

ABSTRACT

Title of Dissertation: NOVEL BIOENGINEERED BIOMATERIALS
AND TISSUE CULTURE MODELS TO
UNDERSTAND MUCUS CLEARANCE
DYSFUNCTION IN ASTHMA

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Airway mucus acts as a protective barrier and vehicle of clearance for inhaled pathogens, providing the lungs with a robust defense mechanism called mucociliary clearance. Airway mucus is composed of two gel-forming mucins, MUC5B and MUC5AC that form a hydrogel that maintains functional clearance in health. However, in asthma, mucus is produced with abnormal properties that result in impaired mucociliary clearance contributing to mucus accumulation and airway obstruction. Recent evidence from clinical studies revealed that mucus obtained from individuals with asthma possesses altered mucin composition as a function of disease severity with a significant shift from MUC5B to MUC5AC as the predominant mucin. However, how these changes alter the functional properties of mucus is not yet fully understood. The overall objective of this dissertation is to understand how an imbalance in the ratio of MUC5B and MUC5AC contributes to abnormal function of mucus in asthma. The central hypothesis is that relative increases in MUC5AC enhances viscoelasticity of the mucus gel, which contributes to the functional

defects of mucus. To study this, we engineered two novel approaches: (i) mucin-based biomaterials with tunable mucin composition and (ii) genetically engineered *in-vitro* systems with targeted knock-out (KO) of each gel-forming mucin. In our first approach, we systematically varied the mucin composition of mucin-based biomaterials and found that a rise in MUC5AC, as observed in asthma, results in increased viscoelasticity, reduced transportability, and impaired barrier function against influenza A virus. Using our second approach, we found that MUC5AC gels produced from MUC5B-KO cultures resulted in impaired mucus clearance, whereas MUC5B gels produced from MUC5AC-KO cultures lacked spatial coordination. Together, these studies suggest that elevated levels of MUC5AC contribute to enhanced viscoelastic properties, while being the key driver of impaired mucociliary clearance in asthma. This work also offers new insight into the contribution of each gel-forming mucin on the dynamic control of mucus transport and flow alignment. This work was motivated by providing a path towards developing new therapeutic targets aimed at normalizing mucus function and improving airway patency in asthma.

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MODELS TO UNDERSTAND MUCUS CLEARANCE DYSFUNCTION IN
ASTHMA

by

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1. INTRODUCTION

Asthma is a chronic inflammatory lung disease that affects an estimated 315 million people worldwide (1–3). Asthma is characterized by the narrowing of the airways, which results from a combination of inflammation-induced smooth muscle constriction and impaired mucus clearance (4, 5). These factors contribute to the primary symptoms of asthma, which include wheezing, shortness of breath, chest tightness, and cough caused by airflow limitation, while worsening of symptoms is usually triggered by factors, such as exercise, allergen exposure, and respiratory infection (3). Failure to effectively control and manage these symptoms can lead to exacerbations, significant obstruction of the conducting airways, and, in fatal cases, death resulting from asphyxiation and respiratory failure (6). With at least 250,000 deaths attributed to the disease, asthma is considered the most common chronic lung disease (7).

The clinical manifestation of asthma ranges from mild to severe, where severity of disease varies significantly from patient-to-patient due to the multiple pathological changes that occur from inflammation to the airways (4). One key pathological feature of asthma is the build-up of intraluminal mucus (8, 9). While the primary function of airway mucus is to protect the underlying epithelium from exposure to inhaled pathogens, in asthma, mucus is produced with significant alterations in composition and viscoelastic properties (10, 11). These changes culminate in mucus aggregation, narrowing of the airways, and increased susceptibility to infection and injury from external assaults (12, 13). While the pathophysiological role of mucus has been recognized by physicians for more than 100 years, the obstructive role of mucus is highly overlooked due to a lack of understanding of

mucus dysfunction, which diverts attention exclusively towards reverting inflammation (3, 7).

Current asthma therapies, such as corticosteroids and bronchodilators, are temporary and incomplete as they do not effectively address the pathological effects of mucus, which, if unaddressed, contributes to recurring symptoms related to airflow limitation (2, 6). In addition, treatments are typically based on empirical approaches that determine clinical severity rather than the underlying mechanism of pathogenesis, further limiting their effectiveness (4, 14). As a result, asthma is still considered to be incurable, where clinical guidelines recommend management of symptoms at best. Despite high-dose pharmacological therapies aiding in asthma symptoms control, an estimate of 20% of asthma patients suffer from uncontrolled, moderate-to-severe asthma, presenting persistent symptoms, with reduced lung functions and recurrent exacerbations (15).

1.1 Objective and Significance

Asthma is a globally burdensome disease that, when left uncontrolled, is potentially life-threatening (3). While the formation of pathologic mucus is a major contributor that can lead to morbidity (16), little is known about mucus dysfunction in asthma. In prior work, biochemical analysis of mucus samples from patients with asthma revealed abnormalities in mucus composition as a function of disease severity with significant alterations in the relative amounts of the two primary gel-forming mucins, mucin 5B (MUC5B) and mucin 5AC (MUC5AC) (11, 17). Further, mucus plugs in fatal asthma are characterized with MUC5AC enrichment (8). However, it is unclear how this contributes to abnormal mucus function in asthma.

The objective of this dissertation is to understand how biomolecular composition of mucus, specifically MUC5B and MUC5AC content, impacts mucus functional properties, such as effectiveness as a barrier, viscoelastic properties, and transportability to provide insight into how alterations in mucus composition contributes to disease progression in asthma. This work is motivated by providing a path towards developing new therapeutic targets aimed at normalizing mucus function and improving airway patency in asthma.

In the United States, the prevalence of asthma is increasing every year. Although asthma affects people of *all* races, ethnicities, and ages, the burden from the disease falls disproportionately on racial and ethnic minority populations (18, 19). Disparities in asthma are particularly evident in hospitalization and mortality rates, where emergency room visits are two to three times higher among people of color and mortality rates are three times higher (19, 20). These disparities limit the overall gains in quality of care and health for the broader population, while increasing the economic burden of asthma. Guideline and actions for addressing asthma disparities have been identified and efforts to reduce asthma disparities have been on-going, such as increasing access to healthcare to everyone. However, increasing coverage and access alone is unlikely to suffice. Additional steps must be taken to improve our understanding of the disease process and develop more effective therapeutic goals (21). These research efforts described in this dissertation to understand asthma pathogenesis and to develop therapeutic goals are imperative for ultimately eliminating existing health disparities associated with asthma.

1.2 Approach and Innovation

Despite the observation and recognition of the pathological features of mucus in asthma, the mechanism by which a typically protective gel leads to occlusion in disease is poorly understood due to the challenges and limitations associated with recapitulating the asthma-specific changes in mucus. While various airway epithelial models have been developed to mimic concentration-dependent changes to mucus observed in diseases, such as cystic fibrosis (22), the compositional changes unique to asthma have been particularly challenging to model.

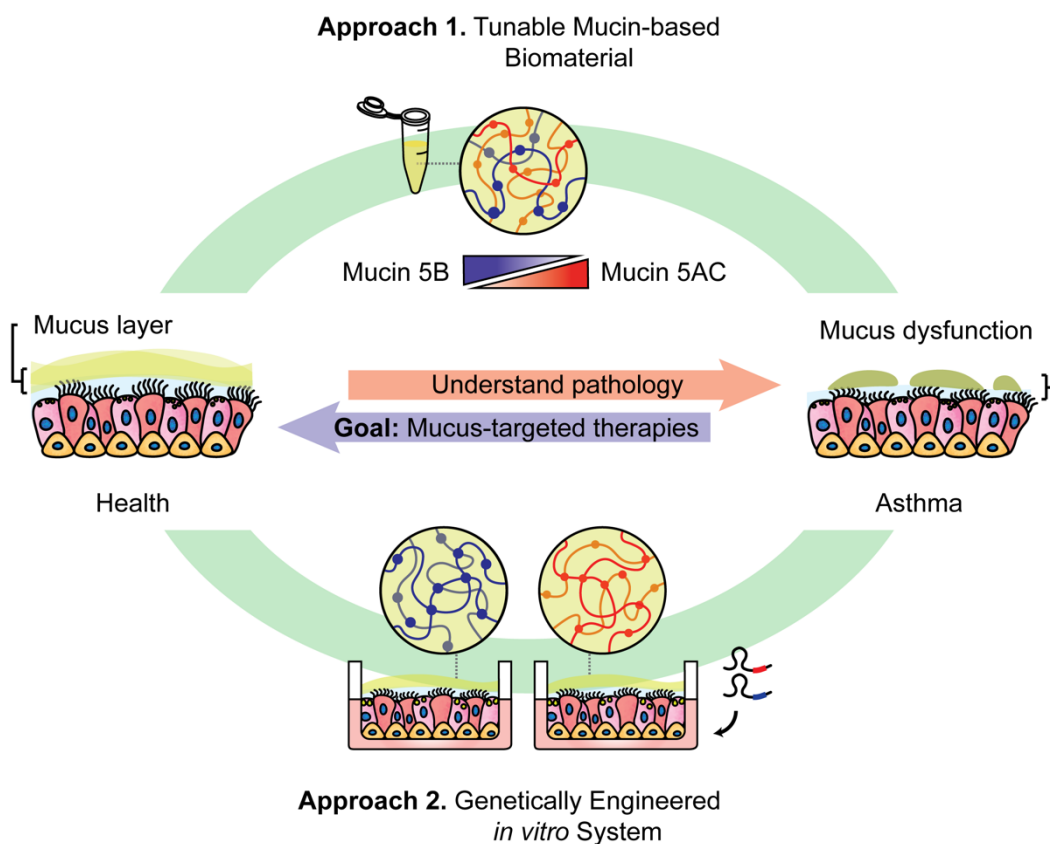


Figure 1.1 Dissertation overview. In this dissertation, we used two approaches: i) highly tunable mucin-based biomaterial and ii) genetically engineered *in vitro* human airway epithelial culture system to investigate the role of mucus composition on impaired mucus clearance. Our goal is to better understand composition dependent mucus dysfunction in asthma to provide a basis for more effective mucus-targeted therapies for individuals suffering with asthma.

The work in this dissertation introduces two novel approaches for better defining the contribution of MUC5B and MUC5AC to mucus dysfunction in asthma (**Fig. 1.1**). In this work, we present:

- (i) Mucin-based biomaterials with controllable mucin composition
- (ii) Genetically engineered *in-vitro* systems with controlled mucin expression

Our first approach utilizes readily available crude mucins to form a tunable biomaterial with varied biomolecular composition for modeling asthma in different stages, while our second approach takes advantage of CRISPR/Cas9-mediated gene-editing technology in human airway epithelial cultures to manipulate mucin secretion.

1.3 Summary and Dissertation Outline

This dissertation is organized as follows: **Chapter 2** provides an overview of the primary defense mechanism of the airways, known as mucociliary clearance. This chapter focuses on the mucin biopolymers that contribute to barrier function at the airway surfaces, while highlighting our current understanding of mucus dysfunction in asthma. **Chapter 3** presents a detailed description of the experimental methods used in this dissertation. **Chapter 4** and **5** contain the results of the studies using the novel mucin-based biomaterials and have been published individually. **Chapter 4** describes a novel approach to forming disulfide cross-linked mucin hydrogels with properties similar to native mucus. **Chapter 5** explores the use of these mucin-based biomaterials as a model for studying mucus dysfunction in asthma. In **Chapter 6**, we report *in vitro* models genetically modified for manipulation of mucin secretion. Using these models, we demonstrate that the right mix of airway mucin proteins is critical for rapid and efficient clearance of pathogens and

particulates from the lungs. **Chapter 7** summarizes the conclusions from this dissertation, while highlighting the scientific contributions of this work and describing the on-going work addressing improved drug delivery methods to the lungs.

2. MUCIN BIOPOLYMERS AND THEIR BARRIER FUNCTION AT AIRWAY SURFACES

2.1 Airway Surface Liquid and Its Role in Airway Defense*

Given the constant exposure of the lungs to potentially harmful substances through inhaled air, the airway surfaces are lined with a protective layer of fluid that is dynamically exchanged to facilitate the removal of foreign pathogens and particulates (23). This fluid lining, commonly termed airway surface liquid, consists of two distinct layers: the secreted mucus layer and the periciliary layer (PCL) (**Fig. 2.1**). The mucus layer is an extracellular hydrogel that protects the airway epithelium by acting as a barrier, while also serving as a vehicle of mechanical transport. The underlying PCL is inhabited by cilia that rapidly beat in coordination exerting shear forces that transport the mucus layer. This process, known as mucociliary clearance (MCC), provides the lungs with an important defense mechanism against infection and injury with the continual removal of pathogens captured within the mucus layer. While effective MCC is essential for innate defense, in the airways

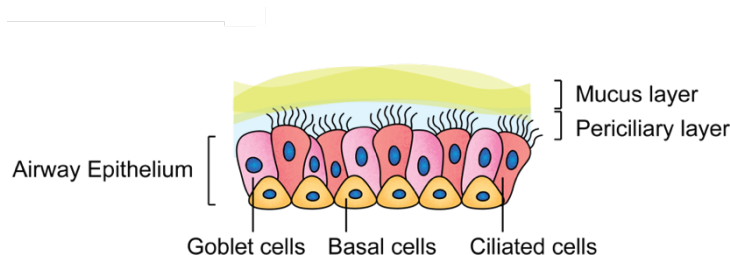


Figure 2.1 Mucociliary clearance system is composed of airway mucus that lies atop PCL.
Ciliary beating propels mucus gel facilitating the removal of pathogens.

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of individuals with pulmonary diseases such as asthma, cystic fibrosis (CF), and chronic obstructive pulmonary disease (COPD), MCC mechanisms are disrupted due to abnormal properties of the airway surface liquid, which include absence or immotility of cilia and biochemical and biophysical alterations to mucus, such as hyperconcentration, increased crosslinking, and altered secretion (24–27). These changes can lead to a build-up of mucus in the airways resulting in obstruction, decreased lung function, and asphyxiation (7).

2.2 Mucin Biopolymers

Mucin biopolymers are the major macromolecular component of mucus gels and the PCL (28, 29). Mucins are large, megadalton (MDa) sized proteins with bottlebrush geometry and extensive glycosylation (28, 29). A diverse array of O-glycans decorate the mucin backbone, which is composed of a variable number of tandem repeats (VNTR). This results in glycan chains that are clustered in specific central regions conferring structural and biological properties, such as hydration and pathogen sequestration (30, 31). There are two sub-families of mucins: secreted mucins and membrane-tethered mucins (32). Secreted mucins are entirely extracellular where polymeric secreted mucins are able to connect via end-to-end bonds forming a continuous gel (32). Tethered mucins are bound to the epithelial surface forming a glycocalyx lining (28, 30, 33). In the airways, the mucus layer is composed of cross-linked secreted mucins that form a selectively permeable gel that protects the airways, while the tethered mucins that inhabit the PCL provide an additional layer of protection and impart lubrication at epithelial surfaces. Given their importance to respiratory function, studies on the physical and chemical properties of mucin biopolymers are essential to our understanding of their barrier function.

2.3 Secreted Mucins and Mucus gel

Human airway mucus is a hydrogel composed of approximately ~98% (w/v) water and ~2% solids (w/v) (34–36). The solid material in airway mucus includes secreted mucins, salts, globular proteins, lipids, DNA, and other cellular debris. Secreted mucins constitute up to 80% of the mass of solid materials of mucus and are the major contributors to the functional and structural characteristics of the mucus gel (37, 38). Assembly of secreted mucins into mucus gels requires the formation of inter- and intramolecular disulfide bonds. To accomplish this, mucin glycoproteins contain cysteine-rich von Willebrand factor (vWF)-like domains at their C- and N-termini (**Fig. 2.2A**), which allow for dimerization or multimerization through disulfide linkages, forming polymers ranging in length (0.5–10 μm) and size (2–50 MDa) (38, 39). Mucins arrange in a network-like formation with a high number of physical entanglements, which are stabilized by covalent and noncovalent interactions (**Fig. 2.2B**) (40). The extensive glycosylation on the mucins creates a bottlebrush-like structure increasing the stiffness and radius of gyration of mucin

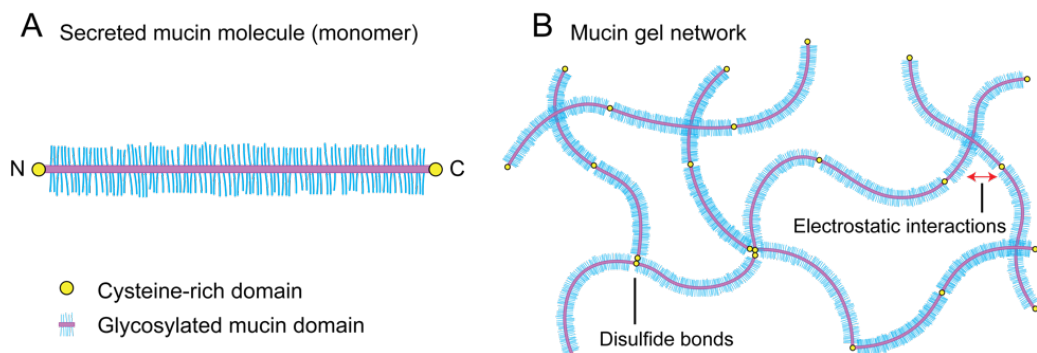


Figure 2.2 Schematic of secreted mucins. (A) Mucin monomers have cysteine-rich regions at the N and C amino terminus and extensive glycosylation that resemble bottlebrushes (B) Schematic of mucin network. Cysteine-rich domains on mucin molecules associate via disulfide linkages forming mucin polymers. The mucin network is stabilized by electrostatic interactions.

chains (41). Terminal sulfation and high sialic acid concentration leads to a strongly negative charge at neutral pH that stabilizes the gel network via repulsive forces (4).

The major gel-forming mucins in the airways are mucin 5B (MUC5B) and mucin 5AC (MUC5AC) (31, 38, 42). Both mucins are products of goblet cells on the surface epithelium, while MUC5B is also produced in submucosal glands (43). Although similar in domain organization and amino acid sequence, MUC5B and MUC5AC mainly differ in charge and shape, which result from differences in their glycosylation pattern (44). Recent evidence has shown that cysteine-rich domains are more prevalent in MUC5AC than MUC5B, which impacts their macromolecular assembly (31). In addition, MUC5B and MUC5AC differ in N-terminal organization, where MUC5B mainly forms dimers, and MUC5AC assembles into dimers as well as higher-order oligomers (45). As the major biopolymers of airway mucus, the physical properties of airway mucus, such as viscoelasticity and network pore size, highly depend on MUC5B and MUC5AC concentrations. Consequently, small changes in mucus concentration or cross-linking density can alter the biophysical properties of mucus.

2.3.1 Viscoelastic properties of airway mucus.

Airway mucus is a complex biological material which behaves like a viscous liquid at high frequency shear deformations and an elastic solid at low frequencies. This enables mucus gels to flow in response to shear forces exerted by the cilia on the airway surface (**Fig. 2.3**). Airway mucus generally shows an elastic dominant behavior ($G' > G''$), which is typical for cross-linked hydrogels (46). The viscoelastic properties of mucus are highly dependent on mucus solid concentration and cross-linking density. Therefore, mucin production is highly regulated resulting in a mucus gel with low elastic and viscous

modulus allowing for efficient transport at airway surfaces at a rate of $\sim 50 \mu\text{m/s}$ to clear inhaled particulates and pathogens (47). However in disease, airway mucus gels are produced with abnormal elasticity, which is attributed to increases in mucus solid concentration and increased cross-linking (36). Studies that explored viscoelastic properties of human airway mucus have relied on various sources and collection methods, including endotracheal tubes used during bronchoscopy, sputum samples that were spontaneously expectorated, and secretions collected from cell cultures (48). Although minor differences are observed between samples acquired using different methods due to sample dilution, the abnormal elasticity in diseased state mucus is observed across all mucus types regardless of source and collection method.

The contribution of mucus solid concentration on viscoelastic properties was investigated by Hill *et al* using particle tracking microrheology (PTM) (49). Hill *et al.* measured viscoelastic properties of mucus samples derived from human airway epithelial (HAE) cultures. Based on the diffusion of $1 \mu\text{m}$ probe particles, frequency dependent viscoelastic moduli were approximated for mucus samples that were reconstituted at systematically varied solid concentrations in a physiological range from 1.5-5%

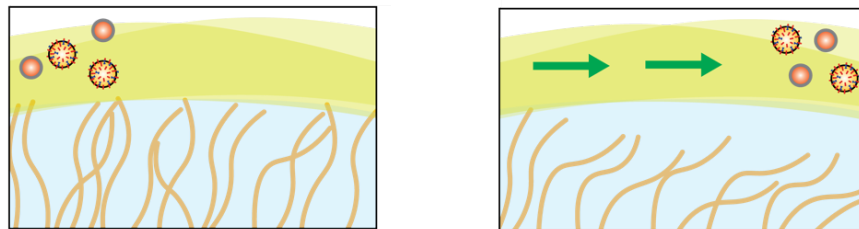


Figure 2.3 Schematic representation of mucociliary clearance. Top layer represents the mucus layer, and the bottom layer represents the PCL. Coordinated ciliary beating results in unidirectional transport of mucus, removing inhaled pathogens and particulates from the airway.

weight/volume. Results indicated that the elastic and viscous modulus increased as a function of increased solids concentration, suggesting that mucus with higher solid concentration has higher resistance to shear deformation. In addition, the study revealed that the critical mucus *wt%* for sol-gel transition, or elastic dominant behavior, occurred at ~ 4 *wt%*. Concentration-dependent rheological properties were further studied in airway mucus collected from the tips of endotracheal tubes (ETT) (35). Markovetz *et al.* performed a comprehensive biophysical and biochemical study using pooled ETT mucus. Using PTM, Markovetz *et al.* measured viscoelastic moduli of mucus tuned to mimic various mucus concentrations similar to those studied by Hill *et al.* and showed concentration-dependent viscoelastic properties, where elastic and viscous modulus increased with increasing solids concentration. Results obtained from ETT mucus were compared to mucus derived from HAE cultures and similar scaling behaviors were observed for the elastic and viscous moduli. Biochemical analysis showed that ETT mucus had significantly higher non-mucin biomacromolecules, such as globular proteins, antibodies, and lipids, compared to HAE mucus. Despite difference in non-mucin protein content no significant differences in viscoelastic properties were observed. These studies indicate that the viscoelastic properties of mucus are largely dependent on mucin concentration, where increased solid concentration results in increased viscoelasticity.

In addition to mucus solid concentration, the effects of cross-linking density on viscoelastic properties of mucus have been investigated. A cone-and-plate rheometer is commonly used for measuring viscoelastic properties of biological fluids. The rheometer is particularly convenient for analysis of non-Newtonian fluids like mucus, since the frequency and shear rate can be varied over a wide range of physiological values. Using a

cone-and-plate rheometer, Innes *et al.* measured the elastic and viscous modulus of induced sputum from healthy subjects (10). Elastic and viscous moduli were measured at frequencies expected to arise from cilia beating (1 Hz) and cough clearance (>10 Hz). Results showed the elastic modulus (~4 Pa at 1 Hz) predominated over the viscous modulus (~1 Pa at 1 Hz), which is characteristic of a lightly cross-linked gel. Healthy airway mucus had low entanglement density (5×10^{20} to 10×10^{22} crosslinks/m³), which was measured by the number of cross-link junctions among polymers in a unit volume. Cone-and-plate rheometer was also used for analyzing induced sputum collected from patients with acute asthma (10). Results indicated that the diseased mucus samples had abnormal rheological properties, namely significantly higher elastic and viscous moduli. The increases in elastic and viscous moduli were primarily attributed to increased entanglement density of mucin polymers. Studies have also demonstrated that the concentration of disulfide cross-linking can significantly alter rheological properties in airway mucus. Yuan *et al.* measured rheological properties of mucus from healthy individuals and individuals with cystic fibrosis (CF) lung disease using a cone-and-plate rheometer and the concentration of disulfide cross-linking within the mucus samples tested using a fluorometric assay (26). CF airway mucus was characterized with higher elasticity and abnormally high disulfide bond concentration compared to healthy airway mucus. To further assess impact of disulfide bond concentration on mucus elasticity, mucus samples from healthy subjects were treated with dimethyl sulfoxide, a mild oxidizing reagent to artificially increase disulfide bond concentration. Results showed that the oxidative stimuli resulted in a marked increase in both disulfide bond concentration and elasticity of airway mucus,

demonstrating that mucus elasticity changes as a function of disulfide cross-linking concentration.

2.3.2 Microscopic Barrier Properties of Airway Mucus

At the microscopic scale, mucus has a heterogeneous and porous architecture constructed by the cross-linked and entangled mucin polymer network (49). This microstructure controls the transport of nano- and microscale entities (e.g. viruses, bacteria, drug delivery vehicles) through physical (steric) and adhesive interactions, such as electrostatic or hydrophobic interactions, that can reduce penetration through mucus (49). PTM has been extensively used to gain insights into the microscale biophysical properties of mucus gels noninvasively by monitoring the diffusion nanoparticle probes. PTM also allows characterization of local microstructural properties of mucus that is often overlooked by bulk rheology (50). Schuster *et al.* demonstrated that polyethylene glycol (PEG)-coated polystyrene (PS) nanoparticles with diameters smaller than the mucus mesh spacings readily diffused through the mucus network, whereas similarly sized uncoated carboxylate modified nanoparticles had reduced transport or were immobilized due to adhesive interactions between particles and the mucus network. These nanoparticles, referred to as muco-inert nanoparticles (MIP), have surfaces that are grafted with neutrally-charged PEG, allowing their transport to be hindered solely through steric obstruction from the mucus mesh (49, 51, 52). Studies have also demonstrated that mucus exhibits size-dependent barrier function by varying the size of the MIP (100, 200, 500 nm in diameter). The rapid diffusion of 100 nm MIP in mucus is attributed to these particles resisting muco-adhesion and experiencing lower viscosity fluid that fills the porous microstructure of mucus (51). In contrast, diffusion of larger sized MIP, 500 nm in diameter, were

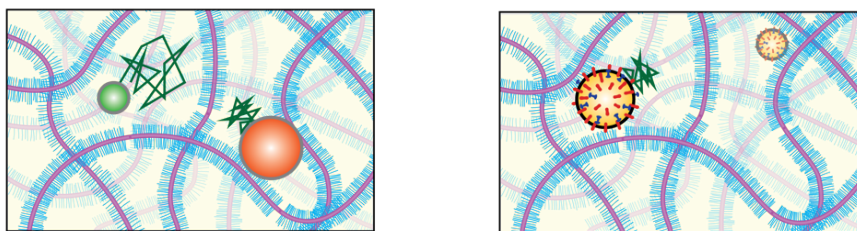


Figure 2.4 Schematic representation of steric and adhesive interactions between nanoparticles and respiratory viruses and mucin networks. Mucin network can sterically obstruct diffusion of nanoparticles based on size, while also immobilizing respiratory viruses through adhesion.

significantly reduced, demonstrating that particles greater than 500 nm in size will be immobilized due to steric obstruction arising from the mucus network (**Fig. 2.4**). As opposed to muco-inert particles, viral particles exhibit adhesive interactions with the mucus network. While the measured pore size of airway mucus ($\sim 200 - 500$ nm) would suggest rapid diffusion of viral particles, adhesive interactions between the glycosylated mucin domains and virus surfaces have shown to significantly reduce viral diffusion (**Fig 2.4**).

Studies have demonstrated that microstructural properties of airway mucus can be altered due to changes in biochemical components, such as increased percent solids and increased disulfide cross-linking (50). Using PTM, Duncan *et al.* measured transport of 100 nm MIP in mucus samples from normal and CF subjects. MIP transport was markedly reduced in mucus samples obtained from CF patients compared to non-CF samples, indicating a tightening of mesh network in CF mucus. In order to determine the effects of biochemical components on microstructure, concentration of mucin and disulfide bridges were measured and showed strong correlation to the decreased MIP transport rates, demonstrating their contribution to the reduction in mucus network. Additionally, Duncan *et al.* developed an assay for quantifying mucus mesh pore size through MIP diffusion.

Based on MIP diffusion rates, pore size of CF mucus was estimated to range from 110-930 nm, which was in accordance to previously reported values.

The contribution of non-mucin biomolecules on mucus microstructure has also been studied using PTM. Although Markovetz *et al.* demonstrated that circulating serum proteins, such as albumin, found in airway mucus do not significantly impact bulk viscoelastic properties (described in the previous section), heterogeneity in PTM measurements indicate that non-mucin molecules may contribute to mucin organization and microstructure of mucus (35). For example, studies have shown that increases in chromosomal DNA found in mucus can significantly alter barrier properties of mucus by elevating mucus adhesive interactions through increased negative charge density (26, 48). This has been demonstrated in cases of CF, where increased DNA, originating from lysed immune cells, in mucus samples correlated with reduced diffusion of MIP and reduced mucus pore size (50). Wagner *et al.* demonstrated that modifications in mucus surfactant and salt content can alter the microstructure of mucin gels by altering mucin-mucin interactions (53). In this study, the changes in microscopic mechanical properties of mucus hydrogels were measured with systematically varied surfactant (1,2-hexanediol) and salt (NaCl) concentration. The addition of a surfactant resulted in increased pore size or loosening of mucin network, as evidenced in decreased MIP diffusion, likely caused by disruptions in hydrophobic interactions between mucins. On the other hand, increasing the salt concentration resulted in the tightening of mucin mesh. The addition of salt results in screening of electrostatic interactions of the glycosylated regions allowing mucins to form tighter networks. These studies demonstrate that microscopic barrier properties of mucus can be altered through modifications in other ionic, chemical, and biomolecular factors.

