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Antimicrobial Peptide Mimicking Primary Amine and Guanidine Containing Methacrylamide Copolymers Prepared by Raft Polymerization

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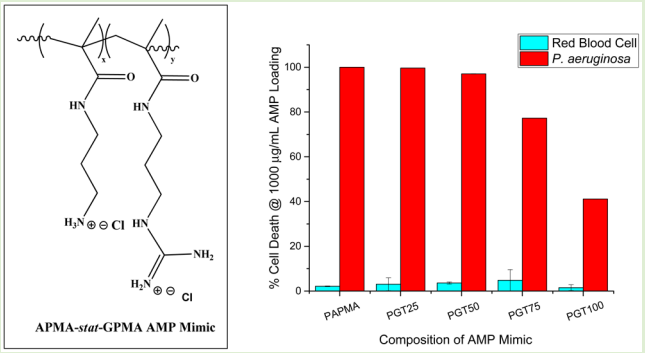
Supporting Information

ABSTRACT: Naturally occurring antimicrobial peptides (AMPs) display the ability to eliminate a wide variety of bacteria, without toxicity to the host eukaryotic cells. Synthetic polymers containing moieties mimicking lysine and arginine components found in AMPs have been reported to show effectiveness against specific bacteria, with the mechanism of activity purported to depend on the nature of the amino acid mimic. In an attempt to incorporate the antimicrobial activity of both amino acids into a single water-soluble copolymer, a series of copolymers containing lysine mimicking aminopropyl methacrylamide (APMA) and arginine mimicking guanadinopropyl methacrylamide (GPMA) were prepared via aqueous RAFT polymerization. Copolymers were prepared with varying ratios of the comonomers, with degree of polymerization of 35−40 and narrow molecular weight distribution to simulate naturally occurring AMPs. Antimicrobial activity was determined against Gram-negative and Gram-positive bacteria under conditions with varying salt concentration. Toxicity to mammalian cells was assessed by hemolysis of red blood cells and MTT assays of MCF-7 cells. Antimicrobial activity was observed for APMA homopolymer and copolymers with low concentrations of GPMA against all bacteria tested, with low toxicity toward mammalian cells.



INTRODUCTION

Antimicrobial peptides (AMPs) are naturally occurring defensive agents that eliminate bacteria and are found in a variety of eukaryotic organisms, including mammals, insects, and plants. Over 500 unique AMPs are catalogued,[1] whose structure is believed to be influenced by specific environmental factors. The peptides display several consistent characteristics, including a composition of 20−50 amino acid residues, distinct hydrophobic and hydrophilic regions, and a net positive charge at physiological pH (7.4). The hydrophilic regions have an abundance of lysine and arginine amino acid residues, which are protonated and positively charged under physiological conditions. It is this net positive charge that enables AMPs to selectively bind to bacteria, as the bacterial membranes are negatively charged, and induce cell death. The host eukaryotic cells have a net neutral charge and, therefore, do not have as high an affinity for AMPs, resulting in an efficient and selective defense against bacteria. Bacteria do not appear to develop resistance to AMP activity as readily as they do to current synthetic antibiotics, but isolation or synthesis of sufficient quantities of AMPs is difficult and costly. Synthetic polymeric mimics of AMPs are a desirable alternative because they offer potential for lower production costs, are amenable to manufacture, and have highly tailorable structures.[2,3]

Several parameters are known to affect the efficacy of AMP mimics. First, polymers of lower molecular weight, around that of natural AMPs, show greater antimicrobial activity than monomers or higher molecular weight systems.[4] Second, a precise amphipathic balance is necessary to impart both antibacterial activity and selectivity,[4−6] where activity is defined as toxicity to bacteria and selectivity is defined as toxicity to bacterial cells with limited toxicity to eukaryotic cells at the concentration required for bacterial cell death. A minimum inhibitory concentration of ∼100 μg/mL is considered to be high antimicrobial activity, while 500−1000 μg/mL is considered moderate. If hydrophobicity is too high, selectivity

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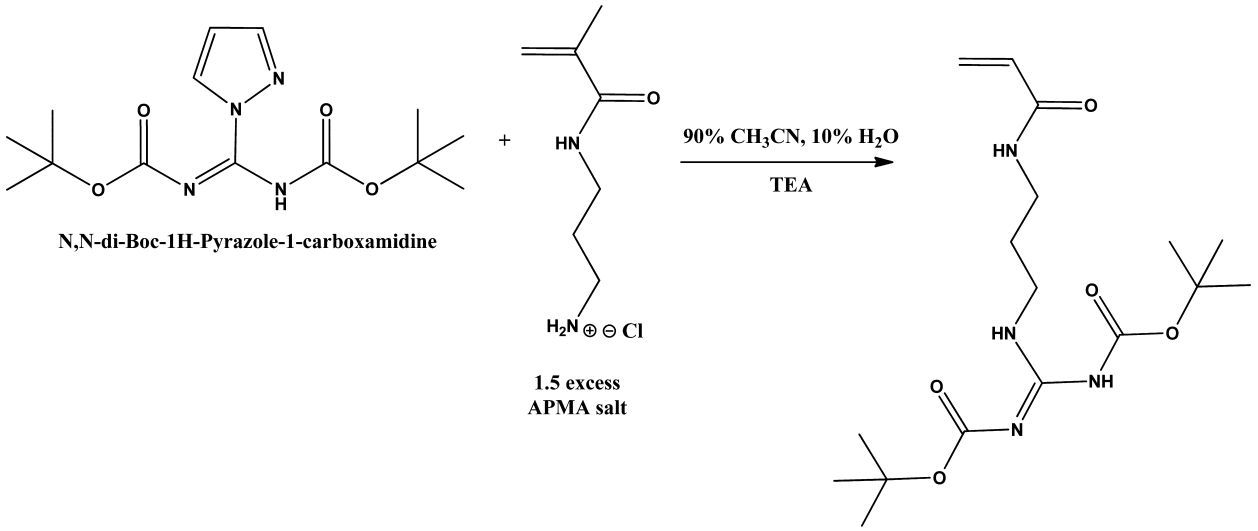
Scheme 1. Synthesis of BOC-Protected GPMA



