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Inhalation delivery technology for genome-editing of respiratory diseases

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Abstract

The clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9) system has significant therapeutic potentials for lung congenital diseases such as cystic fibrosis, as well as other pulmonary disorders like lung cancer and obstructive diseases. Local administration of CRISPR/Cas9 therapeutics through inhalation can achieve high drug concentration and minimise systemic exposure. While the field is advancing with better understanding on the biological functions achieved by CRISPR/Cas9 systems, the lack of progress in inhalation formulation and delivery of the molecule may impede their clinical translation efficiently. This forward-looking review discussed the current status of formulations and delivery for inhalation of relevant biologics such as genes (plasmids and mRNAs) and proteins, emphasising on their design strategies and preparation methods. By adapting and optimising formulation strategies used for genes and proteins, we envisage that development of inhalable CRISPR/Cas9 liquid or powder formulations for inhalation administration can potentially be fast-tracked in near future.

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Keywords: Aerosol formulation, CRISPR, Dry powder inhaler, Gene therapy, mRNA, Nebulisation, Plasmids, Ribonucleoprotein

1. Introduction

Genome editing technologies including zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) and clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9) system have been opening up a new...
era of treatment through precision genome surgery. Compared to ZFN and TALEN, CRISPR/Cas9 has been widely viewed as the preferred candidate for its superior efficiency and specificity, which also comes with a lower cost and does not require dedicated enzyme engineering [1,2]. Originated from bacterial adaptive immune system [3], the CRISPR/Cas9 system comprises a Cas9 endonuclease and a guiding CRISPR RNA (crRNA) to which a trans-activating crRNA (tracrRNA) binds and forms an activated complex. These two short RNAs can also be optimised and combined into one single-guide RNA, or sgRNA. The Cas9 endonuclease then recognises specific region of the target DNA as guided by the sequence of the crRNA, resulting in DNA cleavage or Cas9 endonuclease then recognises speciﬁc optimised and combined into one single-guide RNA, or sgRNA. The forms an activated complex. These two short RNAs can also be viral delivery systems. Viral delivery systems exhibit high transfection efﬁciency genome correction, which can potentially correct innate disease-causing errors of genes. The utility of CRISPR/Cas9 system was initially recognised as a valuable and powerful research tool to understand the usual roles of specific genetic variations, instead of relying on disease models where only a particular disorder is phenocopied [5]. Like other genetic modification techniques such as RNA interference, the potential of this precise genome editing technology as a novel therapeutic strategy has been explored in many diseases, including pulmonary disorders.

Majority of lung congenital diseases, such as cystic ﬁbrosis (CF) and α-1 antitrypsin deﬁciency, are caused by monogenetic mutation, rendering the use of precision genome-editing tool like CRISPR/Cas9 an attractive idea. Moreover, the high prevalence of lung cancers and refractory obstructive respiratory diseases like asthma and chronic obstructive pulmonary disorder remains an enormous socioeconomic burden to even the more developed countries, representing a gargantuan unmet medical need [6–8]. For further details on genome-editing biomacromolecules for lung genetic disorders, the readers are referred to the article by Wan and Ping in this theme issue [9].

Pulmonary delivery of therapeutic genes by inhalation demands the biopharmaceuticals to be delivered in the form of an aerosol, which requires delicate formulation engineering and manufacturing. There have been rapid progresses occurring in the development and understanding in various aspects including the identiﬁcation of genome targets, optimisation of delivery vectors and transfection efﬁciency, reduction of off-target efﬁcacy and improvement in safety proﬁle. Consequently, the lack in the development of CRISPR/Cas9 formulations suitable for inhalation has become one of the major obstacles in translating genome-editing therapeutics to clinical applications.

This review began with an overview on the possible administration routes of gene therapy for pulmonary diseases and the pathophysiologi cal factors that affect aerosol deposition in the lungs. It is followed by a detailed discussion on the current development of two most promising inhalable formulations applicable to the delivery of genome-editing therapeutics, namely liquids for nebulisation and inhalable dry powders. The scope of this article does not include strategies of engineering CRISPR/Cas9 delivery systems as they have already been reviewed [2,10,11]. Instead, we focus on inhalable formulations involving nacked (without any delivery vector) or chemically modiﬁed genes, and non-viral delivery systems. Viral delivery systems exhibit high transfection efficiency[12], but they have been associated with limited loading size and, more critically, potentially fatal immunogenic and carcinogenic effects [13]. As currently there is only one published study on the development of formulation of CRISPR/Cas9 therapeutics for inhalation [14], this review will be a forward-looking article, outlining the present development of some closely related formulations for inhalation of biologics such as genes and proteins, with an emphasis on their design and preparation methods and their relevance for genome-editing agents.

2. Routes of administration of gene therapy for pulmonary disorders

Due to the relative ease of administration and simple formulation design, systemic administration of genome-editing agents for lung diseases are widely used in animal models, especially in those aiming to demonstrate the therapeutic potential of editing certain genomes in proof-of-concept studies [15]. However, systemic administration faces numerous critical challenges for in vivo applications [10], due to the stability of genome-editing complexes and their potential degradation by serum nucleases and proteases and rapid renal clearance. While complication with delivery vectors often partially alleviate the otherwise poor pharmacokinetics, retention of the complexes in the lung often remains suboptimal. Intravenously injected genetic materials are distributed to different organs in the body such as kidney, liver and spleen [16]. They also reach and accumulate in the alveolar region rather than the ciliated epithelial cells in the bronchi. An implication of which is the potential limitation of intravenous administration in treating some pulmonary genetic disorders such as CF, since cystic ﬁbro sis transmembrane conductance regulator (CFTR), the mutation of which causes CF, is primarily expressed in bronchi.

Some of these challenges can be partially mitigated by the local administration of gene-editing therapeutics as aerosols through oral inhalation to the lungs. It enables genes, either in its naked form or complexed with delivery vectors, to be delivered directly and rapidly to the target cells in the lungs at a high concentration with reduced systemic exposure [17]. Local delivery also allows non-invasive access to the lungs and minimises the interactions between genes and serum proteins [18,19]. In fact, the large alveolar area with high vascularisation, coupled with its thin air-blood-barrier [20] and relatively low enzymatic activity, also render pulmonary administration a viable alternative for systemic treatments. In spite of these advantages, the development of inhalable genome-editing therapeutics has been relatively slow due to the complexity involved.

