Article

pubs.acs.org/Biomac

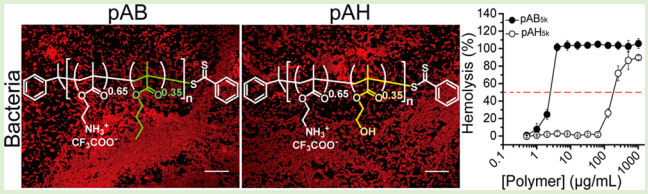
Long Hydrophilic-and-Cationic Polymers: A Different Pathway toward Preferential Activity against Bacterial over Mammalian Membranes

Xin Yang,[§,¶,‡] Kan Hu,[§,¶,‡] Guantai Hu,[§,#] Danyao Shi,[§,#] Yunjiang Jiang,[§,¶,†] Liwei Hui,[§,¶] Rui Zhu,[§,¶] Yuntao Xie,[¶,⊥] and Lihua Yang*[,§,¶,#]

[§]CAS Key Laboratory of Soft Matter Chemistry, [¶]Department of Materials Science and Engineering, [#]Department of Polymer Science and Engineering, [⊥]CAS Key Laboratory of Materials for Energy Conversion, University of Science and Technology of China, Hefei, Anhui 230026 P.R. China

Supporting Information

ABSTRACT: We show that simply converting the hydrophobic moiety of an antimicrobial peptide (AMP) or synthetic mimic of AMPs (SMAMP) into a hydrophilic one could be a different pathway toward membrane-active antimicrobials preferentially acting against bacteria over host cells. Our biostatistical analysis on natural AMPs indicated that shorter AMPs tend to be more hydrophobic, and the hydrophilic-and-cationic mutants of a long AMP experimentally demonstrated certain membrane activity against bacteria. To isolate the effects of antimicrobials' hydrophobicity and systematically examine whether hydrophilic-and-cationic mutants could inherit the membrane activity of their parent AMPs/SMAMPs, we constructed a minimal prototypical system based on methacrylate-based polymer SMAMPs and compared the antibacterial membrane activity and hemolytic toxicity of analogues with and without the hydrophobic moiety. Antibacterial assays showed that the hydrophobic moiety of polymer SMAMPs consistently promoted the antibacterial activity but diminished in effectiveness for long polymers, and the resultant long hydrophilic-and-cationic polymers were also membrane active against bacteria. What distinguished these long mutants from their parent SMAMPs were their drastically reduced hemolytic toxicities and, as a result, strikingly enhanced selectivity. Similar toxicity reduction was observed with the hydrophilic-and-cationic mutants of long AMPs. Taken together, our results suggest that long hydrophilic-and-cationic polymers could offer preferential membrane activity against bacteria over host cells, which may have implications in future antimicrobial development.



INTRODUCTION

Antibiotic-resistant bacteria and their accelerating spread pose an alarming threat to global health.[1−3] Natural antimicrobial peptides—part of the innate immunity of multicellular organisms[4−9]—mainly target the barrier function of bacterial cytoplasmic membranes,[4−13] a generic activity mode which appears to be more difficult for bacteria to circumvent than the specific metabolic targeting modes of antibiotics.[4−12] Two structural characteristics conserved in most AMPs are presumably vital to their membrane activity: They are cationic (i.e., an AMP has net cationic charges) and amphipathic (i.e., the hydrophobic and cationic amino acids of an AMP spatially segregate into discrete patches of the molecule and in doing so confer the peptide cationic and hydrophobic faces). The cationic moiety of an AMP facilitates its association with the anionic bacterial membrane surface via electrostatic interactions, while the hydrophobic moiety of an AMP facilitates the subsequent membrane permeabilization processes, leading to cell death.[4,6,10,12−14] Designed to be simultaneously cationic and hydrophobic, synthetic mimics of AMPs (SMAMPs) including non-natural peptides,[15−24] peptoids,[25] oligomers,[26−32] and polymers[33−45] have demonstrated similar *in vitro* antimicrobial activities as do AMPs. Unlike AMPs, SMAMPs are resistant to proteolysis and relatively cheap to produce, two extra advantages which may overcome the two obstacles hindering the pharmaceutical developments of AMPs.[8] Up to now, several AMPs and SMAMPs have entered clinical trials as antimicrobial or immunomodulatory agents, and among them, some even demonstrated efficacy in phase III clinical trials.[6,8] Clearly, both AMPs and SMAMPs meet minimal structural requirements for membrane-permeabilizing activity against bacteria.

However, how to achieve preferential activity—a critical therapeutic index—for AMPs/SAMPs remains an ongoing challenge. Prior studies indicate that increasing the hydrophobicity (or amphiphilicity) of AMPs and SMAMPs generally results in enhanced antibacterial potency, whereas too high hydrophobicity (or amphiphilicity) may lead to loss of antibacterial activity, indicative of an optimum hydrophobicity (or amphiphilicity) window.[16,23,27,39,45−49] On the other hand,

Received: May 7, 2014
Revised: June 30, 2014
Published: July 28, 2014

dx.doi.org/10.1021/bm5006596 | *Biomacromolecules* 2014, 15, 3267−3277

higher hydrophobicity (or amphiphilicity) in AMPs and SMAMPs is generally correlated with stronger hemolytic toxicity[16,27,30,32,36,37,39,40,45−53]—a widely used toxicity index. Obviously, a trade-off between antimicrobial activity and toxicity exists when increasing the hydrophobicity of AMPs and SMAMPs. Extensive research efforts have therefore been focused on fine-tuning the tacit hydrophobic-to-cationic balance of AMPs and SMAMPs.

In contradiction to AMPs and SMAMPs, certain hydrophilic polycations including poly(aminopropyl methacrylamide),[35] poly(aminoethyl methacrylate),[45] poly(L-lysine),[54] ε-poly-L-lysine,[55] and polyguanidinium oxanorbornene[56] have demonstrated antibacterial activity of varying potency. Inspired by these contradictions, we hypothesized that simply converting the hydrophobic moiety of an AMP or a SMAMP into a hydrophilic one may be a different pathway toward membrane active antimicrobials with the desired differential activity.

In this work, we systematically examined whether the hydrophilic-and-cationic mutants of AMPs and SMAMPs, obtained by simply converting their hydrophobic moiety into a hydrophilic one, could still be membrane active against bacteria and, if that is the case, whether they could offer reduced toxicity and thus enhanced selectivity compared to their parent antimicrobials. Our biostatistical analysis on 397 lysine-rich AMPs revealed a strong increasing trend in peptide hydrophobicity as peptide sequence length decreased, and additional experiments showed that the hydrophilic-and-cationic mutants of a 23-residue AMP successfully captured its membrane activity against bacteria. Using polymethacrylate derivatives as prototypical SMAMPs, we constructed a minimal system and compared the antibacterial membrane activity and hemolytic toxicity of analogues with and without the hydrophobic moiety. Antibacterial assays showed that the hydrophobic moiety of polymer SMAMPs consistently promoted the antibacterial activity but diminished in effectiveness for long polymers and, like the parent SMAMPs, these long hydrophilic-and-cationic polymers were also membrane active against bacteria. Nevertheless, these long mutants demonstrated drastically reduced toxicity and, in doing so, strikingly enhanced selectivity compared to their parent polymer SMAMPs. Similar toxicity reduction was observed with the hydrophilic-and-cationic mutants of AMPs. Taken together, our results demonstrated a different pathway toward membrane-active antimicrobials preferentially acting against bacteria over mammalian cells, which may have implications in future antimicrobial development.

MATERIALS AND METHODS

Materials. Methacryloyl chloride, methyl 3-mercaptopropionate (MMP), and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid were purchased from Sigma-Aldrich (Shanghai, China). Butyl methacrylate (BMA, > 97%), pyrene were purchased from Sinopharm Chemical Reagent Company (Shanghai, China). 2-Hydroxyethyl methacrylate was purchased from Alfa Aesar (Tianjin, China). Di-*tert*-butyl dicarbonate ($(Boc)_2O$), ethanolamine, trifluoroacetic acid (TFA, 99%), 2-phenyl-1-propene, and 2,2-azobis(isobutyronitrile) (AIBN) were purchased from Aladdin-Reagent (Shanghai, China). Peptides used in this work were purchased from ChinaPeptides, Inc. (Shanghai, China). Dehydrated Mueller-Hinton (MH) medium formulation and Tryptic Soy Broth medium formulation were purchased from Qingdao Hope Bio-Technology (Qingdao, China) and used as supplied to prepare cation-adjusted Mueller-Hinton (caMH) broth and Tryptic Soy broth. Live/Dead BacLight bacterial viability kit was purchased from Molecular Probes (Shanghai, China). Bacterial strains used in this work were purchased from American Type Culture Collection (ATCC) (Virginia, USA). *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 29853) were used as representatives for Gram-negative bacteria while *S. aureus* (ATCC 25923) and *B. subtilis* (ATCC 6051) were used as representatives for Gram-positive bacteria. All other reagents were purchased from Sinopharm Chemical Reagent Company (Shanghai, China) unless specified otherwise. AIBN was recrystallized from ethanol before use. All other reagents were used as supplied unless specified otherwise.

Biostatistics Analysis on Lysine-Rich Linear AMPs. Prior studies have quantified the peptide hydrophobicity of AMPs using established hydrophobicity scales of amino acids and related the peptide hydrophobicity to AMP activity.[11,52,57] It should be noted that the average peptide hydrophobicity, <hydrophobicity>, is given as an average hydrophobicity scale of each amino acid residue in a peptide sequence and, as a result, the <hydrophobicity> values do not reflect the peptide sequences and conformations.

