DEVELOPING LIPOSOMES FOR ANTIBIOTIC ENCAPSULATION

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Lay Abstract

When antibiotics are administered, orally or intravenously, they should pass through different tissues to arrive to the site of infection; this can cause dilution and/or intoxication. To overcome these problems, drug delivery vehicles have been used to encapsulate and deliver antibiotics, improving their therapeutic index while minimizing their adverse effects. Liposomes are vesicles composed of at least one lipid bilayer, with an inner aqueous compartment. Liposomes are an attractive vehicle to deliver antibiotics because they can encapsulate both hydrophobic and hydrophilic antibiotics, they have low toxicity, and they can change the bio-distribution of the drug. In my thesis, I addressed two main questions regarding liposomal antibiotic encapsulation: (1) will liposome preparation method affect encapsulation efficiency of antibiotics, and (2) does liposome preparation method adversely affect the efficacy of antibiotics. While investigating these questions, I also identified certain outstanding biases in the liposomal characterization methods.

Abstract

Liposomes are self-assembled lipid vesicles made from phospholipids that are safe and suitable for drug encapsulation and localized drug delivery. Liposomal formulations are characterized by low toxicity and improved therapeutic index (by changing drug biodistribution) and liposomes encapsulating antifungal or anticancer drugs have already been approved by regulatory agencies.

One area of application for liposomes is localized antibiotic delivery. Antibiotics target bacteria, but specific types of infections (namely biofilms or intracellular infections) that required high or prolonged antibiotic administration have long been a challenge for antibiotic treatments. Liposomal delivery of antibiotics can improve their therapeutic index while minimizing their adverse effects. When it comes to methods of antibiotic encapsulation, however, most reports to date follow the methods developed for anticancer drugs for encapsulating antibiotics. This oversight causes discrepancies in the literature, mainly because of the significantly different chemical structures of antibiotics and cancer drugs. Furthermore, most antibiotics are highly sensitive to temperature fluctuations, which is concerning, given most liposomal preparation methods involve extreme temperature fluctuations. The aim of my thesis was to explore these missing links in the literature by answering these questions: (1) will liposome preparation method affect encapsulation efficiency of antibiotics? And (2) does liposome preparation method adversely affect the efficacy of antibiotics? Investigating these questions led to further insight into the optimal process for achieving high encapsulation efficiencies for different antibiotics and for further avoiding damage due to harsh processing conditions. We found that different preparation methods are better for different types of antibiotics, being the one that promotes a large aqueous space better for hydrophilic drugs and the one that creates oligolamellar and large unilamellar vesicles better for more hydrophobic drugs. The steps in liposome preparation methods such as heating and sonication can affect the stability of the antibiotics.

Acknowledgements

I would like to express my deepest gratitude to my supervisor Prof. Zeinab Hosseini-Doust for her support and guidance as well as her invaluable encouragement and her endless energy. I would also like to thank Chenchen Xu, Saif Syed and Kenji Marshal and for their great work and their friendship. Nikki Henriquez, Andy Duft and my HPLC friend Jalees Nasir for always having a great attitude and helping to troubleshoot, even at 1 am in the morning.

I also want to thank all the Biohybrids lab members for their help with the science as well as their friendship and understanding the fact that I see liposome shapes everywhere, specially thanks to Ximena, Claudia and Lei who helped with schematics and some experiments during hectic times.

Last but not least, thanks to my amazing brother for teaching me how to enjoy my life and my work, thanks to both my parents for being supportive, proud and lovely.

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Declaration of Academic Achievement

The experiments reported in this thesis have been designed jointly by myself and my supervisor and carried out in dependently by myself. Furthermore, I independently carried out all the troubleshooting experiments. The following undergraduate interns helped with portions of the lab work, under my direct supervision: Saif Syed, Kenji Marshall, Aileen Liu. Experiments in Chapter 3 where mainly performed by my undergraduate intern, Chenchen Xu, under my direct supervision. I performed data analysis independently and all figures have been prepared by myself, after being designed jointly by my supervisor and myself. The text was written by me and edited by my supervisor.

Chapter 1 - Introduction

1. Introduction to Liposomes

1.1. What are liposomes?

Liposomes are vesicles composed of at least one phospholipid bilayer, with an inner aqueous compartment (**Figure 1.1a**). Liposomes and micelles are both comprised of fatty acid lipids; liposomes have an aqueous core whereas micelles have a hydrophobic core. In the case of liposomes, lipid bilayers are formed due to hydrophobic interactions between the lipid heads and van der Waals forces keep the hydrocarbon tails together; typically, liposomes are formed with phospholipids that have two tails. Micelles are formed with single hydrocarbon chain fatty acids; these fatty acids conform into a spherical shape for lesser steric hindrance. Components of liposomes resemble those of cell membranes; therefore, liposomes are biocompatible and biodegradable. Liposomes can encapsulate hydrophilic compounds in their core and hydrophobic compounds within the bilayer.[1] Liposomes were first described in 1965 by Alec D Bangham, a British hematologist at the Babraham Institute in Cambridge, while he was testing a new electron microscope by adding negative stain to dry phospholipids.[2]

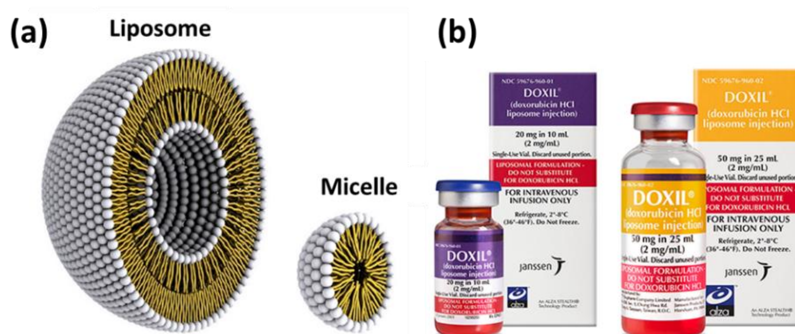

Figure 1.1. ***(a)*** *Liposomes and micelles are both formed of fatty acid vesicles.* ***(b)*** *Doxil, a liposomal formulation encapsulating doxorubicin HCL was the first FDA approved nanodelivered medicine against cancer.*

In 1971, Gregory Gregoriadis proposed the use of liposomes for drug delivery applications.[3] This was followed by the first studies about liposomal fate in the body conducted in the late 70's, where it was shown that liposomes could change the *in vivo* distribution of the drug in the body and promote cellular uptake of the drug.[4] The first injectable liposomal drug, AmBisome®, was made available in Europe in 1990.[5] Doxil® shown in **Figure 1.1b**, the first FDA approved nanodelivered medicine against cancer, was released in 1995 in the USA.[6] Liposomes were attractive as drug delivery systems to many scientists since their discovery due to the low toxicity of their components, the ability to load hydrophilic[7] and hydrophobic[8] cargo, as well as the capacity to create particles of different sizes.

1.2. Liposome building blocks

The building blocks of liposomes are phospholipids. Liposomes are assembled using the natural strong tendency of phospholipids to form membranes when dispersed in aqueous solution.[9] Lipid bilayers are formed due to hydrophobic interactions between the lipid heads and van der Waals forces keep the hydrocarbon tails together.[10] Ultimately, the liposome geometry is determined by the types and amounts of phospholipids used, the ionic and charge properties of aqueous medium, temperature, and hydration time.[11]

The first generation of liposomes was created solely using natural phospholipids, for example: lecithin[12] and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)[13]; however some inconveniences were found; for example, contents leaked out of the vesicles[14] and liposomes were rapidly moved to the liver and spleen increasing toxicity and reducing biodistribution.[14] To affront these problems, cholesterol was added to reduce the leakiness of the liposomes[15,16] and, more recently, PEG was added to the liposomes to increase their half-life circulation in the bloodstream.[17]

The efficacy of liposomes depends highly on the nature of their components, namely their size and charge. Therefore, in order to enhance the activity of liposomes, more components have been added to the original formulations, a variety of the components used nowadays are shown in **Figure 1.2**. Synthetic lipids, such as DOPS, have been used

to create negatively charged liposomes.[18] Charge induced lipids (DODAB, DOTAP) have been used to promote pH-triggered delivered.[19]

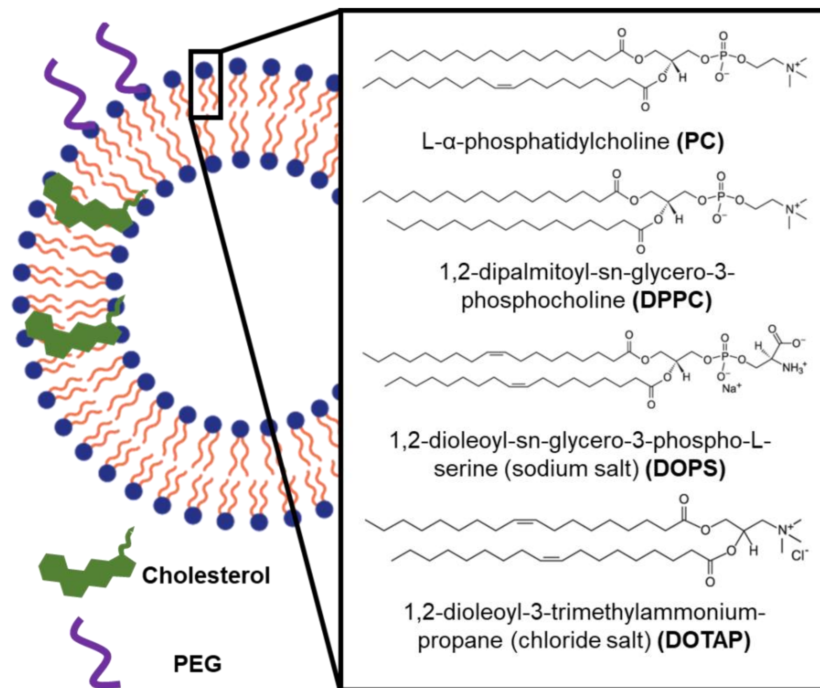

Figure 1.2. *Liposomes can be formed of different types of lipids: natural such as DPPC and PC; synthetic such as DOPS. Charged liposomes made of lipids like DOTAP can be used for specific applications like pH triggered release. Most liposomes contain cholesterol to avoid leakage of the content and PEG is frequently used to promote long blood circulation.*

1.3. Liposomes classification

Liposomes are typically classified in terms of their lamellae and size, or the method of their preparation. Liposomes, as shown in **Figure 1.3**, can have one lipid bilayer (unilamellar) or multiple lipid bilayers (multilamellar vesicles, MLV). Unilamellar vesicles can also be divided in two categories: small unilamellar vesicles (SUV), 25-100 nm in diameter, or large unilamellar vesicles (LUV), 100-400 nm in diameter.[20] Each type of liposome is advantageous for different applications. When encapsulating ketoprofen-cyclodextrin, a poorly soluble drug, it was found that the encapsulation efficiency is as

follows: MLV>LUV>SUV. However, the drug release rate was: SUV>MLV>LUV.[21] The selection on the type of vesicles highly depends on the intended application. Most of the pharmaceutically approved liposomes have diameters of 50–300 nm.[22] Additionally, SUVs typically have a long circulating life.[23]

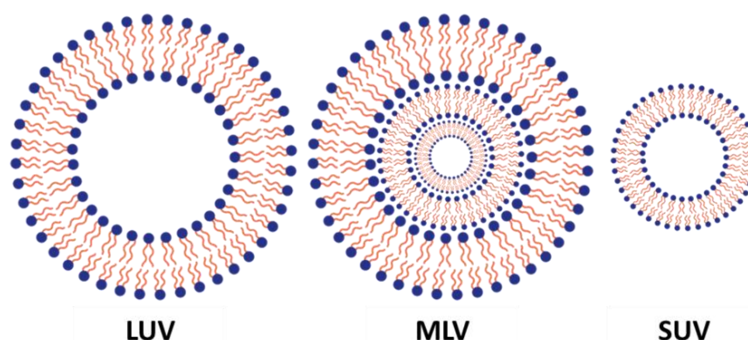

Figure 1.3. *Liposomes can be classified as small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), and multilamellar vesicles (MLV) each type of liposome has advantages and disadvantages for encapsulating different types of drugs.*

1.4. Liposomes preparation methods

The preparation of liposomes involves four main steps: drying a lipid film from organic solvents, dispersing the lipid in aqueous media, resizing the liposomes, and purifying the obtained liposomes. As demonstrated in **Figure 1.4**, multiple methods of preparation exist and the fundamental difference between them is the method of drying down the lipids and re-dispersing them in aqueous media.[24] The methods of preparation can be classified in three major categories: mechanical dispersion, solvent dispersion and detergent removal. Depending on the preparation method, different sizes and lamellarities can be obtained.

The optimal choice of liposome preparation method depends on: the physicochemical characteristics of the material to be entrapped, the desired size and polydispersity of the vesicles, the desired bilayer properties, and the possibility of upscaling. Banghman prepared liposomes using the method, now known as the "thin film method", in which a lipid thin film is formed by adding the lipids and solvents to a round bottom flask and

then evaporating the solvent. An aqueous phase containing the drug of interest is then added to the sample at a temperature above the Tm of the lipids so that they become fluid and liposomes can be produced.[2,25] This technique produces multilamellar vesicles of multiple sizes, and therefore it is fundamental to use size reduction techniques such as sonication or extrusion. Thin film techniques produce multilamellar liposomes, reducing the aqueous space inside. Other techniques have been developed to produce liposomes with a large aqueous core. Two very popular techniques that achieve a large aqueous core are: freezing and thawing and reverse phase evaporation.

The freezing and thawing technique consists on cycles of freezing the liposomes to low temperatures that can range from -20°C to -196°C and thaw them either at room temperature or at the T_m of the lipid.[26-28] It has been reported that repeated cycles of freezing and thawing, of pre-formed multilamellar liposomes, produces a disruption of the bilayer due to the formation of ice crystals during the freezing step, disrupting the closely spaced lamellae of the vesicles and thus increasing the aqueous volume.[28] A 10-50x increase in the internal volume of liposomes after freezing and thawing has been reported, likely as a result of the fusion of small vesicles to form bigger liposomes.[27]

Liposomes prepared using the reverse phase method are also known for producing a high internal aqueous volume. In this technique, an emulsion of micelles is prepared in organic solvents by sonication, which is then collapsed by drying the organic phase to get liposomes. This method produces large unilamellar vesicles.[9] This method in theory encapsulates hydrophilic compounds as well as hydrophobic compounds with high efficiency because it creates oligolamellar vesicles as well as large unilamellar vesicles with large aqueous volumes.

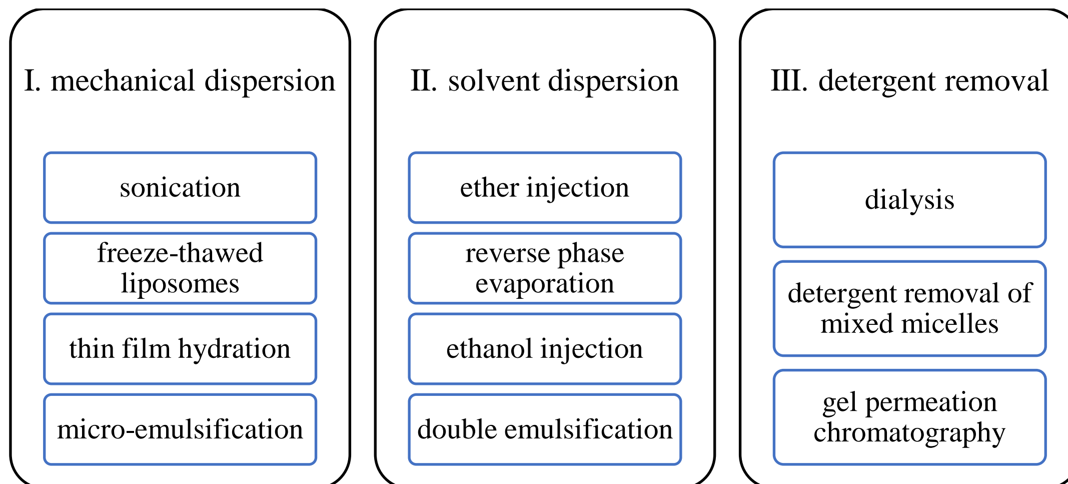

Figure 1.4. *Classification of liposome preparation methods. Each method creates different type of liposomes.*

2. Liposome Characterization Methods

2.1. Size and polydispersity

The size of the liposomes influences their final fate *in vivo* as well as their encapsulation efficiency, which can be measured using dynamic light scattering (DLS, **Figure 1.5a**),[29] electron microscopy techniques,[30] size-exclusion chromatography,[31] and tangential flow,[32] among other techniques.

