Microrheology of gel-forming airway mucins from porcine trachea

Elizabeth M. Engle[1,2]‡, Sydney Yang[1]‡, Allison Boboltz[1], Sahana Kumar[1,2], Alexa Stern[1], Gregg A. Duncan[1,2]
[1]Fischell Department of Bioengineering, [2]Molecular and Cellular Biology Program, University of Maryland, College Park, Maryland, United States of America, 20742

*Correspondences to: Gregg Duncan; email: gaduncan@umd.edu
‡These authors contributed equally to this work.

SUPPLEMENTAL INFORMATION

EXPERIMENTAL

Mucin extraction from porcine tissue

Pig trachea and intestine used for mucin extraction were purchased from Animal Biotech Industries, Inc. Porcine small intestine mucin (PSIM) and porcine trachea mucins (PTM) were extracted based on a previously described protocol.[23] Briefly, tissue samples were dissolved in 0.1 M NaOH overnight. The porcine small intestine was filled with NaOH whereas sections of whole porcine tracheal tissue were fully immersed in NaOH to allow for the solubilization of the mucus layer. Following overnight incubation, the tissue is discarded and the NaOH solution is collected for further processing. Mucins were then isolated from solution by lowering the pH to 4.0 with 1 M HCl where they form a gel phase, then centrifuged at 3500 rpm for 20 minutes to allow for removal of the supernatant. The resulting pellet was resuspended in deionized water and the pH was adjusted to 8.0 to re-solubilize the mucins. Further extraction of mucins is achieved through repeated centrifugation of mucins in the gel phase at pH 4 and solubilization of mucins at pH 8 for 3 cycles in total. Mucins were then treated with DNase I (10 U/mL) at 21ºC overnight. Following DNA removal, the solution was subjected to 4 additional rounds of purification through pH cycling and centrifugation as described above. Following this, the solution was then dialyzed (100 kDa MWCO) in deionized water for 72 hours, and frozen at -80C overnight prior to lyophilization. The lyophilized extracted mucins were solubilized at 2% or 4% (w/v) in a physiological buffer containing 154 mM NaCl, 3 mM $CaCl_2$, and 15 mM NaH_2PO_4 at pH 7.4 prior to usage for further biochemical and biophysical analysis.

BCi-NS1.1 cell culture & mucus collection

The immortalized BCi-NS1.1 human airway epithelial cell line was generously shared by Ronald Crystal (Weill Cornell Medical College) and cultured as previously described.[26] Briefly, BCi-NS1.1 cells were first expanded in a flask with Pneumacult-Ex Plus medium (no. 05040, StemCell Technologies) until confluent. Cells were then seeded (1 x 10^4 cell/cm^2) on rat tail collagen type 1-coated permeable Transwell membranes (12 mm; no. 38023, StemCell Technologies) until confluent. After confluence, only basal Pneumacult-ALI medium was provided in the basolateral compartment (no. 05001, StemCell Technologies) for 4 weeks to allow for polarization to occur at the air-liquid interface (ALI). Culturing at ALI allowed for the formation of an in vivo pseudostratified mucociliary epithelium. Mucus was collected by adding a small volume of pre-warmed PBS to the surface of ALI cultures, incubating at 37ºC for 30 minutes, and gently recovering mucus-containing PBS. Mucus washings are then pooled and concentrated using 100 kDa MWCO Amicon ultra filters, and stored at -80C until usage.

Mass DNA quantification assay
The amount of DNA in commercially available and lab extracted mucins was measured according to a previously established protocol.[24] In brief, 30 µL of 20% (w/v) 3,5-diaminobenzoic acid was added to solubilized mucin samples and incubated at 60°C for 1 hour. The reaction was stopped with the addition of 1 mL of 1.76 M HCl. The fluorescence intensity was measured at 390/530 nm (ex./emis.).

Protein quantification assay
Protein concentration was quantified using a bicinchoninic acid (BCA) assay (no. 23225, ThermoFisher) as described by the manufacturer. In brief, a BCA working reagent was prepared, of which 200 µL was added to 15 µL of each sample, and then incubated for 30 minutes at room 37°C. Absorbance values of the samples were measured at 562 nm and compared to BCA standards.

Relative O-linked glycoprotein content assay
O-linked glycoprotein (mucin) concentration was determined using a cyanoacetamide (CNA) reagent protocol as previously established.[24] In brief, 200 µL of CNA was mixed with 1 mL of 0.15 M NaOH to create the CNA reagent. 60 µL of the CNA reagent were mixed with 50 µL of solubilized mucin samples (2% w/v) and incubated for 30 minutes at 100°C, before addition of 0.5 mL of 0.6 M borate (pH 8.0). The fluorescence intensity of the resulting samples was measured at 336/383 nm (ex./emis.) and compared to a 1 mg/mL solution of bovine submaxillary mucin (BSM; Sigma-Aldrich) as a standard.

