Acta Pharmaceutica Sinica B 2024;14(12):5132–5160

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com

REVIEW

Respiratory delivered vaccines: Current status and perspectives in rational formulation design

Lan Wu[a,b,†], Wenwen Xu[a,b,†], Huiyang Jiang[a,b], Mingshi Yang[a,b,c,*], Dongmei Cun[a,b,d,*]

[a]*Wuya College of Innovation, Shenyang Pharmaceutical University, Shenyang 110016, China*
[b]*Joint International Research Laboratory of Intelligent Drug Delivery Systems, Ministry of Education, Shenyang 110016, China*
[c]*Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen DK-2100, Denmark*
[d]*School of Food and Drug, Shenzhen Polytechnic University, China, Shenzhen 518055, China*

Received 25 May 2024; received in revised form 20 July 2024; accepted 18 August 2024

KEY WORDS

Respiratory vaccines;
Respiratory drug delivery strategies;
Mucosal immunity;
Biomacromolecule delivery systems;
Inhaled formulation;
Particle engineering;
Nucleic acid;
Lung deposition

Abstract The respiratory tract is susceptible to various infections and can be affected by many serious diseases. Vaccination is one of the most promising ways that prevent infectious diseases and treatment of some diseases such as malignancy. Direct delivery of vaccines to the respiratory tract could mimic the natural process of infection and shorten the delivery path, therefore unique mucosal immunity at the first line might be induced and the efficiency of delivery can be high. Despite considerable attempts at the development of respiratory vaccines, the rational formulation design still warrants attention, *i.e.*, how the formulation composition, particle properties, formulation type (liquid or solid), and devices would influence the immune outcome. This article reviews the recent advances in the formulation design and development of respiratory vaccines. The focus is on the state of the art of delivering antigenic compounds through the respiratory tract, overcoming the pulmonary bio-barriers, enhancing delivery efficiencies of respiratory vaccines as well as maintaining the stability of vaccines during storage and use. The choice of devices and the influence of deposition sites on vaccine efficiencies were also reviewed.

*Corresponding authors.
E-mail addresses: mingshi.yang@sund.ku.dk (Mingshi Yang), cundongmei@163.com (Dongmei Cun).
[†]These authors made equal contributions to this work.

Peer review under the responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2024.08.026

1. Introduction

Vaccines are biological substances that can stimulate the body's immune responses against specific or closely related diseases. Most vaccines can be categorized into two broad groups, namely prophylactic vaccines and therapeutic vaccines. Prophylactic vaccine elicits active acquired immunity in healthy individuals, one of the most effective strategies for preventing infectious diseases caused by bacteria or viruses. The wide applications of prophylactic vaccines play an important role in saving lives and lowering the cost of treatments. According to the World Health Organization (WHO), vaccines might prevent 3.5−5 million deaths annually worldwide[1]. With the outburst of COVID-19, the vaccination has exhibited dramatic preventative effects. A recent mathematical analysis claimed that around 14.4 million deaths from COVID-19 infection were prevented in 185 countries and territories in the first year of COVID-19 vaccination[2]. Tuberculosis (TB) is another leading lethal infectious disease. According to WHO, the new TB vaccine will be able to prevent 8.5 to 12.3 million deaths over 25 years[3]. In addition to saving lives, vaccination has reduced $586 billion in treatment costs for 94 low- and middle-income countries[4]. The vaccination could also reduce the infectious disease transmission rate by providing community protection.

On the other hand, with the development of immunotherapy, therapeutic vaccines could also provide novel curing strategies against serious diseases[5]. Unlike traditional therapeutics, vaccines are designed to awaken the patient's immune system, which subsequently initiates and promotes attacks on the malignant cells or molecules. Therefore, therapeutic vaccines could induce a dynamic immune response, which is synchronous with the progress of the disease. In addition, the therapeutic vaccines might induce persistent therapeutic effects by building immunological memory response. As a result, therapeutic vaccines are milestone innovations for some intractable diseases, such as cancer[6], Alzheimer's disease[7], and infection[8].

The human respiratory tract is directly exposed to pathogens and external environmental stresses (*viz.,* pollution, smoking, etc.) and therefore, it is prone to various serious diseases such as lung cancer, COPD, and lethal infectious respiratory diseases. Recently, the respiratory tract has been widely exploited as a promising route for vaccination. As compared to the traditional vaccines administered intramuscularly or subcutaneously, the delivery of vaccines to the respiratory tract would best mimic the natural infection process, which leads to antigen-specific immunity at the mucosal surfaces and directly blocks the invading pathogens and prevents infections at the first-line[9,10]. The inhalable prophylactic cancer vaccine is also capable of avoiding lung cancer metastasis by generating robust mucosal anti-tumor immunity in the lungs[11]. In addition, the route of respiratory administration could ensure the direct deposition of vaccine onto the lung tumors, which would activate the local tissue-resident lymphocytes, facilitate the infiltration of immune cells, produce anti-tumor cytokines, and alleviate the tumor microenvironment (TME) more effectively compared with traditional injected vaccines[12]. Furthermore, the promoted immune cells can reach other mucosal sites through the mucosa-associated lymphoid tissues (MALT) and provide a similar immune response at all the interconnected distal mucosal sites[13,14] such as the female genital tract[15]. The inhalable vaccines are highly effective, needle-free, and amenable to self-administration, which makes them an ideal candidate for fighting against various diseases[16].

Despite the tremendous advantages that respiratory vaccine holds, it is extremely challenging to formulate the vaccine into inhalable dosages. Usually, the high molecular weight and complicated structure of antigens and antigen-encoding nucleic acids make them very fragile and susceptible to the complex environment of the respiratory tract, such as mucosal and macrophage clearance as well as enzyme degradation. Therefore, some particulate engineering technologies need to be developed to maintain the integrity and improve the delivery efficiency of vaccines through the respiratory tract. In addition, from the delivery point of view, the antigen must be taken up by APCs to successfully induce the subsequent humoral or cellular immunity[17]. However, the delivery efficiency of antigen and antigen-encoding nucleic acids to APC is readily compromised by the branched structure of the lung, the presence of mucus or surfactant on the top of epithelia, and the preferential uptake by other non-specific cells[18,19]. The respiratory vaccine particles should be modified with APC targeting motifs or formulated with mucus-penetrating or adhesive excipients to overcome these challenges and enhance their vaccination efficiencies. Besides, environmental stresses during the manufacturing, storage, and aerosolization process pose an extra threat to the vaccine formulation[20]. The lung deposition site and the choice of administration devices for the respiratory tract are other important factors influencing the vaccination effects.

To address the abovementioned formulation issues, it is essential to have an overview of the state of the art of respiratory vaccines and learn from previous research. However, few articles have discussed the research progress in respiratory vaccines or the formulation technologies used for the development of respiratory-delivered vaccines. Therefore, we outlined the current research status of different types of respiratory vaccines, the formulation design strategies facilitating vaccine delivery to the respiratory tract and APC as well as the interactions between respiratory vaccines and mucosal immune systems. The critical factors that influence the stability, deposition, and safety of vaccine formulations are also discussed to provide a reference for the formulation design of promising respiratory vaccines in the future.

2. Respiratory mucosal immune systems

The human respiratory mucosal immune system mainly consists of the nasal cavity, the Waldeyer's ring, and regional lymph nodes[21,22]. The Waldeyer's ring comprises two palatine tonsils, two tubal tonsils, an adenoid, and a lingual tonsil (Fig. 1)[23,24]. These tissues are covered with epithelium layer and mucosa, which serve as the major site for antigen processing. The crypts of the tonsil surface are the main location of respiratory antigen-delivering microfold cells (*i.e.,* respiratory M cells)[25]. These M cells with pocket-like structures harbor the APCs from the lymph nodes. In the human body, the Waldeyer's ring is also regarded as nasal-associated lymphoid tissue (NALT), which is an important part of MALT in the upper airway[23]. In some disease conditions such as lung infections and lung cancer, the patients would develop inducible bronchus-associated lymphoid tissues (iBALT) in their lower respiratory tract, which form a reticular network of lymphocytes and stromal cells beneath the epithelium that lacks cilia. Interestingly, iBALT would not be present in healthy adults.

The mucosal immunization begins with the capture and transport of antigens and vaccine particles by M cells to APCs. The APCs then present the antigen to naïve T cells and activate

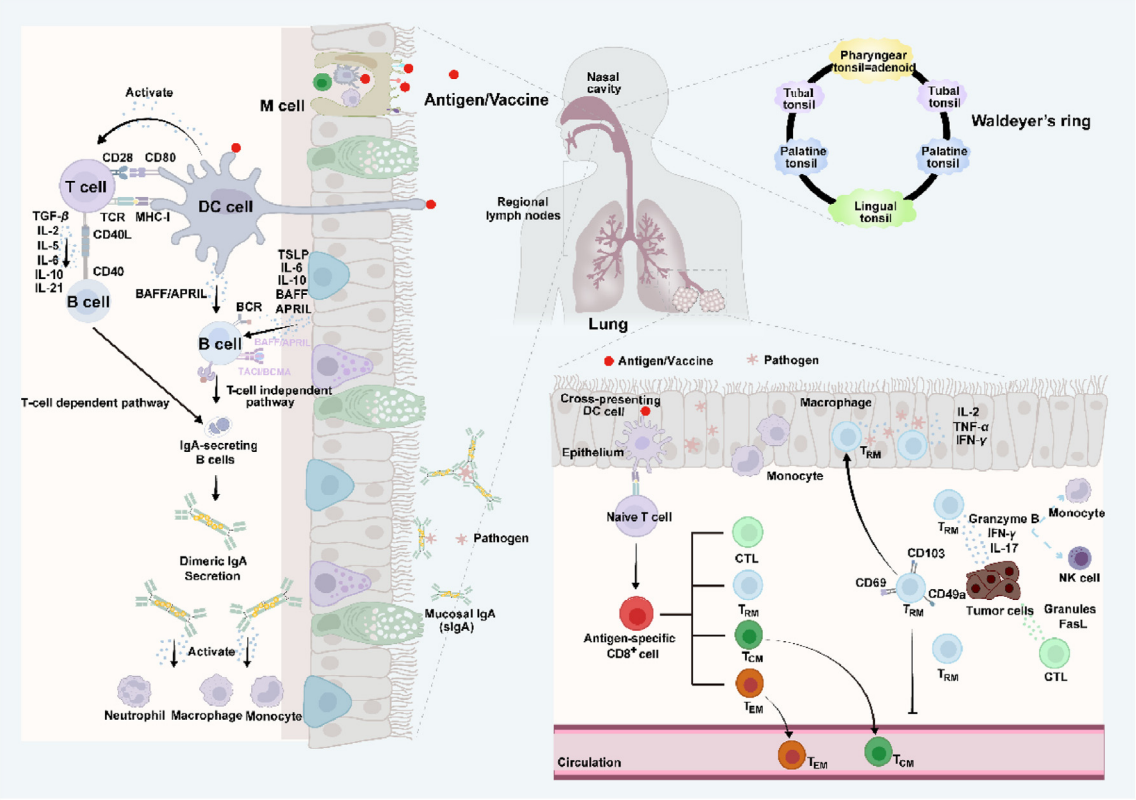

Figure 1 Respiratory immune systems and process of vaccine immunization in respiratory tract. Reprinted with the permission from Refs. 34, 58 and 59. Copyright © 2022 Sánchez Montalvo, Gohy, Rombaux, Pilette and Hox. (Ref. 34); Copyright © 2022 by the author, Licensee MDPI, Basel, Switzerland. (Ref. 58); Copyright © 2021 John Wiley & Sons, Inc or related companies. (Ref. 59).

antigen-specific T cells. The activated T cells would subsequently move to the B cells and convert the IgM-secreting B cells to IgA-secreting B cells with the help of epithelial tissue-derived cytokines including TGF-β, IL-2, IL-5, IL-6, IL-10 and IL-21 in the disease states[26–28], which lead to the production of IgA. Fig. 1 schematically illustrates the adaptive immune response in the mucosal immune system. In addition to the T cell-dependent pathway, the B cells can also be activated and secret IgA by other molecules directly, such as thymic stromal lymphopoietin (TSLP), IL-6, IL-10, B-cell activating factor (BAFF) as well as a proliferation-inducing ligand (APRIL) expressed by epithelial cells and local stromal cells[29–31]. However, different from the T cell-dependent pathway, the IgA produced by the T cell-independent pathway is unmutated with low affinity, which would provide limited protection against the pathogens[32–34]. Notably, the IgA is the major immunoglobulin isotype in most mucosal secretions. It is also the most abundant immunoglobulin in human serum. The IgA in serum is typically present as a monomer, while it exists as a dimer in the mucosal secretions[24]. The mucosal IgA is also known as secretory IgA (sIgA)[35] and it can nebulize the pathogens or toxins at the very front line. In case of pathogen invasion, the sIgA either covers the pathogen in the lumen, preventing its interactions with respiratory epithelial cells or forms a barrier to inhibit the cellular uptake of the pathogens[36]. sIgA can also directly inhibit microbial invasion by neutralizing them[37] or excrete toxins and viruses by intracellular cycling[38]. In addition, sIgA can induce innate immunity by activating monocytes, macrophages, and neutrophils through IgA receptor FcαR (CD89)[39]. As compared to the serum immunoglobulin IgG, the sIgA would not destroy the pathogens through lysis and complement activation, therefore avoiding the inflammation[40]. Thus, the induction of sIgA is very crucial for mucosal vaccination.

The successful induction of T cells and immunological memory is also critical for mucosal vaccination. The DC cells play a major role in activating T cells by presenting antigens to naïve T cells through recognition of antigen-specific major histocompatibility complex (MHC) class I molecules[41]. After recognition, antigen-specific $CD8^+$ T cells would proliferate and differentiate into several subtypes, namely $CD8^+$ cytotoxic T lymphocytes (CTLs), central memory T (T_{CM}) cells, effector memory T (T_{EM}) cells, and tissue-resident memory $CD8^+$ T (T_{RM}) cells (Fig. 1)[42]. The CTLs might kill malignant cells either by releasing granules or inducing FasL-mediated apoptosis[43], which is important for cancer immunotherapy. However, the short life span of CTLs limits their anticancer efficiency. On the other hand, the T_{CM} cells, T_{EM} cells, and T_{RM} cells are essential in building immunological memory. The T_{CM} cells and T_{EM} cells contribute to systemic immunity by being recirculated between blood and lymphoid organs but are of little use in mucosal immunity. The T_{RM} cells are the most abundant memory T cells in the respiratory tract[44,45] and play a significant role in the local defenses against infections and restrict tumor growth. The pulmonary T_{RM} cells are long-lived within the respiratory tract and would be activated rapidly and powerfully after antigen recognition in the lungs. The residence ability of T_{RM} cells is mainly related to the distinct markers on the surface, such as CD103, CD69, and CD49a. The CD103 would facilitate the interactions between T_{RM} cells and epithelial cells[44]. The CD69 might prevent the tissue invasion[45]. The CD49a is a

mucosal integrin and increases $CD8^+$ T cell infiltration[46]. In the case of respiratory infection, T_{RM} cells would promote the production of IL-2, IFN-γ, and TNF-α to induce immune defense[47,48]. At the same time, the T_{RM} cells would produce anti-inflammatory factors such as IL-10 to alleviate the tissue damage by inflammation[49]. T_{RM} cells could also mediate cross-protective immunity against antigenically unrelated pathogens and recruit T cells and B cells to the sites of infection[50,51]. Till now, multiple studies have demonstrated the protection effects of pulmonary T_{RM} cells against respiratory infectious diseases such as influenza[50,51], RSV[52], and SARS-CoV-2[53,54]. In addition to respiratory infections, the T_{RM} cells also play a crucial role in the adoptive immunotherapy against lung cancer. Previous studies showed that the T_{RM} cells in the tumor microenvironment (TME) would produce granzyme B, IFN-γ, and IL-17, which would restrict tumor cell growth and metastasis and enhance the recruitment of monocytes and NK cells[55]. Therefore, the prevalence of T_{RM} cells in the tumor tissues is positively correlated with the improved clinical outcomes of lung cancer[56]. Interestingly, recent studies exhibited that the intranasal route of immunization was much more efficient than that of the intramuscular or systemic routes in inducing T_{RM} cells, which constitutes another critical benefit associated with mucosal vaccination[57].

3. Respiratory vaccine entities

The current respiratory vaccines are promising in protecting against multiple respiratory diseases, such as influenza, tuberculosis (TB), respiratory syncytial virus (RSV), measles, and COVID-19. Therapeutic vaccines against lung cancer can also be administered *via* the respiratory route. Currently, there have been several respiratory vaccines approved for the market (Table 1). The entities of respiratory vaccines in clinical and preclinical stages including whole inactivated vaccines (WIVs), live attenuated vaccines (LAVs), recombinant viral vector vaccines, subunit vaccines, and nucleic acid-based vaccines were summarized in the following sections.

3.1. *Whole inactivated vaccines*

Whole inactivated vaccines (WIVs) are chemically or physically killed pathogens with reserved immunogenicity and lost replication capacity of the source pathogens. Formaldehyde and β-propiolactone, two licensed inactivating agents, are frequently used in the manufacturing of WIVs in industrial[60]. Formaldehyde could inter- and intra-molecularly crosslink the protein or genome of the pathogen by monohydroxymethylating amino acids in proteins and adenine residues in the genome to inactivate the pathogen[61], while β-propiolactone could inactivate pathogens by alkylating the guanine residues in genome[62]. These crosslinking agents will be removed *via* dialysis, thus only the inactivated genomes, polysaccharides, and proteins are retained in the final products. The process of inactivation might lead to a significant conformational change of the surface proteins, which will compromise the immunogenicity of WIV. Insufficient immunogenicity has limited the capacity of WIVs to induce full mucosal immune responses[63] and there are no respiratory-administered WIVs available in the clinic yet.

Mucosal adjuvants are essential for improving the immunogenicity of the respiratory WIVs. However, traditional mucosal adjuvants (cholera toxin and heat-labile enterotoxin) induce severe side effects in humans, such as nasal discharge[64] and facial nerve palsy[65]. Therefore, novel mucosal adjuvants with high efficiency and safety are crucial for the development of respiratory WIVs. Glycolipids were reported to activate natural killer T cells[66], which further increases the immune response. In a related study, α-galactosylceramide, a glycolipid presented by CD1d cell, was co-administered with influenza WIV intranasally to mice[67]. The vaccinated mice demonstrated antigen-specific mucosal IgA production in the nasal tract as well as the boosted population of natural killer T cells related to CXCL16/CXCR6 and IL-4 interactions. In another study, immunostimulant monophosphoryl lipid A (MPL), a glycolipid isolated from *Salmonella minnesota* R595, also mediated enhanced mucosal IgA level in mice after co-administration with influenza WIV intranasally[68]. In addition, the MPL-adjuvanted intranasal influenza WIV provided total protection against the lethal dose challenge of the influenza virus, while the mice who received the influenza WIV without MPL succumbed to the lethal challenge with 100% mortality.

Natural-sourced polymer particles are also potential mucosal adjuvants by simulating macrophages or DC cells and inducing mucosal immune responses. For example, chitin microparticles (CMPs) were co-administered with H1N1 influenza WIV through nasal vaccination to mice[69]. The CMP-aided immunization induced high IgA levels in nasal washes and IgG levels in serum as well as complete protection against the H1N1 influenza challenge in mice. In addition, poly (gamma-glutamic acid) nanoparticles (γ-PGA-NPs) co-administered with influenza WIV also exhibited enhanced IgA levels, and humoral and cellular immune responses since the γ-PGA-NPs activated NF-κB in DC cells. However, the abovementioned mucosal adjuvant candidates are all

Table 1 Respiratory delivered vaccines approved to market.

Vaccine name	Vaccine type	Administration route	Indication	Antigen
Fluenz Tetra	LAV	Intranasal	Influenza	A/H1N1 strain, A/H3N2 strain, two B strains (B/Washington/02/2019 and B/Phuket/3073/2013 lineages)
FluMist quadrivalent	LAV	Intranasal	Influenza	A/H1N1 strain, A/H3N2 strain, two B strains (B/Yamagata/16/88 and B/Victoria/2/87 lineages)
Pandemic influenza vaccine H5N1 by AstraZeneca	LAV	Intranasal	Influenza	A/H5N1 strain
Nasovac-S	LAV	Intranasal	Influenza	A/H1N1 strain, A/H3N2 strain, one B strain (B/56/Brisbane/60/08)
Convidecia air	RVV	Inhalation	SARS-CoV-2	Adenovirus type-5 vector-based vaccine (Ad5-nCoV) against COVID-19

LAV, live attenuated vaccine; RVV, recombinant viral vector vaccine.

well-studied parenteral adjuvants and the preclinical studies were all designed by simply administering them directly to the respiratory tract to examine their mucosal adjuvanticity. Although these studies demonstrated significant mucosal IgA responses in the presence of those adjuvants, their safety for respiratory administration and the mechanisms for mucosal adjuvanticity have not been explored yet. Till now, there is no mucosal adjuvant approved for clinical uses. Thus, the development of safe and effective WIVs warrants further improvement.

3.2. *Live attenuated vaccines*

Live attenuated vaccines (LAVs) are attenuated mutations of pathogens with restricted replication capacity in the human body as compared to wild-type pathogens[70]. Ideal LAVs can induce effective and long-lasting immunity comparable to the original pathogens but without causing infections. Traditionally, LAVs are often developed by culturing the pathogens under a non-physiological condition, such as culturing the pathogens using the technique of cold adaptation[71]. Nowadays, with the advancement in recombinant gene technology, the virulence and replication of pathogens can be edited to produce LAVs as needed. In addition, some naturally attenuated microorganisms can be used as LAVs directly, such as the Bacilli Calmette-Guérin (BCG) vaccine for TB and the cowpox vaccine for smallpox[72].

Unlike WIVs, the LAVs can induce profound immune response. Currently, there are already a few LAVs for respiratory delivery in the market or clinic trials. The most widely used commercial LAV for respiratory delivery is the intranasal seasonal influenza vaccine, *i.e.,* FluMist® or Fluenz™, which is recommended for annual influenza vaccination by the US Centers for Disease Control and Prevention[73]. FluMist® was initially approved as a trivalent vaccine and then was updated as quadrivalent in recent years. Despite its flu-like side effects in adults and children, the FluMist® was proven to be able to provide effective protection against different influenza viruses *via* the stimulation of IgA production, cell-mediated immune response, and systemic immune response[73–75].

BCG, the licensed TB LAV for subcutaneous injection showed significant immune efficacy in different animals when administered *via* respiratory route[76–79]. Inspirited by the positive results in animal models, three Phase I clinical trials have been conducted in British adults who have or do not have a history of BCG vaccination to determine the safety and tolerability of the BCG boost *via* pulmonary administration (clinicaltrials.gov identifier: NCT03912207, NCT02709278, NCT04777721). However, unfortunately, the results have not been published yet. In another phase I clinical trial, measles LAV in the form of inhalable dry powder displayed a strong serological response which was comparable to subcutaneous vaccination and no adverse events were reported. However, the mucosal immune responses and IgA level were not evaluated (NCT01557699; CTRI/2012/02/002447)[80]. Two LAVs *via* intranasal administration against COVID-19, namely COVI-VAC (NCT04619628) and MV-014-212 (NCT04798001), are also in the phase I trial currently. The COVI-VAC is produced by recording the spike protein[81], while the MV-014-212 is an attenuated RSV virus expressing COVID-19 spike protein[82]. However, no results have been reported yet.

Furthermore, some promising results were also reported from preclinical studies. SARS-CoV-2 LAV, attenuated by replacing the key structure of the spike proteins, could induce robust antibody responses and prevent weight loss and the occurrence of pneumonia after the SARS-CoV-2 virus challenge. It only induced negligible lung pathology in both hACE2 transgenic mice and Syrian hamsters due to 100- to 1000-fold lower titers than the original virus. In another study, a cold-adapted, spike protein motif-deleted SARS-CoV-2 LAV also could stimulate robust antibody responses, and no apparent body weight loss and histopathological lesions were detected with the challenge of wild-type SARS-CoV-2 virus[83].

