa, Y. Aramaki, Intranasal administration of cationic liposomes enhanced granulocyte–macrophage colony-stimulating factor expression and this expression is dispensable for mucosal adjuvant activity, BMC Res Notes 11 (2018) 472. https://doi.org/10.1186/s13104-018-3591-3.

[130] N. Filipczak, J. Pan, S.S.K. Yalamarty, V.P. Torchilin, Recent advancements in liposome technology, Adv Drug Deliv Rev 156 (2020) 4–22. https://doi.org/10.1016/j.addr.2020.06.022.

[131] W. Qu, N. Li, R. Yu, W. Zuo, T. Fu, W. Fei, Y. Hou, Y. Liu, J. Yang, Cationic DDA/TDB liposome as a mucosal vaccine adjuvant for uptake by dendritic cells *in vitro* induces potent humoural immunity, Artif Cells Nanomed Biotechnol 46 (2018) 852–860. https://doi.org/10.1080/21691401.2018.1438450.

[132] R. Tada, A. Ohshima, Y. Tanazawa, A. Ohmi, S. Takahashi, H. Kiyono, J. Kunisawa, Y. Aramaki, Y. Negishi, Essential Role of Host Double-Stranded DNA Released from Dying Cells by Cationic Liposomes for Mucosal Adjuvanticity, Vaccines (Basel) 8 (2019) 8. https://doi.org/10.3390/vaccines8010008.

[133] X. Nian, J. Zhang, S. Huang, K. Duan, X. Li, X. Yang, Development of Nasal Vaccines and the Associated Challenges, Pharmaceutics 14 (2022) 1983. https://doi.org/10.3390/pharmaceutics14101983.

[134] EMA, GUIDELINE ON THE PHARMACEUTICAL QUALITY OF INHALATION AND NASAL PRODUCTS, EMEA/CHMP/QWP/49313/2005 Corr (2006).

[135] FDA, Nasal Spray and Inhalation Solution, Suspension, and Spray Drug Products--Chemistry, Manufacturing, and Controls Documentation, DA-1999-D-0060 (2018).

[136] J.Z. Chen, W.H. Finlay, A. Martin, In Vitro Regional Deposition of Nasal Sprays in an Idealized Nasal Inlet: Comparison with In Vivo Gamma Scintigraphy, Pharm Res 39 (2022) 3021–3028. https://doi.org/10.1007/s11095-022-03388-7.

[137] D.L. Swift, N. Montassier, P.K. Hopke, K. Karpen-Hayes, Y.-S. Cheng, Yin Fong Su, Hsu Chi Yeh, J.C. Strong, Inspiratory deposition of ultrafine particles in human nasal replicate cast, J Aerosol Sci 23 (1992) 65–72. https://doi.org/10.1016/0021-8502(92)90318-P.

[138] A. Maaz, I.S. Blagbrough, P.A. De Bank, In Vitro Evaluation of Nasal Aerosol Depositions: An Insight for Direct Nose to Brain Drug Delivery, Pharmaceutics 13 (2021) 1079. https://doi.org/10.3390/pharmaceutics13071079.

[139] H. Xu, R.F. Alzhrani, Z.N. Warnken, S.G. Thakkar, M. Zeng, H.D.C. Smyth, R.O. Williams, Z. Cui, Immunogenicity of Antigen Adjuvanted with AS04 and Its Deposition in the Upper Respiratory Tract after Intranasal Administration, Mol Pharm 17 (2020) 3259–3269. https://doi.org/10.1021/acs.molpharmaceut.0c00372.

[140] E.K. Wasan, J. Syeda, S. Strom, J. Cawthray, R.E. Hancock, K.M. Wasan, V. Gerdts, A lipidic delivery system of a triple vaccine adjuvant enhances mucosal immunity following nasal administration in mice, Vaccine 37 (2019) 1503–1515. https://doi.org/10.1016/j.vaccine.2019.01.058.

[141] Z. Teng, L.-Y. Meng, J.-K. Yang, Z. He, X.-G. Chen, Y. Liu, Bridging nanoplatform and vaccine delivery, a landscape of strategy to enhance nasal immunity, Journal of Controlled Release 351 (2022) 456–475. https://doi.org/10.1016/j.jconrel.2022.09.044.

[142] R. Pabst, Mucosal vaccination by the intranasal route. Nose-associated lymphoid tissue (NALT)—Structure, function and species differences, Vaccine 33 (2015) 4406–4413. https://doi.org/10.1016/j.vaccine.2015.07.022.

[143] Y. Ma, Y. Zhuang, X. Xie, C. Wang, F. Wang, D. Zhou, J. Zeng, L. Cai, The role of surface charge density in cationic liposome-promoted dendritic cell maturation and vaccine-induced immune responses, Nanoscale 3 (2011) 2307. https://doi.org/10.1039/c1nr10166h.

[144] A.R. Nikpoor, M.R. Jaafari, P. Zamani, M. Teymouri, H. Gouklani, E. Saburi, S.A. Darban, A. Badiee, A. Bahramifar, M. Fasihi-Ramandi, R.A. Taheri, Cell cytotoxicity, immunostimulatory and antitumor effects of lipid content of liposomal delivery

platforms in cancer immunotherapies. A comprehensive in-vivo and in-vitro study, Int J Pharm 567 (2019) 118492. https://doi.org/10.1016/j.ijpharm.2019.118492.

[145] E.R. Sherwood, T. Toliver-Kinsky, Mechanisms of the inflammatory response, Best Pract Res Clin Anaesthesiol 18 (2004) 385–405. https://doi.org/10.1016/j.bpa.2003.12.002.

[146] A. Mantovani, A. Ponzetta, A. Inforzato, S. Jaillon, Innate immunity, inflammation and tumour progression: double-edged swords, J Intern Med 285 (2019) 524–532. https://doi.org/10.1111/joim.12886.

[147] J. Korbecki, K. Bajdak-Rusinek, The effect of palmitic acid on inflammatory response in macrophages: an overview of molecular mechanisms, Inflammation Research 68 (2019) 915–932. https://doi.org/10.1007/s00011-019-01273-5.

[148] H. Wajant, D. Siegmund, TNFR1 and TNFR2 in the Control of the Life and Death Balance of Macrophages, Front Cell Dev Biol 7 (2019). https://doi.org/10.3389/fcell.2019.00091.

[149] P. V. Date, A. Samad, P. V. Devarajan, Freeze Thaw: A Simple Approach for Prediction of Optimal Cryoprotectant for Freeze Drying, AAPS PharmSciTech 11 (2010) 304–313. https://doi.org/10.1208/s12249-010-9382-3.

[150] D. Bardoliwala, V. Patel, A. Misra, K. Sawant, Systematic development and characterization of inhalable dry powder containing Polymeric Lipid Hybrid Nanocarriers co-loaded with ABCB1 shRNA and docetaxel using QbD approach, J Drug Deliv Sci Technol 66 (2021) 102903. https://doi.org/10.1016/j.jddst.2021.102903.

[151] W.L.J. Hinrichs, F.A. Manceñido, N.N. Sanders, K. Braeckmans, S.C. De Smedt, J. Demeester, H.W. Frijlink, The choice of a suitable oligosaccharide to prevent aggregation of PEGylated nanoparticles during freeze thawing and freeze drying, Int J Pharm 311 (2006) 237–244. https://doi.org/10.1016/j.ijpharm.2005.12.032.

[152] W.L.J. Hinrichs, N.N. Sanders, S.C. De Smedt, J. Demeester, H.W. Frijlink, Inulin is a promising cryo- and lyoprotectant for PEGylated lipoplexes, Journal of Controlled Release 103 (2005) 465–479. https://doi.org/10.1016/j.jconrel.2004.12.011.

[153] I. Castangia, M.L. Manca, C. Caddeo, A. Maxia, S. Murgia, R. Pons, D. Demurtas, D. Pando, D. Falconieri, J.E. Peris, A.M. Fadda, M. Manconi, Faceted phospholipid vesicles tailored for the delivery of Santolina insularis essential oil to the skin, Colloids Surf B Biointerfaces 132 (2015) 185–193. https://doi.org/10.1016/j.colsurfb.2015.05.025.

[154] C.A. Haselwandter, R. Phillips, Elastic energy of polyhedral bilayer vesicles, Phys Rev E 83 (2011) 061901. https://doi.org/10.1103/PhysRevE.83.061901.

[155] L.G. Sweeney, Z. Wang, R. Loebenberg, J.P. Wong, C.F. Lange, W.H. Finlay, Spray-freeze-dried liposomal ciprofloxacin powder for inhaled aerosol drug delivery, Int J Pharm 305 (2005) 180–185. https://doi.org/10.1016/j.ijpharm.2005.09.010.

[156] R. Mahar, A. Chakraborty, N. Nainwal, The influence of carrier type, physical characteristics, and blending techniques on the performance of dry powder inhalers, J Drug Deliv Sci Technol 76 (2022) 103759. https://doi.org/10.1016/j.jddst.2022.103759.

[157] S. Yaqoubi, H.-K. Chan, A. Nokhodchi, S. Dastmalchi, A.A. Alizadeh, M. Barzegar-Jalali, K. Adibkia, H. Hamishehkar, A quantitative approach to predicting lung deposition profiles of pharmaceutical powder aerosols, Int J Pharm 602 (2021) 120568. https://doi.org/10.1016/j.ijpharm.2021.120568.

[158] Y. Sun, Z. Cui, Y. Sun, L. Qin, X. Zhang, Q. Liu, X. Shen, D. Yu, S. Mao, Exploring the potential influence of drug charge on downstream deposition behaviour of DPI powders, Int J Pharm 588 (2020) 119798. https://doi.org/10.1016/j.ijpharm.2020.119798.

[159] M. Alavi, N. Karimi, M. Safaei, Application of Various Types of Liposomes in Drug Delivery Systems, Adv Pharm Bull 7 (2017) 3–9. https://doi.org/10.15171/apb.2017.002.

[160] J. Wang, M. Zhu, G. Nie, Biomembrane-based nanostructures for cancer targeting and therapy: From synthetic liposomes to natural biomembranes and membrane-vesicles, Adv Drug Deliv Rev 178 (2021) 113974. https://doi.org/10.1016/j.addr.2021.113974.

[161] T. Mazumdar, K. Anam, N. Ali, INFLUENCE OF PHOSPHOLIPID COMPOSITION ON THE ADJUVANTICITY AND PROTECTIVE EFFICACY OF LIPOSOME-ENCAPSULATED LEISHMANIA DONOVANI ANTIGENS, Journal of Parasitology 91 (2005) 269–274. https://doi.org/10.1645/GE-356R1.

[162] J. Wang, P. Li, Y. Yu, Y. Fu, H. Jiang, M. Lu, Z. Sun, S. Jiang, L. Lu, M.X. Wu, Pulmonary surfactant–biomimetic nanoparticles potentiate heterosubtypic influenza immunity, Science (1979) 367 (2020). https://doi.org/10.1126/science.aau0810.

[163] Z. Chroneos, Z. Sever-Chroneos, V. Shepherd, Pulmonary Surfactant: An Immunological Perspective, Cellular Physiology and Biochemistry 25 (2010) 013–026. https://doi.org/10.1159/000272047.

[164] W.L.J. Hinrichs, N.N. Sanders, S.C. De Smedt, J. Demeester, H.W. Frijlink, Inulin is a promising cryo- and lyoprotectant for PEGylated lipoplexes, Journal of Controlled Release 103 (2005) 465–479. https://doi.org/10.1016/j.jconrel.2004.12.011.

[165] P.T. Ingvarsson, I.S. Rasmussen, M. Viaene, P.J. Irlik, H.M. Nielsen, C. Foged, The surface charge of liposomal adjuvants is decisive for their interactions with the Calu-3 and A549 airway epithelial cell culture models, European Journal of Pharmaceutics and Biopharmaceutics 87 (2014) 480–488. https://doi.org/10.1016/j.ejpb.2014.04.001.

[166] C. Loira-Pastoriza, J. Todoroff, R. Vanbever, Delivery strategies for sustained drug release in the lungs, Adv Drug Deliv Rev 75 (2014) 81–91. https://doi.org/10.1016/j.addr.2014.05.017.

[167] J.T. Huckaby, S.K. Lai, PEGylation for enhancing nanoparticle diffusion in mucus, Adv Drug Deliv Rev 124 (2018) 125–139. https://doi.org/10.1016/j.addr.2017.08.010.

[168] J. Akhter, G. Quéromès, K. Pillai, V. Kepenekian, S. Badar, A.H. Mekkawy, E. Frobert, S.J. Valle, D.L. Morris, The Combination of Bromelain and Acetylcysteine (BromAc) Synergistically Inactivates SARS-CoV-2, Viruses 13 (2021) 425. https://doi.org/10.3390/v13030425.

[169] J.G.A. Coelho dos Reis, G.M. Ferreira, A.A. Lourenço, Á.L. Ribeiro, C.P. da S.M. da Mata, P. de Melo Oliveira, D.P. de A. Marques, L.L. Ferreira, F.A. Clarindo, M.F. da Silva, H.P.P. Filho, N.R.R. Oliveira, M.M.D. Sodré, S.R. Gadelha, G.R. Albuquerque, B.M. Maciel, A.P.M. Mariano, M. de M. Silva, R. Fontana, L.J. Marin, R.S.A. Carlos, A.T.S. Lopes, F.B. Ferreira, U.R. dos Santos, Í.T.S. de Santana, H.F. Fehlberg, R.P. Rezende, J.C.T. Dias, E. Gross, G.A.C. Goulart, M.G. Santiago, A.P.M.L. de Lemos, A.O. da Conceição, C.C. Romano, L.D. de Carvalho, O.A.M. Filho, C.A. Quadros, D.L. Morris, S.J. Valle, Ex-vivo mucolytic and anti-inflammatory activity of BromAc in tracheal aspirates from COVID-19, Biomedicine & Pharmacotherapy 148 (2022) 112753. https://doi.org/10.1016/j.biopha.2022.112753.