2.2. Zeta potential

Zeta potential refers to the electrokinetic potential in colloidal dispersions. The zeta potential for a colloidal particle can be used as a good estimate of the stability of the colloidal system. Zeta potential provides a measure of the overall charge that a particle acquires in a particular medium and is largely dependent on pH and ionic strength of the medium. A large negative or positive zeta potential indicates that the particles tend to repel each other, and aggregation would be less likey.[33] Zeta potential can be measured using laser doppler electrophoresis. In this technique the movement of charged particles is measured in an electric field using the doppler effect (**Figure 1.5b**). Particle suspensions with zeta potential >20 mV or <-20 mV are considered stable.[34]

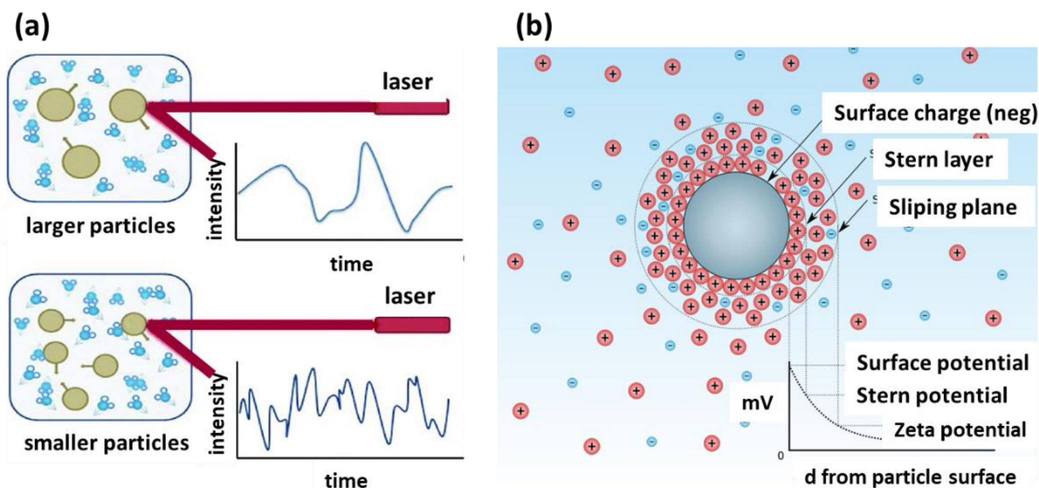

Figure 1.5. (a) *Dynamic light scattering (DLS) is a technique used to measure size and polydispersity. DLS works by illuminating a colloidal suspension using a laser beam and the scattered light is detected and correlated with size and polydispersity of a sample, image from Mike Jones. (**b**) Zeta potential can be used to determine the stability of a sample by measuring the tendency that the samples must repel each other, lase doppler electrophoresis is used to measure zeta potential, image from MJones1984l.*

2.3. Lamellarity

The number of lipid bilayers present in a liposome influence its encapsulation efficiency, release kinetics and intracellular fate, therefore. Liposome lamellarity can be estimated by adding reagents and measuring the change in visible light or fluorescence signal that the lipids emit.[35] For example, it can be accomplished by ^{31}P NMR whereby Mn^{2+} is added to quench the ^{31}P NMR signal of the exterior face of the liposomes.[26] Alternatives to this technique include the use of electron microscopy techniques like cryogenic TEM,[36] small angle X-ray scattering,[37] or adding fluorescently labelled lipids.[38]

2.4. Encapsulation efficiency

Measuring the quantity of material entrapped inside liposomes is necessary to study the behavior of the vesicles. Encapsulation efficiency is defined as the percentage of the drug added that is retained inside the liposome, $EE\% = \frac{total\ drug - free\ drug}{total\ drug}$ x 100. The first step for determining encapsulation efficiency is separating the free and encapsulated

drug; multiple ways of doing so have been reported. Since the size of the liposomes and the free drug are usually vastly different, one of the most popular methods is separation based on size using a mini-column loaded with sephadex (a filtration gel) with[39] or without centrifugation.[40] Dialysis separation[41], ultracentrifugation[42] and ultrafiltration[43] methods all follow the same principle and have also been reported.

The second step consists of finding the total drug in the system; two main methods have been described in the literature: one consists on the use of Triton-X, a detergent, to disrupt the lipid bilayer and allow the content to be released,[44] the other one is the use of solvents to disrupt the lipid bilayer.[41,45] The drug in each phase can then be analyzed by an appropriate detection method such as absorbance, fluorescence, or mass chromatography.

2.5. Cargo Release

As shown in **Figure 1.6**, drug release is typically quantified by using dialysis membranes of an appropriate molecular weight cutoff, whereby the pores should be large enough to allow the passing of the free drug but small enough to retain the liposomes. The buffer for the dialysis can be modified to better mimic the conditions pertaining to the purpose of the liposomes.[46]

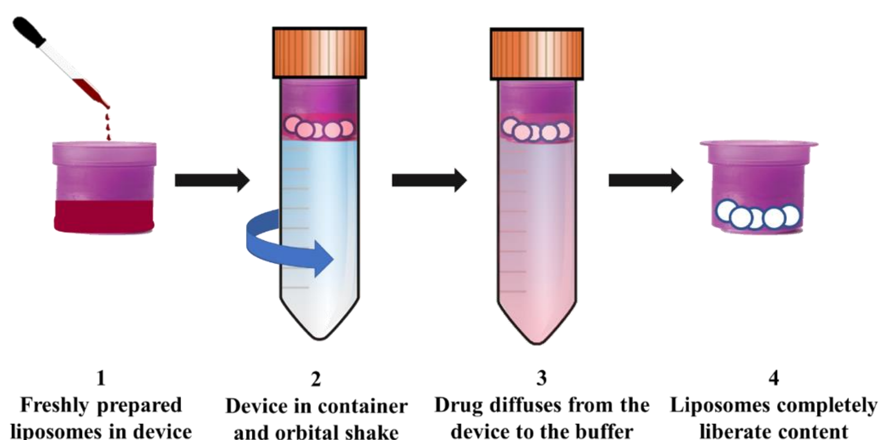

Figure 1.6. *Cargo release profile can be performed by (1) adding the liposomes to dialysis membranes of a cut-off smaller than the liposomes but bigger than the drug so that the drug can pass the membrane. (2,3) The system is subjected to shaking and*

samples are taken at specific times to measure the quantity of the drug that has been released. (4) At the end of the process the liposomes will have liberated all their content.

3. Drug Encapsulation

Over the years, different types of drugs have been encapsulated inside liposomes for various reasons: protection against enzymatic degradation,[47] drug targeting,[48] enhancement of drug solubility,[49] and enhancement of drug uptake,[50] among others. It is important to keep the application in mind when choosing the lipids and the methods that are to be used. Liposome formulations can be tailored to accommodate hydrophilic, lipophilic, charged or uncharged drugs, but it is important to remember that not all types of liposomes perform equally for encapsulate a certain drug.

3.1. Hydrophobicity

The tendency of the drug to interact through polar, non-polar and electrostatic interactions with lipid bilayers determines whether the drug would be incorporated mainly in the aqueous core or in the lipid bilayer. In the case of lipophilic drugs, they have a tendency to be incorporated into the lipid bilayer; therefore, a system that has multiple bilayers (MLV) has shown to be optimal to load this type of drugs.[51] Large unilamellar vesicles have a larger aqueous compartment in their core, making them optimal for a drug that is hydrophilic.[52]

3.2. Charge

A charged cargo can benefit from charged lipids to increase their encapsulation efficiency. Cationic drugs such as doxorubicin have shown to be better loaded in liposomes with negatively charged lipids. A combination of lecithin and PG (7:3M) entrapped a greater amount of doxorubicin when compared with liposomes prepared with lecithin alone.[53]

3.3. Cargo-liposome interaction

There are 4 types of cargo in terms of the interaction they have with the liposome lipid bilayers or core: (1) Water soluble + non bilayer interacting, (2) lipophilic + bilayer

interacting, (3) hydrophilic + electrostatic interactions with bilayer, (4) non water soluble + non bilayer interacting.

Water soluble + not bilayer interacting drugs would exclusively remain in the liposome aqueous core, resulting in their encapsulation efficiency being strongly tied to the total entrapped aqueous volume. This makes large unilamellar liposomes ideal, along with their encapsulation efficiency being relatively independent of the nature of the phospholipid used.[54] Encapsulation of lipophilic + bilayer interacting drugs depends on the total amount and length of the phospholipid used, and other bilayer components, such as cholesterol. For these type of drugs, they are better incorporated in fluid membranes since the fatty acid chain has more movement freedom.[54]

Hydrophilic drugs + electrostatic interactions with bilayer partition between the aqueous phase and the lipid compartments. They are a specific case since pH changes in the aqueous core are related to hydrophilicity changes, thus different pH in the preparation method signifies different partition of the drug in the liposomes. Encapsulation efficiency of tin mesoporphyrin, a competitive inhibitor of microsomal heme oxygenase, was at 90% when the system was at a pH of 5 and at 10% at a pH of 7.[55] If the interaction of the drug with the liposome is based on electrostatic forces, encapsulation is dependent on the bilayer-induced charge and the ionic strength of the aqueous medium.[54] Finally, drugs that are non water-soluble and non bilayer-interacting exhibit a very poor loading efficiency.

3.4. Biodistribution

When a drug is loaded into a liposome, its biodistribution changes, adopting that of the liposomes. When intravenously administered, liposomes are recognized as potential threats and cleared up by phagocytes of the reticuloendothelial system (RES), particularly in the liver and the spleen.[56,57] The rate of phagocytosis is affected by the liposome size, charge, lipids present in the bilayer as well as bilayer fluidity. Liposomes of 360, 230 and 120 nm were analyzed to determine the rate of removal from the bloodstream and it was found that liposomes of 120 nm exhibited the slowest removal rate.[58] Also, charged

liposomes show a higher rate of uptake than neutral liposomes.[59,60] Moreover, small size is sometimes necessary for liposomes to circulate through small intracellular compartments.[61,62]

Interaction of the liposomes with serum proteins highly depends on the lipid composition and the bilayer fluidity. Absence of cholesterol from the liposomes resulted in fast bilayer destabilization by high density lipoproteins.[63] To overcome rapid liposomal clearance, coating of the surface of the liposomes with immobile molecules has been developed; PEG, PHEA, PHEG have been used.[58,64] A periliposomal layer is formed by the flexible chains of the polymer (glycolipid or polyamoniacids). This layer hinders the interaction and binding between plasma proteins to liposomes, thus the phagocytic response is not started.[65]

4. Interaction of Liposomes with Cells Compared to Bacteria

Smaller size nanoparticles have been shown to reduce the minimal inhibitory concentration of some antibiotics, it is believed that this phenomenon is due to the increase in the drug loading inside the bacterial membrane.[66,67] Liposomes interact with the lipid bilayer of eukaryotic cells through four possible mechanisms: fusion, adsorption, lipid exchange or endocytosis, **figure 1.7**. Fusion happens when the liposomes are in close contact to the cell membrane, leading to intermixing of liposomal lipids with the lipids of the cell and allowing the cargo to enter the cell directly. Adsorption occurs when attractive forces such as electrodynamic interactions, van der Waals, hydrogen bonds exceed the repulsive forces between the liposome and the cell membrane and the liposomes are adsorbed within the cell membrane.[68] Lipid exchange consists of the liposomes exchanging lipids for the ones in the lipid bilayer of the cell membranes.[69] There are three proposed mechanisms to explain this phenomenon: (1) transfer of lipid monomers mediated by lipid exchange proteins that naturally exist in the cell surface. (2) The outer layer of the liposomes and the cell undergoes reversible transient merger. (3) Enzymatic exchange of acyl chain between the lipids of the liposomes and the host cell lipids.[4] Endocytosis occurs when phagocytes engulf the liposomes into endosomes, then

the endosomes produce phagosomes and the lysosomal enzymes inside the phagosomes degrade the lipids releasing the liposome content intracellularly.[70]

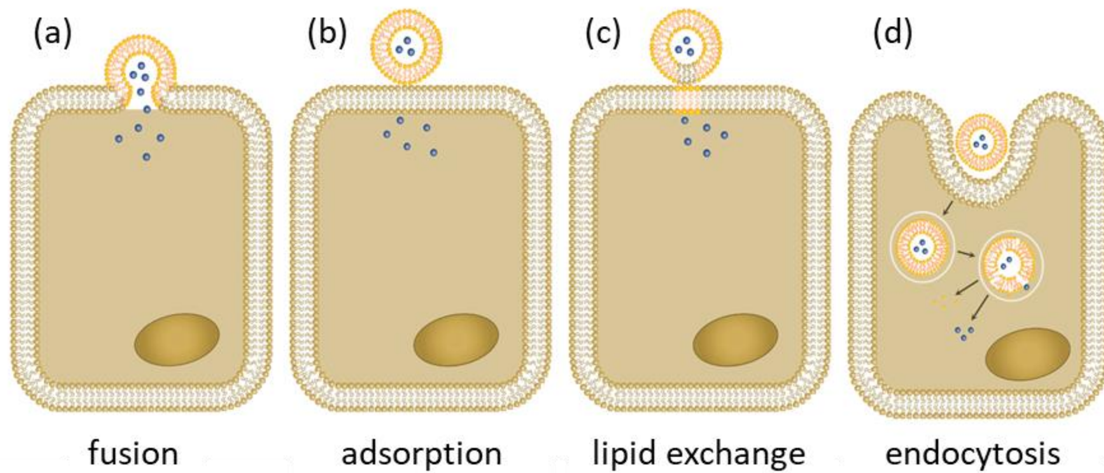

Figure 1.7. *Interaction of liposomes with the lipid bilayer of eukaryotic cells.[71]* ***(a)*** *Fusion merges the lipids of the liposome with the ones in the cell membrane, ultimately releasing the contents of the liposomes inside the cell.* ***(b)*** *Adsorption occurs when the liposomes is attracted to the cell membrane by electrostatic forces ultimately promoting the release of the cargo inside.* ***(c)*** *Lipid exchange happens when the lipids from the cell membrane and the ones from the liposome switch.* ***(d)*** *Endocytosis occurs when the phagocyte cells engulf the liposomes. Reproduced with permission of Taylor & Francis*

The interaction of liposomes with eukaryotic cell membranes has been widely studied; however, the interaction between liposomes and prokaryotic cells has not been studied at the same length. Bacteria have an outer membrane with low permeability to drugs.[72] Their interaction with liposomes needs to be understood in order to assess if they are a good option to deliver antibiotics. Gram negative outer membrane contains lipopolysaccharides and has phosphatidylethanolamine (**Figure 1.8a**). In the case of gram-positive bacteria, (**Figure 1.8b)** they are formed of a single lipid membrane surrounded by a cell wall composed of a thick layer of peptidoglycan.[73]

When *Pseudomonas aeruginosa* was exposed to liposomes made of different lipids vesicles, fusion was observed with eventual disruption of the bacterial membrane and

release of the liposomal content.[74] Chen *et al* studied the mechanisms behind the fusion of fluid liposomes with bacteria and they found that the degree of fusion depends on the bacterial properties. For example, *Escherichia coli* showed a fusion of 66%, and *P. aeruginosa* showed 44% fusion. They believe that this is caused by the presence of phosphatidylethanolamine in the outer membrane of the bacteria, with *E. coli* having 91% and *P. aeruginosa* having 71%, they also found that divalent cations in the media increase the degree of fusion, **Figure 1.8c**.[72] They also studied fusion of gram positive bacteria, *Streptococcus agalactiae* and *Staphylococcus aureus*, and found that the liposome–bacteria interactions correlated well to the membrane's phosphatidylglycerol (PG) content. A 45% fusion between liposomes and *S. aureus* (57% PG) was observed, and 23% fusion for *S. agalactiae* (23% PG) was observed.[72] Furthermore, positively charged liposomes have been shown to have an inherent killing effect against bacteria.[75,76]

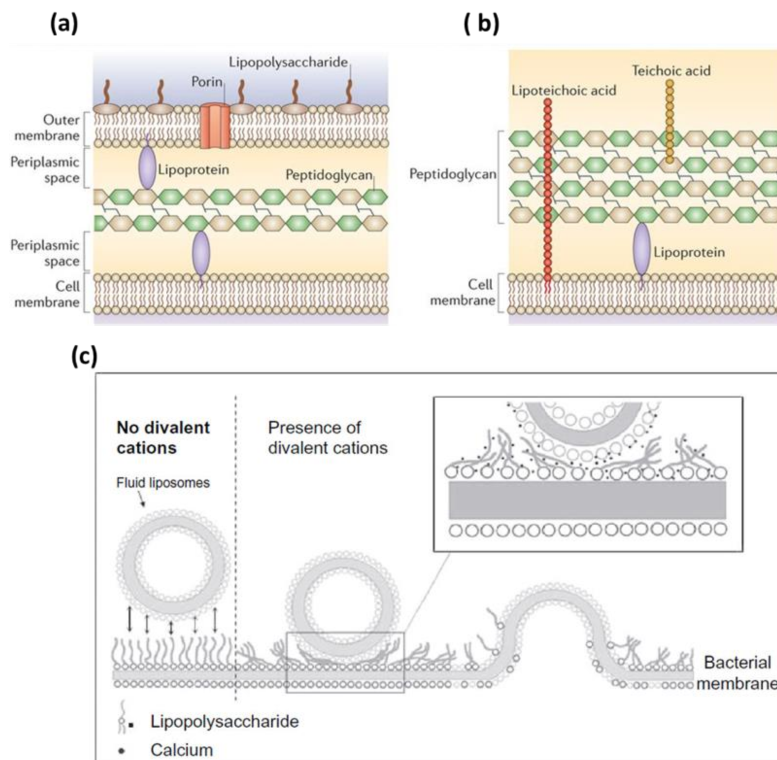

Figure 1.8. ***(a)*** *Gram negative outer membrane contains lipopolysaccharides and has phosphatidylethanolamine;* ***(b)*** *gram-positive bacteria are formed of a single lipid membrane surrounded by a cell wall composed of a thick layer of peptidoglycan. Reproduced with permission of Springer Nature.*[73] ***(c)*** *Calcium-induced fusion between*

fluid liposomes and gram-negative bacterial membrane. When divalent cations are present in the system, the Lipopolysaccharides could re-orient so that negatively-charged polysaccharide chains aggregate and flatten, close to the bacterial membrane, exposing their negatively charged lipid A, creating a connection between the liposomes and the bacteria ultimately leading to fusion. Reproduced with permission of Dove Medical Press[72]

The interaction between liposomes and biofilms was studied with *Staphylococcus epidermidis* and *Proteus vulgaris*. The authors suggested that the hydroxy content of the inositol and glycerol head groups in the liposomes plays a significant role in determining the extent of adsorption from the liposomes to the biofilms.[77] Hydrogen bonding might be promoted when an optimum level of the hydroxy head groups is present in the liposomal surface, this group confers attractive interactions with the negatively charged bacterial cell.[77] Even though hydroxy groups are negatively charged and so are bacteria (under physiological pH), it has been suggested that adsorption can occur because of a balance between self and cross interactions between the surface polymers of the bacterium and the negatively charged lipids of the liposomes.[78]A low energy of interaction, less than a single hydrogen bond, is required between the negatively charged lipids and the surface of the bacterium to produce an interaction profile in which the cross interactions predominate promoting adsorption.[78]

5. Antibiotic Encapsulation

Antibiotics are a type of antimicrobial substance that target bacteria. When antibiotics are administered, they should pass through different tissues to arrive to the site of infection. This potentially causes dilution or inactivation. To assure that enough antibiotics arrive to the site of infection, high concentration of antibiotics for a long period of time are prescribed, increasing intoxication.[79] To overcome these problems, liposomes have been used to encapsulate and deliver antibiotics improving their therapeutic index while minimizing their adverse effects. For example, liposomes have been used to introduce amikacin inside *pseudomonas aeruginosa* cells.[80]

Several liposomal medications have been approved for clinical use, most of them are anticancer treatments such as: Doxil®, DaunoXome®, Depocyt®, Marqibo®, Myocet®, Onivyde™. There are three liposomal formulation used for fungal diseases: Ambisome®, Abelcet® and Ampotec®. Finally, two formulations have been developed for viral vaccination: Epaxal® and Inflexal® V[81]. In general, the application of liposomes has been more oriented to encapsulate antitumor drugs, thus the insight the scientific community has about liposomes is mainly a result of research on encapsulating cancer drugs. However, when encapsulating a different type of drug, the characteristics of the new drug need to be considered and it would be considered a mistake to blindly adopt the same strategies that were successful for other drugs.

