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A quality by design (QbD) case study on liposomes containing hydrophilic API: I. Formulation, processing design and risk assessment

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ABSTRACT

The purpose of this study was to extend QbD principles to liposomal drug products containing a hydrophilic active pharmaceutical ingredient (API) to demonstrate both the feasibility and the advantages of applying QbD concepts to liposome based complex parenteral controlled release systems. The anti-viral drug Tenofovir was selected as a model compound. Desired properties for two of the key liposome drug product qualities, namely the particle size and drug encapsulation efficiency, were defined and evaluated. It was observed that the liposome preparation process significantly affects liposome particle size, and this resulted in considerable variation in the drug encapsulation efficiency. Lipid chain length did not have a significant effect on drug encapsulation efficiency. However, lipid concentration did affect the drug encapsulation efficiency with higher lipid concentrations resulting in higher drug encapsulation. The use of risk assessment in this study assisted the identification of eight high risk factors that may impact liposome drug encapsulation efficiency and particle size.

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1. Introduction

Since the discovery of liposomes in 1965 by Bangham and his colleagues (Bangham et al., 1965; Papahadjopoulos and Bangham, 1966), scientists have applied liposome systems to various fields including chemistry, physics, biology, and medicine. Most notably, the structural similarity of liposome bilayers to cellular membranes has intrigued scientists to explore the potential of using liposomes as drug-carriers to deliver therapeutics with different properties to specific regions of the body since the early 1970s (Gregoriadis, 1976; Gregoriadis et al., 1974; Papahadjopoulos and New York Academy of Sciences, 1978). However, despite the enormous amount of effort spent during the past 44 years (>114,000 scientific publications) and the well formed consensus within the scientific community about the potential of liposomes as drug carriers, currently relatively few products (12 therapeutic products) (Torchilin, 2005) are available on the market and all of these were approved between 1995 and 2004. However, considerable research is underway, and there are several liposome preparations that are in various stages of Clinical Trials (2011). There are several factors that may have contributed to the slow pace of commercialization of liposome drug products during the last decade: (1) the difficulties associated with identifying the formulation and process design critical quality attributes of these complex systems; (2) the high manufacturing cost due to low reproducibility and low entrapment of therapeutic active agents; and (3) the high regulatory burden associated with product safety of these complex parenteral product.

The structural versatility of liposomes allows the incorporation of lipid-soluble and water-soluble materials into the bilayers and the aqueous compartment, respectively. Compared to hydrophobic drugs, the encapsulation of hydrophilic drugs into liposomes presents unique challenges. Specifically, the high water solubility makes it difficult to achieve a high degree of drug entrapment. In addition, it is unknown how different process and product variables impact on product quality and performance. Therefore, an investigation of the application of Quality by Design (QbD) concepts to liposomes containing a hydrophilic compound will provide valuable information on critical formulation and process variables.

Pharmaceutical QbD is a systematic, scientific, risk-based, holistic and proactive approach to pharmaceutical development that starts with predefined objectives and emphasizes product and process understanding as well as process control (Yu, 2008). QbD means that quality-improving scientific methods should be used upstream in the research, development, and design phases, so that quality is designed into product processing at as early stage as possible (Vogt, 1992; Wu et al., 2007). QbD identifies characteristics that are critical to quality from the perspective of patients, translates them into the attributes that the drug product should possess,

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and establishes how the critical process parameters can be varied to consistently produce a drug product with the desired characteristics (Yu, 2008). A complete QbD study should comprise of the following four key elements: (1) define target product quality profile (goals) based on scientific prior knowledge and appropriate *in vivo* relevance; (2) design product and manufacturing processes to satisfy pre-defined goals; (3) identify critical quality attributes, process parameters, and sources of variability to obtain the design space; and (4) control manufacturing processes to produce consistent product quality over time through operation within the established design space, thus assuring that quality is built into the product. In this case study, the first three of these elements were evaluated at a lab scale.

In this case study, a nucleoside reverse transcriptase inhibitor (NRTI), Tenofovir, was selected as the model compound. The current Tenofovir formulations have limited efficacy. This is because the onset of action requires Tenofovir to be intracellularly phosphorylated to the diphosphate form in order to block viral reverse transcriptase (Hawkins et al., 2005). However, due to its high polarity (Log $P = -1.71$), Tenofovir has very low membrane permeability, and therefore its intracellular absorption is extremely low. In an effort to overcome this problem, a commercially available prodrug formulation (disoproxil form) has been developed, which has increased hydrophobocitiy and therefore enhanced intracellular uptake ($F \approx 40\%$) (Barditch-Crovo et al., 2001; Hawkins et al., 2005; Van Gelder et al., 2002). However, this pro-drug formulation suffers from plasma instability issues (hydrolysis). Another issue associated with the current Tenofovir formulations is non-specific drug distribution. Ideally, this drug should be targeted to lymphatic tissues and macrophage rich regions where the viruses are located. However, following parenteral administration of the current formulations, the drug is quickly distributed in the blood stream to every major organ. This results in considerable toxicity (*e.g.* nephrotoxicity due to extensive renal excretion) (Gitman et al., 2007; James et al., 2004). Accordingly, a liposomal Tenofovir formulation is expected to provide a better therapeutic index due to carrier facilitated intracellular transportation as well as the targeting effect.

