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Nitric oxide-releasing alginates as mucolytic agents

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Abstract

The excessive production of thick, viscous mucus in severe respiratory diseases leads to obstruction of the airways and provides a suitable environment for the colonization of pathogenic bacteria. The effect of nitric oxide (NO)-releasing alginates with varying NO release kinetics on the viscoelastic properties of human bronchial epithelial (HBE) mucus was evaluated as a function of the NO-release kinetics using parallel plate rheology. Low molecular weight (~5 kDa) alginates with high NO flux (~4000 ppb/mg) and sustained release (half-life ~0.3 h) proved to be most effective in reducing both mucus elasticity and viscosity (~60% reduction for both). The efficacy of the NO-releasing alginates was shown to be dose-dependent, with high concentrations of NO-releasing alginates (~80 mg•mL⁻¹) resulting in greater reduction of the viscosity and elasticity of the mucus samples. Greater reduction in mucus rheology was also achieved with NO-releasing alginates at lower concentrations when compared to both NO-releasing chitosan, a similarly biocompatible cationic polymer, and *N*-acetyl cysteine (NAC), a conventional mucolytic agent.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information. The following file is available free of charge.

Reaction schemes, molecular weight measurements, elemental analysis, zeta potential measurements, representative ¹H and ¹³C NMR spectra of the biopolymers, representative UV-vis absorbance spectra of control and NO-releasing biopolymers, percent reduction in elasticity and viscosity for all measurements, and control rheology measurements for NO-release kinetics study (PDF)

The authors declare the following competing financial interest(s): Mark Schoenfisch is a cofounder, a member of the board of directors, and maintains financial interest in Novan therapeutics Inc. and Vast Therapeutics, Inc. Both companies commercialize macromolecular nitric oxide storage and release vehicles for multiple clinical indications.

Keywords

nitric oxide; alginates; mucolytic agent; mucus

Introduction

Airway mucus represents the first line of defense in the respiratory tract against invading pathogens (e.g., bacteria), dust, spores, pollen, and other chemical irritants.^{1–3} Mucus is comprised of approximately 95% water, with the balance consisting of proteins, lipids, salts, and mucin glycoproteins (i.e., the main component that determines its gel-like properties).^{4,5} The consistency of mucus is often described in terms of rheological properties, particularly viscosity (resistance to flow) and elasticity (resistance to deformation) due to this complexity.⁵ Excessive, long-term mucus production (i.e., mucus hypersecretion) is a major pathological feature of severe respiratory diseases, such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD).^{1–3,6} Accumulation of highly viscous and elastic mucus obstructs the airways and provides a suitable environment for the colonization of bacteria.⁷ The presence of persistent chronic infections and impeded mucus clearance leads to a decline in lung function and ultimately patient mortality.

Currently used treatment strategies focused on combining therapies that target bacterial infections (i.e., antibiotics) with those that alter mucus rheology (i.e., mucolytics). Nitric oxide (NO), an endogenously-produced free radical, holds promise as a potential therapeutic agent for treating respiratory diseases due to its broad spectrum antibacterial activity against planktonic and biofilm-based bacteria.^{8–15} Present studies indicated that bacteria are unlikely to foster resistance to NO owing to the multi-mechanistic pathways (e.g., nitrosation of thiols on bacterial proteins) involved in NO's antibacterial action.^{16,17} While initial studies have demonstrated the efficacy of exogenous NO in the gas form, the hazards associated with the use of pressurized cylinders and NO's high reactivity in biological media has to date limited its therapeutic utility.¹⁸ Research has thus also focused on the development of water soluble macromolecules capable of storing and releasing NO under specific environmental conditions as an alternative to direct NO inhalation.^{18,19} The use of the *N*-diazoniumdiolate NO donor-modified macromolecular scaffolds is particularly attractive due to the NO donor's ability to spontaneously release NO under physiological conditions (e.g., 37 °C, pH 6.5 in CF airway mucus),^{9,20,21} a property critical for sustained NO delivery in the lungs via intermittent (e.g, twice daily) treatment.¹⁸ Although the activity of NO-releasing macromolecules as antibacterial agents for the treatment of respiratory infections has been proposed,^{8,9} its potential impact on mucus rheology remains unclear. Initial studies with NO-releasing chitosan biopolymers demonstrated NO's ability to damage the three-dimensional mucin network and reduce mucin size (MW).²⁰ While Reighard et al. indicated NO's potential as a mucolytic in this report, a systematic evaluation of the impact of NO-release properties (e.g., release kinetics) and the effect of the macromolecule properties (e.g., molecular weight) of the biopolymer for pulmonary NO delivery is required for further development of NO-release system as a dual-action (i.e., antibacterial and mucolytic) therapeutics. Previous reports have characterized the antibacterial properties of NO-releasing materials as a function of macromolecular water solubility, charge, and NO-

release kinetics.^{22–25} We believe that the same properties are likely to influence mucolytic activity.

Alginate, an anionic biopolymer composed of 1,4-linked α -L-guluronic acid (G) and β -D-mannuronic acid (M) units, holds particular promise as a NO donor scaffold for the development of inhaled NO therapeutics (via nebulization) due to its high water solubility, low toxicity, and favorable biocompatibility.^{26–30} Previous studies with low molecular weight alginate oligosaccharides demonstrated alginate's ability to modulate mucin assembly, and reduce the elastic and viscous properties of CF sputum.^{6,31–33} The biopolymer's ability to alter mucus structure occurs by inhibiting interpolymer crosslinks and interfering with intramolecular interactions between the mucin glycoproteins. We hypothesize that modifying alginate to release NO and thus confer greater antibacterial and mucolytic action (by interfering with the disulfide bonds that stitch mucin monomers into long-chain polymers) may result in a dual-action therapeutic for treating obstructive respiratory diseases plagued by chronic infection.^{20,34}

Bulk rheological properties of mucus are tightly correlated to its clearance from the lung.⁵ Reducing the viscoelastic burden of pathological mucus is thus important in the treatment of muco-obstructive pulmonary diseases such as CF and COPD. Herein, we evaluated the ability of NO-releasing alginates to alter the bulk viscoelastic properties of human bronchial epithelial (HBE) mucus using parallel plate rheology. Alginate modified with different alkyl amine groups provided a platform to tune NO payloads and release kinetics depending on the precursor amine structure. The effect of NO-release kinetics, alginate concentration, and select biopolymer properties (e.g., molecular weight and charge) on mucus rheology were also examined with similar goals.

