pH sensitive liposomes for targeted delivery of antibiotics to localized internal bacterial infection sites

by

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ABSTRACT OF THE THESIS

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Methicillin Resistant *Staphylococcus aureus* (MRSA) causes a myriad of infections ranging from mild skin-infection to more serious infections affecting internal organs. A glycopeptide, Vancomycin, remains the last line of defense against MRSA. The aim of this study is to investigate whether liposomal encapsulated Vancomycin had a better antimicrobial action than free Vancomycin in terms of infection-specific targeting and circulation time. For the same, encapsulation efficiency and release kinetics of the liposomes was evaluated along with Minimum Inhibitory Concentrations (MIC) of the liposomal preparations. The liposomes showcased a 12-15% encapsulation efficiency. Sustained release at pH 6.0 as compared to little to no release at pH 7.4 was demonstrated by the pH sensitive liposomes. Also, in acidic pH, an increase in efficacy was observed with a greater decrease in the MIC of the pH responsive liposomes as compared to the lower decrease in MIC of the non pH-responsive liposomes and increase in MIC of free Vancomycin. Thus, from the results it can be concluded that environmentally sensitive liposomes could be developed as a successful carrier of antimicrobial drugs demonstrating effective antimicrobial action.

ACKNOWLEDGMENTS

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SECTION I: INTRODUCTION

Nanoparticles (NP) are particles ranging in the size range of 1-100 nm and form the building blocks for nanotechnology. Due to their small size they have gained much interest over the past few years for their medical applications. Their usage in the fields of anti-microbial applications[1], bio-sensors[2] and drug delivery applications[3] have been much studied. The results of these studies gave us innovations such as silver nanoparticles for wound healing and metal oxide nanoparticles for imaging to name a few.

In the realm of drug delivery, nanoparticles have been exploited for the delivery of anti-cancer drugs[4], proteins, peptides, nucleic acids etc. The ability to modify and engineer NPs precisely according to need and thus achieve greater drug activity on-site can be attributed to the success of this technology. The aim of designing drug delivery NPs is to improve drug concentration at target sites, reduce loss of activity due to ineffective drug dosing and reduce toxicity. The materials used for the synthesis and surface engineering of the synthesized NPs have resulted in many mechanisms for improved drug delivery, prominent among them been controlled drug release[3], improved circulation time[5], targeting and better molecular-NP interactions[6]. However, studies regarding NP delivery of antibiotics have been plagued with toxicity and pre-dominantly environmentally insensitive surfaces of the particles which has adversely affected the uptake of antibiotic into disease sites[7].

Bacteria have been known to survive in highly diverse environments including acidic environments. A pH drop occurs in surrounding areas of a bacterial infection because of the metabolic activities of the bacteria including anaerobic fermentation[5]. Many antibiotics lose their activity at such low pH and fail to produce the desired effects [8]. As

such, there is a need to develop delivery vehicles for antibiotics which can systemically deliver antibiotics and enhance activity on-site. Radovic-Moreno et. al.[5] developed polymeric nanoparticles composed of Poly lactic-co-glycolic acid (PLGA)- Poly ethylene glycol (PEG) to target bacterial cell walls and deliver Vancomycin to gram-positive bacteria. The NPs remained anionic at pH 7.4 and turned cationic at acidic pH facilitating cell wall binding[5].

Vancomycin is a glycopeptide antibiotic which acts against gram-positive bacteria by forming hydrogen bonds with the terminal amino acid groups of peptides and preventing the formation of N-acetylglucosamine (NAG) and N-acetymuramic acid (NAM) polypeptide crosslinking thus effectively blocking cell wall synthesis[9]. It remains the most sought after antibiotic against Methicillin resistant *Staphylococcus aureus.* This study aims at developing pH sensitive liposomal NPs for the delivery of Vancomycin. Synthesized from lipids, they would be expected to be more biocompatible than most other materials used to synthesize NPs. Previously liposomes have been shown to retain contents at pH 7.4 and release at acidic pH[10]. The same mechanism would be exploited for this study. To effectively deliver the antibiotic to localized infection sites, the extent of encapsulation, retention, and release at pH 7.4 and pH 6.0 has been studied. Furthermore, the formulation would be expected to lower the MIC (Minimum Inhibitory Concentration) at pH 6.0 as compared to pH 7.4. Thus, both pH responsive and non-responsive liposomes have been studied.

SECTION 2: MATERIALS AND METHODS

2.1: Materials

Raw materials: Bioreagent grade Vancomycin Hydrochloride from *Streptomyces orientalis*, Sucrose, Sephadex G-50 and Sepharose 4B were purchased from Sigma Aldrich (St. Louis, MO). *Staphylococcus aureus* (ATCC#25923) was purchased from ATCC (Manassas, VA). BD Tryptic Soy Broth (Soybean Casein Digest Medium) and Hardy Diagnostics Tryptic Soy Agar (Soybean Casein Digest agar Medium) along with innoculatig loops were purchased from VWR. 1,2-dihenarachidoyl-*sn*-glycero-3-phosphocholine (21-PC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dioctadecanoyl-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)] (18 PEG), L-α-lysophosphatidylcholine (Egg-PC) and Cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL).

2.2: Methods

2.2.1: Preparation of Buffer solution

A 2L stock of Phosphate Buffered Saline at pH 7.4 and pH 6.0 was prepared. 1 mM EDTA was added as a chelating agent. The osmolarity was brought to 300 +/- 20 mOsm.

2.2.2: Preparation of Tryptic Soy Broth/Agar

S. aureus cultured via ATCC recommendations. A stock of 1L was prepared by dissolving 30 mg of the powder in 850 mL of distilled water and bringing up the

volume to 1L. The mixture was autoclaved and then cooled till room temperature prior to use.

2.2.3: Preparation of Vancomycin Solution

A 2 mL stock of 50 mg/mL Vancomycin Hydrochloride was prepared was prepared by dissolving the powder in PBS (at 7.4), Tryptic-Soy Broth (at 7.4) or water.

2.2.4: Preparation of Chromatography columns

4 columns each of Sephadex-G50 and 4B were prepared. Of the four two were at pH 7.4 and two at pH 6.0. The buffer at the respective pH was used to make the column. For Sephadex-G50, 1.86 g of the Sephadex G50 powder was added to 35 mL of buffer. The solution was then run through an empty column until the gel settled. For 4B, 60 mL of the solution was taken and vacuum filtered to separate the alcohol. The resultant gel was cleaned with PBS buffer solution and passed through an empty column till it settled.

2.2.5: Preparation of Liposomes- Passive encapsulation

Only non pH-responsive liposomes were prepared by this method. A 10 mM mixture of PC lipid, Cholesterol and PEG in a PC:Cholesterol 7:3 mole ratio with 2% mole PEG was prepared. The mixture was subjected to a rotary evaporator under vacuum to clear all the chloroform and flushed lipid film with nitrogen gas to dry completely. The film obtained was rehydrated with 1ml of Vancomycin in PBS solution at a concentration of 50mg/ml. The solution was left to anneal for 2 hours at 58°C. After annealing, the liposomes were extruded through two 100 nm poly-carbonate membranes. During the extrusion process the mixture was passed through the

membranes 21 times. The resultant solution was made to pass through the column to separate liposomes and free Vancomycin.