In the present study, various Tenofovir liposome formulations were prepared, and the effects of lipid length, lipid concentration, and drug concentration on the drug encapsulation efficiency were explored. With respect to the preparation process, the impact of various processes on the liposome particle size, zeta-potential, as well as drug encapsulation efficiency was analyzed. Lastly, risk analysis on the formulation particle size as well as drug encapsulation efficiency was performed with the goal to identify potential high risk factors for subsequent screening studies.

2. Materials and methods

2.1. Materials

Tenofovir was purchased from Resource Technique Corporation (Laramie, Wyoming). Sodium dodecyl sulfate (SDS) and 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 kD) 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 were weighed into a 50 ml pear-shape flask and ~2 ml of chloroform were added to dissolve the lipids. 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 (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 any larger particles. Then 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). Finally, the samples were 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.

2.2.2. Chromatographic equipment and conditions

The high-performance liquid chromatography (HPLC) system consisted of a Perkin Elmer System (Perkin Elmer Inc, US) equipped with a series 200 pump, a series 200 autosampler, a series 785A UV/VIS detector, and a Peak Simple™ 3.79 chromatography data system for peak identification and integration. The analytical column was a Symmetry C8 column (3.5 μm, 4.6 × 100 mm, Waters Corporation, USA) protected with a Symmetry C8 column (3.5 μm, 2.1 × 100 mm, Waters Corporation, USA). The signal was monitored at 260 nm. The mobile phase consisted of acetonitrile–10 mM sodium dihydrogen phosphate (adjusted to pH 6.5 with 1 N sodium hydroxide) at a ratio of (2.5:97.5; v/v). The flow-rate was set at 1 ml/min and the injection volume was 10 μl. The developed HPLC method was validated as per the ICH guidance (ICH 2005). Linearity was obtained for Tenofovir in the concentration range 0.1–40 μg/ml ($r^2 = 0.9999$) with a calibration curve of Area = 194.5214 × Conc. + 7.4095. The detection and quantitation limits were 65.8 ng/ml and 219.2 ng/ml, respectively.

2.2.3. Determination of encapsulation efficiency (EE%)

10 μl of prepared liposomes (before purification) were withdrawn and diluted with 2 ml 10 mM pH 7.4 HEPES buffer ($n = 3$). 500 μl of this diluted solution was put 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:

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

2.2.4. Purification of liposomes

Prepared liposomes were purified with two PD-10 columns used in series and the following procedures were performed: (1) the top cap of the new column was removed, the column storage solution was poured off, and the sealed end of the column was cut at the notch; (2) each column was filled with equilibration buffer (10 mM pH 7.4 HEPES) and the buffer was allowed to enter the packed bed completely (this process was repeated 4 times for each column and the flow-through was discarded); (3) a maximum of 2.5 ml of sample was added to the column (for sample volumes less than 2.5 ml,

equilibration buffer was added accordingly to adjust the volume up to 2.5 ml after the sample has entered the packed bed completely) and the 2.5 ml flow-through was discarded; (4) a test tube for sample collection was put under the column, 3.5 ml of buffer was added to the column, and the eluate (Tenofovir containing liposomes) was collected; and (5) steps 3–4 were performed on the second equilibrated PD-10 column using the eluate collected. This method (two PD-10 column used in series) resulted in very high purification efficiency of ∼99.9% with a 1.96 times dilution of the sample.

2.2.5. Particle size analysis

Particle size analysis was conducted using a Malvern ZS90 zeta-sizer. Prepared liposome formulations were diluted at least 50 times to obtain a suspension that was below 0.5 mg/ml. All measurements were conducted at 25 °C and it was assumed that suspensions would have similar viscosities to that of water at this temperature (η = 0.89 cp). All measurements were performed in triplicate. In addition, all particle size data were reported as "mean particle size ± width".

2.2.6. Zeta potential analysis

Zeta potential was measured using a Malvern ZS90 zeta-sizer and a folded capillary cell. The same samples used for particle sizing were used for zeta-potential measurement. All tests were conducted at 25 °C and in triplicate.

2.2.7. Risk assessment

Several Ishikawa diagrams (also known as the fish-bone diagram, or cause-and-effect diagram) were constructed to identify the potential risks and corresponding causes. Specifically, two major quality attributes, particle size and drug encapsulation efficiency, were defined and further delineated to identify all potential risks. After the analysis, eight key variables were identified for screening in subsequent studies.

3. Results

3.1. Effect of liposome bilayer thickness on drug encapsulation

To evaluate the effect of lipids, more specifically the carbon tail length of the lipids, on the drug encapsulation efficiency, three liposome formulations were prepared containing DSPC (18 carbons), DPPC (16 carbons) and DMPC (14 carbons). The phase transition temperatures (T_m) of these three lipids are 55 °C, 42 °C and 25 °C, respectively. As shown in Table 1, the EE% for the three formulations increased in the order of DSPC < DPPC < DMPC with very small increments (20–24%).

3.2. Effect of process on drug encapsulation and particle size

To understand how the process would change drug encapsulation inside liposomes, the following experiment was performed. A Tenofovir liposome formulation, containing 50 mg/ml lipid (55:27:18 mole fraction of DSPC:cholesterol:DPTAP) and 1 mg/ml Tenofovir, was prepared and the EE% and particle size distribution were determined after each processing step. As can be seen in Fig. 1, the drug encapsulation was affected greatly by the process, with the largest increase in EE% occurring following freeze-thaw cycling, and the largest decrease in EE% occurring after the extrusion step. With respect to the particle size, the liposomes became smaller after sonication. Further reduction in particle size occurred following 5 cycles of freeze-thaw cycling, and more importantly the particle size distribution became more homogeneous. The most significant size reduction occurred following extrusion. After the extrusion step, the system exhibited a single particle population of 159.2 ± 28.6 nm (Fig. 2). Note that all the values in Fig. 2 are volume-weighted means, which correlates to the mass distribution of the vesicles and the area under the curve of each peak represents the relative percentage of that peak.

