International Journal of Pharmaceutics 434 (2012) 349–359

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Application of quality by design to formulation and processing of protein liposomes

Xiaoming Xu[a,1], Antonio P. Costa[a,2], Mansoor A. Khan[b,3], Diane J. Burgess[a,*]

[a] Department of Pharmaceutical Sciences, University of Connecticut, 69 N Eagleville Rd U3092, Storrs, CT 06269, United States
[b] Division of Product Quality Research, Center for Drug Evaluation and Research, Food and Drug Administration, 10903 New Hampshire Ave, Silver Spring, MD 20993, United States

ARTICLE INFO

Article history:
Received 24 February 2012
Received in revised form 1 June 2012
Accepted 1 June 2012
Available online 7 June 2012

Keywords:
Liposome
QbD
Superoxide dismutase
Freeze-and-thaw unilamellar vesicles (FAT-ULV)
Encapsulation efficiency
D-optimal design
Design space
Risk analysis

ABSTRACT

Quality by design (QbD) principles were explored in the current study to gain a comprehensive understanding of the preparation of superoxide dismutase (SOD) containing liposome formulations prepared using freeze-and-thaw unilamellar vesicles (FAT-ULV). Risk analysis and D-optimal statistical design were performed. Of all the variables investigated, lipid concentration, cholesterol mol%, main lipid type and protein concentration were identified as critical parameters affecting SOD encapsulation efficiency, while the main lipid type was the only factor influencing liposome particle size. Using a model generated by the D-optimal design, a series of three-dimensional response spaces for SOD liposome encapsulation efficiency were established. The maximum values observed in the response surfaces indirectly confirmed the existence of a specific SOD–lipid interaction, which took place in the lipid bilayer under the following optimal conditions: (1) appropriate membrane thickness and curvature (DPPC liposomes); and (2) optimal "pocket size" generated by cholesterol content. With respect to storage stability, the prepared SOD liposomes remained stable for at least 6 months in aqueous dispersion state at 4 °C. This research highlights the level of understanding that can be accomplished through a well-designed study based on the philosophy of QbD.

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1. Introduction

Owing to their unique biological and physico-chemical properties, liposomes are established yet still very promising drug delivery system (Xu and Burgess, 2011). In several areas, including small molecule anti-cancer and anti-fungal therapy (Lasic et al., 1992; Maurer et al., 2001), liposome formulations have been proven extremely effective. In other areas, they show great promise, such as in gene therapy (Web-source, 2011), vaccination (Gregoriadis, 1995; Zhuang et al., 2012), and protein therapeutics (Torchilin and Lukyanov, 2003). In particular, liposomal protein therapeutics have generated great interest. From a clinical point of view, the potential ability of liposomes to deliver protein/enzyme directly into the cytoplasm or lysosomes of live cells is of crucial importance for the treatment of inherited diseases caused by the abnormal functioning of some intracellular enzymes and cancer (Torchilin, 2005). However, from a manufacturing perspective, the extremely low protein encapsulation efficiency has been limiting the broad use of liposome delivery systems, especially in the predominantly used small vesicle size range (50–150 nm). In addition, poor protein stability during preparation elicits concern over the use of harsh processing conditions and/or organic solvents. Furthermore, 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 improve the protein encapsulation efficiency and protein stability as well as understand the sources of variability in order to improve product quality.

Superoxide dismutase (SOD) was used as the model protein in this study. It is one of the most potent antioxidants known in nature. SOD catalyzes the dismutation of the superoxide radical into hydrogen peroxide and oxygen and it has been used for the treatment of oxidative stress diseases such as rheumatoid arthritis, cancer, and respiratory distress syndrome. While SOD has demonstrated great potential as an alternative to conventional therapies (Keele et al., 1971; McCord and Fridovich, 1969; Okado-Matsumoto and Fridovich, 2001; Zhang et al., 2002), its current use is limited by

* Corresponding author. Tel.: +1 860 486 3760; fax: +1 860 486 0538.
E-mail addresses: xiaoming.xu@fda.hhs.gov (X. Xu), antonio.costa@uconn.edu (A.P. Costa), mansoor.khan@fda.hhs.gov (M.A. Khan), d.burgess@uconn.edu, diane.burgess@uconn.edu (D.J. Burgess).
[1] Present address: FDA/CDER/DPQR, 10903 New Hampshire Avenue, Silver Spring, MD 20993, United States. Tel.: +1 301 796 9646.
[2] Tel: +1 860 486 0640.
[3] Tel: +1 301 796 0016.

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http://dx.doi.org/10.1016/j.ijpharm.2012.06.002

several key drawbacks, such as its extremely short circulation time, non-specific tissue distribution, and inability to penetrate through the cellular membrane to the intracellular targets. Accordingly, a liposomal SOD formulation is expected to provide a better therapeutic index due to carrier-facilitated intracellular transportation as well as the targeting effect.

Previously (Xu et al., 2012a), an improved freeze-and-thaw cycling technique was reported where the protein containing liposome preparation process was separated into two steps: the generation of unilamellar vesicles, and freeze–thaw cycling to encapsulate protein. Because the liposomes obtained using this approach remained as unilamellar vesicles and no significant change in particle size was observed, they are referred to as freeze-and-thaw unilamellar vesicles (FAT-ULV). Compared with traditional preparation methods, the FAT-ULV method is very effective in improving protein encapsulation efficiency (up to 50%). However, this process is relatively new. Hence it is very crucial to use the QbD approach to help understand the formulation and processing design space.

Pharmaceutical QbD emphasizes that the product quality should be built (designed) into the product rather than tested (Yu, 2008). This requires that quality-improving scientific methods be used upstream in the beginning stages of the research, development and design phases (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, 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 usually involves the following five stages: (1) define target product quality profile 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 (risk assessment), (4) use a design of experiment (DOE) approach to screen and obtain variable response surfaces in order to establish the product design space (the range of process and/or formulation parameters that have been demonstrated to provide assurance of quality), and (5) 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 (ICH Q8).

The current study focused on the first four stages of QbD implementation in a laboratory setting. Briefly, the desired product quality profiles were defined and risk assessment was conducted to identify potential high risk factors. Subsequently, a D-optimal experimental design was used to screen high-risk variables and to obtain the variable response surfaces (Bodea and Leucuta, 1997; El-Hagrasy et al., 2006). The optimal criterion for D-optimal design is that the determinant of the $\mathbf{X}'\mathbf{X}$ matrix is maximized, where $\mathbf{X}$ is the design matrix (Atkinson et al., 2007). Compared with standard designs (*e.g.* factorial designs), the D-optimal design gives the most precise estimate of the factor effects; however, it requires statistical software to calculate the determinant of the $\mathbf{X}'\mathbf{X}$ matrix. For this reason, JMP software (SAS Institute) was used to create the design. 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

Superoxide dismutase (bovine erythrocytes, 4054 units/mg solid), HEPES sodium salts, stearylamine (SA) and Triton X-100 were purchased from Sigma Aldrich (St. Louis, MO). 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Chloroform, acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Amicon Ultra-0.5 and Ultra-15 centrifugal filter units (50, and 100 kDa) were purchased from Millipore (Billerica, MA). Nanopure™ quality water (Barnstead, Dubuque, IA) was used for all studies.

2.2. Experimental methods

2.2.1. Preparation of empty unilamellar liposomes

Empty unilamellar liposomes were prepared using a film hydration method (Xu et al., 2011). Briefly, the desired amount of lipids was 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 maintained under vacuum overnight to completely remove any residual solvent. The dry lipids were then hydrated with 10 mM pH 7.4 HEPES buffer at 65 °C for 2 h, and this process was followed by four freeze–thaw cycles (10 min at −196 °C and 10 min at 65 °C). Finally, the samples were put into a LIPEX™ extruder (Northern Lipids Inc., Canada) and passed through a stack of polycarbonate membranes (200 nm pore size) to obtain empty unilamellar liposomes with the desired particle size (Z-Ave was approx.150 nm and PDI < 0.1).

2.2.2. Encapsulation of SOD into freeze-and-thaw unilamellar vesicles (FAT-ULV)

Encapsulation of SOD into the empty unilamellar liposomes was achieved using a previously developed freeze–thaw cycling technique (Xu et al., 2012a). Briefly, the desired amount of protein solution was mixed with preformed empty unilamellar liposomes. The mixture was subjected to two to three freeze–thaw cycles (5 min at −196 °C and 5 min at 65 °C), which caused the lipid bilayer to break upon cooling and reform upon heating. After freeze–thaw cycling, samples were extruded using a 200 nm filter (LIPEX extruder) to obtain mono-dispersed liposome samples.