For drugs to be administered through inhalation, they must be formulated into either a liquid or solid aerosol. It follows that the aerosol properties of the formulations need to be precisely controlled for efﬁcient delivery aerosols to the lungs. In pharmaceutics, one of the most relevant measurements of aerosol properties is aerodynamic diameter. The aerodynamic diameter of a single particle is deﬁned as the physical (geometrical) diameter of a spherical particle with density of 1 g cm−3 that has the same settling velocity in air as the particle in question. It is well known that in general inhaled particles with aerodynamic diameter of 1 to 5 μm are suitable for respiratory delivery, whereas lung deposition decreases outside this range either due to impaction loss of large particles in the oropharynx or exhalation of small particles, depending on the inspiratory ﬂowrate and pausing [15,21]. In patients with obstructive airway diseases such as CF, aerosols with aerobic diameter of 2 to 3 μm is considered to provide the greatest therapeutic beneﬁt [22]. The aerodynamic size distribution of an aerosol (i.e. a collection of individual particles) can be measured by cascade impactors in tandem with suitable dispersion devices such as nebulisers or dry powder inhalers (DPI). Cascade impactors separate particles according to their aerodynamic diameters through the principle of inertial momentum. There are two main derived statistics of aerodynamic size distribution, namely the mass median aerodynamic diameters (MMAD) and ﬁne particle fraction (FFP). MMAD refers to the aerodynamic diameter under (and also above) which half of the particles by mass reside, whereas FFP refers to the fraction of particles (by mass) that are ﬁne, the threshold of which is usually taken as 5 μm. Hence, FFP is often regarded as the respirable fraction of a formulation for inhalation. While commercially available DPs had an FFP of about 5% to 40% [23,24], a higher FFP value should be targeted for expensive biomolecules in order to reduce variation in lung deposition [25] and minimise drug wastage. In evaluating the aerosol performance of formulations for inhalation, it should be emphasised that in vivo animal models have limited utilities for such purpose due to signiﬁcant anatomical and
physiological differences between species. Moreover, the different methodologies for pulmonary drug delivery to animals each possess their own strengths and limitations [26]. For example, aerosol administration via passive inhalation such as whole-body exposure or head-/ nose-only exposure systems spare the animal from anaesthesia, yet an accurate control over the delivered dose can be difficult to achieve. Intratracheal administration allows the delivery of a controlled amount of drug, but it might be technically more demanding particularly in smaller animals such as mice. Given these limitations, in vivo animal models should be primarily used to demonstrate the safety, efficacy and possibly pharmacokinetics of the formulations.

In addition to aerodynamic diameter, other particle physicochemical properties such as size, charge, density and surface composition can influence the fate of drug deposition and cell uptake in the lungs [22]. Furthermore, a therapeutic gene formulation must remain inhalable, with sufficient stability and aerodynamic properties during storage and use [27]. Only through the intricate engineering of the biopolymers, excipients in the formulation and the delivery device can a safe and efficient delivery be achieved, yet the final clinical outcomes of which is also inevitably affected by the lung pathophysiology of the patients. As only a limited number of studies in this field has been reported, the following sections will primarily leverage on the prior art in inhalated gene therapy and its potential application for the aerosol delivery of CRISPR/Cas9-based platforms.

3. Pathophysiological factors influencing aerosol delivery of genes in the lungs

Despite numerous advantages afforded by pulmonary administration, successful aerosol delivery of biopharmaceuticals including CRISPR/Cas9-based therapeutics remains highly challenging. There exist several major anatomical and physiological barriers that prevent the successful delivery genome editing via inhalation to the lungs [19,28]. Firstly, oropharyngeal deposition of orally inhalated aerosol particles must be avoided to maximise lung deposition. The highly bifurcated anatomy of the lung together with the constricting airway down the respiratory tract serve as an effective structural barrier to prevent penetration of large particles to the lower respiratory tract [21]. Secondly, the mucus lining secreted by the epithelia has been considered as another major barrier in aerosol delivery. Airway mucus is primarily composed of a gel-type mucin fibre that contains a high density of negatively charged macromolecules [29]. As most nanocomplexes of genes and delivery vectors are cationic, they are prone to accumulation in the mucus layer via a manifold of interactions. These interactions, including hydrophobic forces, hydrogen bonding and electrostatic interactions, not only impair the colloidal stability of vectors and reduce their delivery efficiency, but also make the gene molecules in the cargo vulnerable to degradation by the surrounding nucleases [29]. Thirdly, the situation is further complicated by the disease state, for instance, patients suffering from CF have an accumulation of abnormally thick and stick mucus due to the lack of CFTR functions. Their airways are often found inflamed and infections are commonplace. As a result of the constricted airways, turbulent flow is enhanced which reduces the average travel distance of aerosol particles. Aerosol particle deposition is hence affected as it is more probable for the particles to deposit earlier on large airways by local turbulence [30], making peripheral deposition less favourable [31]. Paradoxically, diseased regions where drug particles should deposit would also receive less air flow and thus a lower dose as a result of obstruction. Prediction of deposition patterns of inhaled genes in lungs of CF patients became less accurate since the airways can differ significantly, depending on the disease states and resulting complications. As stated above, the viscous and dense mucus also act as formidable barriers, further hindering the gene uptake by target epithelial cells [32].

Fourthly, in addition to the mucus gel layer, another barrier in the conducting airways is the periciliary layer (PCL) [33] (Fig. 1). PCL primarily consists of a meshwork of cell-tethered mucins, similar to the mucus gel layer. Upon penetration through the mucus layer, the gene vectors must retain their delivery capacity and stability in the presence of surfactants which are abundant in the airspace of the lungs. Pulmonary surfactant is negatively charged, composed of various phospholipids, cholesterol and surfactant proteins. These anionic components may trigger premature release of gene cargo, or facilitate the aggregation of lipid-based gene delivery vectors because the stability of the cargo and lipid nanoparticle is collapsed and their complexation is disrupted [34,35]. Fifthly, alveolar macrophages residing in the airspace (Fig. 1) also constitute yet another barrier that hampers gene delivery through inhalation [36–39]. Several intracellular barriers, such as endosomes and the nuclear envelope, must be overcome during gene delivery after internalisation by the target cells through the judicious use of delivery vectors [40], of which the latest development for CRISPR/Cas9 has been thoroughly reviewed [2,41–46]. Although non-viral vectors often remain inferior to viral vectors in their transfection efficiency at the trade-off for a safer immunogenic profile, systematic and in-depth study on their structure-function relationships can bring about engineered vectors with improved efficacy [47].

Strategies to overcome these physiological barriers for inhaled gene therapy, such as modification of delivery vectors and modulations of barriers, have been proposed and discussed in detail [19,48]. The incorporation of specific ligands to the vector surface by covalent conjugation or genetic engineering has been widely explored to reduce adhesive interactions with mucus, enhance particle stability, and reduce macropage uptake, which further endow viral and non-viral vectors the ability to be efficiently internalised by target cells [49–54]. The use of mucus-altering agent, including N-acetyl-cysteine and recombinant human DNase, can increase mucus mesh pore size, thereby facilitating the penetration of gene nanocomplexes through the mucus layer [55,56]. Meanwhile, the incorporation of osmotic agents to inhalated formulations, such as the use of hypertonic saline and mannitol, can increase mucus and/or PCL mesh pore size by hydration and thus improve mucociliary clearance [57,58]. Tight junctions disrupting agents, such as sodium caprate, polidocanol and lysophosphatidylcholine, can transiently disrupt tight junctions in the epithelial layer to provide an access to the basolateral surface for gene vectors [59,60]. These well-established strategies will facilitate the rational design of aerosol inhalation delivery for genome-editing agents.

