CHARACTERIZATION OF THE INTERACTION OF DAPTOMYCIN WITH BACTERIAL LIPOSOMAL ANALOGUES

By

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And approved by

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ABSTRACT OF THE THESIS

CHARACTERIZATION OF THE INTERACTION OF DAPTOMYCIN WITH BACTERIAL LIPOSOMAL ANALOGUES

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Efforts to curb the prevalence of antibiotic resistant bacteria are unable to keep up with the aggressive adaptation of these bacterial species to existing antibiotics. Therefore, examining the interaction of existing antibiotics with bacteria may reveal previously unknown bacterial susceptibilities. Daptomycin is a typical second-line treatment for antibiotic-resistant gram positive bacterial strains, like *methicillin-resistant Staphylococcus aureus* (MRSA). Its proposed mechanism of action is to bind to the negatively charged phosphatidyglycerol (PG) head groups of the bacterial cytoplasmic membrane via a calcium ion dependent process. However, the specific mechanisms by which daptomycin exerts its bacteriocidality are currently unknown. It has been hypothesized that bacterial membrane rigidity may have an effect on susceptibility to daptomycin. Additionally, there is evidence to suggest that the charge of the bacterial membrane charge affects daptomycin's mechanism of action. Our study aims to systematically analyze the interaction of daptomycin with liposomal bacterial analogues by varying the rigidity and the zeta potential of the liposomes. Our results show possible mechanisms for targeting daptomycin resistance in gram positive bacteria.

Acknowledgements

I would like to thank my advisor, Professor Stavroula Sofou, for all of her support during both my undergraduate and graduate education. These last four years in her lab have prepared me for my higher education. I am indebted to her for taking me in as an inexperienced student and helping to develop my presentation, research, and analysis skills.

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Table of Contents

List of Figures

List of Tables

Section 1: Introduction

Daptomycin is a lipopetide anionic antibiotic produced by the bacteria, *Streptomyces roseosporus,* and it is a cyclic molecule with a decanoyl fatty acid side chain and a total of 13 amino acid residues [1].

Daptomycin regularly is used as the second line of treatment [2] for gram-positive bacteria, which are a group of bacteria that stain a purple color in a Gram stain. A purple stain, which represents gram-positive bacteria, indicates bacteria that have a thick peptidoglycan layer followed by the cytoplasmic membrane (CM) [3]. Unlike some antibiotics, daptomycin only has bactericidal activity with gram-positive bacteria; this is due to the increased prevalence of the anionic phosphatidylglycerol (PG) in the cell membrane of gram-positive bacteria than in gram-negative bacteria [4]. For example, from bacterial lipid composition analysis, approximately 50-60% of the gram positive bacterial membrane is composed of PG lipids and only about 10-20% of the gram negative bacterial membrane is composed of PG lipids [3]. Since daptomycin and its target PG are anionic, daptomycin cannot interact with the PG lipids due to electrostatic repulsion. As a result, the mechanism of action of daptomycin is dependent upon calcium ions [5]. The calcium ions complex with daptomycin and renders the antibiotic cationic, which then allows the drug to bind to the PG lipids on the cytoplasmic membrane.

It is important to understand the mechanism of action of daptomycin on the CM of gram positive bacteria. After bypassing the cell wall, daptomycin binds to the bacterial CM and the lipophilic acyl tail of daptomycin is inserted into the CM [6]. One theory as to how this mechanism of action works is as follows. Daptomycin first binds to the outer leaflet of the target bilayer membrane via a calcium mediated process. Next, a tetramer of

daptomycin forms in the outer leaflet and this tetramer translocates to the inner lipid leaflet. Lastly, another tetramer of daptomycin once again forms on the outer lipid leaflet and when these tetrameric pores align, an octameric pore is created. This induces permeabilization and depolarization of the CM, and cations flows out of the cell through the octameric pores. This disrupts the cell's metabolic activities, leading to its death [7]. However, another theory posits that daptomycin induces a lipid extracting effect on the bacterial CM. The mechanism by which daptomycin induces lipid extraction is theorized to be due to the molecular aggregation and unbinding of the daptomycin from the lipid bilayer [8]. This lipid extraction effect has also been shown to induce membrane depolarization due to the loss of cations from the bacterial cell.

One type of bacteria that is treated with daptomycin is *Staphylococcus aureus* (*S. aureus*). While the non-resistant strain of *S. aureus* can be treated with antibiotics, like methicillin and vancomycin, antibiotic resistant bacteria prove to be difficult to control and treat. For example, vancomycin and methicillin resistant *S. aureus* (VRSA and MRSA respectively) are becoming more prevalent in domestic and hospital settings [1]. According to a report by Datta and group, it was discovered that patients who harbor MRSA for over one year are at a high risk for morbidity and mortality [9]. In order to treat these particularly aggressive and resistant types of *S. aureus*, it is common to use a second-line of treatment, like daptomycin, in the event that a primary antibiotic does not work.

Unfortunately, *S. aureus* is also susceptible to daptomycin resistance and a startling amount of clinical reports (~35 patients) have been published documenting the emergence of daptomycin resistant *S. aureus* [1]. One of the dominant methods of

daptomycin resistance is seen in the mutation of a variety of single nucleotide polymorphisms (SNPs) [1] . One particular mutation to the *mprF* causes the PG head groups to become lysinylated; as a result, the negatively charged PG becomes positively charged. This positive charge then repels the cationic daptomycin and calcium ions that are needed to bind the daptomycin. Additionally, Mishra and group theorized that membrane rigidity-fluidity may play a role in resistance of *S. aureus* to various drugs, like daptomycin [10].

In the present work, we examine how membrane rigidity and lipid extraction may be responsible for bacteriocidality due to daptomycin. We hypothesize that the addition of daptomycin will show a marked increase in membrane permeability and release of liposomal contents, that a membrane with greater zeta potential will result in more release of liposomal contents, and that the inclusion of rigid lipid domains will elicit more content release.

Section 2: Materials and Methods

2.1 Materials

The lipids L-α-phosphatidylcholine (EggPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol)(sodium salt; DMPG), 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)(sodium salt; DOPG), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and 1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol)(sodium salt; DSPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Calcein, Triton-X 100, phosphate buffered saline (PBS), piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma Aldrich Chemical Company (Milwaukee, WI). The *S. aureus* strain was purchased from ATCC (Manassas, VA). The Tryptic Soy Broth was purchased from Becton Dickinson (San Jose, CA). The daptomycin was purchased from VWR (Radnor, PA).The FAST DiI solid; DiIΔ9,12-C18(3), CBS (1,1'-Dilinoleyl-3,3,3',3'-Tetramethylindocarbocyanine, 4-Chlorobenzenesulfonate) and the DiI Stain (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate ('DiI'; DiIC18(3))) were obtained from Thermo Fisher Scientific (Waltham, MA).

2.2 Killing Curve of S. aureus with Daptomycin

In order to determine the killing curve of *S. aureus*, the bacteria was first cultured in 25 mL of Tryptic Soy Broth (TSB) from a seed culture of 2 mL; the bacterial broth was then incubated at 37°C on a shaker at 200 rpm. After the bacteria reached an optical density (OD_{600}) of 0.002 measured with a Beckman Coulter DU 730 UV/Vis Spectrophotometer

(Indianapolis, IN), 100 µL triplicates of bacteria suspension were added to each of thirteen wells in a 96-well plate. To one of the triplicate sets, 100 µl of water was added as a control for the drug. To another triplicate set, 100 µL of TSB was added as another control. To the other eleven triplicate sets, various concentrations of daptomycin (from 8 mg/L to 0.125 mg/L), dissolved in water containing 1.25 mM calcium ions, were added. Each well was then thoroughly mixed. Before incubating the plates, the other empty wells were filled with water to replicate humidity in order to prevent evaporation from the experimental wells. The plates were then placed in an incubator at 37°C on a shaker at 200 rpm for eight hours. After the eight hours of incubation, absorbance of the bacterial wells was read on a Beckman Coulter DTX 880 (Indianapolis, IN) plate reader with a 595 SL-2 excitation filter.

2.3 Determination of the Standard Curve of Daptomycin

In order to determine the concentration of daptomycin in further experiments, a standard curve for daptomycin was generated. After making serial dilutions of daptomycin from 50 mg/L to 3.125 mg/L, the UV absorbance of each sample at 375 nm was determined using a Beckman Coulter DU 730 UV/Vis Spectrophotometer (Indianapolis, IN).