2.2.3. Determination of encapsulation efficiency (EE%)

20 μL of prepared liposomes were withdrawn and diluted with 1 ml 10 mM pH 7.4 HEPES buffer (working-dispersion). To assess the total protein concentration (C_{Total}), 500 μL of working-dispersion was mixed with 100 μL of 6% (v/v) Triton X-100 and maintained at 65 °C for 5 min to disrupt all the vesicles. To assess the concentration of encapsulated protein (C_{Encap}), 400 μL of working-dispersion was transferred into an Ultra-0.5 centrifugal device (100 kDa MWCO) and centrifuged at 14,000 rpm for 10 min (Eppendorf MiniSpin Plus Microcentrifuge). After the first centrifugation, another 400 μL of fresh HEPES buffer was added on top of the filter and the centrifugation process was repeated (14,000 rpm for 10 min). The final retenate (~20 μL) was transferred to a test tube together with 200 μL of rinse solution (used to clean the filter) as well as 100 μL of 6% (v/v) Triton X-100. The mixture was then maintained at 65 °C for 5 min to disrupt all the vesicles. Note that the volume of these solutions was determined using a Mettler Toledo XS205 balance (assuming a density of 0.997 mg/μL at 25 °C). Both C_{Encap} and C_{Total} were assessed after encapsulation using high-performance liquid chromatography (HPLC). The encapsulation efficiency was calculated as:

$$EE\% = \frac{C_{Encap}}{C_{Total}} \times 100\% \quad (1)$$

Table 1
D-optimal design table and results. X_1 = lipid concentration; X_2 = DPPC% in the main lipid component; X_3 = cholesterol%; X_4 = SOD concentration; and X_5 = freeze–thaw cycles. Particle size is reported as Z-ave mean ± distribution width.

ID	X_1 (mM)	X_2 (mol%)	X_3 (mol%)	X_4 (mg/ml)	X_5 (cycle)	EE%	Particle size (nm)	Zeta-potential (mV)
DOE-1	30	0	20	1	3	13.3 ± 0.8	156.57 ± 35.61	53.30 ± 9.78
DOE-2	30	0	36	1	2	21.3 ± 0.5	145.60 ± 21.29	48.43 ± 8.94
DOE-3	30	25	32	3	3	14.9 ± 0.8	155.47 ± 41.72	51.07 ± 6.29
DOE-4	30	75	24	3	3	12.4 ± 0.3	140.00 ± 43.50	48.83 ± 6.69
DOE-5	30	100	20	1	2	17.1 ± 0.7	134.07 ± 19.50	57.70 ± 9.54
DOE-6	30	100	36	1	3	34.0 ± 1.2	135.57 ± 25.40	45.47 ± 9.99
DOE-7	70	0	36	3	3	34.1 ± 1.2	151.13 ± 25.33	43.50 ± 8.77
DOE-8	70	25	24	1	3	18.1 ± 0.5	145.37 ± 29.79	53.30 ± 6.73
DOE-9	70	75	32	1	2	41.8 ± 0.7	143.20 ± 12.20	44.67 ± 9.97
DOE-10	70	100	20	3	3	20.4 ± 0.3	138.80 ± 21.13	54.97 ± 9.59
DOE-11	110	0	20	3	2	9.1 ± 1.2	157.27 ± 37.63	54.53 ± 7.66
DOE-12	110	0	32	1	3	41.7 ± 1.5	154.63 ± 28.36	47.97 ± 8.84
DOE-13	110	25	36	1	2	37.6 ± 0.7	153.77 ± 27.67	43.10 ± 8.15
DOE-14	110	75	20	1	3	23.7 ± 2.5	139.20 ± 24.32	53.03 ± 8.38
DOE-15	110	100	24	1	2	48.3 ± 1.5	137.47 ± 23.31	55.27 ± 9.14
DOE-16	110	100	36	3	3	44.3 ± 1.0	143.30 ± 40.16	46.33 ± 9.11

2.2.4. Purification of SOD liposomes

Prepared liposomes were purified with an Amicon Ultra-15® centrifugal filtration device (Millipore, Billerica, MA) 100 kDa MWCO. Briefly, 1.5 ml liposome suspension was added to the upper chamber of the ultrafiltration tube and diluted with 13.5 ml of HEPES buffer, which was followed by centrifugation to approximately 3 ml at 4000 × *g* (16 °C) using a Beckman Coulter Allegra® X-15R centrifuge. This resulted in approximately a 5 times concentrating effect, or approximately 80% of free protein removal. Fresh buffer was then added to the upper chamber of the ultrafiltration tube to dilute the partially purified liposomes to 15 ml, and centrifuged to about 3 ml for the second time. In order to remove ~99% of the free protein, at least three passes were required. After the last centrifugation, the purified liposome suspension was collected from the upper chamber and diluted to the desired concentration before storing at 4 °C.

2.2.5. Chromatographic equipment and conditions

The HPLC system consisted of a Flexar System (Perkin Elmer Inc., US) equipped with a quaternary pump, a peltier autosampler (maintained at 4 °C), a UV/vis detector, and a Chromera 3.1 chromatography data system. The analytical column was a Symmetry300 C18 column (3.5 μm, 4.6 mm × 75 mm, Waters Corporation, USA) protected with a Symmetry300 C18 guard column (3.5 μm, 2.1 mm × 10 mm, Waters Corporation, USA). The mobile phase A consisted of 100% DI water with 0.1% (v/v) trifluoroacetic acid (TFA). The mobile phase B consisted of 100% acetonitrile with 0.1% (v/v) TFA. The flow rate was 1 ml/min, the injection volume was 50 μL, and the detection wavelength was 220 nm. Prior to each injection, the column was equilibrated at 20% B for 12 min. The elution gradient was as follows: (1) 3 min linear gradient from 20% B to 36% B, (2) 7 min linear gradient to 43% B, (3) 3 min linear gradient to 95% B, and (4) 3 min isocratic at 95% B to elute out all the content.

2.2.6. Particle size and zeta-potential analysis

Particle size and zeta-potential analysis were conducted using a Malvern ZS90 zeta-sizer. Prepared liposome formulations were diluted at least 50 times to obtain a suspension that was approximately 0.5 mg/ml. All measurements were conducted at 25 °C in triplicate, and were reported as mean ± SD (Z-Ave ± distribution width for particle size)

2.2.7. Risk analysis

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

2.2.8. D-optimal design

D-optimal design gives the most precise estimate of the factor effects and reduces the number of runs compared to standard factorial design, but it requires statistical software to compute the design. In the current study, JMP software (SAS Institute) was used to create the 16-run custom design. Note that all formulations contained three lipid components: main lipid (DPPC, DSPC, or a mixture of two), cholesterol, and stearylamine (charged lipid). The percentage of charged lipid was fixed in all formulations at 10 mol% and the main lipid component percentage was varied according to the percentage of cholesterol. With respect to the design, two responses were evaluated, namely the SOD encapsulation efficiency and SOD liposome particle size. Based on the risk analysis results, five key variables (main effects) were identified: total lipid concentration (X_1), DPPC% in the main lipid component (X_2), cholesterol percentage (X_3), SOD concentration (X_4), and freeze–thaw cycling (X_5). Three two-way interactions of the main effects that may be significant were also included: X_1X_2, X_1X_3, and X_2X_3. Moreover, three second-order terms of lipid concentration, DPPC%, and cholesterol% were also included (X_1^2, X_2^2, X_3^2), where curvatures were observed in a previous study (Xu et al., 2012a). These eleven terms together with the coefficient of the intercept were deemed as "necessary" terms in JMP software and all the other terms (including three third-order terms X_1^3, X_2^3, X_3^3) were set as "if possible". Overall, this design is not saturated (estimation of 12 necessary terms using 16 runs). The end result of the JMP generated design is a *priori* model based on the main effects, quadratic terms, cubic terms, and interactions that can be used to form a response surface (Table 1):

$$y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_5X_5 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 \quad (2)$$

2.2.9. SOD liposome storage stability

To test the stability of SOD liposomes (in terms of protein leakage, particle size and zeta-potential change), one representative formulation was selected (purified DPPC:cholesterol:SA = 6:3:1

