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Nitric Oxide-Releasing Amphiphilic Poly(amidoamine) (PAMAM) Dendrimers as Antibacterial Agents

Yuan Lu, **Danielle L. Slomberg**, **Anand Shah**, and **Mark H. Schoenfisch***
Department of Chemistry, University of North Carolina - Chapel Hill, Chapel Hill, NC 27599

Abstract

A series of amphiphilic nitric oxide (NO)-releasing poly(amidoamine) (PAMAM) dendrimers with different exterior functionalities were synthesized by a ring-opening reaction between primary amines on the dendrimer and propylene oxide (PO), 1,2-epoxy-9-decene (ED), or a ratio of the two, followed by reaction with NO at 10 atm to produce *N*-diazeniumdiolate-modified scaffolds with a total storage of ~1 μmol/mg. The hydrophobicity of the exterior functionality was tuned by varying the ratio of PO and ED grafted onto the dendrimers. The bactericidal efficacy of these NO-releasing vehicles against established Gram-negative *Pseudomonas aeruginosa* biofilms was then evaluated as a function of dendrimer exterior hydrophobicity (i.e., ratio of PO/ED), size (i.e., generation), and NO release. Both the size and exterior functionalization of dendrimer proved important to a number of parameters including dendrimer-bacteria association, NO delivery efficiency, bacteria membrane disruption, migration within the biofilm, and toxicity to mammalian cells. Although enhanced bactericidal efficacy was observed for the hydrophobic chains (e.g., ED), toxicity to L929 mouse fibroblast cells was also noted at concentrations necessary to reduce bacterial viability by 5-logs (99.999% killing). The optimal PO to ED ratios for biofilm eradication with minimal toxicity against L929 mouse fibroblast cells were 7:3 and 5:5. The study presented herein demonstrated the importance of both dendrimer size and exterior properties in determining efficacy against established biofilms without compromising biocompatibility to mammalian cells.

Keywords

Nitric oxide release; dendrimer; anti-biofilm; amphiphilic; hydrophobicity; biocompatibile

Introduction

Bacteria in nature exist in two states – free-floating planktonic bacteria and bacterial biofilms.[1] While many antimicrobial agents have proven effective against planktonic bacteria, medically relevant infections including those associated with medical implants, diabetes mellitus, and cystic fibrosis are often caused by bacterial biofilms.[1–3] In contrast to planktonic bacteria, biofilms are communities of microorganisms and protected by a self-secreted exopolysaccharides (EPS) matrix.[4] Along with inhibiting the penetration of antibiotics, biofilms exhibit several other defense mechanisms including overexpression of stress-responsive genes, oxygen gradients within the EPS matrix, and differentiation of bacteria into resistant dormant species.[4] Collectively, these effects result in greater

*Address correspondence to Dr. Mark H. Schoenfisch, schoenfisch@unc.edu.

Supporting Information
Experimetal details for the confocal microscopy study evaluating the intracellular NO and bacterial membrane disruption of *P. aeruginosa* biofilms are included in supporting information. This information is available free of charge via the internet at http://pubs.acs.org.

resistance of bacteria within biofilms to antimicrobial agents compared to their planktonic counterparts. It has been shown that killing bacteria in biofilms may require up to 1000 times the antibiotic dose necessary for killing planktonic bacteria.[1] As such, new antimicrobial agents capable of eradicating mature biofilms are urgently needed.

Dendrimers, a family of macromolecular scaffolds with hyper-branched architectures and multivalent surfaces,[5–18] exhibit antibacterial activity against both planktonic bacteria[19–23] and biofilms.[24–26] For example, quaternary ammonium-functionalized poly(propylenimine) (PPI) dendrimers[20] and primary amine-functionalized poly(amidoamine) (PAMAM) dendrimers[19] display be biocidal activity against planktonic *Pseudomonas aerugionsa* and *Staphylococcus aureus*. Unfortunately, the inherent toxicity of these scaffolds to eukaryotic (e.g., mammalian) cells has slowed their development as therapeutics.[27] In order to reduce toxicity to mammalian cells, Grinstaff et al. synthesized anionic amphiphilic polyester dendrimers that were effective (bactericidal) against Gram-positive *Bacillus subtilis* but not cytotoxic to human umbilical vein endothelial cells (HUVECs).[21] Likewise, hydroxyl-, carboxyl-, and poly(ethylene glycol) (PEG)-functionalized PAMAM dendrimers have been designed to minimize toxicity towards mammalian cells while retaining their antibacterial efficacy against bacterial species.[22, 28] Although most work to date has focused on the planktonic antibacterial activity of dendrimers, the efficacy of dendritic scaffolds against more challenging systems, including antibiotic-resistant bacteria and biofilms, has become an important focus in the continued development of antibacterial dendrimers. Several studies have demonstrated the dispersion and inhibition of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms by amphiphilic dendritic glycopeptides.[24–26, 28] As opposed to linear antibacterial peptides, dendritic peptides exhibit enhanced inhibition of biofilm growth.[24] However, additional work is required to elucidate the potential of dendrimers as effective agents for both inhibiting biofilm growth and eradicating mature biofilms.

Nitric oxide (NO), an endogenously-produced diatomic free radical, plays a key role in human physiology and our natural immune response to pathogens.[29–31] Both NO and its reactive byproducts (e.g., peroxynitrite and dinitrogen trioxide) exert significant oxidative and nitrosative stress on bacteria to facilitate killing. While nitrosative stress occurs when thiols on proteins and DNA are nitrosated-impairing normal function, oxidative stress is mostly observed through lipid peroxidation, which destroys the integrity of bacterial membrane/envelope.[31] Importantly, NO exhibits broad-spectrum antibacterial activity, making NO-based therapeutics an emerging opportunity. For addressing inadequate development of new antibiotics, we and others have developed macromolecular vehicles (e.g., gold nanoparticles, silica nanoparticles, and dendrimers) that are allowed for controlled NO storage and release.[32–40] These materials have proven effective at killing both Gram–positive and –negative bacteria.[41–46] Nitric oxide-releasing dendrimes in particular exhibit very efficient bactericidal action due to large NO payloads and favorable dendrimer–bacteria association.[44] Sun et al. reported that both the size (i.e., generation) and the exterior functionality significantly influenced biocidal activity.[44] Herein, we report the synthesis of NO-releasing amphiphilic dendrimers with tunable exterior hydrophobicity to eradicate bacterial biofilms with minimal impact to mammalian cells.

Experimental Section

Materials

Phenazine methosulfate (PMS), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), trypsin, phosphate buffered saline (PBS), penicillin streptomycin (PS), rhodamine B isothiocyanate (RITC), Dulbecco's modified Eagle's medium (DMEM), and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Loius, MO). Sodium methoxide (5.4 M solution in

methanol), propylene oxide (PO), and 1,2-epoxy-9-decene (ED) were obtained from Acros Organics (Geel, Belgium). Tryptic soy broth (TSB) and tryptic soy agar (TSA) were obtained from Becton, Dickison and Company (Franklin Lakes, NJ). Spectra/Por Float-A-Lyzers for dialysis of the dendrimers were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). 4,5-Diaminofluorescein diacetate (DAF-2 DA) was purchased from Calbiochem (San Diego, CA). Syto 9 green fluorescent nucleic acid stain was purchased from Life Technologies (Grand Island, NY). Glass bottom microscopy dishes were received from MatTek Corporation (Ashland, MA). Common laboratory salts and solvents were purchased from Fisher Scientific (Pittsburgh, PA). Unless noted otherwise, these and other materials were used as received without further purification.

Synthesis of Secondary Amine- and *N*-Diazeniumdiolate-Functionalized PAMAM Dendrimers

Secondary amine-functionalized PAMAM dendrimers (generation 1 or G1 and generation 3 or G3) were synthesized as described previously.[32] Briefly, primary amine-functionalized G1-PAMAM dendrimer (100 mg) was dissolved in methanol (2 mL). One molar equivalent of PO, ED, or a mixture of PO and ED relative to the primary amines was then added to the G1-PAMAM-NH_2 solution under constant stirring at room temperature for 4 d to yield the secondary amine-functionalized G1-PAMAM conjugates. Similarly, secondary amine-functionalized G3-PAMAM conjugates were formed via the reaction of G3-PAMAM-NH_2 with PO, ED, or a mixture of PO and ED, respectively. Following 3 d of reaction, solvent was removed under reduced pressure. The dendrimers were then washed with ethanol, followed by dialysis against water and lyophilization. The resulting secondary amine-functionalized dendrimers (G1 and G3) were characterized by nuclear magnetic resonance (NMR) spectroscopy in deuterated methanol.