2.4 Tethered Mucins and Periciliary Layer

In contrast to the mucus layer, which is composed entirely of extracellular secreted mucins, the periciliary layer (PCL) consists of tethered mucins that are bound to the surface of epithelial cells. Like secreted mucins, tethered mucins have a rigid brush-like structure that imparts mesh-network properties to the PCL (23). The glycosylation of the tethered mucins also provides a hydrophilic environment conferring hydrating and lubricating properties that facilitate efficient ciliary beating for clearing mucus (54). The three predominant tethered mucins in the PCL include MUC1, MUC4, and MUC16 (33). These tethered mucins have molecular masses that range from 200 kDa to 2.5 MDa and lengths ranging from 200 to 4,000 nm, which are large enough to span the interciliary space ($>1\ \mu\text{m}$) (33, 47). Studies have confirmed distinct localization sites of each tethered mucin, where MUC1 is localized to the microvilli, MUC4 to cilia, and MUC16 to goblet cells (**Fig. 2.5**) (55).

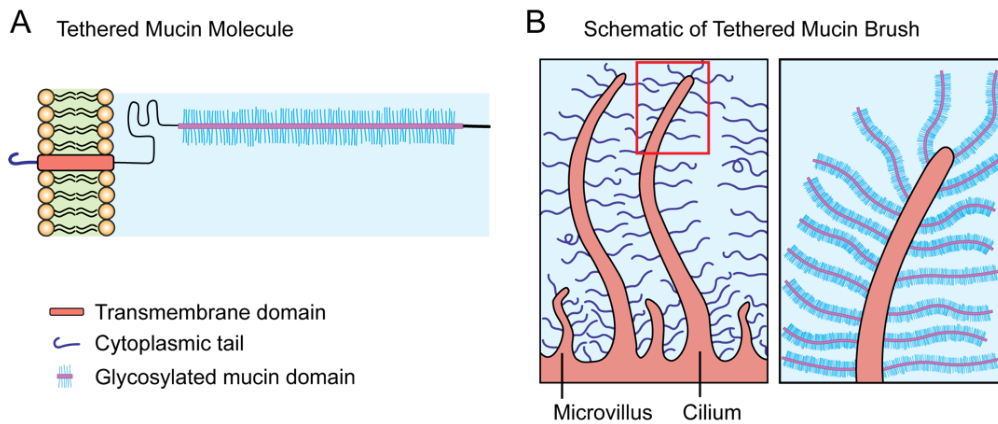


Figure 2.5 Schematic of tethered mucins. (A) The extracellular domain is heavily glycosylated. Tethered mucins also include a transmembrane domain and a cytoplasmic tail, (B) Illustration of tethered mucin brush on the airway epithelium. The PCL consists of densely packed tethered mucins. The glycosylated mucin domain allows the tethered mucins to assume an extended configuration forming a mesh network.

2.4.1 Brush Architecture of Tethered Mucins.

The PCL was classically viewed as a “watery” layer that would promote rapid ciliary beating. However, this view was challenged with the direct measurement of PCL viscosity performed by Derichs *et al* (54). Derichs and colleagues measured viscosity of the airway surface liquid by monitoring the diffusion of fluorescent dextran by fluorescence recovery after photobleaching (FRAP) in HAE cultures. Using computational analyses, relative viscosities of the secreted mucus layer and the PCL were computed using diffusion coefficients of solution standards with specified viscosity, such as water and different percentages of sucrose solutions. The resulting viscosity measurements were substantially higher than that of water or saline. This initial observation that the PCL was highly viscous was followed by inquiries into the molecular composition and organization of the PCL. Using electron and atomic force microscopy, Kesimer *et al.* identified the presence of three tethered mucins, MUC1, MUC4, and MUC16 (33). Kesimer *et al.* probed histological sections of HAE cultures with accumulated mucus secretions and discovered that secreted mucins, MUC5B and MUC5AC were undetectable in the PCL, despite having sufficiently small hydrodynamic radii to enter spaces between cilia. These results suggested that the dense population of tethered mucins excludes larger molecules, including secreted mucins (~250 nm radius of gyration), preventing interpenetration and mixing of the secreted and tethered mucin layers. Barrier functionality of tethered mucin brush was further explored by studying the penetration of viruses into the PCL. Microscopic imaging showed that adeno-associated viruses (~25 nm) penetrated into the PCL, whereas adenovirus particles (~100 nm) were excluded from the PCL, suggesting size-limited penetration into the PCL (**Fig. 2.6**).

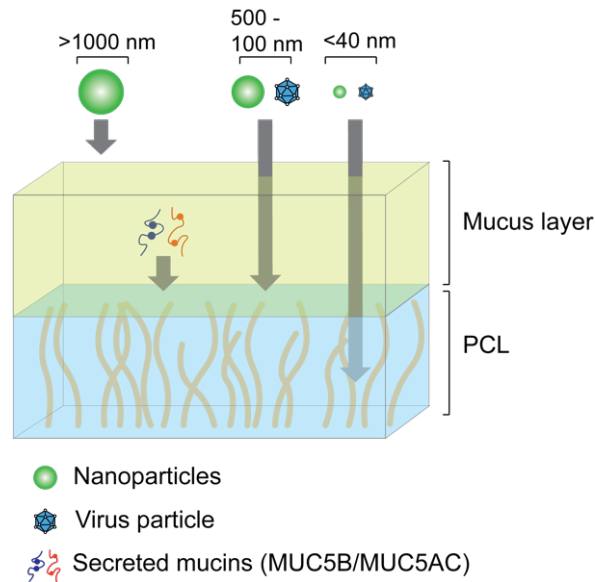


Figure 2.6 Schematic illustration of nanostructure transport through the mucus gel and periciliary tethered mucin brush. Particles with hydrodynamic radii larger than 1000 nm cannot penetrate through the mucus layer. Particles with size in the range of 100 - 500 nm can penetrate through the mucus layer but will be excluded from the denser PCL mesh. Particles less than 40 nm in size can penetrate the PCL.

Seminal work by Button, Rubinstein, and colleagues further investigated size-dependent penetration into the PCL and developed a new physical model describing tethered mucin function at airway surfaces (56). The penetration depth of fluorescent dextran polymers of varying size was determined using XZ-confocal microscopy. Results showed that dextrans with radii of gyration larger than 40 nm were excluded from the PCL, whereas smaller polymers diffused into the mesh network, demonstrating that the mesh size of the PCL is approximately 40 nm. Further, it was determined only probes smaller than 5 nm are able to reach the cell surface. These results demonstrate that smaller sized particles can penetrate to deeper depths within the PCL, indicating that the mucin brush becomes denser towards the cell surface. Based on their studies, the thickness of PCL was also measured to be approximately $\sim 7 \mu\text{m}$.

Through these studies, it was determined that the tethered mucin brush not only confers barrier properties but also imparts hydrating and lubricating functions to the PCL. The gel-on-gel model of the airway surface liquid predicts the distribution of water between the PCL and the mucus layer according to the osmotic pressure of each layer, which is generated by the concentration of the mucins (57). The electrostatic repulsion between high-molecular-weight mucins within each layer generates an osmotic pressure (56). This osmotic pressure within the PCL brush allows resistance to compression and prevents mixing of the airway surface liquid (58). Studies investigating the lubricity of polymer brushes have demonstrated that the resistance to compression generated by the osmotic pressure within a polymer brush substantially reduces friction between two surfaces (59). These observations suggest that the tethered mucins of the PCL substantially reduce the friction between the PCL and mucus layer interface, providing lubricating properties that facilitates effective mucus transport on the airway surface. Studies have also shown that friction is predicted to progressively decrease with increasing brush thickness (60, 61). These observations could explain the varying sizes of tethered mucins and their role in lubrication of the PCL. Increases in mucin concentration, specifically in secreted mucus layer, as observed in respiratory disease, creates an imbalance in osmotic pressure leading to interpenetration of the PCL and mucus layer.

2.5 Airway Surface Liquid Interactions in Health and Disease

The observation that the airway surface is a multi-layered system composed of a secreted mucus gel and a tethered mucin brush has major implications for understanding concentration-dependent abnormalities in airway surface liquid function that are associated

with respiratory diseases. A characteristic of respiratory diseases, such as CF and chronic bronchitis (CB), is the hyperconcentration of the secreted mucus layer. The mucus layer and the PCL are osmotically active species that compete for hydration (56). The airway epithelium maintains an airway surface osmotic pressure (π), where the osmotic pressure of the PCL ($\pi_{PCL} \approx 500 \text{ Pa}$) exceeds that of the mucus layer ($\pi_{Mucus} \approx 100 \text{ Pa}$), which results in a well-hydrated PCL (57). However, elevated concentrations of secreted mucins leads to increased osmotic pressure in the mucus layer, resulting in an uneven distribution of water (22, 62). As the osmotic pressure of the mucus layer increases and exceeds that of the PCL, the mucus layer begins to osmotically compress the PCL, resulting in the failure of cilia-dependent clearance. The relationship between mucin concentration in secreted mucus and osmotic pressure was determined by Henderson *et al.* by comparing mucin concentration and osmotic pressure of mucus obtained from normal individuals and individuals with CF. CF mucus had approximately 3-fold higher mucin concentration compared to normal mucus with a 5-fold increase in osmotic pressure ($>500 \text{ Pa}$), demonstrating that increased mucin concentration results in a significant increase in osmotic pressure (62). Osmotic compression of the PCL due to hydration imbalance was demonstrated by Button *et al.* by measuring PCL height after varying the osmotic pressure of the mucus layer (56). The PCL heights of HAE cultures were measured after application of hydrogels and mucus with known and varied osmotic pressures. PCL compression was not observed in application of gels with comparable osmotic pressure to normal mucus. Gels with osmotic pressure of $\sim 300 \text{ Pa}$ resulted in minor PCL compression, whereas osmotic pressure of $\sim 4000 \text{ Pa}$ resulted in the complete collapse of the PCL (**Fig. 2.7**).

The direct relationship between mucus osmotic pressure and cilia-driven transport was studied by Anderson *et al.* by measuring transport rates of mucus with varying osmotic pressure (22). Anderson *et al.* first measured osmotic pressure and solid concentration of mucus obtained from subjects with CB. Increased solid concentration was strongly correlated with increased osmotic pressure. Results obtained from CB mucus was compared to osmotic pressure of HAE-sourced mucus with systematically varied solid concentrations and similar relationship was observed, suggesting that the rise in osmotic pressure in CB mucus is concentration dependent and not affected by endogenous factors found in mucus that are related to CB. The transport rates of HAE mucus with varying solid concentration were measured by tracking fluorescent microspheres deposited on the mucus layer. Mucus with solid concentration similar to normal mucus had clearance rates ranging from 30-50 $\mu\text{m/s}$. However, percent solids greater than 10% resulted in complete

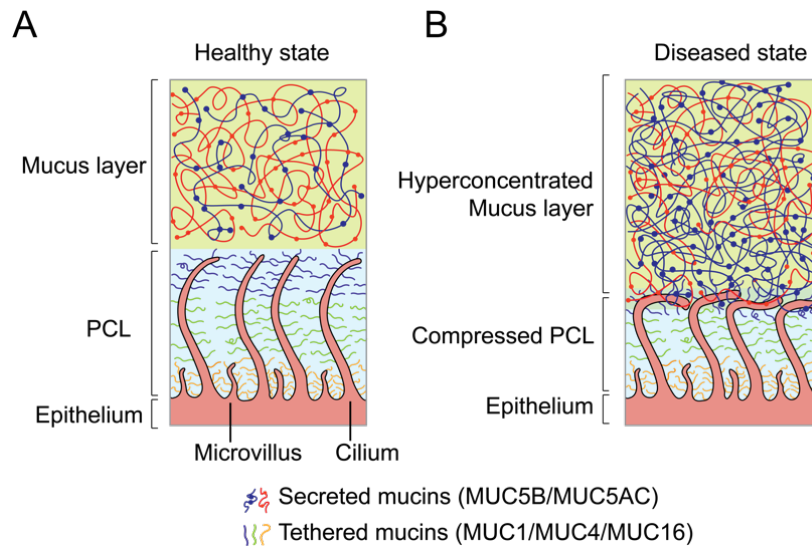


Figure 2.7 Schematic illustration depicting PCL in health and in disease. (A) In health, osmotic pressures within each layer prevents interpenetration of the mucus layer and PCL, providing lubricating properties. (B) In disease, increased osmotic pressure in the mucus layer results in osmotic compression of the PCL.

stoppage of mucus transport. The results obtained from these studies demonstrate that increases in osmotic pressure of the mucus layer generates compression of the PCL that impedes cilia-driven mucus transport and functional airway clearance.

2.6 Abnormalities in Airway Mucus in Asthma

Our current understanding of impaired mucus clearance focuses on the imbalance in osmotic pressure. However, in asthma, mucus solid concentration remains below well below the value reported for PCL compression and collapse and instead, mucus composition and organization has been hypothesized to play a role in MCC impairment. Recent analyses of mucus samples from patients with asthma revealed abnormalities in mucus biomolecular composition, where an increase in MUC5AC and decrease in MUC5B was observed, independent of concentration (11, 17, 63, 64). In addition, in mild and severe asthma, there mucins were observed to localize in distinct sites, forming heterogenous mucus gels with MUC5B- and MUC5AC-rich domains (17). Bonser *et al.* demonstrated that stimulation of HAE cultures with IL-13, a central mediator in allergic asthma, resulted in both changes in mucus composition and organization but also a drastic reduction in MCC rates (17). Results demonstrated that cilia function was preserved, showing that cilia beat frequency was unchanged and cilia height was maintained, confirming no compression in the PCL. Nonetheless, defective MCC is observed in all stages of asthma.

The mechanism by which pathological mucus occludes the airways in asthma is poorly understood due to challenges associated with modeling the specific compositional changes in airway mucus in health and disease.

2.7 Studying Airway Mucus in Asthma

2.7.1 Clinical Mucus Samples Collected *Ex Vivo*.

Studying airway mucus in health and disease has relied heavily on acquiring *ex vivo* human mucus using various collection methods, including sputum expectoration, sputum induction, and collection from endotracheal tubes used during bronchoscopy (50, 51). Expecterated and induced sputum samples have been used to understand mucus barrier properties through biochemical and biophysical analyses (10, 50). However, sputum samples can be diluted during induction from nebulized saline or salivary contamination. Tips of endotracheal tubes (ETT) from patients who have undergone intubation are also a source of *ex vivo* mucus with rheological properties close to physiological values (35). However, ETT-derived mucus samples can be dehydrated due to evaporation during intubation. As a result, clinical samples may possess altered mechanical properties depending on the collection method. In addition, the limited access, sample-to-sample heterogeneity (35), as well as the modest sample volume (~100 μ L) of clinical *ex vivo* samples present a major challenge and limits their use for mechanistic studies.

2.7.2 Mucin-based Biomaterials

Limited availability to native mucus from human origin has stimulated interest in artificial mucus models that can mimic both composition and structural properties of mucus, using commercially available crude mucins, such as porcine gastric mucin (PGM) and bovine submaxillary gland mucin (BSM). Crude mucins when put back into solution fail to form disulfide cross linkages and do not form a hydrogel (**Fig. 2.8**) (65, 66). Therefore, covalent cross-linking strategies have been employed for reestablishing native-like rheological properties. Recent studies have reported mucin-based biomaterials that

combine PGM or BSM with cross-linking agents, such as poly(acrylic) acid (PAA) and thiolated star PEG polymers, producing mucin-based biomaterials that mimic the biophysical properties of mucus found *in vivo* (67, 68). These mucin-biomaterials provide a limitless supply for studying the basic functions of mucus as well as a model for high-throughput permeation studies for more effective drug delivery systems for mucosal surfaces. However, like *ex vivo* mucus samples, their use is limited by the batch-to-batch differences and purity.



Figure 2.8 Inversion test to confirm gelation. 2% mucin solution (left) and 2% mucin hydrogel that used 2% thiolated PEG polymer (right).

2.7.3 *In Vivo* Mice Models

In vivo mouse models have been indispensable for understanding the functional roles of Muc5b and Muc5ac through knock-out (KO) and overexpression (69–71). Specifically, it has been shown in prior work that Muc5b-deficient mice have reduced MCC, increased susceptibility to infection, and reduced survival (69). Conversely, Muc5ac-deficient mice were protected from airway hyperreactivity upon exposure to allergens (70). However, prior work has also shown that overexpression of Muc5ac does not negatively impact MCC and is protective against respiratory infection by influenza A virus (IAV) (71). In addition, overexpression of Muc5b can lead to reduced MCC and

contribute to lung fibrosis (70). A major limitation of these *in vivo* studies are species specific differences that must be complemented with human systems. (72).

2.7.4 *In Vitro* Human Airway Epithelial (HAE) Cultures.

HAE cell cultures have proven to be a valuable model due to their ability to recapitulate the *in vivo*-like microenvironment of the airways at the cellular and extracellular level and have been used extensively as a robust *in vitro* model for studying the airway epithelium. HAE culture systems utilize transwells with porous membrane, where HAE basal stem cells are cultured. Once an air-liquid interface (ALI) is introduced, basal cells can differentiate to into a polarized epithelium with morphological features of the airway, such as mucus production and coordinated ciliary activity (**Fig. 2.9**). HAE cultures have been established as a source of mucus that can be tuned for assessing changes in mechanical properties of mucus that are concentration dependent (27, 35, 36). Therefore, these systems have been particularly useful for understanding MCC mechanisms.

Measuring mucociliary clearance (MCT) rates: HAE cultures develop regions of locally coordinated ciliary beating allowing for mucus to be transported. Transport can be measured by depositing fluorescent microspheres on top of the mucus layer and monitoring the movement of fluorescent microspheres. In addition to mucus solid concentration, cilia

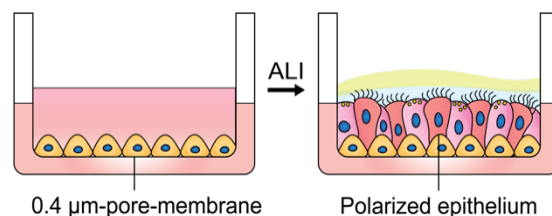


Figure 2.9 *In vitro* HAE culture systems at ALI. HAE culture systems utilize porous membranes for cell attachment. Once ALI is introduced, cells will differentiate into polarized epithelium with mucus production and ciliary beating.

beat frequency has been shown to impact mucus clearance rates, where a reduction in temperature demonstrated a decrease in cilia beat frequency (CBF) and subsequent decrease in MCT (73). MCC studies using HAE cultures have mostly focused on understanding mucus solid concentration and CBF and their effects on MCT rates, while the impact of mucus composition on MCT has been highly overlooked.

IL-13 stimulation of HAE cultures: Recent reports have demonstrated that stimulation of HAE cultures with IL-13, a type 2 cytokine involved in asthma, results in significant compositional changes in mucus, specifically an increase in MUC5AC and a decrease in MUC5B, as observed in asthma (17). These changes were independent of mucus solid concentration. In addition, gel-forming mucins, MUC5B and MUC5AC, formed distinct domains forming a heterogeneous mucus gel with MUC5AC domains that remained tethered to the epithelium, which resulted in a significant drop in MCC rates. These studies demonstrate that stimulation of HAE systems with IL-13 can produce compositional alterations in the mucus gel that closely resemble those observed in fatal asthma.

CRISPR/Cas9 gene editing in HAE cultures: CRISPR/Cas9 gene editing has transformed our ability to investigate gene function and has great potential for therapeutic application. CRISPR/Cas9 gene editing employs a programmable guide RNA (gRNA) as well as a Cas9 nuclease to form a ribonucleoprotein complex, which in turn binds and cleaves the target site in genomic DNA. While different delivery methods exist, viral delivery can allow for selection of transduced cells and can significantly improve transduction efficiencies (74). Gene targeting has enabled mechanistic studies to analyze the contribution of specific genes. CRISPR/Cas9 gene editing with viral delivery of gRNA

and Cas9 transgenes has been demonstrated in primary HAE cultures for knocking-out (KO) mucin, MUC18, revealing proinflammatory role (75). However, gene editing of primary HAE cells has been a challenge due to the limitations in passage numbers after isolation and loss of differentiation capacities. The use of immortalized cell line for gene editing has been limited primarily due to their inability to model differentiation into a polarized monolayer. Recent studies have demonstrated an h-TERT immortalized human airway basal BCI-NS1.1 cell line, an HAE cell line that has been immortalized via retrovirus-mediated human telomerase expression, which also retains its ability to differentiate, enabling gene editing to HAE culture systems (21, 76).

2.8 Therapeutics

The most widely used therapies for asthma target suppression of airway inflammation (anti-inflammatory drugs) or relaxation of airway smooth muscle (bronchodilators). Currently, inhaled anti-inflammatory corticosteroids are used as the first-line therapy for patients with asthma and are used to provide relief to inflamed airways (3). However, inhaled corticosteroids are absorbed from the lungs into the systemic circulation and have adverse effects on children (77). Recent discoveries provide strong arguments for targeting disulfide bonds as a rational mucolytic strategy for mucus-associated diseases in the lungs. Inhaled mucolytic agents are designed to decrease the viscoelasticity of airway secretion by disrupting the mucin intermolecular covalent disulfide bonds to improve mucus clearance. Currently, *N*-acetyl cysteine (NAC) is used clinically as mucolytic therapies. However, the clinical application is limited due to relatively low patency and requirement for high-dose delivery (78). While more effective

reducing agents have been identified, a major limitation for efficient mucolytic treatment in asthma is targeting MUC5AC rich domains that are found in mucus plugs.

3. METHODS

The primary methods used for the work in this dissertation involved characterization of material properties, culture of human airway epithelial cells, assessment of mucociliary clearance function, and gene editing strategies.

3.1 Assessing Biophysical Properties of Complex Biological Fluids

3.1.1 Bulk Rheology

Bulk rheology can be used to assess viscoelastic properties of a complex fluid. Bulk rheological measurements were performed using a rheometer with 20 mm parallel plate geometry or 20 mm cone and plate geometry at 25 °C depending on working volume of sample. Linear viscoelastic regions were determined through a strain sweep from 0.1-10% strain at a frequency of 1 rad s⁻¹. Elastic modulus, G', and viscous modulus, G'', were measured by a frequency sweep within the linear viscoelastic region of the gel at 1% strain amplitude and angular frequencies from 0.1 to 100 rad s⁻¹. The phase angle, δ , was calculated as $\tan^{-1}(G''/G')$.

Bulk Rheology of Mucin-based Hydrogels: For bulk rheological measurements of mucin-based hydrogels, a 20 mm parallel plate geometry was used. Mucin hydrogels were cast in 20 mm-diameter cylindrical hollow molds using 1-2 mL of mucin and cross-linker solution, which resulted in gels with heights of ~1-2 mm. The hollow cylindrical molds were covered with parafilm to which the mucin and cross-linker solution was cast. After gelation the parafilm was gently removed and the gel was transferred to the rheometer for

strain sweep and frequency sweep experiments. The specific parameters used are available in the Materials and Methods sections of **Chapter 4 and 5**.

Bulk Rheology of Mucus from Tissue Cultures: For bulk rheological measurements of mucus gels produced from *in vitro* tissue culture systems, a 20 mm cone-and-plate geometry was used due to the small volume of mucus collected from tissue culture systems (~0.5 mL). Once enough mucus was collected (~2-month biweekly collection), mucus samples were thawed and incubated at 4°C overnight. Samples were pipetted onto the base plate of the rheometer using a high-viscosity pipette. The specific parameters used are available in the Materials and Methods sections of **Chapter 6**.

3.1.2 Particle Tracking Microrheology

Particle tracking microrheology (PTM) has become a vital tool in characterizing local mechanical properties of biological fluids that is often overlooked by bulk rheology (**Fig 3.1**). For PTM, we used carboxylate-modified, fluorescent polystyrene nanoparticles (PS-COOH; Life Technologies) with various diameters and coated them with a high surface density of polyethylene glycol (PEG) via a carboxyl-amine linkage using 5-kDa

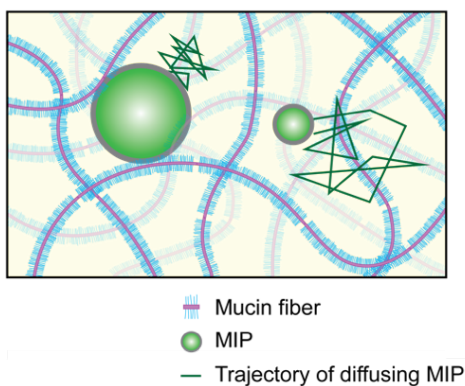


Figure 3.1 Schematice of particle tracking microrheology using muco-inert nanoparticles (MIP). MIP with a diameter smaller than the mucus pore size can diffuse rapidly through the mucus network, probing local microstructural properties. MIPs with diameter larger than the mucus pore size are immobilized through steric obstruction as reflected by the shorter trajectory length.

methoxy PEG-amine (Creative PEGWorks) as previously reported (51, 68). PEG-coated nanoparticle (PEG-NP) size and zeta potential were measured in 10 mM NaCl at pH 7 using a NanoBrook Omni (Brookhaven Instruments). The specific parameters used are available in the Materials and Methods sections of **Chapter 4-6**.

Sample Preparation for Mucin-based Hydrogels and Mucus from Tissue Culture:

Samples were prepared in custom microscopy chambers. Briefly, microscopy chambers were prepared by placing a 5 mm diameter O-ring (McMaster-Carr) on a microscope coverslip and sealing the edges using vacuum grease. 25 μ L of sample was added along with 0.5-1 μ L of $\sim$ 0.002% w/v suspension of PEG-NP ($\sim$ 1:2500 dilution) and gently mixed with a pipette tip. For preparation of mucin-based hydrogels, 25 μ L of mucin hydrogel precursor solution was added and pre-gelation microrheology was assessed after 30 minutes of incubation. In **Chapter 4 and 5**, post-gelation microrheology was consistently measured 15 hours after sample preparation. For preparation of mucus from tissue culture (**Chapter 5 and 6**), 20-25 μ L of mucus was added to the microscopy chamber and incubated for 30 minutes before particle tracking.

Particle Tracking and Analysis: The diffusion of the PEG-coated nanoparticles (PEG-NP) in samples was measured using fluorescence video microscopy. Videos were collected using a Zeiss 800 LSM microscope or Zeiss Axio Observer with a 63x water/oil-immersion objective and an AxioCam 702 camera (Zeiss) at a frame rate of 33 Hz for at least 10 seconds. For each sample, at least 3 high-speed videos were recorded in varying locations. To assess gelation kinetics of mucin-based hydrogels, videos were obtained every hour for 10 hours (**Chapter 4**). To automate video recording on the Zeiss 800 LSM, we used the Experiment Designer module that enables preselection of regions of interest

as well as settings for adjusting time points. The Experiment Designer module can be found as a check box under the ‘Start Experiment’ button in ZEN 2.3 System. After selecting Experiment Designer, we created four loops where three loops were designated each with a region of interest. The last was reserved for a time break that was set to one hour allowing the loop to start every hour.

The particle tracking analysis was performed using a previously developed image processing algorithm (68, 79). Mean squared displacement (MSD) as a function of time lag (τ) was calculated as

$$\langle \Delta r^2(\tau) \rangle = \langle (x^2 + y^2) \rangle$$

for each particle. Using the generalized Stokes-Einstein relation, measured MSD values were used to compute viscoelastic properties of the hydrogels. The Laplace transform of $\langle \Delta r^2(\tau) \rangle$, $\langle \Delta r^2(s) \rangle$, is related to viscoelastic spectrum $\tilde{G}(s)$ using the equation

$$\tilde{G}(s) = 2k_B T / [\pi a s \langle \Delta r^2(s) \rangle]$$

where $k_B T$ is the thermal energy, a is the particle radius, s is the complex Laplace frequency. The complex modulus can be calculated as

$$G^*(\omega) = G'(\omega) + G''(i\omega)$$

with $i\omega$ being substituted for s , where i is a complex number and ω is frequency. Hydrogel network pore size, ξ , is estimated based on G' using the equation,

$$\xi \approx (k_B T / G')^{1/3}.$$

Network pore size was also estimated based on MSD measurements, which describes degree of confinement using the equation,

$$\xi \approx \sqrt{\langle \Delta r^2(\tau) \rangle} + a,$$

where a is nanoparticle radius.

Gelation Kinetics: The sol-gel transition (gel point) was determined by calculating the logarithmic slope of the mean-squared displacement,

$$\alpha = \log_{10}(\langle \Delta r^2(\tau) \rangle) / \log_{10}(\tau)$$

We used a critical value of $n=0.5$ where the gel point is defined as the time when measured $\alpha < n$.

3.2 Human Airway Epithelial (HAE) Cell Culture

The cell line that was used for the studies in this dissertation was BCI-NS1.1, an HAE cell line that was immortalized via retrovirus-mediated human telomerase expression (provided by Dr. Ronald Crystal). BCI-NS1.1 cells originate from a non-smoking donor's human airway basal cells, which are the cells that differentiate to specialized cell populations (i.e. ciliated cells, goblet cells) of the airway epithelium during physiological turnover and repair (76). BCI-NS1.1 cells can be passaged >30 times and maintain the ability to differentiate into a pseudostratified epithelium with coordinated cilia beating and production of mucus.

Cell Culture and Passaging of BCI-NS1.1: BCI-NS1.1 cultures were seeded on plastic at ~3000 cell/cm² and maintained in PneumaCult-Ex Plus (StemCell) at 37°C and 5% CO₂. Media was changed every 2-3 days until 70-80% confluency was reached. Before passaging, cells were initially washed twice with PBS then detached using 0.05% trypsin ethylenediaminetetraacetic acid (EDTA) for 5 minutes at 37°C. Before centrifugation, cells were observed for round morphology and flask was gently taped to detach additional cells. Trypsin was neutralized by pipetting solution containing 10% fetal bovine serum (FBS) and 90% HBSS buffer into the flask. The detached cells were centrifuged at ~1000 RPM

(or ~500xg) for 5 minutes at 4°C. Once centrifuged, the trypsin solution was gently aspirated without disturbing the pellet leaving approximately ~500 µL solution. The pellet was then resuspended first through gentle flicking and then pipetting with PBS. The resuspended cells were centrifuged a second time at 1000 RPM (or ~500 xg) for 5 minutes at 4°C and cells were resuspended in pre-warmed PneumaCult-Ex Plus and plated in a new flask ~3000 cell/cm².

