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Antibacterial Activities of Poly(amidoamine) Dendrimers Terminated with Amino and Poly(ethylene glycol) Groups

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Abstract

Poly(amidoamine) (PAMAM) dendrimer derivatives have been investigated for their biological applications, especially for delivery of drugs, including antimicrobial drugs to eukaryotic cells, but their effects on bacterial cells are largely unexplored. Herein we report that amino-terminated PAMAM dendrimers are highly toxic to the common Gram-negative pathogen *Pseudomonas aeruginosa*. The concentration that kills 50% of the bacteria (EC_{50}) was in the range of ~0.9–1.5 μg/mL for the generation 5, amino-terminated dendrimers with or without partial (43%) coating of poly(ethylene glycol) (PEG). These EC_{50} values were lower than that (~1.9–2.8 μg/mL) for LL-37, a potent antimicrobial peptide expressed in a variety of epithelia. On the contrary, the dendrimers were far less toxic (EC_{50} > 21 μg/mL) to the Gram-positive pathogen *Staphylococcus aureus* than LL-37 (EC_{50} = ~1.9 μg/mL). In agreement with the previous studies on other cell types, the dendrimers were not cytotoxic to human corneal epithelial cells at the concentrations that were toxic to *P. aeruginosa*. Our findings indicate that amino-terminated PAMAM dendrimers and their partially PEG-coated derivatives possess attractive antimicrobial properties, particularly against Gram-negative bacteria, thus expanding the potential biological application of the dendrimers.

Introduction

Dendrimers are highly branched dendritic molecules that possess unique properties including relatively large molecular size, narrow size distribution, well-defined globular structure, and ease of derivatization via the peripheral functional groups. These properties have attracted great interest in exploring their potential biomedical applications such as drug delivery, gene transfection, and imaging.[1] Recent research activities in this area also include the study of antimicrobial activities of dendrimer derivatives.[2] In most cases, dendrimers serve as carriers of biologically active agents by encapsulating them in the interior or, more often, tethering them on the periphery of the dendrimers. For example, most dendrimers displaying antimicrobial activities are terminated with antimicrobial agents, including ferrocene,[3] quaternary ammonium,[2,4] boron complexes,[5] carbohydrates,[6] and peptides.[7]

Poly(amidoamine) (PAMAM) dendrimers (e.g., the generation 3 (G3) PAMAM in Figure 1) are the most extensively studied dendrimers. PAMAM dendrimers with a wide variety of functional groups at the periphery are commercially available. Some of them, including those with amino terminal groups, are shown to have low toxicity to eukaryotic cells.[8] Modification of the amino groups with poly(ethylene glycol) (PEG) or lauroyl chains further improves their biocompatibility.[9,10] As the number of PEG or lauroyl chains increases, the cytotoxicity of

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Supporting Information Available. ^{1}H NMR of PEG–PAMAM. This material is available free of charge via the Internet at http://pubs.acs.org.

PAMAM to human colon adenocarcinoma cells decreases.[10] Shielding of the positive charges of the protonated amino groups on the exterior of the dendrimer by the PEG or lauroyl chains is likely the reason for reduced cytotoxicity.[10] Due to their excellent biocompatibility, PEG-modified PAMAM dendrimers have been used as carriers of imaging agents and pharmaceuticals, including antimicrobial agents such as penicillin V and silver.[1,11,12] Several proprietary PAMAM dendrimer derivatives are shown to be potent antiviral agents.[7f,12b,13] However, their composition has not been disclosed, and it is unclear if the dendrimers alone are inherently antiviral. Despite extensive studies of PAMAM dendrimers for biomedical applications, antimicrobial activity of unmodified PAMAM dendrimers has not been reported.

