Cationic amphiphilic synthetic macromolecules with superior antibacterial activity

Ashish Punia
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CATIONIC AMPHIPHILIC SYNTHETIC MACROMOLECULES
WITH SUPERIOR ANTIBACTERIAL ACTIVITY

by

ASHISH PUNIA

A dissertation submitted to the Graduate Faculty in Chemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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This manuscript has been read and accepted for the Graduate Faculty in Chemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Dr. Nan-Loh Yang

____________________

Date

Chair of Examining Committee

Dr. Brian R. Gibney

____________________

Date

Executive Officer

Dr. Alan M. Lyons

____________________

Dr. David C. Locke

____________________

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK
Abstract

CATIONIC AMPHIPHILIC SYNTHETIC MACROMOLECULES WITH SUPERIOR ANTIBACTERIAL ACTIVITY

by

Ashish Punia

Adviser: Professor Nan-Loh Yang

The increasing prevalence of antibiotic resistant bacteria (superbugs) has created a pressing need for new systems of antimicrobial agents. In this dissertation, I report on my extensive research on the design, synthesis, and development of synthetic amphiphilic macromolecules with antimicrobial activity and very low hemolytic impact. Synthetic amphiphilic polymers attack bacteria directly to rupture the cell membrane through electrostatic and hydrophobic interactions. However, the toxicity of such synthetic amphiphilic polymers against mammalian cells have impeded their therapeutic applications. We investigated the systematic structure/activities relationships for antibacterial and hemolytic activities of amphiphilic polyacrylates and poly(vinyl esters). Acrylate homopolymers with various lengths of alkyl pendant groups displayed high antibacterial activity against Staphylococcus aureus (S. aureus) and very low hemolytic activity toward red blood cells (RBCs). In comparison with polyacrylate homopolymers, random copolymers were highly antibacterial but extremely hemolytic. To further improve antibacterial activity of polyacrylates, while maintaining low hemolytic activity, a series of copolymers with 2-carbon and 6-carbon spacer arm (distance between polymer backbone and cationic center) counits were investigated. Our strategy of controlling charge distribution and mole ratios of 2-carbon (M2)
and 6-carbon counits (M6) resulted in a polymer with >200 fold selectivity toward Escherichia coli (E. coli) over RBCs. Copolymerization of just 10 mole% of shorter spacer arm M2 counits with hydrophobic M6 counits led to a drastic reduction in hemolytic activity by a factor of 850 compared with highly hemolytic M6 homopolymer, without severe deterioration of antibacterial activity. Scanning electron microscopy analysis of bacterial cells established the membrane rupture action of these polymers.

In a second acrylate system, hydrophilic and biocompatible poly(ethylene glycol) (PEG) monomers were copolymerized with M6 monomer to achieve selective (bacteria over RBCs) antibacterial activity in polymers. Incorporation of 30 mole% of PEG monomer led to a polymer with >100 times selectivity toward E. coli over RBCs. The Hydrogen-bonding ability of the PEG segments plays significant roles.

For a third system of poly(vinyl ester), we explored the role of hydrophobic side groups, molecular weight, and amphiphilicity on its activities.

This dissertation investigation has led to one of the most promising synthetic polymer systems reported till today for antimicrobial applications.
Acknowledgements

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I dedicate this dissertation to my
great and beloved family
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Chapter 1

Natural antimicrobial peptide mimesis and overview of structure activity relationships in synthetic amphiphilic polymers

1.1 Introduction

The discovery of penicillin by Alexander Fleming in 1928 and its subsequent mass production and distribution in 1940s has been hailed as one of the greatest medical achievements in modern history. The antibiotic “penicillin” saved thousands of lives worldwide, and the threat to human health from microbial infections seemed surmountable. In his Nobel Prize lecture, Fleming hypothesized that penicillin resistant microbes would result, if the “ignorant man” under-doses himself with penicillin. Unfortunately, Fleming’s hypothesis has become the grim reality of today. More than 20,000 people die in the United States alone each year from infections involving antibiotic drug resistant bacteria. Nosocomial infections add billions of dollars in health care costs every year. There has been an undeterred rise in infections involving multi-antibiotic drug resistant bacteria, which have come to be known as “Super bugs.” Widespread infections involving vancomycin-resistant Enterococci, methicillin-resistant Staphylococcus aureus, and fluoroquinolone-resistant Pseudomonas aeruginosa, among others, have threatened to undo a century of medical advances. The problem of superbugs is further compounded by the ever reducing efforts in the development of new antibiotics. The development of new antibiotic drugs involve huge costs, and then rapid development of bacterial resistance renders them ineffective within a few years. Hence, there is a pressing need to develop new antibacterial agents toward which the development of bacterial resistance would be highly hindered. Minimal toxicity of new antibiotic agents toward host mammalian cells and their facile synthesis to enable cost-effective
production is essential for their wide scale applications. The goal of this dissertation research is to synthesize polymers with high antibacterial activity and concomitant low toxicity toward mammalian cells. We explored structure-activity relationships in amphiphilic polyacrylates and poly(vinyl esters), which mimic the inherent design principles, rather than the exact structural features, of natural host-defense antimicrobial peptides.

1.2 Host-defense antimicrobial peptides and their mode of antibacterial action

Natural host-defense antimicrobial peptides (AMPs) have played an essential role in the successful evolution of multicellular organisms by defending them from microbial infections for millions of years.\(^8\) AMPs form an integral part of multicellular organism’s innate immune response to microbial attack. A large variety of AMPs, such as magainin in African clawed frog,\(^9\) cecropin in silk moth,\(^10\) and defensins\(^11\) in humans have been identified and isolated. Despite the great diversity in peptide sequence and secondary structures, all AMPs share the common design features of small size (10 to 50 amino acids) and amphiphilicity resulting from cationic and hydrophobic side groups.\(^8,12\) In contrast to target specific and enzyme or DNA replication inhibiting action of conventional antibiotics, the amphiphilic structure of AMPs enables them to bind to the negatively charged outer surface of bacterial cell membrane through non-specific electrostatic interactions and the hydrophobic groups of AMPs facilitate their insertion into lipophilic core of lipid bilayer of bacteria.\(^13-16\) This non-specific attack of AMPs on the bacterial membrane leads to bacterial membrane permeabilization, hole formation, and leakage of cytoplasm etc., ultimately leading to the bacterial cell death.\(^8,17\) The development of bacterial resistance toward AMPs has not been documented, and is considered to be highly impeded or improbable, as drastic mutations in bacterial cell membrane morphology and compositions would
be required to gain resistant against the physical nature of membrane rupture and disruption by AMPs.\textsuperscript{8}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{antimicrobial_peptide.png}
\caption{Selective activity of AMPs against bacteria due to significant differences in cell membrane composition of bacteria and mammalian cells. Figure adopted from Zasloff M., \textit{Nature} \textbf{2002}, \textit{415}, 389.}
\end{figure}

The fundamental differences in the cell membrane composition and morphology of bacterial and eukaryotic cells can explain the selective activity of AMPs toward bacterial cells and low toxicity against eukaryotic cells (Figure 1.1).\textsuperscript{8} The lipid bilayer of a bacterial cell is mainly comprised of the negatively charged phospholipids like: phosphatidylserine (PS) and phosphatidyl glycerol (PG).\textsuperscript{18-20} On the other hand, the outer leaflet of eukaryotic cell membrane consists of zwitterionic phospholipids like: phosphatidyl choline (PC) and sphingomyelin (SM), along with cholesterol.\textsuperscript{18-}
Moreover, bacterial cells (with some exceptions) have an additional outer coverage of a cell wall consisting of cross linked negatively charged peptidoglycans (murein layer). Thus, AMPs have strong electrostatic attraction to bind to the negatively charged bacterial cell surface, whereas only weaker hydrophobic interactions could take place between mammalian cells and AMPs, resulting in the selective activity of AMPs against bacteria.

Several mechanism have been proposed for the membrane rupture activity of AMPs. In the toroidal pore forming model, large pores with size ranging from 3 to 10 nm are formed. The hydrophilic components of AMPs cover up the pore surface to create a hydrophilic channels resulting in leakage of cytoplasmic material. The reorientation of hydrophilic phospholipid head groups on the surface of pores have also been proposed. In contrast with the toroidal model, the barrel-stave pore formation model involves certain peptide-peptide interactions to create smaller 1-2 nm in diameter pores. In this model, the phospholipid head groups do not reorient and the surface of the transmembrane pores are lined up by the AMPs alone, thus creating a hydrophilic channel. Both of these models create transmembrane pores leading to leakage of intercellular material and breakdown of membrane potential resulting in the bacterial cell death.

Alternatively, non-pore formation mechanisms of bacterial cell membrane rupture by AMPs have been proposed. At high concentration of peptides relative to membrane phospholipids, AMPs can lie parallel to the membrane surface and form carpets leading to formation of very large size (> 20 nm) defects. “Detergent” or “solubilization” mechanisms in which drastic collapse of bacterial cell membrane take place have also been proposed.
1.3 Synthetic polymers as mimics of antimicrobial peptides

Even though highly selective antibacterial activity and hindered development of bacterial resistance toward AMPs have made them attractive candidates for therapeutic applications, but the large scale application of AMPs face several hurdles. The synthesis or isolation of AMPs from living organisms would be a very expensive and time-consuming process. Furthermore, the oral administration of AMPs would be challenging due to proteolysis, which would drastically reduce the bioavailability of AMPs. Intravenous delivery of AMPs will hamper the large scale implementation of AMPs as antibiotic drugs. Thus, research efforts have been focused in the last decade to obtain synthetic mimics of AMPs, which could be readily synthesized and share the fundamental design features of AMPs. A Majority of AMPs have a secondary α-helical structure. These α-helical amphiphilic peptides adopt a “facially amphiphilic” segregated structures, while interacting with bacterial cell membrane, in which cationic and hydrophobic subunits adopt opposite faces of the helix. Such a segregated amphiphilic structure has been considered vital for the antibacterial activity of AMPs, and initial research endeavors were focused to emulate the α-helical features of AMPs. Other studies mimicking the β-peptide structure demonstrated that highly antibacterial synthetic mimics could be obtained. However, the role of secondary structures in the antibacterial activity was put to test, and it was demonstrated that diastereomeric peptides that were unable to form α-helical structure had potent antibacterial activity. Further studies revealed that overall amphiphilicity rather than the rigid secondary structure is the prerequisite for antibacterial activity.

The approach of amphiphilicity was extended to a range of synthetic polymers with flexible backbones that lacked any ability to form secondary structures, but could be produced on large scale due to their facile and cost effective synthesis. A range of synthetic amphiphilic polymers
including those based on polynorbornenes, \textsuperscript{20} \textsuperscript{21} \textsuperscript{49-53} polymethacrylates, \textsuperscript{54-59} poly(vinyl pyridines), \textsuperscript{60-64} polystyrenes, \textsuperscript{65-67} poly(vinyl alcohols), \textsuperscript{68} Nylon, \textsuperscript{69-72} urea oligomers, \textsuperscript{73-74} among others, have been successfully synthesized. Several studies have shown that the antibacterial activity of these polymers is comparable to those of natural AMPs. The lack of bacterial resistance development toward synthetic amphiphilic polymethacrylates was recently reported, whereas significant bacterial resistance was developed against conventional antibiotics in the same study. \textsuperscript{75}

1.4 Structural parameters affecting the antibacterial and hemolytic activities of synthetic amphiphilic polymers

1.4.1 Polymer amphiphilicity

Much research in the last decade on synthetic amphiphilic polymers has substantiated polymer amphiphilicity, the balance between cationic and hydrophobic groups, as a key structural feature affecting the antibacterial and hemolytic activities of synthetic polymers. Polymers with high cationic charge content would have higher affinity to bind with the bacterial cell membrane, but these polymers would have lower ability to insert through the hydrophobic core of bacterial lipid bilayer. On the other hand, high hydrophobic content in polymers has been shown to result in higher activity toward both bacteria and mammalian cells, leading to a loss of selective antibacterial activity. \textsuperscript{21} \textsuperscript{55} Moreover, reduction in aqueous solubility of highly hydrophobic polymers may lead to polymer aggregate formation. \textsuperscript{54} Formation of polymer aggregates reduces the effective concentration of polymers in solution, resulting in lower antibacterial activity. \textsuperscript{54} Hence an optimum balance between cationic and hydrophobic groups is imperative to achieve selective antibacterial activity in synthetic amphiphilic polymers. Antibacterial activity of synthetic polymers has been widely assessed in terms of minimum inhibitory concentration (MIC), which is often described as the lowest polymer concentration of polymers required to inhibit 100%
(in some reports 90%) bacterial growth within a specified period of incubation (for example 6 h, 12 h, or 18 h). The toxicity of polymers toward mammalian cells has been widely evaluated by various researchers in terms of hemolytic activity - 50% (HC\(_{50}\)), which is the lowest polymer concentration resulting in hemolysis of 50% red blood cells (RBCs), within an incubation period of 1 hour.

**1.4.1.1 Homopolymers**

Hammond et al. described the ring opening polymerization of \(\gamma\)-propargyl-L-glutamate to synthesize amphiphilic polypeptides with quaternary ammonium and hydrophobic alkyl groups (Figure 1.2(a)).\(^7^6\) The amphiphilicity of synthetic polypeptides were optimized through variation in the lengths of hydrophobic tail. Polypeptide with butyl side tail was ineffective against both E. coli and S. aureus, but increasing the length of alkyl tail to 12 carbon atoms led to high antibacterial activity against S. aureus. Amphiphilic polypeptides with 6 and 8 carbons in the alkyl tail displayed high antibacterial activity toward E. coli. Tew and co-workers explored the antibacterial and hemolytic activities of “facially amphiphilic” polynorbornenes (Figure 1.2 (b)).\(^2^1\) “Facially amphiphilic” polynorbornenes have both cationic and hydrophobic groups on the same repeating unit. The polymer amphiphilicity was optimized through variation in alkyl tail length. A polymer with methyl groups had low activity against E. coli and S. aureus. Higher antibacterial activity was observed in a polymer with ethyl side groups. Increase in the length of side groups to propyl led to a substantial increase in antibacterial activity. Further increase in side group lengths resulted in reduction of antibacterial activity, and the polymer having long hexyl side groups was found to be inactive against both E. coli and S. aureus.

An alternative approach of optimizing polymer amphiphilicity through variation of spacer arm distance was reported by Kuroda et al (Figure 1.2(c)).\(^5^7\) Cationic amphiphilic polymethacrylate
homopolymers with various lengths of alkyl spacer arms (distance between polymer backbone and cationic center) and pendent cationic centers were synthesized. The antibacterial and hemolytic activities of these polymers were assessed against E. coli, S. aureus, and RBCs. Homopolymers with 2-carbon and 4-carbon spacer arms were inactive against E. coli and moderately active toward S. aureus, whereas the 6-carbon spacer arm homopolymer was highly active against both E. coli and S. aureus. However, the 6-carbon spacer arm polymer displayed very high hemolytic activity, whereas the 2-carbon and 4-carbon homopolymers were non-hemolytic.

Figure 1.2 a) Amphiphilic polypeptides based on γ-propargyl-L-glutamate with quaternary ammonium and hydrophobic alkyl groups; b) “Facially amphiphilic” polynorbornenes have both the cationic and hydrophobic groups on the same repeating unit; c) amphiphilic polymethacrylates with various lengths of spacer arms. Figures adopted from a) Hammond et al., Biomacromolecules 2011, 12, 1666; b) Tew at al., J. Am. Chem. Soc. 2008, 130, 9836; c) Kuroda et al., Biomacromolecules 2012, 13, 1632.
1.4.1.2 Random copolymers

Kuroda and DeGrado investigated the structure-activity relationships in cationic amphiphilic polymethacrylates (Figure 1.3(a)). Random polymethacrylate derivatives with repeating units of butyl methacrylate (BMA) and amine functionalized ethyl methacrylate were synthesized through free radical polymerization. The mole% of BMA was varied from 0% to 60%, and the MIC and HC\textsubscript{50} values of copolymers were obtained. Both MIC and HC\textsubscript{50} values of copolymers were reduced as the mole% of BMA was increased, signifying the effect of increased hydrophobic content of polymer on their membrane rupture ability. MIC value of polymers reduced till ~30 mole% of BMA after which no further reduction in MIC value was observed. Reduction in water solubility of polymers with increasing mole% of butyl methacrylate was reported. It was proposed that formation of aggregates in polymers with high BMA content could reduce the availability of polymer chains to act on bacterial cells, thus reducing their antibacterial activity.

Palermo and Kuroda reported the synthesis of cationic polymethacrylate copolymers with methyl or butyl side chains at various mole ratios. Random copolymers with small methyl side groups displayed substantially lower hemolytic activity, as compared with random copolymers having butyl side groups. Moreover, copolymers with higher mole% content of methyl counits demonstrated high antibacterial activity against E. coli, resulting in highly selective antibacterial activity in a copolymer with 47 mole% of methyl methacrylate and 53 mole% of amine functionalized ethyl methacrylate repeating units. Tew et al. had also reported the reduction in MIC and HC\textsubscript{50} values with increasing size of alkyl side group in “segregated” amphiphilic polynorbornenes (Figure 1.3(b)). In “segregated” approach, the amine functionalized norbornene comonomer was copolymerized with various hydrophobic comonomers with varying lengths of alkyl tails at 1:1 mole ratio. More than 3 carbon atoms in the side tail led to reduction in the
antibacterial activity, and highest selectivity of ~ 20 times for bacteria over RBCs was reported in copolymer with propyl side group. Similarly, optimization of polymer amphiphilicity through control of cationic and hydrophobic moieties resulted in highly selective (bacteria over RBCs) antibacterial activity in random Nylon-3 copolymers (β-lactams, Figure 1.3(c)).

![Chemical structures](image_url)


### 1.4.2 Molecular weight

The molecular weight of the amphiphilic polymers is a crucial structural parameter, as it affects the number of bioactive moieties on each polymer chain. Generally, MIC and HC<sub>50</sub> values are reported in terms of weight of polymer (usually in µg) per unit volume (mL). Thus, at same mass
level, a polymer with higher average molecular weight would have a smaller number of polymer chains as compared with a lower molecular weight polymer. Kuroda and DeGrado evaluated the effect of molecular weight on the antibacterial and hemolytic activities of polymethacrylate random copolymers.\textsuperscript{54} Higher molecular weight polymers were found to display lower solubility in water, in comparison with lower molecular weight polymers. The smallest polymers displayed the highest antibacterial activity against E. coli. Furthermore, the hemolytic activities of smaller polymers were significantly lower than the higher molecular weight copolymers. Mowery et al. reported that the MIC values of β-lactams were independent of the molecular weight, but the hemolytic activity increased with higher molecular weight.\textsuperscript{71} Lower molecular weight fractions displayed substantially lower hemolytic activity than higher molecular weight polymer fractions (fractions separated by dialysis).\textsuperscript{71}

In contrast to random copolymers, an increase in antibacterial activity with increase in molecular weight or degree of polymerization was observed in case of amphiphilic polymethacrylate homopolymers.\textsuperscript{59} Similarly, synthetic amphiphilic polypeptides (homopolymers) displayed higher antibacterial activity at higher levels of degree of polymerization.\textsuperscript{76} “Facially amphiphilic” polynorbornenes, however, lost activity at higher molecular weight level ($M_n \sim 10,000$ g/mol) against S. aureus, whereas lower molecular weight polynorbornenes were highly antibacterial toward S. aureus.\textsuperscript{21} The formation of irreversible polyion-polyion complex formation was proposed as a probable reason behind this observation.\textsuperscript{21, 77}

### 1.4.3 Incorporation of hydrophilic poly(ethylene glycol) moieties in amphiphilic polymers

Poly(ethylene glycol) (PEG) has been extensively used in a myriad of polymeric materials to impart hydrophilicity, biocompatibility, and antifouling properties.\textsuperscript{78-85} Long PEG chains drastically increase the hydrodynamic radius of polymers, and therefore can substantially improve
the pharmacokinetics of conjugated drugs.\textsuperscript{86-88} In cationic amphiphilic polymers, the inclusion of PEG groups may reduce the hemolytic activity of polymers through reduced hydrophobic interactions with RBCs. Furthermore, the improved water solubility of highly hydrophilic polymers may result in enhanced antibacterial activity. Youngblood et al. explored the effects of Peg content on the antibacterial and hemolytic activities of N-hexylated poly(vinyl pyridine) (Figure 1.4(a)).\textsuperscript{60-64} Random copolymers

![Figure 1.4 (a)](image1.png)

\(n = 1, 4.2, 8.2, \text{ and } 22\)

Figure 1.4 Schematic representation of hydrophilic poly(ethylene glycol) modifications in a) N-hexylated poly(vinyl pyridines); b) amphiphilic polynorbornenes. Figures adopted from a) Youngblood et al., \textit{Biomacromolecules} \textbf{2007}, \textit{8}, 19; b) Colak et al., \textit{Biomacromolecules} \textbf{2009}, \textit{10}, 353.

having varying mole ratios of Poly(ethylene glycol) methyl ether methacrylate (PEGMA) and N-hexylated vinyl pyridine repeating units were synthesized. PEGMA monomers with various lengths of Peg side chains were copolymerized with vinyl pyridine, in order to evaluate the effect of PEG length on biological activities of these polymers. Incorporation of just 1 mole\% of PEGMA-1100 (Mw ~ 1100 g/mol) resulted in significant improvement of antibacterial activity, as compared with N-hexylated poly(vinyl pyridine) with no PEG content.\textsuperscript{61} Similar results were
obtained through copolymerizing hydroxyethyl methacrylate (HEMA) with vinyl pyridine. As compared with N-hexylated poly(vinyl pyridine), copolymer containing up to 10 mole% of HEMA displayed significantly higher antibacterial activity. These observations are interesting as PEG lacks any inherent antibacterial activity. The enhanced wettability of PEG containing poly(vinyl pyridines) was proposed as a reason behind these observations. Higher hydrophilicity of copolymers enables better interactions of polymer with aqueous bacterial assays. However, the reduction in hemolytic activity of these polymers was not observed until 50 mole% of PEGMA was incorporated into the copolymer.

