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Airway mucus in pulmonary diseases: Muco-adhesive and muco-penetrating particles to overcome the airway mucus barriers

Rudra Pangeni[1], **Tuo Meng**[1], **Sagun Poudel**[1], **Divya Sharma**[2], **Hallie Hutsell**[1], **Jonathan Ma**[3], **Bruce K. Rubin**[2,3], **Worth Longest**[1,4], **Michael Hindle**[1], **Qingguo Xu, PhD**[1,5,*]

[1]Department of Pharmaceutics, Virginia Commonwealth University, Richmond, VA, USA

[2]Department of Physiology and Biophysics, Virginia Commonwealth University, Richmond, VA, USA

[3]Department of Pediatrics, Children's Hospital of Richmond, Richmond, VA, USA

[4]Department of Mechanical and Nuclear Engineering, Virginia Commonwealth University, Richmond, VA, USA

[5]Department of Ophthalmology, Massey Cancer Center, Center for Pharmaceutical Engineering, and Institute for Structural Biology, Drug Discovery & Development (ISB3D), Virginia Commonwealth University, Richmond, VA, USA

Abstract

Airway mucus is a complex viscoelastic gel that provides a defensive physical barrier and shields the airway epithelium by trapping inhaled foreign pathogens and facilitating their removal *via* mucociliary clearance (MCC). In patients with respiratory diseases, such as chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), non-CF bronchiectasis, and asthma, an increase in crosslinking and physical entanglement of mucin polymers as well as mucus dehydration often alters and typically reduces mucus mesh network pore size, which reduces neutrophil migration, decreases pathogen capture, sustains bacterial infection, and accelerates lung function decline. Conventional aerosol particles containing hydrophobic drugs are rapidly captured and removed by MCC. Therefore, it is critical to design aerosol delivery systems with the appropriate size

[*]Corresponding authors: Qingguo Xu, PhD, Department of Pharmaceutics, School of Pharmacy, Virginia Commonwealth University, 410 North 12th Street, Smith Building 454C, Richmond, VA 23298, USA, Tell: (804) 828-6431; qxu@vcu.edu.

Declaration of Competing Interest

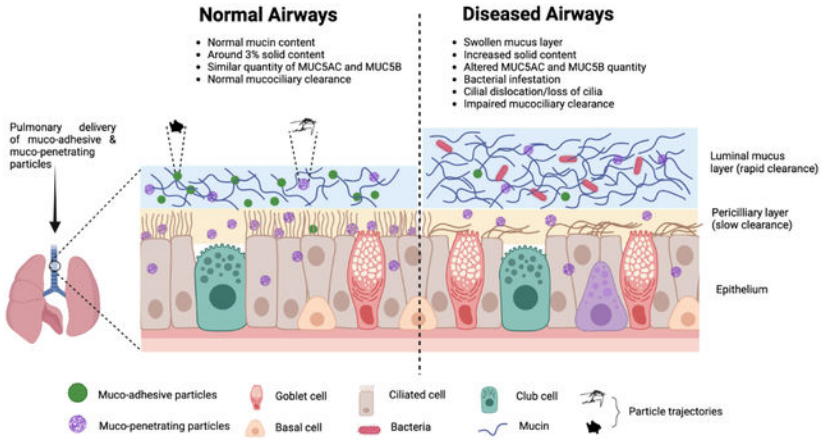
The EEG aerosol technologies described in this publication have been filed as patent applications through Virginia Commonwealth University (VCU). Michael Hindle and Worth Longest are the co-inventors, which are subject to certain rules and restrictions under VCU policy. The terms of this arrangement are being managed by VCU in accordance with its conflict of interest policies.

CRediT authorship contribution statement

Rudra Pangeni: Methodology, Writing – Original draft. **Tuo Meng:** Methodology, Writing – Original draft. **Sagun Poudel:** Writing- review and editing. **Divya Sharma:** Writing- review and editing. **Hallie Hutsell**: Writing- review and editing. **Jonathan Ma**: Writing- review and editing. **Bruce Rubin**: Supervision, Writing- review and editing. **Worth Longest:** Supervision, Writing- review and editing. **Michael Hindle:** Supervision, Writing- review and editing. **Qingguo Xu:** Supervision, Writing- review and editing.

and surface chemistry that can improve drug retention and absorption with the goal of increased efficacy. Biodegradable muco-adhesive particles (MAPs) and muco-penetrating particles (MPPs) have been engineered to achieve effective pulmonary delivery and extend drug residence time in the lungs. MAPs can be used to target mucus as they get trapped in airway mucus by steric obstruction and/or adhesion. MPPs avoid muco-adhesion and are designed to have a particle size smaller than the mucus network, enhancing lung retention of particles as well as transport to the respiratory epithelial layer and drug absorption. In this review, we aim to provide insight into the composition of airway mucus, rheological characteristics of airway mucus in healthy and diseased subjects, the most recent techniques to study the flow dynamics and particle diffusion in airway mucus (in particular, multiple particle tracking, MPT), and the advancements in engineering MPPs that have contributed to improved airway mucus penetration, lung distribution, and retention.

Graphical Abstract



Keywords

Rheology; microrheology; mucociliary clearance; multiple particle tracking; cystic fibrosis; aerosol; pulmonary drug delivery

1. Introduction

Pulmonary drug delivery is a less invasive route for treatment of chronic respiratory airway diseases such as asthma, cystic fibrosis (CF), lung cancer, and chronic obstructive pulmonary disease (COPD) (Fröhlich and Salar-Behzadi, 2021; Mejías and Roy, 2019). This delivery route offers several advantages, including the ability to achieve local drug accumulation in the lungs, providing an alternative route to target systemic diseases, relatively low enzymatic activity, and good permeability due to an extensively vascularized membrane (Azarmi et al., 2008; Labiris and Dolovich, 2003; Yıldız-Peköz and Ehrhardt, 2020). Most inhaled drugs deposited in the conducting airways are subjected to clearance by three mechanisms, namely mucociliary clearance (MCC), macrophage uptake, and/or absorption into the epithelial layer (El-Sherbiny and Smyth, 2012; Matthews et al., 2020). The airway epithelium is the major physical barrier lined by ciliated and secretory cells that

are physically connected with multiple layers of intercellular junctions (Bals and Hiemstra, 2004; Ganesan et al., 2013; Knowles and Boucher, 2002; Pohl et al., 2009).

Airway mucus is a complex viscoelastic gel synthesized and secreted by specialized goblet and mucous glands in the surface epithelium (Birchenough et al., 2015; Rogers and Barnes, 2006). Native airway mucus protects the underlying epithelium by trapping and immobilizing inhaled foreign particles and pathogens, facilitating their removal *via* MCC and cough clearance, and prevents dehydrating the epithelial surface of the lung (Richard A Cone, 2009; Fahy and Dickey, 2010; Shah et al., 2019). Effective MCC mainly depends on the normal structure and function of cilia along with the optimum height, composition, and viscoelasticity of the mucus layer (Houtmeyers et al., 1999; Nawroth et al., 2020). In healthy individuals, mucus is produced continuously to protect the airway and humidify the air. In respiratory diseases, the composition and properties of airway secretions are altered and the airway epithelium often hyper-secretes mucus that disrupts MCC and airflow, resulting mucus retention (Daviskas and Rubin, 2013; Li and Tang, 2021; Livraghi and Randell, 2007; Shen et al., 2018). Defective cystic fibrosis transmembrane conductance regulator (CFTR) protein causes imbalance of sodium, chloride, and bicarbonate ions at the airway epithelial surface and dehydration of the airway surface liquid, increasing percent solids (mucins, salts, lipids, polypeptides, etc.) in the airway mucus (Boucher, 2007; Duncan et al., 2016b; Quinton, 2008). In most cases, mucus secreted by individuals with respiratory diseases has increased viscoelasticity compared to mucus from healthy individuals, causing mucus build-up in the airways leading to deterioration of lung function (Chisholm et al., 2019; Serisier et al., 2009). This is supported by studies that show increased solid mucus content in patients with lung diseases (Anderson et al., 2015; Hill et al., 2014).

Nanoparticles formulation is a promising approach for the treatment of respiratory diseases as it confers significant advantages in terms of sustained or controlled drug release, protection of encapsulated drug from premature degradation or loss-of-function by enzymatic/metabolic activities, and enhanced cellular uptake (D. Chen et al., 2021). However, success of this approach is highly dependent on the ability of nanoparticles to overcome various biological and physiological barriers (Alp and Aydogan, 2020; Porsio et al., 2018). In respiratory diseases, alteration in mucus properties can potentially impact the behavior of nanoparticles when used as a delivery vehicle, requiring careful consideration when designing and formulating nanoparticles (Chen et al., 2010a; Kan et al., 2020). It has been possible to improve the airway mucus penetration of nanoparticles in respiratory diseases using strategies such as modification of physicochemical properties of nanoparticles by coating with polymers, including polyethylene glycol (PEG); and modulation of barrier properties of airway mucus by mucolytics such as N-acetylcysteine (NAC) (Craparo et al., 2016; Kan et al., 2020; Nordgård and Draget, 2018). Nanoparticles densely surface coated with PEG_{5kDa} demonstrated improved diffusion in normal, CF, and COPD airway mucus compared to uncoated nanoparticles (Forier et al., 2013; Schuster et al., 2014; Suk et al., 2009). Similarly, PEG-conjugated polymers (PEG-PEI and PEG-PBAE) based mucus penetrating particles (MPPs) confirmed widespread and uniform airway distribution, and prolonged retention in the mice lungs (Boylan et al., 2012; A. J. Kim et al., 2013).

In this review, we summarize the major functional components of mucus, highlighting the role of the gel-forming mucins as responsible for viscoelastic properties of airway mucus. Thereafter, the impact of mucus macrorheology and microrheology on the normal barrier and clearance function in the airways, and the altered viscoelastic properties of sputum as a biomarker of secondary infection and inflammation is discussed. We also provide an overview on the most recent techniques to study the flow dynamics of nanoparticles using a low volume of mucus. Moreover, we have discussed the contribution of mucus hypersecretion, microstructural alteration, and neutrophil extracellular traps (NETs) in respiratory diseases. Finally, we have introduced several studies focused on the design of muco-adhesive particles (MAPs) and MPPs and aerosols to overcome the airway mucus barrier for effective pulmonary drug delivery.

2. Healthy airway mucus: Composition, macrorheology and microrheology

The major functional component of viscoelastic mucus gel is mucin (1–5%), a family of high molecular weight, heavily glycosylated proteins containing hydrophobic polypeptide backbones connected to multiple hydrophilic oligosaccharide chains (Bansil and Turner, 2018). These mucin polypeptide backbones possess amino acid sequences with positive and negative charge that assist in trapping charged and hydrophobic particles (Lieleg et al., 2010; Suk et al., 2009). Besides mucin, numerous biomacromolecules - including proteins, antibodies, and lipids are present in the mucus and form a mesh-like structure through disulfide cross-linkages and/or physical entanglement, which is referred as the mucus microstructure (Duncan et al., 2016a). As per the HUGO Gene Nomenclature Committee, there are 24 human MUC genes (denoted with capital letters) of which 14 are located in the respiratory tract. Based on the ability to polymerize and if they are cell surface-bound or secreted, airway mucins are classified into three types: secreted monomeric mucins (MUC7, MUC8), secreted gel-forming mucins (MUC2, MUC5AC, MUC5B, MUC6, and MUC19), and non-secreted surface-bound mucins (MUC1, MUC4, MUC13, MUC16, MUC20, MUC21, and MUC22) (Atanasova and Reznikov, 2019; Ma et al., 2018). Each mucin is unique in size, sequence, and shares a common structural motif of tandem repeats that are rich in serine, threonine, and proline residues (Williams et al., 2006). Five of these secreted mucins possess cysteine domains at the terminal ends and can form disulfide bonds that impart a gel-like property. Among them, MUC5AC and MUC5B are the most abundant gel-forming mucins secreted by goblet cells in superficial epithelium and submucosal glands, respectively, which are key to the viscoelastic properties of airway mucus (Atanasova and Reznikov, 2019; Voynow and Rubin, 2009).