2.4 Calcein Release due to Daptomycin from Liposomal Bacterial Analogues

In order to characterize the effects of daptomycin on antibiotic resistant bacterial cells, bacterial analogues were formed using various lipid compositions. It was determined to vary the PG concentration and the rigidity of the liposomal bacterial analogues; the lipids for the bacterial analogues were determined based upon bacterial CM analysis [11]. As a result, DOPC:DOPG, DOPC:DSPG, DSPC:DOPG, and DSPC:DSPG liposomes were each formed at mole ratios of 90:10, 40:60, and 10:90. All of the liposomes were formed

via the thin film hydration method. In this method, lipids are dried in a Buchi Rotovapor R-124 (Flawil, Switzerland) under vacuum. The resultant lipid film is then subsequently dried under a nitrogen stream for two minutes. All of the lipid films were then reconstituted with 1mL of calcein solution at self-quenching concentrations (55 mM, pH 7.4). The liposomal solution is then annealed for two hours at 60°C. The annealed samples are then extruded through two stacked polycarbonate filters twenty one times through 100 nm pore sizes. Following extrusion, the liposomes are passed through a G-50 size exclusion chromatography (SEC) column to collect the liposomal fractions. The vesicles are suspended in 20 mM HEPES buffer at pH 7.4. The Q_{max} of the liposomes was determined before beginning the experiment. In order to do so, the emission of calcein at 515 nm (excitation: 495 nm) was measured using a spectrophotometer, Horiba Scientific Fluromax-3 (Edison, NJ) by taking an aliquot of the liposomes. The emission of calcein for the liposomes is measured before and after adding 50 µL of 5% Triton X-100. The Q_{max} is calculated using Figure 1.

$$\frac{I_{pre-triton}}{I_{post-triton}} = Q_{max} \text{ or } Q_t$$

Figure 1: Equation to calculate the Qmax or Qt

According to David Jung and group [12], daptomycin undergoes two conformational changes in the presence of calcium ions: one in just the presence of calcium ions and then another one before interacting with PG lipid headgroups. As such, before introducing daptomycin to the liposomal bacterial analogues, the daptomycin was preincubated with calcium ions at 37°C for 1 hour. Then, liposomes were added to seven samples containing preincubated daptomycin and calcium ions. The samples had a final liposome concentration of 180 µM and calcium ion concentration of 1.25 mM. Each of the seven

experimental samples had a different final concentration of daptomycin at 0, 2, 4, 8, 16, 24, and 32 mg/L (0, 1.23, 2.47, 4.94, 9.80, 14.82, and 19.76 µM respectively). The control samples had the same concentration of calcium ions and daptomycin as the experimental samples, but did not have liposomes. The experimental and control samples are further incubated at 37°C. Then, at 0.1, 1, 2, 3 and 6 hours, the emission of calcein for aliquots from each of the experimental and control samples were measured. Following each emission measurement, 50 µL of 5% Triton X-100 was added to the samples. For each time point, Figure 1 was used to calculate the Q_t. The relative calcein quenching efficiency was then calculated using Figure 2.

$$\frac{Q_t - 1}{Q_{max} - 1} \; x \; 100 = Relative \; Calcein \; Quenching$$

Figure 2: Equation to calculate the relative calcein quenching efficiency

2.5 Determination of Zeta Potential of Liposomal Bacterial Analogues

The zeta potentials for all of the liposomal bacterial analogues from Section 2.4 were determined using a Zetasizer NanoSeries (Malvern Instruments Ltd.).

2.6 Forming Giant Unilamellar Vesicles of the Liposomal Bacterial Analogues

According to Yen-Fei Chen and group [8], it was possible to microscopically observe the effects that daptomycin had on liposomes by using Giant Unilamellar Vesicles (GUVs). GUVs composed of a mole ratio of 70:30 DOPC:DOPG with 0.5% FAST DiI and 70:30 DOPC:DSPG with 0.5% DiI were formed according to the gentle hydration method [13].

2.7 Imaging the Giant Unilamellar Vesicles

The GUV's were observed using the Olympus IX 70 inverted fluorescent microscope (Olympus America Inc., PA) with a 20x objective. The GUV's were imaged using

Qimaging Retiga 1300 Camera (Surrey, BC, Canada) and analyzed using the Basic Metamorph (7.5.5.0) software (Downingtown, PA).

2.8 Lipid Extracting Effect on Liposomal Bacterial Analogues

GUVs were suspended in 5.0 mM PIPES buffer (50 mM KCl, 1.0 mM EDTA, pH 7.4). After aspirating a single GUV with a CellTram Air/Oil/vario microinjector (Selangor, Malaysia), the GUV was moved to a higher plane in order to reduce background noise. Then, preincubated daptomycin and calcium were added to the buffer with the GUV to get a final concentration of 10 μM daptomycin and 1.25 mM calcium ions. Pictures of the GUV were taken every 30 seconds.

Section 3: Results

3.1 Killing Curve of S. aureus with Daptomycin

The killing curve of *S. aureus* in response to various concentrations of daptomycin can be seen in Figure 3. The concentrations of daptomycin used were 8, 4, 3.6, 3.2, 2.8, 2.4, 2, 1, 0.5, 0.25, and 0.125 mg/L. The higher the absorbance, the denser the bacterial growth is. The sigmoidal line plotted in Figure 3 is an approximate of the killing curve. It appears that the minimum inhibitory concentration (MIC) of daptomycin for this particular strain of *S. aureus* is around 2.6 mg/L, similar to the MIC reported in other studies [4,14]. The MIC is the lowest concentration of an antibacterial drug that will inhibit the visible growth of bacteria after an overnight incubation. The sigmoidal curve fits the data extremely well ($R^2 = 0.9962$).

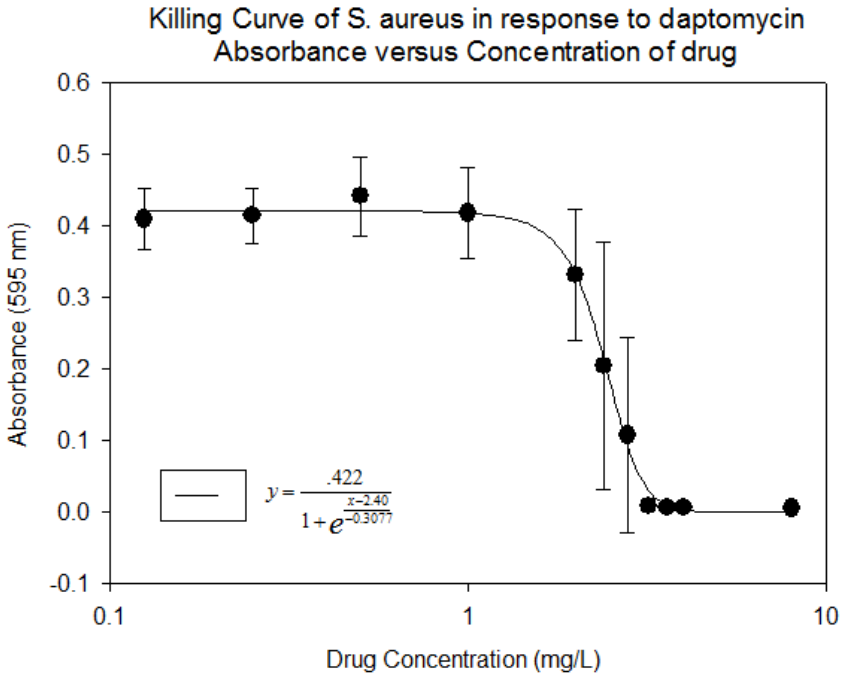

Figure 3: The Killing Curve of S. aureus.

The data represent the arithmetic mean of 3 independent killing studies and the error bars represent the standard deviations.

3.2 Standard Curve of Daptomycin

The standard curve of daptomycin is shown in Figure 2. The approximate equation describing the standard curve is also shown. By measuring the UV absorbance at 375 nm of a solution containing daptomycin, it is possible to use the provided equation to calculate the daptomycin concentration. The standard curve fits the data extremely well (R^2 = .9994).

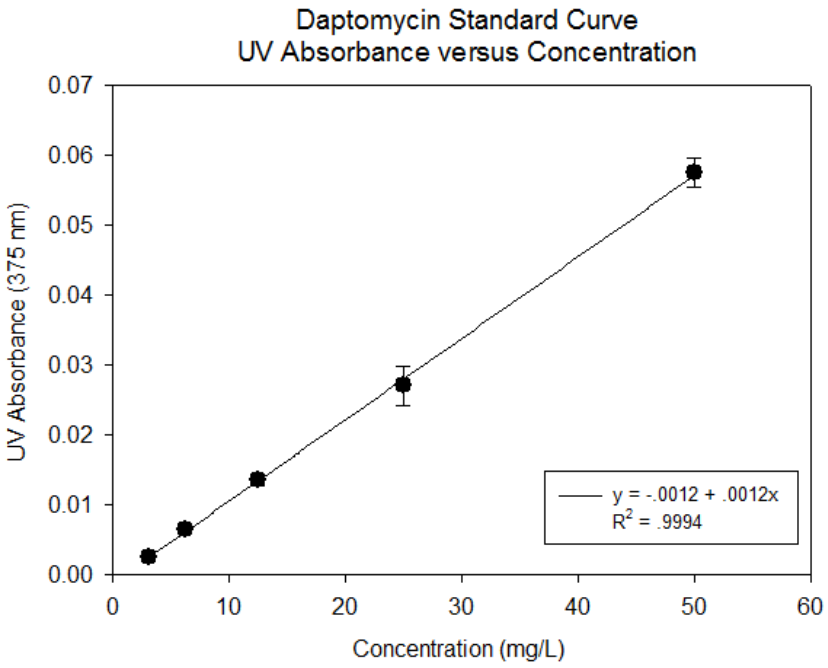

Figure 4: The Standard Curve for Daptomycin

The data represent the arithmetic mean of 2 independent standards and the error bars represent the standard deviations.

