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Mucus, Mucins and Cystic Fibrosis

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Abstract

Cystic fibrosis (CF) is both the most common and most lethal genetic disease in the Caucasian population. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene and is characterized by the accumulation of thick, adherent mucus plaques in multiple organs, of which the lungs, gastrointestinal (GI) tract and pancreatic ducts are the most commonly affected. A similar pathogenesis cascade is observed in all of these organs: loss of CFTR function leads to altered ion transport, consisting of decreased chloride and bicarbonate secretion via the CFTR channel and increased sodium absorption via ENaC channel upregulation. Mucosa exposed to changes in ionic concentrations sustain severe pathophysiological consequences. Altered mucus biophysical properties and weakened innate defense mechanisms ensue, furthering the progression of the disease. Mucins, the high-molecular-weight glycoproteins responsible for the viscoelastic properties of the mucus, play a key role in the disease but the actual mechanism of mucus accumulation is still undetermined. Multiple hypotheses regarding the impact of CFTR malfunction on mucus have been proposed and are reviewed here: 1) dehydration increases mucin monomer entanglement, 2) defective Ca^{2+} chelation compromises mucin expansion, 3) ionic changes alter mucin interactions and 4) reactive oxygen species (ROS) increase mucin crosslinking. Although one biochemical change may dominate, it is likely that all of these mechanisms play some role in the progression of CF disease. This article discusses recent findings on the initial cause(s) of aberrant mucus properties in CF and examines therapeutic approaches aimed at correcting mucus properties.

Keywords

Mucus; Mucins; Polymeric Network; CFTR; Biochemical Interactions; Viscoelastic Properties

Introduction:

CF is a multi-organ disease with symptoms affecting tissues that express CFTR and produce mucus, among those, the lungs and the GI tract[1–3]. Mucus accumulation in the airways, the intestine, and the pancreatic ducts plays a critical role in the disease pathogenesis by compromising airflow and nutrient digestion. Since the resulting progressive lung disease

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can lead to respiratory failure and ultimately death, this review focuses on studies conducted on airway mucus, more specifically on secreted mucins lining the respiratory tree. Mucins are complex macromolecules that govern the biophysical properties of mucus. In the lungs, gel-forming mucins are secreted by goblet cells distributed throughout the conducting airways and submucosal glands located in the large airways[4,5]. Airway goblet cells secrete MUC5B and MUC5AC to produce a thin mucus layer that lines the epithelial surfaces. In contrast, submucosal glands secrete only MUC5B, which is expulsed from the gland ducts in the form of strands intended to sweep the large airways and remove inhaled pathogens[6,7]. Although both airway surfaces and glands produce mucus, the biophysical and biochemical properties of mucus produced by these two compartments may be affected differently by CFTR malfunction and therefore may play distinct roles in the progression of the CF lung disease[7]. In CF animal models that possess submucosal glands (e.g., pigs and rats), gland hypotrophy and plugging occur at different ages (i.e., newborn vs 6 months) and therefore lung/gland maturation may play a critical role in the progression of the CF lung disease[8,9]. Since the specific role of gland mucus strands in disease is still being elucidated, this review will focus on current knowledge of the impact of CFTR malfunction on the ambulatory mucus layer progressing on top of the cilia[8,10].

The viscoelastic properties of the mucin gel lining the airways are critical for proper trapping and clearance of pathogens. Gel properties rely heavily on cys-rich regions scattered throughout the mucin protein backbone to organize a complex disulfide-stabilized polymeric network[11]. Adding to the complexity, the physicochemical properties of mucins are furthermore governed by the large O-linked oligosaccharide chains decorating the apomucin core, which can contribute up to 80% of the mucin molecular mass[12]. The O-glycans are designed to ensure high volume occupancy in solution as well as high water-holding capacity for mucin gels and are important for regulating mucin-mucin, mucin-pathogen, and mucin-mucosal surface interactions[13]. Although mucins are produced to protect the mucosa against pathogens, dysregulation of mucin secretion rate, concentration, expansion, and/or interactions can compromise the protective role of the mucus layer. In this review, we discuss how CFTR malfunction can affect mucin biochemical interactions and alter the viscoelastic properties of mucus. Mucins and mucin gel formation are briefly described herein, followed by in-depth examination of the impact of CFTR on mucin concentration, polymer compaction, mucin-mucin interactions, and the impact of oxidative stress on the mucin network. We conclude by opening the discussion on current and novel pharmacological approaches aimed at altering the mucin network (i.e., mucoactive agents).

1. Mucus and Mucins:

Healthy airway mucus is composed of approximately 90-95% water, 1-5% mucins and other proteins, 1-2% lipids and 1% salt electrolytes[12]. Although hundreds of proteins have been discovered in airway mucus, mucin proteins primarily govern the viscoelastic gel properties of the mucus layer[11,12,14]. Mucins are large (up to 50 MDa) and heavily glycosylated, but polymeric organization and intracellular packaging are tightly regulated to ensure the rapid release of high-quality viscoelastic gels[12,15]. Understanding the structure and organization of a polymeric mucin gel is critical to investigating how CFTR malfunction can affect its biochemical and biophysical properties.

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1.1 Mucin Structure

Mucins are encoded by *MUC* genes, a gene family consisting of 18 different proteins[16]. Mucins can be divided into two groups: the secreted (or gel forming) and the tethered (or membrane-bound) mucins. MUC5B and MUC5AC are the major gel-forming mucins expressed in the airways and are predicted to possess different airway clearance functionalities[6,7]. In the GI tract, MUC2 is the primary gel-forming mucin expressed that, along with lesser amounts of MUC6, protects the epithelium from acidic pH, lubricates the intestine to facilitate transit, and shields the mucosa to prevent pathogen invasion[17]. All gel-forming mucins share a similar core protein, referred to as the apomucin. The protein core features von Willebrand factor (vWF) domains in the N- and C-terminal regions, cysteine-rich domains (CysD) scattered throughout the protein backbone including the cysteine knot (CK) at the C-terminal end, and a variable number of tandem repeat (VNTR) region that undergoes extensive glycosylation and constitutes the bulk of the protein mass (Figure 1).

An important process for the biophysical properties of mucin gels is the polymeric organization of the mucus, a process dependent on the capacity of mucin monomers to form dimers and multimers via C- and N-terminus disulfide bond formation, respectively[11]. CK domains in the C-terminus are responsible for monomer dimerization, while vWF domains in the N-terminus are responsible for the formation of linear polymers and/or more complex multimeric networks[11,15]. In CF, extracellular DNA originating from dying inflammatory cells entrapped in mucus can be present in high concentrations and add to the complexity and entanglement of the polymeric mucin network, increasing gel viscosity and worsening symptoms of the disease[18].

During mucin protein maturation, O-glycosylation of serine and threonine residues distributed through the VNTR occurs in the endoplasmic reticulum. This is a critical step in establishing the biological properties of mucins, as oligosaccharide composition and terminal sugar negative charges contribute to mucus swelling via electrostatic interactions and pathogen trapping via specific binding[13,19,20]. Glycosylation is initiated by the linkage of N-acetyl-D-galactosamine, one of three steps for mucin glycosylation that can yield eight different core structures. Following the glycan core addition, a backbone structure is linked followed by the addition of a peripheral terminal sugar, which is the source for mucin glycan variation. The number of glycans per amino acid, their distribution pattern, and their size vary from mucin to mucin, giving each mucin a unique glycosylation profile and therefore specific biological properties[13].

