A New Class of Safe Oligosaccharide Polymer Therapy To Modify the Mucus Barrier of Chronic Respiratory Disease


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Supporting Information

ABSTRACT: The host- and bacteria-derived extracellular polysaccharide coating of the lung is a considerable challenge in chronic respiratory disease and is a powerful barrier to effective drug delivery. A low molecular weight 12–15-mer alginate oligosaccharide (OligoG CF-5/20), derived from plant biopolymers, was shown to modulate the polyanionic components of this coating. Molecular modeling and Fourier transform infrared spectroscopy demonstrated binding between OligoG CF-5/20 and respiratory mucins. Ex vivo studies showed binding induced alterations in mucin surface charge and porosity of the three-dimensional mucin networks in cystic fibrosis (CF) sputum. Human studies showed that OligoG CF-5/20 is safe for inhalation in CF patients with effective lung deposition and modifies the viscoelasticity of CF-sputum. OligoG CF-5/20 is the first inhaled polymer therapy, represents a novel mechanism of action and therapeutic approach for the treatment of chronic respiratory disease, and is currently in Phase IIb clinical trials for the treatment of CF.

KEYWORDS: mucin, sputum, polymer therapy, alginate, cystic fibrosis, viscoelasticity, safety

1. INTRODUCTION

Chronic respiratory diseases are a major world health issue, with >100 million people affected, and are estimated to cause 4 million premature deaths annually. The polymeric, extracellular surface-coating of the lung represents an important therapeutic target in both the treatment of respiratory disease and in therapeutic delivery of bioactive agents/therapies. In the diseased lung, the surface epithelia are coated with a complex mucus layer composed of mucins, bacterial biofilms (containing extracellular polysaccharide (EPS) and high-molecular weight (Mw) extracellular DNA (eDNA)), inflammatory cells, and keratinocytes. Mucins are a family of 19 polydisperse (250 kDa), heavily glycosylated block copolymers, which are secreted into the mucous layer by specialized epithelial cells that line the aero-digestive and reproductive tracts. Mucins play an important role in innate immunity (as a functional barrier to pathogens and environmental particulates) and epithelial homeostasis (preventing water loss). In many chronic respiratory diseases, such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF), the production of highly viscous/elastic mucus impedes clearance and prolongs infection and airway inflammation. In CF, the difficulties of targeting therapies to the epithelial lung surface across this “mucus barrier” are compounded by the presence of bacterial biofilms on the surface of the epithelia. These biofilms often contain multidrug resistant (MDR) mucoid bacteria (e.g., Pseudomonas and Burkholderia spp.), which secrete polyanionic (high Mw) EPS. The presence of...
these microorganisms, their biofilm structure, and their resistance to conventional antimicrobial/antibiotic therapy is associated with higher morbidity/mortality. In chronic respiratory disease, numerous approaches including nanoparticle- and nanocarrier-based systems have been described in vitro in attempts to modify the structure of mucus and sputum. Previous studies have identified the potential of low Mw alginates to alter the rheological properties of the mucus viscoelastic barrier. Alginate is a biopolymer found in marine algae and bacterial EPS of C-5 epimer guluronic (G) and (1–4)-linked mannuronic (M) acid. Alginates occur in nature as polydispersely high Mw polymers (300–500 kDa), which, following chemical or enzymatic hydrolysis, have been extensively employed in food and biomedical applications (e.g. as gelling agents and wound-dressing materials). The ability to engineer alginate oligomers of defined G-M composition and size/degree of polymerization (DPn) has allowed their development for distinct biological applications. Previous in vitro studies demonstrated that low Mw alginate oligomers (DPn = 10–12; containing >85% G) could alter mucin/alginate and mucin/DNA interactions at concentrations as low as 1.4 μg/mL and alter the structure of mucin by increasing pore-size, thereby facilitating improved nanoparticle diffusion through the mucus layer. Interestingly, in vitro studies have also demonstrated the ability of OligoG CF-5/20 (Figure 1d; Mw 2600 Da, > 85% G residues) to potentiate the action of antibiotics against MDR CF pathogens (including *Pseudomonas* and *Burkholderia* spp.), and in the case of the MDR *P. aeruginosa* enrichment performed using high-speed, density gradient centrifugation and guanidinium treatment of the gel-phase to enrich the MUC5AC and MUC5B component.