3.3 Calcein Release due to Daptomycin from Liposomal Bacterial Analogues

As previously mentioned, there are two theoretical methods of bacterial resistance to daptomycin. In one scenario, the gram positive bacteria lysinylates the PG headgroups; doing so reduces the negative charge on the CM and reduces the affinity for calcium ions, which are needed for daptomycin to insert itself into the CM. In the other scenario, a bacterium can change the relative rigidity of its cytoplasmic membrane. By alternating

the percentage of PG in the liposomal bacterial analogues, it is possible to simulate the lysinylation of PG headgroups; lower percentages of PG in the membrane are analogous to a decreased number of active PG headgroups. In fact, there is evidence to show that the amount of PG in a CM is a defining characteristic of whether daptomycin will work or not [4]. By changing the PG lipid from DOPG to DSPG and also by changing the PC lipid from DOPC to DSPC, it is possible to vary the rigidity of the liposomes. The phase transition temperature for DOPC is -17ºC, for DOPG is -18ºC, for DSPC is 55ºC, and for DSPG is 55ºC [15]**.** The drug to total lipid mole ratio and the drug to PG lipid mole ratio is displayed below in Table 1. These ratios are the same for Figures 5-8.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
Drug:PG lipid 1:9 Compositions	0	6.17×10^{-3}	1.24×10^{-2}	2.47×10^{-2}	4.94×10^{-2}	9.88×10^{-2}	1.98×10^{-1}
Drug:PG lipid 4:6 compositions	0	4.12×10^{-3}	8.23×10^{-3}	1.65×10^{-2}	3.29×10^{-2}	6.59×10^{-2}	1.32×10^{-1}
Drug:PG lipid 9;1 compositions	0	6.86×10^{-4}	1.37×10^{-3}	2.74×10^{-3}	5.48×10^{-3}	1.10×10^{-2}	2.20×10^{-2}
Drug:Total Lipid	0	6.86×10^{-3}	1.37×10^{-2}	2.74×10^{-2}	5.48×10^{-2}	1.10×10^{-1}	2.20×10^{-1}

Table 1: Drug to total lipid and drug to PG lipid ratios for all samples in Figures 5-8

The time dependent release of calcein from all of the liposomal constructs can be found in the Supporting Information (Section 7.1). Figure 5 shows the effect that daptomycin has on DOPC:DOPG liposomes with varying PG lipid. This composition will be a fluid at room temperature.

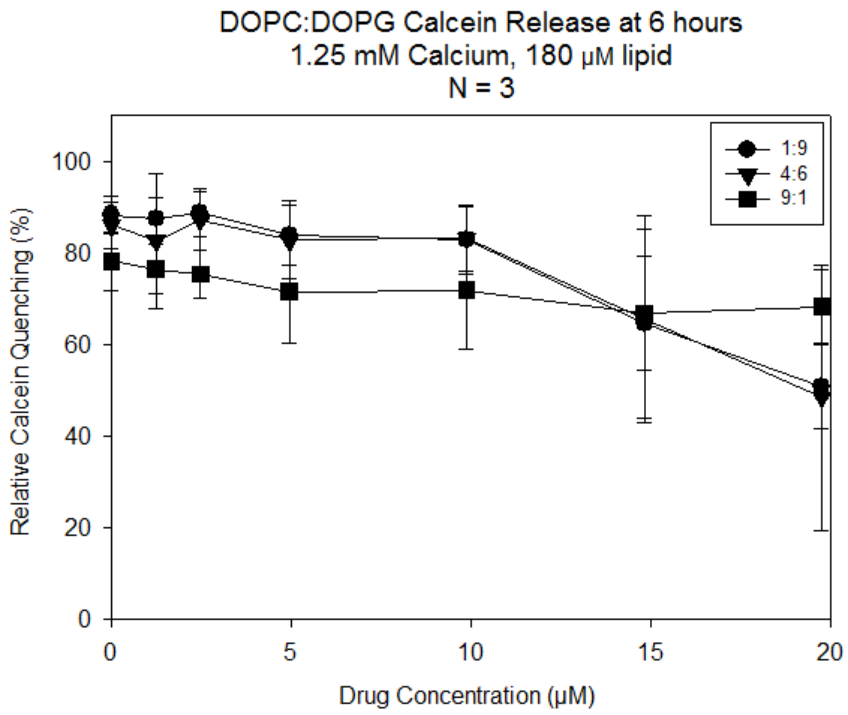

Figure 5: The effects of daptomycin on DOPC:DOPG liposomes.

The data represent the arithmetic mean of 3 independent liposome preparations and the error bars represent the standard deviations.

In Figure 5, all liposome compositions are in the fluid phase. The liposomes with the higher PG content (4:6 DOPC:DOPG and 1:9 DOPC:DOPG) show a trend for increased calcein release as the daptomycin concentration increases; however, the data points are not statistically significant. It was noted that the standard error of the data gets larger as the drug concentration increases, but this may just be an experimental anomaly due to outliers. Figure 6 shows the effect that daptomycin has on DOPC:DSPG liposomes with varying PG lipid. Depending on the mole ratio of the lipids, this composition will have fluid and rigid domains at room temperature.

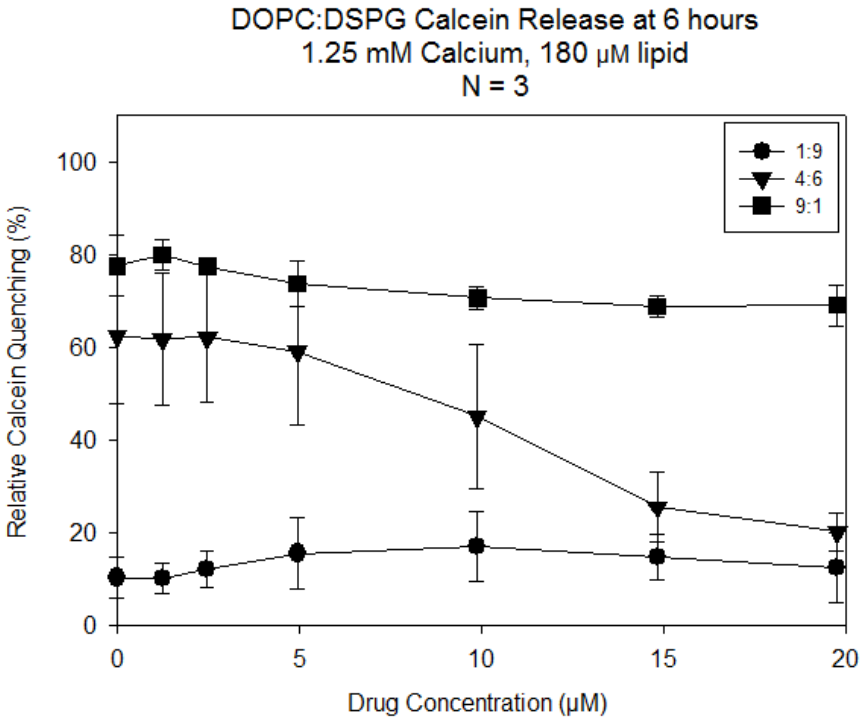

Figure 6: The effects of daptomycin on DOPC:DSPG liposomes.

The data represent the arithmetic mean of 3 independent liposome preparations and the error bars represent the standard deviations.

In Figure 6, the liposomes with the highest PG content (1:9 DOPC:DSPG) shows the greatest calcein release and the liposomes with the lowest PG content (9:1 DOPC:DSPG) shows the lowest calcein release. There is also an intermediate curve representing the 4:6 DOPC:DSPG liposomes. As daptomycin concentration increases, calcein release increases for all liposomal formulations. Figure 7 shows the effect that daptomycin has on DOPC:DSPG liposomes with varying PG lipid. Depending on the mole ratio of the lipids, this composition will have fluid and rigid domains at room temperature.

