International Journal of Pharmaceutics 423 (2012) 410–418

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Predicting hydrophilic drug encapsulation inside unilamellar liposomes☆

Xiaoming Xu[a,1], Mansoor A. Khan[b,2], Diane J. Burgess[a,*]

[a] Department of Pharmaceutical Sciences, University of Connecticut, 69 N Eagleville Rd U3092, Storrs, CT 06269, United States
[b] FDA/CDER/DPQR, 10903 New Hampshire Ave, Silver Spring, MD 20993, United States

ARTICLE INFO

Article history:
Received 23 August 2011
Received in revised form 9 December 2011
Accepted 12 December 2011
Available online 21 December 2011

Keywords:
Liposome
Encapsulation efficiency
Lipid molecular area
Mathematical model
Langmuir balance
Log-Normal distribution

ABSTRACT

A mathematical model has been developed to predict the encapsulation efficiency of hydrophilic drugs in unilamellar liposomes, and will be useful in formulation development to rapidly achieve optimized formulations. This model can also be used to compare drug encapsulation efficiencies of liposomes prepared via different methods, and will assist in the development of suitable process analytical technologies to achieve real-time monitoring and control of drug encapsulation during liposome manufacturing for hydrophilic molecules. Liposome particle size as well as size distribution, lipid concentration, lipid molecular surface area, and bilayer thickness were used in constructing the model. Most notably, a Log-Normal probability function was utilized to account for sample particle size distribution. This is important to avoid significant estimation error. The model-generated predictions were validated using experimental results as well as literature data, and excellent correlations were obtained in both cases. A Langmuir balance study provided insight regarding the effect of media on the liposome drug encapsulation process. The results revealed an inverse correlation between media ionic strength and lipid average molecular area, which helps to explain the phenomenon of inverse correlation between media ionic strength and drug encapsulation efficiency. Finally, a web application has been written to facilitate use of the model allowing calculations to be easily performed. This model will be useful in formulation development to rapidly achieve optimized formulation.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Encapsulation of hydrophilic active pharmaceutical ingredients (APIs) into liposomes presents unique challenges during formulation design and processing. Due to the high aqueous solubility of hydrophilic drugs, they are dissolved in the external aqueous phase during liposome preparation, and become entrapped in the aqueous compartment(s) within the formed liposomes. The proportion of the drug that is entrapped within liposomes is difficult to predict and may depend on the preparation method, the lipid concentration, the media conditions, as well as the liposome size and lamellarity. Typically, liposomes are formed upon hydration of a dry lipid film (Bangham and Papahadjopoulos, 1966; Bangham et al., 1965), upon precipitation of lipids (Du and Deng, 2006; Jahn et al., 2007; Kremer et al., 1977), or upon adsorption of dissolved lipids at liquid interfaces (Peschka et al., 1998; Szoka and Papahadjopoulos, 1978). Depending on their hydrophilicity or hydrophobicity, drugs can either be dissolved in the aqueous medium (hydrophilic) or be dispersed in the lipids (hydrophobic). This manuscript focuses on the encapsulation of hydrophilic molecules, where encapsulation efficiencies are usually very low.

The earliest liposome preparation method (film hydration) utilizes passive diffusion of hydrophilic molecules into the inner aqueous compartment of the liposomes during the vesicle formation process (Bangham et al., 1965). However, because of the presence of large amounts of external aqueous medium, very little drug content can be encapsulated (Szoka and Papahadjopoulos, 1978). To improve the encapsulation efficiency of hydrophilic molecules inside liposomes, various preparation procedures have been developed, such as reverse phase evaporation (Cortesi et al., 1999; Szoka and Papahadjopoulos, 1978), dehydration–rehydration of preformed empty liposomes (Grant et al., 2001; Simıes et al., 2005; Zadi and Gregoriadis, 2000), and freeze–thaw cycling (Mayer et al., 1985). However, lack of understanding of the preparation process as well as inadequate reporting make it extremely difficult to compare the encapsulation efficiency values obtained from different preparation methods, and may even lead to biased conclusions in some cases. It is therefore very important to understand the basic principles behind liposome preparation processes, and more specifically the factors that govern the drug encapsulation

☆ This is the opinion of the authors and does not necessarily reflect the official policy of the Agency.

* Corresponding author. Tel.: +1 860 486 3760; fax: +1 860 486 0538.
E-mail addresses: xiaoming.xu@me.com (X. Xu), mansoor.khan@fda.hhs.gov (M.A. Khan), d.burgess@uconn.edu, diane.burgess@uconn.edu (D.J. Burgess).
[1] Tel.: +1 860 486 0640.
[2] Tel.: +1 301 796 0016.

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.12.019

process. With such knowledge, it may be possible to establish common ground for comparison of various preparation methods, and more importantly to improve drug encapsulation efficiency for hydrophilic molecules. In addition, it would be desirable to predict drug encapsulation for a given formulation and process before conducting any experiments, as this would have significant cost-saving benefits. Therefore, it is the goal of the current work to develop a mathematical model linking formulation specific parameters with product quality attributes (*i.e.* drug encapsulation efficiency). Such a predictive mathematical model will facilitate understanding of the product design space, as well as assist in the development of appropriate process analytical technologies (PAT) (Yu, 2008).

It should be noted, that in addition to passive drug encapsulation, pH induced trans-membrane transport of the drug (also known as the pH remote loading technique) has also been developed (Lasic et al., 1995, 1992). The most successful example of this technique is the commercial product Doxil®, which can encapsulate as much as 95% of the drug into liposomes. However, this active loading approach is only effective for a relatively small group of molecules where the diffusion of the unionized drug molecules is dependent on the pH conditions, and does not apply to all drug candidates (such as nucleic acids, peptides, and enzymes). Hence, it is not suitable for molecules lacking an amine group or larger molecules (such as peptides and proteins). Under these circumstances, passive encapsulation is still the best available method, despite being less effective in terms of encapsulation. The current manuscript focuses on the passive drug loading process, and the proposed model provides accurate prediction of the percentage of drug encapsulation for most hydrophilic molecules prepared via the various passive loading techniques (including direct hydration, reverse phase evaporation, and organic solvent injection method as long as the model assumptions are met as detailed in Appendix A).

