Pharm Res (2012) 29:1919–1931
DOI 10.1007/s11095-012-0720-x

RESEARCH PAPER

Protein Encapsulation in Unilamellar Liposomes: High Encapsulation Efficiency and A Novel Technique to Assess Lipid-Protein Interaction

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Received: 4 January 2012 / Accepted: 22 February 2012 / Published online: 9 March 2012

ABSTRACT

Purpose To encapsulate a large amount of protein (superoxide dismutase, SOD) into unilamellar liposomes using a simple process and to investigate the lipid-protein interaction.

Method To achieve protein encapsulation, preformed unilamellar empty liposomes were mixed with SOD and subjected to freeze-thaw cycling. To investigate the lipid-protein interaction, a novel light scattering technique was used.

Results Up to 50% protein encapsulation was achieved at ~150 nm. There was no significant change in particle size following the freeze-thaw cycling. SOD had a strong interaction with DPPC liposomes containing high concentration of cholesterol. Light scattering data revealed that in some cases the SOD molecules were present inside the lipid bilayer.

Conclusions The method reported here allows great flexibility in the manufacturing process as the liposome preparation and protein-loading operations can be separated. Accordingly, empty liposomes can be prepared without concern about protein stability, making the manufacturing process more flexible and easy to control and ultimately leading to improved product quality. To explain the SOD-lipid interaction, a "pocket-embedding" theory was proposed. The encapsulation method reported here can be applied to hydrophilic small molecules as well as most hydrophilic proteins to achieve high encapsulation efficiency.

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KEY WORDS freeze-and-thaw unilamellar vesicles (FAT-ULV) · high performance liquid chromatography (HPLC) · light scattering · liposome · superoxide dismutase

INTRODUCTION

There are two major challenges in the development of liposomal drug delivery systems for hydrophilic proteins: 1) low protein encapsulation efficiency, especially in the predominantly used small vesicle size range (50~150 nm); and 2) poor protein stability during preparation, especially if harsh processing conditions and/or organic solvents are involved.

The low encapsulation efficiency in small sized liposomes is predominately limited by the low entrapment volume. Various methods have been developed to alleviate this problem, such as reverse phase evaporation (REV) (1,2) and freeze-thaw cycling (3). However the success has been limited, largely due to the fact that the formed liposomes are heterogeneous and hence not directly suitable for pharmaceutical applications. Efforts to overcome this heterogeneity, using downsizing technique such as extrusion or sonication, typically result in significant loss of drug (4), and hence loose the higher encapsulation advantage brought by the REV and freeze-thaw cycling. In addition, the REV process involves the use of organic solvent and high shear rate during the formation of the initial w/o emulsions, and therefore is not suitable for majority of proteins.

Another approach to greatly enhance the drug encapsulation efficiency is through the remote loading technique (5,6). The remote loading method has very high efficiency (>95%), but is only effective for a relatively small group of molecules where diffusion of the unionized drug molecules is dependent on pH conditions. This method is not suitable for

protein therapeutics due to their large molecular size as well as pH stability issues.

Liposome preparation methods can be broadly divided into three different approaches: 1) hydration of a dry lipid film (7,8); 2) precipitation of lipids in aqueous medium, such as the ethanol injection method (9–11); and 3) adsorption of dissolved lipids at liquid/liquid interfaces, such as the reverse phase evaporation method (12). Due to the high drug solubility, the encapsulation of hydrophilic molecules is normally performed during the liposome formation step for convenience. Drug encapsulation is achieved mainly through passive drug diffusion. However, there are two problems associated with this approach: 1) the efficiency of loading is rather low, and 2) for protein molecules the long exposure during the preparation processes can greatly compromise their stability.

To overcome these two problems, in this study the preparation process of protein containing liposomes was separated into two steps. The first step involves the generation of unilamellar vesicles. Since no protein is added in this step, the processing conditions are therefore much more flexible. The second step uses an improved freeze-and-thaw cycling technique to allow protein diffusion and hence achieve encapsulation. Because the liposomes obtained using this approach remained as unilamellar vesicles and no significant change in particle size was observed, they are referred to here as freeze-and-thaw unilamellar vesicles (FAT-ULV).

Superoxide Dismutase (SOD) is an antioxidant enzyme that catalyzes the dismutation of superoxide radical into hydrogen peroxide and oxygen. Being one of the most potent antioxidants known in nature, SOD has been used for treatment of diseases in which oxidative stress is involved such as rheumatoid arthritis, cancer, and respiratory distress syndrome. SOD has been shown to be a promising alternative to conventional therapies (13–16); however, current use of the protein is limited by 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. Conversely, this makes the SOD a perfect candidate for liposome delivery. For this reason, SOD was selected as the model protein in this study.

Interactions between lipids and proteins in liposome system not only affect the physicochemical properties of the protein but also its release rate, hence affecting its bioavailability following administration (17). Moreover, the presence of potential protein-lipid bilayer interaction may increase the protein encapsulation efficiency in addition to the protein that is encapsulated in the interior of the liposome. Accordingly, understanding the distribution of protein inside the liposomes (in the interior aqueous compartment and/or within the lipid bilayer) is very important. To study the interaction between the lipid and protein (*i.e.* the effect of protein on the phase transition behavior of the lipid), calorimetry based analysis is widely used, most notably differential scanning calorimetry (DSC) (18,19). However, this type of analysis is only limited to studying interactions between pure lipid and protein. In cases where high cholesterol content is present, no transition can be observed. To overcome this problem, in the current study, a light scattering based technique was used to study the potential SOD-lipid interaction. This technique was previously used to determine the phase transition temperature of pure lipid: during the phase transition, the amount of scattered light decreases as a result of change in the lipid bilayer thermal expansion coefficient (20,21). In this study, this technique has been adapted to study the interaction between lipid mixtures and protein.

MATERIAL AND METHODS

Materials

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), 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). 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.

Preparation of Empty Unilamellar Liposomes

Empty unilamellar liposomes were prepared using a film hydration method. 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 kept under vacuum overnight to completely remove any residual solvent. Then, dry lipids were 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). Note that all formulations contained three lipid components: main lipid (DPPC, DSPC, or a mixture of two), cholesterol, and charged lipid

(stearylamine or DPTAP). High phase transition temperature main lipids were selected as this provides a great advantage in terms of *in vitro* storage as well as *in vivo* stability. In addition, a minimum of 20 mol% of cholesterol was used in the formulation to further increase formulation stability by enhancing membrane rigidity.

Encapsulation of SOD into Freeze-and-Thaw Unilamellar Vesicles (FAT-ULV)

Encapsulation of SOD into the empty unilamellar liposomes was achieved using a freeze-thaw cycling technique as shown in Fig. 1. Briefly, the desired amount of protein solution was mixed with preformed empty unilamellar liposomes. The mixture was subjected to two or four 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. In most cases, two freeze-thaw cycles were used. However, four freeze-thaw cycles was also attempted to determine whether the increase of the freeze-thaw cycle numbers increase the encapsulation efficiency. After freeze-thaw cycling, samples were extruded using a 200 nm filter (LIPEX extruder) to obtain mono-dispersed liposome samples.

