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Pulmonary vaccine delivery: An emerging strategy for vaccination and immunotherapy

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ABSTRACT

Vaccination has been known as the most successful health strategy for the prevention of infectious diseases, and their subsequent disability conditions. Conventional vaccination is based on parenteral administration and therefore requires needles, cold-chain storage and distribution. To meet these drawbacks, new strategies such as pulmonary vaccination have brought new insights into immunotherapy and vaccination. Among novel strategies, nanoparticulate vaccine delivery systems play an undeniable role in targeting and depositing of antigenic (nano-) microparticles in particular regions of the respiratory tract, engineering the inhalable powders, altering the release profile and pharmacokinetic features of vaccines. This study aims to review pulmonary immune system, pulmonary vaccine delivery, micro and nanocarriers for pulmonary vaccine delivery, and nanotechnology-based pulmonary vaccines.

1. Introduction

Vaccination is one of the most important and efficacious strategies in the prevention of infectious disease transmission. Since vaccination has been converted to public health intervention, the vaccines have played an undeniable role as cornerstone of saving people's lives [1,2]. Vaccines are also argued to be the human's greatest achievement in the field of medicine [3]. Most vaccines are traditionally administered by intramuscular or subcutaneous injection. These administration routes are proven to provide immunity by inducing effective systemic immune responses [4]. However, the antigen-specific immunity at mucosal surfaces (as a gateway for invasion of pathogens) is difficult to be obtained by injection vaccination [5]. Thus, forming a barrier to pathogen invasion at mucosa can be provided hardly by injectable vaccines. Moreover, the injection route is accompanied by drawbacks such as poor compliance due to needle phobia, needle waste, needle sticking, and need to be administered by trained medical professionals [6].

The human body's mucosal surfaces are widely exposed to innocuous, infectious, and pathogenic agents due to their proximity to the external environment. It is approximated that more than 70% of all pathogens invade the host by entering through the mucosa [7]. Although treatment of pulmonary infections is performed by administration of antibiotics, resistance to antimicrobials and antibiotics and critical shortage of innovative antibiotics are found to be the major challenges in the treatment of respiratory infectious diseases [8]. The vast pulmonary mucosal surface provides an enormous interface between inhaled pathogens and the immune system present in the pulmonary system. Thus, the human pulmonary system is equipped with some barriers to protect the lung against the invasion of pathogens [9]. Similar to pathogens and harmful antigens, vaccines can be also administered conveniently by the mucosal route. Also, the mucosal vaccination can induce both systemic and mucosal immunity. Various mucosa-associated lymphoid tissues (MALT) including nasopharynx-associated lymphoid tissue (NALT), rectum-associated lymphoid tissue (RALT), vagina-associated lymphoid tissue (VALT), and gut-associated lymphoid tissue (GALT) are present at mucosal surfaces that make an interconnected immunity among different mucosal sites [2,10–13]. This immune network makes it possible to provide immunity at all interconnected mucosal surfaces by administering a vaccine in just one mucosal site (e.g. pulmonary mucosa) [14].

The mucosal surface of the respiratory system is assumed to be the most vulnerable entry gateway as it is exposed to the external environment and breathed air [15]. Recently, COVID-19 has spread all over the world which is associated with a severe respiratory syndrome, and

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approximately 2.74 million deaths were recorded [16]. Although many countries have taken tough rules on quarantine, travel restrictions, and legislation of social distancing, the results are not satisfying and these facts suggest that making such tough rules may not be adequate to diminish the pandemic, especially, the ones caused by air-borne pathogens with a capability to develop respiratory complications [17]. Vaccination is thought to be the most effective strategy for preventing and diminishing life-threatening diseases and pandemics [18].

Strategies for manufacturing conventional vaccines (e.g. inactivated/killed/attenuated live microbes) are not sufficiently applicable for managing urgent pandemics due to the considerable time needed to develop a vaccine into an industrial product [19]. Recently-developed vaccines approved for the prevention or treatment of pulmonary infections are formulated in liquid form and are administered parenterally. These parenteral vaccines provide low levels of immunity at mucosal surfaces [8]. Furthermore, parenteral vaccination requires cold-chain distribution and storage conditions which may not be available in developing countries [20]. For this reason, developing novel vaccines which provide immunity at mucosal surfaces is thought to be an alternative strategy to manage high-prevalence and life-threatening infectious diseases. Up to date, a few conventional mucosal vaccines including *Salmonella typhi*, cholera, rotavirus, Sabin polio, and influenza vaccines have been approved. Like other conventional vaccines, they are associated with expensive production and tedious quality control. Furthermore, the risk of reversion of virulence is one of the most important drawbacks of conventional vaccines.

As an alternative, subunit vaccines use highly pure antigens to induce immunity. A wide range of antigens including polysaccharides, proteins, peptides, or DNA/RNA can be exploited as immune response inducers in producing subunit vaccines. Recently, developing vaccines based on purified antigens has attracted a lot of attention [21]. Among these novel vaccines, DNA/RNA vaccines with antigen coding capability are tended to be used as expression system makers. Making expression systems results in rapid secretion of antigen required for developing vaccine [22]. For these reasons, these types of vaccines have less risk and a more rapid development rate. Besides these advantages, subunit vaccines often suffer from a lack of immunogenicity, so, the adjuvants are added to increase the immune responses after vaccine administration. Two groups of compounds with different chemical structures present in the adjuvants category: immunopotentiators and delivery systems [15]. Immunopotentiators play the ligand role on pattern-recognition receptors (PRRs) which are present on antigen-presenting cells (APCs), while delivery systems induce the immune response by multiple mechanisms such as by diminishing the release rate of co-delivered antigen and protecting the antigen against degradation [2].

This article aims to review pulmonary vaccination, pulmonary immunization, the barriers to pulmonary delivery of inhalable vaccines, carriers for pulmonary vaccine delivery, and formulation considerations in the development of pulmonary vaccines. The nanotechnology-based carriers are also reviewed.

2. Anatomy of human lung

Structurally, the human lung has five lobes, three lobes on the right and two on the left. Based on function, the human respiratory tract can be divided into two general types of airways: the conducting airways and the respiratory airways. The conducting airway starts with the trachea. According to Fig. 1, with each bifurcation in the respiratory tract, one new generation emerges. The conducting airways are continued up to the 16th generation of the respiratory tract. The lower airways (16th to 23rd generations) are of the respiratory type which perform the gas exchange and play a key role in immune defense (Fig. 1) [23–26].

The pulmonary mucosal surface area is approximated to be ~150 m2 [27]. This surface is also assumed to be the vastest surface for the entry of airborne particles from innocuous to harmful antigens. The structurally diverse barriers including mucociliary escalators [28], epithelial layer conferred with tight junctions [29], and surfactant system [30,31] are present as the first line of structural barriers in the pulmonary system. The immune system cells including cells of the innate and adaptive

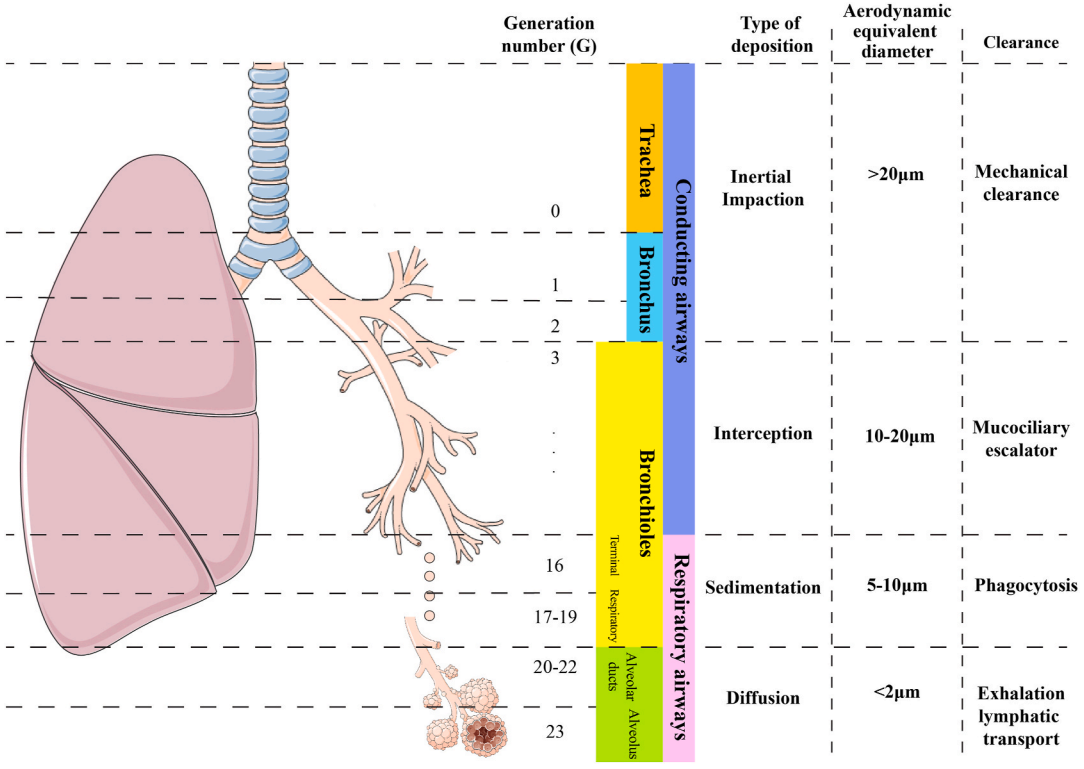

Fig. 1. Generations of human pulmonary system, size-dependent deposition, and clearance.

immune system are located in the respiratory region and play the second line of defense against the harmful pathogen antigens. The immunity cells are positioned at the luminal and basal sides of the epithelial layer. The immunological activities are precisely regulated in direction of protection of the integrity of airways especially the gas exchange region [32,33].

The goblet cells present in the epithelial layer produce thick mucus that covers the surface of conducting airways. The mucus traps the inhaled particulates with a size of more than 10 μm and prevents entry of particles to reach the lower generations of the respiratory tract i.e. respiratory bronchioles and alveolar ducts and alveolar sacs [14,24].

