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## Nitric Oxide-Releasing Chitosan Oligosaccharides as Antibacterial Agents

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### Abstract

Secondary amine-functionalized chitosan oligosaccharides of different molecular weights (i.e., ~2500, 5000, 10000) were synthesized by grafting 2-methyl aziridine from the primary amines on chitosan oligosaccharides, followed by reaction with nitric oxide (NO) gas under basic conditions to yield *N*-diazoniumdiolate NO donors. The total NO storage, maximum NO flux, and half-life of the resulting NO-releasing chitosan oligosaccharides were controlled by the molar ratio of 2-methyl aziridine to primary amines (e.g., 1:1, 2:1) and the functional group surrounding the *N*-diazoniumdiolates (e.g., polyethylene glycol (PEG) chains), respectively. The secondary amine-modified chitosan oligosaccharides greatly increased the NO payload over existing biodegradable macromolecular NO donors. In addition, the water-solubility of the chitosan oligosaccharides enabled their penetration across the extracellular polysaccharides matrix of *Pseudomonas aeruginosa* biofilms and association with embedded bacteria. The effectiveness of these chitosan oligosaccharides at biofilm eradication was shown to depend on both the molecular weight and ionic characteristics. Low molecular weight and cationic chitosan oligosaccharides exhibited rapid association with bacteria throughout the entire biofilm, leading to enhanced biofilm killing. At concentrations resulting in 5-log killing of bacteria in *Pseudomonas aeruginosa* biofilms, the NO-releasing and control chitosan oligosaccharides elicited no significant cytotoxicity to mouse fibroblast L929 cells *in vitro*.

### Keywords

Drug release; Nitric oxide; Chitosan; Anti-bacterial; Biodegradable

### Introduction

Bacteria in nature exist in two states – free-floating planktonic bacteria and bacterial biofilms.[1] While many antimicrobial agents have proved effective against planktonic bacteria, medically relevant infections including the infections associated with medical implants, non-healing wounds, diabetic mellitus, and cystic fibrosis are often caused by bacterial biofilms.[2–5] Biofilms are communities of microorganisms adhered to a surface and surrounded by a self-produced extracellular polysaccharide (EPS) matrix that impedes immune response.[6, 7] Compared to planktonic bacteria, biofilm-based bacteria are more resistant to antibiotics due to several specific defense mechanisms including inefficient penetration of antimicrobial agents cross EPS.[8, 9] For example, the antibiotic dose to kill

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bacteria in biofilms may be 1000 times the dose required to kill planktonic bacteria.[10] As such, new antimicrobial agents capable of eradicating mature biofilms are urgently needed.

Nitric oxide (NO), a diatomic free radical produced endogenously, plays a key role in the mammalian immune response to pathogens.[11–13] The bactericidal properties of NO are attributed to the nitrosative and oxidative stress exerted by its reactive byproducts such as dinitrogen trioxide and peroxyxynitrite, ultimately leading to the disruption of bacteria membrane.[13] Nitric oxide-releasing materials have been widely developed for the use in a number of different biomedical applications, many related to pathogen killing.[14–17] Recent research has demonstrated the antimicrobial efficacy of small molecule (e.g., 1-[2-(carboxylato)pyrrolidin-1-yl]diazene-1-ium-1,2-diolate (PROLI/NO)) and macromolecular (e.g., silica nanoparticles and dendrimers) NO-releasing vehicles against both Gram-positive and Gram-negative bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA).[18–25] Nitric oxide-releasing macromolecular scaffolds (e.g., silica nanoparticles and dendrimers) are particularly attractive due to enhanced bactericidal activity against planktonic bacteria and biofilms compared to small molecule NO donors (e.g., PROLI/NO). [20–23] Although NO-releasing silica particles proved effective at eradicating established biofilms, the lack of biodegradability greatly hinders the clinical utility of silica-based NO-releasing vehicles as antimicrobial agents.

Biodegradable NO-releasing materials have been developed as implant coatings (e.g., poly(diols citrate) elastomers[26] and polyesters [27–29]) to inhibit biofilm formation rather than eradicate established biofilms. To enable more efficient killing of biofilms, the design of new scaffolds is necessary to allow efficient EPS penetration and NO delivery to the bacteria embedded in the biofilms. Chitosan, the second most abundant natural biopolymer, has been widely used for biomedical applications including tissue engineering, drug delivery, and antimicrobial agents due to its biocompatibility, biodegradability and cationic composition.[30–33] The use of chitosan derivatives as NO-releasing scaffolds has also been investigated since these materials contain large concentrations of primary amines, necessary for *N*-diazoniumdiolate NO donor formation.[34–36] Unfortunately, previously reported *N*-diazoniumdiolate-functionalized chitosan polysaccharides have been characterized by low *N*-diazoniumdiolate conversion efficiency and NO storage (~0.2  $\mu\text{mol}/\text{mg}$ ), likely the result of chitosan insolubility under the basic conditions required for *N*-diazoniumdiolate formation.[34–36] Additionally, the effectiveness of chitosan polysaccharides is a concern due to insolubility under physiological conditions.[37],[38] To obtain *N*-diazoniumdiolate-functionalized chitosan derivatives with greater NO storage, we synthesized chitosan oligosaccharides that are soluble under both neutral and basic conditions and highly effective against *Pseudomonas aeruginosa* biofilms.

## EXPERIMENTAL

### Materials and Methods

Medium molecular weight chitosan, 2-methyl aziridine (MAz), rhodamine B isothiocyanate (RITC), poly(ethylene glycol) methyl ether acrylate (average  $M_n = 480$ ) (PEG), fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), phenazine methosulfate (PMS), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), trypsin, phosphate buffered saline (PBS), and penicillin streptomycin (PS) were purchased from the Aldrich Chemical Company (Milwaukee, WI). *Pseudomonas aeruginosa* (ATCC #19143) was obtained from the American Type Culture Collection (Manassas, VA). Tryptic soy broth (TSB) and Tryptic soy agar (TSA) are purchased from Becton, Dickinson, and Company (Franklin Lakes, NJ). L929 mouse fibroblasts (ATCC #CCL-1) were obtained from the University of North Carolina Tissue Culture Facility (Chapel Hill, NC). Distilled water was purified with a Millipore Milli-Q

Gradient A-10 water purification system (Bedford, MA). Syto 9 green fluorescent nucleic acid stain was purchased from Life Technologies (Grand Island, NY). Common laboratory salts and solvents were purchased from Fisher Scientific (Pittsburgh, PA). All materials were used as received without further purification unless noted otherwise. Nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz Bruker instrument. Elemental (carbon, hydrogen, and nitrogen or CHN) analysis was performed using a PerkinElmer Elemental Analyzer Series 2400 instrument (Waltham, MA).

### Synthesis of Chitosan Oligosaccharides

Chitosan oligosaccharides were prepared by oxidative degradation using hydrogen peroxide. Medium molecular weight chitosan (2.5 g) was suspended in a hydrogen peroxide solution (15 or 30 wt%) under stirring for 1 h at 65–85 °C. Following the removal of undissolved chitosan by filtration, chitosan oligosaccharides were precipitated from solution by adding acetone to the filtrate. The precipitate was collected by centrifugation, washed twice with ethanol, and dried under vacuum at room temperature. The viscosity of the chitosan oligosaccharides was measured in a solution of NaCl (0.20 M) and CH<sub>3</sub>COOH (0.10 M) at 25 °C using an Ubbelohde capillary viscometer. Oligosaccharide molecular weight was determined using the classic Mark-Houwink equation (i.e.,  $[\eta]=1.81\times 10^{-3} M^{0.93}$ ).[39]

### Synthesis of Secondary Amine-Functionalized Chitosan Oligosaccharides (Scheme 1)

2-methyl aziridine (MAz) grafted chitosan oligosaccharides were synthesized following a previously reported procedure.[40] Briefly, a mixture of concentrated HCl (11  $\mu$ L), water (100  $\mu$ L) and MAz with a 1:1 (**Chitosan 1**) or 2:1 (**Chitosan 2**) molar ratio to primary amines on the chitosan oligosaccharides was added dropwise to a solution of chitosan oligosaccharides (100 mg) in deionized water (5 mL). The resulting solution was stirred at room temperature for 5 d, and then at 75 °C for 24 h. The product was purified by dialysis and collected by lyophilization. Any high molecular weight poly(2-methyl aziridine) in the product was removed by washing with methanol, and the resulting material was dried under vacuum at room temperature. **Chitosan 2** was then dissolved in water at pH 10.0. The primary amine on the chitosan oligosaccharides was functionalized by adding poly(ethylene glycol) methyl ether acrylate to generate **Chitosan 3**. The resulting PEG-functionalized chitosan oligosaccharide derivative was purified by dialysis and collected by lyophilization.