Recent work has shown that mucin proteins interact with hundreds of other proteins present in the mucus layer, thus further affecting mucus viscoelasticity and giving rise to the term “the mucus interactome” to describe the relationship between globular proteins and mucins[21].

1.2 Mucin Sources

In the lungs, gel-forming mucins are secreted by surface goblet cells and submucosal glands. Unlike goblet cells that produce both MUC5AC and MUC5B, submucosal glands only secrete MUC5B[4,7]. These two mucin sources may play different roles in health and disease,

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but additional work is needed to determine their individual functions in the lungs. Mucins at the surface epithelium are secreted constitutively at basal levels to facilitate airway maintenance but can also be acutely stimulated for extensive degranulation following exposure to a challenge like smoke, allergens, or pathogens[22,23]. Complex cholinergic and purinergic signaling pathways control the rate of mucin granule release from the glands and the epithelial surfaces, respectively. In addition, morphological variation has been noted between mucus released from submucosal glands and goblet cells[6,7]. Under cholinergic stimulation, MUC5B is slowly released from submucosal glands and forms "strands or bundles" with diameters of 5-50μm. These strands are then "coated" with thin MUC5AC sheets following their release from goblet cells, resulting in a MUC5B core bundle enveloped by an outer layer of MUC5AC[6,7]. It has been postulated that these unique structures are tailored for mucociliary transport of large (>300 μm) inhaled particles[10]. In contrast, surface goblet cells possess heterogeneous populations of MUC5AC-rich and MUC5B-rich granules within the same cell, and granule release can be regulated by different exocytotic pathways (i.e., basal vs. stimulated), suggesting that mucin secretion is a delicate event that requires accuracy and precision[22].

Several signaling pathways can initiate mucin granule exocytosis, such as epidermal growth factor (EGFR), toll-like-receptors (TLRs), and cholinergic (Ach) and purinergic (P_2Y_2) stimulation[23]. Mucin production can be signaled by extracellular-regulated kinase (ERK) and activation of nuclear factor κB (NF- κB)[23]. Cytokines like IL-13 and IL-1 are also important regulators of mucin hypersecretion and influence goblet cell hyperplasia[24,25]. If airway challenges persist, goblet cell hyperplasia, metaplasia, and gland hypertrophy can ensue, substantially increasing the amount of airway mucus produced. These phenotypes are commonly observed in muco-obstructive diseases such as chronic bronchitis, COPD, asthma, and CF[26].

1.3 Mucin Network Organization and Interactions

The structure and function of mucin gels lends itself to a diverse array of potential interactions. Although mucus consists of up to 95% water, its viscoelastic gel properties are the result of disulfide bonds, hydration capacity, and non-covalent interactions. The intracellular environment and extracellular milieu dictate how mucin packing, expansion, and interaction transpire. Recent investigations into intracellular arrangement and post-secretory expansion have provided key insights into how disease states can alter mucin interactions.

Mucin exocytosis from goblet cells is extremely rapid and can take only tens of milliseconds to occur once initiated[20]. Prior to exocytosis, these large biopolymers, which can reach up to 10^9 Da, are tightly packed inside granules ranging in size from 0.5 to 2 μm, and, for this reason, mucin storage requires a high level of organization for both packaging and subsequent expansion[15,27]. The packing of linear MUC5B polymers is governed by high Ca^{2+} concentration and acidic pH inside the mucin granules. The presence of calcium and hydrogen ions inside the granules shields the negatively charged glycans decorating the apomucins and prevents electrostatic repulsion, allowing the mucin polymers to organize into nematic arrangements[15,20]. The resulting compacted MUC5B possesses a central N-

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terminus core and outward facing C-termini, allowing for the formation of linear strands upon release, a feature advocated to be essential to its putative function of cleaning large inhaled particles[10]. The intracellular organization of MUC5AC is similar, with C-terminal dimerization occurring via disulfide bonds in the ER followed by covalent linkage of the N-termini in the Golgi and analogous packing[28].

Upon exocytosis, mucin macromolecules undergo dramatic volume expansion (up to 4000-fold), a rapid and critical process that relies on Ca^{2+} chelation and osmotic pressure[20]. To ensure a fast swelling rate upon exocytosis, organized folding and unfolding of the mucins is required to avoid extreme frictional heat generation[20]. The extracellular environment is more alkaline, higher in bicarbonate and sodium ion concentration, and lower in calcium ion concentration. Upon opening of the granule in extracellular milieu, hydrogen bonds that stabilized the mucins intracellularly are broken due to increased pH. Simultaneously, calcium ions are chelated by bicarbonate and exchanged for sodium ions, promoting additional relaxation of the mucin network[20,29]. These ionic changes lead to a large internal osmotic gradient and subsequent influx of water. Swelling stops once an equilibrium between osmotic pressure and the intrinsic elastic component of the matrix is reached.

Once secreted and expanded, mucins are subject to a variety of intermolecular interactions. Acidic side chains and negatively charged polysaccharides contribute to the intermolecular formation of stabilizing salt bridges and electrostatic interactions[30,31]. Adding to the complexity, as the CF disease progresses mucus biophysical properties are altered by reactive oxygen species (ROS) and/or airway inflammation[32].

To summarize, the noncovalent and covalent interactions, coupled with concentration-dependent interpenetration of the mucin polymers, help shape the rheological landscape for mucin gel networks. In the next section, we will discuss how CFTR dysfunction and restoration of function can impact the mucin network with regards to physical, biochemical, and biological interactions.

2. Mucus and Mucin Interactions at the CF Airway Surface:

Static mucus, as observed on CF airway surfaces, is an ideal environment for bacterial colonization as well as inflammation with the recruitment of neutrophils that results in irreversible lung damage[33]. Understanding the biochemical processes that occur once mucins are secreted into the airway surface liquid (ASL) layer and how they relate to the impairment of mucociliary clearance is crucial to developing effective treatments for patients with CF. Several hypotheses have been proposed to explain how CFTR malfunction affects the mucin polymeric network (see Figure 2). Aberrant CFTR-mediated Cl^- and HCO_3^- secretion and dysregulation of ENaC-mediated Na^+ transport results in ASL water hyperabsorption and subsequent dehydration of the mucus layer. Hyperconcentrated mucins in the ASL are subject to increased polymeric entanglement with new/increased solute-mucin interactions resulting from a concentration increase of non-salt molecules in the ASL. In parallel, failure of CFTR to transport bicarbonate can lead to acidification of the ASL and changes in the ionic interactions within the mucus layer. Oxidative stress and inflammation can result in atypical covalent mucin interactions and potentially change the viscoelastic

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properties of the gel. All of these physicochemical changes occur at the airway surface and can alter the viscoelastic properties of the mucus.

