USING COMBINATION THERAPY TO TARGET BIOFILM INFECTIONS

An Honors Thesis Presented

By

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ABSTRACT

Bacteria are single-celled microorganisms that are found in every habitat on Earth. Most bacteria are harmless, but if pathogenic bacteria enter the body, they can cause infection. Most bacterial infections can be cured by antibiotics, however, not infections caused by biofilms. Biofilm infections occur when bacteria infecting a host attach onto a surface and proliferate, producing extracellular polymeric substances (EPS). These EPS form a 3D matrix that serves as extra protection for the bacteria against antimicrobials and host immune response. Most biofilm infections develop a chronic state because biofilms are resistant to antibiotics and lack an effective cure. Combination therapy has recently become an advantageous means to treat antibiotic-resistant infections by decreasing the dosage of antibiotics necessary while utilizing another therapeutic agent. Polymeric nanoparticles have emerged to be potential novel antimicrobials to combat biofilm infections. In this work, engineered polymeric nanoparticles, poly(oxanorbornene-imide)-based polymers (PONI-C11-TMA), have been explored for combination therapy with antibiotics to revive the use of antibiotics against drug-resistant biofilm infections and target biofilm infections more effectively. Preliminary results have shown that the presence of PONI-C11-TMA was able to restore the efficacy of otherwise ineffective antibiotics due to acquired resistance in a planktonic bacteria setting.

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1. Introduction

Each year at least 2.8 million people get antibiotic-resistant infections, and more than 35,000 cases result in death[1]. About 80% of microbial infections are due to bacterial biofilms. Most bacterial infections can be treated with antibiotics, however, not infections caused by biofilms[2]. Biofilms occur when bacteria grow in slime-enclosed clusters, which creates a microenvironment that protects the bacterial cells from antibiotics and a host immune response. The formation of biofilms within a human host leads to a chronic state because biofilm infections are resistant to antibiotics and currently lack an effective cure[3].

When planktonic bacteria make contact with a surface, they become irreversibly attached and begin to form a monolayer, producing an extracellular matrix which acts like "slime" for defense. The matrix consists of extracellular polymeric substances (EPS), such as extracellular polysaccharides, structural proteins, cell debris and nucleic acids[4]. During this step, the extracellular matrix continues to form, and the biofilm grows in a three-dimensional manner. Once the biofilm matures, it becomes impenetrable to antimicrobial agents. The biofilm grows horizontally and vertically to occupy as much of the surface as possible. Some bacterial cells detach, and the biofilm disperses into the environment as planktonic cells to potentially start a new cycle of biofilm formation[5].

Synthetic macromolecules, such as nanoparticles and polymers, have exhibited potential as a therapeutic to strongly bind and destabilize the bacterial outer membrane[6]. Additionally, amphiphilic polymers have demonstrated the ability to penetrate the biofilm matrix[7]. The utilization of the polymers enables membrane sensitizing and biofilm penetration, which offers enhanced efficacy in targeting bacterial and biofilm infections. Using engineered polymeric nanoparticles in combination with antibiotics, it is possible to decrease the dosage of antibiotics

in combating bacterial infections, while reducing the potential side effects of high doses of antibiotics.

Poly(oxanorbornene-imide)-based polymers (PONI-C11-TMA) are synthesized polymeric nanoparticles that have therapeutic properties in biofilm penetration and eradication without affecting mammalian cells[8]. PONI-C11-TMA was engineered with a hydrophobic C11 alkyl chain which bridges a cationic headgroup and polymer backbone. Polymers containing a C11 alkyl chain spontaneously self-assemble into cationic polymeric nanoparticles (PNPs) in an aqueous solution[9].

Through this thesis, combination therapy using polymeric nanoparticles to revive the use of antibiotics against drug-resistant biofilm infections will be performed, as well as understanding the mechanism of interaction between the antibiotic and polymer.

2. Summary of Work of Previous Researchers

2.1 Overview of Antibiotic Resistant Biofilm Infections

The resistance of biofilms to antimicrobials increases with biofilm maturation. This antimicrobial tolerance of biofilms *in vitro* is due to multiple factors, including lack of antibiotic penetration, restricted growth due to low oxygen tension, expression of biofilm specific genes, and the presence of persister cells. Bacterial biofilms prevent the penetration of antimicrobials through the biofilm matrix[10].

Interestingly, lack of biofilm penetration by antibiotics is not the only contributing factor to the antimicrobial resistance of biofilm infections. Studies have shown in *Pseudomonas aeruginosa* biofilms being treated with tobramycin and ciprofloxacin effectively penetrated biofilms but failed to prevent growth of bacteria. A microelectrode analysis was performed, and results concluded that oxygen limitation and low metabolic activity in the interior of the biofilm was the cause of antibiotic tolerance of biofilms[11]. Antibiotics are known to target bacterial cell proliferation, which indicates that biofilm bacteria with low metabolic activity possess high antimicrobial tolerance[10].

Additionally, biofilms express specific genes that have been studied for exhibiting antimicrobial resistant mechanisms[10]. For example, the *ndvB* gene in *Pseudomonas aeruginosa* PA14 encodes for an enzyme that is responsible in periplasmic glucan synthesis which binds to tobramycin to prevent cell death by isolating the antibiotic[10].

Reduced bacterial cell division can also contribute to antimicrobial tolerance of biofilms [10]. Persister cells are the result of bacterial cell differentiation entering a dormant state. Persister cells cause reduced metabolism which prevents antibiotics from exhibiting

antimicrobial functions. This diminished function of antibiotics supplies antimicrobial tolerance to preclude bacterial cell death.

In patients facing *in vivo* biofilm infections, antibiotic resistant mechanisms can be initiated through the immune system, low oxygen tension, and complications of the antibiotics reaching the targeting infection[10]. In studies of patients with cystic fibrosis and chronic *P. aeruginosa* infections, polymorphonuclear (PMN) leukocytes accumulated at the site of the biofilm infections, which consumed oxygen and created anaerobic conditions[12] while preventing bacterial growth[13]. *In vivo*, biofilm formation locates in oxygen-poor areas due to PMN inflammation or physiological conditions. These conditions can cause both reduced bacterial growth and loss of transport of antibiotics across the membrane of biofilms[14]. Dosage of antibiotic treatment varies based on the size and location of the biofilm infection[15] Doses of antibiotics that are under the inhibitory concentration have the potential to increase the risk of antibiotic resistance in biofilms and increase mutagenesis[16].

2.2 Antibiotic Mechanisms

Chloramphenicol is a broad-spectrum, semisynthetic antibiotic with primarily bacteriostatic activity. Chloramphenicol is classified as a macrolide[17]. Chloramphenicol enters the bacterial cell by diffusing through the cell wall and reversibly binding to the bacterial 50S ribosomal subunit. The binding interferes with peptidyl transferase activity, which prevents the growth of peptide chains and blocks peptide bond formation. As a result, bacterial protein synthesis is blocked and intercepts bacterial cell proliferation[18].

