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# Supplementary Materials for

## Oxidation increases mucin polymer cross-links to stiffen airway mucus gels

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**Other Supplementary Material for this manuscript includes the following:**
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## Materials and Methods

### Human Subjects

Eighteen cystic fibrosis subjects and 23 healthy control subjects (Table S1) underwent an initial characterization visit, followed by additional visits to collect samples of expectorated or induced sputum, as previously described (*36*). Patients with cystic fibrosis met the Cystic Fibrosis Foundation criteria for the diagnosis of CF. All study subjects underwent an initial characterization visit, including collection of a sample of induced sputum, followed by 3-7 additional visits (separated by at least 2 days) for collection of multiple samples of expectorated sputum. The studies (NCT01533636, NCT00596232, and NCT00758771) were approved by the Institutional Review Board at UCSF, and informed consent was obtained from all study participants.

### Sputum Induction

Subjects inhaled 3% sterile nebulized saline through a mouthpiece for 20 minutes. During the induction procedure lung function was monitored at 2-4 minute intervals by measurements of peak flow, spirometry, or both. Subjects were instructed to spit saliva into a saliva container before coughing sputum into a sputum container. The inhalation period was interrupted every 2 minutes to allow subjects to cough up sputum.

### Sputum Processing

The volume of sputum sample was determined and an equal volume of 10% Sputolysin (2.5mM dithiothreitol) (EMD Chemicals) was added. The mixture was placed in a 37°C

shaking water bath for 15 minutes. The sample was mixed periodically with a serological pipette to ensure complete homogenization.

**Sputum Cell Counts**

Total cell count: A 10 µL aliquot of homogenized sputum mixed with a 10µL Turk solution was placed in a hemocytometer to determine the total cell count.

Differential cell count: Aliquots of sputum homogenized in Sputolysin were diluted in saline, spun in a Cytospin 4 cytocentrifuge (Thermo Scientifics) at 500rpm for 5 min to generate cytology slides, and stained using with Kwik-Diff (Thermo Scientific). Five hundred non-squamous cells on each sample were read.

**Preparation of Sputum for Imaging Studies**

Sputum was processed for imaging in three steps: (i) Sputum was collected from the container by transfer pipette, gently spread on a slide to prevent alteration of natural structures, and baked at 60ºC overnight to dehydrate liquid from the sample; (ii) the sputum sample was adhered to the slide by swiping quickly over a flame four times; (iii) the sputum slides were lightly fixed for 10 minutes in a 2% glutaraldehyde and 3.7% buffered formalin solution, decontaminated by immersion in a series of 70% ethanol and 1-5% phenol solutions, washed in water, and air dried.

**Immunofluorescence**

Sputum slides were briefly rehydrated in water. Antigen retrieval was first performed in a warm citrate buffer for 5 minutes, followed by immersion in a glycine solution for further

epitope retrieval for 10 additional minutes. Sputum slides were blocked in a 10% normal goat serum (JR Scientific) and 2% IgG-Free BSA (Sigma Aldrich) solution for 1 hour at room temperature. Slides were incubated in a cocktail of MUC5AC/5B mouse anti-human $IgG_1$ antibodies (Table S1) at a final concentration of 0.5 µg/mL overnight at 4□C. After a series of brief washes, slides were incubated in a 2% IgG-free BSA solution with secondary antibody goat anti-mouse Cy3-conjugated $F(ab')_2$ fragments (Jackson ImmunoResearch) at a final concentration of 1.5 µg/mL and DNA stain Yo-Pro-1 Iodide (Life Technologies) at a final concentration of 2 µM for 1 hour at room temperature. After a series of brief washes slides were mounted with Fluoromount-G (Southern Biotech), cover slipped, sealed with nail polish, and left to dry before imaging. Slides were imaged using an Olympus Fluoview FV10i laser scanning confocal microscope. Z-stack images were collected at 0.75 µm intervals at 1024 x 1024 pixels using a 60x phase contrast oil-immersion objective NA 1.35 and 473 nm (12.5 mW)/ 559 nm lasers using a variable barrier filter mechanism to set the fluorescence channels to the appropriate detection wavelengths for fluorophores used. A three-dimensional rendering of the confocal image was generated by the Fluoview Navigator software. Additional processing of the image was performed in Imaris 7.6.0 (Bitplane) to reconstruct the 3D volumes and molecular architecture of both mucins and DNA in the sputum.