is reduced and eukaryotic cell death occurs. A number of different methods have been employed to modify the amphipathic balance in synthetic systems. Researchers increased hydrophobicity through copolymerization of cationic monomers with hydrophobic monomers with alkyl tails of different lengths, which generally resulted in increased bacterial cell death at the expense of selectivity.[7−9] Multiple polymer backbone structures, including methacrylates,[7,8,10,11] β-lactams,[12] norbornenes,[5,13] and methacrylamides,[6,9] with varying solubility and inherent polarity have been evaluated as AMP mimics. Palermo et al. showed that the polarity of the comonomers employed to prepare AMP mimics affected the hydrophobic/hydrophilic balance of the resultant copolymers. In their system, methacrylamide based AMP mimics required a higher concentration of the hydrophobic comonomer to achieve antimicrobial activity than did methacrylate based copolymers.[9,10]

The structure of the cationic moiety also impacts the effectiveness of synthetic AMP mimics. Mostly primary amines,[5,6,8,12] which mimic lysine amino acid residues, have been employed; however, tertiary[6] and quaternary[10] amines have also been investigated. Results indicate, however, that transition from primary to tertiary or quaternary amines reduces activity and selectivity of AMPs.[5,6,8,10,12] In our previous work, we investigated the effect of pendant cation modification with alkyl groups of different structures to alter the amphipathic ratio of methacrylamide AMPs, and found that unmodified primary amine cations display superior antimicrobial activity and selectivity.[6] A high level of antimicrobial activity was exhibited for primary amine methacrylamides against *Escherichia coli* and *Bacillus subtilis*, and it was found that activity was affected by the type of buffer used in the broth microdilution assays.

Researchers have also begun to explore guanidinium, which mimics the amino acid arginine found in natural AMPs, as an alternative cationic moiety. Gabriel et al., using a polynorbornene backbone, reported that substituting guanidinium for primary amines improved selectivity without affecting the antimicrobial activity.[13] Locock et al., using a methacrylate backbone, reported that guanidinium improved both activity and selectivity.[11] These findings suggest the possibility of expanding antimicrobial properties of synthetic systems through incorporation of both lysine and arginine mimics in a single polymer.

Antimicrobial activity of AMP mimics has been tested against both Gram-positive bacteria, which have a cell wall that surrounds an inner plasma membrane, and Gram-negative bacteria, which have a thin cell wall that is sandwiched between two plasma membrane layers. Typically, *E. coli*, Gram-negative, and *B. subtilis*, Gram-positive, are used as representative bacteria in antimicrobial activity testing. Other systems of interest include *Pseudomonas aeruginosa*, which displays an inordinate ability to resist traditional therapeutics and is the most reported nosocomial bacterium,[14,15] and *Staphylococcus aureus*, due to the emergence of methicillin resistant strains.[16] Investigation of the effects of added salt on AMP mimics is also warranted.[17,18]

In this study, statistical copolymers of aminopropyl methacrylamide (APMA) and 3-guanadinopropyl methacrylamide (GPMA) of controlled molecular weight and composition are prepared via aqueous reversible addition−fragmentation chain transfer (RAFT) polymerization for determination of the effects of lysine- and arginine-mimicking pendant groups in a fully water-soluble AMP mimic on antimicrobial activity and selectivity. RAFT is employed to obtain polymers of targeted molecular weights with low molecular weight distributions. Polymers are prepared with molecular weights similar to those of naturally occurring AMPS. The low molecular weights of the synthesized polymers eliminate the need for built-in biodegradability, as small molecules are removed from the body via the renal system.[19] Under the benign aqueous RAFT reaction conditions, *tert*-butyloxycarbonyl (BOC)-protection of the amine-containing monomers is not required. Antimicrobial behavior against *E. coli*, *S. aureus*, and *P. aeruginosa* is reported in solutions of varying salt concentration. Toxicity to mammalian cells is evaluated via hemolysis of red blood cells and MTT assays of MCF-7 cells.