<sup>1</sup>H NMR data of **Chitosan 1** and **Chitosan 2** (400 MHz, CD<sub>3</sub>OD,  $\delta$ ): 0.8–1.1 (NH<sub>2</sub>CH(CH<sub>3</sub>)CH<sub>2</sub>NH), 1.9 (C7: CHNHCOCCH<sub>3</sub>), 2.3–2.7 (NH<sub>2</sub>CH(CH<sub>3</sub>)CH<sub>2</sub>NHCH, C2: NH<sub>2</sub>CH(CH<sub>3</sub>)CH<sub>2</sub>NHCH), 3.3–4.0 (C3, C4, C5, C6: OHCH, OCHCH(OH)CH(NH<sub>2</sub>)CH, OHCH<sub>2</sub>CH, OHCH<sub>2</sub>CH), 4.4 (C1: OCH(CHNH<sub>2</sub>)O). <sup>1</sup>H NMR data of **Chitosan 3** (400 MHz, CD<sub>3</sub>OD,  $\delta$ ): 0.8–1.1 (NH<sub>2</sub>CH(CH<sub>3</sub>)CH<sub>2</sub>NH), 1.9 (C7: CHNHCOCCH<sub>3</sub>), 2.3–2.7 (NH<sub>2</sub>CH(CH<sub>3</sub>)CH<sub>2</sub>NHCH, C2: NH<sub>2</sub>CH(CH<sub>3</sub>)CH<sub>2</sub>NHCH), 3.2 (OCH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>), 3.3–4.0 (OCH<sub>2</sub>CH<sub>2</sub>O and C3, C4, C5, C6: OHCH, OCHCH(OH)CH(NH<sub>2</sub>)CH, OHCH<sub>2</sub>CH, OHCH<sub>2</sub>CH), 4.4 (C1: OCH(CHNH<sub>2</sub>)O).

### Synthesis of N-Diazeniumdiolate-Functionalized Chitosan Oligosaccharides

Secondary amine-functionalized chitosan oligosaccharides (**Chitosan 1**, **Chitosan 2**, and **Chitosan 3**) and 5.4 mM sodium methoxide (75  $\mu$ L) were added to a methanol/water mixture (2 mL) of different v/v ratios (e.g., 1:0, 9:1, 8:2, 7:3, 6:4). The suspension was added to vials in a Parr hydrogenation vessel, which was purged rapidly (5–10 s) with argon three times followed by three longer argon purge cycles (10 min) to remove residual oxygen from the solution. The Parr hydrogenation vessel was then pressurized to 10 atm with NO gas purified over KOH pellets (to remove NO degradation products) and maintained at 10 atm for 3 d. The same argon purging protocol was performed to remove unreacted NO and degradation products from the solution prior to removing the vials from the vessel.

## Characterization of Nitric Oxide Storage and Release

*N*-diazoniumdiolate-functionalized chitosan oligosaccharides (1 mg) (**Chitosan 1/NO**, **Chitosan 2/NO**, **Chitosan 3/NO**) in the water/methanol mixture were added into a sample vessel containing 30 mL deoxygenated phosphate buffered saline (PBS) (10 mM, pH = 7.4) at 37 °C, which initiated NO release. To quantify the NO released, the solution was purged with nitrogen at a flow rate of 70 mL/min to carry the liberated NO to the analyzer. Additional nitrogen flow was supplied to the vessel to match the collection rate of the instrument (200 mL/min). The analysis of NO was terminated when the NO release levels fell to below 10 ppb NO/mg chitosan oligosaccharides. Chemiluminescence data for the NO-releasing chitosan oligosaccharides were represented as: 1) total amount of NO release ( $t[\text{NO}]$ ,  $\mu\text{mol NO/mg}$  of secondary amine-functionalized chitosan oligosaccharides); 2) the maximum flux of NO release ( $[\text{NO}]_{\text{max}}$ , ppb/mg of secondary amine-functionalized chitosan oligosaccharides); and 3) the half-life of NO release ( $t_{1/2}$ ).

## Synthesis of Fluorescently-Labeled Chitosan Oligosaccharides

Fluorescently-labeled chitosan oligosaccharides were prepared following a previously reported procedure.[41] Briefly, chitosan oligosaccharides (50 mg) were dissolved in water (2 mL) at pH 9.0. Rhodamine B isothiocyanate (RITC) was added to the solution in a 1:100 molar ratio to the primary amine of the chitosan oligosaccharides prior to the grafting of 2-methyl aziridine. The solution was stirred at room temperature for 3 d in the dark. Subsequent dialysis and lyophilization yielded the RITC-labeled chitosan oligosaccharides.

## Bactericidal Assays against Planktonic *Pseudomonas aeruginosa*

*P. aeruginosa* bacterial cultures were grown from a frozen (−80 °C) stock overnight in TSB at 37 °C. A 500  $\mu\text{L}$  aliquot of the resulting suspension was added into 50 mL fresh TSB and incubated at 37 °C for ~2 h until the concentration reached  $\sim 1 \times 10^8$  colony forming units (CFU)/mL, as confirmed by the  $\text{OD}_{600}$ , replicate plating and enumeration on nutrient agar. A working bacterial stock was generated by plating the bacterial suspension on TSA and incubating at 37 °C overnight. The TSA bacterial stocks were prepared weekly and stored at 4 °C. For bactericidal assays, colonies of *P. aeruginosa* were taken from the TSA plate, dispersed in 3 mL TSB, and incubated at 37 °C overnight. A 500  $\mu\text{L}$  aliquot of culture was added to 50 mL fresh TSB and incubated to a concentration of  $\sim 1 \times 10^8$  CFU/mL. The bacteria was collected by centrifugation, resuspended in PBS, and diluted to  $\sim 1 \times 10^6$  CFU/mL. The bactericidal efficacy of NO-releasing chitosan oligosaccharides against *P. aeruginosa* was evaluated by incubating the bacteria suspension with NO-releasing chitosan oligosaccharides at 37 °C. At 4 h, 100  $\mu\text{L}$  aliquots of the bacterial suspensions were removed, diluted 10-fold in PBS, plated on TSA, and incubated overnight at 37 °C. The minimum concentration of NO-releasing chitosan oligosaccharides that resulted in a 3-log reduction of bacterial viability was defined as the minimum bactericidal concentration (MBC) for planktonic studies.

## Growth of *P. aeruginosa* Biofilms

A CDC bioreactor (Biosurface Technologies, Bozeman, MT) was used to grow *P. aeruginosa* biofilms over a 48 h period. Briefly, medical grade silicone rubber substrates were mounted in coupon holders prior to assembling the reactor. The assembled reactor was then autoclaved. The reactor effluent line was clamped, and 1% (v/v) sterile TSB (500 mL) was added aseptically. *P. aeruginosa* was then cultured in TSB to  $10^8$  CFU/mL. The reactor was inoculated with an aliquot (1 mL) of this bacterial suspension at a final concentration  $\sim 2 \times 10^5$  CFU/mL. The reactor was incubated at 37 °C for 24 h with slow stirring (150 rpm). Following this “batch phase” growth, the reactor media was refreshed continuously with 0.33% (v/v) TSB at 6 mL/min for another 24 h through the effluent line.