AMP sequences were obtained from the antimicrobial peptide database.[58] Considering the fact that polymer SMAMPs are linear chains and taking into account the finding that arginine facilitates membrane activity via interactions other than simple electrostatic interactions,[11,59,60] linear AMP sequences which exclusively contain the cationic amino acid lysine were used. This yielded 397 lysine-rich linear AMPs. The average hydrophobicity for the *j*th AMP was calculated via

$$<\text{hydrophobicity}>_j \equiv \frac{1}{n}\sum_{i=1}^{n} w_i$$

where n = number of amino acids in the peptide sequence, and w_i = the hydrophobicity of the *i*th amino acid in the peptide, and its value is set by the particular hydrophobicity scale used. A scattergram of peptide <hydrophobicity> versus number of amino acids (i.e., n) was constructed, using the three widely used residue hydrophobicity scales (Kyte−Doolittle,[61] Eisenberg consensus,[62] and Wimley−White[63]).

Preparations of Polymethacrylate Derivatives. The copolymers were prepared via AIBN-initiated free radical copolymerizations or reversible addition−fragmentation chain transfer (RAFT) copolymerizations of *N*-(*tert*-butoxycarbonyl)aminoethyl methacrylate and either butyl methacrylate or 2-hydroxyethyl methacrylate to give the Boc-protected precursor copolymers which, after subsequent TFA-deprotection, yielded the desired copolymer products (Scheme S1 in the Supporting Information [SI]). The homopolymer of aminoethyl methcrylate was prepared via a similar procedure. Methyl 3-mercaptopropionate (MMP) or cumyl dithiobenzoate (CDB) was used as chain transfer agent (CTA) to control the copolymer molecular weight. ^{1}H NMR characterizations were performed using both the final TFA-deprotected polymers and the corresponding Boc-protected precursor polymers. Gel permeation chromatography (GPC) analysis was performed with Agilent 1260 Infinity LC, using tetrahydrofuran (THF) as mobile phase and polystyrene (PS) as the standard for calibration. More experimental and characterization details are described in the SI.

Polymer Hydrophobicity Calculations. We theoretically calculated the hydrophobicities of the copolymers using a method we recently established.[52] Briefly, we used published octanol−H_2O partition coefficients, log *P*'s, for the methacrylate monomers.[64] The free energy of transfer from octanol to water $\Delta G_{\text{oct-w}} = 2.3RT \log P$,[65] where R is the gas constant and T is the temperature. Thus, the log *P* values of BMA and HEMA monomers were converted into $\Delta G_{\text{oct-w}}$ values, ΔG_{BMA} and ΔG_{HEMA}, respectively (Figure S18 in the SI). The $\Delta G_{\text{oct-w}}$ for AEMA has not been measured but was determined to be −1.364 as described previously.[52] The average hydrophobicity, <hydrophobicity>, values of polymers containing AEMA, HEMA, and/or BMA were calculated using the same equation as that for calculating peptide <hydrophobicity>.[52] It should be noted that the polymer <hydrophobicity> is given as an average free energy of octanol-to-water transfer of each monomer residue and, as a result, <hydrophobicity> values do not reflect the polymer sequences, conformations, and chain lengths.

Polymer Critical Micellation Concentration (CMC) Measurements. We determined the critical micelle concentration (CMC) of copolymers by monitoring the changes in fluorescence characteristics of pyrene, a widely accepted fluorescence probe for CMC determinations.[66] Upon partition into the hydrophobic core of a micelle, pyrene has its characteristic low-energy (0,0) band on excitation spectra undergo a blue shift, which was employed to determine the CMC value of dendritic−linear diblock copolymers in water.[66] For amphiphilic polymers used in this work, such a blue shift occurred from 334 to 337 nm. Aliquots of pyrene stock solution (10 μL, 6.0×10^{-5} M) in alcohol were added into glass vials. After removing the solvent via evaporation, the residual was subsequently resuspended into polymer solutions in HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) at expected polymer final concentrations (sonication for 1 h was used to assist pyrene's resuspension); pyrene's final global concentrations in the resultant solutions were kept constant at 6.0×10^{-7} M. After standing still at room temperature for 2 h, the resultant solutions were subjected to fluorescence excitation spectrum measurements (F-4600 spectrofluorometer, Hitachi) using excitation wavelength range of 250−370 nm and emission wavelength λ_{em} = 373 nm. The polymer CMC values were determined as the concentrations where I_{337}/I_{334} drastically increased, where I_{337} and I_{334} are intensities at 337 and 334 nm on the as-measured excitation spectra, respectively.

Bacterial Growth Inhibition Assays. Cation-adjusted Mueller-Hinton (caMH) broth was used as the medium for our bacterial growth inhibition assays unless specified otherwise. The caMH broth was prepared by dissolving dehydrated Mueller-Hinton (MH) medium formulation (4.2 g) in 200 mL deionized water and sterilizing at 121 °C for 20 min, followed by addition of sterilized aqueous solutions of $MgCl_2$ (10 mg/mL, 0.2 mL) and $CaCl_2$ (20 mg/mL, 0.2 mL). For each bacterial strain, 3−5 individual colonies were inoculated into fresh tryptic soy broth (TSB) and incubated at 37 °C for 18 h to stationary phase. A 40 μL culture was diluted with fresh TSB by 100-fold and regrown at 37 °C to mid log phase (OD_{600} = 0.5−0.7). The regrown culture was subsequently adjusted with fresh caMH broth to OD_{600} = 0.001 (approximately 5×10^5 cells/mL), which yielded the final inoculum.

Serial dilutions of polymer stock solutions in Millipore water were made with fresh caMH broth in Eppendorf centrifuge cups. Each polymer dilution (20 μL) was added into each well of a 96-well microplate (Costar, Corning). One hundred and thirty μL final inoculums were inoculated into each well of a preset 96-well flat-bottom microplate and then incubated at 37 °C for 18 h. Bacterial growth was monitored by reading OD595 (iMark, Bio-Rad). Controls include broth only to provide blank values for the assay readings, as well as untreated bacterial suspension samples to indicate 100% bacterial growth. Each bacterial inhibition trial was carried out in triplicate, and the reported results are the averages of two independent trials. The reported MIC values are defined as the minimum polymer concentrations necessary to inhibit 90% bacterial growth.

Bacterial Plate Killing Assays. Bactericidal activity of a polymer was evaluated via plate bacterial killing assays. For each bacterial strain, 3−5 individual colonies were inoculated into fresh tryptic soy broth (TSB) and incubated at 37 °C for 18 h to stationary phase. A 40 μL culture was diluted with fresh TSB by 100-fold and regrown at 37 °C to mid log phase (OD_{600} = 0.5−0.7). Bacterial cells were then harvested and washed twice with sterile HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) via centrifugation (5150 rcf for *S. aureus*, while 10,621 rcf was used for all other bacterial strains) for 5 min and, within 15 min, adjusted with sterile HEPES buffer to $\sim 1.5 \times 10^6$ CFU/mL and inoculated into zero-dilution wells of a preset 96-well microplate.

Serial 2-fold dilutions of antimicrobial stock solutions in Millipore water were made with HEPES buffer. Each antimicrobial dilution (20 μL) was added into each zero-dilution well in a 96-well microplate. Fifty μL adjusted bacteria suspension, was inoculated into each zero-dilution well of a preset microplate, to achieve 5×10^5 CFU/mL in each well (150 μL). The microplate was then incubated at 37 °C for 3 h. Serial 10-fold dilutions were subsequently made with sterile HEPES buffer. Each dilution (20 μL) was plated onto MH agar plates, which were then incubated at 37 °C overnight to give visible colonies. Inoculum size was indicated by control samples containing untreated bacteria. Each trial was performed in triplicate, and the reported results are the averages of two independent trials.

Bacterial Dead/Live Viability Assays. To assess whether a polymer or a peptide was capable of permeabilizing bacterial membranes, we used BacLight Dead/Live bacterial viability kit (Molecular Probes) and examined the staining effects under fluorescence microscopy (IX81, Olympus). For each bacterial strain, 3−5 individual colonies were inoculated into 5 mL of fresh TSB and then incubated at 37 °C for 20 h to stationary phase. The bacterial cells were harvested and washed with sterile HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) via centrifugation at 657 rcf for 1 min. The bacterial pellet was resuspended into PBS buffer supplemented with 0.03% TSB (volume ratio) to achieve $\sim 10^9$ CFU/mL. Ninety μL bacterial solution and 10 μL drug (a polymer or a peptide) stock solution were incubated at 37 °C for 4 h, followed by additions of aqueous solutions of SYTO9 (5 μL) and propidium iodide (5 μL). The resultant mixtures were incubated at 37 °C in the dark for 15 min, and then centrifuged for 1 min to remove the supernatant. The bacterial pellets were then washed with 100 μL HEPES and fixed with 100 μL 4% paraformaldehyde solution. Ten μL of the resultant bacterial suspension was transferred onto a coverslip, air-dried, immersed with 10 μL of mounting oil (Molecular Probes), and imaged under fluorescence microscopy (IX81, Olympus) using a 100× oil-immersion objective lens. FITC and TRITC filters were used for SYTO-9 and propidium iodide, respectively. All bacterial cells were stained green, while only cells with compromised cytoplasmic membranes stained red.

