DEVELOPMENT OF NOVEL COPOLYOXETANES: ANTIMICROBIAL AGENTS

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Virginia Commonwealth University

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DEVELOPMENT OF NOVEL COPOLYOXETANES: ANTIMICROBIAL AGENTS

A dissertation submitted in partial fulfillment of the requirement for the degree of

Doctor of Philosophy at Virginia Commonwealth University

By

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ACKNOWLEDGEMENTS

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ABSTRACT

DEVELOPMENT OF NOVEL COPOLYOXETANES: ANTIMICROBIAL AGENTS

By Allison Lisa King

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy, Engineering at Virginia Commonwealth University

Virginia Commonwealth University, 2011

Major Director: Dr. Kenneth J. Wynne, Professor, Department of Chemical and Life Science Engineering

This thesis focuses on solution antimicrobial effectiveness for copolyoxetanes with quaternary ammonium and PEG-like side chains. Ring opening copolymerization of 3-((4-bromobutoxy)methyl)-3-methyloxetane (BBOx) and 3-((2-(2-methoxyethoxy)ethoxy)methyl)-3-methyloxetane (ME2Ox) yielded random copolymers with 14-100 (m) mole% BBOx designated P[(BBOx-m)(ME2Ox)]. Reaction of P[(BBOx-m)(ME2Ox)] with dodecyl dimethylamine gave the corresponding quaternary P[(C12-m)(ME2Ox)] polycation salts, designated C12-m. Mole ratios and molecular weights were obtained from $^1$H-NMR and end group analysis. Differential scanning calorimetry (DSC) studies showed $T_g$’s between -69 and -34 °C. Minimum inhibitory concentrations (MIC) against Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa showed MIC decreasing with increasing C12 mole% reaching a minimum between C12-43 and C12-60. C12-43 had the lowest MIC for all strains. At 5× MIC (challenge: $10^8$ cfu/ml), C12-43 kills ≥ 99% of the tested strains within 1 hr. C12-m copolyoxetane cytotoxicity toward human red blood cells, HFF (Human Foreskin Fibroblast) and HDF (Human Dermal Fibroblast) was low, indicating good prospects for biocompatibility. Cx-m
copoloyxetane antimicrobial efficacy, hemolytic activity and cytotoxicity were further explored by changing quaternary alkyl chain length. Copolyoxetanes are represented as Cx-50, where 50 is the mole percent quaternary repeat units and ‘x’ is quaternary alkyl chain length (2 to 16 carbons). Reaction of P[(BBOx-m)(ME2Ox)] with a series of tertiary amines yielded the desired quaternary ammonium segment. DSC studies showed $T_g$’s between -40 °C and -60 °C and melting endotherms for C14-50 and C16-50. A systematic dependence of alkyl chain length on MIC was found with C8-50 being the most effective antimicrobial. Kill kinetics for C8-50 (5× MIC, challenge: $10^8$ cfu/ml) effected >99% kill in 1 hour for *S. aureus* (7 log reduction). C8-50 efficacy on biomass and cell viability of *P. aeruginosa* biofilms was investigated. Crystal violet (CV) staining assays demonstrate that C8-50 had no effect on adhesion of already established *P. aeruginosa* biofilms, but reduced biofilm formation by killing cells prior to attachment. For anti-adhesion assays, noticeable reduction in biofilm mass occurred at concentrations greater than 2× MIC. Viability studies show a substantial log reduction of 2.1 at MIC. The low cytotoxicity of Cx-m copolyoxetanes coupled with low MICs and favorable biofilm results indicate good prospects for therapeutic applications.
I. Development of Novel Copolyoxetane Antimicrobial Agents

Introduction

The ongoing threat of antibiotic resistance is an important risk to human health. Conventional antibiotics can kill bacteria by hindering their metabolic pathway through cell wall synthesis, protein synthesis, or transcription. However, many kinds of bacteria are able to adapt and thrive in the presence of antibiotic doses that were once effective. Therefore, there is an increased urgency to develop new classes of antimicrobials. The immune systems of a wide range of organisms encompass certain polypeptides that afford an ability to protect against bacterial infections. These peptides not only are effective against infections, but do so without build up of resistance and are referred to as ‘naturally occurring antimicrobial peptides’ (AMPs). AMPs are believed to function by ionic and hydrophobic interactions with the bacterial membrane, causing cell death by altering the phospholipid bilayer, disruption of ion diffusion through cell wall ion channels, and affecting changes in the cell potential.[2, 3] A major advance would be attained by developing polycations that mimic the charged/neutral structural chain structure of AMPs, but have the potential to be made economically on a large scale. Taking guidance from one structural feature of AMPs, the cation/neutral chain structure, the work in this thesis will develop amphiphilic copolyoxetane antimicrobials that have the potential to overcome bacterial resistance. Encouraging evidence provided in this dissertation shows that these antimicrobials are potent against several strains of bacteria in their planktonic states with low cytotoxicity to red blood cells (RBCs), Human foreskin fibroblasts (HFFs), and Human Dermal Fibroblasts (HDFs). However, when bacteria grow as a biofilm, they are more difficult to treat
than their planktonic counterparts. This dissertation also includes the biocidal efficacy of the most effective copolyoxetane antimicrobial against *P. aeruginosa* biofilms. The long term goal is to develop an antimicrobial that can be used as a therapeutic agent.

**Background and significance:**

**Antimicrobial resistance and hospital acquired infections.** The use of antibiotics in humans, animals, and agriculture has played a significant role in the emergence of resistant bacteria. This well known development of resistant bacterial strains raises the challenge for development of new classes of antimicrobials for which buildup of resistance is curtailed or eliminated.

It is reported by the U.S. Center for Disease Control (CDC) that hospital acquired infections (HAIs) are among the top ten leading causes of death in the US, costing $20 billion annually.[4] Of these HAIs, 60% involve antibiotic resistant bacteria.[5] The majority of HAIs involve the urinary tract, bloodstream, surgical site, and respiratory tract. Many infections are a result of *Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa.*[6]

It should be noted that the resistance of *P. aeruginosa* has been shown for most antimicrobials.[7] Additional lipopolysaccharides and other mechanisms are thought to decrease the interaction of antimicrobials with the cell membrane. Among all Gram (-) bacteria, *P. aeruginosa* has an overall outer cell membrane permeability that is 12-100 times lower than *E. coli.*[8-10] Also, *P. aeruginosa* has a low susceptibility toward antibiotics as a result of efficient efflux pumps.[11]

In addition to the acute infections, microbial biofilms are the cause of many persistent and chronic infections that are also increasingly difficult to treat and eliminate. For example,
many patients with Cystic Fibrosis develop chronic lung *P. aeruginosa* infections that are very complicated to treat with conventional antibiotics because of inherent biofilm formation.[12, 13] Biofilms are surface attached bacteria contained within an exopolysaccharide matrix which further facilitates the antibiotic resistance associated with these persistent infections.[14]

**Bacteria live in Planktonic (free living) and Biofilm states.** Bacteria that are suspended and growing in a fluid environment live in a planktonic state while surface attached bacteria exist in a biofilm state. Being complex communities of surface and/or cell adhered microorganisms, biofilms fundamentally differ from their single celled planktonic counterparts. Biofilms are protected from antimicrobials and the host immune response by an extracellular polymeric substance (EPS) matrix comprised of polysaccharides, proteins and/or nucleic acids, which foster intercellular adhesion and surface attachment.[14] Biofilm growth occurs in sequential stages including i) initial transport of planktonic cells to a surface ii) initial attachment of planktonic cells (iii) formation of the EPS and microcolonies (iv) formation of macrocolonies/ biofilm maturation and (v) dispersion.[15] Because microbial biofilms are the cause of many persistent and chronic infections, they are increasingly difficult to treat and eliminate.

*P. aeruginosa* is one particularly virulent bacterium that can be found to contaminate contact lenses and indwelling medical devices like catheters. Its persistence proves catastrophic to immunocompromised patients particularly burn patients, cystic fibrosis patients, and the elderly. For example, many patients with Cystic Fibrosis develop chronic lung *P. aeruginosa* biofilm infections that are very difficult to eradicate with conventional antibiotics.[12, 13]

3
Antimicrobial Peptides (AMPs) and Synthetic Mimics of Antimicrobial Peptides (SMAMPs). Antimicrobials are found in nature, namely a wide variety of antimicrobial peptides. Zasloff noted that these “naturally occurring antimicrobial peptides – AMPs” provide the first line of defense against bacterial infections.[16] The structure of the AMP magainin 1 consists of positively charged segments (hydrophilic, blue- circled) and neutral segments (hydrophobic, brown).[17] AMPs have relatively low ratios of charged to uncharged units (0.2 for magainins and 0.3 for defensins).[17, 18]

These AMPs have a characteristic amphiphilic structure, are water soluble and bactericidal at relatively low concentrations.[19-21] Amphiphilic means that they have both a hydrophobic and hydrophilic (positively charged units) in the same polypeptide chain. The cationic (hydrophilic) portion of the magainin is believed to act as the antimicrobial moiety. Because this cationic moiety is attracted to anionic phosphatidyl glycerol (PG), a component in bacterial membranes, it has the ability to differentiate between prokaryotic and eukaryotic membranes. The hydrophobic moiety then facilitates bacterial membrane disruption resulting in lysing of the cell and eventual cell death as a result of osmotic shock.[2, 3]
Preliminary data from the Wynne Laboratory: P[AB] copolyoxetanes.

In pioneering research, Kurt synthesized the first water soluble copolyoxetane antimicrobial that had cation and neutral moieties possessed by naturally occurring antimicrobial polypeptides.[22] P[AB] copolyoxetanes (2) are random copolymers containing a quaternary alkylammonium containing side chain ‘A’ and an uncharged PEG-like side chain, ‘B’. Preliminary results showed that these two copolyoxetanes had surprisingly good biocidal properties. They are excellent solution antimicrobials with high water solubility (up to 400 mg/ml). The good water solubility is due to both the presence of the hydrophilic PEG-like side chains and the polar quaternary-salt side chains.

A major advance would be attained by developing polycations that not only mimic the charged/neutral structural chain structure of AMPs, but also have the potential to be made economically on a large scale. Chapter 1 of this dissertation explores the synthesis of copolyoxetanes by changing the amphiphilic balance. Included in this chapter are the effects of these novel copolyoxetanes on antimicrobial activity of planktonic pathogenic bacteria in solution and their hemocompatibility. Chapter 2 further explores the effects of these antimicrobial copolyoxetanes on human cells. In Chapter 3, the synthesis of AMP-like copolyoxetanes with antimicrobial activity in solution is investigated by manipulating the quaternary pendant groups. In view of the sensitivity of biocidal potency to amphiphilic balance, this optimization of the P[AB] copolyoxetanes is achieved by varying the alkyl chain length
(from C2 to C16) while keeping the mole ratio constant at 50 percent. The copolyoxetanes were evaluated for antimicrobial activity and their effects on human cells. Chapter 4 addresses the effect of the most potent antimicrobial copolyoxetane on *P. aeruginosa* biofilms.
Chapter 1

Highly Effective, Water Soluble, Hemocompatible Poly[(3,3-Quaternary/PEG)-Copolyoxetanes]

ABSTRACT

This study focuses on the solution antimicrobial effectiveness of a novel class of copolyoxetanes with quaternary ammonium and PEG-like side chains. A precursor P[(BBOx-m)(ME2Ox)] copolyoextane was prepared by cationic ring opening copolymerization of 3-((4-bromobutoxy)methyl)-3-methyloxetane (BBOx) and 3-((2-(2-methoxyethoxy)ethoxy)methyl)-3-methyloxetane (ME2Ox) to give random copolymers with 14-100 (m) mole% BBOx. Reaction of P[(BBOx-m)(ME2Ox)] with dodecyl dimethylamine gave the corresponding quaternary P[(C12-m)(ME2Ox)] polycation salts, designated C12-m, as viscous liquids in 100% yield. BBOx:ME2Ox and C12:ME2Ox ratios were obtained by $^1$H-NMR spectroscopy. C12-m molecular weights ($M_n$, 3.5-21.9 kDa) were obtained from $^1$H-NMR end group analysis. DSC studies up to 150 °C showed only thermal transitions between -69 and -34 °C assigned to $T_g$’s. Antibacterial activity for the C12-m copolyoxetanes was tested by determining minimum inhibitory concentrations (MIC) against Gram(+) S. aureus and Gram(-) E. coli and P. aeruginosa. MIC decreased with increasing C12 mole percent, reaching a minimum in the range C12-43 to C12-60. Overall, the antimicrobial with consistently low MICs for the three tested pathogenic bacteria was C12-43: (bacteria, MIC, μg/ml) E. coli (6), S. aureus (5) and P. aeruginosa (33). For C12-43, minimum biocidal concentration (MBC) to reach 99.99% kill in 24 hr required 1.5 times MIC for S. aureus and 2 times MIC for E. coli and P. aeruginosa. At 5× MIC against a challenge of $10^8$ cfu/ml, C12-43 kills ≥ 99% S. aureus, E. coli and P. aeruginosa.
within 1 hr. C12-m copolyoxetane cytotoxicity toward human red blood cells was low indicating good prospects for biocompatibility. The tunability of C12-m copolyoxetane compositions, effective antimicrobial behavior against Gram(+) and Gram(-) bacteria, and promising biocompatibility offer opportunities for further modification and potential applications as therapeutic agents.

Introduction

Aqueous solutions of alkylammoniums have long been employed as antimicrobials.[23-25] In 1984, Ikeda noted that “The adsorption of polycations onto the negatively charged cell surface is expected to take place to a greater extent than that of monomeric cations because of the much higher charge density carried by the polycations.”[26] Subsequent research from the Ikeda laboratory explored antimicrobial activity for a number of polycation architectures and compositions.[27-30] The last decade has seen a substantial growth in interest in polycation antimicrobials. Homopolycations,[31-34] copolymers,[35-37] and molecular polyamines[38, 39] have been explored.

Several polycation chain architectures have been shown more effective than their respective low molar mass analogs including side chain polycrylates with biguanide groups[26, 28] and polyphosphonium salts with alkyl side chains.[30, 40, 41] Polycationic dendrimers are also effective antimicrobials.[42, 43] Recent studies have included the development of amphiphilic polycations designated AP which, although very different in terms of molecular structure,[3, 44, 45] are intended to mimic the charged / neutral repeat unit combination of
naturally occurring antimicrobial polypeptides (AMPS).[19, 38, 46] AP have also been
designated as SMAMPs, synthetic mimics of antimicrobial peptides.[47]

Reflecting the wide diversity of polycations, cholic acid derivatives were developed by
Savage to mimic the bactericidal behavior of polymyxin B (PMB).[48] The cationic lipopeptide
PMB is used as a “last resort for the treatment of serious Gram-negative infections caused by
multiresistant strains.”[49] The structure of one of the synthetic cholic acid polycations (CSA-
13) is shown in Table 1.1(16).
Table 1.1. Selected polycation structures and MICs (µg/ml, µM). For a given structural class, structures (and MICs) are for an optimum representative composition.

<table>
<thead>
<tr>
<th>Polycation Structure</th>
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<th>Bacteria</th>
<th>MIC µg/ml, (µM)</th>
<th>Reference</th>
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<tr>
<td><img src="image" alt="Structure 4" /></td>
<td>Methacrylate homopolymers and copolymers with a quaternary amine functionalized side group; optimum quat composition shown; higher DPs, lower MICs</td>
<td>- <em>E. coli</em></td>
<td>&gt;2000 µg/ml (&gt;870 µM)</td>
<td>[36]</td>
</tr>
<tr>
<td><img src="image" alt="Structure 5" /></td>
<td>Homopolymers of acrylate monomers with biguanide groups. 12.1 kDa.</td>
<td>- <em>E. coli</em></td>
<td>660-1000 µg/ml (55-83 µM)</td>
<td>[26]</td>
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<td></td>
<td></td>
<td>- <em>S. aureus</em></td>
<td>100-330 µg/ml (8.4-27 µM)</td>
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<tr>
<td></td>
<td></td>
<td>- <em>P. aeruginosa</em></td>
<td>660-1000 µg/ml (55-83 µM)</td>
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<td>6</td>
<td>Poly(2-methyl-1,3-oxazoline) with terminal quaternary ammonium groups; optimum quat composition; higher DPs, lower MICs.</td>
<td>- S. aureus</td>
<td>200 µg/ml (74 µM)</td>
<td>[37]</td>
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<td>- E. coli</td>
<td>- R = -C$<em>{12}$H$</em>{25}$, 64 µg/ml (2 µM) 128 µg/ml (4 µM) $n = 70$</td>
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<td>- S. aureus</td>
<td>- R = -C$<em>{16}$H$</em>{33}$, 256 µg/ml (8 µM) 32 µg/ml (1 µM)</td>
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<td>- E. coli</td>
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<td></td>
<td>- S. aureus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td>Description</td>
<td>Antibiotics</td>
<td>Dosage</td>
<td>Ref.</td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
<td>-------------</td>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td><img src="image" alt="Pyridinium methacrylate copolymers" /></td>
<td>Pyridinium methacrylate copolymers</td>
<td>- E. coli</td>
<td>- R = -C₄H₉, 50 µg/ml (2.2 µM)</td>
<td>[50]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- B. subtilis</td>
<td>- R = -C₆H₁₃, 30 µg/ml (1.1 µM)</td>
<td>n = 50</td>
</tr>
<tr>
<td><img src="image" alt="Methacrylate copolymers with butyl side groups" /></td>
<td>Methacrylate copolymers with butyl side groups (quaternary amine functionalized)</td>
<td>- E. coli</td>
<td>34 µg/ml, (14 µM)</td>
<td>[36]</td>
</tr>
<tr>
<td>Reaction</td>
<td>Compound Description</td>
<td>Bacteria</td>
<td>Concentration</td>
<td>Ref.</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------</td>
<td>----------</td>
<td>---------------</td>
<td>------</td>
</tr>
<tr>
<td>10</td>
<td>Amphiphilic polynorbornene derivatives</td>
<td>E. coli</td>
<td>25 µg/ml (2.5 µM)</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B. subtilis</td>
<td>25 µg/ml (2.5 µM)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Facially amphiphilic poly(m-phenylene ethynylene) polymers</td>
<td>E. coli</td>
<td>25 µg/ml (4.6 µM)</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B. subtilis</td>
<td>25 µg/ml (4.6 µM)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>PEGylated – quaternary acrylate copolymers</td>
<td>B. subtilis</td>
<td>20 µg/ml (1.02 µM)</td>
<td>[51]</td>
</tr>
<tr>
<td></td>
<td>q = 1 (methyl)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Chemical Structure</td>
<td>Type</td>
<td>Bacterial Activity</td>
<td>MIC</td>
<td>Source</td>
</tr>
<tr>
<td>--------------------</td>
<td>------</td>
<td>--------------------</td>
<td>-----</td>
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</tr>
<tr>
<td><img src="image1" alt="13" /></td>
<td>Oligoguanidines</td>
<td><em>E. coli</em></td>
<td>8 µg/ml (9.5 µM)</td>
<td>[31]</td>
</tr>
<tr>
<td><img src="image2" alt="13" /></td>
<td>Oligoguanidines</td>
<td><em>S. aureus</em></td>
<td>4 µg/ml (4.7 µM)</td>
<td></td>
</tr>
<tr>
<td><img src="image3" alt="13" /></td>
<td>C12-43</td>
<td><em>E. coli</em></td>
<td>6 µg/ml (2.4 µM)</td>
<td>Present study</td>
</tr>
<tr>
<td><img src="image4" alt="13" /></td>
<td>C12-43</td>
<td><em>S. aureus</em></td>
<td>5 µg/ml (2 µM)</td>
<td></td>
</tr>
<tr>
<td><img src="image5" alt="13" /></td>
<td>C12-43</td>
<td><em>P. aeruginosa</em></td>
<td>33 µg/ml (13 µM)</td>
<td></td>
</tr>
<tr>
<td><img src="image6" alt="13" /></td>
<td>Polyoxanorbornene with alkyl pyridinium side chains</td>
<td><em>E. coli</em></td>
<td>4 µg/ml (1.3 µM)</td>
<td>[52]</td>
</tr>
<tr>
<td><img src="image7" alt="13" /></td>
<td>Polyoxanorbornene with alkyl pyridinium side chains</td>
<td><em>B. subtilis</em></td>
<td>4 µg/ml (1.3 µM)</td>
<td></td>
</tr>
<tr>
<td><img src="image8" alt="13" /></td>
<td>Butyl methacrylate-tertiary amine methacrylate copolymers</td>
<td><em>E. coli</em></td>
<td>3.8 µg/ml (2.4 µM)</td>
<td>[36]</td>
</tr>
<tr>
<td>Compound</td>
<td>Description</td>
<td>Organism</td>
<td>Concentration</td>
<td>Reference</td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
<td>----------</td>
<td>---------------</td>
<td>-----------</td>
</tr>
<tr>
<td>16</td>
<td>Dication with a 4,6-dicarboxy pyrimidine core</td>
<td>E. coli</td>
<td>0.8 µg/ml (1.2 µM)</td>
<td>[39]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B. subtilis</td>
<td>0.8 µg/ml (1.2 µM)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>CSA-13 Ceragenin</td>
<td>S. aureus (vancomycin resistant)</td>
<td>1 µg/ml (1.5 µM)</td>
<td>[1, 38]</td>
</tr>
</tbody>
</table>
In antimicrobial studies, the minimum inhibitory concentration (MIC) is the concentration at which bacterial growth is inhibited in nutrient media. The minimum bactericidal concentration (MBC) is the concentration at which all bacteria are killed. Because bacteria have sizes larger than the wavelength of visible light, determining whether bacteria grow is conveniently measured by UV-visible spectroscopy. Growth after 24 hr is signaled by turbidity that results in absorption at 600 nm.[34, 36] At short test times kill kinetics vary substantially depending on the biocide, its concentration, and bacterial target. From the few studies carried out on polycation kill kinetics[26, 38] concentrations 5 or 10 times higher than MICs are necessary to achieve rapid kill. If rapid kill is required, low MICs are desirable for economy and to avoid high antimicrobial concentrations that could lead to adverse effects on human cells.

In addition to low MICs, an important feature emerging from recent studies of amphiphilic polycation antimicrobials is that, at least in some cases, bacteria fail to develop resistance. For example, poly(4-N-alkylpyridinium salts) were still active against *S. aureus* strains that developed resistance to N-hexylpyridinium bromide.[53] Failure to develop resistance may not be a universal characteristic of amphiphilic polycations. Hancock reported that sub-inhibitory levels of polycationic antibiotics such as polymyxins induce resistance toward higher, otherwise lethal, levels of these antimicrobial agents.[54]

By analogy to antimicrobial peptides, the killing mechanism for polycations is explained by a combination of ionic and hydrophobic interactions between the bacterial cellular membrane and multiple ammonium moieties.[27] After or coincident with polycation chemisorption, disruption of the phospholipid bilayer includes diffusion through the cell wall. Polycation diffusion and binding to the cytoplasmic membrane leads to further changes resulting in cell
death. This mechanism was described by Ikeda in the early 1980’s.[26-28] Subsequently, many researchers provided additional details and insights with regard to this mechanism.[38, 55]

Ikeda noted the greater release of K\(^+\) for polycations compared to a single-charge quaternary.[27, 28] With singly charged ammonium species, one mole of K\(^+\) salt may be released from the bacterium \([B(nK^+)nX^-]\) per mole of ammonium (Eq. 1). For polycations the number of moles of K\(^+\) salt released can be as great as the number of charges \(m\) per polycation. Eq. 2 illustrates a molecular polycation with \(m\) charges releasing \(m \times n\) equivalents of K\(^+\).

\[
\begin{align*}
\text{B(nK}^+)\text{nX}^- + n\text{A}^+\text{Y}^- & \rightarrow \text{B(nA}^+)\text{nX}^- + n\text{K}^+\text{Y}^- \quad \text{Eq. 1} \\
\text{B(nK}^+)\text{nX}^- + \text{AP}^{m+}(m\text{Y}^-) & \rightarrow \text{B(AP}^{(m \times n)^+})\text{nX}^- + (m \times n) \text{K}^+\text{Y}^- \quad \text{Eq. 2}
\end{align*}
\]

Polycation effectiveness (PE) can be understood in terms of the entropic driving force generated by polycations binding to bacteria with release of potassium salt (Eq. 2). Potassium ion release disrupts the bacterial cell potential contributing to cell death. While providing a partial explanation for polycation effectiveness, the broad range of polycation activity, ranging from negligible to powerful reflects other important considerations such as amphiphilic balance and polycation architecture, the nature of which are not yet well understood.