By the 20th generation, the alveolar ducts are present, and after three bifurcations the alveolar sacs are recognizable. Two types of cells cover the alveolar sacs: the flat squamous type I cells covers more than 90% of the surface of alveolar sacs. The type II cells have an irregular shape that covers the remaining surface. Although type II cells cover the minor area, they have an undeniable role such as secreting the phospholipid surfactants to facilitate the inhalation by decreasing the surface tension. In the case of tissue damage, type II cells can divide and turn into type I cells to compensate for the damage.

3. The pulmonary mucosal immune system

Based on activity and anatomical localization, the pulmonary mucosal immune system can be classified into inductive and effector sites (Fig. 2). The immune cells of the pulmonary system are activated after recognition and uptake of antigens at an inductive site and finally, a preliminary immune response takes place at an effector site. The most important inductive site for antigen recognition is the MALT where which the memory B and T cells migrate to lamina propria through the lymphatic system. The lamina propria positioned at the upper parts of the respiratory tract plays the effector role [34].

3.1. Innate immunity

The MALT present in the lung screens the mucosal surface for foreign materials. If any pathogen is recognized by MALT, an immune response will be developed [8]. Immunoglobulins (Ig), particularly secretory IgA is produced by epithelial cells positioned on mucosa which attacks inhaled pathogens [35]. Secretory IgA prevents the binding to and passing pathogens through the pulmonary mucosa by forming a complex with the pathogen. The complex is then removed from the pulmonary system by peristaltic activities [36]. The surfactant proteins SP-A and SP-D reside in alveolar sacs play a key role in nonspecific opsonization by setting a linkage to lipopolysaccharides on the outer membrane of Gram-negative bacteria [37]. Moreover, the alveolar fluid contains alveolar macrophages, Ig, T cells, and B cells.

3.2. Adaptive immunity

Antigens are presented to naïve T cells in the lymph nodes by dendritic cells (DC). According to this fact, the immune responses induced by DCs are much stronger compared to B cells and alveolar macrophages [38]. Besides, DCs have higher APC capability and lower phagocytic activity, whereas alveolar macrophages have higher phagocytic activity and lower APC capability [39].

4. APCs in human lung

The surveillance of mucosal surfaces is performed by APCs present in the human pulmonary system. APCs consist of various types of cells including DCs, alveolar macrophages, and B cells. Microfold (M) cells deliver the antigens to APCs. Adaptive immune responses are initiated by APC activation. The vaccine-associated immunity is also mediated by the specific functions of APCs. Antigens are transferred by transcytosis and delivered to DCs by microfold cells. The activation of DCs induces the differentiation of T cell subsets, eventually, after interaction of B cells and differentiated T cells, antibodies are secreted at mucosal sites

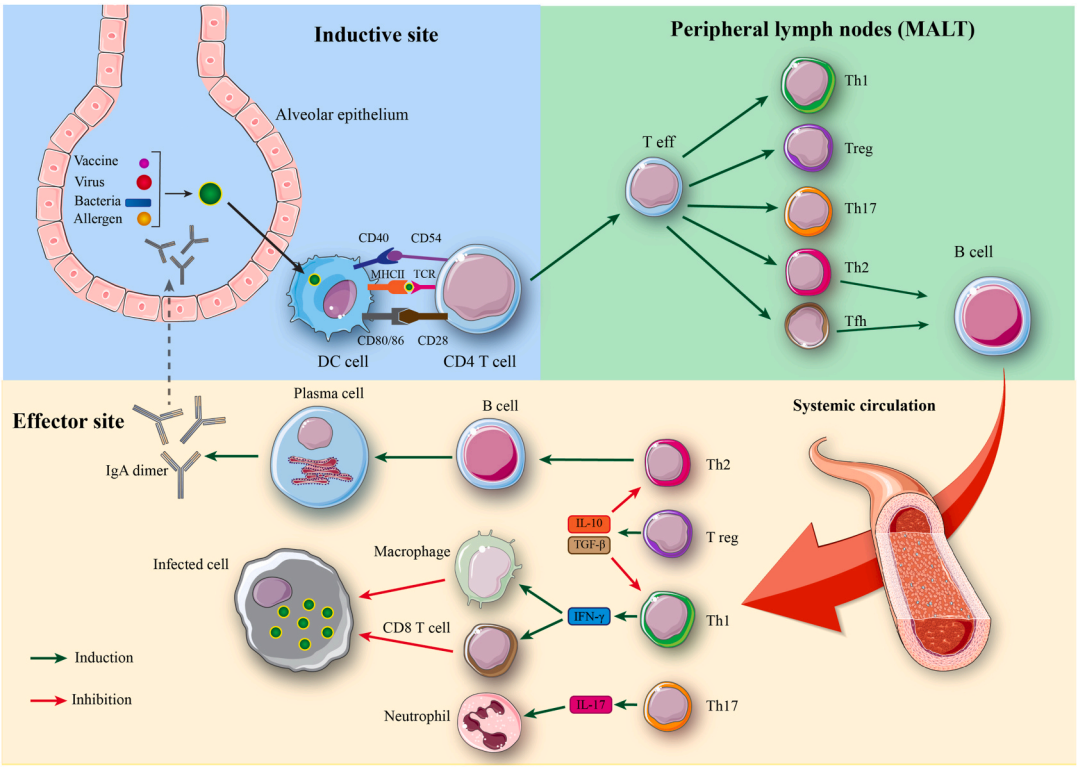

Fig. 2. The function of the immune system in the pulmonary system, MALT, inductive, and effector sites.

[40,41].

4.1. Dendritic cells

4.1.1. DCs present in the respiratory tract

Two types of lung DCs including myeloid MHC class II + CD11c+ and plasmacytoid MHC class II ± B220+ Ly6C + are differentiable [42,43]. Two subsets of myeloid or conventional DCs are present in conducting airways of respiratory system which have different phenotypes: CD11c + DCs which express CD11c + MHC class II + CD11b + CD103- DCs and CD103+ DCs which express CD11c + MHC class II + CD11b- CD103+ proteins. These DCs represent 60 and 40% of total DCs present in airways, respectively [9,43,44].

4.1.2. DCs present in lung

As stated previously, DCs are an important part of the pulmonary immune system that can be found through the conducting airway and lung parenchyma [39]. Based on the localization of DCs in the human lung, DCs have highly diverse cell surface Toll-like receptors and high functional heterogeneity so that DCs in different parts of the pulmonary system are activated by different stimuli. Depending on cell surface receptors, DCs are classified into two functionally diverse groups: myeloid (mDC) and plasmacytoid DCs (pDC) [38]. mDCs predominantly occupy the conducting airways and inactivate the inhaled pathogen by doing phagocytosis. On the other hand, pDCs are present in lung parenchyma and play the APC role [45].

Moreover, the lung DCs have higher phenotypical variation compared to blood DC subsets.

Human lung DCs have an immature phenotype and express MHC class II proteins on their surface and exert high phagocytic activity [46,47]. DCs reside in the epithelium of the lung contain Birbeck granules and also express Langerin which makes the DCs similar to Langerhans cells [48,49].

4.2. Pulmonary alveolar macrophages

Pulmonary alveolar macrophages reside in alveolar sacs provide a key role as one of the first-line defenses against inhaled pathogens that reach the lower parts of the pulmonary system. It is approximated that more than 90% of pulmonary macrophages present in alveolar lining fluid are involved in nonspecific phagocytosis of pathogens [37]. The macrophages contribute to developing an innate immunity against the particulate pathogen which are not recognized in the upper regions of the lung [50,51]. Although the role of alveolar macrophages in developing adaptive T cell-associated immune responses to inhaled pathogens is not fully known, the results of some animal studies suggest that alveolar macrophages can migrate to draining lymph nodes after capturing the inhaled pathogens [52–55]. After the migration of macrophages from the alveolar space to lymph nodes adaptive $CD4^+$ T cell-associated immune responses are initiated [56,57]. After the release of cytokines such as interleukin 8 (IL-8) by alveolar macrophages, leading to an immune response is initiated with the contribution of neutrophils [55].

4.3. B cells

Both in humans and mice, the production of local Ig including IgG, IgA, and IgE is mediated by B cells through the formation of a local germinal center [58]. Secretion of allergen-specific Ig, in particular, IgE has long been the mainstay of the relationship between allergic asthma and atopy [59]. Furthermore, aeroallergens are delivered to $CD4^+$ T cells by B cells via MHC class II protein [60].

5. Pulmonary mucosal vaccination

As stated earlier, parenteral vaccination has many disadvantages including cold-chain requirements for distribution and storage, risk of needle-stick injuries, needle waste disposal issues, lack of sterile water (or other solvents) required for reconstitution of dried powder vaccines, poorer compliance, and vaccination refusal due to needle phobia, and limited mucosal immunity after parenteral vaccination. Moreover, injection vaccination induces a systemic immune system but it elicits a weak mucosal immunity.

The pulmonary route provides a noninvasive needle-free route to deliver bioactives to the lung. Like other mucosal vaccination, pulmonary vaccination can efficiently induce both systemic and mucosal immune responses.

As the function of the systemic immune system differs from the mucosal immune system, understanding the mucosal immune system is necessary for the production of an optimal novel pulmonary vaccine. An interconnected network consists of immune cells that form the mucosal immune system, representing about 80% of the total immune cells [61].

DCs have three major functions in the pulmonary system including the recognition of pathogens, inhibition of pathogen uptake, and prevention of development of deleterious immune responses [62]. Depending on the form and dose of entered antigen, pathogen-associated molecular pattern (PAMP), and exposure frequency, an allergic response, immune response, or tolerance can be developed.

Owing to the importance of DCs in adaptive immunity, the regulatory role of DCs on immune responses to allergens and antigens has been attracted enormous attentions. As described earlier, DCs present in both lung parenchyma and respiratory airway mucosa and, depending on the localization of DCs, serving different immunological homeostasis activities on inhaled antigens [63,64].

The pulmonary system has a thin mucosal membrane and a highly dense vascular network which provides an ideal surface area for rapid absorption of bioactives, also the enzymatic activities are limited in the pulmonary system [65]. For these reasons, delivery to the pulmonary system is assumed to be a non-invasive and efficient strategy for pulmonary vaccination. The unique characteristics of APCs resident in the pulmonary system, make the targeted vaccine delivery a potential strategy to induce specific immune responses. Resident DCs in different parts of the pulmonary system can be targeted in order to achieve an optimal vaccination [43,66–68]. Minor deficiency in population of $CD4^+$ lymphocytes can be compensated by DCs through developing a desirable immune response [69].

