Activity and Mode of Action for Methacrylate Polymers as Antimicrobial Agents against *Staphylococcus aureus*

by

Laura Marjorie Thoma

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Doctoral Committee:

Assistant Professor Kenichi Kuroda, Chair
Associate Professor Anne J. McNeil
Professor Ayyalusamy Ramamoorthy
Associate Professor Chuanwu Xi

DEDICATION

To family and friends for their love, encouragement, and support. Always.

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AEMA	aminoethyl methacrylate
AIBN	azo-bis-isobutyronitrile
CPETC	2-cyanoprop-s-yl ethyl trithiocarbonate
CTAB	cetyltrimethylammonium bromide
Đ	polydispersity
EMA	ethyl methacrylate
FITC	fluorescein isothiocyanate
GPC	gel permeation chromatography
HC_{50}	hemolytic concentration
HDP	host defense peptide
IC_{50}	cytotoxic concentration
MBC	minimum bactericidal concentration
MHB	Mueller Hinton broth
MIC	minimum inhibitory concentration
MMA	methyl methacrylate
MMP	methyl mercaptopropionate
MW	molecular weight
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PEG	polyethylene glycol
PEI	polyethyleneimine
RAFT	reversible addition-fragmentation chain-transfer polymerization
RBC	red blood cell
TFA	trifluoroacetic acid
TSB	tryptone soya broth

Chapter 1 : Introduction

Antibiotic Resistance

There is an urgent need for new antimicrobials due to the increasing number of drug-resistant bacterial infections worldwide.[1, 2, 3, 4] The report from US Centers for Disease Control and Prevention (CDC) in 2013 indicates that in the US each year, at least 2 million people become infected with bacteria that are resistant to antibiotics, resulting in no less than 23,000 deaths and an estimated $20 billion in healthcare costs.[5] With such a tremendous impact on human health and well being, there is a compelling need for antimicrobials to combat antibiotic resistant bacteria due to the lack of new antibiotics in the development pipelines.

Modern antibiotics either act on processes that are unique to bacteria, such as the synthesis of cell walls, or act on bacterium-specific targets.[6] Most current bactericidal antimicrobials inhibit DNA synthesis[7], RNA synthesis[8], cell wall synthesis[9], or protein synthesis[10]. Examples of several antibiotics include Penicillin, Norfloxacin, and Vancomycin (Figure 1-1). While there are a large number of antibiotics available on the market today, many bacteria have already developed antibiotic resistance to these drugs. Drug resistance typically arises through mechanisms that block the interaction of a drug and its target. Bacteria achieve drug resistance through genetic mutation of the antimicrobial target, enzymatic deactivation of the drug, or loss of an enzyme required to activate a prodrug. Bacteria also work to prevent the drug from reaching its target by exporting the drug using efflux pumps or making small structural changes that reduce the bacterial cell's permeability of the drug.

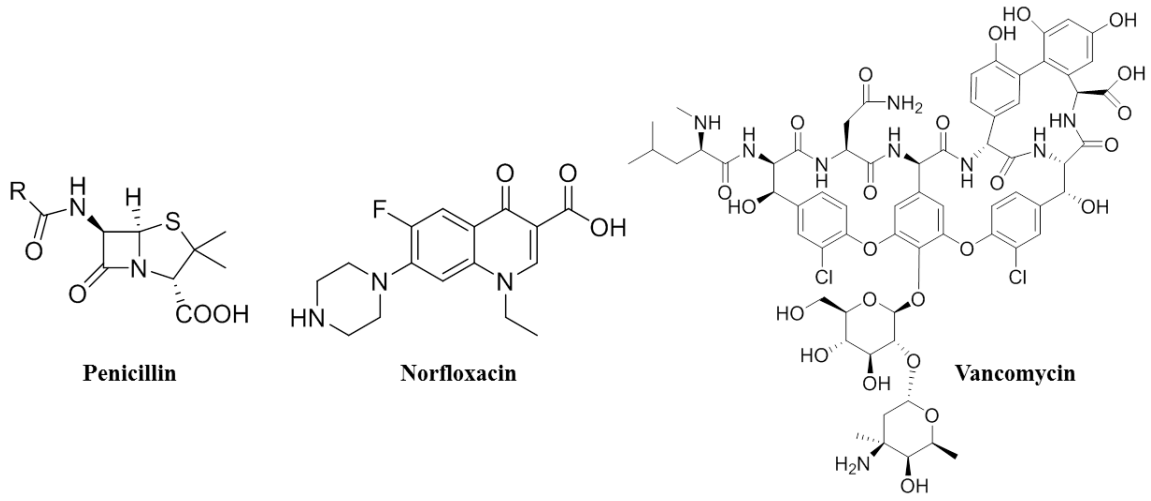


Figure 1-1. Representative antibiotics: penicillin, norfloxacin, and vancomycin.

New Antibiotic Design: Host-Defense Antimicrobial Peptides.

To find inspiration for antimicrobials that are less likely to cause resistance development, scientists looked to nature and found a solution in host-defense peptides.[11] Host-defense peptides are small molecules that are a component of the innate immune system. These peptides present antibacterial, antifungal, antiparasitic, or antiviral activity.[12, 13] Host-defense peptides are attractive as new antimicrobials due to their broad spectrum of activity, rapid bactericidal action, and low propensity for resistance development in bacteria. The host-defense peptides have also been recognized as "antimicrobial peptides" due to their antimicrobial functions. In the field, the peptides are referred to as "host-defense peptides" when the focus is on the function of the peptides in the innate immune system. When the antimicrobial activity of peptides is the interest of research the term "antimicrobial peptides" is used. As our research focuses on the antimicrobial activity of peptides and polymers, the term antimicrobial peptides (AMPs) will be used to refer to the peptides. One AMP is Magainin-2, an antimicrobial peptide found in the African clawed frog Xenopus laevis (Figure 1-2).

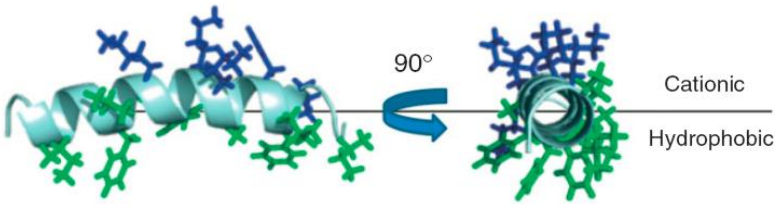


Figure 1-2. Structure of magainin-2, an α-helical cationic antimicrobial peptide. Notice the segregation of cationic and hydrophobic residues along the peptide. Adapted from Zasloff, M. *Proc. Natl. Acad. Sci. U.S.A.*, **1987**, *84*, 5449-5453.[14]

Unlike conventional antibiotics, AMPs are considered to exert their bactericidal effect by causing cell membrane damage and leakage of cellular components, ultimately triggering bacterial cell death. Further contrasting with conventional antibiotics, the antimicrobial mechanisms of AMPs do not involve a specific enzyme or binding site. Several models for the membrane disruption mechanism of AMPs have been proposed in the field; membrane defects induced by peptides include the formation of pores, phase separation, and promotion of non-lamellar lipid structure or disruption of the membrane bilayer (Figure 1-3).[15]

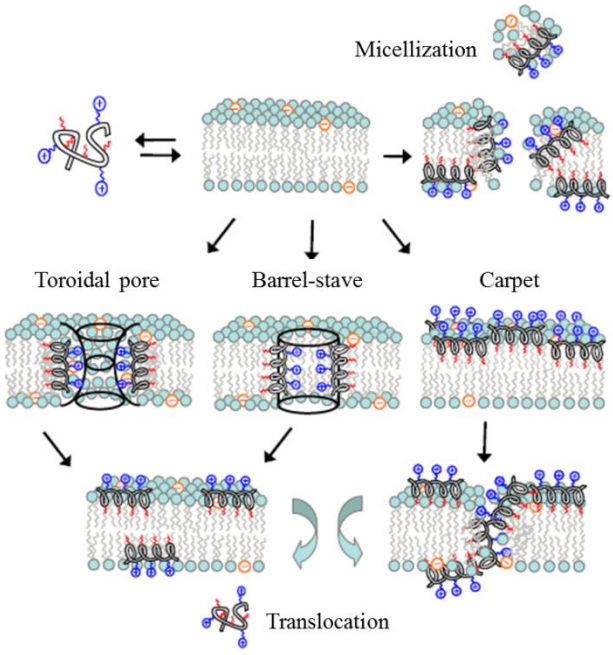


Figure 1-3. Proposed mechanisms of membrane disruption exerted by antimicrobial peptides. The proposed mechanisms include solubilization of membranes due micellization of lipids with peptides, the formation of pores lined with peptides and lipids (toroidal model) or pores lined

with just peptides (barrel-stave model), and the accumulation of peptides followed by non-specific membrane disruption (carpet model). The peptides may ultimately cause translocation of the AMPs through the membrane into the bacterial cell (cytoplasm) and target intracellular components.

AMPs generally consist of 10-50 amino acid residues, and AMPs are classified in different groups depending on amino acid composition, size, and conformation.[16,17] In particular, one of the AMP classes extensively investigated in the field is α-helical peptides. α-helical AMPs are random coils in solution, but form α-helices upon binding to the bacterial membrane. This is followed by membrane disruption and bacterial cell death. The cationic groups on AMPs facilitate the preferential binding of AMPs to bacterial cell surfaces which have higher net negative charges than human cells. This leads to the selective activity of AMPs to bacteria over human cells, resulting in their low toxicity to human cells. However, AMPs and synthetic derivatives are not effective in physiological conditions due to enzymatic degradation as well as the interference of serum salts and proteins. In addition, the high manufacturing cost prevents the translation of AMPs to therapeutic agents. These limitations have led many laboratories to develop synthetic mimics of antimicrobial peptides.[18, 19, 20, 21]

Antimicrobial Polymers

Peptide-mimetics such as β-amino acids were made to emulate the helical conformation of natural AMPs. These peptide backbones are stable in physiological conditions and exert potent antimicrobial activity. These peptidomimetics have defined structures and sequences, which are advantageous for studies clarifying the relationship between structure and antimicrobial activity as well as elucidating their antimicrobial mechanisms. However, these compounds also suffer from labor-intensive preparation and difficulties in large scale production.

The emerging idea is to design random copolymers with cationic and hydrophobic groups which have no defined sequence and molecular size. Based on this concept, polymethacrylates,[22] nylon-3 derived copolymers,[23 24] and poly(norbornene)s [25 26] have been synthesized encompassing the key characteristics of AMPs (Figure 1-4). Our laboratory has previously demonstrated the antimicrobial activity of cationic amphiphilic methacrylate random copolymers which act by disrupting bacterial cell membranes, mimicking the mode of action of AMPs.[27] These polymers have shown potent activity against a broad spectrum of bacteria and a low propensity for resistance development,[28] which are the hallmarks of AMPs. Our laboratory demonstrated that the polymers form a global amphiphilic conformation in bacterial membranes in which the cationic and hydrophobic side chains were segregated onto different sides of the polymer backbone (Figure 1-5). The results suggest that the random copolymers adopt segregated amphiphilic conformations despite their lack of defined intrinsic secondary structures. With the conclusion that random copolymers can exert antimicrobial effects, the design of antimicrobial polymers has been extended to a diverse group of polymers and macromolecular structures.[29]

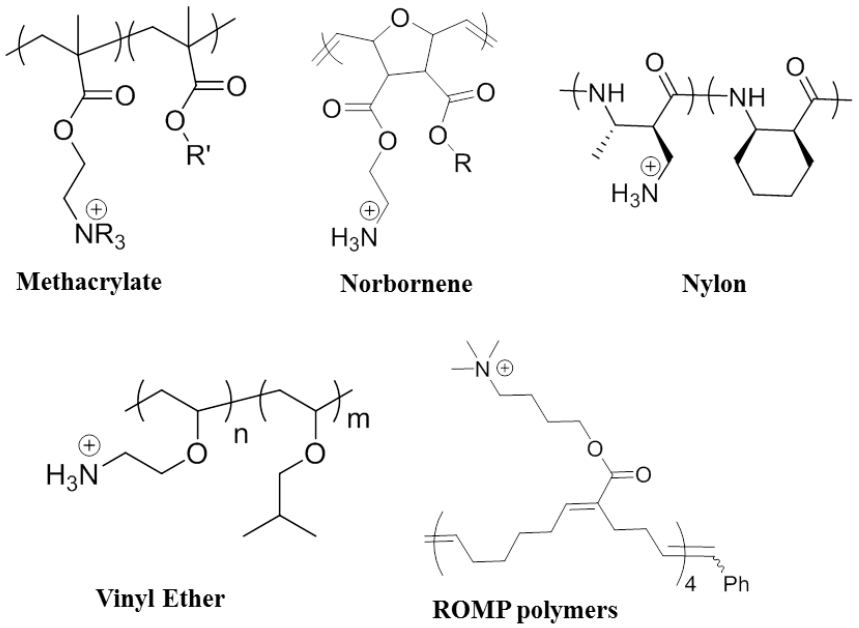


Figure 1-4. Representative antimicrobial Polymers.

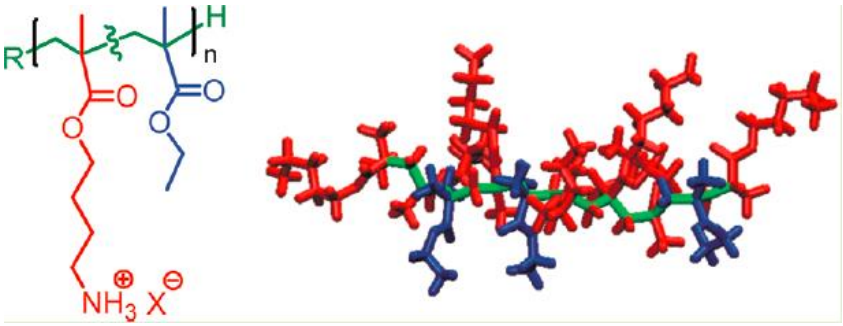
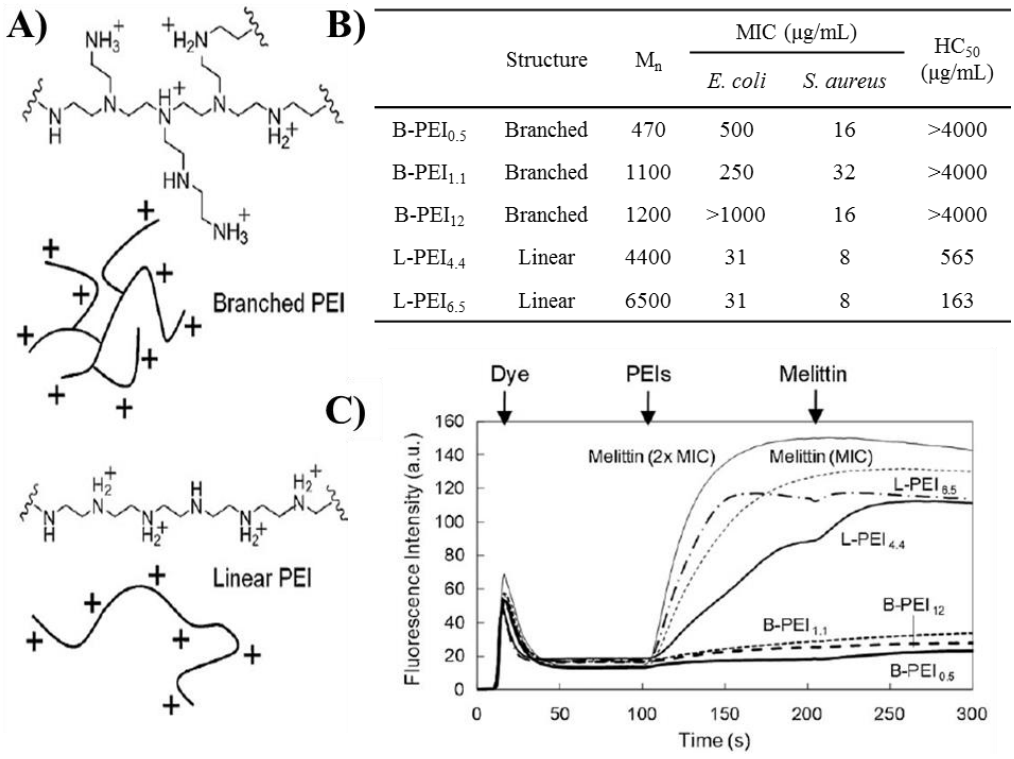


Figure 1-5. Segregated amphiphilic conformation of amphiphilic polymers in bacterial membranes. The effect of the spacer arms on the conformation of polymer chain (left) upon binding to a bacterial membrane. In molecular dynamics simulations, the polymer backbone adopted an extended chain conformation, parallel to the membrane surface (right). A facially amphiphilic conformation at the membrane surface was observed, with the primary ammonium groups (red) localized at the lipid phosphate region and the nonpolar side chains of EMA comonomers (blue) buried in the hydrophobic membrane environment. Adapted from Palermo, E.F. *et al*. *Biomacromolecules*, **2012**, *13*, 1632 – 1641.[27]

Challenge: Antimicrobial Polymers with Cell-specificity

Previously developed antimicrobial copolymers exhibit a broad spectrum of activity, which would be useful to eliminate infectious pathogens. However, this non-selective activity may negatively impact commensal bacterial flora, especially when used for long-term treatment. It would be ideal to develop antimicrobial polymers with cell-selectivity or specificity to bacterial strains which can avoid side effects to commensal flora. Antimicrobial polymers with activity to specific bacteria will complement the treatment of infections by already developed broad-spectrum antimicrobial polymers. However, it has been difficult to develop cell-selective antimicrobial polymers as new modes of action, which is likely to require targeting of specific bacterial components.

To that end, we have previously demonstrated that conventional cationic polymers unmodified branched poly(ethylene imine)s (BPEIs) showed potent antimicrobial activity against *S. aureus*, but did not show any significant activity against *E. coli* (Figure 1-6). The BPEIs are not hemolytic against human red blood cells (RBCs).[30] Furthermore, the BPEIs do not cause membrane permeabilization, indicating that membrane disruption is not the primary mechanism of antimicrobial action.[30] As most amphiphilic antimicrobial peptides and polymers exert their activity through membrane disruption, we were motivated to examine how the polymer's structure and chemical composition provided for a different mode of action and bacterial strain selectivity.



	Structure	M_n	MIC (μg/mL)		HC_{50} (μg/mL)
			E. coli	*S. aureus*	
B-$PEI_{0.5}$	Branched	470	500	16	>4000
B-$PEI_{1.1}$	Branched	1100	250	32	>4000
B-PEI_{12}	Branched	1200	>1000	16	>4000
L-$PEI_{4.4}$	Linear	4400	31	8	565
L-$PEI_{6.5}$	Linear	6500	31	8	163

Figure 1-6. Antimicrobial activity of PEIs. (A) Demonstrates the structural differences between BPEI and LPEI. (B) Examines the MIC and HC_{50} of the polymers. Notice that the BPEIs are selective for *S. aureus* over *E. coli* and RBCs while the LPEIs are not selective. (C) Demonstrates the ability to depolarize *S. aureus* membranes. LPEI is a membrane disrupter similar to melittin, while BPEI does not appear to cause membrane disruption. Data from Gibney, K. *et al*. *Macromol. Biosci.*, **2012**, *12*, 1279-89.[30] Figure adapted from Takahashi, H. *et al*, *Macromol. Biosci.*, **2013**, *13*, 1285-99.[31]

In the literature, other cationic synthetic oligomers and polymers, such as polynorbornenes[26, 32] and oligo-lysins,[33] have shown selective activity against *S. aureus* (Figure 1-7). Similarly, cationic natural polysaccharide chitosan showed potent antimicrobial activity against *S. aureus* without inducing significant membrane disruption.[34] The authors speculate that chitosan may act in the cell wall of *S. aureus*, likely interacting with teichoic acids, and trigger cellular events which result in chitosan's anti-*S. aureus* activity. While these cationic polymers have distinctively different chemical structures, the common cationic groups are likely to play a pivotal role in their antimicrobial action and selective activity against *S. aureus*.[27] Natural

cationic AMPs have been considered to kill bacteria by disrupting bacterial cell membranes. However, recent studies indicate that AMPs also exert their antimicrobial effects by binding to multiple targets of cell walls, rather than only causing membrane disruption.[11] These cationic polymers may also act in the bacterial cell wall, displaying selective activity against *S. aureus* over other bacteria. The polymers will serve as a simple model to dissect the mode of action of antimicrobial polymers and peptides.

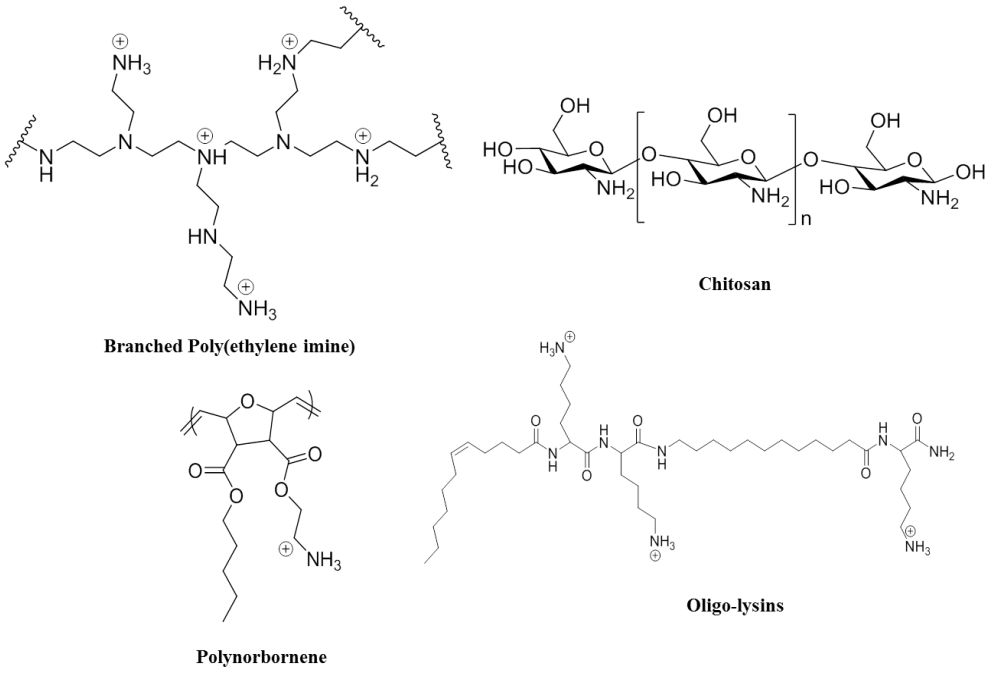


Figure 1-7. Cationic polymers with anti-*S. aureus* activity.

With this in mind, we are interested in the development of cell-selective antimicrobial polymers utilizing the mechanism associated with cationic functionality. In particular, we are interested in anti-*S. aureus* agents because *S. aureus* is a virulent pathogen which causes serious infections in the community and in hospitals. We hypothesize that *cationic polymers would be a new synthetic platform for potent and selective anti-S. aureus agents*. To that end, this dissertation focuses on the development of cationic methacrylate homopolymers for potent and

selective *S. aureus* agents. Chapter 2 will examine the bacterial-strain specificity of cationic methacrylate homopolymers as well as their suitability for *in vivo* treatment of *S. aureus* infections. Chapter 3 discusses the effect of cationic ammonium structures on their activity and their antimicrobial mechanism, focusing on the electrostatic interaction between the cationic polymers and the anionic bacterial cell wall. Chapter 4 examines the antimicrobial mechanism of amphiphilic methacrylate polymers and cationic homopolymers in relation to pore formation in bacterial cell membranes and osmotic lysis. Chapter 5 concludes this dissertation and discusses future directions and challenges.