Colak et al. explored the strategy of PEGylation to improve the selectivity of amphiphilic polynorbornenes (Figure 1.4(b)). The hemolytic activity of polynorbornenes was reduced by increasing the PEG content, however a simultaneous deterioration in antibacterial activity was also reported. Hence, the resulting polymers demonstrated low selectivity (< 5 times) toward bacteria over RBCs. PEGylation was utilized to improve water solubility of amphiphilic polymethacrylates, which were found to be antibacterial toward a gram positive bacteria while displaying low hemolytic activity.

![Diagram](image)
1.4.4 Nature of cationic charge

Cationic charge in AMPs originate from amine functionalized residues such as arginine and lysine. At physiological pH, the primary amine groups on AMPs are positively charged, enabling AMPs to electrostatically bind with the negatively charged cell membrane of prokaryotes. In contrast to AMPs, synthetic quaternary ammonium compounds have permanently charged quaternary ammonium groups. Kuroda et al. investigated the role of cationic group type on the antibacterial and hemolytic activity of amphiphilic polymethacrylate copolymers (Figure 1.5(a)). A series of copolymers with primary, tertiary, or ammonium groups, and with varying mole ratios of hydrophobic and cationic groups were synthesized. Amphiphilic copolymers with primary and tertiary amine groups were found to be highly antibacterial against E. coli and completely inhibited the bacterial growth, while displaying low hemolytic activity toward RBCs. On the other hand, polymers with quaternary ammonium groups were inactive against E. coli. However, an increase in both antibacterial and hemolytic activity was observed after increasing the length of hydrophobic side groups from methyl to butyl. Hammond and co-workers reported similar observations of lack of antibacterial activity in synthetic amphiphilic polypeptides bearing quaternary or tertiary ammonium groups, whereas polypeptides having primary or secondary amine groups displayed antibacterial activity against S. aureus (Figure 1.5(b)). Hence, the nature or type of cationic charge play a crucial role in the antibacterial activities of amphiphilic polymers.

Figure 1.5 Schematic representation of a) cationic amphiphilic polymethacrylates; and b) synthetic amphiphilic polypeptides with various types of cationic groups. Figures adopted from a) Kuroda et al., Biomacromolecules 2009, 10, 1416; b) Hammond et al., Biomacromolecules 2011, 12, 1666.
Several AMPs derive cationic charge from arginine residues bearing guanidinium groups.\textsuperscript{51} In comparison with amino groups, guanidinium groups are highly basic and are approximately completely protonated at physiological pH.\textsuperscript{92} Moreover, guanidinium groups can form hydrogen bonds with phospholipid head groups leading to greater binding affinity with bacterial cell surface.\textsuperscript{93} Gabriel and co-workers synthesized guanidinium functionalized polynorbornenes (Figure 1.6(a)).\textsuperscript{51} In comparison with primary amine charged polynorbornenes, Poly guanidinium oxanorbornenes displayed very high antibacterial activity along with low hemolytic activity. Dye leakage studies revealed the non-membrane rupturing mechanism of antibacterial activity. Intracellular targeting by these cell penetrating guanidinium polymers was proposed as a putative mechanism of antimicrobial activity.\textsuperscript{51} Mattheis et al. reported similar observations of higher antibacterial activity of guanidinium charged amphiphilic polymethacrylates (Figure 1.6(b)).\textsuperscript{94}

Figure 1.6 Guanidinium containing a) amphiphilic polynorbornenes; and b) amphiphilic polymethacrylates. Figures adopted from a) Gabriel et al., \textit{Biomacromolecules} \textbf{2008}, 9, 2980; b) Mattheis et al., \textit{Macromol. Biosci.} \textbf{2013}, 13, 242.
1.4.5 Cationic charge density

AL-Badri et al. investigated the role of cationic charge density on the antibacterial and hemolytic activities of facially amphiphilic polynorbornenes having cationic groups and hydrophobic groups on the same repeating unit (Figure 1.7). Increasing the cationic charge density led to significant reduction in hemolytic activity, while no adverse effect on the antibacterial activity of these polymers was observed. Their study showed that the cationic charge density can be utilized as an effective structural parameter to synthesize antibacterial but non-hemolytic synthetic amphiphilic polymers.

Sampson and co-workers reported the correlation between polymer backbone spacer distance and the biological activities of amphiphilic polymers. Homopolymers with backbone distance of ~ 4 Å were inactive against bacteria or RBCs, whereas polymers with higher backbone spacer distance of ~ 8 Å displayed high antibacterial activity, and a simultaneous increase in hemolytic activity was observed. Thus, higher cationic charge density may reduce the hemolytic activity of
amphiphilic polymers, and an overall balance between charge density and polymer hydrophobicity can lead to polymers with selective antibacterial activity.

1.4.6 Polymer architecture
The antibacterial and hemolytic activities of block and random copolymers were assessed by Oda et al. (Figure 1.8(a)). At similar monomer compositions, both block and random copolymers displayed similar levels of antibacterial activities toward E. coli. However, the hemolytic activities of random copolymers were substantially higher than the block copolymers. Hence, the block copolymer architecture resulted in highly selective (bacteria over RBCs) antibacterial activity of amphiphilic copolymers. The dye leakage studies confirmed the membrane rupture activity of random and block copolymers toward vesicles mimicking the bacterial cell membrane, whereas only random copolymers caused dye leakage mammalian cell type lipid vesicles, confirming the lack of lytic ability of block copolymers toward mammalian cells. However, block copolymers resulted in agglutination (aggregation) of RBCs at a very low polymer concentration, as compared with random copolymers which caused agglutination at much higher polymer concentrations. The minimum bactericidal and hemolytic concentrations were found to be orders of magnitude lower than the critical micelle concentrations, indicating that polymer aggregation in not required for antibacterial activity of these polymers. Similar effects of polymer architecture (random versus block) were reported by Wang et al. (Figure 1.8(b)). Oda and co-workers proposed the formation of intramolecular aggregates in block copolymers with the hydrophobic part of the copolymer at the core surrounded by the hydrophilic cationic part. Such intramolecular aggregates would reduce the hydrophobic interactions of polymers with lipid bilayer of RBCs, resulting in low hemolytic activity.
Figure 1.8 Block copolymer architecture of a) amphiphilic poly(vinyl ether)s; b) amphiphilic polymethacrylates. Figures adopted from a) Oda et al., Biomacromolecules 2011, 12 (10), 3581; b) Wang et al., Macromol. Biosci. 2011, 11, DOI: 10.1002/mabi.201100196.

Intramolecular aggregates would also have enhanced the electrostatic interactions with bacterial cell membrane and may effectively replace the divalent cations from bacterial cell membrane, which are crucial for membrane integrity.\textsuperscript{98}

Sampson and co-workers synthesized alternating and random amphiphilic copolymers and evaluated their antibacterial and hemolytic activities.\textsuperscript{95} They reported 2 to 6 fold lower antibacterial activities in case of random copolymers, in comparison with alternating copolymers. Thus, precise locations of cationic and hydrophobic groups resulted in higher antibacterial activity. A random copolymer does not have consistent local balance of cationic and hydrophobic groups leading to lower activity in regions with higher cationic charge density.\textsuperscript{95}
Chapter 2

Effect of systematic structural variations on the antibacterial and hemolytic activities of polyacrylates

2.1 Introduction

Antimicrobial agents with highly selective (bacteria over human cells) antibacterial activity and an ability to resist the development of bacterial resistance are urgently required. Cationic antimicrobial polymers have attracted significant research interest in the last decade. One of the major class of antimicrobial polymers is quaternary ammonium compounds (QACs), also known as polysurfactants. QACs attach to cell membrane of bacteria through coulombic interactions and the large hydrophobic groups of QACs enable their permeabilization into the hydrophobic core of the bacterial lipid bilayer. However, the high toxicity of polysurfactants toward mammalian cells poses a major hurdle toward their biomedical applications. The other major class of antibacterial polymers are synthetic amphiphilic polymers mimicking the fundamental design principles of natural AMPs. In comparison with polysurfactants, synthetic mimics of AMPs have global amphiphilic nature with a distribution of amino (primary, secondary, or guanidinium) and hydrophobic groups throughout the length of polymer backbone. In the last decade, substantial research has been done to identify factors influencing the antibacterial and hemolytic activity of synthetic amphiphilic polymers. Mole ratio of hydrophobic moiety, length of alkyl side chain, molecular weight, polymer architecture, and identity of amine functionality have been found to play significant roles in the antibacterial and hemolytic activity of synthetic amphiphilic polymers.
Kuroda and DeGrado described the synthesis and biological activities of cationic amphiphilic polymethacrylates.\textsuperscript{54} The random copolymers of cationic and hydrophobic (butyl side group) counits displayed high activity against E. coli, but were found to be highly hemolytic toward human RBCs.\textsuperscript{54} On the other hand, cationic polymethacrylate homopolymer of amine functionalized monomer did not display hemolytic or antibacterial activity (for E. coli).\textsuperscript{54} As hydrophobic groups facilitate the insertion of polymers into hydrophobic lipid bilayer of bacteria,\textsuperscript{17} homopolymers without hydrophobic counits may not readily penetrate through the bacterial cell surface, leading to lower antibacterial activity of homopolymers, as compared with random copolymers. It can be reasonably expected that by increasing the hydrophobicity of polymethacrylate homopolymers, their antibacterial activity could be improved, and due to high charge density (presence of cationic charge on each counit) in homopolymers, they may display low toxicity toward mammalian cells. To this end, we synthesized a selection of polyacrylate homopolymers with varying lengths of the hydrophobic alkyl tail from methyl to butyl, in order to optimize the biological activities of these homopolymers.\textsuperscript{109} Each homopolymer was synthesized at two molecular weight level to explore the effect of polymer size on their biological activities. Random copolymers were synthesized to compare their biological activities with similar homopolymers. We synthesized polymers with an acrylate backbone instead of methacrylate, as the higher chain flexibility of acrylate polymers, due to lack of methyl group at polymer backbone could lead to a greater degree of freedom for side groups resulting in higher membrane permeabilization ability.

Our investigation revealed that amphiphilic polyacrylate homopolymers can show highly selective activity toward bacteria over RBCs, whereas random copolymers were found to be extremely hemolytic and lacked selective (bacteria over RBCs) antibacterial activity.
2.2 Experimental

2.2.1 Materials

(Methylamino)ethanol, 2-(Ethylamino)ethanol, 2-(Propylamino)ethanol, 2-(Butylamino)ethanol, dichloromethane (anhydrous), N,N-diisopropylethylamine, acetonitrile (anhydrous), AIBN, methyl 3-mercaptopropionate, 1-hexanol, cyclohexanol, 3,3-Dimethyl-2-butanol, hexane, and diethyl ether were purchased from Sigma-Aldrich and used without further purification. Acryloyl chloride from Sigma-Aldrich was distilled prior to use. Butyl acrylate was stirred with inhibitor remover packing for 20 minutes and filtered before use. Di-tert-butyl dicarbonate and trifluoroacetic acid were purchased from VWR.

2.2.2 Instrumentation

$^1$H NMR spectra were obtained on 300 MHz and 600 MHz Varian NMR spectrometers using CDCl$_3$ or DMSO-$d_6$ as solvents. Molecular weights ($M_w$ and $M_n$) of Boc protected polymers, and their molecular weight distributions ($M_w/M_n$, PDI) were obtained on Waters alliance GPCV 2000 using linear polystyrene as standard. Tetrahydrofuran (HPLC grade) was used as eluent with a flow rate of 1 mL/min. OD$_{600}$ was obtained to measure bacterial growth on an Agilent 8453 spectrophotometer (using 1 cm path length plastic cuvette). OD$_{595}$ and OD$_{414}$ were obtained on a SpectraMax 340 PC micro plate reader (Molecular devices).
2.2.3 Synthesis of 2-(N-Boc-alkylamino)ethanols

Scheme 2.1 Synthesis of N-Boc protected alklyamino ethanols

\[
\text{H}_2\text{NCH}_2\text{R} + \text{Di-tert-butyl dicarbonate} \rightarrow \text{H}_2\text{NCH}_2\text{Boc} \quad (\text{R} = \text{H, Me, Et, Pr, Bu})
\]

Di-tert-butyl dicarbonate (26 g, 119 mmol) was added in a 250 mL single neck round bottom flask, already charged with 2-(Methylamino)ethanol (8.6 mL, 108 mmol) and H\textsubscript{2}O (110 mL). The reaction mixture was then stirred at 34 °C for 6 hours. After 6 hours, reaction mixture was extracted with ethyl acetate (3*125 mL), dried with sodium sulfate, and ethyl acetate was evaporated under reduced pressure to yield pure product (90 % yield). \(^1\)H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta 1.45 (s, 9H), 2.92 (s, 3H), 3.38 (s, 2H), 3.73 (s, 2H)\).

For Boc protection of 2-(Ethylamino)ethanol, 2-(Propylamino)ethanol, and 2-(Butylamino)ethanol, higher reaction time (1-2 days) and slightly modified work-up procedure was employed. After the completion of reaction, organic and aqueous layers were separated. Ethyl acetate (30 mL) was added into organic layer, and washed with distilled water (3*125 mL). Organic layer was dried over sodium sulfate, and excess solvent removed using rotavapor to yield pure product. **2-(N-Boc-ethylamino)ethanol**: \(^1\)H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta 1.13 (t, 3H), 1.48 (s, 9H), 3.18-3.46 (d, 4H), 3.75 (s, 2H)\); **2-(N-Boc-propylamino)ethanol**: \(^1\)H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta 0.91 (t, 3H), 1.45-1.62 (bs, 11H), 3.2 (s, 2H), 3.4 (s, 2H), 3.77 (s, 2H); 2-(N-Boc-
**butylamino)ethanol**: $^1$H NMR (300 MHz, CDCl$_3$): δ 0.92 (t, 3H), 1.29 (m, 2H), 1.38-1.6 (bs, 11H), 3.21 (s, 2H), 3.37 (s, 2H), 3.71 (s, 2H).

### 2.2.4 Synthesis of amine functionalized monomers

Scheme 2.2 Synthesis of amine functionalized acrylate monomers

2-(N-Boc-butylamino)ethanol (12.84 g, 60 mmol) was added in a 500 mL, 3 neck round bottom flask, loaded with N,N-diisopropylethylamine (17.4 mL, 100 mmol) and dichloromethane (100 mL). Acryloyl chloride (5.52 mL, 68 mmol) was then added drop wise at 0°C, under nitrogen atmosphere. The reaction mixture was allowed to warm to room temperature and stirred overnight. After 18 hours, reaction mixture was washed with distilled water (3 times), 10% citric acid (2 times), 10% potassium carbonate (2 times), and saturated sodium bicarbonate solution (3 times). Organic layer was separated and dried over sodium sulfate followed by removal of excess solvent using rotavapor. The resultant liquid was purified by silica gel column chromatography using hexane/ethyl acetate (1:1) as eluent. 60% yield (Monomer 4). $^1$H NMR (300 MHz, CDCl$_3$): δ 0.92 (t, 3H), 1.30 (m, 2H), 1.48 (bs, 11H), 3.23 (s, 2H), 3.48 (s, 2H), 4.3 (s, 2H), 5.86 (d, 1H), 6.14 (m, 1H), 6.43 (d, 1H). Similar procedure was followed for the synthesis of all other monomers.

**Monomer 1 (R=Methyl)**: $^1$H NMR (600 MHz, CDCl$_3$): 1.41 (s, 9H), 2.88 (s, 3H), 3.48 (s, 2H), 4.23 (s, 2H), 5.81 (d, 1H), 6.09 (q, 1H), 6.38 (d, 1H); **Monomer 2 (R=Ethyl)**: $^1$H NMR (600 MHz, CDCl$_3$): 1.11 (t, 3H), 1.45 (s, 9H), 3.3 (s, 2H), 3.5 (s, 2H), 4.3 (s, 2H), 5.9 (d, 1H), 6.14 (q, 1H).
6.4 (d, 1H); **Monomer 3 (R=Propyl):** ¹H NMR (600 MHz, CDCl₃): 0.86 (t, 3H), 1.39-1.59 (bs, 11H), 3.19 (s, 2H), 3.47 (s, 2H), 4.25 (s, 2H), 5.84 (d, 1H), 6.12 (q, 1H), 6.4 (d, 1H); **Monomer 5 (R=H):** ¹H NMR (600 MHz, CDCl₃): 1.50 (s, 9H), 3.47 (t, 2H), 4.27 (t, 2H), 4.83 (s, 1H), 5.93 (d, 1H), 6.15 (q, 1H), 6.45 (d, 1H); **Monomer 6 (R=n-Hexyl):** ¹H NMR (600 MHz, CDCl₃): 0.86 (t, 3H), 1.28 (m, 4H), 1.34 (m, 2H), 1.63 (m, 2H), 4.11 (t, 2H), 5.77 (d, 1H), 6.08 (q, 1H), 6.35 (d, 1H); **Monomer 7 (R=Cyclohexyl):** ¹H NMR (600 MHz, CDCl₃): 1.18-1.56 (m, 6H), 1.71 (m, 2H), 1.85 (m, 2H), 4.81 (m, 1H), 5.76 (dd, 1H), 6.08 (q, 1H), 6.35 (dd, 1H); **Monomer 8 (R=2,2,3-Trimethylbutyl):** ¹H NMR (600 MHz, CDCl₃): 0.95 (s, 9H), 1.19 (d, 3H), 4.78 (q, 1H), 5.81 (d, 1H), 6.13 (q, 1H), 6.40 (d, 1H).

### 2.2.5 Synthesis of polyacrylate homopolymers

Scheme 2.3 Synthesis of amine functionalized polyacrylate homopolymers

As per literature, a 3 neck 100 mL flask was charged with Monomer 2 (2.25 g, 9.25 mmol), AIBN (15.2 mg), Methyl-3-mercaptopropionate (MMP, 0.205 mL), and Acetonitrile (10 mL). The reaction mixture was degassed with dry nitrogen for 5 minutes, and stirred at 65 °C for 18 hours. Acetonitrile was evaporated using rotavapor, and small amount (1 mL) of dichloromethane was added into reaction mixture, followed by precipitation into hexane (2 times). Resultant polymer was dried under reduced pressure. By varying the mole ratios of MMP and monomer, two series
of molecular weights were obtained ($M_w$ of approximately 6000 g/mol and 1600 g/mol). $^1$H NMR (300 MHz, CDCl$_3$) is as shown in Figure 2.1(a).

