Peptidomimetic polyurethanes disrupt surface established bacterial biofilms and prevent biofilm formation

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ABSTRACT: Over 80% of all chronic bacterial infections in humans are associated with biofilms, which are surface-associated bacterial communities encased within a secreted exopolysaccharide matrix that can provide resistance to environmental and chemical insults. Biofilm formation triggers broad adaptive changes in the bacteria, allowing them to be almost a thousand-fold more resistant to conventional antibiotic treatments and host immune responses. The failure of antibiotics to eliminate biofilms leads to persistent chronic infections and can promote the development of antibiotic-resistant strains. Therefore, there is an urgent need to develop agents that effectively prevent biofilm formation and eradicate established biofilms. Herein, we present water-soluble synthetic peptidomimetic polyurethanes that can disrupt surface established biofilms of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*, all of which show tolerance to the conventional antibiotics polymyxin B and ciprofloxacin. Furthermore, these polyurethanes prevent bacterial attachment and stimulate bacterial surface motility to inhibit biofilm formation of both Gram-positive and Gram-negative bacteria at sub-inhibitory concentrations, without being toxic to mammalian cells. Our results show that these polyurethanes show promise as a platform for the development of therapeutics that target biofilms and modulate surface interactions of bacteria for the treatment of chronic biofilm-associated infections and as antibiofilm agents.

INTRODUCTION

In nature, bacteria generally exist as part of highly organized, surface-attached communities of cells protected by a hydrated, self-secreted polymeric matrix known as biofilms.¹ According to the US National Institutes of Health (NIH), biofilms are associated with approximately 65% of all microbial infections and over 80% of all chronic infections in humans, which include catheter and device-associated infections, endocarditis, cystic fibrosis, and chronic wounds among others.²⁻³ These infections are hard to treat and often recurrent, leading to elevated patient morbidity and mortality, thus posing a significant healthcare burden.⁴⁻⁵ The estimated annual direct cost of biofilm-associated infections in the US is \$94 billion with almost 500,000 deaths annually.⁶ The current standard of care for these chronic infections is long-term antibiotic treatment with multiple antibiotics.⁷ However, treatment of these infections is extremely challenging because within a biofilm bacteria are highly tolerant to conventional antibiotics and biocides and can also evade host immune responses.^{2, 8-10} The extended use of antibiotics to treat such infections also increases the development of antibiotic-resistant strains, further aggravating the problem of bacterial persistence in these infections.¹¹⁻¹² Thus there is an urgent need to develop agents that can address the threat posed by biofilms in healthcare settings.

While significant research has been dedicated to preventing biofilm formation on surfaces,¹³ removing surface established biofilms remains a challenge. Antibiofilm strategies involving debridement,¹⁴⁻¹⁵ bacterial signaling inhibitors,¹⁶⁻¹⁸ EPS degrading agents,¹⁹⁻²¹ bacteriophages,²² combinatorial antibiotic therapies,²³⁻²⁴ and antimicrobial peptides²⁵⁻²⁶ and polymers,²⁷⁻³⁰ are currently being investigated. Of these, antimicrobial peptides and polymers have emerged as promising candidates, due to their broad-spectrum antimicrobial activity, low incidence of

resistance development, and the ability to kill stationary phase ‘persister’ cells through membrane-active mode of action. Host defense antimicrobial peptides like LL-37 and lactoferrin,³¹⁻³² and synthetic peptides reported by Hancock and coworkers³³⁻³⁵ have been shown to disperse mature biofilms and influence bacterial social behaviors like quorum sensing, swarming and twitching motility. However, peptides inherently suffer from high susceptibility to proteolytic degradation, pH sensitivity, and undesired serum-protein interactions, thus making their clinical translation difficult.³⁶ Synthetic peptidomimetic polymers have been shown to overcome some of these challenges while having comparable antimicrobial activities.³⁷ For instance, Halder and coworkers have developed membrane active polymers and antimicrobial compounds that can kill biofilm bacteria at concentrations $\geq 10\times$ MIC (Minimum inhibitory concentration of planktonic bacteria).³⁸⁻³⁹ Antimicrobial polycarbonates reported by Yang and coworkers have been shown to kill planktonic bacteria and disrupt biofilms of *S. aureus* and *E. coli* at concentrations of $8\times$ MIC ($\sim 500\text{ }\mu\text{g/mL}$) or above.²⁹ Boyer and coworkers have incorporated nitric oxide as a biofilm dispersing agent into antimicrobial polyacrylates to show synergism in clearance of immature (grown for 6.5 h) *P. aeruginosa* biofilms at concentrations as low as $16\text{ }\mu\text{g/mL}$.^{28, 40} However, the focus in the examples listed above was on designing polymers that can kill multidrug-resistant planktonic bacteria, and not on disrupting biofilms. The few antimicrobial polymers that have been reported to disrupt biofilms are either active against immature biofilms,^{28, 40} or are needed in high concentrations ($\geq 8\times$ MIC) to disrupt established biofilms,^{29, 38-39} which can potentially lead to high host-cell toxicity due to their membrane-active nature.⁴¹ The mechanism of antibiofilm action of these polymers, which may be different from the antimicrobial mechanism of action, has also not been investigated in detail.²⁵