Introducing Air-Liquid Interface: For introduction of air-liquid interface (ALI), we followed StemCell's protocol. Before plating cells, Costar 12 mm Transwell, 0.4 µm Pore Polyseter Membrane Inserts (Costar or StemCell) were coated with 50 µg/mL type 1-collagen from rat-tail (Corning) at ~10,000 cells/cm². Expansion media, PneumaCult-Ex Plus, was used to feed cells in both the apical and basolateral compartments until 100% confluency. Once the transwell was covered with cells, the apical media was removed to transition from submerged to ALI culture and the basolateral media was replaced with PneumaCult-ALI. Cells were monitored twice daily to account for leakage of media into the apical compartment. If significant media leaked into the apical chamber, cells were resubmerged in PneumcaCult-Ex Plus.

3.2.1 Differentiation into Pseudostratified Epithelium

Full differentiation of BCI-NS1.1 cells takes ~28 days after introduction of ALI. Differentiation into a pseudostratified epithelium was verified using primarily by measuring transepithelial electrical resistance but was also confirmed using immunostaining for visualization of ciliated cells. Once cultures were fully differentiated, mucus was collected using by harvesting the washings and removing excess buffer.

Transepithelial electrical resistance (TEER): We used the Millicell Electrical Resistance System ERS-2 Volt-Ohm Meter (Millipore) and the manufacturer's protocol for measurement of TEER. For stability, a TEER probe was held by clamp on a conventional laboratory stand during measurements. All equipment was sterilized using 70% ethanol, including the TEER probe. Room temperature PBS was added to both the basolateral (1 mL) and apical side (0.5 mL). The electrodes on the TEER probe were immersed so that the shorter tip was in the cell culture insert and the longer tip is in the outer well. Measurements for read for ~10-15 seconds or until resistance values stabilized.

Mucus Collection: Mucus collection was performed every 2-3 days, as this kept the mucus layer well hydrated and did not lead to accumulation in the corners of the inserts. Apical compartments were washed for 30 minutes with 100 μ L of PBS. (Cultures should not be submerged in PBS for more than 1 hour.) After 30 minutes of incubation, PBS was collected using a normal Eppendorf pipette (high viscosity pipette was not used until after reconstituting mucus) and loaded into Amicon Ultra 100 kDa Filters (Millipore-Sigma) and centrifuged at 14,000 $\times g$ for 20 minutes to remove excess buffer. As each Amicon Ultra Filter can only hold up to 500 μ L, filters were reused for the same condition if more than 500 μ L was collected. Collection of reconstituted mucus was performed at 2000 $\times g$ for 2 minutes. Mucus samples were stored at -80 $^{\circ}$ C for long term storage (>2 weeks) or stored at 4 $^{\circ}$ C. Each collection (one plate of 12 mm Transwell) resulted in <50 μ L of mucus. Once enough mucus was collected (~500 μ L), samples were thawed on ice and pooled before performing downstream experiments, such as Western blot analysis, solid concentration analysis, biophysical characterization, and transport experiments. Once thawed, mucus samples were stored at 4 $^{\circ}$ C to prevent continuous freeze-thaws. Optimal frequency for

mucus collection was determined to be biweekly. While 6.5 mm Transwells were also used for mucus production, each collection resulted in significantly lower volumes compared to 12 mm Transwells.

3.2.2 Immunofluorescence and Western Blots

Immunofluorescence of Mucus Layer and Cells: BCI-NS1.1 cultures were fixed using two different fixatives depending on application. Carnoy solution (6:3:1 ratio of 100% ethanol, chloroform, and glacial acetic acid), a non-aqueous fixative that preserves the secreted mucus layer was used for staining of extracellular mucins, while 4% paraformaldehyde was used for cellular markers, such as acetylated alpha tubulin and ZO-1. When fixing with Carnoy solution, cultures were fixed for 30 minutes and then inserts were washed twice with absolute methanol, twice with absolute ethanol, and twice with PBS. When using 4% paraformaldehyde, cultures were fixed for 30 minutes and then immediately washed three times with PBS. For all immunostaining procedures, inserts were blocked with 5% bovine serum albumin (BSA) in PBS for 1 hours at room temperature. When staining for cellular markers, cell membranes were permeabilized using 0.01% triton X-100 in the blocking solution. After blocking, fixed cultures were incubated overnight at 4°C with primary antibodies in 5% BSA in PBS (**Table 3.2**). If secondary antibodies were needed, inserts were incubated with secondary antibody for 1 hour at room temperature. Inserts were washed with PBS 2-3 times and imaged.

Western Blot – SDS-PAGE Gel Electrophoresis: For immunoblotting mucins under *reduced* conditions, SDS-PAGE Western blot system was used adapted from previous studies (80, 81). Western blots were performed using reconstituted washing from BCI-NS1.1 cultures. Protein concentration of samples was quantified using BCA assay (Pierce,

Thermo Scientific) – assay worked best with a dilution of 1:100 in PBS. Samples were run on a 4-20% Tris-Glycine gels (Novex, Invitrogen). Tris-Glycine SDS Running Buffer, Tris-Glycine Transfer buffer, and Tris-Glycine Sample buffer (Novex, Invitrogen) were used for preparing samples and running the gel. Samples were prepared by first diluting samples in ddH₂O to achieve a 10 µg of protein and then adding Tris-Glycine Sample Buffer, and NuPage Sampling Reducing Agent (ThermoFisher) at concentrations outlined in **Table 3.1**. Once samples were prepared, they were incubated at 99°C for 2 minutes before loading into the gel. 6 µL PageRuler Prestained Protein Ladder (ThermoFisher) was added for visualization of protein size. Gels were run at 125 V for 85 minutes. Other types of gels have been explored (NuPage Tris-Acetate gels, agarose) for Western blot analysis. However, NuPage Tris-Acetate gels – although known to be more compatible with large molecular weight proteins – were not able to detect mucin from BCI-NS1.1 cultures, while mucin from human saliva was detected.

Table 3.1: Table of reagents for Western blot sample preparation.

	Mass/ well (µg)	Volume / well (µL)
Tris-Glycine Sample Buffer	-	20
NuPage Sampling Reducing Agent	-	4
Mucus sample Diluted in ddH ₂ O	10	X 16 (Total)
Total		40

Western Blot – Transfer for SDS-PAGE System: A wet transfer was employed for the SDS-PAGE systems. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare). Two Western Blotting Filter Paper (ThermoFisher) were

soaked in Tris-Glycine Transfer Buffer (prepared using manufacturer's protocol) and PVDF membrane cut to the size of the gel was activated in ethanol. Once the gel was retrieved from the cassette, the transfer sandwich was assembled (sponge, filter paper, PVDF membrane, gel, filter paper, sponge). Transfer was run at 75-100 V for 90 minutes. After disassembling transfer sandwich, proteins bands were visualized with ponceau stain.

While different dry and semi-dry transfer systems were attempted, wet transfers were used in the work done in this dissertation. The iBlot 2 (ThermoFisher), a dry transfer system, resulted in poor transfer of BCI-NS1.1 mucins and only successfully transferred MUC5B from human saliva onto a PVDF membrane. The eBlot L1 (GenScript) resulted in efficient and rapid transport of BCI-NS1.1 mucins and should be considered for detection of mucins in the future (81).

Western Blot – Agarose Gel Electrophoreses: For immunoblotting mucins under *non-reduced* conditions, we used an agarose Western blot system adapted from previous studies (82, 83). Agarose Western blots were performed using reconcentrated washing from BCI-NS1.1 cultures as in SDS-PAGE Western blots. 1 L 1xTAE (Tris-acetate-EDTA) buffer was prepared by adding 20 mL of 50x TAE to 980 mL of ddH₂O. 10 mL of loading buffer was prepared by adding 5 mL of 1x TAE buffer to 5 mL of glycerol (50%), 25 mg of bromophenol blue (0.25%), and 100 mg of sodium dodecyl sulfate (SDS) (1%). Mucus samples (10 µg) were mixed with 10x loading buffer. Once samples were prepared, they were vortexed for at least 3 minutes before being incubated at 99°C for 6 minutes and loaded into the agarose gel. The agarose gel was prepared by first preparing 1x TAE-0.1% SDS buffer (1L of 1x TAE buffer and 1 g of SDS). 150 mL of 1x TAE-0.1% SDS buffer was added to a microwavable Erlenmeyer flask and 1.2 g of agarose powder was added to

make 0.8% agarose-SDS gel. After gelation, samples were loaded into each lane and proteins separated by electrophoresis at 12 V overnight (or 16 hours).

Western Blot – Vacuum Transfer: Proteins were transferred using a vacuum transfer system. Two Western Blotting Filter Paper (ThermoFisher) and a large sponge were soaked in transfer buffer, 4x sodium saline citrate (SSC) buffer, which was prepared by diluting 20x SSC in water. PVDF membrane cut to the size larger than the agarose gel was activated in ethanol for approximately 2 minutes. The transfer was performed in a vacuum chamber and the transfer sandwich was assembled (filter paper, filter paper, PVDF membrane, agarose gel, sponge). Transfer was run at 40-75 mmHg for 150 minutes. After disassembling transfer sandwich, proteins bands were visualized with ponceau stain.

Western Blot – Staining and Imaging: Western blots, both SDS-PAGE and agarose method, were blocked with 5% (w/v) fat free milk protein in tris-buffered saline, 0.1% Tween (TBS-T) at room temperature for 1 hours. Primary antibody incubation was over night at 4°C in 5% (w/v) fat free milk protein (**Table 3.2**). After washing in TBS-T, membranes were probed with secondary antibodies for one hour at room temperature in blocking buffer. Imaging was performed five minutes after application of 1 mL of chemiluminescent SuperSignal Dura reagent (Thermo Scientific) on the membrane. It is important to note that the primary antibodies that successfully worked for detecting mucins on a Western blot were not always successful for detecting using immunofluorescence.

Table 3.2: Table of primary and secondary antibodies used in this work.

Name of Antibody	Manufacturer	Dilution (Use*)
Anti-acetylated alpha tubulin AlexaFluor 647 (6-11B-1)	Santa Cruz; sc-23950	1:1000 (IF)
ZO-1 monoclonal antibody AlexaFluor 488 (ZO1-1A12)	Invitrogen; 339188	1:2000 (IF)
Rabbit anti-MUC5B	UNC gift from Dr. Ehre; UNC414	1:1000 (WB, IF)
Mouse monoclonal anti-MUC5B (5B19-2E)	Santa Cruz; sc-21768	1:1000 (IF)
Mouse monoclonal anti-MUC5AC (45M1)	Abcam; ab3649	1:1000 (WB)
Mouse monoclonal anti-MUC5AC (45M1)	Santa Cruz; sc-21701	1:1000 (IF)
anti-rabbit-HRP	Invitrogen; 32460	1:10,000 (WB)
anti-mouse IgG-HRP	Santa Cruz; sc-516102,	1:10,000 (WB)

*WB: Western blot; IF: immunofluorescence

3.3 Assessing Mucociliary Clearance Function

Assessment of mucociliary clearance was a key functional assay for the work in this dissertation. While the assay itself is simple and straight forward, mucus transport rates were highly dependent on hydration level of mucus. For optimal measurement and consistency, mucus was regularly washed from the cultures to prevent accumulation and dehydration.

3.3.1 Measuring transport rate of mucus

Mucociliary transport was measured based on the transport of 2 μm red-fluorescent polystyrene microspheres (Sigma-Aldrich; 1:1000 dilution in PBS) For measurement of

transport rate of mucus, a 4 uL suspension of microspheres was apically applied to native mucus, which was allowed to accumulate for 1-2 days prior to analysis. After equilibration overnight, videos of three regions were recorded at 10x magnification using a Zeiss 800 LSM microscope. Images were collected at a frame rate of 0.5 Hz for 20 seconds on the plane of the mucus gel. The microsphere tracking data analysis is based on an image processing algorithm that was custom written in MATLAB (84, 85). Briefly, the analysis software computes the xy-plane trajectories of each fluorescent microsphere in each frame. Using the trajectory data, displacement of microspheres was computed, and transport rate was calculated by dividing the distance traveled by total elapsed time.

Measuring Transport of Transplanted Mucin-Hydrogels/Mucus: For transport experiments that involved transplantation of exogenous gels, hydrogel or mucus was applied and allowed to equilibrate before depositing fluorescent microspheres. For mucin hydrogels, gels were transplanted 1 hour before measuring transport rate, while for exogenous mucus collected from cell cultures were transplanted at least 12 hours before measurement of transport rates.

3.3.2 Measuring cilia beat frequency

In order to measure ciliary beat frequency (CBF), 10 second videos at a frame rate of 20 Hz were recorded at 20x magnification in ≥ 3 randomly selected regions of each BCi-NS1.1 culture using brightfield. Videos were imported into FIJI/ImageJ and three regions of the video were randomly selected for measurement of intensity change over time. The change in intensity over time data was then imported into MATLAB to determine the local pixel intensity maxima per time, which indicates beating of cilia. Beat frequency was determined by dividing the number of beats over the total elapsed time.

3.4 CRISPR/Cas9-mediated Genome Editing

In our work, we devised a detailed strategy for targeted knockout (KO) of secreted mucins using lentiviral delivery of gRNA and Cas9 transgene as shown in **Fig. 3.2**. Our methods included designing the gRNAs, transfection and lentivirus collection, transduction, and fluorescence-activated cell sorting (FACS) of transduced cells.

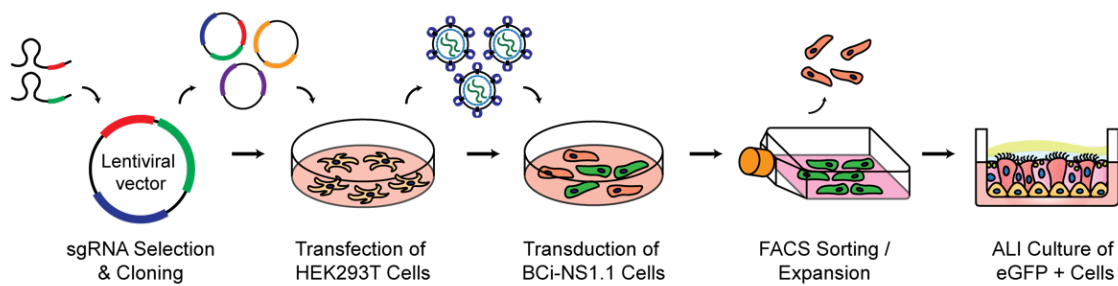


Figure 3.2. Overview of the MUC5B/MUC5AC gene-targeting approach via lentivirus-mediated delivery of sgRNA and CRISPR/Cas9 Strategy. The sgRNAs were cloned into a GFP-expressing lentiviral vector that also encodes the Cas9 nuclease. After lentivirus production, we transduced BCI-NS1.1 cells, an immortalized HAE cell line previously used for manipulating mucin expression. Subsequently, transduced cells were expanded and sorted for GFP expression prior to differentiation.

3.4.1 Designing gRNAs and Cloning into Lentiviral Vector

Designing gRNA for Targeting Secreted Mucins: Online CRISPR design tools (Benchling, CHOPCHOP, IDT) were used to randomly generate ~600 gRNA sequences for MUC5B and MUC5AC. The pool of gRNAs was further narrowed by identifying gRNAs that target earlier exons (exon 1-10) in the mucins to maximize KO efficiency. Lastly, two gRNAs were selected based on favorable targeting using the Doench and Xu scores, self-complementarity, and specificity.

Table 3.3: Table of gRNA for KO of secreted mucins.

Gene	gRNA #	Target	Sequence
MUC5B	gRNA 1	Exon 3	GTGGAACAAAGCTCACGCGC
	gRNA 2	Exon 4	TTCAACGTCCAGCTACGCCG
MUC5AC	gRNA 1	Exon 4	GATGTTAAAATCCTCGTAGG
	gRNA 2	Exon 6	GAGAGGAGCTCGCTGACCAC

Digestion of Lentiviral Vector: Designed gRNAs were inserted into a eGFP-expressing lentiviral vector that encodes for Cas9 nuclease. Before insertion, the lentiviral CRISPR plasmid, pLentiCRISPRv2-GFP (AddGene) was digested using restriction enzyme FastDigest Esp31 (ThermoFisher) for 30 min at 37°C using the reagents found in **Table 3.4**. This step provided the dephosphorylated digested backbone that can be ligated with the gRNAs.

Table 3.4: Table of reagents used for lentiviral vector digest.

	Volume (μL)
pLentiCRISPRv2-GFP (AddGene)	~ 20 (5 μg)
FastDigest Esp31 (<i>BsmI</i>)	3
Fast alkaline phosphatase (Thermo Fisher)	3
10X FastDigest Buffer (Thermo Fisher)	6
ddH ₂ O	X (~ 28 μL)
Total	60ul

Extraction of Digested Lentiviral Vector using Gel Electrophoresis: Digested lentiviral vector was extracted after separation of DNA fragments through gel electrophoresis. 1% agarose gel was prepared by adding 50 mL of TBE (Tris-borate EDTA) buffer and adding 0.5 g agarose. After microwaving for ~40 seconds and dissolving

the agarose, 5 μ L of SYBR Safe DNA gel stain (Invitrogen) was added for visualization of DNA. While still warm, the agarose solution was poured into a mold with a comb to create the wells. A wide gel comb was used for the extraction protocol, while every other lane was left empty as this produced crisp bands and created space for cutting. After gelation, the agarose gel was loaded with 7 μ L of 2log DNA ladder (New England BioLabs), 60 μ L of digested backbone, and 60 μ L of undigested backbone as a size control. The gel was run for 25 minutes at 100-120 V. Subsequently, the gel was imaged on an open UV box (wearing proper UV protection) for visualization of DNA fragments. The digested fragment was extracted by cutting the larger DNA fragment out of the gel. Before cutting, a 2 mL centrifuge tube was weighed. The larger fragment (~11 kB) of digested band was cut and added to the centrifuge tube for calculating the gel's mass, which is directly proportional to the volume. DNA was eluted using QIAquick Gel Extraction Kit (Qiagen) using manufacturer's protocol, which used the volume of cut gel. After extraction, DNA concentration was quantified using a Nanodrop.

Phosphorylation and Annealing of gRNA Oligo Pairs: Designed gRNAs were purchased as Oligos from Integrated DNA Technologies (IDT). Phosphorylation and annealing reaction of gRNA oligo pairs was performed by mixing the reagents found in **Table 3.5** and placing the reaction in a thermocycler at 37°C for 30 minutes, 95°C for 5 minutes with a ramp down to 25°C at 5°C/minutes. The phosphorylated and annealed oligos were subsequently used for ligation with the digested lentiviral vector backbone.

Table 3.5: Table of reagents for annealing of gRNA oligo pairs.

	Volume (μL)
Oligo 1 (100 μM)	1
Oligo 2 (100 μM)	1
10X T4 Ligation Buffer (NEB)	1
ddH ₂ O	6.5
T4 Polynucleotide kinase (NEB M0201S)	0.5
Total	10

Ligation of gRNA and Lentiviral Vector Backbone: Before the ligation reaction, the gRNA (phosphorylated oligo duplex from previous step) was diluted 1:200 into sterile water. The ligation reaction was performed using the Quick Ligation Kit (NEB BioLabs) using manufacturer's protocol with reagents listed in **Table 3.6**. The solution containing digested lentiviral vector and gRNA was gently mixed by pipetting and incubated at room temperature for 10 minutes before transformation in into bacteria.

Table 3.6: Table of reagents for ligation of lentiviral vector backbone.

	Volume (μL)
Digested lentiviral vector (50 ng)	X (~ 0.625 μL)
gRNA (annealed oligo duplex)	1
2X Quick Ligase Buffer (NEB)	5
ddH ₂ O	X (~ 3.375 μL)
Subtotal	10
Quick Ligase (NEB M2200S)	1
Total	11

Transformation into *E. coli* and Collection of Plasmids: A vial of One Shot Stbl3 Chemically Competent *E. coli* (ThermoFisher) was thawed on ice. 1 to 5 μL of DNA (ligated gRNA and lentiviral vector) was added to the vial of cells ($\sim 50 \mu\text{L}$) and mixed gently. As a control, the unmodified lentiviral vector backbone (pLentiCRISPRv2-GFP) was transformed for guiding the colony selection process. The vial was incubated on ice for 30 minutes. After incubation, cells were heat-shocked at 42°C for 45 seconds without shaking. The vial was then placed on ice for 2 minutes. 250 μL of pre-warmed S.O.C medium (ThermoFisher) was added to each vial. 25-100 μL from each transformation mix was spread on a pre-warmed selective agar plate containing ampicillin ($100 \mu\text{g/mL}$) and incubated inverted overnight at 37°C , while the remaining transformation mix was stored at 4°C . For expansion of transformed *E. coli*, single colonies were selected from the agar plates and transferred using a sterile pipette tip or toothpick to a culture test-tube containing 2 mL of liquid LB (ThermoFisher) with ampicillin ($100 \mu\text{g/mL}$ LB). Bacterial cultures were loosely covered and incubated at 37°C for 12-18 hours in a shaking incubator. After incubation, plasmid DNA was recovered using a Miniprep Kit (Qiagen) following manufacturer's protocol. The recovered plasmids were sequenced at Genewiz, where samples were prepared by mixing 1000 ng of DNA ($\sim 2 \mu\text{L}$ of $500 \text{ ng}/\mu\text{L}$ sample) into a PCR tube and adding 5 μL of primer ($5 \mu\text{M}$) and $\sim 8 \mu\text{L}$ ddH₂O was added to reach a total volume of 15 μL . Once DNA of plasmid was sequenced, bacterial cultures were expanded into larger shake flasks and plasmids were isolated using Maxiprep Kit (Qiagen). The plasmids obtained from the Maxiprep, denoted the plentiCRISPR construct, was used for subsequent transfection into human embryonic kidney (HEK) 293T cells.

3.4.2 Transfection of HEK293T Cells

HEK293T cells were seeded onto 6 well plates that were coated with poly-l-lysine at a concentration of $4\text{-}5 \times 10^5$ cells/well and cultured in 1 mL DMEM with 10 % FBS until 60-80% confluency was reached. For transfection, jetPRIME transfection reagent (Polyplus) was used using manufacturer's protocol. Briefly, HEK293T cells were co-transfected with multiple plasmids mixed with jetPRIME buffer and jetPRIME reagent (shown in **Table 3.7**). Plasmid solution was vortexed and added dropwise onto the cells.

Table 3.7: Table of reagents for transfection.

	Mass/ well (μg)	Volume / well (μL)
Media (DMEM, 10 % FBS)	-	2000
jetPRIME Buffer	-	200
jetPRIME Reagent	-	4
Plasmids:		
pCMV-VSV-G (envelope protein)	0.2	X
psPAX2 (packaging protein)	0.7	X
plentiCRISPR Construct	0.7	X
Total	1.6	~2250

HEK293T cells were incubated with plasmids and transfection reagents at 37°C and media was replaced with fresh DMEM with 10% FBS after 6-18 hours. Lentiviral stalks were generated by collecting the media 48 hours after media change and removing cell debris using a $0.45\text{ }\mu\text{m}$ filter. Lentiviral stalks were stored with 20 mM HEPES and $4\text{ }\mu\text{g/mL}$ polybrene at -80°C .

3.4.3 Transduction of BCI-NS1.1 Cells

BCi-NS1.1 cells are seeded onto 12 well plates at a concentration of 10^5 cells/well. Expansion media, PneumCult-Ex Plus (StemCell) was prepared with 20 mM HEPES and 4 μ g/mL polybrene to match the concentration of lentiviral stalk. Appropriate volume of lentiviral stalk and media was mixed to achieve concentrations shown in **Table 3.8**.

Table 3.8: Table of reagents used for transduction.

	Volume (μ L) / Well
Lentiviral stalk	400
PneumaCult-Ex Plus, 20 mM HEPES, 4 μ g/mL Polybrene	2000
Total	2400

Viral stalk and media mixture was added dropwise to BCI-NS1.1 cells. In order to enhance transduction probability, BCI-NS1.1 cells were inoculated in a centrifuge. The centrifuge was warmed up prior to transduction by spinning at 3000 RPM at 37°C for approximately one hour. Cells were spun in the centrifuge for 1 hour at 1000xg and subsequently incubated in an incubator for 6 hours at 37°C. After 6-hour incubation, media was changed. Transduced cells were expanded in PneumaCult-Ex Plus until $\sim 10^6$ cells were reached and subsequently sorted for eGFP⁺.

3.4.4 Fluorescent-Activated Cell Sorting (FACS) of eGFP⁺ Cells

Transduced cells were detached with 0.25% trypsin using standard BCI-NS1.1 cell culture protocol. After centrifugation, cells were resuspended in PBS and filtered through a sterile cell strainer with 40 μ m mesh (Fisherbrand) to remove large debris and obtain uniform single-cell suspension. Cells were centrifuged again and resuspended in ~ 1 mL of sterile filtered FACS buffer, which is PBS with 10 mM HEPES and 0.1% BSA. Depending

on concentration of cells obtained after FACS, eGFP⁺ cells were plated in well plates or tissue culture flasks at a concentration of 3000 cells/cm², expanded in PnemaCult-Ex Plus, and subsequently seeded on transwells for air-liquid interface culture or frozen for storage. Cells were re-sorted after ~4-5 passages after every FACS to maintain a high concentration of eGFP⁺ population.

4. RATIONAL APPROACH TO FORM DISULFIDE LINKED MUCIN HYDROGELS[†]

4.1 Introduction

Mucus is a biological gel that coats and protects epithelial surfaces in tissues throughout the body. Mucins are large, polymeric glycoproteins that are the primary contributor to the viscoelastic properties of mucus (34, 86). The cysteine-rich domains of mucins facilitate their assembly into a mesh-like structure through disulfide cross-linking (38, 87). Reversible, non-covalent bonds mediated through hydrophobic interactions and hydrogen bonds also contribute to the highly dynamic and complex arrangement of mucins within the gel (49, 88). As a result, the physical properties of mucus gels are also influenced by the buffer conditions where pH can vary from acidic (e.g. pH 2 in the stomach) to more neutral (e.g. pH 7 in the lung) conditions (53). This network architecture allows mucus to behave as a viscoelastic gel at the macroscale. On the microscopic scale, mucus acts as physical barrier, entrapping harmful pathogens and particulates to prevent them from reaching the underlying epithelium (50, 89). However, the process by which mucins organize into a mucus gel has been challenging to recreate in the laboratory. This motivates our efforts to develop a new synthetic strategy, that at least in part, mimics the natural assembly of mucins into mucus gels to enable its use as a generalized model for biological studies.

[†] Reproduced from: Joyner K, Song D, Hawkins R, Silcott R, and Duncan, G. “A rational approach to form disulfide linked hydrogels.” *Soft Matter*. 15. 9632-9639. 2019 with permission from Royal Society of Chemistry.

In previous work, it has been observed that commercially available mucins (e.g. porcine gastric mucin, bovine submaxillary mucin) do not form hydrogels at physiological pH or concentrations, presumably as a result of their processing (53, 90). This considerably reduces their utility as a model of natural mucus. It has been shown covalent cross-linking strategies through acrylate- and glutaraldehyde-mediated bonds can be used to form mucin gels (51, 91, 92). However, these chemistries do not mirror the natural thiol-mediated bonds found between mucin polymers in native mucus which may limit their use as a model in biological research. This has led to efforts to develop methods to purify mucins without compromising their capacity to form a gel (93). For example, it has been shown mucins can be purified from porcine stomach scrapings which preserves their natural gel-forming properties when prepared at acidic pH (53, 90, 94, 95). Mucus secreted from tissue culture models has also been collected and purified which has been shown to retain comparable viscoelastic behavior to human mucus (36, 91). However, the requirement of specialized, relatively low-yield processing techniques limits their widespread usage. Methods to enable the formation of gels from crude, partially purified mucins available in large quantities from commercial vendors would allow for its general use for basic and applied biomedical research.

Towards this end, we have developed a simple approach to generate hydrogels using crude, commercially available mucins and thiol-based cross-linkers that re-establish native-like rheological properties. Particle tracking microrheology (PTM) of PEG-coated nanoparticles was used to determine if and when mucins began to form into a gel after addition of a cross-linking reagent. Several thiol-functionalized, polymer-based crosslinkers with varying chemistry and geometry were tested to determine which would

initiate mucin hydrogel network formation. Using an optimized cross-linking strategy, bulk rheological assessment of mucin-based hydrogels was performed to determine if we were able to produce gels with physiological viscoelastic properties. Finally, PTM was used to investigate the kinetics of gel formation as a function of mucin and cross-linker concentration. The results of this work demonstrate how these mucins, incapable of forming gels, may be restored to behave like natural mucus.