Chapter 2 : Assessment of *in vivo* Activity of Methacrylate Homopolymers for Treatment of *Staphylococcus aureus* Nasal Colonization

The work presented in this Chapter is published in Laura M. Thoma, Blaise R. Boles, and Kenichi Kuroda, "Cationic Methacrylate Polymers as Topical Antimicrobial Agents against *Staphylococcus aureus* Nasal Colonization", *Biomacromolecules,* **2014**. DOI: 10.1021/bm500557d

Introduction

Synthetic polymers have been widely investigated as a new molecular platform to create antimicrobial agents which are active against drug-resistant bacteria.[18 19 20 21] The Kuroda lab has demonstrated the antimicrobial activity of cationic amphiphilic methacrylate random copolymers which act by disrupting bacterial cell membranes, mimicking the mode of action of naturally occurring antimicrobial peptides.[27] While these antimicrobial copolymers are potential candidates for therapeutic treatment of antibiotic-resistant bacterial infections, they exhibit a broad spectrum of activity, which may negatively impact commensal bacterial flora during long-term treatment. It would be ideal to develop antimicrobial polymers with cell-selectivity or specificity to bacterial strains which can avoid side effects to commensal flora.

Conventional cationic polymers unmodified branched poly(ethylene imine)s (BPEIs) have shown potent antimicrobials against *S. aureus*, but do not show any significant activity

against *E. coli* nor hemolytic activity against human red blood cells. Furthermore, BPEIs do not cause significant *S. aureus* membrane permeabilization, indicating that membrane disruption is not the primary antimicrobial mechanism of BPEIs. Cationic natural polysaccharide chitosan likewise showed potent antimicrobial activity against *S. aureus* without inducing significant membrane disruption.[34] This contrasts sharply with the antimicrobial action of membrane disruption by AMPs.

Given the fact that several cationic polymers with diverse structures are potent and selectively active against *S. aureus*, cationic functionality seem to be vital to the antimicrobial mechanism of the polymers against *S. aureus*. Accordingly, we hypothesize that *cationic functionality in polymeric structures is the key component for antimicrobial polymers with potent and selective activity towards S. aureus*. To test this hypothesis, we used a methacrylate polymer as our model, as a methacrylate platform has been used for our previous research on broad-spectrum antimicrobial polymers. The living/controlled polymerization of methacrylate monomers allows for precise control of the number of cationic groups, facilitating our investigation on the activity-structure relationship. In this study, we investigate the *in vitro* and *in vivo* antimicrobial activity of cationic synthetic polymers as a potential cell-selective antimicrobial agent against *S. aureus*. We are particularly interested in anti-*S. aureus* agents because *S. aureus* is one of the most common causes of nosocomial and community-acquired infections.[35 36] As cationic functionality appears to be an essential characteristic for *S. aureus* specific activity of antimicrobial polymers, we have designed a series of ammonium ethyl methacrylate homopolymers (AEMPs) with varying numbers of primary ammonium groups in the side chains. This structure contrasts with previously synthesized random copolymers in the Kuroda lab which contain both cationic and hydrophobic side chains and exert their

antimicrobial effect by disrupting bacterial cell membranes. This series of AEMPs serves as a simple model to investigate why the cationic polymers display selective activity to *S. aureus* and to determine if this is a previously uncharacterized mode of antimicrobial action of polymers.

The purposes of this study are to evaluate the *in vitro* efficacy of AEMPs as anti-*S. aureus* agents as well as assess the feasibility and effectiveness of these AEMPs for the treatment of *S. aureus* nasal colonization. We particularly targeted the treatment of *S. aureus* in a nasal colonization as nasal passages have proven to be a prime environment for *S. aureus* colonization and play an important role in *S. aureus* infection.[37, 38] In this study, the spectrum of activity and bactericidal kinetics of the AEMPs will be examined. To assess the activity of AEMPs in physiological conditions, their antimicrobial activity in the presence of serum was also determined. To evaluate the cytotoxicity of AEMPs, cell viability of mammalian cells HEp-2 and COS-7 was determined in the presence of AEMPs using an XTT assay. The propensity for resistance development in *S. aureus* was also evaluated by exposing the bacteria to an AEMP at a sub-inhibitory concentration. Finally, the AEMPs were tested for their *in vivo* activity against a *S. aureus* nasal colonization in a cotton rat model.

Polymer Synthesis and Characterization

Boc-protected amino ethyl methacrylate homopolymers (Boc-P series) were prepared by reversible addition-fragmentation chain-transfer (RAFT) polymerization of Boc-protected aminoethylmethacrylate (AEMA) using 2-cyanoprop-2-yl ethyl trithiocarbonate (CPETC) as a chain transfer agent (Figure 2-1). The protection of amine groups facilitates the polymer synthesis in organic solvents as well as avoids undesirable aminolysis of the RAFT agent and intermolecular amide formation with the ester groups in the monomer. The polymerization proceeded at 80°C and quenched by cooling in a dry ice/ ethanol bath after 48 hours. ^{1}H NMR

spectra of the crude polymerization mixture indicated at least a 96% conversion of monomer for each polymerization. The resulting boc-protected polymers were purified by precipitation into hexane.

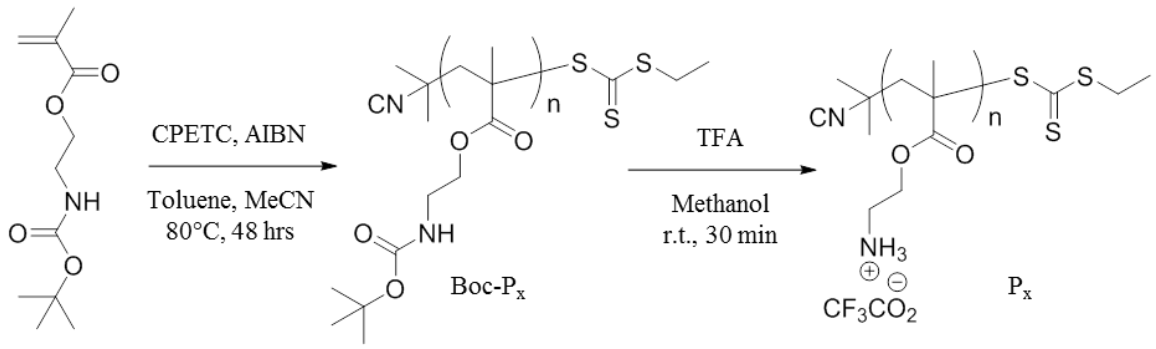


Figure 2-1. Synthesis of ammonium ethyl methacrylate homopolymers (AEMPs).

GPC analysis of these Boc-protected AEMPs indicated that the polydispersity index (M_w/M_n) is ~1.3 (Figure 2-2, Table 2-1). The degree of polymerization (DP) was determined by comparing the integrated intensities of the ^{1}H NMR resonances from the terminal RAFT agent group relative to the monomer side chain (Figure 2-3). In order to examine the effect of molecular weight (MW) on antimicrobial activity and cytotoxicity, Boc-protected AEMPs (Boc-P) were synthesized with different DPs ranging from 9.9 to 19 by varying the ratio of RAFT agent to monomers. The Boc-groups of the polymers were removed by treating the polymers with trifluoroacetic acid (TFA) to give AEMPs with primary ammonium groups, indicated by the disappearance of the peak at 1.5 ppm in the NMR spectrum (Figure 2-3, Appendix B). The DP values of deprotected polymers (P series) were in the range of 7.7 to 12, which represents the average number of ammonium groups in a polymer. The integrated intensities of the methyl groups at the α-end (j) slightly decreased from 3.8 to 2.6 for $P_{7.7}$, for example, relative to the 6 protons of methyl groups at the ω-end after the deprotection procedure (Appendix B). This could

be attributed to the partial decomposition of the trithioester end groups due to low stability of the RAFT agent CPETC under the acidic condition (Appendix C). Because of the decomposition of the RAFT agent, the DPs are calculated based on the signals from the α-terminal group. Accordingly, the AEMPs will be identified as P_x where X is the DP of polymers.

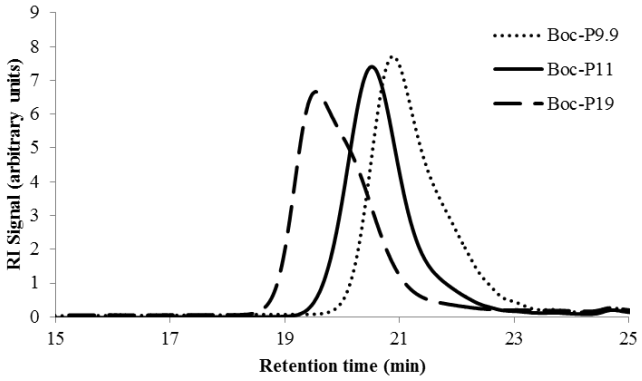


Figure 2-2. GPC Trace of Boc-protected polymers (Waters GPC, RI Detector, THF eluent).

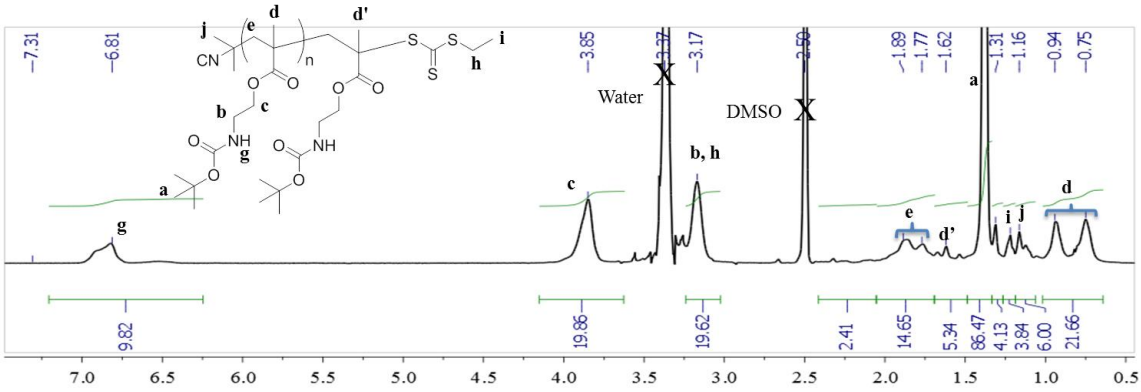


Figure 2-3. ^{1}H NMR spectrum of Boc-$P_{9.9}$ (400 MHz, DMSO-d6).

Table 2-1. Synthesis and Characterization of Boc-protected AEMPs.

Boc-protected Polymer								Deprotected Polymer		
Boc-P	CPETC[a] (%)	Conv.[b] (%)	M_n, GPC[C]	M_w, GPC[c]	Đ[d]	DP[e]	M_n NMR[f]	P	DP[e]	M_n, NMR[f,g]
Boc-$P_{9.9}$	22	97	3,300	4,300	1.32	9.9	2,500	$P_{7.7}$	7.7	1,200 (2,100)
Boc-P_{11}	10	96	5,100	6,700	1.31	11	2,800	P_{10}	10	1,600 (2,700)
Boc-P_{19}	3	97	9,800	13,000	1.35	19	4,600	P_{12}	12	1,800 (3,200)

a) Mole percentage of CPETC relative to the total amount of monomer in polymerization.
b) Monomer conversion was determined by comparing the integrations of peaks from vinyl groups and monomer side chains in ^{1}H NMR spectra.
c) The number and weight average molecular weight (M_n and M_w) were determined by GPC using THF. The molecular weight calibration was based on poly(methyl methacrylate) standards.
d) Polydispersity index is calculated as M_w/ M_n using M_w and M_n values determined by GPC.
e) The degree of polymerization (DP) was determined by comparing the integrated intensity of the ^{1}H NMR resonances from the methyl groups of the α- and ω- terminal RAFT agent group (I and j) relative to the side chain (c).
f) The number-average molecular weights (M_n) were calculated using DP and the molecular weights of monomer and CPETC based on the chemical structure of polymers.
g) The number-average molecular weight (M_n) of deprotected polymers without trifluoroacetate. M_n of polymers including trifluoracetate is presented in parenthesis.

Antimicrobial Activity

The AEMPs were tested for their antimicrobial activity against a panel of clinically relevant bacterial pathogens. The antimicrobial activity of the AEMPs was quantified as the minimum inhibitory concentration (MIC) in which bacterial growth is completely inhibited after an 18 hour incubation (Table 2-2, Figure 2-4). The MIC was determined using a turbidity-based microdilution method.[39] In general, the MIC values of AEMPs against Gram-positive bacteria tested in this study are smaller than those against Gram-negative bacteria. This indicates that the AEMPs were more effective at inhibiting the growth of Gram-positive bacteria when compared to the Gram-negative bacteria tested. The AEMPs are also active against community-acquired methicillin-resistant *S. aureus* (CA-MRSA), with the similar MIC values of AEMPs against susceptible *S. aureus* strain.

Table 2-2. The antimicrobial spectrum of AEMPs and antibiotics.

Bacteria	Gram	MIC[a] (μg/mL) (or μM)					
		$P_{7.7}$	P_{10}	P_{12}	Norfloxacin	Mupirocin	Vancomycin
S. aureus ATCC 25923	+	94 (45)	73 (27)	63 (20)	1.0 (3.1)	0.25 (0.5)	1.3 (0.9)
S. aureus BB2146	+	63 (30)	63 (23)	42 (13)	1.1 (3.3)	0.2 (0.4)	n.d.
S. aureus LAC (CA-MRSA)[b]	+	125 (60)	83 (31)	63 (20)	>8 (>25)	0.25 (0.5)	1.3 (0.9)
S. saprohyticus	+	16 (7.4)	16 (5.8)	16 (5)	0.83 (2.6)	0.13 (0.2)	1.3 (0.9)
B. subtilis	+	83 (40)	63 (23)	63 (20)	1.0 (3.1)	>8 (>16)	1.8 (1.3)
E. faecalis	+	156 (74)	94 (34)	78 (24)	1.5 (4.7)	n.d.	1.8 (1.3)
E. coli	-	>1000 (>476)	1000 (370)	313 (98)	n.d.	>40 (>80)	>40 (>28)
A. baumannii	-	417 (198)	670 (247)	250 (78)	1.3 (4.2)	>8 (>16)	>8 (>5.5)
P. aeruginosa	-	1000 (476)	50 (185)	250 (78)	0.83 (2.6)	>8 (>16)	>8 (>5.5)

a) The MIC in μM is presented in parenthesis, using MW with trifluoroacetate
b) Community-associated methicillin-resistant *S. aureus*

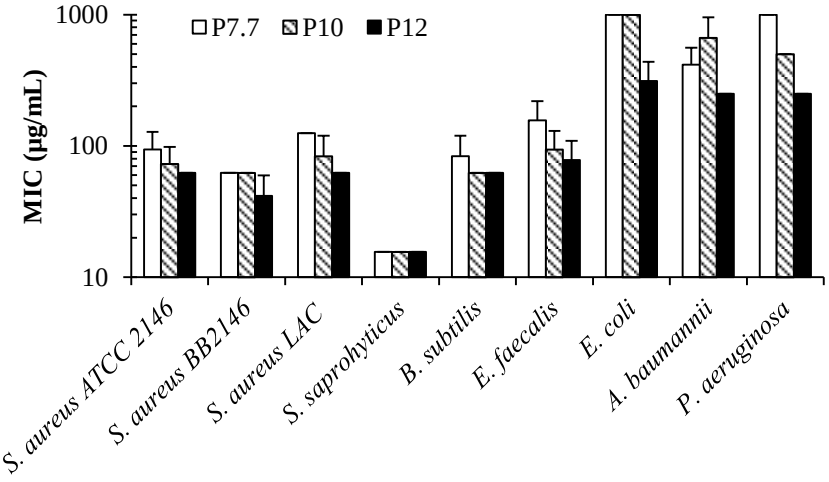


Figure 2-4. Minimum inhibitory concentrations of AEMPs for selected Gram-positive and Gram-negative bacteria.

The molecular weight of AEMPs did not appear to have a large influence on an AEMP's activity against any of the Gram-positive bacterial strains tested as the MICs for all AEMPs tested for a particular bacterium are within a two-fold dilution. This result suggests that increasing molecular weight or the number of cationic groups in the AEMPs has little impact on MIC over the molecular weight range tested here. On the other hand, the MIC values of AEMPs against *E. coli* and *P. aeruginosa* decreased as the molecular weight of AEMPs increased, indicating the AEMPs with higher molecular weights are more active against these bacteria. However, the MIC values of AEMPs against *A. baumannii* do not appear to depend on the molecular weight of AEMPs. The general trend of AEMPs for lower activity towards Gram-negative bacteria may be, at least in part, due to the inability of AEMPs to penetrate the outer membrane of Gram-negative bacteria, preventing AEMP access to the cell wall and cytoplasmic membranes, although the antimicrobial target of AEMPs in bacteria is not clear at this point.

Membrane Depolarization Assay

We have previously demonstrated that conventional cationic polymers of unmodified branched poly(ethylene imine)s (BPEIs) showed potent antimicrobial activity against *S. aureus* without causing significant membrane permeabilization, indicating that membrane disruption is not likely the primary mechanism of antimicrobial action.[30] To elucidate the antimicrobial mechanism and bacterial targets of AEMPs, we attempted to determine membrane permeabilization by the AEMPs using a membrane-potential sensitive fluorophore $DiSC_3(5)$.[33 40] This fluorophore binds to intact *S. aureus* cytoplasmic membranes and undergoes self-quenching. When the membrane is permeabilized through interaction with the polymers, the self-quenching of $DiSC_3(5)$ is alleviated, and the fluorescence is recovered. As a control, the changes

in fluorescence intensity of $DiSC_3(5)$ and polymer mixtures in the absence of bacteria were determined.

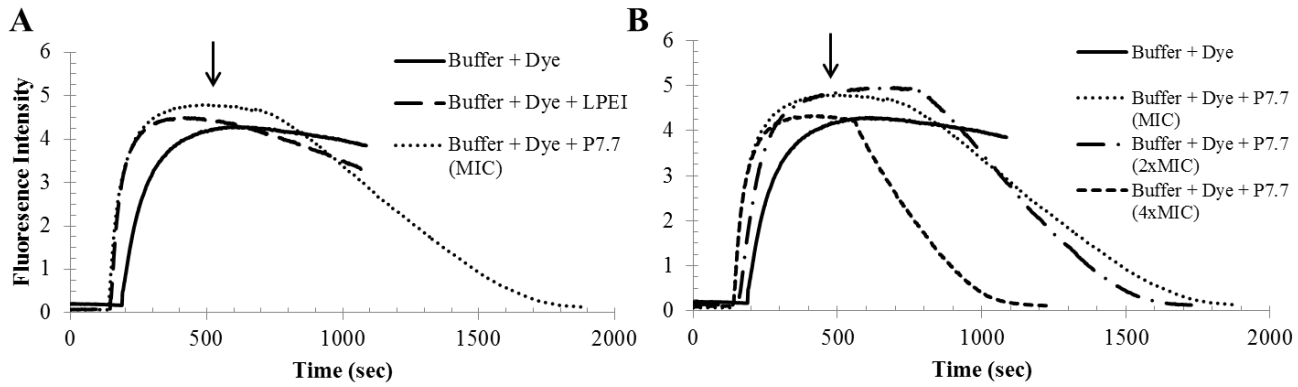


Figure 2-5. Fluorescence intensity of $DiSC_3(5)$ in the presence of AEMP and LPEI. The fluorescence intensity was determined by monitoring the absorbance of the dye mixed with polymers in the absence of *S. aureus*. Dye was added into PBS buffer (final concentration 0.5 μM) and mixed with polymers at their MIC concentrations. A) Shows the dye alone, and mixed separately with LPEI and $P_{7.7}$ at their MICs. B) Shows the dye mixed with $P_{7.7}$ at various concentrations. Open arrows indicate the point of polymer addition.

Figure 2-5 shows the change in absorbance of the dye mixed with polymers in the absence of *S. aureus*. Both LPEI and $P_{7.7}$ caused a decrease in fluorescence at a much faster rate than the dye self-quenches in the absence of bacteria (Figure 2-5A). As the concentration of $P_{7.7}$ increased, the rate of fluorescence quenching also increased (Figure 2-5B). It is likely that the polymer binds to the dye, and the interaction between the polymer and the dye causes quenching of fluorescence from the dye, although the molecular mechanism is not clear at this point.

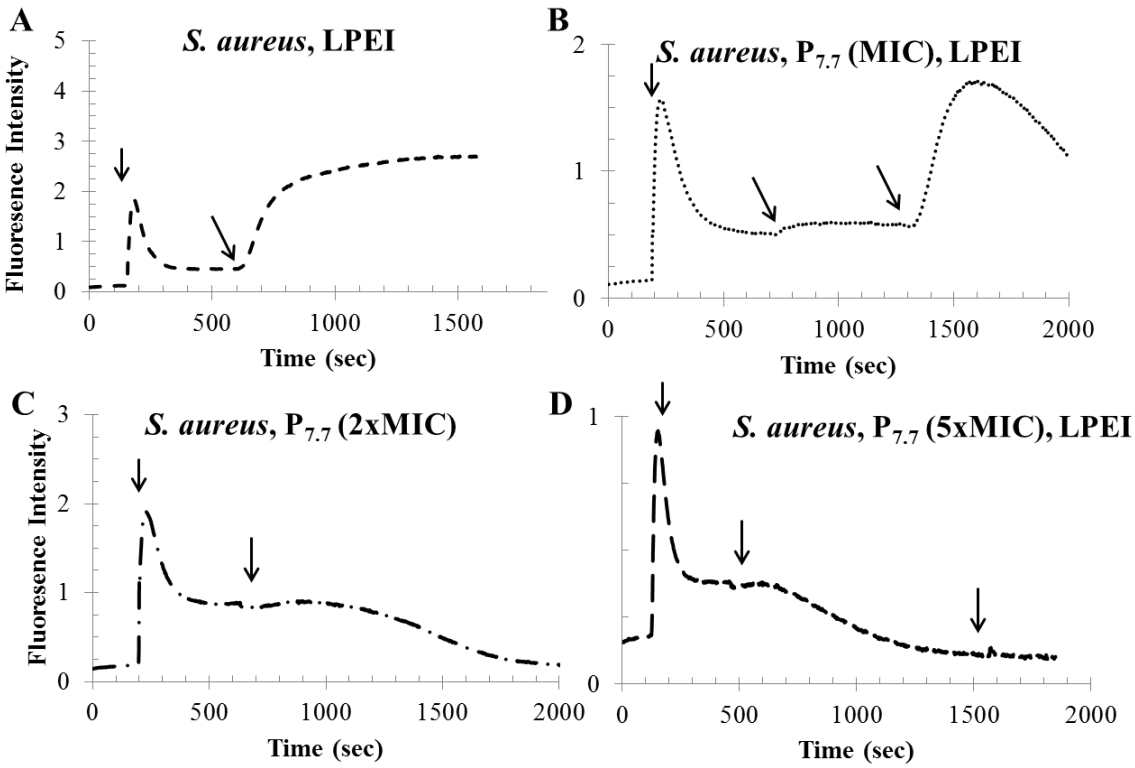


Figure 2-6. *S. aureus* membrane depolarization by LPEI and AEMP.

Figure 2-6 shows the change in fluorescence induced by the polymer mixtures in the presence of *S. aureus*. Figure 2-6A demonstrates the change in fluorescence in the presence of LPEI, with results similar to what we have previously demonstrated.[30] LPEI causes membrane depolarization, as demonstrated by the increase in fluorescence from $DiSC_3(5)$ after the polymer's addition to *S. aureus* solution. Figure 2-6B-D demonstrates the change in fluorescence from $DiSC_3(5)$ bound to *S. aureus* upon the addition of various concentrations of $P_{7.7}$. Only a slight change in fluorescence was observed at polymer concentrations 1x, 2x, or 5x the MIC of $P_{7.7}$. However, as the polymer reduced the fluorescence of $DiSC_3(5)$ in solution (Figure 2-5), the results could be due to fluorescence quenching by the polymer rather than the inability of the polymer to cause membrane depolarization. Therefore, this approach cannot determine if the lack

of substantial change in fluorescence upon introduction of $P_{7.7}$ shows that $P_{7.7}$ doesn't cause membrane depolarization or if it is a result of the fluorescence quenching by the polymer. In addition, closed arrows mark the addition of LPEI, a known membrane depolarizer, for $P_{7.7}$ at the MIC and at 5x the MIC. After the addition of LPEI for $P_{7.7}$ at the MIC, the fluorescence increased, indicating that membrane depolarization does occur. However after the addition of LPEI to $P_{7.7}$ at 5x the MIC there is no change in the fluorescence. This is likely either because the high concentration of $P_{7.7}$ quenches the fluorescence caused by LPEI or $P_{7.7}$ in the cell wall prevents membrane depolarization, possibly by inhibiting the diffusion of LPEI through the cell wall to the cell membrane. It cannot be ruled out that $P_{7.7}$ binds to LPEI, which may sequester LPEI from solution. In conclusion, the membrane depolarization assay using $DiSC_3(5)$ does not provide quantitative results to determine the ability of $P_{7.7}$ to cause membrane depolarization in *S. aureus* due to the strong quenching of fluorescence from $DiSC_3(5)$ by the polymer. It should be noted that LPEI did not cause significant quenching of $DiSC_3(5)$,[30] and we here provide a caution to the use of this assay for cationic polymers.

