Characterization and Evaluation of Freeze-dried Liposomes Loaded with Ascorbyl Palmitate Enabling Anti-aging Therapy of the Skin

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To prepare freeze-dried ascorbyl palmitate (AsP)-containing liposome which can protect the drug from moisture attack and be used instantly by mixing with water for anti-aging and skin whitening therapy, AsP was encapsulated into liposomes and freeze-dried with trehalose. The freeze-dried liposome formulations were characterized by measuring water contents, particle size, time required for complete reconstitution. With the freeze-dried liposomes, we performed the stability test under accelerated conditions, skin permeation and localization test. The measurement of the time to perfect reconstitution showed that the freeze-dried liposomes can be changed to their initial state rapidly and short term stability test of AsP in reconstituted liposomes under accelerated conditions confirmed that the stability of AsP was considerably enhanced as compared to freshly prepared liposomes. The skin permeation and localization properties of AsP in reconstituted liposomes were not significantly different, indicating that the liposomal structures were maintained before and after freeze-drying. In conclusion, the freeze-drying method provided a possible way to overcome the instability issue of AsP induced by the moisture and reproduced similar skin permeation and localization properties as shown by freshly prepared liposomes.

Key Words : Ascorbyl palmitate, Liposome, Freeze-dry, Reconstitution

Introduction

Ascorbic acid (AsA) has been used as a representative anti-aging and skin whitening agent because of its bio-activities such as inhibition of melanin biosynthesis, inhibition of free-radical formation and enhancement of collagen biosynthesis.[1,2] However, practical application of AsA has greatly been limited due to its low chemical stability and hydrophilic nature. To overcome these limitations, ascorbyl palmitate (AsP) has been introduced, which has enhanced chemical stability and lipophilicity, certainly leading to increased skin absorption ability.[3]

For AsP topical delivery, lipid-based delivery systems such as solid lipid nanoparticles[4,5] and liposomes[6] have been considered as they offer high affinity to the skin and high drug encapsulation ability. Of these, liposome systems have demonstrated additional advantages as a lipophilic drug carrier. For instance, transdermally delivered liposomes can be acted as a local depot that can cause an improved local drug action needed for anti-aging and skin whitening therapy. Additionally liposomes facilitate skin permeation of drugs with various polarity.[7-9]

When AsP is encapsulated into liposomes, the stability of the drug could be lowered by the moisture added to form the liposome. To avoid this, freeze-drying process was utilized to produce the liposome devoid of moisture. This liposome prepared in the present study was anticipated to immediately be reconstituted with aqueous media upon skin application as well as increase the stability of AsP.

In general, to protect the liposome being freeze-dried from chemical instability sugars such as sucrose, mannitol and trehalose have been added.[10,11] The protection mechanism exhibited by the sugars (so-called lyo-protective compounds) during freeze-drying process against drying stress was known as forming an amorphous matrix during freezing, and exhibiting a low molecular mobility after drying.[12]

In this study, we characterized and evaluated AsP-containing freeze-dried liposomes to be used as an instantly applicable formulation to the skin by reconstituting them with water. For this purpose, we examined physico-chemical properties of the liposome before and after freeze-drying as well as surface morphology, short-term stability in accelerated condition, and skin permeation and localization properties.

Experimental Procedures

Materials and reagents. Ascorbyl palmitate was provided from Takeda, Co., Ltd (Chuo-ku, Osaka, Japan). Dimyristoylphosphatydilcholine (DMPC) was purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Poloxamer 407 was obtained from BASF (Seoul, Korea) and all other reagents were of analytical grade and used as received.

Liposome preparation. The multilamellar liposome loaded with AsP was prepared by traditional hydration method.[13] Briefly, the lipid (67.8 mg) and AsP (50 mg) were dissolved in chloroform (10 mL). The thin lipid film was obtained by removing the organic solvent under a vacuum condition using a rotary evaporator (Eyela, Tokyo Rikakikai Co., Japan) and rehydrated with 5 mL of PBS (pH 7.0) containing trehalose. The molar trehalose/phospholipid weight ratio studied was 0 : 1, 1 : 1, 3 : 1 and 5 : 1.