4.2 Methods and Materials

4.2.1 Preparation of Nanoparticles

Carboxylate modified fluorescent PS spheres (PS-COOH; Life Technologies) with a diameter of 100 or 500 nm were coated with a high surface density of polyethylene glycol (PEG) via a carboxyl-amine linkage using 5-kDa methoxy PEG-amine (Creative PEGWorks) as previously reported (51, 96). Briefly, PEG-amine was added to a diluted suspension of PS-COOH in ultrapure water. N-hydroxysulfosuccinimide sodium salt (10 mM; Alfa Aesar), borate buffer (pH 8.3) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (2 mM; Thermo Fisher) were then added to activate and link PEG-amine to PS-COOH nanoparticles. The reaction was mixed for at least 4 hours at room temperature and covered to minimize exposure to light. After mixing, excess reagents were removed by washing and centrifuging three times with ultrapure water, then re-suspended to a final volume two times the original. After washing, particle size and zeta potential was measured in 10 mM NaCl at pH 7 using a Malvern Zetasizer Nano ZS90. We measured diameters of 122 nm and 428 nm and zeta potentials of -4.2 and -3.4 mV for 100 nm and 500 nm PEG-coated PS nanoparticles, respectively.

4.2.2 Preparation of Mucin Hydrogels

Solutions of porcine gastric mucin (PGM; Sigma-Aldrich) or mucin from bovine submaxillary glands (BSM; Sigma-Aldrich) were prepared in a physiological buffer (154 mM NaCl, 3 mM CaCl₂, and 15 mM NaH₂PO₄ at pH 7.4) and stirred at room temperature for 2 hours. Crosslinking reagents tested in this work include monofunctional PEG-thiol (PEG-1SH; 10 kDa), bifunctional PEG-thiol (PEG-2SH; 10 kDa), 4-arm PEG-thiol (PEG-4SH; 10 kDa), each purchased from Laysan Bio Inc. and a thiol-functionalized dextran (dextran-SH; 10 kDa; ~60 - 120 thiol groups per polymer) purchased from Nanocs. Crosslinking solutions were prepared separately in buffer directly before mixing with mucin solutions. To initiate gelation, equal volume aliquots of each solution were mixed and either equilibrated for 30 minutes before particle tracking or left for 24 hours before bulk rheological characterization at room temperature. To confirm cross-linker interaction with cysteine rich regions of PGM, thiol-blocking and disulfide cleavage experiments were performed to inhibit or disrupt gel assembly. Iodoacetamide at 50 mM (VWR) was added to the PGM solutions and mixed for 1 hour to alkylate PGM cysteine residues before mixing with the crosslinking polymer (97). As has been previously shown in human mucus, we disrupted disulfide bonds within the gel using the reducing agent, *N*-acetyl cysteine (NAC) (98). NAC was added after gelation (>9 hours) at a final concentration of 50 mM (NAC) and incubated at 37°C for 30 minutes.

Cysteine content in PGM and BSM was measured using a previously described fluorometric assay. Briefly, 50% w/v solutions of PGM and BSM were prepared. The solutions were then diluted 10-fold from the original volume in 8 M guanidine-HCl (Sigma Aldrich). Serial dilutions of 5 mM L-cysteine (Sigma-Aldrich) in 50 mM tris-HCl were

used as a standard. Samples and standards were added to a black Maxisorp plate with an equal volume of 2 mM monobromobimane (mBBBr, Sigma Aldrich) in 50 mM tris-HCl. The fluorescence was measured at excitation and emission wavelengths of 395 and 490 nm, respectively. Based on this analysis, we measured 0.36 μmol and 0.34 μmol cysteine content per gram mucin for PGM and BSM, respectively.

4.2.3 Measurement of Bulk Rheology

Dynamic rheological measurements of the mucin/PEG-4SH hydrogels were performed using an AR2000 rheometer (TA Instruments) with a 20-mm diameter parallel plate geometry at 25°C. To determine the linear viscoelastic region of the fully formed gel, a strain sweep measurement was collected from 0.1-10% strain at a frequency of 1 rad/s. To determine the elastic modulus, $G'(\omega)$, and viscous modulus, $G''(\omega)$, a frequency sweep measurement was conducted within the linear viscoelastic region of the gel, at 1% strain amplitude and angular frequencies from 0.1 to 100 rad/s.

4.2.4 Sample Preparation for Particle Tracking Microrheology

The diffusion of PEG-coated nanoparticles (PEG-NP) in mucin-based hydrogels were measured using fluorescent video microscopy. Samples were prepared with 0.5 μL of ~0.002% w/v suspension of PEG-NP, added into a 25 μL solution of PGM or BSM and cross-linker prior to gel formation. Next, ~25 μL of mucin hydrogel precursor solution containing PEG-NP was added into a custom microscopy chamber, sealed with a cover slip, and equilibrated for 30 minutes at room temperature before imaging. Fluorescent video microscopy experiments were collected using a Zeiss 800 LSM microscope equipped with a $\times 63/1.20$ W Korr UV VIS IR water-immersion objective and an Axiocam 702 camera with pixel resolution of 0.093 $\mu\text{m}/\text{pixel}$. Images were collected at a frame rate of

33.33 Hz for 10 seconds at room temperature. For studies of gel kinetics, videos were taken every hour in order to measure PEG-NP diffusion during hydrogel assembly.

4.2.5 Particle tracking microrheology (PTM)

The particle tracking data analysis used in this work is based on a previously developed image processing algorithm. To briefly describe, an image intensity threshold is set to differentiate nanoparticle signal from background. Nanoparticles above the intensity threshold are then assigned centers based on x and y image location. Particles were tracked for at least 300 sequential frames allowing trajectories to be determined. From these trajectories, time-averaged mean squared displacement (MSD; $\langle \Delta r^2(\tau) \rangle$) as a function of lag time, τ , was calculated as

$$\langle \Delta r^2(\tau) \rangle = \langle [x(t+\tau)-x(t)]^2 + [y(t+\tau)-y(t)]^2 \rangle.$$

Using the generalized Stokes-Einstein relation, measured MSD values were used to compute viscoelastic properties of the hydrogels. The Laplace transform of $\langle \Delta r^2(\tau) \rangle$, $\langle \Delta r^2(s) \rangle$, is related to viscoelastic spectrum $\tilde{G}(s)$ using the equation

$$\tilde{G}(s) = 2k_B T / [\pi a s \langle \Delta r^2(s) \rangle],$$

where $k_B T$ is the thermal energy, a is the particle radius, s is the complex Laplace frequency. The complex modulus can be calculated as

$$G^*(\omega) = G'(\omega) + G''(i\omega),$$

with i being substituted for s , where i is a complex number and ω is frequency. Hydrogel network pore size, ξ , is estimated based on MSD using the equation,

$$\xi \approx \sqrt{\langle \Delta r^2(\tau) \rangle} + a,$$

and also based on G' using the equation,

$$\xi \approx (k_B T / G')^{1/3}.$$

The sol-gel transition (gel point) was determined by calculating the logarithmic slope of the mean-squared displacement, $\alpha = \log_{10}(\langle \Delta r^2(\tau) \rangle) / \log_{10}(\tau)$. We used a critical value of $n=0.5$ where the gel point is defined as the time when measured $\alpha < n$ (99–101).

4.3 Results and Discussion

4.3.1 Design and Characterization of Mucin-based Hydrogel Network Assembly

We aimed to create a viscoelastic gel composed of porcine gastric mucin (PGM) via disulfide-bond mediated crosslinking between the cysteine domains of PGM biopolymers. PTM was employed to evaluate different strategies to make mucin-based gels. We chose to use PTM as it provides a biophysical method to assess the mechanical properties of soft biological gels. Importantly, PTM has been used extensively in previous work to evaluate microrheology and structure of mucus (36, 50, 51). In comparison to bulk rheological methods, PTM can provide additional information on material properties on the micro- to nanoscale such as network pore size and local heterogeneity (102, 103). The diffusion of fluorescently labeled, PEG-coated particles (PEG-NP) with diameter of 100 nm was measured in mixtures of PGM and various thiol-containing polymers to determine which may be a suitable cross-linking reagent. We used a low concentration ($\sim 4 \times 10^{-5}$ % w/v) of nanoparticles coated with a dense layer of low molecular weight PEG in order to ensure nanoparticle probes are minimally adhesive to mucins (104, 105). Thus, PEG-NP probes are unlikely to interfere or influence hydrogel formation. **Fig. 4.1** shows PEG-NP diffusion as measured by the mean squared displacement (MSD; $\langle \Delta r^2 \rangle$) of the particles determined immediately after mixing (hour 0) and 9 hours after mixing. Once the gel has formed, the MSD of PEG-NP will be reduced due to steric interactions with the mesh-like

mucin polymer network. In addition, the logarithmic slope of MSD will be reduced from ~ 1 for a viscous liquid to ≤ 0.5 for a viscoelastic material (106).

Four potential thiol-based crosslinking reagents were tested with a low molecular weight of 10 kDa and a PEG or dextran backbone. Two percent w/v of mucin and crosslinker was used in each case. At these concentrations, the number of thiols in the crosslinkers (1-10 mM for PEG; 120-240 mM for dextran) is far in excess of that of the mucin biopolymers (2-10 μM range) in all cases. As shown in **Fig. 4.1A**, no significant changes in MSD of PEG-NP were observed after mixing PGM with monofunctional PEG-1SH. Since it is monofunctional, it is not able to initiate gel formation. We hypothesized bifunctional PEG-2SH would be able to create disulfide bridges between mucin polymers to facilitate gel formation. However, we found it was unable to form a gel over a 9-hr period (**Fig. 4.1B**). Using a multi-arm PEG-4SH, we observed a substantial decrease in the

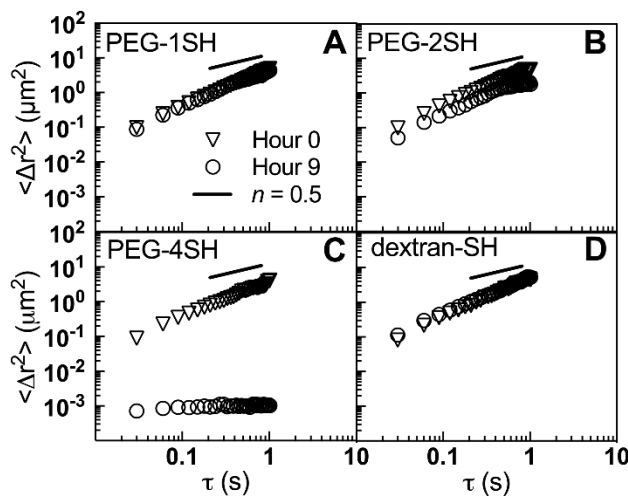


Figure 4.1 Microrheology of mucin mixed with various thiol-based crosslinkers. (A-D) Multiple particle tracking of 100-nm PEG-NP in mucin solutions with varying cross-linking polymers. Each panel represents the ensemble average mean squared displacement as function of time scale τ (MSD; $\langle \Delta r^2 \rangle$) of 100 nm PEG-NP at hour 0 (triangles) and hour 9 (circles) in 2% w/v of PGM and 2% w/v of (A) PEG-1SH, (B) PEG-2SH, (C) PEG-4SH, and (D) dextran-SH. Reference lines with slope $n=0.5$ are included in each panel.

magnitude and slope of MSD after 9 hours of incubation with PGM, indicative of network formation (**Fig. 4.1C**). At present, we can only speculate on the role of PEG linker geometry on mucin hydrogel assembly. A potential explanation is the branched architecture of PEG-4SH enables association between multiple mucin chains whereas PEG-2SH mediated bonds between 2 neighboring chains may only effectively increase polymer molecular weight in these semidilute solution of mucins.

Another complexity in this biohybrid system is discerning the relative contributions of PEG-PEG, mucin-mucin, and PEG-mucin associations to the sol-gel transition. To address this, we considered the potential self-assembly of PEG-4SH, yielding a hydrogel that does not incorporate mucin biopolymers. To address this, we measured the microrheology of a 2% PEG-4SH solution (i.e. in the absence of PGM), where no gel formation was observed in a 9-hour period. We also examined if PEG-4SH self-assembly could be the result of an increased local concentration due to crowding by mucin polymers. To evaluate this, 500 kDa hyaluronic acid (HA) was used to act as a non-thiol containing, high molecular weight crowding agent. Hydrogel network formation was not observed after a 9-hour period in mixtures of 2% HA and 2% PEG-4SH, suggesting crowding did not induce PEG-4SH self-assembly. In addition, we changed the cross-linking polymer backbone from PEG to dextran, but unlike PEG-4SH, the multi-functional dextran-SH did not lead to assembly of mucins into a mesh network (**Fig. 4.1D**). These results suggest that the more flexible PEG backbone may also be important in the ability of PEG-4SH to facilitate hydrogel formation. Based on these results, PEG-4SH was selected as the optimal cross-linking polymer to produce mucin-based hydrogels.

In order to examine the mechanism of gel formation, we tested if alkylation or reduction of cysteines impacted the formation of mucin-based hydrogels. PTM was again employed to monitor changes in the mucin hydrogel network upon treatment with cysteine-blocking or disulfide bond-disrupting agents. Here, we define the point of gelation based on measured MSD of 100 nm PEG-NP as $\alpha = \log_{10}[\text{MSD}]/\log_{10}[\tau] = 0.5$, where as $\alpha < 0.5$ indicates the formation of an elastic network. In order to block all available cysteines, a 2% PGM solution was alkylated with 50 mM iodoacetamide (IAM). As shown in **Fig. 4.2A**, no significant changes in 100 nm PEG-NP diffusion were observed after 9 hours, $\alpha = 0.9$, implying network assembly is inhibited by IAM treatment. While only the PGM solution was treated with IAM, we note that excess IAM would likely block cysteines present on PEG-4SH once the solutions were mixed.

In order to block mucin biopolymers only, the PGM solution was pre-treated with IAM at a decreased concentration of 50 μM . At this concentration, we provide a sufficient excess of IAM to block all cysteines in PGM (7.2 μM in 2% PGM) while the total thiol concentration on PEG-4SH (8 mM in 2% PEG-4SH) is effectively unchanged and available to form disulfide bridges. In this case, we observed a decrease in the magnitude of MSD for 100 nm PEG-NP diffusion after 50 μM IAM treatment (**Fig. 4.2B**). However, based on the measured α of 0.7 at hour 9, we do not see evidence of gel network formation. To determine if the hydrogel network would be disrupted by cleaving disulfide bonds, the reducing reagent, *N*-acetyl cysteine (NAC), was applied to a fully formed PGM/PEG-4SH hydrogel. After 30-minute treatment with NAC, we observed a marked increase in 100 nm PEG-NP diffusion approaching that of the PGM solution alone (**Fig. 4.2C**), indicative of a significant breakdown in the mesh network.

Together, these data would suggest a mechanism where disulfide-mediated formation of mucin hydrogels is promoted through non-covalent PEG-mucin interactions. The reduced MSD observed for PEG-4SH and IAM-capped mucins (**Fig. 4.2B**) indicates PEG-4SH may associate with mucin biopolymers via noncovalent hydrogen bonds and self-associate via disulfide bonds. However, the gel is not able to fully form through these non-covalent PEG-mucin interactions and covalent PEG-PEG disulfide bonds only. Thus, PEG-mucin disulfide bonds also likely contribute to the gel formation process. We reason an initial phase of PEG-4SH adsorption to mucin biopolymers enables hydrogel assembly through both PEG-PEG and PEG-mucin disulfide bonds. PEG-4SH adsorption would also help to explain the high stoichiometric ratio of PEG-4SH necessary to achieve gel formation. An excess of PEG-4SH may be required for a sufficient amount of the crosslinker to adsorb to mucin biopolymers. It is unclear how the geometry of PEG-4SH

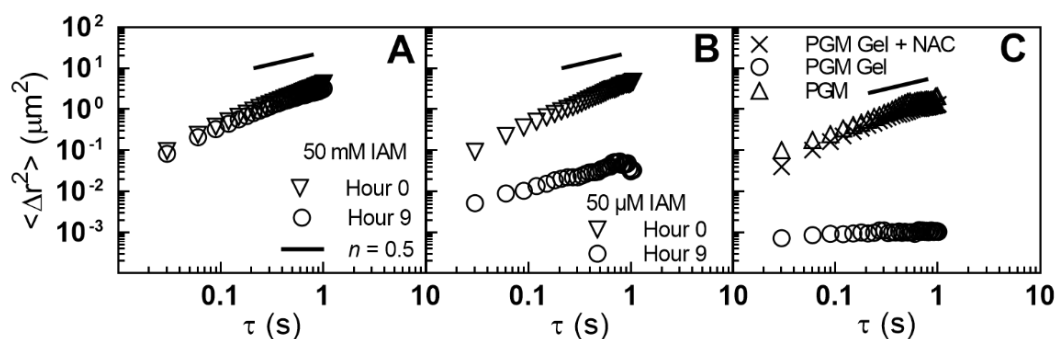


Figure 4.2 Effects of cysteine-blocking and disulfide cleavage on mucin-based hydrogel assembly and disassembly. (A-C) MSD ($\langle \Delta r^2 \rangle$) as a function of time scale (τ) of 100 nm PEG-NP in 2% w/v PGM and 2% w/v PEG-SH-4 solution 0 hours (downward triangles) and 9 hours (circles). α is defined as $\log[\text{MSD}]/\log[\tau]$ where the gelation point defined as $\alpha < 0.5$. (A) MSD of 100 PEG-NP in 2% PGM/PEG-4SH using PGM pre-treated with 50 mM iodoacetamide (IAM) at hour 0 ($\alpha = 1.0$) and hour 9 ($\alpha = 0.9$). (B) MSD of 100 PEG-NP in 2% PGM/PEG-4SH using PGM pre-treated with 50 μ M IAM at hour 0 ($\alpha = 1.1$) and at hour 9 ($\alpha = 0.7$). (C) MSD of 100 nm PEG-NP in 2% w/v PGM solution after 9 hours, $\alpha = 1.0$ (PGM; upward triangles), in 2% PGM/PEG-4SH hydrogel after 9 hours, $\alpha = 0.1$ (PGM Gel; circles), and in 2% PGM/PEG-4SH hydrogel after a 30-minute treatment with *N*-acetyl cysteine (NAC), $\alpha = 0.87$ (PGM Gel + NAC; cross). Reference lines with slope $n=0.5$ are included in each panel.

favors these non-covalent interactions and to study this further, we will explore the effect of geometry on PEG-mucin interactions and hydrogel assembly in future work.

4.3.2 Macro- and Microrheology of Mucin-based Hydrogels

To determine if a viscoelastic gel similar to native mucus was formed, we measured the bulk elastic (G') and viscous (G'') moduli of 2% PGM after mixing and incubation for 24 hours with 2% PEG-4SH. Strain sweep experiments were performed at frequency of $\omega=1$ rad/s from 0.1 to 10% strain as shown in **Fig. 4.3A**. Elastic solid-like behavior of the material was observed with $G' > G''$ and loss tangent ($\tan \delta$) of 8.1, thus confirming the viscoelastic nature of the hydrogel. We did not find evidence of yielding and thus, were within the linear viscoelastic region of the gel. To determine if the behavior of our hydrogel was frequency dependent, a frequency sweep was performed at 1% strain over a physiologically relevant frequency range of 0.1 – 100 rad/s. We confirmed the hydrogel was stable over the full range of frequencies tested (**Fig. 4.3B**). Importantly, we found the elastic modulus of ~ 244 Pa at $\omega=1$ rad/s is within the physiological range for mucus (49, 51, 107). These results suggest that PEG-4SH is able to support gelation of mucins into a viscoelastic network. To determine if this approach was generalizable to other mucin types, we prepared a hydrogel using another commercially available mucin, bovine submaxillary mucin (BSM). We measured the bulk elastic (G') and viscous (G'') moduli of 2% BSM after mixing and incubation for 24 hours with 2% PEG-4SH. We also observed formation of a stable viscoelastic hydrogel with $G' > G''$ and loss tangent ($\tan \delta$) of 1.4 (**Fig. 4.3C, D**).

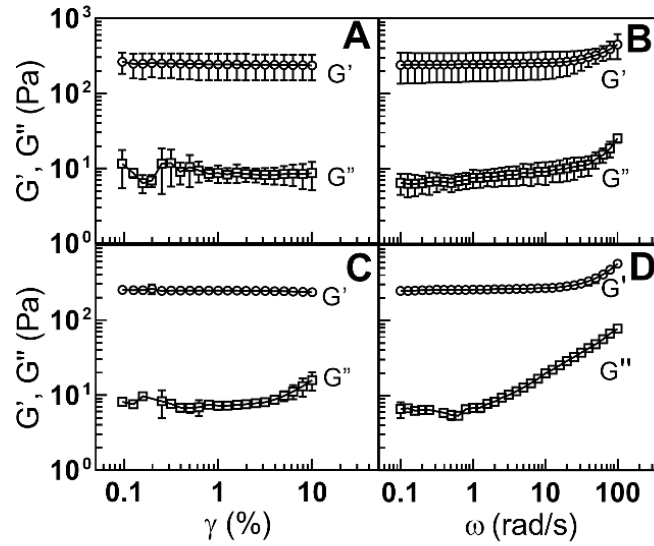


Figure 4.3 Bulk rheological characterization of mucin-based hydrogels. Elastic modulus G' (circles) and viscous modulus, G'' (squares) of PGM-based (A,B) and BSM-based (C,D) mucin hydrogels after a 24-hr gelation time. Data represents average of 3 independently prepared hydrogels. (A) Strain (γ) sweep at frequency (ω) of 1 rad/s and (B) frequency sweep at $\gamma=1\%$ for 2% PGM and 2% PEG-4SH hydrogel. At $\omega = 1$ rad/s, we measured $G' \sim 244$ Pa and $\tan \delta \sim 8.1$. (C) Strain (γ) sweep at frequency (ω) of 1 rad/s and (D) Frequency sweep at $\gamma=1\%$ for 2% BSM and 2% PEG-4SH hydrogel. At $\omega = 1$ rad/s, we measured $G' \sim 270$ Pa and $\tan \delta \sim 1.4$

Next, PTM was used to gain additional insights into the biophysical properties of the material. Given previous reports showing native mucus having network pore sizes ranging from 100-500 nm, we compared microrheology of a 2% PGM and 2% PEG-4SH hydrogel as measured using PEG-NP probes with 100 and 500 nm diameter. PTM measurements were performed each hour for a total of 9 hours to capture local changes in viscosity caused by the organization of mucin fibers into a hydrogel network over time. Figures 4A and 4B show MSD at $\tau = 1$ s for 100 nm and 500 nm PEG-NP, respectively. A decrease in MSD as a function of gelation time was observed for both the 100 and 500 nm PEG-NP, indicating a decrease in diffusion rate as a result of gel network formation. Next, the ratio of elastic and viscous moduli (G'/G'') was calculated based on 100 nm (Fig. 4.4C) and 500 nm (Fig. 4.4D) PEG-NP probes. It should be noted these local measurements of

G' and G'' will strongly depend on probe size as the gel network evolves over time. Based on measured MSD and G' , approximate hydrogel network mesh pore size was found to be on the order of microns at hour 0 and steadily decreasing with median pore sizes on the order of 200-300 nm, consistent with findings in mucus collected from humans (89, 98).

Using nanoparticle probes of different size enabled us to resolve the early and late stages of mucin hydrogel assembly. Based on this analysis, we find evidence of distinct phases in mucin organization into a hydrogel network (i.e. transitions in G'/G'' to values > 1 . After ~ 1 hour, 500 nm probes detect an initial rise in elasticity and reduction of network

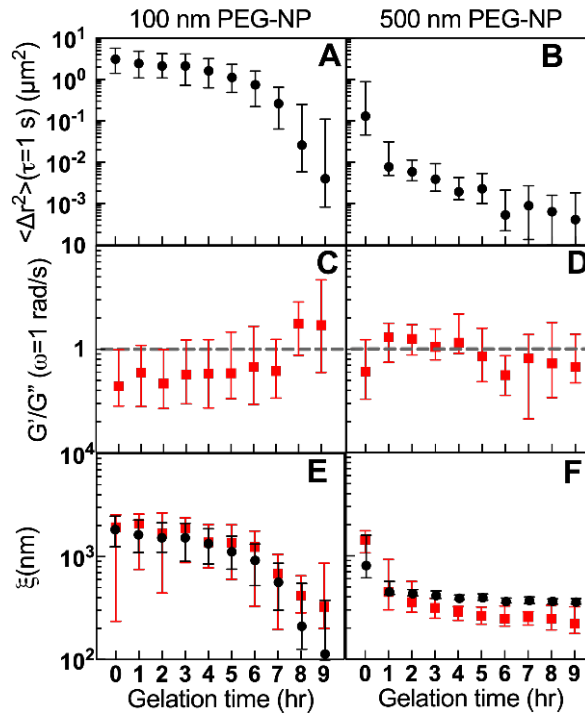


Figure 4.4 Impact of probe size on the microrheology of mucin-based hydrogels. (A-F) Microrheology of a 2% w/v PGM and 2% PEG-4SH gel measured using PEG-NP probes with diameters of 100 and 500 nm. Symbols in each panel are median values and bars indicate the interquartile range. (A,B) The MSD at $\tau = 1$ s ($\langle \Delta r^2(\tau=1 \text{ s}) \rangle$) for (A) 100 and (B) 500 nm PEG-NP during a 9-hour gelation period. (C,D) Elastic to viscous moduli ratio (G'/G'') at $\omega = 1$ rad/sec calculated from measured MSD of (C) 100 nm and (D) 500 nm PEG-NP. (E,F) Estimated pore size based on analysis of MSD at $\tau = 1$ s ($\xi \approx \sqrt{\langle \Delta r^2(\tau) \rangle + a}$; black circles) and G' at $\omega = 1$ rad/s ($\xi \approx (k_B T / G')^{1/3}$; red squares) for (E) 100 nm and (F) 500 nm PEG-NP probes.

pore sizes (**Fig. 4.4D,F**). This is likely indicative of the initial phase of PEG-4SH adsorption to mucins, as discussed earlier. Starting at approximately hour 7, further reductions in network pore size are evident based on the relative increase in G'/G'' measured by 100 nm probes (**Fig. 4.4C, E**), likely as a result of the subsequent phase of disulfide-bond mediated network formation. In future work, microrheological analysis of gel kinetics with greater resolution in time (i.e. measurements taken on the order of minutes) will be used to further interrogate mucin gel assembly. In the next section, we will further discuss gelation kinetics and how we may manipulate the sol-gel transition of these hydrogels.

4.3.3 Concentration Dependence of sol-gel Transition

To understand the influence of mucin to crosslinker ratio on gel assembly, PGM and PEG-4SH concentration were systematically varied to assess their impact on gelation kinetics. The time of gelation was determined based on measured MSD of 100 nm PEG-NP defined as $\alpha = \log_{10}[\text{MSD}]/\log_{10}[\tau] = 0.5$. Given 100 nm PEG-NP captured the latter

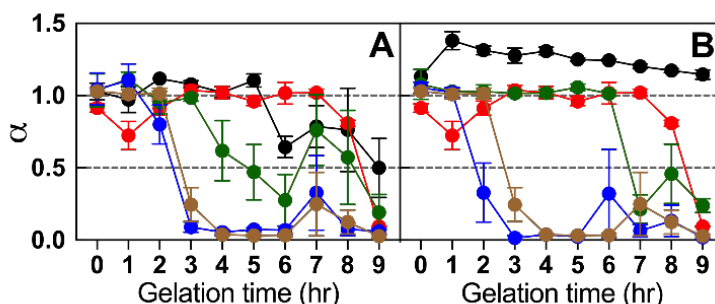


Figure 4.5 Gelation rate of mucin-based hydrogels with varying PGM and PEG-4SH concentration. (A,B) Kinetics of PGM/PEG-4SH gel formation as analyzed by microrheology using 100 nm PEG-NP probes. The gelation point is measured as $\alpha < 0.5$. Each panel displays the mean and standard error of measured α . **(A)** α as a function of gelation time in hydrogels with a constant PEG-4SH concentration of 2% w/v and varying PGM concentrations of 1% w/v (black), 2% w/v (red), 3% w/v (green), 4% w/v (blue), and 5% w/v (brown). **(B)** α as a function of gelation time in hydrogels with a constant PGM concentration of 2% w/v and varying PEG-4SH concentration of 1% w/v (black), 2% w/v (red), 3% w/v (green), 4% w/v (blue), and 5% w/v (brown).

stages of gel assembly (**Fig. 4.4**), probes of this size were used to monitor changes in α every hour for 9 hours as the network assembled. PGM concentration was varied 1-5% w/v while keeping the PEG-4SH concentration constant at 2% w/v. The data in Fig. 5A shows that increasing PGM concentration leads to increases in the rate of gelation evident by measured α values. Next, PEG-4SH concentration was varied from 1-5% w/v, while keeping PGM concentration constant at 2% w/v. We also observed a concentration dependent change in gelation kinetics with a reduction in the time needed to form a gel as PEG-4SH concentration is increased (**Fig. 4.5B**).

These results are consistent with our earlier findings, showing the gelation process is dependent on both PGM and PEG-4SH to create the viscoelastic gel. From control experiments, we did not find evidence of gel formation in solutions of 1-5% PGM (in the absence of PEG-4SH) and 1-5% PEG-4SH (in the absence of PGM) after a 9-hr period. These data suggest variation in gelation kinetics is likely dependent on bond formation between mucins and PEG-4SH. As one would intuitively expect, increasing the concentration of either mucin or crosslinking polymers leads to a decrease in time to form the gel. Starting from either 1% PGM or 1% PEG-4SH, we appear to be below the threshold concentration required to form a gel within a 9-hour period. However, we note the 1% PGM does approach the gel point near hour 9. As PGM concentration increases, a steady decrease in gelation time going is observed from an initial gel point of ~9 hours for 2% w/v PGM, to ~5 hours for 3% w/v PGM and to ~3 hours for 5% w/v PGM (**Fig. 4.5A**). This can be simply explained as PGM concentration increases, the closer proximity between fibers makes PEG-4SH-mediated bridging more probable and as a result, gel assembly is more rapid. Interestingly, a more step-wise response in gelation time is

observed as a function of PEG-4SH concentration with ~8-9 hour for the lower 2% and 3% PEG-4SH concentrations and much faster ~2-3 hr gelation times for the higher 4% and 5% PEG-4SH concentrations (**Fig. 4.5B**). This might be explained as PEG-4SH attach to the mucin fibers at higher density, the rate of assembly is enhanced due to an increase in their multi-valent bridging capacity. We attribute the observed increase in gelation time at 4% compared to 5% to slight variation in start time between experiments and the relatively low resolution in gelation time.