Cell Leakage Assay

In addition to the fluorescence assay, we also performed a cell leakage assay to determine membrane damage by the AEMPs. *E. coli* and S. *aureus* cells were incubated with $P_{7.7}$ for 2 hours and the cells are removed by centrifugation. The absorbance of cell-free supernatant at 260 nm was measured to determine if any UV-absorbing compounds leaked from *S. aureus* cells as a result of any damage to membrane integrity.

Table 2-3. Absorbance of assay solutions.

	E. coli	*S. aureus*
Starting OD_{600}	0.250	0.250
OD_{600} after centrifugation		
PBS	0.0299	0.0288
PBS, bacteria	0.0321	0.0255
PBS, bacteria, CTAB	0.0278	0.0276
PBS, bacteria, Lysostaphin	0.0271	0.0264
PBS, bacteria, Melittin	0.0309	0.0289
PBS, bacteria, $P_{7.7}$	0.0281	0.0277

CTAB, lysostaphin, melittin, and $P_{7.7}$ were tested for their ability to cause leakage of cellular components from *E. coli* or *S. aureus*. PBS buffer without any compounds was used as a control. All test compounds were used at a final concentration of at least 3x their MIC in the representative bacteria. After 2 hours, the assay solution was centrifuged at 14,000 rpm for 10 minutes. The OD_{600} values of assay solutions are close to PBS, indicating that the supernatant was cell-free after centrifugation (Table 2-3). The absorbance of the supernatant was measured at 260 nm to detect UV-absorbing cellular components. As a background, the absorbance of test compounds in PBS without bacteria was determined, and subtracted from the absorbance reading in the presence of bacteria at 260 nm. The corrected absorbance for *E. coli* and *S. aureus* are show in Figure 2-7.

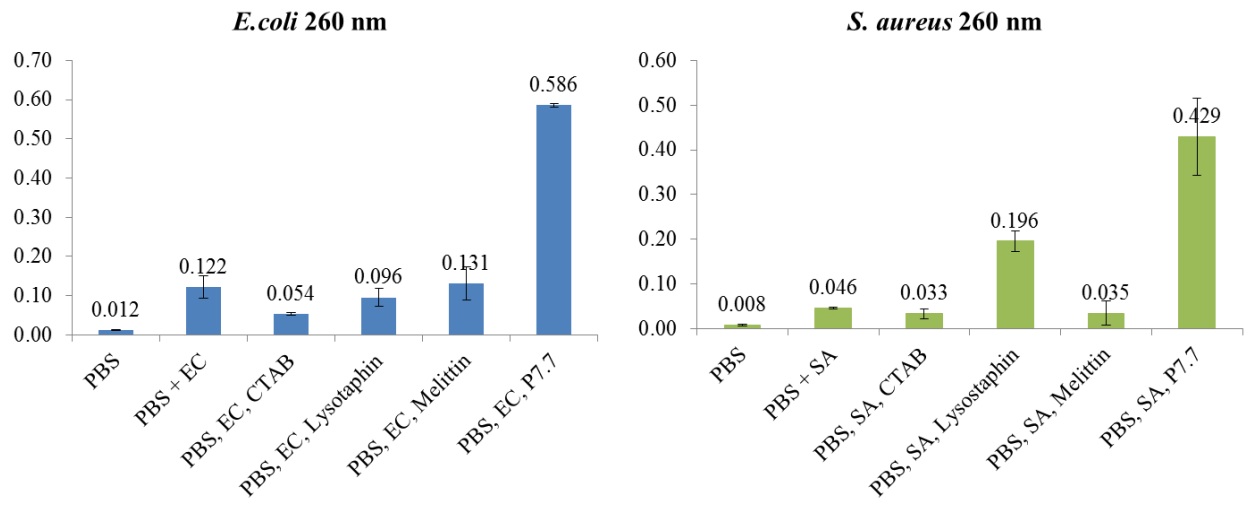


Figure 2-7. Detection of UV-absorbing cellular components.

PBS was used as a negative control, as it should cause no leakage of cellular components from the bacterial membrane, CTAB (a cationic surfactant) and melittin (lytic peptide) were used for comparison. Lysostaphin, an endopeptidase capable of cleaving the crosslinking pentaglycin bridges of *Staphylococci* for cell lysis, was used as a positive control for 100% leakage for *S. aureus*. $P_{7.7}$ showed a large increase in absorbance at 260 nm for both bacteria after 2 hour treatment. It appears that $P_{7.7}$ causes strong membrane permeabilization. However, the absorbance caused by $P_{7.7}$ is significantly higher than the absorbance for lysostaphin and melittin, controls that we expected to give 100% lysis of bacterial cells and subsequent protein leakage. Therefore, because of the amphiphilic nature of the polymer, $P_{7.7}$ is likely to bind to cellular components such as proteins and lipids, which may cause formation of small aggregates. The small aggregates may result in the apparent increase in the UV absorbance due to light scattering. The polymer aggregates may also change the environment of UV-sensitive functional groups of proteins, increasing the absorbance. In conclusion, the membrane permeabilization assay monitoring UV-absorbing cellular components does not provide quantitative results to

determine the ability of $P_{7.7}$ to cause membrane permeabilization in bacteria due to the increased absorbance, which is likely to reflect the aggregation of cellular components with polymer.

Antimicrobial Activity in the Presence of Serum

To evaluate the activity of AEMPs in physiological conditions, the MIC values of AEMPs were determined in the presence of fetal bovine serum (FBS). It has been reported that the activity of antimicrobial peptides is reduced in the presence of serum because the serum salts curtain the electrostatic binding of cationic peptides to anionic bacterial surfaces and serum proteins such as albumin non-specifically bind to peptides.[41,42] Therefore, the activity of AEMPs could also be mitigated in the presence of serum.

AEMP activity was determined in 50% (v/v) FBS in MH broth (MHB). MHB components could non-specifically bind to the polymers, reducing the polymer activity. The reduction of these antagonizing factors in MHB by dilution with PBS buffer would increase their antimicrobial activity (decrease MIC values). To take this dilution effect into account, we used 50% PBS buffer in MHB as a control.

Table 2-4. The antimicrobial activity of AEMPs in the presence of fetal bovine serum.

Bacteria	Condition	MIC (μg/mL)					
		$P_{7.7}$	P_{10}	P_{12}	CTAB	Mupirocin	Norfloxacin
E. coli	MHB	>1000	670	250	35	n.d.	n.d.
	50% PBS	>1000	670	104	35	n.d.	n.d.
	50% FBS	>1000	>1000	>1000	>63	n.d.	n.d.
S. aureus ATCC 25923	MHB	130	63	63	0.98	0.5	1.3
	50% PBS	63	31	16	0.98	0.5	1.0
	50% FBS	20	5.9	6.8	> 7.8	> 4.0	2.0
S. aureus BB 2146	MHB	83	63	42	0.81	0.33	1.0
	50% PBS	31	16	16	0.41	0.25	0.67
	50% FBS	3.9	2.0	2.0	6.5	>4.0	1.0

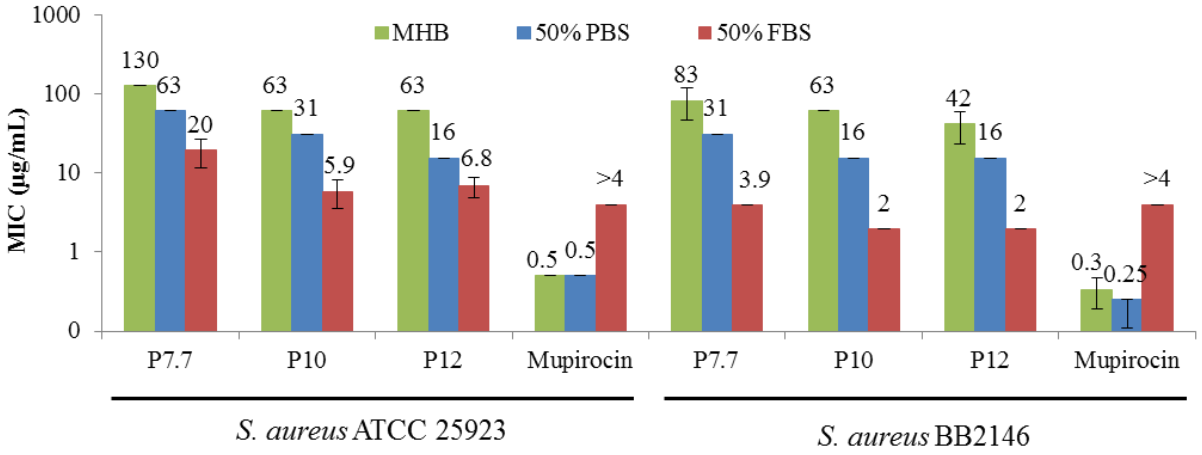


Figure 2-8. The effect of FBS on the activity of AEMPs and mupirocin against *S. aureus* strains.

Against *E. coli*, all of the polymers did not show any activity in the presence of FBS (MIC > 1000 μg/mL), while the high MW polymers P_{10} and P_{12} are active towards *E. coli* in the absence of FBS (Table 2-4). The MIC of cationic surfactant CTAB also increased in 50% FBS, indicating that serum components generally reduce the activity of cationic polymers, AEMPs, and surfactants.

Interestingly, the MICs of AEMPs for *S. aureus* decrease significantly in in the presence of FBS when compared to those in MHB. For example, $P_{7.7}$, which is used for the *in vivo* testing described later, had an MIC of 125 μg/mL in MHB and a MIC of 19.5 μg/mL in FBS, giving 6-fold reduction in MIC (Figure 2-8). The MIC of $P_{7.7}$ showed a 2-fold reduction in PBS/MHB, indicating that the MIC reduction of $P_{7.7}$ in FBS is not just due to the effect of the dilution of MHB described above. The MIC of AEMPs with higher molecular weights also decreased in the presence of serum to give MICs of 5.9 and 6.8 μg/mL for P_{10} and P_{12}, respectively, which is about a 10-fold reduction in their MIC values as compared to those in MHB (Table 2-4). The MIC values of AEMPs in 50% FBS are 6-20 μg/mL (Table 2-4), which are comparable to those of potent antimicrobial polymers reported in literature[43 44 45 46 47] although the literature values

were generally determined in the absence of serum. In addition, caution must be taken when comparing MIC values between research groups as many groups use different assay protocols, and the MIC values depend on multiple assay conditions including bacterial strains, assay plates, and broth media. The MICs of AEMPs for *S. aureus* strain BB2146, the *in vivo* strain used later, are 2 – 4 μg/mL, indicating that the polymers are highly active against this strain of *S. aureus.* The MIC values of the surfactant CTAB and the antibiotic mupirocin, an RNA duplication inhibitor[48] which is widely used in the topical treatment of skin/wound infections, significantly increased in 50% FBS, indicating that FBS does not generally increase *S. aureus* susceptibility to antimicrobials. However, the mechanism of potent activity of AEMPs in FBS is not clear at this point. It should be noted that the decrease in MIC of AEMPs for *S. aureus* was observed for different batches of AEMPs with different lots of FBS, suggesting that the results presented in this report are not batch/lot specific. In summary, the AEMPs showed potent activity against *S. aureus* in the presence of FBS, which contrasts with the activity reduction for CTAB and antibiotics. These results demonstrate that these AEMPs have potential for potent activity against *S. aureus* infections in physiological conditions.

Bactericidal Kinetics

We examined the bactericidal activity exerted by the AEMPs against *S. aureus* ATCC 25923 and their time dependence to assess the rate of killing. Accordingly, we monitored the number of viable *S. aureus* cells in a colony-forming unit (cfu) as a function of exposure time to the AEMPs at concentrations of twice their respective MICs. We used the conventional antibiotics mupirocin and norfloxacin (DNA gyrase and topoisomerase IV inhibitor)[49 50] for comparison.

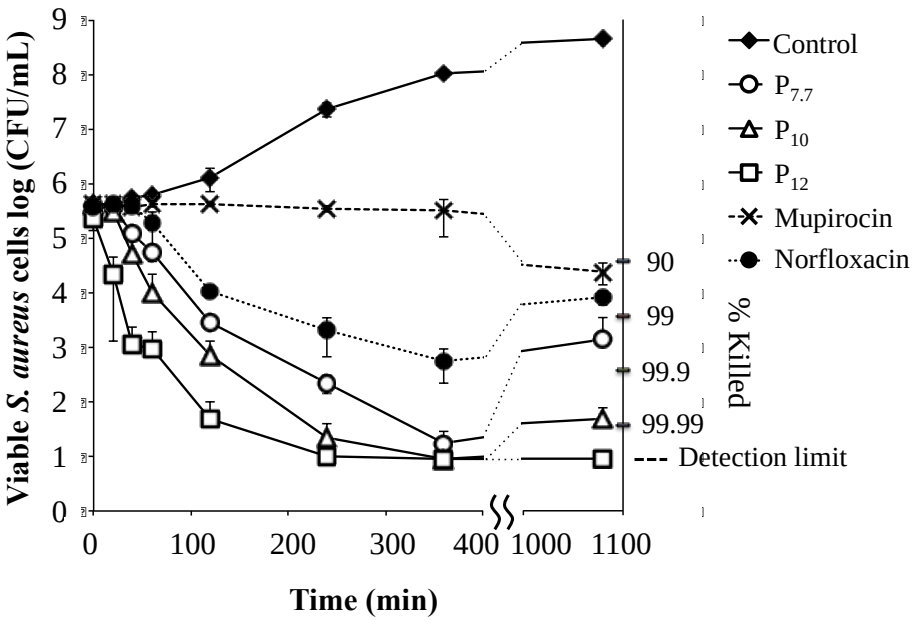


Figure 2-9. Bactericidal kinetics of AEMPs and antibiotics mupirocin and norfloxacin at 2 times MIC. The detection limit is 10 cfu/mL due to the dilution factor in this assay.

At two times the MIC the antibiotic mupirocin caused less than one log reduction in the number of viable *S. aureus* (Figure 2-9), indicating that mupirocin is rather bacteriostatic under this condition, which is in agreement with literature.[51] Norfloxacin did not cause any change in the number of viable *S. aureus* cells while the bacteria were in the lag phase of growth (0 – 40 min.), but after 40 minutes the number of viable cells started to decrease, with a two log reduction after 190 minutes. After 18 hrs, the bacteria treated with norfloxacin grew back slightly which is in agreement with literature.[52]

In contrast to the antibiotics tested, the AEMPs showed killing of *S. aureus* with relatively higher killing rates. All three polymers are bactericidal at 2 times their MICs, and caused four-log reductions in the number of viable bacteria (99.99% killing) within 360 minutes. The highest MW polymer, P_{12}, caused a three-log reduction in the number of viable cells (99.9% killing) after 60 minutes, while P_{10} and $P_{7.7}$ needed at least twice as much time, with 130 and 210

minutes respectively, for the same three-log reduction. This demonstrates that for AEMPs the rate of killing increased as the molecular weight of polymers increased.

After 18 hours, the bacteria incubated with the lower MW polymers $P_{7.7}$ and P_{10} grew back to give a two-fold (99%) or three-fold (99.9%) reduction in the number of initial bacteria. This indicates that some population of bacteria may be resistant to the antimicrobial action of the AEMPs and are still viable. The development of *S. aureus* resistance to the polymer will be discussed in detail below.

Resistance Development

To assess the potential emergence of antibacterial resistance to AEMPs, *S. aureus* was exposed to sub inhibitory concentrations (1/2 MIC) of antimicrobial agents during successive subcultures. We used $P_{7.7}$ as a model compound and two conventional antibiotics norfloxacin and mupirocin as positive controls, as development of antibiotic resistance in *S. aureus* to these antibiotics has been previously reported.[53 54]

The MIC values of $P_{7.7}$ and the antibiotics against *S. aureus* were determined for each passage over 14 successive subcultures (Figure 2-10A). The MIC values of norfloxacin started to increase on the first passage and continuously increased up to 64 times the starting MIC at the 11^{th} passage. The MIC of mupirocin began increasing at the 2nd passage and continued increasing up to 6 times the original MIC at the 11^{th} passage. On the other hand, the MIC of $P_{7.7}$ started to increase at the 4^{th} passage and increased up to 6 times the original MIC after 12 passages, the same final level of resistance development as mupirocin.

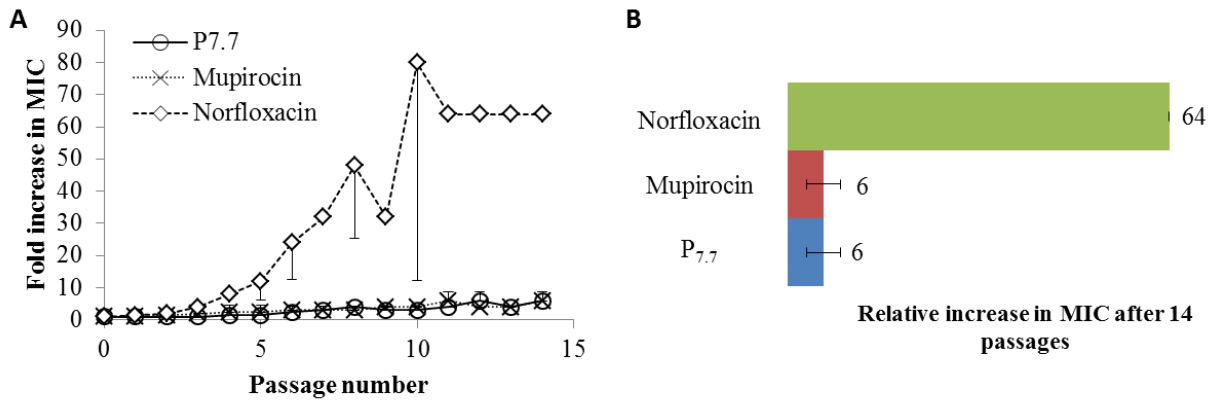


Figure 2-10. Propensity of AEMP $P_{7.7}$ for resistance development in *S. aureus*. (A) Fold increase in MIC against *S. aureus* as a function of passage. One-sided error bars were presented for clarity. (B) Relative increase in MIC after 14 passages.

It has been reported that *S. aureus* have become resistant to cationic antimicrobial peptides by chemically modifying their major cell wall biopolymers, anionic teichoic acids.[55] The bacteria modify the cell walls through alanyl esterification of the teichoic acids. This reduces the net anionic charge in the *S. aureus* cell wall,[56 57] decreasing the electrostatic binding of cationic polymers to the bacterial cell surface. The same resistance mechanism would also reduce the electrostatic binding of cationic polymers to *S. aureus*, resulting in lower AEMP activity or an increase in MIC. While this is a possible resistance mechanism, the antimicrobial target of AEMPs in bacteria, and their actions resulting in resistance development, is not clear at this point.

Hemolytic Activity

As an initial metric of biocompatibility, the lysis of human red blood cells (hemolysis) by AEMPs was measured. The AEMPs showed 1.6% to 7.6% hemolysis at 1000 μg/mL, the highest concentration tested (Figure 2-11), indicating that the AEMPs are not potent hemolysins. For comparison, under the same assay condition, bee venom toxin melittin causes 100%

hemolysis at concentrations as low as 10 μg/mL (data not shown). In corroboration with the antimicrobial activity of AEMPs, the AEMPs are antimicrobial against Gram-positive bacteria over Gram-negative bacteria, but not hemolytic, indicating the AEMPs are cell-selective to Gram-positive bacteria.

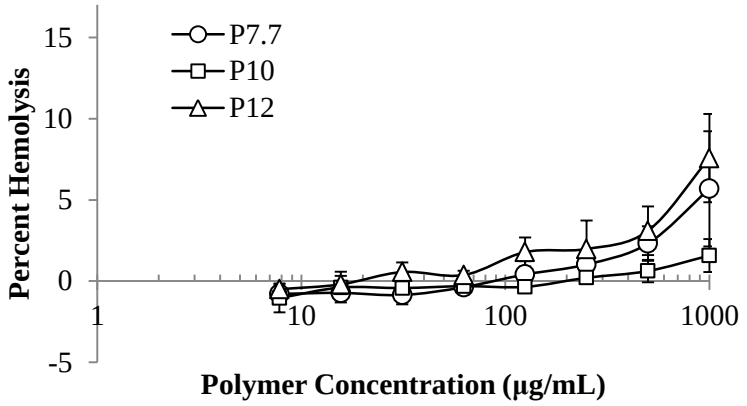


Figure 2-11. Hemolytic activity of AEMPs against human red blood cells, with the hemolysis by Triton X as 100%.

Cell Cytotoxicity

To assess the toxicity profile of AEMPs, the reduction of cell viability of mammalian cells exposed to AEMPs (cytotoxicity) was determined. We used human epithelial HEp-2 cells for cytotoxicity testing as AEMPs could be used as topical antimicrobials for skin and nasal infections. Monkey kidney fibroblast COS-7 cells were also used to test for cytotoxicity in order to examine the cell dependence of AEMP cytotoxicity. The cell viability was determined using the XTT colorimetric assay.[58 59] In addition to the AEMPs, commercially available polymers including neutral non-toxic polyethylene glycol (PEG, MW 2000) as well as branched cationic polymer poly(ethylene imine) (BPEI, MW 1800) were used for comparison.

For both cell lines tested, the cell viability decreased with increasing AEMP and BPEI concentrations, eventually reaching 0% (complete cell death) (Figure 2-12). PEG did not show

any signification reduction in cell viability for either cell line. For the AEMPs, cell viability decreased with increasing polymer concentration, and the cytotoxicity of AEMPs was dependent on the molecular weight of the AEMP. The polymer concentration which caused a 50% reduction in cell viability after 24 hour incubation with AEMPs was defined as the IC_{50} value (Table 2-5).

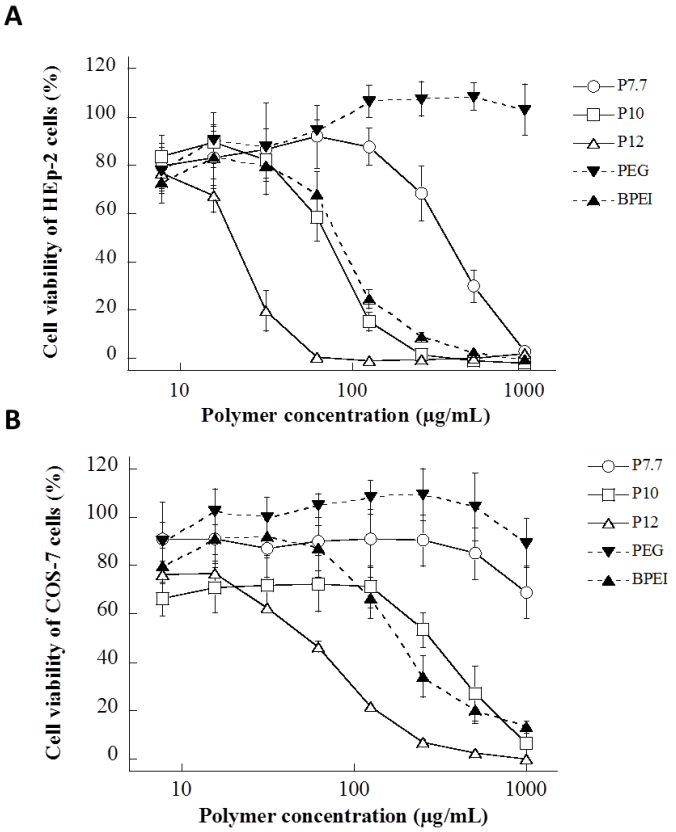


Figure 2-12. Cytotoxicity of AEMPs. Cell viability of HEp-2 (A) and COS-7 (B) cells was determined after 24 hour incubation with AEMPs.

Table 2-5. Cytotoxicity of AEMPs to HEp-2 and COS-7 cells.

Polymer	IC_{50} (μg/mL)[a]	
	HEp-2	COS-7
$P_{7.7}$	250	>1000
P_{10}	75	270
P_{12}	20	55
PEG (MW 2000)	>1000	>1000
BPEI (MW 1800)	85	175

a) Concentration for a 50% reduction in cell viability.