Hemolysis Assays. Mouse red blood cells (mRBC) were used as representative mammalian cells, and hemolysis assays were performed to assess the preliminary toxicity. Polymer or peptide stock solutions were prepared in sterilized Millipore water and stored in aliquots at −20 °C. Serial 2-fold dilutions of a polymer or peptide stock solution were made with sterile HEPES buffer, which yielded polymer or peptide solutions for hemolysis assays. Fresh mouse blood (100 μL) was washed with sterile HEPES buffer (12 mL) and centrifuged at 800 rcf, and the pellet was resuspended into ~20 mL sterile HEPES buffer to yield the mRBC stock suspension ($\sim 2.5 \times 10^4$ cells/μL) for hemolysis assays. Into each centrifuge cup, we added mRBC stock suspension (160 μL) and diluted polymer or peptide solution (40 μL). After incubation at 37 °C for 60 min with shaking at 200 rpm, the centrifuge cups were centrifuged at 800 rcf for 5 min, and the supernatant (50 μL) of each cup was subsequently transferred into a well of a 96-well microplate and diluted with HEPES buffer (140 μL). Absorbance at 414 nm was measured using a microplate reader (Varioskan Flash). Controls included mRBC suspension (160 μL) with HEPES buffer (40 μL) and mRBC suspension (160 μL) treated with triton X-100 (50%, 40 μL) to provide reference for 0% and 100% hemolysis, respectively. Each hemolysis assay trial was carried out in triplicate, and the reported results are the averages of two independent trials.

■ RESULTS AND DISCUSSIONS

In this work, we examined whether simply converting the hydrophobic moiety of an AMP or a SMAMP into a hydrophilic one could lead to membrane-active antimicrobials preferentially acting against bacteria over host cells. As a preliminary test, a biostatistical analysis on 397 lysine-rich linear natural AMPs was performed, to examine how the peptide hydrophobicity of AMPs distributes. Prior studies have quantified the peptide hydrophobicity of AMPs using established hydrophobicity scales of amino acids and related the peptide average hydrophobicity, <hydrophobicity>, to AMP activity.[11,52,57] It should be noted that the average peptide hydrophobicity, <hydrophobicity>, is given as an average hydrophobicity scale of each amino acid residue in a peptide

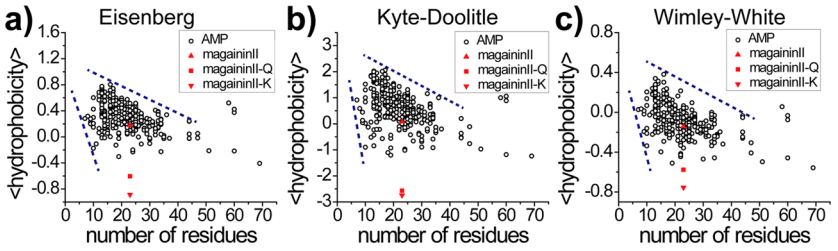


Figure 1. Demonstration of peptide average hydrophobicity, <hydrophobicity>, versus the number of amino acid residues in the peptide sequence based upon 397 lysine-rich cationic AMPs in the antimicrobial peptide database, using three established scales (a) Eisenberg consensus, (b) Kyte–Doolittle, and (c) Wimley–White. Blue dashed lines indicate the boundaries.

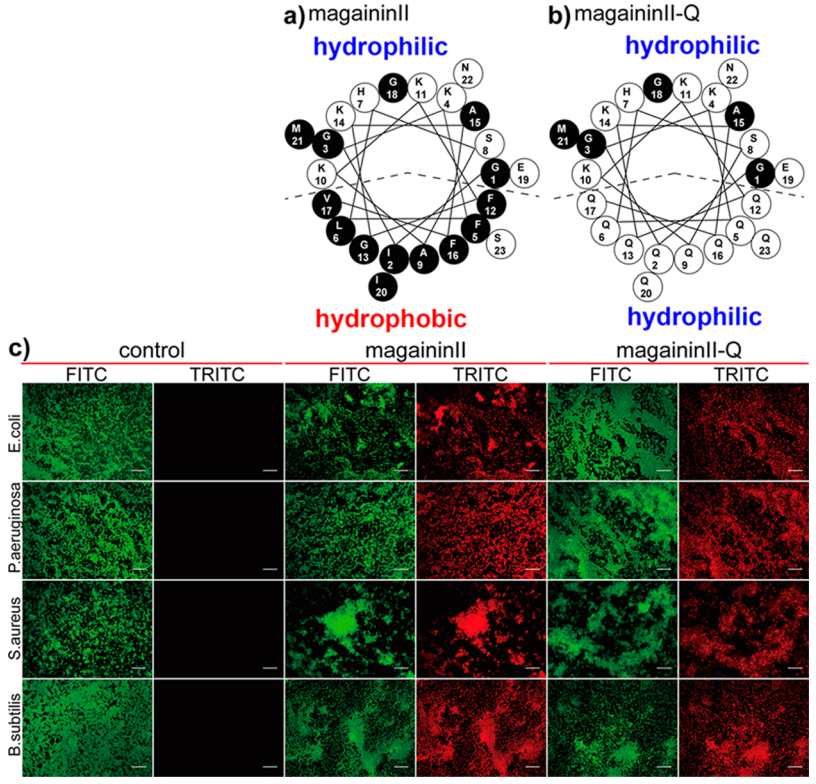


Figure 2. Helical wheel projections of (a) magaininII and (b) magaininII-Q. (c) Fluorescence microscopy images of bacterial strains treated with and without a peptide and subsequently stained briefly (15 min) with SYTO-9 (green) and PI (red). The treatment was carried out with 0.4 mM of magaininII or magaininII-Q for 4 h in 10 mM HEPES buffer (pH 7.4). Controls are those assayed in similar ways but without addition of peptide. Scale bar = 20 μm.

sequence and, as a result, the <hydrophobicity> values do not reflect the peptide sequences and conformations. Figure 1 shows a scattergram of the average peptide hydrophobicity, <hydrophobicity>, versus the number of amino acids in peptide sequence, using three established scales of amino acids (Eisenberg consensus,[67] Kyte–Doolittle,[61] Wimley–White[63]). As peptide sequence length decreased, a strong increasing trend in peptide <hydrophobicity> was discerned, indicating that shorter AMPs tend to be more hydrophobic, whereas longer ones may have more options in peptide <hydrophobicity>.

Indeed, certain membrane activity against bacteria was experimentally observed qualitatively with the hydrophilic-and-cationic mutants of long AMPs, using magaininII—a 23-residue natural AMP with well-characterized membrane-permabilizing activity[68−72]—as a prototypical parent AMP (Figure 2 and Figure S1−3 in the SI). Upon binding a membrane surface, magaininII adopts an α-helical conformation in which cationic and hydrophobic residues are spatially segregated into opposite faces of the helix (Figure 2a). Replacing all residues in its hydrophobic face with hydrophilic ones should lead to hydrophilic-and-cationic mutants, as exemplified by magaininII-Q (Figure 2b and Figure S1 in the SI) and magaininII-K (Figure S2 in the SI). To qualitatively assess whether the as-obtained mutants of magaininII are also membrane active against bacteria, we performed bacterial Dead/Live viability assays, by briefly incubating the peptide-treated bacteria with SYTO-9 and propidium iodide (PI)[24] and examining the staining effects under fluorescence microscopy. SYTO-9 and PI are both nucleic acid stains but differ in both their fluorescence characteristics and capability to permeate bacterial cellular membranes; SYTO-9 is a cell-permeant green-fluorescent stain that labels all bacterial cells, whereas PI is a cell-impermeant red-fluorescent stain that only labels cells with compromised membranes. After brief incubation with SYTO-9 and PI, all peptide-treated bacterial strains stained intensely red (Figure 2c and Figure S3 in the SI), indicative of compromised

dx.doi.org/10.1021/bm5006596 | Biomacromolecules 2014, 15, 3267−3277

cellular membranes; peptide doses were kept the same at 0.4 mM, approximately 10−20 × MIC of magaininII.[73] In contrast, strains treated similarly but without peptide addition (i.e., control) were dark in the red channel, indicative of intact membranes (Figure 2c and Figure S3 in the SI). Thus, the hydrophilic-and-cationic mutants of magaininII, which have the hydrophobic face deprived, still demonstrated certain membrane-permeabilizing activity against bacteria, as did the parent AMP.

Encouraged by these positive initial results, we then constructed a minimal prototypical system and systematically compared the antibacterial membrane activity and toxicity of analogues with and without the hydrophobic moiety. Intuitively, AMPs appear to be the first choice. Nevertheless, they often adopt well-defined secondary structures upon binding a membrane surface, and whether such secondary structure is directly coupled with their antibacterial activity is still under debate.[6,7,49,74] With these concerns in mind, we have turned to random-copolymer-based SMAMPs which, unlike AMPs, are intrinsically heterogeneous and lack well-defined secondary structures. The prototypical parent SMAMPs we used were methacrylate-based polymer SMAMPs, which have well-characterized activity profiles[34,45] and, despite their abiotic and heterogeneous nature, have demonstrated membrane-destabilization mechanisms similar to those by natural AMPs.[52] Specifically, we used random copolymers of aminoethyl methacrylate (AEMA) and butyl methacrylate (BMA) with monomer molar ratio AEMA/BMA = 0.65:0.35 (pABs, Figure 3a, top). Rather than substituting the hydrophobic monomer with the cationic comonomer as normally done for such binary-random-copolymer-based SMAMPs—a practice which increases the hydrophobicity but also inevitably decreases the cationic charges at the same time—we prepared the corresponding hydrophilic-and-cationic mutants, pAHs (Figure 3a, bottom), by keeping AEMA and its molar percentage unchanged but replacing BMA with 2-hydroxyethyl methacrylate (HEMA)—an uncharged but hydrophilic methacrylate monomer[75]—to dissect the effects of hydrophobicity from those of cationicity.