Representative ^{1}H NMR data of secondary amine-functionalized G1-PAMAM conjugates formed via the reactions of G1-PAMAM-NH_2 with PO, ED and PO/ED mixtures yielded the following peaks: **G1-PAMAM-PO**: ^{1}H NMR (400 MHz, CD_3OD, δ): 3.82 ($CH_2NHCH_2CH(OH)CH_3$), 3.10–3.20 ($CONHCH_2CH_2$), 2.75 ($CH_2N(CH_2CH_2CO)_2$), 2.60 ($CH_2NHCH_2CH(OH)CH_3$), 2.55 ($CH_2N(CH_2CH_2CO)_2$), 2.37 ($CH_2N(CH_2CH_2CO)_2$), 1.00 ($CH_2NHCH_2CH(OH)CH_3$). **G1-PAMAM-ED**: ^{1}H NMR (400 MHz, CD_3OD, δ): 5.80 ($CH_2CH=CH_2$), 4.88 ($CH_2CH=CH_2$), 3.58 ($NHCH_2CH(OH)CH_2$), 3.10–3.20 ($CONHCH_2CH_2$), 2.73 ($CH_2N(CH_2CH_2CO)_2$), 2.67 ($NH(CH_2)_2$), 2.55 ($CH_2N(CH_2CH_2CO)_2$), 2.36 ($CH_2N(CH_2CH_2CO)_2$), 1.98 ($CH_2CH=CH_2$), 1.2–1.4 ($(CH_2)_5CH_2CH=CH_2$). **G1-PAMAM-PO/ED**: ^{1}H NMR (400 MHz, CD_3OD, δ): 5.80 ($CH_2CH=CH_2$), 4.88 ($CH_2CH=CH_2$), 3.82 ($CH_2NHCH_2CH(OH)CH_3$), 3.58 ($NHCH_2CH(OH)CH_2$), 3.10–3.20 ($CONHCH_2CH_2$), 2.75 ($CH_2N(CH_2CH_2CO)_2$), 2.60 ($CH_2NHCH_2CH(OH)CH_3$), 2.55 ($CH_2N(CH_2CH_2CO)_2$), 2.37 ($CH_2N(CH_2CH_2CO)_2$), 1.98 ($CH_2CH=CH_2$), 1.2–1.4 ($(CH_2)_5CH_2CH=CH_2$), 1.00 ($CH_2NHCH_2CH(OH)CH_3$).

N-diazeniumdiolate-functionalized PAMAM dendrimers were synthesized by adding one equivalent of 5.4 M sodium methoxide solution in methanol (with respect to the molar amount of primary amine functionality in PAMAM-NH_2 used to synthesize dendrimers) to a vial containing dendrimers (100 mg) in methanol (1 mL). The glass vials were then inserted in a stainless steel reactor, and the headspace in the reactor was subsequently flushed with argon three times followed by three longer purges with argon (3 × 10 min) to remove oxygen from the stirred solution. The reactor was then filled with NO (purified over KOH pellets for 30 min to remove trace NO degradation products) to 10 atm for 3 d. Unreacted NO was then removed using the same argon flushing procedure described above to obtain the *N*-diazeniumdiolate-modified PAMAM dendrimers.

Characterization of NO Storage and Release

Nitric oxide release was measured using a Sievers 280i Chemiluminesce Nitric Oxide Analyzer (Boulder, CO) by adding NO-releasing dendrimers (1 mg) to a sample containing deoxygenated PBS (30 mL) (pH = 7.4, 37 °C). Nitrogen was purged through the sample vessel solution to carry liberated NO to the analyzer at a flow rate of 70 mL/min. Additional nitrogen flow was supplied to the vessel to match the collection rate of the analyzer (200 mL/min). Nitric oxide release was measured in real time, thus allowing for the determination of NO release total (t[NO]), half-life ($t_{1/2}$), and maximum NO flux ($[NO]_{max}$). The analysis was terminated when the NO release levels fell to below 10 ppb NO/mg dendrimer.

Planktonic Bactericidal Assays Under Static Conditions

Bacterial cultures were grown from a frozen (−80 °C) stock overnight in TSB at 37 °C. A 500 μL aliquot of the resulting suspension was added to fresh TSB (50 mL) and incubated at 37 °C for ~2 h until the concentration reached 1×10^8 colony forming units (CFU)/mL, as confirmed by the OD_{600}. A working bacterial stock was generated by plating the bacterial suspension on TSA and incubating at 37 °C overnight. Subsequent TSA bacterial stocks were prepared weekly and stored at 4 °C. For bactericidal assays, colonies of *P. aeruginosa* were taken from the TSA plate, dispersed in TSB (3 mL), and then incubated at 37 °C overnight. A 500 μL aliquot of culture was added to fresh TSB (50 mL) and incubated to a concentration of ~1×10^8 CFU/mL. The bacteria was then collected by centrifugation (3645 × *g* for 10 min), resuspended in PBS, and diluted 100-fold to obtain a final concentration of 1×10^6 CFU/mL. The bactericidal efficacy of NO-releasing dendrimers against the bacteria was evaluated by incubating the bacteria suspension with NO-releasing dendrimers over a range of concentrations in PBS at 37 °C. At 4 h, 100 μL aliquots of the bacteria suspensions were removed, diluted 10-fold in PBS, plated on TSA, and incubated overnight at 37 °C. The minimum concentration of NO-releasing dendrimers that resulted in a 3-log reduction in bacterial viability was defined as the planktonic minimum bactericidal concentration (MBC).

Growth of *P. aeruginosa* Biofilms

A standard US centers for disease control (CDC) bioreactor (Biosurface Technologies, Bozeman, MT) was used to grow *P. aeruginosa* biofilms over a 48 h period. Briefly, medical grade silicone rubber substrates were mounted in coupon holders prior to assembling the reactor. The assembled reactor was then autoclaved. The reactor effluent line was clamped and 1% (v/v) sterile TSB (500 mL) was added aseptically. Subsequently, the reactor was inoculated with an aliquot (1 mL) of *P. aeruginosa* (10^8 CFU/mL in TSB) to achieve a final concentration ~2×10^5 CFU/mL. The reactor was then incubated at 37 °C for 24 h with stirring (150 rpm). Following this “batch phase” growth, the reactor media was refreshed continuously with 0.33% (v/v) TSB at 6 mL/min for another 24 h.

Treatment of *P. aeruginosa* Biofilms with NO-releasing Dendrimers

P. aeruginosa biofilms grown on silicone rubber substrates were exposed to dendrimers in PBS with slight agitation (37 °C, 24 h) to determine the minimum bactericidal concentration (MBC) necessary to elicit a 5-log reduction in viability of the biofilm-based bacteria. At 24 h, samples were sonicated and vortexed to disrupt the biofilm. Aliquots (100 μL) of the cell/dendrimer suspensions were diluted and plated on TSA and incubated overnight. Bacterial viability was determined by counting observed colonies. Of note, the limit of detection for this selected plate counting method is 2.5×10^3 CFU/mL. As such, biofilm growth conditions were selected to accurately represent a 5-log reduction in viability.

In vitro Cytotoxicity

L929 mouse fibroblasts were grown in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1 wt% penicillin/streptomycin, and incubated in 5% (v/v) CO_2 under humidified conditions at 37 °C. After reaching confluency (80%), the cells were trypsinized, seeded onto tissue-culture treated polystyrene 96-well plates at a density of 3×10^4 cells/mL, and incubated at 37 °C for 48 h. The supernatant was then aspirated prior to adding 200 μL fresh DMEM and 50 μL of NO-releasing dendrimers in PBS to each well. After incubation at 37 °C for 24 h, the supernatant was aspirated and a 120 μL mixture of DMEM/MTS/PMS (105/20/1, v/v/v) was added to each well. The absorbance of the resulting colored solution after 1.5 h incubation at 37 °C was quantified using a Thermoscientific Multiskan EX plate reader at 490 nm. The mixture of DMEM/MTS/PMS and untreated cells were used as a blank and control, respectively. Cell viability was calculated as follows (Eq. 1):

$$\text{Cell Viability}=\frac{(\text{Absorbance}_{\text{treated cell}}-\text{Absorbance}_{\text{blank}})}{(\text{Absorbance}_{\text{untreated cell}}-\text{Absorbance}_{\text{blank}})} \quad \text{Eq. 3.1}$$

