Liposomal Nanovesicles for Efficient Encapsulation of Staphylococcal Antibiotics

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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 techniques on the basis of encapsulation efficiency, lipid requirement (important for avoiding lipid toxicity), and mass yield (percentage of mass retained during the preparation process). The moderately hydrophobic (teicoplanin) and highly hydrophobic (rifampin) antibiotics varied significantly in their encapsulation load (max 23.4 and 15.5%, respectively) and mass yield (max 74.1 and 71.8%, respectively), favoring techniques that maximized partition between the aqueous core and the lipid bilayer or those that produce oligolamellar vesicles, whereas vancomycin hydrochloride, a highly hydrophilic molecule, showed little preference to any of the protocols. In addition, we report significant bias introduced by the choice of analytical method adopted to quantify the encapsulation efficiency (underestimation of up to 24% or overestimation by up to 57.9% for vancomycin and underestimation of up to 61.1% for rifampin) and further propose ultrafiltration and bursting by methanol as the method with minimal bias for quantification of encapsulation efficiency in liposomes. The knowledge generated in this work provides critical insight into the more practical, albeit less investigated, aspects of designing vesicles for localized antibiotic delivery and can be extended to other nanovehicles that may suffer from the same biases in analytical protocols.

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, some of the 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 the usual routes of administration is challenging because antibiotics are significantly diluted by the time they reach 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.4,5 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.6 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 nanovehicle by target bacterial cells;7 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 and/or technique used for their preparations. The cargo is typically loaded during the preparation step, resulting in the encapsulation load being strongly affected by the preparation process. The optimal choice of liposome preparation/cargo encapsulation technique depends on the physicochemical characteristics of the material to be encapsulated, the desired size and polydispersity of the vesicles, the desired bilayer properties, and the ease of upsampling the process.8 Multiple techniques for liposome preparation/cargo encapsulation have been reported in the literature. The preparation/encapsulation techniques can be classified into three major categories: mechanical dispersion, solvent dispersion and detergent removal. These techniques have four main steps in common:6,9

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(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 purposes and for accurate determination of encapsulation load.

Multiple reports of antibiotic encapsulation in liposomes have been published using various lipids. A review of the literature on liposomal encapsulation of antibiotics, however, can cause confusion and, in some cases, even shows inconsistencies. A clear example of one of these inconsistencies is the encapsulation of the antistaphylococcal antibiotic rifampin. A group reported 0% encapsulation efficiency using the thin film (TF) method with 1,2-distearoyl-sn-glycero-3-phosphocholine and cholesterol, whereas Manconi et al. reported encapsulation efficiency of 74% with a slightly modified thin-film technique and using a very similar lipid. We hypothesize that these inconsistencies in the literature (most of which we also observed in our lab) are suggestive of a chronic bias in both preparation/encapsulation techniques and the analytical techniques adopted for characterization, partially fueled by the lack of technique development focused on antibiotics. Antibiotics have a different chemical structure, and dissociation constant ($pK_a$) than antitumor drugs. In fact, for antibiotics, very few $pK_a$ values are currently available. This gap in knowledge has resulted in bias when it comes to developing both synthesis and analytical techniques for encapsulation of antibiotics.

We set out to explore the extent of reach for the hypothesized biases by evaluating three of the more common preparation techniques, shown in Figure 1, for three different antistaphylococcal antibiotics with significantly different hydrophobicity: vancomycin hydrochloride, teicoplanin, and rifampin. We used three widely used liposome preparation techniques and evaluated each technique based on the encapsulation efficiency for each antibiotic, lipid usage (important for avoiding lipid toxicity), and antibiotic mass yield (or mass loss during the process), as well as the final liposome size and ζ potential. To decrease scatter, we used the same lipid for all methods and antibiotics. We further developed a technique for quantifying the encapsulation efficiency with minimal bias to address the significant bias observed in techniques reported in the literature for analyzing liposomal antibiotic encapsulation.

**RESULTS AND DISCUSSION**

**Liposomal Preparation Technique Impact on Encapsulation Efficiency and Mass Yield.** Liposomes encapsulating vancomycin hydrochloride, teicoplanin, and rifampin were prepared using the three methods (thin film; freezing, annealing, and thawing (FAT); and reverse-phase evaporation...
vancomycin hydrochloride, (b) rifampin, and (c) teicoplanin. 