Previously, we reported the incorporation of polycation copolyoxetane soft blocks into polyurethane coatings and examined
contact antimicrobial properties.[56] One of the precursors to new soft blocks employed in this coatings effort was 1 with 14 mole percent quaternary bromide (C12-14). The previously reported HMDI/BD(30)-P[(C12-14)(ME2Ox)-8900], is designated C12-14-PU, where the hard block (30 wt %) was derived from 4,4’-(methylen bis(p-cyclohexyl isocyanate) (HMDI) and butanediol (BD). Highly effective contact kill (not biocide release) was demonstrated for neat C12-14-PU coatings and for a blend of C12-14-PU with a base polyurethane.[56] As a polymer surface modifier, only 2 wt% C12-14-PU added to a conventional polyurethane resulted in a coating that provided 100% kill in 30 min exposure to spray challenges (~10\(^7\) cfu/ml) of \textit{E. coli} or \textit{P. aeruginosa} and 98.7% kill for \textit{S. aureus}.[56]

Aqueous solubility for C12-14 (1, m = 0.14), a random copolyoxetane generated by simultaneous addition of comonomers,[57-59] raised interest in investigating solution antimicrobial properties for C12-m copolyoxetanes. To initiate this investigation a series of water soluble C12-m copolyoxetanes was prepared with increasing C12 mole percent. Minimum inhibitory concentrations (MICs) were determined for three bacterial pathogens including Gram (+) \textit{S. aureus} and Gram (-) \textit{E. coli} and \textit{P. aeruginosa}. With optimum compositions, MICs as low as 5 μg/ml were observed. Kill kinetics and MBCs were determined for C12-43, one of the most highly effective C12-m copolyoxetanes. Low cytotoxicity toward human red blood cells indicated good prospects for biocompatibility of C12-m copolyoxetanes. Interestingly, selectivity for antimicrobial behavior against Gram(+) \textit{S. aureus} and Gram(-) \textit{E. coli} was dependent on the mol % C12. For high mol% C12, selectivity was opposite that previously reported.
Experimental

Materials. 3-Bromomethyl-3-methyloxetane (BrOx) was generously provided by OMNOVA Solutions (Akron, OH). N,N-dimethyldodecylamine (C12) was a gift from Lonza (Allendale, NJ). Methylene chloride (CH₂Cl₂), and tetrahydrofuran (THF) were obtained from Aldrich and dried by storing over 4Å molecular sieves. Boron trifluoride etherate (BF₃·O(C₂H₅)₂), tetrabutylammonium bromide (TBAB), dodecyltrimethyl ammonium bromide (DTAB), 3-(hydroxymethyl)-3-methyloxetane, 1,4-dibromobutane and sodium hydride (NaH) were also obtained from Aldrich and used as received. 1,4-Butanediol (BD) and 2-(2-methoxyethoxy)ethanol were purchased from Acros Chemicals and used as received. All M9 media components[60] were purchased from Sigma Aldrich. Luria Broth (LB) was purchased from Fisher Scientific. Human red blood cells (RBCs) were obtained from Innovative Research (Novi, MI) packed and transported on ice in a cooler (~ 4 °C), refrigerated upon receipt and used within one week.

Designations. As noted above, P[(C12)ₘ{(ME2Ox)₁₋ₘ}] copolyoxetanes 1 are designated by C12-ₘ, where m is the mole percent C12-containing repeat unit. Similarly, BBOx-ₘ is used to represent the mole percent BBOx in the P[(BBOx)ₘ{(ME2Ox)₁₋ₘ}] copolyoxetane precursor.

Synthesis and Characterization. Monomers were synthesized as described previously.[22] 3-((4-Bromobutoxy)methyl)-3-methyloxetane (BBOx) and 3-((2-(2-methoxyethoxy)ethoxy)methyl)-3-methyloxetane (ME2Ox) monomers were distilled prior to use (BBOx: 85 °C/5 mmHg, ME2Ox: 100 °C/5 mmHg). Minimum monomer purity was typically 99+ percent (GC-MS). Monomer synthesis and purification was followed by a ring opening
polymerization to BBOx-m copolyoxetane precursors as described by Kurt.[22] Reaction with C12 tertiary amine gave C12-m copolyoxetanes 1. As an example, the preparation of BBOx-42 and the corresponding C12-43 is provided below.

\[ P[(BBOx)_{0.42}(ME2Ox)_{0.58}], \text{ BBOx-42}. \] Through a metering pump, over a period of 3 hr under nitrogen purge, a solution of BBOx (5.74 g, 24.2 mmol) and ME2Ox (10 g, 49 mmol) in 30 mL anhydrous CH\(_2\)Cl\(_2\) was added to a three-necked 100 ml flask containing BF\(_3\)·OEt\(_2\), 1,4-butanediol (0.06 g, 0.67 mmol) and 25 ml anhydrous CH\(_2\)Cl\(_2\). The reaction system was stirred for 15 hr at 0°C. The mixture was quenched with H\(_2\)O followed by stirring for 0.5 hr. The organic phase was washed in sequence with 3 wt% aqueous HCl, 3 wt% aqueous NaCl, and two times with 20 ml distilled water. The solvent was evaporated and the residue was dried at 80°C under vacuum for 48 hours to obtain 15.8 g BBOx-42 copolymer. \(^1\)H-NMR (CDCl\(_3\): \(\delta\) 0.91 ppm (-CH\(_3\), 3H, s), 1.68 ppm (side chain –OCH\(_2\)CH\(_2\)CH\(_2\)Br, –CH\(_2\)-for BBOx, 2H), 1.92 ppm (-OCH\(_2\)CH\(_2\)CH\(_2\)Br, –CH\(_2\)-for BBOx, 2H), 3.19 ppm (main chain –CH\(_2\)-, 4H, m), \(\delta\)3.30 ppm (-CH\(_2\), 2H, s), 3.38 ppm (-OCH\(_3\) 3H, s), 3.4 ppm (-CH\(_2\)Br, 2H), 3.55 ppm (-OCH\(_2\)CH\(_2\)O-, 4H, m), 3.64 ppm (-OCH\(_2\)CH\(_2\)O-, 4H, m).

The preparative procedure was the same for other BBOx-m copolyoxetane precursors, but the amount of the comonomers, catalyst and the co-catalyst was changed to obtain desired copolymer compositions shown in Table 1.2. The value of ‘m’ for the BBOx-m copolyoxetane intermediates and C12-m was determined by \(^1\)H-NMR spectroscopy as described in Appendix A.
Table 1.2. Reactants for BBOx-m synthesis and copolyoxetane compositions.

<table>
<thead>
<tr>
<th>BF₃ etherate catalyst g (mmol)ᵃ</th>
<th>BBOx feed g (mmol)</th>
<th>ME2Ox feed g (mmol)</th>
<th>BBOx/ME2Ox molar feed ratio</th>
<th>BBOx:ME2Ox mole ratioᵇ</th>
<th>BBOx:ME2Ox mole %ᵇ</th>
<th>Mₙ kDaᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.83 (12.9)</td>
<td>3.82 (16.1)</td>
<td>23.08 (113)</td>
<td>1:7</td>
<td>1:6</td>
<td>14:86</td>
<td>7.8</td>
</tr>
<tr>
<td>0.147 (1.04)</td>
<td>3.20 (13.5)</td>
<td>8.27 (40.5)</td>
<td>1:3</td>
<td>1:3</td>
<td>25:75</td>
<td>5.9</td>
</tr>
<tr>
<td>0.185 (1.31)</td>
<td>5.74 (24.2)</td>
<td>10.0 (49.0)</td>
<td>1:2</td>
<td>1:1.4</td>
<td>42:58</td>
<td>2.5</td>
</tr>
<tr>
<td>1.69 (12)</td>
<td>9.5 (40.9)</td>
<td>10.0 (49.0)</td>
<td>1:1.22</td>
<td>1:1</td>
<td>50:50</td>
<td>5.7</td>
</tr>
<tr>
<td>0.77 (5.44)</td>
<td>7.1 (30)</td>
<td>5 (24.5)</td>
<td>1.22:1</td>
<td>1.5:1</td>
<td>60:40</td>
<td>11.5</td>
</tr>
<tr>
<td>0.24 (1.69)</td>
<td>7.5 (31.6)</td>
<td>2.15 (10.5)</td>
<td>3:1</td>
<td>6.7:1</td>
<td>87:13</td>
<td>12.2</td>
</tr>
<tr>
<td>0.24 (1.69)</td>
<td>10 (42.2)</td>
<td>0</td>
<td>NAᵈ</td>
<td>NAᵈ</td>
<td>100:0ᵉ</td>
<td>6</td>
</tr>
</tbody>
</table>

a. The mole ratio of catalyst (BF₃) to co-catalyst (butane diol) was 2:1.
b. The BBOx and ME2Ox mole percents were determined by ¹H-NMR integration.
c. Mₙ was determined by end group analysis employing ¹H-NMR.
d. NA = not applicable
e. P[BBOx]
$P[(C12)_{m}(ME2Ox)_{1-m}]$ copolyoxetanes, C12-m. BBOx-m copolyoxetanes were quaternized with N, N-dimethyldodecylamine to yield the C12-m analogs. Table 1.3 provides reactant quantities. An example follows for C12-43, that is $P[(C12)_{0.43}(ME2Ox)_{0.57}]$. Copolyoxetane BBOx-42 (4 g, 1.6 mmol) in 20 ml acetonitrile was heated to reflux and N, N-dimethyldodecylamine (6.14 g, 28.9 mmol) was added slowly. The mixture was stirred for 15 hours under nitrogen. The solvent and excess N, N-dimethyl dodecylamine were evaporated under vacuum to yield a highly viscous, slightly yellow liquid. [$^1$H-NMR (CDCl$_3$): $\delta$ 0.82 ppm (-CH$_3$, 6H, s), 1.2-1.4 ppm (-CH$_2$(CH)$_2$)$_{10}$CH$_3$, 20H), 1.68-1.92 ppm (-OCH$_2$CH$_2$CH$_2$N-, 4H, broad), 3.19 ppm (main chain –CH$_2$-, 6H, m), 3.30 ppm (-CH$_2$, 2H, s), 3.38 ppm (-OCH$_3$, 3H, s), 3.4 ppm (-CH$_2$N- 2H, CH$_3$-N-CH$_3$, 6H), 3.55 ppm (-OCH$_2$CH$_2$O-, 4H, m), 3.64 ppm (-OCH$_2$CH$_2$O-, 4H, m).

Figure A1 shows the $^1$H-NMR spectrum and assignments for BBOx-42 and C12-43. $^1$H-NMR spectroscopy was used to establish that substitution of C-Br by the amine was complete. The calculations and methods used for confirming the molar ratios are described in Appendix A. The different compositional designations (BBOx-42, C12-43) result from round-off of slightly different $^1$H-NMR integrations. Molecular weights ($M_n$) for BBOx-m copolyoxetanes were determined by $^1$H-NMR end group analysis (Tables 1.2 and 1.3).[57] C12-m copolyoxetane molecular weights ($M_n$) were calculated based on those for BBOx-m precursors (Table 1.3).
Table 1.3. Reactants for quaternionization and C12-m copolyoxetane $T_g$.

<table>
<thead>
<tr>
<th>BBOx/ME2Ox Mole % ratio</th>
<th>BBOx-m, g (mmol)</th>
<th>C12 g (mmol)</th>
<th>C12:ME2Oxa Mole ratio(mole %)</th>
<th>C12-m Mn, kDa</th>
<th>$T_g$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:86</td>
<td>9.41 (1.81)</td>
<td>1.83 (8.6)</td>
<td>0.16 (14:86)</td>
<td>8.9</td>
<td>-69</td>
</tr>
<tr>
<td>25:75</td>
<td>3.95 (0.67)</td>
<td>2.03 (9.54)</td>
<td>0.35 (26:74)</td>
<td>7.4</td>
<td>-67</td>
</tr>
<tr>
<td>43:58</td>
<td>4.00 (1.6)</td>
<td>6.14 (28.9)</td>
<td>0.74 (43:57)</td>
<td>3.5</td>
<td>-57</td>
</tr>
<tr>
<td>50:50</td>
<td>3.00 (0.52)</td>
<td>4 (0.014)</td>
<td>0.99 (50:50)</td>
<td>8.4</td>
<td>-56</td>
</tr>
<tr>
<td>60:40</td>
<td>1.94 (0.17)</td>
<td>3.00 (14.1)</td>
<td>1.5 (60:40)</td>
<td>18</td>
<td>-53</td>
</tr>
<tr>
<td>87:13</td>
<td>3.29 (0.27)</td>
<td>5.27 (24.8)</td>
<td>6.3 (87:13)</td>
<td>21.9</td>
<td>-34</td>
</tr>
<tr>
<td>100:0</td>
<td>2.2 (0.37)</td>
<td>5.38 (25.3)</td>
<td>R.H.S = 0.3b</td>
<td>11.4</td>
<td>-47</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L.H.S = 0.31</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Ratio of C12 to ME2Ox repeats obtained from $^1$H-NMR integration (details in Appendix A Information).
b. Calculations pertaining to reconfirmation of the degree of substitution of the BBOx-m copolyoxetane precursor is explained in the Appendix A.
**GPC.** GPC for C12-60 and C12-87, two representative C12-m copolyoxetanes, was obtained using a Viscotek Model 302 with refractive index, light scattering and viscosity detectors. With THF as the mobile phase and columns for polar polymers (GRAM1000A and GRAM30A, PSS-USA) no signals were obtained suggesting polycation chemisorption on the columns. GPC was tried using acetic acid/water (5:95) as mobile phase[61] with GPC columns recommended for polycations (Viscotek C-series C-MBMMW-3078). Once again, the lack of signals for all detectors indicated column chemisorption. Chemisorption of dendrimer polycations precluded molecular weight determinations by Cooper.[42, 43]

Prior studies used sodium nitrate[62, 63] or other salts and/or buffer solutions[64-68] to adjust ionic strength and prevent aggregation. However, precipitation for C12-60 and C12-87 was observed in the presence of various aqueous salts at several concentrations (0.1 to 1.0 M). As noted below, insolubility in the presence of salts also required the development of special growth media for solution antimicrobial tests. GPC results were finally obtained with pure water as the mobile phase (ViscoGel Polycationic C-MBMMW-3078 column) using a 65 kDa Dextran standard. PEO standards gave irreproducible results with this column. GPC $M_n$ (PD) for C12-60 and C12-87 were 8.2 kDa (4.6) and 20.5 kDa (1.9), respectively.

**Instrumentation:** $^1$H-NMR characterization was done using a Varian Mercury 300 MHz NMR spectrometer, FTIR spectra were obtained with a Magna-IR 760 spectrometer, and Modulated Differential Scanning Calorimetry (MDSC) utilized a DSC Q1000 (TA Instruments). A Perkin-Elmer GC-MS, model G1530A was used for assessing monomer purity.
Antimicrobial Assay: Minimum Inhibitory Concentration (MIC):

**Bacterial suspension preparation.** Strains of *Escherichia coli* (EC) DH5α, *Pseudomonas aeruginosa* (PA) PA01 and *Staphylococcus aureus* (SA) ATCC-25904 were used for the biocidal tests. The cultures were streaked on Luria Agar plates and incubated overnight at 37 °C. A single colony from each strain was used to inoculate 6 ml of Luria Broth (LB), and grown overnight at 37 °C with vigorous shaking (225 rpm). A stock solution of \((10^8 - 10^9)\) colony forming units per milliliter (cfu/ml) of the desired bacteria was prepared from the bacterial solution in steady phase.

**MIC medium.** Initially Luria Broth (LB) was used as the medium for MIC evaluation of monovalent alkylammonium bromide DTAB. However, the C12-m copolyoxetanes precipitated in LB. Different media formulations were tested that would prevent polycation precipitation and at the same time generate substantial bacterial growth during the 24-hr incubation period. M9 growth medium was prepared containing 20 wt% mannitol as the source of carbon for EC and PA. However, significant growth for SA was not observed. A modified Tryptic Soy Broth (TSB) was chosen for MIC evaluation of SA as good growth was observed. Table A1. and Table A2. shows the ingredients used in preparing the respective media.

**MIC Assay.** MIC values for C12-m copolyoxetanes were determined by standard methods.[34, 36, 69] Stock solutions were made by dissolving different concentrations of C12-m copolyoxetanes in distilled water. The medium (6 ml, 1X mannitol for EC and PA or TSB for SA) were placed into Erlenmeyer flasks. Serial dilutions of C12-m copolyoxetane stock solutions
and 60 µL of the bacterial cultures (~ 10^8 cfu/ml) were added sequentially to the flasks. The flasks were then kept in shakers at 37 °C for 24 hours. Bacterial growth was examined by measuring solution optical density (Beckman Coulter DU 640 UV-Visible spectrophotometer, λ = 600 nm) in addition to noting visual turbidity (Figure A3). This MIC assay, which depends on a change in optical density of two orders of magnitude and a visual observation of solution transparency, provides confirmation of complete inhibition, that is, MIC_{100}. [34, 36, 70]

To determine the efficiency of polyvalency, the MIC values were normalized to MIC per quaternary charge bearing repeat unit. An illustration for the above conversion is given in Appendix A-3.

**MBC Assay.** Bacterial suspensions were prepared as described above. C12-43 was selected for MBC assay as overall this was one of the most effective antimicrobial composition. MIC tests were performed at different C12-43 concentrations (0, 1.5, 2 and 3× MIC). After 24 hr, a 1 ml aliquot from each of the flasks was taken and centrifuged at 1400 rpm for 3 minutes. The supernatant formed in this process was gently removed and the pellet was resuspended in 1 ml of saline. This process was repeated 2 times and 100 µl aliquots were taken and then plated on agar plates in triplicate. The plates were incubated at 37 °C for 24 hrs. The number of colonies on each plate was read and MBC for C12-43 was determined.

**Killing Kinetics Assay.** Agar plates were streaked with either *E. coli*, *P. aeruginosa*, or *S. aureus* from a frozen stock culture at -70 °C and incubated at 37 °C for 18-24 hrs. From this plate, a single colony was collected and used to inoculate 6 ml of Luria broth. This culture solution was incubated for 18-24 hrs at 37 °C. A subculture was made by preparing a 1:100 dilution in LB and
incubated at 37 °C until it reached an optical density of ~ 0.5 by UV-VIS (logarithmic growth phase). A 20 ml aliquot of this bacterial culture was taken and spun down to a pellet for 10 min at 10,000 rpm in an ultracentrifuge. The supernatant was discarded and the pellet was dissolved in 20 ml of the respective growth media used for the MIC tests (1X mannitol for E. coli, P. aeruginosa, or TSB for S. aureus). The bacterial culture was divided into two equal volumes; one served as the control, while C12-m copolyoxetane was added to the other at the desired concentration. At selected times (t = 0, 1 hr, 2 hr, 3 hr), 1 ml aliquots were taken from both the control and sample flasks and spun into a pellet in a microcentrifuge for 3 minutes at 1400 rpm. The supernatant was discarded and the pellet was resuspended in saline solution. The same cycle was repeated twice so that the pellet suspended in saline contained no growth media. A serial dilution series was performed with 100 µL of the bacterial culture in saline. 100 µL aliquots were taken from each dilution and plated on Luria agar plates. These plates were incubated at 37 °C for 24 hours. The number of colonies were counted and compared with the control to obtain percent kill as a function of time.

**Cell Cytotoxicity Assay: HC<sub>50</sub> protocol.** The hemolytic toxicity assay followed the procedure described by Palermo and Kuroda.[36] RBCs (1ml) were diluted with 9 ml of phosphate buffered saline, PBS, (i.e. 10mM phosphate, 138 mM NaCl, pH 7.4, Sigma) and then centrifuged at 1000 rpm for 5 min. The resulting supernatant was removed using a pipette making sure not to disturb the RBCs and these steps were repeated two more times. The assay stock (90 µL) was mixed with each of the polymer solutions (10 µL) on a sterile 96 well round bottom polystyrene microplate to give a final solution of 3.3% v/v RBC. Based on a hemacytometer count, this was approximately 10<sup>8</sup> red blood cells per mL. PBS (10 µL) or Triton X-100 (10 µL, 1% v/v) was
added to the assay stock solution as negative and positive hemolysis controls, respectively. Triton X-100, a non-ionic surfactant is commonly used as a positive control in hemolysis assays.[36] For C12-m copolyoxetanes tests, 10 µL of stock solutions were added. The 96 well microplate was secured in an orbital shaker at 37 °C and 250 rpm for 1 hr and then centrifuged at 1000 rpm for 10 min. The resulting supernatant (10 µL) from each well was transferred to a new sterile 96 well round bottom polystyrene microplate and diluted with PBS (90 µL). Absorbance at 405 nm, characteristic of cell lysis,[36] was determined using a Versamax EXT microplate reader (Molecular Devices, Sunnyvale, CA). Negligible absorbance was observed for the negative control (PBS) while the absorbance for the positive control (Triton X) was 3.9. The fraction of hemolysis is defined as the absorbance for C12-m treated wells divided by the average of readings from the positive control wells. Percent RBC hemolysis is plotted as a function of C12-m copolyoxetane concentration in Figure 1.5. Two separate trials were carried out in triplicate for each assay trial. Values reported are the average of the two trials.

Results

The structure and compositions of C12-m copolyoxetanes 1, designated by mole percent C12 (e.g., C12-43), are first discussed followed by minimum inhibitory concentrations (MICs) and kill kinetics. The effect of polyvalency was examined by comparing MICs for 1 against Gram +/- bacteria versus those for dodecyltrimethyl ammonium bromide (DTAB). DTAB does not bear a PEG function, but it is a quaternary salt that was chosen as a monocation reference. Linear charge density, that is, C12 mole percent was systematically increased so as to observe trends and optimum compositions. The results for C12-m copolyoxetanes are compared with related amphiphilic polycation systems.
Polycopolyoxetanes. Copolyoxetanes 1 with 14, 26, 43, 50, 60, 87, and 100 mole % C12 were prepared in two steps. The ring opening copolymerization via simultaneous addition of 2 and 3 provided P[(BBO)x_m(ME2O)x_{1-m}] (BBOx-m) copolyoxetane intermediates. The monomer feed, feed ratio, experimentally determined compositions, and \( M_n \) for each of the six copolyoxetane precursors are in Table 1.2. BBOx/ME2Ox mole ratios were determined by integrating \(^1\)H-NMR spectra (Experimental / Appendix A). GPC on the C12-m copolyoxetanes was hampered by adsorption on columns and insolubility in salt solutions. GPCs were finally obtained for C12-60 and C12-87 in water. \( M_n \) (polydispersity) for C12-60 was 8.2 kDa (4.6) while the corresponding values for C12-87 were 20.5 kDa (1.9). \( M_n \)’s for C12-60 and C12-87 from end group analysis were 18 and 21.9 kDa, respectively. The reason for the differing \( M_n \)’s for C12-60 is not clear. Additional solution studies are required to provide comparative molecular weight data.

