THE INVESTIGATION OF WATER-SOLUBLE POLYURETHANES THAT MIMIC ANTIMICROBIAL PEPTIDES

A Dissertation

Presented to

The Graduate Faculty of the University of Akron

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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May 2018

THE INVESTIGATION OF WATER-SOLUBLE

POLYURETHANES THAT MIMIC ANTIMICROBIAL PEPTIDES

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ABSTRACT

The emergence and increasing incidence of drug resistant strains of bacteria is a serious threat to human health. There is an urgent need for the development of new antimicrobial agents to combat bacterial infections that are different from the currently used small molecule drugs. Antimicrobial peptides, a broad family of peptides that are utilized by virtually every organism, are a promising source for new antimicrobial agents to fight pathogenic microorganisms. However, there are many limitations to the widespread use of these peptides, such as the cost of mass production and their rapid degradation *in vivo* by endogenous proteases. One viable way to overcome these limitations is through using synthetic mimics of these peptides. This work details the design of a new family of antimicrobial, water-soluble polyurethanes that were synthesized to mimic antimicrobial peptides using easily functionalized diols and the subsequent study of their *in vitro* antimicrobial properties. Studies were performed to determine the effects that a broad range of hydrophobic, uncharged polar, and charged polar pendant groups as well as the effects of molecular weight on the structure/property relationships of these antimicrobial polymers and how they impact antimicrobial efficacy, mammalian cell cytocompatibility, and mechanisms of action.

DEDICATION

I would like to dedicate this dissertation to my parents, brothers, and amazing wife. Thank you for all your support and always choosing to put up with me.

ACKNOWLEDGEMENTS

None of this work would have been possible without the amazing support of my mentors Prof. Abraham Joy, Prof. Nita Sahai, and Prof. Hazel Barton. All of your guidance, support, and time has helped me achieve goal after goal and has helped set the course for my future career successes. I am also very grateful for all the support and assistance I have obtained from all my friends and colleagues (past and present) within the Joy, Sahai, and Barton research groups, most especially Dr. Ying Xu; Dr. John Swanson; and Kaushik Mishra for always providing sound synthetic guidance, Dr. Xianfeng Zhou for teaching me sterile technique and mammalian cell culture, Ceth Parker and Olivia Hershey for guidance in the culture of bacteria, Dr. Punam Dalai for imparting her knowledge of liposomes, and Dr. Anna Chamsaz; Chao Peng; Apoorva Vishwakarma; Chin Tantisuwanno; and Nick Nun for constructive discussions about research. Also, beyond the research groups within which I primarily operated, I am extremely grateful for assistance I obtained from various other individuals around campus. Thank you to Prof. Francisco Moore for allowing me to use the plate reader within his lab, Dan Morris for assistance in the operation of the 750 MHz NMR, Dr. Jacob Hill for always providing useful insight, and Dr. Zhao Mankoci for unwavering understanding, support, and patience as well as assistance in statistical analysis.

Lastly, this journey through scientific discovery would not have been possible without all the support I have received from all of my family and all of my friends.

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CHAPTER I

NATURAL AND SYNTHETIC CATIONIC WATER-SOLUBLE ANTIMICROBIAL POLYMERS: A LITERATURE REVIEW OF THESE ALTERNATIVES TO CONVENTIONAL ANTIBIOTICS

1.1 The Need for New Antimicrobials

The world is on the verge of a global health crisis due to the rise of antimicrobial resistance among pathogenic bacteria. In recent years, there have been many reports of outbreaks and individual incidents of bacterial infections that were impossible or very difficult to treat because current antimicrobials were unable to control the infection.[1-4] Within the USA alone, there have been multiple high-profile cases within news reports of patients who were unable to be treated, or difficult to treat, due to resistance to most or all available antimicrobials.[5, 6] In developing countries, the population is disproportionately impacted by antimicrobial resistant infections because of a lack of access to all possible treatment options, while poor hygiene allows resistant organisms to spread more easily. It is estimated that about 700,000 people annually across the world die as a result of infections caused by antibiotic resistant microorganisms in today's world.[7] This number is expected to rise to a staggering 10 million deaths annually by 2050, becoming the greatest single cause for morbidity worldwide if no drastic actions to address this problem are taken.[7]

There are multiple reasons for the increased prevalence of antimicrobial resistance in pathogenic bacteria. Perhaps, the greatest reason for this rise in resistance is just simply the nature of bacteria themselves; they are rather adept at adapting to better suit their environment and have evolved various mechanisms of antimicrobial resistance. Since most clinical antimicrobials are either natural products or derivatives of natural products synthesized by various bacteria and fungi to combat competing microbes, many mechanisms of antimicrobial resistance have been documented in environments that can serve as a reservoir for resistance genes to accumulate, such as soil,[8, 9] and within environments where competition for resources is particularly fierce, such as caves.[10] It is only recently that the length of time that resistance mechanisms to antimicrobials have been in existence has been investigated.[11] While antimicrobials have only been used clinically for less than one hundred years, it has become apparent that bacteria have been producing antimicrobials for millions (or potentially billions) of years.[12]

Due to this long evolutionary history, bacteria have developed many different, sophisticated mechanisms of resistance to antimicrobials. These mechanisms of resistance may be inherent to the bacterial species, especially if it provides an advantage to the fitness of the organism, may be generated through mutation and adaptation, or could be obtained through the acquisition of resistance genes. No matter how the resistance has arose, there are various common mechanisms that bacteria can adopt as a strategy to attenuate the effects of antimicrobials.[13]

One mechanism of resistance is the synthesis of an enzyme that either modifies or inactivates the antimicrobial, such as ß-lactamase. To date, there have been over 800 different ß-lactamase variants identified that work to destroy the ß-lactam ring structure,

which is critical to the function of penicillin and all of its derivatives.[14] Historically, to overcome the action of ß-lactamase, different chemical variants based on penicillin that maintain the same ß-lactam chemical structure have been developed. However, this strategy has been largely unsuccessful because bacteria have been able to adapt and develop resistance to these new antimicrobials rather quickly and it has been shown that it is typically a matter of months after a new ß-lactam antimicrobial is introduced to clinics that a strain of bacteria that is resistant to the new antimicrobial is identified.

Another common mechanism of resistance to antimicrobials is through modification of the permeability of molecules through the cytoplasmic membrane and the outer membrane, in the case Gram negative bacteria. For many small molecule antimicrobials to function, they must somehow gain entrance into the bacterial cell to reach their specific target site. For example, quinolones and fluoroquinolones must enter the cell to inhibit the action of topoisomerases, which are enzymes that are important in controlling the coiling of DNA during replication and transcription, and aminoglycosides and tetracyclines function by inhibiting protein synthesis. To counter the action of these antimicrobials, bacteria have either changed the permeability of antimicrobials through the membrane by modifying the pore proteins in the membrane or by the use of efflux pumps, which expel the antimicrobial outside of the cell before it reaches toxic levels.

Bacteria also resist the action of antimicrobials by modifying target sites. Since many antimicrobials function by affecting very specific target sites, such as a catalytic site of an enzyme vital for metabolism, bacteria can either protect this target site using special proteins or through modification of the target sites. An example of target site protection originally identified among *Streptococcus spp.* involves the use of a protein known as

TetM, which functions to remove tetracycline from its binding site within the ribosome. TetM is able to achieve this by changing the conformation of the target site within the ribosome to which tetracycline binds, dislodging tetracycline from the target site while simultaneously allowing the ternary complex that contains the aminoacyl-tRNA, the complex that carries the amino acid to the active site of the ribosome, to come in and bind and allow for protein synthesis.[15]

The ability to battle antimicrobial resistance is further complicated by the genetic plasticity of bacteria, which allows them to rapidly adapt to any stress imposed by their environment. It is this ability of bacteria to modify and change their genetic information and its expression rather quickly that allows them to adapt to and resist both natural and synthetic antimicrobials. At the genetic level, bacteria adapt to the action of antimicrobials either by 1.) mutation to the gene(s) that code(s) the target site of the antimicrobial or 2.) by acquiring foreign DNA that contains a gene that promotes resistance via horizontal gene transfer.[13] Mutations that occur within populations of bacteria that promote resistance to an antimicrobial can spread rather rapidly because of the exponential growth patterns that most bacteria exhibit under ideal growth conditions. For example, the doubling time of *Escherichia coli* grown using nutrient rich medium at 37 º C is about 40 min.[16] This means that if one cell is grown under these conditions for 24 hr., it could give rise to a population of over 10^7 progenies. Also, with the ability to multiply so rapidly, mutations within the population that arise naturally and through selective pressures can accumulate in a rather rapid timeframe. The rate of mutation and development of resistance within the population that results in the phenotypic expression of antibiotic resistance is dependent on a wide variety of factors, which includes the number of genes and the number of mutations needed

to occur within each gene involved with imparting resistance, the availability of nutrients and/or the presence of the antimicrobial in the surrounding environment, and the effect that the mutation has on the fitness of the organism.[17]

The ability of bacteria to perform horizonal gene transfer (HGT) imparts an additional advantage in combatting the action of antimicrobials. This allows one bacterium that contains a gene that promotes resistance for an antimicrobial, such as for ß-lactamase, to be transferred to another bacterium. HGT across a population of bacteria can be accomplished through multiple different mechanisms.[18] The simplest form of HGT is called transformation, where foreign DNA is taken up by the cell. In order to transform a cell, the cell must enter a special physiological state called competence, which may be induced by environmental conditions such as starvation or high cell density. While a cell is competent, it produces a variety of proteins that assist in the transport of DNA across the membrane(s). However, the role of transformation in the transfer of antimicrobial resistance genes is thought to be rather limited in nature because of the specific requirements needed to establish competence.[13] Transduction, another means of HGT, plays a greater role in the spread of antimicrobial resistance. This process involves the transfer of DNA from one bacterium to another via bacteriophages. When a new bacteriophage virion is formed, bacterial DNA can be incorporated into the phage particle along with the viral genetic information. As a result, when the bacteriophage infects another bacterium, it inserts not only its own nucleic acid but also the bacterial DNA. Another mode of HGT is known as conjugation, which is a process that involves direct contact between two bacteria. During conjugation, a bridge-like connection is made

between the two cells and genetic information from one cell is replicated and transferred to the recipient cell.

Bacteria are well equipped to combat the action of antimicrobials and are quite capable of rapidly adjusting to their environment. However, this is only part of the reason for the huge threat that antimicrobial resistance poses to humanity. This crisis is very much the result of the misuse and overuse of clinically prescribed antimicrobials. According to the Centers for Disease Control and Prevention (CDC), up to 50% of prescribed antimicrobials are either unnecessary or are inappropriate for treatment.[19] For any infection, it is essential for the medical practitioner to implement a course of treatment in a timely manner. In doing so, decisions for the best course of treatment must also be made quickly, which may lead to the prescription of incorrect dosages and/or the incorrect antimicrobial for the type of infection. Responsibility for misuse of antimicrobials may also be attributed to the actions of the patient. When prescribed an antimicrobial, it is essential that the patient continue treatment for the entirety of the prescribed period of treatment. If during the course of treatment, the patient does not use the proper dosage, maintain the duration of treatment, or the incorrect antimicrobial is selected, resistance can develop due to exposure of the pathogenic bacteria to sub-lethal levels of the antimicrobial.[19]

In addition to promoting the development of resistance, the improper use of the antimicrobial may result in resistance among bacteria through a process known as the bystander effect.[20] When an antimicrobial is used to treat an infection, there is no way to control which bacteria are affected. So, antimicrobials target not only the desired pathogenic bacteria within the infection but also may affect many other “bystander”

bacteria that may be naturally occurring or even beneficial to certain parts of the body, *e.g.* the bacteria found within the digestive tract. When these bystander bacteria are killed by the antimicrobial, other bacteria that are unaffected can move into the space that was once occupied, grow, and outcompete other species. When these new bacteria take over, even if they are not originally pathogenic, they can have detrimental effects on the patient who received treatment because of a shift in the natural population of bacteria. *Clostriudium difficile* infections are often times an example of the bystander effect.[19] After a prolonged or a rather intensive antimicrobial course of treatment, many of the over 2,000 different species of bacteria that inhabit the gut can be detrimentally affected. *C. difficile*, which is naturally resistant to many types of antibiotics, can grow rapidly under these conditions. *C. difficile* is an opportunistic pathogen and the toxins produced by this bacterium can irritate and inflame the intestinal tract, causing potentially fatal complications, yet is difficult to treat because of its inherent antimicrobial resistance.

However, the overuse of antimicrobials is not limited in the treatment of human health. Historically, the majority of all antimicrobials are used by the agriculture industry. Since food animals are typically kept together in rather high density, there is a great need to aggressively control the spread of any pathogen within the population. So, antimicrobials are not only utilized to control the spread of infectious disease but also to prevent infections and to promote enhanced growth of the animals.[21] Even though it is difficult to directly link the excessive use of antimicrobials in to contributing to the rise of resistance among pathogens relevant to human health through a single scientific investigation, a body of work that has accumulated clearly indicates the overuse of antimicrobials among livestock is negatively affecting human health.[22, 23] From the accumulated work, there is strong

evidence that bacteria in contact with livestock treated with copious amounts of antimicrobials can result in the development of bacteria that exhibit resistance to antimicrobials relevant to human health, especially when they are treated with those relevant antimicrobials. There are many ways in which humans can encounter these resistant bacteria, including through direct handling and consumption of the produced animal products and through contact with water contaminated with manure runoff. Even though the overuse of antimicrobials among livestock has been identified to pose a significant threat to human health since the late 1960's, it is only in recent years that actions have been taken by world governments to curb the use of antimicrobials in agriculture, especially in the USA.[24] On January 1, 2017, it became illegal to use antimicrobials important to human health in livestock in the USA.[22] Yet, there are still some antimicrobials that are used in raising livestock in the USA and it is predicted to continue for the foreseeable future due to industry dependence lack of available alternatives.

To further compound the effects of the rise of antimicrobial resistance, there has very little action being taken by pharmaceutical companies in recent years to develop and bring to market new antimicrobial drugs.[25, 26] Consequently, there are fewer and fewer antimicrobial drugs being approved for widespread clinical use every year. During the 1980's, there were 30 new antimicrobials approved for use but, between 2005 and 2014, there were less than ten new antimicrobials approved.[27] Also, to make matters even worst, there has been a significant drop off in the development of new antimicrobials that have novel mechanisms of action against bacteria. The reason for such a lack of interest in developing new antimicrobials is due to the economics of new antimicrobial drug development. Among pharmaceutical companies, antimicrobial drug development is not

as profitable as the development of other drugs and includes many more risks.[28] In a recent projection using historical data, it was estimated that it would take 23 years for a new antimicrobial drug developed today to make a net profit. Since bacteria will inevitably develop resistance to any antimicrobial drug created, the amount of profits generated may not be as great in the long term as for a drug that is developed to treat a chronic condition, such as diabetes or heart disease. Additionally, there is increased risk in pursuing antimicrobial drug development because the market of consumers is not very well defined. There are already many antimicrobials that already exist; when the new drug is released, its usage would be rather limited. Also, the process of antimicrobial drug discovery and development is so long and the patterns of antimicrobial resistance among bacteria so unpredictable that it would be difficult to design a project to meet any anticipated futures needs of the market. In recent years there have been various promising attempts by government agencies, nongovernmental organizations, and drug industry leaders to try and jump start the development of much needed novel antimicrobial drugs.[29] Yet, there is still much that needs to be done.

To address the threat of antimicrobial resistance among pathogenic bacteria, the CDC has identified four core actions that are necessary to help combat resistance. These actions are 1.) to prevent the transmission of infections, 2.) track the spread of resistant bacteria, 3.) utilize the antimicrobial tools at our disposal more effectively, and 4.) promote the development of novel antimicrobials and diagnostics.[19] Any of these four actions is a worthy endeavor, however, the action that is perhaps most appealing and suitable for the scientific research community to address is the need for novel antimicrobials and diagnostics. Within the literature, there have been many devices, materials, and chemical

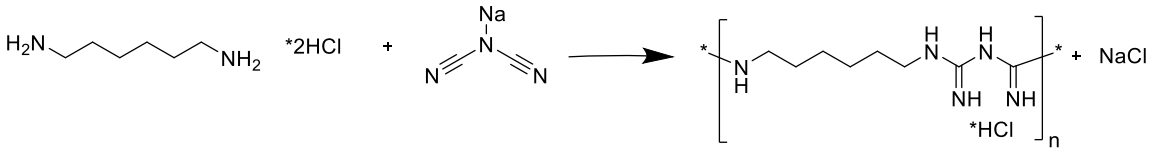
innovations towards addressing antimicrobial resistance. One class of molecules that has been gaining more attention are therapeutic water-soluble natural and synthetic cationic macromolecules that can control bacterial infections. It is clear that to better combat bacterial infections, a new approach must be taken in the types of molecules used as antimicrobials. Cationic antimicrobial macromolecules (CAMs) are different from conventional small molecule antimicrobials in their mode of action. Instead of acting on a specific target site, antimicrobial macromolecules target bacteria in a way that is much broader and less specific, making it more difficult for bacteria to adapt and resist their action. Broadly, most CAMs target the membranes of bacteria and do not require a specific protein target to function. Therefore, the purpose of this chapter is to provide a brief review of what is currently known about CAM's chemistry, function, and mechanisms of action while also providing insight into possible future investigations.

1.2 Biocidal Polymers

There have been many CAMs that have been developed, synthesized, and discovered since the concept of macromolecules and polymers were established in the early 1900's. Broadly, CAMs can be sorted based on the original purposes to which they were applied and the means through which they are synthesized. Within this section, compounds that will be referred to as "biocidal polymers" will be discussed. Historically, this class of CAMs has been known for a long time and includes materials that are completely synthetic in nature. The broad term "biocide" has been selected to describe the action of these polymers because many of them were synthesized with a very simple yet broad objective

of inhibiting the growth or killing bacteria and often times with significantly less regard for the compatibility or survival of other cell types, such as mammalian cells, that may come into contact with the material. So, many of these materials were originally synthesized to be used as disinfectants.

The oldest synthetic material that has been used as a water-soluble CAM is polyhexanide, which is also known as polihexanide and poly(hexamethylene biguanide). It is a synthetic polymer that contains biguanide groups along its backbone that are linked together via a six-carbon hydrocarbon chain. Polyhexanide was originally synthesized and investigated in the early 1950s by two researchers, Rose and Swain, who worked for Imperial Chemical Industries (ICI).[30] The objective of the original research was to try to synthesize novel antimalaria therapeutics since other biguanide containing molecules were found to be active against *Plasmodium spp*, the parasite that causes malaria. Even though polyhexanide is not very effective in the treatment of malaria, it was soon discovered that polyhexanide is rather effective against bacteria. In the original work, it was noted that in order for polyhexanide to function as an antimicrobial, it is essential for the oligomer/polymer to contain at least two biguanide groups and for the hydrocarbon chain between the biguanide groups to contain either 5, 6, or 7 carbons.[30]



Scheme 1.1: A reaction scheme for the synthesis of polyhexanide.

Traditionally, polyhexanide has been produced by the step growth polymerization of hexamethylenediamine and sodium dicyanamide, as shown in Scheme 1.1, to yield number average molecular weights (M_n) in the range of about 1-2 kDa or about 2-12 repeat units.[31-33] Interestingly, resulting polymers (or oligomers) have been shown to have in four different end group functionalities. As expected from the polymerization, polyhexanide has been shown to have a mixture of cyanoguanidine groups and amines. However, it has also been shown that during the synthesis of polyhexanide, there exists a dynamic state of polymerization/depolymerization due to the instability of the biguanide functional group at elevated temperatures.[34] As a result, polyhexanide has also been shown to contain guanidine and cyanoamine terminal groups as well.

Polyhexanide is very soluble (>20 w/v%) in polar solvents like water and alcohols and minimally soluble in less polar solvents.[35] In aqueous solutions, it has been documented that polyhexanide has surfactant-like properties and exhibits a critical micelle concentration of between 20 to 50 mM as determined by surface tension and electrical impedance measurements.[32] Also, the biguanide group is very basic and shows two pKa values for the two imido groups that are present in each repeat unit; pKa_1 = pH 2.0-3.0 and pKa_2 = pH 10.5-11.5.[35] So, at physiological pH, each biguanide group is monoprotonated and carries a charge of 1+ on each repeat unit.

After the initial discovery of polyhexanide in the 1950s, it went on to be used in non-medical consumer products, such as in the preservation of leather, the treatment of pools, a preservative in cosmetics, and in the disinfection of food products.[35] In the 1980s and 1990s polyhexanide regained renewed interest and it was investigated for biomedical applications. It was in the early 1990's that polyhexanide was first used as an antiseptic

during surgical procedures.[35] Since then, there has been various medical products into which polyhexanide has been incorporated rather successfully, such as mouthwashes and wound dressings.[36-38] Polyhexanide has found such widespread application because it's relatively non-toxic, with observed rat oral LD50 values are in the range of ~1-5 g/kg and no apparent ability to cause significant toxicity when exposed chronically in small amounts.[35, 39] Also, polyhexanide has been shown to have a broad spectrum of effectiveness against many different pathogenic bacteria, fungi, and protozoans. Under controlled laboratory conditions, it has been shown that polyhexanide can effectively control the growth (or kill) microbial pathogens at concentrations ranging from 0.1 to 100 µg/mL, depending on the pathogen and the culture conditions.[39] It has additionally been shown to be fast acting and is capable of achieving a 99.999% killing of *E. coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Candida albicans* in less than 5 min when exposed to a concentration of 200 µg/mL.[40]

The primary mechanism through which polyhexanide, and most other CAMs, are thought to affect bacteria is through disruption of bacterial membranes. The membranes of most bacteria are composed of lipid moieties that contain an abundance of anionic charges on the surface presented to the outside environment, especially when compared to mammalian cells. For example, the cytoplasmic membranes of most mammalian cells are composed of phospholipids that have a phosphatidyl choline head group on the side of the bilayer that is presented to the outside environment, which results in a membrane surface that has a net neutral charge, while many bacteria present lipids such as cardiolipin, phospholipids with phosphatidyl glycerol head groups, and, in the case of Gram negative bacteria, lipopolysaccharide, all of which carry a net negative charge across the molecule.

The anionic charge that is present on the surface of the bacteria is what attracts polyhexanide to interact with the membrane. A significant piece of early evidence that polyhexanide disrupts bacterial membranes was provided by Davies *et al*. In their work, they grew *E. coli* spheroblasts, or bacteria that have had their outer membrane and peptidoglycan layers removed, in a medium containing ^{32}P- and ^{13}C- labeled nutrients, then measured the release of the radio-labeled components from the inside of the spheroblasts. The addition of polyhexanide disrupted the membrane over a range of concentrations and, when the concentration is high enough, eventually led to the formation of solid aggregates of biomolecule and polyhexanide.[41] Further investigation with liposomes that contained net neutral and anionic head groups indicated that polyhexanide interacts more selectively towards membranes that contain anionic head groups.[42] This was determined using liposomes that incorporated the dye 1,6-Diphenyl-l,3,5-hexatriene, a dye which exhibits changes in the polarization of it fluorescence with changes in the fluidity of the membrane, and through changes in the phase transition temperature of the lipids measured by differential scanning calorimetry.

However, it has been observed recently that polyhexanide may affect bacteria through a completely different mechanism of action. In contrast to previous experiments that indicate that the primary mechanism of action is through the disruption of bacterial membranes, work performed by Good *et al.* has provided intriguing evidence that the primary mechanism of action is through polyhexanide being taken into the bacterial cell through some method of transmembrane transport and the polymer binding to chromosomes, which causes them to precipitate.[43] The precipitation of chromosomes then leads to an arrest of cell division and the eventual death of the cell. In experiments with the

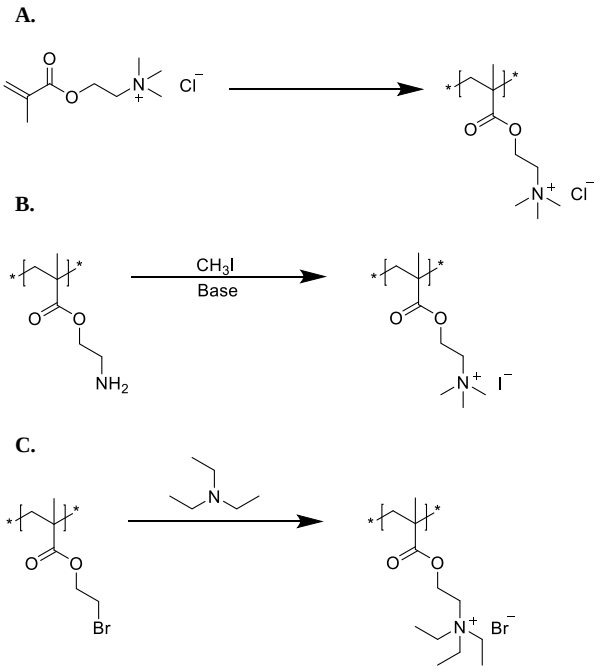
dye SYTOX Green, which is frequently used to determine the integrity of cell membranes, it was shown that the dye is incapable of entering bacteria exposed to polyhexanide, which is in contradiction to various other reports that indicate membrane disruption. Instead, it was observed that polyhexanide that was tagged with FITC accumulated in the cytoplasm of bacterial cells and condensation of the chromosomes was seen microscopically at various concentrations of polyhexanide using bacterial cells that were modified to perform incomplete cell division and stained with DAPI. Experiments performed with various mammalian cell types indicated that polyhexanide was taken up into these cells as well.[43] However, there was no significant toxicity observed because microscopy indicated that FITC-tagged polyhexanide was sequestered and kept in endosomes, preventing the polymer from entering other parts of the cell.

Another class of water-soluble polymeric biocides that have been investigated are those that have been functionalized with quaternary ammonium (QA) functional groups, both in pendant groups and along the backbone, which impart a permanent cationic charge on the molecule, no matter the change in pH. The use of small molecule QA salts in antimicrobial applications can be dated back to the early 20th century when they were originally identified as antimicrobials in 1916 by Jacobs *et al*.[44-46] It wasn't until the 1930's when Domagk investigated QA salts that had at least one long aliphatic chain that this class of molecules began to gain widespread attention and used as antimicrobials.[47] Today, the small molecule QA salts are ubiquitous in consumer products as the main active ingredient in many disinfectant products, such as Lysol. Such effectiveness in controlling the growth of bacteria led to efforts to make synthetic polymers that are functionalized with QAs on pendant groups attached to the polymers to 1.) impart antimicrobial properties to solid

materials and 2.) to help localize the effects of the QAs and prevent the spread of the biocide away from the point of application. As a result, there has been much work in applying polymeric QAs to coatings and to grafting them to surfaces, although it is beyond the scope of this chapter. Instead, attention will be given to the small subclass of QA polymers that were synthesized and investigated as water-soluble polymers that are able to dissolve in solution and are not associated with any surface.

Water-soluble cationic polymers that incorporate QA functional groups into the pendant group can be synthesized in a variety of ways, which are shown in Scheme 1.2 A-C. In most cases, these polymers are synthesized from monomers via chain growth polymerizations of vinyl monomers such as acrylates, methacrylates, functionalized styrenes, and vinyl pyridines. The formation of the QA can be achieved either before or after polymerization of the monomer(s).[48] If the monomer is functionalized with the QA prior to polymerization, care must be taken to ensure that the presence of the functional group does not have any detrimental effects on polymerization or destabilize the monomer in any way. While, if the polymer is functionalized with the QA after polymerization, it can be difficult to completely form the QA, due to unfavorable steric and electrostatic interactions introduced by neighboring groups that ultimately lead to less than 100% functionalization.[49] To form QAs, there are two synthetic strategies that are usually employed. One way to synthesize a QA is through the Menshutkin reaction of a tertiary amine and an alkyl halide, which is an SN_2 reaction where the nucleophilic lone pair of electrons on the nitrogen forms a bond with the electrophilic carbon of the alkyl halide. The other is another SN_2 reaction that involves the reaction of a primary amine with an alkyl halide through exhaustive alkylation. This second method of synthesis involves the

stepwise synthesis of a secondary amine, then a tertiary amine, and then, finally, a QA through the stepwise nucleophilic attack of the alkyl halide by the lone pair of electrons on the nitrogen of the amine.



Scheme 1.2: Methods to synthesize polymers that contain pendant QAs. The three most common methods are through A.) direct polymerization of QA containing monomer(s), B.) exhaustive alkylation of amines, or C.) the Menshutkin reaction.

When making a polymer that has pendant QA functional groups, there are various important parameters that drive their rational design to enable them to exhibit antimicrobial properties, *i.e.* the ability to control the growth of bacteria. For example, just because the monomer exhibits antimicrobial properties does not necessarily mean that the resulting polymer has the same or greater antimicrobial properties, as is the case with 4-vinyl-N-benzylpyridinium chloride and its resulting polymer.[50] While, at the same time, the

opposite is true; the monomer itself doesn't necessarily need to exhibit antimicrobial activity for the polymer to have antimicrobial properties. This can be observed in the behavior of a methacrylate monomer that contains a pendant QA based on a DABCO ring that has either a hexyl or a butyl group; the monomer is not antimicrobial while the resulting polymer is antimicrobial.[51] In synthesizing QA polymers, there are multiple important guiding principles that are important to imparting antimicrobial properties. Perhaps the most important aspect to the design of any QA polymer is simply that the resulting polymer needs to be soluble. A polymer formed by the polymerization of a small molecule biocide does not necessarily result in a polymer that is water-soluble.[52]

Molecular mass has also been indicated in modulating the behavior of QA polymers. It is necessary for the QA polymer to be of a specific molecular weight to have an optimal effect on bacteria, especially in cases where the monomer itself is not antimicrobial. Yet, for the most desirable effect, there often exists a maximum molecular weight at which the antimicrobial properties will begin to decrease; it is believed that the role that molecular mass plays in promoting effectiveness as an antimicrobial is related to how it affects the ability of the molecule to bind to and disrupt the membrane.[53, 54] For instance, when comparing the ability of a charged small molecule species and a polycation to disrupt bacterial membranes, the polycation will bind to the surface more tightly than that of a small molecule. However, if the molecular mass becomes too large, the solubility of the polymer will decrease, which will result in a decrease in antimicrobial activity. Increasing the molecular mass of the polymer also hinders the ability to transcend the outer most layers of the bacteria, *i.e.* the thick peptidoglycan layer of Gram positive bacteria, the outer membrane of Gram negative bacteria, and the glycocalyx of many bacteria. So, to

achieve the most desirable antimicrobial activity, there is typically a range of molecular masses that work most effectively, which can vary between different polymers. Some of the earliest work of this was demonstrated by Ikeda *et al.* where they fractionated poly(vinylbenyl ammonium chloride) into molecular masses of narrow dispersity.[55] When testing the effectiveness of the polymer fractions and the monomer at a fixed concentration in killing *Bacillus subtilis*, the polymers of an intermediate molecular mass of about 16 kDa were most effective.

Another key factor in the synthesis of a successful water-soluble antimicrobial QA polymers is the balance of hydrophobic and cationic character. Although this is related to the need to be water soluble, the need for the polymer to achieve the right amount of hydrophobic character and the right amount of cationic character is important to the polymer's function beyond just making the polymer soluble. Even with the small molecule QA salts, there exists a balance in achieving optimal activity through modification of the length of the alkyl chains attached to the nitrogen. In many instances, it has been found that a small molecule QA salt has optimal effectiveness against bacteria when it has a ~8-16-carbon alkyl chain attached to the nitrogen.[56] There have also been various investigations into the effect of other parameters, such as length of the hydrophobic segment within the backbone, length of the hydrophobic segment connecting the pendant QA, and the length of the alkyl chains attached to the nitrogen of the QA.[53, 54] These investigations suggest that there exists an optimal polymer where the greatest antimicrobial activity efficacy can be achieved by modifying the length of the hydrophobic segment(s). In a study of poly(trialkylbenzylammonium chlorides) by Ikeda *et al*., there were a variety of polymers synthesized that varied the length of the substituents on the nitrogen of the pendant QA.[57]

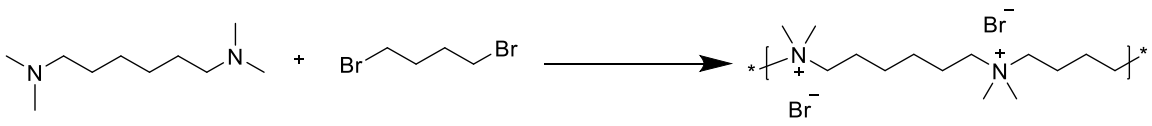
In the investigation, it was found that by increasing the length of one of the alkyl substituents, a greater antimicrobial effect can be achieved. More recently, Fernandez-Garcia *et al.* demonstrated various important relationships in the tuning of the properties of the biocidal polymethacrylates based on quaternized 1,3-thiazole and 1,2,3-triazole side-chain groups.[58] In their investigations, they determined that length and identity of the spacer between the backbone and the quaternized moiety is important; a succinate spacer is not as effective as an aliphatic hydrocarbon spacer and an aliphatic hydrocarbon spacer with an intermediate length works best. Also, with these polymers, it was observed that the polymers quaternized with methyl and butyl groups showed more desirable properties over the analogues that were synthesized with longer hydrocarbon chain substituents.