Materials and Methods

Materials.

Alginic acid sodium salt from brown algae (low viscosity), bis(3-aminopropyl) amine (DPTA), diethylenetriamine (DETA), *N*-propyl-1,3-propanediamine (PAPA), spermine (SPER), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), *p*-anisaldehyde, *p*-toluenesulfonyl chloride, ethanolamine and *N*-acetyl cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO). Medium molecular weight chitosan (95% degree of deacetylation) was purchased from Primex (Siglufjörður, Iceland). Common laboratory salts and solvents were purchased from Fisher Scientific (Fair Lawn, NJ). Unless otherwise specified, all chemicals were used as received without further purification. Argon (Ar), carbon dioxide (CO₂), nitrogen (N₂), nitric oxide (NO) calibration (25.87 ppm, balance N₂), and pure NO (99.5%) gas cylinders were purchased from Airgas National Welders (Raleigh, NC). Distilled water was purified to a resistivity of 18.2 M Ω •cm and a total organic content of 6 ppb using a Millipore Milli-Q UV Gradient A10 System (Bedford, MA).

Instrumentation.

^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker (600 MHz) spectrometer. Elemental (carbon, hydrogen, and nitrogen; CHN) analysis was performed using a PerkinElmer Elemental Analyzer Series 2400 Instrument (Waltham, MA). Zeta potential measurements were collected in phosphate buffer (PB; 10 mM; pH 6.5) using a Zetasizer Nano (Malvern Instruments, UK). Rheological measurements were carried out using a TA Discovery Hybrid Rheometer 3 (DHR3; New Castle, DE) with a 20 mm diameter parallel plate set to a gap thickness of 50 mm. Gel permeation chromatography (GPC) measurements were collected out in 0.1 M sodium nitrate using an aqueous GPC-multi-angle light scattering system equipped with a Waters 2414 refractive index detector (Milford, MA) coupled to a Wyatt miniDawn TREOS multi-angle light scattering detector (Santa Barbara, CA).

Oxidative degradation of biopolymers.

High molecular weight alginate and medium molecular weight chitosan biopolymers were degraded to lower molecular weights following previously published protocols.^{10,25,35} Briefly, the biopolymer (2.5 g) was dissolved in hydrogen peroxide (50 mL) and stirred in an oil bath for 1–4 h. The concentration of the hydrogen peroxide in the solution (15–30 wt% in water), temperature (70–85 °C), and duration of the reaction were varied to obtain alginate oligosaccharides with a range of molecular weights (Table S1). The resulting solution was filtered to remove insoluble material. The alginate oligosaccharides were collected in and washed copiously with ethanol before drying in vacuo to yield a white powder. Alternatively, chitosan was stirred in 15 wt% hydrogen peroxide at 85 °C for 1 h with the resulting oligosaccharides collected in acetone and washed copiously with acetone. *Caution! The direct addition of acetone to hydrogen peroxide solutions is hazardous and prone to detonation!* With each wash step, hydrogen peroxide in the solution was carefully monitored with starch iodide paper. The chitosan oligosaccharides (COS) had an average molecular weight of 4.38 kDa and dispersity of 1.2 as determined by GPC measurements (Table S2).

Synthesis of polyamine-modified alginates (AlgMW-alkyl amine).

The alginate oligosaccharides were modified with either DETA, DPTA, PAPA, or SPER following a previously published protocol.²⁵ Briefly, low molecular weight alginate (100 mg) was dissolved in 10 mL phosphate buffered saline (PBS; 10 mM, pH 6.5) with a 2:1 molar ratio of EDC and a 2:1 molar ratio of NHS with respect to the carboxylic acid moieties on the alginate biopolymer. The reaction was left to stir for 1 h. A 4:1 molar ratio of the alkyl amine with respect to the carboxylic acid groups of the alginate biopolymer was then added dropwise to the mixture. The reaction was allowed to proceed for 24 h at room temperature under constant stirring. The amine-modified alginates were precipitated in methanol and collected by centrifugation, washed twice with methanol, and dried in vacuo to yield a white solid for each modification.

A hybrid alginate system modified with both PAPA and DPTA was also synthesized following similar steps. A 4:1 molar ratio of PAPA with respect to the carboxylic acid groups of alginate were first added dropwise to the solution prior to addition of a 4:1 molar ratio of DPTA with respect to the same groups. The reaction was allowed to proceed for 24 h

at room temperature before being collected and copiously washed with methanol to yield a white powder.

Representative ^1H and ^{13}C NMR of alginate and the polyamine modified alginates included the following peaks, a residual methanol peak at 3.20 ppm may also present:

Alg300, Alg 10, Alg5 and Alg1: ^1H NMR (600 MHz, D_2O , δ) 3.60–4.05 [C2, C3: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$], 4.30 [C4: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$], 4.50–4.60 [C5: OHCOCH], 4.90 [C1: $\text{OCH}(\text{CHOH})\text{O}$]. ^{13}C NMR (600 MHz, D_2O , δ) 65.0–80.0 [C2, C3, C4, C5: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, OHCOCH], 100.0 [C1: $\text{OCH}(\text{CHOH})\text{O}$], 175.0 [C6: OHCOCH].

Alg5-DPTA: ^1H NMR (600 MHz, D_2O , δ) 1.60–1.80 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 2.60–2.30 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 2.80–3.10 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 3.60–4.05 [C2, C3: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$], 4.30 [C4: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$], 4.50–4.60 [C5: OHCOCH], 4.90 [C1: $\text{OCH}(\text{CHOH})\text{O}$]. ^{13}C NMR (600 MHz, D_2O , δ) 22.0–26.0 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 37.0–39.0 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 44.0–46.0 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 65.0–80.0 [C2, C3, C4, C5: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, OHCOCH], 100.0 [C1: $\text{OCH}(\text{CHOH})\text{O}$], 160.0 [amide C6: NHCOCH], 175.0 [carboxylic acid C6: OHCOCH].