**Sputum Supernatant**

(i) Sputum homogenized in 10% Sputolysin (as above) was centrifuged at low speed (300g) for 10 minutes at 4ºC to generate a supernatant for measures of MPO by ELISA.

(ii) Whole sputum (not homogenized or treated with Sputolysin) was ultracentrifuged at high speed (32,000rpm, SW41Ti Beckman Coulter rotor) at 4ºC for 1 hour to separate a sol phase and a gel phase. Measures on the sol phase included ROS, protein-bound 3-nitrotyrosine, 3-chlorotyrosine, 3-bromotyrosine, and o, o'-dityrosine. Measures on the gel phase included total cysteines and cystines (disulfide bonds).

**Synthesis of Methyl 6-deoxy-6-thio-α–D-galactopyranoside (TDG)**

A solution of tosyl chloride (0.540g, 2.83 mmol) in dry pyridine (2.5mL) was added to an ice cooled solution of methyl α-D-galactopyranoside (MTG, 0.500g, 2.57mmol) in dry pyridine (5mL) and the reaction was stirred while allowing to warm up to room temperature. After 4h, additional tosyl chloride (0.442g, 2.32mmol) dissolved in pyridine (2.5mL) was added and the mixture was stirred overnight. MeOH (2mL) was then added and the mixture concentrated and co-evaporated with toluene (3 x 10mL). The residue was taken up into pyridine (4mL), acetic anhydride (2mL) was added and the mixture stirred overnight. The mixture was then concentrated and co-evaporated with toluene (3 x 10 mL), dissolved in EtOAc (30mL), washed with saturated aqueous $NaHCO_3$ (2mL), dried over $MgSO_4$, filtered, and concentrated. The crude residue was purified by FC on silica gel *via* Biotage (toluene-EtOAc) to give methyl 2,3,4-tri-*O*-acetyl-6-deoxy-6-thioacetyl-α-D-galactopyranoside (0.639 g, 52%) as a colourless solid. Potassium

thioacetate (1.70g, 14.9mmol) was added to a solution of this compound (1.77g, 3.73mmol) in DMF (20 mL) and stirred at 90°C overnight. The mixture was cooled down to room temperature, diluted with toluene and EtOAc (1:1, 100mL) and washed with water (50mL) and saturated aqueous $NaHCO_3$ (50mL). The organic layer was dried over $MgSO_4$, concentrated and two times purified by flash chromatography on silica gel (toluene-EtOAc). The obtained slightly yellowish residue was dissolved in $CH_2Cl_2$ (50mL) and stirred with activated charcoal over night. The charcoal was filtered off and the mixture concentrated and dried *in-vacuo* to obtain methyl 2,3,4-tri-*O*-acetyl-6-deoxy-6-thioacetyl-α-D-galactopyranoside as a colourless solid (1.05g, 74%). A solution of sodium methoxide (1N in MeOH) was added to this intermediate (0.272g, 0.719mmol) in dry MeOH (5mL) under a $N_2$ atmosphere until a pH of 13 was reached and the resulting mixture was stirred for 3 h. The mixture was neutralized with Dowex 50 $W^+$ ion exchange resin, the resin filtered off and the solution concentrated. The solid was taken up into water and freeze dried to obtain TDG (137 mg, 91%) as a colourless solid.

