International Journal of Pharmaceutics 387 (2010) 147–153

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

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

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ARTICLE INFO

Article history:
Received 13 October 2009
Received in revised form
24 November 2009
Accepted 6 December 2009
Available online 18 December 2009

Keywords:
Cytarabine
MVLs
Freeze-drying
Freeze-thawing
Stability

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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doi:10.1016/j.ijpharm.2009.12.017

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

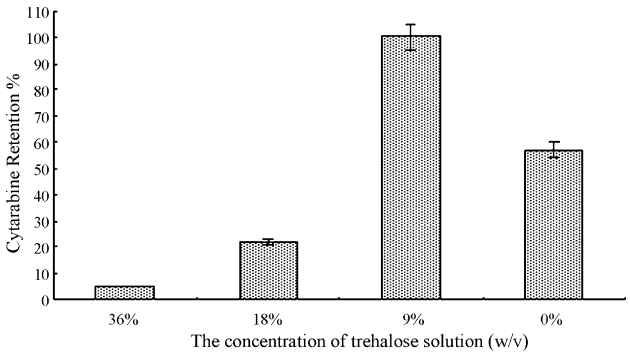

Fig. 1. The influence of the outer trehalose concentration on the stability of EPC–MVLs in an aqueous dispersion at 4 °C for 24 h when no trehalose was added to the inner aqueous phase.

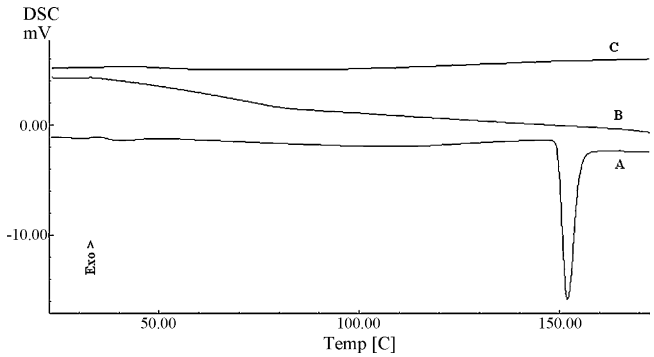

Fig. 2. DSC thermograms obtained from freeze-dried cholesterol (A), the first (B) and second (C) heating scans for DPPC–MVLs (molar ratio of DPPC:DPPG:TO:cholesterol, 12:2.7:19.4:7.05) freeze-dried in the absence of trehalose. The samples contained <1.5 (wt.%) residual water and were scanned at 10 °C/min.

PBS pH 7.4) and analyzed for cytarabine by HPLC as described above. All the experiments were carried out in triplicate.

3. Results and discussion

3.1. Effect of trehalose on the stability of EPC–MVLs

The mass ratio of lyoprotectant to lipid is an important parameter affecting the drug retention after freeze-drying (Crowe and Crowe, 1988a; Crowe et al., 1985). In order to obtain a sufficient interaction between trehalose and lipid, a sugar to lipid mass ratio >9 was used based on optimization of the trehalose concentration in our studies. To choose the optimized outer trehalose concentration of MVLs during freeze-drying/freeze-thawing, a study of cytarabine retention of MVLs in an aqueous dispersion was performed at 4 °C for 24 h. As shown in Fig. 1, almost no drug leaked out from the vesicles when the outer trehalose concentration was 9%, whereas a drug retention of only 5.25% and 22.1% was obtained when the outer trehalose was 36% and 18%, respectively. When the liposomes were suspended in deionized water, approximately 57% cytarabine retention was observed. It is known that vesicles are subjected to shrinkage by the hypertonic outer solution, while the hypotonic solution usually induces vesicle rupture/expansion. So, the drug leakage during this period is possibly due to the osmotic gradient between the two sides of the bilayer. In addition, these findings indicated that the damage produced by hypertonic solutions was greater than that by the hypotonic ones, suggesting that this type of liposome might be much more easily disrupted by hypertonic solutions. In contrast, the MVLs are stable in an outer aqueous phase with the isotonic trehalose solution. This is consistent with other reports, in which MVLs are usually washed with normal saline solution and resuspended in isotonic solution (Ramprasad et al., 2002; Ye et al., 2000).