Overall, the potential of respiratory delivery of various LAVs was proved in studies at different stages. Even though, the main concern about the safety of LAVs is still unresolved. The possibility of virulence recovery and inducing infections is low but exists, and some LAVs might cause transient immunosuppression, which is dangerous for vulnerable people. Therefore, more efforts are urgently needed to facilitate the clinic translation of LAVs for respiratory delivery.

3.3. *Recombinant viral vector vaccines*

Recombinant viral vector vaccines (RVVs) are produced by inserting the DNA encoding immunogenic fragments of the pathogen into viral vectors which are attenuated and unrelated to the pathogens of interest[84]. Because the components of recombinant viral vector vaccines are not directly from the pathogens, the risk of virus reversion can be avoided. However, the viral vector itself may potentially cause danger to the immuno-compromised individuals. In addition, the viral vector would also induce specific immune responses, and the antibodies against the vectors might neutralize the vaccines and weaken their effects. The choice of viral vectors is important for RVVs. The most widely used viral vectors are human adenoviruses (Ads) as their virulence is mild and cell tropism is broad. They could infect various dividing and nondividing cells without causing diseases[85]. In addition to Ads, other viruses such as recombinant influenza virus[86,87], vesicular stomatitis virus[88], vaccinia virus[89], and baculovirus[90] are also under exploitation.

Till now, the most rapidly progressed RVV for respiratory delivery is Convidecia Air, the aerosolized adenovirus type-5 vector-based vaccine (Ad5-nCoV) against COVID-19 by CanSino Biologics (CanSinoBIO). It was granted Emergency Use Authorization for boosting the immune response in Chinese adults who had previously received two doses of intramuscularly (i.m.) administered inactivated COVID-19 vaccines (Sinovac CoronaVac)[91]. The results of the phase III clinical trial (NCT05204589) exhibited that the aerosolized Ad5-nCoV was safe and highly immunogenic in boosting substantial serum antibody response against SARS-CoV-2. It could boost stronger neutralizing antibody responses than injected homologous inactivated vaccine (CoronaVac) and Ad5-nCoV[91]. More interestingly, the aerosolized Ad5-nCoV was proved to be able to induce airway mucosal IgA response and activate resident memory B and T cells in the respiratory mucosa, which could build an effective barrier against infections at virus invasion sites[92].

In addition to the commercialized Ad5-nCoV vaccines, currently, there are several respiratory-delivered RVVs at different stages of clinical trials. The Ad delivered SARS-CoV-2 S protein vaccine ChAdOx1 developed by Oxford and AstraZeneca, which has been approved into the market for injection, exhibited a reduced viral shedding as well as sufficient IgA and IgG production after intranasal administration in sentinel hamsters[93]. However, the use of the injected vaccine has been suspended due to the thromboembolism in vaccinees with an overall lethality of

2.5 in 1,000,000[94–96]. Regrettably, the mechanism of thromboembolism is still uncertain. As a result, the development of the intranasal formulation of SARS-CoV-2 S protein vaccine ChAdOx1 has also been pended simultaneously. Another Ad5 vectored vaccine encoding the receptor-binding domain (RBD) of the SARS-CoV-2 S protein (*viz.,* AdCOVID) displayed significant IgA, IgG, and T cell-based immunity in a mouse model after a single intranasal dose and went into phase I clinical trials (NCT04442230)[97]. Unfortunately, the company has discontinued further development of AdCOVID, and this candidate has been removed from clinical studies. The ad5-based vaccine has also been developed for the prevention of TB. An open-label Phase I clinical trial for aerosol tuberculosis vaccine was conducted by McMaster University Medical Centre (MUMC, 1200 Main St. West, Hamilton L8N 3Z5, Canada). The results indicated that even though the aerosol dose (1×10^6 PFU) was 100 times lower than the intramuscular dose (1×10^8 PFU), it still produced remarkable immunogenicity in human respiratory mucosa[98]. There were no clinically significant abnormalities detected in routine laboratory tests at 2, 4, and 12 weeks after aerosol vaccination.

Overall, RVVs for respiratory delivery are promising, however, their safety and efficacy warrant further investigations. The details of other recombinant viral vector vaccines for respiratory delivery either in preclinical or clinical stages are summarized in Table 2 [99-110].

3.4. Subunit vaccines

Subunit vaccines are split vaccines containing the purified components of pathogens, including proteins, polysaccharides, lipids, or peptides[111], which are immunogenic or necessary to elicit specific immune responses. Traditionally, subunit vaccines were prepared by appropriately splitting the virion particles and collecting the antigenic components. With the development of biotechnology, molecular cloning techniques are extensively used to prepare subunit vaccines which are called recombinant subunit vaccines.

Although no respiratory-delivered subunit vaccines have yet been approved in the market, there are several products in clinical trials. For instance, an intranasal recombinant COVID-19 spike protein subunit vaccine is in phase III clinical trial currently in Iran, which is used as the booster shot for individuals who have received two doses of intramuscular vaccines. In the phase I stage, this vaccine did not show any serious adverse effect but significant IgA response, robust humoral response, and strong T-helper 1 response were observed in a total of 153 participants[112]. In the following phase II stage, the vaccine with a selected dose showed adequate immunogenicity and efficiency after intranasal administration to 500 volunteers[82,113]. ACM-001, another COVID-19 subunit vaccine, containing recombinant spike protein of beta-variant strain SARS-CoV-2 and CpG adjuvant is being tested in Phase I clinical trial (NCT05385991). The ACM-001 was designed to be administered intranasally as a booster vaccine against SARS-CoV-2 after a full primary vaccination and booster (3 doses) schedule with any commercial SARS-CoV-2 vaccines. However, this trial is still ongoing and no results have been reported yet.

In general, this type of vaccine is relatively safer and easier to produce compared to other types, but the lack of additional immunostimulatory molecules could significantly compromise the immunogenicity[114]. Adding adjuvant in the formulation and/or using nano drug delivery systems (NDDS) are the two major strategies to enhance the immunogenicity of subunit vaccine[115–117]. Some adjuvants incorporated into the formulation of respiratory-delivered subunit vaccines for TB[118–120], influenza[121–125], and RSV[126–129] were summarized in Table 3 [130-138]. Some representative NDDS were summarized in Table 4 [139-146].

3.5. Nucleic acid vaccines

Nucleic acid vaccines are plasmid DNA (pDNA), mRNA, and self-amplifying RNA that encodes the distinctive antigenic proteins. Nucleic acids can be produced by some biological synthesis methods, which are relatively simple, fast, and low-cost. However, the unfavorable intrinsic nature of nucleic acids including fragile structure, high hydrophilicity, and high negative charge density of nucleic acids make it extremely difficult to reach the site of action in the cell nucleus or cytoplasm. Therefore, a carrier that can facilitate the delivery of vaccines is an integral part of nucleic acid vaccines. To date, although there are no nucleic acid vaccines

Table 2 Preclinical and clinical studies of recombinant viral vector vaccines delivered *via* respiratory route.

Pathogen	Vector	Administration route	Formulation type	Preclinical or clinical	*In vivo* model	Ref.
TB	Ad5	Pulmonary aerosol and intratracheal	Solution	Preclinical and clinical phase I	*Rhesus macaques*, human	98–100
TB	Simian adenovirus	Aerosol inhalation	Solution	Clinical phase I	Human	101
TB	Recombinant influenza virus	Intranasal	Solution	Preclinical	Mice, guinea pigs	86
TB	Recombinant influenza virus	Intranasal	Solution	Clinical phase I	Human	102
TB	Recombinant influenza virus	Intranasal	Solution	Preclinical	Mice	87
RSV	Recombinant influenza virus	Intranasal	Solution	Preclinical	Mice	103
RSV	Recombinant vesicular stomatitis virus	Intranasal	Solution	Preclinical	Cotton rats	88
RSV	Chimpanzee Ad	Intranasal	Solution	Preclinical	Mice, cotton rats	104
RSV	Baculovirus	Intranasal	Solution	Preclinical	Mice	90
Measles	Human Ad	Intranasal	Solution	Preclinical	Mice, cotton rats	105,106
Measles	Vaccinia virus	Intranasal	Solution	Preclinical	Mice	107
Measles	Parainfluenza	Intranasal	Solution	Preclinical	Syrian hamsters	108
Measles	Parainfluenza	Intranasal	Solution	Clinical phase I	Infants and children	109
Measles	Vesicular stomatitis virus	Intranasal	Solution	Preclinical	Cotton rats	110

TB, tuberculosis; Ad, adenovirus; RSV, respiratory syncytial virus.

Table 3 Preclinical and clinical studies of subunit vaccines delivered *via* respiratory route.

Pathogen	Vaccine component	Adjuvant	Administration route	Formulation type	Preclinical or clinical	*In vivo* model	Ref.
TB	Recombinant *Mycobacterium tuberculosis* proteins	TLR	Pulmonary	Dry powder	Preclinical	Mice	130
TB	Fusion protein containing five *Mycobacterium tuberculosis* proteins (5Ag)	CDN	Intranasal	Solution	Preclinical	Mice	118
Influenza	Membrane-anchored flagellin	VLPs	Intranasal	Solution	Preclinical	Mice	131
Influenza	HA, NA and matrix protein of influenza virus	VLPs	Intranasal	Solution	Preclinical	Mice	132
Influenza	OVA	PLGA nanoparticles	Intranasal	Solution	Preclinical	Mice	133
Influenza	rCTB	−	Intranasal	Solution	Preclinical	Mice	134
Influenza	−	Saponin derives	Intranasal	Solution	Preclinical	Mice	135
COVID-19	CIGB-669 (RBD+AgnHB)	−	Intranasal	Solution	Clinical phase I/II	Human	136
COVID-19	ACM-SARS-CoV-2-beta ACM-CpG vaccine candidate	−	Intranasal	Solution	Clinical phase I	Human	137
RSV	Purified RSV F protein	CpG ODNs	Intranasal	Solution	Preclinical	Mice or cotton rats	126,127
RSV	M2 protein of RSV	Fused peptide	Intranasal	Solution	Preclinical	Mice	128
RSV	RSV-F protein	Influenza virosomes and *Escheriagen* (*Escherichia coli* heat-labile toxin)	Intranasal	Solution	Preclinical	Mice	129
RSV	RSV long strain G protein fused to a fragment of thioredoxin	Protollin	Intranasal	Solution	Preclinical	Mice	138

TB, tuberculosis; TLR, toll-like receptors; CDN, cyclic diguanylate; VLPs, virus-like particles; HA, haemagglutinin; NA, neuraminidase; OVA, ovalbumin; PLGA, poly(lactic-co-glycolic acid); rCTB, recombinant cholera toxin B subunit; RBD, receptor-binding domain; ODNs, oligodeoxynucleotides; RSV, respiratory syncytial virus; −, not applicable.

Table 4 Representative subunit vaccine nanoparticles for respiratory tract delivery.

Indication	Nanoparticle type	Immunogenic compound	Administration route	Excipient	Preclinical or clinical	*In vivo* model	Ref.
TB	Polymeric self-assemblies	Mycolic acid	Intranasal	PEG-PPS copolymers	Preclinical	Mice	139
Pneumococcus	Cationic amphiphilic polysaccharide derivatives	*Pneumococcal* surface protein A	Intranasal	Cationic cholesteryl pullulan nanogel	Preclinical	Macaques	140
Tumor	Chitosan nanoparticles	Gastrin-releasing peptide	Intranasal	Mannosylated chitosan	Preclinical	Mice	141
TB	Amphiphilic peptide self-assemblies	BMDC cognate antigen Ag85B	Pulmonary administration	KFE8, a self-associating peptide containing 8 amino acids	Preclinical	Mice	142
M cell targeting	Polymer-polysaccharide nanoparticles	IgA and protein antigen pertussis toxin	Intranasal	Chitosan−dextran sulphate	Preclinical	Mice	143
M cell targeting	PLGA nanoparticles	Recombinant protein containing HA from influenza virus A, His-tag and perfringens enterotoxin	Intranasal	PLGA	Preclinical	Mice	144
Hepatitis	Chitosan-PLGA nanoparticles	Recombinant hepatitis B surface antigen	Intranasal	Glycol chitosan, PLGA	Preclinical	New Zealand white rabbits	145
TB	Lipidic nanoparticles	*Mycobacterium tuberculosis* antigens	Intranasal, intratracheal	Yellow carnauba wax	Preclinical	Mice	146

TB, tuberculosis; PEG-PPS, poly(ethylene glycol)-bl-poly (propylene sulfide); PLGA, poly (lactic-*co*-glycolic acid); HA, haemagglutinin.

commercially available for respiratory route delivery yet, many attempts have been made to promote the progress in this field. The research progress of nucleic acid vaccines delivered *via* the respiratory tract in both clinical and preclinical stages was summarized in Table 5 [177-189]. Some typical examples of nucleic acid vaccines based on lipid, polymer, or protein/peptide carriers for respiratory delivery were schematically shown in Fig. 2 and discussed in the following.

3.5.1. *pDNA vaccines*

pDNA vaccines are usually constructed by inserting the DNA fragments encoding antigen into a bacterial plasmid. The pDNA has high stability due to the double-chained structure as well as the hydrogen bonds. The stability can be further enhanced when supercoiled[147]. The pDNA is anionic because of the negatively charged phosphate groups with large molecular weight, which hinders the ability to cross cellular and nuclear membranes. Therefore, cationic materials are necessary to be utilized in pDNA vaccine formulations to rearrange their net charge and increase their transmembrane efficiency.

Some cationic lipids, such as 1,2-dioleoyl-3-dimethylammonium chloride (DODAC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) can form complexes with polyanionic pDNA enhance its cellular uptake and endosomal escape. In a preclinical study, cationic lipid DODAC, helper lipid DOPE, and PEGylated lipid PEG-C8 were formulated into cationic liposomes to encapsulate pDNA encoding the HA protein of influenza[148]. The integrity of pDNA against DNAse challenge was retained, and high titers of IgA in BALF and serum as well as a high level of T cell proliferation was induced after intranasal administration to mice. More interestingly, the vaccines rendered animals complete protection against lethal doses of influenza virus. Another cationic liposome encapsulating pDNA encoding mycobacterial 65-kDa heat shock protein (DNA-hsp65) was reported to show a significant reduction of the bacilli amount in lungs together with increased IFN-γ level lung parenchyma preservation against tuberculosis in mice after intranasal administration[149]. The formulation contains egg phosphatidylcholine (EPC), DOPE, and DOTAP. The EPC and DOPE serve as structure lipids, while DOTAP serves as the transfection reagent and complex with pDNA on the surface of liposome through electrostatic interaction. However, the physiochemical stability of liposome vaccines in an aqueous environment after storage still needs improvement for large-scale adoption[150].

Cationic polymers can also serve as net charge adjusters for pDNA vaccine formulations by forming polyplexes with pDNA. Polyethyleneimine (PEI) is an extensively used cationic polymer-based transfection agent for pDNA as it could enhance their transfection efficiency by facilitating endosomal escape. In a previous study, pDNA encoding SARS-CoV spike (S) protein was mixed with PEI and administered to mice intranasally[151]. The immunized mice exhibited significantly higher sIgA levels in the

Table 5 Preclinical and clinical studies of nucleic acid vaccines delivered *via* respiratory route.

Indication	Formulation composition	Nucleic acid vaccine type	Coding antigen	Administration route	Preclinical or clinical	*In vivo* model	Ref.
Hepatitis	PC/DOPE/Cholesterol liposomes	pDNA	S protein	Intranasal	Preclinical	Mice	177
Influenza	DODAC/DOPE/PEG liposomes	pDNA	HA	Intranasal	Preclinical	Mice	148
TB	GAP-DLRIE: DOPE lipoplexes	pDNA	Ag85A	Intranasal	Preclinical	Mice	178
TB	EPC/DOPE/DOTAP liposomes	pDNA	HSP65	Intranasal	Preclinical	Mice	149
Influenza	PEI polyplexes	pDNA	HA	Intranasal	Preclinical	Mice	179
Influenza	dPEI polyplexes	pDNA	HA	Intranasal	Preclinical	Mice	152
SARS-CoV	PEI polyplexes	pDNA	S protein	Intranasal	Preclinical	Mice	151
HIV	PEI polyplexes	pDNA	HXBc2 gp120	Intratracheal	Preclinical	Mice	180,181
TB	Mannosylated chitosan nanoparticles	pDNA	HSP65	Intranasal	Preclinical	Mice	182
SARS-CoV	Chitosan nanoparticles	pDNA	N protein	Intranasal	Preclinical	Mice	153
SARS-CoV-2	Chitosan-gold nanoparticles	pDNA	S protein	Intranasal	Preclinical	Mice	183
RSV	Chitosan nanoparticles	pDNA	M2 protein	Intranasal	Preclinical	Mice	154
Tumor	Protamine nanocomplexes	pDNA	pGRP	Intranasal	Preclinical	Mice	156
Influenza	LNP	mRNA	HA	Intranasal	Preclinical	Mice	184
−	Cyclodextrin−PEI polyplexes	mRNA	OVA	Intranasal	Preclinical	Mice	166
HIV	Cyclodextrin−PEI polyplexes	mRNA	gp120	Intranasal	Preclinical	Mice	185
Influenza	Chitosan nanoparticles	mRNA	HA and M2	Intranasal	Preclinical	Chicken	186
Mice Lewis lung cancer model	Cationic liposome/protamine	mRNA	Cytokeratin 19	Intranasal	Preclinical	Mice	187
HIV	−	mRNA	HT1	Intranasal	Clinical-terminated	Human	188
Influenza	LNPs	saRNA	Influenza A H1N1 Cal/09 strain	Intranasal	Preclinical	Mice	176
SARS-CoV-2	Alphavirus replicon particle	saRNA	CB6	Intranasal	Preclinical	Mice	189
Rabies virus	LNPs, solid lipid nanoparticles and polymeric nanoparticles	saRNA	Glycoprotein	Intranasal	Preclinical	Mice	173

PC, phosphatidylcholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DODAC, 1,2-dioleoyl-3-dimethylammonium chloride; EPC, egg phosphatidylcholine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; PEG, poly(ethylene glycol); HA, haemagglutinin; TB, tuberculosis; PEI, polyethyleneimine; dPEI, deacylated polyethyleneimine; GAP-DLRIE:DOPE, aminopropyl-dimethyl-bis-dodecyloxy-propanaminium bromide−dioleoylphosphatidyl-ethanolamine; HIV, human immunodeficiency virus; LNPs, lipid nanoparticles; Ag85A, tuberculosis antigen 85 A; HSP65, mycobacterial 65-kDa heat shock protein; pGRP, plasmid encoding gastrin-releasing peptide; OVA, ovalbumin; −, not applicable.

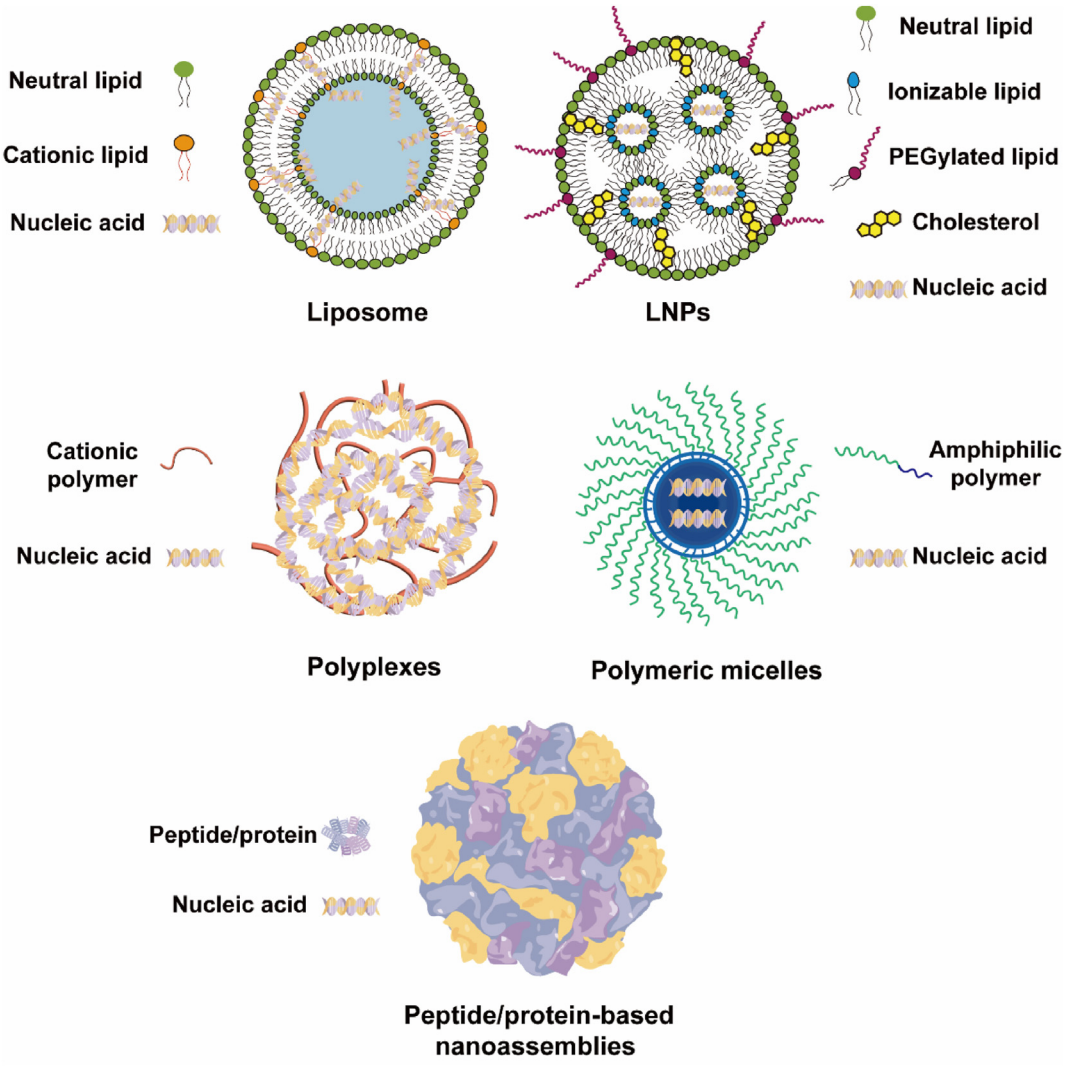

Figure 2 Carrier-based nucleic acid vaccine for respiratory delivery.

lung wash and S-specific IgG levels in the sera than those treated with pDNA alone. In addition, the percentage of IFN-γ, TNF-α, and IL-2-producing cells was also elevated. In another study, Mann et al.[152] have developed a deacylated PEI (dPEI)-pDNA polyplexes-based nasal influenza vaccine. Following administration, influenza HA antigen-specific IgG and IgA responses were significantly induced in mouse lungs, and the dPEI-DNA polyplexes could transfect human mucosal epithelium and traverse epithelial monolayers. Despite the excellent pDNA transfection efficiency PEI provides, the toxicity and non-degradable nature hinder its application in the clinic.

Chitosan is a positively charged natural polysaccharide with great biodegradability and biocompatibility, which can bind with pDNA through electrostatic complexion and facilitate gene transfection. In a related study, pDNA encoding N protein of SARS-CoV was loaded into biotinylated chitosan nanoparticles and given intranasally to mice[153]. The results demonstrated that intranasal vaccination induced enhanced mucosal IgA and systemic IgG levels against N protein. However, the systemic IgG level by intranasal route was lower than that of intramuscular route. In another study, pDNA encoding the CTL epitope from the M2 protein of RSV was formulated with chitosan and immunized to mice through intranasal administration[154]. The RSV-specific CTL responses induced by the intranasal route were comparable to the intradermal route. Moreover, the intranasally immunized mice exhibited great protection effects against RSV challenge.

