Bioconjugate Chemistry

Article

pubs.acs.org/bc

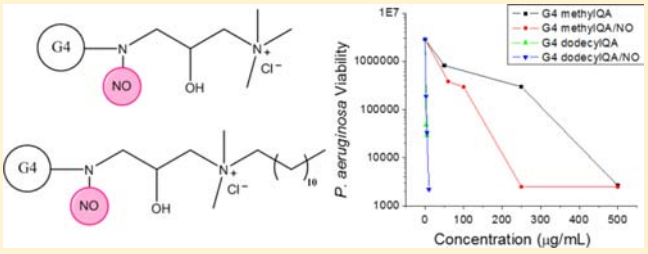
Nitric Oxide-Releasing Quaternary Ammonium-Modified Poly(amidoamine) Dendrimers as Dual Action Antibacterial Agents

Brittany V. Worley, Danielle L. Slomberg, and Mark H. Schoenfisch*

Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States

Supporting Information

ABSTRACT: Herein we describe the synthesis of nitric oxide (NO)-releasing quaternary ammonium (QA)-functionalized generation 1 (G1) and generation 4 (G4) poly(amidoamine) (PAMAM) dendrimers. Dendrimers were modified with QA moieties of different alkyl chain lengths (i.e., methyl, butyl, octyl, dodecyl) via a ring-opening reaction. The resultant secondary amines were then modified with *N*-diazeniumdiolate NO donors to yield NO-releasing QA-modified PAMAM dendrimers capable of spontaneous NO release (payloads of ~0.75 μmol/mg over 4 h). The bactericidal efficacy of individual (i.e., non-NO-releasing) and dual action (i.e., NO-releasing) QA-modified PAMAM dendrimers was evaluated against Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* bacteria. Bactericidal activity was found to be dependent on dendrimer generation, QA alkyl chain length, and bacterial Gram class for both systems. Shorter alkyl chains (i.e., methylQA, butylQA) demonstrated increased bactericidal activity against *P. aeruginosa* versus *S. aureus* for both generations, with NO release markedly enhancing overall killing.



INTRODUCTION

Although antibiotics remain the gold standard in the treatment of bacterial infections, the increased occurrence of antibiotic-resistant bacteria coupled with a decline in the development of new antibiotics has necessitated the development of alternative antibacterial agents.[1] The coadministration of two mechanistically different biocides has been demonstrated to reduce the emergence of bacterial resistance and can be synergistic, where the bactericidal efficacy of the combination is more effective than their individual sums.[2,3] Combining multiple biocides on a single macromolecular scaffold (e.g., nanoparticles, dendrimers) is expected to further increase bactericidal efficacy.[4] Importantly, enhancing the bactericidal efficacy of a scaffold should lower the required therapeutic dose, concomitantly reducing any toxicity to healthy cells and tissue. A promising option for multimodal therapeutics is to modify nondepleting, contact-based antibacterials with chemistries that allow for spontaneous release of a second biocide, thereby increasing the antibacterial sphere of influence.

Nitric oxide (NO) is an endogenously produced, reactive free radical that plays a central role in the host defense against microbial pathogens.[5,6] The broad-spectrum antibacterial activity of NO is derived from the production of reactive byproducts (e.g., dinitrogen trioxide and peroxynitrite) that compromise the bacterial membrane and cell function through both nitrosative and oxidative stresses.[5,7] The ability of NO to act through more than one bactericidal mechanism makes it effective against a multitude of infectious pathogens. Large molecular frameworks capable of storing and controllably releasing NO have been developed as novel antibacterial agents. For example, our laboratory has reported the synthesis and bactericidal efficacy of both NO-releasing silica nanoparticles and dendrimers against several strains of bacteria.[7−10] Benefits of these macromolecular scaffolds include controllable NO payloads and release rates, modifiable surface chemistries (e.g., to allow for the combination of multiple biocides on a single scaffold), and reduced toxicity to mammalian cells.[8,10,11]

Quaternary ammonium (QA) compounds, widely used as antiseptic and disinfectant agents, are popular nondepleting biocides due to their broad-spectrum efficacy.[12] The bactericidal activity of QA compounds stems from the attractive electrostatic interactions between the cationic QA group and the negatively charged bacterial cell membrane, disrupting natural chemical balances by replacing essential metal cations.[12] The addition of long alkyl chains to the QA group promotes bacterial membrane penetration, further amplifying biocidal activity. The bactericidal efficacy of QA compounds is highly dependent on the length of this alkyl chain, with alkyl chains of at least eight carbon atoms demonstrating the greatest bactericidal activity due to increased penetration into the cell membrane.[12−14] The combination of long chain QA moieties with a releasable biocide such as nitric oxide would thus be anticipated to increase bactericidal activity. For instance, it has been demonstrated that QA-functionalized polymers capable of releasing silver ions (Ag^+) exhibit a wider zone of inhibition than QA polymers alone.[15,16] Carpenter et al. similarly reported

Received: February 18, 2014
Revised: April 11, 2014
Published: May 5, 2014

   dx.doi.org/10.1021/bc5000719 | *Bioconjugate Chem.* 2014, 25, 918−927

Scheme 1. Quaternary Ammonium-Modified Dendrimers (C) Were Synthesized via Ring-Opening Reaction of QA Epoxides (B) with PAMAM Primary Amines (A) and Subsequently Reacted with High Pressures of NO to Yield *N*-Diazeniumdiolate NO Donors (D)

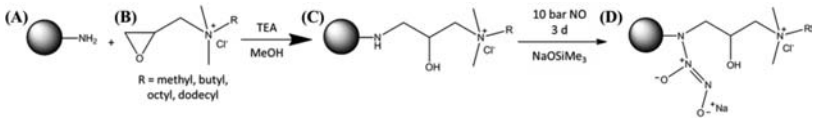


Table 1. Nitric Oxide Release Properties for G1 and G4 QA-Modified Dendrimers in PBS (pH 7.4, 37 °C) as Measured by a Chemiluminescence NO Analyzer[a]

	$[NO]_{max}$[b] (ppb/mg)	t_{max}[c] (s)	$t[NO]$[d] (μmol/mg)	$t[NO]_{4h}$[e] (μmol/mg)	$t_{1/2}$[f] (h)
G1 methylQA/NO	15000 ± 3372	48 ± 7	1.50 ± 0.11	0.73 ± 0.07	4.4 ± 1.1
G1 butylQA/NO	8675 ± 8182	61 ± 9	1.35 ± 0.30	0.78 ± 0.19	3.0 ± 0.5
G1 octylQA/NO	3400 ± 1249	64 ± 5	1.30 ± 0.05	0.69 ± 0.06	3.6 ± 0.9
G1 dodecylQA/NO	5016 ± 1379	71 ± 8	1.07 ± 0.16	0.72 ± 0.12	1.9 ± 0.8
G4 methylQA/NO	5170 ± 252	52 ± 4	1.69 ± 0.22	0.77 ± 0.08	4.9 ± 0.6
G4 butylQA/NO	4550 ± 3097	62 ± 13	1.48 ± 0.32	0.78 ± 0.18	3.8 ± 1.2
G4 octylQA/NO	7721 ± 5372	62 ± 10	1.49 ± 0.41	0.86 ± 0.23	2.9 ± 0.4
G4 dodecylQA/NO	3550 ± 1909	84 ± 4	1.17 ± 0.11	0.78 ± 0.06	1.9 ± 0.1

[a]Each parameter was analyzed with multiple replicates (n = 3). [b]Maximum flux of NO release. [c]Time required to reach maximum flux. [d]Total NO payload released. [e]NO payload released after 4 h. [f]NO release half-life.

on NO donor-modified QA-functionalized silica nanoparticles with enhanced bactericidal efficacy against both Gram-positive and Gram-negative bacteria.[8] Despite improved antibacterial efficacy, however, the NO-releasing QA-modified silica particles demonstrated significant toxicity to mammalian cells at concentrations required to eradicate bacteria, indicating the need for a more suitable molecular framework for further development of these dual action therapeutics.

