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Cationic Pillararenes Potently Inhibit Biofilm Formation without Affecting Bacterial Growth and Viability

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Supporting Information

ABSTRACT: It is estimated that up to 80% of bacterial infections are accompanied by biofilm formation. Since bacteria in biofilms are less susceptible to antibiotics than are bacteria in the planktonic state, biofilm-associated infections pose a major health threat, and there is a pressing need for antibiofilm agents. Here we report that water-soluble cationic pillararenes differing in the quaternary ammonium groups efficiently inhibited the formation of biofilms by clinically important Gram-positive pathogens. Biofilm inhibition did not result from antimicrobial activity; thus, the compounds should not inhibit growth of natural bacterial flora. Moreover, none of the cationic pillararenes caused detectable membrane damage to red blood cells or toxicity to human cells in culture. The results indicate that cationic pillararenes have potential for use in medical applications in which biofilm formation is a problem.

An estimated 17 million new biofilm-associated infections arise each year in the United States alone, resulting in up to 550,000 fatalities annually.[1a] Bacterial biofilms are microbial communities (essentially cities of microbes) that are held together by an extracellular matrix.[1] In recent years, there has been an increasing interest in developing strategies to prevent the formation of bacterial biofilms since biofilms lead to a dramatic enhancement in resistance to antibiotics.[1] Compared with planktonic bacteria (those that grow in suspension), bacteria in biofilms can be up to 3 orders of magnitude less susceptible to antibiotics.[2] Moreover, biofilms account for a large percentage of nosocomial and implanted device-derived microbial infections in patients.[3] Despite the great need, there are currently no clinically approved small molecules that efficiently and specifically inhibit biofilm formation. Identification of small molecules that inhibit biofilm formation without affecting bacterial cell viability will offer a much needed solution to biofilm infections that will not harm important natural bacterial flora.

Biofilm matrices are composed of exopolymeric substances (EPS); these high-molecular weight compounds are secreted by the bacteria into the extracellular environment and are crucial for the integrity of all biofilms.[4] EPS components include polysaccharides (also termed exopolysaccharides), proteins, extracellular DNA, lipids, and bacterial decomposition substances that are held together by a highly complex network of hydrogen bonds as well as ionic and van der Waals interactions. The composition of biofilm matrices varies significantly among different bacterial strains; some matrices contain mainly exopolysaccharides and some mainly proteins.[5] Matrix proteins and exopolysaccharides also vary between different biofilm producing bacterial strains.[6]

Investigation of the biofilm formation process has defined a five-step sequence as illustrated in Figure 1.[7] Negatively

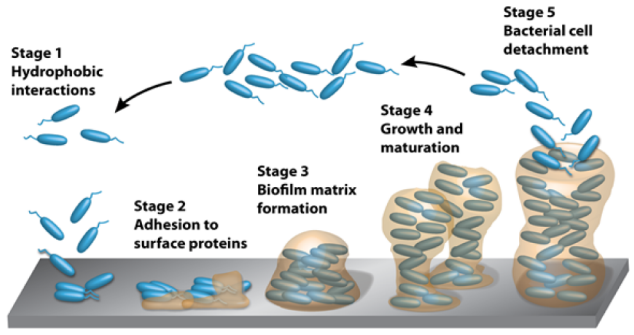

Figure 1. Stages of biofilm formation.

charged polyelectrolytes such as extracellular DNA fragments are important components of matrices of several biofilm-forming Gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Enterococcus faecalis*; in the latter, extracellular DNA is an integral structural component during early biofilm formation stages.[8] DNA efficiently interacts with cationic amphiphiles, suggesting that this type of molecule may be used to disrupt the interactions between extracellular DNA and other components of the bacterial biofilm EPS. Recent studies have provided evidence for antibiofilm activity of cationic amphiphilic compounds: Böttcher et al. reported on a collection of synthetic guanidine- and biguanidine-based cationic amphiphiles that inhibited biofilm formation of *Bacillus subtilis* and *S. aureus* strains.[9] Jennings et al. demonstrated that quaternary ammonium amphiphiles had antimicrobial activity against several Gram-positive and Gram-negative bacterial strains;[10] some of these cationic amphiphiles efficiently broke down existing biofilms of *S. aureus* and *E. faecalis*. Melander and co-workers showed that bromoageliferin analogues inhibit *Pseudomonas aeruginosa* biofilm formation and that aminobenzimidazole conjugates that are positively charged under physiological conditions inhibit formation of biofilms composed of MRSA, vancomycin-resistant enterococci, and *Staphylococcus epidermidis*.[11]