Alg5-DETA: ^1H NMR (600 MHz, D_2O , δ) 2.30–3.30 [$\text{OCNHCH}_2\text{CH}_2$, $\text{OCNHCH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{NH}_2$, $\text{NHCH}_2\text{CH}_2\text{NH}_2$], 3.60–4.05 [C2, C3: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$], 4.30 [C4: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$], 4.50–4.60 [C5: OHCOCH], 4.90 [C1: $\text{OCH}(\text{CHOH})\text{O}$]. ^{13}C NMR (600 MHz, D_2O , δ) 34.0–37.0 [$\text{OCNHCH}_2\text{CH}_2$], 45.0–49.0 [$\text{OCNHCH}_2\text{CH}_2$], 54.0–55.0 [$\text{NHCH}_2\text{CH}_2\text{NH}_2$], 38.0–42.0 [$\text{NHCH}_2\text{CH}_2\text{NH}_2$], 65.0–80.0 [C2, C3, C4, C5: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, OHCOCH], 100.0 [C1: $\text{OCH}(\text{CHOH})\text{O}$], 160.0 [amide C6: NHCOCH], 175.0 [carboxylic acid C6: OHCOCH].

Alg5-PAPA: ^1H NMR (600 MHz, D_2O , δ) 0.70–0.80 [$\text{NHCH}_2\text{CH}_2\text{CH}_3$], 1.52 [$\text{NHCH}_2\text{CH}_2\text{CH}_3$], 1.85 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2$], 2.80–3.10 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{OCNHCH}_2\text{CH}_2\text{CH}_2$], 3.60–4.05 [C2, C3: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$], 4.30 [C4: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$], 4.50–4.60 [C5: OHCOCH], 4.90 [C1: $\text{OCH}(\text{CHOH})\text{O}$]. ^{13}C NMR (600 MHz, D_2O , δ) 9.0–10.0 [$\text{NHCH}_2\text{CH}_2\text{CH}_3$], 23.0–24.0 [$\text{NHCH}_2\text{CH}_2\text{CH}_3$], 34.0–35.0 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2\text{NH}$], 36.0–38.0 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2\text{NH}$], 42.0–45.0 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2\text{NH}$], 48.0–49.0 [$\text{NHCH}_2\text{CH}_2\text{CH}_3$], 65.0–80.0 [C2, C3, C4, C5: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, OHCOCH], 100.0 [C1: $\text{OCH}(\text{CHOH})\text{O}$], 160.0 [amide C6: NHCOCH], 175.0 [carboxylic acid C6: OHCOCH].

Alg5-SPER: ^1H NMR (600 MHz, D_2O , δ) 1.13 [$\text{OCNHCH}_2(\text{CH}_2)_2\text{CH}_2$], 1.56 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2\text{NH}$], 1.80 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2$], 2.20–2.40 [$\text{OCHNHCH}_2\text{CH}_2\text{CH}_2$, $\text{OCNHCH}_2\text{CH}_2\text{CH}_2$], 2.68 [$\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 2.80–3.10 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 3.60–4.05 [C2, C3: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$], 4.30 [C4: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$], 4.50–4.60 [C5: OHCOCH], 4.90 [C1: $\text{OCH}(\text{CHOH})\text{O}$].

^{13}C NMR (600 MHz, D_2O , δ) 22.0–23.0 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2$], 24.0–25.0 [$\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$], 36.0–38.0 [$\text{OCNHCH}_2\text{CH}_2\text{CH}$, $\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 46.0–48.0 [$\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$, $\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 65.0–80.0 [C2, C3, C4, C5: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, OHCOCH], 100.0 [C1: $\text{OCH}(\text{CHOH})\text{O}$], 160.0 [amide C6: NHCOCH], 175.0 [carboxylic acid C6: OHCOCH]. Of note, cross-linking of the two primary amine groups with the carboxylic acid moieties of Alg5-SPER has been reported to occur resulting from the longer alkyl chain of the SPER moiety.²⁵ The peak at 2.95 ppm becomes prominent during cross-linking, attributable to the two protons near the amide.

Alg5-PAPA-DPTA: ^1H NMR (600 MHz, D_2O , δ) 0.70–0.80 [$\text{NHCH}_2\text{CH}_2\text{CH}_3$], 1.48 [$\text{NHCH}_2\text{CH}_2\text{CH}_3$], 1.50–1.70 [PAPA: $\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 2.30–2.40 [DPTA: $\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 2.10 [PAPA: $\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_3$], 2.60–3.10 [DPTA: $\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 3.60–4.05 [C2, C3: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$], 4.30 [C4: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$], 4.50–4.60 [C5: OHCOCH], 4.90 [C1: $\text{OCH}(\text{CHOH})\text{O}$]. ^{13}C NMR (600 MHz, D_2O , δ) 9.0–10.0 [PAPA: $\text{NHCH}_2\text{CH}_2\text{CH}_3$], 22.0–26.0 [DPTA: $\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 23.0–24.0 [PAPA: $\text{NHCH}_2\text{CH}_2\text{CH}_3$], 34.0–35.0 [PAPA: $\text{OCNHCH}_2\text{CH}_2\text{CH}_2\text{NH}$], 36.0–38.0 [PAPA: $\text{OCNHCH}_2\text{CH}_2\text{CH}_2\text{NH}$], 37.0–39.0 [DPTA: $\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 42.0–45.0 [PAPA: $\text{OCNHCH}_2\text{CH}_2\text{CH}_2\text{NH}$], 44.0–46.0 [DPTA: $\text{OCNHCH}_2\text{CH}_2\text{CH}_2$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$], 48.0–49.0 [PAPA: $\text{NHCH}_2\text{CH}_2\text{CH}_3$], 65.0–80.0 [C2, C3, C4, C5: $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, $\text{OCHCH}(\text{OH})\text{CH}(\text{OH})$, OHCOCH], 100.0 [C1: $\text{OCH}(\text{CHOH})\text{O}$], 160.0 [amide C6: NHCOCH], 175.0 [carboxylic acid C6: OHCOCH].

