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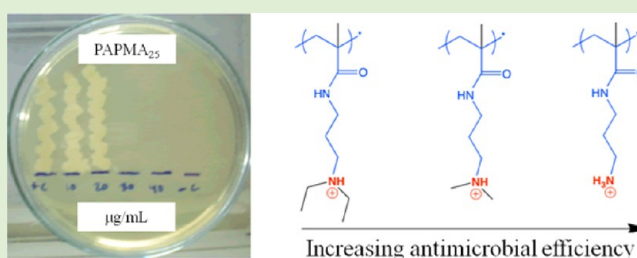
Antimicrobial Poly(methacrylamide) Derivatives Prepared via Aqueous RAFT Polymerization Exhibit Biocidal Efficiency Dependent upon Cation Structure

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ABSTRACT: Antimicrobial peptides (AMPs) show great potential as alternative therapeutic agents to conventional antibiotics as they can selectively bind and eliminate pathogenic bacteria without harming eukaryotic cells. It is of interest to develop synthetic macromolecules that mimic AMPs behavior, but that can be produced more economically at commercial scale. Herein, we describe the use of aqueous reversible addition–fragmentation chain transfer (RAFT) polymerization to prepare primary and tertiary amine-containing polymers with precise molecular weight control and narrow molecular weight distributions. Specifically, *N*-(3-aminopropyl)methacrylamide (APMA) was statistically copolymerized with *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPMA) or *N*-[3-(diethylamino)propyl]methacrylamide (DEAPMA) to afford a range of (co)polymer compositions. Analysis of antimicrobial activity against *E. coli* (Gram-negative) and *B. subtilis* (Gram-positive) as a function of buffer type, salt concentration, pH, and time indicated that polymers containing large fractions of primary amine were most effective against both strains of bacteria. Under physiological pH and salt conditions, the polymer with the highest primary amine content caused complete inhibition of bacterial growth at low concentrations, while negligible hemolysis was observed over the full range of concentrations tested, indicating exceptional selectivity. The cytotoxicity of select polymers was evaluated against MCF-7 cells.



INTRODUCTION

Novel strategies must be employed to combat the ever-growing concern of antibiotic-resistant bacteria. AMPs show great potential as they are small biopolymers (~20–50 amino acids in length) that can selectively bind and eliminate pathogenic bacteria without harming eukaryotic cells within a certain therapeutic range.^{1–3} They are produced naturally by many complex, multicellular organisms and play a role in immunity processes. AMPs have diverse amino acid sequences, however they typically display a net positive charge at physiological pH. This cationic net charge results from an abundance of basic lysine and arginine residues. The AMPs' ability to selectively attack bacterial pathogens rather than their own natural host tissues is governed by fundamental differences in the composition and structure of the phospholipid bilayers found in cell membranes. Bacterial cell membranes typically contain significant concentrations of phosphatidylglycerol, cardiolipin, and phosphatidylserine phospholipids that contribute anionic head groups to the outer membrane surface.² In contrast, eukaryotic cells are commonly rich in zwitterionic phospholipids such as phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin. The net positive charge of AMPs and the

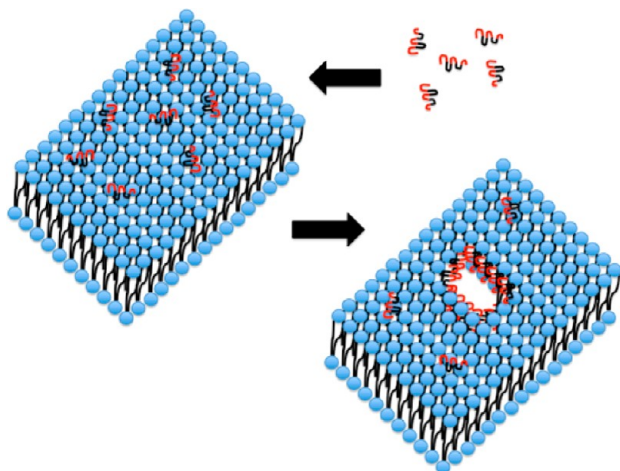
net negative charge of bacterial cell membranes drives the initial attraction of the peptide to the cell surface.^{2,4} Specifically, lysine and arginine residues interact strongly with negatively charged phospholipids, allowing electrostatic interactions to govern initial binding to target cell membranes.^{2,5} After binding to the negatively charged phospholipid, hydrophobic moieties on the peptide interact with the inner hydrophobic core of the bacterial membrane leading to a disruption in integrity and subsequent cell death. One commonly accepted model for the membrane disruption process is depicted in Scheme 1.

AMPs show great promise as therapeutic agents for infections and in general biocidal applications, however they are produced naturally at low levels and synthesizing them in the laboratory is quite expensive. For this reason, there is extensive interest in developing synthetic systems that mimic AMP behavior. Several groups have synthesized low molecular weight oligomers that possess antimicrobial properties,^{6–9} however, this research will focus on polymers which mimic

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Scheme 1. Barrel-Stave Model of AMP Biocidal Mechanism^a

^aModified from Brogden et al.³

the natural size of AMPs. Tew et al.^{10,11} utilized ring-opening metathesis polymerization (ROMP) to prepare polynorbornene derivatives with pendant primary amines resembling the functionality of lysine. To accomplish the polymerization of primary amine-containing monomers, protecting group chemistry was employed. By manipulation of the relative concentration of lipophilic and hydrophilic moieties (amphiphilic balance) via polymer design, hemocompatible polymers that displayed microbial toxicity at low concentration were obtained.

Mowery et al.¹² prepared statistical copolymers of β -lactams to form an amphipathic, antimicrobial polymer. As reported by Tew et al. for polynorbornene derivatives, the authors used reaction design to vary the amphiphilic balance in an attempt to tune the antimicrobial and hemolytic behavior. An interesting conclusion drawn from a later study performed by Epan et al.¹³ was that these β -lactam polymers were able to penetrate through the lipid membrane of vesicles mimicking Gram-positive and Gram-negative bacteria at low concentration; however, when the concentration was raised, the polymers remained bound to the membrane and caused membrane disruption by vesicle aggregation.

In an attempt to further improve the biocompatibility of antimicrobial polymers, Venkataraman et al.¹⁴ used RAFT polymerization to create antimicrobial polyethylene glycol (PEG) functionalized polymers from methacrylate derivatives. The authors statistically copolymerized *N*-[3-(dimethylamino)ethyl]methacrylate (DMAEMA) and oligo(ethylene glycol)-methylether-methacrylate (OEGMA) and then performed post polymerization quaternization of the pendant DMAEMA nitrogen with different halide functional species, testing the relationship of chain length and chemical group to antimicrobial properties. It was concluded that shorter hydrophobic chain lengths (i.e., methyl and ethyl) produced polymers with the best antimicrobial properties.

DeGrado and Kuroda¹⁵ synthesized antimicrobial poly(methacrylate) derivatives via free radical polymerization and obtained a system with microbial toxicity. The effect of pendant amine structure on antimicrobial activity was described for low molecular weight ($DP = 6-10$) random copolymers prepared from similar methacrylate derivatives.¹⁶ Within this system, it was concluded that primary amines helped to maximize antimicrobial properties, while quaternary ammonium-contain-

ing polymers required the addition of excess hydrophobic functionality to realize potent activity. It was reported that, in general, as polymer hydrophobicity increased so too did hemotoxicity. To decrease the overall hydrophobicity, primary amine-containing poly(methacrylamide) derivatives of the above system were prepared to replace acrylate linkages with amide linkages for all side chains.¹⁷ The homopolymer of the primary amine-containing methacrylamide monomer was found to provide significantly higher antimicrobial activity than that of the poly(methacrylate) version, reportedly due to improved hydration of the polymer backbone. Recent work by Kuroda et al. on poly(methacrylate) systems discussed the effect of the spacing of cations from the backbone main chain on antimicrobial properties¹⁸ and the preparation of self-degrading polymers¹⁹ that exploit the inherent properties of acrylate linkages to hydrolyze.