For HEp-2 cells, the IC_{50} values decreased as the molecular weight of the AEMPs increased, indicating that high molecular weight or more cationic groups of AEMPs cause higher cytotoxicity (Figure 2-12A). It has been previously reported that traditional cationic polymers such as PEIs and PAMAM dendrimers likely cause cytotoxicity by increasing the permeability of the cell membrane of mammalian cells[60 61 62] and that membrane permeabilization was strongly dependent on the number of cationic charges presented by the polymer and dendrimer surfaces.[61 62] The structure-function study on the cytotoxicity of cationic amine-poly(vinyl alcohol) polymers demonstrated that the cationic ammonium groups of polymers may bind through multiple points to cell membranes, resulting in membrane disruption or permeabilization.[63] These results indicated that cationic functionality in polymer structures may have an intrinsic toxicity mechanism against host cells through interactions with cell membranes. Similarly, the polycationic AEMPs may also cause cytotoxic effect by permeabilizing cell membranes. The high MW AEMPs have higher cationic densities resulting in the higher cooperative action of cationic groups for membrane binding and permeability as they are able to have multiple-point interaction with lipids. This would result in a higher toxicity (a lower IC_{50} value) than their lower molecular weight counterparts.

For COS-7 cells, all AEMPs reduced cell viability when compared to PEG which showed 100% COS-7 viability across all concentrations tested (7.8 – 1000 μg/mL, Figure 2-12B). The higher MW AEMPs cause a larger reduction in cell viability as P_{12} was the most cytotoxic (IC_{50} = 55 μg/mL) followed by P_{10} (IC_{50} = 270 μg/mL) and $P_{7.7}$ (IC_{50} > 1000 μg/mL). This difference due to molecular weight indicates that more cationic groups on AEMPs cause an increase in toxicity to COS-7 cells, identical to the trend shown for HEp-2 cells. As the IC_{50} values of

AEMPs for HEp-2 cells are lower than those for COS-7 cells, it appears that HEp-2 cells are more susceptible to the AEMPs than COS-7, although the reason for this is not clear (Table 2-5).

Selectivity to Bacteria Over Mammalian Cells

To examine the selective activity of AEMPs to bacterial cells over mammalian cells, we determined the IC_{50}/MIC as a selectivity measure (Table 2-6). In general, due to the low activity of AEMPs against *E. coli* (MICs > 1000 μg/mL), the selectivity indices for *E. coli* are small (< 0.3) for all polymers, indicating that the AEMPs are not selective to *E. coli* over mammalian cells. On the other hand, the low molecular weight AEMPs $P_{7.7}$ and P_{10} showed higher selectivity for *S. aureus* than P_{12}. These data demonstrate that $P_{7.7}$ and P_{10} are more selective for *S. aureus* over both HEp-2 and COS-7 cells. The AEMPs $P_{7.7}$ and P_{10} showed potent activity against *S. aureus* in the presence of FBS (MIC = 2 - 4 μg/mL for *S. aureus* BB2146 and 13 μg/mL for *S. aureus* ATCC 25923), indicating that these two AEMPs would be good candidates for *in vivo* applications for treatment of *S. aureus* infections as demonstrated below.

Table 2-6. Selectivity of AEMPs to *S. aureus* over mammalian cells.

Polymers	HEp-2 [a]			COS-7 [a]		
	E. coli	ATCC 25923	BB 2146	*E. coli*	ATCC 25923	BB 2146
$P_{7.7}$	> 0.3 (< 0.3)	2.0 (13)	4.0 (64)	- (-)[b]	8.0 (> 51)	16 (> 256)
P_{10}	0.1 (< 0.1)	1.2 (13)	1.2 (39)	0.3 (< 0.3)	4.3 (46)	4.3 (139)
P_{12}	0.1 (< 0.1)	0.3 (3.0)	0.5 (10)	0.2 (< 0.1)	0.9 (8.1)	1.3 (28)

a) Selectivity is calculated by IC_{50}/MIC (MHB). The selectivity index for MIC in 50% FBS in MH broth is presented in parentheses. b) Not applicable because IC_{50} > 1000 μg/mL and MIC *E. coli* > 1000 μg/mL.

In vivo Antimicrobial Activity of AEMPs

As a preliminary assessment of the *in vivo* topical treatment of *S. aureus* infections using AEMPs, we choose a cotton rat nasal *S. aureus* colonization model because of the clinic issues associated with nasal *S. aureus* colonization. The nasal cavity is the primary reservoir for *S.*

aureus, with approximately 30% of the human population being asymptomatically colonized.[64 65 66] Patients who are asymptomatically colonized are at significantly higher risk for several infections including bacteremia, post-operative infections, and diabetic foot ulcer infections. Most nosocomial *S. aureus* infections are caused by the patient's own *S. aureus* cells [67 68 69] and the nasal environment serves as a hotbed for drug-resistance development of *S. aureus*.[70 64 65 66] Treatment with the topical antibiotic mupirocin has proven to be effective at reducing nasal colonization and the risk of postoperative infection.[66 68] However the appearance of mupirocin resistance threatens this nasal eradication strategy.[71]

S. aureus strain BB2146[70] colonies were allowed to establish in the nasal passage for 3 days. After 3 days, the rats were given a nasal spray treatment consisting of either polymer ($P_{7.7}$ or P_{10}), mupirocin (antibiotic control), or PBS buffer (control). Mupirocin was added at a treatment concentration of 400 μg/mL, which is 2000 x MIC (MHB) (800 μM), ast his treatment concentration has been demonstrated to eliminate most *S. aureus* strains from the nose.[72] $P_{7.7}$ was added at a treatment concentration of 125 μg/mL. This concentration was used for treatment as it is higher than the MIC of $P_{7.7}$ against *S. aureus* BB2146 (MIC (MHB) = 63 μg/mL, MIC (50% FBS) = 3.9 μg/mL), and HEp-2 and COS-7 are tolerant to the polymer (the cell viabilities of > 90%), giving the maximum polymer concentration for the selective activity to *S. aureus* over mammalian cells. After three days, the animals were sacrificed and the number of *S. aureus* cells in each nasal passage were determined (Figure 2-13). Trial 1 involved 5 animals for $P_{7.7}$, 3 animals for mupirocin, and 3 animals for PBS buffer (control). Trial 2 involved 5 animals for P_{10}, 5 animals for mupirocin, and 5 animals for PBS buffer. The experiments for $P_{7.7}$ and P_{10} were performed independently and the combined data for these controls (PBS control and mupirocin) are presented below (Figure 2-13). All data from these two trials are provided in

Appendix D. As mupirocin has widespread use in the *S. aureus* nasal decolonization,[73 74] mupirocin provides a benchmark for the efficacy of antimicrobials.

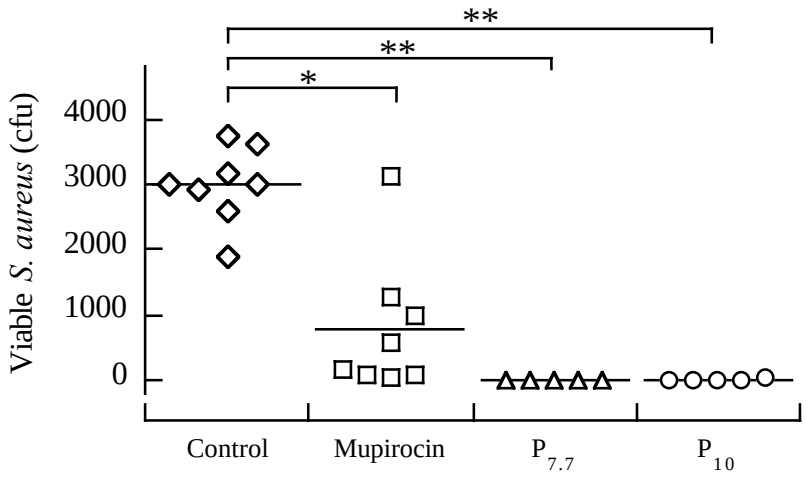


Figure 2-13. *In vivo* assessment in a cotton rat nasal *S. aureus* colonization model. *p < 0.001; **p < 0.0001. *In vivo* experiments were performed by Dr. Blaise R. Boles.

Mupirocin and $P_{7.7}$ reduced the number of viable *S. aureus* cells when compared to the PBS control and the difference in reduction between these two treatments is not statistically significant. Therefore, under these test conditions, $P_{7.7}$ is as effective at reducing the number of viable *S. aureus* cells as mupirocin. It should be noted that the treatment concentration of $P_{7.7}$ is about two times the MIC while mupirocin used a treatment concentration 2000 times the MIC, although lower concentrations of mupirocin may be effective.

AEMP P_{10} was also tested as a treatment for *S. aureus* nasal colonization (MIC for *S. aureus* BB2146 = 63 μg/mL in MHB and 2.0 μg/mL in 50% FBS) at the same polymer treatment concentration (125 μg/mL) as for the testing of $P_{7.7}$, although at this concentration the polymer indicated the significant cytotoxicity to HEp-2 cells (Figure 2-12A). P_{10} also showed a significant reduction in the number of viable *S. aureus* cells when compared to the PBS treatment control.

As a result of the *in vivo* tests, AEMPs demonstrate encouraging treatment outcomes of *S. aureus* in a nasal infection.

Conclusions

In summary, we investigated the *in vitro* and *in vivo* antimicrobial activity of cationic ammonium ethyl methacrylate polymers (AEMPs) with primary ammonium groups in the side chains with varying molecular weights. The AEMPs were active against Gram-positive bacteria when compared to Gram-negative bacteria although the polymers did not show a strong preference to any specific Gram-positive bacteria. The AEMPs also inhibited methicillin-resistant *S. aureus* with similar MIC values against the susceptible strain. The AEMPs also showed potent activity against *S. aureus* in the presence of FBS while their activity against *E. coli* was reduced, suggesting that the AEMPs may be active against *S. aureus* infections in physiological conditions. However, the antimicrobial mechanism of AEMPs and their targets in bacteria is not clear at this point. The AEMPs showed bactericidal activity against *S. aureus* with higher rates as compared to other antibiotics. AEMP $P_{7.7}$ showed similar resistance development as mupirocin (6 times original MIC after 14 passages). In a cotton rat nasal infection model, the AEMP $P_{7.7}$ significantly reduced the number of viable *S. aureus* cells. This AEMP was as effective at reducing the number of viable *S. aureus* cells as mupirocin when compared to the control without polymer treatment. These *in vivo* tests demonstrate the potential of cationic methacrylate homopolymers, particularly with primary ammonium groups, for use in the treatment of *S. aureus* infections. However more work is needed to better understand the antimicrobial mechanism of these polymers to aid in the design of more potent antimicrobials.

The development of antimicrobial polymers have been focused on the design of polymers with both cationic and hydrophobic groups in the side chains for the efficient disruption of bacteria cell membranes, which mimic the function of natural host-defense antimicrobial peptides. However, our study indicates that cationic homopolymer AEMPs without strong hydrophobic moieties can exert antimicrobial activity, and interestingly show potent anti-*S. aureus* activity in the presence of serum. Recent studies indicate that naturally occurring antimicrobial peptides exert their antimicrobial effects by binding to the multiple targets of cell walls, in addition to membrane disruption, which may be one of the components in the antimicrobial mechanism of peptides.[75] The AEMPs will serve as a simple model for the antimicrobial mechanism of cationic polymers as well as may capture the modes of action associated with cationic functionality in the bacterial cell walls. Although not investigated in this study, the antimicrobial activity of AEMPs is likely pH dependent due to the protonation of primary amine groups. We have previously demonstrated that chemical structures of ammonium groups (primary, tertiary, and quaternary) determine the binding of polymers to bacterial cell membranes and antimicrobial activity against *E. coli*.[76] [77] Tuning of cationic functionality of polymers would be of interest in the design of pH-responsive antimicrobial polymers as previously demonstrated using acidic groups.[78] Our finding may provide a new design strategy for antimicrobial polymers effective in physiological conditions and provide new insight into the mode of action of antimicrobial polymers with cell-selective activity.

Chapter 3 : Methacrylate Homopolymers as Cell Wall Targeting Antimicrobials

Introduction

The previous chapter investigated the antimicrobial activity of aminoethyl methacrylate homopolymers (AEMPs). AEMPs showed selective activity to Gram-positive over Gram-negative bacteria and potent activity against *S. aureus* in the presence of serum. However we were unable to identify if the primary antimicrobial mechanism of the polymers was membrane disruption due to limitations in assay conditions or to determine any other underlying mechanism or bacterial targets. As other cationic polymers, including BPEIs[30], oligolysines, and natural polysaccharide chitosan[79] have shown potent *S. aureus* activity without inducing significant membrane disruption. Instead, these polymers may act in the *S. aureus* cell wall by a previously uncharacterized mechanism. Similar in both cationic structure and antimicrobial activity, the AEMPs may also employ a similar antimicrobial mechanism that doesn't rely on the membrane disruption common to AMPs.

In the view of bacterial cell wall as antimicrobial target, Bacteria are surrounded by a complex cell envelope that performs a variety of essential functions.[80] Contrasts between the cell wall of Gram-positive and Gram-negative bacterial cells can be seen in Figure 3-1. While the cell envelope structure of bacteria varies, all cell envelopes contain layers of peptidoglycan (PG), a cross-linked matrix of linear carbohydrate (glycan) chains connected to each other via covalent bonds between attached peptides.[81] This peptidoglycan matrix is essential for survival, and in Gram-positive organisms it is densely functionalized with other glycopolymers.

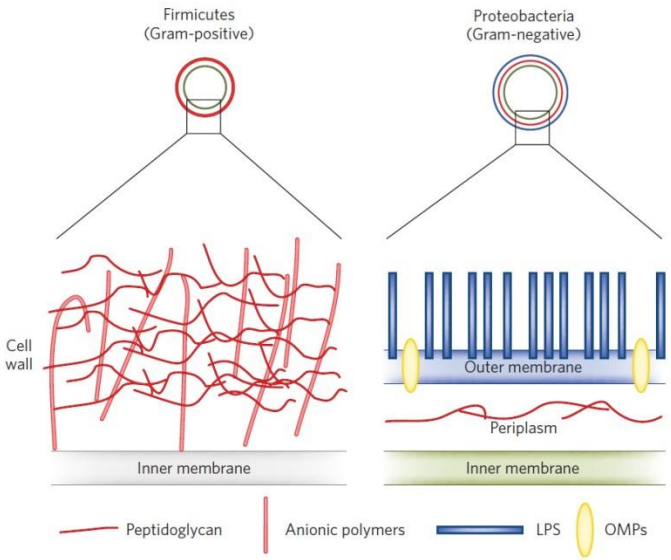


Figure 3-1. Comparison of Gram-positive and Gram-negative bacterial membranes. Adapted from Vollmer, W. *et al*. *FEMS Microbiol. Rev.* **2008**, *32*, 149-167.[82]

The main glycopolymers in Gram-positive organisms are teichoic acids. Teichoic acids describe the cell surface glycopolymers containing phosphodiester-linked polyol repeat units.[83] Teichoic acids present in bacterial cells include lipoteichoic acids (LTAs) which are anchored in the bacterial membrane via a glycolipid, and wall teichoic acid (WTA) which are covalently attached to the peptidoglycan (Figure 3-2).[84,83] Wall teichoic acids (WTAs) are the most abundant glycopolymer in Gram-positive organisms,[84] constituting up to 60% of the total mass of the cell wall.[85,86] The structure of *S. aureus* wall teichoic acids can be seen in Figure 3-3.

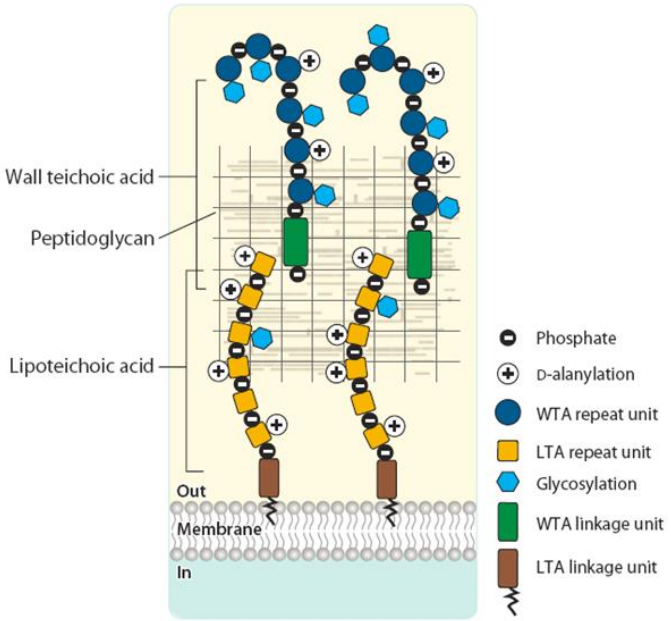


Figure 3-2. Teichoic acid polymers are located within the Gram-positive cell wall. Adapted from Brown, S. *et al. Annu. Rev. Microbiol.*, **2013**, *67*, 313-36.[87]

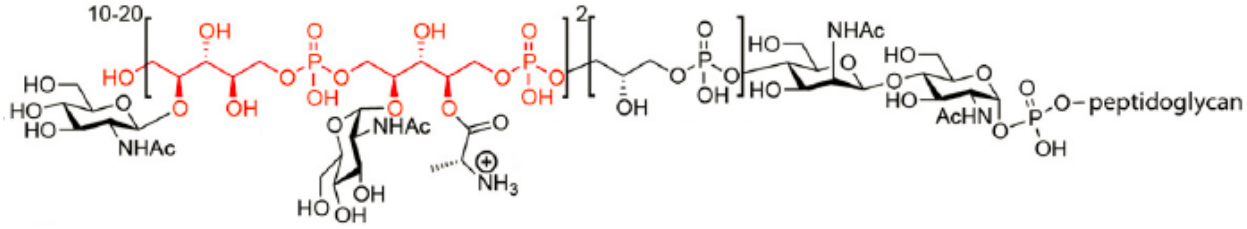


Figure 3-3. *S. aureus* wall teichoic acids (WTAs) are composed of ribitol-phosphate repeats tailored with D-alanines, α-*O*-GlcNAcs, and β-*O*-GlcNAcs. Taken from Brown, S. *et al, Proc. Natl. Acad. Sci. U.S.A.*, **2012**, *109*, 18909-18914.[88]

According to the results from the previous chapter and upon examining the cell wall structure of *S. aureus*, we hypothesize that (1) *electrostatic binding of cationic polymers to the anionic cell wall biopolymers would be the dominant factor in the mechanism of cationic methacrylate homopolymers*, and *(2) therefore, the polymers with quaternary ammonium groups would be more efficient in growth inhibition of S. aureus than their counterparts with primary*

ammonium groups because quaternary ammonium groups are not pH-dependent, providing higher charge density at physiological pH than primary ammonium groups. While quaternary ammonium compounds have previously demonstrated antimicrobial activity, investigation of these compounds usually pairs them with long alkyl chains which aim to disrupt bacterial cell membranes.[89 90 91]

The purposes of this study are to improve the activity of polymers against *S. aureus* as well as to probe the antimicrobial mechanism of these cationic polymers. In this study we prepared a series of cationic methacrylate homopolymers with either primary ammonium or quaternary ammonium groups in the side chains. To assess overall antimicrobial activity, the spectrum of activity of the polymers was determined. To assess the activity of AEMPs in physiological conditions, their antimicrobial activity in the presence of serum was also determined. As an initial assessment of their toxicity, the hemolytic activity of AEMPs was determined. Finally, to determine the role of electrostatic interaction between the polymers and *S. aureus* in the antimicrobial activity, polymers with primary ammonium or quaternary ammonium groups in the side chains were tested for their antimicrobial activity against a *S. aureus* cell wall mutant which has a higher net negative charge in the cell wall than the wild type.

Polymer Synthesis and Characterization

Synthesis of methacrylate homopolymers with primary ammonium groups.

Boc-protected amino ethyl methacrylate was prepared as previously described (see Appendix A).[27] Boc-protected polymers were prepared by free-radical polymerization using methyl 3-mercaptopropionate (MMP) as the chain transfer agent (Figure 3-4). The ratio of MMP to monomer concentration was varied to obtain polymers with a range of degree of

polymerization (DP). The polymerization proceeded at 65°C for 18 hours. The resulting boc-protected polymers were purified by precipitation into hexane. The boc-groups of the polymers were removed by treating the polymers with trifluoroacetic acid (TFA) to give polymers with primary ammonium groups. The polymers with primary ammonium will be identified as P_x where X is the DP of the polymer as determined by comparing the integrated intensities of the ^1H NMR resonances from the terminal group relative to the monomer side chain.

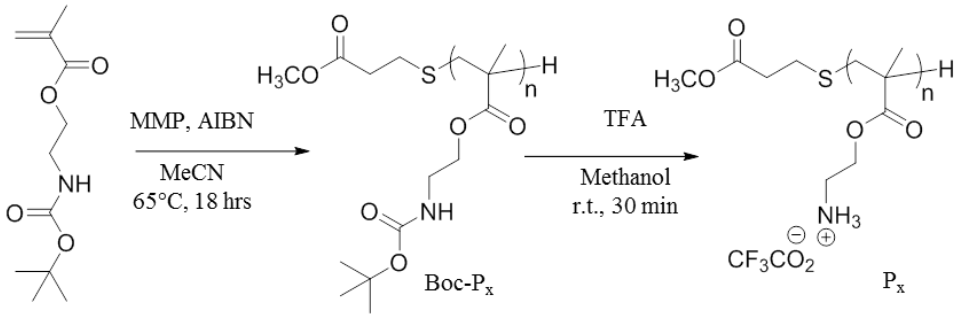


Figure 3-4. Synthesis of ammonium ethyl methacrylate homopolymers with primary ammonium groups.

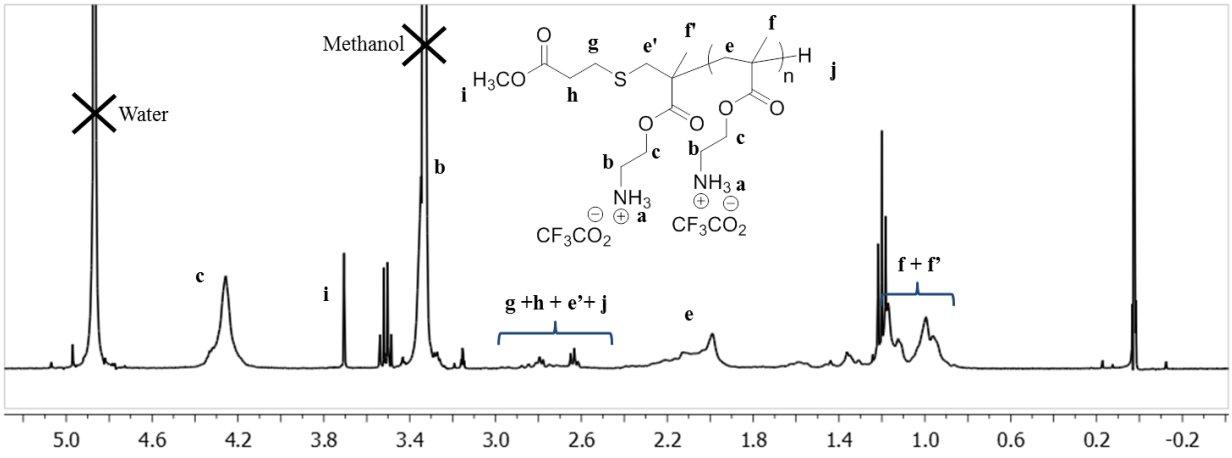


Figure 3-5. ^1H NMR spectrum of Polymer with Primary ammonium groups (400 MHz, Methanol-d4).

Table 3-1. Synthesis and Characterization of Polymers with Primary ammonium groups

	MMP (%)	Boc-Protected		Deprotected	
		DP	M_n	DP	M_n [a]
P_{115}	2	43	10,000	115	28,200 (15,100)
P_{92}	1	60	13,900	92	22,600 (12,100)
P_{27}	20	16	3,800	27	6,700 (3,600)
P_{24}	5	21	4,900	24	5,900 (3,200)
P_{17}	10	16	3,800	17	4,300 (2,300)

a) The number-average molecular weight (M_n) of deprotected polymers without trifluoroacetate. M_n of the polymers including trifluoroacetate are presented in parentheses.