Freeze-drying of liposomes. A hydrated liposome formulation (5 mL) was freeze-dried with a vacuum freeze dryer (Beta type, Martin Christ, Germany). Samples were frozen to a terminal temperature of −45 °C, and dried at −45 °C of the shelf temperature for 48 h.

Measurement of water content. The residual water content of the freeze-dried liposome sample was determined by Karl-Fisher Titrator (Mettler Toledo, Giessen, Germany). The pyridine-free Hydranal-solvent and Hydranal-Titrant 5 were used as solvent and titration medium.

Scanning electron microscopy (SEM). The freeze-dried liposomes were scattered on to 12 mm diameter double-side adhesive carbon pads, which were attached to SEM specimen mounts. The specimens were sputter-coated with a layer of gold/palladium approximately 5 nm in thickness and specimens were examined with an electron microscope.

Measurement of liposome size. The freeze-dried liposome samples were reconstituted with PBS (pH 7.4) at 25 °C and the mean diameter of liposome was determined using dynamic light scattering system (LPA PAR III, Ostuka Electronics, Japan) in the condition of scattering angle of 90° and 12,000 CPS (count per sec).

Measurement of reconstitution time. The ability of reconstitution of the freeze-dried liposomes was evaluated by placing 30 mg of freeze-dried liposome to 10 mL of PBS solution (pH 7.4). We observed the appearance of the reconstituted liposome dispersions at every 1 min and the time required for the completion of reconstitution was estimated by visual inspection.

Accelerated stability test. The freeze-dried liposome was placed in a light-shielded vials and the vials were stored in an incubator maintained at 40 °C and 75% RH. At appropriate time intervals, samples were withdrawn and dissolved with methanol. After vortex-mixing and centrifugation, an aliquot of the filtrate was analyzed using HPLC.

Skin permeation study. The shaved dorsal skin of male Sprague Dawley rats (12-14 weeks old, weighing 220 ± 10 g) was removed carefully leaving the fat tissue and stored in −70 °C deep freezer until skin permeation study. One milliliter of the reconstituted liposome was loaded into the donor compartment of Franz diffusion cell with an effective diffusion area of 2.00 cm^2 and 11 mL of receptor volume. Phosphate buffer solution (pH 7.4) containing 30% ethanol was used as receptor media for AsP having low water solubility. The temperature of receptor phase was maintained at 37 ± 0.5 °C and the receptor compartment was stirred with magnetic stirrer to maintain homogeneous condition. After sampling, an equal volume of receptor medium was added and the sample was analyzed with HPLC.

Skin localization study. After skin permeation study, the rat skin was removed from Franz diffusion cell. The skin was washed with saline solution 3 times. The drug loaded area was cut off with scissors and stored at −70 °C until homogenization. The weight of the skin was measured and minced using Ultra-Turrax homogenizer (Ultra-Turrax T25 basic, IKA Inc., Germany). Methanol (2 mL) was added to the minced rat skin to extract AsP and 0.5 mL of phosphoric acid was added to prevent hydrolytic degradation of AsP by skin esterase The samples were centrifuged (Ecospin 314, Hanil R&D, Korea) at 4 °C, 3,000 × g for 10 min. The supernatant (1 mL) was taken and filtered with 0.45 μm filter and analyzed by HPLC. The localized amount of AsP was calculated as amount of drug per gram of tissue.

HPLC analysis. Normal phase m-Bondapak (Li-NH_2, 3.9 × 300 mm, 10 μm, Waters, USA.) column was used to analyze the amount of AsP. The composition of mobile phase was methanol : phosphate buffer (0.02 M, pH 3.5) = 70 : 30 (v/v). The flow rate was 1.0 mL/min, detection wavelength was 255 nm and injection volume was 20 μL.