From previous research performed on AMPs and AMP mimics, it is clear that any attempts at creating novel systems must meet a few main requirements.²⁰ The system must contain cationic charges, which facilitate binding to anionic bacterial phospholipids, and its amphiphilic balance must be highly tunable to achieve microbial toxicity while maintaining hemocompatibility. Based on the report of Kuroda et al. showing increased antimicrobial activity for methacrylamide-containing polymers, we were interested in determining the degree to which selective toxicity could be controlled in poly(methacrylamide) copolymers through changes of the cation structure and the placement of hydrophobic moieties. In contrast to the poly(methacrylate) systems explored previously,¹⁶ all of the methacrylamide monomers employed in our study are fully water-soluble, with the direct incorporation of hydrophobic moieties onto the hydrophilic monomer side chain. This methodology eliminates the need to add lipophilic comonomers and allows for controlled polymerization to be carried out in aqueous media. Aqueous RAFT polymerization was employed for production of statistical copolymers of precise molecular weight and narrow molecular weight distribution, mimicking that of natural AMPs. Low molecular weights were targeted for these AMP mimics to eliminate the need for biodegradation of polymers within the body, given that small macromolecules are removed from the body by the renal system.²¹

The goal of the study was to define the roles of cation structure and distribution of hydrophobic moieties of protonated amines in determining the selective toxicity behavior of poly(methacrylamide) derivatives. Herein, we describe the use of aqueous RAFT to copolymerize APMA, which mimics the cationic amino acid lysine, with DMAPMA or DEAPMA, both of which impart hydrophobic character to the amines while maintaining water solubility of each of the individual monomers, in a systematic fashion. The rationale for imparting hydrophobic character while maintaining overall hydrophilicity of each monomer is that we hypothesize that eukaryotic cytotoxicity will be decreased as compared to that exhibited by previously reported AMP mimics. To our knowledge, aqueous RAFT polymerization has not yet been reported for preparation of AMP-mimicking antimicrobial polymers. Aqueous RAFT facilitates the polymerization of primary amines without the need for protecting group chemistry or organic solvents.^{22,23} A series of copolymers was prepared with systematic variation of (1) the ratio of primary to tertiary amine and (2) the concentration and structure of hydrophobic groups (DMAPMA and DEAPMA comonomers)

in order to gain greater understanding of the effects of the individual components on selective toxicity. The controlled polymerization of APMA and DMAPMA has been demonstrated previously by our research team,^{24,25} however, using aqueous RAFT to polymerize DEAPMA has, to our knowledge, not yet been reported. The true novelty of this system lies in its controlled distribution of cationic monomers, which allows for a better definition of the structural characteristics necessary to obtain highly selective antimicrobial agents for a (poly)-methacrylamide based system. The antimicrobial activity of the polymers against *E. coli* (EC; Gram-negative) and *B. subtilis* (BS; Gram-positive) was analyzed as a function of buffer type, salt concentration, pH, and time. Hemolytic behavior and cell viability studies with selected polymers were performed.

MATERIALS AND METHODS

Materials. Triethylamine, 99.5% (Sigma Aldrich), methacryloyl chloride, >97% (Fluka), and *N*-[3-(dimethylamino)propyl]-methacrylamide, 99% (Sigma Aldrich) were purchased and further purified via distillation prior to use. 3-Diethylaminopropyl-amine, 99+ % (Acros Organics), ethyl acetate (Fisher Scientific), 4,4'-azobis(4-cyanovaleric acid) (Sigma Aldrich), methanol, anhydrous, 99.8% (Sigma Aldrich), sodium acetate anhydrous (Fisher Scientific), acetic acid, glacial (Fisher Scientific), deuterium oxide, 99.9 atom % D (Sigma Aldrich), chloroform-*d*, 99.8 atom % D (Cambridge Isotope Laboratories, Inc.), *N*-(3-aminopropyl)methacrylamide (Polysciences, Inc.), sodium sulfate, powder, >99%, ACS reagent, anhydrous (Sigma Aldrich), *Escherichia coli* (α DH5) and *Bacillus subtilis* (obtained from the central microbial culture facility, Department of Biotechnology and Biochemical Engineering, Indian Institute of Technology, Delhi, India), Luria broth (Himedia, India), Luria agar (Himedia, India), tris(hydroxymethyl)aminomethane (Sisco Research Laboratories PVT. Ltd., India), potassium dihydrogen phosphate (Merck, India), disodium hydrogen orthophosphate (Qualigens, India), sodium chloride (Qualigens, India), hydrochloric acid (Merck, India), sterile discs, diameter 6 mm (Sigma Aldrich, India), Triton-X 100 (Merck, India), MCF-7 cells (NCCS, Pune, India), Dulbecco's modified Eagle media (DMEM; Himedia, India), fetal bovine serum (Himedia, India), Trypsin-EDTA solution 1 $\times$ (Himedia, India), antibiotic solution 100 $\times$ liquid (Himedia, India), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, India), and dimethyl sulfoxide (DMSO; Merck, India) were purchased and used as received.

Synthesis of DEAPMA. To an ice cooled solution of triethylamine (90 mL), 3-(diethylamino)propyl-amine (7.50 mL, 47.2 mmol) and ethyl acetate (30 mL) were added. Methacryloyl chloride (9.24 mL, 94.5 mmol) was added dropwise over the course of 10 min. An additional 50 mL of ethyl acetate was added and the reaction progressed for 2 h. After the reaction was completed, the mixture was further diluted with ethyl acetate to a final volume of 1 L. The solution was filtered of insoluble salts and the solvents were removed by rotary evaporation. DEAPMA was purified by distillation (115 °C, 0.38 mmHg) and isolated as a light yellow oil (see Figure 1 for ¹H NMR spectrum). The reaction yield calculated after distillation was approximately 54%, however, it is expected that yield can be significantly increased through optimization of the distillation procedure. ¹H NMR (300 MHz, CDCl₃): δ [ppm] 8.12 (s, 1H), 5.67 (s, 1H), 5.25 (s, 1H), 3.45–3.32 (m, 2H), 2.61–2.43 (m, 6H), 1.91 (s, 3H), 1.74–1.57 (m, 2H), 1.00 (t, 6H).

Polymerization of Homopolymers and Copolymers. By way of 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 approximately 30:1 to yield a degree of polymerization of ~30, which mimics the size of naturally occurring AMPs. 4-Cyano-4-(ethylsulfanylthiocarbonylsulfanyl)pentanoic acid (CEP) was used as the CTA and was synthesized via previously published procedures,²⁶ while 4,4'-azobis(4-cyanopentanoic acid) was used as the initiator. For all polymerizations, $CTA_0/I_0 = 5$. The polymerization took place in

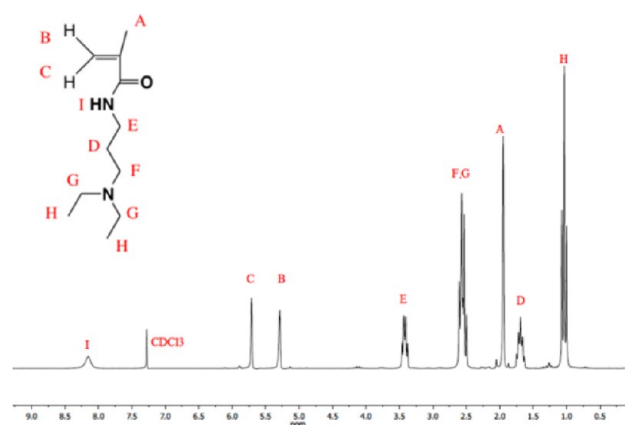


Figure 1. ¹H NMR spectrum of DEAPMA after distillation.

aqueous acetate buffer (pH 5) at 70 °C²⁷ for 6 h (see Scheme 2). 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 followed by lyophilization for 72 h and the samples were then stored in desiccant. A representative yield was calculated to be 75% of the theoretical yield. Some polymer is lost in dialysis due to excessive swelling of the dialysis tubing as a result of internal osmotic pressure. This loss can be minimized by making the water slightly acidic during the dialysis procedure to reduce the extent of swelling.

