Expert Opinion

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Challenges and advances in the development of inhalable drug formulations for cystic fibrosis lung disease

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Introduction: Cystic fibrosis (CF) is a multisystem genetic disorder, which usually results in significant respiratory dysfunction. At present there is no cure for CF, but advances in pharmacotherapy have gradually increased the life expectancy of CF patients. As many drugs used in the therapy of CF are delivered by inhalation, the demand for effective and convenient inhalational CF drug formulations will grow as CF patients live longer. Knowledge of the current limitations in inhalational CF drug delivery is critical in identifying new opportunities and designing rational delivery strategies.

Areas covered: This review discusses current and emerging therapeutic agents for CF therapy, selected physiological challenges to effective inhalational medication delivery, and various approaches to overcoming these challenges. The reader will find an integrated view of the known inhalational drug delivery challenges and the rationales for recent investigational inhalational drug formulations.

Expert opinion: An ideal drug/gene delivery system to CF airways should overcome the tenacious sputum, which presents physical, chemical and biological barriers to effective transport of therapeutic agents to the targets and various cellular challenges.

Keywords: cystic fibrosis, drug delivery system, inhalation, lung disease, sputum

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1. Introduction

Cystic fibrosis (CF) is a life-limiting autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. It affects > 30,000 people in the US [1] and is frequent in the US (1 in 3500 live births [2]), where ~ 3% are heterozygote carriers of a CFTR mutation. Although CF is more common among Caucasians, it is found in every ethnic group [1].

The *CFTR* gene encodes a cAMP-regulated Cl^- channel in the apical membrane of epithelial cells, which is responsible for the regulation of Cl^- secretion and Na^+ reabsorption. So far, ~ 1800 *CFTR* mutations have been reported [3]. They are categorized into six classes based on the specific abnormalities in the resulting protein. Of these, classes 1 and 2, where CFTR synthesis is defective or CFTR folding, processing and trafficking are abnormal, respectively, are generally associated with more severe disease [4]. The most common mutation is the deletion of phenylalanine residue at position 508 (*F508del*, *ΔF508*) (class 2), accounting for ~ 72% of mutations in patients of non-Hispanic Caucasian descent and ~ 66% in the overall CF population [5].

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Article highlights.

- Cystic fibrosis is a genetic disorder caused by mutations in CFTR, a protein that regulates ion and water movement across the epithelium.
- Cystic fibrosis is a multisystem disease, but its morbidity and mortality are mostly a result of how it affects the respiratory system.
- Cystic fibrosis management has been limited to anti-inflammatory and antimicrobial therapies, as well as optimization of airway clearance, but emerging therapies may address the underlying pathophysiology.
- Inhalational drug formulations are likely to provide a promising mode of delivery of existing and emerging therapeutic agents, but dehydrated, tenacious sputum, mucociliary clearance, and several cellular challenges need to be overcome.
- New approaches have been proposed aimed at increasing the mobility of inhaled drug formulations in the sputum or increasing drug access to the therapeutic target.
- An extra challenge in clinical translation of these new approaches is the lack of proper CF animal models.

This box summarizes key points contained in the article.

Mutations in the *CFTR* gene influence several organs, including the liver, pancreas, gastrointestinal and reproductive tracts, sweat glands, and particularly the lungs [6]. Most CF-related morbidity and mortality result from progressive deterioration of the respiratory function [7]. Owing to the absence of or dramatic decrease in the amount of functional CFTR protein, the CF airway develops critical defects. One of the prevailing theories is that reduced Cl^- secretion and uncontrolled Na^+ reabsorption result in a reduction of net amount of salts in the airway surface liquid (ASL) [8] and in a decreased amount of the ASL itself, leading to collapse of cilia and production of adhesive mucus plaques [9]. The stationary mucus in the airway results in poor airway clearance, bringing about chronic bacterial infections, inflammation, and accumulation of large amounts of purulent secretions (phlegm, of which the expectorated form is called sputum [10,11] – as most CF studies are performed with expectorated samples, herein the secretions are referred to as CF sputum throughout), which lead to progressive lung destruction and irreversible respiratory failure [7,12,13]. Some carriers of a single *CFTR* mutation also have sinopulmonary or gastrointestinal symptoms [14-19], but they do not suffer the typical devastating CF-related morbidities.

At present there is no cure for CF. However, concerted efforts including pharmacotherapy, nutrition and specialized medical care have improved the life expectancy and the quality of life of CF patients [1]. The median life expectancy of CF patients has risen steadily, from 32 years in 2000 to 37.4 years in 2008 [1]. With the increase in the number of patients living with CF, there will be a growing demand for convenient and effective delivery methods, such as inhalation, for CF medications. This review focuses on current and emerging therapeutic agents for CF therapy, selected physiological challenges that have hampered effective inhalational drug delivery, and investigational approaches to overcoming these challenges.