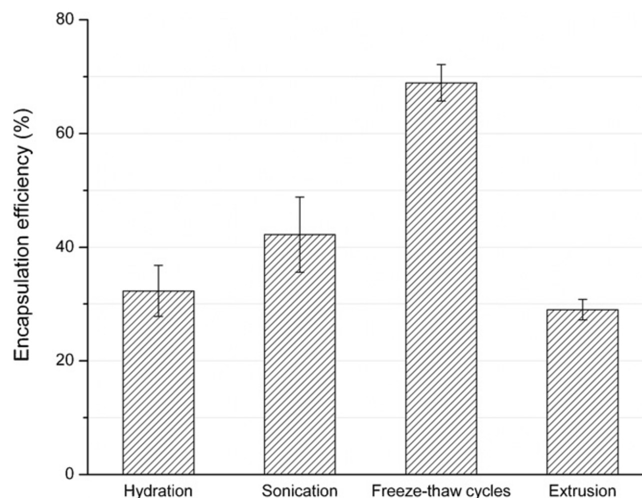


Fig. 1. Effect of preparation process on drug encapsulation.

With respect to surface charge, at 18% mole fraction of the positively charged lipid (DPTAP), the liposomes had a surface charge of 75.81 ± 6.27 mV. The preparation process has no effect on the surface charge.

3.3. Effect of extruder membrane pore size on drug encapsulation

As reported in the previous section, the particle size of the small unilamellar vesicles (SUV) had a significant impact on the EE%. It was therefore crucial to understand how the extruder filter pore size would change the formulation characteristics. To accomplish this, the same formulation (as the previous section) was prepared using the same protocol, except two stacked 100 nm membranes were used instead of two 200 nm membranes.

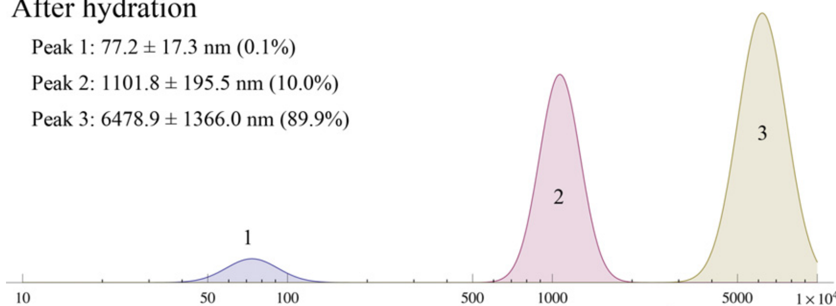
As can be seen in Table 2, the smaller pore size membranes resulted in a 44 nm decrease in liposome particle size and a 14% decrease in EE%.

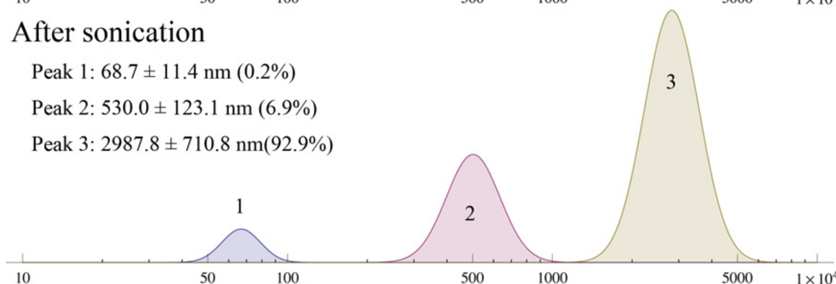
3.4. Effect of lipid and drug concentration on drug encapsulation

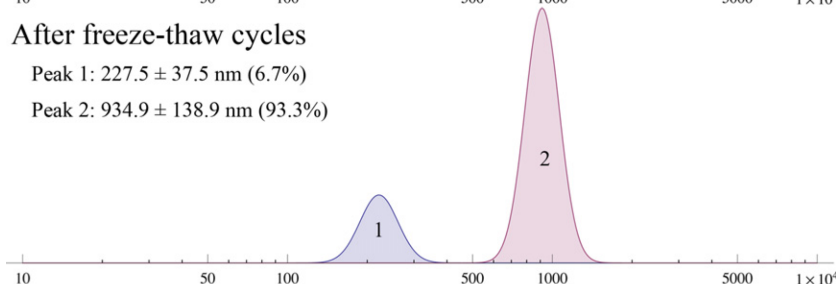
As shown in Table 3, an increase in lipid concentration resulted in an increase in the EE%, however an increase in the drug concentration rendered a decrease in drug encapsulation.

Table 1
Effect of lipids on the EE% (n = 3).