4. Inhalable formulations of CRISPR/Cas9-based therapeutics

Unlike other routes of administration, pulmonary administration consists of not only the drug formulation but also a suitable inhaler device. Nebuliser, pressurised metered dose inhaler (pMDI) and DPI are the three major types of inhaler device that can disperse liquid (nebuliser and pMDI) or solid (DPI) into inhalable aerosols. Among these devices, pMDI is a common device to deliver potent small molecules like beta adrenergic agonists, inhaled cortical steroids and anti-cholinergics for the management of obstructive lung diseases. Apart from some early investigations [61–63], studies on the delivery of biopharmaceutical by pMDI has been limited due to stability issue of biomolecules as they can degrade in propellants and/or be damaged by the high mechanical shear stress through the MDI nozzle during actuation of the device. The low payload of drug in pMDI also posts another major constrain as biopharmaceuticals like genes and proteins are likely to be dosed in milligrams. Consequently, most of the pharmaceutical research on pulmonary delivery of gene therapeutics has been conducted on nebulisation and inhalable dry powder formulations. Since CRISPR/Cas9-based therapeutics can be delivered as cargoes of plasmid DNA encoding Cas9 protein and sgRNA (or crRNA and tracrRNA duplex), RNA (as mRNA encoding the Cas9 protein and sgRNA), or as Cas9 protein-sgRNA complex (ribonucleoprotein RNP) [11,64], the current development of liquid formulations for nebulisation and powder formulations for inhalation for pDNA, mRNA and proteins are discussed below.
4.1. Nebulisation

Nebulisation refers to the process of dispersing liquids into a fine mist of droplets for inhalation. It is often the preferred way of aerosolisation for inhaled biopharmaceuticals especially during the initial phase of investigation because it offers several advantages over dry powder formulations. Formulation of a liquid is relatively simple to develop. It eliminates the needs for drying which can be challenging for thermal labile biomolecules. Nebulisation is also suitable for all clinical situations and patients, including paediatric patients or critically ill patients [65]. Air-jet, ultrasonic and vibrating mesh nebulisers are the three common types of nebulisers [66]. In air-jet nebulisers, compressed air is used to blast the liquid into droplets with sizes controlled by the air flowrate and the presence of baffles to avoid the escape of large droplets. In ultrasonic nebulisers, piezoelectric crystals underneath a liquid reservoir vibrate at ultrasonic frequencies, propagating mechanical energy through the liquid to generate aerosols. Piezoelectric crystals were also used in vibrating mesh nebulisers, in which the ultrasonic vibration is used to drive a mesh plate with many tiny oriﬁces in direct contact with the liquid reservoir, pressuring out a mist of ﬁne droplets. These mechanisms have different signiﬁcance to the formulations. Due to collection of large droplets by the baffles jet nebulisers often result in drug recirculation in which prolonged nebulisation may impair structural integrity of biopharmaceuticals. Ultrasonic nebulisers may not be suitable because the vibration in piezoelectric crystals usually generate heat which can increase the temperature of the liquid signiﬁcantly to cause thermal denaturation of biomolecules [67]. Vibrating mesh nebulisers generate less heat in the liquid, thus reducing the potential for thermal or chemical degradation. However, nebulisers based on ultrasonic vibration may not be powerful enough to aerosolise formulations of pDNA polyplexes even at a moderate concentration of 0.2 mg/ml, because of high liquid viscosity (at 6.3 ± 0.1 cP, whereas water has a viscosity of 1 cP at 20 °C) [68]. Presence of large aggregates in the formulation may also hinder liquid transfer through the micron-sized oriﬁces of vibrating-mesh nebulisers [69]. Regardless of the nebulisation principle, in situ degradation during aerosolization remains a major formulation consideration for nebulisation, as biopharmaceuticals including CRISPR/Cas9 therapeutics are prone to degradation when exposed to hydrodynamic shear stress [70]. Examples of formulation strategies of biomolecules relevant to CRISPR/Cas9 cargoes for nebulisation are presented below.

4.1.1. pDNAs

The feasibility of nebulising pDNA into inhalable aerosols was demonstrated over two decades ago, as Schwarz et al., Eastman et al. and other groups reported aerosolization of pDNA conjugated to cationic lipids using jet nebulisers with bioactivity retained both in vitro [71,72] and in vivo [73,74]. These earlier studies emphasised more on the effects of different nebulisation processes on the transfection efficiency of the pDNA lipoplexes. It was shown that the efﬁciency of transfection decreased over the course of nebulisation, with only about 25% to 35% of initial activity remained at 10 min for both the Aerotech II and the Puritan-Bennett 1600 nebulisers [71]. The degradation could be partially mitigated by increasing reservoir volume or decreasing ﬂow rate. With optimisation of the ratio between the cationic lipid carrier and pDNA, it was possible to maintain the integrity of the complex over the nebulisation process [74]. Manunta et al. reported the nebulisation of nanocomplexes comprising cationic liposomes containing pDNA and receptor-targeting peptides, named as receptor-targeted
nanocomplex (RTN) [75]. A total of three nebulisers were tested, and the RTN system was most effective in terms of transfection efficiency when nebulised by the AeroEclipse® II BAN air-jet nebuliser. The deposition pattern of the RTN aerosol in pigs was then visualised using technetium-99 m labelled radiopharmaceuticals [54]. It was found that the nebulised radio vect oions were primarily deposited in the trachea-main bronchi and in the middle region of the lungs, with the scintigraphy images correlating to the plasmid biodistribution. In a study comparing the performance of four different nebulisers, the breath-actuated AeroEclipse® II nebuliser was also selected as the preferred device in the clinical studies of the pulmonary delivery of the cationic lipid GL67A-conjugated pGM169 plasmid, a CFTR gene therapy in patients with CF [76,77].

Research on formulations for ultrasonic nebulisers also yielded some success [78,79], with majority of subsequent studies on vibrating mesh nebulisers. Utilising a computational fluid dynamics model, Arulimuthu et al. considered the strain rates at the nozzle exit and demonstrated the feasibility of aerosolising plasmid DNA using MicroAIR® mesh nebuliser with a high (10^5 s^-1) strain rate [80]. Although the supercoiled structure of plasmid DNA was retained for smaller plasmids with size 5.7 kb, disintegration of the supercoiled structure was observed in atomic force microscopy for larger plasmids (20 kb), necessitating the use of transfection agents such as polyethyleneimine to protect the genes by electrostatic interactions. As the expression cassette of SpCas9 plasmid is approximately 4.2 kb in size and the total plasmid size is about 7 to 10 kb after the addition of plasmid backbone and other expression elements [11], vibrating mesh nebulisers may be more suitable for nebulising CRISPR/Cas9-based therapeutics. Besides, mannitol (1% w/v) has been shown to protect both the naked pDNA or pDNA conjugated with cell penetrating peptide (CPP) after 4 min of nebulisation. There was an increase in fine particle fraction (<5 µm) of the bioactive supercoiled pDNA of the former and improved robustness towards shear forces of the latter, as evaluated using both the Next Generation Impactor (NGI) and a modified Twin Stage Impinger with Transwell attached [81]. Alternatively, newer form of nebulisers such as the surface acoustic wave nebuliser have improved power efficiency and can be operated at a low power (<1 W), allowing the aerosolization of pDNA of size 6 kb in its naked form without compromising structural integrity [82]. Furthermore, in vivo gene expressions of the nebulised pDNA have been demonstrated in mice, rats and sheep.

Zhang et al. recently published the first proof-of-concept study demonstrating the potential of nebulisation of pSpCas9 plasmids [14]. In this study, poly(ethylene glycol) (PEG) monomethyl ether with different molecular weights was conjugated chitosan. Chitosan is a natural polymer that has been widely used as a non-viral delivery vector because it is biocompatible, biodegradable and is generally non-toxic even in large doses [83]. Its mucoadhesive property has been utilised in oral and nasal gene delivery for vaccination [84]. However, this property has become a disadvantage for pulmonary gene delivery especially in CF patients where there is a hypersecretion of mucus. The conjugation with PEG monomethyl ether was therefore an attempt to overcome its mucoadhesive property. Results have been encouraging as the PEGylated chitosan/DNA nanocomplexes exhibiting mucus permeation capability in a Transwell assay, which was absent in the chitosan/DNA complexes without conjugation. The plasmids were also protected from both the digestion of DNase I and the shear stress induced by a mesh nebuliser during aerosolization, however, the experimental details regarding nebulisation were not given. The transfection efficiency was demonstrated by in vitro assay using HEK293 cell line, even under a slightly acidic environment (pH 6.5 to 6.8) which resembled that of the mucus in the airway. PEGylation was shown to result in a lower cellular transfection efficiency compared to the non-PEGylated counterparts, which was partly attributable to the shielding effect by the PEG molecules. The overall efficiency of the delivery system, however, depends not only on the cellular transfection efficiency but also the stability during aerosolization and the capability of mucus penetration. Regardless, this non-toxic carrier system demonstrated the possibility of inhaled genome editing therapy.