¹H NMR. ¹H NMR was performed with a Varian Mercury^{PLUS} 300 MHz spectrometer in CDCl₃, utilizing delay times of 5 s to determine monomer purity. A 500 MHz NMR equipped with a standard 5 mm ¹H/¹³C probe and operating at 499.77 MHz (¹H) was used to identify the homopolymer structures of PAPMA, PDMAPMA, and PDEAPMA and the statistical structures of the PAPMA-*stat*-PDMAPMA and the PAPMA-*stat*-PDEAPMA copolymer series in D₂O. A total of 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 (see Figures 2 and 3 for representative spectra).

ASEC-MALLS. The molecular weight and polydispersity index (PDI) 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). A total of 1 wt % acetic acid/0.1 M Na₂SO₄ (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, PDMAPMA and PDEAPMA at 25 °C in the eluent (0.2000 mL/g, 0.2086 mL/g and 0.1930 mL/g, respectively) in order to assign absolute molecular weight values to all polymers. 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 NMR. Wyatt ASTRA SEC/LS software was used for molecular weight and PDI calculations.

Antimicrobial Activity: Broth Microdilution Method. Polymer antimicrobial activity was determined against EC (Gram-negative) and BS (Gram-positive) bacteria by modifications of previously published procedures.²⁸ Bacteria glycerol stocks were inoculated into Luria broth (LB) and allowed to grow overnight at 37 °C under shaking conditions. Polymer stock solutions (4–6 mg/mL) for all polymers were prepared in tris(hydroxymethyl)aminomethane (TRIS; 10 mM), TRIS buffered saline (TBS; 10 mM TRIS, 150 mM NaCl), and phosphate buffered saline (PBS; 12 mM NaH₂PO₄, 1 mM K₂HPO₄, 140 mM NaCl). Each buffer was titrated to a pH of either 6.8 or 7.4 with 6 N HCl and further diluted via serial dilution to afford a range of polymer concentrations. The overnight cultures of bacteria were

Scheme 2. Statistical Polymerization of APMA with DMAPMA or DEAPMA to Vary Amine Structure and Hydrophobic Functional Group Size

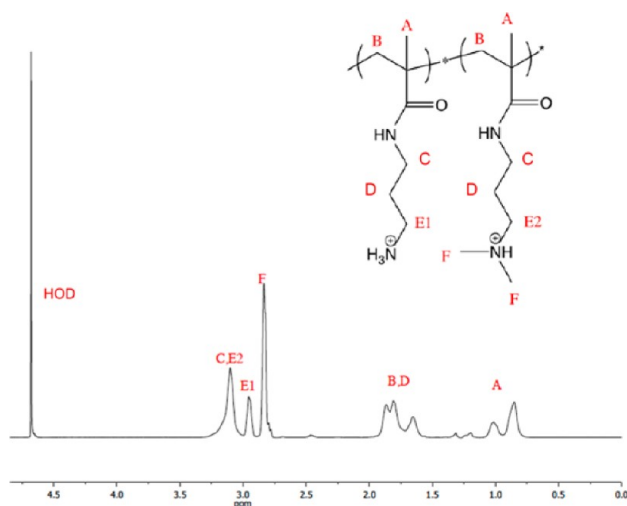
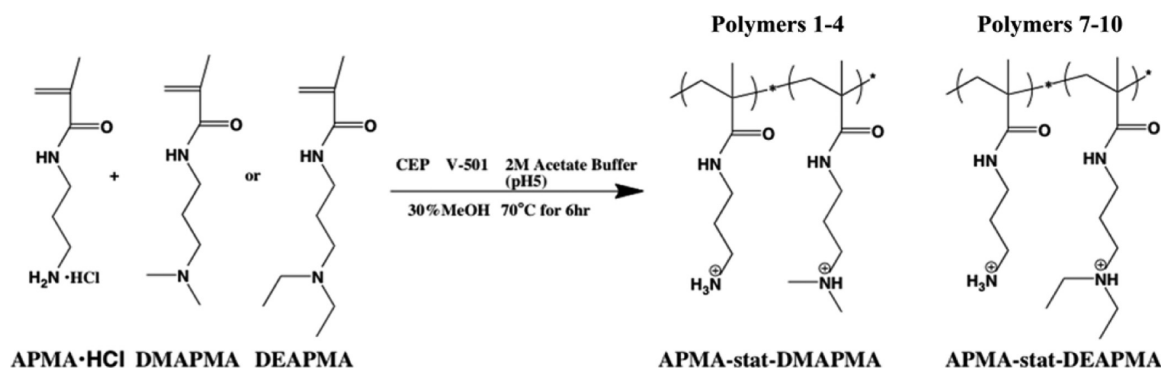


Figure 2. ^1H NMR spectrum of a representative PAPMA-stat-PDMPMA copolymer. Peak integration values indicate 49.7% contribution from APMA (target = 50%).

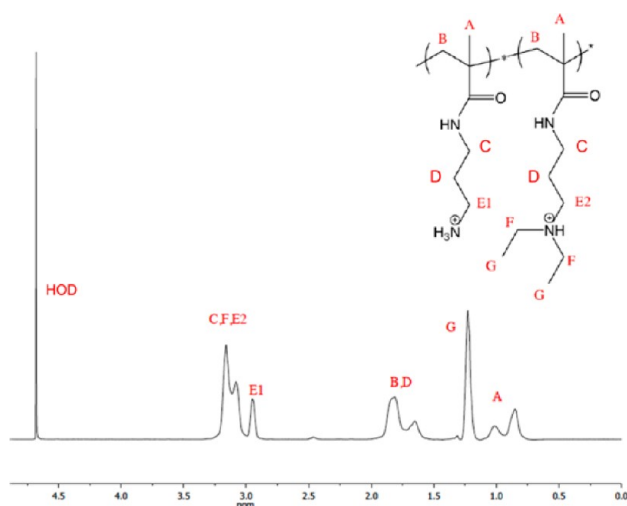


Figure 3. ^1H NMR spectrum of a representative PAPMA-stat-PDEAPMA copolymer. Peak integration values indicate 48.2% contribution from APMA (target = 43%).

diluted with fresh LB to an optical density of 0.001 at 600 nm (OD_{600}) and mixed 1:1 with buffered polymer dilutions in a 96 well plate. These mixtures were then incubated under shaking conditions at 37 °C for 24 h. The OD_{600} was measured using a BioTek PowerWave

XS2 UV–visible plate reader after 6 and 24 h of incubation. Minimum inhibitory concentrations (MIC) are reported for each measurement time as the concentration at which no bacterial growth was observed.

Antimicrobial Assay: Agar Plate Conformation of MIC. Utilizing the broth microdilution method, polymer solutions (TBS, pH 7.4) at concentrations above and below the observed MIC were inoculated with EC and BS for 24 h at 37 °C (shaking). Inoculums from each well were streaked onto sterile LB agar plates and incubated for an additional 18 h at 37 °C. Viable bacteria cells present in the wells are observed as visible bacteria colonies on the agar plates. All experiments were run in duplicate and photographs were taken using a digital camera.

