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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 the thiol nitrosation. The resulting nitric oxide (NO)-releasing chitosan oligosaccharides stored ~0.3 μmol NO/mg 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* (*P. 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.

Keywords

S-nitrosothiol; nitric oxide; chitosan; ascorbic acid; synergy; antibacterial agent

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 nature 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

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Notes

The authors declare the following competing financial interest(s): The corresponding author declares competing financial interest. Mark Schoenfisch is a co-founder and a member of the board of directors, and maintains a financial interest in Novan Therapeutics, Inc. Novan Therapeutics is commercializing macromolecular nitric oxide storage and release vehicles for dermatological clinical indications.

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*-diazeniudmiolate 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) 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),[27] necessitating large dose of chitosan for complete killing of certain bacterial. 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), 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 *P. aeruginosa*.[31] As a reducing agent, ascorbic acid allowed for triggered *S*-nitrosothiols decomposition and subsequent NO release.[32] To date, no study has examined the ability to prepare *S*-nitrosothiol-modified chitosan or evaluate the potential synergistic antibacterial efficacy of such materials with ascorbic acid as a trigger for NO release.

EXPERIMENTAL

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 penicillin, 10,000 μg/mL streptomycin) were purchased from the Aldrich Chemical Company (Milwaukee, WI). *Pseudomonas aeruginosa* (ATCC #19143) was obtained from the American Type Culture Collection (Manassas, VA). Trypic 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. ^{1}H nuclear magnetic resonance (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. 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.

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 ($[\eta]=1.81\times10^{-3}\ M^{0.93}$).[34] Elemental analysis was used to characterize the nitrogen content of the resulting chitosan oligosaccharides using a PerkinElmer CHN/S O Elemental Analyzer Series 2400 (Waltham, MA) instrument.

Synthesis of Thiol-Modified Chitosan Oligosaccharides

(Scheme 1) Primary thiol-modified chitosan oligosaccharides (**Chitosan-TBA**) (**2**) 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**) was added to the solution at a 2:1 molar ratio to the primary amines in chitosan oligosaccharides and stirred for 48 h under nitrogen to yield chitosan 4-thiobutylamidine conjugate (**Chitosan-TBA**) (**2**). 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): 1.9 (C7: $CHNHCOC\mathit{H}_3$), 2.1 ($CH_2C\mathit{H}_2CH_2SH$), 2.65 ($CH_2CH_2C\mathit{H}_2SH$), 3.0 (C2: $NH_2CH(CH_3)CH_2NHC\mathit{H}$), 3.2 ($C\mathit{H}_2CH_2CH_2SH$), 3.3–4.0 (C3, C4, C5, C6: $OHC\mathit{H}$, $OC\mathit{H}CH(OH)CH(NH_2)CH$, $OHCH_2C\mathit{H}$, $OHC\mathit{H}_2CH$), 4.4 (C1: $OC\mathit{H}(CHNH_2)O$).

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**) were synthesized by the reaction of thiolactone (i.e., 3-acetamido-4,4-dimethylthietan-2-one (**NAP)**) (**4**) 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 molar ratio to the primary amines on chitosan oligosaccharides) was then added to this solution. The solution was stirred for 48 h under nitrogen at room temperature. The resulting tertiary thiol-modified chitosan oligosaccharides were precipitated using cold acetone. The precipitate was then collected by centrifugation, redissolved in water and filtered to remove any undissolved material (e.g., **NAP**). The dissolution and precipitation cycle was repeated twice to remove residual thiolactone.

^{1}H NMR data of **Chitosan-NAP** (400 MHz, CD_3OD, δ): 1.3–1.4 ((C*H*$_3$)C(CH)(SH)), 1.9–2.1 (C7: CHNHCOC*H*$_3$, C*H*$_3$CONHCH), 3.0 (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).

Synthesis of Fluorescently-Labeled Chitosan Oligosaccharides

Fluorescently-labeled chitosan oligosaccharides were prepared following a procedure outlined in a previous report.[37] 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 of RITC to the primary amine of the chitosan oligosaccharides, prior to the thiol-modification. The solution was stirred at room temperature for 3 d in the dark. Subsequent dialysis and lyophilization yielded the RITC-labeled chitosan oligosaccharides.

Ellman's Assay

A solution of 2 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) in 50 mM sodium acetate was prepared and refrigerated until use. Chitosan samples were dissolved in water (0.4 mg/mL) and diluted to 3 different concentrations (1:5, 1:10, 1:20). For the assay, 10 μL of the thiolated chitosan sample was incubated with 50 μL of the DTNB sodium acetate solution, 840 μL of ultrapure H_2O, and 100 μL of Tris buffer (1 M, pH 8.0) for 5 min at 37 °C. The optical density of this solution was measured at 412 nm. The absorbance value for each chitosan concentration was divided by the extinction coefficient of the DTNB mixed disulfide complex (13,600 $M^{-1}cm^{-1}$) to determine the concentration of thiol in solution. Thiol concentration was divided by the mass of chitosan in solution to obtain moles of thiol/mg chitosan.

Nitrosation of Thiol-Modified Chitosan Oligosaccharides

Thiol-modified chitosan oligosaccharides (25 mg) were dissolved in 5 M hydrochloric acid (1 mL) and methanol (2 mL). An aqueous solution (1 mL) containing sodium nitrite (5 × molar excess to thiol) and diethylenetriamine pentaacetic acid (DTPA) (500 μM) was then added dropwise. The mixture was stirred for 2 h in the dark and on ice before being cooled in the freezer (−20 °C) overnight. The resulting nitrosated chitosan oligosaccharides were collected by precipitation in cold acetone and isolation via centrifugation. The dissolution and precipitation cycle was repeated twice to remove residual salt. The nitrosated chitosan

oligosaccharides were then dried under vaccum at room temperature for 30 min and stored at −20 °C for future use.

