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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.