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Exploiting dendrimer multivalency to combat emerging and re-emerging infectious diseases

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Abstract

The emergence and re-emergence of bacterial strains that are resistant to current antibiotics reveals the clinical need for new agents that possess broad-spectrum antibacterial activity. Furthermore, bacteriophobic coatings that repel bacteria are important for medical devices, as the lifetime, reliability, and performance of implant devices are hindered by bacterial adhesion and infection. Dendrimers, a specific class of monodisperse macromolecules, have recently shown potential to function as both antibacterial agents as well as antimicrobial surface coatings. This review discusses the limitations with currently used antibacterial agents and describes how various classes of dendrimers, including glycodendrimers, cationic dendrimers, anionic dendrimers, and peptide dendrimers, have the potential to improve upon or replace certain antibiotics. Furthermore, the unexplored areas in this field of research will be mentioned to present opportunities for additional studies regarding the use of dendrimers as antimicrobial agents.

Keywords

Dendrimer; dendritic polymers; bacteria; antimicrobial; infection; antibacterial agents; coatings; carriers; drug delivery

Introduction

A disturbing trend in recent years is the growth of resistant strains of bacteria with the simultaneous dearth of new antimicrobial agents.[1, 2] Few new antimicrobial agents have been developed in the recent past, and those that have been approved, including Cubicin® (injectable daptomycin), Zyvox® (linezolid) and Dificid™ (fidaxomicin), specifically target Gram-positive bacteria. Therefore, there is a need for both new classes of antibiotics that are effective against current resistant strains and new classes that possess broad-spectrum antibacterial activity. Furthermore, bacteriophobic coatings that repel bacteria are highly desirable for medical devices because the lifetime, reliability, and performance of many medical implants are hindered by bacterial adhesion and infection.[3- 6] This review will discuss how dendrimers, a specific class of macromolecules, have recently shown potential to function as both antibacterial agents as well as antimicrobial surface coatings.

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Dendrimers are well-defined, single molecular weight, globular structures 3 to 7 nanometers in diameter containing a central core, branching layers, and numerous end groups.[7, 8] Generally either divergent[9-12] or convergent[13, 14] routes are used to synthesize dendrimer structures, but recent improvements to synthetic methodology have made large-scale production of many of these structures more viable.[15] Since the branching structure of dendrimers was first conceptualized in the early 1970s[16] and synthesized in the mid-1980s,[9, 11] dendrimers, especially poly(amido amine) (PAMAM),[11] polypropylenimine (PPI),[16, 17] and dendritic polylysine structures,[18] have been actively investigated for a wide range of industrial[19-22] and biomedical applications.[23-29] The potential for using dendrimers as antimicrobial agents, both as a drug and as a surface coating, has been recognized over the last decade. In this review, the major concerns with current antimicrobial agents will be discussed, and the various classes of dendrimers that have shown potential to overcome emerging and/or re-emerging infectious diseases will be summarized. While a similar review describing several dendrimer skeletons used as antimicrobial agents has recently been published,[30] this review will instead focus on how dendrimer surface groups affect antimicrobial activity and how these structures can inhibit biofilm formation.

Limitations of current antimicrobial agents

Prior to the development of antibiotics, infection was the leading cause of hospitalization and mortality in the US. However from 1937 to 1953, the rate of infectious diseaserelated mortality decreased, a trend accredited to the first clinical uses of antibiotics, including sulfonamides (1935), β-lactams (1941), and aminoglycosides (1943).[31] Alarmingly, however, this trend was reversed beginning in the 1980s, and for the next 15 years the number of deaths due to infectious diseases increased. While much of this increase was related to acquired immunodeficiency syndrome (AIDS) mortality, the incidence of re-emerging infectious diseases, particularly tuberculosis, increased by more than 20% as well.[32] Moreover, infectious disease is responsible for the greatest number of deaths in developing nations. This spike in emerging and re-emerging bacteria-related deaths emphasizes the continuous evolution of infectious diseases and stresses the limitations of current antimicrobial agents.

Nearly all currently used antibacterial agents can be classified into one of the following categories: (1) agents that target bacterial cell wall biosynthesis, which include β-lactams and vancomycin; (2) agents that target bacterial protein synthesis, which include erythromycin, tetracyclines, aminoglycosides, and oxazolidinones; and (3) agents that target bacterial DNA replication and repair, which include fluoroquinolones.[33] Despite the wide variety of bacterial drug targets, clinically significant resistance is usually observed within months to several years of introduction into the clinic.[34] Furthermore, the ease with which bacteria can collect and exchange antibiotic-resistant genes between other cells and even other species makes the emergence of drug resistance even more troubling.[35] The resistance strategies that bacteria have developed include: (1) effluxing the antibiotic from the bacterial cell; (2) acquiring enzymes capable of deactivating the antibiotic; and (3) altering the target structure of the antibiotic on the bacteria cell surface.[33] Finally, the traditional developers of antimicrobial agents, large pharmaceutical companies, have largely left this space. Consequently, the development of new agents that can target bacteria through alternate strategies is vital. Fortunately, various classes of dendrimers that can target or inhibit virulence factors critical for establishment of microbial pathogens in the host have been designed. Thus, dendrimers have the ability to *prevent* infection. Furthermore, dendrimers have been described which can act either as *antimicrobial agents*, drug-delivery *devices*, or bacteriophobic *coatings*, thus these compounds have the potential to serve multiple, distinct roles in the development of novel antimicrobial strategies.

Types of antimicrobial dendrimers

Glycodendrimers—Interfering with adhesion to eukaryotic cells

The earliest events in host-pathogen interactions involve the microbe securing itself to the host. Bacteria can attach to eukaryotic cells via specific carbohydrate-protein interactions that primarily involve noncovalent binding between bacterial cell surface proteins and eukaryotic glycoproteins or glycolipids.[36] Furthermore, certain bacteria produce secreted virulence factors known as "AB_5 toxins". The A component of the toxin is the catalytic domain, while the B component presents carbohydrate recognition sites to interact with sugars on the surface of eukaryotic cells and facilitate toxin transport into the host cell.[37] By blocking this interaction, bacterial cellular uptake into eukaryotic cells is inhibited (Figure 1). However, because carbohydrate-protein interactions are weak, multivalency is necessary to achieve efficient recognition and binding. By appending carbohydrates on the surface of dendrimers to afford glycodendrimer structures,[38] such multivalency can be achieved to improve antimicrobial activity.[39, 40]

Thompson and Schengrund designed three glycodendrimers based on first- and second-generation polypropylenimine (PPI) and first-generation polyamidoamine (PAMAM) dendrimers to inhibit the activity of cholera toxin and the heat-labile enterotoxin of *E. coli*, both members of the AB_5 toxin family described above (Figure 2).[41] Cholera toxin is a protein secreted by *Vibrio cholerae* that, upon entry into epithelial cells lining the intestine, causes diarrhea, massive dehydration, and possible death of the affected individual if left untreated.[42] The heat-labile enterotoxin of *E. coli* is a structurally similar protein to cholera toxin that, upon exposure to cells in the intestine, causes traveler's diarrhea.[43] The binding site for both toxins is GM1, a ganglioside present on the surface of eukaryotic cells.

Three different dendrimers were conjugated with phenylisothiocyanate derivatized (PITC) galβ1-3galNAcβ1-4[sialic acid α2-3]-galβ1-4glc (oligo-GM1) carbohydrate moieties (Figure 2). The ability of these glycodendrimers to inhibit the binding of both ^{125}I-labeled cholera toxin B subunit and ^{125}I-labeled heat-labile enterotoxin to GM1-coated wells was measured. The results showed that the glycodendrimers were able to inhibit the binding of both proteins to the GM1-coated wells at concentrations 5- to 15-fold lower than was achieved with pre-incubation of the toxins with native GM1. Furthermore, the glycodendrimers inhibited binding at concentrations over 1000-fold lower than was achieved with free oligo-GM1, stressing the importance of dendrimer multivalency.[41]

The following year, Thompson and Schengrund evaluated the activity of the second-generation, oligo-GM1-linked PPI dendrimer to inhibit the binding of cholera toxin and heat-labile enterotoxin to a GM1-treated murine fibroblast cell line (NCTC-2071).[44] The results showed a significant reduction in the adherence of ^{125}I-labeled cholera toxin and ^{125}I-labeled heat-labile enterotoxin to the GM1-treated cells, regardless of whether the toxins were exposed to the cells after the addition of the glycodendrimers or were pre-incubated with glycodendrimer prior to addition to cells. Furthermore, the oligo-GM1-linked dendrimer had no effect on cell viability, suggesting that these glycodendrimer structures can function as effective ligands to inhibit the binding of bacterial toxins.