FAT methods can be attributed to the temperature, which the solution needs to be heated to the lipids melting point. The low mass yield for rifampin and teicoplanin in TF and REV, as shown in Figure 1. Liposome encapsulation efficiency and mass yield during the process of creating the liposomes were then quantified. All liposomes from this section were prepared using an antibiotic concentration corresponding to 18% of vancomycin’s solubility limit in water and 80% of teicoplanin’s and rifampicin’s solubility in water and the lipid was added in a 1:3 ratio of cholesterol/1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC).

As shown in Figure 2, mass yield (percentage of mass retained during the preparation process) varied significantly for each antibiotic depending on the preparation technique. For vancomycin hydrochloride (water solubility 50 mg/mL), FAT retains more mass (93.4 ± 7%) whereas REV results in up to 57% mass loss (Figure 2a). For the highly lipophilic antibiotic rifampin (water solubility 1.3 mg/mL, highly lipid soluble), however, FAT resulted in a mass yield of 44.4 ± 9%, TF showed less than 10% mass remaining, and REV proved to be the most efficient method, resulting in 79.5 ± 3% mass yield (Figure 2b).

For the moderately lipophilic antibiotic, teicoplanin (water solubility 10 mg/mL), FAT resulted in a mass yield of 32.6 ± 9.2% and TF showed less than 50% remaining mass but REV proved to be the most efficient method, resulting in 93.4 ± 3.42% mass retained (Figure 2c).

The low mass yield for rifampin and teicoplanin in TF and FAT methods can be attributed to the “rehydration” step, in which the solution needs to be heated to 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 can take a long time (up to 40 min) resulting in partial evaporation of the aqueous volume; since we used rifampin and teicoplanin solutions at 80% of their solubility in water (1.3 and 10 mg/mL, respectively), evaporation of a fraction of water resulted in precipitation of rifampin or teicoplanin, further decreasing the loading efficiency. Vancomycin hydrochloride, which is highly soluble in water, is not affected in terms of mass yield by this evaporation step, because the concentration we used is 18% of its solubility limit in water, resulting in FAT being the most efficient method for vancomycin. REV, which was highly inefficient in terms of mass yield for vancomycin, proved the most efficient for rifampin. In REV, the aqueous solution with diethyl ether does not fully create a homogeneous dispersion after sonication, probably because 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 yield for vancomycin hydrochloride, also resulted in a 33.4 ± 3% encapsulation efficiency (Figure 2a). 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 disrupt the bilayer due to the formation of ice crystals during the freezing step, disrupting the closely spaced lamellae of the multilamellar liposomes vesicles and increasing the aqueous volume. A 10–50× 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. Additionally, extrusion of FAT liposomes reduces the lamellarity of the liposomes because the disrupted vesicles reassemble into unilamellar vesicles, increasing the internal aqueous volume. It is noteworthy that we conducted theoretical calculation of the aqueous volume for liposomes (model modified from Xu et al.) with the size distribution of our liposomes, which predicts an encapsulation efficiency of 33.72% (calculation given in the Supporting Information), close to that achieved using FAT. It is noteworthy that the adopted theoretical model does not account for the chemistry of lipids, presence of cholesterol, or the interaction of lipid with the antibiotic. It should also be noted that even though REV resulted in a higher encapsulation efficiency (39.4%) for vancomycin, its mass yield was less than 50%, making it a nonoptimal method.

