TRIGGERED RELEASE OF VANCOMYCIN TO BACTERIAL INFECTION SITES USING pH-SENSITIVE LIPID BASED NANOPARTICLES

By

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ABSTRACT OF THE THESIS

Triggered release of Vancomycin to bacterial infection sites using pH-sensitive lipid based nanoparticles

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The appearance of resistant strains of bacteria in community healthcare facilities is a common occurrence with growing severity. Cases of resistance to β-lactam drugs such as Methicillin have been shown in *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermis* (MRSE), among many others, and these resistances limit therapeutic options. The synthetic glycopeptide antibiotic Vancomycin is considered one of the last lines of defense for these types of resistant infections. Failures in antibiotic therapy at this stage come from inadequate drug concentration at the infected sites, reduction of activity due to local acidity, and toxicity associated with accumulation in non-infected tissue. To solve these issues, an environmentally-responsive lipid-based nanoparticle, or liposome, has been developed to deliver Vancomycin to local infection sites. These liposomes retain their drug contents at physiologic pH, increasing antibiotic circulation time. Additionally, they are selectively triggered to release their drug contents by the external stimulus of decreased pH of local infection sites. Encapsulation of Vancomycin in these liposomes was performed, showing stable retention and release between pH 7.4 and 5.5, respectively. Additionally, demonstration of the enhanced antibiotic activity of the pH-triggered nanoparticles was carried out through Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) studies. From the results, there is promising data suggesting that targeted delivery of Vancomycin using environmentally sensitive liposomes is a candidate for sustained and targeted antibiotic therapy in resistant bacterial infections.

ACKNOWLEDGMENTS

I would like to express my sincerest gratitude to my advisor Professor Stavroula Sofou for giving me the opportunity to work in her lab at both the undergraduate and graduate level. I have greatly appreciated her support and have admired her dedication to the work of all graduate students working under her. I would not be completing graduate school if not for her guidance and encouragement. I would also like to thank the members of Dr. Sofou's lab, specifically Charles Zhu, Michelle Sempkowski, Sally Stras, Trevan Locke and Tom Linz for their continued assistance in my graduate research and studies. They have always been available to answer questions and have made me feel like a welcome addition to the lab.

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SECTION 1: INTRODUCTION

Antibiotic resistance has been a growing issue in the treatment of infectious diseases in modern times. While classical antibiotics, such as penicillin and other β-lactams, helped humanity to overcome previously deadly infections, repeated use of these antibiotics has led to the appearance of resistant strains, preventing the effectiveness of antibiotic therapy. These resistant strains of bacteria can range from one resistant cell out of 10^8-10^9 to homogeneous fully resistant colonies, increasing in severity as a result of natural selection of bacteria which have been previously and repeatedly exposed to antibiotics [1]. Community acquired infections originating in hospitals and nursing homes have become a particularly prevalent issue in all hospitals, including those in the developed world. These infections can range from minor skin infections to severe systemic infections leading to toxic shock syndrome and death, and aside from the obvious danger to compromised hospital patients; it is also a costly burden on the healthcare system [2] [3]. Current paradigms of antibiotic therapy are not sustainable, and the field of infectious diseases requires new methods of drug delivery.

Among the growing list of resistant bacterial strains, Methicillin-resistant *Staphylococcus aureus,* or MRSA, stands out as one of the most well known and prevalent community acquired infections. MRSA first appeared in the early 1960s, and has grown to epidemic proportions in community healthcare facilities [4]. Another similar bacterial species which has displayed resistance is *Staphylococcus epidermis*, or MRSE, and this microbe has become responsible for a number of bloodstream infections [5]. As options for antibiotics are limited by resistance, Vancomycin remains as one of the last therapeutic options.

Vancomycin is a synthetic glycopeptide antibiotic which is the main antibiotics used to treat cases of MRSA and MRSE. It is poorly absorbed as an oral formation, so typical administrations

are IV directly into the systemic circulation, with removal occurring via the kidneys within 24 hours [6]. Vancomycin acts by inhibiting penicillin-binding proteins which are responsible for synthesis of the peptidoglycan wall of bacteria [3]. However, resistance to Vancomycin has begun to manifest itself in severe bacterial infections, including MRSA and MRSE. These resistances can be defined by decreased binding affinity to PBP corresponding to inadequate concentration of antibiotic at the infection site [7] [8]. Attempts at increasing doses of Vancomycin have met with concerns of nephrotoxicity and ototoxicity [9] [10]. As a result, doses of free Vancomycin are limited, and prevent aggressive treatment of resistant *Staphylococcal* infections.

In order to circumvent the systemic effects of high Vancomycin doses, novel formulations have been developed in order to encapsulate Vancomycin within nanoparticle formulations and increase circulation time. Additionally, these nanoparticles have also been demonstrated to react to local acidity in order to release internal contents [11] [12] [13] [14] [15]. Use of liposomes as vehicles for nanoparticle encapsulation of chemotherapeutic agents has already been achieved, and has shown increased circulation time by PEGylation of the lipid bilayer as well as environment-specific release of internal contents [16]. Lipids with different head groups and tail lengths can be combined in particular ratios during formulation of liposomes, and decreased pH can result in protonation of a specific lipid head group. This protonation initiates a restructuring of the lipid bilayer into lipid rafts, which in turn enables leakage to occur in the interface of the lipid raft structures [17].

In this proof of concept, it is demonstrated that the concept of pH-mediated lipid based nanoparticle release of drug can be applied to antibiotics. It is demonstrated that Vancomycin can be encapsulated within liposome structures, retained at physiologic pH, and released in

local acidic conditions. Applied to the local acidity observed in bacterial infection sites, it is shown that liposomal Vancomycin can successfully minimize bacterial growth, particularly in decreased pH conditions. This method of delivery could potentially maximize delivery to bacterial infection sites while mitigating the potential adverse effects of high Vancomycin doses.

SECTION 2: MATERIALS AND METHODS

2.1: Materials

Acetonitrile, Sucrose, PBS tablets, Sephadex G-50, Sepharose 4B, Bioreagent grade Vancomycin Hydrochloride from *Streptomyces orientalis*, Hydrochloric acid ACS reagent 37%, and EDTA were purchased from Sigma Aldrich (St. Louis, MO).

Staphylococcus aureus (ATCC#25923) and Staphylococcus epidermis (ATCC#14990) were purchased from ATCC (Manassas, VA).

BD Tryptic Soy Broth (TSB) (Soybean Casein Digest Medium), Hardy Diagnostics Tryptic Soy (TS) Agar (Soybean Casein Digest agar Medium), BD Difco Nutrient Broth, BD Nutrient Agar, Inoculating Loops, and Flat Bottom 96 and 24 Well Plates were purchased from VWR.

1,2-dihenarachidoyl-*sn*-glycero-3-phosphocholine (21:0 PC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phospho-L-serine (DSPS), 1,2-distearoyl-*sn*-glycero-3-phosphate (DSPA), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)] (18PEG), and Cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL).

2.2: Methods

2.2.1: Bacterial Medium and Agar Preparation

Tryptic Soy (TS) broth and #3 Nutrient broth was prepared by dissolving dehydrated broth in distilled water at ratios prescribed in documentation (TS Broth = 30 g/L, Nutrient Broth = 8 g/L). The broth was heated until all dehydrated media was completely dissolved, and then autoclaved for 15 minutes at 121^{o}C.

TS agar and Nutrient agar was prepared by dissolving dehydrated agar in distilled water at a 40 g/L ratio, heating the mixture, and autoclaving while covered. Agar preparations were created and added to petri dishes or well plates 24 hours before use and stored at 37^{o}C.

2.2.2: Preparation of Encapsulation Solution

1 mL of 50 mg/mL Vancomycin Hydrochloride was prepared for each lipid composition by dissolving the Vancomycin powder in DDI H_2O. The pH was adjusted to 7.0 after addition of the Vancomycin using a dilute NaOH solution.

2.2.3: Preparation of Chromatography Columns

Sephadex G-50 columns were prepared by adding 1.86 g powder to 35 mL PBS, adding the volume to the column, and adding buffer until the beads settled. The column was left overnight in order to settle the column and ensure acceptable packing. Six Sepharose 4B Columns were prepared by exchanging the 4B buffer to PBS using vacuum filtration, and then adding 35mL to each column similar to the Sephadex G-50 column. These columns were also left overnight before use. The six columns were each equilibrated with PBS buffer at 7.4, 7.4, 7.0, 6.5, 6.0, and 5.5.