Confocal Microscopy for Association of Dendrimers with Bacteria Cells

Fluorescently-labeled control and NO-releasing dendrimers were prepared following a previously reported procedure.[44] Briefly, G1-PAMAM-NH_2 (100 mg) and rhodamine B isothiocyanate (RITC) (3 mg) were dissolved in 2 mL methanol. The solution was stirred for 3 d in the dark. The product solution was dialyzed against 0.1 M NaCl (2 L) for 24 h, and ultrapure Milli-Q water for 3 d (3 × 2 L). Subsequent lyophilization yielded RITC-labeled G1-PAMAM-NH_2. The fluorescently-labeled G1-PAMAM-NH_2 dendrimers were modified with one molar equivalent of PO or ED alone, or a PO/ED mixture, and further reacted with NO at 10 atm under basic conditions as described above to yield NO-releasing G1-PAMAM dendrimers. *P. aeruginosa* was cultured in TSB to a concentration of 1×10^8 CFU/mL, collected *via* centrifugation (3645 × *g* for 10 min), resuspended in sterile PBS, and adjusted to 1×10^6 CFU/mL. Aliquots of the bacteria solution were incubated in a glass bottom confocal dish for 2 h at 37 °C. A Zeiss 510 Meta inverted laser scanning confocal microscope with a 543 nm HeNe excitation laser and a LP 585 nm filter was used to obtain fluorescence images of the RITC-modified dendrimers. The bright field and fluorescence images were collected using a N.A. 1.2 C-apochromat water immersion lens with a 20× objective. Solutions of RITC-labeled NO-releasing (400 μg/mL) dendrimers in PBS (1.5 mL) were added to the bacteria solution (1.5 mL) in the glass confocal dish to achieve a final concentration of 200 μg/mL. Images were collected at 2 h incubation to characterize the association, if any, of the dendrimers with *P. aerugionsa*. Dendrimer–bacteria association within biofilms was also characterized using confocal microscopy. Established biofilms stained with Syto 9 (10 μM) were incubated with RITC-labeled NO-releasing dendrimers (50 μg/mL) for 1 h before imaging. A 488 nm Ar excitation laser with BP 505–530 nm filter was used to image Syto 9 fluorescence. The bright field and fluorescence images were collected simultaneously using a N.A. 1.2 C-apochromat water immersion lens with a 10× objective.

Confocal Microscopy for the Detection of Intracellular NO and Cell Death

The efficiency of NO delivery and resulting bacteria death were evaluated as a function of dendrimer exterior hydrophobicity using confocal microscopy. Bacteria (*P. aeruginosa*) were cultured in TSB to a concentration of 1×10^8 CFU/mL, collected via centrifugation (3645 × *g* for 10 min), resuspended in sterile PBS, and adjusted to 1×10^6 CFU/mL in PBS supplemented with 10 μM DAF-2 DA and 30 μM PI. The bacteria solution (2.5 mL) was incubated in a glass bottom confocal dish for 45 min at 37 °C. A Zeiss 510 Meta inverted laser scanning confocal microscope with a 488 nm Ar excitation laser and a BP 505–530 nm

filter was used to obtain DAF-2 (green) fluorescence images. Red fluorescence images for PI were obtained using a 543 nm HeNe excitation laser with a BP 560–615 nm filter. The bright field and fluorescence images were collected by a N.A. 1.2 C-apochromat water immersion lens with a 40× objective. An aliquot (1.5 mL) of NO-releasing dendrimers (10 μg/mL) in PBS (supplemented with 10 μM DAF-2 DA and 30 μM PI) was added to the bacteria solution (1.5 mL) in the glass confocal dish. Images were collected every 5 min to observe intracellular NO concentrations (green fluorescence) and compromised bacteria membrane (red fluorescence) temporally. The efficiency of NO delivery to bacteria within biofilms was also evaluated as a function of dendrimer composition by incubating the *P. aeruginosa* biofilm with NO-releasing dendrimers (20 μg/mL) in PBS supplemented with 10 μM DAF-2 DA and 30 μM PI for 1 h. Bright field and fluorescence images were collected using a N.A. 1.2 C-apochromat water immersion lens with a 20× objective.

Results and Discussion

Sun et al. previously reported on the planktonic biocidal activity of NO-releasing dendrimers as a function of exterior functionality.[44] Functionalization with hydrophobic groups at the dendrimer exterior improved bactericidal efficacy but also resulted in significant toxicity towards mammalian cells. Even low concentrations (< 50 μg/mL) of hydrophobic dendrimer resulted in ~80% killing of L929 mouse fibroblast cells.[44] We thus sought to synthesize NO-releasing dendrimers with tunable exterior hydrophobicity in order to fully evaluate the impact of dendrimer structure on both bactericidal action and cytotoxicity.

Synthesis of Nitric Oxide Donor-Modified PAMAM Dendrimers

Secondary amine-functionalized dendrimers with diverse exterior functionalities were prepared using epoxides ring opening reactions with primary amines on the dendrimer exterior.[32] *N*-diazeniumdiolate NO donors were formed by reaction of the resulting secondary amines with NO gas under basic conditions.[32] To synthesize secondary amine-functionalized dendrimer with varied exterior hydrophobicity, poly(amidoamine) (PAMAM) dendrimers were modified by a similar ring opening reaction using either hydrophilic PO exclusively, hydrophobic ED exclusively, or varying molar ratios of PO and ED as shown in Scheme 1.

The PO, ED, and PO/ED mixtures were added in a total of one equivalent with respect to the molar concentration of PAMAM primary amine functionality. The actual ratio of PO and ED conjugated to the PAMAM dendrimers was determined using ^{1}H NMR spectroscopy. As shown in Figure 1, a distinct resonance at 3.82 ppm corresponded to the methyne protons adjacent to the hydroxyl group of the product.

This chemical shift was observed in the products of dendrimer reacting with PO exclusively, as well as those with a PO/ED mixture. The NMR spectra also reveal the presence of a second distinct peak at 5.80 ppm, formed upon reaction of the dendrimer with either ED exclusively or the PO/ED mixtures. The 5.80 ppm peak corresponds to the methyne protons of the unsaturated ED double bond. The integration of the 3.82 and 5.80 ppm peaks allowed for the determination of the molar ratio of PO and ED. For PO/ED bifunctionalized dendrimers, the feed molar ratio of PO and ED was tuned to yield PO/ED ratios of 7:3 (i.e., **G1-PE 73**, **G3-PE 73**), 5:5 (i.e., **G1-PE 55**), and 3:7 (i.e., **G1-PE 37**). In this manner, we were able to study the effects of relative hydrophobicity on bactericidal activity and cytotoxicity (Table 1). In addition, work by Sun et al. indicated the importance of dendrimer size (i.e., dendrimer generation) on bactericidal efficacy, with higher generation (i.e., larger) NO-releasing dendrimers being more effective at killing bacteria due to greater NO payloads.[44] Multivalent dendrimer-bound NO donors with 8 (G1) and 32 (G3) terminal functional groups were thus synthesized to understand the influence of dendrimer size on the

anti-biofilm efficacy against *P. aeruginosa* biofilms. Subsequent reaction of the secondary amine-functionalized dendrimers (e.g., **PO**, **ED**, **PE**) with NO at 10 atm under basic conditions (Scheme 1) produced *N*-diazeniumdiolate-functionalized dendritic scaffolds (e.g., **PO-NO**, **ED-NO**, **PE-NO**).

The dendrimers exhibited similar NO storage (~1 μmol/mg) and NO-release kinetics (i.e., half life ~1 h) regardless of modification (Table 1). Of note, similar total NO storage and NO-release kinetics for the PO/ED (PE)-functionalized dendrimers were expected since both 100% PO or ED-functionalized dendrimers exhibited a total NO storage of ~1 μmol/mg and a half-life of ~1 h. These NO-release properties facilitate the evaluation of how surface hydrophobicity (via the PO to ED ratio) and dendrimer generation impact NO delivery and bacteria killing.

Bactericidal Studies: Planktonic Bacteria

Planktonic Gram-negative *P. aeruginosa* were exposed to control and NO-releasing dendrimers to evaluate the effects of the PO/ED ratio on bacterial killing. The bacterial viability assays were carried out over a 4-h period under static conditions. The concentration of dendrimer required to reduce bacteria viability by 3-logs was determined for each dendrimer structure; hereafter, this concentration is referred to as the planktonic minimum bactericidal concentration or MBC. The bactericidal NO dose for the NO-releasing dendrimers was also determined by multiplying the total NO dose over 4 h (i.e., t[NO][a]) and the corresponding MBC. As shown in Table 2, the amphiphilic control dendrimers functionalized with ED exclusively or a PO/ED mixture (e.g., **G1-ED**, **G1-PE 37**, **G1-PE 55**, **G1-PE 73**) exhibited enhanced biocidal activity against planktonic *P. aeruginosa* compared to the hydrophilic control dendrimer (**G1-PO**). This behavior is in part attributed to the enhanced perturbation and disruption of the bacteria membrane by the amphiphilic structures.[47] Functionalizing 30% of the dendrimer exterior primary amines with ED (i.e., **G1-PE 73**) improved the biocidal action of the dendrimer scaffold by 99% (i.e., the MBC for **G1-PE 73** and **G1-PO** was 30 and 3000 μg/mL, respectively) compared to dendrimers functionalized solely with PO (i.e., **G1-PO**).