The molecules for anti-cancer therapy are very different chemically from antibiotics. Antitumor drugs usually have a molecular weight below 600 g/mol, whereas antibiotics have larger molecular weights. Antibiotics have a complicated structure; for example, teicoplanin and vancomycin are glycopeptides; glycopeptides typically have a cyclic peptide consisting in seven amino acids, to which 2 sugars are bound.[82] Sulfonamides, and the β-lactams are some of the most common antibiotics; sulfonamides have a para-amino group that is essential for their activity and must be unsubstituted, an aromatic ring and a sulfonamide functional group. β-lactams like penicillin, penams and cephalosporins have a β-lactam ring, a four cyclic amide. Antibiotics tend to be sensitive to pH changes, heat or humidity; therefore, special considerations must be taken into account when encapsulating them in liposomes. Another important parameter in drugs is the dissociation constant of the antibiotic molecule (pKa), because it influences the drug's absorption, distribution, metabolism, excretion, toxicity,[83] water solubility, volatility, UV absorption and reactivity with other chemicals. pKa values are also to be considered when encapsulating a drug to avoid unwanted dissociation. However, for antibiotics very few pKa values are currently available.[84]

5.1. *In vitro* studies

Liposomes encapsulating vancomycin were used to target methicillin resistant *S. aureus.* The liposomes showed a MIC two to four fold lower than free vancomycin (**Figure**

1.9a).[85] Cationic liposomes have shown more potent activity against S. *aureus* than anionic or neutral formulations.[7] Similarly, ciprofloxacin cationic liposomes had an increased antibacterial activity towards *P. aeruginosa*, *E. coli* and *Klebsiella pneumoniae.*[19] It has been theorized that the enhanced activity of cationic liposomes is due to their interaction with the negatively charged bacterial cell membrane.[86]

Polymyxin B is an antibiotic used to target gram negative bacterial infections, but its use has been linked with toxic side effects such as ototoxicity and nephrotoxicity.[87] When liposomes encapsulating polymyxin were used against *Bordetella bronchiseptica, P. aeruginosa, E. coli, K. pneumoniae, Acinetobacter lwofii,* and *Acinetobacter baumannii,* antimicrobial activity was improved in the presence of polyanions and sputum (**Figure 1.9b**).[88,89]

Amikacin, netilmicin and tobramycin were loaded into anionic or cationic liposomes, they exhibited consistent release profile in human sera;[90] however, no differences were found between encapsulated and free drugs. The authors argue that this might be because the liposomes were releasing the content gradually rather than in a single hit. Co-encapsulation of bismuth ethanedithiol with tobramycin in liposomes enhanced the penetration into sputum and inhibited biofilm creation in *P. aeruginosa*. Bacterial quorum sensing signal molecules were reduced by bismuth ethanedithiol presence and, the toxic side effects of bismuth ethanedithiol on lung epithelial cells were reduced.[91,92]

Liposomes encapsulating clarithromycin, an antibiotic effective against *P. aeruginosa* showed a lower MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) against clinical isolates of P. *aeruginosa* compared with free clarithromycin (**Figure 1.9c**). Even though neutral liposomes were more effective than the free formulation, positively charged and negatively charged liposomes were more effective in reducing virulence factors and bacterial motility.[93]

5.2. *In vivo* studies

Depending on the liposome size, they can reach and accumulate in different sites of the body, making them optimal to target specific regions. A study that investigated the

distribution of liposomes in mice, found that a large concentration accumulated in the spleen and the lungs.[94] Liposomes encapsulating amikacin were administered intravenously to mice with a *Mycobacterium avium* infection. Accumulation of the drug was found in the liver and the spleen for up to 28 days and a monthly dosage of liposomes containing the antibiotics increased the survival time to 7 months instead of 4 months for the control group (**Figure 1.9d**).[95] Tobramycin encapsulated in liposomes was administered to a mouse model of chronic pulmonary infection was more effective at eliminating *P. aeruginosa* due to an improvement of the pharmacokinetics of tombramycin, sustained concentration of tobramycin at infection site, reduced nephrotoxicity, minimal systemic absorption and a modulation of IL-8 levels in the lungs and sera.[96]

Liposomal vancomycin reduced the infection in a mice MRSA [Methicillin-resistant *Staphylococcus aureus*] model in kidneys and spleen by 2-3 logs when compared to the control.[46] It was hypothesized that the good activity could might be due to the fusion of the liposomes with the cell wall.[97] Another study where they added PEG (polyethylene glycol) to the surface of vancomycin liposomes and challenged MRSA mice infection found that vancomycin was in the system for up to 48 days whereas free vancomycin administered through injection was not detectable after 2 hours.[98]

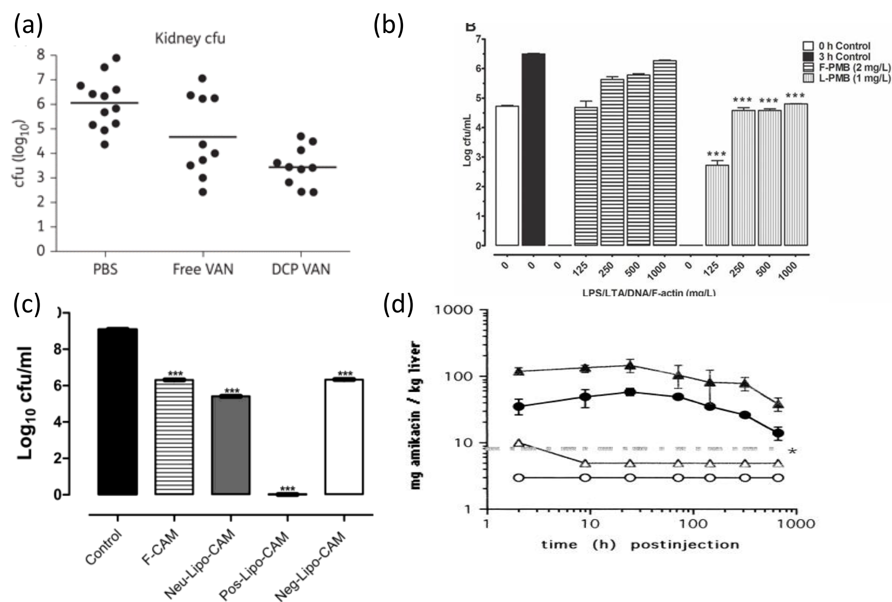

Figure 1.9. (a) *Efficacy of free and DCP liposomal vancomycin in the treatment of MRSA systemic infection. Mice were treated 1 h after infection ($4x10^6$CFU of MRSA) with PBS, DCP liposomal vancomycin (DCP VAN) or free vancomycin (VAN).Reproduced with permission of Oxford University Press*[85] ***(b)*** *Bactericidal concentration of free polymycin B (F-PMB) and liposomal polymycin B (L-PMB) were incubated in presence of DNA/F-actin/LPS/LTA (125 to 1000mg/L) Reproduced with permission of Plos One.*[88] ***(c)*** *MBEC assay performed on P. aureginosa PA-13572 with 64 mg/L of clarithromycin, free drug, neutral, positive and negative liposomes. Reproduced with permission of American Society for Microbiology*[93] ***(d)*** *Concentrations of amikacin when administered freely (open symbols) or in liposomes (closed symbols) in the livers of C57BL/6 mice after a single intravenous injection of 600 mg shown in circles or 2,000 mg shown in triangles. Reproduced with permission of American Society for Microbiology*[95]

6. Aim and Hypotheses

The aim of my thesis was to analyze the possible biases introduced by encapsulation and quantification by methods commonly employed for encapsulating drugs in liposomes. The long-term goal of this research is to develop liposomes that can successfully encapsulate antibiotics/antimicrobial peptides/phage lysins and locally release them at the site of infection, specifically to biofilms. To do so, however requires us to develop methods and fundamental knowledge about both liposomes and antibiotics including identifying possible biases in results introduced by the employed methods.

The current thesis was designed to test two major hypotheses:

- The method of preparation of liposomes and quantification of encapsulation both affect the encapsulation efficiency obtained for antibiotics of hydrophobic and hydrophilic nature.
- Antibiotics, being highly temperature sensitive by nature, may be inactivated during certain steps in the liposome encapsulation process.

During this work, we confirmed that the liposome preparation method strongly influences the encapsulation efficiency of the produced liposomes and that the optimal method differs for antibiotics of different nature. We further identified possible biases in the methods used for quantification of encapsulation in liposomes. In addition, we found that the preparation method affects the efficacy of some drugs and that lipids offer a protective effect against harsh preparation conditions.

7. Contributions to Knowledge

This work resulted in the development of fundamental knowledge related to antibiotic encapsulation in liposomes that will be reported in the form of two manuscripts (I am the first author on both manuscripts):

- Liposome preparation method strongly impacts antibiotic encapsulation efficiency (Chapter 2), to be submitted to Langmuir.
- Preserving antibiotic efficacy during nano-encapsulation in liposomes (Chapter 3), to be submitted to Biomacromolecules (co-first author: Chenchen Xu).

The developed insight and knowledge can be applied for liposomes encapsulating antibiotics to tackle infections in their planktonic or biofilm form. The knowledge obtained in this work can be extended to different kinds of nanoparticles encapsulating antibiotics, as well as antimicrobial peptides and phage lysins, as alternative antimicrobials.

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Chapter 2. Effect of liposome preparation method on encapsulation efficiency of antibiotics

1. Abstract

Liposomes are attractive vehicles for localized delivery of antibiotics. There exists, however, a gap in knowledge when it comes to achieving high liposomal loading efficiencies for antibiotics. To address this issue, we investigated three antibiotics of clinical relevance against staphylococcal infections, with different hydrophilicity and chemical structure, namely vancomycin hydrochloride, teicoplanin, and rifampin. We categorized the suitability of different encapsulation methods based on encapsulation efficiency, lipid requirement (important for avoiding lipid toxicity) and loss in antibiotic mass during the process. The moderately hydrophobic (teicoplanin) and highly hydrophobic (rifampin) antibiotics varied significantly in their encapsulation load (15.5%) and mass loss (28.21%), favoring methods that maximized partition between the aqueous core and the lipid bilayer or those that produce oligolamellar vesicles, while vancomycin hydrochloride, a highly hydrophilic molecule, showed little change between different methods. In addition, we report significant biases introduced by the methods adopted to quantify encapsulation efficiency (underestimation of up to 23% or overestimation by up to 58.82%), and further propose ultrafiltration and bursting by methanol as the method with minimal bias for quantification encapsulation efficiency in liposomes. Finally we investigated the changes in release profile induced by the use of different cholesterol concentrations. The knowledge reported in this work sheds light on the more practical, albeit less investigated, aspects of designing localized antibiotic delivery vehicles and can be extended to other nano-vehicles that may suffer from the same biases.

2. Introduction

Localized delivery of antibiotics is a promising tactic to treat challenging infections such as biofilms (a major challenge with indwelling medical devices) and intracellular

infections (such as Salmonellosis)[1,2]. In fact, even most seemingly resistant infections can be eradicated if a higher dose of antibiotics could be delivered to the site of infection. Delivering such high loads via usual routes of administration is challenging because antibiotics are significant diluted by the time it reaches the site of infection, requiring administration of higher doses that can be dangerously toxic or even deadly.[3]

Liposomes are widely used vehicles for drug delivery, owing to their proven biocompatibility, biodegradability and ability to encapsulate both hydrophilic and hydrophobic compounds.[4] Liposomes are vesicles in which an aqueous volume (which can encapsulate hydrophilic compounds) is enclosed by a spherical lipid bilayer (which can encapsulate hydrophobic compounds) typically composed of phospholipids and additional agents like cholesterol.[5] The liposomal lipid bilayer interacts directly with the lipids comprising the cell/bacteria membrane, thus delivering the cargo directly to the cell membrane without having to rely on active or passive uptake of the nano-vehicle by cells[6]; this makes liposomes specifically advantageous for the localized delivery of high loads of potentially toxic agents that cannot be administered systemically.

Liposomes can be tailored, to a certain degree, to the specific cargo and release conditions by choosing specific phospholipids or methods of preparations. The optimal choice of liposome preparation method depends on: the physicochemical characteristics of the material to be entrapped, the desired size and polydispersity of the vesicles, the desired bilayer properties, and the ease of upscaling.[7] Multiple ways of preparing liposomes have been reported in the literature, three of them are shown in **Figure 2.1**. The methods of preparation can be classified in three major categories: mechanical dispersion, solvent dispersion and detergent removal. Aside from the microfluidic methods, the rest have 4 main steps in common:[5,8] (i) drying the lipids from organic solvents, (iii) dispersing the lipid in aqueous media, (iii) resizing the liposomes and (iv) purifying/cleaning the liposome suspension. The final step is crucial for most real-life applications as well as for quality control steps and determining encapsulation load. The cargo is typically loaded during the preparation step, thus resulting is the encapsulation load being strongly affected by the preparation method.

Multiple reports of antibiotic encapsulation in liposomes have been published using various lipids.[9-16] A review of the literature on liposomal encapsulation of antibiotics, however, reveals outstanding inconsistencies. A clear example of these inconsistencies is the encapsulation of the anti-staphylococcal antibiotic, rifampin: a group reported 0% encapsulation efficiency using the thin film method with DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) and cholesterol,[17] whereas Manconi *et al* reported encapsulation efficiency of 74% with a slightly modified thin-film method and using a very similar lipid.[18] We hypothesize that these inconsistencies in the literature (most of which we also observed in the lab) are suggestive of a chronic method bias in both preparation and characterization methods, partially fueled by the lack of method development for antibiotics, a class of molecules inherently different form cancer drugs for which most liposomal encapsulation and characterization methods have been developed. We set out to explore the extent of reach for the hypothesized method bias by evaluating the encapsulation process for three different anti-staphylococcal antibiotics, namely vancomycin hydrochloride, teicoplanin, and rifampin, with significantly different hydrophobicity. We used three widely-used liposome preparation methods and evaluated each method based on encapsulation efficiency for each antibiotic, lipid usage (important for avoiding lipid toxicity), and antibiotic mass loss during the process, as well as final liposome size and zeta potential. To decrease scatter, we used the same lipid for all different methods and antibiotics. We further evaluated the methods used in the literature for quantifying encapsulation efficiency and detected significant bias in these methods for antibiotics. This led us to propose a new procedure for evaluation of encapsulation efficiency of antibiotics in liposomes. We finally characterized he release profile of liposomes prepared using our proposed method, optimized for each class of antibiotic based on hydrophilicity.