It is interesting to note that MVLs with similar hypertonic trehalose solutions in both the inner and outer aqueous phase also made the vesicles unstable in an aqueous state at 4 °C for 24 h (data not shown), while MVLs with an inner hypertonic trehalose solution (not more than 18%, w/v) and outer isotonic trehalose solution remained stable under the same conditions. It appeared that MVLs were more sensitive to the outer aqueous phase osmotic pressure, which is possibly due to the non-concentric nature of the vesicles (Mantripragada, 2002). These results seem to be counter-intuitive, as trehalose is generally added in large quantities to the outer aqueous phase before freeze-drying, however, Ohtake et al. (2006) showed that high inner and low outer trehalose concentrations could reduce the phase transition temperature (T_m) upon freeze-drying. In the light of these observations, approximately 9% trehalose was used in the outer aqueous phase, while the inner trehalose concentration ranged from 0% (w/v) to 18% (w/v) in our studies.

3.2. Phase transition behavior of freeze-dried formulations

By avoiding a phase transition during lyophilization and rehydration, it is possible to increase the drug retention of liposomes. The degree of phase transition temperature (T_m) depression caused by trehalose also depends on the lipid composition. However, cholesterol-containing liposomes can exhibit multiple phase transitions upon dehydration (Ohtake et al., 2005), depending on the amount of cholesterol added. The effects of trehalose on dehydrated cholesterol-containing liposomes have not been investigated in detail, particularly in the case of complicated compositions.

To determine whether cholesterol crystallization occurred following freeze-drying, DSC for freeze-dried cholesterol with a residual water content <0.3 (wt.%) was carried out (Fig. 2A). We observed two endothermic transitions at approximately 39 and 152 °C, which was slightly different from previous reports (Bach and Wachtel, 2003; Ohtake et al., 2005) possibly due to the different water content. Fig. 2B and C clearly shows that no cholesterol crystals were detected upon dehydration. However, the endothermic transitions of the DPPC–MVLs for the first heating scan were broadened (Fig. 2B), indicating that the distribution of the lipid components in the liposomes was heterogeneous. We also did not observe multiple peaks for the second scan of DPPC–MVLs (Fig. 2C). Similar results were also obtained for DOPC–MVLs and EPC–MVLs in the first and second heating scans (data not shown). These observations were possibly due to the high molar ratio (47 mol%) of the cholesterol in the formulation (Ohtake et al., 2006). The increasing proportion of cholesterol possibly resulted in dilution of the phospholipids and less interactions between the phospholipids, which might lead to the eventual abolition of the heat of transition (Ohtake et al., 2005).

3.3. Effect of lipid composition on the stability of freeze-thawed and freeze-dried MVLs

The freeze-thawing/freeze-drying studies were carried out only when the outer aqueous phase of MVLs was a 9% trehalose isotonic solution. As seen in Figs. 3 and 4, three types of phospholipids (EPC, DPPC, and DOPC) showed a similar response of the drug retention to the changes in the inner trehalose concentration. MVLs with trehalose distributed on both sides of the layers produced a better protective effect than those with trehalose added only to the outer aqueous phase. The results here also showed that the protective effect increased as the inner trehalose concentration ranged from 0% to 9%, and decreased/levelled off when more

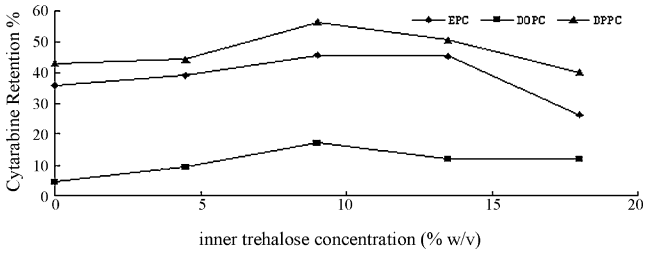

Fig. 3. Effect of the inner trehalose concentration on the cytarabine retention of EPC–MVLs (♦), DOPC–MVLs (■) and DPPC–MVLs (▲) with a 9% outer trehalose concentration following freeze-thawing.

