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A quality by design (QbD) case study on liposomes containing hydrophilic API: II. Screening of critical variables, and establishment of design space at laboratory scale☆

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ABSTRACT

Two statistical designs were used in this case study as part of an investigation into the feasibility and the advantages of applying QbD concepts to liposome-based complex parenteral controlled release systems containing a hydrophilic active pharmaceutical ingredient (API). The anti-viral drug Tenofovir was used as a model compound. First design (Plackett–Burman) was used to screen eight high-risk variables obtained from risk analysis and assess their impact on liposome characteristics (drug encapsulation efficiency, particle size, and physical stability). It was discovered that out of eight high-risk variables only lipid and drug concentration had significant effects on the drug encapsulation efficiency. This allowed the use of a central composite design (CCD) (with more predictive capability) to fully elucidate the relationship between lipid concentration, drug concentration and encapsulation efficiency. On comparing the CCD model generated response surface with additional data points, the accuracy and robustness of the model was confirmed. Using this developed model, the design space for Tenofovir liposomes preparation has been established in a laboratory setting, within which the preparation variability is minimized. With regard to sample storage stability, it was shown that at 4 °C the prepared Tenofovir liposomes, dispersed in aqueous phase, achieved stability for at least 2 years. These principles can be applied to liposomes containing other hydrophilic APIs, and can provide time and cost saving to industrial formulation scientists, and result in a more robust liposome preparation process.

1. Introduction

One of the challenges during the development of liposome formulations for hydrophilic molecules is the low drug encapsulation efficiency, which has been limiting the broad use of this delivery system at a commercial scale. In addition, high manufacturing variability as a result of a lack of understanding of the preparation process means a much more stringent review is necessary in terms of product safety (Rathore and Winkle, 2009; Vogt, 1992). Hence, it is the objective of this study to utilize quality by design (QbD) principles to assist formulation and process design, to help understand the sources of variability in order to improve product quality. The anti-viral drug Tenofovir was used in this study as a hydrophilic model compound. Being a nucleoside reverse transcriptase inhibitor (NRTI), Tenofovir has 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 $P = -1.71$) the intracellular absorption of Tenofovir is extremely low which limits its *in vivo* efficacy. Additionally, due to its non-specific distribution following parenteral administration, the drug quickly distributes in the blood stream to every major organ. This results in considerable nephrotoxicity due to extensive renal excretion (Gitman et al., 2007; James et al., 2004). Ideally, the drug should be targeted to lymphatic tissues and macrophage rich regions where the virus is located. 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. For these reasons, the target profile of the intended Tenofovir liposomes is: (1) relatively high drug encapsulation efficiency (>20%); (2) low and predictable variation in the drug encapsulation efficiency; (3) particle size range of 100–200 nm; and (4) sufficient storage stability (4 °C).

To obtain the above target profile, a systematic QbD approach was used. A complete QbD study (Wu et al., 2007; Yu, 2008) should comprise of the following four key elements: (1) define target

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product quality profile (goals) based on scientific prior knowledge and appropriate *in vivo* relevance; (2) design product and manufacturing processes to satisfy the pre-defined profile; (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 (the range of process and/or formulation parameters that have been demonstrated to provide assurance of quality), thus assuring that quality is built into the product (ICH Q8). The first two elements have already been studied and discussed in a previously published paper (Xu et al., 2011). It was demonstrated that the preparation process has an enormous impact on liposome particle size, and this results in significant variation in drug encapsulation efficiency during preparation. In addition, risk analysis narrowed down the high risk factors that may impact liposome drug encapsulation efficiency and particle size to eight factors. This makes it possible to use an experimental design approach to further study the impact of those eight factors on drug encapsulation as well as particle size and to optimize drug encapsulation.

This manuscript focuses on the third element, which is to identify characteristics that are critical to the final product quality, and establishes how the critical process parameters can be varied to consistently produce a drug product with the desired characteristics. For this purpose, two experimental designs were used. First, a Plackett–Burman screening design (Lewis et al., 1999) was used to identify the most significant factors affecting drug encapsulation and particle size. Next, a central composite design (CCD) was used in the response surface study (Lewis et al., 1999) to obtain the exact relationship between the drug encapsulation and various factors (that have been identified in the screening study). This design contains an imbedded factorial design with center points and is augmented with a group of axial points that allows estimation of curvature and in addition this permits the design to be rotatable (Forbes et al., 1999). After obtaining the response surface the optimal formulation and process conditions were identified. Further experimental tests were performed to test the robustness and accuracy of the generated model.

2. Material and methods

2.1. Material

Tenofovir was purchased from Resource Technique Corporation (Laramie, Wyoming). HEPES sodium salts, and Triton X-100, were purchased from Sigma–Aldrich (St. Louis, MO). 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 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). Subsequently, the samples were put into a LIPEX™ extruder (Northern Lipids Inc., Canada) and passed through a stack of 200 nm polycarbonate membranes to obtain liposomes with the desired particle size. Finally, the samples were purified with two PD-10 columns used in a series configuration.

2.2.2. Chromatographic equipment and conditions

Tenofovir was analyzed using an HPLC method as described previously (Xu et al., 2011). In brief, HPLC was performed on a Symmetry C8 column (3.5 μm, 4.6 mm × 100 mm, Waters Corporation, USA) protected with a Symmetry C8 guard column (3.5 μm, 2.1 mm × 10 mm). 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. Tenofovir was detected at 260 nm using a Perkin-Elmer 785 UV-Vis detector.

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:

$$\text{EE\%} = \left(1 - \frac{C_{free}}{C_{total}}\right) \times 100\% \quad (1)$$

2.2.4. 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 in triplicate. The values were reported as intensity weighted mean ± SD (distribution width).

2.2.5. 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 and reported as mean ± SD.

2.2.6. Drug leakage test

To test drug leakage from liposomes, each formulation was diluted 100 times (100 μl into 10 ml HEPES buffer) in a 15 ml centrifuge tube ($n = 6$). These six samples were then divided into two groups (3 for each group) to be kept at 37 °C and 4 °C, respectively. At predetermined times, 500 μl samples were withdrawn from each centrifuge tube and the amount of free drug was determined.

2.2.7. Plackett–Burman screening study

Plackett–Burman study was used to screen various factors, including: lipid concentration (X_1), drug concentration (X_2),

Table 1
Plackett–Burman study design table and results. X_1: lipid concentration; X_2: drug concentration; X_3: extrusion pressure; X_4: cholesterol%; X_5: HEPES buffer concentration; X_6: hydration time; X_7: sonication time; X_8: freeze–thaw cycles. Particle size is reported as intensity weighted mean ± SD (distribution width).