Ciprofloxacin is used to treat a variety of bacterial infections. Ciprofloxacin belongs to a class of drugs called fluoroquinolone antibiotics. Ciprofloxacin inhibits DNA replication by disrupting bacterial DNA topoisomerase and DNA-gyrase[19]. By interfering with DNA-gyrase, the introduction of negative supercoils in bacterial DNA does not occur, which ultimately inhibits enzyme function and leads to cell death[20]. It is a broad-spectrum antibacterial drug to which most Gram-negative bacteria are highly susceptible *in vitro* and many Gram-positive bacteria are susceptible or moderately susceptible[21].

Ceftazidime is a cephalosporin antibacterial agent which has retained a broad spectrum of *in vitro* antimicrobial activity and clinical utility in serious infections[22]. Ceftazidime binds to and inactivates penicillin-binding proteins (PBP) on the inner membrane of the bacterial cell wall. PBPs play an important role in bacterial cell wall assembly and reconfiguring the cell wall during cell division. However, inactivation of PBPs disturbs the cross-linkage between peptidoglycan chains, which are necessary for cell wall strength. The result of ceftazidime targeting bacteria is weakened bacterial cell walls and cell lysis. Ceftazidime is more active against Gram-negative bacteria than against Gram-positive bacteria[23].

Cefotaxime is classified as a cephalosporin with a broad spectrum of antimicrobial activity. After more than a decade of use, cefotaxime continues to play an important role in the treatment of patients with serious infections, particularly those caused by Gram-negative bacteria[24]. Similar in function to ceftazidime, cefotaxime binds to and inactivates PBPs to interrupt cross-linkages between peptidoglycan chains that make up bacterial cell walls. Cefotaxime depletes cell wall stability and causes cell lysis[25].

2.3 Mechanisms of Traditional Combination Therapy

In a study by Yanagihara et. al.[26], combination therapy was tested in treating chronic Pseudomonas aeruginosa, which is a respiratory infection associated with biofilm formation. This study involved the combination of two antibiotics, clarithromycin and levofloxacin. Researchers began their studies by infecting their model organisms, mice, with Pseudomonas aeruginosa. P. aeruginosa is a pathogen in biofilm-associated infections, which are challenging to eradicate. They then separated the mice into four treatment groups: those that were treated with clarithromycin, those that were treated with levofloxacin, those that were treated with both clarithromycin and levofloxacin, and those that were treated with saline as the control group. Each drug was administered once a day for ten days.

The results showed that treatment with clarithromycin alone or levofloxacin alone had no statistically significant effect on viable bacteria in the lungs. However, use of these two drugs together resulted in a significant decrease in the number of viable bacteria compared to the other three groups. These experiments support the hypothesis that combined use of clarithromycin and levofloxacin improve the therapeutic ability of levofloxacin in biofilm-associated chronic respiratory infection. Because clarithromycin has no antibacterial activity against *P. aeruginosa,* the synergy between the two antibiotics is what contributes to this interaction. The researchers believe that the synergy of clarithromycin and levofloxacin may be caused when clarithromycin removes the polysaccharide glycocalyx in or on bacterial biofilms. While this study demonstrates the combination of antibiotics to effectively treat biofilm infection, this study was only carried out with one strain of bacteria, and other strains of bacteria could potentially build up resistance to clarithromycin, unlike *P. aeruginosa.*

2.4. Targeting Infections with Polymeric Nanoparticles

In a study conducted by Landis et. al.[27], crosslinked polymer scaffolds stabilized by oil-in-water nanocomposites (X-BNCs) were loaded with carvacrol, an antiviral therapeutic to eradicate Gram negative/positive bacteria, including multi-drug resistant strains. The potential of X-BNCs as a therapeutic agent was demonstrated in an in vitro coculture with biofilms grown on top of mammalian cells. These experiments were unique in that X-BNCs do not evoke resistance in bacteria but instead maintain their potency against bacteria.

X-BNCs were able to penetrate and diffuse throughout the biofilm and were observed with fluorescence within the enclosed bacteria. The data revealed the efficiency of X-BNCs in reaching the enclosed pathogen deep within the film's matrix. Additionally, X-BNCs were able to eliminate bacterial cells in four-biofilm species within three hours: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Enterobacter cloacae.* It could be inferred that a polymer scaffold with similar chemical properties could be utilized in this thesis with *Staphylococcus aureus* and *Escherichia coli* to obtain similar results.

This crosslinked polymer-stabilized oil-in-water nanocomposite offers potential as an antimicrobial therapeutic. Interestingly, these nanocomposites maintain their stability, but degrade in the presence of certain biomolecules, preventing them from building up over time. This nanoemulsion technology is very highly effective against Gram negative and positive bacterial biofilms. Additionally, there was no observed toxicity to mammalian cells reported in this study. Another advantage to this treatment is that bacteria were unable to build resistance against the nanoemulsions, making this therapeutic highly promising.

Interestingly, in a paper published in this lab the following year, further investigation showed that the polymer-stabilized carvacrol-in-water nano composite (NC) demonstrated cytotoxicity toward mammalian cells[28]. However, the researchers proposed an alternative method using encapsulated phytochemicals to make the NCs less toxic. Through their experiments, the scientists discovered that the NC delivery strategy improved the antimicrobial efficacy against planktonic bacteria and biofilms.

The researchers hypothesized that other phytochemicals could be stabilized in aqueous media using the nanocomposite technology. The phytochemicals studied included eugenol, carvacrol, linalool, limonene, p-cymene, and a-pinene, which were mixed with DTDS prior to emulsification into Milli-Q water containing the polymer to form the nanocomposite. The data showed that phytochemicals with a lower log P values, such as eugenol with a log P of 2.49 and linalool with a log P of 2.97, had high potential for this delivery system. Additionally, encapsulating phytochemicals with a lower log P value and no phenolic hydroxyl groups equates to low cytotoxicity nanocomposites.

2.5 Combination Therapy Studies with Antibiotics and Polymers

In another study by Ding et. al.[29], the mechanism of antimicrobial guanidinium-functionalized polycarbonates was utilized as a combination therapy with antibiotics to treat drug-resistant bacteria. Polycarbonates are biodegradable polymers that have unique delivery and antimicrobial properties. These researchers studied the mechanism of antimicrobial guanidinium-functionalized polycarbonates to discover that membrane translocation was followed by precipitation of cytosolic material. Multiple experiments with this polymer did not display upregulation or downregulation of gene expression associated with resistance. It was hypothesized that reversing the phenotype of antibiotic resistance could reveal gene expression mechanisms to discover the root cause of resistance and block those modifying genes or proteins.

The results supported the hypothesis that co-delivery of polymer with antibiotics reverses the antibiotic resistance phenotype while improving antibiotic potency. This polymer was shown to allow for the effective repositioning of rifampicin and auranofin meant for Gram-positive bacteria and arthritis, respectively, to be highly effective against multidrug resistant Gram-negative bacteria. By analyzing the phenotypic expression, the researchers discovered more about the polycarbonate pEt_20 affinity to the membrane, transient membrane depolarization, gene binding, intracellular protein precipitation, and subsequent reactive oxygen species (ROS) generation. The polymer/rifampicin combination led to an increased level of intracellular ROS, which led to bacterial death. This combination therapy has been successfully translated in a multidrug resistant *A. baumannii*-caused bacteremia mouse model. This therapy shows great potential in combating Gram-negative bacterial infections.