Recently, we have been interested in the mechanisms of action of antimicrobial peptides (AMPs) associated with the ocular surface. Bacterial keratitis is a major cause of ocular surface morbidity, particularly among contact lens wearers, that often leads to permanently compromised visual function, sometimes necessitating penetrating keratoplasty (corneal transplant) to restore adequate vision. To date, four AMPs, including LL-37 (a 37 amino acid residue peptide), have been detected in the ocular surface epithelia.[14–16] Each of them has potent activity against common ocular pathogens such as *Pseudomonas aeruginosa* (PA) and *Staphylococcus aureus* (SA).[15,16] There is evidence that a threshold concentration of AMP is needed to cause membrane permeation and eventual cell death.[17] We hypothesized that localization of AMP on polymers or substrate surfaces could thus enhance its potency. We selected PAMAM dendrimers to test this idea since we have investigated thin films derived from PAMAM and their functionalization with PEG and ligands.[18] In the course of the investigation, we were surprised to find that PAMAM dendrimers themselves were highly toxic to some bacteria.

In this communication, we report the antibacterial properties of generation 5 (G5) PAMAM dendrimers with or without partial PEG coating. We show that these dendrimers are more toxic than LL-37 against PA, a common Gram-negative ocular pathogen involved in keratitis and conjunctivitis. To our knowledge, this is the first report of the inherent high antimicrobial activity of PAMAM dendrimers unmodified with known antimicrobial agents. PEG-coated PAMAM also exhibited low toxicity to human corneal epithelial cells (HCECs). These results suggest the potential use of PAMAM dendrimers as effective antimicrobial agents.

Experimental Section

Materials

Amino-terminated G5 PAMAM dendrimer (26% w/w in methanol) was purchased from Dendritech. **EG_{11}NHS** (NHS-*m*-dPEG, molecular weight (MW) = 685) was purchased from Quanta Biodesign.

Synthesis of PEG–PAMAM

To each of two vials was added a 26% methanolic solution of G5 PAMAM (38 mg, 0.34 μmol), followed by a 10% w/v solution of **EG_{11}NHS** in CH_2Cl_2 (0.58 mL, 58 mg, 250 equiv). The mixture was stirred vigorously overnight. The solvent was removed under vacuum. The product was isolated by gel filtration chromatography (prepacked Sephadex PD-10 column) with Millipore water as eluent, and dried by lyophilization. The product was further purified by high-performance liquid chromatography with a Nova-Pak C18 column and a gradient eluent from MeCN/H_2O/CF_3COOH 5:95:0.05 to 100:0:0.05. The product was characterized by NMR spectroscopy (see Supporting Information) and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Indole acrylic acid was used as the matrix for MALDI measurement. It was dissolved to a concentration of 0.1 M in a 1:1 water/dimethylformamide (DMF) mixture. The dendrimers were dissolved in DMF to a concentration of ~9 μM. Molar ratios from 100:1 to 6400:1 between the matrix and the dendrimer analytes were tested. The MALDI spectra of the G5 PAMAM starting material and the PEG–PAMAM product are depicted in Figure 2. Ideally, G5 PAMAM has a MW of 28 828 Da and ~128 NH_2 groups at periphery. However, commercial PAMAM dendrimers are known to often contain defects.[19] Indeed, Figure 2a indicates an average MW of 26 575 Da for this starting material, and the number of peripheral amino groups is accordingly estimated to be ~117. The difference of the average MWs of PEG–PAMAM (55 249 Da, Figure 2b) and PAMAM (26 575 Da) indicates that, on average, 50 PEG chains are attached on each PEG–PAMAM, corresponding to ~43% coverage.

Preparation of Bacteria

The strains used were PA ATCC 19660,[20] a clinical isolate of PA (2219) from corneal scrapings of patients with bacterial keratitis, and SA ATCC 29213. All bacterial strains were handled with sterile technique to prevent infection or contamination. Single colonies were used to inoculate 5 mL of Difco's nutrient broth for PA or trypticase soy broth for SA at 37 °C overnight. A small volume (250 μL) of this growth was used to inoculate 50 mL of media at 37 °C for 2.5 h. Harvested cells (3100 rpm, 10 min) were resuspended in sterile nanopure water to an optical density of 0.2 at 600 nm or approximately 10^7 colony-forming units (cfu)/mL.