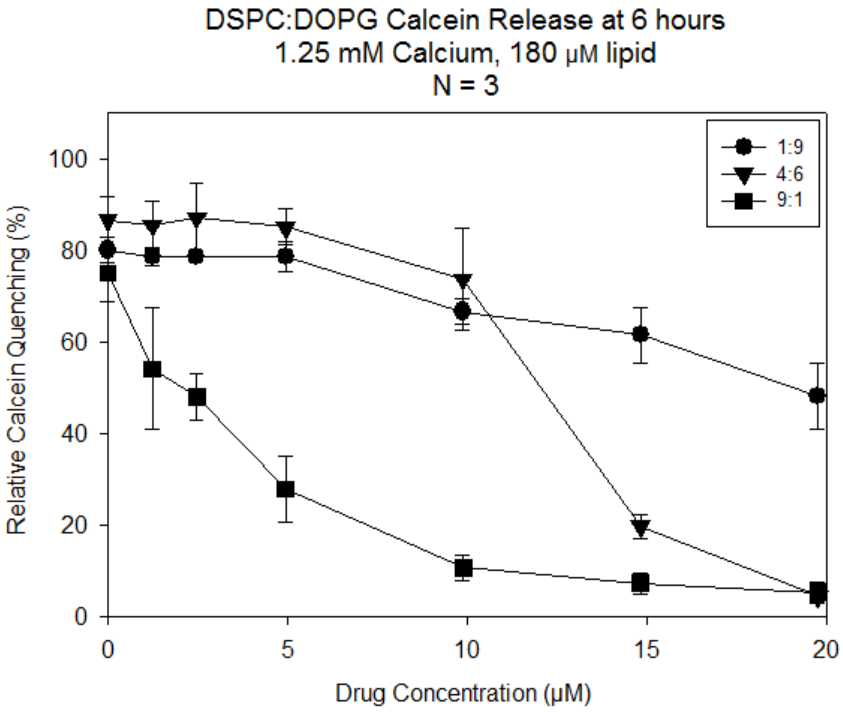

Figure 7: The effects of daptomycin on DSPC:DOPG liposomes.

The data represent the arithmetic mean of 3 independent liposome preparations and the error bars represent the standard deviations.

Like the liposomes from Figure 6, the liposomes from Figure 7 exhibit two extremes and an intermediate release profile. However, the behaviors of the liposomes in Figure 7 nicely contrast that of the liposomes in Figure 6. Figure 8 shows the effect that daptomycin has on DSPC:DSPG liposomes with varying PG lipid. This composition will be in a gel phase at room temperature.

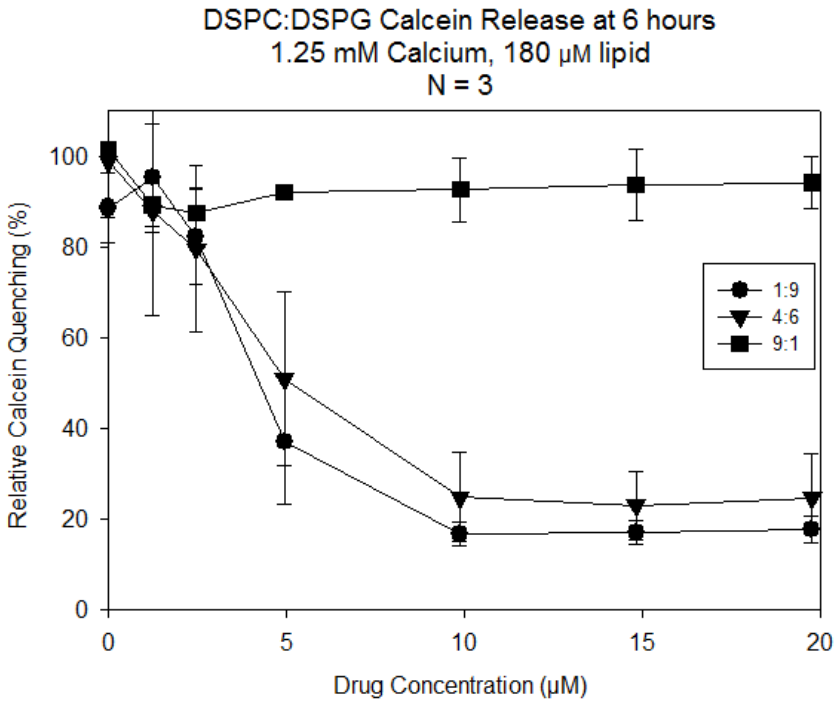

Figure 8: The effects of daptomycin on DSPC:DSPG liposomes.

The data represent the arithmetic mean of 3 independent liposome preparations and the error bars represent the standard deviations.

From Figure 8, the higher the concentration of DSPG, the more relative calcein quenching that was observed. Independent groups t-Test between means to analyze the significance amongst the data in Figures 5-8 can be found in the Supporting Information (Section 7.2).

3.4 Determination of Zeta Potential of Liposomal Bacterial Analogues

In order to objectively show that the charge on the CM affects the interaction of daptomycin with the lipid headgroups, the zeta potentials of the liposomal bacterial analogues were determined. Table 1 shows that as the anionic PG lipid concentration is lowered, the overall zeta potential becomes less negative.

	1:9 DOPC:DOPG	4:6 DOPC:DOPG	9:1 DOPC:DOPG
Average ± Std. Dev.	-39.4 ± 0.52	-35.8 ± 1.54	-20.84 ± 2.74
	1:9 DOPC:DSPG	4:6 DOPC:DSPG	9:1 DOPC:DSPG
Average ± Std. Dev.	-36.6 ± 1.27	-29.53 ± 0.85	-16.47 ± 1.20
	1:9 DSPC:DSPG	4:6 DSPC:DSPG	9:1 DSPC:DSPG
Average ± Std. Dev.	-40.5 ± 3.16	-35.8 ± 3.22	-19.13± 2.92
	1:9 DSPC:DOPG	4:6 DSPC:DOPG	9:1 DSPC:DOPG
Average ± Std. Dev.	-38.6 ± 1.46	-33.4 ± 2.58	-15.28± 1.72

Table 2: Relative zeta potentials for liposomal bacterial analogues.

The data represent the arithmetic mean of 3 independent liposome preparations and the error bars represent the standard deviations.

3.5 Forming GUVs of the Liposomal Bacterial Analogues

By making GUVs of the bacterial analogues, it becomes possible to microscopically view the effect that daptomycin has on the lipid membrane. Both 7:3 DOPC:DOPG and 7:3 DOPC:DSPG GUVs were formed.

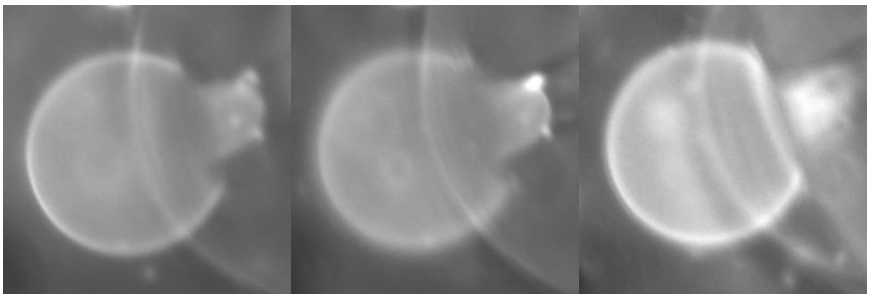

Figure 9: 7:3 DOPC:DOPG GUVs at time = 0 mins.

These three images are all of the equatorial plane of the GUV.

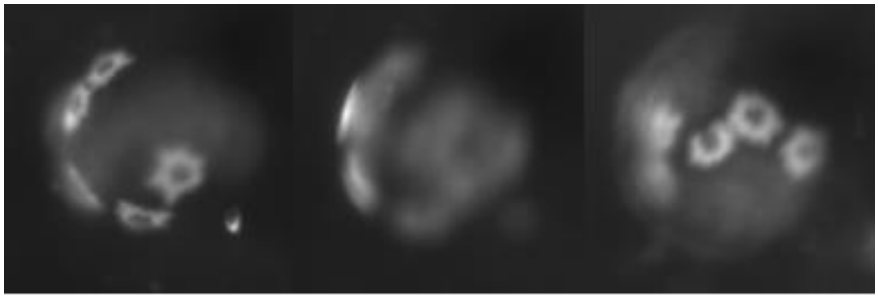

Figure 10: 7:3 DOPC:DSPG GUVs at time = 0 mins.

These images are, respectively, from a lower plane, the equatorial plane, and a higher plane of the GUV.

3.6 Lipid Extracting Effect on Liposomal Bacterial Analogues

One possible mechanism of action for daptomycin involves extracting the PG lipid membrane. This can be visualized by the appearance of bright localized features on the GUV as time progresses. These bright spots can be visualized in Figure 11 for 7:3 DOPC:DOPG and also in Figure 12 and Figure 13 for 7:3 DOPC:DSPG.

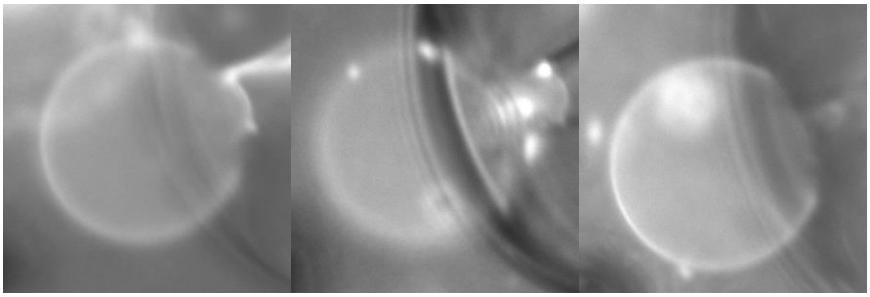

Figure 11: 7:3 DOPC:DOPG GUVs at (from left to right) time = 2 mins, 4.5 mins, 6.5 mins.