We have previously reported a library of synthetic peptidomimetic antimicrobial polymers with tunable selectivity and low cytotoxicity.⁴²⁻⁴⁵ In this study, we focused on variants of these polymers with activity against biofilms and systematically explored the mechanisms behind their antibiofilm characteristics through structure-property relationships. Inspired by natural³¹⁻³² and synthetic^{33-35, 46} antibiofilm peptides reported in the literature, we designed polyurethanes using our pendant-functionalized, amino acid mimetic monomer library to understand how individual amino acid mimetic units would influence the activity of the polyurethane against biofilms, and the surface interactions of bacteria preceding biofilm formation. The polyurethanes were designed to have arginine-mimetic or lysine-mimetic groups as their cationic groups, while the hydrophobicity was modulated by using mimics of phenylalanine and alanine as pendant groups to impart amphiphilicity to the polymer. Our data suggest that differences in the polyurethane pendant functional group significantly alter the surface-associated bacterial behavior like attachment, surface motility, and biofilm formation.

RESULTS AND DISCUSSION

Figure 1. Synthetic scheme of the peptidomimetic polyurethanes with mLys and mArg as the charged pendant groups and mPhe and mAla as the hydrophobic pendant groups. The polymers were synthesized with charge to hydrophobicity ratio of 60/40. Reagents and reaction conditions: (i) Tin (II) octoate, CH₂Cl₂, at room temperature, 12 h; (ii) 4 N HCl in dioxane, CH₂Cl₂, at room temperature, 45 min (iii) 1H-Pyrazole-1-carboxamide hydrochloride, triethylamine, DMF at 60 °C, 6 h.

Polyurethane design and synthesis

The polyurethanes reported in this work were designed to mimic synthetic antibiofilm peptides.^{26, 33-35} While there is no design consensus as to which structural features contribute to the mechanism of action against biofilms, antibiofilm peptides reported in the literature are charged, amphiphilic molecules having 40–50% cationic residues, along with some highly hydrophobic residues present in a short peptide sequence. Based on that, we chose 60/40 ratio of charge/hydrophobicity to mimic structural aspects of these peptides while ensuring polymer solubility in various growth media. The polyurethanes were synthesized using a simple, one-pot step-growth polymerization of *N*-functionalized diethanolamide monomers with hexamethylene diisocyanate using methods previously optimized in our lab, and detailed in the experimental section (Figure 1).⁴³⁻⁴⁴ The monomers were synthesized as reported previously and were designed to be structural mimics of lysine (mLys), alanine (mAla), and phenylalanine (mPhe).^{44, 48} To mimic arginine (mArg), the mLys units were guanylated after the polymerization using 1H-Pyrazole-1-carboxamide hydrochloride in the presence of triethylamine in DMF. The boc-deprotection and guanylation were confirmed using ¹H NMR of the obtained polymers (Figure S1 B). The copolymer ratio of 60/40 charged/hydrophobic units was achieved by appropriate feed ratio of the co-monomers and the composition of the synthesized polymer was confirmed by NMR (Figure S1 A). Table 1 shows that the molar masses (*M_n*) of the polyurethanes range between 9–10 kg/mol. Since the molar mass and hydrophobicity of the polymer directly influence its toxicity towards mammalian cells,⁴³⁻⁴⁴ we minimized the cytotoxicity of the designed polymers by synthesizing polymers with lower molecular weights to compensate for the addition of hydrophobic pendant groups.

Inhibition of biofilm formation and disruption of surface established biofilms

Table 1. Summary of minimum biofilm inhibitory concentration (MBIC) and hemolytic (HC₅₀) activities of polymers and antibiotics against *P. aeruginosa*, *S. aureus* and *E. coli*

Polymer or antibiotic	MBIC (μg/mL) (<i>P. aeruginosa</i>)	MBIC (μg/mL) (<i>S. aureus</i>)	MBIC (μg/mL) (<i>E. coli</i>)	HC ₅₀ (μg/mL)	M _n (g/mol) [Đ]
mLys/mPhe	8	8	2	260.5	10476 [1.77]
mLys/mAla	8	16	4	>1250	9326 [1.72]
mArg/mPhe	4	4	2	197.5	~10476 [1.77] ^a
mArg/mAla	4	4	2	551.6	~9326 [1.72] ^a
Polymyxin B	0.8	625	0.8	-	1301.56
Ciprofloxacin	0.2	0.08	0.09	-	733.93

^amArg polymers were functionalized post-polymerization from their mLys counterparts.

Table 2. Summary of minimum inhibitory concentration (MIC) of polymers and antibiotic controls against *P. aeruginosa*, *S. aureus*, and *E. coli*

Polymer or antibiotic	MIC in MHB (μg/mL)			MIC in BM2 MM (μg/mL)			MIC in LB (μg/mL)
	(<i>P. aeruginosa</i>)	(<i>S. aureus</i>)	(<i>E. coli</i>)	(<i>P. aeruginosa</i>)	(<i>S. aureus</i>)	(<i>E. coli</i>)	(<i>P. aeruginosa</i>)
mLys/mPhe	>250	>250	>250	8	64	2	>250
mLys/mAla	>250	>250	>250	16	>250	16	>250
mArg/mPhe	>250	>250	125	4	4	2	>250
mArg/mAla	>250	>250	>250	4	4	4	>250
Polymyxin B	0.8	625	0.4	0.8	625	0.4	0.8
Ciprofloxacin	1.56	0.08	0.4	0.2	0.08	0.1	1.56

