Pulmonary vaccine delivery

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This review will discuss developments in the field of pulmonary vaccine delivery. The possibilities of adopting aerosol-generation technology and specific pharmaceutical formulations for the purpose of pulmonary immunization are described. Aerosol-generation systems might offer advantages with respect to vaccine stability and antigenicity. Adjuvants and their inclusion in vaccine-delivery systems are described. Other formulation components, such as surfactants, particulate systems and dispersion of the aerosols are detailed in this paper. The noninvasive, relatively safe and low-cost nature of pulmonary delivery may provide great benefits to the public health vaccination campaign.

Expert Rev. Vaccines 6(2), 213–226 (2007)

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KEYWORDS:
aerosol, delivery, device, formulation, vaccine

Vaccination is one of the most powerful strategies to prevent infectious diseases in underserved regions, particularly in developing countries and disaster areas. Most common pathogens enter the body via mucous membranes in the nose, lungs and gastrointestinal tract. Respiratory viral and bacterial infections are a major cause of morbidity and mortality worldwide [1]. Many pathogens involve or utilize the respiratory tract as a portal of entry into the body [2,3]. Therefore, pulmonary aerosol vaccination could be a potentially powerful way to rapidly immunize the population, inducing protection by exposing airways to vaccines. This route of vaccine administration, which follows the natural route of infection, may best mimic the induction of immunity in the respiratory tract by pathogens and may lead to more general systemic immunity. Aerosol vaccination is a noninvasive, nontraumatic method of antigen delivery that avoids the risk of transmitting hepatitis B, HIV and other bloodborne agents through improper injection practices. Therefore, pulmonary immunization is a potential alternative to conventional parenteral delivery approaches.

This review focuses on pulmonary immunization, specific pharmaceutical formulations and their delivery systems for pulmonary vaccines.

Pulmonary vaccination belongs to mucosal immunization

Mucosal immunity is the first line of defense against pathogens entering the body via mucosal surfaces. Pulmonary, nasal and oral immune systems contribute almost 80% of all immunocytes. These cells are accumulated in, or transit between, various mucosal-associated lymphoid tissues (MALT) that together, form the largest mammalian lymphoid organ system [4].

Mucosal immunization can induce systemic immunity: generation of secretory immunoglobulin (Ig)A antibodies, which are able to cross epithelial membranes and prevent future entry of pathogens through the mucosal site. Furthermore, lymphocytes that were stimulated by antigens in the mucosal inductive site migrate via regional lymph nodes and the thoracic duct to the bloodstream and other mucosal effector sites. This migration leads to secretory IgA production at other mucosal sites of MALT (gut, nasal and genitourinary-associated lymphoid tissues) and has been termed the common mucosal immune system [5–8]. Meanwhile, circumstantial evidence indicates the existence of cell-mediated immunity in the common mucosal immune system [9]. The common mucosal immune system appears to have organ selectivity as enhanced memory is seen at the site of mucosal priming compared with that of distant mucosal sites [10].

10.1586/14760584.6.2.213

ISSN 1476-0584

Maintenance of the memory T cells depends on the tissues in which exposure to antigen first occurs [11]. Mucosal vaccination, rather than systemic immunization, gave long-lived cytotoxic T memory lymphocytes [9].

Pulmonary immunization appears very promising since the lungs contain a highly responsive immune system. The pulmonary epithelium has a crucial role in host defense against inhaled pathogens as it presents physical barriers, including mucociliary escalator, secretion of antimicrobial agents [12–14], chemokines [15] and cytokines in the mucus layer covering the airway epithelium to prevent colonization of microorganisms. Pulmonary macrophages and dendritic cells (DCs) play an important role in both innate and adapted immunity. Alveolar macrophages are very abundant, with over a billion in the periphery and interstitium of the lungs [16]. DCs are found in epithelial linings of the conducting airways, submucosa below the airway epithelium, within alveolar septal walls and on the alveolar surfaces [17]. These two cell populations are professional antigen-presenting cells (APCs). They are able to phagocytose, process and present antigens to stimulate T cells. Primary stimulation of T-cell clones within the pulmonary lymphoid tissue is induced when macrophages and DCs migrate to bronchial lymph nodes and home to the T-cell paracortical area [18,19]. In animals and mammals, bronchus-associated lymphoid tissue (BALT) – the respiratory part of MALT – is located mostly at bifurcations of the bronchus. Humoral immune responses elicited by BALT are primarily IgA secretion both locally and by BALT-derived B cells that have trafficked to distant mucosal sites. Local T-cell responses have also been noted. On the basis of these findings, BALT can be thought of as a functional analogue to mucosal lymphoid aggregates in the intestine and, thus, deemed as a component of the common mucosal immune system [20]. There is evidence that mice genetically lacking spleen, lymph nodes and Peyer's patches can generate strong primary B- and T-cell responses to inhaled influenza. These responses appear to be initiated at sites of the induced BALT, which functions as an inducible secondary lymphoid tissue for respiratory immune responses [21]. Exposure of the lungs to various aerosol formulations designed to protect against influenza virus was more effective than either intranasal administration or parenteral injections, indicating that a local response was generated in the respiratory tract [22].

The physiological features of lymphoid tissues in the respiratory tract suggest there is potential for pulmonary immunization. Consequently, pulmonary vaccination is a reasonable strategy for protecting a population from infectious diseases where pathogens enter the body via the lungs.

Current status of pulmonary vaccine delivery

Veterinarians were among the first to recognize the possibilities of mass aerogenic vaccination. Living Newcastle disease vaccine was administered to chickens via inhalation in 1952 [23]. Since then aerosol vaccination against Newcastle disease has been widely and effectively used worldwide to immunize poultry [24,25]. There have been several successful pulmonary immunization trials of fowl and pigs against fowlpox, infectious bronchitis, hog cholera, erysipelas, pseudorabies, gastroenteritis, pasteurellosis and mycoplasmosis [24,26,27].