All images are from the equatorial plane of the GUV.

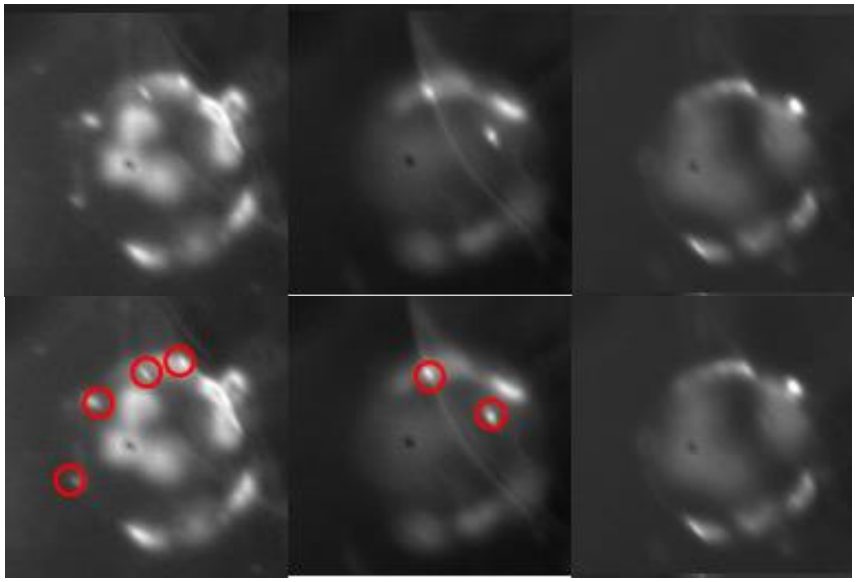

Figure 12: 7:3 DOPC:DSPG GUVs at 3 minutes.

The two rows of images are the same with the bottom row of images marked with lipid extraction sites (red circles). From left to right for both rows, the images were taken from the lower plane, equatorial plane, and the higher plane of the GUV.

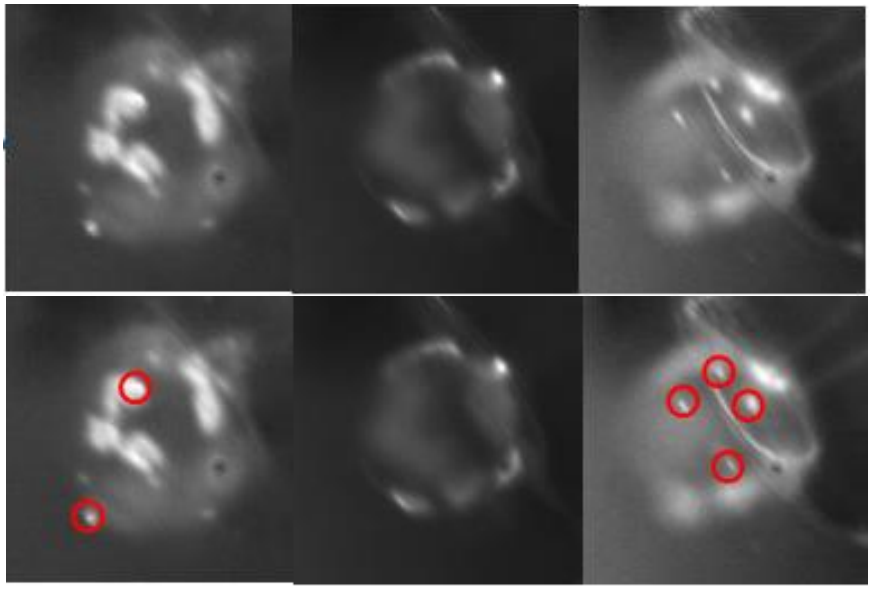

Figure 13: 7:3 DOPC:DSPG GUVs at 3.5 minutes.

The two rows of images are the same with the bottom row of images marked with lipid extraction sites (red circles). From left to right for both rows, the images were taken from the lower plane, equatorial plane, and the higher plane of the GUV.

Section 4: Discussion

The present threat of antibiotic resistant bacteria is not one to be taken lightly. Logistically, we cannot keep up with the natural adaptation of these bacteria by producing newer drugs that can work effectively against these bacterial strains. Even a species like *S. aureus* employs multiple mechanisms of resistance and so a drug that seems to promise bacteriocidality may prove useless once a particular bacterial species develops resistance to that drug. Therefore, it is important to study the bacterial mechanisms of resistance and develop strategies to combat these antibiotic resistant bacteria with existing drugs and technologies. The findings of our study may aid in the development of treatments for antibiotic resistant bacteria.

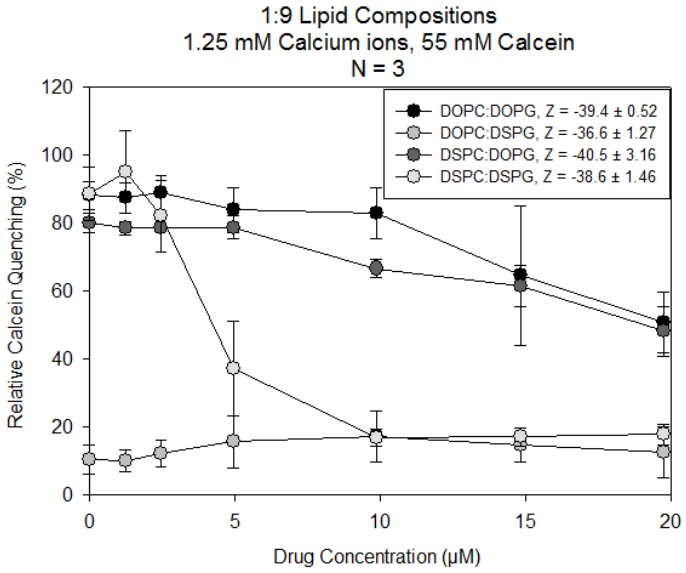

Figure 14: Effect of Daptomycin on 1:9 Liposome Compositions at 6 hours; Z = zeta potential

In general, upon ±comparing Figures 5-8, the more daptomycin that is added to the samples, the more calcein release there is. Additionally, it becomes obvious that daptomycin is more membrane active on more rigid membranes containing DSPG than DOPG; figures 17-19 (Supporting Information Section 7.3) supports this finding. This result also corroborates clinical data that shows daptomycin resistant *S. aureus* develops CM fluidity *in vivo* [16,17]. Alternatively, Mishra and group discovered that *S. aureus* modifies their CM to be more rigid when treated with cationic

antibiotic peptides (CAPs) [10]. Therefore, it may be possible to treat CAP resistant *S. aureus* with daptomycin. Since the mechanism of resistance for CAP's causes *S. aureus*'s membrane to become more rigid, it may be possible to use daptomycin to more effectively treat these CAP resistant bacteria since daptomycin appears to have more activity on rigid membranes.

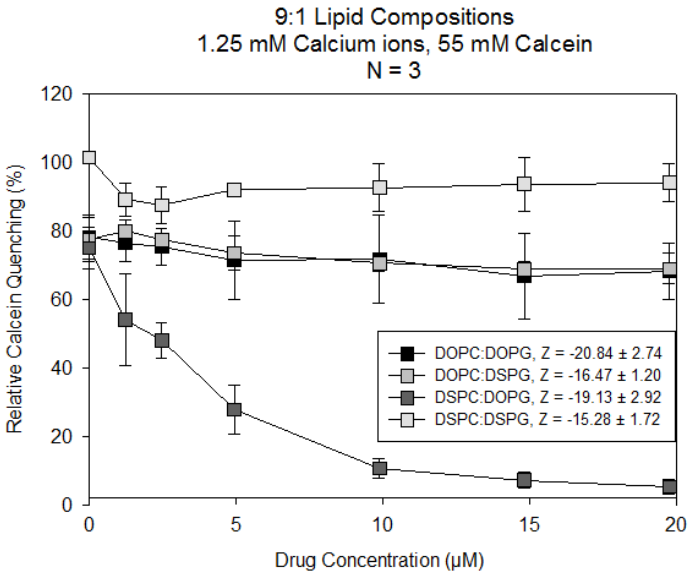

Figure 15: Effect of Daptomycin on 9:1 Liposome Compositions at 6 hours; Z = zeta potential

Figures 14-16 are plotted based upon the relative zeta potentials of all the different liposomal compositions, found in Table 2, in order to examine the role of zeta potential in the release of calcein. Theoretically, as zeta potential becomes more negative (as in the case of 1:9 compositions versus 9:1 compositions), there should be an increase in the amount of daptomycin that binds to the liposomal membranes. However, upon comparing figures 14-16, there does not appear to be any significant trends that would