Cell-specific vaccine delivery is a multistage strategy with specific obstacles for each step. An ideal cell-specific vaccine follows a strategy that overcomes the major obstacles that may reduce the vaccine efficiency. Optimized pulmonary delivery using nanotechnology is almost a new strategy that helps efficient release of the loaded material, protects the loaded material from environmental factors, improves the targeting efficiency, and eventually makes it possible to deliver the bioactive to target in sufficient quantity [70].

5.1. Adjuvants for pulmonary vaccines

The incorporation of adjuvants to the antigen vaccine formulation makes it possible to generate a strong and long-lasting immune response after vaccine administration. Moreover, adjuvants enable the use of a lower antigen dose in vaccine formulations. Some types of vaccines including DNA vaccines and synthetic subunits elicit weak immunogenicity and thus, they need to be administered with adjuvants to evoke strong immune responses [71,72]. Administration of adjuvanted vaccines can cause selective modulations of generated immune responses to MHC class I or II and Th1 or Th2 responses. Such modulations are vital for protection against intracellular pathogens. Three different mechanisms are conceived for the efficacy of adjuvants. The first mechanism is depot generation in which immunostimulant complexes (ISCOMs), aluminum compounds, emulsions, and oil adjuvants such as Freund's complete/incomplete adjuvant act their rule in vaccines. The second

mechanism is immunomodulation by modifying cytokine networks which are accomplished by CpG motif, muramyl dipeptide, cholera toxoid, lipopeptides, monophosphoryl lipid A, and lipopolysaccharides adjuvants. The third and last mechanism is related to adjuvants such as liposomes and biodegradable polymeric microparticles which play the role of a delivery vehicle for targeted delivery to APCs. Among adjuvants, aluminum compounds have acceptable safety, however, they only generate strong humoral but poor Th1 immune responses. The only approved aluminum compound for human use is potassium aluminum sulfate (alum) and others such as aluminum phosphate (adju-phos®) and aluminum hydroxide (alhydrogel®) have been just exploited in preclinical trials [73].

Toxins such as cholera toxin and *Escherichia coli* heat-labile endotoxin can strongly evoke the immune system at mucosal sites. Due to this capability, they are vastly used in the development of intranasal vaccine candidates. These toxins have two subunits: subunit B which helps the endotoxin to link with gangliosides expressed on the cell surface. After establishing the link, the internalization of toxin occurs, then subunit A activates adenyl cyclase that results in elevated cAMP levels. Although the incorporation of toxins in vaccine formulation helps the antigen to accumulate in the olfactory site, the major concern regarding the intranasal administration of toxin-adjuvanted vaccines is that toxins can elicit their neurotoxic effects following accumulation in the olfactory bulb and nerve for an extended period. This may cause serious conditions such as meningitis and other inflammatory responses in the mice [74]. The occurrence of Bell's palsy (temporary facial paralysis) after intranasal administration of Nasalflu (inactivated virosomal-subunit influenza vaccine, Berna Biotech, Switzerland) which contains toxin adjuvant can be explained by this fact [75]. Due to the risks associated with toxin-adjuvanted vaccines, mutated toxins of *Escherichia coli* heat-labile endotoxin (e.g. LTK63) are more acceptable [76].

Since the adjuvants can be toxic to the respiratory tract, dose and therapeutic window need to be clarified to prevent inflammation and other adverse effects. Furthermore, responses to the same adjuvant may be varied in different animal models [77]. Thus, the correlation between responses to adjuvants must be clearly determined to prevent the failure of adjuvanticity of vaccine formulation.

5.2. Cell-targeted vaccine delivery

Based on past studies, the development of inhalational vaccines makes it possible to initiate a much greater mucosal immune response compared to parenteral vaccines [78–83].

Initiation and development of a local or systemic immune response are highly dependent on the site of interaction between immune cells and inhaled antigen. Regarding the different distribution of immune cells in each compartment of the pulmonary system and different immune responses developed after activation of each type of APCs, cell-targeted vaccine delivery can be performed in order to achieve an optimal immune response against infectious diseases. During treatment or control of an allergic condition, cell-targeted delivery aims to induce an active tolerance toward the allergen. In this regard, delivery of allergen to alveolar macrophages is thought to be a potential strategy that can be exploited for obtaining an active tolerance. On the other hand, targeting the DCs resident in conducting airways may represent an applicable approach to develop a specific immune response against respiratory pathogens.

Due to the presence of physical and functional barriers such as mucus, ciliated cells, and surfactant proteins, targeted delivery of bioactives and immunostimulatory agents to specific immune cells in the pulmonary system is a challenging task. The mucociliary barrier makes the DCs-targeted delivery of bioactives as a challenging task. The use of magnetizable aerosols is thought to be an option to reduce the mucociliary clearance of particulate matters in conducting airways.

According to these facts, during the designing of a cell-targeted vaccine delivery system, the aerodynamic diameter of aerosol droplets and particulates must be optimized to achieve optimal immunological and respiratory targeting.

To obtain cell-specific vaccine delivery, passive and active strategies can be used. The passive targeting focuses on the biophysical properties of a particular compound to cause accumulation at a predetermined site. According to different deposition sites of inhaled particles or aerosol droplets based on their size, a size-dependent strategy can be used in developing cell-specific delivery of bioactives to the specific compartments of the pulmonary system [84,85] (Fig. 1).

Basically, three types of size-dependent deposition can occur within the respiratory tract: diffusion, inertial impaction, and sedimentation. Particles or droplets with more than 5 μm diameter are deposited by inertial impaction in upper parts of the respiratory tract (i.e., throat). Both inertial impaction and sedimentation of particles or droplets with 1–5 μm diameter can be found in the main conducting airway regions. Eventually, diffusion with a balanced degree between suspension and exhalation occurs for particles or droplets smaller than 1 μm [85].

Active targeted delivery of bioactives to the pulmonary system has been achieved by fabricating paramagnetic iron oxide nanoparticles embedded in magnetizable aerosol droplets or solid nanocomposites [86]. Moreover, a controlled release pattern for bioactives can be achieved by using nanocarriers. A wide range of nanocarriers, from lipid and polymeric nanoparticles to virus-like particles, can be exploited to design pulmonary vaccines for generating optimal immune responses against pulmonary infectious diseases [87,88].

6. Challenges and barriers to pulmonary vaccine delivery

Although pulmonary vaccine delivery is thought to be advantageous over the parenteral route, there are obstacles and technical challenges which make pulmonary vaccination problematic. However, some challenges can help to induce an active mucosal immune response.

As a prerequisite, the actuation of pressurized metered-dose inhalers (pMDI) must be synchronized with the inhalation pattern. In addition, a minimum inhalation force is required to deaggregate the dry powder mass from dry powder inhalers (DPI) and deliver the particles to the lung [85,89]. A reduced dose delivered to the lung due to incorrect use of inhalational vaccines may lead to vaccine inefficiency and lack of sufficient immunity. Furthermore, the presence of inflammation, local irritation, and exacerbation of asthma makes the pulmonary vaccination a challenging task [90].

Dilution of inhaled vaccine antigens in mucosal secretions may diminish the deposition of particles onto the mucosal epithelia. Furthermore, the inhaled vaccine antigens delivered to mucosal epithelia may be undergone to proteolytic and nucleolytic degradation [5]. According to this fact, the biological efficacy of subunit vaccines containing DNA/RNA, peptides, and proteins may be subtracted or even lost. In this regard, protection strategies are assumed to be a requirement in designing a mucosal vaccine. The protection of mucosal antigen vaccines from detrimental factors helps the vaccines to exert their efficacy by mimicking the physicochemical characteristics of opportunistic pathogens, in particular with respect to particle size, shape, and surface charge [5,91]. Therefore, a successful and efficient mucosal vaccine is designed in order to overcome the mucosal barriers and target the pulmonary mucosal APCs for optimal antigen presentation and processing leading to B cell and T cell activation. Moreover, an optimized mucosal vaccine has the ability to modulate the kinetics of antigen with respect to induce an active immunological memory response [5].

7. Carriers for vaccine delivery to the pulmonary system

Due to the advantages of the pulmonary route, vaccine delivery via inhalational dosage forms has been attracted much attention [92–96]. The first successful clinical trial of pulmonary measles vaccine containing Edmonston-Zagreb strain of attenuated measles was performed on 4 million schoolchildren in Mexico [97]. Delivery of vaccine via

inhalational route produced a higher seroconversion compared to subcutaneous injection [79]. Howbeit, reproducibility of biological stability of reconstituted vaccines can be hardly guaranteed. So the necessity of researching the potential carriers for pulmonary delivery is a mainstay in the field of pulmonary vaccines. Delivering vaccines with a wide range of features from whole bacteria to DNA/RNA sequences needs specific formulation considerations. Nanotechnology-based carriers can facilitate the targeting of immunostimulatory agents to specific compartments or cells in order to generate an appropriate immune response. Furthermore, nanocarriers protect the antigens from detrimental conditions such as enzymatic and escalator activities [91]. Besides the advantages of nanotechnology-based carriers, manufacturing of nanocarriers may increase the cost of the finished product, also nanocarriers are so hard to be scaled up.

The carriers which have been studied for pulmonary vaccine delivery are discussed in the following sections.

7.1. Micro- and nanoparticles

As stated earlier, nanotechnology has provided an advantageous strategy to design targeted pulmonary vaccines. Vaccine antigens can either be loaded on or embedded in nanoparticles. By using nanotechnology-based carriers, some critical characteristics such as solubility, surface properties, and stability of particles can be modified in order to optimize the delivery of vaccines via the pulmonary system [91]. Furthermore, conjugation of antigens onto nanoparticles improves the presentation of antigens to APCs and simulates the natural infection, and eventually generates an active immune response. The high diffusion rate due to the high surface area-to-volume ratio of nanoparticles makes the nanoscaled carrier to be attractive candidates for the delivery of bioactives through pulmonary mucosal sites. Antigen-loaded nanoparticles improve the antigen concentration at the mucosal site and help mucosa to interact with antigen. Subsequently, the increase in antigen exposure to APCs resident in mucosa causes much stronger immune responses against the antigen.