Some of the best characterized and most frequently studied QA polymers are those that are functionalized with pyridinium groups, which are the quaternized form of the nitrogen containing heterocycle pyridine. Originally, it was discovered that insoluble, crosslinked poly(4-vinyl pyridinium bromide) resin was capable of very effectively binding and retaining various Gram negative and Gram positive bacteria, such as *E. coli*, *S. aureus*, and *P. aeruginosa*.[59] Interestingly, it was found that this resin irreversibly bound the bacteria in a nonlethal manner. Further investigation by the same group discovered that water-soluble poly(4-vinyl pyridinium) worked well to kill both Gram positive and Gram negative bacteria.[60] In particular, it was determined that poly(4-vinyl pyridinium) exhibited the greatest antimicrobial activity with an N-benzyl substituent, as opposed to an aliphatic alkyl chain, and demonstrated antimicrobial activity that was comparable to that of widely used small molecule antimicrobial disinfectants benzalkonium chloride and chlorohexidine. Copolymers of poly(4-vinyl pyridinium-co-styrene) have also been

investigated for their antimicrobial properties and toxicity to in an animal model.[61] It was observed that copolymers that had greater incorporation of the 4-vinyl pyridinium group resulted in more efficacious killing of *E. coli*, *S. aureus*, and *Pseudomonas geniculate* and that the copolymer is a non-irritant with low oral toxicity in guinea pigs. Pyridinium functional groups have additionally been added to polymers synthesized via ring opening metathesis polymerization and characterized for their antimicrobial structure/property relationships.[62] The polymers were synthesized to two molar masses, ~3 kDa and ~10 kDa, with the pyridinium group formed using a variety of alkyl bromides and phenylethyl bromide. Antimicrobial testing revealed that there was a very clear difference in performance between the polymers that contained N-alkyl substituents of 4 carbons or less and those that contained the more hydrophobic substituents of 6 carbons or greater, with the polymers that had the more hydrophobic substituents being more potent antimicrobials. However, with the increase in antimicrobial activity among the polymers with the more hydrophobic substituents, there was also a simultaneous decrease in the compatibility of the polymers with human blood cells, indicating that that the increased antimicrobial performance also results in increased activity against all cells.

QA functional groups have also been incorporated into the backbones of polymer chains and investigated for their antimicrobial properties. These polymers are a special class of QA polymers, which are frequently referred to as ionene polymers, that have been studied for a variety of applications in addition to their use as antimicrobials, such as for use as a flocculating agent in the treatment of clay suspensions and waste water, as antistatic agents, and as hair conditioning agents.[63] These polymers were first synthesized in the 1930's but they were not very widely investigated until after Renbaum *et al.*

determined the mechanism through which they are formed and demonstrated a useful means to characterize the resulting polymers' molecular mass.[64-67] The popularity of ionene polymers is in large part due to their rather simple synthesis. The most frequently used method to synthesize ionene polymers is through the step growth polymerization of di-tertiary amines with di-alkyl halides through successive Menshutkin reactions, which is shown in Scheme 1.3. This method of polymerization is rather efficient and polymers with molecular weights in the range of a few thousand Da all the way up into the tens of thousands can easily be synthesized. In these polymerizations it has been observed that 1.) the rate of polymerization increases with the increase polarity of the solvent used in polymerization and 2.) the reactivity of the of the halogen follows I > Br > Cl.[63]



Scheme 1.3: A typical reaction that yields an ionene polymer via a step-growth polymerization using successive Menshutkin reactions.

In terms of antimicrobial properties, ionene polymers have not been as extensively investigated as polymers that have QA functional groups in their pendant groups. However, within the past few years, there have been multiple high-profile publications that have stimulated a resurgence in interest in these polymers by demonstrating their effectiveness against a broad spectrum of microbial pathogens as well as compatibility with mammalian cells .[68-70] Also, there have been various patents filed that claim the superior effectiveness of these QA polymers. Ionene polymers were originally investigated for their antimicrobial

properties by Renbaum *et al*. in the early 1970's.[67, 71] Since then, there have been multiple investigations into how best to tune their hydrophobicity to obtain the most ideal antimicrobial effect, as well as studies that have been performed to try to better understand their mechanism of action. Ikeda *et al*. synthesized a series of ionene polymers that varied the size of aliphatic and aromatic spacers between the QAs of the backbone.[72] It was observed that among all synthesized polymers that each polymer was significantly more effective in controlling the growth of Gram positive bacteria *S. aureus* and *B. subtilis*, with concentrations on the range of 5 - 66 μg/mL effectively inhibiting growth, than against various Gram positive bacteria and fungi, with effective concentrations in the range of 100 - over 1000 μg/mL. When comparing the various alkyl chain lengths and variations in the number of aromatic rings per repeat unit, it was observed that the ionene polymer with two successive o-xylene spacers (both constitutive dihalide and diamine monomers contained the aromatic ring) performed the best in controlling bacterial growth, while the ionene polymer that contained shorter alkyl chains or hydrophilic hydroxyl groups within the spacer performed the poorest. In a mechanistic investigation of these polymers, it was shown that these polymers are capable of disrupting phospholipid membranes. The extent of ability of the ionene polymers to induce disruption appears to be dependent on the identity of the spacers between the QAs along the backbone.[72] A complementary investigation set out to determine the roles that charge density and hydrophobicity have in determining the ability of the ionene polymer to bind to cells and impact their viability.[73] This study determined that the ionene polymers with short alkyl spacers bind to the cells without causing disruption, while ionene polymers with long alkyl spacers cause disruption after binding to the cells. The effectiveness of the ionene polymers can be modulated

though the length of the N-alkyl substituent, with methyl and octyl substituents showing greater antimicrobial efficacy when compared to that of butyl and hexyl substituents. It is believed that this behavior due to the length of the N-alkyl substituents is the result of changes in the conformation of the chain around the QA.

Another important class of biocidal polymers that has been investigated are polymers that contain quaternary phosphonium (QP) functional groups. These polymers are similar to QAs in that they have a permanent positive charge present across the functional group due to bonding of the central phosphate atom with four organic substituents. In fact, the most frequent means in which QPs are synthesized is similar to the Menshutkin reaction that is employed to form QAs, where a tertiary phosphine reacts with an alkyl halide. However, in terms of properties of the functional groups, QAs and QPs have some rather distinct differences. Most significantly, the differences in electronegativity of the nitrogen and phosphorus atoms leads to a very different distribution of electrons and partial charges. In QAs, it has been calculated through *ab initio* calculations that the more electronegative nitrogen atom has a partial negative charge while the surrounding carbon atoms have a partial positive charge.[74] In contrast, when the calculations are performed on QPs, the phosphorus atom has a partial positive charge and the surrounding carbon atoms have a partial negative charge. A significant difference between ammonium and phosphonium moieties is their stability. Phosphonium species tend to be significantly more stable and resistant to degradation than ammonium species, which has led to their utilization in anionic exchange membranes and fuel cells.[75-77]

As biocidal polymers, QP polymers have been well studied. In many respects, the same variables that can modulate the properties of QA polymers also have similar effects

in modulating QP polymers. This was demonstrated in a series of papers by Kanazawa, Ikeda, and Endo, where monomers synthesized from chloroalkylstyrene and tertiary phosphines were polymerized and the effects of various changes in chemical structure on antimicrobial properties measured.[78-83] It was observed from a series of polymers synthesized to have either ethyl, n-butyl, phenyl, or n-octyl substituents attached to the QP that the more hydrophobic substituents lead to more potent antimicrobial activity.[78] However, when the effect of the length of the alkyl spacer between the QP and the backbone of the polymer chain was varied as either a propyl or an ethyl chain, the polymer with the shorter spacer proved to be a better antimicrobial.[82] In terms of molecular mass, when QP polymers that ranged in weight average molecular mass between 16,000 – 94,000 Da, increasing molecular weight resulted in greater antimicrobial efficacy.[79] There have been ionene polymers synthesized to contain a QP group along the backbone of the polymers.[80] These QP ionene polymers showed similar changes in antimicrobial properties when hydrophobicity was adjusted. In a study that measured the effect of a wide range of alkyl chain spacers, the polymers that had the greatest separation of QP groups along the backbone, which was a polymer with alternating hexyl and butyl chain lengths, possessed the greatest activity against *S. aureus*, with all the polymers with fewer carbons in the backbone showing lower activity. There have additionally been investigations into the mechanism of action of QP polymers and they indicate that the polymers function to disrupt membranes of cells.[83]

When compared head-to-head, QP polymers have actually been found to work better as antimicrobials than QA polymers. Although it is not exactly known why QP polymers are stronger antimicrobials, it has been speculated that it has to do with how

tightly anions bind to the quaternary species; since phosphorus is has more electrons than nitrogen, the ionic radius of a phosphonium species is larger than that of an ammonium.[48] Nonetheless, whatever the reason, there is significant experimental evidence of greater activity of QP polymers. Kenawy *et al.* has shown, using methacrylate based polymers functionalized with QA and QP functional groups, that the polymers with incorporated QP functional groups demonstrated greater antimicrobial effectiveness in killing various Gram negative and Gram positive bacteria as well as fungi when compared to a polymer functionalized with a QA.[84] In the work of Kanazawa, Ikeda, and Endo, they compared the antimicrobial ability of a QP polymer to an analogous QA polymer. The results indicated that the QP polymer was able to kill *S. aureus* more quickly than the QA polymer.[78] Other investigations using water insoluble materials that show QP functional groups to have a greater antimicrobial effectiveness than QA functional groups.[85, 86]

1.3 Cationic Antimicrobial Peptides

Another class of important CAMs are cationic antimicrobial peptides (CAPs). Unlike biocidal polymers, CAPs are macromolecules produced naturally by virtually every organism on this planet. There have been over 2,500 different CAPs identified across the entire spectrum of life. When it comes to defining what exactly is a CAP, that can be a little tricky since the definition can vary slightly among researchers within this field. For the purpose of this section, a CAP will be defined as any ribosomally-translated, cationically-charged peptide that has demonstrated antimicrobial activity through non-enzymatic interactions under biologically relevant conditions. Typically, these peptides are about 10-

60 amino acid residues in length and they come is a variety of beta sheet, alpha helix, and extended conformations. Two key characteristics that really ties this entire class of molecules together are 1.) they are composed of an abundance of cationic amino acid residues, *i.e.* arginine, lysine, and histidine, and 2.) that they are composed of around 30% hydrophobic amino acid residues. There are many very good and up-to-date reviews on this topic of CAPs.[87-94] It would be easy to fill an entire book about everything known about CAPs; a single section of a book chapter is insufficient to cover every detail. So, it is the intention of this section to provide a brief introduction to the general features of CAPs while also further exploring in depth some of the best characterized families of these macromolecules.

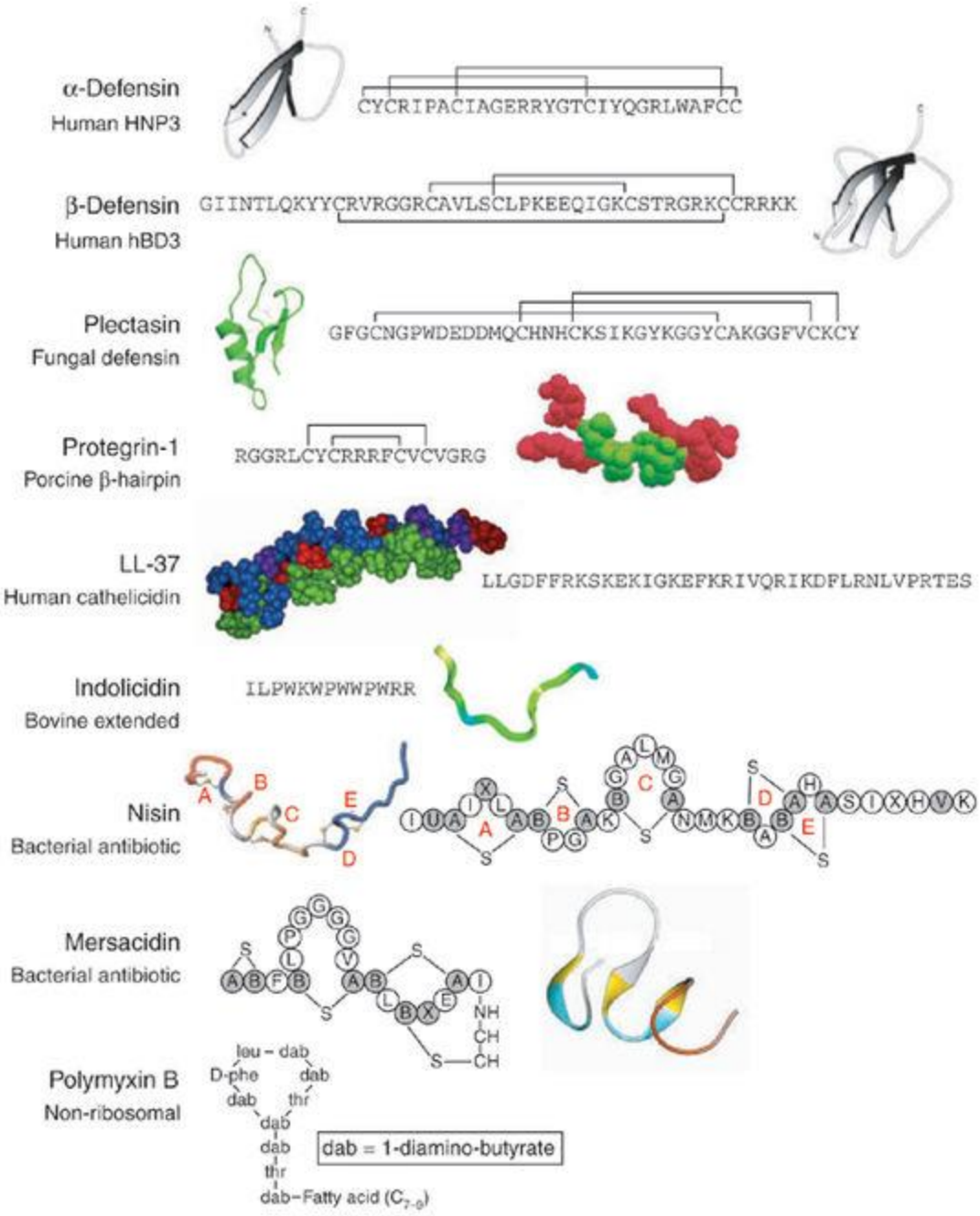


Figure 1.1: The sequences and conformations of some well-studied antimicrobial. Licensed with permission from RightsLink: Springer Nature, *Nature Biotechnology*, 24, (12), Hancock, R. E. W. and Sahl, H.-G., Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies, 1551-1557, copyright 2006.

CAPs have been studied for a very long time. Many believe that the first time a CAP was observed for its antimicrobial action was in 1896 when a substance within wheat flour was shown to kill yeast cells.[95] It wasn't until the 1930s and 1940s that interest in identifying and studying these peptides gained significant interest. In 1942, the substance that killed yeast was isolated from wheat germ and determined to be a peptide.[96] In addition to affecting yeast, this peptide was shown to be effective in killing the pathogenic plant bacteria *Pseudomonas solanacearum* and *Xanthomonas campestris* and would later go on to be named purothionin.[97, 98] During this time period in the 1930's and 1940's various other CAPs were identified that produced by prokaryotic organisms, such as gramicidin.[99, 100] Yet, at the same time that CAPs were beginning to gain interest within the scientific community, small molecule antibiotics, such as penicillin and streptomycin, were discovered and very successfully commercialized to begin the "golden age of antibiotics." It is during this so called "golden age" between 1950 and 1960 that roughly half of the antibiotic drugs used today were discovered and, as a result, CAPs and their function within organisms went largely ignored. It was in 1962 that the first CAP isolated from an animal was identified; a peptide that would be named bombinin was isolated from the frog *Bombina variegate*.[101] Since then, there has been a flourishing of CAP research and a vast amount of information on the function, purpose, and mechanisms of action of these peptides has been discovered.

It is now well known that CAPs are an evolutionarily ancient class of peptides that have been used to combat invading pathogens. For many years it was unknown how organisms that lack an adaptive immune system were capable of fending off pathogenic bacteria. It is now widely accepted that CAPs play a critical role in virtually all organisms

in combating bacteria to prevent infection. Even among mammals, including humans, CAPs play a fundamental role in the innate immunity to protect against bacterial infections.[92] CAPs can be found to be produced and secreted by cells of all multicellular organisms at the interface with the exterior environment and interior environments that come into contact with bacteria, such as the skin and intestinal mucosa. When comparing the sequences of these peptides between organisms, even those that are closely related, there exists differences in the sequence of analogous peptides. For example, the mouse CAP known as CRAMP (Cathelin-Related Anti-Microbial Peptide) shares only 61-66% homology with the analogous human CAP known as LL-37.[102] Also, the number of CAPs as well as the types of CAPs expressed by different organisms varies significantly.[103, 104] It has been reasoned that these differences exist because single substitutions in the amino acid sequence can lead to big changes in the effectiveness of a CAP against a certain microorganism. So, it is believed, that as organisms have evolved, so have their CAPs. The changes that are made to these sequences are reflective of the adaptation of the organism that produces the CAP(s) to the bacteria that they most frequently encounter. This is one important reason for why CAPs are so widely distributed among organisms in nature and so successful in combatting microorganisms.

However, what truly differentiates this class of antimicrobials from other antimicrobials produced naturally is/are the mechanism(s) of action. Unlike small molecule antimicrobials that target very specific sites in bacteria, CAPs take a broader, multifaceted approach to killing bacteria. The best studied and most frequently observed mechanism of action of CAPs is the disruption of bacterial membranes. This attack on the membranes is effective because of its nonspecific approach; there is no specific chirality, peptide

conformation, ligand/substrate interaction, etc. necessary for the CAP to properly function. Just as it is for polymeric biocides, CAPs have been shown to selectively disrupt bacterial membranes through 1.) initial electrostatic attraction of the anionic bacterial membrane and the cationic peptide and then 2.) disruption of the membrane as the hydrophobic portion of the peptide is inserted into the membrane.[93] However, unlike polymeric biocides, there has been extensive investigation into exactly what is occurring on the molecular level with CAPs, in large part because their homogenous, monodisperse structure and the methods of determining molecular level conformations make study of the peptide/membrane interactions somewhat easier. However, it must be noted that there is not one single method that is capable determining the mechanisms of action and what is known about the interaction of CAPs with bacterial membranes is the result of a combination of experimental and computational methods.

With such a diversity of sequences that exist with peptides, there are multiple variables that modulate the effectiveness of CAPs that can indirectly indicate the mechanism of action. For example, there are significant differences in the ability of different CAPs to affect bacteria under different aqueous conditions. The ability of CAPs to function as antimicrobials is very dependent on the complexity of the solution. The presence of high amounts of salts, especially when there is a high concentration of divalent cations Ca^{2+} and Mg^{2+}, and anionically charged macromolecules, such as glycosaminoglycans, can function to screen electrostatic interactions of the CAPs or bind to and prevent the action antimicrobial.[87] This behavior of CAPs indicates that electrostatic interactions are important for the CAP to function as an effective antimicrobial.

The importance of the electrostatic interaction of CAPs with the bacterial membrane has been demonstrated using model phospholipid membranes that are supposed to mimic those of the bacterial cell membranes.[105, 106] CAPs have a greater ability to disrupt membranes that have a greater amount of anionic head groups (*e.g.* phosphatidyl glycerol), which are typically abundant in bacterial cell membranes. However, it must be stressed that all those experiments are oversimplifications of what is truly occurring; there are more complex structures present on the surface of actual cell membranes. The roles that other structures present on bacteria outside of the cell membranes also have a considerable effect on how the CAPs affect membranes and can vary significantly between organisms. For any CAP to interact with the cytoplasmic membrane, it must first diffuse through any glycocalyx that may be present and, in the case of Gram positive bacteria, pass through a rather thick layer of peptidoglycan that is intercalated with polyanionic teichoic acids or, in the case of Gram negative bacteria, find entrance through the outer membrane. It is for this reason that the effectiveness of CAPs can vary among different bacteria and even among different strains of the same bacteria.

When CAPs contact bacterial membranes, there is a concentration dependence on the ability of CAPs to disrupt membranes. A prevailing model that adequately describes this behavior is known as the Two State Model.[107] This model, which was proposed by Huang, contends that there are two distinct physical states that CAPs are capable of presenting when they bind to the surface of bacterial membranes. The physical state that predominates is dependent on the ratio of peptide/phospholipids (P/L) present on the surface of the membrane. At low values of P/L, the CAPs bound to the surface of the membrane are in an inactive state that does not cause any disruption to the membrane,

while, high values of P/L result in the assembly of CAPs that yield the formation of pores in the membrane that are large enough to allow the flow of small molecules and ions. It is at high values of P/L that CAPs actually exhibit antimicrobial properties. At a critical concentration, P/L*, there is a transition from predominately one physical state to another. The strongest evidence for the existence of these two distinctive physical states of AMPs on the surface of model bacterial membranes can be observed through oriented circular dichroism (OCD) spectroscopy, which is a specialized form of circular dichroism spectroscopy capable of detecting the orientation of peptides and determining the secondary structure of peptides.[108, 109] Additional evidence from neutron scattering and solid state NMR further supports this model.[110-112]

After a sufficient concentration of CAPs has accumulated on the surface of the bacterial membrane, there are a wide variety of the structures that CAPs could possibly form to disrupt the membrane that have been observed experimentally and computationally, some of which are shown in Figure 1.2.[93] However, there are three main models that are most frequently discussed and observed to occur and are known as 1.) the toroidal pore model, 2.) the barrel stave model, and 3.) the carpet model. For each of these models, they all result in the disruption of the bacterial membrane by punching holes in the bacterial membrane to disrupt homeostasis but are all different from one another in how the CAPs orient to cause the disruption. In the toroidal pore model, the peptides form a pore in the cytoplasmic membrane in a way where the phospholipids bend through the pore continuously. This means that the phospholipids are part of the pore structure and that the CAPs that form the pore remain on the surface of the phospholipids. Some examples of CAPs that have been demonstrated to form toroidal pores include magainin, melittin, and

protegrins.[113] While, the barrel stave model, which has been demonstrated by alamethicin, a non-ribosomally translated peptide, has been shown to form pores slightly differently than the toroidal pore model.[113, 114] Instead of causing the phospholipids to bend into the pore, the pore that is composed of only the peptide without changing the structure of the phospholipid bilayer. Lastly, the carpet model, which has been demonstrated by the CAP ovispirin, is a detergent-like mechanism.[112] In this model, the CAPs orient parallel to the plane of the phospholipids and cover the surface of the cytoplasmic membrane in a "carpet-like" manner. Once a certain concentration is achieved, the peptides form micelles that strip away the phospholipids from the surface, which eventually results in the formation of transient holes in the surface. Yet, it must be stressed that for all these models that they have been observed under very controlled conditions that these modes of action may or may occur under physiological conditions depending on various factors, such as salinity and concentration. For example, a spectrum of interactions has been demonstrated by various CAPs at different conditions. Low concentrations of the CAPs results in the formation of pores in the membrane through the toroidal pore model, while, high concentrations of the CAPs result in pore formation according to the carpet model.[115]

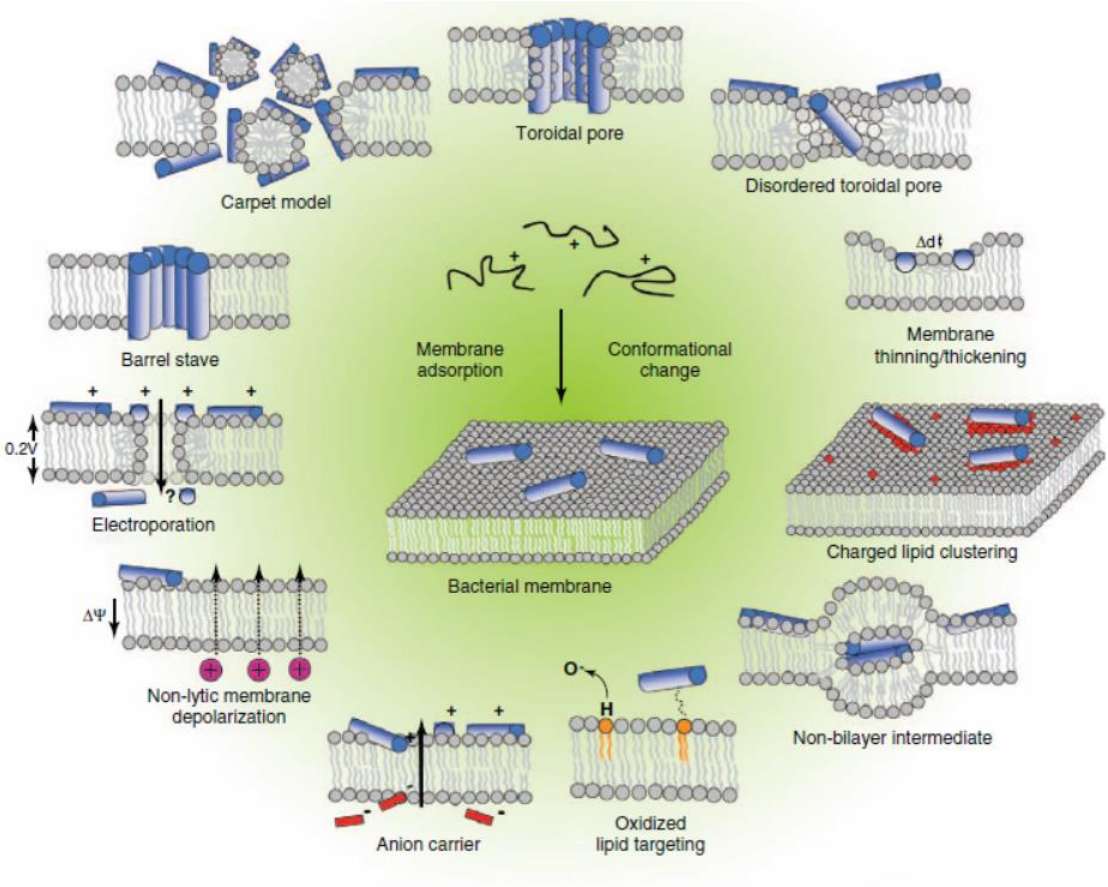


Figure 1.2: The various ways in which CAPs have been found experimentally and computationally to disrupt bacterial membranes. Reprinted from *Trends in Biotechnology*, 29, (9), Nguyen, L. T.; Haney, E. F.; Vogel, H. J., The expanding scope of antimicrobial peptide structures and their modes of action, 464-472, copyright 2011, with permission from Elsevier.

However, some CAPs are capable of killing bacteria through other mechanisms besides membrane disruption. Even though membrane disruption is by far the most studied mechanism of interaction with bacteria and appears to be the most significant cause for any decrease in viability of bacteria for most CAPs, various CAPs demonstrate other mechanism(s) of action.[116] There are CAPs that are known to have multiple mechanisms

of action against bacteria. In some cases, it appears that membrane disruption is not the primary mechanism of action or may be one of multiple mechanisms that are simultaneously acting on bacteria. For example, the CAP pleurocidin, which is produced by the fish *Pleuronectes americanus*, has been shown to significantly decrease the viability of *E. coli* at concentrations that are below what was shown to completely disrupt the cytoplasmic membrane of *E. coli*.[117] Another CAP that appears to have rather difficult to elucidate mechanism(s) of action is indolicidin, which is one of the shortest known naturally occurring CAPs, because it seems to kill bacteria through multiple mechanisms at concentrations relevant to therapeutic activity.[118-122]

One mechanism of action that multiple CAPs have demonstrated is the inhibition of DNA replication and/or translation of DNA into RNA through binding of the peptide to nucleic acid.[122-125] Since DNA is very negatively charged due to the phosphodiester backbone of the molecule, CAPs can have a strong electrostatic affinity for DNA. A CAP that has been rather extensively examined and found to bind to DNA as a mechanism of action is buforin II, an antimicrobial peptide fragment that is derived from a histone of the toad *Bufo gargarizans*.[125-127] It has been reported that buforin II is capable of binding to double stranded DNA within *E. coli* without causing disruption to the membranes of the bacteria, even at five times the MIC.[125] When a fluorescently tagged buforin II was compared to fluorescently tagged magainin 2, a CAP known to form pores in bacterial membranes, at sub-MIC and MIC, it was observed via confocal microscopy that buforin II quickly migrated to the inside of *E. coli* that were exposed to the CAP while magainin 2 remained outside of the cells in the surrounding solution. Along with buforin II, indolicidin has also been indicated in affecting bacteria by interacting with DNA. Indolicidin is

capable of inducing filamentation of *E. coli*, which is the elongation of the cells because they are incapable of properly undergoing cell division.[122] This same behavior where *E. coli* is incapable of successfully dividing is indicative of drugs that inhibit replication of DNA.[128] In an experiment with radioisotope labelled thymidine, which is a nucleotide that only gets incorporated into DNA, it was shown that *E. coli* exposed to 0.5 x and 1x MIC concentrations of indolicidin incorporated significantly reduced amounts of the radio-labelled molecule into their constitutive DNA when compared to a control that did not contain any indolicidin.[122]

Additionally, there are various other mechanisms that CAPs can demonstrate. Nisin, a CAP that is part of the family of peptides produced by bacteria known as lantibiotics, affects the synthesis of the cell wall.[129] It has been observed *in vitro* that nisin binds to multiple intermediates in the biosynthesis of teichoic acid, an important component for the structure of the cell wall. Other CAPs that have gained special interest is a class derived from insects known as apidaecins, which are incapable of disrupting bacterial membranes altogether and function to kill bacteria by inhibiting protein synthesis.[130, 131] These CAPs are unique in that they function like a small molecule antibiotic in their action because they require a specific peptide sequence to bind to bacterial ribosomes and only function if they are constructed with L-amino acids, which indicates that a more specific interaction occurs and is unlike all other CAPs. Also, the mechanisms of action can be much more complex and involve more than two possible mechanisms. For example, a study on the mechanism of action of pleurocidin has identified three possible mechanisms when its action against *E. coli* was investigated.[117] The CAP was observed to simultaneously partially disrupt the cytoplasmic membrane, inhibit DNA

replication, and inhibit protein synthesis at concentrations that kill the bacteria. Perhaps, upon more careful and in-depth investigation, there may be many more CAPs that will be discovered in the future to have more than one mechanism of action that can potentially lead to the death of bacteria.

Yet, the way in which CAPs function to kill bacteria and control infections *in vivo* is not as straightforward as how they have been observed to behave *in vitro*. When measured within the body of mammalian organisms, CAPs are found to exist at concentrations well below their measured *in vitro* MIC.[92] This means that there is some other action through which the CAPs are exerting antimicrobial activity. One possibility that has been proposed is that multiple CAPs within the body work in a synergistic, multi-combinatorial manner. It has recently been observed that this may be true in a study by Rolff *et al.* In this study, six different commercially available CAPs that originated from a variety of organisms were selected and tested to determine whether pairs and trios of the CAPs yielded greater (synergistic), equal, or less antimicrobial activity when combined. In every combination that was tested, it was determined that almost all combinations resulted in a synergistic effect with the tested trios of CAPs showing the greatest effect.[132] Also, to explain these lower concentrations of CAPs observed *in vivo*, it has been suggested that concentrations of CAPs can spike locally when stimulated by pathogenic bacteria. A spike in expression of CAP genes has been shown to occur after injury or the onset of infection.[133]

The greatest role that CAPs play *in vivo* in controlling bacterial infections may not necessarily be the direct role they play in killing bacteria but rather the indirect roles CAPs have in functioning as regulators of immune cell activity, especially in mammals. For instance, it has been shown that various different endogenous CAPs in humans can

stimulate a response and recruit immune cells to a site of infection through chemotaxis.[134-137] This behavior was well demonstrated when observing the chemotactic activity of various fractions that have been isolated from the granules of human neutrophils, which are a type of immune cell that is part of the initial response to an infection.[134] The granules of neutrophils, which are optically visible particles present throughout the cells, contain a cocktail of molecules that are secreted when the cell is activated by an infection or injury. The contents of these granules include multiple peptides that are from the family of CAPs known as alpha defensins. Experiments showed that the fraction of the granule contents that contained the alpha defensins induced the migration of a monocytes, another immune cell type. Further investigation determined that it was more specifically the alpha defensins HNP-1 and HNP-2 that were promoting the migration.[134] Other human defensins have also been found to attract other immune cells, such as macrophages and T cells.[135, 137]

The effects of CAPs on the immune cells are not only limited to chemotaxis. Another important function of CAPs is the modulation of cell signaling pathways that affect inflammation. There have been multiple ways in which CAPs have been found to affect inflammation; both through binding to soluble ligands and through modification of the receptor substrates.[92] For example, it has been shown that human cathelicidin LL-37, a CAP produced within humans, may have a role in reducing the amount of tumor necrosis factor alpha (TNF-α), a potent proinflammatory cytokine, released from monocytes and macrophages when the cells are exposed to lipopolysaccharides (LPS) and teichoic acids (TA), both of which are proinflammatory, anionic macromolecules released by bacteria.[138, 139] LL-37 functions to reduce inflammation through binding to LPS and TA via electrostatic interactions and effectively, as a result, inhibits the ability of the molecules to

induce inflammation.[140, 141] The implications of this effect of LL-37 is quite substantial because the proinflammatory effects of LPS and TA are what typically induce sepsis. In models of contact dermatitis in mice, it was observed that LL-37 and CRAMP, the analog of LL-37 produced by mice, both function *in vivo* to decrease inflammation.[142] This was observed through experiments using wildtype mice and mice that were genetically altered to knockout the gene for CRAMP. After being exposed to a chemical agent that induces dermal inflammation, the mice deficient in CRAMP exhibited a greater degree of swelling than the wildtype mice. Upon intradermal injection of LL-37 at the site of application of the chemical agent, significantly less inflammation was observed when compared to mice that didn't receive the injection in the cases of both the genetically altered and the wildtype mice. It was determined in the investigation that the reason for this effect on inflammation was because the CAPs inhibited toll-like receptor 4 (TLR-4) activity, which is an important cell surface receptor found on dendritic cells that functions to activate a signaling cascade to recruit other immune cells to that location.