4.1.2. mRNAs

Compared to plasmids, the concept of utilising mRNA as a therapeutic agent is relatively new, and therefore studies on the nebulisation of mRNA for pulmonary delivery has been quite limited. Johler et al. investigated the transfection efficiency of nebulised in vitro transcribed (IVT) mRNA in human bronchial epithelial cell lines (16HBE), using cationic lipids (Lipofectamine 2000 and DMRIE-C, a 1:1 (M/M) liposome formulation of DMRIE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methacryloyl]) and cholesterol) and cationic polymers (polyethyleneimine, PEI in branched and linear form) to form lipoplexes and polyplexes, respectively [85]. An air-jet nebuliser (PARI Boy®) was used, and it was shown that upon conjugation with these cationic vectors IVT mRNA could resist the shear stress induced during air-jet nebulisation. Nebulisation did not result in a significant reduction in transfection efficiency for the polyplexes. Interestingly, lipoplexes still exhibited a much higher overall transfection rate than the polyplexes despite the significant reduction after nebulisation. This has been attributed to the stronger affinity between polynucleotides and mRNA which impeded mRNA from the polyplexes and impaired transfection efficiency [86]. Neither the cytotoxicity of the cationic complexes nor the duration of protein expression was adversely affected by the nebulisation process. Patel et al. recently reported the use of a chemically engineered hyperbranched poly(beta amino esters) (hPBAs) as an biodegradable, cationic polymers for the delivery of IVT mRNA by nebulisation [87]. Using a vibrating mesh nebuliser (AeroNeb® from Aerogen Inc.) and a whole-body nebulisation chamber, the group achieved a uniform distribution of luciferase mRNA throughout all five lung lobes in C57BL/6 mice 24 h post administration. The nebulised mRNA was highly localised to the lung with no systemic luminescence observed. This first in vivo study suggested that nebulised delivery of IVT mRNA may serve as a clinically relevant delivery system to lung epithelium. The delivery vectors and nebulisers used to nebulise pDNA and mRNA were summarised in Table 1.

4.1.3. Proteins

Pulmonary delivery of therapeutic proteins has always been of high therapeutic interest primarily because of its non-invasiveness and reduced degradation activity, when compared with other administration routes such as systemic injection and oral administration [88]. Dornase alfa, a recombinant human DNase indicated for patients with CF, was the first and only protein being approved by pulmonary delivery for local therapeutic effects in the lungs. Since its use in 1993 as an adjuvant therapy, the qualities of life of the patients have been drastically improved. Currently, more than 18 inhaled protein therapeutics have reached clinical trials, with indications on both major respiratory disorders such as asthma and pneumonia and rare or orphan lung diseases like CF and alpha-1 antitrypsin deficiency [65]. While the design of formulations suitable for nebulisation of proteins is highly protein specific [89], the formulations can remain relatively simple. By utilising surface acoustic waves, simple aqueous solution of epidermal growth factor receptor (EGFR) monoclonal antibodies at 200 μg/ml can be readily nebulised into fine aerosols with a mass median aerodynamic diameter of about 1.1 μm as measured by the NGI [90]. The protein integrity after nebulisation was evaluated qualitatively using gel electrophoresis. Flow cytometry with A549 cell lines showed that the antigen binding capability of the antibody was retained, and phosphorylation in A431 human epidermoid carcinoma cells overexpressing EGFR was reduced. On the other hand, commercially available preparation of interferon-γ for injection (Imuin® from Boehringer Ingelheim) could be directly nebulised using two types of vibrating mesh nebuliser without sacrificing structural integrity and bioactivity [91]. The post-nebulisation stability of the protein was assessed by both gel electrophoresis and in vitro assay. The overall strategies employed in stabilising monoclonal
antibodies may well be adapted and optimised for Cas9 nuclease formulations [92]. Surfactants act through replacing protein molecules at the air-liquid interface during nebulisation [93,94]. Sugars, such as trehalose and mannitol, as well as polyethylene glycols (PEG) stabilise proteins by steric hindrance, hence preventing protein aggregations [65]. The role of amino acids in improving stability of protein formulations has also been well recognised, although the actual stabilising mechanisms remain to be elucidated [95].

4.2. Dry powder formulations

Whilst nebulisation, due to its simple formulation design, remains the obvious choice for many scientists utilising genome-editing as a research tool to study genetic disorders or explore novel therapeutic options, inhalable dry powder formulations are preferable from the patient usage perspective. Solid aerosols offer distinct advantages over their liquid counterparts in providing i) better chemical stability of the biomolecules, ii) less transport and storage costs, and iii) versatility in the design of formulations by advanced particle engineering techniques to serve a variety of product criteria. Clinically, dry powder inhalers (DPI) are often better accepted by patients because they do not require an external power source, small in size and relatively easy to use. The use of nebulisers has also been discouraged, especially in an clinical setting for patients with respiratory diseases or infection, because of its potential risk of spreading air-borne pathogens including the severe acute respiratory syndrome-related coronavirus (SARS-CoV) [96–98] and potentially the SARS-CoV-2 outbreak from Wuhan, China. The formulation challenges in fabricating biopharmaceuticals into inhalable dry powders arise from various aspects. As the bioactivities of nucleic acids or proteins solely tie to their intricate structures that are prone to chemical denaturation and thermal or mechanical stresses, not all manufacturing processes are suitable. These biopharmaceuticals also differ from small potent molecules that have been dissolved in a miscible organic solvent (SFD) and supercritical fluid (SCF) drying are the three methods that have been mostly investigated [102,103]. SD involves atomising a feed solution into a fine spray followed by drying of droplets in one single continuous step. Different process parameters can be fine-tuned to achieve the desirable particle attributes. As standardised spray dryers are available at various production scales, spray drying represents a popular drying process to pharmaceutical manufacturing [104], including inhalation products of insulin and mannitol. SFD consists of atomisation of the feed solution into a cryogen, followed by lyophilisation of the frozen droplets [105]. The porous nature of the resultant particles improves their aerosol performance by their low density and surface roughness. On the other hand, SCF drying utilises mostly supercritical carbon dioxide as an anti-solvent to precipitate biomolecules that have been dissolved in amiscible polar organic solvent such as alcohol.

Given that there have been no published reports on the preparation of dry powder formulations of CRISPR/Cas9-based therapeutics for inhalation, the following sections shall focus on published literatures on inhalable dry powder formulations of biopharmaceuticals that are highly relevant to the delivery of CRISPR/Cas9 systems, namely plasmid DNA, RNA and protein.