ID	X_1 (mM)	X_2 (mg/ml)	X_3 (psi)	X_4 (%)	X_5 (mM)	X_6 (min)	X_7 (s)	X_8 (cycle)	EE%	Particle size (nm)	Zeta-potential (mV)
PB-1	30	1.00	250	20	10	60	30	4	17.51 ± 2.06	164.4 ± 3.0 (25.7)	67.27 ± 2.60
PB-2	120	1.00	250	20	20	60	90	6	36.25 ± 1.47	166.5 ± 1.2 (34.9)	66.52 ± 3.98
PB-3	120	5.00	250	34	20	180	30	4	30.65 ± 1.27	163.8 ± 0.6 (35.8)	70.17 ± 2.86
PB-4	120	5.00	250	20	10	180	30	6	31.87 ± 1.08	168.0 ± 0.9 (27.6)	67.97 ± 4.40
PB-5	120	1.00	550	34	20	60	30	4	35.37 ± 2.00	160.5 ± 0.8 (35.6)	68.19 ± 4.16
PB-6	30	5.00	550	34	10	60	30	6	8.22 ± 1.58	164.1 ± 1.2 (21.6)	71.09 ± 7.09
PB-7	30	5.00	550	20	20	180	90	4	8.21 ± 1.29	164.6 ± 1.3 (27.9)	67.93 ± 4.18
PB-8	120	1.00	550	34	10	180	90	6	36.54 ± 1.95	167.0 ± 3.1 (28.4)	76.80 ± 3.86
PB-9	120	5.00	550	20	10	60	90	4	30.52 ± 2.85	175.5 ± 1.1 (30.0)	74.99 ± 2.51
PB-10	30	5.00	250	34	20	60	90	6	8.09 ± 1.61	166.8 ± 1.1 (29.3)	64.18 ± 5.12
PB-11	75	3.00	400	27	15	120	60	5	23.66 ± 1.08	170.8 ± 0.8 (29.8)	64.64 ± 6.21
PB-12	30	1.00	550	20	20	180	30	6	16.64 ± 1.89	172.4 ± 0.7 (24.7)	65.05 ± 4.21
PB-13	30	1.00	250	34	10	180	90	4	16.69 ± 1.70	161.4 ± 1.8 (23.7)	61.43 ± 5.02
PB-14	75	3.00	400	27	15	120	60	5	24.61 ± 1.47	164.8 ± 2.1 (37.9)	66.7 ± 10.40
PB-15	75	3.00	400	27	15	120	60	5	23.51 ± 0.21	161.9 ± 1.8 (28.3)	66.31 ± 5.80

cholesterol concentration (X_3), buffer concentration (X_4), hydration time (X_5), sonication time (X_6), freeze–thaw cycles (X_7), and extrusion pressure (X_8). These factors were assessed to be of high importance (risk) compared with others based on the risk analysis (refer to Part I). The first 8 columns of the Plackett–Burman design table were used for the screening study with each factor evaluated at low (−1) and high (+1) levels (Table 1). The selection of the low and high values was based on the preliminary study results. To evaluate the potential curvature, 3 center points were added. The responses evaluated include drug encapsulation efficiency (*Y*1), particle size (*Y*2), and physical stability (drug leakage and aggregation) (*Y*3). Multi-linear regression and ANOVA were performed to analyze the data, and a series of Pareto charts were constructed to demonstrate the influence of each parameter on the responses.

2.2.8. Central composite design

Based on the screening study results, two variables (lipid concentration and drug concentration) were selected for the optimization study, using response surface method (RSM), and more specifically a central composite design. In this two factor CCD design, four axial points were selected so that the distance, α, from the center of the design to any axial point is $\alpha = 2^{2/4} = 1.414$. As shown in Table 5, each of the two factors was tested at 5 different levels and 4 center points were included. Minitab 15.0 software was used for the design and analysis, and Mathematica 7.0 software was used to plot the various 3D and contour graphs.

3. Results

3.1. Influence of various factors on encapsulation efficiency

Eight high risk factors were identified in a risk analysis study to have potential impact on liposome drug encapsulation efficiency (Xu et al., 2011). As shown in Table 1, encapsulation efficiency varied from 8.09% (PB-10) to 36.54% (PB-8) for the various factor combinations. The most significant factors were lipid concentration and drug concentration ($p < 0.05$) relative to other factors influencing encapsulation efficiency as shown in Table 2 and Fig. 1. In Table 2, the "Effect" column determines each factor's relative strength, the higher the absolute value the greater the effect of that factor on the response. A positive effect value indicates an effect that favors the response, and a negative value represents an inverse relationship between the response and the factor. In this study, the results indicated that both increase in lipid concentration and decrease in the drug concentration would contribute to higher drug encapsulation, and lipid concentration had a more dominant effect. The prediction confidence level of the model was 98.18% and a good correlation was obtained between the observed and predicted values as indicated by the r^2 value of 0.9973. Further analysis using ANOVA indicated a significant effect of variables on the response (EE%) ($p < 0.05$) and no curvature was observed ($p > 0.05$).

3.2. Influence of various factors on liposome particle size

As shown in Table 1, for all the formulations the particle size of the liposomes was around 166 nm with very narrow particle size distribution (PDI < 0.1 for all the samples, data not shown) and no factors showed a significant effect on the liposome particle size as shown in Table 3 and Fig. 2. ANOVA confirmed that the model was not significant and independent factors had no relationship with the response ($p > 0.05$).

3.3. Influence of various factors on liposome stability (aggregation and leakage)

The influence of various factors on liposome stability (expressed as percent leaked per month) was a little more complex than the previous two responses. As shown in Fig. 3 and Table 4, at 37 °C

Table 2
Estimated effects and coefficients for EE% (coded units).

Term	Effect	Coef	Std Err Coef	*T*	*P*
Constant		23.223	0.2089	111.15	0.000
Lipid concentration	20.973	10.487	0.2336	44.89	0.000
Drug concentration	−6.907	−3.453	0.2336	−14.78	0.000
Extrusion pressure	−0.927	−0.463	0.2336	−1.98	0.095
Cholesterol%	−0.907	−0.453	0.2336	−1.94	0.100
Buffer concentration	−1.023	−0.512	0.2336	−2.19	0.071
Hydration time	0.773	0.387	0.2336	1.66	0.149
Sonication time	−0.660	−0.330	0.2336	−1.41	0.207
Freeze–thaw cycles	−0.223	−0.112	0.2336	−0.48	0.650

Table 3
Estimated effects and coefficients for particle size (coded units).

