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Nitric oxide-releasing alginates

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Abstract

Low and high molecular weight alginate biopolymers were chemically modified to store and release potentially therapeutic levels of nitric oxide (NO). Carbodiimide chemistry was first used to modify carboxylic acid functional groups with a series of small molecule alkyl amines. The resulting secondary amines were subsequently converted to *N*-diazoniumdiolate NO donors via reaction with NO gas under basic conditions. NO donor-modified alginates stored between 0.4–0.6 $\mu\text{mol NO}\cdot\text{mg}^{-1}$. In aqueous solution, the NO-release kinetics were diverse (0.3–13 h half-lives), dependent on the precursor amine structure. The liberated NO showed bactericidal activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* with pathogen eradication efficiency dependent on both molecular weight and NO-release kinetics. The combination of lower molecular weight (~ 5 kDa) alginates with moderate NO-release durations (half-life of ~ 4 h) resulted in enhanced killing of both planktonic and biofilm-based bacteria. Toxicity against human respiratory epithelial (A549) cells proved negligible at NO-releasing alginate concentrations required to achieve a 5-log reduction in viability in the biofilm eradication assay.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ASSOCIATED CONTENT

Supporting Information. The following file is available free of charge.

Elemental analysis, zeta potential measurements, molecular weight studies, ^1H and ^{13}C NMR spectra of alginates, UV-vis absorbance spectra of control and NO-releasing alginate, NO-release properties of control primary-amine modified alginate, bacterial viability plots for *P. aeruginosa* and *S. aureus* in planktonic and biofilm forms, and controls (PDF)

The authors declare the following competing financial interest(s): Mark Schoenfisch is a cofounder, a member of the board of directors, and maintains financial interest in Novan Therapeutics, Inc. and Novoclem Therapeutics, Inc. Both companies commercialize macromolecular nitric oxide storage and release vehicles for clinical indications.

Keywords

nitric oxide; alginate; antibacterial; biofilm eradication

Introduction

Bacterial infections pose a great challenge to human health in community and hospital settings.^{1–3} Chronic infections associated with implanted devices,⁴ wounds,^{2,5} and cystic fibrosis^{4,6} are frequently caused by biofilm-forming pathogens, including *Pseudomonas aeruginosa* and *Staphylococcus aureus*.^{4,7} Bacteria in biofilms are particularly challenging to address as they form an exopolysaccharide (EPS) matrix as a protective mechanism from the host immune response and antibiotic interventions. Indeed, eradication of bacteria in biofilms may require up to 1000 times greater antibiotic concentrations relative to those needed to kill planktonic bacteria.⁸ Several factors contribute to such protection, including slower bacterial metabolism, limited diffusion of the antibacterial agent (e.g., antibiotic) through the EPS matrix, and altered microenvironments (e.g., regions of nutrient or oxygen depletion).^{4,7,9} Furthermore, bacteria have developed potential cellular mechanisms (e.g., self-alteration of cell membrane targets and the use of pumps to reduce internal concentration of antibiotics) that minimize the effects of antibiotics. New antibacterial agents that can more effectively penetrate and effectively act on biofilm-based bacterial colonies are greatly needed in the fight against emerging antibiotic-resistant pathogens.¹⁰

Nitric oxide (NO) is an endogenous free radical that plays an integral role in the innate immune response to foreign pathogens. Much of NO's broad-spectrum antibacterial activity is accounted for by its reactive byproducts, including peroxynitrite and dinitrogen trioxide, which exert oxidative and nitrosative damage to microbial DNA and membrane structures.^{11–15} As a result of NO's small size and the mechanisms by which it exerts antibacterial action, bacteria are unlikely to foster resistance to NO.¹⁶ The hazards of pressurized cylinders, NO's high reactivity in biological media, and even toxicity concerns have led researchers to develop and study the utility of NO donors – both small molecule and macromolecular systems – as a means for storing and releasing NO under specific environmental conditions.^{12,14,17} *N*-diazeniumdiolate NO donors have garnered much attention due to the spontaneous liberation of NO in aqueous solution at physiological pH.¹⁴ While NO's role in immune cell response to pathogens is clear, developing therapies based on the delivery of NO gas is challenging. Unfortunately, NO payloads for small molecule NO donors can be limited with precursor toxicity risks when larger concentrations are required.¹⁷ To address such concerns, we have focused on the development of macromolecular *N*-diazeniumdiolate NO-release systems with inherently superior NO payloads, control over NO release kinetics, and unique release mechanisms.^{18–24}

Biopolymers such as chitosan, cellulose, and alginate represent attractive potential NO donor scaffolds due to their inherent biodegradability and favorable toxicity profiles.^{25,26} Alginate, a naturally occurring anionic polymer consisting of 1,4-linked α -L-guluronic acid (G) and β -D-mannuronic acid (M) units,^{25,27–30} is water soluble even at high molecular weights (>200 kDa), a critical property for in situ release.^{27,28} As a result of these

properties, investigators have studied alginate's utility for a number of biomedical applications including wound dressings,^{30–32} drug delivery,^{5,30,33} potentiators for antimicrobial agents,²⁸ and mucin modifiers for cystic fibrosis.^{29,30,34,35} The alginate backbone offers straightforward chemical modification at hydroxyl and carboxylic acid functional groups, imparting unique versatility with respect to drug storage and release, and control over solubility, biocompatibility, and degradation rates.^{27,36,37}

Herein, we describe the functionalization of both high and low molecular weight alginates with small molecule alkyl amines for subsequent modification with *N*-diazoniumdiolate NO donors to produce anionic NO-releasing biopolymers. The alkyl amine precursors were selected based on prior literature describing diverse NO-release kinetics related to breakdown of the *N*-diazoniumdiolate.^{38,39} The bactericidal action of the alginates was examined with respect to NO-release kinetics, total NO storage, amine precursor structure, and molecular weight.