Synthesis of tosylated-aminoethyl Schiff base (TES).

The aminoethyl Schiff base was first synthesized following a 1:1 molar ratio reaction between ethanolamine and *p*-anisaldehyde for 24 h with strong continuous stirring. The resulting viscous yellow solution was then placed under vacuo to pull off excess water. The aminoethyl Schiff base was subsequently functionalized with a tosyl group. A 1.411 g aliquot of the viscous solution was added in a solution of dichloromethane (5 mL) and pyridine (1.3 mL) with vigorous stirring. The solution was placed in an ice bath and allowed to cool to 0 °C. Tosyl chloride (2.0 g) was dissolved in dichloromethane (10 mL) before adding dropwise to the Schiff base mixture. The reaction was allowed to proceed for 24 h. The product was then precipitated in diethyl ether and collected via vacuum filtration to yield a yellow powder. ^1H NMR (600 MHz, DMSO-d_6 , δ) 7.84 (d, $J = 8.3$ Hz, 1H), 7.46 (d, $J = 8.0$ Hz, 1H), 7.38 (d, $J = 8.6$ Hz, 1H), 7.01 – 6.87 (m, 1H), 6.10 (s, 1H), 3.83 – 3.69 (m, 2H), 3.59 (s, 1H), 3.48 – 3.32 (m, 1H), 2.43 (s, 2H).

Synthesis of ethylamine-modified chitosan oligosaccharides (COS-EA).

Water-soluble chitosan oligosaccharides were modified with the aminoethyl Schiff base functional group through a tosylate nucleophilic substitution reaction. Briefly, COS (500 mg) was dissolved in 10 mL water and placed in an oil bath at 80°C. The tosylated-aminoethyl Schiff base was then dissolved in dimethyl sulfoxide (DMSO; 12.5 mL) and slowly added dropwise to the chitosan solution. After 1 h, HCl (2 mL; 5 M) was added to the

reaction solution. After 18 h, the solution was neutralized with 1 M NaOH before product isolation via precipitation in ethanol, collected via centrifugation, and washed twice with ethanol to yield a brown powder. ^1H NMR (600 MHz, D_2O , δ) 1.90 [C8: CH_3OCNHCH], 2.90–3.10 [$\text{NH}_2\text{CH}_2\text{CH}_2\text{NHCH}$, C2: $\text{NH}_2\text{CH}_2\text{CH}_2\text{NHCH}$], 3.30–4.00 [C3, C4, C5, C6: OHCH , $\text{OCHCH}(\text{OH})\text{CH}(\text{NH}_2)\text{CH}$, OHCH_2CH , OHCH_2CH], 4.40 [C1: $\text{OCH}(\text{CHNH})\text{O}$].

^{13}C NMR (600 MHz, D_2O , δ) 21.0–22.0 [C8: CH_3OCNHCH], 30.1 [$\text{NH}_2\text{CH}_2\text{CH}_2\text{NHCH}$], 38.6 [$\text{NH}_2\text{CH}_2\text{CH}_2\text{NHCH}$], 53.0–56.0 [C2: $\text{NH}_2\text{CH}_2\text{CH}_2\text{NHCH}$, CH_3OCNHCH], 70.0–89.0 [C3, C4, C5, C6: OHCH , $\text{OCHCH}(\text{OH})\text{CH}(\text{NH}_2)\text{CH}$, OHCH_2CH , OHCH_2CH], 91.0–98.0 [C1: $\text{OCH}(\text{CHNH})\text{O}$], 174.2 [C7: CH_3OCNHCH].

Synthesis of *N*-diazoniumdiolate-modified biopolymers.

Nitric oxide-releasing alginates and chitosan oligosaccharides were synthesized as previously reported.^{10,25} Polyamine-modified alginate (45 mg) was dissolved in 50 mM NaOH solution (3 mL) in a 1-dram glass vial. For the chitosan oligosaccharide sample, COS-EA (45 mg) was dissolved in mixture of water (450 μL), methanol (2.55 mL), and sodium methoxide (5.4 mM in methanol, 75 μL). The open vials were placed in a stainless steel reactor, and solutions stirred continuously via magnetic stirring. Oxygen was removed from the vessel by purging with argon (10 s, 7 bar) three times, followed by three additional long purges with argon (10 min, 7 bar). The vessel was then pressurized to 10 bar with NO gas and allowed to react for 3 d. Afterward, the same argon purging protocol was repeated to remove unreacted NO. The NO-releasing alginates were then precipitated in ethanol, collected by centrifugation, dried overnight in vacuo, and stored at -20°C as a white powder. The NO-releasing COS was collected in acetone via centrifugation and stored similarly as a brown powder.

Characterization of nitric oxide release.

Nitric oxide release was evaluated in real-time using a Sievers 280i Chemiluminescence NO analyzer (NOA; Boulder, CO). Measurements were taken before each use to ensure sample stability. Prior to analysis, the NOA was calibrated with air passed through a NO zero filter (0 ppm NO) and 25.87 ppm of a NO standard gas (balance N_2). In a typical measurement, the NO-releasing biopolymer (1 mg) was dissolved in 30 mL of PBS (10 mM, pH 6.5, 37°C). Nitrogen was passed through the solution at a flow rate of 70 mL/min to carry the liberated NO from the biopolymer to the analyzer. Additional nitrogen flow was supplied to the flask to match the collection rate of the instrument (200 mL/min) to ensure that water soluble interferents (e.g., nitrite and nitrate) that may be present in the sample flask are not transferred to the reaction cell. Nitric oxide analysis was terminated when NO levels fell to below 10 ppb of NO/mg of biopolymer (the limit of detection of the instrument).^{36,37}

Preparation of mucus sample.