## Treatment of *P. aeruginosa* Biofilms with Chitosan Oligosaccharides

*P. aeruginosa* biofilms grown on silicone rubber substrates were exposed to chitosan oligosaccharide in PBS with slight agitation (37 °C, 24 h) to determine the minimum bactericidal concentration (MBC) necessary to elicit a 5-log reduction in viability. At 24 h, samples were then sonicated and vortexed to disrupt the biofilm. Aliquots (100 µL) of the bacteria/chitosan suspensions were diluted and plated on TSA. After incubating the TSA plates overnight at 37 °C, bacteria viability was determined by counting observed colonies. Of note, the limit of detection for this selected plate counting method is  $2.5 \times 10^3$  CFU/mL. As such, biofilm growth conditions were selected to accurately represent a 5-log reduction in viability for biofilms.

## Confocal Microscopy

*P. aeruginosa* was cultured in TSB to a concentration of  $\sim 1 \times 10^8$  CFU/mL, collected via centrifugation ( $3645 \times g$  for 10 min), resuspended in sterile PBS, and adjusted to  $\sim 1 \times 10^6$  CFU/mL. Aliquots of the bacteria solution were incubated in a glass bottom confocal dish for 1.5 h at 37 °C. A Zeiss 510 Meta inverted laser scanning confocal microscope with a 543 nm HeNe excitation laser and a LP 585 nm filter was used to obtain fluorescence images of the rhodamine B isothiocyanate (RITC)-modified chitosan oligosaccharides. The bright field and fluorescence images were collected by a N.A. 1.2 C-apochromat water immersion lens with a 40x objective. Solutions of RITC-labeled NO-releasing chitosan oligosaccharides in PBS (1.5 mL) were added to the bacteria solution (1.5 mL) in the glass confocal dish to achieve a final concentration of 150 µg/mL. Images were collected every 2 min to characterize the association, if any, of the chitosan oligosaccharides with *P. aeruginosa* temporally. To observe the association of chitosan oligosaccharides with bacteria within biofilms, established biofilms stained with syto 9 (10 µM) were incubated with RITC-labeled chitosan oligosaccharides (150 µg/mL in PBS) for 2.5 h. Prior to imaging, samples were rinsed with PBS (3x). A Zeiss 510 Meta inverted laser scanning confocal microscope with 488 nm Ar and 543 nm HeNe excitation lasers, and a BP 505–530 nm and LP 585 nm filters, respectively, was used to obtain all confocal images. Fluorescence images were collected with a 20x objective.

## In Vitro Cytotoxicity Testing of NO-releasing Chitosan Oligosaccharides

L929 mouse fibroblasts were grown in DMEM supplemented with 10% v/v fetal bovine serum (FBS) and 1 wt% penicillin/streptomycin, and incubated in 5% v/v CO<sub>2</sub> under humidified conditions at 37 °C. After reaching confluency (80%), the cells were trypsinized, seeded onto tissue-culture treated polystyrene 96-well plates at a density of  $3 \times 10^4$  cells/mL, and incubated at 37 °C for 48 h. The supernatant was then aspirated prior to adding fresh DMEM (200 µL) and the NO-releasing chitosan oligosaccharides solution in PBS (50 µL) to each well. After incubation at 37 °C for 24 h, the supernatant was aspirated and a mixture of DMEM/MTS/PMS (105/20/1, v/v/v) (120 µL) was added to each well. The absorbance of the resulting colored solution after 1.5 h incubation at 37 °C was quantified using a ThermoScientific Multiskan EX plate reader at 490 nm. The mixture of DMEM/MTS/PMS and untreated cells were used as a blank and control, respectively. Cell viability was calculated as follows (Eq. 1).

$$\text{Cell Viability} = \frac{(\text{Absorbance}_{\text{treated cell}} - \text{Absorbance}_{\text{blank}})}{(\text{Absorbance}_{\text{untreated cell}} - \text{Absorbance}_{\text{blank}})} \quad \text{Eq.1}$$



## RESULTS AND DISCUSSION

Chitosan has been widely used in antitumor, antimicrobial, and wound healing applications due to favorable biocompatibility and the ability to slowly degrade into nontoxic byproducts. [42–45] This polymer has also been modified to store and release NO by functionalizing the primary amines with NO to form *N*-diazoniumdiolates. [34–36] Unfortunately, the NO storage is rather modest (~0.2  $\mu\text{mol}/\text{mg}$ ) due to poor solubility of polysaccharides in basic solutions necessary for NO donor formation. [34–36] To synthesize *N*-diazoniumdiolate-functionalized chitosan derivatives with improved NO storage, we prepared water-soluble chitosan oligosaccharides by the degradation of chitosan polysaccharides in hydrogen peroxide. An additional benefit of the low-molecular weight ( $\text{MW} < 8000$ ) chitosan oligosaccharides involves their ability to more easily penetrate biofilms. [41, 46] In this regard, we predict that low-molecular weight *N*-diazoniumdiolate-modified chitosan oligosaccharides would have even greater bactericidal activity compared to the polysaccharides.

### Synthesis of Secondary Amine-Functionalized Chitosan Oligosaccharides

A number of strategies have been reported for degrading chitosan into oligomer derivatives. [40, 47] Rather than use enzymatic degradation requiring costly enzymes (e.g., chitosanase), we adapted an oxidative degradation strategy using hydrogen peroxide to synthesize chitosan oligosaccharides. [40] Control of the molecular weight ( $M_w$ ) was achieved by varying the concentration of hydrogen peroxide and degradation temperature. The viscosity of the chitosan oligosaccharides was determined in a solution of sodium chloride (0.20 M) and acetic acid (0.10 M) using an Ubbelohde capillary viscometer. [39] In combination with the Mark-Houwink equation (i.e.,  $[\eta] = 1.81 \times 10^{-3} M^{0.93}$ ), molecular weights were determined as a function of processing conditions. Collectively, larger concentrations of hydrogen peroxide and elevated degradation temperatures led to lower molecular weight chitosan. As shown in Table 1, chitosan oligosaccharides of ~10 kD molecular weight were prepared in 15 wt% hydrogen peroxide at 65 °C for 1 h. Increasing the degradation temperature to 85 °C resulted in significantly smaller size ( $\text{MW} \sim 5$  kD). When both a larger concentration of hydrogen peroxide (i.e., 30 wt%) and elevated temperature (i.e., 85 °C) were adopted, the molecular weight of chitosan oligosaccharides decreased further (~ 2.5 kD) were achieved. As shown in Table 2, the CHN elemental analysis of the oligosaccharides indicated an overall nitrogen content of 6.3 wt%.

To enhance NO storage (secondary amines are more readily converted to NO form due to their increased basicity [22, 48, 49]), the primary amines on the synthesized chitosan oligosaccharides were thus modified by a cationic ring opening of MAz to produce secondary amine functionalities (Scheme 1A). By tuning the ratio of MAz and primary amine (e.g., 1:1 **Chitosan 1**, 2:1 **Chitosan 2**), the number of MAz repeating units grafted onto the chitosan oligosaccharides proved tunable (as verified by NMR), leading to a range of secondary amine concentrations and NO storage. To investigate how the local environment surrounding the *N*-diazoniumdiolate NO donors affected the NO-release kinetics, [22] acrylate-functionalized PEG chains were conjugated to the primary amines on **Chitosan 2** by the Michael addition reaction to yield PEG-modified scaffolds (e.g., **Chitosan 3**, Scheme 1B). Grafting of 2-methyl aziridine to the oligosaccharides increased the corresponding nitrogen content from 6.3 to 8.9 and 10.8 wt% for **Chitosan 1** and **Chitosan 2**, respectively. As expected, the PEGylation of **Chitosan 2** led to a corresponding decrease in nitrogen content (3.1 wt%) (**Chitosan 3**).

