ANTIBACTERIAL AND BIOPHYSICAL CHARACTERIZATION OF NITRIC OXIDE-RELEASING CHITOSAN OLIGOSACCHARIDES – TOWARDS A NEW CYSTIC FIBROSIS THERAPEUTIC

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ABSTRACT

Katelyn Patricia Reighard: Antibacterial and Biophysical Characterization of Nitric Oxide-Releasing Chitosan Oligosaccharides − Towards a New Cystic Fibrosis Therapeutic. (Under the direction of Mark H. Schoenfisch)

Cystic fibrosis (CF) is characterized by chronic infections within the highly viscous mucus layer of the airways. Nitric oxide (NO), an endogenously produced free radical involved in the immune response to foreign pathogens, can be delivered exogenously from macromolecular scaffolds to combat bacterial infection. Herein, the ability of NO-releasing chitosan oligosaccharides to eradicate *P. aeruginosa* biofilms and alter the biophysical properties of infected airway mucus was characterized.

As oxygen gradients form within the CF mucus layer, the antibacterial efficacy of NO was determined in aerobic and anaerobic conditions. Against planktonic cultures, NO-releasing chitosan oligosaccharides were more effective antibacterial agents under anaerobic environments due to reduced NO reactions with oxygen. However, the bactericidal NO dose released from the scaffold was the same under both conditions, indicating that the efficacy of NO against planktonic cultures was independent of oxygen concentration. For *P. aeruginosa* biofilms, the minimum biofilm eradication concentration of NO-releasing chitosan oligosaccharides was decreased under anaerobic conditions. Furthermore, the concentration of NO required to eradicate biofilms was 10-fold lower than tobramycin under anaerobic conditions.

Biofilm disruption and eradication were investigated as a function of NO-releasing chitosan oligosaccharide dose, with results compared to non-NO-releasing chitosan

oligosaccharides and tobramycin. Quantification of biofilm expansion/contraction and multiple-particle tracking microrheology were used to assess the biofilm structural integrity before and after antibacterial treatment. While tobramycin had no effect on the physical properties of the biofilm, NO-releasing chitosan oligosaccharides induced biofilm degradation. Control chitosan oligosaccharides actually increased biofilm elasticity, indicating that the scaffold may mitigate the biofilm disrupting power of NO.

The effects of NO on the physical properties of CF mucus were examined to assess the mucolytic potential of NO-releasing chitosan oligosaccharides. When released from a mucoadhesive scaffold, NO increased mucin migration during electrophoretic separation, indicating a reduction in mucin molecular weight. Additionally, NO destroyed the physical structure of mucin networks and reduced the viscosity and elasticity of CF sputum. The mucoadhesive properties of each scaffold dictated the mucolytic efficacy of NO-releasing chitosan oligosaccharides, as non-adhesive scaffolds were incapable of altering mucins in purified mucin solutions and CF sputum.

To my parents,
whose unceasing love has always supported me,

and to my grandfather, Poppy,
who passed his love of chemistry on to me.

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LIST OF ABBREVIATIONS AND SYMBOLS

$	United States dollar(s)
%	percent
~	approximately
<	less than
>	greater than
±	plus or minus
× g	times the force of gravity
≤	less than or equal to
≥	greater than or equal to
°C	degree(s) Celsius
δ	delta
η	eta
τ	tau
µg	microgram(s)
µL	microliter(s)
µM	micromolar
µmol	micromole(s)
17-FTMS	(heptadecafluoro-1,1,2,2-tetrahydrodecyl)trimethoxy silane
^{1}H NMR	proton nuclear magnetic resonance
Ag/AgCl	silver/silver chloride (reference electrode)
Ar	argon
ASL	airway surface liquid

BCC	*Burkholderia cepacia* complex
br	broad
BSA	bovine serum albumin
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
CFU	colony forming units
cGMP	cyclic guanosine monophosphate
cm	centimeter
COS	2-methylaziridine modified chitosan oligosaccharides
d	day(s)
D_2O	deuterium oxide
DAPI	4’,6-Diamidino-2-phenylindole dihydrochloride
DNA	deoxyribonucleic acid
Dnase I	deoxyribonuclease I
DPBS	Dulbecco’s phosphate buffered saline
DPI	dry powder inhaler
DTT	dithiothreitol
e.g.	exempli gratia (for example)
EA	ethyl acrylate
EA	ethyl acrylate
EDTA	ethylenediaminetetraacetic acid
ENaC	epithelial Na+ (sodium) channel
eNOS	endothelium nitric oxide synthase

EPS	exopolysaccharide
et al.	et alia (and others)
FDA	United States Food and Drug Administration
FEV_1	forced air expiratory volume in 1 second
g	gram(s)
GPM	type II gastric pig mucin
h	hour(s)
H. influenzae	*Haemophilus influenzae*
H_2O	water
HBE	human bronchial epithelial
i.e.	id est (that is)
IC_{50}	half maximal inhibitory concentration
IgG	immunoglobulin G
iNOS	inducible nitric oxide synthase
kD	kilodalton
LB	Luria Bertani (broth)
LPS	lipopolysaccharide
M	molar
M	molecular weight
MBC	minimum bactericidal concentration
MBEC	minimum biofilm eradication concentration
MeOH	methanol
mg	milligram(s)

mg	milligram
MIC	minimum inhibitory concentration
min	minute(s)
mL	milliliter(s)
MRSA	methicillin-resistant *Staphylococcus aureus*
MSD	mean squared displacement
MTMOS	methyltrimethoxysilane
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
muc5AC	mucin 5AC
muc5B	mucin 5B
mV	millivolt
mW	milliwatt
mΩ	miliohm(s)
N_2	nitrogen
N2OR	nitrous oxide reductase
NAC	*N*-acetylcysteine
NaNC	nonadrenergic noncholinergic
NAP	3-acetamido-4,4-dimethylthietan-2-one
NAR	nitrate reductase
NBF	neutral buffered formalin
NIR	nitrite reductase
nM	nanomolar

nm	nanometer(s)
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOR	nitric oxide reductase
P. aeruginosa	*Pseudomonas aeruginosa*
PAK	*Pseudomonas aeruginosa* strain K
PB	phosphate buffered
PBS	phosphate buffered saline
PCL	periciliary layer
PEG	polyethylene glycol
PLGA	poly lactic-co-glycolic acid
ppb	part per billion
ppm	parts per million
Pt	platinum
RSNO	*S*-nitrosothiol
s	singlet
s	second(s)
S. aureus	*Staphylococcus aureus*
SDS	sodium dodecyl sulfate
SEM	standard deviation of the mean
SNP	sodium nitroprusside
SPA	sulfopropyl acrylate potassium salt
SSC	saline-sodium citrate

TAE	tris-acetate-EDTA
TBA	2-iminothiolane hydrochloride
TBuA	*tert*-butyl acrylate
UNC	University of North Carolina (at Chapel Hill)
US	United States
UV	ultraviolet
wt/wt	weight to weight ratio

CHAPTER I: INTRODUCTION

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is a life-limiting, genetically inherited disorder affecting 30,000 people in the United States of America (US) and 70,000 people worldwide.[1-2] This disease is caused by mutations in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which in turn controls mucus and ion transport.[3-5] Defects in the CFTR protein result in accumulation of mucus throughout the body and obstruction of normal function in the liver, pancreas, gastrointestinal tract, and the reproductive system. However, buildup of mucus is most damaging in the respiratory system. In healthy airways, CFTR controls the absorption of chloride, sodium, and water across the epithelium surface. Disruption of CFTR function associated with CF results in dehydration of the airway surface liquid (ASL) and excessive absorption of sodium and chloride ions. Depletion of the ASL causes the collapse of cilia, impeding their movement and thus inhibiting mucociliary clearance.[6] Goblet cells in the CF airway continue to secrete mucus, further intensifying the problem. As mucus accumulates and thickens in the CF airway, the bronchial tubes become obstructed, fostering a suitable environment for bacteria colonization. Bacteria are protected from the immune response by the thickened mucus layer and formation of biofilms.[6] Chronic infections develop, causing increased inflammation and degradation of lung health over the lifespan of the patient.[6] Respiratory complications of these chronic infections ultimately result in the death of 85% of patients with CF.[7]

1.1.1 Innate airway defense in healthy lungs

Adult humans inhale over 1,000 liters of air per day with near constant microbe exposure.[8] To combat this, the human airway has developed an innate defense that operates via two mechanisms: the development of a "chemical shield" and the mechanical clearance of foreign particles (i.e., mucociliary and cough clearance). While some controversy over these two mechanisms exists, it is generally recognized that mechanical clearance is more important to preventing microbial colonization.[9]

The chemical shield hypothesis as a mechanism for innate airway defense was first proposed in the mid-1990s. It suggests that inhaled bacteria are eradicated by antimicrobial proteins and peptides such as lysozyme, lactoferrin, and antiporoteases, and defensins in the ASL.[10-13] Many of these molecules are thought to be salt-sensitive and only effective in low salt environments (i.e., < 50 mM sodium chloride).[12,14-15] It is believed that the airway epithelium regulates ionic composition of the ASL to specifically enhance the activity of these antimicrobial proteins and peptides.[16]

The predominant hypothesis of how the innate airway defense functions is mechanical clearance. Bacteria and other foreign particles are physically removed by the flow of mucus in the airway as shown in Figure 1.1 In healthy airways, the ASL consists of two components: the mucus layer and the periciliary layer (PCL). The mucus layer consists primarily of high molecular weight, glycosylated macromolecules called mucins that exhibit an immense diversity of carbohydrate side chains, allowing mucins to adhere to and entrap a wide range of particles. [17-18] Mucins form a gel with turbulent flow that promotes the enmeshment of particles in the tangled mucin network. Below the mucus layer is the PCL (Figure 1.1) serving two primary functions. First, the low viscosity of this layer provides an appropriate media for coordinated ciliary movement, with the

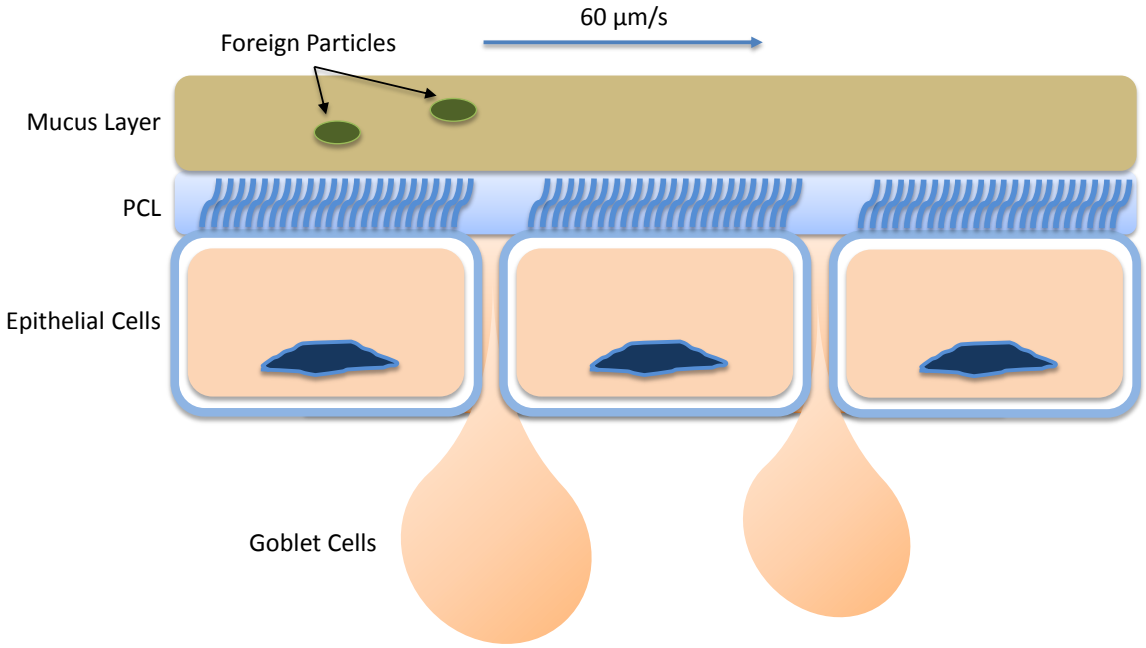


Figure 1.1 In healthy airways, cilia within the hydrated periciliary layer (PCL) beat in a coordinated fashion resulting in mucus flow. Foreign particles entrapped in the mucus layer are removed via this mucociliary transport.

thickness of the PCL directly correlated with cilia height (approximately 7 μm in healthy airways).[19] Secondly, this layer separates and protects the epithelial cell surface from the mucus layer.

Mucociliary clearance requires both a healthy mucus layer and PCL. To move the mucus layer, cilia beat in a coordinated power stroke. During this step, the cilia fully extend and exert force on the undersurface of the mucus layer. Friction between the PCL and the mucus layer causes the PCL to move with the mucus layer.[20] During the recovery stroke, cilia slowly return to their initial position, bending as to not interact with the mucus layer. The recovery stroke does not affect ASL movement.[21] In this concerted manner, the cilia move mucus and entrapped foreign particles up and out of the airway via the larynx.

In order to maintain both the chemical and mechanical mechanisms for airway defense, the ionic strength and volume of the PCL must be carefully regulated. The PCL thickness is maintained by sodium (Na^+) and chloride (Cl^-) ion absorption through the airway epithelial cells.[22] When the PCL is too large, the cilia do not reach the mucus layer during their power stroke, inhibiting mucus transport (Figure 1.2-A). In this situation, the epithelial Na^+ channel (ENaC) is highly active, resulting in absorption of Na^+ and subsequent cellular water intake with PCL dehydration. Conversely, cilia movement is inhibited when the PCL is too thin, as they require low viscosity solutions to beat effectively (Figure 1.2-B).[9] To remedy this, ENaC is inhibited, changing the cell membrane potential and causing the secretion of Cl^- and water. In addition to ion transport, the mucus layer can swell and shrink to release extra liquid into the PCL, helping maintain adequate PCL volume for ciliary movement.[23]

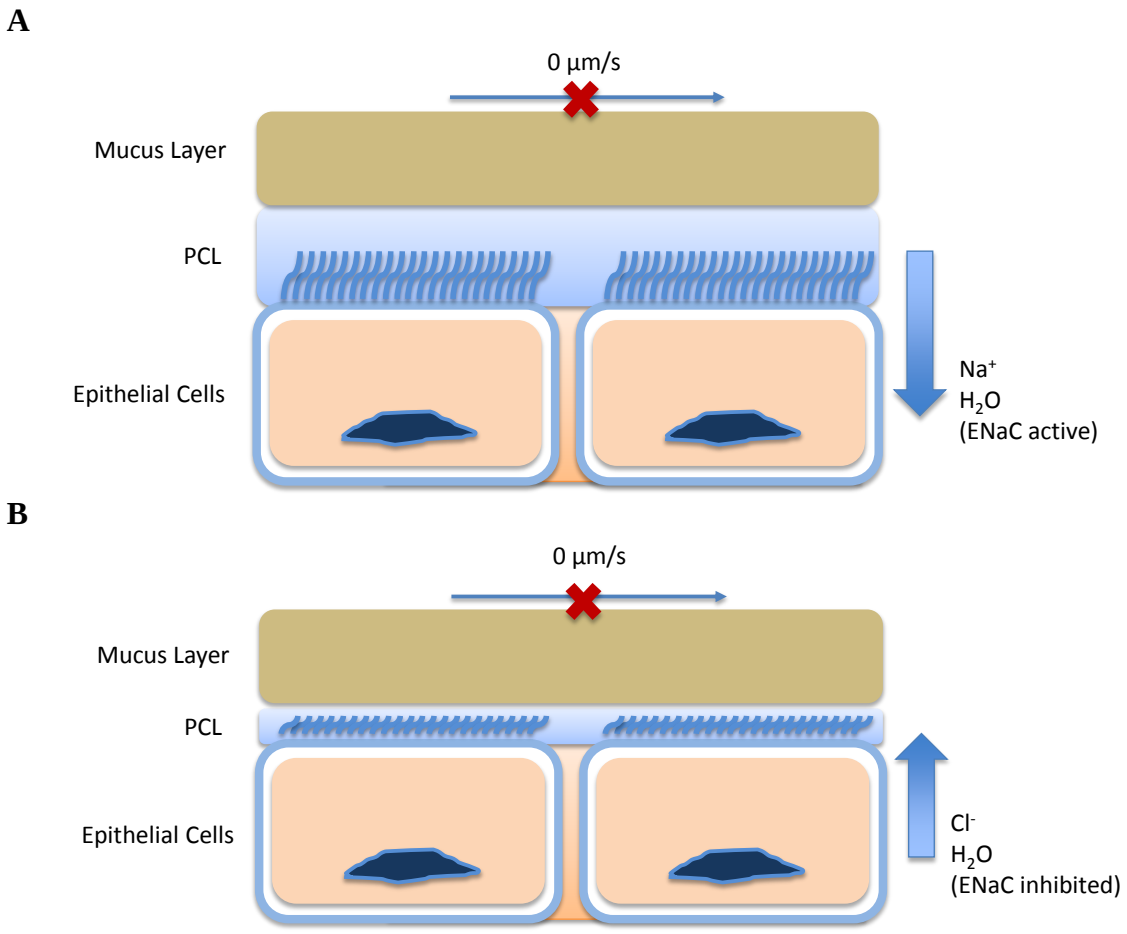


Figure 1.2 Maintenance of PCL volume in healthy airways. (A) When the PCL is too thick, beating cilia do not reach the mucus layer and therefore cannot move it. To remedy this, Na+ absorption is mediated via the ENaC channel, resulting in water absorption. (B) Alternatively, a dehydrated PCL impairs mucus movement by preventing ciliary movement, which is fixed in healthy airways by increasing Cl- and water secretion into the PCL via ENaC inhibition.

1.1.2 Innate airway defense in CF

Disturbances in ion transport in the CF airway result in dehydration of the ASL and increased salt content. In the chemical shield hypothesis, the high salt concentration of the CF airways are purported to render the salt-sensitive antimicrobial peptides and defensins ineffective.[11] However, this hypothesis has largely been discredited as the activities of the most predominant antimicrobial molecules (i.e., lactoferrin and lysozyme) were found to be salt-insensitive.[24] Additionally, ion concentrations of ASL in the upper and lower airways in both CF and non-CF subjects were found to have similar salt concentrations.[25-26] In light of this evidence, the role of salt concentrations in the ASL is believed to be insignificant in CF pathogenesis.

With this knowledge, deficiencies in CF bacterial clearance can be largely explained by defective mechanical clearance as shown in Figure 1.3. As cilia require the low viscosity of the PCL in order to beat properly, depletion of the PCL results in cessation of mucociliary clearance.[19] The CF airways cannot restore the PCL via secretion of water due to defects in the CFTR. Furthermore, the loss of the PCL also results in adhesion of the mucus layer to the airway surface, greatly reducing the efficacy of mucus clearance by coughing.[27] This process is compounded by the continual secretion of mucins by goblet cells, resulting in the accumulation of mucus in the airways (Figure 1.3).

1.1.3 Bacterial lung infections in CF

Lung degradation due to chronic bacterial infections account for 80–95% of CF-deaths.[7] As such, understanding the microbial ecology of the CF lung is vitally important to developing therapeutics. Over the lifespan of a patient, the bacteria strains found in the CF airways change (Figure 1.4).[28] In children, *Staphylococcus aureus* (*S. aureus*) and *Haemophilus influenzae* (*H. influenza*) colonization are most common, but these bacterial strains are associated with low

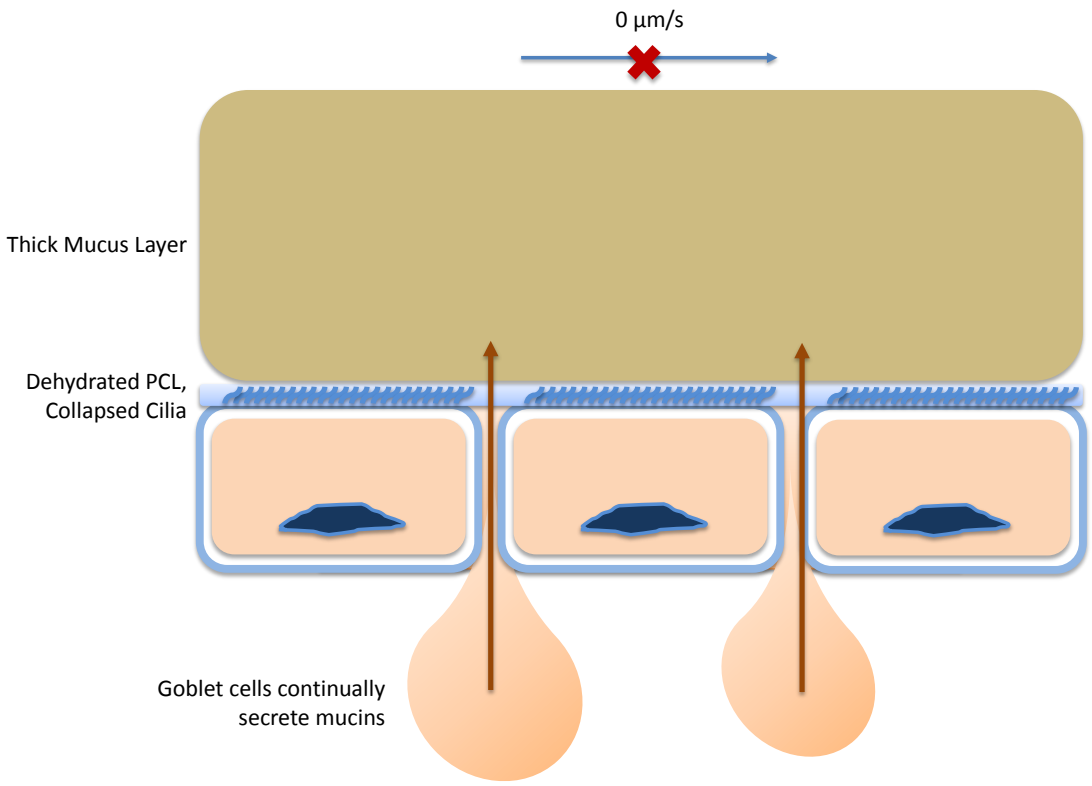


Figure 1.3 Loss of mucociliary clearance in CF airways.

morbidity rates compared to other strains (e.g., *Pseudomonas aeruginosa* and *Burkholderia cepacia*).[7] While *S. aureus* isolates are frequently found in sputum cultures, they are believed to exist primarily in the nasal and upper respiratory tract where their presence is considered benign.[7] However, methicillin-resistant strain colonization has increased in the US over the last 10 years, indicating that emerging drug resistance might increase the toxicity of this pathogen.[29]

Despite low rates of colonization at all ages, the bacteria in the *Burkholderia cepacia* complex (BCC) are linked to high incidences of mortality (3.1% in 2005).[30] The BCC is comprised of at least 17 closely related Gram-negative bacteria species that are intrinsically resistant to antibiotics and disinfectants.[31] One source of this innate resistance is the unusual chemical structure of lipopolysaccharides in the cell membranes of BCC organisms that consist of fewer phosphate groups than typical lipopolysaccharides.[32] The reduced number of anionic phosphate groups renders the bacteria resistance to cationic antibiotics (e.g., aminoglycosides and polymyxins).[32] As a result, BCC infections are often treated with combination therapies based on data from multiple combination bactericidal antibiotic tests (MCBT or "checkerboard" assays).[33] Unfortunately, this type of testing and therapy did not improve clinical or bacteriological outcomes in a recent clinical trial.[34] Due to the seriousness of these infections, patients colonized with BCC organisms are isolated from other patients and ineligible for lung transplants.[30]

The bacteria most associated with morbidity and mortality in the CF population is *P. aeruginosa*.[35] While *P. aeruginosa* colonization rates are low in children, the prevalence of *P. aeruginosa* in the adult CF population is over 80%, making it a nearly universal problem for all patients (Figure 1.4). Additionally, adults are colonized by an average of 2.38 phenotypically distinct *P. aeruginosa* isolates at any given time.[36] One of the main causes for the pathogenicity of *P. aeruginosa* is its ability to genetically adapt to the CF lung in order to evade the host immune

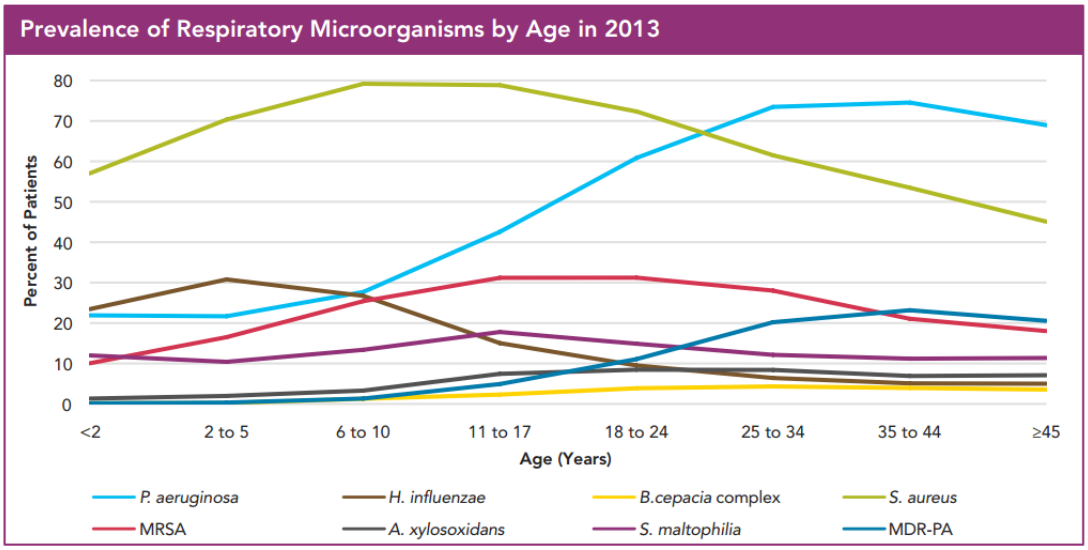


Figure 1.4 Colonizing microbes in CF patients in the US. Reprinted with permission from the 2013 Patient Registry Annual Data Report to the Center Directors (CF Foundation).

response and antibiotic therapy. These adaptations will be outlined in the Section 1.1.4. As *P. aeruginosa* is considered the most clinically important pathogen when treating CF, this dissertation research will focus on *P. aeruginosa*.

1.1.4 Adaptations of P. aeruginosa to the CF airway

The progression of *P. aeruginosa* colonization to chronic infection as a result of the CF mucus environment is outlined in Figure 1.5 (adapted from Worlitsch et al.[37] and Hassett et al.[6]). Inhaled, nonmucoid *P. aeruginosa* respire aerobically and are generally susceptible to antibiotics. In healthy airways, these bacteria are easily removed by mucociliary clearance, as shown in Figure 1.1. In CF airways, depletion of the PCL and accumulation of a highly viscous mucus layer impairs mucociliary clearance (Figure 1.3). Under these conditions, inhaled *P. aeruginosa* utilize their flagella and pili to actively penetrate the mucus layer and move to low oxygen zones (Figure 1.5-A).[38] Once inside the hypoxic or anaerobic plaques of CF mucus, the nonmucoid *P. aeruginosa* undergo several mutations. Bacteria switch from aerobic to less efficient anaerobic respiration and slower growth modes (Figure 1.5-B).[37,39] As most antibiotics (e.g., aminoglycosides, third generation penicillins, and fluoroquinolones) exhibit better eradication against rapidly dividing bacteria, the switch to anaerobic respiration decreases *P. aeruginosa* susceptibility.[6] Conversion to anaerobic metabolism is concomitant with a change to the mucoid phenotype (Figure 1.5-B), a factor that also increases antibiotic resistance.[37] This phenotype is characterized by mutations in the *mucA22* gene, loss of flagella, and the formation of a protective alginate capsule.[40] As flagella stimulate the host immune response, their removal is a mechanism of evasion.[41] The low oxygen conditions of CF mucus stimulate the production of alginate, an exopolysaccharide (EPS) that forms an extra protective capsule around the bacteria, further increasing protection from antibiotics and the host immune response.[37]

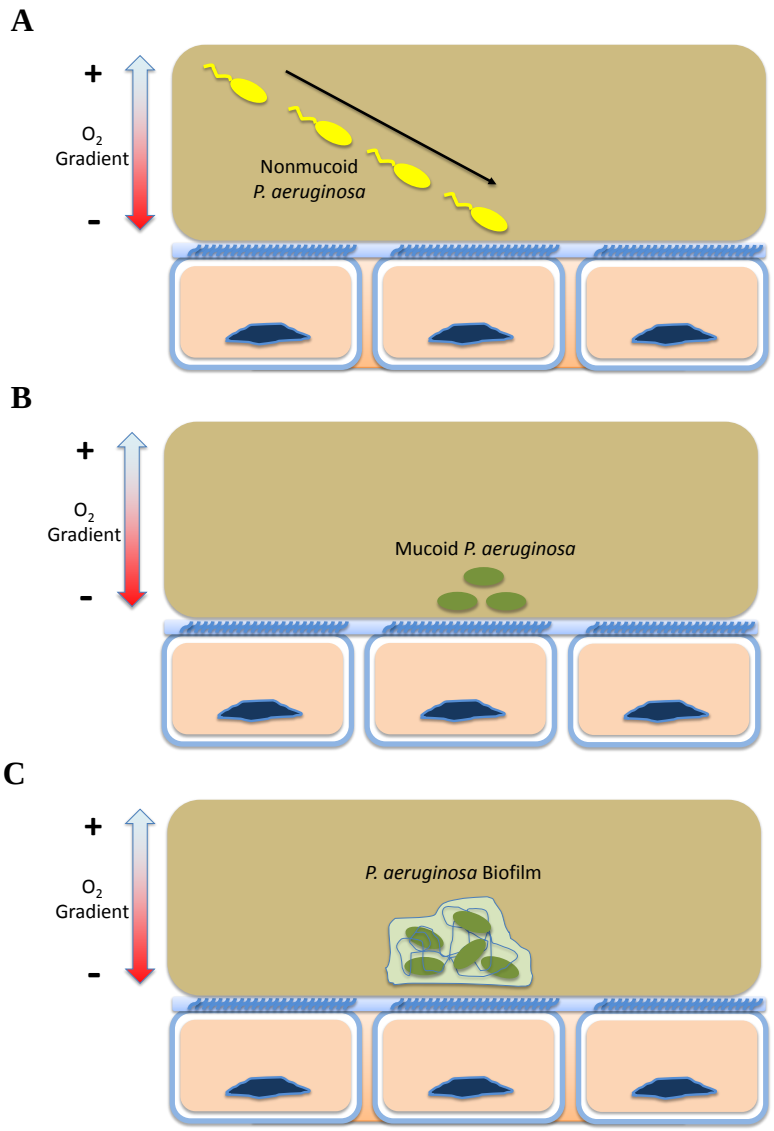


Figure 1.5 Adaptation of *P. aeruginosa* to the CF airways.

The final and most successful *P. aeruginosa* adaptation to the CF airway is the development of bacterial biofilms (Figure 1.5-C).[39] Biofilms are cooperative communities of bacteria encapsulated by a thick EPS matrix that provides additional protection from the host immune response and antibiotics. Bacterial metabolism within biofilms is slowed, further reducing the efficacy of most antibiotics.[39] *Pseudomonas aeruginosa* within biofilms develop genetic adaptations that make them resistant to antibiotics, including modification of lipopolysaccharide (LPS) secretions and drug efflux pumps.[42] In the CF airways, *P. aeruginosa* biofilms frequently develop mutations in the lasR gene, which controls quorum sensing and the formation of acute virulence factors.[42-44] By reducing their virulence, these mutants effectively "hide" from the host immune system, reducing the immune response, and initiating chronic infection.