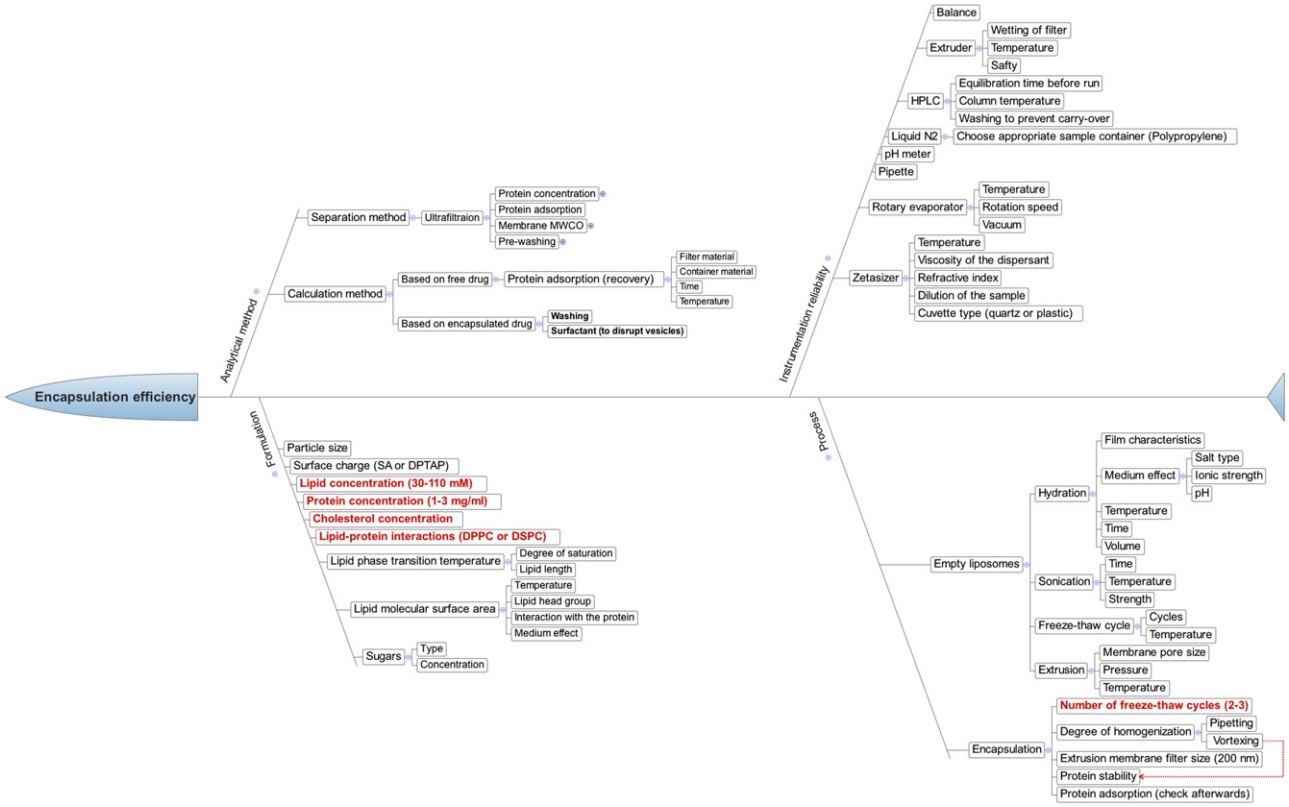

Fig. 1. An Ishikawa diagram illustrating factors that may have impact on the encapsulation efficiency of SOD liposomes.

liposome containing 283.7 ± 18.32 μg/ml SOD, particle size and distribution width = 130.20 ± 15.77 nm, and zeta-potential = 55.57 ± 10.10 mV) and was diluted to *c.a.* 30 μg/ml with respect to SOD in a 6 ml testing tube ($n = 9$). These nine samples were then divided into three groups (3 for each group) to be kept at 37 °C, 25 °C and 4 °C, respectively. At predetermined times, 1 ml samples were withdrawn from each tube and the amount of remaining protein was determined (same method as that used for determination of the encapsulation efficiency).

3. Results

3.1. Risk assessment

Risk identification and risk analysis are two basic components of risk assessment as outlined in the ICH Q9 document. The goal of these two assessments is to obtain the highest risk factors that will be subjected to a more complex DOE study to establish a product or process design space. In the current study, SOD encapsulation efficiency, liposome particle size, and SOD liposome stability are three very critical product qualities and an understanding and awareness of the potential risks is very important. To accomplish this, three cause-and-effect diagrams (Ishikawa diagram) were constructed to identify the potential causes of product variability, as shown in Figs. 1–3.

3.2. Statistical analysis on SOD encapsulation efficiency

As shown in Table 1, SOD encapsulation varied from 9.1% to 48.3% for various factor combinations. Statistical analysis revealed that a good correlation was obtained between the observed and predicted values. The correlation coefficient (r^2) value is 0.9258. Further analysis using ANOVA indicated a significant effect of variables on the response (EE%) ($p < 0.05$) as shown in Table 2. The overall equation describing the effect of various factors on the SOD encapsulation efficiency is:

$$27.71 + 7.64 \times \left(\frac{\text{lipid conc} - 70}{40}\right) + 5.85 \times \left(\frac{\text{DPPC\%} - 50}{50}\right) + 9.50 \times \left(\frac{\text{Chol\%} - 28}{8}\right) - 3.86 \times (\text{SOD conc} - 2) - 3.58 \times \left(\frac{\text{lipid conc} - 70}{40}\right)^2 + 10.24 \times \left(\frac{\text{DPPC\%} - 50}{50}\right)^2 - 8.85 \times \left(\frac{\text{Chol\%} - 28}{8}\right)^2 \quad (3)$$

In comparison to the *priori* model (Eq. (2)), in Eq. (3) none of the interaction terms are present, as they do not show any statistical significance. As shown in Fig. 4a, all the two-way interaction profiles are parallel curves, indicating absence of interactions between factors. Table 3 listed the coefficient and statistical analysis results of all the factors included in Eq. (3). Out of five investigated main factors, four showed statistical significance: cholesterol percentage, lipid concentration, main lipid type, and SOD concentration.

3.2.1. Cholesterol%

Out of all the factors investigated, cholesterol% had the highest impact on SOD EE% ($p < 0.001$) as shown in Table 3. Overall, a negative curvature (concave shape) was observed (Figs. 4b and 5–7), with maximum encapsulation occurring at around 30–32% cholesterol.

3.2.2. Main lipid component (DPPC%)

As shown in Table 1, two types of main lipids (DPPC and DSPC) were evaluated at four different mixing ratios (DPPC:DSPC): 100%:0%, 75%:25%, 25%:75%, and 0%:100%. It was observed that the

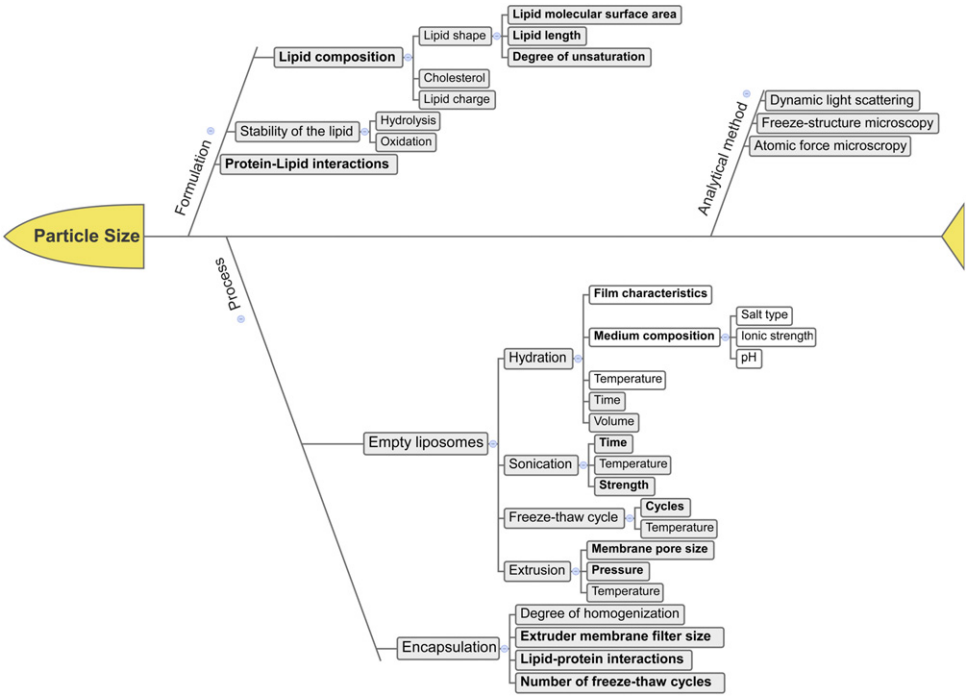

Fig. 2. An Ishikawa diagram illustrating factors that may have impact on the particle size of SOD liposomes.