2.2.4: Dehydration/Rehydration Method of Liposome Preparation

The method of liposome preparation was based on a protocol developed by Anderson, K.E. *et al* [14] [18]. Preparations of lipid were dissolved in chloroform, mixed in a glass round bottom flask, and evaporated in a 60^{o} water bath under vacuum using a Buchi Rotavapor R-124 at 200RPM to create a thin film on the bottom of the flask. The film was then rehydrated in 1 mL DDI H_2O and the lipids were left to anneal for one hour in a 60^{o}C water bath. Next, the lipids were sonicated for two minutes using a Branson 1510 bath sonicator, and then sucrose was added to the volume at a 1:1 w:w lipid/sucrose ratio and dissolved. Sucrose was added in order to stabilize the vesicles during lyophilization [19]. The volume was then transferred to a 5mL culture tube, frozen at -80^{o}C, and lyophilized overnight on a VirTis Lyophilizer. The lyophilized lipids were rehydrated using either 1 mL PBS pH 7.4 for non-loaded liposomes or the 1mL Vancomycin solution, yielding a final osmolality of 300mOsm. After approximately 45 minutes of encapsulation at room temperature, the lipid mixture was extruded 21 times through two 100nm Poly-Carbonate membranes in an 80^{o}C water bath. These resulting 100nm-in-diameter liposomes were separated from unencapsulated Vancomycin and smaller lipid micelles by size exclusion chromatography using either Sephadex G-50 or Sepharose 4B columns equilibrated with PBS at pH 7.4. The liposomes were collected in 2 mL total volume, yielding a known concentration of lipid. The size of these liposomes was characterized for both loaded and non-loaded composition using dynamic light scattering (DLS) for average size and polydispersity.

2.2.5: Vancomycin and Non-loaded Liposome Standard Curve

A standard curve for Vancomycin was created by evaluating serial dilutions of Vancomycin in PBS at 7.4 pH with Acetonitrile in a 1:1 PBS:Acetonitrile ratio. Acetonitrile was added in order to lyse liposomes and achieve an accurate measure of Vancomycin in solution. The absorbance of

Vancomycin was quantified using a Beckman Coulter DU-730 UV/Vis Spectrophotometer at 280 nm, plotted as a function of concentration, and a linear curve fit was applied to convert absorbance at 280 nm to Vancomycin concentration.

In order to eliminate potential interference from lipid molecules during spectrophotometer measurements, a standard curve was created to relate lipid concentration to absorption at 280 nm. This standard curve was achieved by serially diluting a known concentration of each empty lipid and measuring absorbance at 280 nm in a 1:1 PBS:Acetonitrile ratio. For each given concentration of liposomes, this curve was used to eliminate absorption associated with empty liposomes.

2.2.6: Encapsulation-Release Studies

For each liposome construct, the final encapsulated lipid volume was separated into five different pH conditions with PBS (7.4, 7.0, 6.5, 6.0, and 5.5) in a total of 2 mL at 37^{o}C for 24 hours. At the end of the time point, liposomes in 1 mL of the parent suspension were separated from released Vancomycin using size exclusion chromatography in a 4B chromatography column. The volume of 2mL was collected, and equal lipid concentrations were measured in a 1:1 PBS:Acetonitrile mixture in the volume before and after purification. The difference in concentration was associated with the amount of Vancomycin leaked in the 24 hour time period.

2.2.7: Bacterial Growth and pH Curve

A frozen stock of *Staphylococcus aureus* was thawed, streaked onto a TS agar plate and incubated overnight at 37^{o}C. A single colony was selected and inoculated into a 5 mL Falcon culture tube, which was incubated in a shaker incubator at 37^{o}C and 220 rpm overnight. A

volume of this stationary phase bacteria was diluted into an Erlenmeyer flask with 50mL TS broth and brought to a 600 nm optical density in the spectrophotometer of 0.001. Measurements of OD600 were obtained every hour, as well as pH of the media at each time point. Similar methods were applied to *Staphylococcus epidermis*, replacing TS broth and TS agar with #3 Nutrient broth and agar, respectively.

2.2.8: MIC Studies

A flat bottom 96 well plate was prepared for MIC studies with serial dilutions of liposomes or free Vancomycin in triplicate. A single bacterial colony was selected and inoculated in 5mL media overnight at 37^{o}C and 220rpm. This was then inoculated into 5mL media, allowed to enter log growth phase (~3 hours) and diluted to 0.001 at either 7.4 or 6.0 pH in media. The volume of 100µL of this broth was added to each well. The next 100 µL was made up with serial dilutions of liposome or free Vancomycin diluted in sterile water at 300 mOsm. The OD600 was recorded at t=0 using a Beckman Coulter DTX-880 Multimode Detector, and the plate was incubated for 18 hours at 220 rpm. The OD600 was recorded again, and a plot of bacterial density vs. concentration was created to obtain the MIC. In order to normalize bacterial growth, density was visualized as a ratio of OD600 relative to the OD600 of control bacterial growth wells. The concentration at which optical density is 90% lower than control bacterial growth was recorded as the MIC.

2.2.9: MBC Studies

For each well from the MIC studies, an antibiotic-free agar well was prepared in a flat bottom 24-well plate. After the 18 hour time point, an inoculum was streaked in the agar well and incubated overnight at 37^{o}C. MBC was determined to be the lowest drug concentration at which there was no visible colony growth overnight in the agar plate.

SECTION 3: RESULTS

3.1: Elution Profile

Before encapsulation and microbe studies could be conducted, methods of purification of Vancomycin-encapsulated liposomes were qualitatively tested to find the optimal stationary phase for size exclusion chromatography. The two phases studied were Sephadex G-50 and Sepharose 4-B. An elution profile was performed on free Vancomycin, non-loaded liposomes, and Vancomycin-loaded liposomes, and 33 one-mL fractions were collected and tested for absorbance at 280 nm (Figure 1).

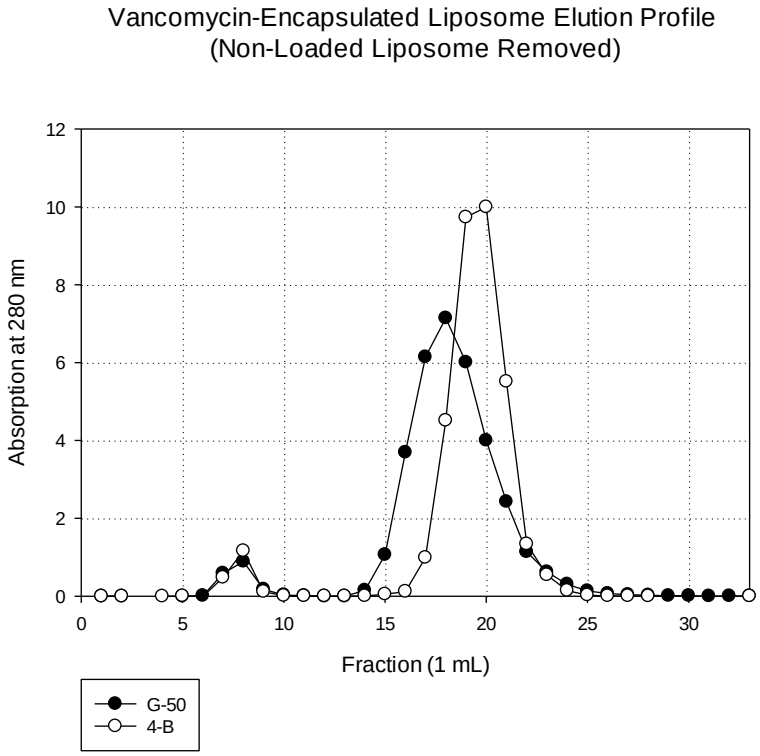


Figure 1: Vancomycin-encapsulated liposome elution profile. The initial peak represents the liposome eluted from the column, and the larger second peak is unencapsulated free Vancomycin

The resulting elution profiles demonstrate that there is a 4 mL fraction between the encapsulated liposomes and free Vancomycin in the G-50 column, and a 5 mL fraction for the 4-

B column. Both chromatography columns appeared in fractions 7 and 8, so future liposome constructs were collected in these fractions. In order to ensure purity of the liposomes, 4-B stationary phase was utilized for all experiments.