Characterization of NO Release from *S*-Nitrosothiol-Modified Chitosan Oligosaccharides

S-nitrosothiol-functionalized chitosan oligosaccharides were added to deoxygenated 500 μM DTPA supplemented phosphate buffered saline (PBS; 30 mL) or regular PBS at 37 °C. Nitrogen was bubbled through the solution at a flow rate of 70 mL/min to carry the liberated NO to the analyzer. Additional nitrogen flow was supplied to the flask to match the collection rate of the instrument at 200 mL/min. The real-time NO-release profiles triggered by copper (50 mg/mL $CuBr_2$), heat (37 °C, shielded from light), or light (37 °C, 200 W, 15 cm above the reaction flask) were recorded. Chemiluminescence data for the NO-releasing chitosan oligosaccharides were represented as maximum NO flux ($[NO]_{max}$, ppb/mg), total amount of NO release (t[NO], μmol NO/mg of secondary amine-functionalized chitosan oligosaccharides), and half-life ($t_{1/2}$, h) under the different conditions.[8, 38] Total NO storage of the *S*-nitrosothiol-modified chitosan oligosaccharides was determined using the Griess assay. For this assay, chitosan oligosaccharides (2 mg) were dissolved in PBS (10 mL) and exposed to light (200 W, 3 cm above the samples) for one week. The resulting solution was subjected to the Griess assay to determine total NO storage.

Bactericidal Assays against Planktonic *Pseudomonas aeruginosa*

P. aeruginosa bacterial cultures were grown from a frozen (−80 °C) stock overnight in tryptic soy broth (TSB) at 37 °C. A 500 μL aliquot of the resulting suspension was added into 50 mL fresh TSB and incubated at 37 °C for ~2 h until the concentration reached ~1×10^8 colony forming units (CFU)/mL, as confirmed by the OD_{600}, replicate plating and enumeration on nutrient agar. Plating the bacterial suspension on TSA and incubating it at 37 °C overnight was used to create a working bacterial stock. The tryptic soy agar (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 μL aliquot of culture was added to 50 mL fresh TSB and incubated to a concentration of ~1×10^8 CFU/mL. The bacteria were collected by centrifugation, resuspended in PBS supplemented with 1 wt% glucose and 0.5 v/v% TSB, and diluted to ~1×10^6 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. After 4 h of exposure, 100 μL 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 and ascorbic acid that resulted in a 4-log reduction of bacterial viability was defined as the minimum bactericidal concentration (MBC) for planktonic studies.

The checkerboard method was employed to determine the antibacterial efficacy of ascorbic acid and NO-releasing chitosan oligosaccharides in combination. Briefly, bacteria at a final innoculum concentration of 1×10^6 CFU/mL were incubated with an array of ascorbic acid and NO-releasing chitosan oligosaccharide concentrations for 4 h at 37 °C. The fractional bactericidal concentration index at 4 h was calculated using Equation 1, where MBC_A and MBC_B are MBC values determined for agents A and B, respectively, in a single-agent assay;

and MBC_{AB} and MBC_{BA} are the concentrations of agent A and B that constituted the effective bactericidal combination for a 4-log reduction of viability. A FBC<0.5 was defined as synergistic.

$$\mathrm{FBC}=\frac{MBC_{AB}}{MBC_{A}}+\frac{MBC_{BA}}{MBC_{B}} \quad \text{Eq. 1}$$

Confocal Microscopy

P. aeruginosa was cultured in TSB to a concentration of ~1×10^8 CFU/mL, collected via centrifugation (3645 × *g* for 10 min), resuspended in sterile PBS, and adjusted to ~1×10^6 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. A 1.2 numerical aperture C-apochromat water immersion lens with a 40× objective was used to collect both bright field and fluorescence images. 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 after 1 h incubation to characterize the association of the chitosan oligosaccharides with *P. aeruginosa*.

In Vitro Cytotoxicity

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_2 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 3×10^4 cells/mL, and incubated for 48 h at 37 °C. The supernatant was then aspirated prior to adding fresh DMEM (200 μL) with NO-releasing/control chitosan oligosaccharides or ascorbic acid 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 at 490 nm using a Thermoscientific Multiskan EX plate reader (Thermo Fischer Scientific, Inc., Waltham, MA). The mixture of DMEM/MTS/PMS and untreated cells were used as a blank and control, respectively. Cell viability was calculated according to Equation 2.

$$\text{Cell Viability}=\frac{(\text{Absorbance}_{\text{treated cell}}-\text{Absorbance}_{\text{blank}})}{(\text{Absorbance}_{\text{untreated cell}}-\text{Absorbance}_{\text{blank}})} \quad \text{Eq. 2}$$

Statistics

All experimental measurements were repeated at least three times to calculate the standard deviation for each data point.

Results and Discussion

Thiol-modified chitosan has attracted much attention for drug delivery applications due to their enhanced mucoadhesive and paracellular junction permeation properties.[39–41] Common synthetic routes for preparing thiol-modified chitosans include functionalization by L-cysteine, thioglycolic acid, and 2-iminothiolane, with the last being most attractive as no catalyst is required.[35] Due to increased reactivity of 2-iminothiolane at high pH, the modification efficiency of primary amines would likely to improve with increasing reaction solution pH. Unfortunately, chitosan polysaccharides are only soluble in acidic conditions, resulting in inherently low thiol modification efficiency.[35] In contrast, chitosan oligosaccharides exhibit excellent solubility in basic conditions, allowing more efficient primary amines functionalization. In this study, chitosan oligosaccharides were synthesized by oxidative degradation. The molecular weight (M_w) was calculated to be 5370 by the classic Mark-Houwink equation ($[\eta] = 1.81\times10^{-3}\ M^{0.93}$),[34] with a nitrogen content of 6.3 ± 0.2 wt% as determined by elemental analysis. Considering the deacetylation degree of ~80%, these chitosan oligosaccharides contain ~4 μmol primary amines/mg chitosan, which was then used to calculate the 2-iminothiolane and **NAP** needed for the synthesis of thiol-modified chitosan oligosaccharides.