Experimental Section

Materials.

Alginic acid sodium salt from brown algae (low viscosity), bis(3-aminopropyl) amine (DPTA), diethylenetriamine (DETA), *N*-propyl-1,3-propanediamine (PAPA), spermine (SPER), phenazine methosulfate (PMS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), trypsin, penicillin streptomycin (PS), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), were purchased from Sigma-Aldrich (St. Louis, MO). Roswell Park Memorial Institute (RPMI) 1640 cell culture medium and common laboratory salts and solvents were purchased from Fischer Scientific (Fair Lawn, NJ). Unless otherwise specified, all chemicals were used as received without further purification. Tryptic soy broth (TSB) and tryptic soy agar (TSA) were obtained from Becton, Dickinson, and Company (Franklin Lakes, NJ). *Pseudomonas aeruginosa* (*P. aeruginosa*; ATCC #19143), *Staphylococcus aureus* (*S. aureus*; ATCC #29213), and A549 human lung epithelial cells (ATCC CCL-185) were obtained from American Type Tissue Culture Collection (Manassas, VA). Argon (Ar), carbon dioxide (CO₂), nitrogen (N₂), nitric oxide (NO) calibration (25.87 ppm, balance N₂) gas cylinders were purchased from Airgas National Welders (Raleigh, NC). Pure NO gas (99.5%) was obtained from Praxair (Sanford, NC). Distilled water was purified to a resistivity of 18.2 M.cm and a total organic content of 6 ppb using a Millipore Milli-Q UV Gradient A10 System (Bedford, MA).

Instrumentation.

¹H and ¹³C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker (600 MHz) spectrometer. Elemental (carbon, hydrogen, and nitrogen; CHN) analysis was performed using a PerkinElmer Elemental Analyzer Series 2400 Instrument (Waltham, MA). Zeta potential measurements were collected in phosphate buffer (10 mM PB; pH 7.4) using a Zetasizer Nano (Malvern Instruments, UK). All absorbance measurements were made in 50 mM sodium hydroxide (NaOH) using a UV-vis Lambda 40 Spectrophotometer (PerkinElmer, Waltham, MA). Gel permeation chromatography measurements were carried

out in 0.1 M sodium nitrate using an aqueous GPC-multi-angle light scattering system equipped with a Waters 2414 refractive index detector (Milford, MA) coupled to a Wyatt miniDawn TREOS multi-angle light scattering detector (Santa Barbara, CA).

Oxidative degradation of alginate.

Alginate was degraded to lower molecular weight alginate oligosaccharides via oxidative degradation following previously published protocols.^{40,41} Briefly, alginate (2.5 g) was dissolved in 15 wt% hydrogen peroxide (50 mL) and stirred at 85 °C for 1 h. The solution was filtered to remove any insoluble material. The resulting alginate oligosaccharides were precipitated from the filtered solution in ethanol, collected via centrifugation, washed copiously with ethanol, and dried in vacuo to yield a white powder. GPC measurements revealed the molecular weight of the starting alginate material, as received, was 283 kDa with a dispersity ($\bar{M}_w/\bar{M}_n$) of 1.11. The average molecular weight of the alginate oligosaccharides (Alg5) was 4.68 kDa with a dispersity of 1.20.

Synthesis of polyamine-modified alginates (AlgMW-alkyl amine).

Alginate starting materials were modified with either diethylenetriamine (DETA), bis(3-aminopropyl)amine (DPTA), N-propyl-1,3-propanediamine (PAPA), or spermine (SPER) through covalent amide bond formation between the carboxylic acid moieties of alginate and the primary amines of the alkyl amine (Scheme 1).²⁷ Briefly, alginate (100 mg) was dissolved in 10 mL phosphate buffered saline (PBS; 10 mM, pH 6.5) together with a 2:1 molar ratio to 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and a 2:1 molar ratio of *N*-hydroxysuccinimide (NHS) with respect to the carboxylic moieties of the alginate scaffold. The resulting solution was allowed to mix at room temperature for 30 min before adding a 4:1 molar ratio of the alkyl amine with respect to the carboxylic acid groups of the alginate scaffold to the reaction solution. After 24 h at room temperature, the amine-modified alginates were precipitated in methanol, collected via centrifugation, washed twice with methanol, and dried in vacuo to yield a white solid for each modification.

Representative ¹H NMR of alginate and the polyamine-modified alginates included the following peaks:

Alg300 and Alg5: ¹H NMR (600 MHz, D₂O, δ) 3.60–4.05 (OCHCH(OH)CH(OH)), 4.30 (OCHCH(OH)CH(OH)), 4.50–4.60 (NHCOCH), 4.90 (OCH(CHOH)O).