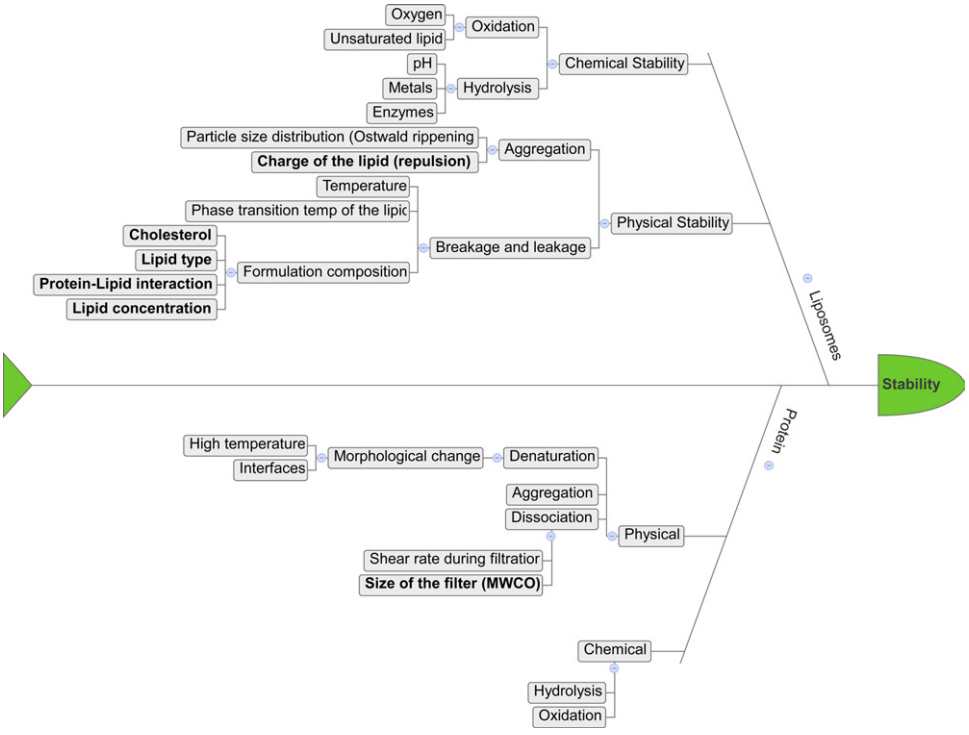

Fig. 3. An Ishikawa diagram illustrating factors that may have impact on the stability of SOD liposomes.

Table 2
Analysis of variance for encapsulation efficiency.

Source	Degree of freedom	Sum of squares	Mean square	*F* ratio
Model	7	2344.9320	334.990	14.2676
Error	8	187.8332	23.479	Prob > *F*
C. Total	15	2532.7652		0.0006

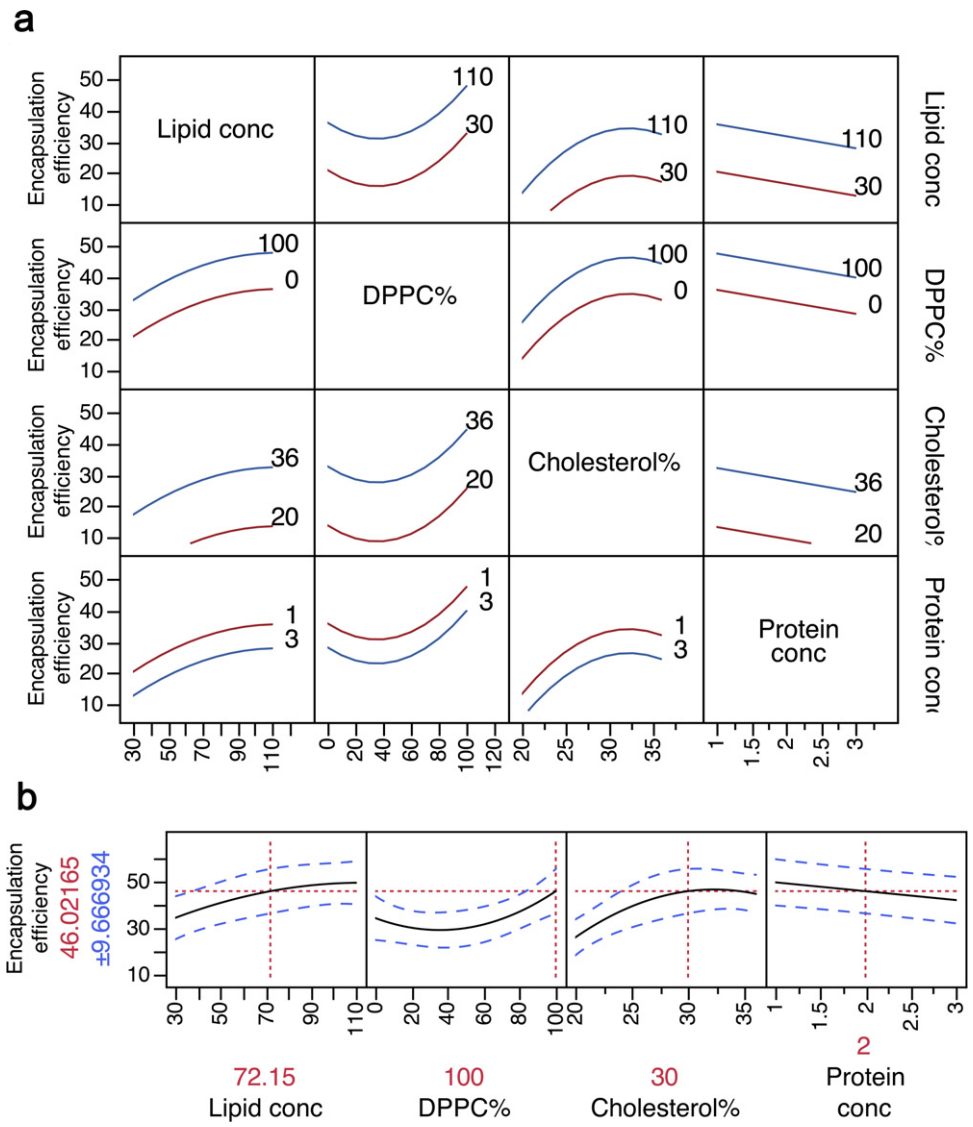

Fig. 4. (a) Two-way interactions for SOD encapsulation efficiency. (b) Prediction profile for SOD encapsulation efficiency.

SOD EE% in DPPC liposomes (100% DPPC) is much higher than that in DSPC liposomes (0% DPPC) (Fig. 4b). This was attributed to a preferential interaction between SOD and DPPC/cholesterol lipid (Xu et al., 2012a). Additionally, a positive curvature (convex shape) was observed in the EE% *vs*. DPPC% as shown in Fig. 4b, suggesting a threshold amount of DPPC (>50%) is required in the DPPC:DSPC mixture before SOD–lipid interaction takes place.

3.2.3. *Lipid and SOD concentration*

As shown in Fig. 4b, SOD EE% was linearly correlated with the lipid concentration at relatively low lipid concentration, but eventually a plateau was reached. This is in agreement with previous findings (Xu et al., 2011, 2012b). With respect to the SOD concentration effect, an increase in SOD concentration slightly reduced the SOD EE%; however, this effect is very insubstantial despite being statistically significant.

3.3. *Validation of the model with additional data*

To evaluate the accuracy and robustness of the obtained model (Eq. (3)), some additional tests were performed. As shown in Fig. 8, very good correlation was obtained between the experimental data

Table 3
Estimated regression coefficients for encapsulation efficiency (uncoded units).

