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A Comparison of the Efficacy of Newly-Synthesized Antimicrobial Polymers and a Preview into their Interaction with the Bacterial Cell Membrane.

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Abstract

Antibiotic-resistant bacteria present an obstacle for physicians and researchers. Over 35,000 people die annually in the U.S. from antibiotic-resistant infections each year. New antibiotics are being developed and studied, and natural products are screened to find potential new antibiotics. In this study, four polymer antibiotics with polyester backbones and peptide functional groups were studied with *Escherichia coli* and *Staphylococcus aureus* to determine the polymers' efficacies on the different bacteria and compare the polymers to each other. Timed fluorescence microscopy experiments were performed to determine how long it takes the antibiotics to compromise the membrane integrity of fluorescently-labelled *E. coli*. The four polymers (designated 115B, 75A, 68B and 81C) compromised cell membranes with average times of 0.59, 2.28, 2.11 and 3.07 minutes, respectively. Faster-acting polymers were more positively charged with a higher percentage of basic side chains. Polymer 115B was 100% functionalized with positive amino acids, polymers 68B and 75A were 50% functionalized with positive amino acids, and polymer 81C was 25% functionalized with positive amino acids. MIC assays revealed that the higher molecular-weight polymers 115B and 75A had minimal inhibitory concentrations of 32 μg/mL, and the lower molecular-weight polymers 81C and 68B had minimal inhibitory concentrations of 128 μg/mL. Observations of the interaction between a labelled polymer and *E. coli* cells showed that the polymer appears to surround the cells at a high concentration before entering the cells. Timed fluorescence microscopy of one polymer with *S. aureus* also demonstrated that the polymer is effective against Gram-positive pathogens. Overall, polymer 115B was the most effective, followed by 75A, 68B, and 81C.

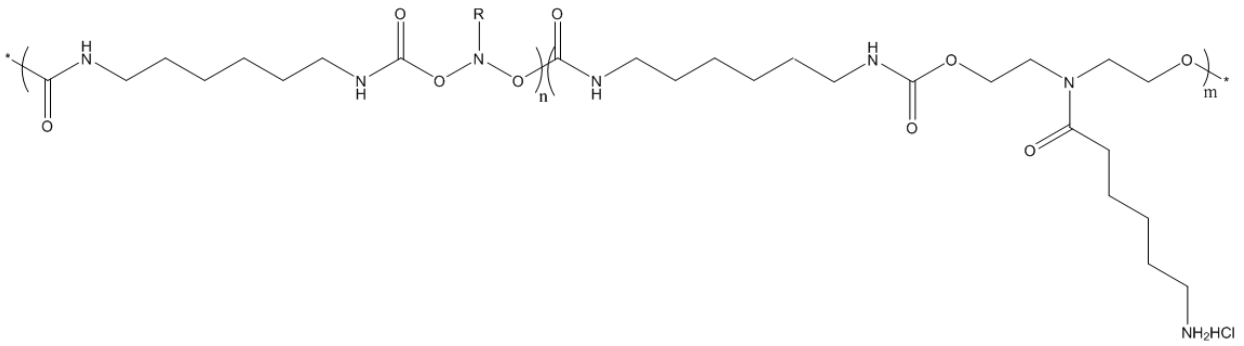
Introduction

The discovery of antibiotics was instrumental to the future of medicine. Antibiotics are useful to the human population because they can be used to control the growth of bacterial populations, or even kill bacteria. However, bacteria can also be resistant to antibiotics and over time, human pathogens have developed more antibiotic resistance.[1-4] In one study, a streptomycin-resistant phenotype occurred in *E. coli* by mutagenesis when bacteria were exposed to the antibiotic.[1] In another, *N. gonorrhoeae* resistance to the antibiotic quinolone increased significantly from 2002 to 2009 among samples of young males with this infection.[2] Yet another study demonstrated that bacteria can use efflux pumps as a resistance mechanism, as seen in quinolone-treated *Pseudomonas aeruginosa*.[3] Unfortunately, while bacteria evolve resistance, the discovery of new antibiotics has declined.[4] In the United States, physicians treat over 2,800,000 patients annually with antibiotic resistant infections and over 35,000 people die annually from these infections.[5] Investigating new ways to kill antibiotic-resistant bacteria is necessary to prevent illness and death caused by such infections.

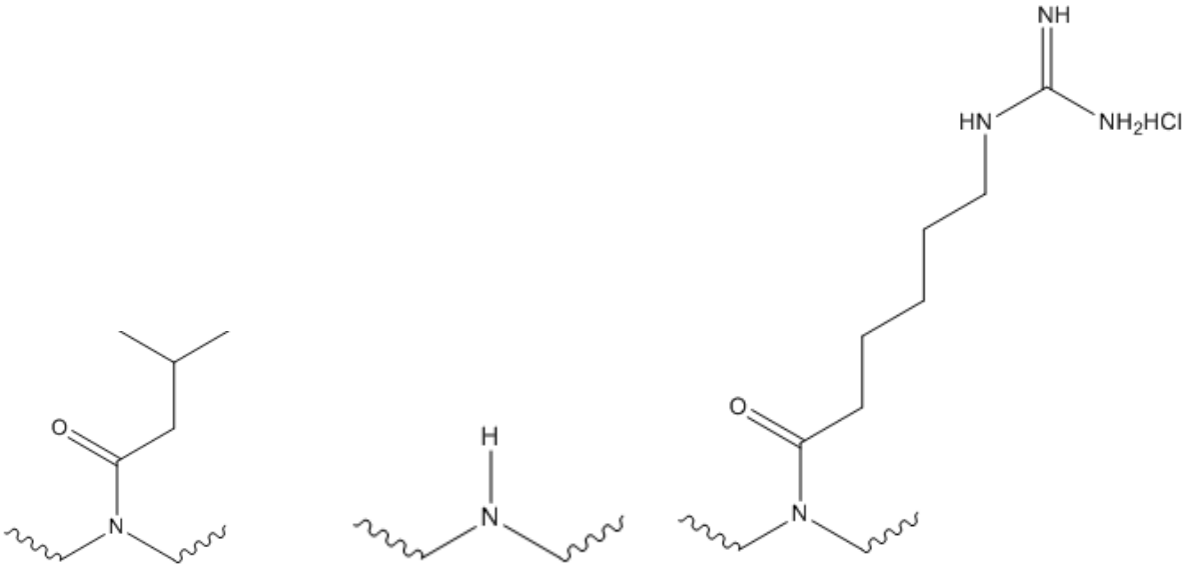
E. coli, N. gonorrhoeae, and *P. aeruginosa* are Gram-negative pathogens. Gram-negative bacteria differ from their Gram-positive counterparts because of their additional outer membrane composed of lipopolysaccharide, a negatively-charged lipid-carbohydrate molecule that is stabilized with divalent cations.[6] Research supports that antimicrobial peptides (AMPs) are shown to destabilize the membrane of the cell by displacing the divalent cations that stabilize the outer membrane, resulting in cell rupture.[7] Therefore, AMPs or molecules similar to them should be investigated for their potential to fight Gram-negative pathogens.