2. Current cystic fibrosis therapy and investigational approaches

The mainstay of the current approach to treating CF has been symptomatic therapy aimed at attenuating disease progression and delaying the onset of irreversible lung damage [12,20,21]. Antibiotics and anti-inflammatory drugs are administered to control the airway infection and inflammation. Bronchodilators are used to control bronchial hyperactivity and improve sputum clearance and airflow through the diseased airway. Mucolytics and osmotic agents followed by aggressive chest physical therapy have been effective in further improving airway clearance. Recently, molecules addressing defects in CFTR transcription, processing, or function have been developed to supplement the impaired CFTR functions or restore normal CFTR structure and function [22,23]. Following identification of the *CFTR* gene [24], gene therapy has also been explored as a fundamental CF therapy at investigational levels. Several new therapies are now in clinical trials [25]. In this section, clinically used drugs and selected exploratory agents are briefly introduced, leading to a discussion of inhalational drug formulations, an attractive mode of delivery of some of these agents.

2.1 Pharmacotherapy

2.1.1 Antibiotics

Staphylococcus aureus and *Haemophilus influenzae* are acquired at an early age, followed by *Pseudomonas aeruginosa*, the most significant bacterial pathogen in CF [26]. CF patients' airways may also harbor *Stenotrophomonas maltophilia*, *Burkholderia cepacia* complex (BCC), non-tuberculous mycobacteria (NTMB) and the fungus *Aspergillus fumigatus*. As in patients with CF bacterial and fungal infections lead to loss of lung function, the initial identification of these organisms triggers aggressive attempts at microbiological eradication. Oral and/or inhaled antibiotics are used for a protracted period of time with limited success. Once chronic infection is established, CF patients suffer from frequent pulmonary exacerbations, which are treated with oral or more frequently intravenous (i.v.) antibiotics. As patients recover, they are frequently given inhaled or oral antibiotics to suppress the growth of the infecting microorganisms.

Amoxicilline-clavulanic acid, clindamycin, linezolid, co-trimoxazole, or fluoroquinolones are used to eradicate *S. aureus*. A combination of at least two antibiotics, including i.v. aminoglycosides, i.v. or oral fluoroquinolones, and i.v. antipseudomonal β-lactams is used to eradicate *P. aeruginosa* [27]. BCC and *S. maltophilia* are treated by oral or i.v. co-trimoxazole, NTMB by oral or i.v. macrolides,

and *A. fumigatus* and other fungi by various oral or i.v. antifungals. The antifungal amphotericin B has also been administered successfully by inhalation [28].

2.1.2 Anti-inflammatories and immunomodulatories

Systemic corticosteroids and high-dose ibuprofen were the first anti-inflammatory drugs studied in CF [29]. At present, the CF Foundation recommends the routine use of ibuprofen in those CF patients over the age of 6 years whose lung function is relatively preserved. The use of inhalable corticosteroids is discouraged in CF patients unless they also display asthmatic features or have allergic bronchopulmonary aspergillosis [30].

Oral administration of azithromycin, a 15-membered macrolide, improves lung function in CF patients [31-35]. Studies suggest that the clinical benefits of azithromycin and other macrolides are independent of antimicrobial properties and rather attributable to immunomodulatory effects [29,34,36], which include modulation of ERK1/2 and downstream transcription factors [37-39].

2.1.3 Bronchodilators and mucus-thinning agents

Airway clearance is central to lung function preservation in CF. To achieve it, CF patients often receive inhalable bronchodilators such as β-agonists and anticholinergics, followed by inhaled mucus-thinning agents. A mucus-thinning agent routinely used in CF patients is human recombinant DNase (Dornase alfa, Pulmozyme®, CA, USA). The recombinant human DNase decreases the viscosity of the CF sputum [40-44] by degrading the DNA, a major component of CF sputum. Small pulmonary hemorrhages are thought to be associated with DNase use in CF patients, and many centers stop its use in patients with hemoptysis [45]; however, there is evidence that massive hemoptysis is less likely with the use of DNase [46]. The CF Foundation recommends that CF patients over the age of 6 years receive daily inhalations of DNase [30]. Unfractionated heparin (UFH) has also been proposed recently as a mucus-thinning agent, as it reduces the elasticity of the CF sputum by disrupting DNA–actin polymer interactions [44,47]. It has been shown that UFH enhances mucolytic activity of DNase, although the utility of UFH as a standalone mucolytic agent is questionable [44].

2.1.4 Airway hydrators

Hypertonic saline hydrates the ASL and decreases the viscosity of the sputum [48-53], making it easier to expectorate. The CF Foundation recommends routine use of inhaled hypertonic saline in CF patients over 6 years old [30]. The inhalable dry powder mannitol, which has recently undergone Phase III trials (ClinicalTrials.gov identifiers: NCT00446680, NCT00630812), also contributes to airway hydration [50,54-57]. Inhaled mannitol dry powder has been effective in patients with non-CF bronchiectasis [56] and CF [55,58].