Composition	Mole fraction (%)	Lipid conc. (mg/ml)	Drug conc. (mg/ml)	EE%
DSPC:cholesterol:DPTAP	55:27:18	50	5.0	20.15 ± 0.13
DPPC:cholesterol:DPTAP	55:27:18	50	5.0	20.25 ± 0.99
DMPC:cholesterol:DPTAP	55:27:18	50	5.0	24.50 ± 2.11







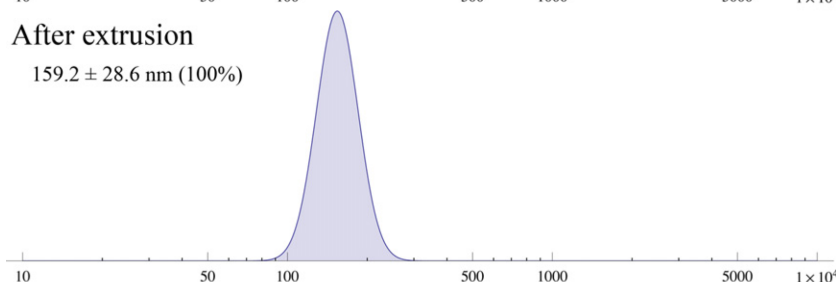


Fig. 2. Particle size distribution (volume weighted) of the liposomes after each step.

Table 2
Effect of extrusion membrane pore size on the EE%.

Composition	Lipid conc. (mg/ml)	Drug conc. (mg/ml)	Membrane pore size (nm)	Particle size (nm)	EE%
DSPC:cholesterol:DPTAP	50	1	100	111.9 ± 17.5	14.86 ± 1.87
DSPC:cholesterol:DPTAP	50	1	200	156.0 ± 28.0	29.01 ± 1.76

3.5. *Risk assessment*

Risk identification and risk analysis are two basic components of risk assessment as outlined in the ICH Q9 document. Both of these components are of vital importance during the application of QbD principles in drug product development. While risk identification focuses on the systematic use of information to identify potential harm, risk analysis deals with qualitative or quantitative linking of the likelihood of occurrence and severity of harm. The goal of these two assessments is to obtain the highest risk factors, which will be subjected to a more complex design of experiment (DOE) study to establish a product or process design space (see part II). In

Table 3
Effect of lipid and drug concentration on EE%.

Composition	Mole fraction (%)	Lipid conc. (mg/ml)	Drug conc. (mg/ml)	EE%
DSPC:cholesterol:DPTAP	55:27:18	50	1	29.01 ± 1.76
DSPC:cholesterol:DPTAP	55:27:18	50	2.5	24.29 ± 1.92
DSPC:cholesterol:DPTAP	55:27:18	50	5	20.15 ± 1.03
DSPC:cholesterol:DPTAP	55:27:18	100	1	45.65 ± 1.55
DSPC:cholesterol:DPTAP	55:27:18	100	5	34.96 ± 1.85

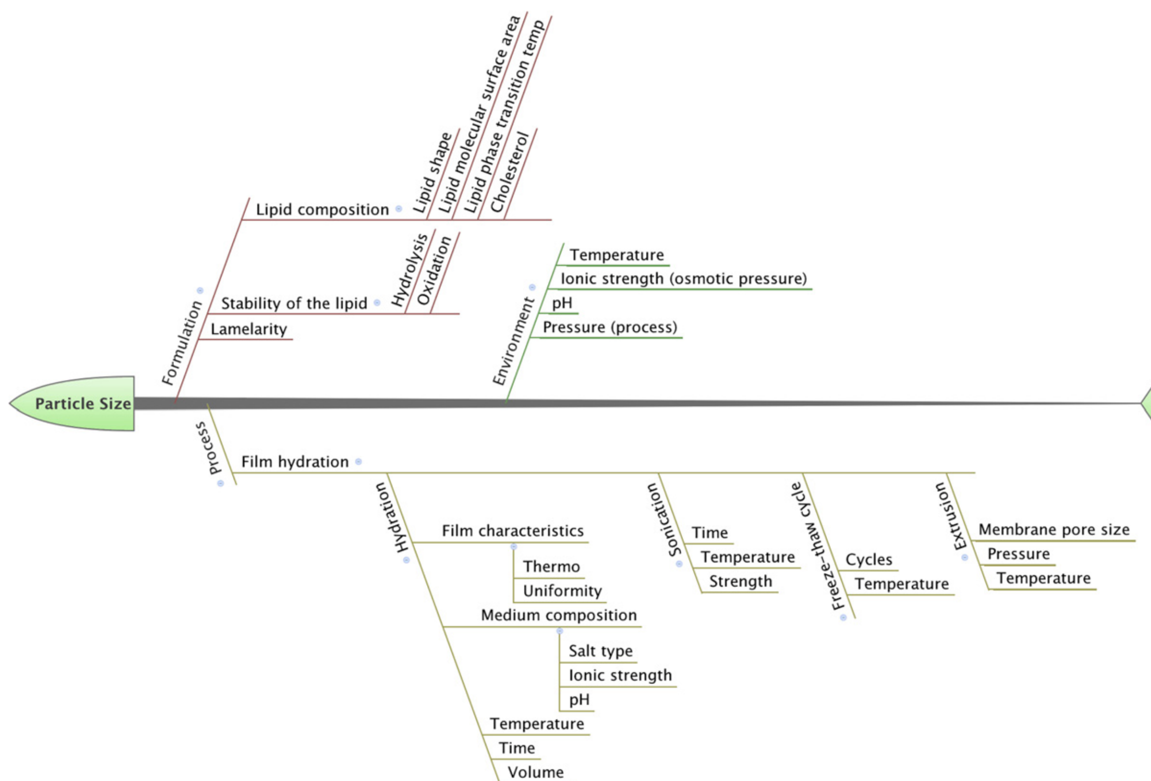


Fig. 3. An Ishikawa diagram illustrating factors that may have impact on the particle size of liposomes.

this section, the risk identification and risk analysis were combined together to identify the potential high risk factors.