Protamine, a cationic protein extracted from mature male fish, is also extensively used as the carrier material for nucleic acid delivery due to the capacity to self-assemble with DNA and target the nuclei *via* the nuclear localization signal-like regions consisting of four to six arginine repeats[155]. Via mannosylation and complexation with pDNA encoding gastrin-releasing peptide (pGRP)[156], the affinity of protamine to macrophage could be increased and consequently, the cellular uptake and transfection efficiency of protamine-based nanoparticles in macrophages were enhanced. Higher pGRP-specific antigen titer and tumor inhibition rates were also observed in mice after nasal vaccination.

Despite the encouraging results of preclinical studies, the potency of pDNA vaccines is disappointing in humans. Firstly, vaccination would benefit from the transient production of antigen instead of long-lasting or even permanent production of protein for gene therapeutics[157], for which the pDNA with great inherent stability may need to be modified to produce the encoded antigen

for a limited time. Secondly, the pDNA vaccines warrant proper boosting or promoting strategies to generate optimal immune responses[158]. Thirdly, the pDNA vaccines need to enter the nuclei, which raises the concern of genomic integration[159].

3.5.2. *mRNA vaccines*

mRNA vaccines have demonstrated remarkable effects in controlling the SARS-CoV-2 pandemic. Compared with pDNA vaccines, the mRNA vaccines would not enter the nucleus and, therefore do not have the risk of insertional mutagenesis and genomic integration[159]. Moreover, the transient gene expression of mRNA would avoid undesired persistent gene expression and provide better control of vaccine activity[160]. Although promising, the mRNA is easily degraded by the RNase in the environment and shear forces during airway nebulization. Therefore, the cargos for respiratory-delivered mRNA vaccines need to be optimized to acquire satisfying immune responses in the respiratory tract.

Lipid nanoparticles (LNPs) have been thoroughly investigated and successfully launched for two authorized coronavirus disease 2019 (COVID-19) vaccines, mRNA-1273[161] and BNT162b2[162]. The typical formulation of LNPs for injection is composed of ionizable or cationic lipids, PEGylated lipids, cholesterol, and helper lipids. When LNPs are applied for the respiratory route, the composition of LNPs should be comprehensively modified according to the aerosol device and local microenvironment in the lung. A recent study exhibited some design principles for aerosolized LNPs through *in vivo* cluster-based iterative screening[163]. According to this study, a higher ratio of PEGylated lipids is required for aerosolized LNPs to ensure better stability and slower lung clearance of mRNAs[164]. It was found that LNPs with higher PEG and cationic lipids molarity could achieve higher transfection efficiency *in vivo* and cause less aggregation during nebulization, but too high amount of PEGylated lipids might reversely compromise the intracellular protein expression level[165]. In addition, the type of PEGylated lipids and helper lipids also have a significant influence on the mRNA transfection and protein expression levels in the lung. The mechanism behind these phenomena warrants further investigation.

The cationic polymers are also appealing for mRNA vaccine delivery to the respiratory tract. In a preclinical case, PEI with different molecular weights (600 Da and 2 KDa) was conjugated with cyclodextrin (CD) respectively. The resultant cationic conjugate CP600 or CP2K was complexed with mRNA encoding OVA protein and intranasally administered to the mice[166]. Compared to CP600-mRNA, CP2K-mRNA complexes more easily migrate to lymph nodes and stimulate dendritic cell maturation *in vivo*, which was probably due to the smaller particle size. The CP2K-mRNA complexes could induce stronger $CD4^+$/$CD8^+$T responses in the lungs and lymph nodes with lower local and systemic toxicities than PEI 25k-mRNA.

A new class of cationic polymer named hyperbranched poly (beta-amino esters) (hPBAEs) showed superior capacities of nebulized delivery of mRNA over PEI as they were less toxic and their hyperbranching architecture could help to maintain structural stability during inhalation. In a related study, the polyplexes of hPBAE and mRNA implied a uniform distribution of mRNA throughout all five lung lobes of mice after nebulization, and the gene expression level in mouse lungs was nearly 20 times greater than that of branched PEI/mRNA complexes. Furthermore, repeated dosing of inhaled hPBAE-mRNA could cause persistent protein production in the lungs without local or systemic toxicities[167].

The use of cationic peptides and proteins in this area is also extensively explored. They can exhibit diverse functions by adjusting the kinds and sequences of amino acids. The incorporation of cationic amino acids (*i.e.,* lysine, histidine, and arginine) would facilitate the peptides or proteins to create complexes with anionic nucleic acids. In addition, the combination of hydrophilic and hydrophobic amino acids would form amphiphilic peptides or proteins, which can be formulated into nanoparticles or nanofibers by self-assembly protocols. Therefore, the peptides and proteins are promising materials for mRNA vaccine delivery. In a previous study, synthetic cationic peptide KL4 was PEGylated for better solubility and formed nanosized complexes with mRNA through electrostatic complexation[168]. The PEG-KL4/mRNA complexes mediated effective transfection onto human lung epithelial cells. The spray-dried complexes were intratracheally administered and exhibited much higher gene expression than naked mRNA and lipofectamine-transfected mRNA. The high gene expression lasted for 24 h post-administration. In addition, the complexes showed no inflammation or toxicity in mice lungs, which indicated that the PEG-KL4 had great potential for pulmonary delivery of mRNA vaccines.

Although mRNA vaccines have exhibited so much potential for respiratory tract delivery, the intrinsic immunogenicity makes it still challenging to transfer respiratory-delivered mRNA vaccines to clinics. mRNA has several immunostimulatory mechanisms, which may either be useful or detrimental for mRNA vaccination or vaccination safety[169,170]. The booming of type I interferon would result in both inflammation and autoimmune responses[171,172]. On the other hand, mRNA can also activate the TLR pathways and exhibit adjuvant activities[157]. The balancing of the potential efficacy and safety of mRNA vaccines needs to be further explored. Furthermore, some side effects of mRNA vaccines cannot be predicted by preclinical studies due to species differences between human and animal models, which indicates that the application of mRNA vaccines should be very careful.

3.5.3. *Self-amplifying RNA vaccines*

Self-amplifying RNA (saRNA) vaccines are generated by combining the gene encoding the antigens with the gene fragments of RNA-dependent RNA polymerases (RDRP)[173]. The RDRP fragments would facilitate the gene amplification, which enables a much lower dose of saRNA to acquire equivalent immune responses as non-amplifying mRNA[174,175]. Similar to other nucleic acid vaccines, the saRNA vaccines also require proper vectors for respiratory tract delivery.

In a recent study, a novel ionizable modified dendron was complexed with saRNA encoding influenza antigen *via* microfluidic mixing. Then the cholesterol, helper lipid DOPE, and PEGylated lipid DMG-PEG2000 were added sequentially to form LNPs[176]. After intranasal vaccination to mice, the LNPs boosted more pulmonary T_{RM} and persisting memory CD8 T cells in BAL fluid and respiratory tract than that of intramuscular vaccination. In contrast, the intranasal vaccination induced less circulating CD8 and CD4 T cell memory than the intramuscular vaccination. Combining intramuscular immunizations with an intranasal boost achieved both high levels of circulating T cell memory and lung T_{RM}, however, the dosage regimen for optimizing long-term protection still warrants further exploration. In addition, this research demonstrated that the modified dendron-based LNPs resulted in negligible material-induced inflammation in the lungs and acceptable liver safety, which were much better than

conventional RNA LNPs. However, the mechanisms of better safety profiles of this novel LNPs were not evaluated.

Despite the promising results discussed above, the immunogenicity of saRNA-loaded LNPs is contradictory in various studies. In another related study, a saRNA encoding the rabies virus glycoprotein (RVG) was encapsulated in LNPs containing helper lipid DOPE, PEGylated lipid DMG-PEG2000, and cationic lipid DOTAP[173]. Regrettably, after intranasal administration to mice, the immune responses generated by the LNPs were lower than those of intramuscular and intradermal administration. In addition, the intranasal-delivered LNPs experienced rapid clearance from the body. The differences might be related to the formulation composition of LNPs as well as the chain length of saRNA. Till now, the saRNA-loaded formulations for respiratory-administered vaccines are still limited. Formulation strategies for saRNA-loaded respiratory administered vaccines require further improvement.

In addition to the abovementioned nucleic acid vaccines, other nucleic acids might also be utilized for vaccines. For instance, small interfering RNA (siRNA), which can mediate sequence-specific gene silencing effects through the RNAi pathway, has great potential in treating diseases caused by gene overexpression. In the case of vaccines, silencing the upstream gene might be able to adjust the levels of immune-related genes. However, the choice of critical genes and their exact mechanism warrants further exploration.

3.6. *Nanobody vaccines*

Nanobodies are naturally the smallest functional single-domain antibodies which consist of two heavy chains of immunoglobulin with ultrahigh affinity to antigens[190]. Compared with traditional monoclonal antibodies, nanobodies are more stable to a wide range of pH and temperatures together with superior hydrophilicity[191], which facilitates them to be aerosolized to the respiratory tract directly[192]. The unique characteristics of nanobodies offer great opportunities for developing both therapeutical reagents and vaccines *via* inhalation.

Till now, there have been several inhaled nanobodies against various lung infection diseases under investigation. The recent research progress of nanobodies for respiratory delivery is summarized in Table 6 [193-199].

For instance, a nanobody targeting the RBD of SARS-CoV-2 S protein was optimized and nebulized to Syrian golden hamsters[192], and the results showed that the virus neutralization capacity of the nanobody was well reserved and the droplet sizes were reproducibly distributed to reach all compartments of airways. When animals were challenged with the SARS-CoV-2 virus intranasally 3 h after the immunization with nebulized nanobodies, almost complete protection with significantly lower weight loss was achieved, as compared with the control group without immunization. For therapeutic purposes, nanobodies administered 24 h after infection also exhibited significant recovering effects on treated animals with much milder weight loss as well as less inflammatory and degenerative changes.

In another study, a trimeric Nanobody that binds the antigenic site II of RSV F protein was administered to cotton rats either intranasally or by nebulization for therapeutic or prophylactic application, respectively[193]. For therapeutic application, the nanobodies were administered intranasally to animals on the second or third day after RSV infection. The treatment exhibited significant viral load reductions in the lungs compared with the untreated group. For prophylactic application, the nanobodies were nebulized to animals 1 h before the RSV challenge, and the RSV replication was almost completely blocked. These results all suggested the great potential of nanobodies to serve as a prophylactic or therapeutic strategy against lung diseases. However, there are still some limitations. Firstly, the protection duration of nebulized nanobodies is too short, mostly in hours, which is insufficient to provide long-term prophylaxis in the human crowd. Secondly, although nanobodies have the potential to be used as therapeutic agents, nanobodies will not be suitable for severe lung infection, since the key pathomechanism of lung infection is hyperinflammation rather than virus replication. Thirdly, the safety profile of nanobodies with multiple dosing regimens in the lungs warrants further investigation.

4. Formulation strategies for enhancing the efficiency of particulate respiratory delivered vaccines

As shown above, the application of nano- or micro-sized particulate drug delivery systems based on various functional materials is an effective way to induce efficient and prolonged immune effects in the respiratory tract. However, the successful delivery of vaccine particles into the airway is still extremely challenging because of the multiple barriers in the respiratory tract. The upper airway of the respiratory tract is covered with a dense and viscoelastic mucus layer, which prevents the vaccines from contacting the lung cells[200–202]. The vaccine particles would be entrapped within mucus and transported towards the mouth, swallowed, or coughed out, which consequently reduces the vaccination effects. In addition, the antigens would only elicit immune responses after being uptake by antigen-presenting cells (APCs)[24,160,166]. However, the majority of cells in the respiratory tract are epithelial cells without antigen-presenting function[203], the preferential uptake by epithelial cells could decrease the immune effects of the mucosal vaccines. To overcome these barriers, more delicate modifications of nanoparticles and microparticles are needed to render them extra functions like targeting profile, mucus penetration, or adhesion ability by controlling the interaction between the vaccine and the microenvironment in the respiratory system[204,205]. In this section, some novel strategies that have been explored to modify particles to promote their performance in the context of respiratory vaccine delivery will be summarized and discussed in detail.

4.1. *Strategies for improving targeting efficiency*

As mentioned above, M cells and APCs that function as antigen capturing and presenting in respiratory mucus immune systems play a critical role in vaccination. Targeted delivery of vaccines to M cells or APCs could increase the immunization efficiency of vaccines, and consequently lead to the reduction of dose and potential side effects induced by broad transportation of proteins or nucleic acids to the somatic cells. In general, targeting can be achieved *via* the way of passive targeting and active targeting (receptor-specific binding).

4.1.1. *Passive targeting*

The preferential uptake of particles with specific sizes and surface charge by APCs could turn into a passive way of targeting APCs (Fig. 3A). Blank et al.[206] have examined the uptake of polystyrene (PS) particles with different sizes by mouse lung APCs after

Table 6 Studies of nanobody vaccines delivered *via* respiratory route.

Pathogens	Nanobody type	Administration route	Prophylactic or therapeutic use	*In vivo* model	Administer regimen	Ref.
RSV	Nanobody that binds the antigenic site II of RSV F protein	Intranasal or nebulized inhalation	Prophylactic and therapeutic	Cotton rats	Prophylactic-1 h before infection; therapeutic-2 or 3 days after infection	193
SARS-CoV-2	RBD-specific nanobody	Nebulized inhalation	Prophylactic and therapeutic	Syrian hamster	Prophylactic-8 h before infection; therapeutic-24 h after infection	194
SARS-CoV-2	Omicron variant RBD-specific nanobody	Nebulized inhalation	Therapeutic	Mice	2 h after infection, once a day for three days	195
SARS-CoV-2	Heterotrimeric RBD-specific nanobody	Intranasal	Prophylactic and therapeutic	Mice	Prophylactic-24 h before infection; therapeutic-24 h after infection	196
SARS-CoV-2	S protein-specific trimer nanobody	Intranasal	Prophylactic and therapeutic	Syrian hamster	Prophylactic-2 h before infection; therapeutic-24 h after infection	197
SARS-CoV-2	Trimerized nanobody cocktail	Intratracheal	Therapeutic	Mice	One day after infection	198
SARS-CoV-2	PiN-21	Nebulized inhalation	Prophylactic and therapeutic	Syrian hamster	Prophylactic-6 h before infection; therapeutic-8 and 24 h after infection	199

RSV, respiratory syncytial virus; RBD, receptor-binding domain; PiN-21, ultrapotent homotrimeric Pittsburgh inhalable nanobody 21.

intranasal administration. The results evidenced that the majority of PS particles were taken up by the alveolar macrophages, independent of particle size. In contrast, DCs preferentially captured 20- and 50-nm particles as compared to 1000-nm particles. The DCs were then activated and triggered an enhanced antigen-specific CD41 T-cell stimulation. In addition, the smaller-sized particles captured by DCs could be translocated to lung-draining lymph nodes (LDLNs), while the particles captured by alveolar macrophages failed to do so.

In addition to size, the surface charges could also influence the uptake of vaccine nanoparticles by DCs (Fig. 3A). It was demonstrated that as compared to anionic scaffolds, cationic nanoparticles bestowed an increased accumulation within DCs of mouse lungs after orotracheal instillation[207]. Furthermore, cationic nanoparticles had an adjuvant-like effect in the lungs by promoting the production of CCL-2 and CXCL-10, which are chemo-attractants involved in DC recruitment to the lungs[208–210] and contributed to the increased IgA production[211].

4.1.2. Receptor-specific binding

The existence of some specific ligands on M cells or APCs offered a way of active targeting *via* specific receptor-ligand interaction (Fig. 3B). An early study has exploited IgA as a targeting ligand for M cells because it can specifically adhere to the apical membrane of M cells[143] (Table 4 [139–146]). When IgA was incorporated into protein antigen pertussis toxin-loaded chitosan−dextran sulfate nanoparticles (IgA-CS-DS NPs, the NPs were preferentially taken up by M cells after intranasal administration to mice. Claudin 4, a tight junction transmembrane protein, that is highly expressed on the surfaces of M cells, was used as a targeting site in another study (Table 4 [139–146]). Since claudin 4 can specifically bind to clostridium perfringens enterotoxin (CPE), the CPE could be employed as a targeting ligand[144]. The investigators built a recombinant protein containing HA from influenza virus A, His-tag (HT), and CPE, in which the HA acted as antigen, and the HT was inserted for purification. The recombinant protein was encapsulated into PLGA nanoparticles using the solvent evaporation/double emulsion method. The recombinant protein was distributed both in core matrices and on the surfaces. The CPE remaining on the surfaces of the nanoparticles served as a targeting ligand. The nanoparticles were readily uptaken by claudin 4-expressing cells *in vitro* and also showed significantly increased cellular uptake by M cells of mice after intranasal administration.

For APCs, the mannose receptors have attracted much interest as these are expressed on most macrophages and DC cells[212]. Therefore, grafting mannose onto the surface of vaccine vectors could improve their targeting of APCs. For example, mannosylated chitosan was shown to enhance the cellular uptake of vaccines by APCs and result in improved immunogenicity[141,156]. In this study, mannosylated chitosan (MCS) was complexed with the pDNA encoding gastrin-releasing peptide (GRP) through electronic interaction to form nanoparticles (MCS-pGRP) (Table 4 [139–146])[141]. The complexes depicted a high macrophage accumulation through mannose-mannose receptor binding, and the levels of anti-GRP antibody were significantly elevated in mice serum after intranasal vaccination. Compared to the non-modified nanoparticles counterpart and free pDNA, the growth of tumors could be dramatically suppressed by MCS-pGRP, when all of them were used as preventative vaccines against tumors. In another study[182], the MCS-DNA nanoparticles (MCS-DNA NPs) also showed specific targeting to alveolar macrophages, and both sIgA titer in BALF and $CD4^+$/$CD8^+$T responses in the lungs were significantly increased relative to the unmodified chitosan-DNA

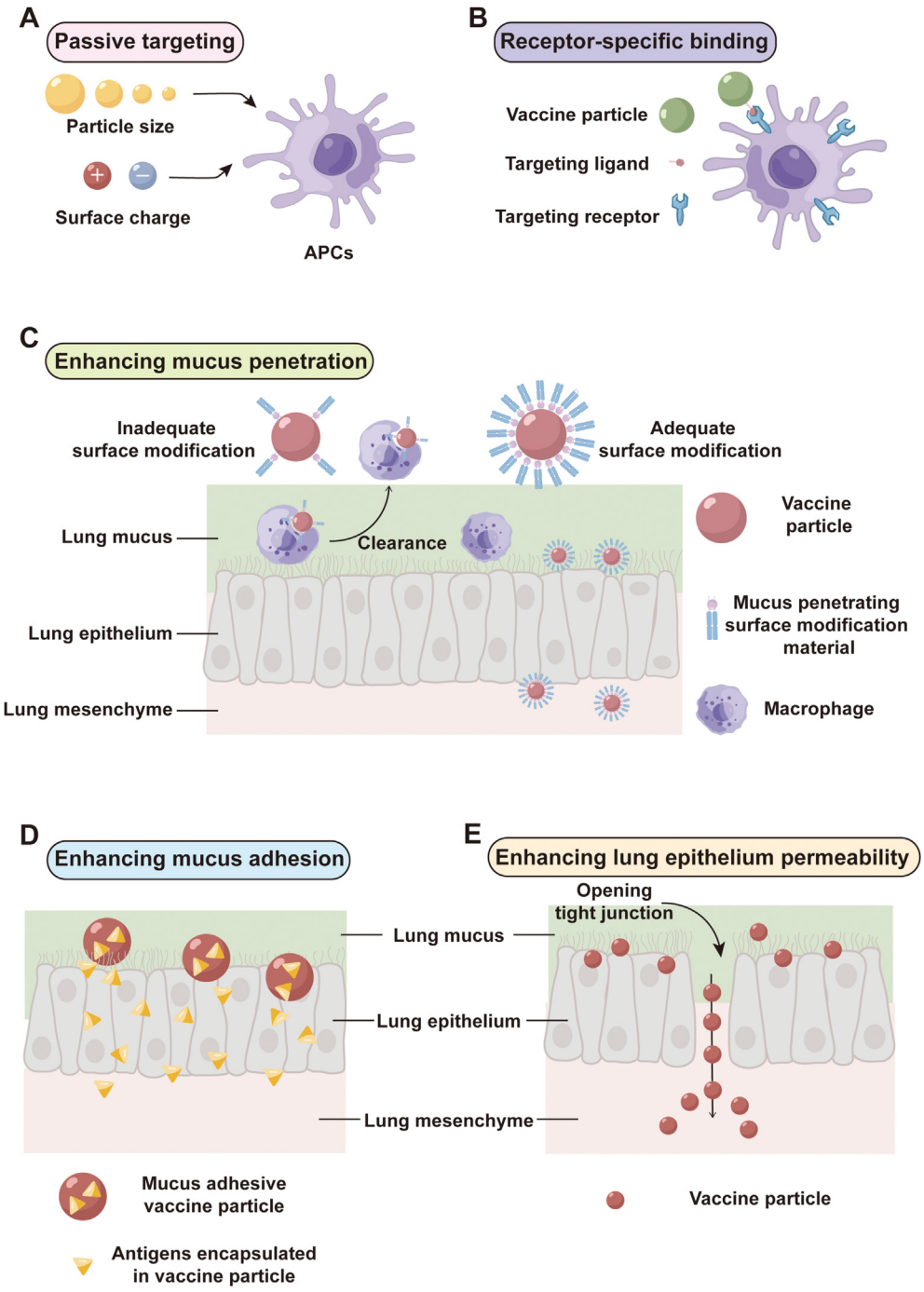

Figure 3 Formulation strategies for enhancing the respiratory delivery efficiencies of vaccines. (A) Facilitating passive targeting; (B) Promoting receptor-specific binding; (C) Enhancing mucus penetration ability; (D) Enhancing mucus adhesion; (E) Enhancing lung epithelium permeability.

nanoparticles or naked DNA. When used as a vaccine for tuberculosis, MCS-DNA NPs could significantly reduce the lung bacterial CFUs comparable to subcutaneous BCG vaccination. However, the protection against bacterial infections in the spleen was less effective.

In addition to mannose, galactose was also used as a ligand to target macrophages because it can selectively bind to the galactose type C-type lectins on the surfaces of macrophages[213]. In a previous study[214], galactose-modified liposomes exhibited higher recognition and endocytosis levels by the macrophages, and induced significantly higher sIgA levels in nasal and lung wash and higher serum IgG antibody level in mice after intranasal administration, as compared to the unmodified liposomes and galactose-mixed liposomes.

Sialic acids are also highly expressed on the surfaces of DCs and widely involved in their functions, such as antigen uptake, migration, and T cell response priming[215]. In an *in vitro* study, glutamic acid-alanine-leucine-alanine (GALA) peptide, a

synthetic peptide with high selectivity binding to sialic acid terminated glycans on DCs, was conjugated with poly uridine (PolyU)/mRNA polyplexes and facilitated mRNA internalization and subsequent cytosolic release, further leading to higher mRNA transfection efficiency in RAW 246.7 macrophages (around 36%) and D1 splenic dendritic cells (around 50%) as compared to the same polyplexes but modified with non-sialic acid targeting peptide[216]. The GALA-modified polyplex could also enhance T-cell responses and DC maturation. However, the *in vivo* DC targeting and immunotherapy effects after respiratory administration warrant further consideration.

4.2. *Strategies for enhancing mucus penetration*

The presence of mucus on the surface of the respiratory tract poses a huge barrier for the respiratory-delivered vaccine. The vaccine particles with excellent mucus penetration abilities could potentially escape from the trap of mucus to avoid the mucociliary clearance effects[217]. The ability to penetrate through mucus is dependent on the adhesive forces between vaccine particles and pulmonary mucus, which are mainly induced through two mechanisms: (1) the interactions between the hydrophobic regions of mucin strands and hydrophobic segments of foreign particles; (2) the electrostatic interactions between mucin and surface-charged particles[218]. Surface modification of vaccine particles with hydrophilic and/or neutrally-charged molecules would be essential to avoid their hydrophobic and electrostatic interactions with mucus and eventually facilitate efficient lung mucus transport (Fig. 3C).