These initial studies, therefore, highlighted the potential of oligomeric structures, based on G-alginate residues, as a treatment in chronic lung disease (e.g., CF). We hypothesized that when inhaled, these low Mw alginate oligosaccharides could interact with (and modify) the structural assembly and rheological properties of mucus (and sputum) in patients with respiratory disease. Initial studies of this potential interaction were undertaken using molecular modeling and Fourier transform infrared spectroscopy (FTIR). Direct imaging and electrophoretic light-scattering (ELS) were then utilized to demonstrate how OligoG CF-5/20 induced the disruption of respiratory mucin networks. Preclinical animal and human studies showed that OligoG CF-5/20 is safe for delivery via an inhaled route in the diseased lung, and this was tested in healthy human Phase I and in CF patients in Phase IIa studies. Further, the potential effect of OligoG CF-5/20 in the management of patients with chronic lung disease was studied by investigating its ability to modify the rheological properties of sputum from a series of CF patients.

### 2. EXPERIMENTAL SECTION

#### 2.1. Preparation of Alginate Biomaterials

Alginate oligosaccharide, OligoG CF-5/20 (>85% guluronic acid content; Mw 2600 Da), was derived from the stem of brown seaweed *Laminaria hyperborea* as previously described.

#### 2.2. Molecular Dynamic (MD) Studies of Mucin–OligoG CF-5/20 Interactions

MD simulations were run on the High Performance Wales Supercomputer (www.hpcwales.co.uk; Supporting Information) using the predominant mucin in the lung (MUC5AC) and taking into account the levels of mucin glycosylation observed in the CF lung. A G-rich alginate molecule of 12 DPn length was utilized to represent OligoG CF-5/20 at a ratio of 100 amino acids to 1 OligoG chain.

#### 2.3. FTIR Spectroscopy of Mucin–OligoG CF-5/20 Interactions

Untreated control sputum samples from the Phase IIa studies were thawed on ice and glycoprotein-enrichment performed using high-speed, density gradient centrifugation and guanidinium treatment of the gel-phase to enrich the MUC5AC and MUC5B component. Samples were then treated with 10% (v/v) distilled water ± 0.2% OligoG CF-5/20 (w/v) and incubated statically at 37°C for 60 min (Supporting Information) prior to FTIR analysis.

#### 2.4. Extraction of Pig Gastric Mucin

Mucin was prepared and purified under nondenaturing conditions as previously described and used in all the following studies.

#### 2.5. Scanning Electron Microscopy (SEM) Imaging of Pig Gastric Mucin

Pig gastric mucin (PGM; 0.2%) was dissolved in distilled water and placed on a roller at 37°C for 2 h prior to being filter sterilized. PGM samples ± 0.2% (1:1) or 2% (1:10) OligoG CF-5/20 (PGM/OligoG, respectively) were placed onto Thermanox glass slides (Agar Scientific) in a 12-well plate (Greiner Bio-One) and incubated at 37°C for 1 h statically before being fixed overnight in 2.5% glutaraldehyde (TAAB Laboratories). Samples were then washed with distilled
water (four times) and freeze-dried prior to imaging using a Hitachi S4800 scanning electron microscope without sputter-coating.

2.6. Atomic Force Microscopy Imaging of Pig Gastric Mucin. PGM (0.004%) ± OligoG CF-5/20 (0.001%) was dissolved in distilled water (4:1, PGM/OligoG) and a 1 μL droplet placed on a freshly cleaved mica plate (Agar Scientific) and left to air-dry. The concentration of OligoG CF-5/20 and PGM was significantly reduced in these studies where the higher in vitro testing concentrations of OligoG CF-5/20 masked the results at the nanoscale level (Supporting Information, Figure 1). Atomic force microscopy (AFM) imaging was carried out on a Dimension 3100 AFM (Veeco) over a 5 μm² area (n = 6). AFM images were analyzed by ImageJ software.