[170] K. Pillai, A.H. Mekkawy, J. Akhter, D.L. Morris, Effect of Nebulized BromAc on Rheology of Artificial Sputum: Relevance to Muco-Obstructive Respiratory Diseases, Adv Respir Med 91 (2023) 146–163. https://doi.org/10.3390/arm91020013.

[171] J. Wang, Y. Dadmohammadi, A. Jaiswal, A. Abbaspourrad, Investigation of the Interaction between *N* -Acetyl- <scp>l</scp> -Cysteine and Ovalbumin by Spectroscopic Studies, Molecular Docking Simulation, and Real-Time Quartz Crystal Microbalance with Dissipation, J Agric Food Chem 68 (2020) 10184–10190. https://doi.org/10.1021/acs.jafc.0c03201.

[172] M. Hassoun, S. Ho, J. Muddle, F. Buttini, M. Parry, M. Hammond, B. Forbes, Formulating powder–device combinations for salmeterol xinafoate dry powder inhalers, Int J Pharm 490 (2015) 360–367. https://doi.org/10.1016/j.ijpharm.2015.05.028.

[173] S.P. Newman, H.-K. Chan, In vitro-in vivo correlations (IVIVCs) of deposition for drugs given by oral inhalation, Adv Drug Deliv Rev 167 (2020) 135–147. https://doi.org/10.1016/j.addr.2020.06.023.

[174] K. Nahar, N. Gupta, R. Gauvin, S. Absar, B. Patel, V. Gupta, A. Khademhosseini, F. Ahsan, In vitro, in vivo and ex vivo models for studying particle deposition and drug absorption of inhaled pharmaceuticals, European Journal of Pharmaceutical Sciences 49 (2013) 805–818. https://doi.org/10.1016/j.ejps.2013.06.004.

[175] S.C. Saha, M.S. Islam, M. Rahimi-Gorji, M.M. Molla, Aerosol particle transport and deposition in a CT-scan based mouth-throat model, in: 2019: p. 040011. https://doi.org/10.1063/1.5115882.

[176] A. V. Kolanjiyil, C. Kleinstreuer, R.T. Sadikot, Computationally efficient analysis of particle transport and deposition in a human whole-lung-airway model. Part II: Dry powder inhaler application, Comput Biol Med 84 (2017) 247–253. https://doi.org/10.1016/j.compbiomed.2016.10.025.

[177] G. Pitcairn, G. Lunghetti, P. Ventura, S. Newman, A comparison of the lung deposition of salbutamol inhaled from a new dry powder inhaler, at two inhaled flow rates, Int J Pharm 102 (1994) 11–18. https://doi.org/10.1016/0378-5173(94)90034-5.

[178] W.D. Bennett, G. Scheuch, K.L. Zeman, J.S. Brown, C. Kim, J. Heyder, W. Stahlhofen, Regional deposition and retention of particles in shallow, inhaled boluses: effect of lung volume, J Appl Physiol 86 (1999) 168–173. https://doi.org/10.1152/jappl.1999.86.1.168.

[179] K. Ahookhosh, S. Yaqoubi, M. Mohammadpourfard, H. Hamishehkar, H. Aminfar, Experimental investigation of aerosol deposition through a realistic respiratory airway replica: An evaluation for MDI and DPI performance, Int J Pharm 566 (2019) 157–172. https://doi.org/10.1016/j.ijpharm.2019.05.058.

[180] N. Joshi, J.M. Walter, A. V. Misharin, Alveolar Macrophages, Cell Immunol 330 (2018) 86–90. https://doi.org/10.1016/j.cellimm.2018.01.005.

[181] A.H. de Boer, D. Gjaltema, P. Hagedoorn, H.W. Frijlink, Can 'extrafine' dry powder aerosols improve lung deposition?, European Journal of Pharmaceutics and Biopharmaceutics 96 (2015) 143–151. https://doi.org/10.1016/j.ejpb.2015.07.016.

[182] M. Corradi, H. Chrystyn, B.G. Cosio, M. Pirozynski, S. Loukides, R. Louis, M. Spinola, O.S. Usmani, NEXThaler, an innovative dry powder inhaler delivering an extrafine fixed combination of beclometasone and formoterol to treat large and small airways in asthma, Expert Opin Drug Deliv 11 (2014) 1497–1506. https://doi.org/10.1517/17425247.2014.928282.

[183] D. Sandell, Bioequivalence assessment of pharmaceutical aerosol products through IVIVC, Adv Drug Deliv Rev 176 (2021) 113895. https://doi.org/10.1016/j.addr.2021.113895.

[184] Q. Liu, X. Zhang, D. Chai, H. Li, S. Li, D. Wu, L. Zhang, Z. Liu, Y. Feng, F. Tang, H. Feng, Enhancement of the immune response via the facilitation of dendritic cell maturation by CD-205 Receptor-mediated Long-circling liposomes acting as an antigen and astragalus polysaccharide delivery system, Int Immunopharmacol 119 (2023) 110242. https://doi.org/10.1016/j.intimp.2023.110242.

[185] B. Koppolu, D.A. Zaharoff, The effect of antigen encapsulation in chitosan particles on uptake, activation and presentation by antigen presenting cells, Biomaterials 34 (2013) 2359–2369. https://doi.org/10.1016/j.biomaterials.2012.11.066.

[186] K. Smith Korsholm, E.M. Agger, C. Foged, D. Christensen, J. Dietrich, C.S. Andersen, C. Geisler, P. Andersen, The adjuvant mechanism of cationic dimethyldioctadecylammonium liposomes, Immunology 121 (2007) 216–226. https://doi.org/10.1111/j.1365-2567.2007.02560.x.

[187] F. Sonvico, V. Chierici, G. Varacca, E. Quarta, D. D'Angelo, B. Forbes, F. Buttini, RespiCellTM: An Innovative Dissolution Apparatus for Inhaled Products, Pharmaceutics 13 (2021) 1541. https://doi.org/10.3390/pharmaceutics13101541.

[188] B. Ruaro, F. Salton, L. Braga, B. Wade, P. Confalonieri, M.C. Volpe, E. Baratella, S. Maiocchi, M. Confalonieri, The History and Mystery of Alveolar Epithelial Type II Cells: Focus on Their Physiologic and Pathologic Role in Lung, Int J Mol Sci 22 (2021) 2566. https://doi.org/10.3390/ijms22052566.

[189] W. Su, Y. Liang, Z. Meng, X. Chen, M. Lu, X. Han, X. Deng, Q. Zhang, H. Zhu, T. Fu, Inhalation of Tetrandrine-hydroxypropyl-β-cyclodextrin Inclusion Complexes for Pulmonary Fibrosis Treatment, Mol Pharm 17 (2020) 1596–1607. https://doi.org/10.1021/acs.molpharmaceut.0c00026.

[190] G. Arango Duque, A. Descoteaux, Macrophage Cytokines: Involvement in Immunity and Infectious Diseases, Front Immunol 5 (2014). https://doi.org/10.3389/fimmu.2014.00491.

[191] S. Arber Raviv, M. Alyan, E. Egorov, A. Zano, M.Y. Harush, C. Pieters, H. Korach-Rechtman, A. Saadya, G. Kaneti, I. Nudelman, S. Farkash, O.D. Flikshtain, L.N. Mekies, L. Koren, Y. Gal, E. Dor, J. Shainsky, J. Shklover, Y. Adir, A. Schroeder, Lung targeted liposomes for treating ARDS, Journal of Controlled Release 346 (2022) 421–433. https://doi.org/10.1016/j.jconrel.2022.03.028.

[192] K. Li, L. Zhang, J. Xue, X. Yang, X. Dong, L. Sha, H. Lei, X. Zhang, L. Zhu, Z. Wang, X. Li, H. Wang, P. Liu, Y. Dong, L. He, Dietary inulin alleviates diverse stages of type 2 diabetes mellitus *via* anti-inflammation and modulating gut microbiota in db/db mice, Food Funct 10 (2019) 1915–1927. https://doi.org/10.1039/C8FO02265H.

[193] B. Watzl, S. Girrbach, M. Roller, Inulin, oligofructose and immunomodulation, British Journal of Nutrition 93 (2005) S49–S55. https://doi.org/10.1079/BJN20041357.

[194] S. Ahmed, S. Fujita, K. Matsumura, A Freeze-Concentration and Polyampholyte-Modified Liposome-Based Antigen-Delivery System for Effective Immunotherapy, Adv Healthc Mater 6 (2017). https://doi.org/10.1002/adhm.201700207.

Chapter 2 – Conclusions

1. In the first study (cutaneous delivery), it was demonstrated that enriched transferosmes (i.e., glycerol-transfersomes, hyaluronan-transfersomes and glycerohyaluronan-transfersomes) can stably encapsulated ovalbumin, a model antigen, in high dose (5 mg/mL) and can be easily manufactured using an eco-scalable method involving the direct sonication of components. All the vesicles had dimension suitable for skin penetration (< 60 nm), were stable up to 9 months and were highly biocompatible (cell viability > 100%). They can be used to imbibed dried disks of polyvinyl alcohol hydrogel creating innovative and advanced systems to facilitate the formulation application on the skin, anticipating real-life applicability. The prepared imbibed disks, especially those imbibed with glycerohyaluronan-transfersomes, improved the antigen deposition in the epidermis and dermis, where the antigen may stimulate immune response. To the best of our knowledge, it is the first time that these hydrogel disks were imbibed with enriched transfersomes, and promising results were obtained in antigen delivery on the skin. Anyways, *in vivo* studies, possibly after association with an adjuvant, are advised to pursue, support and confirm skin immunisation.

2. In the second study (nasal delivery), ovalbumin-encapsulated anionic liposomes and cationic DOTAP-liposomes were successfully prepared and their performances as nanovaccines in the form of nasal spray were confirmed. The eco-friendly manufacturing method of direct sonication, which avoids the use of dissipative preparation steps and organic solvents, was effective in leading to vesicles with high encapsulation efficiencies, good stability overtime, optimal sprayability as well as adjuvanticity and safety. Indeed, the obtained vesicles were able to boost efficaciously antigen delivery (interleukin-2 levels were higher than the soluble antigen used as control at the same dose) while being well-tolerated (no tumor necrosis factor-α was detected at any dose). Even though both formulations allowed to achieve nasal deposition avoiding pulmonary deposition (droplets were way bigger than 5 μm) or any potential risk from antigen delivery to the nervous system, cationic DOTAP-liposomes might be more suitable for this administration route. As pointed out after using the realistic nasal cast Alberta Idealised Nasal Inlet, they were more prone to be deposited in the posterior nasal cavity, where the immune response occurs. In addition, the greater mucoadhesiveness they displayed over the anionic liposomes suggests a better tendency to be retained in situ and thus possibly induce a more prolonged local response. *In vivo* studies are however needed to confirm the great promise of these systems.

3. In the third study (pulmonary delivery), liposomes were studied to deliver the model antigen ovalbumin to alveolar macrophages aiming at improving local protection. Respitose and inulin enabled to obtain dry powders suitable for lung delivery (mass median aerodynamic diameter ~2-3 μm) while successfully preserving the physico-chemical characteristic of the vesicles after re-hydration (re-dispersibility index ~ 1.1). Using the Next Generation Impactor under an enhanced in vitro-in vivo correlation settings, it was demonstrated that the delivery to the lungs was a function of the patient condition in the order healthy > asthmatic ≥ chronic obstructive pulmonary disease profile. Nonetheless, inulin SPC-3 and DPPC liposomes were able to deliver a consistent extra-fine particle fraction under all conditions, which in clinical practice

should translate in the delivery of a consistent dose of antigen to alveolar macrophages regardless of the condition. All the vesicles were well-tolerated and preserved the ability of ovalbumin to stimulate alveolar macrophages, with the Respitose SPC-3 liposomes being even more effective than the solution. Despite vesicles provided slightly higher mucus penetration for ovalbumin than the solution, the mucolytic agent BromAc® was needed to ensure greater and faster antigen penetration and potentially avoid therapeutic failure due to underdosing. To conclude, the antigen-encapsulated liposomes were successfully prepared and characterized for pulmonary delivery. However, this study was limited to the in vitro test on alveolar macrophages, that lie mainly in the deepest region of the lungs and are involved in the local immunisation. Since the obtained powders showcased adequate aerodynamic diameter, Fine Particle Dose and Fine Particle Fraction, these carriers might be studied in the future in a broader context considering different cellular lines (e.g. dendritic cells and T cells) and different immunological scenarios (e.g. in vivo studies and systemic immunisation).