Determination of Encapsulation Efficiency (EE%)

20 μL of prepared liposomes (before purification) were withdrawn and diluted with 1 ml 10 mM pH 7.4 HEPES buffer (working-dispersion). To assess the total protein concentration, 500 μL of working-dispersion was mixed with 100 μL of 6% (*v*/*v*) Triton X-100 and this dispersion was maintained at 65°C for 5 min to disrupt all the vesicles. To assess the concentration of encapsulated protein, 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 using a newly developed high-performance liquid chromatography (HPLC) method (see below). The encapsulation efficiency was calculated as:

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

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 an approximately 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. As an approximation, the remaining free protein %=(1/concentrating factor)^n×100%, where n is the number of passes. 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.

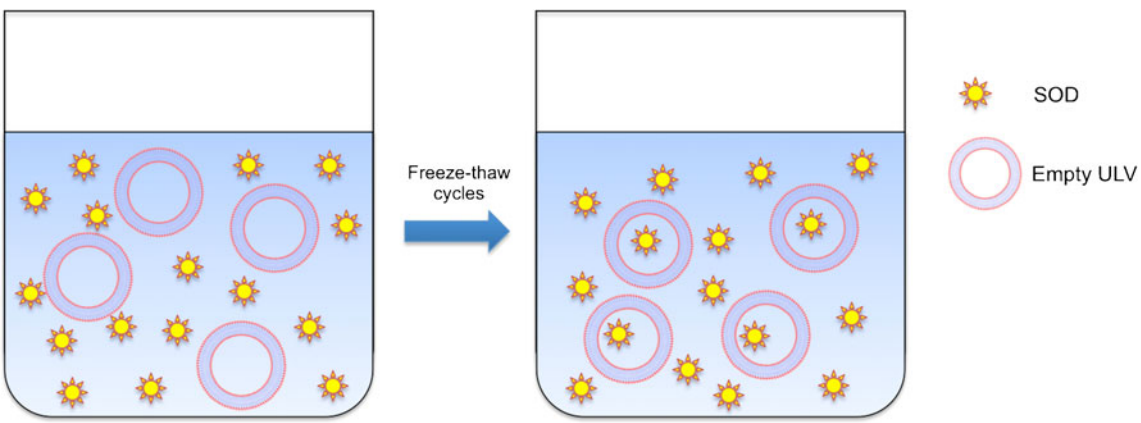

Fig. 1 Schematic diagram illustrating the process of encapsulating SOD into empty unilamellar vesicles (ULV) with freeze-thawing technique.

Chromatographic Equipment and Conditions

The HPLC system consisted of a Flexar System (Perkin Elmer Inc., US) equipped with a quaternary pump, a peltier auto-sampler (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× 75 mm, Waters Corporation, USA) protected with a Symmetry300 C18 guard column (3.5 µm, 2.1×10 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 l 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. The retention time for SOD was around 7 min, and no interferences were observed as shown in Fig. 2. The developed HPLC method was validated as per the ICH guidance (ICH 2005). The obtained detection and quantitation limits for SOD were 0.79 µg/ml and 2.63 µg/ml, respectively.

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)

SOD Activities Under Various Processing Conditions

To evaluate the effect of process conditions (*i.e.* temperature and freeze-thaw cycles) on SOD activities, aliquots of SOD solutions were put into cryovials (1.2 ml capacity), which were incubated at various temperatures (25°C, 37°C, 50°C, and 65°C) as well as subjected to various freeze-thaw cycles (−196°C to 65°C). Each cryovial contained 500 µl of 5.96 Unit/ml (or 1.47 µg/ml) SOD. At designated time intervals, SOD activities were measured using a SOD Assay Kit-WST (Dojindo Molecular Technology, Inc., Rockville, MD). By measuring the change of the absorbance at 440 nm (SpectraMax 190, Molecular device, Inc.), the SOD activity (as inhibition rate) can be quantified using a pre-determined inhibition rate curve (Fig. 3a). The activity of the same SOD solution stored at 4°C was used as a control (normalized as 100%).

Light Scattering to Evaluate the Protein-Lipid Interaction

The liposome samples (with or without protein) were diluted to approximately 5 mM with respect to lipid and put into a small volume quartz cuvette (100 µL), which was then put inside a Malvern ZS90 zeta-sizer. The instrument was set to ramp the temperature from 20~75°C with 1°C

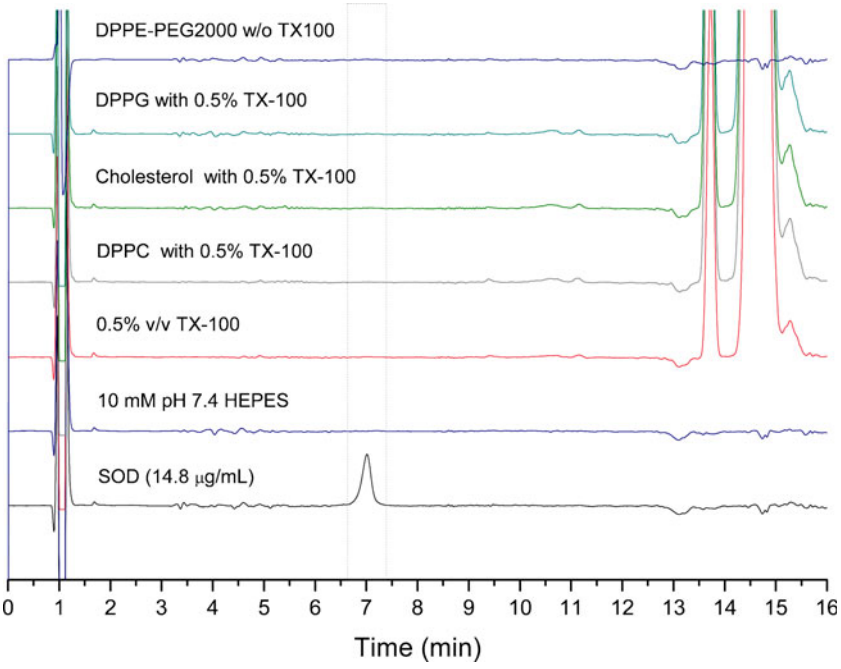

Fig. 2 HPLC-UV/Vis (220 nm) chromatogram showing the profiles of SOD (14.8 µg/ml) and various lipid samples (as negative controls).