Previously, a detailed analysis with related copolyoxetanes using simultaneous comonomer addition provided evidence for a random copolymer architecture.[58, 71] Given the similar reaction conditions and simultaneous comonomer addition, precursor BBOx-m copolyoxetanes are assumed to be a random copolymers having PEG and bromobutoxymethyl side chains.[56] However, compared to feed, the BBOx/ME2Ox mole ratios showed systematic enrichment for BBOx indicating BBOx monomer 3 is preferentially incorporated in the copolyoxetane under the reaction conditions employed.
Figure 1.1. MDSC curves for (A) C12-60 and (B) C12-87

Corresponding C12-m copolyoxetanes 1 were obtained by overnight quaternization with dodecyldimethylamine. Table 1.3 shows the C12/ME2Ox mole ratios determined by $^1$H-NMR spectroscopy. Complete substitution for all C12-m was indicated by an intense peak between 1.2 and 1.4 ppm for –N-(CH$_3$)$_2$CH$_2$(CH$_2$)$_{10}$CH$_3$ and the absence of a peak at 1.9 ppm for CH$_2$ from BBOx-m. Analysis details are in Appendix A. Figure A2 shows the IR spectrum for BBOx-42 and C12-43. The different compositional designations stem from round-off of $^1$H-NMR integrations. BBOx-42 has a C-Br absorption peak at 650 cm$^{-1}$, while no absorption is seen in the 515-690 cm$^{-1}$ range for C12-43 as expected after quaternization.

C12-m copolyoxetanes are slightly yellow, viscous liquids at ambient temperature. Modulated Differential Scanning Calorimetry (MDSC) showed $T_g$’s ranging from -34 to -69 °C (Table 1.3). DSC thermograms are shown in Figure 1.1 for two representative C12-m copolyoxetanes (C12-60 and C12-87) having $T_g$’s at -53 °C and -34 °C, respectively. Although the total number of atoms in the quaternary side chain is 19, there is no evidence of endotherms (0 – 125 °C) typical
of side chain crystallization. The low $T_g$’s may be compared with polycation “molten salts” (-64 to -72 °C) and attest to high molecular mobility. This is attributed to a combination of ME2Ox side chains and ether functionality in the main chain and in the linkage to the C12 side chain.

**MIC Assay.** Several growth media were explored in order to avoid C12-m copolyoxetane precipitation. Luria Broth (LB) solutions were prepared with a range of salt concentrations (0.1-3 wt%). Although adequate bacterial growth was observed, the copolyoxetanes precipitated in media with $\geq 0.5$ NaCl wt%. Subsequently, a formulation was developed using M9 minimum growth medium that contained low salt and a small amount of glucose or mannitol (Table A1). In the M9 medium containing 1 vol% LB, a density of $10^8$ cells/mL was obtained overnight. *E. coli* and *P. aeruginosa* cultures grew well in the M9 mannitol media while *S. aureus* had less growth. A modified Tryptic Soy Broth (TSB), a comparatively richer medium, proved suitable for *S. aureus* growth (Table A2). The salt concentration in both media was similar (~ 5 g/L). Although two different media formulations were used for the MIC tests, the extent of growth for all three bacterial strains was comparable. This mitigates against a C12-m copolyoxetane salt effect for any particular strain.

MIC values were determined by a standard macrodilution method. The results are shown in Figure 1.1 and Table 1.4. Figure 1.2 shows that with increasing C12 mol% MICs decrease and reach a minimum at ~50 mol% C12. Further increases in C12 mol% have either a minimal effect (*E. coli* and *P. aeruginosa*) or a negative effect (*S. aureus*).
Figure 1.2. Log MIC (μg/ml) versus C12 mole percent for C12-m copolyoxetanes: (A) \textit{P. aeruginosa}, (B) \textit{S. aureus}, and (C) \textit{E. coli}.
Table 1.4. MICs for three bacterial strains.

<table>
<thead>
<tr>
<th>C12 (mole %)</th>
<th>MIC, µg/ml, (standard deviation)</th>
<th>MIC per quaternary ammonium moiety (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli</td>
<td>P. aeruginosa</td>
</tr>
<tr>
<td>14</td>
<td>300 (0)</td>
<td>410 (0)</td>
</tr>
<tr>
<td>26</td>
<td>50 (5)</td>
<td>93 (11)</td>
</tr>
<tr>
<td>43</td>
<td>5.7 (0.6)</td>
<td>33 (3)</td>
</tr>
<tr>
<td>50</td>
<td>6.3 (0.6)</td>
<td>28 (2)</td>
</tr>
<tr>
<td>60</td>
<td>5.3 (0.6)</td>
<td>26 (0.6)</td>
</tr>
<tr>
<td>87</td>
<td>5.3 (0.6)</td>
<td>33 (5)</td>
</tr>
<tr>
<td>100</td>
<td>5 (0)</td>
<td>28 (3)</td>
</tr>
<tr>
<td>DTABa</td>
<td>17 (6)</td>
<td>103 (15)</td>
</tr>
</tbody>
</table>

a. DTAB = dodecyltrimethylammonium bromide

*P. aeruginosa* is the bacterium most resistant to C12-m copolyoxetanes. The minimum MIC against *P. aeruginosa* is found for C12-60 (26 µg/ml). The resistance of *P. aeruginosa* has been demonstrated for most antimicrobials.[7] Additional lipopolysaccharides and other mechanisms are thought to decrease the interaction of antimicrobials with the membrane. Among all Gram(-) bacteria, *P. aeruginosa* has an overall outer cell membrane permeability that is 12-100 times lower than *E. coli*. [9]

The optimum C12-m composition range of 40-60 mol% is noteworthy and reflects the interaction of several molecular characteristics. Increasing C12 mole fraction not only increases charge but simultaneously changes amphiphilic balance by a combination of increased...
amphiphilic quaternary ammonium mole fraction and decreased hydrophilic ME2Ox mole fraction.

**Comparative MIC performance.** To facilitate comparison of antimicrobial performance for C12-m copolyoxetanes with related systems, Table 1.1 lists a selection of polycations in order of decreasing MICs in μg/ml, as commonly reported.[31, 32] [26, 33, 34, 36, 37, 39, 51, 52, 69, 75] MIC values in μM, that is MIC/Mₙ are also provided.[36, 37] For medical applications of antimicrobials, mass based units (MICs in μg/ml) are generally favored as the lower amount of material used the better.

The representative polycation structures and MICs in Table 1.1 show biocidal performance varies widely from nil (> 2000 μg/ml) to levels rivaling commonly used antibiotics. For the latter, Tan reported MICs for penicillin of > 50 μg/ml against *E. coli* and < 0.1 μg/ml against *S. aureus,*[76] while MICs for ciprofloxacin are 0.42 μg/ml for *S. aureus*, 0.52 μg/ml for *P. aeruginosa*, and 0.013 μg/ml for *E. coli.*[77] As another reference point, a MIC of 125 μg/mL against *E. coli* was reported by Palermo for the natural host defense peptide magainin-2.[36]

In Table 1.1 structures 4-7 are characterized by relatively high polarity and lack balanced amphiphilicity. Better performing compositions 10-15 have cationic segments accompanied by hydrophobic moieties that may be co-segments (9, 11, 15) or on opposite sides of the same repeat (10, 16).
Structure 13 has a diethylene glycol component in the main chain;[31] it is noteworthy that polycation 13 has high antimicrobial effectiveness similar to C12-m copolyoxetanes. Venkattaraman recently reported a series of polyacrylate copolymers 12 with diethylene glycol (\(\text{CH}_3\text{O(CH}_2\text{CH}_2\text{O})_9\)) and quaternary (\(\text{R(CH}_3)_2\text{NCH}_2\text{CH}_2\text{O}\)) side chains.[51] The ratio of PEG to quaternary ammonium side chains was fixed at ~1:9 which is roughly comparable to C12-87. The MIC for 12 (\(B.\ subtilis\), 20 \(\mu\)g/ml) was minimized for \(R = \text{methyl}\), that is a trimethylammonium ethyl methacrylate repeat. Activity against Gram (+) \(B.\ subtilis\) was comparable to 8, 10, and 11 but considerably higher than 14 (4 \(\mu\)g/ml) and 16 (0.8 \(\mu\)g/ml). The study of 12 against \(B.\ subtilis\) showed that increasing the alkyl group length on the quaternary function resulted in considerably higher MIC. Thus, the presence of a PEG side chain in 12 does not necessarily confer high antimicrobial effectiveness, at least against \(B.\ subtilis\).

Structures with low MICs tend to have primary, secondary, or tertiary alkylammonium charge centers including 10, 11, 13, 15 and 16. In a series of butylacrylate copolymers, one with a charge fraction of 0.77 \(\text{Me}_2\text{HN}^+\) side chains had a MIC of 3.8 \(\mu\)g/mL against \(E.\ coli\), which was the lowest of all butylacrylates (including quat copolyacrylates).[36] MICs for C12-26 to C12-100 against \(E.\ coli\) range from 50 (C12-26) to 5 (C12-100) \(\mu\)g/ml. This comparison demonstrates that the presence of an N-H moiety is not necessary to ensure low MICs. However, broad conclusions are precluded as factors such as testing methods and bacterial strains could influence results.

A systematic antibacterial study was done on an amphiphilic pyridinium methacrylate series of copolymers represented by 8.[50] \(M_n\) for this series of copolymers was in a narrow
range (27 to 33 kDa) and the mole fraction quaternary charge was kept constant at 0.5. The minimum MIC (50 µg/ml) against *E. coli* was observed for a butyl copolymer. The C12-50 copolyoxetane, which has a same linear charge density as 8 has a MIC of 6 µg/ml against *E. coli*.

The butylacrylate series 9 is related to C12-m copolyoxetanes in that both linear charge density and amphiphilic character are changing simultaneously. $M_n$ for the butylacrylate series ranged from 1.2 to 3.5 kDa while the C12-m copolyoxetanes have a broader molecular weight range (3.5 to 21.9 kDa, Table 1.3). For butylacrylates 9, MICs against *E. coli* were similar for quaternary mole fractions from 0.56 to 0.63 (39-34 µg/mL). The MIC for butylacrylate 9 with a charge fraction of 0.63 (34 µg/mL) may be compared with that for C12-60 (5.3 µg/mL). Increasing quaternary mole fraction for 9 from 0.63 to 0.78 resulted in increasing MICs from 34-1700 µg/mL.[36] In contrast, biocidal effectiveness for C12-m against *E. coli* is essentially constant (~ 5 µg/mL) for mole percents C12 ≥ 50. Differences in testing procedures must be taken into account, but it is apparent that a combination of charge balance, amphiphilicity, and polyvalency for C12-50 – C12-87 compositions result in strong biocidal effectiveness against *E. coli*.

**Polycation effectiveness (PE).** The ratio of monocation (dodecyltrimethyl ammonium bromide) to polycation MIC (µM) per alkylammonium provides a measure of polycation effectiveness (PE). Figure 1.3 shows PE versus quaternary mole percent for the three bacterial challenges (Table 1.5).
Figure 1.3. PE (polycation effectiveness) vs. mole fraction C12, where PE = ratio of MIC (μM) for DTAB to MIC (μM) for C12-m copolyoxetanes.
Table 1.5. PE (polycation effectiveness) vs. mole percent C12, where PE = ratio of MIC (μM) for DTAB to MIC (μM) for C12-m copolyoxetanes.

<table>
<thead>
<tr>
<th>C12 (mol%)</th>
<th>PE (MIC\textsubscript{DTAB}/MIC\textsubscript{C12-m copolyoxetane})</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>12</td>
<td>52</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>32</td>
<td>108</td>
<td>235</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>81</td>
<td>86</td>
<td>187</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>145</td>
<td>197</td>
<td>226</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>324</td>
<td>385</td>
<td>364</td>
<td></td>
</tr>
<tr>
<td>87</td>
<td>262</td>
<td>258</td>
<td>315</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>125</td>
<td>134</td>
<td>109</td>
<td></td>
</tr>
</tbody>
</table>

The PE for *E. coli* increases fairly regularly up to C12-60 and then decreases. Similarly, the PE for *P. aeruginosa* increases up to C12-60 and then decreases reaching a maximum of 385 which stands out compared to all C12-m copolyoxetanes. The PE for *S. aureus* also has a maximum at C12-60 (364), but there is a different dependence of PE on composition compared to *E. coli* and *P. aeruginosa*. High PE values from C12-26 to C12-87 reflect the susceptibility of *S. aureus* to C12-m copolyoxetanes even at the low linear charge density C12-26 composition. Compared to DTAB, the combination of lowest quaternary charge fraction, lowest MIC, and highest PE against *E. coli* and *P. aeruginosa* is found for C12-60 which has 324 times higher activity against *E. coli* and 385 times more against *P. aeruginosa* (Table 1.5).

**MBC Assay.** The determination of minimum bactericidal concentration (MBC) is crucial in the development of antibacterial agents. An MBC assay is important in determining concentration multiples (compared to MIC) that are required not just for inhibition of growth, but for bacterial
kill. The MBC assay is the same as the MIC assay, but an aliquot of the test medium is taken after the 24 hr exposure to the antimicrobial. There may be no bacterial growth from this aliquot if MIC and MBC are identical, or more commonly some multiple of MIC is required to affect kill (e.g., 99.99%). As points of reference, a previous study on Pexiganan, a magainin analog, showed that the MBC for some isolates of *S. aureus* was as high as 3 times the MIC,[78] while the MBC for antibiotics Linezolid and Daptomycin is 2 and 4 times MIC, respectively.[1]

**Table 1.6.** Percent lysis for the three strains of bacteria at the respective MIC multiplier.\(^c\)

<table>
<thead>
<tr>
<th>MIC multiplier</th>
<th><em>E. coli</em> (% lysis)</th>
<th><em>P. aeruginosa</em> (% lysis)</th>
<th><em>S. aureus</em> (% lysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Lawn</td>
<td>Lawn</td>
<td>Lawn</td>
</tr>
<tr>
<td>1</td>
<td>had</td>
<td>HD</td>
<td>HD</td>
</tr>
<tr>
<td>1.5</td>
<td>D(^b)</td>
<td>99.99</td>
<td>D</td>
</tr>
<tr>
<td>3</td>
<td>&gt;99.999</td>
<td>&gt;99.999</td>
<td>&gt;99.999</td>
</tr>
</tbody>
</table>

\(^a\) ‘HD’ refers to a very high density of bacterial colonies.  
\(^b\) ‘D’ refers to a high density of bacterial colonies.  
\(^c\) Relative densities are Lawn > HD > D

Copolyoxetane C12-43, one of the most effective compositions, was selected for assessment of MBC. Table 1.6 shows the log reduction in the bacterial colony count as a function of the MIC multiplier. From the MBC assay protocol, Table 1.6 shows that C12-43 affects a 99.99% kill at 1.5 times MIC for *S. aureus*, while *E. coli* and *P. aeruginosa* require 2 times MIC. In summary, susceptibility tests show MBC multipliers that are comparable to antimicrobials and antibiotics noted above.
**Killing Kinetics.** The determination of MIC is an initial step in evaluating antimicrobial behavior. MIC tests are typically done overnight and do not provide information on rate of kill. Kill kinetics are of importance in antimicrobial evaluation, but little information has been reported for polycations. Such “killing curves” are often the subject of studies for antibiotics.[79] Killing curves are often used to evaluate combinations of antibiotics and other moieties such to find synergistic relationships.[80, 81] As an initial evaluation of C12-m copolyoxetanes, C12-43 was again selected for a study of the bacterial kill rate.

For this investigation C12-43 concentrations were 5 and 10 times MIC.[78, 81, 82] The reduction in a bacterial challenge of ~ 10^8 cfu/ml *S. aureus*, *E. coli* and *P. aeruginosa* was monitored for three consecutive hours. At 5× MIC, C12-43 concentrations were 30, 25, and 165 μg/ml for *S. aureus*, *E. coli* and *P. aeruginosa*, respectively. These concentrations are higher than minimum biocidal concentrations. The results in Figure 1.4 show that at 5× MIC, C12-43 kills ≥ 99% *S. aureus*, *E. coli* and *P. aeruginosa* within 1 hr. The log reductions after 1 hr were 2.3, 1.9, and 1.9 for *S. aureus*, *E. coli* and *P. aeruginosa*, respectively (Table 1.7). Additional kill was observed after 3 hours; log reductions increased to 2.8, 2.1 and 2.7 for the respective bacteria.
Figure 1.4. Log reduction of bacterial colony count for C12-43 vs. time. The log cfu/ml at t = 0 is the initial concentration. Solid lines, 5× MIC (Table 1.4); dashed lines, 10 times MIC.

At 10 times MIC, log reductions after 1 hr were 3.6, 2.9, and 4.8 for *S. aureus*, *E. coli* and *P. aeruginosa*, respectively; after 3 hr, log reductions increased to 4.9, 3.4, and 6.1 for the respective bacteria. It is noteworthy that an order of magnitude greater kill for *S. aureus* and *P. aeruginosa* occurs during the additional 2 hr. More rapid kill kinetics are expected for such high concentrations, and parallel 10× MIC data for polycation CSA-13, 17.[38]

While 10× MIC effects rapid kill, the C12-43 concentration is quite high. To place results in perspective (Table 1.7), kill kinetics for copolyoxetane C12-43 at 5× MIC are compared with two other polycations at similar concentrations. Kill kinetics for polycation 5 (Table 1.7) were evaluated against *S. aureus*.[26] Substantial differences in testing procedures are noted below. However, this is the only report we have found for kill kinetics for a polycation; the other
comparison is for a molecular polycation 17 with four charged sites. In view of the minimal information available for polycation kill kinetics, we also compare our observations with selected antibiotics.

The MIC for 5 against *S. aureus* is at least 20 times higher (100-300 µg/ml) than that for C12-43 (5 µg/ml). In testing 5, a much lower concentration of *S. aureus* (< 10⁵ cfu/ml) was used for the determination of MIC compared to that employed herein (~10⁸ cfu/ml). In any event, at 5× MIC (25 µg/ml) C12-43 effects a 2.3 log reduction against *S. aureus* (ATCC-25904) after 1 hr, while 5 effects a 3.6 log reduction. At the end of 2 hours the log reductions are 2.6 for C12-43 and 5.5 for 5 (Table 1.7).

Although the log reduction for polycation 5 is 10 to 1000 times higher than C12-43, the comparison is flawed as the starting inoculum for 5 (3.2 x 10⁵ cfu/ml is a factor of 10³ lower than that for C12-43 (1.9 x 10⁸ cfu/ml, Table 1.7). Savage noted an “inoculum effect” for polycation 17 kill kinetics.1 When the challenge of a vancomycin-resistant strain of *S. aureus* was ~5 x 10⁸, the cfu concentration after 3 hr (4× MIC) was >10⁷, while with an initial cfu concentration of ~8 x 10⁵ cfu the concentration after 3 hr was 10³. Thus, the cfu decrement after 3 hr for the higher challenge was a factor of 10, but for the lower challenge was x 100. From these and other antimicrobial tests against vancomycin-resistant strain of *S. aureus*,¹ we conclude that the lower challenge of *S. aureus* used by Ikeda against 5 accounts in large measure for the faster kill kinetics relative to C12-43.
The MIC for molecular tetracation[1] 17 against a vancomycin-resistant strain of S. aureus is very low, 1 µg/ml.[1] Kill kinetics against S. aureus for 17 at 4× MIC (4 µg/ml) resulted in a 0.2 log reduction after 1 hr and a 1.6 log reduction after 4 hr (Table 1.7).[1, 38] By comparison, after 1 hr C12-43 at 5× MIC (25 µg/ml) effects a 2.3 log reduction against S. aureus (ATCC-25904) and 2.8 after 3 hr. Bacterial inoculate were similar for 17 (3.2 x 10⁸ cfu/ml) and C12-43 (1.9 x 10⁸ cfu/ml). A 2 log reduction corresponds to 99% kill. While the test conditions are similar, the mass per ml C12-43 (25 µg/ml) is six times higher than that for 17 (4 µg/ml).

Table 1.7. Comparison of killing kinetic studies for S. aureus.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>MIC, µg/ml (MIC multiplier)</th>
<th>Challenge (cfu/ml)</th>
<th>Log reduction 1 hr</th>
<th>2 hr</th>
<th>3 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12-43</td>
<td>5 (5)</td>
<td>1.9 x 10⁸</td>
<td>2.3</td>
<td>2.6</td>
<td>2.8</td>
</tr>
<tr>
<td>5</td>
<td>100-330 (0.3-0.1)</td>
<td>3.2 x 10⁵</td>
<td>3.1</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>CSA-13, 17</td>
<td>1 (4)</td>
<td>3.2 x 10⁸</td>
<td>0.2</td>
<td>0.4</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Comparison of molecular and polymer amphiphilic polycations. The molecular, primary polyamines 16[39] and 17[38] share amphiphilic characteristics with C12-m copolyoxetanes. Both 16 and 17 have multiple hydrophilic amine/ammonium and hydrophobic olefinic components. The powerful biocidal activity of 16 and 17 is noteworthy. Unlike quaternary polycations, the amine/ammonium structure is pH dependent, but the ammonium form is stable at physiological pH.[36] Polycations 16[39]and 17[38] share a relatively rigid structure that
favors facile conjugation with negatively charged species on a membrane. It is apparent that unfavorable conformational changes are minimized while strongly favored, entropically driven (Eq. 2), multiple cation-anion interactions are maximized.

Structure 16 is a dimer, which tends to minimize effects of polyvalency. However, 16 requires minimal molecular reorganization in conjugating with the bacterial cell wall. In contrast, the chain structure of C12-m copolyoxetanes may require a more complex and entropically unfavorable reorganization for bacterial wall conjugation.

Many structural variations have been carried out for polycation 17 to provide insight into the mechanism of antimicrobial action.[38] For example, the nature of the group extending from the central multi-ring core influences bactericidal activity of ceragenins against Gram(-) bacteria., such as E. coli.[48] A long alkyl chain confers effective biocidal action against Gram(-) E. coli at relatively low concentrations, whereas without this alkyl chain activity is essentially lost.[83] This finding is similar to the potent activity for the butyl ester copolymer 15 against E. coli but negligible effectiveness for the methyl ester analog.[36]

**Hemolytic activity.** The lytic activity of C12-m copolyoxetanes toward human red blood cells (RBC) was investigated as a measure of biocompatibility. Hemolysis was investigated at concentrations 2-10 times the MIC. Such concentrations are at or above the minimum biocidal concentration (MBC), the minimum concentration that kills all bacteria.

Results from RBC hemolytic assays for C12-m copolyoxetanes, are summarized in Figure 1.5. For all C12-m copolyoxetanes, RBC lysis does not exceed 16% at 50 µg/ml. Interestingly, only modest dependence of percent lysis on concentration is observed. Below 50 µg/ml, RBC lysis does not exceed 10% for C12-26, C12-50 and C12-60. The order of hemolytic activity at ~ 40 µg/ml is C12-26 ~ C12-50, ~ C12-60, > C12-87 ~ C12-100 > C12-43. C12-43 does not fall into
the regular trend of increasing cytotoxicity with increasing mole percent C12. At higher concentrations (≥ 40 µg/ml), C12-43 falls into the group (with C12-87 and C12-100 that have higher (13-15%) RBC cytotoxicity. At low concentrations (~ 10 µg/ml) all C12-m copolyoxetanes have very low hemolytic activity (< 8 %) except for C12-100 (11%). C12-50 and C12-60, which are highly efficient antimicrobials (Figure 1.2) are among the least cytotoxic C12-m copolyoxetanes.

Figure 1.5. Percent RBC lysis as a function of C12-m copolyoxetane concentration.
One metric for defining hemolytic activity is HC\textsubscript{50}, the concentration of antimicrobial that kills 50% of mammalian cells under investigation.[50, 69, 84] At C12-m copolyoxetane concentrations ≤ 50 µg/ml, RBC lysis is less than 16 % for all C12-m copolyoxetanes, precluding an HC\textsubscript{50} determination. Inspection of the slopes for curves in Figure 1.5 shows that much higher concentrations are needed to affect 50% RBC lysis.