Further inspection of the data revealed lower MBCs for NO-releasing dendrimers compared to their non-NO-releasing counterpart, indicating enhanced bactericidal activity with NO. This increase in bactericidal efficacy is attributed to the oxidative and nitrosative stresses resulting from NO.[31] As shown in Table 2, the bactericidal NO dose required to elicit the 3-log reduction in bacterial viability decreased with increasing dendrimer hydrophobicity (i.e., ED content), suggesting enhanced bacterial killing. For example, the bactericidal NO doses for **G1-ED-NO** and **G1-PO-NO** were 3.55 and 182 nmol/mL, respectively. Since dendrimer association with the outer surface (i.e., membrane) of bacteria followed by penetration through the bacteria membrane has been identified previously as an important killing mechanism,[23] we hypothesized that increasing the ED (hydrophobic) content of the dendrimer exterior would improve dendrimer association with the bacteria and facilitate more efficient NO delivery. Confocal microscopy was thus used to characterize the association of RITC-labeled **G1-PE 73-NO**, **G1-PE 55-NO**, **G1-PE 37-NO**, and **G3-PE 73-NO** with planktonic *P. aeruginosa* (Figure 2). Dendrimers were labeled with RITC following a previous report.[44] The impact of the RITC label on the dendrimer–bacteria association was minimized by using a low amount of RITC (i.e., 1:100 molar ratio to total primary amines). As expected, based on hydrophobicity, the red fluorescence intensity was the greatest from RITC-labeled **G1-PE 55-NO** and **G1-PE 37-NO**, indicating more rapid association of these dendrimers with *P. aeruginosa* than **G1-PE 73-NO**. However, the association of **G1-PE 37-NO** and **G1-PE 55-NO** with *P. aeruginosa* were similar, illustrating that the improvement of dendrimer association due to hydrophobicity likely plateaus at ~50% ED functionality (**G1-**

PE-55-NO). Additional ED modification had negligible influence on the dendrimer-bacteria association kinetics. The association of the larger dendrimer (**G3-PE 73-NO**) was similar to that of **G1-PE 73-NO**, indicating that the hydrophobicity of the dendritic scaffolds plays a greater role in bacteria association than size, at least for G1 vs. G3 dendrimers. Other sizes and bacteria may result in altered behavior in dendrimer–bacteria association.

Based on enhanced dendrimer–bacteria association for dendrimers with greater ED character, we predicted more effective NO delivery and greater bacteria damage (i.e., membrane disruption). Fluorescence from DAF-2 (green) and PI (red) molecular probes were employed to study the intracellular NO levels and ensuing membrane disruption, respectively, using confocal microscopy. Green fluorescence (DAF-2) was nearly always observed first, indicating a buildup of intracellular NO. Red fluorescence (PI) followed as the bacteria membrane became compromised, with concomitant diminished green fluorescence. The buildup of measurable intracellular NO for bacteria incubated with **G1-PE 37-NO**, **G1-PE 55-NO**, and **G3-PE 73-NO** was observed at 35 min (Figure 3), significantly earlier than bacteria incubated with **G1-PE 73-NO** (60 min). The more rapid association (**G1-PE 37-NO** and **G1-PE 55-NO**) with bacteria and greater localized NO release (**G3-PE 73-NO**) allowed for faster NO accumulation and biocidal action.

Indeed, red fluorescence (PI) was observed at 55, 75, 135, and 70 min for **G1-PE 37-NO**, **G1-PE 55-NO**, **G1-PE 73-NO**, and **G3-PE 73-NO**, respectively. The confocal images of bacteria incubated with **G1-PE 37-NO**, **G1-PE 55-NO**, **G1-PE 73-NO**, and **G3-PE 73-NO** at 135 min were also used to compare the bactericidal activity of the dendrimers. As shown in Figure 4, bacteria incubated with **G1-PE 37-NO**, **G1-PE 55-NO**, and **G3-PE 73-NO** were characterized as having compromised membranes. Conversely, bacteria incubated with **G1-PE 73-NO** exhibited DAF-2 fluorescence, but maintain their membrane integrity (i.e., low PI fluorescence), confirming the enhanced bactericidal activity of larger generation dendrimers and those with greater exterior hydrophobicity.

Bactericidal Studies: Biofilm Eradication

Although planktonic killing assays are helpful in determining a drug or antibacterial agent's potential biomedical utility, most bacteria establish biofilms as a protective mechanism against therapeutics.[1] To evaluate the effects of PO/ED ratio and dendrimer size on the anti-biofilm activity of NO-releasing amphiphilic dendrimers, *P. aeruginosa* biofilms were exposed to a range of NO-releasing dendrimer concentrations (10–800 μg/mL) for 24 h. Following treatment, the biofilm was forced off of the substrate and dispersed by vortexing and sonication to enable viability quantification. Control experiments were performed to confirm both the growth of *P. aeruginosa* biofilms under the selected conditions and the negligible effect of vortexing/sonication on bacteria viability when incubated only in PBS. The viability of bacteria embedded in biofilms was ~2×10^8 CFU per biofilm. Considering the detection limit of 2,500 CFU/mL for this selected plate counting method, a maximum of 5-log reduction in bacterial viability can be achieved. The lowest concentration for a 5-log reduction in bacterial viability (minimum bactericidal concentration or MBC) was then used to characterize the antibacterial efficacy of the amphiphilic dendrimers. The bactericidal NO dose for each of the NO-releasing dendrimers was also derived by multiplying the total NO release over 24 h ($t[NO]^b$) with the corresponding MBCs (Table 3).

Analogous to the inherent bacterial killing observed for planktonic *P. aeruginosa*, the amphiphilic control dendrimers (**G1-PE 73**, **G1-PE 55**, **G1-PE 37**, and **G1-ED**) proved more effective at killing bacterial biofilms than the hydrophilic dendrimer (**G1-PO**) due to the membrane disruption properties by the amphiphilic structures. Dendrimer MBCs for biofilm eradication (5-log killing) were 10000, 150, 30, 15, and 25 μg/mL for **G1-PO**, **G1-**

PE 73, **G1-PE 55**, **G1-PE 37**, and **G1-ED**, respectively. As expected, NO release improved the bactericidal activity of the dendrimers with reduced MBCs (i.e., 800, 80, 20, 10, and 15 μg/mL for **G1-PO-NO**, **G1-PE 73-NO**, **G1-PE 55-NO**, **G1-PE 37-NO**, and **G1-ED-NO**, respectively). Of the dendrimer systems studied, **G1-PE 37-NO** exhibited the greatest anti-biofilm efficacy indicated by the lowest MBC (10 μg/mL) and corresponding bactericidal NO dose (11 nmol/mL). To evaluate the enhanced bactericidal activity of **G1-PE 37-NO** and whether it could be attributed to more rapid association with bacteria embedded within the biofilms, the association of **G1-PE 73-NO**, **G1-PE 37-NO**, and **G1-ED-NO** with bacteria in *P. aeruginosa* biofilms was characterized using confocal microscopy. As shown in Figure 5, a greater number of bacteria in the biofilms exhibited red fluorescence upon incubation with RITC-labeled **G1-PE 37-NO** compared to **G1-PE 73-NO**. As for planktonic bacteria, the enhanced hydrophobic interaction between the dendrimer and bacteria membrane facilitates more efficient NO delivery and killing bacteria (e.g., membrane disruption). Despite the greater hydrophobicity of **G1-ED-NO**, less dendrimer–bacteria association was noted for **G1-ED-NO** compared to **G1-PE 37-NO**. We currently attribute this result to less efficient EPS penetration due to the greater hydrophobicity of **G1-ED-NO**.[48]

Similarly, Wicke et al. reported inhibited diffusion of hydrophobic organic molecules within microbial biofilms compared to hydrophilic derivatives.[48] **G1-PE 37-NO** proved to be the most effective dendrimer construct for eradicating the biofilm bacteria because of its rapid penetration into the biofilm and association with bacteria. Perhaps unexpectedly, dendrimer size (i.e., dendrimer generation) only slightly impacted bacteria association in the *P. aeruginosa* biofilm studies. Although **G3-PE 73-NO** exhibited similar association with bacteria compared to **G1-PE 73-NO**, the DAF-2 fluorescence from biofilms incubated with the larger (G3) dendrimer indicated greater intracellular NO delivery (Figure 6). As noted by the lower required dose to eradicate the bacteria, this increased NO payload and delivery from the larger generation dendrimer (**G3-PE 73-NO**) proved more bactericidal.

Cytotoxicity of NO-Releasing Dendrimers

Although effective at eradicating biofilms, Sun et al. reported that amphiphilic NO-releasing dendrimers were toxic to mammalian cells.[44] For example, polypropylenimine dendrimers modified with styrene oxide elicited substantial toxicity (i.e., >80% reduction in viability at >34 μg/mL) towards L929 mouse fibroblast cells, whereas dendrimers modified with hydrophilic PO elicited no toxicity at concentrations up to 500 μg/mL. Based on these results, we hypothesized that the ratio of hydrophobic (ED)/hydrophilic (PO) functionalization would influence the cytotoxicity of the amphiphilic NO-releasing dendrimers reported herein. Cytotoxicity to L929 mouse fibroblast cells was thus evaluated at the dendrimer concentrations necessary for 3- and 5-log reductions in bacteria viability measured for the planktonic and biofilm *P. aeruginosa* assays, respectively, using the MTS assay (24 h incubation).