Results and Discussion

After freeze-drying the liposomes, we first measured the water content with Karl-Fisher titration method. The water content of the freeze-dried liposomes was below 1.5% (Fig. 1). The water level of freeze-dried liposomes increased with the addition of trehalose but the level of water content was not dependent upon the amount of trehalose added. The residual water contents of the freeze-dried liposomes were similar to those of freeze-dried product in which the residual water content was generally measured to be in the range of 0.5 to 4%.[14,15]

After freeze-drying, generally a porous matrix is formed. In SEM image analysis, the surface morphology of all freeze-dried liposome samples showed porous structures which can lead to fast reconstitution (Fig. 2).[16]

The particle size was measured after the reconstitution of the freeze-dried liposomes in PBS (pH 7.4) at 25 °C. The particle size (960 ± 50 nm) of reconstituted liposomes was greater than that (795 ± 35 nm) measured before the freeze-drying process when no trehalose was added. However, the particle size of the freeze-dried liposomes was reduced as trehalose was added (Fig. 3). As shown in Table 1, the time required for the completion of reconstitution was estimated to be 1-3 min. The addition of trehalose to the liposome dispersions decreased the reconstitution time. The reason for

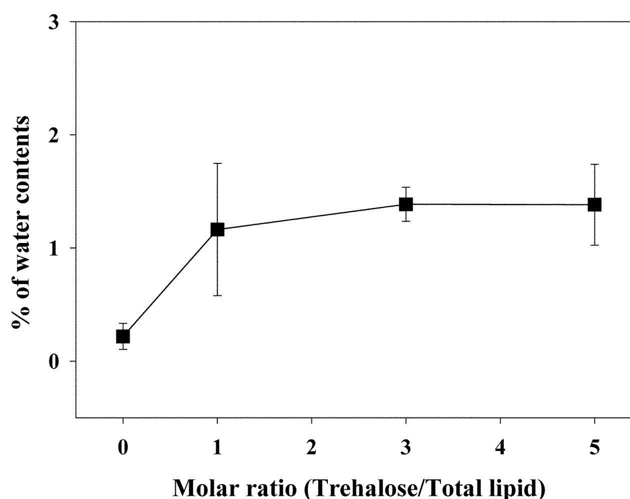

Figure 1. Water contents of freeze-dried liposomes.

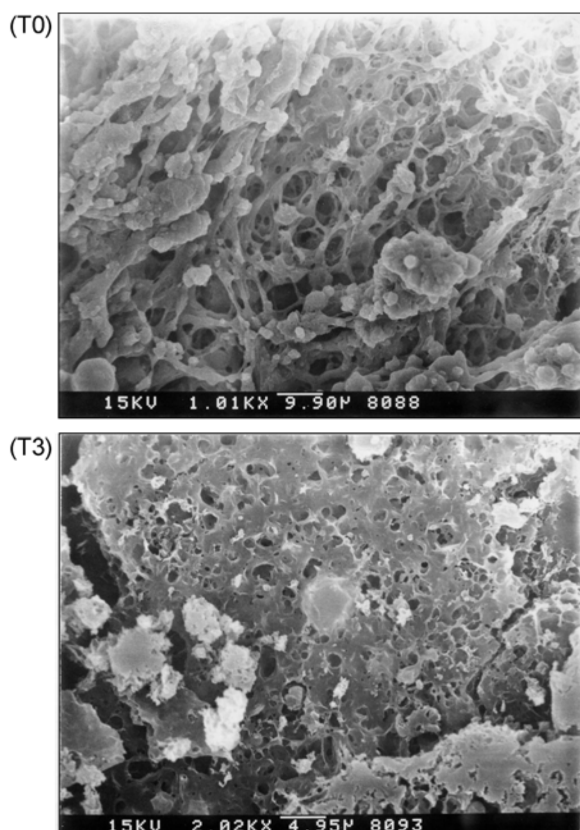

Figure 2. Representative SEM images of freeze-dried liposomes. The numbers in T0 and T3 indicate the molar ratio of trehalose to total lipid.