Synthesis of Thiol-Modified Chitosan Oligosaccharides

Chitosan oligosaccharides were dissolved in aqueous solution (pH 10) and reacted with 2-iminothiolane as shown in Scheme 1. NMR was used to characterize the resulting primary thiol-modified chitosan oligosaccharides (**Chitosan-TBA**). A distinct resonance at 2.65 ppm was apparent, corresponding to the methylene protons adjacent to the sulfhydryl group of the products. Tertiary thiol-functionalized chitosan oligosaccharides (**Chitosan-NAP**) were synthesized by reaction of the chitosan primary amine with thiolactone (**NAP**) via a ring opening reaction. The associated NMR data revealed a resonance at ~1.3 ppm indicative of **NAP**'s methyl groups. The thiol content of the **Chitosan-TBA** and **Chitosan-NAP** as determined with Ellman's assay was 1.1 ± 0.1 and 1.2 ± 0.1 μmol/mg, respectively.

Nitric Oxide-Release Properties

Thiol-modified chitosan oligosaccharides (e.g., **Chitosan-TBA**, **Chitosan-NAP**) were nitrosated with sodium nitrite in 5 M HCl to store NO.[42] *S*-nitrosothiol formation was confirmed by the red and green color of the resulting primary (**Chitosan-TBA**) and tertiary *S*-nitrosothiol-modified (**Chitosan-NAP**) chitosan oligosaccharides, respectively. The total NO storage was assessed indirectly using the Griess assay. Briefly, chitosan oligosaccharides were dissolved in PBS and exposed to light for a week, allowing for complete decomposition. The NO totals for **Chitosan-TBA-NO** and **Chitosan-NAP-NO** were 300 ± 14 and 350 ± 25 nmol/mg, respectively. These results were confirmed using chemiluminescence for NO release triggered by copper.[43] As expected, copper-mediated NO donor decomposition is characterized by more rapid NO release kinetics compared to heat and light. Within the first few minutes, the majority of *S*-nitrosothiols were decomposed, with **Chitosan-TBA-NO** and **Chitosan-NAP-NO** releasing ~90 and 85% of their total payloads at 0.1 h, respectively. Although copper-mediated NO release is unlikely to be clinically useful as blood/tissue does not contain much free copper ions, the rapid NO

release facilitates prompt measurement of total NO storage, particularly for *S*-nitrosothiol NO donors with slow NO-release kinetics under physiological conditions (37 °C, pH=7.4).

Photolysis of **Chitosan-TBA-NO** and **Chitosan-NAP-NO** was characterized using a broad-spectrum white light source to investigate a potentially more clinically relevant NO-release trigger. As shown in Table 1, the total NO release after 24 h of irradiation was ~75 nmol/mg, ~20% of the total available payloads. The NO release data indicated that **Chitosan-TBA-NO** release NO slower than **Chitosan-NAP-NO** (Figure 2, Table 1). These results are somewhat surprising when considering the *S*-nitrosothiol structure alone. Indeed, the primary *S*-nitrosothiol systems (**Chitosan-TBA-NO**) would be expected to release NO faster, not slower. However, the behavior is similar to previously reported *S*-nitrosothiol-modified dendrimers,[44] where increased NO donor stability of **Chitosan-TBA-NO** is likely the result of a more compact structure and less steric hindrance around the *S*-nitrosothiol groups. This phenomenon may result in a greater microviscosity inside the chitosan oligosaccharides and increased frequency of geminate radical pair recombination (caging effect), consequently slowing the rates of NO release.[44]

Thermal decomposition of *S*-nitrosothiols at 37 °C is of greatest interest for biomedical applications where light irradiation is not applicable. Similar to photolytic *S*-nitrosothiol decomposition, thermal irradiation leads to homolytic cleavage of the S-N bond, yielding a thiyl radical and NO.[29] The thermal decomposition of **Chitosan-TBA-NO** and **Chitosan-NAP-NO** was characterized in both DTPA-supplemented and regular PBS at 37 °C, shielded from light (Figure 3).

To eliminate effects from trace copper ion, DTPA was added to PBS as a chelator. As above for photolytic decomposition, primary *S*-nitrosothiol-modified chitosan oligosaccharides (**Chitosan-TBA-NO**) exhibited slower NO release than the tertiary system (**Chitosan-NAP-NO**). The caging effect, resulting in greater geminate radical pair recombination, clearly impacts both light and thermal NO donor breakdown for *S*-nitrosothiol-modified chitosan. The total NO release was ~30 and 13 nmol/mg for **Chitosan-NAP-NO** and **Chitosan-TBA-NO**, respectively. While important for understanding the NO release, DTPA-supplemented PBS is not relevant to clinical applications (e.g., intravenous injection, wound wash, and pulmonary delivery).[45] The NO release of *S*-nitrosothiol-modified chitosan oligosaccharides in regular PBS with and without ascorbic acid was thus investigated to ascertain the influence of a) normal PBS and b) a common reducing agent in blood of importance. Ascorbic acid decomposes *S*-nitrosothiols via two distinct pathways.[32] At low concentrations, ascorbate reduces Cu^{2+} to generate Cu^{+}, which in turn decomposes RSNO to disulfide and NO.[46] Since copper ion is key, the use of a copper ion chelator results in reduced NO release. At higher concentrations, ascorbic acid can also reduce *S*-nitrosothiol NO donors directly, yielding thiol and *O*-nitrosoascorbate, the latter decomposing into dehydroascorbic acid and NO by a free-radical pathway.[32] In contrast to the first mechanism, this reaction is not copper ion dependent.[32] Our goal was to study the effect of ascorbic acid on NO-release kinetics for *S*-nitrosothiol-modified chitosan oligosaccharides in PBS. As shown in Table 2, both **Chitosan-TBA-NO** and **Chitosan-NAP-NO** released greater amount of NO in regular PBS compared to DTPA-supplemented PBS due to the copper-mediated NO release. In regular PBS, trace Cu^{2+} (i.e., 640±50