2.2.6: Preparation of Liposomes- Dehydration/Rehydration Method

This method was developed by combining the methods from papers by Mugabe et.al.[11], Karve et.al.[10], and Muppidi et.al.[12]. A 10 mM mixture of the lipids was prepared. The mixtures prepared were: the non-pH responsive (with gel-phase lipid membrane) DSPC:Cholesterol:PEG in 68.6%:29.4%:2% mole ratio, the non-pH responsive (with fluid lipid membrane) Egg-Pc:Cholesterol:PEG in 66.5%:28.5%:5% mole ratio, the non-pH responsive (with fluid lipid membrane) 21PC:Cholesterol:PEG in 66.5%:28.5%:5% mole ratio and the pH-responsive DSPA:21PC:Cholesterol:PEG in 56%:24%:8%:12% mole ratio. The chloroform in the mixtures was evaporated using a rotovap with vacuum and nitrogen gas. The thin film was dissolved in sucrose solution in a 1:1 w/w lipid:sucrose ratio. Sucrose was added for dissolving as it helps stabilize the liposomes during lyophilization. The volume was made up to 1mL by adding distilled water (DI H_2O). This mixture was annealed for 2 hours at 58°C. At this stage liposomes would have formed. The mixture was frozen at -80°C and lyophilized overnight. The lyophilized sample was then rehydrated with Vancomycin solution in a w/w lipid:Vancomycin ratio of 94.3mg:50mg. The volume was made up to 1 mL by adding PBS at pH 7.4. The mixture was vortexed for 2 minutes and incubated for 45 minutes at room temperature. Following this the mixture was extruded 21 times through two 100 nm Poly-Carbonate membranes. The resultant solution was passed through a 4B column to separate liposomes and free Vancomycin. The liposomes collected were then used for further studies.

2.2.7: Standard Curves and Elution profiles

The standard curve for Vancomycin was generated by making 15 serially diluted 1 mL mixtures of Vancomycin in PBS at 7.4. To this 500 µL of acetonitrile was added. The blank consisted of 1 mL PBS plus 500 µL Acetonitrile. The mixtures were then evaluated on an absorbance spectrophotometer at 280 nm. As Vancomycin is an aromatic compound, it absorbs UV light. Acetonitrile is added to the mixture so as to disassemble the liposomes (when liposomal Vancomycin needs to be evaluated) and release encapsulated Vancomycin in the solution. The readings of the spectrophotometer are plotted on a graph to generate a standard curve for Vancomycin.

For the elution profile, 1mL fractions of the solution from the chromatography columns are collected and evaluated on the spectrophotometer.

2.2.8: Release Studies

The synthesized liposomes were purified on a 4B column and incubated at 37°C for 24 hours. Since the liposomes were prepared at pH 7.4, so the solution was divided in two parts and volume brought up to the required level by adding PBS and keeping one sample at pH 7.4 and adjusting the other to pH 6.0. The solutions were evaluated for Vancomycin release at 10 minutes, 1hr, 3hr and 24 hours. At each time interval, the solution was run through the column to separate free Vancomycin.

2.2.9: Bacterial Growth and Growth Curve

Stock S. aureus was plated on TS agar plates. The plates were incubated overnight at 37°C. A single colony was dissolved in 5 mL of TSB at pH 7.4. Following this, the

broth was added to 50 mL of medium and incubated at the same conditions for 8 hours. A 1mL sample of the broth was evaluated at 600 nm every hour. After 8 hours the broth was transferred to 500 mL and incubated at the same conditions. Readings were taken at every hour at 600 nm till death phase was established. The readings were plotted to obtain the growth curve of *S.aureus.*

For propagation of the bacteria, a colony was dissolved in 50 mL of medium and incubated for 8 hours following which the medium was transferred to 500 mL of medium and incubated for another 8 hours. At this point a mixture of bacterial broth and glycerol was prepared with 20% glycerol in the mixture. The mixture was then frozen at -80°C for future use.

2.2.10: Antibacterial Studies

Antibacterial studies were conducted to obtain MIC for the various Vancomycin-liposome formulations and free Vancomycin. MIC is the minimum concentration of the drug that is needed such that no growth is observed in the medium after 18 hours of incubation. The microplate method was used to obtain the MIC. The 2 mL frozen cultures of *S.aureus* were thawed and suspended in 50 mL of medium at pH 7.4. The broth was incubated till OD_{600} of 0.3 was achieved. The culture was then diluted to an effective OD_{600} of 0.001 in both pH 7.4 and 6.0. The Vancomycin preparations (pH responsive liposomes, non pH-responsive liposomes and free Vancomycin) were serially diluted up to 20 times in a 96 well plate using media at either 7.4 or 6.0. Bacterial broth was then added to these wells to make a final volume of 200 µL. The bacterial concentration was kept constant for each type of Vancomycin preparation. The OD_{600} was measured immediately following the preparation of plates. The plates

were then incubated at 37^{o}C at 220 rpm. OD_{600} reading of the plates was again taken at 18 hours of incubation. The experiment was conducted in duplicates. The readings were plotted on log graph. MIC was calculated by fitting Gompertz's functions to the graph using least square regression technique.

SECTION 3: RESULTS

The study was conducted with non pH-responsive and pH responsive liposomes at pH 6.0 and 7.4 to determine the efficacy of each liposomal delivery system.

3.1 Standard Curves and elution Profiles

The standard curve generated for Vancomycin is depicted in Figure1.

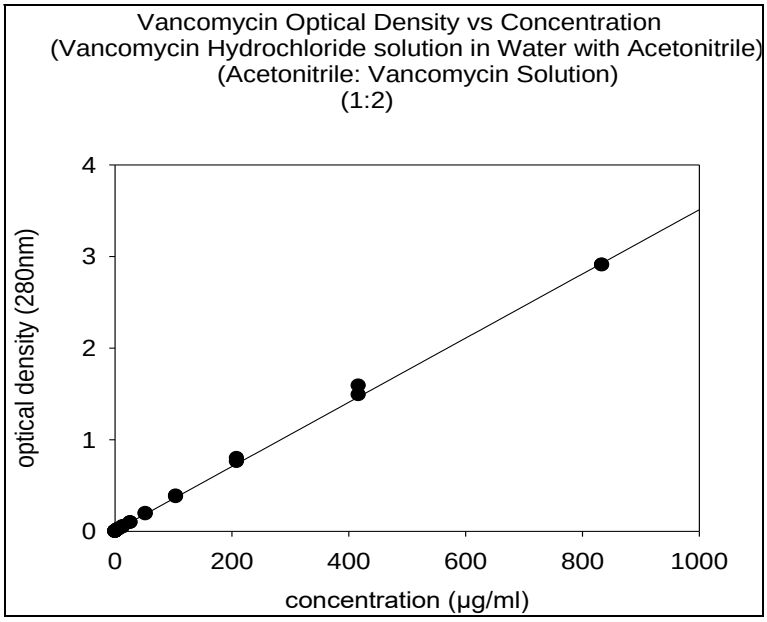

Figure 1: Vancomycin Standard

The graph yields plot parameters which are then further used to extrapolate Vancomycin concentrations in liposomal suspensions.

Elution profiles are important to determine the exact column fractions in which all of the liposome encapsulated Vancomycin is eluted. The elution profiles are depicted in the Figures 2a and 2b.