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In this study we aimed to develop cationic amphiphiles capable of competing with the chemical interactions that stabilize biofilm matrices to prevent biofilm assembly without impairing bacterial cell viability or damaging mammalian cell membranes, as do many types of cationic amphiphiles. In search of such cationic amphiphiles, our attention was drawn to the unique molecular features of the relatively new family of pillararene macrocycles. First reported in 2008 by Ogoshi,[12a] pillararenes have received much attention by the chemical community.[12] For example, pillararenes are used in host−guest and supramolecular chemistry as sensors, drug delivery systems, and scaffolds for supramolecular polymers.[12] The Huang group reported on the first amphiphilic pillararene and showed that it self-assembles into vesicles and microtubes in water.[12i] Since then several amphiphilic and bola-amphiphilic pillararenes have been prepared.[12j,k] Despite the surge in pillararene research in recent years, the biological applications of these unique molecules have been poorly explored. We decided to explore the biofilm inhibition properties of positively charged ammonium- and imidazolium-decorated pillararenes, as these molecules have good water solubility and have a lipophilic, but relatively electron-rich, cavity that may serve as host for a variety of electron-deficient and hydrophobic chemical entities that are found in components of biofilm matrices. In the systems we studied, the positively charged groups are located on the opposite faces of the pillararene backbone.

Following synthetic routes similar to those previously described,[13] we synthesized five positively charged pillar[5]-arene derivatives in which the 10 phenolic positions were substituted by positively charged quaternary ammonium or imidazolium groups (compounds **1**−**5**, Figure 2a, see

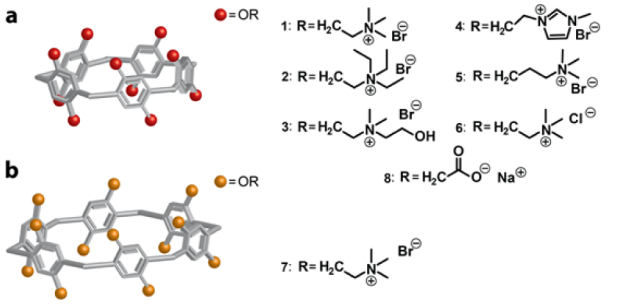

Figure 2. Cationic pillararenes tested for biofilm inhibition properties. (a) Cationic pillar[5]arenes **1**−**6** and anionic pillar[5]arene **8**. (b) Cationic pillar[6]arene **7**.

Supporting Information (SI) for details of synthetic procedures and compound characterization). As a control we prepared the negatively charged pillar[5]arene **8**, which is decorated with 10 carboxylate groups that are negatively charged under physiological conditions.

Each compound was tested for its ability to inhibit biofilm formation by Gram-positive and Gram-negative pathogens.[3,14−17] The mean IC_{50} value for biofilm inhibition ($MBIC_{50}$) was defined as the lowest concentration at which at least 50% reduction in biofilm formation was measured compared to untreated cells[18] (see exact protocol in SI). The results are summarized in Table 1. The most impressive biofilm inhibition properties were observed for deca-trimethylammonium pillar[5]arene **1** and the deca-*N*-methyl-imidazolium pillar[5]arene **4**. The $MBIC_{50}$ values of these compounds against each of the tested biofilm-forming Gram-positive

Table 1. Biofilm Inhibitory Activity: $MBIC_{50}$ (μM) against Gram-Positive Strains[a]

	bacterial strain					
compd	A	B	C	D	E	F
1	0.9	3.5	3.5	0.9	3.5	1.8
2	1.5	5.9	5.9	1.5	5.9	5.9
3	>12	>12	>12	>12	>12	>12
4	0.8	1.6	1.6	0.4	3.2	6.4
5	1.7	6.6	>13	1.7	1.7	6.6
6	1.1	4.4	8.8	1.1	2.2	8.8
7	0.4	1.5	2.9	0.4	0.7	2.9
8	>23	>23	>23	>23	>23	>23
TMA-Cl	>292	>292	>292	>292	>292	>292
TMA-Br	>208	>208	>208	>208	>208	>208

[a]Compounds were evaluated using the double-dilution method for inhibition biofilm formation by (A) *S. aureus* subsp. *aureus* Rosenbach ATCC 33592, (B) *S. aureus* ATCC 29213, (C) *S. aureus* BAA/043, (D) *E. faecalis* ATCC 29212, (E) *S. epidermidis* RP62A, (F) *S. mutans* ATCC 700610. TMA-Cl and TMA-Br are tetramethylammonium chloride and tetramethylammonium bromide, respectively. Each value is a mean of at least three independent experiments each including five replicates of each concentration.

pathogens ranged from 0.4 to 6.4 μM. Inhibition of biofilm formation was selective for Gram-positive strains. None of the cationic pillararenes in this study inhibited the formation of biofilm by Gram-negative strains *E. coli* ATCC 25922 and *P. aeruginosa* PAO1.