Term	Estimate	Std error	*t* ratio	*P*
Intercept	27.711	3.472	7.98	<0.0001
Cholesterol% (20,36)	9.503	1.451	6.55	0.0002
Lipid conc. (30,110)	7.642	1.399	5.46	0.0006
DPPC% (0,100)	5.847	1.451	4.03	0.0038
Protein conc. (1,3)	−3.861	1.272	−3.03	0.0162
DPPC% × DPPC%	10.240	3.794	2.70	0.0271
Cholesterol% × cholesterol%	−8.846	3.794	−2.33	0.0480
Lipid conc. × lipid conc.	−3.578	2.881	−1.24	0.2494

Table 4
Results of additional tests. X_1 = lipid concentration; X_2 = DPPC% in the main lipid component; X_3 = cholesterol%; X_4 = SOD concentration; X_5 = freeze–thaw cycles. Particle size is reported as Z-ave mean ± distribution width.

X_1 (mM)	X_2 (%)	X_3 (%)	X_4 (mg/ml)	X_5 (cycle)	EE%	Pred. value	Particle size (nm)	Zeta-potential (mV)
107.7	100	30	1.7	2	51.9 ± 2.8	50.8	147.23 ± 34.58	48.13 ± 8.18
69.2	100	30	1.7	2	50.0 ± 0.9	46.6	139.47 ± 31.91	43.93 ± 11.11
53.9	100	30	1.3	2	41.5 ± 1.0	44.7	134.70 ± 23.03	50.10 ± 9.29
53.9	100	30	1.7	2	42.4 ± 2.7	43.1	135.83 ± 24.50	51.70 ± 8.99
107.7	50	30	2.6	2	32.8 ± 4.8	31.2	137.57 ± 25.76	50.07 ± 9.22
53.9	50	30	2.6	2	31.9 ± 2.3	23.6	140.20 ± 32.76	46.93 ± 8.60
107.7	0	30	2.6	2	32.4 ± 1.2	35.6	150.57 ± 19.49	52.0 ± 7.61
53.9	100	20	2.6	2	17.7 ± 0.6	19.5	151.17 ± 43.56	54.37 ± 6.38

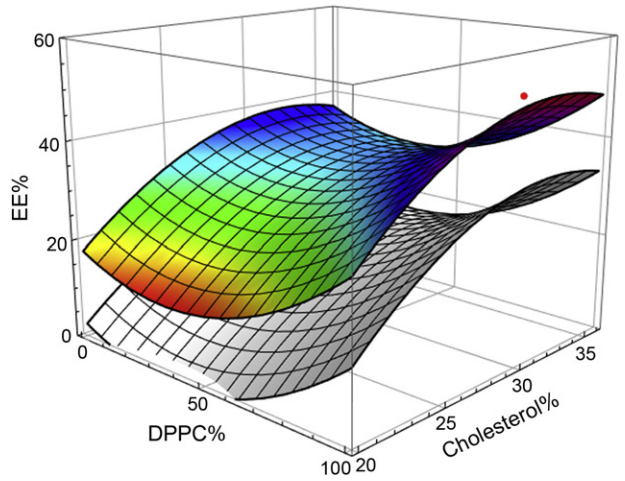

Fig. 5. Response surfaces for predicting EE% with respect to DPPC% and cholesterol%. The top surface represents lipid concentration of 110 mM, while the bottom one represents lipid concentration of 30 mM. SOD concentration is 2 mg/ml.

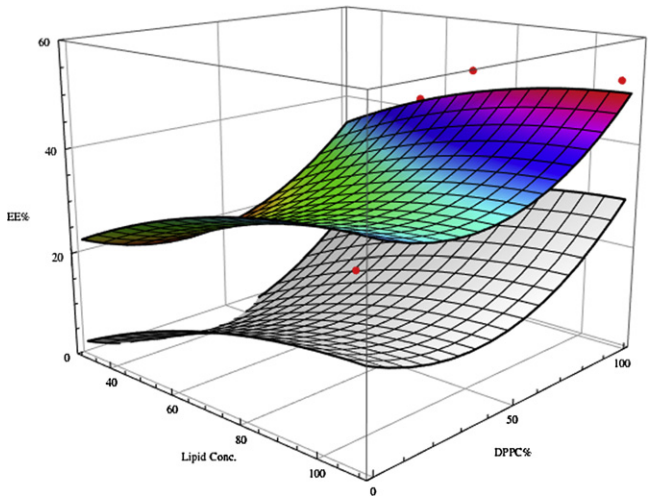

Fig. 7. Response surfaces for predicting EE% with respect to lipid concentration and DPPC%. The top surface represents 30% cholesterol, while the bottom one represents 20% cholesterol. SOD concentration is 2 mg/ml.

and predictions (values inside the white disk are experimental values), and the model was robust and accurate (Table 4). Due to their high prediction accuracy, the countour plots (Fig. 8) that are obtained here for SOD encapsulation also serve as the design space for predicting and controlling SOD encapsulation efficiency.

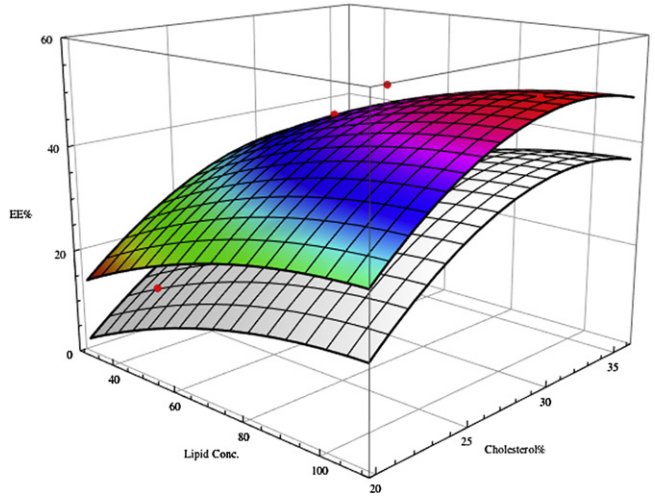

Fig. 6. Response surfaces for predicting EE% with respect to lipid concentration and cholesterol%. The top surface represents 100% DPPC, while the bottom one represents 100% DSPC. SOD concentration is 2 mg/ml.

3.4. Statistical analysis on SOD liposome particle size

As shown in Fig. 9, out of all the variables studied, only DPPC% (*i.e.* type of the main lipid) had a significant effect on SOD liposome particle size. In general, liposome particle size decreases as the DPPC% increases, as shown in Table 5.

3.5. SOD liposome stability

As shown in Fig. 10a, at 37 °C after just 1 week, a substantial amount of protein leaked out into the solution (from 99.23% to 73.20%), accompanied by a dramatic decrease in the liposome surface potential (from 55.57 to 13.87 mV) and an increase in the particle size distribution (from ±15.77 nm to ±42.85 nm). After 3 weeks, near 50% of the protein leaked out, and zeta-potential dropped to near zero and significant particle aggregation was observed (PDI > 0.3). Decrease in the storage temperature to 25 °C increased the stability and no leakage was observed until 70 days later (Fig. 10b). Further decrease in the storage temperature to 4 °C resulted in a much stable formulation, showing no protein leakage even after 6 months

Table 5
Effect of DPPC% on SOD liposome particle size.

DPPC%	Particle size (nm)
0	153.04 ± 4.79
25	151.54 ± 5.41
75	140.80 ± 2.12
100	137.84 ± 3.54