Term	Effect	Coef	Std Err Coef	*T*	*P*
Constant		167.160	1.204	138.79	0.000
Lipid concentration	1.267	0.633	1.347	0.47	0.655
Drug concentration	1.767	0.883	1.347	0.66	0.536
Extrusion pressure	2.200	1.100	1.347	0.82	0.445
Cholesterol%	−4.633	−2.317	1.347	−1.72	0.136
Buffer concentration	−0.967	−0.483	1.347	−0.36	0.732
Hydration time	−0.100	−0.050	1.347	−0.04	0.972
Sonication time	1.433	0.717	1.347	0.53	0.614
Freeze–thaw cycles	2.433	1.217	1.347	0.90	0.401

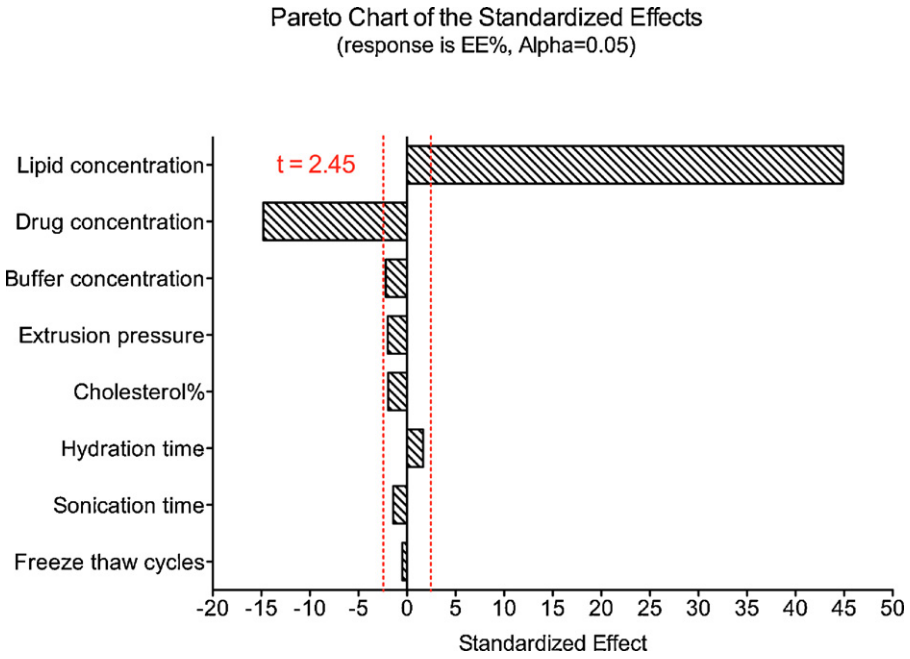

Fig. 1. Pareto chart of the standardized effects of various formulation and process factors on EE%.

after 2 weeks, compared with other factors lipid concentration had the most significant effect ($p < 0.05$). It was noticed that at lower lipid concentration (more dilution), drug leaked faster as compared to at higher lipid concentration. However, after 2 months this effect was nullified due to liposome aggregation (Fig. 4). In terms of temperature effect, more drug contents leaked out at higher temperature than at lower temperature. For example, significant loss of contents were observed for samples stored at 37 °C after 2 months (Fig. 5). As a result of drug leakage, charges initially present on the liposome surfaces decreased to nearly neutral (Fig. 7A). With insignificant electrostatic repulsion forces, liposomes aggregated as evident by increased particle size (Fig. 8A). In comparison, at 4 °C samples remained stable even after 24 months (Figs. 6, 7B, and 8B).

3.4. Central composite design to obtain the response surface for EE%

As shown in Table 5, drug encapsulation varied from 9.17% (lowest lipid concentration and highest drug concentration) to 41.01% (highest lipid concentration and lowest drug concentration) and the rest of the points are distributed evenly across this range. To fully utilize the central composite design and to be able to make accurate prediction for future formulations, three mathematical models were evaluated in order to obtain the highest prediction power. These three models were: a linear model (only main effects); a quadratic model (main effects, interactions, and squared terms); and a linear model with interaction terms. Among these three

Table 4
Estimated effects and coefficients for leakage at 2 weeks (coded units).

Term	Effect	Coef	Std Err Coef	*T*	*P*
Constant		6.610	0.8230	8.03	0.001
Lipid concentration	−6.439	−3.219	0.8566	−3.76	0.020
Drug concentration	−0.658	−0.329	0.8566	−0.38	0.721
Extrusion pressure	−2.062	−1.031	0.8566	−1.20	0.295
Cholesterol%	4.234	2.117	0.8566	2.47	0.069
Buffer concentration	−2.879	−1.439	0.8566	−1.68	0.168
Hydration time	0.723	0.361	0.8566	0.42	0.695
Sonication time	3.690	1.845	0.8566	2.15	0.098
Freeze–thaw cycles	−3.052	−1.526	0.8566	−1.78	0.149

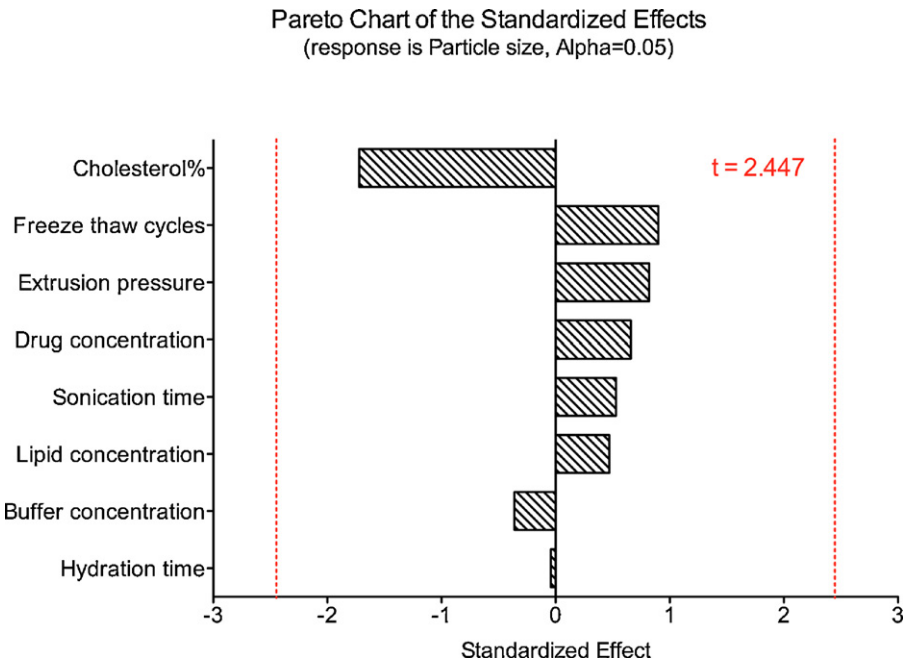

Fig. 2. Pareto chart of the standardized effects of various formulation and process factors on formulation particle size.