In the case of rifampin and teicoplanin, REV method showed the highest encapsulation efficiency, 82.7 ± 0.8 and 84.1 ± 8.3%, respectively (Figure 2b,c). REV liposomes are known for producing a high internal aqueous volume and

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Effect of preparation method on the encapsulation efficiency (black bars) and mass yield (blue markers) for liposomes loaded with (a) vancomycin hydrochloride, (b) rifampin, and (c) teicoplanin. * P < 0.05; ** P < 0.005.
oligolamellar vesicles.\textsuperscript{27} For a drug that can only be loaded in an aqueous space such as vancomycin, a system that produces oligolamellar vesicles is not optimal, 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 oligolamellar vesicles with large aqueous volumes. 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 Bangham et al.)\textsuperscript{28} is known to produce multilamellar vesicles (MLVs) of multiple sizes (reducing aqueous space); therefore, it is necessary to use size reduction techniques, such as sonication or extrusion.\textsuperscript{29,30} This method is believed to be suitable for hydrophobic compounds. Previous reports of rifampin-loaded liposomes (prepared with soy lecithin with a lecithin/cholesterol ratio of 60:40 v/v) created via TF have reported encapsulation efficiencies that range from 53.3 to 79.25%, depending on the composition.\textsuperscript{29,30} However, our results show that the 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 was not low, the amount of rifampin mass loss during the process was remarkably high (>88% in our experiments); thus, we recommend encapsulating rifampin with the REV method. The case for teicoplanin is similar; its encapsulation efficiency was relatively high (63.03 ± 11.34%), a considerably high encapsulation efficiency, comparable with REV; however, its mass loss was significant with an yield of less than 50%.

The hydrodynamic diameter for vancomycin liposomes did not show a significant variation between the different preparation methods (Figure 3a). All liposomes were extruded through a 400 nm membrane; all liposomes are expected to be smaller than 400 nm (representative size distribution presented in Figures S1 and S2). The \( \zeta \) potential (in 1 mM KCl) for vancomycin liposomes was very close to neutral for all preparations (as expected on the basis of the charge of DPPC)\textsuperscript{17} with REV leading to a slightly more negative \( \zeta \) potential. The change in \( \zeta \) potential for REV vancomycin liposomes could be due to the presence of two types of liposomes created by REV methods (namely, multilamellar vesicles, MLVs, and large unilamellar vesicles, LUVs) promoting repulsion between the vesicles.

However, for rifampin, FAT and TF produced significantly bigger liposomes than REV (Figure 3b). Rifampin, being lipophilic, 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 internal space of the unilamellar vesicles is large. 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 also be explained by the antibiotic being exposed in the bilayer.\textsuperscript{9}

Teicoplanin liposomes (Figure 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 moderately hydrophilic and moderately 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 relatively high (63.03 ± 11.34%). Additionally, teicoplanin encapsulation in the bilayer could explain the relatively high negative \( \zeta \) potentials, \(-20.8 \pm 3.5 \) and \(-18.28 \pm 8.6\) 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.

**Solubility Limit of Antibiotics Affects Encapsulation Efficiency and Mass Yield.** Concluding from the previous section that FAT is the most efficient method for

![Figure 3](image-url)
encapsulation of vancomycin and REV is the most efficient method for encapsulation of rifampin and teicoplanin, we prepared FAT-vancomycin liposomes, with different vancomycin concentrations, corresponding to 8, 18, and 25% of vancomycin solubility limit in water, REV-rifampin liposomes with concentrations corresponding to 80, 100, and 120% of rifampin solubility limit in water, and REV-teicoplanin liposomes with concentrations corresponding to 50, 80, and 100% solubility 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.

As shown in Figure 4, changing the amount of vancomycin hydrochloride or teicoplanin used for the preparation of liposomes did not result in a significant change in terms of encapsulation efficiency, mass yield, size, or charge (Figures 4a,c and 5a,c). However, for rifampin, changes in the mass of rifampin used during liposome preparation result in small but statistically significant differences between encapsulation efficiency, they also significantly influence the mass retained, with the largest mass retention achieved at 80% of the rifampicin solubility limit in water (Figures 4b and 5b). This may be explained by the partial evaporation of water in the 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 be considered equally plausible. However, the addition of the mass loss during the process as an additional criterion

Figure 4. Encapsulation efficiency (black bars) and antibiotic mass retained (blue markers) for liposomes prepared with varying (a) vancomycin (FAT), (b) rifampin (REV), and (c) teicoplanin (REV) levels. * P < 0.05; ** P <0.005.

Figure 5. Hydrodynamic size (black bars) and ζ potential (blue markers) of liposomes prepared with varying (a) vancomycin (FAT), (b) rifampin (REV), and (c) teicoplanin (REV) levels. * P < 0.05; ** P <0.005.
for quality control provides extra 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 retained criteria, we can conclude that a concentration of 80% of the solubility limit is the best choice for rifampin encapsulation.