To predict the percentage of water-soluble drugs inside small unilamellar liposomes, a simple mathematical method has been previously reported (Szoka and Papahadjopoulos, 1978). However, in the simple model it was assumed that all liposome particles are of exactly the same size, which is an over simplification as most samples are heterogeneous in size. While this previous model is suitable for approximation purposes, it is incompetent for the purpose of product quality control, which requires a more accurate and predictive tool. In addition, because this simple model underestimates the effect of particle size distribution on drug encapsulation, the model cannot provide a satisfactory explanation regarding the relationship between the solution ionic strength (or media type), lipid molecular area, and drug encapsulation efficiency (Colletier et al., 2002; Szoka and Papahadjopoulos, 1978). It is a hypothesis of the current work that this relationship is due to changes in the lipid polar head group surface area in the presence of various media conditions. Hence a quantitative measure of surface area changes under different media conditions, could potentially confirm the underlying mechanism of media effect on drug encapsulation efficiency, using the proposed model.

In this manuscript, a mathematical model was developed and software was written to predict the encapsulation of hydrophilic APIs in liposomes. Experimentally, Tenofovir liposome formulations were prepared and their drug encapsulation efficiency values were compared with the model predicted values. Tenofovir is a nucleotide reverse-transcriptase inhibitor (NRTI), and is highly water soluble. Lastly, literature reported drug encapsulation efficiency values were used to demonstrate the feasibility of applying the model to predict encapsulation for liposomes containing other hydrophilic APIs.

2. Materials and methods

2.1. Materials

Tenofovir was purchased from Resource Technique Corporation (Laramie, Wyoming). HEPES sodium salts, Triton X-100, were purchased from Sigma–Aldrich (St. Louis, MO). 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-3-trimethylammonium-propane (chloride salt) (DPTAP) and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Chloroform, acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Ultracel YM-50 centrifugal devices (50 kDa) were purchased from Millipore (Billerica, MA). PD-10 desalting columns (Sephadex™ G-25) were purchased from GE Healthcare (Piscataway, NJ). Nanopure™ quality water (Barnstead, Dubuque, IA) was used for all studies.

2.2. Experimental methods

2.2.1. Preparation of Tenofovir liposomes

All the liposome formulations were prepared using a modified thin-film hydration method. Briefly, the desired amount of lipids was weighed into a 50 ml pear-shaped flask and ~2 ml of chloroform was added to dissolve the lipids. The chloroform was then evaporated under vacuum at room temperature for 2 h, after which the flask was kept under vacuum overnight to completely remove any residual solvent. Encapsulation of Tenofovir into liposomes was accomplished during the hydration step where dry lipids were hydrated with 10 mM pH 7.4 HEPES buffer or 10 mM pH 7.4 phosphate buffer (containing the desired amount of drug) at 65 °C for 2 h (vortexed for 30 s every 30 min). After hydration, 1 min of sonication (80 W) was applied to break down bigger particles. Subsequently, the samples underwent several freeze–thaw cycles (10 min at −196 °C and 10 min at 65 °C) to facilitate encapsulation of the drug (30 s vortexing between cycles). The samples were subsequently put into a LIPEX extruder (Northern Lipids Inc., Canada) and passed through a stack of polycarbonate membranes with defined pore sizes to obtain liposomes with the desired particle size characteristics. Finally, the liposomes were purified using two PD-10 gel columns to remove any free drug.

2.2.2. Determination of encapsulation efficiency (EE%)

10 μL of prepared liposomes (before purification) were withdrawn and diluted with 2 ml buffer ($n = 3$). 500 μL of this diluted solution was placed into an Ultracel YM-50 centrifugal device (50 kDa MWCO) and centrifuged at 13,000 rpm for 12 min. The filtrate was collected to determine the free drug concentration (C_{Free}). To assess the total drug concentration (C_{Total}), 1 ml of the same diluted solution was mixed with 200 μL of 6% (v/v) Triton X-100 and the mixture was kept at 65 °C for 10 min to disrupt all the vesicles. Both C_{Free} and C_{Total} were assessed using HPLC. The encapsulation efficiency was calculated as:

$$EE\% = \left(1 - \frac{C_{Free}}{C_{Total}}\right) \times 100 \quad (1)$$

2.2.3. Particle size analysis

Particle size analysis was conducted using a Malvern ZS90 zetasizer. Prepared liposome formulations were diluted at least 50 times to obtain liposome suspensions that were below 0.5 mg/ml. All measurements were conducted at 25 °C and it was assumed that the suspensions had similar viscosities to that of water at the same temperature ($\eta = 0.89$ cP). All measurements were performed in triplicate.

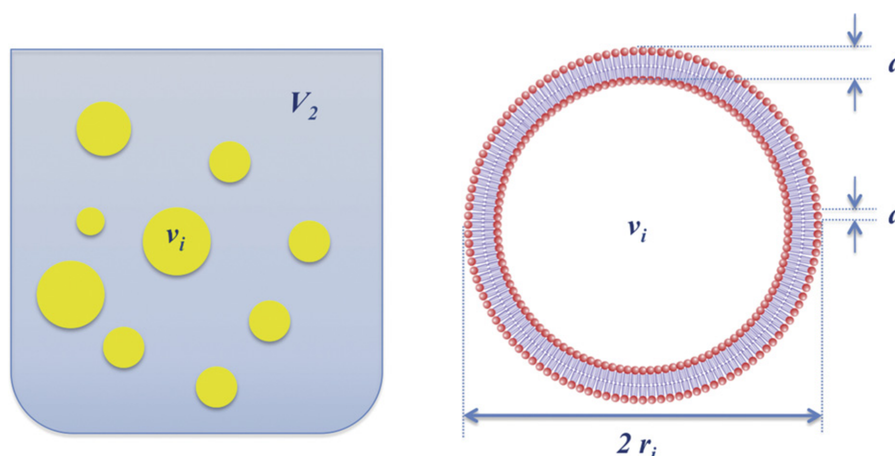

Fig. 1. Schematic drawing of a unilamellar vesicle (right) and vesicles of various sizes distributed in sample medium (left). d, bilayer thickness; a, average lipid molecular area; $2r_i$, mean particle size; v_i, internal volume; V_2, external volume.

2.2.4. Measurement of lipid molecular surface area

A Langmuir mini-trough (KSV instrument) was used in this study to measure the lipid molecular surface area. The effect of media condition on lipid molecular surface area was studied for two groups of lipids: (1) a mixture of three lipids (DSPC:cholesterol:DPTAP in 6:3:2 molar ratio) and (2) three lipids individually (DSPC, cholesterol, and DPTAP). The lipids (either individually or as a mixture) were dissolved in chloroform and spread over the surface of the sub-phase using a 50 μL micro-syringe. Once the lipid was pipetted onto the surface, the chloroform was allowed to evaporate, approximately 10 min, before initiating the movement of the barrier using software. Lipid molecular area was obtained by extrapolating the solid phase isotherm (linear region) to the x-axis (molecular area). Nine different media (sub-phases) were tested for the lipid mixture. These include: DI water, 10 mM and 20 mM pH 7.4 HEPES buffer, 10 mM pH 7.4 HEPES buffer containing 5 mg/ml Tenofovir, 5 mM and 20 mM NaCl solution, as well as 10 mM, 50 mM, and 100 mM pH 7.4 phosphate buffer. Three different media were used for testing the individual lipids: DI water, 10 mM pH 7.4 HEPES buffer, and 10 mM pH 7.4 phosphate buffer. Each test was performed in triplicate except for the sub-phase containing Tenofovir due to the cost of this material.