The potential for immunization of humans by aerogenic vaccination was recognized in the Soviet Union [28] and the USA [29] 40 years ago. Russian investigators employed dry-vaccine preparations containing living vegetative cells or spores of attenuated strains for vaccination to humans and indicated that aerogenic vaccination was as effective as subcutaneous vaccination against plague, tularemia, brucellosis and anthrax [28,30]. Airborne Bacillus Calmette–Guérin (BCG) vaccination was conducted at the University of Illinois (USA) in 1968. BCG were nebulized to people in different age groups [31]. Rosenthal and colleagues indicated that tuberculosis pathology in human beings was similar to that in guinea-pigs rather than mice or rabbits, providing important information on BCG aerosol immunization, as well as animal models, for tuberculosis. Rubella immunizations delivered via pulmonary, nasal and subcutaneous routes were compared in 46 volunteers early in 1973. Humoral immunity via pulmonary delivery was comparable with that of subcutaneous vaccination [32].

Pulmonary immunization is an appealing means of protecting populations in biodefense strategies. *Bacillus anthracis* infection in humans occurs as cutaneous, gastrointestinal or inhalational anthrax depending upon the route of exposure. Inhalational anthrax – the form likely to occur during a bioterrorist attack – is difficult to diagnose early and, despite antibiotic therapy, has a high fatality rate. In 1957, 32 volunteers were exposed to an aerosolized dry spore of live anthrax vaccine composed of a mixture of strains STI-1 and No. 3, for 15 min in the Soviet Union. Few general adverse reactions were recorded [30]. Later, a skin test indicated that the aerosol method induced higher immunity than subcutaneous and scarification methods previously used for immunization in the Soviet Union [33,34]. The pattern of cell-mediated immunity after aerosol anthrax immunization was investigated in human subjects. There were five phases in the kinetic pattern of Phase II (7–15 days postvaccination) showed an exponential rise to a maximum at day 15. In later phases, skin reaction was reduced for up to a year. The loss of skin reaction on day 30 is a characteristic feature of postvaccination anthrax cell-mediated immunity. It may be due to a blockade of macrophages by lethal anthrax toxin released by the multiplying vaccine strain [35].

Francisella tularensis is a facultative intracellular bacterial pathogen. Infection may be caused by inhalation of contaminated air. Therefore, *F. tularensis* is considered a serious biohazard [36]. Live, attenuated *F. tularemia* strain vaccine (LVS) was nebulized and administered to the human lungs [37]. Immunity to aerogenic virulent LVS challenge appeared to be greater than the conventional parenteral vaccine administration.

The only successful clinical case of pulmonary vaccination on a large scale, with characterization of aerosol device technology, is a pulmonary measles immunization study. Measles vaccination via pulmonary aerosol delivery has considerable appeal. Approximately 4 million children in Mexico were exposed to measles

vaccine aerosols and a high rate of successful prevention was achieved [38]. The custom-made system, Classical Mexican Device, used an International Product Inc. (IPI) jet nebulizer driven by an Evans industrial air compressor to deliver aerosols of the reconstituted Edmonston Zagreb (EZ) strain of attenuated measles vaccine virus. The pulmonary delivery system produced 52–64% seroconversion, which compared favorably with the expected seroresponse to subcutaneous administration (4–23% seroconversion) in school children [39]. Interferon (IFN)-γ production in cellular immunity was more robust in infants who received aerosol vaccines [40]. Many children received a much larger dose than that necessary for immunization without any side effects. The necessary immunization dose for different age groups of children has been estimated [41]. The WHO has identified three nebulization devices manufactured by Omron, Trudell and Aerogen that meet the desired performance criteria to replace the classical Mexican device [42]. In order to overcome the cold-chain requirement and maintain the biological stability of measles vaccine, a pulmonary dry-powder aerosol formulation is under development. The attenuated EZ strain of measles was micronized by jet-milling after lyophilization. The measles vaccine was dispersible after blending with carrier lactose [43]. The shift from liquid to powder formulation coupled with appropriate dry-powder inhalation technology potentially strengthens the measles vaccine. The current Measles Aerosol Project by the WHO aims to license at least one method for respiratory delivery of current measles vaccines by 2007 [44].

Clinical trials have demonstrated better or equal immune responses for measles and rubella delivered as inhaled aerosols with respect to alternative routes of administration [45].

Pulmonary DNA immunization is a new and promising vaccination approach. DNA vaccines have the advantage of inducing a strong cellular immunity with a preference to cytotoxic T lymphocyte (CTL) and T helper type (Th)1 T-cell response. Pulmonary delivery of DNA vaccines were recently described in mouse models using plasmids encoding ovalbumin, hepatitis B surface antigen (HBsAg) [46] and HLA-A*0201-restricted T-cell epitopes of *Mycobacterium tuberculosis* [47]. Increased immunity as measured by antibody and cytokine production was achieved.

Aerosol technology available for pulmonary delivery of vaccine

Many aerosol exposure methods have been used to vaccinate animal models by delivery to the lungs. Intratracheal instillation and insufflation allows direct delivery of liquids and powders to the lungs. Animal exposure to aerosols has been achieved through a range of exposure chambers (whole-body and nose-only chambers). For clinical trials, aerosol vaccine delivery requires delivery devices, as well as formulations, in which the antigens are incorporated.