The carriers of interest for developing a pulmonary vaccine must be safe, biocompatible, stable, and nonreactive. Also, nanoparticles must be capable to protect the antigen from harsh conditions and reduce degradation of antigen caused by enzymatic activities [98]. Moreover, they must guarantee the permeability of loaded antigen into the target mucosal site in order to deliver the antigen to the APCs of interest [99,100]. In general, designing a vaccine delivery system with APC-targeting capabilities requires knowledge of antigen uptake and processing, nanoparticle preparation techniques and critical points, pulmonary mucosal immunity, and antigen characteristics and sensitivities [101,102]. Nanoparticles can play either the antigen carrier or adjuvant role. Also, nanoparticles act as a barrier to a deleterious environment. Antigen-loaded nanoparticles can release antigens in a sustained manner in order to enhance the availability of antigen to the pulmonary immune cells [103]. Cross presentation of antigen which is thought to be a prerequisite of generating $CD8^+$ T cell responses against viral infections can be provided by antigen-loaded nanoparticles. Furthermore, inflammatory adverse effects are less likely to be produced due to the lack of microbial danger signals in nanoparticles.

There are several preparation techniques such as dissolvation, chemical and physical conjugation, and encapsulation for the fabrication of nanoparticles. The critical key points of each preparation technique e.g. manufacturing process variables and chemical composition determine the functional characteristics of nanoparticles such as antigen loading efficiency and capacity, antigen release pattern and model, safety, and clinical efficacy [70]. As result, the preparation technique and its critical variables must be optimized with respect to desired functional characteristics [103].

An ideal pulmonary nanoparticulate vaccine has an easy and simple scale-up process with acceptable reproducibility and cost-effective large-scale manufacturing. Also, it has a formulation containing safe, nontoxic, commercial-available, and affordable materials. Moreover, following a single injection of a desired pulmonary vaccine, an active humoral and/or cellular immune response can be induced without the need for booster dose(s). Its formulation also shows acceptable stability in terms of shape, size, surface charge, and particle aggregation during the storage time. Furthermore, the premature leakage/release profile of antigen from nanoparticles must be specified and required secondary actions must be taken into consideration in case any out of specification is observed. An ideal vaccine has a predictable behavior upon secondary processes including sterilization, lyophilization, spray/freeze drying, reconstitution, and packaging. The particle size, distribution, and shape are not distorted through these processes and the quality of immune response generated by the vaccine is held in an acceptable range.

A wide range of materials from polymers to lipids, and natural to synthetic, can be used in the development of pulmonary nanotechnology-based vaccines.

7.1.1. Lipid nanoparticles

7.1.1.1. Liposomes. Among lipid nanoparticles, antigen-entrapped liposomes have been extensively investigated in recent decades [104,105]. They serve nanoscaled carriers for effective antigen delivery beyond the mucosal membrane. Liposomes have a vesicular bilayer structure that can entrap both hydrophilic and lipophilic compounds in the inner aqueous core and lipid bilayer, respectively. Phospholipids and cholesterol are the main constituents of liposomes [106]. Liposomes can be tailored in terms of physicochemical properties and composition in order to optimize the delivery of a wide range of materials (e.g. peptides, proteins, DNA/RNA, etc.) to mucosal membranes. As an example, surface charge inducers such as stearyl amine, asdioleoyltrime thylammoniumpropane (DOTAP), and dimethylaminoethancarbomoyl can modify liposomes into a cationic form which can be exploited to release their cargoes in a pH-dependent manner [101,106]. Cationic liposomes are shown to induce robust mucosal immune responses [107–109]. Moreover, targeting agents such as antibodies, bioadhesive polymers, mannose derivatives, Toll-like receptors, PRR ligands, and Igs can be decorated on the surface of liposomes to increase the intensity and efficiency of the desired immune response [12,110,111]. Adjuvant-entrapped cationic dimethyldioctadecylammonium liposomes bearing trehalose dibehenate (as immunomodulator) are also found to induce protection and mucosal immunity.

Interbilayer cross-linked multilamellar vesicles (ICMVs) which are formed by establishing a cross-linkage between lipid headgroups of multilayered liposomal vesicles are also investigated and it was found that antigen adjuvanted with ICMVs were able to generate a $CD8^+$ T cell response 13-fold stronger compared to simple soluble antigen. Furthermore, long-lasting memory T cells were also induced in vaginal and pulmonary mucosa after pulmonary administration [112].

7.1.1.2. Solid lipid nanoparticles (SLNs). SLNs are the first generation of lipid nanoparticles that are composed of biocompatible and physiologic solid lipids and cholesterol [113,114]. Due to a little void in solid lipid structure, the drug or antigen is incorporated in a molecular form which increases the solubilization capacity, and thus, an increased antigen availability is achieved. Moreover, SLNs improve the permeability of loaded cargoes through the mucosal membranes [115]. Mucosal delivery of hepatitis B antigen-loaded SLNs showed that much stronger systemic and robust mucosal immune responses are induced compared to parenteral vaccine delivery [116]. Cubosomes are a relatively new smart generation of lipid nanoparticles that are composed of cubic phases of lipids in particular proportions [117]. They may contain aqueous channels and serve as a promising carrier for mucosal vaccine delivery [118,119].

7.2. Emulsions

Emulsions are prepared by dispersing two or more immiscible liquid phases, stabilized with an emulsifier. They generally have oleaginous and aqueous phases with versatile proportions depending on the aim of formulation design. Depend on the continuous phase, simple emulsions are generally classified into water-in-oil (w/o) and oil-in-water (o/w) emulsions. Nanoemulsions have nanoscaled droplets dispersed sporadically in the continuous phase. It is well known that emulsions can be used to deliver antigen vaccines through the mucosal membranes. The w/o emulsions have much more ability to efficiently deliver the hydrophilic antigens to mucosal surfaces [120]. The release pattern and rate of actives from emulsions are highly dependent on the type of emulsion (e.g. w/o or o/w), the ratio of oil to the aqueous phase, the viscosity of the oleaginous phase, and the droplet size of the dispersed phase [120]. Emulsion-based adjuvants such as Freund's vaccine adjuvants, MF59, and AS03 are extensively investigated. The latest two adjuvants have been approved for use in influenza vaccines since 1997 and 2009, respectively [121]. Th1- and Th17-dependent cellular immunity in mucosal sites against anthrax [122], human immunodeficiency virus (HIV) [123], influenza virus [123], respiratory syncytial virus [124], methicillin-resistant *Staphylococcus aureus* [125], and hepatitis B virus [126] has been investigated following various mucosal administration of nanoemulsions.

Double emulsions are the second generation of emulsions that can protect the antigen during the emulsification process more efficiently compared to single emulsions. Recently, two studies were conducted on nonviral delivery of MF59 double emulsion self-amplifying mRNA vaccines expressing influenza [127] and HIV [128] surface antigens.

7.3. ISCOMs

ISCOMs are nanoscaled (mean diameter<60 nm), spherical, lipidic micellar delivery systems that can be used as antigen delivery systems. The main constituents of ISCOMs are cholesterol, phospholipid, adjuvant Quil A, and saponin [129]. The antigen-excluded form of ISCOMs is ISCOMATRIX® which has approval for use in veterinary vaccines [130]. Recently, ISCOMs are exploited as carriers of *Mycobacterium tuberculosis* antigen 85 complexes to induce humoral and cellular immune responses following pulmonary administration. In other studies, ISCOMATRIX® adjuvants have been investigated to deliver influenza and human NT cell lymphotropic virus type 1 to the mucosal sites [131,132].

7.4. Polymer-based particulate systems

7.4.1. Natural polymers

7.4.1.1. Chitosan. Chitosan is one of the natural biocompatible and biodegradable polysaccharide polymers which is composed of glucosamine and N-acetylglucosamine units. Chitosan is obtained from a deacetylation reaction involving alkaline hydrolysis of chitin (the second most abundant biopolymer). Based on molecular weight and degree of deacetylation, various grades of chitosan are commercially available. Due to the positive charge of chitosan in physiologic conditions, it exerts mucoadhesive characteristics which make chitosan and its derivatives interesting biopolymers for developing mucosal vaccine delivery systems. Furthermore, chitosan is a nontoxic and noncarcinogenic polymer [133,134]. However, chitosan only dissolves in aqueous acidic solutions and suffers from low solubility in physiologic pH, alkaline solutions, and organic solvents [135]. Due to the solubility matters of chitosan, chitosan derivatives including trimethyl chitosan, hydroxyethyl chitosan, phosphorylated chitosan, and sulfated chitosan have been investigated for mucosal vaccine delivery [136–138].

7.4.1.2. Hyaluronic acid. Hyaluronic acid is a biocompatible, biodegradable, and nontoxic carbohydrate polymer that is composed of glucuronic acid and N-acetyl-glucosamine-6-phosphate units. Due to its mucoadhesiveness properties, it is thought to be an appropriate candidate for mucosal delivery of vaccines and adjuvants. Hyaluronic acid can cause maturation and migration of mucosal DCs and T cell activation. So it plays the role of a multifunctional carbohydrate mediator by modulating the leukocyte trafficking [139]. Mucosal co-administration of heat-labile toxin-based adjuvant (LTK63) conjugated with hyaluronic acid and influenza hemagglutinin antigen was shown to induce active systemic and mucosal immune responses [140]. In another study, mucosal administration of nanocomposites containing F1-V-entrapped cationic liposomes and hyaluronic acid initiated strong humoral and balanced Th1/Th2 immune responses [11].

7.4.1.3. Gelatin. Gelatin is known as the chemical degradation product of collagen. This biodegradable protein is composed of proportionally diverse single or multi-stranded polypeptides rich in glycine, proline, and hydroxyproline. In the human body, gelatin is degraded to its structural units (amino acids). Thus, in order to increase the residence time of gelatin in central circulation, some modifications such as PEGylation of gelatin nanoparticles can be considered. Moreover, it has been found that PEGylated gelatin nanoparticles have a higher tendency to be taken up into cells by endocytosis. cell-specific targeted delivery of vaccines to lymphocytes can be performed by antibody surface modification of gelatin nanoparticles [141].