MATERIALS AND METHODS

Materials. For GPMA synthesis, *N*-(3-aminopropyl) methacrylamide hydrochloride, >98% (APMA·HCl) (Polysciences, Inc.), triethylamine, >99.8% (TEA) (Sigma-Aldrich), *N*,*N*′-di-BOC-1*H*-pyrazole-1-carboxamidine (PCA) (Sigma-Aldrich), acetonitrile, anhydrous, 98% (Sigma-Aldrich), sodium sulfate (Sigma-Aldrich), and 4 M HCl in dioxane (Fischer Scientific) were used as purchased. For all polymerizations, 4,4′-azobis (4-cyanopentanoic acid) (V-501)

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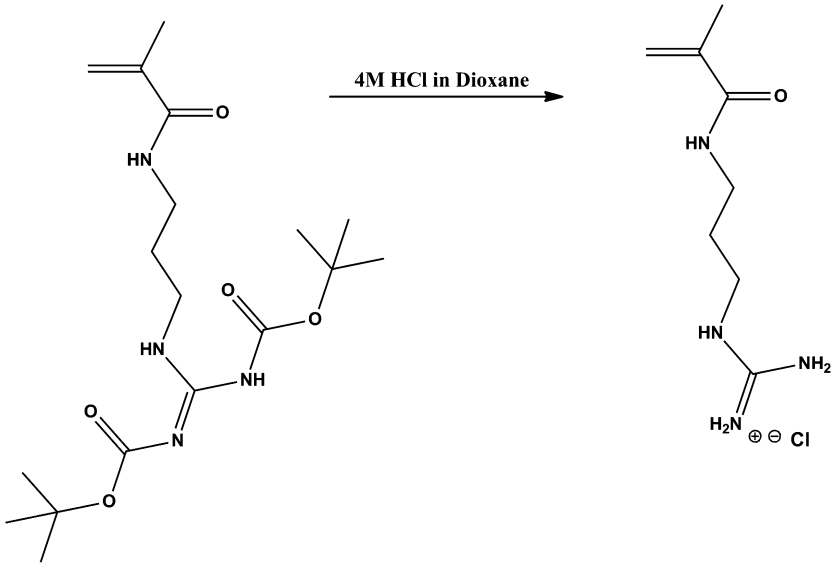
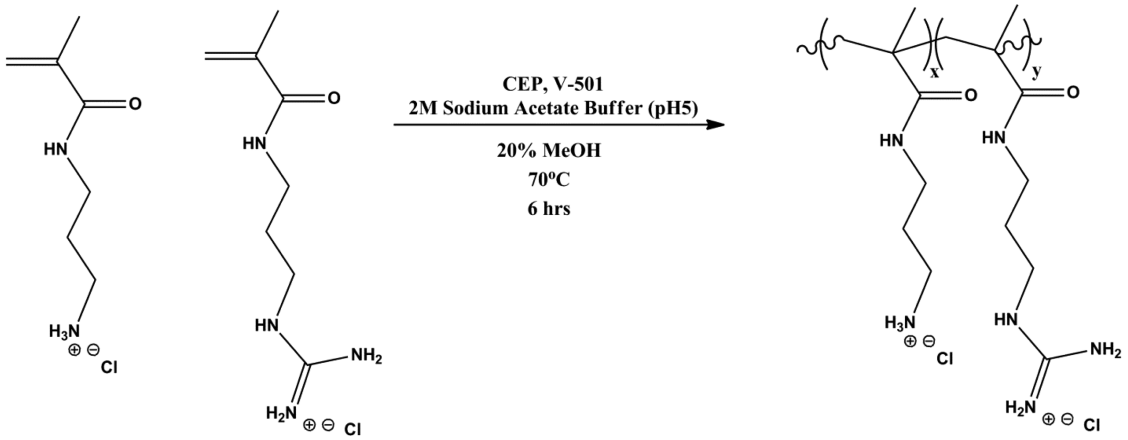
Scheme 2. Deprotection of BOC-Protected GPMA



Scheme 3. Aqueous RAFT Polymerization of Poly(APMA-stat-GPMA)



(Sigma-Aldrich), methanol, anhydrous, 99.8% (Fischer Scientific), sodium acetate, anhydrous (Sigma-Aldrich), acetic acid, glacial (Fisher Scientific), and Spectra/Por dialysis membrane, standard RC tubing (3500 kDa) (Spectrum Laboratories, Inc.) were used as purchased. Judicious choice of RAFT agent is necessary to control the effects of end groups on activity and selectivity.[20] 4-Cyano-4-(ethylsulfanylthiocarbonylsulfanyl)pentanoic acid (CEP) was used as the CTA in all polymerizations and was synthesized via previously published procedures.[21] For antimicrobial susceptibility testing, *Escherichia coli* (DH5α), *Staphylococcus aureus* (RN4220), *Pseudomonas aeruginosa* (PAO1), Mueller-Hinton broth (MHB, 2 g/L beef extract, 17.5 g/L casein hydrolysate, and 1.5 g/L starch, pH 7.4) (Fischer Scientific), and low salt Luria Broth (10 g/L tryptone, 5g/L yeast extract, 0.5 g/L NaCl, pH 7.4) (LB) (Sigma-Aldrich) were used as purchased. Tris(hydroxymethyl) aminomethane (Tris) (Sigma-Aldrich) and NaCl (Sigma-Aldrich) were used to make Tris buffer (10 mM Tris, pH 7.4) and Tris-buffered saline (TBS, 10 mM Tris, 150 mM NaCl, pH 7.4). All testing of mammalian cell toxicity was done with Triton-X 100 (Merck, India), Dulbecco's modified Eagle's media (DMEM) (Himedia, India), fetal bovine serum (FBS), (Himedia, India), trypsin-EDTA Solution 1× (Himedia, India), antibiotic solution 100X Liquid (AS) (made with penicillin and streptomycin) (Himedia, India), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, India), and dimethyl sulfoxide (DMSO) (Merck, India), used as received. Preparation of MCF-7 cells (NCCS, Pune, India) is discussed in the cell viability section of this paper. Isolation of red blood cells (RBC) (AIIMS hospital, New Delhi, India) is discussed in the hemolysis section of this paper.