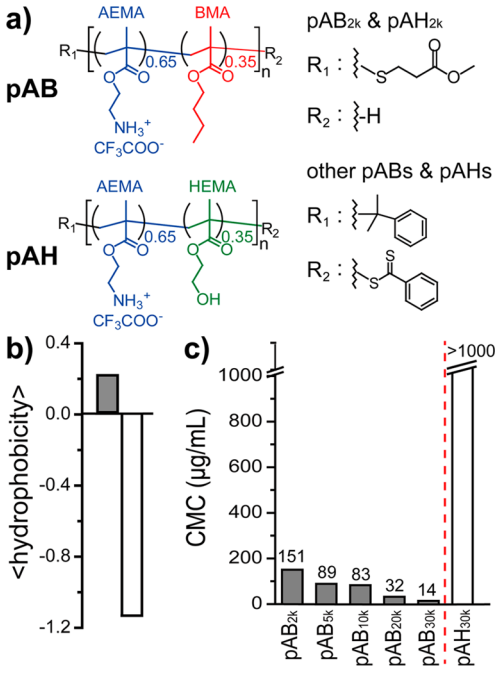


Figure 3. (a) Chemical structures of pABs (top) and pAHs (bottom), which were the prototypical polymer SMAMPs and corresponding hydrophilic-and-cationic mutants, respectively. (b) Theoretically calculated average hydrophobicity, <hydrophobicity>, of pABs (solid) and pAHs (open). (d) Measured critical micelle concentrations (CMC) of pABs (solid) and pAH30k (open), the pAH homologue with the largest polymer molecular weight.

The expected methacrylate-based copolymers were successfully prepared via AIBN-initiated reversible addition−fragmentation chain transfer polymerization or AIBN-initiated free radical polymerization (Scheme S1 in the SI), as confirmed by their ^{1}H NMR spectroscopy and GPC characterizations (Table 1 and Table S1 in the SI). Theoretical calculations on their

Table 1. Monomer Compositions (f) and Molecular Weights (Mw) of Our Copolymers

copolymer	$f_{protected}$[a]	$f_{deprotected}$[a]	Mw[b]
pAB_{2k}	0.65:0.35	0.66:0.34	3216
pAH_{2k}	0.66:0.34	0.64:0.36	3103
pAB_{5k}	0.66:0.34	0.65:0.35	7208
pAH_{5k}	0.65:0.35	0.66:0.34	7490
pAB_{10k}	0.66:0.34	0.66:0.34	9545
pAH_{10k}	0.65:0.35	0.67:0.33	11,233
pAB_{20k}	0.66:0.34	0.64:0.36	16,441
pAH_{20k}	0.65:0.35	0.66:0.34	18,948
pAB_{30k}	0.67:0.33	0.65:0.35	38,453
pAH_{30k}	0.65:0.35	0.64:0.36	37,836

[a] $f_{protected}$ and $f_{deprotected}$ calculated on the basis of ^{1}H NMR spectra of the Boc-protected precursor copolymers and those of the TFA-deprotected final products, respectively. [b] Mw obtained on the basis of GPC of the Boc-protected precursor polymers.

average hydrophobicity, <hydrophobicity>, showed that pABs and pAHs had positive and negative <hydrophobicity>, respectively (Figure 3b), indicating that the former is hydrophobic whereas the latter is hydrophilic. Whether a polymer is amphiphilic can also be deduced by measuring its CMC. Our CMC measurements (Figure S19 in the SI) showed that all pABs exhibited CMC values ranging 14−151 μg/mL whereas even pAH_{30k}, the longest pAH homologue, failed to do so up to 1000 μg/mL (Figure 3c). It is known that, for amphiphilic homologues, longer ones exhibit lower CMC. Therefore, all pAH polymers should not exhibit CMC within 1000 μg/mL, the highest tested polymer concentration. Taken together, both our <hydrophobicity> calculations and CMC measurements consistently indicate that, in our experimental concentration range, all pABs were hydrophobic-and-cationic, whereas all pAHs were hydrophilic-and-cationic.

To assess the antibacterial activity of the as-prepared copolymers, we performed both bacterial growth inhibition assays and plate killing assays. Bacterial growth inhibition assays showed that pAHs exhibited inhibitory activity ranging from inactive (0−10% inhibition) over partially active (~10−90% inhibition) to potently active (≥90% inhibition), depending on both bacterial strain and polymer molecular weight (Figure 4). In stark contrast, all pABs exhibited MIC_{90}—minimum concentration to inhibit 90% bacterial growth—of <64 μg/mL against all bacterial strains tested (Figure 4), consistent with prior studies.[45,76] Notably, though a pAH was generally less inhibitive than its parent pAB against a specific bacterial strain, the gap between their inhibitory activities shrank significantly as

dx.doi.org/10.1021/bm5006596 | Biomacromolecules 2014, 15, 3267−3277

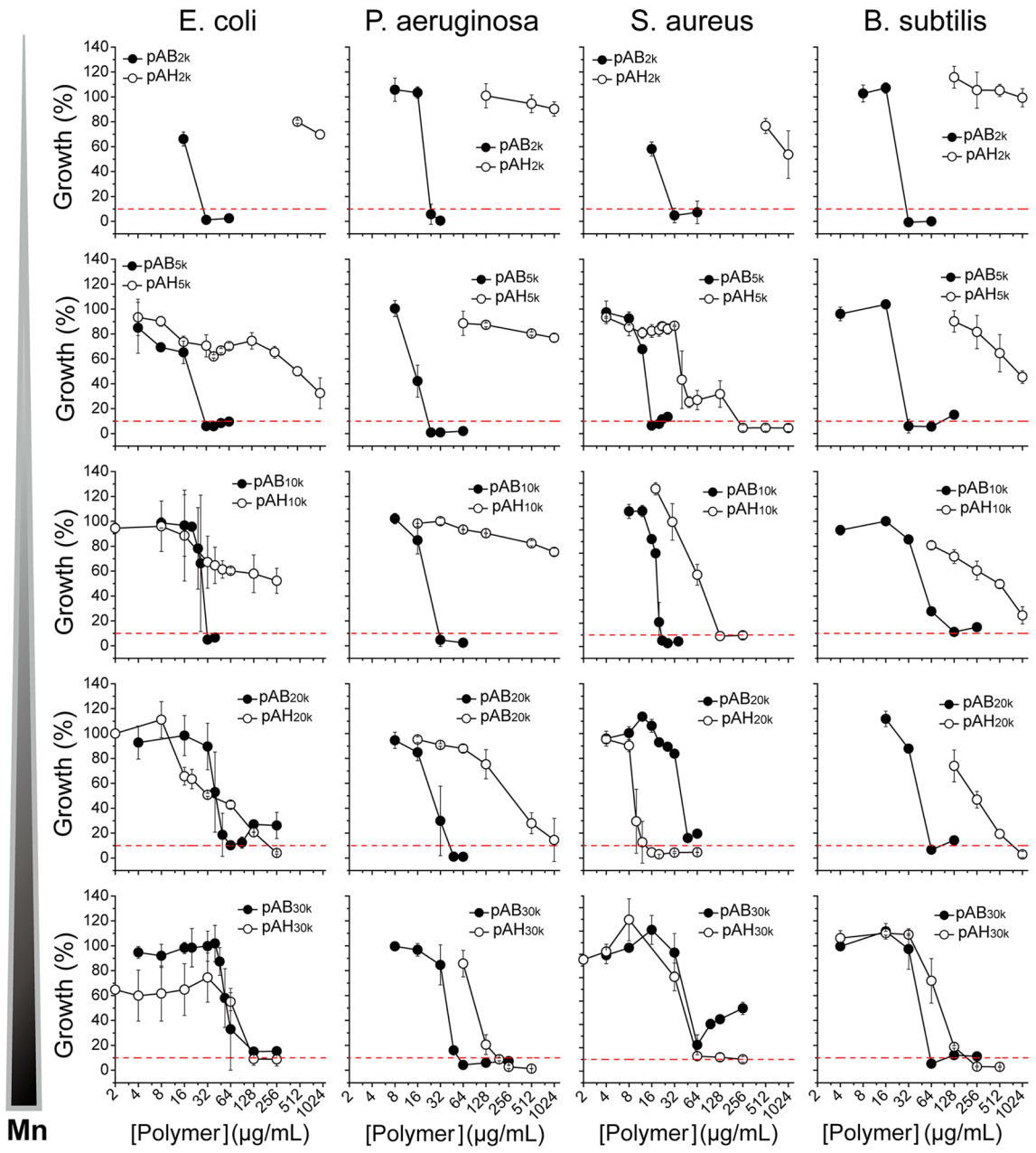


Figure 4. Bacterial growth inhibition assays using pAB (solid) and pAH (open) homologues of varying polymer molecular weights. Red dashed lines indicate 10% bacterial growth. Data points are reported as mean ± standard deviation.

polymer molecular weight increased. Clearly, the hydrophobic moiety of polymer SMAMPs consistently promoted bacterial growth inhibitory activity, as previously expected,[4,6,10,12,13] but its effectiveness diminished as polymer chain length increased. It should be noted that our pAHs contain 65% (molar ratio) cationic monomers. Whether there exists a minimum threshold ratio of cationic monomers for such hydrophilic-and-cationic polymers to exert antibacterial activity is currently unknown.