PEG is one of the most frequently used surface modification materials for mucus penetrating enhancement[219]. Both PEG 2 kDa and 5 kDa were shown to significantly enhance the permeability of PLGA microspheres through artificial lung mucus[220]. In an *in-vitro* model, most of the PEG-modified microspheres penetrated through the mucus layer in 1 h, while the unmodified microspheres were trapped within the mucus. Another research team has formulated neutral PEGylated lipoplexes of pDNA[221,222], which displayed no differences in gene expression between the presence and absence of CF mucin both in *in-vitro* and *in-vivo* conditions. These results suggested the superiority of PEGylation of inhalable particulates to overcome the mucus barrier. However, recent studies revealed that the PEG-specific antibodies have widely spread in humans and animals[223,224], which might not only cause anaphylaxis reactions mediated *via* antigen binding and cross-linking of IgE but also decrease the hydrophilicity and mucus permeability of PEG under *in-vivo* condition[225]. To overcome the shortcomings of PEG, several novel hydrophilic and neutral-charged polymers such as poly(2-alkyl-2-oxazolines) (POZ)[226–228], *N*-(2-hydroxypropyl) methacrylamide (HPMA)[229–232] and zwitterionic polymers[233,234] have been designed. Their penetration abilities through various mucus have been examined, however, their applications in respiratory vaccine delivery have not yet been evaluated.

Other mucus-penetrating agents include hydrating sugars, peptides, endogenous pulmonary surfactants, and so on. For example, mannitol is an osmotically active sugar that could disrupt the interactions between mucins and decrease the viscosity by influxing large amounts of water into the mucus[235]. The conjugation of mannitol with hydrophilic chondroitin sulfate A (CS-A) could combine the mucus disruption ability and hydrophilicity. The ability of pDNA/siRNA-cell penetrating peptide nanocoplexes to penetrate the artificial CF mucus layer was approximately 7−15 times higher than the unmodified counterparts.

An example of a lung mucus penetrating peptide is the recently developed receptor for advanced glycation end products (RAGE) binding peptide (RBP). The RBP was further modified with cis-aconitic anhydride (CA) to form RBP-cis-aconitic amide (RC). The RC exhibits a negative charge in neutral pH and can facilitate diffusion across the mucus layer through electrostatic repulsion toward the mucin glycoproteins. When the surface of siRNA/cationic polymer nanocomplexes was coated with RC, the mean square displacement value (MSD) and the translational motion in CF patient sputum of nanocomplex were improved 1000 fold and around 3 times respectively[236]. After intratracheal administration to mice, the majority of RC-coated nanocomplexes could penetrate through the mucus and reach deeper regions of lung tissues, while the uncoated nanocomplexes mainly stayed over the top of the goblet cells, which was ascribed to their greater entrapment within the mucus layer. The RC-coated nanocomplexes conferred a much lower mucin glycoproteins aggregation effect than that of uncoated nanocomplexes *in vitro*, which implied the mucus penetration abilities of RC.

The effects of some pulmonary surfactants including the negatively charged dipalmitoyl phosphatidylglycerol (DPPG), dipalmitoyl phosphatidylserine (DPPS), and the neutral dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylamine (DPPE) to enhance the penetration of PLGA NPs through artificial lung mucus were also tested[237]. The results elicited that DPPS modification led to increased permeability, but the other three phospholipids failed to do so. However, the mechanisms underlying the results need further investigation.

Combing with some mucolytic agents or permeability enhancers was also effective in enhancing the mucus penetration of particulate vaccine formulations. The mucolytic agents could disturb the gel structure of the mucus layer, therefore decreasing its viscosity and elasticity[238]. *N*-Acetylcysteine (NAC) and dornase alfa are the two major mucolytic agents for pulmonary mucus. The NAC could cleave the disulfide bonds of mucin proteins, destroy the spatial structure of mucin fiber, and decrease the viscosity and the elasticity of the mucus[239,240]. Pretreatment of CF sputum with NAC significantly increased the MSD of PEGylated polystyrene NPs by approximately 10-fold compared with the same NPs in untreated sputum[241]. Nacystelyn, the lysine salt of NAC, also has shown the same enhancement capacity for DNA-EDMPC: Chol lipoplexes to penetrate through the nasal mucus of mice[242]. Dornase alfa could also reduce the mucus viscosity by the DNA degradation in the mucus and is widely used to improve the lung function of CF patients by aerolization[238]. However, its potential abilities to enhance the permeability of particulate systems need to be further examined.

4.3. *Strategies for enhancing mucus adhesion*

Mucus adhesion is another effective strategy to boost the immunogenicity of vaccines since it can increase the opportunity for a vaccine to be taken up by APCs (Fig. 3D). The mucus adhesion of vaccine particles is mainly derived from the use of some mucoadhesive polymers such as chitosan and its derivatives. As shown in a previous study, the recombinant hepatitis B surface antigen (HBsAg) encapsulated PLGA NPs exhibited much higher mucin absorption after their surface was decorated with glycol chitosan[145], which indicated higher mucin adhesion ability. After intranasal administration to New Zealand white rabbits, the glycol chitosan modified PLGA NPs (GC-PLGA NPs) depicted significantly longer nasal retention, higher accumulation levels in both lungs and other organs (blood, lymph nodes, liver, spleen, intestine), and higher levels of both IgA and IgG in mice than PLGA nanoparticles by radiolabeling

the PLGA nanoparticles and investigate the biodistribution through gamma counting, which was attributed to the improved mucoadhesive ability of GC-PLGA NPs[145].

4.4. Strategies for enhancing lung epithelium permeability

The lung epithelium barrier is another challenge for respiratory-delivered vaccines to augment antigen uptake by reaching underlying APCs. The use of permeability enhancers would be a promising way to overcome this barrier (Fig. 3E). Conventional permeability enhancers such as surfactants and bile salts, can open tight junctions and lipid membranes[243]. However, they also have the risk of inducing irreversible damage and even the loss of the integrity of lung epithelium[244]. Therefore, the development of novel permeability enhancers with satisfying safety profiles for pulmonary use would be of great importance for respiratory-delivered vaccines. Cyclodextrin can temporarily open the tight junction by depleting the cholesterol on the epithelial cell membrane and subsequently destroying the tight junction integrity and displacing the tight junction proteins[245]. In an earlier study, the researchers conjugated cyclodextrin with PEI 2K and complexed with mRNA. The complexes significantly lowered the level of tight junction protein ZO-1 in *in-vitro* epithelial MDCK cells after treatment for 6 h. The ZO-1 level could return to the normal state and the tight junction morphology might be recovered after 12 h. Therefore, the complexes could overcome the epithelial barrier by reversibly opening their tight junctions and enhancing the paracellular delivery of mRNA in the nasal cavity with good biocompatibility[185].

In addition, some researchers demonstrated that phospholipids such as DPPC and phospholipid hexadecanol tyloxapol[246,247], polysaccharides such as hydroxypropyl β-cyclodextrin[248], as well as fatty acids[249,250] could be used for enhancing macromolecule absorption *via* respiratory route by opening up the tight junctions. However, few have been used for respiratory delivery, which warrants more investigations.

Collectively, the abovementioned strategies have been demonstrated to be promising in enhancing the immune responses of vaccines by improving delivery efficiency. However, there is still a long distance from clinical. The safety concerns about these new materials, the gap between animal models and human physiological and pathological conditions, and the lack of understanding about the interaction between nanoparticles with the environment in the lung are the key issues that hinder the clinical translation of these vaccine delivery systems, which warrants further investigation in future.

5. Other critical considerations in respiratory vaccine formulation development

Delivering the vaccines directly to the respiratory tract can shorten the delivery path and induce mucosal immune response at the first line. However, to successfully translate the vaccines into clinics, some critical issues must be considered, including the deposition site in the lung, the choice of the respiratory delivery device, the stability of the vaccines during storage and aerosolization, the breath patterns design, and the vaccinee variability.

5.1. Respiratory tract deposition

Controlling vaccine deposition sites in the respiratory tract is critical for enhancing vaccine efficiency and requires careful consideration. For liquid vaccine formulations, the deep lungs seem to be ideal for inducing an immune response. Many studies have exhibited that the immune responses of liquid vaccines were more robust with longer antigen residence time and reduced mucociliary clearance when the vaccine was delivered deep into the lungs[251−255]. However, for powder formulation of vaccines, the effects of the deposition site on the intensity of induced immune response seem to be varied from case to case.

The aerodynamic diameter (D_a) of vaccine particles is the primary factor affecting the deposition pattern of the vaccine in the respiratory tract[256,257]. Generally, particles with D_a in the range of 10−20 μm tend to deposit at the posterior NALT instead of the anterior region of the nose, which is ideal for vaccine nasal delivery[256,257]. For pulmonary delivery, particles with D_a larger than 5 μm tend to deposit in the oropharynx by impaction mechanisms and exhaled afterward, while particles with D_a between 1−5 μm would distribute within the respiratory tract by inertial impaction and sedimentation mechanisms and exert immune effects. In addition, particles smaller than 1 μm can reach the alveoli through diffusion and sedimentation mechanisms[258−260] (Fig. 4A and B).

5.2. Respiratory delivery device

Respiratory delivery device is an integral part of respiratory vaccination and play a critical role. Depending on the physiochemical properties of the formulation and targeted area (nasal cavity or the lung), the device should be designed individually and specifically. An optimal device should coordinate with the formulation to generate appropriate aerosols that can deposit at targeted areas.

Although the nose has an extensive mucous membrane surface, the complex anatomy of the nose and the aerodynamics of the particles still limit the delivery efficiency of nasal spray[261]. Metered-dose spray pumps and nebulizers are the most frequently used devices for nasal vaccine delivery. Metered-dose spray pumps could deliver a precise and constant volume of drug solution or suspension with adjustable particle sizes and geometries[262]. The advantages of low manufacturing costs, portability, simplicity in applications, and safety have made the Metered-dose spray pumps dominate the nasal drug delivery market[263]. For vaccines that need precise dosing, long intervals, and limited inoculation time, single-dose metered-dose spray pumps are preferred. For example, a single-dose metered-dose spray pump produced by BD Accuspray™ device (Fig. 4D) is used for the commercial nasal vaccine FluMist®. The device consists of a single-use prefilled syringe to produce large particles with diameters of 50−200 μm, which contributes to decreased vaccine deposition into the lower respiratory tract and minimizes potential adverse effects[261]. Medical nebulizers like vibrating mesh, air-jet, and ultrasonic nebulizers have also been utilized for intranasal administration. OptiNose is a novel bi-directional nasal nebulizer that consists of a flexible mouthpiece and a sealing nosepiece[264]. The device isolates the nose from the mouth and lungs through automatic closure of the soft palate during oral exhalation and helps the vaccine to deposit in high/deep sites in the nasal, which has been tested for COVID-19 vaccine (NCT05035576) and influenza vaccine delivery in clinical trials[265].

For pulmonary delivery of vaccines, the commercially available devices include pressurized metered-dose inhalers (pMDIs), dry powder inhalers (DPIs), and nebulizers[266,267](Fig. 4C−F). Pressurized metered dose inhalers (pMDIs) use pressurized propellant to deliver a fixed dose of aerosol through a nebulizer

nozzle. The canister, metering valve, actuator, and dose counter are the major components of pMDIs (Fig. 4C)[268]. The canister contains a mixture of propellant, drugs, and excipients. Many drugs are not readily soluble in the propellant, which poses a significant challenge in developing pMDI products[269,270]. Besides, the administration of pMDIs requires specific hand-mouth coordination training of the patients, which is complex and labor-consuming. Therefore, despite some studies on the formulations of pMDIs for DNAs and proteins[271−273], there are no pMDIs for respiratory vaccine delivery on the market or in the clinical research stage yet, which suggests the application of pMDIs as respiratory vaccine delivery devices warrants further consideration.

Soft mist inhalers (SMIs) are propellent-free metered-dose inhalers activated by breathing[274,275]. These deliver aqueous formulations through micro holes, forming soft fog for deep lung deposition[274,275]. The aerosol generated with SMIs generally has a high fine particle fraction (65%−80%), a low velocity, and a more sustained duration than that of pMDIs[276,277]. However, current researches with SMIs mainly focus on their application of COPD[278,279]. Its potential for vaccine delivery in the lungs has not been explored yet.

Unlike pMDIs, DPIs are breath-actuated devices, that can be used for the respiratory administration of either single or multi-dose dry powders[280]. So far, the most promising devices for pulmonary powder vaccines are the BD Solovent® and Aktiv-Dry PuffHaler®. BD Solovent® consists of a 5 mL syringe (without needle), vaccine powder reservoir, and mask. At the time of vaccination, the powder was dispersed into the spacer by depressing the syringe plunger, and then inhaled by the vaccinees[80,281] (Fig. 4F). Aktiv-Dry PuffHaler® consists of a squeeze bulb, powder disperser, and collapsed bag reservoir that contains a mouthpiece[282]. The vaccinees pushes the powder from the disperser into the reservoir by squeezing the bulb and opening the valve. In a study with rhesus macaques, a single dose of measles dry powder vaccine inhaled by these two devices induced durable and fully protective immunity[283]. This result has been further confirmed in the clinical phase I trials (NCT01557699), in which the immunogenicity profile and safety of vaccines administered with PuffHaler® or Solovent® were comparable with subcutaneously injected vaccines[80]. Other disposable DPIs also show promise for inhaled vaccines, including SOLO, TwinCaps, and Occoris[284].

Nebulizers like vibrating mesh, surface acoustic wave (SAW), air-jet, and ultrasonic nebulizers are also promising for delivering vaccines. These generate large volumes of inhalable aerosols, where the loaded formulations do not require propellant as in pMDIs or drying processes as in DPIs. Air-jet nebulizers employ high-velocity gas to pass through a venturi nozzle and convert liquids into a mist. The liquid is drawn from the nebulizer reservoir up to a feed tube and emerges as fine filaments that collapse into aerosol droplets[285]. It has been shown that the immunogenicity of the aerosol measles vaccine produced by the air jet nebulizers on macaques is comparable to vaccination by injection[286]. However, the jet nebulizers are usually noisy and hard to carry. In addition, the lung deposition efficiency of jet nebulizers is as low as 10% of the emitted dose[287,288]. Unlike jet nebulizers, ultrasonic nebulizers exploit a piezoelectric crystal vibrating at high frequency to produce a fountain at the liquid-air interface, which forms droplets that can be aerosolized and exit the nebulizer through ventilation[289]. Ultrasonic nebulizers are compact and silent. However, the heat generated by piezoelectric crystals in the device can inactivate the protein-based vaccines[267,286,290,291]. In addition, the particle size produced by the ultrasonic nebulizers is larger than the air-jet nebulizers, which might affect the deposition sites in the lungs[286]. Similar to ultrasonic nebulizers, mesh nebulizers are more portable and efficient than jet nebulizers, but they are less effective with viscous solutions and suspensions due to their ability to clog the pores. Vibrating mesh nebulizers are most widely used in clinical trials of respiratory-delivered vaccines, which generate aerosols through a perforated plate with micrometric apertures vibrating at frequencies around 100 kHz[292]. Convidecia Air™, the first pulmonary administered COVID-19 vaccine, has used a vibrating mesh nebulizer (Fig. 4E)[91,293,294].

5.3. *Stability of vaccine formulation*

As discussed above, the components of vaccines are mainly biological macromolecules that are prone to multiple stresses, including heat, enzyme, and altered pH value in the external environment as well as the shear stress, particle aggregation, and antigen degradation during storage and respiratory delivery process[20,299−301]. Therefore, stabilization strategies to ensure the integrity of vaccines are crucial for translating the products from bench to bed.

5.3.1. *Stability during storage*

Currently, liquid formulations are commonly used for respiratory-delivered vaccines. The strategies for improving the storage stability of liquid formulation include adjusting pH, regulating ionic strength as well as the application of stabilizers[302]. The optimal pH for maintaining the activity of liquid formulations delivered to the respiratory tract is between 5 to 8[303,304]. It was also found that low osmotic pressure and ionic strength ($I < 0.15$) are conducive to the stability of liquid vaccines[302,304]. Amino acids and amphiphilic molecules are the most commonly used stabilizers[305]. The amino acids could aid the refolding and solubilization of proteins and prevent their aggregation and nonspecific adsorption by preferential hydration[281,305,306]. For example, the addition of 1% L-arginine in the formulation of FluMist® could significantly increase the stability of LAIV strains to enable the liquid preparations to be stable at 2−8 °C for one year[307]. Amphiphilic molecules are capable of preventing the aggregation of proteins by preferential adsorbing to the air/water hydrophobic interface, reducing the free energy of the system, and reducing protein-interface and protein-protein interactions[305,308−310]. These factors should be taken into consideration when designing novel liquid respiratory vaccines.

Compared with liquid formulations, dry powders are less vulnerable to the environmental stresses[20,311]. Spray drying (SD), freeze-drying (FD, also known as lyophilization), and spray freeze drying (SFD) are the most commonly employed techniques to afford vaccine-dry powders for inhalation and enhance their storage stability[312,313]. The application of stabilizers could protect antigens or nucleic acid in the formulation of vaccine during the drying process by two major mechanisms, namely hydrogen bond replacement and vitrification in a glassy matrix[314−316]. The hydrogen bond replacement explains the process that during the drying process, the stabilizers would form hydrogen bonds with the antigens or nano-formulations and replace the water molecules which form the bonds with antigens or nano-formulations previously and maintain a stabilized solid conformation similar to the native solvated antigen[317,318]. Vitrification is a process in which the stabilizers form a glassy matrix around the antigens or nano-

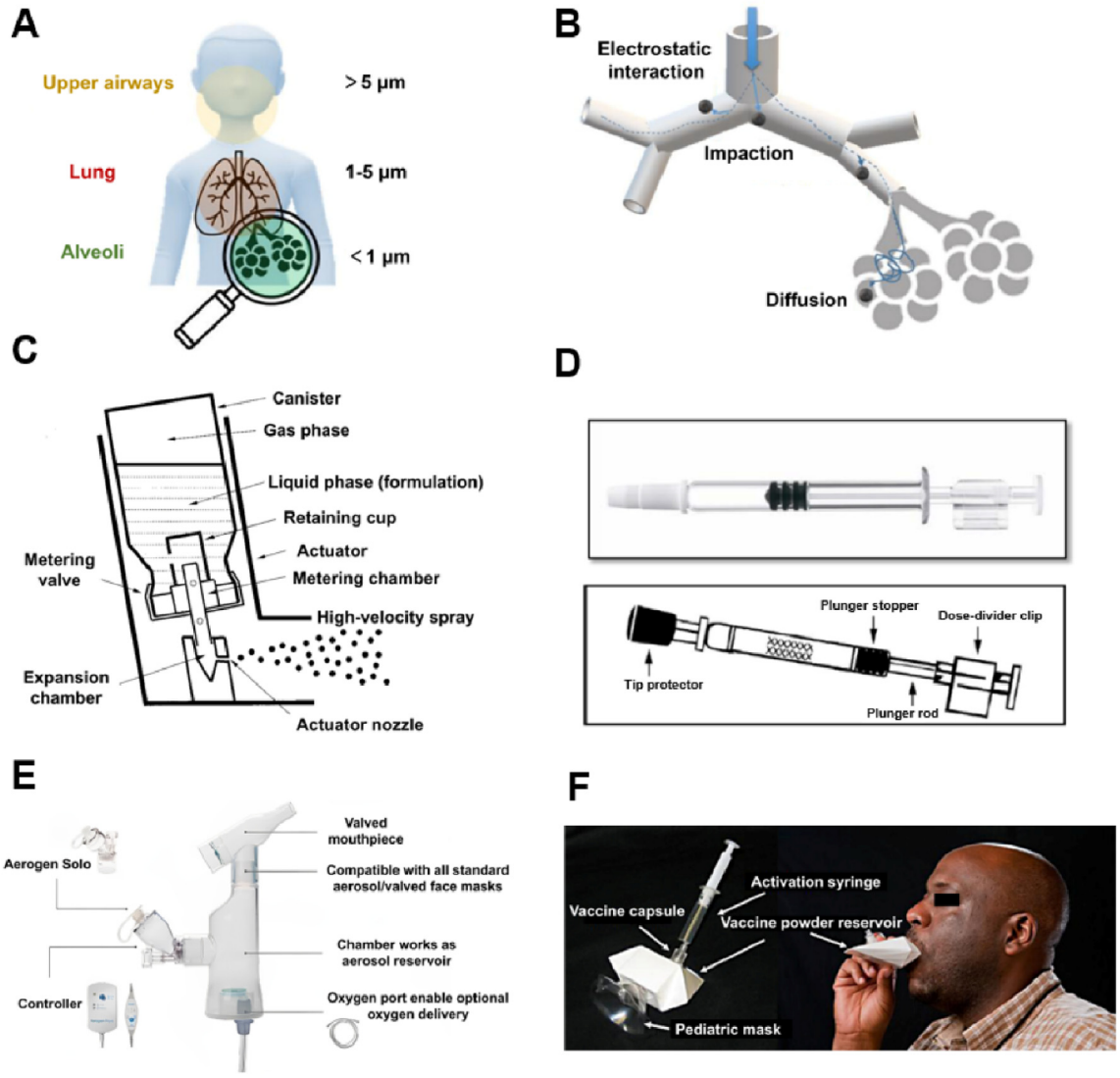

Figure 4 (A) Influence of particle size on lung deposition, (B) Deposition mechanisms, reprinted with the permission from Ref. 295. Copyright © 2022 Elsevier B.V.; (C—F) Some representative devices for medical reagents delivery to the respiratory tract. (C) pMDIs, reprinted with the permission from Ref. 296. Copyright © 2005 Daedalus Enterprises Inc.; (D) Nasal metered-dose spray pump, reprinted with the permission from Ref. 297. Copyright © 2023 Elsevier B.V.; (E) Nebulizer, reproduced with the permission from Ref. 298. Copyright © by Daedalus Enterprises; (F) DPIs, reprinted with the permission from Ref. 80. Copyright © 2014 Elsevier Ltd.

formulations and prevent degradation by inhibiting the molecular movements[319—321]. Given the important role that the stabilizers play in the drying process, the choice of stabilizers is one of the most critical considerations in the dry powder formulation design. In some cases, the combination of two or several stabilizers may have better performance than a single stabilizer. In addition, the addition of other functional excipients such as anti-humidifiers and crystallization inhibitors are also beneficial for formulation optimization[322]. Some examples using combined stabilizer systems for dry powder respiratory vaccines are listed in Table 7 [323-336].

5.3.2. Stability during aerosolization

In addition to storage, aerosolization also brings potential risks to the stability of the vaccine. For liquid vaccines, the shear forces and heat generated during aerosolization would compromise the activities and lead to particle aggregation[337]. Therefore, the delivery devices should be carefully selected to minimize this detriment. For instance, the strong shear force produced by jet nebulizers and rapid temperature rise induced by ultrasound nebulizers would lead to vaccine degradation[338]. In contrast, vibrating mesh nebulizers do not change the solution temperature and generate less shear forces within the drug reservoir, which may be more suitable for vaccine nebulization[98,339—341]. The nanobody vaccine ALX-0171 against RSV, which we have mentioned in Section 3.6, retained purity and potency after being nebulized by a vibrating mesh nebulizer named Akita[2] Apixneb nebulizer[193]. Moreover, the formation of higher-molecular-weight species generated by nebulization was minimal, not exceeding 2%.