3.2: Vancomycin Calibration Curve

In order to convert spectrophotometric absorption data at 280 nm to Vancomycin concentration, a calibration curve was generated by dissolving a known Vancomycin concentration in PBS and measuring absorbance in a 1:1 ratio with acetonitrile, which was used as a liposome membrane disrupting agent to allow full absorption of Vancomycin contained within the liposomes.

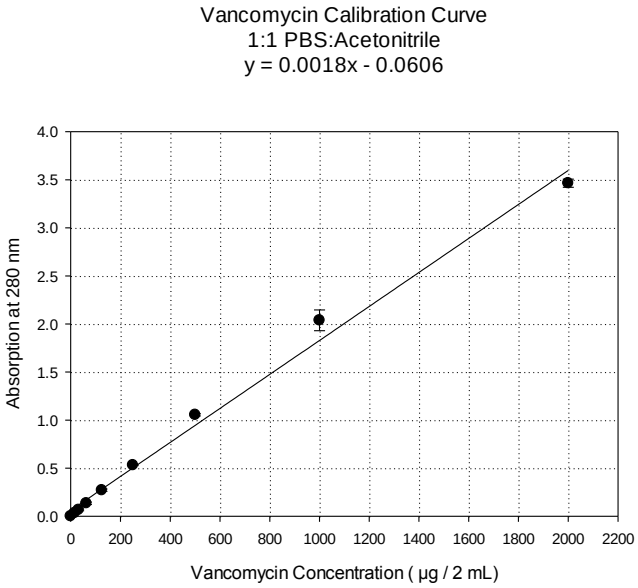


Figure 2: Vancomycin Calibration Curve

3.3: Bacterial Growth and pH vs. Time

In order to determine growth phases of bacteria and to investigate the effect of bacterial growth on broth pH, a growth curve was developed by measuring the absorption every hour until stationary phase was reached.

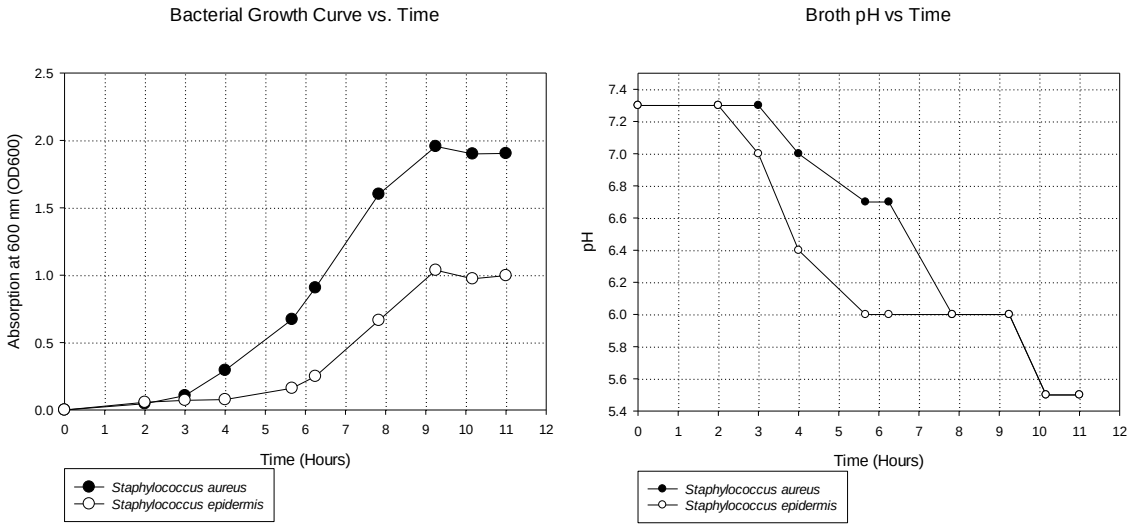


Figure 3: a) Absorbance of *S. aureus* and *S. epidermis* vs. time. b) pH of bacteria broth vs. time. pH and OD600 were measured simultaneously at each time point.

3.4: Vancomycin Encapsulation of Liposome Compositions

The amount of drug encapsulated using the dehydration-rehydration method per µmol lipid is shown in Table 1. Conversions were performed according to the previously established Vancomycin calibration curve. The three compositions of liposomes tested were;

1. 3:1:0.02 DSPC:Chol:18PEG: non-pH responsive liposomes
2. 3:1 21PC:DSPA 8% Chol 5% 18PEG: pH responsive liposomes
3. 3:1 21PC:DSPS 8% Chol 5% 18PEG: pH responsive liposomes

Composition	Vancomycin Encapsulation [Drug/Lipid]	n =
3:1:0.02 DSPC:Chol:18PEG	124.3 ± 19.4 µg/µmol	3
3:1 21PC:DSPA 8% Chol 5% 18PEG	123.0 ± 15.6 µg/µmol	4
3:1 21PC:DSPS 8% Chol 5% 18PEG	91.0 ± 7.6 µg/µmol	4

Table 1: Encapsulation rates for non-pH responsive and pH responsive liposomes

3.5: Characterization of Liposome Compositions

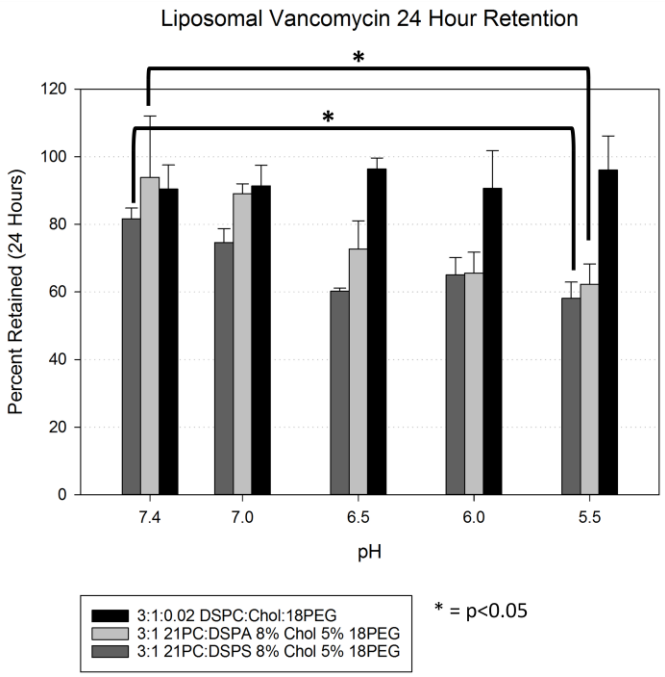
The size and polydispersity of each loaded and non-loaded liposome composition is outlined in Table 2. This confirms the size consistency of each liposome composition.

Composition		Average Size	PDI
DSPC:Chol	Non-Loaded	114.2 ± 1.4	0.028 ± 0.001
	Loaded	103.3 ± 0.3	0.103 ± 0.029
21PC:DSPA	Non-Loaded	100.2	0.013 ± 0.003
	Loaded	102.8 ± 3.2	0.064 ± 0.004
21PC:DSPS	Non-Loaded	104.8 ± 0.6	0.019 ± 0.023
	Loaded	102.2 ± 0.7	0.082 ± 0.004

Table 2: DLS size and PDI of loaded and non-loaded liposomes

3.5: Liposome 24 Hour Retention Rates

The amount of Vancomycin retained in liposomes after 24 hour incubation at 37^{o}C is outlined in Figure 4 a and b.



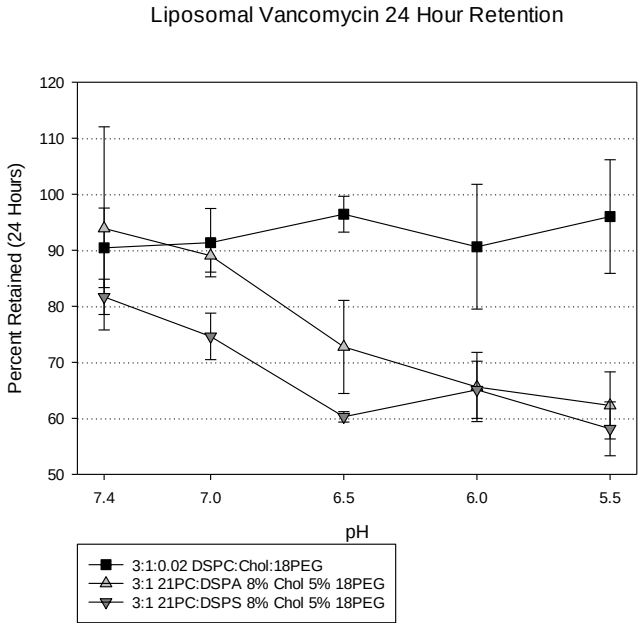


Figure 4a and 4b: Vancomycin retention for pH-responsive and non-pH responsive liposomes in PBS

This retention demonstrates the enhanced release of contents in acidic conditions from pH-responsive compositions as opposed to the non-pH responsive DSPC:Chol liposomes. At a pH of

5.5, the 21PC:DSPA and 21PC:DSPS liposomes released an average of 40% their encapsulated Vancomycin.

