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# Freeze-drying of liposomes using tertiary butyl alcohol/water cosolvent systems

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**Abstract**

The objective of this study was to obtain dehydrated liposomes using a novel procedure that involves freeze-drying (FD) of liposomes with TBA/water cosolvent systems. The effects of TBA on the integrity/stability of vesicles of HSPC (or SPC):Cholesterol (4:1) were investigated. TBA used as a cosolvent was detrimental to SPC liposomes, leading to increased particle size and leakage of trapped calcein. However, this was not the case for HSPC liposomes. The vesicle size and the retention of trapped calcein after lyophilization from cosolvents were similar to those after FD from water alone. Moreover, the addition of TBA can significantly enhance the sublimation of ice resulting in short FD cycles. The resulting lyophilized cake can form a loose powder upon agitation, which flowed well enough to be easily poured from the vial. Thus FD of HSPC liposomes using TBA/water cosolvent systems can provide sterile powder for specialized applications. In addition, in conjunction with a modified injection method, this FD technology might be used to produce dehydrated HSPC liposomes on a large scale.


*Keywords:* Liposomes; Preparation; Freeze-drying; Cosolvents; Tertiary butyl alcohol

## 1. Introduction

The superiority of liposomes as drug carriers has been widely recognized. Ten liposomal and lipid-based formulations have been approved by regulatory authorities and many liposomal drugs are in preclinical development or in clinical trials (Maurer et al., 2001). In order for its clinical benefit to be realized, a liposomal product must meet requirements with respect to the chemical and physical stability of both drug and carrier. Usually, aqueous liposome suspensions may be subject to a series of stability problems such as aggregation, fusion, phospholipid hydrolysis and the leakage of the encapsulated drugs. One approach to resolve this kind of problems is freeze-drying of liposomal products in the presence of protective disaccharides (Crowe and Crowe, 1993). However, the freeze-drying cycles of sugar solutions are excessively long and freeze-drying is capital-, labor- and energy-intensive in comparison with other drying processes, thus limiting the application of this technology largely. Recently, many studies show that the addition of tertiary butyl alcohol (TBA) can considerably enhance the rate of ice sublimation, resulting in short drying cycles of sucrose solutions (DeLuca et al., 1989; Kasraian and DeLuca, 1995b; Oesterle et al., 1998). Therefore, it is desirable to freeze-drying of liposomes using TBA/water cosolvent systems if for economy concerns.

Although freeze-drying of liposome-forming lipids from TBA (or aqueous TBA) has been successfully applied to prepare liposomes (Evans et al., 1982; Amselem et al., 1990; Li and Deng, 2004), to date no studies have been performed on lyophilization of already formed liposomes using TBA/water cosolvent systems. Due to the characteristics of liposomes, the influences of TBA on the structural integrity of liposomes and the retention of the encapsulated materials must be investigated. In the present study, we mainly examined the size of liposomes and the efficiency with which the trapped materials was retained both before and after lyophilization in the presence of TBA. In addition, the influences of TBA on the freezing and drying process and the final freeze-dried cakes were also discussed. Calcein, a frequently used water-soluble marker, was chosen as model drug (Komatsu et al., 2001). Two different kinds of

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vesicles were used, which were composed of HSPC:Chol (4:1) and SPC:Chol (4:1), respectively. The reason why the authors selected these two kinds of vesicles was that previous studies had shown that vesicles of fluid PC such as SPC displayed different behavior during freezing, drying and rehydration process in comparison with vesicles of more saturated HSPC (Crowe and Crowe, 1993).

## 2. Materials and methods

### 2.1. Lipids and chemicals

Soybean phosphatidylcholine (SPC) and hydrogenated soybean phosphatidylcholine (HSPC) were kind gifts from Degussa (Freising, Germany). $MPEG_{2000}$-DSPE was provided by Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Shenyang Medicine Company (Shenyang, China). Calcein was purchased from Sigma (St. Louis, USA). All other chemicals were of analytical reagent grade.

### 2.2. Liposome preparation

Liposomes were prepared using a modified alcohol injection method. Sucrose was chosen as preserving agent, which was added on both sides of liposomes. The initial vesicle size before lyophilization was reduced to about 100 nm using extrusion to omit the influence of size. TBA content in all formulations was not >10% (v/v) because excess TBA may destroy liposomes (Li and Deng, 2004). The preparation process was briefly described as below.