7.4.1.4. γ-polyglutamic acid. γ-polyglutamic acid is a biocompatible and biodegradable polypeptide made by glutamic acid monomers. It also represents a high water solubility. According to studies, mucosal administration of γ-polyglutamic acid nanoparticles and γ-polyglutamic acid/chitosan hybrid nanoparticles loaded by influenza hemagglutinin antigen can induce protective mucosal immune responses against the infection [142,143].

7.4.1.5. Pullulan. Pullulan is an FDA-approved, nontoxic, water-soluble, and biodegradable linear polysaccharide polymer that is obtained from the fermentation of the yeast Aureobasidium pullulans. It is composed of α-1,4-glucose molecule units with maltotriose units which are linked by an α-1,6-glycosidic linkage. pullulan exerts its adjuvant activities by induction of upregulation of maturation and activation markers on DCs [144]. Furthermore, proinflammatory cytokines including IL-6, IL-12p40, tumor necrosis factor-alpha (TNF-α), and TNF-β1 can be induced by pullulan in DCs [145]. It is also shown that strong humoral and Th1 cell-dependent immune responses can be achieved after mucosal administration of antigen-loaded pullulan nanoparticles [146]. Mucosal administration of cationic cholesteryl-pullulan nanogels containing Cholestridium botulinum type-A neurotoxin has been shown to induce active toxoid-specific mucosal and systemic immune responses [147]. Another study showed that intranasal administration of TNF-α –entrapped pullulan nanoparticles initiated a strong immune response against the influenza virus [148]. Nasal administration of pneumococcal surface protein A-containing pullulan nanogels has been shown to activate balanced Th1/Th2 responses in the serum and pulmonary system [149].

7.4.2. Synthetic polymers

7.4.2.1. Poly(lactic-co-glycolic acid) (PLGA). PLGA is a synthetic aliphatic polyester that is composed of lactic acid and glycolic acid units. Due to its biocompatibility, biodegradability, and safety, it has been widely studied for the delivery of vaccine antigens [150]. Upon in vivo administration of PLGA, lactic acid and glycolic acid are produced which are eliminated from the body by the citric acid cycle. Fabrication of various types of PLGA nanoparticles with different release kinetics is available by altering the molecular weight and manipulation of

physicochemical characteristics of particles [150]. Sustained release of antigen-loaded PLGA over several weeks helps to generate effector T cell memory responses. A wide range of loading materials from vaccine antigens to PRRS can be encapsulated in PLGA nanoparticles. Monophosphoryl lipid A (PRR ligand)-loaded PLGA nanoparticles are shown to induce the production of higher mucosal IgA and IgG compared to ligand-free nanoparticles [151]. In a recent study, mucosal administration of monophosphoryl lipid A-loaded PLGA nanoparticles leads to induction of systemic and mucosal immunity against *Mycobacterium tuberculosis* HspX/EsxS fusion antigens [152]. Other PLGA nanoparticles loaded by PRR ligands including TLR2, TLR3, TLR4. TLR7 and TLR9 are investigated and in all of the studies, active immune responses were induced in mice [153,154]. Targeting agents such as lectin [155], claudin [156], and polyethylene glycol [157] were co-formulated with PLGA nanoparticles in order to achieve an M cell-targeted delivery to the mucosal sites.

7.4.2.2. Poly(lactic acid) (PLA). Different types of PLA polymer including PEG [158] or PEG-derived block copolymers [159] have been exploited as carriers for the delivery of vaccine antigens to mucosal sites. Recently, finding of a study has shown that mucosal delivery of nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2-entrapped PLA nanoparticles coated by human immunodeficiency virus (HIV)-1 gag p24 surface antigen initiate both systemic and mucosal immune responses against HIV infection [160]. Moreover, it has been found that PLA nanoparticles can improve mucosal immunity by targeting the DCs [161].

7.4.2.3. Polyethyleneimine (PEI). PEI is a cationic polymer with a hydrophilic nitrogen-rich structure which makes PEI a highly modifiable and customizable polymer [162]. Because of its positive charge and polyplexes formation capabilities, it is one of the main nucleotide delivery reagents [163]. There are linear and branched topologies of PEIs with various molecular weights ranging from 1 to 1000 kDa [164]. One atom of every three atoms of the PEI structural backbone is nitrogen. Due to this property, a "proton sponge" structure is formed as an amorphous net structure which is ideal for work on lysosomes [165] and improves the cross-presentation of phagocytosed antigen after escaping from the vacuole and reacting with histocompatibility complex class I in the cytosol [166]. The cross-presentation of antigen-loaded PEI nanoparticles can induce a strong immune response [167]. Recently, PEI and PEI-derived micro/nanoparticles have been studied for the delivery of vaccine antigens and adjuvants. It has been found that PEI can increase the maturation rate of APCs. Moreover, the proliferation of effector cells can be achieved by using antigen-loaded PEI particles leads to the production of antigen-specific antibodies and various cytokines [163].

Various types of antigens including peptides, proteins, plasmids, etc. can be entrapped into PEI nanoparticles. Mucosal administration of protein antigen-loaded PEI nanoparticle have been shown to induce protective immune responses against the respiratory syncytial virus [168], HIV [169] and influenza [170]. Also, the results of studies have shown that mucosal vaccination by plasmid DNA-loaded PEI nanoparticles provides a strong immunity against coronavirus [171], HIV [172], herpes simplex virus (HSV) [173], and influenza virus [173]. It has been shown that conjugation of cyclodextrin to PEI can be used as an appropriate for nasal delivery of mRNA vaccines which leads to induction of stronger immune response by improving lymph node trafficking [174,175]. In an attempt, pulmonary delivery of DNA vaccine encoding MTB antigen (Rv1733c)-loaded PLGA-PEI hybrid nanoparticles has been shown to have a higher DNA-to-cell transformation capability and to produce antibody and cytokines required for providing stronger immune responses [176].

7.4.2.4. Poly-ε-caprolactone (PCL). PCL is one of the synthetic, hydrophilic, semicrystalline, and biodegradable polymers which has been studied for delivery of DNA and protein antigen vaccines. due to its hydrophilicity and resistance to acidic pH environments, it is one of the major carriers for vaccine delivery through the gastrointestinal tract [177]. Nasal administration of diphtheria toxoid-loaded PCL-PLGA hybrid polymer nanoparticles has been shown to generate higher levels of IgG compared to antigen-free nanoparticles [178]. In another study, it has been found that nasal administration of Streptococcus antigen-loaded PCL nanoparticles induces both systemic and mucosal immune responses [179]. Similarly, chitosan-PCL [180] and PEG-modified-PCL [181] hybrid nanoparticles containing antigens have been shown to lead the generation of strong systemic and mucosal immunity following intranasal administration.

7.4.2.5. Lipid-polymer hybrid nanoparticles. Lipid-polymer hybrid nanoparticles are relatively novel nanoparticulate systems that have several advantages including customizable and controlled release and delivery, high entrapment efficiency, and the possibility for entrapment of both hydrophilic and lipophilic bioactives [182]. They are made of a polymeric core with high antigen loading capabilities and a lipid bilayer shell that covers the polymeric core. In some cases, PEG-surface modification on lipid-polymer hybrid nanoparticles is applied in order to increase the steric stabilization of nanoparticles and prolong the resident time of nanoparticles in systemic circulation [183]. The expression of the reporter protein luciferase was found to be increased in 6 h following intranasal administration of lipid-polymer hybrid nanoparticles with mRNA-entrapped poly(β-amino ester) core covered by a lipid bilayer shell [184]. Recently, Foget et al. found that co-formulation of chitosan with recombinant *Chlamydia trachomatis* fusion antigen CTH522-entrapped lipid-polymer hybrid nanoparticles significantly activated the mucosal immunity after intranasal administration [185].

7.5. Virus-like particles (VLPs)

VLPs are composed of self-assembled virus structural proteins which can mimic live virus. Due to the lack of viral genomes in their structure, they are not pathogenic [186]. Due to the high similarity of conformation of virus-like particles to viruses, these particles can significantly induce strong immune responses. There are some virus-like particle-based vaccines in the market such as Gardasil® (human papillomavirus (HPV) vaccine), Hecolin® (hepatitis E vaccine), and Engerix-B® and Recombivax® (hepatitis B vaccine) and some are under investigation and development. Also, the virus-like particles mimic live viruses, they often need to be co-administered with adjuvants in order to exert their efficacies.

7.6. Virosomes

Virosomes are composed of viral membrane or surface proteins that are integrated into unilamellar vesicles. The vesicles may be constructed of viral lipids, synthetic or natural lipids, and nonionic surfactants [101,104]. In contrast to virus-like particles, the main advantage of virosomes is their intrinsic adjuvanticity [187], however, adjuvants are often required to be administered with virosome vaccines to potentiate the activated immunity [188]. Intranasal administration of HIV-1 gp41 peptide subunit entrapped virosomes was shown to lead to antibody-dependent cellular cytotoxicity and production of mucosal IgA and IgG antibodies [189,190]. Plasmid DNA delivery using virosome carriers was shown to target DCs and induce Th1 immune responses after intranasal administration [21,191,192].

7.7. Gas-filled microbubbles (GFMs)

GFMs are micron-sized spherical structures that are composed of an inert high molecular weight gas enveloped in a polymer or lipid-based shell. Upon ultrasound application, microbubbles are formed by

sonoporation in shell material [193]. Originally, GFMs had been developed as ultrasound contrasting agents for assessing the blood compartments [194]. Thereinafter, researchers were found that GFMs can be exploited to deliver antigens [195], small molecule pharmaceutical bioactives [196], and nucleic acid-based therapeutics [197]. Recently, it has been found that GFMs with cationic lipid shells can be hunt by APCs and eventually can activate T cells by delivering their antigens to APCs without applying ultrasonication [198,199], leading to potentiated immunogenicity of entrapped antigen(s) [196]. Recently, a study was conducted on nasal administration of surface-loaded Salmonella-derived SseB antigen α-galactosylceramide-adjuvanted GFMs showed that an active antibody secretion can occur in the gut mucosa and a reduced bacterial load resulted after Salmonella infection [200]. The results of another study conducted by Bioley et al. showed that nasal administration of allergen-entrapped GFMs to mice can lead to a decreased neutrophil and eosinophil numbers in bronchoalveolar lavage. Furthermore, IgE was lower in the treatment group compared to control group. They also found that $CD4^+$ T cells presents in lung were less likely to secrete Th2 cytokine and IL-17 after administration of allergen-entrapped GFMs [176].