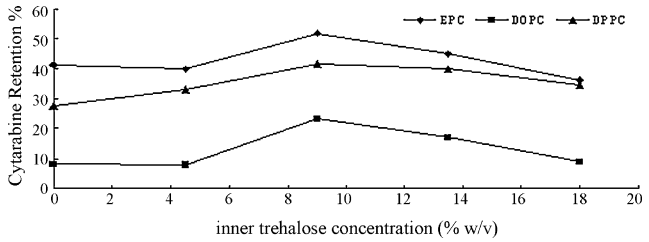

Fig. 4. Effect of the inner trehalose concentration on the cytarabine retention of EPC–MVLs (♦), DOPC–MVLs (■) and DPPC–MVLs (▲) with a 9% outer trehalose concentration following freeze-drying.

trehalose was added to the inner aqueous phase. So, the protective effect is not simply due to the fact that having trehalose on both sides of the bilayer is additive. This protective effect as a function of the inner trehalose concentration is consistent with previous reports (Crowe and Crowe, 1988a), in which only a small amount of trehalose is required inside and adding more inside has no apparent effect on stability. There are several possible reasons to explain why approximately 9% trehalose on both sides of the bilayer achieved the best protective effect during freeze-drying/freeze-thawing. One is that addition of more trehalose may not further improve the protective effect due to the fact that there are sufficient interactions between sugars and phospholipids. Another possible reason is that the distribution of sugars on the two sides of the lipid bilayer might affect the sugar–lipid interactions which influenced the drug retention, or the driving force of drug leakage produced by the osmotic gradient was reduced when a similar isotonic trehalose concentration was added to both sides of the bilayer.

Fig. 3 also showed that DPPC–MVLs and EPC–MVLs produced a better protective effect than DOPC–MVLs when the inner trehalose concentration was the same. During the freeze-thawing process, DPPC (T_m, 41 °C (Hays et al., 2001)) did not undergo a phase transition, but simply went from a gel-like state to the gel state. While for DOPC (T_m, −18 °C (Komatsu et al., 2001)), phase transitions might take place, and this might be from the liquid crystalline state to the gel crystalline state during freezing and from the gel state to the liquid state during thawing. That might be the reason why DPPC–MVLs exhibited the best drug retention and DOPC–MVLs had the lowest retention. We also found that DPPC–MVLs exhibited a higher drug retention than EPC–MVLs during the freezing–thawing process, which is consistent with the previous results for large unilamellar vesicles (Siow et al., 2008). So, it is obvious that the freeze-thawed stability of MVLs depends on both the composition of the preparation and the trehalose distribution.

In the freeze-drying process (Fig. 4), DOPC–MVLs also had the worst protective effect, which might be due to the fact that the interactions between the trehalose and head groups of the phospholipids were markedly reduced in the DOPC–MVLs (Komatsu et al., 2001). However, EPC–MVLs exhibited a higher cytarabine retention (51.65%) than the DPPC–MVLs (41.56%), which was different from the results obtained during the freeze-thawing process. Similar findings, in which EPC–liposomes had a higher retention than DPPC–liposomes during freeze-drying, have also been reported for smaller freeze-dried liposomes in the presence of maltotriose or maltohexaose, depending on the mass ratio of sugar to lipid (Miyajima, 1997). Here, this was possibly due to the distinct phase transition temperature between the DPPC and triolein, so DPPC was in the gel crystalline state while triolein was in the liquid crystalline state at ambient temperature. It was also easy to believe that a phase separation might occur during the drying process. In contrast, both EPC and triolein were in the liquid crystalline state at ambient temperature. The details of this will be discussed later.

3.4. Effect of freeze-thawing and freeze-drying on the particle size of EPC–MVLs

In order to explain the observation that EPC–MVLs produced higher drug retentions than DPPC–MVLs, the changes in vesicle size and morphology upon freeze-drying/freeze-thawing were also monitored. For a vesicle size between 50 and 300 nm, most of the liposomes usually obtained a vesicle size increase due to aggregation of the vesicles after freeze-drying. However, for larger particles, there was a particle size reduction for all the MVLs following freeze-drying. Table 1 shows the mean volume particle size of the EPC–MVLs before and after freeze-thawing/freeze-drying. Before these processes, the vesicle sizes were all about 20 μm, whereas most of them became 7–9 μm after freeze-thawing/freeze-drying. A similar observation of a particle size reduction following freeze-thawing has also been reported by MacDonald et al. (1994). To investigate the effect of the initial size of MVLs on the size changes following freeze-drying, approximately 5 and 50 μm DPPC–MVLs were also prepared, and size reductions were also observed. However, a lower encapsulation efficiency was obtained in the preparation process for 5 μm DPPC–MVLs, and the morphology of MVLs as seen by microscopy following lyophilization and rehydration was not as clear as that of larger vesicles. Hence, we mainly focused on the relatively larger size here in order to investigate the morphological changes more clearly following freeze-drying. It could also be deduced that the vesicle size reduction was highly correlated with the encapsulated drug leakage.