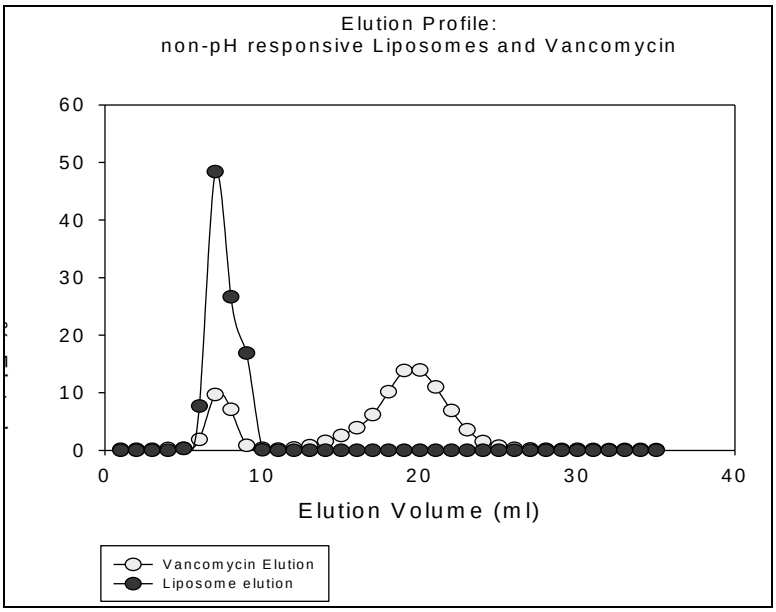

Figure 2a: Elution profile for non pH-responsive liposomes containing Vancomycin. Lipid and Vancomycin were detected independently. Column G50 for DSPC liposomes and 4B for 21PC and Egg-PC liposomes

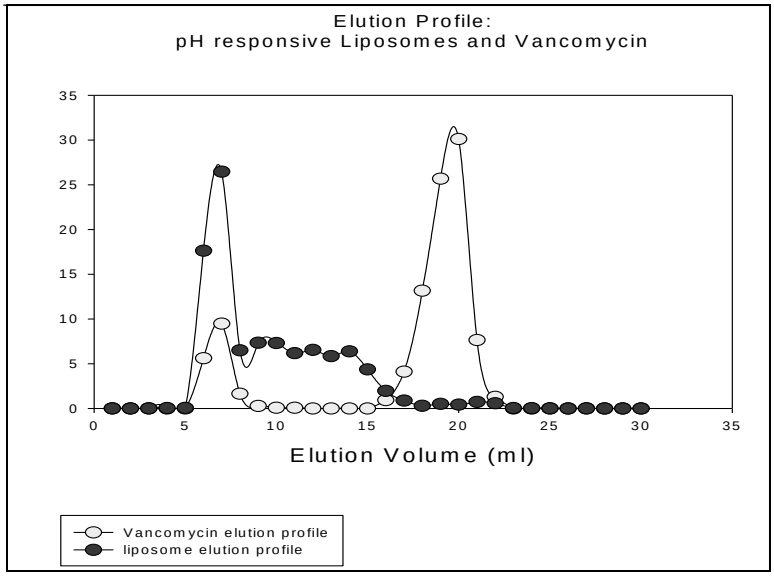

Figure 2b: Elution Profile for pH responsive Liposomes containing Vancomycin. Lipid and Vancomycin were detected independently on 4B Columns

As is clearly seen from the graphs, liposomes were eluted in the 6^{th}, 7^{th} and 8^{th} fractions from the column. Thus, 3 mL of the solution was collected after every purification run on the G50 or 4B columns.

3.2: Drug Encapsulation

Drug encapsulation takes place by passive diffusion through the liposomal surface. As such encapsulation efficiency of the drug needs to be studied to determine the total amount of drug encapsulated by the liposomes. The best liposomal composition would be the one with highest efficiency. Figure 3 summarizes the comparison of loading efficiency of different compositions.

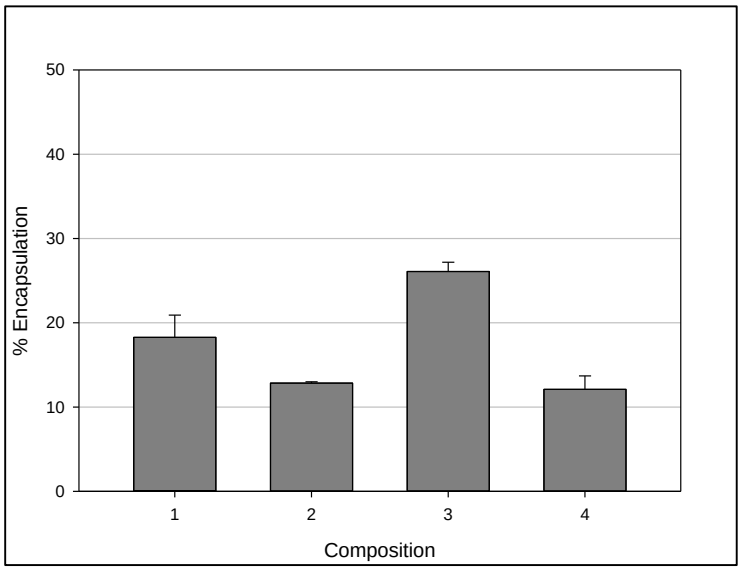

Figure 3: Comparison of Percent Encapsulation of Vancomycin for Different Compositions. Errors correspond to standard deviations of n independent liposomal preparations. Compositions 1, 2, 3 and 4 correspond to DSPC liposomes, Egg-PC liposomes, 21PC liposomes and 21PC:DSPA liposomes respectively.

Table1: Encapsulation efficiency of different liposomal compositions

Number	Composition	% Encapsulated	N
1.	68.6%:29.4% DSPC:Cholesterol +2% PEG	18.26 +/- 2.64	3
2.	66.5%:28.5% Egg-PC:Cholesterol + 5% PEG	12.84 +/-0.16	2
3.	66.5%:28.5% 21-PC:Cholesterol + 5% PEG	26.09 +/-1.10	2
4.	56%:24% 21-PC:DSPA + 8% Cholesterol + 12% PEG	12.15 +/-0.65	3

After evaluating the data sets with Tukey test, it was observed that the p-values of compared means of the non-pH responsive liposomes was less than 0.01 ($p<0.01$) and hence it can be inferred that the difference in the compositions is statistically significant. Further, as seen from Figure 3, the non-pH responsive 21-PC liposomes had the highest encapsulation efficiency as compared to the other non-pH responsive liposomes. Hence, 21PC was selected as the rigid lipid for the formation of the pH-responsive liposomes.

Further, the concentration of Vancomycin in subsequent experiments will depend on the amount of Vancomycin encapsulated. Vancomycin throughout the experiments was added in the ratio of 94.3:50 mg w/w lipid:Vancomycin ratio. To calculate the amount of Vancomycin encapsulated, a 1 mL solution was taken, to which acetonitrile was added in 1:2 solution:acetonitrile ratio. Acetonitrile was added to disassemble the liposome membrane and release encapsulated Vancomycin. The absorbance obtained at 280 nm was then analyzed according to the parameters obtained from standards to give the exact quantity of Vancomycin.