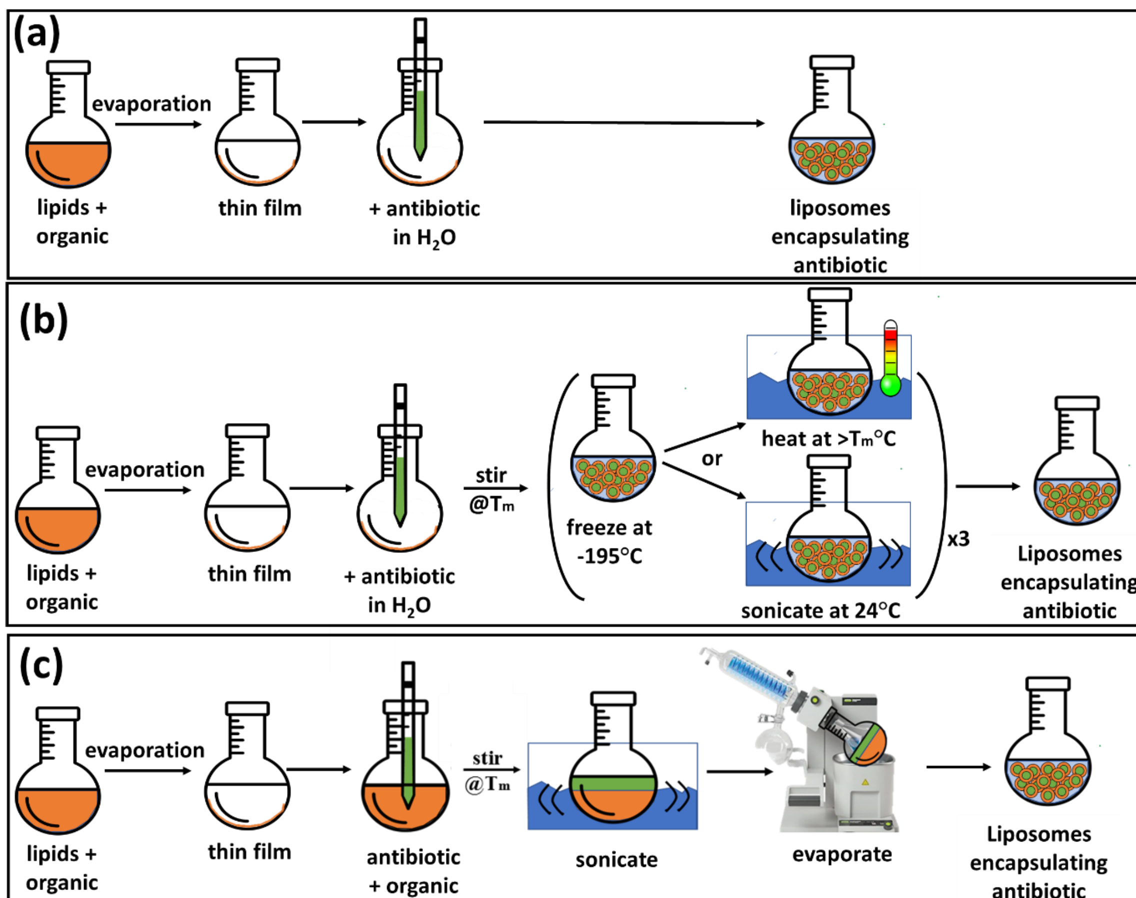

Figure 2.1. *Overview of liposome preparation methods. (**a**)Thin film hydration method, consists on creating a thin film of lipids that will be challenged by water and the drug in a next step at a temperature above the transition temperature of the lipid used, so that the multilamellar liposomes can be formed. (**b**) Freezing and thawing method, it shares the same 3 initial steps but after stirring, three cycles of freezing at –196 and thawing at either RT with sonication or above the transition temperature of the lipid used. (**c**) Reverse phase, the name refers to the fact that the method creates micelles by adding an organic phase and an aqueous phase and sonicating them, then, by evaporating the organic solvent the micelles implode and transform into liposomes.*

3. Experimental Section

3.1. Chemicals and lipids

The lipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (16:0 PC DPPC) (99%, Avanti, Millipore Sigma) was used for all experiments. The antibiotics vancomycin hydrochloride (pharmaceutical grade secondary standard), Rifampin (≥97%), and Teicoplanin (≥80%), as well as Triton X-100 were also obtained from Millipore Sigma. Cholesterol (95%) was obtained from Fischer Scientific. The remaining chemicals (Methanol ≥99.9%, Diethyl ether, and KCl were obtained from VWR.

3.2. Thin film method (TF)

The liposomes were prepared with a modified thin film method, as reported by Meers *et al*.[1] Briefly, DPPC and cholesterol were dissolved in chloroform inside a round bottom flask and evaporated Heidolphat Hei-VAP rotary evaporator at 35˚C. The lipid film was then left overnight in a vacuum desiccator to eliminate traces of chloroform. The film was subsequently rehydrated with MilliQ water resistivity of 18.2 MΩ.cm (at 25°C) containing the corresponding antibiotic (Teicoplanin, vancomycin hydrochloride or rifampin) at 42°C. The lipid vesicles were then extruded, 55-101×, using an Avanti Mini Extruder at 42℃ using a 0.4 nm pore size membrane.

3.3. Reverse phase evaporation method (REV)

The modified reverse-phase liposomes was utilized as reported by Halwani et al.[2] Briefly, appropriate amounts of DPPC and cholesterol were diluted in chloroform in a round bottom flask, a thin film was formed using a rotary evaporator to evaporate the solvent. Diethyl ether (3 mL) and Millipore water (1 mL) were added next. Vancomycin hydrochloride was added to the aqueous phase whereas teicoplanin and rifampin were added to the organic phase. The system was then sonicated for 35 to 40 min at a temperature below 10°C until one phase or a homogeneous dispersion was obtained. Finally, the solvent was removed under reduced pressure at room temperature using a rotary evaporator for 40 min. The formation of bubbles was avoided by increasing the

pressure upon spotting visual signs of bubble or foam formation. The liposome suspension was the extruded as described above.

3.4. Freezing, annealing and thawing method (FAT)

Vesicles were prepared by the thin film method, as described above, but before the extrusion step, 3 freeze-thaw cycles were added. A single freeze-thaw cycle consisted of freezing the vesicles for 5 min at -196°C using liquid nitrogen and thawing them inside a VWR bath sonicator (35 KHz 90W) at room temperature for 5 min. The samples were stored at 4°C for 30 min after the three cycles of freezing and thawing, before annealing at room temperature for 30 min and extrusion, as described above.

3.5. Determination of size and charge

Dynamic light scattering (DLS) was used to evaluate the hydrodynamic diameter of liposomes using the Malvern Instruments Zetasizer Nano-ZSP. Liposomes, prepared with each method, were resuspended in 1 mM KCl and diluted 50× before the measurement. All DLS runs were repeated 3 times on each sample. Malvern Instruments Zetasizer Nano-ZSP was also used for zeta potential measurements using a capillary cell. The zeta potential runs were repeated 3 times for each sample with Non-Invasive Backscatter optics (NIBS) and analyzed with Smoluchowski's model[19].

3.6. Quantification of Encapsulation efficiency and mass loss

Encapsulation efficiency was calculated as the percentage difference between the total antibiotic (encapsulated and non-encapsulated) and the free antibiotic (non-encapsulated). Two methods were used to quantify the total antibiotic: (1) 0.5% methanol was added to the liposome suspension sample. After incubating the sample for 50 min at 4°C, 5 parts Millipore water was added to the system and the suspension was then analyzed via HPLC to quantify the antibiotic. (2) The lipid membranes were disrupted with 2% Triton X-100. Mass kept was determined as the ratio of total antibiotic, as determined in this step, to the initial mass of antibiotic added to the system during preparation stage.

To determine the amount of free antibiotic, 2 methods were used; (1) liposomes were ultrafiltrated (Amicon Ultra centrifugal filters, MWCO 30 K) for 10 min at 10,000×g. (2)

liposomes were centrifuged at 20,000×g for 3 hrs. After separating the free antibiotic and the liposomes, the free antibiotic was analyzed using HPLC. Percentage encapsulation efficiency was calculated after measuring free antibiotic and total antibiotic with the following equation: $EE = \frac{total\ drug - free\ drug}{total\ drug} \times 100.$

3.7. Liposomes quantification and quantification of disruption

The scattered light produced by a colloidal suspension can be measured as the photons per second detected by a detector, when the size of the nanoparticles is close and the attenuator is fixed, this number can be related to concentration.[20] The quantity of liposomes present in each liposomal formulation was estimated by using the photons per second of each formulation with the Malvern Zetasizer Nano ZSP. Then, after the liposomes were separated from the free antibiotic, either by ultrafiltration or centrifugation, the quantity of liposomes in each phase was determined using the Zetaziser.

Liposomes were burst with either methanol or triton and the samples were analyzed via HPLC-micro-TOF to determine the amount of free antibiotic; an increase in the amount of antibiotic indicated more disruption, whereas less antibiotic indicated that some liposomes were not fully releasing their content.

3.8. Drug release

Liposome suspensions (0.5 mL) were subjected to dialysis using the Slide-A-Lyzer 20K MWCO dialysis inserts and antibiotic released was quantified at different timepoints: 2 hr, 4 hr, 12 hr, and every 12 hrs after that for at least 7 days. The samples obtained at each time point were quantified by measuring absorption using the BioTek Synergy Neo plate reader at a wavelength of 280 nm for vancomycin and 470 nm for rifampin. The absorbance reads were converted to concentration using a calibration curve, prepared for each antibiotic.

3.9. High Performance Liquid Chromatography (HPLC) and Time-of-Flight Mass Spectrometry (TOF-MS)

For all HPLC measurements, Agilent 1200 series HPLC with Bruker Mictotof II mass spectrometer was used with the analytical column Agilent XDB-C18 (100 mm×2.1 mm, 3.5 μm). The mobile phase consisted of two solvents; Eluent A: aqueous formic acid (0.1% v/v); Eluent B: acetonitrile containing 0.1% formic acid (0.1% v/v).

For vancomycin and teicoplanin, the column temperature was 40°C with the injection volume of 10 μL. for vancomycin, the run time was 21 min with a flow rate of 0.3 mL/min and a gradient elution program as follows: 97% mobile phase A for 3 min; linear increase to 30% B over 7 min, hold for 2 min; afterwards a linear increase up to 80% mobile phase B within 1 min, hold for 2 min; return to the initial condition within 1 min and re-equilibration for 5 min. For teicoplanin, the run time was 6.5 min at a flow rate of 0.5 mL/min. The gradient elution program as follows: 97% mobile phase A was introduced from initial sample injection hold for 1 min, then switched to 97% mobile phase B over 1 min held for 1 min; returned to initial conditions within half a min and re-equilibration for 3 min.

For rifampin: The column temperature was 25°C and the injection volume was 10 μL. Run time was 12 min at a flow rate of 0.4 mL/min and a gradient elution program as follows: 65% mobile phase A, held for 1 min, increased to 90% solvent B over 4 min, then increased to 95% solvent B, held for 3 min, returned to initial conditions and re-equilibration for 4 min.

Quantification was achieved by TOF-MS positive ion electrospray ionization. Ion detection was performed at m/z 724.7 for vancomycin hydrochloride, at m/z: 939.7, 940.7, 947.8, and 782.4 for teicoplanin components, and m/z 823.4 for rifampin. For teicoplanin quantification, the determination of the area under the curve was obtained as the sum of the four major compounds.

3.10. Statistical analysis

All data presented is the average of at least three independent experiments, presented along with the standard deviation between values obtained for the independent experiments. Statistical significance of differences was tested using a t-test and P-values lower than 0.05 were chosen as the cutoff for significant difference.

4. Results and Discussion

4.1. Liposome preparation methods impact encapsulation efficiency and mass loss

Liposomes encapsulating vancomycin hydrochloride and rifampin were prepared using the three methods (TF, FAT, REV) as shown in **Figure 2.1**. Liposome encapsulation efficiency and mass loss during the process of creating the liposomes was then quantified. All liposomes from this section were prepared using an antibiotic concentration corresponding to 18% of vancomycin's solubility limit in water, 80% of teicoplanin's and rifampicin's solubility in water and a lipid was added in a 1:3 ratio cholesterol:DPPC

As shown in **Figure 2.2**, percentage of mass kept during the preparation process varies significantly for each antibiotic, depending on the preparation method. For vancomycin hydrochloride (water solubility 50 mg/mL), FAT retains more mass (93.4% ± 7) while REV results in up to 60% mass loss (**Figure 2.2a**). For the highly lipophilic antibiotic, rifampin (water solubility 1.3 mg/mL, highly lipid soluble[21]), however, FAT resulted in a mass kept of 44.4% ± 9, TF showed less than 10% mass kept, and REV proved to be the most efficient method, resulting in 79.5 ± 3 mass kept (**Figure 2.2b**).

For the moderately lipophilic antibiotic, teicoplanin (water solubility 10 mg/mL), FAT resulted in a mass kept of 32.6% ± 9.2, TF showed less than 38.1% ± 14.2 mass kept, but and REV proved to be the most efficient method, resulting in 93.4 ± 3.32 mass kept (**Figure 2.2c**).

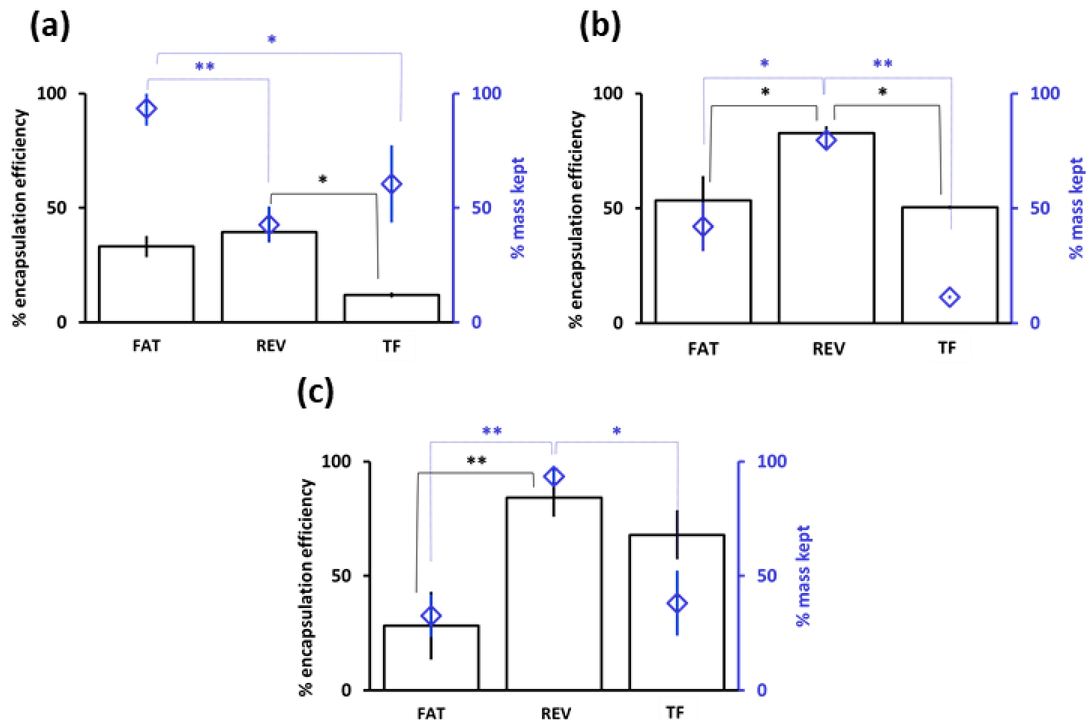

Figure 2.2. *Effect of preparation method on encapsulation efficiency (black bars) and mass kept (blue markers) for lipsomes loaded with* ***(a)*** *vancomycinhydrochloride,* ***(b)*** *rifampin, and* ***(c)*** *teicoplanin. *P < 0.05; **P < 0.005*

The low percentage of mass kept for rifampin and teicoplanin in TF and FAT methods can be attributed to the "rehydration" step, in which the solution needs to be heated at the lipids melting temperature, T_m (42°C for DPPC) until the thin film is completely rehydrated for TF and mostly rehydrated for FAT. This step could take long (up to 40 mins) resulting in partial evaporation of the aqueous volume; since we used rifampin and teicoplanin solutions at 80% their solubility in water (1.3 mg/mL and 10mg/mL, respectively), evaporation of a fraction of water represents precipitation of rifampin or teicoplanin, further decreasing loading. Vancomycin hydrochloride, which is highly soluble in water, is not affected, in terms of mass kept, by this evaporation step, because the concentration we used is 18% its solubility limit in water, resulting in FAT being the most efficient method for vancomycin. REV, however, was highly inefficient in terms of mass kept for vancomycin whereas it proved the most efficient for rifampin. In REV, the

aqueous solution with diethyl ether does not fully create a homogeneous dispersion after sonication, probably since vancomycin is practically insoluble in diethyl ether; this is not the case for rifampin which is equally soluble in water and in diethyl ether.

FAT method, which was very efficient in terms of mass kept for vancomycin hydrochloride, also resulted in the highest encapsulation efficiency, 39.1%±3 (**Figure 2.3a**). This can be explained by the large aqueous space inside this type of liposomes. It has been reported that repeated cycles of freezing and thawing, in the FAT technique, disrupts the bilayer due to the formation of ice crystals during the freezing step, thus disrupting the closely spaced lamellae of the multilamellar liposomes vesicles and increasing the aqueous volume.[22] A10-50x increase in the internal volume of liposomes after freezing and thawing has been reported as a result of the fusion of small vesicles to form bigger liposomes.[23] Additionally, extrusion of FAT liposomes reduces the lamellarity of the liposomes because the disrupted vesicles re-assemble into unilamellar vesicles, increasing the internal aqueous volume.[5,24] It is noteworthy that theoretical calculation of the aqueous volume for liposomes with the size distribution of our liposomes, predicts an encapsulation efficiency of 33.72% (calculation in SI). Encapsulation efficiencies of 40.78%[11], 32.65%[25], and 12.6%[26] have been reported for vancomycin in REV liposomes of 188.4 nm, 220.4 nm, and 245 nm, respectively, all close to the theoretical limit calculated based on aqueous volume.

