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Phosphonium pillar[5]arenes as a new class of efficient biofilm inhibitors: importance of charge cooperativity and the pillar platform†

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Biofilm formation, which frequently occurs in microbial infections and often reduces the efficacy of antibiotics, also perturbs many industrial and domestic processes. We found that a new class of water soluble pillar[5]arenes bearing phosphonium moieties (1, 2) and their respective ammonium analogues (3, 4) inhibit biofilm formation with IC_{50} values in the range of 0.67–1.66 μM. These compounds have no antimicrobial activity, do not damage red blood cell membranes, and do not affect mammalian cell viability in culture. Comparison of the antibiofilm activities of the phosphonium-decorated pillar[5]arene derivatives 1 and 2 with their respective ammonium counterparts 3 and 4 and their monomers 5 and 6, demonstrate that while positive charges, charge cooperativity and the pillararene platform are essential for the observed antibiofilm activity the nature of the charges is not.

According to the reports from the National Institutes of Health, about 65% of infections treated in the developed world involve microbial biofilms.[1] Biofilm-associated diseases in humans include lung infections, ear infections, urinary and gastrointestinal tract infections, chronic and burn wound infections, nosocomial, catheter-related, and dental infections.[2] The formation of biofilms on biomedical devices, surgical implants, urinary tract catheters, and contact lenses, dramatically increases the chances of introducing persistent infections into the human body.[2] Biofilm growth also has detrimental effects in industrial and domestic domains resulting in high costs associated with cleaning and maintenance.[3] In addition, bacteria in biofilms are significantly more resistant to antibiotics than are bacteria grown in suspension.[4] Since prevention of biofilm formation could dramatically reduce effects of infectious diseases and the cost of industrial processes there is a great demand for molecules that will effectively inhibit biofilm formation.[5]

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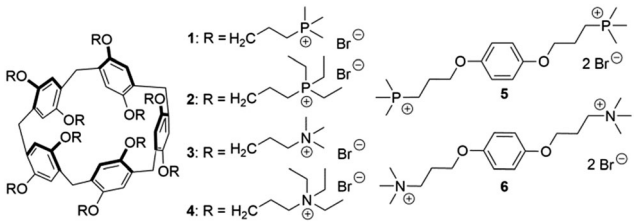
Among various interactions responsible for the formation of biofilms, electrostatic interactions are considered as one of the earliest forces influencing the adherence of bacterial cells to surfaces.[6] The outer surfaces of biofilms consist of an anionic matrix, and disruption of this matrix is thought to be an effective approach for preventing biofilm formation. Cationic amphiphiles therefore appear to be attractive candidates to inhibit early-stage biofilm formation by preventing adhesion of the bacteria to a surface.[7]

In recent years a few inhibitors of biofilm formation based on cationic amphiphiles have been reported.[8] Among the cationic amphiphiles derived from quaternary ammonium and phosphonium salts, the later ones display increased antimicrobial properties compared to their ammonium counterparts.[9] For example, Endo and co-workers demonstrated the antimicrobial properties of a series of phosphonium salts against 11 strains of microorganisms including methicillin-resistant *Staphylococcus aureus* (MRSA).[9a] In recent years, compounds with phosphonium moieties have been used in various biomedical applications and in water treatment including for antifouling purposes.[10] Most of the aforementioned studies report use of phosphonium salts as biocides. The exception is a recent report by Fernández and co-workers describing the antifouling properties of several alkyltriphenylphosphonium salts and their abilities to act as non-toxic quorum sensing disruptors.[11] Indeed, Melander and co-workers pointed out that it is extremely important to develop antibiofilm agents operating *via* non-biocidal mechanisms for several reasons, the most important one is avoiding resistance development.[12]

