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The freeze-thawed and freeze-dried stability of cytarabine-encapsulated multivesicular liposomes

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ABSTRACT

To investigate the stability of cytarabine-encapsulated multivesicular liposomes (MVLs) following freeze-thawing/freeze-drying, three types of phospholipids (EPC, DPPC, and DOPC) were separately employed to prepare MVLs using a double emulsification method. The cytarabine retention (CR), phase transition behavior, aggregation/rupture of vesicles and particle size were monitored using HPLC, differential scanning calorimetry (DSC), digital biological microscopy and a laser diffraction particle size analyzer. The effect of trehalose, the lipid bilayer composition and triglyceride on the drug retention was also investigated. The DPPC–MVLs and EPC–MVLs achieved the best protective effect during freeze-thawing and freeze-drying, respectively, while DOPC–MVLs produced the lowest drug retention during both procedures. Digital biological microscopy showed that most of the MVLs were divided into small irregular and regular vesicles after freeze-thawing and freeze-drying, which was in agreement with the reduction in particle size. The vesicle fragmentations may result from the splitting of triglyceride from the lipid membrane or rupture of the lipid membrane. The rehydrated EPC–MVLs still displayed a controlled-release profile in vitro, and the results presented in this work should help in stabilizing hydrophilic drug-encapsulated liposomes with a large particle size.

1. Introduction

Liposomes can be stored in the freeze-dried/frozen state, or as an aqueous dispersion (Crommelin and van Bommel, 1984; van Bommel and Crommelin, 1984). As an aqueous dispersion, their physical and chemical instabilities (e.g., encapsulated drug leakage, vesicle aggregation and hydrolysis of phospholipids) are major problems for long-term storage (Sharma and Sharma, 1997). Accordingly, a number of liposome formulations, which are now commercially available, are usually stored in the freeze-dried state (Immordino et al., 2006). Freeze-drying is a promising approach to extend the shelf-life of liposomes, however, both freezing and drying may lead to structural and functional damage to liposomes. Furthermore, membrane damage by the ice-crystals during freezing, vesicle rupture/aggregation upon dehydration, and phase transition following rehydration could all possibly contribute to leakage of the encapsulated drug. Hence, optimization of the formulation parameters is always essential to stabilize the liposomes.

Trehalose is the preferred excipient due to its cryoprotection and lyoprotection abilities (Christensen et al., 2007; Quintilio et al., 2000; Siow et al., 2008). It plays an important role in the protection of biological membranes of many organisms that survive osmotic stress, severe dehydration and low temperature stress. To date, two disaccharide-based hypotheses (water replacement model and vitrification model) have been proposed to explain this protective effect. The water replacement hypothesis was first proposed by Crowe et al., in which the sugars maintain the head group spacing and reduce the van der Waals interactions among the acyl chains of phospholipids (Crowe et al., 1996a; Crowe and Crowe, 1988b). In doing so, the sugars reduce the interactions between the water and phospholipids and then replace the water (Strauss et al., 1986). The other mechanism is the vitrification model, in which the sugar solution becomes freeze-concentrated and then becomes a stable glass during the freezing then, finally, the freeze-dried cakes are trapped in the sugar glass matrix upon removal of water (Koster et al., 1994; Sun et al., 1996). Those mechanisms are not mutually exclusive (Crowe et al., 1996a,b; Sun et al., 1996), and also operate in food and biosystems (Patist and Zoerb, 2005).

Cytarabine is a hydrophilic drug that is widely used in the treatment of acute leukaemia and lymphoma (Teijon et al., 1997). Its optimal administration and dosage change with the nature and stage of the disease. There are also some adverse effects, such as myelosuppression and neurotoxicity, particularly at high doses (Ameri et al., 1998). The toxicity of cytarabine is reduced if it is able to maintain an effective therapeutic level for a long period of time and, thus, it is a suitable candidate for administration in a controlled-release dosage form. MVLs, which are composed of non-concentric and close-packed lipid vesicles, have recently

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been developed as a lipid-based depot for controlled-release drug delivery (Mantripragada, 2002). Cytarabine-encapsulated MVLs (DepoCyt™) are now commercially available and a number of other drugs, such as proteins, have also been encapsulated into this type of liposome to obtain a sustained-release (Angst and Drover, 2006; Mantripragada, 2002; Ramprasad et al., 2002; Vyas et al., 2006). In addition, formulation of MVLs with the non-concentric arrangement of small vesicles inside a large particle requires the use of neutral lipids, such as triglycerides. When the neutral lipid is omitted, a single-bilayer unilamellar vesicles or multilamellar vesicles may be formed instead (Kim et al., 1985, 1983). The particle size of MVLs ranges from 1 to 100 μm, providing a good depot for controlled drug release. It is known that the particle size has a great influence on the drug retention following freeze-drying (Crowe and Crowe, 1988a). This might be ascribed to the different curvature and phospholipid packing models on the two sides of the lipid membrane for different vesicle sizes (Komatsu et al., 2001). Recently, for larger vesicles (>1 μm), multilamellar liposomes containing 5-fluorouracil with an average size of 5 μm have been shown to exhibit good drug retention in the presence of sucrose after freeze-drying (Glavas-Dodov et al., 2005). Accordingly, it would be interesting to investigate the stability of liposomes with a larger particle size following freeze-drying.

This paper describes an investigation of the effect of freeze-thawing and freeze-drying on the stability of cytarabine-encapsulated MVLs. The vesicle size, cytarabine retention and morphology of the rehydrated liposomes were monitored. Also, in vitro drug release studies of EPC–MVLs before and after freeze-drying were carried out, and freeze-dried MVLs using medium-chain triglycerides (MCTs) instead of triolein (TO) were prepared to investigate the reasons for drug leakage or a reduction in vesicle size.