## Synthesis of *N*-Diazoniumdiolate-Modified Chitosan Oligosaccharides

Reaction of the secondary amine-functionalized chitosan oligosaccharides (**Chitosan 1**, **Chitosan 2**, and **Chitosan 3**) with NO (10 atm under basic conditions) yielded *N*-diazoniumdiolate NO donor-functionalized chitosan oligosaccharides (**Chitosan 1/NO**, **Chitosan 2/NO**, and **Chitosan 3/NO**). The NO conjugation (“charging”) solvent dictates the charging efficiency and thus total NO storage.[22] Aqueous solutions were necessary in order to adequately dissolve the chitosan oligosaccharides. Despite the high pH that should inhibit NO donor breakdown,[49] NO donor formation due to the presence of water was a concern. To examine the influence of water concentration on *N*-diazoniumdiolate conversion efficiency, mixtures of methanol (a common charging solvent)[22, 50] and water were prepared (10:0, 9:1, 8:2, 7:3, and 6:4 v/v) and the pH was adjusted to above 10 by adding sodium methoxide.

**Chitosan 2/NO-5k** was used as a representative oligosaccharide for these studies with the assumption that lower and greater MW chitosan oligosaccharides would behave similarly. The NO release profile for **Chitosan 2/NO-5k** as a function of the charging solvent are shown in Figure 1. Specific NO-release parameters (e.g., total NO release, maximum flux, and half-life) were extracted from these profiles and are provided in Table 3. The NO storage increased with increasing water/methanol ratios up to 7:3. The maximum NO storage (using the 7:3 methanol/water charging solvent ratio) was 0.87  $\mu\text{mol}/\text{mg}$ , roughly 4x larger than that for previously reported chitosan polysaccharides ( $\sim 0.2 \mu\text{mol}/\text{mg}$ ).[39–41] The improved NO storage may be attributed to the enhanced solubility of **Chitosan 2–5k** in the charging solvents (e.g., methanol/water 9:1, 8:2, 7:3) relative to methanol. In charging solvent where **Chitosan 2–5k** was essentially insoluble (e.g., methanol), only secondary amines on the exterior of the **Chitosan 2–5k** precipitate were accessible to NO, leading to a lower NO donor formation efficiency. In contrast, complete dissolution exposes a significantly greater proportion of secondary amines to the base and NO. At larger water concentrations (i.e., methanol/water 6:4 v/v or 40% water), the NO storage decreased slightly ( $\sim 0.76 \mu\text{mol}/\text{mg}$ ), suggesting destabilization of the *N*-diazoniumdiolate NO donor by water. **Chitosan 2/NO-5k** exhibited similar NO-release kinetics (e.g., half-life of  $\sim 2$  h) regardless of the charging solvent, indicating that while the chemical structure (e.g., the local environment surrounding *N*-diazoniumdiolates) of the NO-releasing scaffolds dictates NO-release kinetics, variation in charging solvent only affects the total NO storage. Overall, the NO-release data revealed that the optimal charging solvent was methanol/water 7:3 (v/v). This solvent composition was used for the functionalization of the other secondary amine-functionalized chitosan oligosaccharides (**Chitosan 1–5k** and **Chitosan 3–5k**).

The antibacterial efficacy of NO-releasing materials has been reported to be dependent on both NO storage (i.e., payload) and release kinetics.[51] 2-methyl aziridine (MAz) was grafted onto the chitosan oligosaccharides at different feed ratios (i.e., 2:1 and 1:1) to alter the secondary amine functionalization and NO storage. As expected, increasing the feed ratio of 2-methyl aziridine to primary amines from 1:1 (**Chitosan 1–5k**) to 2:1 (**Chitosan 2–5k**) resulted in greater NO storage (e.g.,  $\sim 0.30$  to  $0.87 \mu\text{mol}/\text{mg}$ , respectively). As shown in Figure 2, the NO flux and storage of **Chitosan 1/NO-5k** were lower than **Chitosan 2/NO-5k**, a result that is attributable to the smaller amine concentration of **Chitosan 1–5k** ( $\sim 8.9$  wt%) compared to **Chitosan 2–5k** ( $\sim 10.8$  wt%).

To tune the NO-release kinetics of chitosan oligosaccharides further, **Chitosan 2–5k** was modified with acrylate-functionalized PEG chains (**Chitosan 3–5k**). As expected, hydrophilic functionalization of the oligosaccharides with PEG facilitated a more aqueous local environment and result in both larger initial NO flux ( $\sim 12600$  ppb/mg) and faster overall NO release (half-life  $\sim 0.15$  h) relative to **Chitosan 2–5k** (Figure 2).[22] The

PEGylation of primary amines on **Chitosan 2-5k** also affected its ionic characteristics by shielding the amine moieties, a key factor influencing the association of chitosan-based materials with bacteria.[38] The distinct NO-release kinetics and ionic characteristics of the resulting NO-releasing chitosan oligosaccharides allowed for the study of how surface charge and NO release affect anti-biofilm activity.

### Bactericidal Studies: Planktonic Bacteria

Control and NO-releasing chitosan oligosaccharides (e.g., **Chitosan 1/NO -5k**, **Chitosan 2/NO-5k**, **Chitosan 3/NO -5k**) were exposed to Gram-negative *P. aeruginosa*, a pathogen involved in infections associated with burn wounds and cystic fibrosis, to evaluate their ability to kill bacteria.[5, 52] Bacterial viability assays were performed under static conditions to determine the concentration of chitosan required to reduce bacteria viability by 3 logs (i.e., 99.9% killing), which hereafter will be referred to as the minimum bactericidal concentration or MBC. The amount of NO delivered from NO-releasing chitosan oligosaccharides (Table 4) over the time of the assay (4 h) was also examined to quantitatively assess the NO dose necessary for 99.9% bacterial killing. Both MBCs and the bactericidal NO doses required for the chitosan oligosaccharides are provided in Table 5. Each of the NO-releasing chitosan oligosaccharides studied (**Chitosan 1/NO-5k**, **Chitosan 2/NO-5k** and **Chitosan 3/NO-5k**) resulted in 99.9% killing of *P. aeruginosa*. At equivalent concentrations, the control (non-NO-releasing) chitosan did not lead to a significant reduction in bacterial viability, indicating NO as the bactericidal agent (data not shown). Further inspection of the bactericidal NO doses in Table 5 reveals that greater NO levels were required from **Chitosan 3/NO-5k** compared to **Chitosan 1/NO-5k** and **Chitosan 2/NO-5k** to kill *P. aeruginosa*. This behavior may be attributed to decreased interaction between the chitosan oligosaccharides and bacteria membrane. Sun et al. previously reported that positively charged dendrimers associated more readily with bacteria than neutral dendrimers. enhanced bactericidal action was the result of such interactions.[23] The less effective bacteria killing observed for **Chitosan 3/NO-5k** could stem from the ability of the neutral PEG chains to shield the amine moieties. In contrast, **Chitosan 1/NO-5k** and **Chitosan 2/NO-5k** have positively charged primary amines at pH 7.4 on their exterior that enhance their association with the bacteria and facilitate more localized NO delivery.

To further confirm that the lessened antibacterial activity of **Chitosan 3/NO-5k** was the result of decreased interactions with the bacteria membrane, confocal microscopy was utilized to compare the association kinetics of **Chitosan 3/NO-5k** and **Chitosan 2/NO-5k** with bacteria. Rhodamine B isothiocyanate (RITC)-labeled **Chitosan 2/NO-5k** and **Chitosan 3/NO-5k** were synthesized as previously reported.[41] The potential impact of RITC on chitosan-bacteria association was minimized by using small concentration of RITC (i.e., in 1:100 molar ratio to total primary amines). The degree of association of the NO-releasing chitosan oligosaccharides with bacteria was then followed by measuring red fluorescence surrounding the bacteria. As expected, **Chitosan 2/NO-5k** associated with the bacteria more rapidly (within 24 min) than **Chitosan 3/NO-5k** (86 min) (Figure 3). The fluorescence from **Chitosan 2/NO-5k** at 42 min was significantly greater than that of **Chitosan 3/NO-5k** at 110 min, further demonstrating that **Chitosan 3/NO-5k** associated with the bacteria at a much slower rate due to the PEG (neutral) modification. Further inspection of **Chitosan 2/NO-5k** and **Chitosan 3/NO-5k** association with *P. aeruginosa* revealed enhanced bacteria association for **Chitosan 2/NO-5k** (Figure 3-G, H), confirming the benefits of the cationic/positive properties of **Chitosan 2/NO-5k** over **Chitosan 3/NO-5k**.