Mucus has complex and highly variable physical properties. The rheological behavior of mucus changes as a function of shear rate, shear stress, and length scale (Lai et al., 2009c). As the link between altered mucus rheology and difficulty in airway clearance has been established, findings have also correlated abnormal sputum viscoelastic properties as biomarkers of secondary infection (Dulfano and Adler, 1975; Patarin et al., 2020; Puchelle et al., 1985; Serisier et al., 2009). At the macroscopic level (bulk rheology), averaged physical properties of mucus such as flow and deformation can be measured. Macrorheological

analysis of human mucus at low shear yields a viscosity as high as 10^4–10^6 folds to that of water. Depending on the anatomical region in the lungs, the rheological properties of respiratory mucus varies. In particular, small airway mucus has lower elasticity compared to mucus from the trachea (App et al., 1993). In conditions such as during coughing, the viscosity of mucus may be greatly reduced by increased shear stress (Ren et al., 2020).

Microrheology can be defined as the measure of rheology of a small volume of mucus probed by micro- or nanoparticles. Multiple particle tracking (MPT) has been widely used to study the flow dynamics of nanoparticles and gene nanocarriers within the network of mucus (Chai et al., 2020; Dawson et al., 2004; Schuster et al., 2014; Song et al., 2021; Suh et al., 2004). MPT involves studying the motion of individual particles or small groups of particles in real-time using fluorescence video microscopy. This reveals interactions of these particles with the mucus environment and the transport properties of these particles within the mucus network. In addition, post-acquisition analysis of time-resolved particle trajectories can be performed (Chai et al., 2020; Lai et al., 2007; Benjamin S. Schuster et al., 2013). MPT also provides quantitative information such as effective diffusivities, maximum velocity, and directionality along with qualitative information such as the mechanism of particle transport (Lai and Hanes, 2008; Selvaggi et al., 2010; Valentine et al., 2001). In brief, the x and y positions of particle centroids over time are extracted from the MPT movies and the trajectories of particles in the mucus are constructed. The time-averaged mean squared displacement (MSD) of each particle's trajectory is calculated as:

$$(\tau), \left\langle \Delta r^2(\tau) \right\rangle = \left\langle [x(t+\tau) - x(t)]^2 + [y(t+\tau) - y(t)]^2 \right\rangle \quad (1)$$

Where, τ is the time scale, x(t) and y(t) represent the particles coordinates at a given time (Benjamin S Schuster et al., 2013). The particle tracking microrheology is performed by using the generalized Stokes-Einstein equation, which relates the thermal motion of particles to the viscoelastic moduli of the mucus probed. In 2D particle tracking microrheology complex shear modulus, G' and G'' are calculated as explained in detail by (Lai et al., 2009a). The phase angle (δ) determines whether the viscoelastic mucus is more solid ($\delta < 45°$, $G' > G''$) or more liquid like ($\delta > 45°$, $G' < G''$). The following equation determines the phase angle of mucus (Ensign et al., 2012):

$$\delta = \tan(G''/G') \quad (2)$$

Any particles with sizes larger than the length scale of the mucus microstructure may experience steric obstruction and limited diffusion through the mucus. However, particles with lengths up to 55 nm demonstrate similar microviscosity in mucus and water (Lai et al., 2009c; Olmsted et al., 2001; Tan et al., 2020). A study by Lai et al. established that even larger PEG-coated particles up to 500 nm in diameter can diffuse through human mucus with effective diffusivities merely 4-times lower than water (Lai et al., 2007). Later, Schuster et al. studied the effect of particle size and surface chemistry on nanoparticle diffusion through airway mucus from healthy humans and showed that nanoparticles with particle sizes <500 nm in diameter and muco-inert properties can rapidly penetrate normal

airway mucus (Benjamin S. Schuster et al., 2013). These studies highlight the importance that mucus mesh architecture might be more diverse than was recognized in previously reported diffusion experiments. To further understand the microscopic rheological properties of mucus using MPT, complex modulus (G* (ω)) and complex viscosity (|η* (ω)|) can be calculated using the following formulae (Mason et al., 1997; Mason and Weitz, 1995; Benjamin S. Schuster et al., 2013):

$$G^*(\omega) = G'(\omega) + iG''(\omega) \tag{3}$$

$$|\eta^*(\omega)| = |G^*(\omega)|/\omega \tag{4}$$

Muco-inert particles (MIPs) of 100 and 200 nm diameters showed viscosities 16- and 38-fold greater compared to mucus interstitial fluid, respectively (Benjamin S. Schuster et al., 2013). Small-sized MIPs resist muco-adhesion and can diffuse through mucus mesh. However, particles larger than the mucus mesh are sensitive to the biopolymer network elasticity and its bulk rheology (Benjamin S. Schuster et al., 2013). Overall, a proper understanding of macrorheology and microrheology of airway mucus in healthy and diseased subjects might assist in the design of novel drug and gene delivery systems.

3. Airway mucus in respiratory disease and evaluation strategies

Mucin glycoproteins have a vital role in the airway's innate immune system and actively take part in normal MCC (Rose and Voynow, 2006). Healthy airway secretions mainly contain MUC5AC which has a small mass to unit length ratio with shorter oligosaccharide chains, whereas MUC5B is predominant in chronic airway diseases and is present in both low-and high-charge glycoforms, suggesting both MUC5AC or MUC5B have distinct barrier functions in the mucus (Sheehan et al., 1991; Voynow and Rubin, 2009). However, analysis of mucin content in patients with acute and/or chronic airway inflammation, such as CF and COPD, demonstrated upregulation of mucin production, specifically MUC5AC and MUC5B (Kirkham et al., 2002). Esther Jr *et al.* analyzed mucus in the bronchoalveolar lavage fluid (BALF) from children with CF and control group without CF and found that the total airway mucin concentration was higher in CF compared to non-CF samples. Moreover, quantitative assessment of flake density and mucin content by immunohistochemistry (IHC) demonstrated that MUC5B and MUC5AC were mixed in the mucus flakes, and these particular mucins were more in CF samples compared to non-CF samples (Figure 1) (Esther Jr et al., 2019).

In chronic respiratory diseases such as COPD, CF, respiratory syncytial virus (RSV) infection, and non-CF bronchiectasis, a network of copolymerized extracellular DNA and filamentous (F-) actin are released due to NETosis (Kater et al., 2007; McGuckin et al., 2015; Rubin, 2007; Thiam et al., 2020b). NETosis is a program for formation of neutrophil extracellular traps (NETs), characterized by the release of decondensed chromatin and granular contents to the extracellular space, leading to increased viscoelasticity of airway mucus (Thiam et al., 2020a; Vorobjeva and Chernyak, 2020). However, due to a lack of suitable *in vivo* and *in situ* measurement techniques, most of the rheological measurements

of airway sputum are from *in vitro* studies that are subjected to changes in handling, which may not provide a true *in vivo* representation (Chen et al., 2019; Dulfano et al., 1971). Cone and plate rheometers have been used for bulk rheological analysis of the mucus, in which the mucus sample is suspended between a flat plate and an inverted cone. Under oscillation, the plate is rotated with a known amplitude and shear rate which results in a certain torque and input strain. This obtained data is used to calculate shear stress, phase angle, apparent viscosity, viscous modulus (G′′), and elastic modulus (G′) (Lai et al., 2009c; Benjamin S. Schuster et al., 2013). Recently, Abrami et al. proposed the combined use of bulk rheology and low field NMR to study the mesh size distribution in mucus. The study demonstrated that this combination approach can provide a more detailed picture of the mucus three-dimensional architecture along with mesh size distribution of the mucus network (Abrami et al., 2022, 2021).

Scanning electron microscope (SEM) imaging of airway mucus has confirmed a mesh-like architecture with the pore size ranging up to hundreds of nanometers in diameter (Figure 2). Both, normal airway mucus and CF sputum demonstrated similar overall appearances when observed using SEM; however, preparation of SEM samples likely contracts the mucus microstructure (Richard A. Cone, 2009; Benjamin S. Schuster et al., 2013; Suk et al., 2011b). Confocal Raman microscopy also has been used as a label-free and chemically selective technique to visualize the composition and structure of human airway mucus (Vukosavljevic et al., 2017). Despite these advantages, it is a time-consuming technique that uses a laser beam to create heat that dries the mucus sample, resulting in analytical challenges (Alinovi et al., 2020).

In respiratory diseases, increase in mucin disulfide bond density (both inter- and intra-) or impaired secretion of bicarbonates, leads to the formation of mucus with a tighter mesh network (Chen et al., 2010b; Quinton, 2008; van der Vliet et al., 2018). These alterations contribute to the change in permeability and microstructural properties such as decreases in mucus mesh size, which reduces neutrophil migration and decreases bacterial capture, thereby sustaining infection (Duncan et al., 2016b; Linssen et al., 2020; Matsui et al., 2005). Microstructural analysis of mucus may serve as marker of lung disease severity; however, microstructural analysis in its native state is often challenging and necessitates preservation of the hydration state of hydrogels to prevent collapse of the mesh (Duncan et al., 2016b; Vukosavljevic et al., 2017).

Previous studies have shown that the microrheology and mucosal microstructure in respiratory diseases can be quantitatively determined by monitoring transport or diffusion of muco-inert particle (MIP) probes in the mucus *via* MPT, which has been discussed in detail in the previous section (Lai et al., 2011, 2007; Benjamin S. Schuster et al., 2013). Polyethylene glycol (PEG) coated MIPs as large as 500 nm can diffuse through normal airway mucus from healthy subjects, whereas the same size uncoated conventional polymeric particles are restricted by the mucus microstructure due to adhesive interaction between particles and mucus components (Richard A. Cone, 2009; Lai et al., 2007; Benjamin S Schuster et al., 2013). PEG-coated MIPs up to 200 nm in diameter diffused about 90-times faster through sputum from CF patients, compared to uncoated particles of similar diameter (Lai et al., 2011; Suk et al., 2009). However, there is a large variation in

particle diffusion for sputum samples from different CF patients, even while using the same size particle probes, suggesting patient-specific inconsistencies in microstructural properties (Schuster et al., 2014). Studying the effect of key biochemical components of CF sputum on mucus microstructure reveals that the increase in mucin, DNA, and/or solid content is heavily linked with the decrease in mucus mesh size (Duncan et al., 2016b). These real-time dynamics studies using various sized particles have probed the 3D mesh sizes and structures for mucus from healthy, CF, and other respiratory conditions (Lai et al., 2007; Suk et al., 2009). MPT has also been used to study the viscoelasticity of human CF sputum, which demonstrated a lower magnitude for microviscosity compared to macroviscosity, suggesting increased microheterogeneity in particle tracking (Dawson et al., 2003).

4. Characteristics of airway mucus in respiratory diseases and alteration of mucus microstructure

Airway mucus secretions represent the first-line defense of the respiratory tract. In addition, particle permeability through airway mucus depends on the microstructural organization of the mucus, size, and surface chemistry of the particulate matter (Krupa et al., 2020). However, mucus hypersecretion, abnormal secretions, and decreased clearance can cause obstruction of airways, limit airflow, and accelerate lung function decline, leading to obstructive pulmonary diseases. Even though chronic bronchial diseases share distinct origins and mechanisms, the common pathophysiological and clinical manifestations of these diseases are mucus hypersecretion, airway inflammation, and impaired MCC (Fahy and Dickey, 2010; Kirkham et al., 2002). Interestingly, patients with COPD, CF, non-CF bronchiectasis, and asthma have altered mucins and water content at the airway epithelial surface, higher solid percentage, altered viscosity due to the release of DNA and F-actin filaments from stimulated neutrophils and damaged epithelial cells (Daviskas and Rubin, 2013; Thiam et al., 2020a; Williams et al., 2006). Recently, Patarin et al. assessed both quasi-static (linear storage and loss moduli) and dynamic rheological (flow point) properties of sputum samples collected from several chronic bronchial diseases and healthy subjects. Their study demonstrated that respiratory pathologies substantially influence the linear and flow properties of sputum and highlighted the relevance of determining quasi-static and dynamic rheological properties of sputum as a possible marker of chronic respiratory diseases (Patarin et al., 2020). Fahy and Dickey discussed that the conversion of mucus from healthy to pathologic condition occurs through several mechanisms *via* alteration in hydration state, biochemical constituents, and physical properties of mucus, including abnormal secretion of water and electrolytes, neutrophilic inflammation, and bronchovascular permeability (shown in Table 1 (Fahy and Dickey, 2010; Meldrum and Chotirmall, 2021)). The above findings provide broad implications that the characteristics of mucus in airway diseases may be a potential determinant of respiratory diseases.