We first tested the ability of these polymers to prevent biofilm formation on abiotic surfaces against three common, biofilm-forming opportunistic bacterial pathogens, viz., *P. aeruginosa*, *S. aureus*, and *E. coli*, in BM2 minimal medium supplemented with 0.4% glucose and 0.5% casamino acids (which supports biofilm formation of all three species studied). The MBIC experiment as described in the experimental section of the supporting information, was performed by growing the biofilms in increasing polymer concentration, with polymyxin B sulfate and ciprofloxacin as antibiotic controls. All polymer compositions tested showed broad-spectrum biofilm inhibition at low concentrations for *P. aeruginosa* (MBIC = 4–8 μg/mL; Table 1), *S. aureus* (MBIC = 4–16 μg/mL; Table 1) and *E. coli* (MBIC = 2–4 μg/mL; Table 1). While the pendant group hydrophobicity did not have a significant impact on the MBIC values, there was a notable influence of the cationic group on biofilm inhibition, with mArg containing polyurethanes. (mArg/mAla and mArg/mPhe) having a biofilm inhibitory effect at 2–4 μg/mL for all tested species of bacteria compared to their mLys counterparts.

Since these polymers share design features with antimicrobial polymers, we also evaluated their antimicrobial activity against planktonic bacteria using the modified broth microdilution method.^{42–44} The MIC of the polymers was determined in MHB, LB, and BM2 minimal medium. As depicted in Table 2 the polymers showed no inhibitory activity against *P. aeruginosa*, *S. aureus*, or *E. coli* in MHB or LB until 250 μg/mL above which they could not be tested due to their poor solubility in LB and MHB. However, in BM2 minimal media, the polymers inhibited the growth of all three bacteria. mLys polymers had selective activity towards Gram-negative pathogens *P. aeruginosa* and *E. coli* (MIC ~ 2–16 μg/mL) while having high inhibitory concentrations or no activity towards Gram-positive *S. aureus* (MIC = 64 μg/mL for mLys/mPhe and MIC >250 μg/mL for mLys/mAla), while mArg

polymers had a broad-spectrum activity with MIC $\sim$ 2–4 $\mu\text{g/mL}$ for both Gram-positive and Gram-negative bacteria. This trend in the MIC corroborates our previous reports on a similar polyurethane platform.^{43–44} Interestingly, the type of growth media did not affect the inhibitory activity of lipopeptide polymyxin B (MIC = 0.8 $\mu\text{g/mL}$ against *P. aeruginosa* and 0.4 $\mu\text{g/mL}$ against *E. coli*), while ciprofloxacin had higher MIC values in LB and MHB than in BM2 minimal media.

Figure 2. (A) Experimental workflow of biofilm disruption assay (B) Fluorescence confocal micrographs of *preformed P. aeruginosa* biofilms treated with polymyxin B (4 $\mu\text{g/mL}$ $\sim$ 5 $\times$ MBIC), ciprofloxacin (1 $\mu\text{g/mL}$ $\sim$ 10 $\times$ MBIC), and polyurethanes (16–32 $\mu\text{g/mL}$, $\sim$ 4 $\times$ MBIC). Permeabilized cells, indicative of cell death, stain red. Intact cells, indicative of viable cells, stain green. Scale bar represents 100 μm . (50 μm for the inset images).

While the prevention of biofilm formation on surfaces is desirable and has been a major topic of research, the removal of surface established biofilms remains a challenge.⁴⁹ *P. aeruginosa* is a notorious biofilm-forming pathogen, implicated in several biofilm-associated debilitating infections. Under laboratory conditions, *P. aeruginosa* readily forms dense biofilms within 24 h of inoculation in BM2 minimal media. We exposed these surface-established biofilms to our polymers and antibiotics at different concentrations and imaged them using a confocal microscope. As depicted in Figure 2 B, the antibiotics polymyxin B (at 4 $\mu\text{g/mL}$, $\sim$ 5 $\times$ MIC) and ciprofloxacin (at 4 $\mu\text{g/mL}$, $\sim$ 10 $\times$ MIC) were unable to completely disrupt *P. aeruginosa* biofilms. Furthermore, ciprofloxacin exposure led to the formation of elongated, filamentous cells that have been implicated in the development of antibiotic resistance.^{9, 50–51} These observations support what has been well established in literature: while antibiotics are very effective in killing planktonic cells, they fail to adequately target and destroy biofilms, leading to persistent and chronic infections.¹¹ However, the polymers mLys/mPhe and mLys/mAla were able to disrupt the *P. aeruginosa* biofilms, including cell debris, from the surface at 32 $\mu\text{g/mL}$ ($\sim$ 4 $\times$ MBIC). Biofilm treated with mArg/mPhe at 16 $\mu\text{g/mL}$ ($\sim$ 4 $\times$ MBIC) had bacterial aggregates that stained red, indicative of permeabilized cells while mArg/mAla treated biofilm had sparsely distributed viable cells attached to the surface (Figure 2 B). Higher concentrations of the polymers lead to the complete disruption of surface biofilms. The polymers were also able to disrupt *S. aureus* and *E. coli* biofilms as depicted in Figure S2, indicating broad-spectrum action against biofilms. Our results show that while these polyurethanes do not have significant growth inhibitory activity against

planktonic *P. aeruginosa*, *S. aureus*, or *E. coli*, they can inhibit their biofilm formation as well as disrupt established biofilms.