With such a broad spectrum of antimicrobial activities through various different mechanisms of action and with such a diversity of available and known peptide sequences, there has been tremendous interest and activity in trying to adapt these peptides as clinically applied therapeutics to combat increasingly difficult to treat infections. However, there are some very big questions that arise whenever any discussion of CAPs turns to clinical applications, which include 1.) Can bacteria become resistant to CAPs? and 2.) If bacteria do become resistant to CAPs, would there be any measurable impact on our own immune system and our inherent ability to combat infections? These are complicated questions to answer and there are various speculated answers that are supported with evidence to a

certain degree.[89, 143-146] Originally, when CAPs were beginning to be investigated, it was assumed that it was extremely difficult or impossible for bacteria to develop resistance to CAPs. It has been stated many times throughout various publications that it would be extremely difficult for bacteria to develop resistance to CAPs because of their nonspecific mechanisms of action.[143, 144, 147] Since CAPs target the membrane of bacteria in a manner that doesn't require interaction with specific conformations of molecules on the surface of the membrane and the CAPs themselves do not need to conform to a specific chirality, it was reasoned that it was extremely unlikely that bacteria could adapt since it would require such a large change in their membrane structure. The work of Perron, Zasloff, and Bell was the first study to adequately investigate whether these assumptions were correct.[148] Within the study, over the course of 100 days, strains of *E. coli* and *Pseudomonas fluorescens* were allowed to grow for ~600-700 generations while exposed to pexiganan, also known as MSI-78 and a synthetic analog to the CAP magainin, to try to mimic the repeated exposure that these bacteria may experience by the widespread clinical use of the peptide. They found that among the 24 samples exposed to pexiganan, 22 of them developed rather significant resistance to the CAP. The strains exposed to pexiganan grew at concentrations that were nearly 10x greater than those of the control bacteria. Even though it is controversial whether any of these experiments properly mimicked any conditions that may be replicated in the environment, it is indisputable that certain conditions and enough time can allow for resistance to develop.

Resistance to CAPs has been found to be present within naturally occurring bacteria in the environment and there are various mechanisms bacteria have evolved to resist the action of CAPs. Many strains of *Serratia spp.* and *Proteus spp.* are inherently resistant to

the action of CAPs, which is significant because these bacteria are ubiquitous throughout our surroundings and both genera contain species that are opportunistic pathogens.[149, 150] The reason for their enhanced tolerance to CAPs can be attributed to changes in their outer membrane structure (both genera are composed of Gram negative bacteria). The lipids that face the exterior environment that constitute the outer membrane are rich in LPS. Within the structure of LPS, the portion known as lipid A is functionalized with anionic phosphates in typical Gram negative bacteria. However, *Serratia spp.* and *Proteus spp.* have developed ways to modify these anionic charges and neutralize them, which reduces the electrostatic attraction of the CAPs to those membranes.[149, 150] Gram positive bacteria *S. aureus* have adopted a similar approach in resisting membrane disruption by CAPs. The cytoplasmic membranes of *S. aureus* are rich in phosphatidyl glycerol and cardiolipin type phospholipids, both of which carry anionic charges on their head groups that face towards the exterior environment. However, some strains of *S. aureus* have been observed to be capable of functionalizing phosphatidyl glycerol phospholipids with the amino acid lysine.[151-154] As a result, the attachment of lysine, the head group acquires a net cationic charge that reduces the ability of CAPs to disrupt the membrane.

There are also various other mechanisms that bacteria can use to resist the action of CAPs. Many bacteria are capable of resisting the actions of CAPs by secreting enzymes to degrade them.[155] Group A *Streptococcus spp.* of bacteria, which includes the causative agents for strep throat and scarlet fever, secrete an enzyme known as SpeB, which is capable of digesting human cathelicidin LL-37 and human beta defensins.[156, 157] *P. aeruginosa*, *Proteus mirabilis*, and *Enterococcus faecalis* additionally have all been reported to be capable of degrading LL-37.[156] Also, *Neisseria meningitides*, *Neisseria*

gonorrhoeae, and *Klesbsiella pneumoniae* have all been reported to be able of resisting the actions of human CAPs in a different manner; they have been found to use efflux pumps that serve to remove CAPs from the surface of the bacterial membrane after they bind.[158-160] It has even been reported that bacteria have developed sensory pathways through which they can adapt and change the surface chemistry of their membranes when a CAP is detected.[161-165]

There are many mechanisms that many different types of bacteria are capable of using to combat the action of CAPs. However, the exact impact that all these forms of resistance would have on humans and our own inherent ability to combat bacterial infections if they become more prevalent within bacterial populations is not very well studied and the risks are not very well known. There have been studies performed in animals where a certain CAP has been deleted and the ability of the genetically modified animal to combat an infection is observed, which may provide some insight into the impact of widespread CAP resistance. Such an experiment was carried out by Gallo *et at.*, where they observed the ability of mice that were modified to produce defective CRAMP to fight off an infection caused by Group A *Streptococcus*.[166] Their results indicated that the CRAMP deficient mice had a rather significant impairment in their ability to combat the infection when compared to wildtype mice that were able to produce the intact, functional peptide. In the experiments, the bacteria were injected subcutaneously, which resulted in the formation of infected lesions. After 7 days without antibiotic treatment, it was observed that the wildtype control mice were able to naturally clear the infection with no measurable amounts of bacteria present from biopsied tissue at the site of infection. However, when the biopsy was performed on the CRAMP deficient mice, there was still a significant

bacterial load present within the wound. Similar results have also been obtained that indicate impairment of the actions of CAPs leads to impairment of the ability of organisms to combat infections have been shown in multiple other studies.[167, 168]

Even though there is still much work to be done to better understand various aspects of the resistance of bacteria to CAPs, it is very clear that bacteria can develop resistance to CAPs and that weakening of endogenous CAPs can have a detrimental effect on the ability of organisms to fight off infections. In applying any CAP as a clinically used therapeutic, precautions must be made to try to prevent the development of resistance. We must learn from past mistakes made with small molecule antibiotics and prevent the overuse and misuse of CAPs. This is especially important because in recent years there has been much activity in developing CAPs for use as drugs to treat infections. Pexiganan is one of these CAPs that has acquired much attention for use as a clinically applied therapeutic.[169-173] There has been an ongoing attempt to obtain FDA approval of a topical cream that incorporates this CAP for use in the treatment of infected diabetic foot ulcers. Yet, the most recent iteration of a topical product that incorporated pexiganan as the active ingredient, known commercially as Locilex, has recently failed to meet the benchmarks necessary to obtain approval at the end of phase III of FDA clinical trials (NCT01594762).[174] Omiganan, which is a derivative of indolicidin, is another CAP that is in the later stages of FDA trials as a topical treatment. The CAP has been incorporated into a gel and is being investigated for treatment of rosacea, which is currently in phase III of FDA clinical trials (NCT02576847), and for use in the treatment of catheter related infections (NCT00231153).[175] Another noteworthy CAP that entered phase II of clinical trials, yet, did not complete the trial, for use as an intravenous systemic treatment for infection is a

short peptide that is a fragment of the human protein lactoferrin (NCT00430469). This peptide, which consists of the N-terminal 1-11 peptide amino acid residues, is being developed to try to treat the infections that may arise in immunocompromised patients who have recently undergone bone marrow transplants.[176]

However, to date there have not been any CAPs approved for clinical use by the FDA in this most recent push to try and develop new antimicrobials. Even though CAPs hold great promise to be developed as clinically applied antimicrobials, there are still many challenges in adapting CAPs for widespread application. A particularly difficult challenge that has yet to be resolved in adapting CAPs as therapeutics is the fact that CAPs perform quite differently in different aqueous environments; the behavior of CAPs *in vitro* may not match what is observed *in vivo*. This point has been made previously but it bears mentioning again because this performance provides a significant challenge in screening CAPs as potential therapeutics. Moreover, even if a CAP does exhibit all desirable characteristics, their application is still limited by the abundance of proteases that are found throughout the human body as well as by the proteases secreted by bacteria. CAPs typically exist for a very short amount of time before they are degraded. For example, it has been shown that the half-lives of CAPs in the blood stream is a matter of just a few minutes.[177] To further hinder the widespread use of CAPs, it can prove very difficult to manufacture them on a large scale. In making large amounts of any peptide using today's technology, there are two primary strategies: 1.) synthesis of peptides using solid phase peptide synthesizers and 2.) through the use of recombinant DNA to create an organism that over expresses the peptide. In both approaches to making the peptide, it can prove to be rather costly and time consuming to produce. As long as these challenges exist, CAPs will

continue to struggle to gain passage through FDA trials and will remain as simply an academic curiosity.

1.4 Synthetic Mimics of Antimicrobial Peptides

The final category of CAMs is known as synthetic mimics of antimicrobial peptides (SMAPs), which are a class of polymers that have been synthesized to intentionally mimic CAPs. Superficially, this class of polymers appears to be no different than the biocidal polymers discussed within a previous section of this chapter. However, there does indeed exist some important differences that differentiates these two different types of synthetic antimicrobial polymers. Unlike biocidal polymers, which in many ways can be considered unintentionally biomimetic, SMAPs were developed to determine whether synthetic polymers that are designed to mimic CAPs can function in a way that matches or exceeds the antimicrobial properties of CAPs. To further differentiate this class of molecules from biocidal polymers, there are differences in the types of cationic functional groups used by each class of CAM. SMAPs can be characterized by their use of biologically utilized cationic functional groups, such as the primary amine found on the amino acid lysine, the guanidine group found on the amino acid arginine, and the imidazole group found on the amino acid histidine. While, biocidal polymers, which draw inspiration from biguanide antimalaria drugs and small molecule QA salts, typically use functional groups that are not naturally found on CAPs, such as biguanide, quaternary ammonium, and quaternary phosphonium moieties. The differences between polymeric biocides and SMAPs can also be viewed as two different generations of synthetic cationic polymers. Biocidal polymers

are the first generation of synthetic polymers. SMAPs, in contrast, are a newer generation of synthetic polymers that are the result of a greater understanding of how similar CAPs and biocidal polymers truly are in terms of how they affect bacteria.

Investigations of SMAPs have revealed that proper design of any synthetic polymer can result in the creation of polymers that are antimicrobial when they are synthesized in a way that mimics the basic design features of CAPs. As it was discussed in the previous section of this chapter, CAPs come in many shapes, conformations, and lengths of amino acid residues but what really ties them all together is the fact that they are amphiphilic and contain an abundance of cationic amino acids and hydrophobic amino acids. By using these simple specifications that unite all CAPs as design parameters, successful SMAPs can be synthesized that get around some of the disadvantages of CAPs, such as their high cost of production and short *in vivo* lifetime. This section will explore the best studied SMAPs categorized by the chemistry upon which they are synthesized and discuss what is known about their antimicrobial properties and mechanisms of action.

Perhaps the most intuitive selection of backbone chemistry for the synthesis of SMAPs, polyamides have been thoroughly investigated for their antimicrobial properties. There are many synthetic peptides that are synthesized to try and make a better CAP that have been investigated.[89] However, this portion of the chapter will not discuss these molecules, since they should be considered as CAPs. Instead, the focus will be placed on polyamide type polymers that have been assembled from monomers other than α-amino acids. To overcome some of the limitations of CAPs, there have been various polyamide SMAPs that have been innovated, such as ß-peptides, which are constructed from ß- amino acids, and peptoids, which are polyamides constructed from N-functionalized glycines, of

which all the structures of these polyamides are shown in Figure 1.3.[178] By synthesizing polyamides in this manner, they are capable of evading the action of enzymes and, in some cases, still capable of achieving a range of folded structures in solution.

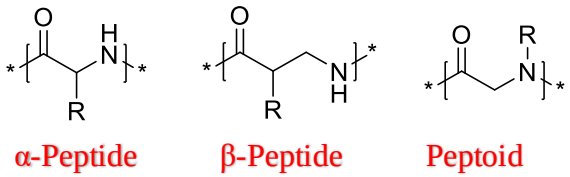


Figure 1.3: The chemical structures of α-peptides, β-peptides, and peptoids.

ß-peptides are one of the earlier types of SMAPs that have been investigated for their antimicrobial properties. Originally, interest in ß-peptides was sparked by the broad need identified by peptide researchers for the development of a more robust means to create molecules that exhibit the ability to fold into the bioactive shapes of peptides but without the detrimental features of peptides, *e.g.* susceptibility to protease degradation. ß-peptides that are synthesized using a solid phase peptide synthesizer, so that they have a well-defined sequence and monodisperse molecular mass distribution, have been well characterized to fold into many peptide-like secondary structures that have been shown to have peptide-like behavior in various applications. DeGrado *et al.* were among the first to investigate whether ß-peptides are able to mimic the structures of CAPs and preserve the antimicrobial properties.[179] Inspired by the α-helical magainins and cecropsins, a series of helical ß-peptides were synthesized that incorporated ß-amino acids that resembled the α-amino acids lysine, leucine, and valine. Their results showed that the ß-peptides that were formed were indeed antimicrobial but also quite toxic towards mammalian cells. It was

speculated that the peptides were too hydrophobic, leading to a decrease in the selectivity of the peptides to disrupt bacterial cells over mammalian cells.[179] In a follow up investigation, the composition of the helical ß-peptides was changed; the ß-amino acid that mimicked leucine was swapped out with a more hydrophilic ß- amino acid that mimicked the amino acid alanine.[180] By swapping out these monomers in the sequence of the ß-peptide, a greater selectivity of the killing of bacteria over mammalian cells was achieved. Using model phospholipid membranes, it was demonstrated that these ß- peptides had a greater affinity to interact with and disrupt membranes that has a greater amount of anionic phospholipids. In a similar approach Gellman *et al.* also designed a successful series of ß-peptides.[181-183] However, in their work, ß-amino acids that imparted a greater degree of rigidity to the synthetic peptide were developed. Their peptides also were designed to form helical structures in solution and they were able to demonstrate a range of antimicrobial effectiveness depending on the functionality of the ß-amino acid. The most noteworthy aspect of this work is that they synthesized a ß-peptide that they named ß-17, which is also referred to as APC40 in later works, that has superior effectiveness against a broad spectrum of bacteria and a selectivity that other ß-peptides have been unable to match.

Due to the success of ß-peptides being applied as SMAPs, peptoids, another type of polyamide SMAP, were investigated for their antimicrobial effectiveness. Peptoids, unlike other polyamide SMAPs, are unable to form hydrogen bonded secondary structures but exhibit other less stable secondary structures through interactions of the N-substituted groups if the sequence of monomers in the chain allow for steric or electrostatic interactions that promote formation.[184] The ability of peptoids to function as successful SMAPs was first observed by Patch and Barron, when they tested peptoids that were constructed to

mimic the helical structure, cationic charge, and size of CAP magainin-2.[185] In a subsequent study, it was determined that not only did the antimicrobial peptoids resemble CAPs in structure and function but also in their mechanism of action.[186] Evidence that antimicrobial peptoids insert themselves into the membranes of phospholipids was obtained through x-ray reflectivity measurements. Even though it could not be determined from this data if the structures that are formed on the surface of the phospholipids are the same as those formed by CAPs, the results obtained were consistent with what was obtained for the CAPs pexiganan and LL-37. It was also noticed within the study that other characteristics of the peptoids were peptide-like as well, such as antimicrobial properties that are independent of the chirality of the helix and dependent on the proper balance of hydrophobic and cationic character.[186] Peptoids have also received attention for having antibiofilm properties.[187] The performance of some peptoids in preventing the formation and inducing the detachment of *P. aeruginosa* biofilms is comparable to or even exceeds the ability of currently available antimicrobial therapeutics.

In much of the earlier work where polyamide SMAPs were investigated for their antimicrobial properties, the SMAPs were designed to not only mimic the chemical structure of the peptides, *i.e.* primary structure, but also the secondary structure of the peptides. However, it was soon realized that it was not necessary for the polyamides to mimic the secondary structures of peptides to have desirable antimicrobial properties. The basic design features that are most important in dictating the antimicrobial properties of SMAPs was outlined within the work of Mor *et al*.[188] They developed a class of SMAPs known as oligoacyl lysines (OAKs), with the chemical structure shown in Figure 1.4. OAKs are oligomers that are composed of lysine amino acids linked together by

hydrocarbon spacers via amide bonds. They demonstrated that there is not one specific hydrophobic amino acid nor is there any specific secondary structure that a peptide forms that is necessary for imparting antimicrobial properties. Instead, the most important feature for a SMAP to have to function as an antimicrobial is the correct balance of cationic and hydrophobic character of the molecule. This was observed with OAKs when they were synthesized with various lengths of hydrocarbon spacers and different lengths of the hydrocarbon on the N-terminal end of the molecule. The OAK that contained an eight -carbon hydrocarbon spacers, seven lysine residues between each spacer, and a 12-carbon hydrocarbon tail at the N-terminal end, which was named $C_{12}K\text{-}7_{\alpha 8}$, functioned as a very potent antimicrobial that killed bacteria at therapeutically relevant concentrations while at the same time demonstrating very good compatibility with mammalian cells. Additionally, at around the same time, Gellman *et al* showed similar results that demonstrated that the hydrophobic and cationic features of the primary structures of peptides are the most important features in imparting antimicrobial properties to the polymer.[189, 190] Within their work, they synthesized random/statistical copolymers of nylon-3, which were synthesized via anionic ring opening polymerization of ß-lactam monomers functionalized with hydrophobic and cationic functional groups to yield polyamides that had the same repeat units of the ß-peptides they previously investigated. The results showed that the lack of regular structure found within the random/statistical copolymers did not reduce the ability of the polymers to affect the bacteria when compared to CAPs but instead lead to the discovery that one of the copolymers actually performed better than all of the CAPs against the clinically isolated strains of *S. aureus* and *Enterococcus faecium* that were used in the study.[189] These findings lead to the proposal that these polymers that do not have any

structure in solution take on what was referred to as a "globally induced amphiphilic conformation," which means that the repeat units of the random/statistical copolymers are flexible enough to segregate into an unorganized, amphiphilic structure that segregates into cationic and hydrophobic domains when interacting with the surface of the bacteria.

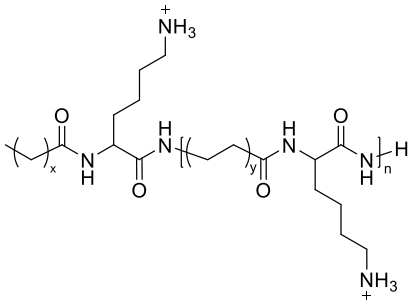


Figure 1.4: A generalized chemical structure of an OAK.[188] The antimicrobial properties of these polymers can be tuned by varying the length of the hydrocarbon spacers between the lysine and by modification of the lipid tail ends.

Since it has been demonstrated that no exact secondary structure is needed for polyamides to acquire antimicrobial properties, there have been many studies that have investigated the structure/property relationships and suitability of polyamides for specific applications. Further investigation of nylon-3 SMAPs has yielded interesting observations about how to modulate the antimicrobial properties of the polymers. In a comparison of the nylon-3 random/statistical copolymers that were composed of a cationic repeat unit and one of four different hydrophobic, cyclic, and non-aromatic repeat units, none of the tested ring sizes, which varied between five and eight membered rings, had a substantial effect on controlling the growth of four different types of bacteria.[190] However, the changes in ring size had a more profound effect on the compatibility of the polymer with mammalian

cells, with the incorporation of larger rings into the repeat unit leading to an increase in lysis of red blood cells. A similar effect on both bacteria and blood cells was observed when comparing the effect of molecular mass on the cells, with molecular mass having very little effect on the activity of the polymers against bacteria and a significant drop off in compatibility of the polymers with red blood cells being observed as the molecular weight is increased over the range of ~10 to ~60 repeat units.[190] Subsequently, it was observed that the use of non-cyclic hydrophobic repeat unit can result in the formation of polyamides that have comparable effectiveness against bacteria and superior compatibility with mammalian cells.[191] However, it appears that just as with their cyclic counterparts, if the noncyclic hydrophobic repeat unit becomes too hydrophobic, there is a decrease in red blood cell compatibility. When comparing the polymers in terms of the distribution of the repeat units along the backbone, the more ideal copolymers were synthesized from random/statistical distributions than from copolymers that have a blockier distribution, such as a diblock copolymer.[190] For each of the copolymers synthesized, the random/statistical copolymers had a significantly lower MIC against all tested bacteria than the diblock copolymers. There have also been nylon-3 terpolymers investigated where a third hydrophilic monomer is added to the random/statistical sequence of the polymers. The addition of polar and uncharged groups to the sequence of the Nylon-3 polymers, no matter whether they replace the hydrophobic repeat unit or the cationic repeat unit or both, resulted in polymers that had significantly greater mammalian cell compatibility without sacrificing antimicrobial activity.[192] In terms of applications of polyamide SMAPs, they have been shown to have a broad spectrum of activity against both Gram negative and Gram positive bacteria. However, best performing polyamide SMAPs have been shown to

have potent activity against the difficult to treat pathogen *C. difficile* that surpasses the *in vitro* performance of vancomycin and LL-37 in being able to affect all strains tested.[193]

Mechanistically, there have been various studies to determine how exactly the nylon-3 SMAPs work to kill bacteria. In experiments where phospholipids were used to form vesicles that mimic the cytoplasmic membranes of *E. coli*, *S. aureus*, and red blood cells, it was observed that nylon-3 SMAPs disrupted the *E. coli*-like and the *S. aureus*-like vesicles to a greater degree than the red blood cell-like vesicles, which is consistent with a CAP-like mechanism.[194, 195] Evidence from single cell time resolved microscopy also indicated that the polymers disrupt the cytoplasmic membrane.[196] The microscope images indicated that the polymers halted growth of the bacteria by inducing severe osmotic shock after translocating across the membrane. Yet, it has been observed with actual cells that membrane disruption is only part of what is occurring on the molecular level. The membrane disruption mechanism of nylon-3 SMAPs does indeed occur at low concentrations of the polymers near MIC. However, at very high concentrations of polymer, bacteria are effectively killed through a different mechanism of action.[194] Once a high enough concentration was achieved with *E. coli*, the cytoplasmic membrane was not disrupted but rather that the cell became unable to transport any solutes into or out of the cell because the cell was encapsulated in an impermeable polymer layer that coated the outer membrane of the bacteria. Evidence for this mechanism of action was indicated in part by live/dead staining that showed that bacteria at high concentrations of nylon-3 SMAP were not permeabilized by the dead stain, even though it was determined that the bacteria were not viable.[194] Also, evidence for the formation of a polymer coating was indicated by the use of the water-soluble molecule ortho-nitrophenyl-ß-galactoside

(ONPG), which is colorless in solution and can be hydrolyzed by the intracellular enzyme ß-galactosidase into galactose and the chromophore ortho-nitrophenol.[194] In experiments where *E. coli* was treated with nylon-3 SMAP, the ability of ONPG to gain entrance into the cell and generate ortho-nitrophenol was progressively reduced with increasing concentrations of polymer.

Besides polyamide SMAPs, there have also been many chain growth polymer (CGP) SMAPs investigated. After it became widely recognized that a well-defined secondary structure is not necessary for a successful SMAP, many conventional CGPs synthesized from readily available libraries of acrylate, methacrylate, and acrylamide monomers were investigated to determine whether they could be used to construct SMAPs that can be effective in controlling the growth of bacteria while having a minimal effect on mammalian cells. The appeal of these types of polymers as SMAPs is driven by the fact that many of these polymers can be made very economically, especially when compared to CAPs. These CGPs that resulted are distinguished from the previously investigated biocidal polymers, which incorporated many similar repeat units, by the fact that these polymers use repeat units that use primary ammonium as the cationic repeat unit and that these polymers are designed to draw inspiration from naturally occurring peptides. The earliest investigation of CGPs that were inspired by CAPs was performed by Kuroda and DeGrado using polymers that were synthesized from aminoethylmethacrylate and butylmethacrylate.[197] Even though their results were not very extraordinary, the SMAPs synthesized had descent antimicrobial activity and poor compatibility with red blood cells, the investigation began a long series of studies to better understand the structure/property

relationships as they relate to antimicrobial activity for CGPs as well as mechanistic investigations into how exactly they affect bacteria.

There have been many investigations into the rational design of CGP SMAPs and their applications. In a subsequent investigation by Kuroda, Caputo, and DeGrado, many methacrylate-based SMAP random/statistical copolymers were synthesized that had different ratios of cationic and hydrophobic methacrylate repeat units, differently functionalized hydrophobic groups, and different molecular masses to determine how all of these variables affect the antimicrobial properties.[198] Just as with polyamide SMAPs, these CGP SMAPs were observed to have antimicrobial activity and mammalian cell compatibility that is dependent on achieving a balance of cationic and hydrophobic functionality. Also, polymers that had a greater molecular mass resulted in greater antimicrobial activity but at the expense of compatibility with mammalian cells. In a similar investigation of polyacrylamide SMAPs, the importance of cationic/hydrophobic balance was also observed.[199] However, since the amide groups found in these polymers are much more polar than the analogous esters of polymethacrylates, the polyacrylamides showed greater effectiveness when copolymerized with hydrophobic acrylamides that had more hydrophobic pendant groups than that of the analogous polymethacrylates. Not only have there been investigations into the effects of hydrophobic groups but also changes in other functionalities. In a head to head comparison of copolymers formed from primary, tertiary, and quaternized ammonium functional methacrylates and a hydrophobic methacrylate, it was actually observed that primary amines and tertiary amines worked better to kill bacteria than quaternized ammonium groups.[200] Guanadinylated cationic functionalized polymethacrylate SMAPs have additionally been investigated for their

antimicrobial properties.[201] Polymers that are functionalized with guanidine functional groups are interesting because they mimic the cationic amino acid arginine. These polymers were shown to be more effective against Gram positive bacteria *S. aureus* and *S. epidermidis*, showed greater efficacy in protein rich medium, and less hemolytic than comparable amine functionalized polymethacrylates. Moreover, there have been CGP SMAPs that have been synthesized that replace the hydrophobic repeat unit with one that has a PEG chain.[202] With a cationic polyacrylate that is sufficiently hydrophobic, the addition of PEG chains can lead to the creation of SMAPs that are potent antimicrobials that have a very limited effect on blood cells if just the right amount of PEG functionality is incorporated. With such wide availability of the monomers used to synthesize these polymers, there have been multiple specific applications for which these polymers have been investigated. Some of the polymethacrylate-based SMAPs have been shown to be effective against many Gram positive bacteria and have shown promising in controlling staph infections.[203] It has been shown that poly(dimethylaminoethyl methacrylate), a polymer that has been functionalized with a tertiary amine pendant group is rather effective in controlling the growth of *Mycobacteria spp.*, which indicates that it may be useful in the treatment of tuberculosis.[204]

Various studies have been performed to determine the mechanism of action of random/statistical CGP SMAPs. There have been multiple experiments performed that have indicated through the use of model phospholipid membranes that have encapsulated fluorescent dyes that these polymers, just as with all other CAMs, have an affinity for phospholipid membranes that are anionically charged.[198, 199] However, beyond probing for rudimentary electrostatic interactions, there have been multiple reports that shed further

light on what is exactly occurring on the molecular level to cause disruption of the membranes of the cells. Small angle x-ray scattering has indicated that CGP SMAPs actually behave very similarly to CAPs in how they destabilize model bacterial membranes.[205] Both CGP SMAPs and CAPs show that they disrupt membranes by generating similar degrees of negative gaussian curvature across the membranes. However, for the CGP SMAPs to induce the same amount of negative gaussian curvature as CAPs, the CGP SMAPs involved in this process need to be significantly more cationic and hydrophobic than the CAPs because they are less effective in binding and inserting themselves into the membranes due to their unordered sequence.[205] Molecular dynamics simulations have been performed using a similar CGP SMAP that shows that the polymer forms micelle-like aggregates in solution and that the ability of these polymers to function as a SMAMP is dependent upon the ability of the polymer chains to dissociate from the aggregate and bind to the surface of the membrane.[206] Upon binding to the surface of the membrane, the flexible polymer chain then adopts a conformation that results in the formation of a hydrophobic domain that is inside the membrane and a cationic domain that is present on the exterior surface. The presence of the polymers within the membrane results in the electrostatic attraction of anionic phospholipids, which causes a change in the distribution of lipids in the membrane, and leads to inconsistencies in the thickness of the phospholipid bilayer, which lowers the membrane permeability barrier. However, these investigations have not only been limited to the effects of CGP SMAPs on bacterial cells; the mechanism of action against mammalian cells has also been investigated.[207] Evidence indicates that once a CGP SMAP achieves a certain degree of hydrophobicity, the polymer causes the formation of nano-sized holes in the cytoplasmic membrane of red blood cells

that ultimately results in colloid-osmotic lysis of the cell, which is lysis of the cell due to an imbalance of osmolarity caused by unregulated diffusion of low molecular mass solutes across the membrane.

In addition to polymers formed via chain growth polymerizations, there have been various other synthetic polymers that have been investigated to determine their suitability as SMAPs. One such family of polymers are those that are formed via ring opening metathesis polymerization (ROMP) of norbornene derivatives, with a representative structure of these polymers shown in Figure 1.5. Just as with the other SMAPs, the application of polymers using this method of polymerization provide a useful means to determine how different structures can affect antimicrobial efficacy. What makes these polymers different from those that have been previously investigated is that careful monomer design can lead to the creation of a polymer with a sequence of alternating functional groups and a more rigid polymer backbone. In the first investigation of the antimicrobial properties of ROMP SMAPs, a series of homopolymers that contained a norbornene-derived repeat unit that had a cationic pendant group as well as a hydrophobic pendant group.[208] The polymers were synthesized to determine how molecular weight and different hydrophobic pendant groups affected antimicrobial performance and compatibility with red blood cells. Unlike previous studies, the SMAPs of these studies were synthesized to molecular masses over a broad range of molecular weights from ~1,600 Da to ~140,000 Da. Over this range of molecular masses, polymers in the range of ~1,600 Da to ~15,000 actually had better antimicrobial activity than those that were of greater molecular masses. None of the homopolymers synthesized exhibited an ideal high effectiveness in controlling bacterial growth while simultaneously having little effect on

red blood cells. Random/statistical copolymers of the ROMP SMAPs were synthesized and, in doing so, a SMAP with an ideal high antimicrobial efficacy with low hemolytic properties was obtained. Subsequent studies demonstrated that ROMP SMAPs can be produced that have very good antimicrobial activity and little effect on red blood cells using ROMP SMAPs that have guanidinylated cationic groups and through changing the location of the hydrophobic groups on the repeat unit.[209, 210] The most interesting effect of changing pendant groups was obtained when ROMP SMAPs that have two pendant primary ammoniums on each repeat unit. These polymers were capable of selectively affecting Gram positive *S. aureus* over Gram negative *E. coli*.[211] Further investigation revealed that this polymer is selective towards *S. aureus* because it is incapable of penetrating the outer membrane of *E. coli*.

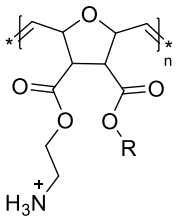


Figure 1.5: The chemical structure of the best characterized family of ROMP SMAPs investigated by Tew *et al.*[209, 211] The R- groups studied have included a wide variety of hydrophobic groups or another amine functionalized pendant group.

There have also been various other polymers that have been investigated for their use as SMAPs. Polymers that have been synthesized via a simultaneous step growth and chain growth polymerization have been studied to determine their usefulness as an antimicrobial.[212] As a result of this polymerization mechanism, the synthesized polymer

incorporated hydrolysable ester bonds interspersed along an aliphatic backbone that had amine functionalized pendant groups. Even though none of the polymers in the series synthesized demonstrated an optimal composition where there was high antimicrobial activity and low hemolysis, these polymers were shown to be degradable and lose their membrane active properties as a result, which could prove useful in certain applications. Polyethylene imines (PEIs) have also been investigated as possible antimicrobials.[213] Even though linear PEIs are well documented as being quite toxic to all cells, low molecular mass (~4,400 Da) linear PEI has been shown to be somewhat more effective against bacteria. However, the introduction of branching points into the architecture of PEI results in a polymer that does not demonstrate significant hemolysis up to very high concentrations and is selective for *S. aureus* over *E. coli*. Branched polymers with a star architecture have additionally been synthesized and demonstrated very desirable properties.[214] The polymers were synthesized using either a generation three or four polyamidoamine dendrimer that served as a macroinitiator for the ring opening polymerization of valine and lysine N-carboxyanhydrides. These polymers demonstrated effectiveness against bacteria at very low concentrations with very good compatibility with mammalian cells.

CHAPTER II

DETERMINATION OF THE FEASABILITY OF USING PENDANT FUNCTIONAL POLYURETHANES AS MIMICS OF ANTIMICROBIAL PEPTIDES

2.1 Introduction

As has been reviewed in the first chapter, many cationic antimicrobial macromolecules (CAMs) have been thoroughly investigated for their ability to affect microbes, their mechanism of action against these microbes, and the feasibility to use these materials in many medical applications. However, even with such a large body of work, there still are many opportunities for improvement, discovery, and innovation beyond what has been previously investigated. One of the greatest needs in combatting antimicrobial resistance will always be the discovery and development of new antimicrobials because bacteria are quite capable of adapting and evolving to meet any challenge to their survival. Due to this fact, there does not exist any single "perfect" antimicrobial that is capable of functioning indefinitely without the development of resistance. Therefore, to continue to successfully treat all infections, it is imperative that there be as broad of a selection of antimicrobials as possible.