Normalized L929 mouse fibroblast cell viabilities of NO-releasing and control dendrimers at the planktonic and biofilm bacteria MBCs are shown in Figure 7A and 7B, respectively. In general, the NO-releasing dendrimers were less toxic than their control counterparts due to the lower concentration of dendrimer required for planktonic killing and biofilm eradication. As expected, dendrimers with increased hydrophobic character (e.g., **G1-ED-NO** and **G1-PE 37-NO**) exhibited greater cytotoxicity (i.e., ~70% cell viability reduction) after 24 h incubation at the MBCs against *P. aeruginosa* biofilms, likely the result of membrane disruption by the large density of hydrophobic chains on the dendrimer periphery.

The most hydrophilic dendrimer, **G1-PO-NO**, was also toxic at its corresponding MBC (800 μg/mL) due to the concentration required to eradicate the biofilm-embedded bacteria. Dendrimers with intermediate ratios of PO/ED (e.g., **G1-PE 55-NO** and **G1-PE 73-NO**) exhibited significantly lower cytotoxicity at the MBCs required to kill the biofilm bacteria. The reduced cytotoxicity is clearly the result of both the lower concentrations of dendrimers required to deliver sufficient levels of NO and the lower ED content on the dendrimer exterior compared to **G1-ED-NO** and **G1-PE 37-NO**. Clearly, the PO/ED ratio at the dendrimer exterior has a significant impact on overall cytotoxicity. Despite the enhanced bactericidal action against biofilms, **G1-PE 37-NO** and **G1-ED-NO** also elicited toxicity towards L929 cells as a result of the ED-induced membrane disruption properties. The larger dendrimer constructs (e.g., **G3-PE 73-NO**) were found to be slightly more toxic (~10% reduction in cell viability) to the L929 mouse fibroblast cells compared to **G1-PE 73-NO** at their respective MBCs, corroborating results by Sun et al. who reported similar generation dependent-cytotoxicity of NO-releasing dendrimers to L929 cells (larger generation being more toxic).[44] The favorable toxicity of the antibacterial **G1-PE 55-NO**, **G1-PE 73-NO**, and **G3-PE 73-NO** dendrimers over previously reported hydrophobic polypropylenimine dendrimers[44] indicate the advantage of using amphiphilic NO-release vehicles with intermediate exterior hydrophobicity (e.g., **G1-PE 55-NO**, **G1-PE 73-NO**) to maximize bacteria killing while minimizing toxicity to mammalian cells.

Conclusions

The utility of amphiphilic NO-releasing dendrimers as antibacterial agents was demonstrated through the systematic study of killing efficiency as a function of NO-releasing dendrimer hydrophobicity and dendrimer size (i.e., dendrimer generation). In particular, the hydrophobicity of dendrimer surface groups were found to significantly influence dendrimer association with *P. aeruginosa* bacteria, the efficiency of intracellular NO delivery, the extent of bacteria membrane disruption, and the cytotoxicity to mammalian cells. Optimal antibacterial activity with minimal toxicity toward mammalian cells was achieved by modifying the dendrimer exterior with a mixture of hydrophilic (PO) and hydrophobic (ED) functionalities. Roughly equal PO/ED modification proved most effectively at eradicating *P. aeruginosa* biofilms with minimal impact on L929 mouse fibroblast cell viability. Future studies should include testing of these materials against polymicrobial biofilms, biofilms formed from clinically-isolated bacteria, and detailed pharmacological toxicity evaluation of the NO-releasing dendrimers using human cells/tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Costerton JW, Stewart PS, Greenberg EP. Science. 1999; 284:1318–1322. [PubMed: 10334980]
2. Nikitkova AE, Haase EM, Scannapieco FA. Appl Environ Microbiol. 2013; 79:416–423. [PubMed: 23144140]
3. Lyczak JB, Cannon CL, Pier GB. Clin Microbiol Rev. 2002; 15:194–222. [PubMed: 11932230]
4. Mah TF, O'Toole GA. Trends Microbiol. 2001; 9:34–39. [PubMed: 11166241]

5. Boas U, Heegaard PM. Chem Soc Rev. 2004; 33:43–63. [PubMed: 14737508]
6. Lee CC, MacKay JA, Frechet JM, Szoka FC. Nat Biotechnol. 2005; 23:1517–1526. [PubMed: 16333296]
7. Mintzer MA, Grinstaff MW. Chem Soc Rev. 2011; 40:173–190. [PubMed: 20877875]
8. Debrabandervandenberg EMM, Meijer EW. Angew Chem Int Ed. 1993; 32:1308–1311.
9. Tomalia DA, Naylor AM, Goddard WA. Angew Chem Int Ed. 1990; 29:138–175.
10. Fox ME, Szoka FC, Frechet JMJ. Acc Chem Res. 2009; 42:1141–1151. [PubMed: 19555070]
11. Bosman AW, Janssen HM, Meijer EW. Chem Rev. 1999; 99:1665–1688. [PubMed: 11849007]
12. Grayson SM, Frechet JMJ. Chem Rev. 2001; 101:3819–3867. [PubMed: 11740922]
13. Carlmark A, Hawker CJ, Hult A, Malkoch M. Chem Soc Rev. 2009; 38:352–362. [PubMed: 19169453]
14. Baleg AA, Jahed NM, Arotiba OA, Mailu SN, Hendricks NR, Baker PG, Iwuoha EI. J Electroanal Chem. 2011; 652:18–25.
15. Huang BH, Kukowska-Latallo JF, Tang SZ, Zong H, Johnson KB, Desai A, Gordon CL, Leroueil PR, Baker JR. Bioorg Med Chem Lett. 2012; 22:3152–3156. [PubMed: 22480432]
16. Mullen DG, McNerny DQ, Desai A, Cheng XM, DiMaggio SC, Kotlyar A, Zhong Y, Qin S, Kelly CV, Thomas TP, Majoros I, Orr BG, Baker JR, Holl MMB. Bioconjugate Chem. 2011; 22:679–689.
17. Zong H, Thomas TP, Lee KH, Desai AM, Li MH, Kotlyar A, Zhang YH, Leroueil PR, Gam JJ, Holl MMB, Baker JR. Biomacromolecules. 2012; 13:982–991. [PubMed: 22360561]
18. Mintzer MA, Dane EL, O'Toole GA, Grinstaff MW. Mol Pharm. 2012; 9:342–354. [PubMed: 22126461]
19. Calabretta MK, Kumar A, McDermott AM, Cai CZ. Biomacromolecules. 2007; 8:1807–1811. [PubMed: 17511499]
20. Chen CZS, Beck-Tan NC, Dhurjati P, van Dyk TK, LaRossa RA, Cooper SL. Biomacromolecules. 2000; 1:473–480. [PubMed: 11710139]
21. Meyers SR, Juhn FS, Griset AP, Luman NR, Grinstaff MW. J Am Chem Soc. 2008; 130:14444–14445. [PubMed: 18842041]
22. Lopez AI, Reins RY, McDermott AM, Trautner BW, Cai CZ. Mol BioSyst. 2009; 5:1148–1156. [PubMed: 19756304]
23. Chen CZS, Cooper SL. Adv Mater. 2000; 12:843–846.
24. Hou SY, Zhou CH, Liu ZG, Young AW, Shi ZH, Ren DC, Kallenbach NR. Bioorg Med Chem Lett. 2009; 19:5478–5481. [PubMed: 19682902]
25. Reymond JL, Darbre T. Org Biomol Chem. 2012; 10:1483–1492. [PubMed: 22274649]
26. Johansson EMV, Crusz SA, Kolomiets E, Buts L, Kadam RU, Cacciarini M, Bartels KM, Diggle SP, Camara M, Williams P, Loris R, Nativi C, Rosenau F, Jaeger KE, Darbre T, Reymond JL. Chem Biol. 2008; 15:1249–1257. [PubMed: 19101469]
27. Hong SP, Bielinska AU, Mecke A, Keszler B, Beals JL, Shi XY, Balogh L, Orr BG, Baker JR, Holl MMB. Bioconjugate Chem. 2004; 15:774–782.
28. Wang B, Navath RS, Menjoge AR, Balakrishnan B, Bellair R, Dai H, Romero R, Kannan S, Kannan RM. Int J Pharm. 2010; 395:298–308. [PubMed: 20580797]
29. Marletta MA, Tayeh MA, Hevel JM. Bio Factors. 1990; 2:219–225.
30. MacMicking J, Xie QW, Nathan C. Annu Rev Immunol. 1997; 15:323–350. [PubMed: 9143691]
31. Fang FC. J Clin Invest. 1997; 99:2818–2825. [PubMed: 9185502]
32. Lu Y, Sun B, Li C, Schoenfisch MH. Chem Mater. 2011; 23:4227–4233. [PubMed: 22053127]
33. Riccio DA, Nugent JL, Schoenfisch MH. Chem Mater. 2011; 23:1727–1735. [PubMed: 21499510]
34. Stasko NA, Schoenfisch MH. J Am Chem Soc. 2006; 128:8265–8271. [PubMed: 16787091]
35. Shin JH, Metzger SK, Schoenfisch MH. J Am Chem Soc. 2007; 129:4612–4619. [PubMed: 17375919]
36. Rothrock AR, Donkers RL, Schoenfisch MH. J Am Chem Soc. 2005; 127:9362–9363. [PubMed: 15984851]
37. Carpenter AW, Schoenfisch MH. Chem Soc Rev. 2012; 41:3742–3752. [PubMed: 22362384]