Antimicrobial Assay: Zone of Inhibition Method. Disc susceptibility tests were used to determine antimicrobial activity as a corroborating test to the broth microdilution method.²⁹ Sterile LB agar plates were prepared, and 100 μL of bacteria inoculums (either EC or BS at an OD_{600} of 0.001) were spread evenly on the surface. Samples were allowed to dry for 5 min. Three sterile discs were fixated on the top of the agar and impregnated with either 10 or 20 μL polymer solution from an initial 6 mg/mL stock (TBS, pH 7.4). The plates were allowed to incubate for 18 and 48 h for BS and EC, respectively, at 30 °C. All experiments were run in duplicate and photographs were taken using a digital camera.

Hemolysis. Hemolysis experiments were performed according to previously published procedures¹⁰ with slight modifications. Human blood was obtained from AIIMS hospital in New Delhi, India. Red blood cells (RBCs) were separated from the whole blood via centrifugation at 1500 rpm for 10 min. RBCs (30 μL) were suspended in 10 mL of TBS (pH 7.4), rinsed three times by centrifugation (10 min at 1500 rpm), and then resuspended in 10 mL of TBS. Polymers were dissolved in TBS (pH 7.4) to create 6.0 mg/mL stock solutions. These solutions were further diluted to 0.1, 0.2, 2.0, 4.0, and 6.0 mg/mL. RBC suspensions were incubated 1:1 at 37 °C (light shaking) for 30 min with polymer dilutions in microcentrifuge tubes to afford final polymer concentrations of 0.05, 0.1, 1.0, 2.0, and 3.0 mg/mL within the total volume of solution. After incubation, the tubes were centrifuged at 1500 rpm for 10 min. The supernatant from each tube was transferred to individual wells in a 96-well plate and the absorbance was measured at 540 nm. Positive and negative controls for 100 and 0% hemolysis were obtained by 1% TRITON-X and TBS, respectively.

Cell Viability. An MTT assay was performed on MCF-7 cell lines using standard procedures³⁰ with slight modification. Cells were cultured on tissue culture polystyrene flacon flasks in DMEM media supplemented with 10% fetal calf serum and 1% antibiotic solution (penicillin and streptomycin) at 37 °C in a 5% CO_2 incubator. After obtaining ~80% confluence, the cells were trypsinized and counted on a hemocytometer. The cells in fresh media were seeded into a 96-well plate (8×10^3 cells/well) and incubated for 24 h at 37 °C in a 5% CO_2 incubator. Next, the media was removed from each well and 100 μL of fresh media was added. To select wells (in triplicate) was added 50 μL of polymer solutions at various concentrations (90, 225, 375, and 6000

Table 1. Molecular Weight and Composition Data for Synthesized (Co)polymers

polymer	mol % DMAPMA (theory)	mol % DEAPMA (theory)	mol % APMA (theory)	mol % APMA (exp) ^a	$M_{n,th}$ (g/mol) ^b	$M_{n,exp}$ (g/mol) ^c	PDI ^c	dn/dc ^d
1 (M30)	37		63	70	4905	5343	1.06	0.2026
2 (M41)	56		44	59	5032	5023	1.06	0.2036
3 (M50)	50		50	50	5100	5110	1.04	0.2045
4 (M72)	79		21	28	5187	5601	1.05	0.2062
5 (M100)	100			0	5328	6006	1.06	0.2086
6 (PAPMA)			100	100	4656	5194	1.05	0.2000
7 (E39)		37	63	61	5287	6674	1.08	0.1973
8 (E43)		47	53	57	5177	6292	1.11	0.1970
9 (E52)		57	43	48	5560	6369	1.12	0.1965
10 (E71)		78	22	29	5844	5263	1.09	0.1950
11 (E100)		100		0	6150	7124	1.10	0.1930

^aDetermined by ¹H NMR. ^bBased on 80% conversion of $[M_0]$. ^cDetermined by ASEC-MALLS. ^dDetermined by a Wyatt Optilab DSP interferometric refractometer.

μg/mL). The resulting solutions had final polymer concentrations of 30, 75, 125, and 2000 μg/mL, respectively. For a positive control, 50 μL TBS (pH 7.4) was used, while 50 μL of 3% Triton-X was used as a negative control. Reported data is normalized for viability observed in the positive control (taken as 100% viability). The plate was incubated for 6 or 12 h at 37 °C in a 5% CO₂ incubator. After incubation, the media was removed from each well and 100 μL of fresh media was added. Next, 10 μL of MTT reagent (MTT dissolved in TBS at 5 mg/mL concentration) was added to each well and samples were incubated for an additional 4 h. The media from each well was then removed and replaced with 100 μL of DMSO to solubilize the formed formazan crystals. Absorbance readings at 570 nm were recorded using a BioTek PowerWave XS2 UV–visible plate reader.

RESULTS AND DISCUSSION

Synthesis of Homopolymers and Copolymers. For this work, methacrylamide monomers were chosen due to their hydrolytic stability, structural similarity to amino acids found in naturally occurring AMPs, incorporation of hydrophobic and hydrophilic moieties, and pK_a values. APMA was chosen due to its similarity to lysine. Many research groups have demonstrated that in addition to electrostatic attraction, hydrophobic functionality is required to realize antimicrobial activity. While ionic bonding facilitates initial polymer–cell interactions, it is the hydrophobic substituents that act to disrupt the lipid membrane of bacteria. DMAPMA and DEAPMA were chosen due to their hydrophobic dimethyl and diethyl amino groups, respectively. Previous literature reports suggested that tuning the amphiphilic balance is important in obtaining selective antimicrobial agents,¹⁰ thus, DMAPMA and DEAPMA monomers were selected to allow manipulation of the relative hydrophobicity of the system. Simultaneously, systematic variation of the copolymer composition utilizing these monomers facilitates study of the effect of cation structure on antimicrobial efficiency in (poly)methacrylamide systems. Additionally, all comonomers studied are expected to remain charged at physiological pH. PAPMA was reported to be essentially fully protonated over a pH range of 6–8.¹⁷ PDMAPMA has been shown to have a pK_a of approximately 8.8,³¹ and thus, following the Henderson – Hasselbalch equation, it is expected that >96% of the monomers will be charged at physiological pH. PDEAPMA is expected to follow a similar trend as PDMAPMA with a slightly higher pK_a . In the poly(methacrylate) copolymers studied previously,¹⁶ because of the lower pK_a values in these systems, partial protonation of the amine groups (and thus greater hydrophobicity) occurred

under physiological pH test conditions. As the PDEAPMA and PDMAPMA copolymers in the present study remain essentially fully charged under the test conditions, it is possible to separate the effects of cation structure from those of charge density on antimicrobial efficiency.

A total of 11 polymers were synthesized using identical polymerization conditions with varying monomer type and monomer feed ratio. APMA, DMAPMA, and DEAPMA were each homopolymerized and unique ¹H chemical shifts were assigned. Copolymers were formed by copolymerizing APMA with DMAPMA and APMA with DEAPMA at varying ratios (Scheme 2). For simplicity of nomenclature, each (co)polymer was given a letter based on its composition of tertiary amine. For example, polymer 1, composed of 70% APMA and 30% DMAPMA is named M30 with “M” representing the dimethyl tertiary amine and the “30” representing its mole percent. Similarly, the letter “E” represents the diethyl tertiary amine (DEAPMA) comonomer. Table 1 summarizes polymer molecular weights and monomer compositions.