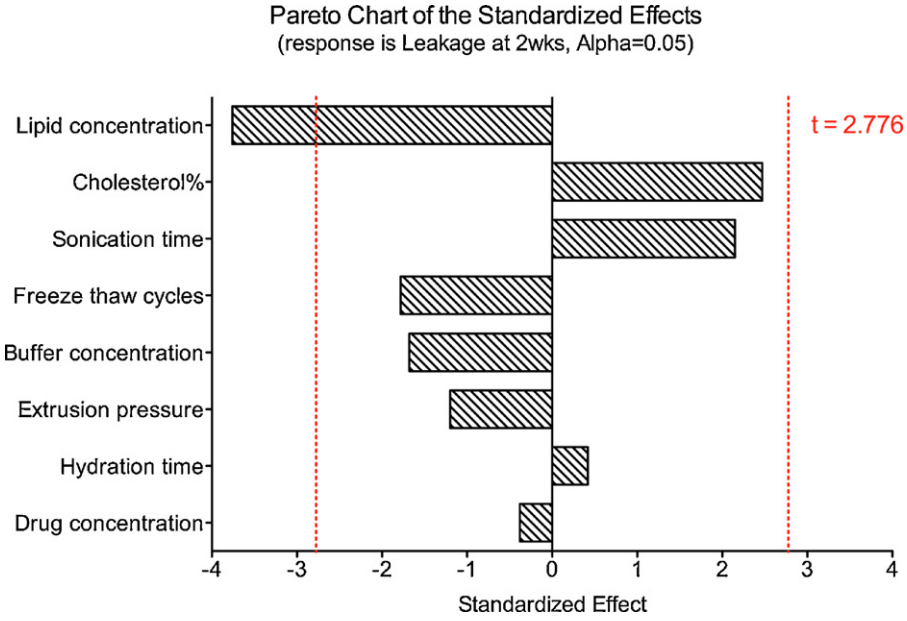

Fig. 3. Pareto chart of the standardized effects of various formulation and process factors on drug leakage from liposomes (2 weeks stored at 37 °C).

models, the first model has significant lack of fit ($p < 0.05$), and the second one over-fits the data (coefficient for the interaction term has a p-value > 0.05). The third one provides the most appropriate fit ($r^2 = 99.57$, $r^2_{\text{prediction}} = 98.48\%$) as shown in Tables 6–7, and Fig. 9. The final equation describing the response surface is: EE% = 11.09 + 0.36 × lipid conc − 3.69 × drug conc − 7.79E−4 × lipid conc^2 + 0.25 × drug conc^2.

3.5. Establishment and evaluation of the design space

Key parameters that had been demonstrated to affect formulation quality were used to construct the design space as shown in Fig. 10. All other parameters that had no impact on formulation performance are listed in Table 8 to illustrate the range of each

Table 5
Design table of the central composite design and results for EE% (mean ± SD).

ID	Type	Lipid conc (mM)	Drug conc (mg/ml)	EE%
CCD-1	Fact	140.96	1.60	41.01 ± 1.68
CCD-2	Center	95	4.25	27.21 ± 1.91
CCD-3	Fact	49.04	1.60	21.73 ± 1.77
CCD-4	Fact	49.04	6.90	14.37 ± 2.13
CCD-5	Axial	95	8.00	24.31 ± 1.13
CCD-6	Center	95	4.25	27.11 ± 1.62
CCD-7	Fact	140.96	6.90	32.03 ± 1.35
CCD-8	Axial	30	4.25	9.17 ± 1.41
CCD-9	Axial	160	4.25	36.80 ± 1.43
CCD-10	Center	95	4.25	25.85 ± 1.41
CCD-11	Axial	95	0.50	35.45 ± 1.93
CCD-12	Center	95	4.25	26.65 ± 1.98

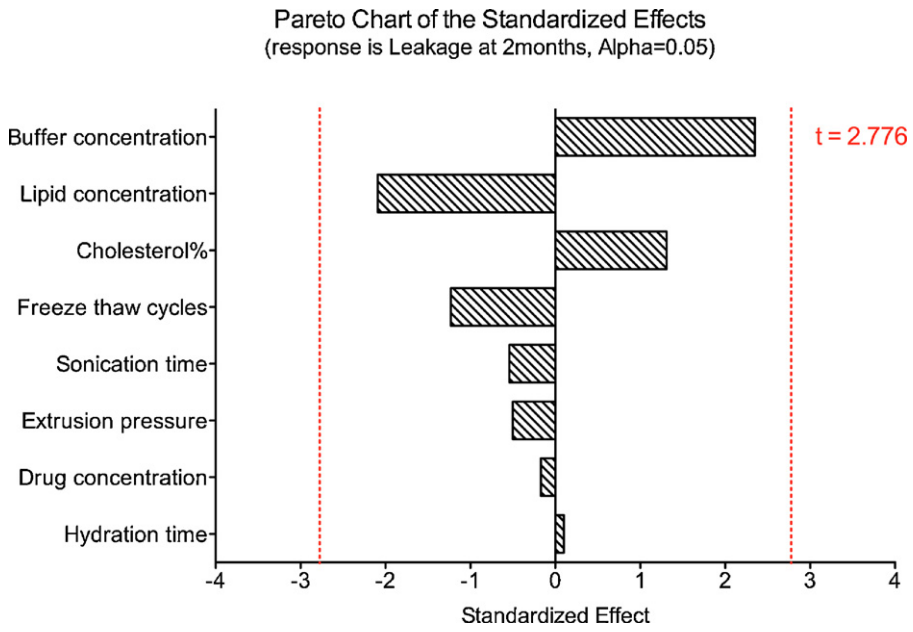

Fig. 4. Pareto chart of the standardized effects of various formulation and process factors on drug leakage from liposomes (2 months stored at 37 °C).

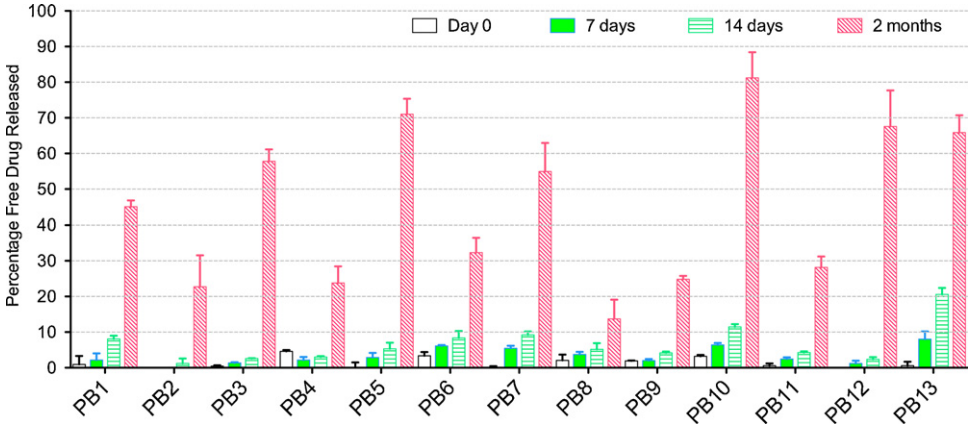

Fig. 5. Drug leakage from various formulations during 2 months storage at 37 °C ($n = 3$).

