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Antimicrobial Activity, Biofilm Disruption Capabilities, and Synergistic Interactions of Novel Amphiphiles

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A thesis submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

In Partial Fulfillment of the Requirements

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Dedication

I would like to dedicate this thesis to my husband and family for encouraging me to continue pursuing a career in biology. They have been supportive emotionally and helped in any way needed through this time.

Acknowledgments

I would like to acknowledge my thesis advisor Dr. Kyle Seifert for guiding me through my research, believing in my abilities as a scientist, and providing me with an opportunity to pursue my career in biology. He has been a great influence as a scientist as well as a human being. I would also like to thank my committee members Dr. Caran and Dr. Herrick. Both contributed greatly to this research project and helped guide the direction of this thesis and provided significant feedback.

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Abstract

The increased prevalence of antimicrobial resistant bacteria requires development of new control strategies. Preventative measures such as development of disinfectants and antiseptics with faster killing and anti-biofilm capabilities would help limit the spread of resistance and reduce the incidence of hospital acquired infection. Several series of novel amphiphiles, including three bis-cationic, four tris-cationic and three tetra-cationic double tailed amphiphile series, as well as one hexa-cationic, triple tailed amphiphile series were synthesized and tested for antimicrobial properties. The amphiphiles in this study were previously tested for MIC value against several Gram-positive and negative bacterial species. This work expanded on the antimicrobial capabilities of these amphiphiles by determining time to kill a population of cells, biofilm disruption activity, and synergistic interactions with other compounds. Bis-cationic amphiphiles were the fastest at killing *S. aureus*, as oX-12,12 killed within 1 minute. Oxacillin, a Gram-positive acting antibiotic, combined with novel amphiphiles against *E. coli*, had FIC's ranging from 0.5 to 0.19, indicating synergistic interactions. The highest biofilm disruption activity disrupted 90% of *P. aeruginosa* preformed biofilms. Synergistic combinations of these amphiphiles with oxacillin and other compounds could prove useful in overcoming bacteria antimicrobial resistant mechanisms. These results contribute to the development of cationic amphiphiles with increased biofilm disruption activity and faster kill time, ultimately better disinfectants and antiseptics that will better reduce the spread of antibiotic resistant pathogens especially in a hospital setting.

Chapter 1: Introduction

1.1 Antimicrobial Resistance and Hospital Acquired Infections

Antimicrobial resistance is a growing problem in the medical field, with antibiotic resistant bacteria responsible for approximately 2 million illnesses annually in the United States alone (CDC, 2013). Widespread use of the first commercially available antibiotic, penicillin, in the early to mid-1940's, lead to antibiotic resistance by the late 1940's (Podolsky, 2015). Alexander Fleming himself, in 1945, predicted antibiotic resistance with increased public use and misuse (Rosenblatt-Farrell, 2007). Since that time, antimicrobial resistance has been a continual problem. Even with the introduction of new antimicrobials, bacteria typically acquire resistance within 10 years of public use (CDC, 2013; Rosenblatt-Farrell, 2007).

The misuse of antibiotics, such as over prescribing antibiotics, use of antibiotics in animal feed, and antibiotics not taken as prescribed, has contributed to the spread of resistant bacteria. Each of these contributing factors provide bacteria with an environment encouraging proliferation of only bacteria that can withstand antibiotics or disinfectants. Those that survive can transfer their genes responsible for resistance to non-resistant bacteria (Marinelli and Genilloud, 2013). It is reasonable to expect that resistance will be gained by bacterial species for every type of antimicrobial at some point. In 2015, resistance was observed in the United States to antibiotics that were considered "last resort drugs", which are reserved for only those infections that harbor antibiotic resistance to all other antibiotics (Mataseje *et al*., 2015). Once fail-safe antibiotics become obsolete, the death toll of antibiotic resistant infections will rise dramatically, potentially leading to a world that is like the time before antibiotics were discovered.

Infections caused by *Staphylococcus* species became the first medical issue involving resistance to antibiotics. In 1946, 14% of hospital acquired (HA) *Staphylococcus* infections were resistant to available antibiotics. Within two years, these numbers rose to 59% (Jevons, 1961; Schaffer, 2013). Methicillin, a new and improved beta-lactam antibiotic, was introduced in 1960, and resistant *Staphylococcus* strains were detected just one year of its introduction (Barber, 1961), and by 1974, 2% of HA staph infections were resistant. This strain is better known as MRSA (Methicillin Resistant *Staphylococcus aureus*). Though a low percentage in 1974, it was an indication that eventually new antibiotic classes could not remain potent against this species. Within 20 years, the percentage rose to 22%, and to 64% by 2004 (Wispinghoff *et al*., 2004; NNIS, 2004). Today MRSA is so prevalent that methicillin is no longer used to treat staph infections. Instead, other antibiotics are used including vancomycin, daptomycin, as well as combination therapy (Mongkolrattanothai *et al*., 2003). Eventually *S. aureus* is likely to become resistant to these antibiotics as well. Other pathogens have a similar history of antibiotic resistance where an antibiotic is heavily used, becomes obsolete due to antibiotic resistance, and is replaced with an antibiotic that has a different mechanism of action only for that bacterial type to acquire a way to become resistant to the new antibiotic. Continued antibiotic discovery as well as discovery of new solutions to treating bacterial infections is needed to keep up with growing antibiotic resistance.

Disinfectant resistance is also a growing problem, especially in hospitals where it is vital to keep all equipment free of pathogens to avoid spread of disease. Immunocompromised patients in hospitals can easily acquire an infection if proper protocols for keeping areas as sterile as possible are not observed. In 2014, 4% of patients had acquired at least one infection while being hospitalized (Magill *et al*., 2014). The best way hospitals reduce the frequency of hospital

acquired infections (HAI) is to increase sterilization/sanitation measures as well as identify sources of infection. Once sources are identified, preventative measures can be used to avoid infecting patients. Sources of most HAI are from person to person contact, surgical sites, and invasive medical devices. Mechanical ventilation equipment, endoscopes, catheters, and intravenous (IV) lines are the most frequently contaminated, as they are the most difficult to sterilize (Djordjevic *et al*., 2016). About 26% of HAI were associated with contaminated equipment (Magill *et al*., 2014). Even though preventative measures are readily used by hospitals and health care facilities, HAI are still one of the top 10 causes of death in the U.S. The costs annually for HAI in the U.S. are more than $5 billion (Peleg, 2010, O'Neill, 2014).

There are two major issues that make HAI prevalent as well as deadly. One is that most patients that are being hospitalized have weakened immune systems and are more susceptible to infection. Second, more bacteria in a hospital setting are likely to be resistant to antibiotics (Dancer *et al*., 2009). Hospitals use antimicrobials more frequently than in other locations, increasing the likelihood of selecting for resistant organisms. If a patient is infected by these bacteria, antibiotics may not be able to clear infection. There is a need for novel disinfectants to prevent the spread of resistant bacteria (McDonnell, 2007). Recent studies have shown that some disinfectants currently used in hospitals around the world cannot kill bacteria as effectively as when they were first introduced (Khan *et al*., 2016). If a solution is not found to prevent the spread of antimicrobial resistance, the projected death toll worldwide due to antimicrobial resistance will rise from 700,000 per year to nearly 10 million per year by 2050, higher than death due to cancer (O'Neill, 2014).

The so-called E.S.K.A.P.E. pathogens (*Enterococcus faecium*, *Staphylococcus aureus*,

Klebsiella pneumoniae, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) are responsible for the majority of serious hospital acquired infections (HAI) that possess extensive antimicrobial resistance (Boucher *et al*., 2009). These pathogens cause ~50% of all hospital acquired infections (Hidron *et al*., 2008). Most of the antimicrobial resistance isolates from these species show multiple antibiotic resistance leaving little or no means of eradicating these infection in patients. (Santajit and Indrawattana, 2016). Approximately 20% of these HAI harbor resistance to multiple commonly used drugs, making these infections difficult to treat (Hidron *et al*., 2008). In 2016 a woman in Nevada was infected with a strain of *Klebsiella pneumoniae* harboring resistance to 26 different antibiotics including even "last resort" antibiotics such as Colistin (Chen *et al*., 2016). The health care workers were unable to treat her infection, therefore she developed sepsis and died. If antibiotic resistance continues spreading, more bacteria will harbor resistance to a similar extent, rendering antibiotics useless for treating infections. Although antibiotic development is needed to combat infections caused by these highly resistant bacteria, preventative measures, including better disinfection methods could help reduce the spread of these bacteria.

1.2 Biofilm

Many bacterial species can form biofilms, which increase antimicrobial resistance. Biofilms are complex structures containing colonies of bacterial cells surrounded by layered extracellular polymeric substance (EPS) matrices connected by water channels for nutrient exchange (Donlan, 2002; Figure 1). Bacterial populations in biofilm communities can be up to 1000 times more resistant to antimicrobials than the corresponding planktonic cells (Ntsama-Essomba *et al*., 1997; Ceri *et al*., 1999). Bacterial cells are densely packed within the biofilm matrix, encouraging exchange of resistance gene via conjugation, which can lead to more than

100 times higher mutation rate (Conibear *et al*., 2009). If one cell in the biofilm harbors a plasmid with an antimicrobial resistant gene, many, if not all, of the cells within the matrix will acquire this gene. (Hausner and Wuertz, 1999; Donlan, 2002).

S. aureus, *E. coli* and *P. aeruginosa* are responsible for most biofilms found within a hospital setting that both cause infections and harbor antibiotic resistance genes. When other bacteria enter these biofilms, they are more likely to acquire antibiotic resistance genes from these species, contributing the spread of antimicrobial resistance (Ntsama-Essomba *et al*., 1997). *P. aeruginosa* is one of the most problematic of these pathogens due to its ubiquitous nature and is inherently more resistant to antimicrobials due to an outer-membrane. *P. aeruginosa* can harbor resistance to readily-used antibiotics because of extensive biofilm formation abilities. More than 60% of infections caused by *P. aeruginosa* strains have multidrug resistance (Ali *et al.*, 2015). Once a *P. aeruginosa* biofilm becomes established, eradication is nearly impossible as regrowth occurs if any biofilm remains after treatment (Vickery *et al*., 2004).

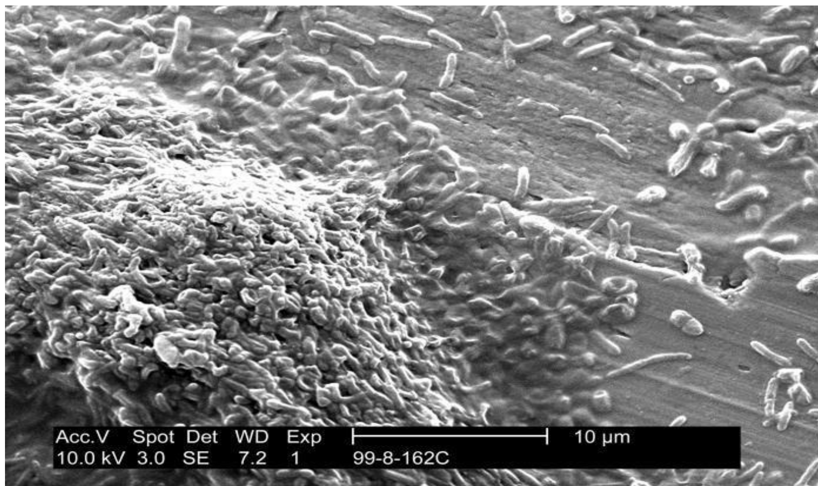

Figure 1: Electron micrograph of a mixed species biofilm containing *P. aeruginosa*, *K. pneumoniae*, *Flavobacterium,* and *L. pneumophila* (Photo from the CDC Public Health Image Library).