Chapter 3 – Appendix

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

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

[1]Department of Scienze della Vita e dell'Ambiente, University of Cagliari, Cagliari, Italy

[2]NanoBioCel Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), Vitoria-Gasteiz, Spain

[3]Biomedical Research Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Vitoria-Gasteiz, Spain

[4]BioAraba, NanoBioCel research Group, Vitoria-Gasteiz, Spain

Expert opinion on Drug Delivery, 2023 Jul-Dec;20(11):1573-1593

IF: 6.6 (2022), Q1

Category: Pharmaceutical science

PMID: 38015659

DOI: 10.1080/17425247.2023.2288856

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.

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 [195]. Vaccination represents indeed the most effective and successful prophylactic intervention ever created to protect people from life-threatening diseases all over the globe [1,2]. Additionally, it plays a significant role in combating antimicrobial resistance and enhancing community resilience and adaptability [2,3]. 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 [196,197]. 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 [4,198]. 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 [6,199,200]. 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 [5]. 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 [7]. 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 [201]. 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 [201,202]. 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 [203]. 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" [203,204]. 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 [205,206]. 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 [207]. 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 [208]. 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 [209].

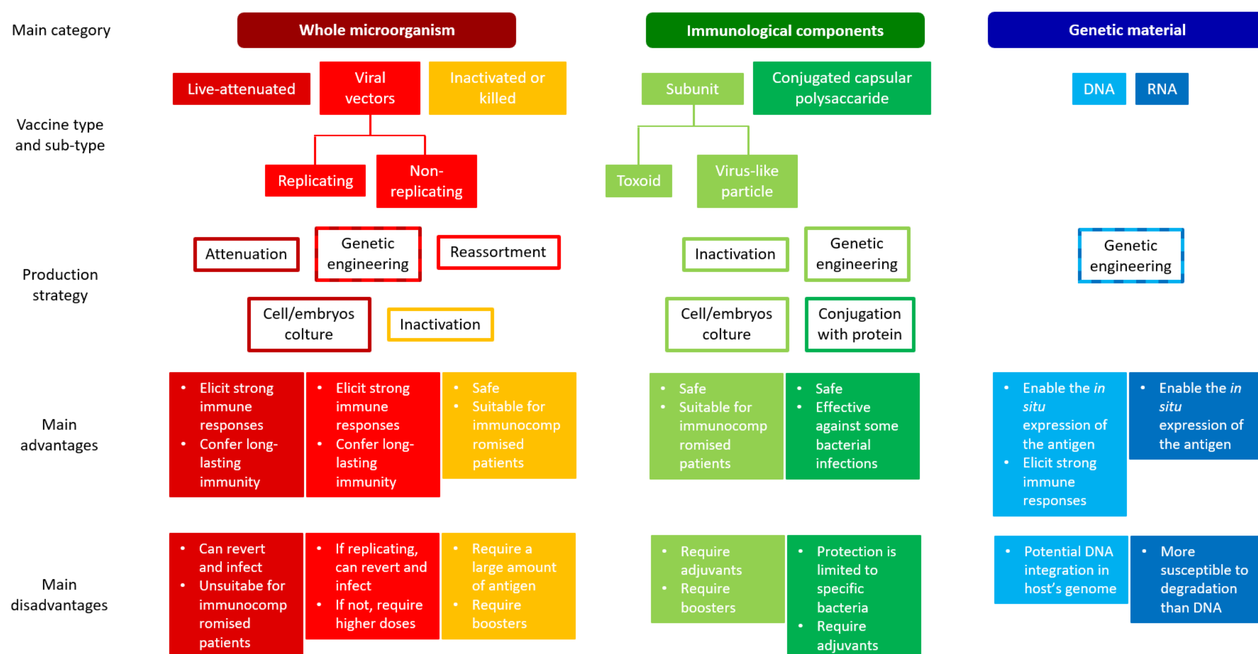
3. Problems encountered in vaccine development and adopted strategies

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 [204,210]. To ensure this stimulation, different strategies have been adopted throughout the century (**Table 1**) [211]. 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 [8]. 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 [212].

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 [213]. 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 [214–218]. Simultaneously, molecules/systems able to improve the magnitude of the immune response to the antigen (adjuvants), have been considered during vaccine design [21]. 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 [23,24]. 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 [25,26,219].

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 [28,220]. 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 [221]. 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 [222]. 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) [223]. 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 [224]. 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.) [225]. All these modifications have been essential to develop therapeutic products now approved for clinical use [226]. To date, these nanoformulated products are

available to treat infections, chronic diseases, pain, autoimmune diseases, mental disorders and even cancer [227]. 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) [25,29,219,228].

5. Liposomes in vaccine development

Liposomes have been identified as one of the most effective carriers for vaccine development [229]. They are nanosized vesicles made of phospholipids, which in water form closed bilayers surrounding an aqueous core and one or more interlamellar spaces [31]. Due to this peculiar structure, liposomes can encapsulate hydrophilic molecules and entrap hydrophobic ones [32]. 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 [29].

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**).

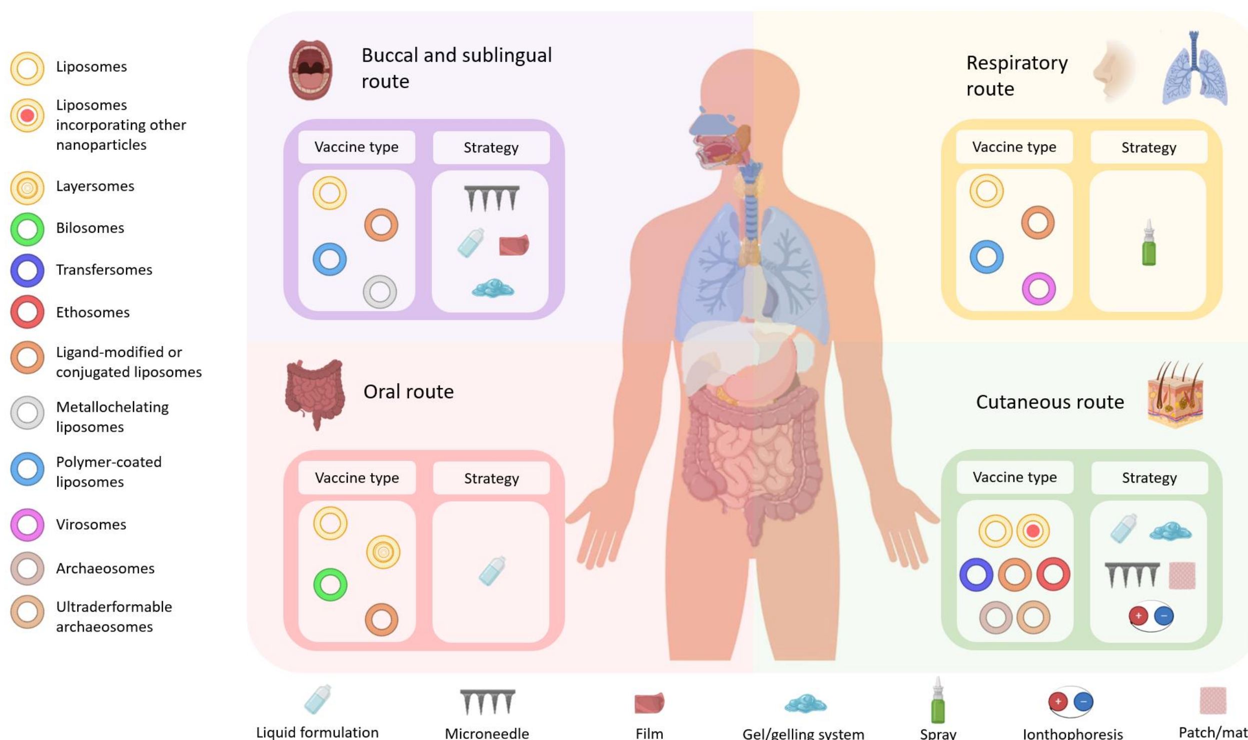


Figure 1. Liposome-based vaccines developed in the last decade (2013-2023) and tested trough minimally or non-invasive administration routes, alone or in combination with medical devices to pursue needle-free immunization.

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 [230]. 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 [231]. Despite all these limitations, several strategies can make this route viable to achieve local or systemic effects [232].

Oral administration also offers important advantages in vaccine formulations, as they enable self-administration, improve compliance and ensure stimulation of the gastrointestinal immune system [233]. 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*) [234]. However, although several vaccine dosage forms have been tested for years, only a small number is licensed and used clinically [11]. 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 [12].

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**) [235].

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 [34]. 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 [236].

In 2015, Harde and co-workers chose tetanus toxoid as model antigen for oral vaccination [35]. 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 [237]. 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 [238].

Accordingly, these carriers were adopted by Wilkhu *et al.* for the oral delivery of recombinant influenza hemagglutinin [36]. 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 [37]. 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	[34]
Cationic liposomes; **Cationic layersomes**	Epikuron 200, cholesterol, and stearylamine; Cationic liposomes composition	Tetanus toxoid	-	-	Oral	[35]

	+ polyacrylic acid sodium salt and polyallylamine hydrochloride					
Bilosomes	Monopalmitoyl glycerol, cholesterol, dicetyl phosphate and sodium deoxycholate	Recombinant hemagglutinin (rHA)	-	-	Oral	[36]
Bilosomes; **Glucomannosylated bilosomes**	Span 80, cholesterol, sodium deoxycholate and stearyl amine; Bilosomes composition + glucomannan-O-Carboxymethyl-Distearyl Phosphatidyl Ethanolamine	Bovine serum albumin	-	-	Oral	[37]

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 [38]. 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 [239]. 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 [39]. 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 [40]. On these premises, the same research group developed a vaccine against hepatitis B virus [41]. 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 [10,240]. Recently, Mašek and colleagues explored multi-layered nanofibrous mucoadhesive films containing liposomes for buccal and sublingual vaccination [42]. 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 [241,242].

Garcia-del Rio and colleagues, developed a muco-adhesive thermogelling hydrogel containing liposomes to be used sublingually after parental prime against *Chlamydia trachomatis* [43]. 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 [243]. 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 [44,244,245]. Nonetheless, no sublingual vaccines against infectious diseases are available on the market [243].

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

Delivery system(s)	Composition	Antigen(s)	Additional adjuvant	Combination with other strategy or technology	Administration route	Reference
PEG-mannosylated cationic liposomes	Mannose-PEG-cholesterol, soy phosphatidylcholine (SPC) and stearyl amine (SA)	Bovine serum albumin (BSA)	Monophosphoryl lipid A	-	Buccal	[38]
PEG-mannosylated cationic liposomes	Mannose-PEG-cholesterol, soy phosphatidylcholine (SPC) and stearyl amine (SA)	Bovine serum albumin (BSA)	Monophosphoryl lipid A	Dis[39]solving microneedles	Buccal	[39]
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	[41]
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)imino diacetic acid)succinyl]	-	-	Mucoadhesive film	Buccal/Sublingual	[42]

	(nickel salt), egg phosphatidylcholine (EPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)					
Cationic liposomes	Trehalose 6,6´dibehenate (TDB), dimethyldioctadecyl ammonium bromide (DDAB)	CTH522	CAF01	Mucoadhesive hydrogel	Sublingual	[43]
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-methoxypolyethylen glycol-2000)-distearoyl-phosphoethanolamine (DSPE-PEG2K)/ N-(carbonyl-methoxypolyethylen glycol-5000)-dipalmitoyl-phosphoethanolamine (DPPE-PEG5K); Liposomes/Pluronic liposomes/PEGylated liposomes	Hemagglutinin (HA, detergent split)	CRX-601	-	Sublingual	[243]

	composition + methylglycol chitosan (MGC)/glycol chitosan (GC)/chitosan 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 [14]. 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 [14]. 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 [246]. 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 nasal or 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 [45]. 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 [46]. 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 [47]. 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 [48]. 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 [49,247]. 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 [50]. 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 [248]. 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 [51]. *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 [249]. 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 [250]. 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 [52,53,251]. 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 [252]. 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* [54]. 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 [253]. 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 [55]. 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 [254]. 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 [255,256]. *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 [257,258]. 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* [56]. 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 [57].