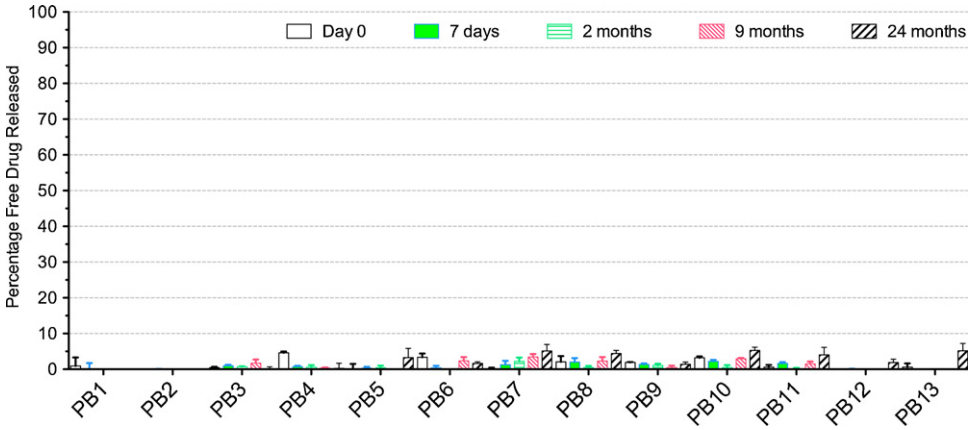

Fig. 6. Drug leakage from various formulations during 24 months storage at 4 °C ($n = 3$).

Table 6
Estimated regression coefficients for EE% (linear + squared terms).

Term	Coefficient (coded)	SE Coef (coded)	Coefficient (uncoded)	*T*	*P*
Constant	26.705	0.3720	11.0936	71.783	0.000
Lipid conc	9.502	0.2631	0.35487	36.120	0.000
Drug conc	−4.012	0.2631	−3.69335	−15.251	0.000
Lipid conc × lipid conc	−1.647	0.2941	−7.79E−4	−5.600	0.001
Drug conc × drug conc	1.801	0.2941	0.25641	6.122	0.000

Table 7
Analysis of variance for EE% (linear + squared term).

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	4	898.640	898.640	224.660	405.81	0.000
Linear	2	851.036	851.036	425.518	768.63	0.000
Square	2	47.604	47.604	23.802	42.99	0.000
Residual error	7	3.875	3.875	0.554		
Lack-of-fit	4	2.722	2.722	0.681	1.77	0.333
Pure error	3	1.153	1.153	0.384		
Total	11	902.515				

Table 8
Range of the variables that can guarantee a successful prediction using the model developed.

Factor	Type	Lower limit	Higher limit	Unit
Cholesterol %	Formulation	20	34	%
Buffer concentration	Formulation	10	20	mM
Hydration time	Process	60	180	min
Sonication time	Process	30	90	s
Freeze–thaw cycles	Process	4	6	cycles
Extrusion pressure	Process	250	550	psi
Lipid concentration	Formulation	30	160	mM
Drug concentration	Formulation	0.5	8	mg/ml

variable. As long as each variable is maintained within its range, the drug encapsulation efficiency can then be successfully predicted and controlled.

4. Discussion

A screening experimental design minimizes the number of experiments required to identify the most critical factors affecting the response. To best use the screening design, a careful examination of all the potential high impact factors is of vital importance. For this reason, in the first part of the case study a risk analysis was performed (Xu et al., 2011), and eight factors were identified

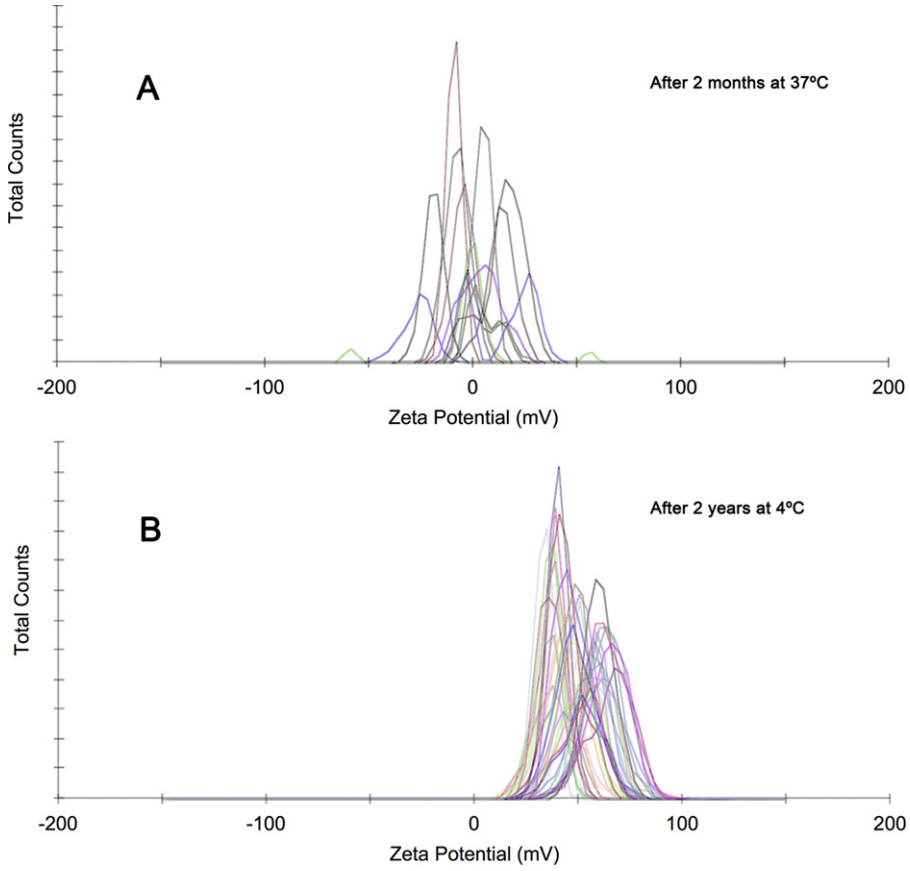

Fig. 7. Zeta-potential of various formulations stored at (A) 37 °C after 2 months and (B) at 4 °C after 24 months.