The site and efficiency of deposition of aerosolized particles in the respiratory tract is critically influenced by their particle size (defined as aerodynamic diameter), size distribution, particle shape and density [48]. If the aerodynamic particle size is greater than 5 µm, inertial impaction is the primary mechanism for deposition of the particles in the upper and central airways. Particles measuring 3 µm or less, which have not been deposited by impaction, deposit in the lower airways by sedimentation. Relatively low velocities, along with longer residence times, in the lower airways favor the deposition of submicron size particles (<1 µm) by the diffusion process. Diffusion and sedimentation are the major mechanisms of deposition in the lower airways of the lungs. Since particles that are subject to diffusion have little mass, this mechanism is not considered important for therapeutic or vaccine aerosol purpose. Aerosol vaccination usually depends on the target pathogen and the sites of inductive immunity. Larger particles (>5 µm) are needed for vaccination to prevent upper respiratory infections by respiratory viruses or bacteria, for example, *Bordetella pertussis* and *Chlamydia pneumoniae* [49], and smaller particles (≤3 µm) for lower respiratory tract infections, such as *Streptococcus pneumoniae* and *B. anthracis* [49].

Inhalers can be classified into three major categories: nebulizer, pressurized metered-dose inhaler (pMDI) and dry-powder inhaler (DPI).

Nebulizers

Two types of nebulizers are commercially available, air jet and ultrasonic nebulizers. Generally, air jet nebulizers can generate smaller particle sizes (mass median aerodynamic diameter 2–5 µm), which penetrate the small airways more easily. Nebulizers have some advantages: constant output can deliver aerosols of most solutions and provide large doses with very little patient co-ordination or skill; larger doses can be delivered than with MDI and DPI devices; disposable nebulizers are inexpensive. However, treatments using these nebulizers are time-consuming and inefficient, resulting in the waste of active ingredient.

Advances in technology have led to novel nebulizers that reduce waste and improve delivery efficiency. An enhanced delivery design, Pari LC Star® (Pari, Germany), increases aerosol output by directing auxiliary air and causing more generated aerosol to be swept out of the nebulizer for inhalation [50]. Breath-actuated nebulizers, such as AeroEclipse® (Trudell Medical International, ON, Canada) and Halolite (Medic-Aid Limited, Bognor Regis, UK) have recently been developed. The AeroEclipse controls an actuator piston to produce aerosol in inspiration and at rest position in patients' expiration [50]. The Halolite monitors a patient's breathing pattern in the first three breaths and then targets the aerosol delivery into the first 50% of each inhalation. This ensures the aerosols are delivered to the patient during inspiration only, thereby eliminating drug loss during expiration [51]. A number of metered-dose liquid inhalers, including AERx® (Aradigm, CA, USA), AeroDose® (Aerogen, CA, USA) and Respimat® (Boehringer Ingelheim, Ingelheim, Germany), have been developed to produce fine aerosols in the respirable range by forcing the drug solution through an array of nozzles, with 30–75% of the emitted dose being deposited in the lungs [52,53].

Pulmonary vaccination studies have been performed by nebulization of live, attenuated pathogens, such as tularemia [37,54], measles [40], BCG [55,56] and rubella [32]. Nebulization delivers vaccine aerosols to the lower respiratory tract. However, there is a

potency-loss problem. It was reported that complex molecules were frequently degraded by the shear force of jet nebulization [57]. The stability of the measles vaccine was determined during nebulization via the Classical Mexican Device. There was a 71% loss of vaccine potency after the nebulizer was run continuously for 20 min. The loss in viral potency was in the order of one third when the nebulizer was run in cycles of 30 s on, 10 s off [41]. Immunity may be elicited even when the number of viable pathogens in the lungs is low. However, the reproducibility issue of vaccine dose in the vaccine mass campaign could not be neglected.

Pressurized metered-dose inhalers

pMDIs represent most pharmaceutical aerosol products. They are comprised of drug formulations filled or packed under pressure along with the energy source, a liquefied propellant, in a canister equipped with a valve, to meter accurate and precise doses, and actuator. A predetermined volume of nonaqueous liquid is discharged per actuation to offer the precise dose delivery on demand. pMDIs deliver only a small fraction of the drug dose to the lungs (10–20% of emitted dose). There is a cold propellant effect owing to the evaporation of propellant when the aerosols impact on the back of the throat, which can be ameliorated by the use of a spacer. Poor hand–mouth coordination is another obstacle in the optimal use of a pMDI. Recently, breath-actuated pMDIs have been developed to eliminate coordination difficulties by firing in response to the patient's inspiratory flow. The Autohaler® (3M Pharmaceuticals, MN, USA), increased lung deposition from 7.2% with a conventional MDI to 20.8% of the dose using the breath-activated pMDI [58]. Recently, Accentia Biopharmaceuticals (FL, USA) launched a new breath-activated, dose-counting inhaler (MD Turbo™) [201]. This device helps to coordinate the press-and-breathe action needed for proper use of an inhaler, apart from counting the remaining doses in the inhaler.

Few vaccines have been delivered as propellant-driven metered dose aerosols. The hydrophobic propellant is not a friendly environment for most vaccine strains or aqueous soluble antigen proteins. Usually, surfactants or cosolvents may be needed for pMDI vaccine formulation. Brown and colleagues delivered *Streptococcus suis* bacteria into the respiratory tract of swine in the presence of surfactants using liquefied dimethylether as a propellant [59]. Approximately 6–12% of bacteria were delivered to the deep lungs. After aerosolization, only 17–38% of cell-wall proteins were associated with the bacteria and 30–50% of antigenicity in the respirable bacteria was retained after actuation. This report demonstrated that small particle aerosols of the bacteria vaccine from pMDI can be generated but with significant loss of antigenicity [59].