Alg300-DETA and Alg5-DETA: ¹H NMR (600 MHz, D₂O, δ) 2.30–3.30 (CH₂CH₂NHCH₂CH₂NH₂), 3.60–4.05 (OCHCH(OH)CH(OH)), 4.30 (OCHCH(OH)CH(OH)), 4.50–4.60 (NHCOCH), 4.90 (OCH(CHOH)O).

Alg300-DPTA and Alg5-DPTA: ¹H NMR (600 MHz, D₂O, δ) 1.60–1.80 (CH₂CH₂CH₂NHCH₂CH₂CH₂NH₂), 2.60–2.30 (CH₂CH₂CH₂NHCH₂CH₂CH₂NH₂), 2.80–3.10 (CH₂CH₂CH₂NHCH₂CH₂CH₂NH₂), 3.60–4.05 (OCHCH(OH)CH(OH)), 4.30 (OCHCH(OH)CH(OH)), 4.50–4.60 (NHCOCH), 4.90 (OCH(CHOH)O).

Alg300-PAPA and Alg5-PAPA: ¹H NMR (600 MHz, D₂O, δ) 0.70–0.80 (NHCH₂CH₂CH₃), 1.52 (NHCH₂CH₂CH₃), 1.85 (CH₂CH₂CH₂NHCH₂CH₂CH₃), 2.80–3.10

($\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{CH}_3$), 3.60–4.05 (OCHCH(OH)CH(OH)), 4.30 (OCHCH(OH)CH(OH)), 4.50–4.60 (NHCOCH), 4.90 (OCH(CHOH)O).

Alg300-SPER and Alg5-SPER: ^1H NMR (600 MHz, D_2O , δ) 1.13 ($\text{NHCH}_2(\text{CH}_2)_2\text{CH}_2\text{NH}$), 1.56 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 1.80 ($\text{C(O)NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 2.20–2.40 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$, $\text{NHCH}_2(\text{CH}_2)_2\text{CH}_2\text{NH}$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 2.68 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 2.80–3.10 ($\text{C(O)NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 3.60–4.05 (OCHCH(OH)CH(OH)), 4.30 (OCHCH(OH)CH(OH)), 4.50–4.60 (NHCOCH), 4.90 (OCH(CHOH)O).

Representative ^{13}C NMR of alginate and the polyamine-modified alginate included the following peaks:

Alg300 and Alg5: ^{13}C NMR (600 MHz, D_2O , δ) 65.0–80.0 ($\text{OCHCH(OH)CH(OH)CH(OH)CH(O)}$), 100.0 (OCHCH(OH)), 175.0 (CHC(O)).

Alg300-DETA and Alg5-DETA: ^{13}C NMR (600 MHz, D_2O , δ) 39.0–47.0 ($\text{C(O)NHCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NH}_2$), 65.0–80.0 ($\text{OCHCH(OH)CH(OH)CH(OH)CH(O)}$), 100.0 (OCHCH(OH)), 160.0 (CHC(O)NH), 175.0 (CHC(O)).

Alg300-DPTA and Alg5-DPTA: ^{13}C NMR (600 MHz, D_2O , δ) 26.9–29.5 ($\text{C(O)NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 37.6–46.0 ($\text{C(O)NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 65.0–80.0 ($\text{OCHCH(OH)CH(OH)CH(OH)CH(O)}$), 100.0 (OCHCH(OH)), 160.0 (CHC(O)NH), 175.0 (CHC(O)).

Alg300-PAPA and Alg5-PAPA: ^{13}C NMR (600 MHz, D_2O , δ) 10.9 ($\text{NHCH}_2\text{CH}_2\text{CH}_3$), 20.0 ($\text{NHCH}_2\text{CH}_2\text{CH}_3$), 31.3–49.0 ($\text{C(O)NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$, $\text{NHCH}_2\text{CH}_2\text{CH}_3$), 65.0–80.0 ($\text{OCHCH(OH)CH(OH)CH(OH)CH(O)}$), 100.0 (OCHCH(OH)), 160.0 (CHC(O)NH), 175.0 (CHC(O)).

Alg300-SPER and Alg5-SPER: ^{13}C NMR (600 MHz, D_2O , δ) 23.2 ($\text{NHCH}_2(\text{CH}_2)_2\text{CH}_2\text{NH}$), 34.8–45.0 ($\text{C(O)NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 65.0–80.0 ($\text{OCHCH(OH)CH(OH)CH(OH)CH(O)}$), 100.0 (OCHCH(OH)), 160.0 (CHC(O)NH), 175.0 (CHC(O)).

Synthesis of NO-releasing alginates.

To form *N*-diazoniumdiolate NO donors on the alginate scaffolds, polyamine-modified alginate (45 mg) was dissolved in 50 mM NaOH (3 mL) in a 1-dram glass vial. The open vials were placed in a stainless steel reactor and stirred continuously using a magnetic stir bar. Oxygen was removed from the vessel by purging with argon (10 s, 7 bar) three times, followed by three additional long purges with argon (10 mins, 7 bar). The vessel was then pressurized to 10 bar with NO gas and allowed to react for 3 d. The same argon purging protocol was then repeated to remove unreacted NO. The NO-releasing alginates were then precipitated in methanol, collected by centrifugation, dried overnight in vacuo, and stored in vacuum sealed bags at $-20\text{ }^\circ\text{C}$ as a dry powder.