4.1 COPD and mucus microstructure

Airways from COPD patients have surface epithelial mucous metaplasia and hyperplasia along with increased submucosal glands and sputum with increased mucin polymerization and elasticity (Yuan et al., 2015). A key risk factor for COPD patients is cigarette smoke which can lead to the activation of pulmonary epidermal growth factor receptor (EGFR)

cascades that enhance mucin production and goblet cell hyperplasia and epithelial barrier dysfunction (Mishra et al., 2016; Mohammad et al., 2013). Mucus obstruction is thought to be due to hypersecretion, airway dehydration, and rheological changes (Chisholm et al., 2019). Radicioni et al. highlighted the role of MUC5AC hypersecretion in the initiation, progression, exacerbation, and pathogenesis of COPD. Published data also suggests a change in MUC5AC concentration as a function of COPD severity (Radicioni et al., 2021). Sputum expectoration in COPD patients assists in the removal of phlegm from the large airways, but small airway mucus obstruction is considered a characteristic abnormality in COPD. Bulk rheological properties of airway mucus in patients with COPD have been widely explored and revealed to have higher viscoelasticity compared to samples from healthy subjects and subjects with CF. In contrast, watery mucus produced from asymptomatic tobacco smokers were cleared faster by cilia than normal mucus (Rubin et al., 1992). However, only limited studies have investigated the correlation between COPD sputum mesh size to sputum composition and lung function.

Chisholm et al. studied sputum microstructure in smokers with and without airway obstruction by using MPT with MAPs and MIPs (Chisholm et al., 2019). In sputum samples from smokers without airway obstruction, 100 nm MIPs highly diffused through sputum, whereas MAPs were largely hindered regardless of their particle diameter. Furthermore, same-sized MIPs and MAPs displayed unhindered diffusion and highly confined trajectories, respectively, in sputum samples from COPD patients with MSD values significantly higher for the MIPs compared to the MAPs. This study demonstrated that MIPs prevented sputum adhesion and the diffusion rates of MIPs can be explored to distinguish the biophysical properties of sputum. However, MIPs and MAPs with particle diameters greater than 300 nm showed confined trajectories in both non-COPD and COPD sputum. This indicated that the MSD values were significantly greater for the MIP_S compared to that of the MAPs, suggesting tighter sputum mesh (smaller mesh size) in COPD. The authors also reported significantly higher solid content and mucin concentration in COPD sputum samples compared to non-COPD samples, whereas DNA content was not statistically significant between these groups.

Lin et al. use MPT to study mucus derived from primary human bronchial epithelial (HBE) cells with or without exogenous cigarette smoke exposure or cholinergic stimulation (Figure 3A) (Lin et al., 2020). As shown in Figure 3B, estimation of the MSD demonstrated decreased transport of MIP (1 μm particles) in mucus from COPD patient's than to the mucus from normal and healthy smoker donors. The microviscosity as well as the solid content of mucus from COPD patients was significantly higher than that of healthy smokers and non-smoking donors (Figure 3C). Sputum collected from COPD donors had higher viscosity at 0.6 Hz and a higher percentage of solid content (both positively correlated) compared to mucus from healthy individuals and smokers (Figures 3D and E). Above data shows that change in mucin structure, mucus mesh size, and mucociliary transport occur in response to chronic exposure to cigarette smoke.

4.2 Microstructural alterations in cystic fibrosis sputum

It is thought that increased mucin concentration in CF sputum is due, in part, to defects in CFTR which leads to an imbalance of water and electrolyte secretion in the CF airway(Chen et al., 2010b; Quinton, 2008). CFTR dysfunction causes decreased airway surface liquid (ASL) leading to increased osmotic pressure in the mucus layer (Button et al., 2012). Another hypothesis suggests that mucus dehydration causes an increase in mucin concentration along with a reduction in mucin pH, increase in cellular debris, reduction in glutathione, and elevation in myeloperoxidase levels, resulting in additional inter-chain cross-links (Perez-Vilar and Boucher, 2004). In contrast to the hydrated normal airway mucus that has a mesh size of around 0.2 – 1 μm, the mesh size in CF sputum is <100 nm (Clunes and Boucher, 2007). The decrease in the mesh size of CF sputum leads to the inability of neutrophils to penetrate through the mucus to capture bacteria (Matsui et al., 2005). Increase in the viscoelasticity of sputum from CF patient's greatly reduced particles diffusion, thereby reducing the efficiency of drug delivery systems to lung epithelial cells (Dawson et al., 2003; Prasher et al., 2022).

Suk et al. estimated that fresh CF sputum has the mesh spacing of approximately 140 ± 50 nm using a fitted transport rate of various sized MIP (100, 200, and 500 nm in diameter) to an obstruction-scaling model (Suk et al., 2009). They also analyzed the extent of impediments to particle penetration in native CF sputum compared to N-acetyl L-cysteine (NAC) treated CF sputum. NAC is a mucolytic drug that cleaves disulfide bonds and so enhance MIP penetration in CF sputum. The diffusion exponent, α (for pure unhindered Brownian diffusion, $\alpha = 1$; as the particle diffusion is obstructed, α approaches to 0) was 0.91 for the 200 nm MIP particles in CF sputum from NAC-treated group but in native sputum the value of α was 0.70 (Suk et al., 2011b). While experimentation of MIP diffusion through sputum can be used to evaluate microrheological and microstructural properties of mucus, there exists an interpatient variability roughly 50-times higher than intra-patient sputum samples. This might be due to differences in disease severity (Duncan et al., 2016b). The same study reported that the decrease in sputum mesh size ($\log_{10}$ [MSD_{1s}]) was linked to the increase in mucin, DNA, and total solid concentration in sputum. Previous studies made several efforts to improve particle diffusion through the CF sputum by decreasing the barrier properties of the sputum or surface coating the particles with non-muco-adhesive polymers such as PEG (Dawson et al., 2003; Duncan et al., 2016b; Forier et al., 2013; Suk et al., 2011b, 2009).

4.3 Mucus hypersecretion in asthma and mucus microstructure

In patients with asthma, the relative proportion of MUC5AC and MUC5B mucins in sputum is altered with disease severity (Bonser and Erle, 2017). Asthmatic subjects had higher levels of MUC5AC compared to MUC5B, while in healthy individuals, MUC5B was more prevalent in respiratory mucus (Bonser and Erle, 2017; Kirkham et al., 2002). Goblet cell hyperplasia, secretory cell hypertrophy, and upregulated expression of MUC5AC might contribute to mucus accumulation and plugging, ultimately causing small airway obstruction (Evans et al., 2015; Ha and Rogers, 2016; Shen et al., 2018). Measurements of bulk rheology of respiratory secretions from asthmatic patients during acute exacerbations showed

increased mucin cross-linking rather than mucin concentrations, which was demonstrated by the marked increase in elastic modulus (Innes et al., 2009; Yuan et al., 2015).

Morgan et al. examined the biophysical properties of airway mucus from asthma patients using the MPT technique (Morgan et al., 2021). To study whether mucus gel structure, MCC, and airway obstruction in asthma can be improved by disulfides disruption of mucus, mucus samples collected from asthma patients were treated with the reducing agent tris(2-carboxyethyl) phosphine (TCEP, 10 mM) and examined by MPT using PEG_{5k}-conjugated 100 nm polystyrene beads. TCEP treatment increased MSD values, decreased the viscoelasticity of mucus, caused rapid depolarization of mucins, normalized homogeneity and level of MSD to that similar to controls. Another study examined the effect of MUC5B:MUC5AC mucin ratios on the macro- and microrheology of synthetic mucus and compared the elastic moduli of mucin-based hydrogels (Song et al., 2021). Higher MUC5AC content resulted in greater elastic and viscous moduli. MPT analysis using 100 nm MIP probes showed that increased MUC5AC content was associated with decreased particle diffusion, suggesting increased viscoelastic and a decrease in the mesh network size. A diffusion study using larger-sized MIP probes of 500 nm demonstrated a further reduction in $\log[MSD_{1s}]$, which suggests an increase in the viscoelasticity with increasing MUC5AC. The authors also evaluated the gel network mesh size via MPT analysis and reported that MUC5B-rich hydrogels had larger mesh sizes (200–400 nm), whereas MUC5AC-rich gels had reduced network mesh sizes (100 nm).

4.4 Hyperconcentration of airway mucus in Non-CF bronchiectasis

Non-CF bronchiectasis is characterized by chronic airway obstruction and infection leading to dilation of the bronchi. These airways become filled with phlegm (Ramos et al., 2015; Rubin, 2009; Van der Schans, 2007). The relationship between secreted mucin levels and the presence of bacterial infection in the airways of stable bronchiectasis patients demonstrated that the highest levels of airway MUC2 was in patients' airways infected with *P. aeruginosa*. Overall, airway mucus from non-CF bronchiectasis patients exhibited increased solid percentage as well as increased major secreted airway mucins compared with sputum from healthy individuals (Kesimer et al., 2017; Ramsey et al., 2020).

5. Contribution of Neutrophil Extracellular Traps (NETs) in respiratory diseases

Neutrophils are the first immune cells recruited to the site of inflammation and infection in pulmonary diseases. Neutrophil defense mechanisms include phagocytosis, degranulation, producing reactive oxygen species (ROS) and pro-inflammatory cytokines, and extruding neutrophil extracellular traps (NETs) (Brinkmann et al., 2004; Tucker et al., 2021). NETs are web-like structures composed of DNA in association with histones, antimicrobial granular, and cytoplasmic proteins such as neutrophil elastase, myeloperoxidase, and α-defensins (Keir and Chalmers, 2022; Porto and Stein, 2016; Tucker et al., 2021). The primary role of NETs is to trap and kill extracellular bacteria and other pathogens during infections (Brinkmann et al., 2004; Lefrançais et al., 2018). NET scaffold consists of chromatin fibers, DNA, and histones as the major structural constituents (Brinkmann et al., 2004). In patients

with lung diseases, production of NETs decorated with histones causes cell cytotoxicity leading to inflammation (Cheng and Palaniyar, 2013; Saffarzadeh et al., 2012). A review by Block and Zarbock discussed the essential steps of NET formation (Block and Zarbock, 2021). As illustrated in Figure 4, different pathogens are capable of inducing NET formation and receptors such as toll-like-, G-protein coupled-, and Fc-receptors communicate with the cells to activate NADPH-oxidase complex, subsequently catalyzing ROS generation. Neutrophil elastase is released from the membranes in a ROS-dependent manner and results in the decondensation and release of chromatins decorated with antimicrobial agents.

NETs have been identified in the sputum of pulmonary disease patients and the ultrastructural characterization of these sputum demonstrated NETs contribute to increased viscosity (Lethem et al., 1990; Saffarzadeh et al., 2012). The contribution of NETs on airway mucus viscoelasticity was recently explored by our team (Linssen et al., 2020). Human airway mucus was collected from endotracheal tubes of healthy adult patients admitted for elective surgery and were coincubated with NETs from phorbol 12-myristate 13-acetate-stimulated neutrophils. The study demonstrated that mucus coincubated with NETs resulted in significant increase in mucus viscoelasticity and decrease in mesh size of the mucus, and decreased movement of MIPs (100 nm and 500 nm) through the mucus as measured by MPT. However, the underlying mechanisms behind the development of NETs and their role in airway inflammation and increased mucus production still remain unclear (X. Chen et al., 2021; Zou et al., 2018). Studies suggest that production of NETs and delayed NET clearance are a contributor to chronic inflammation and lung tissue damage (Grabcanovic-Musija et al., 2015; Keir and Chalmers, 2022; Liu et al., 2017; Uddin et al., 2019). The bactericidal activity of neutrophils and NETosis is affected with pH of airway surface liquid and airway infections (Khan et al., 2019; Moraes et al., 2006; Pezzulo et al., 2012). Increased secretion of pro-inflammatory cytokines in CF lungs might induce NET formation and its release (Dwyer et al., 2014; Marcos et al., 2010). NET formation is also induced by the resistance of *P. aeruginosa* to NET-mediated killing, bacterial mutations in chronic infections, and the release of eicosanoid hepoxilin A3 by lung epithelial cells (Douda et al., 2015; Hurley et al., 2004; Limoli et al., 2014; Oliver et al., 2000; Rahman and Gadjeva, 2014). Dornase alfa can cleave polymerized DNA and reduce the viscosity of CF sputum is a widely accepted therapy for CF. In addition, use of dornase alfa in patients with COPD and non-CF bronchiectasis has shown potentially harmful effects in clinical trials (Aaron, 2017; McShane et al., 2013; Pasteur et al., 2010). Sputum from COPD patients also contains NETs and NET-forming neutrophils (Dicker et al., 2018; Grabcanovic-Musija et al., 2015; Obermayer et al., 2014). COVID-19 patients also have NETs in their airways (Middleton et al., 2020; Radermecker et al., 2020).