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 [58]. 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* [259]. 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 [60]. 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 [59]. 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	Didodecyldimethyl ammonium bromide (DDAB), soy phosphatidylcholine (SPC) and cholesterol	Fel d 1/ crude cat hair extract (cCE)	-	-	Intranasal	[45]
Anionic liposomes	Dipalmitoylphosphatidylcholine (DPPC), cholesterol and cell-penetrating peptides (CPPs)	Synthetic lipopeptide-based antigen (LCP-1)	-	-	Intranasal	[46]
Cationic liposomes	Dipalmitoylphosphatidylcholine	Peptide derived	Cholic acid	-	Intranasal	[47]

	(DPPC), cholesterol and Didodecyldimethyl ammonium bromide (DDAB)	from Group A *Streptococcus*				
Liposomes	Dipalmitoylphosphatidylcholine (DPPC), cholesterol and oligomannose-dipalmitoyl-phosphoethanolamine (Man3-DPPE)	Hemagglutinin-neuraminidase (HN)	Polyriboinosinic-polyribocytidylic acid [poly(I:C)]	-	Intranasal	[48]
Liposomes	Soy lecithin, cholesterol and alpha tocopherol	Pooled influenza A virus peptides	Monosodium urate (MSU) crystals	-	Intranasal	[49]
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	Inactivated H1N1 vaccine	2',3'-cyclic guanosine monophosphate-adenosine monophosphate (cGAMP)	Freeze-drying	Intranasal	[50]
Anionic liposomes	1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG),	SARS-CoV-2 spike protein (S-protein)	2',3'-cyclic guanosine monophosphate-adenosine monophosphate (cGAMP)	-	Intranasal	[51]

	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DPPE-PEG2000) and cholesterol					
Cationic liposomes	1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-chol)	Ovalbumin (OVA)	-	-	Intranasal	[52]
Cationic liposomes	1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-chol)	Ovalbumin (OVA)	-	-	Intranasal	[53]
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	[251]
Cationic liposomes	1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 3β-[N-(N',N'-dimethylaminoeth	Ovalbumin (OVA)	Oligodeoxynucleotides containing immunostimulatory CpG	-	Intranasal	[252]

	ane)-carbamoyl] cholesterol (DC-chol)		motifs (CpG ODNs)			
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	[54]
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	[55]
PEGylated cationic liposomes;	1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1, 2-	mRNA encoding hemaggl	-	-	Intranasal	[254]

PEG-mannosylated cationic liposomes;	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)-Mannose (DSPE-PEG-Mannose)	utinin (HA)				
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	[257]
Chitosan/Carbopol® 974P NF lipogel	Egg phosphatidylcholine (EPC) and	Ovalbumin (OVA)	-	Gel	Intranasal	[258]

	cholesterol + Chitosan/Carbopol ® 974P NF					
Trimethyl chitosan (TMC)-coated liposomes	Dipalmitoylphosphatidylcholine (DPPC), cholesterol and 1-α-phosphophatidyl-DL-glycerol sodium (PG)	Peptide derived from Group A *Streptococcus*	-	-	Intranasal	[56]
Virosomes	Hemagglutinin (HA) and neuramidase (NA) glicoproteins mixed with egg phosphatidylcholine (EPC) and phosphatidylethanolamine (PE)	HIV-1 Gp41 P1 peptide	-	-	Intranasal	[57]
Galactosylated liposomes	Phosphatidylcholine (PC), cholesterol and galactosyl-1,2-didodecanoyl-sn-glycero-3-phosphoethanolamine (galactosyl-DLPE)	Ovalbumin (OVA)	-	-	Intranasal	[58]
Galactosylated liposomes	Phosphatidylcholine (PC), cholesterol and galactosyl-1,2-didodecanoyl-sn-glycero-3-phosphoethanolamine (galactosyl-DLPE)	Ovalbumin (OVA)	-	-	Intranasal	[259]

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 [260]. 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 [261]. 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 [17,262,263]. 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 [264–269].

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 [61]. 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 [270,271]. 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 [272,273]. 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 [62]. Caimi and co-workers enriched archaeosomes with sodium cholate and obtained ultradeformable archaeosomes for the delivery of imiquimod, a topical adjuvant [274,275]. 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 [276]. 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 [63]. 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 [64]. 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 [277,278]. 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 [264,279]. 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 [65]. 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 [280,281]. Yuan-Chuan Chen adopted these devices for the formulation of a vaccine against plague loading the F1 antigen of *Yersinia pestis* into liposomes [66]. 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 [67]. 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 [68]. 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 [282]. Wu and colleagues exploited these devices in combination with ovalbumin-loaded transfersomes [69]. 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 [70]. 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 [283]. 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 [71]. 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 [72]. 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 [73]. 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 [74]. 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;**	Soy phosphatidylcholine (SPC), cholesterol and stearylamine (if cationic); Liposomes composition + ethanol + stearylamine (if cationic);	Ovalbumin (OVA)	Saponin	-	Transcutaneous, transdermal	[61]

Anionic and cationic transfersomes;	Liposomes composition + sodium cholate + stearylamine (if cationic);					
Transfersomes	Soy phosphatidylcholine (SPC) and Span 80	MSP-1_{19}	-	-	Transcutaneous, transdermal	[270,271]
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)	Ovalbumin (OVA)	-	-	Transcutaneous, transdermal	[62]

	and archaetidylglycerol-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	[274]
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	[276]
Anionic transfersomes;	Soy phosphatidylcholine (SPC) and sodium cholate;	Ovalbumin (OVA)	-	Freeze-drying	Transcutaneous, transdermal	[63]

Ultradeformable archaeosomes;	Total polar archaeolipids (TPA) from *H. tebenquichense*, soy phosphatidylcholine (SPC) 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	[64]
Anionic liposomes incorporating silver nanoparticles	Soy phosphatidylcholine (SPC) and 1,2-dioleoylphosphatidylethanolamine (DOPE)	Ovalbumin (OVA)	-	Iontophoresis	Transcutaneous, transdermal	[65]
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	[66]
Cationic liposomes	Egg phosphatidylcholine (EPC), 1,2-dioleoyl-3-trimethylammonium-propane chloride salt	Ovalbumin (OVA)	Polyriboinosinic-polyribocytidylic acid [poly(I:C)]	Hollow microneedles	Transcutaneous, transdermal	[67]

	(DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)					
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	[68]
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	[69,70]
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	[70]

Cationic liposomes	1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol	Leishmania recombinant antigen LiHyp1	Cytosine-phosphate-guanine oligodeoxynucleotide (CpG ODN)	Dissolving microneedles, freeze-drying	Transcutaneous, transdermal	[283]
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	[71]
Cationic liposomes	Hydrogenated egg phosphatidylcholine (HEPC), cholesterol and octadecylamine	Ovalbumin (OVA)	Platycodin (PD)	Dissolving microneedles	Transcutaneous, transdermal	[72]
Anionic/cationic ethosomes	Soy phosphocholine (SPC), cholesterol, ethanol and stearylamine (SA) (if cationic)	Ovalbumin (OVA)	Saponin	Hydrogel	Transcutaneous, transdermal	[73]
Galactosylated chitosan-modified ethosomes	Egg phosphatidylcholine (EPC), cholesterol, octadecylamine, hyaluronic acid and ethanol	Ovalbumin (OVA)	-	Mat	Transcutaneous, transdermal	[74]

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 [284]. 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	Plasmodium Falciparum Malaria Protein	Parenteral (intramuscular)	Walter Reed Army Institute of	**I**

	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*)	FMP012 with liposomal AS01B		Research (WRAIR), United States Agency for International Development (USAID), Military Infectious Diseases Research Program (MIDRP) and GlaxoSmithKline (GSK)	
	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	PAMVAC vaccine antigen	Parenteral (intramuscular)	University Hospital Tuebingen	I

	Malaria Vaccine Candidate PAMVAC Variously Adjuvanted (PAMVAC) (*NCT02647489*)	adjuvanted with GLA-LSQ			
	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 (*NCT04826094*)	HIV Env-C DNA and protein vaccines adjuvanted with ALF43	Parenteral (intramuscular)	National Institute of Allergy and Infectious Diseases (NIAID)	**I**
Not recruiting	Novel RNA-nanoparticle Vaccine for the	Autologous total tumor mRNA-	Parenteral (intravenous)	University of Florida	**I**

	Treatment of Early Melanoma Recurrence Following Adjuvant Anti-PD-1 Antibody Therapy (*NCT05264974*)	loaded DOTAP liposome vaccine			
	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	Parenteral (intramuscular)	U.S. Army Medical Research and Development Command, Armed Forces	I

		glycoprotein HIV-1 adjuvanted with ALFQ		Research Institute of Medical Sciences, ThailandMahidol University, Duke University University of Maryland (Baltimore) and Case Western Reserve University	
	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-IVA Cervical Cancer, the IMMUNOCERV Trial	Liposomal HPV-16 E6/E7 Multipeptide Vaccine PDS0101	Parenteral (subcutaneous)	M.D. Anderson Cancer Center	**II**

	(*NCT04580771*)				
	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	Liposome Formulated mRNA Vaccine in Combination	Parenteral (intravenous)	University Medical Center Groningen	**I**

	Vaccine in Combination With (Neo-) Adjuvant Chemotherapy (OLIVIA) (*NCT04163094*)	With (Neo-) Adjuvant Chemotherapy			
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 [285]. 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 [286]. 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 [9]. 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 [287]. 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 [288]. 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 [289]. 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 [290]. Careful attention must also be paid on the composition of the nanocarrier as it can both affect colloidal/thermostability and cytotoxicity [33]. 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 [256]. 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 [291]. 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 [292]. 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 [75]. 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 [293,294]. 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.

***Interesting introductive chapter on the immune systems.**

[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.

****Extremely detailed review that offers insights into the immune system in the lungs, as well as micro and nanocarriers for pulmonary vaccine delivery.**

[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.

***Intriguing mini-review that delineates the primary instability issues associated with vaccines and explores techniques to enhance their stability.**

[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.

***Interesting review describing how the physical properties of particles can affect the immune response and can be modified to design better vaccines.**

[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.

Updated review highlighting the most recent advances in active and passive delivery through the skin.

[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.

***Review discussing the prospects of liposomal delivery along with innovation and current challenges**.

[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.

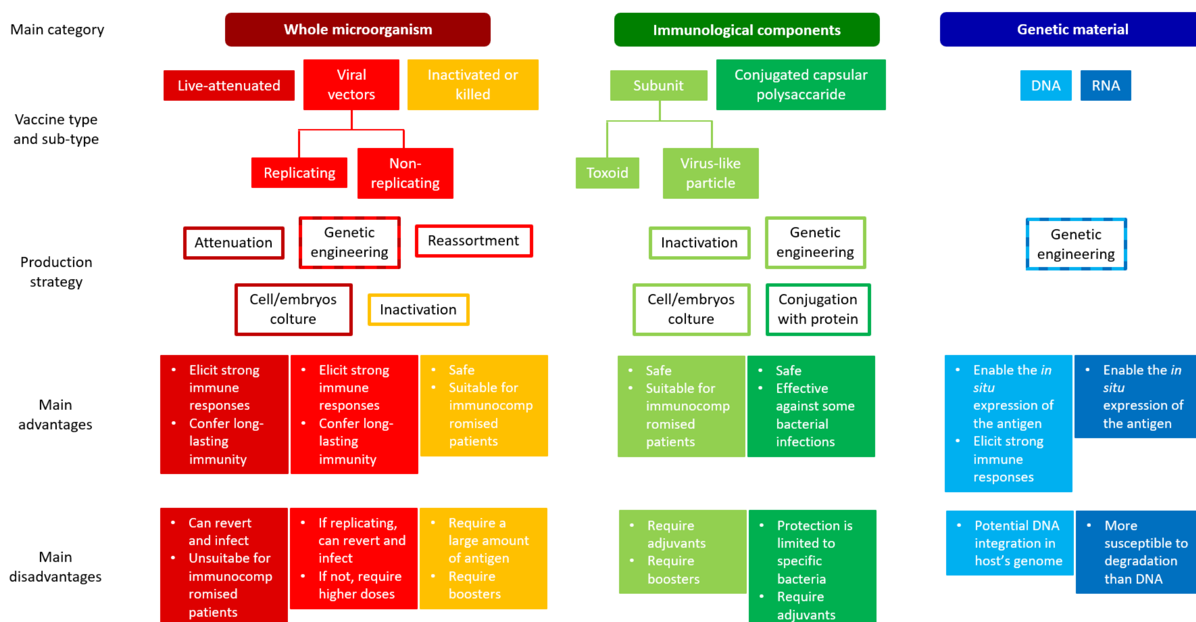
Chapter 4 – Summary

4.1 English summary

Vaccination, which is a milestone in the evolution of public health globally, has represented and continues to represent a true revolution. Its influence extends far beyond simply reducing global deaths caused by infectious diseases; it manifests itself as a bulwark protecting the most vulnerable groups through the powerful mechanism of herd immunity. In this context, vaccination is not only a public health strategy, but also a valuable ally in mitigating the economic pressure on hospitals and health systems worldwide. Its implications therefore go beyond the individual sphere, embracing a far-reaching collective impact. The constant evolution of vaccination practice not only saves lives but helps shape a future in which prevention through immunisation becomes a fundamental lever of social and global well-being.

This practice, today fundamental to the resilience of global communities, has extremely interesting historical roots and evolutionary process. Its origins are intertwined with the older (17th century) practice of variolation and it shares its basic idea: inoculate to prevent. It was Edward Jenner, a century later, who made the technique more effective and safer by inoculating cowpox instead of smallpox. Recognising the greatness of the discovery, Pasteur renamed the practice 'vaccination' (cow = vaccinia).

Table 1. Types of traditional vaccines, production methods, main advantages and disadvantages.



Despite the numerous types of vaccines that can be used, most of them are administered via injection routes (intradermal, subcutaneous or intramuscular). To date, only a handful of vaccines are exceptions (FluMist/Fluenz® and Nasovac™, by the nasal route; Vaxchora®, Dukoral®, Rotarix™, RotaTeq®, Vivotif® and polio vaccine, by the oral route). A clear limitation of traditional vaccines is therefore the mode of administration. Using an injection route of administration means using a needle and, therefore, inevitably running into the following problems: 1) pain, 2) discomfort, 3) limited or no local protection, 4) local irritation, 5) local infection, 6) need for qualified personnel, 7) limited access, 8) difficult disposal, 9) high costs.

Considering the considerable period of time, averaging between 10 and 15 years, required to develop a vaccine from scratch, it is clear that it is of paramount importance for researchers to find viable alternatives. These alternatives have two main objectives: a) to simplify the administration of existing vaccines and b) to enhance their safety and efficacy. The awareness of this time challenge in the vaccine development process emphasises the urgency of exploring innovative avenues and pragmatic strategies.