Unlike the smaller vesicles (such as 100 nm), the morphology of the MVLs after freeze-thawing and freeze-drying can be monitored by microscopy. Furthermore, digital biological/light microscopy is widely used to characterize the morphology of MVLs (Ramprasad et al., 2002; Zhong et al., 2005). As seen in Fig. 5A, for DOPC–MVLs, there was marked vesicle fragmentation, and this might be the reason for the lowest drug retention after freeze-drying. DPPC–MVLs (Fig. 5B) also exhibited vesicle fragmentations and deformations, which caused the larger particles to divide into smaller regular and irregular vesicles. The fragmentation of larger particles might be

Table 1
The particle sizes (mean ± S.E.) of EPC–MVLs with different inner trehalose concentrations (w/v) before and after freeze-thawing (FT) and freeze-drying (FD).

	0%	4.5%	9%	13.5%	18%
Before FD/FT	24.4 ± 13.2 μm	21.8 ± 10.3 μm	19.4 ± 12.7 μm	19.2 ± 11.6 μm	20.7 ± 12.1 μm
After FT	6.57 ± 3.85 μm	7.06 ± 5.83 μm	6.21 ± 4.35 μm	9.48 ± 6.41 μm	8.09 ± 5.02 μm
After FD	8.09 ± 3.49 μm	5.36 ± 3.89 μm	7.21 ± 3.67 μm	5.37 ± 2.98 μm	5.79 ± 4.17 μm