More recently, Pieters, *et al*. have investigated the affinity of carbohydrateconjugated dendrimers based on the 3,5-di-(2-aminoethoxy)benzoic acid repeat unit for the cholera toxin B subunit. Initially the group conjugated first-, second-, and third-generation dendrimers with 2, 4, or 8 lactose groups, respectively, using thiourea linkages (Figure 3).[45] Using a direct fluorescence binding assay, the group observed apparent dissociation constants (K_d values) of 235, 99, and 33 μM for the divalent, tetravalent, and octavalent glycodendrimers, respectively. The modest multivalency effect in this study was related to

the better ability of the higher generation glycodendrimers to bridge the binding sites between toxin molecules compared to the first-generation analogue.

To further improve the inhibitory activity of their glycodendrimer against cholera toxin, Visser, Zuilhof, Pieters, *et al.* synthesized similar structural analogues optimized by two changes: (1) lactose was replaced with the authentic GM1 oligosaccharide ligand, which was linked to the dendrimer using "click" chemistry; and (2) elongated spacer arms with optimal lengths were used to append the carbohydrate moieties to the dendrimers (Figure 3).[46] The ability of these oligo-GM1-linked dendrimers to inhibit the binding of a horseradish peroxidase (HRP)-conjugated cholera toxin B subunit to GM1 ganglioside coated on a 96-well plate was measured using an ELISA assay. The oligo-GM1-linked dendrimers showed unprecedented binding that was up to 380,000-fold stronger than what was observed using the monovalent analogue. Recently, this same trend was reported by Zuilhof and Turnbull, *et al.*, who used the same oligo-GM1-linked dendrimers to inhibit the binding of the B-pentamer of *E. coli* heat-labile enterotoxin.[47] The IC_{50} values for the heat-labile enterotoxin were higher than those measured for cholera toxin since the values were detected using a less-sensitive indirect ELISA assay. However, the *relative* inhibitory activities of the glycodendrimers were similar against both toxins.

Despite the high activity of the oligo-GM1-linked dendrimer structures, Pieters *et al.* pointed out that the ligand is complex and difficult to synthesize on a large scale. Consequently, the group investigated whether binding inhibition of cholera toxin could be achieved using dendrimers linked to simple galactose residues modified with poly(ethylene glycol), thus mimicking the additional sugars of the GM1 oligosaccharide structure, and a lipophilic chain to more closely resemble the structure of the oligo-GM1-linked dendrimers.[48] Inhibitory potencies against cholera toxin of 130, 25, and 12 μM were measured using the divalent, tetravalent, and octavalent galactose-conjugated dendrimers. While these IC_{50} values are only as high as the monovalent oligo-GM1 analogue, the results stress the possibility of designing lower cost ligands (using simple galactose rather than more complicated oligo-GM1) for cholera toxin that still maintain high inhibitory activity.

In addition to using glycodendrimers to target and block the binding of bacterial toxins to eukaryotic cells, groups have also investigated the use of glycodendrimers to inhibit the binding of bacterial cells to human erythrocytes (Figure 1). Pieters, *et al.* synthesized mono-, di-, and tetravalent galabiose (Galα1-4Gal) glycodendrimers based on 3,5-di-(2-aminoethoxy)benzoic acid, as well as an octavalent galabiose glycodendrimer based on G1 PAMAM.[49] The ability of these compounds to inhibit the binding of *Streptococcus suis*, one of the causative agent of meningitis in humans,[50] to human erythrocytes was evaluated in a hemagglutination assay. Results showed that the minimum inhibitory concentration (MIC) for binding of the monovalent glycodendrimer was 900 and 1600 nM against *S. suis* 628 and *S. suis* 836, respectively. By increasing valency to di-, tetra-, and octavalent analogues, the MIC was reduced to 6, 2, and 0.3 nM, respectively. Changes in length, rigidity, or orientation of the dendritic arms did not significantly impact the inhibitory efficacy of the glycodendrimers. These same glycodendrimers, as well as an octavalent 3,5-di-(2-aminoethoxy)benzoic acid dendritic analogue, were also evaluated as agents for blocking the binding of *E. coli* expressing $PapG_{J96}$ to human erythrocytes.[51] PapG adhesin is involved in the establishment of *E. coli* urinary tract infections. The relative potency per carbohydrate residue increased slightly as valency of the glycodendrimer was increased. Conversely, when the carbohydrates on these glycodendrimers, as well as a G2 PAMAM analogue, were replaced with mannose and evaluated for their ability to inhibit the binding of recombinant type I (mannose-specific) fimbriated *E. coli* to a human urinary bladder epithelim (T24) cell line, the multivalency effect was not always observed.[52] While the mono-, di-, and tetravalent glycodendrimers showed reduced IC_{50} values with increased multivalency (IC_{50}

= 337, 204, and 51 μM, respectively), the octavalent analogue showed decreased activity (IC_{50} = 72 μM). A similar trend was previously observed for thiourea-linked mannose glycodendrimers based on PAMAM. In an earlier study conducted by Lindhorst *et al.*, no improvements in binding affinity for type I fimbriated *E. coli* was observed for valencies higher than three, possibly indicating that for this particular binding domain, larger glycoclusters are not accommodated by the binding site.[53]

In addition to using glycodendrimers to target and block the binding of bacteria to eukaryotic cells, groups have also investigated the use of glycodendrimers to inhibit the formation of biofilms. Reymond, *et al.* synthesized a library of 15,625 glycopeptides dendrimers conjugated at the surface with α-C-fucosyl residues.[54] Fucose groups were chosen as ligands because they were previously identified as the target carbohydrate moieties for the lectin LecB, a protein on the surface of *Pseudomonas aeruginosa* that plays a role in bacterial recognition and attachment to eukaryotic cells.[55] *P. aeruginosa* can cause mortality-related infections, particularly in immunocompromised and cystic fibrosis patients, often exhibiting antibiotic resistance that is partially due to biofilm formation.[56] Because *P. aeruginosa* mutants that lack LecB show impaired biofilm formation,[57] blocking this protein with fucose residues should reduce biofilm-associated antibiotic resistance.

Based on binding studies using the initial combinatorial library and LecB, Reymond *et al.* identified a tetravalent glycopeptide dendrimer FD2 (fuc-α-CH_2CO-Lys-Pro-Leu)$_4$(Lys-Phe-Lys-Ile)$_2$Lys-His-Ile-NH_2 that showed high potency (IC_{50} = 0.14 μM) (Figure 4).[54] The group later evaluated the ability of FD2 to inhibit or disperse *P. aeruginosa* biofilms using a steel coupon assay.[58] FD2 showed complete inhibition of biofilm formation at a concentration of 50 μM (IC_{50} = 10 μM) and caused complete dispersion of established biofilms for both the wild-type strain and clinical isolates. Furthermore, the glycopeptides dendrimers showed no cytotoxicity against human kidney-embryonic cells (293T), suggesting that these structures may be relevant in the treatment of *P. aeruginosa* biofilm-based infections in humans. More recently, Reymond *et al.* altered the structure of FD2 to contain D- instead of L-amino acids; while the binding of this compound (D-FD2) to LecB was lower than that observed with the original glycopeptides dendrimer, its ability to inhibit *P. aeruginosa* biofilm formation was retained, and the new analogue showed complete resistance to proteolysis, indicating higher stability.[59]

Cationic dendrimers: Targeting bacterial membranes

In general, the antimicrobial action of many agents can be attributed to cell membrane disruption and permeation; polycationic compounds, and more specifically dendrimers, are particularly adept at promoting such cell permeation (Figure 5).[60, 61] In 1999, Cooper *et al.* quaternized the surface amines of polypropylenimine (PPI) dendrimers by reacting the amines with 2-chloroethyl isocyanate and then treating the intermediate with dimethyldodecylamine to yield the quaternized analogue.[62] The antimicrobial activity of the amphiphilic quaternized dendrimer against the recombinant *E. coli* strain TV 1048 was measured using a bioluminescence assay and compared to the activity of *n*-dodecyltrimethylammonium chloride (DTAC), the small molecule analogue. The concentration of the compound that causes 50% reduction in bioluminescence (EC_{50} value) of the dendrimer was 12 μg/mL while that of the small molecule counterpart was 2000 μg/mL, making the dendrimer over 100-fold more active against *E. coli*, stressing the influence of multivalency.

To further probe the antimicrobial activity of PPI dendrimers, Cooper *et al.* modified various aspects of the dendrimer, including the generation number and the hydrophobic chain length, and evaluated the effect of these changes on the antimicrobial activity against *E. coli*.[63] The effect of generation number on antimicrobial activity showed that G5 > G4 > G1

> G2 > G3. This effect was attributed to the balance between the higher potency achieved with a greater number of quaternary amines and the decreased permeability of the larger dendrimer analogues. The effect of the hydrophobic chain length on antimicrobial activity showed that $C_{10} > C_8 > C_{12} > C_{14} \approx C_{16}$. This trend was attributed to the dual binding site theory, which suggests that on the bacterial cell surface, dual binding sites exist for which the relative binding activities of long and short hydrophobic ligands differ. When the antimicrobial activity of one of the dendrimers was compared to that of a hyperbranched polymer with the same number of functional groups, the dendrimer was significantly more potent.