Very recently, we found that ammonium and methyl imidazolium cationic pillar[*n*]arenes are effective inhibitors of biofilm formation by several strains of Gram-positive bacteria. Interestingly, we observed that this new class of antibiofilm agents shows no antimicrobial activity and no effect on bacterial growth and causes no damage to red blood cells or toxicity to human cells in culture.[13] Therefore in the present work, we prepared a series of phosphonium and ammonium decorated pillar[5]arenes (**1–4**, Scheme 1) and their respective monomers (**5** and **6**, Scheme 1) and studied their anti-biofilm activity with the aim of evaluating the



Scheme 1 Schematic representation of cationic pillar[5]arene conjugates (**1–4**) and monomers (**5** and **6**).

effect of (i) the nature of the positive charges, (ii) the cooperativity of the overall positive charges, and (iii) the pillar[*n*]arene platform on the observed anti-biofilm activity.

Pillar[*n*]arenes, first reported in 2008,[14a] have symmetrical cylindrical structures and relatively large free volumes. Pillar[*n*]arenes can be obtained and functionalized by simple and high yield synthesis routes making them a versatile macrocycles for various applications.[14,15] These macrocycles possess host–guest properties owing to their π-electron rich cavity and crown ether-like arrangement of oxygen atoms at both rims. Hence, in recent years, pillar[*n*]arenes have been used in host–guest chemistry and as sensors and were used to construct supramolecular polymers, interlocked molecules, and hybrid biomolecular materials.[14,15] More recently a phosphonium[16a] and several other phosphorus[16b,c] containing pillararenes were reported. However, despite the significant attention that pillararenes have received from the chemical community, their biological activity is only recently starting to be unravelled.[13,17]

The water-soluble cationic pillar[5]arene derivatives used in this study were synthesized by a four-step process (see Scheme S1 in the ESI†). Briefly, in the first step commercially available hydroquinone was alkylated with 1,3-dibromopropane using potassium carbonate in acetone to afford the monomer **1a** (Scheme S1 in ESI†). The functionalized pillar[5]arene **1b** was obtained by the cyclization of monomer **1a** with paraformaldehyde and boron trifluoride diethyletherate in dichloroethane. Reaction of **1b** with an excess of trimethylphosphine in ethanol under reflux gave the water-soluble compound **1** (Scheme 1). A similar procedure was followed to obtain pillar[5]arene derivatives **2**, **3**, and **4** by reaction with excess of triethylphosphine, trimethylamine, and triethylamine, respectively (Scheme 1 and Scheme S1, ESI†). The control monomers **5** and **6** were synthesized by reacting **1a** with excess trimethylphosphine and trimethylamine, respectively. All the compounds were characterized by ^{1}H and ^{13}C NMR and high-resolution mass spectroscopy (HRMS). Detailed synthetic procedures and the characterization data of compounds **1–6** are presented in the ESI† (see Fig. S1–S8).

The effects of compounds **1–4** were evaluated on biofilm formation by two clinically important Gram-positive bacterial strains, *S. aureus* ATCC 33592 and *Enterococcus faecalis* ATCC 29212. Inhibition of biofilm formation was determined using the crystal violet staining assay.[18] The minimal concentration at which at least 50% reduction in biofilm formation compared to untreated cells ($MBIC_{50}$) was determined, and the results are summarized in Table 1. The dose responses are presented in Fig. 1 (see also Fig. S9–S11 in ESI†).

Table 1 Inhibition of biofilm formation: $MBIC_{50}$ values[a]

Compounds	$MBIC_{50}$[b] in μM (μg mL^{-1})	
	S. aureus ATCC 33592	*E. faecalis* ATCC 29212
1	1.55 (4)	1.55 (4)
2	1.33 (4)	0.67 (2)
3	1.66 (4)	1.66 (4)
4	0.71 (2)	1.41 (4)
5	>317 (160)	>317 (160)
6	>340 (160)	>340 (160)

[a] Compounds were evaluated using the double-dilution method. Each value is the mean of at least three independent experiments that included five replicates at each concentration. [b] Values in parenthesis are of $MBIC_{50}$ in μg mL^{-1}.