4.2.1. pDNAs

The first reported respirable formulation of pDNAs could be dated back to 2002, when Seville et al. proposed the feasibility of utilising SD to manufacture respirable powders of pDNA conjugated with a non-viral lipid-polyacrylamide-liposome delivery vector [106]. Subsequently, the effects of non-condensing sugars [107] or amino acids [108–110] on improving the spray dried powder dispersibility and pDNA stability have been reported. During the same period, Okamoto et al. in Japan began their investigation on employing SCF drying to prepare inhalable dry powder of pDNA. They reported the preparation of chitosan-pDNA complex powders by SCF drying [111], and later confirmed their improved pDNA stability during manufacturing and storage for up to 4 weeks [112]. The in vivo pulmonary delivery and gene expression upon the administration of the pDNA powders in mice was then evaluated using fluorescence label pDNA [113]. On the other hand, while the potential of preparing pDNA dry powder by SFD has been briefly explored by Kuo et al. in 2004 [114], it was not until several years later when Mohri et al. attempted to develop and optimise the formulations of chitosan-conjugated pDNA [115]. Subsequent investigations focused on studying the effect of bovine serum albumin and the amino acid leucine as a lyoprotectant during freeze drying [116,117], as well as the use of alternative biodegradable polycarbonate as the transfection vector [118]. Liang et al. compared the performances of inhalable dry powders of pDNA...
conjugated to cationic pH-responsive peptides prepared by SD and SFD, with a more thorough evaluation on their aerodynamic properties [119]. The in vitro transfection bioactivity of the powders was also demonstrated. Ito et al. recently reported that high in vivo gene expression level could be achieved even by naked pDNA powder by SFD using low-molecular-weight hyaluronic acid (LHA) as an excipient [120]. Possible reasons included the presence of a specific intracellular uptake mechanism via a receptor, and LHA exhibits favourable inhalation characteristics as an excipient for inhalation powder. The storage stability of this formulation in terms of aerosol performance and gene expression has also been recently demonstrated [121]. The aerosol performance, the structural integrity and the in vivo gene expression activity of the powders under 25 °C and a dry condition were found to be stable over a storage period of up to 12 months. As efficient and safe gene delivery vectors are still much sought-after, the abolishment of transfection agents desirably eliminated their associated toxicities.

4.2.2. mRNAs

The other form of CRISPR/Cas9 therapeutics is the administration of mRNA that encodes Cas9 nucleases. In contrast to pDNA, only limited studies on formulating RNAs into dry powders for inhalation have been reported. Most of these studies investigated powder formulations of short interfering RNA (siRNA), a non-coding double-stranded RNA consisting 20–25 base pairs that silences gene expression through RNA interference (RNAi) [122,123]. Inhalable siRNA dry powders have been successfully engineered using SD [124–128], SFD [129–132] or SCF drying techniques [133]. Both the in vitro and in vivo bioactivity have been retained, and satisfactory aerosol performance demonstrated. While some of these formulation strategies can potentially be applied in developing inhalable dry powder formulations of RNA-based CRISPR/Cas9 therapeutics, it should be noted that the fundamental structural differences between siRNA and mRNA or sgRNA, particularly in terms of their length and strandedness, could be decisive to the success of RNA powder formulations. Recently, Qiu et al. have successfully formulated and reported the first inhalable mRNA dry powder by both SD and SFD [134]. PEGylated synthetic cationic KL4 peptides were used as the transfecting agent through electrostatic interactions with the negatively charged mRNAs. While there was no specific optimisation on the drying conditions, by adopting the operation parameters developed for siRNA formulations [124,129], the bioactivity of the peptide/mRNA complexes were preserved upon drying using both techniques. Using a luciferase mRNA as the model gene, intratracheal administration of the peptide/mRNA complexes dry powder resulted in luciferase expression in the deep lung region of mice 24 h post transfection. The powders also exhibited satisfactory aerosol performance, demonstrating a fine particle fraction (fraction of particles with aerodynamic diameters of 5 µm or below) of 41% and 68% for the spray dried powders and the spray freeze dried powders, respectively. Importantly, the powder formulations did not show signs of inflammation in the lungs. This study served as a milestone in demonstrating the feasibility and potential of formulating mRNA therapeutics into inhalable dry powders. Delivery vectors, excipients and preparation methods used to prepare powder formulations of plasmid DNAs and mRNAs which are relevant for pulmonary delivery of CRISPR/Cas9 were summarised in Table 2.

4.2.3. Proteins

Similar to genes, the pulmonary delivery of proteins in dry powder form has been of great interest since it allows both the local treatment of respiratory disorders or as a non-invasive route to systemic drug delivery and elimination of risks of biochemical instability in liquid formulations [135,136]. Earlier investigations include the preparation of recombinant human granulocyte–colony stimulating factor (G-CSF or filgrastim) [137] and recombinant human deoxyribonuclease (rhDNase or dornase alfa) [138–140] by spray drying. rhDNase is the first recombinant human protein approved for therapeutic use through inhalation with a molecular mass of approximately 37 kDa. It is indicated as an adjuvant therapy for the management of CF by hydrolysing the DNA in sputum of CF patients and thus reducing sputum viscoelasticity. The development of insulin into inhalable dry powders has also received much attention and continued to be an area of active research [141–144]. It promised to eliminate subcutaneous injection of insulin in patients of diabetes mellitus, and to date there have been two products commercialised (Exubera® from Pfizer and Afrezza® from MannKind Corporation). Nonetheless, it should be noted that comparing to Cas9 nucleases, insulin is substantially smaller with only 51 amino acids and is considered as a peptide hormone, which can present a significantly different challenge in formulation engineering.

Subsequent research on developing respirable dry powder formulations of proteins has often been focusing on monoclonal antibodies. Omalizumab, a humanised IgG1k monoclonal antibody that binds to human immunoglobulin E (IgE), is another recombinant protein that was indicated for patients with moderate to severe persistent asthma. Maa et al. conducted a series of investigations on the spray drying of omalizumab into inhalable dry powders [145]. The effect of operating conditions, formulation excipients and subsequent processing conditions on protein stability, powder morphology, powder aerosol performance and powder residual moisture has been evaluated [146–150]. Amino acids [151,152], sugars [153–157] [158], surfactants [88,159], cyclodextrin [160,161] and polymers [162] are examples of excipients used to promote protein stability and improve powder characteristics. Faghifi et al. characterised and optimised an inhalable powder formulation of IgG antibodies by a design of experiment approach on the levels of three excipients (sucrose, trehalose and tween 20) [163]. Utilising an ovalbumin-challenged mice model, the group later demonstrated that the bioactivity of infliximab was preserved upon spray drying as reflected by a reduced TNFα (tumour necrosis factor alpha) secretion in lung tissues [164]. As an alternative to SD, SFD has gained popularity recently [157,161]. Monoclonal antibodies like omalizumab and adalimumab has been formulated into inhalable dry powders by SFD technique [145,152]. The presence of amino acids leucine or phenylalanine helped retaining adalimumab stability during SFD and over a 3-month storage under accelerated conditions. Excipients and preparation methods used to prepare dry powder formulations of protein were summarised in Table 3. To date, there has been no published study on the preparation of Cas9 nuclease into inhalable dry powders. Cas9 nuclease has a bilobed structure comprising a target recognition lobe and a nuclease lobe [165], and is morphologically different to monoclonal antibody that consists of two pairs of light chains and heavy chains in an Y-shape formation. The size of Cas9 nuclease (from Streptococcus pyogenes, SpCas9) is 162 kDa, and is larger than that of monoclonal antibodies which typically ranges between 140 and 150 kDa. While the optimal formulation design in fabricating proteins into inhalable powders is largely dependent on the physicochemical properties of the proteins, existing knowledge and experience with monoclonal antibodies can still shed some light on formulating Cas9 nuclease into powder formulations for pulmonary administration. For instance, functional excipients to enhance the physical and chemical stability of amorphous proteins can be harnessed to improve the properties of the powder formulations for Cas9 [166]. Such extrapolation of engineering strategies is also expected to be applicable for formulations of pDNAs and mRNAs. Critically, the distinction of the physicochemical properties, such as size and zeta potential, between the CRISPR/Cas9 cargoes or the cargoes-delivery vector assemblies, and those that have been reported on other biomolecules must be observed and rational adaptation has to be exercised. The size of CRISPR/Cas9 cargoes, as well as model genes and proteins that are commonly used for formulation development in the aforementioned formulation studies are summarised in Table 4. Most of the model biomolecules used for formulation development have a shorter sequence than the CRISPR/Cas9 cargoes, rendering the formulation of the latter more challenging.
5. Conclusion and prospective