However, it should be noted that the higher activity of dendrimers as compared to hyperbranched analogues is not always observed. For instance, Gomez and de la Mata *et al.* have synthesized first- and second-generation ammonium-functionalized carbosilane dendrimers by reacting chloromethylene-terminated dendrimers with 3-dimethylaminophenol followed by treatment with methyl iodide (Figure 6A); the resulting ammonium-functionalized dendrimers were evaluated against *S. aureus* and *E. coli*.[64] Both dendrimers had lower MIC values than the monofunctional counterpart, although the first-generation carbosilane dendrimer was more active than the second-generation structure, particularly against *E. coli* (MIC = 64 mg/L and 4 mg/mL for G2 and G1 dendrimers, respectively). These dendrimers necessitated the use of DMSO as a solubilizing agent, thus the groups later modified the synthesis to involve hydrosilylation of *N,N*-dimethyl-*N'*-allyl-*N'*-ethyl-ethylenediamine with hydride-terminated carbosilane dendrimers (G1-G3) followed by quaternization with HCl or MeI (Figure 6B).[65] Based on measurements of minimum bactericidal concentration (MBC), these water-soluble dendrimers showed increasing activity with higher generation number against both *E. coli* (MBC = 1.65, 1.70, and 3.65 for G3, G2, and G1, respectively) and *S. aureus* (MBC = 0.82, 0.85, and 1.82 for G3, G2, and G1, respectively) strains.

Recently, however, the groups compared the antimicrobial activity of cationic hyperbranched polycarbosilane (PCS 6) with the antimicrobial activity of ammonium-functionalized second- and third-generation carbosilane dendrimers containing quaternized 3-dimethylaminophenol at the periphery.[66] PCS 6 showed higher activity than the third-generation carbosilane dendrimer, which had similar molecular weight and ζ potential, and similar activity to the second-generation structure. Consequently, the hyperbranched structures, which were synthesized in less time and at lower cost than the dendrimer analogues, may offer an appealing alternative that can still achieve suitable antimicrobial activity.

Surprisingly, despite the fact that PAMAM dendrimers were one of the earliest of this class of structures to be synthesized and extensively studied for various biomedical applications, their activity as a cationic antimicrobial agent has only recently been evaluated. In some of the earliest studies, PAMAM was not evaluated as the actual antimicrobial *agent* but rather as an antimicrobial *carrier*. Yang and Lopina described the covalent surface conjugation of Penicillin V to G2.5- and G3-PEG-PAMAM star polymers using amide and ester linkages.[67] While the ester-linked conjugate had activity against *S. aureas*, confirming that the drug was bioavailable through ester hydrolysis, the dendrimer-drug conjugate did not show higher activity than the drug alone, indicating no synergistic effect. The delivery of antibiotics through noncovalent encapsulation in dendrimers has also been described. Cheng *et al.* reported the use of G4-PAMAM dendrimers to solublize the quinolone antimicrobials nadifloxacin and prulifloxacin.[68] The poor solubility of these quinolones prevents their formulation in liquid and topical dosage forms. The authors observed similar or increased potency against *E. coli* for both quinolone-encapsulating dendrimers.

In addition to functioning as a drug *carrier*, several studies have evaluated PAMAM dendrimers as the actual antimicrobial *agent*. Cai *et al*. investigated the antimicrobial activity of G5 PAMAM dendrimers with or without partial poly(ethylene glycol) (PEG) coatings against standard laboratory strains of *P. aeruginosa* and *S. aureus*, as well as a clinical strain of *P. aeruginosa* obtained from a patient with keratitis.[69] Results showed that PAMAM and PEG-PAMAM were inactive against *S. aureus*, but these structures afforded EC_{50} values of 1.5 and 0.9 μg/mL, respectively, against *P. aeruginosa*. While the clinical strain PA2219 showed increased resistance against PEG-PAMAM (EC_{50} = 1.4 μg/mL), the activity of the dendrimer was equally as effective as the antimicrobial peptide cathelicidin (LL-37; EC_{50} = 1.9 μg/mL). Furthermore, both PAMAM and PEG-PAMAM showed lower cytotoxicity against human corneal epithelial cells (HCEC) compared to *P. aeruginosa*.

Cai *et al*. later discovered that by using a G3 PAMAM dendrimer (rather than G5) in which 6% of the surface amines were coated with PEG, the activity against *P. aeruginosa* could be maintained (MIC = 25 μg/mL) while the cytotoxic effects to HCEC could be significantly reduced.[70] To address the problem of implant-associated infection, the authors explored the use of the G5 analog. The G5 PEG-PAMAM structures prevented the colonization of *P. aeruginosa* and *S. aureus* on titanium-based substrates, even after storage of the dendrimer-titanium samples in PBS for 30 days. Furthermore, these G5 PEG-PAMAM-coated titanium substrates did not inhibit cell adhesion or cause cytotoxicity to human bone mesenchymal stem cells (hMSCs), indicating the potential importance of these compounds in preventing implant-associated infections.[71]

Recently, Kannan and Kannan, *et al*. have investigated the inhibitory activity of PAMAM dendrimers in a guinea pig model of chorioamnionitis, an inflammatory response to intrauterine infection that can cause fetal brain injury.[72] For the *in vivo* portion of this study, hydroxyl-terminated G4 PAMAM dendrimers were injected into the cervix of pregnant guinea pigs after *E. coli* inoculation; forty-eight hours later the animals were euthanized, and the amniotic fluid was evaluated for the presence of bacteria.[73] Of the guinea pigs that were inoculated with *E. coli* and that received no treatment, bacterial growth was observed in 57% of the amniotic fluid samples. However, of the animals that were treated with G4 PAMAM dendrimer, no bacterial growth was observed in any of the amniotic samples, indicating that the hydroxyl-terminated G4 PAMAM dendrimer prevented *E. coli* from ascending into the uterus. Furthermore, cytokine levels (tumor necrosis factor [TNFα], IL-6 and IL-1β) in the treated animals were similar to animals with no infection, indicating minimal immune response to treatment, while the animals that were infected and left untreated showed significantly higher levels of both groups of cytokines.

In a similar study, Kannan *et al*. also demonstrated that G4-PAMAM dendrimers encaspsulating amoxicillin with peripheral thiopyridyl groups could be crosslinked via disulfide bonds with thiol-terminated 8-arm PEG polymers to form injectable hydrogels.[74] These gels have the potential to afford antimicrobial activity resulting from the sustained release of amoxicillin from the hydrogel. *In vivo* studies carried out in a pregnant guinea pig model confirmed that hydrogels applied to the cervicovaginal region had a residence time greater than 72 h and did not show toxicity to either the guinea pigs or the fetus (Figure 7). While the *in vitro* studies showed sustained drug release for up to 240 h, further evaluation of both the antimicrobial activity as well as the *in vivo* drug release are underway.

Similar to the encapsulation study conducted by Kannan *et al*., Hammond and coworkers have used a G4-PPO-PAMAM dendrimer as the cationic component, along with negatively charged poly(acrylic acid), of a layer-by-layer device for the slow release of triclosan.[75] The group found that the dendrimer was more effective at encapsulating triclosan as compared to a nondendritic surfactant with similar properties. The release of triclosan could be extended

over 20 days, and a zone of inhibition assay showed killing of *S. aureus*. Consequently, the use of dendrimers as antimicrobial drug carrying devices warrants additional research.

Anionic dendrimers: Imitating detergent activity

Grinstaff and group reported that dendritic amphiphiles synthesized from succinic acid, glycerol, myristal alcohol, and myristic acid inhibited the growth of *B. subtilis* in culture at mM concentrations.[76] An amphiphile with a single hydrophobic strand showed toxicity towards cultured HUVEC cells, but an amphiphile with two hydrophobic chains was relatively non-cytotoxic. Recently variations have been made to the length of the hydrophobic chains to see if improvements to the antimicrobial activity of these dendritic amphiphiles could be realized. Unfortunately, increasing the length of the hydrophobic chains did not improve the dendrimers' antimicrobial activities against clinically relevant bacterial strains.[77] In a similar study starting from a poly(oxypropylene) triamine (Jeffamine®) core, Tulu *et al.* synthesized anionic and cationic PAMAM dendrimers that displayed antimicrobial activity against both Gram-positive and Gram-negative bacteria and a selection of fungi.[78] The anionic dendrimers showed comparable activity to gentamycin (antibiotic) and nystatin (antifungal), but in general the cationic dendrimers were more effective against both bacteria and fungi (Table 1).