All the reported cationic pillar[5]arene derivatives exhibited potent inhibition of biofilm formation against the two tested Gram-positive pathogens. The $MBIC_{50}$ values of the deca-phosphonium pillar[5]arenes **1** and **2** were found to be in the range of 0.67–1.55 μM for both of the tested strains. The corresponding deca-ammonium pillar[5]arene analogues **3** and **4** showed a similar range of $MBIC_{50}$ values, 0.71–1.66 μM. These results indicate that replacement of the ammonium cations by phosphonium cations does not significantly affect the inhibition of biofilm formation by cationic pillararenes. Thus, the positive charges are essential for the observed anti-biofilm activity; however, the nature of the charges has a marginal effect. Note that in our previous study we showed that a negatively charged deca-carboxylate derivative of pillar[5]arene does not significantly inhibit biofilm formation.[13]

To evaluate the effect of hydrophobicity on the biofilm inhibition activity, we compared compounds **2** and **4**, in which the ammonium or phosphonium cations are attached to triethyl moieties, to compounds **1** and **3**, which carry trimethyl moieties. Despite the fact that compounds **2** and **4** have 30 more carbon atoms than do compounds **1** and **3**, their $MBIC_{50}$ values did not significantly differ (Table 1 and Fig. 1a, b). In addition, we found that the dose response for the tested pillar[5]arene derivatives **1–4** (Fig. 1a and b) were also very similar, further corroborating the fact that pillararenes **2** and **4** are as effective as **1** and **3** in preventing biofilm formation by the two tested strains.

To understand the cumulative effect of the positive charges and the advantage of clustering these charges on a pillararene scaffold, we synthesized two monomers, **5** and **6**, corresponding to the repeating units of pillar[5]arenes **1** and **3**, respectively. Compounds **5** and **6** were also tested for their biofilm inhibition properties towards the two bacterial strains. Monomers **5** and **6** were tested at ~5-fold higher concentrations than were compounds **1–4** such that the numbers of charges and ionic strengths of the tested solutions were comparable. Up to 317 μM of **5** and 340 μM of **6** (160 μg mL^{-1} of **5** and **6**), neither **5** nor **6** caused a measurable inhibition of biofilm formation (Fig. 1c), suggesting that in these anti-biofilm agents the cumulative charge organization on the pillar[5]arene scaffold is a crucial factor for the observed activity. This may be the manifestation of the multivalency effect.[19]

Many cationic amphiphiles act as antimicrobial agents that kill bacteria.[9,20] Therefore, to evaluate whether the inhibiting effect of compounds **1–4** on biofilm formation originated from