2.1.5 Modifiers of CFTR activities and other ion channels

The use of modifiers of CFTR and other ion channels, aiming to circumvent the consequences of CFTR dysfunction, remains investigational, but some agents have entered advanced clinical trials [22,59]. Such modifiers include: amiloride, benzamil, or phenamil, which suppress Na^+ reabsorption [60]; the $P2Y_2$ purinergic agonists uridine triphosphate and denufosol tetrasodium, which stimulate an alternative Cl^- channel [61,62]; the benzoquinolizinium compounds and sildenafil, which increase cell-surface expression of defective CFTR [60]; and xanthines, phenanthrolines, benzimidazolones and flavonoids, which stimulate the CFTR [60]. Recently, two new compounds VX-770 (an oral agent that stimulates both the wild-type and the defective CFTR proteins [63]) and Ataluren (a compound that allows complete translation of the CFTR mRNA and induces the formation of a functional CFTR protein in patients with nonsense mutations) have completed Phase II clinical trials with exciting results [64,65]. Both compounds are undergoing Phase III clinical trials at present [66,67].

2.2 Gene therapy

Cystic fibrosis was one of the first diseases considered for gene therapy [68], and most efforts have focused on transferring the normal CFTR cDNA. Recently, anti-sense or RNA interference-mediated gene silencing has also been proposed [68]. Cellular uptake of nucleic acids is hampered by their size, charge and extracellular instability. Therefore, genetic material is delivered by means of vectors derived from viruses or as complexes with synthetic polycations. As bronchiolar epithelium is the main target of gene delivery [69], most formulations are delivered directly to the airways as aerosols [29]. Several exploratory and clinical studies have been performed as summarized below, but no gene delivery system is clinically available at present.

2.2.1 CFTR cDNA delivery

Modified viruses have been widely exploited for CF gene delivery [70]. Although viral vectors are relatively efficient, they are difficult to produce on a large scale, and they may induce a potent immune response and resistance on repeated administrations [71]. A major setback to the viral vector-based gene therapy was the death of a patient receiving adenovirus-based gene therapy in the 1999 trial of gene therapy for ornithine transcarbamylase deficiency (non-CF related) [72]. He succumbed to adenovirus-induced massive systemic inflammatory response syndrome [73]. Non-viral vectors based on cationic lipids and polymers have been developed as a relatively safe alternative. They are less immunogenic, easier to modify, and can be mass-produced. However, they are also less efficient than viral vectors [71], especially in the presence of anionic biological compounds.

Since the initial isolation and cloning of the *CFTR* gene [24], 25 Phase I/II clinical trials involving ~ 400 CF

patients have been carried out using a variety of viral and non-viral vectors [68,74]. Most of the trials used adenovirus, which showed significant adverse effects such as inflammatory response [75], radiographic pulmonary infiltrates [76] and development of humoral immunity [77]. Although the use of the adeno-associated virus was better tolerated, the resulting gene delivery efficiency was unsatisfactory [78,79]. Readers are referred to a recent review by Griesenbach and Alton [68] for more details on CFTR cDNA-based gene therapy.

2.2.2 *RNA interference-mediated gene silencing*

With the recent advances in RNA interference technology, more genetic targets are emerging as alternative therapeutic options. The inhibition targets include: NF-κB, a transcription factor regulating the expression of pro-inflammatory cytokines [80]; B-cell antigen receptor-associated proteins (BAPs), which inhibit normal trafficking of CFTR protein [81]; epithelial Na^+ channel (ENaC) [68], whose overexpression is linked to the CF lung disease [82]; and valosin-containing protein (VCP), which complexes with the CFTR protein during translocation from the endoplasmic reticulum and facilitates its cytosolic degradation [83]. Inhibition of VCP by small interfering RNA (siRNA) results in partial rescue of functional Cl^- channels to the cell surface, improving secretion and decreasing the level of the inflammatory marker interleukin-8 in the primary CF tracheal cell culture model [83]. Suppression of BAP31 protein production also restores Cl^- secretion in various cell types [81].

Although RNA interference is a promising therapeutic option for CF therapy, cellular delivery of siRNA faces several challenges. Owing to the extra hydroxyl group in the ribose backbone, siRNA is more prone to degradation by serum nucleases than DNA [84]. Moreover, it is difficult to form a compact complex between siRNA and a cationic non-viral vector [84,85] owing to the small size, low charge density and stiffness of the siRNA [86,87]. This problem has been overcome recently by forming multimerized siRNAs, which can be condensed with conventional gene carriers and cleaved into monomeric siRNA when taken up by cells [86-88].

3. Challenges in drug delivery to the cystic fibrosis lungs

As the airways are a major therapeutic target in CF, many CF drugs are delivered via inhalation. This mode of delivery ensures deposition of medications at the site of action, increasing their local availability and decreasing their systemic absorption and side effects. At present, tobramycin (TOBI®, Basel, Switzerland) and DNase (Pulmozyme) are available in the US as nebulized solutions. Inhalable dry powders of ciprofloxacin [89,90], gentamicin [91], tobramycin [92-94] and colistin [95-97] have been studied in clinical trials. Inhalable dry powder of tobramycin [92] and mannitol dry powder are expected on the US market soon [25].

On the other hand, several challenges remain to be overcome for efficient inhalational drug delivery to the CF lungs. For example, the tenacious CF sputum functions as both a physical and a chemical barrier to drug delivery into and/or across the sputum. CF sputum also presents a framework for development of bacterial resistance. On the other hand, concomitant use of mucus-thinning agents can enhance the mucociliary clearance of inhaled medications. Although not discussed in this review, several more challenges such as stability and aerodynamic properties of the formulation further complicate inhalational drug delivery to CF lungs. Moreover, the performance of an inhalable drug delivery system depends critically on the effectiveness of an inhaler device. Several efforts to improve the available inhaler devices are continuing [98-108].