Biofilms are involved in over 60% of infections, according to the NIH (Lewis,

2001). Chemical treatments are not effective at eliminating 100% of biofilms (Gawande *et al*., 2008). Currently used chemical biofilm disruptors can only eradicate as much as 75% of the matrix and reduce associated bacterial cells by 4-fold, which is not sufficient since regrowth can readily occur (Vickery *et al*., 2004). These infections include dental plaque conditions, catheter infections, cystic fibrosis, endocarditis, and UTIs (Lewis, 2001). Elimination of biofilms on surfaces and in hospital equipment currently require physical methods in addition to chemical methods. These methods include scrubbing, scraping, and ultrasonication. If performed correctly, biofilms can be eliminated effectively, however this is difficult in practice (Vickery *et al*., 2004)

Two major problems with successfully eliminating biofilm are penetration of antimicrobials into the biofilm matrix to reach bacterial cells, as well as ability to kill cells once they are reached. Penetration of antimicrobials into biofilms is slow, or non-existent. Slow diffusion of antimicrobials is due to various factors (Ishida *et al*., 1998). Negatively charged extracellular DNA as well as polymers within the biofilm matrix bind any cationically charged antimicrobials such as tobramycin and other aminoglycosides used to treat biofilm-based infections (Quirynene *et al*., 2000; Cao *et al*., 2015). Some antimicrobials without a cationic charge, such as ciprofloxacin and daptomycin, can penetrate the biofilm matrix to reach bacterial cells within the biofilm quickly, however may not be able to kill the bacteria once reached (Cao *et al*., 2015; Ishida *et al*., 1998). If an antimicrobial can reach cells within the biofilm, the compound is less concentrated due to binding within the matrix as well as enzyme deactivation. Another contributor of antimicrobial resistance in biofilms is slow-growing cells within biofilms. Most antimicrobials are more potent against fast growing cells, and less effective against slow growing cells (Lewis, 2001).

Several different approaches to enhancing biofilm eradication have been considered, involving the use of combination therapy with currently used disinfectants and antibiotics (Uppu *et al*., 2015). Addition of either specific enzymes that can dissolve biofilms, (Kaplan *et al*., 2012), antimicrobial peptides (AMPs) or other membrane disrupting compounds, (Gopal *et al*., 2014), or quorum-sensing inhibitors (Paza *et al*., 2013) to currently used disinfectants have been considered, and have promising results.

1.3 Amphiphiles as Antimicrobials

Amphiphiles are molecules that possess both hydrophilic and hydrophobic structural features (Ladow *et al.*, 2011; Figure 2). Common examples are molecules that make up the lipid bilayer of cell membranes. The hydrophilic headgroups face the extracellular and intracellular spaces and the hydrophobic carbon tails of the amphiphile face each other to minimize interaction with water.

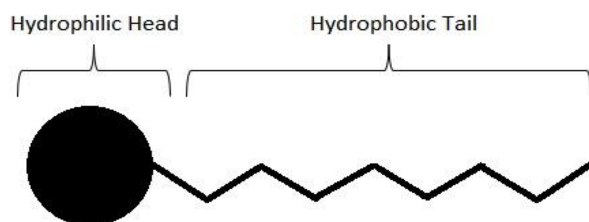

Figure 2: Generic amphiphile structure showing hydrophobic and hydrophilic regions on the same molecule.

Amphiphiles can possess antimicrobial properties. Both anti-microbial peptides (AMPs), and quaternary ammonium compounds (QACs) are amphiphilic molecules with antimicrobial properties currently in use as disinfectants and antibiotics.

AMPs are naturally occurring peptides produced by the innate immune system of most organisms to help eradicate infectious microorganisms in the body. More than 600 different AMPs have been identified in various organ systems (Marr *et al*., 2006). AMPs can also be designed and synthesized in a laboratory. AMPs are potent antimicrobials, however, their complex structure makes them time consuming and expensive to synthesize. Colistin is currently the only used cationic amphiphile antibiotic; it is similar in structure to AMPs. Colistin targets Gram-negative bacteria by binding to lipopolysaccharides (LPS) of the Gram-negative bacterial outer-membrane. This binding disrupts the outer-membrane and leads to cell permeability and death (Benhamou *et al*., 2015). Colistin is more toxic compared to most antibiotics, therefore is reserved for bacterial infections that possess high resistance to other drugs. The use of colistin as an antibiotic further suggests that there should be more research on the broader use of amphiphiles as antibacterials, rather than solely as disinfectants.

QACs are like AMPs but are smaller and less complex, leading to less time and expense in synthesis and production (Zhang *et al*., 2016). In the early 1900's, QACs started to be taken seriously as disinfectants (Domagk, 1935). In the 1930's, benzalkonium chloride (BZC), better known as the active ingredient in Lysol®, became the go-to disinfectant in cleaning household surfaces. New QACs were developed over the past decade, with improved antimicrobial activity and with less toxicity (Forman *et al.*, 2016). Benzalkonium chloride, for example, underwent substitutions of hydrogens on the aromatic ring with methyl, ethyl or chlorine to improve antimicrobial activity. These compounds include BTC 471, and Riseptin.

Today, due to increased concern for QAC toxicity, polymeric QACs with less potency and toxicity were developed. Synergistic interactions between these QACs lowered the

concentration needed for each compound to function, further improving the toxicity profile, and making them popular disinfectants, once again (Merianos, 2001).

AMPs and QACs have some advantages over conventional antibiotics and disinfectants including lower tendency of bacteria to develop resistance and easier avoidance of bacterial resistance mechanisms. Resistance is observed relatively shortly after an antibiotic is used, however, amphiphilic compound exposure must be repeated many times for resistance to occur (Marr *et al*., 2006). For instance, after 30 exposure passages of conventional antibiotics or cationic amphiphiles at sub-MIC concentrations, bacteria became 190-fold more resistant to antibiotics (Steinberg *et al*., 1997), whereas under the same conditions, only a 2 to 4-fold increase in resistance was seen with the amphiphiles (Zhang *et al*., 2005). Even if resistance develops in a bacterial population, amphiphile structure can be changed easily without dramatically altering antimicrobial activity (Marr *et al.*, 2006; Nizet, 2006). Mechanisms of resistance that are plausible to avoid are cell surface charge changes, and efflux pumps. When cell surface charges are altered, amphiphile affinity for the bacterial cell membrane could be weakened (Nizet, 2006). If more or fewer cations are added to the molecule, affinity for the bacterial cell membrane could be restored. To avoid efflux pump triggering, cations on the amphiphile could be spread across a larger surface area, allowing a slower diffusion rate through the bacterial membrane (Forman *et al.*, 2016).

The mechanism of action of amphiphiles against bacteria is hypothesized to be membrane disruption (Hancock and Rozek, 2002). There are different ways antimicrobials can disrupt membranes. If in monomer form, the molecules could intercalate into and even create holes in the bacterial membrane, but if they form micelles, the micelles disrupt by covering the membrane surface, interfering with its integrity in a detergent-like manner (Guillelmelli *et al.*,

2013). Most antibiotics cannot penetrate the outer membrane of Gram-negative bacteria as easily as can an amphiphile because an amphiphile can create its own pore instead of having to enter a porin.

Charge as well as hydrophobicity are important structural features that allow cationic amphiphiles to be highly potent antibacterials. The cationic charge increases the likelihood that the amphiphiles are preferentially targeting bacterial cells rather than eukaryotic cells. The negatively charged LPS of the outer membrane in Gram-negative bacteria, and the negatively charged polysaccharides on the cell walls of Gram-positive bacteria facilitate a strong attraction of cationic antimicrobial compounds to bacteria. Eukaryotic cells are inherently neutrally charged with much less negative charge than bacterial membranes, so positively-charged molecules should preferentially bind to bacteria (Guillelmelli *et al.*, 2013; Denyer, 1995). Amphiphiles act as detergents, aggregating at high concentrations, or as membrane disruptors, working in monomer form at low concentrations. If working as membrane disruptors, the compounds must have enough hydrophobicity in order to interact with the hydrophobic regions of the bacterial cell membrane (Danthe and Wieprect, 1999). This interaction destabilizes the membrane structure which leads to cell death (Cheng *et al*., 2015; Zhang *et al*., 2016; Danthe and Wieprect, 1999). If the compound has high hydrophobicity compared to hydrophilicity, the molecules will aggregate into micelles and become detergents. The exact combination of structural features that have the lowest MIC value continues to be investigated by a number of research groups.

Amphiphiles are some of the most potent biofilm disruptors. Concentrations as low as 25µM can disrupt preformed *S. aureus*, *E. faecalis* and *A. baumannii* biofilms (Jennings *et al.*, 2015; Feng *et al.*, 2013). The mechanism of action is theorized to happen by electrostatic

interactions between the negatively charged biofilm matrix and the positively charged amphiphile. This interaction enables the amphiphiles to reach bacterial cells within the biofilm leading to cell lysis. The most important amphiphile structural features required for biofilm disruption activity are similar to those that increase killing of planktonic cells: at least two cations with at least one hydrocarbon chain on an amphiphilic molecule (Jennings *et al.*, 2015).

1.4 Combination therapy

One solution to antimicrobial resistance is combination therapy. Bacteria resistant to one type of antimicrobial will likely respond to a second antimicrobial with a different mechanism of action with the hope that the antimicrobial resistant bacteria (AMRB) will not be resistant to more than one type of antimicrobial at the same time. Combination therapy also in some cases causes a synergistic effect where the combination of two antimicrobials are more than simply an additive effect. If a synergistic relationship occurs, the combination is better able to overcome resistance (Jorge *et al*., 2017). Combination therapy can be used as an alternative to resorting to antibiotics with harsh side effects or can reduce the concentration of toxic antimicrobials to lessen or eliminate toxicity. Combination therapy can also be used to avoid overusing antibiotics that are considered "last resort" against highly antibiotic resistant bacteria.

Hundreds of studies identifying synergistic interactions between different types of antimicrobials have been explored and have promising results (Zhang *et al*., 2014; Patel *et al*., 2016; Gopal *et al*., 2014; Goswami *et al*., 2015; Ngu-schwemlein *et al*., 2015; Soren *et al*., 2015; Hesje *et al.*, 2009). One widely used combination is a combination of a β-lactam antibiotic and aminoglycosides for use against *Enterococci*, *Streptococcus*, and *Staphylococcus* species. The β-lactam antibiotics interfere with cell wall synthesis, enabling easier aminoglycoside access into the cell (Miller *et al*., 1987; Moellering *et al.*, 1971). One of the most used antibiotic

combinations is amoxicillin and gentamicin to kill Staphylococcus species resistant to each antibiotic (Goldstein *et al*., 2003). Another reason for combination therapy is that a lower concentration of antibiotics reduce toxicity. Colistin, a "last resort drug" is a lipoprotein that can cause nephrotoxicity or neurotoxicity (Lora-Tamayo *et al*., 2014). A combination of polymyxin B, neomycin and bacitracin is used in the topical antibiotic ointment neosporin (Bonomo *et al*., 2014). Colistin acts by disrupting membranes, neomycin as a protein synthesis inhibitor, and bacitracin interferes with bacterial cell wall synthesis.