Dr. Abraham Joy's lab of the Department of Polymer Science at The University of Akron synthesized various new polymers with activities similar to AMPs. The positively-charged

polymers have a backbone of linked ester bonds and side chains of various peptide functional groups. They vary in length and structure, and therefore have different efficacies against bacteria. The molecular weights of polymers 81C, 75A, 68B and 115B are 8 kDa, 30 kDa, 6 kDa and 35 kDa, respectively. Each polymer has an *n* and an *m* subunit that make up the repeating unit (Figure 1A).The functional group present in the *n* subunit varies in each polymer, and is not functionalized in some cases. Non-functionalized *n* subunits contain a hydrogen. The functional group in the *m* subunit is lysine. The *m* and *n* units are present in different proportions for each polymer. Overall for polymer 81C, 50% of the subunits are not functionalized, 25% have valine (*m* subunits) and 25% have lysine (*n* subunits) (Figure 1B). Polymers 75A and 68B have 50% *m* subunits functionalized with lysine, and 50% non-functionalized *n* subunits (Figure 1C). Polymers 75A and 68B differ by their molecular weights. Polymer 115B contains 80% *m* subunits with arginine and 20% *n* subunits with lysine (Figure 1D).



(A)



(B) **(C)** **(D)**

Figure 1. Structure of the synthesized antimicrobial polymers developed by Dr. Joy's lab and the various functional groups in the *m* subunit. A) The repeating subunits *n* and *m,* present in a random pattern in the polymers. Lysine is present in the *m* subunit. The functional group, R, varies in the *n* subunit for each polymer. B) Valine functional group in *n* subunit for polymer 81C. Some *n* subunits are not functionalized. C) Non-functionalized *n* subunit in polymers 75A and 68B. D) Arginine functional group in *m* subunit of polymer 115B.

This project used three approaches to measure the efficacy of the polymer antibiotics against *Escherichia coli*. First, the average time for each antibiotic to reduce the membrane integrity of *E. coli* was determined in a liquid, 37°C system, which closely simulated the conditions of human blood. Second, the zone of inhibition of each antibiotic was measured by performing an antibiotic disk assay with *E. coli* on an agar plate. Kanamycin and carbenicillin were used as controls in this study (Figures 2 and 3). Third, the minimal inhibitory concentration of each antibiotic was measured by observing growth of *E. coli* in different dilutions of the antibiotic in liquid media. polymyxin B and carbenicillin were used as controls for this assay (Figures 3 and 4).

In addition to determining the polymer's efficacies against *E. coli*, the average time for the antibiotics to compromise the membrane of the Gram-negative bacteria *Staphylococcus aureus* was determined. Finally, the interaction of a labelled version of one of the antimicrobial polymers was observed with the *E. coli* membrane.

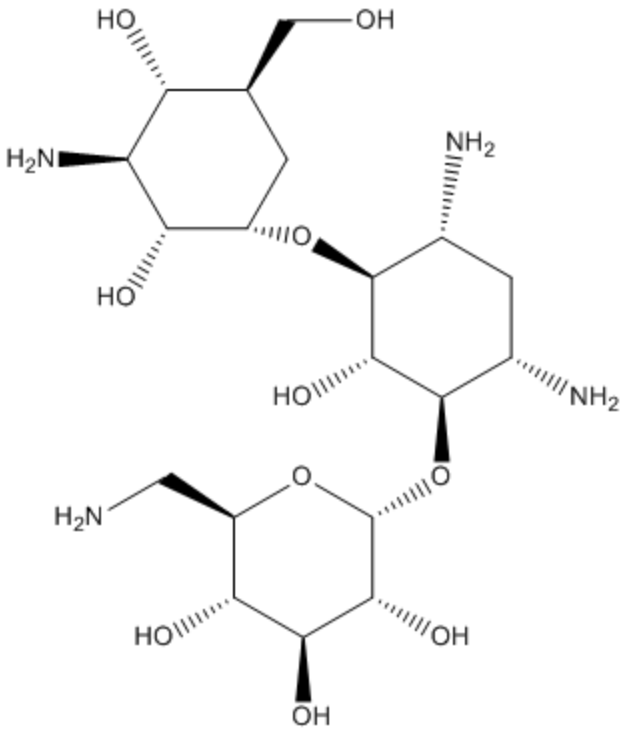


Figure 2. Kanamycin structure.

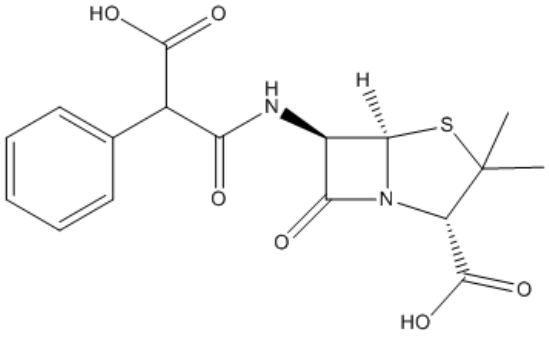


Figure 3. Carbenicillin structure.

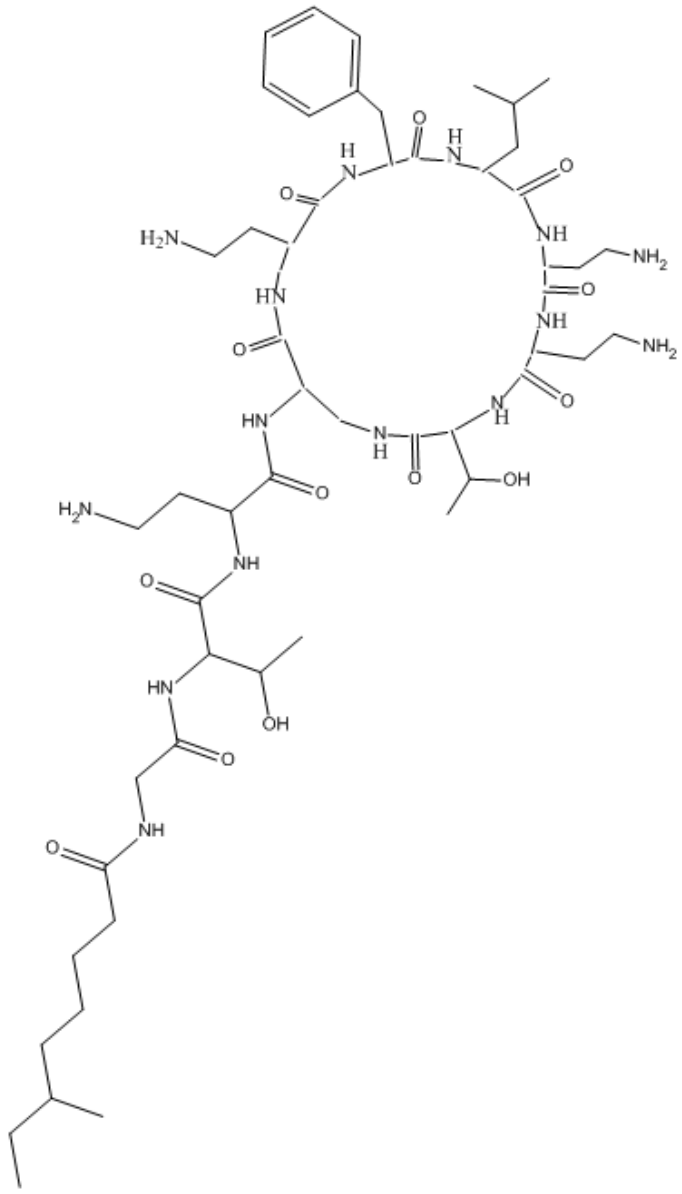


Figure 4. Polymyxin B structure.