Dendrimers are a family of macromolecular scaffolds with hyperbranched architecture and multivalent surfaces that have been widely investigated for use as drug delivery and therapeutic vehicles.[17,18] The ability to functionalize the exterior surface groups of dendrimers allows for their modification with specific bactericidal end groups. This capability, combined with the ability of dendrimers to associate with and/or cross bacterial membranes, provides a potentially more effective scaffold for the development of combination therapeutics.[19,20] Indeed, both amino-terminated (unmodified) and modified poly(amidoamine) (PAMAM) and poly(propyleneimine) (PPI) dendrimers have been shown to exhibit biocidal activity.[17,19−21] Cooper and co-workers demonstrated that PPI dendrimers modified with QA moieties containing alkyl chains of eight or more carbons exhibited antibacterial activity as a function of dendrimer size and QA alkyl chain length.[13,22] Likewise, Charles et al. found that dodecylQA-modified G3 PAMAM dendrimers exhibited an increased zone of inhibition against Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus* compared to butylQA and hexylQA modifications.[23] While these initial studies demonstrate the promise of QA-modified dendrimers for use as antibacterial agents, further investigation into the effect of QA alkyl chain length and bacterial Gram designation on bactericidal efficacy is required. Furthermore, we hypothesize that the modification of QA dendrimer scaffolds with NO release capabilities will result in increased bactericidal efficacy against both Gram-positive and -negative bacteria compared to the QA-modified dendrimers alone.

RESULTS AND DISCUSSION

We have previously reported the synthesis of secondary-amine functionalized dendrimers with diverse exterior functionalities by reacting epoxides with primary amines on the dendrimer exterior.[24] In the present study, generation 1 (G1) and generation 4 (G4) poly(amidoamine) (PAMAM) dendrimers were modified with QA moieties via a ring-opening reaction between the peripheral primary amines (n) and QA epoxides (Scheme 1). To investigate the role of alkyl chain length on NO release and bactericidal activity, PAMAM dendrimers were functionalized with QA moieties containing methyl, butyl, octyl, and dodecyl alkyl chains. While glycidyltrimethylammonium chloride (methylQA epoxide) is available commercially, butylQA, octylQA, and dodecylQA epoxides were synthesized through the reaction of epichlorohydrin with dimethylbutylamine, dimethyloctylamine, or dimethyldodecylamine, respectively.[8]

The resulting QA epoxides were reacted with G1 (n = 8) and G4 (n = 64) PAMAM dendrimers in a 2.5-fold excess to the number of peripheral primary amines in methanol. The number of QA moieties added to the dendrimer scaffold was determined using ^{1}H NMR spectroscopy. On average, 4 and 32 QA moieties were tethered to the G1 and G4 PAMAM scaffolds, respectively, resulting in approximately 50% functionalization of the peripheral primary amines. The addition of the quaternary ammonium to the dendrimer scaffold was confirmed using IR spectroscopy and X-ray photoelectron spectroscopy (XPS) (Supporting Information). A weak IR stretch at 970 cm^{-1} indicative of the QA functional group was present for all QA-modified dendrimers.[25] The N 1s binding energies of the PAMAM dendrimer amines ranged from 397−399 eV, with an additional peak at 402 eV, corresponding to the quaternary ammonium group, present after modification with the QA moiety.[26] Additionally, a doublet at 198 eV, absent for the PAMAM scaffold, was observed for the QA-modified dendrimers, signifying the presence of the chloride counterion.

Both G1 and G4 QA-modified dendrimers were exposed to high pressures of NO in the presence of a base to form *N*-diazeniumdiolate NO donors on secondary amines. *N*-

dx.doi.org/10.1021/bc5000719 | *Bioconjugate Chem.* 2014, 25, 918−927

Diazeniumdiolate formation was confirmed by the presence of an absorbance maximum at 253 nm, a peak that was not observed in the absorbance spectra of QA-modified PAMAM dendrimers prior to diazeniumdiolate formation (Supporting Information).[7] Nitric oxide storage was tunable by adjusting the polarity of the charging solvent (i.e., by increasing the ratio of THF:methanol with increasing alkyl chain length). The resulting NO-releasing QA-modified dendrimers exhibited similar 4 h NO payloads of approximately 0.76 μmol/mg (Table 1). In this regard, the effects of both generation and QA alkyl chain length on bactericidal efficacy could be evaluated independent of NO release totals.

The *N*-diazeniumdiolated QA-modified dendrimers were characterized by an initial maximum burst of NO ($[NO]_{max}$) after introducing the dendrimers into solution, followed by a steady decline in NO release (Supporting Information). For each generation, the time required to reach this maximum flux (t_{max}) increased as the QA alkyl chain length increased from methyl to dodecyl, with a concomitant decrease in the $[NO]_{max}$ values. The increase in t_{max} with increasing alkyl chain length is attributed to the presence of the hydrophobic QA alkyl chains that decrease the rate of water diffusion to the secondary amine-bound NO donors.[8] However, a decrease in NO-release half-life was also observed with increasing alkyl chain length, an opposite phenomenon from what might be expected from reduced water diffusion. The observed decrease in half-life for longer (i.e., octylQA and dodecylQA) QA alkyl chains is likely due to the formation of dendrimer vesicles in aqueous solution, in which the hydrophobic alkyl chains of neighboring dendrimers face inward (i.e., toward each other), exposing the portion of the dendrimer scaffold containing the *N*-diazeniumdiolate to aqueous solution (Figure 1). Indeed,

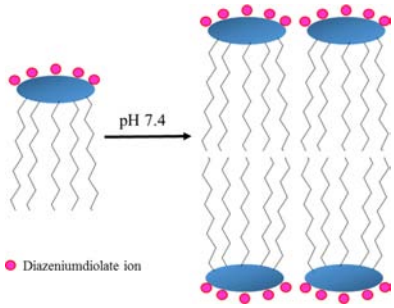


Figure 1. Formation of dendrimer vesicles at pH 7.4. The hydrophobic alkyl chains of neighboring dendrimers face toward each other, exposing the *N*-diazeniumdiolate ions to aqueous solution.

Schenning et al. reported the formation of dendrimer bilayers at pH 7.4 for hydrophobic alkyl chain-modified scaffolds.[27] In this scenario, the hydrophobic alkyl chains align perpendicularly to the dendrimer scaffold, which faces the aqueous phase.[27] To determine if the QA-modified dendrimers were in fact forming supramolecular structures in aqueous solution, the surface tension of phosphate buffered saline (pH 7.4) solutions with increasing concentrations of QA-modified dendrimers was measured to determine the critical vesicle concentration (CVC) for the dendrimer biocides. The CVCs of G4 octylQA and G4 dodecylQA were determined to be 29 ± 11 and 22 ± 4 μg/mL, respectively, suggesting the formation of vesicles in solution above these concentrations. In comparison, the CVC of G4 butylQA was >5 mg/mL, confirming the inability of the shorter QA alkyl chains to readily form vesicles at the concentrations employed herein. Transmission electron microscopy further confirmed the formation of G4 dodecylQA vesicles (Supporting Information).

Bactericidal Efficacy against Planktonic Bacteria. As *P. aeruginosa* and *S. aureus* represent two of the most commonly isolated species in chronic wounds, they were selected to test the antibacterial efficacy of the QA-modified dendrimers.[28] Further, their use allowed us to examine the effect of Gram designation (i.e., Gram-positive or Gram-negative) on the bactericidal efficacy of these antibacterials. Planktonic bacteria viability assays were performed under static conditions to determine the minimum dendrimer concentration required to elicit a 3-log reduction in bacterial viability over 4 h (MBC_{4h}). The bactericidal NO dose for the NO-releasing QA-modified dendrimers was determined by multiplying the 4 h NO payload ($t[NO]_{4h}$) by the corresponding MBC_{4h}.