In another study conducted by Gupta et. al.[30], the researchers hypothesized that by combining the properties of membrane sensitizing and penetration ability of polymers with the selective activity of antibiotics that this would offer a solution of a more effective way to combat multidrug resistant bacterial and biofilm infections. The combination therapy used in this study will closely resemble the experiments for this thesis, however, this study involves both bacterial and biofilm infections with colistin being used as the antimicrobial, while this thesis project will involve several different types of antibiotics and biofilm infections. These researchers used engineered polymeric nanoparticles (PNPs) combined with colistin to target resistant bacteria.

Through these experiments, the scientists observed a 16-to 32-fold decrease in the amount of colistin dosage required to combat both planktonic and biofilm bacteria in combination therapy as compared to colistin alone. The observed synergy was attributed to increased bacterial membrane permeability when antibiotics were combined with PNPs. In addition, the researchers observed a fourfold increase of antibiotics within biofilms in the presence of PNPs, allowing the overall efficacy to increase. This study conveys the therapeutic potential that combination therapies using PNPs holds in restoring antibiotics that antibiotic resistance typically causes.

In a recent study by Tan et. al.[31], the synergistic effect of chlorogenic acid and levofloxacin was tested to observe its effect on *Klebsiella pneumonia* (KPN), which is the most common clinical pathogen. Because KPN can produce biofilm extremely easily and has high resistance to antibiotics, there was a desperate need for an effective treatment. Chlorogenic acid (CA) has been studied for its ability to inhibit biofilm formation of *P. aeruginosa*, however until this study the inhibition effect of CA on KPN was unknown. This study investigated the

antibacterial properties and mechanisms of CA and the combination of CA and levofloxacin (LFX) on KPN in both in vitro and in vivo models.

The results showed that CA with a concentration of 512 μg/mL could effectively inhibit the formation of KPN biofilm in vitro. The researchers also found that the combination of CA and LFX had a good synergistic effect on inhibiting the formation of biofilm in vitro. In addition, the study showed that in mice models that were infected with KPN and treated with the combination therapy of CA and LFX had a significantly reduced mortality and inflammatory rate than those that were not treated. CA can significantly reduce the formation of biofilm in vitro and can diminish the inflammation reaction in KPN infected mice in vivo. The combination of CA and LFX had a synergistic effect in vitro and in vivo.

2.6 Future Implications

These studies have highlighted the capability of combination therapy in targeting bacterial and biofilm infections, while reducing the dosage of antibiotics to reduce inevitable resistance. Each of these techniques described above has potential in targeting infections and decreasing resistance. This thesis project will utilize this functional combination therapy to test the efficacy of cocktails of different antibiotics and polymers against antibiotic resistant biofilm infections.

3. Current Methodology and Goals

3.1 Goals

The main goal of these experiments was to evaluate the efficacy of using antibiotics, PONI-C11-TMA polymer, and antibiotics and polymer combined against the biofilms of antibiotic resistant strains. Over the past year, studies have been ongoing to determine the minimum biofilm eradication concentration (MBEC) and potential synergistic effects between different antibiotics and the polymer.

Firstly, MBEC assays are carried out to determine the lowest concentration of antibiotic or polymer that disrupts the growth of bacterial biofilm. The data is gathered to compare MBECs between both susceptible and resistant strains of both *E. coli* and *S. aureus*. By gathering these results, the inhibitory concentration can be compared to the results gathered in checkerboard titration assays to compare efficacy.

Checkerboard titration assays are then utilized to determine potential synergy when polymer and antibiotics are used in combination with one another.

3.2 Methods

Preparation of PONI-C11-TMA:

The synthesis of the polymer used in these experiments is a multi-step process, which begins from monomer synthesis. Initially, the oxanorbornene derivative was synthesized by *Diels-Alder cycloaddition*. Following this step, the sample was heated, cooled to room temperature, and isolated. Then, the monomer was dissolved in dichloromethane. Upon freeze-pumping and thawing to room temperature three times, the product precipitated out of solution and was filtered through PES syringe filters (0.22 μm pore size) and freeze dried[8].

Preparation of resistant bacteria strains:

Resistant strains of bacteria were generated by exposing strains of *E. coli* (CD2) and *S. aureus* (CD35) to sub-inhibitory concentrations of different antibiotics. By doing this, the bacterial solution was able to grow until resistance was achieved. Seven different strains of resistant bacteria were obtained, which include CD2 Ceftazidime, CD2 Cefotaxime, CD2 Ciprofloxacin, CD35 Chloramphenicol, CD35 Ceftazidime, CD35 Cefotaxime, and CD35 Ciprofloxacin.

Preparation of bacterial biofilm solution:

A single colony of bacteria placed into 3 mL of LB at 37°C in an incubator overnight to produce stationary phase bacteria. To achieve log phase growth of the bacteria, 60 μL of stationary phase bacteria was added to 3 mL of TSB and kept at 37°C for 3 hours, with shaking. Once log phase was achieved, 150 μL of bacteria solution was transferred to each row of a clear 96-well microplate, cover it with a pegged lid, and incubated with 50 rpm speed at 37°C for 5-6

hours. The media used for Gram-negative bacteria (*E. coli*) was M9, and the media used for Gram-positive bacteria (*S. aureus*) was a 95:5 ratio of M9:TSB. The calculations for media preparation for Gram-positive bacteria can be seen in Appendix A Section 1.

Preparation of antibiotic stock solution:

Stock solutions of each antibiotic were prepared, including ceftazidime, cefotaxime, chloramphenicol, and ciprofloxacin. Cefotaxime was dissolved in DMSO, while ceftazidime, chloramphenicol, and ciprofloxacin were dissolved in MQ water. The final concentrations for the antibiotics were 4,700 mg/L for ceftazidime, 5,850 mg/L for cefotaxime, 47,000 mg/L for chloramphenicol, and 24,000 mg/L for ciprofloxacin.

Preparation and Determination of MBEC:

The MBEC is the lowest concentration of antimicrobial agent necessary to disrupt the growth of bacterial biofilm. Biofilms were grown following the protocol described above. Four different MBEC experiments were performed: susceptible bacterial biofilm with polymer only, resistant bacterial biofilm with polymer only, susceptible bacterial biofilm with antibiotic only, and resistant bacterial biofilm with antibiotic only. Upon preparation of the biofilm plate, a material plate is prepared with 150 µL of media and 50 µL of either polymer or antibiotic. The concentration of either polymer or antibiotic is diluted using two-fold sterile dilutions. The growth control row has bacteria and media, while the sterile control row solely has media. The schematic for the MBEC assay plate can be found in the Appendix B Section 1.

Once the biofilm plate is removed from the shaker-incubator, the pegged lid is washed with a PBS plate, which is a 96-well clear plate with 200 µL of PBS in each well. After 30

seconds, the lid is then placed into the material plate, which is then incubated for 16 hours. The plates were analyzed using a SpectraMax M2 plate reader, which measured the absorbance of the plates at a wavelength of 600 nm.