For the deprotection of Boc protecting group, Polybutyl was dissolved in minimum quantity of dichloromethane, and excess quantity of trifluoroacetic acid (TFA) was added. The mixture was stirred for 4 hours at room temperature. TFA was removed under reduced pressure, followed by distillation using methanol (3×40 mL) and Dichloromethane (3×40 mL). Small quantity of acetonitrile (2 mL) was added, and mixture was repeatedly precipitated into diethyl ether to obtain amphiphilic polymers. Polymers were dried under reduced pressure and lyophilized. Complete deprotection of amine groups was confirmed from $^1$H NMR spectra (Figure 2.1(b)).

![Figure 2.1](image-url)
Boc deprotection (600 MHz, CDCl$_3$); and d) Polypropyl_1.6k after Boc deprotection with TFA(600 MHz, CDCl$_3$).

### 2.2.6 Synthesis of random copolymers.$^{54}$

Scheme 2.4 Synthesis of polyacrylate random copolymers

For the synthesis of copolybutyl_6K, butyl acrylate (3.1 g, 24.2 mmol) was charged into a 100 mL, 3 neck round bottom flask, loaded with Monomer 5 (N-(tert-butoxycarbonyl)aminoethyl acrylate, 5.21 g, 24.2 mmol), AIBN (0.08 g), MMP (0.268 mL), and acetonitrile (49 mL). The reaction mixture was purged with dry nitrogen for 5 minutes and stirred at 65 °C for 18 hours. Acetonitrile was evaporated and copolymer was re-dissolved in 2 mL dichloromethane, followed by repeated precipitation in hexane. For the synthesis of Copoly1, Copoly2, and Copoly3; higher mole percentage (60%) of Monomer 5 was used to facilitate the solubility of copolymers in aqueous medium. Deprotection of Boc protected copolymers was carried out as explained above. Relative percentage of monomers in copolymers were confirmed with $^1$H NMR spectra and found to be in agreement with the monomer feed ratio.
2.2.7 Calculation of Degree of polymerization

Figure 2.3 represents the $^1$H NMR spectrum of Polymethyl_6K. A broad single peak at δ 2.9-3.1 (integral value =57.33) belongs to methyl protons in the monomer repeat unit. A single peak at δ 3.68 belongs to methyl proton at the polymer end chain (integral value=3). Degree of polymerization (DP) of polymer is calculated as below:

D. P. = (57.33/3) + (3/3)

D. P. = 20.11

Similarly, DP for all polymers and copolymers were calculated and shown in Table 2.1.
2.2.8 Preparation of Polymer dilutions for antibacterial and hemolysis testing

Stock solution (20 mg/mL) for each polymer was prepared by dissolving in DMSO or in deionized water. Serial dilutions (2 fold) and some intermediate concentrations (14.3 mg/mL, 7.14 mg/mL, and 3.85 mg/mL) were then obtained by further adding distilled water. As described in the antibacterial testing protocol below, a tenfold dilution would further take place in the 96 well assay plates. Control solutions (without polymers) were prepared in a similar way by diluting DMSO with deionized water.

2.2.9 Antibacterial Test.54

Polymers were dissolved in DMSO to prepare stock solutions (20 mg/mL). Subsequent serial dilutions were prepared by adding water. Control solutions without polymers were similarly prepared. An aliquot of E. coli (TOP 10, ampicillin resistant) was incubated in Luria Bertani (LB) broth (containing ampicillin, 100 µg/mL) overnight at 37 °C. To measure bacterial cell growth,
turbidity as optical density at $\lambda=600$ nm ($OD_{600}$) was obtained on an Agilent 8453 spectrophotometer using a disposable plastic cuvette with 1 cm path length. This cell culture was diluted with fresh LB broth to obtain a cell suspension with $OD_{600} = 0.1$ and incubated for approximately 1.5 h at 37 °C. After approximately 1.5 h, the $OD_{600}$ of cell culture increased from 0.1 to 0.45-0.5, indicating a log-phase growth. This cell suspension was further diluted with fresh LB broth to obtain the final stock suspension of bacteria with $OD_{600} = 0.001$. Polymer solution (10 µL) or control solutions were added in triplicate to each well of a 96 well cell culture plate (REF 353916, BD falcon, flat bottom) followed by the addition of E. coli stock suspension (90 µL), and culture plates were incubated at 37 °C for 18 h. Bacterial growth was measured as turbidity at $OD_{595}$ on a SpectraMax 340 PC micro plate reader. Minimum Inhibitory Concentration (MIC) was defined as the lowest polymer concentration resulting in 100% inhibition of bacterial growth. MIC values reported here are the averages of three separate experiments performed on different days. Antibacterial activities of polymers against S. aureus (ATCC 25923) were similarly obtained, except Mueller-Hinton (MH) broth was used in place of LB broth.

2.2.10 Hemolysis Test

Red blood cells were obtained by centrifuging the freshly drawn mouse blood at 3000 rpm for 15 minutes. Plasma and white blood cells were removed as supernatant, and RBCs were washed with TBS buffer (Tris, 10mM, 150 mM NaCl, pH=7) multiple times. 1 ml of RBCs were diluted with 9 mL of TBS buffer. This RBCs suspension was further diluted by a factor of 40 by adding TBS to obtain the final RBCs stock suspension (0.25% RBCs). RBCs stock suspension (120 µL), polymer or control solutions (15 µL), and TBS (15 µL) were added into 600 µL micro centrifuge tubes, and incubated at 37 °C for 1 h. After 1 h, the tubes were centrifuged at 4000 rpm for 5 minutes. Supernatant (30 µL) was added into each well of a 96 well cell culture plate (in triplicate).
Hemoglobin concentration was measured as OD\textsubscript{414}. HC\textsubscript{50} value was defined as the lowest polymer concentration required to lyse 50% of RBCs within an incubation period of 1 h. 1% triton X-100 was used as a reference for 100% hemolysis. HC\textsubscript{50} values reported in this study are the averages of three separate experiments. Percentage hemolysis for each polymer concentration was calculated using the following formula:

\[
\text{% Hemolysis} = \left( \frac{OD\textsubscript{414 Polymer} - OD\textsubscript{414 negative Control}}{OD\textsubscript{414 Triton} - OD\textsubscript{414 negative Control}} \right) \times 100
\]

2.3 Results and discussion

2.3.1 Synthesis of polymers

Polyacrylate homopolymers were synthesized by free radical polymerization of N-Boc protected monomers (Scheme 2.3). Likewise, N-(tert-butoxycarbonyl)aminoethyl acrylate was copolymerized with butyl acrylate (1:1, feed mole ratio, Scheme 2.4) to synthesize random copolymers in order to explore the effect of polymer architecture (homopolymer verses random copolymer) on the biological activities of amphiphilic polyacrylates. In amphiphilic polyacrylate homopolymers, the hydrophobic alkyl tail is directly attached to the cationic center, whereas in random copolymer the cationic group and alkyl side chains are present on separate repeating units. Thus, due to the difference in polymer architecture, homopolymers and random copolymers may display significantly different biological activities. Two series of molecular weights (Mw ~ 6k g/mol (DP~6) and 1.6 g/mol (DP~22)) were synthesized for each homopolymer and random copolymer. Data from \textsuperscript{1}H NMR (600 MHz or 300 MHz) were used to estimate the degree of polymerization (D.P.) of products and to confirm the complete deprotection of the Boc group. Gel permeation chromatography (linear polystyrene standards) was employed to estimate molecular weights and polydispersity (PDI) of precursor polymers (Table 2.1). Polymethyl_6k represents the
homopolymer with methyl chain attached to amine group and having a molecular weight ($M_w$) of approximately 6k g/mol. The nomenclature Copolybutyl_6k is used for the random copolymer with molecular weight (GPC) of approximately 6k g/mol. Scheme 2.4 represents the synthesis of copolymers designed to discern the effect of hydrophobic side chain shape on antibacterial and hemolytic activity of amphiphilic copolymers. Mole percentage (in feed) of hydrophobic comonomer was kept at 40%, because the higher mole% of hydrophobic monomer could result in low solubility of copolymers in aqueous assay mediums.

Table 2.1 Characterization of homopolymers and random copolymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>[MMP]/[Monomer]</th>
<th>$M_w$ (GPC)</th>
<th>$M_n$ (GPC)</th>
<th>PDI</th>
<th>DP$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymethyl_1.6K</td>
<td>0.20</td>
<td>1646</td>
<td>1068</td>
<td>1.54</td>
<td>6.9</td>
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<td>1730</td>
<td>1160</td>
<td>1.50</td>
<td>5.7</td>
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<tr>
<td>Polypropyl_1.6K</td>
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<td>1723</td>
<td>1151</td>
<td>1.50</td>
<td>6.0</td>
</tr>
<tr>
<td>Polybutyl_1.6K</td>
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<td>1637</td>
<td>904</td>
<td>1.80</td>
<td>6.6</td>
</tr>
<tr>
<td>Polymethyl_6K</td>
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<td>7359</td>
<td>5495</td>
<td>1.33</td>
<td>20</td>
</tr>
<tr>
<td>Polyethyl_6K</td>
<td>0.05</td>
<td>6299</td>
<td>4795</td>
<td>1.31</td>
<td>23</td>
</tr>
<tr>
<td>Polypropyl_6K</td>
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<td>3877</td>
<td>1.37</td>
<td>23</td>
</tr>
<tr>
<td>Polybutyl_6K</td>
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<td>4340</td>
<td>1.47</td>
<td>24</td>
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<tr>
<td>Copolybutyl_1.6K</td>
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<td>902</td>
<td>1.57</td>
<td>4.1</td>
</tr>
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<td>Copolybutyl_6K</td>
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<td>4351</td>
<td>1.50</td>
<td>24</td>
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<tr>
<td>Copoly1_6K</td>
<td>0.05</td>
<td>6316</td>
<td>5006</td>
<td>1.26</td>
<td>----</td>
</tr>
<tr>
<td>Copoly2_6K</td>
<td>0.05</td>
<td>5478</td>
<td>3687</td>
<td>1.48</td>
<td>16</td>
</tr>
<tr>
<td>Copoly3_6K</td>
<td>0.05</td>
<td>5350</td>
<td>3982</td>
<td>1.34</td>
<td>22</td>
</tr>
</tbody>
</table>
2.3.2 Antibacterial activity of polymers against E. coli

The antibacterial activity of polymers was obtained against gram negative E. coli in terms of minimum inhibitory concentrations (MIC), and is shown in Table 2.2 and Figures 2.4-2.5. Polymethyl_6k, with methyl tail attached to cationic center, displayed very low antibacterial activity against E. coli (MIC = 1048 µg/mL). Increasing the length of alkyl tail from methyl to ethyl (Polyethyl_6k) did not significantly increase the antibacterial activity against E. coli. Similarly, Polypropyl_6k in which the propyl tail attached to the cationic center, displayed low antibacterial activity (MIC = 810 µg/mL) against E. coli. Further increase in the length of the alkyl tail from propyl to butyl led to an increase in antibacterial activity. Polybutyl_6k, with a butyl chain linked to cationic center displayed moderate antibacterial activity against E. coli (MIC = 417 µg/mL). These observations indicate that the increase in the length of alkyl tail (attached to cationic center) from methyl to butyl in our homopolymers (6k series) does not substantially affect the antibacterial activity of these polymers against E. coli. In comparison with homopolymers, the random copolymer Copolybutyl_6k demonstrated high antibacterial activity against E. coli (MIC = 13 µg/mL). In copolybutyl, the cationic charges and hydrophobic butyl side chains are present on separate repeating units or counits, in contrast with Polybutyl_6k homopolymer, in which hydrophobic butyl tail is attached directly to the cationic center.
Table 2.2 Biological activities of homopolymers and random copolymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>MIC, µg/mL (E. coli)</th>
<th>MIC, µg/mL (S. aureus)</th>
<th>HC50 (RBCs)</th>
<th>Selectivity (HC50/MIC)</th>
</tr>
</thead>
<tbody>
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<td>Polymethyl_1.6K</td>
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<td>1</td>
</tr>
<tr>
<td>Polyethyl_1.6K</td>
<td>&gt;2000</td>
<td>&gt;2000</td>
<td>&gt;2000</td>
<td>1</td>
</tr>
<tr>
<td>Polypropyl_1.6K</td>
<td>&gt;2000</td>
<td>809</td>
<td>&gt;2000</td>
<td>1</td>
</tr>
<tr>
<td>Polybutyl_1.6K</td>
<td>2000</td>
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<td>42</td>
<td>0.02</td>
</tr>
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<td>62</td>
<td>&gt;2000</td>
<td>&gt;2</td>
</tr>
<tr>
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<td>62</td>
<td>&gt;2000</td>
<td>&gt;2.2</td>
</tr>
<tr>
<td>Polypropyl_6K</td>
<td>810</td>
<td>62</td>
<td>&gt;2000</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>Polybutyl_6K</td>
<td>417(^d)</td>
<td>62(^d)</td>
<td>253</td>
<td>0.61</td>
</tr>
<tr>
<td>Copolybutyl_1.6K</td>
<td>13</td>
<td>18</td>
<td>16</td>
<td>1.2</td>
</tr>
<tr>
<td>Copolybutyl_6K</td>
<td>13</td>
<td>34</td>
<td>&lt;7</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Copoly1_6K</td>
<td>16</td>
<td>18</td>
<td>26</td>
<td>1.6</td>
</tr>
<tr>
<td>Copoly2_6K</td>
<td>21</td>
<td>18</td>
<td>&lt;7</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Copoly3_6K</td>
<td>16</td>
<td>18</td>
<td>&lt;7</td>
<td>&lt;0.4</td>
</tr>
</tbody>
</table>
Figure 2.4 Minimum inhibitory concentrations of 6k g/mol series polymers toward E. coli

Figure 2.5 Minimum inhibitory concentrations of 1.6k g/mol series polymers toward E. coli
The effect of reduction in polymer molecular weight on the antibacterial activity of these polymers against E. coli is apparent from Figure 2.5. As compared with the slightly antibacterial 6k series homopolymers (D.P. ~ 23), 1.6k series homopolymers (D.P. ~ 6) did not display antibacterial activity against E. coli till the highest polymer concentration tested (MIC >2000 µg/mL). Hence, there is a significant effect of molecular weight on the antibacterial activity of the polyacrylate homopolymers described here. In contrary to homopolymers, we did not observe the effect of molecular weight on the antibacterial activity of random copolymers at the levels of molecular weight tested in this study. The lower molecular weight random copolymer, Copolybutyl_1.6k, displayed high antibacterial activity (MIC = 13 µg/mL) toward E. coli, similar to its higher molecular weight counterpart Copolybutyl_6k.

Cationic amphiphilic polymers are believed to rupture the bacterial cell membrane through electrostatic and hydrophobic interactions. The presence of the hydrophobic alkyl tail directly on the cationic center may sterically hinder the electrostatic interactions of the cationic group with the negatively charged phospholipid head groups in cell membrane of E. coli, thus leading to low antibacterial activity in polyacrylate homopolymers. Moreover, the presence of cationic charge along with hydrophobic alkyl tail on each counit may hinder the penetration of amphiphilic homopolymers through the hydrophobic core of bacterial lipid bilayer. In random copolymers, the cationic groups and hydrophobic alkyl tails are located on separate counits, thus the cationic charges and hydrophobic groups in the random copolymers can effectively interact with the anionic cell membrane of E. coli. The high charge density in polyacrylate homopolymers may also be one of the reasons for their lower antibacterial activity for E. coli. Sampson and coworkers recently reported that homopolymers with smaller spatial distance (~4 Å) between
cationic centers displayed lower antibacterial activity, as compared with homopolymers with higher (8-10 Å) backbone distance between cationic centers.  

Kuroda et al. recently reported the effect of hydrophobic alkyl spacer arm (distance from polymer backbone to cationic center) lengths on the antibacterial and hemolytic activities of polymethacrylate homopolymers. Whereas the homopolymer with a 2-carbon spacer arm had low activity against E. coli, a homopolymer with a 6-carbon linear spacer arm displayed very high antibacterial activity and hemolytic activity. The effect of longer spacer arm resulting in higher antibacterial activity was attributed to the snorkel effect in which the cationic center can attach to the anionic lipid head groups of bacterial cell membrane and the long hydrophobic spacer arm could extend through the hydrophobic core of lipid bilayer.  

2.3.3 Antibacterial activity of polymers against S. aureus  

Antibacterial activity of polymers were obtained in terms of MIC against S. aureus, and are as shown in Figures 2.6 - 2.7 and Table 2.2. Significantly, all 6k series homopolymers displayed high antibacterial activity against S. aureus (MIC = 62 µg/mL), irrespective of the length of the alkyl tail (methyl to butyl) attached to cationic center. Polymethyl_6k with the shortest alkyl tail in this series of polymers displayed high activity against S. aureus, even though it was not found to be active against E. coli. Copolybutyl_6k having cationic and hydrophobic groups on separate counits also displayed high
Figure 2.6 Minimum Inhibitory Concentrations of 6k g/mol series polymers toward S. aureus

Figure 2.7 Minimum Inhibitory Concentrations of 1.6k g/mol series polymers toward S. aureus
antibacterial activity against S. aureus (MIC = 34 µg/mL). The similar antibacterial activity of polyacrylate homopolymers and random copolymer against S. aureus is in contrast with their activity against E. coli, in which the random copolymer, Copolybutyl_6k, displayed high antibacterial activity, whereas the homopolymers were significantly less active against E. coli.

Significant dissemblance in the cell wall structure of S. aureus and E. coli is a plausible reason behind higher activity of homopolymers against S. aureus than E. coli.\textsuperscript{49,111,112} In E. coli, a thin (8 nm) negatively charged peptidoglycan layer is sandwiched between an outer membrane (lipopolysaccharides) and an inner cytoplasmic membrane. In comparison with this double membrane structure of E. coli, S. aureus has only a single plasma membrane (cytoplasmic) surrounded by a much thicker (15-80 nm) negatively charged peptidoglycan layer (murein layer). Therefore, the double membrane structure of E. coli is more difficult to rupture or penetrate than the single membrane structure of S. aureus. Moreover, the presence of large number of cationic charges in the homopolymer would favor coulombic interactions with the thick negatively charged murein layer of S. aureus. The random copolymer, Copolybutyl_6k, did not show this selectivity between the two classes of bacteria and is highly potent against both E. coli and S. aureus.

The activities of lower molecular weight (~1.6k g/mol) polymers towards S. aureus are as shown in Figure 2.7 and Table 2.2. Polymethyl_1.6k and Polyethyl_1.6k did not show antibacterial activity against S. aureus (MIC > 2000 µg/mL). Further increase in alkyl tail length to propyl and butyl led to increase in antibacterial activity. Polybutyl_1.6k displayed a high antibacterial activity against S. aureus (MIC = 62 µg/mL), which is similar to the activity of its higher molecular weight counterpart Polybutyl_6k. This is in contrast with the activity of homopolymers against E. coli, as all 1.6k series homopolymers were inactive (MIC > 2000 µg/mL), whereas 6k series homopolymers showed moderate activity against E. coli. This observation further
corroborates the higher activity of these homopolymers against gram positive S. aureus, as compared with gram negative E. coli. The random copolymer Copolybutyl_1.6k, manifested high activity against S. aureus (MIC = 18 µg/mL). Hence, the random copolymers at both 1.6k g/mol and 6k g/mol molecular weight levels have high antibacterial activity toward both S. aureus and E. coli, whereas homopolymers in general show an increase in antibacterial activity with higher molecular weight.