3.6: Minimum Inhibitory Concentration (MIC) and Studies

The effect of bacterial growth vs. antibiotic dose was plotted for each case for both *S. aureus* and *S. epidermis*. From these plots, a 4-parameter Gompertz curve was fit to the data points for each pH, and an MIC_{90} was determined by solving for the OD600 at which bacterial growth was 10% that of the control bacteria growth. The curve adhered to the formula outlined on figure 5 using a modified Gompertz curve which was previously established by Lambert *et al* as an effective model of dose response for calculation of MIC [20]. Free Vancomycin was plotted as the concentration per well, and the liposomes were plotted with the x-axis noting the concentration of encapsulated Vancomycin.

$$y = A + Ce^{-e^{B(x-M)}}$$

Figure 5: Modified Gompertz curve [20]. The MIC_{90} is defined where y = 10% the normal control growth. A = lower asymptote of y, B = slope parameter, C = distance between lower and higher asymptote, M = log concentration of the inflection point.

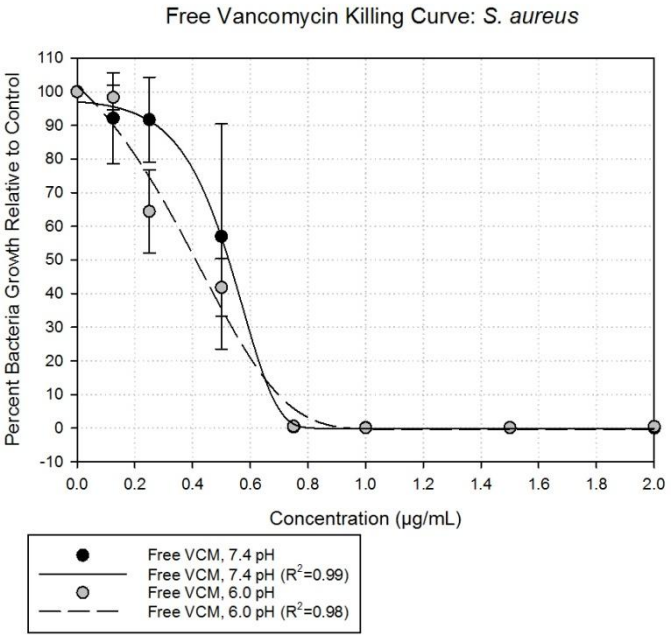


Figure 6a: MIC curve for Free Vancomycin on *S. aureus*

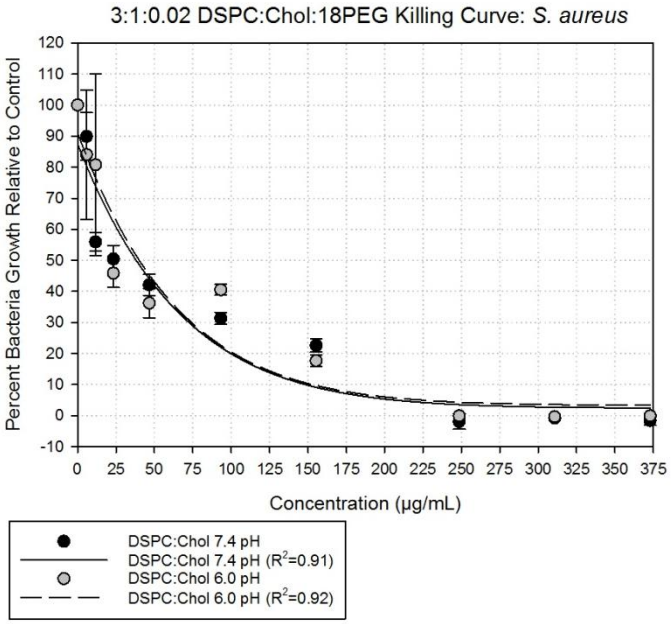


Figure 6b: MIC curve for DSPC:Chol (non-pH responsive) liposomes on *S. aureus*

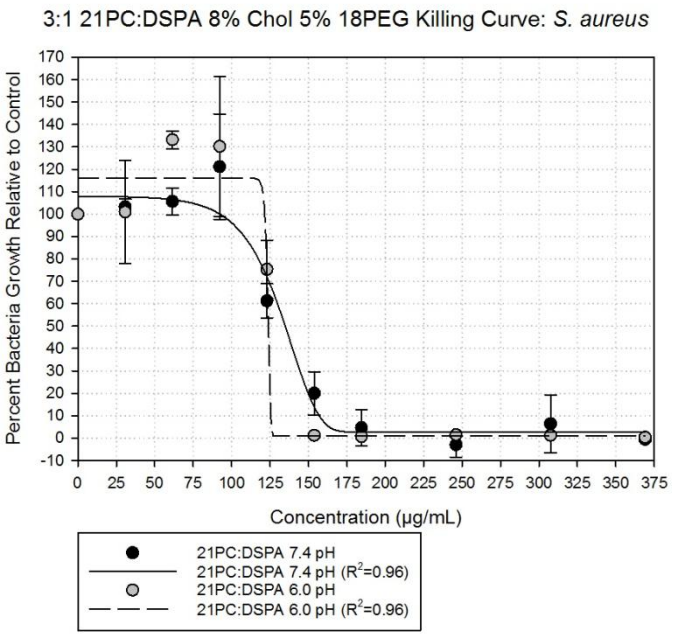


Figure 6c: MIC curve for 21PC:DSPA (pH-responsive) liposomes on *S. aureus*

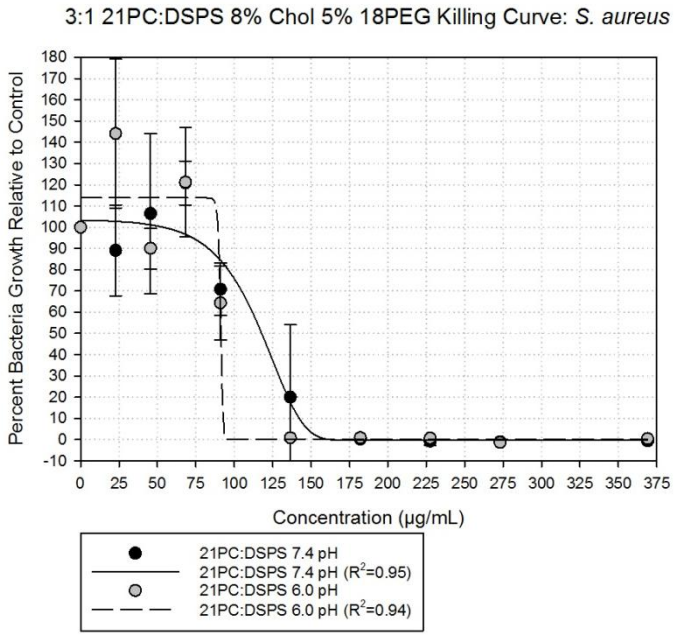


Figure 6d: MIC curve for 21PC:DSPS (pH-responsive) liposomes on *S. aureus*

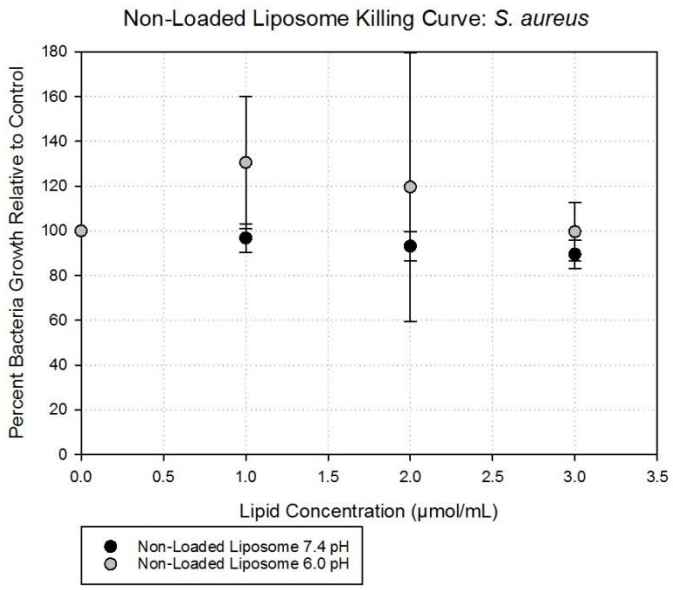


Figure 6e: MIC curve for non-loaded (drug-free) liposomes on *S. aureus*, demonstrating no bacterial growth inhibition

Corresponding MIC_{90} values and errors of estimate were reported and compared for statistical significance. The MIC values recorded showed a decreased MIC_{90} concentration for pH responsive liposomes at a pH 6.0, with the 21PC:DSPS composition indicating a statistically significant difference in inhibitory concentration.