2.1 Hyperconcentration and Mucus Gels

Dysfunctional CFTR regulation of ENaC-mediated Na^+ transport is accepted as the driving force behind the decreased ASL volume in CF airways, which ultimately increases the osmotic pressure of the mucus layer and collapses the cilia[34,35]. In addition to altered ion and fluid transport, the CF airways are characterized by goblet cell and glandular hyperplasia and subsequent overproduction of MUC5B and MUC5AC[26]. As a result, higher mucus concentrations have been reported in both CF patients and CF model systems[9,36–38]. Coupled together, mucus hypersecretion and airway dehydration produce an ASL with percent solids reaching 5-times that of normal levels[37,38].

From a polymer physics point of view, two critical concentration-dependent transition points contribute to the biophysical behavior of mucins in the ASL: the semi-dilute overlap concentration (c^*) and the entanglement concentration (c^e)[34]. The transition from un-interacting oligomers to an un-entangled regime, characterized by mucin chain overlap, is reported to occur at roughly $c^* = 1$ mg/ml for MUC5AC and MUC2[30,39]. These mucins transition from the un-entangled overlapping regime to an entangled regime characterized by mucin chain interpenetration and reptation at c^e of 25 mg/ml and 30 mg/ml for MUC5AC and MUC2, respectively[39]. The incident regimes under normal mucin concentration and pH values physiologically relevant to the lungs (i.e. ~2% solids and $7.0 < pH < 7.2$) reflect a semi-dilute overlapping network, whereas the gastric mucus is normally an entangled gel in the normal mucin concentrations and low pH values of the stomach ($pH < 2$)[38]. MUC5AC viscosity scales with concentration and behaves as a purely viscous fluid at concentrations under 25mg/mL (~3% solids including airway salts). Above 25 mg/mL (~3.5% solids), MUC5AC solutions become viscoelastic, exhibiting both viscous and elastic behavior. Since CF mucus regularly reaches concentrations above 5% solids, it falls into the latter category, i.e., dominated by elastic gel-like behavior[34,37].

The relationship between physiologically relevant mucin concentrations and rheological properties was recently examined and revealed that airway mucus complex viscosity scales with concentration[38]. Furthermore, macro and microrheology performed on bovine submaxillary mucus (BSM), porcine gastric mucus (PGM), and CF and non-CF human bronchial epithelial (HBE) cell-culture mucus over a range of concentrations showed consistency across mucus types[38]. Geometric scaling was consistent between concentration and complex viscosity, and this held true over the pH range of 6-8. This relationship to concentration also correlated to changes in mucociliary transport (MCT) and ASL osmotic properties. Hydration therapy (returning to ~2% solids) profoundly corrected CF sputum samples to near-normal viscoelasticity, reinforcing the clinical findings that administration of hydrating agents such as hypertonic saline yields beneficial results in CF patients[40–42] (see section 3.1).

A secondary consequence of increased mucin concentration in CF airways is that the increased proximity of mucin polymers results in new/stronger interactions with other protein and ionic solutes that can also affect the viscoelasticity of the gel[31]. Indeed, while

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physiological mucus behavior is largely governed by the amount of available solvent, mucin chain dynamics in solution are also dependent on amount of noncovalent interactions (i.e. steric hindrance from charged residues and/or glycans, as well as intermolecular salt bridges). Addition of salt to MUC5AC networks can increase the viscosity of the gel. This has been attributed to stronger intermolecular interactions taking place as a result of reduced electrostatic repulsion of the polysaccharide side chains in the presence of salt[31] (this is discussed in greater detail in section 2.3). Chaotropic agents such as guanidine HCl and urea also reduce mucus viscoelasticity[30]. This is due to the disruption of hydrophobic interactions and the subsequent unfolding of the mucin protein backbone. Accordingly, the next sections discuss the relevant mucin-solute interactions that contribute to airway mucus physiology and CF pathophysiology.

2.2 Ca^{2+} Chelation & Mucus Compaction

While predominantly a Cl^- channel, CFTR also plays a general anion channel role, and therefore, mutations within CFTR can further influence the biophysical properties of mucus beyond dehydration[43]. In particular, much attention has been given to the channel permeability to HCO_3^-, both in terms of pH and polycation chelation.

Inside mucin granules, the environment is rich in Ca^{2+} ions and low in pH. Mucin chains are condensed in the granule as nematic arrays via counterion shielding[15,20]. The negatively charged glycans that would normally repel each other are shielded by Ca^{2+} ions. A single Ca^{2+} ion can neutralize two negative charges, supporting the formation of a compact intragranular mucin matrix with adjacent terminal glycans. It has also been proposed that the vWD domains in the N-terminus selectively bind calcium during granule packing[15]. Upon exocytosis, Ca^{2+} ions are exchanged for two Na^+ ions, as Na^+ concentration predominates by over an order of magnitude in the ASL[20,26]. This local increase in internal cation concentrations due to Na^+ influx draws in water and causes swelling of the matrix via a Donnan effect. Simultaneously, bicarbonate, also abundant in healthy ASL, chelates the Ca^{2+} ions that were exchanged for Na^+, effectively sequestering the Ca^{2+} from reforming an ionic crosslink between charged mucin glycans.

Improper calcium chelation has been noted as a possible cause for CF pathogenesis[3]. Irregular expansion of secreted mucins is seen in CF models and can be attributed to ionic imbalances, particularly related to the lack of bicarbonate and chloride transport through CFTR[29,44,45]. Incompletely unfolded MUC5B was detected in CF saliva, suggesting that terminal unpacking of secreted mucins occurs on longer timescales than previously thought[46]. Persistent deficiency of key counterions in CF likely contributes to keeping the secreted mucins in a semi-expanded state causing mucus stasis and plugging, though hyperconcentration in the ASL was shown to be the predominate cause of incomplete mucin maturation[46].

Counterion donation during unpacking also affects MCT rates. Addition of zwitterionic HEPES buffer had no effect on MCT, whereas Tris buffer or addition of bicarbonate increased MCT in CF rats, and bicarbonate addition to ileum sections of CF mice normalized mucus properties[9,45]. Hence, identifying the counterions involved in the mucin unfolding and subsequent viscoelastic properties of secreted mucins is critical. Addition of

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calcium to pig tracheas increased ASL viscosity, but other divalent ions like magnesium and zinc had no rheological effect[47]. This distinction is important when considering the development of chelating compounds as therapies for CF. Although calcium chelation during mucin exocytosis may be crucial to the unfolding of the mucin, a continuous low-calcium environment is necessary to promote normal rheological properties[47]. If altered ionic fluxes persist after secretion, normally unfolded mucins can begin to interact in an aberrant manner, possibly causing increases in ASL viscosity.

2.3 Acidic pH & Mucus Gels:

The role of CFTR in bicarbonate transport and subsequent acidification in CF airways and models has been known for years[48]. Mucus pH in CF nasal airways has been measured as low as ~6.5, and *in vitro* models of CF have shown greater susceptibility to pH changes than controls[49,50]. Even small changes in hydrogen ion concentration may have important consequences given how resiliently the body regulates pH and have been implicated in the increased susceptibility of CF patients to infection[51].