Synthesis of BOC-Protected GPMA. APMA·HCl, dissolved in a mixture of DI water (12 mL) and TEA (20 mL, 148 mmol), was stirred at 25 °C. PCA (10 g, 32 mmol), dissolved in acetonitrile (108 mL), and then added dropwise, over 30 min, to the stirring APMA·HCl solution via an addition funnel (Scheme 1). The reaction progressed for 24 h. The product was purified via filtration, washed three times with DI water (50 mL), and lyophilized overnight (yield: 91%; Figure S2). BOC-protected GPMA: ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.46 (d, 18H), 1.70 (m, 2H), 2.00 (s, 3H), 3.32 (m, 2H), 3.49 (m, 2H), 5.31 (s, 1H), 5.81 (s, 1H), 7.39 (t, 1H), 8.49 (t, 1H), 11.53 (s, 1H).

Deprotection of BOC-Protected GPMA. Deprotection of GPMA was performed with HCl to obtain Cl^- coordinated with the guanidinium. BOC-protected GPMA was dissolved in 4 M HCl in dioxane (7 equiv of per BOC-protecting group) and allowed to react overnight. The product, a white solid, precipitated from solution and was purified via filtration and washing with anhydrous dioxane (Scheme 2, Figure S3). GPMA: ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.65 (m, 2H), 1.86 (s, 3H), 3.13 (m, 4H), 5.34 (s, 1H), 5.66 (s, 1H), 7.00−7.50 (b, 4H), 7.70 (m, 1H), 8.00 (m, 1H).

Polymerization of Antimicrobial Peptide (AMP) Mimics. Utilizing aqueous RAFT polymerization, polymer molecular weights were targeted by selecting appropriate initial monomer and CTA concentrations. The ratio of M_0:CTA_0 was set to be 30:1 to yield a

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degree of polymerization of ∼30, at 90% conversion, which mimics the size of naturally occurring AMPs. 4,4′-Azobis(4-cyanopentanoic acid) was used as the initiator. For all polymerizations, CTA_0/I_0 = 5. The polymerization was conducted in aqueous acetate buffer (1.5 M sodium acetate and 0.5 M acetic acid, pH 5) at 70 °C for 6 h (Scheme 3).[22] Methanol was added in low quantities (∼30%) to improve the solubility of CEP in the aqueous media. After the reactions were completed, the solutions were exposed to air and quenched in liquid nitrogen. The solutions were then dialyzed against water for 72 h, lyophilized for 72 h, and then stored in desiccant until subsequent testing (representative yield: 75%).

The mol % of GPMA was varied to produce five (co)polymers. The nomenclature PGT represents polyGPMA and the corresponding number indicates the targeted mole fraction in the (co)polymer. For example, PGT25 represents a copolymer composed of 25 mol % GPMA and 75 mol % APMA·Cl. Copolymer compositions were determined by comparing the relative peak areas of the methylene-proton resonance of APMA at 3.00 ppm and methylene-proton resonances of APMA and GPMA at 3.20 ppm.

Nuclear Magnetic Resonance (NMR). ^{1}H NMR was performed with a Varian Mercury^{PLUS} 300 MHz spectrometer in $CDCl_3$ or DMSO-d_6, utilizing delay times of 5 s to determine monomer purity. A 500 MHz NMR equipped with a standard 5 mm ^{1}H/^{13}C probe and operating at 499.77 MHz (^{1}H) was used to identify the structures of PAPMA and PGPMA homopolymers and the structures of the poly(APMA-*stat*-GPMA) copolymers in D_2O. 64 scans were taken for each experiment with a 3.1 s recycle delay. For each of the homopolymers, unique peak assignments were made, and the copolymer compositions were calculated for the statistical polymers via peak integration of methylene bridge E and E′ on the APMA and GPMA monomer residues (Figure S4), respectively.

The polymerization reaction progress was monitored, and the controlled nature of the reaction was ensured for PGT50 through aliquots that were taken every hour from the reaction vial. ^{1}H NMR was utilized to determine [*M*] at time (*t*) by comparing the vinylic hydrogen peak integrations to the integration of an internal standard (MeOH).

Aqueous Size Exclusion Chromatography. The molecular weight and molecular weight distribution (MWD, equal to M_w/M_n) of the polymers were determined by aqueous size exclusion chromatography (ASEC) coupled with multiangle laser light scattering (MALLS). Eprogen CATSEC columns (100, 300, and 1000 Å) were used in combination with a Wyatt Optilab DSP interferometric refractometer (k = 690 nm) and a Wyatt DAWN DSP MALLS detector (k = 633 nm). Acetic acid (1 wt %)/0.1 M Na_2SO_4 (aq) was used as the eluent at a flow rate of 0.25 mL/min. The interferometric refractometer was utilized off-line to determine dn/dc values for PAPMA and PGPMA at 25 °C in the eluent (1 wt % acetic acid/0.1 M Na_2SO_4 (aq)) in order to assign absolute molecular weight values to all homopolymers. For the statistical polymers, the dn/dc values were calculated as the mole-fraction-averaged composites of the measured homopolymer dn/dc values using the copolymer compositions determined by ^{1}H NMR. Wyatt ASTRA SEC/LS software was used for molecular weight and MWD calculations. ASEC-MALLS was also utilized to monitor molecular weight as a function of time. The refractive index (RI) response vs elution volume was collected for aliquots taken at each time interval.