The same approach for determining the MIC for *S. epidermis* was performed, and Figure 7 shows the dose response curves resulting from bacterial studies.

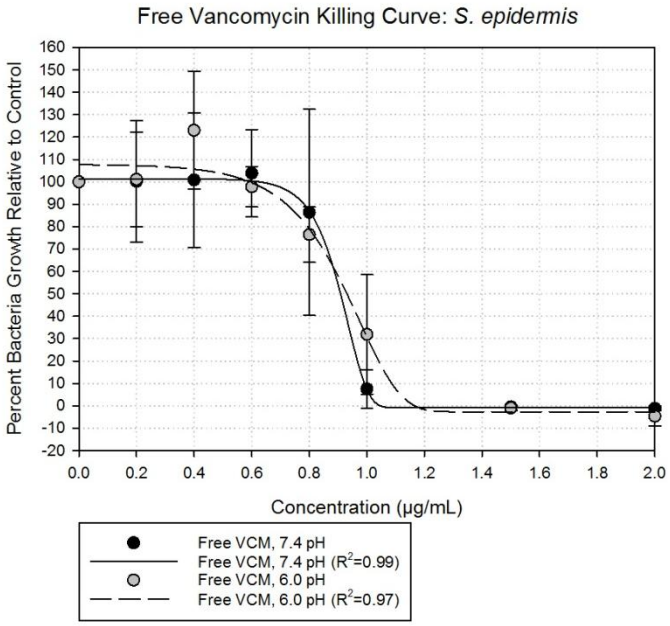


Figure 7a: MIC curve for Free Vancomycin on *S. epidermis*

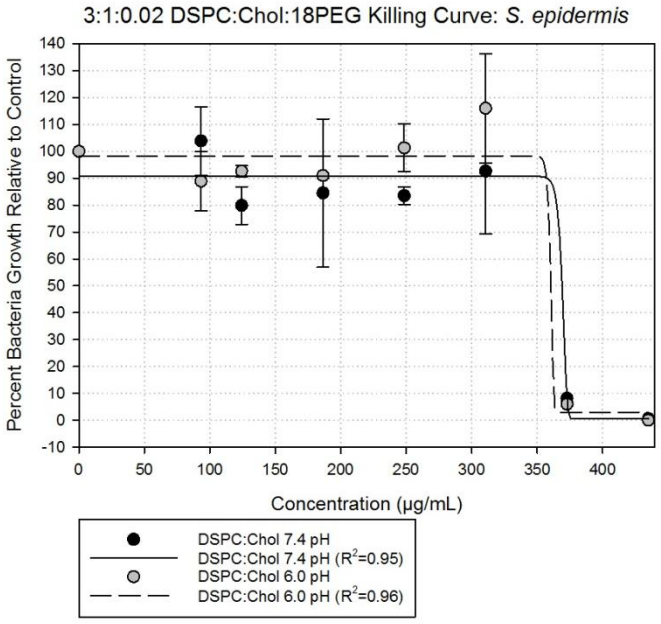


Figure 7b: MIC curve for DSPC:Chol (non-pH responsive) liposomes on *S. epidermis*

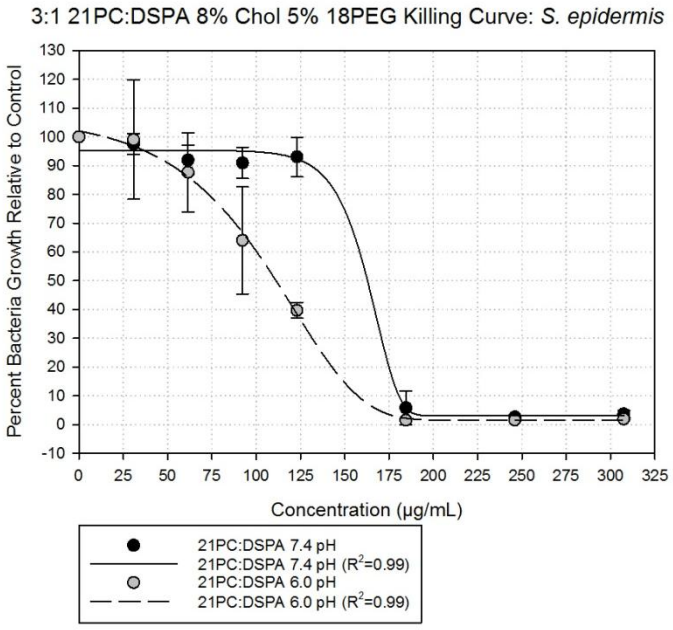


Figure 7c: MIC curve for 21PC:DSPA (pH-responsive) liposomes on *S. epidermis*

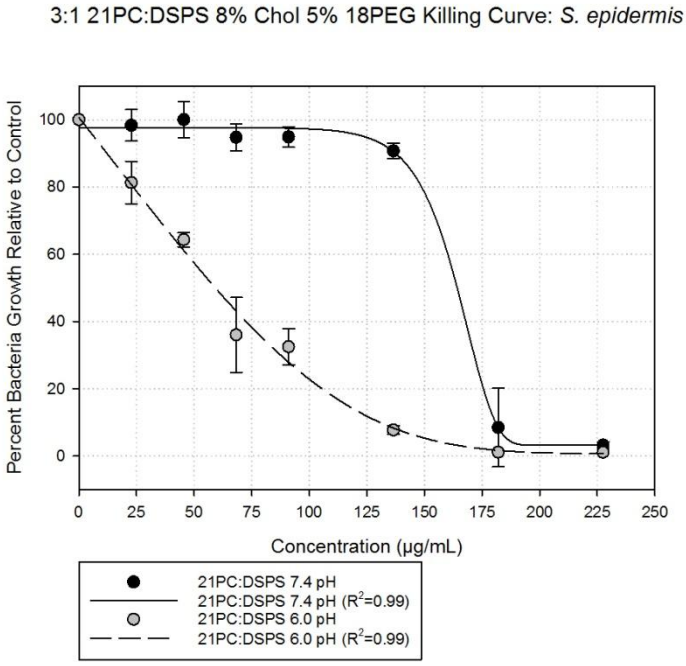


Figure 7d: MIC curve for 21PC:DSPS (pH-responsive) liposomes on *S. epidermis*

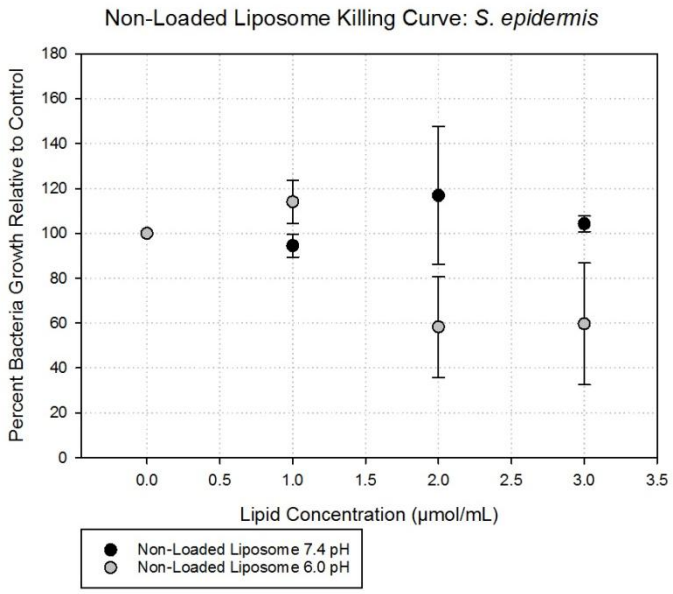


Figure 7e: MIC curve for non-loaded (drug-free) liposomes on *S. epidermis*, demonstrating no bacterial growth inhibition

Following calculation of MIC values for *S. epidermis*, the concentrations were tested for statistical significance, with the 21PC:DSPS composition showing a statistically significant reduction of MIC. The 21PC:DSPA composition also showed a decreased concentration, and the non-pH responsive showed very little change in MIC between the 7.4 and 6.0 pH conditions.