In recent research, synergistic interactions were seen between amphiphilic antimicrobials and several antibiotics. A variety of broad and narrow spectrum antibiotic classes have synergy with cationic amphiphiles or AMPs against bacteria, including tetracyclines, aminoglycosides, macrolides, fluoroquinolones, erythromycin, and chloramphenicol (Zhang *et al*., 2014; Patel *et al*., 2016; Gopal *et al.*, 2014; Goswami *et al*., 2015; Ngu-schwemlein *et al*., 2015). Membrane disrupting compounds, such as cationic amphiphiles, are theorized to create increased permeability of the bacterial cell membranes to allow antibiotic easier access into the cell (Ong *et al*., 2009; Gokel and Negin, 2012). Most antibiotics need access into a bacteria cell to work, therefore, any feature that allows for easier access should lower the MIC value of antibiotics. Tobramycin in combination with both natural and synthetic AMP's, including colistin, had synergy against antibiotic resistant strains of *Enterobacteriaceae* as well as *K. pneumoniae* (Pollini *et al*., 2017; Payne *et al*., 2017; Ozbek *et al*., 2015). Beta-lactam antibiotics, ceftriaxone and ceftazidime had synergy with the AMP novicidin when combined against antibiotic resistant Gram-negative bacteria (Soren *et al*., 2015).

Benzalkonium chloride (BZK) is readily used as a disinfectant in high concentrations and as a preservative in eye drops and nasal sprays in low concentrations (Hesje *et al*., 2009, and

Baron *et al*., 2016). BZK in combination with gatifloxacin, an antibiotic used in eye drops also has synergy (Dyer *et al*., 1998). Gatifloxacin based eye drops contain a small amount of benzalkonium chloride as a preservative, however, the combination was found to have an enhanced antimicrobial effect for the MIC of both benzalkonium chloride and gatifloxacin against *S. aureus* as well as MRSA (Hesje *et al.*, 2009). Combination therapy can also be used to better eradicate biofilms. Several studies have demonstrated enhancement of benzalkonium chloride antimicrobial activity when combined with other antimicrobial substances. Benzalkonium chloride and sodium hypochlorite in combination can remove biofilm forming *E. faecalis* from teeth. (Baron *et al.*, 2016).

Synergy is also observed between antimicrobials and compounds that are not antimicrobials. Sodium metaperiodate is an oxidative agent not used as an antimicrobial, but it has some antimicrobial properties. In recent research, sodium metaperiodate was able to reduce bacterial capsule size, which contributes to increased cell susceptibility to antimicrobials (Klesius *et al*., 2010; Decostere *et al*., 1999). Sodium metaperiodate in combination with antimicrobial citrate-based bio adhesives, used in marine organism studies, had a synergistic effect, lowering the MIC value against bacteria as well as fungus (Guo *et al*., 2015). Sodium metaperiodate in combination with other antimicrobial agents have synergy against Gram negative bacteria as well as biofilm communities. The combination of sodium metaperiodate, sodium bicarbonate, and sodium dodecyl sulfate were better at dispersing a *P. aeruginosa* biofilm than commercially used products, eradicating more than 90% of the biofilm (Gawande *et al*., 2008).

1.5 Compounds in current study

Structural features of amphiphiles such as type of headgroup, spacer length between headgroups, number of tails, tail length, amount of total carbons in tails, and number of charges affect the antimicrobial activity of amphiphiles (Ladow *et al*. 2011; Grenier *et al*., 2012; Jennings *et al.*, 2014; Marafino *et al*., 2015; Minbiole *et al*., 2016; Forman *et al*., 2016; Gallagher *et al*, 2017). In the current study, several features were altered to explore the effect of these changes to antimicrobial activity.

Headgroup substitution:

Four series of tris cationic amphiphiles with two symmetrical hydrocarbon tails, were synthesized. Each amphiphile possessed a mesitylene core, three positively charged head groups, separated evenly around the mesitylene core by a 5-carbon spacer with two symmetrical hydrocarbon tails. Two of these head groups were dimethylalkylammonium groups with attached hydrocarbon tails. The third head group was either with dimethylethanolamine for the M-E n,n series, dimethylaminopyridinium for the M-DMAP n,n series, isoquinolinium for the M-IQ n,n series, or 4-propanol pyridinium for the M-4PP n,n series. Each series had tail lengths ranging from 8 to 16, depending on the series (Figure 3).

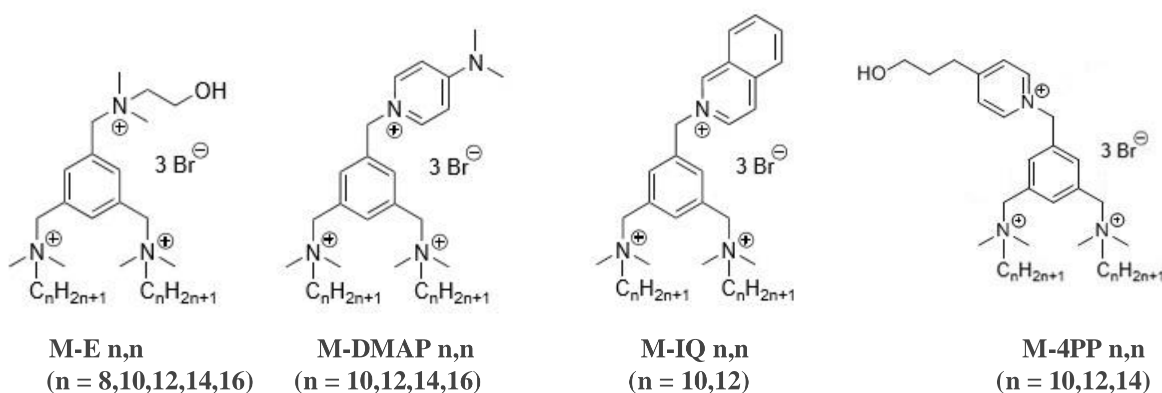

Figure 3: Amphiphile organic structure. M = mesitylene core; n = number of carbons in each symmetrical hydrocarbon tail. E = dimethylethanolammonium, DMAP = dimethylaminopyridinium, IQ = isoquinolinium, and 4PP = 4-propanol pyridinium.

Spacer variation and charge number:

Three bis-cationic amphiphile series and three tetra-cationic series, each with two symmetrical tails were synthesized to determine effect of varied placement of headgroups and tails on the central benzene ring. The bis-cationic amphiphile series consisted of two dimethylalkylammonium residues attached to the central ring in either the ortho (oX-n,n series), meta (mX-n,n series) or para orientation (pX,n,n series). One linear hydrocarbon chain was attached to each of the two dimethylalkylammonium groups, and contained 8-14 carbons for the oX-n,n series, 8-12 carbons for the mX-n,n series, and 8-16 carbons for the pX-n,n series (Figure 4). The tetra-cationic amphiphile series were structured similarly, however two dimethylalkylammonium head groups were attached to each of the same positions on the central ring, connected by a 2-carbon linker. The linear hydrocarbon tail connected to the head group in each of the tetra-cationic series contained 8-12 carbons for the oX-, mX-, and pX-$(2,n)_2$ series (Figure 5).

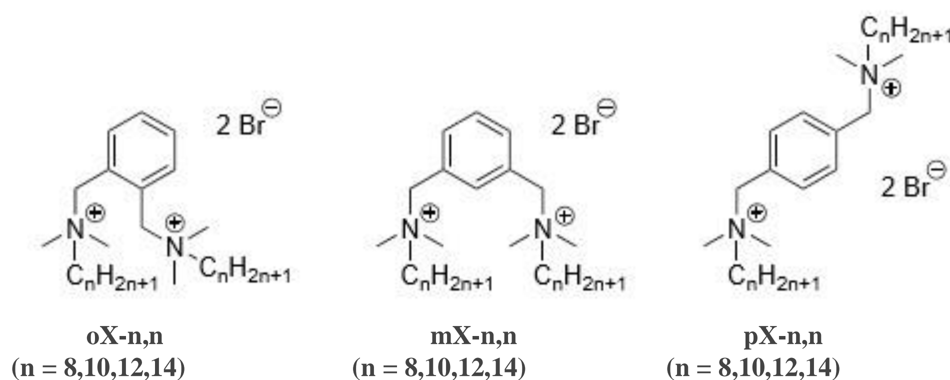

Figure 4: Structure of bis-cationic amphiphiles. n = number of carbons in each symmetrical hydrocarbon tail. oX = ortho-orientation; mX = meta-orientation; pX = para-orientation.

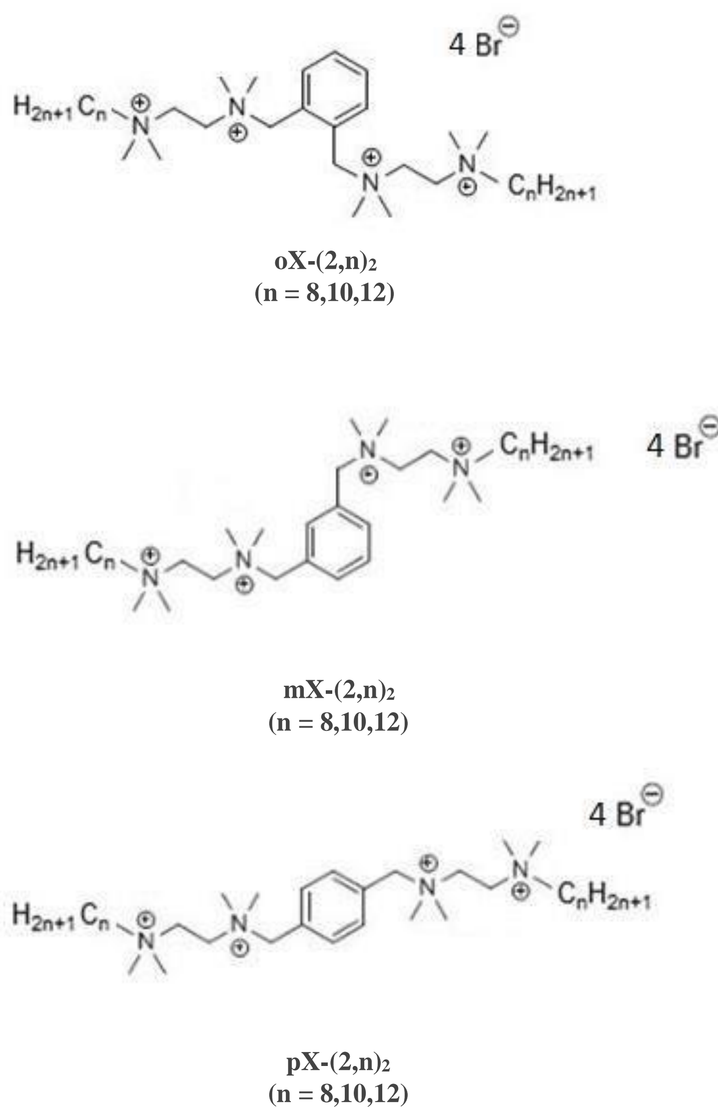

Figure 5: Structure of tetra-cationic amphiphiles. n = number of carbons in each symmetrical hydrocarbon tail. oX = ortho-orientation; mX = meta-orientation; pX = para-orientation.

Tail length variation:

One series of hexa-cationic, triple tailed amphiphiles were synthesized. Each amphiphile possessed 6 dimethylalkylammonium headgroups attached to a benzene ring, and three linear hydrocarbon tails of 8, 10, or 12 carbons in length (Figure 6).