Methods

Time-dependent fluorescence microscopy with *E. coli*

To determine the time for an antibiotic to effectively kill *E. coli*, a fluorescence microscopy technique was used. *E. coli* strain MG1655 were grown to log phase in 0.2% glucose

3-(*N*-morpholino)propanesulfonic acid (MOPS) minimal media at 37°C in a shaking incubator. Log phase was determined as between OD_{600} 0.2 and 0.6. Cells were gently vortexed with 0.01 µM RedoxSensor Green™ (RSG) from ThermoFisher and incubated at 37°C with shaking for 15 minutes. This step allowed the RSG to diffuse to the outer membrane so cells could be visualized using fluorescence excitation. RSG dye was used because it fluoresces upon being reduced, and therefore its signal increased as high concentrations of dye entered the cell after the membrane was compromised by the antibiotic.[8]

At the same time, a treatment condition of 1 mL MOPS minimal media was gently vortexed with the antibiotic and 0.01 µM RSG and incubated at 37°C with shaking for 15 minutes. Four new antimicrobial peptides, 115B, 81C, 75A, 68B, were used in the treatment at a final concentration of 32 µg/mL.

Bacteria-adhesive coverslips were prepared by soaking #1.5 thickness circular coverslips in 0.01% Sigma poly-L-lysine for 5 minutes, then rinsing them in nanopure water and allowing them to dry in a covered container. Prior to imaging, a poly-L-lysine-coated coverslip was secured in a stainless-steel chamber and brought to 37°C in a caged incubator. Twenty microliters of cells with RSG were pipetted onto the center of the coverslip and focused on a Nikon Eclipse Ti-E inverted confocal fluorescence microscope using a 100x Plan Apo λ (NA 1.45) oil objective. Cells remained immobile in the liquid media because they were adhered to the poly-L-lysine on the coverslip. RSG was excited using 488 nm light, and its fluorescent emission was collected using a filter the captured light between 500 nm and 550 nm. The NIS Elements program was set up to collect images automatically every 15 seconds for a duration of thirty minutes. Within one minute of beginning to collect images, one milliliter of treatment

condition was rapidly administered and thoroughly mixed by pipetting, and images continued to be collected until thirty minutes was reached.

For each antibiotic, two or three trials were completed. Experimental methods and analysis methods were kept the same for each trial except where alterations are noted.

Analysis of timed fluorescence imaging

Manual analysis of the images was done using Fiji software by ImageJ. The signal of the RSG in the first image taken at t=0 was reduced until the background noise was eliminated. A threshold RSG signal of six pixels or more within the cell was established. Throughout the time of the experiment, as RSG entered the cells and fluoresced in the reduced environment, individual cells achieved the threshold signal. Once each cell met this threshold, the time was indicated and included in the data set for that trial. Box and whisker plots with the y-axis as time and x-axis as trials were generated for each antibiotic (Appendix B). In these plots, each cell in a trial was plotted at a point along the y-axis to demonstrate the time distribution between trials was similar. One-way analysis of variance (ANOVA) was performed on data sets for multiple trials of the same antibiotic to confirm the data was analyzed from a consistent approach (Appendix B).

Data sets from multiple trials of each antibiotic were combined and the average of time it took cells to reach threshold signal for that antibiotic was reported. ANOVA was used to determine whether the differences between these average times of each antibiotic were statistically significant.

Time-dependent fluorescence microscopy of *E. coli* with polymyxin B (for example images)

Timed-dependent fluorescence microscopy of *E. coli* MG1655 was performed as described above for polymer antibiotics with a few alterations. The RSG concentration was 1 μg/mL. One μg/mL propidium iodide dye (PI) was included in both the treatment and experimental conditions. PI fluoresces upon binding to DNA and therefore suggests cell death.[9] Cells were treated with polymyxin B at a concentration of 5 μg/mL. PI was excited at 488 nm and an additional filter was used to collect PI signal between 663 nm -738 nm. Images from this experiment are included in the results section to demonstrate the increase in RSG signal after exposure to an antibiotic.

Time-dependent fluorescence microscopy with *S. aureus*

The procedure described above for time-dependent fluorescence microscopy of *E. coli* was repeated with *S. aureus* 25923 with the following alterations. The RSG-labelled cells and the antibiotic treatment were prepared in MHB II media, which is a rich media. Only one antibiotic was tested, polymer 75A, at a concentration of 32 μg/mL in the treatment condition. Data sets from two trials were collected using the same method. The cells were given 5 minutes to settle on the poly-L-lysine coverslip once added to the pre-incubated chamber. To analyze the data collected, rather than counting individual cells that reached a threshold of six pixels of signal, clumps of cells were counted once they achieved a six-pixel or greater RSG signal intensity.

Imaging of interaction between Cy5-labelled 75A with *E. coli* membrane

The procedure described above for preparation of *E. coli* for time-dependent fluorescence microscopy of *E. coli* MG1655 was repeated with the following alterations. A treatment condition of 32 μg/mL Cy5-labelled polymer 75A was prepared. The Cy5 dye was excited at 640 nm and its emission was collected using a filter for 663 nm -738 nm light.[10] A timed experiment was not set up on NIS Elements software. Instead, before and after administering the antibiotic treatment, images of the cells labelled with RSG and the antibiotic were taken using NIS Elements.

Antibiotic disk assay

Mueller-Hinton Broth II Agar plates were prepared and *E. coli* MG1655 were grown in Mueller-Hinton Broth II (MHB II) liquid media. Log phase *E. coli* were spread across a plate using a sterile plate spreader and incubated for 24 hours at 37°C. One isolated colony was picked from this plate and used to inoculate a fresh MHB II broth and the culture was grown to an OD_{600} of under 0.5. Of this culture, 200 μL was spread onto a total of 10 plates, the underside of each already pre-labelled with marker to identify the four quadrants. Each antibiotic was diluted in sterile water prior to preparing the disks (BD BBL™ Sensi-Disc™). Concentrations of antibiotics used are in Table 1. All carbenicillin and kanamycin antibiotic disks were prepared by pipetting 10 μL of antibiotic onto a sterile paper disk. Four disks for each antimicrobial polymer were prepared by pipetting 10 μL of antibiotic onto the disk, and four more disks for each antimicrobial polymer were prepared by pipetting 50 μL onto each disk.

One antibiotic disk was placed in the center of each quadrant so that the four disks were equidistant from each other. On two control plates, kanamycin and carbenicillin disks were

pressed gently into quadrants nonadjacent to each other to give a total sample of four quadrants per antibiotic. Kanamycin and carbenicillin were used as controls because their expected zones of clearance were reported by the Clinical and Laboratory Standards Institute for this disk method.[11] On the remaining eight plates, one of each antimicrobial polymer disk was loaded onto each plate so that each quadrant of a plate had either 81C, 115B, 75A, or 68B. The plates were incubated at 37°C for 24 hours. For the zone of clearance of each individual disk, the diameter of the clearance was measured through the center of the disk in two directions. The average of the diameter was calculated for each disk, and then these values were averaged to calculate the diameter of clearance for each antibiotic.