Figure 8 and 9 more clearly outline the MIC for *S. aureus* and *S. epidermis*, respectively.

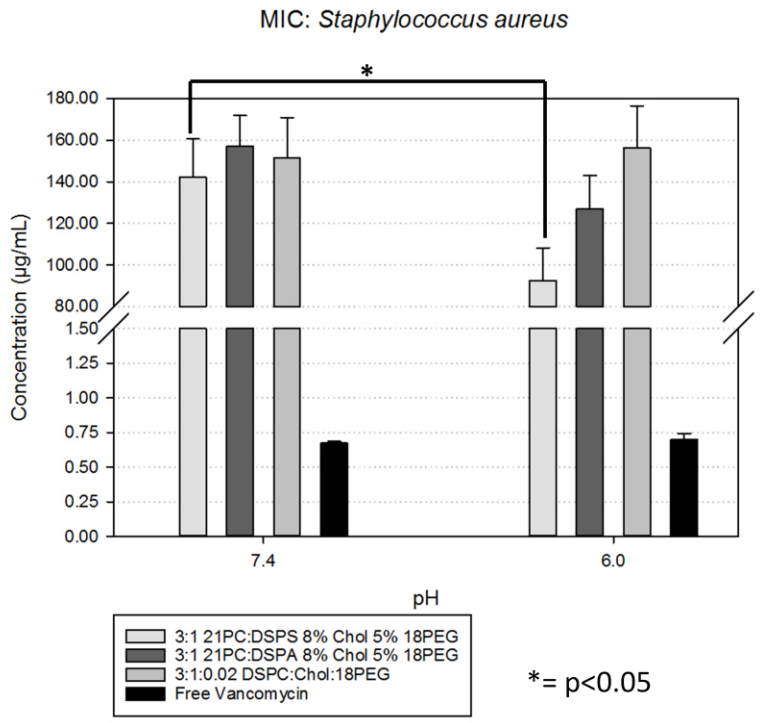


Figure 8: Minimum Inhibitory Concentrations for all drug compositions on *S. aureus*

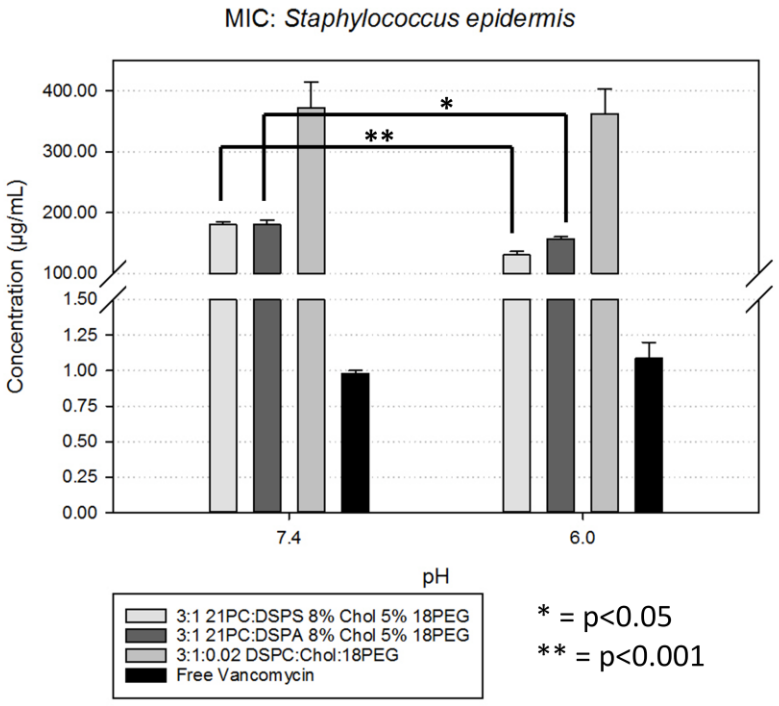


Figure 9: Minimum Inhibitory Concentrations for all drug compositions on *S. epidermis*

3.7: Minimum Bactericidal Concentration (MBC) Study

After recording bacterial growth during the 18 hour incubation for MIC determination, an inoculum of each well exhibiting bacterial growth below 10% of control bacteria were transferred to agar wells and allowed to incubate overnight. The minimum concentration which exhibited no colony growth was recorded as the Minimum Bactericidal Concentration, or MBC. Table 2 shows the MIC and MBC values for each condition.

***Staphylococcus aureus* MIC/MBC**			
Composition	**pH**	**MIC (µg/mL)**	**MBC (µg/mL)**
Free Vancomycin	7.4	0.67 ± 0.01	1.43 ± 0.58
	6.0	0.70 ± 0.04	1.31 ± 0.69
3:1:0.02 DSPC:Chol:18PEG	7.4	151.42 ± 19.45	372.9
	6.0	156.30 ± 20.09	372.9
3:1 21PC:DSPA 8% Chol 5% 18PEG	7.4	156.95 ± 14.87	399.75 ± 43.49
	6.0	126.91 ± 16.23	369
3:1 21PC:DSPS 8% Chol 5% 18PEG	7.4	142.29 ± 18.45	261.625 ± 22.75
	6.0	92.54 ± 15.72	200.20 ± 94.35
Non-Loaded Liposomes	7.4	N/A	N/A
	6.0	N/A	N/A

Table 3a: MIC and MBC values for *S. aureus* antimicrobial studies

***Staphylococcus epidermis* MIC/MBC**			
Composition	**pH**	**MIC (µg/mL) (n=3)**	**MBC (µg/mL) (n=2)**
Free Vancomycin	7.4	0.98 ± 0.02	1.75 ± 0.35
	6.0	1.09 ± 0.11	1.5
3:1:0.02 DSPC:Chol:18PEG	7.4	372.152 ± 42.33	N/A
	6.0	362.74 ± 40.39	N/A
3:1 21PC:DSPA 8% Chol 5% 18PEG	7.4	180.55 ± 6.72	369
	6.0	157.19 ± 3.64	307.5
3:1 21PC:DSPS 8% Chol 5% 18PEG	7.4	180.90 ± 4.65	204.75 ± 32.17
	6.0	130.67 ± 5.97	182
Non-Loaded Liposomes	7.4	N/A	N/A
	6.0	N/A	N/A

Table 3b: MIC and MBC values for *S. epidermis* antimicrobial studies

Unfortunately, MBC results were limited to the testing points due to the binary nature of MBC tests. From a qualitative standpoint, however, MBC studies demonstrate a decrease in Vancomycin required for preventing bacterial growth in the low pH conditions for the two pH-responsive liposome nanoparticles, while being unaffected for the non-pH responsive conditions.

SECTION 4: DISCUSSION

4. 1: Liposome Compositions

The selection of liposomes was determined based on the interaction of the lipid head and tail groups. For the non-pH responsive liposomes, the 3:1:0.02 DSPC:Chol:18PEG composition was selected in order to replicate the protocol utilized by Muppidi *et al* [21]. For the pH responsive compositions, 21PC (Fig 8a) constituted a majority of the liposome surface. This phospholipid has a zwitterionic phosphatidylcholine head group which is miscible in water, and hydrophobic 21-carbon tail groups. The second lipids selected were specifically chosen for their ability to phase partition with 21PC at an acidic pH of 6.0. DSPA (Fig 8b) is composed of a hydrophobic 18-carbon tail group and a phosphatidic acid head group. This head group has pKa values on the phosphate group which allow for protonation under acidic conditions. Similarly, DSPS (Fig 8c) has a phosphatidylserine head group with a carboxyl group capable of protonating and phase partitioning with 21PC in decreased pH.