Changes in the hydrophilic or hydrophobic balance of the cationic pillar[5]arene had general and significant effects on the inhibition of biofilm formation. Elongation of the aliphatic linker between the pillar[5]arene core and the positively charged group from an ethyl in compound **1** to a propyl chain in compound **5** led to a small but significant reduction in the inhibition of biofilm formation. A similar effect was observed when the quaternary trimethylammonium head groups in compound **1** were replaced by more hydrophobic triethyl quaternary ammonium groups in compound **2**. A more pronounced loss of activity was observed when the hydrophilicity of the head groups was increased by the installation of hydroxyethyl-dimethyl quaternary ammonium groups in compound **3**; compound **3** did not inhibit biofilm formation at a concentration of 12 μM, the highest concentration tested.

To further evaluate the structural determinants required for biofilm formation inhibition, we examined antibiofilm activities of several control compounds: Pillar[5]arene **8**, which has carboxylic head groups that are negatively charged under physiological conditions, did not inhibit biofilm formation by any of the tested strains. This showed that the positive charge was important for the observed activity. No inhibition of biofilm formation, up to concentrations of 208 and 292 μM, was observed for tetramethylammonium bromide (TMA-Br) or tetramethylammonium chloride (TMA-Cl), respectively, indicating that neither the quaternary ammonium head groups nor the halogen ions alone are responsible for the inhibition of biofilm formation by compounds **1**−**5**.

The effect of the halogen ion on the inhibition of biofilm formation in the tested strains was further examined by the preparation of compound **6**; the chloride analogue of compound **1**. Compounds **1** and **6** had very similar $MBIC_{50}$ values against four of the tested strains (strains A, B, D, E; Table 1). The halogen ion type did affect the ability of

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pillararene **6** to inhibit *S. aureus* BAA/043 (strain C) and *S. mutans* ATCC 700610 (strain F) biofilm formation; for these strains, the $MBIC_{50}$ values of compound **6** with the chloride counterion were 2- and 4-fold higher than those of compound **1** with the bromide anion, respectively.

Since **1** demonstrated potent inhibition of biofilm formation by all tested strains of Gram-positive pathogens, we reasoned that increasing the quaternary ammonium cluster size and the overall positive charge of the molecule would further improve the inhibition properties. Hence, we synthesized compound **7**, the pillar[6]arene analogue of **1** (Figure 2b, SI). Compared to compound **1** the overall positive charge of compound **7** is 20% higher. Furthermore, the internal cavity diameter of pillar[6]-arene **7** is ∼6.7 Å, whereas that of pillar[5]arene **1** is ∼4.6 Å.[12c] This difference should, in principle, enable compound **7** to bind larger and more structurally diverse molecular guests from the biofilm matrix. Compound **7** was found to be the most potent inhibitor of biofilm formation of all the cationic pillararenes tested strains, as summarized in Table 1 and demonstrated visually in Figure 3. Compared to pillar[5]arene **1**, the $MBIC_{50}$ values of pillar[6]arene analogue **7** were from 2- to 5-fold lower for four of the tested biofilm forming Gram-positive pathogens; no significant difference in the inhibition of biofilm formation between **1** and **7** was observed in *S. aureus* BAA/043 (strain C, Table 1). For *S. mutans* ATCC 700610, however, compound **1** was slightly more active than **7** (strain F, Table 1). The dose-dependent biofilm inhibition ability of compounds **1**−**7** against *E. faecalis* ATCC 29212 and *S. aureus* subsp. *aureus* Rosenbach ATCC 33592 is presented in Figure S16.

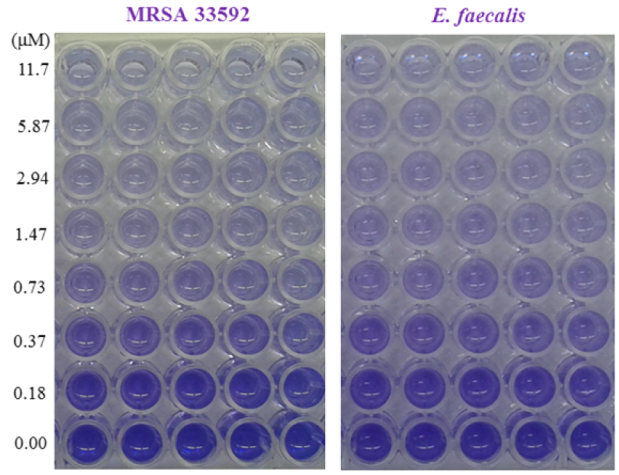

Figure 3. Inhibition of biofilm formation by pillar[6]arene **7**. Biofilms produced by *S. aureus* subsp. *aureus* Rosenbach ATCC 33592 and by *E. faecalis* ATCC 29212 in the presence of increasing concentrations of **7** were stained with crystal violet. Each concentration of compound was tested in five wells.