3. Results and discussion

3.1. Development of the mathematical model

For simplicity, the model proposed in this manuscript deals with spherical unilamellar liposomes (with only one membrane layer separating the internal aqueous compartment and the outer medium) as shown in Fig. 1. Since hydrophilic drug is encapsulated inside the inner aqueous compartment of these liposomes, one can theoretically calculate the drug encapsulation efficiency (EE%) if the internal volume information (or volume distribution across the liposome bilayer) is known. As shall be demonstrated below (refer to Appendix A), the liposome internal volume is directly correlated to liposome particle size, size distribution, lipid molecular surface area, lipid concentration and bilayer thickness. These variables can be measured (*e.g.* sample particle size and distribution, molecular surface area) or can be easily obtained from the literature (*e.g.* bilayer thickness). Hence, it should be possible to calculate the internal volume of liposomes of various sizes and predict drug encapsulation efficiency.

Typically, lipid bilayer thickness can be measured via X-ray analysis (Lewis and Engelman, 1983), neutron scattering (Lemmich et al., 1996), or NMR techniques (Ipsen et al., 1990) due to the presence of highly ordered trans-bilayer profiles within the lipid bilayers. The thickness of a lipid bilayer is affected by four factors: (1) the length and degree of saturation of the fatty-acid chains: the longer the fatty-acid chains and the more saturated they are, the thicker the bilayer will be. (2) The hydration state of the lipid head groups: the less hydrated the thicker the bilayer will be, because dehydration causes the head groups as well as the chains to get closer together and stretch out. (3) The temperature: the higher the temperature, the thinner the bilayer, especially during the gel–liquid phase transition. (4) The presence of cholesterol: at temperatures below the phase transition temperature cholesterol has a negative impact on lipid bilayer thickness, while at higher temperatures it has a positive correlation with lipid bilayer thickness. Despite the fact that these four factors do contribute significantly to the thickness variation of liposomes, their effect on drug encapsulation efficiency is only marginal. It has been demonstrated using an excel program that a 1.2 nm increase in bilayer thickness would only decrease the EE% by 0.73% (refer to the excel program in Supplementary Material), and this is not statistically significant compared with the other factors. Hence, literature reported values could be used for the purposes of these calculations. Bilayer thickness information for lipids with fatty acid chains of 14–18 carbons is summarized in Table 1. Note that all the reported bilayer thickness values are mostly for individual lipids. However, in most liposome formulations, mixtures of two or more lipid components are normally used. Therefore, for calculation purposes, in this study only the main lipid component thickness information was used. For example, for a liposome formulations containing DSPC:cholesterol:DPTAP = 6:3:2, the bilayer thickness of DSPC (5.1 nm) was used.

As shown in Eq. (10) (Appendix A), the obtained model for drug encapsulation efficiency is a function of four material and formulation specific parameters (r_i, d, c, a), and with the help of a computer program one can easily calculate the EE%. As an example, a web application was written to facilitate the calculation of drug encapsulation (www.LiposomeModel.com) and various parameters were measured and used in the above equations to calculate the EE%. As shown in Fig. 2, the particle size of the liposomes in the example is 155.8 ± 19.4 nm as determined by dynamic light scattering. The bilayer thickness is set to 5.1 nm (Table 1) and the average molecular area is 37.56 Å^2 (or 0.3756 nm^2) as determined using a Langmuir balance. The total sample volume is 3 ml and the lipid concentration is 120 mM. The obtained result shows that out of a 3 ml sample, only 0.964 ml is internal volume, which gives an EE% value of 32.13%

Table 1
Summary of lipid bilayer thickness data for various lipids.

Lipid	Carbon number	T_m (°C)	Bilayer thickness (nm)	Method	Condition	Reference
DSPC	18	55	5.1 ± 0.3	Refractive Index	21 °C	Marra and Israelachvili (1985)
			5.15 ± 0.1	X-ray	60 °C	Lewis and Engelman (1983)
DPPC	16	41	4.6 ± 0.3	Refractive Index	21 °C	Marra and Israelachvili (1985)
			4.8 ± 0.1	X-ray	44 °C	Lewis and Engelman (1983)
			4.8	X-ray	20 °C	Nagle and Tristram-Nagle (2000)
			3.9	X-ray	50 °C	Nagle and Tristram-Nagle (2000)
DMPC	14	23	3.8 ± 0.3	Refractive index	30 °C	Marra and Israelachvili (1985)
			3.7	X-ray	30 °C	Nagle and Tristram-Nagle (2000)
			4.2 ± 0.3	Refractive index	16 °C	Marra and Israelachvili (1985)
DSPG	18	53.6	5.28 ± 0.04	Small angle X-ray scattering	pH 7.4, room temperature	Pabst et al. (2008)
DPPG	16	40.2	5.02 ± 0.04	Small angle X-ray scattering	pH 7.4, room temperature	Pabst et al. (2008)
DMPG	14	22.4	4.84 ± 0.04	Small angle X-ray scattering	pH 7.4, room temperature	Pabst et al. (2008)
DPPS	16		5.2 ± 0.8	AFM		Zhang et al. (2009)
			5.55 ± 0.01	X-ray	pH 3.7	Fanucci et al. (2001)
DPTAP	16	45.4	4.0	AFM		Mckiernan et al. (2008)
SOPC	18	−14	4.9 ± 0.1	X-ray	20 °C	Lewis and Engelman (1983)

the experimental result is 32.5 ± 1.5% as shown in Table 3. The significance of the developed model is that a probability function (P) is introduced into the calculation for the first time, permitting a more accurate prediction of drug encapsulation efficiency. It is well known that larger vesicles have much higher internal volume than smaller vesicles ($\sim r^3$). This results in the larger vesicles being able to accommodate more drug than their smaller counterparts. In a liposome formulation, there exists a distribution of sizes around the mean particle size (typically a Log-Normal distribution for particles that have undergone extrusion or sonication). Therefore, if not using this parameter, significant error would occur in predicting the drug encapsulation efficiency.