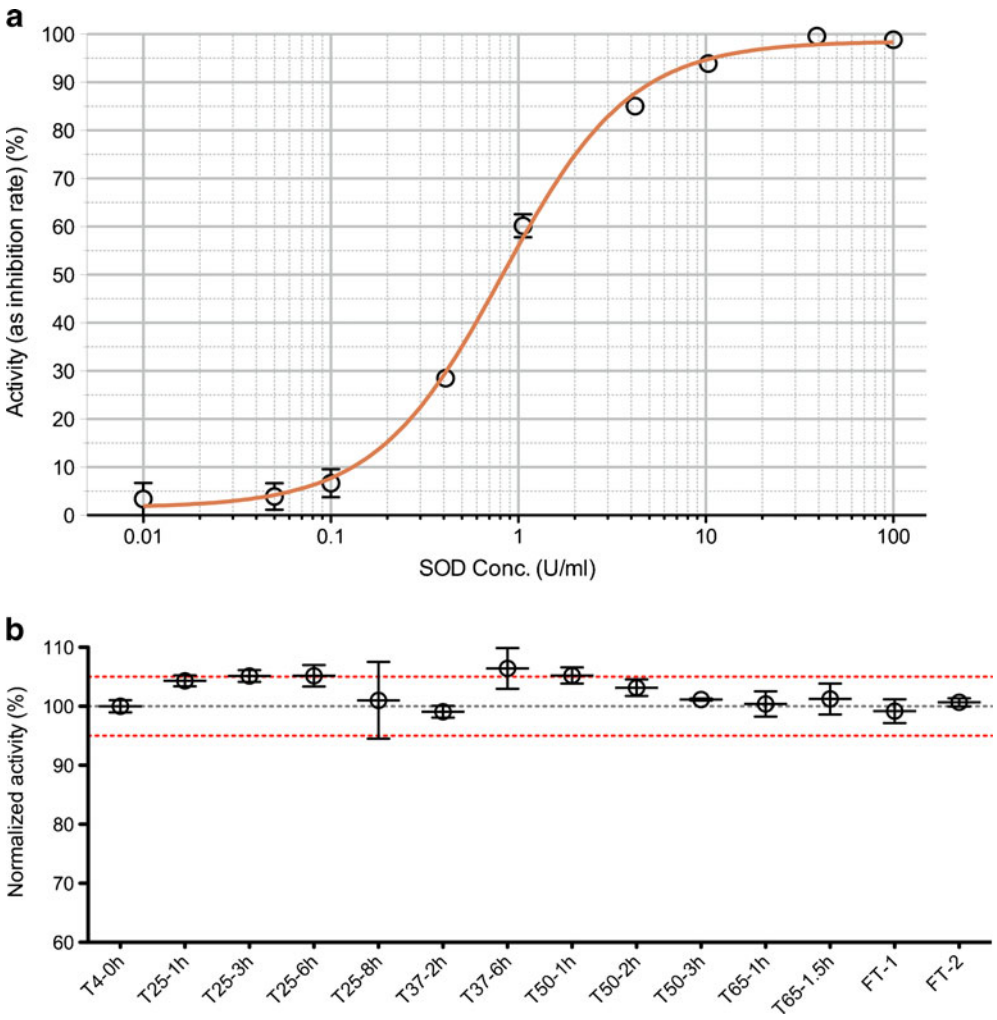

Fig. 3 Evaluation of SOD activities under various conditions. (**a**) SOD activity (expressed as inhibition rate)-concentration curve; (**b**) Normalized activity of SOD standard solution (the activity of samples stored at 4°C as 100%) at different temperatures and after one or two freeze-thaw (FT) cycles (SOD concentration: 5.96 U/ml or 1.47 μg/ml).

increment. 8 min equilibration time was allowed before each measurement. The instrument was run in manual mode at 90° scattering angle (the measurement position was fixed at 4.20 mm and the attenuator setting was 7) to avoid instrument interferences during the experiment. After the run, nonlinear regression and statistical tests were performed to model the data using a Boltzmann sigmoidal model (20).

RESULTS

Effect of Processing Temperature on SOD Activities

As shown in Fig. 3b, under all conditions tested, SOD remained active. Note that even after 1.5 h at 65°C, which is three times longer than the actual processing time (~30 min), SOD activity was unchanged. More importantly, the freeze-thaw cycling process had no impact on the protein activity.

Effect of Freeze-Thaw Cycling on Liposome Particle Size and Protein Encapsulation

To evaluate the effect of freeze-thaw cycling on the protein encapsulation efficiency, the following experiment was performed. 2 ml of preformed empty unilamellar liposomes (70 mM of DPPC:cholesterol:SA in 6:3:1 molar ratio, 153.17±28.22 nm, 56.63±9.04 mV) were mixed with 600 µL of SOD solution (7.4 mg/ml), which resulted in a mixture of 54 mM lipid and 1.7 mg/ml of SOD. To exclude the possibility of encapsulating protein through simple incubation, the mixture was incubated at 55°C for 2 h. As can be seen in Fig. 4, this resulted in zero encapsulation. In contrast, just one freeze-thaw cycle increased SOD encapsulation from near zero to approximately 34% without noticeable change in liposome particle size. After a second freeze-thaw cycle, there was no significant change in the particle size. However, subsequent freeze-thaw cycles induced undesired broadening of the size distribution, and this resulted in a ~5% increased in encapsulation efficiency. Subsequent extrusion of FAT-ULV reduced the mean

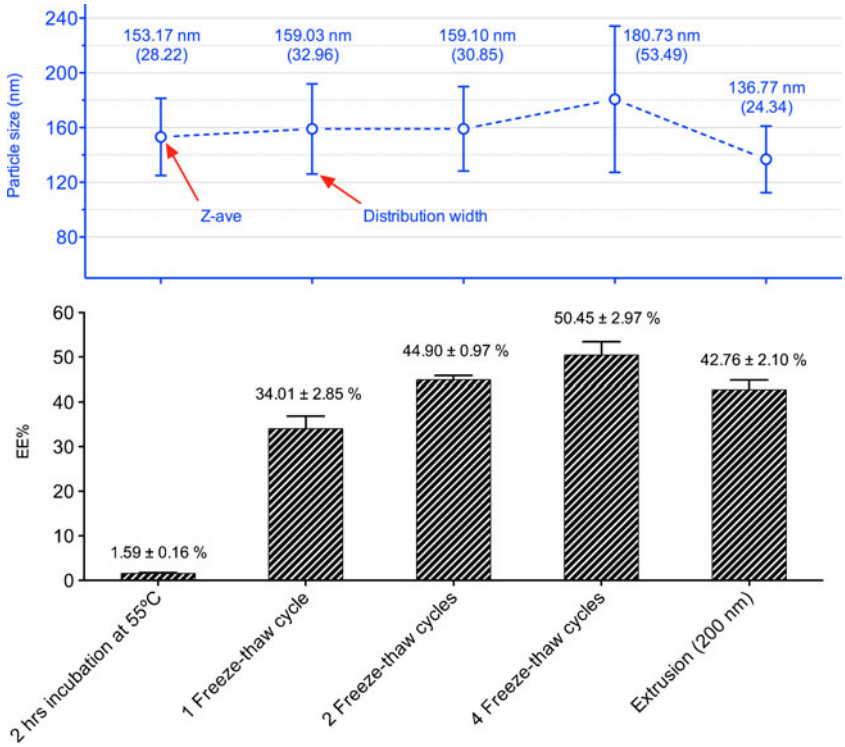

Fig. 4 Effect of freeze-thaw cycles and extrusion on liposome particle size and protein encapsulation efficiency. Top: particle size (Z-ave) and distribution width of SOD liposomes during each process; Bottom: protein encapsulation efficiency after each step. (n=3).

particle size as well as the size distribution, and the EE% was almost identical to that following the second freeze-thaw cycle. Accordingly, two freeze-thaw cycles followed by extrusion through 200 nm filters was sufficient to achieve optimal protein encapsulation in the empty unilamellar liposomes. This process was used in all subsequent studies.

Effect of Formulation Composition on Protein Encapsulation Efficiency

To assess any effect of formulation variables on protein encapsulation efficiency, the following formulation parameters were evaluated: main lipid type (DSPC or DPPC), lipid concentration, charged lipid type, cholesterol percentage, and protein concentration (Table I).