In the case of rifampin and teicoplanin, REV method showed the highest encapsulation efficiency, 82.6%±0.8 and 84.1%±8.3 respectively (**Figure 2.2b and 2.2c**). REV liposomes are known for producing a high internal aqueous volume and oligolamellar vesicles.[4] For a drug that can only be loaded in an aqueous space such a vancomycin, a system that produces oligolamellar vesicles is not ideal, but for a drug that can be loaded in the aqueous space as well as the bilayer, the production of unilamellar and oligolamellar vesicles enhances the encapsulation efficiency. This method could, in theory, encapsulate hydrophilic as well as hydrophobic compounds, because it creates vesicles with large aqueous volumes and oligolamellar vesicles. However, our results

show that it is clearly more advantageous for the moderately and highly hydrophobic antibiotics like teicoplanin and rifampin.

TF technique (introduced by Banghman et al) is known to produce multilamellar vesicles of multiple sizes (reducing aqueous space), therefore it is necessary to use reduction size techniques such as sonication or extrusion. [27,28] This method is believed to be suitable for hydrophobic compounds. Previous reports of rifampin-loaded liposomes (prepared with soya lecithin with a lecithin: cholesterol of 60:40v/v) created via TF have reported encapsulation efficiencies that range from 53.3% to 79.25%, depending on the composition.[16,18,29] However, our results show that TF method may not be the best option for hydrophobic antibiotics. Our TF-rifampin liposomes had an encapsulation efficiency of 50.33%. Even though the encapsulation efficiency is not low, the amount of rifampin mass loss during the process is remarkably high (>88% in our experiments), thus we recommend encapsulating rifampin with REV method. The case for teicoplanin is similar, its encapsulation efficiency using TF was 67.9%±10.7, a considerably high encapsulation efficiency, comparable with REV, however its mass loss was significant with a loss of more than 60%.

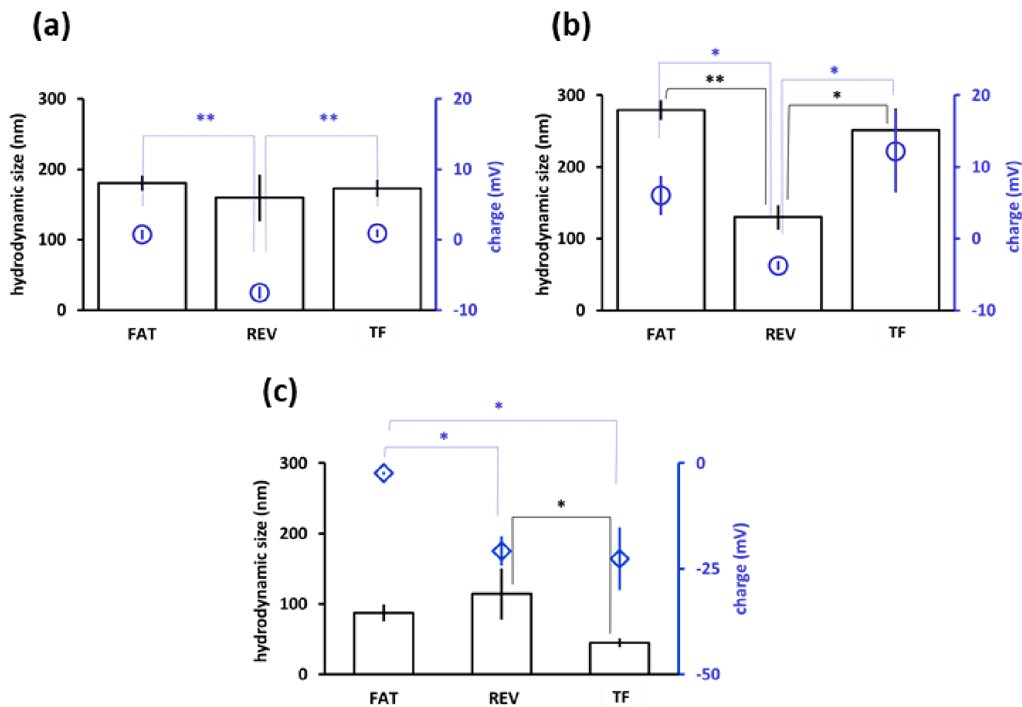

Figure 2.3- *Hydrodynamic size (black bars) and charge (blue markers) are shown in* ***(a)*** *vancomycinhydrochloride,* ***(b)*** *rifampin, and* ***(c)*** *teicoplanin. *P < 0.05; **P < 0.005*

In terms of size and zeta potential, the size for vancomycin liposomes did not show a significant variation between the different preparation methods (**Figure 2.3a**). All the liposomes were extruded through a 400 nm membrane; thus all the liposomes are expected to be smaller than 400 nm (representative size distribution presented in **Fig S2.2**). The zeta potential for vancomycin liposomes was very close to neutral for all preparations (as expected based on the charge of DPPC)[30,31] with REV leading to a slightly more negative zeta potential. The change in charge for REV vancomycin liposomes could be due to the presence of 2 types of liposomes created by REV methods promoting repulsion between the vesicles.

However, for rifampin, FAT and TF produced significantly bigger liposomes than REV (**Figure 2.3b**). Rifampin is likely intercalating in the bilayer for FAT and FT liposomes. REV liposomes with rifampin do not allow for this effect, because multiple bilayers are

created and the inner space of the unilamellar vesicles have a large internal space. In the case of rifampin, positive zeta potential was observed for FAT and REV and close to neutral zeta potential was observed for REV. The change in zeta potential, from neutral and to positive for FAT and TF liposomes with rifampin, may be explained too by the antibiotic being exposed in the bilayer.[8]

Teicoplanin liposomes (**Figure 2.3c**) were smaller than the other two antibiotic liposomes; TF liposomes were smaller than 50 nm, this size would significantly reduce the inner aqueous volume space leading to a low encapsulation efficiency of highly hydrophilic compounds, however since teicoplanin is both slightly hydrophilic and slightly hydrophobic and TF produces multilamellar liposomes it could have been encapsulated in the multiple bilayers not needing a large aqueous internal space. This may explain why TF-teicoplanin liposome encapsulation efficiency was rather high (67.9%±10.7). Additionally, teicoplanin encapsulation in the bilayer could explain the highly negative zeta potentials, -20.8±3.5 and -22.6±7.4, obtained with the methods REV and TF, respectively, methods that promote oligolamellar or multilamellar vesicles. This high zeta potential does not happen in FAT liposomes possibly because the aqueous space is responsible for most of the encapsulation.

4.2. Solubility limit of antibiotic affects encapsulation efficiency and mass loss

Concluding from the previous section that FAT was the most efficient method for encapsulation of vancomycin and REV the most efficient method for encapsulation of rifampin, we prepared FAT-vancomycin liposomes, with different vancomycin concentrations, corresponding to 8, 18, and 25% of vancomycin solubility limit in water, and REV-rifampin liposomes with concentrations corresponding to 80, 100 and 120% of rifampin solubility limit in water. The mass of lipid used was kept constant, thus the different antibiotic concentrations can also be interpreted as different antibiotic to lipid ratios.

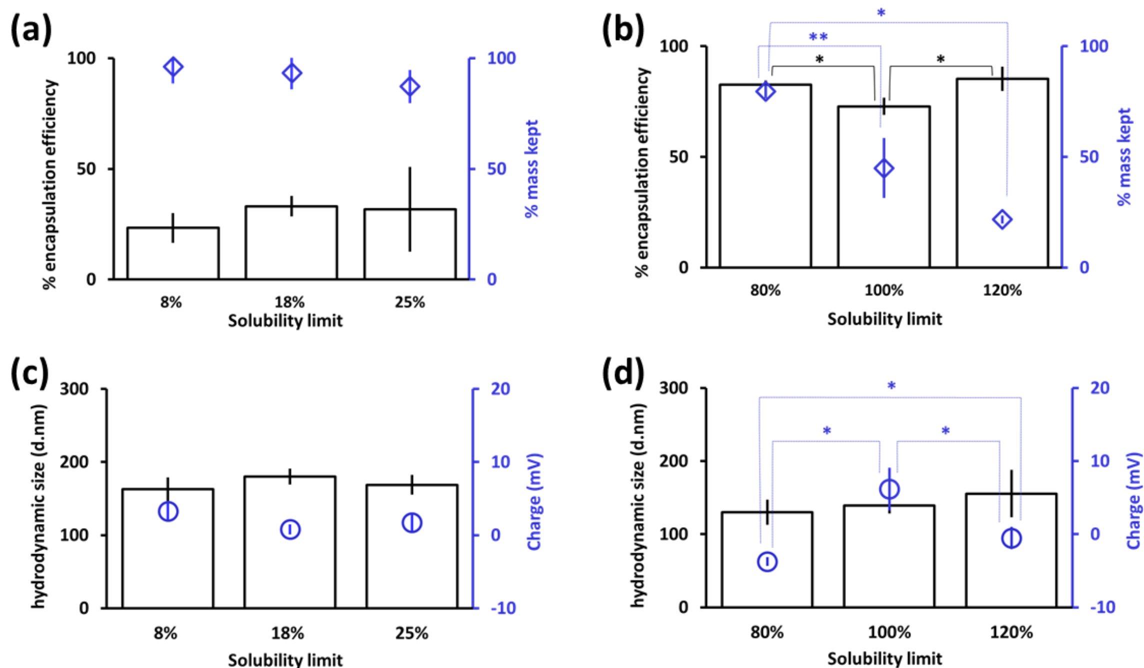

Figure 2.4. *Encapsulation efficiency (black bars) and mass kept (blue markers) of liposomes prepared with varying* ***(a)*** *vancomycin (FAT), and* ***(b)*** *rifampin (REV) initial concentrations. Hydrodynamic size (black bars) and charge (blue markers) of liposomes loaded with varying initial concentrations of* ***(c)*** *vancomycin (FAT),* ***(d)*** *rifampin. *P < 0.05; **P < 0.005*

As shown in **Figure 2.4**, changing the amount of vancomycin hydrochloride used for the preparation of liposomes does not result in a significant change in terms of encapsulation efficiency, mass kept, size, or charge (**Figure 2.4 a,c**). However, for rifampin, changes in the mass of rifampin used during liposome preparation, although it shows small, but statistically significant, differences between in encapsulation efficiency, it also significantly influences mass kept, with the largest mass kept achieved at 80% of rifampicin solubility limit in water (**Figure 2.4 b,d**). This may be explained by the partial evaporation of water in REV method, leading to precipitation of the antibiotic. It is noteworthy that if the only criterion for evaluating the efficiency of a chosen method/concentration was encapsulation efficiency, for the case of rifampin, FAT and TF methods could have been considered equally good. However, the addition of the mass

loss during the process as an additional criterion for quality control, provides an extra piece of information that allows us to determine that FAT is a better method than FT, the same happens when evaluating solubility in which thanks to the mass kept criteria we can conclude that a concentration of 80% of the solubility limit as the best choice for rifampin encapsulation.

4.3. Method bias in liposome cleaning methods

While preforming experiments for the previous, we observed drastic inconsistencies in results obtained for encapsulation efficiency based on the chosen method for cleaning the liposome preparations. Ultimately the encapsulation efficiency is calculated as the fraction of the antibiotic that is inside when compared with the total amount of antibiotic present; therefore, the total amount of antibiotic in the system as well as the free antibiotic need to be determined. Pinpointing possible method bias at this stage is critical for ensuring quality control.

Various methods have been reported for separating encapsulated and free antibiotic; two of the most popular are centrifugation[10,32] and ultrafiltration,[33,34] shown in **Figure 2.5.** In addition, multiple methods can be adopted for releasing the antibiotic encapsulated in the liposomes for the purpose of quantifying total amount of antibiotic, two of the most popular are the use of Triton and methanol to disrupt the lipid bilayer. We focused on the two extremes in hydrophilicity for this section, namely vancomycin hydrochloride and rifampin. All liposomes reported in this section were prepared using FAT, 18% solubility limit (for vancomycin encapsulation) and REV, 80% solubility limit (for rifampin encapsulation).

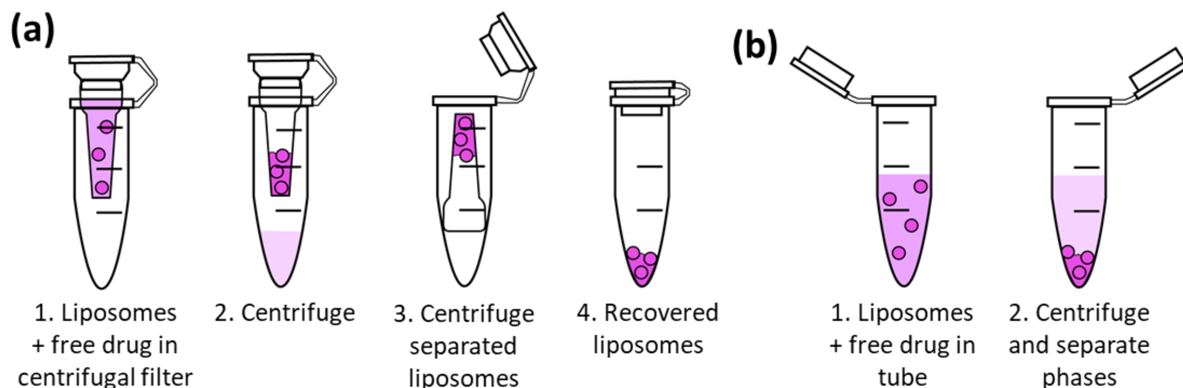

Figure 2.5. ***(a)*** *Ultrafiltration method used to separate free and encapsulated drug; (1) the sample with the freshly made liposomes is added to the ultrafiltration tube and then (2) centrifuged, the free drug will pass through the membrane whereas the liposomes will stay on top,(3) to recovered the liposomes, the filtration tube can be flipped upside down and centrifuged again.* ***(b)*** *Centrifugation to separate free and encapsulated drug; in this method the liposomes are expected to be sun down and form a pellet after a round of centrifugation at high speed and the drug will stay in the liquid on at the top.*

We first analyzed the ability of triton and methanol to disrupt the vesicles by quantifying how much drug was maintained in the system using the best formulations we had, even though this method could be biased if the samples are not keeping a hundred percent of the mass, the comparison between the two methods is still valid. Triton disrupted 76% ± 10 of vancomycin liposomes and only 46.6% ± 2.7 of rifampin liposomes (**Figure 2.6a**); while methanol disrupted the vesicles more efficiently, with 92.4% ± 9 of the vancomycin liposomes and 80% ± 3 of the rifampin liposomes disrupted (**Figure 2.6b**).Triton-X has been commonly to disrupt the liposomes since it changes the phospholipid organization forming highly asymmetrical structures,[35] thus allowing for leakage of the encapsulated antibiotic. Addition of methanol, is believed to alter the planar membrane structure and increases the activation energy required for fusion, possibly due to an increase in membrane fluidity.[36] We observed that methanol outperformed triton-X at disrupting the membrane, however Triton may still be the preferred option for *in vitro* evaluation of antibiotic-loaded liposomes towards bacteria, because it does not represent major toxicity towards bacteria cells.[37]

In addition, we quantified the efficiency of ultrafiltration and centrifugation for separating the liposomes from the un-encapsulated antibiotic. Ultrafiltration effectively separated the antibiotic from the liposomes with no liposomes detected in the free antibiotic phase, and close to 100% (95% ± 9 for vancomycin and 97% ± 4 for rifampin) of liposomes retained in the liposome layer (**Figure 2.6 c**). Centrifugation, however, although widely employed, was not as effective, with liposomes detected in the separated antibiotic phase (**Figure 2.6d**). For vancomycin only 45.4%±12 of the initial liposomes were retained in the liposome layer and 72%±21 stayed in the free antibiotic phase. For rifampin, 42.6%±5 of the initial liposomes were found in the liposomal phase and 66.6%±12.4 were left in the free antibiotic phase. It may be argued that increasing the centrifugation speed may increase the separation effectiveness, however, increased speed significantly increases liposome disruption and is thus not feasible for separating free antibiotic from liposomes. The observation that centrifugation does not effectively separate the two free antibiotics from liposomes suggests that numbers reported for encapsulation efficiency, obtained using centrifugation as the cleaning/separation method may be significantly skewed. As shown in **Figure 2.7**, when centrifugation is used as a cleaning method the encapsulation efficiency calculated is very high (91% and 88% for vancomycin and rifampin respectively) And since triton doesn't fully burst the vesicles the calculated encapsulation efficiency is around 10%. A similar study conducted in 1987 showed that ultrafiltration outperformed centrifugation, airfuge and dialysis at separating free and encapsulated antibiotic; [38] however, a review of the literature shows that centrifugation remains a highly common practice for separate free and encapsulated antibiotic.