Checkerboard Titration for Synergy Testing:

A checkerboard titration assay was performed to determine synergy between antibiotic and polymer combinations. Upon preparation of the biofilm plate, a material plate is prepared with 160 µL of media, 20 µL of polymer, and 20 µL of antibiotic in each well. The concentration of either polymer or antibiotic is diluted using two-fold sterile dilutions. The growth control row has bacteria and media, while the sterile control row solely has media. The schematic for the checkerboard titration plate can be seen in the Appendix B Section 2.

The pegged lid of the biofilm plate is washed with a PBS plate. After 30 seconds, the lid is then placed into the material plate, which is then incubated for 16 hours. The plates were analyzed using a SpectraMax M2 plate reader, which measured the absorbance of the plates at a wavelength of 600 nm.

4. Results and Discussion

Combination therapy of the PONI-C11-TMA polymer along with four different antibiotics, ceftazidime, cefotaxime, chloramphenicol, and ciprofloxacin, were studied for synergistic interactions. While the mechanism behind the synergistic interactions is complex, the mode of action of each component might contribute to synergy[32]. Synergistic interactions lead to increased drug influx, while antagonistic interactions do the opposite[33]. It is hypothesized that upon synergistic combination of polymer and antibiotic, antibiotic permeability of biofilms will increase, causing the inhibition of biofilm growth.

As mentioned, each component of combination therapy has a different function, which may contribute to different results. Below in Figure 1, each antibiotic used in these experiments are listed, including their antibiotic classification and antimicrobial mechanism. Each antibiotic has a unique mechanism in disrupting bacterial cell walls and preventing bacterial biofilm cell replication.

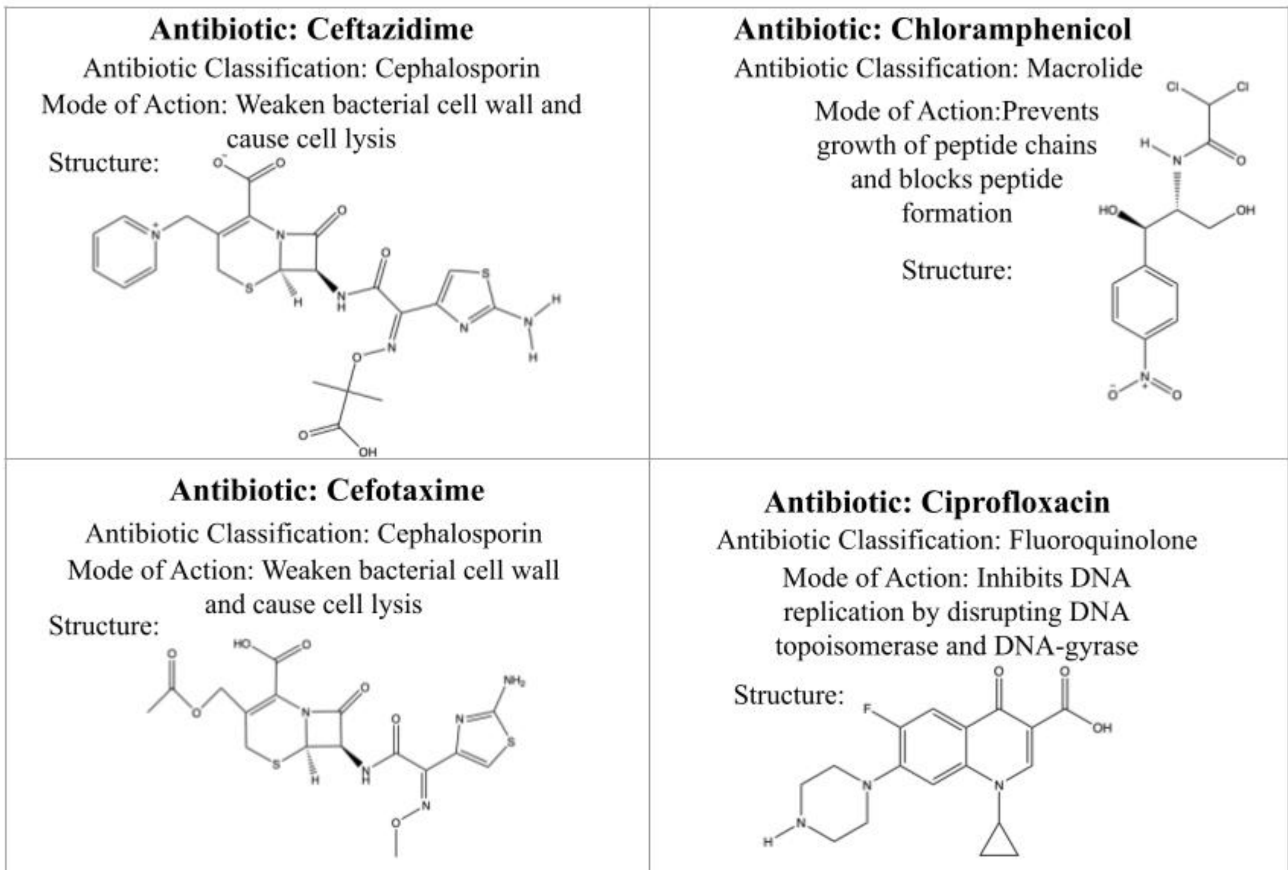

Figure 1: Chemical structures, classifications, and mechanisms of antibiotics: ceftazidime, cefotaxime, chloramphenicol, and ciprofloxacin.

Figure 2 depicts the structure of the PONI-C11-TMA polymer. The polymer contains a hydrophobic C11 alkyl chain and a cationic headgroup. This crosslinked oil-in-water nanocomposite incorporates disulfide and ester crosslinkers, which assist in nanocomposite degradation to prevent accumulation within the body while also providing long-term stability in aqueous environments. Unlike antibiotics, these polymers do not promote resistance in bacteria, and they maintain their potency against pathogenic bacteria. These degradable nanocomposites can penetrate and eliminate multi-drug resistant bacterial without the risk of endangering mammalian cells. Considering the efficacy, biodegradability, and stability of these agents, these crosslinked polymers have therapeutic potential *in vitro* to combat MDR bacterial biofilm infections[27].

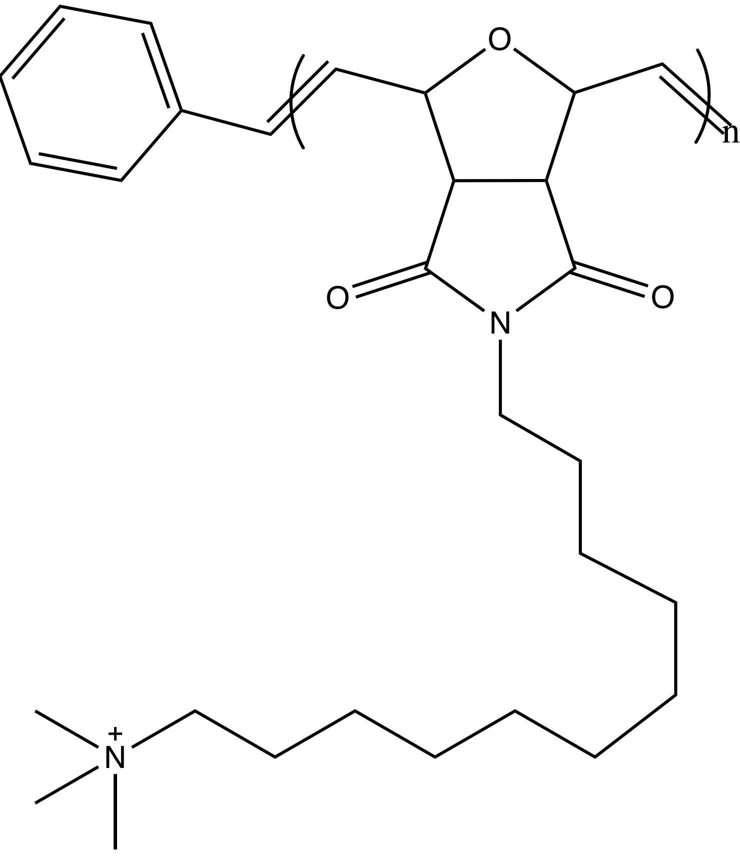

Figure 2: The chemical structure of poly(oxanorbornene-imide)-based polymer with C11 alkyl chain bridging the backbone and cationic head group (PONI-C11-TMA).