3.1 Cystic fibrosis sputum

The thick, tenacious CF sputum presents a significant challenge for effective inhalational delivery of many therapeutic agents [109]. It is important to address this barrier in at least two paradigms. On one hand, in inhalational delivery of antibiotics it is important for the drug to penetrate the sputum and be evenly distributed within the sputum. On the other hand, for the delivery of drugs influencing the epithelium, such as ion-channel regulators or gene therapeutics, it is crucial to traverse the sputum and deliver the drug payload to the underlying cell layer. CF sputum is an important reason for the failure of CF gene therapy in the past 15 years of preclinical and clinical research [110]. This section briefly reviews the physicochemical properties of CF sputum that contribute to its barrier functions. The barrier properties of normal mucus and CF sputum are discussed in greater detail in recent review articles [110-112].

3.1.1 *Cystic fibrosis sputum as a physical barrier*

Normal mucus is 10 – 30 μm thick in the trachea and 2 – 5 μm in the bronchi [113,114]. Whereas gas, ions, nutrients and proteins easily diffuse through mucus, particulate substances can be entrapped and immobilized by the mucus and removed before they contact the underlying epithelial cells [112]. In this manner, mucus protects the body from invasion of foreign substances such as toxins, pathogens and environmental ultrafine particles. A typical mucus sample contains 90 – 95% water by mass [115]. The remaining mass consists of mucins (~ 2% [116]), DNA, lipids, electrolytes, proteins, cells and cell debris [117]. Mucins are high-molecular-mass glycoproteins with alternating glycosylated and cysteine-rich regions [115], produced by the epithelial goblet cells and submucosal glands [118]. Mucins are negatively charged owing to the abundant carboxyl groups at the termini of glycan and form networks via internal disulfide bonds, physical entanglement and non-covalent interactions [111]. Viscoelasticity of normal mucus is mainly attributable to mucins [112], predominantly MUC5AC and MUC5B [119]. On the other hand, CF sputum contains less water (90%)

and intact mucins [119] and more DNA and actin, secreted by necrotic neutrophils, epithelial cells and pathogens in the course of chronic inflammation [110]. DNA and actin co-polymerize to form a polymer network, increasing the viscoelasticity of sputum [40-44].

Mucin fibers are bundled together to form thick cables that create large spaces on the order of hundreds of nanometers [120]. The spaces between mucin cables (mesh spacing) in human cervical mucus are estimated as being from 100 nm [121] to 1000 nm [122], depending on the observation methods. For example, the pore size is estimated to be 100 nm by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) with glutaraldehyde-fixed samples, 500 – 800 nm by TEM with freeze-substitution, and 1000 nm or larger by various conventional electron microscope techniques [120]. The mesh spacing of the CF sputum is smaller, ranging 100 – 400 nm (SEM) [13], 140 ± 50 nm (modeling) [123], or 160 – 1440 nm (atomic force microscopy) [124].

Recent studies found that the spaces between networks of biopolymer cables are filled with low-viscosity fluid [125,126]. On the bulk fluid scale (macroscale rheology), mucus is a shear-thinning gel, whose viscosity decreases markedly as the shear rate increases [111]. This property allows mucus to serve as a lubricating surface on exposure to the vigorous shearing actions of eye blinking, swallowing, coughing, intestinal peristalsis, or intercourse [111]. On the other hand, the local rheology of mucus at nanometer scales (microscale rheology) is quite different from the bulk estimates [112,126]. Lai *et al.* reported that cervicovaginal mucus shows 1 – 4.3 mPa of storage moduli (G′) at the length scale < 500 nm, in contrast to 400 – 154,000 mPa at the scale of > 1 μm [126]. This model explains the observation of Sanders *et al.*, where nanoparticle (NP) diffusion was significantly higher in more viscoelastic mucus samples [13]. This high viscoelasticity (macroscale rheology) reflects a high concentration of biopolymers (DNA, mucins) with an increased number of junctions between them. The increased interactions leave fewer biopolymer chains unengaged and result in more heterogeneous and macroporous networks, through which relatively small NPs travel unimpeded [13]. Perturbation of the biopolymer interactions with a non-ionic detergent reduces the mobility of NP in the cervicovaginal mucus by releasing free polymers and reducing the mesh spacing [126]. The heterogeneous structure of mucus indicates that it is possible to transport NPs through the mucus layer. However, mucus remains a significant steric barrier to most NPs, especially in the case of CF sputum, when the mesh scale is smaller than normal mucus. NPs > 500 nm are almost always immobile in CF sputum [13,123].

3.1.2 *Cystic fibrosis sputum as a chemical and biological barrier*

Owing to the negative charges and hydrophobic regions of the constituent biopolymers, mucus interacts with the charged and/or hydrophobic surfaces of NPs, which is why, for example, the capsid virus-like particles (50 nm) without exposed hydrophobic surfaces diffuse freely through mucus, whereas hydrophobic polystyrene NPs of the same size do not [127]. Moreover, antibodies and other soluble factors in the CF sputum may act as molecular traps. For example, an adenovirus gene vector premixed with the sol phase of the CF sputum showed reduced gene transfection efficiency owing to the presence of adenovirus-specific antibodies [128]. CF sputum also inhibits gene transfection by non-viral liposomal vectors by destabilizing the gene complex [129]. When mixed with linear DNA, a polyanion abundant in the CF sputum, DNA-liposome complexes (lipoplexes) drastically changed the surface charge and size and released plasmid DNA prematurely [129].

