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S-Nitrosothiol-modified nitric oxide-releasing chitosan oligosaccharides as antibacterial agents

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ABSTRACT

S-Nitrosothiol-modified chitosan oligosaccharides were synthesized by reaction with 2-iminothiolane hydrochloride and 3-acetamido-4,4-dimethylthietan-2-one, followed by thiol nitrosation. The resulting nitric oxide (NO)-releasing chitosan oligosaccharides stored ~0.3 μmol NO mg^{-1} chitosan. Both the chemical structure of the nitrosothiol (i.e. primary and tertiary) and the use of ascorbic acid as a trigger for NO donor decomposition were used to control the NO-release kinetics. With ascorbic acid, the *S*-nitrosothiol-modified chitosan oligosaccharides elicited a 4-log reduction in *Pseudomonas aeruginosa* viability. Confocal microscopy indicated that the primary *S*-nitrosothiol-modified chitosan oligosaccharides associated more with the bacteria relative to the tertiary *S*-nitrosothiol system. The primary *S*-nitrosothiol-modified chitosan oligosaccharides elicited minimal toxicity towards L929 mouse fibroblast cells at the concentration necessary for a 4-log reduction in bacterial viability, further demonstrating the potential of *S*-nitrosothiol-modified chitosan oligosaccharides as NO-release therapeutics.

1. Introduction

Bacterial infections are a tremendous challenge to human health [1]. Increased resistance to antibiotics has created a demand for alternative antibacterial therapeutics [1–3]. The endogenously produced diatomic free radical nitric oxide (NO) plays a key role in our body's natural immune response to pathogens [4–6]. Both NO and its reactive byproducts (e.g. peroxynitrite and dinitrogen trioxide) exert significant oxidative and nitrosative stress on bacteria to facilitate their eradication [6]. As such, macromolecular NO-releasing scaffolds have been widely developed for use in a number of biomedical applications, many related to pathogen killing [7–17]. The NO delivery efficiency and antibacterial efficacy are greatly dependent on the morphology, size and chemical composition of the NO donor scaffold [9,10,18]. Chitosan-based materials are particularly attractive due to their biocompatibility, biodegradability and high primary amine precursor content for NO donor modification (i.e. *N*-diazeniumdiolate) [19–24]. Wan et al. reported the synthesis of chitosan polysaccharides as scaffolds for NO storage by reaction of primary amines with NO to form *N*-diazeniumdiolate NO donors [25]. Due to the instability of primary amine-derived *N*-diazeniumdiolate NO donors [26], these materials were characterized by low NO storage (i.e. lower than 0.2 μmol mg^{-1}) and short NO-release duration (~1 h) [25]. To prepare NO-releasing chitosan scaffolds with extended release properties, folate-grafted chitosan was synthesized by the condensation of folic acid with primary amines on chitosan polysaccharides [27]. The resulting secondary amines were reacted with NO to form secondary amine-derived *N*-diazeniumdiolates. As expected, the NO release was extended to >10 h due to enhanced stability of the secondary amine-derived *N*-diazeniumdiolate NO donors [26]. However, total NO storage of the folate-grafted chitosan was low (i.e. <80 nmol mg^{-1}) [27], necessitating a large dose of chitosan for complete killing of certain bacteria. Secondary amine-functionalized chitosan oligosaccharides were thus prepared by grafting 2-methyl aziridine from the primary amines of the chitosan oligosaccharides. The solubility of the chitosan oligosaccharides in basic solutions allowed more efficient *N*-diazeniumdiolate NO donor formation, greater total NO storage (e.g. ~0.87 μmol mg^{-1}), and extended release duration (e.g. ~20 h) [28].

In contrast to their *N*-diazeniumdiolate counterparts, *S*-nitrosothiol NO donors can decompose through multiple mechanisms, including photo and thermal irradiation, transnitrosation and chemical reduction [29,30]. Ascorbic acid or vitamin C is a natural antioxidant present in the body, which has demonstrated synergistic antibacterial efficacy with antibiotics, including chloramphenicol, kanamycin, streptomycin and tetracycline, against *Pseudomonas aeruginosa* [31]. As a reducing agent, ascorbic acid allowed for triggered *S*-nitrosothiol decomposition and subsequent NO release [32]. To date, no study has examined the ability to

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prepare *S*-nitrosothiol-modified chitosan or evaluated the potential synergistic antibacterial efficacy of such materials with ascorbic acid as a trigger for NO release.

2. Experimental

2.1. Materials and methods

Medium-molecular-weight chitosan, 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, diethylene triamine pentaacetic acid (DTPA), phosphate buffered saline (PBS) used for cell culture and penicillin streptomycin (PS) solution (10,000 u ml^{-1} penicillin, 10,000 μg ml^{-1} streptomycin) 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) were 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). Common laboratory salts and solvents were purchased from Fisher Scientific (Pittsburgh, PA). Unless noted otherwise, all materials were used as received without further purification. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a 400 MHz Bruker instrument. Elemental analysis was carried out using an inductively coupled plasma (ICP) optical emission spectrometer (Prodigy High Dispersion ICP-OES, Teledyne Leeman Labs, Hudson, NH). The emission intensity at 324.75 nm was monitored for copper. The standard addition of a copper reference standard (TraceCERT®, Sigma–Aldrich, St. Louis, MO) was employed to determine the concentration of dissolved copper in PBS buffer.