The formulation design of delivery vehicles is also important for maintaining vaccine stability and avoiding particle aggregation during aerosolization, especially for mRNA nanocarriers. Including the PEGylated lipid is the most common strategy for enhancing the stability of mRNA-loaded LNPs by minimizing the interactions between particles. For respiratory tract delivery, the

Table 7 Stabilizer systems for solidified respiratory delivered vaccines.

Pathogen	Vaccine type	Drying technology	Prescription composition and function	Key stability findings (stability-indicating assay)	Ref.
Influenza	RVV	SD	Inulin: stabilizers	Stable for 3 years at 20 °C (reversed-phase chromatography, bioassay, particle size, and T*g*)	323
Influenza	WIV	SD	Trehalose dihydrate: stabilizers L-leucine: dispersibility enhancers	Stable for 2 months at 40 °C (particle size, bioassay, antigen content, T*g*)	319
Influenza	WIV	SD	Trehalose dihydrate: stabilizers	Stable for 3 months at 60 °C (particle size, bioassay, T*g*)	322
TB	RVV	SD	Mannitol: anti-humidifier Cyclodextrin and dextran: crystallization inhibitor Trehalose: stabilizers	Stable for 12 months at 25 °C and 5 weeks at 37 °C (bioassay)	324
TB	Subunit	SD	Trehalose: stabilizers Trileucine: dispersibility enhancers	Stable for 7 months at 25 °C and 3 mouths at 40 °C and 50 °C (particle size, moisture content, *in vitro* aerosol performance, antigen content)	325
TB	RVV	SD	Trehalose: stabilizers Mannitol: anti-humidifier Dextran: stabilizers	Stable for least 72 h at 45 °C (particle size, bioassay, thermal aging)	326
TB	Subunit	SD	Trehalose: stabilizers	— (bioassay)	327
TB	LAV	SD	Trehalose: stabilizers Trileucine: dispersibility enhancers Mannitol: anti-humidifier PVP: improve T*g* BSA: resuspend stabilizer	Stable for 24 months at 25 °C (particle size, bioassay, T*g*)	301
Pertussis	Subunit	SD	Trehalose dihydrate: stabilizers	Stable for 4 weeks at 40 °C and 65 °C (bioassay, particle size, electrophoresis, fluorescence spectroscopy)	321
Pneumococcal	Subunit	SD	Lactose: stabilizers Leucine: dispersibility enhancers	— (particle size, bioassay)	328
Influenza	WIV	SFD	Dextran: cryoprotectant Trehalose: stabilizers Inulin: stabilizers	Stable for 3 months at 30 °C (particle size, bioassay, aerosol characterization, electrophoresis)	329,330
Influenza	Subunit	SFD	Inulin: stabilizers	— (particle size, aerosol characterization, bioassay)	320
Influenza	WIV	SFD	Trehalose: stabilizers	Stable for 12 weeks at 25 °C (bioassay)	331
Plague	Subunit	SFD	D-Mannitol: stabilizers Myo-inositol: stabilizers L-Leucine: dispersibility enhancers Poloxamer 188: stabilizers	— (particle size, T*g*, bioassay, electrophoresis, aerodynamic diameter)	332
Anthrax	RVV	SFD	Trehalose: stabilizers Dextran: cryoprotectant	Stable for 15 days at 25 °C, 29 days at 40 °C (electrophoresis, reversed-phase chromatography)	333,334
Botulinum neurotoxin	Subunit	SFD	D-Mannitol: dispersibility enhancers Myo-inositol: stabilizers L-Leucine: dispersibility enhancers Poloxamer 188: stabilizers	— (electrophoresis, bioassay, aerosol characterization)	335
Ricin	Subunit	FD	Trehalose: stabilizer 10 mmol/L histidine-HCl: buffer	Stable for 12 months at 4 °C (reversed-phase chromatography, bioassay, electrophoresis)	336

TB, tuberculosis; SD, spray drying; FD, freeze drying; SFD, spray freeze drying; LAV, live attenuated vaccine; RVV, recombinant viral vector vaccine; WIV, whole inactivated vaccine; −, not applicable.

type and content of PEGylated lipids should be carefully optimized. In a previous study, researchers found that after aerosolization, the particle size of mRNA LNPs using DSPE-PEG as PEGylated lipid increased from 50 nm to 1000 nm. In contrast, LNPs with DMG-PEG or DMPE-PEG exhibited little size change[165]. The reason why different PEGylated lipids have different abilities to maintain the size of LNPs during the aerosolization process warrants further investigation. In addition, the higher molar ratios of PEGylated lipids would make the mRNA LNPs to be more resistant to shear stress. In a related study, more PEGylated lipids in LNPs contributed to the lesser increase of particle size after aerosolization[342]. However, the excessive PEGylated lipids would be detrimental to the mRNA encapsulation efficiency. In addition, the increased PEGylated lipid would inhibit the receptor-mediated endocytosis by reducing the interaction of LNP with the cell membrane, which significantly restricts the intracellular delivery of mRNA[342,342]. Therefore, the successful respiratory delivery of mRNA by LNP aerosolization requires the balancing of the positive effect of PEG content on stability and their negative effect on transfection efficiency.

The maintenance of encapsulation efficiency of LNPs during aerosolization is also crucial for retaining mRNA vaccine stability since the leaking of mRNA would lead to degradation by environmental stresses mentioned above. According to a previous study, the encapsulation efficiencies of the LNP formulations significantly decreased after aerosolization[165]. The LNP formulations with DOPE showed a significantly higher encapsulation efficiency compared to LNP formulations with either DSPC or DPPC. The highest encapsulation efficiency of aerosolized LNPs with DOPE was 79.9%, while the lowest encapsulation efficiency was 15.5%, which was from LNPs with DSPC. The results suggested that the type of phospholipid in the LNPs significantly influenced the encapsulation efficiencies during aerosolization, and the inclusion of DOPE in LNPs would enhance the mRNA vaccine stability during the aerosolization process. Furthermore, the type and content of ionizable lipids should not be overlooked when nebulizing mRNA LNPs. The LNPs with four double-bonds ionizable lipid MC3 exhibited better structural integrity than the LNPs with less constrained ionizable lipid SM-102, with no double bonds against aerosolization. However, the LNPs with SM-102 demonstrated higher mRNA transfection efficiency than the LNPs with MC3, the reason was still not clear[163,343].

Moreover, the mRNA LNPs are more fragile to aerosolization than other kinds of vaccines. For instance, as mentioned above, the nanobody vaccines exhibited excellent stability after being aerosolized by a vibrating mesh nebulizer. However, a related study showed that conventional mRNA LNPs aerosolized by a similar vibrating mesh nebulizer demonstrated increased size, decreased mRNA encapsulation efficiency, and loss of transfection ability[344]. To increase the stability of LNPs during aerosolization, the researchers optimized the components of the nebulizing buffer. The results suggested that a slightly acidic environment (pH 5.2) would improve the aerosolization stability and transfection efficiency of mRNA vaccines. This might be attributed to the protonation of the ionizable lipid, which leads to a reduction in LNP aggregation *via* electrostatic repulsion[344]. In addition, adding hydrophilic polymers such as bPEG20K into the buffer also inhibits LNP aggregation after aerosolization by reducing steric hindrance.

In addition to LNPs, polyplexes with mRNA are also promising in respiratory delivery, as we discussed above. Compared with lipid-based formulations, the mRNA polyplexes have more rigid structures because the high molecular weight and entangled structures of polymer chains would lead to less mobility. Therefore, the polyplexes would provide an attractive method for maintaining mRNA vaccine stability after aerosolization. The mRNA-hPBAE polyplexes, which we have presented in Section 3.5.2, exhibited stable particle size before (137 ± 21 nm) and after (146 ± 40 nm) aerosolization[167]. In addition, the transfection efficiency of mRNA after nebulization to mouse lungs was also retained. In addition, the stability of mRNA polyplexes could be further enhanced through crosslinking[345]. However, the excessive stability of polyplex may also inhibit the intracellular delivery of mRNA, which suggests the maintenance of stability during aerosolization needs limitation[346].

The stability threat of liquid protein vaccines is mainly the air/water hydrophobic interface-induced aggregation. Since the proteins are amphiphilic and surface active, they tend to be adsorbed at the air/water interface. This adsorption would cause conformational changes that expose hydrophobic residues at the interface to avoid contact with water, leading to aggregation and unfolding of protein[347]. The air/water interface effect also exists during storage, as we discussed above. However, this progress could be accelerated by the aerosolization process[337]. Therefore, the type and amounts of amphiphilic molecules for aerosolized protein vaccines should be carefully optimized. In a related study, researchers demonstrated that adding 0.001% polysorbate 20 to the monoclonal antibody formulation reduced the percentage of large aggregates (>2 μm) by almost 6-fold after aerosolization compared with the formulations without polysorbate 20[348], which should be taken into considerations.

For dry powder vaccines, the shear forces generated by the airflow would not be the primary threat to the vaccine stability. However, the strong binding force of DPIs generated by the energy during inhalation would form agglomerates of powders and possess poor aerosol performance without being properly administered to the lungs[349]. The addition of dispersibility enhancers such as leucine and tri leucine would have the potential to improve the aerosol properties of dry powder vaccines[301,325,328,335], which warrants consideration. The details of the application of dispersibility enhancers for respiratory-delivered dry powder vaccines are summarized in Table 7 [323–336].

5.4. *Other considerations*

In addition to the deposition site controlling and delivery technologies optimization, the breathing pattern of vaccines also needs consideration in detail. Although natural breathing pattern varies widely among individuals, breathing patterns are controllable for desired purposes. Deep breathing and then retaining the breath are the most common methods for increasing the tidal volume and prolonging the respiratory retention time within the lungs, respectively[350]. The first world's inhaled COVID-19 vaccine, Convidecia™, takes three steps to breathing patterns: "exhale, inhale, and breath-holding". In general, the first step is to take a deep exhale away from the atomizing cup and then take a deep inhale with the atomizing cup nozzle in the mouth until there is no fog in the cup. Finally, hold your breath for at least 5 s to ensure successful inoculation[91]. The study on immune persistence indicated that the high level of neutralizing antibody was still maintained after 6 months of inoculation with inhaled Convidecia™[351].

We should also take into consideration that the lung function of vaccinees would significantly affect the effectiveness of respiratory vaccines. Respiratory diseases such as asthma and chronic obstructive pulmonary disease would obstruct the airways and

increase the risk of overdose[352−354]. The respiratory vaccines need to overcome this challenge and provide sufficient immune responses across different vaccinee populations safely. The fate and safety profiles of respiratory vaccines after administration are also critical for dosage regimen optimization to acquire strong and long-lasting immune responses with minimal side effects. Collectively, achieving optimized immune responses in the respiratory tract should consider all the abovementioned factors, which is a complex process that requires multidisciplinary collaboration.

6. Current challenges and future perspectives

Despite a lot of attention that has been paid and rapid progress achieved in the field of respiratory-delivered vaccines as we discussed above in recent years, only a few products have entered different stages of clinical trial and few of them could successfully be launched in the market. In our opinion, there are several reasons for this dilemma. Firstly, the complicated microenvironment in the respiratory tract could potentially compromise the efficacy of vaccines. To overcome these barriers, elegant and rational formulation design specifically for respiratory delivery is essential, but most of the formulations tested are just simply taken from the parental formulations without further modification. Secondly, the unsatisfactory predictive ability of *in vivo* animal models used in preclinical studies also contributed to the failure of some clinical trials. Thirdly, in most current preclinical studies, some well-studied parenteral adjuvants were frequently used, but the research mainly focused on evaluating their mucosal adjuvanticity, while their respiratory safety and mucosal adjuvant mechanisms were rarely evaluated. Moreover, the lack of fundamental exploration of the fate of vaccines in the lung and how the respiratory-delivered vaccines stimulate mucosal and systemic immune responses has also severely restricted the progress of respiratory-delivered vaccines. Last but not least, nanomedicine is emerging as a powerful tool to overcome the barriers to vaccine delivery in the respiratory tract. However, the safety of these novel nanomaterials for lung tissue is still a big concern. To sum up, formulation design, the selection of animal models, and the safety of new adjuvants and nanomaterials are the key issues that have to be addressed to make widespread market access for respiratory vaccines become a reality in the near future.

7. Conclusions

Respiratory delivery of vaccines is a relatively new approach to the prevention/treatment of pulmonary diseases. Respiratory delivery of vaccines can induce specific and strong mucosal immune reactions and systemic immunity. Several commercially available vaccines have exhibited the potential of delivery through the respiratory route. However, the complicated environment within the respiratory tract and the unfriendly properties of vaccines make it challenging to acquire satisfying effects. To overcome these barriers, a formulation with the capacity to protect the vulnerable macromolecules of vaccines, enabling APC-specific delivery and promoting respiratory mucus penetration is highly desirable. In addition, the proper deposition within the respiratory tract should be achieved by the optimization of formulations and delivery devices. Although respiratory-delivered vaccines are currently booming, the rational formulation design is still lagging. Thus, it would be beneficial for researchers to fully understand the principles of respiratory formulations and focus on optimizing the formulation engineering strategies, which might facilitate the development of more advanced and promising vaccines and expedite their translation to the clinic.

Acknowledgments

This work was financially supported by the Liaoning Pan Deng Xue Zhe Scholar (No. XLYC2002061, China), the National Natural Science Foundation of China (No. 82173768), and the Overseas Expertise Introduction Project for Discipline Innovation ("111 Project") (No. D20029, China). D.C. acknowledges financial support from the Science and Technology Foundation of Liaoning Province (NO. 2022-MS-241, China), and the Ministry of Education Chunhui Program (2020). L.W. acknowledges the financial support from the National Natural Science Foundation of China (No. 82204316) and the China Postdoctoral Science Foundation (No. 2021TQ0219 and 2022MD713776, China).

Author contributions

Lan Wu and Wenwen Xu drafted the manuscript. Lan Wu prepared the figures in the manuscript. Lan Wu, Wenwen Xu, and Huiyang Jiang collected the information. Mingshi Yang and Dongmei Cun edited the manuscript and supervised the project. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest in this work.

References

1. World Health Organization. Vaccine and immunization. Available from: https://www.who.int/health-topics/vaccines-and-immunization#tab=tab_1.
2. Watson OJ, Barnsley G, Toor J, Hogan AB, Winskill P, Ghani AC. Global impact of the first year of COVID-19 vaccination: a mathematical modelling study. *Lancet Infect Dis* 2022;**22**:1293−302.
3. The United Nations. WHO launches council to develop TB vaccines, hoping to save millions of lives. Available from: https://news.un.org/en/story/2023/01/1132552.
4. Ozawa S, Clark S, Portnoy A, Grewal S, Brenzel L, Walker DG. Return on investment from childhood immunization in low- and middle-income countries, 2011-20. *Health Aff* 2016;**35**:199−207.
5. Melero I, Gaudernack G, Gerritsen W, Huber C, Parmiani G, Scholl S, et al. Therapeutic vaccines for cancer: an overview of clinical trials. *Nat Rev Clin Oncol* 2014;**11**:509−24.
6. Hu ZT, Ott PA, Wu CJ. Towards personalized, tumour-specific, therapeutic vaccines for cancer. *Nat Rev Immunol* 2018;**18**:168−82.
7. Herline K, Drummond E, Wisniewski T. Recent advancements toward therapeutic vaccines against Alzheimer's disease. *Expert Rev Vaccin* 2018;**17**:707−21.
8. Offersgaard A, Bukh J, Gottwein JM. Toward a vaccine against hepatitis C virus. *Science* 2023;**380**:37−8.
9. Huang MW, Zhang MM, Zhu HB, Du XJ, Wang J. Mucosal vaccine delivery: a focus on the breakthrough of specific barriers. *Acta Pharm Sin B* 2022;**12**:3456−74.
10. Masjedi M, Montahaei T, Sharafi Z, Jalali A. Pulmonary vaccine delivery: an emerging strategy for vaccination and immunotherapy. *J Drug Deliv Sci Tec* 2022;**69**:103184.
11. Costa CP, Moreira JN, Sousa Lobo JM, Silva AC. Intranasal delivery of nanostructured lipid carriers, solid lipid nanoparticles and

nanoemulsions: a current overview of *in vivo* studies. *Acta Pharm Sin B* 2021;**11**:925−40.
12. Hamid O, Ismail R, Puzanov I. Intratumoral immunotherapy-update 2019. *Oncologist* 2020;**25**:423−38.
13. Wang N, Wei CL, Zhang Z, Liu T, Wang T. Aluminum nanoparticles acting as a pulmonary vaccine adjuvant-delivery system (VADS) able to safely elicit robust systemic and mucosal immunity. *J Inorg Organomet Polym Mater* 2020;**30**:4203−17.
14. Fan YC, Sahdev P, Ochyl LJ, Akerberg J, Moon JJ. Cationic liposome-hyaluronic acid hybrid nanoparticles for intranasal vaccination with subunit antigens. *J Control Release* 2015;**208**:121−9.
15. Lindsay KE, Vanover D, Thoresen M, King H, Xiao P, Badial P, et al. Aerosol delivery of synthetic mRNA to vaginal mucosa leads to durable expression of broadly neutralizing antibodies against HIV. *Mol Ther* 2020;**28**:805−19.
16. Tu B, Gao YR, An XR, Wang HY, Huang YZ. Localized delivery of nanomedicine and antibodies for combating COVID-19. *Acta Pharm Sin B* 2023;**13**:1828−46.
17. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. *Nat Rev Immunol* 2006;**6**:148−58.
18. van Riet E, Ainai A, Suzuki T, Kersten G, Hasegawa H. Combatting infectious diseases; nanotechnology as a platform for rational vaccine design. *Adv Drug Deliv Rev* 2014;**74**:28−34.
19. Muralidharan P, Mallory E, Malapit M, Hayes DJ, Mansour HM. Inhalable PEGylated phospholipid nanocarriers and PEGylated therapeutics for respiratory delivery as aerosolized colloidal dispersions and dry powder inhalers. *Pharmaceutics* 2014;**6**:333−53.
20. Dumpa N, Goel K, Guo Y, McFall H, Pillai AR, Shukla A, et al. Stability of vaccines. *AAPS PharmSciTech* 2019;**20**:42.
21. Randall TD. Structure, organization, and development of the mucosal immune system of the respiratory tract. *Mucosal Immunol* 2015: 43−61.
22. Du GS, Qin M, Sun X. Recent progress in application of nanovaccines for enhancing mucosal immune responses. *Acta Pharm Sin B* 2023;**13**:2334−45.
23. Debertin AS, Tschernig T, Tönjes H, Kleemann WJ, Tröger HD, Pabst R. Nasal-associated lymphoid tissue (NALT): frequency and localization in young children. *Clin Exp Immunol* 2003;**134**:503−7.
24. Shakya AK, Chowdhury MYE, Tao WQ, Gill HS. Mucosal vaccine delivery: current state and a pediatric perspective. *J Control Release* 2016;**240**:394−413.
25. Cesta MF. Normal structure, function, and histology of mucosa-associated lymphoid tissue. *Toxicol Pathol* 2006;**34**:599−608.
26. Heida R, Hinrichs WL, Frijlink HW. Inhaled vaccine delivery in the combat against respiratory viruses: a 2021 overview of recent developments and implications for COVID-19. *Expert Rev Vaccin* 2022;**21**:957−74.
27. Bi QJ, Song X, Zhao YY, Hu XY, Yang H, Jin RR, et al. Mucus-penetrating nonviral gene vaccine processed in the epithelium for inducing advanced vaginal mucosal immune responses. *Acta Pharm Sin B* 2023;**13**:1287−302.
28. Chiu C, Openshaw PJ. Antiviral B cell and T cell immunity in the lungs. *Nat Immunol* 2015;**16**:18.
29. He B, Xu WF, Santini PA, Polydorides AD, Chiu A, Estrella J, et al. Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. *Immunity* 2007;**26**:812−26.
30. Hardenberg G, van Bostelen L, Hahne M, Medema JP. Thymus-independent class switch recombination is affected by APRIL. *Immunol Cel Biol* 2008;**86**:530−4.
31. Berland R, Wortis HH. Origins and functions of B-1 cells with notes on the role of CD5. *Annu Rev Immunol* 2002;**20**:253−300.
32. Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 2004;**303**: 1662−5.
33. Wijburg OLC, Uren TK, Simpfendorfer K, Johansen FE, Brandtzaeg P, Strugnell RA. Innate secretory antibodies protect against natural *Salmonella typhimurium* infection. *J Exp Med* 2006;**203**:21−6.
34. Sánchez Montalvo A, Gohy S, Rombaux P, Pilette C, Hox V. The role of IgA in chronic upper airway disease: friend or foe? *Front Allergy* 2022;**3**:852546.
35. Tang J, Cai L, Xu CF, Sun S, Liu YH, Rosenecker J, et al. Nanotechnologies in delivery of DNA and mRNA vaccines to the nasal and pulmonary mucosa. *Nanomaterials* 2022;**12**:226.
36. Woof JM, Kerr MA. The function of immunoglobulin A in immunity. *J Pathol* 2006;**208**:270−82.
37. Phalipon A, Cardona A, Kraehenbuhl JP, Edelman L, Sansonetti PJ, Corthésy B. Secretory component: a new role in secretory IgA-mediated immune exclusion *in vivo*. *Immunity* 2002;**17**:107−15.
38. Kaetzel CS, Robinson JK, Chintalacharuvu KR, Vaerman JP, Lamm ME. The polymeric immunoglobulin receptor (secretory component) mediates transport of immune complexes across epithelial cells: a local defense function for IgA. *Proc Natl Acad Sci U S A* 1991;**88**:8796−800.
39. Lusuardi M, Capelli A, Di Stefano A, Donner CF. Lung mucosal immunity: immunoglobulin-A revisited. *Eur Respir J* 2002;**19**:785−6.
40. Kurono Y. The mucosal immune system of the upper respiratory tract and recent progress in mucosal vaccines. *Auris Nasus Larynx* 2022; **49**:1−10.
41. Corgnac S, Boutet M, Kfoury M, Naltet C, Mami-Chouaib F. The emerging role of $CD8^+$ tissue resident memory T (T_{RM}) cells in antitumor immunity: a unique functional contribution of the CD103 integrin. *Front Immunol* 2018;**9**:1904.
42. van Der Gracht ETI, Behr FM, Arens R. Functional heterogeneity and therapeutic targeting of tissue-resident memory T cells. *Cells* 2021;**10**:164.
43. Farhood B, Najafi M, Mortezaee K. $CD8^+$ cytotoxic T lymphocytes in cancer immunotherapy: a review. *J Cel Physiol* 2019;**234**:8509−21.
44. Skon CN, Lee JY, Anderson KG, Masopust D, Hogquist KA, Jameson SC. Transcriptional downregulation of S1pr1 is required for the establishment of resident memory $CD8^+$ T cells. *Nat Immunol* 2013;**14**:1285−93.
45. Mackay LK, Rahimpour A, Ma JZ, Collins N, Stock AT, Hafon M-L, et al. The developmental pathway for $CD103^+CD8^+$ tissue-resident memory T cells of skin. *Nat Immunol* 2013;**14**:1294−301.
46. Sandoval F, Terme M, Nizard M, Badoual C, Bureau MF, Freyburger L, et al. Mucosal imprinting of vaccine-induced $CD8^+$ T cells is crucial to inhibit the growth of mucosal tumors. *Sci Transl Med* 2013;**5**:172ra20.
47. Wein AN, McMaster SR, Takamura S, Dunbar PR, Cartwright EK, Hayward SL, et al. CXCR6 regulates localization of tissue-resident memory CD8 T cells to the airways. *J Exp Med* 2019;**216**: 2748−62.
48. McMaster SR, Wilson JJ, Wang H, Kohlmeier JE. Airway-resident memory CD8 T cells provide antigen-specific protection against respiratory virus challenge through rapid IFN-γ production. *J Immunol* 2015;**195**:203−9.
49. Kumar BV, Ma WJ, Miron M, Granot T, Guyer RS, Carpenter DJ, et al. Human tissue-resident memory T cells are defined by core transcriptional and functional signatures in lymphoid and mucosal sites. *Cell Rep* 2017;**20**:2921−34.
50. Pizzolla A, Nguyen THO, Smith JM, Brooks AG, Kedzieska K, Heath WR, et al. Resident memory $CD8^+$ T cells in the upper respiratory tract prevent pulmonary influenza virus infection. *Sci Immunol* 2017;**2**:eaam6970.
51. Wu T, Hu YH, Lee YT, Bouchard KR, Benechet A, Khanna K, et al. Lung-resident memory CD8 T cells (T_{RM}) are indispensable for optimal cross-protection against pulmonary virus infection. *J Leukoc Biol* 2014;**95**:215−24.
52. Jozwik A, Habibi MS, Paras A, Zhu J, Guvenel A, Dhariwal J, et al. RSV-specific airway resident memory $CD8^+$ T cells and differential disease severity after experimental human infection. *Nat Commun* 2015;**6**:10024.
53. Liao MF, Liu Y, Yuan J, Wen YL, Xu G, Zhao JJ, et al. Single-cell landscape of bronchoalveolar immune cells in patients with COVID-19. *Nat Med* 2020;**26**:842−4.