3.1.3 *Cystic fibrosis sputum as a stage for bacterial resistance*

The tenacious, stationary CF sputum sets the stage for bacterial resistance [130]. *P. aeruginosa* develops an impressive armamentarium of strategies to evade antibiotic therapies. In one of the strategies, *P. aeruginosa* changes into mucoid strains and forms biofilms, which are resistant to phagocytosis and to penetration by antibiotics [130]. In addition, the biofilm center contains very little O_2 and few nutrients, which slows down the growth of the bacteria there and reduces their susceptibility to some antibiotics [131]. Biofilm formation is significantly enhanced by the presence of DNA and actin polymers in the CF sputum [40,43], which leads to the consideration of using anionic polymers and DNase to oppose biofilm formation [43].

3.2 Mucociliary clearance effect

In normal airways, the respiratory cilia transport mucus at a rate of 2.5 – 5 mm/min [132,133] towards the oropharynx, where it is either swallowed or expectorated [12]. Mucociliary clearance of mucus-trapped foreign substances is an important pulmonary defense mechanism against inhaled pathogens and particles [134]. However, it is a challenge to drug/gene delivery to the airway epithelia, as the delivery vehicles are similarly cleared. Sinn *et al.* reported that gene delivery by means of viral vectors to normal Balb/c mouse airways is significantly improved by inhibiting the mucociliary clearance using methylcellulose gels [135]; because in CF patients the mucociliary clearance is reduced [136,137], it is less of a challenge to inhalational medication delivery in that population. However, when mucus-thinning agents are co-administered to enhance transmucus diffusion of other medicines, the effect of improved mucociliary clearance should be considered.

3.3 Cellular challenges

3.3.1 *Bacterial drug resistance*

As mentioned, bacteria seen in the CF airway often develop antibiotic resistance, making it difficult to eradicate them. In addition to forming biofilms and developing a mucoid phenotype, drug-resistant strains of *Pseudomonas* lack outer

membrane porins, through which some antibiotics would normally diffuse [138-141], or develop active drug-efflux machinery [142]. Moreover, many Gram-negative CF pathogens develop resistance if treated with a single antibiotic; therefore, two or more agents of different classes are often used in combination [143-148]. Recent studies report the production of inhalable particles co-encapsulating multiple antibiotics, such as ciprofloxacin and ceftazidime [149] or ciprofloxacin and doxycycline [150,151], to assure their airway co-deposition at the intended doses. The combination particle system is a promising approach because it can treat patients who harbor several types of microorganism that may not be killed by a single antibiotic [152]. If the antibiotics show a synergistic effect, it can also reduce the amount of particles to inhale.

3.3.2 *Challenges in gene delivery*

The failure of gene delivery to the airway epithelia is largely attributed to cellular barriers [69]. For example, apical surface glycocalyx is a significant barrier to adenovirus-mediated gene transfer [153]. The sialic acid in the glycocalyx on the apical surface of airway epithelial cells interferes with gene delivery by affecting the interactions of adenovirus with its receptors [153]. In addition, specific receptors on the surface of the epithelial cells are required for the uptake of viral vectors; therefore, viral gene delivery can be challenging if required receptors are unavailable [154]. Moreover, repeated administration of adeovirus or adeno-associated virus induces humoral and cellular immune responses to the viruses [155-157], which provides more molecular traps, as described in Section 3.1.2.

Although non-viral vectors can avoid these problems, they still face other cellular challenges, such as degradation of the genetic materials during intracellular trafficking [68]. Unprotected DNA, for example, is degraded in the lysosomes [158] or by cytoplasmic nucleases [159,160]. Another intracellular barrier, especially in quiescent cells, is the nuclear envelope, which limits the entry of exogenous DNA [158,161,162]. Achieving a balance between extracellular protection and intracellular unpacking of DNA is also important for efficient gene transfection [163-165]. Critical cellular challenges in gene delivery are discussed in more detail in recent review articles [68,158,161,163,164].

4. New drug delivery approaches

4.1 Liposomal antibiotics

Bacterial resistance to antibiotics has been addressed by encapsulating the antibiotics in liposomal formulations. Liposomal gentamicin shows significantly lower minimal inhibitory concentration (MIC) values against drug-resistant strains of *P. aeruginosa* than free gentamicin [166,167]. Liposomal tobramycin inhibits growth of various bacteria at concentrations equivalent to sub-MIC levels of free tobramycin [168,169]. Such enhancement of antibacterial activity is attributed in part to the ability of liposomes to penetrate the bacterial membrane [166,168]. Halwani *et al.* demonstrated using TEM and flow cytometry that liposomal aminoglycosides can penetrate *B. cenocepacia*, effectively killing highly resistant strains [170]. Moreover, liposome encapsulation protects tobramycin in the polyanionic environments such as DNA, actin and bacterial endotoxins that reduce its bioactivity [171].