**Conclusion**

Ring opening copolymerization of 2 and 3 followed by quaternization of the BBOx-m intermediate gives C12-m copolyoxetanes 1 having PEG-like and C12 quaternary side chains with m = 14, 26, 43, 50, 60, 87, and 100 mole%. By analogy with prior work,[57-59] copolyoxetanes 1 are random copolymers (except for homopolymer C12-100). C12-m copolyoxetanes are polycation molten salts that are slightly yellow, viscous liquids with $T_g$’s ranging from -34 to -69 °C (Table 1.3). $^1$H-NMR end group analysis gave relatively low molecular weights in the 4-20 kDa range.

Although having long side chains (19 atoms), there is no DSC evidence (0 – 125 °C, Figure 1.1) for endotherms that are characteristic of side chain crystallization.[72] The low $T_g$’s may be compared with polycation molten salts reported by Ohno ($T_g = -64$ to -72 °C)[73, 74] and attest to high mobility of the –CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}O- main chain. In addition, low $T_g$’s are favored by a combination of ME2Ox side chains and ether functionality in the linkage to the C12 side chain. Polycations listed in Table 1.1 are typically reported as “solids” (no thermal transitions were found for 12).[33] It appears that C12-m copolyoxetanes are the first polycation molten salts examined as antimicrobials.
A systematic trend in composition versus antimicrobial activity was found for water soluble copolyoxetanes 1 with C12 alkylammonium side chains. This finding stands in contrast to recently reported polyvinyl amines where no direct relationship was found for antimicrobial activity and charge density.[85] As shown by other polycationics when compared to monovalent analogs,[29, 86, 87] C12-m copolyoxetanes had higher effectiveness against both Gram(+) and Gram(-) bacteria compared to monovalent DTAB. This polycation effectiveness (PE) can be understood in terms of the proposed entropic driving force described by Eq 2, whereby multiple equivalents of potassium salt are liberated by conjugation of a single polycation. Potassium ion release disrupts the bacterial cell potential contributing to cell death. A broad range of polycation activity, ranging from negligible to powerful reflects other important interactions such as amphiphilic balance and polycation architecture, the nature of which are not yet well understood.

For C12-m copolyoxetanes, increasing quaternary alkylammonium content improves biocidal activity up to the compositional range C12-43 to C12-60 (Figure 1.2). A comparison with other antimicrobial polycations (Table 1.1) shows that C12-43 to C12-60 copolyoxetanes are among the most effective antimicrobials known against the tested Gram(+) and Gram(-) bacteria. For C12-m (m > 60), a static or gradual decrease in MIC is observed. A charge shielding or steric effect by the long alkyl chains due to increased occurrence of C12 diads, triads and higher sequences may be the reason for this gradual decrease in biocidal activity. Alternatively, the changes in antimicrobial effectiveness above m = 60 may be due to decreased hydrophilic character due to the increased prevalence of long alkyl chains.
Despite different compositions and different molecular weights for C12-43 (3.5 kDa), C12-50 (8.4 kDa), and C12-60 (18 kDa) copolyoxetanes, MICs against *S. aureus* are similar and low (5.3 – 10 µg/ml). This is a remarkable result considering the MIC sensitivity to compositions found in other polycations.[36, 51]

Tew has noted that “Gram selectivity is rare” for amphiphilic polycations.[88] Inspection of Figure 1.2 shows that copolyoxetanes with maximum biocidal activity (C12-43 to C12-60) indeed have similar antimicrobial effectiveness against Gram(+) *S. aureus* and Gram(-) *E. coli*. However, MICs for Gram(+) *S. aureus* and Gram(-) *E. coli* diverge as mole % C12 increases, reaching a maximum selectivity of 2.6 for Gram(-) *E. coli* at C12-100. Interestingly, this selectivity for *E. coli* compared to *S. aureus* is opposite that found for poly(oxanorbornene)s described by Tew.[88] For poly(oxanorbornene)s selectivity for *S. aureus* was driven by MICs against *E. coli* that were > 20 times those for the C12-m copolyoxetanes (m > 43). Comparisons must be qualified in light of different testing procedures and the different structures, notably the primary ammonium functionality for poly(oxanorbornene)s versus the quaternary C12-m copolyoxetanes. The selectivity of C12-m copolyoxetanes for Gram(-) *E. coli* may be compared with elegantly designed polypeptides that had very high Gram(-) selectivity.[89, 90]

Few studies have been reported on polycation antimicrobials against *P. aeruginosa*. This Gram(-) bacteria has a low susceptibility toward antibiotics due to the presence of efficient efflux pumps.[11] Resistance to polycation antimicrobials may be due in part to low cell wall permeability.[9] Polycations studied by Ikeda showed that acrylates with pendant biguanide groups have MICs between 660-1000 µg/ml.[28] Poly(methyloxazoline) polycations have MICs
from 4000 to 45000 µg/ml.[91] Studies performed on Carbenicillin, an antibiotic belonging to the carboxypenicillin group, gave a strain-dependent MIC between 6-25 µg/ml.[92] The minimum MIC against *P. aeruginosa* was 26 µg/ml for C12-60 (Figure 1.2, Table 1.4). Also of note is the insensitivity of MIC (29.5, sd = 3.2) against *P. aeruginosa* for copolyoxetanes with ≥ 43 mol% C12. This is noteworthy considering the range of molecular weights and linear quaternary charge density.

For C12-43 at 5 times MIC a killing kinetics study showed ≥ 99% kill for *S. aureus*, *E. coli* and *P. aeruginosa* in the first hour. C12-43 has a minimum bactericidal concentration that is 1.5 times MIC for *S. aureus* and 2 times MIC for *E. coli* and *P. aeruginosa*. This performance for C12-43 against *S. aureus* is comparable to some common antibiotics such as linezolid and Daptomycin.

The biocompatibility of PEG-functionalized biomaterials is widely recognized[93-96] but few studies of PEGylated polycations have been carried out. Structure **13** has a diethylene glycol component in the main chain[31] and has high antimicrobial effectiveness similar to C12-m copolyoxetanes, but cytotoxicity against human cells is unknown.

An optimized copolyacrylate **12** with [CH$_2$O(CH$_2$CH$_2$O)$_y$-] and (CH$_3$)$_3$N-CH$_2$CH$_2$-OC(O)- side chains (~1:9 mole ratio) showed moderate antimicrobial effectiveness against *B. subtilis* (MIC 20 µg/ml) compared to **14** and **16**.[51] The same copolyacrylate **12** had low cytotoxicity against mouse RBCs (< 10% at 100 µg/ml), but **12** with longer alkyl chains (C8, C12) caused 100% lysis at 50 µg/ml.[51] We did not test C12-m copolyoxetanes against
Gram(+) *B. subtilis*, a common soil bacterium which is rarely found to be a human pathogen.[97] However, C12-87, which has a similar ratio of PEG-like:C12 side chains to 12 (with C12 side chains, 100% mouse RBC lysis), has relatively low human RBC cytotoxicity (~ 14% at 50 μg/ml). While the tests conducted for 12 are quite different than those reported on C12-m, the conclusion reached by Yang that longer alkyl groups on quaternary side chains correlate with increased cytotoxicity[51] is certainly not general.

C12-m copolyoxetanes 1 have antimicrobial effectiveness that is exceeded by few other polycations (Table 1.1). The molecular polycations 16 and 17 have important advantages of low MICs against well known pathogens and well defined chemical structures. The MIC for 16 against *E. coli* is 0.8 μg/ml while the HC<sub>50</sub>, which is the concentration that kills 50% RBCs, is 14 μg/ml. The MIC for C12-m against *E. coli* is ~ 5 μg/ml over the range C12-43 to C12-100, about 6 times higher than 16. The HC<sub>50</sub> for C12-m was not measured as low % RBC lysis (< 15%) was found up to 50 μg/ml for all compositions (Figure 1.5). For example, RBC lysis for C12-50 and C12-60, two of the optimum *E. coli* antimicrobials, was ~ 7% at 2× MIC (10 μg/ml). From the maximum % lysis observed for C12-50 and C12-60 (Figure 1.5) the selectivity for *E. coli* compared to RBCs must be much greater than 10.

While C12-m copolyoxetanes have high antimicrobial effectiveness and low RBC cytotoxicity, the fundamental reasons behind these observations are not clear. As noted above for several polycations, what seem to be modest structural variations cause dramatic changes in antimicrobial effectiveness or cytotoxicity. C12-m copolyoxetanes with m > 43 have similar overall antimicrobial effectiveness but subtle changes in selectivity for the pathogenic bacteria
employed. Although not general, the presence of the PEG-like ME2Ox side chain seems to play an important role in minimizing C12-m cytotoxicity. A recent study supportive of this contention has shown hemocompatibility of positively charged PAMAM dendrimers was greatly enhanced by PEGylation.[93]

There are important issues that our initial study has raised concerning C12-m copolyoxetanes as antimicrobials. Among these are the need to control molecular weights, chain architecture, and composition to bring structure property relationships into better focus. However, the favorable performance of this easily prepared new class of biocompatible antimicrobials that are novel polymer ionic liquids is encouraging. Our results may lead to new options for applications such as wound care, lotions, and cosmetics that will combat continually emerging resistance to traditional antibiotics.

Chapter 2

Cytocompatibility of Poly[(3,3-Quaternary/PEG)-Copolyoxetanes]

ABSTRACT

The determination of cytocompatibility for a novel class of copolyoxetanes with quaternary ammonium and PEG-like side chains is investigated. A precursor P[(BBOx-m)(ME2Ox)] copolyoxetane was prepared by cationic ring opening copolymerization of 3-((4-bromobutoxy)methyl)-3-methyloxetane (BBOx) and 3-((2-(2-methoxyethoxy)ethoxy)methyl)-3-methyloxetane (ME2Ox) to give random copolymers with 26-100 (m) mole% BBOx. Reaction of P[(BBOx-m)(ME2Ox)] with dodecyl dimethylamine gave the corresponding quaternary P[(C12-m)(ME2Ox)] polycation salts, designated C12-m. A series of C12-m copolyoxetanes were obtained (m = 26 to 100). Cytotoxicity studies were performed with HFF (Human Foreskin Fibroblast) and HDF (Human Dermal Fibroblast) cell lines. From previously determined minimum inhibitory concentrations (MIC) for strains of E. coli, S. aureus and P. aeruginosa, HFF and HDF selectivity ratios (EC50/MIC) were determined. Previous studies have shown relatively low RBC values (HC50) and moderate to high selectivity ratios (HC50/MIC). The results of cytotoxicity studies show that the P[(C12-m)(ME2Ox)-MW] copolyoxetanes have relatively low cytotoxicity for HFF and HDF cell lines. The excellent antimicrobial behavior and human cell compatibility makes these novel copolyoxetanes potential candidates for therapeutic applications.
Introduction

Cytotoxicity studies are an essential parameter in the evaluation of a compound as a potential therapeutic. Polymer disinfectants are frequently toxic to human cells or their cytotoxicities are not reported. [98] If researchers attempt to address the question of cytotoxicity, studies usually focus exclusively on hemocompatibilities. Prior antimicrobial and hemocompatibility studies discussed in Chapter 1 showed the effectiveness of C12-m copolyoxetanes as antimicrobials and hemocompatibility with relatively low RBC values (HC<sub>50</sub>). [99] The present study attempts to further evaluate C12-m copolyoxetane therapeutic potential by examining their toxic effects on two types of human fibroblast cells: Human foreskin fibroblasts (HFFs) and human dermal fibroblasts (HDFs).

Experimental

Materials. Copolyoxetanes with PEG and quaternary pendant groups were synthesized and characterized as previously reported. [99] Copolyoxetanes were dissolved in Dulbecco's modified Eagle's medium (DMEM) in sterilized centrifuge tubes and vortexed to obtain homogenous stock solutions. Serial dilutions were prepared using DMEM.

Immortalized human foreskin fibroblast cells and immortalized human dermal fibroblast cells were generously provided by Drs. Shawn Holt, Department of Pathology, and Dr. Valerie Kristoffer, Department of Radiation Oncology, both at Virginia Commonwealth University School of Medicine.
**Cell Cytotoxicity Assays.** Cytotoxicities are reported as EC\(_{50}\) values which indicate the concentration which brings about 50% lysis of the respective cell line. Cytotoxicity was tested using a CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) and were run according to the manufacturer’s protocol.[100] Human foreskin fibroblasts (HFFs) and human dermal fibroblasts (HDFs) were used to enhance relevance to medical applications and therapeutics. HFFs or HDFs were seeded in 96 well plates at a seeding density of 1.5 × 10\(^3\) cells/cm\(^2\). After incubation at 37 °C and 5% CO\(_2\) for 24 hr, the cells were exposed to sample solutions at different concentrations for 24 hr, followed by the addition of the tetrazolium dye solution to the wells and incubation for 4 hr. During this period, living cells convert tetrazolium into a formazan product. A stop solution was added to formazan and the plate absorbance was read at 570 nm using a Biomate3 Spectrophotometer (Thermo Electron Corporation). EC\(_{50}\) values are determined by using concentration vs. absorbance plots.[101] From concentration vs. absorbance plots, a maximum absorbance (plateau) was obtained. EC\(_{50}\) values were determined by locating the concentration corresponding to one-half of the maximum.

**Results**

In view of the effective antimicrobial performance for C12-m copolyoxetanes and low human red blood cells (RBCs) cytotoxicity reported previously [99] it was decided to further explore cytotoxicity on human foreskin fibroblasts (HFFs) and human dermal fibroblasts (HDFs). No known studies for antimicrobial AMPs or their mimics have focused on HFF or HDFs. Cytotoxicity studies have been limited to RBC assays and were discussed in detail in Chapter 1. Below, cytotoxicity of C12-m copolyoxetanes are assessed in relation to changing mole percent
quaternary charge. Cytotoxicity assays with C12-m where m = 26, 43, 60, 87 and 100 were performed and the results are reported below.

**C12-m copolyoxetanes.**

*Cytotoxicity for HFF and HDF cell lines.* Cytotoxicity of C12-m copolyoxetanes was examined using the MTT assay. MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), is a yellow dye that is used as an indicator for cell viability. Mitochondrial enzymes present in living cells convert MTT dye into purple formazan which is solubilized. Absorbance of the resulting solution is determined at 570 nm. In a control with no polymer (i.e. cells and media only), the MTT dye is completely converted to formazan which is solubilized resulting in negligible MTT absorbance. With increasing concentration of C12-m copolyoxetanes, more cells are lysed so that less MTT is converted to formazan. This leads to increased MTT absorbance at higher C12-m concentrations. According to this protocol, the concentration of C12-m copolyoxetane at half the maximum absorbance corresponds to 50% cell lysis. A representative determination of EC$_{50}$ for C12-43 for the HDF cell line is shown in Figure 2.1. 
Figure 2.1. Absorbance at 570 nm of tetrazolium vs. C12-43 concentration; 4 hr exposure of human dermal fibroblast (HDF) cell line. The concentration at half maximum absorbance (EC$_{50}$) is circled.

EC$_{50}$ cytotoxicity results for HFF and HDF cell lines are shown below in Table 2.1. It is evident that all C12-m copolyoxteanes are relatively nontoxic toward both human fibroblast cell lines with C12-25 and C12-43 being the least non-toxic. HDFs are relatively insensitive to mol% C12, but HFFs exhibit lower EC$_{50}$ values for $\geq$ 60 mol%. Cytotoxicity is still considered minimal when compared to the bacterial MIC values.
Table 2.1. Cytotoxicities (HFF and HDF cell lines) and MIC values for three bacterial strains for C12-m copolyoxetanes, where m= mol% C12.

<table>
<thead>
<tr>
<th>Copolyoxetane</th>
<th>EC₅₀ (µg/ml)</th>
<th>Minimum Inhibitory Concentration (MIC, µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HFF</td>
<td>HDF</td>
</tr>
<tr>
<td>C12-26</td>
<td>43000</td>
<td>38000</td>
</tr>
<tr>
<td>C12-43</td>
<td>44000</td>
<td>57000</td>
</tr>
<tr>
<td>C12-60</td>
<td>9000</td>
<td>55000</td>
</tr>
<tr>
<td>C12-87</td>
<td>10000</td>
<td>33000</td>
</tr>
<tr>
<td>C12-100</td>
<td>9000</td>
<td>23000</td>
</tr>
</tbody>
</table>

Selectivities for C12-m copolyoxetanes. Based on previous MIC results for E. coli, S. aureus and P. aeruginosa [99] revisited in Table 2.1. Selectivity, S, defined by Eq.1, is a measure of the concentration required to lyse human cells compared to the minimal concentration for inhibiting the growth of bacteria (MIC). As a measure of cytotoxicity, it is important to recognize that selectivities provide an optimistic estimate of antibiotic effectiveness, as the MIC is typically 2-4 times lower than the minimal biocidal concentration, MBC.[99] Nevertheless, the measurement of selectivity provides a guide for potential use of antimicrobials as antibiotics. High selectivities mean effective antimicrobial activity while minimizing effects on human cells.
Amphiphilic C12-m copolyoxetanes have low cytotoxicity and high selectivities. Table 2.2 shows selectivities for HFF and HDF cell lines are highest for C12-43 and C12-60 copolyoxetanes. For the HFF cell line, C12-43 have selectivities of 7820 (E. coli), 8310 (S. aureus) and 1340 (P. aeruginosa). For the HDF cell line, C12-60 has selectivities of 10400 (E. coli) and 2170 (P. aeruginosa) but lower selectivity for S. aureus (5540). Based upon prior C12-m copolyoxetane RBC assays[99], although it is difficult to make direct comparisons between different cell lines, it is apparent that HFFs and HDFs are more robust when compared to RBCs. This leads to much higher selectivity values for the former.

**Table 2.2.** Selectivities for C12-m copolyoxetanes for HFFs, and HDFs.

<table>
<thead>
<tr>
<th>Copolyoxetane</th>
<th><strong>E. coli</strong></th>
<th><strong>S. aureus</strong></th>
<th><strong>P. aeruginosa</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HFF</td>
<td>HDF</td>
<td>HFF</td>
</tr>
<tr>
<td>C12-26</td>
<td>860</td>
<td>765</td>
<td>3110</td>
</tr>
<tr>
<td>C12-43</td>
<td>7820</td>
<td>10130</td>
<td>8310</td>
</tr>
<tr>
<td>C12-60</td>
<td>1700</td>
<td>10400</td>
<td>906</td>
</tr>
<tr>
<td>C12-87</td>
<td>1890</td>
<td>6230</td>
<td>1010</td>
</tr>
<tr>
<td>C12-100</td>
<td>1800</td>
<td>4600</td>
<td>750</td>
</tr>
</tbody>
</table>
Conclusion.

Previous work has shown that the C12-m copolyoxetanes are promising antimicrobials. The present investigation of cell compatibility has shown that certain compositions (particularly, C12-43 and C12-60) have low toxicity towards human fibroblast cells and have high selectivities (E. coli, S. aureus and P. aeruginosa). The water solubility of these polymers coupled with excellent antimicrobial behavior and cell compatibility makes these novel copolyoxetanes potential candidates for therapeutic applications.
Chapter 3

PEG/quaternary copolyoxetanes: antimicrobial effectiveness, hemolytic and cytotoxic activity in response to alkyl side chain length

ABSTRACT

This study concerns the effect of changing quaternary alkyl chain length of quaternary ammonium/PEG copolyoxetanes on solution antimicrobial efficacy, hemolytic activity and cytotoxicity. Copolyoxetanes have an oxetane repeat unit with a quaternary side chain and a PEG-like hydrophilic repeat unit utilizing the monomers 3-((4-bromobutoxy)methyl)-3-methyloxetane (BBOx) and 3-((2-(2-methoxyethoxy)ethoxy)methyl)-3-methyloxetane (ME2Ox). A precursor P[(BBOx)(ME2Ox)-50:50-5.7] copolyoxetane was generated via ring opening polymerization of BBOx and ME2Ox monomers with a BBOx mole fraction of 0.50. The $M_n$ of 5.7 kDa is obtained from $^1$H-NMR end group analysis. The desired quaternary ammonium segment results from a substitution of C-Br with a series of tertiary amines. The copolyoxetanes are represented as Cx-50, where 50 is the mole percent of quaternary repeat units and ‘x’ is quaternary alkyl chain length (2, 6, 8, 10, 12, 14 or 16 carbons). The BBOx: ME2Ox ratio was confirmed by $^1$H-NMR spectroscopy. Modulated differential scanning calorimetry (MDSC) studies showed $T_g$’s between -40 °C and -60 °C and melting endotherms for C14-50 and C16-50. Minimum inhibitory concentration (MIC) tests were determined for *E. coli*, *S. aureus* and *P. aeruginosa*. A systematic dependence of alkyl chain length and MIC was found. The most lethal antimicrobial was C8-50 with MIC values of (MIC in μg/ml, bacteria) 4 μg/ml, *E. coli*, 2 μg/ml, *S. aureus* and 24 μg/ml, *P. aeruginosa*.) Kill kinetics for C8-50 were obtained.
at 5 times MIC. This concentration effected >99% kill in 1 hour for a challenge of $10^8$ cfu/ml with log reductions of 7, 3, and 5 for S. aureus, E. coli and P. aeruginosa, respectively, at the end of 1 hour.

Cx-50 copolyoxetane cytotoxicity toward human red blood cells, human dermal fibroblasts (HDF), and human foreskin fibroblasts (HFF) was low indicating good prospects for biocompatibility. This novel class of copolyoxetanes with tunable compositions offers opportunities for applications as therapeutic agents.
Introduction

Over the past two decades, several naturally occurring peptides have been isolated and found to possess antimicrobial properties. These naturally occurring antimicrobial peptides (AMPs) have an amphiphilic architecture, are water soluble and inhibit the growth of bacteria at low concentrations.[19, 20] These AMPs provide the first line of defense against bacterial infections and participate in innate immune response.[21, 102] In humans, AMPs are found primarily in tissues that are exposed to the most bacteria (skin, mucous membranes, and gastrointestinal tract). The widespread occurrence of AMPs in a diverse range of organisms further suggest these innate components of immunity defense control bacterial growth.[103]

Although promising as therapeutics, AMPs are difficult and cost prohibitive to isolate and many have insufficient biostability.[104] Inspired by the amphiphilic nature of these naturally occurring antimicrobial peptides, synthetic analogs have been prepared that mimic AMP structure and properties.[105] These AMP analogs can have increased potency by one to two orders of magnitude for inhibiting the growth of microbes.[106]

In connection with exploring amphiphilic soft blocks for contact-kill coatings, a novel class of copolyoxetanes 1 was developed.[22, 56] These copolyoxetane soft blocks were used to create copolyoxetane soft block

![Diagram of copolyoxetane soft block](image-url)
polyurethane surface modifiers.[56, 107] An interesting feature of copolyoxetanes 1 (when \(x = 12\)) regardless of mole \%, is they are viscous, molten salts with low glass transition temperatures.

With charged and uncharged segments, 1 has AMP-like characteristics. Copolyoxetanes 1 have quaternary alkyl side chains and PEG-like ME2Ox side chains.

Water solubility for P[(C12-m)(ME2Ox)] copolyoxetanes led to an investigation of solution biocidal properties. C12-m copolyoxetanes with m = 14, 25, 42, 50, 60, 87 and 100 were tested against *E. coli*, *P. aeruginosa* and *S. aureus*. Standard minimum inhibitory concentration (MIC) assays showed that C12-m copolyoxetanes with m = 40-60 had maximum biocidal efficiency. C12-43 had the lowest MIC for *E. coli* (5.7 \(\mu\)g/ml) and *S. aureus* (5.3 \(\mu\)g/ml) while C12-60 had the lowest MIC for *P. aeruginosa* (26 \(\mu\)g/ml). Killing kinetic assays with the C12-43 at a concentration of 5 times MIC gave >99% kill in 1 hr for all the three bacterial strains with a starting inoculum ~ 10\(^8\) cfu/ml.