Characterization of NO storage and release.

Nitric oxide release was evaluated in real-time using a Sievers 280i Chemiluminescence NO analyzer (NOA; Boulder, CO). Each sample was analyzed before use (e.g., in a biological study) to ensure accurate NO levels. Of note, the NO levels did not change appreciably for alginates stored in moisture-free environments. Prior to analysis, the NOA was calibrated with air passed through a NO zero filter (0 ppm NO) and 25.87 ppm of NO standard gas (balance N₂). In a typical measurement, NO-releasing alginates (1 mg) were dissolved in 30 mL of PBS (10 mM, pH 7.4, 37 °C). The solution was purged with nitrogen gas at a flow rate of 70 mL/min to carry liberated NO from the solution to the analyzer. Additional nitrogen flow was supplied to the flask to match the collection rate of the instrument (200 mL/min). Nitric oxide analysis was terminated when NO levels fell below 10 ppb of NO/mg of alginate (the limit of detection of the instrument).

Planktonic bactericidal assays.

P. aeruginosa and *S. aureus* bacterial cultures were grown from a frozen (−80 °C) stock overnight in TSB at 37 °C. A 500 µL aliquot of culture was grown in 50 mL of fresh TSB to a concentration of 10⁸ colony forming units per mL (CFU/mL). 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* and *S. aureus* were taken from the TSA plate, inoculated in 3 mL TSB overnight at 37 °C, and recultured in fresh TSB (50 mL) to a concentration of 10⁸ CFU/mL. These cultures were centrifuged, resuspended in PBS, and diluted to 10⁶ CFU/mL. Weighed samples of NO-releasing alginates and controls (non-NO-releasing alginates) were added to a 1-dram vial. Corresponding volumes of the 10⁶ CFU/mL bacteria were then added to obtain a range of alginate concentrations (0.5 to 16 mg/mL). These solutions were incubated for 4 h at 37 °C. Blanks or untreated bacterial solutions were included in each experiment to ensure bacteria viability over the duration of the experiment. Following treatment, the bacterial solutions were diluted serially (10- and 100-fold dilutions), spiral plated on TSA using an Eddy Jet spiral plater (IUL; Farmingdale, NY), and incubated overnight at 37 °C. Bacterial viability was determined using a Flash & Go colony counter (IUL; Farmingdale, NY). The minimum bactericidal concentration after a 4-hour exposure period (MBC_{4h}) was defined as the minimum concentration required to achieve a 3-log reduction in bacterial viability relative to untreated cells (i.e., reduced bacterial counts from 10⁶ to 10³ CFU/mL). The limit of detection for this counting method is 2.5 × 10³ CFU/mL.⁴² The corresponding NO dose was calculated by multiplying the MBC_{4h} of the alginate samples (mg/mL) with the measured NO released in PBS (pH 7.4; µmol NO/mg alginate) over the 4 h experimental window.

Biofilm eradication assays.

Similar to planktonic experiments, *P. aeruginosa* and *S. aureus* bacterial cultures were grown overnight in TSB at 37 °C and recultured in fresh TSB to a concentration of 10⁸ CFU/mL. These solutions were diluted to 10⁶ CFU/mL in sterile media (*P. aeruginosa*, TSB; *S. aureus*, TSB + 0.1% glucose) and grown for 48 h at 37 °C with gentle shaking. The formed biofilms were separated from the growth media by pipetting the biofilm mass. Biofilms (250 µL)

were combined with 750 μL of PBS and added to 1-dram vials containing premeasured samples of NO-releasing and control alginates, with final alginate concentrations ranging from 4 to 64 mg/mL. The samples were incubated with gentle shaking for 24 h at 37 °C. Blanks were included in each experiment to ensure bacterial viability over the duration of the experiment. The dispersed biofilms were vortexed, serially diluted (10-, 100-, 1000-, and 10,000-fold dilutions), plated on TSA plates using an Eddy Jet spiral plater, and incubated overnight at 37 °C. Bacterial viability was assessed using a Flash & Go colony counter. The minimum biofilm eradication concentration at 24 h (MBEC_{24h}) was defined as the minimum concentration required to achieve a 5-log reduction in bacterial viability compared to untreated cells (i.e., reduced bacterial counts from 10⁸ to 10³ CFU/mL). The corresponding NO dose was calculated by multiplying the MBEC_{24h} of the alginate samples (mg/mL) with the NO released in PBS (pH 7.4; μmol NO/mg alginate) over the testing period.

Time-based biofilm eradication assay.

P. aeruginosa biofilms were grown and treated with NO-releasing alginates (8 mg/mL) following the same protocol as for the biofilm eradication assays. Blanks were included to ensure bacteria viability over the duration of the experiment. The samples were incubated with gentle shaking at 37 °C for 1–24 h before plating on TSA plates using an Eddy Jet spiral plater and incubating overnight at 37 °C. Bacterial viability was assessed using a Flash & Go colony counter. A similar experiment was carried out at the MBEC_{24h} for Alg-PAPA/NO and Alg-SPER/NO for comparison to time-based killing at 8 mg/mL.

In vitro cytotoxicity assay.