In addition, an excel program was developed to demonstrate the statistical significance of each of the four parameters, *i.e.* particle size, bilayer thickness, lipid molecular area, and lipid concentration. The distribution value was fixed at 20% of the mean particle size value. This is very similar to a typical factorial design but only it is simulated without performing any experiments. Each of the four factors has 4 different levels covering the usual range of formulation conditions. For example, the particle size has 100 nm, 130 nm, 160 nm, and 190 nm; the bilayer thickness has 4 nm, 4.4 nm, 4.8 nm, and 5.2 nm; the lipid molecular area has 0.36 nm^2, 0.39 nm^2, 0.42 nm^2, and 0.45 nm^2; the lipid concentration has 50 mM, 80 mM, 110 mM, and 140 mM. This resulted in a total of 256 possible combinations. After testing them in the mathematical model, it was discovered that out of the four parameters, lipid bilayer thickness has the least statistical significance on the drug encapsulation efficiency ($P > 0.05$) while all the other three factors contribute significantly toward the drug encapsulation process.

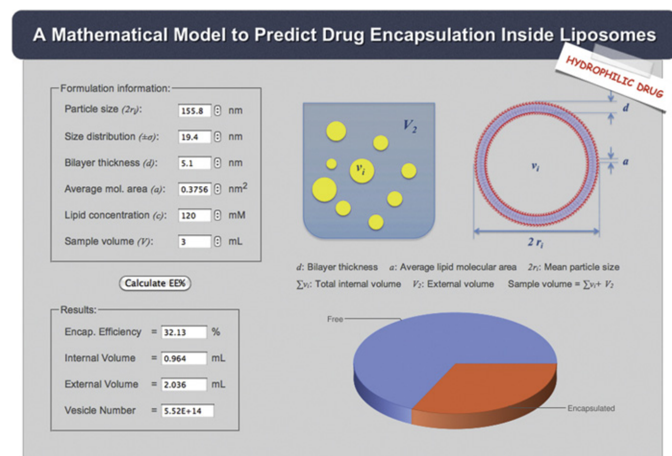

Fig. 2. Program interface for prediction of the liposome drug encapsulation efficiency (www.LiposomeModel.com).

3.2. Media effect on lipid molecular surface area

Among the four variables identified in Eq. (10), lipid molecular surface area is a key parameter that is sensitive to environmental changes. For example, the lipid area may change under different media ionic strength conditions since most lipids are ionic in nature (negative, positive or zwitterions). Therefore, it is crucial to experimentally determine the lipid molecular area and use this value in the mathematical model to predict potential changes in drug encapsulation efficiency as a result of changing the liposome preparation medium. To accomplish this, a Langmuir mini-trough was used for this purpose and the results are summarized in Table 2. As mentioned earlier, due to the ionic nature of most lipids, different buffer systems may affect the head group size of the lipids differently, which may or may not change the drug encapsulation efficiencies. To address this question, the effect of various media on lipid molecular surface area was studied, for both the lipid mixture and the individual lipids.

For the lipid mixture, in the presence of HEPES buffer, the lipid molecular surface area increased significantly ($\sim$3 Å^2) as compared to that in the DI water (Table 2). Addition of 5 mg/ml of Tenofovir and doubling of the HEPES buffer concentration both caused a slight decrease in the molecular area. Replacing the HEPES buffer system with phosphate buffer resulted in a significant decrease in the lipid molecular area. However, it is interesting to note that increase in the ionic strength of the inorganic buffer had no effect on the lipid molecular area but only on the inter-molecular pressure (Fig. 3) at intermediate distance (approximately 40–80 Å^2 in terms of intermolecular area or 6.4–9 Å in terms of distance).

Regardless of the medium used, all of the individual lipids exhibited a larger molecular surface area than the mixtures as shown in Table 2. This may be attributed to the following: (1) packing of molecules of different sizes results in a smaller void area, and hence tighter packing and (2) the presence of cholesterol in the mixture contributes to a tighter packing due to the compression effect. Various media conditions had very different effects on these individual lipid molecular areas. Changing the medium from DI water to 10 mM phosphate buffer resulted in a significant decrease in the surface area of DPTAP ($P < 0.05$), while the surface area of the other two lipids remained almost the same. On the other hand, a change of medium from DI water to 10 mM HEPES buffer resulted in a slight increase in the molecular surface area of cholesterol and DSPC, but not that of DPTAP.

Table 2
Molecular surface area of the lipid mixture and individual lipids in various sub-phases at 25 °C.

Lipid sample	Sub-phase	Mol area (Å^2)
DSPC:cholesterol:DPTAP, 6:3:2	DI water	36.92 ± 0.37
DSPC:cholesterol:DPTAP, 6:3:2	10 mM 7.4 HEPES	39.76 ± 0.56
DSPC:cholesterol:DPTAP, 6:3:2	10 mM HEPES with 5 mg/ml Tenofovir	39.13
DSPC:cholesterol:DPTAP, 6:3:2	20 mM HEPES	38.79 ± 0.62
DSPC:cholesterol:DPTAP, 6:3:2	5 mM NaCl	37.81 ± 0.81
DSPC:cholesterol:DPTAP, 6:3:2	20 mM NaCl	37.28 ± 0.72
DSPC:cholesterol:DPTAP, 6:3:2	10 mM phosphate	37.56 ± 0.35
DSPC:cholesterol:DPTAP, 6:3:2	50 mM phosphate	37.64 ± 0.10
DSPC:cholesterol:DPTAP, 6:3:2	100 mM phosphate	37.65 ± 0.45
DSPC	DI water	50.00 ± 0.34
DSPC	10 mM HEPES	51.15 ± 0.16
DSPC	10 mM phosphate	50.09 ± 0.19
Cholesterol	DI water	37.62 ± 0.73
Cholesterol	10 mM HEPES	39.85 ± 0.39
Cholesterol	10 mM phosphate	37.94 ± 0.09
DPTAP	DI water	56.78 ± 0.48
DPTAP	10 mM HEPES	57.04 ± 0.88
DPTAP	10 mM phosphate	53.54 ± 0.69

To explain the phenomenon observed above, it is necessary to understand the various forces involved in these systems. It is well known that the following forces may be present at the lipid/aqueous interface: hydrophobic, hydration, electrostatic as well as van der Walls forces. Among them, hydrophobic and van der Walls forces are attractive in nature, while hydration and electrostatic forces are normally repulsive. Of the two repulsive forces, hydration forces are short-range force (operating between the head groups at a distance of a few water molecules) while electrostatic interaction is relatively long range. For the positively charged DPTAP used in this study, a bigger head group may form in the presence of a larger counter ion (*e.g.* HEPES ion compared with phosphate ion) and hence cause an increase in the lipid molecular area. On the other hand, for neutral lipids as well as cholesterol, as the concentration of the buffer increases, the ionic strength also increases, which inversely reduces the lipid molecular surface area due to shrinking of the hydration sphere (lipid head group together with tightly bound immobile water molecules). Consequently, addition of 5 mg/ml Tenofovir (ionized drug) or increasing the buffer concentration both decreased the molecular surface area of the lipid mixture. Further increase in the medium ionic strength did not significantly reduce the molecular surface area (Table 2 and Fig. 4). The combination of these two effects is believed to have resulted in the observation of a slightly higher surface area in HEPES buffer and an inverse correlation between the medium ionic strength and the surface area.