Peptide-based dendrimers: Mimicking antimicrobial peptides

Antimicrobial peptides, generally composed of short sequences of cationic and hydrophobic amino acids, participate in innate immune response by killing bacteria through binding to and permeabilizing the bacteria cell membrane.[79] As a specific class of dendrimers first synthesized in the 1980s,[80] dendrimeric peptides offer a potential structural scaffold to mimic the activity of antimicrobial peptides. Tam *et al.* synthesized tetravalent (D4) and octavalent (D8) polylysine dendrimer cores and decorated the surface of the cores with either R4 tetrapeptides (Arg-Leu-Tyr-Arg) or R8 octapeptides (Arg-Leu-Tyr-Arg-Lys- Val-Tyr-Gly) (Figure 8).[81] These structures were evaluated for antimicrobial activity against several Gram-negative and Gram-positive bacteria strains. Regardless of the peptide sequence length (R4 versus R8), the tetravalent dendrimers showed improved broad-spectrum antimicrobial activity as compared to the divalent analogues. While increasing the dendrimer branching to the octavalent analogue only improved activity slightly compared to the tetravalent structures, the R8 octavalent dendrimer showed significantly better activity under high-salt conditions (i.e. 100 mM NaCl). Furthermore, peptide dendrimers displaying either the R4 or R8 peptides showed low hemolytic activity against human erythrocytes, indicating low cytotoxicity.

More recently, Pini *et al.* used phage display library selection to identify a peptide sequence (Gln-Lys-Lys-Ile-Arg-Val-Arg-Leu-Ser-Ala) that was subsequently appended to a tetravalent polylysine dendrimer core.[82] The peptide dendrimer structure showed high activity against a range of bacterial strains (particularly Gram-negative), low cytotoxicity towards human keratinocyte (HaCaT) cells, low hemolytic activity towards human erythrocytes, and good stability in human plasma and serum (Table 2). More recently, the structure of the peptide dendrimer was modified to include a lipophilic amino valeric acid chain to enhance membrane affinity and pyroglutamic acid (rather than glutamine) at the N-terminus to avoid cyclization.[83] The new peptide dendrimer showed higher antimicrobial activity against Gram-negative strains and better stability in solution when compared to the original peptide dendrimer.

With high activity shown for a number of different peptide dendrimers, it is reasonable to postulate if structures with shorter peptide lengths can still exhibit antibacterial properties. Kallenbach *et al.* synthesized a tetravalent dendrimeric peptide using a lysine dendrimer core

and appending Trp-Arg dipeptides to the surface.[84] The peptide dendrimer displayed activity against both ampicillin- and streptomycin-resistant *E. coli* (MIC_{50} = 4.5 μg/mL) and multidrug resistant *S. aureus* (MIC_{50} = 16 μg/mL), while showing low hemolytic activity. Furthermore, the tetravalent peptide dendrimer was more effective as an antimicrobial agent and less cytotoxic than linear brush-like peptide or polymeric structures conjugated to the Trp-Arg peptide sequence. The dendrimer also elicited lower levels of resistance compared to traditional antibiotics, including ciprofloxacin, vancomycin, chlorohexidine, and gentamicin.[85]

Kallenbach *et al.* also assessed the tetravalent peptide dendrimer for antimicrobial activity against *E. coli* RP437 biofilms.[86] The initial results showed that at a concentration of 40 μM, the peptide dendrimer reduced the planktonic growth rate of *E. coli* by 33.5% and inhibited biofilm formation in 96-well plates by 93.5%. Ren *et al.* showed that, at low concentrations, the same peptide dendrimer is effective in killing *E. coli* persister cells, strains of bacteria that are tolerant to antibiotic stress even over long treatment periods.[87] Furthermore, at a concentration of 80 μM, the tetravalent peptide dendrimer reduced the number of viable *E. coli* biofilm cells by 99.3%, and caused the persister cells of the biofilm to become highly sensitive to ofloxacin. Consequently, the synergistic effect between peptide dendrimers and antibiotics is a topic that warrants further investigation.

Surface Adsorbed Dendrimers: Coating metal implants to inhibit biofilm formation

In an alternative route to combating biofilm formation, Grinstaff *et al.* appended titanium-binding peptides (TBP; SKHKGGKHKGGKHKGSSG) to the surface of mono-, di-, and tetravalent lysine dendrimer cores with a single PEG chain appended to a cysteine residue at the C-terminus of the lysine dendrimer (Figure 9).[88] These studies built off of an earlier report with a coating composed of a mono-TBP-PEG that exhibited inhibition of bacteria biofilms on surfaces, but the coating possessed limited stability.[89] The tetravalent analogue showed high coating performance on titanium beads, with 90% of the coating remaining after the beads were shaken in serum media for 2 weeks compared to 55% and 5% for the di- and mono analogs. Next, the three coatings were evaluated in a series of *in vitro S. aureus* biofilm assays to determine the effect of multivalency on coating performance. *S. aureus* is one of the most common causes of hospital-acquired infections and post-surgical wound infections and is often transferred to the implanted devices during handling.[90-92] For these studies, Ti-coated slides were used as model substratum and a pathogenic strain of *S. aureus* (MZ100) was added to the wells at a concentration of ~5×10^7 CFUs/well, which is ≈ 10,000 times greater than that present on human skin. The tetravalent TBP-PEG dendrimer formed a serum-resistant layer that out performed the mono- and di- analogs, and afforded a 90% reduction in *S. aureus* biofilm formation as compared to the uncoated titanium control beads (Figure 9). These results indicate the potential for these macromolecule structures to inhibit *in vivo* orthopedic implant infections.

Dendrimer-metal conjugates: Enhancing the effects of antimicrobial elements

Silver in its ionized form (Ag^+) is well-established as a broad-spectrum antimicrobial agent.[93] The mechanism of action is believed to involve disruption of protein structure through interaction with thiols, but the mechanism of action is not fully agreed upon. In 2001, Hagnauer and McManus *et al.* demonstrated that PAMAM dendrimers with tris(2-hydroxymethyl)-amidomethane (TRIS) or carboxylate termini could be used to complex silver ions, which upon exposure to light resulted in dendrimer conjugates containing silver nanoparticles (AgNPs).[94] The materials showed activity comparable to silver nitrate solutions against *S. aureus, P. aeruginosa*, and *E. coli*, with improved solubility and stability. Murugan and Vimala reported the use of a three component system containing multiwalled carbon nanotubes (MWCNTs), an amphiphillic poly(propyleneimine) dendrimer (APPI),

and AgNPs.[95] Covalent conjugation of APPI to the MWCNTs increased the bacteriocidal activity against *B. subtilis, S. aureus*, and *E. coli* as compared to unfunctionalized MWCNTs. The addition of AgNPs also increased the activity. Saha *et al*. prepared and investigated the antibacterial properties of Zn/Te/dendrimer nanocomposites (ZnTe DNCs).[96] They found that the ZnTe DNCs were active against clinical isolates of the Gram (-) bacteria *V. cholerae* and *E. coli*, but were inactive against Gram (+) bacteria. In addition, they demonstrated that the ZnTe DNC were non-toxic to human erythrocytes at 8-times the MBC, whereas bulk ZnTe is cytotoxic.

Other organic polymer and supramolecular antimicrobial systems

The dendrimers described in this review are just one class of organic nanostructures capable of functioning as antimicrobial agents. Other classes of nanostructures include polymers and liposomes. Because these structures have been actively investigated for decades, they are further developed, with examples in clinical use. For instance, the number of different linear polymer systems that display antimicrobial activity is large and includes polynorbornene[97-99] and polyacrylate[100-102] derivatives, poly(arylamide),[103] poly(β-lactam)[104] and pyridinium copolymers.[105] These structures, many of them cationic, mimic antimicrobial peptides but can be synthesized more easily, scaled up readily, and produced at low cost. Moreover, the polymers that possess an amphiphilic structure exhibit greater cytotoxicity towards bacteria over erythrocytes.[105] Micro- and nanoparticles can also be formulated from polymers to function as antibiotic carriers; several such synthetic polymer systems have shown promising results as antimicrobial agents *in vivo*, and this area has been extensively reviewed.[106] Finally, a recently designed biodegradable polymer that can function as an antimicrobial amphiphilic self-assembling system has sparked further interest in this class of structures for *in vivo* applications.[107, 108]

Similarly, liposomes as vehicles to encapsulate antimicrobial drugs have exhibited remarkable antimicrobial activity by improving both drug half-life and drug efficacy.[109- 111] For instance, liposome-aminoglycoside antimicrobial systems have been evaluated in preclinical and clinical trials against various infections including intracellular *B. canis*,[112] *B. abortus*,[112, 113] *B. melitensis*,[114] *S. tryphimurium*,[115] *S. dublin*,[116] *S. enteritidis*,[117] *M. avium-intracellulare*,[118] and *M. tuberculosis*.[119] Furthermore, antimicrobial liposomes can be prepared with free fatty acids (FFAs), which are found naturally in the human body, making these nanostructures particularly biocompatible. A variety of these structures have shown antimicrobial activity, most notably against Gram-positive bacterial cell lines.[120-122] In a recent study, liposomes containing the FFA oleic acid showed antimicrobial activity against MRSA cells *in vitro* and could be used to effectively target MRSA infections *in vivo* without causing skin toxicity.[123] The documented successful use of liposomal formulations in the clinic[110] provides an example for going forward with other systems to combat infections. The current clinical success of polylysine-based dendrimers as an antiviral agent indicates that dendrimers have the potential to be very successful antimicrobial agents in the near future with continued research/clincial efforts.[124]

Conclusion

Based on the reports summarized in this review, it is apparent that dendrimers can be used both as antibacterial drug delivery *systems*, bacteriophobic *coatings*, and antibacterial *agents* capable of targeting bacterial cells or bacterial toxins. Due to their globular shape and the positioning of reactive groups at the surface, dendrimers are well suited to engage in multivalent interactions, allowing these compounds to interfere with the function of critical bacterial virulence determinants. Whereas glycodendrimers offer an opportunity for high specificity, cationic dendrimers are promising as broad-spectrum antibiotics, either acting

alone or in synergy with known drugs. Antimicrobial peptide dendrimers represent a middle ground between the two strategies. Being composed of a combination of cationic and hydrophobic residues, they act through membrane disruption, but are more selective than classic cationic dendrimer because of their polypeptide nature.