Effect of Protein and Lipid Concentration

As shown in Fig. 5, protein concentration had very little effect on the encapsulation efficiency. For both DPPC and DSPC liposomes, only a small drop in the encapsulation efficiency was observed. With regard to the lipid concentration, it was observed that for both DPPC and DSPC liposomes, protein EE% was linearly correlated with the lipid concentration at relatively low lipid concentration (below 60 mM), but reached a plateau above 70 mM (Fig. 6).

Effect of Formulation Composition

The main lipid type (namely DSPC or DPPC) had a significant effect on protein encapsulation efficiency. As shown in Figs. 5 and 6, DPPC liposomes (DPPC:cholesterol:SA) exhibited about 20% higher EE% than DSPC liposomes (DSPC:cholesterol:SA) throughout the lipid concentration range. Replacing half of the DPPC with DSPC (DPPC:DSPC:cholesterol:SA=30:30:30:10) resulted in a 10% reduction in the EE% compared to the DPPC liposomes (Fig. 7). In addition, it was observed that the percentage of cholesterol also had a significant effect on protein EE%. As shown in Fig. 7, for DPPC liposomes, a 10% decrease in cholesterol (from 30% to 20%) resulted in a 20% reduction in the protein EE%. One factor that did not show any significance was the type of charged lipid (DPTAP or SA) as shown in Fig. 7.

Evaluation of SOD-Lipid Interaction Using Light Scattering

The difference in the encapsulation efficiencies of liposomes with different main lipids suggested that SOD might have an interaction with certain lipid bilayers. It was speculated that the presence of SOD inside the lipid bilayer can result in changes in the bilayer thermal expansion coefficient and hence the refractive index. Accordingly, it was considered that it might be possible to adapt a light scattering technique (used to determine the lipid phase

Table I Encapsulation Efficiency, Particle Size and Zeta-Potential Values of Various SOD Formulations (n=3)

Composition	Lipid conc. (mM)	SOD conc. (mg/ml)	Particle size (nm)		Zeta-potential (mV)		EE%	
			Z-ave	Dist. width	Mean	SD	Mean	SD
DPPC:Chol:SA (6:3:1)	34.62	1.70	138.90	33.49	40.20	6.43	26.52	1.17
DPPC:Chol:SA (6:3:1)	53.85	1.70	135.83	24.50	51.70	8.99	42.40	2.69
DPPC:Chol:SA (6:3:1)	69.23	1.70	139.47	31.91	43.93	11.11	49.95	0.87
DPPC:Chol:SA (6:3:1)	107.69	1.70	147.23	34.58	48.13	8.18	51.87	3.24
DPPC:Chol:SA (6:3:1)	53.85	1.30	132.80	22.15	48.83	8.52	41.52	1.01
DPPC:Chol:SA (6:3:1)	53.85	2.60	134.70	23.03	50.10	9.29	39.62	4.16
DPPC:Chol:SA (7:2:1)	53.85	2.60	151.17	43.56	54.37	6.38	17.68	0.55
DPPC:Chol:DPTAP (6:3:1)	53.85	2.60	138.30	34.88	57.10	8.46	41.05	1.68
DSPC:Chol:SA (6:3:1)	53.85	1.30	148.23	31.95	48.83	9.72	22.18	3.45
DSPC:Chol:SA (6:3:1)	53.85	2.60	154.83	35.55	48.30	6.30	18.63	2.15
DSPC:Chol:SA (54:36:10)	70.00	3.00	151.13	25.33	43.50	8.77	34.07	1.18
DSPC:Chol:SA (6:3:1)	107.69	2.60	150.57	19.49	52.00	7.61	32.39	1.20
DSPC:DPPC:Chol:SA (3:3:3:1)	53.85	2.60	140.20	32.76	46.93	8.60	31.91	2.32
DSPC:DPPC:Chol:SA (3:3:3:1)	107.69	2.60	137.57	25.76	50.07	9.22	32.76	4.80

transition temperature (20)) to investigate the SOD-lipid interaction in the liposomes. Two representative formulations were selected (DPPC:cholesterol:SA=6:3:1 and DSPC:cholesterol:SA=6:3:1), and each formulation was tested against the corresponding empty liposome formulations to evaluate the effect of SOD on the thermo-optical properties.

For empty DSPC liposomes a very broad transition was observed (Fig. 8) and the transition temperature was 49.8°C. For empty DPPC liposomes, a similar broad transition was observed (Fig. 9) but with a much earlier transition temperature (39.3°C). Note that despite the fact that the transitions were broadened, the observed transition temperatures were very close to those of the pure lipids (DSPC=55°C, and DPPC=41°C). To exclude the possibility of protein interfering with the observation of the transitions, pure SOD solution (50 μg/ml) was tested under the same testing conditions and no change in the mean count rate was observed (Fig. 9).

Encapsulation of SOD into DSPC liposomes resulted in very little change in the sigmoidal profile of mean count rate *vs.* temperature (transition temperature= 50.6°C) as compared to empty liposomes (Fig. 8). However, for DPPC liposomes, encapsulation of SOD resulted in a distinctively different profile with two sigmoidal transitions (Fig. 10). The first transition occurred at around 34°C while the second transition was around 49°C. The two transitions were attributed to two possible domains, one enriched with SOD and the other enriched with lipids.

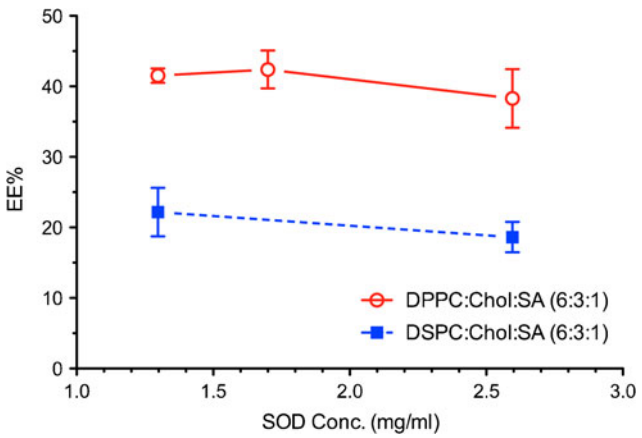

Fig. 5 Effect of SOD concentration and lipid type on protein encapsulation efficiency.

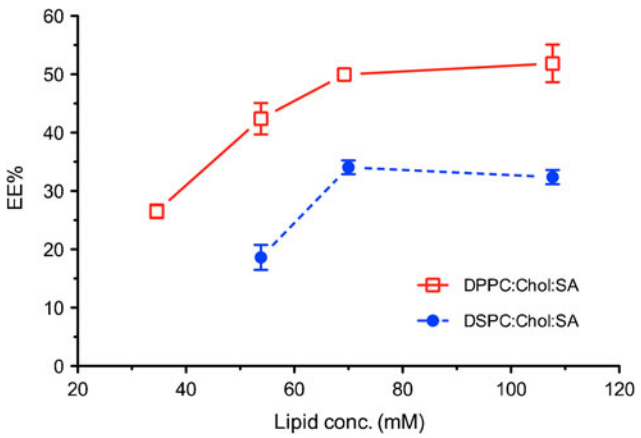

Fig. 6 Effect of lipid concentration and lipid type on protein encapsulation efficiency.