**Method Bias in Liposome Cleaning Methods.** While performing experiments with different preparation techniques, we observed drastic inconsistencies in results obtained for encapsulation efficiency depending on the chosen method for cleaning the liposome preparations. Ultimately, the encapsulation efficiency is calculated as the percentage of the antibiotic that is inside liposomes 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 a possible method bias at this stage is critical for ensuring proper quality control.

Various methods have been reported for separating encapsulated and free antibiotic; three of the most popular methods are centrifugation, ultrafiltration, and dialysis shown in Figure 6. In addition, multiple methods can be adopted for releasing the antibiotic encapsulated in the liposomes for the purpose of quantifying the total amount of antibiotics; two of the most popular methods are the use of Triton and methanol to disrupt the lipid bilayer. We focused on the two extremes in hydrophilicity/hydrophobicity 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).

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 optimal formulations obtained in the previous section. Even though this method could be biased if the samples do not retain 100% of the mass, the comparison between the two methods is still valid. Triton disrupted 77 ± 8.5% of vancomycin liposomes and only 53.31 ± 5.8% of rifampin liposomes (Figure 7a), whereas methanol disrupted the vesicles more efficiently, with 92.4 ± 7% of the vancomycin liposomes and 94 ± 1.73% of the rifampin liposomes disrupted (Figure 7b). Triton X has been commonly used to disrupt the liposomes since it changes the phospholipid organization, forming highly asymmetrical structures, 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. 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 toward bacteria because, unlike methanol, it does not represent major toxicity toward bacterial cells.

In addition, we quantified the efficiency of ultrafiltration and centrifugation for separating the liposomes from the unencapsulated 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 the liposomes were retained in the liposome layer (Figure 7c). Centrifugation, however, although widely employed, was not as effective, with liposomes detected in the separated antibiotic phase (Figure 7d). 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, 105.52 ± 6.5% of the initial liposomes were found in the liposomal phase and 57.31 ± 1.4% were left in the free antibiotic phase. The fact that these numbers do not add up to 100% indicates a clear method bias and suggests that centrifugation is probably disrupting the liposomes and breaking them into smaller vesicles. 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 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 7, when centrifugation was used as a cleaning method, the calculated encapsulation efficiency was very high for vancomycin (91%). For rifampin, however, the outcome was very different. Centrifugation lead to a calculated encapsulation efficiency of 45% and Triton (which does not fully burst the liposomes) led to a calculated encapsulation efficiency of 15%, both much lower than that calculated when ultrafiltration was used. A similar study conducted in 1987 showed that ultrafiltration outperformed centrifugation, air-fuge, and dialysis at separating free and encapsulated antibiotics; unfortunately, a review of the literature shows that centrifugation remains a common practice for separation of free and encapsulated antibiotics.

The final method that we used to calculate encapsulation efficiency was through dialysis. This method also separates unencapsulated drug from encapsulated drug by filtering the content through a membrane. The results obtained are similar to the encapsulation efficiency numbers calculated with methanol + ultrafiltration. The calculated efficiency for vancomycin was 41 ± 5% whereas for rifampin it was 63.7 ± 11%. That is why this is a popular method in the literature; however, there are two limitations that are worth mentioning: (1) the criteria for determining the time point in which all unencapsulated drug has been released is subjective, and (2) it is a lengthy method (Figure 8).

Figure 7. Liposome disruption with (a) Triton and (b) methanol. The percentage of disruption was obtained by measuring the amount of initial antibiotic existent in the system. Efficiency of separation of liposomes form unencapsulated antibiotic using (c) ultrafiltration and (d) centrifugation. The percentage of liposomes in the free antibiotic phase (ideally zero) is represented in gray, whereas the percentage of liposomes in the separated liposome layer is shown in white. For (c), after centrifugation for 2 h at 30k relative centrifugal force (RCF), the pellet and the supernatant were separated and quantified with dynamic light scattering (DLS) to determine the amount of vesicles in dilution in each phase. For (d), the samples were ultrafiltered at 10k RCF for 10 min using Amicon filters before quantification.