The key findings from this review that highlight the importance of dendrimers as antimicrobial agents are summarized in Figure 10. However, it is important to point out that some unexplored areas remain in this field of research, presenting opportunities for additional studies regarding the use of dendrimers as antimicrobial agents. The number and variety of chemical structures investigated is limited, with PAMAM, PPI, and dendritic polylysine derivatives being the most widely explored. In most cases, insufficient data is available to obtain structure-activity relationships necessary to guide future developments. Generally, only a few papers are reported on each particular dendrimer and the variety in strains of bacteria tested is usually limited. Despite the significant *in vitro* antimicrobial activity data collected for a variety of dendrimer analogues, the effects of these structures on non-model bacteria systems, including MRSA strains or TB, have been evaluated in only a handful of studies, and only a few studies can be found with regard to the activity of dendritic polymers against fungi.[39] Furthermore, the potential for these compounds to be used as drug delivery *devices* rather than *agents* via the formation of hydrogels has only been evaluated in a few studies. The ability to use dendrimers to form complex supramolecular structures gives researchers the opportunity to exploit the synergistic effects of dendrimer-drug systems. Finally, in a clinical setting, the potential of biofilm formation poses a serious risk of infection for patients due to the limited activity of many current antimicrobial drugs. The use of dendrimers as a method to target and disrupt these bacterial aggregates on surfaces may allow for reduced prevalence of resistant strains and should be further probed in future studies. In summary, unlike the area of drug and gene delivery where there has been a significant research effort by many groups, the use of dendritic polymers to combat emerging and re-emerging infectious diseases is less established, and, as such, we welcome and encourage others to invest their knowledge, time, and resources to solving this unmet clinical challenge.

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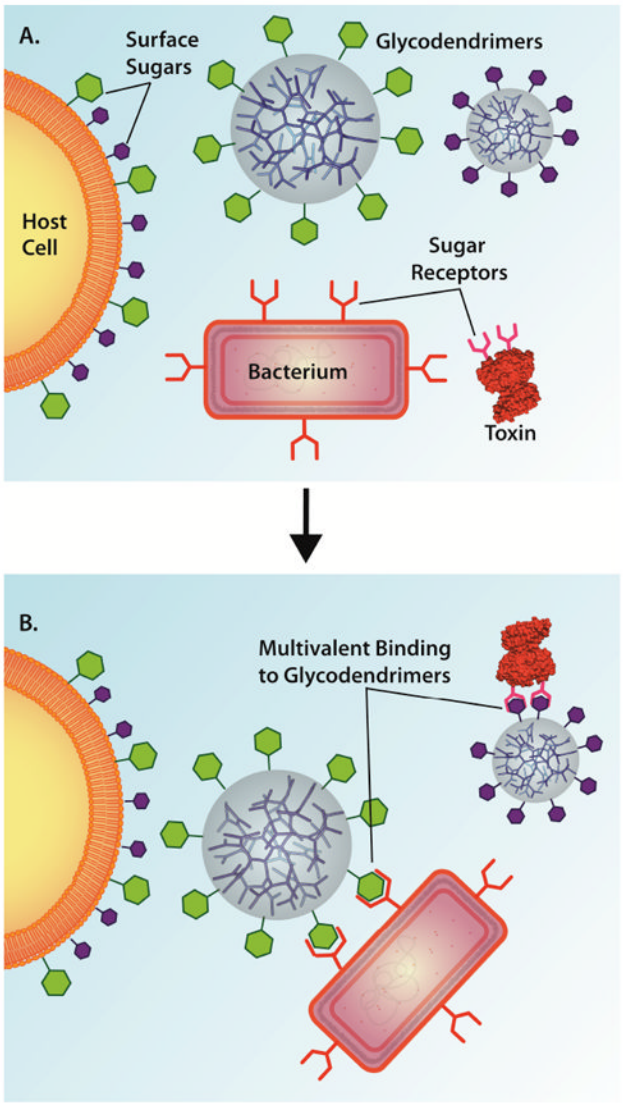

Figure 1.

Multivalent binding of a bacterium or a bacterial toxin to a glycodendrimer. (A) Depiction of a host eukaryotic cell with surface sugar groups, two glycodendrimers with surface sugars groups, a bacterial toxin with receptors for the purple sugar groups, and a generic bacterium with receptors for the green sugar groups. (B) Binding of the bacterium and the toxin to the glycodendrimer through multivalent interactions to prevent infection of the host cell. Structures not draw to scale.

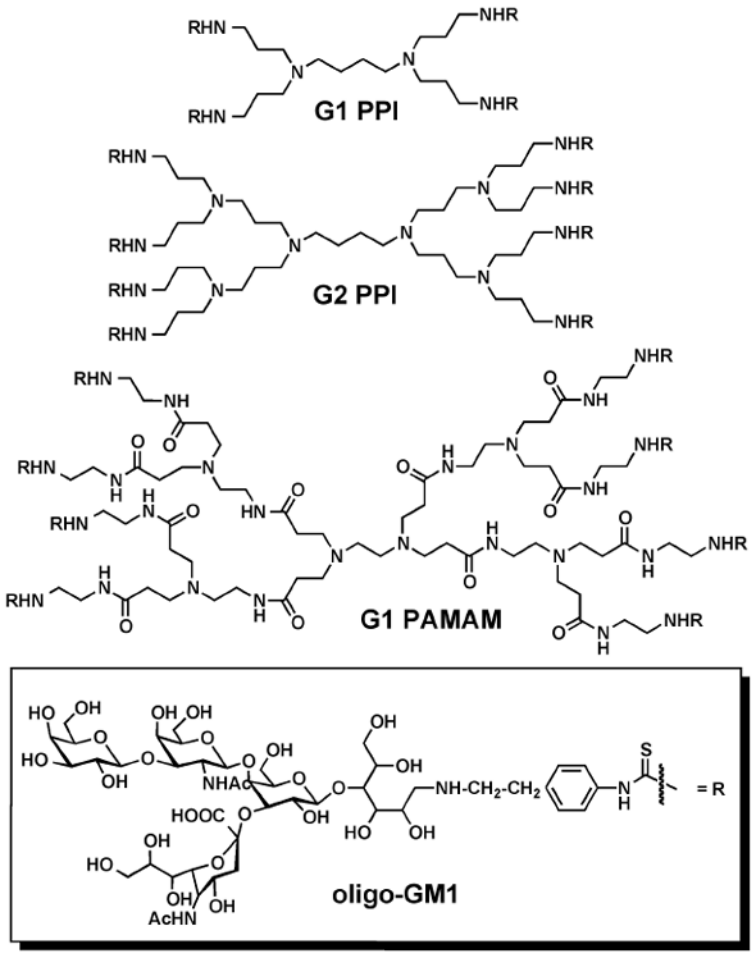

Figure 2.
Structures of oligosaccharide dendrimers based on PAMAM and PPI cores with oligo-GM1 sugar appended to the surface.