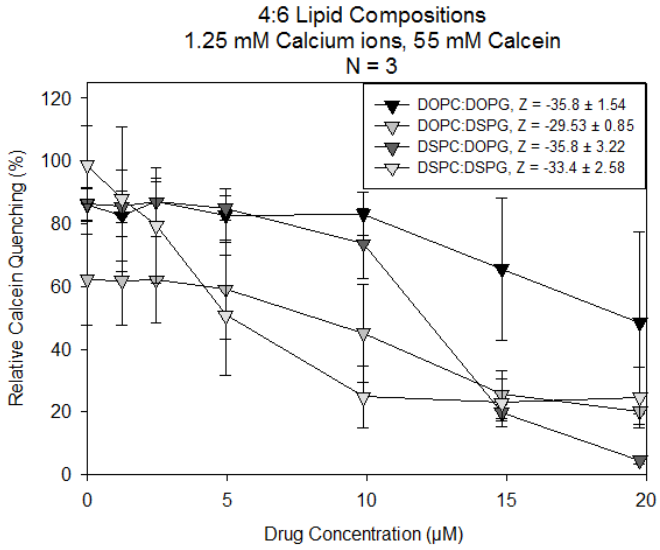

Figure 16: Effect of Daptomycin on 4:6 Liposome Compositions at 6 hours; Z = zeta potential

indicate that daptomycin activity increases due to a more negative zeta potential. Additionally, it is apparent that even when zeta potential is held constant, there doesn't appear to be any consistent trend with calcein release. As a result, while electrostatics does impact the mechanism of action of daptomycin, the release of liposomal contents seems to have little to do with membrane zeta potential.

The lipid extraction effect in Figure 11 corroborates results from previous studies [8]. In our additional experiments, we found that DOPC:DSPG also exhibited lipid extraction. In Figures 12 and 13, it is evident that the lipid extraction is occurring from the liquid ordered phase [18,19]. The DOPC:DSPG GUVs have three different phases as evidenced by the radial gradient caused by the partitioning of the DiI. There is a dark fluid phase corresponding to the DOPC lipids, a liquid ordered phase, corresponding to both DOPC and DSPG and another dark phase, corresponding to the tight packing DSPG lipids. It is possible that there is lipid extraction from the tightly packed DSPG phase, but since the DiI did not partition in this region, it is not possible to conclude. However, lipid extraction did occur in both DOPC:DOPG and DOPC:DSPG GUV's, but the two compositions exhibited different amount of calcein release as liposomes (Figures 5-8). In similar experiments with DOPC:DOPG GUVs encapsulated with Texas Red sulfonyl chloride, there was little to no release of the dye in the presence of daptomycin [8]. So lipid extraction does not solely explain why daptomycin causes molecular leakage. However, the mechanism for molecular leakage may be due in part to the presence of rigid domains. From our results, it appears that daptomycin is extracting from the liquid ordered phase of the lipid membranes. This liquid ordered phase is at the interface of the rigid and fluid membranes where there are an increased line tensions caused by the

curvature of the membranes. As a result, extraction may cause increased instability at this interface, causing molecular leakage. This could explain why there is the most calcein release from the mixed liposomal compositions (Figures 6 and 7). However, this theory needs to be further evaluated.

Our study also elucidates another way to study bacteria; by simplifying bacterial CM into liposomal analogues in the form of GUVs, it was possible to microscopically study the mechanism of action of daptomycin in real time. This process can be applied to other bacteria and antibiotics. In fact, such a process may help to quickly validate the efficacy of certain antibiotics on drug resistant bacteria. This method may also enable other researchers to better understand current methods of resistance and quickly study newer drug resistances. Understanding such a complex resistance pathway may also aid in the development of better drugs.

Despite the findings in this study, one inherent problem in treating antibiotic resistant bacteria is the presence of the cell wall of these bacteria. While it may be beneficial to bypass the cell wall using antibiotics like penicillin, bacterial resistances to even these antibiotics are prevalent. The killing curve presented in Figure 3 shows that the MIC for *S. aureus* was approximately 2.6 mg/L. Although the MIC will increase due to daptomycin resistance [20], by encapsulating daptomycin into lipid nanocarriers, it may be possible to deliver high concentrations of daptomycin to a bacterial site, overcoming the increased MIC.

Section 5: Conclusion

Through the systematic analysis of daptomycin on liposomal bacterial analogues, it was possible to quickly study the effect of the antibiotic. By using the bacterial liposomal analogues and the GUVs, it was easier to simplify the complex mechanisms of drug resistance and focus on a few variables at a time. As a result, it was possible to gain insight into the mechanisms of resistance of daptomycin resistant bacteria. Our results show that daptomycin is more effective on membranes that are more rigid. Additionally, there is preliminary evidence to suggest that rigidity or fluidity may be the stronger contributing factor towards daptomycin resistance than zeta potential. Lastly, knowing this information, it is possible to develop new engineering methods to combat the emergence of antibiotic resistant bacteria.