Synthesis of methacrylate homopolymers with quaternary ammonium groups.

Ethyl methacrylate homopolymers with quaternary ammonium groups were prepared by the same method as described above for homopolymers with primary ammonium groups (Figure 3-6). The resulting polymers were purified by precipitation from methanol into diethyl ether. Polymers were then lyophilized to afford homopolymers bearing quaternary ammonium groups in the form of chloride salts. The polymers with quaternary ammonium side chains will be identified as Q_x where X is the DP of the polymer as determined by comparing the integrated intensities of the ^{1}H NMR resonances from the terminal group relative to the monomer side chain ^{1}H NMR.

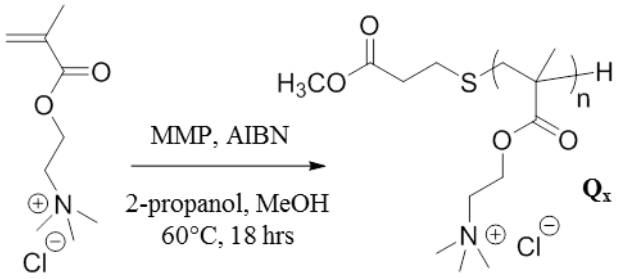


Figure 3-6. Synthesis of ammonium ethyl methacrylate homopolymers with quaternary ammonium groups.

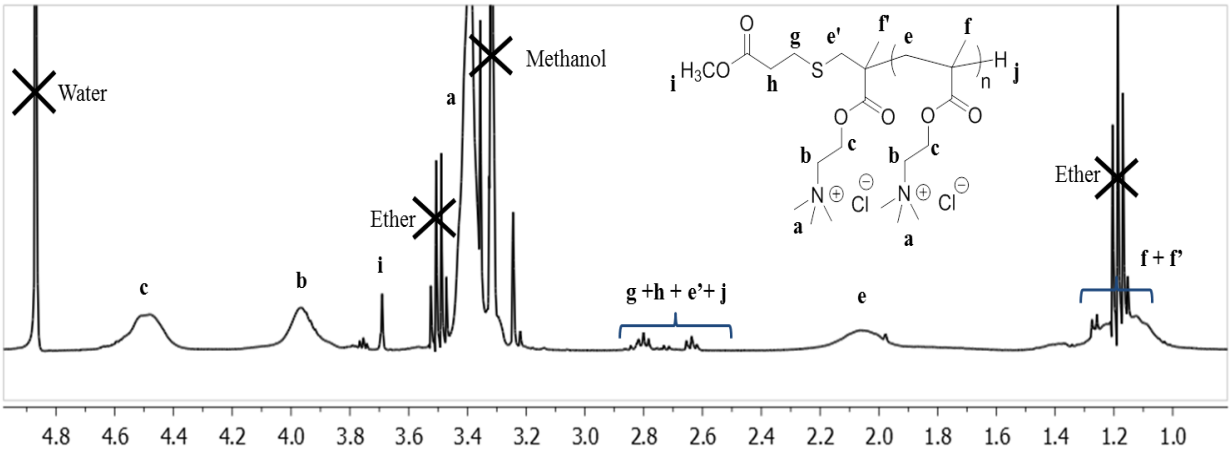


Figure 3-7. [1]H NMR spectrum of Polymer with quaternary ammonium groups.

Table 3-2. Synthesis and Characterization of Polymers with Quaternary ammonium groups.

	MMP (%)	DP	M_n
Q_{37}	9.4	37	7,800
Q_{32}	15	32	6,800
Q_{19}	5.6	19	4,100
Q_{11}	15	11	2,400
Q_{10}	3.7	10	2,200
Q_9	14	9	2,000

Antimicrobial Activity

The series of polymers with quaternary ammonium groups (Q-polymers) and the series of polymers with primary amine groups (P-polymers) were tested for their antimicrobial activity against a panel of clinically relevant bacterial pathogens. The antimicrobial activity of the prepared polymers was quantified as the minimum inhibitory concentration (MIC) in which bacterial growth is completely inhibited after an 18 hour incubation. The MIC was determined using a turbidity based microdilution method. [39] Both P-polymers and Q-polymers were more effective at inhibiting the growth of *S. aureus* strains when compared to other bacteria. The polymers were active against community-acquired methicillin-resistant *S. aureus* (CA-MRSA),

with similar MIC values against the susceptible *S. aureus* strain. The general trend of the polymers for lower activity towards Gram-negative bacteria may be, at least in part, due to the inability of polymers to penetrate the outer membrane of Gram-negative bacteria. This would prevent polymer access to the cell wall and cytoplasmic membranes, although the antimicrobial target of polymers in bacteria is not clear at this point. The P-polymers were not active against *E. faecalis* while the series of polymers (AEMPs) studied in Chapter 2 were active. It is not clear at this point why the results contradict each other. It could be due to the difference in the end groups of polymer chains, which have been reported to impact the antimicrobial activity of polymers.[24 92] The hydrophobic or hydrophilic nature of end-groups may affect the binding of polymers to bacterial cell walls and possibly cell membranes, resulting in different antimicrobial activities. To determine this, systematically changing the polymer's end groups would provide new insight into the role of the end groups of polymers on antimicrobial activity. It would be possible to incorporate different end groups such as varied lengths of alkyl groups using the corresponding RAFT agents.[92] Changing end groups of polymers likely results in changes to the polymers' hydrophobicity and subsequently changes their interaction with bacterial cell walls.

Table 3-3. The antimicrobial spectrum and hemolysis of polymers with primary and quaternary ammonium groups.

Polymer	MW	MIC (μg/mL) (or μM)[a]					
		Gram-positive bacteria			Gram-negative bacteria		
		S. aureus	*S. aureus* (CA-MRSA	*E. faecalis*	*E. coli*	*P. aeruginosa*	*A. baumannii*
P_{115}	28,200	47 (1.7)	59 (2.1)	708 (25)	219 (7.8)	188 (6.7)	188 (6.7)
P_{92}	22,600	39 (1.7)	26 (1.2)	>1000 (>44)	156 (6.9)	167 (7.4)	125 (5.5)
P_{27}	6,700	109 (16)	42 (6.3)	729 (109)	625 (93)	438 (65.4)	750 (112)
P_{24}	5,900	109 (19)	55 (9.3)	708 (120)	625 (106)	313 (53.1)	500 (85)
P_{17}	4,300	55 (13)	52 (12.1)	750 (174)	500 (116)	438 (102)	750 (174)
Q_{37}	7,800	20 (2.6)	8 (1.0)	>1000 (>128)	>1000 (>128)	271 (35)	nd
Q_{32}	6,800	22 (3.2)	12 (1.8)	>1000 (>147)	>1000 (>147)	500 (74)	nd
Q_{19}	4,100	23 (5.6)	10 (2.4)	>1000 (>244)	>1000 (>244)	>1000 (>244)	nd
Q_{11}	2,400	16 (6.7)	13 (5.4)	>1000 (>417)	>1000 (>417)	>1000 (>417)	nd
Q_{10}	2,200	29 (13)	10 (4.5)	>1000 (>455)	>1000 (>455)	688 (313)	nd
Q_9	2,000	26 (13)	16 (8.0)	>1000 (>500)	>1000 (>500)	>1000 (>500)	nd

a) MIC values are the average of at least two experiments, performed in triplicate. The MIC in μM is presented in parenthesis.
b) Community –associated methicillin resistant *S. aureus*

The MIC values of the P-polymers do not appear to significantly depend on the polymers' DP, similar to the polymers prepared by RAFT polymerization (Chapter 2). Both the P-polymers and Q-polymers showed lower MIC values for *S. aureus* than other bacteria. Furthermore, the activity of the Q-polymers likewise does not appear to be dependent on the DP or the number of cationic groups in a polymer. Similar to the P-polymers, Q-polymers are also selective to *S. aureus* over other bacteria. When compared to the P-polymers, the Q-polymers showed larger MICs for Gram-negative bacteria, indicating that they are less active towards these bacteria than

the P-polymers. While some of the P-polymers are active against *E. coli*, none of the Q-polymers are active against *E. coli*.

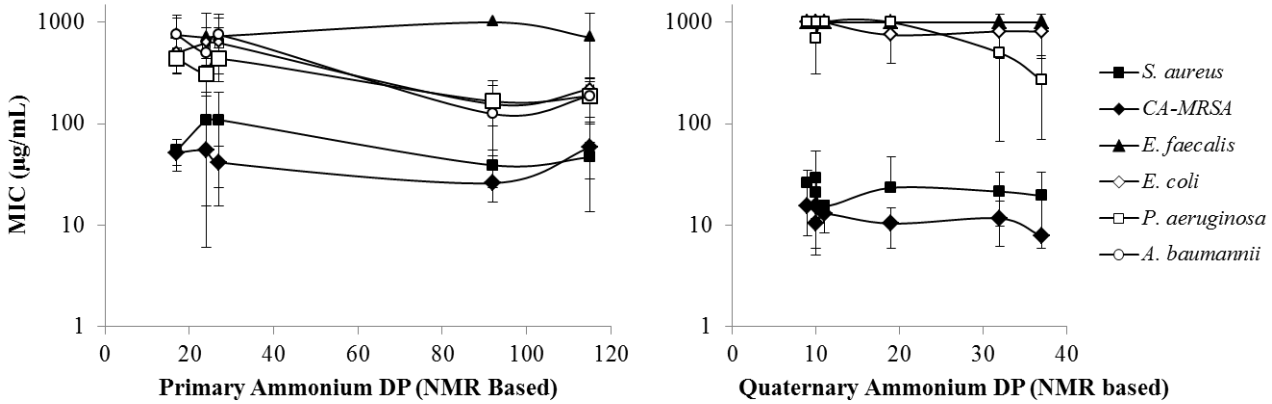


Figure 3-8. Activity spectrum of A) Polymers with Primary Ammonium groups (P-polymers) and B) Polymers with Quaternary Ammonium groups (Q-polymers).

The effect of the difference in the ammonium structures of the polymers (primary vs. quaternary ammonium groups) on their antimicrobial activity were compared more specifically by examining the MIC values of polymers P_{17} and Q_{19}, which have similar degrees of polymerization (DP) (Figure 3-9). Because of differences in molecular weights of these polymers, the comparison of MIC in µg/mL does not reflect the activity of each polymer chain. To end, the MIC values are given in µM (Table 3-3, Figure 3-9).

Against *S. aureus*, the polymers P_{17} and Q_{19} showed an MIC of 12.7 ± 3.6 µM and 5.7 ± 5.9 µM, respectively, indicating that there was no difference in the antimicrobial activity of P- and Q-polymers. The P-polymers and Q-polymers also show similar levels of antimicrobial activity against the other bacterial strains tested – *E. faecalis*, *E. coli*, and *P. aeruginosa*. Against *E. coli*, the polymers showed similar activity with the MIC of P_{17} = 116 ± 0 µM and MIC of Q_{19}

= 213 ± 61 μM. These MICs are on the same order of magnitude and are not considered to be significantly different.

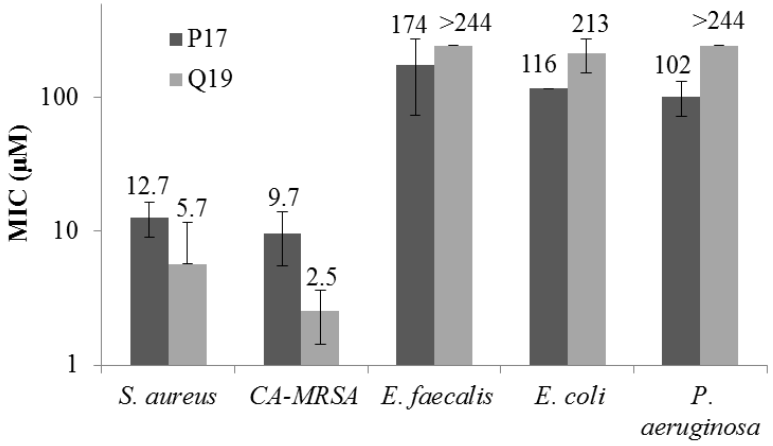


Figure 3-9. Minimum inhibitory concentrations of polymer with primary ammonium groups P_{17} and polymer with quaternary ammonium groups Q_{19} against selected bacteria. The MIC values are presented in μM.

These series of P and Q-polymers are likely to bind to the anionic phosphate groups of teichoic acids found in the *S. aureus* cell wall. Our laboratory has previously demonstrated that the primary amine groups of polymers have a higher binding affinity for the phosphate head groups of lipids when compared to Q-polymers. This is possibly due to combination of hydrogen bonding and electrostatic interaction between primary ammonium groups of polymers with phosphate groups of lipids, resulting in the formation of more stable complexes, as the quaternary ammonium groups are not capable of forming hydrogen bonds.[77] We would expect this relationship to be reflected in the polymers' MIC values, with the P-polymers being more active than the Q-polymers. Alternatively, quaternary ammonium groups are cationic at any pH, while not all primary amine groups are cationic (protonated) at neutral pH. From this trait, we would expect there to be stronger attraction between the bacteria and the Q-polymers due to the increased charged differential, resulting in a lower MIC (or more potent activity against bacteria)

for the Q-polymers over the P-polymers. However, the similarity in antimicrobial activities of P-polymers and Q-polymers demonstrated above do not clearly show which cationic polymer characteristic is more important for antimicrobial activity. Both polymer groups maybe equally attracted to and able to bind with the teichoic acids in *S. aureus*. However, the polymers did not show significant activity against Gram-positive *E. faecalis*, which also has teichoic acids in the cell wall. Teichoic acids may not be the only component involved in the polymers' antimicrobial mechanism. It is possible that the polymers have multiple targets – with teichoic acid being just one component of their antimicrobial mechanism. Alternatively, the polymers may target other intercellular targets such as lipid II, or processes synthesizing nucleic acid or DNA. [11]

Antimicrobial Activity in the Presence of Serum

To evaluate the activity of AEMPs in physiological conditions, the MIC values of AEMPs were determined in the presence of fetal bovine serum (FBS). It has been reported that the activity of antimicrobial peptides is reduced in the presence of serum because the serum salts curtain the electrostatic binding of cationic peptides to anionic bacterial surfaces and serum proteins such as albumin non-specifically bind to peptides.[41,42] Therefore, the activity of AEMPs could also be mitigated in the presence of serum.

AEMP activity was determined in 50% (v/v) FBS in MH broth (MHB). MHB components could non-specifically bind to the polymers, reducing the polymer activity. The reduction of these antagonizing factors in MHB by dilution with PBS buffer would increase their antimicrobial activity (decrease MIC values). To take this dilution effect into account, we used 50% PBS buffer in MH broth as a control.

Table 3-4. The antimicrobial activity of P_{20} and Q_{19} in the presence of fetal bovine serum.

Bacteria	Condition	MIC (μg/mL)				
		P_{17}	Q_{19}	CTAB	Mupirocin	Norfloxacin
E. coli	MHB	500	>500	7.8	nd	nd
	50% PBS	187.5	62.5	3.9	nd	nd
	50% FBS	> 500	>500	62.5	nd	nd
S. aureus	MHB	104.2	7.8	0.975	0.5	1.3
	50% PBS	26.0	2.93	0.975	0.5	1.0
	50% FBS	7.8	13	>7.8	>4.0	2.0

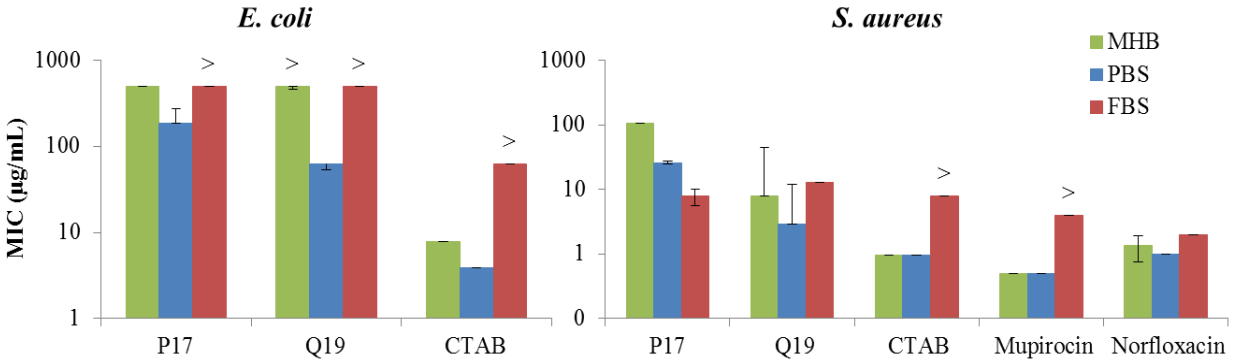


Figure 3-10. The effect of FBS on the activity of P_{17} and Q_{19} against *E. coli* and *S. aureus*.

Against *E. coli* both P_{17} and Q_{19} did not show any activity in the presence of FBS (MIC > 500 μg/mL), although neither polymer was especially active against *E. coli* in MHB. The MIC of the cationic surfactant CTAB also increased in 50% FBS, indicating serum components generally reduced the activity of cationic polymers and surfactants.

Interestingly, the effects of FBS on the MICs for *S. aureus* are different for P_{17} and Q_{19}. The MIC of P_{17} decreased in the presence of FBS (MIC = 7.8 μg/mL) when compare to its activity in MHB (MIC = 104.2 μg/mL) or PBS (MIC = 26.0 μg/mL), indicating an increase in the antimicrobial activity of P_{17}. We should note that the mechanism of activity enhancement of

the polymers with primary ammonium groups in FBS is not clear at this point. The MIC of P_{17} showed a 4-fold reduction in PBS/MHB, indicating that the MIC reduction of P_{17} in FBS is not due only to the effect of dilution of MH broth as described above. On the other hand, the MIC of Q_{19} appears to show little variance regardless of whether the polymer is in MHB (MIC = 7.8 μg/mL), PBS (MIC = 2.93 ± 0.98 μg/mL), or FBS (MIC = 13 ± 2.3 μg/mL). This indicates that serum components have little effect on the antimicrobial activity, and possibly the antimicrobial mechanism, of Q_{19}. For comparison, the MIC values of both the surfactant CTAB and the antibiotic mupirocin significantly increased in 50% FBS, indicating that FBS does not generally increase *S. aureus* susceptibility to antimicrobials.

Hemolytic Activity

As an initial metric of biocompatibility, the lysis of human red blood cells (hemolysis) by AEMPs was measured. In general, the Q-polymers showed no hemolytic activity while the P-polymers showed less than 40% hemolysis up to the highest concentration tested (Figure 3-12). The P-polymer (P_{17}) showed 38% hemolysis at 500 μg/mL, the highest concentration tested. It should be noted that other P-polymers, with different end groups, showed less than 5% hemolysis at 500 μg/mL (Chapter 2). It is known that deprotection of Boc groups by TFA produces carbocations in peptide synthesis, which may react with amino acid side chains including the aromatic rings of tryptophan and tyrosine, giving t-butyl compounds

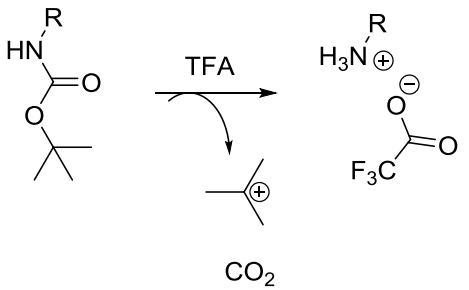


Figure 3-11 Boc deprotection by TFA

Although the polymers have no aromatic groups, the carbocation may also react with the polymer backbone or side chains. This side reaction would modify the polymers with t-butyl groups although the modification of polymers cannot be identified in ^{1}H NMR spectra. The t-butyl modification would be likely increase the hydrophobicity of polymers, which increase the hemolytic activity of polymers.

P-polymers were also slightly hemolytic, with polymers P_{92}, P_{27}, and P_{24} causing 13%, 2%, and 3% hemolysis at the same concentration. For comparison, Q_{19} showed 0.6% hemolysis at 500 μg/mL while Q_{37}, Q_{32}, and Q_{10} showing similar values (0.6%, 0.9%, and 0.5%, respectively) (Figure 3-12). This indicates that the P-polymers are slightly more potent hemolysins than the Q-polymers, although neither is as hemolytic as the bee venom toxin melittin (100% hemolysis at 33 μg/mL).

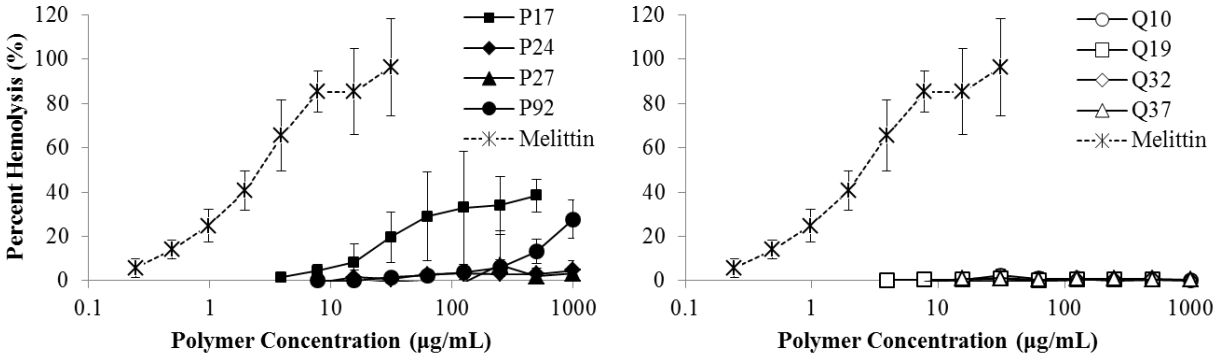


Figure 3-12. Hemolytic activity of AEMPs against human red blood cells, with the hemolysis by Triton X as 100%.

Activity against *S. aureus* Mutants

The results described above indicated that the polymer with primary ammonium groups P_{17}, as well as the quaternary ammonium polymer, Q_{19}, showed potent antimicrobial activity against *S. aureus.* Our central hypothesis is that *electrostatic binding of cationic polymers to the*

anionic cell wall biopolymers is the dominant factor in the mechanism of cationic methacrylate homopolymers. According to this hypothesis, a higher net negative charge density of *S. aureus* cell walls should increase the antimicrobial activity of cationic polymers. Accordingly, the antimicrobial activity of the polymers was assessed against *S. aureus* strain SA113 (parent) and a dltA strain (mutant) in the same SA113 background. DltA is a D-alanine-D-alanyl carrier protein that is responsible for the biosynthesis and degradation of murein sacculus and peptidoglycan.[93] The products of *dlt* genes catalyze D-alanylation which is necessary for the modification of both lipoteichoic acids and wall teichoic acids. [94,56,95] Alanylation adds positive charge onto the teichoic acids (Figure 3-3). The dltA mutant lacks this protein, and is no longer able to add positive charge to the teichoic acids, making the mutant cell more negatively charged than the wild type. It has been reported that *S. aureus* has become resistant to cationic antimicrobial peptides by this alanylation.[46] The alanyl esterification of the teichoic acids reduces the net anionic charge in the *S. aureus* cell wall,[47,48] which decreases the electrostatic binding of cationic polymers to the bacterial cell surfaces. Therefore, we expected that the cationic polymers would show higher activity against the mutant than the parent due to the higher net negative charge in the cell wall of mutant if electrostatic binding is a key determinant in the antimicrobial mechanism.

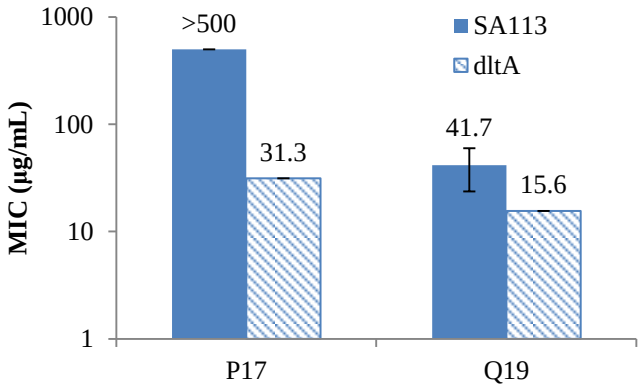


Figure 3-13. MIC of P_{17} and Q_{19} against *S. aureus* and bacterial mutants.