54. Szabo PA, Dogra P, Gray JI, Wells SB, Connors TJ, Weisberg SP, et al. Longitudinal profiling of respiratory and systemic immune responses reveals myeloid cell-driven lung inflammation in severe COVID-19. *Immunity* 2021;**54**:797−814.
55. Amsen D, van Gisbergen KPJM, Hombrink P, van Lier RAW. Tissue-resident memory T cells at the center of immunity to solid tumors. *Nat Immunol* 2018;**19**:538−46.
56. Djenidi F, Adam J, Goubar A, Durgeau A, Meurice G, de Montpréville V, et al. $CD8^+CD103^+$ tumor-infiltrating lymphocytes are tumor-specific tissue-resident memory T cells and a prognostic factor for survival in lung cancer patients. *J Immunol* 2015;**194**: 3475−86.
57. Nizard M, Roussel H, Diniz MO, Karaki S, Tran T, Voron T, et al. Induction of resident memory T cells enhances the efficacy of cancer vaccine. *Nat Commun* 2017;**8**:15221.
58. Joseph J. Harnessing nasal immunity with IgA to prevent respiratory infections. *Immuno* 2022;**2**:571−83.
59. Knight FC, Wilson JT. Engineering vaccines for tissue-resident memory T cells. *Adv Ther* 2021;**4**:2000230.
60. Sanders B, Koldijk M, Schuitemaker H. Inactivated viral vaccines. In: Nunnally BK, Turula VE, Sitrin RD, editors. *Vaccine analysis: strategies, principles, and control*. Berlin: Springer Inc.; 2014. p. 45−80.
61. Delrue I, Verzele D, Madder A, Nauwynck HJ. Inactivated virus vaccines from chemistry to prophylaxis: merits, risks and challenges. *Expert Rev Vaccin* 2012;**11**:695−719.
62. Uittenbogaard JP, Zomer B, Hoogerhout P, Metz B. Reactions of beta-propiolactone with nucleobase analogues, nucleosides, and peptides: implications for the inactivation of viruses. *J Biol Chem* 2011;**286**:36198−214.
63. Sano K, Ainai A, Suzuki T, Hasegawa H. Intranasal inactivated influenza vaccines for the prevention of seasonal influenza epidemics. *Expert Rev Vaccin* 2018;**17**:687−96.
64. van Ginkel FW, Jackson RJ, Yuki Y, McGhee JR. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. *J Immunol* 2000;**165**:4778−82.
65. Mutsch M, Zhou WG, Rhodes P, Bopp M, Chen RT, Linder T, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *New Engl J Med* 2004;**350**:896−903.
66. Taniguchi M, Seino KI, Nakayama T. The NKT cell system: bridging innate and acquired immunity. *Nat Immunol* 2003;**4**:1164−5.
67. Kamijuku H, Nagata Y, Jiang X, Ichinohe T, Tashiro T, Mori K, et al. Mechanism of NKT cell activation by intranasal coadministration of alpha-galactosylceramide, which can induce cross-protection against influenza viruses. *Mucosal Immunol* 2008;**1**:208−18.
68. Baldridge JR, Yorgensen Y, Ward JR, Ulrich JT. Monophosphoryl lipid A enhances mucosal and systemic immunity to vaccine antigens following intranasal administration. *Vaccine* 2000;**18**:2416−25.
69. Hasegawa H, Ichinohe T, Strong P, Watanabe I, Ito S, Tamura SI, et al. Protection against influenza virus infection by intranasal administration of hemagglutinin vaccine with chitin microparticles as an adjuvant. *J Med Virol* 2005;**75**:130−6.
70. Okamura S, Ebina H. Could live attenuated vaccines better control COVID-19? *Vaccine* 2021;**39**:5719−26.
71. Belshe R, Lee MS, Walker RE, Stoddard J, Mendelman PM. Safety, immunogenicity and efficacy of intranasal, live attenuated influenza vaccine. *Expert Rev Vaccin* 2004;**3**:643−54.
72. Mak TW, Saunders ME. Vaccines and clinical immunization. *The Immune Response* 2006:695−749.
73. Carter JN, Curran MP. Live attenuated influenza vaccine (FluMist®; Fluenz™): a review of its use in the prevention of seasonal influenza in children and adults. *Drugs* 2011;**71**:1591−622.
74. Boyce TG, Gruber WC, Coleman-Dockery SD, Sannella EC, Reed GW, Wolff M, et al. Mucosal immune response to trivalent live attenuated intranasal influenza vaccine in children. *Vaccine* 1999;**18**:82−8.
75. Forrest BD, Pride MW, Dunning AJ, Capeding MRZ, Chotpitayasunondh T, Tam JS, et al. Correlation of cellular immune responses with protection against culture-confirmed influenza virus in young children. *Clin Vaccin Immunol* 2008;**15**:1042−53.
76. Scriba TJ, Nemes E. Protection against tuberculosis by mucosal BCG administration. *Nat Med* 2019;**25**:199−201.
77. White AD, Sarfas C, West K, Sibley LS, Wareham AS, Clark S, et al. Evaluation of the immunogenicity of mycobacterium bovis BCG delivered by aerosol to the lungs of macaques. *Clin Vaccin Immunol* 2015;**22**:992−1003.
78. Rosenthal SR, McEnery JT, Raisys N. Aerogenic BCG vaccination against tuberculosis in animal and human subjects. *J Asthma Res* 1968;**5**:309−23.
79. Middlebrook G. Immunological aspects of airborne infection: reactions to inhaled antigens. *Bacteriol Rev* 1961;**25**:331−46.
80. Cape S, Chaudhari A, Vaidya V, Mulay R, Agarkhedkar S, Shermer C, et al. Safety and immunogenicity of dry powder measles vaccine administered by inhalation: a randomized controlled Phase I clinical trial. *Vaccine* 2014;**32**:6791−7.
81. Wang Y, Yang C, Song YT, Coleman JR, Stawowczyk M, Tafrova J, et al. Scalable live-attenuated SARS-CoV-2 vaccine candidate demonstrates preclinical safety and efficacy. *Proc Natl Acad Sci USA* 2021;**118**:e2102775118.
82. Pilicheva B, Boyuklieva R. Can the nasal cavity help tackle COVID-19? *Pharmaceutics* 2021;**13**:1612.
83. Abdoli M, Shafaati M, Ghamsari LK, Abdoli A. Intranasal administration of cold-adapted live-attenuated SARS-CoV-2 candidate vaccine confers protection against SARS-CoV-2. *Virus Res* 2022; **319**:198857.
84. de Vries RD, Rimmelzwaan GF. Viral vector-based influenza vaccines. *Hum Vaccin* 2016;**12**:2881−901.
85. Tatsis N, Ertl HCJ. Adenoviruses as vaccine vectors. *Mol Ther* 2004; **10**:616−29.
86. Buzitskaya Z, Stosman K, Khairullin B, Kassenov M, Nurpeisova A, Abylai Sansyzbay A, et al. A new intranasal influenza vector-based vaccine TB/FLU-04L against tuberculosis: preclinical safety studies. *Drug Res (Stuttg)* 2022;**72**:255−8.
87. Flórido M, Muflihah H, Lin LCW, Xia YJ, Sierro F, Palendira M, et al. Pulmonary immunization with a recombinant influenza A virus vaccine induces lung-resident $CD4^+$ memory T cells that are associated with protection against tuberculosis. *Mucosal Immunol* 2018; **11**:1743−52.
88. Binjawadagi B, Ma YM, Binjawadagi R, Brakel K, Harder O, Peeples M, et al. Mucosal delivery of recombinant vesicular stomatitis virus vectors expressing envelope proteins of respiratory syncytial virus induces protective immunity in cotton rats. *J Virol* 2021; **95**:e02345-20.
89. Russell MS, Thulasi Raman SN, Gravel C, Zhang WY, Pfeifle A, Chen WX, et al. Single immunization of a vaccine vectored by a novel recombinant vaccinia virus affords effective protection against respiratory syncytial virus infection in cotton rats. *Front Immunol* 2021;**12**:747866.
90. Lee JY, Chang J. Recombinant baculovirus-based vaccine expressing M2 protein induces protective $CD8^+$ T-cell immunity against respiratory syncytial virus infection. *J Microbiol* 2017;**55**:900−8.
91. Li JX, Wu SP, Guo XL, Tang R, Huang BY, Chen XQ, et al. Safety and immunogenicity of heterologous boost immunisation with an orally administered aerosolised Ad5-nCoV after two-dose priming with an inactivated SARS-CoV-2 vaccine in Chinese adults: a randomised, open-label, single-centre trial. *Lancet Respir Med* 2022; **10**:739−48.
92. Afkhami S, D'Agostino MR, Zhang A, Stacey HD, Marzok A, Kang A, et al. Respiratory mucosal delivery of next-generation COVID-19 vaccine provides robust protection against both ancestral and variant strains of SARS-CoV-2. *Cell* 2022;**185**:896−915.
93. van Doremalen N, Purushotham JN, Schulz JE, Holbrook MG, Bushmaker T, Carmody A, et al. Intranasal ChAdOx1 nCoV-19/AZD1222 vaccination reduces viral shedding after SARS-CoV-2 D614G challenge in preclinical models. *Sci Transl Med* 2021;**13**: eabh0755.
94. D'Almeida S, Markovic S, Hermann P, Bracht H, Peifer J, Ettrich TJ, et al. Thromboembolism after Astra Zeneca COVID-19 vaccine: not

always PF4- antibody mediated. *Hum Vaccin Immunother* 2023;**19**:2252239.
95. Østergaard SD, Schmidt M, Horváth-Puhó E, Thomsen RW, Sørensen HT. Thromboembolism and the Oxford−AstraZeneca COVID-19 vaccine: side-effect or coincidence? *Lancet* 2021;**397**:1441−3.
96. Mueller B. The U.K. Reports more blood-clotting cases in people who received the Astra Zeneca shot. New York Times. Published [2021 Apr 2]. Accessed [2021 May 1]. Available from: https://www.nytimes.com/2021/04/02/world/uk-astrazeneca-blood-clots.html.
97. ClinicalTrials.gov. NasoVAX in patients with early coronavirus infectious disease 2019 (COVID-19). Available from: https://clinicaltrials.gov/search?cond=NCT04442230.
98. Jeyanathan M, Fritz DK, Afkhami S, Aguirre E, Howie KJ, Zganiacz A, et al. Aerosol delivery, but not intramuscular injection, of adenovirus-vectored tuberculosis vaccine induces respiratory-mucosal immunity in humans. *JCI Insight* 2022;**7**:e155655.
99. Damjanovic D, Khera A, Afkhami S, Lai R, Zganiacz A, Jeyanathan M, et al. Age at *mycobacterium bovis* BCG priming has limited impact on anti-tuberculosis immunity boosted by respiratory mucosal AdHu5Ag85A immunization in a murine model. *PLoS One* 2015;**10**:e0131175.
100. Jeyanathan M, Shao ZQ, Yu XF, Harkness R, Jiang R, Li JQ, et al. AdHu5Ag85A respiratory mucosal boost immunization enhances protection against pulmonary tuberculosis in BCG-primed non-human primates. *PLoS One* 2015;**10**:e0135009.
101. Stylianou E, Griffiths KL, Poyntz HC, Harrington-Kandt R, Dicks MD, Stockdale L, et al. Improvement of BCG protective efficacy with a novel chimpanzee adenovirus and a modified vaccinia Ankara virus both expressing Ag85A. *Vaccine* 2015;**33**:6800−8.
102. ClinicalTrials.gov. Reactogenicity, safety and immunogenicity of a TB/FLU-04L tuberculosis vaccine. Available from: https://clinicaltrials.gov/search?cond=NCT02501421.
103. De Baets S, Schepens B, Sedeyn K, Schotsaert M, Roose K, Bogaert P, et al. Recombinant influenza virus carrying the respiratory syncytial virus (RSV) F85-93 CTL epitope reduces RSV replication in mice. *J Virol* 2013;**87**:3314−23.
104. Taylor G, Thom M, Capone S, Pierantoni A, Guzman E, Herbert R, et al. Efficacy of a virus-vectored vaccine against human and bovine respiratory syncytial virus infections. *Sci Transl Med* 2015;**7**:300ra127.
105. Fooks AR, Jeevarajah D, Lee J, Warnes A, Niewiesk S, ter Meulen V, et al. Oral or parenteral administration of replication-deficient adenoviruses expressing the measles virus haemagglutinin and fusion proteins: protective immune responses in rodents. *J Gen Virol* 1998;**79**:1027−31.
106. Lobanova LM, Baig TT, Tikoo SK, Zakhartchouk AN. Mucosal adenovirus-vectored vaccine for measles. *Vaccine* 2010;**28**:7613−9.
107. Etchart N, Wild F, Kaiserlian D. Mucosal and systemic immune responses to measles virus haemagglutinin in mice immunized with a recombinant vaccinia virus. *J Gen Virol* 1996;**77**:2471−8.
108. Durbin AP, Skiadopoulos MH, McAuliffe JM, Riggs JM, Surman SR, Collins PL, et al. Human parainfluenza virus type 3 (PIV3) expressing the hemagglutinin protein of measles virus provides a potential method for immunization against measles virus and PIV3 in early infancy. *J Virol* 2000;**74**:6821−31.
109. Karron RA, Wright PF, Newman FK, Makhene M, Thompson J, Samorodin R, et al. A live human parainfluenza type 3 virus vaccine is attenuated and immunogenic in healthy infants and children. *J Infect Dis* 1995;**172**:1445−50.
110. Schlereth B, Rose JK, Buonocore L, Ter Meulen V, Niewiesk S. Successful vaccine-induced seroconversion by single-dose immunization in the presence of measles virus-specific maternal antibodies. *J Virol* 2000;**74**:4652−7.
111. Gomez M, McCollum J, Wang H, Ordoubadi M, Jar C, Carrigy NB, et al. Development of a formulation platform for a spray-dried, inhalable tuberculosis vaccine candidate. *Int J Pharm* 2021;**593**:120121.
112. Dodaran MS, Banihashemi SR, Es-Haghi A, Mehrabadi MHF, Nofeli M, Mokarram AR, et al. Immunogenicity and safety of a combined intramuscular/intranasal recombinant spike protein COVID-19 vaccine (RCP) in healthy adults aged 18 to 55 years old: a randomized, double-blind, placebo-controlled, phase I trial. *Vaccines (Basel)* 2023;**11**:455.
113. Heidary M, Kaviar VH, Shirani M, Ghanavati R, Motahar M, Sholeh M, et al. A comprehensive review of the protein subunit vaccines against COVID-19. *Front Microbiol* 2022;**13**:927306.
114. Cossette B, Kelly SH, Collier JH. Intranasal subunit vaccination strategies employing nanomaterials and biomaterials. *ACS Biomater Sci Eng* 2021;**7**:1765−79.
115. Bachmann MF, Jennings GT. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat Rev Immunol* 2010;**10**:787−96.
116. Hou YY, Chen M, Bian Y, Zheng X, Tong RS, Sun X. Advanced subunit vaccine delivery technologies: from vaccine cascade obstacles to design strategies. *Acta Pharm Sin B* 2023;**13**:3321−38.
117. Irvine DJ, Aung A, Silva M. Controlling timing and location in vaccines. *Adv Drug Deliv Rev* 2020;**158**:91−115.
118. van Dis E, Sogi KM, Rae CS, Sivick KE, Surh NH, Leong ML, et al. STING-activating adjuvants elicit a Th17 immune response and protect against *Mycobacterium tuberculosis* infection. *Cel Rep* 2018;**23**:1435−47.
119. Amini Y, Tebianian M, Mosavari N, Fasihi Ramandi M, Ebrahimi SM, Najminejad H, et al. Development of an effective delivery system for intranasal immunization against *Mycobacterium tuberculosis* ESAT-6 antigen. *Artif Cell Nanomed Biotechnol* 2017;**45**:291−6.
120. Dockrell HM, Brahmbhatt S, Robertson BD, Britton S, Fruth U, Gebre N, et al. A postgenomic approach to identification of *Mycobacterium* leprae-specific peptides as T-cell reagents. *Infect Immun* 2000;**68**:5846−55.
121. Koppolu B, Zaharoff DA. The effect of antigen encapsulation in chitosan particles on uptake, activation and presentation by antigen presenting cells. *Biomaterials* 2013;**34**:2359−69.
122. Jabbal-Gill I, Watts P, Smith A. Chitosan-based delivery systems for mucosal vaccines. *Expert Opin Drug Deliv* 2012;**9**:1051−67.
123. Carroll EC, Jin L, Mori A, Muñoz-Wolf N, Oleszycka E, Moran HBT, et al. The vaccine adjuvant chitosan promotes cellular immunity *via* DNA sensor cGAS-STING-dependent induction of type I interferons. *Immunity* 2016;**44**:597−608.
124. Amidi M, Romeijn SG, Verhoef JC, Junginger HE, Bungener L, Huckriede A, et al. N-trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit antigen for intranasal vaccination: biological properties and immunogenicity in a mouse model. *Vaccine* 2007;**25**:144−53.
125. Bal SM, Slütter B, Verheul R, Bouwstra JA, Jiskoot W. Adjuvanted, antigen loaded N-trimethyl chitosan nanoparticles for nasal and intradermal vaccination: adjuvant- and site-dependent immunogenicity in mice. *Eur J Pharm Sci* 2012;**45**:475−81.
126. Prince GA, Mond JJ, Porter DD, Yim KC, Lan SJ, Klinman DM. Immunoprotective activity and safety of a respiratory syncytial virus vaccine: mucosal delivery of fusion glycoprotein with a CpG oligodeoxynucleotide adjuvant. *J Virol* 2003;**77**:13156−60.
127. Garlapati S, Garg R, Brownlie R, Latimer L, Simko E, Hancock REW, et al. Enhanced immune responses and protection by vaccination with respiratory syncytial virus fusion protein formulated with CpG oligodeoxynucleotide and innate defense regulator peptide in polyphosphazene microparticles. *Vaccine* 2012;**30**:5206−14.
128. Simmons CP, Hussell T, Sparer T, Walzl G, Openshaw P, Dougan G. Mucosal delivery of a respiratory syncytial virus CTL peptide with enterotoxin-based adjuvants elicits protective, immunopathogenic, and immunoregulatory antiviral $CD8^+$ T cell responses. *J Immunol* 2001;**166**:1106−13.
129. Cusi MG, Zurbriggen R, Correale P, Valassina M, Terrosi C, Pergola L, et al. Influenza virosomes are an efficient delivery system