In addition to charge effect, we sought to study the role of molecular weight in *P. aeruginosa* killing. Chitosan oligosaccharides of ~2.5, 5, and 10 kD were synthesized and functionalized with NO (**Chitosan 2/NO-2.5 k**, **Chitosan 2/NO-5 k**, **Chitosan 2/NO-10 k**). As expected, these materials exhibited similar NO-release kinetics (Table 4) due to the similar nitrogen content, allowing for the study of how molecular weight impacts bactericidal efficacy. Regardless of size (i.e., molecular weight), each of the NO-releasing chitosan oligosaccharides (**Chitosan 2/NO-2.5 k**, **Chitosan 2/NO-5 k**, **Chitosan 2/NO-10 k**) exhibited similar bactericidal NO concentrations (i.e., ~10  $\mu\text{mol NO/mL}$ ) for 3-log killing (Table 5).

### Bactericidal Studies: Biofilms Eradication

As previously reported, NO-releasing scaffolds have proven effective against both planktonic and biofilm bacteria. [20, 23, 53, 54] For example, Barraud et al. reported that exposing established biofilms to NO (from small molecule NO donors) greatly enhanced the anti-biofilm efficacy of antimicrobial compounds including tobramycin, hydrogen peroxide, and sodium dodecyl sulfate.[54] Hetrick et al. demonstrated the use of macromolecular NO-releasing scaffolds (e.g., *N*-diazoniumdiolate-modified silica particles) to eradicate a broad-spectrum of biofilms with comparable or reduced toxicity (~80% viability reduction) against mammalian cells versus currently administered antiseptics (e.g., povidone iodine or chlorhexidine).[53] Based on these prior studies, we expected the NO-releasing chitosan oligosaccharides to also be effective against biofilms with perhaps less toxicity against mammalian cells than other scaffolds (e.g., silica, dendrimers).[55]

To evaluate the anti-biofilm activity of NO-releasing chitosan oligosaccharides (e.g., **Chitosan 1/NO-5k**, **Chitosan 2/NO-5k**, **Chitosan 3/NO-5k**), *P. aeruginosa* biofilms were exposed to 0.2–1.3 mg/mL NO-releasing chitosan oligosaccharides for 24 h (corresponding to ~0.17–0.46  $\mu\text{mol NO/mL}$ ). After treatment, the biofilms were removed from the silicone rubber substrates by vortexing and sonication to enable viability quantification.[54] Control experiments were performed to confirm the growth of *P. aeruginosa* biofilms using the selected protocol. As shown in Figure 4, the viability of *P. aeruginosa* in the biofilm was  $\sim 2 \times 10^8$  CFU when exposed only to PBS. The chitosan concentrations for 5-log reduction of biofilm bacteria viability (MBC) were 400, 700, and 1000  $\mu\text{g/mL}$  for **Chitosan 2/NO-5k**, **Chitosan 1/NO-5k**, and **Chitosan 3/NO-5k**, respectively. **Chitosan 2/NO-5k** exhibited the greatest anti-biofilm efficacy, a likely result due to both increased NO storage/release and rapid association with the negatively charged bacteria.

Although **Chitosan 1/NO-5k** and **Chitosan 3/NO-5k** stored similar levels of NO (~0.3  $\mu\text{mol/mg}$ ), **Chitosan 1/NO-5k** was more effective at eradicating the biofilm bacteria (MBC 700  $\mu\text{g/mL}$ ) compared to **Chitosan 3/NO-5k** (MBC 1000  $\mu\text{g/mL}$ ). The decreased antibiofilm efficacy for **Chitosan 3/NO-5k** may result from the shielding of the amine moieties by the neutral PEG chains, thus impeding association with the negatively charged exterior of the bacteria. To confirm this hypothesis, the association of **Chitosan 2/NO-5k** and **Chitosan 3/NO-5k** with *P. aeruginosa* biofilm was evaluated using confocal microscope. As shown in Figure 5, biofilms exposed to **Chitosan 2/NO-5k** exhibited more intense red fluorescence compared to **Chitosan 3/NO-5k**, again confirming the enhanced association of the positively charged **Chitosan 2/NO-5k** with the bacteria. The efficient association of chitosan oligosaccharides with bacteria in biofilms is a great advantage over previously reported NO-releasing polysaccharides which are not expected to penetrate the exopolysaccharides matrix due to their insolubility under physiological conditions.

Although chitosan molecular weight was not observed to play a significant role in planktonic killing, less effective bacteria killing was observed when using **Chitosan 2/**

**NO-10k**, the largest chitosan oligosaccharides (600  $\mu\text{g}/\text{mL}$  vs. 400  $\mu\text{g}/\text{mL}$  for **Chitosan 2/NO-10k** and **Chitosan 2/NO-2.5k**) against biofilms. The exopolysaccharides matrix is likely slowing the diffusion of the largest chitosan structure. Takenaka et al., previously reported less efficient EPS penetration of high (vs low)-molecular weight dextran,[55] further supporting this hypothesis that the impeded diffusion of **Chitosan 2/NO-10k** led to slow association with bacteria within the biofilms (Figure 5). Control chitosan oligosaccharides (e.g., **Chitosan 1**, **Chitosan 2**, and **Chitosan 3**) did not lead to a significant reduction in bacterial viability at the same concentrations as their NO-releasing counterparts, indicating that the observed bacterial killing was due to NO from the *N*-diazoniumdiolate-functionalized chitosan oligosaccharides rather than the chitosan scaffolds themselves.

### Cytotoxicity of NO-releasing Chitosan Oligosaccharides to Mammalian Fibroblasts

The benefit of utilizing chitosan as NO-releasing vehicles includes its non-toxic nature to mammalian cells. The cytotoxicity of control and NO-releasing chitosan oligosaccharides were compared by exposing mouse fibroblast cells to the oligosaccharides at the MBCs against *P. aeruginosa* biofilms noted above.

The normalized cell viabilities of control and NO-releasing chitosan oligosaccharides after 24 h incubation are shown in Figure 6. Regardless of size (i.e., molecular weight), the control and NO-releasing chitosan oligosaccharides were non-toxic against mouse fibroblast cells at the MBCs for the NO-releasing scaffolds, indicating an advantage of these materials as anti-biofilm agents compared to other antibacterial agents.[53] Of note, the NO-releasing chitosan oligosaccharides exhibited lower cytotoxicity than the chitosan controls, corroborating previous reports that certain levels of NO may promote cell proliferation.[56, 57] Indeed, treatment of mouse lung endothelial cells and human aortic endothelial cells with small molecule NO donors such as *S*-nitroso-L-glutathione (GSNO), *S*-nitroso-N-acetylpenicillamine (SNAP), *N*-diazoniumdiolate-diethyltriamine (DETA/NO) has been shown to promote proliferation.[58, 59] Collectively, the antimicrobial activity against planktonic and biofilm bacterial and the lack of toxicity of NO-releasing chitosan oligosaccharides suggests that NO-releasing chitosan oligosaccharides may serve as ideal antimicrobial agents for applications including wound healing and cystic fibrosis.

## CONCLUSION

This study focused on the synthesis of *N*-diazoniumdiolate-modified chitosan oligosaccharides with controlled NO storage and tunable NO-release kinetics. The water solubility of chitosan oligosaccharides allows for both beneficial diffusion into biofilms and subsequent association with bacteria, resulting in efficient eradication of biofilm bacteria at concentrations eliciting minimal toxicity to L929 mouse fibroblast cells. Our study demonstrates the potential of NO-releasing chitosan oligosaccharides as antimicrobial agents. Experiments are underway to evaluate the biocidal efficacy of these materials against a broad spectrum of bacteria strains including methicillin-resistant *Staphylococcus aureus* and cystic fibrosis-related *P. aeruginosa* strains (e.g., mucoid/alginate-producing strains).