Dry-powder inhalers

The development of DPIs was driven largely by the Montreal protocol to eliminate chlorofluorocarbons from traditional pMDIs. There is a wide range of DPI devices on the market, from single-dose devices (Aerolizer® [Novartis, Basel, Switzerland] and Handihaler® [Boehringer Ingelheim, Ingelheim, Germany]) to multiunit dose devices provided in a blister pack (Diskhaler® and Diskus® [GlaxoSmithKine, Greenford, UK]) or reservoir-type system (Turbuhaler® [AstraZeneca, London, UK]) [60]. Generally, inert carriers are needed for dispersion of small particles of the active ingredients; lactose is commonly used. Aerosols are created by directing the air through loose powders. The lung deposition varies from 12–40% of the emitted dose among different DPIs [60]. Insufficient deaggregation of the active ingredient from coarse carrier particles contributes to the low active ingredient deposition. Active DPIs are being investigated to reduce patients' inspiratory effort to disperse the fine particles. Aspirair™ (Vectura, Wiltshire, UK) is triggered by a patient's inhalation. This inhaler generates an aerosol plume significantly slower than most currently available inhalers. Therefore, the use of Aspirair reduces the amount of drug that is unintentionally deposited in the mouth and throat, and subsequently swallowed, rather than reaching the lungs [61]. Spiros® (Dura Pharmaceuticals, CA, USA) uses a battery-driven propeller to aid the dispersion of powders. The Inhance™ Pulmonary Delivery System (Nektar, CA, USA) uses compressed air to aerosolize the powder and then converts it into a standing cloud in a holding chamber. This makes the generation of aerosol independent of patients' inspiratory effort.

In addition to the general advantages of dry-powder vaccination, a unique feature is that the alveolar APCs (especially macrophages and/or DCs) are phagocytic and respond to small-size particulates by eliciting cell-mediated and humoral immunity. These particulates could be whole vaccine strains, subunit proteins or DNAs formulated in particulates.

Dry-powder aerosol vaccination has been in use to immunize humans and animals. In the early 1960s, Russian investigators used dry-powder vaccines of attenuated bacterial strains to immunize experimental animals against plague, tularemia, brucellosis and anthrax [62]. Large particle aerosols of a live, temperature-sensitive recombinant influenza virus were generated by a spinning-top aerosol generator to immunize the mice [63]. These dry-powder particles of influenza virus provided 89% survival after challenge.

Dry-powder measles formulation was suggested for delivery by Spiros inhalers [64]. They are durable, handheld and could be either single or multidose inhalers. The delivery efficacy of Spiros technology was demonstrated by pulmonary imaging with radiolabelled albuterol sulfate. Scintigraphy results showed uniform deposition of radiolabelled drug throughout the tracheobronchial region and significant and uniform deposition in the alveolar region [65]. The studies performed with nebulized measles vaccine provide evidence that the aerosol vaccine to the lungs need not be greater than the currently accepted 50% tissue culture infective dose ($TCID_{50}$). The estimated measles vaccine concentrations could vary from 3 to 40% in lactose, depending on $TCID_{50}$ from different manufacturers. This blending is in the good range of mixture with inert lactose for dispersion [64].

Components of pulmonary vaccine formulations

Formulation is an important factor that can affect the stability of subunit antigens or live, attenuated/inactivated vaccine strains. The formulations also dictate the aerosol delivery device for vaccine administration. Different delivery devices need different formulation strategies to meet the criteria to generate the respirable aerosols.

Adjuvants

Adjuvants help to elicit early, high and long-lasting immune responses with limited quantities of antigen. They are the focus of vaccine research as purified, synthetic subunits and DNA vaccines are frequently poor immunogens and require adjuvants to evoke immune responses [66,67]. With the use of adjuvants, an immune response can be selectively modulated to major histocompatibility complex (MHC) class I or II and Th1 or Th2 type [68], which is very important for protection against diseases caused by intracellular viruses, parasites and bacteria.

There are several mechanisms of action for adjuvants:

- Depot generation (aluminum compounds, immunostimulant complexes [ISCOM], emulsions, and oil adjuvants [Freund's complete/incomplete adjuvant]);
- Immunomodulation (modification of cytokine networks, includes lipopolysaccharide [LPS], monophosphoryl lipid A [MPL], lipopeptides, CpG motif, muramyl dipeptide [MDP], and cholera toxoid [CT]);
- Delivery vehicles (liposomes and biodegradable polymer microspheres) for antigen in targeting to APCs.

The adjuvants of the former two mechanisms used in research reports of respiratory vaccines are listed in TABLE 1. The delivery vehicle function of adjuvants will be discussed in detail in the Particulate systems section. The only approved adjuvant for humans is potassium aluminum sulfate (alum). Aluminum hydroxide (alhydrogel) and aluminum phosphate (adju-phos) are also used in research and preclinical trials. Aluminum compounds have a good safety record in vaccination history but are usually only able to elicit a strong humoral, but poor Th1, response. MF59 and MPL have also been used in human trials [69–71] but there are no experimental reports in pulmonary vaccine delivery yet. Cholera toxin and *Escherichia coli* heat-labile enterotoxin (LT) are very potent mucosal adjuvants, which are frequently used in intranasal vaccination. The B subunit of these toxins binds to gangliosides on the cell surface, leading to internalization, and the A subunit is responsible for the activation of adenyl cyclase, leading to elevated cAMP levels [72]. However, after intranasal administration of antigen in mice, both antigen and toxin were found in the olfactory nerve and olfactory bulb for an extended period; this antigen accumulation did not occur in the absence of toxin. In addition, the intranasal toxin induced an inflammatory response in the olfactory sites that led to meningitis in the mice. Thus, there is concern regarding neurotoxic effects of intranasal administration of vaccines containing an enterotoxin adjuvant [72]. This might also explain the occurrence of Bell's Palsy after intranasal delivery of Nasalflu™ (inactivated virosomal-subunit influenza vaccine, Berna Biotech, Switzerland) in Switzerland [73]. This factor might preclude the clinical use of cholera toxin as an adjuvant [74]. While some other mutated toxins, especially those based on the *E. coli* LT, such as LTK63, have been reported to be safe [75]. Although not many adjuvants have been used in pulmonary vaccine delivery yet, some other mucosal immunization adjuvants may be tried for aerosol vaccination.