#### 2.2.1. Liposomal calcein with different sugar/lipid ratio

Mixtures of HSPC (or SPC) and cholesterol (4:1, w/w) were dissolved in TBA at 60 °C to form an optically clear solution. The total lipid concentration in TBA solution was 0.2 g total lipid per mL TBA. About 1 mL dissolved lipid solution was transferred to a conical flask containing 20 mL of 2 mM calcein solution equilibrated to 60 °C. The calcein solutions used here contained 0.5%, 1%, 2.5% and 5% sucrose (w/v), respectively. Then the mixture was mixed continuously for 1 h while maintaining the temperature using a 60 °C water bath to form oligolamellar TBA hydration liposomes. The resulting heterogeneous liposomes were extruded 10 times through 100 nm pores using a 10 mL thermobarrel extruder (Northern Lipids Inc., Vancouver, Canada). The free calcein was removed by centrifugation at 50,000 × *g* for 30 min using CS120GX ultracentrifuge (Hitachi Koli Co., Japan). After three-time washing, the precipitates were resuspended using initial external phases. A total of seven samples were prepared. Because when the sugar/lipid weight ratio was up to 5, it was hard to separate SPC liposomes from free calcein using centrifugation due to the high viscosity of sucrose solution, this sample was not prepared. The lipid concentrations of all final liposome preparations were 1% (w/v) and the sucrose/lipid weight ratios ranged from 0.5 to 5 for HSPC samples and from 0.5 to 2.5 for SPC liposomes. After sample preparation, 1.5 mL liposomal calcein was instantly filled into 10 mL vials and subjected to lyophilization or freeze-thawing.

#### 2.2.2. Liposomal calcein with different TBA content

The samples were prepared in the same way. But upon mixing, 1.0 mL (0.200 g/mL), 1.5 mL (0.133 g/mL) and 2.0 mL (0.100 g/mL) lipid solutions were added to 20 mL of 2 mM calcein solution contained 2.5% (w/v) sucrose. A total of six samples were prepared, namely, three SPC samples and three HSPC samples.

In order to prepare liposome samples without TBA, mixtures of HSPC (or SPC) and cholesterol (4:1, w/w) were dissolved in TBA to form clear solutions, which were subjected to freeze-drying. Following lyophilization, 0.2 g dry lipid powder was hydrated using 20 mL of 2 mM calcein solution contained 2.5% (w/v) sucrose at 60 °C for 1 h. The resulting heterogeneous liposomes were further treated as described in Section 2.2.1. Here one SPC sample and one HSPC sample were prepared.

#### 2.2.3. Samples for size measurement

For the purpose of determining the changes of vesicle size before and after freeze-drying, four different samples were prepared using above preparation procedure. The main difference among these four samples was that upon mixing different hydration buffers were used. All the buffers contained 150 mM sucrose. For SPC samples, the sucrose solutions were buffered using 50 mM glycine and Tris–HCl, respectively. For HSPC samples, the hydration buffers were buffered with 50 mM glycine and 50 mM oxalic acid, respectively. After mixing and extrusion, the samples were directly filled into the vials, and separation step was omitted. The lipid concentrations of all final liposome preparations were 1% (w/v), the sucrose/lipid weight ratio was 5 and TBA/water volume ratio was 1:20. A total of four samples were prepared.

### 2.3. Freeze-drying process

A laboratory freeze-drier (Bioking Technology Co. Ltd., China) was used. The freeze-drying process was as follows: (1) the samples were cooled from 25 to −40 °C at a rate of 0.5 °C/min and then maintained at −40 °C for 8 h; (2) primary drying was performed at −40 °C for 48 h; (3) the samples were heated from −40 to 25 °C at a rate of 1 °C/min and dried at 25 °C for 10 h. The chamber pressure was maintained at 20 Pa during the drying process.

### 2.4. Freeze-thawing process

The samples were cooled in the freeze-drier at a rate of 0.5 °C/min from 25 to −40 °C and then maintained at −40 °C for 8 h to allow for complete solidification. After freezing, the vials were inserted into 25 °C water bath until the melting process finished. The heating rate was about 2 °C/min.