With the goal of broadening the selection of antimicrobials, a new family of water soluble antimicrobial polyurethanes were investigated for their feasibility to be adapted as

a CAM, with the chemical structure of these polyurethanes shown in Figure 2.1. These polyurethanes were designed using versatile N-functionalized diol monomers, which were previously developed within the Joy research group, and synthesized to contain amine functionalized cationic repeat units. In the initial report of these novel monomers, they were used to synthesize a broad range of pendant functionalized polyesters and demonstrated a range of physical properties by modulation of these pendant groups.[215] Since this initial report, further investigation has shown that these polyesters can be tuned by changing the pendant group identity and adapted for application as thermoresponsive polymers that show great potential for use in drug delivery,[216-218] as a solvent-free 3D printer ink that can potentially be used in the construction of tissue engineering scaffolds,[219] as a mussel-inspired adhesive that can be used to adhere substrates while submerged under water,[220] and as sugar-functionalized polymers that can interact with protein lectins.[221] Also, beyond polyesters, these diol monomers have been shown to be useful in the creation of other step-growth polymers, such as polyurethanes.[222] This chapter details the preliminary data that was used to indicate that further investigation of polyurethanes constructed from these novel diol monomers is indeed a promising research direction that warrants further investigation. Included in these preliminary results are the synthetic routes used for the successful post-polymerization deprotection of amine functionalized polyurethanes as well as their initial testing for their antimicrobial efficacy.

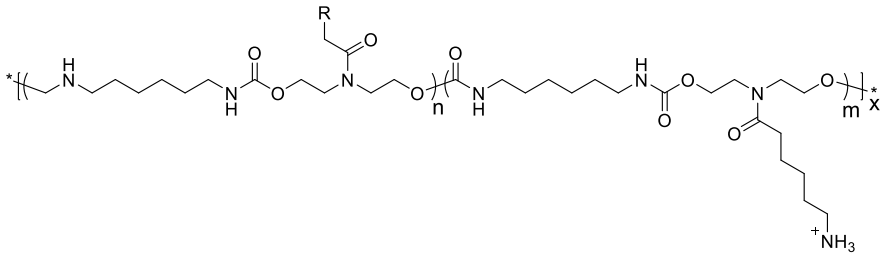


Figure 2.1: The chemical structure of antimicrobial polyurethanes proposed for further investigation as a CAM.

2.2 Experimental Procedures

2.2.1 Materials

All the polyurethanes used in this investigation were synthesized previously and are detailed in the thesis and lab notebook of RLK.[222] The chemicals N,N-dimethylformamide (DMF), methylene chloride, diethyl ether, and trifluoroacetic acid (TFA) were obtained from Fisher Scientific (Waltham, MA, USA) and the 4 M HCl in dioxane and triisoproyl silane (TIPS) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The DMF and methylene chloride was dried by distillation after preliminary drying with CaH_2 and stored over molecular sieves. The Mueller Hinton Broth (MHB) was purchased from Himedia (Mumbai, India). The Trypticase Soy Broth (TSB) was purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). Agar was added separately to the medium and was purchased from Sigma. The bacteria used to determine antimicrobial efficacy were *Escherichia coli* K12 (ATCC 10798) and *Staphylococcus aureus* (25923).

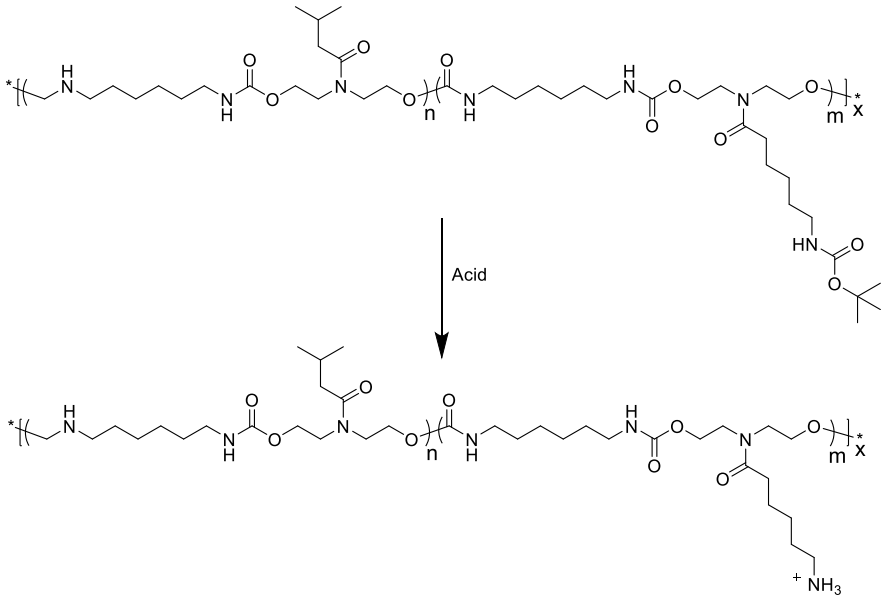
2.2.2 Instrumentation

^{1}H NMR spectra were acquired using a 300 MHz Varian Mercury spectrometer. The chemical shifts are reported in ppm relative to the signal of the residual protons of the deuterated solvent. Molar mass determinations were performed via size exclusion chromatography on a Tosoh EcoSec HLC-8320 instrument equipped with two PSS Gram Analytical SEC columns in series using 25 mM LiBr in N,N-dimethylformamide as the mobile phase at a flow rate of 0.8 mL/min. The column and detector were kept at 50 °C for the experiments. All molar mass values were obtained using a standard curve generated from polystyrene standards. All absorbance values obtained in the measurements of turbidity were obtained using a Hach DR 2800 spectrophotometer.

2.2.3 Amine-functionalized Polyurethane Post-polymerization Deprotection

Deprotection of the *tert*-butyloxycarbonyl (Boc)- protected amines on the pendant groups of the polyurethanes was performed according to Scheme 2.1 and optimized for complete deprotection without degradation of the polymer. In a typical reaction, 100-150 mg of the polyurethane was weighed into a round bottom flask with a magnetic stir bar, capped with a rubber septum, and purged and backfilled with nitrogen three times on a Schlenk line or performed open to ambient atmosphere. The polyurethane was then dissolved in either anhydrous DMF or anhydrous methylene chloride (0.5- 3 mL) and allowed to dissolved. Upon complete dissolution of the polymer, either TFA or 4 M HCl

in dioxane (0.5- 3 mL) was added and allowed to react for a range of tested times (45 min – 24 hr). After reaction, the resulting deprotected polyurethane was either precipitated into cold diethyl ether, the precipitate collected, and kept under reduced pressure to remove residual solvent or directly placed under reduced pressure and the polymer collected after complete solvent removal. Evidence of successful or unsuccessful deprotection was obtained using ^{1}H NMR spectroscopy.



Scheme 2.1: The general reaction scheme for the amine deprotection of the polyurethanes.

2.2.4 Measurement of Antimicrobial Efficacy

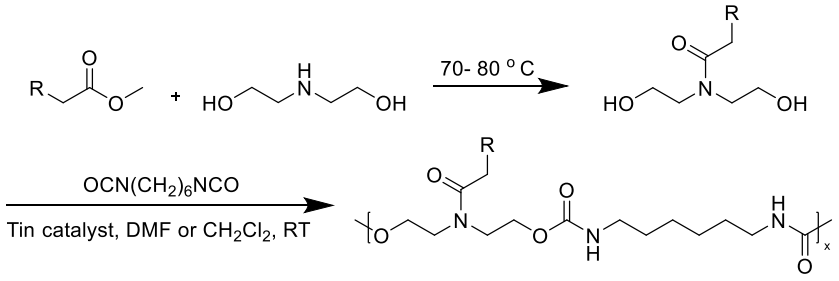
The experiments were performed under the guidance set forth by the Clinical and Laboratory Standards Institute (CLSI) for the Macrobroth Dilution Method.[223] For each experiment, either *E. coli* or *S. aureus* was streaked onto a Trypticase Soy Agar (TSA) plate and incubated overnight at 37 º C. After incubation, three colonies of bacteria were

selected from the plate and used to inoculate 3 mL of TSB in a test tube and placed on a shaker to incubate at either room temperature or 30 º C for ~12-18 hr overnight followed by adjustment of the turbidity of the resulting suspension to $OD_{600\ nm}$ of about 1.0. This adjusted suspension of bacteria was diluted 100 x into MHB to yield ~1 x 10^6 CFU/mL stock suspension. This stock suspension was added to test tubes that were prepared to contain antimicrobial polyurethane dissolved in DI water in a 1:1 ratio (1.5 mL + 1.5 mL). These tubes were measured for their initial $OD_{600\ nm}$ turbidity and then placed to incubate at 30 º C for ~12-18 hr overnight. After incubation, the tubes were measured again for their turbidity and 100 µL removed from each tube and spread onto a TSA plate, the plates incubated overnight at 37 º C, and the colonies on each plate counted and photographed to determine the viability of the bacteria after exposure to the polyurethanes.

2.3 Results and Discussion

The first step in determining the feasibility of these novel pendant functionalized polyurethanes as antimicrobials is to optimize their synthetic route. The synthesis of the N-functionalized diol monomers has been well characterized and the ability to polymerize these monomers into polyurethanes has already been established. The reaction scheme for the monomer synthesis and polymerization is shown in Scheme 2.2.[222] However, the procedure for the successful deprotection of the pendant Boc-protected amines of the Boc-6-aminohexyl pendant functionalized repeat units, which is synthesized to mimic a lysine residue of a peptide, had not been well characterized previously within the Joy lab. The protection of the amine during polymerization is necessary because the amine would

otherwise react with the diisocyanate comonomer during the polymerization to yield either low molecular mass polymer, polymer with a branched architecture, or a crosslinked gel, depending on the stoichiometry of the diol and the diisocyanate monomers. Boc is a commonly used protecting group for amines and is easily removed under a variety of conditions. Most frequently, the protecting group is removed under acidic conditions, with the reaction scheme shown in Scheme 2.1. The Boc- group under these conditions is removed via the acid catalyzed cleavage of the *tert*-butyl group to produce a *tert*-butyl cation as well as carbamic acid. Due to the instability of the carbamic acid and the relative stability of the *tert*-butyl cation, the reaction is driven forward by the degradation of the carbamic acid into an amine and carbon dioxide and the evolution of the carbon dioxide gas.



Scheme 2.2: The general method used for the synthesis of N-functionalized diol monomers and their polymerization into polyurethanes.

With the polyester analogues developed within the Joy lab, there has been success in deprotection of Boc-protected amine containing polyesters using a procedure that involves the dissolution of the polymer in dichloromethane followed by the addition of TFA and TIPS and allowing the reaction to proceed at room temperature for about 1.5 to 2

hr.[215, 224] To test whether this was a feasible synthetic route that could be used with the polyurethanes, a variety of polyurethanes functionalized with the Boc-protected amine pendant group that were synthesized previously by RLK were used for these experiments. The experiments were designed to test the ability of this method to work using 1:1 and 2:1 ratios of solvent:acid. However, in both cases, these experiments appeared to be unsuccessful; it appeared that this deprotection method lead to the degradation of the polyurethanes. Even though the ^{1}H NMR spectrum indicated a successful deprotection by the reduction of the peaks that correspond to the Boc group, with a typical ^{1}H NMR spectrum shown in Figure 2.2, the resulting polymer acquired a strange orangish-yellow liquid-like appearance. This was unexpected because the original polymer was a brittle, uncolored material. The reason for this appearance wasn't explored further but it was hypothesized that this was the result of some unknown side reaction due to oxidation or the presence of water since the procedure used involved performing the reaction open to ambient air.

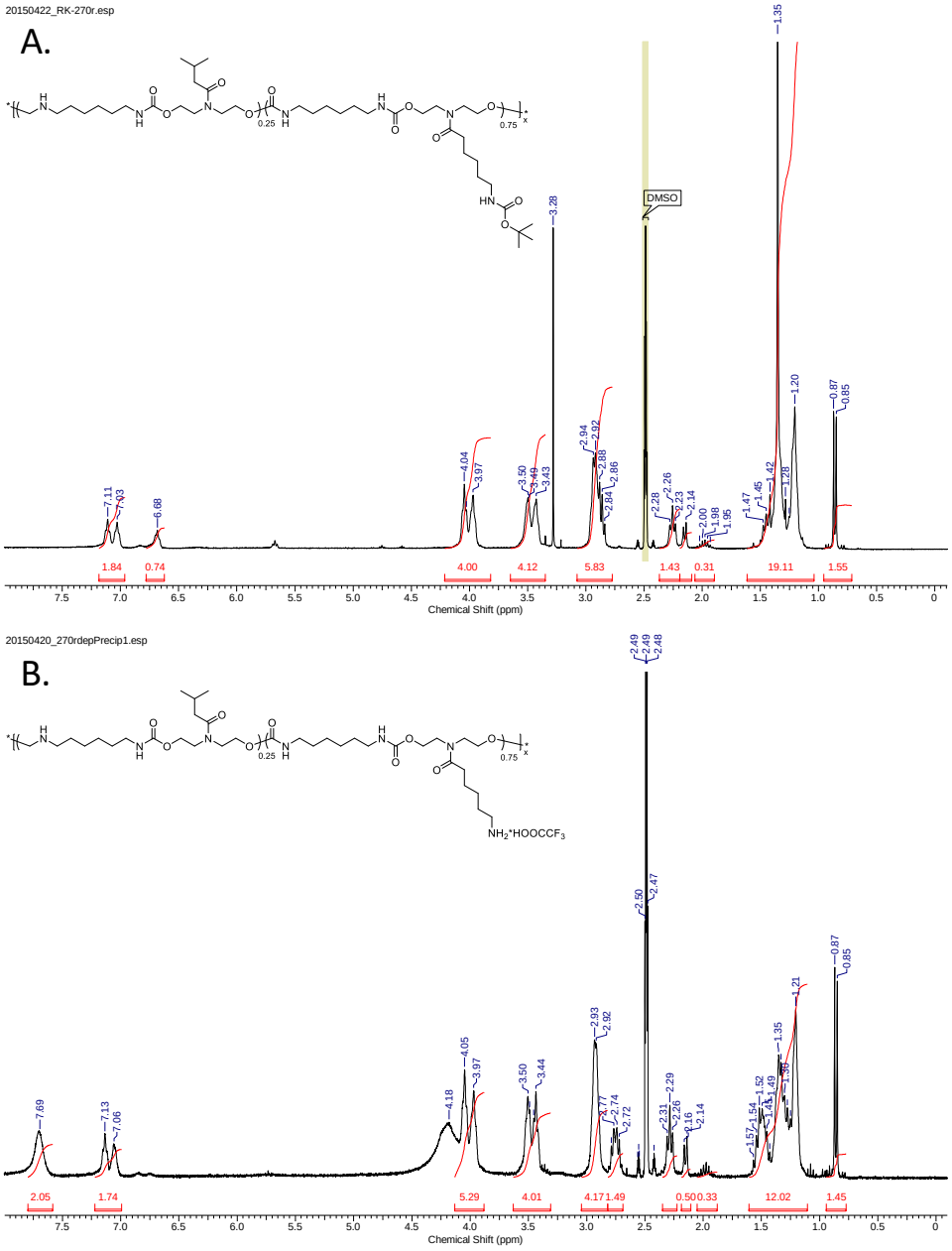


Figure 2.2: The NMR spectra of a polyurethane before (A.) and after (B.) deprotection using TFA.

The failure to deprotect the pendant amine of the polyurethanes using the methylene chloride/ TFA combination lead to a reevaluation of the deprotection procedure. It was decided that further experimentation should involve procedures that use anhydrous

technique and inert, oxygen-free atmosphere. To achieve this, TFA was replaced by a different, anhydrous acid reagent. Within the thesis of RLK, there were preliminary results obtained in trying to deprotect pendant amines on the polyurethanes using anhydrous hydrogen chloride dissolved in dioxane.[222] This alternative approach to deprotection was originally pursued by RLK not because of problems encountered by TFA catalyzed deprotection but instead to produce a different pendant amine salt from the deprotection reaction. The usage of TFA with peptides, which these polyurethanes are synthesized to mimic, is discouraged since peptides that contain the TFA/ amine salt have been shown to interfere with FTIR spectroscopy peptide conformation measurement, which is one of the most frequently used methods to measure peptide secondary structure.[225] Even though this is not relevant to the polyurethanes, this method was used to try and make the polyurethanes more closely resemble peptides. The procedure in the thesis of RLK involved the dissolution of the polyurethane in DMF followed by the addition of 4M hydrogen chloride in dioxane and reaction for 45 min at room temperature.[222] Therefore, this method, along the aforementioned anhydrous technique, was attempted. However, this procedure resulted in inconsistent deprotection of the amine; it was difficult to obtain completely deprotected polyurethane and repeatable results were not produced. Experiments were performed using different ratios of solvent and hydrogen chloride solution and different concentrations of dissolved polyurethane. Yet, none of these parameters had a significant effect in increasing the consistency of the degree of amine deprotection. It is believed that the reason for these inconsistencies may be due to the acid/base instability of DMF. So, the DMF was replaced with methylene chloride and repeatable, successful deprotection of amines was obtained. The final optimized procedure for the amine deprotection uses a 2:1 ratio of methylene

chloride: 4M HCl in dioxane with an optimal concentration of ~65 mg/mL polymer reacted for 45 min. During the reaction the polyurethane precipitates out of solution and can be used without any further purification besides removal of solvent from the sample by placing it under reduced atmosphere. The precipitation during the reaction did not cause any problems with incomplete deprotection as indicated by ^{1}H NMR, with representative before and after deprotection spectra shown in Figure 2.3.

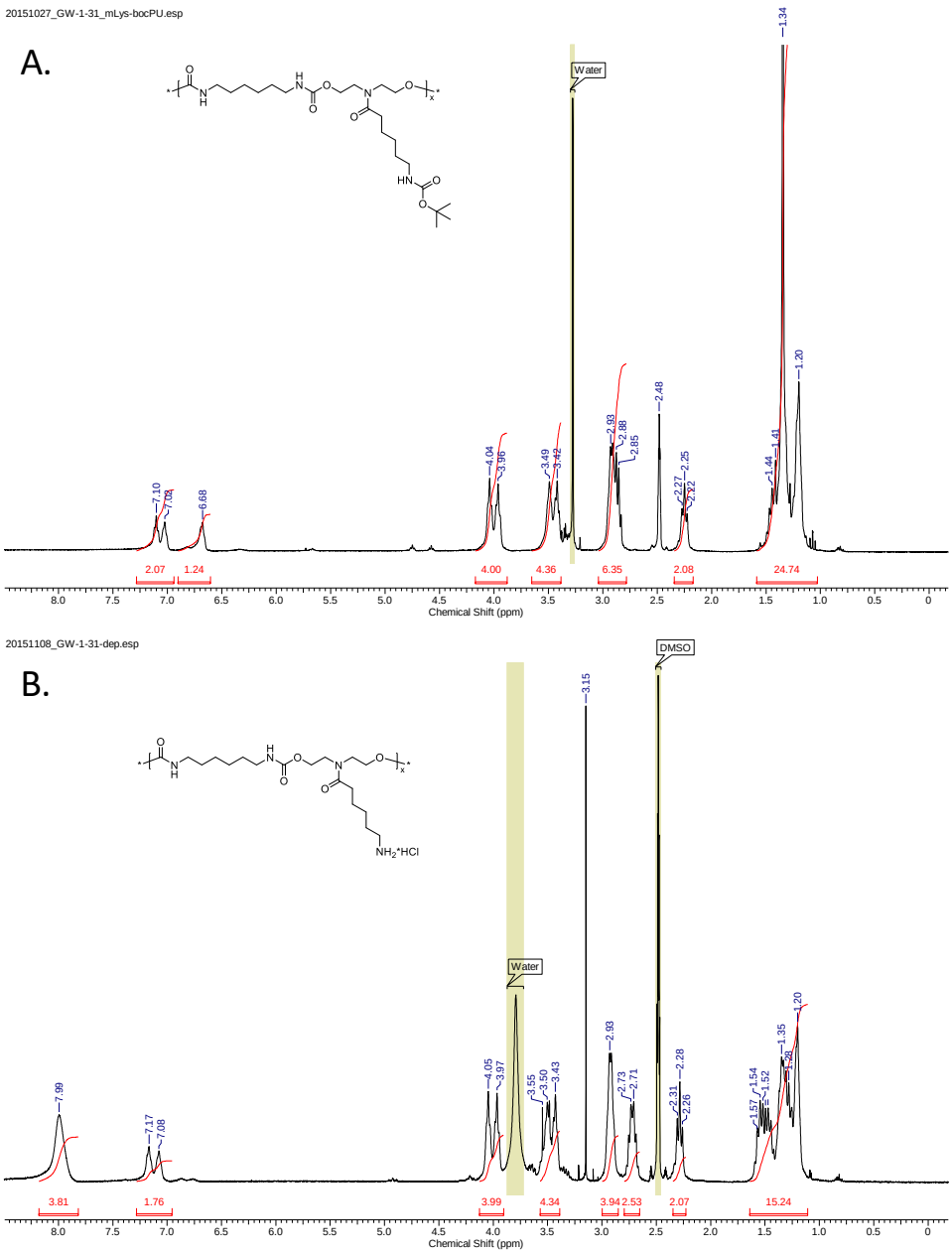


Figure 2.3: The NMR spectra of a polyurethane before (A.) and after (B.) deprotection using 4 M HCl in dioxane.

Additionally, another important factor to consider in making these pendant functionalized polyurethanes was made apparent when remeasuring the molecular masses of those synthesized by RLK. For many of the polyurethanes, their measured molecular

mass did not match with what was recorded shortly after they had been synthesized. A comparison of the originally recorded molecular mass and the molecular mass observed by the most recent measurement is shown in Table 2.1. Note, the time between these measurements was ~1-1.5 years and during this time the polyurethanes were kept neat in a 5 ° C refrigerator in a glass screw cap vial. Although it is difficult to determine the exact reason for the discrepancy in molecular masses, it has been surmised that this may have been the result of the reactivity of the isocyanate end groups of the polyurethane since there were no reagents added at the end of the reaction to cap these reactive functional groups, according to the procedures of RLK. Therefore, it must be noted that future polymerizations need to have a step where these reactive end groups are terminated with a less reactive moiety.

Table 2.1: The number average molecular mass (M_n) of polyurethanes synthesized by RLK as they were initially reported and the values obtained after remeasurement. Note, all of the polyurethanes contained the pendant Boc-protected amine and were copolymers of the same chemical structure shown in Scheme 2.1.

Polyurethane Name (According to RLK)	***Initially Reported M_n (kDa)***	***Actual M_n (kDa)***
RK-1138	33	39
RK-274	33	42
RK-370r2	30	30
RK-170r	18	54
RK-270r	17	14
RK-3138	17	15
RK-170r2	4.3	17
RK-270r6	3.7	4.0
RK-370r4	3.8	4.0

To determine whether these polyurethanes can perform as an antimicrobial, two polyurethanes were selected from what was synthesized by RLK for this preliminary investigation. These polyurethanes selected were of two different molecular masses, one is ~8 kDa and the other is ~16 kDa. Both of these polyurethanes were synthesized to contain 50% of repeat units functionalized with the 6-aminohexyl pendant group while the remaining 50% of the repeat units were functionalized with an isobutyl pendant group, which mimics the valine residue of peptides. These two polyurethanes were selected as candidates for this initial evaluation because they both were synthesized using the same procedure according to the notes of RLK, were in the range of molecular masses that have been found to be most useful among other polymers that use different backbone chemistry, and contained some repeat units with a hydrophobic pendant group, which has been indicated as an important feature for having antimicrobial activity.[198, 208]

Multiple different tests and procedures have been developed for the determination of chemicals as antimicrobials. However, the method that seemed to be the most easily adapted for the evaluation of the antimicrobial efficacy of these polyurethanes is known as the broth dilution method. This method involves the testing of many concentrations of the antimicrobial dissolved in the medium of a suspension culture of bacteria. The method is designed to determine the smallest concentration of antimicrobial that is necessary to prevent the growth of bacteria, which is known as the minimum inhibitory concentration (MIC). The growth of bacteria in this type of testing is determined through turbidity measurements of the medium, where greater growth is indicated by increasing amounts of turbidity, and can be quantified through the measurement of optical density at 600 nm ($OD_{600\ nm}$). This method was applied to the two selected polyurethanes and concentrations

ranging between 10 and 100 μg/mL of each polymer were tested against *E. coli* and *S. aureus*, with the results of this testing shown in Figure 2.5. As shown in this figure, there are two points at which the measurement of $OD_{600\,nm}$ have been performed for each sample; after the initial addition of bacteria and polyurethane together and after overnight (12-18 hr) incubation. These two measurements were performed, as opposed to just a single measurement after incubation, because a significant amount of initial turbidity was observed upon addition of the higher concentrations of the polyurethane due to precipitation/complexation of the polyurethane induced by interaction with the components within the culture medium. By comparing the initial and after overnight incubation turbidity values, it can be observed that these polyurethanes demonstrated an MIC of 25 and 50 μg/mL against *E. coli* for the 16 kDa and 8 kDa polyurethanes, respectively, and had very little effect against *S. aureus* with no MIC observed over the range of tested concentrations. These findings are promising, as it shows that these polyurethanes are able to exert an antimicrobial effect and may have some unique property that allows them to selectively affect one type of bacteria over another.

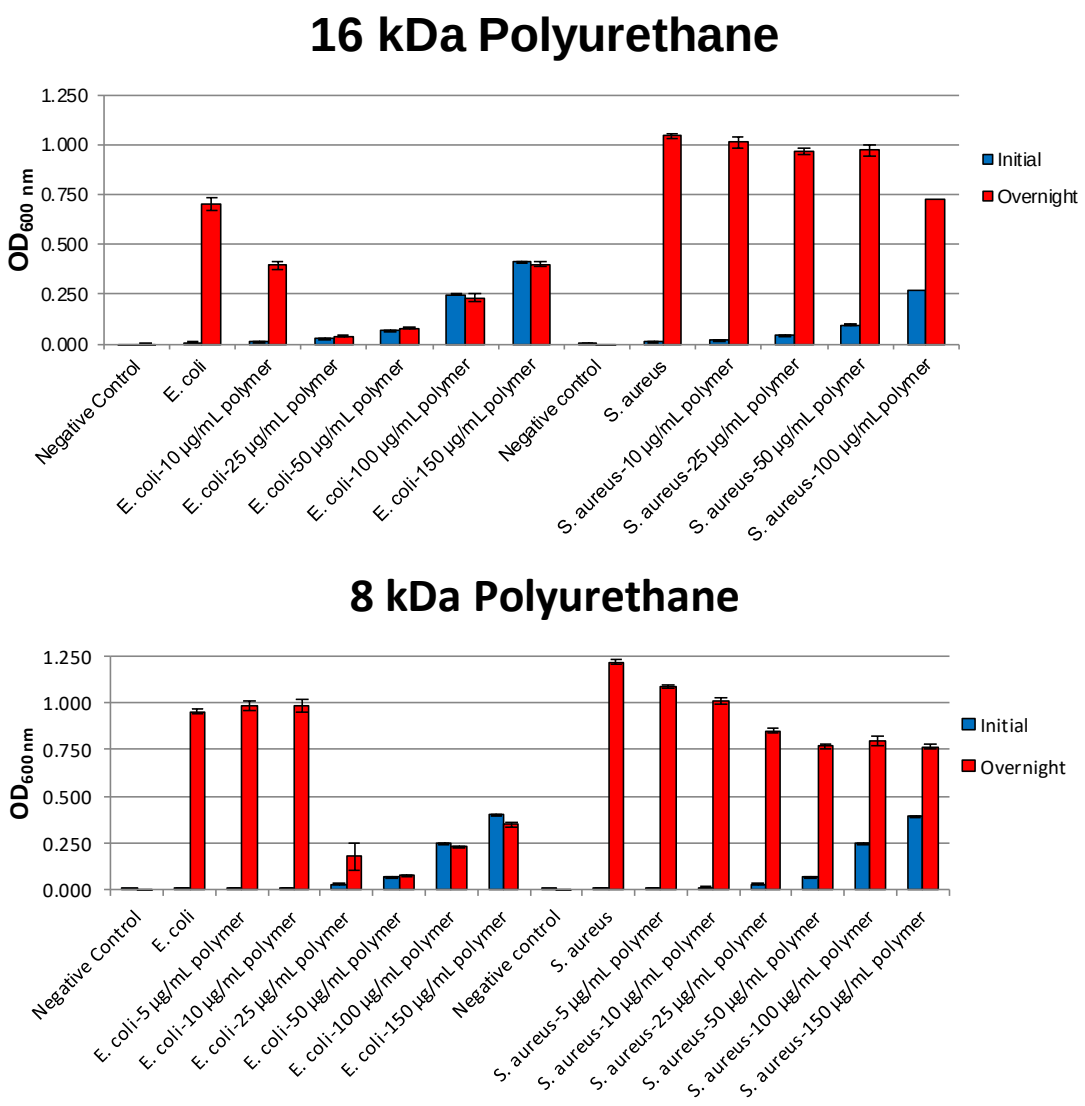


Figure 2.4: The measured $OD_{600\ nm}$ turbidity values obtained after initial addition of either *E. coli* or *S. aureus* to solutions of either 16 kDa or 8 kDa polyurethane and after overnight incubation of the bacteria with the polyurethanes.

To further characterize the antimicrobial behavior of the polyurethanes, a sample was removed from each tested concentration of polyurethane after the MIC experiment was completed, spread onto a TSA plate, incubated overnight at 37 º C, and the resulting plate was photographed. This was done to try and provide a general understanding as to

whether the polyurethane is just preventing the growth of bacteria, *i.e.* exerting a bacteriostatic effect, or is capable of killing the bacteria, *i.e.* showing bactericidal behavior, which cannot be indicated by MIC measurement. The images of each plate after overnight incubation are shown in Figure 2.6 and 2.7 for both *E. coli* and *S. aureus* and for each polyurethane at each concentration tested. The sample that was spread onto each plate was a 10x dilution of each tested concentration of polyurethane. Since the number of colonies that can be counted on a single plate is on the range of ~300-500 and the concentration of bacteria used in the initial inoculation of the plate is ~$5x10^5$ CFU/mL, it can be estimated that if the number of colonies on the plate are countable, then ~99% of the bacteria have been killed. While, if there are no bacteria present on the plate, it indicates that greater than 99.998% of bacteria had been killed. So, from the images within the figure, it can be observed that the polyurethanes do exhibit bactericidal behavior against *E. coli* at some concentrations of the polyurethane. Note, the plates that are completely covered in a hazy growth of bacteria are uncountable since they contained too many bacteria and, therefore, the sample taken did not show a reduction of viability to less than 1% of the initial inoculation concentration of bacteria. Just as with the MIC values, this data indicates that there appears to be a minimum concentration at which significant killing of ~99% of bacteria can be achieved. For the 8 kDa polyurethane, the plates show a countable number of colonies at 100 µg/mL, where the counted colonies indicated that ~98.5-99.3% of bacteria were killed. In contrast, for the 16 kDa polyurethane, a countable number of colonies was observed at 50 µg/mL, where the counted colonies indicated that ~99.8-99.9% of bacteria were killed. However, for both of the polyurethanes there was not any significant action against *S. aureus*.

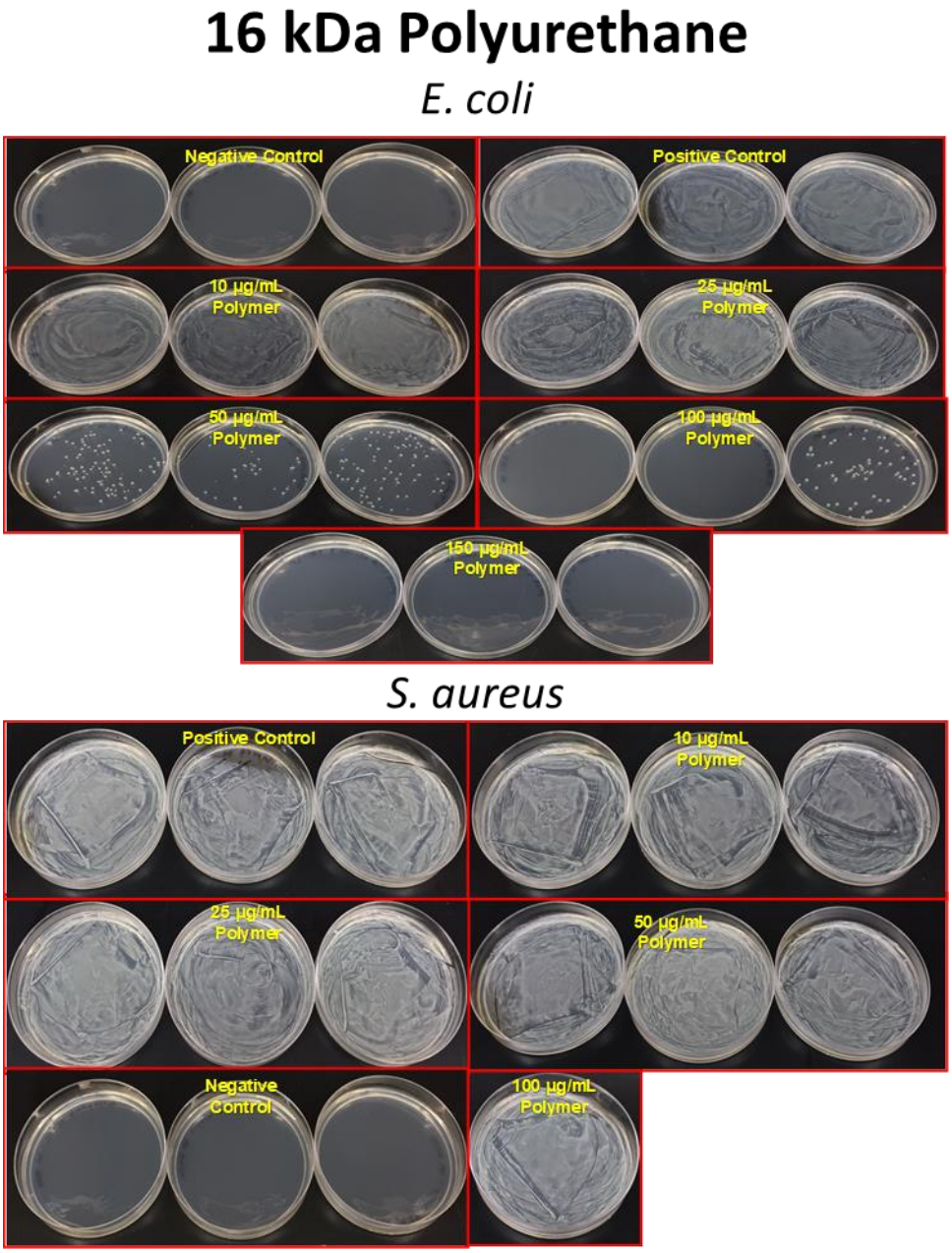


Figure 2.5: Images of bacterial growth on TSA plates after spreading 100 µL of medium removed from overnight liquid cultures of bacteria that were exposed to various concentrations of the 16 kDa polyurethane.