The bactericidal efficacy of individual action (i.e., non-NO-releasing) QA-modified dendrimers was first assessed to determine the effects of QA alkyl chain length, dendrimer generation, and bacteria Gram designation on antibacterial activity prior to evaluating the effects of NO release. All QA alkyl chain lengths exhibited a generation dependence on bactericidal efficacy against *P. aeruginosa*, with G4 QA-modified dendrimers resulting in improved killing (i.e., decreased MBC_{4h} values) relative to their G1 counterparts (Table 2). The

Table 2. Minimum Bactericidal Concentrations (MBC_{4h}) and Bactericidal NO Doses against *S. aureus* and *P. aeruginosa* for Control and NO-Releasing QA-Modified Dendrimers[a]

	S. aureus		*P. aeruginosa*	
	MBC_{4h} (μg/mL)	NO dose (μmol/mL)	MBC_{4h} (μg/mL)	NO dose (μmol/mL)
G1 PAMAM	>8000		250	
G4 PAMAM	1000		30	
G1 methylQA	>8000		1500	
G1 methylQA/NO	500	0.37	300	0.22
G1 butylQA	3500		1500	
G1 butylQA/NO	500	0.39	300	0.23
G1 octylQA	30		75	
G1 octylQA/NO	30	0.02	50	0.03
G1 dodecylQA	10		20	
G1 dodecylQA/NO	10	0.01	10	0.01
G4 methylQA	2000		500	
G4 methylQA/NO	300	0.23	250	0.19
G4 butylQA	3500		1000	
G4 butylQA/NO	500	0.39	250	0.20
G4 octylQA	30		30	
G4 octylQA/NO	20	0.02	30	0.03
G4 dodecylQA	10		10	
G4 dodecylQA/NO	10	0.01	10	0.01

[a]Each parameter was analyzed with multiple replicates (n = 3).

generation-dependent increase in antibacterial activity against *P. aeruginosa* is attributed to the greater concentration of alkyl chains afforded by the increased functional group density of the G4 scaffold over the more sparsely functionalized G1 dendrimers. Alternatively, only the methylQA-modified dendrimers exhibited a generation dependence against *S. aureus*. While the bactericidal concentration of G1 methylQA against *S. aureus* was greater than 8.0 mg/mL, G4 methylQA dendrimers resulted in complete bacterial killing at one-fourth that

dx.doi.org/10.1021/bc5000719 | *Bioconjugate Chem.* 2014, 25, 918−927

concentration (2.0 mg/mL). Although the shortest QA alkyl chain (methylQA) seemed to benefit from the increased functional group density of the G4 scaffold, no generation effect was observed for the remaining QA alkyl chain lengths. This indicates that for antibacterials containing these longer QA alkyl chains (i.e., butylQA to dodecylQA), the mechanism of action against the Gram-positive pathogen *S. aureus* is not influenced by functional group density.

The Gram-designation dependence on bactericidal efficacy was highly dependent on the combination of both dendrimer generation and QA alkyl chain length. While Gram-negative bacteria possess a lipid-rich outer membrane and a thin peptidoglycan sheet, the outer wall of Gram-positive bacteria is composed of a thicker and more resistant peptidoglycan layer.[29] For the short alkyl chains, both G1 and G4 scaffolds were more effective against Gram-negative *P. aeruginosa*. The antibacterial activity of the short alkyl chain QAs is attributed to positively charged ammonium groups interacting with the negatively charged bacterial cell membranes. Once associated, the QA functionality can induce cell death through a number of pathways, including disrupting membrane functions, replacing essential metal cations, interrupting protein activity, and damaging bacterial DNA.[12,14] The increased bactericidal action toward *P. aeruginosa* indicates greater association of the dendrimers with the outer membrane layers present in the Gram-negative bacterium as opposed to with the thick peptidoglycan layer of Gram-positive *S. aureus*. Indeed, RITC-labeled G4 methylQA dendrimers associated with *P. aeruginosa* more rapidly than with *S. aureus* bacterial cells (Figures 2 and 3). While association with *P. aeruginosa* was noted at 10 min, G4 methylQA dendrimer association with *S. aureus* was not observed until after 28 min. In contrast, G1 dendrimers modified with longer QA alkyl chains more effectively killed *S. aureus* than *P. aeruginosa*, but neither modification demonstrated a Gram dependence with the G4 scaffold. We believe that the penetration of the longer QA alkyl chains tethered to the G1 scaffold exerts greater physical damage to the thicker peptidoglycan layer of *S. aureus* compared to the outer membrane of *P. aeruginosa*. This hypothesis, however, does not hold true for the G4 dendrimers, for which the increased functional group densities afforded by the G4 scaffold result in similar killing against both strains of bacteria.

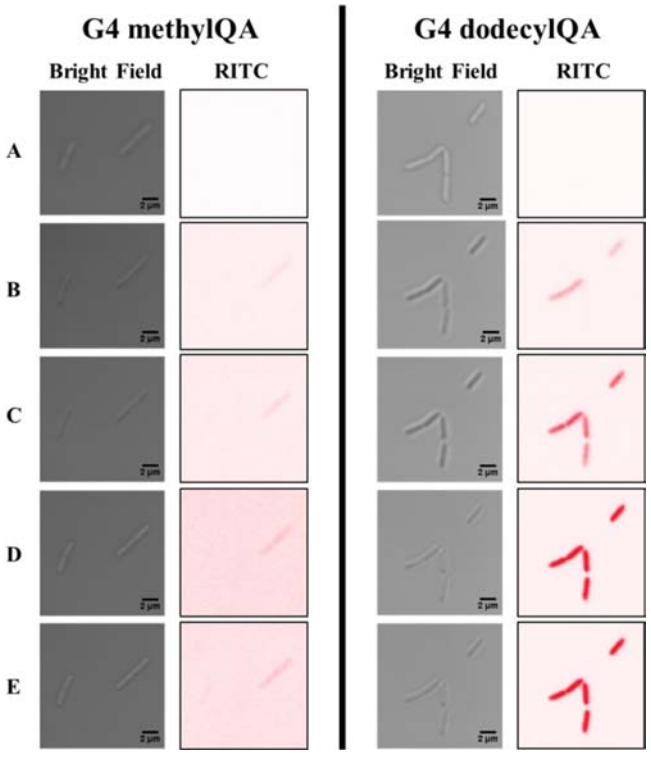


Figure 2. Confocal microscopy images of *P. aeruginosa* exposed to 50 μg/mL RITC-labeled G4 methylQA and G4 dodecylQA dendrimers at (A) 0, (B) 4, (C) 6, (D) 8, and (E) 10 min after dendrimer addition. Threshold reversed for clarity.

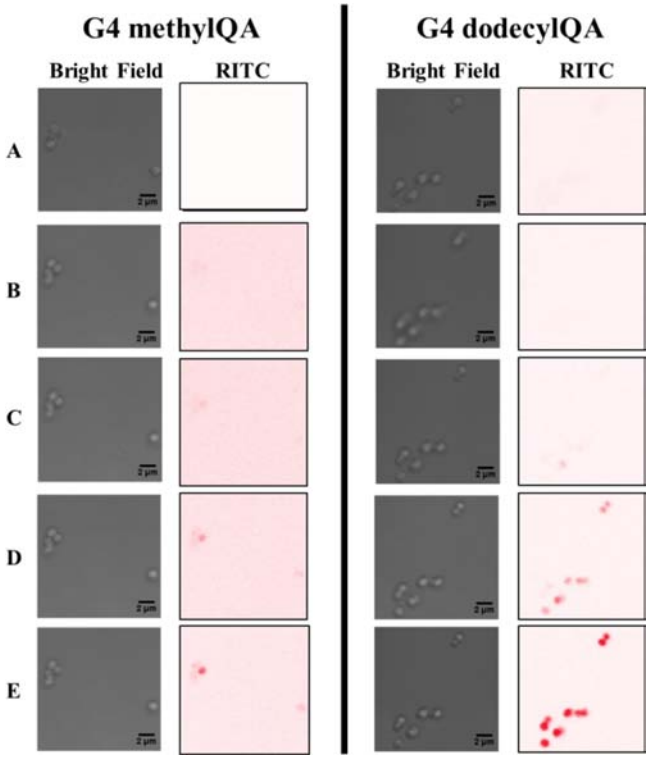


Figure 3. Confocal microscopy images of *S. aureus* exposed to 50 μg/mL RITC-labeled G4 methylQA and G4 dodecylQA dendrimers at (A) 0, (B) 20, (C) 24, (D) 28, and (E) 32 min after dendrimer addition. Threshold reversed for clarity.