A549 human respiratory epithelial cells were grown in RPMI 1640 media supplemented with 10 vol% FBS and 1 wt% penicillin streptomycin. Cells were incubated in 5 vol % CO₂ under humidified/aerobic conditions at 37 °C. After reaching 80% confluency, the cells were seeded onto 96-well polystyrene plates at a density of 2×10^3 cells/well, and incubated for 24 h at 37 °C. The supernatant was then aspirated and replaced with 100 μL of alginate in fresh growth medium with a final concentration of test material equivalent to MBEC_{24h} against *P. aeruginosa* or *S. aureus* biofilms. After 24 h incubation at 37 °C, the supernatant was aspirated and the wells containing the cells were washed with PBS. A 100 μL mixture of RPMI 1640/MTS/PMS (105/20/1, v/v/v) was added to each well. After an additional 90 min incubation period (37 °C), the absorbances of the wells were measured at 490 nm using a ThermoScientific Multiskan EX plate reader (Waltham, MA). A blank mixture of RPMI 1640/MTS/PMS and untreated cells were used as the blank and control, respectively. Cell viability for each sample was calculated as follows:

$$\% \text{ cell viability} = \frac{(Abs_{490} - Abs_{blank})}{(Abs_{control} - Abs_{blank})} \times 100 \quad (\text{eq.1})$$

Results and Discussion

Alginates are found in nature as polydisperse, water soluble high molecular weight polymers (200–500 kDa).^{26,27} Prior research has demonstrated the ease by which these polymers

undergo oxidative degradation to form lower molecular weight oligosaccharides.^{41,43} In this regard, the oxidative degradation reaction conditions were optimized to yield alginate oligosaccharides of ~5 kDa molecular weight to allow for comparison to other previously reported water-soluble NO-releasing biopolymer systems.⁴⁰ The addition of secondary amine-bearing functional groups to the alginate backbone allows for the subsequent formation of *N*-diazoniumdiolate NO donors. This class of NO donor facilitates spontaneous NO release in physiological media. In the present study, a series of small molecule NO donor precursors were linked to the carboxylic acid functional groups of both high molecular weight (~300 kDa) and low molecular weight (~5 kDa) alginates via EDC/NHS reactions (Scheme 1) to enable tuning of the NO-release kinetics. Of note, pH 6.5 phosphate buffer saline was employed to avoid gelation of alginate.³⁰ The reaction was optimized to maximize alkyl amine modification while minimizing the possibility of crosslinking between the alginate backbone and the diamine functional groups (i.e., DETA, DPTA, SPER). The average molecular weight (Mw) was dependent on the molar excess of the alkyl amine added to the reaction solution (Table S4). For example, maintaining the molar ratio at 1:1 resulted in a significant increase in the molecular weight of the alkyl amine-modified alginate (e.g. Alg300-SPER with Mw ~700 kDa), suggesting possible crosslinking with the biopolymer. Increasing the molar ratio of the diamine functional group minimized this effect (Table S4). Of note, no crosslinking was observed with the low molecular weight systems regardless of the molar ratio employed. The gel phase was avoided entirely when using the optimized reaction conditions. Polyamine functionalization was also confirmed using ¹H NMR, ¹³C NMR, and elemental analysis (Figures S1, S2, S3, and S4). The nitrogen content of the alginates increased from 0 to 5–8 wt% with amine modification (Table S1). This value translates to ~40–50 % modification of the carboxylic acid functional groups. A positive shift in zeta potential was also noted upon amine modification, corresponding to the formation of protonated amine groups at pH 7.4 (Table S1).

Synthesis of *N*-diazoniumdiolate-functionalized alginate.

The secondary amine-modified alginates were charged with high pressures of NO (10 bar) in basic aqueous solution (50 mM NaOH) to allow efficient formation of *N*-diazoniumdiolate NO donors (Scheme 2). The formation of the NO donors was confirmed using UV-vis spectroscopy and the appearance of a characteristic absorbance band at 253 nm (Figure S5).

Representative NO-release profiles of the *N*-diazoniumdiolate-functionalized alginates in PBS (pH 7.4) at 37 °C are shown in Figure 1. Both high and low molecular weight alginates were found to have similar NO totals that were comparable to other macromolecular NO-releasing biopolymeric scaffolds (0.3–0.6 μmol/mg).^{40,44–47} A broad range of NO-release kinetics was also observed for the alginate scaffolds with Alg300-PAPA/NO and Alg5-PAPA/NO having the shortest NO-release half-lives (~0.5 and ~0.3 h, respectively) or fastest NO donor breakdown. In contrast, the NO release of Alg300-DETA/NO and Alg5-DETA/NO was highly prolonged (NO-release half-lives of ~40 and 29 h, respectively). The NO-release duration varied as a function of amine precursor structure, following trends previously reported for the corresponding small molecule NO donors (i.e., DETA, DPTA, PAPA, and SPER).^{48,49} A decrease in the NO-release half-life (i.e., faster NO release) was clearly observed upon using the longer chain length alkyl amines.^{38,48,49} The terminal,

positively charged primary amine appeared to stabilize the negative charge of the *N*-diazoniumdiolate NO donor, increasing the NO-release half-life for DETA-, DPTA-, and SPER-modified alginates compared to the PAPA-modified biopolymer (Scheme 2).⁴⁹ The molecular weight of the alginate also dramatically influenced the NO release. For example, the Alg5 oligosaccharides released NO more rapidly than the larger Alg300 analogs modified with the same amine precursor (Table 1). In addition, negligible NO release was observed for control alginates modified with ethylenediamine (EDA), resulting in a biopolymer containing only amide and primary amine groups. These results confirmed that neither the amide nor primary amine functional groups are able to react with NO under the conditions used for *N*-diazoniumdiolate formation (Table S5). Overall, these experiments demonstrate exquisite NO-release tunability by simply varying the molecular weight of the scaffold and/or the chemical structure of an amine precursor grafted onto the alginate backbone.

