Supplementary Materials:

**Biophysical Measurements: Technical Considerations**

Due to the non-linear nature of the rheological properties of mucus, measurement techniques have a profound impact on the reported mucus viscosity or elasticity [1]. Macroscopic viscosities of CF sputum measured by cone and plate rheology have been reported to be as high as 100 – 1,000 Pa·s at low shear rates, <1s⁻¹ [2]. However, the same study found viscosities between 1 – 10 Pa·s at higher shear rates typical of normal ciliary beat frequencies, ~ 10 s⁻¹ [3], reflecting shear thinning properties of mucus. In addition to shear rate dependence, the length scales of measurement have a profound effect on the reported physical properties of mucus [4, 5] (Figure 2A). Biologically relevant mucus samples contain high molecular weight mucin polymers that are sufficiently concentrated in solvent to interpenetrate, *i.e.*, be in overlap conditions. Nanoscopic probes (*e.g.*, ~ 5 nm), like those employed by FRAP studies, typically measure the solvent viscosity of mucus (polymeric) solutions because the probe diameter is much smaller than the correlation lengths of mucin polymers, *i.e.*, polymer mesh size [4, 6, 7]. This scenario is almost certainly applicable to normal mucus where the correlation length is on the order of 100’s nm [4, 6, 7]. The length scale dependent properties of mucus is one possible explanation for why CF sputum viscosities are often times reported 1,000 – 10,000 times larger macroscopically [2] than those reported by FRAP [8].

Microbead rheology may act as a bridge between the length scales of FRAP and macrorheology (Figure 2A). Particles smaller than the mesh size of mucus (100 – 500 nm [9]) will report viscosities similar to those measured by FRAP, while particles larger than the mesh size will report rheological properties more closely aligned with the mesh [10]. Particles with diameters near the mesh size will see their diffusive properties change depending on the timescale of measurement [11-13] and bead surface chemistry [4].

**Supporting Data**
Buffer Capacity.

Changes in mucus pH in response to acid administration are dictated by the buffer capacity of the mucus. Our data in PGM and HBE mucus confirmed the prior results of Kim [14] and Holma [15, 16] that the buffer capacity of mucus is a function of both mucin / mucus (Figure 1) and bicarbonate concentrations (Figure S2). Importantly, increasing the mucin concentration from 0 to 20 mg/ml (or 8 mM mucin, assuming a molecular weight of the mucins monomer to be 2.5 MDa [17]) had a greater effect on the buffer capacity than increasing the bicarbonate concentration from 0 mM to 20 mM (or 0 – 1.8 mg/ml). This finding indicates that mucins are able to buffer the airways against changes in pH occurring during changes in pCO₂ during tidal breathing. Further, the buffer capacity of PGM, BSM, as well as normal and CF HBE mucus, all increased with concentration, again consistent with the results of Holma and Kim [14-16]. Notably, the higher the concentration of mucus, the larger the proton addition required to produce a change in pH, indicating that pathologically concentrated mucus is more resistant to pH changes than healthy mucus.

As a first step to understanding pH interactions with airway mucus, detailed mucus pH titration studies were performed. These pH titrations studies (Figure S1) were noteworthy as they showed that mucus itself has a high intrinsic buffer capacity, confirming the earlier results of Holma [15, 16] and Kim [14]. Further, our data indicate that the chemical constituents within mucus play a greater role in the buffer capacity of mucus over physiologic ranges than bicarbonate, with bicarbonate adding to the buffer capacity of mucus at all concentrations. Interestingly, our results indicate that hyperconcentrated CF mucus (i.e., 5-8% solids) will require an addition of substantially more protons to change its pH than healthy, 2% mucus, i.e., it may be more difficult to perturb mucus pH in disease.

Figure S 1:
To explore the effect of bicarbonate on the buffer capacity of mucus, we performed titration experiments utilizing multiple concentrations of porcine gastric mucin (PGM) and sodium bicarbonate. Titration experiments were performed by adding 5uL of 100mM HCl to 2mL of a mucus sample at atmospheric CO₂. Concentrations of PGM ranging between 0 and 100mg/ml were equilibrated with 0, 5, and 20mM bicarbonate. At each PGM concentration, the presence of bicarbonate required a greater
concentration of protons be added to reach the same pH (i.e. the buffer capacity) as PGM without added bicarbonate. Note, adding 20mg/ml of PGM to PBS had a greater impact on the buffer capacity than the addition of 20 mM bicarbonate. Our data demonstrating that mucus acts as a buffer are consistent with previous studies of Holma [15, 16] and Kim [14] (Figure S2)

![Graph showing pH changes with varying concentrations of PGM and bicarbonate](image)

Figure S2 Titration of PGM (0 – 100mg/mL) with 0 - 20 mM bicarbonate.