2.7. Electrophoretic Light Scattering of Pig Gastric Mucin—OligoG CF-5/20 Interactions (Surface Charge). At low concentrations, the zeta-potential of the OligoG CF-5/20-only control (at both pH 5 and 7) was undetectable. Therefore, in these experiments, the concentrations of OligoG CF-5/20 and the OligoG CF-5/20:PGM ratio was reduced to visualize the mucin—oligomer interactions, which allowed the change in mucin surface charge to be detected without “masking” by OligoG CF-5/20. PGM (0.03%) ± OligoG CF-5/20 (0.02%) was made up in NaCl buffer (0.001 M) at pH 5 and pH 7 (3:2, PGM/OligoG) and filter sterilized (0.22 μm). Samples were incubated at room temperature for 20 min prior to electrophoretic light scattering (ELS) analysis as previously described. A Zetasizer Nano ZS (Malvern Instruments) and disposable capillary cells (DTS1061) were used for the zeta potential analysis. Electrophoretic mobility of samples was analyzed using Smoluchowski’s equation.

2.8. ImageJ Analysis of AFM and SEM Images. AFM images of PGM were converted into binary mode and the percentage area covered analyzed. SEM images of CF sputum samples were enhanced prior to adjustment of threshold to highlight dark porous areas within the images. These were then converted into binary mode to allow measurement of surface-area coverage.

2.9. Patients and Samples. Noninduced sputum samples (n = 23) were collected by expectoration from seven patients with CF attending the Cystic Fibrosis Service, Cardiff and Vale University Health Board (Supporting Information Table 1; Ethics number: 11/ WA/0318). Mean patient age was 26 years (range 17–39), and mean forced expiratory volume in 1 s (FEV1) was 0.80–1.93 L (46% of the predicted value; range 20–65%).

2.10. SEM Imaging of CF Sputum. Noninduced sputum samples (0.1 mL) were incubated following treatments with water (control), 100 nM rhDNase I (Pulmozyme; Genentech Inc.), OligoG CF-5/20 (0.2% or 2%), or 100 nM rhDNase I ± OligoG CF-5/20 (0.2, 2%) for 20 min (as previously described) at 37 °C. Nonhomogenized CF sputum samples (0.1 mL) were then placed on freshly cleaved mica plates (Agar Scientific) and left for 5–10 min to air-dry. Nonadherent sputum was washed from the slides using PBS (pH 7.4). The slides were then fixed in 1 mL of 2.5% glutaraldehyde in a sterile 12-well plate (24 h). Drying of the samples was achieved via the ethanol dehydration method and imaging performed using an Hitachi S4800 SEM without sputter coating.

2.11. Animal and Human Inhalation Studies. Animal toxicity studies with OligoG CF-5/20 were performed in Sprague–Dawley rats. See Supporting Information.

The human Phase I study was a single center, randomized, placebo controlled, 72 h, dose escalation study to test the in vivo safety and tolerability of OligoG CF-5/20 in humans (www.clinicaltrials.gov, Identifier: NCT00970346), and the phase II study was a multicenter randomized placebo controlled crossover study with 28 days treatment periods (www.clinicaltrials.gov, Identifier: NCT01465529). See Supporting Information.

2.12. Scintigraphy Studies Were Conducted To Investigate Lung Deposition of Radiolabeled OligoG. For this, an open label two-way randomized crossover study in 10 CF patients was conducted (www.clinicaltrials.gov, Identifier: NCT01991028). See Supporting Information.

2.13. Rheological Analysis of CF Sputum. Noninduced sputum from CF patients (Supporting Information, Table 1) was collected and studied following written, informed consent and study approval by the Local Research Ethics Committee (11/ WA/0318). Initial observational studies demonstrated that 2% OligoG CF-5/20 induced differences in the extensional thinning behavior of CF sputum (Supporting Information, Video 1). Due to the large heterogeneity found within sputum samples, bulk rheology was subsequently carried out and quantified using dynamic shear rheometry (Supporting Information).

3. RESULTS

3.1. Predicting Molecular Interactions between Guluronate Alginate Oligosaccharides and Mucin in CF Sputum. MD modeling was initially utilized to provide information on potential molecular interactions between OligoG CF-5/20 and mucin (Supporting Information, Tables 2–8). MD modeling techniques were employed to study the interaction of 12-mer guluronate oligosaccharides with a glycosylated 100 amino acid sequence of the central motif of MUCSAC (the predominant human airway mucin). The glycan structures on the MUCSAC model peptide backbone were core one and two glycan structures: core 1 (consisting of N-acetylgalactosamine [GalNAc] and glucose) and core 2 (consisting of GalNAc, glucose, and N-acetyl-D-glucosamine [GlcNAc]). These studies predicted binding of the guluronate alginate oligosaccharide to both glycan structures and the peptide backbone of the MUCSAC amino acid structure. Specifically, bonding was found between the hydroxyl groups in the G-alginic oligosaccharide and N- or O-atoms on the peptide backbone; N-atoms from the amide group in GalNAc and hydroxyl groups in the sugar rings constituting all components of the glycan structures. This resulted in decreased flexibility and linearization of the local region of the MUCSAC molecule (P = 0.0009; Figure 2a,b).