### 2.5. Reconstruction of freeze-dried samples

Prior to use, 1.5 mL of purified water was added into the vial. The rehydration was performed at 25 °C, and following the addition of water the sample was equilibrated at 25 °C for 30 min.

### 2.6. Measurement of entrapment efficiency

Calcein trapped in the liposomes was assayed before and after lyophilization (or freeze-thawing), thus providing an estimate of the efficiency with which calcein was retained. The encapsulation efficiency of calcein was determined using a method developed by Oku et al. (1982). Briefly, the fraction of encapsulated calcein was obtained as the fraction of the fluorescence that remained after adding cobalt$^{2+}$ ions which, when chelated by calcein, quench its fluorescence. For each sample, at least three independent measurements were performed.

### 2.7. Size measurement

The size measurement was performed using two different methods and each sample was measured three times. Firstly, the size of liposomes was determined at 25 °C using LS 230 Particle Size Analyzer (Beckman Counter Inc., USA). LS 230 adopts a patented PIDS technology for enhanced resolution of submicron size particles and permits analysis of particles without the risk of missing either the largest or the smallest particles in a sample. The calculation of the data was performed using the LS32 (version 3.19) software. A PSL optical model was used. For accurately determining the size of submicron liposomes, a submicron particle analyzer—Zetasizer Nano ZS (Malvern Instruments, UK) was also used. The measurement was performed at 25 °C. A DTS software (version 4.0) was used to collect the data that were analyzed using "multinarrow modes".

### 2.8. Thermal analysis

Differential scanning calorimetry (DSC) was carried out with Perkin-Elmer 7 series thermal analysis system that was equipped with a mechanical cooling. A total of six liquid samples (c1, s2, slc3, hlc4, sls5 and hls6) were analyzed. Sample c1was a mixture of TBA and water. Sample s2 was a solution of 5% (w/v) sucrose in TBA/water cosolvent. Sample slc3 was a suspension of 1% (w/v) SPC liposomes in TBA/water cosolvent. Sample hlc4 was a suspension of 1% (w/v) HSPC liposomes in TBA/water cosolvent. Sample sls5 was a mixture of TBA and water containing 5% sucrose and 1% SPC liposomes (w/v). Sample hls6 was a mixture of TBA and water containing 5% sucrose and 1% SPC liposomes (w/v). In all cases, the TBA/water volume ratio was equal to 1:20.

For analysis, an aliquot of the sample solution (ca. 10 mg) was pipetted into an aluminum pan and the pan was capped with a hand press to prevent the evaporation of the solution. The scanning procedure was performed as the following. The solutions were frozen to −50 °C at a rate of 0.5 °C/min and then heated at a rate of 2 °C/min to 30 °C.

### 2.9. Cryomicroscopy

A cryomicroscope was created by interfacing a cold stage with an optical microscope. This system was used to observe the freezing process of the liposome suspensions. Typically, the sample solution was cooled to −40 °C at a cooling rate of 0.5 °C/min and a polaroid camera was used to photograph the changes.

### 2.10. Mass loss analysis

To investigate the influence of TBA on the sublimation of ice, two formulations were used. Formulation 1 was a suspension of 1% (w/v) liposomes (HSPC:Chol = 4:1, w/w) in 250 mM sucrose solution. Formulation 2 had the same composition as formulation 1 but with 5% (w/v) TBA. For analysis, preweighed 10 mL vials were filled with 1 mL solution, reweighed and transferred to the freeze-drier. The solutions were cooled at −40 °C for 8 h to allow for the complete solidification. Primary drying of the solutions was undertaken at −40 °C and the chamber pressure was maintained at 20 Pa during the drying process. Samples were removed at various stage of drying and weighed. The mass loss at each time point was determined using three vials of samples.

### 2.11. Residual solvent content

The total amount of residual solvents was determined by thermogravimetric analysis (TGA), which was conducted on 3–5 mg samples in a nitrogen atmosphere at a heating rate of 10 °C/min from 25 to 150 °C using TGA 50 (Shimadzu Co., Japan). The weight loss was used to calculate the total amount of residual solvents. The residual levels of TBA were measured using gas chromatography method described by Gogineni et al. (1993). Thus, the weight percentage of water in the lyophilized cakes can be calculated by subtraction of the residual level of TBA from the total amount of residual solvents. Using above method, the amount of residual solvents in all typical formulations were determined.