38. Coneski PN, Schoenfisch MH. Chem Soc Rev. 2012; 41:3753–3758. [PubMed: 22362308]
39. Nichols SP, Storm WL, Koh A, Schoenfisch MH. Adv Drug Deliv Rev. 2012; 64:1177–1188. [PubMed: 22433782]
40. Riccio DA, Schoenfisch MH. Chem Soc Rev. 2012; 41:3731–3741. [PubMed: 22362355]
41. van ‘t Hof W, Veerman ECI, Helmerhorst EJ, Amerongen AVN. Biol Chem. 2001; 382:597–619. [PubMed: 11405223]
42. Lu Y, Slomberg DL, Sun B, Schoenfisch MH. Small. 2013; 9:2189–2198. [PubMed: 23362159]
43. Carpenter AW, Slomberg DL, Rao KS, Schoenfisch MH. ACS Nano. 2012; 5:7235–7244. [PubMed: 21842899]
44. Sun B, Slomberg DL, Chudasama SL, Lu Y, Schoenfisch MH. Biomacromolecules. 2012; 13:3343–3354. [PubMed: 23013537]
45. Hetrick EM, Shin JH, Stasko NA, Johnson CB, Wespe DA, Holmuhamedov E, Schoenfisch MH. ACS Nano. 2008; 2:235–246. [PubMed: 19206623]
46. Hetrick EM, Shin JH, Paul HS, Schoenfisch MH. Biomaterials. 2009; 30:2782–2789. [PubMed: 19233464]
47. Denyer SP. Int Biodeter Biodegr. 1995; 36:227–245.
48. Wicke D, Bockelmann U, Reemtsma T. Environ Sci Technol. 2008; 42:1990–1996. [PubMed: 18409626]

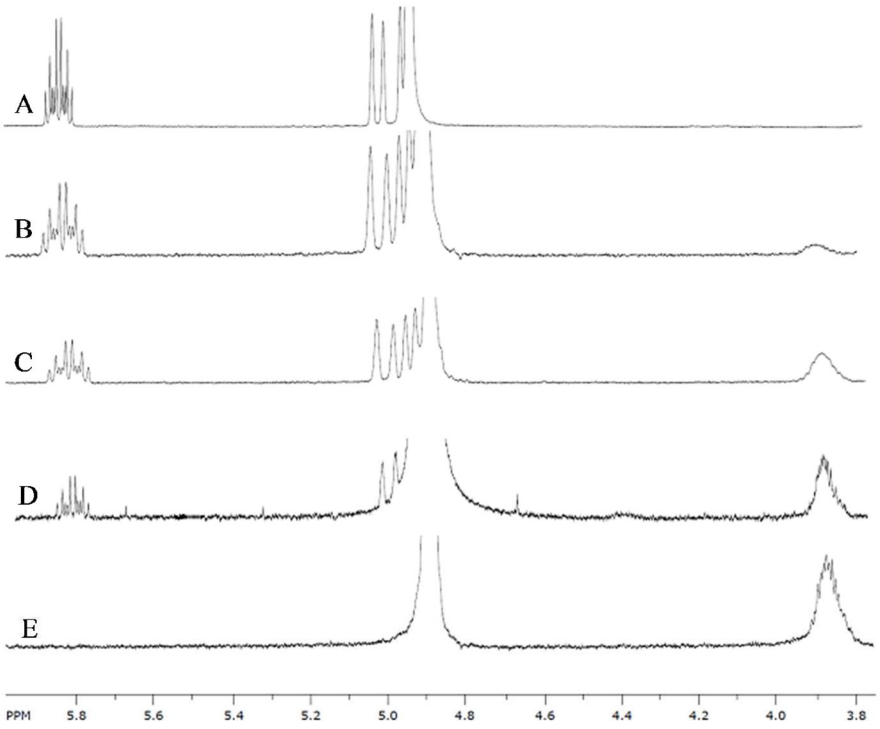

Figure 1.

^{1}H NMR spectra of A) **G1-PAMAM-ED**, B) **G1-PAMAM-PE 37**, C) **G1-PAMAM-PE 55**, D) **G1-PAMAM-PE 73**, and E) **G1-PAMAM-PO**. The actual composition of ED and PO was determined by the integration of peaks at 5.80 (-C*H*=CH_2) and 3.82 (-C*H*(OH)CH_3) ppm.

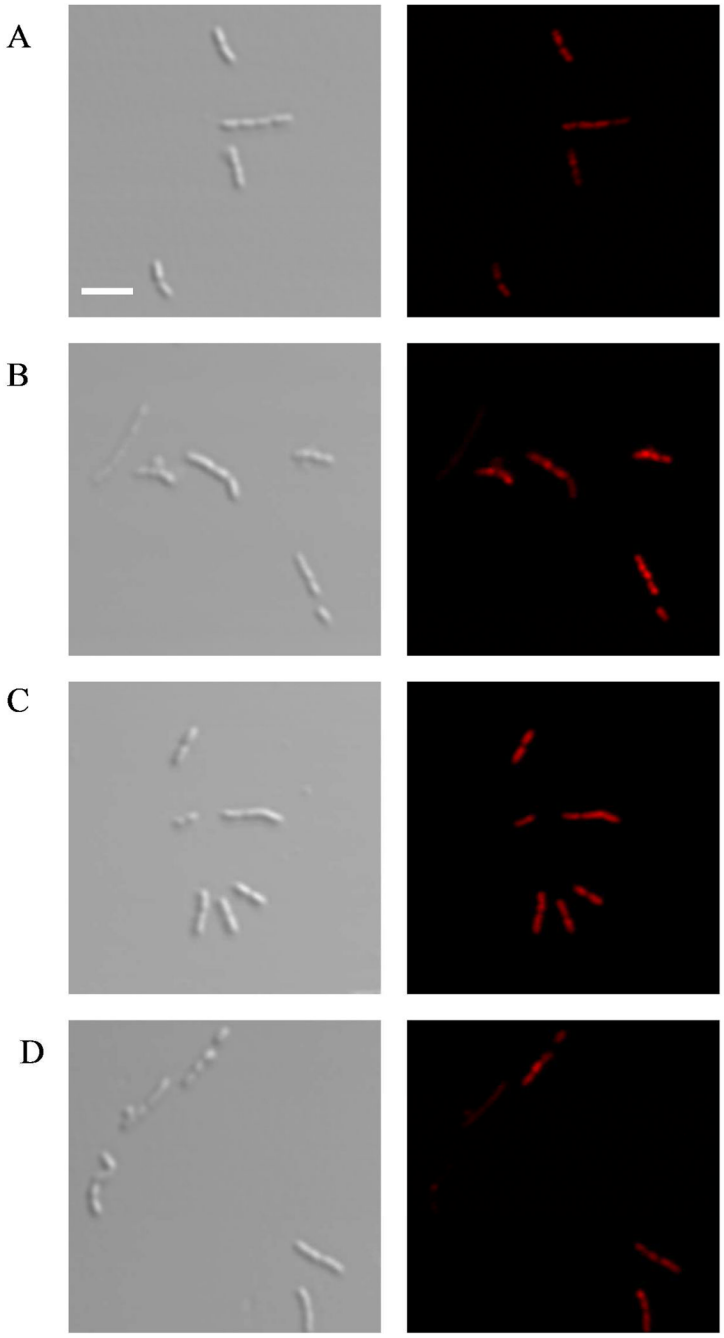

Figure 2.
Bright field/fluorescent overlay and fluorescent images of RITC-label NO-releasing dendrimers association with planktonic *P. aeruginosa*. A) **G1-PE 73**; B) **G1-PE 55**; C) **G1-PE 37**; D) **G3-PE 73**. Scale bar 5 μm. Image on the right are color and best observed electronically.