To determine whether this predicted mucin binding was evident in vivo, attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) was used to characterize the electrostatic interactions between the mucins and the OligoG CF-5/20. CF sputum was obtained (www.clinicaltrials.gov, Identifier: NCT01465529) with written, informed consent and following study approval by the Local Research Ethics Committee (10/ H1005/88). Absorbance spectra showed the distinct peak positions of mucin and OligoG CF-5/20 (Figure 3a); the interaction between mucin and OligoG CF-5/20 was evident in the Amide I region of the second-derivative spectra, involving the carbonyl group within the peptide link of the mucin protein backbone (Figure 3b). Post-treatment, absorbance was decreased at the random coil associated wavenumber.
1652 cm\(^{-1}\) and \(\beta\)-sheet associated wavenumber 1637 cm\(^{-1}\), with a shift in peak positions to higher frequencies at 1650 and 1634 cm\(^{-1}\) as previously described.\(^9\)\(^{28}\)\(^{30}\) FTIR analysis predicted widespread H\(^{+}\)-bonding between the oligosaccharide and peptide, leading to reduced flexibility of the mucin molecule post-treatment, as predicted by the MD studies.

3.2. OligoG CF-5/20 Modulation of the Surface Charge and Structural Assembly of Mucin Networks \textit{ex Vivo}. Following these initial results, the interaction of OligoG CF-5/20 was directly observed \textit{ex vivo} with porcine mucin samples by employing AFM, SEM, and ELS to characterize the surface charge using zeta potential analysis. PGM has been extensively employed, and its biophysical features characterized as a homologue of MUCSAC in previous studies.\(^9\)\(^{28}\)\(^{30}\) Furthermore, AFM imaging has been extensively utilized to characterize mucin networks \textit{in vitro},\(^27\) investigating glycoprotein structure and interactions.\(^31\)

AFM imaging revealed that treatment with OligoG CF-5/20 significantly modified the mucin network by changing its morphology and causing increased pore size of the network with a reduction in mucin interlinking networks. Unsurprisingly, there was an increase in surface area due to the addition of OligoG CF-5/20 (52.1\%) into the system compared to the control (28.8\%) when analyzed using ImageJ \((P < 0.0001; \text{Figure 4a})\). SEM showed that at 0.2\% OligoG CF-5/20, there appeared to be minimal effect on porosity, with OligoG CF-5/20 instead seeming to coat the surface of the mucin. At 2\%, however, OligoG CF-5/20 induced marked morphological changes in the mucin networks with treated samples exhibiting significantly increased porosity compared to the control (\textit{Figure 4b}). These \textit{in vitro} structural changes reflected those predicted by initial MD modeling and FTIR studies, with alterations in bonding between mucin chains resulting in a more open mucin network. ELS (zeta-potential) analysis also revealed that treatment with OligoG CF-5/20 induced marked alterations in mucin surface charge. Significantly increased negative surface charge of the mucin was evident after treatment with OligoG CF-5/20 at 0.001 M NaCl, pH 5 (-16.0 mV vs -24.2 mV) and pH 7 (-16.7 mV vs -23.1 mV) \((P < 0.0001; \text{Figure 5a,b})\). Because of the low concentration of the OligoG CF-5/20-only control (at pH 5 and 7), only multiple, weak unstable peaks were seen confirming the mucin—oligomer interaction. These studies, therefore, confirmed the ability of OligoG CF-5/20 to modify both the surface charge and the structural assembly of the mucin biopolymer.

3.3. Optimizing Chronic Pulmonary Administration of the Polymer in the CF Lung. Preclinical animal and human respiratory studies demonstrated that OligoG CF-5/20 could be reliably and safely administered via inhalation to the lung. Initial dose-escalation animal studies were undertaken for up to 28 days (with estimated achieved dose levels of 288–467 mg/
kg/day; Supporting Information, Tables 9–12). These demonstrated no adverse clinical signs or test-item related changes in body weight, food consumption, respiratory, hematological, or biochemical parameters. A separate animal study with tritiated OligoG CF-5/20 showed that following oral administration of OligoG CF-5/20, the majority was excreted via the gastrointestinal (GI) tract (82.6% within the first 24 h). Furthermore, intravenous administration of the ^3H-labeled OligoG CF-5/20 in animals revealed standard drug-like properties with rapid elimination via urine (80.3% within the first 24 h).