6. Muco-adhesive and muco-penetrating particles designed to overcome the respiratory mucus barrier

Particles with sizes larger than the airway mucus meshwork are trapped by steric obstruction while smaller particles can diffuse through the mucus pores (Huckaby and Lai, 2018). Negatively charged mucins can capture particles with cationic domains. This strategy has been applied to develop muco-adhesive particles (Figure 5) (Huck et al., 2022). The high density of glycans in the mucin fibers can also form hydrogen bonds with carboxyl and

hydroxyl groups on the particle surfaces (Lele and Hoffman, 2000) and the non-glycosylated domain of the mucin forms a hydrophobic backbone thereby providing hydrophobic interactions with particles (Richard A. Cone, 2009; Murgia et al., 2018). Therapeutic aerosol delivery has been a primary approach to treat lung conditions such as asthma, CF, and COPD, however, it is important to design nanoparticles with appropriate sizes and surface chemistry for improved drug delivery to the lungs.

As discussed above, pulmonary drug delivery is an effective approach to treating lung diseases, however, the mucus layer in the airways has evolved in protecting the lungs by rapidly trapping and removing foreign particles through the MCC (Da Silva et al., 2023; Ji et al., 2020; Osman et al., 2018). Several formulation-based approaches have been developed to extend the drug residence time in the lungs of poorly water-soluble drugs (Table 2). Among them, surface-modified biodegradable nanoparticles that can adhere to the mucus, namely MAPs (muco-adhesive particles), have been developed. Since mucus has highly glycosylated proteins, MAPs have been engineered to possess a positive surface charge to enhance electrostatic interactions with mucin fibers (Linden et al., 2008). Chitosan is one of the most commonly used polymers for developing MAPs through chemical conjugation or physical absorption on particle surfaces (M Ways et al., 2018; Rawal et al., 2018; Vieira et al., 2018). Another approach to enhancing the muco-adhesive properties of nanoparticles is to engineer nanoparticles using thiol-derived chitosan that can form disulfide bonds with mucin strands via thiol groups (Makhlof et al., 2010). It has been demonstrated that the chitosan-based MAPs can provide higher permeability and a better pharmacokinetic profile in healthy rat lungs than uncoated particles; however, it is still controversial whether MAPs can support the clinical application in some chronic inflammatory airway diseases with secretion retention (Bustamante-Marin and Ostrowski, 2017; Morrison et al., 2019). These cationic and thiol-coated nanoparticles are usually formulated with polymeric cores indicating that the muco-adhesive properties of the nanoparticles may also be due to the hydrophobic interactions of the nanoparticles and the mucus (Benjamin S Schuster et al., 2013; Suk et al., 2009). An alternative approach to achieving muco-adhesion is to use polymers that can physically entangle with mucin fibers, such as polyacrylic acid, alginates, and hyaluronic acid-coated nanoparticles (Cui et al., 2006; H. Kim et al., 2013; Müller et al., 2012). Despite this, MAPs mainly stick to the superficial layer of the airway mucus and are quickly cleared due to the rapid MCC and steady airway mucus turnover (~10–20 min). Therefore, it is expected that fewer MAPs will reach the underlying periciliary layer (PCL), potentially decreasing their bioavailability (Ali and Pearson, 2007).

Mucins are condensed and/or bundled into a thick cable through hydrophobic interaction, so with MAP treatment the structure of the mucus might be altered via interaction with MAPs (Lai et al., 2010). Wang et al. engineered MIP by dense coating of carboxylated polystyrene particles (1 μm in diameter) with PEG_{2k} (surface charge −6.1 ± 0.9 mV), whose diffusion in the mucus is hindered only by steric obstruction. These MIP were used to determine the microstructural changes of mucus caused by MAP (200 nm PS-NH_2, surface charge 13 ± 1.1 mV at pH 4). The study demonstrated that a high concentration (0.24% w/v) of MAP induced increased average effective diffusivity of the probe particles (MIP). In addition, use of obstruction-scaling model showed that the higher MAP concentration increased the average mucus mesh size from 380 nm to 470 nm, which was not observed at

low concentration of MAP (0.0006% w/v) (Wang et al., 2011). Although the MAPs did not alter the rheology of the mucus, the MAPs may have cross-linked with the mucus fibers and caused them to bundle together, enlarging mucus meshwork and compromising its ability to trap foreign particles and pathogens. Since larger nanoparticles can provide higher drug loading and longer drug release profiles, the authors proposed to use “Sacrificial MAPs” to transiently enlarge the mucus pores facilitating the later administration of larger drug-loaded nanoparticles. Unfortunately, this may not be applicable for pulmonary drug delivery considering there is much faster airway mucus clearance (10~20 min) than cervicovaginal mucus (up to hours) (Ali and Pearson, 2007; Lai et al., 2009b).

Mucus penetration particles (MPP) or muco-inert particles (MIP) engineered by dense PEG-coating can minimize the interaction with airway mucus (Craparo et al., 2016; Schneider et al., 2017). Study conducted by Forier et al. to characterize the mobility of 100 nm and 200 nm PS-PEG MPP and PS-COOH MAP in CF sputum and biofilm suggested that PEGylation increased diffusion of nanoparticles in CF sputum and a better accumulation in the biofilm (Forier et al., 2013). Later studies further demonstrated that nanoparticles that are densely coated with high molecular weight PEG (e.g. 10 kDa and 40 kDa) can also prevent the mucus interactions, as long as the PEG coating is a dense brush conformation ($\Gamma/\Gamma^{*}>2$) (Maisel et al., 2016; Xu et al., 2013). These results highlighted the importance of engineering MPPs with suitable physiochemical properties for characterizing the microstructure of airway mucus and effective drug delivery to the lungs.

Schneider et al. compared the behavior of PS-PEG_{5k} (MPPs) and PS-COOH (MAPs) particles in mouse lungs after intranasal administration (Schneider et al., 2017). MPPs as large as 300 nm were uniformly distributed and retained in the airway while MAPs, regardless of particle sizes, formed large aggregates that were poorly distributed through the airway. In order to facilitate future clinical translation, biodegradable MPPs and MAPs were further engineered using poly(lactic-co-glycolic acid) (PLGA) polymers, first using a PLGA-PEG_{5k} polymer. Uncoated PLGA nanoparticles (biodegradable MAPs control) and MPPs showed a particle size around 130–140 nm. However, the average zeta potential of uncoated and PEG_{5k} coated nanoparticles were −73 mV and −6 mV, respectively. A previous study demonstrated that dense F127 coated PLGA/F127 nanoparticles can rapidly penetrate through human cervicovaginal mucus (CVM), while F68 with insufficient PEG coating limited the trajectories of nanoparticles in the CVM (Yang et al., 2011). Based on these findings, the authors then engineered b-MPPs and b-MAPs with comparable particle sizes around 180 – 200 nm using PLGA/F127 (densely PEG coating) and PLGA/F68 (non-densely PEG coating), respectively. Similar to the PS-PEG MPP, the biodegradable PLGA/F127 MPPs diffused rapidly in CF sputum *ex vivo* and had more uniform distribution and enhanced retention time in mouse lungs than biodegradable PLGA/F68 MAPs. Moreover, the dexamethasone sodium phosphate-loaded PLGA/F127 nanoparticles significantly reduced LPS-induced murine lung inflammation when compared to the drug-loaded PLGA/F68 nanoparticles.

Uniform distribution and expression of genes through the airway epithelial layer is important for gene therapy of lung diseases, however there are barriers that limit the effective penetration of gene carriers to the lungs (Suk et al., 2016). Bolyan et al. engineered

highly compacted DNA-nanoparticles using CK30 polymer (poly-L-lysine) conjugated with various PEG MW (2kDa, 5kDa, and 10 kDa) forming $CK_{30}PEG_{2k}$, $CK_{30}PEG_{5k}$, and $CK_{30}PEG_{10k}$ nanoparticles with particle sizes around 220, 300, and 350 nm, respectively and nearly neutral surface charges (Boylan et al., 2012). $CK_{30}PEG_{10k}$ nanoparticles presented a flexible-rod shape structure (Figure 6A). Although the hydrodynamic diameters were 4-fold less than that of the $CK_{30}PEG_{2k}$ nanoparticles, all the DNA-nanoparticles formulations were immobilized and the diffusion in CF sputum was 40-fold slower than muco-inert PS-PEG_{2k} nanoparticles (Figure 6B). To improve the diffusive rate of $CK_{30}PEG$ DNA-nanoparticles, mucolytic agents such as dornase alfa, NAC or a combination of NAC, and dornase were added to CF sputum before the $CK_{30}PEG_{10k}$ DNA-nanoparticles' incubation (Suk et al., 2011a). Treatment alone with dornase did not enhance gene carrier diffusion; however, NAC and NAC plus dornase increased the average diffusive rates by 6-fold and 13-fold, respectively. While a mucolytic can improve the sputum penetration of $CK_{30}PEG_{10k}$ DNA nanoparticles, many DNA nanoparticles were restrained in the CF sputum. This suggest that adhesive interactions rather than steric interactions are the primary factor that limits the mobility of the $CK_{30}PEG_{10k}$ DNA nanoparticles in the sputum. The PEG surface density on all the $CK_{30}PEG$ DNA nanoparticles ranges from 4 to 10 molecules/100 nm^2, which is 12–30-fold less than that of the muco-inert PS-PEG_{2k} nanoparticles. A previous study reported that 40% reduction in the surface PEG density might cause 700-fold decrease in the average mobility of the PS-PEG in the CVM, suggesting insufficient PEG coating on the surface of the $CK_{30}PEG$ DNA-nanoparticles is responsible for the DNA-nanoparticles trapped in the CF sputum (Wang et al., 2008). However, to effectively minimize nanoparticles binding with mucin, a dense brush conformation is required ($[\Gamma]/[\Gamma^*]>2$), which reflects how densely packed and constrained the PEG molecules are on the nanoparticle surface. Where, $[\Gamma]$ is the number of PEG chains (molecules) on the nanoparticle surface per 100 nm^2 surface area and $[\Gamma^*]$ represents the number of unconstrained (mushroom conformation) PEG molecules that occupy a surface area of 100 nm^2 on the nanoparticle surface (Shi et al., 2021; Xu et al., 2015).

A dense PEG surface coating is important for minimizing the muco-adhesion of gene carriers (Craparo et al., 2020). Nonetheless, a high degree of PEG-grafting on the cationic polymer impedes the DNA compaction and leads to larger particle sizes, due to the fewer positive charges available for DNA compaction and the higher degree of steric interferences from PEG. Loss of DNA compaction and large nanoparticle sizes can further compromise the surface PEG density causing poor sputum penetration. To overcome this dilemma, Suk et al. developed highly compacted small DNA-nanoparticles with a dense PEG-coating by using the mixed polymers of PEI and PEG_{5K}-PEI at a 1:3 ratio (PEI-MPP) (Suk et al., 2014). A similar MPP formulation was also developed by using poly-l-lysine (PLL-MPP) polymers. Both PEI-MPPs and PLL-MPPs have particle sizes around 50 nm with a near neutral surface charge. Although both the conventionally coated particles (PEI-CCPs) and the PEI-MPPs have spherical-shaped structures, the PEI-MPPs have a more compact structure compared with the PEI-CCPs (Figures 6C and 6D). The PEI-MPPs can penetrate through human CF sputum, whereas the PEI-CCPs and un-PEGylated PEI-nanoparticles (PEI-UCP) with similar particle sizes are immobilized (Figure 6E). PEI-MPPs had more

uniform distribution and more than 70% retention in mouse airways 6 hours after intranasal administration, in contrast with a restrained distribution and faster clearance of conventional PEGylated nanoparticles with 20% remaining at 2 hours after administration.