The most studied non-invasive routes of administration include oral, sublingual, buccal, inhalation (nasal and pulmonary) and dermal. These options offer numerous advantages, including (1) significantly improved patient compliance, (2) avoidance of unwanted effects at the site of administration (such as pain, irritation or infection), (3) reduced dependence on trained personnel, and, most importantly, (4) guaranteed local as well as systemic protection. This revolutionary approach not only responds to current needs but opens up new perspectives for an effective and accessible dissemination of immunisations worldwide.

However, research in the field of vaccination has also gone beyond the exclusive investigation of alternative routes of administration, as it has become apparent that these approaches alone are not sufficient to guarantee a significant improvement in the efficacy and safety of vaccines. In response to this need, the simultaneous exploration of new systems and technologies proved necessary. Among the many options, nanotechnologies have emerged as a promising prospect for raising the standards of vaccination, and among them, liposomes emerge as one of the most widely studied transport nanosystems.

Liposomes, structured as spherical phospholipid systems, have a phospholipid bilayer enclosing an aqueous core. Their most distinctive and advantageous feature in the context of vaccination lies in their functional duality. First, they act as effective transport and protection systems for the antigen, facilitating its precise delivery and preserving its integrity. Secondly, they can play an active role in enhancing the immune response to the antigen, acting as intrinsic adjuvants that can further activate the immune system and increase the magnitude of the response. This ability to simultaneously act as transport vectors and modulators of the immune response gives them a crucial role in innovating vaccine strategies. In this context, exploring the potential of liposomes represents a key chapter in the progress towards more advanced and efficient vaccination.

It is undeniable, however, that liposomes, while representing a promising frontier in vaccine delivery, structurally present certain limitations. The assessment of these limitations depends, to a large extent, on the route of administration selected for their use. Therefore, research has embarked on a path of considerable complexity, evolving from an initial phase of using generic liposomes to a subsequent phase of fervent and continuous innovation in compositions, aimed at optimising the performance of these vesicular systems and making them more resistant, mucoadhesive, etc. The crucial role of the route of administration, in this regard, becomes clear as the liposomal formulation has to interact with different enzyme systems and overcome specific barriers, each with the potential to affect their structural integrity to a greater or lesser extent. Consequently, it is imperative to customise and enrich the composition of these systems, adapting them to the specific challenges posed by the chosen route of administration.

This need for targeted adaptation is crucial to ensure that liposomes are able to perform their function effectively following administration. This is why, from the literature review covering the last decade (2013-2023), a wide variety of modified liposomes produced following years of research studies can be seen (Fig. 1). Specifically, one can identify anionic, neutral or cationic liposomes (whose charge can affect cell interaction, mucoadhesivity, etc.), liposomes containing other nanoparticles (used as shields and stabilisers), stratosomes (stabilised and reinforced with multiple layers, of various natures, mainly to cope with the oral route), bilosomes (made gastro-resistant with bile salts), transfersomes (made ultra-deformable with edge activators to overcome the skin), ethosomes (made deformable with ethanol to overcome the skin), ligand-conjugated liposomes (to pursue active, specific targeting of target cells and tissues), metal-chelating liposomes (to exploit electricity as a means of transport through the skin) polymer-matrix-coated liposomes (to achieve adhesiveness or resistance in harsh environments), virosomes (with viral-like structure), archaeosomes and ultra-deformable archaeosomes (obtained from particular lipids, more resistant under particular conditions, either ultra-deformable or not).

In the realm of vaccine administration, considerations extend beyond the formulation to encompass storage stability and delivery methods. While liquid liposomal formulations offer versatility across various administration routes, their storage presents significant challenges. The susceptibility to temperature fluctuations and the need for specialized storage conditions make liquid vaccines complex and potentially less viable for widespread use. In response, there is a growing inclination towards employing solid or semi-solid formulations. These alternatives, such as lyophilized powders or tablets, offer enhanced stability during storage, addressing concerns related to degradation and facilitating ease of transportation, especially to remote or resource-limited areas. The use of solid forms not only extends the shelf life of vaccines but also aligns with evolving technologies, including nanoparticle encapsulation and innovative delivery methods such as microneedle patches. These advancements collectively contribute to a more robust and accessible landscape for vaccine development and distribution, ensuring the efficacy and availability of vaccines on a global scale.

Nonetheless, a third element must be taken into account: a true optimization of mass vaccination campaigns can be achieved only by integrating pharmaceutical formulations with medical devices. Notably, the incorporation of inhalers and nasal spray devices stands out as viable alternatives for large-scale immunization initiatives. Inhalers provide an efficient, needle-free method for precise vaccine delivery, catering to individuals averse to needles. Similarly, nasal spray devices offer a convenient, non-invasive option, potentially simplifying the administration process and expanding acceptability. Concurrently, wearable technologies like smart patches, featuring microneedles or electronic monitoring, continue to play a crucial role in refining vaccination approaches. This strategic blending of pharmaceuticals with advanced medical devices, including inhalers and nasal spray devices, not only streamlines vaccination procedures but also advances a holistic approach to public health monitoring, promoting efficiency and positive health outcomes.

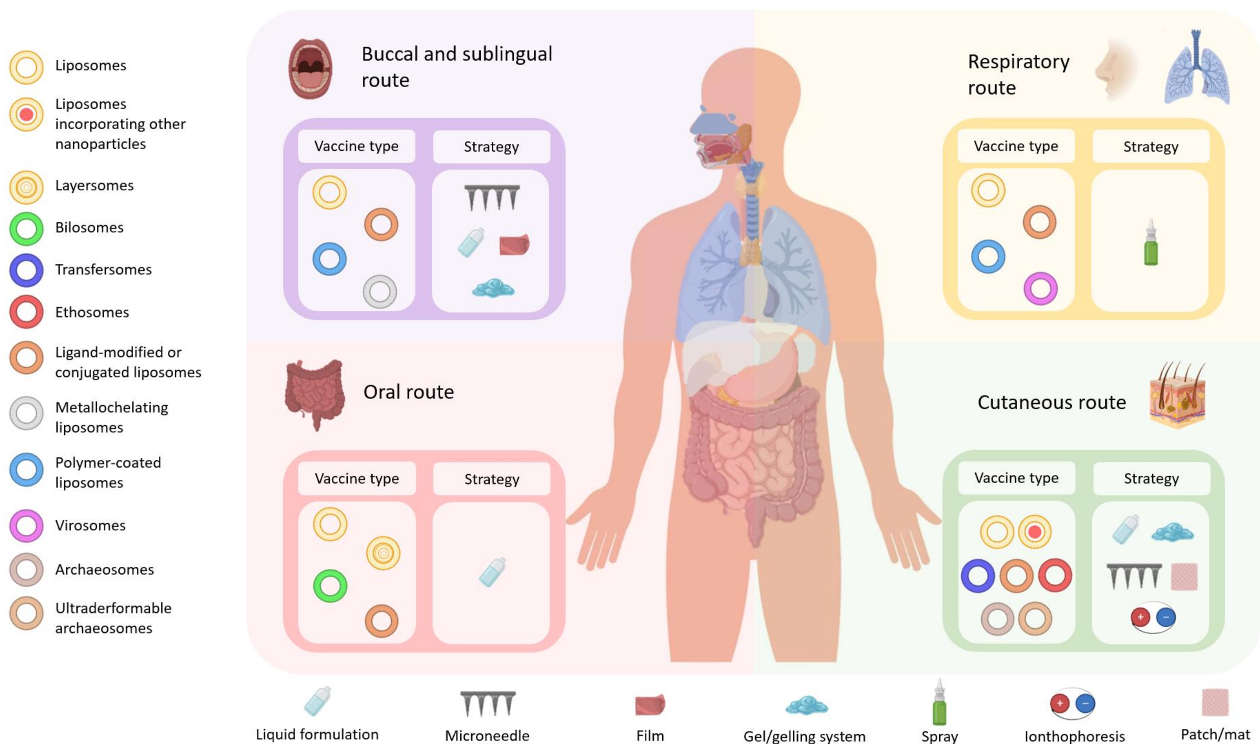


Figure 1. Liposome-based vaccines developed in the last decade (2013-2023) and tested, through minimally or non-invasive routes of administration, alone or in combination with medical devices.

In the light of all this, this thesis sought to combine liposomes with strategies or devices capable of favouring the use of non-invasive routes of administration such as the skin (via hydrogel), nasal (via nasal spray) and pulmonary (via dry powder inhalation device), aiming to enhance local protection precisely at those main entry points into the body exploited by pathogens. Particular attention was paid to the composition of the vesicles in each study, adapting and modifying it from time to time in order to achieve optimal transport performance according to the selected route. As innovative elements, we sought to use novel compositions, adapted and researched according to the route of administration and favouring the use of lipids as natural as possible, and to exploit more eco-friendly preparation methods. Most studies, in fact, involve the use of synthetic and particularly expensive lipids and the use of methods such as lipid film evaporation, ethanol injection and microfluidics, all of which exploit organic solvents that could generate toxicity following in vivo administration. Instead, in this thesis, direct sonication was used as an alternative method that, in a single step, makes it possible to obtain phospholipid vesicles of comparable size and efficiency to the aforementioned methods, but without the need to remove the organic solvent in an additional step.

In the first study, formulations for skin use were created. Transfersomes prepared with sodium deoxycholate and Lipoid S75 were enriched with glycerol, hyaluronic acid or their combination to improve ovalbumin accumulation in the dermis and epidermis. All formulations were highly biocompatible and stable for up to 9 months. No inflammatory effects were detected during exposure of antigen-presenting cells to these formulations. Antigen presentation, on the other hand, was not enhanced as the response did not differ significantly from that of unencapsulated antigen. However, in a real-world context, they are expected to perform better

on the skin. Transfersomes, in fact, resulted in higher ovalbumin deposition than solution in any condition (non-occlusive, occlusive with adherent film and occlusive with hydrogel) when studied with Franz's cells. Transfersomes enriched with hyaluronic acid and glycerol-hyaluronic acid were more effective than transfersomes with glycerol alone when compared under non-occlusive and occlusive (adherent film) conditions. Finally, enriched transfersomes hydrogels were found to be the most effective strategy, further enhancing ovalbumin deposition at the dermis and epidermis level, and could be promising in a real-world setting if properly applied.

In the second study, nasal formulations were produced. Anionic and cationic DOTAP liposomes were produced using Phospholipon 90G and cholesterol, with and without 1,2-diolyl-3-trimethylammonium-propane (DOTAP), respectively. The dispersions remained stable for up to 3 months. The cationic DOTAP liposomes had superior interactions and mucus uptake compared to the anionic liposomes. Furthermore, they showed high adjuvanticity, leading to increased production of interleukin-2. No inflammation was detected. Although both anionic and cationic DOTAP liposomes had a good sprayability (90% of the droplets had a size of about 100 μm), the latter showed a higher deposition in the turbinates, where the immune response takes place. Considering the higher deposition and mucoadhesivity, DOTAP liposomes were the most suitable formulation to research local nasal protection.

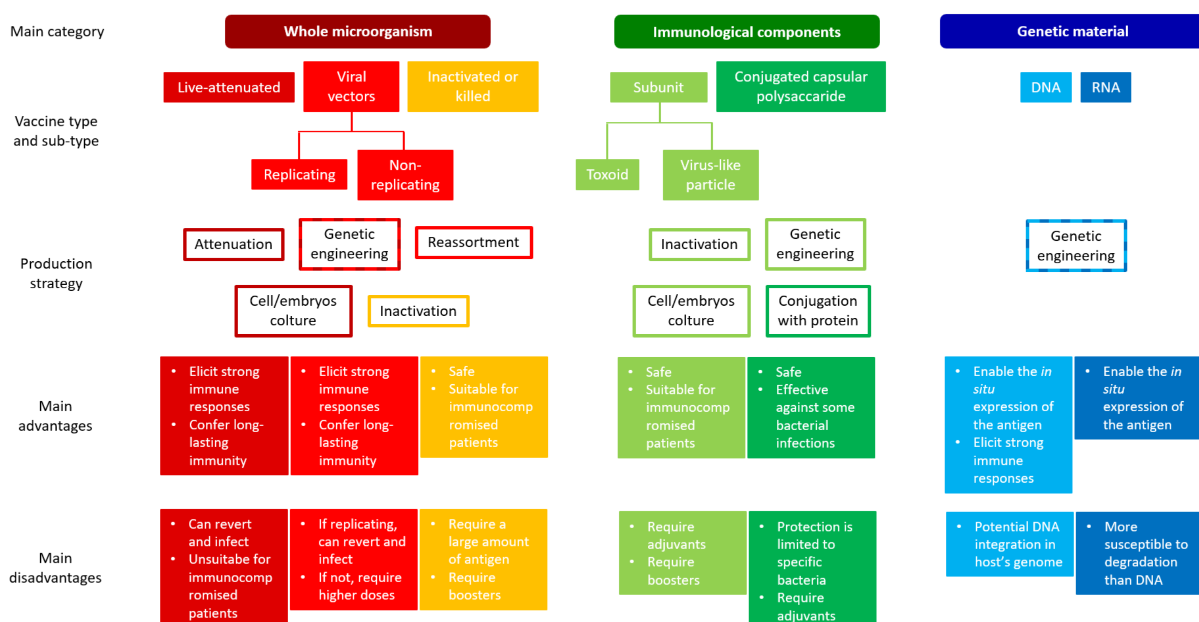
In the third and final study, lung formulations were made. After an initial screening on the lipids and cryoprotectants to be used, anionic liposomes prepared with Lipoid S PC-3 and DPPC (SPC3 liposomes and DPPC liposomes, respectively) were selected as the best candidates. These were then lyophilised with Respitose® or inulin to improve their stability and to be able to take advantage of pulmonary administration via a commercial dry powder inhaler (Aerolizer®). A further dilution of these powders with Respitose® was necessary to adjust the dose of ovalbumin. By means of laser diffraction, it was shown that this dilution increased the fraction of particles suitable for pulmonary administration as it increased the volume of particles with a diameter < 5 μm, thus confirming the anti-dusting effect of Respitose. Profiles of healthy people, asthmatics and people with chronic obstructive pulmonary disease were then studied, showing that the fine particle fraction, i.e. the percentage of the dose that actually reaches the lungs, was higher for the healthy condition than for the pathological condition.

