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Solidification of liposomes by freeze-drying: The importance of incorporating gelatin as interior support on enhanced physical stability

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ABSTRACT

The main purpose of this study was to investigate the effect of gelatin as interior support on the physical stability of freeze-dried liposomes. Anticancer agent paclitaxel (PTX) was selected as a model drug. Freeze-dried liposomes containing interior gelatin support (GLs) were prepared by thin-film dispersion/freeze-drying method. Several properties of the GLs, including entrapment efficiency, particle size and gelation temperature, were extensively characterized. Encapsulation efficiency of conventional liposomes (CLs) and liposomes containing lyoprotectants as interior support dropped to lower than 20% after reconstitution, while GLs still maintained an entrapment efficiency of over 84%. Scanning electron microscopy revealed well preserved liposomal structure of GLs after reconstitution. Meanwhile, the particle size and entrapment efficiency of GLs were also well preserved after reconstitution. In contrary, deformation of CLs and recrystallization of PTX were observed, as well as significant changes in particle size and entrapment efficiency. Taken together, interior gelatin support obviously enhanced the physical stability of liposomes against the lyophilization stress.

1. Introduction

Liposomes, with the fundamental structure of a water phase enclosed within vesicles of phospholipid bilayers, have been extensively studied as carriers for efficient delivery of therapeutic agents since the first report by Bangham et al. (Bangham et al., 1965). The superiority of liposomes as drug carriers is now widely recognized and advances in this field have resulted in the development of a few approved liposomal products (Fan and Zhang, 2013; Sen and Mandal, 2013). However, the potential application of liposomes as therapeutic tools is still being challenged by their inherent physical and chemical instability, which may result in increased bilayer permeability, drug leakage, vesicle aggregation/fusion and precipitation (du Plessis et al., 1996; Kang et al., 2013). This instability may aggravate due to bilayer defects induced by chemical degradation (e.g., lipid oxidation and hydrolysis) as well as physical factors such as heating and freezing, or during a phase transition (Ingvarsson et al., 2011). The most efficient approaches to resolve this kind of problems are to dry the liposomal products so as to render them stable, such as air drying (Wolkers et al., 2004), freeze drying (lyophilization) (Liu et al., 2013; van den Hoven et al., 2012), spray drying (van den Hoven et al., 2012), spray-freeze drying (Sweeney et al., 2005), and drying using supercritical fluid technology (Karn et al., 2013). Among these, lyophilization is the main approach used to extend the shelf-life of liposomes, especially for liposomes containing thermo-sensitive drugs. However, drying of liposomes is not an easy task. The ideal drying techniques should be able to preserve not only the physical structure of liposomes but also their physicochemical properties such as particle size, zeta-potential and encapsulation capacity. The dried liposomes are expected to be able to reconstitute into their original counterparts upon hydration without significant variation in their fundamental properties.

However, the art of liposome drying is far below our expectation up to now. It remains to be a challenge to prepare liposomes into dried formulations with well-preserved physical structure and properties. It is reported that the freeze-drying procedures might strongly affect liposome structure (Benjakul et al., 2011; Misra et al., 2009; Stark et al., 2010). Shrinking of the bilayers during freezing can result in damage of the liposomal membranes, dehydration can render vesicle fusion/aggregation and a phase transition may occur during rehydration (Wessman et al., 2010). Usual approach to overcome these problems is to use lyoprotectants such as carbohydrates to form stable boundaries between the vesicles. The effect of lyoprotectants has been attributed to their ability to replace the bound water around the

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bilayers via interaction with the polar region of the lipid head groups (water replacement hypothesis) (Chen et al., 2010). Alternatively, the formation of a vitreous layer of the lyoprotectants around the bilayer, with a high viscosity and low mobility, prevents liposome fusion/aggregation, avoids the phase transition and protects lipid bilayers from damage by ice crystals (Chen et al., 2010).

Until now, many physiologically inert substances such as saccharides, polyols, cyclodextrins, amino acids and many other additives have been used as lyoprotectants (Chen et al., 2010; Huang et al., 2013; Mohammed et al., 2007; Stark et al., 2010). Despite of the protective effect of lyoprotectants, the liposomal structure can hardly be restored after reconstitution due to collapse and rupture of the liposomes during drying. To perform better freeze-drying of liposomes, our hypothesis in this study is to incorporate some kind of support in the liposomal interior to preserve the liposomal structure to survive the freeze-drying stress.

Herein, we prepared liposomes containing gelatin in the inner water phase with the aim to preserve the structure of the liposomes during freeze-drying. The rationale of this technique is that gelatin solution incorporated at higher temperature, over its gelling temperature (T_{gel}), will undergo sol–gel transition when the temperature drops down to its T_{gel} to form gelliposomes (liposomes with gelated interior), which are assumed to better preserve the liposome structure during freeze-drying than liposomes without interior support. At this stage of proof-of-concept study, we employ gelatin as the interior support because of not only its gelation properties but also its amorphous nature. It is reported that crystalline materials in the interior of liposomes will form large crystals during freezing, which is assumed to affect the integrity of the lipid bilayers during the freeze-drying process (Wang et al., 2006). In this study, gelatin-containing liposomes (GLs) and their freeze-dried counterparts were prepared and characterized, focusing on the effect of gelation incorporation on preservation of the physical state and fundamental properties of the freeze-dried liposomes. Paclitaxel is a mitotic inhibitor for cancer chemotherapy that belongs to the taxane family. Encapsulation of paclitaxel into liposomes aims to enhance the therapeutic effect and reduce systemic toxicity. Since it is a commonly used poorly water-soluble drug to study the performance of liposomes, it is also used as a model drug in this study (Wei et al., 2014; Yin et al., 2013).