By analogy with AMPs, the killing mechanism for polycations is explained by ionic and hydrophobic interactions between the bacterial cellular membrane and multiple ammonium moieties.[108] Disruption of the phospholipid bilayer includes ion diffusion through cell wall ion channels and a change in cell potential that results in cell death.[55]

Previous research on biocidal polycations shows biocidal performance varies widely from nil (> 2 mg/ml) to levels rivaling commonly used antibiotics. For the latter, Tan reported MICs for penicillin of > 50 \(\mu\)g/ml against *E. coli* and < 0.1 \(\mu\)g/ml against *S. aureus*. [76] While MICs for ciprofloxacin are 0.42 \(\mu\)g/ml for *S. aureus*, 0.52 \(\mu\)g/ml for *P. aeruginosa*, and 0.013 \(\mu\)g/ml for *E. coli*. [77] As another reference point, a MIC of 125 \(\mu\)g/mL against *E. coli* was reported by Palermo for the natural host defense peptide magainin-2.[36]
A previous analysis of antimicrobial polycations showed poor performance for structures with high polarity and a lack of balanced amphiphilicity.[99] Better performing compositions were found to have cationic segments accompanied by hydrophobic moieties that may be co-segments or on opposite sides of the same repeat.

Amphiphilic balance effecting biocidal activity can be very sensitive to what appears as a modest structural change. For example, it is remarkable that changing butyl acrylate in structure 2 to a methyl acrylate results in a 100 times increase in MIC for *E. coli*. [36]

Another example is found for “CSA-13”, 3, a molecular polycation.[38] An octyl substituent (shown) results in a low MIC against Gram(-) *E. coli*, whereas without this group, activity is lost.[48]

In view of the sensitivity of biocidal potency to amphiphilic balance, we have investigated the effect of changing alkyl chain length on the antimicrobial properties of copolyoxetanes 1. Based on prior work that showed the highest antimicrobial potency for C12-m with m = 40-60,[99] a series of Cx-50 copolyoxetanes were prepared (1, x = 2, 6, 8, 10, 12, 14 or 16 carbons). Minimum inhibitory concentration (MIC) assays were carried out to assess relative antimicrobial effectiveness. Kill kinetics were established for C8-50, which is the most
antimicrobial Cx-50 copolyoxetane. At 5 times MIC, for a starting inoculum of $10^8$ cfu/ml, C8-50 affected a kill >99% in 1 hour. Details of this investigation are reported below.

**Experimental**

**Materials.** 3-bromomethyl-3-methyloxetane (BrOx) was generously provided by OMNOVA Solutions (Akron, OH). N,N-dimethyl ethylamine (C2), N,N-dimethylhexylamine (C6), N,N-dimethyl octylamine (C8), N,N-dimethyldecylamine (C10), N,N-dimethyltetradecylamine (C14), and N,N-dimethylhexadecylamine (C16) were obtained from Aldrich. N,N-dimethyldodecylamine (C12) was a gift from Lonza (Allendale, NJ). Methylene chloride (CH$_2$Cl$_2$), and tetrahydrofuran (THF) were obtained from Aldrich and dried by storing over 4Å molecular sieves. Boron trifluoride dietherate (BF$_3$O(C$_2$H$_5$)$_2$), tetrabutylammonium bromide (TBAB), 3-(hydroxymethyl)-3-methyloxetane, 1,4-dibromobutane and sodium hydride (NaH) were also obtained from Aldrich and used as received. 1,4-Butanediol (BD) and 2-(2-methoxyethoxy)ethanol were purchased from Acros Chemicals and used as received. All M9 media components were purchased from Sigma Aldrich. Luria Broth (LB) was purchased from Fisher Scientific.

Human red blood cells (RBCs) were obtained from Innovative Research (Novi, MI) packed and transported on ice in a cooler (~ 4 °C), refrigerated upon receipt and used within one week. Immortalized human foreskin fibroblast (HFF) cells and immortalized human dermal fibroblast (HDF) cells were generously provided by Drs. Shawn Holt, Department of Pathology,
and Dr. Valerie Kristoffer, Department of Radiation Oncology, both at Virginia Commonwealth University School of Medicine.

**Synthesis and Characterization.** The monomers 3-((4-bromobutoxy)methyl)-3-methyloxetane (BBOx) and 3-((2-(2-methoxyethoxy)ethoxy)methyl)-3-methyloxetane (ME2Ox) were synthesized and purified as described previously.[99] Copolyoxetane precursor P[(BBOx)(ME2Ox)] was analyzed by $^1$H-NMR to determine the co-repeat mole ratio. End group analysis was employed using trifluoroacetic anhydride (TFAA) for determining the number average molecular weight ($M_n$).[57]

**P[(BBOx)(ME2Ox)] copolyoxetane precursor synthesis.** The target copolyoxetane intermediate was synthesized by cationic ring opening polymerization in CH$_2$Cl$_2$ by a procedure previously described.[99] ME2Ox (10g, 49 mmol), BBOx (9.5g, 40.9 mmol), 1,4-butanediol (0.538g, 6 mmol) and BF$_3$·OEt$_2$ (1496 µl, 12 mmol) were used for P[(BBOx)(ME2Ox)-50:50] preparation. A higher ME2Ox feed molar ratio was used (55 mol%) as BBOx is somewhat more reactive.

P[(BBOx)(ME2Ox)-50:50-5.7] characterization: $^1$H-NMR (CDCl$_3$): δ 0.91 ppm (-CH$_3$, 3H,s), 1.68 ppm (Side chain –OCH$_2$CH$_2$CH$_2$CH$_2$Br, –CH$_2$-for BBOx, 2H), 1.92 ppm (-OCH$_2$CH$_2$CH$_2$CH$_2$Br, –CH$_2$-for BBOx, 2H), 3.19 ppm (main chain –CH$_2$-, 4H,m), δ3.30 ppm (-CH$_2$, 2H, s), 3.38 ppm (-OCH$_3$ 3H, s), 3.4 ppm (-CH$_2$Br-, 2H), 3.55 ppm (-OCH$_2$CH$_2$O-, 4H, m), 3.64 ppm (-OCH$_2$CH$_2$O-, 4H, m). Molecular weight ($M_n$) by $^1$H-NMR end group analysis was 5.7 kDa. The mole percent of the bromobutoxy group was calculated using equations described in Appendix A.
**Cx-50 copolyoxetane synthesis. Safety Note:** Dimethylalkylamines are toxic; C2, C4, and C6 are hazardous due to volatility. Reactions were carried out in a well ventilated hood using appropriate personal safety equipment. C2-50 was made with caution using small quantities of dimethylethylamine. Dimethylbutylamine, the amine precursor for C4-50 was not commercially available.

\[ \text{P[(BBOx)(ME2Ox)-50:50-5.7]} \] was quaternized with tertiary amines to obtain Cx-50 copolyoxetanes by a method described previously for C12-m copolyoxetanes.[99] The copolyoxetanes are represented as Cx-50, where 50 is the mole percent of quaternary repeat units and ‘x’ is quaternary alkyl chain length (2, 6, 8, 10, 12, 14 or 16 carbons). The quantities of the P[((BBOx)(ME2Ox))] precursor and the respective tertiary amines used for quaternization is provided in Table 3.1. After removing solvent and excess amine *in vacuo* (liquid nitrogen trap), Cx-50 copolyoxetanes were isolated as highly viscous liquids.
Table 3.1. Reactants for quaternization of P[(BBOx)(ME2Ox)-50:50-5.7] copolyoxetanes

<table>
<thead>
<tr>
<th>x</th>
<th>P[(BBOx-50)(ME2Ox)] g (mmol)</th>
<th>Cx amine g (mmol)</th>
<th>Cx/ME2Ox&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>2.5 (0.439)</td>
<td>1.42 (11.0)</td>
<td>49.5/50.5</td>
</tr>
<tr>
<td>8</td>
<td>2.18 (0.382)</td>
<td>1.55 (9.87)</td>
<td>50/50</td>
</tr>
<tr>
<td>10</td>
<td>2.5 (0.439)</td>
<td>1.75 (9.46)</td>
<td>50/50</td>
</tr>
<tr>
<td>12</td>
<td>3 (0.526)</td>
<td>4 (13.6)</td>
<td>50/50</td>
</tr>
<tr>
<td>14</td>
<td>2 (.0351)</td>
<td>2.17 (10.1)</td>
<td>50/50</td>
</tr>
<tr>
<td>16</td>
<td>1.51 (0.265)</td>
<td>1.84 (6.84)</td>
<td>50/50</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ratio of Cx to ME2Ox repeats obtained from <sup>1</sup>H-NMR integration.

**Characterization:** Figure 3.1 shows the <sup>1</sup>H-NMR spectrum for P[(BBOx-50)(ME2Ox)-50:50] and the C8-50 copolyoxetane. The <sup>1</sup>H-NMR chemical shifts for C8-50 are: <sup>1</sup>H-NMR (CDCl<sub>3</sub>): δ 0.91 ppm (-CH<sub>3</sub>, 6H, s), 1.2~1.4 (-CH<sub>2</sub>(CH<sub>2</sub>)<sub>6</sub>CH<sub>3</sub>, 12H), 1.68~1.92 ppm (-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N-, 4H, broad), 3.19 ppm (main chain –CH<sub>2</sub>-, 6H, m), 3.30 ppm (-CH<sub>2</sub>, 2H, s), 3.38 ppm (-OCH<sub>3</sub> 3H, s), 3.4 ppm (-CH<sub>2</sub>N- 2H, CH<sub>3</sub>-N-CH<sub>3</sub>6H), 3.55 ppm (-OCH<sub>2</sub>CH<sub>2</sub>O-, 4H, m), 3.64 ppm (-OCH<sub>2</sub>CH<sub>2</sub>O-, 4H, m).

**Instrumentation:** <sup>1</sup>H-NMR characterization was done using a Varian Mercury 300 MHz NMR spectrometer, FTIR spectra were obtained with a Magna-IR 760 spectrometer, Modulated Differential Scanning Calorimetry (MDSC) utilized a DSC Q1000 (TA Instruments).
Antimicrobial Testing. Bacterial suspension preparations, media for minimum inhibitory concentration (MIC) tests, MIC tests, and the procedure for kill kinetics assays were identical to those described previously.[99] and are discussed below.

Antimicrobial activity assay.

Bacterial strains. Three strains, which represent potential pathogens, were used to enhance relevance to medical applications and therapeutics. For the biocidal tests, strains used were *Escherichia coli* DH5α, *Pseudomonas aeruginosa* PA01 and *Staphylococcus aureus* ATCC-25904. The cultures were streaked on Luria Agar plates and incubated overnight at 37 ºC. A single colony from each strain was used to inoculate 6 ml of Luria Broth (LB), grown overnight at 37 ºC with vigorous shaking (225 rpm). A sample was diluted in fresh medium and incubated to $10^8 – 10^9$ colony forming units per milliliter (cfu/ml) of the desired bacteria to generate a culture in the logarithmic growth phase.

Media. Luria Broth (LB) was the standard medium for MIC evaluations. However, the Cx-50 copolyoxetanes precipitated in LB. Different media formulations were tested that would prevent polycation precipitation and at the same time generate ample bacterial growth during the 24-hr incubation period. M9 growth medium was prepared containing 20 wt% mannitol as a carbon source for *E. coli* and *P. aeruginosa* and is referred to as “M9 mannitol” medium. However, significant growth for *S. aureus* was not observed. A modified Tryptic Soy Broth (TSB) was chosen for MIC evaluation of *S. aureus* as good growth was observed.

Several growth media were explored in order to avoid Cx-50 copolyoxetane precipitation. Luria Broth (LB) solutions were prepared with a range of salt concentrations (0.1 - 3 wt%).
Although adequate bacterial growth was observed, the copolyoxetanes precipitated in media with ≥ 0.5 NaCl wt%. Subsequently, a formulation was developed M9 mannitol medium that contained low salt and a small amount of mannitol. In the M9 mannitol medium containing 1 vol% LB, a density of 10^8 cfu/ml was obtained overnight. *E. coli* and *P. aeruginosa* cultures grew well in the M9 mannitol media while *S. aureus* had less growth. A modified Tryptic Soy Broth (TSB), a comparatively richer medium, proved suitable for *S. aureus* growth. The salt concentration in both media was similar (~ 5 g/L). Although two different media formulations were used for the MIC tests, the extent of growth for all three bacterial strains was comparable. This mitigates a Cx-50 copolyoxetane salt effect for any particular strain.

**MIC Assays.** MIC values for Cx-50 copolyoxetanes were determined by standard methods. [34, 36, 69] Stock solutions were made by dissolving different concentrations of Cx-50 copolyoxetanes in distilled water. The media (6 ml of M9 mannitol for *E. coli* and *P. aeruginosa* or TSB for *S. aureus*) were placed into Erlenmeyer flasks. Serial 1:2 dilutions of Cx-50 copolyoxetane stock solutions and 60 µl of the bacterial cultures (~ 10^8 cfu/ml) were added sequentially to the flasks to establish an initial range for MIC values. In order to determine MIC values with more precision, series were re-run within the initial range at smaller concentration differences (1µg/ml). The flasks were then kept in shakers at 37 °C for 24 hours. Each series of tests were performed in triplicate. Bacterial growth was examined by measuring solution optical density at λ = 600 nm (OD_{600}) as well as examination of turbidity visually. This MIC assay, which depends on a change in optical density of two orders of magnitude and a visual observation of solution transparency, provides confirmation of complete inhibition, that is, MIC.[34, 36, 70]
Kinetics of C8-50 copolyoxetane on bacterial killing. Agar plates were streaked with either *E. coli*, *P. aeruginosa*, or *S. aureus* from a frozen stock culture at -70 °C and incubated at 37 °C for 18-24 hrs. From this plate, a single colony was collected and used to inoculate 6 ml of Luria broth. This culture solution was incubated for 18-24 hrs at 37 °C. A subculture was made by preparing a 1:100 dilution in LB and incubated at 37 °C until it reached an optical density of ~ 0.5 by UV-VIS (logarithmic growth phase). A 20 ml aliquot of this bacterial culture was spun down to a pellet for 10 min at 10,000 rpm in a centrifuge. The supernatant was discarded and the pellet was dissolved in 20 ml of the respective growth media used for the MIC tests (M9 mannitol for *E. coli*, *P. aeruginosa*, or TSB for *S. aureus*). The bacterial culture was divided into two equal volumes; one served as the control, while C8-50 copolyoxetane was added to the other at the desired concentration. C8-50 was chosen for the kinetic kill study as it had the lowest MIC values. At selected times (t = 0, 1 hr, 2 hr, 3 hr) 1 ml aliquots were taken from both the control and sample flasks and spun into a pellet in a microcentrifuge for 3 minutes at 1400 rpm. The supernatant was discarded and the pellet was resuspended in saline solution. The same cycle was repeated twice until the pellet suspended in saline contained no growth media. A serial dilution series was performed with 100 μL of the bacterial culture in saline. 100 μL aliquots were taken from each dilution and plated on Luria agar plates. These plates were incubated at 37 °C for 24 hours. The number of colonies were counted and compared with the control to obtain percent kill as a function of time.
**Cytotoxicity Testing.**

**Hemolytic activity assay.** One metric for defining hemolytic activity is HC$_{50}$, the concentration of antimicrobial that kills 50% of RBCs under investigation.[50, 69, 84] A red blood cell lysis assay was developed following the procedure described by Palermo and Kuroda.[36] Red blood cells, RBCs (1ml), were diluted with 9 ml of phosphate buffered saline, PBS, (i.e. 10mM phosphate, 138 mM NaCl, pH 7.4, Sigma) and then centrifuged at 1000 rpm for 5 min. The resulting supernatant was removed using a pipette making sure not to disturb the RBCs and these steps repeated two more times. The assay stock (90 µL) was mixed with each of the polymer solutions (10 µL) on a sterile 96 well round bottom polystyrene microplate to give a final solution of 3.3% v/v RBC. Based on a hemacytometer count, this was approximately $10^8$ red blood cells per ml.

PBS (10 µL) or Triton X-100 (10 µL, 1% v/v) was added to the assay stock solution as respective negative and positive hemolysis controls. Triton X-100, a non-ionic surfactant is commonly used as a positive control in hemolysis assays.[36] For tests employing C12-m copolyoxetanes, 10 µL of stock solutions were added. The 96-well microtiter plate was secured in an orbital shaker at 37 °C and 250 rpm for 1 hr and then centrifuged at 1000 rpm for 10 min. The resulting supernatant (10 µL) from each well was transferred to a new sterile 96-well round bottom polystyrene microplate and diluted with PBS (90 µL).

Absorbance at 405 nm, characteristic of cell lysis,[36] was determined using a Versamax EXT microplate reader (Molecular Devices, Sunnyvale, CA) Negligible absorbance was
observed for the negative control (PBS) while the absorbance for the positive control was 3.9. The fraction of hemolysis is defined as the absorbance for copolyoxetane treated wells divided by the average of readings from the positive control wells. Hemolysis was plotted as a function of Cx-50 concentration to obtain HC_{50}, the copolyoxetane concentration that causes 50% hemolysis relative to the positive (Triton-X) control. Two separate trials were carried out in triplicate for each assay trial. Values reported are the average of the two trials.

**Human cell cytotoxicity assay.** Cytotoxicities are reported as EC_{50} values which indicate the concentration which brings about 50% lysis of the respective cell line. Cytotoxicity was tested using a CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) and were run according to the manufacturer’s protocol.[100] Human foreskin fibroblasts (HFFs) and human dermal fibroblasts (HDFs) were used to enhance relevance to medical applications and therapeutics. HFFs and HDFs were seeded in 96 well plates at a seeding density of 1.5 × 10^3 cells/cm^2. After incubation at 37 °C and 5% CO_2 for 24 hours, the cells were exposed to sample solutions at different concentrations for 24 hours, followed by the addition of the tetrazolium dye solution to the wells and incubation for 4 hours. During this period, living cells convert tetrazolium into a formazan product. A stop solution was added to formazan and the plate absorbance was read at 570 nm using a Biomate3 Spectrophotometer (Thermo Electron Corporation). EC_{50} values are determined by using concentration vs. absorbance plots.[101] From concentration vs. absorbance plots, a maximum absorbance (plateau) was obtained. EC_{50} values were determined by locating the concentration corresponding to one-half of the maximum.
Results

Biocidal efficacy of water soluble C12-m copolyoxetanes was previously demonstrated, wherein the quaternary alkyl chain length was 12 carbon atoms (1, x = 12, m = C12 mole fraction). The optimum antibacterial activity was observed for copolyoxetanes containing 40-60 mole % C12.[99] To explore further amphiphilic balance, the present study investigates the effect of alkyl chain length for Cx-50 copolyoxetanes, where x = 2, 6, 8, 10, 12, 14 and 16. These copolyoxetanes were prepared via the intermediate P[(BBOx)(ME2Ox)-50:50-5.7]. The mole percent alkylammonium was constant and was in the middle of the range found most effective for C12. Preparation and characterization are briefly described followed by results from solution biocidal studies.

Cx-50 copolyoxetanes. Cx-50 copolyoxetanes 1 were prepared in two steps. First the intermediate P[(BBOx)(ME2Ox)-50:50-5.7] was prepared. $^1$H-NMR confirmed mole ratios while end group analysis with TFAA (trifluoroacetic anhydride) provided a molecular weight ($M_n$) of 5.7 kDa. The second step was quaternization of the copolyoxetane precursor with the respective tertiary amines. The substitution reaction was carried out at 60 °C except for the reaction with C2 amine. For C2, the reaction was carried out at 10 °C and utilized an ice jacketed condenser.

Complete substitution of the C-Br in the P[(BBOx)(ME2Ox)-50:50-5.7] precursor to the respective Cx-50 copolyoxetanes was obtained as shown via $^1$H-NMR spectroscopy (Table 3.1). Representative $^1$H-NMR spectra for the P[(BBOx-50)(ME2Ox)] precursor and C8-50 are shown in Figure 3.1 A and B, respectively. In the spectrum of C8-50 the absence of precursor peaks at
1.9 ppm (–CH₂CH₂CH₂CH₂Br) is noteworthy. New peaks at 1.2-1.3 ppm (–CH₂(CH₂)₆CH₃ from C8) are characteristic for the C8 substitution.

![Chemical Shift Graph](image)

**Figure 3.1.** $^1$H-NMR of (A) - P[(BBOx)ME2Ox]-50:50 and (B) - C8-50

Cx-50 copolyoxetanes are viscous liquids at ambient temperature with $T_g$’s ranging from -40 to -60 °C (Figure 3.2). Cx-50 copolyoxetanes have $T_g$’s that are 10-20 °C higher than P[(BBOx)(ME2Ox)-50:50-5.7] due to presence of strong polar interactions from quaternary salt functionality. The low Cx-50 $T_g$’s compare favorably with polycation salts that are molten salt
analogs (-64 °C to -72 °C).[73, 74] The low $T_g$’s for Cx-50 copolyoxetanes attest to high molecular mobility. This is attributed to a combination of ME2Ox side chains and ether functionality in the main chain and in the linkage to the alkyl side chain.

![Figure 3.2. MDSC thermograms for the Cx-50 polyoxetanes: (A) C2-50 (B) C6-50 (C) C8-50 (D) C10-50 (E) C12-50 (F) C14-50 (G) C16-50 and (H) P[(BBOx)(ME2Ox)-50:50-5.7).](image-url)
The MDSC thermograms for the C14 and the C16 show endotherms at -7 °C and at 10 °C, respectively (Figure 3.2). These endotherms are attributed to melting of phases formed by C14 and C16 side chains. Compared to poly(n-alkyl)acrylate)s, for which side chains with 8 atoms crystallize,[110] the onset of crystallization is x = 14 for the Cx-50 copolyoxetanes. The low \( T_m \)’s and higher chain length for crystallization for the Cx-50 copolyoxetanes are due to the lower linear density of hydrocarbon side chains.

**MIC Assays.** Three common pathogenic bacteria were the subject of minimum inhibitory concentration (MIC) tests: Gram(-) *E. coli* and *P. aeruginosa* and Gram (+) *S. aureus*. The effect on MIC for changing the Cx-50 quaternary alkyl side chains is shown in Figure 3.3. The most potent antimicrobials are C6-50, C8-50, and C10-50. C8-50 is at the center of a shallow minimum in the bar-graphed data. MICs for C8-50 are 4 \( \mu \)g/ml (*E. coli*), 2 \( \mu \)g/ml (*S. aureus*) and 24 \( \mu \)g/ml (*P. aeruginosa*). Overall, C8-50 is the most effective antimicrobial against the three bacterial strains tested.

Relatively high MIC values are observed for *P. aeruginosa*, which is the most resistant bacterium to all Cx-50 copolyoxetanes. C8-50 (24 \( \mu \)g/ml) and C10-50 (25 \( \mu \)g/ml) have similar MICs against *P. aeruginosa*, Previously, a minimum MIC (26 \( \mu \)g/L) against *P. aeruginosa* was found for C12-60.[99] The resistance of *P. aeruginosa* has been demonstrated for most antimicrobials.[7] Additional lipopolysaccharides and other mechanisms are thought to decrease the interaction of antimicrobials with the membrane. Among all Gram(-) bacteria, *P. aeruginosa* has an overall outer cell membrane permeability that is 12-100 times lower than *E. coli*.[8-10]
The least effective Cx-50 copolyoxetane is C2-50 with MICs >90 µg/ml for Gram(-) E. coli and Gram(+) S. aureus and > 120 µg/ml for P. aeruginosa (Figure 3.3). Depending on the bacterial strain, an increase in MIC of 4-45 times is observed for C2-50 compared to C8-50. For C2-50 the amphiphilic balance is clearly shifted in a way that precludes effective conjugation with bacterial membranes.