The significance of these findings lies in the fact that they can help to explain how media conditions affect drug encapsulation efficiencies. For example, for Tenofovir liposomes prepared using DSPC:cholesterol:DPTAP (6:3:2 molar ratio) at a concentration of 120 mM/L, a change in the preparation media from 10 mM pH 7.4 phosphate buffer to 10 mM pH 7.4 HEPES buffer resulted in an increase in the drug encapsulation efficiency by ~4% (Table 3). This can be attributed partly to a change in the lipid molecular area (from 37.56 Å^2 in phosphate buffer to 39.76 Å^2 in HEPES buffer). Of course, it should be noted that the decrease in the drug encapsulation efficiency in this example is not solely caused by the surface area change, since the particle size of the formulation also decreased slightly.

3.3. Evaluation of the mathematical model

To test the mathematical model, experimentally obtained drug encapsulation efficiency values as well as literature reported values were compared with the model-generated results. Using Tenofovir as a model compound, various liposome formulations were prepared. To ensure that the prepared liposomes are unilamellar and hence satisfy the assumptions of the model, several techniques were used. These include sonication, freeze–thaw cycles, and extrusion repeatedly through a stack of filters with uniform pore size. As shown in various publications (Hope et al., 1986; Szoka et al., 1980), after the extrusion most of the vesicles should be unilamellar. For all the formulations, sample particle sizes as well as size

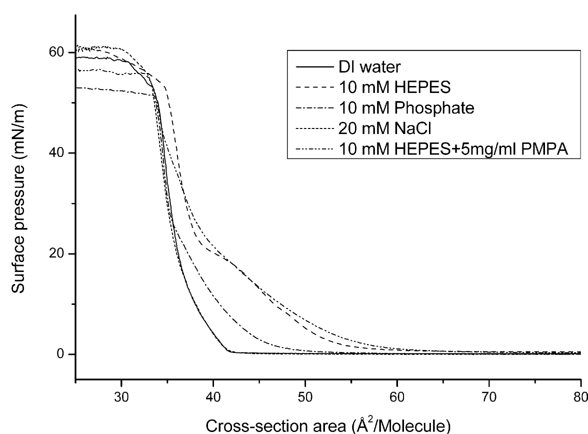

Fig. 3. Langmuir isotherm of the lipid mixture (DSPC:cholesterol:DPTAP, 6:3:2 molar ratio) in various sub-phases (25 °C).

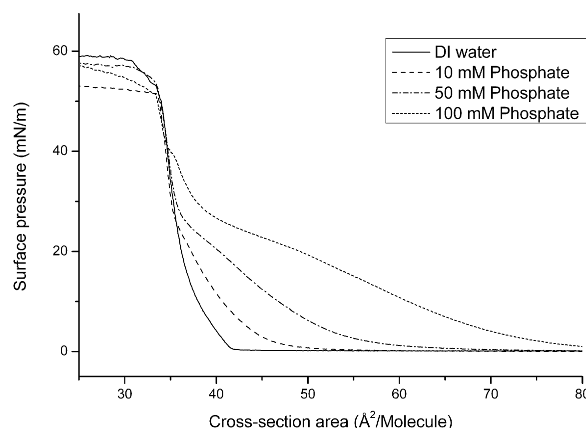

Fig. 4. Effect of buffer ionic strength on the Langmuir isotherm of the lipid mixture (DSPC:cholesterol:DPTAP, 6:3:2 molar ratio) at 25 °C.

Table 3
Comparison of model predicted EE% with experimental data.

Drug conc. (mg/ml)	Lipid conc. (mM)	Buffer	Particle size (distribution) (nm)	Area (nm^2)	Bilayer thickness (nm)	Model prediction (%)	Experimental EE (%)
0.5	30	10 mM pH 7.4 HEPES	147.4 (30.7)	0.3976	5.1	8.66	9.2 ± 1.4
2.5	76.45	10 mM pH 7.4 HEPES	153.6 (25.0)	0.3976	5.1	22.02	24.3 ± 1.9
1	76.45	10 mM pH 7.4 HEPES	111.9 (17.5)	0.3976	5.1	15.15	14.9 ± 1.9
1	76.45	10 mM pH 7.4 HEPES	157.4 (30.5)	0.3976	5.1	23.37	29.0 ± 1.8
6.9	49.04	10 mM pH 7.4 HEPES	163.5 (32.7)	0.3976	5.1	15.76	14.4 ± 2.1
1	49.04	10 mM pH 7.4 HEPES	166.9 (35.6)	0.3976	5.1	16.38	21.7 ± 1.8
0.5	95	10 mM pH 7.4 HEPES	131.0 (34.4)	0.3976	5.1	25.85	29.1 ± 1.3
0.5	95	10 mM pH 7.4 HEPES	170.6 (36.8)	0.3976	5.1	32.61	35.5 ± 1.9
1	152.9	10 mM pH 7.4 HEPES	162.1 (39.3)	0.3976	5.1	51.34	45.7 ± 1.7
1	120	10 mM pH 7.4 HEPES	169.4 (15.9)	0.3976	5.1	36.68	36.5 ± 2.0
1	120	10 mM pH 7.4 Phosphate buffer	155.8 (19.4)	0.3756	5.1	32.13	32.5 ± 1.5

distributions were determined by dynamic light scattering, average lipid surface areas were measured using a Langmuir balance, and membrane thickness values were obtained from the literature (Table 1). All the information was input into the software and the results are shown in Table 3. A very good correlation between the experimental and predicted values was obtained ($r^2 = 0.933$) as shown in Fig. 5. Note that the buffer effect on drug encapsulation efficiency can be predicted if the changes in the lipid molecular surface area can be accurately measured.

The feasibility of applying the developed model to other small unilamellar vesicles (SUVs) or large unilamellar vesicles (LUVs) containing water-soluble agents was also evaluated, and the results are summarized in Table 4. Note that only limited information could be obtained from the literature data (such as particle size and lipid concentration), other information was approximated (such as lipid molecular area and bilayer length). In some cases where no distribution data were reported, it is assumed that the distribution is about 20% of the mean particle size value. This is a very typical of a unimodal size distribution. As shown in Fig. 6, a very good correlation was obtained between the reported and predicted values.