Recent work showed an inverse correlation between the pH and viscosity of MUC5AC gels[30,31]. MUC5AC begins to act more like a solid at low pH levels (pH < 4), shown rheologically by a decrease in tan (δ), the ratio of the loss modulus (G”) to the storage modulus (G’). The storage modulus increases with decreasing pH, indicating that the mucus becomes more gel-like, evidence of additional cross-linking and/or increased mucin interaction in the gel-phase. Biochemically, this can be explained by protonation of the carboxyl side chains of residues such as glutamic and aspartic acid, leading to disruption of intramolecular salt bridges and causing the mucin to unfold. Unfolding of the mucin exposes hydrophobic sites hidden in the native conformation of the protein. The newly exposed hydrophobic domains govern the interaction of the now-neutrally charged mucin molecules, reducing the elasticity and increasing the viscosity of the MUC5AC gel. This is in agreement with additional studies on microrheology of gastrointestinal mucins (MUC2) at low pH levels[30]. Salt bridge protonation and hydrophobic interactions are likely the driving forces behind the sol-gel transition of mucins below pH 2.

These pH ranges are physiologically relevant to the stomach and portions of the GI tract but not to human airways. Therefore, a few important distinctions must be made when comparing data from recent publications. While the protonation of carboxyl side chains in the protein backbone is a probable mechanism for increasing intermolecular interactions and gel viscosity, it is crucial to note that the pKa’s of glutamatic and aspartic acid are 4.15 and 3.71, respectively. At pH values of ~2, such as in the stomach, these side chains are ~99% protonated. However, with hydrogen ion concentrations 10^4-10^6 lower (i.e., pH 6.5), such as in the lungs, the carboxyl side chains of the acidic amino acids remain deprotonated. Examination of porcine ASL viscosity in the pH range of ~6.5-8.0, much more physiologically relevant to human airways, revealed that ASL viscosity at the small length-scales probed by fluorescence recovery after photobleaching (FRAP) assays increased slightly under acidified conditions[47]. However, since increased ASL viscosity as a function of acidity did not correlate with disulfide bond formation nor bicarbonate concentration, it is likely due to altered electrostatic interactions between mucin molecules. Although viscosity

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changes were modest (e.g., 4-6 fold) compared to the several-log fold changes with respect to concentration reported in GI and airway mucus studies, small changes in pH can have critical physiological consequences and contribute to CF pathogenesis by altering MCT[30,38].

2.4 ROS & Mucus Crosslinking:

Evidence of increased levels of ROS in CF patients has been documented for decades and is thought to play a role in the progression of CF pulmonary dysfunction[52]. Inflammatory immune cells, notably polymorphonuclear (PMN) neutrophils, produce oxidants (such as superoxide anion, hydrogen peroxide, and hypochlorous acid) when defending against infection[52]. Myeloperoxidase (MPO), a pro-oxidant enzyme secreted by PMN neutrophils, is present in the ASL of CF patients and methionine sulfoxide, a byproduct of MPO oxidation, has been correlated with early CF lung disease and bronchiectasis[52,53]. This is of particular interest because recent findings showed that neutrophil-dominated inflammation occurs in the absence of infection[36,54]. Additionally, lower concentrations of the antioxidant glutathione (GSH) were measured in CF bronchoalveolar lavage fluids, furthering the potential for oxidative damage[55].

Limited data exists on the effects of ROS on mucins. While cysteine residue distribution for the same mucus concentration was similar between CF and non-CF controls, disulfide bond concentration was higher in the CF population and correlated with ROS levels[32]. Furthermore, simulated crosslinking of healthy mucus samples with DMSO or oxygen (O_2 gas mimicking treatment for hypoxia) increased the elastic modulus of the solution[32]. Disulfide bond formation is a crucial step to proper mucin synthesis and function; however, excessive disulfide bridging may play a role in the pathophysiology of muco-obstructive diseases. The likely explanation is that high levels of MPO induce posttranslational oxidative modifications of cysteine residues. In CF and asthma, inflammatory enzymes released by neutrophils and eosinophils, respectively, initiate the oxidation of free thiols/cysteine to form additional disulfide bridges or cystines[32,56]. The reaction is speculated to involve myeloperoxidase (MPO) or eosinophil peroxidase (EPO) entrapped in mucus that catalyzes the reaction of H_2O_2 with thiocyanate to generate potent local oxidants. The subsequent increase in mucin disulfide interactions causes polymer network crosslinking and stiffening of the mucus gel. The viscoelastic changes resulting from mucus oxidization were of a similar magnitude to that of 5-fold concentration changes. These bonds are potential therapeutic targets and can be broken by reducing agents such as NAC, dithiothreitol (DTT), and tris(2-carboxyethyl)phosphine (TCEP) (see section 3.5).

3. Pharmacological Approaches Targeting Mucus:

Improvement of mucus clearance in CF is key to preventing declines in lung function. CFTR-directed therapies have been shown to improve MCC *in vivo*, with ivacaftor producing a ~10% increase in MCC in patients with a G551D mutation[57,58]. In the age of CFTR-corrective therapies, patients with mutations not responsive to modulator compounds (e.g., nonsense mutations) will require symptomatic treatment via CFTR-independent approaches. In addition, patients treated with CFTR modulators may still benefit from therapies improving clearance as mucus plugging will persist due to residual infections and

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permanent lung damage. In recent years, therapeutic strategies aimed at changing the viscoelastic properties of mucus have gained momentum. Mucus provides a relevant therapeutic target for all patients regardless of genotype (unlike CFTR modulators) and/or inflammatory status (unlike dornase alfa). Compounds that change the biophysical properties of mucus are commonly called “mucoactive” but do not always work directly on mucins (e.g., dornase alfa breaks down extracellular DNA). As a result of the complexity of mucus and the intricate interactions of the mucin network, a wide range of pharmacological approaches, including osmotic agents, ion channel potentiators and inhibitors, Ca^{2+} chelators, surfactants, and reducing agents are currently being tested or have been approved and are discussed in this section.

3.1 Osmotic Agents

Osmotic agents, compounds that cause water to be drawn into the airway surfaces, have the potential to treat muco-obstructive diseases like CF and COPD by “reversing” the effects of water hyperabsorption via the ENaC channel. Inhalation of hypertonic saline (HS) is clinically efficacious although the precise mechanism of action has not been completely elucidated[40–42]. The current hypothesis is that administration of HS causes fluid influx into the airway lumen. Once water flows into the lumen, it hydrates and swells the ASL, with the magnitude of swelling being governed by mucus concentration (i.e., the higher the mucus concentration, the longer the duration of ASL swelling)[42]. Hence, mucus hyperconcentration in CF provides an additional osmotic driving force, potentially via counterions on the mucin glycans. Interestingly, ENaC inhibition increased the response to HS *in vitro*, indicating that sodium reabsorption likely diminishes the effect of aerosolized HS, which establishes another therapeutic target in CF (see section 3.2).