Table 2: Amount of Vancomycin Encapsulated

Composition of Liposomes	Amount of Vancomycin encapsulated
21PC+Cholesterol+18-PEG	990µg+/-15
DSPA+21PC+Cholesterol+18-PEG	450µg+/-15

To determine whether the liposomes could encapsulate more Vancomycin, the initial amount of Vancomyicin added to the liposome mixture was increased to 50 mg. Also, the incubation time with Vancomycin at room temperature was varied. However, each time only a pre-determined amount of Vancomycin got encapsulated resulting in an overall decrease in efficiency. Thus, about 1 mg and 0.5 mg of Vancomycin was encapsulated in the non pH-responsive and non pH –responsive liposomes each.

3.3 Release Studies

The release studies were necessary to determine the exact time of profile of drug release from the liposomes. For the liposomes to be effective, it is necessary for the liposomes to retain at pH 7.4 and release gradually at pH 6.0.

Figure 4 shows the release profile for non-pH-responsive EggPC:Cholesterol liposomes.

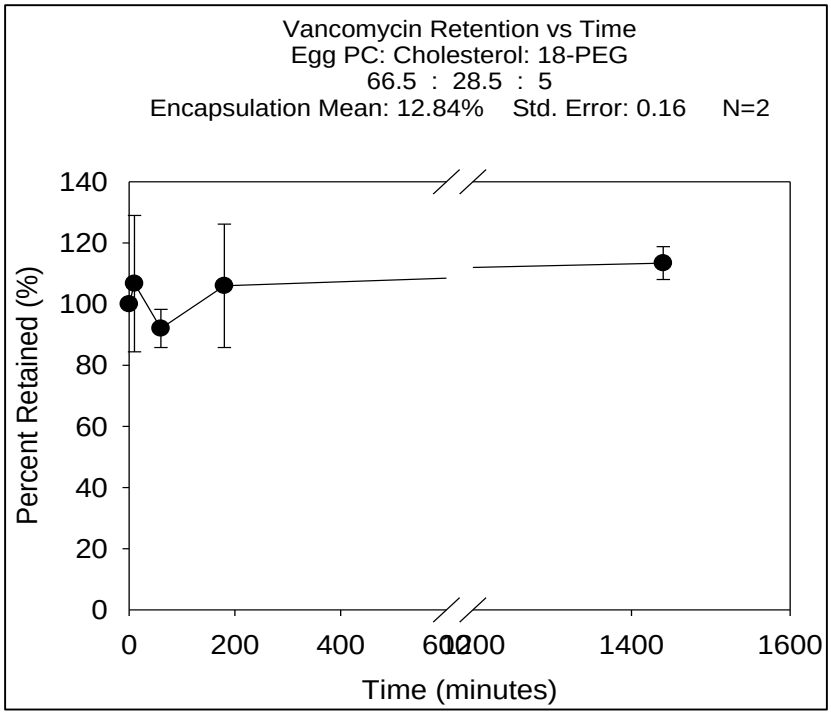

Figure 4: Release Profile of EggPC:Chol liposomes at physiologic pH 7.4. Errors Correspond to standard deviations of repeated measurements (n=2)

The release profiles for the Egg-PC liposomes demonstrate relatively stable retention over time. However, the liposomes were prone to aggregation and hence further use was restricted. When Egg-PC was substituted with DSPC, release profile is depicted in Figure 5, the retention of Vancomycin decreased over time. Retention decreased to 60% after 24 hours of incubation.

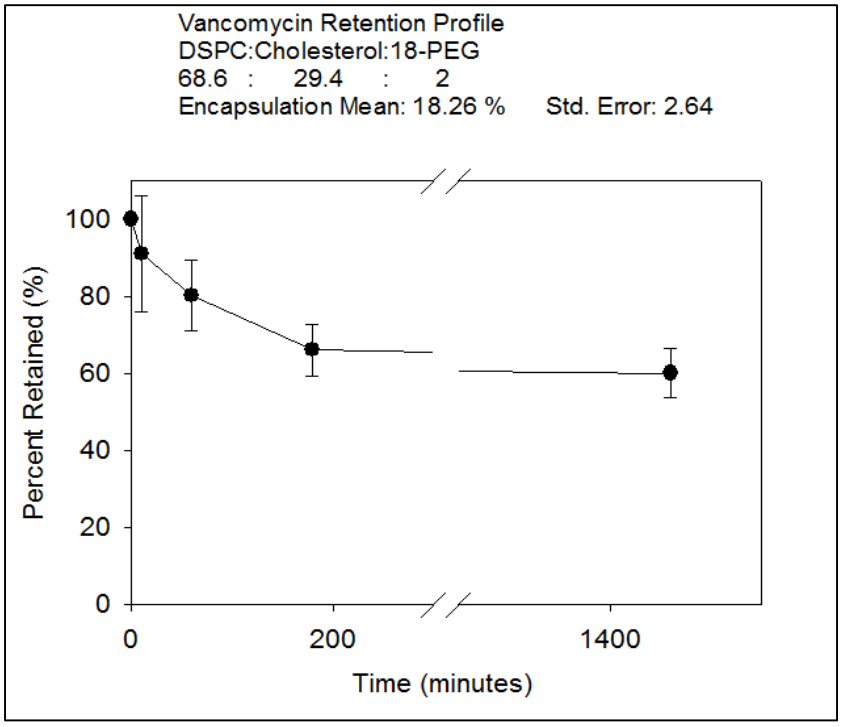

Figure 5: Release Profile of DSPC:Chol liposomes at physiologic pH 7.4. Errors Correspond to standard deviations of repeated measurements (n=3)

Figure 5 shows that the concentration of Vancomycin in the liposomes at each time point followed a logical path. However, complete retention of drug at pH 7.4 is required so as to maximize drug retention at physiologic pH and have maximum effect at the infection site. The DSPC:Chol liposomes start leaking within 10 minutes of incubation and release about 40% of their contents at pH 7.4 in 24 hrs. The DSPC was substituted with 21PC and the results analyzed. The release profile for the 21PC:Chol liposomes is as shown in Figure 6a.

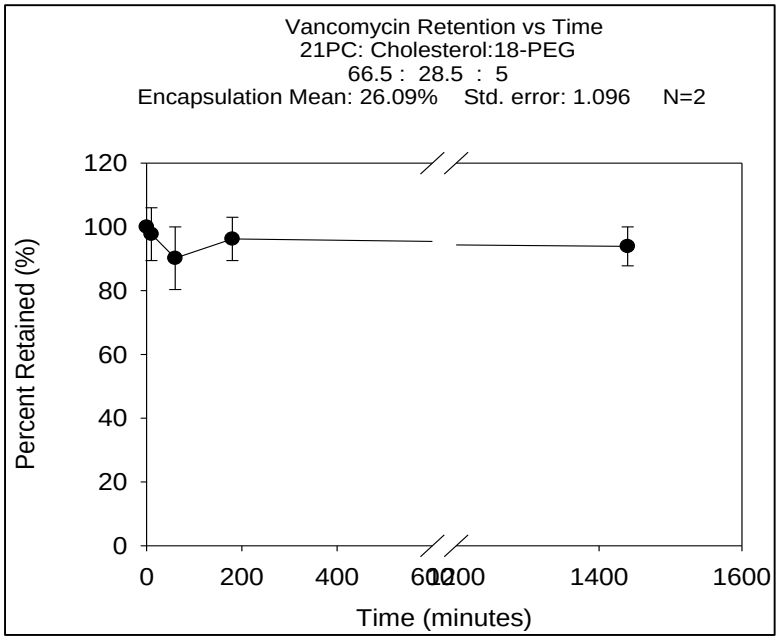

Figure 6: Release Profile of 21PC:Chol liposomes at physiologic pH 7.4. Errors Correspond to standard deviations of repeated measurements (n=2)

From Figure 6a it can be seen that 21PC liposomes offer the ideal vesicles for transport of Vancomycin. Even at 24 hours almost all of the Vancomycin was retained by the liposomes at pH 7.4. Thus, it can be concluded that the liposomes will deliver Vancomycin with maximum retention through the blood stream (pH 7.4) to the site of infection. However, PC-Cholesterol liposomes by themselves have no infection site specific attributes and would not show maximal effect at infection site. Thus, to incorporate pH sensitivity, PC-PA liposomes were synthesized and tested at both pH 7.4 and pH 6.0. The release profiles of the same are depicted in Figure 7.