Antimicrobial Susceptibility Assay. Antimicrobial susceptibility was measured using the broth microdilution method according to the CLSI guidelines.[23] In order to assess separately the effects of the medium and the effects of added salt on polymer antimicrobial effectiveness, bacterial strains were incubated in both MHB and low salt LB. Polymer solutions were prepared in both Tris and Tris buffered saline solutions. Each experiment consisted of three replicates (three wells per each strain), and each experiment was replicated three times (resulting in nine replicates of each individual condition). Briefly, overnight cultures of bacteria were prepared in 15 mL polystyrene culture tubes containing 5 mL of MHB. The bacterial cells were diluted 1:10 times in fresh MHB medium and allowed to grow for 3 h before normalizing to OD_{600} of 0.008. The same procedure was followed for preparation of bacterial cultures in low salt LB. Twofold serial dilutions of each polymer solution (1000 μg to 15.6 μg/mL) were prepared in a 96-well Costar microtiter plate (using either Tris or Tris buffered saline solution as delineated in the Results and Discussion section). An additional well containing 750 μg of polymer solution was included between the 1000 μg/mL and 500 μg/mL wells. The microtiter wells containing polymer dilutions were then inoculated with 5×10^5 colony forming units per milliliter of each test strain and incubated overnight at 37 °C.[24] MHB medium (or low salt LB medium) without any polymer solution was inoculated with each strain and used as a positive control for microbial growth, and MHB medium (or low salt LB medium) without any bacteria was used as a negative control and as a blank for absorbance readings. After overnight incubation at 37 °C, wells were analyzed for bacterial growth both by visual inspection and by measuring OD_{580} using a BioTek plate reader. The lowest concentration (μg/mL) of the polymer that completely prevented bacterial growth was considered the minimum inhibitory concentration (MIC).

Hemolysis Assay. Hemolysis testing was done in accordance with the procedure previously published by Paslay et al.[6] Whole blood (received from AIIMS hospital in New Delhi, India) was centrifuged at 1500 rpm for 10 min to pellet the red blood cells (RBCs). The serum was decanted and RBCs (30 μL) were suspended in TBS (10 mL) and then centrifuged at 1500 rpm for 10 min. Suspension in TBS and centrifugation was repeated a total of three times to adequately rinse RBCs before suspending them in TBS (10 mL). Simultaneously, stock solutions were made of all homo- and copolymers in TBS at concentrations relevant to determine MICs.

RBC and polymer solutions were incubated together in microcentrifuge tubes at a 1:1 ratio in a total volume of 1 mL, at 37 °C for 30 min. The microcentrifuge tubes were then centrifuged at 1500 rpm for 10 min to separate the intact, healthy RBCs from the rest of the solution. The supernatant, containing hemoglobin released from lysed RBCs, was transferred to a 96-well plate (100 μL in triplicate) where absorbance at 540 nm was determined in a Biotek PowerWave X S2 UV−vis plate reader. Percent hemolysis was determined through normalization of the observed absorbance for individual polymer wells to that of positive (100% hemolysis) and negative (0% hemolysis) controls.

MTT Assay. Further selectivity testing was performed as previously described against MCF-7 cancer cells.[13] Cells were grown to 80% confluence at 37 °C in a 5% CO_2 incubator, using tissue culture polystyrene Falcon flasks and DMEM medium that was supplemented with 10% fetal calf serum and 1% antibiotic serum.[6] Trypsin was added to dissociate cells from the Falcon flask; cells were counted in a hemocytometer to determine cell concentration. Fresh medium was then added to the Falcon flask so that cells could be seeded into a 96-well plate (100 μL per well). Seeded cells were incubated for 24 h at 37 °C in a 5% CO_2 incubator. The old medium was then removed from each well and replaced with fresh medium (100 μL per well). Polymer solutions (50 μL per well) were added to appropriate wells in triplicate, resulting in final polymer concentrations of 25, 100, 200, and 2000 μg/mL, respectively. TBS (50 μL per well) and 3% Triton-X (50 μL per well) were added, in triplicate, as the positive and negative controls, respectively. Plates were incubated for either 6 or 12 h at 37 °C in a 5% CO_2 incubator. Old medium was then removed and replaced with new medium (100 μL per well). MTT in TBS (10 mg/mL, 10 μL per well) was added and allowed to incubate for 4 h at 37 °C in a 5% CO_2 incubator. Old medium was removed, and DMSO (100 μL per well) was added to dissolve formazan crystals produced by living MCF-7 cells. Absorbance of each well was read at 570 nm using a Biotek PowerWave X S2 UV−vis plate reader. Percent cell viability was determined by normalizing the measured absorbance in polymer-containing wells with the absorbance in the positive and negative control wells.