Sialic acid concentration assay
Total sialic acid concentration was measured for lab extracted and commercial mucins by utilizing a modified Warren method centered on the thiobarbituric acid reaction (Sigma-Aldrich, MAK314) and following the manufacturer protocol. Briefly, bound sialic acid was hydrolyzed, then oxidized to form formyl pyruvic acid which formed a measurable colored solution with the addition of thiobarbituric acid. The fluorescence of the colored solution was measured at 555/585 nm (ex./emis.) and compared to measurements in serially diluted solutions of sialic acid as a standard.

Disulfide bond concentration assay
The disulfide bond concentration in the various mucin types was determined using a previously described protocol.[17] In brief, 8 M guanidine hydrochloric acid was added to 50 - 70 µL of sample for a final volume of 500 µL. 10% (v/v) of 500 mM iodoacetamide was added and samples were kept at room temperature for 1 hour. 10% (v/v) of 1 M DTT was added, and samples incubated at 37°C for 2 hours. Samples were then filtered and buffer exchanged in a 7 KDa MWCO Zebra desalting column with 50 mM Tris-HCl (pH 8.0). As a standard, solutions of L-cysteine with concentration ranging from 0 µm – 5000 µM were prepared. Samples were diluted 1:1 with 2 mM monobromobimane in a flat black plate 96-well plate and incubated in the dark at room temperature for 15 minutes before reading the fluorescence at 395/490 nm (ex./emis.).

Multiple particle tracking and microrheology analysis

Using a previously established protocol,[17] nanoparticle (NP) probes were prepared for use in multiple particle tracking experiments by modifying 100 nm diameter carboxylate-modified polystyrene NP (ThermoFisher) with a polyethylene glycol (PEG) coating to render these particles non-adheseive to mucus. We then constructed custom microscopy chamber consisting of a vacuum grease coated O-ring that was then filled with 20 μL of the mucin / mucus sample of interest and 1 μL of muco-inert NPs (~0.002% w/v) before being sealed with a coverslip. Slides were then incubated at room temperature for 30 minutes in the dark prior to fluorescence imaging (Zeiss Confocal LSM 800, 63x water-immersion objective) to allow for sample equilibration. NP diffusion was imaged for 10 seconds at 33.3 frames per second and then tracked and analyzed using a custom MATLAB code that calculated the mean squared displacement, $\langle MSD(\tau)\rangle = \langle(x^2 + y^2)\rangle$, for each particle. The MSD values were used to estimate the microrheological properties of the gel through the Stokes-Einstein relation, $G(s) = \frac{2k_BT}{(\pi as\langle\Delta r^2(s)\rangle)}$, where k_BT is the thermal energy, a is the radius, and s is the complex Laplace frequency. The frequency-dependent complex modulus (G*) was calculated as, $G^*(\omega) = G'(\omega) + G''(i\omega)$ where $i\omega$ is substituted for s, i is the complex number, and ω is the frequency. The pore size (ξ) at ω = 1 Hz was estimated from the G' using the following expression: $\xi = \left(\frac{k_BT}{G'}\right)^{1/3}$. The complex microviscosity (η*) at ω = 1 Hz was calculated using the following expression: $\eta^*(\omega) = G^*(\omega) / \omega$.

Dynamic light scattering of mucin solutions

Semi-dilute solutions of PSIM and PGM were prepared at concentration of 0.5 mg/mL in 10 mM NaCl. Prior to measurements, the suspensions were filtered using a 100 kDa Amicon Ultra centrifugal filter. The filtrate containing species below 100 kDa MW was discarded and the sample containing high MW species (>100 kDa) was resuspended in 10 mM NaCl. The sample was then subjected to dynamic light scattering analysis (NanoBrook Omni; Brookhaven Instruments) to determine hydrodynamic radii.

SUPPLEMENTAL FIGURES

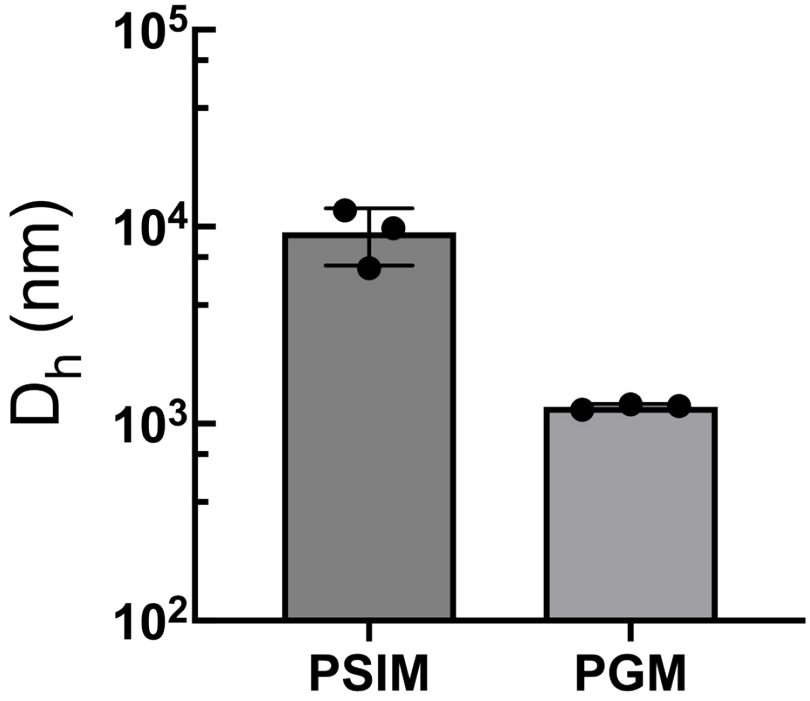

Figure S1. **Dynamic light scattering of PSIM and PGM solutions.** Effective hydrodynamic diameter (D_h) of 0.5 mg/mL PSIM and PGM solutions in 10 mM NaCl.