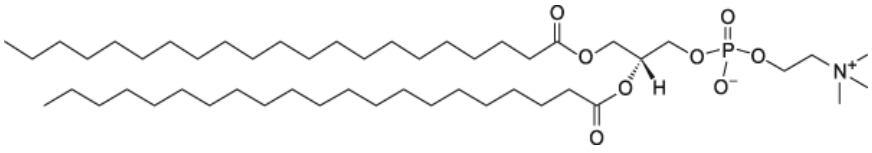


Figure 10a: 21PC Lipid. Protonation of the phosphate group is unlikely and the head group remains miscible in water

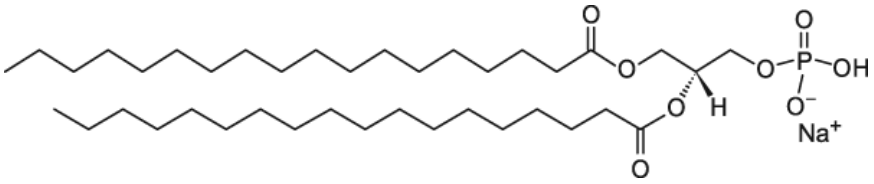


Figure 10b: DSPA Lipid. Protonation of the phosphate group is responsible for phase partitioning with 21PC

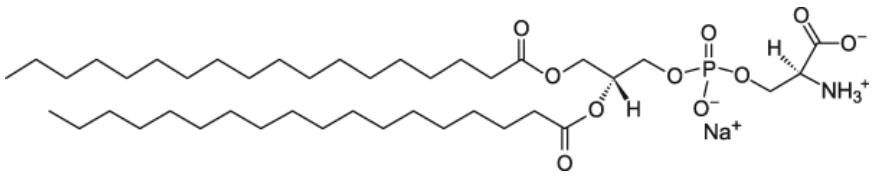


Figure 10c: DSPS Lipid. Protonation can occur in the phosphate and carboxyl group, resulting in phase partitioning

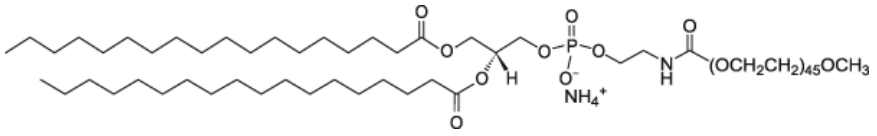


Figure 10d: 18PEG-DSPE Lipid-polymer. The long polymer chain serves to shield the liposome membrane and increase circulation time.

In addition to these main lipids, cholesterol was also included in order to contribute to membrane rigidity structure, and 18PEG, or polyethyleneglycol, is added to shield the surface of the liposome, add stability, and increase circulation time.

The concept of pH-responsive liposomes relies on the phenomena of phase separation of two or more lipid components of the lipid bilayer. When the pH decreases sufficiently and one species of lipid becomes protonated, the lipid head groups undergo phase partition, with the lower concentration of lipid forming “rafts” of similar phospholipid heads. At the interface of these rafts, there is increased membrane permeability due to mismatching tail lengths, slow acyl-tail molecular dynamics and different tilts- with respect to the membrane normal- of each of the lipid types comprising the bilayer. From these interfaces, Vancomycin exhibited increased permeability through the liposome membrane.

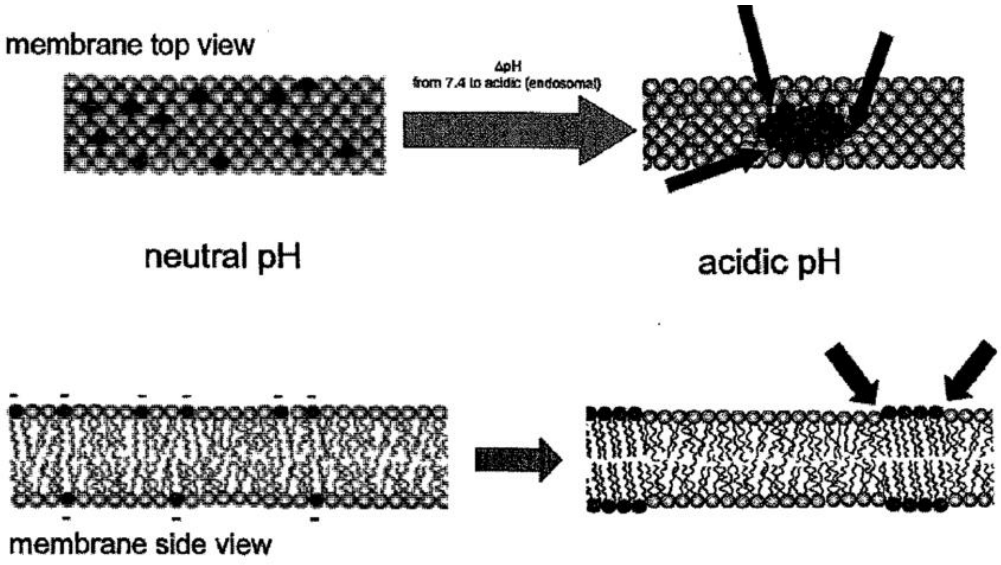


Figure 11: Formation of Lipid rafts and leakage sites of liposome membrane [15]

Several techniques were employed and compared in order to achieve the maximum concentration of Vancomycin encapsulated within the nanoparticles. Initial attempts at dissolving Vancomycin Hydrochloride in PBS resulted in issues with osmolality values well above the accepted 300 mOsm point as a result of dissociation of the Vancomycin salt as well as sucrose prior to lyophilization. This resulted in unfavorable loading of drug into the nanoparticles. Instead, Vancomycin was dissolved in distilled water and the osmolality was corrected to 300 mOsm with NaCl based on the amount of sucrose necessary for lyophilization. Experiments were performed to investigate the loading of liposomes without lyophilization, but this technique resulted in lower encapsulation per lipid than those while utilizing the dehydration-rehydration method.

Another important consideration regarding Vancomycin loading was the pH of the hydration solution after lyophilization. The dissolution of the Vancomycin hydrochloride salt significantly decreased the pH of the solution, with measurements observing a final pH of 4.0 for the Vancomycin solution dissolved in distilled water. For the lipid head groups, this significantly

lowered pH and could have an effect on the membrane, interfering with passive loading and extrusion procedures. Maximum loading was achieved when the Vancomycin solution was adjusted to pH 7.0 after dissolving the Vancomycin powder in the distilled water. There were concerns over lack of a buffering agent, but this loading period did not experience a change in pH, and separation during size exclusion chromatography exchanged the buffer to PBS of pH 7.4.

4.2: Bacterial Growth

Both *S. aureus* and *S. epidermis* experienced very similar growth curves with a stationary phase being achieved within 12 hours. In order to maintain a consistent growth phase, both bacteria lines were able to be taken at the same time and used for MIC studies. As seen on Figure 3a, the log-growth phase was observed between hours two and five, so bacteria was selected for studies at this point.

The effect of bacterial growth on the pH of broth identifies the increase in acidity within a broth medium which is not being replenished over time. Anaerobic activity of bacteria decreases the overall pH of broth, and this is physiologically relevant considering that decreased pH is indicative of worsening bacterial infection [12]. #3 Nutrient broth is originally formulated to exist at a pH of around 6.8 after dissolution and autoclaving, and the more rapid decrease in pH for *S. epidermis* may be due to this factor. Regardless, both bacteria demonstrated an ability to decrease environmental pH to as low as 5.5, which has also been observed in infection sites. As a result, the precedent for pH-responsive nanoparticle delivery is justified by the decreasing pH observed in this experiment.

4.3: Liposome Retention Profile

The studies conducted and highlighted in Figure 4 confirm concepts outlined previously, and shows that Vancomycin is capable of being encapsulated, stably retained, and released under specific pH conditions. Both of the pH-responsive compositions exhibited retention at physiologic pH and release of a statistically significant concentration of Vancomycin at 5.5 pH. Conceptually, this would suggest that the pH-responsive liposomes release more Vancomycin on the bacteria in decreased pH conditions than at physiologic pH.