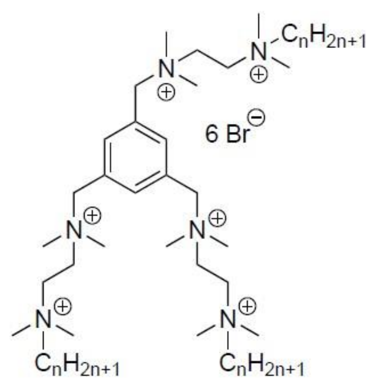

Figure 6: Structure of the 6-headed, triple-tailed M-$(2,n)_3$ series. M = mesitylene core; n represents the number of carbons per tail (8,10,12).

Chapter 2: The effect of head group substitution and tail length variation on synergistic combinations, kill time, and biofilm disruption

2.1 Introduction

Antimicrobial resistance is a growing problem in the medical field, with antibiotic resistant bacteria responsible for approximately 2 million illnesses annually in the United States alone (CDC, 2013). If a solution is not found to prevent the spread of antimicrobial resistance, the projected death toll worldwide due to antimicrobial resistance will rise from 700,000 per year to nearly 10 million per year by 2050, higher than death due to cancer (O'Neill, 2014; Figure 1). Preventative measures such as development of disinfectants and antiseptics that kill quickly and have antibiofilm capabilities would likely help prevent the spread of resistance.

Cationic amphiphiles are one possible solution to antimicrobial resistance, as they can be used as effective disinfectants and antiseptics. Amphiphiles are molecules that possess both hydrophobic and hydrophilic structural features, and can have extensive antimicrobial properties (Ladow *et al*., 2013). Structural features effect antimicrobial activity of amphiphiles such as type of headgroup, placement of headgroup on molecule, number of tails, tail length, amount of total carbons in tails, spacer length between headgroups, and number of charges. Charge as well as hydrophobicity are important structural features that allow cationic amphiphiles to be highly active against bacteria (Marafino *et al*., 2015; Ladow *et al*., 2014).

Amphiphiles are some of the most potent biofilm disruptors. Concentrations as low as 25µM can disrupt preformed *S. aureus*, *E. faecalis* and *A. baumannii* biofilms (Jennings *et al*., 2015; Feng *et al*., 2013). The ideal structure for biofilm disruption is not known, however thus far, the most important structural features required for biofilm disruption activity is similar to that of killing planktonic cells; at least two cations with at least one hydrocarbon chain on an amphiphilic molecule (Jennings *et al*., 2015).

Combination therapy could be used with amphiphiles to help combat antibiotic resistance. Bacteria with resistance mechanisms against one type of antimicrobial will respond to a second antimicrobial with a different mechanism of action with the expectation that the antimicrobial resistant bacteria will not be resistant to more than one type of antimicrobial at the same time. Combination therapy also, in some cases causes a synergistic effect where the combination of two antimicrobials are more effective at killing bacteria than alone. If a synergistic effect happens, combination therapy can overcome resistance to both compounds by combination (Jorge *et al*., 2017). Recent research involving amphiphilic antimicrobials and certain types of antibiotic reveal that these compounds have synergistic interactions when combined. A variety of broad and narrow spectrum antibiotic classes have been determined to have synergy with cationic amphiphiles or AMPs against certain bacteria, including tetracyclines (Ngu-schwemlein *et al*., 2015), aminoglycosides, macrolides, fluoroquinolones, erythromycin (Patel *et al*., 2016; Gopal *et al.*, 2014; Goswami *et al*., 2015), and chloramphenicol (Zhang *et al*., 2014). Membrane disrupting compounds, such as cationic amphiphiles, are theorized to make bacterial cell membranes more permeable, allowing an antibiotic easier access into the cell (Ong *et al*., 2009; Gokel and Negin, 2012). Most broad-spectrum antibiotics need access into a bacteria cell to work, therefore any feature that allows for easier access should lower the MIC value of antibiotics.

Four series of tris-cationic, double tailed amphiphiles were synthesized. Each series possessed three cationic head groups connected to a mesitylene core. Two of the head groups were dimethylalkylammonium groups, both with attached hydrocarbon tails, symmetrical in length. The third head groups were an dimethylethanolammonium (M-E series), dimethylaminopyridinium (M-DMAP series), isoquinolinium (M-IQ series), or 4-propanol pyridinium (M-4PP series; Figure 4). Hydrocarbon tail lengths varied from 8 to 16. The MIC of

each series with varying tail lengths was determined for several different bacterial species (Rogers, 2017). Tails with 12 carbons had the lowest MIC value for each series. Three of the four series with the 12-carbon tail length also possessed biofilm disruption capabilities on par with tobramycin at 65% biofilm disruption (Rogers, 2017). In this study, biofilm disruption activity was further explored for effect of tail length variation. Time to kill *S. aureus* and *P. aeruginosa* as well as combination studies with tobramycin, oxacillin, sodium metaperiodate and benzalkonium chloride against *S. aureus* and *E. coli* were performed to further explore amphiphile capabilities.

2.2 Results and Discussion

Time Kill

Amphiphiles in the following series were tested against *P. aeruginosa* and *S. aureus* for amount of time needed to eradicate all bacterial cells in the sample. Only amphiphiles in the four tris-cationic series with low MIC values against both Gram-positive and negative species were used. All amphiphiles tested killed all *S. aureus* cells in sample between 15 minutes and 1 hour, and reduced cell number 2000-fold between one and five minutes. The Gram-negative Species *P. aeruginosa* took 24 hours or longer to kill.

Amphiphiles with a twelve-carbon chain length had the fastest kill time (Table 1). MDMAP 12,12, M-E 12,12, M-4PP 12,12, and M-IQ 12,12, reduced cell number 2000-fold within one minute, and could kill all bacteria in the *S. aureus* sample in 15 minutes. M-4PP 10,10 and M-DMAP 14,14 reduced cell number 2000-fold within five minutes. M-4PP 10,10 killed all cells in sample within one hour, whereas M-DMAP 14,14 did so in 30 minutes.

Table 1: Time kill results (in hours) of each amphiphile tested against *P. aeruginosa* and *S. aureus* at 100µM concentration. MIC values for each amphiphile/bacteria pair are indicated in µM.

Compound: (M-X n,n)	***P. aeruginosa***		***S. aureus***	
	Time Kill (Hours)	MIC (µM)	Time Kill (Hours)	MIC (µM)
M-DMAP 12,12	24	16	0.25	4
M-DMAP 14,14	72+	63	0.5	4
M-4PP 10,10	24	16	1	4
M-4PP 12,12	24	16	0.25	2
M-IQ 12,12	24	16	0.25	4
M-E 12,12	72	16	0.25	4

Amphiphiles with a twelve or ten carbon chain length, M-DMAP 12,12, M-E 12,12, M4PP 12,12, M-4PP 10,10, and M-IQ 12,12, reduced *P. aeruginosa* cell numbers 2000-fold within two hours with the exception of M-E 12,12, which took between three and 24 hours. The time to kill all cells in the sample was 24 hours for all amphiphiles with the exception of M-E 12,12, which took 72 hours, and M-DMAP 14,14 which did not reduce cell number within 72 hours. The MIC value of M-DMAP 12,12, M-4PP 12,12 and M-E 12,12 were the lowest at 16µM, however M-E 12,12 took longer to kill by four-fold. M-IQ 12,12 and M-4PP 10,10 had an MIC of 31µM, but had a time kill the same as M-DMAP 12,12 and M-4PP 12,12. M-DMAP14,14 had an MIC value of 63µM which was not potent enough to kill *P. aeruginosa* cells within 72 hours. In summary, the 12-carbon tail length amphiphiles for each tris-cationic series tested killed the quickest, with M-DMAP 12,12 and M-IQ 12,12 having the fastest reduction in *S. aureus* cells within one minute.

Combination Studies

Tris-cationic amphiphiles differing only in a single head group were tested for synergy when combined with either tobramycin, oxacillin, sodium metaperiodate ($NaIO_4$) or benzalkonium chloride (BZK) against *S. aureus* and *E. coli*.

The combination of benzalkonium chloride and M-DMAP 12,12 or M-IQ 12,12 against *E. coli* and *S. aureus* had synergy, with an FIC of 0.5, which is considered a synergistic interaction (Table 2). The combination of tobramycin and M-E 12,12 against *S. aureus* had an FIC of 0.5 indicating synergy. The rest of the combinations were not synergistic.

Table 2: FIC values between amphiphile and tobramycin, benzalkonium chloride (BZK), sodium metaperiodate ($NaIO_4$) or oxacillin against *E. coli* and *S. aureus*. X = head group (E = dimethylethanolammonium; DMAP = dimethylaminopyridinium; IQ = isoquinolinium; 4PP = 4-propanol pyridinium), n= hydrocarbon tail length. Values are FIC of each combination where ≤ 0.5 indicates synergy and are in bold.

	E. coli				***S. aureus***			
Compounds: (M-X n,n)	tobramycin	BZK	$NaIO_4$	oxacillin	tobramycin	BZK	$NaIO_4$	oxacillin
M-E 12,12	1	1	0.75	1.02	**0.5**	0.63	1	0.75
M-DMAP 12,12	0.75	**0.5**	0.75	0.52	0.63	**0.5**	0.63	0.75
M-IQ 12,12	0.75	**0.5**	0.51	0.53	0.75	**0.5**	0.63	0.63
M-4PP 12,12	1	1.5	1	0.52	1.25	0.71	1	1

Oxacillin was further tested with amphiphiles varying in tail length against *E. coli*, a representative Gram-negative bacterial species. All tail length variations were tested for MDMAP series (16, 14, 12, and 10 hydrocarbons). A tail length of 10 hydrocarbons had synergy with oxacillin with an FIC of 0.19. A 10-carbon length tail was not synergistic with oxacillin for all series tested. M-IQ 10,10 had synergy however M-4PP 10,10 only had an additive effect when combined with oxacillin. The FIC values were 0.31 and 0.52 respectively.

An additive effect was found with all 12-carbon length amphiphiles tested with the exception of M-E 12,12, which had an FIC of 1.02 (Table 3).

Table 3: FIC values between amphiphile and oxacillin against *E. coli*. X = head group (E = dimethylethanolammonium; DMAP = dimethylaminopyridinium; IQ = isoquinolinium; 4PP = 4-propanol pyridinium), n=hydrocarbon tail length. Values were FIC of each combination where ≤ 0.5 indicates synergy. FIC with values ≤ 0.5 were bolded. ND=Not done. MIC values in µM.

Compound	FIC	MIC
M-DMAP 10,10	**0.19**	63
M-DMAP 12,12	0.52	8
M-DMAP 14,14	0.55	16
M-DMAP 16,16	1.06	31
M-4PP 10,10	0.52	8
M-4PP 12,12	0.52	2
M-4PP 14,14	ND	16
M-IQ 10,10	**0.31**	8
M-IQ 12,12	0.53	8
M-E 8,8	ND	>250
M-E 10,10	ND	125
M-E 12,12	1.02	4
M-E 14,14	ND	8
M-E 16,16	ND	63

Biofilm Disruption

Tris-cationic, double tailed amphiphile series M-DMAP n,n, was used to determine the effect of tail length on biofilm disruption activity. Four different tail lengths derivatives of MDMAP n,n were tested including 10, 12, 14 and 16 (Figure 7). Tail length had an effect on biofilm disruption, but only at the 250µM concentration. M-DMAP 10,10 and M-DMAP 12,12 had 71% biofilm disruption at 250µM concentration, however M-DMAP 14,14 and M-DMAP 16,16 had 85% biofilm disruption at the same concentration.