ppb/mL by inductively coupled plasma) was reduced by residual thiolate ions on the chitosan oligosaccharides to yield Cu^{+} and more NO than the thermal decomposition in DTPA-supplemented PBS. **Chitosan-NAP-NO** released ~240 nmol NO/mg in regular PBS, significantly greater than the ~78 nmol NO/mg by thermal decomposition alone. The NO release half-life was ~0.82 and 0.52 h for **Chitosan-TBA-NO** and **Chitosan-NAP-NO**, respectively, a significantly faster overall release than that observed with DTPA-supplemented PBS (Table 2). The **Chitosan-TBA-NO** also exhibited slower NO release due to the caging effect, as described above.

Nitric oxide release in PBS as a function of ascorbic acid concentrations (i.e., 0.1–0.5 mg/mL) was characterized to better understand the role of this reducing agent on NO-release kinetics. Enhanced *S*-nitrosothiol decomposition was observed in the presence of ascorbic acid as indicated by shorter half-life and greater maximum NO flux (Table 2). The half-life of NO release from **Chitosan-NAP-NO** was ~0.52, 0.10 and 0.07 h at 0, 0.1 and 0.5 mg/mL ascorbic acid, respectively. Compared to the copper ion-mediated NO release in regular PBS, ascorbic acid proved to be a stronger reducing agent than thiolate ions, leading to faster *S*-nitrosothiol NO donor decomposition.[32] Along with the copper ion-mediated NO release, the direct reduction of *S*-nitrosothiol NO donor by ascorbic acid may also contribute to *S*-nitrosothiol decomposition.[32] The NO release of **Chitosan-NAP-NO** and **Chitosan-TBA-NO** at different concentrations of ascorbic acid (i.e., 0.1 and 0.5 mg/mL) was thus characterized in DTPA-supplemented PBS. As shown in Figure 4, the use of copper ion chelator had little impact on the total NO release in 0.1 and 0.5 mg/mL ascorbic acid, indicating the presence of *S*-nitrosothiol decomposition via direct reduction by ascorbic acid. Of note, the NO release in DTPA-supplemented PBS did show lower maximum NO flux and slower decay compared to the NO-release behavior in regular PBS. This difference in *S*-nitrosothiol breakdown to NO indicates that both *S*-nitrosothiol decomposition pathways play a role in NO release from *S*-nitrosothiol-modified chitosan oligosaccharides by ascorbic acid in regular PBS.

Bactericidal studies

Nitric oxide releasing materials (e.g., silica particles, dendrimer) have been demonstrated to be effective at killing bacteria.[9, 47] However, their lack of biodegradability may be problematic for clinical applications. In this study, control and NO-releasing chitosan oligosaccharides (e.g., **Chitosan-TBA-NO**, **Chitosan-NAP-NO**) 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,.[48, 49] Bacterial viability assays were performed in nutrient conditions to determine the concentration of chitosan required to reduce bacteria viability in 4 h by 4 logs (i.e., 99.99% killing), which hereafter is referred to as the minimum bactericidal concentration or MBC. Given that ascorbic acid is bactericidal against *P. aeruginosa* with synergistic efficacy depending on the antibiotic,[31, 50] we also evaluated the combination of *S*-nitrosothiol-modified chitosan oligosaccharides and ascorbic acid. Both the MBC for the NO-releasing chitosan oligosaccharides and the fractional bactericidal concentration index (FBC) of NO-releasing chitosan at different concentrations of ascorbic acid (i.e., 0.1 and 0.5 mg/mL) are provided in Table 3. The MBC for ascorbic acid alone was 2.5 mg/mL. Control thiol-modified chitosan oligosaccharides with ascorbic

acid at 0.1 and 0.5 mg/mL elicited no significant reduction (<1 log) in bacterial viability at the MBCs for their NO-releasing counterparts. Of note, 0.1 and 0.5 mg/mL ascorbic acid in the PBS (pH=7.4) slightly lowered the pH to 7.3 and 6.8, respectively. As shown in Table 3, **Chitosan-TBA-NO** proved more effective against *P. aeruginosa* than **Chitosan-NAP-NO** despite the lower total NO storage. This effect is likely the result of better association of **Chitosan-TBA-NO** with bacterial membrane.