8. Formulation considerations

8.1. The physical form of the vaccine and drying technique

Pulmonary vaccines can be formulated in solid or liquid states. Most of them are unstable in liquid form. The occurrence of various degradation reactions can affect the nature of protein vaccines in the liquid state. Among them, oxidation, hydrolysis, and deamidation are important degradation reactions that can inactivate the protein vaccines. Hence, such vaccines preferably being formulated in solid forms by using various drying technologies [38,201,202]. Solid-state vaccines with particle sizes in the range of 1–5 μm can be produced by using freeze drying, spray drying, and spray freeze drying. Moreover, supercritical fluid technology, a more complex and expensive technology can be exploited to produce powdered vaccines. Powders obtained by these technologies have the ability of being deposited by sedimentation in respirable regions of the pulmonary system which makes it possible to deliver the antigens to APCs. Parenteral solid-state vaccines such as live-attenuated measles virus vaccine need to be reconstituted and administered by a healthcare professional. Recently, a stable dry-state live-attenuated measles virus vaccine for pulmonary delivery has been produced by using carbon dioxide-assisted nebulization with a bubble dryer which makes it possible to administer the vaccine by a needleless and noninvasive route. The incorporation of myoinositol as a cryoprotectant in the formulation helps to obtain a powder mass with a water content of less than 0.5% [203,204]. In the following, the top technologies for developing solid-state vaccines are discussed.

8.1.1. Freeze drying

Freeze drying technology comprises three major steps: freezing, preliminary or primary drying, and secondary drying. It is a well-established method used in the pharmaceutical and food industries. All steps of the freeze drying process have deleterious effects due to the nature of proteins. Proteins can lose their secondary and tertiary structures upon freezing and drying. The formation of ice crystals from the buffer of the formulation can directly affect the stability of vaccines. Moreover, a subsequent pH variation results in vaccine destabilization and potential degradation. To address these issues, stabilizers or cryoprotectants can be incorporated into formulation to stabilize it through freeze drying. Amorij et al. have studied the effect of HEPES and phosphate buffers on the stability and antigenic properties of a pH-sensitive influenza subunit vaccine upon freezing [205]. They concluded that the conformation of the vaccine containing HEPES buffer and cryoprotectant can be stabilized upon rapid freezing. Since a cake-like mass is obtained from the freeze drying, further processing steps are required to produce an inhalable form.

8.1.2. Spray drying

Spray drying is a single-step method for achieving a potentially inhalable powder from a liquid starting material. Through the spray drying, a solution, emulsion, or suspension is subjected to atomization through a nozzle in an insulated hot air chamber, and eventually, after rapid evaporation of the solvent, a dried powder is obtained. It is a versatile method that makes it possible to obtain a physically and chemically stable powder mass with customized properties suitable for pulmonary delivery. With the manipulation of various parameters including the composition of formulation, inlet and outlet temperature, pumping rate, and vacuum pressure, powder masses with various particle sizes and morphology can be achieved [206,207]. Dried powders obtained by spray drying have a mean diameter less than 10 μm and uniform characteristics. Furthermore, powders obtained by spray drying with the same parameter adjustments have minimum batch-to-batch variation which makes it possible to provide a reproducible and repeatable therapeutic effect. In some cases, subjecting drug solution to spray drying results in low yield and possible aggregation or inactivation. To address these issues, excipients such as L-leucine or lactose are incorporated to enhance the stability of formulation upon processing and storage. Similar to freeze drying, spray drying also imposes some stresses that may affect the stability of protein and peptide vaccine formulation [206]. The most crucial concern is to maintain the native conformation of powder vaccine over mechanical and thermal stresses of the spray drying process. The incorporation of glass-forming excipients such as glycols, organic acids, and sugars is well-established to increase the stability of macromolecular vaccines upon the spray drying process and its stresses [103].

8.1.3. Spray freeze drying

Spray freeze drying is a relatively new approach to obtain inhalable dry powders. During this process, a liquid starting material is sprayed through the nozzles in a cryogenic medium with a controlled inner vacuum instead of spraying in a hot air chamber. Spray freeze drying is a selective method for obtaining thermal sensitive biopharmaceuticals. Due to freezing conditions and risk of buffer crystallization, cryoprotectants are incorporated into the formulation. Patil et al. have used inulin as a cryoprotectant in a pulmonary inactivated influenza virus vaccine [208].

8.2. Particle size

Inhaled particles deviate and deposit in the pulmonary system as their linear momentum predominate the lines of airflow. Fundamentally, particles are deposited in the pulmonary system by three mechanisms including inertial impaction, sedimentation, and diffusion [209]. Particle mean diameter determines in which lung region an inhaled particle or droplet will be deposited (Fig. 1). Particle size may also represent a crucial determinant on the fate of inhaled vaccine. The pharmacokinetic and pharmacodynamic of delivered particles including the efficacy of translocation into lung parenchyma, targeting of a specific pulmonary APC, draining of delivered antigen(s) to the lymphatic system are highly dependent on particle mean aerodynamic diameter. Deposition of a particular dosage of a vaccine in a specific region may seem straightforward in theory, but in reality, there are several obstacles to deliver an adequate vaccine dose to the pulmonary system [85]. As shown in Fig. 1, particles larger than 10 μm are deposit mostly in the upper airways, larynx, and pharynx. Particles with aerodynamic diameter below 2 μm are subjected to diffusion and maybe exhaled from the respiratory region. To target the upper parts of respiratory regions, particles must have a 5–10 μm size range. Generally, 2–5 μm is the ideal size range for the delivery of particles or droplets to alveolar regions [210]. Conventional vaccine antigens possess a wide range of three-dimensional sizes from 10 nm to 20 μm. viral subunit antigens,

peptides, and proteins have the smallest sizes and therefore often combined with adjuvants to form larger aggregates. In the next order, supramolecular structures such as VLPs and nanoparticulate antigens have a mean size diameter between 100 and 200 nm. Incorporation of such small antigens into liposomes, emulsions (e.g. Freund's adjuvant as a w/o emulsion and MF59 adjuvant as an o/w emulsion), mineral salts (e.g. alum) and liposomes forms a larger antigen delivery system up to 20 μm [211].

The uptake of antigens by APCs is highly size-dependent. Larger particulate antigens such as VLPs, virosomes, whole-cell vaccines, antigen-adjuvant hybrid microparticles, or liposomes provide larger surfaces that are appropriate for endowing with properties required for generating active immune responses. Larger surfaces also permit the particulate antigens to be endowed with charged, hydrophobic, and/or receptor-interacting properties which facilitate the interaction of particulate antigens with APCs. Furthermore, repetitive antigenic surfaces of pathogens provide a facilitated binding to natural immunoglobulins due to high avidity interactions. This leads to the activation of complement cascades due to high avidity interactions.

Pathogen-sized particulate antigens mimic the normal uptake by the APCs and lead to generate an active immune response [212,213]. A study conducted on the effect of particle size of antigens on the activated immune response has shown that smaller particles in the virus size range can be taken up by DCs, whereas the bacterial-size particle is hunted by alveolar macrophages following by the production of IgG [214]. Another survey conducted by Gutierro has reported that a stronger IgG-dependent immune response was generated following administration of 1000-nm size particles compared to particles with 200 and 500 nm mean diameters. No significant difference was reported regarding the immune responses activated by 200 nm and 500 nm particles [170].

As described earlier, adaptive immune responses are mainly developed in secondary lymphatic organs. Particle size must be considered throughout the trafficking of antigens from peripheral tissues to lymphatic organs. According to the hydrodynamic diameter and net surface charge, submicron particles have different migration, clearance, and biodistribution pathways, and eventually, they have different biofates after administration. So it seems that size plays a critical role in designing a particulate vaccine. Particles with hydrodynamic diameter below 6 nm and zwitterionic surface characteristics are rapidly translocated from alveolar air space to central circulation and then subjected to a fast filtration by the renal system. Larger particles with a size between 6 and 34 nm shown to be ideal for developing a vaccine antigen with aim of targeting lymph nodes. These particles are subjected to rapid routing to the regional draining lymphatic system and translocated from the alveolar luminal membrane to the septal interstitium. Particles larger than 34 nm are transferred to lymph nodes by APC trafficking [215]. Due to the difference between the optimal particle size for deposition to a particular lung compartment and the ideal size for targeting a specific APC, the antigen vaccine with cell-specific delivery characteristics may reach draining lymph nodes prior to deposit in respiratory system compartments. This may affect the efficacy of the antigen vaccine. A promising approach to overcome this issue is to incorporate nanosized structures into microparticles in order to form microstructured "Trojan" carriers with nano and microsized virtues. Being microscale allows the particles to be deposited into predetermined lung compartment. Then, after the release of nanoparticles from microscaled structures, the nanoparticles exert their therapeutic effects on the pulmonary system [216]. Besides the suggested approaches, a challenging issue is the required intensive energy to produce droplets (from a liquid dosage form) or deaggregate a powder mass [217,218]. In regard to the need for a low-cost and also highly-efficient liquid and solid pulmonary delivery system, large porous particles have been developed. Rendering particles to more porous allow to prepare a relatively new type of particles with a reduced mass, large geometric (>5 μm), and small aerodynamic diameters (≤5 μm) with high deposition capabilities. Since inertia is the predominant mechanism for the deposition of porous particles in the lung, as they become larger, their deposition also decreases. Another significant advantage of large porous particles is the reduced energy required for deaggregation of powder mass [103].

8.3. Surface charge, shape, and characteristics

The interaction of vaccine antigen with APCs has been also reported to be influenced by surface charge. Mayumi et al. have reported that cationic liposomes can generate stronger immune responses compared with neutral and anionic liposomes [219]. In a more recent study, the results obtained by Kumar et al. showed that small spherical particles with a mean diameter of 193 nm induced a Th1-biased immune response, while larger rod-shaped particles with a mean diameter of 1530 nm generated a Th2-biased response [217].