A clinical Phase I study (Supporting Information, Table 13) also demonstrated that inhalation of OligoG CF-5/20 (dosing up to 540 mg/day over 3 days) was well-tolerated, with no toxicity/adverse events or clinically significant changes in hematology, biochemistry, urinalysis, or vital signs (blood pressure, pulse, respiration rate, body temperature, SpO2, ECG, or spirometry) observed.

The administration of the agent OligoG CF-5/20 to the diseased lung was planned as an inhalational therapy.32 A lung scintigraphic study was, therefore, performed to characterize the pulmonary deposition of ^99mTc-labeled OligoG CF-5/20 in both nebulized solution and as an inhaled, dry-powder formulation in the diseased lungs of patients with CF. This single-dose study demonstrated that both formulations were well-tolerated. The dry-powder inhalation exhibited a significantly increased whole lung deposition compared to the nebulized solution (38.6% vs 17.1%; P = 0.001; Figure 6a,b) and a significantly lower deposition in the oro-pharyngeal (mouth, pharynx, and gastric) region (11.3% vs 19.9%; P = 0.033; Supporting Information, Table 14).

3.4. OligoG CF-5/20 Induced Disruption of the Mucin Hydrogel Network of CF Sputum. The effects of OligoG CF-5/20 on patient sputum samples were studied ex vivo using a combination of imaging and rheological analysis. These experiments employed the OligoG CF-5/20 alone and in combination with recombinant human rhDNase I (Pulmozyme, a common mucolytic treatment employed in CF).33,34 SEM

Figure 4. Effect of mucin–alginate interactions on polymer morphology. (a) AFM imaging (5 μm) of PGM (0.004% PGM) ± OligoG CF-5/20 (0.001%), z-scale 9 nm. (b) SEM imaging of PGM ± 0.2–2% OligoG CF-5/20, scale bar 5 μm.

Figure 5. Effect of mucin–alginate interactions on surface charge. Zeta-potential measurements of PGM (0.03%; solid line) ± OligoG CF-5/20 (0.02%; dashed line) performed at 0.01 M NaCl (a) pH 5 and (b) pH 7.
demonstrated the previously reported anisotropic (directional) nature of the sputum with marked variation in pore-size (Figure 7a). The SEM results were, unsurprisingly, not as pronounced as those observed in AFM analysis of PGM alone due to the lack of contrast with the substratum, the lower OligoG CF-5/20 concentration used (0.001%), and artifactual contraction of the sample during freeze-drying. Treated samples demonstrated a marked dose-dependent increase in porosity when treated with OligoG CF-5/20 (Figure 7a), where the difference in percentage surface area of pores between the control (8.91 ± 4.49%) and 2% OligoG CF-5/20-treated sputum (16.61 ± 3.00%) was significantly different (P = 0.007). Interestingly, while DNase I alone demonstrated alteration in the structure of sputum (in keeping with previous studies24), marked increases in the porosity of CF sputum networks after treatment with rhDNase I and the OligoG CF-5/20 were evident. Imaging demonstrated synergy between OligoG CF-5/20 and the rhDNase I in disrupting the three-dimensional network of the sputum (Figure 7a).

3.5. OligoG CF-5/20 Induced Improvement of the Viscous and Elastic Properties of CF Sputum. The ability of OligoG CF-5/20 to modify the viscoelasticity of CF sputum was tested using extensional and shear rheology. The studies were performed using noninduced sputum from seven patients (n = 23 samples; Supporting Information, Table 1). Initial differences were observed in video imaging and extensional rheology (Figure 7b), with OligoG CF-5/20 inducing rapid “extensional thinning” behavior of CF sputum compared to the control (Supporting Information, Video 1).