![Figure 3.3](image)

**Figure 3.3.** Minimum Inhibitory Concentrations as a function of quaternary chain length for Cx-50 copolyoxetanes. - *E. coli*, - *S. aureus*, and - *P. aeruginosa*.

This attenuation of biocidal efficiency resulting from shortening the alkyl chain can be compared with two related cases cited above. Palermo noted that changing the butyl group in structure 2 to a methyl group resulted in a 100 times increase in MIC for *E. coli*,[36] while Savage found that a
long alkyl chain for the molecular polycation “CSA-13”, 3 was essential with a low MIC against E. coli. [48]

The results shown in Figure 3.3 may be compared with those for a series of amphiphilic polyoxanorbornenes 4 with varying quaternary alkyl side chains.[52]. With R = ethyl or butyl the MIC was 200 µg/ml for E. coli, while for hexyl and octyl analogs the MIC decreased to 12.5 and 4 µg/ml, respectively. For R = decyl, the MIC increased to 12.5 µg/ml. A similar trend was observed for Gram (+) B. subtilis. Considering the different molecular structures of 1 and 4, the trends are remarkably similar.

A MIC minimum was also observed for amphiphilic pyridinium methacrylate copolymers by Sen.[50] The highest antimicrobial efficiency against E. coli was observed for the butyl copolymer 5 (50 µg/ml), but the MIC was 550 µg/ml for the ethyl analog. Above butyl, MICs increased with increasing alkyl chain length, reaching 1000 µg/ml for the decyl analog. However, against Gram (+) B. subtilis a drop in MIC occurs from ethyl (300 µg/ml) to hexyl (30 µg/ml). Increasing the chain length to decyl increased the MIC to 300 µg/ml. Thus, compared with the optimum C8-50 copolyoxetane 1 and polyoxanorbornenes 4, polycation 5 has moderate antimicrobial efficiency that varies with bacterial strain.
Molecular polycation 3, polycation 5, and Cx-50 copolyoxetanes 1 share poor biocidal efficacy with short alkyl chain lengths. At longer chain lengths near the optimum C8-50, Cx-50 copolyoxetanes 1 are not very sensitive to alkyl group chain length. This relative potency toward Gram(-) *E. coli* and *P. aeruginosa* and Gram(+) *S. aureus* differs little in the C6/C10-50 compositional range is advantageous. The uniformly high antimicrobial effectiveness of C8-50 may portend low MICs for a broad range of pathogenic bacteria.

**Killing Kinetics Assay.** Rapid kill of pathogenic bacteria is of practical value and is important for developing model(s) of polycation biocidal action. Thirty years ago, Ikeda carried out pioneering research on antimicrobial effectiveness of several polycations.[26, 28] Since these studies, surprisingly little has been reported on polycation kill kinetics. Based on Ikeda’s method, a kill kinetic assay was performed to determine the biocidal effectiveness of the most effective copolyoxetane (C8-50) as a function of time.

Figure 3.4 shows bacterial kill over three hours for C8-50 at a concentration of 5 times MIC. Three separate experiments were done in triplicate yielding low standard deviations. The bacterial challenges were ~ 10⁸ cfu/ml Gram(-) *E. coli* and *P. aeruginosa* and Gram(+) *S. aureus*. Log reductions of 7 (0), 3.0 (0.18) and 5.0 (0.13) were obtained for *S. aureus*, *E. coli* and *P. aeruginosa*, respectively, at the end of 1 hour.
The kill kinetics study shows that the C8-50 kills >99% of the bacterial cells during the first hour. The order of MICs is S. aureus < E. coli << P. aeruginosa. From these MICs, it might be anticipated that P. aeruginosa would exhibit slower kill kinetics than either S aureus or E. coli and that E. coli would have the fastest kill kinetics. However, the order of rate of kill is S. aureus >> P. aeruginosa >> E. coli. This order must result from a nonlinear dependence of C8-50 concentration on rate of kill. Choosing 5× MIC resulted in a C8-50 concentration of 125 µg/ml against P. aeruginosa. While for E. coli the concentration was only 20 µg/ml.

Figure 3.4. Killing kinetic assays for (A) E. coli, (B) P. aeruginosa, and (C) S. aureus
Figure 3.4 shows that 100% kill, that is a 7 log reduction, for *S. aureus* was observed in 1 hr at 10 µg/ml (5× MIC). This rate may be compared with Ikeda's result for the polyacrylate 6, which is the only polymer polycation for which kill kinetics have been reported.[28] Polycation 6 has a relatively high MIC:[26, 99] 660-1000 µg/ml for *E. coli*, 100-330 µg/ml for *S. aureus* and 660-1000 µg/ml for *P. aeruginosa*. For comparing kill rates, the original data of Ikeda have been converted to log reduction of the original challenge (Table B1). From these data, a concentration of 29 µg/ml 6 against *S. aureus* gave a log reduction of 3.1 in 1 hour.[26] Considering that 29 µg/ml is at the most ~ 0.3 MIC, the 3 log reduction/1h is noteworthy. However, the bacterial challenge was 10⁵ cfu/ml which is 3 log lower than those employed for testing C8-50.

Kill kinetics for C8-50 may be compared with those recently obtained for the molecular polycation 3, designated CSA-13.[38] CSA-13 was tested at 10 times MIC (10 µg/ml) against a clinical isolate of vancomycin-resistant *S. aureus*. Kill rates were 3.8 log reduction/4 hr and 4.8 log reduction/8 hr.[1, 38] For these tests the bacterial challenges were 3.2 x 10⁸ cfu/ml. This concentration is similar to those used for C12-43 which is 1.9 x 10⁸ cfu/ml. By comparison, C12-43 at 10x MIC (53 µg/ml) effects a log reduction of 3.6 against *S. aureus* (ATCC-25904) at 1 hr while 3 effects a 3.2 log reduction in 3 hours.

Finally, in view of the limited development of heuristics for bacterial kill rate, the kill rates for antibiotics are noted. Ciprofloxacin, ofloxacin, sparfloxacin and trovafloxacin were
tested against 2 strains of *S. aureus*. [111] For both ciprofloxacin and ofloxacin, at 8 times MIC (4 µg/ml), a log reduction of <2.5 was observed at the end of 2 hours for a starting inoculum of ~10^6 cfu/ml.

**Cytotoxicity for HFF and HDF cell lines.** Cx-50 copolyoxetanes with x = 6, 8 and 10 carbon atoms were employed to investigate cytotoxicity toward mammalian cell lines as they were the most effective compositions with regard to antimicrobial activity. From Table 3.2 it is seen that among the Cx-50 copolyoxetanes, C6-50 has the least toxicity towards fibroblast cells (60,000 µg/ml for HFF cells and 61000 µg/ml for HDF cells). Table 3.2 shows that toxicity towards fibroblast cells increases with increasing length of the alkyl chain from 6 to 10 carbon atoms. Even though C10-50 has relatively low values for EC_{50}, i.e., 36000 µg/ml for HFF cells and 37000 µg/ml for HDF cells, cytotoxicity is minimal considering bacterial MICs.

**Table 3.2.** Cytotoxicity against HFFs, HDFs and RBCs as a function of the alkylammonium chain length of the Cx-50 copolyoxetanes.

<table>
<thead>
<tr>
<th>Copolyoxetane</th>
<th>EC_{50} (µg/ml)</th>
<th>HFF</th>
<th>HDF</th>
<th>RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6-50</td>
<td>60000</td>
<td>61000</td>
<td></td>
<td>762</td>
</tr>
<tr>
<td>C8-50</td>
<td>42000</td>
<td>41000</td>
<td></td>
<td>303</td>
</tr>
<tr>
<td>C10-50</td>
<td>36000</td>
<td>37000</td>
<td></td>
<td>353</td>
</tr>
</tbody>
</table>
**Selectivities.** Selectivity, $S$, defined by Eq. 1 is a measure of the concentration required to lyse 50% human cells (EC$_{50}$) compared to the minimal concentration for inhibiting the growth of bacteria (MIC). High selectivities mean effective antimicrobial activity while minimizing effects on human cells. As a measure of cytotoxicity, selectivities provide an optimistic estimate of antibiotic effectiveness, as the MIC is typically 2-4 times lower than the minimal biocidal concentration, MBC.[99] Nevertheless, the measurement of selectivity provides a guide for potential use of antimicrobials as antibiotics.

$$S = \frac{\text{EC}_{50}}{\text{MIC}}$$

Eq. 1

The selectivities follow the EC$_{50}$ trend with C6-50 and C8-50 being more selective towards human fibroblasts compared to bacterial cells than C10-50 (Table 3.3). The general trend of selectivity for Cx-50 copolyoxetanes is evident with an inverse dependence on the alkyl chain length. The absolute values for selectivities follow the order $S.\text{aureus}$ (x=6, 22600 to x=10, 11000) > $E.\text{coli}$ (x = 6, 9200 to x = 10, 5870) >> $P.\text{aeruginosa}$ (x = 6, 1990 to x = 10, 1500). The lower selectivities for $P.\text{aeruginosa}$ stem from MICs that are 5-6 times higher than those for $E.\text{coli}$ and $S.\text{aureus}$. For comparison, an evaluation of 3 yielded a relatively low MIC (4 µg/ml) for $P.\text{aeruginosa}$.[81] However, 3 has an HC$_{50}$ of ~120 µg/ml for RBCs giving a selectivity of 30.[1] As noted previously in several studies on other antimicrobials, $P.\text{aeruginosa}$ has an outer membrane permeability that is 12-100 times lower than that of other gram negative bacteria.[9] Hence, $P.\text{aeruginosa}$ is known to be resistant to most antimicrobials.[7]
**Hemolytic activity assay.** Hemolytic assays were performed using relatively low Cx-50 copolyoxetanes concentrations but at least 10 times the MIC for *S. aureus*. Percent lysis of human RBCs vs. Cx-50 concentration is shown in Figure 3.5. At the highest concentration tested, C6-50 has the lowest lysis (9 %) whereas C8-50 affects 20 % lysis. The modest trend for increasing lysis with increasing Cx-50 concentration indicates that the concentration affecting 50% human RBC lysis (HC₅₀) would be much higher than 50 µg/ml. To correlate with literature studies on antimicrobials, high concentrations (to 5000 µg/ml) Cx-50 copolyoxetanes with *x* = 6, 8 and 10 were tested on RBCs.

*Figure 3.5.* Percent lysis of RBCs as a function of concentration of the Cx-50 copolyoxetanes. (A) C6-50, (B) C8-50, (C) C10-50, and (D) C12-50
These hemolytic assays with high concentrations confirmed the benign nature of the Cx-50 copolyoxetane towards human RBCs (Figure 3.6). The C6-50 copolyoxetane was the least hemolytic with an HC50 of 762 µg/ml (Table 3.2). The potent antimicrobial C8-50 was the most hemolytic with an HC50 of 303 µg/ml. Selectivity, $S$, defined by Eq. 2 is a measure of the concentration required to lyse 50 % of red blood cells (HC50) compared to the minimal concentration for inhibiting the growth of bacteria (MIC). The RBC selectivity for C6-50 was high for the tested strains of bacteria. From Table 3.3 it is seen that the highest RBC selectivity of 283 has been observed against $S. aureus$ for C6-50.

$$S = \frac{HC_{50}}{MIC}$$

Eq. 2

Table 3.3. Selectivities for Cx-50 copolyoxetanes for RBCs, HFFs, and HDF

<table>
<thead>
<tr>
<th>Copolyoxetane</th>
<th>$E. coli$</th>
<th>$S. aureus$</th>
<th>$P. aeruginosa$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBC</td>
<td>HFF</td>
<td>HDF</td>
</tr>
<tr>
<td>C6-50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>114</td>
<td>9050</td>
<td>9200</td>
</tr>
<tr>
<td>C8-50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>9330</td>
<td>9110</td>
</tr>
<tr>
<td>C10-50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>5710</td>
<td>5870</td>
</tr>
</tbody>
</table>

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Comparative antimicrobial effectiveness and cytotoxicity.

The above results for Cx-m copolyoxetanes are compared with selected studies on molecular and macromolecular polycation antimicrobials in Table 3.4. The selected antimicrobials are optimum compositions from the cited references. The compositions follow an order that places those with highest selectivities toward the top of the table. Some judgment was exercised in this regard, as a given polycation may have variable antimicrobial effectiveness and cell selectivity depending on the tested organisms.

Figure 3.6. Percent lysis of RBCs as a function of concentration of the Cx-50 copolyoxetanes
Phenylene ethynelene molecular polycation 7 has an HC\textsubscript{50} of 88 µg/ml against human RBCs.[84] This molecular polycation has two primary alkylammonium functions per uncharged group, which is about double the linear charge density of the Cx-50 copolyoxetanes, but achieved with quaternary alkylammonium function. The selectivity for \textit{S. aureus} (440) is somewhat greater than that found for C6-50 (283). On the other hand, 7 has relatively low selectivity for other cell lines tested (60 for 3T3; 70 for HepG2). This comparison shows how radically different structures such as 7 (molecular aromatic, primary amine, 2:1 charge per neutral group, two C-Br bonds)[84] and 8, C6-50, P[(C6-50)(ME2Ox)-50:50-5.7] (aliphatic, quat, 1:1 charge per neutral group) are both good antimicrobials with low cytotoxicity. This comparison emphasizes how little is understood about fundamental interactions of polycation antimicrobials with bacteria and human cells and how these interactions compare with those of AMPs.

Like molecular polycation 7, 9 has primary alkylammonium functionality but is a low molecular weight polymethacrylamide (Table 3.4). Polymer 9 has an HC\textsubscript{50} of 6400 µg/ml for human RBCs and an EC\textsubscript{50} of 34 µg/ml for Hep2 cells.[69] This polymer without alkyl side chains on nitrogen has the least toxicity towards mammalian cells, while copolymers with a butyl or hexyl pendant group exhibit high cytotoxicity. Copolymer 9-a with 49 mole % cationic charge has a comparable charge density and alkyl chain length to that of the C6-50 copolyoxetane. However, copolymer 9-a also has a low HC\textsubscript{50} (<12
µg/ml) compared to 762 µg/ml for C6-50. The selectivity of the hexyl copolymer 9-a for human RBCs compared to *E. coli* and *S. aureus* is 0.15 compared to 114 (*E. coli*) and 283 (*S. aureus*) for C6-50. Once again the imperfect knowledge of polycation / cell interactions makes correlations difficult.

The pyridinium methacrylate copolymers 10 have a similar charge density compared to the Cx-50 copolyoxetanes.[50] Copolymer 10 with butyl N-side chains has the least cytotoxicity towards human red blood cells (HC₅₀ = 1709 µg/ml). However, relatively high MICs of 50 µg/ml (*E. coli*) and 30 µg/ml (*B. subtilis*) make the copolymer less selective (selectivity 34 for *E. coli* and 57 for *B. subtilis*) toward human red blood cells compared to the microbes. Copolymers 10 with hexyl and octyl side chain have HC₅₀ values of 500 µg/ml and 450 µg/ml respectively. The relatively high MICs (40 µg/ml for R = hexyl and 100 µg/ml for R = octyl) leads to low selectivity values of ~ 12 and ~ 5 respectively for the copolymers compared to 114 for C6-50 and 65 for C8-50 against *E. coli*.

Methacrylate copolymers 11 with quaternary amine functionalized side group exhibit high HC₅₀ (1200 µg/ml).[36] A high MIC (46 µg/ml) against a strain of *E. coli* leads to a low selectivity (26). Copolymer 11 with a 56 mole % quaternary ammonium charge has an HC₅₀ of 67 µg/ml that gives a selectivity of 2, while the lowest selectivity (65) for the Cx-50 copolyoxetane series was for C8-50 (HC₅₀ = 303 µg/ml). As above, subtle and as-yet not understood interactions for amphiphilic copolycation antimicrobials confound an easy interpretation of such comparisons.
Biocidal properties and cytotoxicity of polyoxanorbornenes 12 with alkyl pyridinium side chains gave a high HC$_{50}$ of 4030 µg/ml ($R$ = ethyl) but again, selectivity was low (20) because of relatively high MICs for against *E. coli* and *B. subtilis*.[52] For $R$ = hexyl, octyl and decyl side chains, polyoxanorbornenes 12 with a 10 kDa molecular weight had lower HC$_{50}$ values (202 µg/ml, <50 µg/ml, <50 µg/ml, respectively) compared to the corresponding 3 kDa polymer. Amphiphilic cationic polynorbornenes 13 with a molecular weight of 1.6 kDa was the least hemolytic towards human red blood cells compared to its other structural analogs.[34] Related structure 13 has an HC$_{50}$ of >4000 µg/ml but rather poor antibacterial characteristics leads to low selectivities of >20 (*E. coli*) and >13 (*B. subtilis*).

Polyamine oxanorbornene copolymer 14 with a 50 mole percent of cationic charge gave an HC$_{50}$ of 500 µg/ml against human red blood cells and a selectivity of 7 (*E. coli*) and 20 (*S. aureus*). Primary amine functionalized pyrimidine oligomer 15 has good antimicrobial properties with low MIC but it is also toxic towards human red blood cells (HC$_{50}$ = 14 µg/ml) which gave a selectivity of 17.5.[39]

Finally, the biocidal effectiveness and cytotoxicity of an arylamide compound, PMX 30063, is noted.[112] The nature of PMX 30063 is not disclosed except for general structure 16. PMX 30063 is reported to have low toxicity toward mammalian cells with an HC$_{50}$ of >500 µg/ml, an EC$_{50}$ of 430 µg/ml against 3T3 cells and 1031 µg/ml against HepG2 cells. A low MIC against *S. aureus* (1 µg/ml) leads to high selectivity for RBCs (>500), 3T3 cells (430) and HepG2 cells (1031 (HepG2).
Conclusion

From previous studies we observed that increasing the linear charge density on C12-m copolyoxetanes resulted in optimum biocidal activity at a charge fraction of ~ 0.5.[99] Changing the alkyl chain length at the site of cationic charge in the copolyoxetane side chain had a profound influence on its antimicrobial property. The studies have shown that the Cx-50 copolyoxetanes acquire an optimum antimicrobial characteristic when there are 8 carbon atoms on the site of cationic charge (x = 8). For x > 8, the biocidal efficacy decreases gradually. The antimicrobial properties of C6-50 is comparable to that of C8-50, while for x < 6, there is a sudden decrease in activity. Another key issue in side chain aggregation is the presence of the methyl groups at the side of the cationic charge (tertiary amine groups).

The present investigation of cell compatibility has shown that the Cx-50 series have low toxicity towards human fibroblast cells and have high selectivities (E. coli, S. aureus and P. aeruginosa). The Cx-50 copolyoxetanes, particularly C6-50, exhibit low hemolysis of human red blood cells and have high selectivities. The water solubility of these polymers coupled with excellent antimicrobial behavior and cell compatibility makes these novel copolyoxetanes potential candidates for therapeutic applications.
Table 3.4. Comparison of cell toxicity and selectivity for polymeric and molecular polycations.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Description</th>
<th>MIC (µg/ml)</th>
<th>EC$<em>{50}$ or HC$</em>{50}$ (µg/ml)</th>
<th>Selectivity</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Molecular phenylene ethynylene diammonium chloride" /></td>
<td>Molecular phenylene ethynylene diammonium chloride</td>
<td>0.21 µg/ml (S. aureus)</td>
<td>88 µg/ml (RBC) 12 µg/ml (3T3 cells) 14 µg/ml (HepG2 cells)</td>
<td>440</td>
<td>[84]</td>
</tr>
<tr>
<td><img src="image2.png" alt="C6-50 copolyoxetane" /></td>
<td>C6-50 copolyoxetane M$_n$ – 5.7 kDa m = 50</td>
<td>2.7 µg/ml (S. aureus) 31 µg/ml [P. aeruginosa] 6.7 µg/ml [E. Coli]</td>
<td>762 µg/ml (RBC) 60000 µg/ml (HFF) 61000 µg/ml (HDF)</td>
<td>283 22000 [1960] 22600 [1990]</td>
<td>[99]</td>
</tr>
<tr>
<td>9</td>
<td>Methacrylamide homopolymer with primary amine groups</td>
<td>n = 17</td>
<td>118 µg/ml (E. coli)</td>
<td>~ 6400 µg/ml (RBC)</td>
<td>&gt; 54 [&gt; 640]</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>10</td>
<td>Pyridinium methacrylate copolymers</td>
<td>n = 50</td>
<td>50 µg/ml (E. coli)</td>
<td>1709 µg/ml (RBC)</td>
<td>34 [57]</td>
</tr>
<tr>
<td></td>
<td>R = buty group</td>
<td></td>
<td>30 µg/ml [B. subtilis]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Methacrylate copolymers with a quaternary amine functionalized side group</td>
<td>R = buty group</td>
<td>46 µg/ml (E. coli)</td>
<td>1200 µg/ml (RBC)</td>
<td>26</td>
</tr>
<tr>
<td>12</td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td>Polyoxanorbornene with alkyl pyridinium side chains</td>
<td>R = ethyl group</td>
<td>200 µg/ml (E. coli)</td>
<td>200 µg/ml (B. subtilis)</td>
</tr>
<tr>
<td>13</td>
<td><img src="image2" alt="Chemical Structure" /></td>
<td>Amphiphilic cationic polynorbornene derivatives</td>
<td>200 µg/ml (E. coli)</td>
<td>&gt; 4000 µg/ml (RBC)</td>
<td>&gt; 20 (&gt; 13)</td>
</tr>
<tr>
<td>14</td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>Polyamine oxanorbornene copolymer</td>
<td>R = propyl group</td>
<td>75 µg/ml (E. coli)</td>
<td>500 µg/ml (RBC)</td>
</tr>
<tr>
<td>Compound</td>
<td>Concentration (µg/ml)</td>
<td>Species</td>
<td>Concentration (µg/ml)</td>
<td>IC50</td>
<td>Reference</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------</td>
<td>---------</td>
<td>-----------------------</td>
<td>------</td>
<td>-----------</td>
</tr>
<tr>
<td>Pyrimidine oligomer</td>
<td>0.8 (E. coli)</td>
<td>0.8 (B. subtilis)</td>
<td>14 (RBC)</td>
<td>17.5</td>
<td>[39]</td>
</tr>
</tbody>
</table>
Chapter 4
Effects of a Poly[(3,3-quaternary/PEG)-copolyoxetane] Antimicrobial on Pseudomonas aeruginosa biofilms

ABSTRACT
Effects of a polycation antimicrobial, poly[(3,3-quaternary/PEG)-copolyoxetane] (i.e. C8-50), on biomass and cell viability of Pseudomonas aeruginosa biofilms were investigated. Crystal violet (CV) staining assays demonstrate that C8-50 has no effect on adhesion of already established P. aeruginosa biofilms, but reduced biofilm formation by killing planktonic cells prior to attachment. For anti-adhesion assays, there is a noticeable reduction in biofilm mass at concentrations greater than MBC (~2× MIC). Viability studies show a substantial log reduction of 2.1 at MIC. These results suggest that C8-50 shows promise as a potential therapeutic.