Antibacterial action against planktonic bacteria.

The antibacterial activity of control and NO-releasing alginates was evaluated against *P. aeruginosa* and *S. aureus*, two prevalent pathogens associated with infections.^{6,50} Bacterial viability assays were performed under static conditions to extract the minimum bactericidal concentration of the test agent required to elicit a 3-log reduction (i.e., 99.9% killing) in bacterial viability over 4 h (MBC_{4h}). The non-NO-releasing controls did not significantly impact bacterial viability up to 16 mg/mL for both *P. aeruginosa* and *S. aureus* (Figure S6). In contrast, low concentrations (< 8mg/mL), led to a 3-log reduction (99.9%) in bacterial viability for each of the NO-releasing alginate scaffolds (Table 2), with the less negatively charged alkyl amine-modified alginate requiring the least amount to achieve bactericidal activity (Table S1). Other studies have reported that positively charged scaffolds associate more readily with bacteria with a concomitant enhancement in bactericidal action.^{40,51} The alginates herein followed a similar trend, with Alg300-SPER/NO (zeta potential -13.0 mV) eradicating bacteria at the lowest concentration while Alg300-PAPA/NO (-33.4 mV) necessitated the largest (Table S1). Nitric oxide-release kinetics also influenced the bactericidal action for the alginate materials, with the fastest (Alg300-PAPA/NO) and slowest (Alg300-DETA/NO) NO-releasing systems requiring greater concentrations relative to alginates with moderate release kinetics. Despite larger overall NO payloads (~0.6 µmol/mg), the faster NO release associated with Alg300-PAPA/NO may liberate NO prematurely prior to the alginate associating with the bacteria, which could then facilitate more efficient delivery of lethal NO doses to the bacteria. Indeed, other macromolecular NO-release systems (e.g., silica) required 20 min to elicit NO build up within *P. aeruginosa* as studied via confocal microscopy.¹⁵ The elevated concentrations of NO-releasing alginates needed for eradication using Alg300-DETA/NO are likewise attributed to insufficient NO released over the 4h MBC assay (~0.10 µmol/mg) compared to the other NO donor modifications (i.e., DPTA, PAPA, SPER).

A similar dependence on NO-release kinetics was observed for the low molecular weight alginates with both Alg5-SPER/NO and Alg5-DPTA/NO having the lowest MBC_{4h}. Of note, the concentrations of NO-releasing alginate oligosaccharides required to eradicate bacteria were significantly lower than their high molecular weight counterparts. Similar size-

dependent effects were reported for NO-releasing silica, a denser (i.e., harder) material.^{20,52} For biopolymers specifically, Liu et al. reported more effective antibacterial action with lower molecular weight NO-releasing chitosan.⁵³ Our results indicate that the use of lower molecular weight alginates also allows for more efficient NO delivery, in turn reducing the required NO dose for eradicating planktonic bacteria.

For both high and low molecular weight alginates, greater concentrations of NO-releasing derivations were necessary to achieve bactericidal activity against *S. aureus* relative to *P. aeruginosa* (Table 2). The thicker peptidoglycan layer of *S. aureus* likely decreases the diffusion of NO into the bacterium, necessitating larger doses of the macromolecule to achieve equivalent killing.⁵⁴ Gram-negative bacteria such as *P. aeruginosa* generally have a more lipid-rich outer membrane and thinner peptidoglycan sheets, increasing their susceptibility to NO and other permeating antimicrobials.⁵⁴

Anti-biofilm efficacy.

In addition to exhibiting bactericidal action against planktonic bacteria, the NO-releasing alginate scaffolds were also highly effective against *P. aeruginosa* and *S. aureus* biofilms. Similar to the planktonic assays, the concentrations of NO-releasing alginate oligosaccharides were lower for eradicating the biofilms relative to the higher molecular weight systems (Table 3), further emphasizing the role of NO delivery scaffold on bactericidal action. Greater biocidal action was also noted against *P. aeruginosa* over *S. aureus* biofilms. Worley et al. previously reported reduced penetration of NO-releasing poly(amido amine) dendrimers into *S. aureus* biofilms relative to *P. aeruginosa*.²² Such differences can be attributed to the biofilm architectures. The exopolysaccharide matrix of *S. aureus* biofilms produce greater levels of polysaccharide and extracellular proteins that hinder the diffusion of antibacterial agents such as NO.^{22,55} Coupled with the peptidoglycan differences, these structural advantages account for the lower susceptibility of *S. aureus* biofilms. Nevertheless, the broad-spectrum action of NO is clearly capable of eradicating both Gram-positive and Gram-negative bacterial biofilms.