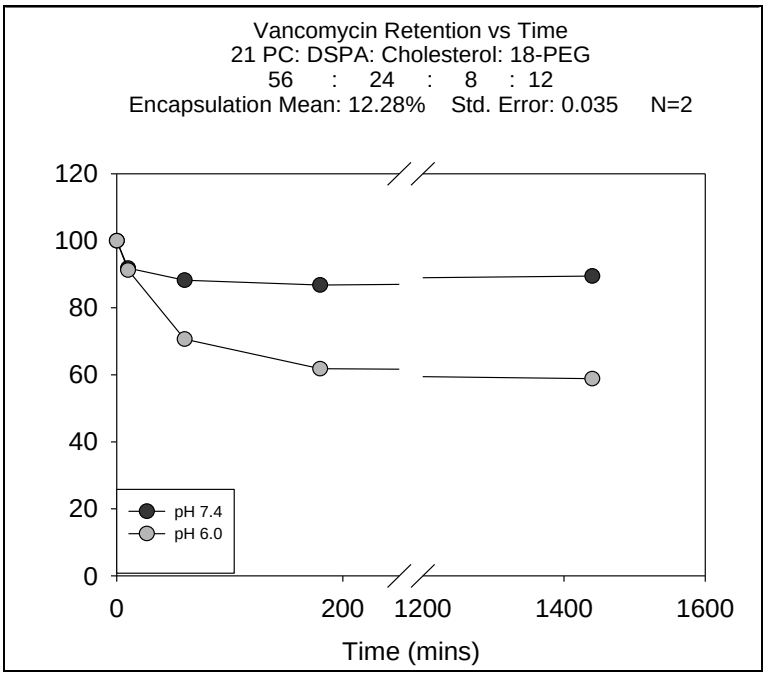

Figure 7: Release Profile of DSPA:21PC liposomes at physiologic pH 7.4 and acidic pH 6.0. Errors Correspond to standard deviations of repeated measurements (n=2)

DSPA equips the liposomes with pH sensitivity. As seen in Figure 7, the liposomes have minimal release (~90%) at pH 7.4. On the other hand, almost 40% of the contents were released at pH 6.0 at 24 hours. Hence, it can be concluded that the liposomes release at a slow rate at acidic pH. This is desired. Thus, the PC-PA liposomes were used for the antimicrobial studies.

3.4 Bacterial Growth

To propagate bacteria, it is important to understand growth kinetics. The Growth curve of *S. aureus* is as shown in Figure 8.

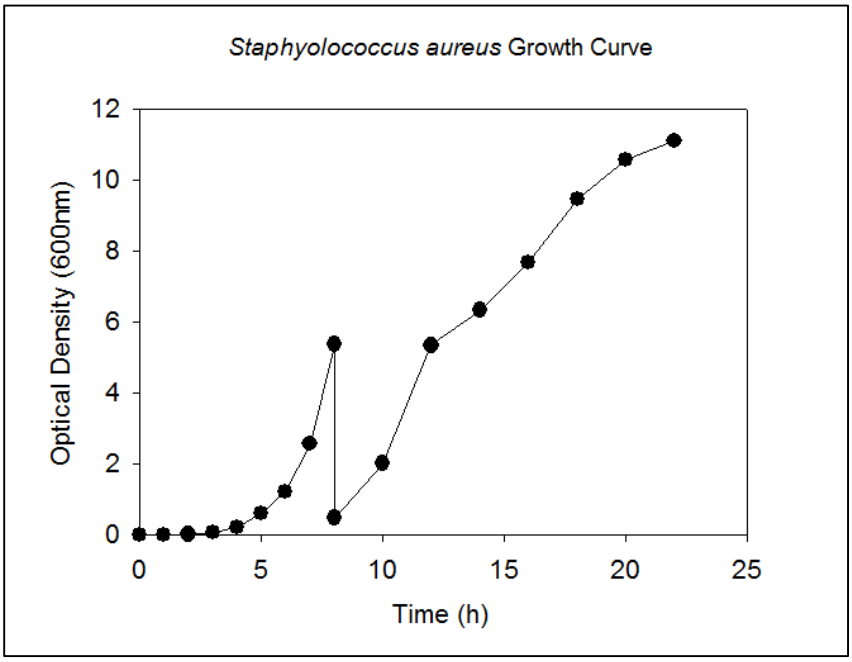

Figure 8: Growth Curve of *Staphylococcus aureus.* A drop in OD after 8 Hours was observed because the 50 ml bacterial culture was transferred to 500 ml of medium indicating dilution of the bacterial concentration by 11 times. Thereon, bacteria followed exponential growth with minimal lag phase till the establishment of stationary phase (as they were already accustomed to the surrounding media)

Bacterial colonies were observed as circular masses on the surface of the agar. A single colony is then picked up for further propagation. A single colony ensures homogenous characteristics of the cells (include why). In the initial culture the lag phase extended to about 4 hours. Whereas in the 500 mL culture the bacteria entered log phase almost immediately. The initial log phase was achieved at an OD_{600} of 0.3 in approximately 3 hours. The bacteria were therefore harvested at this point for the antibacterial experiments.

3.5 Antibacterial Studies

To calculate the MIC of the liposomal preparations, the 96 well plate dilution method was used. The Vancomycin concentration was determined according to the antibiotic encapsulated within the liposomes at the start of the experiments. Serial dilutions of free Vancomycin, PC:Chol liposomes and PC:PA liposomes were prepared. Incubations for all formulations were under the same conditions. The pH of the bacterial-liposomal solution was checked during the 18 hour of incubation time. No change in pH throughout the incubation period was observed. The killing curves of all three formulations are depicted in Figure 9, Figure 10 and Figure 11.