Antimicrobial Activity. The biocidal nature of the polymers was probed first by the broth microdilution method. All polymers were tested against EC and BS, and MIC values were reported as a function of saline concentration, incubation time (6 and 24 h), polymer concentration (0–2 mg/mL), polymer composition (effect of hydrophobic functionality and cation architecture), and bacterial cell line. The presence of salt is known to induce the antipolyelectrolyte effect,³² thus, it was necessary to determine if physiological salt conditions would influence polymer–cell electrostatic interaction. Table 2 provides a summary of MIC data after 24 h of incubation. The data show that activity depends on the bacterium studied, the buffer solution in which the test was performed, and, for the tests using BS, the copolymer composition.

In general, high antimicrobial activity (defined as an MIC value of 100 μg/mL or lower) was exhibited by all of the polymers against EC. Exceptionally high activity was observed in TRIS buffer, which does not contain additional NaCl. In the TBS buffer system (TRIS plus 150 mM NaCl), the MIC values increased slightly, however all polymers demonstrate very high activity against EC. Similar trends were observed when the polymers were dissolved in PBS buffer. Against the Gram-positive bacteria, BS, on the other hand, generally lower antimicrobial efficiency is observed. Other than the obvious difference in outer cell structure (Gram-positive vs Gram-negative), the most observable difference between EC and BS

Table 2. Minimum Inhibitory Concentration, 24 h, Values as Determined by Microdilution Assays^a

polymer	MIC $\mu\text{g/mL}$					
	<i>E. coli</i> (Tris)	<i>E. coli</i> (TBS)	<i>E. coli</i> (PBS)	<i>B. subtilis</i> (Tris)	<i>B. subtilis</i> (TBS)	<i>B. subtilis</i> (PBS)
1 (M30)	10	10	25	50	100	100
2 (M41)	10	25	25	75	125	125
3 (M50)	10	25	25	75	250	125
4 (M72)	10	25	25	100	500	250
5 (M100)	10	25	25	250	500	500
6 (PAPMA)	5	25	25	50	50	100
7 (E39)	10	25	25	75	250	500
8 (E43)	10	25	25	75	250	250
9 (E52)	10	25	25	100	250	250
10 (E71)	10	50	125	250	2000	2000
11 (E100)	5	25	50	500	>2000	>2000

^aMIC = concentration of polymer required to completely inhibit bacterial growth. The addition of salt to the solution media weakens polymer/cell interactions. Against BS, as the amount of tertiary amine increases within each copolymer series the amount of polymer needed to achieve complete inhibition also increases. (pH = 6.8).

was their speed of growth in micodilution assays. BS control samples consistently displayed higher OD₆₀₀ values after 6 h

incubations, indicating a faster growth rate. This will be discussed further in later sections. Biocidal activity against BS was influenced to a large extent by polymer composition and buffer type. Polymers M30 and PAPMA (PAPMA₂₀-stat-PDMPMA₉ and the PAPMA₂₅ homopolymer, respectively) showed the lowest MIC values against BS in each of the three buffers. As the concentration of tertiary amine-containing monomer (DMPMA or DEAPMA) increased within each copolymer series the effectiveness against BS decreased. Figure 4 provides a more detailed analysis of the relationship of the activity against BS.

Both series show a decrease in MIC with an increase in primary amine content (Figure 4A). Series E (PAPMA-*stat*-PDEAPMA copolymers) shows a sharp increase in MIC below 48% (by number) APMA, whereas series M (PAPMA-*stat*-PDMPMA copolymers) shows a less severe increase below 28% APMA. We attribute the differences in MIC behavior to the weaker association of the diethyl-substituted tertiary amine of the DEAPMA copolymers with the bacterial membrane than the association of the dimethyl-substituted tertiary amine of the DMPMA copolymers, primarily due to greater steric hindrance. This leads to a requirement for higher primary amine ratios in the DEAPMA polymers to achieve comparable MIC values. Figure 4B shows MIC as a function of the weight fraction of hydrophobic groups, calculated as the total

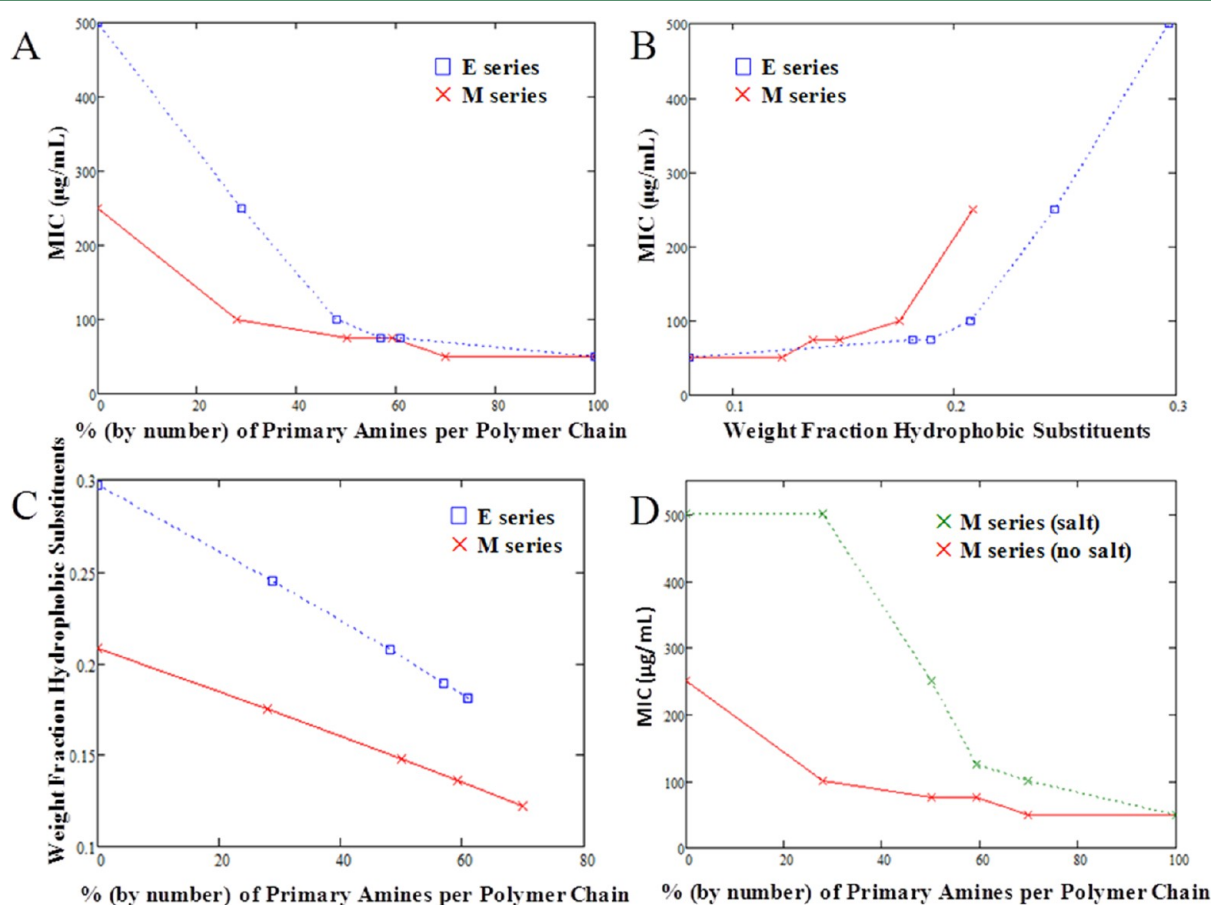


Figure 4. Analysis of activity against BS as a function of copolymer composition; M series is PAPMA-*stat*-PDMPMA copolymers, while E series is PAPMA-*stat*-PDEAPMA copolymers. The solvent for (A), (B), and (C) is Tris buffer, while Tris buffer and Tris buffered saline were used for (D). (A) Relationship between MIC and primary amine content. (B) Relationship between the weight fraction of hydrophobic groups of each polymer and the observed MIC. (C) Depicts the linear relationship between % primary amine and the weight fraction of hydrophobic groups for each polymer system. (D) Isolates the role of amine structure on MIC by the introduction of salt into the solution media.