## 3. Results and discussion

### 3.1. Influences of TBA on calcein retention and liposome size

Although lyophilization of liposomes from water alone has been carefully investigated (Crowe and Crowe, 1989; van Winden, 2003; van Winden et al., 1997), there are almost no available data on freeze-drying of liposomes with TBA/water cosolvent systems. Accordingly, in this study we mainly examine the effects of following factors: sugar/lipid mass ratio, lipid composition, and TBA content.

#### 3.1.1. Effects of sugar/lipid mass ratio and lipid composition

As shown in Table 1, if the sugar/lipid ratio was >0.5, the retention of calcein by HSPC liposomes after freeze-thawing and freeze-drying had no marked changes. However, things became different for SPC liposomes. Although FT samples still showed no difference in the retention of calcein, for the FD samples, the percentage retention of calcein increased with the increasing sugar/lipid ratio when the sucrose/lipid ratio ranged from 0.5 to 2.5. To our knowledge, by means of determining the percentage retention of calcein after freeze-thawing, the injury that

Table 1
Retention (%) of calcein after freeze-drying (FD) and freeze-thawing (FT)

| Lipid composition, mass ratio | Sucrose/lipid, mass ratio | %Retention, after FT ± S.D. | %Retention, after FD ± S.D. |
|---|---|---|---|
| HSPC/Chol, 4:1 | 0.5 | 67.7 ± 2.8 | 60.2 ± 2.5 |
| | 1 | 74.5 ± 6.1 | 60.5 ± 5.0 |
| | 2.5 | 71.7 ± 3.0 | 58.3 ± 6.3 |
| | 5 | 74.3 ± 2.4 | 61.4 ± 2.1 |
| SPC/Chol, 4:1 | 0.5 | 58.2 ± 5.9 | 43.0 ± 1.9 |
| | 1 | 50.4 ± 8.6 | 47.2 ± 3.4 |
| | 2.5 | 60.0 ± 4.0 | 52.5 ± 6.3 |

Each value is shown as mean ± S.D., $n = 3$.

liposomes suffered during the freezing process could be roughly evaluated. Similarly, the injury induced by freeze-drying process could also be determined through the measurement of calcein retention following freeze-drying and rehydration (Crowe and Crowe, 1989). It was found that for HSPC liposomes, ~30% injury resulted from freezing process and ~10% from drying process. In comparison with HSPC liposomes, SPC liposomes were much sensitive to the freezing process. For example, after freeze-thawing of a sample with a sugar/lipid ratio of 2.5, about 40% calcein was lost. Therefore, it was concluded that freeze-drying of liposomes using cosolvent systems was suitable for HSPC liposomes, not for SPC liposomes.

### 3.1.2. Effect of TBA content

As shown in Fig. 1, the presence of a small amount of TBA (<10%, v/v) had no significant influence on the retention of calcein by HSPC liposomes. The retention of calcein almost remained constant irrespective of whether TBA was supplemented or not. This result showed that during the lyophilization process, HSPC liposomes might suffer the same injury as in the absence of TBA. However, it was not the case for SPC liposomes. It could be found in the same figure that the retention of calcein by SPC liposomes was strongly influenced by TBA content. The leakage of calcein from SPC liposomes significantly increased with the increasing TBA content.

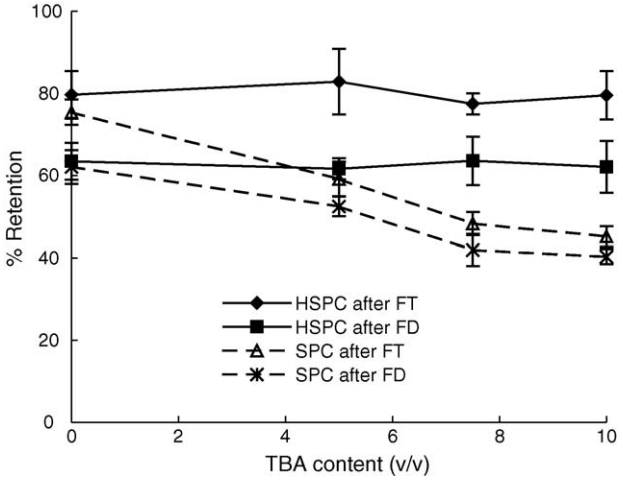


Fig. 1. Influences of TBA content on retention of calcein by HSPC and SPC liposomes. Each value is shown as mean ± S.D., $n = 3$.