Indeed, **Chitosan-TBA-NO** is more positively charged due to the presence of amine on the TBA unit. It would be thus expected to associate more extensively with the negatively charged outer bacterial membrane. To confirm this hypothesis, confocal microscopy was used to characterize the association of both **Chitosan-TBA-NO** and **Chitosan-NAP-NO** with *P. aeruginosa*. As shown in Figure 5, bacteria incubated with **Chitosan-TBA-NO** exhibited more red RITC fluorescence, indicating greater association with the bacterial membrane compared to **Chitosan-NAP-NO**. The bactericidal efficacy was found to be synergistic for ascorbic acid (0.1 mg/mL) and **Chitosan-NAP-NO** (FBC≤0.5). While the combination of **Chitosan-TBA-NO** and ascorbic acid resulted in a FBC=0.51, a value some consider moderately synergistic,[51] it was significantly greater than the additive effect (FBC=1) of single antibacterial agents (i.e., ascorbic acid or **Chitosan-TBA-NO** alone). These results demonstrate the benefit of ascorbic acid as a NO-release trigger for *S*-nitrosothiol-modified chitosan oligosaccharides in bacterial killing. The enhanced bactericidal efficacy is likely the result of a greater initial NO level induced by the ascorbic acid, corroborating previous observations.[10]

Cytotoxicity to Murine Fibroblasts

The benefit of chitosan as an NO-releasing vehicle includes its non-toxic nature.[28] However, the effects of both NO and ascorbic acid on mammalian cells are unknown. Thus, the cytotoxicity of ascorbic acid, control and NO-releasing chitosan oligosaccharides were evaluated against L929 mouse fibroblast cells.

Normalized cell viabilities in the presence of ascorbic acid, and control and NO-releasing chitosan oligosaccharides after 24 h incubation are shown in Figure 6. Ascorbic acid at both 0.1 and 0.5 mg/mL elicited minimum toxicity (~15%) to the L929 fibroblast cells. Of note, significantly reduced cell death for **Chitosan-TBA-NO** was observed compared to **Chitosan-NAP-NO**. A key difference between these two NO-release materials is the initial NO flux. Nitric oxide is a well known bifunctional regulator of apoptosis.[52] High dose of NO can interact with superoxide ions to produce peroxynitrite and induce apoptotic DNA fragmentation and p53-dependent apoptosis. In contrast, low doses of NO inhibit apoptosis via multiple mechanisms (e.g., cGMP-mediated interruption of apoptotic signaling and direct inhibition of caspase activity).[52] This bifunctional role of NO may likely explain the greater cytotoxicity of **Chitosan-NAP-NO** over **Chitosan-TBA-NO**. In this regard, **Chitosan-TBA-NO** may serve as a more suitable antibacterial agent for biomedical applications such as wound healing due to being more tolerable to mammalian cells.

Conclusions

A new class of NO-releasing chitosan oligosaccharides with tunable NO-release kinetics is described using *S*-nitrosothiols NO donors and ascorbic acid. The combination of *S*-nitrosothiol-modified chitosan oligosaccharides and ascorbic acid allows for synergistic bactericidal action against *P. aerugionsa*. At concentrations eliciting a 4-log reduction in bacterial viability under nutrient condition, primary *S*-nitrosothiol-modified chitosan oligosaccharides showed minimal toxicity against L929 mouse fibroblast cells. This study demonstrates the potential of *S*-nitrosothiol-modified chitosan oligosaccharides as antibacterial agents. The biodegradability of chitosan makes this NO-releasing material a viable therapeutic candidate for a range of biomedical applications including chronic wounds, nasal infections, and cystic fibrosis. Experiments are underway to evaluate the biocidal efficacy of these materials against a broad spectrum of bacteria strains including *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, and cystic fibrosis-related *P. aeruginosa* strains (e.g., mucoid/alginate-producing strains).

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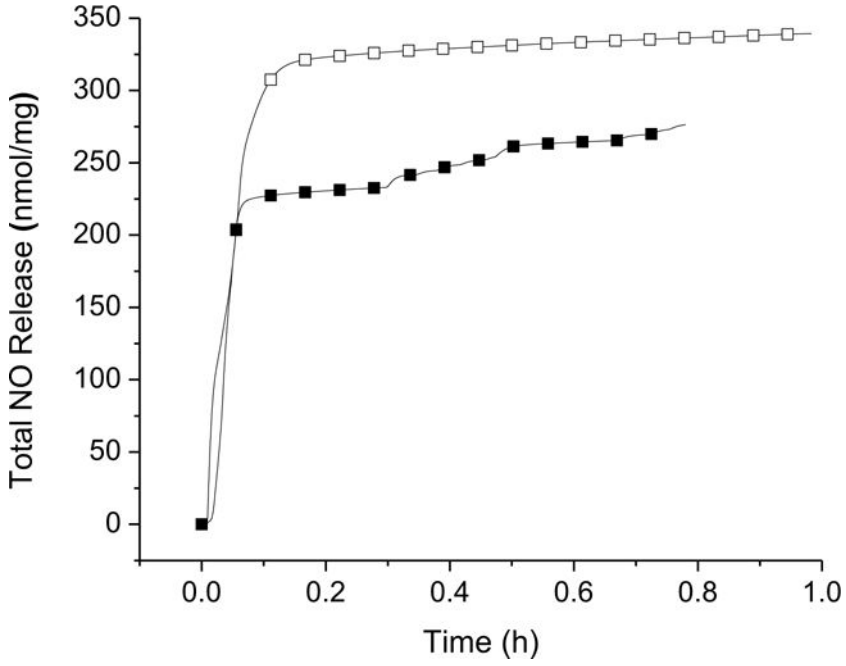


Figure 1.

Nitric oxide release from **Chitosan-TBA-NO** (solid square) and **Chitosan-NAP-NO** (open square) in 50 mg/mL $CuBr_2$ aqueous solution at 37 °C as measured by chemiluminescence.