molecular weight of alkyl chains on the polymer available to interact with the bacterial membrane (defined as the methyl groups pendant to the polymer backbone and the methyl and ethyl substituents of the tertiary amines) divided by the total molecular weight of the polymer (determined by ASEC-MALLS). This parameter provides a relative value for the degree of hydrophobic modification (by weight) in our methacrylate copolymers as the copolymer composition shifts. The observation made clear in Figure 4B is that as the weight fraction of hydrophobic substituents is increased within each series, MIC increases. However, there is no direct correlation between hydrophobic substituent weight fraction and MIC when comparing M and E series. At equivalent weight fractions of hydrophobic substituents, E series has a lower fraction of tertiary amine, as its substituents are of a larger mass. Therefore, because a lower MIC is observed for E series than M series at equivalent hydrophobic substituent fractions, the evidence suggests that amine type is the primary factor influencing MIC. However, it is difficult to completely separate the contributions of hydrophobicity and cation structure on antimicrobial activity, as they are linearly correlated in our copolymer systems, as shown in Figure 4C. To further define the effects of cation structure on antimicrobial activity, MIC was evaluated in the presence of added salt. The addition of salt is known to weaken or shield electrostatic interaction. Figure 4D shows that the introduction of NaCl causes an increase in MIC for M series polymers. This shift is greatest when the primary amine fraction (by number) drops below 60%, indicating that the tertiary amine moieties are more susceptible to electrostatic shielding. PAPMA (100% primary amine) is unaffected by added NaCl, indicating a strong attraction to BS. A similar trend was observed for E series polymers (graph not shown).

Steric hindrance, due to the bulky methyl and ethyl substituents on the tertiary amines, limits interactions between the polymers and the bacteria. It is likely that the primary amine of the APMA monomer is more effective at binding (and staying bound) to negatively charged phospholipids. Palermo et al.³³ reported a similar finding, in which they concluded that ammonium containing poly(methacrylate) derivatives interacted more strongly with lipid membranes than did tertiary or quaternary amines. Because the PAPMA homopolymer shows the highest level of activity, it appears that the methyl group on the PAPMA backbone adds sufficient lipophilic character to cause membrane disruption and the additional hydrophobic groups of the PDMAPMA and PDEAPMA pendant amines are not necessary. Conversely, if the biocidal activity for this system is realized through a cellular aggregation mechanism,¹³ then the additional hydrophobic groups may not influence antimicrobial activity. In this case, the strong binding affinity between negatively charged phospholipids and protonated primary amines,⁵ serving to agglomerate cells, is the main driving force.

Against BS, under all buffering conditions, DEAPMA-containing (co)polymers show reduced biocidal activity compared to DMAPMA-containing polymers. Previous systems reported in the literature rarely show a decrease in activity with increasing hydrophobicity as long as the system maintains solubility, therefore, we attribute the observed decrease in activity toward BS to the tertiary amine's weakened ability to interact with the bacterial membrane. All polymers are effective against the relatively slower growing EC, yet when the rate of growth is increased, the polymers with the highest content of primary amine show higher performance. It appears that the

rapidity and strength of the initial polymer ionic attachment to the bacteria controls the biocidal effectiveness against BS.

MIC levels were evaluated for each polymer against BS after 6 and 24 h incubation (TBS pH 6.8) to gain a better understanding of activity as a function of time (Table 3). In all

Table 3. MIC Values Measured after 6 and 24 h^a

polymer	MIC ($\mu\text{g/mL}$)	
	<i>B. subtilis</i> (6 h)	<i>B. subtilis</i> (24 h)
1 (M30)	25	100
2 (M41)	25	125
3 (M50)	25	250
4 (M72)	75	500
5 (M100)	50	500
6 (PAPMA)	25	50
7 (E39)	50	250
8 (E43)	25	250
9 (E52)	25	250
10 (E71)	250	2000
11 (E100)	100	>2000

^aBuffer = TBS, pH 6.8.

cases, the concentration required to inhibit growth for only 6 h was lower. This indicates that inhibiting growth or at least keeping growth below an observable level for a given period of time does not necessarily mean that complete bacterial death is obtained.

While it is difficult to compare reported antimicrobial activity of copolymers analyzed in separate studies because of differences in testing protocols (i.e., buffer conditions, bacterial strain and bacterial concentration), it is clear that the most potent (co)polymers in our study yield MIC values at least as effective as those of other successful systems presented in the literature. Polymers M30, PAPMA, and E39 were chosen for further characterization because they demonstrated the greatest antimicrobial activity in the above studies. When the microdilution method was used, antimicrobial activity was determined for the polymers after they had been dissolved in TBS at a more physiologically relevant pH of 7.4 (the pH of blood). Table 4 summarizes the MIC values determined for EC

Table 4. MIC Values after 6 and 24 h for a Physiologically Relevant pH^a

polymer	MIC ($\mu\text{g/mL}$)			
	<i>E. coli</i> (6 h)	<i>E. coli</i> (24 h)	<i>B. subtilis</i> (6 h)	<i>B. subtilis</i> (24 h)
1 (M30)	30	30	20	70
6 (PAPMA)	30	30	20	70
7 (E39)	40	50	30	170

^aBuffer = TBS, pH 7.4.

and BS at 6 and 24 h time intervals. Against EC, the MIC for polymers M30 and PAPMA was 30 $\mu\text{g/mL}$, while polymer E39 showed a MIC of 40 $\mu\text{g/mL}$ after 24 h. For the same time of incubation, polymers M30 and PAPMA exhibited a MIC of 70 $\mu\text{g/mL}$, while polymer E39 showed a MIC of 170 $\mu\text{g/mL}$ against BS. MIC values obtained at pH 6.8 and pH 7.4 were similar, indicating that, within the chosen pH range, the polymers maintain activity.

To illustrate the biocidal activity of polymers M30, PAPMA, and E39, inoculums from 24 h incubations with polymer