1.2 Treatment of Cystic Fibrosis

Despite significant advances in research, CF remains a disease with no cure. Most therapeutics have focused on treating the symptoms of the disease as it progresses (Figure 1.6). As disease pathogenesis is rooted in deficient or defect CFTR proteins, CFTR modulators are employed to slow CF progression. Unfortunately, CFTR modulators are only available to a small percentage of CF patients, as will be discussed in Section 1.2.1. As highly viscous mucus accumulates in the airways, hypertonic saline and mucolytic agents are utilized to rehydrate the airway and decrease mucus viscoelasticity. If these treatments do not sufficiently improve mucociliary clearance, bacterial infection and inflammation develop, necessitating the need for antibiotic and anti-inflammatory agents. When all other treatment options have failed, lung replacement is necessary for patients with advanced lung disease. Together, improvements in

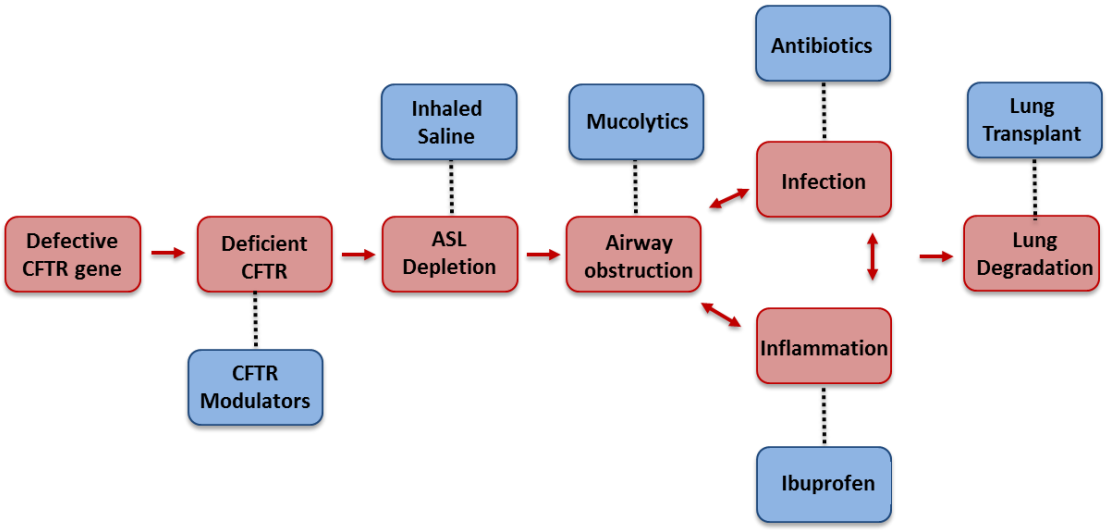


Figure 1.6 Therapeutics associated with CF. As symptoms develop in CF, different treatment options mitigate each new symptom.

these treatments have raised the life expectancy of CF patients from 28 years in 1988 to 38 years in 2012.[28]

1.2.1 CFTR modulators

While most therapeutics (e.g., mucolytics, antibiotics, and hypertonic saline) treat the symptoms of CF, CFTR modulators aim to correct the underlying cause of the disease (i.e., defects in the CFTR protein). Currently, Kalydeco (generic name Ivacaftor) is the only CFTR modulator approved for the treatment of CF.[45] Kalydeco is a CFTR potentiator that repairs the function of the CFTR protein in people with the G551D *CFTR* mutation.[46] Approval of this drug by the US Food and Drug Administration (FDA) in 2012 was extremely exciting; however, the G551D gene is only found in 4-5% of patients with CF.[47] While this drug is highly effective for a small portion of the CF population, the occurrence of over 1800 mutations in the CFTR gene make development of gene modulators for a majority of CF patients highly unlikely.[48]

1.2.2 Drugs enhancing mucociliary clearance: Inhaled saline and mucolytics

The primary step in CF disease pathogenesis is the development of a dehydrated, highly viscous ASL that prevents mucociliary clearance and leads to airway obstruction, infection, and inflammation. Therefore, one of the key steps in treating CF is restoration of mucociliary clearance. To this end, inhalation of hypertonic saline is prescribed to hydrate the ASL while mucolytic therapies aim to reduce mucus viscosity. Hypertonic saline inhalation has been shown to increase mucus transport[49-51] and cough clearance[52] in patients with mucociliary dysfunction. The exogenous addition of water to the ASL improves lung function and decreases pulmonary exacerbations.[53-55] In a long-term study where CF patients inhaled hypertonic saline twice daily for 48 weeks, dramatic improvements were seen in lung function (as measured by increased lung forced vital capacity) and a reduction in pulmonary exacerbations compared to non-treated groups

over the course of the trial.[55] Of note, the benefits of this therapy are acute and disappear within 24 h of administration, necessitating daily maintenance.[52]

Mucolytics are designed to decrease the viscoelasticity of the mucus layer, thereby improving mucociliary clearance. Currently, dornase alfa, a highly purified solution of recombinant human deoxyribonuclease I (rhDNase I) is the only FDA-approved mucolytic to treat CF. Dornase alfa (proprietary name Pulmozyme), reduces mucus viscosity by cleaving DNA strands in the ASL, decreasing their entanglement, and reducing mucus viscoelasticity. The use of dornase alfa as a mucolytic in CF has been linked to improved lung function and reduction in pulmonary exacerbations via the removal of hyperviscous airway secretions.[56-57] In reviewing 19 studies consisting of more than 3,000 patients, the CF Foundation concluded that dornase alfa treatment provided a significant improvement in patient health over placebos and highly recommends its use for patients with moderate to severe lung disease.[58] The European Cystic Fibrosis Society currently recommends that all CF patients $\geq$ 6 years old receive treatment with dornase alfa regardless of lung function.[59] As such, dornase alfa is one of the most successful and a highly recommended therapeutics for managing CF.

N-Acetylcysteine (NAC) has also been studied as a potential mucolytic agent. The mucolytic mechanism of NAC is to reduce mucin molecular weight by breaking disulfide bonds within the polymer and replacing it with a sulfhydryl group.[60] In this way, the long mucin polymers are disrupted and the overall viscosity of the mucus layer is reduced. In in vitro studies of mucin solutions, NAC greatly reduces the viscosity of the solution; however, its efficacy has never been proven in clinical trials.[61] Additionally, NAC is poorly tolerated and incompatible with aminoglycoside antibiotics. Thus, it has not been recommended for the treatment of CF.[62]

1.2.3 Anti-inflammatory treatment

Inflammation represents a chronic and serious component of CF pathogenesis. As shown in Figure 1.6, airway obstruction, infection, and inflammation are involved in a perpetual cycle. Bacterial infections trigger inflammation as part of the host immune response. In turn, byproducts of the inflammatory response stimulate mucus secretion, further decreasing the ability of the airways to remove the infection. Administration of anti-inflammatory therapeutics interrupts this cycle and reduces lung degradation.[63] Ibuprofen is currently the only anti-inflammatory therapeutic that has been clinically proven to improve lung function over a two year period. Unfortunately, ibuprofen exhibits reduced efficacy in adults and children over the age of 13, indicating that it is more effective at slowing disease progression than reversing it.[58,64]

1.2.4 Antibiotics

As lung damage resulting from chronic bacterial infection is the leading cause of morbidity and mortality in patients with CF, effective antibiotic treatments are essential to maintaining lung function and patient quality of life.[65-67] Due to the large variety of antibiotics available, such therapeutic intervention is tailored to the species of colonizing bacteria (e.g., *P. aeruginosa, B. cepacia, S. aureus*) and the severity of the infection.

Current antibiotic treatment regimens for CF have two primary goals: eradicate early colonization and manage chronic infections. Early eradication is essential to delay chronic infection and prevent deterioration of lung function. During the early stages of colonization, *P. aeruginosa* are easier to eradicate as they have not yet fully adapted to the CF airways as outlined in Section 1.1.4. Aggressive antibiotic treatment is therefore warranted in the early stages of colonization. Alternatively, treatment of chronic infections is aimed at reducing exacerbations caused by bacterial infection and improving lung function as eradication is unlikely.[58]

For both initial colonization and chronic infections, developments in antibiotic treatments over the last decade have focused on improving the delivery of approved antibiotics rather than development of new classes of antibiotics.[65] By developing inhalable formulations of antibiotics, the effective dose in the airways can be increased dramatically (Table 1.1). As a result of increased drug concentration at the site of infection, inhalable antibiotics exhibit improved efficacy in vivo. This attribute was first proven with aerosolized formulations of carbencillin and gentamicin.[68] Localized delivery of antibiotics to the lungs decreased both systemic absorption and the risk of toxicity.

Tobramycin was the first FDA approved aerosolized antibiotic for CF. Prior to the development of inhalable formulations, tobramycin was administered intravenously as it does not pass through the gastrointestinal tract. Development of a nebulized form of tobramycin (i.e., tobramycin inhalation solution or TOBI) increased the concentration of tobramycin in sputum by two orders of magnitude while simultaneously decreasing the concentration in blood.[69] The delivery of tobramycin to the CF airways was further improved by the development of a dry inhalation powder. Administration of tobramycin via dry powered inhalation increased tobramycin concentrations in CF sputum by 3 orders of magnitude compared to intravenous administration (Table 1.1).[69]

Inhalation of tobramycin is currently the most highly recommended treatment for eradication of initial *P. aeruginosa* colonization and maintenance of lung health after the development of chronic infections. The CF Foundation recommends inhalable tobramycin doses of 300 mg to be administered twice daily for 28 days.[62,70] During early colonization, administration of additional antibiotics either by inhalation, ingestion, or intravenously is recommended if tobramycin fails to eradicate *P. aeruginosa* from the airways.

Table 1.1 Antibiotic concentrations in serum and sputum. Adapted from Chmiel et al.

Drug Formulation	Dosage	Mean Peak Serum Concentrations (µg/g)	Mean Peak Sputum Concentration (µg/g)
Tobramycin			
Intravenous	8 mg/kg/d	29.4 (range 23.1-35.5)	3.88 (range 1.8-5.7)
Nebulized Solution	300 mg	1.04 ± 0.58	737 ± 1028
Inhalable Powder	112 mg	1.02 ± 0.53	1048 ± 1080
Aztreonam			
Intravenous	2 g	80.1	5.2
Nebulized Solution	75 mg	0.622 (range 0.31-1.7)	537 (range 0.2-3010)
Levofloxacin			
Oral	500 mg	6.5	5.1
Nebulized Solution	240 mg	1.71 ± 0.62	4691 ± 4516

Due to the success of inhalable tobramycin formulations, other antibiotics have been adapted for inhalation. Since 2007, inhalable azithromycin has been highly recommended by the CF Foundation for the management of chronic *P. aeruginosa* infections.[58,62] A nebulized formulation of aztreonam lysine, marketed under the name Cayston (Gilead, Seattle, WA) was approved in 2010. [71] By 2013, the CF Foundation recommended inhalable aztreonam for the treatment of chronic *P. aeruginosa* infections in patients with severe to moderate lung disease.[58] Both inhalable tobramycin and aztreonam solutions are currently still in clinical trials to optimize dosages and treatment regimes.[72] As lung infections are the primary cause of morbidity and morality in CF patients, the development of new antibacterial treatments is crucial to improving quality of life and expanding life expectancy.[65-67] Additionally, expansion of research into non-conventional antibiotics aimed at eradicating bacteria in the biofilm mode of growth is necessary as antibiotic resistance continues to be problematic, particularly for CF.[73]

1.2.5 Lung transplantation

When all other treatment options have failed, lung transplants are required to replace the weakened and diseased lungs of CF patients. Lung transplants are not a cure for CF— they are only considered for patients with advanced lung disease. In addition to the risk associated with serious surgery, the immunosuppressive drugs required post-transplantation weaken the immune system of the patient and leave them vulnerable to infection. In CF, immunosuppression is particularly harmful as infection is the leading cause of mortality. In fact, these hazards are so great that it is unclear if lung transplantation benefits children with CF.[74] These factors along with the low supply of donor organs mean this treatment will always be limited to end of life care. In 2012, only 205 patients in the US received lung transplants.[28]

1.3 Nitric Oxide

Nitric oxide (NO) is an endogenously produced, highly reactive free radical involved in a wide variety of physiological processes. In normal function, NO is produced by endothelium NO synthase (eNOS) and neuronal NO synthase (nNOS) at low concentrations (pM-nM) to regulate processes such as vasodilation, angiogenesis, and neurotransmission.[75-77] Larger concentrations (μM) are produced by inducible NO synthases (iNOS) as an antimicrobial in macrophages and neutrophils in response to bacterial colonization and infection.[78] As the antimicrobial properties of NO are essential to developing a CF therapeutic, further details are provided below.

1.3.1 Molecular targets of NO antimicrobial activity

Nitric oxide is a broad-spectrum antimicrobial molecule capable of eradicating bacteria, biofilms, and fungus.[78-79] The bactericidal activity of NO is complex and multi-mechanistic (Figure 1.7).[79-80] Due to its uncharged, lipophilic state, NO can readily penetrates bacteria cell membranes and exerts both extracellular and intracellular stresses. Many of NO's bactericidal mechanisms involve the formation of reactive intermediates.[81] The reaction of NO with oxygen and superoxide results in the formation of peroxynitrite ($^{-}$ONOO), nitrogen dioxide (NO_2), and dinitrogen trioxide (N_2O_3). Of note, some of these congener molecules, especially peroxynitrite, are more potent antimicrobials than NO alone.[79]

The molecular targets of NO and its reactive intermediates include DNA, lipids, thiols, heme-containing proteins, and metalloenzymes. The main bactericidal mechanism of NO is indirect deamination of DNA by the reactive N_2O_3 intermediate.[82-83] Additionally, $^{-}$ONOO and NO_2 have been linked to DNA strand breaks and abasic site formation.[79,84] Lipid peroxidation has been noted as another primary source of NO's toxicity and is mediated by both $^{-}$ONOO and NO_2.[78]

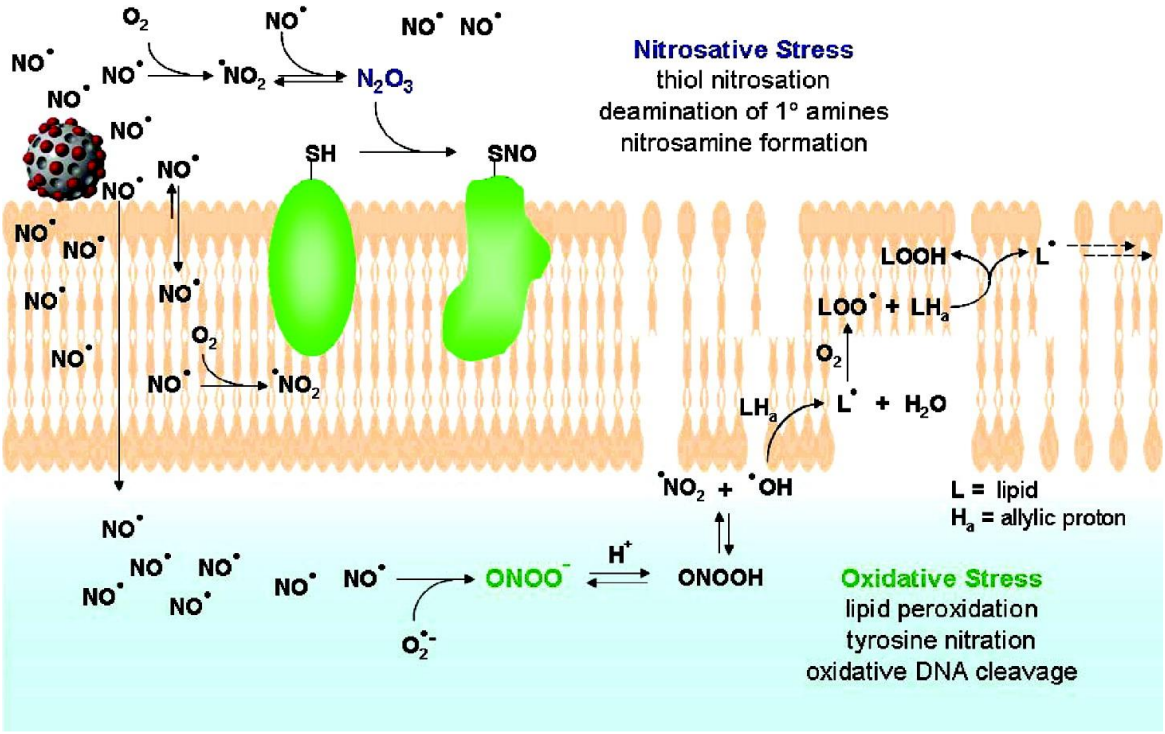


Figure 1.7 Antimicrobial reactions of NO with likely mechanisms of action. Nitric Oxide reacts with oxygen and superoxide to form reactive intermediates (e.g., N_2O3, ONOO-). Together these molecules exert oxidative and nitrosative stresses on bacteria. Reprinted with permission from Hetrick et al.[81]

Protein deactivation can occur either by *S*-nitrosylation of sidechain thiols or NO binding to the Fe(II) centers in heme-containing proteins.[79,85] For proteins that contain iron-sulfur clusters, reaction with NO causes release of iron and subsequent loss of protein function.[79,85] Deactivation of these enzymes impairs nearly every cellular process including metabolism, respiration, RNA modification, and DNA repair and replication.

Due to multi-mechanistic antimicrobial activity, NO exhibits killing action against both Gram-positive and Gram-negative bacterial strains.[86-87] Gram-positive species such as *S. aureus* are generally believed to be less susceptible to NO than Gram-negative species due to their thick, protective peptidoglycan membrane layer.[78,81] Nevertheless, successful eradication of *S. aureus* in both planktonic and biofilm states has been demonstrated, albeit at greater concentrations.[88-90] Nitric oxide is effective at eradicating methicillin-resistant *S. aureus* (MRSA),[91-92] which holds particular importance as MRSA infections continue to rise in the CF community (Figure 1.4), necessitating the development new broad-spectrum antimicrobial treatments.

Pseudomonas aeruginosa, a Gram-negative pathogen, is particularly susceptible to NO-mediated eradication.[78,81,87,93-99] As the pathogen most associated with lung degradation and eventual death,[35] eradication of *P. aeruginosa* is particularly important in the context of CF. Unfortunately, *P. aeruginosa* eradication by NO has only been reported under aerobic conditions, [78,81,87,93-99] which do not adequately the environment of the CF mucus layer (Section 1.1.4). As shown in Figure 1.8, *P. aeruginosa* both produces and consumes NO as part of its metabolic cycle during anaerobic growth.[6,39,100] Located in the bacterial cytoplasmic membrane, nitrate reductase enzymes (NAR) reduce nitrate (NO_3^-) to nitrite (NO_2^-), which is quickly moved to the periplasmic space between the inner and outer membrane via NarK2 efflux pumps. In the periplasm, NO_2^- is

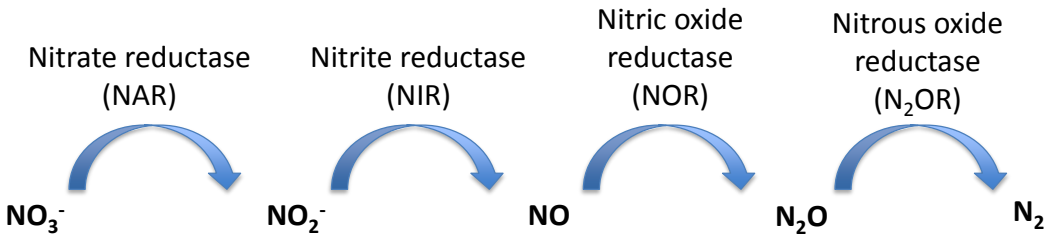


Figure 1.8 Anaerobic respiration of *P. aeruginosa*.

further reduced via nitrite reductase (NIR) to NO. Complementary to its generation, NO is eliminated from the cell by nitric oxide reductase (NOR) to yield N_2O. In the final stage of denitrification, N_2O is reduced to N_2 by nitrous oxide reductase (N_2OR) subsequently diffuses out of the cell. During this process, NIR and NOR act in concert so that NO levels do not exceed 65 nM.[101-102] While NO-releasing therapeutics are generally effective against *P. aeruginosa*, the ability to enzymatically reduce NO may alter the antibacterial efficacy in anaerobic or low oxygen conditions (e.g., CF mucus).

1.3.3 Anti-biofilm efficacy of NO against P. aeruginosa

In addition to being toxic to planktonic bacteria, NO has been shown to both disperse and eradicate *P. aeruginosa* biofilms. Biofilm dispersal is a naturally occurring phenomenon that allows for the colonization of new environments while limiting overcrowding in the mature biofilm.[103-104] The Kjelleberg lab discovered that *P. aeruginosa* biofilms utilize NO as a chemical signal to initiate dispersal upon biofilm maturation.[105-107] Biofilms grown with strains of *P. aeruginosa* incapable of NO production (i.e., lacking NIR) did not exhibit coordinated dispersal following maturation.[105] Furthermore, strains that produced NO but could not scavenge it (i.e., lacking NOR) exhibited increased dispersal over wild-type strains. Exposure to exogenous NO released from sodium nitroprusside (SNP) also resulted in biofilm dispersal, indicating that NO-releasing materials can be used to trigger biofilm dispersal.[105] Together, these results demonstrate that accumulation of NO within *P. aeruginosa* biofilms, whether from endogenous or exogenous sources, triggers dispersal. Treatment with exogenous NO also increases the efficacy of co-administered antibiotics against *P. aeruginosa* biofilms. By triggering dispersal and a transition to the planktonic state, treatment with NO has been shown to increase bacterial susceptibility to traditional antibiotics. [105] For example, pretreatment of biofilms with sub-bactericidal

concentrations of SNP (500 mM) increased the bactericidal efficacy of tobramycin by two-logs compared to biofilms that were not exposed to SNP.[105]

While biofilm dispersal requires low concentrations of NO (e.g., pM-nM), biofilm eradication typically requires higher NO concentrations (e.g., μM).[108] Achieving these large local NO concentrations requires the use of exogenous NO donors to store and controllably release NO. The mechanisms for NO-mediated biofilm toxicity are similar to planktonic bacteria; however, higher doses of NO are required for eradication of biofilms due to the protective EPS matrix. The development of NO-releasing materials capable of biofilm eradication will be discussed in more detail in Section 1.5.

1.4 Nitric Oxide in Cystic Fibrosis

In the lungs and airways, NO controls many regulatory processes, including bronchomotor control, inflammation, and the immune response.[109] The human airway produces NO from L-arginine via three isoforms of NO synthase (i.e., nNOS, eNOS, and iNOS). Bronochomotor control is regulated by nNOS in nonadrenergic noncholinergic (NANC) nerves and disruption of nNOS activity results in airway obstruction.[110] Pulmonary circulation is regulated by eNOS produced in the pulmonary blood vessels. When inhibited, vasoconstriction and vessel wall thickening results.[111] Finally, macrophages, neutrophils, and bronchial epithelial cells enzymatically produce NO via iNOs when stimulated by pro-inflammatory cytokines (i.e., interleukin-1 tumor necrosis factor and interferon-gamma) or by lipopolysaccharides secreted by bacteria.[112]

1.4.1 Causes of low exhaled NO in CF patients

In airway diseases associated with inflammation or chronic infections (e.g., asthma and COPD), exhaled NO concentrations are increased, especially during exacerbations. Surprisingly,

exhaled NO levels in CF patients are similar to or decreased compared to healthy individuals, despite the intense inflammation associated with CF.[109] Two main hypotheses are used to explain the decreased levels of exhaled NO in patients with CF. First, many scientists believe that high levels of NO are produced in CF airways but that NO cannot be detected in the exhaled breath of CF patients because the highly viscous mucus layer prevents NO from reaching the gas phase.[109] In this case, a significant fraction of NO is converted into its stable metabolites (i.e., nitrate, nitrite, and nitrotyrosine) in the CF mucus prior to NO measurement.[109] In one study, exhaled concentrations of NO were similar in CF patients and healthy individuals, but nitrate and nitrotyrosine concentrations in sputum were significantly higher in the CF subjects, supporting this hypothesis.[113] Moreover, no differences in exhaled NO were measured when stable (i.e., non-exacerbated) CF patients were compared to those with acute respiratory infections while elevated nitrate and nitrite concentrations were detected in patients with active infections.[114] Finally, it has been reported that NO consumption by *P. aeruginosa* during anaerobic metabolism reduced levels of exhaled NO (Figure 1.8).[115] Together, these studies suggest that NO production is not diminished in the CF airway, but rather that NO is consumed or degraded in CF mucus prior to detection in exhaled breath.

Alternatively, others hypothesize that NO production is indeed curtailed in CF patients. Bio-available concentrations of L-arginine, the substrate for NO production by NOS, are decreased in CF, resulting in diminished NO production.[116-117] The NO production may be reduced due to downregulation of iNOS in CF airways.[118] In a CF mouse model, total NOS activity was downregulated in the trachea of CF mice compared to wild type.[118] While NO activity was stimulated with LPS and L-arginine in wild-type mice, NO production was not affected in the CF model, indicating that iNOS cannot be upregulated during bacterial colonization and infection.[118]

It is believed that the failure to upregulate iNOS during infection is related to the CFTR mutations and not chronic infection, as depressed NO levels are seen in infants with CF who have never experienced infection or prolonged bouts of inflammation.[118] As evidence exists to support both views, it is likely that NO production is limited in CF airways and that measurements of exhaled NO do not accurately assess NO production.

1.4.2 Implications of low exhaled NO levels in CF

Reduced concentrations of exhaled NO have been associated with poor pulmonary health in CF patients.[109,116,119] Most notably, the loss of iNOS activity decreases the ability of the host immune response to prevent infection by bacteria and viruses. Mice with impaired iNOS upregulation were shown to be less able to eradicate *P. aeruginosa* from the airways than wild-type mice.[118] Limited NO production by iNOS also diminishes the ability to fight viral infection, as high levels of NO are also required to inhibit viral replication.[120-121]

Decreased NO in the airways also likely affects the chronic hydration of CF airways. Soluble guanylate cyclase, which produces cyclic guanosine monophosphate (cGMP), is activated by NO.[122] As cGMP downregulates amiloride-sensitive sodium absorption, chronically low levels of NO may contribute to inhibited cGMP availability and the resulting hyperabsorption of sodium in the CF airways.[109,122] While research into the causes and implications of low exhaled NO concentrations in CF patients is still ongoing, new therapeutic approaches are being designed to supplement NO levels in CF airways. These technologies will be discussed in Section 1.5.

1.5 Nitric Oxide as a Cystic Fibrosis Therapeutic

To augment NO concentrations in the airways, two main approaches have been investigated: L-arginine supplementation and inhalation of NO gas. Both approaches are active

research areas; however, neither approach is currently FDA approved as a CF therapeutic. As discussed in Section 1.4.1, L-arginine is a substrate for NO production that is found in reduced concentrations in the CF airways. In order to correct this deficiency, L-arginine can be supplemented orally, intravenously, or via inhalation. Both oral and intraveneous administration of L-arginine have been shown to increase concentrations of exhaled NO compared to placebo treatment in CF and healthy patients,[123-124] but no increase in pulmonary function was observed. Despite increased exhaled NO concentrations, NO levels in CF patients were still lower than untreated healthy controls, indicating that L-arginine supplementation alone does not fully compensate for deficiencies in NO production.

Inhalation of nebulized solutions is more effective than intravenous administration of L-arginine. After a single dose of nebulized L-arginine (18 mL of 7% L-arginine hydrochloride solution), CF patients exhibited increased exhaled NO concentrations as well as improved pulmonary function, as characterized by increased forced air expiratory volume in 1 second (FEV_1) and improved oxygen saturation.[125] In a separate study inhalation of nebulized L-arginine twice daily for two weeks improved exhaled NO levels but not FEV_1 compared to inhalation of saline solution over the same period.[126] Together these trials suggest that L-arginine supplementation, particularly via inhalation, is a potential therapeutic for increasing exhaled NO concentrations in people with CF. However, currently no evidence exists indicating L-arginine supplementation improves pulmonary function over longer periods. Additionally, L-arginine supplementation will never fully restore NO concentrations in CF airways if iNOS is downregulated in CF, as currently hypothesized.[118]

While L-arginine supplementation attempts to increase NO concentrations by upregulating host NO-production, NO levels can be more easily supplemented by the direct inhalation of NO

gas. As such, several studies have investigated the utility of gaseous NO as a pulmonary therapeutic. In adults with COPD, NO gas inhalation has been shown to reduce pulmonary hypertension.[127] One early study on non-exacerbated CF patients showed no immediate improvement in lung function after inhalation of 0.1−40 ppm NO.[128] However, inhalation of NO gas at higher concentrations or during exacerbations may improve the utility of this therapeutic.

Since these early studies, the use of NO gas as a CF therapeutic has changed focus to its potential as an antibacterial treatment. As summarized in Table 1.2, three separate research groups have measured decreased bacterial loads in rats infected with *P. aeruginosa* following inhalation of NO gas. It was first demonstrated that inhalation of NO gas (40 ppm, 24 h) could significantly reduce *P. aeruginosa* colonization in intratracheally injected rats compared to those treated with room air.[129] As inhalation of 40 ppm for 48 h resulted in death in a sizable minority of rats,[129] lower doses (10 ppm) of NO were investigated next.[130] This reduced NO dose was only effective at bacterial eradication when balanced with pure oxygen instead of room air, which increased airway inflammation.[130] In an attempt to approve bactericidal efficacy while reducing toxicity, Miller's group administered higher NO doses (160 ppm) intermittently.[131] This regimen resulted in similar decreases in bacterial load with the added advantage of preventing inflammation.

Based on these studies, it is certain that NO has the potential to be an effective antibacterial agent for pulmonary *P. aeruginosa* infections. As such, inhaled gaseous NO is being investigated as a supplemental treatment to current antibiotic therapies. In an initial clinical trial, the addition of NO inhalation (5-10 ppm for 8 h/day) to intravenous tobramycin or ceftazidime treatment reduced the number of biofilm aggregates and improved FEV_1 compared to inhaled placebo gas in CF patients.[132]

Table 1.2 Inhalation of NO gas to treat *P.aeruginosa* airway infections in rat models.

Reference	Year	Corresponding Author	NO Concentration	*P. aeruginosa* Reduction (log_{10})
[129]	2000	Sanjay Mehta	40 ppm in air (continuous administration)	1.7
[130]	2002	Christophe Delclaux	10 ppm in oxygen (continuous administration)	2
[131]	2013	Christopher Miller	160 ppm in air (30 min every 4 h)	2

These initial studies demonstrate that NO has the potential to become an effective therapeutic for reducing bacterial infections and improving pulmonary function in patients with CF. While these studies are important proofs of concept, the potential utility of inhaled NO gas is limited by convenience, patient compliance, and toxicity concerns. The clinical trial described above required patients to be connected to a pressurized NO gas tank for 8 hours a day, a treatment that is impractical for young or active patients. Inhalation of NO gas is also costly ($125/h or $1000/d) for chronic treatment.[82,133] As such, new research into the development of NO-releasing materials that can more conveniently deliver NO to the airways are being developed. These materials will be discussed in Section 1.6.

1.6 Nitric Oxide-Releasing Materials as Potential Cystic Fibrosis Therapeutics

While NO holds promise as a potential CF therapeutic, materials must be developed to effectively store and controllably release NO over an extended period of time as the expense, inconvenience, and potential toxicity associated with NO gas inhalation limit its clinical utility. Macromolecular scaffolds designed to store and release NO are generally more effective at eradicating bacteria than gaseous NO or low-molecular weight donors.[78,81,134-135] By allowing for localized delivery, the minimum NO-dose required for bacterial eradication can be reduced, thus improving the safety of NO therapy. Additionally, inhalation of NO-releasing materials is a more convenient therapeutic strategy than inhalation of gaseous NO. As NO can be released from macromolecular scaffolds for several hours, continuous NO delivery to the airways can be achieved by intermittent treatment rather than continuous inhalation from a pressurized gas cylinder.

Many NO-releasing materials have been developed for a range of medical applications. However, most scaffolds are unsuitable for pulmonary delivery. Silica nanoparticles[81,88-89,93,136]

and dendrimers[90,95,98-99,137-138] have been developed as effective NO-release vehicles for dermatological and dental applications, but toxicity concerns and ineffective clearance from the lungs make these scaffolds unsuitable for inhalation. Acidified nitrite and NO-releasing chitosan oligosaccharides are currently the primary NO-releasing materials being evaluated as potential inhalable therapeutics.

1.6.1 Acidified nitrite

Under acidic conditions (i.e., pH < 6.5), nitrite (NO_2^-) is converted to nitrous acid (HNO_2) and can be used as non-enzymatic NO source.[139-140] This release strategy has been used to prevent infections in cutaneous wounds via the co-application of creams containing acetic acid and sodium nitrite ($NaNO_2$).[82,141-144] In CF, sodium nitrite can be inhaled directly and protonated in the acidic environment of the CF mucus layer to form HNO_2.[145-146] Generation of NO from 15 mM $NaNO_2$ at pH 6.5 (the reported pH of CF mucus) has been measured electrochemically in bacterial growth medium to yield a NO formation rate of 4 μM/h with a maximum NO concentration of 500 nM.[145] These results confirm that NO can be generated from $NaNO_2$ under CF-mimicking in vitro conditions.

As a potential CF therapeutic, acidified nitrite has been investigated as a broad-spectrum antibacterial agent, particularly for the eradication of bacteria in hypoxic or anaerobic environments typical of CF mucus. Yoon et al.[145] determined that sodium nitrite could eradicate *P. aeruginosa* only when culture conditions were below pH 7, indicating that the acidification of nitrite was required for antibacterial activity. At this pH, the ability of acidified nitrite to eradicate mucoid *P. aeruginosa* was demonstrated for clinical strains, anaerobically grown biofilms, and in a mouse model.[145] Of particular relevance to CF, mucoid *P. aeruginosa* showed increased susceptibility to acidified nitrite compared to nonmucoid strains. This increased vulnerability is

likely caused by the substantially lower NOR activity (required for NO detoxification during anaerobic respiration) of mucoid *P. aeruginosa* compared to the nonmucoid phenotype.[145] Growth of *S. aureus*, MRSA, and several *B. cepacia* strains was also inhibited by 15 mM sodium nitrite at pH 6.5, and biofilm viabilities of these strains were reduced by 1–2 logs after two days of treatment.[146] As with *P. aeruginosa*, the toxicity of acidified nitrite to *S. aureus* and *B. cepacia* was attributed to its conversion to acidified nitrite and subsequent reduction to NO.