RESULTS AND DISCUSSION

GPMA Synthesis and (Co)polymerization. Guanidinium functionalized synthetic AMP mimics were prepared to

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Table 1. Molecular Weight and Composition Data for APMA and GPMA Homo- and Copolymers

polymer	mol % GPMA (theory)	mol % GPMA (exp)[a]	$M_{n,th}$ (g/mol)[b]	$M_{n,exp}$ (g/mol)[c]	M_n/M_w	DP	dn/dc[d]
PAPMA	0	0	5600	6500	1.06	35	0.200
PGT25	25	30	5700	7000	1.11	36	0.183
PGT50	50	44	6300	7400	1.12	36	0.169
PGT75	75	67	6600	8100	1.16	37	0.152
PGT100	100	100	6900	9200	1.11	40	0.132

[a]Determined by ^{1}H NMR. [b]Based on 90% conversion of $[M]_0$. [c]Determined by ASEC-MALLS. [d]Determined by a Wyatt Optilab DSP interferometric refractometer.

compare the broad-spectrum antimicrobial effectiveness of arginine and lysine analogues when incorporated as pendant groups into fully water-soluble, hydrolytically stable, methacrylamide copolymers (Figure S1). We previously demonstrated high antimicrobial activity against *E. coli* (Gram-negative) and *B. subtilis* (Gram-positive) for lysine-mimicking APMA polymers, with low toxicity to mammalian cells.[6] These findings, combined with literature reports of antimicrobial effectiveness obtained with incorporation of guanidinium moieties in other polymeric systems,[11,13] and the McCormick group's previous report of GPMA copolymer synthesis and eukaryotic cell penetration behavior,[25] provided the motivation for the current study. Methacrylamide based monomers were chosen for their previously demonstrated antimicrobial activity and selectivity, hydrolytic stability, high degree of water solubility, and pK_a values (ensuring ionization at physiological pH). Increased monomer purity and yield for GPMA were obtained through modifications of the synthetic methods reported by Treat et al.[25] and Gabriel et al.[13] Specifically, APMA·HCl was deprotonated in situ with TEA, allowing nucleophilic attack on the PCA, as shown in (Scheme 1, Figure S2). The resulting BOC-GPMA precipitated at high yield (~91%) from solution, with byproducts removed through subsequent filtration and washing with DI water. This reaction was more facile than the previous synthesis by Treat et al., who reported deacidification of purchased APMA followed by dropwise addition to a stirring solution of 2-ethyl-2-thiopseudourea and TEA in acetonitrile. Additionally, that reaction required column chromatography for product purification and resulted in a lower (72%) yield. In our current procedure, GPMA was deprotected, shown in (Scheme 2, Figure S3), before polymerization through a well-established BOC-deprotection protocol[13] with HCl.

GPMA copolymers of controlled molecular weight, narrow molecular weight distribution, and desired composition were prepared via aqueous RAFT polymerization as described in the experimental section (Table 1). Measured values of molecular weight and GPMA incorporation are in good agreement with predicted values. All polymers show narrow molecular weight distributions, with M_w/M_n values smaller than 1.2. Kinetic plots for the polymerization of the 1:1 molar ratio of APMA:GPMA are shown in Figure 1. A plot of $\ln([M]_0/[M])$ as a function of reaction time shows linear pseudo-first-order kinetic behavior, with no apparent induction time (Figure 1a). The SEM chromatogram with overlay of traces obtained at specified times within the polymerization (Figure 1b) shows a steady increase in MW and narrowing of MWD with no evidence of a high molecular weight shoulder as a function of reaction time, suggesting controlled polymerization. Plots of MWD and theoretical and experimental M_n as a function of percent conversion provide further evidence of controlled polymerization, with low MWD and linear increase in M_n to 50%