Inhalation delivery of CRISPR/Cas9 as an aerosol is a manifold challenge, demanding a holistic consideration and engineering in the CRISPR/Cas9 cargoes and the accompanied delivery vector, the formulation and its manufacture, the dose required and the aerosol performance and biochemical stability (including long-term storage) of the delivery platform. Undoubtedly, these factors substantially interact with each other, and the selection of them will be affected or dictated by other factors, depending on the priorities of the formulation. For instance, the dose of the CRISPR/Cas9 therapeutics is dependent on the cargos used, and in turn by the extent of control over Cas9 expression one has to achieve, as cargos that rely on cellular transcription will result in a less predictable Cas9 activity compared to direct administration of nucleic acid. The dose will also be affected by the delivery efficiency of the vectors and will be limited by the capacity (such as the extent of mechanical stress exerted on the biomolecules during manufacturing / aerosolization and the drug payload) of the delivery platform. However, inhalation formulation and delivery technology has advanced rapidly over the past 25 years. What have been learned about aerosol delivery of biological molecules such as genes and proteins can be adapted and optimised for delivery of CRISPR/Cas9 therapeutics. A proof-of-concept study of CRISPR/Cas9 has already confirmed the feasibility of its delivery by nebulisation [14]. As the potential in CRISPR/Cas9 genome-editing tool has just been unveiled, its applications for pulmonary diseases are expected to expand. There are already investigations with propitious tool has just been unveiled, its applications for pulmonary diseases are expected to expand. There are already investigations with propitious

Table 2
Delivery vectors, excipients and preparation methods used to prepare powder formulations of plasmid DNAs and mRNAs which are relevant for pulmonary delivery of CRISPR/Cas9.

| Genes          | Delivery Vectors | Excipients | Preparation | Reference |
|----------------|------------------|------------|-------------|-----------|
| Plasmid DNA    | pCMV-Luc         | Chitosan   | Mannitol    | SFD       | [111,112] |
| pCMV-Luc       | Chitosan         | Mannitol   | SD          | [115]     |
| pCMV-Luc       | Chitosan         | Lactose    | SFD         | [113]     |
| pEGFP-N1       | DOTAP, cholesterol | Bovine serum albumin, leucine, mannitol | SFD | [116] |
| pEGFP-N1       | DOTAP, protamine sulphate | Lactose | SD, FD | [106] |
| pEGFP-N1       | DOTAP, protamine sulphate | Leucine, lactose | SD | [108] |
| pEGFP-N1       | DOTAP, protamine sulphate | Arginine, aspartic acid, phenylalanine, threonine | SD | [109] |
| pEGFP-N1       | DOTAP, protamine sulphate | Dimethyl-β-cyclodextrin, sodium taurocholate, carntine hydrochloride, trehalose | SD | [110] |
| pEGFP-N1       | Low molecular weight chitosan | Lactose, leucine | SD, FD | [171] |
| pSGSlocZ       | PEI               | Mannitol   | Succrose    | SD        | [107] |
| pCMV-Luc       | PEI, PEG          | pH responsive peptides | Mannitol | SFD | [119] |
| pGWI™ Luc      | pH responsive peptides | Mannitol | SFD | [118] |
| pCAG-Luc       | PAsp(DET), PEG-PAsp(DET) | Leucine, mannitol | SFD | [120,121] |
| pCAG-Alu       | Naked             | Hyaluronic acid | SFD |
| mRNA           | Luciferase        | PEG-KL4 peptide | Mannitol | SFD |

Table 3
Excipients and preparation methods used to prepare powder formulations of proteins relevant for pulmonary delivery of CRISPR/Cas9.

| Proteins | Excipients | Preparation | Reference |
|----------|------------|-------------|-----------|
| IgG1     | Mannitol   | SD          | [154]     |
| IgG      | Cysteine, phenylalanine | SD | [151] |
| IgG1, κ-cyclodextrin, hydroxypropyl-α-triazinurab | Mannitol, trehalose | SD | [155,156] |
| IgG      | Cysteine, trehalose, tween 20 | SD | [163] |
| IgG      | Mannitol, trehalose | SD | [172] |
| IgG      | Cysteine, trehalose, hydroxypropyl-α-cyclodextrin, mannitol, trehalose | SFD | [161] |
| IgG      | Lactose, mannitol, trehalose | SFD | [157] |
| Infliximab | Cysteine, trehalose, tween 20 | SFD | [164] |
| Adalimumab | Leucine, phenylalanine | SFD | [152] |

IgG: Immunoglobin G

DOTAP: N-(2,3-Dioleoyloxy-1-propyl)trimethylammonium methyl sulphate; EGFP: enhanced green fluorescent protein; Luc: luciferase; PAsp(DET): poly(N-(2-aminoethyl)-2-aminooethyl)aspastamide); PEG: polyethylene glycol; PEI: polyethylenimine.
and dosage regimen. In addition, knowledge on the potential impacts of aerosolised CRISPR/Cas9 therapeutics to the environment and unintended exposure to people has been scarce, yet such an understanding is essential should the inhaled form of CRISPR/Cas9 be translated to clinical use. Last but not the least is the commercial and pharmacoeconomic aspect as developing new products of CRISPR/Cas9 therapeutics depends on the stage of product development, competition, and alternative treatments available in the market, health-care providers and health cost reimbursement arrangement. It is worth to note that genome-editing therapeutics may become the most cost-effective treatment approach if it indeed can correct the underlying disease cause without the need for repeated treatments.

Acknowledgements

H-K Chan is grateful to Mr. Richard Stenlake for the generous financial support of his research.