In animal models, locally administered liposomal antibiotics achieve a higher concentration and longer lung exposure than does the free drug [172-175]. Compared with the corresponding free drug, intratracheal liposomal tobramycin shows enhanced antibacterial activity [174,176], and nebulized liposomal amikacin significantly reduces bacterial counts in the lungs of rats infected with a mucoid strain of *P. aeruginosa* [175].

4.2 Application of external forces

Magnetic forces have been shown to improve the delivery of NPs to the lung [177]. Application of external magnetic gradient fields during inhalation significantly increased the deposition of aerosol droplets containing superparamagnetic NPs in the desired regions of mouse lungs [177]. This suggests that the magnetic force can aid in the diffusion of NPs through the airway mucus layer. However, scaling up the magnetic gradient field to the human scale would be challenging in clinical application of this technology.

A recent review mentions an unpublished study that used low-frequency ultrasound (20 – 100 kHz) for NP delivery through the airway mucus [110]. The transport of negatively charged polystyrene NPs (500 nm) was enhanced 10-fold by the application of low-frequency ultrasound. However, ultrasound application to the lungs may not be simple owing to interference by the air in the lungs. Moreover, the duration of ultrasound application should be controlled carefully to avoid generation of excessive heat.

4.3 Pretreatment with mucolytics

Various mucolytic agents have been administered before NP drug formulation to reduce the steric hindrance of mucus, but the outcomes have been mixed. On the one hand, NP transport across a layer of isolated CF sputum is enhanced by premixing the NPs with DNase [13,178], and adenoviral gene delivery to normal mouse airways is enhanced by pretreatment with *N*-acetylcysteine [179,180]. On the other hand, pretreatment of mucous tissues with *N*-acetylcysteine does not improve the gene transfection efficiency in a CF mouse model [180], perhaps owing to the enhanced removal of the gene carriers secondary to the increased mobility of the mucus. In addition, the degrading mucus may release free biopolymers, increasing viscous drag and delaying NP diffusion [110].

4.4 Surface-protected nanoparticles

To decrease the interactions between mucus components and NPs, Hanes and co-workers proposed modifying the NP

surface with low-molecular-mass polyethylene glycol (PEG) to prevent the interactions between the NPs and the mucus components (**Figure 1A**) [123,181-183]. Densely PEGylated NPs can diffuse through the cervicovaginal mucus [181,183] or through the CF sputum [123,183]. The molecular mass of PEG and the extent of PEGylation of the NP surface are critical in controlling the mucus–NP interactions. Polystyrene NPs densely coated with 2 or 5 kDa PEG penetrate the undiluted cervicovaginal mucus relatively fast, whereas the NPs with 10 kDa PEG do not because of the PEG–mucin entanglement [184]. For the NPs coated with 2 kDa PEG, a 40% decrease in PEG coverage results in a 700-fold decrease in the average transport rate within the mucus [184]. When sufficiently covered with PEG, even relatively large NPs (500 nm) diffuse through the cervicovaginal mucus layer [181]. The diffusion coefficient of PEGylated 500 nm NPs in mucus is only 4 times lower than that in water, and ~ 70% of PEGylated NPs (500 nm) are mobile in the mucus, whereas 45% of uncoated NPs of the same size remain immobile [181]. On the other hand, when the surface is not sufficiently PEGylated, smaller NPs (100 nm) cannot diffuse as effectively as larger PEGylated NPs [181]. These studies suggest that when the NPs are smaller than a certain threshold, the interactions between them and the biomolecules in the mucus are the main obstacle to their migration through the mucus [120]. The threshold NP size for CF sputum is much smaller than for cervicovaginal mucus. Two hundred-nanometer PEGylated NPs move through undiluted CF sputum at an average speed 90-fold higher than uncoated particles [123]. However, movement of the 500 nm NP is significantly hindered, irrespective of the PEG surface [123]. When the diffusion rates of NPs of various sizes are fitted in an obstruction-scaling model, the mesh spacing in CF sputum is estimated to be in the range 60 – 300 nm, with an average of ~ 140 ± 50 nm [123].

The benefit of PEGylation in transmucosal NP delivery is not limited to the enhancement of their penetration. PEGylated NPs are less likely to aggregate and be taken up by alveolar macrophages. Moreover, PEGylation can improve the stability of the gene-vector complex in the mucus. A cationic DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane) lipoplex displays a significantly lower gene transfection activity on exposure to albumin, linear DNA, or mucin [129]; however, PEGylation protects the lipoplex from destabilization and loss of transfection activity owing to the anionic environment [185,186]. Recently, PEGylated NPs have been used to deliver PS-341, a ΔF508-CFTR corrector and chronic inflammation inhibitor, to the lungs of CF mice [187].

PEGylated NPs are also not without disadvantages. The PEGylated surface can interfere with the NP–cell interactions and the efficient cellular entry by NPs [188-193], and it can decrease endosomal escape [110]. Moreover, complete protection from mucus or sputum components may require very dense PEG coverage, because molecules such as albumin or phospholipids can penetrate through the uncoated gaps on the NP (liposome) surface and destabilize the NPs [185,186,194].