As expected, longer QA alkyl chains (i.e., octylQA, dodecylQA) were significantly more bactericidal than the shorter alkyl chains (i.e., methylQA, butylQA) for both dendrimer generations against both strains of bacteria. The increased efficacy of octylQA- and dodecylQA-modified dendrimers against *S. aureus* and *P. aeruginosa* is likely due to the insertion of the longer alkyl chain groups into the peptidoglycan layer, resulting in physical disruption of the cell membrane.[12,30] Furthermore, the greater hydrophobicity of the octyl and dodecyl groups versus the shorter alkyl chains may allow for enhanced association of the dendrimers with the bacteria cell membranes. This is evident in the confocal microscopy images of RITC-labeled dendrimers with both *P. aeruginosa* and *S. aureus*, with G4 dodecylQA dendrimers associating to a greater extent in a shorter period of time than the G4 methylQA dendrimers (Figures 2 and 3). The increased association efficiency combined with the potential for greater membrane damage results in the dramatically enhanced killing for these biocides. While the dodecylQA-modified dendrimers are slightly more effective than the octylQA-modified dendrimers for both generations, the same trend does not

dx.doi.org/10.1021/bc5000719 | *Bioconjugate Chem.* 2014, 25, 918−927

apply to the methylQA- and butylQA-modified dendrimers. For G1 dendrimers, the butylQA modification was more effective than methylQA against *S. aureus*, with a decrease in MBC_{4h} from >8.0 to 3.5 mg/mL, respectively. These results indicate that the slight increase in alkyl chain length may allow for better association of the G1 butylQA dendrimers, resulting in greater antibacterial activity against *S. aureus*.

The same trend in bactericidal efficacy was not observed for the G4 scaffold. In fact, G4 methylQA dendrimers were almost twice as effective as G4 butylQA against both *S. aureus* and *P. aeruginosa*. The lowered bactericidal efficacy is attributed to decreased dendrimer−bacteria association for the G4 butylQA dendrimers relative to the G4 methylQA system (Figure 4). A

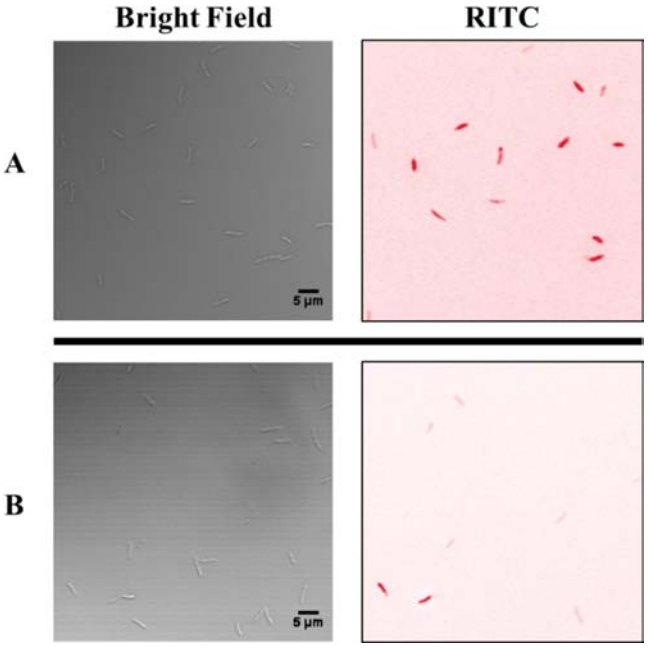


Figure 4. Confocal microscopy images of *P. aeruginosa* exposed to 50 μg/mL RITC-labeled (A) G4 methylQA and (B) G4 butylQA dendrimers after 20 min exposure. Threshold reversed for clarity.

number of factors may play a role in this decreased association. Normally, the positively charged ammonium group enhances association of the biocide with the bacteria membrane. However, back-folding of the peripheral functional groups toward the dendrimer interior may shield the QA from the bacterial membrane, resulting in less membrane damage.[31] Such shielding effects may be further amplified for the G4 scaffold (as compared to G1) due to the closer proximity of terminal functional groups. Although the bactericidal concentration of G4 butylQA was below the CVC (>5.0 mg/mL; Supporting Information), a decrease in surface tension was still observed at these concentrations, which may be indicative of dendrimers assembling at the air−water interface instead of associating with bacteria in solution.[27,32] We hypothesize that the slightly longer alkyl chain amplifies both QA shielding and the formation of supramolecular assemblies for the G4 butylQA scaffold, reducing its bactericidal action from that of the methylQA system.

The coadministration of active-releasing antibacterial agents with contact-based QA biocides has been shown to result in a more effective antibacterial treatment.[15,16] We thus sought to combine the multimechanistic killing of nitric oxide with the contact-based biocidal attributes of QA-modified dendrimers. The combination of NO release with the short QA alkyl chains increased the bactericidal efficacy for both G1 and G4 scaffolds considerably, resulting in a 2−7-fold decrease in MBC_{4h} values over non-NO-releasing QA-modified dendrimers. For most of the scaffolds, the bactericidal NO doses required to kill *S. aureus* was nearly twice that required for *P. aeruginosa* (Table 2). The larger NO dose for *S. aureus* killing is attributed to the more robust peptidoglycan layer inhibiting NO diffusion into the bacteria. The outer membrane and thinner peptidoglycan layer of *P. aeruginosa* do not provide the same barrier for NO diffusion. Similarly, NO release resulted in increased bactericidal efficacy for the G1 octylQA/NO and G1 dodecylQA/NO dendrimers against *P. aeruginosa* compared to their non-NO-releasing counterparts, but had no effect on their bactericidal action against *S. aureus*. Imparting NO release on the G4 dodecylQA dendrimers did not affect the efficacy against either pathogen; both the individual and dual action dendrimers exhibited bactericidal action against *S. aureus* and *P. aeruginosa* at equivalent concentrations (10 μg/mL). For this system, the long dodecyl chains likely cause increased physical disruption of the cell membrane at the expense of intracellular NO buildup. To confirm this, we investigated the levels of intracellular NO and membrane disruption generated by these scaffolds using specific fluorescent probes (i.e, DAF-2 and PI) and confocal microscopy. In cases where NO serves as the lone antibacterial, a bright green fluorescent signal from DAF-2 (indicative of intracellular NO buildup) is generally observed prior to red fluorescence (PI) signifying membrane disruption and subsequent cell death.[7] As this red fluorescence increases, the green fluorescence is concomitantly diminished as the DAF-2 diffuses out of the membrane-compromised bacteria. Bacteria incubated with G4 dodecylQA/NO, however, exhibited considerable membrane disruption before any significant NO accumulation (Figure 5). For both *P. aeruginosa* and *S. aureus*, some intracellular NO was briefly observed after 25 min, but

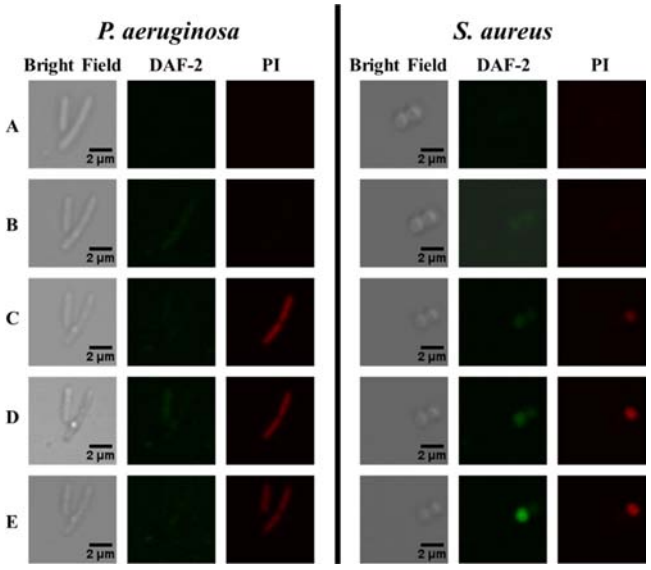


Figure 5. Confocal microscopy images of *P. aeruginosa* and *S. aureus* exposed to 20 μg/mL G4 dodecylQA/NO dendrimers at (A) 0, (B) 25, (C) 35, (D) 45, and (E) 60 min after dendrimer addition. DAF-2 green fluorescence designates the presence of intracellular NO, while PI red fluorescence indicates compromised membranes (cell death).

dx.doi.org/10.1021/bc5000719 | *Bioconjugate Chem.* 2014, 25, 918−927

the significant membrane disruption observed for both strains shortly thereafter (at 35 min) supports our hypothesis that the dodecylQA modification compromises the cell membrane to such an extent it precludes the buildup of intracellular NO.