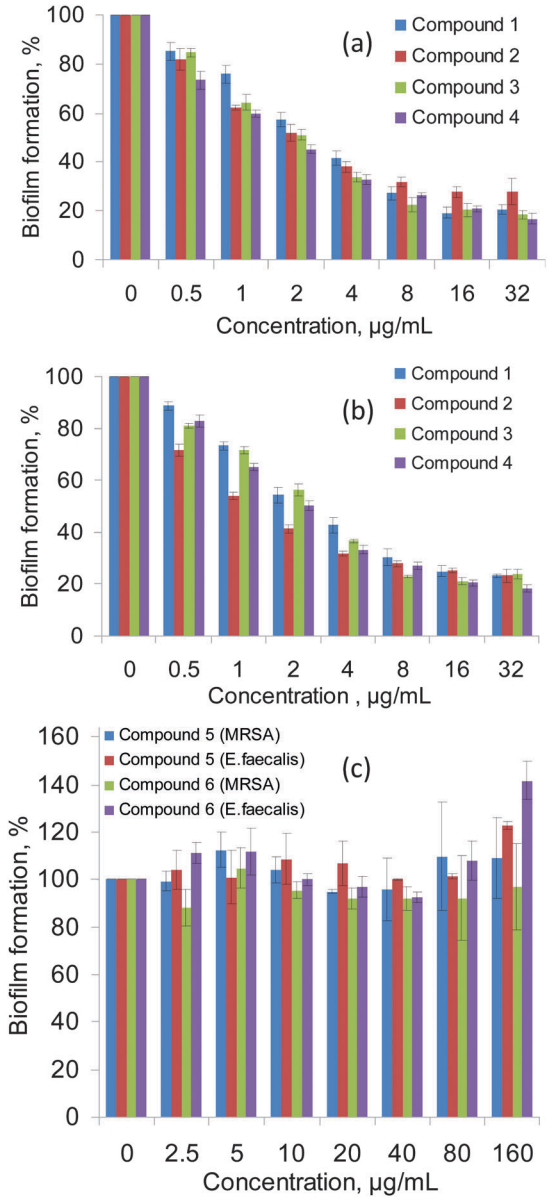


Fig. 1 (a and b) Biofilm formation by (a) *S. aureus* ATCC 33592 (MRSA) and (b) *E. faecalis* ATCC 29212 evaluated using the double-dilution method with starter inoculum of 1 : 100 (OD600 = 0.01) in the presence of compounds **1–4**. (c) Biofilm formation in the presence of compounds **5** and **6**. Concentration ranges of the tested compounds were: (**1**) 0.19–12.40, (**2**) 0.17–10.66, (**3**) 0.21–13.27, (**4**) 0.18–11.30, (**5**) 4.95–317 and (**6**) 5.3—340 μM.

a possible antimicrobial activity of these compounds we measured the minimal inhibitory concentrations (MICs) against the tested strains. The MIC values for compounds **1–4** were found to be 25, 21, 27, and 23 μM, respectively, more than 16 fold higher than the highest $MBIC_{50}$ values measured for these compounds against the two tested strains. These results demonstrate that the inhibition of biofilm formation by the phosphonium-decorated pillararenes **1** and **2** did not originate from antibacterial activity.

The stability of a bioactive compound may affect the molecule performance. To address this issue the stability of the new phosphonium and ammonium pillar[5]arene derivatives was evaluated by incubation for 4 hours in solutions at different pH values. Thereafter the materials were freeze dried, inspected by ^{1}H-NMR and tested for their biofilm inhibition properties. No significant decomposition was observed in the ^{1}H-NMR spectra recorded after exposure to acidic or alkaline pH (see Fig. S11 in ESI†). More importantly the anti-biofilm activities of compounds **2** and **4** remained unchanged after these exposures as seen in Fig. S12 and Table S1 (ESI†).

Finally, it is well established that many cationic amphiphiles disrupt mammalian cell membranes, which limits potential for clinical utility.[8b] We therefore determined the haemolytic effect of pillar[5]arenes **1** and **2** on rat red blood cells (RBCs). Up to a concentration of 85 μM, none of the phosphonium-decorated pillar[5]arenes caused measurable haemolysis of RBCs. In addition, compounds **1–3** were found to have no effect on mammalian cell viability up to a concentration of 128 μg mL^{-1} as shown in Fig. S13 (see ESI†). These findings are in accordance to our previous results.[13]

To conclude, we synthesized four water-soluble cationic pillar[5]arenes capable of inhibiting biofilm formation at sub μM concentrations without affecting the tested bacterial cell and mammalian cell viability or causing measurable damage to the membranes of mammalian RBCs. The phosphonium-decorated pillar[5]arenes showed similar potencies as inhibitors of biofilm formation as their corresponding ammonium analogues, demonstrating that the number of positively charged groups and not their chemical identity are key to their antibiofilm activity. The pillararene platform appears to be important and positive charges operating cooperatively are needed for effective antibiofilm activity as shown by our finding that the respective cationic monomers were completely inactive. We also demonstrated that the reported cationic pillar[5]arene derivatives retained their antibiofilm capability even after 4 hours of exposure to acidic or alkaline pH. More studies are undergoing in our laboratory to understand the mechanistic aspects and the role of host–guest properties of pillar[*n*]arene derivatives in the inhibition of biofilm formation.

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