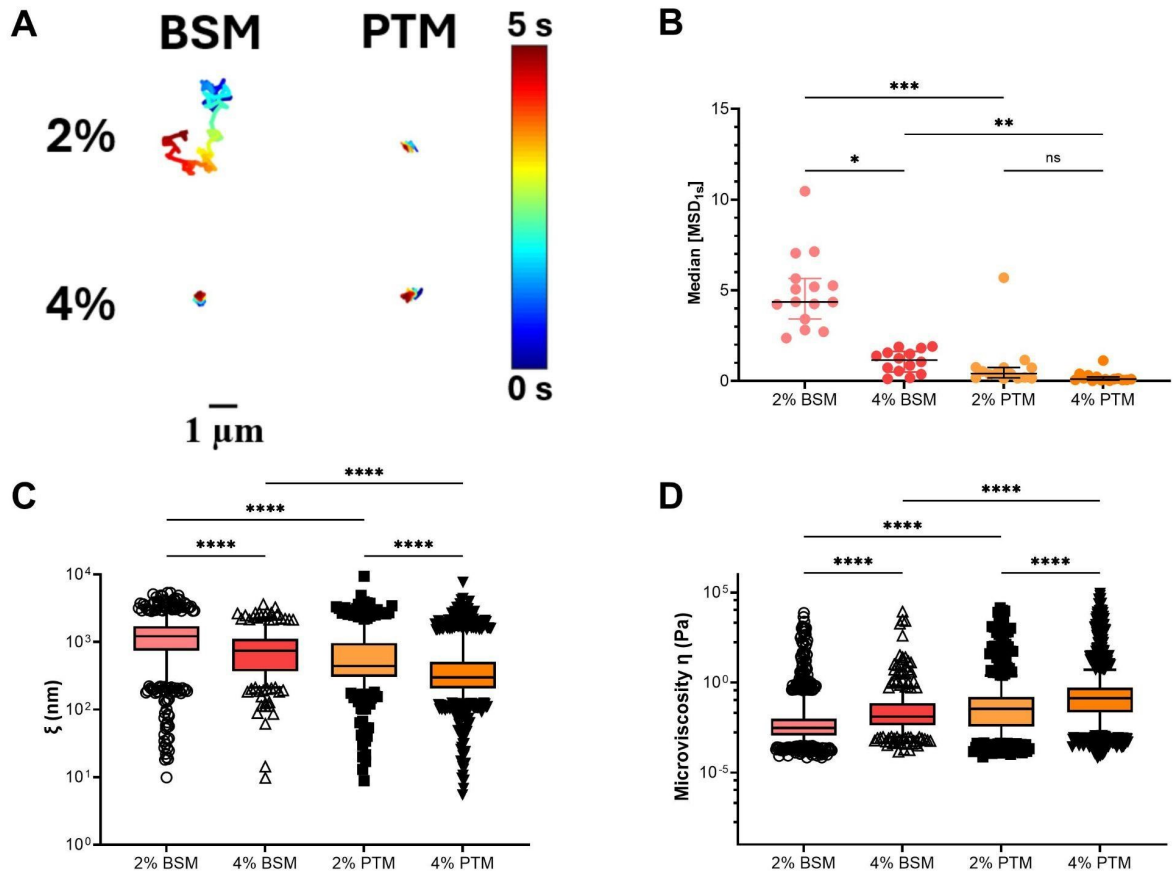

Figure S2. **Microrheology of bovine submaxillary (BSM) and porcine tracheal mucin (PTM)**. (A) Representative trajectories of 100 nm (diameter) NP diffusion in 2% and 4% w/v of BSM and PTM. Trajectory colors change as a function of time with 0 s indicated by dark blue and 5 s indicated by dark red. Scale bar = 1 µm. (B) Calculated median MSD at a time scale of 1 second (MSD1s) for NP diffusion in 2% and 4% w/v of BSM and PTM. Each data point represents the median calculated MSD1s in each video with 3 - 5 videos from 3 technical replicates. Black lines indicate interquartile range. (C) Estimated pore size (ξ) from NP diffusion. (D) Estimated microviscosity (η) from NP diffusion. (C, D) Each data point is an estimated pore size or microviscosity for individual particles. (C,D) Data was analyzed with Kruskal-Wallis test with Dunn's test for multiple comparison: ns = not significant, **** $p < 0.0001$, ** $p < 0.01$.