Our results demonstrate how the mucin-based hydrogel may be tuned to assemble in a user-defined time frame. This presents the opportunity for our hydrogel system to be designed for various biomedical applications where slower or more rapid kinetics may be desirable. This system may also be used for fundamental studies on the role of kinetics in mucus gel formation and its biological function, which due to the lack of available models has not been previously investigated. While the results here motivate the continued optimization of the system, there are limitations in the present work which should be addressed in the future. First, we have yet to explore the effects of buffer conditions (e.g. ionic strength, pH) which can vary significantly between mucosal tissues and it will be important to evaluate this in our future work. Second, we have yet to determine if our synthetic system functionally behaves like mucus in the body. For example, we plan to investigate properties such as tissue adhesion, pathogen capture, and transportability of the gel in response to shear as these are all important aspects of mucus function.

4.4 Conclusion

A mucin-based synthetic hydrogel was designed and characterized using particle tracking microrheology. We determined that hydrogel assembly was mediated by hydrogen bonds and disulfide linkages between mucin and 4-arm PEG-thiol polymers. This provides the unique opportunity to control the degree of cross-linking within these gels which will directly influence their functional properties. Bulk rheology confirmed the formation of a solid-like viscoelastic material, with an elastic modulus resembling naturally produced mucus. During the assembly process, the evolution of microscale structure in the mucin gel network was apparent based on microrheological measurements using PEG-coated nanoparticle probes of different sizes. We also found gelation kinetics can be tailored by changing mucin or cross-linker concentrations. In future work, we hope to further investigate how changes in gelation kinetics may influence mucin fiber structure and network heterogeneity. The results in this work establish a simple means to produce mucin-based hydrogels which may be useful for biological and medical applications.

5. MODELING AIRWAY DYSFUNCTION IN ASTHMA USING SYNTHETIC MUCUS BIOMATERIALS[‡]

5.1 Introduction

Asthma affects ~300 million people worldwide of all racial and ethnic backgrounds with cases documented from childhood to adulthood (108). As lung disease worsens in asthma, chronic airway inflammation commonly leads to excess production of mucus (3, 4). Mucus is continuously cleared from the lung through high frequency, coordinated beating of ciliated cells on the surface of the airway epithelium (33, 56). This process, known as mucociliary clearance (MCC), provides the lung with an important defense mechanism against infection and injury with the continuous removal of inhaled environmental particles captured within the mucus layer (109). However, in all stages of asthma, MCC mechanisms become impaired, resulting in the accumulation of mucus in the airway (63). Persistent mucus accumulation not only leads to inflammation and infection by providing an environment for microbial growth, but also results in obstruction of conducting airways potentially causing asphyxiation in severe cases of asthma (7, 110). Further, many individuals with asthma suffer from recurrent pulmonary exacerbations characterized as an acute worsening of symptoms requiring emergency department visits and hospitalizations (111, 112). Exacerbations in asthma are often associated with infections by respiratory viruses such as rhinovirus, respiratory syncytial virus (RSV), and influenza (12, 13, 113). Accordingly, it is of importance to understand how the changes to

[‡] Reprinted (adapted) with permission from: Song D, Iverson E, Kaler L, Bader S, Scull MA, Duncan GA. “Modeling Airway Dysfunction in Asthma Using Synthetic Mucus Biomaterials”. *ACS Biomaterials Science & Engineering*. 7(6), 2723-33, 2021. Copyright 2021. American Chemical Society.

mucus produced in asthma leads to dysfunctional MCC and further examine its potential role in susceptibility to infection.

For effective MCC, the mucus gel must be a soft, shear-thinning, viscoelastic material. The primary building blocks of respiratory mucus are the gel-forming mucins, mucin 5B (MUC5B) and mucin 5AC (MUC5AC) (*114*). These large (~MDa) and heavily glycosylated proteins form the mucus gel network primarily through reversible associations (e.g. entanglements, hydrogen bonds) and disulfide crosslinks. The resulting mesh-like microstructure acts as a selectively permeable barrier that allows passage of certain substances while restricting diffusion of others (*114, 115*). The assembly of the mucin network gives rise to the viscoelastic properties of mucus, enabling unidirectional transport in response to shear forces exerted by the cilia on the airway surface. Although similar in general structure and amino acid sequence, MUC5B and MUC5AC differ in charge, which is a direct result of differences in their glycosylation pattern. MUC5B is heavily sialylated, whereas MUC5AC has a high fucose content (*4, 116*). These mucins also possess different amounts and arrangement of hydrophobic and cysteine-containing domains which can further influence hydrogel assembly (*114*). Recent evidence has also shown that MUC5B and MUC5AC are capable of forming polymer fibers with distinct morphologies (*4, 117*).

In individuals with asthma, biochemical analysis of mucus produced by cough has revealed mucin composition is altered as a function of disease severity, with a shift from MUC5B to MUC5AC as the predominant secreted mucin (*11*). Further, computerized tomography (CT) imaging has revealed an increased likelihood and number of mucus plugs within the airway in asthma patients with MUC5AC-enriched airway mucus (*9*). These

data suggest overproduction of MUC5AC may predispose the airways to mucus accumulation in asthma. MCC defects in asthma have been attributed to oxidation-induced crosslinking of mucins in the asthmatic lung and physical tethering of MUC5AC to the airway surface (10, 17, 26), both as a result of exaggerated inflammatory responses in asthma. However, it is not yet fully understood how an imbalance in the ratio of MUC5B to MUC5AC contributes to the biological function of mucus in asthma.

Studying airway mucus in health and disease has relied heavily on acquiring *ex vivo* human mucus samples through sputum induction or endotracheal tubes from patients who have undergone intubation (50, 51). However, the limited availability of clinical samples as well as the modest sample volume (~100 μ L) using these collection methods presents challenges in mechanistic studies on how altered mucus composition influences its function. In addition, samples acquired from humans can often possess altered mechanical properties due to sample dilution after sputum induction (35). These limitations have stimulated interest in synthetic mucus biomaterials that have similar physical properties to airway mucus. Recent studies have reported mucus biomaterials that use commercially available crude mucins, sourced from porcine stomach or bovine submaxillary gland for studying functional properties, such as rheology and transport (26, 73). In suspension, reconstituted mucins fail to establish covalent bonding and do not form mucin gel networks (65). Combining mucins with cross-linking agents, such as poly(acrylic) acid (PAA) and thiolated star polyethylene glycol (PEG) polymers re-establishes covalent crosslinking producing mucin-based biomaterials with *in vivo*-like biophysical properties (67, 68).

In our work, we report a synthetic mucus biomaterial capable of modeling transport and barrier function of airway mucus. Specifically, we have adapted an approach

established in our previous work (68) to create disulfide-linked mucin-based hydrogels composed of MUC5B and MUC5AC with viscoelastic properties comparable to airway mucus. This model system provides the capability to systematically vary the composition of mucus to capture how these changes impact its physiological function. Using this model, we examined the rheology of mucin-based hydrogels with varied and specified MUC5B:MUC5AC ratio to understand the impact of mucin composition on its biophysical properties. Transport of synthetic mucus on fully differentiated human airway epithelial (HAE) tissue cultures were then compared to natively secreted mucus in normal cultures and IL-13 exposed cultures to establish an asthma-like inflammatory condition. To understand the barrier function of synthetic mucus towards respiratory viruses, we directly measured the diffusion of fluorescent IAV within synthetic mucus gels and the extent of infection in synthetic mucus-coated human airway tissue cultures. The results of work establish a new biomaterial-based approach to create *in vitro* models of asthmatic airways and identify potential mechanisms of lung dysfunction.

5.2 Methods and Materials

5.2.1 Preparation of Synthetic Mucus

Synthetic mucus hydrogels were prepared using a previously established method (68). Briefly, porcine gastric mucin (PGM; Sigma-Aldrich) and mucin from bovine submaxillary glands (BSM; Sigma-Aldrich) are mixed with a cross-linking reagent, 4-arm PEG-thiol (PEG-4SH; Laysan Bio Inc.) to mediate cross-linking between mucin biopolymers. A solution of 4% (w/v) PGM and/or BSM is dissolved in a physiological buffer solution containing 154 mM NaCl, 3 mM CaCl₂, and 15 mM NaH₂PO₄ at pH 7.4 at

4 % (w/v) and stirred at room temperature for 2 hours. The cross-linking reagent, PEG-4SH, is initially prepared at 4 % (w/v) in the same buffer then mixed with equal volumes of mucin solutions to achieve a final concentration of 2 % (w/v) mucin and 2 % (w/v) cross-linking reagent. For experiments with varying ratios of MUC5B and MUC5AC, solutions of PGM and BSM were mixed at varying MUC5B:MUC5AC ratios (75:25, 50:50, and 25:75) prior to addition of the cross-linking reagent. The mixed solution of mucin and cross-linker were incubated at room temperature for ≥ 15 hours to allow gelation. For bulk rheology experiments, synthetic mucus hydrogels were prepared in petri dishes with 40 mm diameter with 2 mm thickness. For PTM experiments, hydrogels were prepared in custom microscopy chambers prior to gelation. For mucus transport and influenza viral challenge studies, hydrogels were prepared 24 hours prior to experiments and UV disinfected for 15 minutes before being transferred to HAE tissue cultures.

5.2.2 Collection of Human Mucus Sample

Human mucus samples were collected using the endotracheal tube (ETT) method in accordance with an IRB-approved protocol at the University of Maryland Medical Center (Protocol#: HP-00080047). ETT were collected from patients after intubation as a part of general anesthesia at UMMC. To collect the ETT-derived *ex vivo* samples, the last 10 cm of the tubes were cut, including the balloon cuff, and suspended in a 50-mL centrifuge tube using a syringe needle. The centrifuge tube containing the ETT was spun at 220 xg for 30 seconds for mucus collection, which resulted in 100-300 μ L of ETT mucus. Samples were stored at -80°C immediately after collection and thawed (no more than three times) prior to use for experiments.

5.2.3 Particle Tracking Microrheology in Human Mucus and Synthetic Mucus

Fluorescent carboxylate modified fluorescent polystyrene nanoparticles (PS-COOH; Life Technologies) with a diameter of 100 and 500 nm were coated with a high surface density of polyethylene glycol (PEG) via a carboxyl-amine linkage using 5-kDa methoxy PEG-amine (Creative PEGWorks) as previously reported (51, 68). Particle size and zeta potential was measured in 10 mM NaCl at pH 7 using a NanoBrook Omni (Brookhaven Instruments). We measured diameters of 122 nm and 491 nm and zeta potentials of -0.54 ± 1.16 and 1.09 ± 0.51 mV for 100 nm and 500 nm PEG-coated PS nanoparticles, respectively. The diffusion of the PEG-coated nanoparticles (PEG-NP) in human and synthetic mucus gels were measured using fluorescent video microscopy. Mucus samples were prepared in custom microscopy chambers that consisted of O-rings coated in vacuum grease on a microscope slide. Twenty μL of human mucus was added to the chamber along with 1 μL of $\sim 0.002\%$ w/v suspension of PEG-NP 30 minutes prior to PTM experiments. Synthetic mucus was prepared by adding 25 μL of mucin and cross-linker solution to the microscopy chamber. 1 μL of $\sim 0.002\%$ w/v suspension of PEG-NP was added to the mucin and cross-linker solution and sealed with a cover slip. Imaging was performed 24 hours later, after gelation. Fluorescent video microscopy videos were collected using a Zeiss 800 LSM microscope with a 63x water-immersion objective and an Axiocam 702 camera (Zeiss). High-speed videos were recorded at a frame rate of 33 Hz for 10 seconds at room temperature. For each sample, at least 3 high-speed videos were recorded. The particle tracking analysis was performed using a previously developed image processing algorithm. (79) Mean squared displacement (MSD) as a function of time lag (τ) was calculated as

$$\langle \Delta r^2(\tau) \rangle = \langle (x^2 + y^2) \rangle,$$

for each particle. Pore size of sample was calculated by

$$\xi = \sqrt{\text{MSD}_{1s}} + a,$$

where MSD_{1s} is the measured MSD at $\tau = 1$ second and a is the nanoparticle radius. For *N-acetyl cysteine* (NAC) experiments, NAC was added after gelation at a final concentration of 50 mM and incubated for 30 minutes prior to imaging.

5.2.4 Measurement of Bulk Rheology

Dynamic rheological measurements of the synthetic mucus gels were performed using the ARES G2 rheometer (TA Instruments) with a 20-mm diameter parallel plate geometry at 25°C. To determine the linear viscoelastic region of the fully formed gel, a strain sweep measurement was collected from 0.1-10% strain at a frequency of 1 rad s⁻¹. To determine the elastic modulus, $G'(\omega)$, and viscous modulus, $G''(\omega)$, a frequency sweep measurement was conducted within the linear viscoelastic region of the gel, at 1% strain amplitude and angular frequencies from 0.1 to 100 rad s⁻¹.

5.2.5 Culture of BCI-NS1.1 cells and Preparation for Immunostaining

The h-TERT immortalized human airway basal BCI-NS1.1 cell line was generously provided by Dr. Ronald Crystal which their group established in previous work (76). Cells were initially seeded on plastic at ~3000 cells/cm² in PneumaCult-Ex Plus media (StemCell) and incubated at 37°C, 5% CO₂. Once cells reached 70-80% confluency, they were dissociated using 0.05% trypsin ethylenediaminetetraacetic acid (EDTA) for 5 minutes at 37°C. For differentiation at air-liquid interface (ALI), cells were seeded on 12 mm diameter transwell inserts (Corning Costar) coated with 50 µg/mL collagen from human placenta (Sigma Aldrich) at ~10,000 cells/cm². Expansion media, PneumaCult-Ex

Plus, was used to feed cells in both the apical and basolateral compartments until 100% confluency. After reaching confluency, the apical media was removed, introducing ALI, and the basolateral media was replaced with PneumaCult-ALI (StemCell). All cells were grown for 28 days to reach differentiation with media exchanged every other day. Collection method of BCI-NS1.1 mucus was adapted from a previously described protocol (36, 118). BCI-NS1.1 mucus was allowed to accumulate for 7 days. *In vitro* mucus samples were harvested by washing apical compartments with PBS for 30 minutes at 37°C. After 30 minutes incubation, the solution of mucus and PBS in the apical compartment was collected. Samples were loaded into Amicon Ultra 100 kDa filters (Millipore-Sigma) and centrifuged at 14,000 *xg* for 30 minutes to remove excess PBS. After filtration, protein concentration was measured using a BCA assay and samples were reconstituted into physiological concentrations (~2 mg/mL)(119) using physiological buffer used to prepare mucin hydrogels. To establish an asthma-like human tissue culture model, BCI-NS1.1 cultures were maintained in medium with IL-13 (100 ng/mL, Peprotech, Rocky Hill, NJ) for 7 days prior to *en face* staining and MCC experiments.

For *en face* immunohistochemical staining and fluorescence imaging of MUC5B and MUC5AC, cultures were prepared by aspirating culture medium from the basolateral compartment and adding Carnoy solution (6:3:1 ratio of 100% ethanol, chloroform, and glacial acetic acid), a non-aqueous fixative that preserves the secreted mucus layer, to the basolateral (1 mL) and apical (0.5 mL) compartments (17). After 30 minutes of fixation at room temperature, inserts were washed twice with absolute methanol, twice with absolute ethanol, and twice with PBS. For blocking non-specific background in all imaging experiments, inserts were incubated in 3% bovine serum albumin (BSA) in PBS, which

had been filter sterilized, for 1 hour at room temperature. The primary antibodies that were used were mouse monoclonal anti-MU5AC (Santa Cruz, sc-21701, 1:100) and mouse monoclonal anti-MUC5B (Santa Cruz, sc-21768, 1:100). Primary antibody incubation was over night at 4°C in 1% BSA in PBS. After overnight incubation, inserts were washed in PBS twice and incubated in secondary antibody staining solution for 1 hour at room temperature. The secondary antibody used was Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Thermo Fisher Scientific, A-21202, RRID AB_141607). Inserts were washed with PBS twice and imaged at 10x magnification using a Zeiss Axio Observer and Axiocam 503 mono camera (Zeiss). For the non-primary control, Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (Thermo Fisher Scientific, catalog # A-31573, RRID AB_2536183) was used and background intensity was subtracted from anti-mucin images. MUC5B and MUC5AC staining was performed in separate BCI-NS1.1 cultures as same species for secondary antibody was used. Relative fluorescence intensities of MUC5B and MUC5AC in unstimulated and IL-13-stimulated cultures were quantified by normalizing the average fluorescence intensity of mucin staining to the percentage of fluorescence positive area. Quantification of mucin staining intensity and fluorescence positive area was performed in FIJI using the automated thresholding function.

5.2.6 Measurement of Mucus Transport and Cilia Beat Frequency

Mucociliary transport was measured based on the transport of 2 µm red-fluorescent polystyrene microspheres (Sigma-Aldrich). For measurement of transport rate of native BCI-NS1.1 mucus, a 4 uL suspension of the fluorescent polystyrene microspheres (1:1000 dilution in PBS) was added on top of the native mucus, which was allowed to accumulate

for 7 days. After 30 minutes of equilibration at 37°C, videos of three regions were recorded at 10x magnification using a Zeiss 800 LSM microscope. Images were collected at a frame rate of 0.5 Hz for 10 seconds on the plane of the mucus gel. Images were acquired centrally within cultures and away from the edges, where mucus tends to accumulate. For measurement of transport of synthetic mucus, BCI-NS1.1 native mucus was first removed from the cultures by washing the apical surfaces of cultures with PBS for 30 minutes. Forty μ L of the hydrogel was added to the apical surface ($\sim$ 40 μ m thickness) of fully differentiated BCI-NS1.1 cells and equilibrated for 30 minutes at 37°C. A suspension of 4 μ L of 2 μ m red-fluorescent polystyrene microspheres was added and allowed to equilibrate for an additional 30 minutes. For NAC experiments, fluorescent polystyrene microspheres were diluted in PBS containing 50 mM NAC in order to prevent further dilution of hydrogels. After 30 minutes of incubation, videos of at least three regions were recorded as described for the transport rates of BCI-NS1.1 native mucus. The microsphere tracking data analysis is based on an image processing algorithm that was custom written in MATLAB (The MathWorks). Briefly, the analysis software computes the xy-plane trajectories of each fluorescent microsphere in each frame. Using the trajectory data, displacement of microspheres was computed, and transport rate was calculated by dividing the displacement of microsphere by total time elapsed. In order to measure ciliary beat frequency (CBF), 10 second videos at a frame rate of 20 Hz were recorded at 10x magnification in ≥ 3 randomly selected regions of BCI-NS1.1 cultures using brightfield. Using a custom written algorithm in MATLAB, the number of local pixel intensity maxima were counted, which indicates beating of cilia. Beat frequency was determined by dividing the number of beats over the total elapsed time.

5.2.7 Measurement of Disulfide Bond Concentration in Synthetic Mucus

Concentration of disulfide bonds were measured using a previously described fluorometric assay (26). Synthetic mucus hydrogels were resuspended in 8 M guanidine-HCl (Sigma) to 10 times the original hydrogel volume. In order to block free cysteines, samples were treated with 10% (v/v) of 500 mM iodoacetamide for 1 hour at room temperature. To quench excess iodoacetamide and reduce existing disulfide bonds, samples were treated with 10% (v/v) of 1 M DTT at 37°C. Samples were loaded into Amicon Ultra 10 kDa filters (Millipore-Sigma) and centrifuged at 14,000 $\times g$ for 20 minutes for removal of excess DTT and quenched iodoacetamide. Recovered samples were resuspended in 50 mM tris-HCl (pH 8.0) and mixed with equal volumes of 2 mM monobromobimane (mBBr; Sigma). After 15 minutes of incubation in mBBr at room temperature, fluorescence was measured at 395-nm excitation/490-nm emission on a Spark Multimode Microplate reader (Tecan). Serial dilutions of 5 mM L-cysteine in 50 mM tris-HCl were used as standards. Disulfide bond concentration was calculated against the standard curve.

5.2.8 Preparation of IAV and IAV Challenge Studies

The plasmid-based reverse genetics system for influenza A virus (IAV) A/Puerto Rico/8/34 (PR8; H1N1) was a gift from Dr. Adolfo Garcia-Sastre. Infectious virus was generated from cloned cDNAs in 293T and Madin-Darby canine kidney (MDCK) cell co-cultures and purified by ultracentrifugation through a 20% sucrose onto a 50% sucrose cushion as previously described (72). The infectivity of resulting virus stocks was quantified by standard plaque assay on MDCK cells (120), yielding a viral titer of 3.9×10^9 plaque-forming units (pfu)/mL. IAV was subsequently labeled with a lipophilic dye, 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (DiI; Invitrogen). The

labeled virions were then purified and concentrated via haemadsorption to chicken red blood cells (120), IAV stock was aliquoted and stored at -80°C. This strain exhibits a primarily spherical morphology and diameter of roughly 100 nm. DiI-labeled virus stocks were counter-stained with an anti-H1N1 IAV HA antibody (NR-3148; antiserum, goat; BEI Resources, NIAID, NIH). Diffusion of IAV was assayed similarly to PTM studies. Mucin hydrogels were prepared in microscopy chambers and 1 µL of DiI -labeled IAV labeled was applied to the surface of the hydrogel and equilibrated for 30 minutes before imaging. The same tracking analysis employed in PTM experiments was used to compute the MSD of IAV. For experiments with IAV infection, BCI-NS1.1 cultures were initially washed with PBS to remove native mucus. Forty µL of hydrogels with 75:25 and 25:75 MUC5B:MUC5AC ratio were added to the apical surface and allowed to equilibrate for 30 minutes. IAV (PR8) was then applied (4 µL; 7500 plaque-forming units (pfu)) centrally to HAE cultures and incubated for 2 hours at 37°C. After inoculation, cultures were rinsed with PBS prior to a 10 minutes wash at 37°C to remove hydrogel and non-absorbed virus. Cultures were imaged 48 hours after IAV and hydrogel were removed. To image IAV infection, cultures were fixed in 1:1 methanol:acetone solution overnight at 4°C. Afterwards, cultures were washed in PBS and then permeabilized in 2.5% Triton X-100 for 15 minutes. After blocking, the primary antibody that was used was mouse monoclonal anti-IAV nucleoprotein A1 and A3 blend (EMD Millipore, MAB8251, 1:250). Images were taken at 10x magnification using a Zeiss Axio Observer and AxioCam 503 mono camera (Zeiss). Percentages of infection area for uncoated and synthetic mucus coated cultures were determined by using the automated thresholding function in FIJI.

5.2.9 Statistical Analysis

All graphing and statistical analyses were performed using GraphPad Prism 8 (GraphPad Software). Two-group comparisons were performed using 2-tailed Student's *t*-test (normally distributed data) or Mann-Whitney *U* test. For comparisons between groups, one-way analysis of variance (ANOVA) followed by a Tukey *post hoc* correction was performed. Kruskal-Wallis with Dunn's correction was used for comparison of multiple groups with non-Gaussian distributions. Bar graphs show mean and standard deviation and box and whiskers plots show median value and 5th percentile up to 95th percentile of the data and outliers were not included. Differences were considered statistically different at the level of $p < 0.05$.

5.3 Results

5.3.1 Engineering Synthetic Mucus Biomaterials with Properties like Mucus

In this study, we aimed to create a synthetic mucus biomaterial with similar rheological and transport properties to human respiratory mucus. In our previous work, we reported the design of a hydrogel system that employs a thiol-based cross-linking strategy and verified that hydrogel assembly was mediated by hydrogen and disulfide bonding (68). Using this approach, we prepared hydrogels with 2% (w/v) mucin and 2% (w/v) cross-linking reagent, PEG-4SH, using two types of mucins: porcine gastric mucin (PGM), containing MUC5AC (90, 121), and bovine submaxillary gland mucin (BSM), containing MUC5B (Fig. 5.1A,B) (122, 123). Given the limited volume (~50-100 μ L) collected *ex vivo* from patients and generated *in vitro*, we chose to use particle tracking microrheology (PTM) to characterize the samples collected to compare to our synthetic model. Our results

showed that MUC5B hydrogels had similar microrheology and pore network size to HAE-derived mucus, in range of previously reported values (~200-300 nm) (50, 51). Therefore, we used PTM to compare the physical properties of our synthetic mucus biomaterial to natural human airway mucus collected *ex vivo* from endotracheal tubes (ETT) and *in vitro* collected from BCI-NS1.1 HAE cultures. Based on 100 nm PEG-NP diffusion, as measured by logarithm based 10 of mean squared displacement at time lag 1 second ($\log_{10}\text{MSD}_{1s}$), we find that *ex vivo* ETT mucus had similar microstructural properties to *in vitro* mucus samples derived from BCI-NS1.1 cultures. Comparing our synthetic mucus biomaterials to human samples, we found that MUC5B (BSM) hydrogels had comparable microstructural properties to human mucus samples collected *in vitro* (**Fig. 5.1C**). However, MUC5B hydrogels showed significantly different microstructural properties compared to ETT-sourced mucus (**Fig. 5.1C**) as indicated by a reduction in 100 nm PEG-NP diffusion. The MUC5AC (PGM) gels had significantly reduced 100 nm PEG-NP diffusion compared to human mucus (*ex vivo* and *in vitro*) and MUC5B gels, indicative of a significantly smaller network pore size (**Fig. 5.1C**). Using PTM analysis, we estimated gel network pore sizes (ξ) in synthetic and human mucus. Similar to particle diffusion, MUC5B hydrogels showed similarities in pore size to the *in vitro* mucus samples ($\xi \approx 200\text{-}400$ nm), whereas MUC5AC gels had significantly reduced network pore sizes ($\xi \approx 100$ nm) (**Fig. 5.1D**). We also observed varying distributions in nanoparticle diffusion and pore size in different samples, where MUC5AC hydrogels showed a smaller MSD and pore size distribution compared to MUC5B hydrogels and human mucus samples. Based on the size of nanoparticles used (100 nm) for microrheological analysis, we are only able to detect network sizes ≥ 100 nm. Thus, MUC5AC hydrogels likely possess pore sizes of 100 nm or less.

Next, we measured and compared the transport rate of synthetic mucus and native mucus produced from BCI-NS1.1 HAE cultures. Although MUC5B hydrogels had similar microrheological properties to BCI-NS1.1 mucus, MUC5B gels showed significantly lower transport rates compared to native BCI-NS1.1 mucus. Alternatively, the MUC5AC hydrogel, which showed significantly reduced pore size, had significantly higher transport rates than MUC5B gels. Given native mucus contains both MUC5B and MUC5AC, we prepared a hydrogel composed of a blend of both mucins with relative concentrations

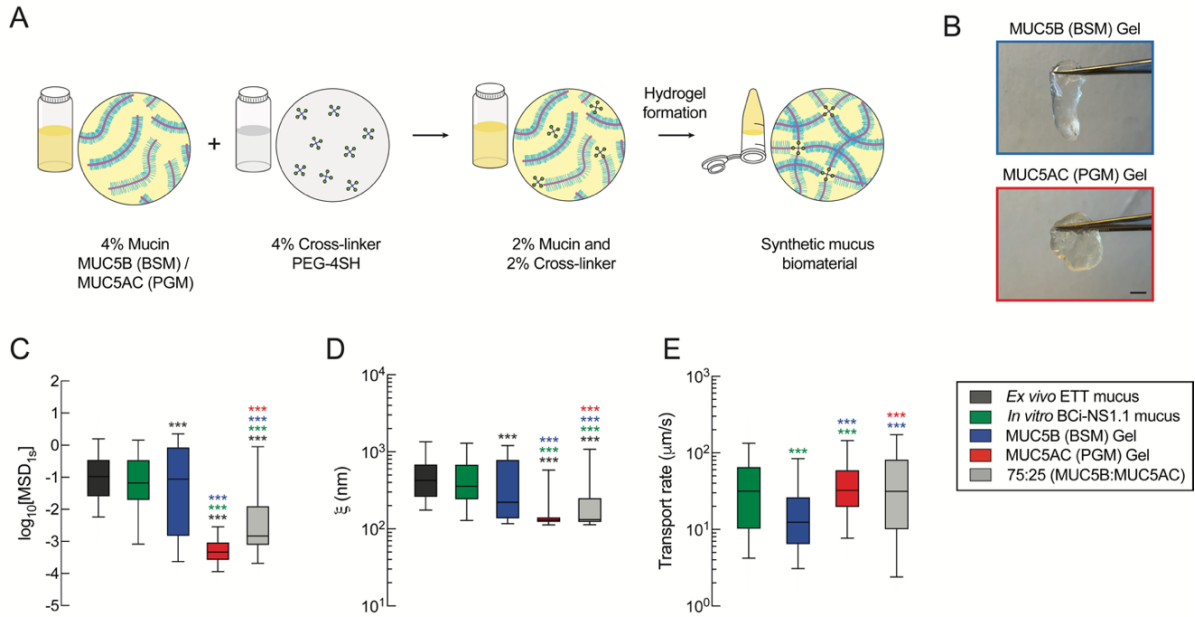


Figure 5.1 Comparison of the biophysical and transport properties of native human mucus to synthetic mucus biomaterials. (A) Schematic illustration of the preparation steps for the synthetic mucus biomaterial. 4% (w/v) mucin solution is mixed with 4% (w/v) cross-linker to form a mucin-based hydrogel with 2% (w/v) mucin and 2% (w/v) cross-linker. (B) Images of MUC5B (BSM) hydrogel (blue) and MUC5AC (PGM) hydrogels (red). Scale bar = 2 mm. (C) Box-and-whisker plots of measured $\log_{10}[\text{MSD}_{1s}]$ for 100 nm PEG-NP in human mucus samples (*ex vivo* and *in vitro*) and synthetic mucus biomaterials with varying mucin composition. (D) Box-and-whisker plots of estimated pore size (ζ) of human mucus samples and synthetic mucus biomaterials calculated based on 100 nm PEG-NP diffusion. (E) Box-and-whisker plots of microsphere transport rate of BCI-NS1.1 native mucus and synthetic mucus biomaterials on BCI-NS1.1 cultures. *** $p < 0.001$ by Kruskal-Wallis test with Dunn's correction. Color of asterisk indicates the comparison group.

representative of healthy airway mucus. Previous studies have shown that normal (non-diseased) human mucus is primarily composed of MUC5B (75-85%) and smaller amount of MUC5AC (~15-25%).(11) To test whether a mixed ratio of mucins, specifically 75% MUC5B (BSM): 25% MUC5AC (PGM), could mimic airway mucus, we measured microrheological properties (**Fig. 5.1C,D**) and transport rates (**Fig. 5.1E**) of blended mucin hydrogels to compare to *ex vivo* and *in vitro* human mucus. Interestingly, we found the blended gels had network sizes smaller than human samples based on PTM but had the most similar transport rate to the native mucus gels produced in BCI-NS1.1 HAE cultures.