Unlike the conventional PEG conjugation method, Kim et al. designed PEG-dendrons through polyamidoamine (PAMAM) dendrimers which have a high density of primary amines on their surface and a cleavable disulfide core for preparing densely coated gene carriers that can penetrate through CF sputum (A. J. Kim et al., 2013). The PEG-dendrons were first synthesized by conjugating PEG_{5k} to the terminal amine groups on the PAAM dendrimer surface, followed by PAMAM S-S disulfide bond reduction to two single-site sulfhydryl functional PEG-dendrons (-SH). Then the cationic polymers, either PEI or the G4 PAMAM polymer was connected to PEG-dendrons(-SH) through cross-linking, forming PEG-dendron conjugated cationic polymers. The plasmid DNA vector was encapsulated into nanoparticles using the synthesized PEG-dendron conjugated cationic polymers, namely $dPEG_{5k}$-PAMAM/DNA or $dPEG_{5k}$-PEI/DNA. Both nanoparticles have a spherical shape with particle sizes less than 80 nm and nearly neutral surface charges. Compared with conventional PEGylated PAMAM/DNA or PEI/DNA, the PEG-dendron systems provided more compact DNA nanoparticles with smaller particle sizes. Additionally, $dPEG_{5k}$-PAMAM/DNA and $dPEG_{5k}$-PEI/DNA have high surface PEG densities around 33 PEG/100 nm^2 and 28 PEG/100 nm^2, respectively. The surface PEG density was comparable to the muco-inert nanoparticles and was 6–8 fold higher than that of $CK_{30}PEG_{10k}$, which was unable to penetrate through CF sputum. The high surface PEG density on $dPEG_{5k}$-PAMA/DNA and $dPEG_{5k}$-PEI/DNA contributes to their uniform distribution and rapid penetration in CF sputum. In human bronchial epithelial cells (BEAS-2B), $dPEG_{5k}$-PAMA/DNA and $dPEG_{5k}$-PEI/DNA showed higher luciferase activity compared to plasmid DNA control, however, gene transfection efficiencies were lower compared to uncoated counterparts, which might be due to decreased cellular uptake. PEG-dendron/DNA can stably induce the expression of CFTR genes in CF bronchial epithelial cells. This might be due to sterically shielding of particle core by hydrophilic PEG as well as reducing the particles uptake by surrounding immune cells.

Another approach to develop MPP nanoparticles is using zwitterionic polymers, such as poly(phosphobetaine), poly(sulfobetaine), and poly(carboxybetaine) (Cao and Jiang, 2012). Zwitterionic polymers are polyelectrolytes that have both positively and negatively charged groups but have an overall neutral surface charge (Zhang et al., 2019). Poly(zwitterionic) molecules display strong binding with water molecules through electrostatic interactions, forming a super hydrophilic surface that contributes to their non-fouling characteristic against protein and biofilms (Jiang and Cao, 2010; Zhang et al., 2019). Studies have shown that zwitterionic poly(carboxybetaine) (PCB) and poly(sulfobetaine) (PSB) coated nanoparticles can resist nonspecific protein absorption in plasma (Li et al., 2015; van Andel et al., 2017). Hu et al. evaluated the ability of PLGA-based nanoparticles with different surface modifications such as PEG, polyvinyl alcohol (PVA), F127, and zwitterionic polydopamine (PDA) to penetrate mucus (Hu et al., 2022). Bare PLGA-NPs were first prepared using the nanoprecipitation method, followed by dropwise addition of 20 mL of 1% PVA, PEG, or F127 solution. For PDA nanoparticles, PLGA nanoparticles were resuspended in dopamine solution modified with Tris buffer (pH 10) and allowed to react for

4 h under magnetic stirring. All the nanoparticles had particle sizes ranging from 190 to 270 nm, whereas surface zeta charges for PLGA/F127, PLGA/PDA, and PLGA nanoparticles were −26.9 ± 0.9, −26.3 ± 2.8, and −13.0 ± 2.7 mV, respectively, except for PLGA-PEG nanoparticles which had near-neutral surface charges (−6.2 ± 1.7 mV). Particle tracking studies revealed that PDA-coated PLGA-nanoparticles can penetrate through porcine mucin, showing similar trajectories to muco-inert particles including PLGA/F127 and PLGA-PEG nanoparticles, whereas PLGA nanoparticles were immobilized. However, the particle tracking was conducted using commercialized porcine mucus mixed with agarose gel and thus the study cannot mimic airway mucus in chronic pulmonary diseases that generate compacted mucus (Bustamante-Marin and Ostrowski, 2017; Morrison et al., 2019).

Although, MPPs penetrate mucus more than MAPs and the residence time is less affected by MCC, MPPs may have back-diffusion due to the particle's concentration gradient (Netsomboon and Bernkop-Schnürch, 2016). Another issue with MPPs may be the limited endocytosis by the epithelial cells due to neutral charges and hydrophilic surfaces. Therefore, design of nanoparticles should be such that they penetrate the mucus layer and then get endocytosed by epithelial cells. This can be achieved by varying the PEG coating percentage on nanoparticle surface along with coating of polymers such as poly(2-ethyl-2-oxazoline) (POZ), polydopamine (PDA), or N-(2-hydroxypropyl) methacrylamide copolymer (PHPMA) (Liu et al., 2016; Mansfield et al., 2015; Poinard et al., 2019). Fasquelle et al. designed lipidated maltodextrin nanoparticles by incorporating anionic phospholipid core (NPL) in the nanoparticles and studied the interaction of cationic maltodextrin based nanoparticles, PEG-coated MPPs and chitosan-coated MAPs to the airway mucins and epithelial cells (Fasquelle et al., 2020). There was increased mobility of NPLs in airway mucin as well as unhindered uptake and delivery of proteins into the airway epithelial cells. In another study, the diffusion behavior of PDA-coated polystyrene nanoparticles and PEGylated polystyrene nanoparticles through reconstituted mucin solution was similar (Poinard et al., 2019). However, the cellular uptake of PDA-coated nanoparticles in T24 mucosal cells was three folds higher compared to that of PEGylated nanoparticles, suggesting enhanced muco-penetration by minimizing interaction with the negatively charged mucins and increased cellular uptake by interaction with positively charged choline groups on the lipid membrane.

PCB and its derivatives can also delay biofilm formation and inhibit biofilm adhesion (Cheng et al., 2009, 2008). Cheng et al. constructed zwitterionic poly (sulfobetaine methacrylate) (pSBMA) coated surfaces through atom transfer radical polymerization and evaluated the adhesion of *S. epidermidis* and *P. aeruginosa* using a laminar flow chamber (Cheng et al., 2007). Compared with bare glass and the methyl-terminated (CH3) coated glass surfaces which provided strong biofilm adhesion, pSBMA reduced the short-term (3h) *S. epidermidis* and *P. aeruginosa* adhesion by 92% and 96%, respectively. In long-term biofilm resistant studies, pSBMA coating can inhibit biofilm attachment up to 48 h for *S. epidermidis* and 24 h for *P. aeruginosa*. Zwitterionic polymers mainly conjugate to proteins or polymers, forming polymer-protein complexes or copolymers that can be formulated into muco-inert nanoparticles or liposomes (Tsao et al., 2020). Currently, zwitterionic polymers are mainly applied for oral drug delivery. Considering that they can resist non-specific

protein binding and biofilm formation, the zwitterionic polymers may also have a broad application in developing muco-inert nanoparticles for pulmonary delivery.

7. Aerosolized muco-adhesive and muco-penetrating particles for pulmonary delivery

Inhalable systems employing a carrier excipient offer numerous advantages, for example, reduced particle size, increased surface area, and altered drug release in the lung tissues (Chenthamara et al., 2019; Maurya et al., 2020; Pontes and Grenha, 2020; Youngren-Ortiz et al., 2017). In particular, biodegradable MAPs and MIPs can be delivered into the lungs *via* nebulization of colloidal dispersions or pressurized metered dose inhalation (pMDI) or dry powder inhalation (DPI) (Kumar et al., 2022; Mangal et al., 2017; Wiedmann et al., 1997; Yang et al., 2008). Polymer-based carriers, liposomes and lipid-based carriers, emulsions, and dendrimers have been used as carrier systems; however, colloidal dispersion formulations have limitations such as strong interparticle interactions within a nanosystem, agglomeration and settling of particles, and chemical instability leading to carrier hydrolysis and drug degradation (Ahmad et al., 2005; Bivas-Benita et al., 2004; Cryan et al., 2006; Joshi and Misra, 2001; Plumley et al., 2009). To address these issues, dry powder-based carriers containing nanoparticles or microparticles with better particle size, surface morphology, aerodynamic diameter, and interparticulate interactions were designed (Xu et al., 2011). DPI are an attractive pulmonary delivery platform for the treatment of lung diseases (Son et al., 2013). However, commercial DPIs often deliver particles in the size range of 2 – 5 μm which produce extra-thoracic losses of 40~70% of the dose, in part due to incomplete particle deaggregation during inhalation (Hindle and Longest, 2012). The excipient enhanced growth (EEG) technology has been used to deliver particles with an initial size of <2 μm to reduce mouth-throat loss. The particles increase in size to 2–5 μm by hygroscopic growth after inhalation to achieve lung deposition and more uniform delivery throughout the airways (Boc et al., 2021; Hassan et al., 2020). Nevertheless, sputum and mucus are still critical barriers that limit effective drug penetration into the lung.

Muco-adhesive powder microspheres loaded with a poorly soluble model drug were developed using hydroxypropylcellulose polymers (HPC) (Sakagami et al., 2001). The crystalline HPC microspheres showed sustained absorption, whereas amorphous HPC microspheres demonstrated rapid absorption. Moreover, microspheres prepared using HPC-H (high viscosity) and a drug-HPC ratio of greater than 1:4 successfully enhanced absorption and reduced MCC compared to microspheres containing HPC-SL (the least viscous) and the lowest drug-HPC ratio of 1:1. Dong et al. compared muco-adhesive and muco-penetrating characteristics of baicalein-phospholipid complex loaded polymeric nanoparticles that were surface coated with either chitosan (CS-BA nanoparticles) and Pluronic F127 (F127-BA nanoparticles) (Dong et al., 2020). Due to the cationic amino group of chitosan, CS-BA nanoparticles had muco-adhesive properties, whereas the Pluronic F127 coating enhanced muco-penetration in the F127-BA nanoparticles, thereby generating a higher diffusion rate across porcine airway mucus *ex-vivo*. Findings from this study indicated that the F127-BA nanoparticles-based formulation could deliver baicalein with a

higher diffusion rate to allow deeper penetration in healthy airway mucus in mouse model compared to the muco-adhesive formulation.

Our group conducted a proof-of-concept study using densely PEG coated muco-inert nanoparticles and uncoated polystyrene nanoparticles (PS-PEG nanoparticles and PS nanoparticles) with a particle size of 100 nm, which were further formulated as a powder using an EEG strategy (Chai et al., 2020). EEG is a recently introduced strategy that uses the powder formulation to produce aerosol size growth and minimizes extra thoracic losses by delivering micrometer-sized particle formulation (Walenga et al., 2017). PS-nanoparticles, both colloidal suspension or powdered EEG aerosols were highly hindered in sputum from CF patients, whereas PEG-coated nanoparticles had greater diffusion as shown in Figure 7A. Representative single particle trajectory showed greater movement for PEG-coated nanoparticles in the CF sputum compared with PS nanoparticles (Figure 7B). Moreover, the diffusion rate distribution study showed similar movement for the PS-PEG nanoparticle colloidal suspension and muco-inert nanoparticles loaded into the EEG dry powder aerosol (Figure 7C). Although, the diffusion of the aerosol was slightly slower compared to the suspension, this study showed that the muco-inert nanoparticles are released from the aerosol and maintain rapid mucus diffusion, which are still 22-fold faster than uncoated PS nanoparticles loaded EEG powder aerosols. Besides, hygroscopic growth of the EEG aerosols in the lungs will create a suspension before deposition and supply additional water, which could enhance mucus diffusion. In addition, densely PEG-coated nanoparticles of particle size smaller than mucus or sputum meshwork diffused efficiently. Overall, muco-adhesive and muco-penetrating aerosols can be successfully designed to achieve reduced drug loading for inhalation, superior aerosol performance, and deep lung deposition.

8. Conclusions and perspectives

Pulmonary drug delivery is made more challenging, in part due to airway barrier functions that either trap and remove inhaled drugs or inactivate them when they are deposited. Over the last decades, the role of airway mucus in health and disease has been extensively investigated and it has been found that airway mucus hypersecretion, decreased mucus clearance, upregulation of mucin production (particularly MUC5AC and MUC5B), pulmonary infection and inflammation, and the formation of NETs are critical factors in airway obstruction and lung function decline. In addition, an increase in the disulfide bond density and the total solid contents of mucus leads to the formation of a tighter mesh network in subjects with respiratory disease, which directly contributes to the alteration in microstructural properties of mucus such as mesh size and viscosity. SEM and magnetic microrheometry measurements have their unique advantages in studying the rheology of airway mucus. More recently, MPT has been widely accepted for investigating the interaction of nanoparticles and their mucus environment along with quantitative determination of the effective diffusivities, maximum velocity, and time-resolved particle trajectories. Substantial progress has been made in terms of formulations and delivery devices to improve lung deposition of aerosol drugs. Therefore, it is important to understand the fate of aerosol drugs or carrier systems after deposition in the lungs as well as the potential barriers to pulmonary delivery.