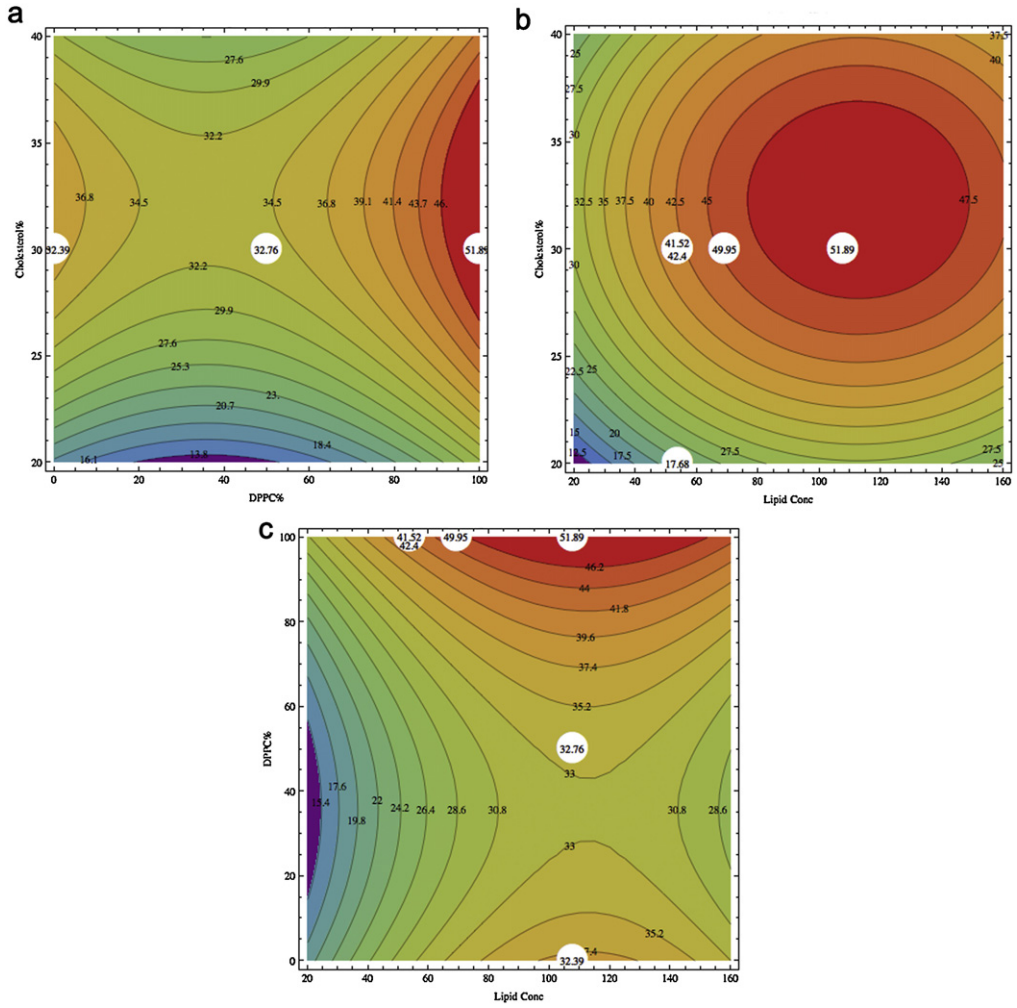

Fig. 8. Contour plot (design space) for EE% (a) with respect to cholesterol% and DPPC% (lipid concentration is 110 mM and SOD concentration is 2 mg/ml); (b) with respect to cholesterol% and lipid concentration (100% DPPC, SOD concentration is 2 mg/ml); and (c) with respect to DPPC% and lipid concentration (cholesterol% is 30%, SOD concentration is 2 mg/ml). Additional data points (white disks) are included to evaluate the accuracy and robustness of the design spaces.

(Fig. 10c), and no change in zeta-potential or particle size was observed.

4. Discussion

The current study focused on three key product qualities: (1) protein encapsulation efficiency, (2) liposome particle size, and (3) SOD liposome stability. A higher percentage of protein encapsulation could significantly reduce the manufacturing cost and increase drug concentration in the final formulation allowing greater flexibility in dosing. Accordingly, the first goal of the current study was to maximize drug encapsulation, in doing so, liposome formulations that resulted in encapsulation efficiencies below 5% were excluded from further analysis. The second goal was to achieve a product particle size range between 100 and 200 nm since this size range would allow sterile filtration of the final product. The third goal was to maintain SOD liposome stability, especially in terms of protein retention inside liposomes, both *in vitro* and *in vivo*. This is very important because for *in vivo* applications, free SOD by itself has very limited access to the intracellular space due to its large size and polarity whereas liposome-encapsulated SOD can be internalized along with the liposome carrier. It is therefore very crucial for SOD to remain inside the liposome throughout circulation before internalization. It is equally important that prepared SOD liposomes possess reasonable storage stability. For these reasons, a complete risk analysis was performed to assess the risks associated with the three key product qualities.

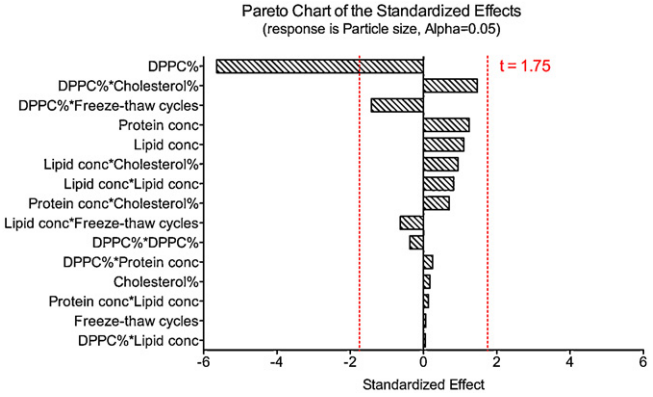

Fig. 9. Pareto chart of the standardized effects of various formulation and process factors on SOD liposome particle size.

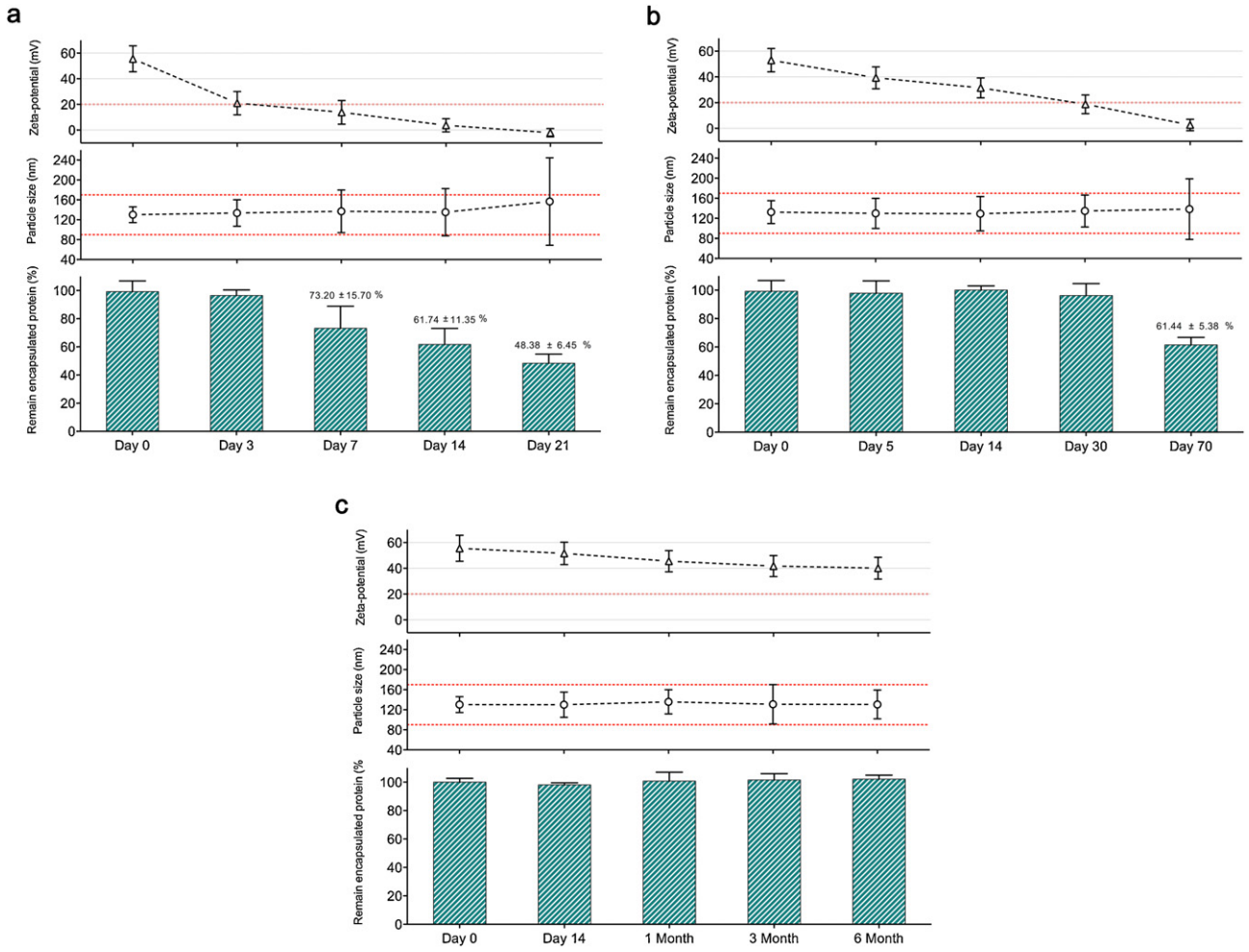

Fig. 10. SOD liposome storage stability at (a) 37 °C, (b) 25 °C, and (c) 4 °C. Top: zeta-potential changes (mean ± SD); middle: particle size and distribution width (shown as error bar); and bottom: remaining SOD percentage (mean ± SD).