Influence on bacterial adhesion and surface motility

Figure 3. (A) Live/dead fluorescence images of *P. aeruginosa* cells attached to glass coverslips in presence of control, antibiotics, and polymers. (B) Graphical representation of attachment of *P. aeruginosa* on glass in presence of sub-MBIC antibiotics and polymers (mLys/mPhe and mLys/mAla are at 2 µg/mL and mArg/mPhe and mArg/mAla are at 1 µg/mL (0.25× MBIC). Polymyxin B is at 0.5 µg/mL and ciprofloxacin at 0.1 µg/mL (0.5× MBIC). The cell counts per mm² area are shown as mean ± SD. Green bars in the graph correspond to number of bacteria fluorescing green, indicative of intact cells, while red bars correspond to bacteria fluorescing red, indicating permeabilized cells.

Biofilm formation is a dynamic, multistep process that begins with surface detection, followed by attachment of the bacteria to the surface.⁴⁹ Filamentous protein structures such as pili and flagella are employed by the bacteria for surface detection, translocation, and attachment.⁸ We hypothesized that the ability of the polymers to inhibit biofilm formation and disrupt established biofilms of a broad spectrum of bacteria could be due to the influence of the polymers on these surface interactions. To study surface-attachment, *P. aeruginosa* PAO1 cells were allowed to adhere to a glass substrate in BM2 minimal media containing known concentrations of polymers or antibiotics. As depicted in Figure 3 B, *P. aeruginosa* in BM2 minimal media rapidly attached to the glass surface within 3 h, reaching approximately 2×10^4 cells/mm². In presence of 0.5 µg/mL (about 0.5× MBIC) of polymyxin B, the number of attached bacteria reduced to approximately 1×10^4 cells/mm², with a significant number of viable cells that fluoresced green. Bacteria in the presence of sub-inhibitory concentrations of ciprofloxacin (0.1 µg/mL, 0.5× MBIC) also had viable cells that were almost 100× elongated and appeared as filaments. For Gram-negative bacteria, filamentation in the presence of sub-inhibitory concentrations of ciprofloxacin has been reported in the literature to occur via the SOS pathway, a state of rapid DNA repair and mutagenesis, implicated in the development of antibiotic resistance.⁵⁰ The elongation of attached cells was not observed in the case of polymers or polymyxin B (Figure 3 A). On the other hand, the polymers were able to reduce the attachment of bacteria to only a few hundred cells/mm² at as low as 0.25× MBIC (~1–2 µg/mL). Among the polymers, mLys/mPhe at 2 µg/mL had the least number of surface-attached cells, all of which fluoresced red, indicating non-viability. mArg/mAla at 1 µg/mL had the most surface-attached cells, with $\sim 1 \times 10^3$ cells/mm², having a mix of both viable

and non-viable cells. At concentrations higher than 2 $\mu\text{g/mL}$, no cells were observed to attach to the glass surface in the presence of polymers within the 3 h period of study. At concentrations higher than 2 $\mu\text{g/mL}$ bacteria did not attach to the surface and hence biofilm formation was prevented, which could explain why the polymers inhibit biofilm formation at concentrations of 4–8 $\mu\text{g/mL}$.

Figure 4. (A) Experimental workflow for the motility assays. (B) Twitching motility of *P. aeruginosa* in presence of polymers. All polymers were added at 4 $\mu\text{g/mL}$ in LB with 1% agar and circular twitching zones were measured after 20 h incubation at 37 °C. (C) Swarming motility of *P. aeruginosa* PAO1 in presence of polymers. All polymers were added at 4 $\mu\text{g/mL}$ in BM2 swarm plates and circular swarm zones were measured after 16 h incubation at 37 °C. The twitching or swarming distance of each polymer represents the mean $\pm$ SD of three biological replicates. Statistical significance was determined using one-way ANOVA by comparing swarm diameter of each polymer to control. (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$)

Once surface attached, bacteria can either rapidly multiply and secrete extracellular polysaccharides (EPS) to initiate biofilm formation, or they can migrate over the surface to seek better nutrient availability, using mechanisms like twitching or swarming motility.⁵²⁻⁵³ Hence, we also investigated the influence of polymers on bacterial twitching and swarming motilities since they are both social behaviors and have considerable overlap in genes that also regulate the development and maintenance of biofilms.^{52, 54-56} Moreover, both twitching and swarming motility have been well studied for *P. aeruginosa*.^{53, 57} Twitching motility provides a means for bacteria to rapidly colonize surfaces under conditions of high nutrient availability through ‘grappling hook’ like motions of fibrillar protein structures known as type IV pili. Twitching has been known to play a role in the early stages of biofilm formation and in the disassembly/dispersion of mature biofilms.⁵⁵ Figure 4 B depicts the influence of the polymers on the twitching motility of *P. aeruginosa* at sub-inhibitory concentrations. Polymers mLys/mAla and mLys/mPhe showed a notable increase in twitching compared to the control, with mLys/mPhe causing about 40%

increase in the twitching of *P. aeruginosa*. Polymers with mArg pendant groups had either minimal or no effect on twitching, while polymers with mLys pendant groups caused an increase in twitching motility. These results align with the observations of the adhesion assay, where mLys/mPhe had the least bacteria attached to the surface, which could be in part due to the polymer's ability to stimulate twitching in *P. aeruginosa*. Additionally, twitching motility had a dose-dependent response to concentration for all polymers, and at higher concentrations around 16–32 $\mu\text{g/mL}$, twitching motility was significantly reduced (Figure S3).