As mentioned above, liposome particle size and drug encapsulation efficiency are two very critical product qualities, and an understanding and awareness of the potential risks is very important. To accomplish this, two cause-and-effect diagrams (Ishikawa diagram) were constructed to identify the potential causes of product variability.

After the risk analysis, the following eight variables were identified as high risk factors affecting liposome drug encapsulation and particle size: lipid concentration, drug concentration, cholesterol concentration, buffer concentration, hydration time, sonication time, number of freeze-thaw cycles, and extrusion pressure as labeled in Fig. 4.

4. Discussion

The first (and the most important) element in using the QbD concept to assist formulation and process design is to pre-define the desired final product quality profiles. This study focused on two critical formulation qualities, namely the drug encapsulation efficiency and formulation particle size. These two product qualities are very important for both manufacturers and patients. A higher percentage of drug encapsulation could reduce the manufacturing cost and increase drug concentration in the final formulation allowing greater flexibility in dosing. Depending on the pharmacokinetics, higher drug concentration in the formulation can result in increased dosing intervals and hence improved patient compliance. Accordingly, a goal of the current study is to maximize the drug encapsulation, and liposome formulations that resulted in encapsulation efficiencies below 5% were excluded from further analysis. Another goal was to achieve a particle size range between 100 and 200 nm, since this size range would allow sterile filtration of the final product. In addition, a liposome size range of 100 and 200 nm has been reported to result in maximum uptake by macrophages (Oussoren et al., 1997; Velinova et al., 1996). Liposome stability (or drug retention inside liposomes) is also a critical product attribute. Since the liposomes used in this study are intended for targeting to the macrophages and lymphatic tissues, there should be no drug leakage until cellular uptake. Therefore, the designed formulation should have reasonable *in vitro* and *in vivo* stability, and resist drug leakage as well as liposome aggregation. To achieve this, the following formulation design strategies were used: (1) a saturated long alkyl chain PC lipid, DSPC, is used as the main lipid component to increase physical stability as well as reduce possible chemical degradation (oxidation); (2) at least 20% of cholesterol was used in the formulations to reduce the membrane permeability; and (3) positively charged lipids, such as 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP), were used to increase the drug–membrane association to reduce drug leakage, since Tenofovir is negatively charged at neutral pH conditions ($pKa_1 = 1.61 \pm 0.14$, $pKa_2 = 4.11 \pm 0.50$). As a result, all the prepared formulations were stable at 4 °C over a 2-year test period, with no change in particle size and no leakage of drug (data not shown). Finally, the drug concentration was set at 8 mg/ml and lower to reduce the possibility of precipitation and re-crystallization. These desired product characteristics limited the range of formulation and processing conditions investigated.

4.1. Effect of lipid length

Theoretically, lipids with shorter chain length will contribute to a thinner liposome membrane, which will result in a higher internal volume given the same amount of lipid molecules and the same particle size distribution. However, compared with other factors that may also alter the drug encapsulation process (*e.g.* drug concentration, lipid concentration, particle size, etc.) differences in membrane thickness were not expected to be significant. This is because a 1 nm change in the membrane