Pillararenes **1** and **7**, the most potent inhibitors of biofilm formation, did not eradicate mature biofilms (SI). In addition, we determined the $MBIC_{50}$ of compound **7**, which demonstrated potent biofilm inhibition properties, against *S. aureus* subsp. *aureus* Rosenbach ATCC 33592 and *E. faecalis* in cultures that were 2-, 4-, and 10-fold the standard starting inoculum (OD = 0.001). No significant change in $MBIC_{50}$ values was observed indicating that there is no significant inoculum effect for this compound (Figure S17 and Table S1 in SI).

Several families of both synthetic and natural antimicrobial agents are composed of cationic amphiphiles.[19] These compounds bind to negatively charged bacterial membrane lipids and lead to enhanced and uncontrolled membrane permeability and bacterial cell death. Since all of the pillararenes in this study are cationic amphiphiles, we evaluated the antimicrobial activity of the most potent inhibitor of biofilm formation pillar[6]arene **7** to determine whether the capability to inhibit biofilm formation results from bactericidal activity. Minimal inhibitory concentration (MIC) experiments were performed following the double-dilution protocol.[20]

The MIC values against the examined Gram-positive strains were higher than ∼47 μM, at least 16-fold higher than the highest $MBIC_{50}$ value measured for pillar[6]arene **7** against the tested strains. We therefore concluded that the observed inhibition of biofilm formation did not result from a bactericidal effect. The possibility that pillar[6]arene **7** had a bacteriostatic effect was examined by comparing the growth curves of two of Gram-negative strains (*E. coli* ATCC 29522 and *P. Aeruginosa* PAO1) and two Gram-positive strains (*S. aureus* subsp. *aureus* Rosenbach ATCC 33592 and *E. faecalis* ATCC 29212) in the absence and presence of 32 and 64 μg/mL of **7** for 24 h. These concentrations are ∼15- and ∼30-fold higher than the $MBIC_{50}$ values measured for this compound against the two Gram-positive strains. The growth curves clearly indicated that, at a concentration significantly higher than the $MBIC_{50}$, this compound had no effect on bacterial growth (Figure S15). Thus, the anti-Gram-positive biofilm properties of this compound do not result from a bacteriostatic effect.

Finally, many families of antimicrobial cationic amphiphiles disrupt mammalian cell membranes as well as bacterial cell membranes.[19] Rat red blood cells serve as a standard model for the evaluation of the ability of compounds to lyse mammalian cell membranes. Up to a concentration of 94 μM, none of the cationic pillararenes caused any measurable hemolysis of red blood cells obtained from laboratory rats following a previously reported protocol.[19b] The toxicity of compound **7** toward human monocytic THP1 cells (ATCC TIB 202) and cystic fibrosis human bronchial epithelial cells IB3-1 (ATCC CRL-2777) was also evaluated. No effects on viability were observed after 72 h incubation with concentrations up to 46.96 μM, about 50 times the $MBIC_{50}$ values measured for this compound against the two Gram-positive strains (Figure S18).

In conclusion, we demonstrated that decoration of pillararene scaffolds with positively charged quaternary ammonium or imidazolium groups resulted in compounds that efficiently inhibited assembly of biofilms formed by several clinically important Gram-positive pathogens. The pathogens in the tested panel are responsible for a broad spectrum of biofilm-associated infections that are challenging to treat with the current repertoire of clinically used antibiotics. Our investigation indicates that the ammonium and imidazolium groups and the pillararene structure are required for the inhibition of biofilm formation. Moreover, our findings suggest that positive charges, their accessibility, and the inner diameter of the pillararene, which determines the host−guest properties, all contribute to the observed inhibition of biofilm formation by these compounds. The unique and potent biofilm inhibition properties of the reported cationic pillararenes suggest that these compounds will find utility in the clinic as well as in industrial applications.

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■ ASSOCIATED CONTENT

Supporting Information

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Experimental procedures, spectroscopic characterization data, and detailed protocols for the biological assays (PDF)

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Notes

The authors declare no competing financial interest.

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