Antibiotic	Concentration (µg/µL)	Volume pipetted onto sterile paper disk (µL)	Total mass antibiotic (µg)	Number of disks plated
Carbenicillin	10	10	100	4
Kanamycin	3	10	30	4
Polymer 81C	3.8	10	38	4
Polymer 75A	3.8	10	38	4
Polymer 68B	3.8	10	38	4
Polymer 115B	3.8	10	38	4
Polymer 81C	3.8	50	190	4
Polymer 75A	3.8	50	190	4
Polymer 68B	3.8	50	190	4
Polymer 115B	3.8	50	190	4

Table 1. Concentration of antibiotics for disk preparation and volumes loaded onto disks.

Minimal inhibitory concentration (MIC)

For each polymer antibiotic, an MIC was found using the dilution method. Carbenicillin and polymyxin B were used as controls. A culture of *E. coli* MG1655 was grown to an OD 0.5 in MHB II for each dilution of antibiotic. Four or five concentrations of each antibiotic were prepared in a 1:1000 diluted cell culture, and the cultures were incubated at 37°C with shaking for 22 hours. The concentrations of antibiotics used are listed in Table 2.

Antibiotic	Concentration 1 (µg/mL)	Concentration 2 (µg/mL)	Concentration 3 (µg/mL)	Concentration 4 (µg/mL)	Concentration 5 (for controls) (µg/mL)
Carbenicillin	8.0	16.0	32.0	64.0	128.0
Polymyxin B	0.5	1.0	2.0	4.0	8.0
Polymer 81C	16.0	32.0	64.0	128.0	
Polymer 75A	16.0	32.0	64.0	128.0	
Polymer 68B	16.0	32.0	64.0	128.0	
Polymer 115B	16.0	32.0	64.0	128.0	

Table 2. Concentration of antibiotic for each dilution during MIC assay.

Following incubation of the cell cultures with the various concentrations of antibiotic, OD measurements were taken on cultures that did not exhibit visual growth. The minimal inhibitory concentration was determined as the minimal concentration that exhibited no growth with an $OD_{600} \leq 0.050$.

Results

Time-dependent fluorescence microscopy of *E. coli* with polymyxin B (for example images)

This timed microscopy experiment was performed to serve as an example for how RSG intensity increased when exposed to antibiotic. In this example, *E. coli* were labelled with both RSG and PI, immobilized on a coverslip, then exposed to the antibiotic polymyxin B. Propidium iodide was used because it serves as an indicator for *E. coli* cell death, since it binds to DNA.[9] Figure 5 illustrates the RSG and PI signal increase after addition of polymyxin B. The green signal represents RSG, and the purple signal represents PI. Although PI was not used with the polymer antibiotics, it would give insight to the timing of the PI signal appearance compared to the RSG signal. This would explain how soon cells die after their membranes become permeated.

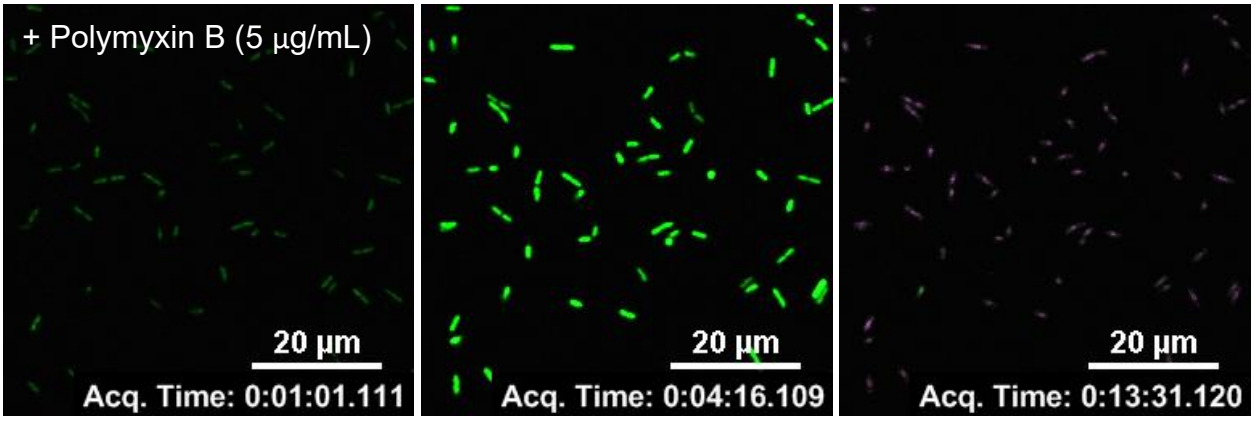


Figure 5. *E. coli* MG1655 RSG and PI signal upon treatment of 5 µg/mL PMB. Green signal represents RSG and purple signal represent PI. RSG signal was observable before adding antibiotic, but its intensity was greater at 4 minutes (after PMB exposure). PI signal appeared after antibiotic exposure.

Time-dependent fluorescence microscopy with polymer antibiotics

The first study on the polymers measured the average time for each antimicrobial polymer to compromise *E. coli* cell membranes in liquid media conditions at 37°C. Multiple trials with each polymer antibiotic were completed and analyzed. There was no significant difference between trials of a single antibiotic (Table B1, Appendix B) indicating that the data was collected and analyzed from a consistent approach.

The average efficacy for each antibiotic was determined as the mean time it took all cells from all trials to reach a threshold intensity of RSG signal (Table 3). The average times for polymers 81C, 75A, 68B, and 115B were 3.07 minutes, 2.28 minutes, 2.11 minutes, and 0.59 minutes, respectively. ANOVA between polymers supports that polymers 81C, 75A, 68B, and 115B differed significantly from each other, with the exception of 68B and 115B. The F-values and F-critical values from ANOVA between polymers are reported in Table B2 of Appendix B.

Antimicrobial Polymer	Average effective time (minutes):	Differed significantly from:
Polymer 81C	3.07	75A, 68B, 115B
Polymer 75A	2.28	81C, 68B, 115B
Polymer 68B	2.11	81C, 75A
Polymer 115B	0.59	81C, 75A

Table 3. Average time for each polymer to compromise *E. coli* cell membranes. The average was calculated as the mean of the time it took each cell to reach threshold RSG signal once exposed to antibiotic.

Further analysis of each polymer's efficacy was made by looking at the distribution of cells that met signal threshold over time for each polymer. While 73% of *E. coli* cells reached threshold within four minutes of exposure to polymer 81C, cell membranes continued to be

compromised until 14 minutes after exposure (Figure 6). For polymer 75A, 90% of all cells reached threshold within four minutes of polymer addition and all cells were compromised after seven minutes (Figure 7). Polymer 68B compromised 82% of the cells within the first four minutes, and cells continued to reach threshold until 14 minutes after exposure (Figure 8). The most rapid antibiotic was polymer 115B, which compromised all of the cells within three minutes (Figure 9).