Despite the published antibacterial efficacy of sodium nitrite, the control of NO generation poses a significant challenge. The dosage of NO is dependent upon the concentration of NO_2^- inhaled and the pH of the airway, which has been reported to range from 6.39 ± 0.04[145] to 7.1 ± 0.2.[147] As such, the rate of NO generation from sodium nitrite cannot be controlled. It has previously been demonstrated that the kinetics of NO generation or release are important for the effective eradication of bacteria and biofilms.[78,88-89,96,135] Therefore, controllable NO release from macromolecular scaffolds represents the next generation of NO-based therapeutics for the treatment of CF.

1.6.2 Nitric oxide-releasing chitosan oligosaccharides

Chitosan is a naturally derived polymer comprised of repeating units of *N*-acetylglucosamine and D-glucosamine (Figure 1.9). The molecular weight of chitosan depends on the chitin from which it is derived and generally ranges from 20 kD to 375 kD.[148] Chitosan is easily broken down to smaller units via oxidative or enzymatic degradation.[149-150] The degree of deacetylation (i.e., the percentage of D-glucosamine monomers) ranges from 0 (chitin) to 100% and determines the number of primary amines available per linear polymer chain. Both the molecular weight and deacetylation degree affect the solubility, toxicity, and mucoadhesion of the polymer.

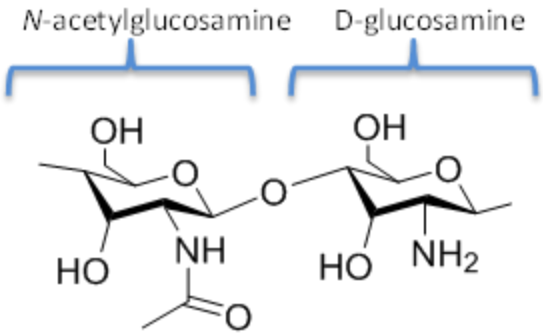


Figure 1.9 Chemical structure of chitosan. Chitosan is comprised of repeating units of N-acetylglucosamine and D-glucosamine monomers.

Mucoadhesion is an important property in pulmonary drug delivery as it has been correlated with increased drug retention times.[151] As reported by Yamamoto et al., poly lactic-*co*-glycolic acid (PLGA) copolymer nanospheres loaded with calcitonin were more slowly eliminated from the lungs of guinea pigs when modified with chitosan, allowing for sustained drug release.[152] The mucoadhesive properties of chitosan are related to its polycationic nature, as primary amines on chitosan interact with the negatively charged sialic acid residues of mucins.[153-154] Highly deacetylated chitosan is thus more mucoadhesive than chitosan polymers with a low degree of deacetylation.[154] In addition to electrostatic attractions, hydrogen bonding and hydrophobic interactions contribute to chitosan mucoadhesion.[154]

As with all drug delivery systems, pulmonary excipients must be nontoxic. Based on its low toxicity, chitosan has been approved for dietary applications in Japan, Italy, and Finland,[155] and for use in wound dressings in the United States.[156] While chitosan is generally considered nontoxic, the degree of deacetylation may alter the inherent toxicity of chitosan scaffolds. In two different studies, it was found that exposure to highly deacetylated chitosan (~80% deacetylation) was significantly more toxic to human lung epithelial cells than exposure to chitosan with lower degrees of deacetylation (~40%).[151,157] In addition to having more primary amines, highly deacetylated chitosan polymers have extended conformations due to intramolecular electrostatic repulsion, which further increase their ability to bind to cell membranes.[156]

The biodegradability of chitosan sets it apart from many other NO delivery scaffolds and makes it particularly suitable for pulmonary deliver in stagnant CF mucus. Polymeric chitosan with molecular weights less than 30 kD are cleared by the liver. In contrast, chitosan polymers larger than 30 kD need to be enzymatically degraded by lysozyme in vivo prior to elimination.[148] Lysozyme is secreted in the airways at 10-20 mg/day, making biodegradation of chitosan in the

lungs possible;[158] however, systematic studies on the biodegradation and clearance of chitosan from the airways are lacking.

The low toxicity, mucoadhesive properties, and biodegradability of chitosan make it suitable for the development of an inhalable, NO-releasing scaffold. Chitosan is only soluble in acidic aqueous solutions (pH < 6.5), limiting the number of delivery mechanisms available. To make chitosan suitable for use with dry powder inhalers, it has been formulated into drug-releasing macroparticles (1–3 μm).[151,155] Upon degradation of these macroparticles, large chitosan polymers are deposited in the airways and require further degradation by enzymes. To solve both the solubility and degradation problems associated with chitosan polymers, chitosan may be exogenously degraded into oligosaccharides prior to therapeutic use. Chitosan oligosaccharides are low molecular weight (i.e., less than 10 kD). In vitro degradation to oligosaccharides improves the solubility of chitosan in water, making it possible for delivery to the airways via nebulization.[150] As dehydration of the ASL is a symptom of CF, nebulization provides an advantage over dry powder inhalers because it hydrates the ASL during drug delivery. Due to these inherent advantages, we have pursued the use of chitosan oligosaccharides as an alternative to polymeric chitosan for the development of inhalable NO-releasing scaffolds.

The formation of *S*-nitrosothiol or *N*-diazeniumdiolate NO donors on the scaffold is readily achieved to impart NO storage and release capabilities to the chitosan oligosaccharides. As shown in Figure 1.10, *S*-nitrosothiols (RSNOs) are NO donors that are formed by the reaction of sulfhydryl groups with a nitrosating agent (e.g. alkyl nitrite, dinitrogen trioxide, nitrous acid).[159] Release of NO from RSNOs is triggered by heat, light, copper, or ascorbic acid (Figure 1.10).[134,159] In the airways, NO release from RSNOs may occur through thermal cleavage of the S-N bond to generate NO and a thiyl radical. While reaction with ascorbic acid may generate NO in vivo,

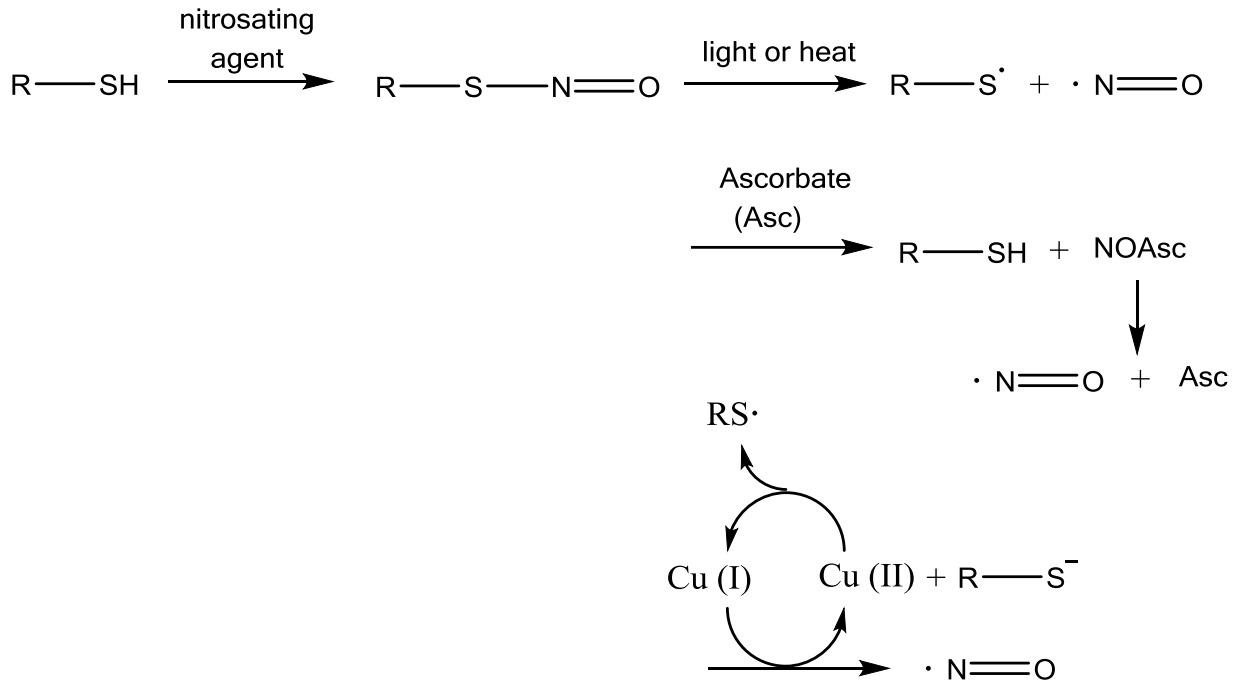


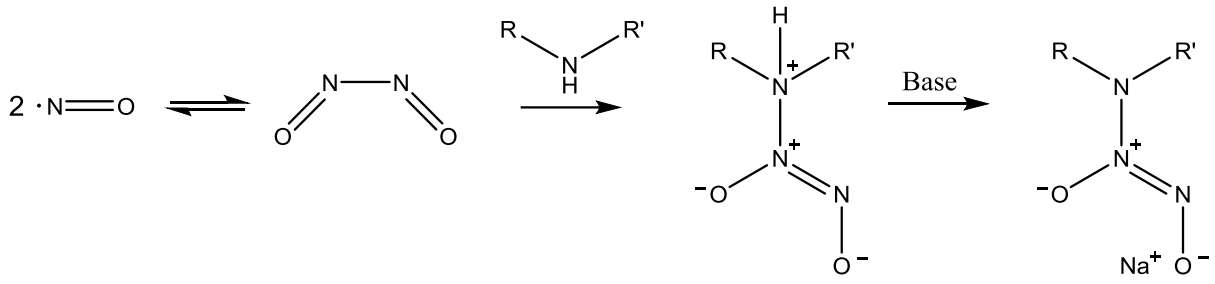
Figure 1.10 Reactions for the formation and decomposition of *S*-nitrosothiols. In the airways, the thermal decomposition pathway is predominant.

ascorbic acid depletion is a known characteristic of the ASL in CF patients.[160]

Lu et. al. previously reported the synthesis, NO-release characteristics, and antibacterial activity of RSNO-modified chitosan oligosaccharides.[161] Thiolation of the chitosan oligosaccharide scaffold was achieved with 2-iminothiolane hydrochloride and 3-acetamido-4,4-dimethylthietan-2-one to form chitosan-TBA and chitosan-NAP, respectively. Both thiolated chitosan oligosaccharides were nitrosated with acidified nitrite to form RSNOs. Under release conditions simulating that which is likely in the airways (i.e., 37 °C, without ascorbic acid or light), chitosan-TBA-NO and chitosan-NAP-NO liberated low levels of NO (35 ± 5 and 240 ± 40 nmol NO/mg chitosan, respectively). Due to such low NO payloads, the concentration of chitosan-TBA-NO and chitosan-NAP-NO required to eradicate planktonic cultures of *P. aeruginosa* were significant (15 and 30 mg/mL, respectively). *N*-diazeniumdiolate-modified chitosan oligosaccharides may represent a more suitable NO-release system for overcoming the low bactericidal action of RSNO modified chitosan oliogosaccharides.[97]

The *N*-diazeniumdiolate NO donor is formed by exposure of amines to high pressures of NO gas, typically under basic conditions.[134] The most widely accepted mechanism of *N*-diazeniumdiolate NO-donor formation is shown in Figure 1.11-A, in which NO dimerizes to N_2O_2 at elevated pressures and subsequently reacts with a secondary amine to form an *N*-diazeniumdiolate.[162] While *N*-diazeniumdiolates may form on primary amines, these NO donors are highly unstable and therefore not suitable for extended release.[137] In physiological environments, *N*-diazeniumdiolates decompose to release two equivalents of NO (Figure 1.11-B). The NO-release kinetics in aqueous solutions are controlled by pH, temperature, and the hydrophobicity of the scaffold surrounding the *N*-diazeniumdiolate.[97,134]

A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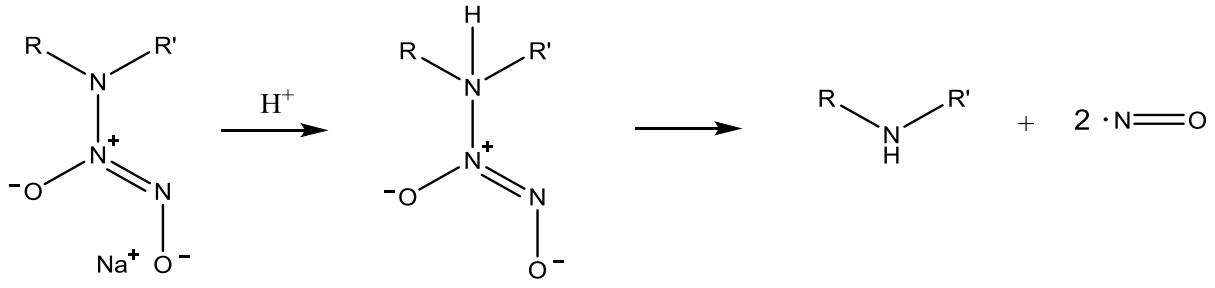
B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Utilizing the *N*-diazeniumdiolate moiety to impart NO storage and release to chitosan oligosaccharides, Lu et al. reported increased NO payloads and bactericidal efficacy compared to RSNO-modified counterparts.[97] To form the most stable *N*-diazeniumdiolates, existing primary amines on native chitosan oligosaccharide scaffolds were modified with 2-methylaziridine to yield secondary amines (Figure 1.12). The 2-methylaziridine-modified chitosan oligosaccharides were then exposed to high pressures of NO under basic conditions to yield *N*-diazeniumdiolate-modified chitosan oligosaccharides (Figure 1.12). These NO-donor scaffolds were capable of releasing NO payloads approaching 1 μmol NO/mg chitosan, and having improved bactericidal action. Only 0.2 mg/mL of *N*-diazeniumdiolate-modified chitosan oligosaccharides were required to eradicate planktonic bacteria, a > 99% decrease from the RSNO-modified chitosan oligosaccharides.[161] The improved bactericidal efficacy was attributed to both the increased NO payloads and the highly cationic nature of the 2-methylaziridine modified chitosan oligosaccharide scaffold. As cationic scaffolds likely associate with the negatively charged bacteria membrane to a greater extent than neutral or negatively charged scaffolds,[163-164] the pendant amine on the 2-methlyaziridine modification enhances localized delivery of NO. Indeed, the antibacterial efficacy of the *N*-diazeniumdiolate modified chitosan oligosaccharides was great enough to eradicate *P. aeruginosa* biofilms at concentrations found to be non-toxic to mammalian cells.[97]

In summary, the low toxicity, mucoadhesive properties, and biodegradability of chitosan make it a suitable scaffold for pulmonary NO delivery. By degrading the chitosan polymer to oligosaccharides (<10 kD), water solubility is achieved, allowing for future nebulization. When modified with *N*-diazeniumdiolate NO donors, chitosan oligosaccharide scaffolds were capable of controlled NO release and eradication of *P. aeruginosa* biofilms at concentrations non-toxic to mammalian cells.[97] These materials hold great potential as alternatives to inhalation of NO gas

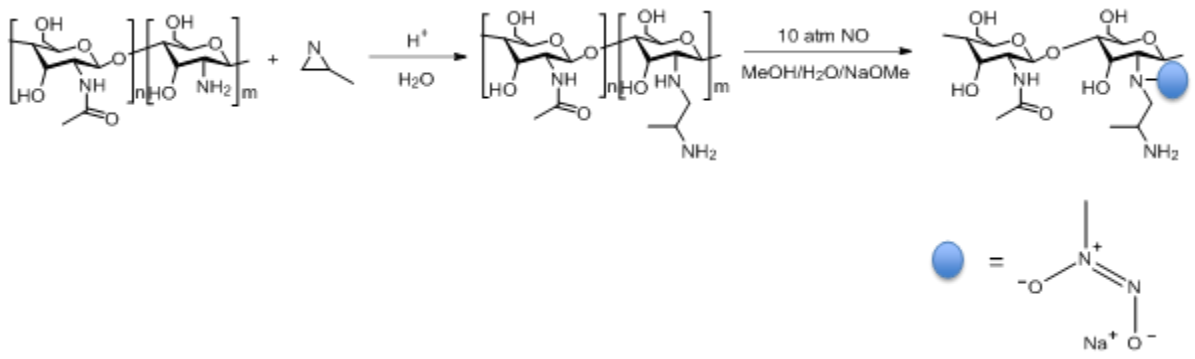

Figure 1.12 Synthesis of N-diazeniumdiolate modified chitosan oligosaccharides.

delivered from pressurized cylinders or the use of acidified nitrite because the mucoadhesion, NO payload, and release kinetics are all easily modified synthetically to maximize delivery and killing action. Of importance, the ability to eradicate mucoid *P. aeruginosa* in reduced oxygen environments needs remains unknown. Additionally, biophysical investigation of the effects of NO and NO-releasing chitosan oligosaccharides on CF biofilms and mucus are lacking. These important properties must be determined before NO-releasing chitosan oligosaccharides may be advanced a therapeutic for CF.

1.7 Summary of Dissertation Research

The goal of my dissertation research was to evaluate NO-releasing chitosan oligosaccharides in their capacity as potential CF therapeutics. As such, my thesis work focused on the antibacterial efficacy of NO in CF-relevant conditions, and determining its effects on the physical properties of CF ASL components (i.e., bacterial biofilms and mucus). The following specific aims represent the goals of my work:

1) Determining the antibacterial efficacy of NO-releasing chitosan oligosaccharides against relevant *P. aeruginosa* strains under culture conditions which accurately mimic CF airways;
2) Quantifying the effects of NO-releasing and non-NO-releasing chitosan oligosaccharides on the biophysical properties of *P. aeruginosa* biofilms; and,
3) Evaluating the ability of NO-releasing chitosan oligosaccharides to reduce mucin molecular weight and the rheological properties of CF sputum.

In this chapter, CF pathogenesis was introduced as a function of the biophysical properties of the airways. As defective chloride ion transport decreases ASL hydration, the resulting

accumulation of highly viscous mucus promotes bacterial infection and subsequent lung degradation. Motivation was also provided for the development of NO-based therapies for the treatment of CF. Inhalation of NO gas has proven effective in small animal studies and is safe for healthy adults. However, the inconvenience and high cost of gaseous administration warrants the development of new delivery mechanisms. To this end, NO-releasing chitosan oligosaccharides have been proposed as a potential CF therapeutic. As these materials are water soluble and exhibit prolonged NO release, inhalation of NO-releasing materials via nebulization would allow for prolonged NO delivery to the airways. In Chapter 2, I will describe the use of NO-releasing chitosan oligosaccharides for determining the antibacterial efficacy of NO against *P. aeruginosa* under CF-relevant conditions. In these studies, alterations in the antibacterial activity of NO as a function of bacteria metabolism, phenotype, and oxygen availability were investigated. Chapter 3 details the effects of NO-releasing chitosan oligosaccharides and tobramycin treatment on the viscoelasticity of *P. aeruginosa* biofilms as determined via multiple particle tracking microrheology. Chapter 4, describes the synthesis of NO-releasing chitosan oligosaccharides with different modifications to create materials with a range of mucoadhesive strengths. Furthermore, the use of these materials to examine how the mucoadhesion of the scaffold alters the ability of NO to degrade mucins from human bronchial epithelial cell cultures and clinical samples of CF sputum are detailed. Lastly, Chapter 5 provides a summary of my dissertation work and suggests future directions for the evaluation of NO-release scaffolds for the treatment of CF.

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CHAPTER 2: ANTIBACTERIAL ACTION OF NITRIC OXIDE-RELEASING CHITOSAN OLIGOSACCHARIDES AGAINST PSEUDOMONAS AERUGINOSA IN CYSTIC FIBROSIS-RELEVANT CONDITION

2.1 Introduction

Cystic fibrosis (CF) is a genetic autosomal recessive disorder that affects > 70,000 people worldwide.[1] Genetic mutations in the transmembrane conductance regulator (CFTR) gene disrupt the movement of water and ions across the airway surface, resulting in a dehydrated, highly viscous mucus layer. In addition to preventing mucociliary clearance (i.e., the main mechanism of the airways to prevent bacterial infection), this mucus layer also provides an ideal environment for bacterial biofilm formation.[2-3] Disease and degradation of the lungs caused by such biofilms represent the leading causes of morbidity and mortality in CF patients.[4] As more than 80% of adults with CF suffer from chronic *Pseudomonas aeruginosa* (*P. aeruginosa*) infections, the development of an effective treatment capable of efficient biofilm eradication would dramatically improve their life span.[5]

Several interrelated factors contribute to the poor efficacy of current antibiotics against *P. aeruginosa* in CF airways. Chronic CF infections are characterized predominately by the mucoid phenotype, resulting in increased alginate production.[6] Alginate encapsulates bacteria, providing a physical barrier against the host immune system and antibiotics.[7] Increased neutrophil activity in CF mucus also lowers oxygen levels and increases nitrate concentrations in the airways.[8-9] Consequently, bacteria metabolism occurs through anaerobic respiration via denitrification. This process is inefficient compared to aerobic respiration and ultimately slows bacterial growth. The reduced metabolic activity of *P. aeruginosa* in anaerobic CF mucus protects the bacteria against

traditional antibiotics that are most effective against rapidly dividing cells, including aminoglycosides and β-lactams.[9-10]

The failure of conventional antibiotics to treat *P. aeruginosa* infections in CF airways necessitates the development of new antibacterial agents. Nitric oxide (NO), an endogenously produced free radical that can disperse[11-12] and eradicate[13-14] biofilms, holds particular promise as a CF treatment. In aerobic environments, NO reacts with molecular oxygen, superoxide, and hydrogen peroxide to form highly reactive intermediates (peroxynitrite, nitrogen dioxide, and dinitrogen trioxide). These molecules cause DNA deamination, nitrosation of membrane and intracellular proteins, and membrane damage via lipid peroxidation through both nitrosative and oxidative stresses, culminating in bacteria death.[15-18] Some of these congener molecules, especially peroxynitrite, are more potent antimicrobials than NO alone.[16] In anaerobic environments such as CF mucus, NO toxicity is less understood. Ren et al. reported the bacteriostatic mechanisms to include modification of iron-sulfur proteins.[19] As these proteins are linked to nearly every cellular process including metabolism, respiration, RNA modification, and DNA repair and replication, their alteration greatly influences bacteria viability.[20]

Diminished airway and exhaled NO are characteristic for patients with CF, possibly correlating to greater susceptibility to infection.[21-22] While the administration of exogenous NO holds promise as a therapeutic, direct inhalation of gaseous NO is highly impractical and dangerous as NO mediates other physiological processes (e.g., vasodilation and blood clotting).[23-24] Macromolecular scaffolds capable of effectively storing and releasing NO have been developed to enable local delivery.[25-26] The most promising NO-release vehicles to date include NO donor-modified *N*-diazeniumdiolate silica nanoparticles,[27-29] dendrimers,[30-34] and chitosan.[14] While silica nanoparticles[13,35-37] and dendrimers[30-32] are effective as antimicrobials, they do not easily

break down and thus have limited potential as inhaled therapeutics. Chitosan-based oligosaccharides represent attractive scaffolds for pulmonary NO delivery as they are biodegradable and of low toxicity to mammalian cells.[38-39] We have previously reported that NO-releasing chitosan oligosaccharides are capable of NO storage/release and eradicating *P. aeruginosa* biofilms under aerobic environments at concentrations non-toxic to mammalian cells.[14] Herein, we evaluate the antibacterial efficacy of NO-releasing chitosan oligosaccharides using *P. aeruginosa* phenotypes (i.e., nonmucoid, mucoid, and biofilm) and oxygen concentrations that mimic those of the CF airway to more fully elucidate the potential of these materials as CF therapeutics.

2.2 Materials and Methods

2.2.1 Materials

Medium molecular weight chitosan, 2-methylaziridine, and tobramycin were purchased from Sigma-Aldrich (St. Louis, MO). Methyltrimethoxysilane (MTMOS) was purchased from Fluka (Buchs, Switzerland). (Heptadecafluoro-1,1,2,2-tetrahydrodecyl)trimethoxysilane (17 FTMS) was purchased from Gelest (Morrisville, PA). Nitric oxide gas was purchased from Praxair (Sanford, NC). Standardized NO gas (26.85 ppm, balance N_2), Argon (Ar), and nitrogen (N_2) gases were purchased from Airgas National Welders (Durham, NC). Sodium methoxide was purchased from Acros Organics (Geel, Belgium). Distilled water was purified using a Millipore Milli-Q UV Gradient A-10 system (Bedford, MA). All common laboratory salts and reagents were purchased from Fisher Scientific (Pittsburgh, PA). All materials were used without further purification unless otherwise specified.

2.2.2 Bacteria strains and media

The laboratory *P. aeruginosa* strain used in this study was strain K (PAK). The nonmucoid phenotype and mucoid phenotype (*mucA22*) were a gift from Prof. Matthew Wolfgang, UNC Department of Microbiology and Immunology (Chapel Hill, NC). Clinical isolates were collected from patients at the UNC Hospital Clinical Microbiology Lab (Chapel Hill, NC). Clinical isolates were screened for tobramycin resistance using the Kirby Bauer disk diffusion method according to standards published by the Clinical and Laboratory Standards Institute in document M100-S23.[40] All bacteria were grown in Luria Bertani (LB) broth (BD Biosciences, San Jose, CA) with the pH adjusted to 6.5 using 10 mM sodium phosphate. When indicated, potassium nitrate (15 mM) was added to the broth. Phosphate buffered saline (PBS) was adjusted to pH 6.5 with 10 mM sodium phosphate. Anaerobic media was kept in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) with the lid loosened for one week prior to use.

2.2.3 Synthesis of 2-methylaziridine modified chitosan oligosaccharides

2-Methylaziridine modified chitosan oligosaccharides were synthesized as previously published.[14] Briefly, medium molecular weight chitosan (2.5 g) was oxidatively degraded to ~5 kD in 15% hydrogen peroxide for 1 h at 85 °C. Non-degraded chitosan was removed by filtration. The remaining chitosan oligosaccharides were precipitated from solution with acetone, collected via centrifugation, and dried in vacuo. The ~5 kD chitosan oligosaccharides (500 mg) were then dissolved in water (10 mL). Concentrated hydrochloric acid (27.6 µL), water (250 µL), and 2-methylaziridine (356 µL) were then added to this solution. The reaction was stirred for 5 d at 25 °C followed by 24 h at 75 °C. The modified chitosan oligosaccharides were again precipitated with acetone, collected via centrifugation, and dried in vacuo. ^{1}H NMR data of COS: (400 MHz, D_2O, δ): 0.8–1.1 ($NH_2CH(CH_3)CH_2NH$), 1.9 (C7: $CHNHCOCH_3$), 2.3–2.9

($NH_2CH(CH_3)CH_2NHCH$, C2: $NH_2CH(CH_3)CH_2NHCH$), 3.3–4.0 (C3, C4, C5, C6: OHC*H*, OC*H*CH(OH)CH(NH_2)CH, $OHCH_2CH$, $OHCH_2CH$), 4.4 (C1: OC*H*($CHNH_2$)O).

2.2.4 Synthesis of NO-releasing chitosan oligosaccharides

In order to impart NO storage and release, *N*-diazeniumdiolates were formed on the secondary amines of COS.[14] Briefly, COS (15 mg) was dissolved in a solution of water (300 µL), methanol (700 µL) and 5.4 M sodium methoxide (25 µL) in a 1 dram vial equipped with a stir bar. The open vial was placed in a 160 mL Parr general purpose stainless steel pressure vessel and rigorously stirred. Oxygen was removed from the reaction vessel by purging with argon (10 s, 8 bar) 3 times, followed by 3 additional long argon purges (10 min, 8 bar). The vessel was then filled with potassium hydroxide-purified NO gas (10 bar) for 72 h at room temperature. Afterwards, the argon purging procedure was repeated to remove unreacted NO. The *N*-diazeniumdolate-modified chitosan oligosaccharides (COS-NO) were precipitated in acetone, collected via centrifugation, dried in vacuo, and stored at -20 °C as a yellow powder.

2.2.5 Chemiluminescence detection of NO

A Sievers 280i Chemiluminescence Nitric Oxide Analyzer (Boulder, Colorado) was used for chemiluminescence detection of NO from COS-NO (1.0 mg) in 30 mL of deoxygenated PBS (pH 6.5) at 37 °C. Released NO was carried by N_2 gas to the reaction vessel/detector at a flow rate of 80 mL/min. Additional N_2 flow was supplied to the sample flask at 200 mL/min to match the collection rate of the instrument. The analysis was terminated when NO concentrations fell below 10 ppb NO/mg COS-NO. Prior to analysis, the instrument was calibrated with air passed through a NO zero filter (0 ppm NO) and 26.8 ppm of NO standard gas (balance N_2).

2.2.6 Electrochemical detection of NO

Home built NO-selective electrochemical sensors were fabricated as previously reported.[41] Briefly, polished polycrystalline Pt disk electrodes (2mm) sealed in Kel-F (CH Instruments, Austin, TX) were coated with a NO-selective xerogel membrane prepared by mixing MTMOS (60 µL), ethanol (300 µL), 17 FTMS (15 µL), water (80 µL), and 0.5 M hydrochloric acid (5 µL) for 1 h at 25°C. The resulting sol was spreadcast over the Pt electrode and dried overnight at room temperature. Amperometric NO measurements followed using a three-electrode set up with the NO-selective membrane-modified Pt electrode as the working electrode, a Pt-coiled counter electrode, and a Ag/AgCl reference electrode. The applied potential for NO oxidation was +700 mV vs. Ag/AgCl. Immediately prior to use, NO sensors were calibrated by adding a known amount of PBS saturated with NO gas (1.9 mM) into deoxygenated PBS (pH 6.5). Saturated NO solutions were made on the day of use by degassing PBS (pH 6.5) for 30 min with Ar followed by 20 min purging with NO gas. The sensors were immersed in 10.0 mL of PBS or LB broth (stirred, 37 °C), and polarized at +700 mV vs. Ag/AgCl until a stable baseline was achieved prior to the addition of COS-NO. The NO oxidation current was measured every 0.1 s and ceased when the current returned to its background value. Measurement of NO release in anaerobic media was carried out in a Coy Anaerobic Chamber. Total NO for 1.0 mg COS-NO/mL solutions are reported as the average ± the standard deviation for 4 or more separate measurements.

2.2.7 Planktonic bactericidal assays

Bacteria were grown as overnight cultures, diluted 1:100 in fresh LB broth (with or without nitrate supplementation), and grown to mid-log phase (2×10^8 CFU/mL). These cultures were centrifuged, resuspended in PBS, and diluted to 2×10^6 CFU/mL in PBS. Each suspension was then added to vials containing COS-NO or COS-controls, and incubated at 37°C for 4 h with gentle shaking. Following treatment, bacteria solutions were serially diluted, spiral plated on LB Agar,

and incubated for 24 h at 37°C. Colonies were enumerated using a Flash & Go colony counter (IUL, Farmingdale, NY). The minimum bactericidal concentration (MBC_{4h}) was defined as the minimum concentration required to achieve a 3-log reduction in viable bacteria (from 10^6 to 10^3 CFU/mL). The plating counting method employed has a limit of detection of 2.5 x10^3 CFU/mL.[42]

2.2.8 Planktonic inhibition assays

Bacteria were grown as overnight cultures, diluted 1:100 in fresh LB broth, grown to mid-log phase (2×10^8 CFU/mL), and diluted to 2×10^6 CFU/mL in LB. Bacteria cultures were then added to vials containing COS-NO or COS controls, and incubated at 37 °C for 18 h with gentle shaking. The minimum inhibitory concentration (MIC) was determined to be the minimum concentration that inhibited visible growth, defined as an optical density < 0.1 at 600 nm. Nitrate-supplemented LB was used for all stages of bacteria growth and exposure. Anaerobic experiments were performed in a Coy anaerobic chamber.

2.2.9 Biofilm eradication assays

Bacteria were grown as overnight cultures, diluted 1:100 in fresh LB broth, and grown to mid-log phase (2×10^8 CFU/mL). Bacteria cultures were then diluted to 10^6 CFU/mL in diluted (25%) LB Broth supplemented with 15 mM KNO_3 (pH 6.5), and grown for 72 h at 37 °C with gentle shaking. The viscous microcolony biofilms formed were easily separated from the growth media via pipetting. The biofilms were harvested by placing a pipette tip near the center of the biofilm and applying suction. The biofilms were then washed by injection into PBS and extracted using the same pipetting procedure to remove planktonic or loosely-associated bacteria. The freshly washed biofilms (250 µL) were combined with 750 µL of PBS (pH 6.5), added to vials containing COS, COS-NO or tobramycin, and incubated with gentle shaking for 18 h at 37 °C.

After treatment, biofilms were washed via pipetting in PBS to remove excess antibacterial agent, transferred to 750 µL of PBS (pH 6.5), and gently sonicated to disrupt the biofilm matrix. Dispersed biofilms were vortexed, serially diluted, plated, and enumerated on LB Agar. The minimum biofilm eradication concentration ($MBEC_{18h}$) was defined as the concentration which caused a 5-log reduction in viable bacteria (i.e., 10^8 to 10^3 CFU/mL) after the 18 h treatment.