Supported by a straightforward mechanism of action, many clinical trials have tested the effect of osmotic agents on lung function and MCC in patients with obstructive lung disease. Hypertonic saline (7% NaCl) was first shown to be clinically effective over a decade ago[40,41]. Administration of HS was shown to increase lung function (FVC and FEV_1) and decrease pulmonary exacerbations in CF patients[41]. HS was also shown to increase MCC in a sustained fashion *in vivo* and expedited symptom resolution during hospitalization[40,41].

Another osmotic agent, mannitol, has also shown clinical potential to treat obstructive airway diseases and has been approved in the E.U., Australia, Israel, and recently the USA. Mannitol is an inert sugar that is not absorbed by the GI tract, not metabolized, and does not cross the blood-brain barrier. The mechanism of action is postulated to be similar to HS, exerting its effects by creating an osmotic gradient and drawing water into the airway lumen. In contrast to the Na^+ in HS, mannitol has no transcellular absorption pathway. While Na^+ is hyperabsorbed in CF, mannitol absorption rates are unaffected by the ion transport defects in CF, possibly explaining why it contributes to decreased paracellular small-molecule probe absorption rates as measured using gamma scintigraphy[59]. Inhalation of dry powder mannitol increased FEV_1 in CF patients and improved MCC in healthy, asthmatic, bronchiectasis, and CF subjects[60,61]. Since mannitol can be administered as a dry powder, there is no need for nebulization. This added convenience may reduce treatment time and improve adherence to treatment plans.

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3.2. Non-CFTR Ion Channel Agents

In addition to therapies that directly affect the osmolarity of the ASL, ENaC blockers have the potential to increase ASL hydration by inhibiting active Na^+ absorption via the ENaC channel. While inhibition of ENaC has been shown to positively affect ASL hydration, ENaC-blocking small molecule therapies have, in general, been unsuccessful at producing long-term benefits in CF patients[62]. Lack of efficacy of these drugs can be attributed to their short half-life, while effects on renal ion transport is the key dose-limiting safety concern[63,64]. Currently, several ENaC modulators are being tested in clinical trials. Compound AZD5634 from AstraZeneca recently completed two Phase I trials. AZD5634 was well tolerated both intravenously and inhaled at all dose levels during a Phase Ia pharmacokinetic evaluation, however, data regarding its effect on MCC has not yet been released from the completed Phase Ib trial[65]. VX-371 from Vertex (formerly P-1037) is under investigation as well, being tested alone, in combination with HS, or in combination with orkambi for CF patients and has also been tested in patients with primary ciliary dyskinesia[66].

In CF, ENaC upregulation is partly due to the proteolytic degradation of SPLUNC1, an inhibitory defense protein responsible for cellular internalization of the ENaC protein[67,68]. By this mechanism, SPLUNC1 reduces active sodium absorption via ENaC and, in turn, prevents ASL dehydration. Neutrophil elastase, present in high concentrations in CF sputum, degrades SPLUNC1 and therefore favors ENaC upregulation and/or fluid hyperabsorption[69]. SPX-101, an ENaC blocker created by Spyryx Biosciences, is a peptidomimetic compound that resembles the active ENaC-inhibiting region of the SPLUNC1 protein and is not degraded by proteases found in CF sputum[70]. SPX-101 has been tested in clinical trials in patients with CF, and non peer-reviewed data from a Phase II study shows a 5.2% increase in FEV_1 in SPX-101 treated patients compared to the placebo control group[71].

Another approach is to potentiate other chloride channels already expressed in the lungs. TMEM16A or anoctamin-1 (ANO1) is a voltage-sensitive calcium-activated chloride channel (CaCC) expressed in epithelial tissues such as the gut and the airways. Like CFTR, TMEM16A has the capacity to regulate Cl^- currents directly or indirectly via the control of CFTR-mediated Cl^- secretion[72]. Counterintuitively, inhibition of TMEM16A is speculated to promote clinical benefits, as TMEM16A expression was linked to goblet cell metaplasia[73,74]. In parallel, TMEM16A potentiators are currently being tested *in vitro* by Enterprise Therapeutics; the drugs were reported to positively stimulate anion conductance and fluid secretion in CF cultures[75]. With the same objective of increasing Cl^- secretion, denufosol, a P_2Y_2-receptor agonist that stimulates Ca^{2+} elevation and activates CaCC channels, made its way to clinical trials in 2008. However, inhalation of denufosol for 48 weeks failed to improve pulmonary function in CF patients[76,77]. TMEM16A may provide an alternate route to address muco-obstructive lung disease but warrants further investigation. Another anion channel, SLC26A9, is also being studied as a potential therapeutic target in CF, although limited data exists at this time[78,79].

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3.3 Chelating Agents

In CF, the secretion of bicarbonate, an alkalinizing but also a chelating agent, is diminished[80]. As a result, calcium chelation, a critical step during mucin granule exocytosis, may be hindered and mucin maturation (i.e. transition from a compacted to an expanded form) may be compromised[46]. The addition of high concentrations of bicarbonate to CF airway model systems like the CF rat and pig trachea has been shown to increase MCT rates and change the proportion of condensed/expanded mucins[9,46]. Conversely, the addition of free calcium ions to pig tracheal surfaces was found to increase ASL viscosity, supporting that calcium sequestering is necessary for healthy airway mucus rheology[47]. Zinc and magnesium, both also divalent cations, do not change the ASL viscosity; therefore, chelation therapies specifically targeting calcium are of particular interest[47].

Ethylenediaminetetraacetic acid (EDTA), a hexadentate ligand capable of chelating metal ions, has been used in CF models to bind calcium and normalize mucus properties[45]. Compared side by side on mucus from the ileum of CF mice, EDTA induced rheological changes that were similar to bicarbonate at roughly 6-fold lower concentrations (20 vs. 115 mM)[29]. A similar compound more selective for calcium chelation, ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), has also been used in CF research to provide calcium chelation at even lower concentrations than EDTA[47]. Unfortunately, tissue integrity was found to be compromised by both 20 mM EDTA and 115 mM bicarbonate, suggesting that these compounds are likely not suitable for human treatment[29].

OligoG, a guluronate-rich alginate with a high affinity for calcium ions, facilitated the removal of adherent mouse ileum mucus at concentrations as low as 1.5%, while showing no effect on tissue integrity at concentrations as high as 6%[81]. It has been speculated that concentrations of 1.5% can be reached in the lungs via dry powder inhalation[81]. One Phase II clinical trial was recently completed and another trial is currently underway for the treatment of CF patients with OligoG[82]. Preliminary data from a recent Phase II trial showed that although there was not a significant difference in MCC, a strong trend towards a more peripheral deposition of tracer particles was observed in subjects treated with OligoG, suggesting the opening of previously occluded airways[82].

Recently, a polycationic biopolymer, poly (acetyl, arginyl) glucosamine (PAAG) was used in *in vitro* and *in vivo* models of CF to displace Ca^{2+} and promote optimal expansion and linearization of mucins upon exocytosis[83]. The high-molecular-weight polyglucosamine significantly improved the viscoelastic properties of CF sputum and CF HBE mucus. In addition, PAAG increased ciliary beat frequency and MCT in CF HBE cultures, which correlated with the alteration of the MUC5B network ultrastructure towards a more linear organization. Treatment of CF mice via oral gavage and CFTR-KO ferrets via aerosolization resolved intestinal and airway plugging, respectively. Furthermore, PAAG-treated ferrets revealed significantly lower levels of inflammatory markers, suggesting Ca^{2+}-chelating agents may be a valid therapeutic approach to treat patients with CF.