5.3.2 Macro- and Microrheology of Synthetic Mucus Biomaterials

Previous studies have demonstrated the relative increase in the abundance of MUC5AC compared to MUC5B in asthmatic mucus from adults and children where the percentage of MUC5AC reaches up to ~70% of the total mucin content (11, 64). Therefore, we sought to determine the effects of mucin ratio, MUC5B:MUC5AC, on macro- and microrheological properties of synthetic mucus biomaterials. We systematically varied the MUC5B:MUC5AC ratio starting hydrogels composed of: (i) 75% MUC5B and 25% MUC5AC, representative of health, (ii) 50% MUC5B and 50% MUC5AC representative of stable asthma and (iii) 25% MUC5B and 75% MUC5AC, representative of exacerbated

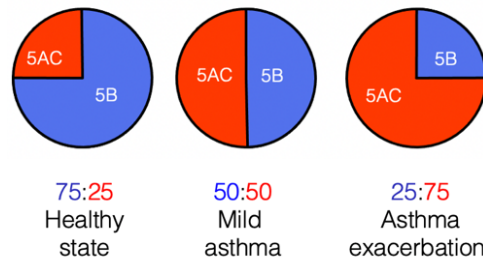


Figure 5.2 MUC5B:MUC5AC ratios studied. 75% MUC5B and 25% MUC5AC, representative of health, 50% MUC5B and 50% MUC5AC representative of stable asthma, and 25% MUC5B and 75% MUC5AC, representative of exacerbated asthma.

asthma (11) (**Fig 5.2**). For simplicity, we will refer to 75:25 MUC5B:MUC5AC ratio as “MUC5B-rich” hydrogels and 25:75 MUC5B:MUC5AC ratio as “MUC5AC-rich” hydrogels. We then compared the elastic moduli of mucin-based hydrogels at $\omega = 1 \text{ rad s}^{-1}$ which revealed higher elastic and viscous moduli for hydrogels with higher MUC5AC content (**Fig. 5.3A,B**). PTM analysis showed that a relative increase in MUC5AC content resulted in reduced particle diffusion, as measured by $\log_{10}[\text{MSD}_{1s}]$ of 100 nm PEG-NP, indicative of tightening of mesh network of the hydrogels with increased MUC5AC content (**Fig. 5.3C**). We also assessed diffusion of 500 nm PEG-NP, as PEG-NP with diameter greater than the pore size can probe physical properties closer to bulk scale.(49) The

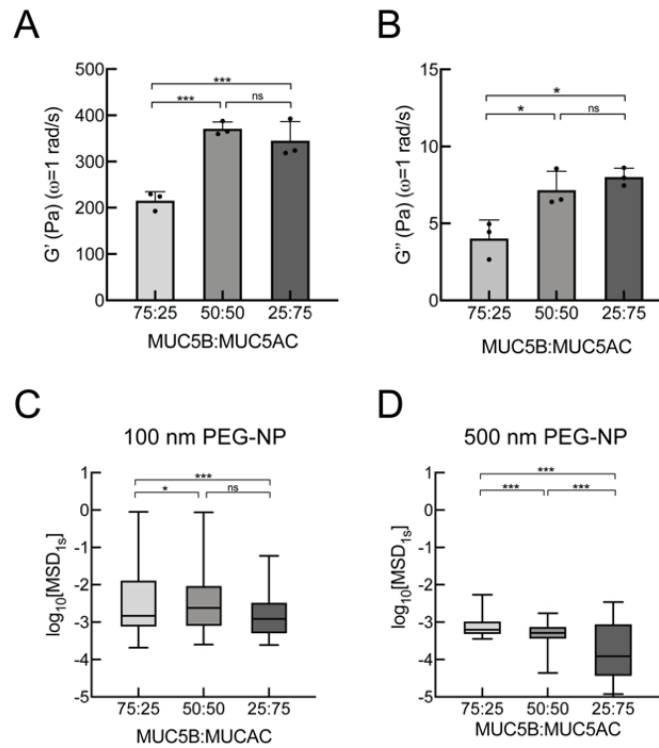


Figure 5.3 Macro- and microrheology of synthetic mucus with varied concentrations of MUC5B and MUC5AC. (A) Mean elastic and (B) viscous modulus (G' , G'') of hydrogels with varying MUC5B:MUC5AC ratio at $\omega = 1 \text{ rad/s}$ measured using bulk rheology. (C) Box-and-whisker plots of measured $\log_{10}[\text{MSD}_{1s}]$ for 100 nm PEG-NP in hydrogels with varying MUC5B:MUC5AC ratio. (D) Box-and-whisker plots of measured $\log_{10}[\text{MSD}_{1s}]$ for 500 nm PEG-NP in hydrogels with varying MUC5B:MUC5AC ratio. *** $p < 0.001$, * $p < 0.05$ by (A,B) one-way ANOVA and (C,D) by Kruskal-Wallis test with Dunn's correction.

diffusion of 500 nm PEG-NP showed a systematic reduction in $\log_{10}\text{MSD}_{1s}$ with increasing MUC5AC content, suggesting increased viscoelastic properties with increasing MUC5AC concentration (**Fig. 5.3D**).

5.3.3 Measurement of Transport Rates of Synthetic Mucus Biomaterials

Previous studies have shown that stimulation of primary HAE cultures with IL-13, a key mediator in asthma, results in increased MUC5AC production and markedly reduced mucus transport rates.^(17, 124) In order to establish and confirm asthmatic phenotype in our culture system, we stimulated BCI-NS1.1 HAE tissue cultures with IL-13 and performed *en face* staining to examine MUC5B and MUC5AC expression (**Fig. 5.4A**). IL-13 stimulation in BCI-NS1.1 cultures consistently increased MUC5AC expression, whereas no significant changes were observed for MUC5B expression. We observed a significant reduction in transport rates of 2 μm microspheres in IL-13 stimulated cultures (**Fig. 5.4B,C**). We also measured the cilia beat frequencies (CBF) of BCI-NS1.1 cultures to confirm that the reduction of transport rate was not due to changes in ciliary activity. We also confirmed that CBF was unaffected after IL-13 treatment (**Fig. 5.4D**). We next sought to test whether mucin composition of synthetic mucus affects mucociliary transport rates by applying hydrogels with varying MUC5B:MUC5AC ratios on apically-washed, mucus-free BCI-NS1.1 cultures. Based on microsphere trajectories, we found the transport rate of MUC5AC-rich gels were significantly reduced in comparison to gels composed of equal MUC5B/MUC5AC concentrations or MUC5B-rich gels (**Fig. 5.4B,E**). CBF was unaffected by the introduction of synthetic mucus with varied MUC5B:MUC5AC ratio (**Fig. 5.4F**).

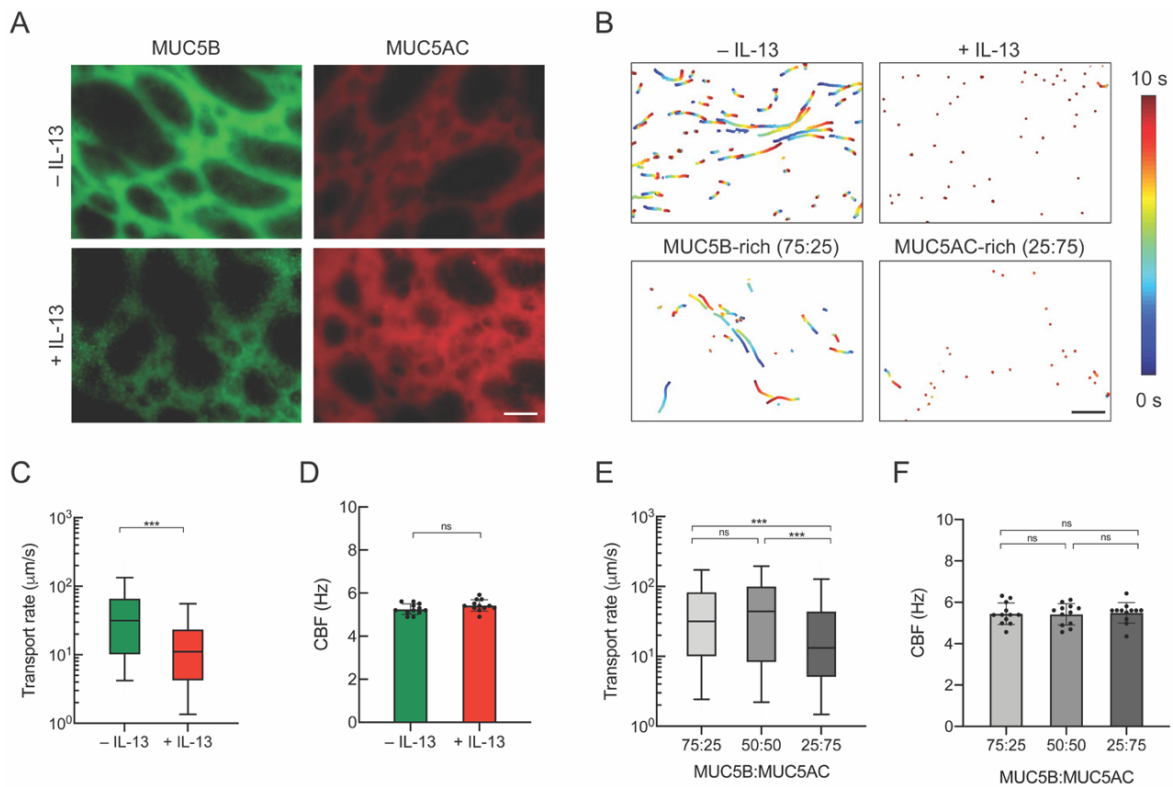


Figure 5.4 Mucociliary transport in human airway epithelial (HAE) cultures for native mucus in IL-13 stimulated HAE and synthetic mucus with varied MUC5B:MUC5AC ratio. (A) *En face* immunostaining of mucus gels produced from unstimulated (–IL-13) or IL-13-stimulated (+IL-13) BCI-NS1.1 HAE cultures. Scale bar = 20 μm. (B) Paths of fluorescent microspheres deposited onto gels from –IL-13 and +IL-13 BCI-NS1.1 cultures and synthetic mucus gels (shown conditions are MUC5B-rich (75:25) and MUC5AC-rich (25:75) gels). Trajectories show 10 seconds of motion with color scale that indicates time elapsed. Scale bar = 100 μm. (C) Box-and-whisker plots of microsphere transport rate on –IL-13 and +IL-13 BCI-NS1.1 cultures. *** $p < 0.001$ by Mann-Whitney U test. (D) Mean ciliary beat frequency of –IL-13 and +IL-13 BCI-NS1.1 cultures. (E) Box-and-whisker plots of microsphere transport rate on BCI-NS1.1 cultures with hydrogels with varying MUC5B:MUC5AC ratio. *** $p < 0.001$ by Kruskal-Wallis test with Dunn’s correction. (F) Mean ciliary beat frequency BCI-NS1.1 cultures with hydrogels with varying MUC5B:MUC5AC ratio.

5.3.4 Impact of Disulfide Bonds on the Transport of Synthetic Mucus Biomaterials

While a constant PEG-4SH concentration was used throughout our work, we hypothesized enhanced viscoelasticity of MUC5AC-rich synthetic mucus may be a result of the higher number of available cysteines on MUC5AC noted in a previous study (31).

Therefore, we measured the disulfide bond concentration in hydrogels that showed significant differences in macro- and microrheological properties as well as transport rates for MUC5B-rich and MUC5AC-rich gels (**Fig. 5.5A**). We found that the disulfide bond (S-S) concentration in MUC5AC-rich hydrogels was markedly higher than in MUC5B-rich hydrogels. As enhanced elasticity resulting from increased disulfide crosslinking is considered a major factor in impairing mucus clearance (26), we tested the effectiveness of N-acetylcysteine (NAC), a reducing agent that is used clinically as a mucolytic agent, on restoring transport rates of MUC5AC-rich gels on BCI-NS1.1 cultures. We observed that treatment with 50 mM NAC on MUC5AC-rich hydrogels significantly increased transport rates (**Fig. 5.5B**), whereas CBF remained unchanged (**Fig. 5.5C**). While MCC was improved with NAC treatment for the asthma-like MUC5AC-rich gels, transport rates were not restored to transport values of hydrogels composed of MUC5B-rich gels, representative of health (**Fig. 5.5B**).

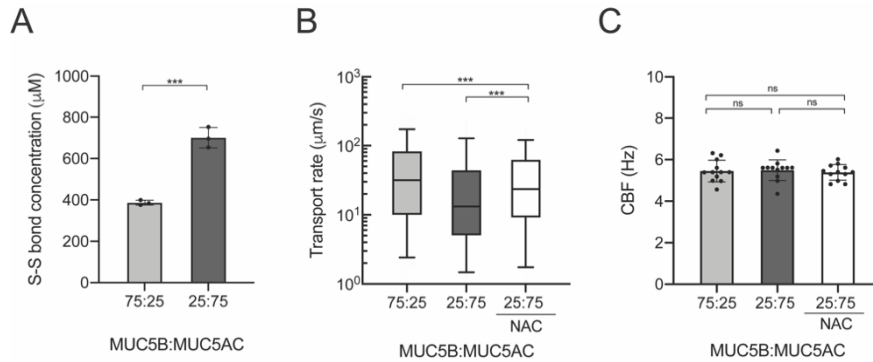


Figure 5.5 Influence of disulfide crosslinking on asthma-like synthetic mucus transport function. (A) Mean disulfide bond (S-S) concentration in MUC5B-rich (75:25) and MUC5AC-rich (25:75) gels. *** $p < 0.001$ by unpaired student *t*-test. (B) Box-and-whisker plots of microsphere transport rates on BCI-NS1.1 HAE cultures with MUC5B-rich (75:25) and MUC5AC-rich (25:75) hydrogels and MUC5AC-rich hydrogel (25:75) with NAC treatment. *** $p < 0.001$ by Kruskal-Wallis test with Dunn's correction. (C) Ciliary beat frequency of BCI-NS1.1 HAE cultures.

5.3.5 Barrier Properties of Synthetic Mucus Biomaterials against Influenza A

Viral Infection

Previous studies have shown that individuals with asthma are more susceptible to viral infections (125) which can often cause an exacerbation of disease symptoms (113). To determine if mucin composition could influence the barrier function of synthetic mucus towards influenza A virus (IAV), we measured the diffusion rate of fluorescently labeled IAV in synthetic mucus with different MUC5B:MUC5AC ratios using PTM. We found IAV had a higher average diffusion rate in MUC5AC-rich synthetic mucus compared to MUC5B-rich gels (**Fig. 5.6A,B**). However, the frequency distribution of $\log_{10}[\text{MSD}_{1s}]$ of individual IAV showed that only a small fraction of IAV could rapidly penetrate MUC5B-rich (~7%) and MUC5AC-rich gels (~9%) (**Fig. 5.6C**). We further tested if synthetic mucus with variable mucin content were more or less effective at blocking IAV infection in BCI-NS1.1 HAE cultures. In these studies, BCI-NS1.1 cultures were coated with MUC5B-rich (75:25) and MUC5AC-rich (25:75) synthetic mucus and challenged with 7500 pfu of IAV (multiplicity of infection ~ 0.15). IAV were introduced in minimal volume (4 μL) in order to avoid diluting mucus and the inoculum were kept on the cultures for 2 hours. Two days post infection, staining for IAV nucleoprotein revealed a significant reduction in successful infection by IAV when coated with MUC5B-rich synthetic mucus whereas MUC5AC-rich, asthma-like mucus had a similar amount of staining for IAV nucleoprotein to uncoated controls (**Fig. 5.6D,E**).

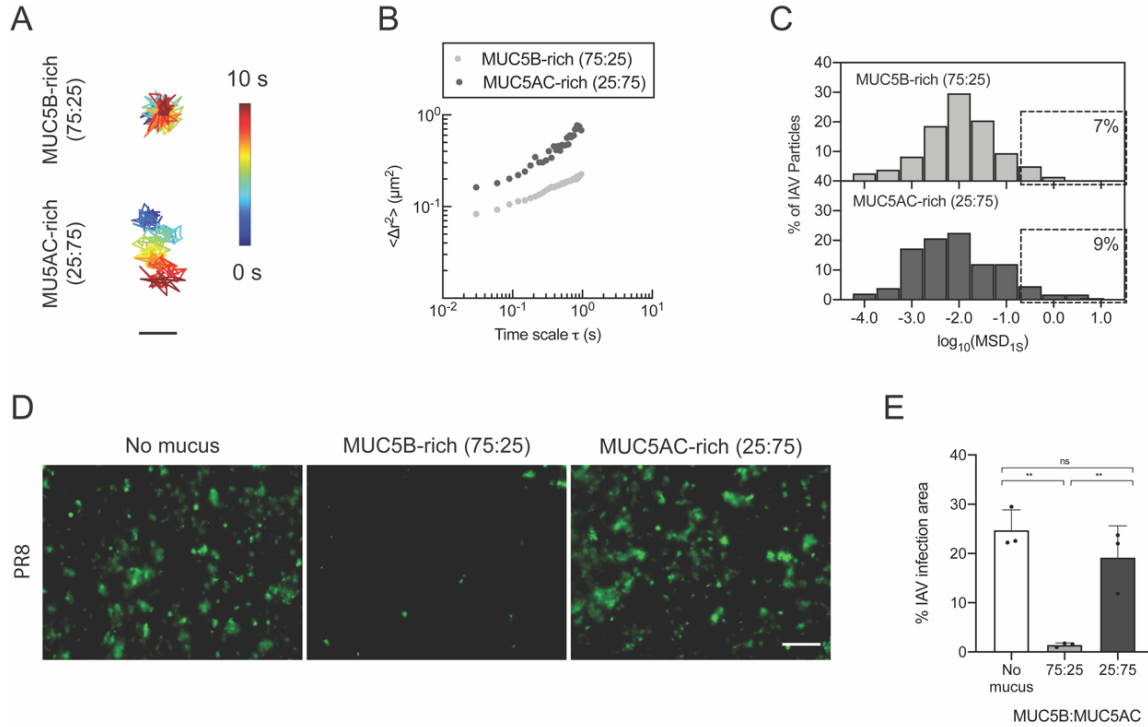


Figure 5.6 Barrier function of normal and asthma-like synthetic mucus towards influenza A virus (IAV). (A) Trajectories of IAV diffusion in MUC5B-rich (75:25) and MUC5AC-rich (25:75) gels. Trajectories show 10 seconds of motion with color scale that indicates time elapsed. Scale bar = 200 nm. (B) Ensemble average MSD ($\langle \Delta r^2 \rangle$) of DiI labeled IAV as a function of time scale (τ) in MUC5B-rich (75:25) and MUC5AC-rich (25:75) gels. (C) Frequency distribution of $\log_{10}[\text{MSD}_{1s}]$ of individual IAV in MUC5B-rich (75:25) and MUC5AC-rich gels (25:75). (D) Fluorescent micrographs of uncoated and synthetic mucus coated BCI-NS1.1 cultures infected apically with IAV (PR8) 48hpi after 2-hour inoculation. Green indicates staining for IAV nucleoprotein. Scale bar = 20 μm . (E) Percentage of infection area as determined by IAV nucleoprotein staining for uncoated and synthetic mucus coated BCI-NS1.1 cultures.

5.4 Discussion

In order to create synthetic mucus biomaterials with native-like function, we formulated mucin hydrogels using BSM, composed of MUC5B, and PGM, composed of MUC5AC, combined with a star PEG-thiol cross-linking agent to establish rheological properties similar to human airway mucus. We directly compared our synthetic mucus model to human mucus collected (i) from individuals without lung disease and (ii) from well-differentiated HAE tissue cultures. Although MUC5B gels were most similar to HAE-

derived mucus, we observed significant differences between MUC5B gels and ETT-sourced mucus. We expect these differences to arise due to heterogeneity in microrheological properties in clinical samples of mucus as these samples possess high amounts of non-mucin biomolecules that can contribute to gel organization and microstructure (35). MUC5AC hydrogels, on the other hand, had significantly smaller pore sizes (~100 nm) compared to ‘normal’ *ex vivo* and *in vitro* human mucus that were comparable to previously reported properties of mucus in diseases, such as in CF (10, 50). To account for the potential effect mucin concentration and MW on the observed differences in hydrogel elasticity, we measured total protein content as well as hydrodynamic radii of mucin solutions. We found no significant differences between protein concentration and hydrodynamic size of MUC5B (BSM) and MUC5AC (PGM) solutions.

Due to the similarities in microrheological properties between MUC5B gels and HAE-derived mucus, we expected comparable transport rates on HAE cultures. Interestingly, transport rates of MUC5B gels were significantly lower compared to native HAE mucus, whereas MUC5AC gels exhibited significantly faster transport rates compared to MUC5B hydrogels, despite differences in microrheological properties. Our results would suggest MUC5B and MUC5AC gel transport could be influenced by factors other than network elasticity, such as friction or surface adhesion (47, 91). Of note, frictional coupling of the mucus gel to beating cilia can also contribute to its transport (126). One would expect more densely crosslinked gels will possess a higher degree of friction due to reduced water permeation at the gel-tissue interface. This may, in part, explain why the more highly crosslinked MUC5AC gels achieve faster transport rates than

MUC5B gels due to an enhanced coefficient of friction. However, additional studies are necessary to explore these potential effects on mucus transport. Overall, neither MUC5AC or MUC5B hydrogels alone resembled both the biophysical and transport functions of native human mucus.

As native airway mucus is composed of both MUC5B and MUC5AC, we prepared hydrogels with both mucins at a physiological MUC5B:MUC5AC ratio of 75:25 (MUC5B-rich). Our results showed that blending of the mucins resulted in mucus clearance rates that were comparable to the transport of BCI-NS1.1 HAE mucus and also in range of physiological values reported in literature ($\sim 50 \mu\text{m/s}$) (47). We should note the bulk elastic moduli measured here, on the order of 200-400 Pa, are higher than previously reported moduli for human airway mucus (~ 10 -50 Pa) (10, 35, 36). With the similarities in transport rates of synthetic and native human mucus on airway surfaces, we expect synthetic mucus is partially diluted and as a result, its elasticity is reduced when equilibrated for 30 minutes on HAE tissue culture prior to experiments. Another limitation in our synthetic model is our use of non-human mucins which possess species-specific differences in glycosylation. For example, BSM possesses similar terminal sialic acid groups to human MUC5B, but also contains a small percentage of sialic acid glycoforms, such as N-glycolylneuraminic acid, not found in human tissues (127). In addition, these mucins are only partially purified and contain other non-mucin components (e.g. albumin, immunoglobulin). However, mucin is the predominant polymeric component of our gels and as such, likely dictates the mechanical and transport properties of these gels. In the airways, MUC5B and MUC5AC are known to be secreted in distinct cell types and reside in distinct regions of the epithelial surface (128). As mucins are mixed homogeneously prior to gelation and application to

HAE cells, it is unlikely our present model fully captures these effects. However, we have demonstrated that our synthetic mucus biomaterial exhibits transport rates in range of physiological values in health and disease and thus provides a model system complementary to the *in vitro*, *ex vivo*, *in vivo* models of mucociliary transport traditionally used.

Our results suggest, independent of concentration, increases in MUC5AC, as observed in sputum samples from patients with asthma, could be sufficient to enhance the bulk viscoelastic properties of mucus. In the past, these changes in viscoelastic properties in asthma have been attributed to increased mucin concentration and excess plasma protein that may interfere with normal degradation and turnover of mucins in the airways (10). While we presume transport in IL-13 stimulated HAE will also be influenced by MUC5AC tethering to HAE as previously shown (17), our data on synthetic mucus transport suggests increasing MUC5AC in mucus gels may progressively slow MCC. We also found the enhanced elasticity and reduced transport of MUC5AC-rich gels is likely due to increased disulfide cross-linking mediated by PEG-4SH. Given the importance of disulfide bonds in mucus gel elasticity, reducing agents such as N-acetylcysteine (NAC) have been used as an inhaled treatment to breakdown hyper-viscous mucus in chronic lung diseases like cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) (70, 129). Using NAC, we also found reducing the disulfide bonds can significantly improve transport of asthma-like synthetic mucus with increased MUC5AC.

Given susceptibility to infection in those with underlying diseases like asthma (12), we examined the role of MUC5B:MUC5AC ratio in the barrier function of synthetic mucus towards IAV. We expected the tighter microstructure of MUC5AC-rich gels to reinforce

barrier properties against IAV, restricting IAV diffusion. In contrast, we observed faster average diffusion of IAV in asthma-like, MUC5AC-rich gels compared to MUC5B-rich gels/ However, in both MUC5B and MUC5AC-rich gels, we estimate only a small fraction (7-9%) of IAV particles can diffuse rapidly enough to penetrate a ~40 μm mucus layer during the 2-hour period of exposure to viral inoculum used in our studies. Given the similarities in IAV penetration, we expect IAV diffusion within MUC5B-rich gels is influenced by mucin-associated glycan binding to a greater extent than the more heavily crosslinked MUC5AC-rich gels which present a greater physical barrier to IAV diffusion. While these gel thicknesses and exposure times are more reflective of diseased airways, we note thinner mucus layers and shorter clearance times (on the order of 30 minutes) would be expected in healthy lungs *in vivo* (47). However, we kept gel thickness and inoculation time consistent for both MUC5B and MUC5AC-rich to enable direct comparisons. Notably, we found in HAE cultures challenged with IAV that the barrier properties of MUC5AC-rich gels towards IAV are significantly compromised, whereas MUC5B-rich gels are effective in neutralizing IAV. It has been established in prior studies that IAV interacts with mucin glycans, primarily sialic acid (130). In comparing mucin glycosylation profiles, MUC5B has been shown to contain a high concentration of sialic acid whereas MUC5AC contains greater fucose content (4, 117). Thus, we expect that the reduction in sialic acid content and transport rates of MUC5AC-rich gels could render the airway surface more susceptible to widespread and increased frequency of infection. We anticipate that the barrier properties of MUC5B-rich and MUC5AC-rich gels can be further understood by studying IAV diffusion through and extent of infection in synthetic mucus-coated cultures using gels with modified glycosylated domains. Modification of mucin

glycans, specifically removal of sialic acids, of BSM gels through enzymatic cleavage using sialidase has been demonstrated in prior work (131) and will be pursued in our future studies. In addition, prior work has demonstrated mucosal antibody mediated trapping of IAV which could also be explored using our synthetic model in the future (132).

5.5 Conclusion

Our results establish a new biomaterial capable of recapitulating native airway mucus properties where mucus composition can be precisely controlled to study how its functions may be altered in disease. Using bioengineered synthetic mucus, we have found that the changes observed in mucus composition in asthma can have a major impact on gel viscoelasticity which in turn reduces its capacity to be effectively cleared. We also observed asthma-like mucus biomaterials were less capable of protecting the airway surface from infection by IAV. To confirm the broader relevance of these findings to human disease, future studies on mucus harvested *in vitro* from primary asthmatic tissue cultures and *ex vivo* isolated from patients with asthma will be performed to determine how rheology and barrier function towards respiratory viruses may be affected by changes to mucin composition. The model established herein could also be applied to other lung diseases (e.g. COPD, CF, pulmonary fibrosis) to understand airway dysfunction and uncover disease mechanisms that provide the basis for new therapeutic interventions.

6. MUC5B MOBILIZES AND MUC5AC SPACIALLY ALIGNS MUCOCILIARY TRANSPORT

6.1 Introduction

Airway mucus is a complex biological fluid that protects the lung from environmental insults through a process known as mucociliary clearance (MCC). The mucus layer, which coats the airways, protects the underlying epithelium by acting as a physicochemical barrier immobilizing inhaled pathogens and particulates (7, 33). Unlike other mucosal tissues, the airways can dynamically clear mucus through shear forces exerted by the rapid beating of cilia on the epithelium allowing removal of trapped particulates (56, 133). Normal MCC is essential for innate defense and is facilitated by a transportable mucus layer and coordinated ciliary activity (134).