4.2 Italian summary

La vaccinazione, una pietra miliare nell'evoluzione della salute pubblica a livello globale, ha rappresentato e continua a rappresentare una vera e propria rivoluzione. La sua influenza si estende ben oltre la semplice riduzione dei casi di morte globale causati da malattie infettive; essa si manifesta come un baluardo che protegge le categorie più vulnerabili attraverso il potente meccanismo di immunità di gregge. In questo contesto, la vaccinazione si configura non solo come una strategia di salute pubblica, ma anche come un prezioso alleato nel mitigare la pressione economica che grava su ospedali e sistemi sanitari a livello mondiale. Le sue implicazioni vanno pertanto oltre la sfera individuale, abbracciando un impatto collettivo di vasta portata. La costante evoluzione della pratica vaccinale non solo salva vite, ma contribuisce a plasmare un futuro in cui la prevenzione attraverso l'immunizzazione diventa una fondamentale leva di benessere sociale e globale.

Questa pratica, oggi fondamentale per la resilienza delle comunità mondiali, ha delle radici storiche ed un processo evolutivo estremamente interessanti. Le sue origini si intrecciano con la più antica pratica (17th secolo) della variolazione e ne condivide l'idea di base: inoculare per prevenire. Fu Edward Jenner, un secolo più tardi, a rendere la tecnica più efficace e sicura inoculando cowpox al posto di smallpox. Riconoscendo la grandezza della scoperta, Pasteur rinominò la pratica "vaccinazione" (cow = vaccinia). Con l'avanzare della tecnologia e della ricerca scientifica poi, nel corso degli anni, vennero pian piano sviluppati innumerevoli tipologie di vaccini (Table 1). Si è progressivamente passati dall'uso di vaccini contenenti microrganismi a quelli contenenti loro frazioni, per giungere infine all'impiego di vaccini preparati con del materiale genetico. Tali differenze nella composizione si sono rese necessarie per migliorare efficacia e sicurezza del vaccino, nonché per ottimizzarne il processo produttivo e migliorarne il rapporto costo-beneficio.

Table 1. Tipi di vaccini tradizionali, metodiche di produzione, principali vantaggi e svantaggi.



Nonostante le numerose tipologie di vaccini utilizzabili, la maggior parte di essi viene somministrata attraverso vie iniettive (intradermica, sottocutanea o intramuscolare). Ad oggi, fanno eccezione solamente una manciata di vaccini (FluMist/Fluenz® and Nasovac™, per via nasale; Vaxchora®, Dukoral®, Rotarix™, RotaTeq®, Vivotif ® e vaccino per la poliomielite, per via orale). Un chiaro limite dei vaccini tradizionali è pertanto rappresentato dalla modalità di somministrazione. Utilizzare una via di somministrazione iniettiva significa utilizzare un ago e, quindi, inevitabilmente andare incontro ai seguenti problemi: 1) dolore, 2) disagio, 3) limitata o assente protezione locale, 4) irritazione locale, 5) infezione locale, 6) necessità di personale qualificato, 7) accesso limitato, 8) smaltimento difficoltoso, 9) costi elevati.

Considerando il notevole periodo di tempo, mediamente compreso tra 10 e 15 anni, richiesto per lo sviluppo ex novo di un vaccino, è evidente che risulta di primaria importanza per i ricercatori individuare valide alternative. Queste alternative mirano a due obiettivi principali: a) semplificare la somministrazione dei vaccini già esistenti e b) potenziarne sicurezza ed efficacia. La consapevolezza di questa sfida temporale nel processo di sviluppo vaccinale enfatizza l'urgenza di esplorare vie innovative e strategie pragmatiche.

Le vie di somministrazione non invasive maggiormente studiate comprendono la via orale, sublinguale, buccale, inalatoria (nasale e polmonare) e cutanea. Queste opzioni offrono numerosi vantaggi, tra cui 1) il significativo miglioramento della compliance del paziente, 2) l'evitamento di effetti indesiderati nella sede di somministrazione (quali dolore, irritazione o infezione), 3) la riduzione della dipendenza da personale qualificato e, soprattutto, 4) la garanzia di una protezione sia locale che sistemica. Questo approccio rivoluzionario non solo risponde alle esigenze attuali, ma apre nuove prospettive per un'efficace e accessibile diffusione delle immunizzazioni in tutto il mondo.

La ricerca nell'ambito della vaccinazione si è però spinta anche oltre l'indagine esclusiva delle vie di somministrazione alternative, poiché è emerso che tali approcci da soli non sono sufficienti per garantire un miglioramento significativo nell'efficacia e nella sicurezza dei vaccini. In risposta a questa esigenza, si è rivelata necessaria l'esplorazione contemporanea di nuovi sistemi e tecnologie. Tra le molteplici opzioni, le nanotecnologie si sono affermate come una prospettiva promettente per elevare gli standard della vaccinazione, e tra queste, i liposomi emergono come uno dei nanosistemi di trasporto più ampiamente studiati.

I liposomi, strutturati come sistemi fosfolipidici sferici, presentano un doppio strato fosfolipidico che racchiude un core acquoso. La loro caratteristica più distintiva e vantaggiosa nel contesto della vaccinazione risiede nella loro dualità funzionale. In primo luogo, agiscono come efficaci sistemi di trasporto e protezione per l'antigene, facilitandone la veicolazione precisa e preservandone l'integrità. In secondo luogo, possono svolgere un ruolo attivo nel potenziare la risposta immunitaria all'antigene, agendo come adiuvanti intrinseci in grado di attivare ulteriormente il sistema immunitario e incrementare la grandezza della risposta. Questa capacità di agire simultaneamente come vettori di trasporto e modulatori della risposta immunitaria conferisce loro un ruolo cruciale nell'innovazione delle strategie vaccinali. In questo contesto, l'esplorazione delle potenzialità dei liposomi rappresenta un capitolo fondamentale nel progresso verso una vaccinazione più avanzata ed efficiente.

È innegabile però che i liposomi, pur rappresentando una promettente frontiera nella somministrazione di vaccini, presentino strutturalmente alcuni limiti. La valutazione di questi limiti dipende, in larga misura, dalla via di somministrazione selezionata per il loro impiego. Pertanto, la ricerca ha intrapreso un percorso di notevole complessità, evolvendo da una fase iniziale di utilizzo di liposomi generici a una successiva fase di fervente e continua innovazione nelle composizioni, finalizzata a ottimizzare le performance di questi sistemi vescicolari e a renderli più resistenti, mucoadesivi ecc. Il ruolo cruciale della via di somministrazione, a tal proposito, emerge con chiarezza poiché la formulazione liposomiale si trova a dover interagire con diversi sistemi enzimatici e a superare barriere specifiche, ciascuna con il potenziale di influire in maniera più o meno marcata sulla loro integrità strutturale. Di conseguenza, si rivela imperativo personalizzare e arricchire la composizione di tali sistemi, adattandoli alle sfide specifiche poste dalla via di somministrazione prescelta.

Questa necessità di adattamento mirato è fondamentale per garantire che i liposomi siano in grado di svolgere efficacemente la loro funzione a seguito della somministrazione e sono pertanto fondamentali al fine di garantirne l'efficacia. È per questo che, dall'analisi fatta in letteratura e che copre l'ultimo decennio (2013-2023), è possibile constatare un'ampia varietà di liposomi modificati prodotti a seguito di anni di studi di ricerca (**Fig. 1**). Nello specifico, si possono individuare liposomi anionici, neutri o cationici (la cui carica può influire per quanto concerne interazione cellulare, mucoadesività ecc.), liposomi contenenti altre nanoparticelle (impiegati come scudi e stabilizzanti), stratosomi (stabilizzati e rinforzati con molteplici strati, di varia natura, soprattutto per far fronte alla via orale), bilosomi (resi gastro-resistenti con sali biliari), transfersomi (resi ultra-deformabili con edge activators per superare la cute), etosomi (resi deformabili con etanolo per superare la cute), liposomi coniugati a ligandi (per perseguire il targeting attivo, specifico su cellule e tessuti bersaglio), liposomi chelanti metalli (per sfruttare l'elettricità come mezzo di trasporto attraverso la pelle), liposomi rivestiti da matrici polimeriche (per ottenere adesività o resistenza negli ambienti più duri), virosomi (con struttura simil virale), archeosomi e archeosomi ultradeformabili (ottenuti con lipidi particolari, più resistenti in particolari condizioni, ultradeformabili o meno).

Come per i farmaci, anche i vaccini hanno bisogno di strategie e dispositivi per poter essere somministrati adeguatamente. Se la formulazione liposomiale liquida può essere adeguata potenzialmente per tutte le vie, è anche vero che quelle liquide sono le formulazioni più problematiche in termini di conservazione. Strategie che adottano forme solide o semisolide vengono quindi sempre più utilizzate.

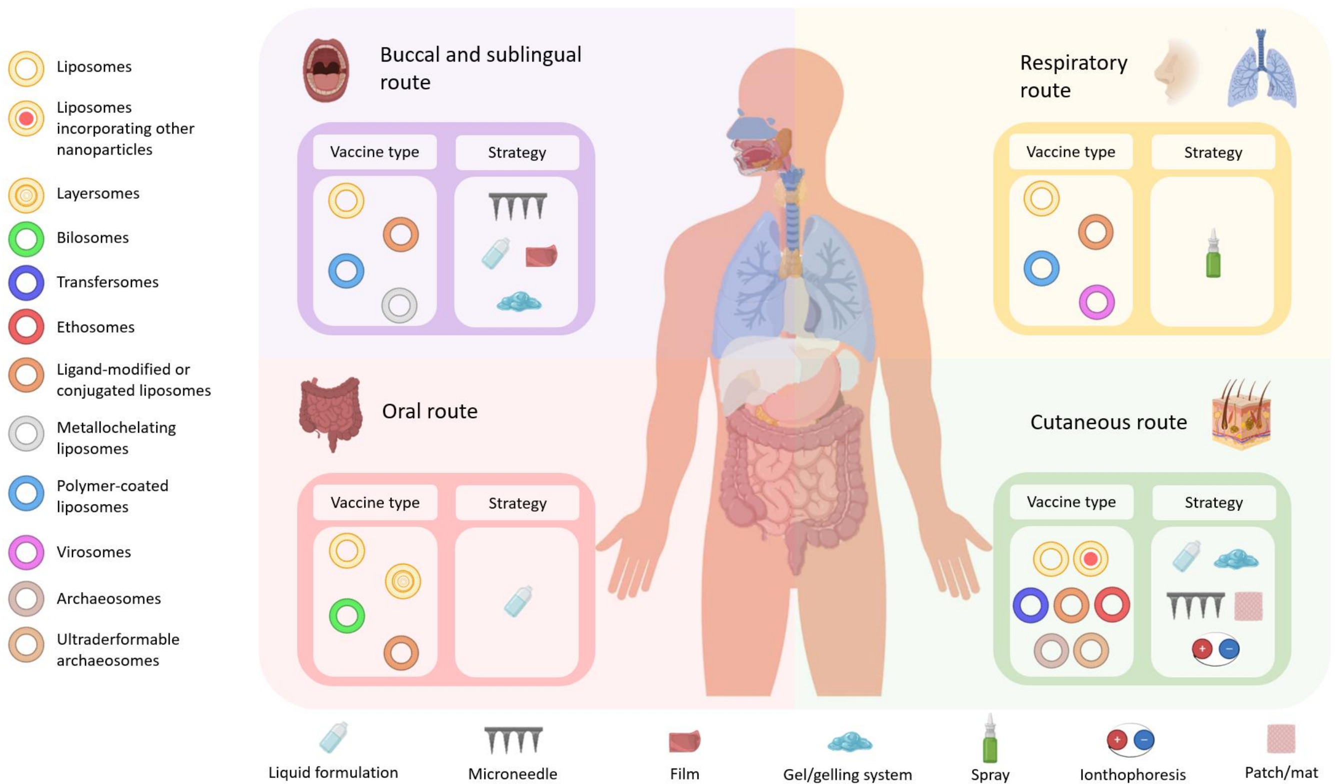


Figura 1. Vaccini basati su liposomi sviluppati nell'ultimo decennio (2013-2023) e testati, attraverso vie di somministrazione poco o non invasive, da soli o in combinazione con dispositivi medici.