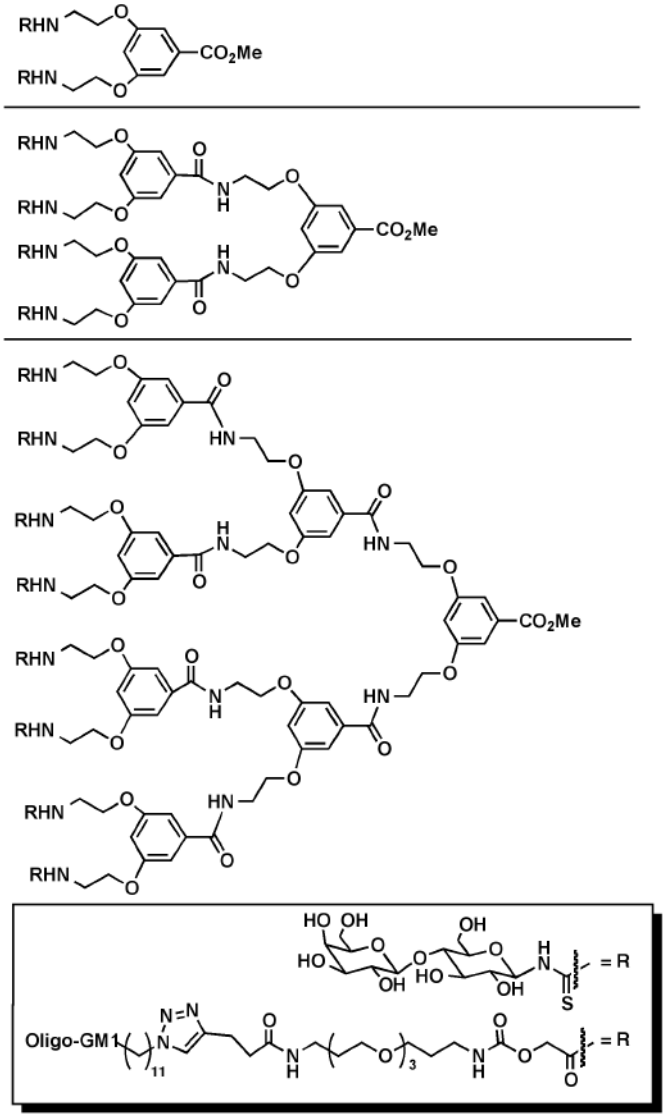

Figure 3.
Dendrimers based on the 3,5-di(2-aminoethoxy)benzoic acid repeat units with 2, 4, or 8 oligo-GM1 or lactose groups appended to the surface.

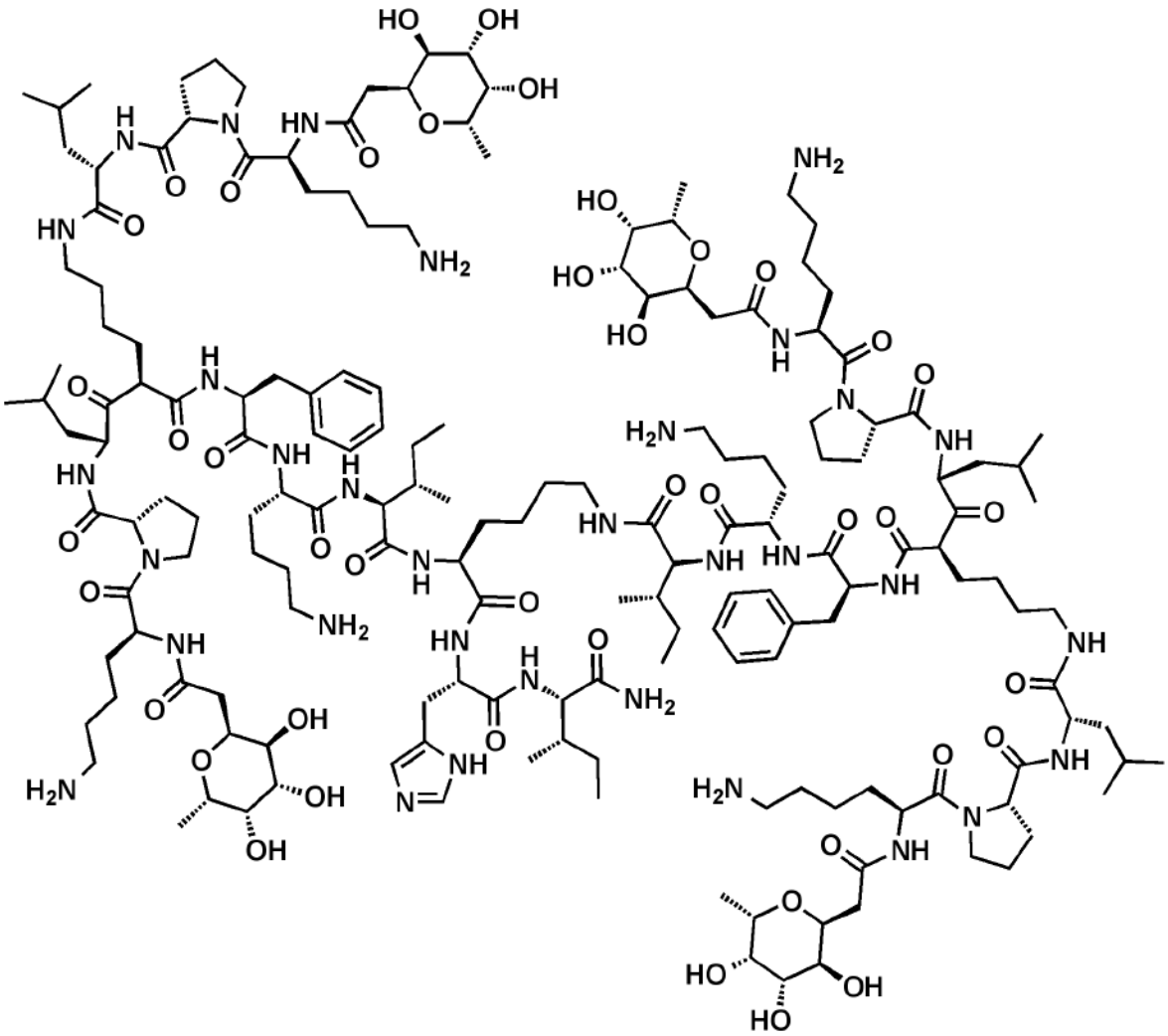

Figure 4.
Structure of glycopeptide dendrimer FD2.

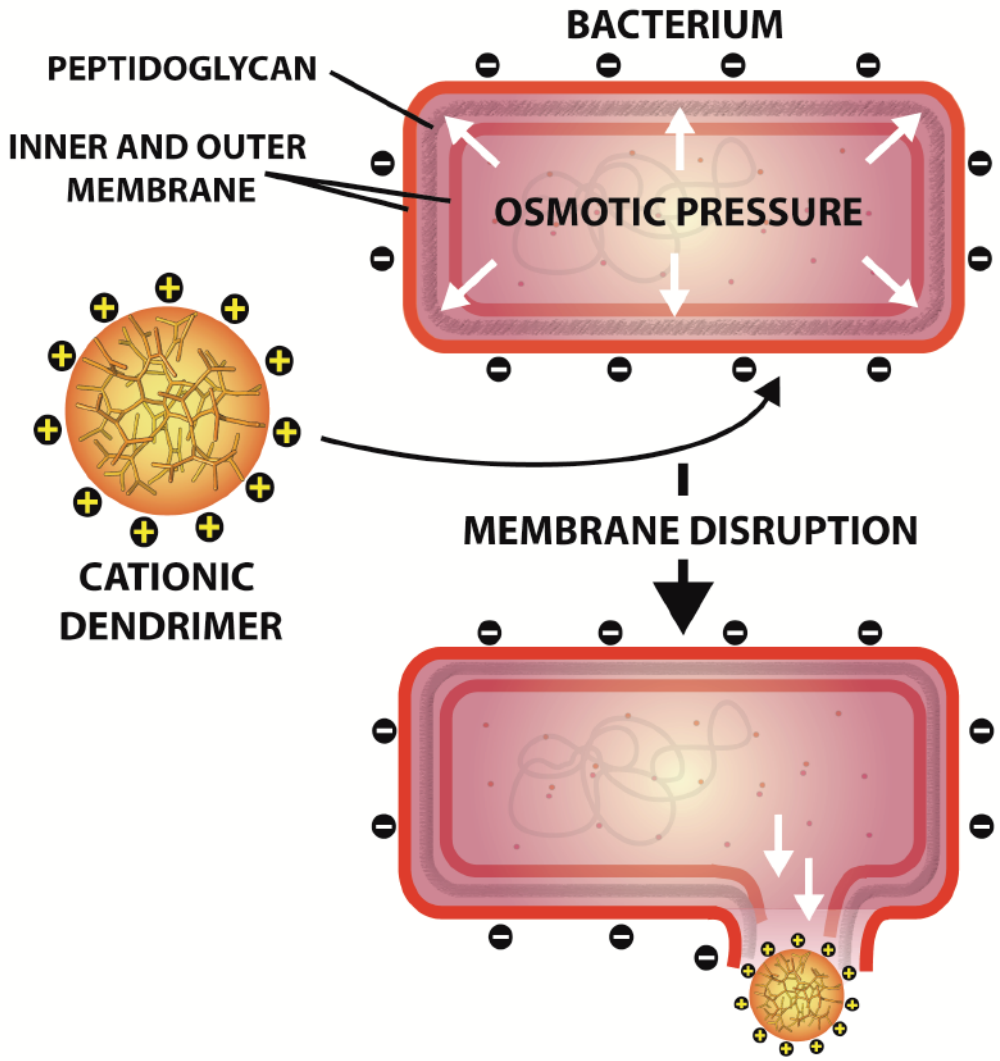

Figure 5.
Proposed mechanism of action of cationic dendrimers via initial electrostatic attraction to the negatively charged bacterium followed by membrane and peptidoglycan disruption. Structures not drawn to scale.