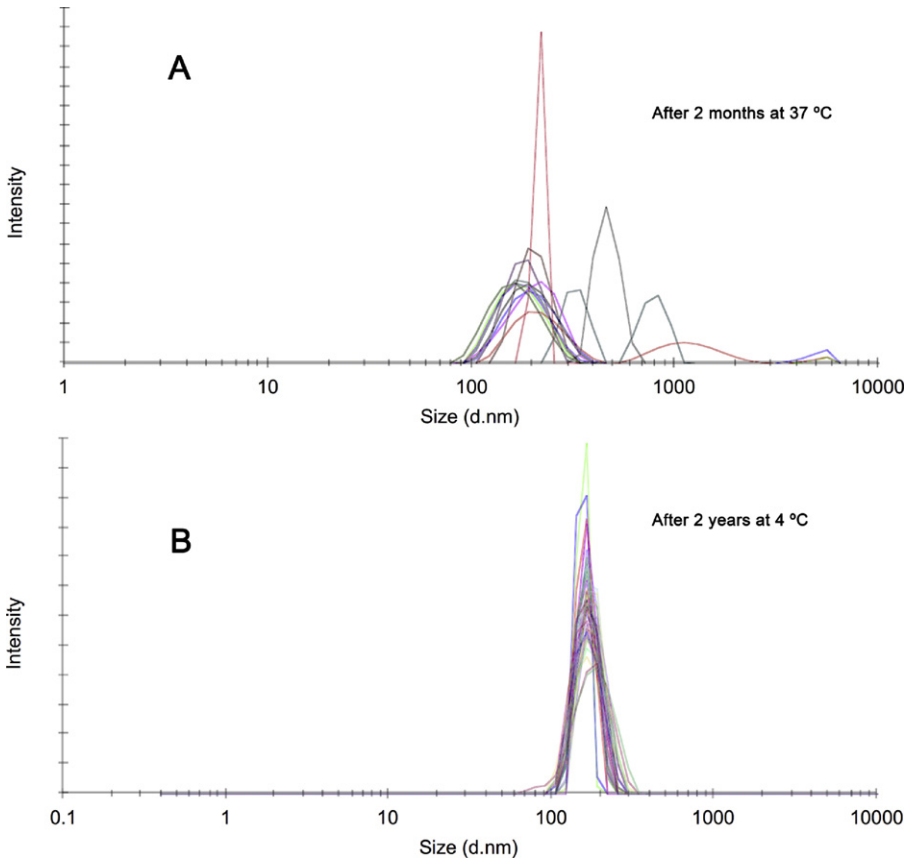

Fig. 8. Particle size distribution of various formulations stored at (A) 37 °C after 2 months and (B) at 4 °C after 24 months.

as potential high impact factors. These include lipid concentration, drug concentration, cholesterol concentration (percentage), buffer concentration, hydration time, sonication time, freeze–thaw cycles, and extrusion pressure. The screening design used in the current study was a Plackett–Burman design. It is a two level factorial design that involves a large number of factors and relatively few runs (12 runs for up to 11 variables). Being a Resolution-3 design, Plackett–Burman can estimate the significance of the main effects

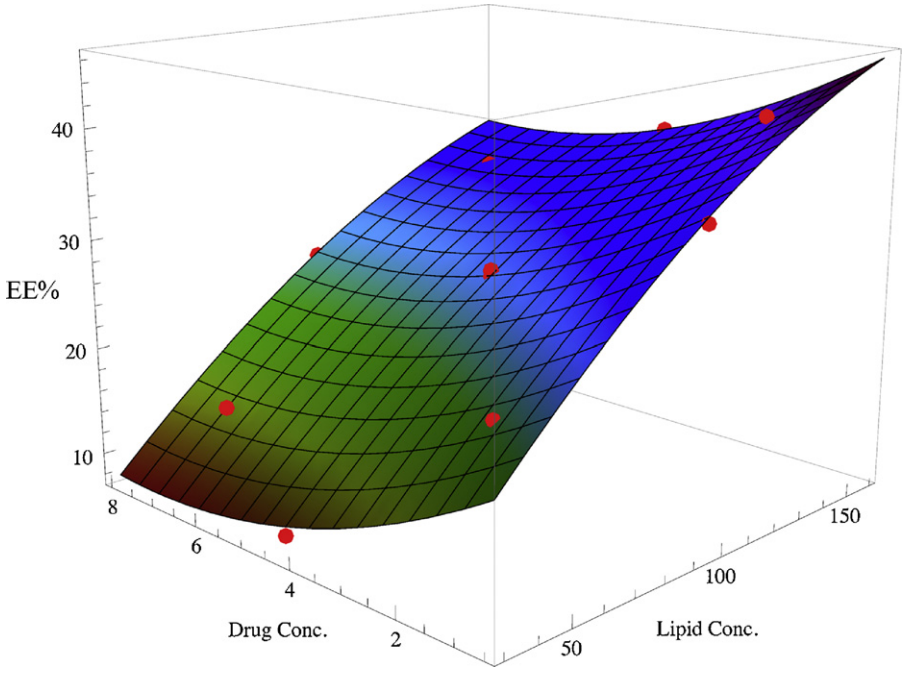

Fig. 9. Response surface for predicting EE% (red points are experimental data). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