Antimicrobial Assays

We used a standard colony count assay to determine the concentration of dendrimer that kills 50% of the bacteria (EC_{50}).[21] Reaction mixtures (100 μL total) consisted of 20 μL of 10^7 cfu/mL PA and PAMAM dendrimers over a range of dendrimer concentrations (0.01–50 μg/mL). These mixtures were incubated for 2 h at 37 °C. Samples were serially diluted and plated on agar for growth overnight at 37 °C. The number of colonies on agar were counted on a light board and compared to negative controls (no dendrimer). Data were fit to sigmoidal curves in Origin 6.1 (OriginLab, Northampton, MA) to calculate the EC_{50}. At least three independent experiments were performed.

Cytotoxicity Assays

The cytotoxicity of PEG–PAMAM to SV40-transformed HCECs,[22] a gift from Kaoru Araki-Sasaki (Tane Memorial Eye Hospital, Osaka, Japan), was determined. Cells were maintained in SHEM-X media with 10% fetal bovine serum and 30 μg/mL gentamicin. HCECs (8000/well) were grown for 24 h at 37 °C to confluence in 96-well plates.[16,23] PEG–PAMAM (1–100 μg/mL) diluted in serum-free media was incubated at 37 °C with HCECs for 24 h. Controls were culture medium incubated for 24 h (negative control) or 0.002% benzalkonium chloride incubated for 30 min (positive control). Cytotoxicity was assessed according to the manufacturer's instructions with an MTT assay kit (Chemicon International, Temecula, CA), which relates the number of surviving cells with the absorbance signal from a colorimetric indicator. Data were collected at 590 nm on a Perkin-Elmer HTS 7000 bioassay reader with a reference wavelength of 635 nm.

Results and Discussion

Antimicrobial Activity of PEG–PAMAM

In this work, we used a standard colony count assay to assess the antimicrobial activity of PAMAM to the common ocular pathogens PA and SA. We focused on G5 PAMAM, with ~117 amino groups at its periphery, and its derivative (PEG–PAMAM), where 43% of the amino groups were modified with monodisperse PEG chains consisting of 11 ethylene glycol units (EG_{11}, Figure 1). We evaluated PA19660 and SA29213, which are standard lab strains used in animal keratitis models, and a clinical strain, PA2219, that was isolated from the cornea of a patient with keratitis. PAMAM and PEG–PAMAM were both effective against PA19660 in a concentration-dependent manner, as shown in Figure 3. The EC_{50} values, obtained from the data presented in Figure 3, are 1.5 ± 0.1 and 0.9 ± 0.1 μg/mL for PAMAM and PEG–PAMAM, respectively. Significantly ($p \leq 0.05$), the value for PEG–PAMAM is 3 times lower than the previously published value of 2.8 ± 1.3 μg/mL for LL-37.[15] The clinical strain, PA2219, showed a slightly increased resistance to PEG–PAMAM as compared to the lab strain ($p \leq 0.05$), with an EC_{50} value of 1.4 ± 0.2 μg/mL. This value is comparable to the EC_{50} (1.9 ± 1.3 μg/mL) of LL-37 against this strain.[15]

In contrast to Gram-negative PA, the Gram-positive pathogen SA29213 exhibited resistance to PEG–PAMAM up to the highest concentration tested (50 μg/mL) as shown in Figure 4. The unmodified PAMAM was still effective at killing SA29213, but the EC_{50} (20.8 ± 3.4 μg/mL) was an order of magnitude higher than the EC_{50} values for the Gram-negative PA19960 and PA2219. In comparison, LL-37 maintains similar toxicity to SA29213 (EC_{50} = 1.6 ± 1.5 μg/mL)[15] versus PA19960 and PA2219.