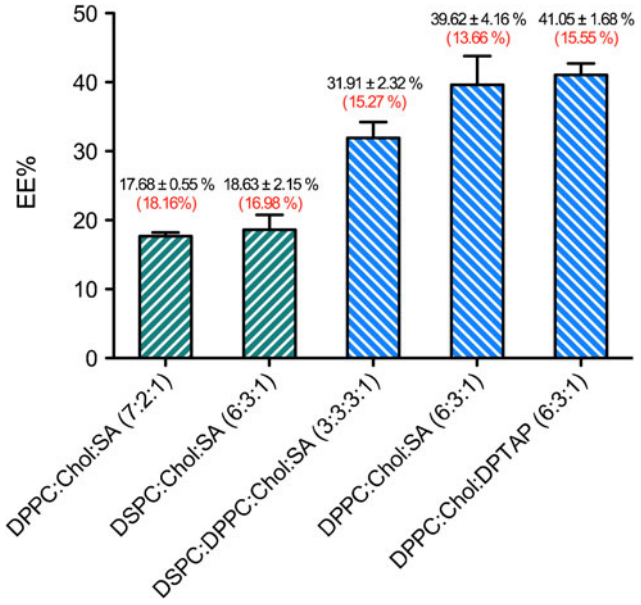

Fig. 7 Effect of formulation composition on protein encapsulation efficiency (lipid concentration=53.85 mM, SOD concentration=2.596 mg/ml). The values inside the parentheses are the mathematical model predicted values, which are only accurate when there is no lipid-drug interaction (*i.e.* the first two formulations).

DISCUSSION

Maintaining protein activity throughout the preparation process is of vital importance to ensure product quality and safety. In the current study, an improved freeze-thaw cycling technique was developed allowing a simple process to encapsulate SOD into preformed unilamellar liposomes. This method significantly reduced total processing time (to about 30 min), eliminated exposure of protein to organic solvent, as well as shortened exposure of the protein to high temperature.

As shown in Fig. 3, SOD remained active after two freeze-thaw cycles. It is worth noting that this was achieved without addition of any cryo-protectant. SOD is a relatively stable protein and may be less sensitive to freeze-thaw cycling stress compared to other proteins. In addition, it should be noted that the rate of cooling/heating in the freeze-thaw cycling process is relatively rapid, and this may have minimized any potential damage to the protein (22). For application of this freeze-thaw cycling technique to other proteins that may be more prone to thermo destabilization, it may be necessary to include stabilizing sugars (such as sucrose or trehalose) in the protein solution prior to mixing with empty unilamellar liposomes to prevent potential denaturation. Though not tested, the addition of cryo-protectants is not expected to cause any significant negative effect on the protein encapsulation process.

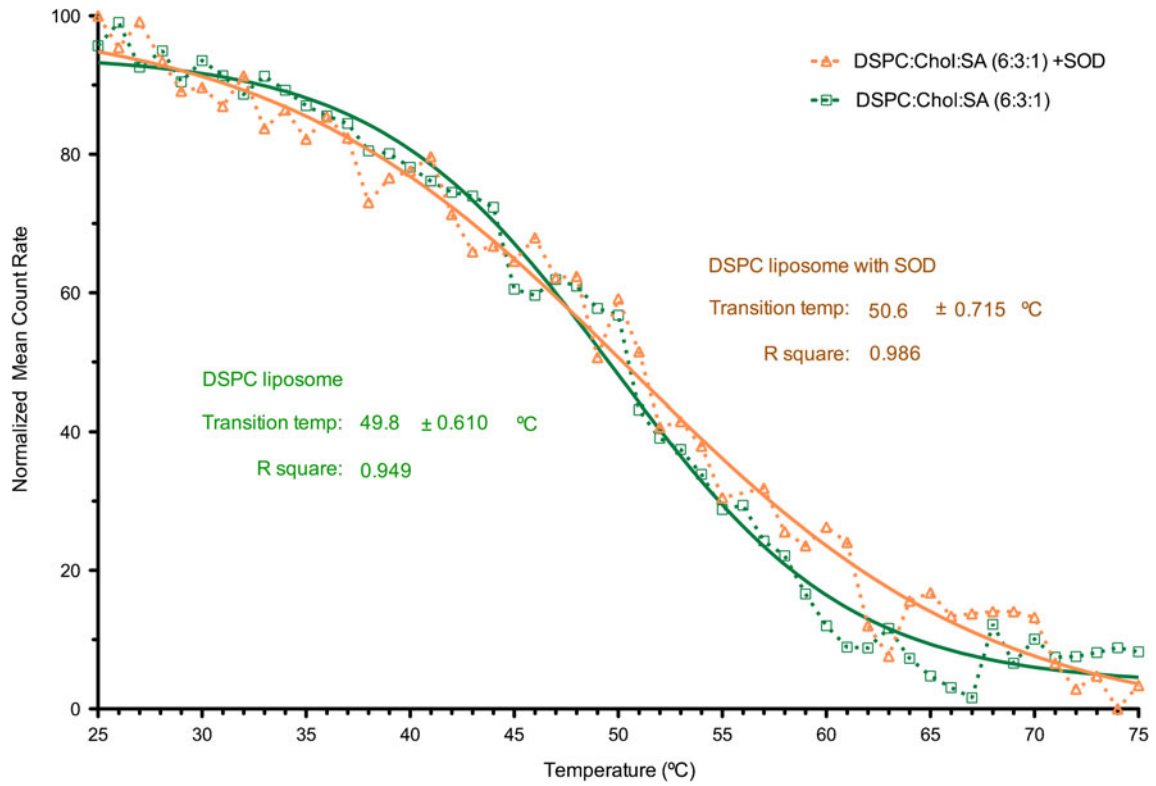

Fig. 8 Mean count rate of scattered light as a function temperature for DSPC liposomes (with and without SOD). The transition temperatures were determined using the Boltzmann sigmoidal model. All experiments were performed in 10 mM pH 7.4 HEPES buffer.