References

[1] D. Gupta, O. Bhattacherjee, D. Mandal, M.K. Sen, D. Dey, A. Dasgupta, T.A. Kazi, R. Gupta, S. Sinharoy, K. Achariya, D. Chattopadhyay, V. Ravichandiran, S. Roy, D. Ghosh, CRISPR/Cas9 system: a new-fangled dawn in gene editing, Life Sci. 232 (2019) 116036.
[2] A.P. Chandrasekaran, M. Song, K.S. Kim, S. Ramakrishna, Different methods of delivering CRISPR/Cas9 into cells, Prog. Mol. Biol. Transl. Sci. 159 (2018) 157–176.
[3] R. Barrangou, C. Fremeaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, D.A. Raouf, P. Horvath, CRISPR provides acquired resistance against viruses in prokaryotes, Science 315 (2007) 1709–1712.
[4] D.R. Rodriguez-Rodriguez, R. Ramírez-Solís, M.A. Garza-Elizondo, M.L. Garza-Rodriguez, H.A. Herrera-Saldaña, Genome editing: a perspective on the application of the CRISPR/Cas9 to study human diseases (review), Int. J. Mol. Med. 43 (2019) 1559–1574.
[5] P.D. Hsu, E.S. Lander, F. Zhang, Development and applications of CRISPR/Cas9 for genome engineering, Cell 157 (2014) 1262–1278.
[6] L. Zhang, J. Zhou, D. Gao, S. Ehteshami-Afshar, J.M. FitzGerald, M.M. Doyle-Waters, M. Sadatsafavi, The global burden of COPD: the effects of risk factor exposure: an update of a previous report from the Global Lung Initiative 2017, Eur. Respir. J. 52 (2018) 1151–1159.
[7] A.H. de Boer, P. Hagedoorn, F. Buttini, F. Grasmeijer, H.W. Frijlink, Dry powder inhalation: past, present and future, Expert. Opin. Drug Deliv. 14 (2017) 499–512.
[8] L. Borgstrom, B. Olsson, L. Thorsen, Degree of throat deposition can explain the variability in lung deposition of inhaled drugs, J. Aerosol. Med. 19 (2006) 473–483.
[9] C.A. Brandsma, M. Van den Berge, T.L. Hackett, G. Brusselle, W. Timens, Recent advances in chronic obstructive pulmonary disease pathogenesis: from disease mechanisms to precision medicine, J. Pathol. 250 (5) (2020) 624–635, https://doi.org/10.1002/path.5364 Epub 2019 Dec 3.
[10] L. Li, S. Hu, X. Chen, Non-viral delivery systems for CRISPR/Cas9-based genome editing: challenges and opportunities, Biomaterials 171 (2018) 207–218.
[11] C.A. Lino, J.C. Harper, J.P. Carney, J.A. Timlin, Delivering CRISPR: a review of the challenges and approaches, Drug Deliv. 25 (2018) 1234–1257.
[12] C.L. Xia, M.Z.C. Ruan, V.B. Mahajan, S.H. Tsang, Viral delivery systems for CRISPR, Vi- nes 11 (2019).
[13] K. Chen, S. Jiang, Y. Hong, Z. Li, Y.-L. Wu, C. Wu, C. Catonic polymer nanofoma- tion: recent advances in material design for CRISPR/Cas9 gene therapy, Progress Nat. Sci. Mater. Int. 29 (2019) 617–627.
[14] H. Zhang, T.F. Bahamonde-Canas, Y. Zhang, J. Leal, H.D.C. Smyth, PEGylated chito- san for nonviral aerosol and mucosal delivery of the CRISPR/Cas9 system in vitro, Mol. Pharm. 15 (2018) 4814–4826.
[15] O.M. Merkel, I. Rubinstein, T. Kissel, siRNA delivery to the lung: what's new? Adv. Drug Deliv. Rev. 75 (2014) 112–128.
[16] C. Loira-Pastoriza, J. Todoroff, R. Vanbever, Delivery strategies for sustained drug release in the lungs, Adv. Drug Deliv. Rev. 75 (2014) 81–91.
[17] W.D. Bennett, J.S. Brown, K.L. Zeman, S.C. Hu, G. Scheuch, K. Sommerer, Targeting delivery of aerosols to different lung regions, J. Aerosol. Med. 15 (2002) 179–188.
[18] U. Griesenbach, E.W. Alton, Expert opinion in biological therapy: update on develop- ments in lung gene transfer, Expert. Opin. Biol. Ther. 13 (2013) 345–360.
[19] Y. Liu, H. Li, N. Ding, N. Wang, D. Wen, Functional status assessment of patients with COPD: a systematic review of performance-based measures and patient-reported measures, Medicine (Baltimore) 95 (2016), e3672.
[20] S.A. Cryan, N. Sivadas, L. Garcia-Contreras, In vivo animal models for drug delivery across the lung mucosal barrier, Adv. Drug Deliv. Rev. 59 (2007) 1133–1151.
[21] H.L. Li, W. Liang, L. Han, Pulmonary delivery of therapeutic siRNA, Adv. Drug Deliv. Rev. 64 (2012) 1–15.
[22] W. de Kruijf, B. Olsson, L. Thorsen, Overcoming biological barriers-therapeutic possibilities and technological challenges, Lancet Respir. Med. 1 (2013) 401–403.
[23] B. Button, L.H. Cai, C. Ehre, M. Kesimer, D.B. Hill, J.K. Sheehan, R.C. Boucher, M. Rubinstein, A periciliary brush promotes the lung health by separating the mucus layer from airway epithelia, Science 337 (2012) 937–941.
[24] J.E. Duncan, J.A. Whitset, A.D. Horovitz, Pulmonary surfactant maintains cationic liposome-mediated gene delivery to respiratory epithelial cells in vitro, Hum. Gene Ther. 9 (1997) 4341–4347.
[25] S.A. Cryan, N. Sivadas, L. Garcia-Contreras, In vivo animal models for drug delivery across the lung mucosal barrier, Adv. Drug Deliv. Rev. 59 (2007) 1133–1151.
[26] H.L. Li, W. Liang, L. Han, Pulmonary delivery of therapeutic siRNA, Adv. Drug Deliv. Rev. 64 (2012) 1–15.
[27] Y. Jónsson, P.W. Longest, G. Tian, M. Hindle, Evaluation and modification of commer- cial dry powder inhalers for the aerosolization of a submicrometer excipient en- hanced growth (EEG) formulation, Eur. J. Pharm. Sci. 49 (2013) 390–399.
[109] H.Y. Li, P.C. Seville, J.I. Williamson, J.C. Birchall, The use of amino acids to enhance the aerosolisation of spray-dried powders for pulmonary gene therapy, J. Gene Med. 3 (2001) 433–441.

[110] H.Y. Li, P.C. Seville, J.I. Williamson, J.C. Birchall, The use of absorption enhancers to enhance the dispersibility of spray-dried powders for pulmonary gene therapy, J. Gene Med. 7 (2005) 1035–1043.

[111] H.Y. Li, P.C. Seville, T. Okuda, Y. Sakakura, K. Iida, K. Danjo, Pulmonary gene delivery by chitosan-PDNA complex powder prepared by a supercritical carbon dioxide process, J. Pharm. Sci. 92 (2003) 371–380.

[112] H. Okamoto, Y. Sakakura, K. Shiraiki, K. Oka, S. Nishida, H. Todo, K. Iida, K. Danjo, Stability of chitosan-PDNA complex powder prepared by supercritical carbon dioxide process, Int. J. Pharm. 290 (2005) 73–81.

[113] T. Mizuno, K. Mohri, S. Nasu, K. Danjo, H. Okamoto, Dial imaging of pulmonary delivery and gene expression of dry powder inhalant by fluorescence and bioluminescence assays, J. Control. Release 134 (2009) 149–155.

[114] J.H. Koo, R. Hwang, Preparation of DNA dry powder for non-viral gene delivery by spray-freeze drying: effect of protective agents (polypeylineiminium and sugars) on the stability of DNA, J. Pharm. Pharmacol. 56 (2004) 27–33.

[115] T. Ito, T. Okuda, A. Mori, K. Danjo, H. Okamoto, Optimized pulmonary gene transfection in mice by spray-freeze dried powder inhalation, J. Control. Release 144 (2010) 221–226.

[116] M. Tsukamoto, T. Okuda, H. Okamoto, Y. Higuchi, S. Kawakami, F. Yamashita, M. Hashida, Bovine serum albumin as a lyoprotectant for preparation of DNA dry powder formulations using the spray-freeze drying method, Bio Pharm. Bull. 35 (2012) 1178–1181.