To analyze bulk changes, shear rheology was then utilized, employing a frequency range of 0.1–10 Hz (relevant to biological processes in the airway). The effect of OligoG CF-5/
20 at a range of concentrations (0.2–2%) on CF sputum samples was compared to those of distilled water (Figure 8a) and rhDNase I controls (Figure 8b, 9a,b). Ionic strength variations were minimized by preparing all samples in distilled water. OligoG CF-5/20 treatment exhibited marked reductions in both elastic ($G'$) and viscous response ($G''$) (Figure 8a) of CF sputum compared to the water-treated control (0.16 Hz; $P < 0.0001$). A significant reduction in the elastic response, $G'$ ($P = 0.0005$), and viscous response, $G''$ ($P = 0.0175$), was also seen at 10 Hz (Supporting Information, Figure 2). To monitor clinical reproducibility of potential changes in the efficacy of OligoG CF-5/20 with disease-state, a longitudinal study of sputum rheology was also conducted using successive samples ($n = 9$) from a single subject (receiving 7% hypertonic saline, rhDNase I, and intravenous antibiotics). This patient demonstrated marked intraindividual variation (at 0.16 Hz) in control values of elastic response (Figure 9a) and viscous response (Figure 9b), with marked heterogeneity in the observed viscoelastic properties of their sputum. In these experiments, samples were treated with rhDNase I (the standard mucolytic treatment employed in CF clinical practice$^{33,36}$), and a final rhDNase I concentration of 2.5 $\mu$g/mL (approximately 100 nM) was chosen in accord with previous studies.$^{37}$ The distinct mechanism of action of rhDNase I induces degradation of the large, viscous polyanionic eDNA and subsequent changes in the viscoelasticity.$^{37}$ In these studies, samples treated with rhDNase I in conjunction with 2% OligoG CF-5/20 showed statistically significant differences in both $G'$ ($P = 0.004$) and $G''$ ($P = 0.001$; Figure 8b). The structural changes in CF sputum following OligoG CF-5/20 treatment were reflected in the ability of OligoG CF-5/20 to modify the viscoelastic properties ($G'$ and $G''$) of CF sputum with significant, reproducible changes induced over the entire frequency range.

4. DISCUSSION

These studies demonstrate that a novel oligosaccharide polymer therapy$^{38}$ from nature can disrupt mucin hydrogel networks in sputum and represent a novel and highly safe mechanistic approach in the treatment of chronic lung disease. The initial MD modeling and FTIR results in this study predicted the ability of a 12-mer G-block structure (based on OligoG CF-5/20) to exhibit mucin-binding and demonstrated how this binding might result in a reduction in the potential sites of interaction with both mucin chains and molecules. The subsequent in vitro studies (using AFM and SEM) went on to demonstrate that this binding did induce physical disruption of the glycoprotein-enriched, mucin polymer network. This finding was potentially clinically important, as workers have previously demonstrated that “branching” of CF mucin (associated with increased glycosylation) impairs drug diffusion in vitro.$^{29}$

SEM previously has been used to investigate the effects of nanoparticle systems on mucin aggregation.$^{15,39}$ Moreover, these changes reflect recent ex vivo studies, which demonstrated similar charge alterations in mucins when PGM was treated with the heteropolysaccharide pectin.$^{23}$ While the structural changes observed here (in porosity and branching) are
significant, the ionic charge distribution of the mucin polymer network is also a key determinant of penetration both in vitro and in vivo. The ability of OligoG CF-5/20 to modify the anionic surface-charge of the mucin was also important and may explain the previously reported increase in mobility of nanoparticle diffusion observed in low molecular weight alginate-treated mucus. As the spatial arrangement of charge and structural assembly within the mucin network is pivotally important in determining the transport of materials in vivo, these changes indicated that OligoG CF-5/20 may exhibit clinically significant effects in modifying the mucus barrier in the lungs in vivo.

A number of inhaled therapies have already been identified to potentially modify mucus clearance and increase diffusion through the mucin barrier for gene-, drug-, and protein-delivery, employing both mucolytic and decreased muco-adhesive approaches. Examples include rhDNase I, a mucolytic showing high efficacy in vivo in altering the viscoelasticity of CF sputum, and hypertonic saline, which modifies the rheological properties of mucus and stimulates the cough reflex. Safety is of pivotal importance, as a number of theoretical nanomedicine approaches described in the literature have been either ineffective in vivo or associated with side-effects or difficulty of use.