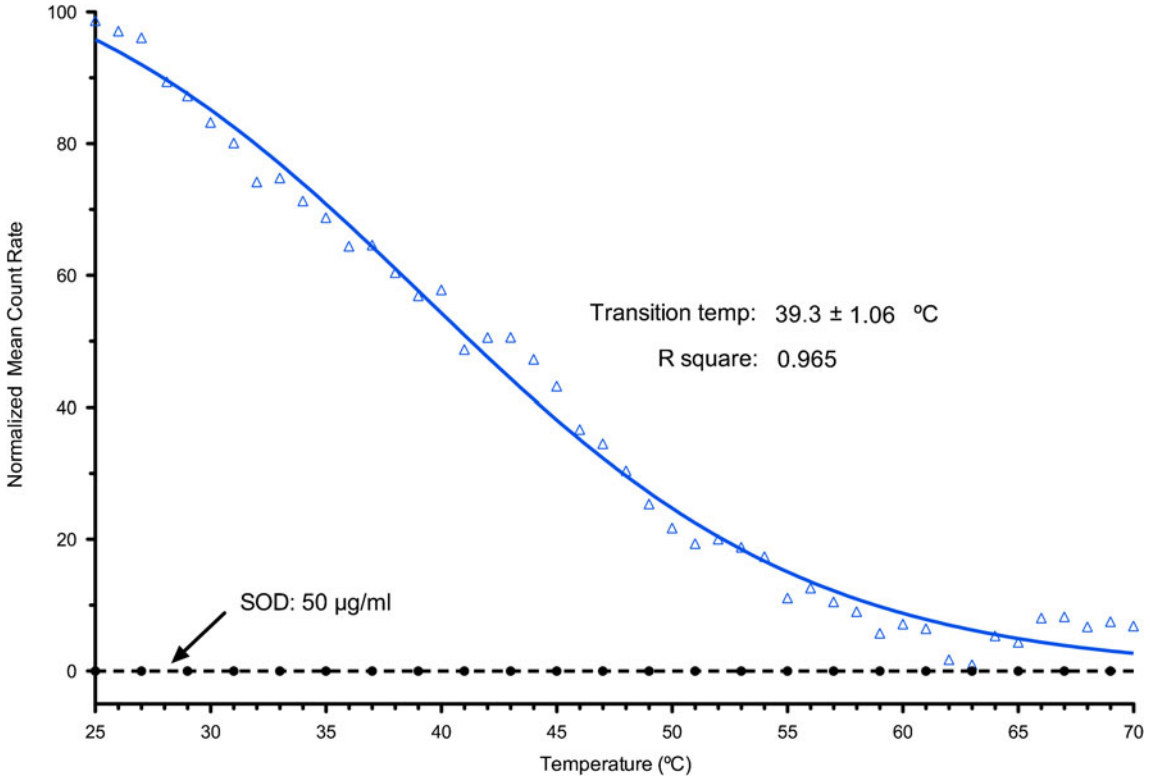

Fig. 9 Mean count rate of scattered light as a function temperature for empty DPPC liposomes and pure SOD solutions (50 μg/ml). All experiments were performed in 10 mM pH 7.4 HEPES buffer.

Encapsulation of SOD into FAT-ULV

At a first glance, the freeze-thaw cycling (FAT-ULV) technique used in the current study appears to be similar to the traditional freeze-thaw cycling approach, where samples are subjected to fast cooling and heating. However, the main difference, which is advantageous over the traditional method, is that mono-dispersed homogeneous empty liposome vesicles are mixed with protein prior to performing the freeze-thaw cycling. Compared with the traditional method that involves freeze-thaw cycling of multilamellar liposome containing the drug, the new FAT-ULV method is advantageous in that there is no need for further size-reduction following freeze-thaw cycling and hence loss of the encapsulated drug. Also since the protein is introduced at the freeze-thaw cycling stage, the exposure of the protein to hash processing conditions is significantly reduced.

In the FAT-ULV method, which involves freeze-thaw cycling of empty unilamellar liposomes with protein, it is speculated that protein passively diffuses through the lipid bilayer following bilayer fragmentation. It is known that lipid bilayers fragment during the freezing process, where ice crystals form across the bilayers at temperatures much below the phase transition temperature (around −196°C) (23). At such low temperatures, the bilayers are brittle and breakage can easily occur. During the heating process, it is speculated that water channels form following melting of the ice crystals, and these channels allow passive diffusion of the protein due to the concentration gradient across the bilayer. Upon heating above the lipid phase transition temperature, the fragmented bilayers reform as vesicles, since the open ends of the fragmented lipid bilayers cannot be tolerated in aqueous environment.

For the traditional freeze-and-thaw cycling approach used with multilamellar vesicles, it is not unusual to require more than four freeze-thaw cycles to facilitate drug encapsulation into the multilamellar vesicles (24). The newly developed FAT-ULV method is much more efficient as only one lipid bilayer needs to be fragmented before drug diffusion. Accordingly, the FAT-ULV approach only requires two freeze-thaw cycles to reach equilibration concentration and hence completion of the protein encapsulation process. Furthermore, for small hydrophilic molecules, which usually have a higher diffuse rate, it is speculated that one freeze-thaw cycle may be sufficient. This FAT-ULV method will significantly reduce the complexity of liposome processing.

After two freeze-thaw cycles, there is no significant change in liposome particle size compared to before the freeze-thaw treatment, as shown Fig. 4. This means that the liposome entrapment volume remained almost constant throughout the process. Therefore researchers can quickly predict the drug encapsulation efficiency using a previously

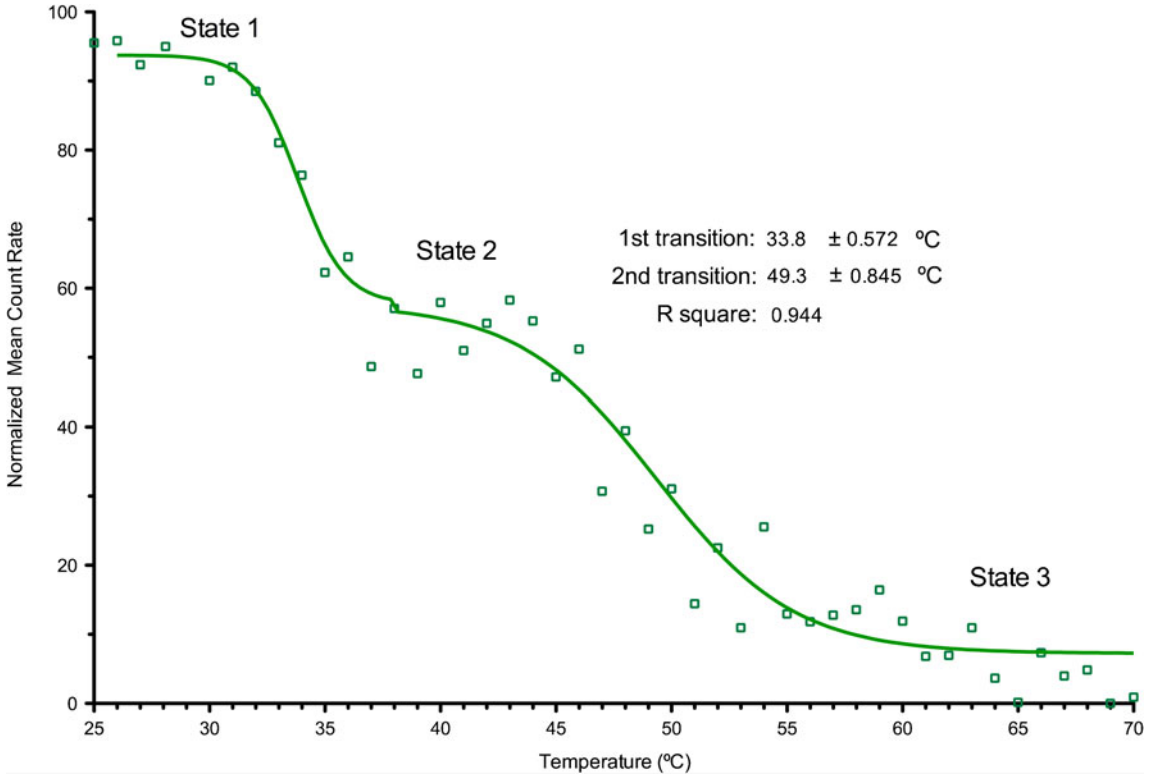

Fig. 10 Mean count rate of scattered light as a function temperature for DPPC liposomes containing SOD.

developed mathematical algorithm (25). This is because the drug encapsulation efficiency is largely defined by the internal to external volume ratio (or entrapment volume to total sample volume), which is mainly dependent on the lipid concentration and the liposome particle size as well as size distribution. These parameters are largely fixed once the empty liposomes have been mixed with the water-soluble drug. Indeed, a very good correlation was obtained in the current study for several formulations when no interactions between the lipids and the SOD occurred, such as DSPC liposomes (DSPC:cholesterol: SA=6:3:1) and DPPC liposomes with low cholesterol content (DPPC:cholesterol:SA=7:2:1). However, the accuracy of the mathematical prediction decreased dramatically when protein-lipid interaction took place as shown in Fig. 7.