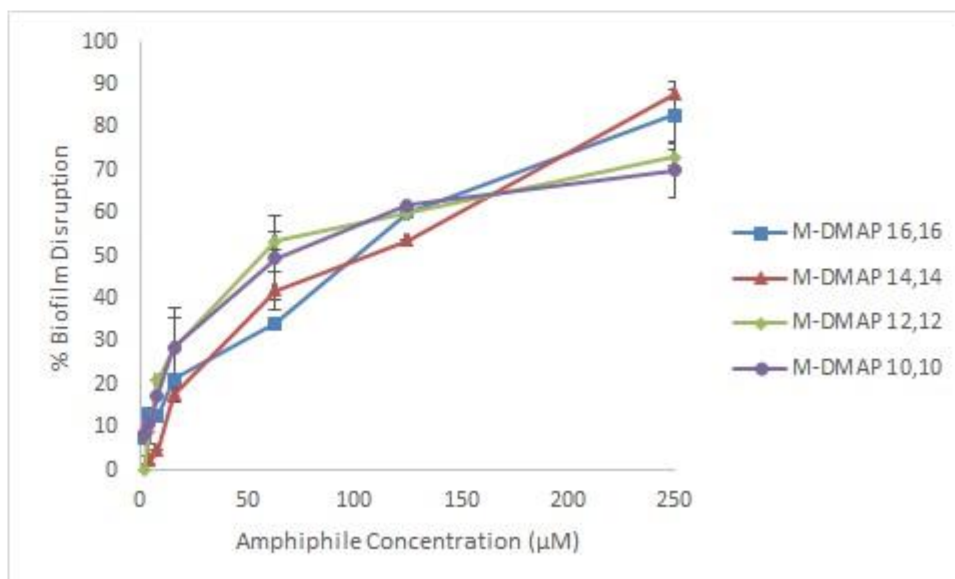

Figure 7: Difference in percent biofilm disruption activity by 10,12,14 and 16 hydrocarbon tail length derivatives of M-DMAP(n,n) amphiphile series. n=hydrocarbons in tail. Error bars represent standard deviation of at least two independent trials.

2.3 Conclusions

Several trends emerged from this work that will help with developing amphiphiles with better biofilm disrupting activity, that kill faster, and interact synergistically with other compounds. For double tailed, tris-cationic amphiphiles, longer tail lengths at 250µM concentration contributed to higher biofilm disruption activity. In general, double-tailed cationic amphiphiles have decreased MIC with tail lengths higher than 12 hydrocarbons. A balance between increased hydrophobicity of the amphiphile and altering the headgroups to keep MIC value low could help with creating amphiphiles with low MIC against planktonic bacteria, while also disrupting a high percentage of preformed biofilms.

The 12 hydrocarbon tail length derivatives of all four series were the amphiphiles that killed the fasted, killing *S. aureus* within 15 minutes and *P. aeruginosa* within 24 hours. Head group substitution did not affect time kill value.

Synergistic combinations were seen primarily with M-DMAP n,n and M-IQ n,n series. Both M-DMAP 12,12 and M-IQ 12,12 had synergy with benzalkonium chloride against both *S. aureus* and *E. coli.* Both of these amphiphiles also had an additive effect when combined with tobramycin, oxacillin and sodium metaperiodate against both *S. aureus* and *E. coli*.

When combining oxacillin with varied tail length derivatives of each tris-cationic amphiphile series, synergy was observed only with 10 hydrocarbon tail length derivatives of MDMAP and M-IQ. We can take from this data that a tail length of 10 hydrocarbons was more synergistic with oxacillin, and therefore likely has more synergy with other compounds, than 12, 14, or 16 hydrocarbon tail length amphiphiles with similar structure. We can also conclude that M-DMAP and M-IQ series was more synergistic with all compounds tested than M-4PP and ME series. The reason for this difference is unknown and needs further investigation.

2.4 Methods and Materials

Bacterial Strains and Growth Conditions

The Gram-negative bacterial strains used in this study were *Escherichia coli* ATCC® 25922™, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC® 27853™ for experiments with planktonic cells, and hyper-biofilm forming *Pseudomonas aeruginosa* strain PAO2 for biofilm disruption studies (Holloway, 1955). The Gram-positive bacterial strains used were *Staphylococcus aureus* subsp. aureus ATCC® 29213™, *Enterococcus faecalis* ATCC® 29212™, *Bacillus anthracis* Sterne, *Streptococcus agalactiae* J48 (Seifert *et al*., 2006). All bacterial strains, except for PAO2 were grown in MHB. For biofilm studies, PAO2 was grown in Luria-Bertani (LB) broth.

Minimum Inhibitory Concentration

The MIC was determined for each amphiphile against four Gram-positive and three Gram-negative bacterial strains as previously described (CLSI, 2012). Bacterial cultures were grown overnight at 37°C and diluted in MHB to a concentration of $5x10^6$ cells/ml. Aliquots of 100µl were added to 96-wells plates along with 100µl of amphiphile being tested at 2-fold dilutions, from 500µM to 2µM in triplicate, yielding a final concentration of $5x10^5$ cells/ml in each well. Sterile deionized water was used as a control for bacterial growth. The plates were incubated at 37°C for 72 hours. The wells with lowest concentration of amphiphile without visual growth were used to determine the MIC. Each amphiphile was tested a minimum of 2 trials.

Time-kill

Time-kill assays were performed as previously described (Ladow *et al.*, 2011). Overnight cultures of *S. aureus and P. aeruginosa* were diluted with Mueller-Hinton broth and amphiphile to $2.5x10^6$ cells/ml. Amphiphiles added to the broth culture had a final concentration of 100µM and were incubated at room temperature to mimic conditions when disinfectants are normally used. Aliquots of 100µ1 were plated on THB (Todd Hewitt Broth) agar plates at 0, 1, 5, 15, 30 minutes, 1, 2, 24 and 48 hours after incubation at room temperature for *S. aureus*, and 30 minutes, 1, 2, 3, 24, 48 and 72 hours after incubation at room temperature for *P. aeruginosa*, and incubated at 37°C for 24 hours, and colonies counted. Values are reported as the time when no colonies were observed.

Checkerboard Assay

A checkerboard assay using 96-well microtiter plates were used to determine if synergy exists when amphiphiles are combined with other compounds against *E. coli* ATCC® 25922™ and *S. aureus* ATCC® 29213™ (ATCC, Manassas, VA, USA). Bacteria diluted to $5x10^6$ cell/mL

was added to each well followed by 100µL of treatment, yielding $5x10^5$ cell/mL in each well. Wells with sterile deionized H_2O treatment used as a control for bacterial growth and a comparison for turbidity, respectively. In the checkerboard section of the assay, 50µl of each compound at 2-fold dilutions at concentrations below the previously determined MIC of each compound. The Fractional Inhibitory Concentration (FIC) was determined by comparing the MIC value of each compound alone and in combination using the following equation:

$FIC = FIC_A + FIC_B$

$FIC_A = A/MIC_A$

$FIC_B = B/MIC_B$

MIC_A and MIC_B represent the MIC of compound A and B alone and A and B represent the MIC values of compound A and compound B combined. Synergy was defined as an FIC value of <0.5, and antagonism was defined as an FIC value of >4. Synergy assays for each combination was performed at least 3 times.

Biofilm Disruption Assay

Biofilm disruption was determined as previously described (O'Toole, 2011). *P. aeruginosa* strain PAO2 was incubated overnight in LB broth at 37°C and diluted to $5x10^6$ cells/ml. Aliquots of 100µl were added to each well and incubated at 37°C for 24 hours for biofilm to grow. Serial 2-fold dilutions of amphiphile were added to wells containing bacteria and plates were incubated at 37°C for 24 hours. Tobramycin was used as a positive control for biofilm disruption. Bacterial cells were stained using the crystal violet method as previously described. Wells were rinsed with dH_2O and allowed to air dry completely. Crystal violet at a concentration of 0.01% was added to each well at 100µl aliquots and plates were incubated at room temperature for 15 minutes. Crystal violet stain was then removed and wells were rinsed

3-4 times with gently running deionized water, allowed to dry for 40 minutes, followed by addition of 100µl of 95% ethanol into each well. Plates were incubated with gentle shaking at room temperature for 1 hour. The ethanol/stain resulting mixture was then transferred into a clean 96-well microtiter plate and absorbance read with a plate reader at 570 nm. Absorbance values of treated wells were compared to the control to determine % biofilm disruption.

Chapter 3: The effect of spacer variation and tail length variation on bis- and tetra-cationic double-tailed amphiphile biofilm disruption, time kill and synergistic combinations with oxacillin.

3.1 Introduction

Antimicrobial resistance is a growing problem in the medical field, with antibiotic resistant bacteria responsible for 2 million illnesses annually in the United States alone (CDC, 2013). If a solution is not found to prevent the spread of antimicrobial resistance, the projected death toll worldwide due to antimicrobial resistance will rise from 700,000 per year to nearly 10 million per year by 2050, higher than death due to cancer (O'Neill, 2014). Preventative measures such as development of disinfectants and antiseptics with faster killing and antibiofilm capabilities would prevent the spread of resistance.

Cationic amphiphiles are one possible solution to antimicrobial resistance, as they can be used as effective disinfectants and antiseptics. Amphiphiles are molecules that possess both hydrophobic and hydrophilic structural features, and can have extensive antimicrobial properties (Ladow *et al*., 2013). Structural features affect antimicrobial activity of amphiphiles such as type of headgroup, placement of headgroup on molecule, number of tails, tail length, amount of total carbons in tails, spacer length between headgroups, and number of charges. Charge as well as hydrophobicity are important structural features that potentially allow cationic amphiphiles to be highly active against bacteria (Marafino *et al*., 2015; Ladow *et al*., 2014).

Amphiphiles are some of the most potent biofilm disruptors. Concentrations as low as 25µM can disrupt preformed *S. aureus*, *E. faecalis* and *A. baumannii* biofilms (Jennings *et al*., 2015; Feng *et al*., 2013). The ideal structure for biofilm disruption is not known; however thus far, the most important structural features required for biofilm disruption activity are similar to

those for killing planktonic cells: at least two cations with at least one hydrocarbon chain on an amphiphilic molecule (Jennings *et al*., 2015).

Combination therapy could be used with amphiphiles to help combat antibiotic resistance. Bacteria with resistance mechanisms against one type of antimicrobial will respond to a second antimicrobial with a different mechanism of action. The expectation is that the antimicrobial resistant bacteria will not be resistant to more than one type of antimicrobial at the same time. Combination therapy also in some cases causes a synergistic effect where the combination of two antimicrobials are more effective at killing bacteria than each is alone. In some cases, combination therapy can overcome antimicrobial resistance mechanisms (Jorge *et al*., 2017). Recent research involving amphiphilic antimicrobials and certain types of antibiotic revealed synergy when these were combined. A variety of broad and narrow spectrum antibiotic classes have synergy with cationic amphiphiles or AMPs against bacteria, including tetracyclines (Ngu-schwemlein *et al*., 2015), aminoglycosides, macrolides, fluoroquinolones, erythromycin (Patel *et al*., 2016; Gopal *et al.*, 2014; Goswami *et al*., 2015), and chloramphenicol (Zhang *et al*., 2014). Membrane disrupting compounds such as cationic amphiphiles are theorized to make bacterial cell membranes more permeable, allowing an antibiotic easier access into the cell (Ong *et al*., 2009; Gokel and Negin, 2012). Most broad-spectrum antibiotics need access into a bacteria cell to work, therefore any feature that allows for easier access should lower the MIC value of antibiotics.