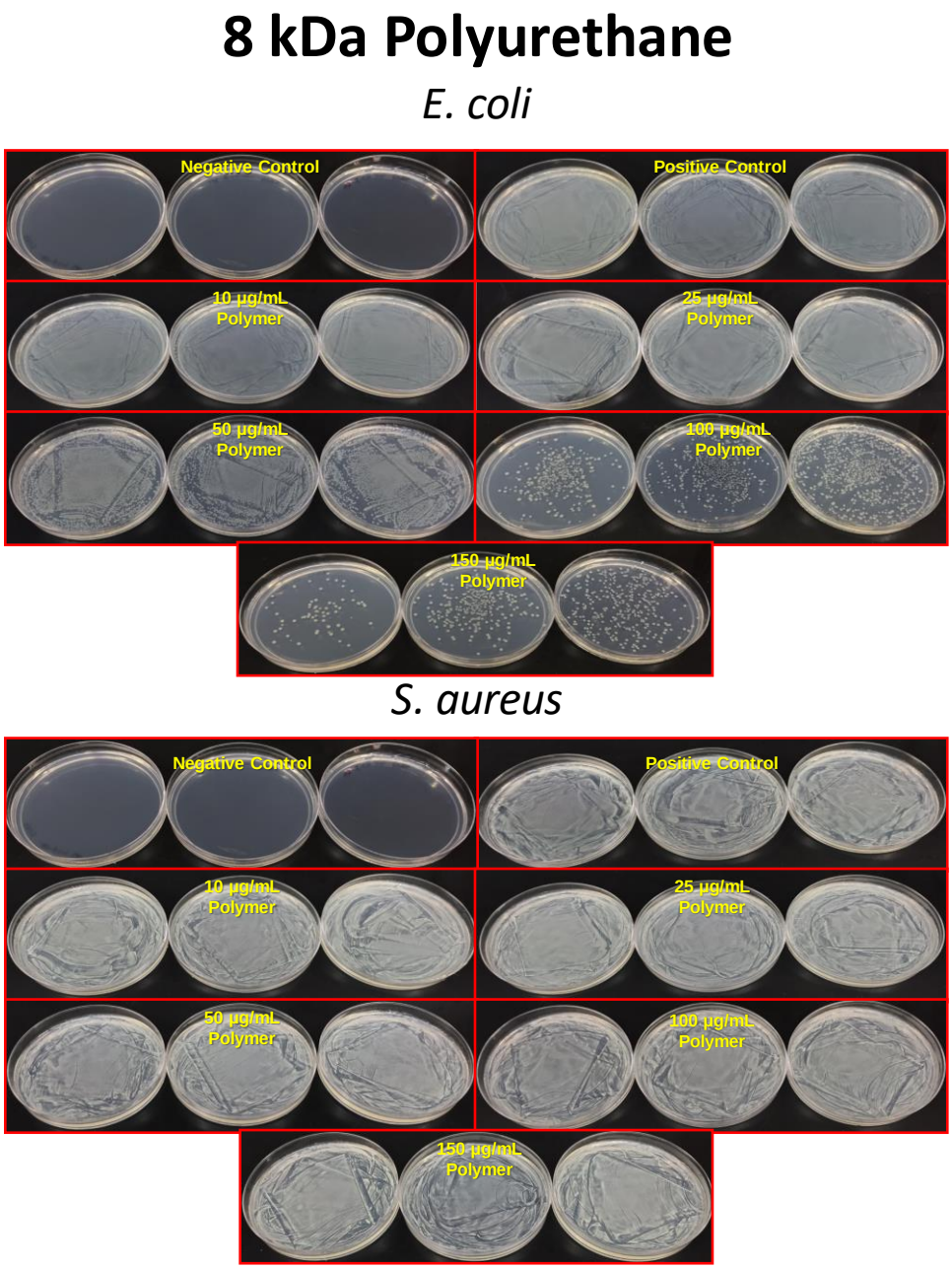


Figure 2.6: Images of bacterial growth on TSA plates after spreading 100 µL of medium removed from overnight liquid cultures of bacteria that were exposed to various concentrations of the 8 kDa polyurethane.

2.4 Conclusion

An adequate method for the deprotection of Boc-protected amines on pendant groups of polyurethanes has been optimized and the resulting cationic polyurethanes were shown to have some rather unique antimicrobial properties. Successful, complete deprotection of the pendant amine groups on the polyurethanes was achieved by dissolving of the polyurethane in methylene chloride, subsequent addition of 4 M HCl in dioxane, and allowing the reaction to proceed at room temperature for 45 min. The polyurethanes that resulted from this deprotection were found to be water-soluble and capable of demonstrating a bactericidal effect on *E. coli* but had very little effect on *S. aureus*. Future studies of these polymers should focus on gaining a better understanding of the structure/property relationships and how different pendant groups can be used to modulate their effectiveness as an antimicrobial. Also, investigation of the mechanism of action of the polyurethanes on bacteria as well as their effects on mammalian cells should be part of any future study.

CHAPTER III

BACTERICIDAL PEPTIDOMIMETIC POLYURETHANES WITH REMARKABLE SELECTIVITY AGAINST *ESCHERICHIA COLI*

*The following chapter was previously published. It has been reprinted and adapted from ACS Biomaterials Science and Engineering, 2017, 3 (10), pp 2588-2597. Copyright 2017 American Chemical Society. It was originally published with the following co-authors:

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3.1 Introduction

The rise and increasing prevalence of drug resistant and multidrug resistant strains of pathogenic bacteria is leading to a crisis in our ability to treat infectious disease.[19] The Centers for Disease Control estimates that annually within the United States there are two million infections caused by drug resistant bacteria, of which ~23,000 die due to an inability to successfully treat the infection.[19] These numbers are expected to rise dramatically in the near future due to an increasing prevalence of drug-resistance in bacterial pathogens, such as methicillin-resistant *Staphylococcus aureus* and carbapenem-

resistant *Enterobacteriaceae* (CRE).[226, 227] This problem will be further compounded by the emergence of new forms of resistance, such as the colistin-resistance observed in a Chinese strain of *Escherichia coli*, and the potential movement of such resistance into other pathogens by horizontal gene transfer, particularly into strains where it remains the treatment of last resort, such as CRE.[228] The development of new antimicrobials is urgently needed because of the lack of new classes of antimicrobials within the development pipeline that are capable of overcoming current antibiotic resistance profiles.[229] It is predicted that in the current scenario deaths as a result of antibiotic resistant infections could exceed 10 million annually by 2050.[7]

One promising source for new antimicrobials are antimicrobial peptides (AMPs). These peptides are typically ~10-50 amino acid residues in length and amphipathic; they contain an abundance of charged amino acid residues and are composed of at least 30% hydrophobic amino acid residues.[87] Such AMPs have since been found in virtually all forms of life as an innate resistance to bacterial infection. AMPs are thought to express their antimicrobial activity through the abundance of cationic amino acid residues that allows for their selective interaction with bacterial membranes, leading to membrane disruption.[88,93] Specific AMPs have also been implicated in inhibiting bacterial cell wall formation,[129] DNA replication and protein synthesis,[122] and selectively binding and inhibiting proteins involved in critical cell function.[230] Despite this impressive arsenal of antimicrobial activities, to date there are no AMPs that have successfully been developed and approved for clinical use.[89, 231-233] Pexiganan, a synthetic AMP which is derived from Magainin found in *Xenopus laevis*, has come the closest to clinical application out of all

AMPs, even though it hasn't been able to pass Phase III of FDA clinical trials as a treatment for diabetic foot ulcers.[174]

In order to circumvent some of the challenges of commercializing AMPs, such as their high cost of production and susceptibility to rapid proteolytic degradation, a variety of synthetic oligomers and polymers which mimic AMPs have been investigated. Various polyacrylates and polymethacrylates,[58, 197, 200-203, 205-207, 234-242] polyacrylamides,[199, 243] synthetic polyamides,[189-193, 195, 196, 244, 245] polycarbonates,[246-248] metathesis ring opened polymers,[209, 210] and various other polymers and copolymers[212, 249-257] have been explored for their antimicrobial properties and compatibility with mammalian cells *in vitro* and *in vivo*. Such data indicate that an ideal balance of cationic and hydrophobic nature of the polymer must be achieved for optimal antimicrobial activity. For example, from studies with polymethacrylates, Kuroda *et al* suggest that there is an optimal length for the hydrocarbon linker between the pendant cationic amine and the backbone.[241] Additionally, Mor *et al* have reported simple acyl-lysine oligomers that show effective broad spectrum antimicrobial activity, demonstrating that in some cases minimal design features can confer antimicrobial activity.[188] Polymer molar mass also influences antimicrobial activity, with higher molar mass corresponding with higher antimicrobial activity, but with the trade-off that toxicity also increases with molar mass.[258] Additional parameters, such as the addition of a polar uncharged moiety to improve compatibility with mammalian cells, has been demonstrated.[192, 202]

In previous work, we have reported the development of a modular 'peptide-like' polyester platform that is distinguished by the ability to design in various amino acid-like pendant groups by the use of pendant functionalized diols.[215] In the current work, we have

applied diols functionalized with mimics of lysine (mLys) and valine (mVal) pendant groups to synthesize cationic, non-segmented polyurethanes and examined their effect as AMP mimics. In these novel polyurethanes, the balance of hydrophobic and cationic character was modulated by varying the pendant groups to gain a better understanding of how this balance affects the antimicrobial properties of these polymers. We varied the monomers to synthesize mLys/mVal polyurethanes composed of 100/0, 90/10, 70/30, and 50/50 ratios of each repeat unit and synthesized two molar mass ranges, a ~9-13 kDa lower molar mass range (LM) and a ~22-31 kDa higher molar mass range (HM), for each composition. These non-segmented polyurethanes were tested for their antimicrobial efficacy by determining each of their minimum inhibitory concentrations, or smallest amount necessary to prevent the growth of *E. coli* and *S. aureus,* along with growth assays, hemocompatibility, and eukaryotic cell viability to understand the impacts of various polymer chemistries on antibacterial activity.

3.2 Experimental Procedures

3.2.1 Materials

All solvents and reagents used were purchased from Fisher Scientific (Waltham, MA, USA) unless otherwise specified. The 6-aminohexanoic acid, diethanolamine, and methyl isovalerate were obtained from Alfa Aesar (Ward Hill, MA, USA); hexamethylene diisocyanate (HDI), 2-nitrophenyl-β-D-galactopyranoside (ONPG), and the anhydrous 4N HCl in dioxane were obtained from Acros Organics (Fair Lawn, NJ, USA); tin 2-

ethylhexanoate and dibutyltin dilaurate were obtained from Sigma-Aldrich (St. Louis, MO, USA); di-tert-butyl dicarbonate was obtained from Oakwood Chemical (Estill, SC, USA); and thionyl chloride was obtained from TCI (Tokyo, Japan). Methylene chloride was dried by distillation after preliminary drying with CaH_2 and stored over molecular sieves. Dry methanol was obtained from EMD Millipore (Billerica, MA, USA). The HDI was used as received. For the preparation of phosphate buffered saline (PBS) and M9 minimal medium (M9MM), sodium phosphate dibasic, potassium phosphate monobasic, and potassium chloride were obtained from Sigma (St. Louis, MO, USA); magnesium sulfate from EMD Millipore; and calcium chloride from Sigma-Aldrich. The Mueller Hinton Broth (MHB) was purchased from Himedia (Mumbai, India). The Trypticase Soy Broth (TSB) was purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). Agar was added separately to the medium and was purchased from Sigma. The bacteria used were *Escherichia coli* K12 (ATCC 10798), *E. coli* (ATCC 25922), and *E. coli* P1C4 (isolated from a healthy 28-year-old male and verified for its identity via Gram staining and a BBL Crystal Enteric/Non-Fermenter kit from Becton-Dickinson), and *Staphylococcus aureus* (ATCC 25923). For the hemolysis assays, defibrinated sheep blood was purchased from Hardy Diagnostics (Santa Maria, CA, USA) and the Triton X-100 was acquired from Fisher Scientific. For the culture of the cells, penicillin/streptomycin, fetal bovine serum, and DMEM were obtained from HyClone (Chicago, Il, USA).

3.2.2 Instrumentation

^{1}H NMR spectra were acquired using a 300 MHz Varian Mercury spectrometer. The chemical shifts are reported in ppm relative to the signal of the residual protons of the deuterated solvent. Molar mass determinations were performed via size exclusion chromatography on a Tosoh EcoSec HLC-8320 instrument equipped with two PSS Gram Analytical SEC columns in series using 25 mM LiBr in N,N-dimethylformamide as the mobile phase at a flow rate of 0.8 mL/min. The column and detector were kept at 50 °C for the experiments. All molar mass values were obtained using a standard curve generated from polystyrene standards. All absorbance and fluorescence spectroscopy for the biological experiments were obtained using either a BioTek Synergy H1 or a Molecular Devices Spectramax M2 multimode plate reader.

3.2.3 Synthesis of Amino Acid-like Diol Monomers

The mLys monomer was synthesized according to previously reported methods.[215] In brief, 6-aminohexanoic acid (10 g, 76.2 mmol) was dissolved in a large excess of anhydrous methanol (125 mL) and thionyl chloride (22.9g, 193 mmol) was added dropwise while submerged in an ice bath. After overnight reaction at room temperature, the excess methanol was removed and the methyl-6-aminohexanoate product was rinsed multiple times with methylene chloride. The partially purified product along with triethylamine (19.2 g, 190 mmol) was then dissolved in 1:1 (by volume) dioxane and water and di-tert-butyl dicarbonate (20.5 g, 91 mmol), also dissolved in 1:1 (by volume) dioxane and water,

was added dropwise (200 mL of total solvent). The reaction was allowed to stir overnight and the methyl-6-(amino-N-tert-butoxycarbonyl)hexanoate product was purified by removing the solvent, performing ethyl acetate and water extractions, and drying under vacuum (17.8 g, 95.5%). The obtained, pure product was reacted with diethanolamine (15.9 g, 151 mmol) neat and under vacuum at 80°C overnight to yield mLys monomer. Following the reaction, purification of the mLys monomer was performed via silica gel liquid column chromatography using 9:1 methylene chloride:methanol followed by drying of the pure mLys fractions under vacuum (17.5 g, 75.7%). The purified product was characterized by ^{1}H NMR, shown in Figure 3.1.

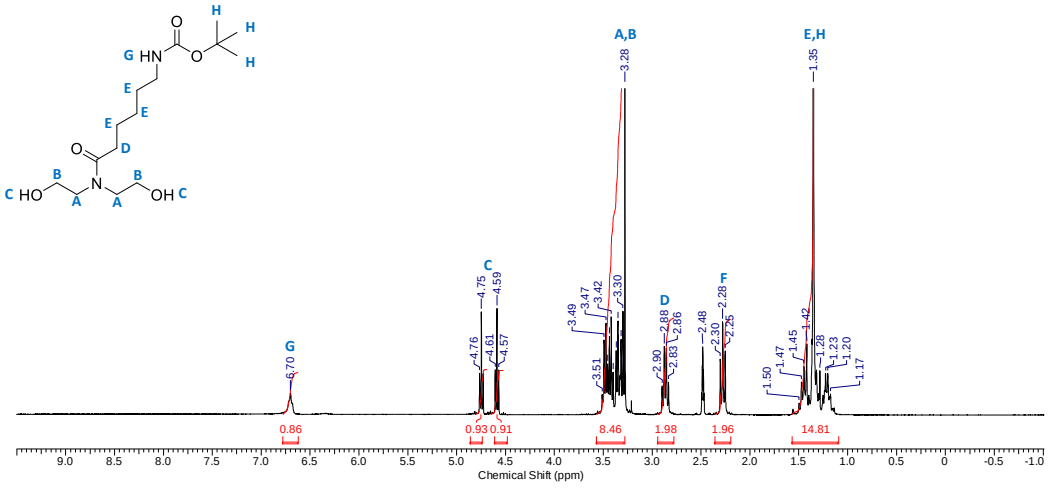


Figure 3.1: ^{1}H NMR spectrum of mLys monomer.

The mVal monomer was synthesized by reacting methyl isovalerate (4.26 g, 36.8 mmol) with diethanolamine (7.72 g, 74 mmol) neat in a microwave reactor set to 60 W and 70 °C for two hours under vigorous stirring. The obtained mVal monomer was purified from the reaction mixture via silica gel liquid column chromatography using 9:1 methylene

chloride:methanol followed by drying of the pure mVal fractions under vacuum (3.57 g, 51%). The purified product was characterized by ^{1}H NMR, shown in Figure 3.2.

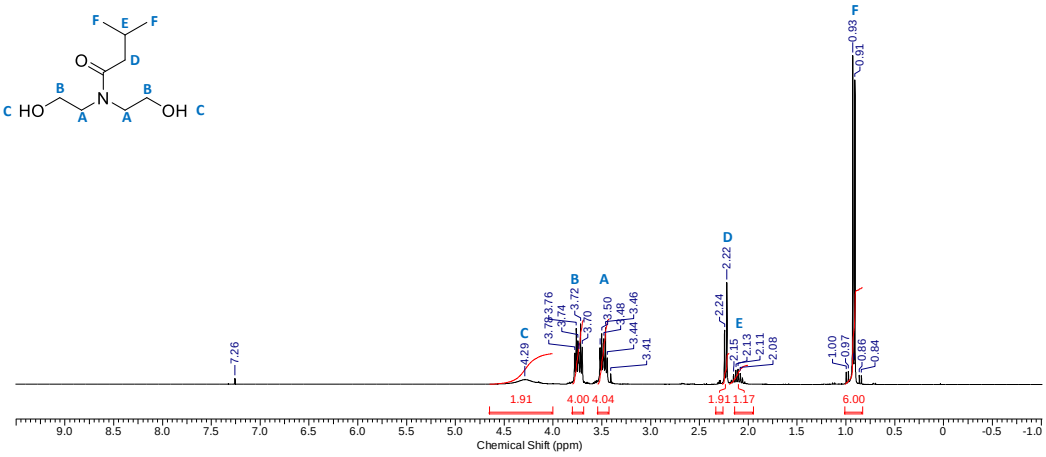


Figure 3.2: ^{1}H NMR spectrum of mVal monomer.

3.2.4 Polymerization and Post-polymerization Deprotection of Antimicrobial Polyurethanes

For a typical polymerization, mLys and/or mVal (1.56 mmol) was loaded along with hexamethylene diisocyanate (250 µL, 1.56 mmol) and either tin(II) 2-ethylhexanoate or dibutyltin dilaurate (5 µL) into a 1 necked round bottom flask. The flask was then capped with a rubber septum and vacuum purged and backfilled with dry N_2. Anhydrous methylene chloride (4 mL) was added to the flask and the reaction was allowed to stir at room temperature under N_2 overnight. After overnight reaction, methanol (0.5 mL) was added and the reaction mixture was allowed to stir at room temperature and open to ambient air for at least 1 hour. The polymer was isolated and purified by precipitation into cold ether. The obtained polymer was dried under vacuum and characterized via ^{1}H NMR and SEC. A typical ^{1}H NMR spectrum is shown in Figure 3.3.

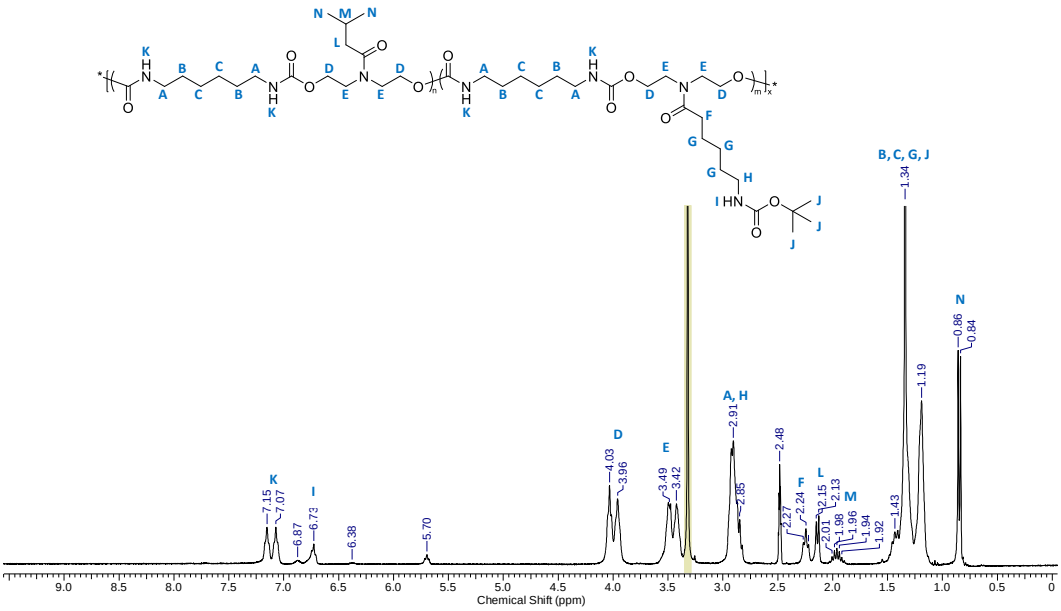


Figure 3.3: A typical ^{1}H NMR spectrum of a synthesized polyurethane.

All post polymerization amine deprotection reactions were performed in a similar manner as previously reported.[259] A typical reaction was performed by weighing ~250 mg of polyurethane into a 50 mL round bottom flask. The flask was capped with a rubber septum and vacuum purged and back filled with dry N_2. Anhydrous methylene chloride (2 mL) was added to the flask and allowed to stir until the polymer was completely dissolved. Then, anhydrous 4N HCl in dioxane (1 mL) was added dropwise to the flask and allowed to react at room temperature under N_2 for 45 minutes. After reaction, the reaction mixture was placed under vacuum overnight. The resulting deprotected polyurethane was dissolved in 18.2 MΩ*cm H_2O, transferred to a container for storage, and lyophilized. The obtained amine functionalized polyurethane was characterized via ^{1}H NMR. The shift in peak positions between the protected and deprotected polyurethanes is shown in Figure 3.4.

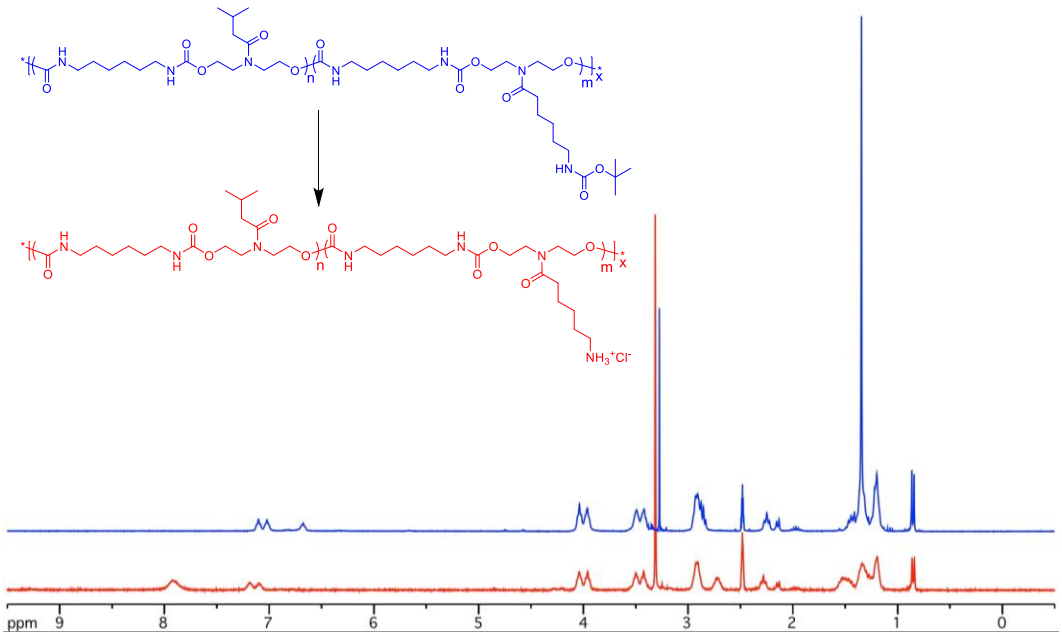


Figure 3.4: Typical ^{1}H NMR spectra obtained before and after acid catalyzed amine deprotection. The blue spectrum was obtained prior to deprotection and the red spectrum was obtained after deprotection.

3.2.5 Minimum Inhibitory Concentration (MIC) Testing

The MIC of the antimicrobial polyurethanes was performed according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) with some modifications.[223] An overnight culture of either *E. coli* or *S. aureus* was used to inoculate 3 mL of TSB and incubated at 37 °C with shaking (130 rpm) until the culture reached an OD_{600} of 0.4-0.7. This culture was then diluted to OD_{600} ~0.10-0.12 and diluted 100x further in either MHB or 2x M9MM for a working suspension of ~$1x10^{6}$ colony forming units (CFU)/mL. This bacterial suspension was mixed in a 1:1 ratio with the antimicrobial polyurethanes in a 96-well plate and controls dissolved in sterile deionized (DI) water. The

plates were then sealed, measured for initial turbidity (OD_{600}), and incubated at 37 °C overnight (~16 hours). After overnight culture, turbidity was measured and MIC determined as the lowest concentration of polymer that prevented bacterial growth.

3.2.6 Time/kill assays

A cell suspension of *E. coli* K12 at ~$1x10^6$ CFU/mL was set up as described above. This suspension was added 1:1 to test tubes containing solutions of either antimicrobial polyurethane or Pexiganan dissolved in sterile DI water to a final concentration of 2X MIC. Sterile DI water and a 100 μg/mL solution of ampicillin served as growth and bactericidal controls, respectively. The cultures were then incubated in triplicate with shaking at 37 °C and after 0.5, 1, 2, 4, 8, and 24 hours of incubation samples were removed, serially diluted in PBS for plate counts of CFUs.

3.2.7 Cytoplasmic Membrane Permeability Assays

A cell suspension of *E. coli* K12 at OD_{600} ~0.10-0.12 was set up as described above with one modification; it was cultured in suspension in TSB supplemented with 2% lactose. The suspension was centrifuged at 2000 x g for 10 min., the supernatant decanted, and the cell pellet resuspended in PBS. This *E. coli* suspension was mixed in a 1:1 ratio with solutions that contained various concentrations of antimicrobial and ONPG dissolved in PBS in a 96-well plate (final concentration of ONPG was 1.5 mM after mixing). Promptly

after addition, the 96-well plate was placed onto a multi-mode plate reader set to measure the absorbance at 420 nm every 5 min. for 200 min. at 25 ° C.

3.2.8 Sheep Blood Hemolysis Assays

The hemolysis assays performed were adapted from a previously reported method.[260] The sheep blood was prepared for the assay by isolating the intact cells via centrifugation at 500 x *g* for 10 minutes at 5°C. The supernatant was aspirated carefully without disrupting the cell layer and replaced with an equivalent volume of 150 mM NaCl, followed by gentle cell suspension. This process was repeated until the supernatant no longer contained any observable hemoglobin (typically, 5-7 washes were required). After the final centrifugation, the bottom layer was gently resuspended in PBS. This rinsed suspension was then diluted 25x into PBS to yield the working suspension of sheep blood. This working suspension was added in a 1:1 ratio to wells of a 96 well conical bottomed plate which contained antimicrobial polyurethane and control samples dissolved in PBS. The antimicrobial polyurethane and control solutions were prepared just before preparing the stock suspension of blood by performing a 1:1 serial dilution within the well plates. For the experiment, wells which contained a final concentration of 1% Triton X-100 and only PBS were used to prepare 100% and 0% hemolysis controls, respectively. After addition of the working blood suspension to the well plate, the plate was sealed and placed into a 37°C incubator for 1 hour. Following incubation, the plate was centrifuged 500 x *g* for 10 minutes at 5 °C. The supernatant was removed from each plate and the amount of hemolysis was quantified at an absorbance of 450 nm.

3.2.9 Eukaryotic Cell Viability Assays

Mouse fibroblast NIH 3T3 cells were cultured using high glucose DMEM medium supplemented with 10% FBS and 1% of a 10,000 U/mL penicillin and 10,000 μg/mL streptomycin solution. The cells were grown at 37 °C in 5% CO_2 until reaching 80-90% confluence. The cells were harvested using 0.25% trypsin solution and seeded into 96 well plates at a density of 25,000 cells/cm^2. After allowing the cells to adhere overnight (~16 hours), the medium was replaced with a 2:1 mix complete growth medium: antimicrobial polyurethane dissolved in PBS. The antimicrobial polyurethanes dissolved in PBS were prepared to various concentrations by performing a 1:1 serial dilution, with the control prepared using only PBS. The cells were incubated for either 1 hour or 24 hours in the polyurethane mix before determining viability using a CellTiter Blue assay kit according to the instructions of the manufacturer, Promega (Madison, WI, USA). After addition of the assay reagent, the well plates were incubated at 37 °C for 1 hour and then the fluorescence was measured at 560/590 nm excitation/emission with the percent viability calculated using the PBS control.

3.2.10 Statistical Analysis

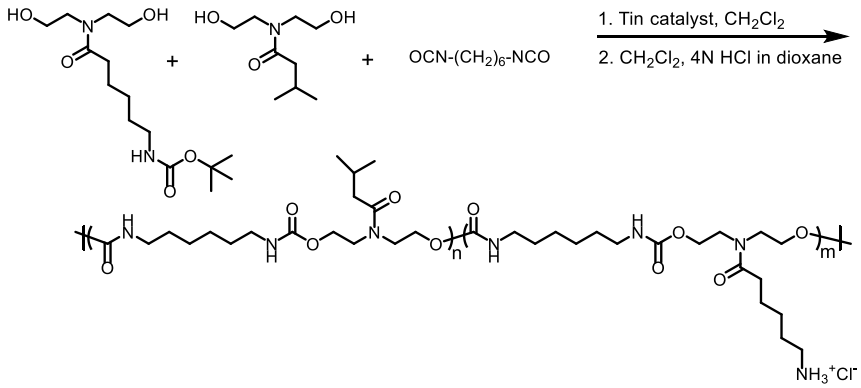
All experiments were performed at least twice independently and all were performed using at least three replicates. Values shown are expressed as the mean with their standard deviation of a single experiment. Comparisons were made among

experimental data using one way ANOVA with MATLAB. Groups of data were considered significantly different for $p < 0.05$.

3.3 Results and Discussion

We have previously reported the polymerizations of novel amide functionalized diols to obtain peptidomimetic polyesters.[215, 216, 219] The non-segmented, peptidomimetic polyurethanes synthesized in the current work are an expansion of this modular platform of monomers. For this initial investigation of the usefulness of these peptidomimetic polyurethanes, the monomers mLys and mVal that structurally mimic cationic lysine and hydrophobic valine residues, respectively, were selected to determine the feasibility of adapting these polymers as mimics of antimicrobial peptides. The composition of mLys and mVal was varied to determine the ideal balance of cationic / hydrophobic character necessary for optimal antimicrobial activity. The polyurethanes were synthesized by polymerization of hexamethylene diisocyanate and diols functionalized with the appropriate pendant groups (Scheme 3.1). Polymerizations were mediated by use of either dibutyl tin dilaurate (DBTDL) or tin(II) 2-ethylhexanoate (TEH). Using these two catalysts, two molar mass ranges were obtained for each mLys / mVal composition (Table 3.1); DBTBL resulted in polyurethanes in the number average molar mass range of ~21-31 kDa (high molar mass, HM) while TEH resulted in polyurethanes in the range of ~9-13 kDa (low molar mass, LM) under the same reaction conditions. These different molar mass ranges are believed to be the result of different catalytic activities of the tin catalysts. It has been reported that the catalytic activity of tin catalysts is variable depending on the tin

species as well as the counterion.[261] The composition of the synthesized polyurethanes closely matched the feed ratios of each diol (Table 3.2).



Scheme 3.1: Synthesis of peptidomimetic polyurethanes with mLys and mVal pendant groups.

Table 3.1: Polyurethanes and peptide used for antimicrobial investigation.

Polyurethane or Peptide* **(Lys/Val) (High M_n/Low M_n)**	**M_n (kDa)****	**Đ**
100/0 HM	31	1.6
100/0 LM	11	1.8
90/10 HM	24	1.6
90/10 LM	9	1.7
70/30 HM	21	1.6
70/30 LM	9	1.7
50/50 HM	27	1.7
50/50 LM	14	1.6
Pexiganan	2.477	1.0

* Ratios of mLys/mVal are based on monomer feed ratios. ^{1}H NMR analysis indicated no significant deviation in expected composition of the final polymers. **Polyurethane molecular mass was determined via size exclusion chromatography using polystyrene standards.

Table 3.2: Calculated composition of each copolymer.

Feed Ratio mLys/mVal	Catalyst	M_n (kDa)	mLys Repeat Unit Composition*	mVal Repeat Unit Composition*
90/10	DBTDL	24	89%	9%
90/10	TEH	9	93%	8%
70/30	DBTDL	21	71%	27%
70/30	TEH	9	73%	28%
50/50	DBTDL	27	50%	47%
50/50	TEH	14	51%	47%

*These values were calculated from the integration values obtained from ^{1}H NMR. mLys and mVal were calculated from peaks F and N, respectively, in Figure S3 for each polymer.