Previous studies have demonstrated pH-dependent changes to the rheological properties of mucus [9, 18-20] over pH ranges of 1 to 9. To expand on the study of physiologically relevant ranges that were the focus of our studies, we performed macroscopic rheological assays on 20 and 50mg/mL PGM over a broader pH range, 4 to 8. Our data (Figure S3) show that, over pH physiologically relevant pH ranges (6-8), there was little pH-dependent change to the complex viscosity of mucus. However, at pH 4, there was a notable increase in the viscosity of both concentrations of PGM
mucus. It is important to note that, even at this low pH, the complex viscosity of 20mg/ml mucus is still much less than the complex viscosity of 50mg/ml PGM over physiologically relevant pH.

Figure S3: Complex viscosity of 20 and 50mg/ml PGM over pH 4-8.

**Effect of Pooling and Homogenization on Sputum Rheology**

Pooling and homogenization of CF sputum samples is requisite to ensure a sample is stable, present in large enough volumes to allow for the suite of assays presented in this work, and be as easily divisible as possible. However, it is an open question as to the effect of homogenization on the biophysical properties of mucus. We find that the pH (7) and concentration (5.2%) of our pooled CF sputum samples matches the average pH and % solids of all the subject samples (pH = 7.1 +/- 0.42, % solids = 5.6 +/- 2.6) as well as the mean of the samples chosen to be pooled (pH 7.1 +/- 0.28, % solids = 4.9 +/- 1.5) (Figure S4A, B). However, the viscosity of the pooled sample, 0.74 +/- 0.46 Pa·s, was nominally lower than the viscosity of either the entire subject
pool, 2.4+/- 1.5 Pa·s, or the mean of the pool samples, 1.8 +/- 1.9 Pa·s. While we note this decrease in the complex viscosity, the decrease was not significant (p = 0.08). When comparing the pooled sputum to the mean of the samples include in the pool, we likewise did not find statistical significance (p = 0.23). Further, when comparing the % solids (p = 0.6), pH (p = 1), and complex viscosity (p = 0.56), there was no statistically significant difference between the sputum samples from the overall patient cohort group and the samples chosen to be pooled. Therefore, we conclude that the pooled sample is representative of the sputum used in this study and that homogenization via trituration did not overly affect the rheological properties of sputum.

**Figure S4: Effect of pooling on sputum.** A) The concentration of P, pooled, homogenized sputum), A, all sputum samples included in this study, and A_p, Sub-set of samples that were pooled. B) pH of P, A, and A_p, and C) complex viscosity of A, P, and A_p. All error bars and standard deviations. No statistical significance was determined between A, P, and A_p.

**Methods**

**Sample Preparation:** Porcine gastric mucin (PGM) type III and bovine submaxillary mucin (BSM) (Sigma Aldrich) were prepared at concentrations between 10mg/ml and 100mg/ml in buffers of PBS with varying bicarbonate (0 – 20 mM) following methods similar to those of Celli [20]. PBS was chosen to most closely mimic the native ionic composition of the ASL [21]. HBE cultures for mucus harvesting and MCT studies were generated from excess surgical tissue procured by the UNC Marsico Tissue Core Facility. Approval was received from the UNC IRB for research use of
human tissue specimens in this study. Normal and CF human bronchial epithelial cells were cultured on a 0.4 µm pore-sized Millicell filter (Millipore, Billerica, MA) coated with collagen and maintained in air-liquid interface media (UNC Tissue Core) as described in [22]. HBE mucus was harvested from both normal and CF HBE cell cultures as previously described [7, 23-25]. In brief, over a 6-week interval, confluent cultures developed cilia, generated a periciliary layer (PCL) surrounding the cilia, formed a mucus layer, and exhibited rotational mucus transport. Washings from >100 cultures were pooled in 3,500 kDa cut off dialysis tubing (Fischer Scientific) and concentrated against Spectra/Gel (Spectrum Labs) to the designated % solids. Concentrated mucus was dialyzed against the designated buffers overnight prepared to desired pH to control ionic balance and concentrations of mucus. Concentrations of 1.5 - 2.5 % solids served as models of healthy mucus, whereas higher concentrations (3 – 8 % solids) served as models of disease states [1, 13, 18]. The mucus concentrations, pH, and ionic composition were varied to provide mucus for designated experiments.

**Titration**: PGM and BSM concentrations ranged between 20 mg/ml (3% solids) to 50 mg/ml (6% solids), while HBE mucus concentrations ranged between 2% solids and 6% solids (each with 0.9% solids as salt). Preliminary titration curves were generated by slowly adding small volumes of 100 mM HCl to 2 mL aliquots of mucus specimens employing methods similar to those of Holma [15, 16] and Kim [14]. All titrations and pH measurements were performed on a Mettler Toledo SevenCompact pH meter (Mettler Toledo, Columbus, OH) with an InLab ISM electrode capable of measuring the pH of ~ 50 µL of sample.