[117] Y. Suzuki, T. Okuda, H. Okamoto, Development of new formulation dry powder for pulmonary delivery using amino acids to improve stability, Bio Pharm. Bull. 39 (2016) 394–400.

[118] T. Okuda, Y. Suzuki, Y. Kobayashi, T. Ishii, S. Uchida, K. Itaka, K. Kataoka, H. Okamoto, Development of biodegradable polycation-based inhalable dry gene powder formulation by spray drying vs. freeze drying, Pharm. Res. 26 (2009) 231–234.

[119] W. Liang, P.C. Kwok, M.Y. Chow, P.T. Tung, A.J. Mason, H.K. Chan, J.K. Lam, Formulation of pH responsive peptides as inhalable dry powders for pulmonary delivery of nucleic acids, Eur. J. Pharm. Biopharm. 86 (2014) 64–73.

[120] T. Ito, T. Okuda, Y. Katsukawa, H. Okamoto, Naked siRNA inhalation powder composed of hyaluronic acid exhibits high gene expression in the lungs, Mol. Pharm. 16 (2019) 489–497.

[121] T. Ito, M. Fukuhara, T. Okuda, H. Okamoto, Naked cDNA powder shows excellent long-term storage stability and gene expression in murine lungs, Int. J. Pharm. 574 (2020), 118880.

[122] Y. Qiu, J.K. Lam, S.W. Leung, W. Liang, Delivery of RNAi therapeutics to the airways: from bench to bedside, Molecules 21 (2016).

[123] M.Y.T. Chow, Y. Qiu, F.F.K. Lo, H.H.S. Lin, H.K. Chan, P.C.L. Kwok, J.K.W. Lam, Inhaled siRNA-containing PLGA nanoparticles intended for inhalation, J. Control. Release 314 (2019) 102–119.

[124] V. Ramezani, A. Vatanara, A.R. Najafabadi, M.A. Shokrgozar, A. Khabiri, M. Amini Pouya, S. Aghababaie, A. Vatanara, Preparation, characterization, and pharmacodynamics of insulin-loaded fumaryl diketopiperazine microparticle dry powder inhalation drug, Drug Deliv. 26 (2019) 650–660.

[125] K.T. Ung, N. Rao, J.G. Weers, D. Huang, H.K. Chan, Design of spray-dried insulin microparticles to bypass deposition in the extrathoracic region and maximize total lung dose, Int. J. Pharm. 511 (2016) 1070–1079.

[126] H.Y. Li, P.C. Seville, I.J. Williamson, J.C. Birchall, The use of amino acids to enhance the aerosolisation of spray-dried powders for pulmonary gene delivery, J. Gene Med. 3 (2001) 433–441.

[127] H.K. Chan, A. Clark, I. Gonda, M. Mummenthaler, C. Hsu, Spray dried powders and powder blends of recombinant human deoxyribonuclease (rhDNase) for aerosol delivery, Pharm. Res. 14 (1997) 431–437.

[128] H.K. Chan, I. Gonda, Solid state characterization of spray-dried powders of recombinant human deoxyribonuclease (rhDNase), J. Pharm. Sci. 87 (1998) 647–654.

[129] S.S. Zijlstra, B.J. Possenius, S.A. Hummel, N. Sanders, W.L. Hinrichs, A.H. de Boer, H.W. Frijlink, Formulation and process development of (recombinant human) deoxyribonuclease I as a powder for inhalation, Pharm. Dev. Technol. 14 (2009) 358–368.

[130] E.M. Wilson, J.C. Luft, J.M. DeSimone, Formulation of high-performance dry powder aerosols for pulmonary protein delivery, Pharm. Res. 35 (2018), 195.

[131] A.C. Anselmo, Y. Gokarn, S. Mitragotri, Non-invasive delivery strategies for biologics, Nat. Rev. Drug Discov. 18 (2019) 19–40.

[132] O.N. O’Gain, F.D. Lott, J.M. Cribbs, Pulmonary delivery of powders and solutions containing recombinant human granulocyte colony-stimulating factor (rhG-CSF) to the rabbit, Pharm. Res. 11 (1994) 1101–1109.

[133] H.K. Chan, A. Clark, I. Gonda, M. Mummenthaler, C. Hsu, Spray dried powders and powder blends of recombinant human deoxyribonuclease (rhDNase) for aerosol delivery, Pharm. Res. 14 (1997) 431–437.

[134] K. Miwata, H. Okamoto, T. Nakashima, D. Ihara, Y. Horimasu, T. Masuda, S. Y. Qiu, R.C.H. Man, Q. Liao, K.L.K. Kung, M.Y.T. Chow, J.K.W. Lam, Effective mRNA delivery of high-performance dry powder aerosols for pulmonary protein delivery, Pharm. Res. 35 (2018), 195.
[163] H. Faghihi, A.R. Najafabadi, A. Vatanara, Optimization and characterization of spray-dried IgG formulations: a design of experiment approach, Daru 25 (2017), 22.

[164] H. Faghihi, A.R. Najafabadi, Z. Daman, E. Ghasemian, H. Montazeri, A. Vatanara, Respiratory administration of infliximab dry powder for local suppression of inflammation, AAPS PharmSciTech 20 (2019), 128.

[165] H. Nishimasu, F.A. Ran, P.D. Hsu, S. Konermann, S.I. Shehata, N. Dohmae, R. Ishitani, F. Zhang, O. Nureki, Crystal structure of Cas9 in complex with guide RNA and target DNA, Cell 156 (2014) 935–949.

[166] L. Chen, T. Okuda, X.Y. Lu, H.K. Chan, Amorphous powders for inhalation drug delivery, Adv. Drug Deliv. Rev. 100 (2016) 102–115.

[167] C. Ghosh, P. Sarkar, R. Issa, J. Haldar, Alternatives to conventional antibiotics in the era of antimicrobial resistance, Trends Microbiol. 27 (2019) 323–338.

[168] Z. Xu, M. Li, Y. Li, H. Cao, L. Miao, Z. Xu, Y. Higuchi, S. Yamasaki, K. Nishino, P.C.Y. Woo, H. Xiang, A. Yan, Native CRISPR-Cas-mediated genome editing enables dissecting and sensitizing clinical multidrug-resistant P. aeruginosa, Cell Rep. 29 (2019) 1707–1717 (e1703).

[169] A.C. Greene, CRISPR-based Antibacterials: transforming bacterial Defense into offense, Trends Biotechnol. 36 (2018) 127–130.

[170] A. Guillon, T. Secher, L.A. Dailey, L. Vecellio, M. de Monte, M. Si-Tahar, P. Diot, C.P. Page, N. Heuze-Vourc'h, Insights on animal models to investigate inhalation therapy: relevance for biotherapeutics, Int. J. Pharm. 536 (2018) 116–126.

[171] N. Mohajel, A.R. Najafabadi, K. Azadmanesh, M. Ansari, A. Vatanara, E. Moazeni, A. Rahimi, K. Gilani, Drying of a plasmid containing formulation: chitosan as a protecting agent, Daru 20 (2012), 22.

[172] V. Carli, L. Menu-Bouaouiche, P. Cardinael, L. Benissan, G. Coquerel, Immunoglobulin G particles manufacturing by spray drying process for pressurised metered dose inhaler formulations, Ann. Pharm. Fr. 76 (2018) 291–298.