Three bis-cationic amphiphile series and three tetra-cationic series, each with two symmetrical tails, were synthesized to determine the effect of varied placement of headgroups and tails on the central benzene ring. The bis-cationic amphiphile series consisted of two dimethylalkylammonium residues attached to the central ring in either the ortho (oX-n,n series),

meta (mX-n,n series) or para orientation (pX,n,n series). One linear hydrocarbon chain was attached to each of the two dimethylalkylammonium groups, and contained 8 to 14 carbons for the oX-n,n series, 8 to 12 carbons for the mX-n,n series, and 8 to 16 carbons for the pX-n,n series (Figure 5). The tetra-cationic amphiphile series were structured similarly, however two dimethylalkylammonium head groups were attached to each of the same positions on the central ring, connected by a 2-carbon linker. The linear hydrocarbon tail connected to the head group in each of the tetra-cationic series contained 8 to 12 carbons for the oX-, mX-, and pX-$(2,n)_2$ series. MIC values were determined previously (Rogers, 2017). Consistent with previous research on similar amphiphiles, tail length had more of an effect on MIC value than position of hydrocarbon tail or head group around benzene ring. The 12-carbon tail length for each series had the lowest MIC value (Rogers, 2017).

One series of hexa-cationic, triple tailed amphiphiles were synthesized. Each amphiphile possessed 6 dimethylalkylammonium headgroups attached to a benzene ring, and three linear hydrocarbon tails of 8, 10, or 12 carbons in length. In previous research the hexa-cationic amphiphile with 10 hydrocarbon tail lengths had the lowest MIC (Rogers, 2017). Disruption of preformed *P. aeruginosa* biofilms were determined for effect of tail length variation, charge number, and spacer variation. Time kill studies on *S. aureus* and *P. aeruginosa* as well as combination studies with oxacillin against *E. coli* were performed to further explore amphiphile capabilities.

3.2 Results and Discussion

Time Kill:

Bis, tetra and hexa-cationic amphiphiles were tested for time kill values against *S. aureus* and *P. aeruginosa*. Number of charges, tail length, number of tails, and spacer variation were

tested for their effect of time kill values. Spacer variation and tail length influenced time kill value against *S. aureus*. Ox-12,12 reduced cell number by 2000-fold immediately after addition to *S. aureus* sample and was able to kill all cells within 1 minute. Px-12,12 and mX-12,12 reduced cell number by 2000 fold within one minute, and all cells within 5 minutes. The MIC value of pX-12,12 was lowest compared to mX- and oX-12,12, at 2μM compared to 8μM but did not demonstrate faster bacterial killing. Px-10,10 was slightly slower, reducing cell number by 2000-fold within 5 minutes, and all cells within 15 minutes (Table 4).

Tetra-cationic amphiphiles with 12 carbon length tails were fastest at killing bacteria in the series. mX-$(2,12)_2$ killed *S. aureus* in 30 minutes, whereas pX-$(2,12)_2$ killed *S. aureus* in an hour of exposure. Both started working within 5 minutes, reducing cell count by 2000-fold. Bis-cationic amphiphiles with similar structure were faster at killing *S. aureus* cells than tetra-cationic derivatives.

Hexa cationic amphiphiles were similar to tetra cationic amphiphiles in time kill. M-$(2,10)_3$ was able to kill *S. aureus* in one hour, and reduced cell count by 2000-fold within 5 minutes. M-$(2,8)_3$, the only 8 carbon chain amphiphile with a low MIC against *S. aureus* was able to reduce cell count by 2000-fold within 24 hours and killed all cells in the sample within 72 hours. Despite having a very low MIC against *S. aureus*, 1μM, both hexa cationic amphiphiles were slow at killing bacterial cells (Table 4). MIC value therefore is not always correlated with time kill value.

All amphiphiles tested needed 24 hours or more to kill all *P. aeruginosa* cells in the sample. Only four out of seven amphiphiles tested could kill all cells in the sample within 72 hours. The only bis-cationic amphiphile with a time kill of under 72 hours was mX-12,12. Cells in the sample were reduced 2000-fold in 24 hours and killed all cells in 48 hours. pX-12,12, oX-

12,12 and pX-10,10 did not reduce cell number within 72 hours of exposure.

Both tetra-cationic amphiphiles tested reduced cell number within 24 hours and eradicated all cells in sample within 48 hours. One hexa cationic amphiphile was tested. M-$(2,10)_3$ reduced cell number between 3 and 24 hours and had the lowest time kill at 24 hours (Table 4).

Table 4: Results of time kill assay (in hours) with bis-cationic compounds oX (n,n), mX (n,n), pX (n,n), tetra-cationic compounds oX-$(2,n)_2$, mX-$(2,n)_2$, pX-$(2,n)_2$, (oX = ortho orientation; mX = meta orientation; pX = para orientation and n = the number of carbons per tail), and tris-cationic compound M-$(2,n)_3$, against *P. aeruginosa* and *S. aureus* at 100µM concentration. MIC values for each amphiphile/bacteria pair are indicated in µM. 0.017 hours = 1 minute, and 0.083 hours = 5 minutes. Time kill values of less than 10 minutes are bolded. ND = not done

	P. aeruginosa		***S. aureus***	
Compound	Time Kill (Hours)	MIC (µM)	Time Kill (Hours)	MIC (µM)
oX-12,12	>72	16	**0.017**	8
mX-12,12	48	16	**0.083**	8
pX-10,10	>72	63	0.25	8
pX-12,12	>72	16	**0.083**	2
mX-$(2,12)_2$	48	31	0.5	2
mX-$(2,10)_2$	>72	>250	72	16
pX-$(2,12)_2$	48	31	1	2
oX-$(2,12)_2$	48	31	1	2
M-$(2,8)_3$	ND	>500	72	1
M-$(2,10)_3$	24	16	1	1

In conclusion, the number of charges had an effect on time kill values against *S. aureus*. Bis-cationic amphiphiles tested had the fastest time-kill value against *S. aureus*, with the lowest time kill of 1 minute, seen with oX-12,12. Tail length also had an effect on time kill, as the 12 carbon derivatives of bis- and tetra-cationic amphiphiles had a faster time kill compared to 10. A tail length of 10 hydrocarbons was better than 8 for hexa-cationic amphiphiles with three

tails. Interestingly, the 8-carbon variation of the hexa-cationic series had the same MIC value as the 10-carbon variation against *S. aureus*, however the time kill values were vastly different between these two amphiphiles showing that MIC is not the only predictor. Spacer variation had some effect on time kill, as bis-cationic amphiphiles in the ortho position killed *S. aureus* faster than those in the meta or para position on the benzene ring.

Combination Studies

Oxacillin, a beta-lactamase resistant antibiotic, is normally only effective at killing Gram-positive bacterial species. Here we test if the addition of amphiphile will lower the MIC of oxacillin against Gram-negative bacteria. Amphiphiles with bis, tetra or hexa cations were tested in combination with oxacillin against *E. coli*, a representative Gram-negative species, to determine FIC. Synergy was defined as an FIC value of less than or equal to 0.5. Combinations had an additive effect if between 0.51 and 1, no effect between 1.01 and 4 and antagonistic if greater than 4. Bis-cationic amphiphiles with 8 or 10 carbon chain length had synergy with oxacillin. Ox-8,8, mX-10,10, and pX-10,10 had FICs of 0.19, 0.32 and 0.38 respectively. The 8-carbon tail length had the lowest FIC. Two 12 carbon tail length amphiphiles tested, oX-12,12, and mX-12,12 had an additive effect when combined with oxacillin with FICs of 0.63, and 0.52 respectively. Both 14 carbon tail length amphiphiles tested had no effect when combined (Table 5a).

All tetra-cationic amphiphiles tested had synergy with oxacillin, which included one amphiphiles with an 8-carbon length tail, three with 10-carbon length tails, and 2 with 12-carbon length tails. The lowest FIC values were seen with the 8 and 10 carbon tail length amphiphiles, ranging between 0.19 and 0.38 (Table 5b).

All hexa-cationic amphiphiles tested had synergy with oxacillin. Only 8 and 10 carbon tail length amphiphiles in the series was tested. M-$(2,8)_3$ had an FIC of 0.19, and M-$(2,10)_3$ had a slightly higher FIC of 0.27 (Table 5c).

Table 5: Results of combination studies with oxacillin and **a)** bis-cationic compounds oX-n,n, mX-n,n, pX-n,n, or **b)** Tetra-cationic compounds oX-$(2,n)_2$, mX-$(2,n)_2$, pX-$(2,n)_2$, (oX = ortho orientation; mX = meta orientation; pX = para orientation and n = the number of carbons per tail), or **c)** tris-cationic compound M-$(2,n)_3$, against *E. coli*. Combination values in FIC where ≤0.5 indicates synergy. Synergic combinations were highlighted. MIC values for each amphiphile/bacteria pair are indicated in µM. ND=Not done

a.

Compounds	FIC	MIC
oX-8,8	**0.19**	250
oX-10,10	ND	16
oX-12,12	0.63	4
oX-14,14	1.02	63
mX-8,8	ND	>250
mX-10,10	**0.32**	31
mX-12,12	0.52	4
pX-8,8	ND	250
pX-10,10	**0.38**	16
pX-12,12	1.02	4
pX-14,14	1.02	31

b.

Compound	FIC	MIC
oX-$(2,8)_2$	**0.19**	>250
oX-$(2,10)_2$	**0.25**	31
oX-$(2,12)_2$	ND	8
mX-$(2,8)_2$	ND	>250
mX-$(2,10)_2$	**0.19**	31
mX-$(2,12)_2$	**0.38**	16
pX-$(2,8)_2$	ND	250
pX-$(2,10)_2$	**0.19**	31
pX-$(2,12)_2$	**0.38**	16

c.

Compound	FIC	MIC
M-$(2,8)_3$	**0.19**	16
M-$(2,10)_3$	**0.27**	8
M-$(2,12)_3$	ND	31

Although M-$(2,10)_3$ FIC was not the lowest seen when combined with oxacillin against *E. coli*, the reduction in oxacillin MIC was the most dramatic. The MIC of oxacillin against *E. coli* species was reduced 64-fold, from 1000µM to 16µM, near clinical strength when combined with M-$(2,10)_3$ (Figure 8; Table 5c).