Section 6: References

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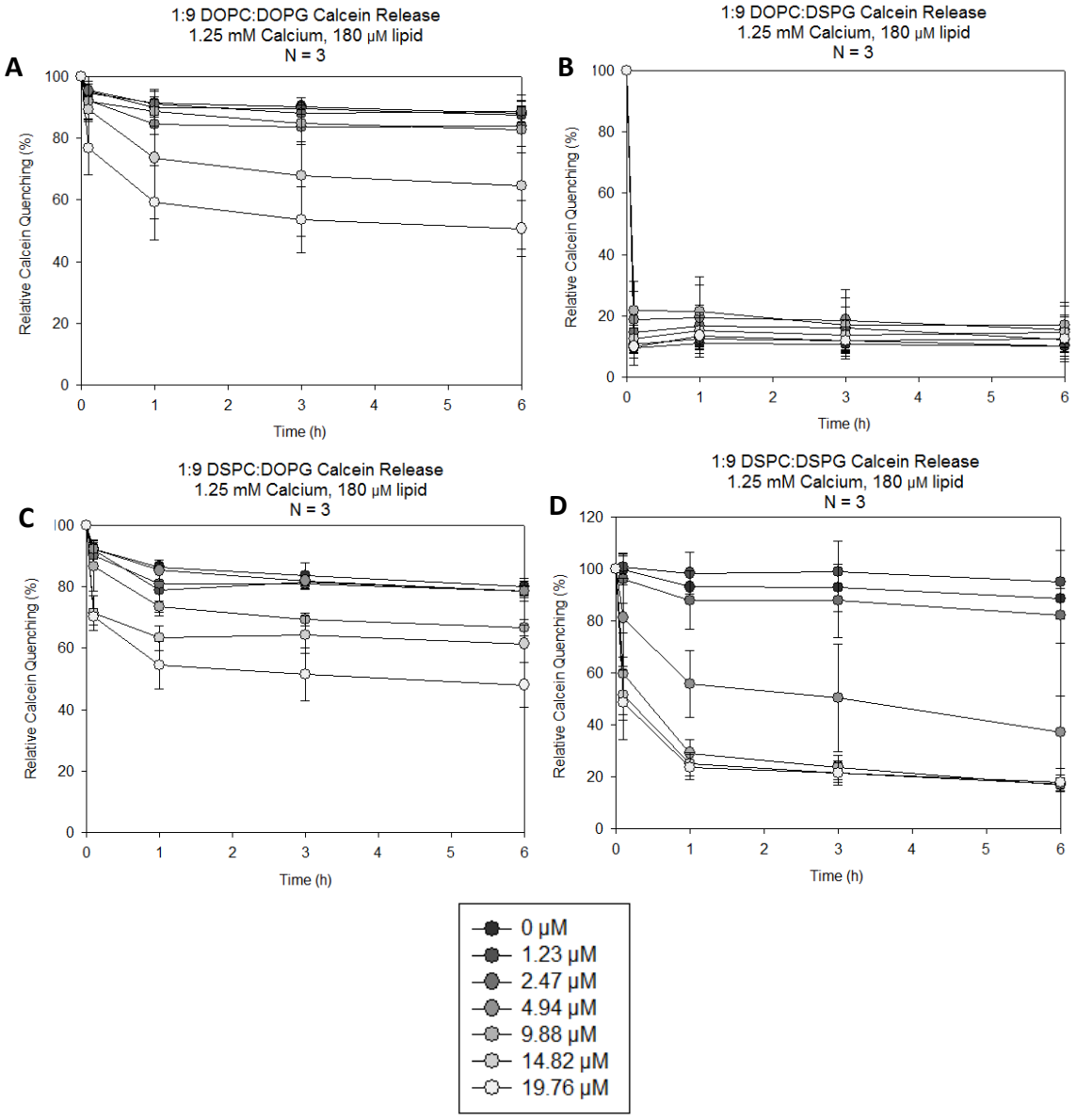
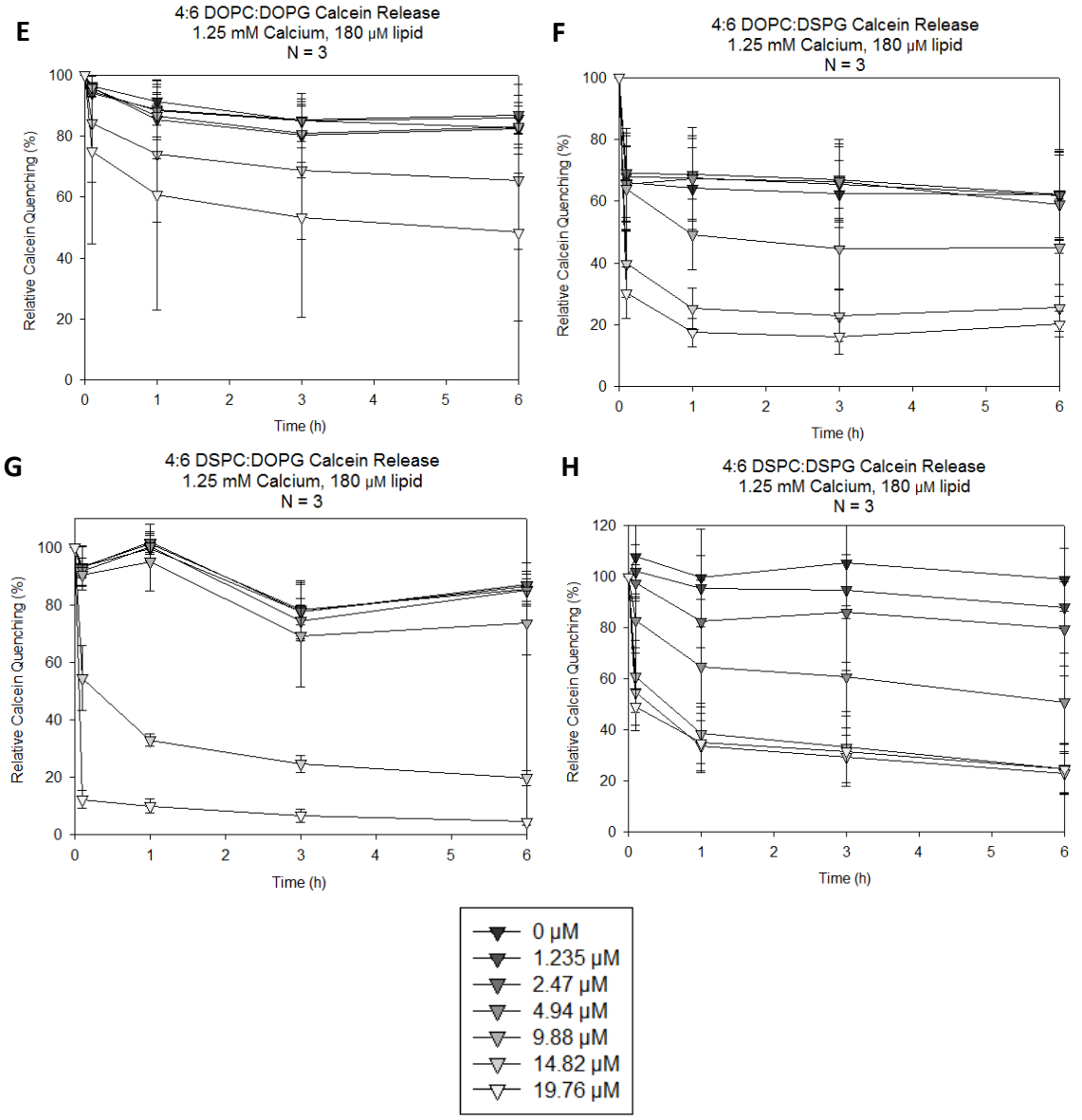
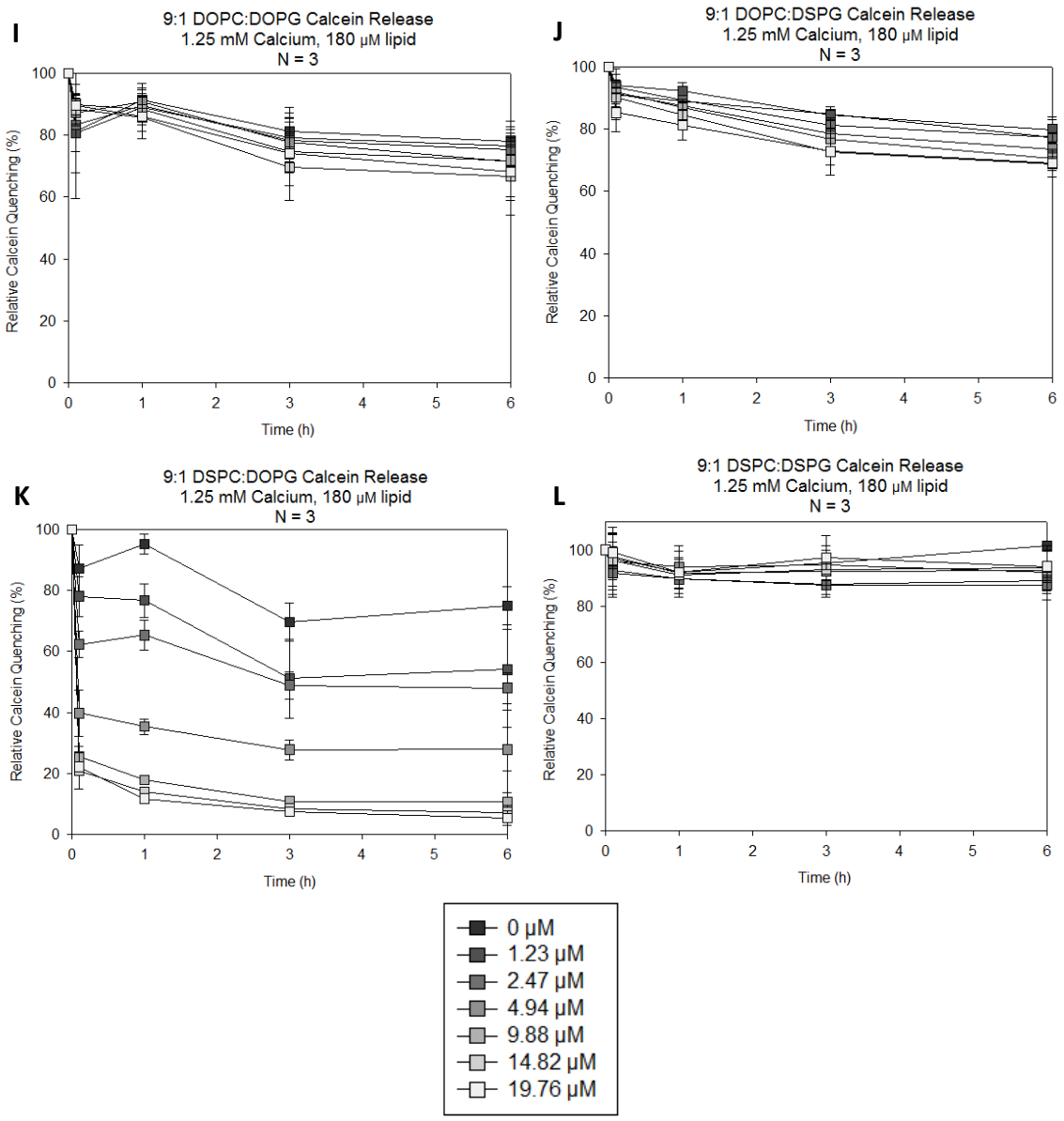
Section 7: Supporting Information

7.1 Calcein Release from Liposomal Bacterial Analogues over time




E
4:6 DOPC:DOPG Calcein Release
1.25 mM Calcium, 180 μM lipid
N = 3
Relative Calcein Quenching (%)
Time (h)
F
4:6 DOPC:DSPG Calcein Release
1.25 mM Calcium, 180 μM lipid
N = 3
Relative Calcein Quenching (%)
Time (h)
G
4:6 DSPC:DOPG Calcein Release
1.25 mM Calcium, 180 μM lipid
N = 3
Relative Calcein Quenching (%)
Time (h)
H
4:6 DSPC:DSPG Calcein Release
1.25 mM Calcium, 180 μM lipid
N = 3
Relative Calcein Quenching (%)
Time (h)
0 μM
1.235 μM
2.47 μM
4.94 μM
9.88 μM
14.82 μM
19.76 μM



Figures A-L show the release of calcein over a time period of 6 hours in the presence of various concentrations of daptomycin. This is shown for the 1:9, 4:6, and 9:1 DOPC:DOPG, DOPC:DSPG, DSPC:DOPG, and DSPC:DSPG liposomal compositions.

7.2 Independent groups t-Test between means for Figures 5-8

The null hypothesis for Tables 3-6 are to set the means of the 1:9 liposomes equal to the means of 4:6 liposomes, the means of 1:9 liposomes equal to the means of 9:1 liposomes,

and the means of 1:9 liposomes equal to the means of 9:1 liposomes. Subsequent t-Test analysis determined the significance of the null hypothesis.