The activity of Q_{19} increased about 2.5 times from an MIC of 41.7 µg/mL against the SA113 parent to an MIC of 15.6 µg/mL against the dltA mutant. The activity of P_{17} increased approximately 16 times from an MIC > 500 µg/mL against the parent to an MIC of 31.3 µg/mL against the mutant. These results indicate that both polymers were more active against the dltA mutant than the SA113 parent strain (Figure 3-13). This suggests that an increase of net negative charge increases the activity of the polymers. This result supports the proposed mechanism that the electrostatic binding of cationic polymers to anionic cell walls is a key factor in the mechanism. Interestingly, although P_{17} did not show any significant activity against the parent SA113, P_{17} and Q_{19} are both active against the dltA mutant with similar MIC values.

Conclusion

In summary, we investigated the *in vitro* activity of series of polymers with primary (P-polymers) or quaternary ammonium groups (Q-polymers) in the side chains to determine the effect of cationic group on a polymer's antimicrobial activity. Both P-polymers and Q-polymers

were more active towards *S. aureus*, than the other bacteria tested (*E. faecalis*, *E. coli*, and *P. aeruginosa*). There was little difference in the activity between P-polymers and Q-polymers for the tested bacterial strains. Both polymers showed similarly potent activity in the presence of serum. P-polymers were somewhat hemolytic at higher concentrations (38% hemolysis at 500 μg/mL), while most P-polymers showed less than 10% hemolysis at the same concentration. Q-polymers were nonhemolytic, showing less than 0.6% hemolysis at the same concentration. The polymers were more active against the cell wall mutant which has a more negatively charged cell wall than the wide type. This result supports our hypothesis that electrostatic binding of cationic polymers to the anionic cell wall biopolymers is an important factor in the mechanism of cationic methacrylate polymers, although whether electrostatic binding is the only factor in the antimicrobial mechanism of these polymers remains to be proven. While we hypothesized that polymers with quaternary ammonium groups would be more effective against *S. aureus* than polymers with primary ammonium groups, this hypothesis is not supported by these results. Q-polymers showed similar, but not better, activity than P-polymers against *S. aureus* both in MHB and in FBS. While these assays failed to distinguish the differences, if any, in antimicrobial mechanism of the P-polymers and Q-polymers, additional research may reveal differences. It would be beneficial to determine if the polymers localize at different areas of bacterial cells through fluorescence imaging of dye-labeled polymers with bacteria. This would provide information on the bacterial structures where the polymers act. Furthermore, data on the bactericidal kinetics of each polymer would reveal if the polymers are effective on different time scales. This could then be correlated to the polymers targeting different cell processes including DNA replication, cell wall growth, and cell splitting, as each event occurs at a specific time in the bacteria's growth cycle. These results could help determine if the amine structure of the

cationic side chain has an impact on antimicrobial activity, or if just having a cationic charge is the most important characteristic.

Chapter 4 : Antimicrobial Action of Amphiphilic Copolymers Targeting Bacterial Cell Membranes

Background and Previous Results: Osmolysis of Red Blood Cells

To gain insight into a new design strategy for nontoxic antimicrobial polymers, our laboratory previously studied the mechanism of toxicity of amphiphilic copolymers (Fig. 4-1) to human red blood cells.

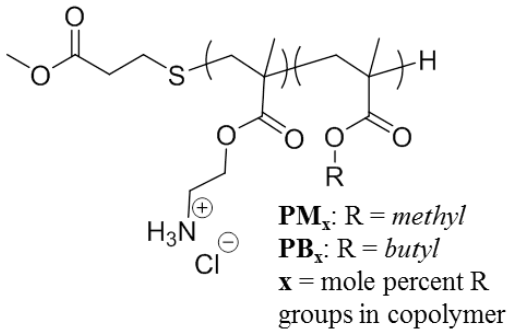


Figure 4-1. Structure of Amphiphilic Methacrylate Random Copolymers. A series of polymethacrylate copolymers with primary ammonium side chains and methyl or butyl hydrophobic side chains were synthesized by free-radical polymerization by Dr. Ed Palermo.

Colloid-osmotic lysis results when pores are formed in a membrane and there are differences in ions or other suspended particles (colloids) between the inside and outside of the membrane. Colloid-osmotic lysis of red blood cells (RBCs) is a common cause of hemolysis by many peptides,[96, 97, 98] bacterial toxins,[99, 100, 101] and synthetic polymers[102, 103]. Initially, these peptides and polymers produce pores in cell membranes which create an osmotic imbalance between the inside (cytosol) and outside of the cell. While these pores are too small to allow the efflux of macromolecules from the cytosol, including hemoglobin in red blood cells, they do allow for the

passage of small solutes such as water and ions. While these small solutes are allowed to establish equilibrium between the cytosol and surrounding environment, larger molecules are unable to equilibrate, as they are too large to fit through the formed pores. This results in the osmolarity of the cytosol exceeding that of the external buffer solution, which contains only small molecules. This colloid-osmotic imbalance (hypotonic condition) causes an influx of water through the small pores as the system attempts to dilute the macromolecules in the cytosol, resulting in cell swelling and compromise of membrane integrity. The loss of hemolytic activity of peptides or polymers upon the addition of large osmolytes such as high molecular weight PEG or "osmo-protectants" to the external media is evidence of a colloid-osmolytic mode of action, as these large compounds balance the colloid-osmotic pressure across the membrane.

My contribution to this project was to use an osmoprotection assay to determine if the lytic peptide melittin induced hemolysis by colloid-osmotic lysis as a result of pore formation. My secondary objective was to estimate pore size caused by melittin if pores were indeed formed. The hemolysis of melittin is concentration dependent, with higher concentrations of melittin resulting in more hemolysis. PEGs with molecular weights > 1000 protected RBCs against hemolysis by melittin (Figure 4-2 and Figure 4-3). PEGs with >1000 MW were able to protect the RBCs as their hydrodynamic sizes were larger than the pores in the membrane, reducing the osmolarity difference between the cytosol and the buffer solution. The result suggests that melittin caused colloid-osmotic hemolysis by forming pores with a diameter of ~ 2.0 nm in the cell membranes of RBCs which is in agreement with previous reports.[97]

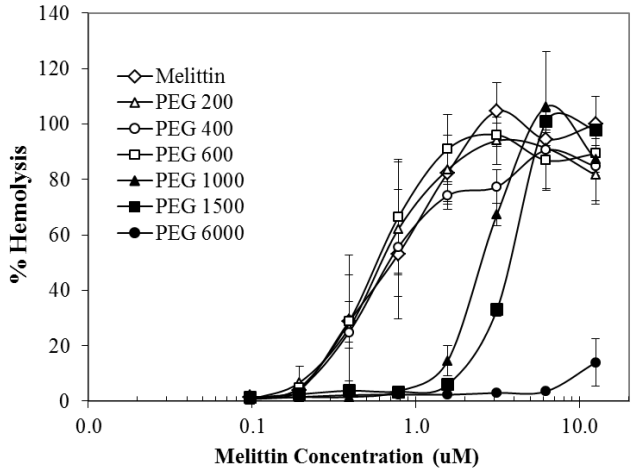


Figure 4-2. Osmoprotection against hemolysis induced by melittin.[104] Hemolysis was performed at least three times in triplicate in TBS, and determined relative to the positive lysis control Triton X-100 (0.2% v/v in water). PEGs were prepared in PBS buffer and added to give a final concentration of 30 mM in the assay.

The same assay method was used to determine the osmotic hemolysis by the cationic amphiphilic copolymers presented in Figure 4-1. All the assays were conducted by Dr. Iva Sovadinova, who is the first author of the published article.[104] Protection against hemolysis induced by PB_{27} started to be effective in the range of PEG MWs between 600-1000 (Figure 4-3B). At a polymer concentration of 31 μg/mL, the percent hemolysis decreased as PEG MW (and subsequently the hydrodynamic radius of the osmoprotectant) increased (Figure 4-3C). The same trend of decreasing hemolysis with increasing PEG MW was observed for melittin at a concentration of 4.5 μg/mL. PEG 600 and 1000 have hydrodynamic radii of 0.8 and 1.0 nm respectively in water,[105] indicating that PB_{27} produced pores 1.6 – 2.0 nm in diameter at a polymer concentration of 31 μg/mL. Similarly, PEG 1500 (2.4 nm in diameter) provided osmoprotection against hemolysis induced by PM_{63} while sucrose (0.92 nm diameter) did not

affect the hemolysis (Figure 4-3B). These results indicate that the polymers produce nanosized pores in the RBC membranes, leading to an influx of small solutes and osmotic lysis of RBCs.

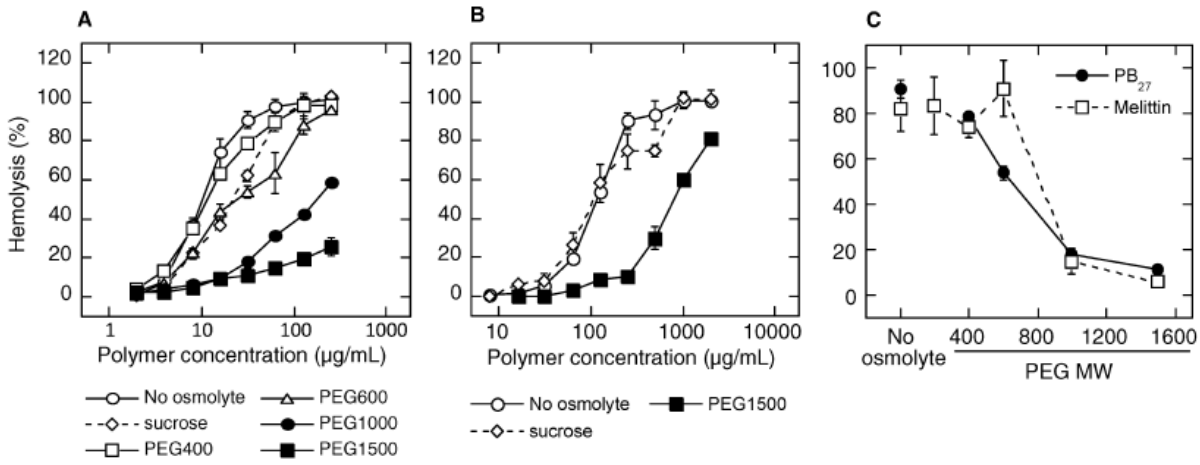


Figure 4-3. Osmoprotection against hemolysis induced by the copolymers and melittin. (A) PB_{27}, (B) PM_{63}, (C) PEG MW dependence of osmoprotection against hemolysis induced by PB_{27} at 31 μg/mL or melittin at 4.5 μg/mL. The assays were performed and previously reported by Dr. Iva Sovadinova.[104]

Current Efforts

Several models for the mechanism of antimicrobial peptides have been proposed, including pore formation, non-specific disruption, and lipid phase separation (Figure 1-3). Previous research in our laboratory, outlined above, demonstrated that cationic amphiphilic methacrylate random copolymers induce hemolysis by the formation of nano-sized pores (~1-2nm) in cell membranes, resulting in osmolysis.[104] It has been also previously reported that Another AMP mimic, a synthetic antimicrobial peptide (CM15) which is a hybrid AMP of cecropin A and melittin, exert its antimicrobial effect through the formation of nano-sized pores (2.2-3.8 nm) in bacterial cell membranes, followed by osmolysis.[106] Based on these results, *we hypothesized that*

the mechanism of antimicrobial action exerted by the amphiphilic copolymers involves membrane pore formation, followed by osmolysis.

To test our hypothesis, we used an osmoprotection assay described below, which determines the effect of osmolytes on the antimicrobial activity of polymers. We used a variety of cationic methacrylate polymers with primary ammonium side chains. A random copolymer, a cationic homopolymer, and a block copolymer were used to determine the effect of polymer architecture on the antimicrobial mechanism of polymers.

Polymer Synthesis and Characterization

A series of cationic amphiphilic copolymers containing primary amine groups as cationic functionality and ethyl groups as hydrophobic side chains were prepared by Dr. Enrico Nadres using RAFT polymerization. To determine if the amphiphilic structures of the polymers had any impact on its antimicrobial mechanism, the series of polymers included a random copolymer (EN-A), a homopolymer (EN-B), and a block copolymer (EN-C). Branched (BPEI) and linear (LPEI) poly(ethylene imine)s were also tested for comparison. These PEIs have been reported to show antimicrobial activity against *E. coli* and *S. aureus*.[30] Dr. Haruko Takahashi evaluated the antimicrobial and hemolytic activities of the RAFT polymers. The random copolymer EN-A showed potent activity, but also high hemolytic activity (HC_{50} = 6 μg/mL), indicating this polymer is not cell-selective. On the other hand, homopolymer EN-B did not show any activity against *E. coli*, but did show activity to *S. aureus*, in agreement with our previous results (Chapters 2 and 3). The block copolymer EN-C also showed similar selective activity to *S. aureus* over *E. coli*. Neither EN-B nor EN-C showed any significant hemolytic activity up to the

highest concentration tested (1000 µg/mL), indicating that these polymers are selective to *S. aureus* over *E. coli* and RBCs. The BPEI and LPEI are active against both *E. coli* and *S. aureus*.

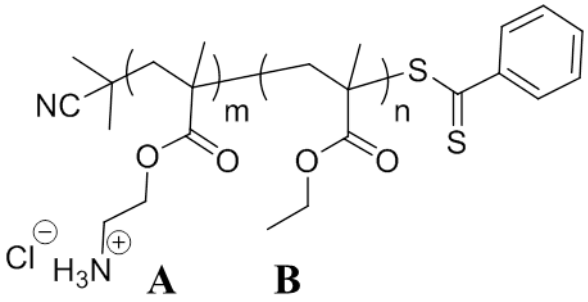


Figure 4-4. Structure of Amphiphilic Methacrylate Polymers. The polymers were previously syntheised by Dr. Enrico Nadres.

Table 4-1. Characterization and Activity of Amphiphilic Methacrylate Polymers and Controls.[a]

Polymer	%A	%B	DP[b]	Mn[c]	MIC (µg/mL) *E. coli*	MIC (µg/mL) *S. aureus*	HC_{50} (µg/mL)
EN-A	50	50	23.5	3,300	7.8	15.6	6
EN-B	100	0	26.2	3,600	>1000	62.5	>1000 (4.8%)
EN-C	46	54	80	9,900	>1000	62.5	> 1000 (30.9%)
BPEI	-	-	-	1,800	250[d]	32[d]	>4000 (2%)[d]
LPEI	-	-	-	2,500	31[d]	8[d]	565 ± 104[d]

a) The polymers were synthesized and characterized by Dr. Enrico Nadres. The MIC and HC_{50} were previously determined by Dr. Haruko Takahashi.
b) The degree of polymerization was determined by comparing the integrated intensities of the ^{1}H NMR resonances from the terminal RAFT agent group relative to the monomer side chain.
c) The number-average molecular weights (M_n) were calculated using DP and the molecular weights of monomer and RAFT agent based on the chemical structure of polymers.
d) Values taken from paper Gibney, K.A. *et al*.[30]

The Effect of Osmolytes on Antimicrobial Activity of Polymers

To determine if the polymers exert their antimicrobial activity by osmotic lysis of bacterial cells, the effect of osmolytes on the growth inhibition of bacteria by the polymers was determined. If the inhibitory effects of the polymers were antagonized by osmolytes or the bacteria were protected, an increase in MIC would be detected. As demonstrated for the

mechanism of polymer-induced hemolysis, if the polymers form nano-sized pores the increase in MIC would also depend on the size of osmolytes. In these experiments, the polymers were incubated with either *E. coli* or *S. aureus* in MH broth containing a 10 mM concentration of an osmolyte listed in Table 4-2. After incubation for 18 hours at 36°C, MIC was determined as the polymer concentration necessary to completely inhibit bacterial growth.

Table 4-2. Osmolytes and their hydrodynamic radii

Osmolytes	hydrodynamic radii (nm)[107]
glucose	0.72
sucrose	0.98
PEG 400	1.12
PEG 600	1.38
PEG 1000	1.78
PEG 1500	2.2
PEG 2000	2.54
PEG 4000	3.2

For both *E. coli* and *S. aureus* the presence of osmoprotectants in the growth media had little effect on the MIC value for any of the polymers tested (Figure 4-5). For *E. coli* the MIC of the random copolymer EN-A is 9.1 μg/mL in the presence of sucrose (r_h = 0.72 nm), and the MIC value remains the same even in the presence of the osmoprotectant with the largest hydrodynamic radius, PEG4000 (r_h = 3.2nm). MIC values were likewise maintained upon exposure to each osmoprotectant for the remaining polymers. Likewise, MICs against *S. aureus* in the presence of osmoprotectants seem to vary very little, regardless of hydrodynamic radius.

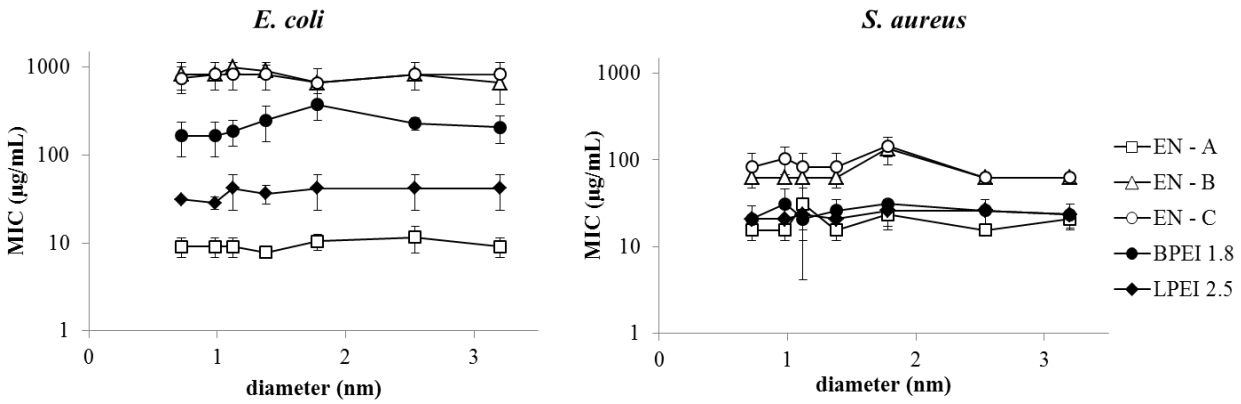


Figure 4-5. Osmoprotection against hemolysis induced by the polymers and PEIs.

If the chief antimicrobial mechanism of any of the polymers tested here were colloid-osmotic lysis, we would expect the polymers to show less activity in solutions of osmoprotectants with hydrodynamic radii larger than the pores formed by the polymer. As this assay result does not demonstrate any change in MIC in the presence of a variety of osmoprotectant solutions, these results seem to reject our hypothesis of antimicrobial activity as a result of colloid-osmotic lysis. It is possible that these polymers may disrupt bacterial membranes in a non-specific manner rather than through the formation of discrete membrane pores.

Another possibility is that these assay results were obscured by the contents of MHB. While performing the assay in MHB does enable us to observe the effect of osmoprotectants on the MIC of bacteria under a growing condition, MHB is comprised of many components including acid hydrolysate of casein, beef extract, and starch.[108] It may be advantageous to repeat this experiment in a buffer solution, such a phosphate buffered saline (PBS), where the exact environmental composition is known in case any components of MHB or PBS have an effect on the antimicrobial activity of the polymers in the presence of osmoprotectants.

Conclusion

Although previous studies have shown methacrylate copolymers causing colloid-osmotic lysis of RBCs, it does not necessarily follow that the polymers also cause colloid-osmotic lysis as their primary mechanism of antimicrobial activity. Varying the hydrodynamic radius of osmoprotectants in the system did not cause a significant change in the minimum inhibitory concentration of the polymers when tested against either *E. coli* or *S. aureus*. This assay rejects our hypothesis that the copolymers cause colloid osmotic lysis of bacterial cells. Going forward, it is important to clarify if the polymers exert their bactericidal activity through membrane disruption as a result of pore formation, or if another mode of action is more prominent. Since the polymers have different structures, a random copolymer, a cationic homopolymer, and a block copolymer, as well as different antimicrobial activities, we expected that they would show differences in their antimicrobial mechanism. It is possible that MHB obfuscated the results, as MHB consists of various ions and proteins that may interfere with the osmoprotection. To avoid this, the osmoprotection assay should be repeated in PBS buffer or another more controlled buffer. Further studies to reveal the antimicrobial mechanism, as presented in the conclusion, would enable us to better correlate the structure/ activity relationships of antimicrobial polymers.

Chapter 5 : Conclusions and Future Directions

At the outset of my research, the central hypothesis was that *cationic polymers would be a new synthetic platform for potent and selective anti-S. aureus agents*. These new anti-*S. aureus* agents would have limited toxicity and a antimicrobial mechanism that did not rely on membrane disruption. Toward that end, the antimicrobial activity and mechanism of cationic methacrylate homopolymers were investigated.

Chapter 2 examined the bacterial-strain specificity of cationic methacrylate homopolymers, as well as their suitability for *in vivo* treatment of *S. aureus* infections. The methacrylate homopolymers with primary ammonium groups in the side chains (ammonium ethyl methacrylate homopolymers, AEMPs) were more active against *S. aureus* than *E. coli* without causing hemolysis of red blood cells. They also showed potent activity against *S. aureus* in the presence of serum, suggesting that the polymers may be active against *S. aureus* infections in physiological conditions. The exposure of AEMPs to *S. aureus* bacteria resulted in the development of resistance in *S. aureus* (6 times increase in MIC), which was comparable that caused by the conventional antibiotic mupirocin. Under the same conditions, the exposure of *S. aureus* to norfloxacin resulted resistance development (64 times increase in MIC). The cationic homopolymer AEMPs without strong hydrophobic moieties can exert antimicrobial activity, especially in the presence of serum. In a cotton rat nasal infection model, the AEMPs significantly reduced the number of viable *S. aureus* cells when compared to the non-treatment control.

Chapter 3 discussed the antimicrobial mechanism of cationic methacrylate homopolymers, focusing on efforts to improve upon the electrostatic interaction between the cationic homopolymers and the anionic bacterial cell walls. To that end, the antimicrobial activity of methacrylate polymers with primary ammonium side chains (P-polymers) and polymers with quaternary ammonium side chains (Q-polymers) were examined. The hypothesis was that the polymers with quaternary ammonium groups would be more efficient in growth inhibition of *S. aureus* than their counterparts with primary ammonium groups because quaternary ammonium groups are not pH-dependent. While both polymer groups were more active towards *S. aureus* strains than other bacteria tested, there was little difference in activity between the P-polymers and the Q-polymers. As both groups of polymers were active against *S. aureus* in serum, the polymers could be used *in vivo* without losing antimicrobial activity. These polymers are both more active against a *S. aureus* mutant deficient in cell wall alanylation which has higher net negative charge in the cell wall than the wild type. This result supports our hypothesis that electrostatic binding of cationic polymers to the anionic cell wall biopolymer is an important factor in the mechanism of cationic methacrylate polymers. However, it remains unclear if electrostatic binding is the dominant factor in the mechanism. Our hypothesis that polymers with quaternary ammonium groups would be more effective anti-*S. aureus* agents was not validated, as the Q-polymers showed similar, but not better, activity when compared to P-polymers.

Chapter 4 examined the antimicrobial mechanisms of amphiphilic methacrylate polymers in relation to pore formation in bacterial cell walls and subsequent osmotic lysis. While amphiphilic methacrylate polymers have been shown to cause colloid-osmotic lysis of red blood cells, our experiments rejected the hypothesis that polymers cause pore formation and subsequent colloid-osmotic lysis in bacterial cells. While bactericidal activity through colloid-osmotic lysis was one

hypothesis for the antimicrobial activity of these polymers, many other possible modes of action exist. It would be beneficial to test other membrane permeabilization methods as well as methods targeting intracellular components and processes.

These results indicate that AEMPs can serve as a new design strategy for antimicrobial polymers effective in physiological conditions. While many other AMPs may lose their activity in serum, the antimicrobial activity of our polymer improves in serum, enabling lower dosing for effective treatment than other compounds. Considering their activity *in vitro* and preliminary *in vivo* investigations, it is reasonable to propose that these polymers have potential for applications in topical anti-infective creams, wound dressings, and implants or biomedical devices.