Swarming motility is a group translocation involving a coordinated and rapid movement of the bacteria on a semi-solid surface. It involves both the flagella and pili along with the secretion of biosurfactants and is often oppositely regulated to biofilm formation.⁵⁸ Interestingly mLys/mPhe did not affect the swarming of *P. aeruginosa*, while mLys/mAla caused a slight increase in swarming (Figure 4 C). However, polymers with mArg pendant group, namely mArg/mAla and mArg/mPhe, markedly reduced the swarming of *P. aeruginosa*. Increasing polymer concentrations had no significant effect on the swarming up until high concentrations of about 64–128 $\mu\text{g/mL}$, at which point there was a reduction in swarming diameter, likely due to inhibition of bacterial growth by the polymers (Figure S4). Additionally, swarming motility involves the secretion of biosurfactants that facilitate bacterial motion by reducing the surface tension. The polymers in our study are amphiphilic molecules and may behave like surfactants. To ascertain that the differences in swarming in presence of these polymers were not due to differences in their surface-active properties, we measured the surface tension of the polymer solutions at different concentrations (Figure S5). Up until 32 $\mu\text{g/mL}$, there was no significant decrease in the surface tension of the polymer solutions compared to that of pure water. The surface tension decreased only at much higher concentrations, plateauing close to 5 mg/mL with a surface tension of about 55 mN/m for all polymers tested. This suggests that the differences in swarming of *P. aeruginosa* observed in the presence of mArg and mLys polymers are not due to differences in their surfactant-like behavior since these polymers do not significantly alter the surface tension of water at concentrations below 500 $\mu\text{g/mL}$.

Hemolysis and cytotoxicity

Figure 5. Cytotoxicity to Human Dermal Fibroblasts (HDFs) measured by LDH assay after exposure to polymers for (A) 1 h (B) 24 h. Percent cytotoxicity relative to controls is represented as mean $\pm$ standard deviation of three biological replicates. (C) Hemolysis of sheep red blood cells in presence of polymers over a broad range of concentrations (2–1250 $\mu\text{g/mL}$). HC_{50} , polymer concentration to cause 50% hemolysis is reported in the inset. mLys/mAla caused $<2\%$ hemolysis for all concentrations tested and so HC_{50} could not be determined.

To understand the potential of these polymers as therapeutics, mammalian cell toxicity of the polymers was measured by red blood cell hemolysis (Figure 5 C) and HDF cytotoxicity (Figure 5 A, B) at different polymer concentrations. Hemolysis in the presence of the polymers was evaluated by quantifying the relative hemoglobin released at the test concentration compared to 1% Triton X-100, which was assumed to cause 100% hemolysis. HC_{50} , defined as the polymer concentration leading to 50% hemolysis, was determined as shown in Figure 5 C, with lower HC_{50} values indicating higher toxicity to red blood cells. Polymer mLys/mAla caused <2% hemolysis even at 1250 $\mu\text{g/mL}$, beyond which it began to precipitate out of blood, rendering its HC_{50} indeterminable. All tested polymers showed significant hemolytic activity only at higher concentrations (HC_{50} for mLys/mPhe $\approx$ 260.5 $\mu\text{g/mL}$, HC_{50} for mLys/mAla > 1250 $\mu\text{g/mL}$, HC_{50} for mArg/mPhe $\approx$ 197.5 $\mu\text{g/mL}$, HC_{50} for mArg/mAla $\approx$ 551.6 $\mu\text{g/mL}$) compared to their biofilm-inhibitory concentrations, showing desirable activity within a potential therapeutic window. However, in a very clear trend, polymers with mArg caused significantly more hemolysis than their mLys counterparts, which corroborated our previous reports on a similar polyurethane platform. In accordance with multiple reports in the literature,^{42, 59-61} the hemolysis was observed to be significantly higher for polymers with the more hydrophobic mPhe pendant group compared to the polymers with mAla, indicating that even with the same overall cationic charge, increasing hydrophobicity increases the hemolytic activity of the polymers.

To further understand the toxicity posed by the polymers to mammalian cells, we investigated the cytotoxicity of the polymers on HDFs (Figure 5 A, B). HDF cytotoxicity, as measured by the LDH assay, followed a similar trend as hemolysis, with the more hydrophobic mPhe polymers showing higher toxicity than the mAla containing polymers. Interestingly, polymers with mArg showed lower toxicity than the mLys polymers as opposed to the results of the hemolysis assay. Furthermore, the polymers were much less cytotoxic to HDFs than they were to red blood cells, with a maximum cytotoxicity value of 38% shown by mLys/mPhe at 500 $\mu\text{g/mL}$ for 1 h, which remained the same after 24 h of exposure. The lower toxicity of the polymers can be attributed to HDFs being more resilient to damage than the red blood cells. While mLys/mAla did not show any cytotoxicity at 1 h for all concentrations tested, it did cause significant cytotoxicity at 24 h (51.5% at 500 $\mu\text{g/mL}$). This indicates that the polymers have a time and concentration dependent cytotoxicity only at concentrations much higher than their biofilm-active concentrations against both HDFs and RBCs, which indicates that their potential as suitable candidates for antibiofilm therapeutics merits further investigation.