Figure 8. Encapsulation efficiency calculated using different separation techniques (ultrafiltration or centrifugation) and different total release methods (methanol and Triton, dialysis).

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 the 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 flasks or (2) in terms of the ratio of cargo found inside the vesicles to the total antibiotic (encapsulated and unencapsulated) in the system after the preparation of the vesicles. Both methods represent a measure of the encapsulation efficiency; the former represents the combined effect of efficacy of drug encapsulation and possible mass loss during the process, whereas the latter (which is the method that we adopted) represents the absolute capacity of the preparation method to encapsulate the cargo; we analyzed mass yield 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 nanodrug 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 yield separately. Otherwise, it will not be clear if a low encapsulation efficiency is the result of mass lost during an unsuitable preparation method or other factors (e.g. lipid chemistry) for encapsulating a specific cargo.

Liposomal Antibiotic Release Profile. As a final assessment of the 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.\(^{38}\) The release profiles for vancomycin hydrochloride and rifampin are presented in Figure 9. For vancomycin, the total content of the drug was released in 13 days, as shown in Figure 9a, whereas for rifampin it was released in 7 days, as shown in Figure 9b. Adding more cholesterol showed lower release, increasing the release time for both vancomycin and rifampin. Interestingly, the concentration of cholesterol influences the release rate 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 h, 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 encapsulation efficacy (EE) = 39.1 ± 3%. The same case happens with rifampin: in 4 h, 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 h is most probably the unencapsulated drug, presenting dialysis as a good but time-consuming alternative to cleaning liposomes. After the cleaning stage, vancomycin burst released 55% of the remaining drug in the next 12 h, after which it constantly released 6.26 μg/h for 12 days. After the cleaning stage, rifampin showed an almost linear release (\(R^2 = 0.98\)) for 48 h with a constant release of 27.4 μg/h and then it plateaued. Vancomycin took considerably more time than rifampin to release the entire content of the liposomes; this could be due to the fact that vancomycin is allocated in the aequous core whereas rifampin intercalates in the bilayer making it more readily available. Optimizing the antibiotic release rate is of interest for antibiotics. For example, rifampin is used in antituberculosis therapy. Due to various systemic side effects, its treatment involves prolonged oral administration of high systemic doses over a period of 4–10 months.\(^{19}\) Thus, a delivery system that can release the antibiotic for a prolonged period in high dosages without affecting other organs would be highly desirable.

**CONCLUSIONS**

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 can be designed to maintain sustained release. Although liposome preparation methods have been thoroughly studied and optimized for maximal encapsulation of cancer drugs, antibiotics are a different class of compounds that require further method development for efficient liposomal encapsulation. Our investigation highlights the importance of the methods of preparation of the liposomes and its impact on the encapsulation of antibiotics, with different methods leading to optimal results for hydrophilic versus hydrophobic antibiotics. Methods leading to multilamellar vesicles (MLVs) are preferred for antibiotics that are highly hydrophobic, and large unilamellar vesicles (LUVs) are preferred for hydrophilic antibiotics. In addition, we highlight that significant bias can be introduced into quantification of antibiotic encapsulation efficacy through (i) capsule disruption and cleaning methods and (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 mass yield as a criterion for evaluating the suitability of antibiotic encapsulation methods. In summary, our results point to the importance of evaluating the methods for nanoencapsulation of antibiotics and being mindful of potential biases in methods and analysis.

**EXPERIMENTAL SECTION**

**Chemicals and Lipids.** The lipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (16:0 PC DPPC) (99%, Avanti, MilliporeSigma) 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 MilliporeSigma. Cholesterol (95%) was obtained from Fischer Scientific. The remaining chemicals (methanol ≥99.9%, diethyl ether, and KCl) were obtained from VWR.

**Thin Film (TF) Method.** The liposomes were prepared with a modified thin film method, as reported by Meers et al.2 Briefly, DPPC (10 mg) and cholesterol (1.75 mg) were dissolved in chloroform (1 mL) inside a round bottom flask and evaporated using a Hei-VAP rotary evaporator (Heidolph) 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 Milli-Q water with 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×,

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**Figure 9.** Release profile of vancomycin (a) and rifampin (b) with liposomes created with different ratios of cholesterol. (Inset) the first 3 days of release profile for vancomycin-loaded liposomes.
using an Avanti mini extruder at 42 °C using a 0.4 nm pore size membrane.