Finally, we compared the bactericidal action of NO-releasing QA-modified dendrimers against control (i.e., unmodified) G1 and G4 PAMAM scaffolds to determine if the combinatorial effects were indeed more efficacious. Both G1 and G4 PAMAM dendrimers were more effective against *P. aeruginosa* than *S. aureus* (Table 2). For the G1 scaffold, the individual and dual-action octylQA- and dodecylQA-modified dendrimers were more bactericidal than "bare" (unmodified) G1 PAMAM against both pathogens. A similar result was found for the G4 modifications, with the exception of the individual and dual action G4 octylQA dendrimers, which exhibited equivalent bactericidal efficacy compared to the bare G4 scaffold against *P. aeruginosa*. Modifying the G1 and G4 scaffolds with short QA alkyl chains (e.g., methylQA and butylQA) greatly decreased their antibacterial action, with only G1 butylQA dendrimers exhibiting greater bactericidal efficacy against *S. aureus* than their corresponding bare PAMAM scaffold. The antibacterial efficacies of the NO-releasing methylQA and butylQA systems were dependent on bacterial Gram designation. G4 methylQA/NO and G4 butylQA/NO exhibited an almost 10-fold reduction in bactericidal action against *P. aeruginosa* versus the bare G4 scaffold, while the G1 methylQA/NO and G1 butylQA/NO systems were similar to bare G1 PAMAM. All of the dual action QA-modified dendrimers displayed significantly greater bactericidal action against Gram-positive *S. aureus* than the unmodified PAMAM scaffolds, demonstrating the utility of these scaffolds as broad-spectrum antibacterial agents.

In Vitro Cytotoxicity. The relative toxicity against mammalian cells at bactericidal doses is critical to the design of any new antibacterial agent. Although the clinical utility of long chain quaternary ammonium salts has been somewhat limited to topical applications due to their inherent toxicity, tethering QA moieties to larger scaffolds has been shown to mitigate their toxic effects.[14,33] The toxicity of the QA-modified dendrimers prepared herein was thus evaluated against L929 mouse fibroblast cells. As shown in Figure 6, the resulting dendrimer cytotoxicity was dependent on dendrimer generation, QA alkyl chain length, and biocide concentration. While the G4 methylQA dendrimers were nontoxic at 500 μg/mL (4% decrease in cell viability), increasing the biocide concentration 4-fold to 2.0 mg/mL reduced L929 cell viability by 20%. In contrast, the toxicity of G4 butylQA dendrimers was more or less independent of concentration (both 1.0 and 3.5 mg/mL reduced cell viability by 40%). However, the toxicity of the butylQA functionality was generation dependent, with the G4 scaffold accounting for an additional 10% decrease in cell viability from the G1 butylQA system at 3.5 mg/mL. Of significance, the low (but highly antibacterial) concentrations (i.e., 10 μg/mL) of the dodecylQA-modified dendrimers were relatively nontoxic, only reducing L929 cell viability by 15% and 2% for G1 and G4, respectively.

While the large initial NO burst characteristic of these macromolecular scaffolds may yield toxic levels of NO at substantially greater concentrations (i.e., those required to eradicate biofilms), the concentrations tested herein did not exhibit significant toxicity to mammalian cells. Indeed, the NO-releasing QA-modified dendrimers proved to be relatively nontoxic, exhibiting >80% L929 cell viability at the effective bactericidal concentrations. Furthermore, NO release resulted

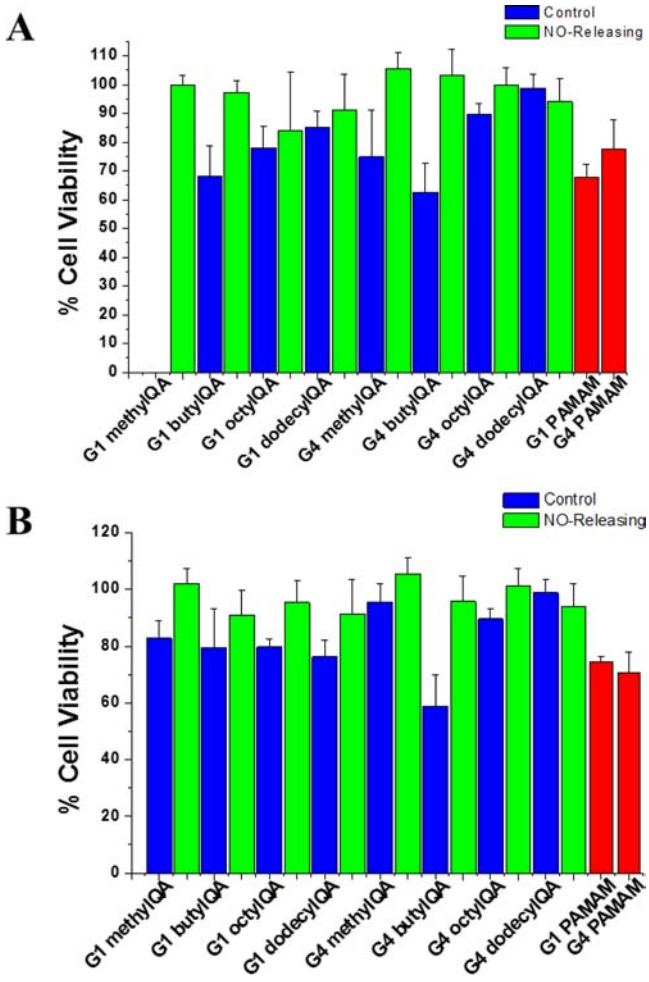


Figure 6. Viability (%) of L929 mouse fibroblast cells following 4 h exposure to control (blue) and NO-releasing (green) QA-modified dendrimers, as well as unmodified PAMAM dendrimers (red), at the MBC_{4h} against (A) *S. aureus* and (B) *P. aeruginosa* compared to untreated control cells. Each parameter was analyzed with multiple replicates (n = 3) with error bars representing standard deviation of the mean.

in increased cell viability versus controls for nearly all of the QA modifications. For several of the scaffolds that exhibited the same bactericidal concentration for both the individual and dual action QA-modified dendrimers, the addition of NO-release still resulted in increased fibroblast cell viability. For example, the G4 octylQA and G4 octylQA/NO dendrimers resulted in cell viabilities of 90% and 101%, respectively, at 30 μg/mL doses. Of significance, these results indicate that the combination of low NO concentrations with QA moieties may be beneficial in decreasing the overall toxicity of QA dendrimers to mammalian cells. It is important to note that the bactericidal doses for all of the NO-releasing QA-modified dendrimers resulted in greater L929 cell viability than the unmodified G1 and G4 PAMAM dendrimers, demonstrating the advantage of dual action therapeutics in reducing scaffold toxicity.

■ CONCLUSIONS

Both individual and dual action QA-modified PAMAM dendrimers exhibited biocidal activity against *P. aeruginosa* and *S. aureus*, with longer QA alkyl chains (i.e., octylQA,

dx.doi.org/10.1021/bc5000719 | *Bioconjugate Chem.* 2014, 25, 918−927

dodecylQA) proving more effective than shorter chain (i.e., methylQA, butylQA) modifications for both G1 and G4 dendrimer scaffolds. While previous work has suggested that QA compounds are more potent against Gram-positive versus Gram-negative bacteria due to the additional outer membrane barrier characteristic of Gram-negative bacteria, this observation has lacked a mechanistic understanding.[13,22] Our work indicates that the potency of QA moieties against Gram-positive and Gram-negative bacteria is highly dependent on both the QA alkyl chain length and functional group density. In contrast to a prior report,[23] G4 dendrimers modified with shorter QA alkyl chains demonstrate reasonable bactericidal action against both *P. aeruginosa* and *S. aureus*, albeit at higher concentrations than those required for longer QA alkyl chain lengths.