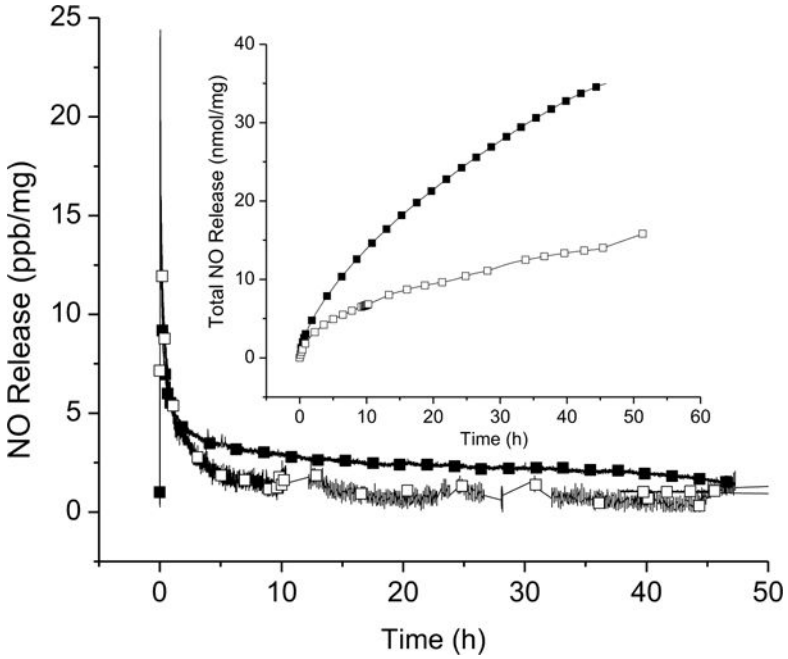


Figure 2.

Nitric oxide-release profiles for **Chitosan-TBA** (open square) and **Chitosan-NAP** (solid square) under light irradiation (200 W, 15 cm above) as measured by chemiluminescence. Inset: total NO release with time (nmol/mg).

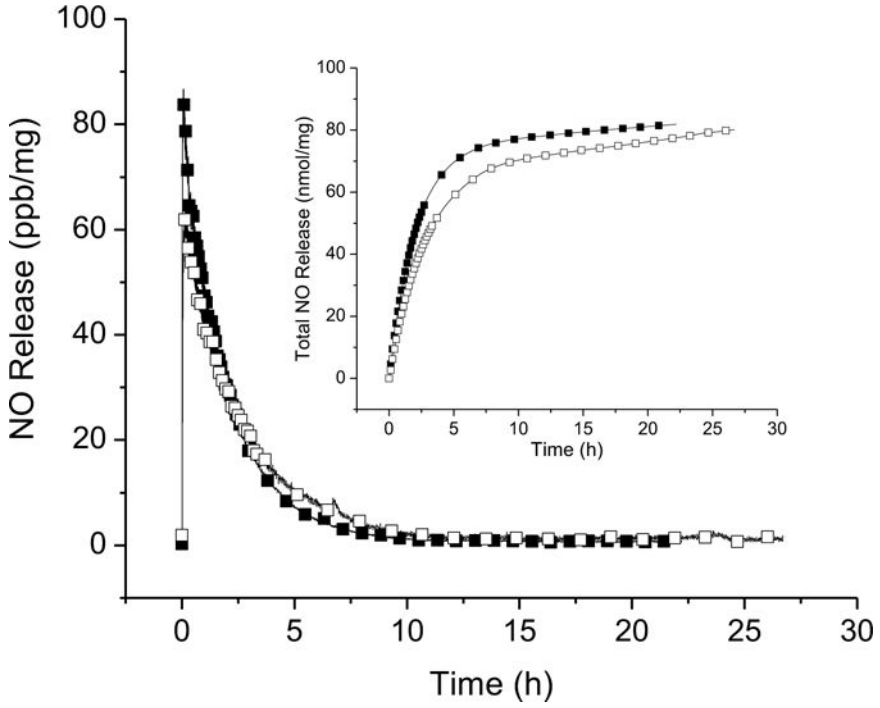


Figure 3.

Nitric oxide-release profiles for **Chitosan-TBA** (open square) and **Chitosan-NAP** (solid square) in DTPA-supplemented PBS at 37 °C shielded from light as determined using chemiluminescence. Inset: total NO release with time (nmol/mg).