CONCLUSIONS

We report on a class of synthetic, peptidomimetic polyurethanes that disrupt surface established biofilms of a broad spectrum of clinically relevant bacteria without being toxic to mammalian cells. At sub-inhibitory concentrations, these polyurethanes modulate surface interactions of bacteria by influencing motility, such as swarming and twitching, and prevent attachment of the bacteria to surfaces, thus inhibiting the biofilm development. While all the polymers tested had antibiofilm activity, their ability to modulate bacteria-surface interactions was a function of their cationic pendant group. mArg polymers reduced bacterial swarming while mLys polymers enhanced twitching of *P. aeruginosa* at sub-MBIC concentrations. Since the development of resistance in bacteria remains a challenge with antimicrobial and bactericidal agents, the studies described here can serve as the basis for designing polymers that can disperse biofilms without inducing resistance in bacteria. We also urge the antimicrobial polymer community to look beyond the membrane-active bactericidal properties of synthetic cationic polymers. Our results show these polymers may influence bacterial interactions with their environment even at sub-inhibitory concentrations. While not effective in killing bacteria, the polymers can still be used as a viable strategy to reduce or inhibit the expression of virulence factors that lead to surface attachment, biofilm formation, or surface motility and have the potential to increase severity of an infection. Further investigation can give us insights into the mechanisms by which these polymers influence bacteria-surface interactions, and the role of different chemical functionalities present in the polymers in modulating such interactions. Our findings present a potential strategy not only for the development of biofilm disrupting agents but also for manipulating the bacterial behavior on surfaces with applications in the field of biomaterials, antimicrobial development, bioenergy, and biofouling.

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Supporting Information

EXPERIMENTAL

Materials

All materials and solvents were used as received unless described otherwise. Tin(II) 2-ethylhexanoate ($\text{Sn}(\text{Oct})_2$), 1*H*-Pyrazole-1-carboxamidine hydrochloride, *N,N*-diethylethanamine, sodium phosphate dibasic, potassium phosphate monobasic, potassium chloride, calcium chloride, iron(II) sulfate, ammonium sulfate, magnesium sulfate, crystal violet, polymyxin B sulfate, and Triton X-100 were purchased from Sigma-Aldrich (St Louis, MO, USA). Di-*tert*-butyl dicarbonate was purchased from Oakwood Chemical (Estill, SC, USA). Diethanolamine, 3-phenylpropionic acid, 6-aminohexanoic acid, and D(+)-glucose were obtained from Alfa Aesar (Ward Hill, MA, USA). Hexamethylene diisocyanate (HDI) and anhydrous 4 N HCl in dioxane were purchased from Acros Organics (Fair Lawn, NJ, USA). Dimethyl sulfoxide (DMSO) and sodium chloride were purchased from BDH Chemicals (Dubai, UAE). Anhydrous methanol, triethylamine, and dimethylformamide (DMF) were purchased from EMD Millipore (Billerica, MA, USA). Thionyl chloride and ciprofloxacin hydrochloride were obtained from TCI Chemicals (Tokyo, Japan). Methylene chloride was dried by distillation after drying with CaH_2 and stored over molecular sieves. Mueller Hinton broth (MHB) was purchased from Himedia (Mumbai, India), trypticase soy broth (TSB) was purchased from Becton-Dickinson (Franklin Lakes, NJ, USA) while lysogeny broth (LB) and casamino acids were obtained from Fisher Scientific (Hampton, NH, USA). Agar was added separately to the medium and was purchased from Sigma-Aldrich (St Louis, MO, USA). Viability/cytotoxicity assay kit for staining bacteria was purchased from Biotium (Fremont, CA, USA). *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), and *Pseudomonas aeruginosa* (PAO1) were obtained from the American Type Culture Collection (ATCC). For the hemolysis assay, defibrinated sheep blood was purchased from Hardy Diagnostics (Santa Maria, CA, USA). For mammalian cell culture, penicillin/streptomycin, fetal bovine serum (FBS), and Dulbecco's Modified Eagle Medium (DMEM) were obtained from HyClone (Chicago, IL, USA). The Pierce™ LDH cytotoxicity assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Human Dermal Fibroblasts (HDFs) were kindly donated by Dr. Nic Leipzig, University of Akron.

Instrumentation

^1H NMR spectra were acquired using a Varian Mercury 300 MHz spectrometer. The ^1H NMR chemical shifts are reported in ppm relative to the residual proton signal in the deuterated solvent. Polymer molecular weight was determined via size exclusion chromatography (SEC) on a TOSOH EcoSec HLC-8320 instrument equipped with an RI detector and two PSS Gram Analytical SEC columns in series with 25 mM LiBr in DMF as the mobile phase at a flow rate of 0.8 mL/min. For all the measurements, the column and detector were kept at 50 °C, and the molecular weights were estimated using a polystyrene standard curve. BioTek Synergy H1 microplate reader was used for all UV-visible absorbance measurements. Olympus FLUOVIEW FV1000 laser scanning microscope was used to image bacteria and biofilm samples. Ramé-hart contact angle goniometer was used for surface tension experiments. For microbiology experiments, the optical density of bacterial suspension at 600 nm (OD_{600}) was determined using a Hach DR 2800™ spectrophotometer.