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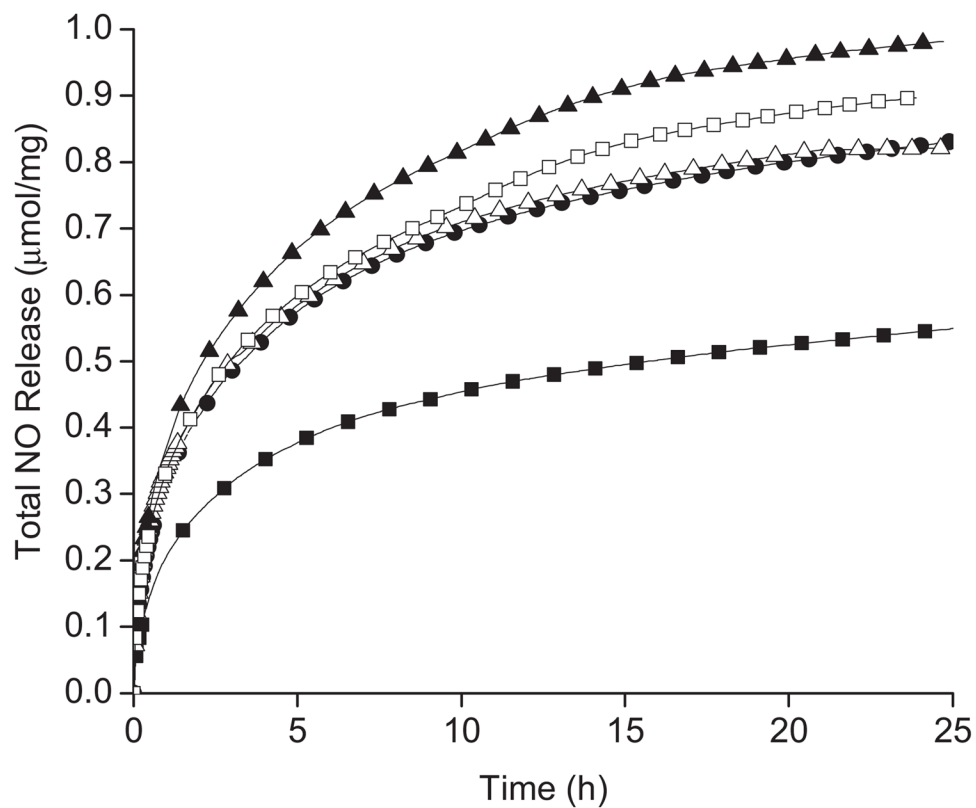
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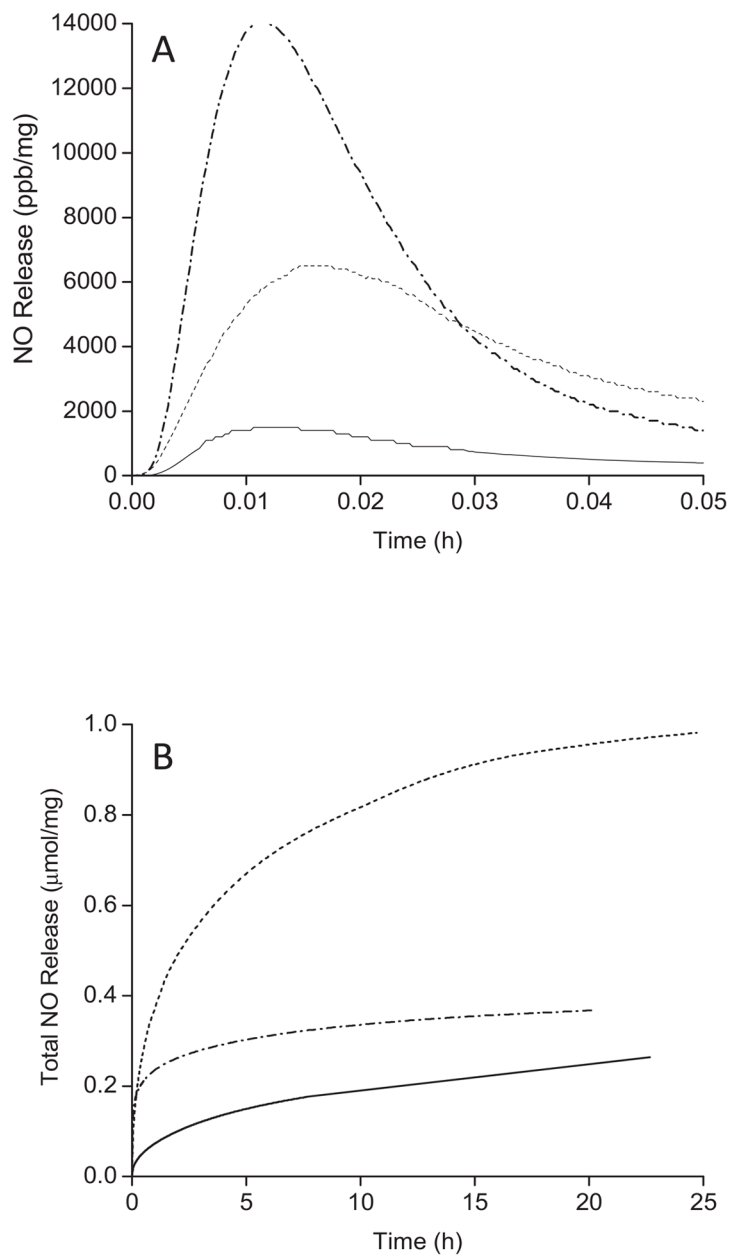
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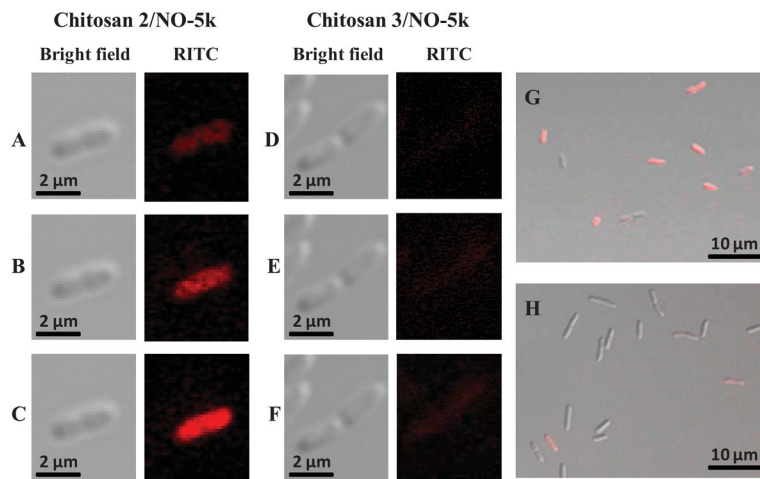