For cationic non-viral vectors, anionic polymers are often used to mask the surface charge and reduce the interaction with the anionic environment. Hyaluronic acid [165,195], alginic acid [196] and poly(propylacrylic acid) [197] have been shown to protect the gene carriers from anionic proteins and preserve their ability to transfect cells. The same principle may be applicable for overcoming the interaction between the gene carriers and the biopolymer network in the mucus or sputum.

4.5 Co-formulation with agents that influence the cystic fibrosis sputum

To overcome the CF sputum barrier that insulates bacteria from inhaled antibiotics [12,43,198], co-administering antibiotics and agents that degrade the sputum was proposed. For example, anti-pseudomonal activities of liposomal and free aminoglycosides in CF sputum were enhanced by the addition of DNase and/or alginate lysate, which decreased the alginate level in the biofilm [199]. An inhalable dry powder system co-delivering DNase and ciprofloxacin has been developed to enhance the penetration of ciprofloxacin (**Figure 1B**) [200]. These particles decrease the viscoelasticity of the artificial sputum, which resembles the CF sputum in chemical composition and rheological properties. Moreover, these particles kill the bacteria contained in the artificial sputum more efficiently than the particles containing ciprofloxacin alone. This study suggests that co-delivery of antibiotics and mucus-thinning agents using a single inhalable particle system may be a promising strategy for local antibacterial therapy in the CF airways.

On the other hand, the use of DNase as a way of overcoming the sputum barrier may not be a viable option for delivery of genetic therapeutics. Moreover, DNase is expensive, a further challenge to its routine use. Yeo *et al.* have explored mannitol as an alternative agent to influence the sputum [201], based on several continuing clinical studies using it in that capacity [50,54-57]. They observed that mannitol improves the antibacterial efficiency of ciprofloxacin against *P. aeruginosa* in the artificial sputum, most probably because of its ability to increase the local water content in the sputum, increasing the heterogeneity of the network and thereby enhancing drug transport (**Figure 1C**) [201]. Adi *et al.* have proposed using mannitol in a similar context and investigated its potential to form dry particles with mannitol and ciprofloxacin [202].

Alternatively, a recent study proposed utilizing particle–biopolymer network interaction to facilitate transmucus drug diffusion [203]. McGill and Smyth observed that penetrations of fluorescein and rhodamine through artificial mucus models are significantly enhanced after treatment of the mucus with particles (200 nm or 1 μm) [203]. This effect is attributed to the collapse of the mucin network mediated by particle–network interactions, which leads to an increase of the mesh size (**Figure 1D**) [203]. Chen *et al.* also reported

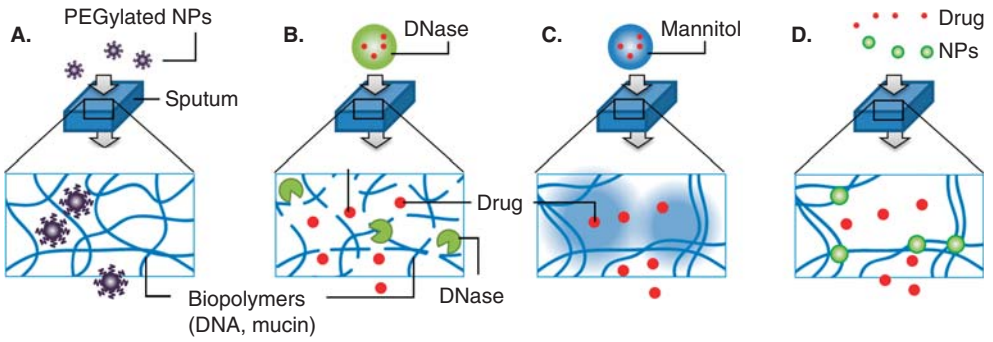

Figure 1. Approaches to enhance transport of NPs or drug molecules through cystic fibrosis sputum. A. Surface-protected NP. **B.** Co-formulation with DNase. **C.** Co-formulation with mannitol (airway hydrator). **D.** Co-treatment of drug and NPs.
NP: Nanoparticles.

cationic NP-mediated mucus aggregation, but cautioned that the NPs can impede mucus hydration, thereby worsening the CF airway obstruction [204].

5. Remaining challenges

With the recent advances in inhalational drug delivery technologies, the authors anticipate that more strategies to address the remaining challenges in inhalational drug delivery will emerge in the near future. A significant challenge in clinical translation of these new approaches is the lack of proper CF animal models. Several CFTR-knockout mice have been developed; however, most of them rapidly develop CF-related bowel problems and die in infancy owing to cecal obstruction without ever developing lung disease [205]. Therefore, the existing CF mouse models are not appropriate for routine evaluation of inhalable formulations. Several more differences between the CF mice and humans with CF need to be pointed out. First, murine airway epithelium expresses an alternative Cl^- channel, which complements the CFTR deficiency and saves mice from severe lung disease [206]. In addition, interspecies differences such as lung architecture, physiology and airway cell composition may be physiologically and pharmacologically important contributors to the difference [205].