While these AEMPs have potential for use as polymeric antimicrobials, there are still problems and questions that need to be addressed. While the AEMP with the least number of cationic groups was the most nontoxic against HEp-2 and COS-7 cells, it would be ideal design a polymer with even lower toxicity. One approach to lower toxicity would be to incorporate non-toxic segments into the polymer structure. For example, the methacrylate could be copolymerized with polyethylene glycol (PEG) or another nontoxic polymer, ideally imparting more cytocompatibility to the cationic methacrylates. PEGs itself shows little or no antimicrobial activity, and direct incorporation into the polymer may have little effect on the polymer's antimicrobial activity if the number of cationic charges remain the same. However depending on the method of incorporation of PEG, spacing between cationic charges may change, which could have an effect on the antimicrobial activity. An optimal PEG/ cationic polymer structure would have to be determined. Another approach would be to decrease the number of cationic charges on the polymer chain. $P_{7.7}$, with 7.7 amine groups and possibly 7.7 positive charges depending on the pH of the environment, was the least nontoxic of the AEMPs

synthesized. It would be interesting to determine if P_6 or P_5 or smaller polymers, perhaps even oligomers, still retain their selectivity to *S. aureus* while decreasing or eliminating toxicity to HEp-2 cells. Previous studies have indicated that cytotoxicity is likely related to the spacing of the cationic charge of the polymer when it interacts with the mammalian cell. Spacing between cationic charges should be manipulated, both increasing and decreasing spacing, to determine the optimal spacing distance for antimicrobial activity and biocompatibility.

While *in vitro* experiments are an important tool to examine cytotoxicity, ultimately the commercialization of the drug relies on *in vivo* experiments. Although preliminary *in vivo* experiments were performed and no animals perished during treatment, more information about the pharmacokinetics or the movement of drugs within the body, of the antimicrobial is necessary before commercialization. Furthermore, we need to understand the bio distribution of the polymer to ensure dangerous complications do not arise from the use of this antibiotic.

Upon completion of this research project, it still remains unclear how the AEMPs exert their antimicrobial mechanism. We continue to hypothesize that our AEMPs act using an antimicrobial mechanism that does not cause membrane disruption. Cationic BPEIs have not demonstrated membrane depolarization, as previously discussed.[30] Furthermore, chitosan, a cationic natural polysaccharide active against *S. aureus* without causing membrane damage, likely impacts membrane-bound energy generation pathways that ultimate lead to cell death.[34]

The first steps in our investigation would be to confirm that AEMPs do not cause membrane disruption. Monitoring the potassium ion flux in suspensions of bacterial cells using a K^+-specific ion electrode would allow us to detect membrane impairment and leakage of cellular components upon exposure to different concentrations of AEMPs. Typical pore-formers (membrane disruptors) such as nisin typically show complete leakage of K^+ ions from bacterial

cells, while gradual or non-leaking compounds are not likely to have pore-formation as their primary mechanism of antimicrobial activity.[11] Further evidence about membrane disruption could be gathered by using liposomes as a simple model to observe the interaction between the AEMPs and model bacterial lipid membranes. We could encapsulate fluorescent compounds of various sizes in the liposomes and monitor leakage of fluorescent components upon exposure to various AEMP concentrations. If the AEMPs formed pores, we would expect an efflux of molecules smaller than the pores from the liposomes as the system works to reestablish equilibrium. We could compare results from AEMP/liposome interactions with liposomes exposed to pore-formers, such as nisin or melittin, to identify if AEMPs cause membrane disruption through pore formation.

If we confirm that AEMPs do not cause membrane disruption as we hypothesize, additional actions could help determine the polymers' antimicrobial mechanism. Due to the cationic charge of the AEMPs, interactions between the polymers and teichoic acids may be important. Preliminary investigations in Chapter 3 determined that the cationic homopolymers were more attracted to a *S. aureus* mutant deficient in alanination in the cell wall teichoic acid, a compound that normally makes *S. aureus* cell walls less negatively charged. It would be interesting to expand upon this research, testing the antimicrobial activity of the polymers against other mutants lacking one or more genes involved in the biosynthesis of teichoic acid. If the polymers do target teichoic acid, a negatively charged biopolymer, due to electrostatic interactions, we would want to confirm that the polymers are less active against a more positively charged *S. aureus* mutant – perhaps a mutant with a higher percentage of alanine. Monitoring the activity of the AEMPs against *S. aureus* without teichoic acids would reveal if teichoic acids are essential for the AEMPs' antimicrobial mechanism,[109] or if teichoic acid is just one of many components

the polymers target in bacterial cells. To get an even more detailed picture of the polymer's antimicrobial mechanism, analysis of the transcriptional response pattern through the use of genome-scale microarrays would identify the fine-tuned responses of bacteria to stress.[11] Identifying which genes are up or down regulated would give us an indication of the intracellular targets of these AEMPs. This information could be compared to the gene expression information of other antimicrobials, such as chitosan, to determine the relationship between antimicrobial mechanism and structure of the antimicrobial.

We hypothesize that amphiphilic copolymers are likely to act by membrane disruption. The amphiphilic copolymers are more structurally similar to membrane disrupting AMPs, having both hydrophobic and hydrophilic segments. Additionally, the amphiphilic random copolymers show a broad spectrum of activity, whereas the cationic homopolymers show more selective antimicrobial activity. It is likely that the increased hydrophobicity of the amphiphilic copolymer is responsible for this difference in antimicrobial activity, as the hydrophobicity enhances insertion of the polymers into the hydrophobic region of cell membranes. Osmoprotection assays in Chapter 4 rejected the hypothesis that the polymers caused colloid-osmotic lysis of bacterial cells. While it would be beneficial to repeat the assay in a more defined buffer such as PBS, experiments monitoring potassium leakage from cells as well as pore formation on liposomes could help identify the antimicrobial mechanism of random amphiphilic copolymers. Identifying the mechanism of both cationic homopolymers and random amphiphilic copolymers would better help us understand the relationship between a polymer's structure and its antimicrobial activity, providing scaffolds for future polymeric antimicrobial design.

Overall, this research demonstrated that AEMPs have potential for use as scaffolds in the design of strain-specific antimicrobials that are active *in vivo*. These AEMPs lack a distinct

hydrophobic segment found in many AMPs and synthetic polymers, but despite this omission they still are effective against bacteria. This work will provide inspiration for the development of other polymer antimicrobials with antimicrobial activity specific to target bacterial strains, and may eventually help stem the tide of the development of drug-resistant bacteria.

APPENDIX A

Materials and Methods

Materials

2,2'-Azobisisobutyronitrile (AIBN) was purchased from Sigma-Aldrich, and ethanolamine and di-tert-butyldicarbonate were purchased from Acros and used without further purification. Methacryloyl chloride was purchased from Acros and was freshly distilled prior to each use. Trifluoroacetic acid and reagent grade solvents were purchased from Fisher, and the bee venom toxin melittin (>85%) was purchased from Sigma. Poly(ethylene glycol) (PEG, MW = 2,000, cat. 81221, lot 1237799 14006162) was purchased from Sigma-Aldrich and branched PEI (BPEI, MW = 1800, cat. 06089, lot 559792) was purchased from Polysciences, Inc. (Warrington, PA, USA). The remaining PEGs were purchased from Acros Organics. Human RBCs (leukocytes reduced adenine saline added) were obtained from the American Red Cross Blood Services Southeastern Michigan Region and used prior to the out date indicated on each unit. Heat-inactivated fetal bovine serum (HI FBS), certified and of US origin (cat 10082-147, lot 1382252) was purchased from Life Technologies (Gibco). RAFT agent 2-cyanoprop-2-yl ethyl trithiocarbonate (CPETC) was synthesized as previously described.[25] For cytotoxicity testing, HEp-2 (ATCC® CCL-23) and COS-7 (ATCC® CRL-1651) cell lines were used. Gel permeation chromatography (GPC) was performed using a Waters 1515 HPLC instrument equipped with Waters Styragel® (7.8 x 300 mm) THF HR 0.5, THF HR 1, and THF HR 4 type columns in sequence and analyzed with a differential refractometer (RI) at 40°C in THF. Samples were dissolved in THF and passed through a 0.2 μm PTFE filter prior to analysis. The number-

averaged molecular weight (M_n) and weight-average molecular weight (M_w) were calculated using a calibration curve based on 10 standard samples of poly(methyl methacrylate), MW 500 – 50,000 (Agilent Technologies, M-L-10, No. PL2020-0100). ^{1}H NMR was performed using a Varian MR400 (400 MHz) and analyzed using VNMRJ 3.2 and MestReNova.

Monomer Synthesis

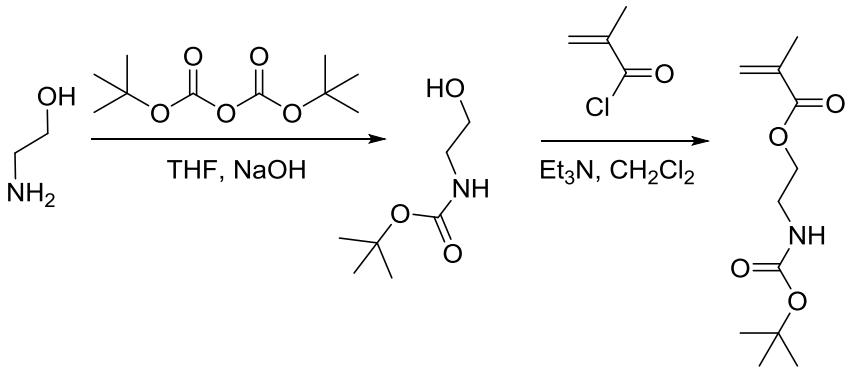


Figure A-1. Synthesis of monomer 2-(tert-butoxycarbonylamino) ethyl methacrylate

The monomer was prepared in the same procedure reported previously.[27] To a solution of ethanolamine (116 mmol, 7 mL) in a biphasic mixture of THF (150 mL) and NaOH (aq) (5M, 30 mL) in an ice bath di-tert-butyldicarbonate (118 mmol, 25.67 g) was added, and the mixture was stirred at room temperature overnight. The solution was concentrated under reduced pressure and the resulting N-Boc ethanol amine was washed with ethyl acetate, 10% citric acid, saturated $NaHCO_3$ (aq), and brine. The organic layer was dried over magnesium sulfate and filtered. Ethyl acetate was removed under reduced pressure. ^{1}H NMR (400 MHz, $CDCl_3$): δ1.367 (s, 9 H), 3.182 (t, 2H), 3.587 (t, 2H), 5.287 (bs, 1H).

Freshly distilled methacryloyl chloride (51.2 mmol, 5.0 mL) in dichloromethane (20 mL) was added dropwise to a solution of the N-Boc-ethanolamine (44.8 mmol, 7.22 g) and triethylamine (89.7 mmol, 12.5 mL) in dichloromethane (50 mL) at 0°C, and the mixture was allowed to stir

overnight at room temperature. The solution was then filtered, and the filtrate was concentrated under reduced pressure and washed with water, 10% citric acid, 10% K_2CO_3, saturated $NaHCO_3$ (aq), and brine. The organic layer was dried over magnesium sulfate, and filtered. The monomer 2-(tert-butoxycarbonylamino) ethyl methacrylate, was recrystallized from hexanes at -20°C to give a white solid in 70.3% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.44 (s, 9 H), 1.94 (s, 3 H), 3.43 (d, 2 H), 4.20 (t, 2 H), 4.77 (bs, 1H), 5.58 (s, 1 H), 6.12 (s, 1 H).

Polymer Synthesis

Synthesis of Methacrylate Polymers with Primary Amine Groups by RAFT polymerization

Reversible addition-fragmentation chain transfer (RAFT) polymerization of N-(tert-butoxycarbonyl)aminoethyl methacrylate (Boc-AEMA) was carried out using the RAFT agent 2-cyanoprop-2-yl ethyl trithiocarbonate (CPETC). Boc-AEMA (1.1947 g, 5.211 mmol) and AIBN (8.6 mg, 0.05211 mmol) were placed in a 20 mL flask. After evacuating and refilling the flask with nitrogen three times, dry toluene (total toluene volume 7.526 mL, including CPETC solution) and dry acetonitrile (5.211 mmol, 0.272 mL) were added to maintain a monomer concentration of 0.67M. The mixture was stirred at room temperature until contents were dissolved. Finally CPETC (0.5M in toluene) was added at 22, 10, or 3 mol % relative monomer (2.3, 1.05, 0.3 mL, respectfully). The reaction flask was then placed in oil bath at 80°C for 48 hrs with stirring. After 48 hrs, the polymerization was quenched by cooling the reaction mixtures in a dry ice/ ethanol bath. The percent conversion of monomer was determined by ^{1}H NMR by monitoring the disappearance of the hydrogens at δ 6.1 and 5.6 in $CDCl_3$. The polymerization was concentrated under reduced pressure and the polymer was then precipitated twice in hexane. The precipitates were collected by centrifugation and dried under high vacuum overnight to give Boc-protected AEMPs (1g, Yield 83%). Boc-protected polymers were characterized by ^{1}H NMR

and GPC. ^{1}H NMR (400 MHz, DMSO-d6) for $P_{7.7}$: δ 0.85 (d), 1.16 (s), 1.19 (s), 1.31 (bs), 1.62 (bs), 1.80 (m), 3.17 (bs), 3.85 (bs), 6.81 (bs). See APPENDIX B, or Figure 2-3, for peak assignment.

The Boc-protected polymers (1 g) were dissolved in methanol (0.5 mL). TFA (3 ml) was added to the polymer solution, and the reaction mixture was stirred at room temperature for 10 min. TFA and methanol was reduced by N_2 flushing, and the polymers were twice precipitated from methanol into diethyl ether. The precipitates were collected by centrifugation and dried under high vacuum. The resultant polymers were further dissolved in water (~ 10 mL) and frozen in a freezer (0°C) for at least 3 hours and a -80°C freezer overnight. These polymers were then lyophilized under high vacuum using Labconco's FreeZone® 1 Liter Benchtop Freeze Dry System (Model 7740021) overnight, affording a powder of homopolymers bearing primary ammonium groups in the form of trifluoroacetate salts (0.750, Yield 59%). The polymers were characterized by ^{1}H NMR. ^{1}H NMR (400 MHz, DMSO-d6) for $P_{7.7}$: δ δ 0.85 (d), 1.16 (s), 1.25 (s), 1.63 (bs), 1.91 (m), 3.12 (bs), 4.07 (bs), 8.27 (bs). See APPENDIX B for NMR spectra and peak assignment.

Synthesis of Methacrylate Polymers with Primary ammonium Groups by Free-radical polymerization

Free-radical polymerization of Boc-AEMA was carried out in acetonitrile using AIBN as the initiator and MMP as a chain transfer agent to control the average degree of polymerization (DP). Boc-AEMA (200 mg, 0.872 mmol) was dissolved in acetonitrile (0.6 mL) in a borosilicate glass test tube. MMP (x ml, x mmol, as a 1.0M solution in acetonitrile) and AIBN (87 µL of 0.1M solution in acetonitrile, 0.0087 mmol) were added. The test tubes were sealed with a rubber septa, and the solutions were deoxygenated with N_2 bubbling for 5 minutes. The tubes were then placed

in a 60 -70°C oil bath. After 18 hours, the mixtures, viscous yellow-brown oils, were removed from the oil bath. Dichloromethane (0.3 mL) was added to each tube to dissolve the polymer then the polymers were then precipitated twice in hexane. The precipitates were collected by centrifugation and dried under high vacuum overnight. Boc-protected polymers were characterized by ^{1}H NMR.

The Boc-protected polymers (1 g) were dissolved in methanol (0.5 mL). TFA (3 ml) was added to the polymer solution, and the reaction mixture was stirred at room temperature for 10 min. TFA and methanol was reduced by N_2 flushing, and the polymers were twice precipitated from methanol into diethyl ether. The precipitates were collected by centrifugation and dried under high vacuum. The resultant polymers were dissolved in water and lyophilized to afford homopolymers bearing primary ammonium groups in the form of TFA salts. The polymers were characterized by ^{1}H NMR.

Synthesis of Methacrylate Polymers with Quaternary Ammonium Groups by Free-radical polymerization

Living radical polymerization using methyl 3-mercaptopriopionate (MMP) as the chain transfer agent. 2-Methacryloxyethyltrimethylammonium chloride (100 mg, 0.481 mmol, 70% solution in water), AIBN (48 μL of a 0.1M solution in methanol, 0.00481 mmol), and MMP (9.02M in methanol) was added at 2, 5, 10, or 15 mol% relative monomer (2, 3, 5, 8 μL respectively). The reaction vessel was purged with N_2 and then placed in an oil bath at 60°C for 18 hours. After 18 hours, a white gel had formed. The polymers were twice precipitated from methanol into diethyl ether. The precipitates were collected by centrifugation and dried under high vacuum. The resultant polymers were dissolved in water and lyophilized to afford homopolymers bearing

quaternary ammonium groups in the form of chloride salts. The polymers were characterized by ^{1}H NMR.

Antimicrobial Assay

The minimum inhibitory concentration (MIC) of polymers was determined by a turbidity-based microdilution assay according to the procedure reported previously.[27] Assays were performed in Muller Hinton (MH) broth for all bacteria. *Escherichia coli* (ATCC® 25922), *Staphylococcus aureus* (ATCC® 25923), *Enterococcus faecalis* (ATCC® 29212), *Pseudomonas aeruginosa* (ATCC® 27853), *Staphylococcus saprohyticus* strain 710826, *Bacillus subtilis* (ATCC® 6633), *Acinetobacter baumannii* (ATCC® 17978), *S. aureus* strain LAC (MRSA USA300-0114)[48] and *S. aureus* strain BB2146[70] were used. Each polymer was dissolved in DMSO, and this DMSO solution was diluted by 0.01% acetic acid to give 2-fold serial dilutions. An overnight bacterial culture was prepared by taking 2-3 bacterial colonies from a petri dish and placing them into 7 mL of MHB with shaking at 37°C. After overnight growth, the bacterial culture was diluted by MH broth (OD_{600} = 0.1) and incubated at 37°C until the bacteria reached the mid-log growth phase, OD_{600} = 0.5-0.6 (1.5 -4.5 hours depending on bacterial strain). The bacteria culture in the midlog growth phase were diluted to the final concentration of OD_{600} = 0.001, which contains approximately 5 x 10^5 cfu/mL. This stock (90 μL) was mixed with a polymer solution (10 μL) in a 96-well polypropylene microplate (Corning #3359). After incubating for 18 h at 37°C, the plate was examined visually to determine bacterial growth as the formation of bacterial palettes or increases in solution turbidity. The MIC was defined as the lowest polymer concentration at which no turbidity increase was observed for at least 2 out of 3 wells relative to the negative control, broth. As an additional negative control, 2-fold serial dilutions of DMSO, without polymer, were tested in the same conditions and showed no inhibitory effects, even at the highest

DMSO concentration (10%). All assays were performed in triplicate at least three independent experiments. The polymers in MH broth without bacteria did not show any precipitation, as their solubility was determined to be > 1000 μg/mL.

Membrane Depolarization Assay

Cytoplasmic membrane disruption was evaluated for polymers against *S. aureus* using the membrane potential sensitive dye $DiSC_3(5)$. A single colony of *S. aureus* was inoculated in MHB for 18h at 37°C, and mid-logarithmic phase cells (OD_{600} = 0.5 – 0.6) were collected. Cells were then resuspended in buffer (5 mM HEPES, 5 mM sucrose, 100 mM KCl, pH 7.2) to OD_{600} = 0.05. A stock solution of $DiSC_3(5)$ in ethanol was added to *S. aureus* suspension (3 mL). The final dye concentration is 0.5 μM. The cell suspension with $DiSC_3(5)$ (0.5 μM) was stirred at room temperature until the stable reduction in fluorescence intensity was achieved due to quenching upon accumulation of dye on the *S. aureus* membrane. At 100 s, a solution of melittin (7.3 mg/mL, 5 μL) or polymer (various concentrations, 5 μL) in HEPES buffer was added to the bacterial suspension to give the final concentration (0.5 – 8x MIC). The fluorescence intensity was monitored with excitation and emission wavelengths of 622 and 670 nm, respectively. Finally, melittin was added after 200s to a final concentration of 2x MIC. As a control, the changes in fluorescence intensity of dye and polymers mixtures in buffer were determined. As a control, the changes in fluorescence intensity of $DiSC_3(5)$ and polymer mixtures in the absence of bacteria were determined

Cell Leakage Assay

Leakage of UV-absorbing cellular components from *S. aureus* and *E. coli* upon treatment with $P_{7.7}$ was measured as a measure of membrane disruption by the polymer. The bacteria were regrown in MHB to give an OD_{600} > 0.8. The bacterial cells were harvested, and the pellets were

washed with PBS. The cells were then resuspended in PBS buffer. Cells were then incubated with the compound of interest ($P_{7.7}$, lysostaphin, melittin, CTAB, or PBS for control) with shaking at 37°C. 2 mL of solution was removed at 2 hours, and centrifuged at 14,000 rpm for 10 minutes to afford cell-free supernatant. The absorbance of cell-free supernatant was measured at 260 and 600 nm.

Antimicrobial Assay in Fetal Bovine Serum

The minimum inhibitory concentration (MIC) of the AEMPs in the presence of fetal bovine serum (FBS) was determined as described above with modifications. Bacteria were grown overnight, diluted and regrown in MH broth at 37°C until the bacteria reached the mid-log growth phase. The bacteria culture in the midlog growth phase were diluted to the final concentration of OD_{600} = 0.00225. This stock (40 μL) was mixed with a polymer solution diluted in 0.01% acetic acid as for MIC (10 μL) and FBS or PBS (50 μL, 50% of total volume), and bacteria in MHB (40μL, OD_{600} 0.00225) in a 96-well polypropylene microplate. After incubating for 18 h at 37°C, the plate was examined visually to determine bacterial growth as the formation of bacterial palettes or increases in solution turbidity. MIC was determined as described above.

Hemolytic Assay

Toxicity to human red blood cells (RBCs) was assessed by a hemoglobin release assay. RBCs (1 mL) were diluted into PBS (9 mL) and then centrifuged at 2000 rpm for 5 min. The supernatant was carefully removed using a pipet. This procedure was repeated two additional times. The resulting stock (10% v/v RBC) was diluted 100x in PBS and the number of red blood cells was counted using a hemocytometer. The original stock (10% v/v) was then diluted to 1.11×10^8 RBC/mL with PBS. This RBC suspension (90 μL) was then mixed with each of the polymer serial dilutions (10 μL) on a sterile 96-well round-bottom polypropylene microplate (RBC

concentration on plate is 1.0 x 10^8 RBC/mL). PBS (10 μL) or Triton X-100 (10 μL, 1% v/v) were added instead of polymer solution as negative and positive hemolysis controls, respectively. The microplate was incubated at 37°C in an orbital shaker at 180 rpm for 60 min. The plate was then centrifuged at 1000 rpm for 5 minutes. The supernatant (6 μL) was diluted into PBS (110 μL) in a 96-well flat-bottom polystyrene microplate (Corning #3370) and the absorbance at 405 nm was recorded using a microplate reader (Perkin-Elmer Lambda Reader). The fraction of hemolysis was defined as $H = (A - A_0)/(A_{TX} - A_0$where A is the absorbance reading of the sample well, A_0 is the negative hemolysis control (PBS buffer), and A_{TX} is the positive hemolysis control (Triton X-100). Hemolysis was plotted as a function of polymer concentration and the HC_{50} was defined as the polymer concentration which causes 50% hemolysis relative to the positive control. The data were obtained in at least three experiments performed in triplicate.

Bactericidal Kinetics

Bactericidal kinetics was determined by inoculation of the polymers with bacteria in MH broth and colony counting on agar plates. An overnight culture of *S. aureus* ATCC® 25923 was regrown to the exponential growth phase (OD_{600} of 0.5 to 0.6) and diluted to an OD_{600} 0.001 to give 8 mL of bacterial solution in a 15 mL centrifugation tube. To this solution, the polymer solution was added to a final concentration of two times the MIC value of each polymer. The tubes were incubated at 37°C in an orbital shaker at 180 rpm, and aliquots of solution (100 μL) were drawn at appropriate time intervals. After dilution by 10^5, 10^4, 10^3, 10^2, or 10-fold into PBS buffer, the dilutions were streaked onto agar plates and incubated at 37°C overnight. The colonies were then counted to determine the number of viable cells as colony-forming unit (cfu)/mL in the polymer solution. The data and errors are the average and standard deviation of two experiments, each performed in doublet (n = 4).