2.3.4 Hemolytic activity of polymers

The toxicity of polymers toward mammalian cells were assessed in terms of hemolytic concentration-50% (HC<sub>50</sub>), which is the lowest polymer concentration required to lyse 50% of RBCs within an incubation period of 1 h. Our study found that majority of our homopolymers are non-hemolytic (HC<sub>50</sub> > 2000 µg/mL, Figures 2.8 - 2.9), whereas random copolymers were highly hemolytic against RBCs. Hemolytic activity of Polybutyl_6K is 36 times lower than the random copolymer, copolybutyl_6k. Cell membranes of RBCs lack the net negative charge, and hemolytic activity of amphiphilic polymers primarily arises from the hydrophobic interactions of polymers with RBCs’ cell membrane.<sup>17, 21, 54</sup> Higher hydrophobicity of polymers increases the ability of the polymers to permeate into the hydrophobic domain of lipid bilayer, leading to high hemolytic activity. The high cationic charge density in homopolymers would reduce the overall hydrophobicity of the homopolymers, and the presence of a cationic group along with every alkyl side tail in homopolymers would not favor hydrophobic interactions of homopolymers with the lipid bilayer of RBCs. However, in random copolymers (Scheme 2.4), cationic charges and hydrophobic alkyl side groups are placed on separate repeat units, and thus side groups can readily insert into lipid membrane of RBCs. In homopolymers, increasing the length of alkyl tail from
Figure 2.8 Hemolytic activity (HC50) of 6k g/mol series polymers against RBCs

Figure 2.9 Hemolytic activity (HC50) of 1.6k g/mol series polymers against RBCs
propyl to butyl led to marked increase in hemolytic activity. Therefore, three carbon atoms in the alkyl tail is the threshold for hydrophobicity, above which these homopolymers have higher hemolytic activity. Such a dramatic increase in hemolytic activity by increasing the length of alkyl tail from 3 to 4 carbon atoms has been reported by Sen and coworkers.\textsuperscript{113}

**2.3.5 Selectivity of polymers**

The selectivity of polymers toward bacteria over RBCs is defined as the ratio of hemolytic activity, HC\textsubscript{50}, to minimum inhibitory concentration, MIC, of polymers. High selectivity of polymers toward bacteria over mammalian cells is desired for therapeutic applications of polymers. All homopolymers in 6k g/mol series displayed selective activity toward bacteria over RBCs (Figure 2.10 and Table 2.2). However, homopolymers demonstrated lower selectivity for E. coli over RBCs, in comparison with selectivity toward S. aureus over RBCs. Among homopolymers, Polypropyl\textsubscript{6k} showed the highest selectivity of >2.5 times toward E. coli over RBCs. All homopolymers in 6k g/mol series, except Polybutyl\textsubscript{6k}, displayed a high selectivity of >32 times toward S. aureus over RBCs. Polybutyl\textsubscript{6k} due to its high hemolytic activity (HC\textsubscript{50} = 253 µg/mL) has the lower selectivity of 4.1 times for S. aureus over RBCs. Moreover, all homopolymers in 6k g/mol series were more selective towards S. aureus than E. coli. We found Polymethyl\textsubscript{6k} to be 17 times more active against S. aureus over E. coli. Hence, the polyacrylate homopolymers reported here are doubly selective; i.e. selective for bacteria over RBCs, and selective for one type of bacteria over other. As apparent from Figures 2.10 - 2.11 and Table 2.2, random copolymers have highly potent antibacterial and hemolytic activity, and thus lack selectivity for bacteria over RBCs. The lack of selectivity of random copolymers may limit their practical applications. We found the copolymers to have similar high activity against both E. coli and S. aureus.
Figure 2.10 Biological activity (MIC and HC₅₀) of 6k g/mol series polymers.

Figure 2.11 Biological activity (MIC and HC₅₀) of 1.6k g/mol series polymers.
2.3.6 Effect of alkyl side group shape on the biological activities of random copolymers

The pertinence of the role of the hydrophobic side chain on optimizing the antimicrobial and hemolytic activity amphiphilic copolymers has been well established, but the role of the hydrophobic side chain shape on antibacterial and hemolytic activity of random copolymers has not been explored, to the best of our knowledge. To this end, we synthesized polyacrylate random copolymers having different shapes of hydrophobic alkyl chain while keeping the same number of carbon atoms in side chain. Copoly1—6K, Copoly2—6K, and Copoly3—6K were synthesized (Scheme 2.4) to explore the role of the hydrophobic side chain shape on biological activity of SMAMPs. As evident from the data compiled in Figure 2.12 and Table 2.2, all three copolymers exhibited similar antibacterial activities against E. coli and S. aureus. Hence, we did not observe any effect of shape of alkyl side chain on antibacterial activity of amphiphilic copolymers. Copoly2_6K and Copoly3_6K were highly hemolytic (HC$_{50}$<7 µg/mL), whereas slightly lower hemolytic activity in case of Copoly1_6K (HC$_{50}$ = 26 µg/mL) was observed.

![Biological activity (MIC and HC$_{50}$) of random copolymers with various shapes of alkyl side group.](image)

Figure 2.12 Biological activity (MIC and HC$_{50}$) of random copolymers with various shapes of alkyl side group.
2.4 Conclusion

In conclusion, the position of the hydrophobic alkyl side group with respect to the cationic center has substantial effect on the antibacterial and hemolytic activity of amphiphilic polymers. In the “same centered” homopolymers, the presence of the alkyl tail on the cationic center resulted in selective activity toward S. aureus over RBCs and S. aureus over E. coli. We found that the molecular weight of our polymers has a significant effect on their antibacterial activities. Higher molecular weight homopolymers demonstrated higher antibacterial activity in comparison with lower molecular weight homopolymers. In contrary to homopolymers, “separate center” random copolymers having alkyl side groups and cationic centers separately on different repeating units displayed high antibacterial and hemolytic activity, leading to non-selective antibacterial activity. Random copolymer displayed similar antibacterial activity at the molecular weight levels investigated in this study. The role of shape of hydrophobic alkyl side groups was investigated. We observed that the biological activity of random polyacrylate copolymers described here does not depend on the shape of the alkyl side chain having six carbon atoms, or the molecular weight of random copolymers.
Chapter 3

Synthesis of Cationic Amphiphilic Polyacrylates with Superior Bactericidal Activity and Concomitant Low Hemolytic Activity

3.1 Introduction

One of the major challenges toward therapeutic applications of synthetic amphiphilic polymers is their toxicity to mammalian cells. The biocompatibility of synthetic polymers has been widely assessed in terms of hemolytic activity toward red blood cells (RBCs). Some of the factors affecting the hemolytic activity of synthetic amphiphilic polymers have been recently explored. Compared with random copolymers, block copolymer architecture can show lower toxicity toward RBCs.\textsuperscript{96, 97} “Same centered” polymers, having an alkyl tail attached to their cationic center, demonstrated lower hemolytic activity in comparison with “separate center” copolymers.\textsuperscript{109, 113} Incorporation of hydrophilic poly(ethylene glycol) side groups reduced hemolytic activity in N-hexylated poly(vinyl pyridines).\textsuperscript{60-63} Mole ratios and size of hydrophobic groups were varied to optimize the antibacterial and hemolytic activity of polymers.\textsuperscript{52, 54, 55, 71, 76, 113}

In chapter 2, we reported that amphiphilic polyacrylates with 2-carbon spacer arms and various lengths of alkyl tail attached to the cationic center have low hemolytic activity and high activity against S. aureus. Kuroda and coworkers recently reported an amine functionalized polymethacrylate homopolymer with a 6-carbon linear spacer arm to be highly active against both E. coli and S. aureus.\textsuperscript{57} However, the hemolytic activity of this polymer was also extremely high, and it did not display selectivity toward bacteria over RBCs.\textsuperscript{57} Thus, by copolymerizing a longer spacer arm monomer with a short spacer arm monomer, copolymers with high antibacterial activity and lower hemolytic activity could be expected. The “snorkel effect”\textsuperscript{114-117} from longer spacer arms
in which the cationic charges attach to the bacterial cell membrane and the long alkyl spacer arms can extend through the hydrophobic core of lipid bilayer may lead to high antibacterial activity, whereas the high cationic charge density and lower hydrophobicity of short spacer arms lower the hemolytic ability of polymers.

Here we report the synthesis of highly antibacterial but selective (bacteria over RBCs) polyacrylates by copolymerization of a monomer (M2) having a 2-carbon spacer arm (distance from polymer backbone to cationic center) with a 6-carbon spacer arm monomer (M6), in varying mole ratios (Scheme 3.3). All copolymers in the range of 10 to 90 mole% of M6 displayed highly selective activity toward bacteria over RBCs. A copolymer having 90 mole% of M6 demonstrated 208 times more selectivity towards E. coli over RBCs (Table 3.2 and Figure 3.6).

3.2 Experimental

3.2.1 Materials

2-(Methylamino)ethanol, methyl 3-mercaptopropionate (MMP), acetonitrile (anhydrous), N,N-diisopropylethylamine, 2,2’-Azobis(2-methylpropionitrile) (AIBN), tetrahydrofuran (THF), and 6-Amino-1-hexanol were purchased from Sigma-Aldrich and used without further purification. Acryloyl chloride was purchased from Sigma-Aldrich and distilled prior to use. Di-tert-butyl dicarbonate, trifluoroacetic acid, hexane, and diethyl ether were purchased from Alfa Aesar and used as received. Dichloromethane and ethyl acetate were purchased from BDH. All other chemicals and reagents were used without further purification.
3.2.2 Instrumentation

$^1$H and $^{13}$C NMR spectra of polymers were obtained on a Varian Unity NMR spectrometer (600 MHz) using CDCl$_3$ or DMSO-d$_6$ as solvents. Molecular weights and polydispersities (PDI) of Boc-protected polymers were estimated on an EcoSec HLC-83220 gel permeation chromatography instrument (RI detector) using linear polystyrene standards and THF as eluent. Bacterial growth in E. coli and S. aureus assays were determined by measuring turbidity as optical density at $\lambda=600$ nm (OD$_{600}$), on an Agilent 8453 spectrophotometer using 1 cm path length plastic cuvette. SpectraMax 340 PC micro plate reader from Molecular Devices was used to measure OD$_{595}$ (Antibacterial test) and OD$_{414}$ (Hemolysis test).

3.2.3 Synthesis of N-Boc protected 6-hexanol-1-amine$^{57}$

Scheme 3.1 Synthesis of tert-butyl (6-hydroxyhexyl)carbamate.

\[
\begin{align*}
\text{HO-} & \quad \text{(Boc)$_2$O, room temp.} \\
& \quad \text{1M NaOH : THF (1:1)} \quad \text{24 h} \\
\text{NH$_2$} & \quad \text{60\% yield.} \\
\text{HO-} & \quad \text{N$_2$Boc}
\end{align*}
\]

9.16 g (42 mmol) di-tert-butyl dicarbonate in 20 mL THF was added drop wise to a 250 mL round bottom flask already charged with 4.92 g (42 mmol) 6-hexanol-1-amine, 30 mL THF, and 50 mL 1M NaOH. The biphasic reaction mixture was stirred at room temperature for 24 hours. The reaction mixture was extracted with ethyl acetate, and subsequently washed with water and saturated sodium bicarbonate solution. The resulting organic layer was dried with sodium sulfate, and solvent was evaporated using a Rotavapor. 60% yield. $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 1.26-1.38 (m, 4H), 1.39-1.43 (s, 9H), 1.43-1.48 (m, 2H), 1.50-1.57 (m, 2H), 3.08 (t, 2H), 3.6 (t, 2H).
3.2.4 Synthesis of 6-((tert-butoxycarbonyl)amino)hexyl acrylate (Monomer M6)\textsuperscript{57}

Scheme 3.2 Synthesis of 6-((tert-butoxycarbonyl)amino)hexyl acrylate.

N-Boc protected 6-hexanol-1-amine (5 g, 23 mmol), triethyl amine (4 mL), and dichloromethane (50 mL) were added into a 100 mL round bottom flask. The flask was sealed with septa and reaction mixture was purged with nitrogen (using a stainless steel needle) for 5 minutes. A solution of acryloyl chloride (5 mL, 23 mmol) in 5mL dichloromethane was added drop-wise to the reaction mixture at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred overnight, followed by washing with distilled water and saturated sodium bicarbonate solution. The organic layer was dried with sodium sulfate and solvent was evaporated using a Rotavapor. The resultant liquid was purified by silica gel chromatography using 9:1 hexane/ethyl acetate as eluent. 60% yield. \textsuperscript{1}H NMR (600 MHz, CDCl\textsubscript{3}): δ 1.28-1.36 (m, 4H), 1.37-1.42 (s, 9H), 1.42-1.48 (m, 2H), 1.63 (m, 2H), 3.06 (s, 2H), 4.10 (t, 2H), 5.77 (dd, 1H), 6.07 (q, 1H), 6.35 (dd, 1H). \textsuperscript{13}C NMR (151 MHz, CDCl\textsubscript{3}): δ 25.6, 26.35, 28.37, 28.49, 29.93, 40.39, 64.42, 78.95, 128.51, 130.50, 156, and 166.26.

3.2.5 Synthesis of 2-((tert-butoxycarbonyl)methylamino)ethyl acrylate (Monomer M2)

Monomer M2 was synthesized as described in Chapter 2.
3.2.6 Synthesis of amphiphilic Polymers

Homopolymers and random copolymers were synthesized using a slightly modified literature procedure. Monomer M6 (0.814 g, 3 mmol) and Monomer M2 (0.688 g, 3 mmol) were added into a 100 mL round bottom flask containing AIBN (9.85 mg), MMP (33.2 µL), and 6 mL acetonitrile (anhydrous). The flask was sealed with septa and the reaction mixture was purged with nitrogen (using a stainless steel needle) for 5 minutes, followed by stirring at 65 °C for 18 hours. Solvent was then evaporated under reduced pressure, and polymer was re-dissolved in 2 mL THF and precipitated in hexane twice. The resulting polymer was dissolved in excess trifluoroacetic acid and left under stirring for 3 hours. TFA was removed under reduced pressure and polymer was dissolved in methanol and precipitated in diethyl ether 3 times. The resulting polymer was kept under vacuum for 3 days and lyophilized. $^1$H NMR (600 MHz, D$_2$O): δ 1.19-1.33 (bs, 53H), 1.41-2.45 (bm, 131H), 2.56 (s, 2H), 2.62-2.72 (bs, 42H), 2.81-2.89 (bs, 26H), 3.20-3.31 (bs, 24H), 3.57 (s, 3H), 3.84-4.05 (26H), 4.10-4.33 (24H). $^1$H NMR of all other cationic amphiphilic polymers and GPC curves of precursor polymers are as shown in section 3.5 and 3.6.
3.2.7 Antibacterial and hemolytic activity of polymers

Antibacterial activity of polymers in terms of Minimum Inhibitory Concentrations (MIC) and hemolytic activity of polymers in terms of HC\textsubscript{50} were obtained as described in Chapter 2.

3.2.8 Field-Emission Scanning Electron Microscopy Analysis (FESEM) of bacterial membrane disruption\textsuperscript{118 (a), 119}

Bacteria (E. coli and S. aureus) were cultured in nutrient media (LB broth for E. coli and MH broth for S. aureus) at 37 °C for 24 h. 1 mL of bacterial suspension was dissolved in 9 mL fresh nutrient broth and incubated at 37 °C to obtain bacterial cell suspension in log phase growth (OD\textsubscript{600} = 0.45 to 0.5). This cell suspension was further diluted with fresh nutrient broth to obtain a final cell suspension with OD\textsubscript{600} = 0.1 (~ 10\textsuperscript{8} CFU/mL). 900 µL of this cell suspension was added into 100 µL aqueous solution of PM6-90% (6 × MIC), followed by incubation at 37 °C for 2 h under constant shaking (200 rpm). Untreated cell suspension (without polymer) was used as a control. Bacteria were obtained by centrifuging the suspension at 12000 rpm for 2 minutes. Cells were washed with PBS (twice), and fixed by treating with glutaraldehyde (2.5%) for 60 minutes. Cells were then washed with deionized water, and sequentially dehydrated with 30, 50, 70, 80, 90, and 100% aqueous ethanol solutions. The dehydrated cells were dried at room temperature for two days before being mounted on a carbon tape. The samples were sputter coated with Au/Pd (60:40). SEM analysis was performed on an AMRAY 1910 Field Emission Scanning Microscope at an operating voltage of 5 kV.

3.2.9 Time dependent killing efficiency study of polymer toward E. coli\textsuperscript{119}

Bacteria (E. coli) were cultured in nutrient broth overnight. This cell suspension was diluted with fresh nutrient broth and incubated at 37 °C for a period of approximately 1.5 h to achieve log-
phase growth (OD$_{600}$ ~ 0.5). Cell culture in log-phase growth was further diluted with fresh nutrient broth to obtain stock cell suspension with OD$_{600}$ = 0.001 (~ $10^5$ CFU/mL). This stock cell suspension was treated with polymers at two concentrations (1×MIC and 2×MIC), and bacterial cell suspension without polymer treatment was used as a control. Bacterial samples were taken out at regular time intervals (0 h, 1 h, 2 h, 4 h, 6 h, and 8 h), and were serially diluted using several 10 fold dilutions. 20 µL of final cell dilution was streaked on to an Agar plate for incubation at 37 °C for 24 h, followed by counting of Colony Forming Units (CFU).

3.3 Results and Discussion

3.3.1 Synthesis of polymers

We synthesized a series of polyacrylate random copolymers via free radical copolymerization of a monomer having a 2- carbon spacer arm (M2), with a 6-carbon spacer arm monomer (M6). AIBN was used as an initiator and methyl 3- mercaptopropionate was used as a chain transfer agent for molecular weight control. The mole % (in feed) of M6 was increased in steps of 10%, giving polymers with compositions that closely match the feed mole% (Table 3.1). Our synthetic approach was expected to result in random copolymers of M2 and M6, rather than the homopolymers of M2 and M6, because M2 and M6 are both acrylate monomers with similar structures and can be expected to have similar monomer reactivity ratios (r) close to 1, leading to the formation of copolymers with random counit placement. For example, glycidyl acrylate (r = 1.18) and 2-ethylhexyl acrylate (r = 1.12) have similar monomer reactivity ratios close to 1 for free radical copolymerization. The mole ratios found in our resulting copolymers are close to the feed mole ratios of monomers over the entire range of polymer compositions, including the cases of PM6-10% and PM6-90% after 18 h of reaction time (Table 3.1). Thus, the homopolymerization of one of the two comonomers is not likely. The molecular weights of precursor copolymers, before
deprotection with TFA, were estimated using Gel Permeation Chromatography. The average degree of polymerization (DP) of polymers was obtained from $^1$H NMR, using end group analysis. The DP of all polymers was found to be in close proximity of 30. PM6-x%, denotes the cationic amphiphilic copolymer having x mole% (in feed) of M6 monomer.