It is interesting to note that the developed model can make very accurate predictions for liposome formulations prepared via different processing methods. Most notably, accurate predictions were observed for liposomes prepared via the reverse phase evaporation method (Szoka and Papahadjopoulos, 1978). As mentioned by the authors (Szoka and Papahadjopoulos, 1978), a typical formulation should contain ~66 μmol lipid in 1 ml aqueous medium mixed with 3 ml organic solvent to form a w/o emulsion. After evaporation of the organic solvent, phase inversion occurs following which LUVs form. At this point in the processing the lipid concentration is very high (≥66 μmol/ml) which makes possible the encapsulation of large amount of drug. Subsequent dilution with buffers reduces the lipid as well as the drug concentration. However since dilution does not disrupt the structure of the formed LUVs, it has minimal effect on the drug encapsulation efficiency. This analysis brings an interesting comparison of the reverse phase evaporation method with liposomes prepared via the film-hydration–extrusion process. For a film-hydration–extrusion liposome formulation, the concentration of lipid depends on the volume of buffer added into the dry lipid film, and remains constant throughout the processing. Therefore, when comparing the two methods with formulations containing the same final lipid concentration, much higher drug encapsulation efficiency would be obtained for the reverse phase evaporation method. Another very important parameter that should be considered when comparing the two methods is the size and size distribution of the final liposome product. Liposomes prepared via reverse phase evaporation are generally heterogeneous LUVs while extrusion of film hydrated MLVs can produce either SUVs or LUVs

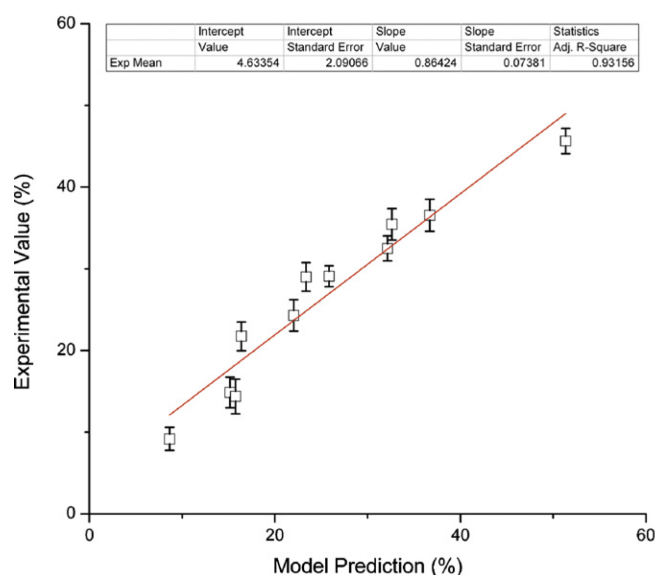

Fig. 5. Comparison of model predicted drug encapsulation efficiency values with experimental data for Tenofovir liposomes. All the formulations were prepared using a modified thin-film hydration method and all the data are summarized in Table 3.

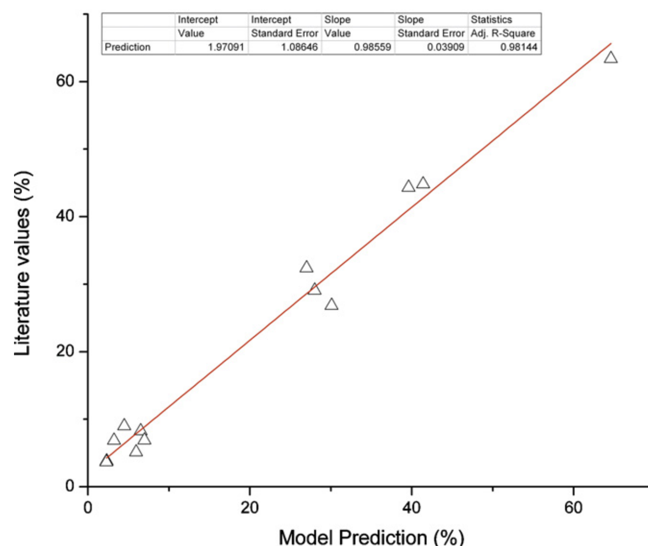

Fig. 6. Comparison of model predicted drug encapsulation efficiency with literature data (refer to Table 4 for detailed experimental conditions).

Table 4
Comparison of model predicted EE% with literature data.

Particle size (nm)	Area (nm^2)	Bilayer thickness (nm)	Lipid conc. (mM)	Experimental EE (%)	Model prediction (%)	Drug	Reference
450	0.4000	4.8	66	64.6	63.40	Sucrose	Szoka and Papahadjopoulos (1978)
~200	0.4000	4.8	66	30.1	26.82	Sucrose	Szoka and Papahadjopoulos (1978)
138.6	0.4000	4.2	30	6.51	8.25	Hemoglobin blood substitutes	Arifin and Palmer (2003)
91.4	0.4000	4.2	30	5.95	5.12	Hemoglobin blood substitutes	Arifin and Palmer (2003)
71.4	0.4000	4.2	30	2.33	3.79	Hemoglobin blood substitutes	Arifin and Palmer (2003)
~115	0.4000	5.1	150	~27	32.39	Calcein	Berger et al. (2001)
~105	0.4000	5.1	150	~28	29.08	Calcein	Berger et al. (2001)
229 ± 35	0.4000	4.8	20	4.5	8.99	5,6-CF	Elorza et al. (1993)
189 ± 5	0.4000	4.8	20	3.24	6.84	5,6-CF	Elorza et al. (1993)
109 ± 5	0.4000	4.8	20	2.3	3.65	5,6-CF	Elorza et al. (1993)
99 ± 32.3	0.4000	5.1	150 mg/g	39.6	44.33	Iodine	Schneider et al. (1995)
107 ± 30.3	0.4000	5.1	150 mg/g	41.4	44.83	Iodine	Schneider et al. (1995)
110 ± 60	0.4000	4.8	16	7	6.88	SOD	Corvo et al. (1999)

that are both homogeneous. As a consequence of their size differences and therefore enclosed volumes, LUVs have much higher drug encapsulation efficiencies than SUVs. Therefore, size and lipid concentration information should be provided in order to make a comparison regarding which method is better in terms of drug encapsulation efficiency. As demonstrated in the model, the drug encapsulation efficiency is mainly dependent on: the geometry of the prepared vesicles (size and lamellarity), the lipid concentration used, and drug–lipid interactions (if applicable). Accordingly, for a sample of unilamellar liposomes of size 450 nm (assuming a 20% deviation in the size distribution) and with lipid concentration of 66 μmol/ml, the drug encapsulation efficiency should be approximately 64% regardless of the method used.