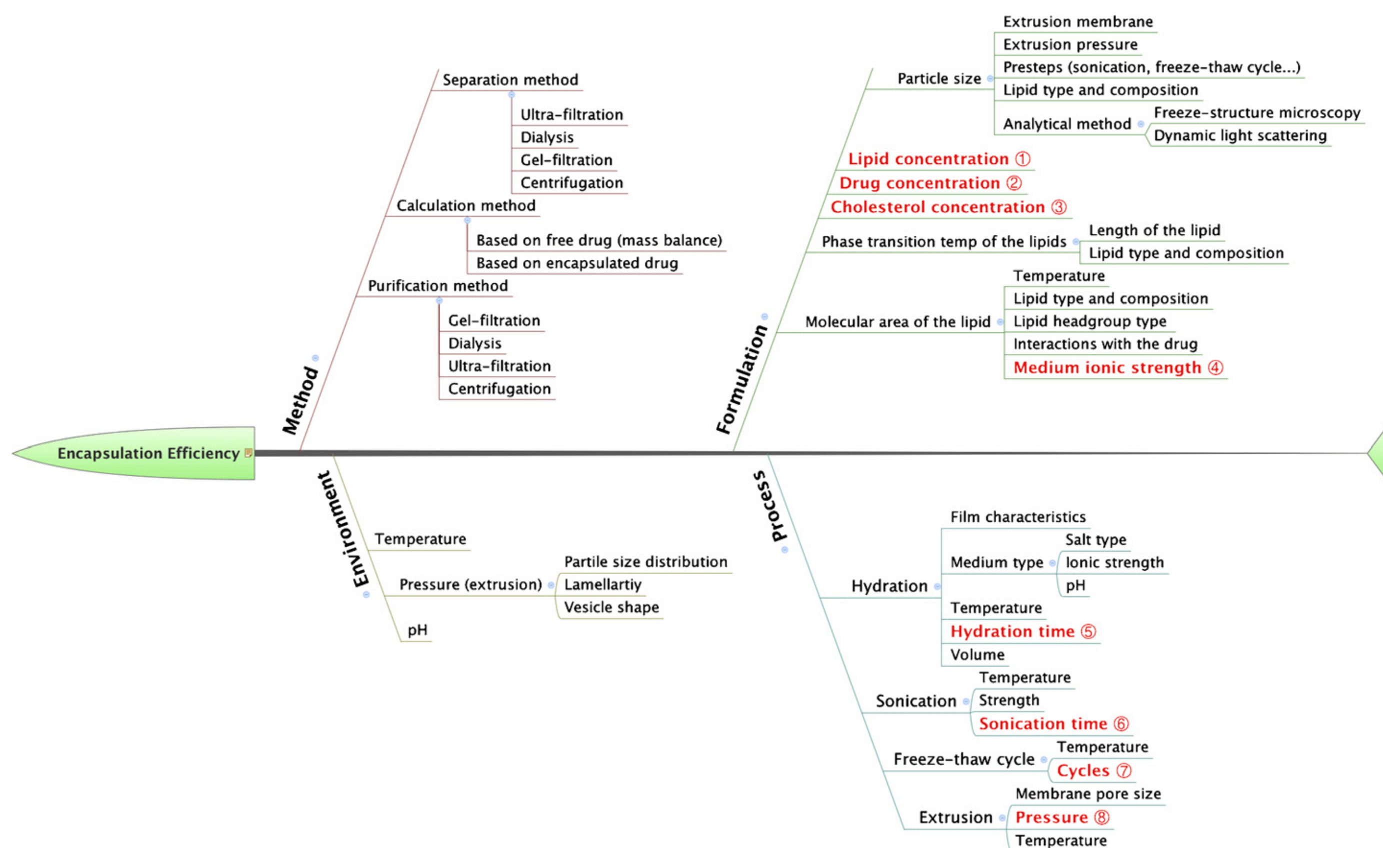


Fig. 4. An Ishikawa diagram illustrating factors that may have impact on the encapsulation efficiency of liposomes.

thickness would only change the EE% by ∼0.7% (according to our previously published mathematical model), and in this study the maximum difference in length among the three lipids is less then 1 nm. However, statistic analysis did show a very small but statistically significant difference in EE% between the formulations containing DPPC and DMPC ($p = 0.022$). It was considered that this small difference was not due to the lipid tail length, but due to variations that may have occurred during preparation, such as small differences in lipid concentration, and liposome particle size.

4.2. Effect of process on drug encapsulation and particle size

In this study, hydration of the dry lipids produced a suspension of large multilamellar vesicles. These vesicles are heterogeneous with regard to their size, since a large portion of vesicles (∼90% by volume) is in the micron size range (6 μm) while a small population (<1% by volume) of vesicles is smaller than 100 nm. Because of their extensive lamellar structures, large particles have very high percentage of lipid but very little internal aqueous volume for drug entrapment. Hence, these freshly formed MLVs typically are not suitable for pharmaceutical use and post-hydration processing, *i.e.* sizing, is required. After one minute of sonication, and five cycles of freeze-thaw cycling, vesicle particle size decreased, the system become less and less heterogeneous (Fig. 2), and the EE% increased. The reason for the increase in the EE% was speculated to be due to break-down of the liposome multi-lamellar structure. Consequently, the lipid molecules rearrange resulting in changes in the lamellar structures, and this contributes to a higher internal volume:lipid ratio, which allows higher drug encapsulation. Compared to one minute of sonication, five cycles of freeze-thaw cycling showed a more significant effect on the EE%. This is considered to be due to the freeze-thaw cycling process not only breaking down the multi-lamellar structure but also causing homogenization of the drug distribution inside and outside liposomes. It is expected that during freeze thaw cycling, larger vesicles are broken and smaller vesicles may fuse together to result in a reduction in particle size distribution. Finally, after passing through two stacked 200 nm polycarbonate membranes, the sample became homogeneous and showed a single particle size distribution of 156.0 ± 28.0 nm. This dramatic change in particle size resulted in a decrease in liposome entrapment volume and hence a reduction in the EE%. In summary, the preparation process could affect sample particle size as well as lamellar structure considerably, and both of these played a very important role in changing drug encapsulation inside the liposomes. It was concluded that a larger particle size and uni-lamellar structure would result in liposome formulations with higher drug encapsulation.

4.3. Effect of extruder membrane pore size

From a formulation point of view, it should be noted that the selection of the appropriate membrane pore size was critical in optimizing drug encapsulation efficiency. For an extruded liposome formulation, the particle size of the final product is mainly determined by the extruder membrane as well as the pressure applied. If the selected membranes are too small (≤100 nm), it would be very difficult to encapsulate a reasonable amount of drug inside the liposomes. On the other hand, if the membranes are too large (≥400 nm), then it would be impossible to sterilize the liposomes using a 0.22 μm filter without losing their structural integrity. Accordingly, 200 nm is an appropriate membrane pore size to achieve Tenofovir liposomes with a size range of 150–158 nm.