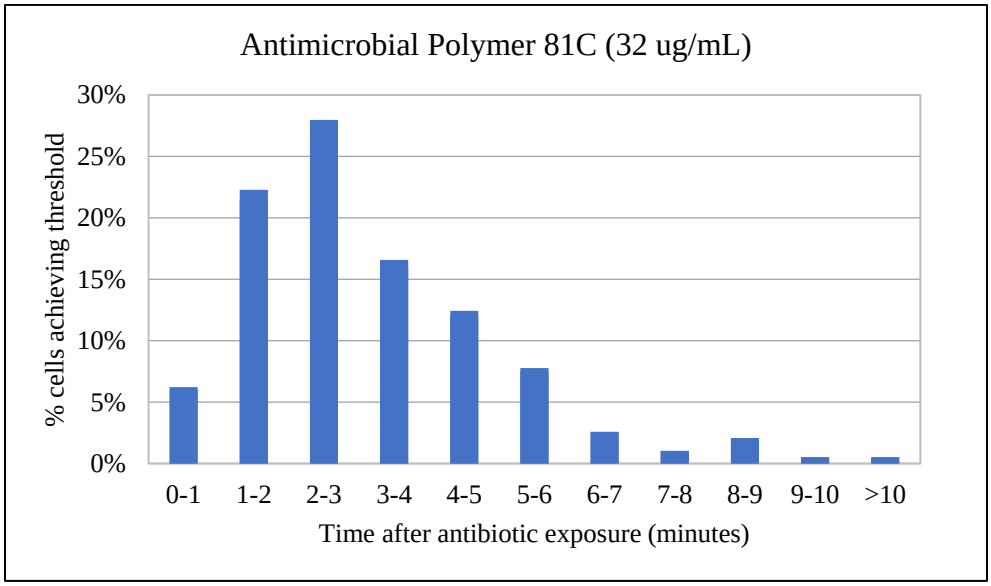


Figure 6. Antimicrobial polymer 81C effective time. Polymer 81C was used to treat *E. coli* MG1655 at a concentration of 32 µg/mL. This antibiotic effectively compromised 73% of the cells within 4 minutes. A total of 193 cells were counted from two trials.

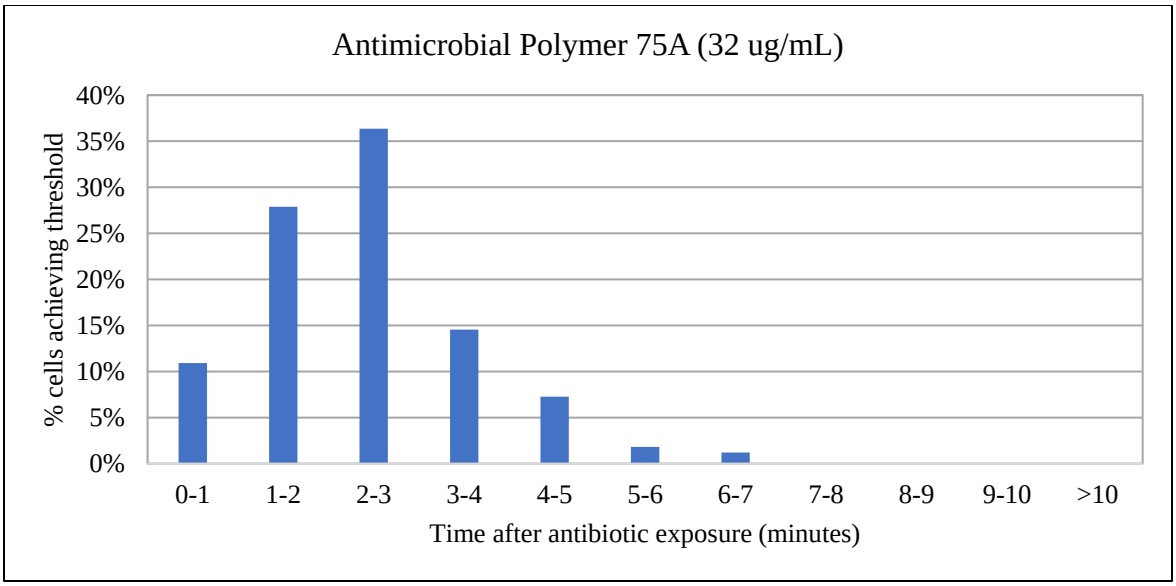


Figure 7. Antimicrobial polymer 75A effective time. Polymer 75A was used to treat *E. coli* MG1655 at a concentration of 32 µg/mL. This antibiotic effectively compromised 90% of the cells within 4 minutes. A total of 116 cells were counted from two trials.

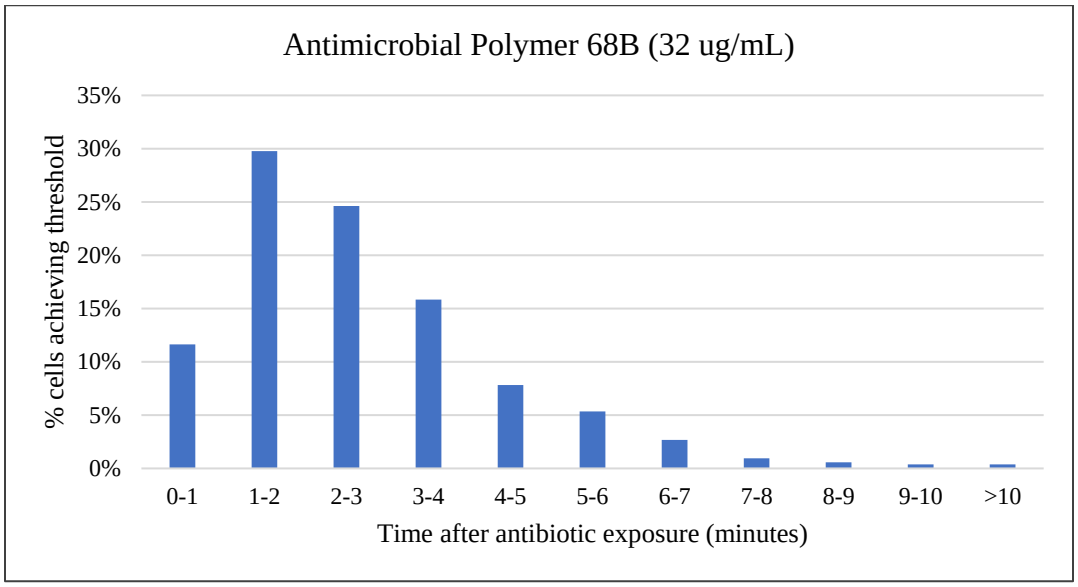


Figure 8. Antimicrobial polymer 68B effective time. Polymer 68B was used to treat *E. coli* MG1655 at a concentration of 32 µg/mL. This antibiotic effectively compromised 82% of the cells within 4 minutes. A total of 524 cells were counted across three trials.