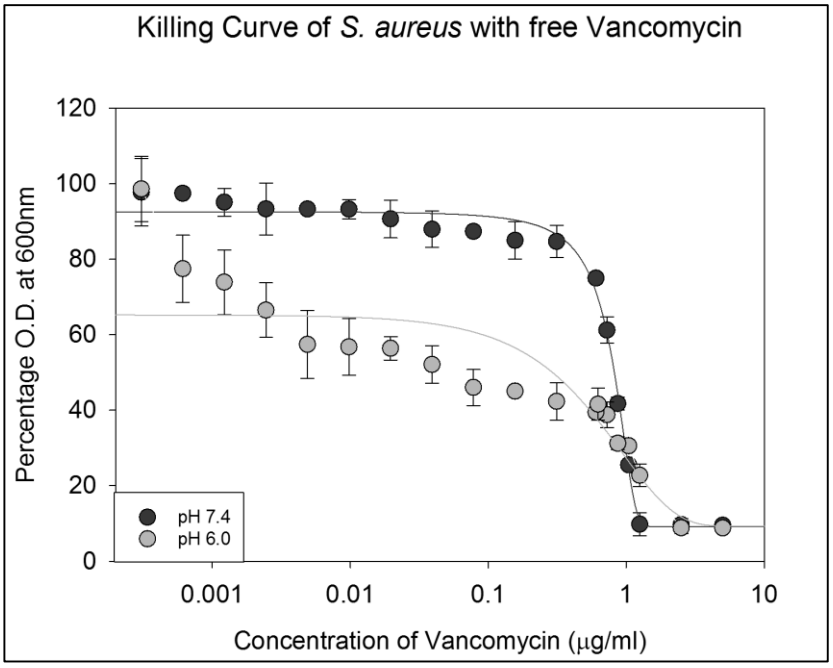

Figure 9: Killing Curve of S. aureus with free Vancomycin at pH 7.4 and pH 6.0. Errors correspond to standard deviations of repeated measurements (n=4)

As observed from Figure 9, the MIC for free Vancomycin was calculated to about 2 +/- 0.23 µg/mL at pH 7.4. However, the MIC increased by two-folds at acidic pH. At pH 6.0, the MIC was observed to be 3.98 +/- 0.16µg/mL.

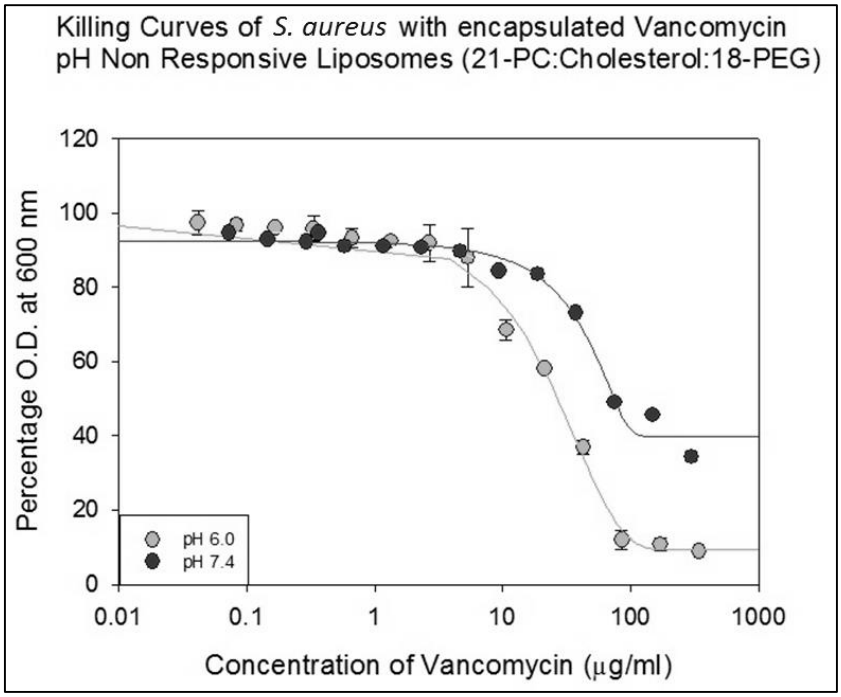

Figure 10: Killing Curve of S. aureus with non pH-responsive liposomes. Errors correspond to standard deviations of n measurements (n=4)

Experiments for MIC of 21PC:Chol liposomes at pH 7.4 did not yield satisfactory results. Going by the trend of the curve it can be assumed that MIC would be more than 1000 µg/mL. The MIC visibly decreased in acidic conditions going down to a 90 µg/mL.

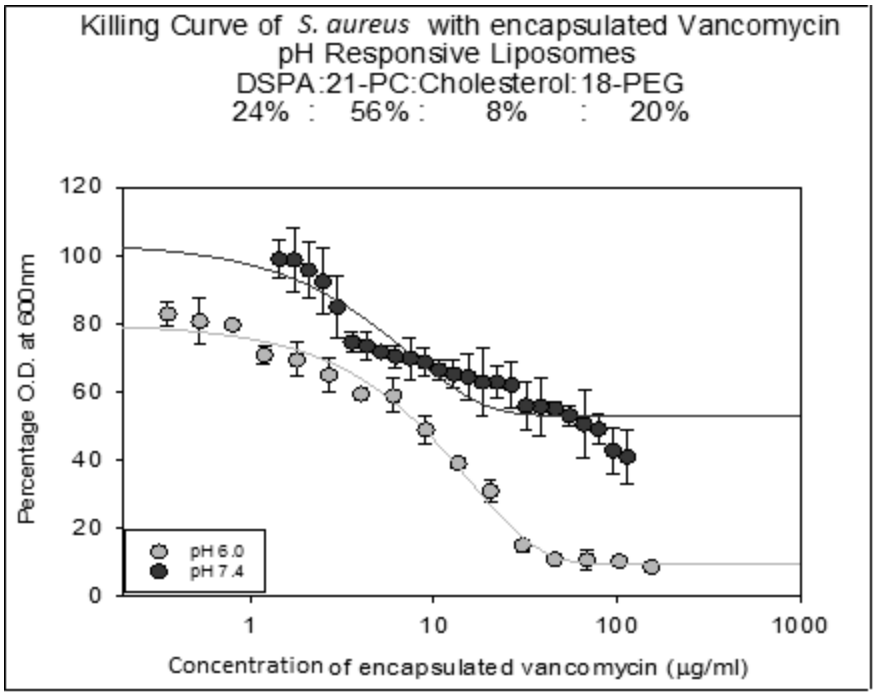

Figure 11: Killing Curve of S. aureus with pH responsive liposomes. Errors correspond to n measurements (n=4)

As seen in the figure, even at very high concentrations of Vancomycin complete inhibition of the bacteria was not possible at pH 7.4. But at pH 6.0, MIC was as low as 43 +/- 1.8 µg/mL which is almost half of the non pH-responsive liposomes.

SECTION 4: DISCUSSION

4.1 Formulation

Stability of Vancomycin in different solutions was tested. Vancomycin was prepared in PBS, distilled water and TSB. They were stored at 4°C. TSB preparation lost its activity after two weeks. However, the other two preparations were viable till almost 3 months, with the PBS preparation outliving the D/W preparation. The PBS preparation was used for almost all the experiments to maintain osmolality. However, the distilled water preparation was used once for antimicrobial studies at pH 6.0. The results obtained were similar to those obtained for the PBS preparation.

The liposomes were at first prepared by the passive encapsulation method. On rehydration of film with Vancomycin solution and subsequent incubation a thick viscous mixture with blobs was produced. Analysis showed very little Vancomycin encapsulation. A lot of the Vancomycin remained in the solution itself. Thus, a new modified dehydration-rehydration method was adopted for their preparation. This method had shown much success previously in the works of Mupidi et.al. and Mugabe et.al. In this, the completely formed liposomes are disrupted during lyophilization and then reconstructed during rehydration with a concentrated solution of the drug[11]. The drug gets encapsulated in the Dried Reconstituted Vesicles (DRV's) with a high efficiency. The liposomes prepared by this method did not develop any of the previously seen undesirable characteristics. The amount of Vancomycin introduced for rehydration of lyophilized liposomes was determined according to the lipid: Vancomycin w/w ratio used by Mugabe et.al.[11] and Muppidi et.al.[12]. Mugabe et.al. achieved an encapsulation of 13% with DSPA:Chol liposomes[12]. The encapsulation of liposomes prepared for the present study was comparable, and in

some cases, better than that. As seen in results, the encapsulation achieved by 21PC liposomes was the highest reaching up to 26% for non pH-responsive and 12% for pH responsive liposomes.