4.4. Effect of lipid concentration and drug concentration

At high lipid concentrations the population of the vesicles in the system is high, and hence there is more internal volume for drug encapsulation. When not considering drug–lipid interactions, the encapsulation efficiency was dependent on the internal to external volume ratio of the liposomes for any drug concentration. A higher internal to external volume ratio resulted in higher drug encapsulation. However, due to drug-lipid interactions, a small portion of the free-drug associates with the liposome surface, causing a small increase in apparent drug encapsulation. This additional increase in drug encapsulation is largely dependent on the liposome surface area as well as the free-drug concentration in the medium. At low drug concentrations, a higher percentage of free-drug is associated with the liposome surface. At very high drug concentrations, the percentage of surface attached drug becomes negligible, and hence any additional increase in the drug concentration would not make any appreciable difference in drug encapsulation. This explains the second observation of an inverse relationship between drug concentration and drug encapsulation efficiency.

4.5. Risk analysis

The liposome formulation was optimized based on the encapsulation efficiency, since the liposome particle size was determined by the extruder filter pore size. The following eight variables with potential to affect liposome drug encapsulation were identified in the risk analysis: lipid concentration, drug concentration, cholesterol concentration, buffer concentration, hydration time, sonication time, number of freeze-thaw cycles, and extrusion pressure as labeled in Fig. 4. This information can be used by scientists in formulation and process screening studies to identify the most significant variables for liposome formulation and process optimization. These significant variables can then be evaluated using a response surface methodology to obtain the appropriate design space. Some of the key factors affecting particle size and encapsulation efficiency are discussed below.

4.6. Risk analysis—liposome particles size

As shown in Fig. 3, the three major categories for the Ishikawa diagram can be divided into formulation, processing and environment conditions, and under each category all factors were listed. Of these, several key factors were identified and discussed here: (1) lipid shape; (2) lipid charge; (3) processing method; and (4) membrane pore size. The shape and hence the packing parameter of the lipids will affect the intrinsic curvature of the liposome membrane, and hence the liposome size (the packing parameter is defined as $P = v/(a \times l)$, where v is the molecular volume, a is the surface area, and l is the molecular length (Kumar, 1991)). However, this effect will only become dominant when the liposome particle size is small (<100 nm). Liposome surface charge will play an important role on stability as the electrostatic repulsive force will prevents particle aggregation (Nakamori et al., 1993). Surface charge can be introduced by incorporating charged lipids, such as DPTAP, stearylamine, and DPPG. The mean particle size and the size distribution of the final liposome product largely depend on the processing method (extrusion, homogenization and sonication). As shown in Section 3.2, each of these steps may cause significant change in the mean particle size and size distribution. The final product size is mainly dependent on the extruder membrane pore size.

4.7. Risk analysis—drug encapsulation efficiency

The major categories of factors affecting liposome drug encapsulation are formulation, manufacturing process, environmental conditions, and analytical methodologies. Under each category, all possible factors were identified as shown in Fig. 4. Of these, several key factors were identified and discussed here: (1) hydration time; (2) cholesterol concentration; (3) hydration medium; and (4) analytical method. To prepare the liposomes, drug-containing solutions are used to hydrate the dry lipids. During this process the lipid molecules re-arrange into spherical vesicles with the drug molecules entrapped inside. Therefore, the hydration process is considered to be a vital step. It is possible that a longer hydration time may result in a higher percentage drug encapsulation. Temperature is also a critical factor during the hydration process, since hydration only occurs at temperatures above the T_m of the lipid. Cholesterol is usually used to increase liposome stability, since it reduces bilayer fluidity and hence permeability (Kirby et al., 1980; Lee et al., 2005). It is unknown whether increase in stability will also increase drug encapsulation since this may improve drug retention inside the liposomes. Lipid molecular surface area is also believed to have an impact on drug encapsulation. In the case of lipid molecules with smaller surface area, more molecules will be required per unit surface area to form the liposomes. Accordingly, the number of vesicles formed will be reduced compared to lipid molecules with higher surface area, and this will affect the capacity of the liposomes to entrap drug. The lipid molecular surface area may change with change in the ionic strength of the medium, which may lead to a change in drug encapsulation. To determine the EE% of liposome drug products, drug-containing liposomes need to be separated from free drug; this generally involves a separation process. There are currently two different approaches to do this. The method used in this study (determination of the free and total drug concentration and the percentage drug encapsulation is calculated based on mass balance) represents one approach. Another approach involves direct measurement of the amount of drug encapsulated inside the liposomes ($C_{\text{encapsulated}}$) and the EE% is calculated as the ratio of encapsulated drug amount to total drug amount ($\text{EE\%} = C_{\text{encapsulated}}/C_{\text{Total}} \times 100\%$). To determine the encapsulated drug amount, a separation process is needed to obtain the amount of drug-containing liposomes (same process as purification). It is worth noting that the accuracy of this approach largely depends on the completeness and robustness of the separation process. Following incomplete separation, even very small amounts of free-drug will result in a very inaccurate estimation of the EE% (the estimation error could reach 120% (data not shown) or even higher if free-drug concentration or percentage is higher). Consequently, extra care should be taken when using the second approach to estimate the EE%, especially during selection of the appropriate separation method.