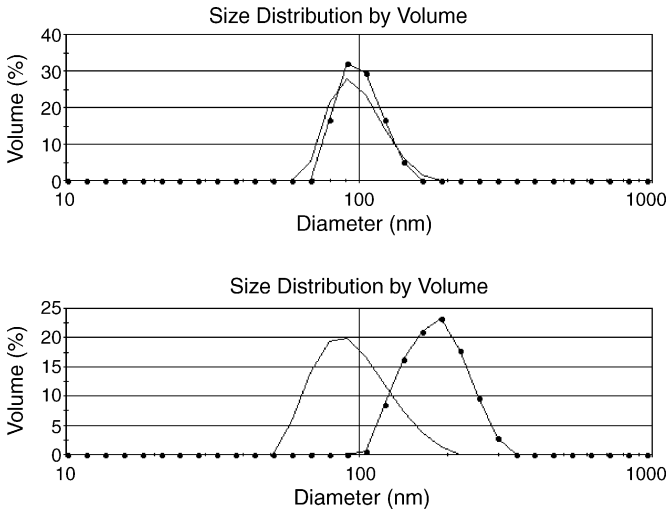


Fig. 2. Typical size distribution graphs of SPC and HSPC liposomes before and after lyophilization. A 1% HSPC (or SPC) vesicles are suspended in 50 mM glycine and 150 mM sucrose solution. Upper inset: HSPC liposomes; bottom inset: SPC liposomes. Solid line (—): samples before freeze-drying; solid line with dot (•—•): samples after freeze-drying. Note: for SPC sample after freeze-drying, there are large particles that cannot be detected by Nano ZS.

### 3.1.3. Size change before and after freeze-drying

Fig. 2 provides typical size distribution graphs of both SPC and HSPC liposomes before and after freeze-drying. As shown in this figure, freeze-drying of liposomes in the presence of TBA had remarkable influence on the size of SPC liposomes. Usually, the size of SPC liposomes after freeze-drying would significantly increase in comparison with the initial vesicle size. However, this process exerted almost no influences on the size and size distribution of HSPC liposomes. These data were in agreement with the results from calcein leakage. Both experiments showed that this freeze-drying technology might be suitable for the production of dehydrated HSPC liposomes.

## 3.2. Influences of TBA on the freeze-drying process and final cakes

In accordance with the phase diagram of TBA/water mixtures, cooling TBA/water cosolvent systems with a TBA weight percentage <20% would result in the crystallization of ice and eutectic A in turn (Kasraian and DeLuca, 1995a). In our studies, the TBA content was usually <10%, thus cooling the corresponding systems should also lead to the formation of these two kinds of crystals. To prove this, the freezing characteristics of relevant samples were investigated using DSC technology. Data on DSC assay were presented in Fig. 3. It was shown that when sample c1 was cooled, DSC thermogram displayed two peaks; one was related to the melting of eutectic A, and the other corresponded to the melting of ice. When the systems contained liposomes (samples slc3 and hlc4), there were still two peaks; furthermore, the shapes and positions of the peaks had no obvious changes. However, when the systems contained sucrose, the positions and relative height of eutectic A peak changed significantly, thus revealing the strong interaction between sucrose and eutectic A. Because in comparison with freeze-drying using