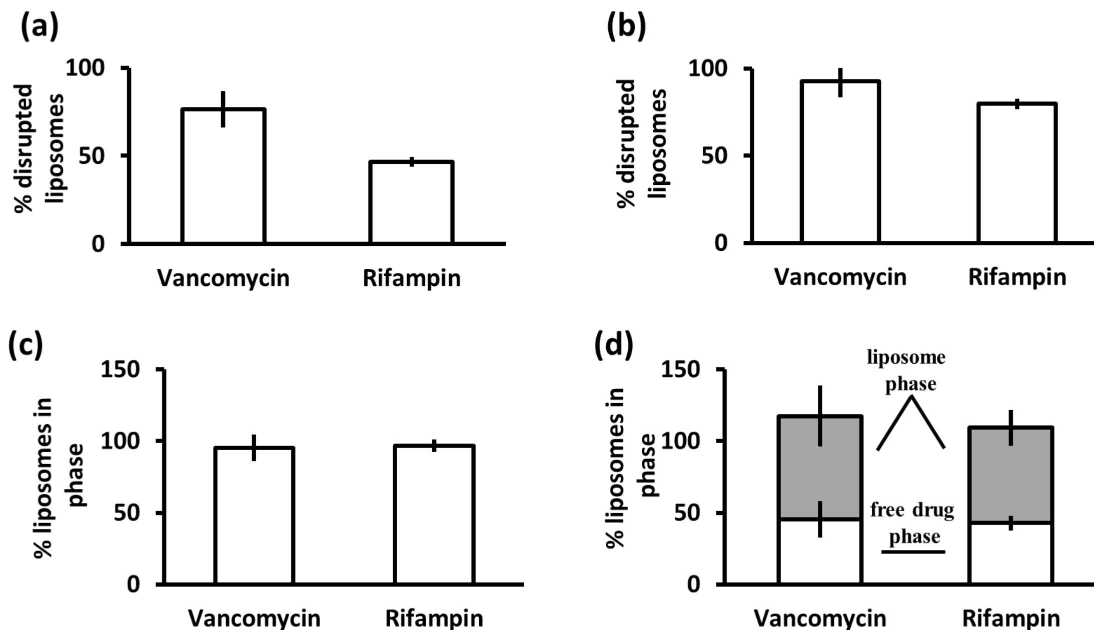

Figure 2.6. *Liposomes disruption with (**a**) Triton and (**b**) methanol. Percentage disruption was obtained by measuring the amount of initial antibiotic existent in the system. Efficiency of separation of liposomes form un-encapsulated antibiotic using (**c**) ultrafiltration and (**d**) centrifugation. The percentage of liposomes in the free antibiotic phase (ideally zero) is represented in grey whereas the percentage of liposomes in the separated liposome layer is shown in white. For (**c**) after centrifugation for 2 hours at 30k RCF the pellet and the supernatant were separated and quantified with DLS to determine the amount of vesicles in dilution in each phase. For (**d**) the samples were ultrafiltrated at 10k RCF for 10 min using Amicon filters before quantification.*

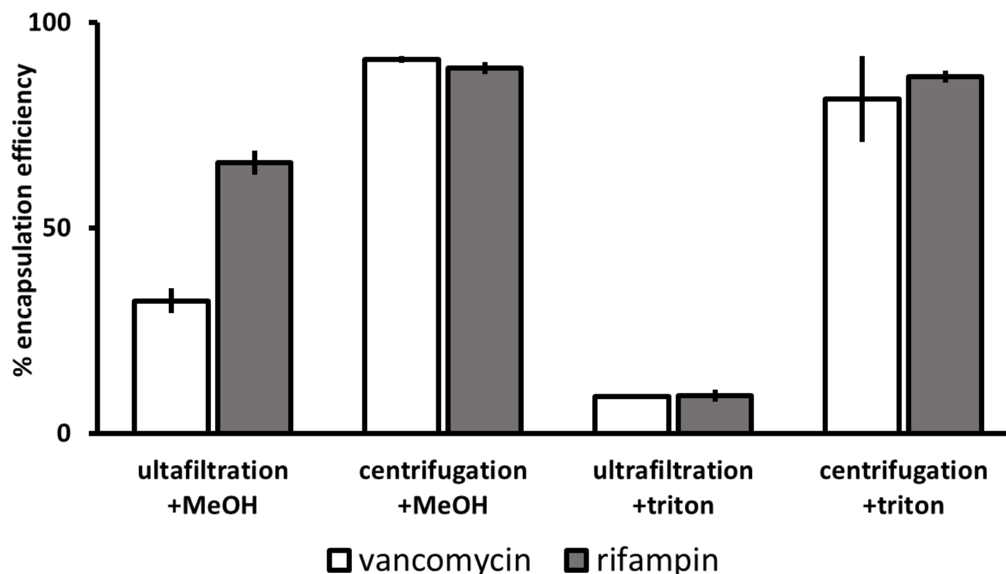

Figure 2.7. *Encapsulation efficiency calculated using different separation techniques (ultrafiltration or centrifugation) and different total release methods (methanol and triton)*

An additional bias is introduced in values calculated for encapsulation efficiency in the process of analyzing the experimental results. There are two major methods adopted in the literature to calculate encapsulation efficiency: (1) in terms of the ratio of cargo found inside the vesicles to the amount of cargo that was initially added to the preparation flask, or (2) in terms of the ratio of cargo found inside the vesicles to the total antibiotic (encapsulated and un-encapsulated) in the system after the preparation of the vesicles. Both methods represent a measure of encapsulation efficiency; the former represents the combined effect of efficacy of drug encapsulation and possible mass loss during the process, while the latter (which is the method that we adopted) represents the absolute capacity of the preparation method to encapsulate the cargo, we analyzed mass kept separately. This lack of consistency in analysis means that the values reported in the literature span a wide range for very similar systems and are sometimes even contradictory. It is, therefore, important to adopt a standard method for reporting encapsulation efficiency in liposomes and other nano drug delivery vehicles. We propose

decoupling encapsulation efficiency (partitioning of drug inside and outside the liposomes) from mass lost during liposome preparation, by calculating encapsulation efficiency in terms of the total drug present in the liposome preparation (and not the amount added at the beginning), and report mass loss separately. Otherwise a it will not be clear if a low encapsulation efficiency is a result of mass lost during preparation unsuitable method/lipid chemistry for encapsulating a specific cargo.

4.4. Effect of cholesterol on antibiotic release profile

As a final assessment of optimal liposome composition for antibiotics, we prepared liposomes with vancomycin or rifampin using the optimal preparation method and compositions determined in the previous sections, with the addition of various concentrations of cholesterol, namely 2:1, 3:1, and 4:1, DPPC : cholesterol (molar ratio). Cholesterol has long been added to liposomal preparations to decrease leaking of the lipid bilayer and allow for sustained release. Cholesterol works by inducing conformational ordering of the lipid chains.[39] The release profiles for vancomycin hydrochloride and rifampin are presented in **Figure 2.8.** For vancomycin, the total content of the drug was released in 13 days, **Figure 2.8a**. Whereas for rifampin it was released in 5 days, **Figure 2.8b**. More cholesterol added showed lower release, increasing the release time for both vancomycin and rifampin. Interestingly the concentration of cholesterol influences the release *rate* significantly for the samples containing 2:1 DPPC : cholesterol for the case of vancomycin. This was not the case for rifampin in which the increasing cholesterol did not result in a significant difference in release. This could have two possible reasons: (1) rifampin randomly intercalates in the bilayer making the release random or (2) rifampin liposomes, due to the fact that they were created using REV, are more polydisperse in size thus its release profile has more variability.

Vancomycin liposomes show burst release; in the first 4 hrs they release almost 50% of the content, which is close to the quantity of the drug that was previously identified as not being encapsulated, FAT-Vancomycin EE = 39.1%±3. The same case happens with rifampin, in 4 hrs it releases 23%, and in our previous experiments it was shown that rifampin encapsulates 82.6%±0.8, so the released drug for the first 4 hours is most

probably the unencapsulated drug, presenting dialysis as a good but time-consuming alternative to cleaning the liposomes. After the cleaning stage, vancomycin burst releases 55% of the remaining drug in the next 12 hrs, after which it constantly releases 6.26 μg/hr for 12 days. After the cleaning stage, rifampin shows an almost linear release (R^2=0.98) for 48 hrs with a constant release of 27.4 μg/hr, and then it plateaus. Vancomycin takes considerably more time than rifampin to release the entire contests of the liposomes, this could be due to the fact that vancomycin is allocated in the aqueous core whereas rifampin intercalates in the bilayer making it more readily available. Optimizing antibiotic release rate, is of interest for antibiotics. For example, rifampin is used in anti-tuberculosis therapy. Due to various systemic side effects, its treatment involves prolonged oral administration of high systemic doses over a period of 4–10 months.[18] Thus, a delivery system that can release the antibiotic for a prolonged period in high dosages without affecting other organs would be highly desirable.

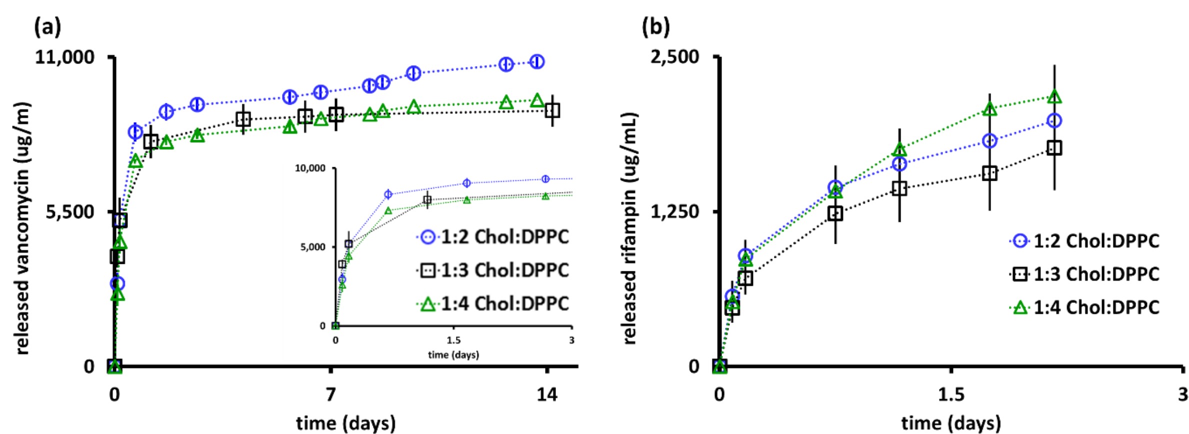

Figure 2.8- *Release profile of vancomycin* ***(a)*** *and rifampin* ***(b)*** *with liposomes created with different ratios of cholesterol.* ***(inset)*** *close-up to the first 3 days of vancomycin's release profile.*

5. Conclusion

Liposomes are attractive vehicles for delivering antibiotics because they can be made of physiologically compatible lipids that can interact with the bacterial cell membrane, delivering the drug via direct interaction and maintain sustained release. Our investigation

highlights the importance of the methods of preparation of the liposomes/encapsulation of antibiotic on the effective encapsulation of antibiotics, with different methods leading to optimal results for hydrophilic versus hydrophobic antibiotics. Methods leading to multilamellar vesicles (MLV) are preferred for antibiotics that are highly hydrophobic and large unilamellar vesicles (LUV) are preferred for hydrophilic antibiotics. In addition, we demonstrate that significant bias can be introduced into quantification of antibiotic encapsulation efficacy through (i) liposome disruption and cleaning methods, (ii) analysis of results, by not accounting for mass loss of antibiotics during the preparation process. Regardless of the preparation and cleaning methods adopted, we strongly recommend evaluating/reporting percentage mass kept as a criterion for evaluating the suitability of antibiotic encapsulation methods. In summary, our results point to the importance of evaluating the methods for nano-encapsulation of antibiotics and being mindful of potential biases in methods and analysis.

6. Supplementary information

7.1 Calculation of estimated encapsulation efficiency:

The calculation efficiency done in this work was done for FAT-Vanco liposomes. **Figure 3.6** with an average size of 215 nm and a polydispersity index (PDI) of 0.246. The formula used was:

$$EE\% = \frac{\sum_j V_{RI_J} \cdot \left(\frac{c \cdot V \cdot N_A}{(0.635) \sum_k \left(\frac{V_{SE_k} + V_{SI_K}}{V_{lipid}} \cdot P_k \right)} \right) P_j}{V} \cdot 100$$

EE = encapsulation efficiency

c = lipid concentration

V = volume of liquid added

NA = Avogadro’s constant

VRI = volume ring internal

VSE= volume sphere external

VSI = volume sphere internal

Vlipid = volume lipid

P = liposome distribution

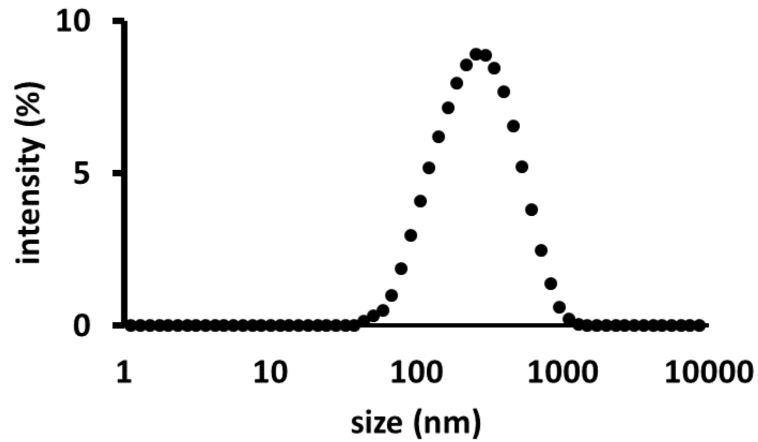

Figure S2.1. *Size distribution of FAT-Vanco liposomes, 18% solubility in water, obtained by dynamic light scattering.*

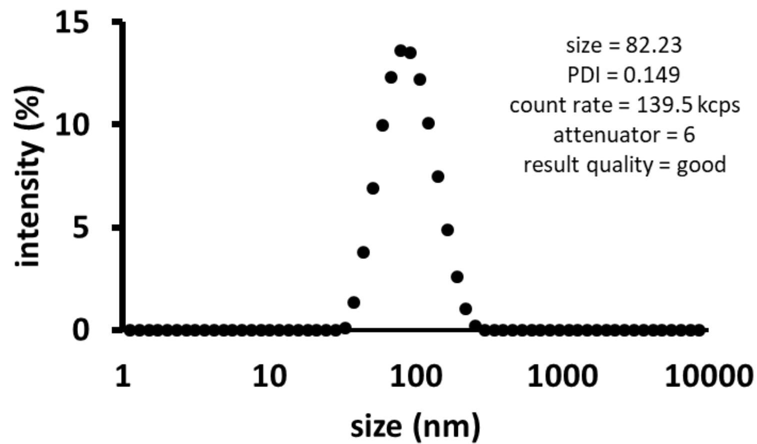

Figure S2.2 *DLS results of teicoplanin liposomes created by REV with 80% of teicoplanin's solubility in water.*

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Chapter 3 – Preserving antibiotic efficacy during nano-encapsulation in liposomes

1. Abstract

Antibiotics, in general, are extremely sensitive to elevated temperatures (sometimes degrading during prolonged incubation at physiological temperatures) and temperature fluctuations. Liposomes are attractive vehicles for encapsulation and localized delivery of antibiotics, but the liposomal preparation and encapsulation process involves numerous cycles of freeze-thaw as well as heating/sonication steps, all of which can potentially deactivate or degrade antibiotics. We investigate the extent of antibiotic deactivation during the liposomal preparation method, using two different antibiotics commonly used for *Staphylococcus* infections, namely vancomycin hydrochloride and teicoplanin. Both antibiotics, in the non-encapsulated state, were found to be sensitive to freeze-thaw cycles (representative of liposomal preparation methods), with vancomycin completely losing efficacy after three cycles of freeze-thaw/sonication cycles and teicoplanin losing some efficacy in the initial heating step. However, this effect was significantly mitigated when the antibiotics were encapsulated in liposomes, with the released antibiotic showing full potency against bacterial cultures of *Staphylococcus aureus*. Differential Scanning Calorimetry liposomes and mass spectrometry suggest that liposomes had a protective effect on the encapsulated antibiotic. The protective effect of lipid vesicles towards the antibiotics serves as an additional advantage or antibiotic encapsulation, mitigate the need for frequent administration of highly heat sensitive drugs.

2. Introduction

The discovery of antibiotics was one of the most significant medical achievements of the 20th century. However, eradication of infections in biofilm mode and intracellular infections has long been a challenge for antibiotic therapy.[1,2] That, combined with the rise of highly antibiotic-tolerant bacterial mutants, calls for employment of methods that can deliver very high doses of antibiotics, directly to the site of an infection; concentrations

that would be toxic or even deadly if delivered systemically. Drug delivery vehicles, have been used to encapsulate and deliver antibiotics, improving their therapeutic index while minimizing their adverse effects.[3] The encapsulation of antibiotics in drug delivery vehicles is not trivial because antibiotics are sensitive molecules and may degrade during the encapsulation process. Therefore, methods of preparation need to be evaluated to assess the possible degradation caused by preparation and encapsulation steps.

Liposomes are attractive vehicles for encapsulation of antibiotics. Cargo is encapsulated in liposomes during preparation of the lipid vesicles. When liposomes are created, cargo (plus carrier) is mixed with the phospholipids and heated above bilayer melting temperate (T_m). Heat-sensitivity of the antibiotics is a major factor to be evaluated in this process, because a significant number of phospholipids used for pharmaceutical purposes have a T_m above 37°C (DSPC-55°c, DPPG-41°C, DPPC-41°C); therefore, the antibiotics might be degraded or deactivated during liposomal preparation/encapsulation process.

Furthermore, most non-microfluidic liposomal preparation methods also involve extreme temperature fluctuations and/or the use of ultrasonication. Freeze-thaw cycles and ultrasonication are essential steps in creating liposomes with high cargo encapsulation efficiencies and uniform lamellarity and size distribution. However, extreme temperature fluctuations are also known to lead to antibiotic degradation,[4] and sonication can be deleterious to the fragile antibiotic molecule.[5]

In this work, we investigated the effect freeze-thaw cycles as well as sonication on the efficiency of three anti-staphylococcal antibiotics (vancomycin hydrochloride and teicoplanin, and rifampin) in the non-encapsulated free state and once encapsulated in liposomes. Motivated by the significant difference in efficacy of free and encapsulated liposomes, we investigated the possibility of a thermo-protective effect from the lipid vesicles, using DSC and mass spectrometry. We hypothesized that this chemoprotective effect could be beneficial for preserving efficacy for extremely heat-sensitive antibiotics at physiological temperatures, potentially increasing their half-life in the body.