5. Conclusions

One of the biggest challenges for designing a liposome formulation for hydrophilic molecules is the low encapsulation efficiency, which has been limiting the broad use of this type of delivery systems at a commercial scale. In addition, high manufacturing variability as a result of lack of understanding in the preparation process means a much more stringent review process is necessary for the safety concern. Accordingly, it is the objective of current study to utilize the QbD principles to assist formulation and process design, to help understand the sources of the variability in order to improve the product quality. To accomplish this, in the study the anti-viral drug Tenofovir was used as a model compound. Desired profiles for two of the key product qualities, namely the particle size

and drug encapsulation efficiency, were defined and evaluated. The goals were to achieve as high as possible the encapsulation, while maintaining the particle size distribution as narrow as possible. It was observed that the liposome preparation process has enormous impact on the liposome particle size, and this results in significant variation in drug encapsulation efficiency during preparation. However, through appropriate process design, such as optimizing the extruder filter size and the use of freeze-thaw cycling to increase the homogeneity of the system, very consistent particle size distribution as well as drug encapsulation efficiency can be achieved. With regard to the drug encapsulation efficiency, it is concluded that a larger particle size and uni-lamellar structure would result in liposome formulations with higher drug encapsulation. To further increase the encapsulation, higher lipid concentration should be used. It is discovered that lipid chain length has a minimal effect on drug encapsulation efficiency. Finally, the use of risk assessment assisted the identification of eight high risk factors that may impact liposome drug encapsulation efficiency and particle size. This information will be useful for a more comprehensive experimental design to better understand the interactions among all the variables and to obtain the appropriate design space.

Disclaimer

The views expressed are those of authors and do not necessarily represent the official position of the Agency.

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References

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.

Barditch-Crovo, P., Deeks, S., Collier, A., Safrin, S., Coakley, D., Miller, M., Kearney, B., Coleman, R., Lamy, P., Kahn, J., McGowan, I., Lietman, P., 2001. Phase I/II trial of the pharmacokinetics, safety, and antiretroviral activity of tenofovir disoproxil fumarate in human immunodeficiency virus-infected adults. Antimicrob. Agents Chemother. 45, 2733–2739.

Gitman, M., Hirschwerk, D., Baskin, C., Singhal, P., 2007. Tenofovir-induced kidney injury. Expert Opin. Drug Saf. 6, 155–164.

Gregoriadis, G., 1976. The carrier potential of liposomes in biology and medicine (second of two parts). N. Engl. J. Med. 295, 765–770.

Gregoriadis, G., Wills, E.J., Swain, C.P., Tavill, A.S., 1974. Drug-carrier potential of liposomes in cancer chemotherapy. Lancet 1, 1313–1316.

Hawkins, T., Veikley, W., St Claire, R., Guyer, B., Clark, N., Kearney, B., 2005. Intracellular pharmacokinetics of tenofovir diphosphate, carbovir triphosphate, and lamivudine triphosphate in patients receiving triple-nucleoside regimens. J. Acquir. Immune Defic. Syndr. 39, 406–411.

James, C., Steinhaus, M., Szabo, S., Dressler, R., 2004. Tenofovir-related nephrotoxicity: case report and review of the literature. Pharmacotherapy 24, 415–418.

Journal of Gene Medicine Clinical Trials Website, 2011. http://www.wiley.co.uk/genmed/clinical/.

Kirby, C., Clarke, J., Gregoriadis, G., 1980. Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. Biochem. J. 186, 591.

Kumar, V.V., 1991. Complementary molecular shapes and additivity of the packing parameter of lipids. PNAS 88, 444.

Lee, S.C., Lee, K.E., Kim, J.J., Lim, S.H., 2005. The effect of cholesterol in the liposome bilayer on the stabilization of incorporated Retinol. J. Liposome Res. 15, 157–166.

Nakamori, K., Nakajima, T., Odawara, M., Koyama, I., Nemoto, M., Yoshida, T., Ohshima, H., Inoue, K., 1993. Stable positively charged liposome during long-term storage. Chem. Pharm. Bull. 41, 1279.

Oussoren, C., Zuidema, J., Crommelin, D.J.A., Storm, G., 1997. Lymphatic uptake and biodistribution of liposomes after subcutaneous injection. II. Influence of liposomal size, lipid composition and lipid dose. Biochim. Biophys. Acta: Biomembranes 1328, 261–272.

Papahadjopoulos, D., Bangham, A.D., 1966. Biophysical properties of phospholipids. II. Permeability of phosphatidylserine liquid crystals to univalent ions. Biochim. Biophys. Acta 126, 185–188.

Papahadjopoulos, D., 1978. Liposomes and Their Uses in Biology and Medicine. New York Academy of Sciences, New York.

Torchilin, V.P., 2005. Recent advances with liposomes as pharmaceutical carriers. Nat. Rev. Drug Discov. 4, 145–160.

Van Gelder, J., Deferme, S., Naesens, L., De Clercq, E., Van den Mooter, G., Kinget, R., Augustijns, P., 2002. Intestinal absorption enhancement of the ester prodrug tenofovir disoproxil fumarate through modulation of the biochemical barrier by defined ester mixtures. Drug Metab. Dispos. 30, 924–930.

Velinova, M., Read, N., Kirby, C., Gregoriadis, G., 1996. Morphological observations on the fate of liposomes in the regional lymph nodes after footpad injection into rats. Biochim. Biophys. Acta: Lipids Lipid Metab. 1299, 207–215.

Vogt, N., 1992. Quality by design: managing research and development. Chemometr. Intell. Lab. Syst. 14, 93–101.

Wu, H., Khan, M., Hussain, A.S., 2007. Process control perspective for process analytical technology: integration of chemical engineering practice into semiconductor and pharmaceutical industries. Chem. Eng. Commun. 194, 760–779.

Yu, L.X., 2008. Pharmaceutical quality by design: product and process development, understanding, and control. Pharm. Res. 25, 781–791.