Alla luce di tutto questo, nella tesi in questione si è cercato di combinare liposomi e strategie o dispositivi in grado di favorire l'utilizzo di vie di somministrazione non invasive quali quella cutanea (tramite idrogel), nasale (tramite spray nasale) e polmonare (tramite dispositivo inalatorio di polvere secca) mirando a potenziare la protezione locale proprio a livello di quelli che sono i principali punti di ingresso nell'organismo sfruttati dagli agenti patogeni. Particolare attenzione è stata rivolta alla composizione delle vescicole in ogni studio, adattandola e modificandola di volta in volta in modo da ottenere performance ottimali nel trasporto secondo la via selezionata. Come elementi di innovazione si è quindi cercato di utilizzare delle composizioni nuove, adattate e studiate in base alla via di somministrazione e prediligendo l'uso di lipidi quanto più naturali possibile, e di sfruttare delle metodiche di preparazione più eco-sostenibili. La maggior parte degli studi, infatti, prevede l'uso di lipidi sintetici e particolarmente costosi e l'utilizzo di metodiche quali evaporazione del film lipidico, iniezione di etanolo e microfluidica che sfruttano tutte solventi organici che potrebbero generare tossicità a seguito di somministrazione in vivo. In questa tesi si è ricorso invece alla sonicazione diretta come metodo alternativo che, in un unico step, consente di ottenere delle vescicole fosfolipidiche dalle dimensioni ed efficienze paragonabili alle suddette metodiche senza però la necessità di dover rimuovere il solvente organico con un ulteriore step.

Nel primo studio sono state realizzate delle formulazioni ad uso cutaneo. I transfersomi preparati con sodio deossicolato e Lipoid S75 sono stati arricchiti con glicerolo, acido ialuronico o la loro combinazione per migliorare l'accumulo di ovalbumina in derma ed epidermide. Tutte le formulazioni sono risultate altamente biocompatibili e stabili fino a 9 mesi. Non sono stati rilevati effetti infiammatori durante l'esposizione delle cellule presentanti

l'antigene a queste formulazioni. La presentazione dell'antigene invece non è stata potenziata poiché la risposta non differiva significativamente da quella dell'antigene non incapsulato. Tuttavia, in un contesto reale, ci si aspetta che abbiano prestazioni migliori sulla pelle. I transfersomi, infatti, hanno portato ad una maggiore deposizione di ovalbumina rispetto alla soluzione in qualsiasi condizione (non occlusiva, occlusiva con pellicola aderente e occlusiva con idrogel) quando studiati con le celle di Franz. Gli transfersomi arricchiti con acido ialuronico e glicerolo-acido ialuronico sono risultati più efficaci rispetto agli transfersomi con solo glicerolo quando confrontati in condizioni non occlusive e occlusive (pellicola aderente). Gli idrogel imbevuti di transfersomi arricchiti sono infine risultati la strategia più efficace, potenziando ulteriormente la deposizione di ovalbumina a livello di derma ed epidermide, e potrebbero essere promettenti in un contesto reale se opportunamente applicati.

Nel secondo studio sono state realizzate delle formulazioni nasali. Sono stati prodotti liposomi anionici e cationici utilizzando Phospholipon 90G e colesterolo, con e senza 1,2-dioloile-3-trimetilammonio-propano (DOTAP), rispettivamente. Le dispersioni sono rimaste stabili fino a 3 mesi. I liposomi cationici DOTAP hanno avuto interazioni e assorbimento di muco superiori rispetto ai liposomi anionici. Inoltre, hanno mostrato un'elevata adiuvanticità, portando a una maggiore produzione di interleuchina-2. Non sono state rilevate infiammazioni. Nonostante entrambi i liposomi DOTAP anionici e cationici avessero una buona spruzzabilità (il 90% delle gocce aveva una dimensione di circa 100 μm), questi ultimi hanno mostrato una maggiore deposizione nei turbinati, dove si svolge la risposta immunitaria. Considerando la maggiore deposizione e mucoadesività, i liposomi DOTAP sono risultati la formulazione più adatta per ricerca una protezione nasale locale.

Nel terzo e ultimo studio sono state realizzate delle formulazioni polmonari. Dopo un iniziale screening sui lipidi e i crioprotettori da usare, sono stati selezioni come migliori candidati i liposomi anionici preparati con Lipoid S PC-3 e DPPC (rispettivamente SPC3 liposomi e DPPC liposomi). Questi sono quindi stati liofilizzati con Respitose® o inulina per migliorarne la stabilità e poter sfruttare la somministrazione polmonare tramite un inalatore a polvere secca commerciale (Aerolizer®). Un'ulteriore diluizione di queste polveri con Respitose® è stata necessaria per regolare la dose di ovalbumina. Tramite diffrazione laser, si è dimostrato che tale diluizione aumentava la frazione di particelle adatte alla somministrazione polmonare poiché aumentava il volume delle particelle di diametro < 5 μm, confermando quindi l'effetto antiaderente del Respitose sulle polveri. Profili di persone sane, asmatiche e con malattia polmonare ostruttiva cronica sono poi stati studiati evidenziando che la frazione di particelle fini, ovvero la percentuale della dose che effettivamente raggiunge i polmoni, era più alta per la condizione di salute rispetto a quella patologica. Allo stesso modo, la frazione di particelle extrafini è stata influenzata dalla condizione, ad eccezione dei liposomi liofilizzati con inulina. Tutti gli studi *in vitro* sono poi stati condotti su una linea cellulare di macrofagi alveolari (RAW 264.7). Tutte le formulazioni sono risultate biocompatibili. L'assorbimento è stato maggiore per le vescicole rispetto all'ovalbumina non incapsulata, specialmente dopo le prime ore di esposizione, come confermato da citometria a flusso e microscopia a fluorescenza. Tutte le formulazioni hanno garantito la risposta dei macrofagi all'ovalbumina, rimanendo ben tollerati. Gli SPC3 liposomi liofilizzati con Respitose sono però stati l'unico tipo di formulazione in grado di potenziare la produzione di interleuchina-6, nota per favorire la differenziazione delle cellule

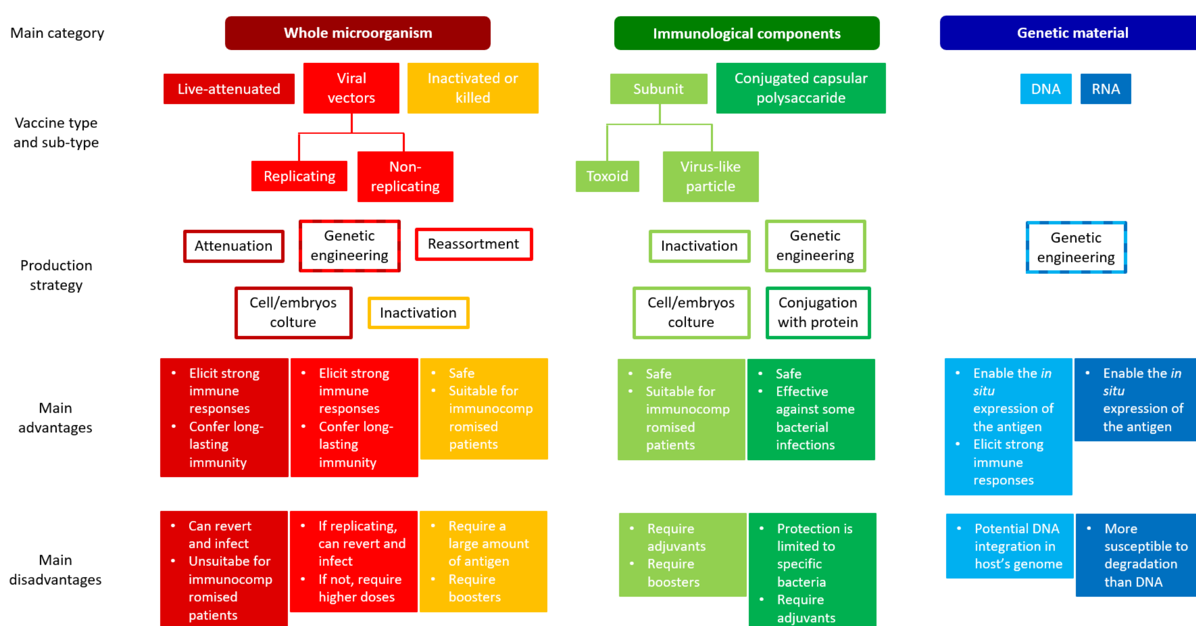
B in plasmacellule e l'attivazione delle cellule T citotossiche. Allo stesso modo, la frazione di particelle extrafini è stata influenzata dalla condizione, ad eccezione dei liposomi liofilizzati con inulina. Tutti gli studi in vitro sono stati condotti su una linea cellulare di macrofagi alveolari (RAW 264.7). Tutte le formulazioni sono risultate biocompatibili. L'assorbimento è stato maggiore per le vescicole rispetto all'ovoalbumina non incapsulata, soprattutto dopo le prime ore di esposizione, come confermato dalla citometria a flusso e dalla microscopia a fluorescenza. Tutte le formulazioni hanno garantito la risposta dei macrofagi all'ovalbumina e sono rimaste ben tollerate. Tuttavia, i liposomi SPC3 liofilizzati con Respitose sono stati l'unico tipo di formulazione in grado di aumentare la produzione di interleuchina-6, nota per promuovere la differenziazione delle cellule B in plasmacellule e l'attivazione delle cellule T citotossiche.

4.3 Spanish summary

La vacunación, un hito en la evolución de la salud pública mundial, ha representado y sigue representando una auténtica revolución. Su influencia va mucho más allá de la simple reducción de las muertes globales causadas por enfermedades infecciosas; se manifiesta como un baluarte que protege a los grupos más vulnerables a través del poderoso mecanismo de la inmunidad de rebaño. En este contexto, la vacunación no es sólo una estrategia de salud pública, sino también un valioso aliado para mitigar la presión económica que sufren los hospitales y los sistemas sanitarios de todo el mundo. Sus implicaciones, por tanto, van más allá de la esfera individual, abarcando un impacto colectivo de gran alcance. La constante evolución de la práctica de la vacunación no sólo salva vidas, sino que contribuye a forjar un futuro en el que la prevención a través de la inmunización se convierta en una palanca clave del bienestar social y global.

Esta práctica, hoy fundamental para la resiliencia de las comunidades de todo el mundo, tiene unas raíces históricas y un proceso evolutivo sumamente interesantes. Sus orígenes se entrelazan con la práctica más antigua (siglo XVII) de la variolación y comparte su idea básica: inocular para prevenir. Fue Edward Jenner, un siglo más tarde, quien hizo la técnica más eficaz y segura al inocular la viruela vacuna en lugar de la viruela. Reconociendo la grandeza del descubrimiento, Pasteur rebautizó la práctica como "vacunación" (vaca = vaccinia). A medida que la tecnología y la investigación científica fueron avanzando con el paso de los años, se desarrollaron gradualmente innumerables tipos de vacunas (Tabla 1). Se pasó gradualmente del uso de vacunas que contenían microorganismos a las que contenían sus fracciones y, por último, al uso de vacunas preparadas a partir de material genético. Estas diferencias de composición eran necesarias para mejorar la eficacia y la seguridad de las vacunas, así como para optimizar el proceso de producción y mejorar su relación coste-beneficio.

Tabla 1. Tipos de vacunas tradicionales, métodos de producción, principales ventajas e inconvenientes.



A pesar de los numerosos tipos de vacunas que pueden utilizarse, la mayoría de ellas se administran por vía inyectable (intradérmica, subcutánea o intramuscular). Hasta la fecha, sólo un puñado de vacunas constituyen excepciones (FluMist/Fluenz® y Nasovac™, por vía nasal; Vaxchora®, Dukoral®, Rotarix™, RotaTeq®, Vivotif® y la vacuna antipoliomielítica, por vía oral). Una clara limitación de las vacunas tradicionales es, por tanto, el modo de administración. Utilizar una vía de administración inyectable significa utilizar una aguja y, por tanto, toparse inevitablemente con los siguientes problemas: 1) dolor, 2) incomodidad, 3) protección local limitada o nula, 4) irritación local, 5) infección local, 6) necesidad de personal cualificado, 7) acceso limitado, 8) difícil eliminación, 9) costes elevados.

Teniendo en cuenta el considerable periodo de tiempo, entre 10 y 15 años de media, que se necesita para desarrollar una vacuna desde cero, está claro que es de vital importancia que los investigadores encuentren alternativas viables. Estas alternativas tienen dos objetivos principales: a) simplificar la administración de las vacunas existentes y b) mejorar su seguridad y eficacia. La conciencia de este reto temporal en el proceso de desarrollo de vacunas pone de relieve la urgencia de explorar vías innovadoras y estrategias pragmáticas.

Las vías de administración no invasivas más estudiadas son la oral, la sublingual, la bucal, la inhalatoria (nasal y pulmonar) y la dérmica. Estas opciones ofrecen numerosas ventajas, como (1) una mejora significativa del cumplimiento terapéutico por parte del paciente, (2) la evitación de efectos no deseados en el lugar de administración (como dolor, irritación o infección), (3) una menor dependencia de personal cualificado y, lo que es más importante, (4) una protección garantizada tanto local como sistémica. Este enfoque revolucionario no sólo responde a las necesidades actuales, sino que abre nuevas perspectivas para una difusión eficaz y accesible de las inmunizaciones en todo el mundo.