It is also worth mentioning that the model developed here not only can provide a prediction of the drug encapsulation efficiency for unilamellar liposomes, but may also provide additional insight into other aspects of the formulation characteristics and process. First, the model can provide insight with regard to "interactions with the lipids" despite the fact that the model assumes that there is no drug–lipid interaction (a necessary simplification to calculate the volume distribution of the solute (drug) inside and outside the liposome vesicles). This is because in the event of a lipid–drug interaction (mostly attractive in nature), the model would generate a significantly lower value for encapsulation efficiency due to failure to account for the drug that is present in the lipid bilayer. The lack of correlation between the predicted and actual encapsulation efficiency would suggest a potential lipid–drug interaction to the researcher. Secondly, the model can facilitate formulation optimization. For example, formulation changes (such as addition of cholesterol, or PEGylation to enhance stability) may result in alteration of the lipid molecular area and lipid bilayer thickness, which in turn may change the encapsulation efficiency. Through using the model provided here, the formulation scientist can quickly assess the impact of such formulation changes on the encapsulation efficiency without the need to perform many labor intensive experiments thus saving time and expense. Thirdly, if it is necessary to account for the osmotic pressure induced by the encapsulated agent, researchers can modify the model. Since the current model can already predict the concentration and volume of the encapsulated agent inside the liposomes, the researchers only need to supply the MW of the molecule and the buffer concentrations, *etc.*

4. Conclusions

The mathematical model presented in this work will be useful during early stage formulation and processing design to predict drug encapsulation efficiency for hydrophilic molecules in unilamellar liposomes. The model is most accurate when there is no interaction between the drug molecule and the lipid. Otherwise deviation can occur if there is significant drug–lipid association (charge–charge interaction) or if the drug has a relatively high Log *P*. However, even under these circumstances, the model provides relative trends for changes made to the formulation. In addition, depending on the degree of deviation of experimental results from the model predicted values, it may be possible to detect the existence of drug–lipid interactions and this will help researchers to understand the encapsulation processes. One of the findings of the present work is that lipid concentration and liposome particle size are the two most critical variables in determining the final formulation drug encapsulation efficiency, and should always be reported together with the drug encapsulation efficiency to avoid any biased comparison of the preparation method. Unfortunately, many publications do not report both the lipid concentration and the liposome particle size. The model can be used to compare various liposome preparation methods as long as the assumptions are met. Moreover, the Langmuir balance study together with the use of the mathematical model provided insight regarding the media effect on the liposome drug encapsulation process. Most importantly, from a manufacturing perspective, the model described in this manuscript can be useful in designing suitable PAT tools for monitoring and controlling the liposome preparation process. For example, appropriate instrumentation can be developed to monitor the sample particle size and size distribution changes during the preparation process through inline size measurement. This type of PAT implementation would allow an accurate estimation of formulation changes during preparation in real-time, and would provide enormous cost–benefit as well as improve product quality.

Acknowledgement

This research was supported by FDA funding (HHSF223201010484P).

Appendix A. Derivation of the mathematical model

The model is developed for unilamellar liposomes containing a hydrophilic drug. The system is schematically illustrated in Fig. 1. The assumptions of the model include the following:

(1) all liposomes (vesicles) are spherical in shape;
(2) each individual vesicle contains an inner aqueous core separated from the outer medium with a single lipid bilayer;
(3) there are no drug–lipid interactions at the interface;
(4) the drug is water soluble and distributes evenly across the lipid bilayer, *i.e.* the inside drug concentration is identical to the outside drug concentration;
(5) the particle size follows a Log-Normal distribution, $f(\mu, \sigma)$, which is very typical for systems prepared through size reduction processes, *e.g.* sonication and extrusion.

Given the above assumptions, for a liposome vesicle i with known size ($2r_i$) and membrane thickness (d), the outer and inner surface are, respectively,

$$A_{outer} = 4\pi {r_i}^2 \tag{2}$$

$$A_{inner} = 4\pi (r_i - d)^2 \tag{3}$$

With known average lipid molecular area (a), one can calculate how many lipid molecules (k_i) are required to form a vesicle of this size:

$$k_i = \frac{A_{inner} + A_{outer}}{a} = \frac{4\pi[{r_i}^2 + (r_i - d)^2]}{a} \tag{4}$$

Given the liposome formulation mean particle size μ, and the size distribution σ, the probability (P_i) of vesicle in this size can be obtained,

$$P_i = \frac{1}{\sigma \cdot r_i \cdot \sqrt{2\pi}} e^{-(1/2)((\ln r_i - \ln \mu)/\sigma)^2} \tag{5}$$

With known lipid molar concentration (c), the total sample volume (V) the total vesicle number (m) can be calculated:

$$m = \frac{c \cdot V \cdot N_A}{\sum_i k_i P_i} \tag{6}$$

Using again the probability function, the exact number of vesicles for each particle size can be obtained:

$$n_i = m \cdot P_i \tag{7}$$

This allows calculation of the total internal volume (V_{in}):

$$V_{in} = \sum_i v_i \cdot n_i \tag{8}$$

where v_i is the internal volume of vesicle

$$v_i = \frac{4}{3}\pi (r_i - d)^3 \tag{9}$$

Since the volume of the sample is V ($V = V_{in} + V_{out}$), the encapsulation efficiency (EE%) is the ratio of the internal volume and total sample volume as shown below:

$$\mathrm{EE\%} = \frac{V_{in}}{V} \times 100\% = \frac{\sum_i \left((4/3)\pi (r_i - d)^3 \cdot (c \cdot V \cdot N_A) / \sum_i \left(4\pi \left[r_i^2 + (r_i - d)^2 \right] \cdot P_i / a \right) \cdot P_i \right)}{V} \times 100\% \tag{10}$$

As shown in Eq. (10), the drug encapsulation efficiency is proportional to the vesicle particle size (distribution), lipid molecular surface area, and lipid concentration, and is inversely proportional to the lipid bilayer thickness.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.12.019.

References

Arifin, D.R., Palmer, A.F., 2003. Determination of size distribution and encapsulation efficiency of liposome-encapsulated hemoglobin blood substitutes using asymmetric flow field-flow fractionation coupled with multi-angle static light scattering. Biotechnol. Prog. 19, 1798–1811.

Bangham, A.D., Papahadjopoulos, D., 1966. Biophysical properties of phospholipids. I. Interaction of phosphatidylserine monolayers with metal ions. Biochim. Biophys. Acta 126, 181–184.