Table 1. Potential adjuvants for use in pulmonary vaccine delivery.

Vaccine	Adjuvant	Adminstration route	Species studied	Ref.
Ricin toxoid	Aluminum hydroxide	it.	Rats	[91]
Staphylococcal enterotoxin B	Aluminum hydroxide	in.	Mice	[94]
rPA	Aluminum hydroxide, CpG motif	in.	Mice	[129]
Respiratory syncytial virus envelope antigen	ISCOM	in.	Mice	[130]
HIV-Tat protein	MALP-2	in.	Mice	[131]
rPA	MPL	in.	Rabbits	[132]
Phosphate transport protein-1	Cholera toxin	in.	Mice	[133]
rPA	Cholera toxin	in.	Mice	[134]
Respiratory syncytial virus F protein	Cholera holotoxin-CT-E29H	in.	Mice	[77]
Influenza A H3N2 virus	*E. coli* heat-labile enterotoxin LT(R192G)	in.	Mice	[135]
Respiratory syncytial virus F antigen	Escheriagen	in.	Mice	[78]

Escheriagen: *Escherichia coli* heat-labile toxin; in.: Intranasal; ISCOM: Immunostimulant complexes; it.: Intratracheal; MALP-2: Macrophage-activating lipopeptide-2; MPL: Monophosphoryl lipid A; rPA: *Bacillus anthracis* protective antigen.

The adjuvant dose and toxicity to the respiratory tract need to be clarified to prevent unexpected inflammation by overdosing.

In adjuvant selection, attention should be given to the animal model used. Different animal species can respond differently to the same adjuvants [76]. Biological differences between animal models and humans may lead to the failure of promising formulations to show adjuvanticity in clinical trials.

Stabilizers in liquid-based formulation

Liquid formulations for nebulizers and pMDIs include solutions, suspensions, liposomes and micro- or nanoparticle suspensions. The vaccine components could be live, attenuated or inactivated pathogen strains, subunit antigen proteins or DNAs. Owing to the shear force produced in aerosol generation, the large molecules and the whole virus or bacteria usually require a surfactant present for structure stability and antigenicity. The surfactants also help prevent agglomeration of micronized particles in suspension. BCG bacterial strains were stabilized in 0.01% of Tween 80® for nebulization [62]. Phosphatidylcholine, including lysophosphatidylcholine (LPC), egg phosphatidyl-choline (EPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphocholine (DSPC), tyloxapol [22], Tween 20®, caprylic/capric glyceride [77] and octaethylglycol [78] have been used in solution or microparticulate suspension formulations. Pluronic F127, a nonionic polyoxyethylene–polyoxypropylene copolymer, can stabilize proteins and be administered in a liquid form. It acts as a sustained-release gel depot at body temperature owing to its property as a reverse thermal gelatin [79]. This surfactant was also reported to enhance the immune response to tetanus toxoid [80]. However, some caution must be taken before using surfactants. The existence of some surfactants, such as DPPC, may reduce the number of alveolar macrophages, which engulf the particles, or the number of particles entering a macrophage [81].

In addition, nebulization frequently damages DNA structure. DNA aerosols with a polyethyleneimine (PEI)-based formulation have been shown to be stable during nebulization resulting in 100% transfection [82].

Particulate systems

Microparticulate systems

Microparticulate systems have been a common theme in the vaccine delivery field. They enhance the immune response greatly in comparison with soluble antigens. They have efficient adjuvant functions and stimulate the immune response to help Th1 induction [83]. Microparticulate systems include liposomes, lipid-based and biodegradable polymer-based microparticles. Some subunit antigens and DNA vaccines use viral vectors for pulmonary delivery.

Liposomal aerosols composed of phospholipids have some advantages, including sustained release, potential targeting functions and prevention of local irritation and reduced toxicity [84,85]. The most commonly used phospholipids are the phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylglycerols (PG), cationic lipids and cholesterol mixtures. Liposomal vaccines can be prepared for aerosols in liquid or dry-powder form [86]. Unexpected vaccine release occurs during nebulization and MDI actuation [87]. Dry-powder liposomes have been produced by lyophilization followed by milling [86,88] or by spray drying [89]. Dried liposomes overcome the storage instability often associated with aqueous systems. De Haan and colleagues demonstrated that, in the lungs, the target cells of liposomes are alveolar macrophages [90]. Pulmonary liposome delivery of vaccine to animals illustrated that they produced a higher titer of specific antibodies than alhydrogel vaccine and vaccine solution [91], and stronger protection of animals after challenge [92].

Virosomes, liposomes with the addition of viral membrane proteins, have been developed without requiring additional immunostimulators. Influenza virosomes are unilamellar liposomes mainly composed of PC, with influenza hemagglutinin intercalated into the membrane. The use of viral membrane proteins in the formation of virosomes offers the opportunity to exploit the targeting and fusogenic properties of the native viral membrane proteins, perhaps resulting in effective delivery of entrapped antigens into the cytosol for CTL induction [93]. Respiratory syncytial virus-F antigen immunization to mice by intranasal delivery of a virosome developed a mucosal IgA and a high-level serum IgG response. A balanced Th1/2 cytokine profile was observed. However, the same antigen with no virosome delivery system only had a Th2 response [78]. Vaccines in the meningitis outer-membrane proteosome also offered the targeting and adjuvant activities for staphylococcal enterotoxin B (SEB) toxoid [94,95].