Factors Affecting the Protein Encapsulation

Factors affecting protein encapsulation efficiency in liposomes can be broadly divided into two categories, the process and formulation variables. The former has already been discussed in the previous section. Based on the results obtained in the current study, the following formulation variables were shown to affect the protein encapsulation efficiency: lipid concentration, cholesterol, and main lipid type, as shown above.

It is known that lipid concentration affects the liposomal encapsulation efficiency of most hydrophilic drugs (small or large molecules). 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 (4). The total internal volume is determined by two factors: the entrapment volume of individual vesicles (which is a function of vesicle size and size distribution, as well as vesicle lamellarity) and the total vesicle number. In the current study, it was shown that the encapsulation process had very little effect on the liposome entrapment volume. Therefore, the increase in the protein encapsulation efficiency (Fig. 6) as a result of increase in lipid concentration is primarily attributed to the increase in the total vesicle number.

The other two effects (the cholesterol and main lipid type) observed in this study are considered to be a special case, resulting from an interaction between SOD and lipid. As discussed below, SOD preferentially interacts with DPPC liposomes containing high cholesterol content (30 mol%), displaying much higher protein encapsulation efficiency than both DSPC liposomes and DPPC liposomes containing low cholesterol content (20 mol%) as shown in Fig. 7. This phenomenon has not been reported before and it is believed that the freeze-thaw cycling technique used in this study may have facilitated this interaction. During the passive diffusion process, a cross-section of the lipid bilayers was exposed to the SOD molecules, allowing a much higher probability for lipid-protein interaction.

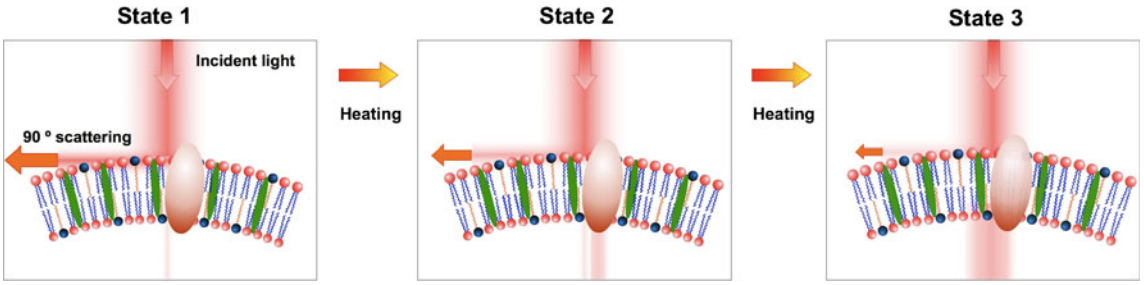

Fig. 11 Schematization of three macroscopic states of lipid bilayer/SOD during heating.

Light Scattering to Study the Interaction Between SOD and Lipids

An interesting discovery of the current study is that SOD had a preferentially strong interaction with DPPC/cholesterol (30%) liposomes, compared to weak or no interaction with DSPC/cholesterol liposomes. This preferential interaction phenomenon is considered to be due to a favorable insertion of SOD into the DPPC/cholesterol (30%) liposome bilayers. Indirect evidence in support of this speculation was obtained using a light scattering technique, where it was observed that in the presence of SOD two phase-transitions were present compared to only one phase-transition without SOD, whereas with other liposome formulations, no change in the phase transition was observed using this light scattering method. As suggested by Michel *et al.*, an alteration in the scattered light mean count rate (average number of photons detected per second) for pure lipid supramolecular dispersions, measured using dynamic light scattering, can be correlated to the refraction and absorption coefficients. Accordingly, changes in the measured scattering intensity are directly related to changes in the optical properties of the material during temperature variations. This light scattering method allows the determination of the phase transition behavior of various lipids.

In the current study, this technique was adapted to study lipid-protein interaction based on the assumption that the presence of protein inside the lipid bilayer will alter the refraction and absorption coefficients of the liposome dispersions. As shown in Figs. 8 and 9, a broad transition was observed in both DPPC/cholesterol (30%) and DSPC/cholesterol (30%) empty liposome systems. However, in the presence of SOD, a two-phase sigmoidal transition was observed, as shown in Fig. 10. The two transitions were attributed to two possible domains, one enriched with SOD and the other enriched with lipid. The protein-enriched region is considered to be responsible for the first transition (State 1 to State 2) observed at around 34°C, and the lipid-enriched region is responsible for the second transition at around 49°C (State 2 to State 3), as shown in Fig. 11. The appearance of a broadened transition as opposed to a sharp transition (lamellar gel phase L_β to liquid crystalline phase L_α) reported by Michel *et al.* is attributed to the *liquid-ordered* (*lo*) phase stabilized by cholesterol (26).

The location of SOD inside the bilayer is speculated to be dependent on the protein structure as well as the bilayer composition. The shape of SOD resembles that of an ellipsoid (27) (the two equatorial diameters are 4.29 and 4.28 nm, and the polar diameter is 6.49 nm) as shown in Fig. 12. In comparison, the thickness of the

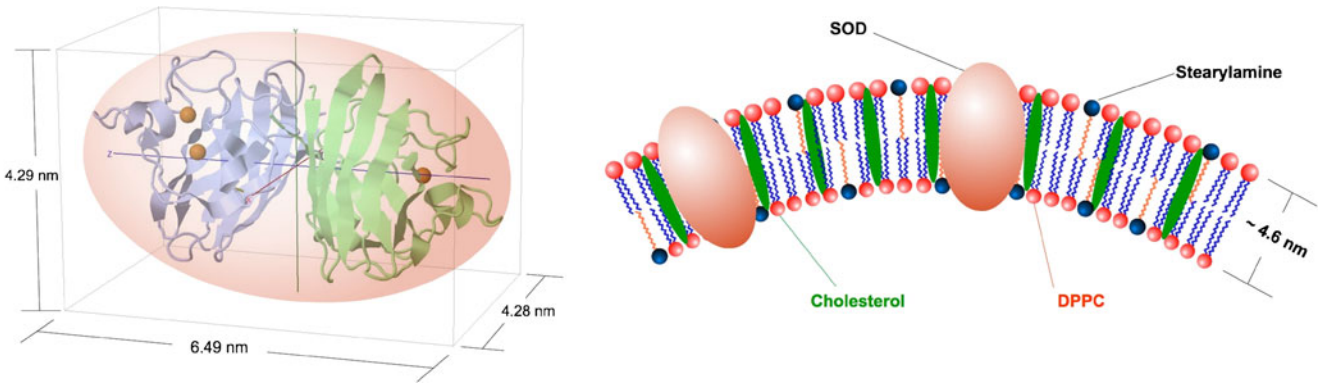

Fig. 12 A 3D structure of SOD molecule (left) and schematic drawing of possible SOD location inside DPPC/cholesterol/SA liposome bilayer (right).