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3.4 Surfactants

Surfactants are surface-active agents that reduce the surface tension between a liquid and another substance (e.g., liquid, gas or solid) and work by interfacing between the hydrophilic and hydrophobic components of a solution. Relevant to polymer gels, the addition of surfactants can interact with the hydrophobic regions of the mucin network as well as the interface of the ASL. The disruption of hydrophobic interactions thus disperses the mucin-rich fraction of the gel, creating a more homogenous or a single phase gel with lower viscosity[31]. Adding surfactant to an electrostatically charged system like a mucin gel initiates the formation of polymer-surfactant aggregates and, at a critical concentration, a single phase is obtained. Mucin polymers then no longer interact with each other via hydrophobic interactions, reducing surface affinity and increasing water solubility. Hence, adding surfactant increases the homogeneity of a mucin-rich solution and decreases hydrophobic crosslinking of the gel.

Nonionic surfactants such as 1,2-hexanediol effectively reduce both the storage and loss moduli of MUC5AC gels and prevent assembly of mucins via hydrophobic interactions[31]. The elastic modulus decreases with increasing concentrations of surfactant until a single population is governed by Brownian motion[31].

Surfactant interaction with the mucus layer could be relevant as both a treatment option for muco-obstructive diseases as well as a pertinent area of research for drug delivery through mucus gel systems. For example, combining proteolytic enzymes such as papain-palmitate with surfactant compounds in the form of self-emulsifying drug delivery systems increased enzyme activity likely due to improved mucus permeability[84]. Clinical data on surfactants is conflicting, with studies showing positive results in patients with chronic bronchitis, negative results in healthy patients with rhinosinusitis, and neither benefit nor harm in CF patients[85–87].

3.5 Reducing Agents

Another approach different from hydration therapies is fragmentation of the mucin network itself. Therapies targeted at chemically breaking down mucus (mucolytics) have been used for decades. The most common and most effective “mucolytic” used in CF is inhaled dornase alfa, a recombinant human DNase that works by enzymatically digesting extracellular DNA released by dying neutrophils entrapped in mucus[88]. Due to its target, dornase alfa was shown to be most effective in CF patients presenting with inflammation and was found to be ineffective in patients with other obstructive airways diseases (e.g., COPD)[89,90]. Despite targeting a macromolecule other than mucins, the clinical benefits of rhDNase confirmed the notion that an inhaled drug affecting the rheology of airway secretions could improve health outcomes. However, NAC, a thiol-based compound that directly affects the mucin network by cleaving intra- and inter-molecular disulfide bonds, showed only limited *in vivo* efficacy[91,92]. Nevertheless, the concept that reducing mucin disulfide bonds decreases the viscoelastic properties of mucus has been broadly demonstrated and can be explained by basic polymer physics. To facilitate clearance or lubrication, mucins interact by forming loosely interwoven mucus networks comprised of linear disulfide-linked mucin polymers able to reptate[20,30]. Reducing disulfide bonding was

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shown to lower the viscoelastic properties of the mucus because network stability is dependent on the second power of the length of the polymers that form the network[30,93]. Breaking mucin polymers into smaller oligomers therefore significantly decreases the random walk time and allows for greater axial diffusion[30].

The concept of a stiffer mucus dominated by disulfide bridges is of great interest as it unveils a commonly overlooked target for therapeutic intervention. Diseased mucus may require greater reducing power than healthy mucus to achieve similar results. Despite the lack of clinical efficacy, NAC has been the only reducing agent approved for inhalation since the 1960's.

Our recent study described the limitations of NAC as an inhaled reducing agent[94]. In brief, we showed that NAC possesses a low intrinsic reducing activity, as it mostly remains in its inactive protonated form at physiological pH and cycles slowly between active and inactive forms. As a result, NAC requires long periods of time to react to completion and the drug is rapidly cleared and/or absorbed from the epithelial surfaces. Consequently, the limited potency and slow kinetics of NAC, coupled with the off-target irritation effects including cough and bronchospasm, appear responsible for its failure in clinical pulmonary medicine as an inhaled mucolytic. Identifying the deficiencies of NAC is the first step towards preclinical testing of the next generation of reducing agents.

Other reducing agents are commonly used in laboratories but have limited or poor toxicity data. Dithiothreitol (DTT) exhibited a faster reaction time and increased potency compared to NAC. However, due to its cell toxicity, DTT cannot be used *in vivo* for the treatment of CF patients[95]. Similarly, Tris(2-carboxyethyl)phosphine (TCEP) is also used in laboratories and has the advantage of being odorless, but limited toxicological data exists on this compound.

TCEP was tested for MCT activity *ex vivo* and *in vivo* in newborn pigs following stimulation of submucosal gland secretion with methacholine. Mucus clearance rate was assessed by tracking the velocity of large metal particles (>300μm) across the airway surface. Although TCEP did not affect the overall velocity of the particles, it decreased the percentage of particles in movement, which correlated with a delay in clearance via the action of mucus strands originating from submucosal ducts[10]. Hence, designing novel reagents will be a balancing act between increasing potency, improving kinetics, and preventing off target effects and cell toxicity.

Parion Sciences designed several new thiol reagents (e.g., P-3001, P-2062, and P-2119) that were tested in *in vitro* and *in vivo* models. Compared to NAC, P-3001 showed superior reducing activities including faster reaction rates[94]. P-3001 required lower concentrations to alter the viscoelasticity of patient sputa and reduced mucus burden in βENaC mice, a model of CF lung disease. Drug effects were achieved without evidence of *in vitro* or *in vivo* toxicity. Similarly, P-2062 was more effective than NAC and rhDNase at dissolving mucus flakes collected via bronchoalveolar lavages performed on CF preschoolers[36]. Mucus flake integrity is also affected by treatment with TCEP (Figure 3). Although TCEP treatment of pig trachea delayed the mobilization of microdisks, P-2062 treatment accelerated tracheal

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mucus velocity in a sheep model of muco-obstructive lung disease, suggesting that the overall MCT is not negatively affected[36].

MUC5B overexpression has been shown to be a risk factor for developing idiopathic pulmonary fibrosis (IPF)[96]. In the bleomycin-challenged Muc5b-overexpressing mouse model of IPF, P-2119 restored MCT and minimized fibrosis following lung injury[97].

These studies demonstrated that reducing the viscoelasticity of airway mucus with reducing agents may be an achievable therapeutic goal and provides unique insights into new mucolytic agents as inhaled therapies to treat a broad range of muco-obstructive diseases.