It is generally accepted that cationic AMPs promote the disruption of anionic bacterial cell membranes initially through electrostatic interactions.[24] Similarly, being a polycationic molecule, PAMAM is expected to strongly bind to bacteria. It has been shown that PAMAM causes the formation of nanoscale holes in supported lipid bilayers.[25] Upon capping the peripheral amino groups on PAMAM with acetyl groups, PAMAM is no longer able to cause expansion of defects in supported lipid bilayers. Therefore, the protonated amino groups on PAMAM are necessary for the rupture of the lipids,[25b] although other subsequent interactions might also be necessary (see below). The reason for the substantially lower toxicity of PAMAM to the Gram-positive over the Gram-negative bacteria is not clear. It could be due to the much thicker cell wall that covers the membrane of the Gram-positive bacteria. The cell wall may restrict penetration and so impair the interaction between the cell membrane and the amino groups on the highly branched PAMAM. Partial shielding of the amino groups by the PEG chains on PEG–PAMAM may further reduce the direct interactions of the amino groups with the Gram-positive cell membrane. In addition, the thick cell wall on Gram-positive bacteria is permeable to molecules of MWs lower than 30 000–57 000 Da.[26] Hence the amino-terminated PAMAM (MW 26575 Da) may penetrate through the cell wall to interact with the cell membrane, while the PEG–PAMAM (MW 55250 Da) may be blocked by the cell wall. For the Gram-negative species, the function of their outer membrane may be significantly impaired, and hence bacterial viability may be compromised before PAMAM reaches the cell membrane. Furthermore, the actual composition of the cell membrane is known to differ between Gram-positive and Gram-negative species, and this may markedly affect interactions with PAMAM. It should also be noted that, while several AMPs, such as LL-37, show comparable effects on Gram-negative and Gram-positive bacteria, others, such as human β-defensin-2, have potent activity against Gram-negative organisms but are minimally effective against Gram-positive species.[14b]

Toxicity of PEG–PAMAM to HCECs

We tested the cytotoxicity of PAMAM to SV40-transformed HCECs with a MTT toxicity assay. The results are summarized in Figure 5. At low concentrations (0.001–1 μg/mL), neither the unmodified PAMAM nor the PEG–PAMAM showed significant toxicity to HCECs (shown as % Survival). At the highest concentration tested (10 μg/mL), PAMAM showed 25% survival of HCECs, comparable to the benzalkonium chloride positive control. This result is consistent with the findings that, at high concentrations, PAMAM can lead to the formation of nanoscale holes in eukaryotic membranes.[25e] We have previously reported the toxicity of LL-37 to primary cultured HCECs, showing that significant toxicity occurs at 25 μg/mL LL-37.[16] The reason that G5 PAMAM is more toxic to HCECs than LL-37 might be due to the fact that highly branched cationic polymers permeate eukaryotic membranes better than linear molecules, such as LL-37.[25a] This property of branched polymers has been used to transfect eukaryotic cells with foreign DNA and to deliver other compounds into the cell.[1b] Permeability is also dependent on the charge density on the polymer. In fact, capping the amino groups on the exterior of PAMAM with acetyl groups reduces eukaryotic cell permeation.[25a] Accordingly, the PEG-coated PAMAM showed only a slight decrease to 91% survival. This result is in agreement with the previous reports that PEG coating improves the biocompatibility of PAMAM and reduces the toxicity of PAMAM to mammalian cells.[9,10]

Conclusions

In this work, we show that amino-terminated G5 PAMAM dendrimers are effective antimicrobial agents against common Gram-negative and Gram-positive pathogens PA and SA. These pathogens, especially PA, are often associated with contact lens-related bacterial keratitis, a condition that can ultimately lead to blindness if not treated.[27] We tested the toxicity of G5 PAMAM to HCECs as a first step in developing these compounds as therapeutics with low resistance for bacterial keratitis. Although unmodified, amino-terminated PAMAM is toxic to HCECs, partial coating of the dendrimers with PEG reduces cytotoxicity. The partial PEG coating maintains a high toxicity to the Gram-negative pseudomonal species, although it results in a large decrease in toxicity to Gram-positive staphylococcal species. Pseudomonal strains, however, are the most frequently isolated from patients with contact lens-associated keratitis.[27] These findings show that PAMAM derivatives could be an excellent candidate for a new class of antimicrobial compounds that could be incorporated to contact lenses to combat pseudomonal keratitis.