The presence of targeting moieties such as antibodies which can be adjoined to the particle can affect the intensity of activating immune responses by improving the interaction of antigen with APCs [218]. Moreover, hydrophobicity of surfaces such as those of pluronic-coated particulate antigens can improve the uptake of antigens by APCs and also activate the alternative pathway of complement cascade [220].

9. Instrumental characterization of pulmonary vaccine candidates

Various high-resolution molecular imaging techniques including magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), positron emission tomography (PET) can be exploited to evaluate and characterize pulmonary vaccine candidates. The most important advantage of mentioned techniques is that they are noninvasive and clinically translatable [221,222]. Moreover, combined multi imaging modalities such as SPECT-CT, PET-MRI, and PET-CT enabling to obtain more accurate and precise knowledge of the pharmacokinetic aspects of vaccine candidates [223]. Furthermore, the immune responses evoked by the pulmonary vaccine or anticancer vaccine can be tracked by PET-CT and SPECT-CT [224–226].

10. Review on pulmonary vaccine formulations

Pulmonary nanoparticulate systems mimic pathogens in order to provide mucosal vaccination in a safe manner. Besides all advantageous aspects of nanoparticulate systems, various technical challenges such as selecting a safe adjuvant for pulmonary administration, targeting of nanoparticles to particular compartment of respiratory tract to induce optimal immunogenicity against infectious agents, difficult optimization of particle mean diameter with aerosolization capability, avoidance of tolerance induction and designing an appropriate and affordable device are the obstacles for entry of pulmonary vaccine candidates from preclinical to clinical setups [8].

The need for cold-chain storage and distribution and the stability of pulmonary vaccine formulations can be reduced by turning the liquid forms into solid dosage forms. As described earlier, techniques such as spray drying and freeze-drying can be used as scalable methods to dry a liquid formulation. Other physicochemical parameters such as particle size, shape, surface charge, etc. can affect the fate of pulmonary vaccine candidates. In the following, pulmonary vaccine candidates are reviewed Table 1.

11. Recent patents and clinical trials on pulmonary vaccine delivery

Recent patents on pulmonary vaccine development have exploited different strategies from design a novel or hybrid nano/microparticle to use of adjuvants (see Table 2). However, the number of patents on development of pulmonary vaccination are still limited. Recent patents or Patent Cooperation Treaties (PCTs) are presented in Table 2.

Table 1
Nano- and microparticulate systems for pulmonary vaccine delivery and immunotherapy.

Immunostimulatory agent (vaccine form)	Adjuvant	Targeting agent	Nano/micro particle system	Technique of preparation	In vitro/in vivo study	Year	Outcome	Ref.
DNA plasmid encoding eight HLA-A*0201-restricted T-cell epitopes from *Mycobacterium tuberculosis*	–	–	Chitosan nanoparticles	complexation-coacervation method	HLA-A2 transgenic mouse	2004	Plasmid-loaded chitosan nanoparticles were found to be able to induce the maturation of dendritic cells (DCs). Nanoparticles loaded by DNA also was shown to act as a booster for IFN-γ secretion.	[227]
human papillomavirus (HPV) type 16	–	–	Virus-like particle (VLP)	–	Human	2005	Volunteers vaccinated by pulmonary HPV VLP formulation were shown to have suitable serum antibody titers. Also, pulmonary delivery of the vaccine led to IgA secretion and subsequent mucosal immunity.	[228]
Measles antigen	–	–	–	Spray drying	Macaque	2007	Inhalation of measles antigen powder was found to be less effective compared to the injectable measles vaccine.	[229]
Influenza subunit vaccine	–	–	–	Spray freeze drying	BALB/c mouse	2007	Dry powder vaccine-induced superior systemic humoral (IgG), cell-mediated (IL-4, IFN-γ) and mucosal (IgA, IgG) immune responses, as compared with the parenteral route.	[230]
Monovalent A/Panama/2007/99H3N2 influenza split virus vaccine	–	Ovalbumin	–	Spray drying	BALB/c mouse	2007	Site-dependent IgG and IgA immune responses were achieved. It was found that deep lung deposition of the vaccine was correlated with superior immune responses.	[231]
Bacillus Calmette-Gurerin (BCG)	–	–	–	Spray drying	–	2007	The spray-dried vaccine displayed improved viability as compared with a lyophilized vaccine.	[232]
Live-attenuated tuberculosis vaccine bacille Calmette–Guérin (BCG)	–	–	Rod-like nanomicroparticle	Spray drying	Guinea pig	2008	A significant reduction in bacterial burden was observed after vaccination. Dry powder vaccine was able to produce comparable tuberculin reaction compared with subcutaneous or intradermal routes and maintain viability after 9 months in refrigeration	[233]
Bacillus Calmette-Gurerin (BCG)	–	–	–	Spray drying	Female BALB/c mouse	2009	A safe and reproducible procedure that enables the efficient delivery of the powdered vaccine to the lungs of mice was developed.	[234]
Recombinant hepatitis B surface antigen (rHBsAg)	–	–	PLGA/PEG hybrid nanoparticles	Spray drying	Guinea pig	2010	Higher IgA titers and higher IgG titers were observed following intrapulmonary and intramuscular administration of vaccine formulations, respectively.	[235]
Diphtheria CRM-197 Antigen (CrmAg)	–	–	formalin-treated/untreated CrmAg nanoaggregates	Spray drying	Guinea pig	2010	Intramuscular and intrapulmonary administration of vaccine formulation to guinea pig were correlated to higher IgG and IgA titers, respectively.	[236]
Tuberculosis recombinant antigen 85B (rAg85B)	–	–	poly(lactic-*co*-glycolic acid) microparticles	Spray drying	Guinea pig	2010	Pulmonary delivery of the spray-dried powder was found to be able to reduce the extent of granuloma and necrosis growth in the lung and spleen upon challenge.	[237]
Whole inactivated influenza virus vaccine	–	–	–	Spray freeze drying	BALB/c mouse	2011	A transient influx of neutrophils and a concomitant decrease in the number of macrophages were found following both powder and liquid formulations. Pulmonary administration of powder formulation was found to induce higher mucosal IgA responses.	[238]
Hepatitis B surface antigen (HBsAg)	–	–	Porous poly(l-lactic acid) (PLA) Porous poly(lactic-co-glycolic acid) (PLGA) nanoparticles	Double emulsion solvent evaporation technique	Female Sprague-Dawley rats	2011	Pulmonary delivery of vaccine formulations led to IgA secretion in mucosal sites and cytokines in the spleen. The immune. Large hydrophobic particles were more efficiently internalized by rat alveolar macrophages compared to smaller hydrophilic particles.	[239]
Mycobacterium tuberculosis antigen 85A	CpG oligonucleotide	–	Poloxamer 407 nanoparticles	–	BALB/c mouse	2012	Deep deposition of antigen in the lung was shown to generate more intense specific immunity. Also, the combination of poloxamer 407 and CpG led to humoral	[240]

(*continued on next page*)

Table 1 (*continued*)

Immunostimulatory agent (vaccine form)	Adjuvant	Targeting agent	Nano/micro particle system	Technique of preparation	In vitro/in vivo study	Year	Outcome	Ref.
							immune responses *in vitro*. The adjuvant CpG boosted the Th-1 immune responses with a high IgG2a/IgG1 ratio, high IFN-γ and TNF-α productions *in vitro*.	
Tuberculosis subunit combining with cutinase-like proteins 1 and 6 and MPT83	Lipokel			Spray drying	female C57BL/6 mouse	2013	Recruitment of neutrophils and APCs, induction of antigen-specific effector T lymphocyte, and subsequent production of INF-γ were achieved after pulmonary administration of powder vaccine.	[241]
Inactivated influenza virus antigen	–	–	–	Spray drying	Female Sprague-Dawley rats	2015	Spray drying led to the formation of an amorphous glassy matrix. The incorporation of antigen into the matrix affected the water content and glass transition temperature without any impact on the efficacy of the vaccine. Both mucosal and systemic immunizations with no inflammatory reaction were achieved following pulmonary administration of the spray-dried formulation.	[242]
Pneumococcal surface protein A (PspA)	–	–	L-leucine encapsulated nanocomposites	Spray drying	Cellular uptake of particles by DCs	2015	It was found that spray-dried nanoparticles can deposit in the broncho-alveolar region and can potentially be uptaken by DCs.	[243]
Human papillomavirus vaccine	–	–	–	Nebulization	Human healthy volunteers	2016	An optimized and safe droplet deposition in lung compartments was achieved.	[244]
Neisseria meningitis serogroup B	amorphous aluminum hydroxyphosphate sulfate aluminum hydroxyphosphate	–	–	Spray drying	–	2016	Combining the Design of Experiment strategies with accelerated stability studies was shown to potentially develop an accelerator of vaccine formulation.	[245]
Whole inactivated influenza virus vaccine	–	–	–	Spray drying	–	2016	Optimization of a vaccine formulation by the Design of Experiment method led to a thermostable and efficient formulation suitable for pulmonary vaccination.	[246]
Whole inactivated virus vaccine	Advax® (delta inulin)	–	–	Spray freeze drying	female BALB/c mouse	2018	Higher systemic, cellular, and mucosal immunities were achieved following the pulmonary administration of Advax®-adjuvanted liquid and powder vaccine formulations compared to non-adjuvanted vaccine formulations.	[247]
Live-attenuated *Flavivirus* vaccine	–	–	–	Freeze drying	–	2018	The combining of semi-empirical excipient screening and Design of Experiment strategies led to achieving vaccine powder with better physicochemical characteristics.	[248]
SHetA2 (for treatment of tuberculosis)	–	–	–	Spray drying	–	2018	Deposition of microparticles in the alveolar region was provided due to narrow particle size distribution. Also, the dissolution rate was found to be applicable for pulmonary delivery system development.	[249, 250]
Outer membrane vesicle pertussis vaccine (omvPV)	–	–	–	Spray drying	Female BALB/c mice	2018	Mucosal (IgA and Th17-dependent) and systemic (IgG and Th1/Th17-dependent) responses were observed.	[251]
Tuberculosis subunit vaccine (fusion antigen H56)	Liposome-based cationic adjuvant formulation (CAF01)	–	Liposome	Thin-film hydration method	Female CB6F1 hybrid mice	2018	Parenteral prime and intrapulmonary boost immunization led to the induction of strong mucosal and systemic IgA and polyfunctional Th1 and Th17 responses.	[223]
Tuberculosis subunit vaccine (fusion antigen H56)	Liposome-based cationic adjuvant formulation (CAF01)	–	Liposome	Thin-film hydration method Spray drying	Female CB6F1 hybrid mice	2018	humoral and cell-mediated immune responses were provided after pulmonary administration of dried powder vaccine formulation.	[252]

Table 2
Recent patents on pulmonary vaccine development and delivery.