3. Experimental section

3.1. Chemicals and lipids

The lipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (16:0 PC DPPC) (99%, Avanti, Millipore Sigma) was used for all experiments. The antibiotics, vancomycin hydrochloride (pharmaceutical grade secondary standard), Rifampin (≥97%), and Teicoplanin (≥80%), as well as Triton X-100, XTT sodium salt (2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) (>90%), Menadione (2-Methyl-1,4-naphthoquinone) (98%) were obtained from Millipore Sigma. Cholesterol (95%) was obtained from Fischer Scientific. The remaining chemicals; PBS, Methanol ≥99.9%, Diethyl ether, and KCl, acetone, and Mueller Hinton Broth (MHB), were obtained from VWR.

3.2. Bacteria and culture conditions

Staphylococcus aureus ATCC 25923 from frozen glycerol stock, was inoculated into Mueller Hinton Broth (MHB) and grown aerobically at 37 ℃ to approximately 5×10^5 CFU/mL (or 0.5 McFarland Standard). This culture was then used in microdilution assays with free antibiotics or liposome-encapsulated antibiotics. All cultures were used freshly prepared.

3.3. Microdilution for minimal inhibitory concentration (MIC)

Stock solutions of vancomycin hydrochloride and teicoplanin, and rifampin were prepared at 1mg/mL, 0.5mg/mL, and 0.5mg/mL, respectively and filter sterilized. Both vancomycin and teicoplanin solutions were prepared with milliQ water (resistivity of 18.2 MΩ.cm at 25°C) while rifampin solution was prepared in DMSO. A series of two-fold micro-dilutions were conducted for respective ranges of 0.08 to 40μg/mL, 0.06 to 60 μg/mL, and 0.004-128 μg/mL. 500mL of bacteria solution was pipetted into microcentrifuge tubes before inoculation with the same volume of antibiotic solution. Each microcentrifuge tube was sealed with parafilm to minimize chances of evaporation and contamination. The solutions were then incubated overnight in the shaking incubator at 37°C, 200rpm. MIC was determined for each antibiotic as the minimum antibiotic

concentration that inhibited growth, as quantified by measuring OD_{600} for the cultures using a BioTek Plate reader.

3.4. Liposome preparation and quantification of loading

Liposomes with vancomycin and teicoplanin were prepared using freeze-thaw thawing, and reverse phase respectively, as described in chapter 2. For teicoplanin **(Figure 3.1a)**, a thin film was formed and diethyl ether (3mL) and Millipore water (1 mL) were added with the teicoplanin in the organic phase. The system was sonicated for 40 minutes at a temperature below 10ªC and then the solvent was removed under reduced pressure for 40 min. For vancomycin **(Figure 3.1b)**, a thin film was produced using DPPC and cholesterol and it was dried overnight, the film was rehydrated with MilliQ water containing vancomycin hydrochloride at 42°C and underwent 3 cycles of freezing at -196ºC and thawing at room temperature with sonication or at 50ºC. The lipid vesicles were then extruded, 55×, using an Avanti Mini Extruder at 42°C using a 0.4 nm pore size membrane.

The concentration of antibiotic encapsulated inside was determined using High Performance Liquid Chromatography (HPLC) as described in Chapter 2. Briefly, the liposomes were cleaned using ultra-filtration, then they were burst using methanol. The amount of free drug was obtained by quantifying the free drug phase in HPLC-Micro-TOF and the total content was obtained by quantifying the methanol sample. The encapsulation efficiency was calculated using: $EE = \frac{total\ drug - free\ drug}{total\ drug} \times 100$

3.5. Freeze-thaw, sonication, and temperature challenge

For freeze-thaw challenge, antibiotics at stock concentrations were loaded in microcentrifuge tubes. All the tubes were flash frozen with liquid nitrogen and subsequently thawed in a water bath at 50°C, as shown in **Figure 3.1c**. For sonication challenge, antibiotics in microcentrifuge tubes were thawed using sonication at 27°C. The cycles were repeated up to three times. Each sample was then diluted to the corresponding MIC, added to *S. aureus* cultures, and incubated aerobically at 37°C for 24 hrs. The level of bacterial growth was quantified using the XTT assay.

As positive control for degradation, vancomycin and teicoplanin and rifampicin were incubated at 50°C overnight. The degradation products were analyzed using HPLC-TOF-MS. In addition, teicoplanin was sonicated with 3 cycles of 5-minute sonication in a bath sonicator (35 KHz 90W).

3.6. XTT assay

XTT sodium salt was prepared at 1 mg/mL in phosphate-buffered saline, filter sterilized and stored at −70°C, protected from light. Menadione, an electron carrier used to increase sensitivity of assay, was prepared as a 10 mM stock solution in acetone and stored at −70°C. A ratio of 10:1 of XTT:Menadione was used as a stock solution for the assay. This stock solution was added to bacterial (20µL XTT stock: 200µL bacterial culture) and incubated in the dark for 1 hr. The colorimetric signal was quantified by measuring the adsorption peak at 490nm using a BioTek Synergy Neo plate reader.

3.7. Efficacy and degradation of liposome-encapsulated antibiotic

Liposome stocks were diluted to achieve desired antibiotic concentration. Liposomes of each dilutions were then added to *S. aureus* cultures at a 1:1 ratio and the mixture was incubated overnight in a shaking incubator at 37°C. The assay was repeated for liposomes when burst with Triton-X, to not disturb the liposomes, in a 8:2 (liposome:triton-X) ratio. Bacterial growth in both cases was quantified using the XTT assay.

In addition, both liposomes were incubated overnight in the incubator at 37°C. The liposomes were burst on the next day with methanol (1:20 water:methanol). HPLC-TOF-MS was used to analyze the degradation products.

3.8. High Performance Liquid Chromatography (HPLC) and Time-of-Flight Mass Spectrometry (TOF-MS)

For all HPLC measurements, Agilent 1200 series HPLC with Bruker Mictotof II mass spectrometer was used with the analytical column Agilent XDB-C18 (100 mm×2.1 mm, 3.5 µm). The mobile phase consisted of two solvents; Eluent A: aqueous formic acid (0.1% v/v); Eluent B: acetonitrile containing 0.1% formic acid (0.1% v/v).

For vancomycin and teicoplanin, the column temperature was 40°C with the injection volume of 10 μL. for vancomycin, the run time was 21 min with a flow rate of 0.3 mL/min and a gradient elution program as follows: 97% mobile phase A for 3 min; linear increase to 30% B over 7 min, hold for 2 min; afterwards a linear increase up to 80% mobile phase B within 1 min, hold for 2 min; return to the initial condition within 1 min and re-equilibration for 5 min. For teicoplanin, the run time was 6.5 min at a flow rate of 0.5 mL/min. The gradient elution program as follows: 97% mobile phase A was introduced from initial sample injection hold for 1 min, then switched to 97% mobile phase B over 1 min held for 1 min; returned to initial conditions within half a min and re-equilibration for 3 min.

For rifampin: The column temperature was 25°C and the injection volume was 10 μL. Run time was 12 min at a flow rate of 0.4 mL/min and a gradient elution program as follows: 65% mobile phase A, held for 1 min, increased to 90% solvent B over 4 min, then increased to 95% solvent B, held for 3 min, returned to initial conditions and re-equilibration for 4 min.

Quantification was achieved by TOF-MS positive ion electrospray ionization. Ion detection was performed at m/z 724.7 for vancomycin hydrochloride, at m/z: 939.7, 940.7, 947.8, and 782.4 for teicoplanin components, and m/z 823.4 for rifampin. For teicoplanin quantification, the determination of the area under the curve was obtained as the sum of the four major compounds.

3.12. Dynamic Scattering Calorimetry (DSC)

Differential scanning calorimetry (DSC, Q200 model from TA Instruments, New Castle DE) was used to measure the thermal properties of antibiotics, lipids, and liposomes. Of each sample, 10mg, were placed in an aluminum crucible, while an empty crucible was placed in the reference cell. The antibiotics and liposomes were in liquid solutions. A heat ramp from 30 ˚C to 230 ˚C at 10 ˚C/min was applied to each sample. Tzero pans and Hermatic lids (TA Instruments) were used for the measurements. The melting point was determined as the maximum of the endothermic melting peak.

3.13. Statistical analysis

All data presented are the average of at least three independent experiments, presented along with the standard deviation between values obtained for the independent experiments. Significance of difference between the different conditions was analyzed using Student's t test with Bonferroni correction, and P values of <0.05 were considered significant.

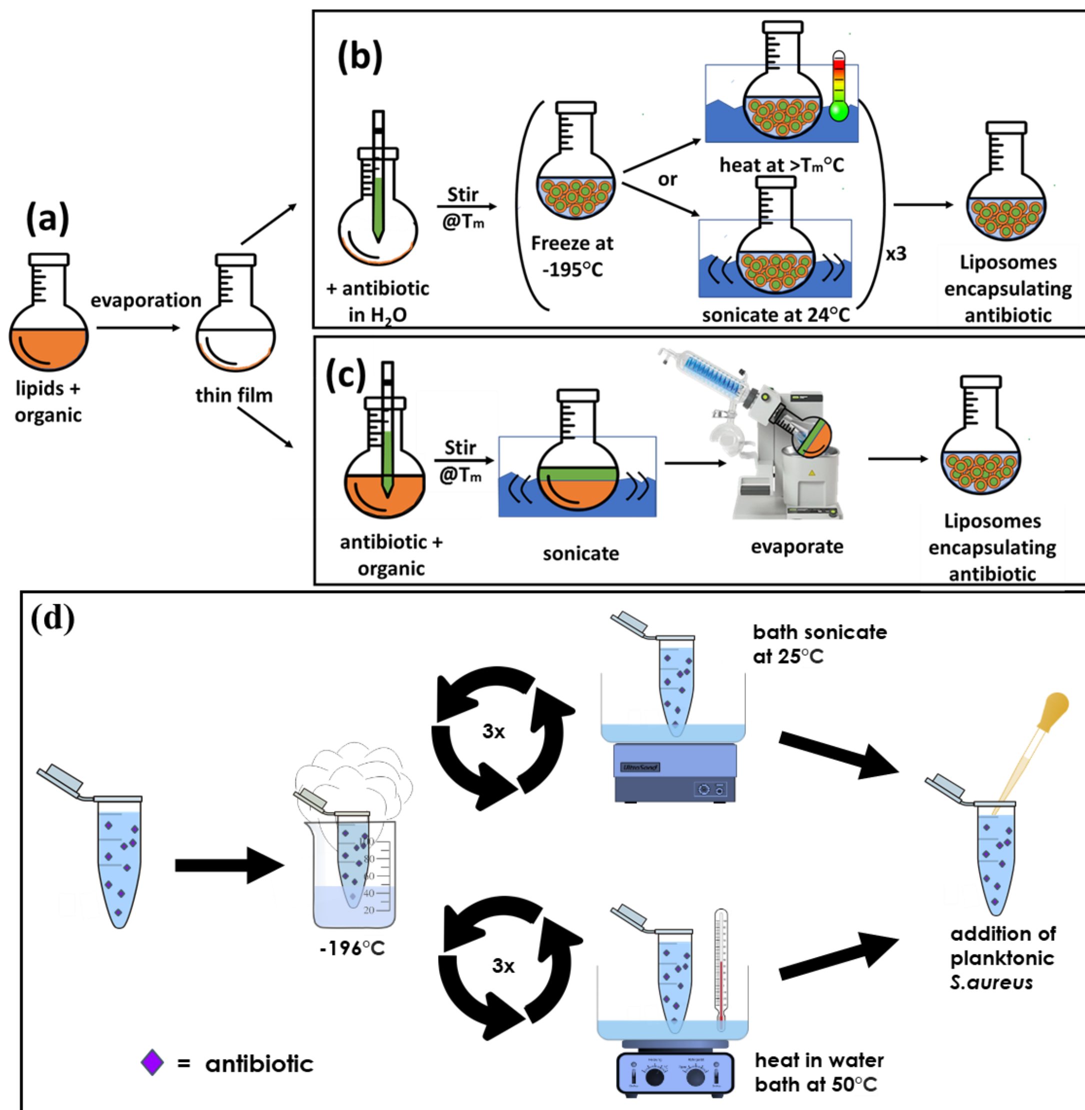

Figure 3.1. ***(a)*** *Producing a thin film is frequently the first step of producing liposomes, to rehydrate said thin film it is necessary to heat the formulation above the temperature of melting of the lipid (T_m).* ***(b)*** *Freezing and thawing liposome preparation method. In this method a lipid film is produced and afterwards it is rehydrated at the lipids T_m (42°C for*

*DPPC), then it is subjected to three freeze and thawing cycles and finally unilamellar liposomes are produced. **(c)** Reverse phase liposomal preparation has a long sonication step (40 min) and reduced pressure step (40 min) **(d)** Experimental design, antibiotics were subjected to cycles of freezing and thawing at 50°C and freezing and thawing at room temperature in a bath sonicator for 1, 2 and 3 cycles. Then their antimicrobial activity against S. aureus was evaluated.*

4. Results and Discussion

Vancomycin, an antibiotic that has been encapsulated vastly in liposomes[6-9], showed degradation after freezing and thawing when it was in free drug form, however, when the antibiotic was encapsulated in liposomes the degradation effect did not happen and the liposomal formulation showed more activity against an staphylococci infection. We also studied teicoplanin, obtaining different results, the drug did not show high sensitivity to the cycles of freezing and thawing and a thermo-protective effect was also observed; a reduction of teicoplanin's efficacy was still observed and it might be caused by a degradation provoked by sonication as the degradation products of the liposomes observed via mass spectrometry match the ones due to sonication.

4.1. Effect of freezing and thawing on antibiotic efficacy

The MIC for vancomycin and teicoplanin against *S. aureus* 25932 was determined to be 5 µg/mL and 62.5 µg/mL respectively. When Vancomycin and teicoplanin were subjected to three cycles of freezing and thawing at room temperature with sonication (FAT+S) and in a separate experiment they were subjected to three cycles of freezing and thawing at a temperature above the T_m of the lipid used, namely 50°C (FAT+50ºC). *S. aureus* cultures were then challenged with the antibiotics and the bacterial metabolic activity was measured using XTT assay. As shown in **Figure 3.2a,** vancomycin lost antimicrobial activity against *S. aureus* after being subjected to freezing and thawing, with each freeze-thaw cycle adding to the efficacy decline (as represented by the increase in bacterial growth), and the cycles that included heating represented the largest decrease in vancomycin efficacy. In the case of teicoplanin, **Figure 3.2b**, the cycles of freezing and thawing with sonication (and not with heating) affected antibiotic efficacy the most; interestingly, freezing and thawing showed small decrease in teicoplanin's activity.

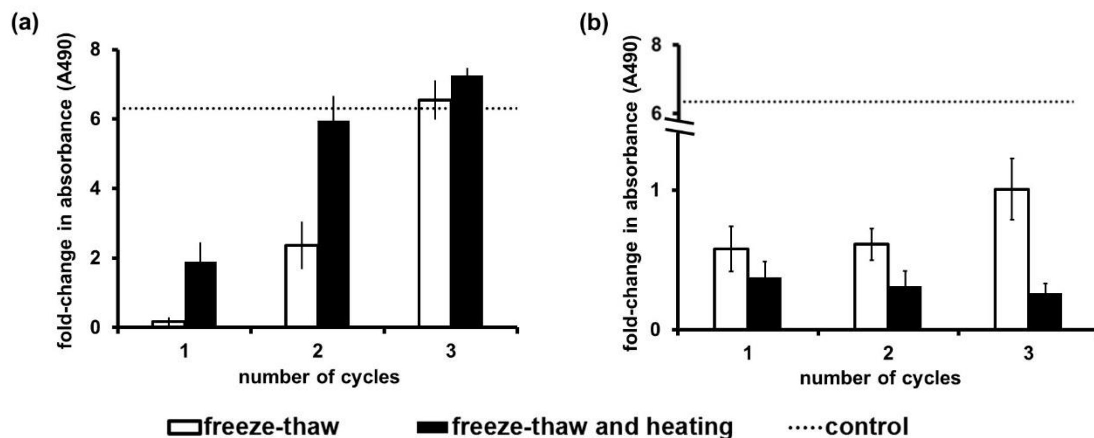

Figure 3.2. *Metabolic activity of S. aureus in the presence of **(a)** vancomycin and **(b)** teicoplanin challenged by freeze-thaw cycles with the thawing step done at room temperature with sonication or freeze-thaw cycles with the thawing step done at 50°C.*

Previously it has been shown that MIC of vancomycin against S. *aureus* 25923 increased by less than 2 fold when heated at up to 120°C for 15 mins,[10] and by less than a 1 fold when exposed to temperatures up to 100°C for 15 min.[11] However, freezing vancomycin samples at -20°C (typical storage temperature) and then thawing at room temperature or at 4°C was reported to have no effect on antimicrobial activity.[12] We employed a much lower freezing temperature of -196°C, representative of temperatures used during liposome preparation, and thawed the samples at either room temperature or 50°C in less than 30 minutes.

Teicoplanin showed a very different trend in that cycles of freezing and thawing did not affect teicoplanin's efficiency against *S. aureus* to the same extent as vancomycin. Interestingly, teicoplanin's activity did decrease significantly after 15 min of heating at 120 ºC,[13] and in our case does not decrease after cycles of flash freezing followed by thawing at 50°C. However, when the sample was thawed by sonication at room temperature, the activity of teicoplanin decreased, suggesting possible degradation of teicoplanin.