2.2.10 Statistical analysis

All data are expressed as the mean ± one standard deviation and were analyzed for significance ($p < 0.05$) with a two-tailed Student's *t*-test.

2.3 Results

2.3.1 Nitric oxide release from COS-NO in media

Nitric oxide release from COS-NO was measured in deoxygenated PBS (pH 6.5) at 37 °C to yield total NO release payloads of 0.86 ± 0.05 µmol NO/mg with an overall release duration 10.2 ± 2.7 h (Figure 2.1, Table 2.1). While NO release from macromolecular scaffolds are generally measured in deoxygenated media, measuring the amount of bioavailable (i.e., non-scavenged) NO is critical for elucidating the biocidal dose-response relationship of NO under intended conditions as NO is rapidly scavenged by oxygen and proteins in biological media [43]. Unfortunately, foaming associated with nutrient rich media makes chemiluminescence detection difficult and irreproducible.[41] Thus, we turned to amperometric NO detection to carry out NO measurements in broth (LB).

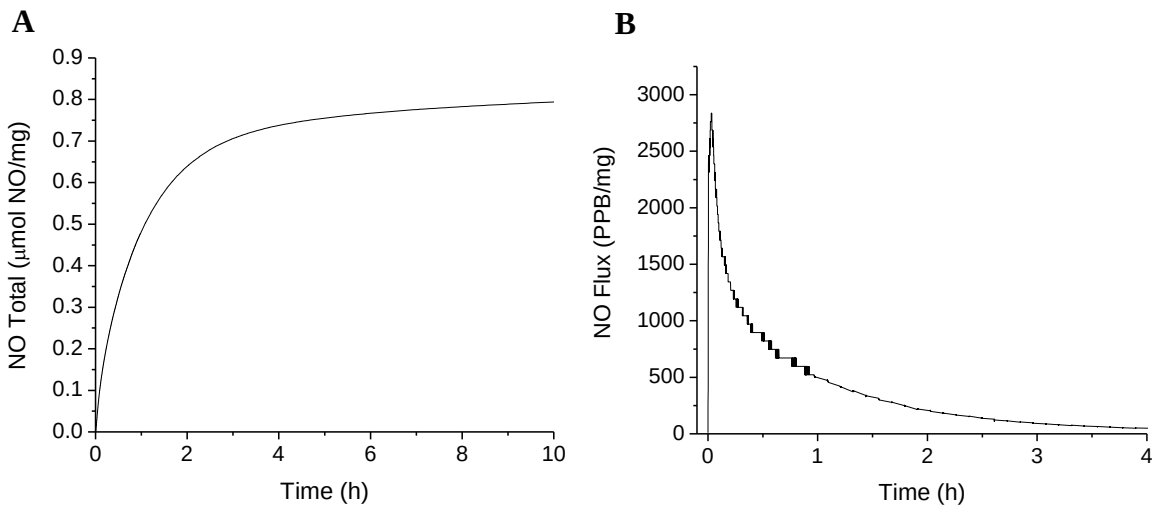

Figure 2.1 Chemiluminescence detection of NO release from COS-NO. The NO release from 1.0 mg of COS-NO was determined in deoxygenated PBS (pH 6.5) for comparison with other NO-releasing systems. (A) The NO total and (B) NO flux of representative measurements.

Table 2.1 Nitric oxide-release properties of COS-NO in PBS (pH 6.5, 37 °C) as determined by chemiluminescence detection. Values are presented as means ± standard deviations for n=3 pooled experiments.

NO Total (μmol NO/mg COS-NO)	Duration (h)	Half-Life (h)	Max Flux (PPB/mg)
0.86 ± 0.05	10.2 ± 2.7	0.63 ± 0.09	3100 ± 800

In the absence of NO scavenging (i.e., in deoxygenated PBS), amperometric measurements revealed a total NO payload of 0.34 ± 0.17 μmol/mL from the 1.0 mg/mL solution of COS-NO over 4 h (Figure 2.2). As might be expected, both the total NO payload and release duration of COS-NO measured via amperometry were reduced compared to chemiluminescence detection. These decreases are common for electrochemical sensors that are based on the diffusion of NO to the working electrode and the inherent loss of NO to the ambient atmosphere.[41]

Under aerobic conditions, oxygen scavenging reduced the amount of free NO available in PBS by approximately 35% (0.22 ± 0.08 μmol NO/mL). Nutrient broth (LB) further diminished the available NO payload via scavenging of the NO by proteins in the broth. The amount of NO available in anaerobic LB Broth was reduced to 0.14 ± 0.08 μmol NO/mL, a 66% reduction relative to anaerobic PBS. Further reductions (0.027 ± 0.017 μmol/mL) were observed in aerobic LB broth due to reaction of NO with oxygen.

2.3.2 Bactericidal action of the COS scaffold

To confirm NO was responsible for the observed bacteria killing and not the scaffold, all bacteria assays were performed using NO-releasing and control (i.e., non-NO-releasing) chitosan oligosaccharides. In MBC_{4h} assays of planktonic cells, COS did not influence bacteria viability at 1x or 10x the MBC_{4h} of COS-NO, indicating that the chitosan oligosaccharide alone was not bactericidal (Figure 2.3). Similarly, bacteria viability was not reduced upon treatment of the biofilms with 4.0 mg COS/mL (1x $MBEC_{18h}$) for 18 h under both aerobic and anaerobic conditions (Figure 2.4). With this data, the bactericidal activity of COS-NO is attributed solely to the effects of NO and not to toxicity of the COS scaffold.

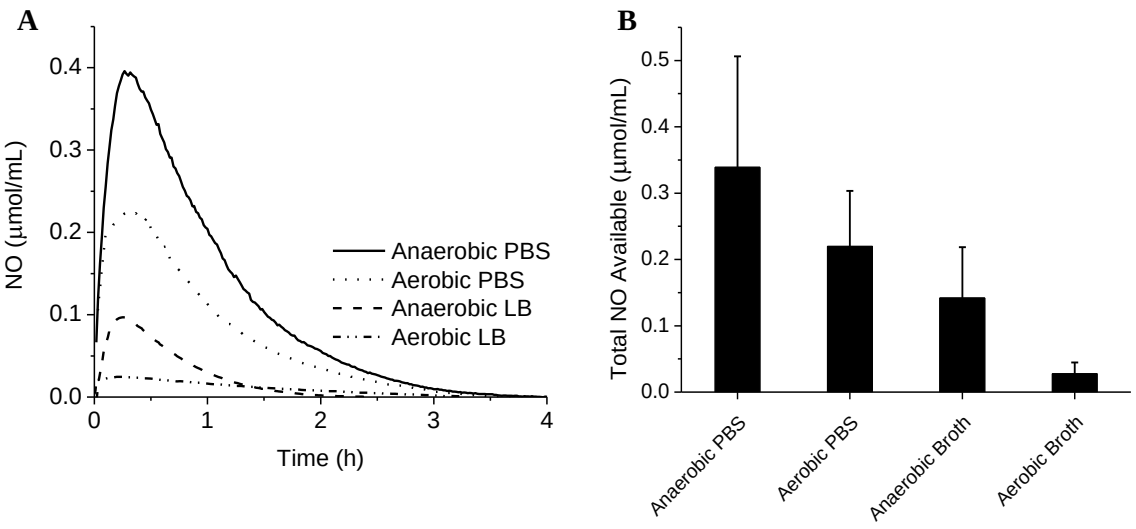

Figure 2.2 Electrochemical measurements of available NO in media. (A) Representative NO-release profiles for 1.0 mg/mL of COS-NO in anaerobic PBS, aerobic PBS, anaerobic LB broth, and aerobic LB Broth are shown. These values were integrated to find the (B) total concentration of available NO in a 1.0 mg/mL solution of COS-NO in biological media over 4 h.

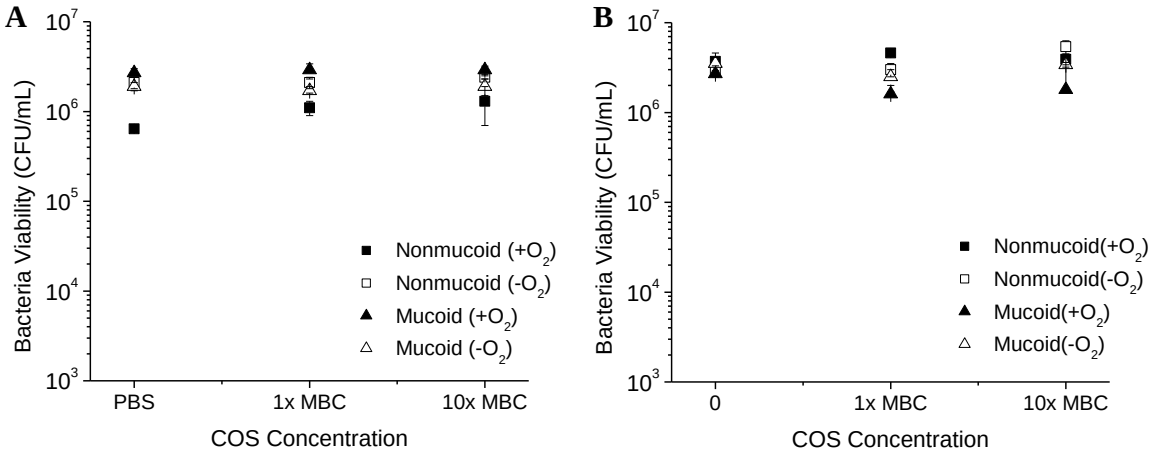

Figure 2.3 Planktonic bacteria viability (in CFU/mL) after a 4 h exposure to COS in PBS. Bacteria were grown (A) aerobically and (B) anaerobically in LB (with nitrate supplementation) and exposed to COS in PBS (pH 6.5).

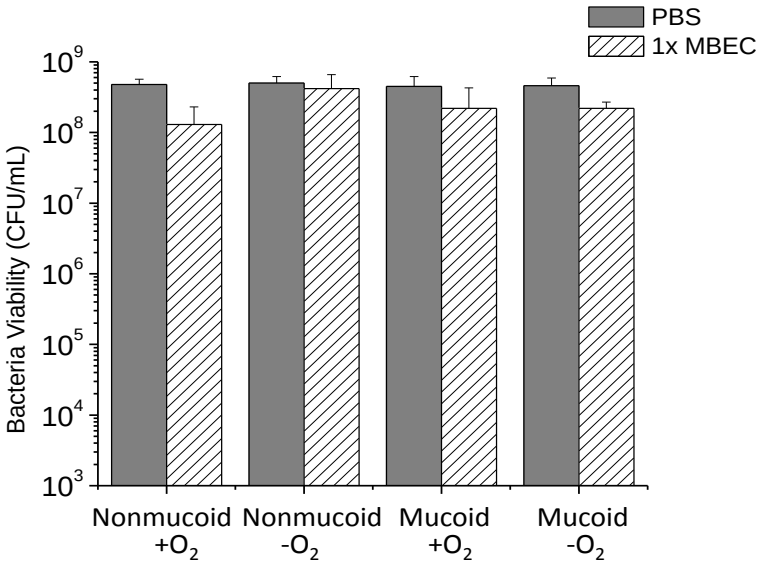

Figure 2.4 Biofilm viability (in CFU/mL) after exposure to COS. Bacteria biofilms were grown for 72 h, then exposed to COS under aerobic or anaerobic conditions for 18 h.

2.3.3 *Effect of oxygen on bactericidal action of NO against planktonic P. aeruginosa*

The biocidal action of NO was evaluated with respect to oxygen concentration in the treatment media by exposing planktonic cultures to COS-NO in both aerobic and anaerobic PBS (pH 6.5) (Table 2.2). While the same concentration of COS-NO was required to kill both bacteria phenotypes under aerobic and anaerobic exposure conditions, the NO dose delivered in anaerobic PBS was slightly greater due to reduced NO reaction with oxygen. For example, the bactericidal NO dose for nonmucoid *P. aeruginosa* was 0.022 ± 0.008 μmol NO/mL under aerobic conditions versus 0.034 ± 0.017 μmol NO/mL in deoxygenated (anaerobic) media. Differences in NO-payloads between aerobic and anaerobic environments were not statistically significant.

Bacteria cultures were also grown aerobically and anaerobically to determine if oxygen in the growth media affected *P. aeruginosa* susceptibility to NO. As anaerobic growth requires nitrate, all assays were carried out using nitrate-supplemented LB to enable direct comparison. The absence of oxygen in the growth media had no effect on the susceptibility of nonmucoid *P. aeruginosa* to NO. However, strict anaerobic growth of mucoid *P. aeruginosa* increased the tolerance of this strain to NO by 2-fold (MBC_{4h} of 0.044 ± 0.016 and 0.022 ± 0.008 μmol NO/mL for anaerobic and aerobic growth conditions, respectively) (Table 2.2). The increased tolerance to NO was observed under both aerobic and anaerobic exposure conditions.

2.3.4 *Inhibition of planktonic P. aeruginosa growth by COS-NO*

Minimum inhibitory concentration (MIC) assays were performed to evaluate the efficacy of COS-NO during bacterial growth under aerobic and anaerobic environments (Table 2.3). Under aerobic conditions, the nonmucoid phenotype was more tolerant to COS-NO than the mucoid strain with inhibitory doses of 800 μg COS-NO/mL (0.022 ± 0.014 μmol NO/mL) versus 400 μg COS-NO/mL (0.011 ± 0.007 μmol NO/mL), respectively. Anaerobic conditions decreased the

Table 2.2 Effect of oxygen on non-growing planktonic cultures. *P. aeruginosa* cultures were grown in LB (+15 mM KNO3) under aerobic or anaerobic conditions, then exposed to COS-NO in PBS (pH 6.5) for 4 h under aerobic or anaerobic conditions.

		Aerobic Exposure		Anaerobic Exposure	
	Growth Media	MBC (µg COS-NO/mL)	NO Dose (µmol NO/mL)	MBC (µg COS-NO/mL)	NO Dose* (µmol NO/mL)
Nonmucoid	Aerobic	100	0.022 ± 0.008	100	0.034 ± 0.017
	Anaerobic	100	0.022 ± 0.008	100	0.034 ± 0.017
Mucoid	Aerobic	100	0.022 ± 0.008	100	0.034 ± 0.017
	Anaerobic	200	0.044 ± 0.016	200	0.068 ± 0.033

*Determined via amperometry. Values are presented as means ± standard deviations for n=3 or more pooled experiments.

Table 2.3 Influence of oxygen on the inhibitory efficacy of COS-NO. Bacteria cultures in mid-log growth were diluted to 2x106 CFU/mL in LB (+15 mM KNO3) with COS-NO and grown for 18 h under aerobic or anaerobic conditions. The MIC was determined as the concentration of COS-NO which visibly inhibited growth.

	Nonmucoid		Mucoid	
	MIC (μg COS-NO/mL)	NO Dose (μmol NO/mL)	MIC (μg COS-NO/mL)	NO Dose* (μmol NO/mL)
Aerobic	800	0.022 ± 0.014	400	0.011 ± 0.007
Anaerobic	100	0.014 ± 0.008	100	0.014 ± 0.008

*Determined via amperometry. Values are presented as means ± standard deviations for n=3 or more pooled experiments.

MIC to 100 μg COS-NO/mL (0.014 μmol NO/mL) for both phenotypes. While the COS-NO dose required to inhibit growth was reduced in anaerobic environments, the NO dose delivered was not significantly lower, indicating that NO lost to reaction with oxygen accounts for the increased MICs against COS-NO under aerobic conditions.

2.3.5 Inhibition of growth by COS-NO for clinical isolates, including tobramycin–resistant strains.

To ensure that the increased inhibition of bacterial growth by COS-NO under anaerobic conditions was not solely a function of the laboratory *P. aeruginosa* strain used, 10 clinical isolates of *P. aeruginosa* were tested including mucoid, nonmucoid, and tobramycin-resistant isolates. The amount of COS-NO required to inhibit growth under anaerobic conditions was less than or equal to those under aerobic conditions for all 10 isolates (Figure 2.5A). There was no statistical difference in the NO-dose required to inhibit growth for most of the strains tested (Figure 2.5B). However, two isolates (denoted with * in Figure 2.5B) showed a statistically significant increase in NO payload required to inhibit growth under anaerobic conditions relative to aerobic conditions. Overall, COS-NO was more effective at inhibiting growth under anaerobic environments but the efficacy of NO was unchanged for most of the isolates tested.

As with the laboratory strain, differences were seen between the mucoid and nonmucoid phenotypes (Figure 2.5). Mucoid strains were more susceptible to COS-NO as indicated by the low MIC range (25-200 μg COS-NO/mL) relative to nonmucoid strains (MIC range 100-400 μg COS-NO/mL). No apparent differences were observed between tobramycin-susceptible and tobramycin-resistant strains of the same phenotype; however, more isolates would be needed to confirm the statistical significance of these trends.

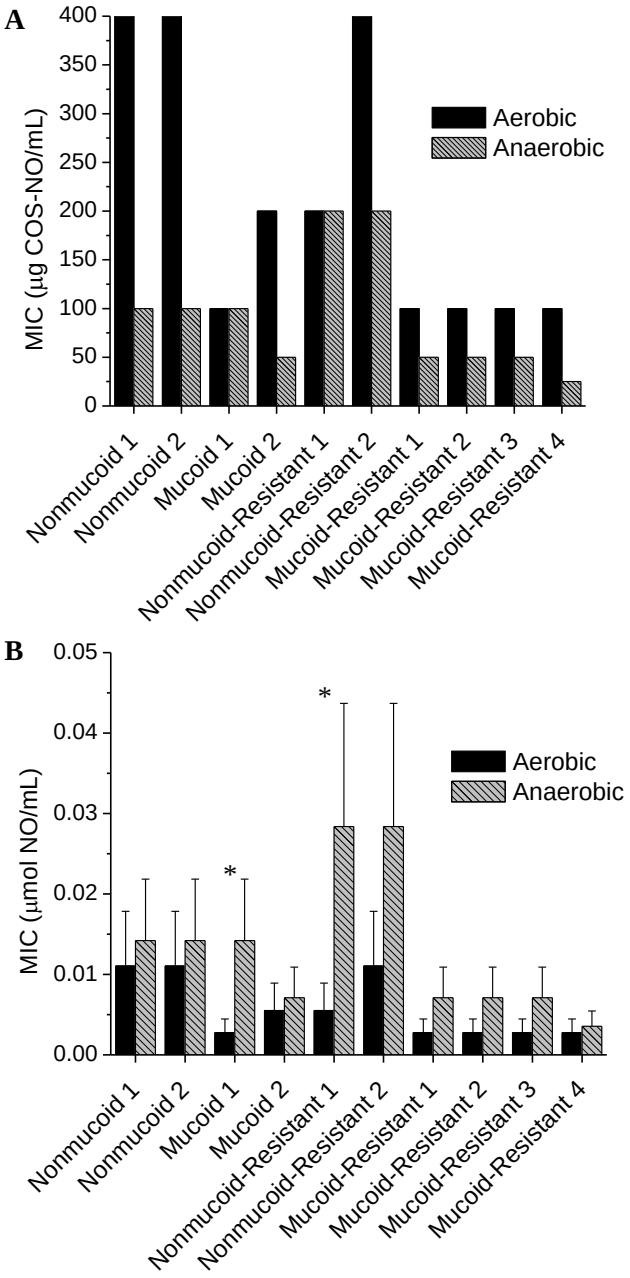

Figure 2.5 MICs against clinical isolates of *P. aeruginosa*. (A) The minimum concentration of COS-NO that visibly inhibited bacterial growth was determined for nonmucoid, mucoid, and tobramycin resistant isolates and (B) the corresponding NO dose for each isolate under aerobic and anaerobic environments. All bacteria were grown and exposed in LB supplemented with nitrate (pH 6.5) for 18 h. Statistically significant differences ($p < 0.05$) between aerobic and anaerobic NO payloads are indicated (*).

2.3.7 Biofilm eradication by COS-NO and tobramycin

As bacteria biofilms form predominantly in anaerobic pockets of mucus in the CF airways,[2] it was important to determine how oxygen concentrations affected the anti-biofilm activity of COS-NO. Under aerobic conditions, highly viscous microcolony biofilms were formed (~250 µL in volume) with bacteria viability of $4.0 \pm 0.6 \times 10^8$ and $2.5 \pm 0.5 \times 10^8$ CFU/mL for nonmucoid and mucoid phenotypes, respectively. Of note, nitrate supplementation was required to prevent phenotypic switching from the mucoid to the nonmucoid phenotype.[44] Under anaerobic growth conditions, we were unable to form robust biofilms even after 7 d of growth.

Bacteria biofilms were exposed to COS-NO for 18 h in PBS (pH 6.5) under aerobic or anaerobic conditions. The $MBEC_{18h}$ for both phenotypes was 4000 µg COS-NO/mL and 1000 µg COS-NO/mL (0.88 ± 0.33 and 0.34 ± 0.17 µmol NO/mL) in aerobic and anaerobic conditions, respectively (Table 2.4). These results indicate that NO is equally effective at eliminating biofilms derived from nonmucoid and mucoid strains. Moreover, NO is significantly more effective at eliminating biofilms in the absence of oxygen.

The $MBEC_{18h}$ of tobramycin against biofilms was also determined to allow comparison of the NO treatment to current antibiotic therapies. Under aerobic environments, the nonmucoid strain was eradicated at lower concentrations of tobramycin than the mucoid strain (200 and 800 µg/mL, respectively) (Table 2.5). Both strains required greater tobramycin levels (1600 µg/mL for both strains) to eradicate bacteria biofilms under anaerobic conditions.

2.4 Discussion

We have previously reported on the antibacterial activity of NO against planktonic and biofilm based *P. aeruginosa*.[13-14,37] However, little is understood regarding how oxygen and bacteria phenotype impact NO's efficacy. Such knowledge is critical in the development of NO-

Table 2.4 Bactericidal efficacy of COS-NO against *P. aeruginosa* biofilms. Biofilms were exposed to COS-NO in PBS (pH 6.5) for 18 h under aerobic or anaerobic conditions. The MBEC18h was reported as the concentration of COS-NO required for 5-log reduction in biofilm viability.

	Aerobic Exposure		Anaerobic Exposure	
	MBEC (μg COS-NO/mL)	NO Dose (μmol NO/mL)	MBEC (μg COS-NO/mL)	NO Dose* (μmol NO/mL)
Nonmucoid	4000	0.88 ± 0.33	1000	0.34 ± 0.17
Mucoid	4000	0.88 ± 0.33	1000	0.34 ± 0.17

*Determined via amperometry. Values are presented as means ± standard deviations for n=3 or more pooled experiments.

Table 2.5. Bactericidal efficacy of tobramycin against *P. aeruginosa* biofilms. Biofilms were exposed to tobramycin in PBS (pH 6.5) for 18 h under aerobic or anaerobic conditions. The $MBEC_{18h}$ was reported as the concentration of COS-NO required for 5-log reduction in biofilm viability.

	Aerobic Exposure		Anaerobic Exposure	
	MBEC (μg/mL)	MBEC (μmol/mL)	MBEC (μg/mL)	MBEC (μmol/mL)
Nonmucoid	200	0.43	1600	3.42
Mucoid	800	1.71	1600	3.42

based therapeutics for CF. Water soluble NO-releasing chitosan oligosaccharides were used as the NO-release scaffold in the studies described herein due to the requirement that a macromolecular CF therapeutic be well tolerated and biodegradable. Although chitosan is a known bactericidal agent, the reduced molecular weight (to ensure water solubility) and 2-methylaziridine modification (for NO donor addition) resulted in a material with no bactericidal activity (Figures 2.3 and 2.4).

It is well-known that NO reacts with oxygen and superoxide to form highly reactive intermediates that facilitate bacteria killing through oxidative and nitrosative stresses.[16,45] As oxygen plays an integral role in the antibacterial action of NO, anaerobic environments may reduce the biocidal efficacy of NO.[17,46] However, NO also reacts with oxygen to form nitrate and nitrite. These seemingly paradoxical roles of oxygen on NO-mediated killing are not fully understood. To this end, we carried out electrochemical measurements of NO under aerobic and anaerobic conditions to quantify the amount of bioavailable NO. Under aerobic conditions, the measured NO decreased by 35% compared to anaerobic conditions (Figure 2.2). To elucidate the effects of oxygen availability in treatment media, bacteria were first grown aerobically and then exposed to COS-NO in aerobic or anaerobic PBS. Identical concentrations of COS-NO were required to kill *P. aeruginosa* regardless of the treatment conditions. Due to reaction of NO with oxygen the bioavailable concentration of NO was slightly, but not significantly, higher under anaerobic conditions (Table 2.2). As such, oxygen availability in the treatment media has no overall effect on the biocidal activity of NO-releasing chitosan oligosaccharides against planktonic *P. aeruginosa*.

While oxygen concentration in the exposure media did not alter the bactericidal efficacy of NO, the presence of oxygen during bacterial growth did influence *P. aeruginosa* susceptibility

to NO. Anaerobic growth conditions reduce the efficacy of current antibiotics by altering certain properties of the bacteria such as alginate production[2] and metabolic rates.[9] To separate these factors, MBC assays were performed under non-nutritive conditions to minimize the effects of bacteria metabolism on the bactericidal activity of NO. When bacteria were grown under anaerobic conditions, the efficacy of NO was decreased against the mucoid but not the nonmucoid phenotype, indicating that growing mucoid bacteria without oxygen significantly alters its defense against NO (Table 2.2). Worliztsch et al. previously reported that *P. aeruginosa* produce a protective alginate exopolysaccharide that is 50% thicker when grown under anaerobic versus aerobic conditions.[2] The increased thickness of this protective layer, characteristic of the mucoid phenotype, thus likely requires larger NO concentrations to penetrate the exopolysaccharide compared to nonmucoid *P. aeruginosa.*

To study the role of anaerobic growth on the efficacy of NO, we evaluated the inhibition of *P. aeruginosa* growth by COS-NO in nutrient rich media under both aerobic and anaerobic conditions. In contrast to the static conditions of MBC assays, bacteria are actively growing during inhibition assays. Comparison of MIC values in oxygen and oxygen-free environments shows that the efficacy of COS-NO was enhanced under anaerobic conditions while there was no statistical difference in the NO dose. This behavior was observed in the laboratory strains (Table 2.3) and most of the clinical isolates tested (Figure 2.5), indicating that the efficacy of NO did not change but that the ability of COS-NO to deliver NO to the bacteria was improved in anaerobic conditions. Current antibiotic treatments, including aminoglycosides and β-lactams, are less effective under anaerobic treatments because their mechanism of action requires actively dividing cells.[47-48] In this respect, NO release provides a clear advantage over current treatments as the antibacterial action is not influenced by anaerobic environments.

Regardless of the antibacterial action against planktonic bacteria, the greatest challenge in reducing infection rates is the eradication of biofilms.[1,49] As shown in Table 2.5, mucoid biofilms are significantly more resilient against tobramycin. Hentzer et al. previously attributed decreased antibiotic efficacy against mucoid strains to the overproduction of alginate.[50] As has been previously reported,[51-52] the low oxygen conditions of CF mucus further decreases the effectiveness of tobramycin ($MBEC_{18h}$ to 1600 μg/mL under anaerobic conditions). While tobramycin is a highly effective anti-*Pseudomonal* agent, these factors compromise its ability to kill bacteria in CF airways. In contrast, NO was equally effective at eradicating mucoid and nonmucoid biofilms and exhibited increased antibacterial activity in anaerobic versus aerobic conditions (Table 2.4).

Direct comparison of tobramycin with the NO-releasing chitosan oligosaccharides shows that NO is a more effective anti-biofilm agent under anaerobic conditions. Indeed, the NO-dose required for biofilm eradication under anaerobic conditions is 0.34 ± 0.17 μmol NO/mL, tenfold lower than that of tobramycin (3.42 μmol/mL) (Tables 2.4 and 2.5). Equally important, the amount of NO-releasing scaffold required for biofilm eradication is less than that of tobramycin. Together, this data suggests that NO may be a more effective therapeutic for eradicating *P. aeruginosa* biofilms in the CF lung, particularly under the low-oxygen conditions characteristic of CF mucus.

2.5 Conclusions

These studies examined the susceptibility of *P. aeruginosa* to NO-releasing chitosan oligosaccharides in conditions relevant to CF. The antibacterial activity of NO-releasing chitosan oligosaccharides was enhanced in oxygen-free environments, despite a concomitant decrease in the number of possible mechanisms available to kill bacteria (i.e., less toxic byproducts from the

reactions of NO and oxygen). Furthermore, the anti-biofilm action of NO was more effective than tobramycin and was not influenced by bacteria phenotype. When combined with NO's significant biocidal action against *P. aeruginosa*, these results suggest that NO-releasing chitosan oligosaccharides may represent a highly effective CF therapeutic. We are currently seeking to enhance the NO payloads and study the effects of NO-release kinetics to improve the antibacterial efficacy of chitosan oligosaccharides while simultaneously evaluating cytotoxicity against human bronchial epithelial cultures.

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CHAPTER 3: DISRUPTION AND ERADICATION OF *PSEUDOMONAS AERUGINOSA* BIOFILMS USING NITRIC OXIDE-RELEASING CHITOSAN OLIGOSACCHARIDES

3.1 Introduction

Cystic fibrosis (CF) lung disease is caused by defective chloride transport, resulting in thickened, dehydrated mucus with altered biophysical properties such as increased viscoelasticity and osmotic pressure.[1-2] One pathological consequence of the altered CF mucus is the inhibition of mucociliary clearance, ultimately resulting in increased airway inflammation and infection.[3-4] In addition to preventing the removal of pathogens, thickened CF mucus restricts bacteria motility and promotes *P. aeruginosa* biofilm formation.[1] While biofilms are traditionally defined as antibiotic resistant, cooperative communities of bacteria within a protective matrix,[5] they also constitute viscoelastic materials with well-defined physical and mechanical properties.[6-7] Strategies for treating *P. aeruginosa* biofilms and infections in the CF airways to date have focused on reducing bacteria viability through antibiotic treatment, specifically through the use of inhalable tobramycin. Inhaled tobramycin is currently the only antibiotic recommended for both the treatment of initial[8] and chronic[9] *P. aeruginosa* infections in patients with CF. While inhaled tobramycin is effective at eradicating bacteria within biofilms, it fails to physically remove the structural remnants of the biofilm from the airways. Any bacteria that survive antibiotic treatment (e.g., persister cells) may initiate biofilm regrowth and the development of antibiotic-resistant infections.[5,10-11] As such, degradation of the biofilm and its removal from the airway are essential to preventing recolonization.[11-12] Physical disruption of the biofilm also increases the anti-biofilm efficacy of co-administered antibiotics, as antibiotic diffusion becomes enhanced in mechanically

weakened biofilms.[13-15] Therefore, an ideal anti-biofilm therapeutic for CF would both eradicate bacteria and physically degrade the biofilm, facilitating clearance from the airway.

In light of the importance of the viscoelastic properties of biofilms, much recent research has focused on quantifying how chemical and antibiotic treatments alter the mechanical properties of biofilms. Lieleg et al.[7] reported that gentamicin, colistin, ofloxacin, ethanol, and bleach did not alter *P. aeruginosa* biofilm elasticity when measured via rheometry. In contrast, ciprofloxacin was shown to reduce the elasticity of *P. aeruginosa* biofilms to that of a viscous fluid.[12] As each treatment elicits different effects, it is important to probe how antibacterial agents alter biofilm viscoelasticity for the development of any new therapies.

Nitric oxide (NO) is an endogenously produced diatomic free radical with significant antibacterial activity against *P. aeruginosa* biofilms.[16-18] At sub-bactericidal concentrations, NO has biofilm dispersing properties.[19-20] The antibacterial efficacy of NO is derived from its ability to exert both nitrosative and oxidative stresses to bacterial membrane components (e.g. proteins, lipids, DNA) directly or via reactive byproducts including and dinitrogen trioxide and peroxynitrite.[21-22] As the *P. aeruginosa* biofilm matrix is composed of proteins, extracellular DNA, and polysaccharides, it is likely that NO would alter or disrupt the structural integrity of these biofilms.[23-24] Furthermore, atomic force microscopy has revealed that NO exposure causes structural damage to the membranes of planktonic Gram-negative bacteria, including *P. aeruginosa.*[25] To determine the effects of NO on the viscoelastic properties of *P. aeruginosa* biofilms, macromolecular scaffolds capable of storing and controllably releasing NO were employed to locally deliver NO to bacterial biofilms.[26-27] Chitosan oligosaccharides represent an attractive scaffold for pulmonary NO delivery due to several attractive properties including biodegradability, tolerability to mammalian cells, and ease of NO donor functionalization.[17,28]

Herein, we evaluate the utility of NO-releasing chitosan oligosaccharides to both eradicate and physically alter *P. aeruginosa* biofilms, with comparison to tobramycin.