While the addition of NO release markedly improves the bactericidal action of short alkyl chain QA-modified G1 and G4 dendrimers against both bacteria strains, the longer alkyl chain QA dendrimers do not benefit from NO release in the same manner. For these systems, the long alkyl chains induce significant damage to the bacteria membrane, greatly increasing their biocidal action but precluding the buildup of intracellular NO. These dual action antibacterial agents hold great therapeutic potential as they exhibit minimal toxicity to mammalian cells at the dendrimer concentrations required to illicit a three-log reduction in bacterial viability. Experiments are underway to investigate the activity of NO-releasing QA macromolecules on the eradication of biofilms, bacteria that are considerably more difficult to eradicate than their planktonic counterparts.[34,35]

EXPERIMENTAL SECTION

Materials. Phenazine methosulfate (PMS), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt (MTS), trypsin, phosphate buffered saline (PBS) used for cell culture, penicillin streptomycin (PS), glycidyltrimethylammonium chloride, epichlorohydrin, dimethylbutylamine, dimethyloctylamine, dimethyldodecylamine, trimethylsilanolate, triethylamine (TEA), rhodamine B isothiocyanate (RITC), and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Loius, MO). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), ethylenediamine (EDA), and methyl acrylate were purchased from the Aldrich Chemical Co. (Milwaukee, WI). 4,5-Diaminofluorescein diacetate (DAF-2 DA) was purchased from Calbiochem (San Diego, CA). Common laboratory salts and solvents were purchased from Fisher Scientific (Fair Lawn, NJ). Unless noted otherwise, these and all other materials were used as received without further purification. Tryptic soy broth (TSB) and tryptic soy agar (TSA) were obtained from Becton, Dickison and Company (Franklin Lakes, NJ). *Pseudomonas aeruginosa* (*P. aeruginosa*; ATCC #19143) and *Staphylococcus aureus* (*S. aureus*; ATCC #29213) were obtained from American Type Tissue Culture Collection (Manassas, VA). L929 mouse fibroblasts were obtained from the UNC Tissue Culture Facility (Chapel Hill, NC). Nitrogen (N_2), argon (Ar), carbon dioxide (CO_2), and nitric oxide (NO) calibration (26.81 PPM, balance N_2) gases were purchased from National Welders (Raleigh, NC). Pure nitric oxide (NO) gas (99.5%) was purchased from Praxair (Sanford, NC). Cellulose ester dialysis membranes (500−1000 MWCO) were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Glass bottom microscopy dishes were received from MatTek Corporation (Ashland, MA). Distilled water was purified using a Millipore Milli-Q UV Gradient A-10 system (Bedford, MA), resulting in a total organic content of ≤6 ppb and a final resistivity of 18.2 mΩ·cm.

Instrumentation. ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker (400 MHz) spectrometer. X-ray photoelectron spectroscopy (XPS) analysis was performed on a Kratos Axis Ultra DLD X-ray Photoelectron Spectrometer with a monochromatic Al Kα X-ray source (150W). Electrons were collected at an angle of 90° from the sample surface from a 300 × 700 μm^2 area on the sample. The pass energy was set to 20 eV to obtain high resolution spectra. All spectra were acquired with a step size of 0.1 eV and calibrated to the C 1s peak at 284.6 eV. Surface tension measurements were made using an Attension Sigma 701 tensiometer with a standard Du Noüy ring.

Synthesis of Quaternary Ammonium-Functionalized PAMAM Dendrimers. Poly(amidoamine) (PAMAM) scaffolds were synthesized as described previously,[18,36,37] by repeated alkylation/amidation steps using methyl acrylate and EDA from an EDA core. The addition of QA functionalities to the dendrimer scaffold necessitated the synthesis of quaternary ammonium epoxides (QA-epoxides), as described previously.[8] Briefly, 0.04 mmol epichlorohydrin was reacted with 0.01 mmol *N,N*-dimethylbutylamine, *N,N*-dimethyloctylamine, or *N,N*-dimethyldodecylamine at room temperature overnight (~18 h). The mixture was then added dropwise to cold ether while sonicating, and the solid/viscous liquid QA-epoxides were collected via centrifugation (810 *g*, 5 min). The supernatant was decanted, and the QA-epoxides were washed with 50 mL of cold ether and sonicated extensively. This washing procedure was repeated three times before drying the product in vacuo.

A ring-opening reaction was then carried out between the QA-epoxides and the terminal primary amines of the PAMAM dendrimers. G1 or G4 PAMAM (50.0 mg) was dissolved in 5 mL of methanol. One equivalent of triethylamine (e.g., with respect to the molar amount of primary amines) and 2.5 mol equiv of QA-epoxide were then added to the vial. The solution was stirred at room temperature for 4 d. Solvent was then removed in vacuo. The dendrimers were subsequently dissolved in water, followed by dialysis against water overnight and lyophilization.

Representative ^{1}H NMR data of QA-functionalized G4 PAMAM via the reactions of G4 PAMAM with glycidyltrimethylammonium chloride (methylQA), glycidyldimethylbutylammonium chloride (butylQA), glycidyldimethyloctylammonium chloride (octylQA), and glycidyldimethyldodecylammonium chloride (dodecylQA) included the following peaks. G4 methylQA: ^{1}H NMR (400 MHz, D_2O, δ) 3.07 (s, $CH_2N^+(CH_3)_3$), 2.31 (s, $NCH_2CH_2C(O)NH$). G4 butylQA: ^{1}H NMR (400 MHz, CD_3OD, δ) 2.30 (s, $NCH_2CH_2C(O)NH$), 1.81 (s, $CH_2N^+(CH_3)_2CH_2CH_2CH_2CH_3$), 1.36−1.30 (q, $CH_2N^+(CH_3)_2CH_2CH_2CH_2CH_3$), 0.93 (t, $CH_2N^+(CH_3)_2CH_2CH_2CH_2CH_3$). G4 octylQA: ^{1}H NMR (400 MHz, CD_3OD, δ) 2.31 (s, $NCH_2CH_2C(O)NH$), 1.80 (s, $CH_2N^+(CH_3)_2CH_2CH_2(CH_2)_5CH_3$), 1.31−1.23 (m, $CH_2N^+(CH_3)_2CH_2CH_2(CH_2)_5CH_3$), 0.83 (t, $CH_2N^+(CH_3)_2CH_2CH_2(CH_2)_5CH_3$). G4 dodecylQA: ^{1}H NMR (400 MHz, CD_3OD, δ) 2.31 (s, $NCH_2CH_2C(O)NH$), 1.81 (s, $CH_2N^+(CH_3)_2CH_2CH_2(CH_2)_9CH_3$), 1.32−1.22 (m, $CH_2N^+(CH_3)_2CH_2CH_2(CH_2)_9CH_3$), 0.83 (t, $CH_2N^+(CH_3)_2CH_2CH_2(CH_2)_9CH_3$).

***N*-Diazeniumdiolation of QA-Functionalized PAMAM Dendrimers.** To form *N*-diazeniumdiolate NO donors on the

dendrimer scaffold, QA-modified G1 and G4 PAMAM (20 mg) were added to varying ratios of anhydrous methanol (MeOH) to tetrahydrofuran (THF) (1.5 mL solvent) depending on QA modification (i.e., methylQA: 100% MeOH; butylQA: 100% MeOH; octylQA: 3:1 MeOH:THF; dodecylQA: 1:1 MeOH:THF). Trimethylsilanolate was then added in a 10-fold excess relative to secondary amines.

The dendrimer solutions were placed in a stainless steel reactor with continuous magnetic stirring and connected to an in-house NO reactor. Prior to NO exposure, the solutions were flushed 6 times with 5 bar Ar to remove oxygen. The reactor was then pressurized to 10 bar with NO gas prescrubbed with KOH. The pressure was maintained at 10 bar for 3 d, after which the solutions were again purged with Ar to remove unreacted NO. Solvent was removed in vacuo, and the resulting dendrimers were dissolved in anhydrous methanol in a 1 dram glass vial, capped and parafilmed, and stored at −20 °C.