4.1. Risk analysis

4.1.1. Risk analysis: SOD encapsulation efficiency

Factors affecting SOD encapsulation efficiency were divided into four categories: formulation, process, analytical method, and instrumentation reliability, as shown in Fig. 1. Overall, five key factors were identified as having high impact on SOD encapsulation efficiency (marked bold): four of these were formulation parameters and one was a process parameter. No factors were selected from the "analytical method" and "instrument reliability" categories, mostly because all of these can be very well controlled. However, the importance of factors from these two categories should not be overlooked. For instance, under the "analytical method", using an inappropriate method will result in an erroneous calculation of encapsulation efficiency, and this would jeopardize any subsequent statistical experimental design aimed to optimize encapsulation efficiency. For non-adsorptive small molecules, it was concluded that calculation based on free drug are more accurate and robust (Xu et al., 2011). However, for very adsorptive proteins, using free drug can result in significant under-estimation of the free protein concentration due to protein adsorption. Indeed, it was discovered that as much as 30% of the protein may be retained (Fig. 11) by ultrafiltration filter (100 kDa). This can cause significant over-estimation of the SOD encapsulation efficiency. For this reason, in this study the SOD encapsulation efficiency was calculated

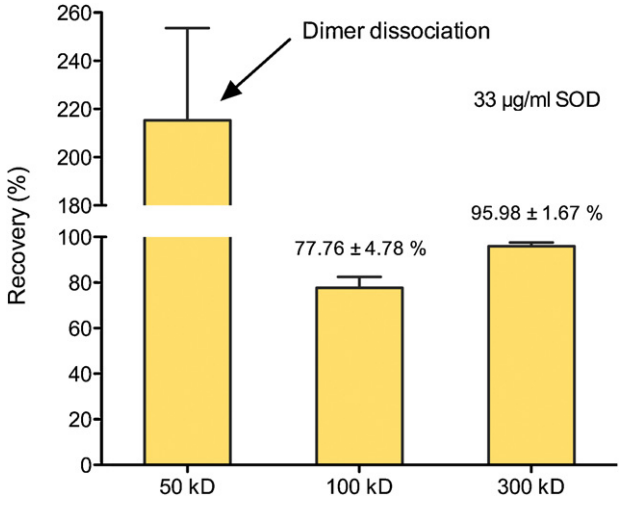

Fig. 11. Effect of ultrafiltration membrane molecular weight cut-off on SOD recovery.

based on encapsulated drug (Eq. (1)) and extra-attention was taken to ensure that the remaining free SOD was washed away thoroughly to avoid any erroneous results (Xu et al., 2011). In addition, using an inappropriate ultrafiltration filter can also sacrifice protein stability; for example, when using a 50 kDa filter, SOD dimer dissociation was observed (Fig. 11). With respect to instrumentation reliability, for example, the temperature of the extruder should be kept under at least 10 °C above the main lipid phase transition temperature so that the lipid bilayers were flexible enough to pass through the filter pores. In addition, appropriate container material should be selected to avoid container cracking and sample leakage during freeze thaw cycling.

4.1.2. Risk analysis: liposome particle size

As shown in Fig. 2, factors potentially affecting liposome particle size were divided into three categories: formulation, process, and analytical method. Note that it has been demonstrated that the extrusion membrane pore size greatly affects the final product size (Xu et al., 2011). However, in the current study, only one extruder membrane size was suitable; hence, the membrane pore size was not considered a variable here. For this reason, the same variables identified for the D-optimal design were selected to evaluate their effect on SOD liposome particle size, *i.e.* main lipid type (lipid length), and cholesterol%.

4.1.3. Risk analysis: SOD liposome stability

Factors affecting the stability of SOD containing liposomes were separated into two categories, those affecting the protein and those affecting the liposome carrier. Factors in the first category are beyond the scope of the current research and our focus was mainly on the liposome carrier stability, in particular the physical stability of the protein containing liposomes. Due to time and cost constraints, no statistical design study was performed with respect to the liposome physical stability. Instead, various strategies were combined together to achieve a stable formulation. These strategies include: (1) saturated long alkyl chain PC lipids such as DPPC or DSPC were used as the main lipid component to increase physical stability as well as reduce possible chemical degradation (mainly oxidation), (2) a minimum of 20% cholesterol was used in the formulations to reduce the membrane permeability, and (3) positively charged lipids such as SA or DPTAP were used to introduce charge to the liposome surface preventing particle aggregation.

4.2. Influence of various factors on SOD encapsulation efficiency

The generated statistical model (Eq. (3)) suggests that the SOD encapsulation efficiency of FAT-ULV liposomes is mainly dependent on four main effects (in the order of significance level from high to low): cholesterol% (X_3), lipid concentration (X_1), DPPC% (X_2), and SOD concentration (X_4); as well as three second-order terms: X_2^2, X_3^2, and X_1^2. Note that X_1^2 was included in the model despite having a p-value of 0.25. This is because in a previous study, the evidence for the curvature of lipid concentration effect response surface was very substantial (Xu et al., 2012a). It is speculated that the reason for lack-of-significance of response surface curvature in this study was due to insufficient data levels with respect to lipid concentration. Indeed, after including eight additional data points (Table 4) into the existing model, the p-value for the X_1^2 dropped to $p < 0.1$ (results not shown).

4.2.1. Effect of cholesterol%

It is widely known that incorporation of cholesterol into the lipid bilayer can increase liposome stability as it reduces bilayer fluidity and hence permeability (Kirby et al., 1980; Lee et al., 2005). This may contribute to higher protein retention inside the liposomes and a higher degree of encapsulation. In addition, it was observed that SOD containing liposomes had a much higher EE% than our mathematical model predicted, suggesting the presence of strong lipid–protein interaction (Xu et al., 2012b). For example, at 30% cholesterol content, DPPC liposomes exhibited a 26% higher EE% than predicted (assuming no interaction). This strong interaction was explained previously using a "pocket" theory (Xu et al., 2012a), which states that inside the lipid bilayer various sizes of pockets are generated in between the cholesterol molecules. These pockets allow for a favorable interaction of the lipid with SOD since the structure of cholesterol is similar to a flat sheet, which enables a better interaction with the protein. One critical property of these pockets is that their sizes are largely defined by the percentage of cholesterol. Higher cholesterol content leads to smaller pocket size while lower cholesterol content generates larger pockets. It is reasonable to believe that the SOD–lipid interaction requires an optimal sized pocket (relative to the size of the protein). This may explain why the encapsulation exhibited a maximum with respect to cholesterol percentage (Figs. 4b and 5).

4.2.2. Effect of main lipid component (DPPC%)

The main lipid component accounts for more than 50 mol% of the total lipid in a liposome formulation. Lipids that are suitable for the main lipid component are mostly saturated long chain neutral lipids, such as DMPC (14 carbons), DPPC (16 carbons), and DSPC (18 carbons). A lipid with a longer chain length will have a higher lipid phase transition temperature. Normally, the difference in the bilayer thickness does not translate to variation in the drug encapsulation efficiency (Xu et al., 2011, 2012c); however, as shown in Fig. 6, the response surface for DPPC liposomes (100% DPPC) is much higher than DSPC liposomes (0% DPPC). This was attributed to a strong interaction between SOD and DPPC/cholesterol, which may not exist (or is very weak) between SOD and DSPC/cholesterol. This specific lipid–protein interaction is speculated to arise from the "hydrophobic coupling" between the protein's hydrophobic domains and the bilayer hydrophobic core (Andersen and Koeppe II, 2007). A variety of lipid bilayer properties might be responsible for this "hydrophobic coupling", such as bilayer thickness, intrinsic lipid curvature, the elastic compression and bending moduli.

4.2.3. Effect of lipid concentration

Generally, a higher lipid concentration leads to higher drug encapsulation efficiency. This is attributed to its positive impact on the total internal volume of liposomes (Xu et al., 2011, 2012c) that is determined by two factors, the entrapment volume of individual vesicles and the total vesicle number. It was shown previously that protein containing FAT-ULV liposomes had a similar entrapment volume to the empty liposomes (Xu et al., 2012a). Therefore, the increase in the protein encapsulation efficiency as a result of an increase in lipid concentration was primarily attributed to an increase in the total vesicle number. The plateau occurred at relatively high lipid concentrations (Figs. 4b, 6 and 7) was attributed to the loss of protein samples as well as lipid on the extruder filter due to high sample viscosity.