3.2 Materials and Methods

3.2.1 Materials

Tobramycin, medium molecular weight chitosan, and 2-methylaziridine were purchased from Sigma Aldrich (St. Louis, MO). Sodium methoxide was purchased from Acros Organics (Geel, Belgium). FluoSpheres carboxylate-modified microspheres (1 μm diameter) for use as tracer particles in microrheology experiments were purchased from Molecular Probes (Life Technologies, Carlsbad, CA). Nitric oxide gas was purchased from Praxair (Sanford, NC). Calibration standard NO gas (26.85 ppm, balance N_2), nitrogen (N_2), and argon gases were purchased from Airgas National Welders (Durham, NC). *P. aeruginosa* strain K (PAK) and the mucoid derivative (PAK*mucA22*) were a gift from Prof. Matthew Wolfgang from the University of North Carolina at Chapel Hill, Department of Microbiology and Immunology. Phosphate buffered saline (PBS) was made with 10 mM sodium phosphate and adjusted to pH 6.5 to more closely resemble the CF airway.[29] Luria-Bertani (LB) broth and agar were purchased from Becton, Dickinson, and Company (Franklin Lakes, NJ). Biofilm growth media was prepared by diluting LB Broth 1:4 in water after which the pH was adjusted to 6.5 with 10 mM sodium phosphate. Milli-Q water with a resistivity of < 18.2 mΩ cm and a total organic content of < 6 ppb was prepared by purifying distilled water using a Millipore Milli-Q UV Gradient A-10 system (Bedford, MA). All common laboratory salts, solvents, and reagents were purchased from Fisher Scientific (Pittsburgh, PA). All materials were used without further purification.

3.2.2 Synthesis of water-soluble 2-methylaziridine- modified chitosan oligosaccharides (COS)

Water-soluble chitosan oligosaccharides were synthesized by degrading medium molecular weight chitosan (2.5 g) in 50 mL of hydrogen peroxide (15 wt. %) for 1 h at 85 °C. The resulting oligosaccharides were filtered to remove insoluble oligosaccharides, precipitated with acetone, collected via centrifugation (6500 × *g*, 10 min), and dried in vacuo. The viscosity of the chitosan oligosaccharides was determined using an Ubbelohde viscometer in a solution of sodium chloride (0.20 M) and acetic acid (0.10 M) at 25 °C. The classic Mark-Houwink equation ($\eta = 1.81 \times 10^{-3} M^{0.93}$) was used to determine the molecular weight.[30]

2-Methylaziridine was grafted to the chitosan oligosaccharides as previously described.[17] Briefly, the water soluble chitosan oligosaccharides (0.5 g) were dissolved in water (10 mL) after which a solution of concentrated hydrochloric acid (27.5 µL), water (250 µL), and 2-methylaziridine (356 µL) was added dropwise at room temperature. The solution was stirred for 5 d at 25 °C followed by 24 h at 70 °C. The 2-methylaziridine-modified chitosan oligosaccharides (COS) were then collected via precipitation in acetone, washed copiously with ethanol, and dried in vacuo at room temperature. ^{1}H NMR data of COS: (400 MHz, D_2O, δ): 0.8–1.1 ($NH_2CH(CH_3)CH_2NH$), 1.9 (C7: $CHNHCOCH_3$), 2.3–2.9 ($NH_2CH(CH_3)CH_2NHCH$, C2: $NH_2CH(CH_3)CH_2NHCH$), 3.3–4.0 (C3, C4, C5, C6: $OHCH$, $OCHCH(OH)CH(NH_2)CH$, $OHCH_2CH$, $OHCH_2CH$), 4.4 (C1: $OCH(CHNH_2)O$).

3.2.3 N-diazeniumdiolate modification of chitosan oligosaccharides

N-diazeniumdiolate NO donors were formed on the secondary amines of COS via exposure to high pressures of NO gas.[17] Briefly, 2-methylaziridine-modified chitosan oligosaccharides (45 mg) were dissolved in water (900 µl), methanol (2.10 mL), and sodium methoxide (5.4 M, 75 µL) in 1 dram glass vials. The vials containing the COS solution were placed in a stainless steel reactor. Oxygen was removed from the system by purging with argon via three short purges (15 s, 8 bar)

followed by three long purges (10 min, 8 bar). After the final purge, the vessel was filled with NO gas (10 bar) that had been purified in a potassium hydroxide chamber. Solutions were stirred at room temperature for 72 h. Following *N*-diazeniumdiolate formation, unreacted NO was removed from solution via the same argon purging procedure used to remove oxygen. The resulting solutions of NO-releasing chitosan oligosaccharides (COS-NO) were centrifuged (6500 × *g*, 15 minutes), precipitated with 3 mL of acetone, collected via centrifugation, and dried in vacuo overnight at room temperature. The solid COS-NO was stored in a vacuum sealed bag at -20°C until use.

3.2.4 Characterization of nitric oxide release

Real-time NO-release kinetics from COS-NO were determined using at a Sievers Nitric Oxide Analyzer (Boulder, CO). Prior to analysis, the instrument was calibrated with air passed through a NO zero filter (0 ppm NO) and 25.87 ppm of NO standard gas (balance N_2). Solid COS-NO (1 mg) was added to 30 mL of deoxygenated PBS (37 °C, pH 6.5). Nitric oxide released from COS-NO was carried to the analyzer using nitrogen gas flowing through the solution at rate of 80 mL/min. Additional nitrogen was supplied to the reaction flask to achieve the required instrument collection rate of 200 mL/min. Analysis was terminated when NO levels decreased below 10 ppb/mg chitosan oligosaccharide.

3.2.5 Biofilm growth and eradication assays

Frozen cultures of *P. aeruginosa* were grown overnight in LB broth, diluted 1:100 in 50 mL of fresh LB broth, and grown to mid-log phase (OD_{600}=0.25). Rapidly growing cultures were diluted to 10^6 CFU/mL (1:100 dilution) in biofilm growth media. Viscous biofilms were grown in 12-well microtiter plates at 37°C for 72 h with gentle shaking (100 rpm). Biofilms appeared as

viscous bacterial aggregates (i.e. microcolonies) floating in the growth media. These biofilms were mechanically robust (i.e. they could not be disrupted by vigorous pipetting), indicating the formation of a suitable experimental matrix.[31] Biofilms were extracted from the growth media via pipetting and then washed by ejection into PBS (pH 6.5). The biofilms were subsequently added to a solution of PBS (pH 6.5) containing COS-NO, COS, or tobramycin and incubated for 18 h at 37 °C with gentle shaking (100 rpm). The biofilms were again washed in PBS (pH 6.5) to remove excess antibacterial agent prior to further analysis.

Following exposure to the test agents, biofilms were plated and enumerated to determine the minimum biofilm eradication concentration (MBEC), defined herein as the minimal concentration of drug required for a 5-log reduction in bacteria viability. Freshly washed biofilms were gently sonicated for 10 min and vortexed to disrupt the matrix. The resulting solutions were serially diluted in PBS, spiral plated on LB Agar, and incubated at 37°C for 24 h. Bacteria colonies were quantified using a Flash & Go colony counter (IUL, Farmingdale, NY). This method has an inherent limit of detection of 2.5×10^3 CFU/mL.[32]

3.2.6 Multiple-particle tracking microrheology

Biofilms were grown as described above except with the incorporation of fluorescent tracer particles. Fluorescent tracer particles were diluted 1:1000 from their stock solution (2 wt. %) into the biofilm growth media prior to the addition of planktonic bacterial. Biofilms were exposed to antibacterial agents (i.e. COS-NO, COS, or tobramycin) as in the MBEC assays. Following treatment, the biofilms were placed in a transparent sample holder. Specifically, the biofilms were sealed between a glass microscope slide and coverslip with two sheets of parafilm acting as a spacer. The sample holders were sealed on using parafilm to minimize sample evaporation. Tracer particle movement was recorded at 60 frames/s for 30 s with a Flea3 grey scale camera (Point

Grey, Richmond, Canada) mounted on a Nikon Eclipse TE2000-E inverted microscope at 40× magnification. The tracer particle displacement as a function of time was quantified using Video Spot Tracker software (Center for Computer Integrated Systems for Microscopy and Manipulation, University of North Carolina at Chapel Hill).

The mean squared displacement (MSD) of each tracer particle was calculated from the displacement of individual particles as a function of time as previously described.[33] Briefly, the MSD was determined according to:

$$\mathrm{MSD} = \frac{1}{N-\tau}\sum_{i=t}^{N-\tau}\left[\left(x(t_i+\tau)-x(\tau)\right)^2+\left(y(t_i+\tau)-y(\tau)\right)^2\right] \quad \textbf{(Eq. 1)}$$

where τ represents time lag, t_i is the time at the start of the video (0.00 s), and N is the total number of frames in a video (1800 for all experiments).

Due to biofilm heterogeneity, the MSD of all tracer particles were ensemble averaged to achieve meaningful MSD curves.[33] For each condition tested, tracer particle displacement was measured in 15 different viewing areas of three separate biofilms, resulting in ensemble averaging of at least 200 particles per treatment. The ensemble averaged MSD was calculated as previously reported.[33] For clarity, MSD values were analyzed at τ = 0.83 s. At this time point ($MSD_{\tau=0.83s}$), the accuracy of the camera speed does not affect the measurement, and thermal drift is minimized. All MSD values are reported as the mean ± standard error of the mean (SEM). Due to the large sampling size, the error is often too small to be visible on the figures presented herein.

3.2.7 Measurement of distance between nearest neighbor particles

The average distance between nearest neighbor particles was determined to quantify contraction and expansion of the bacterial biofilms.[34] Distances between nearest neighbor tracer particles were calculated according to the equation:

$$R_{12} = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2} \qquad \textbf{(Eq. 2)}$$

where R_{12} represents the distance between beads, x_1 and y_1 are the location of tracer particle 1, and x_2 and y_2 are the location of tracer particle 2. The smallest R_{12} value for each bead was then selected as the distance between nearest neighbor particles.

3.3 Results

Water soluble chitosan oligosaccharides were synthesized from chitosan via oxidative degradation.[35] The molecular weight of the chitosan oligosaccharides was determined to be 4.410 ± 0.037 kDa by the classic Mark-Houwink Equation.[30] To impart NO-release capabilities, 2-methylaziridine-modified chitosan oligosaccharide scaffolds (COS) were reacted with NO gas at high pressure. The resulting *N*-diazeniumdiolate-modified chitosan oligosaccharides (COS-NO) released a total of 0.78 ± 0.09 μmol NO/mg over a duration of 10.7 ± 1.1 h in PBS (pH 6.5, 37 °C) (Figure 3.1, Table 3.1). The half-life of NO release was 0.62 ± 0.08 h.

3.3.1 Viscoelastic properties of P. aeruginosa biofilms

Fluorescent tracer particles were incorporated into *P. aeruginosa* biofilms during biofilm formation to determine the viscoelastic properties of bacterial biofilms (Figure 3.2). Tracer particles within the biofilms exhibited decreased diffusion compared to tracer particles in solutions of planktonic bacteria (Figure 3.3). The diffusion exponent (α) was derived to approximate viscoelasticity according to $MSD \propto t^{\alpha}$. Through this relationship, purely viscous solutions are defined as having diffusion exponents of one, while purely elastic solids exhibit diffusion coefficients of zero. Therefore, diffusion coefficients of viscoelastic materials range from zero to one.[36] The displacement of tracer particles in solutions of planktonic bacteria increased linearly

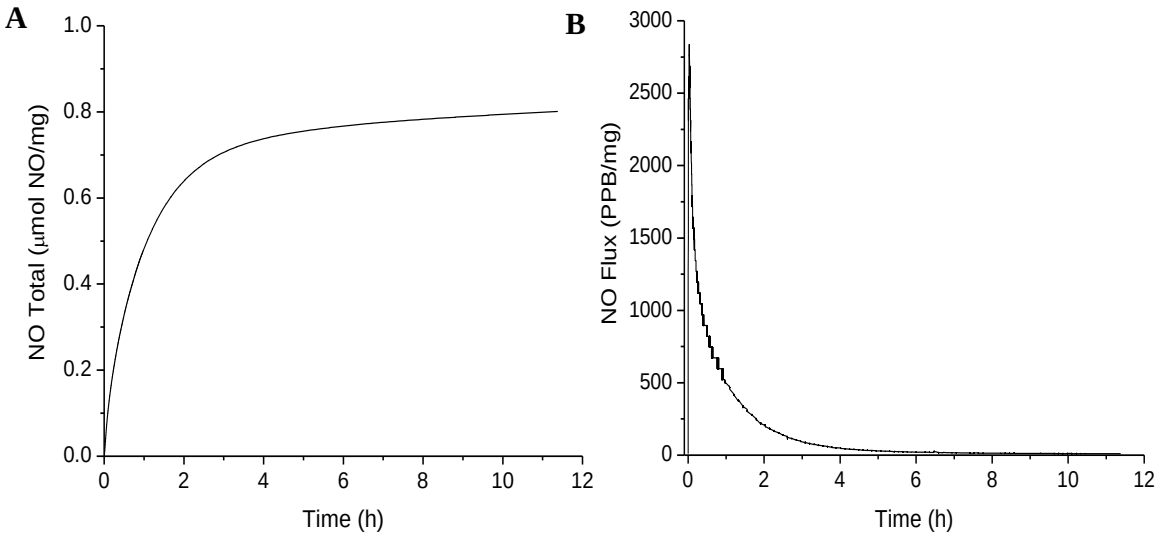

Figure 3.1 Chemiluminescence detection of NO release from COS-NO. The NO release from 1 mg of COS-NO was determined in deoxygenated PBS (pH 6.5) for comparison with other NO-releasing systems. The (A) NO total and (B) NO flux of representative measurements are shown.

Table 3.1 Nitric oxide-release properties of COS-NO in PBS (pH 6.5, 37 °C) as determined by chemiluminescence detection. Values are presented as means ± standard deviations for n=3 pooled experiments.

NO Total (μmol NO/mg COS-NO)	Duration (h)	Half-Life (h)	Max Flux (PPB/mg)
0.78 ± 0.09	10.7 ± 1.1	0.62 ± 0.08	3200 ± 600

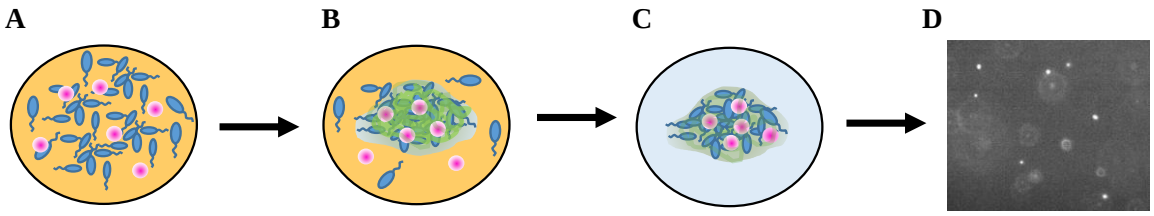

Figure 3.2 Incorporation of fluorescent tracer particles in *P. aeruginosa* biofilms. (A) Planktonic bacteria (10^6 CFU/mL) and fluorescent tracer particles were incubated in 25% LB at pH 6.5. (B) After 72 h of growth at 37 °C, microcolony biofilms formed with tracer particles embedded throughout the biofilm. (C) Biofilms were washed in PBS and exposed to antibacterial treatments for 18 h prior to (D) analysis by particle tracking microrheology.

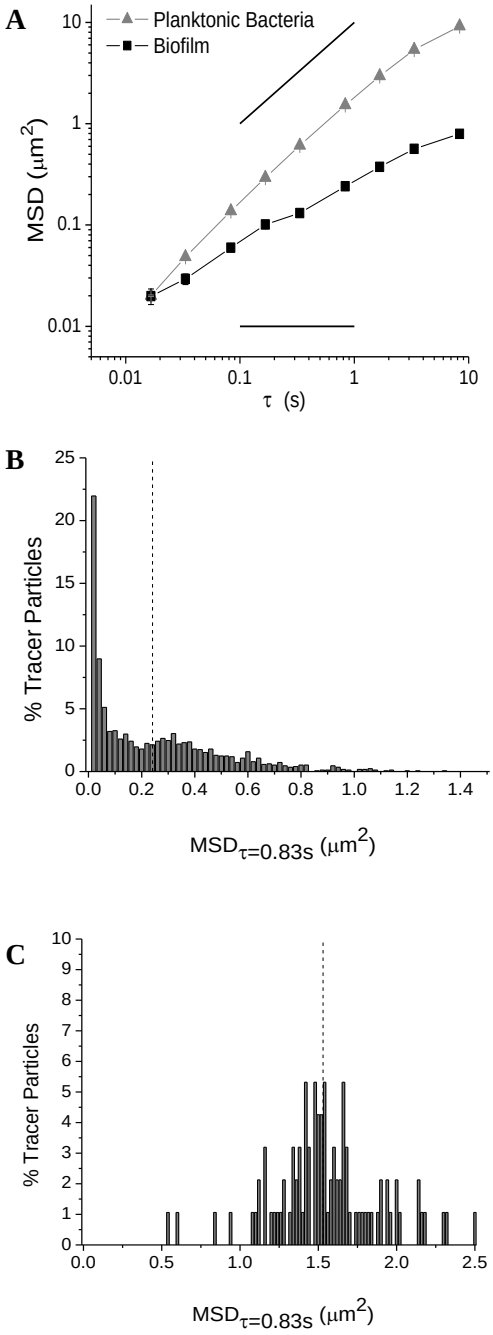

Figure 3.3 Microrheology of *P. aeruginosa* biofilms. (A) Ensemble average MSD of tracer particles in solutions of planktonic bacteria and biofilms show incorporation of particles into the biofilm. Diffusion coefficients of viscous solutions ($\alpha = 1$) and elastic solids ($\alpha = 0$) are superimposed on MSD plots for reference. Distributions of $MSD_{\tau=0.83s}$ for tracer particles in (B) biofilms compared to (C) solutions of planktonic bacteria. The mean of each distribution is marked with a vertical dotted line.

with time ($\alpha = 1.05 \pm 0.13$), indicating that they were moving in a purely viscous solution. In contrast, the measured slope for tracer particles within *P. aeruginosa* biofilms was significantly lower ($\alpha = 0.62 \pm 0.14$), indicating that the medium surrounding the particles was both viscous and elastic. *P. aeruginosa* biofilms were thus determined to be viscoelastic materials with properties distinct from solutions of planktonic bacteria.

Histograms were used to visualize the distribution of the $MSD_{\tau=0.83s}$ and analyze biofilm heterogeneity. The $MSD_{\tau=0.83s}$ of tracer particles within *P. aeruginosa* biofilms were highly heterogeneous, as indicated by the non-Gaussian distribution of $MSD_{\tau=0.83s}$ (Figure 3.3 B). The average $MSD_{\tau=0.83s}$ was heavily influenced by fast moving particles with the mean (0.24 ± 0.01 μm^2) substantially greater than the mode ($0.00 - 0.02$ μm^2). This specific type of heterogeneity indicates that some tracer particle were moving inside large pores or water channels within the biofilms.[37] Tracer particles in solutions of planktonic bacteria exhibited a Gaussian distribution of $MSD_{\tau=0.83s}$ with mean (1.53 ± 0.04 μm^2) and mode ($1.40 - 1.68$ μm^2) approximately equal (Figure 3.3 C).

3.3.2 Tobramycin treatment of P. aeruginosa biofilms

As tobramycin is the current standard for the treatment of *P. aeruginosa* infections in CF patients,[8-9] the effects of tobramycin on the mechanical properties of *P. aeruginosa* biofilms were determined as a comparison to NO therapy. Tobramycin was found to eradicate biofilms (i.e. to reduce bacteria viability by 5 logs over 18 h) at a concentration of 200 µg/mL (Figure 3.4 A.).

The MSD plots of biofilms treated with tobramycin appeared similar to untreated controls, indicating that tobramycin did not alter the viscoelastic properties of the biofilm (Figure 3.3 B). Over a timescale of 3 decades (0.017 – 3.33 s), no significant differences in the diffusion coefficients of untreated and tobramycin-treated biofilms were observed (Table 3.2). While the

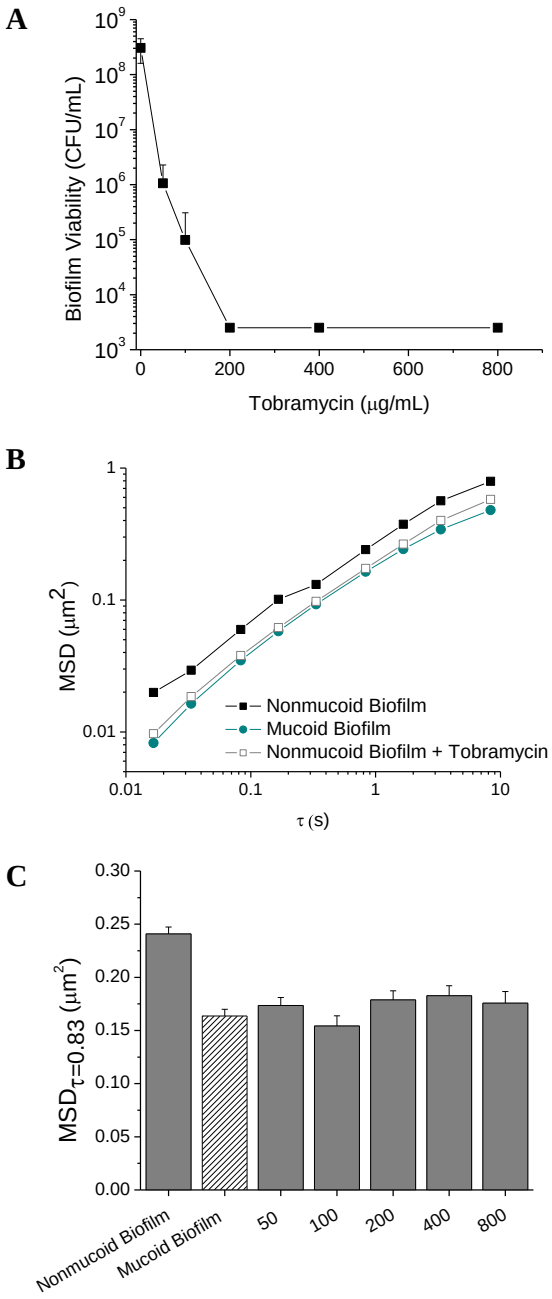

Figure 3.4 Treatment of *P. aeruginosa* biofilms with tobramycin. (A) Biofilm viability after exposure to tobramycin for 18 h. The MBEC of tobramycin was 200 μg/mL. (B) MSD values of tracer particles in biofilms grown with nomucoid (motile) *P. aeruginosa*, mucoid (non-motile*) P. aeruginosa*, and nonmucoid *P. aeruginosa* treated with 50 μg/mL of tobramycin. (C) MSDτ=0.83s untreated nonmucoid and mucoid *P. aeruginosa* biofilms as well as nonmucoid *P. aeruginosa* biofilms exposed to tobramycin for 18 h. All MSD were determined by tracking particles in 15 distinct areas of 3 separate biofilms and are plotted as ensemble average MSD ± SEM.

temporal dependency of particle diffusion was unchanged by tobramycin treatment, the magnitude of the MSD was reduced at all time points for treated biofilms. This decrease was independent of tobramycin concentration (Figure 3.4 B). For tobramycin-treated biofilms, the $MSD_{\tau=0.83}$ ranged from 0.154 ± 0.009 μm^2 to 0.183 ± 0.009 μm^2, compared to 0.241 ± 0.007 μm^2 for untreated biofilms (Figure 3.4 C). We hypothesized that the decrease in MSD magnitude was due to loss of bacteria motility upon treatment with tobramycin. Unlike traditional macro- and microrheology, which solely measure the bulk viscoelastic properties of a material, multiple-particle tracking microrheology quantifies the movement of particles within the biofilms. Tracer particle movement is influenced by the movement (i.e. swimming or motility) of bacteria as well as the viscoelastic properties of the biofilm matrix. Therefore, cessation of bacteria motility upon killing would reduce the MSD of particles within the biofilm.

To confirm that the decrease in MSD was related to reduced bacteria motility, biofilms were grown with a strain of non-motile, mucoid *P. aeruginosa*.[38] The magnitude of the MSD of tracer particles in biofilms comprised of non-motile bacteria (i.e. bacteria that are mucoid or have lost motility due to tobramycin-induced cell death) was indeed lower than that of tracer particles in biofilms of motile bacteria (Figure 3.4 B). The diffusion coefficient of mucoid biofilms was 0.70 ± 0.17, similar to the tobramycin-treated biofilms (range 0.56 ± 0.02 to 0.70 ± 0.12) (Table 3.2). While biofilms grown from mucoid *P. aeruginosa* have a different matrix composition than the nonmucoid strain, the similar diffusion coefficients for all three biofilms (i.e. nonmucoid, mucoid, and tobramycin-treated) indicated that the reduction in $MSD_{\tau=0.83}$ magnitude upon treatment with tobramycin was the result of bacteria motility loss, and not tobramycin-induced alterations in the biofilm viscoelasticity. The decrease in the $MSD_{\tau=0.83}$ was independent of tobramycin concentration over the range of ¼ – 4x the MBEC (Figure 3.4 C). At the lowest

Table 3.2. Diffusion exponents (α-values) of *P. aeruginosa* biofilms treated with tobramycin.

Tobramycin (μg/mL)	α
0	0.62 ± 0.14
50	0.70 ± 0.12
100	0.56 ± 0.02
200	0.66 ± 0.08
400	0.68 ± 0.10
800	0.59 ± 0.06

concentration tested (50 μg/mL), ~99% of the bacteria in the biofilm were no longer viable and therefore lacked motility. Therefore, the decrease in bacteria viability was substantial enough to alter the $MSD_{\tau=0.83}$ of tracer particles within the tobramycin-treated biofilms, even at the lowest concentration tested. Overall, the bacteria motility accounted for 31.8 ± 1.6% of the $MSD_{\tau=0.83}$ based on the decrease in the magnitude of $MSD_{\tau=0.83}$ of tobramycin-treated and mucoid *P. aeruginosa* biofilms

3.3.3 Treatment of P. aeruginosa biofilms with COS and COS-NO

At the largest concentration tested (16 mg/mL), the COS scaffold decreased the bacteria viability by three logs without fully eradicating the *P. aeruginosa* biofilm (Figure 3.5 A). While chitosan in general is a known antimicrobial,[39] the anti-biofilm efficacy of the chitosan oligosaccharides was significantly reduced, as has previously been demonstrated.[40] Addition of NO to the COS scaffold significantly improved of anti-biofilm activity, eradicating biofilms at 4 mg/mL, which corresponds to a bactericidal NO dose of 3.1 ± 0.4 μmol/mL (Figure 3.5 A).

Despite low biofilm eradication capabilities, the COS scaffold significantly altered the viscoelasticity of *P. aeruginosa* biofilms. Treatment of biofilms with 2 mg/mL COS decreased the magnitude of the $MSD_{\tau=0.83s}$ by two logs (to 0.0031 ± 0.0002 μm^2) compared to untreated controls ($MSD_{\tau=0.83s}$ = 0.241 ± 0.007 μm^2), with no further reduction in the $MSD_{\tau=0.83}$ when the concentration of COS was increased to 16 mg/mL (Figure 3.5 B). While the MSD (at all τ) of untreated biofilms exhibited a temporal dependency (revealing partial viscosity), treatment with COS over the concentration range of 1 – 16 mg/mL eliminated this time dependence (Figure 3.5 C). The diffusion coefficients of COS treated biofilms ranged from 0.03 ± 0.02 to 0.14 ± 0.02, a significant reduction compared to untreated biofilms (0.62 ± 0.14) (Table 3.3). Treatment with

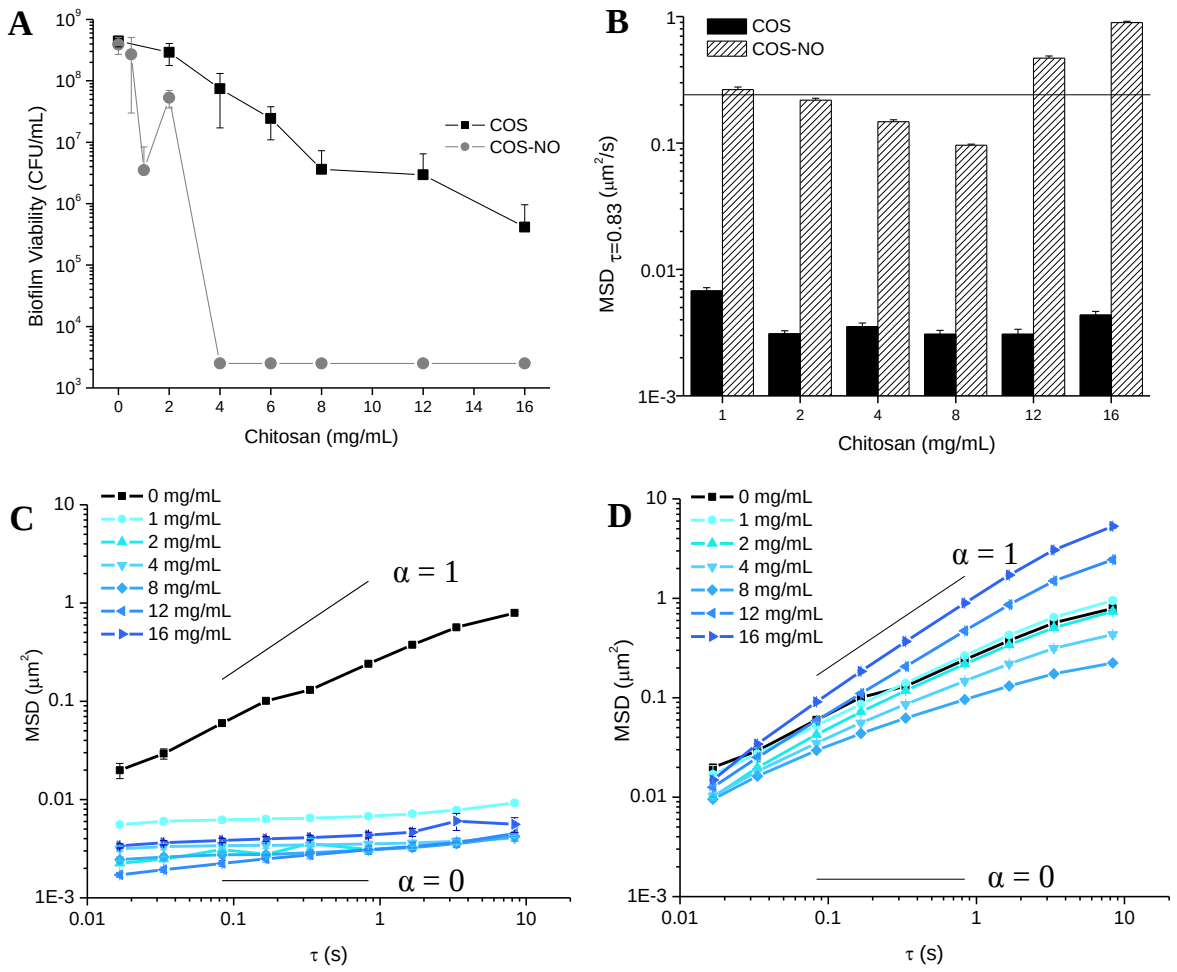

Figure 3.5 Treatment of *P. aeruginosa* biofilms with COS and COS-NO. (A) Biofilm viability following exposure to COS or COS-NO for 18 h. While COS did not fully eradicate biofilms, the MBEC of COS-NO was 4 mg/mL (NO dose of 3.1 ± 0.4 µmol). (B) $MSD_{\tau=0.83s}$ of biofilms treated with COS and COS-NO. The $MSD_{\tau=0.83s}$ of untreated biofilms is indicated as a horizontal line. Note the logarithmic scale of the y-axis. (C) The MSD values of biofilms treated with COS and (D) COS-NO. All MSD were determined by tracking particles in 15 distinct areas of 3 separate biofilms and are plotted as ensemble average MSD ± SEM. Diffusion coefficients of viscous solutions (α = 1) and elastic solids (α = 0) are superimposed on MSD plots for reference.

Table 3.3 Diffusion exponents (α) of *P. aeruginosa* biofilms treated with COS and COS-NO. The total NO released from COS-NO at each concentration is also provided.

Dose (mg/mL)	COS α	COS-NO α	NO μmol/mL
0	0.62 ± 0.14	0.62 ± 0.14	n/a
1	0.07 ± 0.04	0.68 ± 0.04	0.8 ± 0.1
2	0.10 ± 0.20	0.74 ± 0.14	1.6 ± 0.2
4	0.03 ± 0.02	0.64 ± 0.10	3.1 ± 0.4
8	0.07 ± 0.04	0.55 ± 0.13	6.2 ± 0.7
12	0.14 ± 0.02	0.91 ± 0.06	9.4 ± 1.1
16	0.11 ± 0.12	1.00 ± 0.11	12.5 ± 1.4

COS clearly transformed *P. aeruginosa* biofilms into nearly elastic solids with almost no viscous component.