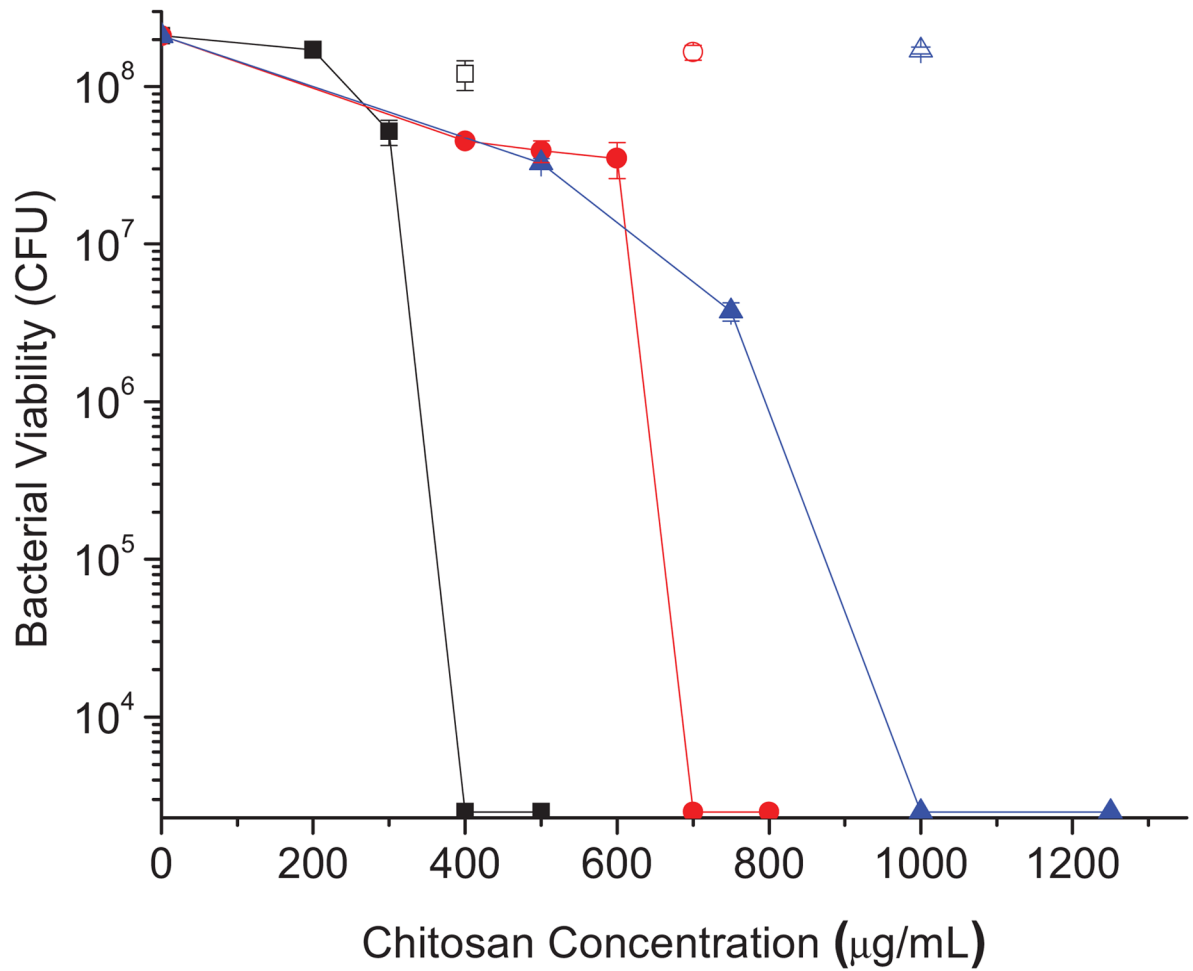
**Figure 1.** Nitric oxide release profiles of **Chitosan 2/NO-5k** in methanol (solid square), methanol/water 9:1 (solid circle), 8:2 (open triangle), 7:3 (solid triangle), and 6:4 v/v (open square).



**Figure 2.** Real-time NO release profiles (A) and plot of  $t[\text{NO}]$  vs time (B) for NO-releasing chitosan oligosaccharides (e.g., **Chitosan 1-5k** (solid line), **Chitosan 2-5k** (dot line), and **Chitosan 3-5k** (dash dot line)).

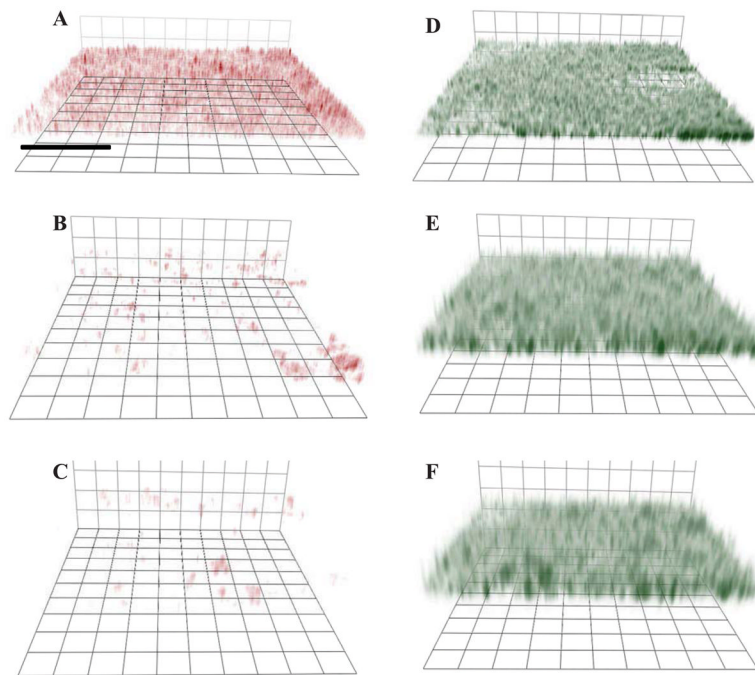


**Figure 3.** Bright field and fluorescent images of RITC-modified **Chitosan 2/NO-5k** ( $150 \mu\text{g mL}^{-1}$ ) at A) 24, B) 28, C) 42 min and **Chitosan 3/NO-5k** ( $150 \mu\text{g mL}^{-1}$ ) at D) 82, E) 86, F) 110, H) 120 min association with *P. aeruginosa*. Overlay images of *P. aeruginosa* incubated with G) **Chitosan 2/NO-5k** at 44 min and H) **Chitosan 2/NO-5k** at 120 min.