4.3. Influence of various factors on SOD liposome particle size

As shown in Fig. 9, the particle size of SOD containing liposomes is dependent on the type of the main lipid used (*i.e.* DPPC%). Based on the mechanics of thin materials, it is well known that the bending modulus (K_c) of the material is proportional to the area compression modulus (K_a) multiplied by the square of thickness (h), *i.e.* $K_c \propto K_a h^2$ (Halet et al., 1993; Rawicz et al., 2008). Under normal conditions, the area compression moduli of the lipid bilayer remain almost constant, while the square root of the

bending modulus (stiffness) varies proportionally to the thickness of the bilayer. In this study, DSPC liposomes (0% DPPC) have a relatively thicker bilayer in comparison to DPPC liposomes (100% DPPC). This may result in a stiffer DSPC lipid bilayer, and subsequently a lower surface curvature as compared to the DPPC liposomes, which can explain the differences in their particle size.

4.4. SOD liposome stability

At low temperatures, the prepared SOD liposomes remained stable (no leakage, no particle size change, and no zeta-potential change) at 4 °C for at least 6 months. In comparison, at an elevated temperature (37 °C), a substantial amount of the protein leaked out after two weeks storage (~40%), accompanied by near neutral surface charge and larger aggregated particles (Fig. 10a). It is believed that the cause of the instability (protein leakage) is due to the high lipid molecular mobility at higher temperatures (close to the phase transition temperature, T_m, of the lipid, *e.g.* 41 °C for DPPC). The enhanced lipid mobility can result in an increased lipid bilayer permeability (Xu et al., 2012c) that may lead to higher protein partitioning and faster diffusion; hence, rapid protein leakage. In addition, the increased lipid mobility can also accelerate collision and coalescence rates of liposome particles. For charged lipids, this translates to a much faster dissipation of the liposome surface charge that is necessary for electrostatic stabilization. Consequently, particle aggregation and lipid fusion occurs leading to more protein leakage. In addition, it is speculated that the leaked protein content is largely from inside the lipid bilayer. Due to the relatively large size of the protein molecule, leakage from the liposome internal aqueous compartment is less likely to occur (slow diffusion) unless the liposome bilayer integrity is lost under stress (such as sonication, freeze–thaw cycling, or enzyme/surfactant degradation).

5. Conclusions

The current study demonstrated the usefulness of the application of quality by design to gain a comprehensive understanding of formulation and processing parameters affecting protein liposome formulations prepared *via* FAT-ULV. Lipid concentration, cholesterol mol%, main lipid type and protein concentration were identified as critical parameters affecting SOD encapsulation efficiency. Moreover, the D-optimal statistical design was shown to be very beneficial as a highly predictive model was obtained from a small number of experiments. Using the generated model, a design space for SOD liposome preparation was established, within which preparation variability is minimized and product quality can be assured. Furthermore, the maximum values observed in the response surfaces indirectly confirmed the possibility of the existence of a specific SOD–lipid bilayer interaction under the following optimal conditions: (1) appropriate membrane thickness and curvature (DPPC liposomes), and (2) optimal "pocket size" generated by cholesterol content. With respect to storage stability, at 4 °C storage temperature, the prepared SOD liposomes remained stable for at least 6 months in the aqueous dispersion state. This is significant since expensive and potentially damaging lyophilization of the liposomes can be avoided. At room temperature, the SOD liposomes are expected to have at least one-month stability. Further increase in storage temperature may greatly compromise liposome stability. Lastly, the methods and principles used in the current study can be applied to liposomes containing other protein molecules and can provide time and cost savings to industrial formulation scientists, which will result in a more robust liposome preparation process.

Disclaimer

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

Acknowledgement

This research was supported by FDA critical path funding (HHSF223201011124P).

References

Andersen, O.S., Koeppe II., R.E., 2007. Bilayer thickness and membrane protein function: an energetic perspective. Annu. Rev. Biophys. Biomol. Struct. 36, 107–130.
Atkinson, A.C., Donev, A.N., Tobias, R.D., 2007. Optimum Experimental Designs with SAS. Oxford Univeristy Press, pp. 1–527.
Bodea, A., Leucuta, S.E., 1997. Optimization of hydrophilic matrix tablets using a D-optimal design. Int. J. Pharm. 153, 247–255.
El-Hagrasy, A.S., D'Amico, F., Drennen, J.K., 2006. A process analytical technology approach to near-infrared process control of pharmaceutical powder blending. Part I: D-optimal design for characterization of powder mixing and preliminary spectral data evaluation. J. Pharm. Sci. 95, 392–406.
Gregoriadis, G., 1995. Engineering liposomes for drug delivery: progress and problems. Trends Biotechnol. 13, 527–537.
Halet, F.R., Marsh, J., Nickel, B.G., Wood, J.M., 1993. Mechanical properties of vesicles. II. A model for osmotic swelling and lysis. Biophys. J. 64, 435–442.
Keele, B.B., McCord, J.J.M., Fridovich, I., 1971. Further characterization of bovine superoxide dismutase and its isolation from bovine heart. J. Biol. Chem. 246, 2875–2890.
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.
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.
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.
Maurer, N., Fenske, D., Cullis, P., 2001. Developments in liposomal drug delivery systems. Expert Opin. Biol. Ther. 1, 923–947.
McCord, J.M., Fridovich, I., 1969. Superoxide dismutase. J. Biol. Chem. 2444, 6049–6055.
Okado-Matsumoto, A., Fridovich, I., 2001. Subcellular distribution of superoxide dismutases (SOD) in rat liver. J. Biol. Chem. 276, 38388–38393.
Rathore, A.S., Winkle, H., 2009. Quality by design for biopharmaceuticals. Nat. Biotechnol. 27, 26.
Rawicz, W., Olbrich, K.C., Mcintosh, T., Needham, D., Evans, E., 2008. Effect of chain length and unsaturation on elasticity of lipid bilayers. Biophys. J. 79, 328–339.
Torchilin, V.P., 2005. Recent advances with liposomes as pharmaceutical carriers. Nat. Rev. Drug Discov. 4, 145–160.
Torchilin, V.P., Lukyanov, A.N., 2003. Peptide and protein drug delivery to and into tumors: challenges and solutions. Drug Discov. Today 8, 259–265.
Vogt, N., 1992. Quality by design: managing research and development. Chemom. Intell. Lab. Syst. 14, 93–101.
2011. Gene Therapy Clinical Trials Worldwide. http://www.wiley.co.uk/genmed/clinical/.
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.
Xu, X., Burgess, D.J., 2011. Liposomes as carriers for controlled drug delivery. In: Wright, J.C., Burgess, D.J. (Eds.), Long Acting Injections and Implants. , 1st ed. Springer, New York, pp. 195–220.
Xu, X., Costa, A., Burgess, D.J., 2012a. Protein encapsulation in unilamellar liposomes: high encapsulation efficiency and a novel technique to assess lipid-protein interaction. Pharm. Res. 29, 1919–1931.
Xu, X., Khan, M.A., Burgess, D.J., 2011. A quality by design (QbD) case study on liposomes containing hydrophilic API: I. Formulation, processing design and risk assessment. Int. J. Pharm. 419, 52–59.
Xu, X., Khan, M.A., Burgess, D.J., 2012b. Predicting hydrophilic drug encapsulation inside unilamellar liposomes. Int. J. Pharm. 423, 410–418.
Xu, X., Khan, M.A., Burgess, D.J., 2012c. 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. Int. J. Pharm. 423, 543–553.
Yu, L.X., 2008. Pharmaceutical quality by design: product and Process Development, Understanding, and Control. Pharm. Res. 25, 781–791.
Zhang, Y., Wang, J., Wu, Y., Li, W., 2002. Anti-inflammatory effect of recombinant human superoxide dismutase in rats and mice and its mechanism. Acta Pharmacol. Sin. 23, 439–444.
Zhuang, Y., Ma, Y., Wang, C., Hai, L., Yan, C., Zhang, Y., Liu, F., Cai, L., 2012. PEGylated cationic liposomes robustly augment vaccine-induced immune responses: role of lymphatic trafficking and biodistribution. J. Control. Release 159, 135–142.