Following the size-dependent trend observed in the planktonic studies, the less negatively charged alginate systems required lower alginate concentrations to elicit biocidal activity, with the exception of Alg300-SPER/NO. Despite having the least negative charge of the evaluated alginate systems (Table S1), the large NO flux and rapid release half-life for Alg300-SPER/NO (Table 1) likely results in undesirable NO donor breakdown similar to that noted for both Alg300-PAPA/NO and Alg5-PAPA/NO. In this respect, the overall biocidal efficacy of the NO-releasing scaffold depends on both the charge of the scaffold and the NO-release kinetics. Alginate scaffolds with moderate to extended NO-release durations ($t_{1/2}$ ~ 2 h) consistently require lower alginate concentrations and NO doses to eradicate biofilm-based bacteria, regardless of the alginate molecular weight or bacteria Gram class. In this regard, the slower, sustained NO release ensures more effective NO delivery. This result is in agreement with previous studies using different NO-releasing systems.^{20,23,24}

P. aeruginosa biofilms were treated with equal concentrations (8 mg/mL) of each high molecular weight NO-releasing alginate (Figure 2A) to study bacterial viability as a function of release kinetics. The high molecular weight alginate scaffolds were selected for this

experiment as they demonstrated the largest range in NO-release kinetics. A concentration of 8 mg/mL represented the lowest MBEC_{24h} for the Alg300 systems. At 1 h, treatment with either Alg300-PAPA/NO or Alg300-SPER/NO resulted in a 1- to 2-log reduction in biofilm bacterial viability, as expected based on the NO-release half-lives for the two NO-releasing alginates (~0.5–1.4 h). However, the low NO levels released from the fast NO-releasing alginate at longer durations were insufficient for bacterial killing, with *P. aeruginosa* viability recovering to ~10⁸ CFU/mL after 4 h. In contrast to the fast NO-release systems, the slower NO-releasing alginate scaffolds (~4 and 13 h for Alg300-DPTA/NO and Alg300-DETA/NO, respectively) elicited a more gradual decrease in bacteria viability at this same concentration (i.e., 8 mg/mL), eventually leading to a 5-log reduction after 8 h of treatment. At the larger concentrations required to achieve the MBEC_{24h}, both Alg300-PAPA/NO (32 mg/mL) and Alg300-SPER/NO (16 mg/mL) elicited a 5-log reduction in bacterial viability after only 1 and 4 h exposures, respectively, but resulted in observable bacterial growth after 24 h (Figure 2B). The greater alginate (and NO) concentrations improved the antibacterial efficacy of the faster NO-releasing alginates through rapid killing induced by the large initial NO burst. Overall, these results suggest that slower, more sustained NO release is preferred for biofilm eradication at the smaller alginate concentrations.

In Vitro Cytotoxicity against A549 cells.

An attractive property of alginates for biomedical applications is the perceived favorable toxicity to mammalian cells. Of course, the influence of the *N*-diazoniumdiolate modification on cytotoxicity might elicit unexpected toxicity. Thus, the cytotoxicity of the alginate scaffolds was evaluated using human respiratory epithelial (A549) cells at the alginate MBECs determined to kill *P. aeruginosa* and *S. aureus*.^{56–60} As shown in Figure 3, neither control nor the NO-releasing alginates exhibited significant toxicity (i.e., cell viabilities >70%) against A549 cells at the determined MBEC_{24h} values, regardless of size (i.e., molecular weight) or charge, highlighting the advantage of these biopolymers as anti-biofilm agents. The low to negligible toxicity of the NO-releasing alginates at the bactericidal concentrations shows promise for the use of these macromolecules as antibacterial and anti-biofilm treatment for wound healing and respiratory infections, for example.

Conclusions

N-diazoniumdiolate-modified alginate scaffolds were synthesized with diverse NO-release kinetics (0.5–13 h half-lives) and antibacterial properties. The biocidal efficacies of the NO-releasing alginate materials were demonstrated against both planktonic and biofilm-based forms of *P. aeruginosa* and *S. aureus*. Alginate modifications that exhibited slow and sustained NO release (e.g., Alg300-DPTA/NO and Alg300-DETA/NO) proved to be highly effective against bacterial biofilms, requiring lower NO-releasing alginate to elicit eradication. The molecular weight of the scaffold proved important with the oligosaccharides eliciting the greatest antibacterial action. Both amine-modified (control) and NO-releasing alginates exerted minimal toxicity against A549 cells, demonstrating the biocompatible properties of the bare scaffold. Nitric oxide-releasing alginates may prove useful as novel antibacterial agents for treating acute and chronic infections. Experiments

are currently underway to evaluate the biocidal efficacy of these materials against a broader spectrum of bacteria strains, including clinical isolates that are resistant to conventional antibiotics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Real time NO-release profiles for the first 1 h and (B) plot of total NO release vs. time for Alg300-DETA/NO (solid), Alg300-DPTA/NO (dash), Alg300-SPER/NO (dash-dot), and Alg300-PAPA/NO (dot) measured via chemiluminescence in pH 7.4 PBS.

Figure 2.

Time-based bactericidal efficacy of Alg300-DETA/NO (square), Alg300-DPTA/NO (circle), Alg300-SPER/NO (triangle), and Alg300-PAPA/NO (diamond). Comparison of all high molecular weight NO-releasing alginates at equivalent concentrations of 8 mg/mL is presented in (A), and the time-based killing of the fast NO-releasing systems (Alg300-SPER/NO and Alg300-PAPA/NO) at their respective MBEC_{24h} is presented in (B). Studies consisted of at least three experiments with error bars representing the standard deviation.