These peptidomimetic polyurethanes and pexiganan, an analogue of an antimicrobial peptide, were tested for their antimicrobial efficacy by determining each of their minimum inhibitory concentrations (MICs) necessary to prevent the growth of Gram negative *E. coli* K12 (ATCC 10798), *E. coli* (25922), *E. coli* P1C4, or Gram positive *Staphylococcus aureus* (ATCC 25923). Growth assays, *E. coli* cytoplasmic membrane permeability assays, hemocompatibility, and eukaryotic cell viability were also used to understand the impacts of various polymer chemistries on antibacterial activity and potential mammalian toxicity and compared with the performance of pexiganan.

The MIC experiments were performed using representative Gram negative (*E. coli*) and Gram positive (*S. aureus*) bacterial species. A 50% MHB formulation (Muller-Hinton broth) was used as it allowed for preparation of various concentrations of polyurethane solutions while maintaining the same salt and protein concentrations, the variability of which has been shown to have a significant impact on the antimicrobial activity of peptides and synthetic polymers.[262] *E. coli* was additionally tested in M9 Minimal Medium (M9MM), a defined medium that contains just essential salts and dextrose, to determine whether antimicrobial activity can be enhanced in a simpler medium.

The most striking observation from the MIC data of the polyurethanes, ampicillin, and pexiganan is the large difference in MIC values for *E. coli* and *S. aureus* (Figure 3.5 A, B, and C), with the polymers having higher inhibitory effects against *E. coli* at lower concentrations, 8-31 μg/mL, similar to pexiganan. Contrary to this, the polyurethanes were not very effective against *S. aureus*, with MICs of 125-250 μg/mL. To the best of our knowledge, such large selectivity of *E. coli* over *S. aureus* has not been reported with synthetic antimicrobial polymers. Previous studies with other synthetic polymers have been able to show an opposite result where a large selectivity of the polymer for *S. aureus* over *E. coli* was achieved.[211, 213] Also, from the results, it was observed that the composition of the polyurethanes does not significantly influence the MIC (Figure 3.5 A, B, and C) tested in 50% MHB, all of which exhibited an MIC of 16-31 μg/mL for *E. coli*. The independence of MIC on the identity of the pendant group functionality was unexpected as past literature on synthetic polymeric antimicrobials suggests modulation of MIC with the modulation of the hydrophobic / hydrophilic balance.[263] Perhaps, this is because the backbone of the polyurethane is already sufficiently hydrophobic for antimicrobial activity. This lack of modulation may also be the result of the experimental conditions such as the use of 50% MHB in these experiments. Methods for MIC determination for polymeric antimicrobials can vary significantly between research groups and are frequently modified to suit the materials and bacteria being tested. This observed independence of MIC on pendant group identity also holds true for the MIC of *S. aureus* in 50% MHB and the MIC of *E. coli* in M9MM. The MICs for *E. coli* in M9MM were observed to be 8 μg/mL for all of the HM polyurethanes and the 70/30 LM polyurethane and 16 μg/mL for remaining LM polyurethanes. When the polymers are plotted in terms of molarity (μM) of each

antimicrobial rather than as the weight of antimicrobial per volume of medium (μg/mL), the performance of all of the polyurethanes exceeds that of ampicillin and pexiganan in controlling the growth of *E. coli* (Figure 3.5 B). Additionally, there does not appear to be a significant difference in MIC amongst three different strains of *E. coli* (Figure 3.5 C). Therefore, these results do not appear to be strain specific.

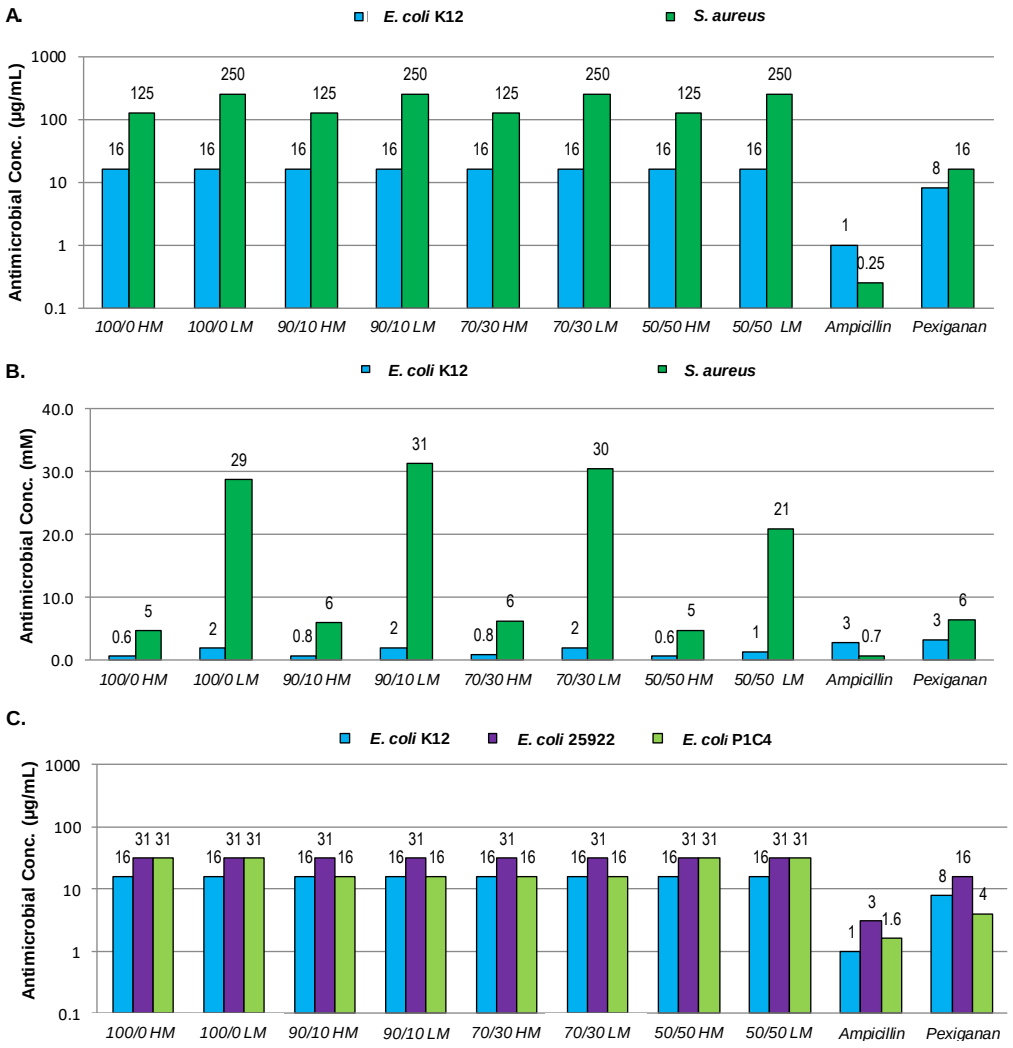


Figure 3.5: The MIC values of *E. coli* K12 vs. *S. aureus* for each antimicrobial A.) in terms of µg/mL antimicrobial B.) mM antimicrobial, and C.) the MIC values obtained with three different strains of *E. coli*.

To differentiate between bacteriostatic and bactericidal activities of the polyurethanes, *E. coli* K12 growth in the presence of the 100/0 and 50/50 polyurethanes was tested. The antimicrobial polyurethanes and pexiganan were tested at 2x MIC and ampicillin at 100 µg/mL. The results show that the polyurethanes are bactericidal in both

50% MHB and M9MM media (Figure 3.6 A and B, respectively). It was observed that the rate of killing for the polyurethanes depends on the medium used; faster bactericidal kinetics were observed in the simpler, defined M9MM than in the more complex 50% MHB, requiring about 8h for the 50/50 polyurethanes to achieve 99.9% killing of *E. coli* in 50% MHB and less than 0.5 hr in M9MM. A similar yet less extreme reduction of the time necessary to kill *E. coli* was observed using the 100/0 polyurethanes. The observed difference may be due to the more hydrophobic polyurethanes binding to proteins in the MHB. A previous study has shown that complex, protein-rich media had a greater impact on the ability of synthetic mimics of antimicrobial peptides to interact with cells and had little to no impact on the activity of antimicrobial peptides themselves.[264] From the assay performed in 50% MHB, it was observed that molar mass of the antimicrobial polyurethanes appears to have very little effect on the bactericidal kinetics and that the rates of killing are very dependent on the chemical composition of the polymers. Contrary to this observation, in M9MM the bactericidal kinetics of both the 100/0 polyurethanes are dependent on the molar mass.

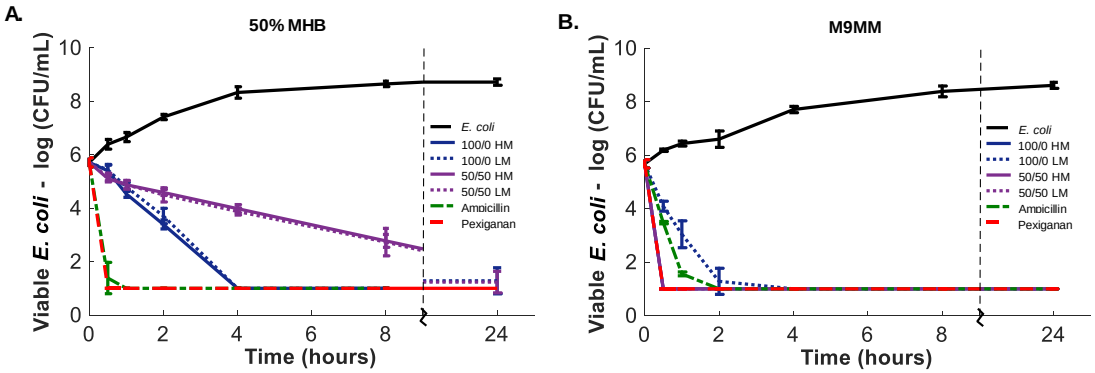


Figure 3.6: The growth curves of *E. coli* K12 when exposed to antimicrobials in A.) 50% MHB and B.) M9MM.

Most AMPs have been characterized to act on bacteria by disrupting their membranes as their primary mode of interaction.[88, 93] In order to test whether the antimicrobial and peptidomimetic polyurethanes are acting on *E. coli* K12 in a similar manner as AMPs, a cytoplasmic membrane permeabilization assay was performed on the 100/0 and 50/50 LM and HM polyurethanes at concentrations of 25, 50, and 100 μg/mL. For comparison and to serve as a positive control, pexiganan was also used in the experiments at the same concentrations. Membrane permeabilization was measured by assaying the release of cytoplasmic β-galactosidase by its ability to hydrolyze ONPG to produce ortho-nitrophenol, the formation of which can be tracked by measuring absorption at 420 nm. These assays were performed over the course of 200 minutes and are shown in Figure 3.7. From the results, it can be observed that all four of the polymers and pexiganan showed enhanced β-galactosidase activity as a result of membrane disruption when compared to the negative control, which is *E. coli* suspended in PBS with just ONPG. There is some production of ortho-nitrophenol observed in the negative control due to the natural influx of the ONPG into *E. coli*. Also, from the results, it can be observed that each polyurethane shows a slightly different β-galactosidase release profile. The polyurethanes that contained a greater amount of mVal and the polyurethanes that are higher in molecular weight result in greater damage to the cytoplasmic membrane of the *E. coli*. (The ONPG assay is not applicable to *S. aureus* as it does not produce β-galactosidase.)

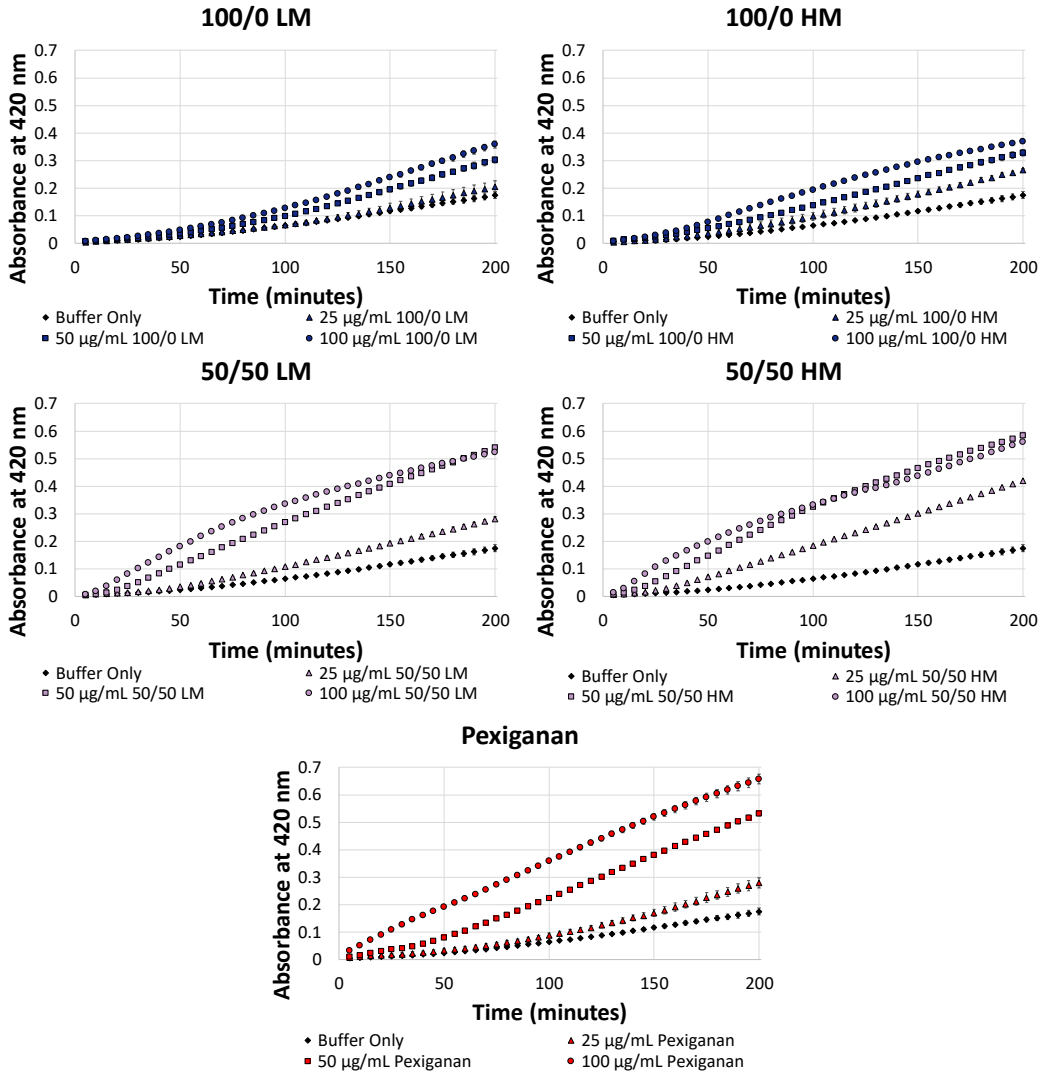


Figure 3.7: *E. coli* K12 cytoplasmic membrane permeabilization by antimicrobial polyurethanes and pexiganan over the course of 200 minutes at three different concentrations (25, 50, and 100 µg/mL). The hydrolysis of ONPG into lactose and ortho-nitrophenol due to β-galactosidase released as a result of membrane disruption was monitored by measuring absorbance at 420 nm.

For an antimicrobial to be clinically viable, it should not display any negative effects against mammalian cells. Previous studies using water soluble cationic polymers

have shown that the balance of cationic and hydrophobic character of the polymer is important in determining the antimicrobial effectiveness and mammalian cell toxicity.[205] Typically, polymers that are very hydrophobic or highly cationic are either weakly antimicrobial and/or highly cytotoxic to mammalian cells.[263] Also, molar mass has been shown to play an important role in determining the antimicrobial activity of the polymers on mammalian cells,[258] with a larger molar mass being more effective in killing both bacterial and eukaryotic cells.

We investigated the polyurethane compatibility with mammalian cell membranes using sheep blood cells (Figure 3.8). The results show that exposure of blood cells to the polyurethanes for 1 h at various concentrations showed very low hemolysis. The tolerance of these materials at high concentrations was unexpected, especially for the 100/0 and 90/10 polyurethanes. For the 100/0 and 90/10 HM polyurethanes, less than 10% hemolysis was observed up to about 625 μg/mL. This is significant because these two polymers have Mn~ 30 kDa and polymers of such high molar mass would be expected to be quite hemolytic. Perhaps, this greater tolerance of the HM polyurethanes is due to the more hydrophilic nature of the backbone. Previous studies have shown that the addition of polar, uncharged pendant groups to antimicrobial polymers decreased their hemolytic activity.[192, 202] Also, the 100/0 and 90/10 LM polyurethanes maintained ~5% hemolysis across the entire range of concentrations tested. This is impressive because the hemocompatibility is comparable to pexiganan, which was tested under the same conditions alongside the antimicrobial polyurethanes. Pexiganan has previously shown a range of hemolytic activity depending on the method of testing. The hemocompatibilities have been shown within the literature to range from exceeding 50% hemolysis at a concentration of 124 μg/mL, as

reported by Mor *et al*, to observing less than 50% hemolysis up to the highest concentration tested, 500 µg/mL, as reported by Barron *et al*.[186, 188] In our investigation, where no organic solvent was used and exposure was limited to 1 h, pexiganan was found to be less than 10% hemolytic up to the highest concentration tested, 2.5 mg/mL. The 70/30 and 50/50 polyurethanes showed less desirable results; both HM and LM 50/50 as well as the HM 70/30 polyurethanes all exceeded 10% hemolysis at concentrations around 100 µg/mL.

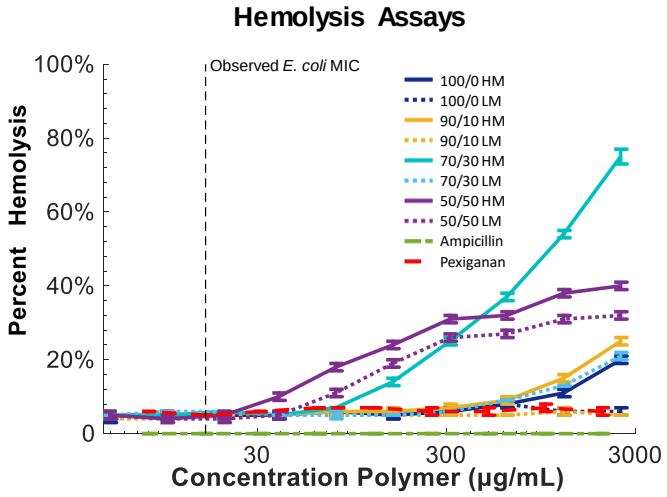


Figure 3.8: The hemocompatibility of the antimicrobial polyurethanes, pexiganan, and ampicillin over a broad range of concentrations (5-2500 µg/mL).

To further evaluate mammalian cell compatibility, cell viability was investigated using mouse fibroblast NIH 3T3 cells. These cells were exposed to the polymers, either for 1 or 24 h to determine the short term and long term effects of the antimicrobial polyurethanes on their viability. At concentrations where *E. coli* is inhibited (8-16 µg/ml), the cells remained viable for both the 1 h and 24 h time points. At higher concentrations, the results of the 1 h assay (Figure 3.9 A) corroborated multiple trends and observations

which were apparent in the hemolysis assay. The 1 h assay indicated that the higher molar mass and more hydrophobic polyurethanes were the least compatible with mammalian cells. After 1 h of exposure, all the antimicrobial polyurethanes were significantly more compatible with the cells than pexiganan; a significant drop in viability of cells exposed to 125 μg/mL of pexiganan is observed while the cells exposed to 125 μg/mL of all tested antimicrobial polyurethanes still remained viable. Figure 3.9 B shows the results of the 24 h cell viability assay and there appears to be a time-dependent aspect to the interaction of the cells with the antimicrobial polyurethanes. Higher concentrations of antimicrobial polyurethane which were compatible with the cells after 1 h of exposure were not compatible after 24 h of exposure to the antimicrobial polyurethanes. This observation is most apparent when observing the HM polyurethanes. After 1 h of exposure, the HM polyurethanes exhibited a range of compatibilities dependent upon the ratio of mLys to mVal. However, after 24 h of exposure to the antimicrobial polyurethanes, the HM polyurethanes showed a significant decrease in cell viability at the tested concentrations of 63 μg/mL and above. Although the range of compatibility for the LM polyurethanes decreased after 24 h of exposure, there still remained a considerable difference in viability between the different compositions of mLys and mVal monomer. At higher concentrations, even though there was a significant decrease in viability after 24 h, the 100/0 LM polyurethane had a minimal impact on cell viability up to about 125 μg/mL, a concentration at which pexiganan shows significant toxicity. Interestingly the 70/30 HM polyurethane showed the greatest hemolysis and the highest toxicity, compared to the 100/0 and 50/50 polyurethanes. Therefore, for these polyurethanes, an increasing hydrophobic nature is not linearly related to toxicity. Also, it must be noted that when comparing the viability of cells

exposed to pexiganan for 1 h and 24 h, there is not a significant decrease in cell viability with time. This is consistent with other data within this study that indicated that the interaction of pexiganan with cells is quite rapid.

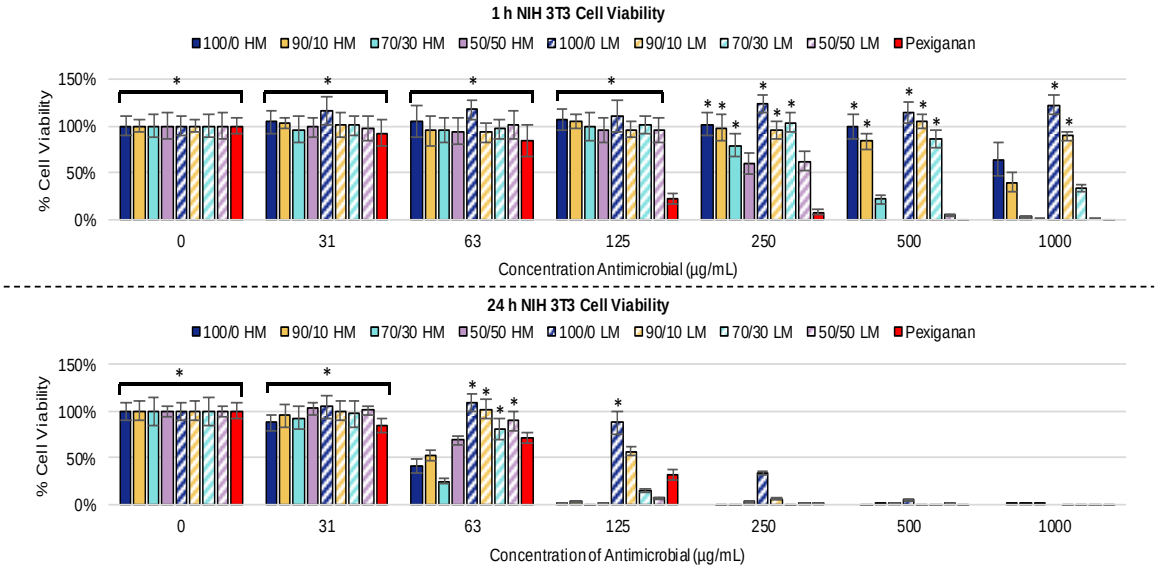


Figure 3.9: NIH 3T3 mouse fibroblast cell viability after A.) 1 h and B.) 24 h of exposure to an antimicrobial polyurethane or pexiganan. * Indicates 90% cell viability or greater, $p < 0.05$.

3.4 Conclusion

In conclusion, we show that a new class of AMP-mimicking polyurethanes based on our platform of modular amide-functionalized diols demonstrated high efficacy against *E. coli.* These peptidomimetic polyurethanes are especially noteworthy in that they are capable of selectively targeting *E. coli* over *S. aureus*. Such selectivity has not been

previously reported by any other synthetic polymer mimic of AMPs. The use of antimicrobials that non-discriminately target all bacteria is non-ideal, due to both the bystander effect on important commensal species and the likelihood of the development of antimicrobial resistance. Surprisingly, the hydrophilic and hydrophobic ratios of the lysine-like and valine-like pendant groups did not result in the modulation of the MIC of the polyurethanes, which again is contrary to most reports in the literature of synthetic antimicrobial polymers. However, it was seen that the ratios of the hydrophobic and hydrophilic groups influence blood cell hemolysis and NIH 3T3 cell viability, with the more hydrophilic polyurethanes showing very low hemotoxicity and cytotoxicity. The MIC and bactericidal studies showed that the lysine containing polyurethanes had comparable performance to pexiganan, while demonstrating lower mammalian cell toxicity. The higher toxicity of pexiganan may result from its non-selective nature and its rapid killing activity compared to the polyurethanes. From these studies, it was inferred that lowering the hydrophobic nature of the polyurethanes while maintaining a high cationic charge are important design elements to achieve high bactericidal activity and low mammalian cell toxicity.

Overall, the results reported here demonstrate that the AMP-mimicking polyurethanes are a promising new class of synthetic antimicrobials. Furthermore, when compared to peptides, the higher stability of such polyurethanes, their synthetic scalability and manufacturing reproducibility are important advantages that may enable their translation to several applications in the biomedical field.

CHAPTER IV

BACTERIAL MEMBRANE SELECTIVE ANTIMICROBIAL PEPTIDE MIMETIC POLYURETHANES: A STUDY OF THEIR STRUCTURE/PROPERTY RELATIONSHIPS AND MECHANISMS OF ACTION

*The following chapter will be submitted for publication with the following co-authors:

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4.1 Introduction

The historic misuse and overuse of antibiotics along with a waning interest in antibiotic discovery by major pharmaceutical companies has led to a great crisis within the biomedical community where bacterial infections are becoming more difficult to treat. Some of the most worrisome "super bugs" emerging are resistant to virtually all frequently used antibiotics. It is estimated that there are about 700,000 deaths annually worldwide as a result of antibiotic resistance.[7] However, it is projected that this number will continue to

grow in the coming years and, if no action is taken to curb this trend, a staggering 10 million deaths annually are estimated to be attributable to antibiotic resistant bacteria by 2050, which would exceed estimated annual deaths due to all forms of cancer.[7]

As a response to this crisis, water-soluble polycations are emerging as an alternative to conventional small molecule antibiotics to combat antimicrobial resistance. To date, there have been a wide variety of natural and synthetic polycations that have been characterized for their abilities to control the growth of bacteria, examples of which include antimicrobial peptides (also known as host defense peptides),[87, 265, 266] synthetic polymers that are intended to mimic host defense peptides,[178, 263, 267] and various other polycationic synthetic polymers that have been traditionally used as biocides.[48, 53, 54] All of these antimicrobial polycations, no matter whether they are synthetic or natural and no matter their exact chemical composition, are united by their membrane active mode of action.[268] This ability of polycations to disrupt bacterial membranes is achieved upon attaining just the right amount of balance in the amphiphilic cationic and hydrophobic character of the molecules so that they are electrostatically attracted to bacterial membranes, which typically are negatively charged, and insert themselves into the membrane to cause disruption.

There have been various structure/property relationships that have been established among all antimicrobial polycations that can be used to tailor their effectiveness in disrupting cell membranes. For example, many studies have shown that the balance of amphiphilic cationic and hydrophobic character of any antimicrobial polycation is important in determining the capability of the polycation to disrupt bacterial membranes.[58, 241, 254] This cationic and hydrophobic balance of antimicrobial polycations is also important

in determining their compatibility with mammalian cells. Typically, the greater the incorporation of hydrophobic portions into the polycation, the greater the toxicity of the polycation towards mammalian cells. The incorporation of uncharged polar groups can increase the compatibility of the antimicrobial polycations with mammalian cells without having a very detrimental effect on bacterial cells.[192, 202, 254] However, a less frequently encountered subset of antimicrobial polycations that have fewer established structure/property relationships are polycations that exhibit a selectivity of one bacteria or type of bacteria over another. Lienkamp and Tew *et al* synthesized multiple oxynorbornene derived polymers and found them to have a greater effectiveness against Gram positive *S. aureus* over Gram negative *E. coli*, which was found to be attributable to the inability of the polymer to penetrate the outer membrane of the *E. coli*.[211] Caputo and Kuroda *et al* found that polyethyleneimine that had a branched architecture was able to also selectively affect *S. aureus* over *E. coli*, which was not observed with linear analogs.[213] Therefore, any information that is obtained about how to modify their behavior can have very significant implications on how to rationally design polycations.

One such polycation that has demonstrated a unique selectivity amongst bacteria are a new class of water-soluble antimicrobial polyurethanes that were previously reported by our group.[269] Within our previous study, it was observed that all of the antimicrobial polyurethanes were effective in controlling the growth of *Escherichia coli* but not *Staphylococcus aureus,* while, at the same time having very desirable compatibilities with mammalian cells. This study was designed to further explore the antimicrobial potential of this family of antimicrobial polymers by 1.) trying to gain a better understanding of the structure/property relationships of these polymers when the pendant functional groups are

expanded beyond those that were previously investigated, 2.) the effect of molecular mass across a broader range than previously investigated, and 3.) to gain a better understanding of the mechanism of how the antimicrobial polyurethanes interact with the membranes of cells. To do this, the antimicrobial polyurethanes synthesized for this investigation were tested for their minimum inhibitory concentration, which is the smallest concentration necessary to prevent overnight bacterial growth, against an expanded panel of microorganisms. Also, the compatibility of these polymers with mammalian cells was probed by performing hemolysis assays. Lastly, the mechanism of action of the antimicrobial polyurethanes was investigated by performing membrane permeabilization assays on live bacteria and with phospholipid vesicles constructed to mimic bacterial and mammalian cells.

4.2 Experimental Procedures

4.2.1 Materials

All solvents and reagents used were purchased from Fisher Scientific (Waltham, MA, USA) unless otherwise specified. The anhydrous 4N HCl in dioxane and cyanamide were obtained from Acros Organics (Fair Lawn, NJ, USA); hexamethylene diisocyanate, tin 2-ethylhexanoate and dibutyltin dilaurate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methylene chloride and N,N-dimethylformamide were dried by distillation after preliminary drying with CaH_2 and stored over molecular sieves. For the preparation of phosphate buffered saline (PBS) and HEPES buffered saline (HBS), HEPES (4-(2-

hydroxyethyl)piperazine-1-ethanesulfonic acid), bovine serum albumin, sodium phosphate dibasic, potassium phosphate monobasic, and potassium chloride were obtained from Sigma (St. Louis, MO, USA). The Mueller Hinton Broth (MHB) was purchased from Himedia (Mumbai, India). The Trypticase Soy Broth was purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). The nutrient broth was purchased from Ward's Science (Rochester, NY, USA). Agar was added separately to the medium and was purchased from Sigma. The antimicrobial control ampicillin was purchased from Alfa Aesar (Ward Hill, MA, USA), melittin from Cayman Chemical (Ann Arbor, MI, USA), and polymyxin B from Calbiochem (San Diego, CA, USA). The bacteria used for MIC testing were *Staphylococcus* epidermidis (ATCC 12228), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis*, *Escherichia coli* K12 (ATCC 10798), *Pseudomonas aeruginosa* PAO1, *Stenotrophomonas maltophilia* (ATCC 13637), and *Serratia marcescens* (ATCC 13880). The bacteria used for the outer membrane permeability assay was *E. coli* K12 (ATCC 10798). The bacteria used in the cytoplasmic membrane depolarization assays were *E. coli* UB1005 and *S. aureus* (ATCC 25923). For the hemolysis assays, defibrinated sheep blood was purchased from Hardy Diagnostics (Santa Maria, CA, USA). The phospholipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), and1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG) were received dissolved at a known concentration in chloroform and purchased from Avanti Polar Lipids (Alabaster, AL, USA). Isolated and purified lipopolysaccharide (LPS) from *E. coli* 011:B4 was obtained from Sigma Aldrich. The indicator dyes 3,3'-Dipropylthiadicarbocyanine

Iodide (diSC$_3$-5) and N-phenyl-1-naphthylamine (NPN) were purchased from TCI (Tokyo, Japan) and 5(6)-carboxyfluorescein from Acros Organics.

4.2.2 Instrumentation

^{1}H NMR spectra were acquired using a 300 MHz Varian Mercury spectrometer or a 750 MHz Varian INOVA spectrometer. The chemical shifts are reported in ppm relative to the signal of the residual protons of the deuterated solvent. Molar mass determinations were performed via size exclusion chromatography on a Tosoh EcoSec HLC-8320 instrument equipped with two PSS Gram Analytical SEC columns in series using 25 mM LiBr in N,N-dimethylformamide as the mobile phase at a flow rate of 0.8 mL/min. The column and detector were kept at 50 °C for the experiments. All molar mass values were obtained using a standard curve generated from polystyrene standards. All absorbance and fluorescence spectroscopy for the biological experiments were obtained using either a BioTek Synergy H1 or a Molecular Devices Spectramax M2 multimode plate reader.