Table 3.1 Characterization of polymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$^a$ Mol% M6</th>
<th>$^a$ Mole % M2</th>
<th>$M_w$ (kDa)</th>
<th>$M_n$ (kDa)</th>
<th>PDI</th>
<th>$^b$D.P.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM6-0%</td>
<td>0</td>
<td>100</td>
<td>6.2</td>
<td>4.3</td>
<td>1.45</td>
<td>30</td>
</tr>
<tr>
<td>PM6-10%</td>
<td>11</td>
<td>89</td>
<td>6.6</td>
<td>4.5</td>
<td>1.47</td>
<td>39</td>
</tr>
<tr>
<td>PM6-20%</td>
<td>21</td>
<td>79</td>
<td>6.3</td>
<td>4.3</td>
<td>1.46</td>
<td>33</td>
</tr>
<tr>
<td>PM6-30%</td>
<td>30</td>
<td>70</td>
<td>6.6</td>
<td>4.5</td>
<td>1.46</td>
<td>33</td>
</tr>
<tr>
<td>PM6-40%</td>
<td>40</td>
<td>60</td>
<td>6.8</td>
<td>4.6</td>
<td>1.46</td>
<td>30</td>
</tr>
<tr>
<td>PM6-50%</td>
<td>52</td>
<td>48</td>
<td>6.9</td>
<td>4.6</td>
<td>1.49</td>
<td>25</td>
</tr>
<tr>
<td>PM6-60%</td>
<td>61</td>
<td>39</td>
<td>6.7</td>
<td>4.6</td>
<td>1.48</td>
<td>30</td>
</tr>
<tr>
<td>PM6-70%</td>
<td>71</td>
<td>29</td>
<td>7.1</td>
<td>4.8</td>
<td>1.47</td>
<td>25</td>
</tr>
<tr>
<td>PM6-80%</td>
<td>81</td>
<td>19</td>
<td>7.8</td>
<td>5.4</td>
<td>1.46</td>
<td>31</td>
</tr>
<tr>
<td>PM6-90%</td>
<td>91</td>
<td>9</td>
<td>7.2</td>
<td>5.1</td>
<td>1.42</td>
<td>29</td>
</tr>
<tr>
<td>PM6-100%</td>
<td>100</td>
<td>0</td>
<td>7.5</td>
<td>5.8</td>
<td>1.28</td>
<td>36</td>
</tr>
</tbody>
</table>

$^a$Actual mole% of monomer in polymers, as calculated by $^1$H NMR. $^b$As calculated from $^1$H NMR
Figure 3.1 Graph showing that the actual mole % of M6 counits in the copolymers closely matches the feed mole % of M6 monomer

3.3.1.1 Determination of degree of polymerization and actual mole percentage of repeating units in polymer

In Figure 3.2, a single peak at \( \delta 3.57 \) belongs to the methyl protons (q) at chain end. A broad single peak at \( \delta 3.84-4.05 \) (f) belongs to methylene protons in M6 monomer repeat unit. Methylene protons in M2 monomer repeat unit (k) give a chemical shift at \( \delta 4.10-4.33 \). Degree of polymerization of polymer is calculated as below:

Degree of Polymerization \( = [(25.98/2) + (24.29/2)]/ (3/3) = 25 \)

Mole % of M6 repeat units: \( 25.98/ (25.98+24.29) = 52\% \)

Mole % of M2 repeat units: \( 24.29/ (25.98+24.29) = 48\% \)
3.3.1.2 Calculation of ratio of rate of chain transfer to rate of propagating chain coupling

Due to high rate constant for termination by coupling (~$10^6$ L/mol/s),\textsuperscript{118(b)} coupling between propagating species is possible resulting in two methyl esters per chain. However, in our case we expect chain transfer to dominate (>97%) due to: the very low concentration of propagating species in free radical polymerization (~$10^{-6}$ M); high rate constant for chain transfer of acrylates to MMP (~$1.6 \times 10^3$ L/mol/s using chain transfer constant as 2 and rate constant for propagation as 800 L/mol/s);\textsuperscript{118(b)} as well as high concentration of chain transfer agent ($5 \times 10^{-2}$ M) used in our polymer synthesis. A conservative estimate of the ratio of rate of chain transfer to chain coupling is at least

\[ \frac{\text{Rate of Chain Transfer}}{\text{Rate of Chain Coupling}} \approx \frac{10^{-6} \times 0.05 \times 1.6 \times 10^3}{10^{-6} \times 10^{-6} \times 10^6} = 45.4 \]

3.3.2 Antibacterial activity of polymers toward E. coli

The antibacterial activities of polymers were determined in terms of minimum inhibitory concentration (MIC) against E. coli. MIC is defined as the minimum polymer concentration required to inhibit 100% bacterial growth after an incubation period of 18 h. As is apparent from Figure 3.3 and Table 3.2, MIC values of polymers reduced substantially as the mole % of M6 was increased in the polymers. Whereas a homopolymer of 2-carbon spacer arm monomer M2, PM6-0%, did not display antibacterial activity against E. coli, incorporation of just 20 mole % of M6 monomer led to substantial (~ 6 times) decrease in MIC value. Further reduction in MIC value was observed with increasing the mole % of M6 monomer. PM6-90% and PM6-100% demonstrated the lowest MIC values against E. coli in our series of polymers. Sampson et al. reported the effect of backbone spacer distance between attach points of side groups (having pendent cationic charge).\textsuperscript{95} Homopolymers 11 and 12 with a smaller backbone spacer arm distance between cationic
centers (~ 4 Å) lacked antibacterial activity, whereas homopolymers 13 and 14 with higher backbone spacer arm distance (~ 8 Å) displayed high antibacterial activity. It was reported that the charge density in homopolymers 11 and 12 could be too high to cause bacterial killing, hence a higher backbone spacer distance (hydrophobic spacer) of 4 Å to 8 Å is required for antibacterial activity in that polymer system. However, the increase in backbone spacer distance led to increase in hemolytic activity also. Whereas homopolymers 11 and 12 were found to be non-hemolytic (HC$_{50}$ > 2000 µg/mL), homopolymers 13 and 14 showed high hemolytic activity (HC$_{50}$ = 379 µg/mL and 443 µg/mL). Our approach of copolymerizing the 2-carbon spacer arm monomer M2 with the 6-carbon spacer arm monomer M6, changes the spatial charge distribution in polymer, even though the backbone spacer distance remains the same thus reducing the overall charge density in the polymer. As both cationic charge and hydrophobic groups are required in the polymer to

Table 3.2 Biological activities of polymers toward bacteria and RBCs.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>MIC (µg/mL) E. coli</th>
<th>MIC (µg/mL) S. aureus</th>
<th>HC$_{50}$ (µg/mL) RBCs</th>
<th>Selectivity HC$_{50}$/MIC</th>
<th>E. coli</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM6-0%</td>
<td>1428</td>
<td>104</td>
<td>&gt;2000</td>
<td>&gt;1.4</td>
<td>&gt;19</td>
<td></td>
</tr>
<tr>
<td>PM6-10%</td>
<td>809</td>
<td>62</td>
<td>&gt;2000</td>
<td>&gt;2.5</td>
<td>&gt;32</td>
<td></td>
</tr>
<tr>
<td>PM6-20%</td>
<td>250</td>
<td>62</td>
<td>&gt;2000</td>
<td>&gt;8</td>
<td>&gt;32</td>
<td></td>
</tr>
<tr>
<td>PM6-30%</td>
<td>125</td>
<td>52</td>
<td>1667</td>
<td>13</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>PM6-40%</td>
<td>62</td>
<td>52</td>
<td>1619</td>
<td>26</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>PM6-50%</td>
<td>52</td>
<td>52</td>
<td>&gt;2000</td>
<td>&gt;38</td>
<td>&gt;38</td>
<td></td>
</tr>
<tr>
<td>PM6-60%</td>
<td>41</td>
<td>31</td>
<td>&gt;2000</td>
<td>&gt;49</td>
<td>&gt;64</td>
<td></td>
</tr>
<tr>
<td>PM6-70%</td>
<td>26</td>
<td>31</td>
<td>&gt;2000</td>
<td>&gt;80</td>
<td>&gt;64</td>
<td></td>
</tr>
<tr>
<td>PM6-80%</td>
<td>16</td>
<td>26</td>
<td>&gt;2000</td>
<td>&gt;125</td>
<td>&gt;80</td>
<td></td>
</tr>
<tr>
<td>PM6-90%</td>
<td>7.8</td>
<td>16</td>
<td>1619</td>
<td>208</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>PM6-100%</td>
<td>5.8</td>
<td>16</td>
<td>&lt;1.9</td>
<td>&lt;0.33</td>
<td>&lt;0.12</td>
<td></td>
</tr>
</tbody>
</table>
permeate and disrupt the bacterial cell membrane, a balance is required between cationic and hydrophobic groups in the polymers. PM6-0% with a short 2-carbon spacer arm has a higher charge density and less hydrophobicity thus reducing its ability to penetrate through the hydrophobic core of the lipid bilayer. High charge density and small distance between cationic charges may restrain the conformational freedom of side groups, thus affecting the lipid bilayer permeability of the polymers. Incorporation of the 6-carbon spacer arm monomer M6 with its long hydrophobic spacer arm increases the overall hydrophobicity of polymer. Addition of M6 also increases the distance between cationic groups on adjacent repeating units, thus reducing the repulsion between cationic charges.
3.3.3 Antibacterial activity of polymers toward S. aureus

Antibacterial activities of polymers in terms of MIC were determined against the gram positive S. aureus (ATCC 25923) and are as shown in Table 3.2 and Figure 3.4. All polymers in this series displayed high antibacterial activity against S. aureus. The homopolymer of M2, PM6-0%, manifested an MIC of 104 µg/mL toward S. aureus, whereas PM6-0% was found to be inactive against E. coli (MIC = 1428 µg/mL). The effect of increasing monomer M6 mole% on the antibacterial activity of polymers toward S. aureus was less pronounced as compared with E. coli (Table 3.2 and Figure 3.3). We did not observe substantial increase in antibacterial activity against S. aureus till incorporating 50 mole% of M6 monomer. Compared with the MIC values of PM6-0%, PM6-90% displayed 7 times lower MIC value against S. aureus, and 183 times lower MIC value against E. coli.

Figure 3.4 Minimum inhibitory concentrations of polymers toward S. aureus
3.3.4 Hemolytic activity of polymers toward mouse red blood cells (RBCs)

Hemolytic activities of polymers were determined in terms of hemolytic concentration – 50% (HC\textsubscript{50}), which is the minimum polymer concentration resulting in 50% lyses of mouse RBCs within an incubation period of 1 hour. HC\textsubscript{50} values of polymers are as shown in Table 3.2 and Figure 3.5. Significantly, all polymers, except PM6-100%, displayed very low hemolytic against RBCs. In comparison with extremely hemolytic PM6-100%, polymer PM6-90% (HC\textsubscript{50} = 1619 \mu g/mL) was found to be approximately 850 times less hemolytic toward RBCs, even though its antibacterial activities are similar to PM6-100%. We found that majority of our polymers have HC\textsubscript{50} value in excess of 2000 \mu g/mL, which is the highest polymer concentration tested in this study.

RBCs’ cell membrane consists of zwitterionic phospholipid head groups, and thus lacks net negative charge on its outer surface. Amphiphilic polymers can penetrate the RBCs’ cytoplasmic membrane through hydrophobic interactions. The observation that all of our polymers in the range of PM6-0% to PM6-90% are non-hemolytic but PM6-100% is highly hemolytic, indicates that even a small mole% of the shorter spacer arm counits (~3 per chain) can prevent the insertion of these polymers into lipid bilayer of RBCs. Monomer M6 has a long hydrophobic spacer arm, and increasing the mole% of M6 should have inevitably led to a rapid increase in hemolytic activity.

Incorporation of 20-30 mole% of hydrophobic monomer in polymers was reported to drastically increase the hemolytic activity of polymers.\textsuperscript{54,55,59} In our polymers, the presence of positive charge on each counit and a combination of longer and shorter cationic spacer arms led to high antibacterial but low hemolytic activity.
3.3.5 Selectivity of polymers

The selectivity of a polymer toward bacteria over RBCs is defined in terms of the ratio of hemolytic activity to minimum inhibitory concentration of the polymer (HC$_{50}$/MIC). The selectivity of polymers toward bacteria over RBCs is apparent from Figure 3.6 and Table 3.2. PM6-100% was highly antibacterial as well as hemolytic, whereas PM6-90% was found to be 208 times more selective toward E. coli over RBCs, and 108 times selective toward S. aureus over RBCs. PM6-80% is >133 times selective toward E. coli over RBCs and >80 times more selective toward S. aureus over RBCs. Both polymers displayed high antibacterial activity similar to PM6-100%. All copolymers in the range of 0 to 90 mole% of M6 monomer manifested highly selective antibacterial activity.
A majority of these polymers displayed doubly selective antibacterial activity i.e. bacteria over RBCs and one type of bacteria over another. Polymer containing 0 to 60 mole% of M6 monomer displayed selective antibacterial activity against S. aureus over E. coli. PM6-0%, a homopolymer of M2, is inactive against E. coli, but it displayed high activity against S. aureus. Significant differences in the cell envelope structure of gram negative E. coli and gram positive S. aureus may be a probable reason behind this observation.\textsuperscript{49, 111, 112} The double membrane structure of the gram negative E. coli is more difficult to penetrate than the single membrane structure of the gram positive S. aureus. Furthermore, S. aureus has a 15-80 nm thick negatively charged murein layer (peptidoglycans) covering the lipid bilayer; whereas E. coli has a thin 6 nm thick peptidoglycan layer, sandwiched between the outer and inner membranes. This may result in higher coulombic interactions between PM6-0% and S. aureus, as compared with E. coli.
Figure 3.6 Biological activity (MIC and HC$_{50}$) of polymers

Figure 3.7 Schematic showing the cell envelope structure of bacteria and red blood cell.
3.3.6 Scanning electron microscopy analysis of bacterial membrane disruption

The MIC assay does not distinguish between the bacteriostatic (growth inhibiting) and bactericidal (killing) ability of polymers. Also, MIC assay does not provide details about the mechanism of antibacterial action of amphiphilic polymers. The membrane rupturing mechanism of antibacterial action of our polymers was confirmed using the FE-SEM analysis. E. coli and S. aureus were treated with 6×MIC concentration of polymers for 2 hours. The cell morphologies of bacterial cells treated with polymers were analyzed using SEM. Bacterial cells without polymer treatment were used as controls. As shown in Figure 3.8, control E. coli cells (without polymer treatment) showed intact cell surface morphologies. Similarly, control S. aureus cells show no damage to the cell surface (Figure 3.10). As evident from Figure 3.9, E. coli treated with PM6-90% displayed severe damage and rupture of the cell surface. Most of the E. coli cells were covered with cell lysate and the rod shaped morphology of E. coli was not recognizable. Similar results were obtained after treating E. coli with PM6-60% and PM6-30% (Figures 3.12 and 3.14), confirming that these polymers disrupt and rupture the cell membrane of bacteria.

Figures 3.11, 3.13, and 3.15 show the S. aureus treated with polymers PM6-100%, PM6-60%, and PM6-30%, respectively. Similar to our observation in case of E. coli, S. aureus cells treated with polymers displayed extensive cell surface damage and leakage of intercellular material.
Figure 3.8 FE-SEM image of E. coli cells without polymer treatment (control) show intact cell surface morphology

Figure 3.9 FE-SEM image of E. coli cells treated with PM6-90% show severe damage and rupture of bacterial cell surface
Figure 3.10 FE-SEM image of S. aureus cells without polymer treatment (control) show intact cell surface morphology

Figure 3.11 FE-SEM image of S. aureus cells treated with PM6-90% show severe damage and rupture of bacterial cell surface
Figure 3.12 FE-SEM image of E. coli cells treated with PM6-60% show severe damage and rupture of bacterial cell surface.

Figure 3.13 FE-SEM image of S. aureus cells treated with PM6-60% show severe damage and rupture of bacterial cell surface.
Figure 3.14 FE-SEM image of E. coli cells treated with PM6-30% show severe damage and rupture of bacterial cell surface

Figure 3.15 FE-SEM image of S. aureus cells treated with PM6-30% show severe damage and rupture of bacterial cell surface
3.3.7 Time dependent killing efficiency study of polymer PM6-90% against E. coli

In order to further explore the bactericidal ability of these polymers, PM6-90%, the polymer with highest selectivity toward E. coli over RBCs in this series, was used as a representative polymer. E. coli at an initial concentration of ~ 10^5 CFU/mL were treated with PM6-90% for various time intervals. Within 2 hours of incubation, more than 90% of bacterial colony forming units were found to be killed (Table 3.3 and Figure 3.16). At both 1 × MIC and 2 × MIC concentrations of PM6-90%, 100% killing of bacterial colony forming units was observed within an incubation period of 6 hours. This 5-log reduction in bacterial CFUs within few hours of incubation with PM6-90% demonstrates the highly potent bactericidal ability of these polymers. This test also confirmed that the minimum bactericidal concentration i.e. the minimum polymer concentration to result in 100% killing of bacterial CFUs is equal to the MIC value of PM6-90%.

Table 3.3 Time-dependent killing efficiency of PM6-90% toward E. coli. Viable cells (CFU/mL) are shown after incubating with PM6-90% or without PM6-90% (control), for various time intervals

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>E. coli (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 × MIC</td>
</tr>
<tr>
<td>0</td>
<td>6.0 × 10^4</td>
</tr>
<tr>
<td>1</td>
<td>3.0 × 10^4</td>
</tr>
<tr>
<td>2</td>
<td>4.7 × 10^3</td>
</tr>
<tr>
<td>4</td>
<td>6 × 10^2</td>
</tr>
<tr>
<td>6</td>
<td>nil</td>
</tr>
<tr>
<td>8</td>
<td>nil</td>
</tr>
</tbody>
</table>
Figure 3.16 Time-dependent killing efficiency curve of PM6-90% against E. coli

Figure 3.17 Confirmation of bactericidal activity of PM6-90% against E. coli at various time intervals using Agar plate assay. The final polymers concentration was equal to 1×MIC. E. coli without polymer treatment was used as positive control and is shown in a) at 1 h, b) at 6 h time interval. E. coli treated with PM6-90% at c) 0 h, d) 1h, e) 2 h, f) 4 h, g) 6 h, and h) 8 h time interval
3.4 Conclusions

In conclusion, we have synthesized cationic amphiphilic acrylic random copolymers having 2- and 6-carbon spacer arm counits as antibacterial agents. These amphiphilic copolymers (DP ~ 30) displayed superior antibacterial activities with concomitant low hemolytic effect. The homopolymer with a 6-carbon spacer arm (PM6-100%) has high cell membrane disruption ability and is non-selective (high antibacterial and hemolytic activity). Incorporation of a 2-carbon spacer arm counits in the copolymers resulted in highly selective antibacterial activity. We found that with just 10 mole% of M2 counits, PM6-90% polymer displayed drastically reduced hemolytic activity, by a factor of 850, without serious deterioration of antibacterial activity, in comparison with highly hemolytic PM6-100% homopolymer. Unlike the case of strong electrostatic interactions between the negatively charged cell surface of bacteria and cationic polymers, the selectivity of cationic amphiphilic polymers toward bacteria over RBCs may arise from the weaker electrostatic interactions between cationic polymers and zwitterionic lipid head groups of the RBC membrane. Incorporation of only three M2 counits into a chain with DP of ~30 can significantly impact the conformation of the polymer during the process of membrane rupture. M2 with a four carbon shorter hydrophobic spacer arm than M6, can lead to a level of polymer conformation with fewer degrees of freedom, thus substantially reducing the “snorkel effect” of a longer spacer arm of the copolymer. This copolymer system represents one of the most promising systems in synthetic polymeric antibacterial agents. The control of spacer arm lengths and copolymer composition can serve as one of the effective structural parameters in the synthesis of polymers with highly selective (bacteria over mammalian cells) antibacterial activity.
3.5 $^1$H NMR spectra of cationic amphiphilic polymers

Figure 3.18 $^1$H NMR spectrum of polymer PM6-0% (600 MHz, D$_2$O)

Figure 3.19 $^1$H NMR spectrum of polymer PM6-10% (600 MHz, D$_2$O)
Figure 3.20 $^1$H NMR spectrum of polymer PM6-20% (600 MHz, D$_2$O)

Figure 3.21 $^1$H NMR spectrum of polymer PM6-30% (600 MHz, D$_2$O)
Figure 3.22 $^1$H NMR spectrum of polymer PM6-40% (600 MHz, D$_2$O)

Figure 3.23 $^1$H NMR spectrum of polymer PM6-50% (600 MHz, D$_2$O)
Figure 3.24 $^1$H NMR spectrum of polymer PM6-60% (600 MHz, D$_2$O)

Figure 3.25 $^1$H NMR spectrum of polymer PM6-70% (600 MHz, D$_2$O)
Figure 3.26 $^1$H NMR spectrum of polymer PM6-80% (600 MHz, D$_2$O)

Figure 3.27 $^1$H NMR spectrum of polymer PM6-90% (600 MHz, D$_2$O)
Figure 3.28 $^1$H NMR spectrum of polymer PM6-100% (600 MHz, D$_2$O)
3.6 Gel Permeation Chromatography curves of precursor polymers (before Boc deprotection)

Figure 3.29 GPC traces of precursor (Boc protected) polymers.
Figure 3.30 GPC traces of precursor (Boc protected) polymers.
Chapter 4

Effect of Incorporating Non-ionic Hydrophilic Poly(ethylene glycol) Counits on the Biological Activities of Amphiphilic Polymers Having 6-carbon Spacer Arm Counits as Bioactive Component

4.1 Introduction

In chapter 3, we described the synthesis of highly antibacterial but non-hemolytic polyacrylates through copolymerization of a 6-carbon spacer arm amphiphilic monomer with a 2-carbon spacer arm amphiphilic monomer.\textsuperscript{120} Whereas the homopolymer of M6 was highly hemolytic and antibacterial, a random copolymer with just 10 mole% of M2 displayed dramatically lower hemolytic activity than the M6 homopolymer while maintaining similar antibacterial activity, resulting in a greater than 200 fold selectivity toward E. coli over RBCs. As compared with these copolymers of M6 and M2, in which every repeating unit bears a cationic charge, copolymers of M6 and non-ionic counits with hydrophilic poly(ethylene glycol) (PEG) side groups may be expected to show selective (bacteria over RBCs) antibacterial activity (Scheme 4.1). In contrast with short cationic M2 counits, incorporation of monomers with long (~ 12 to 57 atoms) hydrophilic PEG side groups may lead to considerably different biological activity. Biocompatible linear PEG polymers are FDA-approved for internal consumption and are known to exert protective effect on RBCs.\textsuperscript{78-82, 121-122} Long hydrophilic PEG side groups would have highly extended confirmation in an aqueous environment and may reduce hydrophobic interactions of polymers with the RBC lipid bilayer.\textsuperscript{86-88, 122} Hydrophilic PEGs have been widely used to improve biocompatibility and hydrophilicity in a myriad of polymeric materials.\textsuperscript{78-85} Youngblood et al. reported the improvement in
biocompatibility of N-hexylated poly(vinyl pyridines) through incorporation of Poly(ethylene glycol)methyl ether methacrylate (PEGMA) monomers with various lengths of PEG side groups. Colak et al. reported the reduction in hemolytic activity through incorporation of non-ionic and hydrophilic repeat units in amphiphilic polynorbornenes, but the antibacterial activity of PEGylated polynorbornenes reduced drastically with increasing content of PEG, resulting in very low selectivity values (< 5). PEGylated polymethacrylates were found to be antibacterial toward a gram positive bacteria while displaying low hemolytic activity. In our present study, as compared with

Scheme 4.1 a) Synthesis of cationic amphiphilic polymers. b) Biological activity of PM6 polymers in comparison with copolymers having non-ionic hydrophilic poly(ethylene glycol) counits.
highly hemolytic PM6-100, the copolymerization of the appropriate mole % of non-ionic hydrophilic PEGMA monomer led to substantial reduction in hemolytic activity of polymers resulting in highly selective antibacterial activity.