In this review, we discussed some approaches for engineering nanoparticles that can overcome the heterogenous airway mucus barrier. These nanoparticles can be modified for particle size, surface morphology, surface charge, and targeting moieties as each of these characteristics are critical for distinguishing muco-adhesive or muco-penetrating properties of nanoparticles. MAPs coated with chitosan, polyacrylic acid, hyaluronic acid, and surfactant phospholipids provide higher permeability and better pharmacokinetic profiles compared to non-coated nanoparticles and extend the drug residence time in the lungs. However, the clinical efficacy in the case of chronic pulmonary diseases with mucus over production is still poor. Unlike MAPs that adhere to mucus polymers, nanoparticles densely coated with muco-inert chemicals with particle size below the mesh network size can easily diffuse through the mucus and achieve a higher absorption of drug into the lungs, which is an effective alternative to conventional therapy. This has encouraged researchers to explore biodegradable polymers to design MAPs and MPPs and the use nanotechnology to engineer nanoparticles with higher efficacy to treat pulmonary disease.

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Abbreviations

AMP	Antimicrobial peptide
ASL	airway surface liquid
BALF	bronchoalveolar lavage fluid
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
COPD	chronic obstructive pulmonary disease
CVM	cervicovaginal mucus
DPI	dry powder inhaler
EEG	excipient enhanced growth
EGFR	epidermal growth factor receptor
HBE	human bronchial epithelial
IHC	immunohistochemistry
MAP	muco-adhesive particles
MCC	mucociliary clearance
MIP	muco-inert particles
MPP	mucus penetrating particles

MPT	multiple particle tracking
MSD	mean squared displacement
NAC	N-acetyl L-cysteine
PAMAM	polyamidoamine
PCB	poly(carboxybetaine)
PCL	periciliary layer
PDA	polydopamine
PEG	polyethylene glycol
PEI	polyethylenimine
PLGA	poly(lactic-co-glycolic acid)
PS	polystyrene
PSB	poly(sulfobetaine)
PSBMA	poly (sulfobetaine methacrylate)
PTM	particle tracking microrheology
PVA	poly vinyl alcohol
rhDNase	recombinant human deoxyribonuclease
RSV	respiratory syncytial virus
SEM	scanning electron microscopy
TCEP	tris(2-carboxyethyl) phosphine

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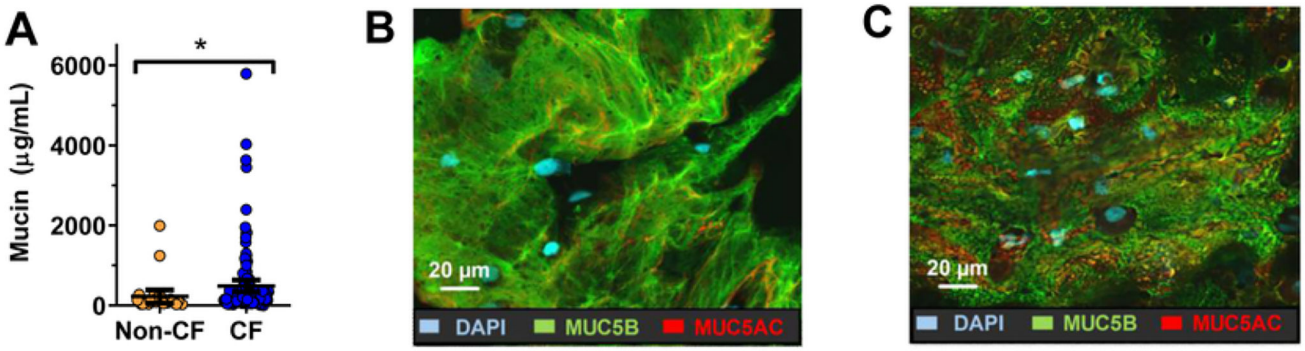

Figure 1.

(A) BALF total mucin concentration from CF patients and non-CF controls; (B) & (C) represents IHC of MUC5B (green) and MUC5AC (red) in a cytospin from non-CF and CF BALF, respectively. *$p<0.05$ after multivariate analysis. Reproduced with permission from (Esther Jr et al., 2019).

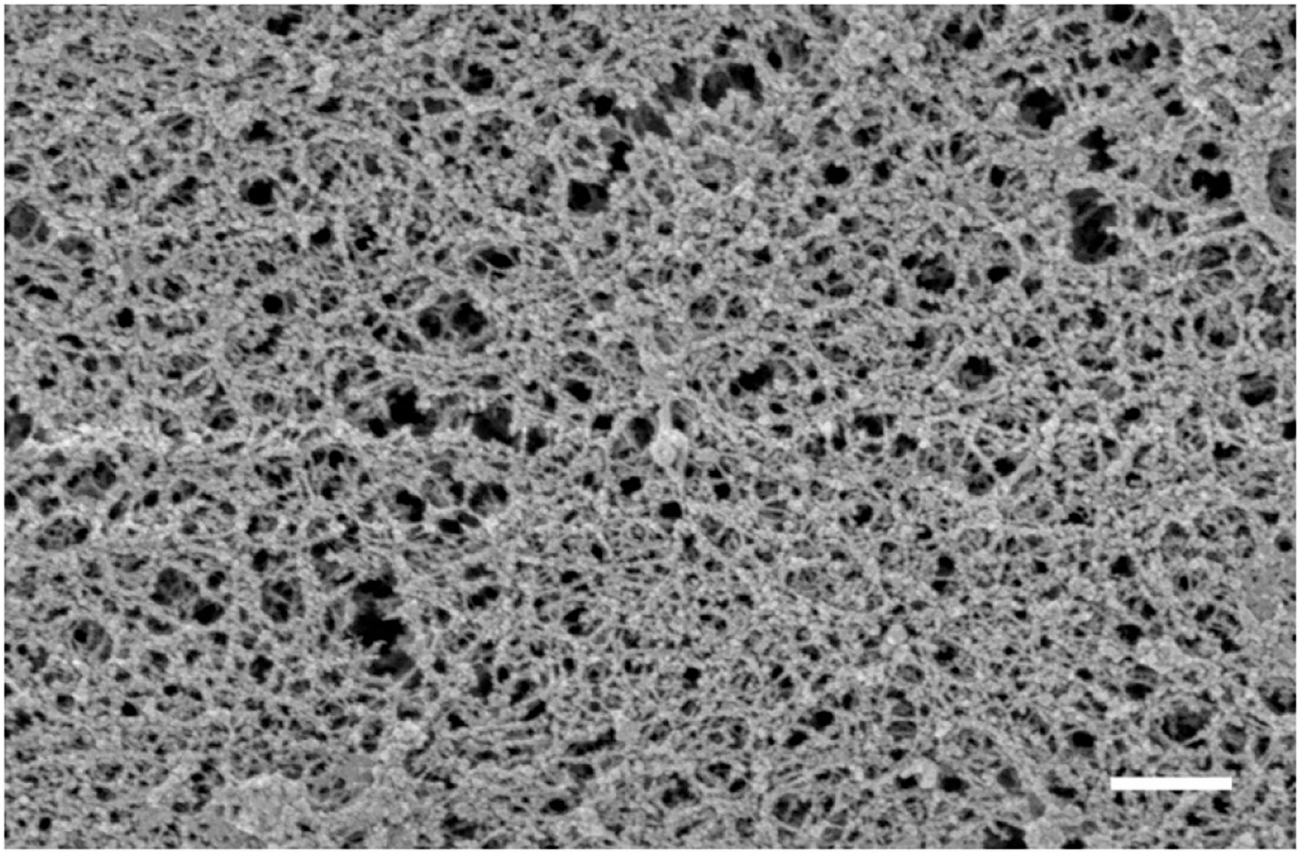

Figure 2.
SEM of airway mucus that shows a mesh-like architecture with the pore size ranging up to hundreds of nanometers in diameter (scale bar 500 nm). Reproduced with permission from (Benjamin S. Schuster et al., 2013).

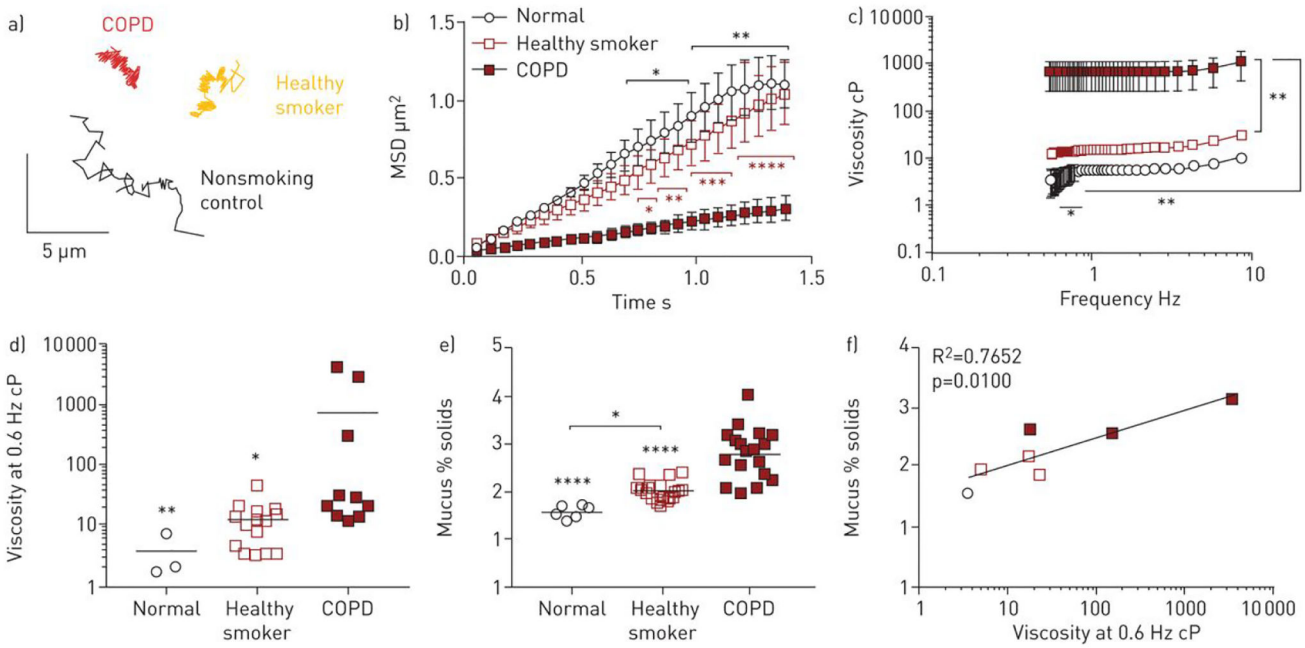

Figure 3.