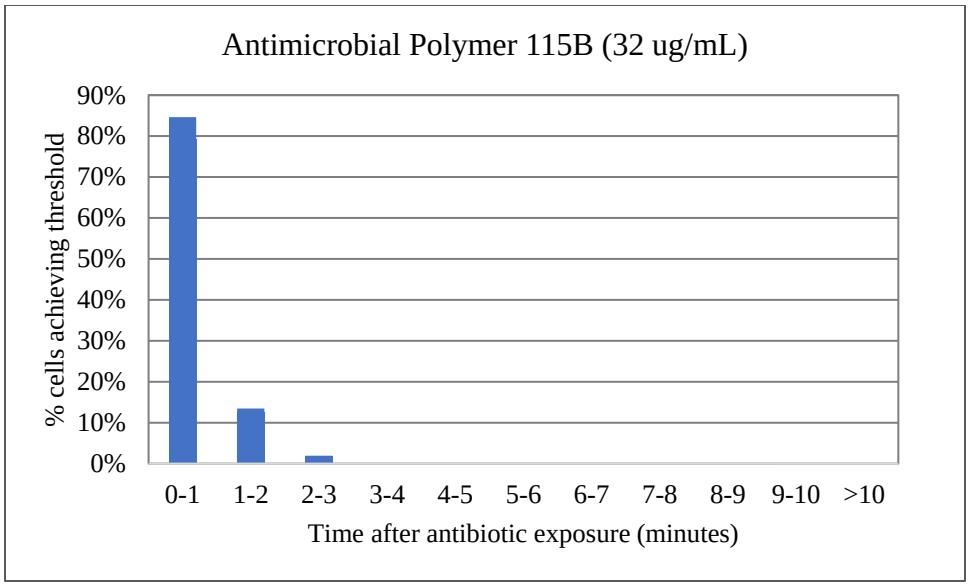


Figure 9. Antimicrobial polymer 115B effective time. Polymer 115B was used to treat *E. coli* MG1655 at a concentration of 32 µg/mL. This antibiotic effectively compromised 100% of the cells within 3 minutes. A total of 52 cells were counted over two trials.

Antibiotic disk assay

The next part of this research evaluated *E. coli* MG1655 growth on agar plates with antibiotic disks. Kanamycin and carbenicillin disks were used as controls and have expected zones of inhibition of 17–25 mm and 23-29 mm, respectively.[11] The zones measured in this experiment (n=4) was 21.1 mm for kanamycin and 22.4 mm for carbenicillin. These measurements are within or close to the expected ranges.

The polymer antibiotic disks loaded with 10 µL of antibiotic did not exhibit any clearance around them. The polymer antibiotic disks loaded with 50 µL of antibiotic had clearances with uneven edges around them, within 2 mm of each disk. These results were inconclusive. It was speculated that the polymer did not diffuse in the MHB II agar.

Minimal inhibitory concentration

The minimal inhibitory concentration for each polymer antibiotic and two controls was measured in liquid MHB II media (Table 4). The controls used were carbenicillin and polymyxin B, which inhibited *E. coli* MG1655 growth at concentrations of 64 µg/mL and 0.5 µg/mL, respectively. The antimicrobial polymers 81C and 68B inhibited the growth of *E. coli* MG1655 in MHB II media at a concentration of 128 µg/mL. The antimicrobial polymers 75A and 68B inhibited the growth of *E. coli* MG1655 in MHB II media at a concentration of 32 µg/mL.

Antibiotic	Minimal Inhibitory Concentration for *E. coli* MG1655 in MHB II (µg/mL)
Carbenicillin	64
Polymyxin B	0.5
Polymer 81C	128
Polymer 75A	32
Polymer 68B	128
Polymer 115B	32

Table 4. MIC of polymer antibiotics 81C, 75A, 68B, and 115B.. Carbenicillin and polymyxin B were used as controls.

Time-dependent fluorescence microscopy with *S. aureus*

Staphylococcus aureus was exposed to the same concentration of polymer antibiotic 75A as *E. coli*, but due to its tendency to form clusters, analysis was done on clusters rather than individual cells. Individual clusters were marked and counted as the RSG signal reached threshold. The average time for clusters to reach threshold was 0.704 minutes, and 245 clusters were analyzed. Of all of the clusters, 99% reached threshold signal within three minutes (Figure 10).

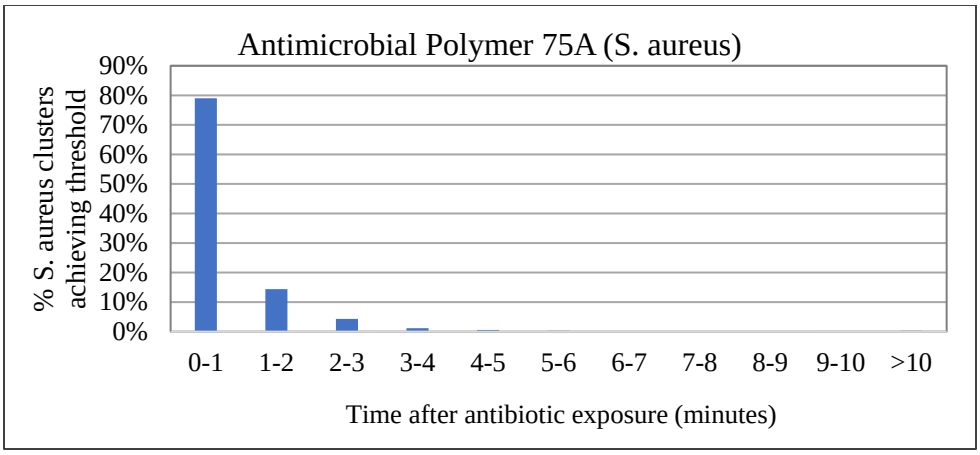
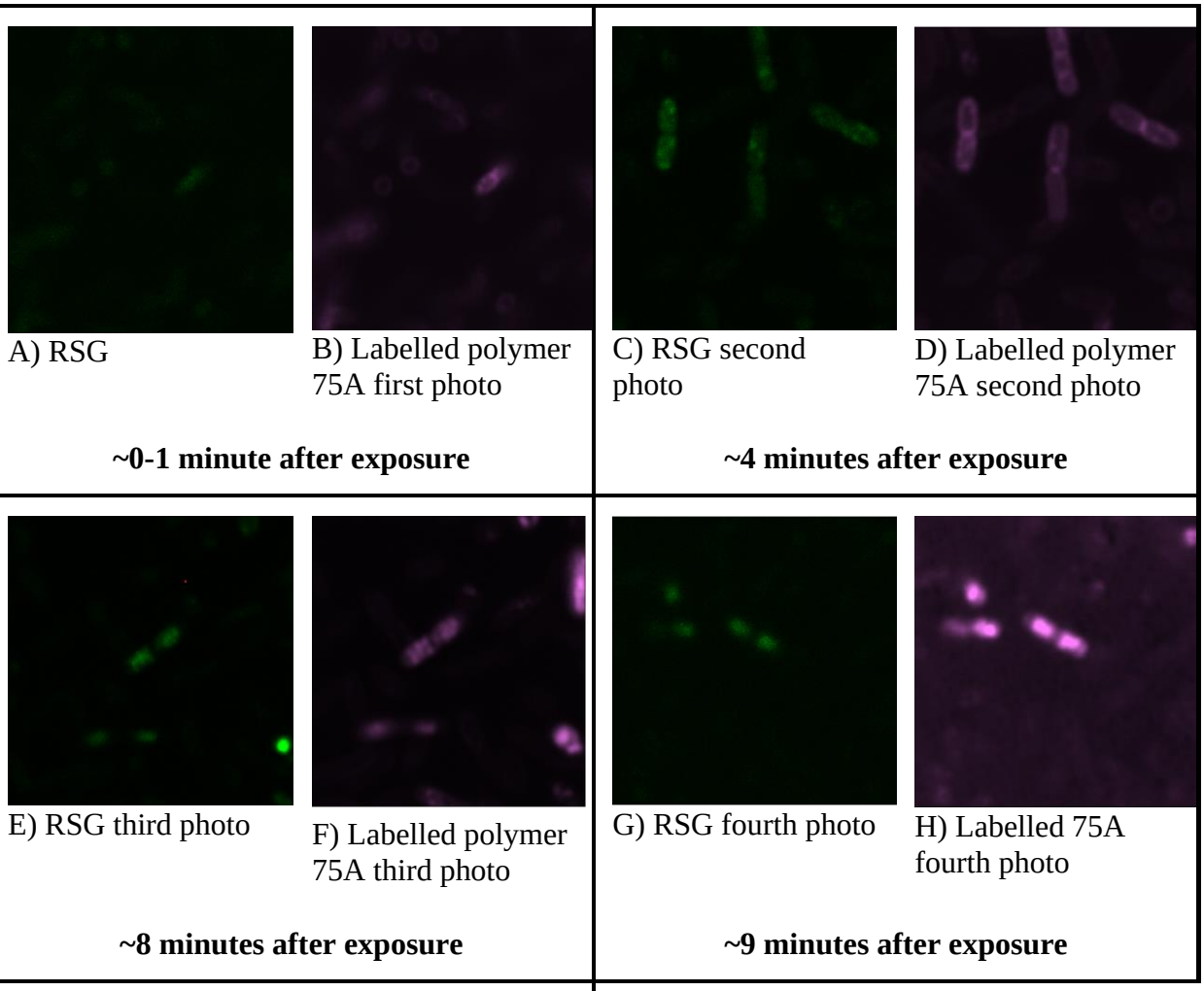