TDG: $R_f$ 0.53 (EtOAc-MeOH-$H_2O$ 4:1:1); $[\alpha]_D^{20}$ +139° (*c* 1.00, $H_2O$); $^1H$ NMR (400 MHz, $D_2O$) δ 4.85 – 4.83 (m, 1H, H-1), 4.08 – 4.06 (m, 1H, H-4), 3.90 – 3.85 (m, 1H, H-5), 3.84 – 3.82 (m, 2H, H-2, H-3), 3.47 (s, 3H, -OC$H_3$), 2.80 (dd, $J_{6a,b}$ = 13.7, $J_{5,6a}$ = 8.1 Hz, 1H, H-6a), 2.74 (dd, $J_{6a,b}$ = 13.7, $J_{5,6b}$ = 5.8 Hz, 1H, H-6b) ppm. $^{13}C$ NMR (100 MHz, $D_2O$) δ 99.4 (C-1), 72.1 (C-5), 69.5 (C-4), 69.4 and 68.0 (C-2, C-3), 55.07 (-O$CH_3$), 23.87 (C-6) ppm. ES-HRMS calcd for $C_7H_{14}O_5S$ $[Na]^+$ 233.0460 found 233.0471.

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# Supplementary Tables

## Table S1: Clinical characteristics of the healthy and CF subjects.

| | Healthy (n= 23) | Cystic Fibrosis (n=18) | p value |
|---|---|---|---|
| Age, mean (SD) | 38.0 (11.3) | 36.1 (13.0) | 0.61 |
| Female, n (%) | 12 (52.2) | 5 (27.8) | 0.12 |
| Non-Hispanic, n (%) | 22 (95.7) | 16 (88.9) | 0.41 |
| BMI, mean (SD) | 26.3 (5.4) | 22.2 (3.6) | 0.01 |
| FEV1 %, mean (SD) | 98.0 (8.5) | 50.0 (16.7) | <0.0001 |
| FEV1/FVC %, mean (SD) | 79.0 (7.0) | 59.4 (10.0) | <0.0001 |
| Sputum infected with Pseudomonas Aeruginosa, n (%) | - | 10 (55.6) | - |
| Sputum infected with Staphylococcus Aureus, n (%) | - | 6 (33.3) | - |
| Sputum infected with Stenotrophomonas maltophilia, n (%) | - | 3 (16.7) | - |

| | | | |
|---|---|---|---|
| Sputum infected with Burkholderdia cepacia, n (%) | - | 0 (0) | - |
| Sputum infected with Aspergillus fumigatus, n (%) | - | 3 (16.7) | - |

**Table S2: Mucin antibodies.**

| Protein | Clone | Mfg. | Isotype |
|---|---|---|---|
| Muc5AC | MG-31 | Abnova | mouse IgG1 |
| Muc5AC | 2H7 | Abnova | mouse IgG1 |
| Muc5AC | 45M1 | Santa Cruz Biotechnology | mouse IgG1 |
| Muc5AC | 1-13M1 | Santa Cruz Biotechnology | mouse IgG1 |
| Muc5AC | 2-11M1 | Santa Cruz Biotechnology | mouse IgG1 |
| Muc5AC | SPM297 | Santa Cruz Biotechnology | mouse IgG1 |
| Muc5AC | 2Q445 | Santa Cruz Biotechnology | mouse IgG1 |
| Muc5AC | CLH2 | Santa Cruz Biotechnology | mouse IgG1 |
| Muc5AC | 2X123 | Santa Cruz Biotechnology | mouse IgG1 |
| Muc5B | 4H310 | Santa Cruz Biotechnology | mouse IgG1 |
| Muc5B | 19.4E | Abcam | mouse IgG1 |
| Muc5B | 6F10-E4 | Abcam | mouse IgG1 |
| Muc5B | 5B19-2E | Invitrogen | mouse IgG1 |

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## Supplementary Videos

**Video S1: 3D architecture of CF sputum**. Laser-scanning confocal microscopy movie showing the overall three-dimensional architecture of cystic fibrosis sputum in phase contrast (grey), DNA (green) and mucins (red). Cells (green) are dispersed throughout the sputum sample.

**Video S2: 3D rendering of the architecture of CF sputum**. Reconstruction of the three-dimensional cystic fibrosis sputum structure showing the organization of DNA (green) and mucins (red). A dense mucin core is overlaid by a web-like structure of DNA polymers.