4.4: Anti-microbial Studies

Exploration of the MIC for both bacteria lines resulted in promising data that confirms the successful delivery of Vancomycin in decreased pH for pH-responsive liposomes compared to the same liposomes at physiologic pH and the non-pH-responsive liposome compositions. In the 21PC:DSPS condition, a statistically significant decrease in total encapsulated Vancomycin was achieved for 6.0 pH compared to the 7.4 pH condition for both bacteria types. This suggests that the encapsulated Vancomycin is more leaked at this condition and the liposomes have greater antimicrobial activity. The 21PC:DSPA liposomes also demonstrated a lower MIC in the 6.0 pH bacteria, with statistically significant reduction in MIC_{90} occurring on the *S. epidermis* bacteria. The DSPC:Chol liposomes were unaffected by the decreased pH. Although the MIC values were promising for the pH-responsive liposomes across the different pH values, there was a noticeable increase in all liposome MIC compared to that of free Vancomycin. This was expected, however, due to the fact that free Vancomycin is more readily available than liposomes, and only released Vancomycin is able to act on bacteria cell walls. Conceptually, the benefit of liposome delivery of Vancomycin is that larger doses are able to be provided without the risk of antibiotic being distributed to tissue where there are no bacterial infections.

Although previous studies have suggested that Vancomycin has a greater activity at higher pH, it was not deemed statistically significant for either bacteria line in the free Vancomycin condition [22]. The MIC for both *S. aureus* and *S. epidermis* were both slightly higher for 6.0 than 7.4, but this was not significant enough to confidently attribute to decreased activity of the antibiotic. However, MIC values were generally higher for all conditions when testing vs. *S. epidermis*, which may have been due to the more rapidly decreased pH over time during growth which was observed in the bacterial growth curves. In the *S. epidermis* condition, there was an extremely high MIC value for the DSPC:Chol liposomes compared to the *S. aureus*, and no colony growth was prevented in MBC studies. This may have been due to unexpectedly decreased loading for these conditions, although repeated iterations observed similar results.

The results for the MBC studies suggest that the pH-responsive liposomes were able to prevent bacterial growth with lower encapsulated antibiotic concentrations compared to physiologic pH. Additionally, the non-pH responsive conditions showed no effect on the MBC. This study became more qualitative due to the limited concentration data points, but averaging of several tests yielded results which could be considered comparable.

SECTION 5: CONCLUSION

This study successfully demonstrated the potential application of pH-responsive nanoparticles in order to treat bacterial infections. MIC and MBC studies confirm the ability of liposomal Vancomycin to escape the interior space of liposomes and kill bacteria, and retention studies demonstrate the stable retention of Vancomycin at physiologic pH. Compared to non-pH responsive constructs, enhanced killing was achieved at low pH for the pH-responsive liposomes, and prevention of bacterial colony formation was also improved in the long term after removal of drug from the bacteria environment. Further work involving more applicable infection sites, focus on specific MRSA and MRSE infection sites, and improved knowledge regarding the release kinetics and interaction of Vancomycin with liposome membranes would allow researchers to tailor nanoparticle membranes to efficiently deliver antibiotics to infection sites with efficacy which exceeds that of current IV administration of free Vancomycin.

Encapsulation of antimicrobial agents within nanoparticles presents challenges and has many unanswered questions, but the application of this delivery mechanism has the potential to provide benefits to infectious disease therapy which are physiologically relevant in bacterial infections. Studies have determined that PEGylated liposome delivery allows for greater concentration of antibiotics in lung tissue, which could aid in treating previously resistant lung infections [13]. Additionally, studies have demonstrated the decreased nephrotoxicity potential of liposomal Vancomycin due to the more selective release of drug contents within the body [23]. Liposomal antibiotic formulations have demonstrated stability in physiologic conditions, and stable release at a given pH. Continued innovation will allow clinicians to specifically tailor nanoparticle therapies in order to optimize existing antibiotic therapies and change the landscape of infectious disease treatment to combat drug resistance in community infections.

REFERENCES

1. Hryniewicz, W., *Epidemiology of MRSA.* Infection, 1999. **27**(2): p. S13-S16.
2. Cunningham, J.B., G. Kernohan, and T. Rush, *Bed occupancy, turnover intervals and MRSA rates in English hospitals.* British journal of nursing, 2006. **15**(12): p. 656-660.
3. Stapleton, P.D. and P.W. Taylor, *Methicillin resistance in Staphylococcus aureus: mechanisms and modulation.* Science progress, 2002. **85**(Pt 1): p. 57.
4. Batabyal, B., G. Kundu, and S. Biswas, *Methicillin-resistant Staphylococcus aureus: a brief review.* International Research Journal of Biological Sciences, 2012. **1**(7): p. 65-71.
5. Miragaia, M., I. Couto, and H.D. Lencastre, *Genetic diversity among methicillin-resistant Staphylococcus epidermidis (MRSE).* Microbial Drug Resistance, 2005. **11**(2): p. 83-93.
6. Rybak, M.J., *The pharmacokinetic and pharmacodynamic properties of vancomycin.* Clin Infect Dis, 2006. **42 Suppl 1**: p. S35-9.
7. Neu, H.C., *The crisis in antibiotic resistance.* Science, 1992. **257**(5073): p. 1064-1073.
8. Hartman, B.J. and A. Tomasz, *Low-affinity penicillin-binding protein associated with beta-lactam resistance in Staphylococcus aureus.* Journal of Bacteriology, 1984. **158**(2): p. 513-516.
9. Lodise, T.P., et al., *Larger vancomycin doses (at least four grams per day) are associated with an increased incidence of nephrotoxicity.* Antimicrobial agents and chemotherapy, 2008. **52**(4): p. 1330-1336.
10. Forouzesh, A., P.A. Moise, and G. Sakoulas, *Vancomycin ototoxicity: a reevaluation in an era of increasing doses.* Antimicrobial agents and chemotherapy, 2009. **53**(2): p. 483-486.
11. Sande, L., et al., *Liposomal encapsulation of vancomycin improves killing of methicillin-resistant Staphylococcus aureus in a murine infection model.* J Antimicrob Chemother, 2012. **67**(9): p. 2191-4.
12. Radovic-Moreno, A.F., et al., *Surface charge-switching polymeric nanoparticles for bacterial cell wall-targeted delivery of antibiotics.* ACS nano, 2012. **6**(5): p. 4279-4287.
13. Muppidi, K., et al., *PEGylated liposome encapsulation increases the lung tissue concentration of vancomycin.* Antimicrob Agents Chemother, 2011. **55**(10): p. 4537-42.
14. Anderson, K.E., et al., *Formulation and evaluation of a folic acid receptor-targeted oral vancomycin liposomal dosage form.* (0724-8741 (Print)).
15. Sofou, S., *Liposome drug carriers with pH-Sensitivity*. 2008, Google Patents.
16. Karve, S., et al., *The pH-dependent association with cancer cells of tunable functionalized lipid vesicles with encapsulated doxorubicin for high cell-kill selectivity.* Biomaterials, 2010. **31**(15): p. 4409-4416.
17. Karve, S., G. Bajagur Kempegowda, and S. Sofou, *Heterogeneous Domains and Membrane Permeability in Phosphatidylcholine− Phosphatidic Acid Rigid Vesicles As a Function of pH and Lipid Chain Mismatch.* Langmuir, 2008. **24**(11): p. 5679-5688.
18. Mugabe, C., A.O. Azghani, and A. Omri, *Preparation and characterization of dehydration-rehydration vesicles loaded with aminoglycoside and macrolide antibiotics.* Int J Pharm, 2006. **307**(2): p. 244-50.
19. Bedu-Addo, F.K., *Understanding lyophilization formulation development.* Pharmaceutical Technology, 2004. **20**: p. 10-19.
20. Lambert, R.J. and J. Pearson, *Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values.* J Appl Microbiol, 2000. **88**(5): p. 784-90.

21. Muppidi, K., et al., *Development and stability studies of novel liposomal vancomycin formulations.* ISRN Pharm, 2012. **2012**: p. 636743.
22. Mercier, R.-C., C. Stumpo, and M.J. Rybak, *Effect of growth phase and pH on the in vitro activity of a new glycopeptide, oritavancin (LY333328), against Staphylococcus aureus and Enterococcus faecium.* Journal of Antimicrobial Chemotherapy, 2002. **50**(1): p. 19-24.
23. Pumerantz, A., et al., *Preparation of liposomal vancomycin and intracellular killing of meticillin-resistant Staphylococcus aureus (MRSA).* Int J Antimicrob Agents, 2011. **37**(2): p. 140-4.