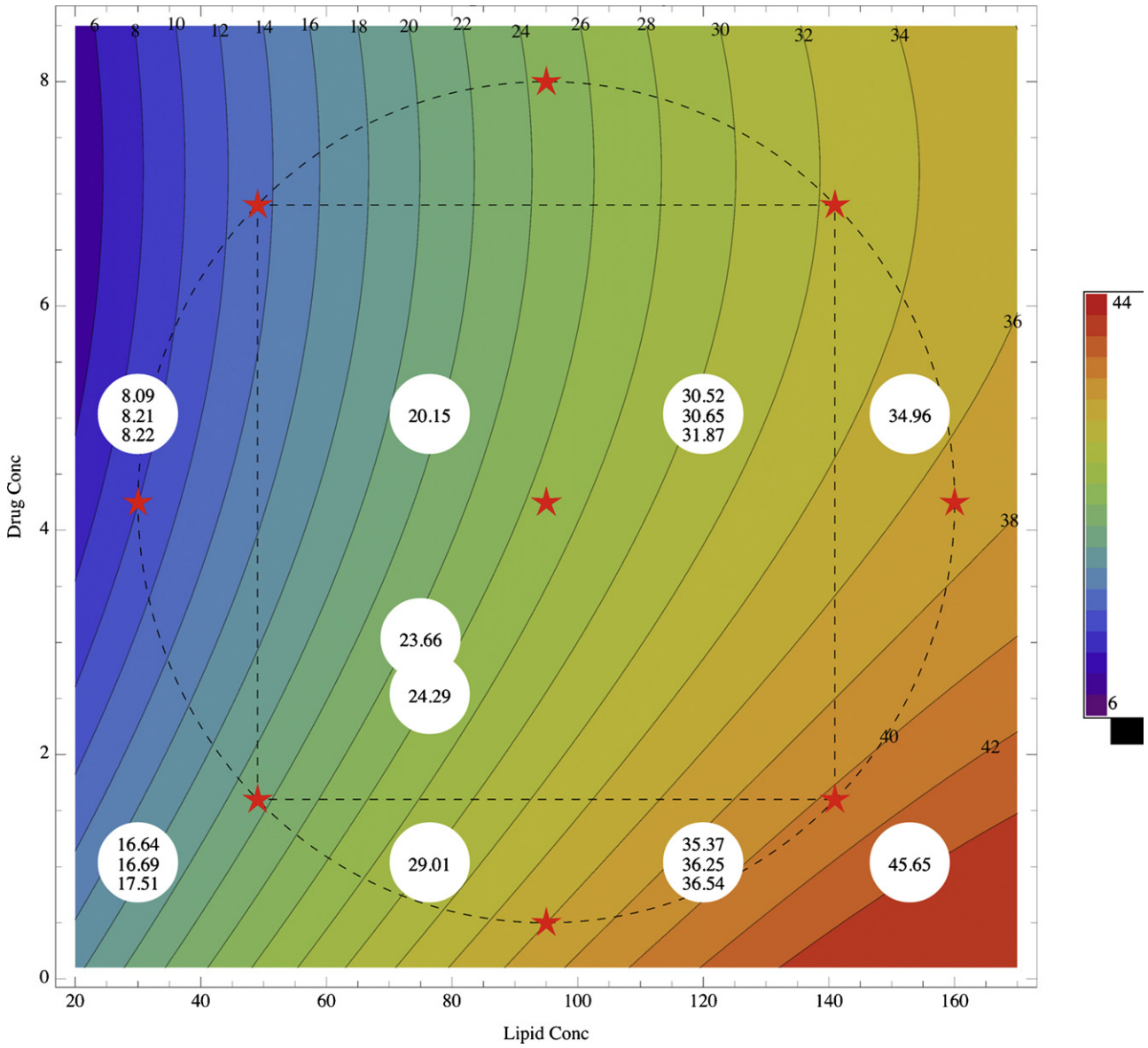

Fig. 10. Contour plot for EE% with respect to lipid concentration and drug concentration. Red stars are central composite design points. Within the circle (red stars) is the design space for prediction of EE%. Additional data points (white disks) are included to evaluate the accuracy and robustness of the design space. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(Deng and Tang, 1999) with very high efficiency and accuracy, but it cannot separate the main effects from the possible interactions. However, as the goal of this design is to quickly reduce the high risk factors to be studied in the next step, such a design is sufficient. As shown in Table 2, out of eight factors only two are statistically significant, namely the lipid concentration and drug concentration. Of the two factors, lipid concentration has a positive effect on drug encapsulation. This can be attributed to the larger population of vesicles in the system and consequently larger internal volume for drug encapsulation. On the other hand, increase in the drug concentration leads to a decrease in drug encapsulation efficiency, which maybe due to the charge–charge interactions between lipid and drug. At low drug concentration, the percentage of surface bound drug is much higher than that at high drug concentration, under the assumption that liposome surface area remains the same. One surprising finding was that all the other six factors (including 4 process factors) had no effect on drug encapsulation efficiency. One possible explanation is that the impact of different processes on drug encapsulation is mostly reflected by their effect on liposome particle size and size distribution. However, during the last preparation step, *i.e.* extrusion, heterogeneity of the system is significantly reduced; no effect from other process parameters can be observed. Particle size analysis data supports this speculation. As shown in Table 1, the mean particle size of all the formulations was 166.6 ± 4.4 nm, and statistical analysis reveals no effect from various formulation and process factors (Fig. 2).

In terms of the storage stability, at low temperature the prepared Tenofovir liposomes remained stable (no leakage, no particle size change, and no zeta-potential change) at 4 °C for at least 24 months. In comparison, at elevated temperature (37 °C), a substantial amount of the drug leaked out after just 2 months storage (Fig. 5) accompanied by near neutral surface charge (Fig. 7A) and larger aggregated particles (Fig. 8A). It is believed that the cause of the instability (drug leakage) is the high lipid molecular mobility at higher temperatures (close to the phase transition temperature, T_m, of the lipid, *e.g.* 55 °C for DSPC). The first consequence of the enhanced lipid mobility is increased lipid bilayer permeability (Magin and Niesman, 1984), which may lead to higher drug

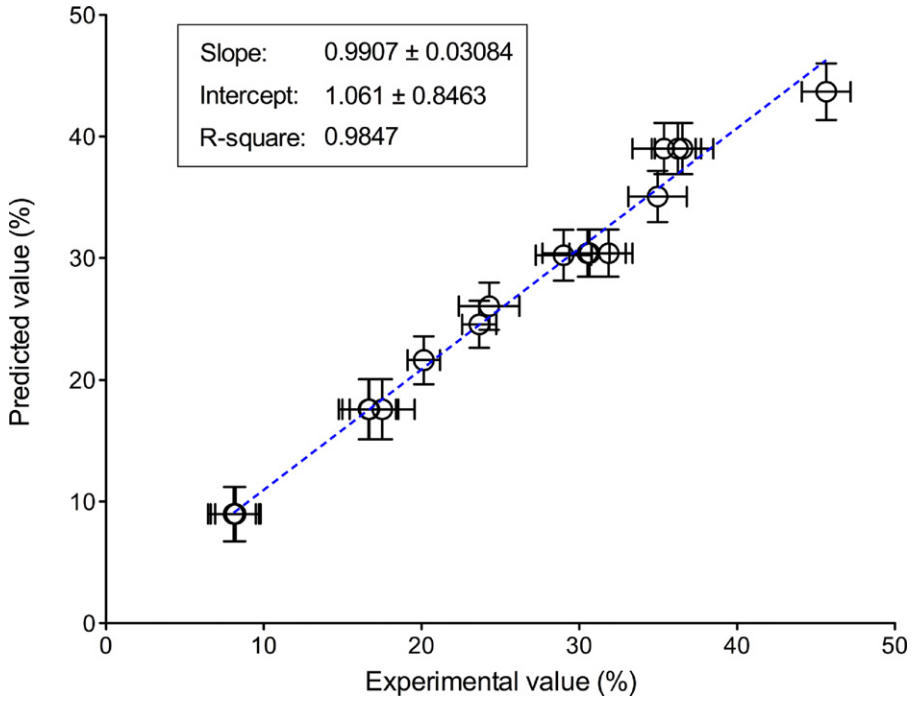

Fig. 11. Comparison of the predicted and experimental values for additional data points inside and outside the design space.

partitioning and faster diffusion, and hence more rapid drug leakage. Two other controlling factors of drug leakage are the partition coefficient of the API (Bemporad et al., 2004; Orsi, 2010) and the concentration gradient across the lipid bilayer. The higher the partition coefficient of the hydrophilic API (for Tenofovir Log $P = -1.7$) and the more dilute the sample, the faster the rate of drug leakage.