4.2.3 Synthesis of Monomers and Antimicrobial Polyurethanes

The synthesis of all monomers and the polyurethanes has been detailed in multiple previous studies with the exception of the guanidine functionalized polyurethanes.[215, 216, 269, 270] In brief, all of the diol monomers were synthesized via the amidation of diethanolamine with the corresponding methyl or ethyl ester for each monomer that would yield the polymers shown in Figure 1. The resulting monomers were purified via silica gel

liquid column chromatography using a mixture of dichloromethane and methanol. In a typical polymerization, the desired diol monomers (1.56 mM) were loaded along with hexamethylene diisocyanate (250 μL, 1.56 mmol) and either tin(II) 2-ethylhexanoate or dibutyltin dilaurate (5 μL) into a 1 necked round bottom flask. The flask was then capped with a rubber septum and vacuum purged and backfilled with dry N_2. Anhydrous methylene chloride or anhydrous N,N-dimethylformamide (4 mL) was added to the flask and the reaction was allowed to stir at a temperature between room temperature and 40 ºC under N_2 overnight. After overnight reaction, methanol (0.5 mL) was added and the reaction mixture was allowed to stir at room temperature and open to ambient air for at least 1 hour. The polymer was isolated and purified by precipitation into cold ether. The obtained polymer was dried under vacuum and characterized via ^{1}H NMR and SEC. Following the polymerization, the pendant amine groups were deprotected using the same acid catalyzed method that has previously been used.[269] In a typical post-polymerization amine deprotection reaction, 100 mg of polyurethane was dissolved in 2 mL of anhydrous methylene chloride followed by the addition of 1 mL of 4 M HCl in dioxane and allowed to react for 45 min. After completion of the reaction, the polymer was isolated under reduced pressure. Confirmation of successful deprotection was indicated by ^{1}H NMR spectroscopy. Typical NMR spectra before and after deprotection are shown in Figure 4.1.

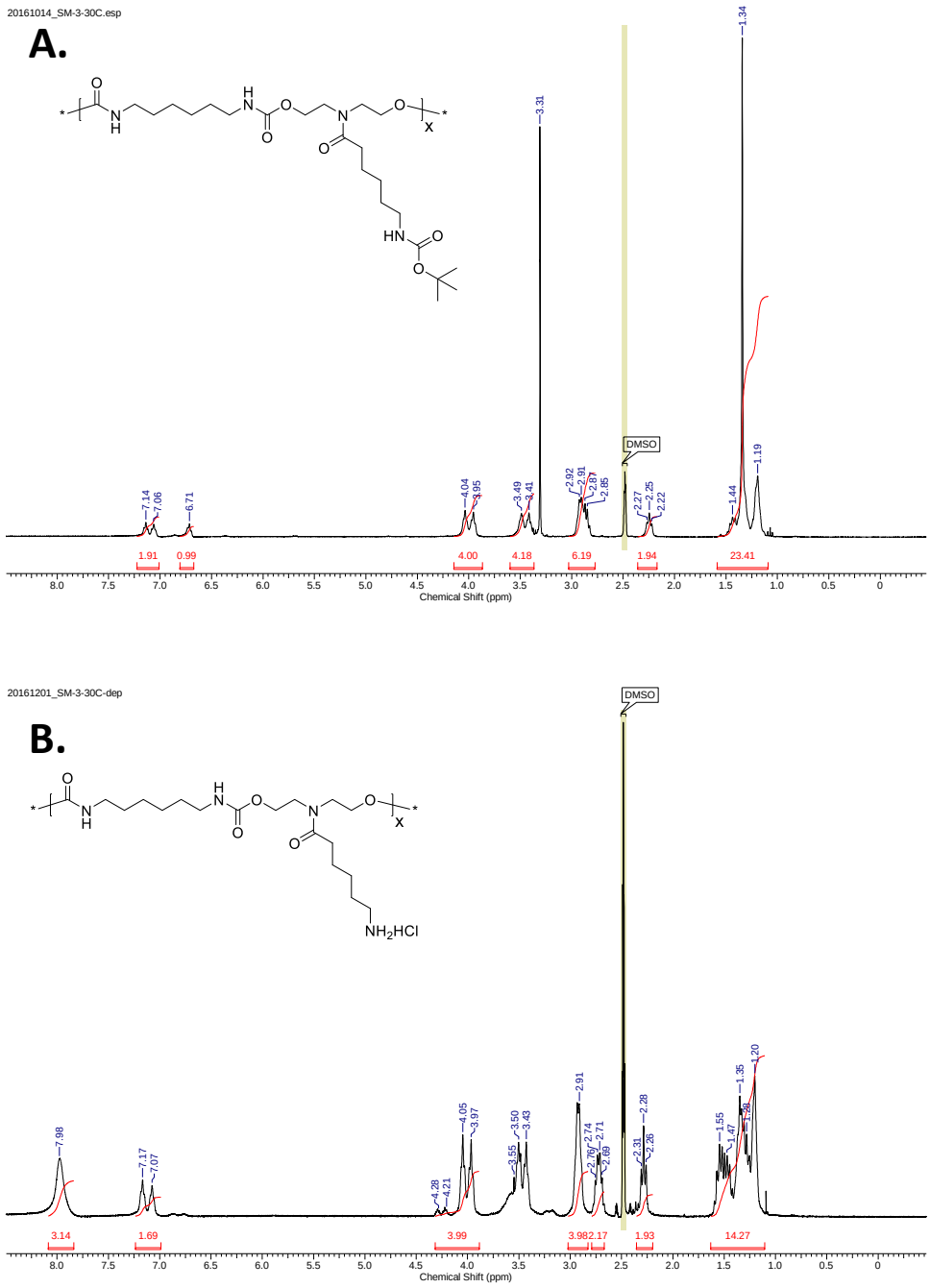
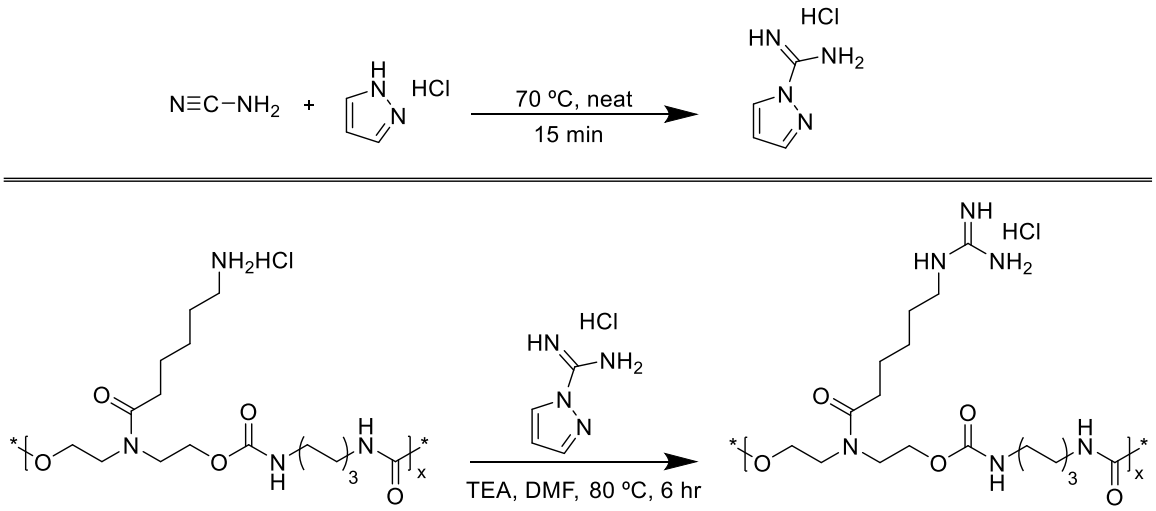


Figure 4.1: A typical ^{1}H NMR spectrum of an antimicrobial polyurethane A.) after polymerization and B.) after post-polymerization amine deprotection.

The guanidine functionalized polyurethanes, or the polyurethanes synthesized to mimic the amino acid arginine (mArg), were synthesized through the process shown in

Scheme 4.1. This process involved the post polymerization reaction of antimicrobial polyurethane that contains the lysine-like repeat unit (mLys HDIPU) with 1-amidinopyrazole hydrochloride to transform the primary amine into a guanidine, which is a method that has been previously shown to be effective in guanylating amines.[201, 271] The 1-amidinopyrazole hydrochloride was synthesized by the reaction of cyanamide (110 mg, 2.6 mmol) with pyrazole hydrochloride (200 mg, 1.9 mmol) neat in a scintillation vial while stirring at 70 ºC under ambient atmosphere. After about 15 min of reaction, the reagents that had initially melted under the heat resulted in the formation of a white, crystalline product. The obtained 1-amidinopyrazole hydrochloride was used without purification and used immediately after synthesis in a reaction with the mLys HDIPU. In a typical reaction, the 1-amidinopyrazole was weighed into a scintillation vial and dissolved in 2 mL of DMF and 0.5 mL triethylamine followed by addition of the polyurethane (200 mg, 0.47 mmol equiv amine) and reacted for six hours while heated at 60 ºC and stirring. To control the degree of functionalization of the mLys HDIPU with mArg, the mole equivalents of amine was calculated and the amount of 1-amidinopyrazole was adjusted accordingly to obtain the desired degree of functionality; 0.2x, 0.5x, and 1.5x the mol equiv amine yielded ~20%, ~50%, and ~100% mArg functionalized polyurethane, respectively, and the calculated values are shown in Table 4.1. After reaction, the reaction mixture was dialyzed in methanol and isolated under reduced pressure. ^{1}H NMR was used to characterize the resulting mArg containing polyurethanes polyurethane, with the spectra shown in Figure 4.2.



Scheme 4.1: The synthetic route for the post-polymerization guanylation of mLys HDIPU into mArg HDIPU.

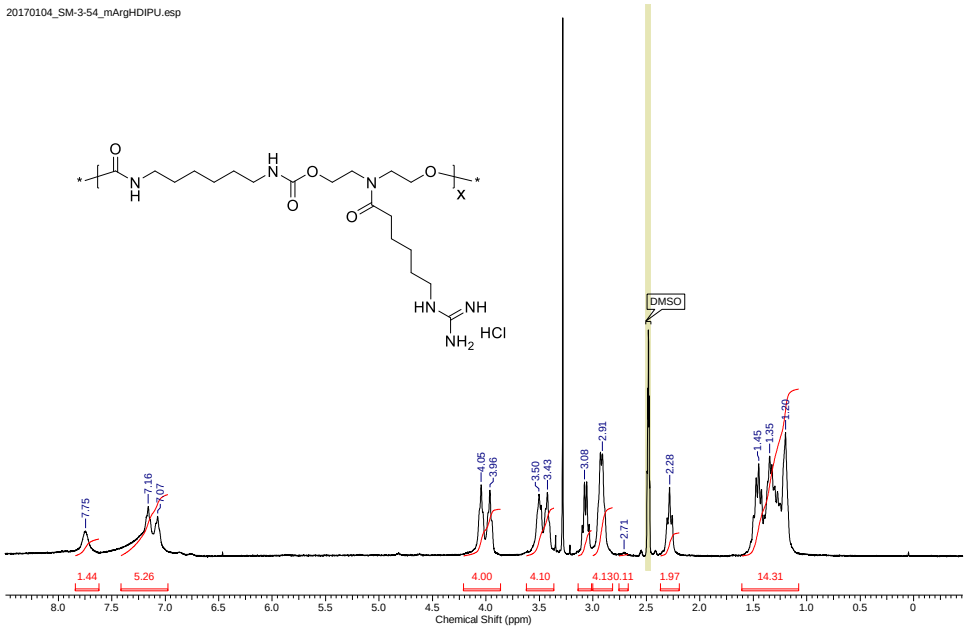


Figure 4.2: A typical ^{1}H NMR spectrum of an antimicrobial polyurethane after reaction with 1-amidinopyrazole to yield a polyurethane with an mArg repeat unit.

Table 4.1: The theoretical vs. actual degrees of guanylation of mArg containing polyurethanes.

Polyurethane	Theoretical mArg %	Actual mArg %*
80/20 mLys/mArg HDIPU	20	15-20
50/50 mLys/mArg HDIPU	50	50-60
100/0 mArg HDIPU	100	95-100

* These values were calculated from ^{1}H NMR spectroscopy from peaks that correspond to the mLys and mArg repeat units.

4.2.4 Minimum Inhibitory Concentration (MIC) Testing

All experiments were performed using the same procedure as previously reported.[269] In brief, all experiments commenced by selecting 3 colonies of bacteria from an overnight culture plate and used to inoculate a liquid culture in a test tube. *S. epdiermidis, S. aureus, E. faecalis, E. coli*, and *P. aeruginosa* were grown at 37 ºC with trypticase soy broth/agar (TSB/TSA) and *S. maltophilia* and *S. marcescens* were grown at 30 ºC with nutrient broth/agar. After inoculation of the liquid culture, the test tube was incubated at 30 ºC or 37 ºC while shaken at 150 rpm to reach mid-logarithmic growth phase (about 4 hrs). The culture was removed and diluted to $OD_{600\ nm}$ ~0.1, or about $1x10^{8}$ CFU/mL, and further diluted 100x into Mueller Hinton broth to yield a working concentration of ~$1x10^{6}$ CFU/mL. This working suspension of bacteria was added in a 1:1 ratio to solutions of antimicrobial polyurethane that had been dissolved in sterile DI water and serially diluted in a polypropylene 96 wellplate. Following addition of the bacteria to the polymer solution, the plates were measured for their initial $OD_{600\ nm}$ turbidity, incubated overnight at either 30 ºC or 37 ºC, and then measured again for turbidity. The MIC was determined as the lowest concentration that prevented a measurable growth of bacteria.

4.2.5 Hemolysis Assays

All experiments were performed using the same procedure as previously reported.[224, 269] In brief, the sheep blood cells to be used were prepared for the experiment by centrifugation of the cells at 500 x g for 10 min. at 5 ºC, careful removal of the supernatant via aspirator, resuspension of the pellet into an equivalent volume of 150 mM NaCl solution, and repeated until no more observable or very little hemoglobin was present in the supernatant after centrifugation. After the last centrifugation step, the pellet was resuspended in an equivalent amount of phosphate buffered saline (PBS) and then diluted 25x into additional PBS to yield the working suspension of blood cells. The working suspension was added in a 1:1 ratio to the wells of a 96 wellplate that contained antimicrobial polyurethane dissolved in PBS prepared to various concentrations by serial dilution. After addition of the blood to the antimicrobial polyurethane, the plate was incubated for 1 hour at 37 ºC, centrifuged at 500 x g at 5 ºC for 10 min, and the supernatant from each well removed and transferred to a new 96 well plate without disrupting the pellet. The 96 well plate that contained the supernatant was measured for absorbance at 450 nm on a plate reader. The absorbance values were normalized to blood cells exposed to 1% Triton X-100 and PBS, which resulted in 100% and 0% hemolysis, respectively.

4.2.6 *E. coli* and *S. marcescens* Outer Membrane Permeability Assays

The assays were performed according to methods used previously by Helander and Mattila-Sandholm with a few modifications.[272] Three colonies of either *E. coli* K12 or *S. marcescens* were selected from an overnight TSA plate and used to inoculate a 50 mL centrifuge tube containing TSB. The tube was incubated at 37 ºC while shaken at 150 rpm until reaching $OD_{600\ nm}$ = 0.5. The tube was then centrifuged at 2000 x g for 10 min while at room temperature. The supernatant was decanted and replaced with an equivalent volume of pH 7.3 10 mM HEPES + 150 mM NaCl (HBS). The suspension of bacteria in HBS was added (100 µL) in a 2:1:1 ratio to a black 96 wellplate that had been prepared to contain 1 part (50 µL) antimicrobial polyurethane dissolved in HBS and 1 part (50 µL) 40 µM NPN dissolved in HBS. Various concentrations of the polyurethane were tested by performing a serial dilution within the wellplate. After addition of all of the components within the wellplate, fluorescence at 350/420 nm ex/em was promptly measured on a plate reader.

4.2.7 *E. coli* and *S. aureus* Cytoplasmic Membrane Depolarization Assays

The assays in these experiments were performed using the same procedure that was developed by Strahl *et al* with a few modifications.[273] Three colonies of *E. coli* UB1005 or *S. aureus* were selected from a TSA plate cultured overnight and used to inoculate TSB in a 50 mL centrifuge tube. The bacteria were allowed to grow at 37 ºC while shaken at 150 rpm until $OD_{600\ nm}$ = 1.0 was achieved. The centrifuge tubes were then centrifuged 2000 x

g for 10 min at room temperature, the supernatant decanted, and the pellet resuspended in 10 mM pH 7.3 HEPES buffer + 4 µM $diSC_3$-5 + 1% DMSO. The bacteria were then allowed to incubate for either 45 min (*S. aureus*) or 60 min (*E. coli*) at 37 ºC while shaken at 150 rpm to allow for uptake of the membrane potential sensitive fluorescent dye. After incubation, the bacterial suspensions were diluted 2x into buffer to yield the working suspension of bacteria in a solution composed of 10 mM pH 7.3 HEPES buffer + 150 mM NaCl + 2 µM $diSC_3$-5 + 1% DMSO. This working suspension was allowed to equilibrate to the added salt while incubated at 37 ºC and shaken at 150 rpm for either 10 min (*S. aureus*) or 60 min (*E. coli*). The working suspension of bacteria was added in a 1:1 ratio to a black 96 wellplate that was prepared to contain various concentrations of antimicrobial polyurethane dissolved in the same buffer as the bacteria but without $diSC_3$-5 and with 1% BSA (added to prevent attenuation of the $diSC_3$-5 fluorescence due to it binding to the surface of the wellplate). Immediately after addition of the bacteria suspension to the antimicrobial polyurethane, the wellplate was placed into a plate reader set to measure fluorescence at 610/660 nm ex/em every minute for each well over the course of two hours while incubated at 37 ºC and continuously shaken between measurements. The obtained values were plotted as relative fluorescence units with time and compared with values obtained for 100 µg/mL melittin, 1% Triton X-100, unexposed bacteria, and buffer only controls.

4.2.8 Phospholipid Vesicle Dye Release Assays

These experiments were performed using procedures similar to what has been previously reported.[274] All phospholipids were prepared for use by pipetting the desired amount of phospholipid dissolved in chloroform into a small vial, evaporating the chloroform under N_2 airstream, and further removing the solvent completely by placing the vial under reduced pressure for 2-3 hrs. The phospholipid film that remained in the vial after drying was suspended to a concentration of 10 mM phospholipid in HBS + 40 mM carboxyfluorescein by repeated vortexing and sonication of the suspension until there was no more apparent phospholipid film remaining on the surface of the vial. The resulting vesicle suspension was subjected to three freeze/thaw cycles by submerging the vial in liquid N_2 and then allowing the contents of the vial to thaw to room temperature. After the final freeze/thaw cycle, the vial was placed onto a rotating shaker and kept there overnight at room temperature. The following morning, the vesicles were extruded 15x through a 0.2 μm pore size membrane and allowed to equilibrate for about 2 hours afterwards while on the rotating shaker. Vesicles were separated from the free carboxyfluorescein dye using a column packed with Sephadex G-50 beads using HBS as the mobile phase. Fractions from the separation were collected into a black 96 wellplate using a Gilson FC 204 fraction collector. After separation, the collected fractions that contained vesicles were determined using a plate reader to measure fluorescence at 495/530 nm. Once the vesicle containing fractions were identified, the fractions were removed from the plate, combined together into a vial, and the concentration of phospholipid quantified via ^{1}H NMR spectroscopy using the same procedure used by Hennig *et al.*[275] In brief, a sample of the vesicle

suspension was removed and dissolved in a mixture of methanol-D_6, chloroform-D, and D_2O that was spiked with 5 mM 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid (TMSP-D_4) as an internal standard. After obtaining the ^1H NMR spectrum of the mixture with pre-saturation of the water peak, the concentration of phospholipid was calculated using the integration values obtained from the peak corresponding to the internal standard and the peak corresponding to the methyl protons of the fatty acid chains of the phospholipid. After quantifying the concentration of the phospholipid, the vesicle suspension diluted to 0.2 mM phospholipid with HBS, combined in a 1:1 ratio with solutions of antimicrobial polyurethane prepared in a black 96 wellplate, and fluorescence measured at 495/530 nm ex/em every 1.5 minutes over the course of 30 min. Percent rupture of the vesicles was calculated from the 1% Triton X-100 and buffer only controls, which indicated 100% and 0% rupture, respectively. In the experiments that used *E. coli* lipopolysaccharide (LPS), the LPS was prepared into the plate along with the antimicrobial polyurethanes immediately before the experiment.

4.2.9 Statistical Analysis

All experiments were performed at least twice independently and all were performed using at least three replicates. Values shown are expressed as the mean with their standard deviation of a single experiment. Comparisons were made among experimental data using one way ANOVA with MATLAB. Groups of data were considered significantly different for $p < 0.05$.

4.3 Results and Discussion

One of the greatest strengths of the functionalized diol monomers developed within our labs is the ability to attach virtually any pendant group desired so that they can be adapted to fit any application. These monomers have been utilized for many biomaterial applications; from forming degradable 3D printed polyester scaffolds to making innovative thermoresponsive polymers.[216, 219] As a result of such extensive investigation, there have been many different pedant groups that have been designed and there exists a broad library of possible monomers that can be incorporated into the antimicrobial polyurethanes. From this vast library, there were a total of ten different pendant groups that were selected for further investigation and incorporation into antimicrobial polyurethanes, which is shown in Figure 4.3. The pedant groups selected can be categorized as either charged polar (mArg and mAsp), uncharged polar (nPrDEA, cPrDEA, QL1, and mSer) or as hydrophobic (mVal, mAla, mTrp, and mPhe). These pendant groups were selected for investigation to capture the broadest spectrum of hydrophobic, hydrophilic, and charged pendant groups as possible.

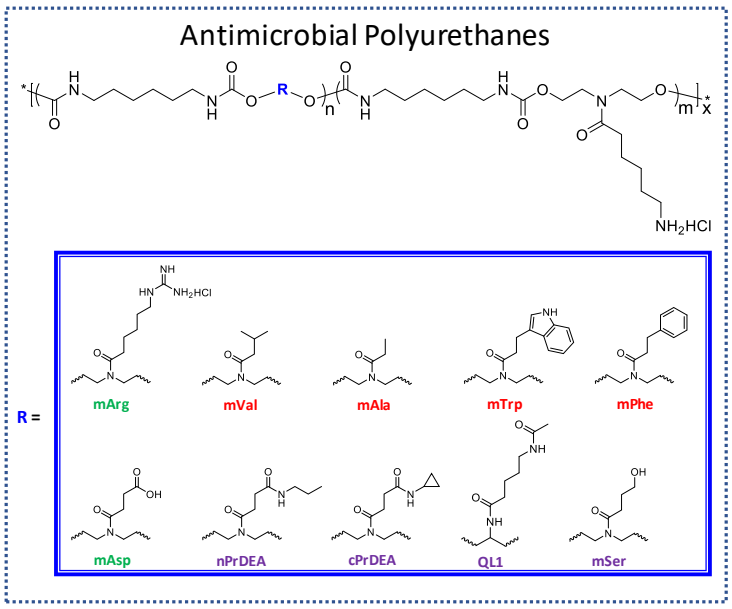


Figure 4.3: The chemical structures of the antimicrobial polyurethanes with different pendant groups.

The molecular masses and dispersities of all polymers investigated are shown in Table 4.2. In designing the polyurethanes for this investigation, it was decided to investigate 80/20 copolymers that had a molecular mass of about 30 kDa. The composition ratio was selected to allow the co-monomer that is polymerized with mLys to have a measurable effect while still retaining solubility of all synthesized polyurethanes, *i.e.* the polymers that incorporated the most hydrophobic repeat units may have solubility issues at compositions of greater incorporation. Since the guanidine functionality was of particular interest, additional 50/50 and 100/0 mArg/mLys compositions were also synthesized. Since the guanylation chemistry selected is a post-polymerization reaction, all of the mArg containing polyurethanes are derived from 35 kDa 100/0 mLys HDIPU. Also, since our previous investigation, we have been able to synthesize a broader range of molecular masses of polyurethanes. It was reported previously that the molecular mass can

be controlled by using different catalysts.[269] It has been discovered that temperature can exert an effect as well. Polymerizations that are performed at a temperature above room temperature can result in larger molecular masses. So, a series of 100/0 mLys HDIPUs between the molecular masses of 6 – 86 kDa were included in this study.

Table 4.2: The molecular mass and dispersity of the synthesized antimicrobial polyurethanes.

Polyurethane	M_n (kDa)	Đ
100/0 mLys HDIPU	6.0	1.2
100/0 mLys HDIPU	24	1.4
100/0 mLys HDIPU	35	1.4
100/0 mLys HDIPU	86	1.7
80/20 mLys/mAla HDIPU	36	1.4
80/20 mLys/mVal HDIPU	26	1.4
80/20 mLys/mPhe HDIPU	33	1.4
80/20 mLys/mTrp HDIPU	28	1.4
80/20 mLys/mSer HDIPU	45	1.5
80/20 mLys/QL1 HDIPU	33	1.4
80/20 mLys/cPrDEA HDIPU	29	1.4
80/20 mLys/nPrDEA HDIPU	32	1.4
80/20 mLys/mAsp HDIPU	27	1.4
80/20 mLys/mArg HDIPU	*	*
50/50 mLys/mArg HDIPU	*	*
100/0 mArg HDIPU	*	*

*These polyurethanes were synthesized via a post-polymerization reaction on35 kDa mLys HDIPU.

To gauge the performance of this entire series of polyurethanes as antimicrobials, all were tested for their MIC against Gram positive *S. epidermidis*, *S. aureus*, and *E. faecalis* as well as Gram negative *E. coli*, *P. aeruginosa*, *S. maltophilia*, and *S. marcescens*. The results of all these experiments are shown in Table 4.3. In general, the most significant trend that emerges from this data is that all the polyurethanes appear to be more effective against Gram negative bacteria than Gram positive bacteria, with a couple noteworthy exceptions. The addition of the mArg increased the effectiveness of the polyurethanes

against Gram positive bacteria when compared to the 35 kDa 100/0 mLys HDIPU from which all the mArg containing polyurethanes are derived. This is especially noticeable when comparing the *S. aureus* and *E. faecalis* MIC values of the three mArg containing copolymers to all other polyurethanes. The other exception to this observed trend is the diminished effectiveness of these polyurethanes against *S. marcescens*. This diminished effectiveness of the polyurethanes against these bacteria was not entirely unexpected because *S. marcescens* is well known to resist the action of many conventional antibiotics and membrane active antimicrobial peptides.[276, 277] This has been confirmed with ampicillin and polymyxin B, the control antimicrobials used in this investigation, which showed MIC values of 31 μg/mL and >250 μg/mL, respectively. The mechanism through which *S. marcescens* resists membrane active antimicrobials is well characterized and this knowledge has helped in guiding the investigation into why exactly these polyurethanes are more effective against Gram negative bacteria, which is further discussed later.

Table 4.3: The MIC values observed for the antimicrobial polyurethanes against various bacteria.

	Gram Positive Bacteria			Gram Negative Bacteria			
Polyurethane or Antimicrobial	*S. epidermidis*	*S. aureus*	*E. faecalis*	*E. coli*	*P. aeruginosa*	*S. maltophilia*	*S. marcescens*
6 kDa 100/0 mLys HDIPU	250	>250	>250	31	125	31	250
24 kDa 100/0 mLys HDIPU	63	125	250	16	31	16	63
35 kDa 100/0 mLys HDIPU	63	125	250	16	31	16	63
86 kDa 100/0 mLys HDIPU	63	63	125	16	31	16	63
80/20 mLys/mAla HDIPU	63	250	>250	31	31	31	125
80/20 mLys/mVal HDIPU	63	250	250	16	63	16	125
80/20 mLys/mPhe HDIPU	63	125	250	16	63	16	125
80/20 mLys/mTrp HDIPU	63	125	250	31	63	16	125
80/20 mLys/mSer HDIPU	63	250	250	31	63	31	125
80/20 mLys/QL1 HDIPU	63	>250	>250	31	63	31	125
80/20 mLys/cPrDEA HDIPU	63	250	>250	31	63	31	125
80/20 mLys/nPrDEA HDIPU	63	250	>250	31	63	31	125
80/20 mLys/mAsp HDIPU	125	>250	>250	31	125	31	250
80/20 mLys/mArg HDIPU	31	63	125	16	63	16	63
50/50 mLys/mArg HDIPU	31	63	63	8	63	16	63
100/0 mArg HDIPU	31	63	63	8	63	4	31
Ampicillin	3.1	0.2	1.6	3.1	125	125	31
Polymyxin B	-	-	-	1.6	1.6	1.6	>250

There are also multiple other trends that can be picked out from the MIC data. The addition of either a hydrophobic, uncharged polar, or anionic repeat unit to the polyurethanes either did not have a significant effect on MIC or slightly attenuated the effectiveness of the antimicrobial polyurethane when compared to the homopolymers of similar molecular mass across all bacteria investigated. The performance of the polyurethanes in controlling the growth of Gram negative bacteria was slightly reduced with the addition of any of the uncharged polar or anionic repeat units by at least one dilution within the serial dilution of concentrations. However, among polyurethanes that

incorporated a repeat unit that had a hydrophobic pendant group, the performance of the polyurethanes was split between having no significant impact and slightly decreasing effectiveness in controlling Gram negative bacterial growth. Among these polyurethanes that incorporated a hydrophobic pendant group, there were no discernable patterns that emerged to indicate one hydrophobic pendant group performs better than another. Overall, these results are consistent with what was previously observed with the polyurethanes that were composed of various ratios of mLys and mVal repeat units. With the previously investigated antimicrobial polyurethanes, there was not a very significant effect noticed among the different compositions of varying hydrophobic/cationic balance on the MIC against *E. coli* or *S. aureus*.

However, molecular mass does appear to have some effect on the ability of the polyurethanes to control the growth of bacteria. In our previous investigation of the effect of molecular mass, there was no observable difference in the ability of the two molecular mass ranges of the investigated polyurethanes on *E. coli* but a slight effect was observed against *S. aureus*. In this expanded investigation, similar results can be observed. With *E. coli*, *P. aeruginosa*, *S. maltophilia*, *S. marcescens*, and *S. epidermidis*, it appears that once the polymers attain a molecular mass greater than 6 kDa, the MIC values reach a plateau that is observed for the other three polyurethanes that are of greater molecular mass. However, a slightly different behavior is observed with *S. aureus* and *E. faecalis*. The MIC values associated with these bacteria show a gradual increase in effectiveness with increasing molecular weights. In the context of all other water-soluble cationic antimicrobial polymers, these polymers are somewhat unique in their behavior. In most accounts of antimicrobial polymers, there is indeed an increase in effectiveness of the

polymer with increasing molecular mass but there is a point at which this trend reverses and the effectiveness against bacteria drops off.[53, 54] This effect has been attributed to the polymer becoming less soluble or due to the polymer becoming too large to permeate through the peptidoglycan layer of bacteria at higher molecular masses. However, in the case of these polyurethanes, it appears that a molecular mass up to 86 kDa is still quite effective and more effective than lower molecular weights when used against *S. aureus* and *E. faecalis*. Perhaps, this behavior may be due to the inherent hydrophilicity along the backbone allowing greater solubility at larger molecular masses when compared to other polymers.

In the design of any membrane active antimicrobial, it is important to not only characterize how well the antimicrobial to affects bacteria but also verify that it is indeed an antimicrobial and not a substance that is toxic to every cell it encounters, including mammalian cells. A frequent means to measure the compatibility of any substance is through measuring the hemolytic activity, or the ability of the substance to lyse blood cells. The ability of all the antimicrobial polyurethanes to induce hemolysis is shown in Figure 4.4. These graphs were constructed to show how the different pendant groups (Figure 4.4 A), the incorporation of mArg (Figure 4.4 B), and different molecular masses (Figure 4.4 C) affected the compatibility with blood cells. Unlike the MIC results, the incorporation of different pendant groups into the polyurethane had a profound effect on hemocompatibility. Among all the 80/20 copolymers, the copolymers that incorporated the more hydrophobic pendant groups appeared to be more hemolytic, while, the copolymers that incorporated the uncharged polar pendant groups tended to be less hemolytic than the comparable mLys homopolymer that has similar molecular mass. These trends are not

unexpected; it has been previously shown that the addition of hydrophobic groups can lead to increased toxicity to mammalian cells and uncharged polar groups can reduce toxicity.[192, 202, 254] The hemocompatibility of some of these polyurethanes is unprecedented for polymers of molecular weights in the range of about 30 kDa. The polyurethanes that incorporated the mAla, nPrDEA, cPrDEA, and QL1 repeat units did not exceed 5% hemolysis up to a concentration of 2.5 mg/mL. Even more impressively, the polyurethane that incorporated the anionic repeat unit mAsp did not show any hemolysis up to a concentration of 2.5 mg/mL. However, the addition of the mArg repeat unit into the polyurethane resulted in a significant amount of hemolysis. Among the 80/20 copolymers, 80/20 mLys/mArg HDIPU was the most hemolytic. Increasing the content of mArg above 20% resulted in even greater hemolysis. This increase in hemolysis because of the addition of mArg to the polyurethane is opposite to what has been observed with other polymers that have incorporated guanidine groups. Polymethacrylates functionalized with guanidine and polyguanidinium oxanorbornene both have greater hemocompatibility than their primary amine analogues along with potent antimicrobial activity.[201, 210] There appears to be other factors that also play a role in determining the membrane disruptive abilities of polymers functionalized with guanidine and that simple generalizations of this functional group alone cannot be made. When observing the effect of molecular mass on hemolysis, the data indicates that increasing the molecular mass leads to a greater amount of hemolysis.