Similar effects were observed with the bactericidal activity of the copolymers, according to plate killing assays performed using the short copolymer pair (pAH_{5k} versus pAB_{5k}) and the longest copolymer pair (pAH_{30k} versus pAB_{30k}) as two representative extreme cases. For the short pair, pAB_{5k} exhibited MBC_{100}—minimum concentration to kill 100% inoculated cells—of 4–8 μg/mL against all tested bacterial strains, whereas pAH_{5k} exhibited varying bactericidal potency depending on bacterial strain tested (see Figure 5a–d); obviously, pAB_{5k} was significantly more active than pAH_{5k}. In striking contrast, the longest copolymers—pAH_{30k} and pAB_{30k}—exhibited close or even overlapping MBC_{100} against all four test bacterial strains (Figure 5e–h). Taken together, both types of antibacterial assays consistently showed that the hydrophobic moiety of polymer SMAMPs facilitated the antibacterial activity, but its effectiveness diminished as polymer chain length increased.

Of note, pAB_{5k} and pAB_{30k}, despite their approximately 6-fold difference in polymer molecular weight and CMC, demonstrated almost overlapping MBC_{100} values (Figure 5), and their observed MBC_{100} values were also well below their respective CMC values (Figure 3c). Similarly, pAH_{5k} and pAH_{30k}, though incapable of forming micelles within tested concentration range, still exhibited bactericidal activity against

dx.doi.org/10.1021/bm5006596 | Biomacromolecules 2014, 15, 3267–3277

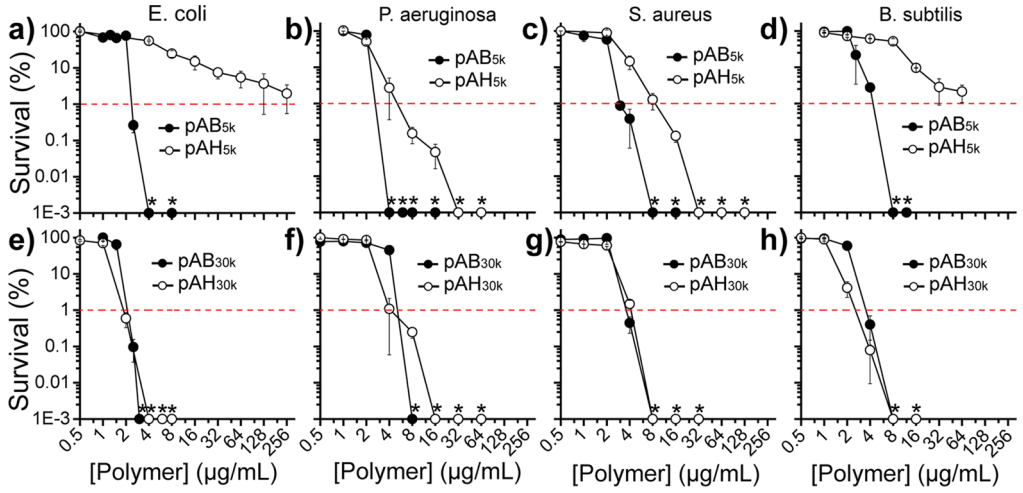


Figure 5. Plate killing assays using (a–d) the short copolymer pair, pAH_{5k} (open) and pAB_{5k} (solid), and (e–h) the longest copolymer pair, pAH_{30k} (open) and pAB_{30k} (solid), as two representative extreme cases. * indicates bacterial survival percentage of 0%. Red dashed lines indicate 1% bacterial survival. Data points are reported as mean ± standard deviation.

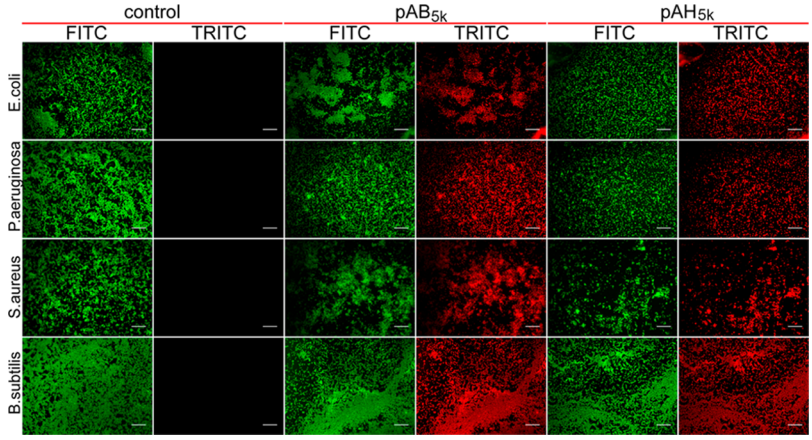


Figure 6. Fluorescence microscopy images of bacterial strains treated with and without a copolymer and subsequently stained briefly (15 min) with SYTO-9 (green) and PI (red). The treatment was carried out with 48 μg/mL of either pAB_{5k} or pAH_{5k} for 4 h in HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.4). Controls are those assayed in comparable ways but without addition of copolymer. Scale bar = 20 μm.

all four bacterial strains tested. These observations suggest that polymer micellation is not a prerequisite for polymethacrylate derivatives to exert bactericidal activity and their MBC_{100} values correlate with their free polymer concentrations. Consistent with plate killing assays, bacterial growth inhibition assays showed that, against the same bacterial strain, pAB_{2k} and pAB_{5k} demonstrated very close MIC_{90} values (Figure 4) and their observed MIC_{90} values were also well below their respective CMC values (Figure 3c). Nevertheless, pAB_{20k} and pAB_{30k} were less active and their MIC_{90} values were above their respective CMC (Figure 4). These results indicate that polymer micellation is not a prerequisite for polymethacrylate derivatives to exert bacterial growth inhibitory activity; instead, its occurrence resulted in decreased effective free polymer concentrations, likely rendering longer pABs less active in bacterial growth inhibition assays.

Intriguingly, the observed MIC_{90} of a polymethacrylate derivative is significantly higher than its MBC_{100}, as observed previously with amphiphilic poly(vinyl ether) derivatives.[41] This may arise because of the following two reasons. One is the difference between these two antibacterial assays. A plate killing assay is normally performed in buffer or saline which lack nutrients necessary to support bacterial growth, whereas a growth inhibition assay is normally performed in nutrient broth. Therefore, during a growth inhibition assay, there exist not only competition between polymers' bactericidal activity and bacterial growth but also polymer aggregations due to their interactions with broth components (e.g., nutrient proteins), leading to the observation that MIC_{90} is higher than MBC.[41] Another possible explanation is the Mg^{2+} and Ca^{2+} ions present in caMH broth, the medium used in our growth inhibition assays, considering the finding that 0.75 mM Mg^{2+} or 0.5 mM Ca^{2+} ions inhibited the antibacterial activity of magaininII[77] and taking into account that both magaininII and methacrylate-based polymer SMAMPs act by permeabilizing bacterial membranes.[52,69,71,72]

Antibacterial activity is not necessarily correlated with membrane-permeabilizing activity against bacteria.[78] Although pABs have well-characterized membrane activity,[52] whether or not pAHs are also membrane active remains uncharacterized. To clarify this, we performed bacterial Dead/Live viability assays and qualitatively compared the membrane-permeabilizing activity of pAHs and pABs. The short copolymer pair (pAH_{5k} versus pAB_{5k}) and the longest copolymer pair (pAH_{30k}

dx.doi.org/10.1021/bm5006596 | Biomacromolecules 2014, 15, 3267–3277

Table 2. Biological Activity Data of Representative Copolymer Pairs

	MBC_{100} (μg/mL)					selectivity (HC_{50}/MBC_{100})			
polymer	E. c.[a]	P. a.[a]	S. a.[a]	B. s.[a]	HC_{50} (μg/mL)	E. c.[a]	P. a.[a]	S. a.[a]	B. s.[a]
pAB_{5k}	4	4	8	8	2−4	0.5−1	0.5−1	0.25−0.5	0.25−0.5
pAH_{5k}	−	32	32	−	128−256	−	4−8	4−8	−
pAB_{30k}	3	8	8	8	2−4	0.67−1.33	0.25−0.5	0.25−0.5	0.25−0.5
pAH_{30k}	4	16	8	8	256−512	64−128	16−32	32−64	32−64

[a]E.c., *E. coli*; P.a., *P. aeruginosa*; S. a., *S. aureus*; B.s., *B. subtilis*.

versus pAB_{30k}) were used as two representative extreme cases. After brief incubation with SYTO-9 and PI, all pAH_{5k}-treated strains stained intensely red as did the pAB_{5k}-treated ones, indicative of compromised cellular membranes (Figure 6); copolymer doses were kept the same at 48 μg/mL. In contrast, strains treated without a copolymer were dark in the red channel, indicative of intact membranes (Figure 6). Thus, pAH_{5k} significantly permeabilized bacterial membranes as did pAB_{5k}. Similar results were observed with pAH_{30k} and pAB_{30k} (Figure S22 in the SI). Taken together, these results showed that the long hydrophilic-and-cationic polymers, with average molecular weight ≥5 kDa, successfully captured the antibacterial membrane activity of their hydrophobic-and-cationic counterparts.