Figure 3. Viability of A549 human respiratory epithelial cells exposed to control and NO-releasing (white) and control (dash) alginates at the MBEC_{24h} against (A) *P. aeruginosa* and (B) *S. aureus*. Studies consisted of at least three experiments with error bars representing the standard deviation.

Scheme 1.
Synthesis of amine-functionalized alginate.

Scheme 2.
Synthesis of *N*-diazeniumdiolate-modified alginates.

Table 1.Nitric oxide-release properties of *N*-diazoniumdiolate-functionalized alginates in PBS (pH 7.4, 37 °C).^a

Scaffold	t[NO] ^b (μmol/mg)	[NO] _{max} ^c (ppb/mg)	t _{1/2} ^d (h)	t _d ^e (h)	t[NO] _{4h} ^f (μmol/mg)
Alg300-DETA/NO	0.40 ± 0.04	232 ± 78	13.1 ± 5.8	40.3 ± 14.2	0.10 ± 0.01
Alg300-DPTA/NO	0.42 ± 0.04	428 ± 124	3.4 ± 0.6	16.1 ± 2.9	0.23 ± 0.01
Alg300-PAPA/NO	0.61 ± 0.13	2110 ± 1240	0.5 ± 0.1	6.1 ± 1.0	0.59 ± 0.11
Alg300-SPER/NO	0.65 ± 0.16	1236 ± 240	1.3 ± 0.4	14.6 ± 3.5	0.49 ± 0.11
Alg5-DETA/NO	0.33 ± 0.03	101 ± 30	9.8 ± 0.1	29.3 ± 1.7	0.08 ± 0.03
Alg5-DPTA/NO	0.48 ± 0.02	1157 ± 227	1.2 ± 0.1	8.7 ± 0.5	0.45 ± 0.10
Alg5-PAPA/NO	0.55 ± 0.11	3543 ± 908	0.3 ± 0.0	3.9 ± 0.3	0.55 ± 0.11
Alg5-SPER/NO	0.38 ± 0.03	477 ± 307	1.6 ± 0.2	11.1 ± 0.8	0.25 ± 0.04

^aError represent standard deviation for n = 3 experiments.^bTotal NO released.^cMaximum flux of NO release.^dNO-release half-life.^eDuration of NO release.^fTotal NO released after 4 h.

Table 2.

Minimum bactericidal concentrations (MBC_{4h}) of NO-releasing alginates against planktonic *P. aeruginosa* and *S. aureus*.^a

Scaffold	<i>P. aeruginosa</i>		<i>S. aureus</i>	
	MBC _{4h} (mg/mL)	NO dose ^b (μmol/mL)	MBC _{4h} (mg/mL)	NO dose ^b (μmol/mL)
Alg300-DETA/NO	4	0.40 ± 0.04	8	0.80 ± 0.08
Alg300-DPTA/NO	2	0.45 ± 0.01	4	0.88 ± 0.02
Alg300-PAPA/NO	2	1.18 ± 0.24	8	4.75 ± 0.95
Alg300-SPER/NO	1	0.49 ± 0.11	4	1.96 ± 0.45
Alg5-DETA/NO	2	0.20 ± 0.02	4	0.40 ± 0.04
Alg5-DPTA/NO	0.25	0.11 ± 0.01	2	0.92 ± 0.01
Alg5-PAPA/NO	0.5	0.30 ± 0.06	8	4.69 ± 0.88
Alg5-SPER/NO	0.25	0.07 ± 0.01	2	0.54 ± 0.09

^aError represent standard deviation for n = 3 experiments.

^bNO dose was calculated from NO totals of each tested sample at 4 h in PBS (10 mM, pH 7.4, 37 °C).

Table 3.

Minimum biofilm eradication concentrations (MBEC_{24h}) of NO-releasing alginates against *P. aeruginosa* and *S. aureus* biofilms.^a

Scaffold	<i>P. aeruginosa</i>		<i>S. aureus</i>	
	MBEC _{24h} (mg/mL)	NO dose ^b (μmol/mL)	MBEC _{24h} (mg/mL)	NO dose ^b (μmol/mL)
Alg300-DETA/NO	8	2.6 ± 0.5	16	6.2 ± 0.2
Alg300-DPTA/NO	8	3.4 ± 0.0	16	6.8 ± 0.1
Alg300-PAPA/NO	32	19.6 ± 3.8	64	39.2 ± 7.6
Alg300-SPER/NO	16	10.3 ± 1.8	32	20.7 ± 3.6
Alg5-DETA/NO	4	1.8 ± 0.5	8	3.6 ± 1.1
Alg5-DPTA/NO	4	1.7 ± 0.2	4	1.7 ± 0.2
Alg5-PAPA/NO	16	9.8 ± 2.0	16	9.8 ± 2.0
Alg5-SPER/NO	4	1.3 ± 0.2	8	2.8 ± 0.2

^aError represent standard deviation for n = 3 experiments.

^bNO dose was calculated from 24 h NO totals of each tested sample in PBS (10 mM, pH 7.4, 37 °C).