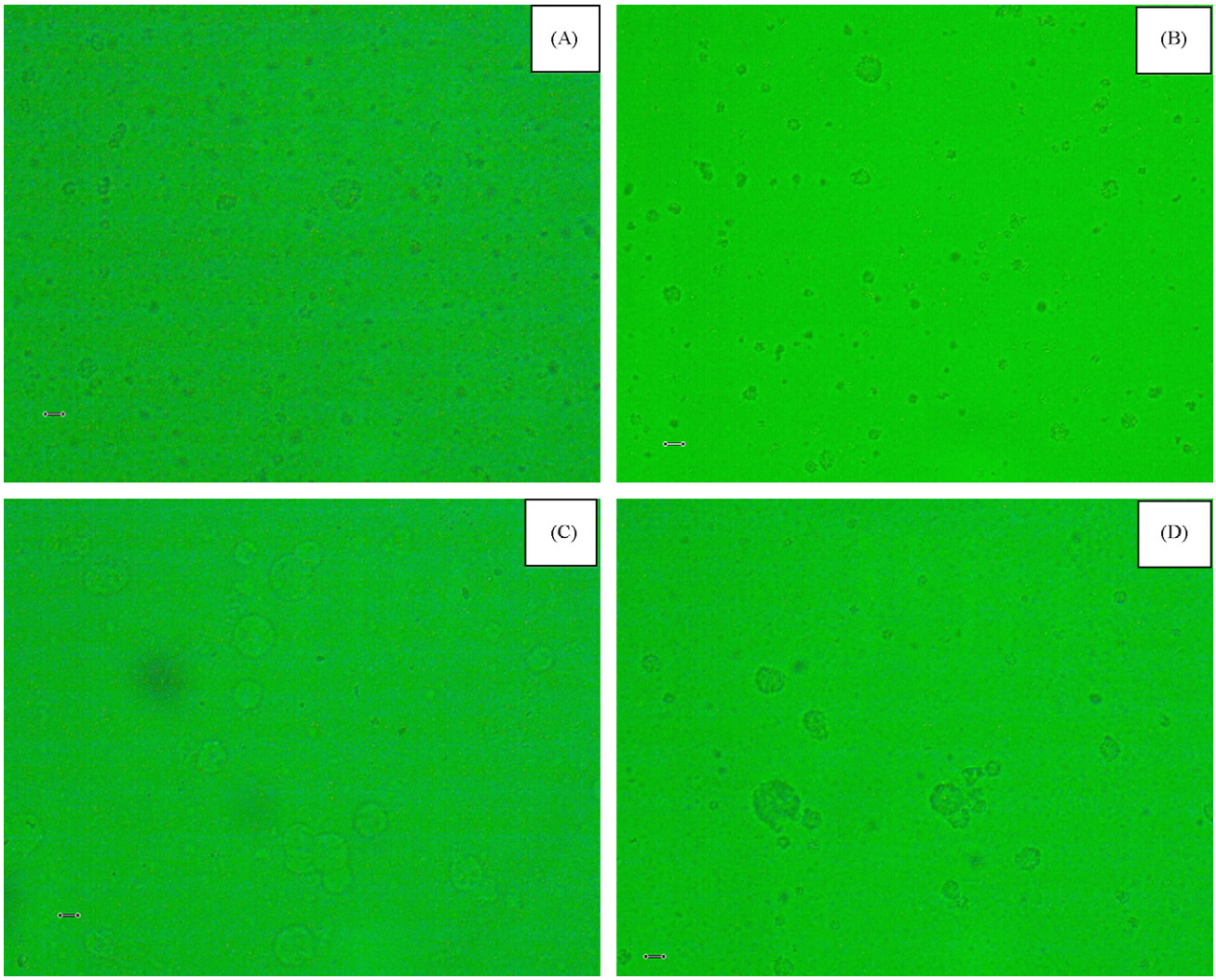

Fig. 5. Photomicrographs of the different types of MVLs in aqueous dispersions at 400× magnification: (A) freeze-dried DOPC–MVLs after rehydration, (B) freeze-dried DPPC–MVLs after rehydration, (C) EPC–MVLs before freeze-drying, and (D) freeze-dried EPC–MVLs after rehydration. The inner trehalose concentration is 9% for all the samples here. The bar in all photomicrographs is equal to 10 μm.

due to the damage caused by ice-crystals during freezing, because both the freeze-thawed and freeze-dried MVLs exhibited a size reduction. In addition, Ellena et al. (1999) have reported that most of the triglyceride in MVLs was located in, and filled spaces at, the bilayer intersection points. So, it appears that triglycerides retain the non-concentric structure of MVLs and connect the smaller vesicles together. These connections may be destroyed due to the removal of triglyceride from the layer intersection points upon freezing. This connection may be also destroyed due to the distinct phase transition temperatures and weak van der Waals interactions between triolein and lipid membrane during drying. That might be the reason why EPC–MVLs have a higher drug retention than their DPPC counterparts during the freeze-drying process. Some other factors, such as phase transition during rehydration and the weak interactions between the trehalose and phospholipids, may also contribute to the drug leakage. It is interesting to note that there were still some vesicles retaining most of their integrity for DPPC–MVLs and EPC–MVLs following freeze-drying. These results were to some extent consistent with the drug retentions shown in Fig. 4. Most of them belong to relatively smaller vesicles, suggesting that vesicle size has a great influence on the drug retention during freeze-drying.

3.5. *In vitro cytarabine release*

To see whether smaller regular and irregular vesicles are still able to provide a controlled drug release, in vitro release was examined. The drug leaked from rehydrated EPC–MVLs was removed by washing with normal saline solution after rehydration of the vesicles. The release profile of EPC–MVLs before freeze-drying was compared with that after freeze-drying. Fig. 6 shows that the EPC–MVLs after freeze-drying had a slightly higher initial burst release in comparison with that before freeze-drying. This burst release might be due to the increased ratio of surface area to volume with the vesicle size reduction or deformation of the vesicles upon freeze-drying. Before freeze-drying, the MVLs consisted of non-concentric smaller vesicles inside a larger particle, so the controlled drug release could be due to the longer diffusion pathway or erosion of the small vesicles at the outer region of the larger particles. When the vesicle size got smaller after freeze-drying, the diffusion pathway became shorter and the erosion became easier, resulting in relatively quick release of the drug. According to the release profiles, the integrity of the vesicles and the non-concentric structure might still be retained to some extent and, thus, provide a controlled drug release.

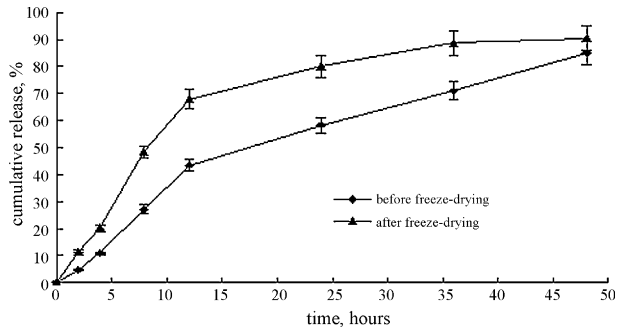

Fig. 6. In vitro cytarabine release profiles of EPC–MVLs before freeze-drying (♦) and after freeze-drying (▲) in PBS (pH 7.4) at 37 °C as a function of time. The points were expressed as the mean value on the basis of three experiments.