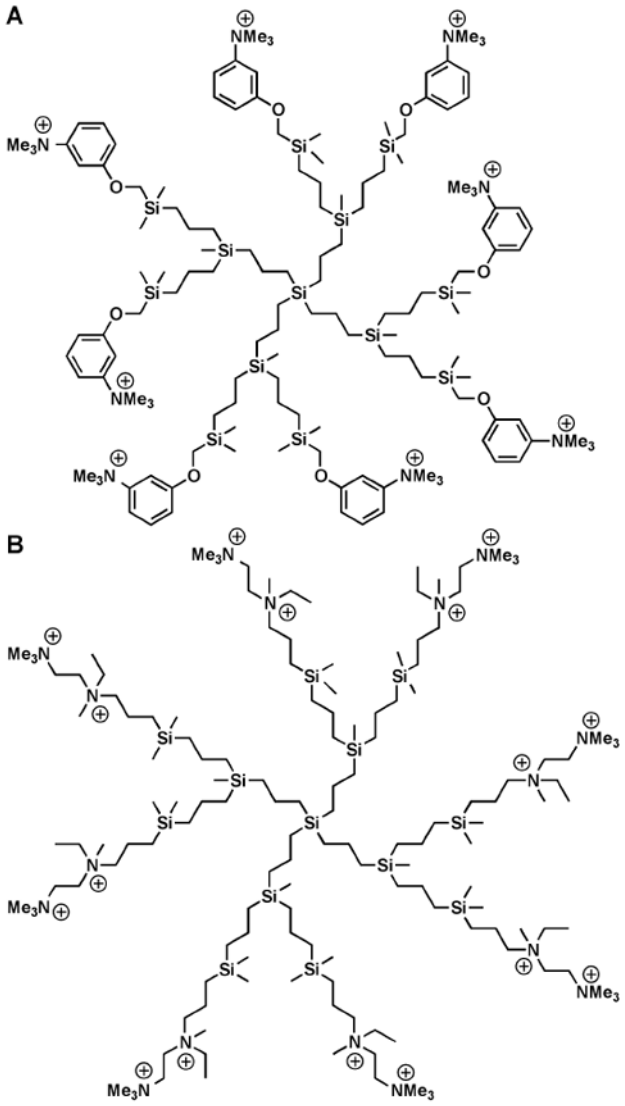

Figure 6.
(A) Structure of initial carbosilane used for antimicrobial studies. (B) Structure of modified carbosilane dendrimer with improved solubility in aqueous conditions.

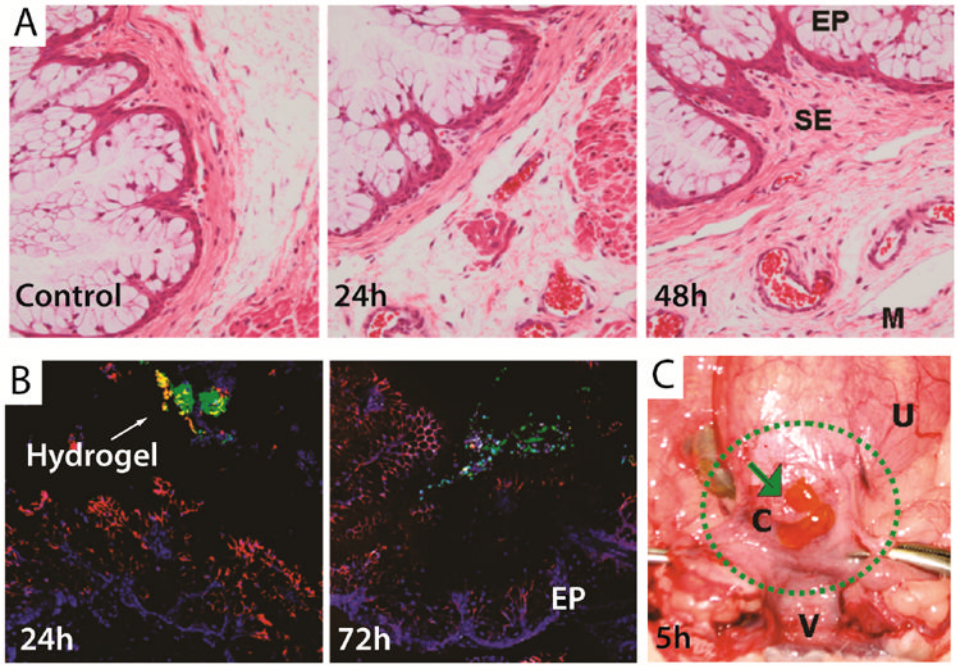

Figure 7.

(A) Histological sections of the cervix of pregnant guinea pigs not treated (control) and treated with hydrogel after 24 and 72 hours. The epithelial cells do not show signs of damage or inflammation and are comparable to the control. (B) Confocal image of the guinea pig cervix treated with the hydrogel (green) after 24 and 72 hours confirming that the gel remains on the mucosal layer (red) after 3 days. (C) Hydrogel (green arrow) 5 hours after being placed on the guinea pig cervix. EP = epithelial cells, SE = subepithelium, M = muscular layer, C = cervix, V = vaginal cavity, U = uterus with pups. Adapted from Ref. 73.

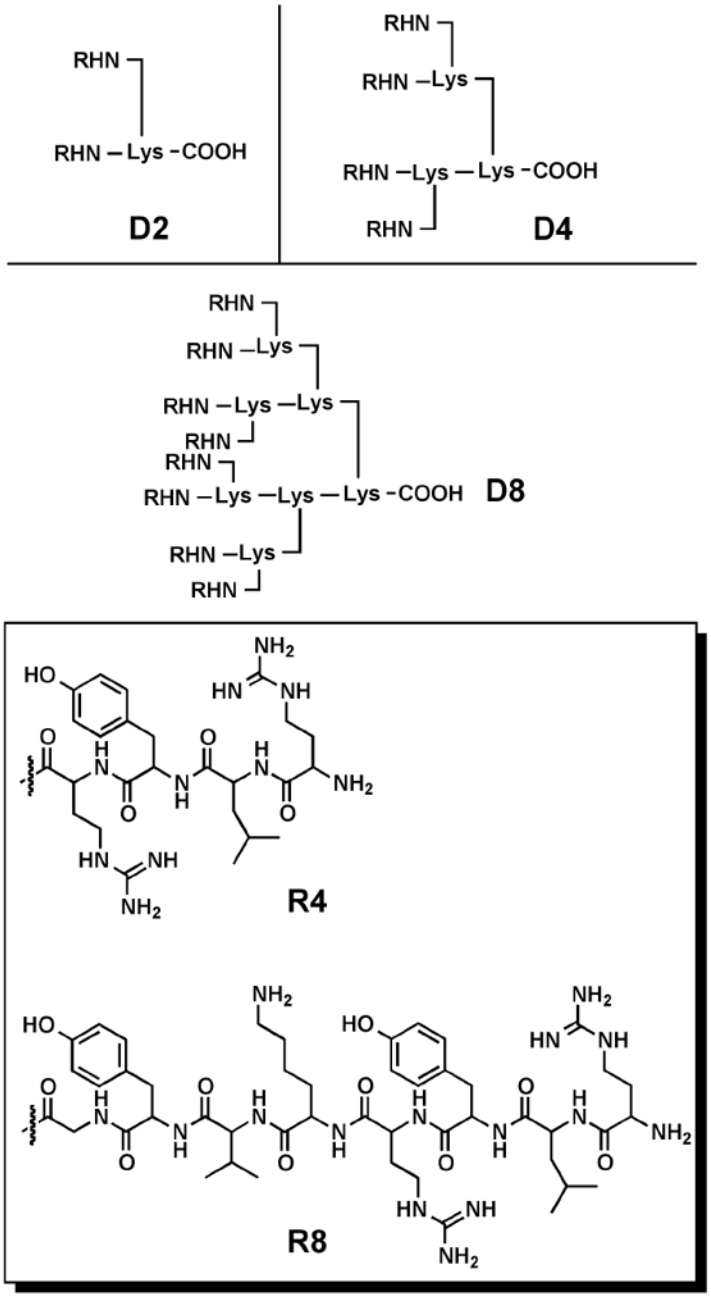

Figure 8.
Schematic representation of three types of peptide dendrimer cores (D2, D4, and D8) and the structures of two short peptide sequences (R4 and R8) appended to the surface of the cores.