HEMA residue comprises 34−35% of monomers in a pAH homologue, and long HEMA homopolymers are amphiphilic. May the as-observed antibacterial membrane activity of a long pAH homologue originate in potential residual hydrophobicity conferred by its HEMA monomers? To exclude this possibility, we also executed the experiments using AEMA homopolymers (pAs, Table S2 in the SI), an extremely hydrophilic-and-cationic mutants of pABs which contains no HEMA, and observed same results (Figures S23−24 in the SI). Therefore, the as-observed antibacterial membrane-permeabilizing activity of long pAHs must originate in their hydrophilic-and-cationic nature, rather than their HEMA monomers, suggesting that long hydrophilic-and-cationic polymers may be membrane-active antibacterial agents, as are their hydrophobic-and-cationic counterparts.[79]

It is known that decreasing hydrophobicity of AMPs and SMAMPs generally reduces toxicity. Now our hydrophilic-and-cationic mutants completely lack the hydrophobic moiety. How would that affect the toxicity? To address this, we performed hemolysis assays, using both the short copolymer pair (pAB_{5k} versus pAH_{5k}) and the longest copolymer pair (pAB_{30k} versus pAH_{30k}) as representative extreme cases. Against mouse red blood cells (mRBC), pAB_{5k} demonstrated an HC_{50}—minimum concentration to lyse 50% of treated red blood cells—of 2−4 μg/mL, consistent with prior studies,[34,45,76] whereas pAH_{5k} demonstrated HC_{50} of 128−256 (Figure S25a in the SI), indicative of ≥64-fold reduction in hemolytic toxicity (Table 2). Even enlarged toxicity reduction was observed with the longest copolymer pair, pAH_{30k} versus pAB_{30k} (Figure S25b in the SI); pAB_{30k} exhibited HC_{50} of 2−4 μg/mL, whereas pAH_{30k} exhibited HC_{50} of 256−512 μg/mL (Table 2), indicative of ∼100-fold reduction in hemolytic toxicity. Notably, the observed HC_{50} values of all four tested polymers are well below their respective CMC values (for pAHs, CMC >1000 μg/mL if there is), indicating that polymer micellation is neither a prerequisite for polymethacrylate derivatives to exert hemolytic activity and their observed HC_{50} values correlate with their free polymer concentrations. Similar degree of reduction in hemolytic toxicity was observed with the hydrophilic-and-cationic mutants of natural AMPs, using melittin[80]—a 26-residue bee venom toxin—as a prototypical AMP (Figure S26−27 in the SI).

Combined with plate killing assays, hemolysis assays showed that, compared to pAB_{5k}, pAH_{5k} achieved ∼10-fold enhancement in selectivity, HC_{50}/MBC_{100}, against *P. aeruginosa* and *S. aureus* (Table 2). Increase in polymer molecular weight further enlarged such enhancement; compared to pAB_{30k}, pAH_{30k} demonstrated 32−128-fold enhancement in selectivity against all four bacterial strains tested (Table 2). Taken together, these results suggest that long hydrophilic-and-cationic polymers (synthetic and peptidic), obtained by simply converting the hydrophobic moiety of a long polymer SMAMP or AMP into a hydrophilic one, may be a new pathway toward preferential activity against bacteria over mammalian cells.

CONCLUSIONS

In summary, we have studied whether simply converting the hydrophibic moiety of an AMP or a SMAMP into a hydrophilic one is a new pathway toward membrane-active antimicrobials with preferential activity against bacteria over host cells. Our biostatistical analysis on natural AMPs revealed that short AMPs are more hydrophobic, and the hydrophilic-and-cationic mutants of a 23-residue membrane-active AMP was experimentally confirmed to be also membrane active against bacteria. Using polymethacrylate derivatives as prototypical SMAMPs, we constructed a minimal prototypical system with isolated effects of antimicrobials' hydrophobicity and systematically compared the activity and toxicity of analogues with and without the hydrophobic moiety. Antibacterial assays showed that the hydrophobic moiety of polymer SMAMPs facilitated the antibacterial activity but diminished in effectiveness for long ones, and those long hydrophilic-and-cationic mutants were also membrane active against bacteria. Compared to the hydrophobic-and-cationic parent SMAMPs, those long hydrophilic-and-cationic mutants demonstrated strikingly reduced toxicity and, in doing so, drastically enhanced selectivity. Similar toxicity reduction was observed with the hydrophilic-and-cationic mutants of natural AMPs. Taken together, our results suggest that long hydrophilic-and-cationic polymers could be a different pathway toward the desired preferentially membrane-active antimicrobials, which may have implications in future antimicrobial development.

ASSOCIATED CONTENT

Supporting Information

Details on polymer preparations and characterizations, as well as additional results and discussions. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*(L.Y.) E-mail: lhyang@ustc.edu.cn

Present Address

[†]Department of Metallurgical and Materials Engineering, Colorado School of Mines, 1500 Illinois Street, Golden, CO 80401, U.S.A.

Author Contributions

[‡]X.Y. and K.H. contributed equally to this work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Gerard C. L. Wong, Hongjun Liang, and Ghee Hwee Lai for critical comments and helpful suggestions, Zhishen Ge for helpful discussions, and Ji-Gang Piao and Peng Chen for technical assistance. The ^{1}H NMR, GPC, and CMC measurements were carried out in the Analytical Center of School of Chemistry and Materials Science at USTC. This work was supported in part by the National Natural Science Foundation of China (11074178, 21174138, J1030412), Ministry of Education of China (NCET-13-0547, SRF for DPHE 20090181120046, FRF for CU WK2060140008 and WK2060200012), and Anhui Education Department (KJ2013A267).

ABBREVIATIONS

HEMA, 2-hydroxyethyl methacrylate; AEMA, aminoethyl methacrylate; BMA, butyl methacrylate; CDB, cumyl dithiobenzoate; MMP, methyl 3-mercaptopropionate; NMR, nuclear magnetic resonance; GPC, gel permeation chromatography; CTA, chain transfer agent; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; ATCC, American Type Culture Collection; *E. coli*, *Escherichia coli*; *S. aureus*, *Staphylococcus aureus*; *B. subtilis*, *Bacillus subtilis*; *P. aeruginosa*, *Pseudomonas aeruginosa*; caMH, cation-adjusted Mueller-Hinton broth; TSB, tryptic soy broth; MIC_{90}, minimum concentration to inhibit 90% bacterial growth; MBC_{100}, minimum concentration to kill 100% inoculated bacterial cells; HC_{50}, minimum concentration to cause 50% hemolysis

REFERENCES

(1) Tomasz, A. Multiple-Antibiotic-Resistant Pathogenic Bacteria: A Report on the Rockefeller University Workshop. *N. Engl. J. Med.* **1994**, *330*, 1247−1251.

(2) Taubes, G. The Bacteria Fight Back. *Science* **2008**, *321* (5887), 356−361.

(3) McKenna, M. Antibiotic resistance: The last resort. *Nature* **2013**, *499*, 394−396.

(4) Zasloff, M. Antimicrobial Peptides of Multicellular Organisms. *Nature* **2002**, *415*, 389−395.

(5) Hancock, R. E. W.; Lehrer, R. Cationic Peptides: A New Source of Antibiotics. *Trends Biotechnol.* **1998**, *16* (2), 82−88.

(6) Fjell, C. D.; Hiss, J. A.; Hancock, R. E. W.; Schneider, G. Designing antimicrobial peptides: Form follows function. *Nat. Rev. Drug. Discovery* **2012**, *11* (1), 37−51.

(7) Boman, H. G. Antibacterial peptides: Basic facts and emerging concepts. *J. Int. Med.* **2003**, *254* (3), 197−215.

(8) Hancock, R. E. W.; Sahl, H.-G. Antimicrobial and Host-Defense Peptides As New Anti-Infective Therapeutic Strategies. *Nat. Biotechnol.* **2006**, *24* (12), 1551−1557.

(9) Shai, Y. From Innate Immunity to de-Novo Designed Antimicrobial Peptides. *Curr. Pharm. Des.* **2002**, *8* (9), 715−725.

(10) Brogden, K. A. Antimicrobial Peptides: Pore Formers or Metabolic Inhibitors in Bacteria? *Nat. Rev. Microbiol.* **2005**, *3* (3), 238−250.

(11) Schmidt, N. W.; Mishra, A.; Lai, G. H.; Davis, M.; Sanders, L. K.; Tran, D.; Garcia, A.; Tai, K. P.; McCray, P. B.; Ouellette, A. J.; Selsted, M. E.; Wong, G. C. L. Criterion for Amino Acid Composition of Defensins and Antimicrobial Peptides Based on Geometry of Membrane Destabilization. *J. Am. Chem. Soc.* **2011**, *133* (17), 6720−6727.

(12) Wimley, W. C. Describing the Mechanism of Antimicrobial Peptide Action with the Interfacial Activity Model. *ACS Chem. Biol.* **2010**, *5* (10), 905−917.

(13) Yeaman, M. R.; Yount, N. Y. Mechanisms of Antimicrobial Peptide Action and Resistance. *Pharmacol. Rev.* **2003**, *55* (1), 27−55.

(14) Matsuzaki, K. Why and How Are Peptide-Lipid Interactions Utilized for Self-Defense? Magainins and Tachyplesins As Archetypes. *Biochim. Biophys. Acta, Biomembr.* **1999**, *1462*, 1−10.

(15) Oren, Z.; Shai, Y. Selective Lysis of Bacteria but Not Mammalian Cells by Diastereomers of Melittin: Structure-Function Study. *Biochemistry* **1997**, *36* (7), 1826−1835.