Bangham, A.D., Standish, M.M., Watkins, J.C., 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13, 238–252.

Berger, N., Sachse, A., Bender, J., Schubert, R., Brandl, M., 2001. Filter extrusion of liposomes using different devices: comparison of liposome size, encapsulation efficiency, and process characteristics. Int. J. Pharm. 223, 55–68.

Colletier, J.-P., Chaize, B., Winterhalter, M., Fournier, D., 2002. Protein encapsulation in liposomes: efficiency depends on interactions between protein and phospholipid bilayer. BMC Biotechnol. 2, 9.

Cortesi, R., Esposito, E., Gambarin, S., Telloli, P., Menegatti, E., Nastruzzi, C., 1999. Preparation of liposomes by reverse-phase evaporation using alternative organic solvents. J. Microencapsul. 16, 251–256.

Corvo, M.L., Boerman, O.C., Oyen, W.J.G., Van Bloois, L., Cruz, M.E.M., Crommelin, D.J.A., Storm, G., 1999. Intravenous administration of superoxide dismutase entrapped in long circulating liposomes: II. In vivo fate in a rat model of adjuvant arthritis. Biochim. Biophys. Acta (BBA)—Biomembr. 1419, 325–334.

Du, S., Deng, Y., 2006. Studies on the encapsulation of oxymatrine into liposomes by ethanol injection and pH gradient method. Drug Dev. Ind. Pharm. 32, 791–797.

Elorza, B., Elorza, M.A., Sainz, M.C., Chantres, J.R., 1993. Comparison of particle size and encapsulation parameters of three liposomal preparations. J. Microencapsul. 10, 237–248.

Fanucci, G.E., Backov, R., Fu, R., Talham, D.R., 2001. Multiple bilayer dipalmitoylphosphatidylserine LB films stabilized with transition metal ions. Langmuir 17, 1660–1665.

Grant, G., Barenholz, Y., Piskoun, B., Bansinath, M., Turndorf, H., Bolotin, E., 2001. DRV liposomal bupivacaine: preparation, characterization, and in vivo evaluation in mice. Pharm. Res. 18, 336–343.

Hope, M.J., Bally, M.B., Mayer, L.D., Janoff, A.S., Cullis, P.R., 1986. Generation of multilamellar and unilamellar phospholipid vesicles. Chem. Phys. Lipids 40, 89–107.

Jahn, A., Vreeland, W.N., Don, L.D., Locascio, L.E., Gaitan, M., 2007. Microfluidic directed formation of liposomes of controlled size. Langmuir 23, 6289–6293.

Kremer, J., Esker, M., Pathmamanoharan, C., Wiersema, P., 1977. Vesicles of variable diameter prepared by a modified injection method. Biochemistry 16, 3932.

Lasic, D.D., Ceh, B., Stuart, M.C.A., Guo, L., Frederik, P.M., Barenholz, Y., 1995. Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery. Biochim. Biophys. Acta 1239, 145–156.

Lasic, D.D., Frederik, P.M., Stuart, M.C.A., Barenholz, Y., McIntosh, T., 1992. Gelation of liposome interior A novel method for drug encapsulation. FEBS Lett. 312, 255–258.

Lemmich, J., Mortensen, K., Ipsen, J.H., Honger, T., Bauer, R., Mouritsen, O.G., 1996. Small-angle neutron scattering from multilamellar lipid bilayers: theory, model, and experiment. Phys. Rev. E 53, 5169–5180.

Lewis, B.A., Engelman, D.M., 1983. Lipid bilayer thickness varies linearly with acyl chain length in fluid phosphatidylcholine vesicles. J. Mol. Biol. 166, 211–217.

Ipsen, J.H., Mouritsen, O.G., Bloom, M., 1990. Relationships between lipid membrane area, hydrophobic thickness, and acyl-chain orientational order. The effects of cholesterol. Biophys. J. 57, 405–412.

Marra, J., Israelachvili, J., 1985. Direct measurements of forces between phosphatidylcholine and phosphatidylethanolamine bilayers in aqueous electrolyte solutions. Biochemistry 24, 4608–4618.

Mayer, L., Hope, M., Cullis, P., Janoff, A., 1985. Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. Biochim. Biophys. Acta 817, 193–196.

Mckiernan, A.E., Ratto, T.V., Longo, M.L., 2008. Domain growth, shapes, and topology in cationic lipid bilayers on mica by fluorescence and atomic force microscopy. Biophys. J. 79, 2605–2615.

Nagle, J.F., Tristram-Nagle, S., 2000. Structure of lipid bilayers. Biochim. Biophys. Acta 1469, 159–195.

Pabst, G., Grage, S.L., Danner-Pongratz, S., Jing, W., Ulrich, A.S., Watts, A., Lohner, K., Hickel, A., 2008. Membrane thickening by the antimicrobial peptide PGLa. Biophys. J. 95, 5779–5788.

Peschka, R., Dennehy, C., Szoka Jr., F., 1998. A simple in vitro model to study the release kinetics of liposome encapsulated material. J. Control. Release 56, 41–51.

Schneider, T., Sachse, A., Robling, G., Brandl, M., 1995. Generation of contrast-carrying liposomes of defined size with a new continuous high pressure extrusion method. Int. J. Pharm. 117, 1–12.
Simıes, S.I., Delgado, T.C., Lopes, R.M., Jesus, S., Ferreira, A.A., Morais, J.A., Cruz, M.E.M., Corvo, M.L., Martins, M.B.F., 2005. Developments in the rat adjuvant arthritis model and its use in therapeutic evaluation of novel non-invasive treatment by SOD in transfersomes. J. Control. Release 103, 419–434.
Szoka, F., Olson, F., Heath, T., Vail, W., Mayhew, E., Papahadjopoulos, D., 1980. Preparation of unilamellar liposomes of intermediate size (0.1–0.2 μm) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes. Biochim. Biophys. Acta (BBA)—Biomembr. 601, 559–571.
Szoka, F., Papahadjopoulos, D., 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. U.S.A. 75, 4194–4198.
Yu, L.X., 2008. Pharmaceutical quality by design: product and process development, understanding, and control. Pharm. Res. 25, 781–791.
Zadi, B., Gregoriadis, G., 2000. A novel method for high-yield entrapment of solutes into small liposomes. J. Liposome Res. 10, 73–80.
Zhang, Y., Cheng, X., Wang, J., Zhou, F., 2009. Bi-and tetra-layered dipalmitoyl phosphatidylserine (DPPS) patterns produced by hydration of Langmuir–Blodgett monolayers and the subsequent enzymatic digestion. Colloids Surf. A: Physicochem. Eng. Aspects 337, 26–32.