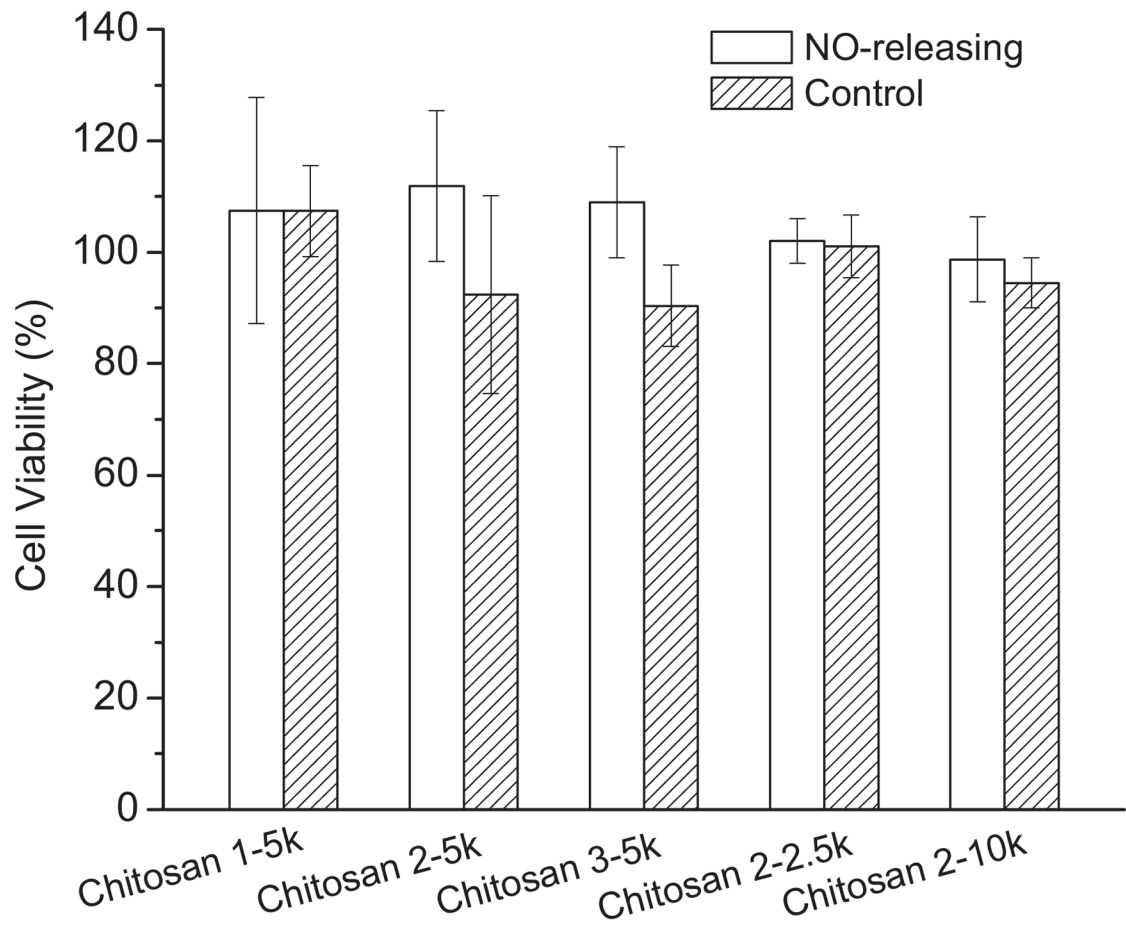
**Figure 4.**

Anti-biofilm efficacy of NO-releasing (solid symbols) and control (open symbols) chitosan oligosaccharides (**Chitosan 1-5k** (sphere), **Chitosan 2-5k** (square), and **Chitosan 3-5k** (triangle)) against established *P. aeruginosa* biofilms. Control chitosan oligosaccharides did not affect the bacteria viability.

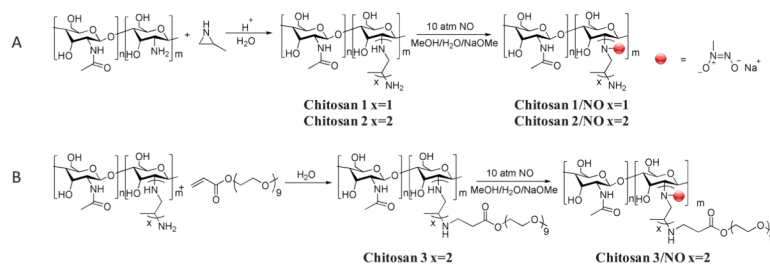


**Figure 5.** Confocal fluorescence images of RITC-labeled chitosan oligosaccharide association with *P. aeruginosa* in biofilms: A) **Chitosan 2/NO-5k**, B) **Chitosan 3/NO-5k**, C) **Chitosan 2-10k** and images of syto 9 labeled biofilms incubated with D) **Chitosan 2/NO-5k**, E) **Chitosan 3/NO-5k** and F) **Chitosan 2/NO-10k**. Scale bar: 40  $\mu\text{m}$ . The green fluorescence of syto 9 indicates that the *P. aeruginosa* bacteria are embedded within the biofilms. Red fluorescence of RITC indicates the association of RITC-labeled chitosan oligosaccharides with *P. aeruginosa* in biofilms.





**Figure 6.** Viability of L929 mouse fibroblasts exposed to control and NO-releasing chitosan oligosaccharides at concentration required to kill biofilm bacteria (MBC). Studies consisted of at least three experiments with error bars representing standard deviation.

**Scheme 1.**

Synthesis of secondary amine- and *N*-diazeniumdiolate-functionalized chitosan oligosaccharide derivatives. A) Grafting of 2-methyl aziridine onto primary amines of chitosan oligosaccharides (**Chitosan 1** and **Chitosan 2**) and subsequent *N*-diazeniumdiolate formation of the resulting materials (**Chitosan 1/NO** and **Chitosan 2/NO**); B) PEGylation of 2-methyl aziridine-grafted-chitosan oligosaccharide (**Chitosan 3**) with NO donor functionalization (**Chitosan 3/NO**).

**Table 1**

Degradation conditions of chitosan oligosaccharides of different molecular weights.

Chitosan oligosaccharides	$M_v^a$	T (°C)	[H <sub>2</sub> O <sub>2</sub> ] (wt%)
2.5 k	2657	85	30
5 k	5370	85	15
10 k	10142	65	15

<sup>a</sup> viscosity average molecular weight as determined by classic Mark-Houwink equation (i.e.,  $[\eta] = 1.81 \times 10^{-3} M^{0.93}$ ).

**Table 2**

Elemental (CHN) analysis of chitosan oligosaccharides and secondary amine-functionalized derivatives.

<b>Materials</b>	<b>C (%)</b>	<b>H (%)</b>	<b>N (%)</b>
Chitosan oligosaccharides <sup>a</sup>	42.2 ± 1.6	6.8 ± 0.1	6.3 ± 0.2
Chitosan 1–5k	43.5 ± 1.2	7.7 ± 0.3	8.9 ± 0.1
Chitosan 2–5k	44.7 ± 1.8	8.4 ± 0.2	10.8 ± 0.8
Chitosan 3–5k	51.0 ± 0.2	9.0 ± 0.2	3.1 ± 0.1
Chitosan 2–2.5k	43.7 ± 0.7	8.5 ± 0.2	10.9 ± 0.1
Chitosan 2–10k	44.7 ± 1.8	8.4 ± 0.2	10.8 ± 0.8

<sup>a</sup> chitosan oligosaccharides before the grafting of 2-methyl aziridine. Each parameter was analyzed with multiple replicates (n=3).

**Table 3**

Influence of charging solvent on nitric oxide-release properties for secondary amine-functionalized chitosan oligosaccharides (**Chitosan 2/NO-5k**) in PBS (pH = 7.4, 37 °C) as measured using a chemiluminescence NO analyzer.

MeOH/H <sub>2</sub> O	10:0	9:1	8:2	7:3	6:4
t[NO] <sup>a</sup> (μmol/mg)	0.58 ± 0.09	0.74 ± 0.12	0.81 ± 0.14	0.87 ± 0.16	0.75 ± 0.18
[NO] <sub>max</sub> (ppb/mg)	2648 ± 120	4150 ± 70	4350 ± 484	5500 ± 414	5000 ± 572
Half-life (h)	2.40 ± 0.13	2.25 ± 0.02	2.05 ± 0.07	2.20 ± 0.14	2.05 ± 0.25

<sup>a</sup> total NO storage per mg chitosan. Each parameter was analyzed with multiple replicates (n=3).



**Table 4**

Nitric oxide-release properties of different *N*-diazoniumdiolate NO donor-functionalized chitosan oligosaccharides in PBS (pH = 7.4, 37 °C) as measured using a chemiluminescence NO analyzer.

Material	t[NO] <sup>a</sup> (μmol/mg)	t[NO] <sup>b</sup> (μmol/mg)	[NO] <sub>max</sub> (ppb/mg)	t <sub>1/2</sub> (h)
Chitosan 1/NO-5 k	0.30 ± 0.04	0.16 ± 0.03	1600 ± 215	3.60 ± 0.13
Chitosan 2/NO-5 k	0.87 ± 0.16	0.52 ± 0.15	5500 ± 414	2.20 ± 0.14
Chitosan 3/NO-5 k	0.35 ± 0.02	0.29 ± 0.01	12600 ± 2121	0.15 ± 0.01
Chitosan 2/NO-2.5 k	0.84 ± 0.04	0.49 ± 0.02	7500 ± 550	2.06 ± 0.10
Chitosan 2/NO-10 k	0.81 ± 0.05	0.47 ± 0.03	7350 ± 672	2.04 ± 0.05

<sup>a</sup> total NO released and

<sup>b</sup> NO released over 24 and 4 h (μmol) per milligram of chitosan oligosaccharides. Charging solvent used was methanol/water 7:3 v/v. Each parameter was analyzed with multiple replicates (n=3).

**Table 5**

Minimum bactericidal concentration (MBC) and NO doses of NO-releasing chitosan oligosaccharides for 3-log reduction in planktonic *P. aeruginosa* viability.

Chitosans	MBC ( $\mu\text{g/mL}$ )	NO dose ( $\mu\text{mol/mL}$ )
Chitosan 1/NO-5k	2000	0.32
Chitosan 2/NO-5k	200	0.10
Chitosan 3/NO-5k	1500	0.45
Chitosan 2/NO-2.5k	250	0.12
Chitosan 2/NO-10k	250	0.12