Among other approaches, lipid-based microparticles have been examined as carriers for pulmonary vaccines delivery in order to overcome the instability associated with the use of liposomes (TABLE 2). Usually, solvent evaporation or spray-drying methods are used for the manufacture of lipid microparticles. The encapsulation efficiency and stability are much improved compared with liquid liposomes. Lipid microparticles with biocompatible lipids, such as DPPC and DSPC, were poorly phagocytosed [81]. Surface modification of lipid microparticles can greatly improve phagocytosis by APCs. IgG encapsulated in the spray-dried lipid particles with influenza virus provided receptor-mediated targeting to APCs. These delivery systems were internalized in a Fc receptor-dependent manner by phagocytic APCs that were then able to efficiently present a dominant, MHC class II-restricted epitope to specific T cells [96]. Lipid microparticle–cationic stearylamine complexes can generate stronger binding with the mucosa owing to ionic interactions with the negatively charged sialic groups of mucus, therefore generating a stronger mucosal response (IgA) [97]. The addition of surfactant Tyloxapol (a biocompatible detergent approved for pulmonary use in humans) into DPPC microparticles, can abolish the negative interference of the lipid matrix with the virus hydrophobic envelope and allow more effective presentation of epitopes to specific T cells [22].

Table 2. The aerosolized vaccines delivered via microparticulate systems.

Vaccine	Delivery system	Route of delivery	Species	Ref.
Ricin toxoid	Liposome	it. instillation	Rats	[91]
Ricin toxoid and ricin A chain	Liposome	it. instillation	Rats	[92]
Respiratory syncytial virus envelope antigen	Liposome	in.	Mice	[130]
Measles	Liposome	in.	Mice	[136]
Influenza subunit anigen	Liposome	in.	Mice	[137]
Respiratory syncytial virus F antigen	Influenza virosome	in.	Mice	[78]
SEB toxoid vaccines	Proteosome	in., it.	Rabbits, monkeys	[95,138]
Influenza virus	Lipid particle	in., it.	Rats	[96]
HBsAg	Lipid microparticle	in.	Rats	[97]
Influenza virus	Lipid microparticle	it.	Rats	[22]
EAST-6	PLA microsphere	in.	Mice	[139]
Anthrax rPA	PLA microsphere	in.	Mice	[98]
Yersinia pestis subunit F and V	PLA microsphere	in., it.	Mice	[99,100]
VEE virus	PLGA microparticle	i.t.	Mice	[140]
Bordetella pertussis FHA	PLGA microparticle	in.	Mice	[141]
Respiratory syncytial virus DNA for F protein	Macroaggregated albumin particle	in.	Mice	[116]

FHA: Filamentous haemagglutinin; HBsAg: Hepatitis B surface antigen; in.: Intranasal; it.: Intratracheal; PLA: Polylactide; PLGA: poly(lactic-co-glycolic acid); rPA: *Bacillus anthracis* protective antigen; SEB: Staphylococcal enterotoxin B; VEE: Venezuelan equine encephalitis.

Biodegradable polymeric microspheres demonstrated good adjuvant activity. Antigen uptake by APCs was enhanced by the association of the antigen with polymeric microparticulates or by encapsulation of the antigen in the polymers. The biodegradable and biocompatible polyesters, polylactide-co-glycolides (PLGA) and polylactide (PLA) are the primary candidates for the development of microparticles since they have been used in humans for many years as suture material and as controlled-release delivery systems (TABLE 2). The microsphere particle sizes (1–10 µm) can be manipulated to target macrophages. Macrophages can present the antigens 100–1000-fold more efficiently to MHC class I and II pathways than soluble antigens alone when antigens are attached to small particles [83]. There is a greater possibility for the microparticulates residing in the lungs for an extended period of time to be taken up by APCs. These microparticles can protect live vaccine strains or subunit antigens against rapid degradation by extracellular enzymes. They may also offer controlled-release dissolution profiles for antigens, which allow the development of single-dose vaccines. In contrast to alum, PLGA microparticles have been shown to be effective for the induction of a CTL response [83]. *Yersinia pestis* subunit and anthrax *B. anthracis* protective antigen have been reported to be delivered intranasally or intratracheally in PLA formulations leading to an improvement in immune response with respect to other formulations [98–100].

Nanoparticulate system

Nanoparticle-delivery systems have been reported for vaccine delivery targeting the peripheral and lymph node DCs in subcutaneous and intradermal vaccination [101]. Macropinocytosis is used to internalize extracellular fluid and smaller solutes, such as macromolecules, and particularly small nanoparticles (<50 nm), whereas phagocytosis occurs when larger nanoparticles and microparticles (>500 nm) are taken up by DCs. DCs also use lectin-like surface receptors to endocytose ligands with a terminal sugar, such as mannose [102]. Copland and colleagues showed that mannosylated liposomes 260 nm in size were internalized through receptor-mediated endocytosis to a higher degree and presented antigens to T cells more efficiently than neutral liposomes or free antigens [103]. Thus, both the physicochemical and biochemical character of biomaterial vehicles can be adjusted to tailor DC uptake.

Few studies of pulmonary vaccines in nanoparticulate systems delivered to the lungs have been conducted. Bivas-Benita and colleagues have reported that pulmonary delivery of chitosan–DNA nanoparticles (375 ± 59 nm) enhanced the immunogenicity of a DNA vaccine encoding HLA-A*0201-restricted T-cell epitopes of *M. tuberculosis* [47]. Their DNA formulation was able to induce maturation of DCs ,while chitosan solution alone could not, indicating the DNA was released from the particles and able to stimulate DCs [47]. Their endotracheal delivery of 200-nm fluorescene nanoparticles had evidenced the nanoparticles deposited into the small airways [104].

Nanoparticle aggregates made of biodegradable materials have been approved as suitable for aerosol delivery in therapeutic drugs [105–107]. The nanoparticle-delivery systems are very promising vaccine delivery carriers for aerosol administration to target DCs.

Precoating the particulate surfaces with bovine γ-globulin, human fibronectin and gelatin can enhance APC uptake [108].