Diffusion of MIPs (1 μm) in mucus from primary HBE cells. (A) Representative particle trajectories of 1 μm MIPs in mucus from normal control, healthy smoker, and COPD patients. Scale bar, 5 μm. (B) Ensemble-averaged geometric mean square displacements (<MSD>) as a function of time scale. (C) Effective viscosity of mucus from normal control, healthy smoker, and COPD patients. (D) Comparative effective viscosity of each mucus samples at 0.6 Hz. (E) Mucus percent solids content by weight in each mucus samples. (F) Plot between mucus solid content and effective viscosity for mucus from each group. Reproduced with permission from (Lin et al., 2020)

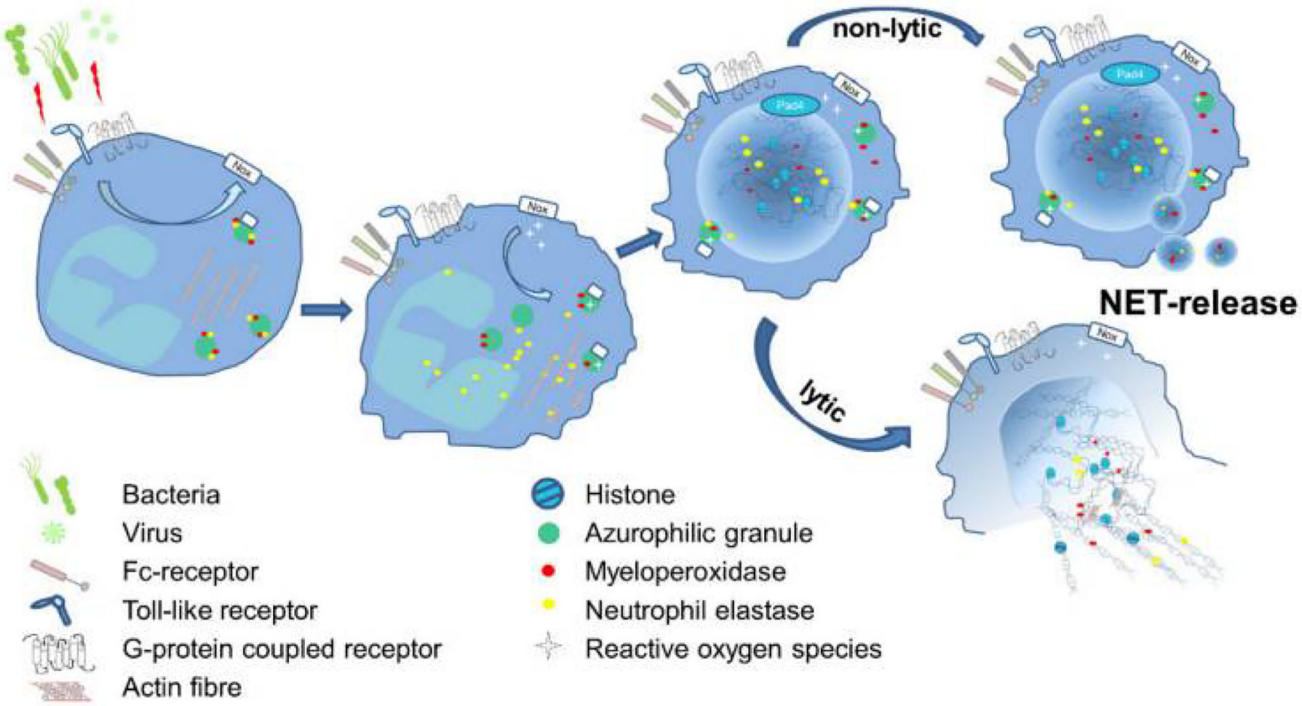

Figure 4.

Schematic illustration of the essential steps of NET formation. Reproduced with permission (Block and Zarbock, 2021).

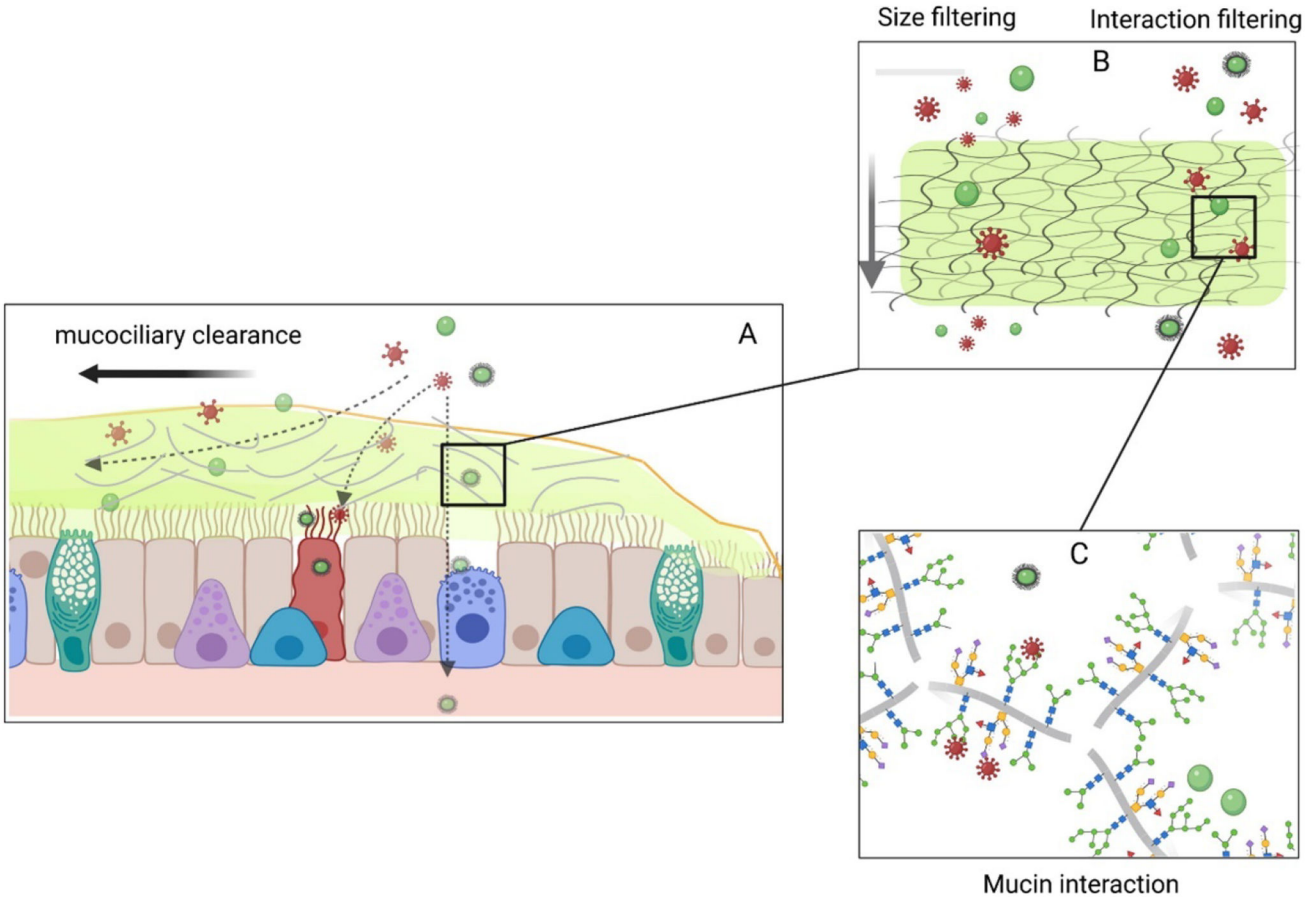

Figure 5.

Schematic on the role of mucus barrier. (A) As shown in figure, airway mucus entraps hazardous (red spiked structures) and therapeutic (green structures) inhaled particles. (B) Depending on the size and surface charge, the inhaled particles either penetrate the mucus barrier or (C) interacts with mucin backbone. Reproduced with permission from (Huck et al., 2022).

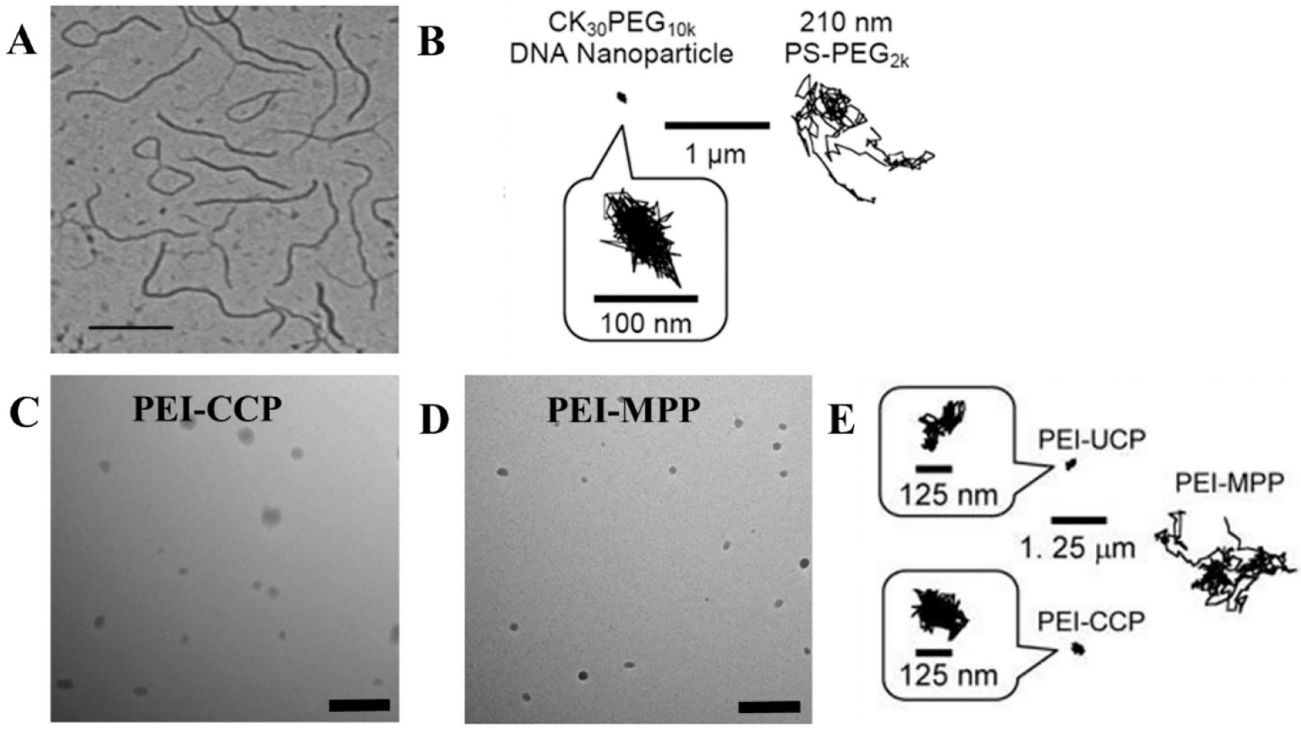

Figure 6.

Diffusion of gene carriers in CF sputum. (A) TEM image of $CK_{30}PEG_{10k}$ DNA-nanoparticles with hydrodynamic diameter of 60 nm which presented a rod-shape structure. (B) Representative trajectories of $CK_{30}PEG_{10k}$ DNA-nanoparticles which are immobilized in CF sputum, and muco-inert PS-PEG_{2K} nanoparticles with larger particle size. (C) TEM image of conventionally coated PEI/DNA NP (PEI-CCP) which had a loose spherical shape. (D) TEM image of PEI-MPP presented a more compacted spherical structure. (E) Representative trajectories of PEI-CCP nanoparticles, un-PEGylated PEI-nanoparticles (PEI-UCP) and PEI-MPP. PEI-CCP and PEI-UCP nanoparticles were restrained in CF sputum, while PEI-MPP can penetrate through CF sputum. Reproduced with permission from (Boylan et al., 2012) and (Suk et al., 2014).

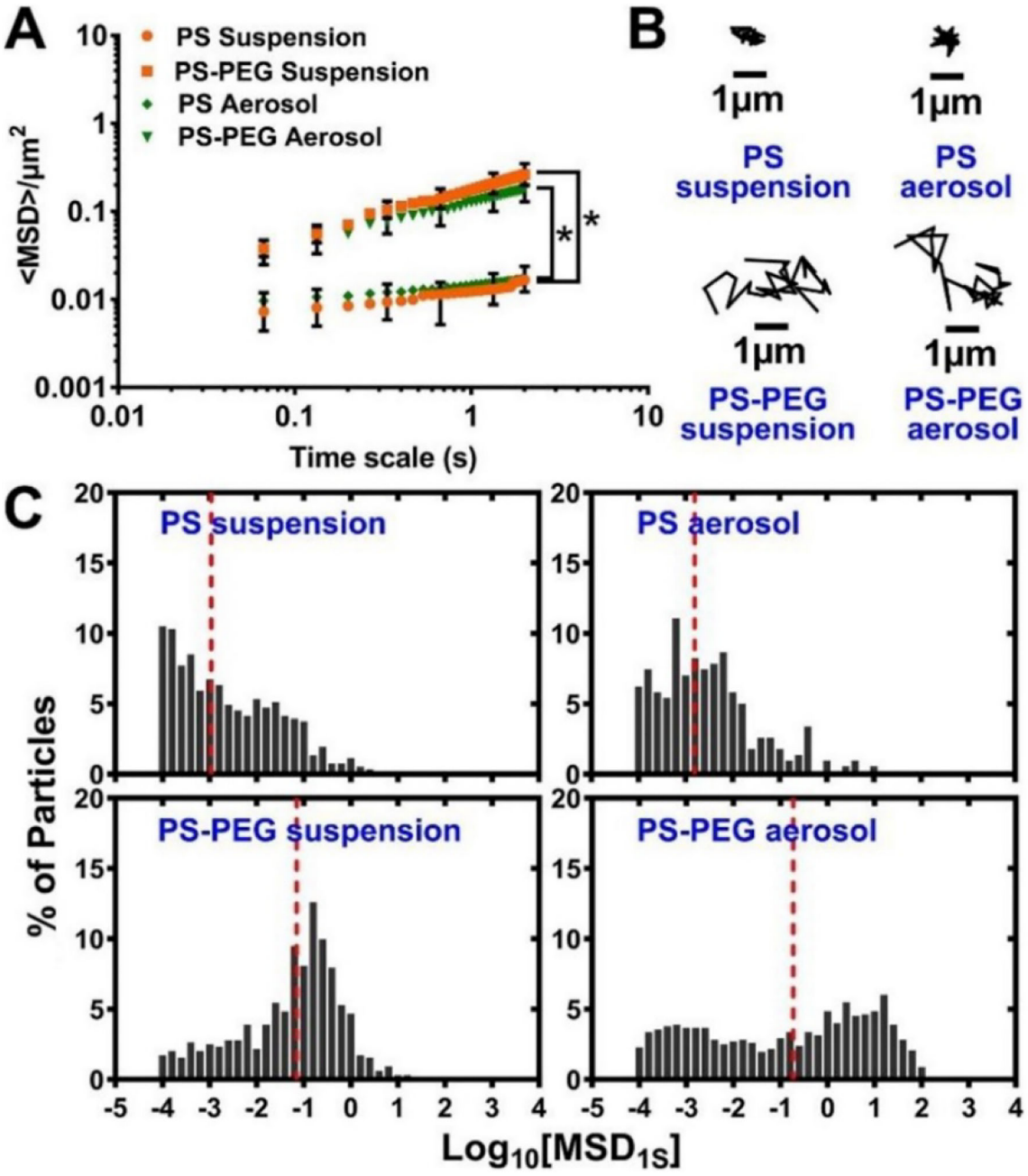

Figure 7.