Resistance development in *S. aureus*

The first MIC determination of AEMPs and two antibiotics Norfloxacin and Mupirocin against *S. aureus* ATCC® 25923 was performed as described above. Bacteria samples from triplicate wells (60 µL) at the concentration of one-half the MIC were removed, combined, and added to fresh MH broth (1.5 mL). The bacterial culture was regrown at 37°C to the mid-log growth phase (OD_{600} = 0.5 – 0.6). This culture was used to determine the MIC values of polymers again as described above. This experiment was repeated for 14 successive passages. This was repeated, for a total of two resistance experiments.

Cell Culture

Cytotoxicity experiments were carried out using the HEp-2 cell line and the COS-7 cell line. HEp-2 cells are human epithelial cells isolated from larynx carcinoma. It should be noted that the HEp-2 cell line is contaminated with HeLa cells derived from cervical cancer. HEp-2 cells were grown in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), pyruvate (1 mM), and nonessential amino acids (0.1 mM) at 37°C, 5% CO2, and 95% relative humidity. The doubling time of the HEp-2 cells in this supplemented medium is about 22-24 h. COS-7 cells are fibroblast-like cells derived from monkey kidney tissue. COS-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, high glucose (25 mM), L-glutamine (4 mM), sodium pyruvate (1 mM) at 37°C, 5% CO2, and 95% relative humidity. The doubling time of the HEp-2 cells in this supplemented medium is about 35 – 48 hours.

XTT Cell Viability Assay

HEp-2 cells or COS-7 cells were seeded into the 96-well cell culture plates (Falcon #3072, USA) at a density of 1 x 10^4 cells per well. After 20 h incubation, 24 h for COS-7 cells, the cell

confluence was about 50-60%, and the cell culture medium was replaced with serial dilutions of polymer stock solutions in an antibiotic- and scrum-free MEM. The viability of cells exposed to the polymers was assessed using a commercial kit (Cell Proliferation Kit II, Roche, USA). After a 24 hr exposure to polymers, the cells were washed once with PBS and then PBS (100 μL) was added to each well. A solution of sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis-(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) and an electron-coupling reagent N-methyl dibenzopyrazine methyl sulfate (PMS) were added to each well to give the final concentration of 0.2 mg/mL and 1.5 μg/mL, respectively. To assess the effect of polymers on the conversion of substrate, control wells containing only PBS and XTT with PMS were included. After a 4 hr incubation for HEp-2 cells or 6 hr incubation for COS-7 cells at 37°C in the presence of 5% CO_2, the formation of orange colored formazan derivative produced by the metabolic cellular activity was determined by the absorbance at 450 nm (test wavelength) and 650 nm (background wavelength). The spectrophotometer was calibrated to zero absorbance using PBS without cells. The cell viability was determined relative to that of a control containing intact cells, which were exposed only to solvent. The data are means and standard errors of 3 independent experiments performed in triplicate (n = 9).

In vivo Animal Testing

The cotton rat nasal colonization model described by Kokai-Kun was utilized to determine the ability of polymers to decolonize *S. aureus* from the nasal environment.[110, 70] Non-transgenic cotton rats (strain Hsd) were purchased from Harlen Laboratories. The protocol was approved by the Committee on Use and Care of Animals (UCUCA) of the University of Michigan (Permit Number: 10394). *S. aureus* (strain BB2146)[111] was grown overnight in TSB, harvested by centrifugation, washed and resuspended in phosphate buffered saline (PBS). Cotton rats (3 - 5 for

each condition) were anesthetized, and a 10-µl aliquot containing 1 x 10^8 colony forming units (CFUs) were intranasally instilled drop-wise equally between the two nostrils. After 3 days the animals were intranasally treated with an AEMP ($P_{7.7}$ or P_{10}) at 125 µg/mL or mupirocin at 400 µg/mL (MIC = 0.2 µg/mL). Three days later the animals were sacrificed, the noses surgically removed, and *S. aureus* CFUs were determined. As a negative control, animals were exposed to PBS without polymer. Mupirocin was used as a benchmark for effective decolonization. The experiments for $P_{3.1}$ and $P_{5.5}$ were performed independently along with mupirocin and PBS controls, and the data for these controls were combined and used for statistical analysis.

Osmoprotection Assay

To estimate the functional diameter of the pores formed by the polymers, a MIC assay was performed in the presence of sucrose and polyethylene glycols (PEGs) with different molecular weights (400 – 4000). Sucrose and PEGS were prepared in PBS buffer and added to give a final concentration of 10 mM in the assay. Higher concentrations of PEG were not used because of possible difficulties concerning solubility and the viscosity of the assay media.

Statistical analyses

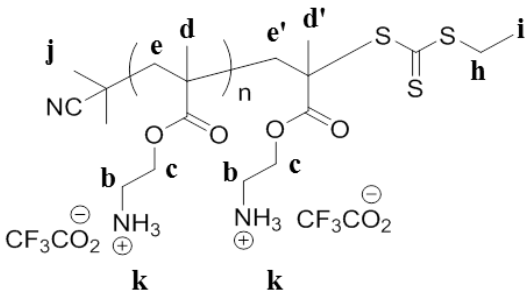
Statistics were performed using Student's t-test. Results are expressed as mean ± standard deviation, unless otherwise indicated.

APPENDIX B

Polymer Characterization

^{1}H NMR Characterization of Homopolymers synthesized by RAFT polymerization

Figure B-1 shows the chemical structure of the cationic methacrylate homopolymers and their ^{1}H NMR peak assignments presented in Chapter 2. The NMR spectra of both the Boc-protected polymers as well as the deprotected polymers are shown below.



ppm	peak	Assignment
8.5	bs	k
4.07	bs	c
3.12	s & bs overlap	b + h
1.9	mult	e
1.63	s	d'
1.33	s	i
1.19	t	j
0.86	d	d + d’

Figure B-1. ^{1}H NMR peak assignments for polymers with primary ammonium side chains synthesized by RAFT polymerization.

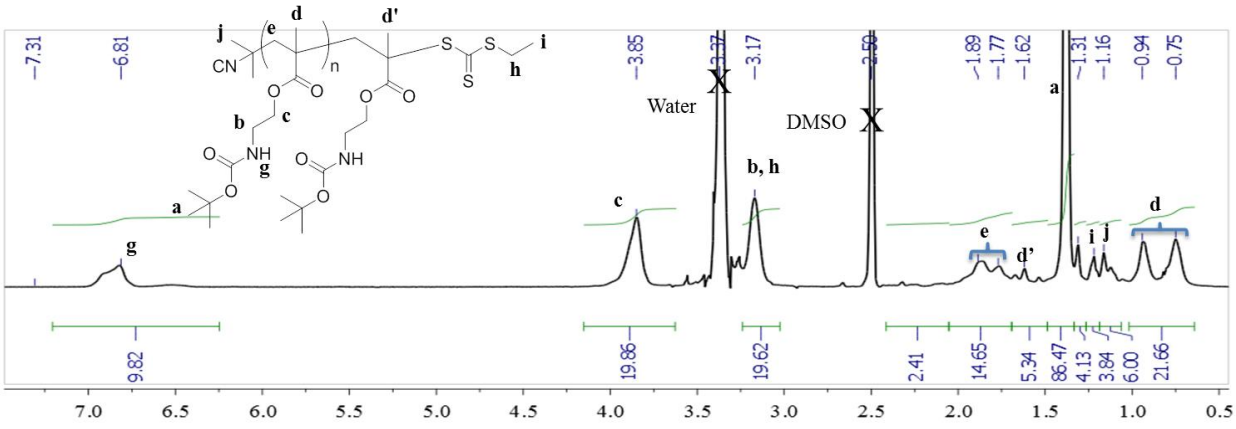


Figure B-2. ^{1}H NMR of Boc-$P_{9.9}$ (DMSO-d6)

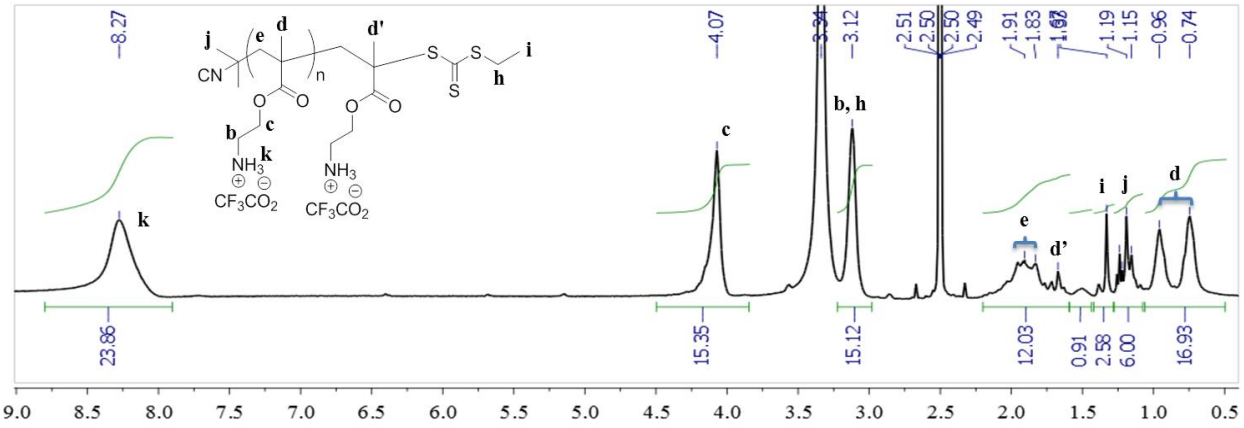


Figure B-3. ^{1}H NMR of $P_{7.7}$ (DMSO-d6)

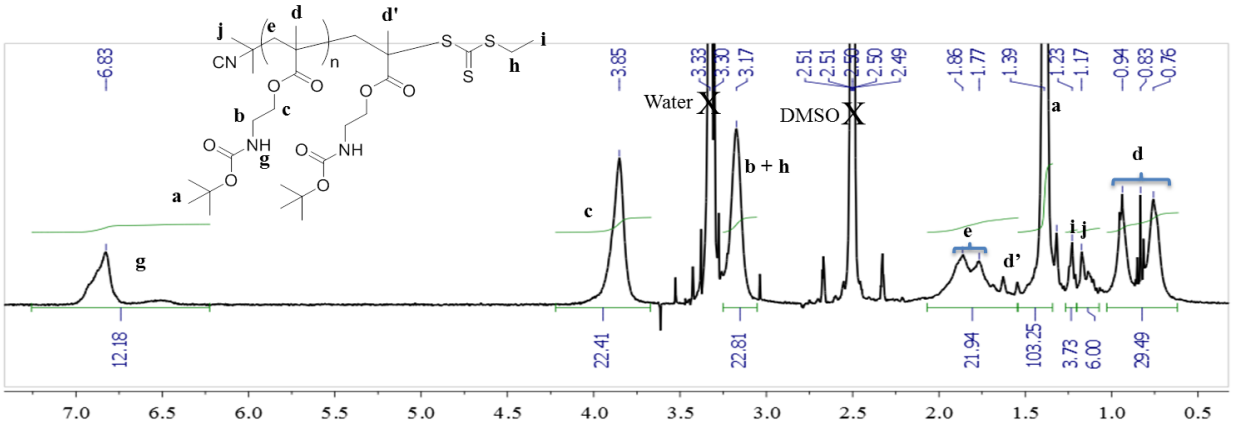


Figure B-4. ^{1}H NMR of Boc-P_{11} (DMSO-d6)

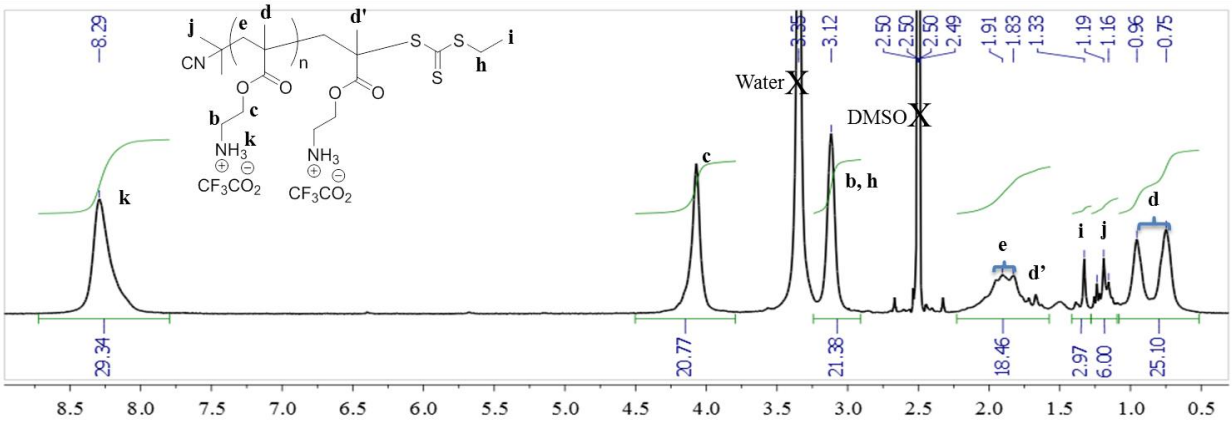


Figure B-5. ^{1}H NMR of P_{10} (DMSO-d6)

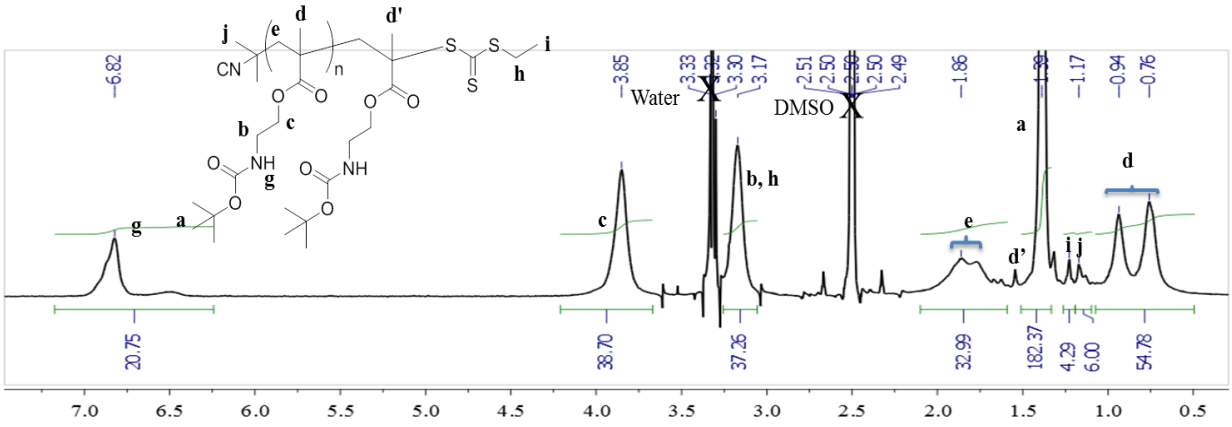


Figure B-6. ^{1}H NMR of Boc-P_{19} (DMSO-d6)

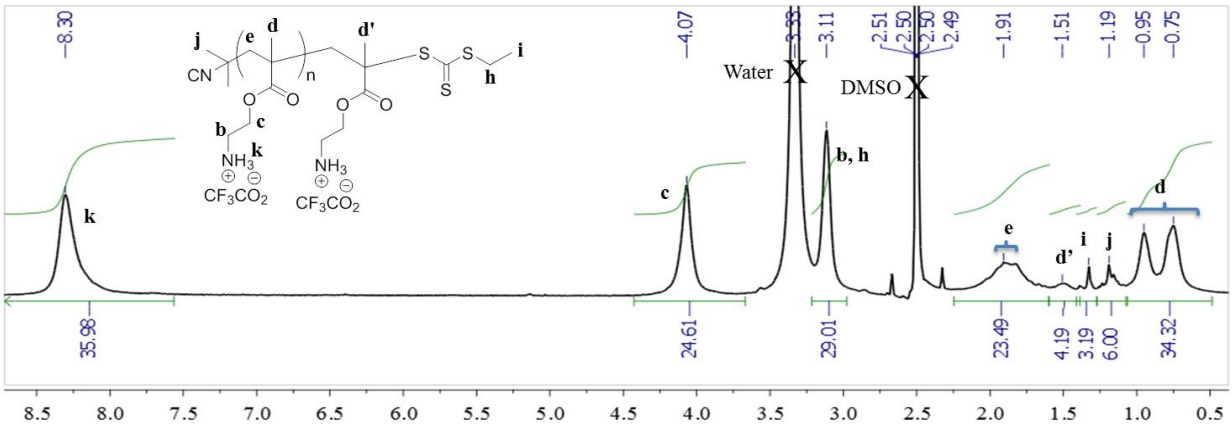


Figure B-7. ^{1}H NMR of P_{12} (DMSO-d6)

APPENDIX C

Stability of Polymer Endgroups: CPETC after exposure to TFA

Trifluoroacetic acid (TFA) (100 μL) was added to 2-cyanoprop-2-yl ethyl trithiocarbonate (CPETC) (20.5 mg, 0.1 mmol). The reaction mixture was stirred at room temperature. Aliquots were removed at 0, 5 and 30 minutes, and the reaction was monitored by ^{1}H NMR analysis in chloroform.

The peaks of CPETC (3.35, 1.88, and 1.35 ppm) were monitored upon the addition of TFA. After 5 minutes, additional peaks appeared, indicating the formation of products likely due to degradation of CPETC. After 30 minutes, the multiplets around 3.3 and 1.34 ppm remain, while the singlet at 1.88 ppm has disappeared. This demonstrates that the trithioester end groups of polymers might decompose during the deprotection of boc groups in the side chains of polymers under the acidic condition, possibly as a result of hydrolysis although the detailed mechanism of reaction is not clear at this point.

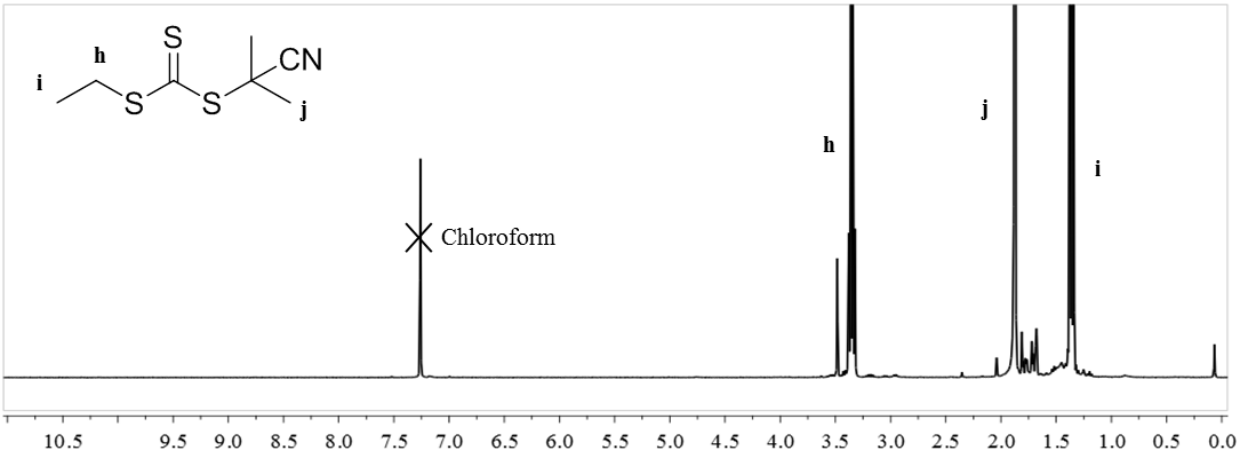


Figure C-1. NMR of CPETC at t = 0 ($CDCl_3$)

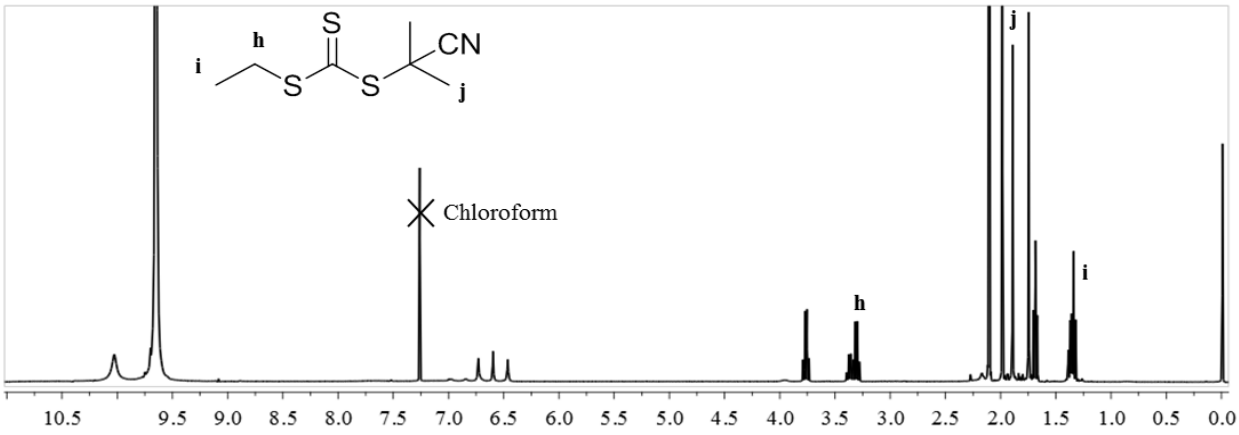


Figure C-2. CPETC + TFA at t = 5 ($CDCl_3$)

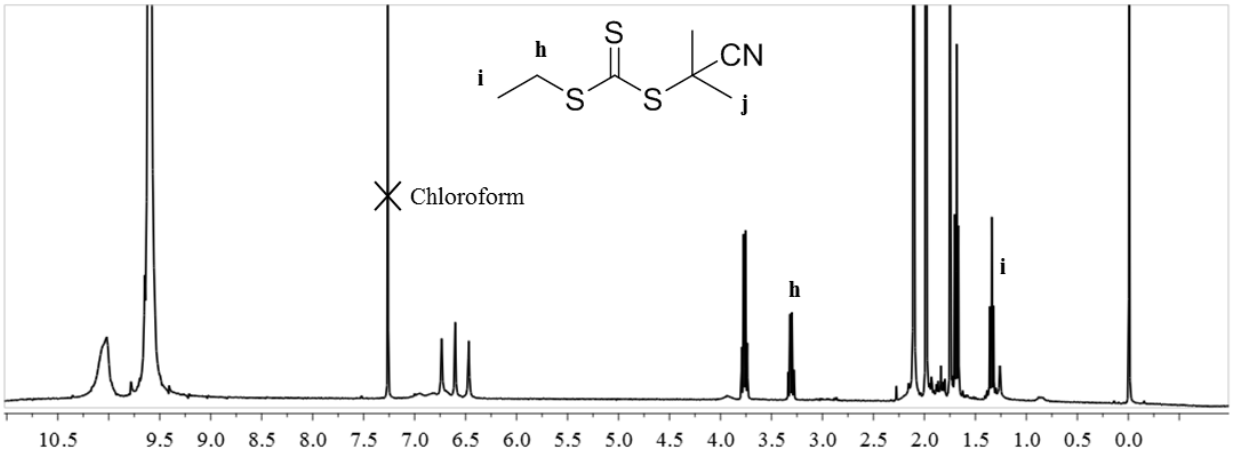


Figure C-3. CPETC + TFA at t = 30 min ($CDCl_3$)

APPENDIX D

In vivo trial data

As a preliminary assessment of the *in vivo* topical treatment of *S. aureus* infections using AEMPs, we chose a cotton rat nasal *S. aureus* colonization model. Two trials were performed: trial 1 involved 5 animals for $P_{7.7}$, 3 animals for mupirocin, and 3 animals for PBS buffer (control); trial 2 involved 5 animals for $P_{10.4}$, 5 animals for mupirocin, and 5 animals for PBS buffer (control).

Table D-1. *In vivo* Testing

Number of *S. aureus* BB2146 cells isolated				
	PBS Control	**Mupirocin**	**$P_{7.7}$**	**$P_{10.4}$**
Trial 1	3169	50	0	-
	2901	179	0	-
	3595	102	6	-
	-	-	0	-
	-	-	19	-
Trial 2	2019	3120	-	0
	2593	77	-	0
	3740	983	-	11
	1902	587	-	0
	2983	1276	-	65

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