for respiratory syncytial virus-F antigen inducing humoral and cell-mediated immunity. *Vaccine* 2002;**20**:3436−42.
130. Gomez M, Ahmed M, Das S, McCollum J, Mellett L, Swanson R, et al. Development and testing of a spray-dried tuberculosis vaccine candidate in a mouse model. *Front Pharmacol* 2022;**12**:799034.
131. Wang BZ, Xu R, Quan FS, Kang SM, Wang L, Compans RW. Intranasal immunization with influenza VLPs incorporating membrane-anchored flagellin induces strong heterosubtypic protection. *PLoS One* 2010;**5**:e13972.
132. Ren ZG, Zhao YK, Liu J, Ji XL, Meng LN, Wang TC, et al. Intramuscular and intranasal immunization with an H7N9 influenza virus-like particle vaccine protects mice against lethal influenza virus challenge. *Int Immunopharmacol* 2018;**58**:109−16.
133. Knight FC, Gilchuk P, Kumar A, Becker KW, Sevimli S, Jacobson ME, et al. Mucosal immunization with a pH-responsive nanoparticle vaccine induces protective $CD8^+$ lung-resident memory T cells. *ACS Nano* 2019;**13**:10939−60.
134. Prabakaran M, Velumani S, He F, Karuppannan AK, Geng GY, Yin LK, et al. Protective immunity against influenza H5N1 virus challenge in mice by intranasal co-administration of baculovirus surface-displayed HA and recombinant CTB as an adjuvant. *Virology* 2008;**380**:412−20.
135. Liu H, Patil HP, de Vries-Idema J, Wilschut J, Huckriede A. Evaluation of mucosal and systemic immune responses elicited by GPI-0100- adjuvanted influenza vaccine delivered by different immunization strategies. *PLoS One* 2013;**8**:e69649.
136. International Clinical Trials Registry Platform. Available from: https://rpcec.sld.cu/en/trials/RPCEC00000345-En.
137. ClinicalTrials.gov. Booster dose study to assess the safety and immunogenicity of ACM-001 administered intramuscularly or intranasally. Available from: https://clinicaltrials.gov/search?cond=NCT05385991.
138. Huang Y, Cyr SL, Burt DS, Anderson R. Murine host responses to respiratory syncytial virus (RSV) following intranasal administration of a Protollin-adjuvanted, epitope-enhanced recombinant G protein vaccine. *J Clin Virol* 2009;**44**:287−91.
139. Shang SB, Kats D, Cao L, Morgun E, Velluto D, He Y, et al. Induction of *mycobacterium tuberculosis* lipid-specific T cell responses by pulmonary delivery of mycolic acid-loaded polymeric micellar nanocarriers. *Front Immunol* 2018;**9**:2709.
140. Nakahashi-Ouchida R, Uchida Y, Yuki Y, Katakai Y, Yamanoue T, Ogawa H, et al. A nanogel-based trivalent PspA nasal vaccine protects macaques from intratracheal challenge with pneumococci. *Vaccine* 2021;**39**:3353−64.
141. Yao WJ, Peng YX, Du MZ, Luo J, Zong L. Preventative vaccine-loaded mannosylated chitosan nanoparticles intended for nasal mucosal delivery enhance immune responses and potent tumor immunity. *Mol Pharm* 2013;**10**:2904−14.
142. Files MA, Naqvi KF, Saito TB, Clover TM, Rudra JS, Endsley JJ. Self-adjuvanting nanovaccines boost lung-resident $CD4^+$ T cell immune responses in BCG-primed mice. *Npj Vaccin* 2022;**7**:48.
143. Sharma S, Benson HAE, Mukkur TKS, Rigby P, Chen Y. Preliminary studies on the development of IgA-loaded chitosan−dextran sulphate nanoparticles as a potential nasal delivery system for protein antigens. *J Microencapsul* 2013;**30**:283−94.
144. Rajapaksa TE, Stover-Hamer M, Fernandez X, Eckelhoefer HA, Lo DD. Claudin 4-targeted protein incorporated into PLGA nanoparticles can mediate M cell targeted delivery. *J Control Release* 2010;**142**:196−205.
145. Pawar D, Mangal S, Goswami R, Jaganathan KS. Development and characterization of surface modified PLGA nanoparticles for nasal vaccine delivery: effect of mucoadhesive coating on antigen uptake and immune adjuvant activity. *Eur J Pharm Biopharm* 2013;**85**:550−9.
146. Hart P, Copland A, Diogo GR, Harris S, Spallek R, Oehlmann W, et al. Nanoparticle-fusion protein complexes protect against *mycobacterium tuberculosis* infection. *Mol Ther* 2018;**26**:822−33.
147. Middaugh CR, Evans RK, Montgomery DL, Casimiro DR. Analysis of plasmid DNA from a pharmaceutical perspective. *J Pharm Sci* 1998;**87**:130−46.
148. Wang DQ, Christopher ME, Nagata LP, Zabielski MA, Li HD, Wong JP, et al. Intranasal immunization with liposome-encapsulated plasmid DNA encoding influenza virus hemagglutinin elicits mucosal, cellular and humoral immune responses. *J Clin Virol* 2004;**31**:99−106.
149. Rosada RS, Torre LGDL, Frantz FG, Trombone AP, Zárate-Bladés CR, Fonseca DM, et al. Protection against tuberculosis by a single intranasal administration of DNA-hsp65 vaccine complexed with cationic liposomes. *BMC Immunol* 2008;**9**:38.
150. Chen CJ, Han DD, Cai CF, Tang X. An overview of liposome lyophilization and its future potential. *J Control Release* 2010;**142**:299−311.
151. Shim BS, Park SM, Quan JS, Jere D, Chu H, Song MK, et al. Intranasal immunization with plasmid DNA encoding spike protein of SARS-coronavirus/polyethylenimine nanoparticles elicits antigen-specific humoral and cellular immune responses. *BMC Immunol* 2010;**11**:65.
152. Mann JFS, McKay PF, Arokiasamy S, Patel RK, Klein K, Shattock RJ. Pulmonary delivery of DNA vaccine constructs using deacylated PEI elicits immune responses and protects against viral challenge infection. *J Control Release* 2013;**170**:452−9.
153. Raghuwanshi D, Mishra V, Das D, Kaur K, Suresh MR. Dendritic cell targeted chitosan nanoparticles for nasal DNA immunization against SARS CoV nucleocapsid protein. *Mol Pharm* 2012;**9**:946−56.
154. Iqbal M, Lin W, Jabbal-Gill I, Davis SS, Steward MW, Illum L. Nasal delivery of chitosan-DNA plasmid expressing epitopes of respiratory syncytial virus (RSV) induces protective CTL responses in BALB/c mice. *Vaccine* 2003;**21**:1478−85.
155. Sorgi FL, Bhattacharya S, Huang L. Protamine sulfate enhances lipid-mediated gene transfer. *Gene Ther* 1997;**4**:961−8.
156. Zeng ZY, Dai S, Jiao Y, Jiang L, Zhao YK, Wang B, et al. Mannosylated protamine as a novel DNA vaccine carrier for effective induction of anti-tumor immune responses. *Int J Pharm* 2016;**506**:394−406.
157. Liu MA. A comparison of plasmid DNA and mRNA as vaccine technologies. *Vaccines (Basel)* 2019;**7**:37.
158. Campbell JD. Development of the CpG adjuvant 1018: a case study. *Methods Mol Biol* 2017;**1494**:15−27.
159. Sahin U, Karikó K, Türeci Ö. mRNA-based therapeutics-developing a new class of drugs. *Nat Rev Drug Discov* 2014;**13**:759−80.
160. Kim J, Eygeris Y, Gupta M, Sahay G. Self-assembled mRNA vaccines. *Adv Drug Deliv Rev* 2021;**170**:83−112.
161. Corbett KS, Edwards DK, Leist SR, Abiona OM, Boyoglu-Barnum S, Gillespie RA, et al. SARS-CoV-2 mRNA vaccine design enabled by prototype pathogen preparedness. *Nature* 2020;**586**:567−71.
162. Skowronski DM, Serres GD. Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine. *N Engl J Med* 2021;**384**:1576−7.
163. Lokugamage MP, Vanover D, Beyersdorf J, Hatit MZC, Rotolo L, Echeverri ES, et al. Optimization of lipid nanoparticles for the delivery of nebulized therapeutic mRNA to the lungs. *Nat Biomed Eng* 2021;**5**:1059−68.
164. Kaczmarek JC, Patel AK, Kauffman KJ, Fenton OS, Webber MJ, Heartlein MW, et al. Polymer-lipid nanoparticles for systemic delivery of mRNA to the lungs. *Angew Chem Int Ed Engl* 2016;**55**:13808−12.
165. Zhang HR, Leal J, Soto MR, Smyth HDC, Ghosh D. Aerosolizable lipid nanoparticles for pulmonary delivery of mRNA through design of experiments. *Pharmaceutics* 2020;**12**:1042.
166. Li M, Li Y, Peng K, Wang Y, Gong T, Zhang ZR, et al. Engineering intranasal mRNA vaccines to enhance lymph node trafficking and immune responses. *Acta Biomater* 2017;**64**:237−48.
167. Patel AK, Kaczmarek JC, Bose S, Kauffman KJ, Mir F, Heartlein MW, et al. Inhaled nanoformulated mRNA polyplexes for protein production in lung epithelium. *Adv Mater* 2019;**31**:1805116.

168. Qiu YS, Man RCH, Liao QY, Kung KLK, Chow MYT, Lam JKW. Effective mRNA pulmonary delivery by dry powder formulation of PEGylated synthetic KL4 peptide. *J Control Release* 2019;**314**:102−15.
169. Feng JY, Johnson AA, Johnson KA, Anderson KS. Insights into the molecular mechanism of mitochondrial toxicity by AIDS drugs. *J Biol Chem* 2001;**276**:23832−7.
170. Moyle G. Toxicity of antiretroviral nucleoside and nucleotide analogues: is mitochondrial toxicity the only mechanism? *Drug Saf* 2000;**23**:467−81.
171. Pepini T, Pulichino AM, Carsillo T, Carlson AL, Sari-Sarraf F, Ramsauer K, et al. Induction of an IFN-mediated antiviral response by a self-amplifying RNA vaccine: implications for vaccine design. *J Immunol* 2017;**198**:4012−24.
172. Theofilopoulos AN, Baccala R, Beutler B, Kono DH. Type I interferons (alpha/beta) in immunity and autoimmunity. *Annu Rev Immunol* 2005;**23**:307−36.
173. Anderluzzi G, Lou G, Woods S, Schmidt ST, Gallorini S, Brazzoli M, et al. The role of nanoparticle format and route of administration on self-amplifying mRNA vaccine potency. *J Control Release* 2022;**342**:388−99.
174. Vogel AB, Lambert L, Kinnear E, Busse D, Erbar S, Reuter KC, et al. Self-amplifying RNA vaccines give equivalent protection against influenza to mRNA vaccines but at much lower doses. *Mol Ther* 2018;**26**:446−55.
175. Blakney AK, McKay PF, Hu K, Samnuan K, Jain N, Brown A, et al. Polymeric and lipid nanoparticles for delivery of self-amplifying RNA vaccines. *J Control Release* 2021;**338**:201−10.
176. Künzli M, O'Flanagan SD, LaRue M, Talukder P, Dileepan T, Stolley JM, et al. Route of self-amplifying mRNA vaccination modulates the establishment of pulmonary resident memory CD8 and CD4 T cells. *Sci Immunol* 2022;**7**:eadd3075.
177. Khatri K, Goyal AK, Gupta PN, Mishra N, Mehta A, Vyas SP. Surface modified liposomes for nasal delivery of DNA vaccine. *Vaccine* 2008;**26**:2225−33.
178. D'Souza S, Rosseels V, Denis O, Tanghe A, De Smet N, Jurion F, et al. Improved tuberculosis DNA vaccines by formulation in cationic lipids. *Infect Immun* 2002;**70**:3681−8.
179. Torrieri-Dramard L, Lambrecht B, Ferreira HL, Van den Berg T, Klatzmann D, Bellier B. Intranasal DNA vaccination induces potent mucosal and systemic immune responses and cross-protective immunity against influenza viruses. *Mol Ther* 2011;**19**:602−11.
180. Bivas-Benita M, Bar L, Gillard GO, Kaufman DR, Simmons NL, Hovav AH, et al. Efficient generation of mucosal and systemic antigen-specific $CD8^+$ T-cell responses following pulmonary DNA immunization. *J Virol* 2010;**84**:5764−74.
181. Bivas-Benita M, Gillard GO, Bar L, White KA, Webby RJ, Hovav AH, et al. Airway $CD8^+$ T cells induced by pulmonary DNA immunization mediate protective anti-viral immunity. *Mucosal Immunol* 2013;**6**:156−66.
182. Wu ML, Zhao HX, Li M, Yue Y, Xiong SD, Xu W. Intranasal vaccination with mannosylated chitosan formulated DNA vaccine enables robust IgA and cellular response induction in the lungs of mice and improves protection against pulmonary mycobacterial challenge. *Front Cel Infect Microbiol* 2017;**7**:445.
183. Kumar US, Afjei R, Ferrara K, Massoud TF, Paulmurugan R. Gold-nanostar-chitosan-mediated delivery of SARS-CoV-2 DNA vaccine for respiratory mucosal immunization: development and proof-of-principle. *ACS Nano* 2021;**15**:17582−601.
184. Zhuang XY, Qi YX, Wang MP, Yu N, Nan FL, Zhang H, et al. mRNA vaccines encoding the HA protein of influenza A H1N1 virus delivered by cationic lipid nanoparticles induce protective immune responses in mice. *Vaccines(Basel)* 2020;**8**:123.
185. Li M, Zhao MG, Fu Y, Li Y, Gong T, Zhang ZR, et al. Enhanced intranasal delivery of mRNA vaccine by overcoming the nasal epithelial barrier *via* intra- and paracellular pathways. *J Control Release* 2016;**228**:9−19.
186. Hajam IA, Senevirathne A, Hewawaduge C, Kim J, Lee JH. Intranasally administered protein coated chitosan nanoparticles encapsulating influenza H9N2 HA2 and M2e mRNA molecules elicit protective immunity against avian influenza viruses in chickens. *Vet Res* 2020;**51**:37.
187. Mai YP, Guo JS, Zhao Y, Ma SJ, Hou YH, Yang JH. Intranasal delivery of cationic liposome-protamine complex mRNA vaccine elicits effective anti-tumor immunity. *Cell Immunol* 2020;**354**:104143.
188. ClinicalTrials.gov. iHIVARNA Clinical Trial in HIV Infected Individuals (iHIVARNA-01). Available from: https://clinicaltrials.gov/search?cond=nCT02888756.
189. Li JQ, Zhang ZR, Zhang HQ, Zhang YN, Zeng XY, Zhang QY, et al. Intranasal delivery of replicating mRNA encoding neutralizing antibody against SARS-CoV-2 infection in mice. *Signal Transduct Target Ther* 2021;**6**:369.
190. Valenzuela-Nieto G, Miranda-Chacon Z, Salinas-Rebolledo C, Jara R, Cuevas A, Berking A, et al. Nanobodies: COVID-19 and future perspectives. *Front Drug Discov* 2022;**2**:927164.
191. Mei YX, Chen YZ, Sivaccumar JP, An ZQ, Xia NS, Luo WX. Research progress and applications of nanobody in human infectious diseases. *Front Pharmacol* 2022;**13**:963978.
192. Aksu M, Kumar P, Güttler T, Taxer W, Gregor K, Mußil B, et al. Nanobodies to multiple spike variants and inhalation of nanobody-containing aerosols neutralize SARS-CoV-2 in cell culture and hamsters. *Antivir Res* 2024;**221**:105778.
193. Detalle L, Stohr T, Palomo C, Piedra PA, Gilbert BE, Mas V, et al. Generation and characterization of ALX-0171, a potent novel therapeutic nanobody for the treatment of respiratory syncytial virus infection. *Antimicrob Agents Ch* 2016;**60**:6−13.
194. Esparza TJ, Chen YZ, Martin NP, Bielefeldt-Ohmann H, Bowen RA, Tolbert WD, et al. Nebulized delivery of a broadly neutralizing SARS-CoV-2 RBD-specific nanobody prevents clinical, virological, and pathological disease in a Syrian hamster model of COVID-19. *mAbs* 2022;**14**:2047144.
195. Li C, Zhan WQ, Yang ZL, Tu C, Hu GW, Zhang X, et al. Broad neutralization of SARS-CoV-2 variants by an inhalable bispecific single-domain antibody. *Cell* 2022;**185**:1389−401.
196. Wu XL, Cheng L, Fu M, Huang BL, Zhu LJ, Xu SJ, et al. A potent bispecific nanobody protects hACE2 mice against SARS-CoV-2 infection *via* intranasal administration. *Cel Rep* 2021;**37**:109869.
197. Huo JD, Mikolajek H, Le Bas A, Clark JJ, Sharma P, Kipar A, et al. A potent SARS-CoV-2 neutralising nanobody shows therapeutic efficacy in the Syrian golden hamster model of COVID-19. *Nat Commun* 2021;**12**:5469.
198. Nagata K, Utsumi D, Asaka MN, Maeda R, Shirakawa K, Kazuma Y, et al. Intratracheal trimerized nanobody cocktail administration suppresses weight loss and prolongs survival of SARS-CoV-2 infected mice. *Commun Med* 2022;**2**:152.
199. Nambulli S, Xiang YF, Tilston-Lunel NL, Rennick LJ, Sang Z, Klimstra WB, et al. Inhalable nanobody (PiN-21) prevents and treats SARS-CoV-2 infections in Syrian hamsters at ultra-low doses. *Sci Adv* 2021;**7**:eabh0319.
200. Ruge CA, Kirch J, Lehr CM. Pulmonary drug delivery: from generating aerosols to overcoming biological barriers-therapeutic possibilities and technological challenges. *Lancet Respir Med* 2013;**1**:402−13.
201. Olsson B, Bondesson E, Borgström L, Edsbäcker S, Eirefelt S, Ekelund K, et al. Pulmonary drug metabolism, clearance, and absorption. In: Smyth HDC, Hickey AJ, editors. *Controlled pulmonary drug delivery*. New York: Springer Inc.; 2011. p. 21−50.
202. Munkholm M, Mortensen J. Mucociliary clearance: pathophysiological aspects. *Clin Physiol Funct* 2014;**34**:171−7.
203. Stone KC, Mercer RR, Gehr P, Stockstill B, Crapo JD. Allometric relationships of cell numbers and size in the mammalian lung. *Am J Respir Cel Mol Biol* 1992;**6**:235−43.
204. Ding L, Tang SY, Wyatt TA, Knoell DL, Oupický D. Pulmonary siRNA delivery for lung disease: review of recent progress and challenges. *J Control Release* 2021;**330**:977−91.

205. Wang CC, Muttil P, Lu DM, Beltran-Torres AA, Garcia-Contreras L, Hickey AJ. Screening for potential adjuvants administered by the pulmonary route for tuberculosis vaccines. *AAPS J* 2009;**11**:139−47.
206. Blank F, Stumbles PA, Seydoux E, Holt PG, Fink A, Rothen-Rutishauser B, et al. Size-dependent uptake of particles by pulmonary antigen-presenting cell populations and trafficking to regional lymph nodes. *Am J Respir Cel Mol Biol* 2013;**49**:67−77.
207. Fromen CA, Rahhal TB, Robbins GR, Kai MP, Shen TW, Luft JC, et al. Nanoparticle surface charge impacts distribution, uptake and lymph node trafficking by pulmonary antigen-presenting cells. *Nanomedicine* 2016;**12**:677−87.
208. Rose CEJ, Sung SSJ, Fu SM. Significant involvement of CCL2 (MCP-1) in inflammatory disorders of the lung. *Microcirculation* 2003;**10**:273−88.
209. Pesce I, Monaci E, Muzzi A, Tritto E, Tavarini S, Nuti S, et al. Intranasal administration of CpG induces a rapid and transient cytokine response followed by dendritic and natural killer cell activation and recruitment in the mouse lung. *J Innate Immun* 2010;**2**:144−59.
210. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res* 2009;**29**:313−26.
211. Suzuki Y, Suda T, Furuhashi K, Shibata K, Hashimoto D, Enomto N, et al. Mouse CD11b high lung dendritic cells have more potent capability to induce IgA than $CD103^{+}$ lung dendritic cells *in vitro*. *Am J Respir Cel Mol Biol* 2012;**46**:773−80.
212. Chen PM, Zhang XP, Jia L, Prud'homme RK, Szekely Z, Sinko PJ. Optimal structural design of mannosylated nanocarriers for macrophage targeting. *J Control Release* 2014;**194**:341−9.
213. van Vliet SJ, Saeland E, van Kooyk Y. Sweet preferences of MGL: carbohydrate specificity and function. *Trends Immunol* 2008;**29**:83−90.
214. Wang HW, Jiang PL, Lin SF, Lin HJ, Ou KL, Deng WP, et al. Application of galactose-modified liposomes as a potent antigen presenting cell targeted carrier for intranasal immunization. *Acta Biomater* 2013;**9**:5681−8.
215. Crespo HJ, Lau JTY, Videira PA. Dendritic cells: a spot on sialic acid. *Front Immunol* 2013;**4**:491.
216. Lou B, De Koker S, Lau CYJ, Hennink WE, Mastrobattista E. mRNA polyplexes with post-conjugated GALA peptides efficiently target, transfect, and activate antigen presenting cells. *Bioconjug Chem* 2019;**30**:461−75.
217. Lai SK, Wang YY, Hanes J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. *Adv Drug Deliv Rev* 2009;**61**:158−71.
218. Norris DA, Sinko PJ. Effect of size, surface charge, and hydrophobicity on the translocation of polystyrene microspheres through gastrointestinal mucin. *J Appl Polym Sci* 1997;**63**:1481−92.
219. Khutoryanskiy VV. Beyond PEGylation: alternative surface-modification of nanoparticles with mucus-inert biomaterials. *Adv Drug Deliv Rev* 2018;**124**:140−9.
220. Li JQ, Zheng HL, Xu EY, Moehwald M, Chen L, Zhang X, et al. Inhalable PLGA microspheres: tunable lung retention and systemic exposure *via* polyethylene glycol modification. *Acta Biomater* 2021;**123**:325−34.
221. Sanders NN, De Smedt SC, Demeester J. Mobility and stability of gene complexes in biogels. *J Control Release* 2003;**87**:117−29.
222. Sanders NN, De Smedt SC, Cheng SH, Demeester J. Pegylated GL67 lipoplexes retain their gene transfection activity after exposure to components of CF mucus. *Gene Ther* 2002;**9**:363−71.
223. Yang Q, Lai SK. Anti-PEG immunity: emergence, characteristics, and unaddressed questions. *Wires Nanomed Nanobi* 2015;**7**:655−77.
224. Li BW, Yuan ZF, Hung HC, Ma JR, Jain PY, Tsao C, et al. Revealing the immunogenic risk of polymers. *Angew Chem Int Ed Engl* 2018;**57**:13873−6.
225. Li BW, Jain P, Ma JR Y, Smith JK, Yuan ZF, Hung HC, et al. Trimethylamine N-oxide-derived zwitterionic polymers: a new class of ultralow fouling bioinspired materials. *Sci Adv* 2019;**5**:eaaw9562.
226. Hoogenboom R. Poly(2-oxazoline)s: a polymer class with numerous potential applications. *Angew Chem Int Ed Engl* 2009;**48**:7978−94.
227. Sedlacek O, Monnery BD, Filippov SK, Hoogenboom R, Hruby M. Poly(2-oxazoline)s-are they more advantageous for biomedical applications than other polymers? *Macromol Rapid Commun* 2012;**33**:1648−62.
228. Viegas TX, Bentley MD, Harris JM, Fang ZH, Yoon K, Dizman B, et al. Polyoxazoline: chemistry, properties, and applications in drug delivery. *Bioconjug Chem* 2011;**22**:976−86.
229. Kopecek J, Kopecková P. HPMA copolymers: origins, early developments, present, and future. *Adv Drug Deliv Rev* 2010;**62**:122−49.
230. Tucker BS, Sumerlin BS. Poly(*N*-(2-hydroxypropyl) methacrylamide)-based nanotherapeutics. *Polym Chem* 2014;**5**:1566−72.
231. Talelli M, Rijcken CJF, van Nostrum CF, Storm G, Hennink WE. Micelles based on HPMA copolymers. *Adv Drug Deliv Rev* 2010;**62**:231−9.
232. Du N, Guo WX, Yu QS, Guan SL, Guo LY, Shen T, et al. Poly(D,L-lacticacid)-block-poly(*N*-(2-hydroxypropyl)methacrylamide) nanoparticles for overcoming accelerated blood clearance and achieving efficient anti-tumor therapy. *Polym Chem* 2016;**7**:5719−29.
233. Jiang SY, Cao ZQ. Ultralow-fouling, functionalizable, and hydrolyzable zwitterionic materials and their derivatives for biological applications. *Adv Mater* 2010;**22**:920−32.
234. Shao Q, White AD, Jiang SY. Difference of carboxybetaine and oligo(ethylene glycol) moieties in altering hydrophobic interactions: a molecular simulation study. *J Phys Chem B* 2014;**118**:189−94.
235. Yang Y, Tsifansky MD, Shin S, Lin QG, Yeo Y. Mannitol-guided delivery of Ciprofloxacin in artificial cystic fibrosis mucus model. *Biotechnol Bioeng* 2011;**108**:1441−9.
236. Yang JD, Duan SZ, Ye H, Ge CG, Piao CX, Chen YB, et al. Pro-eptide-reinforced, mucus-penetrating pulmonary siRNA delivery mitigates cytokine storm in pneumonia. *Adv Funct Mater* 2021:2008960.
237. Liu QY, Xue JW, Zhang XR, Chai JJ, Qin L, Guan J, et al. The influence of a biomimetic pulmonary surfactant modification on the *in vivo* fate of nanoparticles in the lung. *Acta Biomater* 2022;**147**:391−402.
238. Henke MO, Ratjen F. Mucolytics in cystic fibrosis. *Paediatr Respir Rev* 2007;**8**:24−9.
239. Gallon AM. Evaluation of nebulised acetylcysteine and normal saline in the treatment of sputum retention following thoracotomy. *Thorax* 1996;**51**:429−32.
240. Sheffner AL, Medler EM, Jacobs LW, Sarett HP. The *in vitro* reduction in viscosity of human tracheobronchial secretions by acetylcysteine. *Am Rev Respir Dis* 1964;**90**:721−9.
241. Suk JS, Lai SK, Boylan NJ, Dawson MR, Boyle MP, Hanes J. Rapid transport of muco-inert nanoparticles in cystic fibrosis sputum treated with *N*-acetyl cysteine. *Nanomedicine (Lond)* 2011;**6**:365−75.
242. Ferrari S, Kitson C, Farley R, Steel R, Marriott C, Parkins DA, et al. Mucus altering agents as adjuncts for nonviral gene transfer to airway epithelium. *Gene Ther* 2001;**8**:1380−6.
243. Sou T, Meeusen EN, de Veer M, Morton DAV, Kaminskas LM, McIntosh MP. New developments in dry powder pulmonary vaccine delivery. *Trends Biotechnol* 2011;**29**:191−8.
244. Weers JG, Tarara TE, Clark AR. Design of fine particles for pulmonary drug delivery. *Expert Opin Drug Deliv* 2007;**4**:297−313.
245. Deli MA. Potential use of tight junction modulators to reversibly open membranous barriers and improve drug delivery. *Biochim Biophys Acta* 2009;**1788**:892−910.
246. Hoyer H, Perera G, Bernkop-Schnürch A. Noninvasive delivery systems for peptides and proteins in osteoporosis therapy: a retro-perspective. *Drug Dev Ind Pharm* 2010;**36**:31−44.