Characterization of NO Storage and Release. Real-time NO release in deoxygenated PBS (pH 7.4, 37 °C) was monitored using a Sievers NOA 280i chemiluminescence NO analyzer (NOA, Boulder, CO). Prior to analysis, the NO analyzer was calibrated with air passed through a NO zero filter (0 ppm NO) and a 26.80 ppm of NO standard gas (balance N_2). One milligram aliquots of *N*-diazeniumdiolate-functionalized PAMAM in methanol were added to 30 mL deoxygenated PBS to initiate NO release. Nitrogen was flowed through the solution at a flow rate of 80 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. Nitric oxide analysis was terminated when NO levels decreased to 10 ppb NO/mg dendrimer.

Planktonic Bactericidal Assays. *P. aeruginosa* and *S. aureus* were cultured in tryptic soy broth to a concentration of 10^8 colony forming units per mL (cfu/mL), collected by centrifugation (2355 *g*), resuspended in sterile PBS, and diluted to 10^6 cfu/mL. Premeasured samples of QA-modified or NO-releasing QA-modified dendrimer in methanol were added to a 1 dram glass vial and dried under vacuum for 2 h prior to the bacteria assays. Corresponding volumes of 10^6 cfu/mL bacteria were then added to obtain a range of dendrimer concentrations (37 °C). Untreated controls (blanks) were included in each experiment to ensure the bacteria remained viable (at 10^6 cfu/mL) over the 4 h assay. The blanks and dendrimer-treated bacteria were then spiral-plated at 10- and 100-fold dilutions on tryptic soy agar plates using an Eddy Jet spiral plater (IUL; Farmingdale, NY). Bacterial viability was assessed by counting the number of colonies formed on the agar plate using a Flash & Go colony counter (IUL; Farmingdale, NY). Minimum bactericidal concentrations (MBC) were determined to be the minimum concentration of dendrimers that resulted in a 3-log reduction in bacterial viability compared to untreated cells (i.e., reduced bacterial counts from 10^6 to 10^3 cfu/mL). Of note, the plate counting method used has an inherent limit of detection of 2.5×10^3 cfu/mL.[38]

Confocal Microscopy to Assess Dendrimer−Bacteria Association. Fluorescently labeled G4 PAMAM dendrimers were synthesized as described previously.[10,36,39] Briefly, 100 mg G4 PAMAM was added to a vial containing one molar equivalent of RITC (3.8 mg) in 2 mL methanol. One equivalent of triethylamine (with respect to the molar amount of primary amines) was then added to the vial. The solution was stirred for 24 h in the dark, after which solvent was removed in vacuo. Dendrimers were dissolved in water, dialyzed against water (3 d), and then lyophilized. The above procedure for QA functionalization was performed in the dark to modify the fluorescently labeled G4 PAMAM with methylQA, butylQA, and dodecylQA moieties. *P. aeruginosa* and *S. aureus* were cultured as described above and diluted to 10^6 cfu/mL. Aliquots of the bacteria solutions were incubated in a glass bottom confocal dish for 45 min at 37 °C. A Zeiss 510 Meta inverted laser scanning confocal microscope (Carl Zeiss, Thornwood, NY) with a 543 nm HeNe excitation laser (1.0 mW, 25.0% intensity) and a BP 560−615 nm filter was used to obtain fluorescence images of the RITC-modified dendrimers. Both bright field and fluorescence images were collected using an N.A. 1.2 C-apochromat water immersion lens with a 40× objective. Solutions of RITC-labeled dendrimers (100 μg/mL) in PBS (1.5 mL) were added to 1.5 mL of the bacteria solution in the glass confocal dish to achieve a final concentration of 50 μg/mL. Images were collected every 2 min to temporally monitor association of the dendrimers with the bacteria.

Confocal Microscopy for Detection of Intracellular NO and Cell Death. *P. aeruginosa* and *S. aureus* were cultured as described above and diluted to 10^6 cfu/mL in PBS containing 10 μM DAF-2DA and 30 μM PI. Aliquots of the bacteria solution were incubated in a glass bottom confocal dish for 45 min at 37 °C. A Zeiss 510 Meta inverted laser scanning confocal microscope (Carl Zeiss, Thornwood, NY) with a 488 nm Ar excitation laser (30.0 mW, 2.0% intensity) and a BP 505−530 nm filter was used to obtain DAF-2 (green) fluorescence images. A 543 nm HeNe exciation laser (1.0 mW, 25.0% intensity) with a BP 560−615 nm filter was used to obtain PI (red) fluorescence images. Both bright field and fluorescence images were collected using an N.A. 1.2 C-apochromat water immersion lens with a 40× objective. Solutions of G4 methylQA/NO, G4 butylQA/NO, G4 octylQA/NO, or G4 dodecylQA/NO (40 μg/mL) in 1.5 mL PBS (containing 10 μM DAF-2DA and 30 μM PI) were added to 1.5 mL of the bacteria solution in the glass confocal dish to achieve a final concentration of 20 μg/mL. Images were collected every 5 min to temporally observe intracellular NO concentrations and bacteria cell death.

In Vitro Cytotoxicity. L929 mouse fibroblasts were grown in DMEM supplemented with 10 vol % FBS and 1 wt % PS and incubated in 5 vol % CO_2 under humidified conditions at 37 °C. After reaching 80% confluency, the cells were trypsinized, seeded onto tissue culture-treated polystyrene 96-well plates at a density of 2×10^4 cells/mL, and incubated at 37 °C for 72 h. The supernatant was then aspirated and replaced with 200 μL of fresh growth medium and 50 μL of dendrimer in PBS at the determined MBCs against *P. aeruginosa* or *S. aureus*. Dimethyl sulfoxide (10%) and 50 μL PBS were used as positive and negative controls, respectively. After 4 h incubation at 37 °C, the supernatant was aspirated and 120 μL of a mixture of DMEM/MTS/PMS (105/20/1, v/v/v) was added to each well. After 1.5 h incubation at 37 °C, the absorbance of the colored solutions was quantified at 490 nm using a Thermoscientific Multiskan EX plate reader (Waltham, MA). The mixture of DMEM/MTS/PMS and untreated cells were used as a blank and control, respectively. Results were expressed as percentage of relative cell viability as follows:

$$\%\text{Cell Viability} = [(\text{Abs}_{490} - \text{Abs}_{\text{blank}}) / (\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}})] \times 100\% \quad (1)$$

dx.doi.org/10.1021/bc5000719 | *Bioconjugate Chem.* 2014, 25, 918−927

■ ASSOCIATED CONTENT

Supporting Information

FTIR, ^{1}H NMR, and XPS (N 1s and Cl 2p) of the QA-modified dendrimers. UV−vis spectra, real-time NO release plots, surface tension plots and CVC values, TEM images, and confocal microscopy images of the NO-releasing QA-modified dendrimers. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: schoenfisch@unc.edu.

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.

■ ACKNOWLEDGMENTS

Financial support was provided by the National Science Foundation (DMR 1104892). The authors thank Carrie Donley for assistance with XPS, and Amar Kumbhar for assistance with TEM. We also thank Dr. Neal Kramacy at the Michael Hooker Microscopy Facility at the University of North Carolina at Chapel Hill for microscopy assistance. We acknowledge support for the purchase of instrumentation (FTIR) from UNC EFRC Center for Solar Fuels ("Energy Frontier Research Center" funded by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences under Award Number DE-SC0001011) and from UNC SERC ("Solar Energy Research Center Instrumentation Facility" funded by the U.S. Department of Energy, Office of Energy Efficiency and Renewable Energy under Award Number DE-EE0003188).

■ ABBREVIATIONS

NO, nitric oxide; QA, quaternary ammonium; PAMAM, poly(amidoamine); G1, generation 1; G4, generation 4; MBC, minimum bactericidal concentration; CVC, critical vesicle concentration

■ REFERENCES

(1) Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., Scheld, M., Spellberg, B., and Bartlett, J. (2009) Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin. Infect. Dis. 48*, 1−12.

(2) Cottarel, G., and Wierzbowski, J. (2007) Combination drugs, an emerging option for antibacterial therapy. *Trends Biotechnol. 25*, 547−555.

(3) Eliopoulos, G., and Eliopoulos, C. (1988) Antibiotic combinations: should they be tested? *Clin. Microbiol. Rev. 1*, 139−156.

(4) Fischbach, M. A. (2011) Combination therapies for combating antimicrobial resistance. *Curr. Opin. Microbiol. 14*, 519−523.

(5) Fang, F. C. (1997) Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J. Clin. Invest. 99*, 2818.