**Fluorescence Recovery after Photobleaching (FRAP)**: Mucus for FRAP studies was harvested from well-differentiated HBE cultures as previously described [26]. Briefly, HBE cultures were allowed to accumulate mucus on apical surfaces for 3 weeks. Mucus was lavaged from each culture surface after a 10-minute incubation in 30 µl of PBS. Lavaged samples were pooled and concentrated to designated concentrations using spin concentrators (Amicon Ultra, 3 kDa). The final mucus concentration of each preparation was determined using a previously reported
technique [27]. Samples (HBE mucus or spontaneous sputum) were incubated with FITC-Dextran, 10 kDa MW, 5 mg/ml, for 30 minutes prior to analysis. For each experiment, a 10 μl aliquot was deposited on a 35-mm coverslip and imaged with a confocal microscope (Leica SP5; Germany). Sequential images were taken before and after photobleaching (with 488 and 594 nm lasers). Leica’s FRAP Wizard software was used to perform the experiments and calculate single exponential time-constants of recovery. To distinguish whether the pH effect on FRAP reflected an effect of the mucin polymer matrix or the small protein component of the complex milieu of mucus, sputum was filtered to remove the very large (> 1MDa [17, 28]) mucin polymer matrix. Raw sputum samples were spin filtered with a protein concentrator (Corning Costar Spin-X with a poly ether sulfone (PES) membrane) supplied with a 100-kDa filtration membrane. Samples were centrifuged at 16,000 x g for 3hrs (at 4˚C). The resulting retentate (mucin-rich fraction > 100 kDa) was discarded and the filtrate (sputum supernatant material < 100 kDa) was used for FRAP measured viscosity studies. The concentration of mucins in both the raw sputum sample and filtered sputum was measured by combination gel permeation chromatography with refractive index and multi-angle laser light scattering [29]. These data show that the mucins present in the raw sputum sample are absent in filtered sputum (Figure S5).

![Figure 55](image)

Figure 55: Multi-angle light scattering (Red Curves) and refractive index measurements (Blue Curves) of A: raw and B: filtered sputum. Samples are diluted 1:200 and eluded through a CL2B
column. Mucins pass through the column and generate a peak at ~20 minutes. Smaller proteins interact with the pores in the CL2B column and generate a peak after ~50 minutes. The lack of a mucin peak is evident in the filter sample.

**Sputum collection:** Spontaneous sputum samples were collected as detailed previously [26, 30]. All patients who provided sputum granted prior written consent to participate in this study. All procedures employed in this study conformed to the regulations put forth by the UNC IRB. The mucus concentration of each sputum sample was determined as previously described [26, 30]. For experiments on homogenized CF sputum, 500 µL of PBS with protease inhibitor tablets (4 tablets per 10 mL PBS, cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Sigma-Aldrich) was added to 750 µL of sputum and mixed via titration. Homogenized, inhibited sputum from 4 patients was then mixed by titration and then prepared to desired pH and concentration.

**Microrheology:** Protocols established in [25] for diffusive microrheology were employed. Briefly, carboxyl-functionalized polystyrene particles, radius 1 µm, were mixed overnight on a rotator at 4°C. 1 µm particles were used to ensure that the study probes were larger than the correlation length (mesh size) of mucus, thereby ensuring that the probes sampled the entangled polymeric nature of the gel-forming mucin molecules in mucus [4, 6, 7, 25]. Microbeads were added to mucus at very low (1:600 vol/vol) concentrations to ensure that the addition of particles did not dilute mucus and alter its rheological properties. Image sequences were collected at 60 fps for 30 s. Particle trajectories were tracked using video spot tracker (http://cismm.web.unc.edu/software) and analyzed using custom Matlab scripts (The MathWorks, Natick Massachusetts). For simplicity, comparisons of MSD data are made at 1 sec [9, 31].

**Macrorheology:** Macrorheological properties of mucus specimens were measured by performing high-resolution stress amplitude sweeps, frequency, and creep recovery experiments on a TA Discovery Hybrid Rheometer 3 (TA Instruments, New Castle, Delaware), with a 20mm diameter 1° cone. All analyses were performed at 23°C and employed a solvent trap to minimize sample dehydration [17]. Once loaded, the following assays were run on each sample: 1) 0.1 and 10 Hz
stress sweeps ranging from 0.01 Pa to 10 Pa to ascertain the linear viscoelastic regime (LVR); and 2) 0.01 – 10 Hz frequency sweeps at stresses below the nonlinear threshold, yielding macroscopic linear moduli. Macroscopic rheological data were analyzed via TA Trios software. Complex viscosity ($\eta^*$) measured by amplitude sweeps were directly reported by Trios. Determination of the linear regime of specimens was done as previously described [24, 32]. The LVR is defined by a range of strain rates ($\gamma$) at which the rheological properties of the fluid are consistent (Figure S6A). Once the LVR is determined, the frequency sweep measurement is performed at a strain rate that falls within the LVR (Figure S6B). The mean of the technical repeats is calculated, and then the mean and standard deviation of the biological repeats is calculated and presented in bar-graph form (Figure S6C) and used for further statistical analysis.