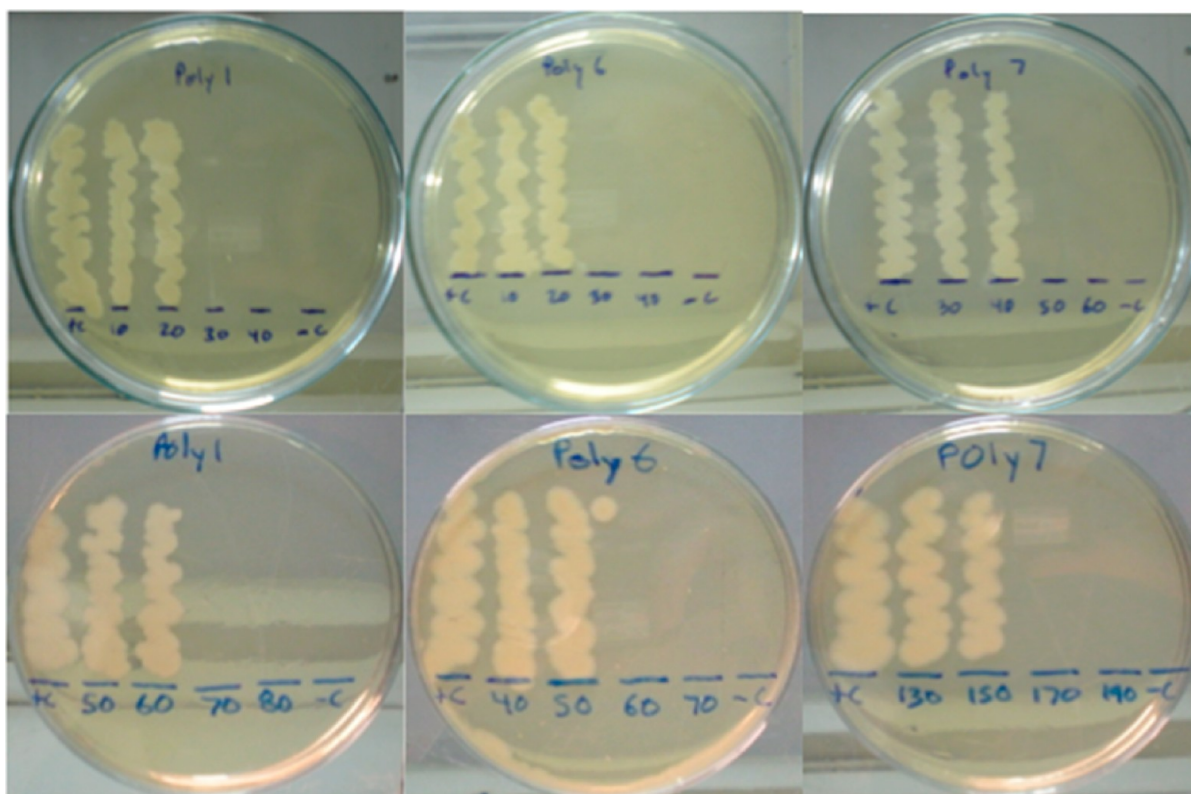


Figure 5. MIC confirmation via agar plate visualization (polymer solvent = TBS pH 7.4). +C is positive control (bacteria in growth media plus TBS without polymer) and –C is negative control (polymer in TBS and bacterial growth media without the presence of bacteria). Numbers refer to final polymer concentration in $\mu\text{g/mL}$. EC (top) and BS (bottom) growth is observed below MIC, however, complete cell death is obtained above MIC. As observed in microdilution assays, polymers 1 (M30) and 6 (PAPMA) show similar MIC levels, where polymer 7 (E39) requires a slightly higher concentration to cause complete inhibition. Each experiment was run in duplicate.

concentrations ranging from below to above their respective MICs were streaked onto freshly prepared agar plates and incubated for 24 h at 37 °C. Figure 5 gives pictorial evidence that live colony forming units are not present in inoculums derived from broth microdilutions above the determined MIC values. Thus, it can be concluded that the polymers induce cell death rather than solely inhibit cell growth. In the case of BS, polymer PAPMA showed slight growth at 60 $\mu\text{g/mL}$, thus, the MIC is reported as 70 $\mu\text{g/mL}$ to be conservative.

To confirm antimicrobial activity by a secondary technique, zone of inhibition measurements were performed. Sterile agar plates were prepared and inoculated with either EC or BS and sterile disks loaded with varying amounts of polymer were fixated onto the top of the agar and incubated at 30 °C. Figure 6 shows the results of disk susceptibility tests.

Against EC (top images), obvious inhibition zones were observed for all polymers when 120 μg of material were deposited. Against BS (bottom images), smaller zones were observed at equivalent concentrations. The amount of growth observed for EC (top images) was reached after 40 h of incubation where BS shows overgrowth in the plates after only 18 h under the same incubation condition and starting OD_{600} . Thus, not only did BS show faster growth rates in the microdilution assays, but also in zone of inhibition studies. The immobilization of the polymers onto sterile discs demonstrate that they need not be free-floating in solution to realize antimicrobial activity, indicating potential application in surface modification and wound dressings where immobilization is required.

In summary, polymers M30, PAPMA, and E39 demonstrate antimicrobial activity against EC and BS over a broad range of conditions. In microdilution assays, polymers M30 and PAPMA (polymers with the highest content of primary amine) display the highest potency against BS. Against the faster growing BS, cation structure is important to the resulting activity in solution assays. Increasing the amount of tertiary amine or the size of hydrophobic functional groups surrounding the amine leads to weaker interaction between the polymers and microorganisms. Weakened interactions, caused by either the cation structure or the addition of saline, give bacteria with high turnover rates an edge in overwhelming the antimicrobial agent.

Hemolysis. An effective way to test eukaryotic cell toxicity is to study the hemolytic behavior of a system. While not conclusive, hemolysis experiments are used to indicate the possibility of eukaryotic cell toxicity.³⁴ Polymers M30, PAPMA, E39, and E100 were dissolved in TBS at pH 7.4 and incubated with isolated red blood cells for 30 min at 37 °C. It was found that, at the highest concentration tested (3 mg/mL), minimal hemolysis was observed (Chart 1). E100 has the highest weight fraction of hydrophobic groups (Figure 4B) of the polymers synthesized, however, hemolysis was avoided. The high selectivity of these polymers is attributed to the use of fully water-soluble monomers, which allow incorporation of hydrophobic moieties while maintaining water solubility of the polymer.

A common practice within the antimicrobial polymer community is to report polymer selectivity toward bacterial cells over eukaryotic cells as a ratio of $\text{HC}_{50}/\text{MIC}$, where HC_{50}

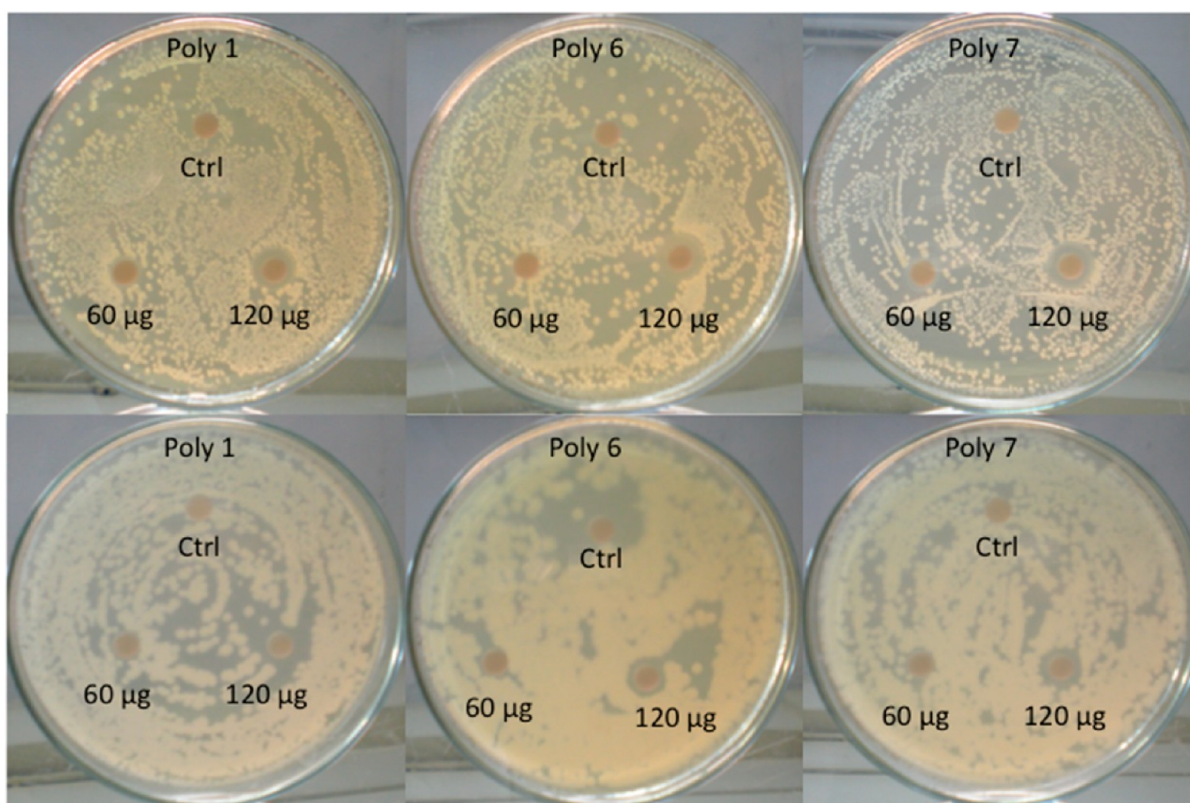
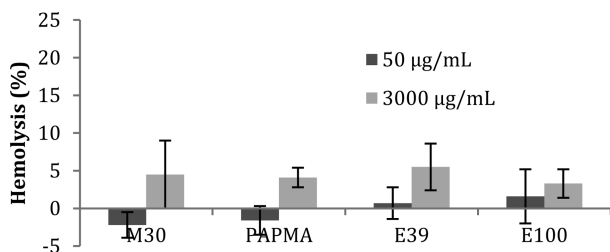