2.2. Synthesis of chitosan oligosaccharides

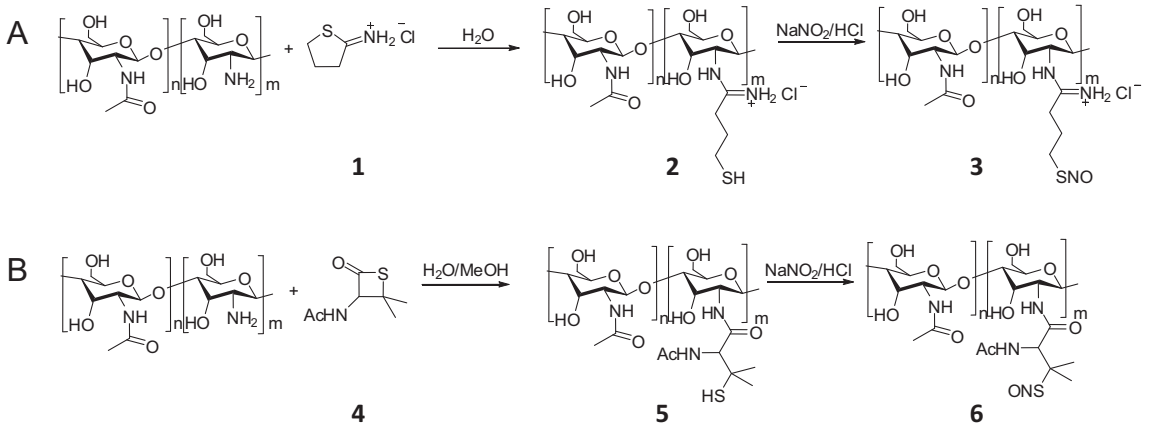
Chitosan oligosaccharides were prepared by oxidative degradation using hydrogen peroxide [33]. Medium-molecular-weight chitosan (2.5 g) was suspended in a hydrogen peroxide solution (15 wt.%) under stirring for 1 h at 85 °C. Following removal of undissolved chitosan by filtration, chitosan oligosaccharides were precipitated out of solution using acetone. The precipitate was collected by centrifugation and washed twice with ethanol before drying under vacuum at room temperature. The viscosity of the chitosan oligosaccharides was measured in a solution of sodium chloride (0.20 M) and acetic acid (0.10 M) at 25 °C using an Ubbleohde capillary viscometer. The molecular weight of the chitosan was determined using the classic Mark–Houwink equation ($[\eta] = 1.81 \times 10^{-3}\ M^{0.93}$) [34]. Elemental analysis was used to characterize the nitrogen content of the resulting chitosan oligosaccharides using a Perkin Elmer CHN/S O Elemental Analyzer Series 2400 (Waltham, MA) instrument.

2.3. Synthesis of thiol-modified chitosan oligosaccharides

Primary thiol-modified chitosan oligosaccharides (chitosan-TBA) (see (2) in Scheme 1) were synthesized according to a previous report [35]. Briefly, chitosan oligosaccharides (25 mg) were dissolved in aqueous solution (1 ml) at pH = 10. 2-Iminothiolane hydrochloride ((1), Scheme 1) was added to the solution at a 2:1 M ratio to the primary amines in chitosan oligosaccharides and stirred for 48 h under nitrogen to yield chitosan 4-thiobutylamidine conjugate (chitosan-TBA) ((2), Scheme 1). The resulting thiol-modified chitosan oligosaccharides were precipitated by cold acetone and redissolved in water. This procedure was repeated twice to remove residual 2-iminothiolane.

^{1}H NMR data of chitosan-TBA (400 MHz, CD_3OD, δ) are as follows: 1.9 (C7: CHNHCOC*H*$_3$), 2.1 ($CH_2CH_2CH_2SH$), 2.65 (CH_2CH_2-CH_2SH), 3.0 (C2: $NH_2CH(CH_3)CH_2NHCH$), 3.2 ($CH_2CH_2CH_2SH$), 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).

3-Acetamido-4,4-dimethylthietan-2-one (NAP) was synthesized according to a previous report [36]. Briefly, acetic anhydride (5.9 ml, 63 mmol) was added dropwise over a period of 30 min to an ice-cooled solution of *N*-acetyl-D,L-penicillamine (4.0 g, 21 mmol) dissolved in anhydrous pyridine (10 ml) under N_2. The solution was warmed to room temperature and stirred for 18 h. The stirring mixture was diluted with chloroform (150 ml), washed with 0.5 M hydrochloric acid (3 × 50 ml), and the organic layer dried over magnesium sulfate. The chloroform was concentrated under reduced pressure and the crude thiolactone product was precipitated and triturated in petroleum ether (100 ml), filtered, rinsed with ether and dried to yield a white crystalline solid (2.1 g). Tertiary thiol-modified chitosan oligosaccharides (chitosan-NAP) ((5), Scheme 1) were synthesized by the reaction of thiolactone (i.e. 3-acetamido-4,4-dimethylthietan-2-one (NAP)) ((4), Scheme 1) with chitosan's primary amines. First, the chitosan oligosaccharides (25 mg) were dissolved in aqueous solution (1 ml) at pH 10. Thiolactone in 0.5 ml methanol (2:1 M ratio to the primary amines on chitosan oligosaccharides) was then added



Scheme 1. Synthesis of *S*-nitrosothiol-modified chitosan oligosaccharides. (1) 2-iminothiolane; (2) chitosan-TBA; (3) chitosan-TBA–NO; (4) thiolactone (NAP); (5) chitosan-NAP; (6) chitosan-NAP-NO.

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