While the COS scaffold decreased both the $MSD_{\tau=0.83s}$ and diffusion coefficients of tracer particles within *P. aeruginosa* biofilms at all concentrations, NO-releasing COS (i.e., COS-NO) induced a dose-dependent response in biofilm viscoelasticity. Both the $MSD_{\tau=0.83s}$ and diffusion coefficients decreased with increasing COS-NO at concentrations ≤ 8 mg/mL (Figure 3.5 B**,** Table 3.3). Tracer particles in biofilms exposed to COS-NO exhibited substantially higher $MSD_{\tau=0.83s}$ and diffusion coefficients than those in biofilms exposed to the same concentrations of the COS scaffold (Figure 3.5 B). At concentrations ≥ 12 mg/mL COS-NO, both the $MSD_{\tau=0.83s}$ and diffusion coefficients increased substantially above those of untreated biofilms (Figure 3.5 B, Table 3.3). The increase in both of these parameters indicates degradation of the viscoelastic properties of the biofilm. As biofilm degradation occurred above the MBEC of COS-NO (4 mg/mL), destruction of the physical properties of the biofilm required a larger dose of NO (9.4 ± 1.1 μmol/mL) than biofilm eradication (3.1 ± 0.4 μmol/mL).

3.3.4 Heterogeneity of treated P. aeruginosa biofilms

Sample heterogeneity has been correlated with increased viscoelasticity for complex biological materials such as sputum.[33,41] The effect of antibacterial treatment on biofilm heterogeneity was thus evaluated at concentrations in excess of the MBEC. As expected, tobramycin had no observable effect on the heterogeneity of biofilms. While tobramycin treatment decreased the average $MSD_{\tau=0.83s}$, concentrations of tobramycin in excess of the MBEC did not alter the shape of the $MSD_{\tau=0.83s}$ distributions (Figure 3.6 A-C) compared to untreated biofilms (Figure 3.3-B);

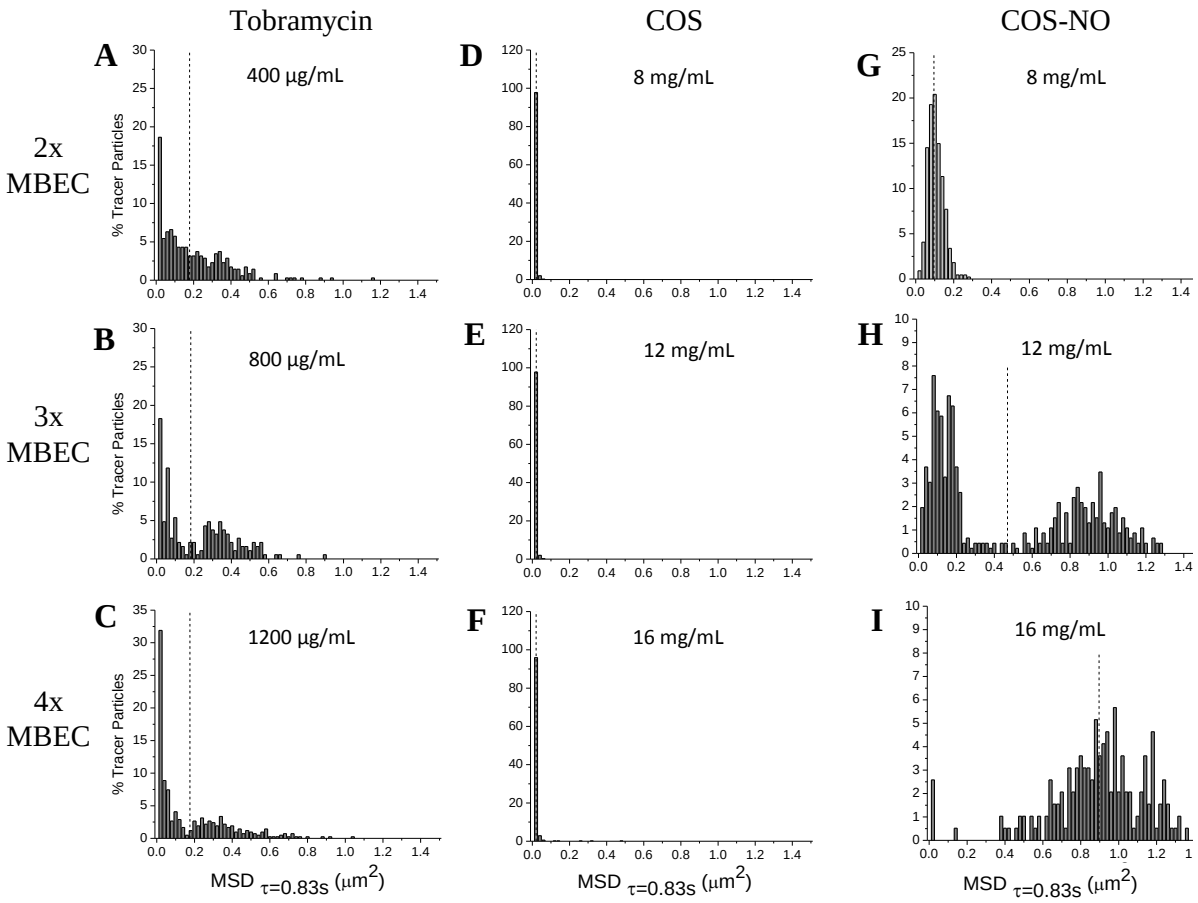

Figure 3.6. Histograms of biofilm heterogeneity after treatment. The distributions of $MSD_{\tau=0.83s}$ of individual tracer particles in (A-C) biofilms treated with tobramycin, (D-F) COS, and (G-I) COS-NO at concentrations above the MBEC values. The mean of each distribution is marked with a vertical dotted line.

both exhibited $MSD_{\tau=0.83s}$ distributions skewed such that the modes were substantially lower than the mean.

Treatment with COS and COS-NO resulted in opposing effects on the $MSD_{\tau=0.83s}$ distributions of *P. aeruginosa* biofilms. The COS scaffold constrained tracer particle movement, decreasing the width of the $MSD_{\tau=0.83s}$ distribution and reducing biofilm heterogeneity (Figure 3.6 D-F). Alternatively, treatment with COS-NO at concentrations above the MBEC resulted in degradation of the biofilm and increased heterogeneity. Treatment with 8 mg/mL COS-NO showed little alteration in the $MSD_{\tau=0.83s}$ distribution (Figure 3.6 G) compared to untreated controls (Figure 3.3-B). However, exposure to 12 mg/mL COS-NO resulted in a bimodal $MSD_{\tau=0.83s}$ distribution (Figure 3.3-H). When treated with 16 mg/mL COS-NO, the $MSD_{\tau=0.83s}$ distribution was Gaussian and centered around 1 μm^2 (Figure 3.6-I).

3.3.5 Quantification of biofilm contraction

The average distance between nearest neighbor particles was determined to quantify biofilm expansion and contraction after treatment. For untreated biofilms, the average distance between nearest neighbor particles was 21.7 ± 0.8 μm (indicated by the horizontal line in Figure 3.7). At concentrations ranging from ½ to 4-times the MBEC, tobramycin exposure did not alter the average distance between nearest neighbor particles compared to the untreated biofilm (Figure 3.7 A), indicating that tobramycin treatment had little effect on the contraction or expansion of the biofilm.

In contrast, COS and COS-NO treatment resulted in significant alterations to the biofilm structure. The COS scaffold reduced the average distance between nearest neighbor tracer particles compared to untreated biofilms with ~50% biofilm contraction that was independent of COS concentration from 1 – 16 mg/mL (Figure 3.7 B). Treatment with COS-NO exhibited a

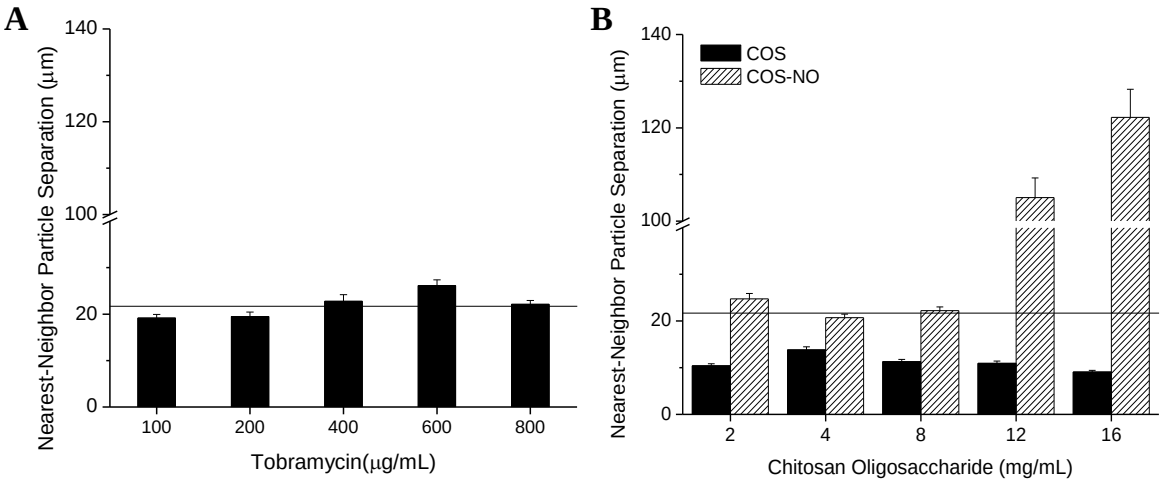

Figure 3.7 Average distances between nearest neighbor particles following treatment with (A) tobramycin and (B) chitosan oligosaccharides. Average separation between tracer particles in untreated biofilms is indicated by a horizontal line. All separation distances were quantified in 15 distinct areas of 3 separate biofilms and are plotted as ensemble average MSD ± SEM.

dose-dependent response on tracer particle separation (Figure 3.7 B). At concentrations below 8 mg/mL, treatment with COS-NO had no effect on the distance between nearest neighbor tracer particles. At larger concentrations (i.e. ≥ 12 mg/mL), the average particle separation increased significantly. As expected, tracer particle separation increased as the biofilm degraded. The concentration of COS-NO resulting in increased separation of nearest neighbor particles correlated with the concentration required for partial destruction of the viscoelastic properties of the biofilm (12 mg/mL).

3.6 Discussion

An ideal treatment for *P. aeruginosa* biofilms in CF patients would reduce bacteria viability while physically disrupting the biofilm in order to ease biofilm removal and prevent regrowth. As such, it is important to characterize the effects of antibacterial therapeutics on the viscoelastic properties of *P. aeruginosa* biofilms. While highly effective at eradicating bacteria, tobramycin does not alter the biophysical properties of *P. aeruginosa* biofilms. Indeed, our results indicate that treatment with tobramycin neither alters the distance between nearest neighbor tracer particles (Figure 3.7) nor the diffusion coefficient of tracer particles within biofilms (Table 3.2). Of note, a decrease in particle diffusion (i.e., the MSD at all τ) was observed and attributed to the loss of bacteria motility rather than alterations in the physical properties of the biofilm (Figure 3.4).

In comparison to tobramycin, NO released from the chitosan oligosaccharides both eradicated bacteria and degraded the biofilm physical properties (Figure 3.5). At concentrations above the MBEC (i.e., ≥ 12 mg/mL), the COS-NO significantly compromised the structural integrity of the biofilms, as evidenced by increases in the $MSD_{\tau=0.83s}$ and diffusion coefficients of tracer particles within treated biofilms (Figure 3.5, Table 3.3). The increased tracer particle

movement may be attributed to either an overall loosening of the biofilm or heterogeneous destruction of the biofilm matrix. In the case of uniform biofilm loosening, the diffusion of all particles would increase, shifting $MSD_{\tau=0.83s}$ distributions to larger values without changing the overall shape of the distribution. However, the observed bimodal distribution is consistent with destruction of discrete segments of the biofilm, where some of the tracer particles remained constrained in the biofilm while a second population of tracer particles experienced increased diffusion outside of the biofilm (Figure 3.6-H). Further increasing the concentration of COS-NO resulted a Gaussian $MSD_{\tau=0.83s}$ distribution centered around 1 μm^2, suggestive of complete biofilm destruction and free diffusion of the tracer particles in PBS (Figure 3.6-I). These results indicate that NO does not cause a gradual loosening of the biofilm but rather destroys segments of the biofilms and is capable of complete biofilm destruction.

While the mechanism of NO-mediated destruction of the biofilm is likely complex (i.e. due to multiple factors), NO has been shown to alter the biological macromolecules that constitute the majority of the biofilm matrix.[21,23-24,27] For example, DNA is essential to the formation of *P. aeruginosa* biofilm[42] with cleavage of DNA by DNase decreasing the structural integrity of such biofilms.[43] As in vitro studies have shown that exogenous NO damages and cleaves DNA,[44-45] it is likely that NO-mediated destruction of DNA would physically degrade *P. aeruginosa* biofilms. Nitric oxide and its reactive intermediates also depolymerize polysaccharides,[46] which may further explain NO's ability to reduce biofilm viscoelasticity through damage to alginate, Psl, and Pel structural biofilm polysaccharides.[47]

While NO represents a promising therapeutic for the physical degradation of *P. aeruginosa* biofilms, it is currently limited by the effects of the COS scaffold as the chitosan treatment alone results in biofilm contraction regardless of concentration (0.5 – 16 mg/mL). Such contraction

(Figure 3.7) parallels increased biofilm elasticity and decreased particle diffusion (Table 3.3, Figure 3.5), which correlates to physical entanglement of polymers and effective cross-linking (e.g. covalent or hydrogen bonding, electrostatic interactions) in both ideal polymer and biological systems.[47-49] As the *P. aeruginosa* biofilm matrix is comprised predominantly of anionic macromolecules,[23] the introduction of cationic chitosan alters the electrostatics of the biofilm and potentially enhances cross-linking. To further improve the efficacy these NO-releasing therapeutics, scaffolds that do not increase the elasticity of bacterial biofilms should be investigated. As the cationic nature of chitosan likely contributes to biofilm cohesion, modifying the chitosan oligosaccharide scaffold with anionic moieties may reduce these effects. Furthermore, the biofilm disrupting capabilities of NO-releasing chitosan oligosaccharides may be improved by adjusting the NO payload. For example, increasing the NO storage should reduce the concentration of COS-NO required for biofilm degradation, thereby minimizing any underlying effects of the scaffold.

3.7 Conclusions

This work describes the ability of NO-releasing chitosan oligosaccharides to act as dual-functioning therapeutics which eradicate and physically degrade *P. aeruginosa* biofilms. At concentrations in excess of those required for eradication, NO segmentally destroyed *P. aeruginosa* biofilms. As the physical properties of biofilms are related to antibiotic efficacy and potential for regrowth, the ability to physical degrade the bacterial biofilms represents a significant improvement over tobramycin, which failed to alter biofilm viscoelasticity. The chitosan oligosaccharides used as NO-donor vehicles actually enhanced biofilm elasticity, potentially mitigating the biofilm degrading activity of NO. Therefore, future studies investigating the biophysical effects of NO on biofilms should utilize more inert scaffolds.

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CHAPTER 4: NITRIC OXIDE-RELEASING CHITOSAN OLIGOSACCHARIDES AS MUCOLYTIC AGENTS

4.1 Introduction

Cystic fibrosis disease pathogenesis is primarily dictated by the viscoelastic properties of airway mucus. Dysfunction of the cystic fibrosis transmembrane regulator (CFTR) protein results in sodium hyperabsorption and subsequent airway dehydration, ultimately leading to inhibited mucociliary clearance.[1] Accumulation of a concentrated mucus layer with increased viscosity and elasticity cultivates bacterial infections.[2-3] While antibiotic treatments attempt to eradicate bacteria in the airways, mucolytic agents are designed to prevent bacterial colonization by disrupting the stagnant mucus layer, thereby improving clearance.[4] Conventional mucolytics such as L-cysteine, *N*-acetylcysteine (NAC), and dithiothreitol improve clearance by reducing mucin disulfide bonds, thus decreasing entanglement of high molecular weight mucins.[5-6] The most popular thiol-based mucolytic, NAC, has proven to reduce mucus viscoelasticity by this pathway both in vitro[7-9] and in vivo.[10] Unfortunately, improvements in lung function have not been demonstrated in clinical trials.[11] The only mucolytic agent currently available for the treatment of CF is dornase alfa, a recombinant human deoxyribonuclease (DNase) enzyme that depolymerizes extracellular DNA in CF mucus. Dornase alpha has been shown to both reduce mucus viscoelasticity[12-14] and improve pulmonary function.[13,15-17]

Unfortunately, dornase alfa is only effective in 10% of CF patients.[18] As such, the development of new mucolytic agents capable of reducing mucus viscosity and elasticity remains

critical. Inhalation of nitric oxide (NO) gas has been proposed as a mucolytic therapeutic.[19] In thoroughbred horses suffering from excess mucus accumulation in the airways, inhalation of NO gas (100 breaths, 5,000-10,000 ppm) resulted in the secretion of non-viscous fluids from the nares.[19] Multiple treatment cycles over several weeks decreased airway mucus accumulation in 25 of 27 horses. Of note, a lack of controls (i.e., measurement of mucus accumulations in non-treated horses) reduced the impact of this study.

While the in vivo efficacy of NO has been demonstrated,[19] the potential mechanisms of NO's mucolytic activity have not been characterized systematically. Disulfide bond cleavage has been proposed as NO reacts with thiols to form *S*-nitrosothiols.[19-20] Nitric oxide also depolymerizes *O*-linked polysaccharides,[21] thereby likely altering the glycosylated domains of mucins. While disruption of the glycosylated domains would alter intra-mucin interactions, the effects of NO on gel formation and mucus viscoelasticity are not clear. Similar to dornase alfa, NO could potentially reduce mucus viscoelasticity by damaging and/or cleaving DNA.[22-23]

Strategies for controllably storing and releasing NO are required to effectively develop NO as a mucolytic therapeutic. Compared to small molecule donors, NO released from macromolecular scaffolds has been demonstrated to improve bactericidal efficacy against both planktonic cultures and biofilms.[24-26] Additionally, continuous NO delivery to the airways may be achieved by intermittent treatment rather than continuous inhalation (i.e., from a pressurized gas cylinder) due to prolonged NO release afforded by macromolecular scaffolds.

Chitosan oligosaccharides have been purposed as scaffolds for pulmonary drug delivery due to their inherent mucoadhesive properties.[27-28] Specifically, primary amines on chitosan oligosaccharides associate with the negatively charged sialic acid residues of mucins via electrostatic interactions.[27,29-30] As drug delivery vehicles, mucoadhesive scaffolds increase the

retention time in the airways by preventing drug removal via coughing or particle mucociliary clearance.[27,31] In fact, chitosan has already been incorporated into antibiotic-releasing particles to enhance pulmonary delivery.[32-36] Alternatively, Klinger-Strobel et al. reported the development of non-mucoadhesive drug delivery scaffolds.[31] Most commonly, polyethylene glycol (PEG) modifications have been used to create non-adhesive or "muco-inert" drug-releasing particles that exhibit improved particle penetration in human cervicovaginal mucus,[37-38] bacterial biofilms,[39] and CF sputum[37-40] via the hydrophilicity and charge neutrality of the PEG moiety. Herein, chitosan oligosaccharides with various mucoadhesive properties but similar NO storage and release properties were synthesized to investigate the role of scaffold mucoadhesion on the mucolytic efficacy of NO-releasing therapeutics.

4.2 Materials and Methods

4.2.1 Materials

Medium molecular weight chitosan (viscosity 200-800 centipoise), 2-methylaziridine, ethyl acrylate, *tert*-butyl acrylate, sulfopropyl acrylate potassium salt, sodium dodecyl sulfate (SDS), dithiothreitol (DTT), bovine serum albumin (BSA), and type II gastric pig mucin (GPM) were purchased from Sigma-Aldrich (St. Louis, MO). Nitric oxide gas was purchased from Praxair (Sanford, NC). Standardized NO (26.85 ppm, balance N_2), Argon (Ar), and nitrogen (N_2) gases were purchased from Airgas National Welders (Durham, NC). Sodium methoxide was purchased from Acros Organics (Geel, Belgium).

Tris-acetate-EDTA (TAE) buffer (10x) was purchased from Mediatech, Inc. (Manassas, VA) and diluted 1:10 in distilled water prior to use. Saline-sodium citrate (SSC) buffer (20x) was purchased from Promega Corporation (Madison, WI) and diluted 1:5 to obtain 4x SSC buffer.

Dulbecco's phosphate buffered saline (DPBS, 1x) was purchase from Life Technologies (Carlsbad, CA). Powdered milk (Drink 'n Mix) was purchased from Walmart (Durham, NC). Neutral buffered formalin (NBF, 10 vol.%) was purchased from Sigma Aldrich (St. Louis, MO). Phosphate buffer (10 mM, pH 6.5) was prepared in house using common laboratory salts and reagents.

Anti-MUC5B antibody (H-300) was purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-MUC5AC antibody (45M1) was purchased from Abcam (Cambridge, MA). Secondary antibodies (IRDye 800CW Donkey anti-Mouse IgG and IRDye 680RD Donkey anti-Rabbit IgG) were purchased from LI-COR Biosciences (Lincoln, NE). 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Life Technologies (Carlsbad, CA).

Distilled water was purified using a Millipore Milli-Q UV Gradient A-10 system (Bedford, MA). All common laboratory salts and reagents were purchased from Fisher Scientific (Pittsburgh, PA). All materials were used without further purification unless otherwise specified.

Mucus was harvested from primary human bronchial epithelial (HBE) cell cultures as previously described.[41] Briefly, primary cell cultures obtained from excess surgical tissue (UNC Chapel Hill Tissue Core Facility) were grown on 0.5 mm pore-sized Millicell cell culture inserts (Millipore, Bedford, MA) in air-liquid interface media (UNC Chapel Hill Tissue Core Facility) for a minimum of 6 weeks until the cultures developed cilia, and well-defined periciliary liquid (PCL) and mucus layers. Mucins were harvested from one continuously grown primary cell culture at distinct time points and stored at 3 °C prior to use.

Sputum samples were collected from CF patients by spontaneous expectoration. All studied were approved by the UNC Chapel Hill Institutional Review Board with informed consent obtained from all subjects. All samples were stored in sterile containers at 3 °C prior to use.

4.2.2 Synthesis of 2-methylaziridine modified chitosan oligosaccharides

Polymeric chitosan was oxidatively degraded into chitosan oligosaccharides as previously described.[42] Briefly, medium molecular weight chitosan (2.5 g) was dissolved in 15 wt. % hydrogen peroxide (50 mL) and stirred at 85 °C for 1 h. Insoluble, non-degraded chitosan was removed by filtration. Water-soluble oligosaccharides were collected via precipitation in acetone, washed copiously with ethanol, and dried in vacuo. A Ubbelohde viscometer was used to measure the viscosity of the chitosan oligosaccharides in a solution of sodium chloride (0.20 M) and acetic acid (0.10 M) at 25 °C. The molecular weight was calculated to be 4.410 ± 0.037 kD using the classic Mark-Houwink equation ($\eta = 1.81 \times 10^{-3} M^{0.93}$).[43]

The water-soluble chitosan oligosaccharides were then modified with 2-methylaziridine (Figure 4.1). Chitosan oligosaccharides (0.50 g) were dissolved in water (10.00 mL). While stirring, hydrochloric acid (12.1 M, 27.5 µL), water (250 µL), and 2-methylaziridine (178 µL, 1:1 molar ratio to primary amines on the unmodified chitosan oligosaccharide) was added to the solution followed by stirring for 5 d at 25 °C and 24 h at 85 °C. The resulting 2-methylaziridine-modified chitosan oligosaccharides (COS) were precipitated in acetone, washed with methanol to remove excess 2-methylaziridine, and dried in vacuo.

4.2.3 Acrylate modifications of chitosan oligosaccharides

The mucoadhesive properties of COS were altered by the Michael addition of acrylates to the amino groups (Figure 4.1). Ethyl acrylate (EA, 2.08 mL) and tert-butyl acrylate (TBuA, 2.78 mL) were added to COS (500 mg) in a solution of water (6.00 mL), methanol (14.00 mL), ammonium hydroxide (1.00 mL). Methanol was excluded from the reaction solvent for the addition of sulfopropyl acrylate (SPA) as it is water soluble. Therefore, SPA (4.43 g) was added to COS (500 mg) in a solution of water (20.00 mL) and ammonium hydroxide (1.00 mL). A

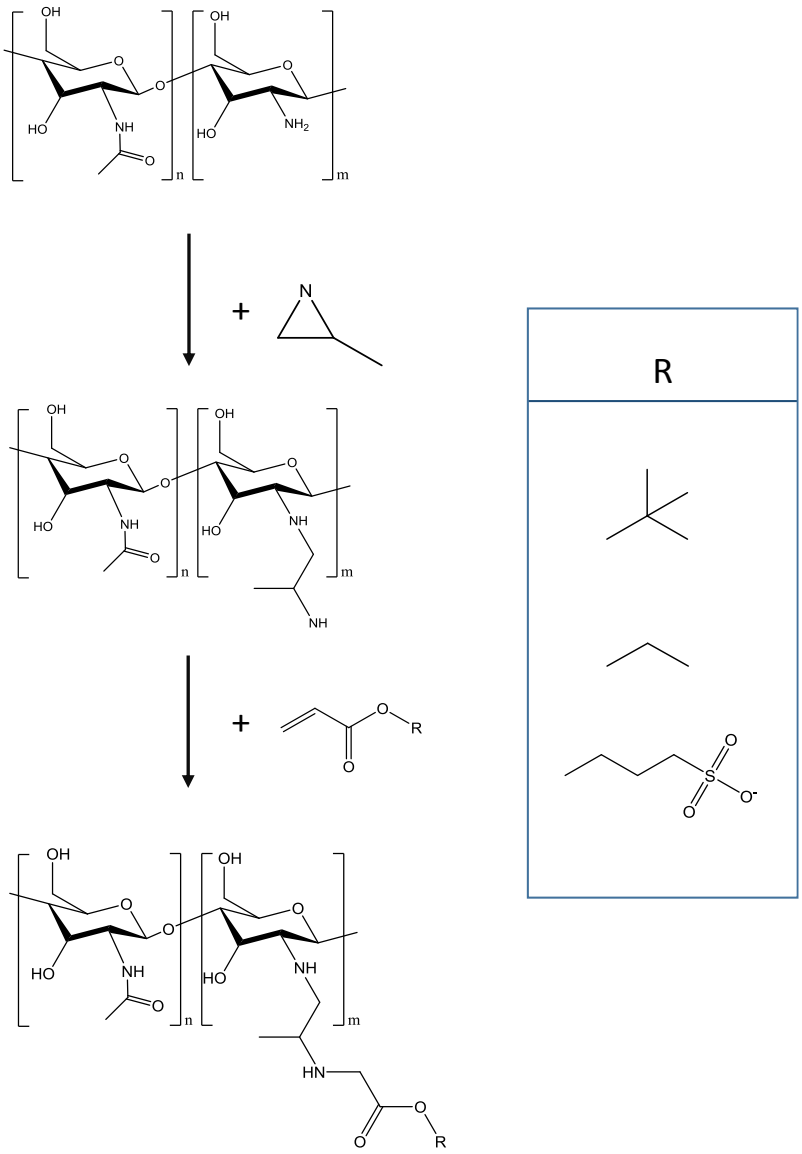

Figure 4.1 Chitosan oligosaccharide modification with 2-methylaziridine and subsequent functionalization with acrylates.

10-fold molar excess of acrylate (vs. primary amine) was used in all reactions to maximize the efficiency of the acrylate addition. After 72 h, acrylate-modified COS was precipitated with acetone, collected via centrifugation, and washed with methanol to remove excess reagent. As SPA is not soluble in methanol, this product was washed copiously with ethanol. The resulting EA-, TBuA-, and SPA-modified COS (COS-EA, COS-TBuA, and COS-SPA, respectively) were dried in vacuo overnight and stored at room temperature. Removal of unreacted acrylate was verified by the disappearance of the vinyl protons for the products in ^{1}H NMR spectra.

4.2.4 ^{1}H NMR characterization

Acrylate-modified COS were characterized by ^{1}H NMR to determine the degree of substitution and product purity. Modified COS products (6.0 mg) were dissolved in D_2O (600 µL), followed by the collection of NMR spectra using a Bruker 400 MHz DRX spectrometer. Representative spectra with peak designations of COS, COS-EA, COS-TBuA, and COS-SPA are provided in Figures 4.2-4.5. As described previously,[44] the degree of substitution was determined from the peak area of δ 4.1 ppm (s, OC*H*$_2$CH$_3$ of EA), 1.3 ppm (br, OCC$_3$*H*$_9$ of TBuA), and 4.1 ppm (s, OC*H*$_2$CH$_2$CH$_2$SO$_3^-$ of SPA) against 4.4 ppm (s, OC*H*(CHNH$_2$)O of COS) for COS-EA, COS-TBuA, and COS-SPA, respectively. All calculations for the degree of substitution were the deacetylation level of the oligosaccharide backbone (85% as reported by manufacturer), as the *N*-acetylglucosamine monomer (15%) does not facilitate modification.

4.2.5 Synthesis of NO-releasing chitosan oligosaccharides

To impart NO storage and release, *N*-diazeniumdiolate NO donors were formed on the secondary amines of the COS and acrylate-modified COS.[42] Modified chitosan oligosaccharides (15 mg) were dissolved in a solution of water (300 µL), methanol (700 µL) and 5.4 M sodium

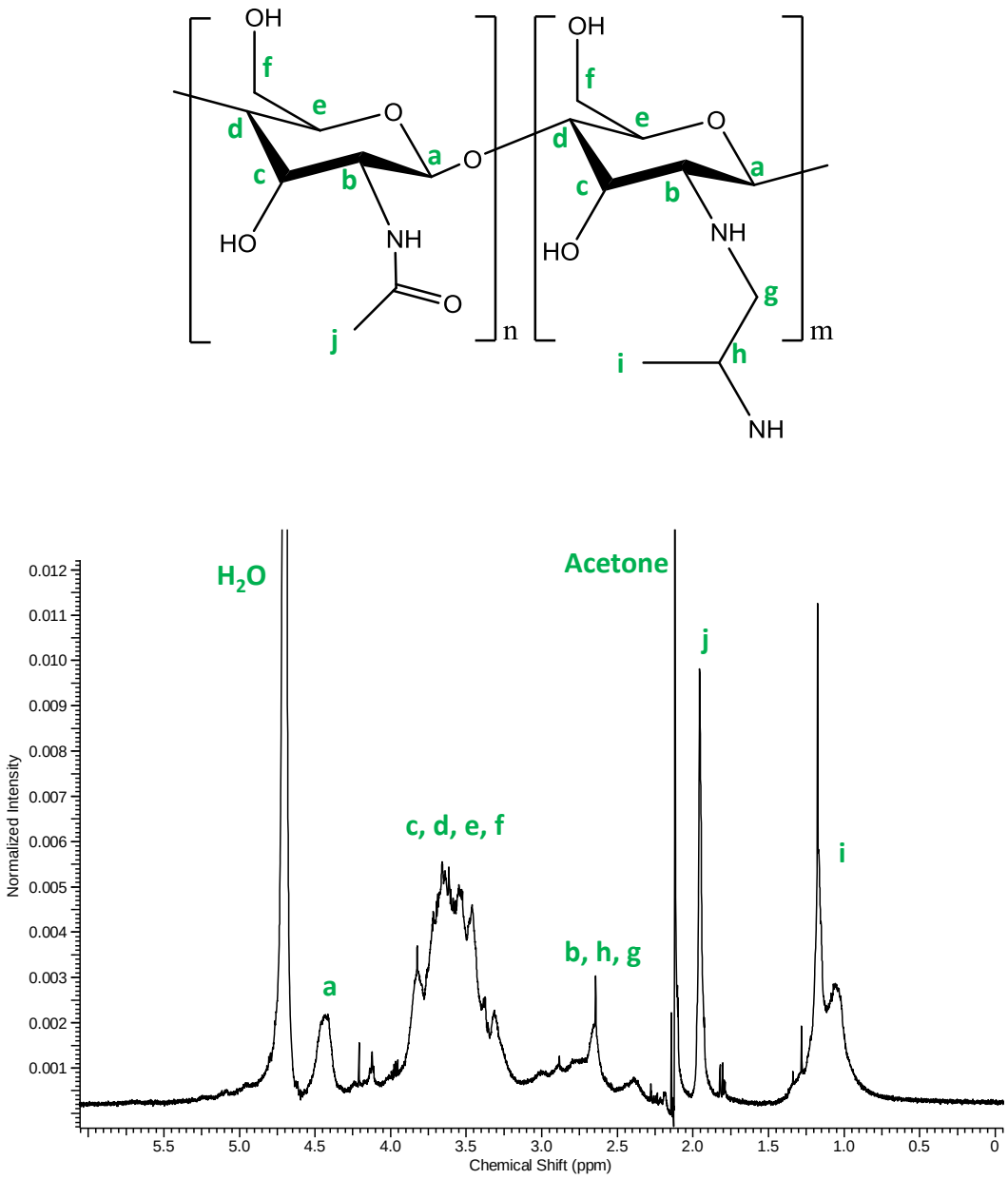

Figure 4.2 ^{1}H NMR Characterization of COS.