Patent number	Immunostimulatory agent	Aim/Novelty of invention	Year	Ref.
WO2009077732A2	–	The invention aimed to provide a slow release composition for development of micron or submicron particles comprising water-soluble crystals (such as metal salt is calcium phosphate, calcium pyrophosphate, aluminum hydroxide, aluminum phosphate, calcium carbonate, iron hydroxide, magnesium phosphate, magnesium carbonate, zinc phosphate) with release-controller coated shells.	2008	[253]
EP2144626B1	Inactivated whole *Mycobacterium tuberculosis*	The invention aimed to establish adequate protection against TB by induction and of T-cell immune responses in the lung mucosa and maintaining it in adult life.	2013	[254]
WO2014066856A1	Inhalable influenza (type A, B, C or any other subtype of influenza A) VLPs compromised hemagglutinin and/or a neuraminidase protein and/or a matrix protein	The invention aimed to induce systemic immunity through mucosal immunization (IgG and long lasting cell mediated immune response) And also local (s-IgA) immunity at the site of mucosal entry.	2013	[255]
US8481055B2	Bacillus anthracis (Anthrax)	The invention aimed to enhance the immunogenicity of a vaccine against Bacillus anthracis in a subject, comprising administering to the subject a therapeutically effective amount of an oligodeoxynucleotide consisting of the nucleic acid sequence set forth as SEQ ID NO: 23, an oligodeoxynucleotide consisting of the nucleic acid sequence set forth as SEQ ID NO: 24, and an oligodeoxynucleotide consisting of the nucleic acid sequence set forth as SEQ ID NO: 25; and administering to the subject a therapeutically effective amount of the vaccine against Bacillus anthracis, wherein the vaccine is (a) Anthrax Vaccine Adsorbed (AVA) or (b) recombinant Protective Antigen or Protective Antigen and an aluminum salt.	2010	[256]
US20150283069	–	The method comprises the administration of an inhalable composition comprising virus-like particles and/or specific protein/s or antigenic peptides, peptide fragments and/or derivatives thereof using a dry powder drug delivery system.	2015	[257]

Table 3
Pulmonary delivery of liquid-based viral vaccines.

Virus	Vaccine strain/type	Humoral response measured	Outcome	Ref.
HPV	HPVL1 VLP	IgA and IgG	High-dose aerosol provides a comparable immunization to those who received intramuscular vaccine. Also, inhalational vaccination led to a significant greater immunity against HPV compared to intranasal vaccination.	[258]
Influenza	Bivalent WIV vaccine containing B/Massachusetts/66 and A/Aichi/68/H3N2	Not specified	Statistically significant greater protection against influenza infection compared to control group was reported.	[263]
	Bivalent WIV vaccine containing A/Japan/62/H2N2; A/Taiwan/64/H2N2; B/Massachusetts/66 vs. monovalent A/Hong Kong/68/H3N2	Not specified	Authors have reported greater immunity after aerosol immunization compared to subcutaneous immunization.	[264]
	Bivalent WIV vaccine containing A/Japan/62/H2N2; A/Taiwan/64/H2N2; B/Massachusetts/66vs. monovalent A/Hong Kong/68/H3N2	Not specified	Two-dose aerosolized H3N2 vaccination of adults was reported to be more efficient.	[265]
Measles/mumps/rubella	MMR-SII vaccine (Edmonston-Zagreb live-attenuated measles vaccine, Leningrad-Zagreb live-attenuated mumps vaccine and RA 27/3 live-attenuated rubella vaccine); MMR-II vaccine (Attenuvax strain (comparable to Schwarz live-attenuated measles vaccine), Jeryl-Lynn live-attenuated mumps vaccine and RA 27/3 live-attenuated rubella vaccine)	Not specified	Aerosol administration of booster dose to seropositive children led to almost 100% seropositivity. No statistical differences between immunization after injection and aerosolized vaccine were not reported.	[266, 267]
Measles	Edmonston-Zagreb live-attenuated measles vaccine	IgA and IgG	Serum IgG was reported to be significantly greater after pulmonary administration compared to subcutaneous injection. Serum IgA titers increased in both groups with no statistically significant differences. Remarkable increases in IgG and specific IgA in nasal secretions after aerosol administration were reported.	[268]

The majority of clinical trials on pulmonary vaccines has focused on aerosolized liquid formulations. Several studies have reported that inhalational vaccines can provide broader immunity against infections as a booster, compared to vaccination via injection. A clinical trial performed on nebulized VLP against human papillomavirus (HPV) type-16 has shown that specific IgA antibodies were produced in cervical mucosal secretions [258]. This may reflect the immune cell trafficking to different areas of MALT. Although studies on aerosolized and liquid vaccine formulations have been progressed, there are still stability and logistical issues about them [259–261]. Regarding this aspect, dry-powder inhalable vaccines are stable and reliable candidates for delivery of immunostimulatory agents to pulmonary system. The clinical studies on pulmonary liquid vaccine formulations have been presented in Table 3.

However, the number of clinical trials on dry-powder inhalable vaccines is still limited. To date, only one clinical study has performed

on inhalational dry-powder measles vaccine in adult males.

Although pulmonary system provides numerous advantages for vaccine delivery, there is only one licensed intranasal vaccine in market. FluMist® is a two-dose intranasally-administered live viral reassortant with trivalent mix of H1, H3, and B strains of hemagglutinin and neuraminidase genes in an attenuated donor strain. It provides hemagglutinin and neuraminidase-specific mucosal IgA and systemic IgG immune responses. Its efficacy against influenza type A and B virus infection among young children is reported to be more than %85 [262].

12. Expert opinion and future perspectives

According to the estimation of the World Health Organization (WHO), approximately 2 million children died from vaccine-preventable diseases. Based on the WHO global immunization coverage report, in 2019, about 85% of infant populations (approx. 116 million infants) have received 3 doses of diphtheria-tetanus-pertussis (DTP3) vaccine around the world, which protect them against the serious illness and debilitating conditions of the infectious diseases. In 2019, 125 countries have vaccinated more than 90% of infant populations against diphtheria, tetanus, and pertussis [269]. One of the major obstacles to achieving a wider immunization coverage is that most developed vaccines are manufactured for injection and they need to have cold-chain storage and distribution. Providing sterile water for injection, cold-chain conditions, and trained professionals are the most important limitations of vaccination in developing countries [207]. For these reasons, the development of pulmonary vaccines which have solid-state formulations can be a mainstay to enhance global immunization coverage.

Solid-state pulmonary vaccines have higher stability and more capability for inducing both mucosal and systemic immune systems. Moreover, the spray-drying technique presents the opportunity of engineering particles that cannot be readily performed by other processes. Also, antigen stabilizing excipients can be incorporated to preserve the integrity of antigenic molecules. The presence of an interconnected immune system network in different mucosal sites makes it possible to induce desired immune responses against infectious diseases originating from a remote mucosal site by administration of vaccine via the pulmonary route. Moreover, the unique features of the pulmonary system such as enormous mucosal surfaces provide a desired condition for vaccine delivery. Nevertheless, conducted studies have obtained controversial results regarding the optimum particle size ranges for inducing strong and long-lasting immune responses. for these reasons, although a number of intranasal and oral vaccines have been approved, there is still no approved vaccine for pulmonary delivery [270].

Nanoparticulate systems have played an undeniable role in improving pharmacokinetic aspects of pulmonary drug and vaccine delivery. Nanoparticles confer several advantages over conventional vaccines as they alter the release and improve the targeted delivery of antigens to APCs at mucosal sites. New generations of mucosal vaccines utilize the components of bacteria or viruses to induce the immune system. Targeted antigen delivery to the M cells present at the mucosal epithelium of the respiratory tract can be performed using antigen-loaded nanoparticles. Nevertheless, unmet needs and challenges toward mucosal delivery of antigens by using nanoparticulate systems still remain. One of the still-standing challenges against the development of nanoparticulate pulmonary vaccines is the incomplete understanding of the mechanism of action of existing vaccines. Moreover, the correlation between the immune responses evoked by vaccines and by mucosal adjuvants is not well established.

Another important limitation is the variation of the effective and the toxic dose of the same adjuvant in different animal models which leads to a poor correlation between preclinical and clinical trials of pulmonary vaccines. Advances in high-resolution molecular imaging have greatly contributed to the development of efficacy studies of pulmonary vaccine candidates. Moreover, more studies should be performed on developing new adjuvants with appropriate features for pulmonary use. In addition, further studies should be directed towards understanding the features of evoked immune responses after antigen delivery to different compartments and regions of the respiratory system [231,271].

13. Conclusion

From the pharmaceutical and immunological point of view, the pulmonary route has been demonstrated to be an efficient means for inducing the immune system against airborne pathogens and various infectious diseases. Although the pulmonary vaccine development seems to be a promising approach to vaccinate different populations in developed and developing countries equally, technological and scientific obstacles such as high cost of pulmonary devices, incomplete knowledge toward adjuvants, and immune responses evoked in different regions of the respiratory tract still remain. Nanotechnology has brought new insights into pulmonary vaccines. Nanoparticulate vaccine delivery systems allow scientists to engineer the particles supposed to be delivered to the pulmonary system and also alter the pharmacokinetic aspects of formulations in direction of improving the efficacy of vaccination. However, there are still several avenues to be explored.

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Authors contribution

In this study, Writing the original draft, reviewing, and editing was conducted by Dr. Moein Masjedi. Dr. Talieh Montahai, Dr. Atefe Jalali and Dr. Zeinab Sharafi revised the manuscript.

Declaration of competing interest

Authors have no conflict of interest to declare.

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