First, the susceptibility of the antibiotics and polymers towards biofilms of the selected strains were assessed separately. Table 1 below shows the results from assays of both susceptible and resistant strains of bacteria paired with different antibiotics. The 2 strains of bacteria that were tested in these studies were clinical isolates of *E. coli* (CD2), and *S. aureus* (CD35). The minimum biofilm eradication concentration of ciprofloxacin and CD2 was 1.56 mg/L, while the MBEC against the resistant strain greater than 1000 mg/L. The MBEC of CD2 with cefotaxime was 3.125 mg/L, while the MBEC of the resistant strain was double that at 6.25 mg/L. The MBEC of ceftazidime with CD2 was 1 mg/L, while that against the resistant strain was greater than 1000 mg/L. These data support the hypothesis that antibiotic resistant biofilm infections cannot be treated with solely antibiotics.

Likewise, the results with CD35 indicated a similar conclusion. The minimum biofilm eradication concentration of CD35 with ciprofloxacin was 2.5 mg/L while that of the resistant strain was greater than 50 mg/L. CD35 chloramphenicol had the same results with the MBEC of the susceptible strain being 2.5 mg/L and the MBEC of the resistant strain being greater than 50. Similarly, the MBEC of CD35 and ceftazidime was 0.156 mg/L, while the resistant strain of CD35 ceftazidime and ceftazidime was greater than 50. The MBEC of cefotaxime vs CD35 was 31.25 mg/L while that of the resistant strain was greater than 50.

Table 1: MBEC values of antibiotics against susceptible and resistant stains

Susceptible Strains with Antibiotic			Resistant Strains with Antibiotic		
Bacteria	Antibiotic	MBEC (mg/L)	Bacteria	Antibiotic	MBEC (mg/L)
CD2	Ciprofloxacin	1.56	CD2 Ciprofloxacin	Ciprofloxacin	>1000
CD2	Cefotaxime	3.125	CD2 Cefotaxime	Cefotaxime	6.25
CD2	Ceftazidime	1	CD2 Ceftazidime	Ceftazidime	>1000
CD35	Ciprofloxacin	2.5	CD35 Ciprofloxacin	Ciprofloxacin	>50
CD35	Cefotaxime	31.25	CD35 Cefotaxime	Cefotaxime	>50
CD35	Chloramphenicol	2.5	CD35 Chloramphenicol	Chloramphenicol	>50
CD35	Ceftazidime	0.156	CD35 Ceftazidime	Ceftazidime	>50

*CD2= *E.coli* CD35= *S.aureus*

Table 2 below shows the results from assays of both susceptible and resistant strains of bacteria paired with polymer. The same strains of bacteria were used in these experiments: *E. coli* (CD2) and *S. aureus* (CD35). The MBEC of CD2 with the polymer was 0.5 μM while the MBEC of CD2 with resistant strains was 1 μM for CD2 ciprofloxacin, cefotaxime, and ceftazidime. Interestingly, the MBEC of CD35 did not change when the polymer was paired with a susceptible strain vs the resistant strain, which was 16 μM, indicating that the polymer remains effective even against resistant bacteria.

Table 2: MBEC values of PONI-C11-TMA against susceptible strains

Susceptible Strain with Polymer		Resistant Strain with Polymer	
Bacteria	MBEC (μM)	Bacteria	MBEC (μM)
CD2	0.5	CD2 Ciprofloxacin	1
CD35	16	CD2 Cefotaxime	1
		CD2 Ceftazidime	1
		CD35 Ciprofloxacin	16
		CD35 Cefotaxime	16
		CD35 Chloramphenicol	16
		CD35 Ceftazidime	16

*CD2= *E.coli* CD35= *S.aureus*

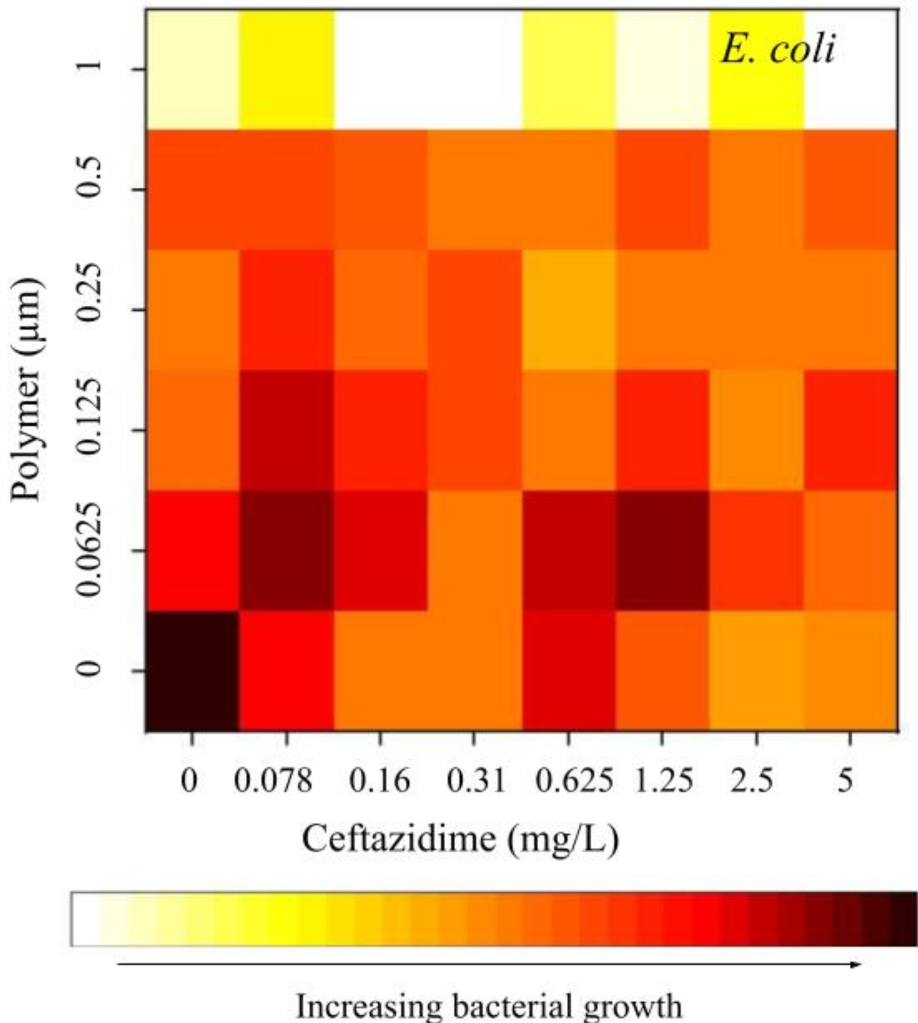

Figure 3: Checkerboard broth microdilution assay with ceftazidime and polymer against ceftazidime-resistant E. coli.