In this chapter, we describe the antibacterial and hemolytic activities of amphiphilic polymers synthesized by copolymerizing a 6-carbon spacer arm monomer M6 with the hydrophilic and biocompatible PEGMA monomers. Our strategy of copolymerizing counits having cationic and hydrophobic spacer arm with non-ionic hydrophilic monomers resulted in polymers with as high as >100 times selectivity toward E. coli over RBCs (Table 4.2). Similar to our previous findings, copolymerization of M6 with a small mole% of PEGMA resulted in significant reduction in hemolytic activity of polymers, but to a lesser extent. We also observed an abrupt increase in antibacterial activity of these polymers at certain mole%’s of PEGMA monomers (Figures 4.1, 4.2, and Table 4.2), rather than a gradual increase in antibacterial activity as previously reported for the case of copolymers of M6 and M2. Moreover, PEGMA copolymers displayed higher antibacterial activity toward gram negative E. coli over gram positive S. aureus, which is in contrary to copolymers of M6 and M2. The bactericidal activities of these copolymers were confirmed by time-kill studies, and Field Emission Scanning Electron Microscopy (FE-SEM) analysis ascertained the membrane disruption mechanism of antibacterial activity of copolymers reported here.

4.2 Experimental

4.2.1 Materials

Methyl 3-mercaptopropionate (MMP), acetonitrile (anhydrous), N, N-diisopropylethylamine, 2, 2’-Azobis(2-methylpropionitrile) (AIBN), tetrahydrofuran (THF), and 6-amino-1-hexanol were purchased from Sigma-Aldrich and used without further purification. Poly(ethylene glycol) methyl
ether methacrylates ($M_n \sim 300$ and 950) was purchased from Sigma-Aldrich and were treated with inhibitor remover prior to use. Acryloyl chloride was purchased from Sigma-Aldrich and distilled prior to use. Di-tert-butyl dicarbonate (t-Boc), trifluoroacetic acid (TFA), hexane, and diethyl ether were purchased from Alfa Aesar and used as received. Dichloromethane and ethyl acetate were purchased from BDH. All other chemicals or reagents were used as obtained without further purification.

4.2.2 Instrumentation

$^1$H and $^{13}$C NMR spectra of monomers and polymers were obtained on a Varian Unity NMR spectrometer (600 MHz) using CDCl$_3$ or D$_2$O as solvents. Molecular weights and polydispersities (PDI) of precursor polymers (Boc protected) were estimated on an EcoSec HLC-83220 gel permeation chromatography instrument (RI detector) using linear polystyrene standards and THF as eluent. Bacterial growth in E. coli and S. aureus assays was determined by measuring the turbidity as optical density at $\lambda=600$ nm (OD$_{600}$), on an Agilent 8453 spectrophotometer using 1 cm path length plastic cuvette. A SpectraMax 340 PC micro plate reader from Molecular Devices was used to measure OD$_{595}$ (Antibacterial test) and OD$_{414}$ (Hemolysis test). Scanning electron microscopy images were obtained on an AMRAY 1910 field emission scanning microscope at an operating voltage of 5 kV.

4.2.3 Synthesis of tert-butyl (6-hydroxyhexyl)carbamate and 6-((tert-butoxycarbonyl)amino)hexyl acrylate (monomer M6)$^{120}$

Boc protected hexanolamine (tert-butyl (6-hydroxyhexyl)carbamate) and monomer M6 (6-((tert-butoxycarbonyl)amino)hexyl acrylate) were synthesized as described in Chapter 3.
4.2.4 Synthesis of polymers

Scheme 4.2 Synthesis of amphiphilic polymers having 6-carbon spacer arm and non-ionic hydrophilic counits.

0.9 g (3 mmol) poly(ethylene glycol) methyl ether methacrylate ($M_n \approx 300$) and 0.814 g (3 mmol) Monomer M6 were added to a 100 mL round bottom flask containing 9.85 mg (0.06 mmol) AIBN, 33.3 µL MMP, and 6 mL anhydrous acetonitrile. The flask was sealed and purged with nitrogen for 5 minutes. The reaction mixture was stirred at 65 °C for 18 h. Acetonitrile was evaporated using a rotavapor and the polymer was re-dissolved in THF and repeatedly precipitated in hexane. The molecular weight of the polymer was estimated using GPC. The resultant polymer was dissolved in excess TFA and left under stirring for 4 hours. TFA was removed under reduced pressure and polymers was dissolved in methanol and repeatedly precipitated in diethyl ether. The polymer was kept under vacuum for 2 days to remove solvent traces followed by lyophilization. Yield 0.82 g. $^1$H NMR (600 MHz, D$_2$O): δ 0.63-1.12 (bm, 33H), 1.15-2.48 (bm, 134H), 2.67-2.77 (s, 2H), 2.79-2.92 (bs, 20H), 2.92-3.1 (bm, 2H), 3.2-3.35 (bs, 30H), 3.38-3.75 (bm, 153H), 3.77-4.29 (bm, 44H). $^1$H NMR spectra of all other cationic polymers are as shown in section 4.5.
4.2.5 Antibacterial and hemolytic activity of polymers

Antibacterial activity of polymers in terms of Minimum Inhibitory Concentrations (MIC) and hemolytic activity of polymers in terms of HC50 were obtained as described in Chapter 2.

4.2.6 Field-Emission Scanning Electron Microscopy (FE-SEM) analysis and time-dependent killing efficiency studies of polymers

The membrane disruption mechanism of antibacterial activity and time dependent killing efficiency studies were performed as per protocols described in Chapter 3.

4.3 Results and Discussion

4.3.1 Synthesis of polymers

We synthesized a series of random copolymers via free radical polymerization of monomer 6-((tert-butoxycarbonyl)amino)hexyl acrylate (M6) and PEGMA-300 (300 g/mol is molecular weight) or PEGMA-950, followed by treatment with excess trifluoroacetic acid to remove Boc protecting groups. The feed mole ratios of comonomers were varied from 0% to 100% in order to optimize the amphiphilic balance of copolymers. Actual mole ratios of comonomers in copolymers were confirmed using 1H NMR spectroscopy, and were found to be in close agreement with feed mole ratios (Table 4.1). Two series of copolymers were synthesized by copolymerizing monomer M6 with PEGMA-300 or PEGMA-950 in order to explore the effect of PEG side chain lengths on the biological activity of polymers. Due to insolubility of cationic polymers in THF, the molecular weights of precursor polymers (Boc protected) were estimated using GPC and found to be similar for all polymers (Table 4.1). Degree of polymerization (DP) of approximately 20 was targeted for
all polymers, because a DP of 20 had been shown to result in superior antibacterial and low hemolytic activities in cationic polymethacrylates. DPs of deprotected polymers (cationic charge) were calculated using end group analysis of $^1$H NMR spectra assuming chain transfer as mode of termination, and found to be around 20 for all polymers. Notation of PM6-X-PEG300 is used to represent a polymer with X mole% of M6 Monomer (feed percentage) copolymerized with 100-X mole % of PEGMA-300.

Table 4.1 Characterization of polymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Mol% M6</th>
<th>Mole % PEGMA</th>
<th>Wt % PEGMA</th>
<th>$M_w$ kDa</th>
<th>$M_n$ kDa</th>
<th>PDI</th>
<th>$^b$D.P.</th>
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</thead>
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<tr>
<td>PM6-0-PEG300</td>
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<td>100</td>
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<td>6.2</td>
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<td>PM6-10-PEG300</td>
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<td>89</td>
<td>93</td>
<td>9.2</td>
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</table>

4.3.2 Antibacterial activity of polymers

The antibacterial activities of polymers against E. coli (Top 10, ampicillin resistant) and S. aureus (ATCC 25923) were obtained in terms of Minimum Inhibitory Concentration (MIC), which is the lowest polymer concentration to result in 100% inhibition of bacterial growth after an incubation period of 18 hours. MIC values of polymers are as shown in Table 4.2 and Figures 4.1 & 4.2.
Copolymers with up to 30 mole% of 6-carbon spacer arm counits (M6) were found to be inactive against bacteria. We observed an abrupt increase in antibacterial activity toward both E. coli and S. aureus at 50 mole% of M6 suggesting that there is an optimum mole% of M6 counits at which polymers display antibacterial activity. This observation is in contrast with our previous findings (Chapter 3) in which we observed a gradual increase in antibacterial activity with increase in mole% of M6 monomer. One of the reasons behind this observation may be the reduction in number of cationic charges and hydrophobic moieties on polymer with increasing amount of PEGMA, leading to lower electrostatic and lipophilic interactions between the polymers and negatively charged bacterial cell surface. In comparison with the homopolymers of 2-carbon spacer arm monomer M2 (PM6-0%, chapter 3), which has high antibacterial activity toward
Figure 4.1 Minimum Inhibitory Concentrations (MIC) values of polymers against E. coli as a function of mole % of M6 monomer in polymers.

Figure 4.2 Minimum Inhibitory Concentrations (MIC) values of polymers against S. aureus as a function of mole % of M6 monomer in polymers.
S. aureus and is slightly antibacterial against E. coli, PEGMA homopolymer lacks antibacterial activity. Moreover, long hydrophilic PEG side groups can obstruct the interactions of M6 counits with the bacterial membrane in aqueous solution. This phenomena would be more effective in preventing M6 counit insertion into bacterial membranes at higher mole% of PEGMA-300. Thus, we did not observe antibacterial activity, up to the highest polymer concentration tested (2000 µg/mL) in copolymers containing higher than 50 mole% of PEGMA-300. Increase of M6 mole% above 50% led to rapid increase in antibacterial activity against both E. coli and S. aureus. PM6-90-PEG300 with 10 mole% of PEGMA-300, demonstrated similar level of activity against bacteria as compared with PM6-100%.

As is apparent from Table 4.2 and Figures 4.1 and 4.2, PM6-X-PEG950 series polymers, with longer length of PEG side groups, displayed lower antibacterial activity in comparison with PM6-X-PEG300 series at same mole% of PEGMA counits. Whereas PM6-50-PEG300 displayed significant antibacterial activity, PM6-50-PEG950 was found to be inactive against both E. coli and S. aureus. Also, PM6-70-PEG300 showed low MIC value toward bacteria, whereas PM6-70-PEG950 displayed substantially higher MIC values (lower antibacterial activity). It can be concluded from these observations that at same mole%, shorter PEG side groups results in higher antibacterial activity than longer PEG side groups.

In order to assess the effect of PEG lengths independent of the weight % (wt %) or content of overall PEG composition in the copolymer, we analyzed the biological activities of PM6-70-PEG300 and PM6-90-PEG950, which have similar wt % (~43 %) or content of PEG side groups. As is evident from Table 4.2, we did not observe a significant difference in the antibacterial activities of these two polymers. Similarly, both PM6-30-PEG300 and PM6-50-PEG950, which have around 80 wt % PEG composition, demonstrated lack of antibacterial or hemolytic activity,
whereas PM6-50-PEG300 with lower wt % of PEG (64%) displayed antibacterial activity. It appears from these observations that overall PEG composition but not the length of PEG side groups affects the antibacterial activities of these polymers.

MIC measurements and time-kill studies demonstrate that these polymers are more active against E. coli than S. aureus. These polymers displayed higher MIC values toward S. aureus as compared with E. coli. As described later in this chapter, at 2×MIC polymer concentration, > 99.9 % killing efficiency was achieved against E. coli after an incubation period of 8 h, but substantial S. aureus CFUs survived when challenged with 1×MIC concentration of polymers suggesting a higher value of minimum bactericidal concentration (MBC) than MIC toward S. aureus. This is in contrast with our previously reported polymer analogues of M6 and M2 that were in general more active against S. aureus than E. coli.\(^{120}\) Similarly, other researchers have also reported higher antibacterial activity of amphiphilic polymers against S. aureus than E. coli, as the double membrane structure of E. coli is considered more difficult to penetrate and disrupt as compared with the single membrane structure of S. aureus.\(^{49, 57, 123}\) One of the reason behind lower activity of these PEGylated polymers against S. aureus than E. coli, may be the significant differences in the cell wall structure of E. coli and S. aureus. S. aureus has up to 80 nm thick outer cell wall composed of negatively charged cross linked peptidoglycans (polysaccharides and amino acids). Long PEG side groups of polymers can make a network of hydrogen bonds with the polysaccharides in the bacterial cell wall, and may get stuck in the thick polysaccharide cell wall of S. aureus. In contrast to S. aureus, E. coli has only a 6-8 nm thick polysaccharide (peptidoglycan) layer, which is sandwiched between the outer cell membrane and inner cell membrane. Thus the degree of hydrogen bonding would be lower between these PEGylated copolymers and E. coli cell surface,
resulting in greater ability of polymers to permeate through the cell envelope of E. coli (Figure 4.3).

Figure 4.3 Schematic representation of E. coli and S. aureus cell wall and membranes. Long hydrophilic PEG side groups may form polyion complex through H-bonds with polysaccharides in the thick negatively charged cell wall murien layer (peptidoglycans) of S. aureus.
4.3.3 Hemolytic activity of polymers toward mouse red blood cells (RBCs)

Hemolytic activities of polymers were obtained in terms of HC$_{50}$, which is the lowest polymer concentration resulting in 100% lyses of red blood cells within an incubation period of 1 h (Figures 4.4 & 4.5 and Table 4.2). As compared with the highly hemolytic PM6-100% homopolymer, PM6-70-PEG300 with 33 mole% of PEGMA-300 counits displayed the HC$_{50}$ value of 1809 µg/mL, which is three orders of magnitude higher than that of PM6-100%. Thus, addition of 33 mole% of non-ionic hydrophilic PEGMA monomer led to >950 times reduction in hemolytic activity. All other copolymers in the PEG300 series with higher than 30 mole% content of PEGMA did not show significant hemolytic activity till the highest concentration tested (HC$_{50}$ >2000 µg/mL). Similarly, PM6-90-PEG950 with just 12 mole% PEGMA950 showed substantially lower hemolytic activity (HC$_{50}$ = 809 µg/mL) than the PM6-100% homopolymer. Copolymers with more than 10 mole% of PEGMA-950 demonstrated very low hemolytic activity (HC$_{50}$ >2000 µg/mL).

These observations buttress the biocompatibility advantages of PEG side groups. In the absence of blood plasma, RBCs are more exposed to foreign bodies.$^{124}$ Similar to blood plasma, PEG is believed to protect RBCs from foreign body contact.$^{62}$ It has been reported that PEG may interact with RBC cell membrane through hydrogen bonds and weakly adsorb on the surface of RBCs, thus enhancing its protective effect.$^{122}$ Cationic amphiphilic polymers cause hemolysis primarily through hydrophobic interactions. The presence of bulky hydrophilic PEG side groups would not favor the insertion of amphiphilic copolymers through the hydrophobic core of RBC lipid bilayer thus resulting in lower hemolytic activity.

At the same mole % of PEGMA monomer, longer PEG side chains displayed lower hemolytic activity (Table 4.2 and Figures 4.4 &4.5). In order to assess the effect of PEG side group lengths independent of the
Figure 4.4 Hemolytic activity of PM6-X-PEG300 series polymers against RBCs as a function of mole % of M6 monomer

Figure 4.5 Hemolytic activity of PM6-X-PEG950 series polymers against RBCs as a function of mole % of M6 monomer
weight % or content of overall PEG composition in the polymer, we analyzed the biological activities of PM6-70-PEG300 and PM6-90-PEG950, which have similar wt % (~43 %) or content of PEG side groups. We observed a difference in the hemolytic activity of PM6-70-PEG300 and PM6-90-PEG950, even though both polymers have similar wt % PEG composition (~ 43 wt %, Table 4.1). PM6-70-PEG300 with 30 mole % of shorter PEG chains showed significantly lower hemolytic activity (HC$_{50}$ = 1809 µg/mL) as compared with PM6-90-PEG950 (HC$_{50}$ = 809 µg/mL) with 10 mole % of longer PEG chains. Hemolytic activity of amphiphilic polymers mainly arise from the hydrophobic interactions of polymers with lipid bilayer of RBCs. Shorter but spread out hydrophilic regions throughout the polymer backbone may deter the permeability of polymers into the hydrophobic domain of RBCs’ lipid bilayer, whereas in polymers with same wt % of PEG but longer PEG chains at lower mole %, wider regions of highly hydrophobic and hemolytic M6 counits may exist, resulting in higher ability of these polymers to lyse the lipid bilayer of RBCs (Figure 4.6). This observation is in contrast with the previous report in which hemolytic activity of hydrophilic N-hexylated vinyl pyridine copolymers was found to be a function of total PEG composition and not dependent on the length of PEG side groups.$^{62}$
Figure 4.6 Schematic representing the interactions of RBC cell membrane with polymers having similar wt% of PEG but different lengths of PEG side groups.

4.3.4 Selectivity of polymers toward bacteria over RBCs

Selectivity of polymers is defined as the ratio (HC$_{50}$/MIC) of HC$_{50}$ to MIC value of polymers. High selectivity of polymers toward bacteria over mammalian cells would be highly advantageous for healthcare and therapeutic applications of synthetic amphiphilic polymers. PM6-100% without PEG was highly antibacterial and hemolytic, and thus lacked selective (bacteria over RBCs) antibacterial activity, which would hamper its practical therapeutic applications. Incorporation of hydrophilic PEG side groups led to an increase in selectivity of these polymers. We found that Polymer PM6-70-PEG300 is >113 times more selective toward E. coli over RBCs and >29 times more selective toward S. aureus over RBCs (Table 4.2). PM6-50-PEG300 with 50 mole% content of
Figure 4.7 Antibacterial and hemolytic activities of PM6-X-PEG300 series polymers

Figure 4.8 Antibacterial and hemolytic activities of PM6-X-PEG950 series polymers
PEGMA-300 demonstrated >19 times selectivity toward E. coli (MIC = 104 μg/mL) over RBCs (HC<sub>50</sub> > 2000 μg/mL). Similarly, PM6-90-PEG950 was found to be 31 times more selective toward E. coli over RBCs, and 16 times selective toward S. aureus over RBCs. However, incorporation of more than 50 mole% of PEGMA-300 and more than 30 mole% of PEGMA-950 monomer resulted in complete loss of antibacterial activity, thus leading to non-selective polymers.

4.3.5 FE-SEM analysis of bacterial membrane disruption by polymers

FE-SEM analysis of bacterial cell morphology was performed to ascertain the membrane disruption mechanism of antibacterial action of these polymers. PM6-70-PEG300 and PM6-90-PEG950 were chosen as representative polymers as these polymers displayed the highly selective (bacteria over RBCs) antibacterial activity. Control E. coli and S. aureus cells, without polymer treatment, show intact cell surface morphologies (Figures 4.9 & 4.11). E. coli treated with PM6-70-PEG300 and PM6-90-PEG950 show severe cell damage and surface rupture, confirming the cell membrane disruption mechanism of antibacterial activity (Figures 4.10 & 4.13). Similar cell surface damage and rupture was observed in case of S. aureus treated with PM6-70-PEG300 and PM6-90-PEG950 (Figures 4.12 & 4.14).
Figure 4.9 FE-SEM image of E. coli cells without polymer treatment (control) show intact cell surface morphology.