Synthesis of monomers and polymers

The synthesis of all the monomers and polymers was done as previously described.^{43, 47-48} Briefly, the monomeric diols were synthesized by amidation of diethanolamine with methyl esters of desired pendant group and purified by silica gel column chromatography using a mixture of methylene chloride and methanol as the mobile phase. The purified diol monomers were solution polymerized with hexamethylene diisocyanate using tin(II) 2-ethylhexanoate as the catalyst and anhydrous methylene chloride as the solvent for 12 h at room temperature under N_2 flow to yield polymers described in **Scheme 1**. The polymers were dialyzed against methanol for 24 h and dried under vacuum overnight and later characterized using ^1H NMR and SEC. Deprotection of pendant amine groups was done using 4N HCl in dioxane as previously described. Guanylation of the amine groups was performed as described previously by reacting the polymers with 1*H*-Pyrazole-1-carboxamidine hydrochloride in presence of triethylamine at 60°C for 6 h in DMF.⁴³

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Biofilm Inhibitory Concentration (MBIC)

The broth microdilution method as reported previously was used to measure MIC and MBIC with some modifications.^{33, 44} In brief, TSB (3 mL), was inoculated with an overnight culture of bacteria and incubated at 37 °C with shaking (200 rpm) for 3 h until the culture reached the mid-log phase. The culture was then diluted into either 2× MHB, 2× LB, or 2× BM2 minimal medium (124 mM pH 7; potassium phosphate buffer, 4 mM MgSO₄, 20 μM FeSO₄, 0.8% (w/v) glucose, 0.5% (w/v) casamino acids) to yield a working stock of approximately 1×10^6 CFU/mL. The bacterial stock was added in a 1:1 ratio to serially diluted polymer solutions in sterile polypropylene 96-well plates. After initial determination OD₆₀₀, the plates were sealed with breathable membrane and incubated for 16–18 h at 37 °C under stationary conditions after which the OD₆₀₀ was measured again. The lowest concentration that prevented bacterial growth, measured by an increase in OD₆₀₀ was reported as the MIC. For the determination of MBIC, the bacteria were grown in 2× BM2 minimal medium in the presence of polymers. Biofilms were then allowed to form under static conditions for 18 h at 37 °C. Post incubation, planktonic cells were gently removed by washing with 1× phosphate-buffered saline (PBS) using a pipette, and the adhered biofilm was stained with 0.1% crystal violet solution for 20 min. After staining, the excess dye was removed and crystal violet from the biomass was extracted with 100% ethanol. The absorbance at 595 nm was measured using a BioTek Synergy H1 microplate reader. Minimum polymer concentration leading to 90% decrease in biofilm biomass compared to the untreated control was reported as the MBIC. Each experiment was done in triplicate and repeated at least three times.

Biofilm disruption assay and microscopy

Bacterial stocks of about 1×10^6 CFU/mL as described above, were prepared in BM2 minimal medium and glass coverslips of 12 mm diameter were used as substrates for biofilm formation. These coverslips were sterilized by UV irradiation and placed inside the wells of a sterile 12-well plate. The bacterial suspension (2 mL) was added to each well and the well plate was incubated at 37 °C under stationary conditions for 24 h. Post incubation, the biofilm containing coverslips were gently washed with pH 7, 1× PBS to remove planktonic bacteria and then transferred to a sterile 12-well plate. A solution of the polymer in BM2 minimal medium (2 mL) at the desired concentration was added to each well and the well plate was incubated again for 24 h at 37 °C under stationary conditions. BM2 minimal media (2 mL) without polymers served as the negative control for the experiment. After 24 h, the coverslips were washed with 1× PBS to remove planktonic bacteria and stained with Biotium viability/cytotoxicity assay kit according to the manufacturer's instructions. The samples were mounted on a slide and imaged using Olympus FLUOVIEW FV1000 laser scanning microscope with 488 nm and 559 nm laser lines. The confocal stacks were processed with FLUOVIEW 3.0 and ImageJ.

Determination of bacterial adhesion in presence of polymers

Glass coverslips of 12 mm diameter were used as substrates to study bacterial adhesion. The stock culture of *P. aeruginosa* PAO1 with about 1×10^6 CFU/mL in 2× BM2 minimal medium served as the inoculant. The bacterial inoculant (500 μL) and an aqueous polymer solution of the desired concentration (500 μL) were added in 1:1 (v/v) ratio to each well containing a sterile glass coverslip in a 12-well plate. The well plate was incubated at 37 °C under stationary conditions for 3 h to allow the cells to adhere to the glass. Post incubation, the glass coverslips were rinsed twice with 1× PBS to remove unattached cells, stained with Biotium viability/cytotoxicity assay kit, and imaged using Olympus FLUOVIEW FV1000 laser scanning microscope with 488 nm and 559 nm laser lines. The attached cells were enumerated using ImageJ for both channels separately and reported as the number of cells attached per unit surface area.

Swarming and twitching motility assays

Bacterial swarming assays were performed with *P. aeruginosa* PAO1 on BM2 swarming agar plates (62 mM; pH 7 potassium phosphate buffer, 2 mM MgSO₄, 10 μM FeSO₄, 0.4% (w/v) glucose, 0.25% (w/v) casamino acids, 0.5% (w/v) agar) in the presence or absence of different concentrations of polymers. The autoclaved agar solution was cooled to 60 °C and 10 mg/mL aqueous polymer solution was diluted in it to achieve the desired concentration of the polymer in agar. This mixture was then poured into the wells of 6-well plate and was allowed to cool in a sterile laminar flow hood for 1 h. Then, 1 μL of mid-log phase culture of *P. aeruginosa* PAO1 grown in BM2