Figure 6. Zone of inhibition. Against EC (top) and BS (bottom), polymers 1 (M30), 6 (PAPMA), and 7 (E39) show similar zone of inhibition patterns. The control discs, where no polymer was present, come into contact with bacteria. When the disk was loaded with 60 μg polymer, little inhibition zone was evident, however, when 120 μg polymer was used, an additional zone of inhibition was observed. Each experiment was run in duplicate.

Chart 1. Polymers Cause Negligible Hemolysis up to 3000 $\mu\text{g/mL}$ ^a



^aHC₅₀ is reported as >3000 $\mu\text{g/mL}$ to estimate polymer selectivity. Negative values are interpreted as zero hemolysis and are considered to be within the error of the experiment.

is the polymer concentration required to lyse 50% of red blood cells. At 3 mg/mL, percent hemolysis was near 5% for each polymer, which is well below 50%. Table 6 provides a summary of polymer selectivity toward EC and BS.

Cell Viability. MTT assays were performed to further investigate the eukaryotic cytotoxicity of polymers M30 and E39. Chart 2 shows cell viability as a function of polymer concentration after 6 and 12 h for polymers M30 and E39. Although these polymers caused little hemolysis, a reduction in MCF-7 cell viability was observed.

At 30 $\mu\text{g/mL}$ the reduction is minimal at each incubation time, however viability decreases with increasing concentration. In the lower concentration range, polymer E39 appears to be less toxic than polymer M30. It is difficult to compare MIC data for bacterial toxicity with eukaryotic cell viability results because

Table 6. Hemolysis and MIC Data Suggest that Polymers M30, PAPMA, and E39 Display Selective Toxicity^a

polymer	MIC ($\mu\text{g/mL}$)		HC ₅₀ ($\mu\text{g/mL}$)	selectivity (HC ₅₀ /MIC)	
	<i>E. coli</i>	<i>B. subtilis</i>		<i>E. coli</i>	<i>B. subtilis</i>
1 (M30)	30	70	>3000	>100	>42
6 (PAPMA)	30	70	>3000	>100	>42
7 (E39)	50	170	>3000	>60	>17

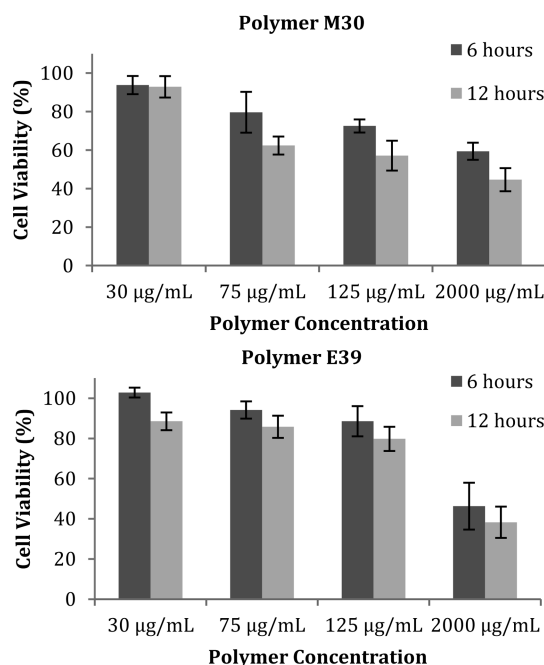
^aTBS buffer, pH 7.4.

each testing procedure utilizes different techniques to compare the number of living cells. For antimicrobial assays, OD₆₀₀ is monitored to visualize the presence of bacterial growth directly, whereas in a MTT assay, the conversion of MTT to purple formazan by mitochondrial reductase is utilized to quantify the mitochondrial activity of the cells rather than directly visualizing the number of living cells. Also, bacterial cells have a much faster growth rate than MCF-7 cells in vitro. Under optimal growth conditions the number of EC cells doubles approximately every 40 min,³⁵ whereas MCF-7 cells (29 h doubling time)³⁶ do not have an opportunity to multiply within the given time frame of polymer incubations. This fact puts MCF-7 cells at a disadvantage in a direct comparison. The goal of future work should be to outline a therapeutic concentration range in which these polymers are considered safe.

CONCLUSION

A series of amine-functionalized methacrylamide (co)polymers with systematic variation of the ratio of primary/tertiary amines

Chart 2. Cell Viability of MCF-7 Cells after Incubation with Polymers M30 (Top) and E39 (Bottom) for 6 and 12 h^a



^aData were normalized based on (+) and (−) controls. For 12 h samples, (+) control = 100 ± 5% and (−) control = 0 ± 0%.

and hydrophobic content was synthesized via aqueous RAFT polymerization. As the PDEAPMA and PDMAPMA copolymers produced remain positively charged and fully water-soluble at physiological pH, it was possible to study the effects of cation structure on antimicrobial efficiency independently from those of charge density. Very low MIC values (≤ 100 µg/mL) were observed against both Gram-negative EC and Gram-positive BS in a range of buffers for those polymers having the highest primary amine content and the lowest concentration of hydrophobic groups. Approximately 50% primary amine content was required when evaluated in TRIS buffer and approximately 60% when evaluated in TRIS with 150 mM NaCl to achieve the highest levels of antibacterial activity. Bacterial toxicity was observed both when polymers were allowed to float freely in solution and when they were immobilized on surfaces. The excellent biocidal performance of these AMP mimics is attributed to high binding efficiency of the primary amines with bacterial phospholipids and a resultant high rate of bacterial cell destruction. The reduced performance of the copolymers with high tertiary amine content is attributed to the bulky methyl and ethyl amine substituents, which limit interactions between the cations and the bacteria. Greater reductions in antimicrobial activity were observed for the bulkier DEAPMA copolymers. The degree of hydrophobicity of the copolymers appeared to be of lower importance than the concentration of primary amine groups in determining antibacterial effectiveness. Selective toxicity performance was also observed. Polymers M30 and PAPMA (PAPMA₂₀-stat-PDMAPMA₉ and PAPMA₂₅, respectively) displayed excellent toxicity toward EC and BS while showing little disruption in membrane integrity of red blood cells. In cell assays, polymer M30 killed 100% of EC and BS cells at 30 and 70 µg/mL, respectively, while approximately 50% of MCF-7 cells remained viable at 2000 µg/mL. To authenticate the results from

eukaryotic cell viability studies, MTT assays using additional cell lines should be performed. These studies demonstrate the importance of cation structure and solution environment in the design and performance of polymeric biocidal agents.

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Notes

The authors declare no competing financial interest.

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