A secondary effect of the increased lipid mobility is accelerated collision and hence coalescence rates. For charged lipids, this translates to a much faster dissipation of the liposome surface charge that is necessary for electrostatic stabilization. Consequently, particles aggregate and lipid fusion occurs leading to more drug leakage. To overcome drug leakage issues, higher phase transition temperature lipids should be used, and if possible charged lipids should also be added into the formulation to create electrostatic stabilization. Most importantly, samples should be stored at least 30 °C below the T_m of the lipid. However, liposome samples should not be stored at freezing temperature as this would cause breakage and therefore lipids with phase transition temperatures lower than approximately 32 °C should be stored at 2–8 °C.

On another note, an optimally designed liposome formulation should have an appropriate *in vivo* release profile. Over-stabilizing the formulation just to achieve high storage stability (>2 year shelf life) is not recommended as it may actually decrease the therapeutic efficacy of the delivery system. From this perspective, the increased lipid permeability observed at elevated temperatures may indeed be beneficial to the *in vivo* drug release.

Following the screening study, to optimize the drug encapsulation efficiency the two most significant factors (lipid concentration and drug concentration) were evaluated using a response surface method. In contrast to the screening design, where the generated model is only sufficient for qualitative determination of the main effects, the response surface design would allow generation of a more predictive model. The accuracy of the response surface design is much higher than even a full factorial design in terms of prediction variance (Atkinson et al., 2007). As shown in Fig. 9, the response surface is curved with respect to both factors. At any given lipid concentration, an increase in drug concentration leads to a decrease in EE%. This effect is more predominant at lower drug concentrations than at higher concentrations, as eventually EE% no longer decreases. As mentioned earlier, it is believed that the drug–lipid interaction is the reason behind this effect.

In cases where there is no drug–lipid interaction, the encapsulation efficiency is dependent on the internal to external volume ratio of the liposomes for any drug concentration, and a higher internal-to-external volume ratio results in higher drug encapsulation. However, due to the drug–lipid interactions, a small portion of the free drug associates with the liposome surfaces, causing a small increase in 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 surfaces. At very high drug concentrations, the surface attached drug percentage becomes negligible; hence any additional increase in the drug concentration would not make any appreciable difference in drug encapsulation.

The effect of lipid concentration on drug encapsulation showed a different type of curvature. At relatively low lipid concentrations (<100 mM), an increase in the lipid concentration causes a proportional increase in the encapsulation efficiency. But as the lipid concentration continues to increase, a plateau is eventually reached. The non-linear curvature exhibited at higher lipid concentrations is believed to be a result of increased sample viscosity. As the viscosity increases, processing (extrusion) becomes more and more difficult and a small portion of the lipid is retained on the extruder membrane, causing a decrease in lipid concentration. For this reason, higher than 160 mM lipid concentration is not recommended, as it can no longer provide increase in drug encapsulation.

Fig. 10 illustrates the countour plot for prediction of drug encapsulation efficiency. Every single point corresponds to a combination of lipid concentration and drug concentration. The space inside the dashed circle is the testing domain of the central composite design, and the imbedded square is the full factorial design domain. Prediction variance is at a minimum inside the central composite design domain, and as the data point moves outward the prediction variance increases (lower prediction confidence). As shown in Fig. 10,

drug encapsulation efficiency can be accuratly predicted using the contour line ($r^2_{pred} = 98.48\%$). To test the accuracy and robustness of the developed model outside the testing domain (extrapolation), some additional data points were added (white disks). As shown in Fig. 10, some of these points were inside the testing domain and some were outside. As can be observed, all the points were very close to their predicted values even for those data points outside the testing domain (values inside white disks represent experimental values) and the model was robust and accurate (Fig. 11). Due to its high prediction accuracy, the countour plot that is obtained here for drug encapsulation also serves as the design space for predicting and controlling drug encapsulation efficiency.

In this study, attention was mainly focused on illustrating the principles of design space at laboratory setting by focusing on design space for drug encapsulation efficiency since this is one of the most important as well as the most difficult properties to predict and control for liposomes containing hydrophilic drugs. In addition, the pre-set formulation and process ranges limited the investigation of the other properties (particle size, and stability). Even though a quantitative relationship between these two properties and the variables investigated was not obtained due to these study design limitations, it was still possible to control these two variables to achieve optimal formulation and processing conditions. Two examples follow: (1) liposome particle size can be precisely controlled by the pore size of the extrusion membrane and has been demonstrated to be not affected by any of the other variables investigated; and (2) liposome aggregation and drug leakage issues can be avoided by storing the samples at low temperature (4 °C) as mentioned earlier.

5. Conclusions

Quality by design principles were used in the current case study to improve the fundamental understanding of the liposome preparation process as well as to assist in the identification of critical formulation and process parameters that affect liposome drug product quality. The Plackett–Burman and central composite statistical designs were shown to be very beneficial in these experiments as highly predictive models were obtained from small numbers of experiments. Using these models, formulation scientists can obtain a design space for liposome preparation, within which preparation variability is minimized, and product quality can be assured. The studies conducted here were based on a specific API, however the formulation and process design for liposomes containing other hydrophilic APIs is very similar. In addition, the *in vivo* distribution of these liposomes is dependent on the liposome properties rather than the drug properties. Accordingly, the information obtained in this QbD case study will be useful for the development of liposomes containing other hydrophilic APIs. This design approach will have significant benefit to the industry in terms of time and cost saving and may reduce the regulatory burden and hence promote the use of liposome formulations for innovator as well as generic companies. Lastly, with regard to sample storage stability, through an understanding of the formulation properties (*e.g.* Log *P*, and T_m) as well as control of the environmental conditions (*e.g.* temperature), liposomes containing hydrophilic APIs can be prepared which remain stable in solution state for at least 2 years. This may eliminate the need to lyophilize the product, which not only complicates the process and increases production cost but also imposes reconstitution stability issues.

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