Diffusion experiment using MIP suspension or MIP-containing EEG dry powder in CF sputum. (A) Ensemble-averaged geometric mean square displacements (<MSD>) as a function of time scale for PS suspension, PS-PEG suspension, PS aerosol, and PS-PEG aerosol in the CF mucus. (B) Representative particles trajectories of PS suspension, PS-PEG suspension, PS aerosol, and PS-PEG aerosol in the CF mucus in CF mucus. Scale bar, 1 μm. (C) The diffusion rate distribution of PS suspension, PS-PEG suspension, PS aerosol, and PS-PEG aerosol in the CF mucus. Reproduced with permission from (Chai et al., 2020).

Table 1

Characteristics of mucus in airway mucosal diseases

Airway mucus	Mucus characteristics	References
Healthy airway mucus	• Normal mucin production from airway epithelium • Gel with deformable, elastic solid and viscous liquid • Contains 97% water and 2−4% solid contents (mucins, salts, lipids, polypeptides, etc.) • Similar quantity of MUC5AC (proximal airways) and MUC5B (surface secretory cells) in airway mucus	(Fahy and Dickey, 2010; McGuckin et al., 2015; Rubin, 2007)
Asthma	• Airway mucin stores increase due to goblet cell hypertrophy and hyperplasia • Surface epithelial mucous metaplasia and uncertain hyperplasia • Population of subepithelial bronchial microvessels increased but remodeling is not prominent • In severe disease conditions there is alteration in submucosal glands • Increased expression of MUC5AC and decreased expression of MUC5B gene	(Bonser et al., 2016; Bonser and Erle, 2017; Kuyper et al., 2003; Lachowicz-Scroggins et al., 2016)
Chronic obstructive pulmonary disease (COPD)	• Increase in mucin stores • Surface epithelial mucous metaplasia and some hyperplasia • Volume/number of submucosal glands increase • Concentration of MUC5AC increases rather than MUC5B	(Anderson et al., 2015; Lin et al., 2020; Radicioni et al., 2021)
Cystic fibrosis (CF)	• The levels of epithelial mucin stores are near normal • Submucosal glands are very prominent • More than 10% of solid content in mucus • Characterized by a predominant increase in MUC5B	(Button et al., 2016; Livraghi-Butrico et al., 2017; Martens et al., 2011)

Table 2

Formulation strategies for the design of muco-adhesive and muco-penetrating particles to overcome airway mucus barrier

Type of Formulation	Drug/Formulation details	Formulation characterization	Findings	References
Muco-adhesive nanoparticles	• Bedaquiline (BDQ)-loaded chitosan nanoparticles • Ionic gelation method • Sodium tripolyphosphate (TPP) used as a cross-linking agent	• Particle size: 109.7 ± 9.3 nm; zeta potential: 36 ± 2.1 mV • Sustained release and improved safety profile	• No ex-vivo particle diffusion data • Target disease: tuberculosis • Nanoparticle loaded dry powder inhaler enhanced delivery in the lungs	(Rawal et al., 2018)
	• Rifampicin (RIF)-loaded chitosan coated solid lipid nanoparticles (C-SLNs-RIF) • Hot ultrasonication method • Chitosan coating by gentle physical adsorption procedure	• Particle size of uncoated SLN-RIF: 245 ± 20 nm; C-SLN-RIF: 344 ± 11 nm • C-SLN-RIF zeta potential: 35 ± 2 mV • Encapsulation efficiency (EE)>90% and drug loading ~ 4.5%	• C-SLN-RIF: higher in vitro mucoadhesion and permeability in alveolar epithelial cells • No ex-vivo particle diffusion data • Target disease: tuberculosis	(Vieira et al., 2018)
	• Calcitonin-loaded glycol chitosan nanoparticles (GCS-NP) and GCS-thioglycolic acid conjugate nanoparticles (GCS-TGA NPs) • Ionic gelation method • TPP: cross-linking agent	• Both nanoparticles particle size: 230 − 330 nm, zeta potential: 22.3 − 27.4 mV • Entrapment efficiency of GCS and GCS-TGA NPs: 54.2% and 63.6%, respectively	• GCS-TGA NPs: 2-fold higher muco-adhesion to lung tissues compared to non-thiolated NPs • Calcitonin-loaded GCS and GCS–TGA NPs: pronounced hypoglycemic effect at least 12 and 24 h	(Makhlof et al., 2010)
	• Dexamethasone (Dexa)-loaded Flt1 peptide-hyaluronic acid (HA) conjugate nanoparticle • Self-assembly of nanoparticles by conjugation of Flt1 peptide to HA	• Dexa/Flt1peptide-HA: average diameter 110.2 ± 17.4 nm; zeta potential −20 mV • Drug loading: 12% • Around 50% drug release in first 6 h and remaining drug over 18 h	• Ex vivo imaging: enhanced retention of Flt1peptide-HA nanoparticles in deep lungs (mucoadhesion) • Improved efficacy in eosinophilic and neutrophilic asthma murine model	(H. Kim et al., 2013)
Muco-penetrating nanoparticles	• Fluorescent, carboxylatemodified polystyrene spheres (PS-COOH) of diameters 100, 200, and 500 nm • Nanoparticles densely coated with low molecular weight PEG (PS-PEG) (covalent modification)	• PS-COOH diameter around 100, 200, and 500 nm demonstrated highly negative zeta potential of −50 ± 3, −54 ± 4, and −73 ± 3 mV, respectively • PS-PEG nanoparticles exhibited zeta potential near neutral <-5 mV • multiple-particle tracking (MPT) for nanoparticles diffusion studies	• Airway mucus from healthy individuals • 100 nm PS-COOH: hindered trajectories • 200 and 500 nm PS-COOH: immobilized • 100 and 200 nm PS-PEG: diffusive	(Benjamin S. Schuster et al., 2013)

Type of Formulation	Drug/Formulation details	Formulation characterization	Findings	References
			• 500 nm PS-PEG: immobilized	
	• Fluorescent particles (PS-COOH) of similar diameter were used • PS-COOH covalently modified with diamine PEG 3.4 kDa at a ratio of 3:1 (PEG:COOH)	• Diamine PEG-coated PS nanoparticles of sizes 100, 200, and 500 nm have zeta potential values of −2.5 ± 1.2, −1.9 ± 3.7, −4.0 ± 1.3 mV, respectively • Amine-modified nanoparticles (PS- • NH_2) exhibited −5.6 ± 0.1 mV Transport of PEG-coated nanoparticles in airway mucus measured by MPT	• Airway sputum from CF patients • PS-NH_2 nanoparticles: strongly hindered transport • PEG-coated nanoparticles (100 nm and 200 nm): improved transport (Brownian diffusion) • PS-PEG nanoparticles (500 nm): immobilized and hindered	(Suk et al., 2009)
	• PS-nanoparticles densely coated with 5 kDa PEG (PS-PEG) forming mucus penetrating particles (MPPs) • Muco-adhesive particles (MAPs) or MPPs using poly(lactic-*co*glycolic acid) (PLGA), namely PLGA MAPs and PLGA-PEG MPPs, respectively • Pluronics F127 and F68 used as noncovalent surface coating polymers to form PLGA/F127 MPPs and PLGA/F68 MAPs	• Dense PEG coating: marginally increased particle size, zeta potential: significantly increased from −34 to −83 mV for uncoated to <-5 mV • Both nanoparticles exhibited particle sizes: 130 to 140 nm, • PLGA MAPs and PLGA-PEG MPPs exhibited zeta potential of −73 and −6 mV, respectively • PLGA/F127 mucus penetration particles (MPPs) and PLGA/F68 MAPs: comparable particle size of 180 to 200 nm; zeta potential of −23 and −64 mV, respectively	• Airway sputum from CF patients • PLGA-PEG MPPs and PLGA/F127 MPP efficiently demonstrated higher diffusion in CF sputum • PLGA MAP and PLGA/F68 MAPs both exhibited restricted particle trajectories • PLGA-PEG MPPs and PLGA/F127 MPPs exhibited Brownian-diffusion • Even PS-PEG MPPs with a particle size of 300 nm demonstrated greater diffusion in CF sputum compared to 300 nm uncoated PS-MAPs	(Schneider et al., 2017)
	• DNA-loaded nanoparticles compacted with CK_{30} peptide conjugated to PEG of varying molecular weights forming $CK_{30}PEG_{2k}$-, $CK_{30}PEG_{5k}$-, and $CK_{30}PEG_{10k}$ DNA nanoparticles • Reduce muco-adhesion by coating CK_{30}PEG DNA nanoparticles with LMW PEG	• Percentage yield for $CK_{30}PEG_{2k}$- and $CK_{30}PEG_{5k}$ nanoparticles were >95% and $CK_{30}PEG_{10k}$ DNA nanoparticles was around 85% • $CK_{30}PEG_{2k}$-, $CK_{30}PEG_{5k}$-, and $CK_{30}PEG_{10k}$ DNA demonstrated average dimensions of ~ 220 × 15 nm, ~ 300 × 13 nm, and ~ 350 × 11 nm, respectively; zeta potential ñeutral	• CF sputum • Well-PEGylated PS-PEG_{2k} nanoparticles of diameter: ~ 210 nm exhibited markedly higher diffusion • $CK_{30}PEG_{5k}$ and $CK_{30}PEG_{10k}$ DNA nanoparticles: trapped in CF sputum • Insufficient PEG coating: particle's muco-adhesion, regardless of molecular weight of PEG	(Boylan et al., 2012)
	• Polymeric gene carrier that can penetrate through CF sputum • First, uncoated PEI/DNA nanoparticles designed by conjugating	• Average hydrodynamic diameter of PEI-CCP was 15% higher than uncoated PEI/DNA nanoparticles	• Particle tracking using airway CF sputum • PEI-UCP: strongly hindered in CF sputum due to positive surface charge	(Suk et al., 2014)

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	• PEG 5kDa to branched PEI 25 kDa (PEI-UCP) • Second, plasmid DNA loaded nanoparticles (PEI-UCP) were densely coated with PEG 5kDa forming PEI-CCP • Free PEI with different percentages of PEG_{5k}-PEI (0%, 50%, 75%, and 100%) to yield nanoparticles with reduced particle size and neutral charge	• Zeta potential: uncoated nanoparticles has highly positive compared to PEG coated nanoparticles: ~ 3.5 mV • TEM: dense spherical morphology for uncoated nanoparticles and larger and more diffused particles for coated nanoparticles • Nanoparticles with 25% PEI and 75% PEG_{5k}-PEI (PEI-MPP) were highly compacted with a diameter around 50 nm and near neutral zeta potential	• PEI-CCP: strongly stuck in the sputum (conventional coating does not provide sufficient muco-inert surface coating to enable diffusion in CF sputum) • Diffusion of PEI-MPP was bimodal with most fractions rapidly diffused through CF sputum and a small fraction of gene carriers were trapped	