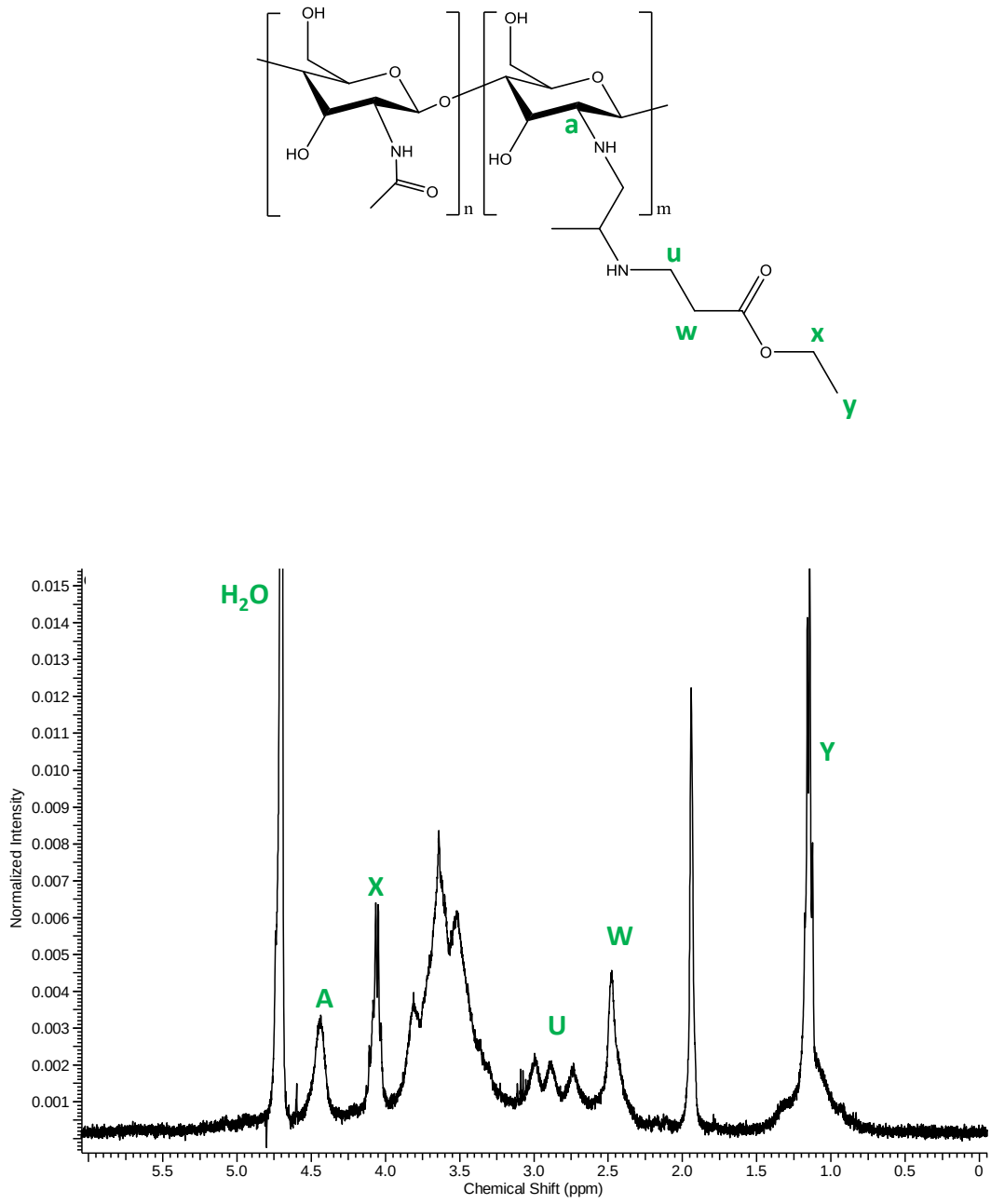

Figure 4.3 ^{1}H NMR Characterization of COS-EA. The degree of substitution is characterized by integration of the peak X against peak A.

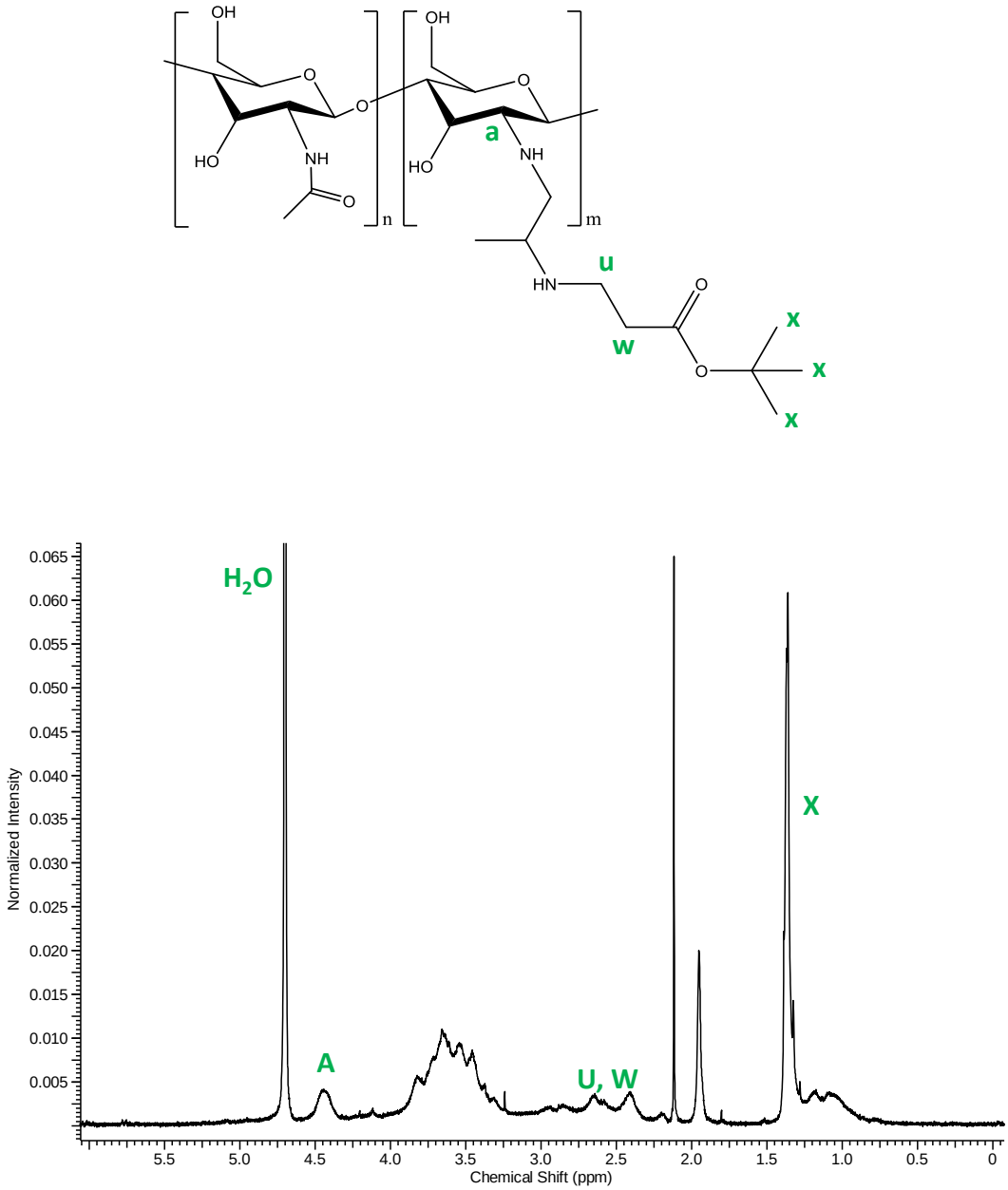

Figure 4.4 ^{1}H NMR Characterization of COS-TBuA. The degree of substitution is characterized by integration of the peak X against peak A.

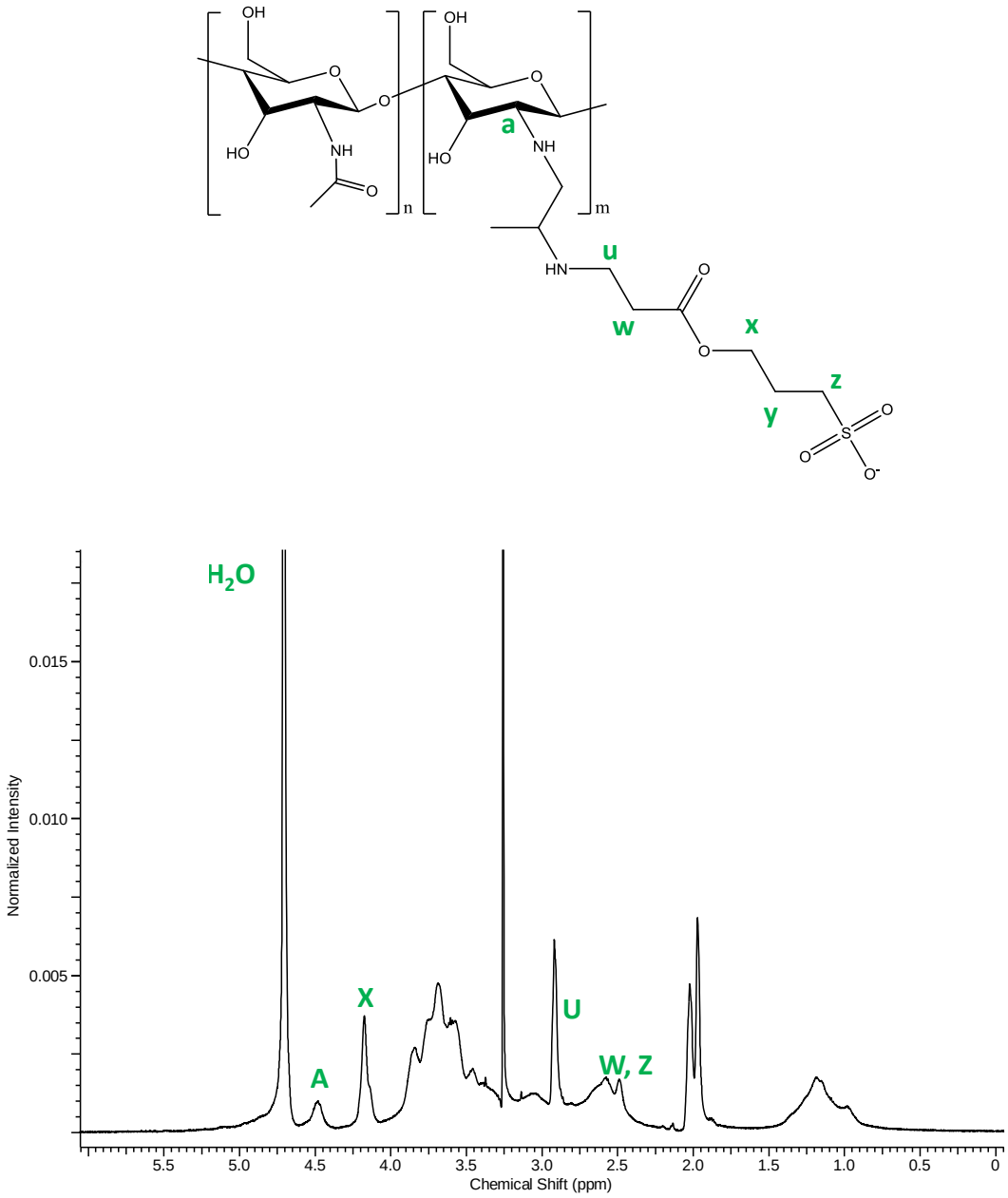

Figure 4.5 ^{1}H NMR Characterization of COS-SPA. The degree of substitution is characterized by integration of the peak X against peak A.

methoxide (25 µL) in a 1 dram vial equipped with a stir bar. The open vial was placed in a 160 mL Parr general purpose stainless steel pressure vessel and stirred vigorously. Oxygen was removed from the reaction vessel by purging with argon (10 s, 8 bar) thrice, followed by three longer argon purges (10 min, 8 bar). The vessel was then filled with potassium hydroxide-purified NO gas (10 bar) for 72 h at room temperature. Afterwards, the argon purging procedure was repeated to remove unreacted NO. *N*-diazeniumdolate-modified chitosan oligosaccharides (COS-NO, COS-EA-NO, COS-TBuA-NO, and COS-SPA-NO) were precipitated in acetone, collected via centrifugation to yield a yellow powder, dried in vacuo, and stored at -20 °C until use.

4.2.6 Chemiluminescence detection of NO release

A Sievers 280i Chemiluminescence Nitric Oxide Analyzer (Boulder, Colorado) was used to quantify NO release. Prior to analysis, the instrument was calibrated with air passed through a NO zero filter (0 ppm NO) and 26.8 ppm of NO standard gas (balance N_2). The *N*-diazeniumdiolate modified chitosan oligosaccharides (1.0 mg) were immersed in 30 mL of deoxygenated PBS (pH 6.5) at 37 °C whereupon released NO was carried by N_2 gas to the detector at a flow rate of 80 mL/min. Additional N_2 flow was supplied to the sample flask at 200 mL/min to match the collection rate of the instrument. Analysis was terminated once NO concentrations fell below 10 ppb NO/mg COS-NO.

4.2.7 Turbidimetric titrations of mucins

Gastric pig mucin (960 mg) was dissolved in 250 mL of sterile phosphate buffer (PB) at 4 °C for 18 h. The mucin suspension was centrifuged (1,500 × g, 4 °C, 0.5 h) to remove insoluble components. The resulting mucin solution was stored in sterile containers at 4 °C for up to 1 week prior to use. Solutions of mucin and acrylate-modified chitosan oligosaccharides were prepared by combining 236 µL of the purified mucin solution with 34 µL of the chitosan oligosaccharide

solutions (3.6 - 54.5 mg/mL in sterile PB) in a 96-well plate. The mucin and chitosan solutions were incubated for 1 h at 37 °C with gentle shaking (100 rpm) after which the absorbance was read at 540 nm using a Thermoscientific Multiskan EX plate reader. Corrected absorbances were obtained after subtracting the absorbance of the chitosan oligosaccharides (i.e., without mucin) from the COS-mucin absorbance.

4.2.8 Zeta potentials of mucin-chitosan oligosaccharide aggregates

Gastric pig mucin (10.0 mg) was dissolved in 10.00 mL sterile PB at 4 °C for 18 h. The mucin suspension was centrifuged (1,500 × g, 4 °C, 0.5 h) to remove insoluble components. The resulting mucin solution was stored in sterile containers at 4 °C for up to 1 week prior to use. Modified chitosan oligosaccharide solids were added to the mucin solution, vortexed until dissolved, and incubated for 1 h at 37 °C. The zeta potential (i.e., surface charge) of the mucin-chitosan aggregate was determined using a Malvern Zetasizer Nano-ZS equipped with a 10 mW HeNe laser (633nm) and a NIBS detector at an angle of 173°.

4.2.9 Gel Electrophoresis

Concentrated stocks of chitosan oligosaccharides (COS and COS-SPA) and NO-releasing chitosan oligosaccharides (COS-NO and COS-SPA-NO) or DTT (20 µL) were added to HBE mucus (40 µL), stirred gently, and incubated at room temperature for 2 h with gentle rocking. As CF sputum contains proteolytic enzymes, the incubation time was decreased to 1 h to reduce enzymatic degradation of the sample.

Following treatment, samples (40 µL) were loaded onto a 0.7% agarose gel in 1x TAE buffer with 1% SDS for electrophoretic separation of MUC5AC and MUC5B at 80 V for 90 min. The gel was reduced with 10 mM DTT for 20 min. Mucins were transferred by vacuum (45 mbar, 1.5 h) in 4x SSC buffer onto a nitrocellulose membrane with a pore size of 45 µm (Optitran BA-S

85 membrane, GE Healthcare Life Sciences, Piscataway, NJ). Following blocking of nonspecific interactions with powdered milk (3 wt% in DPBS) for 1 h, mucins were detected by exposure to diluted primary antibodies raised against MUC5AC and MUC5B (0.1 μg/mL in 3% milk) overnight (3 °C). The membranes were washed thrice with DPBS (10 min) and revealed with fluorescently labeled secondary antibodies (0.2 μg/mL in 3% milk, 1 h, 25 °C). The gels were subsequently washed in DPBS again and were analyzed on a LI-COR Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Migration distances were quantified using Image J (US National Institute of Health, Bethesda, MD). For CF sputum samples, migration distances were normalized to the PBS-treated sample to account for the large degree of heterogeneity.

4.2.10 Confocal microscopy

Prior to being loaded into the agarose gel, CF sputum samples (5μL) exposed to COS, COS-NO, COS-SPA, or COS-SPA-NO were carefully smeared on glass microscopy slides to prevent mechanical disruption of mucin and DNA network. The samples were fixed with NBF (10 vol. %), washed with DPBS, and blocked with BSA (3 wt. % in DPBS) for 1 h at room temperature. Mucins networks were visualized by immunohistochemical detection. First, MUC5AC and MUC5B were identified via exposure to primary antibody solutions (0.4 and 0.2 μg/mL for MUC5AC and MUC5B, respectively). The slides were washed with DPBS three times (10 min) prior to exposure to secondary antibodies (1 μg/mL) and DAPI (5 μg/mL) for 1 h at 25 °C to facilitate quantitative measurement. The slides were then washed with DPBS (10 min) and mounted with Fluorsave. Confocal images were obtained with an Olympus FV 1000 (Olympus, Hamilton, Bermuda) with a 20x objective.

4.2.11 Parallel plate rheology

Spontaneously expectorated sputum from one CF patient collected at a single time point was used for rheological measurements. Concentrated solutions of COS-NO (27.8 µL) were added to 250 µL aliquots of sputum to achieve final COS-NO concentrations of 0, 5, 10, and 20 mg/mL. Sputum samples were slowly rotated at room temperature for 1 h. The rheological properties of the treated samples were measured via amplitude sweep experiments over a stress range of 0.025-50 Pa at a single frequency (1 Hz) on a Bohlin Gemini Rheometer (Malvern Instruments, Worcestershire, UK) with a 20 mm diameter parallel plate set to a gap thickness of 50 mm. 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.[45] All values are reported as the mean ± standard error of the mean (SEM) for a minimum of three separately evaluated aliquots of the treated sputum sample.

4.2.12 Statistical analysis

All values are reported as the mean ± standard deviation for three or more pooled experiments unless otherwise noted. Statistical significance was determined by two-tailed student's t-test.

4.3 Results and Discussion

4.3.1 Synthesis of Mucoadhesive Chitosan Oligosaccharides

To determine if mucoadhesion altered the mucolytic efficacy of NO-releasing chitosan oligosaccharides, 2-methylaziridine-modified chitosan oligosaccharides (COS) were modified with three structurally distinct acrylates (Figure 4.1). These modifications were chosen to sterically block the primary amine on COS, as primary amines are the main source of chitosan's

mucoadhesive properties.[46] Reaction conditions for the Michael addition of acrylate moieties to the COS scaffold were optimized to maximize modification efficiency. Specifically, the synthesis was carried out under basic conditions to increase primary amine nucleophilicity and reactivity towards the β-carbon of the acrylate vinyl group. For example, the modification efficiency was 10.8 ± 1.1% when the reaction between COS and TBuA was carried out under neutral conditions (pH 7) versus 71.5 ± 13.5 % at pH 12. All reactions were carried out at room temperature as heating to 50 °C produced undesirable side-reactions between ethyl acrylate and the chitosan backbone (data not shown). These optimized reaction conditions resulted in similar modification efficiencies of ~ 80% for all three acrylate moieties (Table 4.1).

4.3.2 Nitric oxide-release properties

To impart NO storage and release, *N*-diazeniumdiolates were formed on the modified chitosan oligosaccharides via exposure to high pressures of NO gas.[42] Nitric oxide storage was tuned by maintaining constant solvent ratios and base concentrations for all scaffolds. Modification of the COS scaffold acrylate did not alter NO payloads ($[NO]_{total}$ ~ 0.4 μmol/mg) or release kinetics ($t_{1/2}$ ~ 30 min) regardless of the acrylate modification (Table 4.1). While NO release from *N*-diazeniumdiolates may be altered by charge stabilization or hydrophobicity imparted from local functional groups,[47] the data indicates that NO-release kinetics from acrylate-modified chitosan oligosaccharides are primarily influenced by the hydrophobicity of the chitosan backbone. Indeed, the NO-release kinetics of these materials were somewhat expected as the acrylate modifications did not significantly alter the hydrophobicity of the materials.

4.3.3 Mucoadhesion of acrylate- and 2-methylaziridine-modified chitosan oligosaccharides

Table 4.1 Acrylate modification and NO-release characteristics of modified chitosan oligosaccharides in PBS (pH 6.5, 37 °C). All values are reported as the mean ± standard deviation for 3 or more pooled experiments.

	Degree of substitution[a] (%)	$[NO]_{total}$[b] (μmol/mg)	$[NO]_{max}$[c] (ppb/mg)	$t_{(1/2)}$[d] (min)	t_d[e] (h)
COS	—	0.44 ± 0.11	2800 ± 600	36.1 ± 2.8	7.85 ± 0.75
COS-EA	76.3 ± 3.7	0.42 ± 0.16	3200 ± 1100	28.8 ± 5.2	7.62 ± 0.67
COS-TBuA	71.5 ± 13.5	0.42 ± 0.09	2500 ± 400	25.3 ± 8.6	7.74 ± 0.74
COS-SPA	90.6 ± 13.9	0.39 ± 0.12	3300 ± 1100	28.9 ± 4.3	6.72 ± 1.92

[a] relative to primary amines, [b] Total NO, [c] Max NO Flux, [d] Half-life, [e] Duration of NO-release

Turbidimetric titrations of mucin with acrylate-modified COS were carried out to determine the mucoadhesion of the chitosan oligosaccharide scaffolds (Figure 4.6). In the presence of mucoadhesive polymers, mucins form self-assembled complexes which scatter light.[30,48] As such, the turbidity of mucin solutions (as measured by corrected absorbance) increases with the addition of mucoadhesive scaffolds but remains constant with muco-inert scaffolds. Turbidity was monitored at 540 nm to maximize sensitivity towards mucin-chitosan complexes while minimizing the absorbance of free chitosan oligosaccharides in solution (λ_{max} = 375 nm).

The turbidity of mucin solutions increased rapidly at low chitosan concentrations for all scaffolds tested with the exception of COS-SPA, demarcating it as the least mucoadhesive scaffold (Figure 4.6). As electrostatic interactions between protonated amines on the chitosan scaffold and negatively charged residues on mucins are the primary cause of chitosan mucoadhesion,[29-30,46] electrostatic repulsion between mucins and the negatively charged sulfonate group of COS-SPA likely prevented the formation of chitosan-mucin aggregates. While the TBuA and EA modifications sterically block amines on the COS scaffold, this steric hindrance did not significantly reduce mucoadhesion compared to the unmodified COS scaffold (Figure 4.6).

Zeta potential measurements of chitosan-mucin aggregates corroborated the findings from turbidimetric titrations (Figure 4.7). At pH 6.5, GPM exhibited a zeta potential of 9.8 ± 0.5 mV. Adding positively charged chitosan oligosaccharides (i.e., COS, COS-TBuA, and COS-EA) to dilute mucin solutions increased the zeta potential, indicating the adherence of chitosan oligosaccharides to the mucins. The change in zeta potential as a function of chitosan oligosaccharide concentration plateaued as the charge of the mucin was neutralized, verifying the electrostatic nature of the interactions. Treatment with negatively charged COS-SPA did not

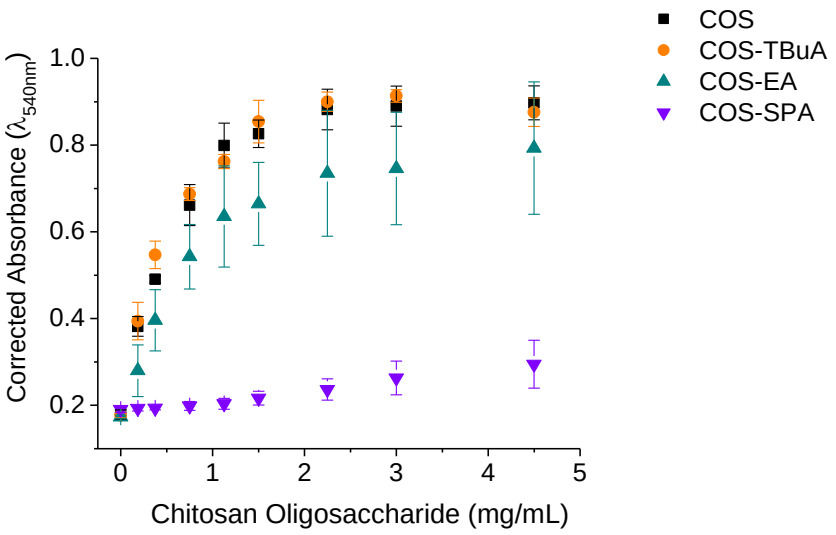

Figure 4.6 Turbidimetric titration of gastric pig mucin (GPM) with modified chitosan oligosaccharides.

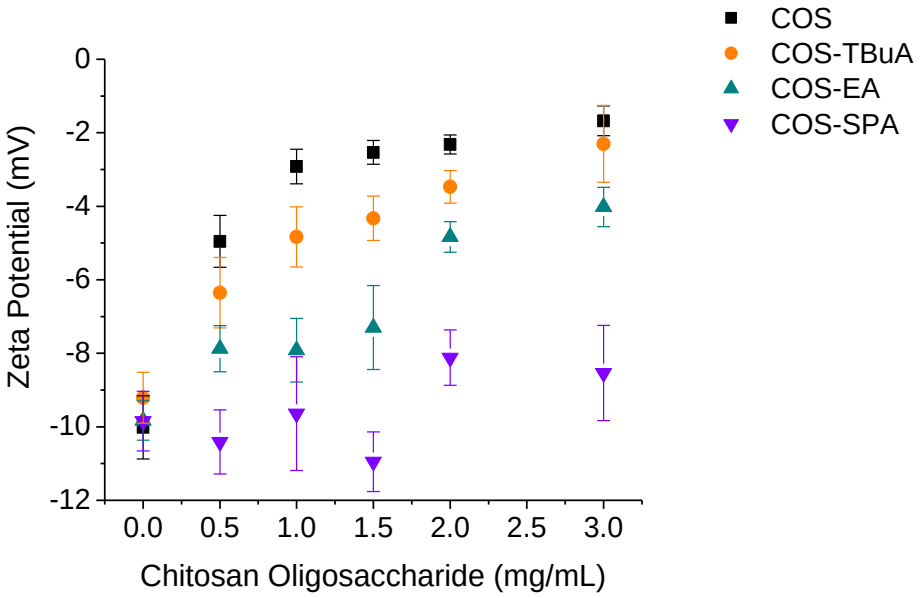

Figure 4.7 Zeta potential measurements of GPM-chitosan aggregates formed upon the addition of chitosan oligosaccharides to mucin solutions (1 mg/mL in 10 mM PB, pH 6.5).

significantly alter the zeta potential at any of the tested concentrations, further supporting electrostatic repulsion between COS-SPA and mucin.

4.3.4 Electrophoretic separation of purified mucus following treatment with chitosan oligosaccharides.

To determine the effects of NO on mucin size (i.e., molecular weight), mucus collected from HBE cultures was treated with control (i.e., non-NO-releasing) and NO-releasing chitosan oligosaccharides and evaluated using gel electrophoresis (Figure 4.8). The most and least mucoadhesive chitosan oligosaccharides (i.e., COS and COS-SPA, respectively) were compared to determine the importance of scaffold mucoadhesion. Treatment with control scaffolds did not alter mucin migration compared to samples treated with PBS regardless of the mucoadhesive properties of the scaffold. Retained mucin monomers were visible in mucus secretions treated with 10-20 mg/mL COS (Figure 4.8). As shown in Figure 4.7, treatment with COS reduced the magnitude of the mucin zeta potential, which accounts for the decreased migration of the monomers in the applied electric field (Figure 4.8).

Treatment with COS-NO increased the migration of both MUC5AC and MUC5B during electrophoretic separation (Figure 4.9). This increase in migration was statistically significant ($p \leq 0.05$) for both mucins at 10 mg/mL COS-NO. As treatment with the COS scaffold did not alter migration, we are able to attribute this effect to NO. While the polarity of NO on the COS scaffold may alter mucin electrophoretic mobility, the inability of the negatively charged COS-SPA scaffold to alter migration suggests that NO released from COS-NO reduced the size of mucin multimers, leading to faster migration.

Mucins from HBE mucus were also treated with DTT for comparison to the mucolytic properties of NO (Figure 4.10). While DTT is too toxic for clinical use,[49-50] it is commonly used

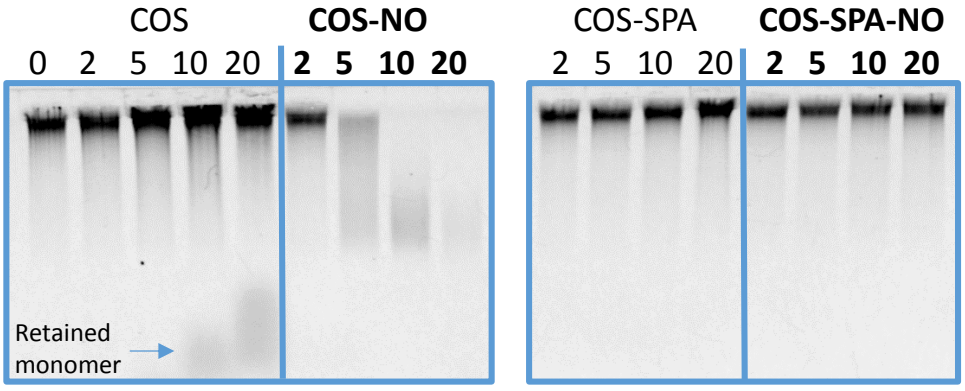

Figure 4.8 Representative western blot of Muc5AC mucins from HBE mucus treated with COS, COS-NO, COS-SPA, and COS-SPA-NO for 2 h at 25°C at concentrations ranging from 0-20 mg/mL. Similar trends were observed for Muc5B.

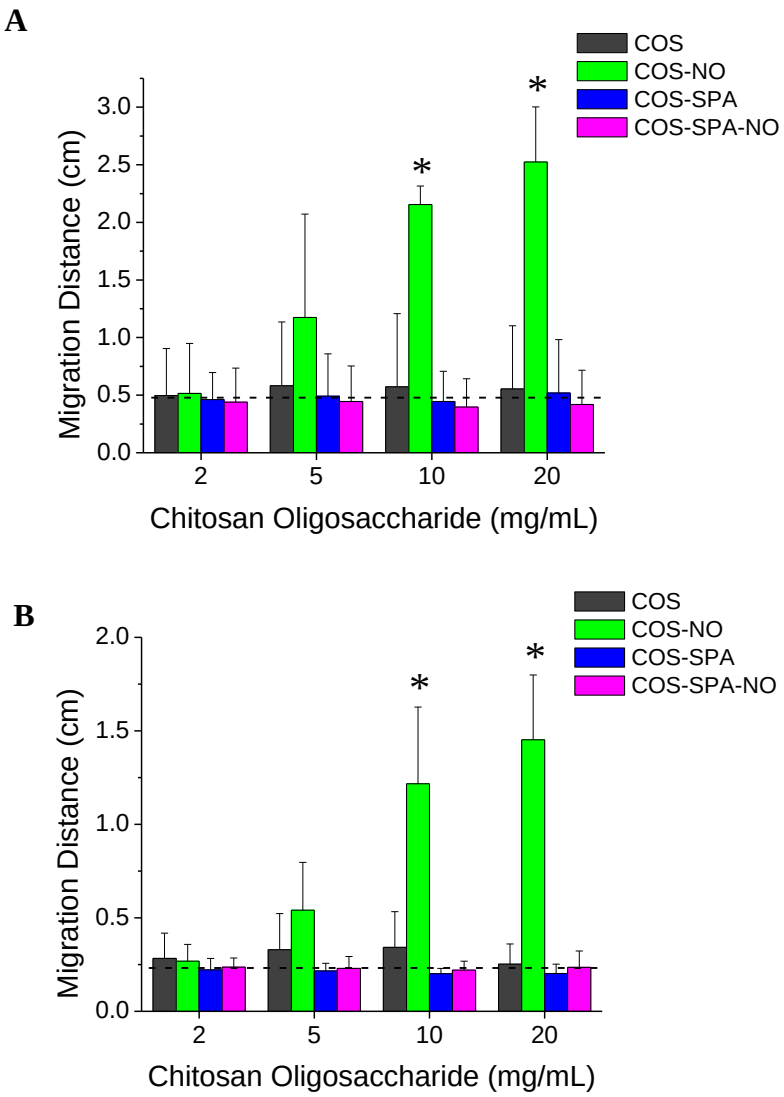

Figure 4.9 Migration distances of (A) Muc5AC and (B) Muc5B mucins in HBE Mucus following treatment with modified chitosan oligosaccharides for 1 h at room temperature. The migration distances of mucin treated with an equal volume of PBS are denoted with dashed horizontal lines. All values are presented as the mean ± standard deviation for 3 or more pooled experiments. Asterisks (*) indicate significant differences ($p < 0.1$) relative to treatment with PBS.

for mucin reduction in in vitro assays and acts by cleaving mucin disulfide bonds. Exposure to DTT at concentrations ≥ 5 mM fully reduced both MUC5AC and MUC5B mucins (Figure 4.10). Following DTT exposure, completely reduced MUC5AC and MUC5B migrated distances of 2.52 ± 0.02 and 1.30 ± 0.09 cm, respectively, while treatment with 20 mg/mL COS-NO resulted in migration distances of 2.52 ± 0.48 and 1.45 ± 0.35 cm, respectively. The similarity between migration distances for both treatments demonstrates that treatment with 20 mg/mL COS-NO fully degraded both mucins, indicating its effectiveness as a mucolytic agent.