An alternative animal model widely used for evaluation of microbial virulence and host defense mechanisms is a murine model with chronic *P. aeruginosa* lung infection, which develops the mucopurulent matrix seen in the lungs of CF patients [207,208]. In this model, agar or alginate beads containing a mucoid strain of *P. aeruginosa* are implanted into the airways of mice or rats via intratracheal instillation [208]. The infection is usually established 3 – 4 days after inoculation [207]. On a histological level, the infected lungs show lesions similar to those of chronically inflamed CF lungs [207]. This model is relatively inexpensive and useful for evaluating formulations designed for *trans*- or intramucus drug delivery, but its utility in testing medications that affect the genotype or bioelectric phenotype of the airway epithelium is limited.

A CFTR-knockout mouse model with nasal epithelium that mimics ionic transport of the airway epithelium has been widely used for proof of concept studies of CFTR gene delivery [68]. However, according to a recent study, expression of human CFTR in the nasal epithelia fails to change the nasal bioelectrics of the transgenic mice, raising questions about the validity of the nasal epithelium as a model for airway gene delivery [209].

Transgenic mice overexpressing subunits of ENaC develop a CF-like lung disease [82]. The animals develop several phenotypes pertinent to CF lung disease, such as viscoelastic mucus, delayed mucus transport, lung infection and inflammation. This model may be useful for the evaluation of new drug formulations designed to address the mucus barriers [68].

Other larger animal models such as pigs and sheep are considered because of the similarity of their airways to the human airway [205]. Domestic ferrets are also considered to be a promising model because they have similarities to humans in lung physiology, airway morphology and cell types [205,210]. In addition, the CFTR expression in the ferret submucosal gland is almost identical to that in humans [211]. Recently, Sun and co-workers [212,213] and Rogers *et al.* [214] have reported cloning of the eagerly-awaited CF ferrets and pigs, respectively. It remains to be seen whether the two models simulate the human CF lung disease.

6. Conclusion

CF is a significant genetic disorder with major deleterious influences on respiratory function. CF management relies largely on symptomatic therapy, but new therapeutic agents are emerging for treatment of the underlying pathophysiology. Although many drugs are delivered by means of inhalation to increase their local availability, several physiological barriers interfere with effective delivery of medications. For example, tenacious CF sputum presents a physical, chemical and biological barrier to effective drug delivery. Several drug delivery approaches have been proposed aimed at altering the sputum and/or drug carriers, so as to increase the carrier

mobility within the sputum. Bacterial resistance to antibiotics is addressed by liposomal formulations. Further advancement of new drug formulations hinges critically on expeditious development of animal models that simulate the human CF lung disease with high fidelity.

7. Expert opinion

With the advancement of new pharmacological agents for CF and the growing number of CF patients needing chronic therapy, there will be an increasing demand for drug carriers that deliver the medications effectively. As the lung is often the most affected organ in CF, inhalational drug delivery systems, which ensure deposition of medications in the lungs, are widely used for drug administration. Several drugs are administered as nebulized solutions and are now being developed as inhalable dry powder formulations. On the other hand, CF sputum, tenacious and dehydrated owing to the CFTR dysfunction and chronic infection/inflammation, presents a formidable barrier to penetration by drugs and/or drug carriers, especially the NPs used for delivery of genetic therapeutics.

Years of studies on CF sputum have identified its physical, biological and chemical properties that influence the transport of drugs and NPs and laid the groundwork for rational delivery strategies. One of the promising approaches relies on shielding the surface of NPs with a protective coating such as PEG to reduce their interactions with the biomacromolecular network in the CF sputum. A significant enhancement in movement of PEGylated NPs has been demonstrated in CF sputum samples. However, their clinical effectiveness, which will rely on the timescale of penetration and stability of PEGylated NPs, remains to be seen. In particular, an optimal balance between dense PEGylation and particle uptake would be the key to the success of this approach. An alternative approach to reducing the untoward interactions between NPs and mucus is the use of anionic, hydrophilic polymers such as hyaluronic acid or alginic acid. A few recent studies explored the potential of these polymers to prevent destabilization of cationic nanocarriers, but they have not been studied in the context of drug/gene delivery to the CF airways. Instead of the NPs, the structure of sputum can be modified by the drug formulations. In recent studies, agents affecting sputum, such as DNase or mannitol, have been incorporated in addition to an active ingredient, and they have been shown to enhance the dissolution and diffusion of the drug in an artificial sputum model. This approach, still in its infancy, requires further studies on the clinical CF sputum and appropriate animal models. The development of new mucus-thinning agents and airway hydrators will provide more options for excipients, making this strategy applicable to a broader range of drugs, including gene therapeutics. For the ultimate success of inhalable CF therapeutics, such chemical enhancement of formulations should be accompanied by the achievement of optimal aerodynamic properties of the formulations and the effectiveness of inhaler devices.

In translating innovative drug delivery strategies into clinical benefits, it is critical to obtain proof of concept in relevant animal models in the early stages. A significant challenge in the advancement of CF drug delivery research is the lack of economical small animal models that represent the pathological features of human CF airways. The development of CF model animals with CF lung diseases would be one of the most awaited technological breakthroughs in CF drug delivery research.

Declaration of interest

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