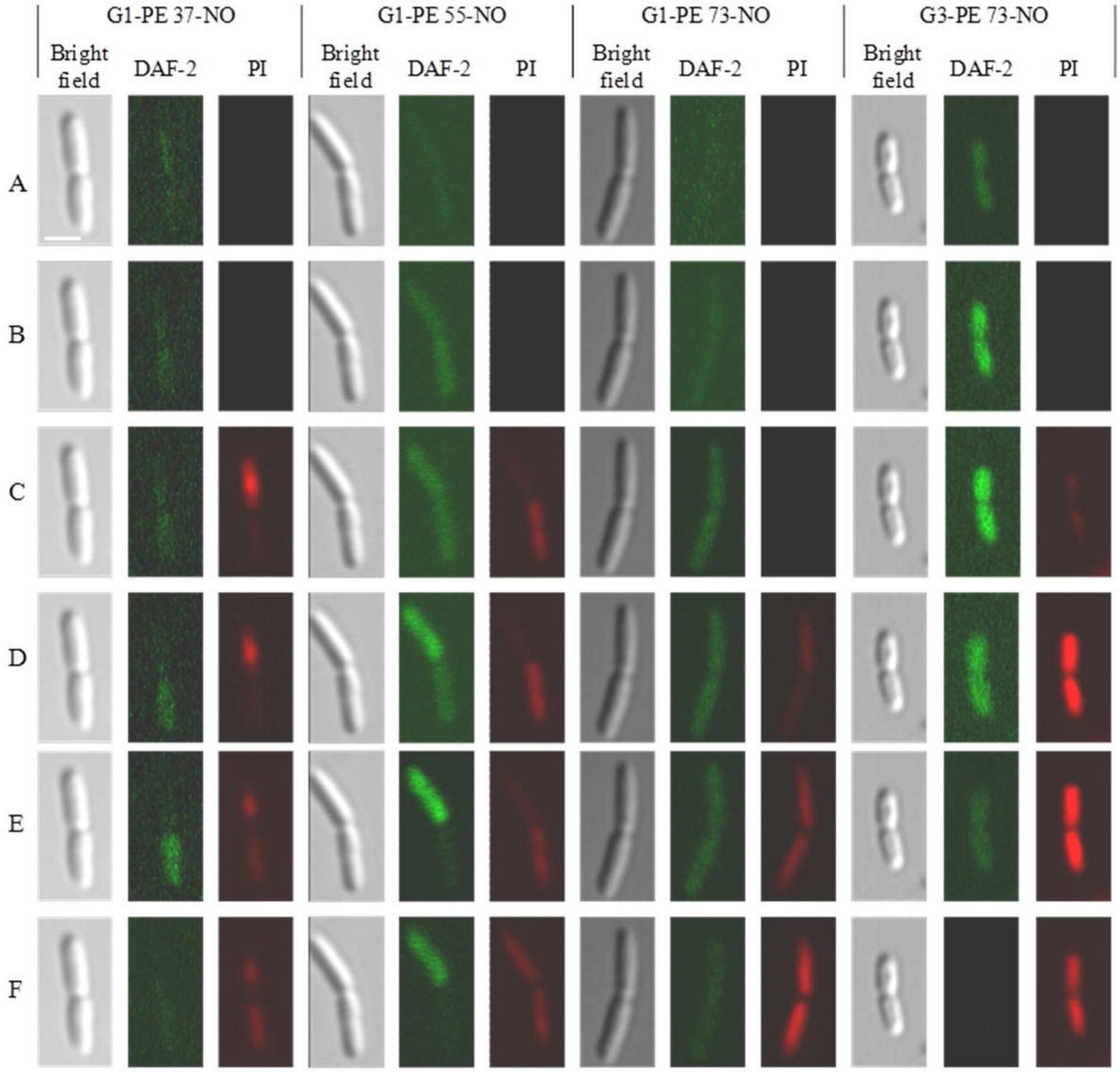

Figure 3.

Intracellular DAF-2 (green) and PI (red) fluorescence from planktonic *P. aeruginosa* exposed to NO-releasing dendrimers **G1-PE 37-NO** at A-35, B-50, C-55, D-65, E-105, F-110 min, **G1-PE 55-NO** at A) 35, B) 60, C) 75, D) 85, E) 115, F) 150 min, **G1-PE 73-NO** at A) 60, B) 85, C) 125, D) 135, E) 140, F) 150 min, and **G3-PE 73-NO** at A) 35, B) 50, C) 65, D) 70, E) 100, F) 130 min. Intracellular NO is indicated by the DAF-2 green fluorescence, whereas PI red fluorescence points to compromised membranes. Scale bar: 2 μm.

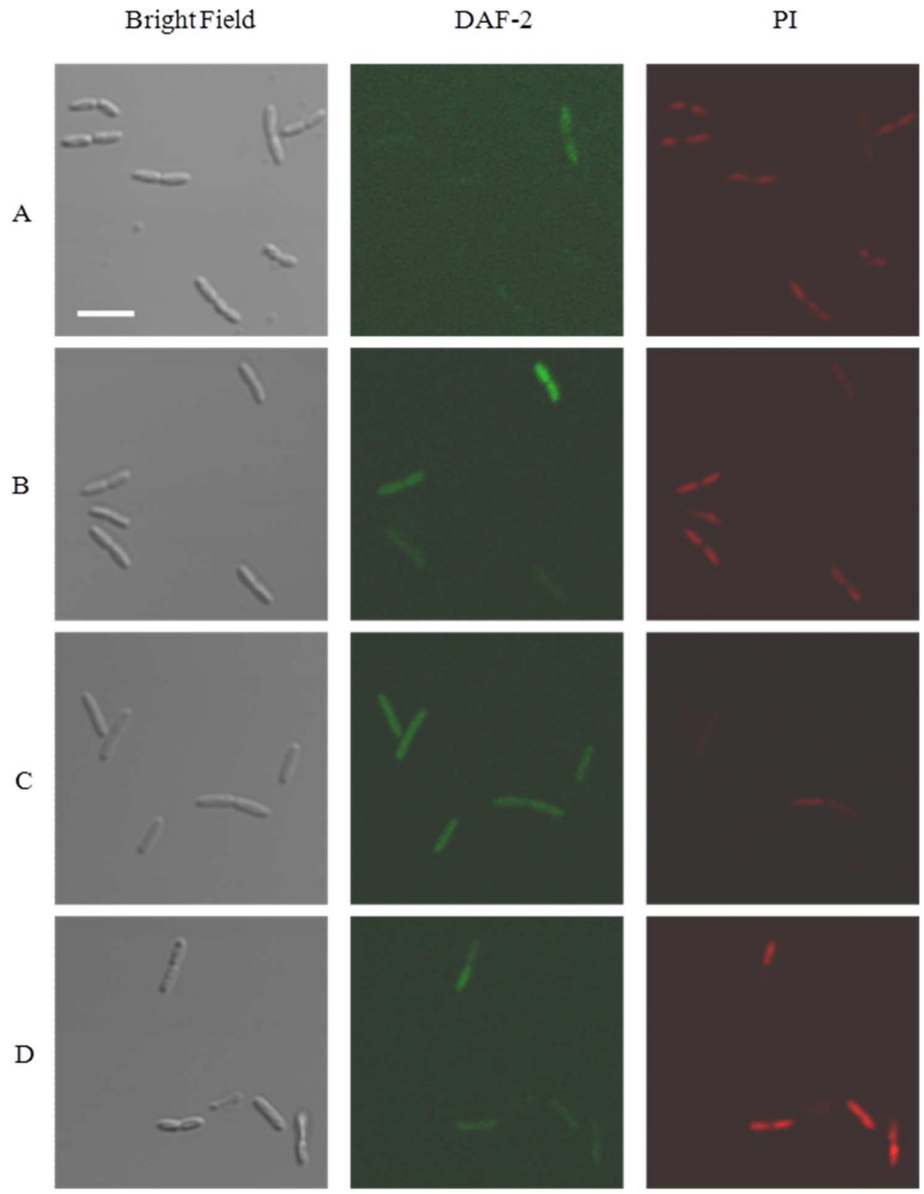

Figure 4.
Intracellular DAF-2 (green) and PI (red) fluorescence from planktonic *P. aeruginosa* exposed to NO-releasing dendrimers at 135 min incubation A) **G1-PE 37-NO**; B) **G1-PE 55-NO**; C) **G1-PE 73-NO**; and D) **G3-PE 73-NO**. Scale bar: 5 μm.

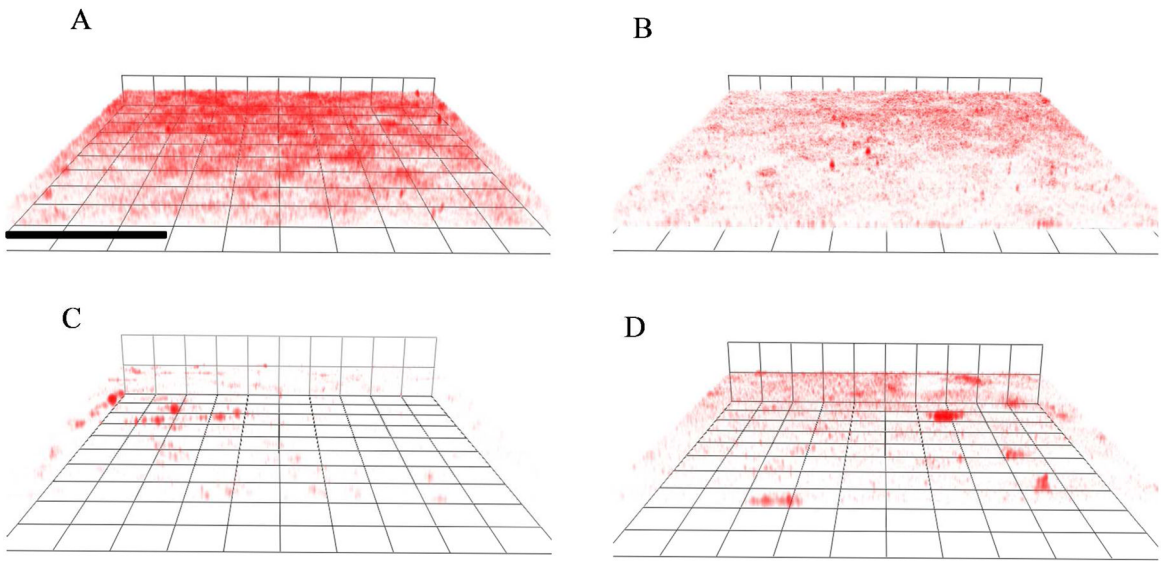

Figure 5.