Figure 10. Antimicrobial polymer 75A effective time against S. aureus. *S. aureus* 25923 were treated with 32 µg/mL polymer 75A. This antibiotic brought 99% of cell clusters to threshold RSG signal within three minutes. A total of 245 cell clusters were counted over two trials.

Interaction of the polymer antibiotic with the *E. coli* outer membrane.

A Cy-5 labelled version of polymer 75A was utilized to obtain images of the polymer localizing around *E. coli*. RSG was used to label the *E. coli* to provide additional understanding of when the membranes were compromised compared to where the polymer was located. Images of RSG-labelled *E. coli* as they were exposed to Cy-5-labelled polymer 75A showed that immediately after the polymer was added, it surrounded the *E. coli* (Figure 11A-B). As the polymer signal became more concentrated around the membrane approximately four minutes after its addition, the RSG signal inside the cell increased (Figure 11C-D). This could indicate that the polymer caused openings in the membrane through which RSG entered the cell. By around eight minutes, the signal of the polymer increased inside the cell (Figure 11E-H). Eleven minutes after its addition, the polymer signal intensity decreased inside the cell, but the RSG signal increased (Figure 11I-J).



A) RSG

B) Labelled polymer 75A first photo

~0-1 minute after exposure

C) RSG second photo

D) Labelled polymer 75A second photo

~4 minutes after exposure

E) RSG third photo

F) Labelled polymer 75A third photo

~8 minutes after exposure

G) RSG fourth photo

H) Labelled 75A fourth photo

~9 minutes after exposure

I) RSG fifth photo

J) Labelled 75A fifth photo

~11 minutes after exposure

Figure 11. Labelled polymer 75A localization around RSG-labelled *E. coli* over time. A-D) The polymer surrounded the cell, then the RSG signal increased. E-J) Intracellular RSG signal increased, intracellular polymer signal increased then decreased.

Discussion

The results from the timed fluorescence microscopy experiments and MIC assay provided sufficient data to compare the efficacy of polymers 81C, 75A, 68B, and 115B in terms of their effective times and effective concentrations for *E. coli*. The microscopy experiments demonstrated that polymer 115B was the most rapid antibiotic in terms of average time to compromise membranes, followed by both 68B and 75A, and lastly 81C. When cells were exposed to polymers 81C and 68B it took up to 14 minutes for cells to be compromised. Comparatively, *E. coli* exposed to 115B had compromised membranes within 3 minutes and *E. coli* exposed to 75A had compromised membranes within 7 minutes. Polymers 81C (8 kDa) and 68B (6 kDa) had higher MICs and lower molecular weights than polymers 75A (30 kDa) and 115B (35 kDa). This indicates that molecular weight might contribute to the minimal inhibitory concentration of an antibiotic. Polymer 115B was 80% functionalized with the positive groups arginine and 20% functionalized with the positive group lysine. This was the only polymer that was completely functionalized with positive functional groups, making it more positive than the others. The least effective polymer, 81C, was only 25% functionalized with positive amino acids. Polymers 75A and 68B were 50% functionalized with positive amino acids. Possibly, the positively-charged polymers functionalized with more basic side chains are faster acting because of their attraction to the more negative surface of the *E. coli* membrane.

The antibiotic disk assay results were inconclusive for the polymers. The kanamycin and carbenicillin controls yielded expected zones of clearance, demonstrating that the procedure used for this assay was effective. Therefore, it is suspected that the polymer antibiotics did not diffuse well through the solid agar. Even increasing the overall amount of antibiotic per disk by using 50 µL rather than 10 µL did not give significant or consistent zones of clearance around the disk.

The time-dependent fluorescence microscopy experiment with polymer 75A and *S. aureus* demonstrated that the same concentration of polymer antibiotic used on *E. coli* was also effective against *S. aureus*, a Gram-positive pathogen. The RSG-labelled *S. aureus* cells were adhered to the coverslip as were the *E. coli*, however, they were in clusters. This made it difficult to determine boundaries between cells and therefore the time for RSG signal to reach threshold was recorded for clusters, rather than individual cells. For this reason, the average time for *S. aureus* clusters to reach threshold was not compared to the average time for *E. coli* cells to achieve threshold signal.

Images of Cy5-labelled polymer 75A interacting with the RSG-labelled *E. coli* were provided. General observations were made as images were collected after antibiotic exposure. These images show the polymer first accumulated around the cell, which suggested that the polymer diffused through the liquid media and either surrounded or inserted itself in the outer membrane. The higher concentrations of polymer and RSG in the cell indicate that the membrane was no longer intact. Therefore, these polymers are likely effective by destabilizing the outer membrane, similar to the mechanism of polymyxin B and AMPs.[6,7]

Conclusion

The most rapid antibiotic that required the lowest concentration to be effective against *E. coli* was polymer 115B, with an average time of 0.59 minutes and an MIC of 32 μg/mL. Therefore, polymer 115B, which was the most positive polymer and also the heaviest in terms of molecular weight, was the most effective. Polymer 75A was the second most effective, with an MIC of 32 μg/mL and an average time to compromise *E. coli* of 2.28 minutes. Its average time was only slightly longer than that of polymer 68B, which was 2.11 minutes. Both polymers were

less positive than 115B but more positive than 81C due to their functional groups. Polymers 68B and 81C had a much higher MIC of 128 μg/mL. Polymer 81C had the longest average time of 3.07 minutes. This polymer was therefore the least effective of the four polymers tested and was also the least positive. There is a notable trend in a higher composition of positive amino acids corresponds with a higher efficacy for a given polymer. It is also notable that lower MIC's correspond with higher molecular weights for the antibiotics. The antibiotics demonstrated similar effects against Gram-positive *S. aureus*, affecting entire clusters of cells with an average time of 0.704 minutes. The polymers do not diffuse well through solid media.