Human bronchial epithelial cell culture washings containing mucus were collected from excess surgical tissue (sourced from UNC-Chapel Hill Tissue Core facility). Briefly, primary HBE cells were grown on 0.4 μm pore-sized Millicell cell culture inserts (Millipore; Bedford, MA) coated with collagen in air-liquid interface media (UNC Chapel Hill-Tissue Core Facility) for a minimum of 6 w until the cultures developed cilia, well-defined

periciliary liquid (PCL), and mucus layers.³⁸ Mucus was harvested from the cell cultures following previously published protocols.^{39,40} Washings were pooled in 3,500 kDa cut off dialysis tubing (Fisher Scientific; Fairlawn, NJ) and concentrated against Spectra/Gel (Spectrum Labs; Rancho Dominguez, CA) to 3 wt% solids, which served as the minimum mucus concentration for a disease state model.⁴⁰ Subsequently, these washings were treated immediately with either alginate, chitosan, or *N*-acetyl cysteine.

Parallel plate rheology.

Concentrated solutions of both control (non-NO-releasing) and NO-releasing biopolymer samples (5 μ L) were added to 50 μ L aliquots of 3 wt% HBE mucus to achieve a final concentration of 20 mg/mL prior to collecting rheological data. Mucus samples were slowly rotated at room temperature for 18 h. The rheological properties of both treated and untreated (blank) mucus samples were measured via amplitude sweep experiments over a stress range of 0.025–50 Pa at a single frequency (1 Hz) using the rheometer equipped with a 20 mm diameter parallel plate set to a gap thickness of 50 μ m. Rheological measurements were performed at 23 °C to minimize sample dehydration. The elastic modulus (G') and viscous modulus (G'') were determined from the linear regimes as previously reported.⁴¹

A time-based study with control and NO-releasing biopolymers was conducted following the aforementioned protocol. Rheological data were collected at 1 h, 3 h, and 5 h timepoints using the same parameters. Similarly, a nitric oxide dose-dependence study (20–80 mg/mL) using control and NO-releasing Alg5-PAPA-DPTA, COS-EA, and *N*-acetyl cysteine (1–200 mg/mL) was also carried out following the same protocol.

Statistical Analysis.

All values either numerically or with error bars are reported as the mean $\pm$ standard deviation of the mean for a minimum of three or more pooled experiments. Statistical significance was determined using the two-tailed Student's *t*-test.

Results and Discussion

Synthesis of *N*-diazoniumdiolate-modified biopolymers.

Alginate oligosaccharides (~5 kDa) with different secondary amine functional groups (i.e., DETA, DPTA, PAPA, and SPER) were prepared via EDC/NHS reactions as reported previously (Supporting Information, Scheme 1).²⁵ The alkyl amine modification was confirmed via ¹³C NMR by the appearance of the amide bond peak (~160 ppm; Figure S3, Figure S4). The nitrogen content for the alkyl amine-modified materials also increased from 0 to 6–11%, providing further evidence of successful modification (Table S2).²⁵ The protonated amines at pH 6.5 (Table S2) resulted in a positive shift of the zeta potential (from –40 mV to between –12 to –30 mV), making the alkyl amine-modified alginates less negatively charged than the native alginate biopolymer.

In addition to the monosubstituted polymer, a hybrid system was also prepared to design a NO-releasing alginate system with potentially high initial NO flux and more sustained NO release by making use of NO donors having both fast and slow NO-release properties.

Specifically, the PAPA and DPTA polyamine modifications were selected for the hybrid system based on the reported NO-release half-lives of the small molecule alkyl amines (0.2 and 3.0 h, respectively).⁴² Integration of the peak related to the methyl terminal group of PAPA indicated that the alginate was 43% modified with this functional group. The percent DPTA modification for the hybrid alginate system (50%) was extrapolated from the total nitrogen content of the modified alginate biopolymer, subtracting out the predicted nitrogen contribution from PAPA (calculations provided in Supporting Information). These percentages suggest that the two-alkyl amine functional groups have similar alginate modification efficiencies.

The secondary amine-modified alginates were subsequently functionalized with NO donors by exposure to high pressures of NO gas under basic conditions. Formation of the *N*-diazoniumdiolate NO donors was confirmed by UV-vis spectroscopy and the appearance of a characteristic absorbance band at 253 nm (Figure S8).¹⁶ The resulting *N*-diazoniumdiolate-modified alginates possessed a broad range of NO payloads and release kinetics (Table 1), with Alg5-PAPA/NO and Alg5-DETA/NO having the fastest and shortest NO-release half-lives (0.1 and 2.0 h, respectively). Overall, the NO payloads were comparable to that of previously reported water-soluble systems (0.3–0.6 $\mu\text{mol}/\text{mg}$; Table 1).^{10,11,25,43–45} While the alginate hybrid system (Alg5-PAPA-DPTA/NO) had a similar NO payload to that of Alg5-PAPA/NO, both the NO-release half-lives and durations resembled Alg5-DPTA/NO. This result was unexpected given the greater number of secondary amine groups for the hybrid system. It is likely that steric hindrance from the crowded functional groups limits *N*-diazoniumdiolate formation.

The NO-release properties of the anionic alginate biopolymers were also compared to chitosan, another naturally derived biopolymer, comprised of repeating units of *N*-acetylglucosamine and D-glucosamine units. To date, chitosan has been used for many biomedical drug delivery applications due to its tolerability (i.e., biocompatibility) and mucoadhesive properties.^{10,11,44–47} Lu et al. reported on the synthesis of low molecular weight (~ 5 kDa) NO-releasing chitosan oligosaccharides (COS).¹⁰ A similar protocol was adopted in this work to prepare water soluble starting material. The COS primary amines were then modified with ethanolamine via a tosylate nucleophilic substitution reaction (Supporting Information, Scheme 2) to yield secondary amines that could subsequently be functionalized with NO. The resulting NO-donor-modified COS was found to have greater NO payloads than the alginate systems (~ 0.7 $\mu\text{mol}/\text{mg}$) but comparable NO-release kinetics ($t_{1/2} \sim 0.8$ h). The lower NO payloads for alginate compared to chitosan may be due to the conformation adapted by alginate's monomers. The repeating units of G monomers on the alginate are described to have a buckled conformation resulting in a diamond-shaped hole while the repeating units of M monomers are described as an extended ribbon, similar to that of chitosan.^{48,49} Both monomeric units can be modified with alkyl amine functional groups via carbodiimide chemistry, however, the conformation adapted by G residues could limit NO donor formation due to steric hindrance.