(16) Chen, Y.; Mant, C. T.; Farmer, S. W.; Hancock, R. E. W.; Vasil, M. L.; Hodges, R. S. Rational Design of a-Helical Antimicrobial Peptides with Enhanced Activities and Specificity/Therapeutic Index. *J. Biol. Chem.* **2005**, *280* (13), 12316−12329.

(17) Won, H.-S.; Jung, S.-J.; Kim, H. E.; Seo, M.-D.; Lee, B.-J. Systematic Peptide Engineering and Structural Characterization to Search for the Shortest Antimicrobial Peptide Analogue of Gaegurin 5. *J. Biol. Chem.* **2004**, *279* (15), 14784−14791.

(18) Fernandez-Lopez, S.; Kim, H.-S.; Choi, E. C.; Delgado, M.; Granja, J. R.; Khasanov, A.; Kraehenbuehl, K.; Long, G.; Weinberger, D. A.; Wilcoxen, K. M.; Ghadiri, M. R. Antibacterial Agents Based on the Cyclic D,L-[α]-Peptide Architecture. *Nature* **2001**, *412* (6845), 452−455.

(19) Hamuro, Y.; Schneider, J. P.; DeGrado, W. F. De Novo Design of Antibacterial β-Peptides. *J. Am. Chem. Soc.* **1999**, *121* (51), 12200−12201.

(20) Porter, E. A.; Wang, X.; Lee, H.-S.; Weisblum, B.; Gellman, S. H. Antibiotics: Non-haemolytic β-Amino-Acid Oligomers. *Nature* **2000**, *404*, 565−565.

(21) Liu, D.; DeGrado, W. F. De Novo Design, Synthesis, and Characterization of Antimicrobial β-Peptides. *J. Am. Chem. Soc.* **2001**, *123* (31), 7553−7559.

(22) Porter, E. A.; Weisblum, B.; Gellman, S. H. Mimicry of Host-Defense Peptides by Unnatural Oligomers: Antimicrobial β-Peptides. *J. Am. Chem. Soc.* **2002**, *124* (25), 7324−7330.

(23) Hayouka, Z.; Chakraborty, S.; Liu, R.; Boersma, M. D.; Weisblum, B.; Gellman, S. H. Interplay among Subunit Identity, Subunit Proportion, Chain Length, and Stereochemistry in the Activity Profile of Sequence-Random Peptide Mixtures. *J. Am. Chem. Soc.* **2013**, *135* (32), 11748−11751.

(24) Rapireddy, S.; Nhon, L.; Meehan, R. E.; Franks, J.; Stolz, D. B.; Tran, D.; Selsted, M. E.; Ly, D. H. RTD-1Mimic Containing γPNA Scaffold Exhibits Broad-Spectrum Antibacterial Activities. *J. Am. Chem. Soc.* **2012**, *134* (9), 4041−4044.

(25) Patch, J. A.; Barron, A. E. Helical Peptoid Mimics of Magainin-2 Amide. *J. Am. Chem. Soc.* **2003**, *125* (40), 12092−12093.

(26) Tew, G. N.; Liu, D.; Chen, B.; Doerksen, R. J.; Kaplan, J.; Carroll, P. J.; Klein, M. L.; DeGrado, W. F. De Novo Design of Biomimetic Antimicrobial Polymers. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99* (8), 5110−5114.

(27) Liu, D.; Choi, S.; Chen, B.; Doerksen, R. J.; Clements, D. J.; Winkler, J. D.; Klein, M. L.; DeGrado, W. F. Nontoxic Membrane-Active Antimicrobial Arylamide Oligomers. *Angew. Chem., Int. Ed.* **2004**, *43* (9), 1158−1162.

(28) Tew, G. N.; Clements, D.; Tang, H.; Arnt, L.; Scott, R. W. Antimicrobial Activity of an Abiotic Host Defense Peptide Mimic. *Biochim. Biophys. Acta, Biomembr.* **2006**, *1758* (9), 1387−1392.

(29) Choi, S.; Isaacs, A.; Clements, D.; Liu, D.; Kim, H.; Scott, R. W.; Winkler, J. D.; DeGrado, W. F. De Novo Design and in Vivo Activity