Further work should evaluate the efficacy of these antibiotics against other Gram-negative pathogens such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* The time-dependent microscopy experiments and MIC study should be completed with strains such as these. Evaluation of the polymers' effectiveness in solid media could be completed by plating bacteria cultures on agars of various concentrations of antibiotic. This could give insights to how effective the polymers are in solid surfaces. Lastly, a high-level overview of the mechanism of polymer antibiotics was given in this experiment. It appears that they attack the membrane or cell wall of the bacteria. Since they were effective against *S. aureus*, which have no outer membrane, further work would definitely need to be done to determine their exact target.

Overall, the work from this experiment provided sufficient results to compare four new polymer antibiotics. Further research needs to be completed to determine how these antibiotics might be used safely against infectious bacteria and understand their mechanism. These types of antibiotics should continue to be developed and studied given their potential to prevent or treat antibiotic resistant infections.

Appendix A,

Safety Precautions

This project involved working with safety hazards, including bacteria and sharps, for example. Therefore, multiple things were done to ensure safety. When working with cells, nitrile gloves were worn and all cells were handled near a flame or in a sterile hood. When working with *Staphylococcus aureus*, additional precautions were taken in accordance with laboratory biosafety level two standards. This included wearing lab coats and eye protection, and clothes that entirely covered the entire body and extremities.

To safely dispose of cells, they were bleached thirty minutes prior to disposal with a 50% bleach/water solution that was freshly prepared. All glassware used in this experiment was washed with detergent and rinsed in deionized then nanopure water. All media and glassware were autoclaved prior to use to ensure proper sterility. The stainless-steel chamber for microscopy was cleaned in 50% ethanol and rinsed in nanopure water. Counters were cleaned with bleaching wipes or flamed using 50% ethanol. Sharps were disposed of in a designated sharps disposal, and biohazard materials were disposed of in a designated container as well.

Appendix B,

Statistical Analysis

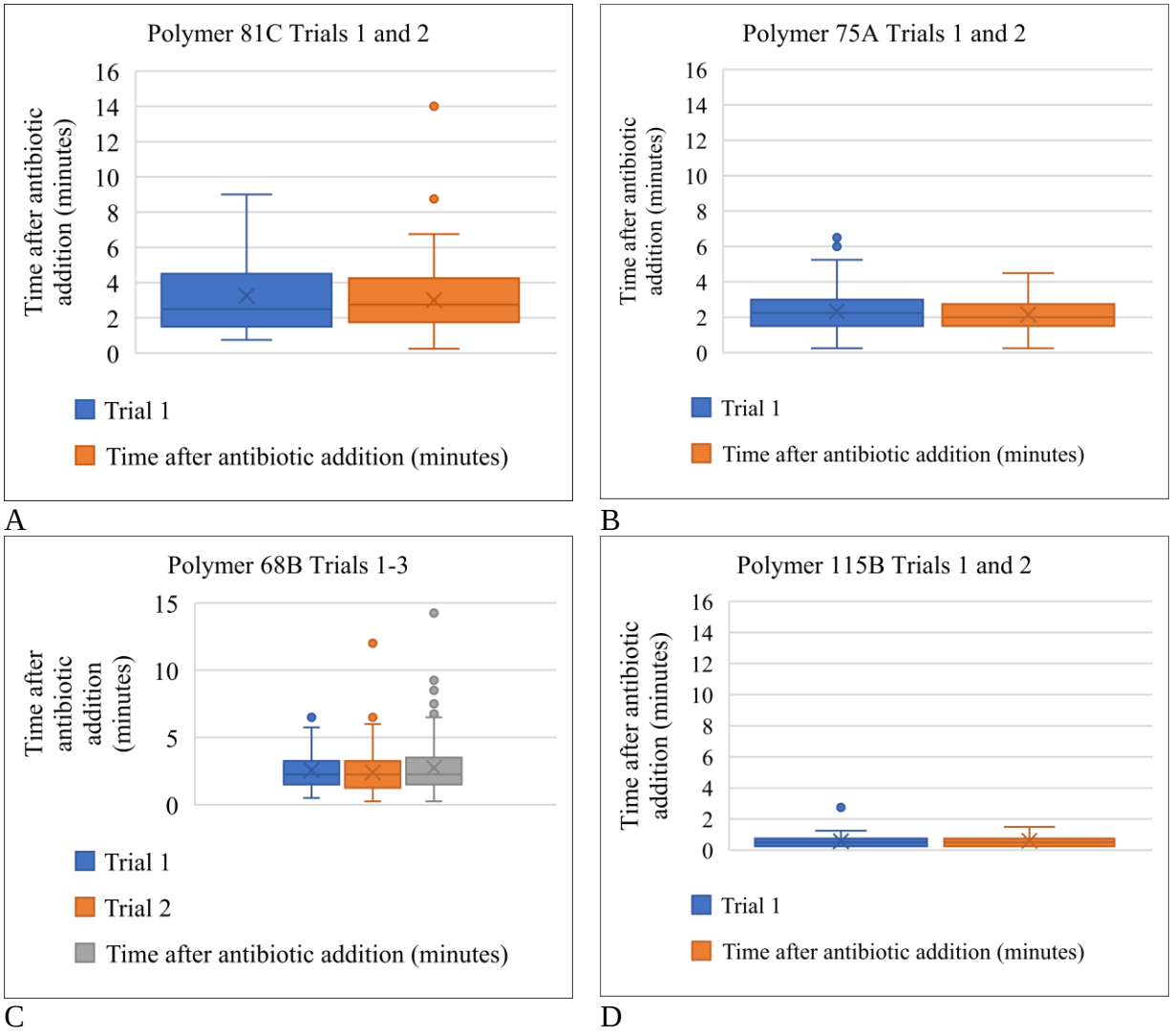


Figure B1. Time distributions across trials one and two for fluorescence microscopy of *E. coli* treated with antimicrobial polymer. The time indicated is the time at which a cell reached the RSG fluorescence intensity threshold. This time indicates when the cell was compromised. A) Cells treated with 32 µg/mL Polymer 81C. B) Cells treated with 32 µg/mL Polymer 75A. C) Cells treated with 32 µg/mL Polymer 68B. D) Cells treated with 32 µg/mL Polymer 115B. Each box and whisker plot demonstrates the data range across trials for a given antibiotic is consistent.

Antibiotic	# trials completed	F-value	F-critical value
Polymer 81C	2	0.657505878	3.890606301
Polymer 75A	2	0.689117649	3.899142394
Polymer 68B	3	2.109113687	3.012957475
Polymer 115B	2	1.510071402	4.030392595

Table B1. ANOVA F- and F-critical values between trials of a polymer antibiotic from time-dependent fluorescence microscopy of *E. coli.*

Polymers	F-value	F critical value	Significant difference?
81C v. 75A	20.98378013	3.867712204	Yes
81C v. 68B	50.33792677	3.856900595	Yes
81C v. 115B	84.58122657	3.880011169	Yes
75A v. 68B	2.026157516	3.85765358	No
75A v. 115B	99.1909791	3.885074063	Yes
68B v. 115B	64.38133713	3.86162144	Yes

Table B2. ANOVA F- and F-critical values between polymer antibiotics from time-dependent fluorescence microscopy of *E. coli*.

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