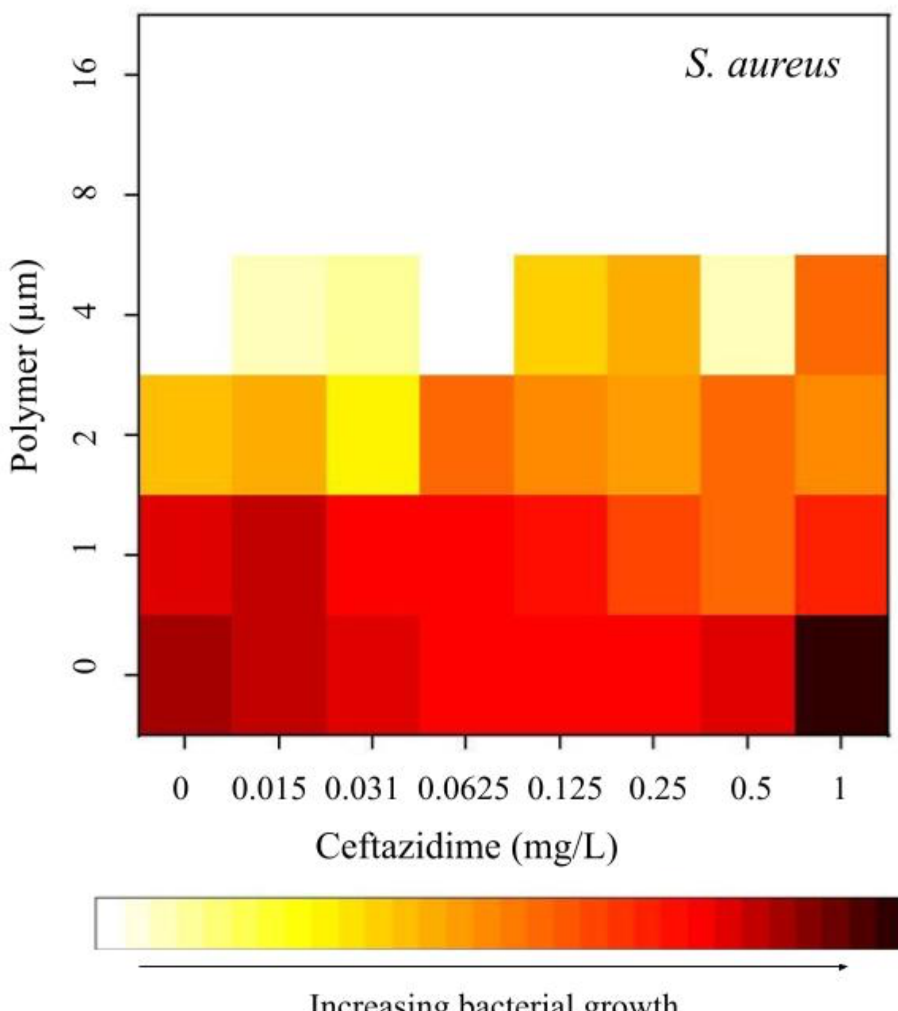

Figure 4: Checkerboard broth microdilution assay with ceftazidime and polymer against ceftazidime-resistant S. aureus.

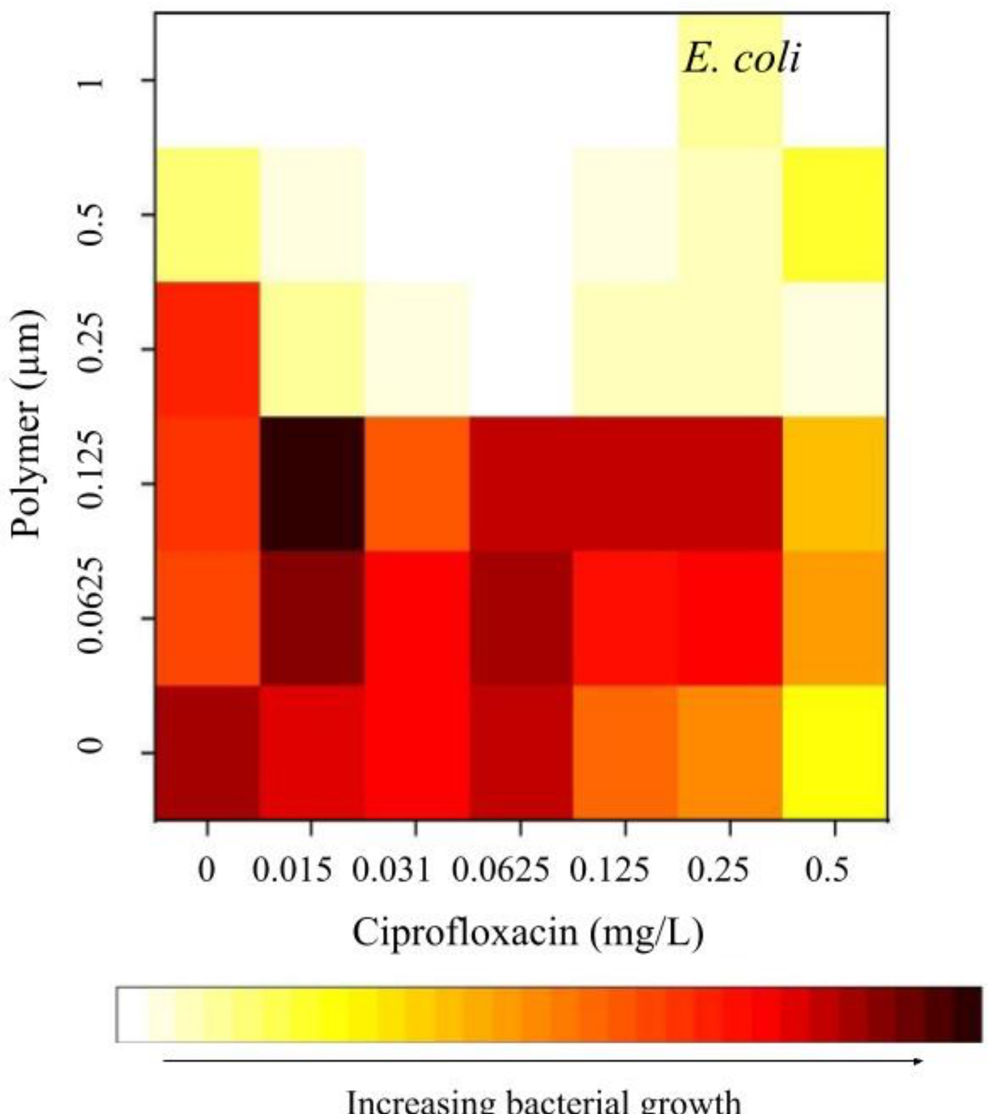

Figure 5: Checkerboard broth microdilution assay with ciprofloxacin and polymer against ciprofloxacin-resistant E. coli.