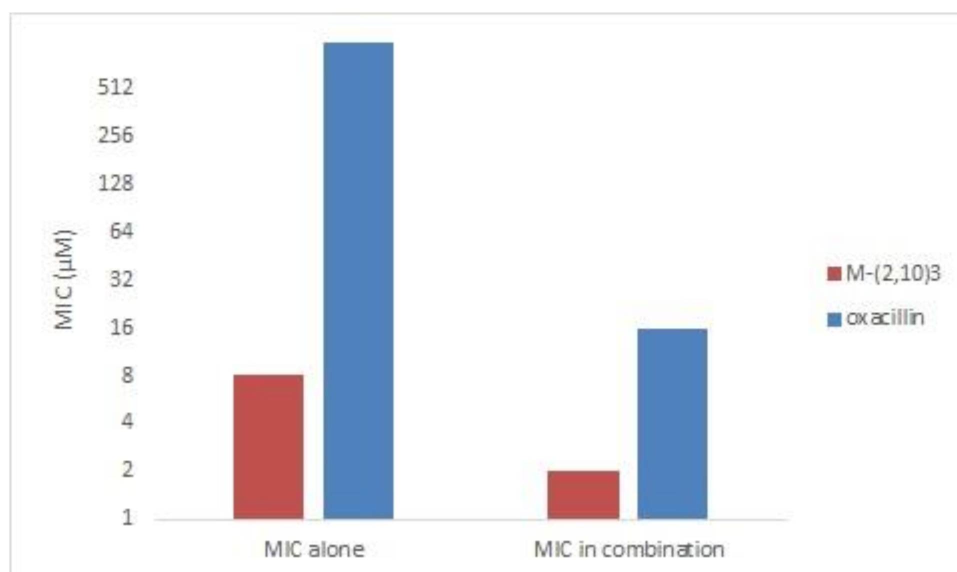

Figure 8: MIC values of oxacillin and M-$(2,10)_3$ when used alone and in combination to kill *E. coli*.

Biofilm Disruption

Effect of spacer length

Bis-cationic amphiphiles pX-12,12, mX-12,12 and oX-12,12, and tetra-cationic amphiphiles pX-$(2,12)_2$, mX-$(2,12)_2$ and oX-$(2,12)_2$ biofilm disruption activity were compared to determine effect of spacer length. *P. aeruginosa* preformed biofilms were exposed to 2-fold serial dilutions of each amphiphile and stained with crystal violet to find the percentage biofilm disruption compared to negative control. For bis-cationic amphiphiles, biofilm disruption increased in a concentration dependent manner, peaking at 125µM with a maximum of 58% disruption (Figure 9). Bis-cationic amphiphiles tested were identical in biofilm disruption at each concentration, indicating spacer variation had no effect on biofilm disruption activity.

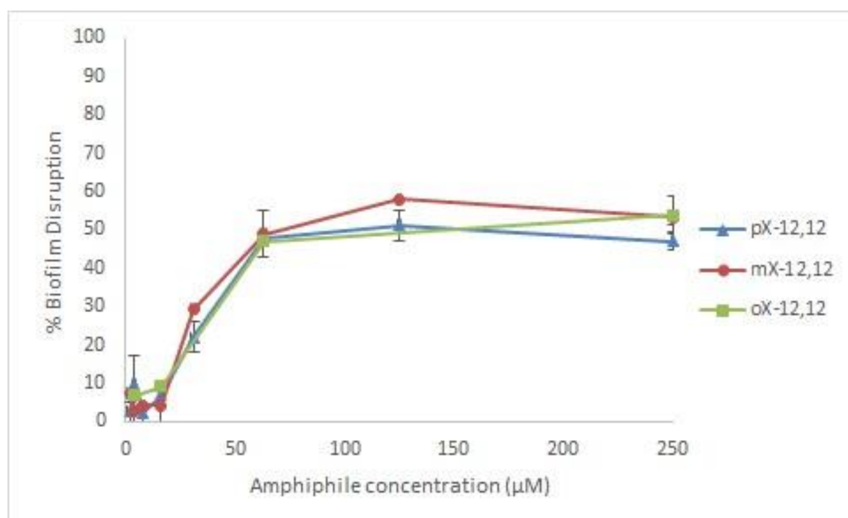

Figure 9*:* Percent biofilm disruption of 12 hydrocarbon tail length derivatives of bis-cationic amphiphile series at the para, meta, or ortho position on a benzene ring. Error bars represent standard deviation of at least two independent trials.

Tetra-cationic amphiphiles tested did not have the same trend as with bis-cationic amphiphiles (Figure 10). There was no difference in biofilm disruption activity between pX-$(2,12)_2$ and mX-$(2,12)_2$, however oX-$(2,12)_2$ was different at the 250µM concentration. All three amphiphiles had an increase in biofilm disruption in a concentration dependent manner, peaking at 63µM with a maximum of 62% disruption, followed by a slight decline at 125µM, and a steep decline at 250µM for pX-$(2,12)_2$ and mX-$(2,12)_2$. At 63µM and higher, oX-$(2,12)_2$ did not decline, but stayed the same. Spacer variation for tetra-cationic amphiphiles effects biofilm disruption activity at 125µM concentration and higher.

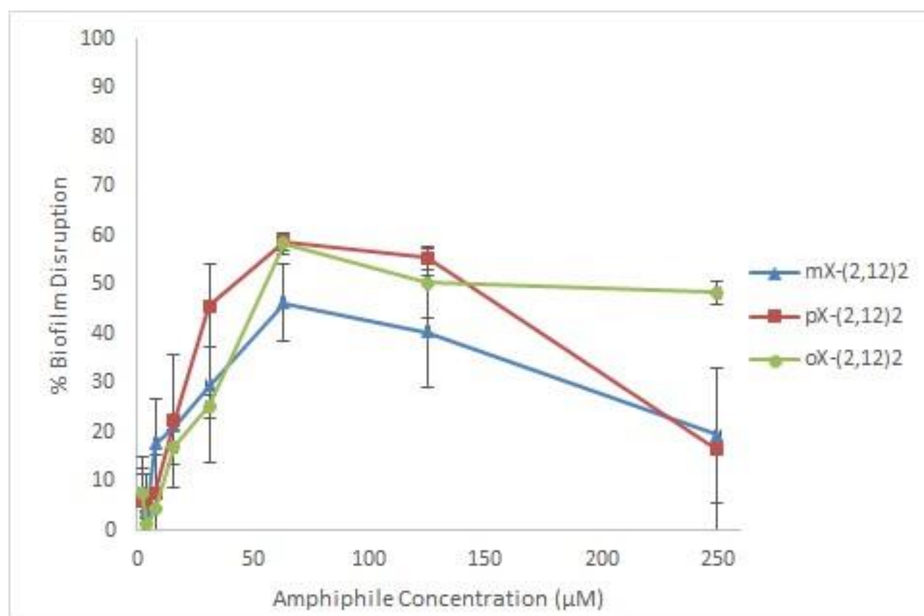

Figure 10: Percent biofilm disruption of 12 hydrocarbon tail length derivatives of tetra-cationic amphiphile series at the para, meta or ortho position on a benzene ring. Error bars represent standard deviation of at least two independent trials.

Effect of tail length

Bis-cationic and hexa-cationic amphiphiles were tested to determine if biofilm disruption activity was correlated with tail length (Figure 11). The bis-cationic amphiphile pX-n,n series with 10, 12, and 14 carbon tail length was tested. Tail length had an effect on biofilm disruption activity. pX-10,10 had the highest biofilm disruption activity of 62% at a 63µM concentration, however percentage biofilm disruption dropped dramatically at 250µM. At 250µM, tail length had the most effect on biofilm disruption activity; biofilm disruption increased with increase in tail length: pX-14,14 disrupted 57%, pX-12,12 47% and pX-10,10 20%. pX-10,10 had maximum biofilm disruption at 63µM (63%), pX-12,12 at 125µM (58%) and pX-14,14 at 250µM (57%).

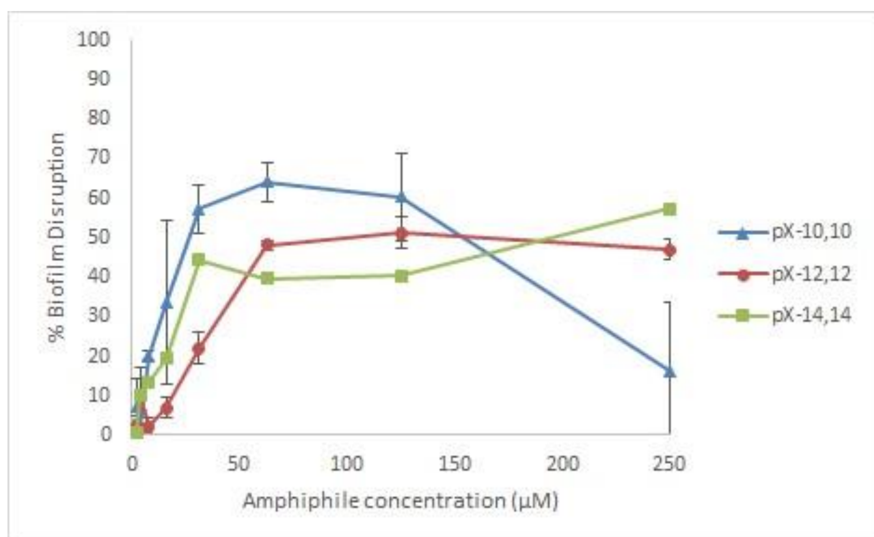

Figure 11: Percent biofilm disruption activity of bis-cationic amphiphile series with variation in tail length (10,12 or 14 hydrocarbons). Error bars represent standard deviation of at least two independent trials.

Two hexa-cationic amphiphiles from the M-$(2,n)_3$ series were tested for differences in biofilm disruption activity between 8 and 10 carbon tail length derivatives (Figure 12). As with other amphiphiles tested, biofilm disruption increased for both hexa-cationic amphiphiles in a concentration dependent manner, however, peak disruption was observed at 125µM with an average biofilm disruption of 65%. Little difference in biofilm disruption was observed between the 8 and 10 hydrocarbon versions. Since only two tail lengths were tested for this series, it can be concluded that there was no difference in biofilm disruption between the 8 and 10 hydrocarbon tail length derivatives.

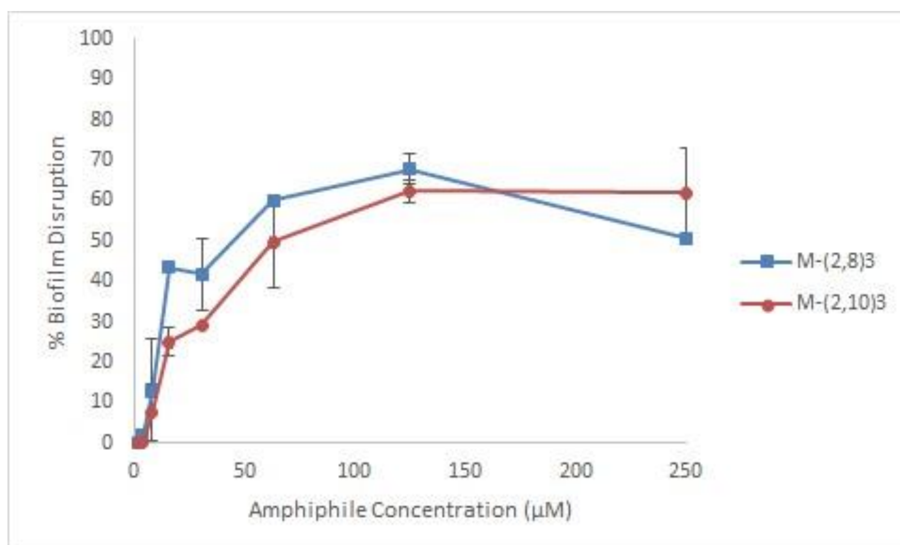

Figure 12: Percent biofilm disruption activity of 8 and 10 hydrocarbon tail length derivatives of hexa-cationic amphiphile series. Error bars represent standard deviation of at least two independent trials.