2. Materials and methods

2.1. Materials

Cytarabine (purity 99.6%) was obtained from Peking University Pharmaceutical Co., Ltd. (Beijing, China). Dipalmitoyl-phosphatidylcholine (DPPC), egg phosphatidylcholine (EPC), and dioleoyl-phosphatidylcholine (DOPC) were all purchased from Lipoid GmbH (Ludwigshafen, Germany) and used without further purification; medium-chain triglyceride (MCT) was obtained from Lipoid KG (Ludwigshafen, Germany). Trehalose dihydrate was obtained from Nanning Sinozyme Biotechnology Co., Ltd., and L-lysine monohydrate was purchased from Alfa Aesar China (Tianjin) Co., Ltd. Triolein (TO) was obtained from Sinpharm Chemical Reagent Co., Ltd.; cholesterol of analytical grade was obtained from Tianjin Chemical Reagent Co., Inc. All other materials and solvents were of analytical grade.

2.2. Preparation of liposomes

MVLs were prepared by the previously published water–oil–water (w/o/w) emulsification procedure (Kim et al., 1983, 1985). Briefly, an aqueous solution of cytarabine (35 mg/ml) containing selected amounts of trehalose (0–18%, w/v) was emulsified with an equal volume of chloroform–diethyl ether (volume ratio 1:1) solution containing 12.0 mM lipids (DOPC/EPC/DPPC), 2.7 mM negatively charged lipid (DPPG), 19.4 mM cholesterol, and 7.05 mM triolein at ambient temperature (DPPC–MVLs at 45 °C) for 8 min at 14,000 rpm. Then, this initial emulsion was mixed with a second aqueous solution containing 1.5% glycine and 40 mM lysine at 3000 rpm for 0.5 min to form the w/o/w emulsion. Chloroform was removed by flushing nitrogen over the surface of the mixture at 37 °C (DPPC–MVLs at 45 °C). The resulting cytarabine-encapsulated MVLs were harvested by centrifugation for 5 min at 600 × *g*, washed with normal saline solution and finally resuspended in approximately 9% isotonic trehalose solution. Aliquots (0.5 ml) of the MVLs were placed in 10 ml glass vials. In the freeze-thaw process, samples were stored in a refrigerator (−20 °C) for 24 h and then thawed in a 37 °C water bath for 10 min. In the freeze-drying procedure, the samples were freeze-dried for 36 h in a Virtis Advantage ES-53 (NY, USA) freeze-dryer at a pressure of 60 mTorr and a condenser temperature of −55 °C.

2.3. Liposome characterization

Particle size was measured by a laser diffraction particle size analyzer (LS 230, Beckman Coulter Inc.). The morphology was determined by a digital biological microscopy, equipped with a computer-controlled image analysis system (DMBA 450, Motic China Group Co., Ltd.). Residual water measurements on freeze-dried samples were made using moisture analysis equipment (SC69-02C, Shanghai Precision & Scientific Instrument Co., Ltd.), and all the freeze-dried samples contained <1.5 wt.% water. For freeze-dried samples, a TA-60WS DSC (SHIMADZU, Japan) was used to measure the phase transition temperatures.

2.4. Cytarabine retention

The preparations of MVLs (0.5 ml), to which 2 ml of normal saline was added, were centrifuged at 600 × *g* for 5 min to separate the free cytarabine (in the supernatant) from the MVLs containing cytarabine (in the pellets), then the pellets were dissolved in 2 ml methanol and sonicated for 10 min. Finally, the amounts of cytarabine in the supernatant and in the pellets were determined by HPLC. The encapsulation efficiency (EE) was calculated according to the following formula:

$$\mathrm{EE}\,(\%) = \frac{\text{encapsulated drug}}{\text{encapsulated drug} + \text{free drug}} \times 100$$

The HPLC system consisted of a Jasco PU-1580 pump and a Jasco UV-1575 detector set at 280 nm. The cytarabine was determined at ambient temperature on a 250 mm × 4.6 mm, 5 μm ODS-2HYPERSIL column (Thermo Electron Corporation). The mobile phase consisted of water (containing 0.15% triethylamine and 0.15% acetic acid)–methanol (95:5, v/v) and was pumped through the system at a flow rate of 1.0 ml/min. The retention time of the drug was 4.9 ± 0.3 min and the calibration curve was rectilinear over the concentration range of 1–80 μg/ml with a correlation coefficient of 0.999. The cytarabine retentions (CR) after freeze-drying and freeze-thawing were calculated using the following formula:

$$\mathrm{CR}\,(\%) = \frac{\mathrm{EE}_2}{\mathrm{EE}_1} \times 100$$

where EE_1 and EE_2 are before and after freeze-drying/freeze-thawing, respectively. For example, if the EE_1 = 80% and the EE_2 = 32%, the CR is approximately 40%.

2.5. In vitro drug release assays

The free drug of rehydrated EPC–MVLs was removed by washing with normal saline solution after rehydration of the vesicles. EPC–MVLs suspensions (1 ml) were sealed in dialysis bags (Sigma, 14,000 MW cutoff) and immersed in PBS pH 7.4 (9 ml) contained in 15 ml glass vials. The glass vials were maintained at 37 °C in a water bath shaker (ZHWY-110X30, Shanghai ZhiCheng Machinery Equipment Co., Ltd.) at 100 rpm. Samples were withdrawn at predetermined time intervals (replaced with an equivalent volume of