Introduction

Bacteria that are suspended and growing in a fluid environment live in a planktonic state while surface attached bacteria are sessile in a biofilm.[114] Being complex communities of surface and/or cell adhered microorganisms, biofilms fundamentally differ from their single celled planktonic counterparts. Biofilm growth occurs in sequential stages including i) transport of planktonic cells to a surface ii) initial attachment of planktonic cells (iii) formation of the extracellular polymeric substance, or exopolysaccharides, EPS, matrix and microcolonies (iv)
formation of macrocolonies/ biofilm maturation and (v) dispersion, that is, release of bacteria.[15]

The biofilm matrix of a fully hydrated biofilm is composed of water (50-90%) and EPS. This three dimensional matrix provides the means by which these bacterial communities are held together and forms a protective barrier for the adherent population.[115] The formation of bacterial biofilms aids survivability for a variety of intractable infections in humans.[116] The EPS is a mixture of polysaccharides, proteins, extracellular DNA and nucleic acids that have not been fully characterized but are believed to foster intercellular adhesion and surface attachment. [14, 117] Because of this matrix, bacteria living within a biofilm are less susceptible to environmental stresses, host immune responses, and antimicrobial agents.[12] Adherent bacteria are up to 1000 times more resistant than their planktonic counterparts.[14]

This important virulence mechanism is estimated to comprise more than 80% of all human microbial infections.[116] Bacterial biofilms on medical devices are a steady source of infection as bacteria released from the biofilm invade surrounding tissues.[103] Another factor that aids in bacterial survival in biofilms is the presence of persistent cells that are completely resistant to antimicrobials that are effective against planktonic bacterial populations.[103]

*Pseudomonas aeruginosa* is particularly virulent and persistent pathogen for immunocompromised patients, including burn patients, cystic fibrosis patients, and the elderly. *P. aeruginosa* contaminates contact lenses and indwelling medical devices such as catheters and is known to cause more than 200,000 hospital acquired infections in the United States annually.
More than 80% of cystic fibrosis patients develop chronic lung *P. aeruginosa* biofilm infections that are difficult to eliminate with traditional antibiotics.[12, 13, 118]

*P. aeruginosa* resistance has been shown for most antimicrobials, even planktonic populations.[7] The presence of added lipopolysaccharides and other mechanisms are believed to decrease the interaction of antimicrobials with the cell membrane. Among all Gram (-) bacteria, *P. aeruginosa* has an overall outer cell membrane permeability that is 12-100 times lower than *E. coli*.[8-10] Due to the presence of efficient efflux pumps, this bacteria has a low susceptibility toward antibiotics.[11] Inbuilt resistance coupled with the increased threat of multi-drug resistant strains is problematic as conventional treatments for *P. aeruginosa* infections rely on long term multi- antibiotic treatment.[118]

A key component of immune function in a higher organism includes the ability to recognize and eradicate bacteria though naturally occurring antimicrobials. Antimicrobial peptides (AMPs) are an important element of the host immune system in many organisms including insects, plants, and animals.[103, 118] These antimicrobials do not demonstrate buildup of resistance because AMPs killing action is confined to attacking the cell wall membrane.[98] AMPs have chain structures with cationic moieties on one side and hydrophobic moieties on the opposite side leading to the term “facially amphiphilic”. This amphiphilic balance of naturally occurring AMP enables selective binding to bacterial cell membranes with no effect on mammalian cells.[51] AMP design is, therefore, an attractive model for synthetic mimics.[51]
Isolating AMPs is complicated and cost prohibitive with regard to practical application.[103] Synthetic antimicrobial peptides have been synthesized which have proven even more potent than their naturally occurring counterparts. An emerging class of polycation antimicrobials take inspiration from these AMPs. These synthetic polycations promise economy via large scale production.[98] A range of chemical structures have been reported with widely varying effectiveness against planktonic populations.

One subgroup of these polymer antimicrobials is the poly[(3,3-quaternary/PEG)-copolyoxetanes]. The copolyoxetanes have quaternary alkyl side chains and PEG-like ME2Ox side chains. The copolyoxetane used in this study is C8-50 where ‘50’ is the mole percent of quaternary repeat units and ‘8’ is quaternary alkyl chain length.

\[
\text{C8-50}
\]

MIC (minimum inhibitory concentration) assays have shown that C8-50 is a potent antimicrobial against several strains of bacteria.[99] MIC is defined as the lowest concentration of an antimicrobial that will inhibit visible growth of an organism after overnight incubation. MIC tests are a first step to assess antimicrobial effectiveness against planktonic (single cell) bacterial populations and determine feasibility as a potential therapeutic.
To further evaluate the antimicrobial effectiveness, a test against biofilms was carried out. The most potent copolyoxetane antimicrobial, C8-50, was chosen for testing against \textit{P. aeruginosa} biofilms to determine biocidal efficacy. The first part of this study addresses whether C8-50 has an effect on adhesion of established biofilms and a second study addresses whether C8-50 can prevent biofilm formation. Finally, a study is presented that focuses on viability of established biofilms in the presence of C8-50.

\textbf{Experimental}

\textbf{Determination on Minimum Bactericidal Concentration (MBC)} MBC is defined as the concentration that kills the bacterial population (i.e. 99.99\%). The MBC was determined according to previously reported methods.[99]

\textbf{Adhesion studies: biofilm detachment assay in 96 well plates.} C8-50 was tested against pre-existing biofilms as an assessment of effectiveness. Agar plates were streaked with \textit{P. aeruginosa} (PA01) from a frozen stock culture at -70 °C and incubated at 37 °C for 18-24 hrs. From this plate, a single colony was collected and used to inoculate 6 ml of Luria broth. This culture solution was incubated for 24 hrs at 37 °C. A subculture was made by diluting it with M9 mannitol medium to an OD$_{600}$ of 0.02 and inoculating each cell with 100 µL of the subculture. Known protocols for growing biofilms in 96-well plates typically use Luria Broth.[12, 118] As noted earlier, C8-50 precipitates in this medium. Initial studies to optimize conditions for MIC tests established M9 mannitol as the medium of choice. The 96 well plates were covered with an air-permeable foil, put into a shallow glass dish lined with paper towels immersed in DI water,
and allowed to incubate at 37 °C for 24 hr. After this initial incubation, the biofilms contained within the 96 well plates were exposed to 40 µL of the C8-50 solutions prepared in M9 mannitol using a 2 fold dilution series. The initial stock solution concentration was at the solubility limit of C8-50 (400 mg/ml). Final C8-50 concentrations take into account the initial biofilm volume. An initial C8-50 concentration of 400 mg/ml is 114 mg/ml when diluted in the 96 well plates. For the *P. aeruginosa* controls, 40 µL of M9 mannitol media was added to keep volumes consistent with the biocidal challenge wells. Bacterial suspension, media, and no antimicrobial served as positive controls. Three wells contained bacterial suspension, media, and ethanol as negative controls. After subsequent 24 hr incubation at 37 °C, the cells were washed with a saline solution to remove nonadherent bacteria, stained with 0.1% crystal violet dye, incubated at room temperature for 20 min, washed with saline solution, and dried in air. Visual inspection qualitatively confirmed biofilm adherence because adsorbed dye correlated directly with biofilm density. Desorption of the dye into 30% acetic acid provided a semiquantitative measurement of optical density at 550 nm using a Versamax EXT microplate reader (Molecular Devices, Sunnyvale, CA). A minimum of four 96 well plates were used for each assay with at least three replicates of each C8-50 concentration per plate. Values in Figure 4.2 are the mean of twelve replicates with associated standard deviations.

**Biofilm formation assay: anti-biofilm adhesion studies.** The same preparative procedure was used as described above with the exception that C8-50 solutions were introduced into the 96 well plates at the same time as the *P. aeruginosa* subculture. This assay was used to determine if C8-50 would prevent biofilm formation. Values in Figure 4.5 are the mean of twelve replicates with associated standard deviations.
**Viability Assays.** Live cell counts (cfu) using standard plating techniques were used as a measure of viability. Because of the difficulty associated with removing live biofilms from 96 well plates, biofilms were grown in 5 ml polystyrene test tubes. Agar plates were streaked with *P. aeruginosa* from a frozen stock culture at -70 °C and incubated at 37 °C for 18-24 hrs. From this plate, a single colony was collected and used to inoculate 6 ml of Luria broth. This culture solution was incubated for 18-24 hrs at 37 °C. A subculture was made by preparing a 1:100 dilution in M9 mannitol medium and inoculating each test tube with 500 µL of the subculture. The preparative procedure used for the adhesion studies was scaled up 5× to account for the larger volume of the test tube compared to a 96 well plate. The test tubes were covered with an air-permeable foil, placed in a test tube rack within a shallow dish lined with paper towels soaked in DI water, covered loosely with aluminum foil, and allowed to incubate at 37 °C for 24 hrs. Stock solutions of the C8-50 were prepared using M9 mannitol media then introduced to the individual test tubes at a volume of 200 µL and a final copolyoxetane with biofilm concentration at MIC, 5×MIC, and 10×MIC. These concentrations were chosen as they correspond to the earlier planktonic MIC studies and killing kinetic assays.[119] For the *P. aeruginosa* control, 200 µL of M9 mannitol media was added to keep volumes consistent with the biocidal challenge test tubes.

As there was no precedent to predict if the C8-50 would be effective against biofilms, a concentration of 1000× MIC was included. Each C8-50 test and the control consisted of four replicates. After 24 hr, each test tube was decanted, washed with saline three times to remove any unattached cells and media components and 1 ml saline was added. *P. aeruginosa* is a motile
organism that typically forms biofilms at the air-liquid interface. Preliminary studies showed that vortexing alone did not adequately dislodge biofilms from the test tube sides. Therefore, the sides of the test tubes were manually scraped with sterile micropipette tips for 30 s to remove attached cells. Each test tube was recapped with a fresh sterile test tube caps and vortexed for 20 min each. Crystal violet (CV) staining of the polystyrene test tubes confirmed the complete removal of biofilm mass. From these initial 1 ml aliquots, 10-fold serial dilution series were plated on Luria agar in duplicate and incubated at 37 ºC for 24 hrs. The mean number of colonies were counted and compared with the mean control to obtain percent kill and log reductions according to Eq.1 and Eq. 2.

Percent Kill Formula:

\[
\%\text{kill} = 1 - \frac{CFU_{\text{sample}}}{CFU_{\text{control}}} \times 100
\]  
(Eq. 1)

Log Reduction Formula:

\[
\text{Log Reduction} = \log\left(\frac{CFU_{\text{control}}}{CFU_{\text{sample}} + 1}\right)
\]  
(Eq. 2)

Results

**Biocidal activity of C8-50 against planktonic bacteria.** C8-50 MIC for *P. aeruginosa* is 24 μg/ml with bacterial challenges of ~ 10^8 cfu/ml. MBC is 48 μg/ml (2× MIC). Killing kinetic

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studies at a concentration of 5× MIC showed C8-50 killed > 99.9% *P. aeruginosa* bacteria within the first hour. Log reductions were: 5.0, 5.2 and 5.5 at 1 hr, 2 hr, and 3 hr, respectively. [119]

**Adhesion Assays.** To determine if C8-50 would eradicate established biofilms, crystal violet (CV) staining assays were performed. Visual inspection of the 96 well plates after CV staining showed little variance in color among the positive *P. aeruginosa* controls and the antimicrobial challenge wells (Figure 4.1) indicating that C8-50 has no effect on adhesion of pre-existing biofilms. Further analyses of CV staining assays using UV-visible spectroscopy demonstrated that C8-50 did not reduce the biomass of established biofilms regardless of concentrations employed (Figure 4.2).

A similar effect was shown when Kapoor tested several AMPs (LL-37 and Pexaganin) that did not reduce biomass of pre-existing biofilms. [118] With the exception of LB used as the medium rather than M9 mannitol, CV staining assay procedures were similar to C8-50. It should be mentioned that CV staining can be used to measure biomass removal, but not disinfection.[121] Although the researchers do not provide an explanation as to why AMPs did not reduce biofilm mass, the inability of both C8-50 and AMPs tested to reduce biomass could be the result of limited diffusion into the exopolysaccharide matrix. Also, it is known that cationic moieties in AMPs and their synthetic analogs bind to DNA in the extracellular matrix.[118] Reaction of the antimicrobials, in this case, C8-50 and AMPs, or sorption to the biofilm matrix components can hinder the ability of an antimicrobial to penetrate the cells within the biofilm.[122]
Figure 4.1. Representative 96 well plate for C8-50 adhesion assay
Figure 4.2. Effect of C8-50 antimicrobial on established *P. aeruginosa* biofilms
For comparison, Kapoor demonstrated that conventional antibiotic tobramycin had no effect on established *P. aeruginosa* (PA 14) biofilms at MIC (0.7 µg/ml) and a ~30% reduction at ~16× MIC [118]). Consistent with the results shown above, *P. aeruginosa* biofilm matrix slows down the diffusion of the positively charged aminoglycoside antibiotics, like tobramycin.[115]Ciprofloxacin showed ~60% reduction in biofilms at values between MIC (0.1µg/ml) and ~31× MIC. Suci et al reported that ciprofloxacin penetration is significantly reduced in a *P. aeruginosa* biofilm.[123] Fluoroquinolone antibiotics, like ciprofloxacin, are one of the most effective classes of antibiotics used to treat *P. aeruginosa* biofilm infections. This suggests even though ciprofloxacin also demonstrates delayed diffusion, other factors must be involved in ciprofloxacin’s removal efficacy.

Kapoor has tested *P. aeruginosa* (PA 14) biofilms against a class of synthetic antimicrobial peptoids. Peptoids, i.e. oligo-N-substituted glycines, are isomers of peptides with side chains attached to the backbone nitrogen instead of the α-carbon. Most peptoids tested were able to reduce biofilms by ~40% at values near their MICs. The most effective peptoid, “Peptoid 1”, H-(NLys-Nspe-Nspe)_4-NH₂, having repeat units shown in Figure 4.3, reduced established *P. aeruginosa* biofilms ~ 60% at MIC (22.7 µg/ml). Being facially amphiphilic, Peptoid 1 allows for more effective biofilm detachment because additional stabilization can be provided by the presence of aromatic side chains along one side of the structure. This may allow for increased localization of cationic charge on the opposite side of the structure near the cell membrane; therefore, increased activity which may contribute to biofilm detachment.
**Figure 4.3.** Repeat units for Peptoid 1, H-\((\text{MLys-Nspe-Ns})_4\)-NH$_2$

**Anti-adhesion Assays.** To determine if C8-50 could be used to inhibit biofilm formation, a crystal violet (CV) staining assay was used. On visual inspection, wells with the highest concentration of C8-50 had a paler purple color signifying reduced biomass compared to the darker purple wells with lower concentration of C8-50 and the *P. aeruginosa* controls (Figure 4.4).
Figure 4.4. Representative 96 well plate for C8-50 anti-adhesion assay
Subsequent analyses of CV staining assays using UV-visible spectroscopy measured whether C8-50 eliminated biofilms prior to formation. Exact MIC values for C8-50 and their multipliers were not tested for the adhesion assays but were included in the expanded range tested (3.5 µg/ml to 114 mg/ml). The protocol typically calls for a 6-8 dilution series of the initial stock solution.[12] To keep tested concentration values within a narrower rage, a 2 fold dilution series was chosen. This was done so it would be easier to establish a trend for C8-50 concentrations near the MIC (Figure 4.6). From Figures 4.5 and 4.6, it is evident that C8-50 kills planktonic bacteria that could eventually form biofilms as there is a noticeable reduction in biofilm mass at concentrations greater than 27.9 µg/ml, a concentration that approximates the MIC. That is, biofilm mass is markedly diminished at 55.8 µg/ml, a concentration slightly higher than 2 ×MIC.
Figure 4.5. Effect of C8-50 antimicrobial as a preventative to *P. aeruginosa* biofilm formation
For comparison, ciprofloxacin had ~ 60% reduction in concentrations up to 4×MIC (0.4 µg/ml) while tobramycin did not show a significant reduction in biofilm formation until 8×MIC (~70%, 5.6 µg/mL).[118] The MBC value for ciprofloxacin is ~ 4-8× MIC [124] while tobramycin is ~ 2× MIC.[125] Peptoid 1 had a ~70% reduction in biomass at MIC and other tested peptoids were ~40%.[118] C8-50 has no effect on P. aeruginosa biofilms at MIC but a biomass reduction of ~80% at concentrations ~2× MIC (i.e. MBC) and above.

Figure 4.6. Effect of C8-50 antimicrobial as a preventative to P. aeruginosa biofilm formation at concentration values near MIC to 5× MIC.
Viability Studies. Adhesion and anti-adhesion (CV staining) assays provide an estimate of biomass but do not provide information on viability. Bacterial plating was employed to determine if C8-50 could kill *P. aeruginosa* biofilms. Bacterial biofilm challenges were ~ $10^6$ cfu/ml (based on *P. aeruginosa* control samples). Cell viability studies show that C8-50 kills >99.2% of *P. aeruginosa* biofilms at MIC (24 µg/ml) and higher percentages at greater concentrations (Table 4.1 and Figure 4.7). These results demonstrate that although C8-50 has no effect on adhesion of pre-existing biofilms, adherent components in the biofilm show greatly reduced activity. Log reductions of 2.1, 3.3, 4.5, and 6.8 were obtained for MIC, 5× MIC, 10× MIC, and 1000× MIC, respectively, after 24 hrs (Figure 4.8).

It is important to mention that previous killing kinetic studies on planktonic *P. aeruginosa* at a concentration of 5× MIC showed C8-50 killed > 99.9% bacteria within the first hour.[119] C8-50 also killed > 99.9% of *P. aeruginosa* biofilms, but the challenges were $10^6$ cfu/ml compared to $10^8$ cfu/ml. For viability studies, a log reduction of 3.3 was shown after 24 hr compared to a log reduction of 5.0 at 1 hr for planktonic studies. Although viability test results are promising, it is evident that C8-50 was less effective in killing biofilms than in killing planktonic populations. As mentioned earlier, this could be a result of limited diffusion and, or, sorption into the biofilm matrix hindering the antimicrobial’s ability to penetrate the cells within the biofilm.[122] In addition, the presence of efficient efflux pumps in *P. aeruginosa* further complicates cell penetration in both biofilm and planktonic populations and, thus, antibiotic susceptibility.[11]
Figure 4.7. Representative agar plates for *P. aeruginosa* biofilm viability assays with C8-50 (no dilution) (A) *P. aeruginosa* (control), challenge concentration $\sim 6.6 \times 10^6$ cfu/ml (B) MIC (C) 5× MIC (D) 10× MIC

Figure 4.8. Log reduction of *P. aeruginosa* biofilms vs. C8-50 concentration. MIC is 24 µg/ml. Antimicrobial exposure time is 24 hr.
Table 4.1. Percent kill and log reductions of *P. aeruginosa* biofilms (~ 10^6 cfu/ml) at several C8-50 copolyoxetane concentrations

<table>
<thead>
<tr>
<th>C8-50 Concentration (µg/ml)</th>
<th>MIC Multiplier</th>
<th>Percent (%) Kill</th>
<th>Log Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>1</td>
<td>&gt;99.2</td>
<td>2.1</td>
</tr>
<tr>
<td>120</td>
<td>5</td>
<td>&gt;99.9</td>
<td>3.3</td>
</tr>
<tr>
<td>240</td>
<td>10</td>
<td>&gt;99.99</td>
<td>4.5</td>
</tr>
<tr>
<td>24000</td>
<td>1000</td>
<td>100</td>
<td>6.8</td>
</tr>
</tbody>
</table>

There is limited research on the effect of antimicrobial peptides, molecular polycations, and polymer antimicrobials on biofilm viability. Biofilms can be grown under a wide variety of conditions and the method employed will affect antimicrobial performance.[103] It is difficult to make a direct comparison between antimicrobials, but relevant studies are summarized below and in Table 4.2.

Kapoor tested peptoids, AMPs, and conventional antibiotics for viability at concentrations close to peptoid MIC values and MBC concentrations (~8× MIC). The initial *P. aeruginosa* (PA14) biofilm challenge was ~10^10 cfu/ml (in Luria broth) compared to ~ 10^6 cfu/ml for C8-50 (M9 mannitol). Ciprofloxacin and tobramycin caused significant reductions in biofilms ~2 and ~1 log reductions but these values translate to 31× MIC and 8× MIC. AMPs tested (LL-37 and Pexaganin) had no effect. Peptoid-1 had a ~1 log reduction and 1-C13₄MER showed a roughly 2 log reduction (Table 4.2). It is suggested that 1-C13₄MER, repeat units in Figure 4.9, had greater biocidal activity against biofilms than Peptoid-1 and AMPs, due to the presence of a Ntridec which has a long tridecyl, surfactant, hydrophobic tail which can disturb
the hydrophobic extracellular matrix and cause increased penetration of antimicrobial into the biofilm matrix.[118] The long alkyl chain in C8-50 has a similar effect.

**Figure 4.9.** Repeat units for 1-C13\textsubscript{4}\textsubscript{MER}, H-Ntridec-Nlys-Nspe-Nspe-Nlys-NH\textsubscript{2}\[118]

In another study, the molecular polycation ceragenin CSA-13 was tested for *P. aeruginosa* (PA01) biofilm viability and showed complete eradication at 32 µg/ml (7 log reduction, ~13xMIC, MIC 2.5 µg/ml). CSA-13 had comparable concentration effectiveness to ciprofloxacin against gram negative bacterial biofilms tested (7 log reduction, reported ciprofloxacin MIC much lower at 0.15 µg/ml; translates to 213×MIC).[103] C8-50 showed >99.99% kill (4.5 log reduction) at 10x MIC and complete eradication (100% kill, 6.8 log reduction) at 1000×MIC. The testing procedure for CSA-13 included growing biofilms using TSB (trypic soy broth) in a bioreactor where the procedure for C8-50 used M9 mannitol in polystyrene test tubes. Initial *P. aeruginosa* biofilm challenge for CSA-13 was ~10^8 cfu/ml compared to ~ 10^6 cfu/ml for C8-50. Antimicrobial solutions remained static for 72 hrs for the ceragenins before final cell counts compared to 24 hrs for C8-50.[103]
The molecular, primary polyamine CSA-13[38] shares amphiphilic characteristics with C8-50. Polycation CSA-13 has a rather rigid structure that favors facile conjugation with negatively charged species on a bacterial membrane or in the biofilm EPS. Both structures have long alkyl chains that favor EPS matrix and cell penetration which further aids killing inside the biofilm. MIC for CSA-13 (2.5 µg/ml) is comparatively lower than C8-50 MIC (24µg/ml). Unlike C8-50 with a flexible oxetane backbone, it is apparent that, for CSA-13, unfavorable conformational changes are minimized while multiple cation-anion interactions are maximized. This also translates to higher log reductions for CSA-13 compared to C8-50.
Table 4.2. Log reductions for *P. aeruginosa* biofilm viability studies of polycations, peptoids, and conventional antibiotics.

