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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, 10,000) 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*-diazeniumdiolate 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*-diazeniumdiolates (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 (P. aeruginosa)* biofilms, the NO-releasing and control chitosan oligosaccharides elicited no significant cytotoxicity to mouse fibroblast L929 cells *in vitro*.

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1. 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 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 peroxynitrite, 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]diazen-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.

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Biodegradable NO-releasing materials have been developed as implant coatings (e.g., poly(diol 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*-diazeniumdiolate NO donor formation [34–36]. Unfortunately, previously reported *N*-diazeniumdiolate-functionalized chitosan polysaccharides have been characterized by low *N*-diazeniumdiolate conversion efficiency and NO storage (~0.2 μmol/mg), likely the result of chitosan insolubility under the basic conditions required for *N*-diazeniumdiolate formation [34–36]. Additionally, the effectiveness of chitosan polysaccharides is a concern due to insolubility under physiological conditions [37,38]. To obtain *N*-diazeniumdiolate-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.

2. Experimental

2.1. 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). *P. 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).

2.2. 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_3COOH (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].

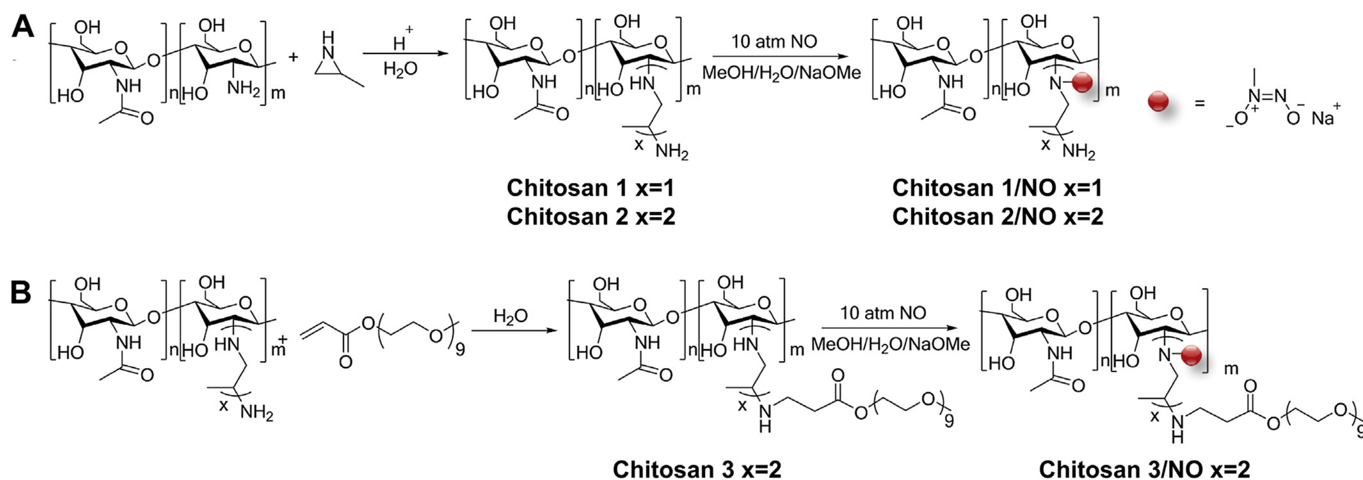
2.3. Synthesis of secondary amine-functionalized chitosan oligosaccharides (Scheme 1)

2-Methyl aziridine (MAz) grafted chitosan oligosaccharides were synthesized following a previously reported procedure [40]. Briefly, a mixture of concentrated HCl (11 μL), water (100 μL) 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.

^{1}H NMR data of **Chitosan 1** and **Chitosan 2** (400 MHz, CD_3OD, δ): 0.8–1.1 ($NH_2CH(CH_3)CH_2NH$), 1.9 (C7: $CHNHCOCH_3$), 2.3–2.7 ($NH_2CH(CH_3)CH_2NHCH$, 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). ^{1}H NMR data of **Chitosan 3** (400 MHz, CD_3OD, δ): 0.8–1.1 ($NH_2CH(CH_3)CH_2NH$), 1.9 (C7: $CHNHCOCH_3$), 2.3–2.7 ($NH_2CH(CH_3)CH_2NHCH$, C2: $NH_2CH(CH_3)CH_2NHCH$), 3.2 ($OCH_2CH_2OCH_3$), 3.3–4.0 (OCH_2CH_2O and 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).

2.4. 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 μL) 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.



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**).

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