247. Zheng JH, Zheng Y, Chen JY, Fang F, He JK, Li N, et al. Enhanced pulmonary absorption of recombinant human insulin by pulmonary surfactant and phospholipid hexadecanol tyloxapol through Calu-3 monolayers. *Pharmazie* 2012;**67**:448−51.
248. Chavanpatil MD, Vavia PR. The influence of absorption enhancers on nasal absorption of acyclovir. *Eur J Pharm Biopharm* 2004;**57**:483−7.
249. Ghadiri M, Canney F, Pacciana C, Colombo G, Young PM, Traini D. The use of fatty acids as absorption enhancer for pulmonary drug delivery. *Int J Pharm* 2018;**541**:93−100.
250. Coyne CB, Ribeiro CMP, Boucher RC, Johnson LG. Acute mechanism of medium chain fatty acid-induced enhancement of airway epithelial permeability. *J Pharmacol Exp Ther* 2003;**305**:440−50.
251. Minne A, Louahed J, Mehauden S, Baras B, Renauld JC, Vanbever R. The delivery site of a monovalent influenza vaccine within the respiratory tract impacts on the immune response. *Immunology* 2007;**122**:316−25.
252. Bolton DL, Song KM, Tomaras GD, Rao S, Roederer M. Unique cellular and humoral immunogenicity profiles generated by aerosol, intranasal, or parenteral vaccination in rhesus macaques. *Vaccine* 2017;**35**:639−46.
253. Vujanic A, Sutton P, Snibson KJ, Yen HH, Scheerlinck JPY. Mucosal vaccination: lung *versus* nose. *Vet Immunol Immunopathol* 2012;**148**:172−7.
254. de Swart RL, de Vries RD, Rennick LJ, Van Amerongen G, McQuaid S, Verburgh RJ, et al. Needle-free delivery of measles virus vaccine to the lower respiratory tract of non-human primates elicits optimal immunity and protection. *Npj Vaccin* 2017;**2**:22.
255. Todoroff J, Ucakar B, Inglese M, Vandermarliere S, Fillee C, Renauld JC, et al. Targeting the deep lungs, Poloxamer 407 and a CpG oligonucleotide optimize immune responses to *Mycobacterium tuberculosis* antigen 85A following pulmonary delivery. *Eur J Pharm Biopharm* 2013;**84**:40−8.
256. Gao JJ, Xia ZT, Vohidova D, Joseph J, Luo JN, Joshi N. Progress in non-viral localized delivery of siRNA therapeutics for pulmonary diseases. *Acta Pharm Sin B* 2023;**13**:1400−28.
257. Praphawatvet T, Peters JI, Williams IIIRO. Inhaled nanoparticles—an updated review. *Int J Pharm* 2020;**587**:119671.
258. Zhong WH, Zhang XY, Zeng YX, Lin DJ, Wu J. Recent applications and strategies in nanotechnology for lung diseases. *Nano Res* 2021;**14**:2067−89.
259. Wauthoz N, Rosière R, Amighi K. Inhaled cytotoxic chemotherapy: clinical challenges, recent developments, and future prospects. *Expert Opin Drug Deliv* 2021;**18**:333−54.
260. Osman N, Kaneko K, Carini V, Saleem I. Carriers for the targeted delivery of aerosolized macromolecules for pulmonary pathologies. *Expert Opin Drug Deliv* 2018;**15**:821−34.
261. Xu HY, Cai L, Hufnagel S, Cui ZG. Intranasal vaccine: factors to consider in research and development. *Int J Pharm* 2021;**609**:121180.
262. Djupesland PG. Nasal drug delivery devices: characteristics and performance in a clinical perspective-a review. *Drug Deliv Transl Res* 2013;**3**:42−62.
263. Li BV, Jin FY, Lee SL, Bai T, Chowdhury B, Caramenico HT, et al. Bioequivalence for locally acting nasal spray and nasal aerosol products: standard development and generic approval. *AAPS J* 2013;**15**:875−83.
264. Djupesland PG, Skretting A, Winderen M, Holand T. Breath actuated device improves delivery to target sites beyond the nasal valve. *Laryngoscope* 2006;**116**:466−72.
265. Bakke H, Samdal HH, Holst J, Oftung F, Haugen IL, Kristoffersen AC, et al. Oral spray immunization may be an alternative to intranasal vaccine delivery to induce systemic antibodies but not nasal mucosal or cellular immunity. *Scand J Immunol* 2006;**63**:223−31.
266. Savvateeva EN, Rubina AY, Gryadunov DA. Biomarkers of community-acquired pneumonia: a key to disease diagnosis and management. *BioMed Res Int* 2019;**2019**:1701276.
267. Rau JL. Design principles of liquid nebulization devices currently in use. *Respir Care* 2002;**47**:1257−78.
268. Stein SW, Sheth P, Hodson PD, Myrdal PB. Advances in metered dose inhaler technology: hardware development. *AAPS PharmSciTech* 2014;**15**:326−38.
269. Sheth P, Stein SW, Myrdal PB. Factors influencing aerodynamic particle size distribution of suspension pressurized metered dose inhalers. *AAPS PharmSciTech* 2015;**16**:192−201.
270. Zhu B, Traini D, Young P. Aerosol particle generation from solution-based pressurized metered dose inhalers: a technical overview of parameters that influence respiratory deposition. *Pharm Dev Technol* 2015;**20**:897−910.
271. Brown AR. Propellant-driven aerosols of proteins. *Aerosol Sci Technol* 1996;**24**:45−56.
272. Williams IIIRO, Liu J. Formulation of a protein with propellant HFA 134a for aerosol delivery. *Eur J Pharm Sci* 1999;**7**:137−44.
273. Bains BK, Birchall JC, Toon R, Taylor G. *In vitro* reporter gene transfection *via* plasmid DNA delivered by metered dose inhaler. *J Pharm Sci* 2010;**99**:3089−99.
274. Tamura G. Comparison of the aerosol velocity of Respimat® soft mist inhaler and seven pressurized metered dose inhalers. *Allergol Int* 2015;**64**:390−2.
275. Brand P, Hederer B, Austen G, Dewberry H, Meyer T. Higher lung deposition with Respimat® Soft Mist™ Inhaler than HFA-MDI in COPD patients with poor technique. *Int J Chron Obstruct Pulmon Dis* 2008;**3**:763−70.
276. Dalby R, Spallek M, Voshaar T. A review of the development of Respimat® soft Mist™ inhaler. *Int J Pharm* 2004;**283**:1−9.
277. Hochrainer D, Hölz H, Kreher C, Scaffidi L, Spallek M, Wachtel H. Comparison of the aerosol velocity and spray duration of Respimat® Soft Mist™ Inhaler and pressurized metered dose inhalers. *J Aerosol Med* 2005;**18**:273−82.
278. Zhang W, Xu LL, Gao S, Ding N, Shu P, Wang Z, et al. Technical evaluation of soft mist inhaler use in patients with chronic obstructive pulmonary disease: a cross-sectional study. *Int J Chron Obstruct Pulmon Dis* 2020;**15**:1471−9.
279. Ge Y, Tong ZB, Li RJ, Huang F, Yu JQ. Numerical and experimental investigation on key parameters of the Respimat® Spray Inhaler. *Processes* 2020;**9**:44.
280. Berkenfeld K, Lamprecht A, McConville JT. Devices for dry powder drug delivery to the lung. *AAPS PharmSciTech* 2015;**16**:479−90.
281. Tonnis WF, Lexmond AJ, Frijlink HW, de Boer AH, Hinrichs WL. Devices and formulations for pulmonary vaccination. *Expert Opin Drug Deliv* 2013;**10**:1383−97.
282. Jahan N, Archie SR, Shoyaib AA, Kabir N, Cheung K. Recent approaches for solid dose vaccine delivery. *Sci Pharm* 2019;**87**:27.
283. Lin WH, Griffin DE, Rota PA, Papania M, Cape SP, Bennett D, et al. Successful respiratory immunization with dry powder live-attenuated measles virus vaccine in rhesus macaques. *Proc Natl Acad Sci U S A* 2011;**108**:2987−92.
284. Ye YQ, Ma Y, Zhu J. The future of dry powder inhaled therapy: promising or discouraging for systemic disorders? *Int J Pharm* 2022;**614**:121457.
285. O'Callaghan C, Barry PW. The science of nebulised drug delivery. *Thorax* 1997;**52**(Suppl 2):S31−44.
286. de Swart RL, Kuiken T, Fernandez-de Castro J, Papania MJ, Bennett JV, Valdespino JL, et al. Aerosol measles vaccination in macaques: preclinical studies of immune responses and safety. *Vaccine* 2006;**24**:6424−36.
287. Zarogoulidis P, Chatzaki E, Porpodis K, Domvri K, Hohenforst-Schmidt W, Goldberg EP, et al. Inhaled chemotherapy in lung cancer: future concept of nanomedicine. *Int J Nanomedicine* 2012;**7**:1551−72.
288. de Pablo E, Fernández-García R, Ballesteros MP, Torrado JJ, Serrano DR. Nebulised antibiotherapy: conventional *versus* nanotechnology-based approaches, is targeting at a nano scale a difficult subject? *Ann Transl Med* 2017;**5**:448.
289. Flament MP, Leterme P, Gayot A. Study of the technological parameters of ultrasonic nebulization. *Drug Dev Ind Pharm* 2001;**27**:643−9.

290. Berlinski A, Waldrep JC. Effect of aerosol delivery system and formulation on nebulized budesonide output. *J Aerosol Med* 1997;**10**:307−18.
291. Pillai R, Petrak K, Blezinger P, Deshpande D, Florack V, Freimark B, et al. Ultrasonic nebulization of cationic lipid-based gene delivery systems for airway administration. *Pharm Res (N Y)* 1998;**15**:1743−7.
292. Dhand R. Nebulizers that use a vibrating mesh or plate with multiple apertures to generate aerosol. *Respir Care* 2002;**47**:1406−18.
293. Wu SP, Huang JY, Zhang Z, Wu JY, Zhang JL, Hu HN, et al. Safety, tolerability, and immunogenicity of an aerosolised adenovirus type-5 vector-based COVID-19 vaccine (Ad5-nCoV) in adults: preliminary report of an open-label and randomised phase 1 clinical trial. *Lancet Infect Dis* 2021;**21**:1654−64.
294. Huang T, Zhang S, Dai DF, Wang BS, Zhuang L, Huang HT, et al. Safety and immunogenicity of heterologous boosting with orally aerosolised or intramuscular Ad5-nCoV vaccine and homologous boosting with inactivated vaccines (BBIBP-CorV or CoronaVac) in children and adolescents: a randomised, open-label, parallel-controlled, non-inferiority, single-centre study. *Lancet Respir Med* 2023;**11**:698−708.
295. Forest V, Pourchez J. Nano-delivery to the lung-by inhalation or other routes and why nano when micro is largely sufficient? *Adv Drug Deliv Rev* 2022;**183**:114173.
296. Newman SP. Principles of metered-dose inhaler design. *Respir Care* 2005;**50**:1177−90.
297. He XY, Chen XY, Wang HR, Du GS, Sun X. Recent advances in respiratory immunization: a focus on COVID-19 vaccines. *J Control Release* 2023;**355**:655−74.
298. Sarhan RM, Elberry AA, Abdelwahab NS, Rabea H, Salem MN, Abdelrahim ME. Effect of oxygen flow on aerosol delivery from a nebulizer with a holding chamber. *Respir Care* 2019;**64**:1508−15.
299. Elphick M, Von Hollen D, Pritchard JN, Nikander K, Hardaker LE, Hatley RH. Factors to consider when selecting a nebulizer for a new inhaled drug product development program. *Expert Opin Drug Deliv* 2015;**12**:1375−87.
300. Wu JY, Wu L, Wan F, Rantanen J, Cun DM, Yang MS. Effect of thermal and shear stresses in the spray drying process on the stability of siRNA dry powders. *Int J Pharm* 2019;**566**:32−9.
301. Price DN, Kunda NK, Ellis R, Muttil P. Design and optimization of a temperature-stable dry powder BCG vaccine. *Pharm Res (N Y)* 2020;**37**:11.
302. Brandau DT, Jones LS, Wiethoff CM, Rexroad J, Middaugh CR. Thermal stability of vaccines. *J Pharm Sci* 2003;**92**:218−31.
303. Andre EF. Stability of vaccines. *BMJ* 1993;**307**:939.
304. Sou T, Bergström CAS. Contemporary formulation development for inhaled pharmaceuticals. *J Pharm Sci* 2021;**110**:66−86.
305. Kamerzell TJ, Esfandiary R, Joshi SB, Middaugh CR, Volkin DB. Protein−excipient interactions: mechanisms and biophysical characterization applied to protein formulation development. *Adv Drug Deliv Rev* 2011;**63**:1118−59.
306. Arakawa T, Ejima D, Tsumoto K, Obeyama N, Tanaka Y, Kita Y, et al. Suppression of protein interactions by arginine: a proposed mechanism of the arginine effects. *Biophys Chem* 2007;**127**:1−8.
307. White JA, Estrada M, Flood EA, Mahmood K, Dhere R, Chen D. Development of a stable liquid formulation of live attenuated influenza vaccine. *Vaccine* 2016;**34**:3676−83.
308. Li JJ, Krause ME, Chen XD, Cheng Y, Dai WG, Hill JJ, et al. Interfacial stress in the development of biologics: fundamental understanding, current practice, and future perspective. *AAPS J* 2019;**21**:44.
309. Tomar J, Tonnis WF, Patil HP, de Boer AH, Hagedoorn P, Vanbever R, et al. Pulmonary immunization: deposition site is of minor relevance for influenza vaccination but deep lung deposition is crucial for hepatitis B vaccination. *Acta Pharm Sin B* 2019;**9**:1231−40.
310. Mossadeq S, Shah RJ, Shah V, Bagul M. Formulation, device, and clinical factors influencing the targeted delivery of COVID-19 vaccines to the lungs. *AAPS PharmSciTech* 2022;**24**:2.
311. Xu ZQ, Bera H, Wang HZ, Wang JW, Cun DM, Feng Y, et al. Inhalable ciprofloxacin/polymyxin B dry powders in respiratory infection therapy. *Acta Mater Med* 2023;**2**:142−56.
312. Chan HK. Dry powder aerosol drug delivery-opportunities for colloid and surface scientists. *Colloids Surf, A* 2006;**284**:50−5.
313. Vehring R. Pharmaceutical particle engineering *via* spray drying. *Pharm Res (N Y)* 2008;**25**:999−1022.
314. Mensink MA, Frijlink HW, van der Voort Maarschalk K, Hinrichs WLJ. How sugars protect proteins in the solid state and during drying (review): mechanisms of stabilization in relation to stress conditions. *Eur J Pharm Biopharm* 2017;**114**:288−95.
315. Ingvarsson PT, Yang MS, Nielsen HM, Rantanen J, Foged C. Stabilization of liposomes during drying. *Expert Opin Drug Deliv* 2011;**8**:375−88.
316. Tonnis WF, Mensink MA, de Jager A, van der Voort Maarschalk K, Frijlink HW, Hinrichs WLJ. Size and molecular flexibility of sugars determine the storage stability of freeze-dried proteins. *Mol Pharm* 2015;**12**:684−94.
317. McAdams D, Chen DX, Kristensen D. Spray drying and vaccine stabilization. *Expert Rev Vaccin* 2012;**11**:1211−9.
318. Crowe JH, Carpenter JF, Crowe LM. The role of vitrification in anhydrobiosis. *Annu Rev Physiol* 1998;**60**:73−103.
319. Sou T, Morton DAV, Williamson M, Meeusen EN, Kaminskas LM, McIntosh MP. Spray-dried influenza antigen with trehalose and leucine produces an aerosolizable powder vaccine formulation that induces strong systemic and mucosal immunity after pulmonary administration. *J Aerosol Med Pulm Drug Deliv* 2015;**28**:361−71.
320. Amorij JP, Saluja V, Petersen AH, Hinrichs WLJ, Huckriede A, Frijlink HW. Pulmonary delivery of an inulin-stabilized influenza subunit vaccine prepared by spray-freeze drying induces systemic, mucosal humoral as well as cell-mediated immune responses in BALB/c mice. *Vaccine* 2007;**25**:8707−17.
321. Kanojia G, Raeven RHM, van der Maas L, Bindels THE, van Riet E, Metz B, et al. Development of a thermostable spray dried outer membrane vesicle pertussis vaccine for pulmonary immunization. *J Control Release* 2018;**286**:167−78.
322. Kanojia G, Willems GJ, Frijlink HW, Kersten GFA, Soema PC, Amorij JP. A Design of Experiment approach to predict product and process parameters for a spray dried influenza vaccine. *Int J Pharm* 2016;**511**:1098−111.
323. Saluja V, Amorij JP, Kapteyn JC, de Boer AH, Frijlink HW, Hinrichs WLJ. A comparison between spray drying and spray freeze drying to produce an influenza subunit vaccine powder for inhalation. *J Control Release* 2010;**144**:127−33.
324. Jin TH, Tsao E, Goudsmit J, Dheenadhayalan V, Sadoff J. Stabilizing formulations for inhalable powders of an adenovirus 35-vectored tuberculosis (TB) vaccine (AERAS-402). *Vaccine* 2010;**28**:4369−75.
325. Gomez M, McCollum J, Wang H, Bachchhav S, Tetreau I, Gerhardt A, et al. Evaluation of the stability of a spray-dried tuberculosis vaccine candidate designed for dry powder respiratory delivery. *Vaccine* 2021;**39**:5025−36.
326. Manser M, Morgan BA, Feng XY, Rhem RG, Dolovich MB, Xing Z, et al. Dextran mass ratio controls particle drying dynamics in a thermally stable dry powder vaccine for pulmonary delivery. *Pharm Res (N Y)* 2022;**39**:2315−28.
327. Thakur A, Ingvarsson PT, Schmidt ST, Rose F, Andersen P, Christensen D, et al. Immunological and physical evaluation of the multistage tuberculosis subunit vaccine candidate H56/CAF01 formulated as a spray-dried powder. *Vaccine* 2018;**36**:3331−9.
328. Mehanny M, Boese A, Bornamehr B, Hoppstädter J, Presser V, Kiemer AK, et al. Spray-dried pneumococcal membrane vesicles are promising candidates for pulmonary immunization. *Int J Pharm* 2022;**621**:121794.
329. Murugappan S, Patil HP, Kanojia G, ter Veer W, Meijerhof T, Frijlink HW, et al. Physical and immunogenic stability of spray freeze-dried influenza vaccine powder for pulmonary delivery: comparison of inulin, dextran, or a mixture of dextran and trehalose as protectants. *Eur J Pharm Biopharm* 2013;**85**:716−25.
330. Audouy SAL, van der Schaaf G, Hinrichs WLJ, Frijlink HW, Wilschut J, Huckriede A. Development of a dried influenza whole

inactivated virus vaccine for pulmonary immunization. *Vaccine* 2011; **29**:4345−52.
331. Garmise RJ, Staats HF, Hickey AJ. Novel dry powder preparations of whole inactivated influenza virus for nasal vaccination. *AAPS PharmSciTech* 2007;**8**:2−10.
332. Zhang W, Song XL, Zhai LN, Guo JS, Zheng XY, Zhang LL, et al. Complete protection against yersinia pestis in BALB/c mouse model elicited by immunization with inhalable formulations of rF1-V10 fusion protein *via* aerosolized intratracheal inoculation. *Front Immunol* 2022;**13**:793382.
333. Jiang G, Joshi SB, Peek LJ, Brandau DT, Huang J, Ferriter MS, et al. Anthrax vaccine powder formulations for nasal mucosal delivery. *J Pharm Sci* 2006;**95**:80−96.
334. Wang SH, Kirwan SM, Abraham SN, Staats HF, Hickey AJ. Stable dry powder formulation for nasal delivery of anthrax vaccine. *J Pharm Sci* 2012;**101**:31−47.
335. Gan CJB, Luo W, Yu YZ, Jiao ZG, Li S, Su D, et al. Intratracheal inoculation of AHc vaccine induces protection against aerosolized botulinum neurotoxin A challenge in mice. *NPJ Vaccin* 2021;**6**:87.
336. Smallshaw JE, Vitetta ES. A lyophilized formulation of RiVax, a recombinant ricin subunit vaccine, retains immunogenicity. *Vaccine* 2010;**28**:2428−35.
337. Bodier-Montagutelli E, Mayor A, Vecellio L, Respaud R, Heuzé-Vourc'h N. Designing inhaled protein therapeutics for topical lung delivery: what are the next steps? *Expert Opin Drug Deliv* 2018;**15**: 729−36.
338. Hertel S, Pohl T, Friess W, Winter G. That's cool!-nebulization of thermolabile proteins with a cooled vibrating mesh nebulizer. *Eur J Pharm Biopharm* 2014;**87**:357−65.
339. Hertel SP, Winter G, Friess W. Protein stability in pulmonary drug delivery *via* nebulization. *Adv Drug Deliv Rev* 2015;**93**:79−94.
340. Martin AR, Finlay WH. Nebulizers for drug delivery to the lungs. *Expert Opin Drug Deliv* 2015;**12**:889−900.
341. Goodson JL, Seward JF. Measles 50 years after use of measles vaccine. *Infect Dis Clin North Am* 2015;**29**:725−43.
342. Kim J, Jozic A, Lin YX, Eygeris Y, Bloom E, Tan XC, et al. Engineering lipid nanoparticles for enhanced intracellular delivery of mRNA through inhalation. *ACS Nano* 2022;**16**:14792−806.
343. Lewis MM, Soto MR, Maier EY, Wulfe SD, Bakheet S, Obregon H, et al. Optimization of ionizable lipids for aerosolizable mRNA lipid nanoparticles. *Bioeng Transl Med* 2023;**8**:e10580.
344. Jiang AY, Witten J, Raji IO, Eweje F, MacIsaac C, Meng S, et al. Combinatorial development of nebulized mRNA delivery formulations for the lungs. *Nat Nanotechnol* 2024;**19**:364−75.
345. Deng ZY, Liu SY. Emerging trends in solution self-assembly of block copolymers. *Polymer* 2020;**207**:122914.
346. Wang YY, Ye MZ, Xie RS, Gong SQ. Enhancing the *in vitro* and *in vivo* stabilities of polymeric nucleic acid delivery nanosystems. *Bioconjug Chem* 2019;**30**:325−37.
347. Bandi S, Singh SM, Shah DD, Upadhyay V, Mallela KMG. 2D NMR analysis of the effect of asparagine deamidation *versus* methionine oxidation on the structure, stability, aggregation, and function of a therapeutic protein. *Mol Pharm* 2019;**16**:4621−35.
348. Respaud R, Marchand D, Parent C, Pelat T, Thullier P, Tournamille JF, et al. Effect of formulation on the stability and aerosol performance of a nebulized antibody. *mAbs* 2014;**6**:1347−55.
349. Liu TT, Tong SQ, Liao QQ, Pan L, Cheng MS, Rantanen J, et al. Role of dispersion enhancer selection in the development of novel tratinterol hydrochloride dry powder inhalation formulations. *Int J Pharm* 2023;**635**:122702.
350. Kim CS, Jaques PA. Total lung deposition of ultrafine particles in elderly subjects during controlled breathing. *Inhal Toxicol* 2005;**17**:387−99.
351. Xu F, Wu SP, Yi LN, Peng SD, Wang F, Si WX, et al. Safety, mucosal and systemic immunopotency of an aerosolized adenovirus-vectored vaccine against SARS-CoV-2 in rhesus macaques. *Emerg Microbes Infect* 2022;**11**:438−41.
352. Kim CS. Physiological factors affecting lung deposition. *J Aerosol Med Pulm Drug Deliv* 2021;**34**:147−54.
353. Horváth A, Farkas Á, Szipőcs A, Tomisa G, Szalai Z, Gálffy G. Numerical simulation of the effect of inhalation parameters, gender, age and disease severity on the lung deposition of dry powder aerosol drugs emitted by Turbuhaler®, Breezhaler® and Genuair® in COPD patients. *Eur J Pharm Sci* 2020;**154**:105508.
354. Clarà PC, Jerez FR, Ramírez JB, González CM. Deposition and clinical impact of inhaled particles in the lung. *Arch Bronconeumol* 2023;**59**:377−82.