Figure 4.10 FE-SEM image of E. coli cells treated with PM6-70-PEG300 show severe damage and rupture of bacterial cell surface.
Figure 4.11 FE-SEM image of S. aureus cells without polymer treatment (control) show intact cell surface morphology.

Figure 4.12 FE-SEM image of S. aureus cells treated with PM6-70-PEG300 show severe damage and rupture of bacterial cell surface.
Figure 4.13 FE-SEM image of E. coli cells treated with PM6-90-PEG950 show severe damage and rupture of bacterial cell surface.

Figure 4.14 FE-SEM image of S. aureus cells treated with PM6-90-PEG950 show severe damage and rupture of bacterial cell surface.
4.3.6 Time dependent killing efficiency studies of polymers

$10^5$ CFU/mL bacterial colonies were treated with polymers for various time intervals in order to determine the time-dependent bacterial killing efficiency of polymers. Within 2 hours of incubating E. coli with PM6-70-300PEG, > 95% of bacterial colony forming units were eliminated. A 5 log reduction (> 99.99 % killing efficiency) in E. coli CFU/mL was achieved within 8 h of incubation at 2×MIC concentrations of PM6-70-PEG300 and PM6-90-PEG950 copolymers. The time-kill studies further confirmed the bactericidal action of these polymers, rather than just a bacteriostatic action. As is clear from Figures 4.19 & 4.20 and Table 4.4, PM6-70-PEG300 and PM6-90-PEG950 polymers demonstrated lower antibacterial activity against S. aureus, in comparison with E. coli. Whereas, 99.9% killing efficiency was achieved against E. coli at 1×MIC concentration of these polymers, significant percent of S. aureus cells survived even after 8 h incubation with polymers. This indicates that minimum bactericidal concentration is higher than the minimum inhibitory concentration of these polymers against S. aureus. This observation is in agreement with the MIC studies reported in section 4.3.2, in which higher MIC values of these polymers were found against S. aureus, as compared with E. coli.
Table 4.3 Time-dependent killing efficiency of polymers toward E. coli. Viable cells (CFU/mL) are shown after incubating with polymers, or without polymers (control), for various time intervals.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>PM6-70-PEG300</th>
<th>PM6-90-PEG950</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 × MIC</td>
<td>2 × MIC</td>
</tr>
<tr>
<td>0</td>
<td>7.0 × 10⁴</td>
<td>9.2 × 10⁴</td>
</tr>
<tr>
<td>1</td>
<td>1.8 × 10⁴</td>
<td>1.2 × 10⁴</td>
</tr>
<tr>
<td>2</td>
<td>2 × 10³</td>
<td>1 × 10²</td>
</tr>
<tr>
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<td>5 × 10²</td>
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</tr>
<tr>
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<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>8</td>
<td>nil</td>
<td>nil</td>
</tr>
</tbody>
</table>

Table 4.4 Time-dependent killing efficiency of polymers toward S. aureus. Viable cells (CFU/mL) are shown after incubating with polymers, or without polymers (controls), for various time intervals.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>PM6-70-PEG300</th>
<th>PM6-90-PEG950</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 × MIC</td>
<td>2 × MIC</td>
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<tr>
<td>8</td>
<td>5.2 × 10⁴</td>
<td>nil</td>
</tr>
</tbody>
</table>
Figure 4.15 Time-dependent killing efficiency curve of PM6-70-PEG300 against E. coli
Figure 4.16 Time-dependent killing efficiency curve of PM6-90-PEG950 against E. coli

Figure 4.17 Confirmation of bactericidal activity of PM6-90-PEG950 at various time intervals using Agar plates. The final polymer concentration was equal to MIC value. E. coli without polymer treatment was used as positive control and is shown in a) at 1 h, b) at 6 h time interval. E. coli treated with PM6-90-PEG950 at c) 0 h, d) 1h, e) 2 h, f) 4 h, g) 6 h, and h) 8 h time interval
Figure 4.18 Time-dependent killing efficiency curve of PM6-70-PEG300 against S. aureus

Figure 4.19 Confirmation of bactericidal activity of PM6-70-PEG300 against S. aureus at various time intervals using Agar plates. The final polymer concentration was equal to 2×MIC value. S. aureus without polymer treatment was used as positive control and is shown in a) at 1 h, b) at 6 h time interval. S. aureus treated with PM6-90-PEG950 at c) 0 h, d) 1h, e) 2 h, f) 4 h, g) 6 h, and h) 8 h time interval
Figure 4.20 Time-dependent killing efficiency curve of PM6-90-PEG950 against S. aureus

Figure 4.21 Confirmation of bactericidal activity of PM6-90-PEG950 against S. aureus at various time intervals using Agar plates. The final polymers concentration was equal to 2×MIC value. S. aureus without polymer treatment was used as positive control and is shown in a) at 1 h, b) at 8 h time interval. S. aureus treated with PM6-90-PEG950 at c) 0 h, d) 1h, e) 2 h, f) 4 h, g) 6 h, and h) 8 h time interval
4.4 Conclusions

In conclusion, a series of amphiphilic copolymers manifesting antibacterial activity against E. coli and S. aureus were synthesized. Copolymerization of biocompatible PEGMA monomers with a 6-carbon spacer arm amphiphilic acrylate monomer led to substantial reduction in hemolytic activity resulting in polymers with highly selective (bacteria over RBCs) antibacterial activity. We observed that the length of PEG groups appears to affect the hemolytic activity of our polymers, whereas only the overall wt % of PEG composition instead of the length of PEG side groups has effect on the antibacterial activity of these polymers. FE-SEM analysis showed severe damage to bacterial cell surface morphology confirming the cell membrane disruption mechanism of antibacterial activity. Bactericidal activity of polymers was further confirmed by time-kill studies. Due to their low hemolytic activity and highly selective bactericidal activity, these polymers may contribute to widespread therapeutic applications for fighting infections caused by antibiotic drug resistant bacteria.
4.5 $^1$H NMR spectra of cationic amphiphilic polymers

Figure 4.22 $^1$H NMR spectrum of polymer PM6-90-PEG300 (600 MHz, D$_2$O)

Figure 4.23 $^1$H NMR spectrum of polymer PM6-70-PEG300 (600 MHz, D$_2$O)
Figure 4.24 $^1$H NMR spectrum of polymer PM6-50-PEG300 (600 MHz, D$_2$O)

Figure 4.25 $^1$H NMR spectrum of polymer PM6-30-PEG300 (600 MHz, D$_2$O)
Figure 4.26 $^1$H NMR spectrum of polymer PM6-20-PEG300 (600 MHz, D$_2$O)

Figure 4.27 $^1$H NMR spectrum of polymer PM6-10-PEG300 (600 MHz, D$_2$O)
Figure 4.28 $^1$H NMR spectrum of polymer PM6-0-PEG300 (600 MHz, D$_2$O)

Figure 4.29 $^1$H NMR spectrum of polymer PM6-90-PEG950 (600 MHz, D$_2$O)
Figure 4.30 $^1$H NMR spectrum of polymer PM6-70-PEG950 (600 MHz, D$_2$O)

Figure 4.31 $^1$H NMR spectrum of polymer PM6-50-PEG950 (600 MHz, D$_2$O)
Figure 4.32 $^1$H NMR spectrum of polymer PM6-30-PEG950 (600 MHz, D$_2$O)

Figure 4.33 $^1$H NMR spectrum of polymer PM6-20-PEG950 (600 MHz, D$_2$O)
Figure 4.34 $^1$H NMR spectrum of polymer PM6-10-PEG950 (600 MHz, D$_2$O)

Figure 4.35 $^1$H NMR spectrum of polymer PM6-0-PEG950 (600 MHz, D$_2$O)
Chapter 5

Structural Parameters Affecting the Antibacterial and Hemolytic Activities of Cationic Amphiphilic Poly(vinyl esters)

5.1 Introduction

Incidents of infections involving multi drug-resistant bacteria are increasing worldwide at an alarming rate.\textsuperscript{125} Outbreaks of drug-resistant bacteria in hospitals are becoming dangerously common and lead to thousands of casualties and add billions of dollars in health-care costs in the United States alone.\textsuperscript{3,126-127} Hospital-acquired infections are mostly associated with surface growth of bacteria on catheters and other medical devices.\textsuperscript{128} Coating of medical devices and other surfaces with antibacterial agents may prevent the proliferation of harmful bacteria.\textsuperscript{129} Antibacterial polymers that kill bacteria by leaching bioactive agents such as heavy metals,\textsuperscript{130-134} halogen species,\textsuperscript{135-137} and small biocide molecules,\textsuperscript{138-139} among others, have attracted significant research interest in the last couple of decades. These polymers suffer from limitations because they release toxic agents into environment, and leaching of bioactive agents renders them inactive after some time.\textsuperscript{126} In another approach to synthesize antibacterial materials, conventional antibiotics have been attached to polymer backbone to achieve antibacterial activity.\textsuperscript{140-143} These polymers may not be active against multi drug resistant bacteria, and widespread use of such materials may further enhance the rate of bacterial resistance development.

As described in previous chapters, synthetic amphiphilic polyacrylates mimicking the structural features of natural antimicrobial peptides (AMPs) can show high antibacterial activity. Scanning electron microscopy analysis showed that amphiphilic polyacrylates disrupt the cell membranes of both gram negative E. coli and gram positive S. aureus bacteria. Thus, due to the membrane
rupture mechanism of antibacterial action, the development of bacterial resistance toward synthetic cationic amphiphilic polymers is highly hindered or improbable. Poly(vinyl acetate), a biocompatible material, has been widely used in paints, coatings, adhesives, and as binder for non-woven materials.\textsuperscript{144-145} Cationic amphiphilic poly(vinyl esters) could be used in coatings with antibacterial properties, and thus may have large scale applications in homecare and healthcare settings, where nosocomial bacterial infections takes a huge toll on patient lives and healthcare costs. However, to the best of our knowledge, the antimicrobial activities of cationic amphiphilic poly(vinyl esters) have not been reported yet. Here we report the synthesis and antibacterial activities of cationic amphiphilic poly(vinyl esters) bearing quaternary ammonium groups. We synthesized a library of amphiphilic poly(vinyl ester) random copolymers with various molecular weights, lengths of alkyl side chain, and mole ratios of lipophilic repeating units. We found that majority of our copolymers manifested high antibacterial activity towards both E. coli and S. aureus.

One of the disadvantages of poly(vinyl esters) such as poly(vinyl acetate) is their propensity to undergo base-catalyzed hydrolysis, which severely affects their weather resistant properties. Moreover, the relatively high glass transition temperature of poly(vinyl acetate) also limits its film-forming applications. Vinyl versatate (trade name VeoVa-10) is a commercially available vinyl ester monomer with highly branched and hydrophobic ten carbon atom side group. Vinyl versatate is a mixture of several structural isomers with different shapes of the decyl side group. Such a highly branched and hydrophobic side group would protect the esters linkages from alkali catalyzed hydrolysis.\textsuperscript{146-148} Furthermore, the highly hydrophobic side groups can provide the necessary hydrophobicity to the polymer chain, which is required for polymer
insertion in bacterial cell membrane. We synthesized a series of copolymers with varying mole ratios of vinyl versatate and counits with pendent cationic groups. The antibacterial and hemolytic activities of these polymers were assessed against bacteria and mouse Red blood cells (RBCs). A copolymer with just 10 mole% of vinyl versatate and 90 mole% of cationic counits displayed 46 times more activity against gram S. aureus over RBCs.

5.2 Experimental Section

5.2.1 Materials

Butyric acid (>99%), heptanoic acid (>99%), mercuric acetate (98%), vinyl acetate (>99%), vinyl propionate (>98%), sulfuric acid (99.99%), silica gel, tetrahydrofuran (anhydrous), 2,2’-azobis(2-methylpropionitrile) (99%), methyl 3-mercaptopropionate (98%), acetonitrile (anhydrous), and N,N-dimethylethylamine (99%) were purchased from Sigma-Aldrich. Vinyl acetate and vinyl propionate were stirred with inhibitor remover for 20 minutes and filtered prior to use. All other chemicals were used without further purification. Valeric acid (99%) and hexanoic acid (>98%) were obtained from Alfa Aesar and were used as received. Dichloromethane, hexanes, and diethyl ether were purchased from BDH Chemicals. Vinyl versatate (VeoVa-10) was generously provided by Hexion Specialty Chemicals (now Momentive).

5.2.2 Instrumentation

1H and 13C NMR spectra for monomers and polymers were obtained on a Varian unity NMR spectrometer (600 MHz) using CDCl3 or DMSO-d6 as solvents. Molecular weights of precursor polymers were estimated using polystyrene standards on EcoSec HLC-83220 gel permeation chromatography instrument by Tosoh Bioscience, using tetrahydrofuran as eluent. To measure bacterial growth in E. coli and S. aureus cell culture, OD600 was obtained on Agilent 8453
spectrophotometer using 1 cm path length plastic cuvette. OD\textsubscript{595} (antibacterial test) and OD\textsubscript{414} (hemolysis test) were obtained on a SpectraMax 340 PC micro plate reader from Molecular Devices.

### 5.2.3 Synthesis of Monomers

Vinyl butyrate, vinyl valerate, vinyl hexanoate, and vinyl heptanoate were synthesized by transvinylation reaction between vinyl acetate and corresponding acid, using mercuric acetate as catalyst (Scheme 5.1(a)).\textsuperscript{149-153} The representative synthesis procedure is described as follows. Heptanoic acid (20 mL, 141 mmol) was added into a 250 mL single round bottom flask, already charged with 0.4 mercuric acetate (1.26 mmol) and vinyl acetate (78 mL, 846 mmol). Sulfuric acid (0.07 mL, 1.32 mmol) was then added drop wise under stirring. The reaction mixture was stirred at 30 °C for 30 hours. Excess sulfuric acid was neutralized by adding 0.2 g sodium acetate, followed by filtration. Vinyl acetate was evaporated under reduced pressure, and the resultant liquid was purified using silica gel chromatography with dichloromethane eluent. Solvent was evaporated using a rotavapor and pure monomer was obtained as clear liquid (12.2 g, 55.4% yield).

All other monomers were similarly synthesized. \textsuperscript{\textit{1}H NMR (600 MHz, CDCl\textsubscript{3}, \deltat)} of vinyl heptanoate: 7.27 (q, J = 8 Hz, 1H), 4.86 (d, J = 13.9 Hz, 1H), 4.55 (d, J = 6.4 Hz, 1H), 2.37 (t, J = 7.5 Hz, 2H), 1.64 (m, 2H), 1.24-1.36 (m, 6H), 0.88 (t, J = 7 Hz, 3H); \textsuperscript{\textit{13}C NMR (600 MHz, CDCl\textsubscript{3}, \deltat)} of vinyl heptanoate: 170.89, 141.23, 97.38, 33.92, 31.36, 28.68, 24.52, 22.42, 13.96;

\textsuperscript{\textit{1}H NMR (600 MHz, CDCl\textsubscript{3}, \deltat)} of vinyl hexanoate: 7.27 (q, J = 6.2 Hz, 1H), 4.85 (d, J = 14.2 Hz, 1H), 4.54 (d, J = 6.3 Hz, 1H), 2.36 (t, J = 7.7 Hz, 2H), 1.65 (m, 2H), 1.27-1.37 (m, 4H), 0.89 (t, J = 7 Hz, 3H); \textsuperscript{\textit{13}C NMR (600 MHz, CDCl\textsubscript{3}, \deltat)} of vinyl hexanoate: 170.89, 141.23, 97.38, 33.89, 31.18, 24.28, 22.20, 13.84; \textsuperscript{\textit{1}H NMR (600 MHz, CDCl\textsubscript{3}, \deltat)} of vinyl valerate: 7.28 (q, J = 7.6 Hz, 1H), 4.87 (dd, J = 13.9 Hz, 1H), 4.56 (dd, J =
Scheme 5.1 a) Synthesis of monomers; b) synthesis of random copolymers followed by quaternization with N, N-dimethylethylamine.

\[
\text{O} = \text{O} \quad + \quad \text{R} \quad \text{O} \quad \text{OH} \quad \xrightarrow{\text{Mercuric acetate}} \quad \text{O} \quad \text{O} \quad \text{R} \quad + \quad \text{OH} \\
\text{Sulfuric acid, 30 °C} \quad 30 \text{ h} \\
R = \text{a) Propyl} \\
\text{b) Butyl} \\
\text{c) Penyl} \\
\text{d) Hexyl}
\]

\[
\text{O} \quad \text{R} \quad \quad \text{+} \quad \quad \text{AlBN, MMP} \quad \text{A} \quad \text{OH} \quad \text{Cl} \quad \text{Furan} \quad 65 \text{ °C}, 20 \text{ h, } \text{N}_2 \\
\text{CH}_3 \text{CN, 72 h room temp.}
\]

\( R = \text{a) Methyl} \)
\( \text{b) Ethyl} \)
\( \text{c) n-Propyl} \)
\( \text{d) n-Butyl} \)
\( \text{e) n-Pentyl} \)
\( \text{f) n-Hexyl} \)
\( \text{g) Decyl} \)

* AlBN is 2,2'-azobisisobutyronitrile, MMP is Methyl 3-mercaptopropanoate

6.4 Hz, 1H), 2.39 (t, J = 7.6 Hz, 2H), 1.65 (m, 2H), 1.34-1.41 (m, 2H), 0.93 (t, J = 7.3 Hz, 3H);

\(^{13}\text{C NMR (600 MHz, CDCl}_3, \delta)\) of vinyl valerate: 170.89, 141.23, 97.38, 33.68, 26.58, 22.20, 13.63;

\(^{1}\text{H NMR (600 MHz, CDCl}_3, \delta)\) of vinyl butyrate: 7.28 (q, J = 7.6 Hz, 1H), 4.87 (dd, J = 13.9 Hz, 1H), 4.56 (dd, J = 6.4 Hz, 1H), 2.39 (t, J = 7.6 Hz, 2H), 1.65 (m, 2H), 1.34-1.41 (m, 2H), 0.93 (t, J = 7.3 Hz, 3H);

\(^{13}\text{C NMR (600 MHz, CDCl}_3, \delta)\) of vinyl butyrate: 170.58, 141.23, 97.38, 35.77, 18.02, 13.63.
5.2.4 Synthesis of random copolymers

A representative synthesis procedure is described here (Scheme 5.1(b)). Vinyl heptanoate (1.56 g, 10 mmol) and vinyl chloroacetate (1.20 g, 10 mmol) were added into a 100 mL 3 neck round bottom flask already charged with AIBN (0.0328 g), tetrahydrofuran (12 mL), and methyl 3-mercaptopropionate (0.110 mL, 1 mmol) as chain transfer agent. The reaction flask was sealed and purged with nitrogen using a stainless steel needle for 5 minutes, followed by stirring under reflux at 65 °C for 20 hours. Excess solvent was evaporated under reduced pressure, and the resulting viscous polymer was dissolved in tetrahydrofuran and precipitated in hexane 3 times. The mole percentage of comonomers was calculated from $^1$H NMR and found to be in close agreement with feed mole percentage. Degree of polymerization was calculated using end group analysis from $^1$H NMR. Molecular weights were estimated using gel permeation chromatography. $^1$H NMR (600 MHz, CDCl$_3$, $\delta$): 4.71-5.19 (bm, 37H), 3.97-4.14 (bs, 47H), 3.69 (s, 3H), 2.59-2.86 (m, 4 H), 2.17-2.37 (bs, 35H), 1.48-2.09 (bm, 129H), 1.21-1.36 (bs, 107H), 0.83-0.92 (bs, 53H). Copolymers of vinyl versatate and vinyl chloroacetate were synthesized using similar procedures. CP-decy15.5k-30% (before quaternization) $^1$H NMR (600 MHz, CDCl$_3$, $\delta$): 4.64-5.19 (bm, 36H), 4.00-4.17 (bs, 65H), 3.70 (s, 3H), 2.51-2.91 (m, 4H), 1.71-2.09 (bm, 87H), 0.70-1.69 (bm, 287H).
Figure 5.1 $^1$H NMR (600 MHz, CDCl$_3$) spectra of precursor copolymers (before quaternization reaction).
5.2.5 Quaternization of Copolymers

0.4 g of poly(vinyl heptanoate-Co-vinyl chloroacetate), as synthesized as described in section 5.2.4, was dissolved in 30 mL anhydrous acetonitrile and added into a 100 mL single neck round bottom flask. The reaction mixture was sealed and degassed with nitrogen for 5 minutes. N, N-dimethylethylamine (6 mL) was added while stirring, and the mixture was left under stirring at room temperature for 72 hours. Excess solvent was evaporated using a rotavapor under reduced pressure, and the polymer was re-dissolved in 2 mL methanol and precipitated in diethyl ether three times. The resulting polymer was kept under vacuum for 2 days and lyophilized to obtain quaternized polymer as a white powder (0.456 g). Complete quaternization was observed within the detection limit of NMR (Figure 5.3). $^1$H NMR (600 MHz, DMSO-$d_6$, δ): 4.43-5.15 (bs, 68H), 3.54-3.79 (bs, 39H), 3.15-3.38 (bs, 101H), 2.04-2.34 (bs, 41H), 1.35-2.01 (bm, 113H), 1.12-1.31 (bs, 158H), 0.77-0.88 (bs, 53H). CP-decyl5.5k-30% (after quaternization) $^1$H NMR (600 MHz, DMSO-$d_6$, δ): 4.37-5.61 (bm, 99H), 3.50-3.88 (bs, 66H), 3.13-3.40 (bs, 190H), 0.58-2.24 (bm, 415H).