DPPC liposome bilayer is about 4.6 nm and for DSPC liposomes it is about 5.1 nm. It is possible that SOD molecules vertically insert into the DPPC/cholesterol (30%) lipid bilayer, such that the beta-sheet part (flatter structure) of SOD is embedded inside the bilayer, and the alpha-helix part is extended outside. Inside the lipid bilayer, various sized of pockets (spacing) are generated in between the cholesterol molecules. These pockets allow 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 (beta-sheet). In summary, it is believed that for the SOD-DPPC/cholesterol interaction to take place, two conditions need to be both met: 1) an optimal lipid bilayer thickness (that of DPPC), and 2) the existence of optimally sized "pockets" generated by cholesterol (30%). The first condition can explain why DPPC showed a stronger interaction then DSPC. The second condition can explain why previously no SOD-DPPC interaction was found (18), as in that case only pure DPPC liposomes were tested.

CONCLUSIONS

In the current study, an improved freeze-and-thaw cycling technique is reported that is capable of encapsulating a large amount of protein in a simple step. Compared with traditional liposome preparation procedures, the newly reported process does not affect the liposome particle size, and hence does not affect the drug entrapment volume. This allows much greater flexibility in the manufacturing process, as the preparation of the empty liposomes and the protein loading operations can be separated. Accordingly, empty liposomes can be prepared without concern about protein stability, thus making the manufacturing process more flexible (with respect to solvent, temperature, *etc.*) and easy to control. Ultimately this will result in enhancement in product quality. The method reported in this article can be used for majority of the hydrophilic protein molecules as well as all the hydrophilic small molecules. Using the adapted light scattering technique, the interaction between SOD and DPPC/cholesterol (30%) liposomes is confirmed. This technique can be very useful in future studies of potential protein-lipid interactions.

ACKNOWLEDGMENTS & DISCLOSURES

The authors acknowledge the helpful discussions with Dr. Mansoor Khan at FDA. This research was supported by FDA critical path funding (HHSF223201011124P).

REFERENCES

1. Szokaand F, Papahadjopoulos D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc Natl Acad Sci U S A. 1978;75:4194–8.
2. Cortesi R, Esposito E, Gambarin S, Telloli P, Menegatti E, Nastruzzi C. Preparation of liposomes by reverse-phase evaporation using alternative organic solvents. J Microencapsul. 1999;16:251–6.
3. Hope M, Bally M, Webb G, Cullis P. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. Biochim Biophys Acta. 1985;812:55–65.
4. Xu X, Khan MA, Burgess DJ. A quality by design (QbD) case study on liposomes containing hydrophilic API: I. Formulation, processing design and risk assessment. Int J Pharm. 2011;419:52–9.
5. Lasic DD, Ceh B, Stuart MCA, Guo L, Frederik PM, Barenholz Y. Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery. Biochim Biophys Acta. 1995;1239:145–56.
6. Lasic DD, Frederik PM, Stuart MCA, Barenholz Y, McIntosh T. Gelation of liposome interior A novel method for drug encapsulation. FEBS Lett. 1992;312:255–8.
7. Banghamand AD, Papahadjopoulos D. Biophysical properties of phospholipids. I. Interaction of phosphatidylserine monolayers with metal ions. Biochim Biophys Acta. 1966;126:181–4.
8. Bangham AD, Standish MM, Watkins JC. Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol. 1965;13:238–52.
9. Duand S, Deng Y. Studies on the encapsulation of oxymatrine into liposomes by ethanol injection and pH gradient method. Drug Dev Ind Pharm. 2006;32:791–7.
10. Jahn A, Vreeland WN, DeVoe DL, Locascio LE, Gaitan M. Microfluidic directed formation of liposomes of controlled size. Langmuir. 2007;23:6289–93.
11. Kremer J, Esker M, Pathmamanoharan C, Wiersema P. Vesicles of variable diameter prepared by a modified injection method. Biochemistry. 1977;16:3932.
12. Peschka R, Dennehy C, Szoka Jr F. A simple *in vitro* model to study the release kinetics of liposome encapsulated material. J Control Release. 1998;56:41–51.
13. Keele BB, McCord JJM, Fridovich I. Further characterization of bovine superoxide dismutase and its isolation from bovine heart. J Biol Chem. 1971;246:2875–90.
14. McCordand JM, Fridovich I. Superoxide dismutase. J Biol Chem. 1969;2444:6049–55.
15. Okado-Matsumotoand A, Fridovich I. Subcellular distribution of superoxide dismutases (SOD) in rat liver. J Biol Chem. 2001;276:38388–93.
16. Zhang Y, Wang J, Wu Y, Li W. Anti-inflammatory effect of recombinant human superoxide dismutase in rats and mice and its mechanism. Acta Pharmacol Sin. 2002;23:439–44.
17. Gasset M, Albar J, Lacadena J. Membrane interaction of a beta-structure-forming synthetic peptide comprising the 116-139th sequence region of the cytotoxic protein alpha-sarcin. Biophys J. 1995.
18. Loand YL, Rahman YE. Protein location in liposomes, a drug carrier: a prediction by differential scanning calorimetry. J Pharm Sci. 1995;84:805–14.
19. Papahadjopoulos D, Moscarello M, Eylar E. Effects of proteins on the thermotropic phase transitions of phospholipid membranes. Biochim Biophys Acta. 1975;401:317–35.

20. Michel N, Fabiano A-S, Polidori A, Jack R, Pucci B. Determination of phase transition temperatures of lipids by light scattering. Chem Phys Lipids. 2006;139:11–9.
21. Mertins O, Lionzo M, Micheletto Y. Chitosan effect on the mesophase behavior of phosphatidylcholine supramolecular systems. Mater Sci Eng C. 2009;29:463–9.
22. Cao E, Chen Y, Cui Z, Foster PR. Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. Biotechnol Bioeng. 2003;82:684–90.
23. Castileand JD, Taylor KM. Factors affecting the size distribution of liposomes produced by freeze–thaw extrusion. Int J Pharm. 1999;188:87–95.
24. Colletier J-P, Chaize B, Winterhalter M, Fournier D. Protein encapsulation in liposomes: efficiency depends on interactions between protein and phospholipid bilayer. BMC Biotechnol. 2002;2:9.
25. Xu X, Khan MA, Burgess DJ. Predicting hydrophilic drug encapsulation inside unilamellar liposomes. Int J Pharm. 2012;423:410–8.
26. Vistand MR, Davis JH. Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: 2H. Biochemistry. 1990;29:451–64.
27. Hough MA, Strange RW, Hasnain SS. Conformational variability of the Cu site in one subunit of bovine CuZn superoxide dismutase: the importance of mobility in the Glu119-Leu142 loop region for catalytic function. J Mol Biol. 2000;304:231–41.