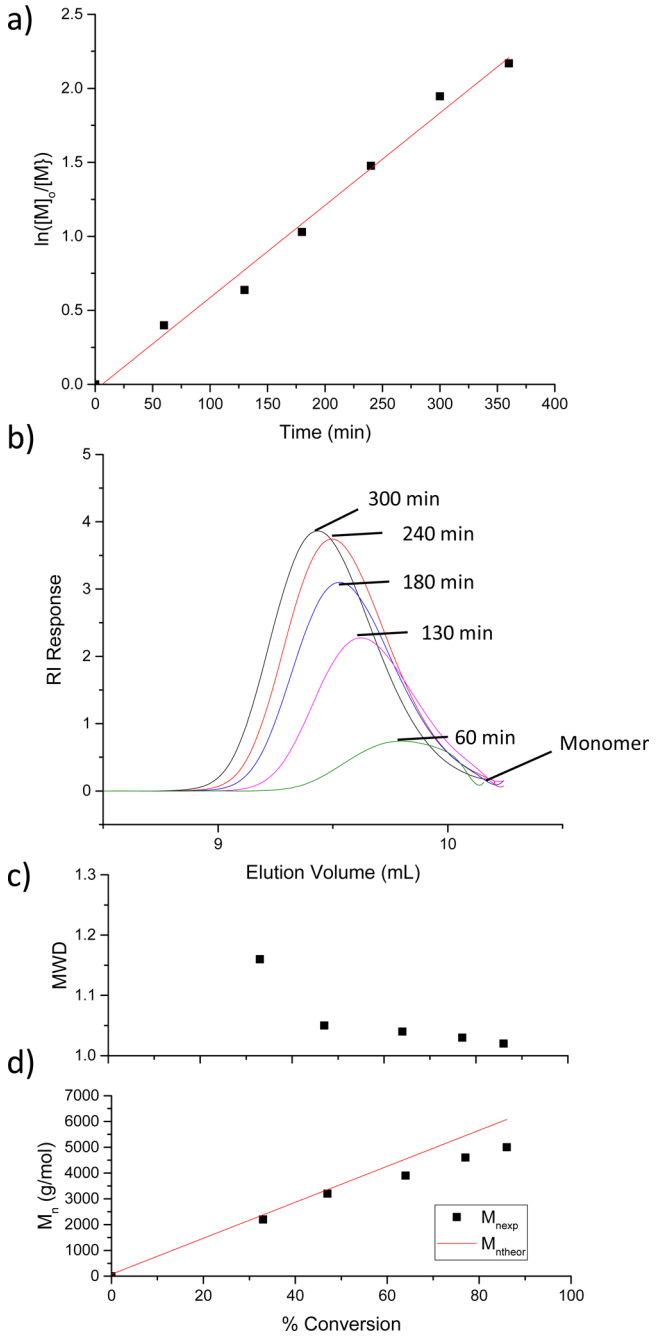

Figure 1. Kinetic plots for the polymerization of 1:1 molar ratio of APMA:GPMA: (a) $\ln([M]_0/[M])$ as a function of time, (b) SEC overlay at specified reaction times, (c) MWD vs % conversion, and (d) theoretical and experimental M_n vs % conversion.

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Table 2. Minimum Inhibitory Concentrations (MIC) Values (μg/mL) of AMP-Mimics Determined by Microbroth Dilution Method (24 h) with Different Media and Buffers[a]

	MIC (μg/mL)								
	MHB (Tris buffer)			MHB (TBS buffer)			low salt LB (Tris buffer)		
polymer	*E. coli*	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. aureus*	*P. aeruginosa*
PAPMA	125	250	250	500	500	500	250	500	500
PGT25	250	500	750	1000	1000	1000	250	500	500
PGT50	1000	1000	1000	>1000	>1000	>1000	500	750	750
PGT75	1000	1000	1000	>1000	>1000	>1000	500	750	1000
PGT100	1000	1000	1000	>1000	>1000	>1000	500	750	1000

[a]MIC = concentration of (co)polymer required to completely inhibit bacterial growth. Experiments performed in triplicate with three replications.

conversion. Deviations from linearity at longer reaction times have been reported previously for acrylamide RAFT polymerizations.[26] A representative ^{1}H NMR spectrum of poly(APMA-*stat*-GPMA) targeted at a 50:50 mol ratio is provided in the Supporting Information (Figure S4). Copolymer composition was determined as described in the Materials and Methods, and in general experimental composition is close to target. Similar kinetic plots, GPC traces, and ^{1}H NMR analyses were obtained for all copolymer compositions. It was important to ensure that the targeted MW and copolymer composition were achieved in order to separate the effects of MW and MWD from those of polymer composition on antimicrobial activity and selectivity.

Antimicrobial Activity. Broth microdilution testing, as described in the experimental section, was completed to determine the antimicrobial activity of the copolymers against *E. coli*, *S. aureus*, and *P. aeruginosa* under specific solution conditions, and the results are summarized in Table 2. Activity was determined based on the minimum inhibitory concentration (MIC), which is the polymer concentration at which 100% cell death occurs. Polymer solutions were prepared in either Tris or TBS, and activities were evaluated against bacteria incubated in either MB or low salt LB, as indicated in the table. Each experiment was performed in triplicate and replicated three times, and MIC values were found to be identical for the three replicates.

The highest activity is observed for the copolymers suspended in Tris buffer and tested in MHB medium. Under these conditions, PAPMA demonstrates the highest antimicrobial activity against *E. coli* (125 μg/mL), followed by *S. aureus* (250 μg/mL) and *P. aeruginosa* (250 μg/mL), indicating PAPMA's broad range activity against both Gram-negative and Gram-positive bacteria. PAPMA's effectiveness against *S. aureus* and *P. aeruginosa* is of particular significance because of the reported extreme resistance of these bacteria to traditional therapeutics.[14,16] Under these same conditions, antimicrobial activity decreases with increasing GPMA content, and only the copolymer with the lowest GPMA concentration (PGT25)) shows moderate activity against the bacteria tested, *E. coli* (250 μg/mL), *S. aureus* (500 μg/mL), and *P. aeruginosa* (750 μg/mL). The other copolymers with increasing GPMA content (PGT50, PGT75, and PGT100) show antimicrobial activity in the range of 1000 μg/mL. Reduced antimicrobial activity is observed when the copolymers are evaluated in the presence of NaCl, by suspending them in TBS (150 mM NaCl) and testing in the MHB medium (Table 2). Under these conditions, only APMA homopolymer and PGT25 show moderate activity against all three bacteria, with MICs of 500 and 1000 μg/mL, respectively. These findings suggest that salt interferes with the interaction of the AMP-mimics with the bacterial membrane, and that copolymers with higher GPMA concentrations are affected to a greater extent. Intermediate activities are observed for copolymers in the low salt LB medium (8.5 mM NaCl). PAPMA and PGT25 show the highest antimicrobial activity, with both polymers yielding MICs of 250 μg/mL against *E. coli* and 500 μg/mL against *S. aureus* and *P. aeruginosa*. Under these conditions the copolymers with higher GPMA concentrations show moderate activity against Gram-negative *E. coli* and *P. aeruginosa* (MICs of 500 and 750 μg/mL) but lower activity against Gram-positive *S. aureus* (MICs of 1000 μg/mL). These findings suggest that both the medium and the salt concentration are critical factors in determining the antimicrobial activity of AMPs, and demonstrate the importance of utilizing identical conditions when comparing performance of polymers of different structures. Under all conditions tested, APMA homopolymer shows the highest antimicrobial activity, with PGT25 showing equivalent or slightly lower activity.