3.3 Methods and Materials

Bacterial Strains and Growth Conditions

The Gram-negative bacterial strains used in this study were *Escherichia coli* ATCC® 25922™, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC® 27853™ for experiments with planktonic cells, and hyper-biofilm forming *P. aeruginosa* strain PAO2 for biofilm disruption studies (Holloway, 1955). The Gram-positive bacterial strains used were *Staphylococcus aureus* subsp. aureus ATCC 29213™, *Enterococcus faecalis* ATCC® 29212™, *Bacillus anthracis* Sterne, and *Streptococcus agalactiae* J48 (Seifert *et al*., 2006). All bacterial strains, except for PAO2 were grown in MHB. For biofilm studies, PAO2 was grown in Luria Bertani (LB) broth.

Minimum Inhibitory Concentration

The MIC was determined for each amphiphile against four Gram-positive and three Gram-negative bacterial strains as previously described (CLSI, 2012). Bacterial cultures were

grown overnight at 37°C and diluted in MHB to a concentration of $5x10^6$ cells/ml. Aliquots of 100µl were added to 96-wells plates along with 100µL of the amphiphile being tested at 2-fold dilutions, from 500µM to 2µM in triplicate, yielding a final concentration of $5x10^5$ cells/ml in each well. Sterile deionized water was used as a control for bacterial growth. The plates were incubated at 37°C for 72 hours. The wells with lowest concentration of amphiphile without visual growth were used to determine the MIC. Each amphiphile was tested in a minimum of 2 trials.

Time-kill

Time-kill assays were performed as previously described (Ladow *et al*., 2011). Overnight cultures of *S. aureus and P. aeruginosa* were diluted with Mueller-Hinton broth and amphiphile to $2.5x10^6$ cells/ml. Amphiphiles added to the broth culture had a final concentration of 100µM and were incubated at room temperature to mimic conditions when disinfectants are normally used. Aliquots of 100µl were plated on THB (Todd Hewitt Broth) agar plates at 0, 1, 5, 15, 30 minutes, 1, 2, 24 and 48 hours after incubation at room temperature for *S. aureus*, and 30 minutes, 1, 2, 3, 24, 48 and 72 hours after incubation at room temperature for *P. aeruginosa*, and incubated at 37°C for 24 hours, and colonies counted. Values are reported as the time when no colonies were observed.

Checkerboard Assay

A checkerboard assay using 96-well microtiter plates were used to determine if synergy exists when amphiphiles are combined with other compounds against *E. coli* ATCC® 25922™ and *S. aureus* ATCC® 29213™ (ATCC, Manassas, VA, USA). Bacteria diluted to $5x10^6$ cell/mL were added to each well followed by 100µl of treatment, yielding $5x10^5$ cell/ml in each well. Wells with sterile deionized H_2O treatment used as a control for bacterial growth and a

comparison for turbidity, respectively. In the checkerboard section of the assay, 50µl of each compound at 2-fold dilutions at concentrations below the previously determined MIC of each compound. The Fractional Inhibitory Concentration (FIC) was determined by comparing the MIC value of each compound alone and in combination using the following equation:

$FIC = FIC_A + FIC_B$

$FIC_A = A/MIC_A$

$FIC_B = B/MIC_B$

MIC_A and MIC_B represent the MIC of compound A and B alone and A and B represent the MIC values of compound A and compound B combined. Synergy was defined as an FIC value of ≤0.5, and antagonism was defined as an FIC value of >4. Checkerboard assays for each combination were performed at least 3 times.

Biofilm Disruption Assay

Biofilm disruption was determined as previously described (O'Toole, 2011). *P. aeruginosa* strain PAO2 was incubated overnight in LB broth at 37°C and diluted to 5 x 10^6 cells/ml. Aliquots of 100µl were added to each well and incubated at 37°C for 24 hours to allow biofilm to grow. Serial 2-fold dilutions of amphiphile were added to wells containing bacteria and plates were incubated at 37°C for 24 hours. Tobramycin was used as a positive control for biofilm disruption. Bacterial cells were stained using the crystal violet method. Wells were rinsed with dH_2O and allowed to air dry completely. Crystal violet at a concentration of 0.01% was added to each well at 100µl aliquots and plates were incubated at room temperature for 15 minutes. Crystal violet stain was then removed and wells were rinsed 3-4 times with gently running deionized water, allowed to dry for 40 minutes, followed by addition of 100µl of 95% ethanol into each well. Plates were incubated with gentle shaking at room temperature for 1

hour. The ethanol/stain resulting mixture was then transferred into a clean 96-well microtiter plate and absorbance read with a plate reader at 570 nm. Absorbance values of treated wells were compared to the control to determine % biofilm disruption.

3.4 Conclusion

The goal of this research was to design amphiphiles with low MIC values against bacteria while also retaining high biofilm disruption activity, a time kill of less than 5 minutes, and the ability to act synergistically with other antimicrobial compounds. Data gathered in the current study contributes to designing amphiphiles with increased antimicrobial properties. Amphiphiles possessing low MIC values against both Gram-positive and negative species had low time kill values as well. Bis-cationic amphiphiles with 12 carbon length hydrocarbon tails could kill *S. aureus* in 5 minutes or less. Of particular interest, oX-12,12 killed *S. aureus* within one minute. This data suggests that amphiphiles with two cations and two tails can kill Gram-positive bacteria quicker than amphiphiles of similar structure with more than two cations. It can also be concluded that *P. aeruginosa* cell count was not reduced significantly within 10 minutes of amphiphile exposure. In order for amphiphiles to be used as disinfectants, Gram-negative bacteria must be killed within 10 minutes, or at least reduced significantly within 10 minutes since currently used disinfectants kill within this time. In studies involving similar amphiphiles, *E. coli* was killed within 15 minutes or less. Although not tested, it is possible that the amphiphiles in the current study are able to kill other Gram-negative species in similar times.

The maximum amount of biofilm disruption with these compounds was approximately 65% at a concentration of 63µM with one bis-cationic amphiphile pX-10,10 and both hexa cationic amphiphiles M-$(2,8)_3$ and M-$(2,10)_3$. Biofilm disruption at the same concentration was seen with Tobramycin, an antibiotic with known biofilm disruption capabilities, used to treat

P. aeruginosa infections. Development of even more biofilm active amphiphiles is likely. In research conducted simultaneously in our lab, similar amphiphiles were able to disrupt approximately 90% of pre-established *P. aeruginosa* biofilms at a concentration of 250µM (unpublished). Further adjustment of amphiphile structural features will likely identify amphiphiles that can eradicate 100% of biofilms with even lower concentrations. Complete biofilm eradication of *S. aureus* and *E. faecalis* biofilms at 25µM has been seen with amphiphiles of similar structure (Jennings *et al*., 2014).

Synergistic combinations between these amphiphiles and oxacillin against *E. coli* were surprisingly abundant. Oxacillin is not normally active against Gram-negative bacterial species. It was successfully proven that bacteria resistant to oxacillin, could become more susceptible if combined with a membrane disrupting compound. It is possible that the amphiphile allowed oxacillin easier access to the cell wall by acting on the outer membrane of the Gram-negative bacteria. The 10 and 8 hydrocarbon chain length amphiphiles had better synergy with oxacillin than 12 in this study. The reason for this trend requires further investigation to understand.

In conclusion, this study identified important amphiphile structural features for more rapid bacterial killing and better biofilm disruption activity. This information will help with design of compounds that could be used as disinfectants. The ability of these compounds to have synergistic interactions with oxacillin against Gram-negative bacteria expands on how these antimicrobials could be used. The amphiphiles in this study could be added to other antibiotics or disinfectants to possibly overcome bacterial resistance mechanisms, making them susceptible.

Chapter 4: Conclusions

4.1 Conclusions

The goal of this research was to extend knowledge of novel amphiphile antimicrobial capabilities beyond MIC values. MIC values may determine the potency of antimicrobials against bacterial species, however, MIC assays typically allow 72 hours for compounds to work. In order for amphiphiles to be useful as antimicrobials, the time to kill bacteria must take only a few minutes. Currently-used disinfectants often advertise as killing 99.9% bacteria, however, the amount of time needed to do this is usually at least 10 minutes. In reality, while disinfecting a surface, exposure time would likely be less than 10 minutes due to user oversight, allowing for more bacteria to continue proliferating than expected. It is necessary to develop disinfectants with faster killing speed so the expected amount of bacteria are eliminated, preventing infection as well as the spread of antibiotic resistant bacteria. Three amphiphiles in this study were capable of killing *S. aureus* in less than 10 minutes: oX-12,12, pX-12,12 and mX-12,12 with kill times between 1 and 5 minutes. *P. aeruginosa* was not eliminated within 10 minutes of exposure with these amphiphiles, however this particular bacteria is more resistant to disinfectants (Russel and Chopra, 1996). Previous studies with similar amphiphiles were able to kill the Gram-negative *E. coli* in the shortest amount of time tested, 15 minutes (Ladow *et al*, 2011). It is likely the amphiphiles in the current study are able to kill *E. coli* and other Gram-negative bacteria in a similar amount of time as Gram-positive bacteria. Further testing is needed with a greater number of bacterial species to assess killing speed of the bis-cationic amphiphile series in this study.

Synergy when combining two compounds against bacteria is a rarity. Surprisingly, many synergistic combinations were identified with amphiphiles against both *E. coli* and *S.*

aureus. The most notable combinations were 10 or 8 hydrocarbon tail length amphiphile derivatives combined with oxacillin against *E. coli*, a Gram-negative species. Oxacillin, a narrow spectrum antibiotic which is normally only active against Gram-positive species, was able to kill a Gram-negative species at a 64-fold reduced MIC value. The MIC of oxacillin against *E. coli* was reduced from >1000μM to 16μM when combined with the amphiphile M-$(2,10)_3$. The exact mechanism causing synergy is not well understood and requires further investigation. It is likely the amphiphiles are acting on bacterial membranes, increasing membrane permeability to the antibiotic. Without addition of the amphiphile, oxacillin cannot penetrate the outer-membrane of Gram-negative bacteria, blocking the oxacillin action of disrupting cell wall synthesis.

Biofilm disruption capabilities of some amphiphiles in this study warrant further investigation. Tris-cationic amphiphiles M-DMAP 14,14 and M-DMAP 16,16 could disrupt nearly 100% of established *P. aeruginosa* biofilms at a 250μM concentration. Biofilms generally inhibit diffusion of cationic antimicrobials through the protective matrix, making it difficult to reach embedded bacteria. This inhibition is due to the overall anionic charge of polysaccharides within the matrix. A higher concentration of antimicrobials is needed in order for the MIC of the antimicrobial to reach cells within the biofilm. M-DMAP 14,14 and 16,16 both have high MIC values against *P. aeruginosa* planktonic cells, 63μM and 250μM respectively, therefore biofilm disruption is likely only at high concentrations. M-DMAP 16,16 was able to disrupt nearly 100% of the pre-established biofilm at MIC. The ideal structure for biofilm disruption is not known, however thus far, the most important structural features required for biofilm disruption activity is similar to that of killing planktonic cells: at least two cations with at least one hydrocarbon chain on an amphiphilic molecule (Jennings *et al*., 2015). Further

research is needed to understand biofilm disruption activity of these amphiphiles, and why MDMAP 14,14 and 16,16 had higher biofilm disruption activity than amphiphiles with lower MICs.

Several compounds of interest were identified with high biofilm disruption activity, fast killing speed and synergistic when combined with other compounds. These compounds could be used as powerful disinfectants. Those with fast killing speeds could be useful as an active ingredient in disinfectants, reducing the required exposure time. The development of novel antimicrobials with fast action and biofilm disruption will help reduce the spread of resistant bacteria.

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