4. Conclusion and Future Directions:

Mucus is a complex polymeric gel that serves as a critical defense mechanism in multiple organ systems. In muco-obstructive diseases like CF, restoring proper mucus viscoelasticity and clearance in the lungs remain major goals. The advancement of CFTR correctors and modulators provide an exciting glimpse at how CFTR correction can change the course of CF pathogenesis. However, some patients remain ineligible for modulator therapies and rely on symptomatic treatments to control their disease. While knowledge surrounding mucin biochemistry, ASL interaction, and MCT has expanded rapidly in the past decade, there is still much to learn. Understanding the precise biochemical and biophysical mechanisms of both normal and aberrant mucus will help guide treatment efforts and ensure that all patients suffering from muco-obstructive diseases receive effective therapies.

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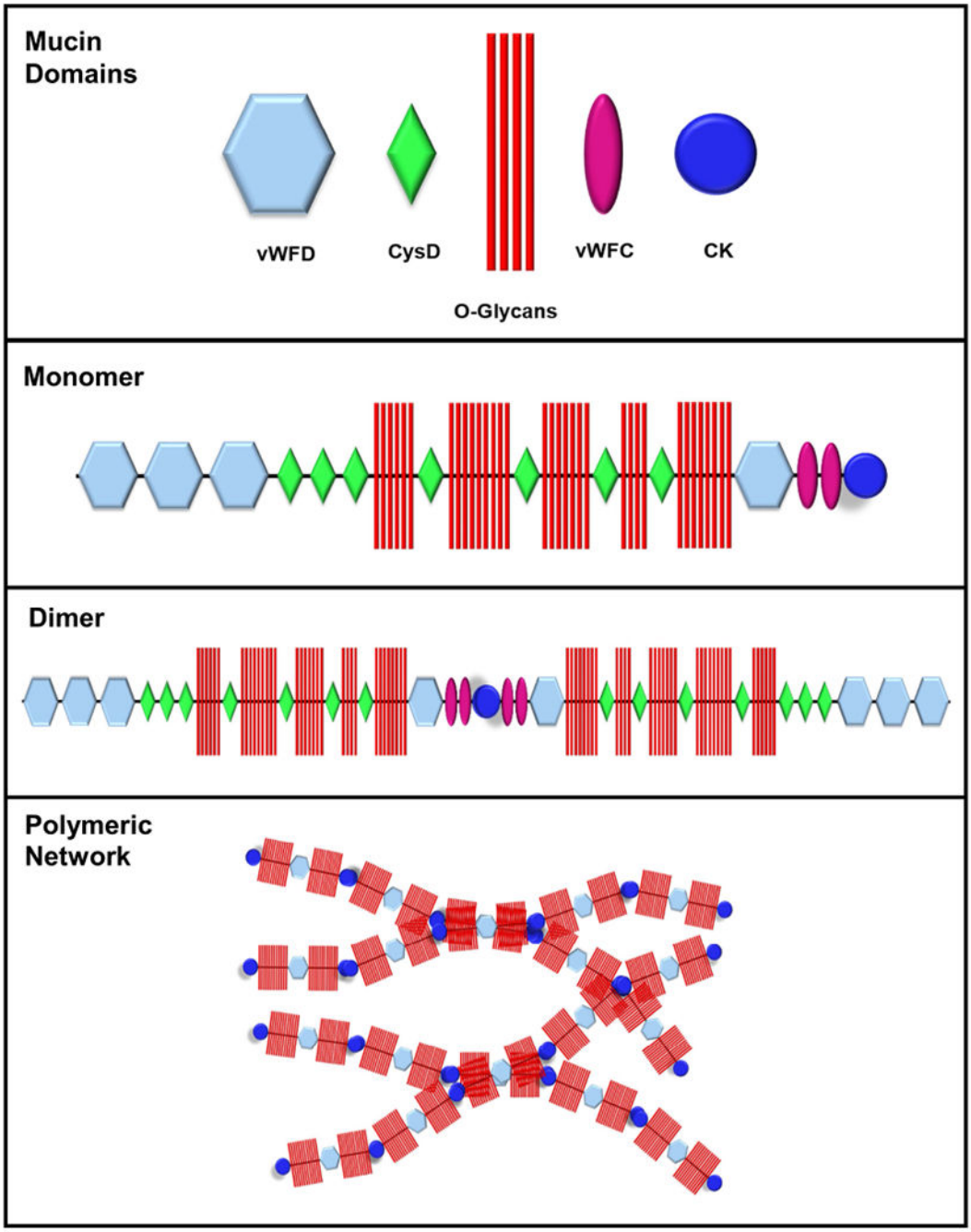

Figure 1. Mucin domains govern polymeric network organization.

Mucin structure and assembly rely on von Willerbrand Factor (vWF) D and C domains in the N- and C-terminal regions and cysteine-rich domains (CysD) scattered throughout the protein backbone including the cysteine knot (CK) at the C-terminal end. The protein core, which is rich in serine and threonine residues, undergoes post-translational O-glycosylation (O-glycans) to form mature mucin monomers. Dimer formation ensues in the endoplasmic reticulum and is mediated by disulfide linkage of the CK domains to form linear, interwoven polymeric networks.

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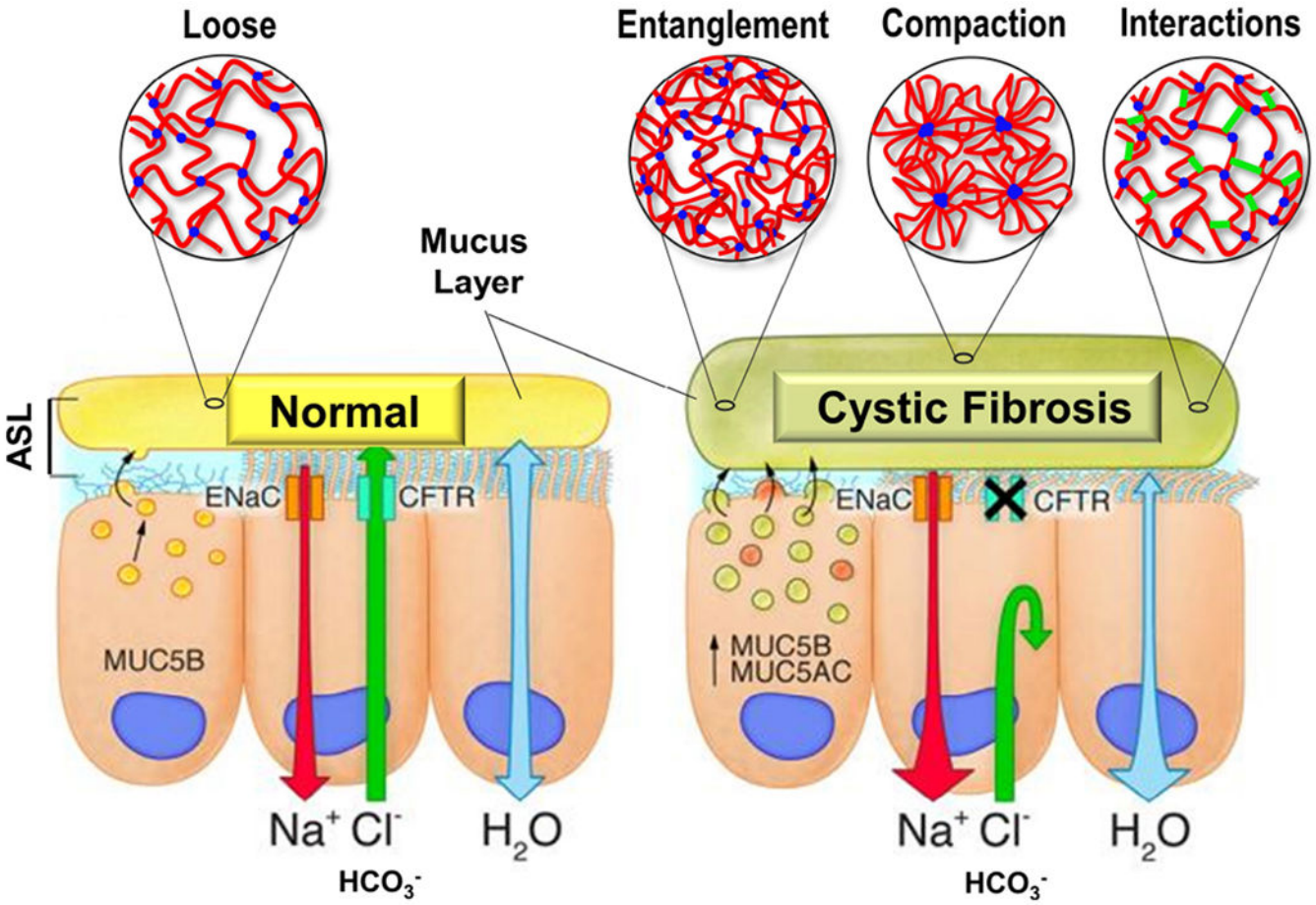