Effect of molecular weight on alginate-treated mucus rheology.

The diffusion of therapeutic agents through mucus depends on both the size and the mucoadhesive properties of the material.⁴ Regardless of charge, low molecular weight polymers (< 10 kDa) usually diffuse through the mucus layer, allowing for more efficient drug delivery to the site of interest (e.g., biofilms embedded in the mucus).⁵⁰ In contrast, larger molecular weight polymers (> 10 kDa) have increased interactions with mucins (e.g., chain entanglements, increased H-bonding interactions). These interactions form mucin-polymer aggregates and increase the viscoelastic properties of the mucus.^{46,50} To study the impact of alginate molecular weight on the viscoelasticity of mucus, the elastic (G') and viscous (G'') moduli were measured using parallel plate rheology. Mucus was treated with 20 mg/mL of bare (unmodified) alginate systems of different molecular weights (1, 5, 10, and 300 kDa) for 24 h. The alginate-treated samples reduced both the elastic and viscous moduli of mucus relative to the untreated blanks (Figure 1), with < 5 kDa MW alginates reducing both elasticity and viscosity by 60–70% (Table S4). In agreement with previous studies,^{28,31–33} the lower molecular weight materials (Alg1 and Alg5) proved to be the most effective at reducing the viscoelastic properties of the mucus. Alg300 (i.e., the high molecular weight biopolymer) was the least effective, as might have been expected based on size.^{28,31–33} Alginate molecular weight is a crucial factor that controls the biopolymer's gel-forming ability.^{29,30,49} While the Alg300 system still reduced rheological properties, lower molecular weight biopolymers avoid the potential for mucin chain entanglements by having less repeating monomer units and reactive functional groups that would interact with the glycoprotein (Table S4).³¹

Effect of varying functional group and biopolymer charge on mucus.

In addition to size and chain entanglement, the mucoadhesivity of the biopolymer (impacted most directly by the electrostatic interactions between charged pendant chains of mucins and the polymers) will clearly influence drug diffusion through the mucus layer.⁵¹ Positively charged biopolymers, including chitosan, are generally mucoadhesive, an advantageous property for facilitating localized drug delivery to mucus-coated regions as in the lungs.^{52–55} However, studies have also shown that increased mucoadhesivity may promote the formation of polymer-mucin aggregates that, in turn, increase the viscoelastic properties of the mucus layer and limit drug diffusion.⁵³ Neutral and negatively charged polymers (e.g., polyethylene glycol, alginate) reported to exhibit mucus layer penetration are being considered as alternatives for drug delivery in mucus coated regions.^{32,50,53} Amine modification on alginate introduces a positive charge on the biopolymer that may influence its mucoadhesive property. Thus, the impact of these functional groups on the elastic and viscous properties of the mucus might prove equally relevant.

The effect of biopolymer charge was studied after modifying Alg5 with different alkyl amine functional groups (i.e., DETA, DPTA, PAPA, and SPER). The Alg5 systems were selected for this study to allow for a direct comparison with the previously developed NO-releasing chitosan biopolymers of similar molecular weight. As expected, modification of the alginate backbone with alkyl amine functional groups resulted in a distinct positive charge (Table S2), although increases noted in the viscoelastic properties of mucus treated with amine-modified alginates were not statistically significant with respect to the untreated

sample (Figure 2). Alkyl amine-modified alginates with a less negative zeta potential (e.g., Alg5-SPER) increased both the mucus elasticity and viscosity (~17 and 43%, respectively; Figure 2 and Table S4). Negligible changes to the mucus was observed with more negatively charged alginate (i.e., Alg5-PAPA) compared to the reductive effect of the unmodified biopolymer. The increase in both elasticity and viscosity for Alg5-SPER, Alg5-DPTA, Alg5-DETA biopolymers suggests that the addition of amine-based chemical modification resulting in cationic ammonium groups at physiological pH, facilitates interaction with the net negatively charged mucin glycoproteins via electrostatic interactions.^{4,56}

Treatment of mucus with the NO-releasing biopolymers resulted in a decrease in both the elastic (~30–60%) and viscous (~20–60%) moduli, with reductive ability dependent on the NO donor (Table S5). Of the different alginate modifications, the hybrid Alg5-PAPA-DPTA/NO biopolymer resulted in the greatest reduction among the different alginate systems (~60% for both elastic and viscous moduli). The enhanced action of the hybrid alginate biopolymer suggests that the mucolytic ability of the alginates depends on both the NO payloads and the NO-release kinetics, with higher NO totals and slower release resulting in greater reduction in the elastic and viscous moduli. While addition of the secondary amine-bearing functional groups counteracts the effect of the native alginate biopolymer, the NO release influence the mucolytic activity to a greater extent. As such, the results suggest the potential of using alginate biopolymers as an attractive biopolymer for NO storage and delivery through mucus-coated airways.

Relative to unmodified Alg5, native COS did not significantly change mucus rheology (Figure 2). However, the elasticity and viscosity of mucus treated with COS-EA increased by ~40 and 60%, respectively (Table S4). These results re-emphasize the effect of biopolymer charge on mucus rheology. In contrast, NO-releasing chitosan decreased both the elastic and viscous moduli to that of the mucus treated with the unmodified COS. Similarly, Reighard et al. reported a reduction in the size of mucin multimers after treatment with NO-releasing COS compared to treatment with blank buffer or COS controls (non-NO-releasing).²⁰

While the results of this study indicate that NO has potential as a mucolytic agent, the charge of the delivery system may further influence mucus rheology. Interactions between the biopolymer and mucin glycoproteins can enhance NO delivery, as reported for mucoadhesive COS.²⁰ However, electrostatic interactions between highly charged biopolymers and mucin might facilitate unfavorable results (i.e., greater viscoelasticity). For NO-releasing therapeutics, the positive charge of the secondary amine-modified biopolymer has the potential to mitigate NO's effect as a mucolytic by promoting the formation of chitosan-mucin aggregates. The use of a net negatively charged biopolymer, such as alginate, may more favorably act on mucus to improve mucociliary clearance in diseased airways. Of course, such hypothesis must be further evaluated pre-clinically and clinically in human trials.