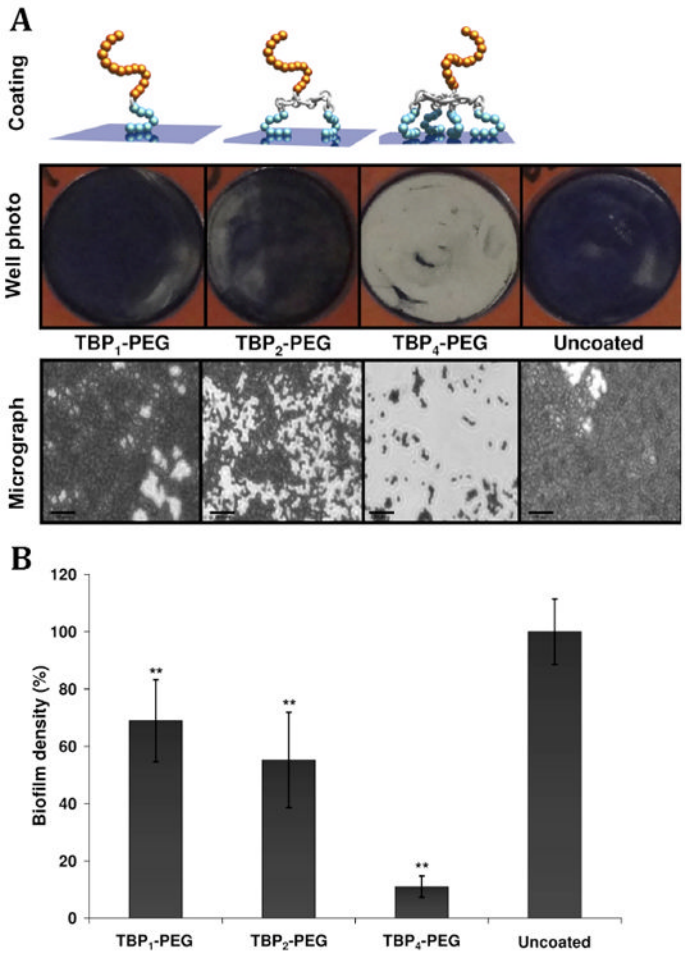

Figure 9.

(Top) Illustration of the bacteriophobic coatings possess one or more bottom TBPs (blue), a peptide linker (silver), and a top PEG domain (gold). Schematic of three bacteriophobic coatings under investigation where one (left), two (middle), or four (right) titanium-binding peptides (TBPs) are covalently attached to a polyethylene glycol (PEG). Digital photographs and (bottom) phase-contrast micrographs (Magnification = 630X) of coated and uncoated Ti wells following a 5 h exposure to *S. aureus* cultures (starting inoculum of ~5×10^7 CFUs/mL). Bacteria were stained with 0.1% crystal violet to aid visualization. Scale bars = 20 μm. (Bottom) Quantification of biofilm formation on coated and uncoated Ti surfaces following a 5 h exposure to *S. aureus* (starting inoculum of ~5×10^7 CFUs/mL) cultures. (N=3, ** P <0.01). Adapted from Ref. 87.

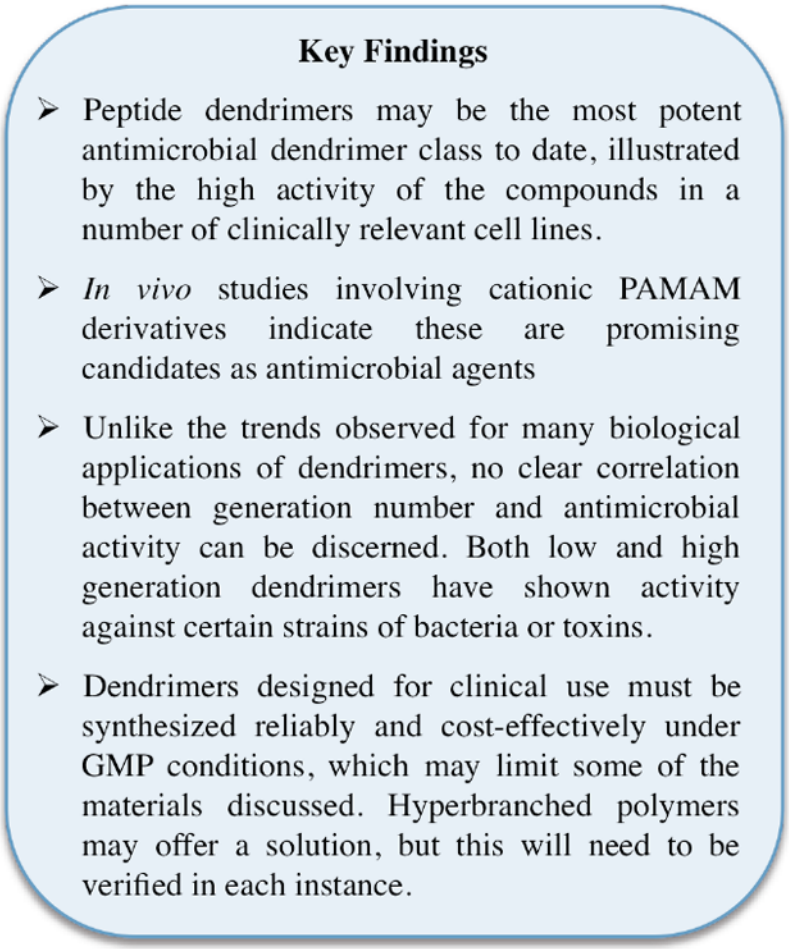

Figure 10.
Key findings in the review of dendrimers for use as antimicrobial agents, coatings, or drug delivery devices.

Table 1

In vitro minimum inhibitory concentration (MIC, μg mL^{-1}) of dendrimers[78]

	Dendrimer Surface Groups						
Microbe	**$(NH_2)_3$**	**$(CO_2H)_6$**	**$(NH_2)_6$**	**$(CO_2H)_{12}$**	**$(NH_2)_{12}$**	**$(CO_2H)_{24}$**	**Gentamycin**
E. coli	3.1	25.0	6.3	12.5	12.5	12.5	6.3
S. aureus	1.6	12.5	6.3	12.5	6.3	12.5	25.0
K. pneumoniae	6.1	12.5	12.5	12.5	12.5	25.0	6.3
B. cereus	6.3	12.5	12.5	25.0	25.0	12.5	6.3
M. luteus	1.6	6.3	6.3	6.3	12.5	12.5	25.0
P. vulgaris	1.6	12.5	3.1	6.3	6.3	12.5	6.3
M. smegmatis	12.5	12.5	12.5	25.0	12.5	12.5	12.5
L. monocytogenes	6.3	6.3	12.5	12.5	12.5	12.5	12.5
P. aeruginosa	6.3	12.5	6.3	25.0	25.0	12.5	6.3

Table 2

In vitro minimum inhibitory concentration of antimicrobial peptide dendrimer against gram-positive and gram-negative bacteria[82]

Microbe	Relevant features	MIC (μg mL^{-1})
Escherichia coli ATTCC 25922	Reference strain	8
Escherichia coli W99FI0077	FQ, ESC (ESBL/SHV type)	8
Escherichia coli W03BG0025	FQ, AG, ESC (ESBL/CTX-M-15)	8
Escherichia coli W03NO0013	FQ, ESC (ESBL/CTX-M-1)	8
Pseudomonas aeruginosa ATCC 27853	Reference strain	4
Pseudomonas aeruginosa 885149	FQ, AG, ESC, CP (MBL/IMP-13)	8
Pseudomonas aeruginosa 891	FQ, AG, ESC, CP (MBL/VIM-2)	8
Pseudomonas aeruginosa VA463/98	FQ, AG, ESC (ESBL/PER-1)	4
Klebsiella pneumoniae W99FI0057	ESC (ESBL/SHV type)	4
Klebsiella pneumoniae W03NO0078	ESC (ESBL/CTX-M-1)	16
Klebsiella pneumoniae W03BG0019	AG, ESC (ESBL/CTX-M-15)	8
Klebsiella oxytoca W99FI00049	ESC (ESBL/SHV-12)	64
Proteus mirabilis W99FI0089	FQ	>128
Proteus mirabilis W03VA1144	FQ, AG, ESC (ESBL/PER-1)	64
Enterobacter aerogenes W03BG0067	AG, ESC (ESBL/SHV-5)	8
Enterobacter cloacae W03AN0041	ESC (ESBL/SHV-12)	4
Morganella morganii W03VA1342	FQ, ESC (ESBL/CTX-M-1)	>128
Acinetobacter baumannii AB1MG	FQ, AG, ESC (ESBL/TEM-92)	16
Acinetobacter baumannii AB7MG	FQ, AG, ESC	32
Citrobacter freundii W99FI00007	ESC (ESBL/SHV-12)	16
Chryseobacterium meningosepticum CCUG4310	Reference strain	>128
Burkholderia cepacia SMC71	FQ, AG, ESC	64
Serratia marcescens W99FI0111	FQ, AG, ESC (ESBL/SHV-5)	>128
Stenotrophomonas maltophilia PT4/99	Wild-type profile	>128
Providencia stuartii W03FI0001	AG, ESC (ESBL/PER-1)	>128
Staphylococcus aureus ATCC 25923	Reference strain	>128
Staphylococcus aureus MIU-68A	MS	128

Relevant resistance phenotypes and resistance mechanisms are indicated by FQ (resistance to fluoroquinolines), AG (resistance to aminoglycosides), ESC (resistance to extended-spectrum cephalosporins), CP (resistance to carbapenems), ESBL (extended-spectrum β-lactamase), MBL (metallo-β-lactamase), and MS (methicillin susceptible).