Figure 2. Model of normal vs. CF airway mucus layer illustrating changes within the mucus network (i.e., polymer entanglement, mucin compaction, and/or changes in molecular interactions) in response to altered ionic fluxes.

In normal individuals, CFTR function ensures proper Cl^- and HCO_3^- secretion as well as regulates Na^+ absorption via the downregulation of the ENaC channel, controlling water flux through the epithelium. A thin mucus layer is produced by airway goblet cells with optimal biophysical properties (e.g., loose transportable) for airway clearance. In CF, reduced Cl^- and HCO_3^- secretion and increased Na^+ absorption can alter the biochemical interface of the mucin network in different ways. Mucus layer hyperconcentration causes decrease in mucus mesh size (e.g., entanglement). Impaired Ca^{2+} sequestering prevents mucin expansion (e.g., compaction). Changes in hydration, pH, and oxidative stress can introduce additional ionic, hydrogen, hydrophobic, and disulfide bonds (e.g., interactions).

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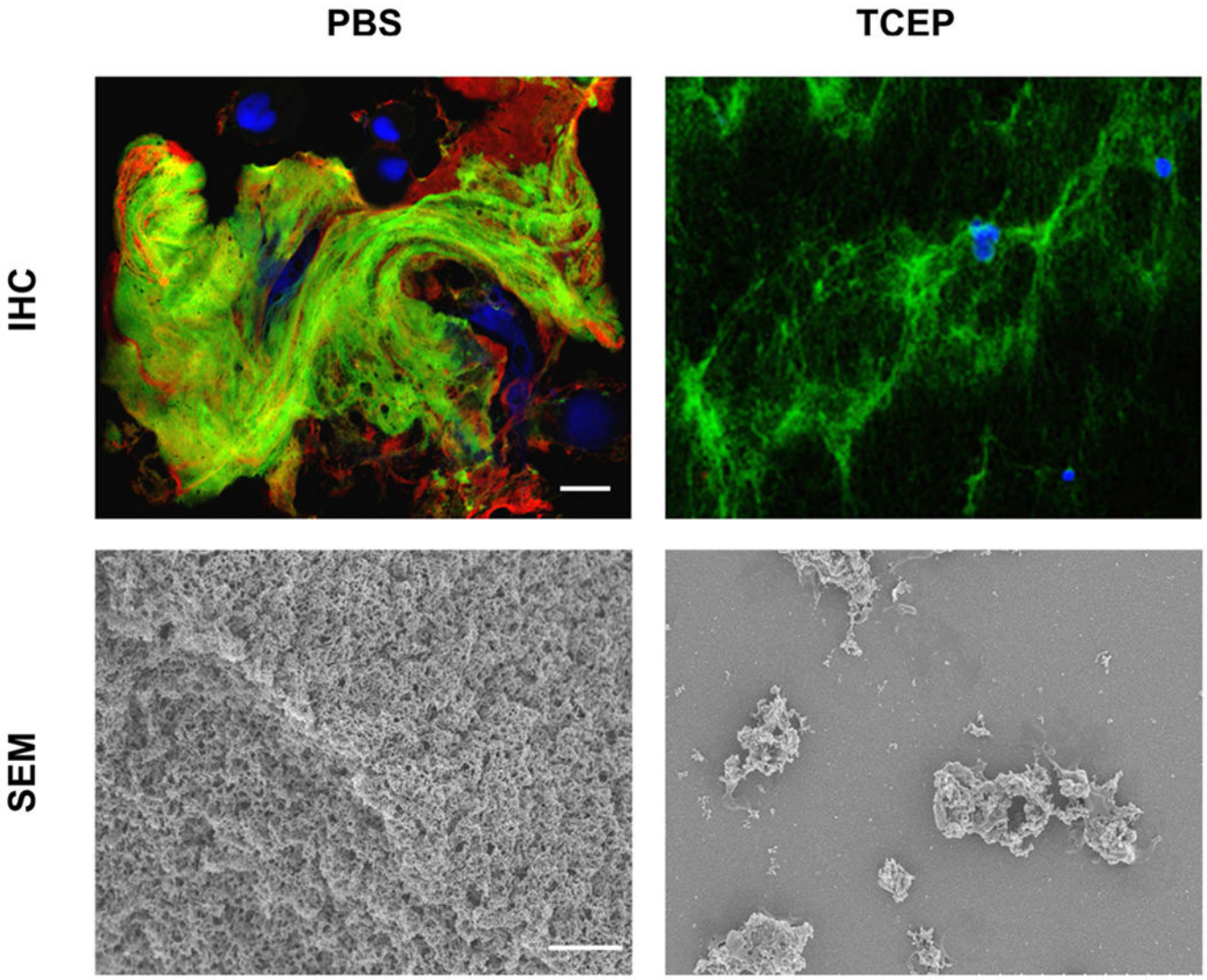

Figure 3. Images of mucus flakes collected via bronchoalveolar lavage (BAL) from a CF preschooler and treated *ex vivo* with PBS or TCEP.

Top images show the mucin polymeric network of a mucus flake treated with PBS or TCEP (5 mM for 10 min). BAL samples were stained with anti-MUC5B (green) and anti-MUC5AC (red) antibodies. DAPI (blue) was used to stain the nuclei of entrapped inflammatory cells. Bottom images reveal the organization of the mucin mesh via scanning electron microscopy (SEM) following treatment with PBS or TCEP. Scale bar is 4 μm.