<table>
<thead>
<tr>
<th>Antimic</th>
<th>MIC (µg/ml)</th>
<th>MIC Multiplier</th>
<th>Challenge (cfu/ml)</th>
<th>Log Reductions</th>
</tr>
</thead>
<tbody>
<tr>
<td>C8-50</td>
<td>24</td>
<td>1</td>
<td>$10^6$</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>$10^6$</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>$10^6$</td>
<td>4.5</td>
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<td></td>
<td></td>
<td>1000</td>
<td>$10^6$</td>
<td>6.8</td>
</tr>
<tr>
<td>Peptoid-1[118]</td>
<td>22.7</td>
<td>1</td>
<td>$10^{10}$</td>
<td>1</td>
</tr>
<tr>
<td>1-C13_{4MER}[118]</td>
<td>10.4-20.8</td>
<td>1</td>
<td>$10^{10}$</td>
<td>2</td>
</tr>
<tr>
<td>Ciprofloxacin[118]</td>
<td>0.1</td>
<td>31</td>
<td>$10^{10}$</td>
<td>2</td>
</tr>
<tr>
<td>Tobramycin[118]</td>
<td>0.7</td>
<td>8</td>
<td>$10^{10}$</td>
<td>1</td>
</tr>
<tr>
<td>CSA-13[103]</td>
<td>2.5</td>
<td>~13</td>
<td>$10^8$</td>
<td>~7</td>
</tr>
<tr>
<td>Ciprofloxacin[103]</td>
<td>0.15</td>
<td>213</td>
<td>$10^8$</td>
<td>~7</td>
</tr>
</tbody>
</table>

**Conclusion**

CV staining assays demonstrate that C8-50 has no measurable effect on adhesion of already established *P. aeruginosa* biofilms, but can prevent biofilm formation by killing planktonic cells. For anti-adhesion assays, there is a measurable reduction in biofilm mass at concentrations greater than ~2× MIC which shows that C8-50 can be used as a biofilm preventative at values around MBC. MIC values only apply to free floating planktonic environments and, it is apparent, cannot be easily translated to sessile communities. Viability studies show a substantial log reduction of 2.1 at MIC which is similar to the result for C13_{4MER}. Both antimicrobials possess long cationic tails that can easily penetrate into the EPS matrix and further facilitate killing inside the biofilm matrix.
Chapter 5
Conclusion and Further Exploration

Water soluble $P[(Cx-m)(ME2O_x)]$ copolyoxetanes have been studied with regard to antimicrobial effectiveness. These compositions are designated Cx-m, where $x$ is the number of carbons in the quaternary group on the Cx side chain and $m$ is the mole percent quaternary segment. The remaining mole percent is a segment containing a PEG-like side chain (Chapter 3, 1). Chapters 1 and 2 explored solution antimicrobial effectiveness and human cell cytotoxicity of Cx-m copolyoxetanes as a function of amphiphilic balance, that is, the segment ratio of C12 to PEG. The length of the quaternary pendant group, dodecyltrimethylammonium butoxymethyl ($x = 12$) was kept constant while charge density ($m$) was altered. MIC decreased with increasing C12 mole percent, reaching a minimum in the range C12-43 to C12-60. Overall, C12-43 had consistently low MICs for the three tested pathogenic bacteria: (bacteria, MIC, $\mu$g/ml) $E. coli$ (6), $S. aureus$ (5) and $P. aeruginosa$ (33). At $5 \times$ MIC against a challenge of $10^8$ cfu/ml, C12-43 kills $\geq 99\%$ $S. aureus$, $E. coli$ and $P. aeruginosa$ within 1 hr. C12-m copolyoxetane cytotoxicity toward human red blood cells, human dermal fibroblasts (HDFs), and human foreskin fibroblasts (HFFs) was low indicating good prospects for biocompatibility. The tunability of C12-m copolyoxetane compositions, effective antimicrobial behavior against Gram (+) and Gram (-) bacteria, and promising biocompatibility offered opportunities for further modification.

In view of the sensitivity of biocidal potency to amphiphilic balance, optimization of Cx-m copolyoxetanes was explored in Chapter 3 by varying the alkyl chain length (from $x = 2$ to 16) while keeping the mole ratio constant near the biocidal optimum for C12-m ($m = 50$). A systematic dependence of alkyl chain length and MIC was found. The most lethal antimicrobial
was C8-50 with MIC values of (bacteria, MIC, μg/ml) E. coli (4), S. aureus (2) and P. aeruginosa (24). 4 μg/ml, E. coli, 2 μg/ml, S. aureus and 24 μg/ml, P. aeruginosa. Kill kinetics for C8-50 at 5 × MIC affected > 99% kill in 1 hour for a challenge of 10⁸ cfu/ml. Most notable is a log reduction of 7 for S. aureus at 1 hour. Cell cytotoxicity studies on the three most effective antimicrobials (C6-50, C8-50, C10-50) also yielded favorable results.

Chapter 4 addressed the effect of C8-50, the most potent antimicrobial copolyoxetane, on biomass and viability of P. aeruginosa biofilms. Crystal violet (CV) staining assays demonstrated that C8-50 has no effect on adhesion of already established P. aeruginosa biofilms, but reduced biofilm formation by killing planktonic cells prior to attachment. For anti-adhesion assays, there was a clear reduction in biofilm mass at concentrations greater than the minimum biocidal concentration (MBC, ~2× MIC). Viability studies show a substantial log reduction of 2.1 at MIC.

P. aeruginosa biofilms are not fully eradicated at 10× MIC but there is 100% kill at 1000× MIC. The 4.5 log reduction at 10× MIC suggests that MBC is much closer to the C8-50 10× MIC concentration (240 μg/ml) than the 1000× MIC concentration (24000 μg/ml). The question of the exact MBC concentration remains unanswered and will be the subject of future studies. To broaden the scope of this study, future studies will include new strains (such as PA14) to see if results comparable to P. aeruginosa (PA01) are obtained.

There are a number of additional avenues for future research. Studies to ensure that bacteria do not build up a resistance to Cx-m copolyoxetanes are desirable to confirm prior studies on AMP-like models and other reported work.[84, 126] The logical choice for studies of bacterial resistance is C8-50 due to superior biocidal effectiveness. Common antibiotics and commercially available AMPs, e.g. magainin, would be used for controls.
Exploration of permeation for model membranes would give a better understanding of mechanisms involved in bacterial activity. Dr. Yasuhara of Nara Institute of Science and Technology has shown that lipid membrane disruption can be visualized directly by fluorescent spectroscopy.[127] A recently initiated collaboration with Dr. Yasuhara will provide evidence concerning the question as to whether the kill mechanism is primarily a surface initiated process.

A more complete evaluation of copolyoxetane biological activity is necessary to determine antimicrobial efficacy. In-vitro antibiotic sensitivity testing can be performed using Kirby-Bauer antibiotic testing (KB testing) For KB testing, wafers having surface films of Cx-m copolyoxetanes would be employed.[128, 129] Bacteria of a known concentration are grown on agar plates and exposed to these wafers. As the Cx-m copolyoxetane diffuses from the perimeter of the disk, a zone of inhibition is established where bacteria are killed. Regions where bacteria cannot grow will occur around the wafer. This zone of inhibition is generally inversely proportional to the MIC (i.e. the lower the MIC, the greater the zone of inhibition). Thus, the extent of formation of the zone of inhibition is a measure of biocidal effectiveness. This qualitative test is useful and is expected to be sensitive to the concentration of the Cx-m copolyoxetane film on the disk.

Moving ahead, evaluation of the copolyoxetane as an antimicrobial using in vivo testing is desirable. In vivo animal studies could include testing for skin/sensitivity with a view to topical therapeutic use or even gastrointestinal effects of Cx-m copolyoxetanes on mice populations for possible oral antibiotic applications.

Kuroda’s work[69, 126] has shown that block copolymers have much higher selectivity than random copolymers. It is possible to prepare block copolyoxetanes,[130] but it is not known whether the block copolyoxetane precursors to Cx-m systems can be made. If this is possible,
future work could explore whether the block copolymers would have even higher antimicrobial effectiveness compared to the random copolymer analogs.
REFERENCES


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APPENDICES
Appendix A-1: \(^1\)H-NMR determination of BBOx-m copolymer ratios

BBOx-42 is chosen to illustrate the analysis method in detail. A similar analysis was done for all BBOx-m copolyoxetanes. These ratios are compared with monomer feed ratios in Table 1.2.

Figure A1 shows the \(^1\)H-NMR spectrum of BBOx-42 and P[(C12-43)(ME2Ox)-2.5].

Assignments: ‘a’ = peaks for [-O-CH\(_2\)(CH\(_2\))\(_2\)CH\(_2\)-Br], ‘b’ = peak for [-CH\(_3\) from ME2Ox plus BBOx], ‘c’ = peak for [-CH\(_2\)(CH\(_2\))\(_{10}\)CH\(_3\) for C12] and ‘d’ = peaks for [-CH\(_3\) from ME2Ox plus C12].

All mole percents were determined as described below for BBOx-42. The \(^1\)H-NMR peak at 0.91 ppm (Figure A1, b) corresponds to the 2-methyl group on each repeat unit, while the peaks at 1.68 and 1.92 ppm (a) are the middle methylene groups, -O-CH\(_2\)CH\(_2\)CH\(_2\)-Br, in the BBOx side chain. Considering ME2Ox as ‘x’ and BBOx as ‘y’, Equation (1a) describes the relationship of these peak areas:

\[
\frac{\text{Area}_{0.91\text{ppm}}}{\text{Area}_{1.68-1.92\text{ppm}}} = \frac{3x + 3y}{4y} \quad \text{Equation (1a)}
\]

Where \(\text{Area}_{1.68-1.92\text{ppm}}\) = area of the peaks between 1.68 and 1.92 ppm, and \(\text{Area}_{0.91\text{ppm}}\) = area of the peak at 0.91 ppm. From Equation (1a) we obtain:
\[
\frac{x}{y} = \frac{4\text{Area}_{0.91\text{ppm}} - 3\text{Area}_{1.68-1.92\text{ppm}}}{3\text{Area}_{1.68-1.92\text{ppm}}} \quad \text{Equation (1b)}
\]

From Equation (1b), a value for \(x:y\) is obtained. Using Equation (1c), the value of ‘1-\(m\)’ is calculated and likewise the value of \(m\) is obtained.

\[
1 - m = \frac{x}{x + y} = \frac{4\text{Area}_{0.91\text{ppm}} - 3\text{Area}_{1.68-1.92\text{ppm}}}{4\text{Area}_{0.91\text{ppm}}} \quad \text{Equation (1c)}
\]

The integrals for the peaks at 0.91 ppm [-\(\text{CH}_3\) from ME2Ox plus BBOx] and 1.68 and 1.92 ppm [-\(\text{O-CH}_2(\text{CH}_2)_2\text{CH}_2\)-Br] are, respectively, 12.52 (= 3\(x\)+3\(y\)) and 7.04 (=4\(y\)). From Equation (1b), the value for \(x:y\) is 1.37. Similarly, from Equation (1c), the value of ‘1-\(m\)’ is 0.58 and ‘\(m\)’ is 0.42. The composition of the copolyoxetane thus obtained is (BBOx-42). The ratio of (BBOx)/(ME2Ox) determined by \(^1\text{H-NMR}\) (1:1.4) compared to the feed ratio of 1:2 indicates that BBOx is incorporated preferentially into the copolyoxetane.
Figure A1. $^1$H-NMR of A, BBOx-42 and B, C12-43, for the above chemical structures, alphabetical designations refer to hydrogen protons exclusively.
Appendix A-2. Copolymer ratios for C12-m copolyoxetanes.

C12-43 is chosen to describe the method of determining C12 mole percent in detail. A similar analysis was done for all C12-m copolyoxetanes to provide redundancy in the determination of mole% C12. The agreement with independently determined mole percent BBOx and C12 provides confirmation of complete substitution. The copolymer ratios determined for BBOx-m (Table 1.2) and C12-m (Table 1.3) are combined in a Table A3 below for facile comparison.

The method to determine substitution for C12-100 is described in this section. 

$^1$H-NMR spectroscopy was used to establish complete substitution of C-Br by C12 amine for every C12-m composition. This was ascertained by the absence of the BBOx peak at 1.9 ppm in every C12-m. As an example, Figure A1.1 provides the $^1$H-NMR spectrum for BBOx-42 and C12-43. The absence of the 1.9 ppm BBOx peak in the spectrum of C12-43 is evident. Also, the prominent peak “c” between 1.2 and 1.4 ppm due to $(\text{CH}_2)_{10}$ appears in the spectrum of C12-43 (and every C12-m copolyoxetane). This peak is absent in the $^1$H-NMR spectrum of the BBOx precursor.

Details for the determination of the ME2Ox/C12 ratio for C12-m copolyoxetanes by $^1$H-NMR are now provided. The $^1$H-NMR peak at 0.82 ppm corresponds to -CH$_3$ in the 2-position of each repeat unit plus the -CH$_3$ group at the end of the quaternary alkyl chain. The peaks “c” between 1.2 and 1.4 ppm are unique to the C12 quaternary side chain. These peaks are due to the following (bolded) protons: -CH$_2$(CH$_2$)$_{10}$CH$_3$. Considering ME2Ox as ‘x’ and C12 as ‘y’, Equation (2a) describes the relationship of these peak areas:
\[
\frac{\text{Area}_{0.82\text{ppm}}}{\text{Area}_{1.2-1.4\text{ppm}}} = \frac{3x + 6y}{20y}
\]
Equation (2a)

Where \(\text{Area}_{1.2-1.4\text{ppm}}\) = area of the peaks between 1.2 and 1.4 ppm, and \(\text{Area}_{0.82\text{ppm}}\) = area of the peak at 0.82 ppm. From Equation (2a) we obtain:

\[
\frac{x}{y} = \frac{20\text{Area}_{0.82\text{ppm}} - 6\text{Area}_{1.2-1.4\text{ppm}}}{3\text{Area}_{1.2-1.4\text{ppm}}}
\]
Equation (2b)

From Equation (2b), a value for \(x:y\) is obtained. Using Equation (2c), we calculate ‘1-m’ and obtain \(m\).

\[
1 - m = \frac{x}{x + y} = \frac{20\text{Area}_{0.82\text{ppm}} - 6\text{Area}_{1.2-1.4\text{ppm}}}{20\text{Area}_{0.82\text{ppm}} - 3\text{Area}_{1.2-1.4\text{ppm}}}
\]
Equation (2c)

The integrals for the peaks at 0.82 ppm [-CH\(_3\) from ME2Ox plus C12] and between 1.2 to 1.4 ppm [-CH\(_2\)(CH\(_2\))\(_{10}\)CH\(_3\) for C12] are, respectively, 12.9 (= 3x+6y) and 25.96 (=20y). From Equation 2b, the value of \(x:y\) is 1.32. Similarly, from Equation 2c, the value of ‘1-m’ is 0.57 and ‘m’ is 0.43. The composition of the copolyoxetane thus obtained is C12-43.

C12-100 does not have ME2Ox repeats. Putting \(x = 0\), the R.H.S of Equation (2a) becomes \(6y/20y = 0.3\), which gives us the theoretical ratio of the peak areas. The actual ratio of peak areas \((\text{Area}_{0.82\text{ppm}}/\text{Area}_{1.2-1.4\text{ppm}} = 11.07/35.7 = 0.31)\) from \(^1\)H-NMR is given by the L.H.S of Equation 2a. The actual ratio of the hydrogen atoms obtained from \(^1\)H-NMR thus complies with the calculated ratio and hence the composition C12-100 is reconfirmed.
Appendix A-3. Calculation for determination of MIC per quarternary charge repeat unit

For C12-14;

\[ M_n = 7870 \text{ g/mol}, \text{MIC (}E.\text{ coli)} = 0.3 \text{ mg/ml} \]

The MIC is converted to µmol/ml.

\[ \text{MIC} = (0.3 \text{ mg/ml})(\text{mol/7870 g})(g/1000 \text{ mg})(10^6 \text{ µmol/mol}) = 0.0381 \text{ µmol/ml} \]

C12-14 contains 14% quaternary charge bearing moiety.

MIC per quaternary charge repeat unit = 0.0381 µmol/ml (0.14) = 0.0053 µmol alkylammonium/ml.
Appendix A-4. FTIR spectrum (% transmittance) for (A) BBOx-42 and (B) C12-43 in acetonitrile.

Figure A2. FTIR spectrum (% transmittance) for (A) BBOx-42 and (B) C12-43 in acetonitrile.
Appendix A-5. Luria broth solutions with (A) bacterial growth (turbid) and (B) no bacterial growth (clear).

Figure A3. Luria broth solutions with (A) bacterial growth (turbid) and (B) no bacterial growth (clear).
Appendix A-6. Media formulation for *P. aeruginosa* and *E. coli* cultures.

Table A1. Media formulation for *P. aeruginosa* and *E. coli* cultures.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>30</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>15</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>5</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>0.015</td>
</tr>
</tbody>
</table>

The above salt solution is diluted 5 times to prepare the final growth media.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume in ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X M9 salt solution</td>
<td>20</td>
</tr>
<tr>
<td>Sterilized water</td>
<td>80</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O (1M)</td>
<td>0.1</td>
</tr>
<tr>
<td>Mannitol (20 wt%)</td>
<td>1</td>
</tr>
<tr>
<td>Luria Broth</td>
<td>1</td>
</tr>
</tbody>
</table>
Appendix A-7. Tryptic Soy Broth (TSB) medium formulation for *S. aureus* cultures.

**Table A2.** Tryptic Soy Broth (TSB) medium formulation for *S. aureus* cultures.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>20</td>
</tr>
<tr>
<td>Dextrose</td>
<td>27.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
</tbody>
</table>
Appendix A-8. A comparison of mole ratios from $^1$H-NMR spectroscopy for BBOx-m (Table 1.2) and C12-m copolyoxetanes (Table 1.3).

**Table A3.** A comparison of mole ratios from $^1$H-NMR spectroscopy for BBOx-m (Table 1.2) and C12-m copolyoxetanes (Table 1.3).

<table>
<thead>
<tr>
<th>BBOx/ME2Ox mole ratio</th>
<th>C12/ME2Ox mole ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:86</td>
<td>14/86</td>
</tr>
<tr>
<td>25:75</td>
<td>26/74</td>
</tr>
<tr>
<td>42:58</td>
<td>43/57</td>
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<td>50:50</td>
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<td>87:13</td>
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<td>100:0</td>
<td>100:0</td>
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</tbody>
</table>

Appendix B-1. $^1$H-NMR analysis for P[(BBOx-50)(ME2Ox)] copolyoxetane.

The $^1$H-NMR peak at 0.91 ppm corresponds to the -CH$_3$ side chains on each repeat unit, while the peaks at 1.68 and 1.92 ppm are the middle methylene groups, -O-CH$_2$CH$_2$CH$_2$CH$_2$-Br, in the BBOx side chain. Considering ME2Ox as ‘x’ and BBOx as ‘y’, Equation 1a describes the relationship of these peak areas:

$$\frac{\text{Area}_{0.91\text{ppm}}}{\text{Area}_{1.68,1.92\text{ppm}}} = \frac{3x + 3y}{4y}$$

Equation (1a)

Where $\text{Area}_{1.68,1.92\text{ppm}}$ = area of the peaks between 1.68 and 1.92 ppm, and $\text{Area}_{0.91\text{ppm}}$ = area of the peak at 0.91 ppm. From Equation 1a we obtain:

$$\frac{x}{y} = \frac{4\text{Area}_{0.91\text{ppm}} - 3\text{Area}_{1.68,1.92\text{ppm}}}{3\text{Area}_{1.68,1.92\text{ppm}}}$$

Equation (1b)

From Equation 1b, a value for $x:y$ is obtained. Using Equation 1c, the value of ‘1-m’ is calculated and likewise the value of m is obtained.

$$1 - m = \frac{x}{x + y} = \frac{4\text{Area}_{0.91\text{ppm}} - 3\text{Area}_{1.68,1.92\text{ppm}}}{4\text{Area}_{0.91\text{ppm}}}$$

Equation (1c)

The integrals for the peaks at 0.91 ppm [-CH$_3$ from ME2Ox plus BBOx] and 1.68 and 1.92 ppm [-O-CH$_2$(CH$_2$)$_2$CH$_2$-Br] are, respectively, 15.65 (= 3x+3y) and 10.45 (=4y). From Equation 1b, the value for $x:y$ is 0.996. Similarly, from Equation 1c, the value of ‘1-m’ is 0.498 and ‘m’ is 0.501. The composition of the copolyoxetane thus obtained is P[(BBOx-50)(ME2Ox)]. The ratio of (BBOx)/(ME2Ox) determined by $^1$H-NMR (1:1) compared to the feed ratio of 1:1.22 indicates that BBOx is incorporated preferentially into the copolyoxetane.
Appendix B-2. ¹H-NMR analysis for Cx-50 copolyoxetanes.

¹H-NMR spectroscopy was used to establish that substitution of C-Br by the amine was complete. This was ascertained by the absence of the characteristic BBOx peak at 2 ppm. The Cx/ME2Ox ratio for P[(C12-m)(ME2Ox)] copolyoxetanes was also determined by ¹H-NMR. An example of ¹H-NMR analysis of C8-50 copolyoxetane is as follows: The ¹H-NMR peak at 0.91 ppm corresponds to -CH₃ side chains on the 2-position of each repeat unit plus the -CH₃ group at the end of the quaternary alkyl chain. The peaks between 1.2 and 1.4 ppm are unique to the C8 quaternary side chain. These peaks are due to the following (bolded) protons: -CH₂(CH₂)₆CH₃.

Considering ME2Ox as ‘x’ and C8 as ‘y’, Equation 2a describes the relationship of these peak areas:

\[
\frac{Area_{0.91\, pm}}{Area_{1.2,1.4\, ppm}} = \frac{3x + 6y}{12y} \tag{2a}
\]

Where \(Area_{1.2,1.4\, ppm}\) = area of the peaks between 1.2 and 1.4 ppm, and \(Area_{0.91\, ppm}\) = area of the peak at 0.91 ppm. From Equation (2a) we obtain:

\[
\frac{x}{y} = \frac{12Area_{0.91\, ppm} - 6Area_{1.2,1.4\, ppm}}{3Area_{1.2,1.4\, ppm}} \tag{2b}
\]

From Equation (2b), a value for x:y is obtained. Using Equation 3, we calculate ‘m’ and obtain 1-m.

\[
1 - m = \frac{x}{x + y} = \frac{12Area_{0.91\, ppm} - 6Area_{1.2,1.4\, ppm}}{12Area_{0.91\, ppm} - 3Area_{1.2,1.4\, ppm}} \tag{2c}
\]
Figure 3.1 shows the $^1$H-NMR spectrum of P[(BBOx)(ME2Ox)-50:50] and the C8-50. Here, ‘a’ = peaks for [-O-CH$_2$(CH$_2$)$_2$CH$_2$-Br], ‘b’ = peak for [-CH$_3$ from ME2Ox plus BBOx], ‘c’ = peak for [-CH$_2$(CH$_2$)$_{10}$CH$_3$ for C12] and ‘d’ = peaks for [-CH$_3$ from ME2Ox plus C12].

The integrals for the peaks at 0.91 ppm [-CH$_3$ from ME2Ox plus C8] and between 1.2 to 1.4 ppm [-CH$_2$(CH$_2$)$_6$CH$_3$ for C8] are, respectively, 13.08 (= 3x+6y) and 17.42 (=12y). From Equation 2b, the value of x/y is 1.00. Similarly, from Equation (2c), the value of ‘1-m’ is 0.5 and m is 0.5. The composition of the copolyoxetane thus obtained is C8-50. The $^1$H-NMR analyses for the other substituted copolyoxetanes are also done and are listed in Table 1.2.
Appendix B-3. FTIR spectra (% transmittance) for (A) P[(ME2Ox)(BBOx)-50:50-5.7] and (B) C8-50 in acetonitrile.

Figure B1. FTIR spectra (% transmittance) for (A) P[(ME2Ox)(BBOx)-50:50-5.7] and (B) C8-50 in acetonitrile.

The C-Br peak appears at 650 cm$^{-1}$ for the precursor while no absorption peak is observed for the C8-50 copolyoxetane in the 515 – 690 cm$^{-1}$ region, as expected for complete quaternization.
Appendix B-4. Comparing the log reductions of some common polycations against *S. aureus*.

**Table B1.** Comparing the log reductions of some common polycations against *S. aureus*.

<table>
<thead>
<tr>
<th>Antimic</th>
<th>MIC (MIC multiplier) (µg/ml)</th>
<th>Challenge (cfu/ml)</th>
<th>Log reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 hr</td>
</tr>
<tr>
<td>C8-50</td>
<td>2 (5)</td>
<td>10^7</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>100-330 (0.3-0.09)</td>
<td>3.2 x 10^8</td>
<td>3.1</td>
</tr>
<tr>
<td>CSA-13, [1]</td>
<td>1 (10)</td>
<td>3.2 x 10^8</td>
<td>0.9</td>
</tr>
</tbody>
</table>
VITA

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