Effect of nitric oxide-release kinetics.

Previous reports demonstrated the importance of NO-release kinetics on the antibacterial activity of macromolecular NO-release systems.^{22–25} For example, slow and sustained NO-

releasing alginate oligosaccharides were proved most favorable for eradicating bacterial biofilms at low (8 mg/mL) biopolymer concentrations.²⁵ In a similar mode, NO's ability to alter mucus may also depend on NO-release kinetics. To evaluate this hypothesis, mucus samples were treated with equal concentrations (20 mg/mL) of Alg5-DPTA, Alg5-PAPA-DPTA, and Alg5-PAPA biopolymers, selected based on their diverse NO-release kinetics

As anticipated, the secondary amine-modified oligosaccharides did not alter the viscoelastic properties of the mucus samples regardless of exposure time, with COS-EA being the only exception. Treatment with COS-EA resulted in an increase in the elastic and viscous moduli starting at 1 h (Figure S11). The mucus viscoelasticity remained constant even upon treatment with COS-EA/NO, regardless of the exposure time (Figure 3), suggesting that treatment with COS-EA/NO has no overall impact on mucus rheology.

The greatest initial reductions in both elasticity and viscosity were observed after 1 h following treatment with the Alg5-PAPA/NO and Alg5-PAPA-DPTA/NO biopolymers (60–70% and 70–80%, respectively). The large initial NO fluxes characteristic of these materials compared to the slower NO-releasing biopolymers (i.e., Alg5-DPTA/NO and COS-EA/NO), induced mucolysis via delivery of a large NO dose (Table 1). However, the faster NO-releasing system (Alg5-PAPA/NO, half-life ~0.1 h) had a depleted NO payload (i.e., NO levels were below the limit of detection of the NOA) at longer exposure times (e.g., 3 and 5 h post exposure), causing the mucus viscoelasticity to return to pre-treatment levels. The biopolymers remained in the mucus sample without NO release capacity with potential for charge-based mucus interactions facilitated by the positively charged Alg5-PAPA and the negatively charged residues of mucin glycoproteins.^{4,56}

In contrast, samples treated with Alg5-PAPA-DPTA/NO did not significantly vary across treatment exposure periods (Figure 3). The slower NO-release properties of the hybrid alginate biopolymer enabled by the DPTA modification allowed for near continuous NO release through 5 h (Table 1). Even after 24 h, when NO was depleted, the rheological properties of the mucus were comparable to that observed at 1 h, resulting in a significant reduction in viscoelasticity compared to the non-NO-releasing control. Similarly, the slower sustained NO release from Alg5-DPTA/NO (half-life ~0.4 h) maintained the initial reduction in elasticity and viscosity (~50 and 60%, respectively) over the course of the experiment (Figure 3). Similar to the controls, no additional changes were observed up to 24 h once Alg5-DPTA/NO and Alg5-PAPA-DPTA/NO were both depleted of NO. For both Alg5-PAPA-DPTA/NO and Alg5-DPTA/NO, two competing mechanisms occur as NO is released by the biopolymer. Electrostatic interactions between amine groups of the modified alginates can potentially interact with the mucins over the treatment period. However, the release of NO is steady over this same period, thereby acting as a mucolytic. Overall, these results suggest that while a large initial NO flux is favorable for greater reduction in mucus viscoelasticity, slower and more sustained NO release is required to maintain a mucolytic effect over extended exposure periods.

Dose-dependent effects.

A range of concentrations using both control and NO-releasing biopolymers were used to treat mucus samples to evaluate dosing effects. The alginate system selected for this study

was Alg5-PAPA-DPTA due to its ability to greatly reduce mucus rheology. For comparison, we also evaluated COS-EA at varying concentrations.

Both control and NO-releasing Alg5-PAPA-DPTA biopolymers reduced the rheological moduli of mucus linearly as a function of concentration (Figure 4). At the lowest concentration tested (20 mg/mL), Alg5-PAPA-DPTA/NO was able to achieve a greater reduction in elasticity and viscosity (60 and 80 %, respectively) compared to the Alg5-PAPA-DPTA control (~10% for both elastic and viscous moduli), clearly demonstrating the enhanced mucolytic activity with NO. At the greatest concentration (80 mg/mL), the control biopolymer reduced the elasticity and viscosity up to 90 and 80%. The NO-releasing form was able to further decrease these moduli nearly 100%, suggesting an additive effect between the biopolymer and NO for the alginate system that could allow for greatly improved mucociliary clearance in mucus producing respiratory disorders.

Treatment of mucus with COS-EA controls showed an increase in both elasticity and viscosity from 20–40 mg/mL, but then less effect above 40 mg/mL (Figure 4). Previous reports have indicated that COS concentrations above 4.5 mg/mg mucin (i.e., the COS to mucin ratio at 40 mg/mL) lead to disaggregated chitosan-mucin complexes.^{20,46} Imparting NO-release capabilities minimized the impact of chitosan's attractive interaction with mucus at lower concentrations (i.e., 20–40 mg/mL). At concentrations greater than 40 mg/mL, the NO-releasing COS-EA reduced the mucus elasticity and viscosity up to 70 and 80%, respectively (Figure 4). These results suggest that at larger COS-EA/NO concentrations, NO overcomes the electrostatic effects between the positively charged chitosan biopolymer and negatively charged mucin glycoproteins, resulting in a therapeutic action similar to that of the NO-releasing alginate systems. Regardless, the additive effect of the alginate biopolymer with NO at increasing doses, combined with the lower concentrations required to achieve substantial reduction in mucus rheology demonstrates an advantage for NO-releasing alginates over chitosan oligosaccharides with respect to mucolytic action.

Efficacy compared to *N*-acetyl cysteine (NAC).