The primary secreted mucins expressed in the lung are mucin 5B (MUC5B) and 5AC (MUC5AC) (11, 135). These mucins are large (~MDa) glycoproteins that have cysteine-rich domains at their N- and C-termini that cross-link and polymerize via disulfide bonds (28, 133). The resulting network acts as a barrier by controlling the transport of nano- and microscale entities through physical and steric interactions while also giving rise to the viscoelastic nature of mucus (10, 35, 36, 50, 51). While MUC5B and MUC5AC have a high degree of similarity in amino acid sequence and domain organization, recent evidence shows that MUC5B and MUC5AC differ in assembly and N-terminal organization, with MUC5B mainly forming dimers, and MUC5AC assembling into dimers as well as higher-order oligomers (4, 45). These subtle differences in macromolecular organization impact the functional properties of secreted mucins, such as effectiveness as a barrier and transportability, which have been studied in *in vivo* animal models (32). In

Muc5ac/b knockout (KO) mice, Muc5b deficiency leads to impaired mucus transport and pathogen accumulation, while Muc5ac is shown to be dispensable for transport (69, 78). Nonetheless, in health, the two gel-forming mucins form a mucus gel that is optimized for providing proper protection to the airways.

In health, MUC5B is the predominant mucin, while MUC5AC is expressed at lower levels (11, 135). While the expression of MUC5B and MUC5AC is tightly regulated to ensure efficient MCC, production of MUC5AC is increased in chronic respiratory diseases due to type I and II immune responses, resulting in a mucus gel with abnormal mucin composition (17, 135–137). Elevated MUC5AC in mucus has been identified as the major driver in impairment of MCC and is correlated with enhanced viscoelastic properties, decreased lung function in chronic bronchitis, and significant mucous plugging in fatal asthma (9, 17, 135, 138). However, how MUC5AC, in health, contributes to effective mucus clearance, while driving the formation of stagnant mucus in disease is unknown. As a result, there is growing interest in understanding how these two secreted mucins with similar domain structures but varying functional properties work in concert to enable efficient airway clearance. Moreover, the spatial coordination of transport on airway surfaces has been largely attributed to cilia organization and activity (139). However, the contribution of secreted mucins to tissue-scale alignment in MCC has not been widely considered.

In our studies, we developed *in vitro* airway epithelial systems deficient for one of the two gel-forming mucins to investigate the role and contribution of MUC5B and MUC5AC on MCC mechanisms. We used an *in vitro* human airway epithelium model to harvest mucus gels composed of single mucins for rheological assessment. Using our

cultures, we measured mucociliary transport rates, while also assessing flow orientation in real-time, demonstrating that MUC5B deficiency leads to impaired mucus clearance, whereas MUC5AC deficiency leads to transport that lacks spatial coordination. Accordingly, we observed that that supplementation of KO cultures with the absent mucin could improve these irregular mucociliary transport phenotypes. Taken together, our data demonstrates the functional significance of both mucins for promoting effective MCC in health, while providing insight into the mechanism of composition-dependent MCC impairment.

6.2 Methods and Materials

6.2.1 CRISPR/Cas9-mediated Knockout of MUC5B and MUC5AC

We utilized lentivirus for CRISPR/Cas9-mediated gene targeting of MUC5B and MUC5AC in BCI-NS1.1 cells, an h-TERT immortalized human airway basal cell line, generously provided by Dr. Ronald Crystal, which their group established and characterized in previous work (140). Two single guide (sg) RNAs that target different regions of MUC5B and MUC5AC were selected based on favorable targeting using the Doench and Xu scores. To generate negative control, we designed sgRNA sequences with no matching sequence in the genome (non-targeting control). Selected gRNAs were cloned into a GFP-expressing lentiviral vector, pLENTICRISPRv2-GFP, (a gift from Dr. Feng Zhang (Addgene plasmid #52961; <http://n2t.net/addgene:52961>; RRID:Addgene_52961)), which also encodes Cas9 nuclease. Lentiviral stalks were generated by co-transfecting plentiCRISPRv2, pCMV-VSV-G (a gift from Dr. Bob Weinberg (Addgene plasmid #8454; <http://n2t.net/addgene:8454>; RRID:Addgene_8454), and psPAX2 (a gift from Dr. Didier

Trono (Addgene plasmid #12260; <http://n2t.net/addgene:12260>; RRID:Addgene_12260)) into HEK293T cells with jetPrime (Polyplus) in cell media, DMEM (Gibco) supplemented with 10% fetal bovine serum using manufacturer's protocol. BCI-NS1.1 cells were transduced at 40-60% confluence using harvested lentiviruses in media with a final concentration of 20 mM HEPES (Gibco) and 4 µg / mL Polybrene (American Bio). BCI-NS1.1 cells were then centrifuged (1,000 g for one hour at 37°C) and incubated at 37°C overnight. At 60-80% confluence cells were passaged and expanded and sorted for eGFP expression. DNA from transduced, sorted (eGFP+) cells was extracted using QuickExtract DNA Extraction Solution (Lucigen). EnGen Mutation Detection Kit (New England BioLabs) was used for amplification of target DNA, heteroduplex formation, and heteroduplex digestion, using manufacturer's protocol. Final DNA products were separated and visualized in a 1% agarose gel through gel electrophoresis.

6.2.2 Cell Culture and Mucus Collection

Transduced and sorted (eGFP+) BCI-NS1.1 cells were seeded on plastic at ~3000 cells/cm² in PneumaCult-Ex Plus media (StemCell) and incubated at 37°C, 5% CO₂. Once cells reached 70-80% confluency, they were dissociated using 0.05% trypsin ethylenediaminetetraacetic acid (EDTA) for 5 minutes at 37°C. To establish well-differentiated human airway epithelial (HAE) tissues cultures grown at an air-liquid interface (ALI), BCI-NS1.1 cells were seeded on 12 mm diameter transwell inserts (Corning Costar) coated with 50 µg/mL type 1-collagen from rat-tail (Corning) at ~10,000 cells/cm². Expansion media, PneumaCult-Ex Plus, was used to feed cells in both the apical and basolateral compartments until 100% confluency. After reaching confluency, the apical media was removed to transition from submerged to ALI culture and the basolateral

media was replaced with PneumaCult-ALI (StemCell). All cells were grown for 28 days to reach differentiation with media exchanged every other day. Transepithelial electrical resistance (TEER) was quantified cultures using the Millicell Electrical Resistance System ERS-2 Volt-Ohm Meter (Millipore).

Once fully differentiated, mucus was allowed to accumulate prior to collection every 3-4 days. Mucus samples were harvested by washing apical compartments with PBS for 30 minutes at 37°C. After 30 minutes incubation, the solution of mucus and PBS was collected (35, 36). Samples were loaded into Amicon Ultra 100 kDa filters (Millipore-Sigma) and centrifuged at 14,000 $\times g$ for 20 minutes to remove excess PBS for mucin isolation. Mucus samples were stored at -80 °C for long-term storage (> 2 weeks) or stored at 4 °C (84). Pooled mucus samples were used for Western blot analysis, solids concentration analysis, biophysical characterization, and transport experiments.

6.2.3 Western Blot Analysis

Mucus samples from BCI-NS1.1 cell washings were used for Western blot analysis. Protein concentration of samples was quantified using BCA assay (Pierce, Thermo Scientific) and equal amounts (10 μg) were loaded into each lane and run on a 4-20% Tris-Glycine gel (Novex, Invitrogen) under reducing conditions. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare) and blocked with 5% (w/v) fat free milk protein in tris-buffered saline, 0.1% Tween (TBS-T) at room temperature. Primary antibody incubation was over night at 4°C in 5% (w/v) fat free milk protein. The primary antibodies that were used were mouse anti-MU5AC (1:1,000; cat. no. ab3649; Abcam) and rabbit anti-MUC5B UNC414 (1:1,000), generously gifted by Dr. Camille Ehre. After washing in TBS-T, membranes were probed with secondary antibodies for one

hour at room temperature in blocking buffer. The secondary antibodies that were used were anti-mouse IgGk-HRP (sc-516102, Santa Cruz, 1:10,000), anti-rabbit-HRP (32460, Invitrogen, 1:10,000). Imaging was performed with chemiluminescent SuperSignal Dura reagent (Thermo Scientific) on an iBright 1500 (Thermo Fisher).

6.2.4 Measurement of Mucus Solids and Total Mucin Concentration

We measured solids concentrations of mucus samples by aliquoting 50 μ L of mucus on weighing paper of known mass and recording the combined mass of the mucus sample and paper. The samples were then placed on a heat plate at 60°C for 4-5 hours. The final mass of the dried sample and weighing paper was recorded and used to calculate % solids concentration.

Mucin concentration was quantified by measuring the total O-glycosylated glycoproteins in mucus samples using a previously described method (51, 141). Samples were diluted 20-fold with physiological buffer (154 mM NaCl, 3 mM CaCl₂, and 15 mM NaH₂PO₄ at pH 7.4). 50 μ L of diluted mucus sample were mixed with alkaline 2-cyanoacetamide reagent, which was prepared by mixing 200 μ L of 0.6M 2-cyanoacetamide with 1 mL of 0.15M NaOH. Samples were then incubated at 100 °C for 30 minutes. After cooling, 0.5 mL of 0.6 borate buffer was added since maximum fluorescence is displayed in the presence of borate ions. As a standard, serial dilutions of mucin from bovine submaxillary gland (Sigma) dissolved in physiological buffer was used. The fluorescence was measured at excitation and emission wavelength of 336 nm and 383 nm, respectively.

6.2.5 Measurement of Bulk Rheology

Dynamic rheological measurements of mucus gels harvested and pooled from ALI cultures were performed using the ARES G2 rheometer (TA Instruments) with a 40-mm diameter 2° cone and plate geometry at 25°C. To determine the linear viscoelastic region of the fully formed gel, a strain sweep measurement was collected from 0.1-10% strain at a frequency of 1 rad s⁻¹. To determine the elastic modulus, $G'(\omega)$, and viscous modulus, $G''(\omega)$, a frequency sweep measurement was conducted within the linear viscoelastic region of the gel, at 1% strain amplitude and angular frequencies from 0.1 to 10 rad s⁻¹.

6.2.6 Particle Tracking Microrheology (PTM)

Carboxylate-modified, fluorescent polystyrene nanoparticles (PS-COOH; Life Technologies) with a diameter of 100 nm were coated with a high surface density of polyethylene glycol (PEG) via a carboxyl-amine linkage using 5-kDa methoxy PEG-amine (Creative PEGWorks) as previously reported (51, 68). Particle size and zeta potential was measured in 10 mM NaCl at pH 7 using a NanoBrook Omni (Brookhaven Instruments). We measured diameters of 122 nm and zeta potentials of -0.54 ± 1.16 for 100 nm PEG-coated PS nanoparticles, respectively. The diffusion of the PEG-coated nanoparticles (PEG-NP) in mucus gels was measured using fluorescence video microscopy. Twenty five μ L of mucus was added to the chamber along with 1 μ L of $\sim 0.002\%$ w/v suspension of PEG-NP 30 minutes prior to particle tracking microrheology (PTM) experiments. Videos were collected using a Zeiss 800 LSM microscope with a 63x water-immersion objective and an Axiocam 702 camera (Zeiss) at a frame rate of 33 Hz for 10 seconds at room temperature. For each sample, at least 3 high-speed videos were recorded. The particle tracking analysis was performed using a previously developed image processing algorithm

(68, 79). Mean squared displacement (MSD) as a function of time lag (τ) was calculated as

$$\langle \Delta r^2(\tau) \rangle = \langle (x^2 + y^2) \rangle$$

for each particle. Using the generalized Stokes-Einstein relation, measured MSD values were used to compute viscoelastic properties of the hydrogels. The Laplace transform of $\langle \Delta r^2(\tau) \rangle$, $\langle \Delta r^2(s) \rangle$, is related to viscoelastic spectrum $\tilde{G}(s)$ using the equation

$$\tilde{G}(s) = 2k_B T / [\pi a s \langle \Delta r^2(s) \rangle]$$

where $k_B T$ is the thermal energy, a is the particle radius, s is the complex Laplace frequency. The complex modulus can be calculated as

$$G^*(\omega) = G'(\omega) + G''(i\omega)$$

with i being substituted for s , where i is a complex number and ω is frequency. Hydrogel network pore size, ξ , is estimated based on G' using the equation,

$$\xi \approx (k_B T / G')^{1/3}.$$

6.2.7 Mucociliary Transport and Ciliary Beat Frequency

For measurement of mucociliary transport rate, a 4 μ L suspension of 2 μ m red-fluorescent polystyrene microspheres (Sigma-Aldrich; 1:1000 dilution in PBS) was apically applied to native mucus, which was allowed to accumulate for 1-2 days prior to analysis. After equilibration overnight, videos of three regions were recorded at 10x magnification using a Zeiss 800 LSM microscope. Images were collected at a frame rate of 0.5 Hz for 20 seconds on the plane of the mucus gel. The microsphere tracking data

analysis is based on an image processing algorithm that was custom written in MATLAB (84, 85). Briefly, the analysis software computes the xy-plane trajectories of each fluorescent microsphere in each frame. Using the trajectory data, displacement of microspheres was computed, and transport rate was calculated by dividing the distance traveled by total elapsed time. In order to measure ciliary beat frequency (CBF), 10 second videos at a frame rate of 20 Hz were recorded at 20x magnification in ≥ 3 randomly selected regions of each BCI-NS1.1 culture using brightfield. Using a custom written algorithm in MATLAB, the number of local pixel intensity maxima were counted, which indicates beating of cilia. Beat frequency was determined by dividing the number of beats over the total elapsed time.

6.2.8 Immunostaining

BCI-NS1.1 cultures were fixed using 4% paraformaldehyde for 30 minutes at room temperature. Inserts were washed with PBS and blocked with 5% bovine serum albumin (BSA) in PBS and 0.01% triton X-100 for 1 hour at room temperature. After blocking, fixed cultures were incubated overnight at 4°C with AlexaFluor conjugated antibodies in 5% BSA in PBS. The antibodies that were used were anti-acetylated alpha tubulin AlexaFluor 647 (cilia marker, clone 6-11B-1, sc23950, Santa Cruz, 1:2000) and ZO-1 monoclonal antibody AlexaFluor 488 (tight junction marker, ZO1-1A12, Invitrogen, 1:2000). Inserts were washed with PBS three times and imaged at 63x magnification using a Zeiss 800 LSM microscope (Zeiss). Quantification of % tubulin positive area was performed in FIJI using the automated thresholding function.

6.2.9 Transplantation of Mucus

Reconstituted mucus samples from KO culture washings were used for transplantation studies. 20 μ L of mucus was applied to the apical surface of ALI cultures that had been washed with PBS for 30 minutes (84, 142). 2 μ m red-fluorescent polystyrene microspheres were applied immediately after transplantation of exogenous mucus. Mucociliary transport rates were measured after equilibration overnight as described in the methods above.

6.2.10 Statistical Analysis

All graphing and statistical analyses were performed using GraphPad Prism 8 (GraphPad Software). For comparisons between groups, one-way analysis of variance (ANOVA) followed by a Tukey *post hoc* correction was performed. Kruskal-Wallis with Dunn's correction was used for comparison of multiple groups with non-Gaussian distributions. Bar graphs show mean and standard deviation and box and whiskers plots show median and interquartile range. Differences were considered statistically different at the level of $p < 0.05$.

6.3 Results

6.3.1 Generation of MUC5B/MUC5AC-KO BCI-NS1.1 Cultures

We used CRISPR/Cas9-mediated genome editing to generate *in vitro* HAE models genetically deleted for one of the two gel-forming mucins. We designed two single guide (sg)RNAs that target each mucin gene, MUC5B and MUC5AC, at different exons (**Table 6.1**). Earlier exons were targeted to maximize knock-out efficiency, while two sgRNAs were designed to account for potential off-target effects. As our control, we used a sgRNA

with no predicted target. The sgRNAs were cloned into a GFP-expressing lentiviral vector that also encodes the Cas9 nuclease. After lentivirus production, we transduced BCI-NS1.1 cells, an immortalized HAE cell line, which has previously been used for manipulating mucin expression (21, 84, 140). Subsequently, transduced cells were expanded, and sorted for GFP expression prior to differentiation (**Fig 6.1A**).

Table 6.1: Table of gRNA sequence and exon target.

Gene	gRNA #	Designation	Target	Sequence
MUC5B	gRNA 1	B KO1	Exon 3	GTGGAACAAAGCTCACGCGC
	gRNA 2	B KO2	Exon 4	TTCAACGTCCAGCTACGCCG
MUC5AC	gRNA 1	AC KO1	Exon 4	GATGTTAAATCCTCGTAGG
	gRNA 2	AC KO2	Exon 6	GAGAGGAGCTCGCTGACCAC

Using a T7 endonuclease cleavage assay, we confirmed editing at the correct genomic loci in undifferentiated cells, evidenced by heteroduplex fragments in all KO conditions (**Fig 6.1A, B**). To verify that lentiviral transduction did not impact normal differentiation of BCI-NS1.1 cultures, we measured the transepithelial electrical resistance

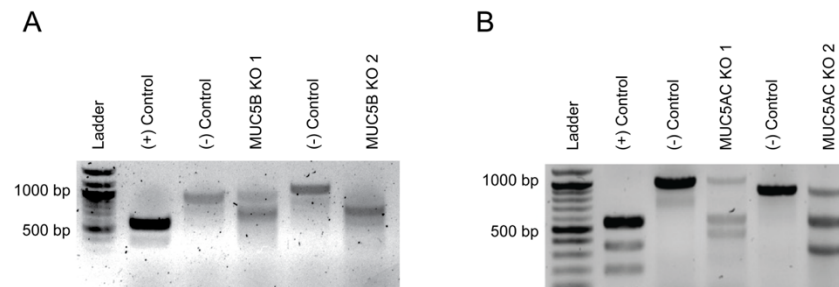


Figure 6.1 Validation of on-target editing using T7 endonuclease cleavage assay. Gel images show PCR products amplified from target sites where digested fragments indicate on-site editing. (+) control corresponds to a control template and primer mix provided with the assay kit. (-) control corresponds to BCI-NS1.1 cells treated with a non-targeting sgRNA. T7 endonuclease cleavage assay using genomic DNA from (A) MUC5B-KO cultures and (B) MUC5AC-KO cultures.

(TEER) 28 days into air-liquid interface (ALI) culture and confirmed TEER values that were within reported ranges in all conditions, indicative of cell polarization and differentiation into a pseudostratified epithelium (143). Using mucus samples collected from apical washes, we confirmed KO expression on Western blot analysis demonstrating loss of MUC5B and MUC5AC expression in KO cultures using respective sgRNA (**Fig 6.2D**). These data also confirmed that mucus secretions from MUC5B-KO cultures are primarily composed of MUC5AC, while secretions from MUC5AC-KO cultures are composed of MUC5B, allowing us to study the native properties of mucus composed of single mucins.

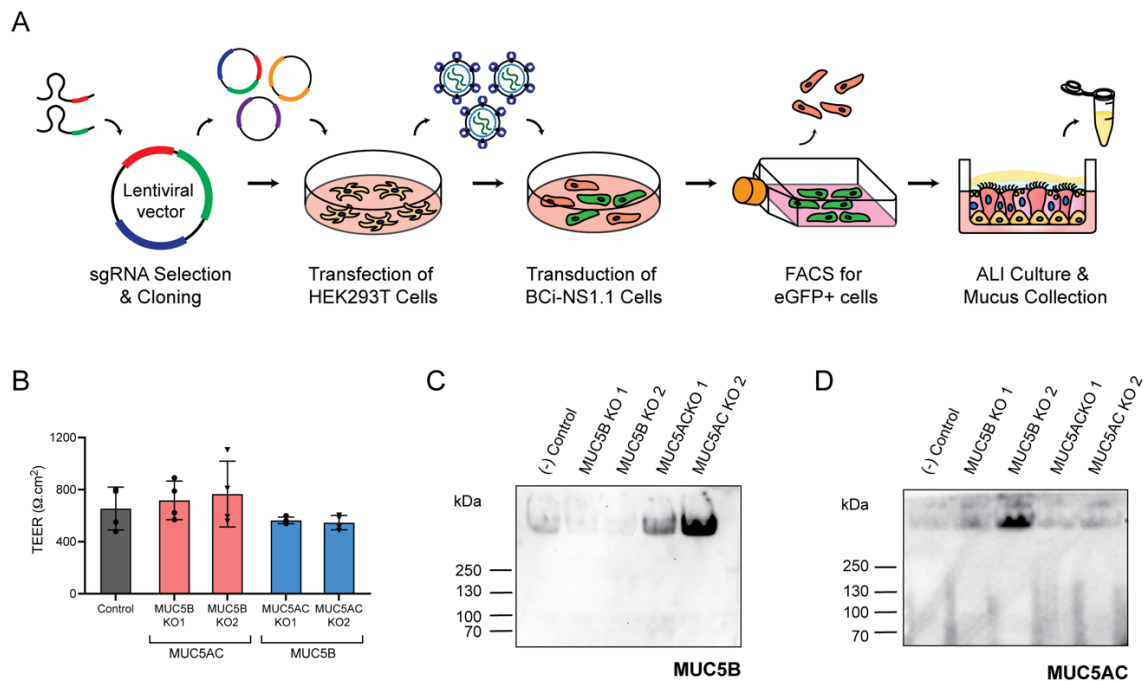


Figure 6.2 Generation and validation of MUC5B/AC-KO cultures. (A) Overview of the MUC5B/MUC5AC gene-targeting approach via lentivirus-mediated delivery of sgRNA and CRISPR/Cas9. (B) Transepithelial electrical resistance (TEER) measurements in differentiated MUC5B/MUC5AC KO cultures. KO1 corresponds to BCI-NS1.1 cells transduced with sgRNA1 and KO2 corresponds to cells transduced with sgRNA2. (C, D) Western blot analysis for (C) MUC5B and (D) MUC5AC in mucus collected from apical washings from control and KO cultures.

6.3.2 Viscoelasticity of MUC5B and MUC5AC Gels

To evaluate the biophysical properties of gels composed of either MUC5B and MUC5AC, we performed macro- and microrheology on pooled mucus samples collected from control and KO cultures. Using macrorheology, we observed elastic dominant properties for all mucus samples ($G' > G''$), confirming a cross-linked polymer network. We also demonstrated that samples exhibited rheological properties in range of previously reported values for mucus collected *in vitro* and *ex vivo* (**Fig 6.3A**) (10, 35, 36). While MUC5AC gels resulted in slightly higher mean complex viscosities than other gel types, differences were not statistically significant (**Fig 6.3B**). We next measured percent solids concentration of each sample as the biophysical properties of a polymer gels are highly sensitive to changes in solids concentration. We found that the solids concentration of pooled mucus samples were within a physiological range (2-4%) (**Fig 6.3C**) (36, 133). While macrorheological properties have major implications for flow and transport, mucus microstructural network allows mucus to act as a barrier to inhaled material by controlling the diffusion of nano- and microscale entities through physical and adhesive interactions (**Fig 6.3D**) (133). Therefore, we used particle tracking microrheology (PTM) to assess diffusion of muco-inert nanoparticles (MIP) through mucus with varying mucin composition to probe mucus microstructure. Results showed that MUC5AC gels had significantly reduced MIP diffusion, as measured by mean square displacement (MSD), and reduced pore size compared to MUC5B gels and mucus collected from control cultures (**Fig 6.3E, F**). Comparing the frequency distributions of MIP diffusion, we found that MUC5B gels resulted in MIP diffusion that was similar to mucus obtained from control cultures, whereas MUC5AC gels resulted in a significant reduction in MIP diffusion with

multiple peaks indicative of mucus gels with a tighter mesh network and heterogenous structure (**Fig 6.3E, F, G, I**). Importantly, we also found that there were no significant differences in macro- and microrheology measurements of MUC5B and MUC5AC gels harvested from cultures with equivalent KO condition targeted using different sgRNAs.

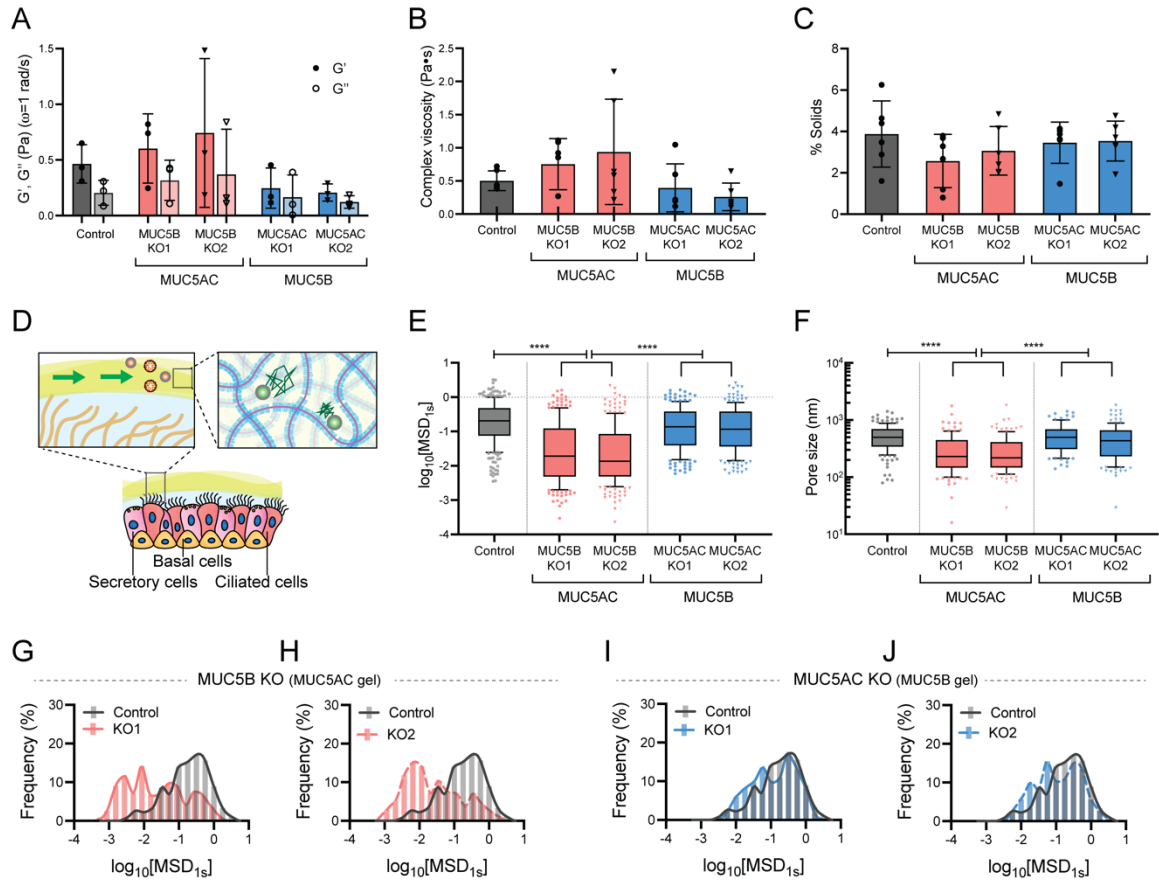


Figure 6.3 Viscoelastic properties of mucus produced from MUC5B/AC-KO cultures. (A) Elastic and viscous moduli (G' , G'') at $\omega = 1$ rad/s measured using macrorheology. Moduli show G' -dominant mucus gels. (B) Complex viscosity measured using macrorheology. (C) Concentration of % solids in pooled mucus samples from MUC5B/AC-KO and control cultures. (D) Airway mucus acts as a vehicle of clearance of pathogens, providing the lungs with a robust defense mechanism. Mucus also has a microstructural network that can immobilize diffusing particles. (E) Diffusion of 100 nm PEG-NP in mucus samples from KO cultures as measured by logarithm mean squared displacement (MSD). (D) Estimated pore sizes of mucus samples. (G-J) Frequency distribution of $\log_{10}[\text{MSD}_{1s}]$. Particle distribution in control and (E) MUC5B-KO1, (F) MUC5B-KO2, (G) MUC5AC-KO1, and (H) MUC5AC-KO2 samples.

6.3.3 MUC5B-KO Cultures Exhibit Reduced Mucociliary Transport and MUC5AC-KO Cultures Exhibit Discoordinated Transport

We next examined the mucociliary transport rates of mucus produced from MUC5B or MUC5AC KO cultures to investigate the contribution of each gel-forming mucin on mucociliary clearance. To do so, we deposited 2- μ m microspheres onto KO cultures and tracked the movement of particles in real-time (**Fig 6.4A**). We observed that movement of microspheres on MUC5B-KO cultures were significantly reduced compared to the non-targeted control cultures. While functional transport was maintained in MUC5AC-KO cultures, we observed loss of unidirectional flow (**Fig 6.4B**). Quantifications of microsphere speed confirmed that MUC5B-KO cultures had significantly reduced transport rates compared to control and MUC5AC-KO cultures (**Fig 6.4C**). We next quantified the directionality of mucus flow on control and MUC5AC-KO cultures by measuring the angle, $\Delta\theta$, which was calculated by measuring the angle between individual microsphere trajectory and ensemble average flow direction (θ_{Average}) (**Fig 6.4D**). We found that control cultures resulted in an overall alignment of flow direction, where 95% of the microspheres moved within 10° to -10° of overall flow (**Fig. 6.4E**). However, MUC5AC-KO cultures resulted an irregular flow pattern that resembled circular swirls, where only 35-50% of microspheres aligned with the ensemble average flow direction (**Fig 6.4F,G**). We confirmed that these differences in transport rate and directionality of mucus flow were not likely the result of changes in the periciliary layer properties by confirming normal cilia beat frequency and normal cilia expression as demonstrated in +tubulin % area (**Fig 6.4H-J**).