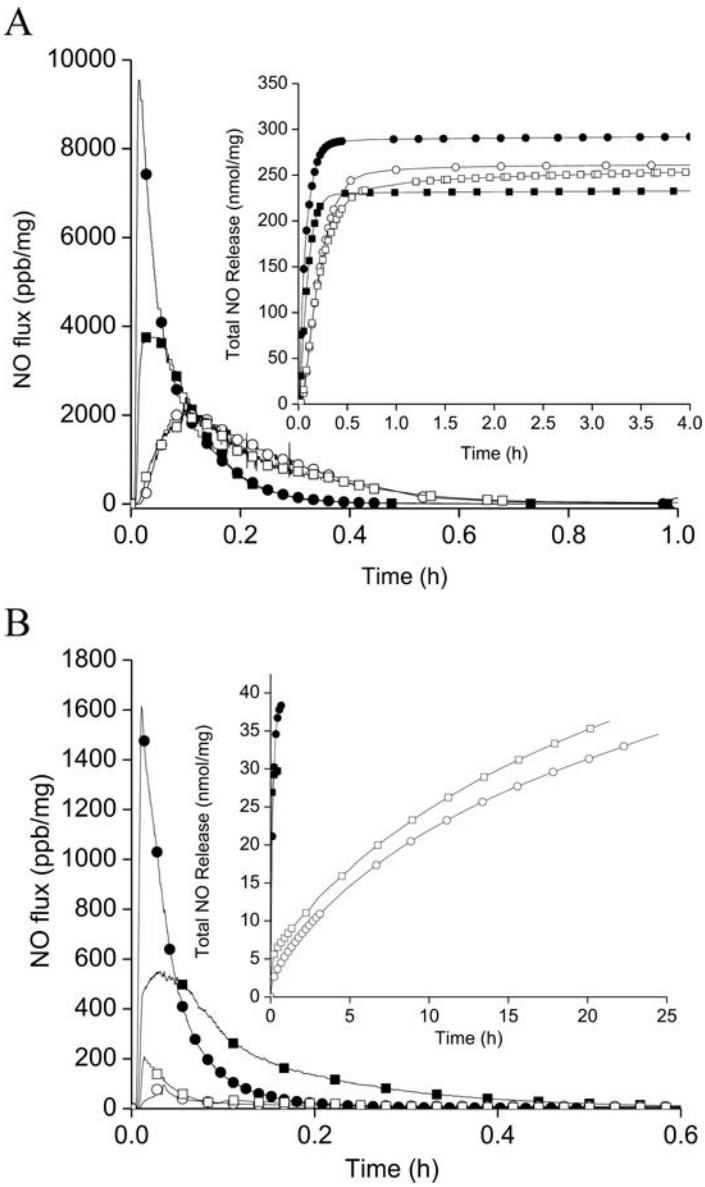


Figure 4.

Nitric oxide-release properties for (A) **Chitosan-NAP-NO** and (B) **Chitosan-TBA-NO** in 0.1 (square) and 0.5 (circle) mg/mL ascorbic acid with (open) and without (solid) DTPA. Inset: total NO release with time (nmol/mg).

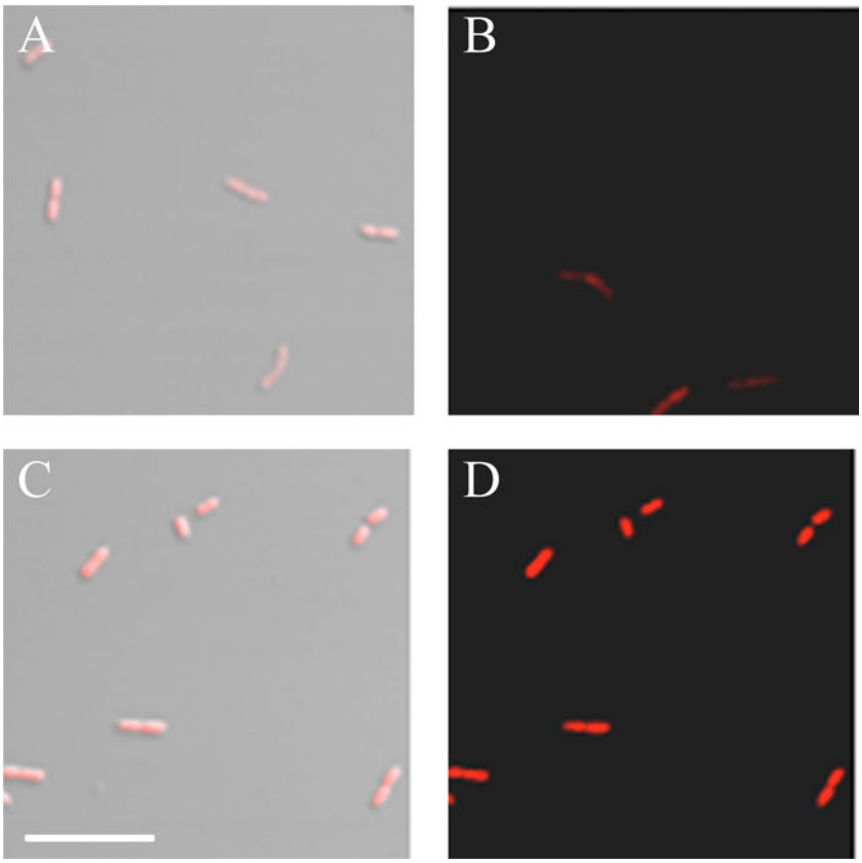


Figure 5.

Bright field/fluorescent overlay images (A and C) and fluorescent images (B and D) of RITC-label NO-releasing chitosan oligosacchrides association with planktonic *P. aeruginosa*. A) and B) **Chitosan-NAP-NO**; C) and D) **Chitosan-TBA-NO**. Scale bar 10 μm.