While the mucolytic action of NO-releasing alginate materials was demonstrated, a comparison of relative efficacy with conventional mucolytic drugs such as *N*-acetylcysteine (NAC) is necessary to fully evaluate its potential as a therapeutic.^{57–60} For this work, the effect of NAC on mucus rheology was evaluated at comparable doses to the lowest NO dose delivered by the oligosaccharide biopolymers (1–2 mg/mL or 8–16 μ mol/mL NAC) and currently nebulized therapeutic doses (100–200 mg/mL or 613–1220 μ mol/mL NAC).^{57,58,60} At equivalent doses to NO, NAC reduced the mucus elasticity by ~2–8%, which is minimal compared to that observed using any of the NO-releasing alginate systems (~40–60%; Table S4). At the inhaled therapeutic dose of NAC, the small molecule mucolytic reduced mucus elasticity at an equivalent level to the lowest concentration of alginate (~40%) but at the expense of increasing viscosity (Figure 5). As a mucolytic, NAC reduces disulfide bonds of high molecular weight mucins (resulting in sulfhydryl groups).^{61,62} We hypothesize that the thiol-bearing small molecule at high concentrations may cause undesirable entanglement within the mucin network through interactions with the mucin disulfide bonds (i.e., inducing both inter- and intra- mucin disulfide bond formation), resulting in greater viscosity over the

24-h exposure period.^{4,56} Despite an overall positive effect on mucus viscoelasticity (Figure S12), the result of this study indicates that above a threshold NAC concentration, the mucolytic action becomes limited (i.e., increased viscosity despite decreasing elasticity), leading to no discernable change with respect to the untreated sample. The opposite appears true for the NO-releasing alginates. Indeed, the mucolytic action of both control and NO-releasing alginate is dose dependent. Moreover, lower NO-releasing alginate concentrations are needed to exhibit the same reductive effect as that of high concentrations of NAC. Combined with the previously reported antibacterial properties of NO-releasing alginates,²⁵ the results of this study highlight the potential utility of these materials as dual-action therapeutic agents for the treatment of chronic respiratory diseases such as cystic fibrosis.

Conclusions

Low molecular weight (5 kDa) alginate oligosaccharides having high initial NO fluxes and sustained NO-release (e.g., Alg5-PAPA-DPTA/NO) greatly reduced both the elasticity and viscosity of mucus, suggesting a high potential utility for improving mucociliary clearance in diseased lungs. Regardless of chemical modifications, each alginate biopolymer investigated was more efficacious relative to the cationic biopolymer chitosan, indicating that a negatively charged scaffold is beneficial for reducing mucus viscoelasticity. As the NO-releasing dose of the alginate oligosaccharides required is even lower than that of current standard of care mucolytic agents (NAC) and the levels of NO released are antibacterial,²⁵ this work highlights the utility of NO-releasing alginates as a highly unique therapeutic agent for treating chronic respiratory diseases such as cystic fibrosis. While the exact mechanism by which NO alters the rheological properties is yet to be fully elucidated, the results presented herein suggest the addition of NO dramatically increases the mucolytic potency of the alginate biopolymers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Elastic and viscous moduli of 3 wt% human bronchial epithelial (HBE) mucus following treatment with 20 mg/mL Alg300 (black solid), Alg10 (black diagonal), Alg5 (gray solid), and Alg1 (gray diagonal). Single asterisks (*) indicate significant differences ($p < 0.05$) relative to untreated sample (hollow).

Figure 2. Elastic and viscous moduli of 3 wt% human bronchial epithelial (HBE) mucus following treatment with 20 mg/mL unmodified (solid), modified (horizontal stripe), and NO-releasing (diagonal stripe) (A) Alg5-DETA, (B) Alg5-DPTA, (C) Alg5-PAPA, (D) Alg5-SPER, (E) Alg5-PAPA-DPTA, and (F) COS-EA. Values presented as the mean standard error of the mean for n = 3 experiments. Asterisks (*) indicate significant differences (p < 0.05) relative to untreated sample (hollow).

Figure 4. Elastic (circle) and viscous (square) moduli of 3 wt% HBE mucus following treatment with 20, 40, 60, and 80 mg/mL concentrations of control (hollow) and NO-releasing (solid) (A) Alg5-PAPA-DPTA and (B) COS-EA.

Figure 5. Elastic and viscous moduli of 3 wt% HBE mucus following treatment with NAC at 0.1 wt% (black solid), 0.2 wt % (black diagonal), 10 wt % (gray solid), and 20 wt% (gray diagonal). Values presented as the mean standard error of the mean for n = 3 experiments. Asterisks (*) indicate significant differences ($p < 0.05$) relative to untreated sample (hollow).

Table 1.

Nitric oxide-release properties of *N*-diazoniumdiolate-functionalized biopolymers in PBS at pH 6.5 and 37 °C.^a

Biopolymer	t[NO] ^b (μmol/mg)	[NO] _{max} ^c (ppb/mg)	t _{1/2} ^d (h)	t _d ^e (h)
Alg5-DETA/NO	0.34 ± 0.15	570 ± 23	2.40 ± 1.37	16.1 ± 3.5
Alg5-DPTA/NO	0.40 ± 0.02	1000 ± 230	2.37 ± 0.10	4.4 ± 0.4
Alg5-SPER/NO	0.39 ± 0.04	2900 ± 330	0.29 ± 0.13	3.0 ± 0.8
Alg5-PAPA/NO	0.57 ± 0.02	11900 ± 3900	0.13 ± 0.06	2.9 ± 0.3
Alg5-PAPA-DPTA/NO	0.53 ± 0.05	4900 ± 1300	0.30 ± 0.08	4.5 ± 1.3
COS-EA/NO	0.74 ± 0.07	1500 ± 300	0.82 ± 0.01	10.2 ± 1.5

^aError represents standard deviation for n = 3 experiments.

^bTotal NO released.

^cMaximum instantaneous flux of NO release.

^dNO-release half-life.

^eDuration of NO release. All measurements were collected in 10 mM PBS using ~1.0 mg of the biopolymer.