DNA vaccine-delivery vectors

Microparticulate delivery systems for DNA vaccines are categorized into viral and nonviral vectors. Viral DNA vectors mainly focus on adenoviruses and vaccinia viruses [15]. Intranasal immunization with adenoviral HIV antigens [109] and recombinant adenovirus-expressing Ag85A of *M. tuberculosis* provided potent protection [110]. However, the detection of infection in the CNS could limit intranasal delivery of adenoviral systems. Highly attenuated vaccinia viral vector (modified vaccinia Ankara [MVA]) presented no serious side effects in clinical trials [111]. Recently, Sindbis, a virus-based DNA vaccine expressing antigen 85B, induced similar protective immunity to BCG against *M. tuberculosis* after subcutaneous administration [112].

Nonviral vectors are biocompatible and less toxic delivery systems, although their efficiency is not as good as viral systems. Liposomal complexes (lipoplexes) and PLGA have reportedly been used for DNA delivery. Cationic PLGA microparticles bearing the cationic agent cetyltrimethylammonium bromide (CTAB) enhanced immune response to the HIV-1 Gag protein after intransal administration to mice [113]. Chitosan DNA plasmid encoding eight T-cell epitopes from *M. tuberculosis* encapsulated in nanoparticles were delivered intratracheally to the lungs of mice and induced the maturation of DCs, increased levels of IFN-γ secretion compared with DNA plasmid solution alone [47]. Nebulization of the PEI–DNA systems resulted in a high level of pulmonary transfection (10–100-fold greater than many cationic lipids) and DNAs were stable during nebulization [114]. Caution must be exercised in that PEI alone is able to activate the immune system and activate genes involved in cellular processes, such as cell-cycle regulation, oncogenesis and differentiation [115]. Consequently, there might be toxic effects when using PEI in formulations.

Macroaggregated albumin (MAA), commonly used to view pulmonary blood flow in humans, has been effective as a mucosal DNA vaccine delivery agent. After pulmonary delivery, MAA accumulates in the alveolar interstitium without inducing inflammation and targets pulmonary interstitium macrophages and DCs. MAA–PEI–DNA of respiratory syncytial virus F protein induced substantially improved anti-F antibody response and balanced Th1 and Th2 intracellular cytokine responses [116].

Dispersion of vaccines & microparticulates

Dry-powder aerosol delivery is an attractive delivery method of pulmonary immunization since it has many advantages over nebulizer and MDI delivery of aqueous and nonaqueous droplets. However, dry-powder vaccines are not currently commercially available. LiCals and colleagues tested a live, attenuated EZ measles vaccine and observed that it maintained significant viral potency (31–89%) after being milled into respirable particles and dispersed from a lactose mixture [43]. Whether these particles can be dispersed is an important issue in proving the concept of the dry-powder vaccine delivery. The interaction energy between micron-sized particles needs to be overcome to efficiently generate an aerosol. Sugars are frequently added as carriers for active ingredient dispersion. Lactose is the only excipient approved for this use in the USA. Other carriers, such as mannitol, trehalose, glucose, sorbitol, malitol and xylitol, also have the potential to be used as carriers in DPIs [117]. However, most of these carriers are hygroscopic in nature and agglomeration may occur, leading to a change in particle size distribution [118].

In peptide and protein delivery, sodium chloride (NaCl) has been used to improve dispersion. Recombinant human DNase powder was spray-dried with NaCl. A monolayer-like adhesion of the fine drug particles to NaCl at a drug content of 50% was responsible for the improved aerosol properties of the drug powder [119].

Redistribution of protein particles from coarse particles to the fine particle component can improve protein aerosol dispersion [120]. It has been reported that addition of fine particles addition to the carrier can improve the dispersion of the active pharmaceutical ingredient [121].

The spray freeze-drying technique can provide light and porous particles that have superior dispersion performance [122]. Some hygroscopic growth inhibitors, such as the polymer maltodextrin, and hydroethyl starch can help reduce the aggregation problem of the fine particle of drugs [123].

Self-dispersion is another approach for aerosol particles. Large porous particles have a mean geometric diameter of up to 30 µm, with good flow and dispersion properties [124]; however, they evade phagocytosis by macrophages. Recently, van der Walle and colleagues reported that PLGA microspheres could be manufactured with novel dimpled surfaces for the pulmonary delivery of DNA [125]. The hollow microspheres, with a low density (0.24 g/cm^3) and dimples, were produced by the addition of high molecular weight hydrophobic blocks of pluronic (FIGURE 1). The calculated aerodynamic diameter of microspheres was 3.8 µm and the dimpled microspheres showed good aerosol property. Porous particles larger than 10 µm are not suitable formulations for vaccine delivery when the targeting APC is alveolar macrophages. However, there is an exception to this observation: a particle aggregate that, upon deposition, disassembles into its smaller component particles.

There is a dearth of research into dispersion of vaccines in dry-powder states. The methods reviewed here provide some dispersion choices. Consideration of excipient compatibility with the vaccines and their combined safety with response to pulmonary delivery is required.

Conclusions

Besides small-scale human vaccination trials in the Soviet Union, the measles vaccine is the only successful use of pulmonary immunization on a large scale. From the immunological,