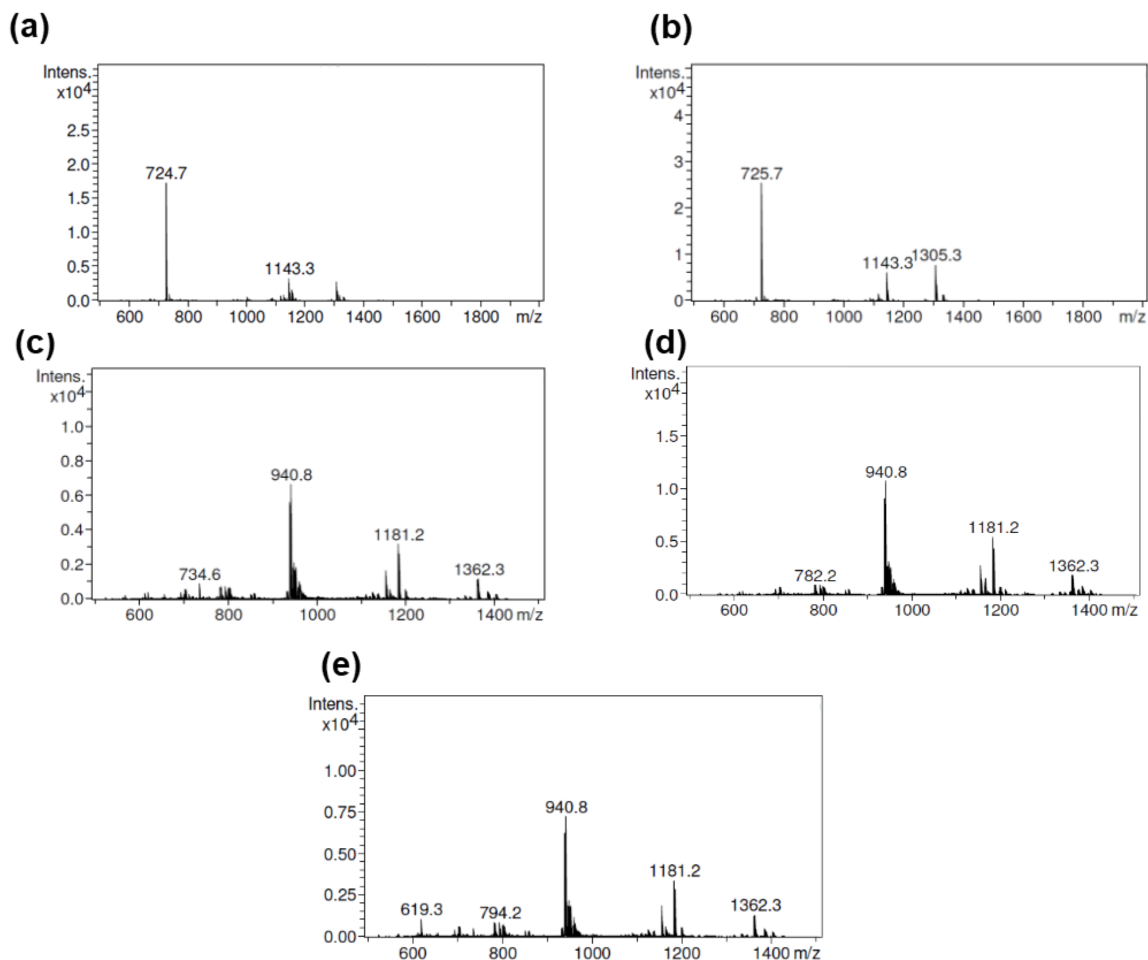

***Figure 3.3 .** (**a**) Mass spectrometer of vancomycin. (**b**) Mass spectrometer of vancomycin after incubation at 50°C overnight. (**c**) Mass spectrometer of teicoplanin. (**d**) Mass spectrometer of teicoplanin after incubation at 50°C overnight. (**e**) Mass spectrometer of teicoplanin after 3 cycles of sonication for 5 minutes each.*

Vancomycin is known to degrade into crystalline degradation product-1 (CDP-1) after only three hours of incubation at room temperature, with 50% of it being degraded after 16 hrs, and 90% being degraded to CDP-1 after 40 hrs.[14] In this experiment we identified the presence of CDP-1 after overnight incubation at 50°C (**Figure 3.3b)**, non existant in the control **(Figure 3.3a).** Teicplanin did not show new peaks when compared to the control **(Figure 3.3c).** When heated overnight at 50ºC (**Figure 3.3d**), however new peaks appeared after 3 cycles of sonication for 5 minutes each **(Figure 3.3e).**

4.2. Efficacy of antibiotics when encapsulated inside liposomes.

When vancomycin and teicoplanin, encapsulated with liposomes, were compared with the same concentration of free antibiotics in terms of efficacy against bacterial cultures of *S. aureus*, the free drug outperformed the liposomes in both cases (**Fig 3.4**). We suspected possible bias in these results because of the slow release nature of the liposomal vehicles and so the experiment was repeated with burst liposomes. When the liposomes were burst, releasing their entire content, the antibiotic released from the liposomes showed full potency. Once the liposomes were burst, the released vancomycin showed a better effect at reducing bacterial growth than the free antibiotic, even at concentrations 25% of MIC (**Figure 3.4a**). This potentiation of the killing capacity produced by neutral liposomes has been observed and reported previously with other antibiotics[15-17] and also for negatively charged vancomycin liposomes.[8] Halwani et al. found that gentamicin neutral liposomes required a twofold lower MIC than free gentamicin against *Pseudomonas aeruginosa* and *Klebsiella oxytoca*[16]. However, these liposomes had been burst so the increase in activity could be because the antibiotic was protected and delivered for a longer period of time, this theory seems plausible because potentiation was not detected for teicoplanin liposomes; in these liposomes the drug gets intercalated in the lipid bilayer being more readily available.

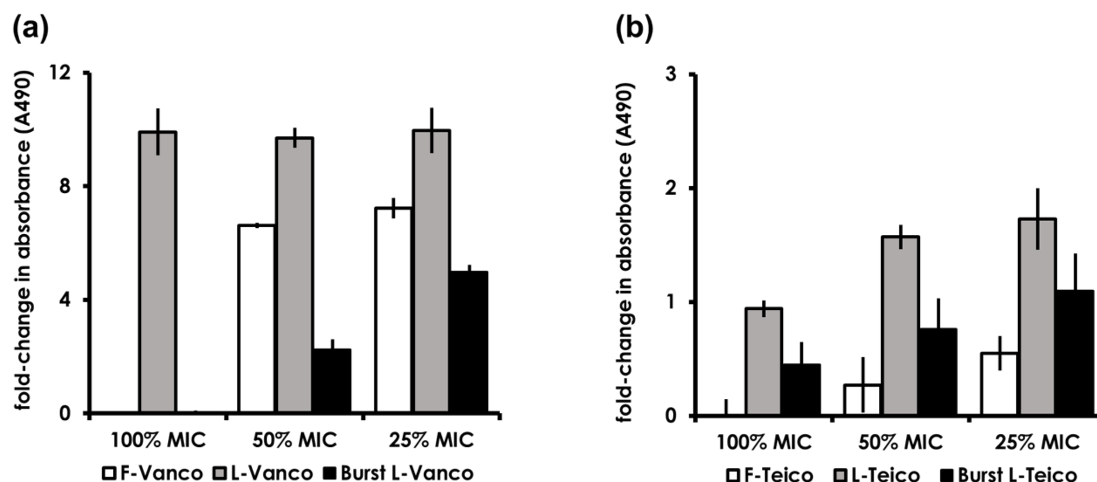

Figure 3.4. ***(a)*** *Metabolic activity of S. aureus measured by XTT assay when challenged with free vancomycin (F-Van), liposome-encapsulated vancomycin (L-Van) and burst liposomal vancomycin (Burst L-Van), all changes were statistically significant (p <0.05). except for 100% F-Vanco vs. Burst L-Vanco, and 25% F-Vanco and L-vanco.* ***(b)*** *Metabolic activity of S. aureus 25923 measured by XTT assay when confronted with free teicoplanin (F-Tei), liposome-encapsulated (L-Tei) and burst liposomal teicoplanin (Burst L-Tei), all changes were statistically significant (p <0.05).*

In case for teicoplanin, even after bursting induced by triton-X, bacterial growth control is not as effective as that with the free antibiotic (**Figure 3.4b**). We theorized two possible reasons for this, one could be that even when bursting with triton the entire content wasn't fully released, since teicoplanin is contained both in hydrophilic core and the hydrophobic bilayers of the liposomes (unlike vancomycin that is completely contained in the aqueous core), thus even when the vesicles are burst, some teicoplanin could be contained in the lipid aggregates. The other reason could be a forfeit of activity due to sonication in the process of creating the liposomes, this reason seems plausible since after three cycles of freezing and thawing with sonication at room temperature a one-fold increase in metabolic activity of bacteria was observed with a teicoplanin's MIC concentration **(Figure 3.2b)**.

4.3. Thermo protective effect of liposomes on encapsulated antibiotics

The relatively high efficacy of vancomycin encapsulated in liposomes against *S. aureus* cultures, compared to when free vancomycin that was put through the same temperature fluctuations as the liposome, lead us to investigate possible chemoprotective effect of the lipid vesicles. We evaluated this by using differential scanning calorimetry (DSC), where the peak for vancomycin encapsulated in liposomes was compared with the peaks for all the components for the liposomes, including the free antibiotic.

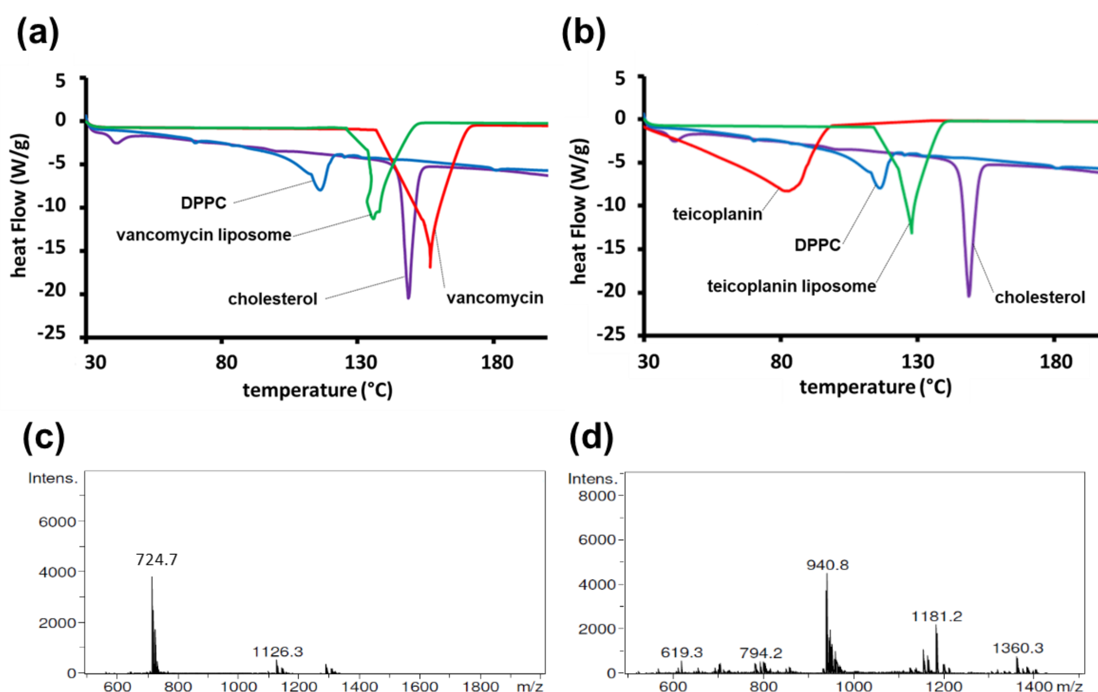

Figure 3.5. ***(a)*** *DSC showing the protective effect of vancomycin when loaded in liposomes.* ***(b)*** *DSC showing the protective effect of teicoplanin when loaded in liposomes.* ***(c)*** *Mass spectrometer of vancomycin liposomes kept overnight at 37°C, no degradation peaks are observed.* ***(d)*** *Mass spectrometer of teicoplanin liposomes kept overnight at 37°C, peaks similar than the ones observed with teicoplanin subjected to sonication are observed.*

When heating up phospholipid bilayers, the sample undergoes a thermotropic gel to liquid crystal phase transition, that is represented as an enthalpy change. Cholesterol's thermogram shows a large endothermic peak at 148.6°C (**Figure 3.5a,b**). Liposomes

show a broadened peak that is also shifted to the lower range, representative of liposomes (**Figure 3.5 a,b**).[18] The endothermic peak for vancomycin appears at 156.7°C and in vancomycin liposomes is eliminated (**Figure 3.5 a**), which suggests incorporation of the drug inside the liposomes. The same case occurs with teicoplanin with its original peak being eliminated when encapsulated in liposomes. These results only confirm incorporation of antibiotic in the liposome. To confirm thermopreotective effect we conducted mass spectrometry to detect possible degradation compounds. As shown in **Figure 3.5c**, the vancomycin molecule does not degrade in the process of creating the liposomes (compare peaks to **Figure 3.3a**), but the teicoplanin liposomes do show a degradation, and the spectrophotogram of the teicoplanin present in the liposomes after incubation at 37°C is very similar to the antibiotic that was degraded by sonication (**Figure 3.3e**) not to the one observed when heating the sample (**Figure 3.3d)**, suggesting that the loss in efficiency of the drug observed in **Figure 3.4b** to be due to a sonication effect. Therefore, the liposome does not seem to offer a protective effect against sonication.

Liposomal thermo-protective effect and prolonged release of drugs makes the system particularly attractive to drugs that are heat sensitive. Some antibiotics are so sensitive that even prolonged incubation at body temperature 37°C times has been studied; Beta-lactam antibiotics were found to degrade quite rapidly at 37°C whereas aminoglycosides, glycopeptides, tetracyclines and quinolones showed low degradation.[13]

5. Conclusion

Liposomal encapsulation of antibiotics is not trivial, and the techniques previously used to encapsulate other drugs like anticancer drugs cannot blindly be translated to antibiotic encapsulation. In a study, 25 out of 62 antibiotics, among them them beta lactams were extensively or totally inactivated by autoclaving; they also studied aminoglycosides and quinolones which proved to be heat stable, 21 antibiotics were found to loose activity after being exposed to 121°C for 15 minutes.[10] Liposomal preparation methods involve extreme temperatures and temperature fluctuations; therefore, the study of the impact of methods used in creating of the liposomes on antibiotic efficacy is fundamental to

designing effective antibiotic delivery vehicles. In this work we found that even though vancomycin is sensitive to freeze-thaw cycles and sonication, once encapsulated in lipid vesicles (as confirmed by DSC), it seems to be protected from degradation (as confirmed by mass spectrometry) as well as its potency against *S. aureus* cultures. Teicoplanin, an antibiotic with a different chemical structure and different level of hydrophilicity, also lost antimicrobial activity during the encapsulation process, but due to freeze-thaw-sonication and not freeze-thaw-heating. However, the lipid vesicles so not seem to offer protective affect for this antibiotic against the detrimental effects of sonication, leading to a decline of potency against *S. aureus* cultures. These results highlight the importance of being mindful of the effect that the preparation/encapsulation process has on the drug. The results further point to a potential added benefit of antibiotic encapsulation, namely heat protection, especially for antibiotics that are known for degrading at physiological temperatures.

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Chapter 4 – Future perspective

1. Challenges

This project started because of the difficulty of replicating experiments that were reported in the literature; different publications reported different methods to be ideal for the encapsulation of a specific substance, the encapsulation efficiencies were calculated using different formulas which created biases in the methods as being good or bad. This variability could be because antibiotics are have different chemical and structure and properties compared to other drug; thus, making extension of methods developed for other cargo to antibiotics challenging. A similar situation occurs with lipids, with more than 20 lipids available commercially for liposomal production and the possibly to mix and match (to an extent), identifying the ideal methods for creating liposomes for all cases is an extremely difficult task. We also found papers that reported the use of absorbance to quantify a drug even though we found interferences between the drug and the lipid present in the liposomes at the drug absorption wavelength. A similar problem occurred when we tried evaluating the MIC of liposomes by reading optical density; we encountered interferences with some antibiotics. That led us to use colorimetric methods like, XTT, but we again found interference, this time not with the lipids but with one of our antibiotics: rifampin.

2. Impact

We reported three main actions that can be taken to avoid biases in the assessing of different methods of encapsulation of antibiotics in liposomes: (1) Mass kept should be taken in consideration when encapsulating a drug because in some cases a lot can be encapsulated but the process promotes drug loss. (2) Encapsulation efficiency should be calculated with a method that does not generate interference, like HPLC. (3) The separation of free and encapsulated drug as well as the bursting of the liposomes needs to be evaluated to determine if the methods used efficiently convey their objective.

We showed that antibiotics are susceptible to heat or sonication and it should be taken in consideration when encapsulating them inside liposomes, furthermore we showed a protective effect of the liposomes to the encapsulated antibiotic, this is very attractive for long term delivery of antibiotics that are heat sensitive, such as rifampin.

3. Future Directions

Liposomes are promising since they show low toxicity, they improve therapeutic index and some formulations have already been approved by regulatory agencies. We also found that they offer a thermo-protective effect that is essential for the prolonged deliver of heat-sensitive antibiotics. In order to deliver big quantities of antibiotics where they are needed, a biofilm for example, we could target the antibiotics specifically to the site of infection; however, it is necessary to understand about the interactions of liposomes with planktonic bacteria and biofilms, an area less explored compared to interaction of lipid vesicles (and nanoparticles in general) with mammalian cells.