of Conformationally Restrained Antimicrobial Arylamide Foldamers. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106* (17), 6968−6973.
(30) Radzishevsky, I. S.; Rotem, S.; Bourdetsky, D.; Navon-Venezia, S.; Carmeli, Y.; Mor, A. Improved Antimicrobial Peptides Based on Acyl-Lysine Oligomers. *Nat. Biotechnol.* **2007**, *25* (6), 657−659.
(31) Thaker, H. D.; Cankaya, A.; Scott, R. W.; Tew, G. N. Role of Amphiphilicity in the Design of Synthetic Mimics of Antimicrobial Peptides with Gram-Negative Activity. *ACS Med. Chem. Lett.* **2013**, *4* (5), 481−485.
(32) Yang, L.; Gordon, V. D.; Mishra, A.; Som, A.; Purdy, K. R.; Davis, M. A.; Tew, G. N.; Wong, G. C. L. Synthetic Antimicrobial Oligomers Induce a Composition-Dependent Topological Transition in Membranes. *J. Am. Chem. Soc.* **2007**, *129* (40), 12141−12147.
(33) Arnt, L.; Nüsslein, K.; Tew, G. N. Nonhemolytic Abiogenic Polymers as Antimicrobial Peptide Mimics. *J. Polym. Sci. A, Polym. Chem.* **2004**, *42* (15), 3860−3864.
(34) Kuroda, K.; DeGrado, W. F. Amphiphilic Polymethacrylate Derivatives as Antimicrobial Agents. *J. Am. Chem. Soc.* **2005**, *127* (12), 4128−4129.
(35) Palermo, E. F.; Sovadinova, I.; Kuroda, K. Structural Determinants of Antimicrobial Activity and Biocompatibility in Membrane-Disrupting Methacrylamide Random Copolymers. *Biomacromolecules* **2009**, *10* (11), 3098−3107.
(36) Lienkamp, K.; Madkour, A. E.; Musante, A.; Nelson, C. F.; Nüsslein, K.; Tew, G. N. Antimicrobial Polymers Prepared by ROMP with Unprecedented Selectivity: A Molecular Construction Kit Approach. *J. Am. Chem. Soc.* **2008**, *130* (30), 9836−9843.
(37) Ilker, M. F.; Nüsslein, K.; Tew, G. N.; Coughlin, E. B. Tuning the Hemolytic and Antibacterial Activities of Amphiphilic Polynorbornene Derivatives. *J. Am. Chem. Soc.* **2004**, *126* (48), 15870−15875.
(38) Mowery, B. P.; Lee, S. E.; Kissounko, D. A.; Epand, R. F.; Epand, R. M.; Weisblum, B.; Stahl, S. S.; Gellman, S. H. Mimicry of Antimicrobial Host-Defense Peptides by Random Copolymers. *J. Am. Chem. Soc.* **2007**, *129* (50), 15474−15476.
(39) Mowery, B. P.; Lindner, A. H.; Weisblum, B.; Stahl, S. S.; Gellman, S. H. Structure−Activity Relationships among Random Nylon-3 Copolymers That Mimic Antibacterial Host-Defense Peptides. *J. Am. Chem. Soc.* **2009**, *131* (28), 9735−9745.
(40) Sambhy, V.; Peterson, B. R.; Sen, A. Antibacterial and Hemolytic Activities of Pyridinium Polymers as a Function of the Spatial Relationship between the Positive Charge and the Pendant Alkyl Tail. *Angew. Chem., Int. Ed.* **2008**, *47* (7), 1250−1254.
(41) Oda, Y.; Kanaoka, S.; Sato, T.; Aoshima, S.; Kuroda, K. Block versus Random Amphiphilic Copolymers as Antibacterial Agents. *Biomacromolecules* **2011**, *12* (10), 3581−3591.
(42) Qiao, Y.; Yang, C.; Coady, D. J.; Ong, Z. Y.; Hedrick, J. L.; Yang, Y.-Y. Highly Dynamic Biodegradable Micelles Capable of Lysing Gram-Positive and Gram-Negative Bacterial Membrane. *Biomaterials* **2012**, *33* (4), 1146−1153.
(43) Jiang, Y.; Yang, X.; Zhu, R.; Hu, K.; Lan, W.-W.; Wu, F.; Yang, L. Acid-Activated Antimicrobial Random Copolymers: A Mechanism-Guided Design of Antimicrobial Peptide Mimics. *Macromolecules* **2013**, *46* (10), 3959−3964.
(44) Lienkamp, K.; Kumar, K.-N.; Som, A.; Nüsslein, K.; Tew, G. N. "Doubly Selective" Antimicrobial Polymers: How Do They Differentiate between Bacteria? *Chem.—Eur. J.* **2009**, *15* (43), 11710−11714.
(45) Kuroda, K.; Caputo, G. A.; DeGrado, W. F. The Role of Hydrophobicity in the Antimicrobial and Hemolytic Activities of Polymethacrylate Derivatives. *Chem.—Eur. J.* **2009**, *15* (5), 1123−1133.
(46) Chen, Y.; Guarnieri, M. T.; Vasil, A. I.; Vasil, M. L.; Mant, C. T.; Hodges, R. S. Role of Peptide Hydrophobicity in the Mechanism of Action of α-Helical Antimicrobial Peptides. *Antimicrob. Agents Chemother.* **2007**, *51* (4), 1398−1406.
(47) Sgolastra, F.; deRonde, B. M.; Sarapas, J. M.; Som, A.; Tew, G. N. Designing Mimics of Membrane Active Proteins. *Acc. Chem. Res.* **2013**, *46* (12), 2977−2987.
(48) Thaker, H. D.; Cankaya, A.; Scott, R. W.; Tew, G. N. Role of Amphiphilicity in the Design of Synthetic Mimics of Antimicrobial Peptides with Gram-Negative Activity. *ACS Med. Chem. Lett.* **2013**, *4* (5), 481−485.
(49) Porter, E. A.; Weisblum, B.; Gellman, S. H. Mimicry of Host-Defense Peptides by Unnatural Oligomers: Antimicrobial β-Peptides. *J. Am. Chem. Soc.* **2002**, *124* (25), 7324−7330.
(50) Wieprecht, T.; Dathe, M.; Krause, E.; Beyermann, M.; Maloy, W. L.; MacDonald, D. L.; Bienert, M. Modulation of Membrane Activity of Amphipathic, Antibacterial Peptides by Slight Modifications of the Hydrophobic Moment. *FEBS Lett.* **1997**, *417* (1), 135−140.
(51) Fernández-Vidal, M.; Jayasinghe, S.; Ladokhin, A. S.; White, S. H. Folding Amphipathic Helices Into Membranes: Amphiphilicity Trumps Hydrophobicity. *J. Mol. Biol.* **2007**, *370* (3), 459−470.
(52) Hu, K.; Schmidt, N. W.; Zhu, R.; Jiang, Y.; Lai, G. H.; Wei, G.; Palermo, E. F.; Kuroda, K.; Wong, G. C. L.; Yang, L. A Critical Evaluation of Random Copolymer Mimesis of Homogeneous Antimicrobial Peptides. *Macromolecules* **2013**, *46* (5), 1908−1915.
(53) Kondejewski, L. H.; Jelokhani-Niaraki, M.; Farmer, S. W.; Lix, B.; Kay, C. M.; Sykes, B. D.; Hancock, R. E. W.; Hodges, R. S. Dissociation of Antimicrobial and Hemolytic Activities in Cyclic Peptide Diastereomers by Systematic Alterations in Amphipathicity. *J. Biol. Chem.* **1999**, *274* (19), 13181−13192.
(54) Colville, K.; Tompkins, N.; Rutenberg, A. D.; Jericho, M. H. Effects of Poly(L-lysine) Substrates on Attached Escherichia coli Bacteria. *Langmuir* **2009**, *26* (4), 2639−2644.
(55) Yoshida, T.; Nagasawa, T. ε-Poly-L-lysine: Microbial Production, Biodegradation and Application Potential. *Appl. Microbiol. Biotechnol.* **2003**, *62* (1), 21−26.
(56) Gabriel, G. J.; Madkour, A. E.; Dabkowski, J. M.; Nelson, C. F.; Nüsslein, K.; Tew, G. N. Synthetic Mimic of Antimicrobial Peptide with Nonmembrane-Disrupting Antibacterial Properties. *Biomacromolecules* **2008**, *9* (11), 2980−2983.
(57) Tossi, A.; Sandri, L.; Giangaspero, A. Amphipathic, α-Helical Antimicrobial Peptides. *Pept. Sci.* **2000**, *55* (1), 4−30.
(58) http://aps.unmc.edu/AP/main.php.
(59) Schmidt, N. W.; Lis, M.; Zhao, K.; Lai, G. H.; Alexandrova, A. N.; Tew, G. N.; Wong, G. C. L. Molecular Basis for Nanoscopic Membrane Curvature Generation from Quantum Mechanical Models and Synthetic Transporter Sequences. *J. Am. Chem. Soc.* **2012**, *134* (46), 19207−19216.
(60) Mishra, A.; Lai, G. H.; Schmidt, N. W.; Sun, V. Z.; Rodriguez, A. R.; Tong, R.; Tang, L.; Cheng, J.; Deming, T. J.; Kamei, D. T.; Wong, G. C. L. Translocation of HIV TAT Peptide and Analogues Induced by Multiplexed Membrane and Cytoskeletal Interactions. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108* (41), 16883−16888.
(61) Kyte, J.; Doolittle, R. F. A simple Method for Displaying the Hydropathic Character of a Protein. *J. Mol. Biol.* **1982**, *157* (1), 105−132.
(62) Eisenberg, D.; Weiss, R. M.; Terwilliger, T. C.; Wilcox, W. Hydrophobic Moments and Protein Structure. *Faraday Symp. Chem. Soc.* **1982**, *17* (0), 109−120.
(63) Hessa, T.; Kim, H.; Bihlmaier, K.; Lundin, C.; Boekel, J.; Andersson, H.; Nilsson, I.; White, S. H.; von Heijne, G. Recognition of Transmembrane Helices by the Endoplasmic Reticulum Translocon. *Nature* **2005**, *433* (7024), 377−381.
(64) Fujisawa, S.; Masuhara, E. Determination of Partition Coefficients of Acrylates, Methacrylates, and Vinyl Monomers Using High Performance Liquid Chromatography (HPLC). *J. Biomed. Mater. Res.* **1981**, *15* (6), 787−793.
(65) Kim, A.; Szoka, F., Jr. Amino Acid Side-Chain Contributions to Free Energy of Transfer of Tripeptides from Water to Octanol. *Pharm. Res.* **1992**, *9* (4), 504−514.
(66) Ananthapadmanabhan, K. P.; Goddard, E. D.; Turro, N. J.; Kuo, P. L. Fluorescence Probes for Critical Micelle Concentration Determination. *Langmuir* **1985**, *1* (3), 352−355.
(67) Eisenberg, D.; Weiss, R. M.; Terwilliger, T. C.; Wilcox, W. Hydrophobic Moments and Protein Structure. *Faraday Symp. Chem. Soc.* **1982**, *17* (0), 109−120.

(68) Ludtke, S. J.; He, K.; Heller, W. T.; Harroun, T. A.; Yang, L.; Huang, H. W. Membrane Pores Induced by Magainin. *Biochemistry* **1996**, *35* (43), 13723−13728.
(69) Matsuzaki, K.; Murase, O.; Fujii, N.; Miyajima, K. An Antimicrobial Peptide, Magainin 2, Induced Rapid Flip-Flop of Phospholipids Coupled with Pore Formation and Peptide Translocation. *Biochemistry* **1996**, *35* (35), 11361−11368.
(70) Matsuzaki, K.; Murase, O.; Fujii, N.; Miyajima, K. Translocation of a Channel-Forming Antimicrobial Peptide, Magainin 2, across Lipid Bilayers by Forming a Pore. *Biochemistry* **1995**, *34* (19), 6521−6526.
(71) Matsuzaki, K.; Sugishita, K.-i.; Harada, M.; Fujii, N.; Miyajima, K. Interactions of an Antimicrobial Peptide, Magainin 2, with Outer and Inner Membranes of Gram-Negative Bacteria. *Biochim. Biophys. Acta, Biomembr.* **1997**, *1327* (1), 119−130.
(72) Matsuzaki, K.; Sugishita, K.-i.; Ishibe, N.; Ueha, M.; Nakata, S.; Miyajima, K.; Epand, R. M. Relationship of Membrane Curvature to the Formation of Pores by Magainin 2. *Biochemistry* **1998**, *37* (34), 11856−11863.
(73) Zasloff, M.; Magainins, A. Class of Antimicrobial Peptides from Xenopus Skin: Isolation, Characterization of Two Active Forms, and Partial cDNA Sequence of a Precursor. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84* (15), 5449−5453.
(74) Rathinakumar, R.; Walkenhorst, W. F.; Wimley, W. C. Broad-Spectrum Antimicrobial Peptides by Rational Combinatorial Design and High-Throughput Screening: The Importance of Interfacial Activity. *J. Am. Chem. Soc.* **2009**, *131* (22), 7609−7617.
(75) Fujisawa, S.; Masuhara, E. Determination of Partition Coefficients of Acrylates, Methacrylates, and Vinyl Monomers Using High Performance Liquid Chromatography (HPLC). *J. Biomed. Mater. Res.* **1981**, *15* (6), 787−793.
(76) Wei, G.; Liu, X.; Yuan, L.; Ju, X.-J.; Chu, L.-Y.; Yang, L. Lipid Composition Influences the Membrane-Disrupting Activity of Antimicrobial Methacrylate Co-polymers. *J. Biomater. Sci. Polym. Ed.* **2011**, *22* (15), 2041−2061.
(77) Darveau, R. P.; Cunningham, M. D.; Seachord, C. L.; Cassiano-Clough, L.; Cosand, W. L.; Blake, J.; Watkins, C. S. Beta-Lactam Antibiotics Potentiate Magainin 2 Antimicrobial Activity in Vitro and in Vivo. *Antimicrob. Agents Chemother.* **1991**, *35* (6), 1153−1159.
(78) Hancock, R. E. W. Alterations in Outer Membrane Permeability. *Annu. Rev. Microbiol.* **1984**, *38* (1), 237−264.
(79) Klibanov, A. M. Permanently Microbicidal Materials Coatings. *J. Mater. Chem.* **2007**, *17* (24), 2479−2482.
(80) Raghuraman, H.; Chattopadhyay, A. Orientation and Dynamics of Melittin in Membranes of Varying Composition Utilizing NBD Fluorescence. *Biophys. J.* **2007**, *92* (4), 1271−1283.