As the in vitro experiments appeared promising, toxicity studies were undertaken in vivo. The initial oral toxicity studies demonstrated the relatively rapid excretion and lack of systemic absorption of OligoG CF-5/20 (see Supporting Information). Importantly, while OligoG CF-5/20 was eliminated via the GI tract, these studies revealed no clinical evidence of modification of the GI mucin barrier even following chronic, high-dose administration. The lack of toxicity was confirmed in subsequent Phase IIa human studies, which established the safety of inhaled OligoG CF-5/20 in both healthy and chronically diseased lungs.

The lack of safe nanomedicine-based inhalation therapies that efficiently penetrate the mucin barrier gives the natural oligomer, OligoG CF-5/20, an inherent advantage. These toxicity and pharmaco-distribution studies demonstrated that OligoG CF-5/20 could be delivered to the diseased CF lung at doses that were deemed effective in vitro. OligoG CF-5/20 has been granted orphan status by the European Medicines Agency (EU/3/07/475) for the treatment of CF. The lack of toxicity for OligoG CF-5/20 was anticipated based on existing knowledge of sodium alginate, which is registered in the European Pharmacopoeia and US Pharmacopeia.

While the “simplistic” in vitro mucin studies were encouraging, the sputum of the diseased lung is a highly complex therapeutic target, being composed of <5% mucin, >90% water, and containing variable amounts of polyanions such as eDNA and EPS. Previous studies have shown that the rheological changes induced by guluronate oligomers in the treatment of CF sputum are the result of disruption in interpolymer cross-links and not simply the result of varying ionic strength. The marked heterogeneity in the elastic and viscous properties of sputum samples in our longitudinal observations from a single patient further highlight the complexity of sputum as a therapeutic target. The predictable decrease in the elastic and viscous responses of CF sputum, which were observed when the results with OligoG CF-5/20

Figure 9. Longitudinal study of CF sputum samples obtained from a single patient over 9 days (AM, morning; PM, evening) showing change in (a) elastic response, $G'$, and (b) viscous response, $G''$, at 0.16 Hz (compared to the untreated control) for sputum treatments ±2% OligoG CF-5/20 or rhDNase I (100 nM).
were compared with rhDNase I, reflected the universal alteration in the structural assembly of mucus in OligoG CF-S/20-treated CF sputum and its value as a therapeutic target.\textsuperscript{45} Importantly, for clinical utilization, these results showed that, in these experiments, following OligoG CF-S/20 treatment, the ratio of viscous to elastic response was maintained (the values of $G'$ and $G''$ decreasing by similar proportions), allowing optimum clearance of mucus by both cough and mucociliary action.\textsuperscript{36,37} These studies, while important, were short-term (<10 days) and are being extended in ongoing, longer term (28 days) Phase Ib clinical trials in CF patients (www.clinicaltrials.gov, Identifier: NCT02157922).

5. CONCLUSION
These studies highlight, at a nano- and macro-scale, the ability of OligoG CF-S/20 to alter the structure of mucus, modulate mucin assembly, and reduce the elastic and viscous properties of sputum from CF patients and potentially other chronic respiratory diseases. Employing such an approach may enable physicians to utilize the high surface-area and noninvasive nature of pulmonary administration\textsuperscript{34} to effectively deliver therapies across the mucin “barrier” in the treatment of a range of human diseases. This study describes the first-in-class of a new generation of inhaled polymer therapies. It has clear potential for the management of chronic lung disease such as CF and offers a novel mechanism to disrupt mucin hydrogel networks in a range of other applications including drug delivery and gene- and reproductive-therapy.

ASSOCIATED CONTENT
2 Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.5b00794.

Additional experimental results; detailed experimental procedures, including human and animal studies (PDF)
Reconstructed video of sputum extensional rheology (AVI; Control)
Reconstructed video of sputum extensional rheology (AVI; 2% OligoG CF-S/20)

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Author Contributions
D.W.T. and K.E.H. directed the research and wrote the manuscript. Dynamic modeling and FTIR were performed by G.M. and P.L. SEM, AFM, and ELS studies were performed by M.F.P. and L.C.P. The AFM was provided by C.W. Rheology was by M.F.P. and K.H. Patient sample collection was by I.D. M.F.P. and L.C.P. The AFM was provided by C.W. Rheology networks in a range of other applications including drug formulation development studies were by E.O., P.D.R., A.H.M., A.D., and R.M. The scintigraphic studies were by H.N.E.S., L.A.H., G.M.C., and J.B.N. All authors contributed to, revised, and approved the final manuscript.

Notes
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