Nonetheless, the encapsulation efficiency needs to be improved some more. Increasing the amount of initial Vancomycin during rehydration had no effect. The amount been encapsulated remained the same and the relative efficiency decreased. Thus, it can be concluded that the liposomes formed under the given conditions are capable of encapsulating only a certain amount of solute. One of the reasons for this could be the presence of multilamellar liposomes during the rehydration process. Further, sucrose concentration during lyophilzation can also be changed to determine its effect on extent of encapsulation. Conversely, other methods could also be used for liposome preparation such as the emulsion method, the pH gradient method, the electrostatic association method etc.

4.2 Liposomal Characteristics

Apart from encapsulation, release profiles of liposomes would be the most important characteristic determining their potential as a therapeutic formulation. As seen in Results, the release profile for Egg-PC liposomes were riddled with problems on account of their tendency to aggregate. This preparation couldn't be used further on and hence, Egg-PC was replaced with DSPC. DSPC liposomes had good encapsulation efficiency, however, when incubated at 37°C at pH 7.4 they released about half of their contents within 24 hours. This was not advantageous. Minimal release at pH 7.4 is desirable to achieve maximum retention in blood stream and provide maximum release/supply of drug at the localized infection sites. DSPC was replaced by 21-PC. The non pH-responsive formulation of 21PC yielded a desirable release profile. Almost all of the drug was retained in the liposomes at

24hours. This would lead to the conclusion that minimal drug loss would be expected in-vivo making this the most favorable formulation. The subsequent pH responsive liposomes were made with DSPA as the domain-forming lipid with titratable headgroups. The release profile showed almost 90% retention at pH 7.4. About 40% of the antibiotic was released in 24 hours at acidic pH. The profile showed slow and steady release. This is consistent with the release obtained by Radovic-Moreno et.al. from their polymeric nanoparticles. This release over an elongated period of time is desirable as for the formulation to be effective against localized infections it needs to have a larger Area Under Curve (AUC) at the site of infection in order to facilitate Liposome-bacterium interactions and provide antibiotic over a larger period of time. Thus, it can finally be concluded that 21PC:DSPA liposomes had the highest encapsulation and desired release.

Once, the raw lipids for the liposome synthesis were decided, it was necessary to understand their stability over prolonged periods of time. Both the 21PC preparations along with DSPC liposomal preparations were stored at 24°C. After a period of two weeks, cloudiness started developing in the DSPC liposomal preparation indicating that the liposomes may not be stable. Vancomycin analysis of the 21PC liposome showed very little loss of antibiotic from liposomes. Also, they were active against bacteria after 2 weeks of storage. However, loss of activity was observed after 3 weeks of storage. Thus, for all purposes these liposomes could be considered to be stable at -18°C and 4°C for up to 2 weeks.

One of the reasons for 21PC liposomes to be so effective is because of the lipids that go into their formation. 21PC and DSPA are long saturated lipids with high transition temperatures which ensures lipid stability in the liposomes. Furthermore, DSPA is anionic

and PC zwitterionic at physiological pH causing electrostatic repulsion amongst them leading to a homogenous liposome surface. This ensures a stable retention of antibiotic at pH 7.4. At acidic pH, DSPA starts getting non-ionized and the liposome becomes cationic. DSPA is a domain forming lipid, hence, as and when it gets protonated, the electrostatic repulsion amongst the two lipids decreases leading to domain formation[13]. Antibiotic might be gradually released from areas around these domains at a steady rate. The lipid characteristics explain the higher encapsulation and the slow release profiles of these liposomes.

Even after the advantages conferred on the liposomes by virtue of the constituent lipids, the liposomes may still not be effective. They may get cleared from the body before ever reaching sites of infection. Thus, PEG is used in the preparation. Due to its hydrophilicity it can be assumed that during synthesis, PEG would rise and form the outermost layer of the liposome. PEG ensures longer circulation times preventing non-target interactions and increases the probability for site-specific targeting.

Therefore, it can be concluded that the charge switching capabilities of the liposomes along with surface PEGylation would be the reason for the effectiveness of the system and its desirable characteristics.

4.3 Anti-microbial Studies

Bacteria are hard to eliminate due to the presence of a cell wall, especially Gram-positive bacteria which have an extremely thick peptidoglycan layer. Therefore, the easiest way to kill bacteria is to attack the cell wall. *S. aureus* is an extra-cellular organism, but it has been known to invade mammalian cells and cause infection. Vancomycin is one of the

antibiotics which has shown success against *S. aureus* even after the organism developed resistance against other antibiotics. Vancomycin is a glycopeptide which acts against the NAM-NAG polypeptides and prevents formation of cell wall making the bacteria highly susceptible. However, in case of localized internal infections, Vancomycin falls short of killing the bacteria. In the case of internal infections, a region of acidic pH develops around the bacteria due to a number of reasons which include, but are not limited to, anaerobic metabolism and production of acidic by-products[5]. Vancomycin loses much of its activity in such acidic environments. Moreover, it's difficult for the antibiotic to penetrate mammalian cells and eliminate the bacteria. Due to its advantages as an antibiotic and its growing resistance, it was chosen for this study.

S. aureus like most bacteria have a negatively charged cell wall and inhabit positively charged environments. Thus, delivery vesicles which could exploit this were needed. Liposomes have been shown to enter mammalian cells, especially phagocytic cells, with ease [10]. Liposomes in themselves are negatively charged due to the constituent lipids. However, they can be made environmentally sensitive by introducing titrable head groups. Hence, 21PC:DSPA liposomes were chosen to carry Vancomycin to the bacterial cells.

The results of antimicrobial studies proved that the liposomal formulation was indeed effective against *S. aureus*. Amongst all the tested formulations, free Vancomycin emerged as the most potent having an MIC of 2+/-0.23 µg/mL. However, the MIC rapidly increased at pH 6.0 going up to 3.98+/-0.16µg/mL. This was almost a two-fold increase. This result was consistent with the MIC's obtained for free Vancomycin by Radovic-Moreno et.al.[5]. For non pH-responsive liposomes the MIC was obtained to the tune of 1000µg/mL and 90µg/mL at pH 7.4 and pH 6.0 respectively which stands true according to the release

profile of the 21PC:Chol liposomes. The MIC decreased as compared to the increase observed for free Vancomycin. The results were favorable, however, it was still too high. This makes sense since the non pH-responsive liposomes release very little Vancomycin at pH 7.4 and at 18hrs a higher concentration of Vancomycin would be required to kill the bacteria. The pH responsive liposomes showed an MIC of 45µg/mL at pH 6.0 and at pH 7.4 complete inhibition was not observed even at very high concentrations. Again, according to the release profiles for 21PC:DSPA liposomes this trend is justifiable. 21PC:DSPA liposomes release very little antibiotic at pH 7.4 and almost half of the encapsulated antibiotic at pH 6.0 in the same time. Hence, the decrease in MIC. Furthermore, the MIC is lower than that obtained for 21PC:Chol liposomes at the same pH. The MIC's obtained were much higher than that obtained by Radovic-Moreno et.al. for polymeric nanoparticles. But, it can be argued that liposomal nanoparticles have very low toxicity and pose little threat to the body. Also, there are similarities between their surface and eukaryotic cell membrane surfaces which guarantees easier penetration into infected cells thus proving their superiority over polymeric nanoparticles. Figure 12 shows the comparison of MIC's of different formulations.