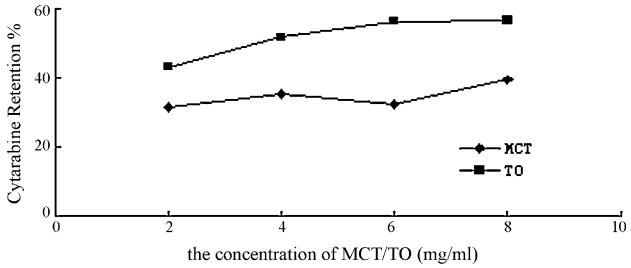

Fig. 7. Effect of TO/MCT concentration on the cytarabine retention of EPC–MVLs upon freeze-drying.

3.6. Effect of triolein or MCT on the cytarabine retention upon freeze-drying

To investigate the contribution of triglyceride to the stability of MVLs upon freeze-drying, samples using MCT instead of TO were prepared and freeze-dried. MCT is a mixed composition of triglycerides including saturated or unsaturated fatty acids from 6 to 14 carbon atoms, preferably from 6 to 12 carbon atoms, while triolein is a triglyceride which is a 16 carbon atom unsaturated fatty acid ester. As shown in Fig. 7, approximately 31% drug retention was observed for MVLs with an MCT concentration of 2 mg/ml during the sample preparation process. The increased concentration of MCT did not have a noticeable effect on improving the stability of MVLs after freeze-drying. It is obvious that the preparation using TO had a better protective effect than that using MCT. For preparations using TO, the drug retention increased with the TO concentration from 2 to 6 mg/ml, and then levelled off from 6 to 8 mg/ml. This improved lyoprotective effect is possibly related to the stronger van der Waals interactions between the lipid layers and TO. So, it can be inferred that the fragmentation of the vesicles is, to some extent, due to the separation of triglycerides from the lipid membrane intersection points. This is consistent with the results that the van der Waals interactions between the triglycerides and phospholipids retain the non-concentric and close-packed structure which influences the drug retention and release profiles (Ellena et al., 1999; Mantripragada, 2002). Also, these fragmentations led to encapsulated drug leakage after freeze-drying or freeze-thawing. The results here demonstrate that both the content and the choice of triglycerides affect the stability of MVLs during freeze-drying, and the fragmentation of vesicles is more closely related to the choice of triglyceride.

4. Conclusions

EPC, DOPC and DPPC were separately employed as the main component of the lipid membrane to prepare MVLs. It is known that the composition plays a key role in ensuring the protective effect of lyoprotectants on the retention of the encapsulated drug. The stability of all the MVLs here following freeze-drying/freeze-thawing depended on both the composition of the lipid membrane and the type of triglyceride used. Both DPPC–MVLs and EPC–MVLs exhibited better drug retentions than DOPC–MVLs during freeze-thawing and freeze-drying, and the preparations using TO had better protective effects than those using MCT following freeze-drying. It was also found that MVLs with a similar isotonic trehalose concentration on the two sides of the lipid membrane exhibited the best protective effect during the freeze-drying/freeze-thawing process.

All types of MVLs here exhibited a particle size reduction upon freeze-thawing/freeze-drying. The reason why the particle size decreased could be: (1) the separation of triglycerides from the lipid layer intersections by the ice-crystals during freezing; (2) according to the photomicrographs, the vesicles ruptured after freeze-drying. So, the rupture of the lipid membrane might also contribute to the vesicle fragmentation. It should be noted that the vesicles after rehydration still displayed a controlled drug release, which demonstrated that the controlled-release function might not be totally lost upon freeze-drying even although the large particles split into smaller ones. It could be assumed that the smaller vesicles still retained their non-concentric and close-packed structure which provided a controlled drug release. Taken together, the findings here should help in the formulation of large vesicle freeze-dried liposomes containing hydrophilic solute for drug delivery applications.

Acknowledgements

We would like to thank Li Zheng from the Laboratory Center of Shenyang Pharmaceutical University for her kind help in the experimental work. Dr. David B. Jack is gratefully thanked for correcting the manuscript.

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