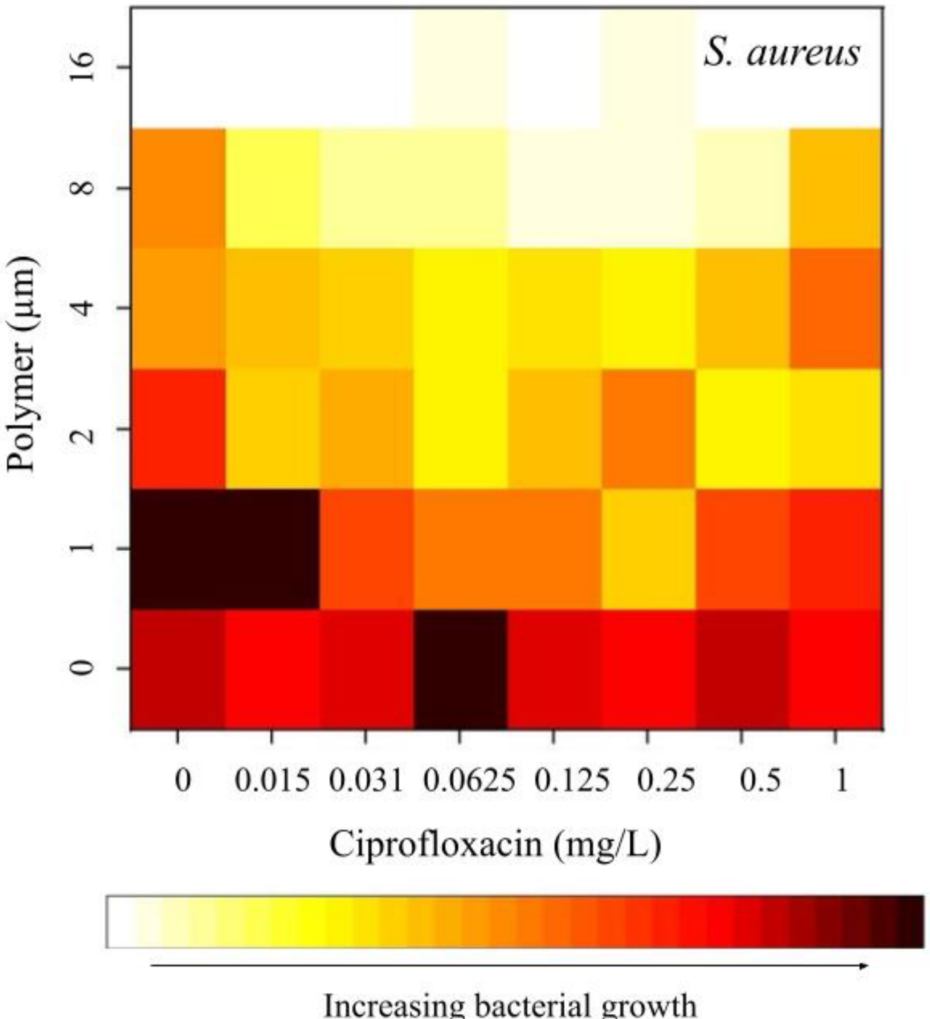

Figure 6: Checkerboard broth microdilution assay with ciprofloxacin and polymer against ciprofloxacin-resistant S. aureus.

Combination experiments of various antibiotics and PONI-C11-TMA polymer provided insight on therapeutic potential of various combinations against both Gram-negative and Gram-positive bacterial biofilm strains. In Figure 5, Ciprofloxacin in combination with PONI-C11-TMA displayed a combination effect in targeting biofilm growth in *E. coli* at concentrations of 0.25 μM and less of polymer and 0.0625 mg/L and less of ciprofloxacin. When referring to table 1, the concentration of ciprofloxacin necessary to eradicate resistant *E. coli* biofilm was >1000 mg/L. This concentration of ciprofloxacin to inhibit biofilm growth was resumed active by >16,000 times less of the concentration when combined with the polymer, which indicates that the combination of polymer and antibiotics effectively eradicated biofilms while reducing the dosage of antibiotics.

However, in Figures 3, 4, and 6, no combination effect was identified. For example, in Figure 6, bacterial growth occurred more at low concentrations of polymer and antibiotic than in Figure 5. Additionally, a higher concentration of polymer is needed in Figure 6 to have the same low bacterial growth effects, as seen in Figure 5. Furthermore, increasing the concentrations of the antibiotics could more effectively target and eliminate the biofilm.

One factor that may contribute to this variation in effectiveness of combination therapy is the chemical differences in the make-up cell wall of Gram-positive and Gram-negative bacteria. As shown in Figure 7, Gram-positive bacteria have a thick peptidoglycan layer and an absence of a lipid membrane, while Gram-negative bacteria have a thin peptidoglycan layer and an outer lipid membrane[34]. The molecules on the outer membranes, including the peptidoglycan layer embedded with teichoic and lipoteichoic acids in Gram-positive bacteria and lipopolysaccharides in Gram-negative bacteria, contribute to the overall negative charge of the bacterial membrane[35].

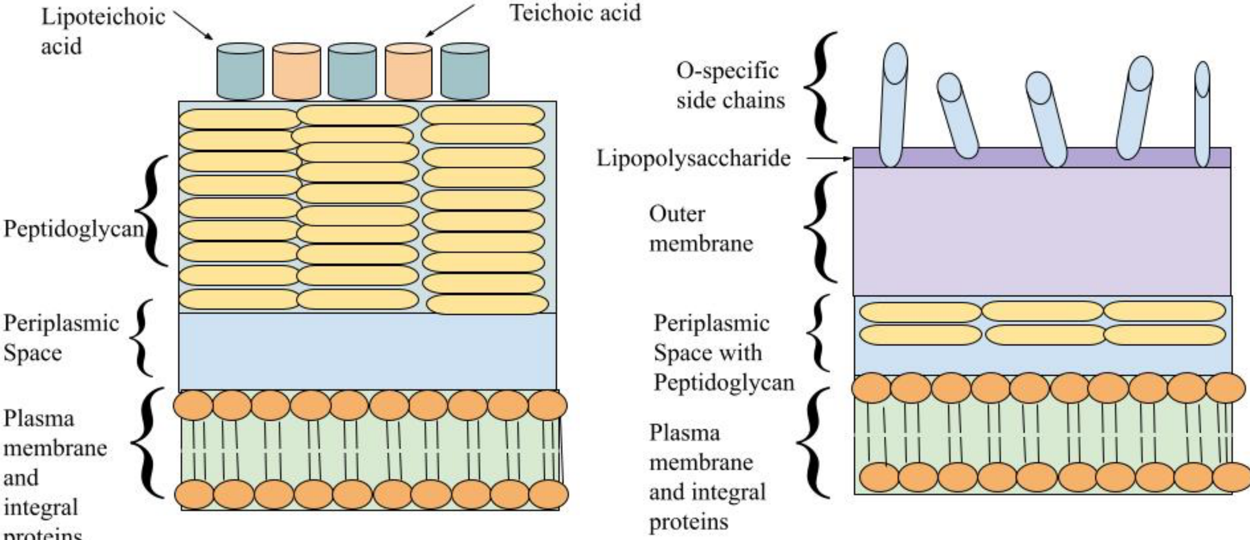

Figure 7: Structures of cell wall structures of Gram-positive (left), and Gram-negative (right) bacteria.