Sin embargo, la investigación en el campo de la vacunación también ha ido más allá de la investigación exclusiva de vías alternativas de administración, ya que se ha puesto de manifiesto que estos enfoques por sí solos no bastan para garantizar una mejora significativa de la eficacia y la seguridad de las vacunas. En respuesta a esta necesidad, se hizo necesaria la exploración simultánea de nuevos sistemas y tecnologías. Entre las muchas opciones, las nanotecnologías han surgido como una perspectiva prometedora para elevar los estándares de vacunación, y entre ellas, los liposomas emergen como uno de los nanosistemas de transporte más ampliamente estudiados.

Los liposomas, estructurados como sistemas esféricos de fosfolípidos, tienen una bicapa de fosfolípidos que encierra un núcleo acuoso. Su característica más distintiva y ventajosa en el contexto de la vacunación reside en su dualidad funcional. En primer lugar, actúan como sistemas eficaces de transporte y protección del antígeno, facilitando su administración precisa y preservando su integridad. En segundo lugar, pueden desempeñar un papel activo en la potenciación de la respuesta inmunitaria al antígeno, actuando como adyuvantes intrínsecos que pueden activar aún más el sistema inmunitario y aumentar la magnitud de la respuesta. Esta capacidad de actuar simultáneamente como vectores de transporte y moduladores de la respuesta inmunitaria les confiere un papel crucial en la innovación de estrategias vacunales. En este contexto, explorar el potencial de los liposomas representa un capítulo clave en el avance hacia una vacunación más avanzada y eficaz.

Sin embargo, es innegable que los liposomas, aunque representan una frontera prometedora en la administración de vacunas, presentan estructuralmente ciertas limitaciones. La evaluación de estas limitaciones depende, en gran medida, de la vía de administración seleccionada para su uso. Así, la investigación ha emprendido un camino de considerable complejidad, evolucionando desde una fase inicial de utilización de liposomas genéricos hasta una fase posterior de ferviente y continua innovación en las composiciones, destinada a optimizar las prestaciones de estos sistemas vesiculares y hacerlos más resistentes, mucoadhesivos, etc. A este respecto, resulta evidente el papel crucial de la vía de administración, ya que la formulación liposomal tiene que interactuar con diferentes sistemas enzimáticos y superar barreras específicas, cada una de las cuales puede afectar en mayor o menor medida a su integridad estructural. En consecuencia, es imperativo personalizar y enriquecer la composición de estos sistemas, adaptándolos a los retos específicos que plantea la vía de administración elegida.

Esta necesidad de adaptación específica es crucial para garantizar que los liposomas puedan desempeñar su función de forma eficaz tras su administración. Por ello, de la revisión bibliográfica que abarca la última década (2013-2023) se desprende una amplia variedad de liposomas modificados producidos tras años de estudios de investigación (Fig. 1). En concreto, se pueden identificar liposomas aniónicos, neutros o catiónicos (cuya carga puede afectar a la interacción celular, mucoadhesividad, etc.), liposomas que contienen otras nanopartículas (utilizados como escudos y estabilizadores), estratosomas (estabilizados y reforzados con múltiples capas, de diversa naturaleza, principalmente para hacer frente a la vía oral), bilosomas (hechos gastrorresistentes con sales biliares), transferomas (hechos ultradeformables con activadores de bordes para superar la piel), etosomas (que se deforman con etanol para atravesar la piel), liposomas conjugados con ligandos (para dirigirse de forma activa y específica a células y tejidos diana), liposomas con quelantes metálicos (para aprovechar la electricidad como medio de transporte a través de la piel) liposomas recubiertos de matriz polimérica (para lograr adhesividad o resistencia en entornos agresivos), virosomas (con estructura similar a la viral), arqueosomas y arqueosomas ultradeformables (obtenidos a partir de lípidos particulares, más resistentes en condiciones particulares, ultradeformables o no).

Al igual que los medicamentos, las vacunas también necesitan estrategias y dispositivos para administrarse correctamente. Aunque la formulación liposomal líquida puede ser potencialmente adecuada para todas las vías, también es cierto que las formulaciones líquidas son las más problemáticas en términos de almacenamiento. Por ello, cada vez se recurre más a estrategias que adoptan formas sólidas o semisólidas.

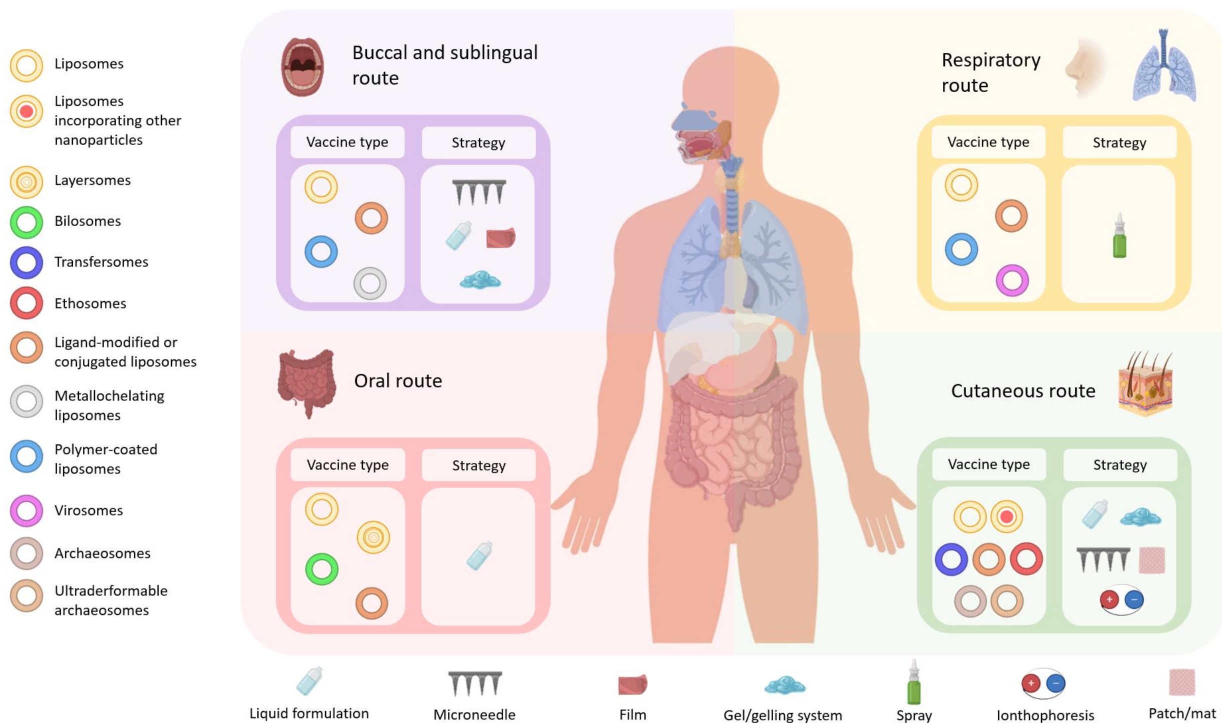


Figura 1. Vacunas basadas en liposomas desarrolladas en la última década (2013-2023) y probadas, a través de vías de administración mínimamente o no invasivas, solas o en combinación con dispositivos médicos.

Por todo ello, en esta tesis se buscó combinar liposomas con estrategias o dispositivos capaces de favorecer el uso de vías de administración no invasivas como la cutánea (vía hidrogel), nasal (vía spray nasal) y pulmonar (vía dispositivo de inhalación de polvo seco), con el objetivo de potenciar la protección local precisamente en aquellos principales puntos de entrada en el organismo explotados por los patógenos. En cada estudio se prestó especial atención a la composición de las vesículas, adaptándola y modificándola de vez en cuando para lograr un rendimiento óptimo en el transporte según la vía seleccionada. Los elementos innovadores fueron, por tanto, la utilización de composiciones novedosas, adaptadas y diseñadas en función de la vía de administración y favoreciendo el uso de lípidos lo más naturales posible, y la explotación de métodos de preparación más respetuosos con el medio ambiente. En efecto, la mayoría de los estudios implican el uso de lípidos sintéticos y particularmente costosos, así como la utilización de métodos como la evaporación de películas lipídicas, la inyección de etanol y la microfluídica, todos los cuales explotan disolventes orgánicos que podrían generar toxicidad tras la administración in vivo. En cambio, en esta tesis se utilizó la sonicación directa como método alternativo que, en un solo paso, permite obtener vesículas de fosfolípidos de tamaño y eficacia comparables a los métodos mencionados, pero sin necesidad de eliminar el disolvente orgánico en un paso adicional.

En el primer estudio se crearon formulaciones para uso cutáneo. Los transfersomas preparados con desoxicolato sódico y Lipoid S75 se enriquecieron con glicerol, ácido hialurónico o su combinación para mejorar la acumulación de ovoalbúmina en la dermis y la epidermis. Todas las formulaciones eran altamente biocompatibles y estables hasta 9 meses. No se detectaron efectos inflamatorios durante la exposición de las células presentadoras de antígeno a estas formulaciones. Por el contrario, la presentación del antígeno no mejoró, ya

que la respuesta no difirió significativamente de la del antígeno no encapsulado. Sin embargo, en un contexto real, se espera que se comporten mejor sobre la piel. De hecho, los transferomas dieron lugar a una mayor deposición de ovoalbúmina que la solución en cualquier condición (no oclusiva, oclusiva con película adherente y oclusiva con hidrogel) cuando se estudiaron con células de Franz. Los transferomas enriquecidos con ácido hialurónico y glicerol-ácido hialurónico fueron más eficaces que los transferomas con glicerol solo cuando se compararon en condiciones no oclusivas y oclusivas (película adherente). Por último, se observó que los hidrogeles enriquecidos con transferomas eran la estrategia más eficaz, ya que mejoraban la deposición de ovoalbúmina a nivel de la dermis y la epidermis, y podrían ser prometedores en un entorno real si se aplican correctamente.

En el segundo estudio, se produjeron formulaciones nasales. Se produjeron liposomas aniónicos y catiónicos utilizando fosfolipón 90G y colesterol, con y sin 1,2-diolil-3-trimetilamonio-propano (DOTAP), respectivamente. Las dispersiones permanecieron estables hasta 3 meses. Los liposomas catiónicos DOTAP presentaron interacciones y captación mucosa superiores a las de los liposomas aniónicos. Además, mostraron una elevada adyuvanticidad, lo que condujo a una mayor producción de interleucina-2. No se detectó inflamación. No se detectó inflamación. Aunque tanto los liposomas DOTAP aniónicos como los catiónicos presentaban una buena pulverizabilidad (el 90% de las gotitas tenían un tamaño de unos 100 µm), estos últimos mostraron una mayor deposición en los cornetes, donde tiene lugar la respuesta inmunitaria. Teniendo en cuenta la mayor deposición y mucoadhesividad, los liposomas DOTAP fueron la formulación más adecuada para investigar la protección nasal local.

En el tercer y último estudio, se realizaron formulaciones pulmonares. Tras un cribado inicial sobre los lípidos y crioprotectores a utilizar, se seleccionaron como mejores candidatos los liposomas aniónicos preparados con Lipoid S PC-3 y DPPC (liposomas SPC3 y liposomas DPPC, respectivamente). A continuación, se liofilizaron con Respitose® o inulina para mejorar su estabilidad y poder aprovechar la administración pulmonar mediante un inhalador comercial de polvo seco (Aerolizer®). Fue necesaria una nueva dilución de estos polvos con Respitose® para ajustar la dosis de ovoalbúmina. Mediante difracción láser, se demostró que esta dilución aumentaba la fracción de partículas aptas para la administración pulmonar, ya que aumentaba el volumen de partículas con un diámetro < 5 µm, confirmando así el efecto antipolvo de Respitose. A continuación, se estudiaron los perfiles de personas sanas, asmáticas y con enfermedad pulmonar obstructiva crónica, y se demostró que la fracción de partículas finas, es decir, el porcentaje de la dosis que llega realmente a los pulmones, era mayor en el caso de las personas sanas que en el de las patológicas. Asimismo, la fracción de partículas extrafinas se vio afectada por la condición, a excepción de los liposomas liofilizados con inulina. A continuación, se realizaron todos los estudios in vitro en una línea celular de macrófagos alveolares (RAW 264.7). Todas las formulaciones resultaron biocompatibles. La captación fue mayor en el caso de las vesículas que en el de la ovoalbúmina sin encapsular, especialmente tras las primeras horas de exposición, como confirmaron la citometría de flujo y la microscopía de fluorescencia. Todas las formulaciones garantizaron la respuesta de los macrófagos a la ovoalbúmina y fueron bien toleradas. Sin embargo, los liposomas SPC3 liofilizados con Respitose fueron el único tipo de formulación capaz de potenciar la producción

de interleucina-6, conocida por promover la diferenciación de las células B en células plasmáticas y la activación de las células T citotóxicas. Asimismo, la fracción de partículas extrafinas se vio afectada por la condición, a excepción de los liposomas liofilizados con inulina. Todos los estudios in vitro se realizaron en una línea celular de macrófagos alveolares (RAW 264.7). Todas las formulaciones eran biocompatibles. La captación fue mayor en el caso de las vesículas que en el de la ovoalbúmina no encapsulada, especialmente tras las primeras horas de exposición, como confirmaron la citometría de flujo y la microscopía de fluorescencia. Todas las formulaciones garantizaron la respuesta de los macrófagos a la ovoalbúmina y fueron bien toleradas. Sin embargo, los liposomas SPC3 liofilizados con Respitose fueron el único tipo de formulación capaz de aumentar la producción de interleucina-6, conocida por promover la diferenciación de células B en células plasmáticas y la activación de células T citotóxicas.