(6) Fang, F. C. (2004) Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat. Rev. Microbiol. 2*, 820−832.

(7) Hetrick, E. M., Shin, J. H., Stasko, N. A., Johnson, C. B., Wespe, D. A., Holmuhamedov, E., and Schoenfisch, M. H. (2008) Bactericidal efficacy of nitric oxide-releasing silica nanoparticles. *ACS Nano 2*, 235−246.

(8) Carpenter, A. W., Worley, B. V., Slomberg, D. L., and Schoenfisch, M. H. (2012) Dual action antimicrobials: nitric oxide release from quaternary ammonium-functionalized silica nanoparticles. *Biomacromolecules 13*, 3334−3342.

(9) Privett, B. J., Deupree, S. M., Backlund, C. J., Rao, K. S., Johnson, C. B., Coneski, P. N., and Schoenfisch, M. H. (2010) Synergy of nitric oxide and silver sulfadiazine against gram-negative, gram-positive, and antibiotic-resistant pathogens. *Mol. Pharmaceutics 7*, 2289−2296.

(10) Sun, B., Slomberg, D. L., Chudasama, S. L., Lu, Y., and Schoenfisch, M. H. (2012) Nitric oxide-releasing dendrimers as antibacterial agents. *Biomacromolecules 13*, 3343−3354.

(11) Park, J., Kim, J., Singha, K., Han, D.-K., Park, H., and Kim, W. J. (2013) Nitric oxide integrated polyethylenimine-based tri-block copolymer for efficient antibacterial activity. *Biomaterials 34*, 8766−8775.

(12) Simoncic, B., and Tomsic, B. (2010) Structures of novel antimicrobial agents for textiles-a review. *Text. Res. J. 80*, 1721−1737.

(13) Chen, C. Z., Beck-Tan, N. C., Dhurjati, P., van Dyk, T. K., LaRossa, R. A., and Cooper, S. L. (2000) Quaternary ammonium functionalized poly (propylene imine) dendrimers as effective antimicrobials: structure-activity studies. *Biomacromolecules 1*, 473−480.

(14) Thorsteinsson, T., Másson, M., Kristinsson, K. G., Hjálmarsdóttir, M. A., Hilmarsson, H., and Loftsson, T. (2003) Soft antimicrobial agents: synthesis and activity of labile environmentally friendly long chain quaternary ammonium compounds. *J. Med. Chem. 46*, 4173−4181.

(15) Li, Z., Lee, D., Sheng, X., Cohen, R. E., and Rubner, M. F. (2006) Two-level antibacterial coating with both release-killing and contact-killing capabilities. *Langmuir 22*, 9820−9823.

(16) Song, J., Kang, H., Lee, C., Hwang, S. H., and Jang, J. (2011) Aqueous synthesis of silver nanoparticle embedded cationic polymer nanofibers and their antibacterial activity. *ACS Appl. Mater. Interfaces 4*, 460−465.

(17) Duncan, R., and Izzo, L. (2005) Dendrimer biocompatibility and toxicity. *Adv. Drug Delivery Rev. 57*, 2215−2237.

(18) Tomalia, D. A. (2005) Birth of a new macromolecular architecture: dendrimers as quantized building blocks for nanoscale synthetic polymer chemistry. *Prog. Polym. Sci. 30*, 294−324.

(19) Jevprasesphant, R., Penny, J., Attwood, D., McKeown, N. B., and D'Emanuele, A. (2003) Engineering of dendrimer surfaces to enhance transepithelial transport and reduce cytotoxicity. *Pharm. Res. 20*, 1543−1550.

(20) Wiwattanapatapee, R., Carreño-Gómez, B., Malik, N., and Duncan, R. (2000) Anionic PAMAM dendrimers rapidly cross adult rat intestine in vitro: a potential oral delivery system? *Pharm. Res. 17*, 991−998.

(21) Meyers, S. R., Juhn, F. S., Griset, A. P., Luman, N. R., and Grinstaff, M. W. (2008) Anionic amphiphilic dendrimers as antibacterial agents. *J. Am. Chem. Soc. 130*, 14444−14445.

(22) Chen, C., Cooper, S., and Tan, N. B. (1999) Incorporation of dimethyldodecylammonium chloride functionalities onto poly (propylene imine) dendrimers significantly enhances their antibacterial properties. *Chem. Commun.*, 1585−1586.

(23) Charles, S., Vasanthan, N., Kwon, D., Sekosan, G., and Ghosh, S. (2012) Surface modification of poly (amidoamine)(PAMAM) dendrimer as antimicrobial agents. *Tetrahedron Lett. 53*, 6670−6675.

(24) Lu, Y., Sun, B., Li, C., and Schoenfisch, M. H. (2011) Structurally diverse nitric oxide-releasing poly (propylene imine) dendrimers. *Chem. Mater. 23*, 4227−4233.

(25) Beyth, N., Houri-Haddad, Y., Baraness-Hadar, L., Yudovin-Farber, I., Domb, A. J., and Weiss, E. I. (2008) Surface antimicrobial activity and biocompatibility of incorporated polyethylenimine nanoparticles. *Biomaterials 29*, 4157−4163.

(26) Shi, Z., Neoh, K., Kang, E., and Wang, W. (2006) Antibacterial and mechanical properties of bone cement impregnated with chitosan nanoparticles. *Biomaterials 27*, 2440−2449.

(27) Schenning, A. P., Elissen-Roman, C., Weener, J.-W., Baars, M. W., van der Gaast, S. J., and Meijer, E. (1998) Amphiphilic dendrimers as building blocks in supramolecular assemblies. *J. Am. Chem. Soc. 120*, 8199−8208.
(28) Bjarnsholt, T., Kirketerp-Møller, K., Jensen, P. Ø., Madsen, K. G., Phipps, R., Krogfelt, K., Høiby, N., and Givskov, M. (2008) Why chronic wounds will not heal: a novel hypothesis. *Wound Repair Regen. 16*, 2−10.
(29) Beveridge, T. J. (1999) Structures of gram-negative cell walls and their derived membrane vesicles. *J. Bacteriol. 181*, 4725−4733.
(30) Yamanaka, M., Hara, K., and Kudo, J. (2005) Bactericidal actions of a silver ion solution on Escherichia coli, studied by energy-filtering transmission electron microscopy and proteomic analysis. *Appl. Environ. Microbiol. 71*, 7589−7593.
(31) Maiti, P. K., Cagin, T., Wang, G., and Goddard, W. A. (2004) Structure of PAMAM dendrimers: Generations 1 through 11. *Macromolecules 37*, 6236−6254.
(32) Fréchet, J. M. (2002) Dendrimers and supramolecular chemistry. *Proc. Natl. Acad. Sci. U. S. A. 99*, 4782−4787.
(33) Lv, H., Zhang, S., Wang, B., Cui, S., and Yan, J. (2006) Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Controlled Release 114*, 100−109.
(34) Dunne, W. M. (2002) Bacterial adhesion: seen any good biofilms lately? *Clin. Microbiol. Rev. 15*, 155−166.
(35) Lindsay, D., and Von Holy, A. (2006) Bacterial biofilms within the clinical setting: what healthcare professionals should know. *J. Hosp. Infect. 64*, 313−325.
(36) Lu, Y., Slomberg, D. L., Shah, A., and Schoenfisch, M. H. (2013) Nitric oxide-releasing amphiphilic poly (amidoamine)(PAMAM) dendrimers as antibacterial agents. *Biomacromolecules 14*, 3589−3598.
(37) Tomalia, D., Baker, H., Dewald, J., Hall, M., Kallos, G., Martin, S., Roeck, J., Ryder, J., and Smith, P. (1985) A new class of polymers: starburst-dendritic macromolecules. *Polym. J. 17*, 117−132.
(38) Breed, R. S., and Dotterrer, W. (1916) The number of colonies allowable on satisfactory agar plates. *J. Bacteriol. 1*, 321.
(39) Slomberg, D. L., Lu, Y., Broadnax, A. D., Hunter, R. A., Carpenter, A. W., and Schoenfisch, M. H. (2013) Role of size and shape on biofilm eradication for nitric oxide-releasing silica nanoparticles. *ACS Appl. Mater. Interfaces 5*, 9322−9329.