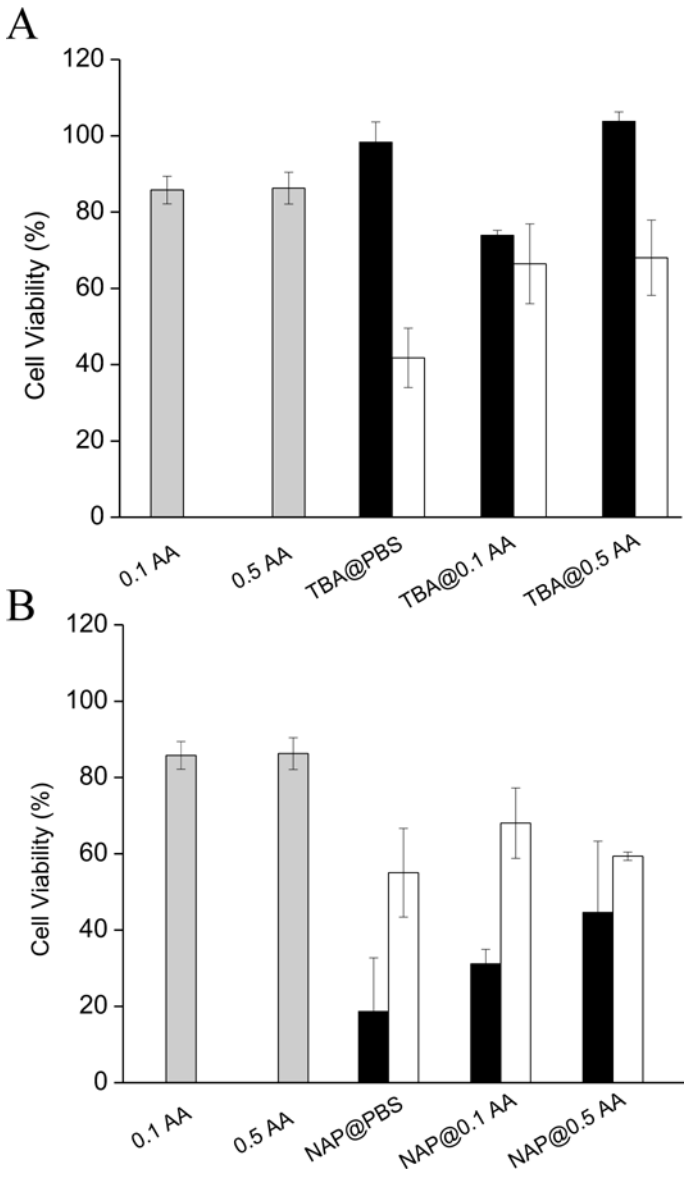
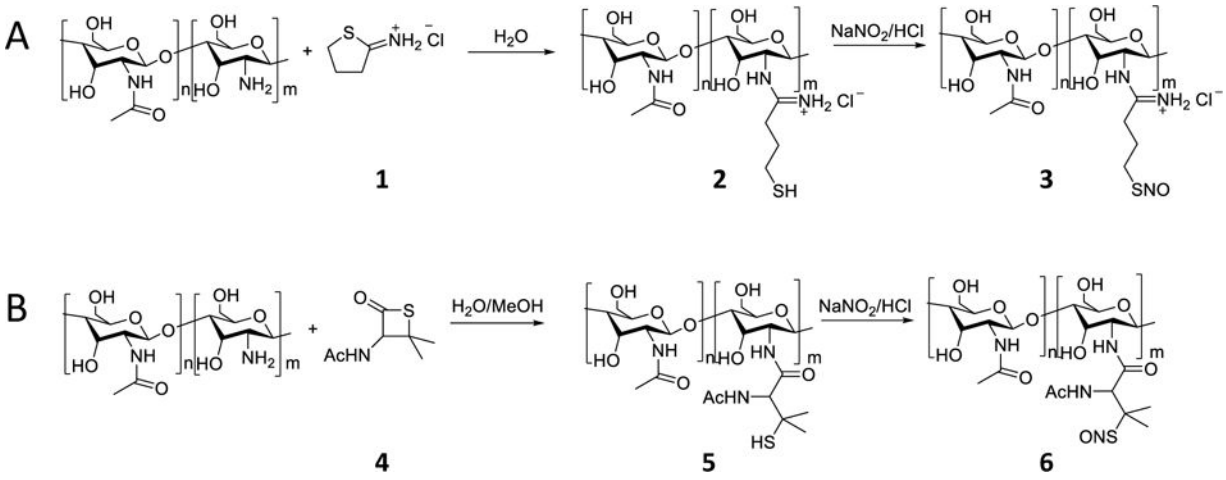


Figure 6.

Cytotoxicity of ascorbic acid (AA) at 0.1 and 0.5 mg/mL (grey), and NO-releasing (black) and control (white) chitosan oligosaccharides in 0.1 and 0.5 mg/mL ascorbic acid solutions against L929 mouse fibroblast cells at 24 h.



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**.

Table 1

Nitric oxide-release properties for *S*-nitrosothiol-modified chitosan oligosaccharides in copper chelator diethylene triamine pentaacetic acid (DTPA)-supplemented PBS at 37 °C as measured via chemiluminescence.

	Chitosan-TBA-NO			Chitosan-NAP-NO		
Light	**t[NO] (nmol/mg)**	**$[NO]_{max}$ (ppb/mg)**	**$t_{1/2}$ (h)**	**t[NO] (nmol/mg)**	**$[NO]_{max}$ (ppb/mg)**	**$t_{1/2}$ (h)**
Yes	75 ± 5	54 ± 9.0	2.3 ± 0.2	78 ± 6	87 ± 6.5	1.5 ± 0.3
No	13 ± 5	5.5 ± 1.2	21 ± 1.2	31 ± 4	36 ± 3.4	14 ± 0.8

Table 2

Nitric oxide-release properties for *S*-nitrosothiol-modified chitosan oligosaccharides in regular PBS at 37 °C with and without ascorbic acid as characterized by chemiluminescence.

Ascorbic acid (mg/mL)	Chitosan-TBA-NO			Chitosan-NAP-NO		
	t[NO] (nmol/mg)	$[NO]_{max}$ (ppb/mg)	$t_{1/2}$ (h)	t[NO] (nmol/mg)	[NO] (ppb/mg)	$t_{1/2}$ (h)
0	35 ± 5	90 ± 40	0.82 ± 0.02	240 ± 40	1005 ± 100	0.52 ± 0.02
0.1	38 ± 3	332 ± 81	0.14 ± 0.07	230 ± 20	3280 ± 660	0.10 ± 0.03
0.5	37 ± 1	509 ± 48	0.09 ± 0.01	230 ± 10	9000 ± 800	0.07 ± 0.03

Table 3

Minimum bactericidal concentration (MBC) and fractional bactericidal concentration index (FBC) for *S*-nitrosothiol-modified chitosan oligosaccharides and ascorbic acid against planktonic *P. aeruginosa*.

Ascorbic acid (mg/mL)	Chitosan-TBA-NO		Chitosan-NAP-NO	
	MBC[a] (mg/mL)	FBC	MBC[a] (mg/mL)	FBC
0	15	–	30	–
0.1	7	0.51	10	0.37
0.5	5	0.53	10	0.53

[a] concentration of *S*-nitrosothiol-modified chitosan for 4-log reduction in *P. aeruginosa* viability. Each parameter was analyzed with multiple replicates (n=3).