A remarkable result of this work is that partial modification of amino-terminated PAMAM with PEG does not reduce toxicity to Gram-negative bacteria, while it does greatly reduce toxicity to epithelial cells. To rationalize this observation, we note that the polycationic PAMAM molecules prefer to bind to bacteria cells that carry a higher density of negative charges on their surfaces than the eukaryotic cells. Following the initial electrostatic interaction, further interactions, including hydrophobic interactions between the dendrimers and cell membrane, are likely necessary to cause cell lysis, since it has been shown that surfaces presenting a high density of amino groups have no appreciable effect on the membrane of the attached bacteria.[28] Both the polypeptide branches of PAMAM and the PEG side chains on PEG–PAMAM possess a dual hydrophilic–hydrophobic characteristic, thus they might be able to disrupt the membranes at a high local concentration. To test this hypothesis, a systematic study of the antibacterial activities of a series of G3–G7 PEG–PAMAM dendrimers with varied coverages and chain lengths of PEG is currently under way in our laboratories.

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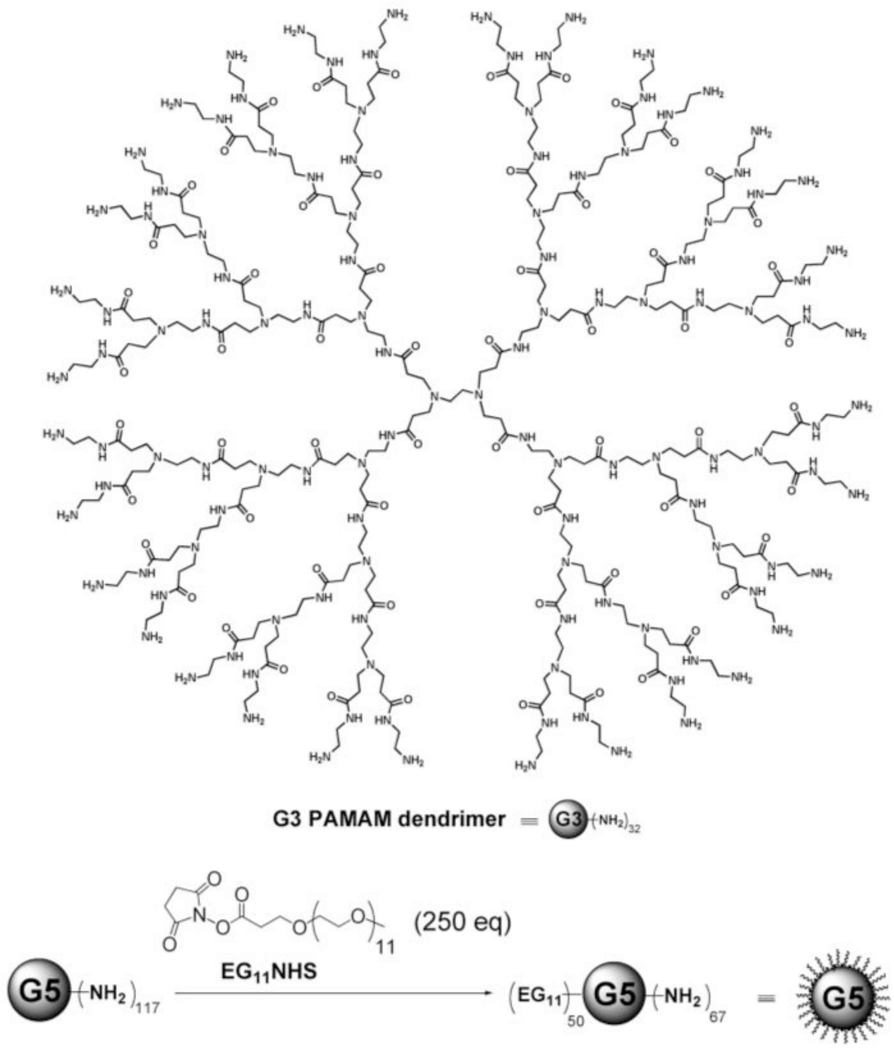

Figure 1.

Structure of the G3 PAMAM dendrimer possessing ~32 amino groups at the periphery, and the reaction used in the present study for modifying the commercial G5 PAMAM dendrimer, possessing ~117 amino groups at its periphery, with PEG consisting of 11 ethylene glycol units (EG_{11}).