Reverse-Phase Evaporation (REV) Method. The modified reverse-phase liposomes were utilized as reported by Halwani et al.39 Briefly, previously indicated amounts of DPPC and cholesterol were diluted in chloroform in a round bottom flask and 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 mixture was then sonicated for 35–40 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 extruded as described above.

Freezing, Annealing, and Thawing (FAT) Method. Vesicles were prepared by the thin film method, as described above, but before the extrusion step, three 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, 90 W) 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 extruding, as described above.

Determination of Size and Charge. Liposomes, prepared with each method, were resuspended in 1 mM KCl and diluted 50× before size and ζ potential measurements. Dynamic light scattering (DLS) was used to evaluate the hydrodynamic diameter of liposomes using the Malvern Instruments Zetasizer NanoZSP. All DLS runs were repeated three times on each sample. Malvern Instruments Zetasizer Nano-ZSP was also used for ζ potential measurements using a capillary cell. The ζ potential runs were repeated three times for each sample with noninvasive backscatter optics and analyzed with Smoluchowski’s model.40

Quantification of Encapsulation Efficiency and Mass Yield. Encapsulation efficiency was calculated as the percentage difference between the total antibiotic (encapsulated and nonencapsulated) and the free antibiotic (non-encapsulated). Three methods were used to quantify the total antibiotic: (1) Methanol (0.5%) was added to the liposome suspension. After incubating the sample for 50 min at 4 °C, five parts Millipore water was added to the system and the suspension was then analyzed via high-performance liquid chromatography (HPLC)-micro-time-of-flight (TOF) to quantify the antibiotic. (2) The lipid membranes were disrupted with 2% Triton X-100. (3) Liposome suspensions (0.5 mL) were subjected to dialysis using the Slide-A-Lyzer 20k molecular-weight cutoff (MWCO) dialysis inserts, and the total antibiotic release was 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. Mass yield was determined as the ratio of total antibiotic, as determined in this step, to the initial mass of antibiotic added to the system during the preparation stage.

To determine the amount of free antibiotic, three methods were used: (1) Liposomes were ultrafiltrated (Amicon Ultra centrifugal filters, MWCO 30k) for 10 min at 10.000g. (2) Liposomes were centrifuged at 20.000g for 3 h. (3) Liposome suspensions (0.5 mL) were subjected to dialysis using the Slide-A-Lyzer 20k MWCO dialysis inserts, and nonencapsulated antibiotic was measured after 2 and 4 h. After separating the free antibiotic from the liposomes, the free antibiotic was analyzed using HPLC-micro-TOF. The percentage encapsulation efficiency was calculated after measuring free antibiotic and total antibiotic with the following equation:

\[ \text{EE} = \frac{\text{total drug} - \text{free drug}}{\text{total drug}} \times 100 \]

Liposome 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.41 The quantity of liposomes present in each liposomal formulation was estimated by using the photons per second of each formulation with the Malvern Zetasizer NanoZSP. 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 Zetasizer. After the liposomes were burst with either methanol or Triton, 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.

High-Performance Liquid Chromatography (HPLC) and Time-of-Flight Mass Spectrometry (TOF-MS). For all HPLC measurements, Agilent 1200 series HPLC with a Bruker microOTOF-II mass spectrometer was used with the Agilent XDB-C18 analytical column (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; afterward, 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 was as follows: 97% mobile phase A was introduced from the initial sample injection hold for 1 min, then switched to 97% mobile phase B over 1 min, then 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. The run time was 12 min at a flow rate of 0.4 mL/min with 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.

**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. Statistical significance of differences was tested using a t-test, and P values lower than 0.05 were chosen as the cutoff for statistically significant differences.

**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00825.

Calculation of estimated encapsulation efficiency, size distribution of FAT-vancomycin liposomes (Figure S1); DLS results of teicoplanin liposomes (Figure S2) (PDF)

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**Notes**
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