Three dimentional scanning confocal microscopy images of *P. aeruginosa* biofilms exposed to A) **G1-PE-37-NO**; B) **G1-ED-NO**; C) **G1-PE 73-NO**; and D) **G3-PE 73-NO** RITC-labeled NO-releasing dendrimers for 1 h incubation. Greater red fluorescence indicates more efficient dendrimer–bacteria association. Scale bar: 300 μm.

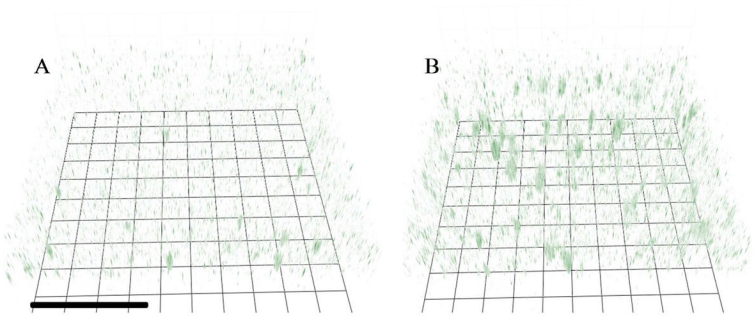

Figure 6.
Three dimensional intracellular DAF-2 fluorescence images of *P. aeruginosa* biofilms incubated with A) **G1-** and B) **G3-PE 73-NO** for 1 h. Scale bar: 50 μm. Green fluorescence indicates the intracellular NO levels.

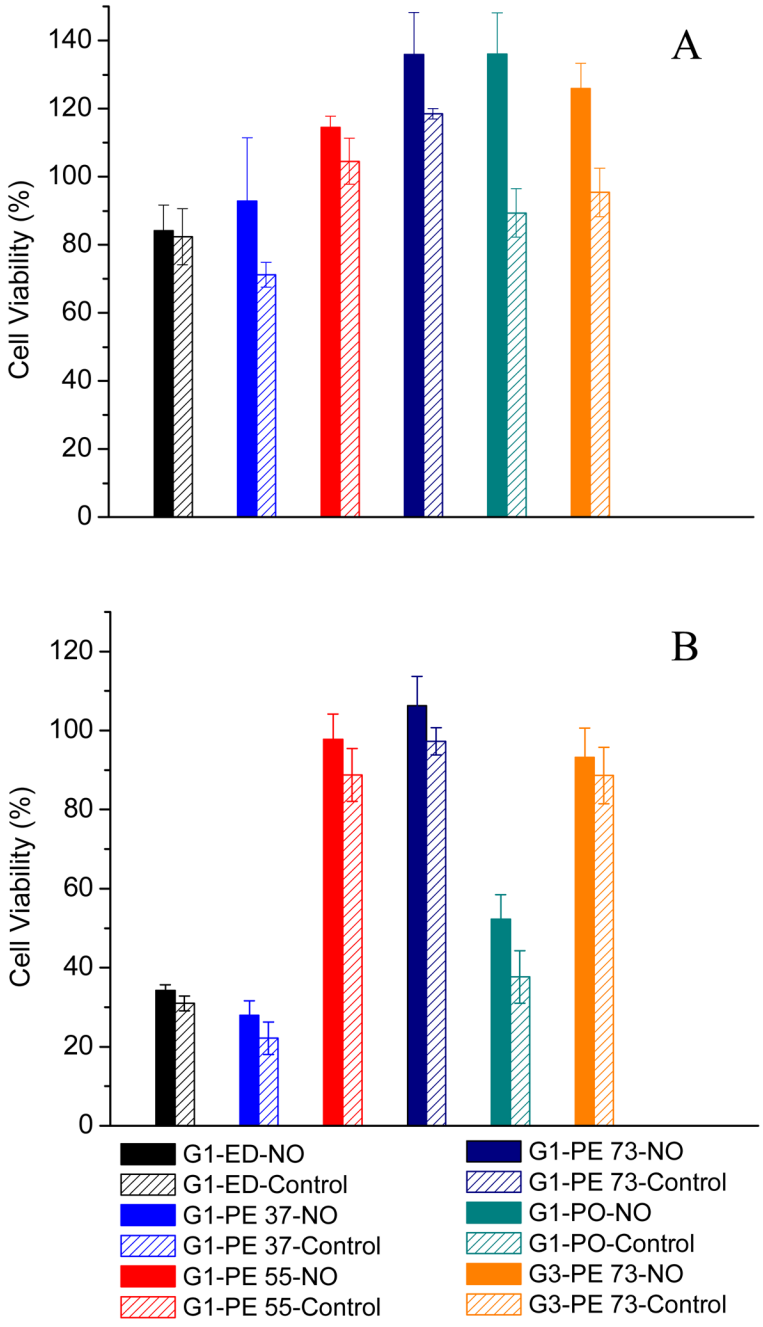

Figure 7.
Cytotoxicity of NO-releasing dendrimers to L929 fibroblast cells at the MBCs against A) planktonic and B) biofilm-based *P. aeruginosa*.

Scheme 1.

Synthesis of secondary amine- and *N*-diazeniumdiolate-functionalized PAMAM conjugates for which n represents the number of primary amines on the periphery of PAMAM dendrimers (n = 8, 32).1

Table 1

Nitric oxide-release properties for G1 and G3 PAMAM dendrimers in PBS (pH = 7.4 at 37 °C) as measured by a chemiluminescence NO analyzer.

	Feed molar ratio (PO/ED)	t[NO]^a (μmol/mg)	t[NO]^b (μmol/mg)	$[NO]_{max}$ (ppb/mg)	$t_{1/2}$ (h)
G1-ED	0:1	0.71±0.15	1.23±0.18	3295±435	0.94±0.04
G1-PE 37	5:5	0.93±0.16	1.10±0.18	5666±562	0.87±0.03
G1-PE 55	7:3	1.10±0.15	1.23±0.17	5000±575	0.95±0.03
G1-PE 73	9:1	0.88±0.18	1.04±0.19	3000 ±356	1.01±0.02
G1-PO	1:0	0.92±0.13	1.03±0.15	3800±379	0.75±0.04
G3-PE 73	9:1	0.89±0.09	1.06±0.12	2400±314	1.02±0.03

^{a}the NO released over 4 h (μmol per milligram of secondary amine-functionalized dendrimers).

^{b}the NO released over 24 h (μmol per milligram of secondary amine-functionalized dendrimers).

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

Table 2

Comparison of the minimum bactericidal concentration (MBC) and bactericidal NO doses of control and NO-releasing dendrimers against planktonic Gram-negative *P. aeruginosa* after 4 h exposure for 3-log reduction in bacterial viability.

Dendrimers	MBC[a] (μg/mL)	MBC[b] (μg/mL)	Bactericidal NO Doses (nmol/mL)
G1-ED	8	5	3.55
G1-PE 37	5	4	3.72
G1-PE 55	10	7	7.70
G1-PE 73	30	20	17.6
G1-PO	3000	200	182
G3-PE 73	20	15	13.4

[a] concentration of control dendrimer required to reduce bacterial viability by 3-logs.

[b] concentration of NO-releasing dendrimer required to reduce bacterial viability by 3-logs.

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

Table 3

Comparison of the minimum bactericidal concentration and bactericidal NO doses of control and NO-releasing dendrimers required to achieve 5-log reduction in bacteria viability of Gram-negative *P. aeruginosa* biofilms after 24 h exposure.

	MBC[a] (μg/mL)	MBC[b] (μg/mL)	Bactericidal NO Doses (nmol/mL)
G1-ED	25	15	18.5
G1-PE 37	15	10	11.0
G1-PE 55	30	20	24.6
G1-PE 73	150	80	83.2
G1-PO	10000	800	824
G3-PE 73	150	60	63.6

[a] concentration of control dendrimer required to reduce bacterial viability by 5-logs.

[b] concentration of NO-releasing dendrimer required to reduce bacterial viability by 5-logs.

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