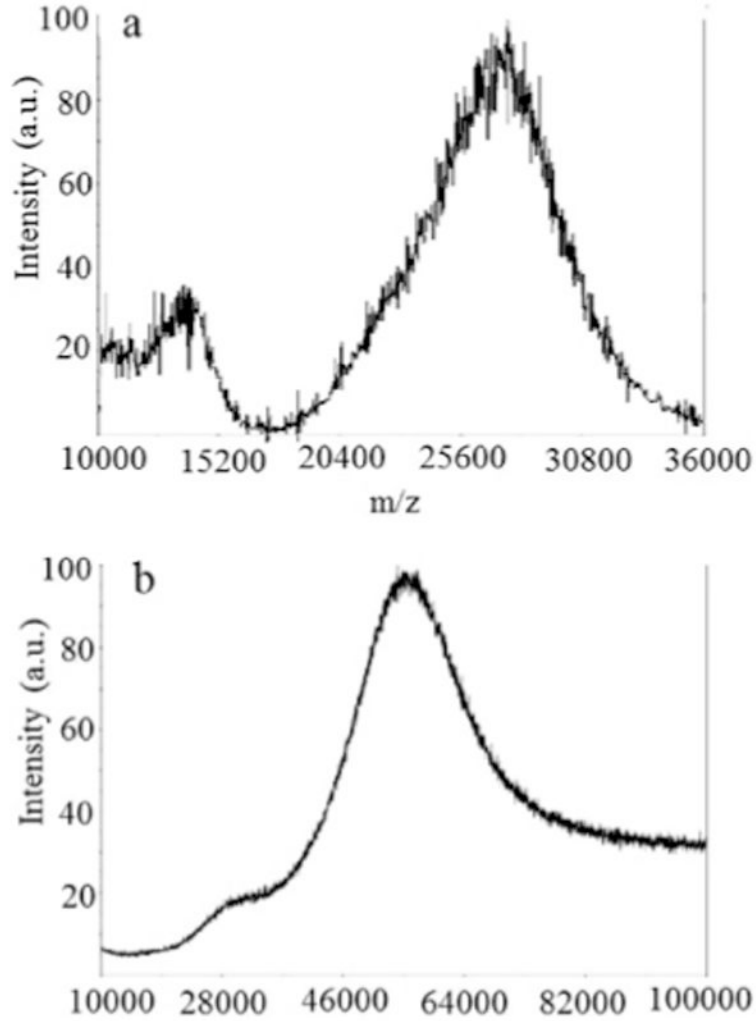

Figure 2.
MALDI-TOF mass spectra of the G5 PAMAM (a) and PEG–PAMAM (b), showing their average MWs to be 26 575 and 55 249 Da. The peak at ~13 290 (a) and the shoulder at ~27 600 Da/charge (b) are likely from the double-charged molecular ions.

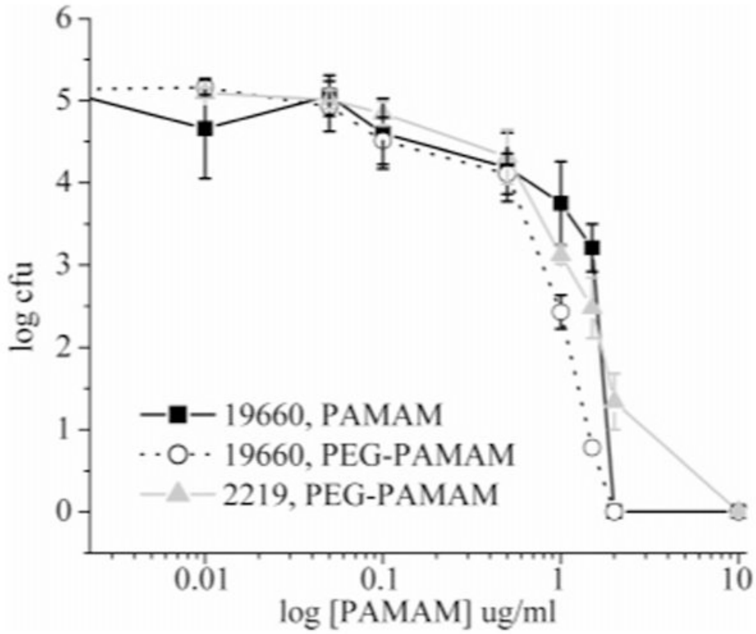

Figure 3.
Concentration (cfu/mL) of PA, including PA19960 (lab strain) and PA2219 (clinical strain), upon incubation with PAMAM and PEG–PAMAM at various concentrations for 2 h. The data points are the mean of at least three separate experiments, and the error bar represents the standard deviation.