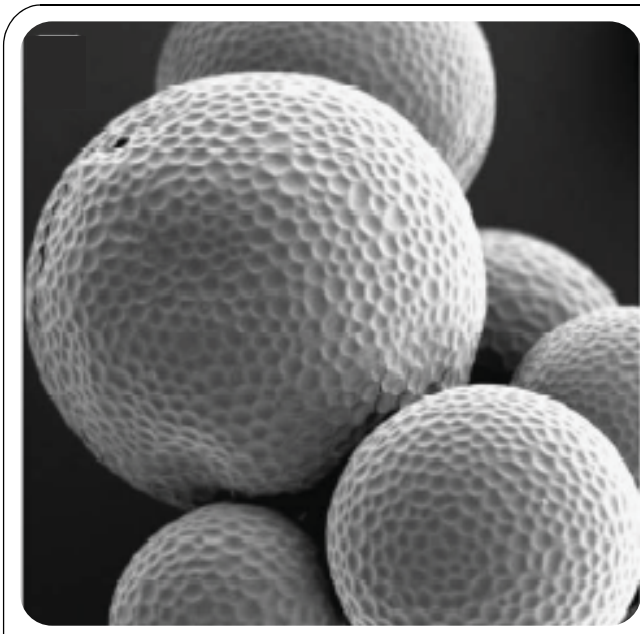

Figure 1. Surface morphology of microspheres fabricated using polyvinyl alcohol in secondary emulsion and Pluronic L92 (magnification 3500×). Reprinted from [125] with permission from Elsevier.

pharmaceutical and technological point of view, the proof of concept for pulmonary immunization has been demonstrated to protect against infectious diseases and bioterrorism attack, especially for airborne pathogens, such as measles, tuberculosis, influenza, rubella, respiratory syncytial virus, avian influenza and anthrax. Dry-powder vaccination has the potential for a successful mass vaccination campaign. Appropriate selection of a delivery device and vaccine formulation for delivery via the pulmonary route would have broad benefits for the welfare of mankind.

Expert commentary & five-year view

Pulmonary delivery of aerosols is a promising and potentially effective means of mucosal vaccination to prevent infectious diseases caused by airborne pathogens. There are good immunological, aerosol technological and pharmaceutical possibilities for pulmonary vaccine delivery (stated in the *Pulmonary vaccination belongs to mucosal immunization*, *Aerosol technology available for pulmonary delivery of vaccine* and *Components of pulmonary vaccine formulations sections*). However, some concerns and limitations related to formulation, adjuvants and delivery device approaches need to be clarified.

Safety

One of the main concerns regarding pulmonary immunization is the potential to exacerbate respiratory diseases, such as bronchitis, pneumonia and allergic asthma. The excipients in aerosol formulations may be allergenic and irritating, inducing unanticipated and undesirable inflammation. The vaccines containing egg protein may induce allergy, especially in infants [126]. Inflammation would induce swelling of the lining of the airways that leads to narrowing and obstruction of the airways. The inflammation also stimulates mucus production, which may further cause obstruction of the airways. The virion strains in aerosol may exacerbate asthma by infection to the airway tract.

Attention also needs to be given to immunization of high-risk populations with pulmonary disease, such as asthma, chronic obstructive pulmonary disease and emphysema. Owing to the restriction of airways in asthma, bronchitis or alveolar dysfunction in emphysema, aerosol deposition in the lungs will be much less predictable than in the healthy population. Vaccine dosing adjustment may be required to achieve effective immunization for high-risk patients. Indeed, some subjects may be considered ineligible for aerosol vaccination.

Delivery device selection may change the deposition pattern and reduce the possibility of inducing undesirable side effects. CNS side effects from olfactory uptake are significant concerns in intranasal delivery. Aerosol delivery to the nasal cavity was quantified in adults who inhaled aerosols administered via a nasal spray pump and a nasal nebulizer. The vault of the nasal cavity (where the olfactory region is located) has been shown to be inaccessible by spray pump delivery but accessible by nasal nebulizer aerosolization [127]. Although there is no vaccine deposition data to support the absence of CNS absorption, this may be an approach to reduce side effects.

Pulmonary vaccination in HIV patients may elicit a range of undesirable effects depending on the nature of the antigen (whole organism vs protein or DNA vaccines), which is a common issue for all routes of immunization.

Precise dosing

There are some difficulties in the ability to deliver precise quantities of live pathogen strains, protein subunit antigens or DNA vaccines to the respiratory tract of the lungs. If conventional nebulizers and MDIs are used for vaccine delivery, the operating conditions and aerosol output must be characterized and standardized. With the emergence of the metered-dose liquid inhalers (AERx, AeroDose and Respimat), the reproducibility of precise dosing and regional deposition in the lungs could be improved. Precise dosing can help the selection of vaccine dose needed for effective immunization and reduce the possible side effects and potential immunological tolerance.

Immune responses

Different animal models may provide different immune responses to the same aerosol formulations from the same device owing to the physiological, spatial and, possibly, functional differences of immune inductive and effector sites of the respiratory tract. For human clinical trials, environmental factors and cross-immunization need to be considered. The observed immune responses are subject to the influence of genetic polymorphisms, MHC-related variations in immune responses to antigen and potential variations in the effect of adjuvants between species and within diverse populations [128].

Stability & storage

Although dry-powder vaccine products are not yet available, this approach to immunization is very promising. Advanced DPI technology, particularly active aerosol dispersion devices and systems containing novel formulation techniques, make dry-powder vaccination a viable proposition and an exciting prospect on the prophylactic horizon. In addition, solid-state vaccines may be more stable in storage and potentially avoid expensive and inconvenient cold-chain storage requirements.

Acknowledgement

The authors greatly appreciate Pavan Muttil's insights into the subject and editorial suggestions.

Key issues

- Pulmonary aerosol vaccination may be employed for mucosal immunization. Lymphatic systems are present in the lower respiratory tract. Pulmonary immunization has been shown to be effective in animal studies and clinical trials.
- Technologies and their current status for aerosol vaccine delivery: nebulizer, pressurized metered-dose inhalers and dry-powder inhaler.
- Aerosol formulations are important: adjuvants, stabilizers and particulate systems for aerosol vaccine delivery may be used to improve vaccine stability and immunogenicity.
- Dry-powder delivery has exciting potential for pulmonary vaccination. Dispersion is important for the efficient delivery of aerosols to the lower respiratory tract.
- Safety issues, precise dosing and differences in immune responses seen in animal models need to be considered in pulmonary aerosol vaccine development.

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