The lower than expected activity observed for copolymers with high GPMA content may be related to the molecular weight of the polymers. Previous reports involved lower molecular weight guanidinium containing copolymers.[11,13] Locock et al.[11] demonstrated in their systems that MIC for both Gram-positive and Gram-negative bacteria increased with increasing molecular weight of the guanidinium based polymers. This was not the case for primary amine based AMP mimics, also observed by Mowery et al.[27] Locock suggested that this behavior was indicative of an alternate mechanism of inducing cell death for guanadinium AMP mimics.

Hemolysis Assay. Selectivity testing against red blood cells (RBCs) was performed for all polymers at concentrations of 50, 500, 1000, and 3000 μg/mL (Figure 2). Hemolysis testing monitored the percent release of hemoglobin from lysed RBCs in comparison to a positive and negative control. For all polymers, at all concentrations, RBC lysis is below 10%, indicating low mammalian toxicity at desired antimicrobial concentrations (≤1000 μg/mL). In general, hemolysis increases with polymer concentration, and the GPMA-containing polymers show greater hemolysis than the PAPMA homopolymer. Hemolysis also increases with increasing GPMA content. Molecular weight may also be a factor in mammalian cell toxicity. The copolymers in this study were designed to have DP of 30, close to that of naturally occurring antimicrobial peptides. Locock reported that lower DPs for their guanadinium copolymers correlated with decreased hemolysis.[11]

MTT Assay. The MTT assay utilized MCF-7 breast cancer cells as the representative mammalian cell for selectivity testing. Table 3 summarizes percent viability of MCF-7 cells as a

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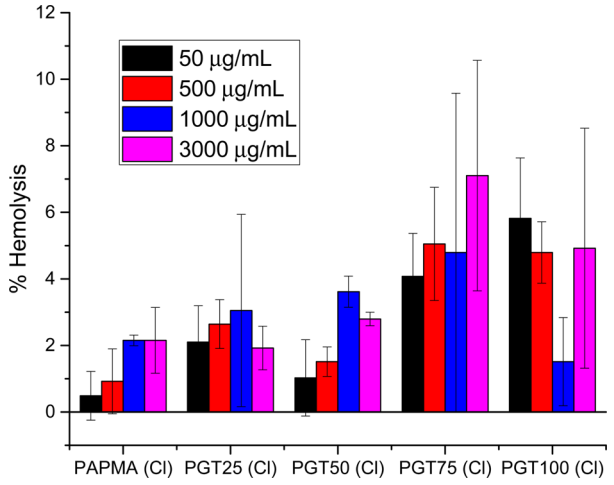

Figure 2. Hemolysis testing, where percent hemolysis of red blood cells as a function of relevant antimicrobial concentrations is shown for all synthesized AMP mimics.

Table 3. MTT Assay for Select AMP Mimics, Where Percent Cell Viability of MCF-7 Cancer Cells as a Function of Relevant Antimicrobial Concentrations Is Shown after Incubation for 6 and 12 h[a]

	6 h		12 h	
polymer	200 μg/mL	2000 μg/mL	200 μg/mL	2000 μg/mL
PAPMA·Cl	90 ± 5	71 ± 3	85 ± 4	73 ± 13
PGT50·Cl	77 ± 5	71 ± 3	73 ± 6	64 ± 5
PGT100·Cl	54 ± 9	34 ± 6	58 ± 2	8 ± 1

[a]Data displayed are averages with ± values representing one standard deviation.

function of polymer concentration after incubation for 6 and 12 h. After 6 h, greater than 70% cell viability is demonstrated for solutions of PAPMA homopolymer and PGT50 copolymers at concentrations up to 2000 μg/mL. Exposure to the PGT homopolymers, however, results in dramatically reduced cell viability. Similar results are observed after 12 h of incubation, however the PAPMA homopolymer displays slightly higher cell viability than the PGT50 copolymers.

CONCLUSION

GPMA was synthesized and polymerized with APMA via aqueous RAFT to produce homopolymers and copolymers with narrow molecular weight distribution, DP of 30−40, and desired copolymer composition. Antimicrobial activity was evaluated against Gram-negative *E. coli* and *P. aeruginosa* and Gram-positive *S. aureus* under different solution conditions. Toxicity to mammalian cells was evaluated via hemolysis and MCF-7 cell viability testing. The APMA homopolymer demonstrates the greatest antimicrobial activity and lowest toxicity to mammalian cells of the copolymers evaluated, indicating its potential use as a broad spectrum antibiotic. The antimicrobial activity of the APMA homopolymer was least affected by changes in salt concentration or broth type of the polymers tested. The copolymer with 25% GPMA incorporation showed similar, but slightly reduced, antimicrobial activity. Copolymers with higher concentrations of GPMA showed substantially lower antimicrobial effectiveness against all bacteria tested and higher sensitivity to salt concentrations. Mammalian cell death increased with increasing GPMA content. Antimicrobial activity against Gram-negative and Gram-positive bacteria with low levels of mammalian cell toxicity are demonstrated for fully water-soluble, primary-amine containing, methacrylamide polymers, without the need for incorporation of guanidinium adducts, indicating the potential of this synthetic system for broad spectrum antibiotic development. Evaluation of their effectiveness in preventing bacterial biofilm formation is underway.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.5b01162.

Structures of amino acids and mimics and ^{1}H NMR spectra of protected monomers, deprotected monomers, and copolymer (PDF)

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Notes

The authors declare no competing financial interest.

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