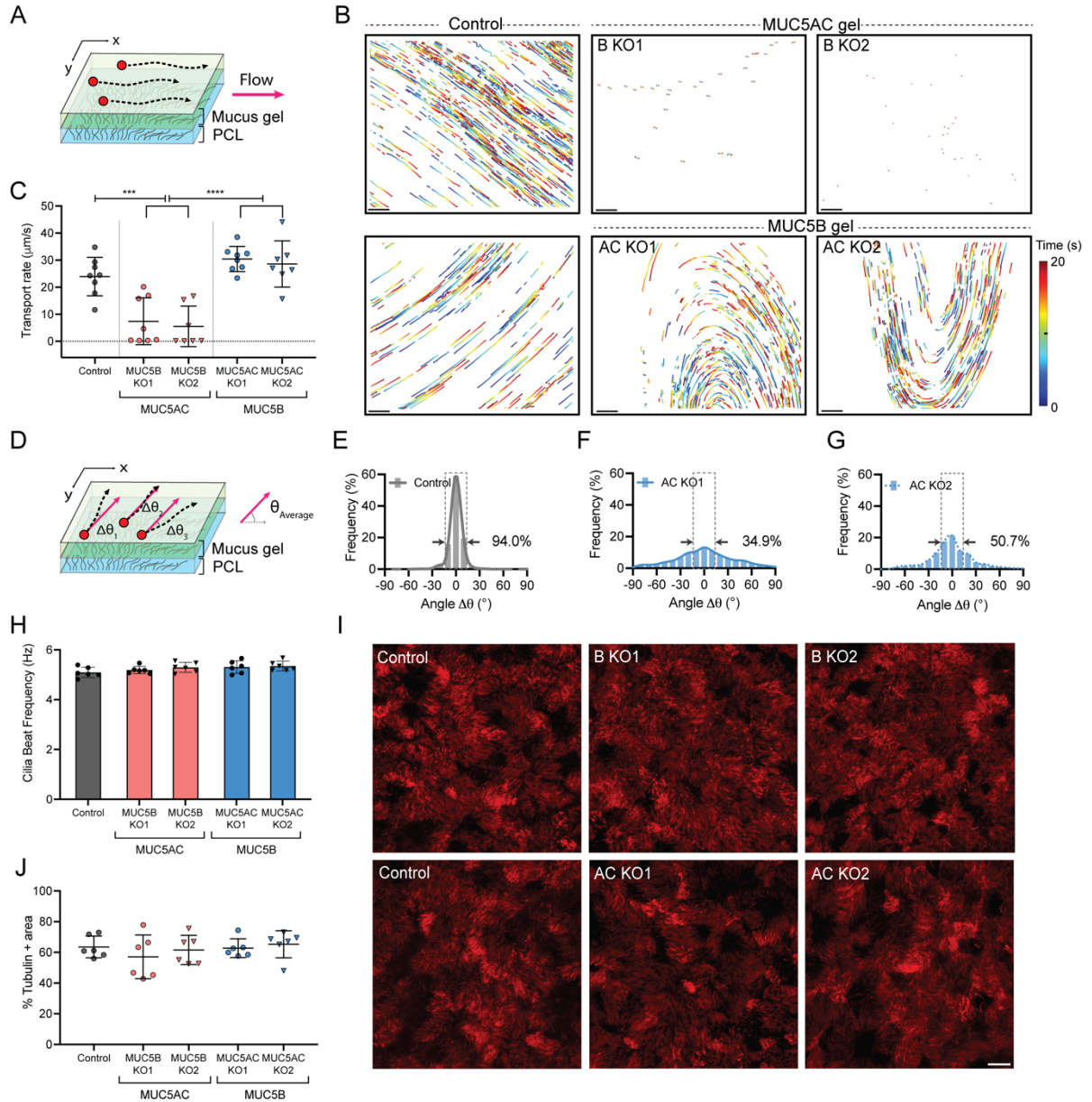


Figure 6.4 Mucociliary clearance dynamics of MUC5B/AC-KO cultures. (A) Mucociliary transport rate is measured by apically applying 2-μm microspheres to cultures and measuring particle displacement over time. (B) Representative trajectory images of microspheres over time. Trajectories show 20 s of motion with a color scale that indicates elapsed. Scale bar = 100 μm. (C) Mucociliary transport rates of MUC5B/AC KO cultures. n=8 cultures. (D) Trajectory angle, $\Delta\theta$, was calculated by measuring the angle between and the overall flow direction ($\theta_{Average}$) and instantaneous individual microsphere trajectory. $\theta_{Average}$ was set to 0°. (E-G) Frequency distribution of $\Delta\theta$. (E) Control (F) MUC5AC-KO1 (G) MUC5AC-KO2. (H) Cilia beat frequencies of MUC5B/AC KO cultures. (I) Representative image of cilia expression of MUC5B/AC-KO cultures by immunostaining of acetylated alpha tubulin. Scale bar: 10 μm. (J) Quantification of +tubulin % area. (E-H, J) n=6 cultures. Data shows mean and standard deviation.

6.3.4 Transplantation Exogenous Mucus Rescues Impaired Clearance

Our previous data showing abnormal transport resulting from loss of one of the two mucins led us to hypothesize that supplementing KO cultures with the absent mucin could restore stalled and/or irregular mucociliary transport phenotypes. To test this, we transplanted exogenous MUC5B and MUC5AC gels onto KO cultures and tracked the movement of microspheres on the cultures after equilibration (**Fig 6.5A**). We chose MUC5B-KO1 and MUC5AC-KO2 as representative cultures for transplantation experiments. We found that transplantation of MUC5B onto MUC5B-KO cultures resulted in a significant increase in transport rates, while also resulting in unidirectional flow (**Fig 6.5B-D**). However, transplantation of MUC5AC gels onto their native MUC5B-KO cultures did not result in a significant change in transport rate. Interestingly, the addition of either MUC5B or MUC5AC gels onto MUC5AC-KO cultures did not result in any significant change to transport rates (**Fig 6.5E**) but resulted in improved directionality as shown in the trajectory images (**Fig 6.5F,G**).

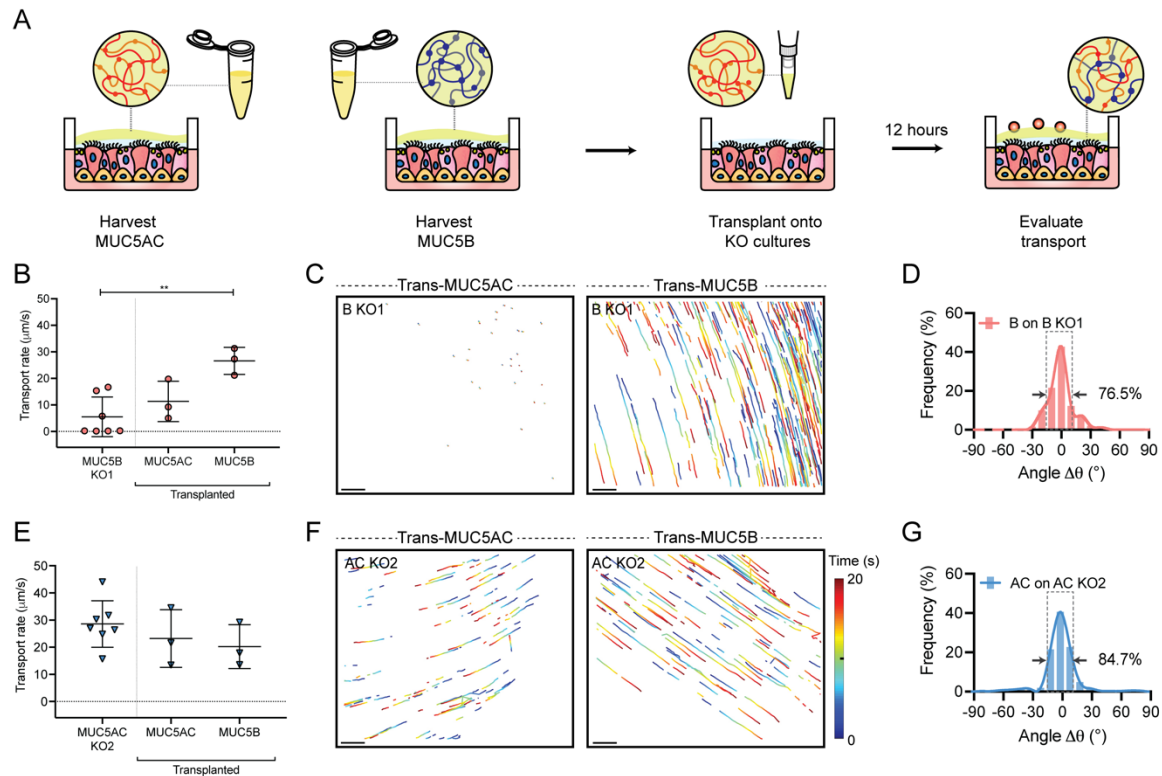


Figure 6.5 Mucociliary clearance dynamics of transplanted mucus on MUC5B/AC-KO cultures. (A) Exogenous mucus collected from KO cultures was transplanted onto KO cultures to supplement KO cultures deficient mucin. (B) Mucociliary transport rates of transplanted gels on B-KO culture (MUC5B-KO1 was used). (C) Representative trajectory images of microspheres trajectories on transplanted mucus on B-KO cultures. Scale bar = 100 μm . (D) Frequency distribution of $\Delta\theta$, demonstrating directional flow. (E) Mucociliary transport rates of transplanted gels on AC-KO culture (MUC5AC-KO2 was used). (C) Representative trajectory images of microspheres trajectories on transplanted mucus on AC-KO cultures. Scale bar = 100 μm . (D) Frequency distribution of $\Delta\theta$, demonstrating directional flow. (B, E) $n=3$ transplantation on cultures. Data shows mean and standard deviation.

6.4 Discussion

In these studies, we report *in vitro* HAE models that are genetically depleted for one of the two gel-forming mucins expressed in airway mucus to understand their role and overall contribution in mucociliary clearance mechanisms. We targeted MUC5B and MUC5AC genes in the BCI-NS1.1 human airway basal cell line and demonstrated successful editing at targeted genomic loci in undifferentiated cultures, while confirming

KO expression in pooled mucus washings post-differentiation. MUC5B-KO cultures produced gels composed primarily of MUC5AC, while MUC5AC-KO cultures produced MUC5B gels, which allowed us to study each mucin independently. We note that our Western blot analysis indicates that KO cultures produce slightly elevated levels of respective mucins compared to control cultures, while overall percent solids and protein concentration are conserved. These results may indicate that MUC5B and MUC5AC secretion is hydrodynamically controlled, regardless of mucin type. However, the pathways that regulate secretion of each mucin after KO will need to be explored in future studies.

Using our KO cultures, we assessed the biophysical properties of mucus samples with varying mucin composition. Our results indicate that gels composed of MUC5AC have altered microstructural properties compared to MUC5B gels, where pore size is significantly reduced and heterogenous. These results are consistent with recent findings that show that MUC5AC secreted from CALU3 cells form tight networks with high degrees of branching, while MUC5B organizes in linear strands (45). The smaller pore size in MUC5AC gels have implications for their functional role as a protective gel against pathological and chemical insults to the airway. In past work, MUC5AC has been shown to protect against viral respiratory pathogens such as influenza and prevent parasite infiltration (71, 137, 144). We also observed major differences in transportability of gels composed of different mucin types. MUC5AC gels produced from MUC5B-KO cultures were poorly cleared, while MUC5B gels from MUC5AC-KO cultures were more easily transported. These data are consistent with previous mouse models that showed that *Muc5b*-KO in mice, but not *Muc5ac*-KO, resulted in impaired mucociliary clearance and

mucus accumulation in the airways (69). Furthermore, in humans, new evidence has shown that congenital absence of MUC5B results in impaired mucus clearance (145). This may be due in part to macromolecular organization of mucins, where MUC5B forms linear gels that can be easily transported via ciliary activity, while MUC5AC forms a tighter gel, which increases friction between gel surfaces, resulting in poor clearance (45, 56). While bulk rheological properties are known to impact transport properties of the mucus gels, our data showed no resolvable differences in viscosities between gels of varying mucin types (133). Previous studies have predicted MUC5AC to form more elastic gels than MUC5B and we note that MUC5AC gels had higher complex viscosities compared to MUC5B gels, despite having lower percent solids concentration (45). We expect solids concentration-matched MUC5B and MUC5AC gels would possess even greater differences in their viscoelastic properties and this will be explored in subsequent work.

Despite functional transport of MUC5B gels on MUC5AC-KO cultures, we observed that MUC5AC deficiency led to poor directionality with local swirls, while control cultures that expressed both mucins showed global order. These transport patterns have been previously observed in HAE *in vitro* models and attributed to hydrodynamic active coupling between mucus and cilia, where changes in mucus properties and cilia density can alter the overall orientation of mucus flow (146, 147). While the role of MUC5AC in mucociliary transport has been largely overlooked, our data suggests that MUC5AC-enhanced mucus viscoelasticity plays a significant role in hydrodynamic coupling between the mucus and periciliary layers, driving long-range transport and effective clearance. This can also be explained by our transplantation experiments with exogenous mucus, where we demonstrated that supplementation of KO cultures with the

absent mucin can rescue and improve irregular transport phenotypes. We note supplementation of MUC5B to MUC5B-expressing cultures also improved transport coordination likely by increasing mucin concentration and mucus gel viscoelasticity leading to enhanced hydrodynamic coupling. However overall, these experiments demonstrate the biological importance of both gel-forming mucins, MUC5B and MUC5AC for efficient mucociliary transport in the airways.

Our current understanding of impaired mucus clearance has primarily focused on the imbalance in osmotic pressure between the mucus and periciliary layer, which results from a dehydrated or hyper-concentrated mucus gel that is not easily cleared (27, 56). However, our findings shed light on the mechanisms of defective mucociliary clearance dictated by mucus composition and independent of concentration. While increased MUC5AC may contribute to protection against inhaled pathogens through physical obstruction, disproportionate increases have been associated with pathogenesis of chronic obstructive lung diseases and airway obstruction (17, 135). Mucous plugs from individuals with fatal asthma show MUC5AC enrichment and localization close to the epithelium. In mice, *Muc5ac* mediates mucous occlusion after allergic stimulation, while *Muc5ac*-KO resulted in significant protection against mucus plugging. Taken together, elevated levels of MUC5AC on the epithelial surface could be a significant contributor to mucus accumulation and airway obstruction due to its poor transportability as demonstrated in our studies.

6.5 Conclusion

In conclusion, our study provides unique insight into the contribution of each gel-forming mucin, MUC5B and MUC5AC on mucociliary clearance mechanisms, demonstrating the significance of each mucin. Our findings suggest secretion of MUC5B and MUC5AC offer a means for the airway epithelium to dynamically control mucus transport velocity and flow alignment. While MUC5AC has been considered to be a potential therapeutic target for allergic asthma (70), we predict that preserving baseline levels of MUC5AC could be important in maintaining flow orientation to facilitate effective removal of pathogens. To resolve MUC5AC-induced MCC dysfunction, ongoing work on mucolytic strategies that target the disulfide bonds in mucus gels for normalizing clearance may prove useful as MUC5AC-enriched gels are likely to be more extensively crosslinked (45, 129). This work also establishes the premise for future development of new precision therapies aimed at restoring an optimal balance of MUC5B and MUC5AC secretion in airway diseases.

7. CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Summary and Conclusions

Despite the widespread impact of asthma and collective efforts to prevent, diagnose, and treat the disease, asthma remains incurable (2). Patients with asthma have recurring symptoms of airway obstruction that result from diverse pathogenetic mechanisms (148). Current therapies primarily target inflammation of the airways, while the pathological impact of mucus has been widely ignored due to a lack of understanding of mucus dysfunction in asthma.

The objective of this dissertation was to understand the contribution of MUC5B and MUC5AC to mucus dysfunction by developing novel model systems that recapitulate mucus composition in asthma and allow systematic studies on the impact of mucin composition on mucus properties. The overarching motivation was to contribute to the body of knowledge that is focused on better understanding asthma pathology with the hopes of providing a path towards developing new and effective treatment strategies that can normalize mucus function and increase airway patency in asthma patients.

7.1.1 Summary of Work using Mucin-Based Hydrogels

In **Chapter 4**, we reported the design of a novel mucin hydrogel created using a thiol-based cross-linking strategy. By using a cross-linking reagent capable of forming disulfide linkages between mucins, we were able to produce mucin-based hydrogels possessing viscoelastic properties similar to natural mucus as measured by bulk rheology and particle tracking microrheology. We found that the rate of gel formation could be tuned by varying the mucin to crosslinker ratio, producing network pore sizes in the range

measured previously in human mucus. The results of this work provided a new, simple method for creating mucin hydrogels with physiologically relevant properties and became the foundation for the work in **Chapter 5 (Fig 7.1)**.

In **Chapter 5**, we systematically varied the mucin composition to closely mimic the properties of native mucus in health and asthma. We found that mucus gel viscoelasticity is enhanced when predominantly composed of MUC5AC, as is observed in asthma. As a result, asthma-like synthetic mucus gels are more slowly transported on the surface of HAE tissue cultures. We also discovered the barrier function of asthma-like synthetic mucus towards influenza A virus was impaired as evidenced by the increased frequency of infection in MUC5AC-rich hydrogel coated HAE cultures. Together, this work establishes a biomaterial-based approach to understand airway dysfunction in asthma and related muco-obstructive lung diseases.

7.1.2 Summary of Work *In vitro* HAE system KO

In **Chapter 6**, we generated human airway epithelial tissue cultures deficient in either MUC5B or MUC5AC to understand their individual contributions to mucus

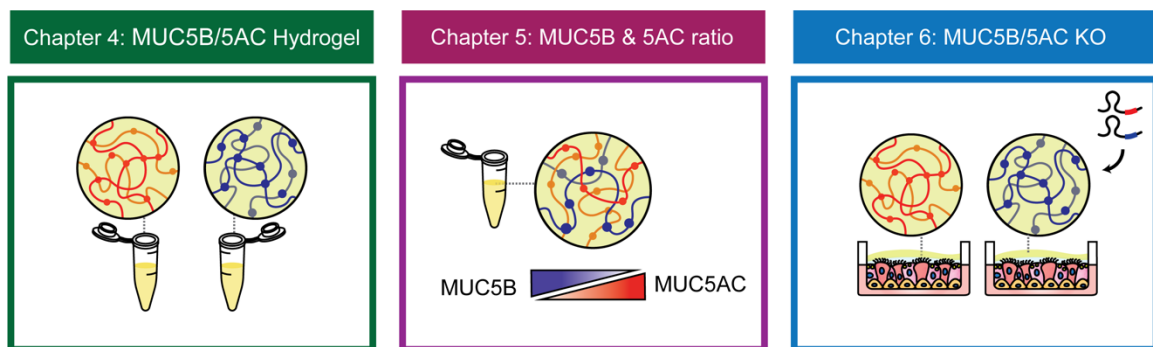


Figure 7.1 Summary of work. **Chapter 4** present a novel mucin-based hydrogel with native like properties. **Chapter 5** examines the impact of varied MUC5B:MUC5AC ratio on mucus barrier properties. **Chapter 6** presents *in vitro* HAE models for studying the contribution of MUC5B and MUC5AC on mucociliary clearance.

transport. We found that MUC5B and MUC5AC deficiency results in impaired and dis-coordinated mucociliary transport, respectively, demonstrating the importance of each mucin to airway clearance. Together, this work provides insight into the contribution of each gel-forming mucin, MUC5B and MUC5AC on mucociliary clearance mechanisms and highlights the importance of both mucins for dynamic control of mucus transport and flow alignment on the airway epithelium. In addition, mucociliary clearance dysfunction observed in MUC5B-KO (MUC5AC-expressing) cultures demonstrates that elevated levels of MUC5AC could be a significant contributor to mucus accumulation and airway obstruction due to its poor transportability, shedding light on a new mechanism of mucus dysfunction that is dictated by mucus composition (**Fig 7.1**).

In summary, our work suggests that elevated MUC5AC contributes to enhanced viscoelastic properties, which in turn reduces its ability to be efficiently cleared and increases likelihood for mucus plug formation in the lungs. These altered properties could also lead to compromised barrier function of mucus resulting in increased susceptibility to viral infections. Our work also provides insight into the functional roles of individual mucins, MUC5B and MUC5AC, where MUC5B is required to maintain functional transport and MUC5AC is required for spatial alignment of mucus flow on the airway epithelium.

These findings provide strong motivation for designing novel mucus-targeted therapeutics for asthma. While further work is required, we believe that the novel approaches provided by the work in this dissertation can be used to have a clinical impact.

7.2 Future Research Directions

7.2.1 Synthetic Mucus Array for Screening Mucolytics Agents

The mucosal barrier poses a significant challenge for inhaled therapeutics intended to reach the underlying epithelium due to the inability to traverse the mucus layer (114). In addition, increased viscoelasticity and reduced pore size of mucus as observed in diseases often make drug delivery to the airways even more challenging. Recent studies have demonstrated disulfide disruption through mucolytic treatment can reverse pathological effects of mucus accumulation in a mouse allergic asthma model demonstrating potential for mucus-targeted treatments (129, 149). We hypothesize that co-delivery of mucolytic agents and therapeutics can enhance inhaled drug delivery to the lungs.

Currently, *N*-acetylcysteine (NAC) is only Food and Drug Administration-approved reducing agent (26, 129). However, the efficacy of NAC is limited due to low patency and requirement for high dose delivery. Therefore, novel mucolytics demonstrating effective reducing capabilities should be identified. Multiple mucolytic agents, such as tris(2-carboxyethyl) phosphine (TCEP) and mucolytic compounds, P-3001 and P-2119, have been shown to depolymerize MUC5B and MUC5AC, reverse mucus plugging in mouse models (149). However, efficacy has not been compared to NAC. Ongoing work will be focused on using mucin-based biomaterials (developed in **Chapter 4 and 5**) to assess the efficacy of aerosolized mucolytic agents on disrupting mucin intermolecular bonds and improving drug delivery.

In our preliminary work, we designed a 3D-printed synthetic mucus array compatible with a 96-well plate capable of high-throughput assessment of mucolytic agents (**Fig 7.2**). The synthetic mucus array allows mucin-based hydrogels to be cast in individual

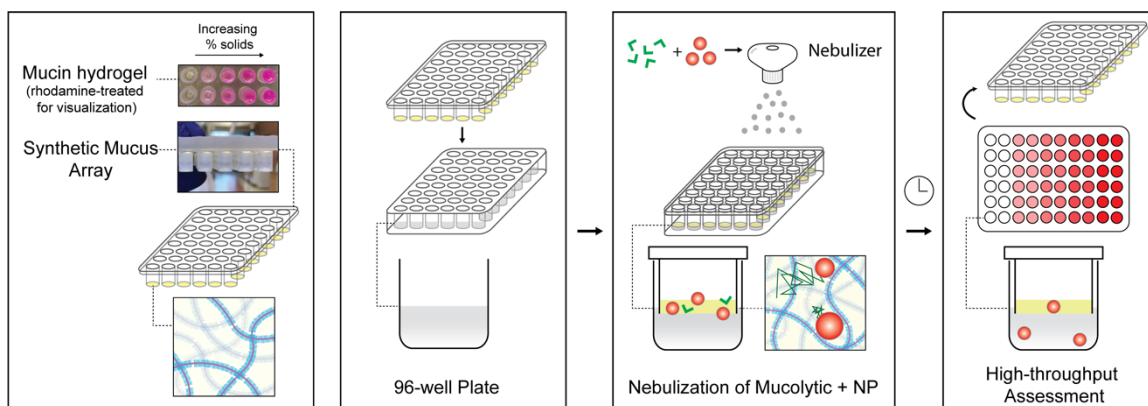


Figure 7.2 Overview of high-throughput assessment of mucolytics using synthetic mucus array. 3D-printed synthetic mucus array (compatible with a 96-well plate) was designed high-throughput assessments of mucolytics. Mucin hydrogels can be cast into the synthetic mucus array with varying concentration or composition. Varying concentrations of nanoparticles (NP) and mucolytics can be nebulized on the synthetic mucus hydrogels and incubated. Diffusion of nanoparticle can be assessed using fluorescence.

wells. These disulfide-linked hydrogels can serve as a physicochemical barrier for nanoparticles diffusion. In future studies, we will deposit fluorescent muco-inert nanoparticles (MIP), similar in size to drug delivery vehicles, onto the synthetic mucus array together with mucolytic agents through nebulization. The synthetic mucus arrays will be incubated with the MIPs and mucolytic agent to allow disulfide disruption and diffusion through the mucin gels. We will calculate efficacy of mucolytic by estimating the percentage of MIP diffusion through the mucin hydrogels using fluorescence reading in a conventional plate reader. The synthetic mucus array will be useful in determining mucolytic efficacy, while also allowing assessment of diffusion of various nanosized entities through mucus gel. Importantly, the use of our mucin-based biomaterial will allow control of mucin composition of gels to model different stages of asthma, while also allowing control of the percent solids concentration. This work will be able to contribute to identifying effective mucolytic agents that can reverse pathological effects of mucus accumulation, while enhancing efficacy of drug delivery.

7.2.2 MUC5AC-targeted Gene-Therapy Strategies

Current treatments for asthma focus on the management of symptoms rather than addressing the progressive and permanent pathological changes to the airways. While mucolytic strategies could offer temporary relief by directly targeting mucus-related obstruction and enhancing nanoparticle delivery to the airways, alternative methods that can reverse the development of life-threatening pathologic features of asthma should be explored. Gene-therapy has the potential means to intervene and control disease progression in asthma by providing modulatory expression in a therapeutic manner.

Recent studies in MUC5AC knock-out mice have demonstrated that the absence of MUC5AC reduces mucus plug formation after allergic stimulation (78). In HAE cultures, gene targeting of SAM pointed domain containing ETS transcription factor (SPDEF) prevented IL-13 induced mucociliary clearance impairment (124) and blocking NOTCH 3, a key mediator in airway epithelial cell differentiation, reduced production of MUC5AC in HAE cells from asthmatic patients (150). These studies highlight the pathological impact of excessive MUC5AC expression, while also demonstrating the possibility of MUC5AC modulation and its therapeutic effects.

Our studies in **Chapter 6** demonstrate direct modulation of mucins in a human airway epithelial model is possible and provides significant motivation for developing MUC5AC-targeting gene therapy strategies. We hypothesize that selective modulation of MUC5AC expression could prevent mucus aggregation and mucus plugging but also have broader therapeutic applications in other respiratory diseases, such as cystic fibrosis and chronic obstructive pulmonary disease.

7.2.3 Addressing Asthma-related Health Disparities through Research

The prevalence of asthma is significantly greater among racial and ethnic minorities and the populations with the highest asthma prevalence tends to be the least responsive to commonly prescribed asthma therapies (20). In addition, a recent study examining pediatric asthma outcomes showed that there are racial/ethnics differences in eligibility for asthma biologics among pediatric populations demonstrating differential effects as well as limitations of current biologics. (151). While these differences shed important insight into the need for future studies of asthma biologic therapies in racially/ethnically diverse populations, these studies also highlight the need for continued research efforts that improve understanding of disease process that can contribute to more effective assessment of clinical severity in patients and identify universal therapeutic goals that can address the key pathological changes to the airways in asthma.

7.3 Contributions

The work presented in this dissertation has resulted in multiple publications, conference proceedings, and awards. **Chapters 2, 4, and 5** are adapted into this dissertation with permission from journals as indicated and **Chapter 6** has been submitted for publication. I have also presented the related work at 3 conferences and am preparing for two upcoming conferences. My research was partially supported by the Ann G. Wylie Dissertation Fellowship and the Graduate School Summer Research Fellowship.

Peer-reviewed Journal Publications:

- Iverson, E., Griswold, K., **Song, D.**, Gagliardi, T., Hamidzadeh, K., Kesimer, M., Sinha, S., Perry, M., Duncan, G., Scull, M. “Membrane-Tethered Mucin 1 is stimulated by interferon in multiple cell types and antagonizes Influenza A virus Infection in Human Airway Epithelium.” *mBio*. e01055-22. 2022
- Kaler, L., Iverson, E., Bader, S., **Song, D.**, Scull, M., Duncan, G. “Influenza A virus diffusion through mucus gel networks.” *Communications Biology*, 5 (249). 2022.
- Gagliardi, T., Goldstein, M., **Song, D.**, Gray, K., Jung, J., Ignacio, M., Stroka, K., **Duncan, G.**, Scull, M. “Rhinovirus C replication is associated with the endoplasmic reticulum and triggers cytopathic effects in an in vitro model of human airway epithelium”. *PLOS Pathogens*. 18(1), e1010159. 2022.
- **Song, D.**, Iverson, E., Kaler, L., Bader, S., Scull, M., Duncan, G. “Modeling airway dysfunction in asthma using synthetic mucus biomaterials.” *ACS Biomaterials Science & Engineering*. 7(6), 2723-33, 2021.
- Iverson, E., Kaler, L., Agostino, E., **Song, D.**, Duncan, G., Scull, M. “Leveraging 3D Model Systems to Understand Viral Interactions with the Respiratory Mucosa.” *Viruses*. 12(12). 1425, 2020.
- **Song, D.**, Cahn, D., Duncan, G. “Mucin Biopolymers and Their Barrier Function at Airway Surfaces.” *Langmuir*. 12773-12783, 2020. (*Selected as ACS Editor’s Choice*)
- Joyner, K. *, **Song, D.** *, Hawkins, R., Silcott, R., Duncan, G. “A rational approach to form disulfide-linked mucin hydrogels.” *Soft Matter*. 15, 9632-9639, 2019.

(* denotes equal contribution)

In Preparation:

- **Song, D.**, Iverson, E., Kaler, L., Scull, M., Duncan, G. “MUC5B mobilizes and MUC5AC spatially aligns mucociliary transport on human airway epithelium.”
Submitted. 2022.

Conference Presentations:

- **American Thoracic Society 2022** – *Poster*
- **Society for Biomaterials 2022** – *Oral*
- **American Thoracic Society 2020** – *Poster*
- **Biomedical Engineering Society 2019** – *Oral*
- **Gordan Conference on Cilia, Mucus, and Mucociliary Interactions 2019** –
Poster

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