Null Hypothesis	0 μM	1.23 μM	2.47 μM	4.94 μM	9.88 μM	14.82 μM	19.76 μM
1:9 = 4:6	NS	NS	NS	NS	NS	NS	NS
1:9 = 9:1	NS	NS	S	NS	NS	NS	NS
4:6 = 9:1	NS	NS	NS	NS	NS	NS	NS

Table 3: DOPC:DOPG liposomes t-Test analysis

NS = not significant (p > 0.05) and S = significant (p <0.05)

Null Hypothesis	0 μM	1.23 μM	2.47 μM	4.94 μM	9.88 μM	14.82 μM	19.76 μM
1:9 = 4:6	S	S	S	S	S	NS	NS
1:9 = 9:1	S	S	S	S	S	S	S
4:6 = 9:1	NS	NS	NS	NS	S	S	S

Table 4: DOPC:DSPG liposomes t-Test analysis

NS = not significant (p > 0.05) and S = significant (p <0.05)

Null Hypothesis	0 μM	1.23 μM	2.47 μM	4.94 μM	9.88 μM	14.82 μM	19.76 μM
1:9 = 4:6	NS	NS	NS	NS	NS	S	S
1:9 = 9:1	NS	S	S	S	S	S	S
4:6 = 9:1	NS	S	S	S	S	S	NS

Table 5: DSPC:DOPG liposomes t-Test analysis

NS = not significant (p > 0.05) and S = significant (p <0.05)

Null Hypothesis	0 μM	1.23 μM	2.47 μM	4.94 μM	9.88 μM	14.82 μM	19.76 μM
1:9 = 4:6	NS	NS	NS	NS	NS	NS	NS
1:9 = 9:1	S	NS	NS	S	S	S	S
4:6 = 9:1	NS	NS	NS	S	S	S	S

Table 6: DSPC:DSPG liposomes t-Test analysis

NS = not significant ($p > 0.05$) and S = significant ($p < 0.05$)

7.3 Relative calcein quenching versus rigidity of liposomes

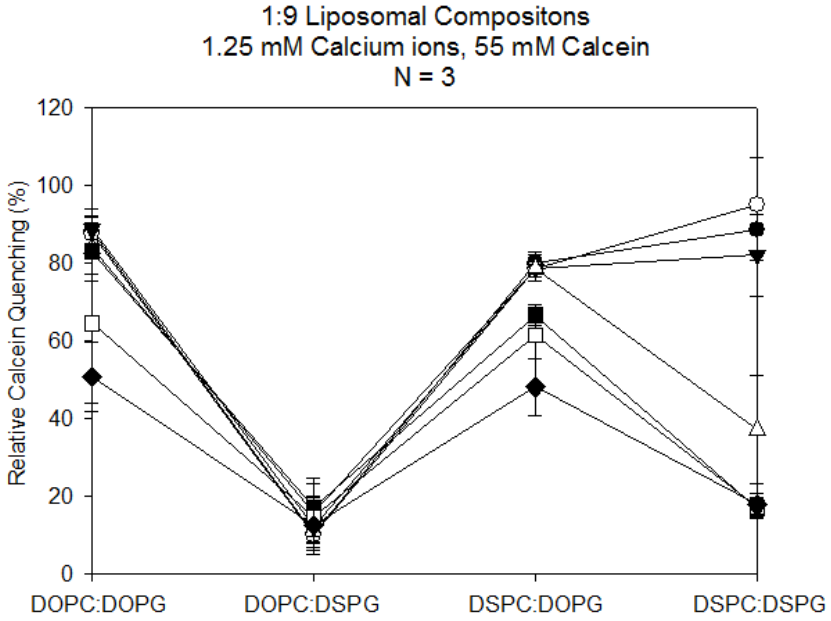


Figure 17: Liposomal Rigidity and Fluidity versus Relative Calcein Quenching (1:9 liposomes)

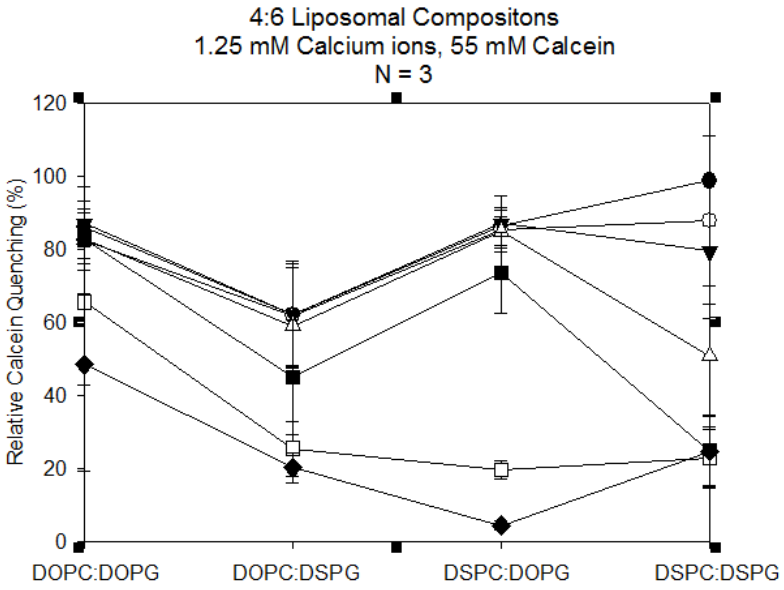


Figure 18: Liposomal Rigidity and Fluidity versus Relative Calcein Quenching (4:6 liposomes)

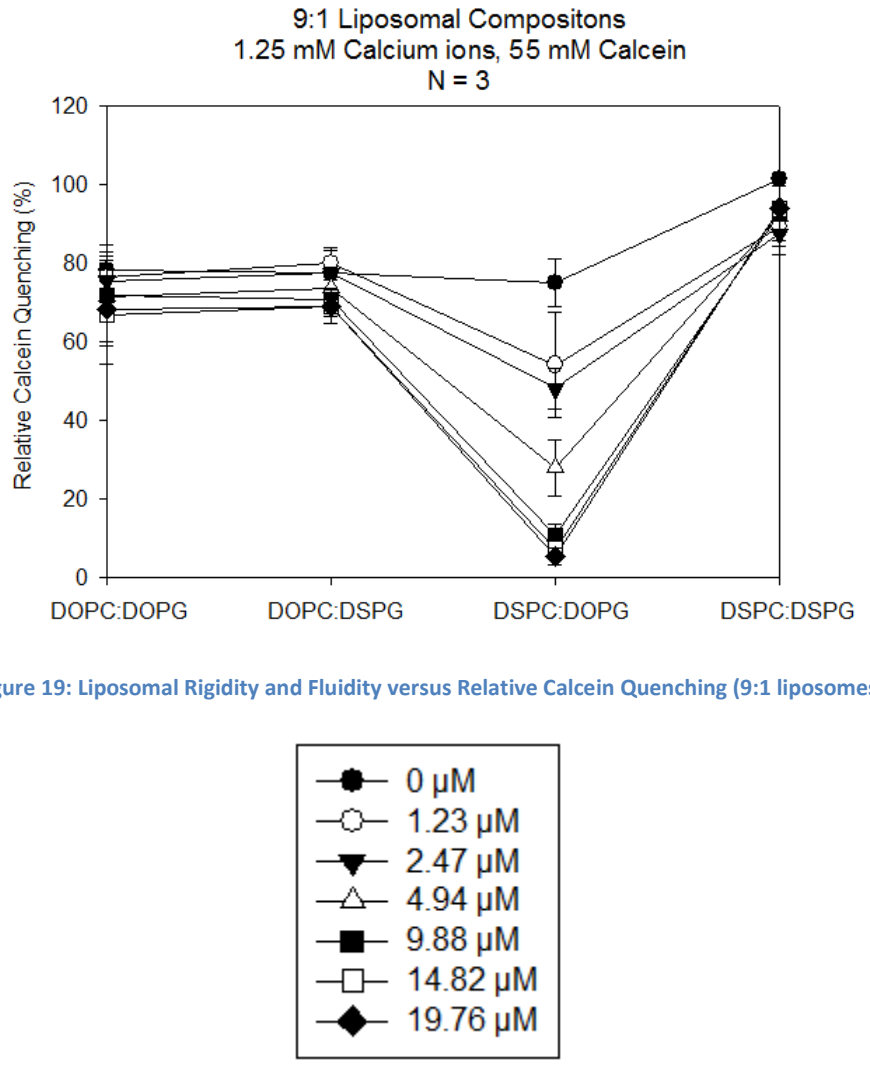


Figure 19: Liposomal Rigidity and Fluidity versus Relative Calcein Quenching (9:1 liposomes)

Figures 17 – 19 are plotted according to the rigidity of the liposomes and the relative calcein quenching. The legend above corresponds to the various drug concentrations used in the experiments.