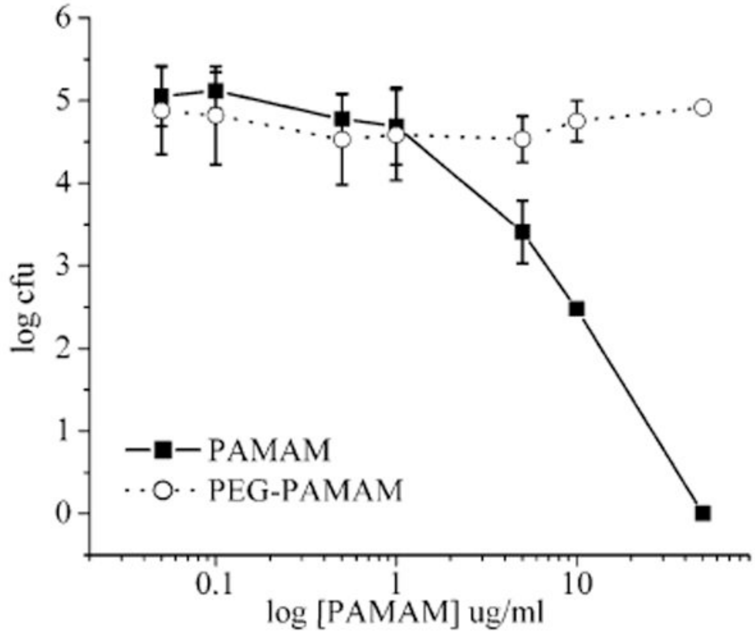

Figure 4.
Concentration (cfu/mL) of bacteria SA ATCC 29213 upon incubation with G5 PAMAM and 43% PEG-coated PAMAM (PEG–PAMAM) at various concentrations for 2 h. The data points are the mean of at least three separate experiments, and the error bar represents the standard deviation.

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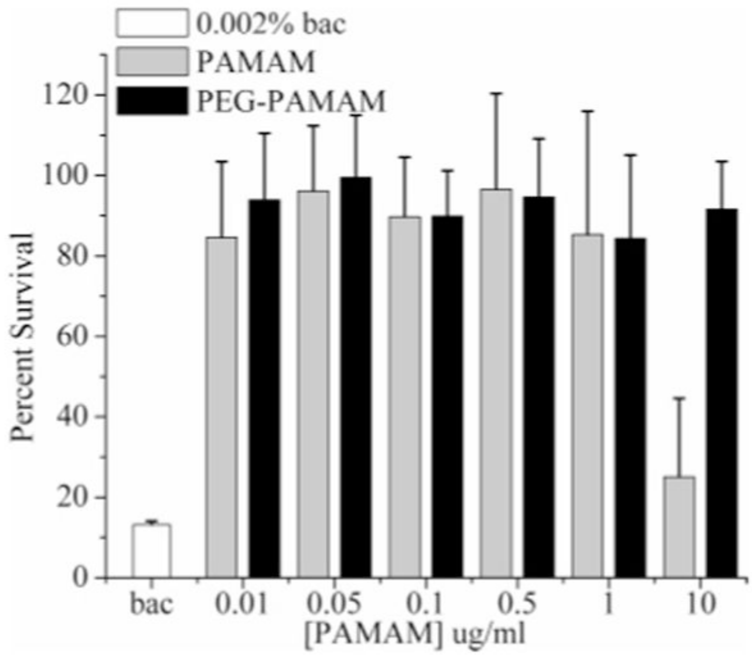

Figure 5.
PAMAM cytotoxicity to HCECs measured by MTT survival assay with 0.002% benzalkonium chloride (bac) as the positive control. Percent survival of HCECs upon treatment with PAMAM and PEG–PAMAM at various concentrations is based on an untreated control. The data show the mean from two separate experiments with four replicates per condition, and the error bar represents a standard deviation.