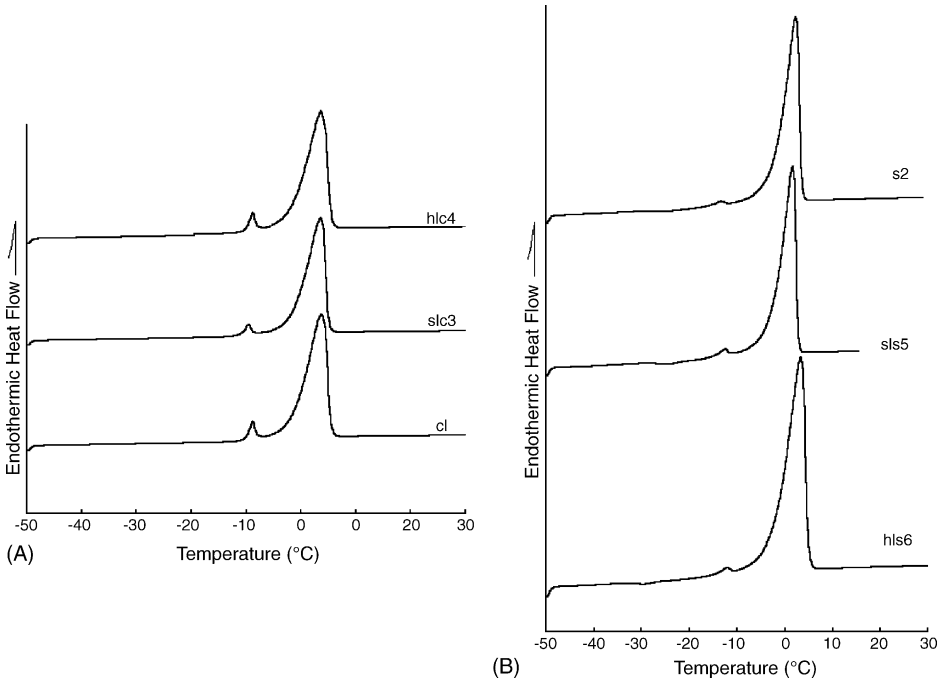


Fig. 3. DSC thermograms for (A) c1, slc3 and hlc4; (B) s2, sls5 and hls6. Without sucrose (inset A), the enthalpy value for ice crystallization is 316.208 ± 1.771 J/g and that for TBA/water eutectic A crystallization is about 21.562 J/g. With sucrose (inset B), the enthalpy value for ice peak is 279.494 ± 3.805 J/g and that for TBA/water eutectic A crystallization is reduced to about 9.962 J/g.

water as solvent, an additional eutectic A crystal would arise during the cooling process, it is reasonable to conclude that the extra injury that SPC liposomes suffered might be attributed to the formation of eutectic A. It might be just because HSPC liposomes were not sensitive to the crystallization of eutectic A that the existence of TBA had no obvious influences on HSPC liposomes. Simultaneously, it is seen from Fig. 3 that sucrose can significantly inhibit the crystallization of eutectic A, which might expound why increasing sucrose/lipid ratio induced the increased retention of calcein by SPC liposomes.

The addition of low concentrations of TBA has been shown to influence the crystal habit of ice. It is also the case in our study. Based on our observation, in the presence of TBA, needle-shaped ice crystals were formed when the liposome suspensions were cooled. Fig. 4 presents a typical microscopic view of frozen liposome suspensions. The needle-shaped ice crystals formed in the presence of TBA had a substantially larger surface area than the spherical ice crystals formed when TBA was not present (Kasraian and DeLuca, 1995b). The higher specific area contributed to the faster removal of solvents, which was in agreement with the results from mass loss analysis. Referring to Fig. 5, when the liposome suspensions were supplemented with a small fraction of TBA, the sublimation rate of solvents from the frozen solutions was enhanced. The acceleration of sublimation of solvent resulted in short drying cycles. Thus freeze-drying of liposomes using TBA/water cosolvent systems is economical in comparison with the traditional process.

A remarkable influence of TBA on the final liposomal products was that the lyophilized cakes were friable. Manual shaking of the vials or tapping the vials against a hard surface led to the formation of a loose powder that can be freely poured from the vials. This quality attribute of dried cakes may result from the modification of the ice habit in the presence of TBA. As mentioned above, when TBA was present, needle-shape ice crystals other than spherical ice crystals were formed. After solvent removal, a porous dried product with a high specific surface area left. Therefore, the products were more friable than the same systems when lyophilized from water alone (Wittaya-Areekul et al., 2002). This attribute may be helpful for specialized applications where the sterile liposomal drug powders are preferable. Accordingly, the freeze-drying of liposomes using TBA/water cosolvent systems is a useful means for the production liposome powders for topical use.