2. Materials and methods

2.1. Materials

Soybean phosphatidylcholine (SPC) was purchased from Shanghai Taiwei Pharmaceutical Industry Co., Ltd. (Shanghai, China). Cholesterol (Chol) was obtained from Shanghai Toshisun Enterprise Co., Ltd. (Shanghai, China). Paclitaxel (PTX) was obtained from Shanghai Jinhe Bio-Technology Co., Ltd. (Shanghai, China). Rousselot 250PS gelatin (250 ± 10 g bloom) was kindly gifted from Rousselot Gelatin Co., Ltd. (Wenzhou, China). Sephadex G-50 was purchased from Pharmacia (Uppsala, Sweden). Carboxyfluorescein (CF) was purchased from Sigma–Aldrich Chemicals Co., Ltd. (St. Louis, MO, USA). Ultra-pure water was prepared by a Milli-Q purification system (Millipore, USA). HPLC-grade methanol and acetonitrile was supplied by TEDIA Company, Inc. (Fairfield, OH, USA). All other chemicals were of analytical grade.

2.2. Preparation of liposomes

The PTX-loaded GLs and conventional liposomes (CLs) were prepared by a thin-film hydration method as reported previously with slight modifications (Zhang et al., 2013). Briefly, SPC, Chol and PTX in different ratios were dissolved in dichloromethane in a round bottom flask. Then the organic solvent was removed by a rotary evaporator (RV 10 digital, IKA Works, German) at 30 °C in a water bath (HB 10 digital, IKA Works, German) under vacuum, and the so-dried lipid films were maintained under reduced pressure to remove residual solvent. The lipid film was then hydrated with a phosphate buffer (50 mM, pH 7.4) containing different concentrations of gelatin (phosphate buffered saline was used for CLs) for 30 min at 45 °C, while small glass beads were added to facilitate hydration. Non-entrapped gelatin was removed by centrifugation (TJ-25Centrifuge, Beckman Coulter Inc. CA, USA) for 30 min at 21,000 × *g* for three times. The liposome pellet was re-suspended in phosphate buffer and stored at 4 °C until use.

2.3. Freeze-drying of liposomes

Hydrated liposome formulations (1 mL) in vials were freeze-dried using a vacuum freeze-dryer (Alpha type, Martin Christ, Germany) immediately after preparation. Appropriate amount of lyoprotectant was added to the vials. Samples were rapidly frozen to a terminal temperature of −70 °C for 12 h and dried at 0.10 mbar for 24 h. Secondary drying was performed at 25 °C under vacuum for another 12 h, after which the vials were filled with nitrogen and sealed.

2.4. Reconstitution of freeze-dried liposomes

To each lyophilized liposome sample, suitable amount of ultra-pure water was added under mild shaking to reconstitute into its original form at room temperature.

2.5. Entrapment efficiency

Entrapment efficiency of PTX in GLs was determined by a Sephadex G-50 chromatographic method. Briefly, Sephadex G-50 gel in column (20 cm × 1.0 cm) was loaded with 100 μL blank liposomes to saturate the column and minimize adsorption of actual sample (PTX-loaded GLs). Subsequently, the PTX-loaded GLs suspension was introduced into the column and eluted by phosphate buffer (pH 7.4) at a flow rate of 0.5 mL/min to separate free PTX from the liposome-entrapped drug. The eluted sample, which contained entrapped liposomal PTX, was dissolved with 80% methanol and analyzed for PTX using Agilent 1200HPLC system (Agilent Technologies, Santa Clara, CA). Acetonitrile/methanol/ammonium acetate buffer solution (10 mM, pH 4.5) in volume ratio of 54/18/28 was used as the mobile phase, which was pumped at a flow rate of 1.0 mL/min (Wang et al., 2003). PTX were separated at 37 °C by a C18 column (Diamonsil, 5 μm, 4.6 × 150 mm, Dikma, China) guarded with a refillable precolumn (C18, 2.0 mm × 20 mm, Alltech, USA) and detected at 227 nm. The entrapment efficiency (EE %) was determined by comparing the PTX concentration of the eluted sample to that of bulk GLs samples prior to separation. The following equation was used to calculate EE%:

$$EE\% = \frac{W_c}{W_t} \times 100\%$$

where W_c and W_t denote the drug content in the GLs and the total drug in suspension before centrifugation.

2.6. Particle size measurements

The mean particle size of the prepared PTX-loaded GLs was determined by dynamic light scattering instrument (NICOMP 380 ZLS Particle Sizing Systems, Santa Barbara, CA). Data were collected and processed with the ZPW388 software program. The particle size was expressed with volume-based Gaussian