As treatment with COS-SPA-NO did not alter the migration of MUC5AC or MUC5B, scaffold mucoadhesion was determined to be necessary for effective NO delivery (Figure 4.9). This conclusion is consistent with previous reports as association between NO-releasing scaffolds and drug targets leads to localized drug delivery and enhances therapeutic efficacy.[25] In contrast, poor scaffold-target association results in distal NO release, thereby lowering any therapeutic activity. For example, dendrimers[51-53] and silica nanoparticles[26,54-56] that associate with bacteria membranes improve the bacteria killing of the macromolecular NO release scaffolds compared to non-associating NO donors, suggesting that mucoadhesion would also advantageous in designing NO-releasing scaffolds to alter mucolytic activity.

4.3.5 Electrophoretic separation of CF sputum following treatment with chitosan oligosaccharides

While NO released from COS-NO altered mucin migration in HBE mucus, the complexity of CF sputum (e.g., high concentrations of DNA, proteins, bacteria, and neutrophils) may influence NO delivery and action. We thus evaluated the effects of COS, COS-NO, COS-SPA, and COS-SPA-NO on mucins in CF sputum using gel electrophoresis (Figure 4.11). As with HBE mucus, treatment with controls (i.e., non-NO-releasing chitosan oligosaccharides) did not affect mucin

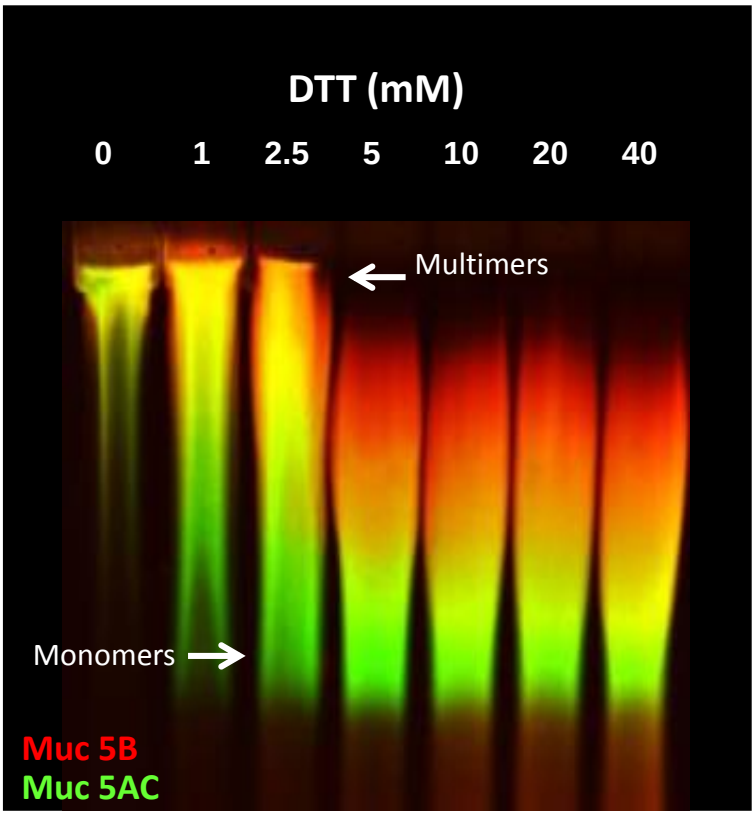

Figure 4.10 Representative western blot of HBE mucus treated with DTT for 10 min at room temperature. This figure was generously provided by Prof. Camille Ehre of the Marsico Lung Institute, University of North Carolina at Chapel Hill, Chapel Hill, NC (unpublished data).

migration. In contrast, mucin migration was enhanced relative to controls following treatment with COS-NO at concentrations ≤ 10 mg/mL for both MUC5AC and MUC5B (Figure 4.12), further indicating the mucolytic activity of this scaffold. While it has been argued that muco-inert scaffolds are more effective for drug delivery in CF airways due to improved penetration,[31] treatment with COS-SPA-NO failed to influence mucin migration. In both HBE mucus and CF sputum, the mucolytic activity of NO-releasing chitosan oligosaccharides was thus dependent upon the mucoadhesive properties of the scaffold.

4.3.6 Fluorescent microscopy of CF sputum

To visualize NO-mediated changes in the network formed by mucins and DNA in CF sputum, samples imaged with a confocal laser scanning microscope (Figure 4.13). As expected, networks of MUC5AC (red) and MUC5B (green) intertwined to form three-dimensional architectures characterized by thick filaments of mucins and web-like mucin sheets in the PBS-treated sample. Intact neutrophils (blue) were embedded within the three-dimensional mucin network. Treatment with the chitosan oligosaccharides at 20 mg/mL caused neutrophil apoptosis, regardless of the chitosan composition, as evidenced by the increase in extracellular DNA (Figure 4.13). Chitosan oligosaccharide-induced neutrophil apoptosis is proposed to be responsible for the anti-inflammatory properties of this scaffold.[57-58] Treatment with COS-SPA and COS-SPA-NO had no other discernable effects on the CF sputum architecture.

Both COS and COS-NO, the positively charged chitosan variant, significantly altered the appearance of the mucin networks in CF sputum. While the filaments and webs of the elastic network were still visible, treatment with COS induced aggregation of the mucin networks as was apparent by the increased fluorescence (i.e., brightness) of the images. Additionally, the presence of large sheets of flowing mucin was decreased in sputum treated with COS. These images show

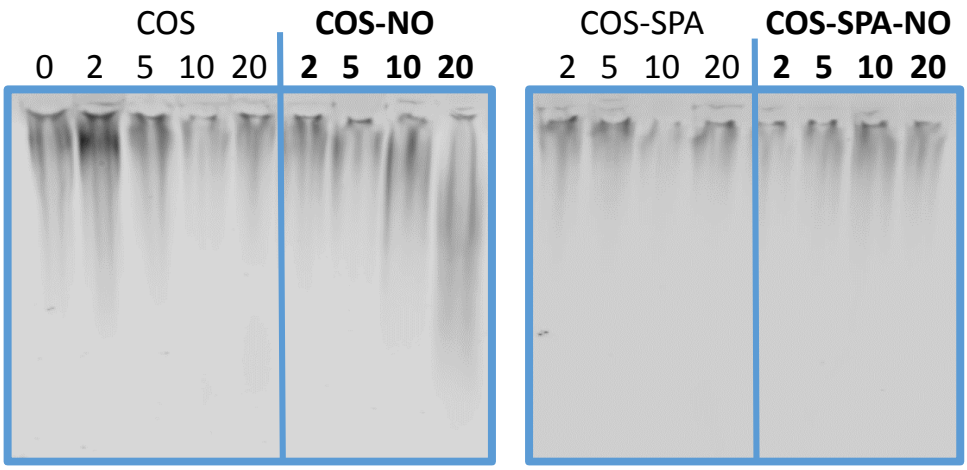

Figure 4.11 Representative western blot of MUC5AC mucins from CF sputum treated with COS, COS-NO, COS-SPA, and COS-SPA-NO for 1 h at 25°C at concentrations ranging from 0-20 mg/mL. Similar trends were observed for MUC5B.

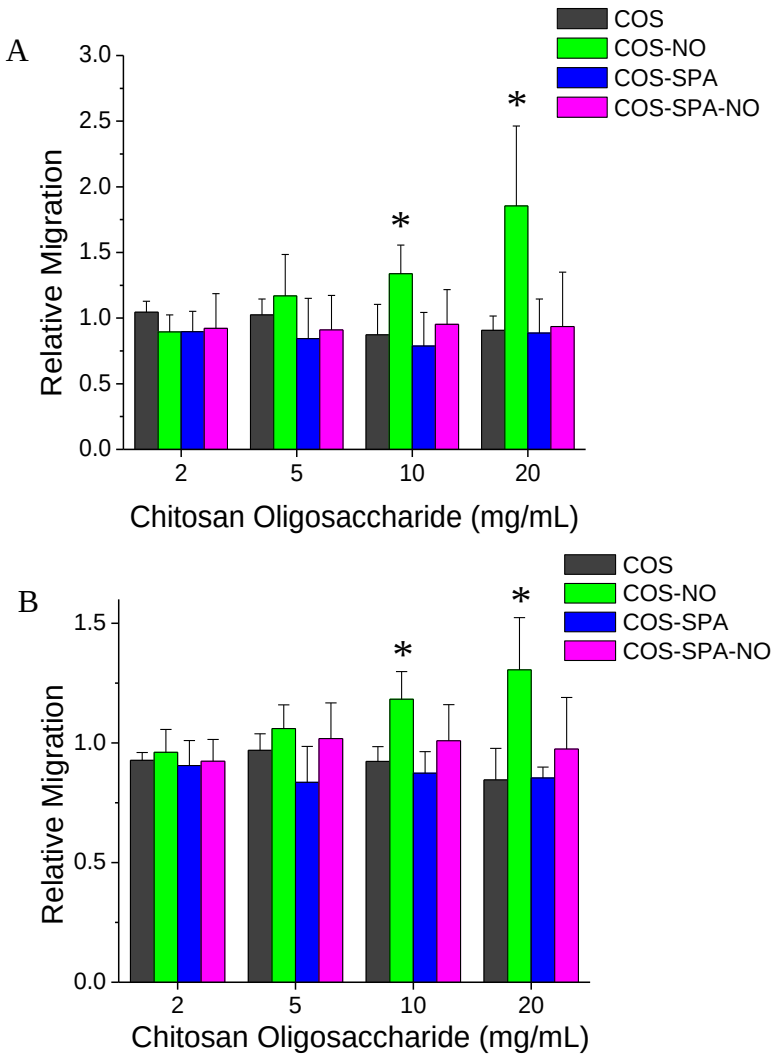

Figure 4.12 Relative migration distances of (A) MUC5AC and (B) MUC5B mucins from CF sputum following treatment with modified chitosan oligosaccharides for 1 h at room temperature. Migration distances were normalized to CF sputum samples treated with an equal volume of PBS. All values are presented as the mean ± standard deviation for n=3 or more pooled experiments. Asterisks (*) indicate significant differences ($p < 0.1$) relative to treatment with PBS.

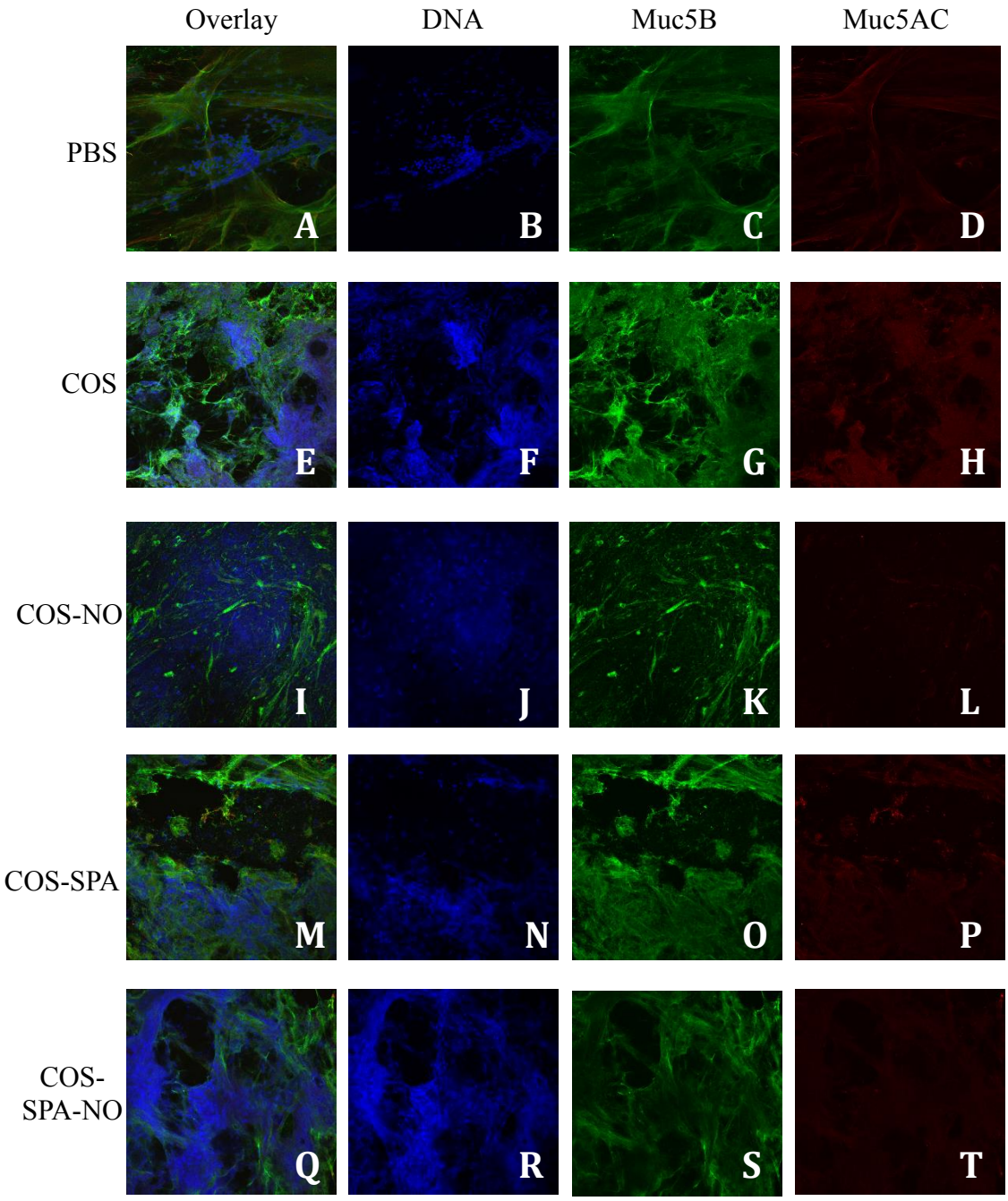

Figure 4.13 Confocal microscopy images of CF sputum treated with PBS (A-D)or 20 mg/mL COS (E-H), COS-NO (I-L), COS-SPA (M-P), or COS-SPA-NO (Q-T) for 1 h at 25 °C.

that COS-mucin aggregation persists even in the complex viscoelastic CF sputum network. In contrast to treatment with COS alone, COS-NO greatly degraded the mucin network. Following treatment, the length of mucin filaments was decreased and the mucin network appeared relaxed with no dimensionality (Figure 4.13). As the presence of highly intertwined mucin structures correlate with sputum elasticity,[59] the NO-mediated disruption of this network suggests NO's ability to reduce sputum elasticity.

4.3.7 CF sputum rheology

The ultimate test of a mucolytic agent is its ability to decrease mucus viscosity and elasticity, as these rheological parameters are the predominant predictors of mucociliary and coughing clearance.[7,60-61] Due to superior efficacy in vitro, COS-NO was chosen for rheological analysis. Dose-dependent reductions in sputum viscosity and elasticity were observed following treatment with COS-NO for 1 h at room temperature (Figure 4.14). At the lowest concentration tested (5 mg/mL), COS-NO reduced sputum elasticity by 74.5% and viscosity by 66.4 % compared to PBS-treated controls while treatment with 20 mg/mL reduced sputum elasticity and viscosity by an order of magnitude.

While it is difficult to compare rheological data across literature reports due to the diversity of exposure conditions and measurement parameters,[62] treatment with COS-NO produced similar changes in sputum viscoelasticity as conventional therapeutics. For example, Seagrave et al. reported that NAC treatment for 24 h decreased the viscosity and elasticity of HBE mucus by an order of magnitude versus controls.[45] Similarly, Shah et al. observed reduced sputum viscosity and elasticity by 59% and 68%, respectively, following treatment with dornase alfa for 10 min.[13] While the concentration of COS-NO required to alter sputum rheological properties is greater than

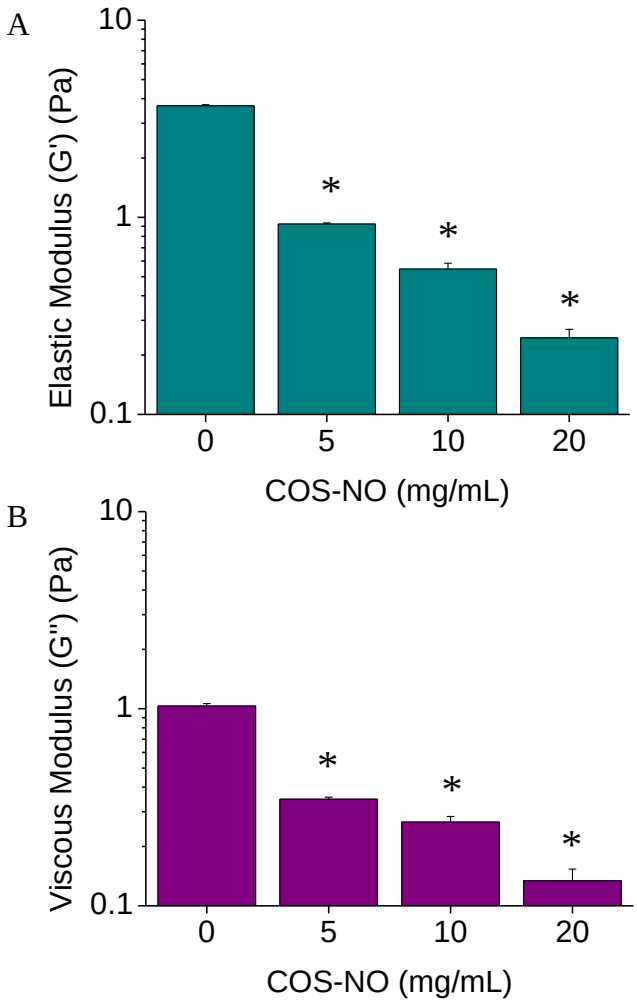

Figure 4.14 (A) Elastic and (B) viscous moduli of CF sputum following treatment with COS-NO for 1 h at 25 °C. Values presented as the mean ± standard error of the mean for n=3 triplicate measurements.

the NAC and dornase alfa used in these reports, longer exposure conditions and/or greater NO payloads would decrease the required dose. The development of mucoadhesive NO-releasing chitosan oligosaccharides with improved NO payloads for enhanced mucolytic efficacy is currently underway in our laboratory.

4.4 Conclusions

The antibacterial properties of NO-releasing scaffolds are well documented,[25,63-64] however the utility of such materials as mucolytic agents is just beginning to be developed. The work reported herein sought to determine the biophysical effects of NO released from *N*-diazeniumdiolate-modified chitosan oligosaccharides on HBE mucus and CF sputum as well as the importance of mucoadhesion in effective drug delivery. Mucoadhesive COS-NO increased mucin migration during electrophoresis in both HBE mucus and CF sputum by reducing the molecular weight of multimeric mucin. Control chitosan oligosaccharides did not alter mucin migration, indicating that NO is the only mucoactive agent. In addition to decreasing the molecular weight of mucins, the NO released from COS-NO is capable of destroying the mucin network of CF sputum. Confocal microscopy of CF sputum treated with COS-NO revealed substantial alterations in the mucin networks, including decreased length of mucin fibers and the loss of three-dimensional structures.

Both the reduction in mucin size and the destruction of the three-dimensional mucin network by NO ultimately decreases the viscoelasticity of CF sputum. While the diminished elastic and viscous moduli of CF sputum could be attributed to alterations in the DNA network via NO-mediated DNA cleavage, electrophoretic separations and confocal microscopy indicate that NO actively alters the mucin network. Due to the poor clinical efficacy of NAC and an undesirable inflammatory response associated with DTT treatment, NO holds unique potential as a

combination antibacterial and mucolytic agent. As such, NO-based therapies could also prove complimentary to dornase alfa treatments as dornase alfa only alters the DNA network of CF mucus.

While inhalation of NO gas has been proposed as a mucolytic therapy, our work indicates that NO released from mucoadhesive scaffolds may be more effective my improving NO delivery in CF mucus. In all experiments, the mucoadhesive properties of the chitosan oligosaccharide scaffolds greatly influenced NO's action. As has been previously demonstrated in with other NO-release vehicles and applications,[25] the ability to localize NO delivery in CF mucus would likely improve the efficacy of NO treatment compared to NO gas while simultaneously improving the convenience of treatment and overall clinical utility.

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CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

5.1 Summary

The preceding chapters have examined the utility of NO-releasing chitosan oligosaccharides as potential CF therapeutics. In Chapter 1, the pathogenesis of CF disease was described as a cascade of biophysical processes. Inherited defects in the cystic fibrosis transmembrane conductance regulator (CFTR) gene cause sodium hyperabsorption and depletion of the periciliary layer (PCL).[1] Without a hydrated PCL, mucociliary clearance fails with the accumulation of highly viscoelastic mucus layer.[1] The physical properties of the CF mucus layer decrease the ability to eliminate pathogens while fostering the development of highly pathogenic and drug resistant infections.[2] These chronic infections elicit a strong immune response, leading to lung disease and degradation.[3] The NO-releasing chitosan oligosaccharides are proposed as a therapeutic treatment to potentially both eradicate bacteria biofilms and alter the biophysical properties of the CF mucus layer. *N*-diazeniumdiolate-NO-donor modified chitosan oligosaccharides were chosen as the NO-release scaffold for study due to their low toxicity, ease of functionalization, mucoadhesive properties, biodegradability, and large NO payloads.

In Chapter 2, the bactericidal efficacy of NO-releasing chitosan oligosaccharides was determined under in vitro conditions that simulate CF airways. As reactions between oxygen and NO produce several reactive species that enhance NO's bactericidal action,[4-6] it was hypothesized that NO may be less effective under the low oxygen conditions common to CF mucus.[7-8] Importantly, such behavior was not observed. Rather NO released from chitosan oligosaccharides was equally effective at inhibiting growth and eradicating planktonic *P.*

aeruginosa under aerobic and anaerobic conditions, indicating that the efficacy of NO would not be reduced in the CF airways. Furthermore, the NO treatment was 10-fold more effective at eradicating *P. aeruginosa* biofilms under anaerobic conditions than tobramycin. As the low oxygen conditions of the CF mucus layer curtail the effectiveness of traditional antibiotics,[9-12] the enhanced anti-biofilm activity of NO in these environments make it attractive as a therapeutic for treating bacterial infections in CF airways.

In Chapter 3, the effects of NO-releasing chitosan oligosaccharide treatment on the physical properties of *P. aeruginosa* biofilms were determined using multiple particle tracking microrheology. Unlike tobramycin, which is only capable of eradication and not dispersal, NO-releasing chitosan oligosaccharides were capable of both killing and physically degrading *P. aeruginosa* biofilms. Treatment with NO-releasing chitosan oligosaccharides resulted in the destruction of biofilm segments, rather than only loosening the biofilms. Of note, the chitosan oligosaccharide scaffold alone increased biofilm elasticity, caused physical contraction of the biofilm, and ostensibly mitigated the destructive power of NO. Despite the negative effects of the scaffold, NO was capable of fluidizing *P. aeruginosa* biofilms, thus likely decreasing the contributions of *P. aeruginosa* biofilms to CF mucus viscoelasticity.

The effects of NO-releasing chitosan oligosaccharides on the physical properties of mucins from human bronchial epithelial cells and CF sputum were determined as a function of scaffold mucoadhesion in Chapter 4. For each assay, NO that was released from non-mucoadhesive scaffolds had no effect on the mucin networks. In contrast, NO released from a mucoadhesive scaffold (COS-NO) both decreased mucin molecular weight and degraded mucin networks in CF sputum. Destruction of the sputum network ultimately reduced the viscous and elastic moduli of CF sputum as determined by parallel plate rheology. Collectively, the work in

my thesis demonstrates the potential utility of NO as a dual-action therapeutic as NO is capable of both bacterial biofilm eradication and mucolysis.

5.2 Future Directions

While the antibacterial effects of NO-releasing chitosan oligosaccharides have been reported previously,[13-14] my work is the first to characterize the biophysical activity of NO released from macromolecular scaffolds. Nitric oxide was determined to decrease the viscoelastic properties of both bacterial biofilms and CF sputum. These results are preliminary and further research is required to characterize the interactions between NO-releasing chitosan oligosaccharides and CF airways.

5.2.1 In vitro cytotoxicity testing

The inherently low toxicity of chitosan makes it an attractive scaffold for pulmonary delivery.[15] In previous work, NO-releasing chitosan oligosaccharides were capable of eradicating *P. aeruginosa* biofilms grown in a Center for Disease Control bioreactor at concentrations that were non-toxic to L929 mouse fibroblast cells.[14] While L929 mouse fibroblast cells are widely used to assess the cytotoxicity of NO-releasing materials,[13-14,16-20] this cell line does not accurately model CF airways. Therefore, cytotoxicity assays should be performed using human bronchial epithelial (HBE) cell cultures in mucus. The mucoadhesive properties of the chitosan oligosaccharides will likely determine how much NO is delivered to the epethilum in vivo. Highly mucoadhesive scaffolds may not effectively penetrate the CF mucus barrier, resulting in NO release only at the mucus surface.[15,21-22] In order to determine the validity of this hypothesis, cytotoxicity assays using NO-releasing chitosan oligosaccharides with a range of mucoadhesive properties should be examined.

Ideally, HBE cultures should be grown under conditions that would allow them to differentiate, form cilia, and secrete their own mucus layer.[23] Unfortunately, the expense of this cell culture technique is prohibitive for determining the half maximal inhibitory concentrations (IC_{50}) of multiple NO-releasing chitosan oligosaccharide scaffolds. In order to study the protective properties of the mucus layer, mucus harvested from larger HBE cultures can be added to the cells in high throughput assays performed in 96-well plates. Following treatment with chitosan oligosaccharides, cell viability could be determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, a reagent that forms a colored product in the presence of active mitochondria (i.e., MTS assay).[14,19-20]

5.2.2 Combined mucolytic and bactericidal efficacy of nitric oxide-releasing chitosan oligosaccharides

While the anti-biofilm action of NO-releasing chitosan oligosaccharides in CF relevant conditions was determined in Chapter 2, the effects of mucoadhesion on bactericidal properties remains unknown. Ideal chitosan oligosaccharides should penetrate both CF mucus and bacterial biofilms to effectively deliver bactericidal concentrations of NO. As such, the mucoadhesive properties of NO-releasing chitosan oligosaccharides may significantly alter the antibacterial activity in vivo.

Biofilm exopolysaccharide (EPS) matrix and CF mucus are highly complex and heterogeneous, but both can be generalized as intertwining networks of polyanionic polymers with large hydrophobic domains.[24-26] As such, altering the cationic amines of chitosan oligosaccharides with negatively charged or hydrophilic groups will likely decrease association with biofilm EPS and the mucus network. Therefore, chitosan oligosaccharides modified with 2-

methylaziridine (COS), sulfopropyl acrylate (COS-SPA), PEG (COS-PEG), and thiols (COS-SH) should be evaluated using similar bacterial assay formats.

In order to determine the mucolytic and bactericidal efficacy NO-releasing chitosan oligosaccharides, multiple particle tracking rheology and minimum bactericidal concentration assays should be employed concurrently. Growing *P. aeruginosa* biofilms in HBE mucus supplemented with fluorescent tracer particles accurately mimics in vivo growth conditions and allows for the rheological characterization of both biofilms and mucus. In order to differentiate between the biofilms and mucus, *P. aeruginosa* biofilms should be grown with bacteria that express green fluorescent protein (GFP). Samples treated with chitosan oligosaccharides can be aliquoted for simultaneous rheological and bactericidal assays since both methodologies require small sample volumes (i.e. ≤ 10 μL).

5.2.3 Susceptibility of other CF pathogens to nitric oxide-releasing chitosan oligosaccharides

While *P. aeruginosa* is the most clinically relevant CF pathogen due to its association with patient morbidity and mortality,[27] pulmonary infections result from a wide variety of bacterial species. The first bacterial species to typically infect the airways is *S. aureus* with >70% of all CF patients being colonized by age 10.[28] Airway colonization by *S. aureus* results in increased inflammation and low body mass index of infants with CF.[29] Colonization with organisms within the *Burkholderia cepacia* complex (BCC) is relatively low (i.e., 3.5% versus 80% colonization by *P. aeruginosa* in adult CF patients).[30] However, the pathogenicity of BCC species is high and has been linked to the development of necrotizing pneumonia (frequently termed "cepacia syndrome"), sepsis, and death.[30-31] The pathogenicity of BCC species is furthered by high incidences of antibiotic resistance as a result of their enhanced biofilm

formation, anaerobic growth capacity, and unusual lipopolysaccharide composition. Currently, BCC infections are treated by co-administration of antibiotics with limited success.[30-31]

The susceptibility of these CF pathogens (i.e., *S. aureus* and BCC organisms) to NO-releasing chitosan oligosaccharides should be determined under conditions that closely mimic CF airways. While minimum inhibitory assays against planktonic bacteria are used clinically, these assays do not reliably predict biofilm susceptibility.[32] All bactericidal assays should be performed using macrocolony biofilms grown in artificial sputum media (ASM) to better mimic in vivo physiological conditions.[33] Of note, BCC species do not readily form biofilms in vivo and therefore may not form biofilms in ASM. Regardless, these conditions will most accurately mimic the CF airway.[34] Finally, assays against BCC should be performed both aerobically and anaerobically as BCC are frequently found within the hypoxic zones of the CF mucus layer.[34]

5.2.4 Co-administration of nitric oxide-releasing chitosan oligosaccharides with antibiotics

Additive or synergistic activity between NO and commonly prescribed CF antbiotics may increase the effectiveness of antibacterial treatment regimens. Co-administration of several antibiotics is currently used to treat drug-resistant infections, but the success of this approach is debated.[35-38] Unlike traditional antibiotics that only kill bacteria, the unique biofilm degrading capabilities of NO make it uniquely suited for co-administration. For example, combination therapy with the NO donor sodium nitroprusside (SNP) and tobramycin was shown to increase *P. aeruginosa* biofilm eradication in vitro compared to either treatment alone.[39] Inhalation of NO gas (5-10 ppm for 8 h/day) by CF patients has been demonstrated to reduce bacterial biofilms in the airways when combined with antibiotic therapy (i.e., tobramycin or ceftazidime). While these results are promising, systematic in vitro studies quantifying the effect of NO (i.e., additive or synergistic) are lacking. Therefore, multiple combination bactericidal antibiotic tests

(MCBT or "checkerboard" assays) should be performed on CF-relevant biofilms. As inhalable formulations of tobramycin, aztreonam, and levofloxacin have already been developed, the potential synergy between NO-releasing chitosan oligosaccharides and these antibiotics should be evaluated first. As outlined in Section 2.5.4, *P. aeruginosa*, *S. aureus*, and BCC biofilms are the most clinically relevant organisms and therefore should be evaluated under aerobic and anaerobic conditions.

Section 5.3 Conclusions

This dissertation describes the potential utility of NO-releasing chitosan oligosaccharides to both eradicate *P. aeruginosa* biofilms and alter the biophysical properties of CF mucus. Nitric oxide-releasing chitosan oligosaccharides exhibit several advantages over tobramycin as antibacterial agents. First, the antibacterial efficacy of NO-releasing chitosan oligosaccharides was enhanced under anaerobic conditions due to reduced reactions with oxygen (i.e., oxygen scavenging) in the media. In addition to eradicating *P. aeruginosa* biofilms, NO was shown to degrade the physical properties of the biofilm while tobramycin failed to alter the rheological properties of *P. aeruginosa* biofilms. By reducing the biofilm viscoelasticity, NO-releasing chitosan oligosaccharides may improve the ability to clear infections, particularly as the physical properties of CF mucus dictate disease pathogenesis.

Nitric oxide-releasing chitosan oligosaccharides clearly alter the physical properties of HBE mucus and CF sputum, indicating NO's potential as a mucolytic agent. For example, NO released from mucoadhesive scaffolds increased mucin migration during electrophoretic separation, altered mucin networks in CF sputum, and decreased the viscous and elastic moduli of CF sputum. The chitosan oligosaccharide scaffold also significantly altered the biophysical properties of CF mucus components. While the cationic nature of the chitosan oligosaccharides

increases the scaffold mucoadhesion and NO's mucolytic efficacy, it also negatively impacts (i.e. increases) the elasticity of *P. aeruginosa* biofilms. Future experiments should determine the relative contributions of bacterial biofilms and mucus to the rheological properties of infected CF sputum to further guide the development of NO-releasing scaffolds as CF therapeutics. While this dissertation research represents an initial step, it also provides motivation for the development of NO-releasing materials as dual-action antibacterial and mucolytic therapies for the treatment of CF.

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