**Figure S6: Determining the linear viscoelastic regime (LVR).** A) Two strain-sweep experiments were run at frequencies of 0.1 and 10 Hz. The LVR (Black Box) is determined to be the region of the strain sweep at which the least change is observed across $G'$, $G''$, and $\eta^*$ at both frequencies. A strain (in this case, 1%) that falls near the center of the LVR is then selected for B) Frequency sweep experiment ranging from 0.1 Hz to 10 Hz. The average of the complex viscosities at 1 Hz (Black Box) for each loading of the rheometer is then compiled to make a C) Bar plot of the mean and standard deviation of the biological replicates ($n=3$) for a given mucus sample, concentration, and pH.

**Mucus osmotic pressure measurements:** The osmotic pressure of HBE mucus were measured with a direct-membrane oncometer as previously described [27]. HBE mucus preparations (at
6.5% solids) was titrated to acidic and basic pHs as described above. For each measurement, 250 μl samples were measured in triplicate.

**Measurement of PCL height:** Parallel HBE mucus samples used for osmotic pressure samples were used to assay the effect of pH on PCL height. In these studies, PCL height was measured by the exclusion of large (>40 nm) green-fluorescent dextrans using the two-dye technique previously reported [27]. Here, 100 μl-samples were applied to freshly washed HBE cultures. The exclusion height of the large dextran was monitored by XZ confocal microscopy as previously described [27]. At each pH, a total of 12-20 images were obtained and average PCL height measured analyzed using a custom Matlab script.

**In vitro mucociliary clearance:** Well-differentiated HBE cultures that exhibited coordinated transport were allowed to accumulate mucus over a period of 7 days. Green fluorescent microspheres (1 μm) in PBS were nebulized in a small volume (~100 nL) onto the surface of the HBE cultures 2 hours prior to the initiation of the study. During this time, cells were returned to the incubator, and the added fluid was reabsorbed [33]. Cultures were then placed on an inverted epifluorescence microscope (TE2000, Nikon) outfitted with an environmental chamber to control temperature, humidity, and [CO₂]. At the initiation of the study, CO₂ was maintained at 5% and humidity > 80%. MCC rates were measured every 2 minutes. After baseline MCC rates were measured, the pH of the mucus covering the culture was either lowered by increasing the CO₂ from 5 to 15%. At the end of each assay, the effect of increasing mucus concentration was assessed by decreasing the humidity within the environmental chamber. Mucus concentration was determined after each maneuver as previously described [27].

**In vitro HBE mucus pH measurements:** During the MCC experiments, HBE cultures were maintained in a tissue culture incubator outfitted with a custom pH measurement system. Throughout the MCC assays, designated environmental conditions were maintained (i.e., 0.5%, 5%, or 15% CO₂, 37°C, 95% humidity). pH was measured using a micro-pH meter (0.65 mm
diameter; Innovative Instruments, Inc., USA) which does not require full immersion to measure pH. During the MCC measurement studies, a micromanipulator (Siskiyou, USA) was used to remotely advance the micro-pH probe into the thin airway surface layer. Positioning of the probe was visually monitored and confirmed using a magnifying camera (90X; Dino-Lite) outfitted with a right-angle objective. ASL pH values were recorded once steady-state values were obtained, typically within 1–2 min following immersion of the probe into the ASL. The pH meter was regularly calibrated with pH standards (Fisher Scientific).

**Mucus Reduction by dithiothreitol (DTT):** The effect of rheology on mucolytic agents that cleave disulfide bounds was tested by treating 5% normal HBE mucus at pH 7 with dithiothreitol (DTT) at 0, 1, and 10mM final concentrations. 10 µL of PBS with DTT 10 fold higher than desired final concentration was added to 90 µL and rotated at 37°C for 1 hour and then immediately loaded onto the rheometer for measurement as previously described [34].

**Statistics:** The dependence of rheology data and MCC data on concentration and pH were determined using two-way ANOVA and principal component analyses using Matlab. The dependence of FRAP data on concentration for both HBE mucus and CF sputum were determined from the analysis of the covariance of linear regressions. Dependence of osmotic pressure and PCL height on pH was quarried by paired t test. P values of < 0.05 were considered statistically significant.

**Study Approval:** Mucus harvested from human bronchial epithelial cell cultures are considered non-human subject protocol #03-1396. Sputum samples were collected under IRB #15-2431.