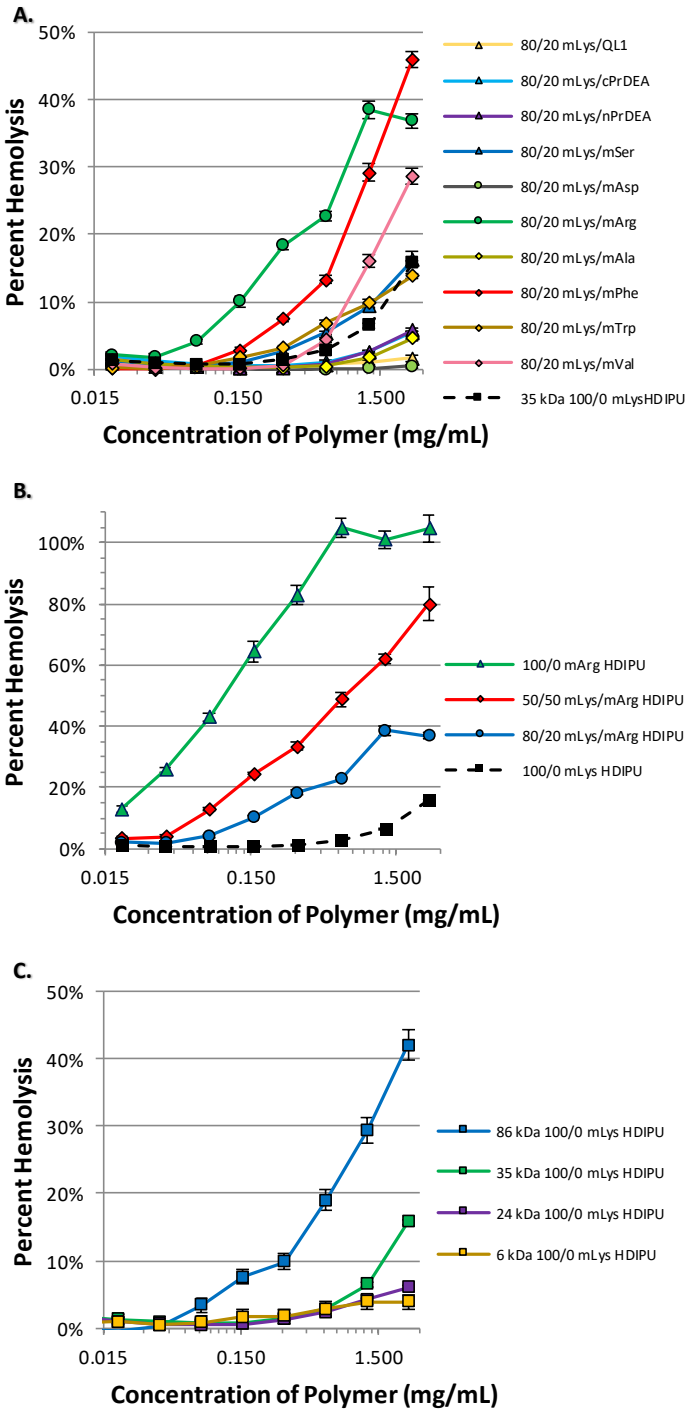


Figure 4.4: Hemocompatibility of the antimicrobial polyurethanes that have A.) incorporated a variety of pendant groups, B.) functionalized to contain various ratios of

mLys and mArg repeat units, and C.) different molecular masses over a range of concentrations (20 μg/mL – 2.5 mg/mL).

To gain a better understanding of how these antimicrobial polyurethanes work and why exactly the different pendant groups do not appear to have a very great effect on modulating antibacterial efficacy as well as why it appears that these polymers have a greater propensity to affect Gram negative bacteria, a series of experiments were performed to try to understand the mechanism of action using a smaller set of the antimicrobial polyurethanes. Chosen for further investigation were the 80/20 mLys/mVal, 80/20 mLys/mAsp, and the 80/20 mLys/mArg copolymers as well as the 35 kDa 100/0 mLys and 100/0 mArg HDIPUs. These selection of antimicrobial polyurethanes captures a diverse range of polymers that demonstrated the greatest antimicrobial effectiveness, superior compatibility with blood cells, and a balance of both factors.

A piece of evidence that provided some insight into how the polyurethanes affect bacteria was provided by the MIC values obtained with *S. marcescens*. As mentioned, this bacterium is resistant to many antimicrobial peptides. *S. marcescens* has been shown to exhibit multiple mechanisms of action against them, including the secretion of enzymes to degrade peptides and through functionalization of the LPS of their outer membrane to prevent the peptides from binding.[149, 278] It is this latter mechanism of action that caught our interest as the most probable mechanism to impart resistance to the action of the antimicrobial polyurethanes. It is widely recognized that *S. marcescens* is resistant to polymyxins and it has recently been determined that a significant contribution to this resistance is through functionalization of the LPS that reduces the net negative charge on

the molecule.[149] We hypothesized that the reason that these polymers were able to affect the other Gram negative bacteria tested was as a result of the polymer's inability to penetrate the outer membrane. To test this hypothesis, the ability of polyurethanes to penetrate the outer membranes of *E. coli* and *S. marcescens* was determined in an assay using the fluorescent dye NPN. It has been previously shown in various studies that this dye is capable of indicating a disruption in the outer membrane of Gram negative bacteria.[272, 279] This hydrophobic dye functions to indicate permeation of the outer membrane by showing an increase in fluorescence at 350/420 nm ex/em when the dye is able to gain entrance into the hydrophobic space between the lipid bilayer, which it is unable to access in intact membranes. The results of this assay when testing both *E. coli* and *S. marcescens* are shown in Figure 4.5, which displays the NPN uptake factor, a dimension-less value that indicates the fold increase in fluorescence from the bacteria only control, with concentration of all tested antimicrobial polyurethanes along with a known outer membrane permeabilizer control polymyxin B. The most striking observation that can be made from this data is the difference in the ability of the polyurethanes to disrupt the outer membrane of *E. coli* and *S. marcescens*. All the antimicrobial polyurethanes tested affect the outer membrane of *E. coli* to a greater degree at lower concentrations than *S. marcescens*. Within *E. coli*, it seems that the ability of each polyurethane to disrupt the membrane is chemistry dependent. From these results, it appears that the polyurethanes that incorporate non-cationic repeat units mVal and mAsp are attenuated in their ability to disrupt the outer membrane when compared to the polyurethanes that contain only contain the cationic repeat units mLys and mArg. Yet, this behavior is only observable at lower

concentrations of polyurethane and once a high enough concentration is reached, the NPN uptake factors for all polyurethanes approach the same values.

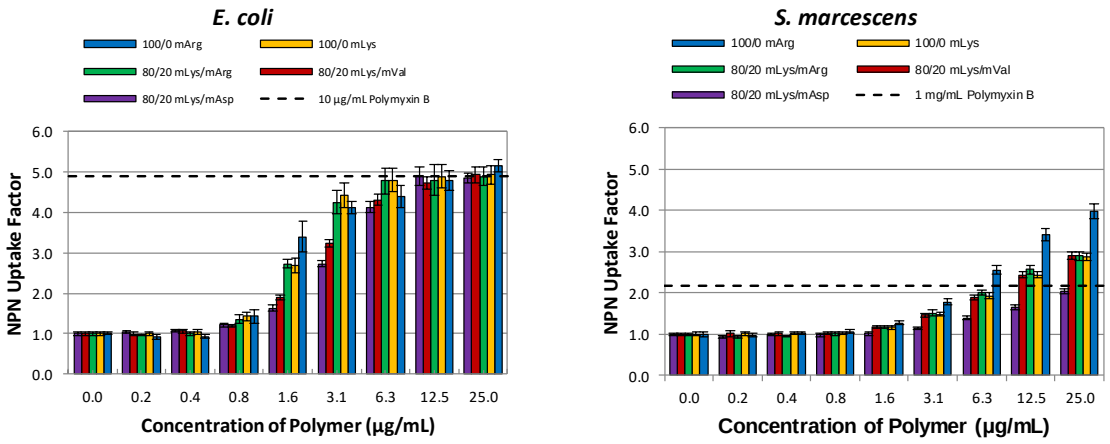


Figure 4.5: Disruption of the outer membranes of *E. coli* and *S. marcescens* caused by the addition of antimicrobial polyurethanes as indicated by measurement of the fluorescence of the lipophilic dye NPN at 495/530 nm ex/em.

Disruption of the outer membrane of Gram negative bacteria alone by any chemical or antimicrobial has never been shown to be a lethal mode of action. The mechanism of action that seems to be most consequential in determining whether an antimicrobial peptide or its synthetic mimic is an effective antimicrobial is its ability to disrupt the cytoplasmic membranes of bacteria. To determine the ability of the five select antimicrobial polyurethanes to disrupt bacterial cytoplasmic membranes in this investigation, the membrane potential indicator dye $diSC_3$-5 has been used. This dye has found widespread use for this application because it is capable of detecting the depolarization of the membrane potential as a direct result of the dissipation of important ionic or proton gradients across the membrane due to the formation of holes or pores in the membrane.[273,

[280] For the experiments, *E. coli* and *S.* aureus were selected as representative organisms that are very susceptible to and not very affected by the antimicrobial polyurethanes, respectively. Since it is believed that any antimicrobial polymer's most important mechanism of action to control the growth of bacteria and ultimately kill the bacteria is through cytoplasmic membrane disruption, it can stand to reason that bacteria that are less affected by any antimicrobial polyurethane must also be the result of differences in the ability of the polymer to disrupt the bacterial membrane. This appears to be evident in the results shown in Figure 4.6 A-E, which shows diSC_3-5 fluorescence due to membrane depolarization with time over the course of two hours for all of the selected antimicrobial polyurethanes at various different concentrations for both bacteria. When observing these graphs, it is evident that most of the antimicrobial polyurethanes demonstrate a greater ability to disrupt the cytoplasmic membranes of *E. coli* over *S. aureus*, which is indicated by the increase in observed fluorescence with increasing polymer concentration in *E. coli* when compared to what is observed with *S. aureus*. The one exception to this is 100/0 mArg HDIPU, which showed significant depolarization of both *E. coli* and *S. aureus* cytoplasmic membranes. When compared to the MIC values and hemolytic capabilities of this polymer, it does seem reasonable that it, as well as any other polymers that incorporate the mArg repeat unit, should effectively disrupt the membranes of both bacteria since the incorporation of this monomer lead to a greater effectiveness against almost all bacteria tested and was shown to induce significant hemolysis. When comparing all the polymers in their ability to induce depolarization of the *E. coli* cytoplasmic membrane, there are slight differences between each antimicrobial polyurethane tested. The results indicate that the polyurethane that appears to result in the greatest degree of depolarization, according

to the fluorescence values, is 100/0 mArg HDIPU, followed by 80/20 mLys/mArg and 80/20 mLys/mVal HDIPU, which both demonstrated similar depolarization profiles, 100/0 mLys HDIPU, and 80/20 mLys/mAsp, which showed the least amount of depolarization.

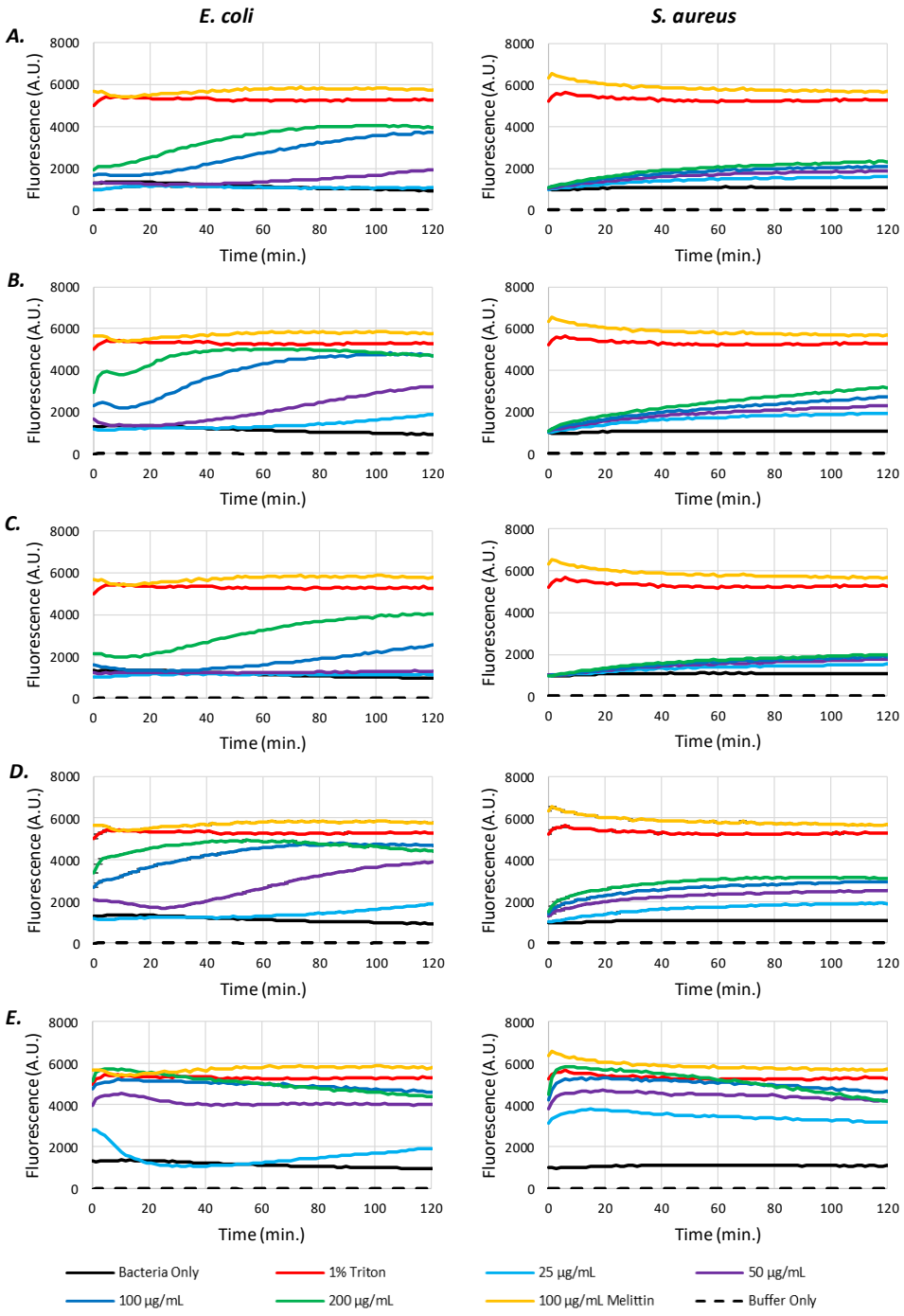


Figure 4.6: *E. coli* and *S. aureus* cytoplasmic membrane depolarization as a result of exposure to A.) 35 kDa 100/0 mLys HDIPU, B.) 80/20 mLys/mVal HDIPU, C.) 80/20 mLys/mAsp HDIPU, D.) 80/20 mLys/mArg HDIPU, and E.) 100/0 mArg HDIPU, which

was monitored by tracking the fluorescence of the membrane potential sensitive dye $diSC_3$-5 at 610/660 nm ex/em.

To further evaluate how the antimicrobial polyurethanes work mechanistically, experiments were performed using phospholipid vesicles to model different cell types that have encapsulated, water-soluble dyes, such as carboxyfluorescein, that are released upon disruption of the phospholipid bilayer. A difficult to understand aspect that model vesicles can help better understand is how membrane active antimicrobials interact in a direct comparison of mammalian cells and bacterial cells. Since it can be difficult to measure both bacteria and mammalian cells under the same conditions *in* vitro, it is difficult to determine whether membrane active antimicrobials are indeed selective for the membranes of one cell type over another. So, these experiments were conducted to gain greater insight into how the antimicrobial polyurethanes interact with vesicles that are composed of the phospholipid POPC, which have a net neutral charge across the surface of the membrane and mimic mammalian cells, and vesicles that are composed of an 80/20 ratio of POPE/POPG, which have a net anionic charge across the surface of the membrane and mimic the composition that would typically be found in bacterial cytoplasmic membranes. In these experiments, the interactions of the antimicrobial polyurethanes with the vesicles was quite rapid and the maximum amount of rupture was observed to have been achieved within about 30 min of mixing the vesicles with the antimicrobial polyurethanes. So, all model vesicle data shown is after incubating the vesicles with the antimicrobial polyurethanes for 30 min. The results of the experiments are shown in Figure 4.7, which shows the percent of dye released from both vesicle compositions when exposed to 5

μg/mL antimicrobial polyurethane. Most significantly, these results show that at the same concentration and within the same medium that each of the antimicrobial polyurethanes have a propensity to rupture and cause the release of dye from the bacteria-like vesicles when compared to the mammalian cell-like vesicles, except for 100/0 mArg HDIPU. Just as it has been evident in all the other data, the 100/0 mArg HDIPU appears to have very little selectivity over the membranes it disrupts and actually appears to have a greater propensity to disrupt mammalian cell-like POPC vesicles over bacteria-like POPE/PG vesicles.

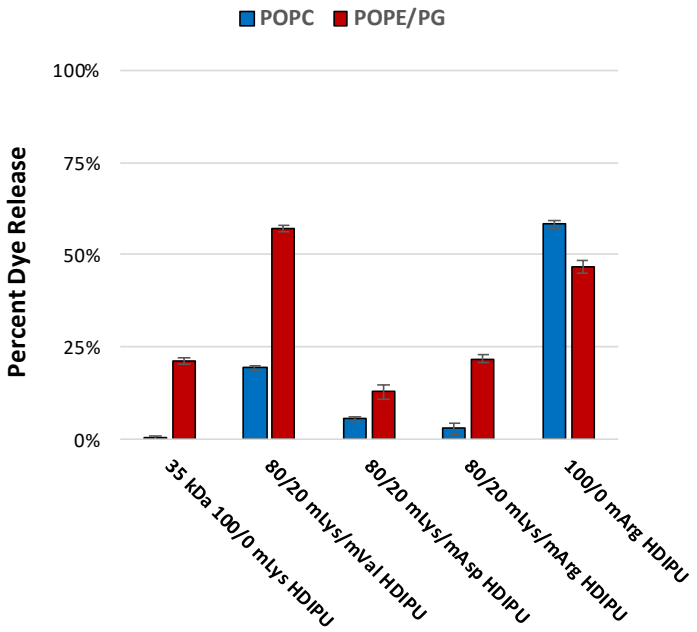


Figure 4.7: Antimicrobial polyurethane induced disruption of vesicles constructed to mimic mammalian cells (POPC) and bacterial cells (POPE/PG) by measuring the release of the encapsulated fluorescent dye carboxyfluorescein.

Another aspect to the mechanism as to how these antimicrobial polyurethanes work that can be aided using model vesicles is related to how the polymers interact with the two membranes of Gram negative bacteria. From our data, it appears that the polyurethanes can disrupt the outer membrane at concentrations that are much lower than what can cause a measurable drop in membrane polarization. However, even though these experiments are carried out using the same final concentration of bacteria, to draw comparisons between these two different methods is not correct because they are performed in different buffer solutions and the exact sensitivity of these tests are not completely known. Intuitively, it would make sense that the outer membrane would be affected to a greater degree by exogenous antimicrobials but the extend to how much it affects interaction of the antimicrobial with the cytoplasmic membrane is difficult to elucidate using bacterial cells. Therefore, these experiments with model vesicles were designed to try and more directly measure the effect of the outer membrane on the ability of the polyurethanes to disrupt the cytoplasmic membrane of bacteria. To do this, the experiments were designed to determine the effect of added LPS, which is the major lipid component at the extracellular interface of the outer membrane, on the ability of the polyurethanes to disrupt the 80/20 POPE/POPG vesicles. The results of these experiments with the selected antimicrobial polyurethanes is shown in Figure 4.8, which shows the percent dye released because of membrane disruption for each polyurethane plotted with and without the addition of LPS to the mixture after initial, 15 min, and 30 min of mixing the polyurethane with the vesicles. From the data, it is evident that the addition of LPS slows the rate of interaction of the polyurethane with the vesicles but does not prevent interaction. With all the antimicrobial polyurethanes, the difference between the percent dye released from the vesicles with and

without LPS is the greatest at the initial measurement but this difference narrows at greater time points. However, the chemical composition of the antimicrobial polyurethanes appears to influence how much the LPS slows the interaction of the polyurethanes with the vesicles. After 15 minutes of incubation, there is no statistical difference in the percent of dye released from the vesicles when exposed to the 80/20 mLys/mArg and100/0 mArg HDIPUs with and without LPS. While, with the 100/0 mLys HDIPU, the differences between percent dye released with and without LPS is the greatest for this polyurethane. All the 80/20 compositions of the polyurethanes fall in between the 100/0 mLys and 100/0 mArg HDIPUs in the effect that LPS has on the ability of the polyurethanes to disrupt the vesicles.

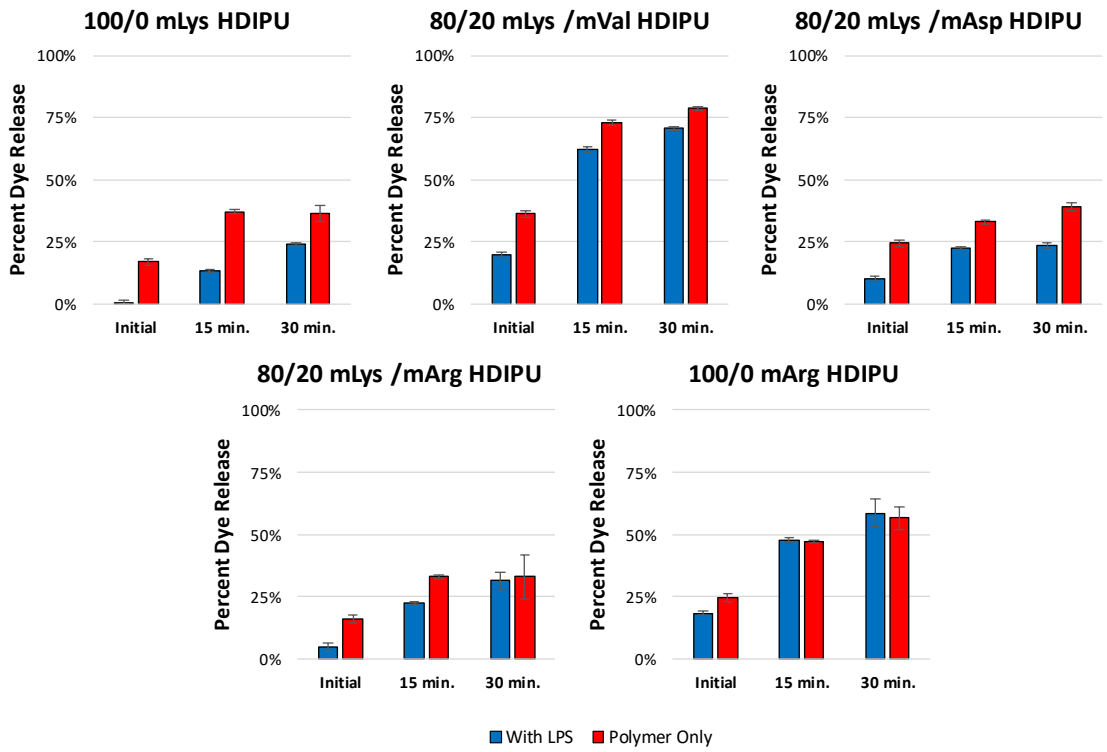


Figure 4.8: Antimicrobial polyurethane induced disruption of bacteria-like vesicles (POPE/PG) with and without added LPS measured by the release of encapsulated fluorescent dye carboxyfluorescein.

Overall, when considering all the data collected concerning the different pendant groups of all the antimicrobial polyurethanes within this investigation, there are some interesting implications on how the various chemical structures of these polymers can dictate their behavior. At first glance, it appears rather peculiar how the antimicrobial polyurethanes do not see a wide fluctuation in their antimicrobial properties when adjusting the balance of amphiphilic cationic and either hydrophobic, uncharged polar, and charged polar chemical structures, which previous literature has implicated as crucial factors that can cause large fluctuations in MIC values. However, upon further investigation of how these polymers are functioning mechanistically, it reveals that among Gram negative

bacteria, which appear to be most affected by the antimicrobial polyurethanes, that the different chemical moieties on the pendant groups affect different aspects of how the polyurethanes disrupt the membranes of bacteria. Collectively, the data indicates that the antimicrobial polyurethanes that have the greatest effect on the outer membrane contain a greater amount of cationic repeat units, such as the 100/0 mLys or 100/0 mArg HDIPUs and any copolymers of mLys and mArg, while, substitution of the cationic group results in a decrease in affinity for the outer membrane. In contrast, it appears that the polyurethanes that are more effective in disrupting the cytoplasmic membranes either have a greater amount of hydrophobic repeat units or a greater amount of guanidine repeat units while the polyurethanes that incorporate the primary amine or the carboxylic acid reduce the ability of the polyurethane to disrupt the cytoplasmic membrane. Perhaps, the reason for the inability of the pendant groups explored in this investigation to significantly modify the MIC values among Gram negative bacteria is partially due to this modulation of affinities for the two different membranes. It appears in the case of the hydrophobic pendant group substitutions that the resulting polyurethanes have a lower affinity for the outer membrane but a simultaneous increase in affinity for the cytoplasmic membrane that may lead to no net change in its effectiveness as an antimicrobial. Even though it is rather clear that the disruption of the outer membrane is by no means a lethal mode of action on its own but it has been shown that the penetration of it is an important step for membrane-active antimicrobials to reach the cytoplasmic membrane and destabilization of the outer membrane can make the cell more sensitive to damage. Therefore, it would be reasonable to believe that any reduction in the polyurethane's ability to permeate the outer membrane may lead to lower antimicrobial effect. While, for the uncharged polar pendant groups and

the anionic pendant group, it appears that addition of these pendant groups leads to a slightly lower antimicrobial effectiveness since it reduces the affinity of the polyurethane for both the membranes, which is reflected in the slightly lower MIC values.

Collectively from these results, it also becomes clear that the reason for the effectiveness of these polymers against Gram negative bacteria is because of their strong ability to disrupt both the outer membrane and the cytoplasmic membrane. It has been well established within the literature that this is indeed necessary for both membranes to be disrupted for membrane active antimicrobials to influence the viability of Gram negative bacteria. This is made especially evident by the significant decrease in the MIC values against *S. marcescens* that can be correlated with a decrease in the ability of the antimicrobial polyurethanes to permeate the outer membrane. However, it is not completely known why exactly the polyurethanes have an overall decreased efficacy against the Gram positive bacteria that were tested. With most polycations, if they can disrupt anionic bacterial membranes, they tend to have a broad spectrum of antimicrobial effectiveness. Yet, our results indicate that the mLys containing polyurethanes are much better at causing disruption and depolarization of the cytoplasmic membrane of Gram negative *E. coli* over Gram positive *S. aureus*. It is possible that the Gram positive bacteria tested have some sort of inherent ability to resist the membrane-active action of the antimicrobial polyurethanes, especially *S. aureus* and *E. faecalis*. Previous studies have shown that strains of *S. aureus* are capable of resisting the actions of antimicrobial peptides by changing the net charge of anionic phosphatidylglycerol head groups of phospholipids within the cytoplasmic membrane to a net positive charge by conjugation of the amino acid lysine to yield lysyl phosphatidylglycerol head groups.[151, 153, 154] Also, it has been reported

that polycations that are ineffective against Gram positive bacteria because they are unable to reach the cytoplasmic membrane due to limitations caused by their ability to translocate through the pores of the thick peptidoglycan layer.[54, 211] With the antimicrobial polyurethanes, there remains much that needs to be done to understand their interactions with Gram positive bacteria and whether there are any modifications to the structure of the polyurethane that may result in increased efficacy. Nonetheless, with our current understanding of the antimicrobial polyurethanes, there are many promising applications to which they can be applied to provide a novel tool that is capable of selectively killing certain bacteria.

4.4 Conclusions

Within this chapter, a series of antimicrobial polyurethanes were synthesized, investigated for their antimicrobial properties, and mechanisms of action. The collected data indicated that most of the antimicrobial polyurethanes were effective primarily in controlling the growth of Gram negative bacteria over Gram positive bacteria and that many of the different hydrophobic and polar pendant groups that were added to the polymers did not exert much effect in controlling the growth of the bacteria. The one pendant group that proved to be an exception to the general trend were those that were synthesized to contain a pendant guanidine functional group so that it mimics the amino acid arginine. The antimicrobial polyurethanes also exhibited very desirable hemolysis profiles, with many of the polyurethanes showing very little hemolysis, especially for the molecular weights investigated. It was observed that increasing the molecular mass, adding

hydrophobic pendant groups, or increasing the content of the guanidine functionalized pendant group lead to greater hemolysis while either decreasing the molecular mass or the addition of uncharged polar and anionic pendant groups lead to less hemolysis. Experiments that probed the mechanism of action of the antimicrobial polyurethanes revealed that they are membrane active and that the different pendant groups can modulate the effectiveness of each polyurethane on both the outer membrane and cytoplasmic membrane of Gram negative bacteria as well as the cytoplasmic membrane of Gram positive bacteria. From the data, it is believed that the reason that the polyurethanes are quite effective against Gram negative bacteria is because of their strong propensity to disrupt both membranes. Overall, the antimicrobial polyurethanes have shown many desirable properties and have great potential for further development as an antimicrobial.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

The work within this this dissertation detailed the synthesis and investigation of a novel family of polyurethane antimicrobials that mimic cationic antimicrobial peptides. These peptide-mimetic polyurethanes were designed using modular N-functionalized diol monomers that have been developed within the Joy research lab and polymerized with hexamethylene diisocyanate. While synthesizing these polymers, it was observed that the molecular mass can be controlled by the type of catalyst used and the temperature at which the polymerization is performed. Also, in the process of synthesizing these polyurethanes, an optimized method for the post polymerization deprotection of the Boc-protected amines has been developed to ensure complete, consistent deprotection.

Investigation of the antimicrobial properties of these polyurethanes revealed that they had some rather unique antimicrobial properties. The preliminary investigation of these polyurethanes showed that they had a greater propensity to affect *E. coli* over *S. aureus* and demonstrated an ability to kill *E. coli* at concentrations above the MIC. When the investigation was expanded to a greater number of polyurethanes that had a gradient of different cationic and hydrophobic repeat units, it was revealed that the balance of cationic and hydrophobic repeat units did not have a very significant effect on the ability of the polyurethanes to affect *E. coli* or *S. aureus* but had a profound effect on the compatibility of the polyurethanes with blood cells and fibroblast cells. Similarly, with these polyurethanes, it was observed that they did not have a very significant effect on *S. aureus*

yet were very effective against *E. coli*. To further investigate the effects of pendant group identity on the performance of the antimicrobial polyurethanes, another series of polyurethanes were synthesized to have a wide variety different hydrophobic, polar uncharged, and polar charged pendant groups. This series of polyurethanes was tested against a larger panel of bacteria and it revealed that the polyurethanes typically have a greater effect against Gram negative bacteria over Gram positive bacteria, with a few exceptions. Among the tested antimicrobial polyurethanes, those that contained mArg repeat unit were found to increase efficacy against most bacteria, independent of cell wall structures. While, the only exception to this generalization of greater effectiveness against Gram negative bacteria was *S. marcescens*, which is known to resist the action of many antimicrobial peptides.[149, 276, 278] The blood cell compatibility testing of the antimicrobial polyurethanes functionalized with the different pendant groups revealed that, in general, the polyurethanes that incorporated the hydrophilic pendant groups have greater compatibility while those that contain hydrophobic pendant groups have decreased compatibility when compared to an analogous polyurethane that just contained the mLys repeat unit. Also, the effect of molecular mass on antimicrobial efficacy was tested and it was observed that it has a rather slight effect on antimicrobial efficacy while having a greater effect on the compatibility with mammalian cells.

Mechanistically, it appears that the antimicrobial polyurethanes function through the disruption of the bacterial membranes. This was initially indicated by the assays performed with ONPG and *E. coli*, which showed that exposure of the bacteria to the polyurethanes resulted in a faster degradation of the ONPG due to an enzyme released from inside the cell. Further investigation of the mechanism of the polyurethanes revealed that

the pendant groups do have some effect in controlling the ability of the polyurethanes to interact with the membranes of bacteria. Assays using the indicator dye NPN revealed that the polyurethanes that contained a greater amount of cationic repeat units have a greater ability to disrupt the outer membrane of Gram negative *E. coli* as compared to those that incorporate hydrophobic or anionic repeat units. While, assays using the membrane potential-sensitive dye diSC_3-5 indicated that the polyurethanes that incorporated hydrophobic repeat units or the mArg repeat unit were more capable of disrupting the cytoplasmic membrane of Gram negative *E. coli*. For all polyurethanes, except for those that contain the mArg repeat unit, it appeared that the reason for the ineffectiveness of the polyurethanes against *S. aureus* may be because they are incapable of disrupting the cytoplasmic membrane. Experiments with vesicle membranes that mimicked the cytoplasmic membranes of bacteria and mammalian cells further corroborated what was found with living cells; all the polyurethanes, except those that contain the mArg repeat unit, have a greater proclivity to disrupt bacteria-like vesicles over mammalian cell-like vesicles. Experiments with the bacteria-like vesicles with and without added LPS showed that its addition effectively slows the rate at which the polymers can disrupt the vesicles.

This body of work clearly indicates that these new antimicrobials possess great potential for future investigations to gain a better understanding of how they work mechanistically and how they can be adapted towards clinical applications. Even though there has been much determined already about the mechanism of action of these antimicrobials, there are still many uncertain aspects concerning how they work. One big question that remains about these polyurethanes is about how they behave in aqueous solution. Are there any structures that are formed by the polymers that promote their

antimicrobial efficacy either in solution or upon binding to the surface of bacteria? What exactly is happening on the molecular level with these polymers when they interact with the membranes of bacteria and mammalian cells? Previous studies with other polymers have provided interesting insight but the unique antimicrobial selectivity of these polymers would make them very interesting for similar in-depth analysis.[194, 206] Also, investigation of 1.) why these polymers are not very effective against Gram positive bacteria and 2.) whether there are any chemical structures that can be used to broaden the spectrum of effectiveness to include Gram positive bacteria would provide further meaningful future research directions. Nonetheless, as they are, these antimicrobial polyurethanes in their current form still hold immense potential for development towards specific applications, most especially those where Gram negative bacteria are the most frequent causative agents of infection.

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Publication: ACS Biomaterials Science & Engineering

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