The Gram-negative bacteria used in these experiments, *E. coli*, is structurally unique in that lipopolysaccharides (LPS) contain more charge per unit of surface area than any phospholipid, and most of that charge is anionic at neutral pH because of exposed phosphoryl and carboxyl groups that are readily ionized. The outer membrane is also highly charged and is highly interactive with cations[36]. Therefore, the cationic headgroup of the PONI-C11-TMA polymer can easily interact with the bacterial cell-wall of *E. coli* to disrupt the membrane. Additionally, the hydrophobic alkyl chain of the polymer enables further interaction with the membrane, which contributes to membrane disruption. Of the fluoroquinolone antibiotics, ciprofloxacin is the most potent in combating Gram-negative bacteria[19].

The combination of these two molecules enables the polymer to disturb the bacterial membrane for ciprofloxacin to penetrate the layers of the bacteria to effectively target the infection. As mentioned in Figure 1, ciprofloxacin obstructs bacterial growth by inhibiting DNA replication by disrupting DNA topoisomerase and DNA-gyrase. These results indicate that using the cationic and hydrophobic polymer in combination with ciprofloxacin can increase the accumulation of antibiotics inside the biofilm. Therefore, the combination therapy of both PONI-C11-TMA and ciprofloxacin has the potential to revive antibiotics that are previously ineffective due to antibiotic resistance.

5. Conclusions and Implications for Future Research

Successful combination therapies were observed between PONI-C11-TMA and ciprofloxacin against ciprofloxacin-resistant *E. coli*. Against the bacterial biofilm of the resistant strain, the presence of the polymer in combination with the antibiotic facilitated penetration across the biofilm to target resident bacteria. However, further research is needed to fully understand the properties contributing to the discrepancy between interactions of the polymer with Gram-positive and Gram-negative bacteria.

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7. Appendix

7.1 Appendix A: Sample Calculations

Section 1: 95:5 of M9:TSB

To prepare 20 mL of media:

Amount of M9 required= 0.95 x 20 = 19 mL

Amount of TSB required= 0.05 x 20 = 1 mL

Section 2: Volume of bacterial biofilm required for MBEC

Volume of bacterial biofilm required for one plate/6 rows=50 μL x 6 rows x 9 columns=2700 μL

Prepared amount required is 3500 μL, in case excess is needed

Section 3: Preparation of biofilm for MBEC

$C_1V_1=C_2V_2$

Where C_1 is the target highest concentration of the biofilm multiplied by 4

Where C_2 is the stock concentration

Where V_1 is the targeted volume

Where V_2 is the volume of stock needed

If the highest target concentration for an antibiotic is 50 mg/L

(200 mg/L) (400 μL) = (V_2) (47,000 mg/L)

V_2 = 1.7 μL of stock volume needed

Section 4: Preparation of Checkerboard Titration Assay Materials

Final Volume: 200 μL = 20 μL of polymer + 20 μL of antibiotics + 160 μL of media

Prepare 10 times this amount

Polymer:

5 concentrations must be prepared

If the highest concentration= 1 µM

Prepare:

((1 µM x 10) (400 µL))/ (stock concentration of polymer) = Volume of polymer needed

Antibiotic:

7 concentrations must be prepared

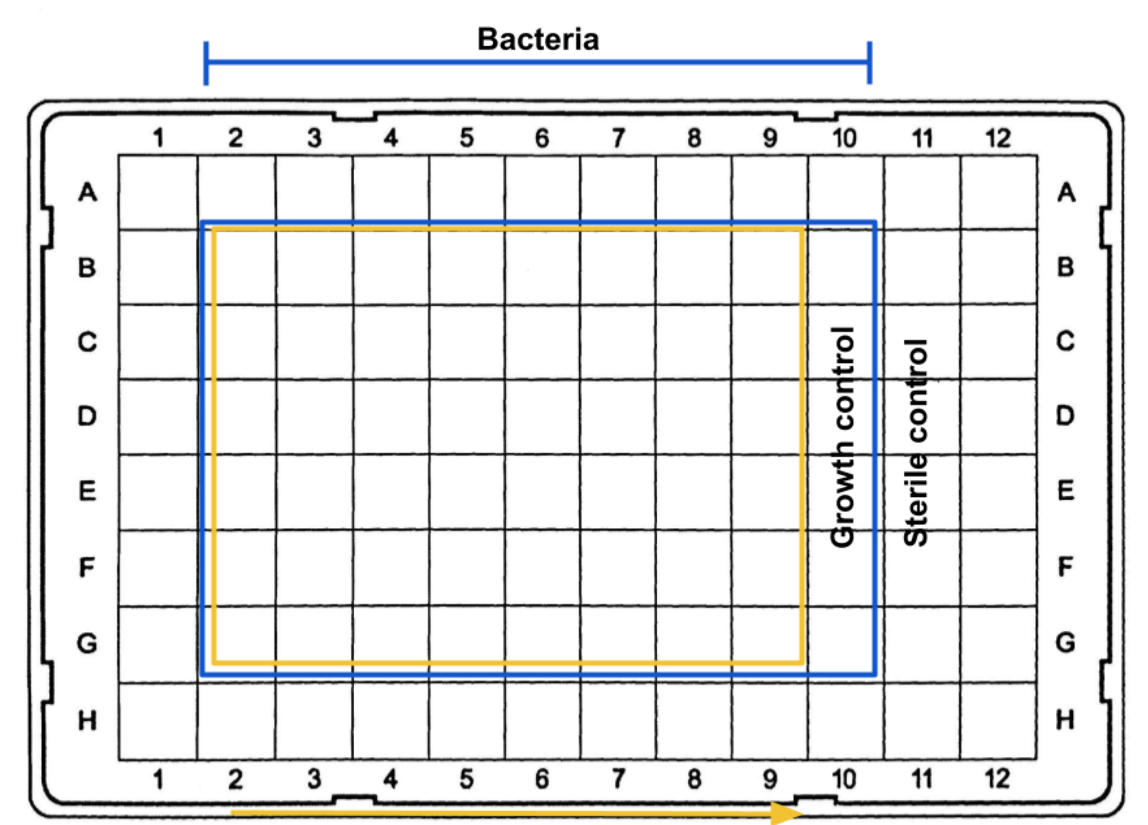
If the highest concentration= 1 mg/mL

Prepare:

((1 mg/mL x 10) (400 µL))/ (stock concentration of antibiotic) = Volume of polymer needed

7.2 Appendix B: Plate Layouts

Section 1: MBEC Assay Layout



Section 2: Checkboard Assay of Polymer and Antibiotic Layout