minimal medium ($OD_{600} = 0.5$) was used to inoculate the agar surface at the center of each well and the well plate was incubated at 37 °C for 16 h. All resulting circular swarms were analyzed by measuring the swarm diameter using ImageJ and plotted against polymer concentration. Each experiment was performed in triplicate and repeated twice. For studying twitching motility in presence of polymers, 10 mL of LB supplemented with 1% (w/v) agar and the desired polymer concentration was poured into each well of a 6-well plate. A mid-log phase culture of *P. aeruginosa* PAO1 ($OD_{600} = 0.5$) was stabbed through the agar, to the plastic interface using a needle. The agar plate was then incubated at 37 °C for 20 h. Post incubation, the agar was gently removed using a pair of tweezers to expose the twitching zone at the agar-plastic interface. The twitching zone was stained with 0.1% crystal violet solution and analyzed by measuring the twitching diameters with ImageJ. Each experiment was performed in triplicate and repeated twice.

Hemolysis Assay

Hemolysis assay was performed using defibrinated sheep red blood cells (RBCs). The cells were centrifuged at 500g for 10 min at 5 °C and then washed with pH 7.4, 1× PBS until no visually observable hemoglobin was present in the supernatant. The RBC suspension was then diluted in a ratio of 1:50 (v/v) in PBS, which was added to serially diluted aqueous polymer solutions in a 1:1 (v/v) ratio and incubated at 37 °C for 1 h. The wells containing pH 7.4, 1× PBS were used as the negative control and 1% (w/v) aqueous solution of Triton X-100 was used as the positive control. Post incubation, the well-plate was centrifuged at 500g for 10 min at 4 °C and the hemoglobin released was quantified at 450 nm using a BioTek Synergy H1 plate reader. Each sample point was performed in triplicate and percent hemolysis was calculated by using the equation: $\left[\frac{H - H_0}{(H_{Max} - H_0)} \right] \times 100$, where H , H_0 and H_{Max} represent the absorbance of the sample well, negative control, and positive control at 450 nm, respectively. The percent hemolysis derived from the equation was plotted as a function of polymer concentration. The polymer concentrations which caused 50% hemolysis relative to the positive control were reported as HC_{50} .

Lactate Dehydrogenase (LDH) Assay for Human Dermal Fibroblast (HDF) cytotoxicity

The Pierce™ LDH Cytotoxicity Assay was used to determine the cytotoxicity of the polymers against HDFs as the representative mammalian cells. Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin streptomycin at 37 °C in 5% CO₂ until reaching about 90% confluency. Then, the cells were harvested using a 0.25% trypsin solution and seeded into a 96-well plate at a cell density of 1×10^4 cells per well. The HDFs were allowed to attach to the wells overnight before adding the polymer solutions. Post polymer addition, the cells were incubated at 37 °C for 24 h. 50 µL medium was pipetted from the wells to determine the cytotoxicity at 1 and 24 h timepoints according to the instructions provided on the assay kit.

Surface tension measurements

The surface tension of aqueous polymer solutions at different concentrations was determined using a ramé-hart contact angle goniometer equipped with DROPimage software. Milli-Q Ultrapure water and cetrimonium bromide (CTAB) at 625 µg/mL were used as controls and the surface tension was measured to be 72.7 ± 1.2 mN/m and 38.0 ± 0.2 mN/m respectively. The surface tension of the polymers was reported as mean $\pm$ standard deviation of minimum of five measurements and plotted against concentration. Data was fit using a first-order exponential decay function in the Origin software.

Figure S1. ^1H NMR spectrum of (A) Boc-protected mLys/mAla (B) Deprotected mLys/mAla and post polymerization guanylation to obtain mArg/mAla.

S. aureus

E. coli

Figure S2. Fluorescence confocal micrographs of preformed *S. aureus* and *E. coli* biofilms exposed to antibiotics and polymers. (A) *S. aureus* biofilms were exposed to ciprofloxacin (0.8 µg/mL), mLys/mPhe (16 µg/mL), mLys/mAla (32 µg/mL), mArg/mAla (16 µg/mL), mArg/mPhe (16 µg/mL) for 24 hours. (B) *E. coli* biofilms were exposed to polymyxin B (1 µg/mL), ciprofloxacin (1 µg/mL), mLys/mPhe (8 µg/mL), mLys/mAla (32 µg/mL), mArg/mAla (16 µg/mL), mArg/mPhe (16 µg/mL) for 24 hours. Biofilms were stained with Live/Dead bacterial viability kit and imaged on a confocal microscope. Permeabilized cells, indicative of cell death fluoresce red, while intact cells, indicative of viable cells fluoresce green. Scale bar represents 100 µm.

Figure S3 Dependence of polymer concentration on *P. aeruginosa* PAO1 twitching motility. Statistical significance was determined using one-way ANOVA by comparing twitching zones of *P. aeruginosa* in presence of respective polymer concentration to the control. (***) p-value < 0.001)

Figure S4. Dependence of polymer concentration on *P. aeruginosa* PAO1 swarming motility. Statistical significance was determined using one-way ANOVA by comparing the swarm zones of *P. aeruginosa* in presence of each polymer concentration to the control. (** $p < 0.005$, *** p -value < 0.001)

Figure S5. Surface tension as a function of polymer concentration. Each data point represents mean $\pm$ SD of minimum five measurements. Milli-Q Ultrapure water was used as control and the surface tension was measured to be 72.7 ± 1.2 mN/m.