Figure 5.2 Gel permeation chromatography curves of copolymers prior to quaternization reaction.
Figure 5.3 $^1$H NMR (600 MHz, DMSO-$d_6$) spectra of quaternized copolymers.
5.2.6 Antibacterial and hemolytic activity of polymers

Antibacterial activity of polymers in terms of Minimum Inhibitory Concentrations (MIC) and hemolytic activity of polymers in terms of HC50 were obtained as described in Chapter 2.

5.3 Results and Discussion

5.3.1 Synthesis and Characterization of Copolymers

We synthesized a library of cationic amphiphilic poly(vinyl ester) random copolymers by free radical polymerization of vinyl ester monomers (Scheme 5.1 and Table 5.1). Vinyl chloroacetate was copolymerized in an equimolar ratio (1:1) with a range of hydrophobic vinyl ester monomers with varying side group lengths from methyl to decyl. The length of side group was varied from methyl to decyl in order to explore the effect of amphiphilic balance of copolymers on their biological activities. The balance of hydrophobic to cationic moieties in polymers is essential for bacterial cell membrane permeability of polymers. Vinyl chloroacetate counits in the precursor copolymers were quaternized with N, N-dimethylethylamine to generate cationic groups in the copolymers. Precursor copolymers were treated with excess N, N-dimethylethylamine at room temperature for 72 hours to ensure complete quaternization. Complete quaternization was observed within the detection limit of NMR. The molecular weights of the precursor polymers (before quaternization) were assessed using GPC (Table 5.1 and Figure 5.2). Notation of CP-alkylxk represents different copolymers. For example, CP-butyl7k denotes the copolymer having n-butyl group in the side chain and molecular weight (Mw, GPC) around 7k g/mol. 1H NMR was used to calculate the degree of polymerization, mole ratio of hydrophobic to cationic repeating units, and to characterize precursor and quaternized copolymers.
The monomer vinyl versatate has a highly hydrophobic side chain of ten C-atoms. Vinyl versatate monomer is a mixture of several structural isomers with various shapes of decyl side chain. Such a highly hydrophobic side group is expected to protect ester functional groups from hydrolysis, thus improving the stability of polymers. Moreover, hydrophobic decyl side groups provides hydrophobicity to the copolymers, which is essential for membrane rupturing ability of these copolymers. Vinyl versatate was copolymerized with vinyl chloroacetate at various mole ratios in order to explore the effect of relative compositions of hydrophobic and cationic groups in copolymers on their biological activities. The feed mole% of hydrophobic vinyl versatate was varied from 0% to 60%, in increments of 10 mole%. Cationic charge was generated in the copolymers through quaternization reaction with N,N-dimethylethylene amine. Quaternized copolymers with more than 60 mole% were found to be insoluble in water, and their biological activities were not evaluated. Each copolymer was synthesized at three molecular weight levels by adjusting the feed mole ratio of monomers to methyl 3-mercaptopropionate, used as a chain transfer agent (Table 5.1). $^1$H and $^{13}$C NMR were used to characterize the precursor and quaternized copolymers. The notation CP-decyl$_{xk}$-Y% is used to represent the copolymer with Y mole% (in feed) of vinyl versatate counits and has an estimated molecular weight of xk g/mol.
Table 5.1 Characterization and biological data of quaternized random copolymers containing different lengths of alkyl side chain at various molecular weights.

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>P_{alkyl}^{a)}</th>
<th>MMP^{b)}</th>
<th>M_w [g/mol]</th>
<th>DP^{c)}</th>
<th>MIC E. coli [µg/mL]</th>
<th>MIC S. aureus [µg/mL]</th>
<th>HC_{50} RBC [µg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-methyl_{2.3k}</td>
<td>0.39</td>
<td>0.443</td>
<td>1878</td>
<td>6</td>
<td>1810</td>
<td>2000</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>CP-ethyl_{2.3k}</td>
<td>0.43</td>
<td>0.443</td>
<td>2076</td>
<td>7</td>
<td>2000</td>
<td>1620</td>
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</tr>
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</table>

^{a)} & ^{c)} Calculated from {^1}H NMR; ^{b)} MMP is Methyl 3-mercaptopropionate
5.3.2 Biological activity of poly(vinyl esters) with various lengths of alkyl side chains

5.3.2.1 Antibacterial Activity

Antibacterial activities of quaternized copolymers were assessed against gram negative E. coli and gram positive S. aureus in terms of minimum inhibitory concentrations (MIC). MIC values of 2.3k g/mol series copolymers are as shown in Figure 5.4 and Table 5.1. CP-methyl\textsubscript{2.3k} and CP-ethyl\textsubscript{2.3k} were found to be inactive against E. coli. However, increasing the length of the side chain to three carbon atoms (CP-propyl\textsubscript{2.3k}) led to substantial increase in antibacterial activity against E. coli. This observation is in accordance with the membrane rupture mechanism of cationic amphiphilic polymers, as polymer hydrophobicity promotes bacterial cell membrane rupture through lipophilic interactions with the hydrophobic core of lipid bilayer. Further increase in the length of hydrophobic side chain led to a gradual increment in antibacterial activity with CP-hexyl\textsubscript{2.3k} displaying the highest antibacterial activity in 2.3k g/mol series. CP-decyl with highly hydrophobic side groups displayed lower antibacterial activity (MIC 62 µg/ml) than CP-hexyl.

In 4k g/mol and 7k g/mol series copolymers, CP-methyl and CP-ethyl were found to be inactive against E. coli (Figures 5.5 and 5.6), similar to their lower molecular weight counterparts (CP-methyl\textsubscript{2.3k} and CP-ethyl\textsubscript{2.3k}). Increase in side group length to propyl led to substantial increase in antibacterial activity against E. coli, reached a maxima at butyl, but further increase in side group length resulted in lower antibacterial activity. Similar observations of attaining a maxima of antibacterial activity at a certain length of alkyl side chain, and lower antibacterial activity at lower or higher length of alkyl side chain have been reported in “facially amphiphilic” polynorbornens.\textsuperscript{21} An optimum level of overall hydrophobicity is required in polymers for lipophilic interactions with bacterial cell membrane. However, increasing the length of the alkyl side chain may decrease the
water solubility. At higher lengths of alkyl side chains, the lower water solubility of polymers may lead to associations of polymer chains leading to reduction in the concentration of polymer chains available to bind to the bacterial cell surface. Moreover, a balance of cationic and hydrophobic components in polymers is required to effectively interact with the negatively charged murein layer and lipophilic lipid bilayer (phospholipids) core of bacterial cell surface. In 4k g/mol and 7k g/mol series copolymers, CP-butyl demonstrated the highest antibacterial activity for E. coli, but CP-hexyl\textsubscript{2.3k} showed the highest activity against E. coli in 2.3k g/mol series copolymers. Smaller molecular weight polymer chains may require longer side chains to achieve sufficient overall hydrophobicity.

![Figure 5.4](image.png)

**Figure 5.4** Antibacterial activity of 2.3k g/mol series copolymers against E. coli and S. aureus.
Figure 5.5 Antibacterial activity of 4k g/mol series copolymers against E. coli and S. aureus.

Figure 5.6 Antibacterial activity of 7k g/mol series copolymers against E. coli and S. aureus.
Figure 5.7 Hemolytic activities of copolymers against RBCs.

for bacterial membrane rupture ability. Moreover, CP-hexyl$_{2.3k}$ displayed highest antibacterial activity toward E. coli among all polymers in this study. A copolymer with lower molecular weight has a higher molar concentration of active polymer molecule in a given weight as compared with higher molecular weight copolymers, leading to higher antibacterial activity.

The MIC values of our quaternized copolymers against S. aureus are as shown in Figures 5.4 to 5.6 and Table 5.1. Similar to activity against E. coli, a combination of low molecular weight (2.3k g/mol) and long alkyl side chain led to highest antibacterial activity against S. aureus. CP-pentyl$_{2.3k}$, CP-hexyl$_{2.3k}$, and CP-decyl$_{2.3k}$ manifested the lowest MIC value against S. aureus, among all of our copolymers. CP-methyl$_{2.3k}$ and CP-ethyl$_{2.3k}$ were inactive against S. aureus, in congruence with their activity against E. coli. However, with increase in molecular weight (4k and 7k g/mol), CP-ethyl displayed some antibacterial activity against S. aureus. Similar to activity against E. coli, an increase in alkyl side chain length from ethyl to propyl led to substantial increase in antibacterial activity against S. aureus at all molecular weight levels. Therefore, three carbon
atoms in the side chain is the threshold, below which no significant antibacterial activity was observed in these copolymers.

Copolymers with alkyl side chains from propyl to hexyl are more antibacterial towards S. aureus as compared to E. coli. As described in previous chapters, gram negative E. coli and gram positive S. aureus have significantly different cell wall structures. Double membrane structure of E. coli may be more difficult to penetrate as compared to the single membrane structure of S. aureus. Moreover, the negatively charged peptidoglycan or murein layer is only 6-8 nm thick in E. coli, and is also sandwiched between the outer and inner membrane. In comparison to E. coli, the surface of S. aureus is covered by a 20-80 nm thick negatively charged murein layer. Therefore, cationic copolymers may have more electrostatic interactions with S. aureus as compared to E. coli. Hence, one or both these factors may have contributed to higher antibacterial activity of our copolymers against S. aureus.

5.3.2.2 Hemolytic Activity

In order to ascertain the cytotoxicity of our quaternized copolymers toward mammalian cells, we evaluated the hemolytic activity of quaternized copolymers against freshly drawn mouse RBCs. \( HC_{50} \) values for quaternized copolymers are shown in Figure 5.7 and Table 5.1. All copolymers, except CP-methyl and CP-ethyl, displayed high toxicity towards RBCs. The lack of significant hemolytic and antibacterial activity in CP-methyl and CP-ethyl copolymers suggests that they do not have ability to rupture cell membranes of either erythrocytes or prokaryotes. The outer surface of RBCs’ cytoplasmic membrane consists of zwitterionic phospholipid head groups and thus lack net charge on its outer surface. Therefore, hemolytic activity of cationic amphiphilic polymers primarily arise from the hydrophobic interactions between lipophilic part of lipid bilayer, and the
hydrophobic moieties in amphiphilic copolymers. CP-propyl\textsubscript{2.3k} and CP-butyl\textsubscript{2.3k} displayed lower hemolytic activity as compared to their higher molecular weight counterparts, but CP-pentyl, CP-hexyl, and CP-decyl were highly hemolytic (HC\textsubscript{50} < 7 µg/mL) at all three molecular weight levels.

5.3.3 Effect of cationic and hydrophobic counits composition on the biological activity of poly(vinyl esters)

5.3.3.1 Antibacterial Activity

Vinyl versatate was copolymerized with vinyl chloroacetate at various mole ratios in order to explore the effect of relative compositions of hydrophobic and cationic groups in copolymers on their biological activities. The notation CP-decyl\textsubscript{xk}-Y\% is used to represent the copolymer with Y mole\% (in feed) of vinyl versatate counits and has an estimated molecular weight of xk g/mol. As is apparent from Figures 5.8 to 5.10, homopolymers of vinyl chloroacetate are not active against E. coli (MIC > 1000 µg/mL). Incorporation of just 10 mole\% of vinyl versatate counits led to significant reduction in MIC value. CP-decyl\textsubscript{11k}-10\% displayed substantial antibacterial activity against E. coli. Further increments in vinyl versatate mole\% resulted in higher antibacterial activity and copolymers with ~ 30-40 mole\% of vinyl versatate displayed highest antibacterial activity in the 2.3k and 5.5k g/mol series copolymers. In 2.3k g/mol series polymers, further increase in vinyl versatate mole\% did not result in higher antibacterial activity, whereas in 5.5k g/mol series copolymers we observed a reduction in antibacterial activity of copolymers with greater than 40 mole\% of vinyl versatate counits. Similarly, antibacterial activity decreased with higher than 30 mole\% of vinyl versatate in 11k g/mol series polymers.

Hence, 20-30 mole\% of vinyl versatate is the optimum mole\% of vinyl versatate at which the highest activity against E. coli was observed. Quaternized homopolymers of vinyl chloroacetate
have low overall hydrophobicity and high cationic charge density, which would impede the ability of polymers to penetrate through the hydrophobic core of lipid bilayer. Incorporation of up to 30 mole% of lipophilic vinyl versatate groups led to higher antibacterial activity due to higher lipophilic interactions of polymers with cell membrane. However, further incorporation of hydrophobic comonomer led to a reduction in antibacterial activity. Number of cationic groups in the polymer reduces with increasing mole% of vinyl versatate, resulting in lower electrostatic interactions of polymers with bacterial cell surface. Furthermore, highly hydrophobic decyl side groups of vinyl versatate would reduce the solubility of polymers in the aqueous assay medium, particularly at higher mole%’s of vinyl versatate. The reduction in polymer solubility may facilitate the hydrophobic association of polymer chains resulting in lower effective concentration of polymer available to attack bacterial cells. Hence, a balance between hydrophobic and cationic groups in amphiphilic copolymers is required to achieve high antimicrobial activity.
Table 5.2 Characterization and biological data of quaternized random copolymers containing various mole ratios of vinyl versatate counits.

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<th>Copolymer</th>
<th>MMPb) [mL]</th>
<th>M&lt;sub&gt;w&lt;/sub&gt; [g/mol]</th>
<th>PDI</th>
<th>MIC E. coli [µg/mL]</th>
<th>MIC S. aureus [µg/mL]</th>
<th>HC&lt;sub&gt;50&lt;/sub&gt; [µg/mL]</th>
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<td>2409</td>
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<td>&lt;7</td>
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Figure 5.8 Antibacterial and hemolytic activities of 2.3k g/mol series quaternized Poly(vinyl versatate-co-vinyl chloroacetate) polymers.

Figure 5.9 Antibacterial and hemolytic activities of 5.5k g/mol series quaternized Poly(vinyl versatate-co-vinyl chloroacetate) polymers.
Figure 5.10 Antibacterial and hemolytic activities of 11k g/mol series quaternized Poly(vinyl versatate-co-vinyl chloroacetate) polymers.

MIC values of polymers were obtained against gram positive S. aureus (Figure 5.8-5.10 and Table 5.2). Except CP-decyl_{2.3k}-0%, all polymers displayed high antimicrobial activity toward S. aureus. In 2.3k g/mol series, addition of 10 mole% of vinyl versatate led to a substantial increase in activity against S. aureus. Antimicrobial activity toward S. aureus increases with further increase in vinyl versatate mole% with CP-decyl_{2.3k}-30% to CP-decyl_{2.3k}-60% showing very high antibacterial activity (MIC = 12 µg/mL). This observation is in contrast with antibacterial activity of 11k g/mol series copolymers, in which a significant reduction in antibacterial activity was observed in polymers with higher than 30 mol% of vinyl versatate. Hence, lower molecular weight polymers require higher mole% composition of hydrophobic counits in copolymers to achieve high antibacterial activity. It is interesting to note that the antibacterial activity of polymers with 0% and 10% vinyl versatate increased with higher molecular weight, whereas the antibacterial activity of copolymers with 20-60 mole% vinyl versatate decreased with increase in molecular weight. The
antibacterial activity of the CP-decyl\textsubscript{11k} series polymers is significantly lower than the antibacterial activity of the CP-decyl\textsubscript{2.3k} series polymers in the range of 20-60 mole% vinyl versatate. In polyacrylate homopolymers (chapter 2), we observed the similar effect of molecular weight as higher molecular weight homopolymers were antibacterial, but lower molecular weight homopolymers were inactive against bacteria.

5.3.3.2 Hemolytic activity

CP-decyl\textsubscript{2.3k}-0\% and CP-decyl\textsubscript{2.3k}-10\% showed very low hemolytic activity toward mouse RBCs. 2.3k g/mol series copolymers with higher than 10 mole\% of vinyl versatate counits displayed very high hemolytic activity (Table 5.2 and Figures 5.8 - 5.10). Similarly, CP-decyl\textsubscript{5.5k}-0\% and CP-decyl\textsubscript{5.5k}-10\% demonstrated very low hemolytic activity, but copolymers in 5.5k g/mol series with higher than 10 mole\% vinyl versatate were found to have extremely high lytic ability toward RBCs (HC\textsubscript{50} < 7 µg/mL). In 11k g/mol series copolymers, except homopolymer CP-decyl\textsubscript{11k}-0\%, all copolymers displayed 100\% hemolysis at the lowest polymer concentration tested (HC\textsubscript{50} < 7 µg/mL). Low hemolytic activity of homopolymers and copolymers with up to 10 mole\% vinyl versatate could be attributed to lower hydrophobicity of polymers. As discussed earlier, the lipid bilayer of RBCs consist of zwitterionic phospholipids, thus lacking net negative charge on the outer surface of cytoplasmic membrane. High hemolytic activity of amphiphilic polymers arises from the hydrophobic interactions of polymer chains with RBC lipid bilayer. Quaternized homopolymers displayed selective (selectivity = HC\textsubscript{50}/MIC) antibacterial activity toward S. aureus over RBCs. CP-decyl\textsubscript{11k}-0\% was found to be 21 times more active against S. aureus over RBCs. CP-decyl\textsubscript{5.5k}-10\% was 46 times more active against S. aureus over RBCs.
5.4 Conclusions

In this study, we synthesized a library of cationic amphiphilic poly(vinyl ester) copolymers, and evaluated their antibacterial and hemolytic activities. The majority of the quaternized copolymers displayed high antibacterial activity towards E. coli and S. aureus. The role of alkyl side group length was investigated on the antibacterial and hemolytic activities of poly(vinyl esters). We found that the combination of lower molecular weight and six carbon atoms in the side group resulted in highest antibacterial activity towards both E. coli and S. aureus. In case of higher molecular weight copolymers (4k and 7k series), maximum antibacterial towards E. coli was achieved with 4 carbon atoms in the side chain, and further increase in the length of side group from four to six carbon atoms led to reduction in activity against E. coli. On the other hand, quaternized copolymers with very short alkyl side groups (methyl and ethyl) were inactive against both E. coli and S. aureus. Therefore, the molecular weight and length of alkyl side group is critical to achieve superior antibacterial activity of these quaternized poly(vinyl esters). We found that most of the copolymers were more active towards S. aureus as compared to E. coli. Such difference in activity may have resulted from significant difference in the cell surface morphology of E. coli and S. aureus. All copolymers, except CP-methyl and CP-ethyl, were highly hemolytic against mouse RBCs. Copolymers with varying degrees of amphiphilicity were synthesized through copolymerizing lipophilic vinyl versatate monomer with vinyl chloroacetate, at various mole ratios. Most of the polymers displayed high activity against S. aureus. CP-decyl5.5k-10% with 90 mole% cationic counts and 10 mole% hydrophobic counits displayed 46 times more activity against S. aureus over RBCs. Thus, incorporation of around 10 mole% of highly hydrophobic monomer led to highly selective antibacterial activity. These amphiphilic polymers with excellent antibacterial activity against both gram negative E. coli and gram positive S. aureus, makes them
promising candidates to curb the burgeoning problem of multidrug resistant bacteria on surfaces in hospitals, in sanitation, and numerous other surfaces that do not come in direct contact with human tissues, but contribute significantly to the spread of drug-resistant bacterial infections.
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