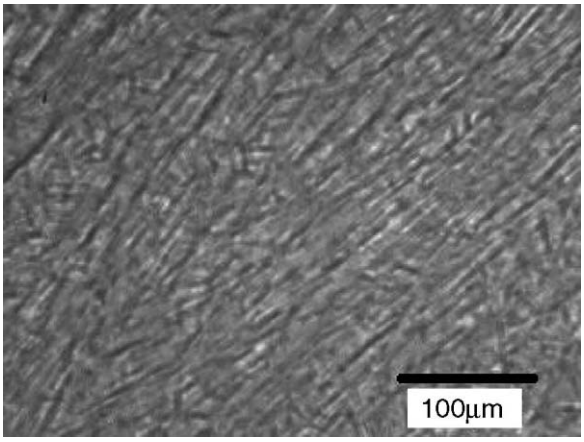


Fig. 4. Microscopic view of frozen HSPC liposomes. Formulation composition: HSPC/Chol (4:1, w/w), lipid concentration (1%, w/v), 50 mM glycine, 150 mM sucrose, TBA/water (5%, v/v).

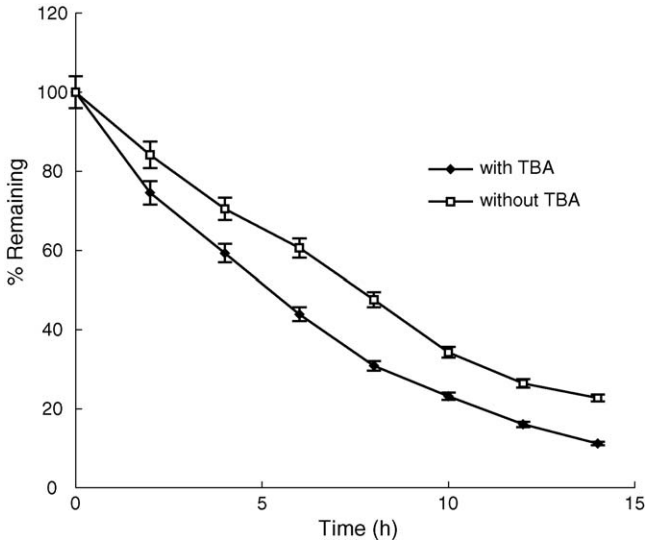


Fig. 5. Sublimation of solvents from two different liposome preparations. Formulation 1 (□): 1% (w/v) liposomes (HSPC/Chol = 4:1, w/w) in 250 mM sucrose solution. Formulation 2 (♦) has the same composition but with 5% (v/v) TBA. Each value is shown as mean ± S.D., $n = 3$.

### 3.3. Residual solvents

In general, the residual TBA levels were 0.8% or less and the residual water levels were less than 2% under all formulation and processing conditions. Although TBA was not listed in the International Conference on Harmonization (ICH) guidance document on impurities, it is likely to fall in the category of a class 3 solvent based on the similarity of acute $LD_{50}$ toxicity data for other class 3 solvents (Teagarden and Baker, 2002). Therefore, the low level of TBA in the lyophilized cakes should not be harmful to both animal and human. And this freeze-drying technology might be acceptable even for safety concerns.

### 3.4. Possible application of this freeze-drying technology

As described in the experimental section, the freeze-drying technology might be especially effective in conjunction with a modified alcohol injection method for production of dehydrated liposomal products. The full procedure should be composed of mixing, extrusion, removal of free drug and freeze-drying. It should be noted that step 3 could be omitted if the encapsulation efficiency of pharmaceuticals is acceptable. In order to remove free drug, cross-flow dialysis can be employed in large-scale production procedure; for laboratory preparation, both gel filtration chromatography and centrifugation are feasible. If all the operations are performed under sterile circumstances, the sterile liposomal products can be obtained.

## 4. Conclusions

Based on above data, it is possible to produce dehydrated HSPC liposomes by means of freeze-drying of HSPC liposomes with TBA/water cosolvent system. The addition of a small amount of TBA not only has no obvious influences on the HSPC vesicle size and the retention of trapped calcein, but also can result in short freeze-drying cycles. Moreover, freeze-drying of HSPC liposomes from TBA/water cosolvent systems can provide sterile powder for specialized applications. In conjunction with a modified alcohol injection method, this technology may be used to produce dehydrated HSPC liposomes on a large scale.

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