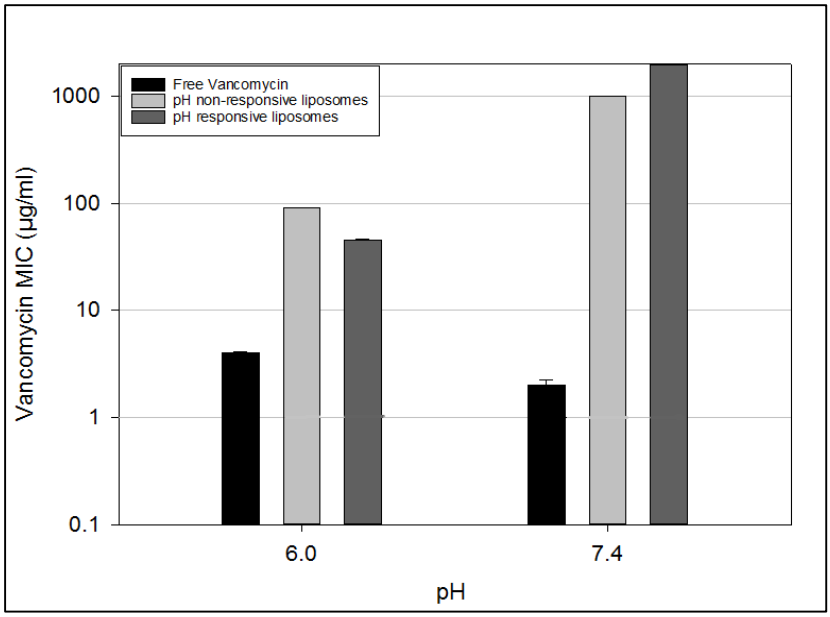

Figure 12: Comparison of MIC obtained for free Vancomycin, non pH-responsive liposomes and pH responsive liposomes. Errors correspond to N measurements

A Tukey test analysis of the MICs of different compositions yielded a p-value of less than 0.01 showing that the values were statistically significant in a 99% confidence interval. Further, as can be seen from Figure 12, PCPA liposomes emerged as the most effective formulation for Vancomycin delivery and subsequent elimination of MRSA. Apart from relative decrease in MIC of the liposomal formulation, it also provides the advantage of a larger AUC and longer circulation times. For complete treatment of MRSA, a ratio of AUC of release over 24 hrs to the MIC should be ≥400min[12]. The ratio obtained in this experiment was up to 2000min. The slow steady release over a period of 24 hours ensures complete elimination of the bacteria. Thus, it can be concluded that liposomal encapsulation helps in alleviating the loss of activity faced by Vancomycin at acidic pH.

Even though the experiments showed positive results, the experiments themselves were riddled with several drawbacks. At the start of the study, evaporation from the plates was a major problem. The media from the edge wells would get evaporated within hours of

incubation at 220rpm resulting in erratic readings. To overcome this, beakers of water were placed all around the plates. This resulted in increased humidity of the incubator. However, the wells farthest from the air dispenser of the incubator were still affected by evaporation. This might be because there were no beakers placed on the other end of the incubator. The rotation speed was lowered to almost 100rpm and a combination of lowered speeds and increased humidity finally resulted in negligible evaporation. However, the low rotation speeds translated to a decrease in the rate of growth of the bacteria and hence the results couldn't be conclusive. Some studies suggested the use of Hermetic containers or humidity chambers with a level of water in it and the plates placed over it. However, due to the small size of the incubator, such chambers couldn't be placed in it. Finally, a modification of the same was used. The plates were placed in a pipette tip box. The plates were covered with tissue or cloth soaked in water. This led to a decrease in evaporation and media levels remained constant in all wells. Figure 13 shows the humidity chamber proposed by Walzl et.al.[14].

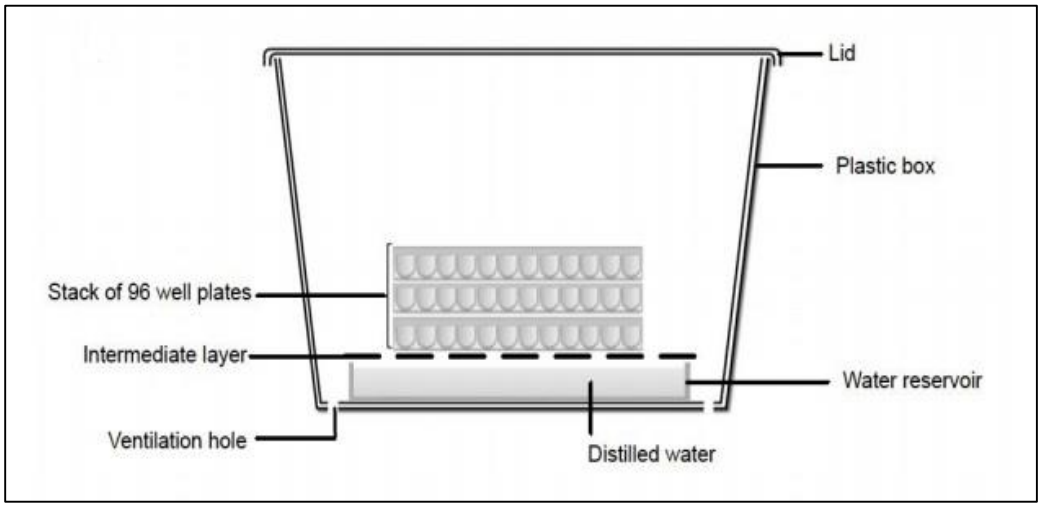

Figure 13: Humidity Chamber [14]

SECTION 5: CONCLUSION

The study showed that Vancomycin delivery by liposomes to localized internal MRSA infection sites is a promising alternative to administering the antibiotic directly. 21PC liposomes, both non pH-sensitive (21PC:Cholesterol) and pH sensitive (21PC:DSPA) were tested for their encapsulation, release and antimicrobial activity. Results showed high encapsulation by liposomes prepared by dehydration-rehydration method. Almost no release at physiological pH and slow and steady release over a period of at least 24 hours at acidic pH was observed. The antimicrobial experiments showed that the MIC of the PCPA liposomes decreased by a large extent as compared to the higher MIC of 21PC:Cholesterol liposomes and the 2 fold increase of MIC of free Vancomycin at pH 6.0. Thus, it was concluded that the liposomal formulation can overcome loss of activity of Vancomycin at acidic pH. Further work regarding the encapsulation efficiency of liposomes needs to be conducted. Other types of lipids or different methods of synthesis should be explored. Tests regarding the exact nature of liposome-bacteria interactions can be conducted to improve efficacy. Future work may comprise of testing the validity of this system in-vivo. All in all, this proof-of-concept study exploited the natural characteristics of bacteria with promising results and hence it can be applied for delivery of various other drugs to a myriad of bacterial infection sites.

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