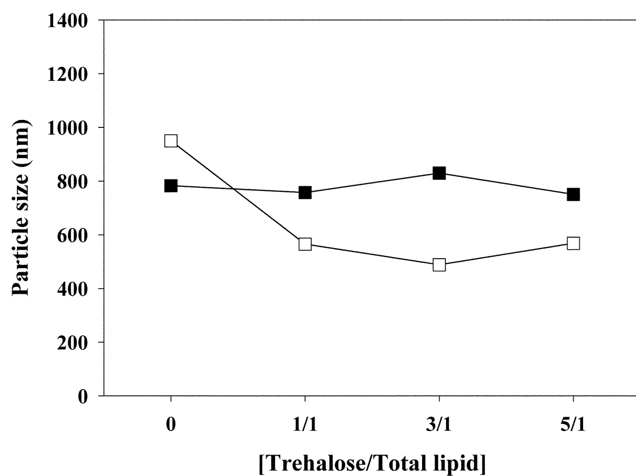

Figure 3. Particle size of liposomes. ■ ; before freeze-drying, □ ; after freeze-drying.

the reduced reconstitution time caused by trehalose may largely be due to the increased opportunity to contact the reconstitution media made by hydrophilic trehalose molecule.

In the stability test performed under accelerated conditions of 40 °C, 75% RH, as expected the freeze-dried liposome clearly showed enhanced stability of AsP compared to that encapsulated in liposomal solution (Fig. 4). This increased stability of AsP in freeze-dried liposomes was probably

Table 1. Reconstitution time (min) of freeze-dried liposomes in PBS (pH 7.4) at 25 °C (Mean ± S.D. n = 3).

	Molar ratio of trehalose to phospholipid			
	0 : 1	1 : 1	3 : 1	5 : 1
Reconstitution time	2.9 ± 0.4	1.1 ± 0.3	0.6 ± 0.2	0.7 ± 0.2

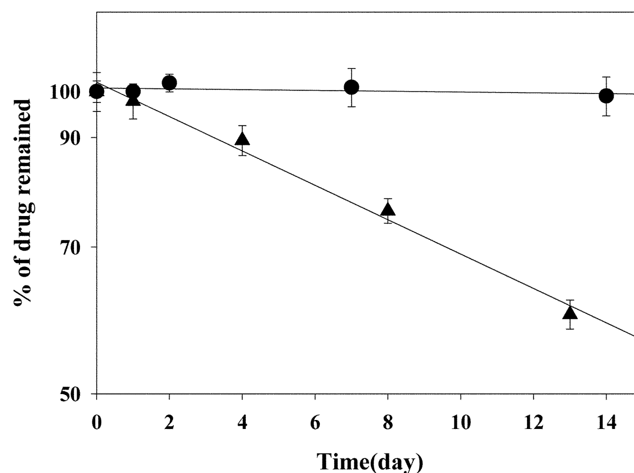

Figure 4. Stability of liposomally encapsulated ascorbyl palmitate in accelerated condition of 40 °C, 75% RH. ● ; reconstituted freeze-dried liposome, ▲ ; freshly prepared liposome.

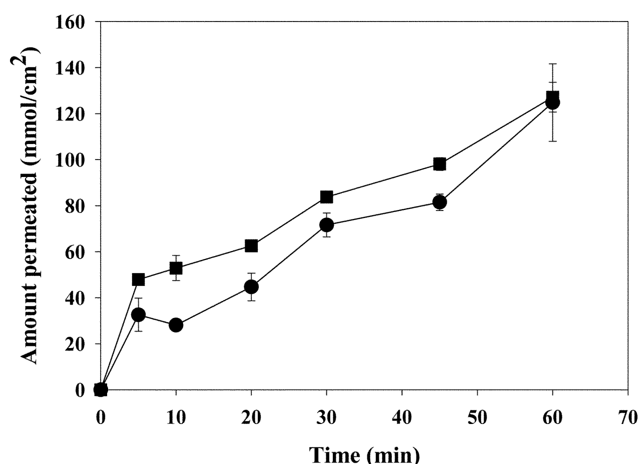

Figure 5. The skin permeation profile of ascorbyl palmitate loaded liposomes. ■ ; before freeze-drying, ● ; after freeze-drying.

caused by the protection of AsP from the moisture that essentially added in the liposomal formulation. In general, drug stability increases in a solid state as compared to a solution state especially a drug is degraded by a hydrolysis mechanism. Thus, from the results obtained so far, it is believed that the freeze-dried liposomes can be used as its initial liposomal dispersion by simple agitation but it offers a considerably increased stability.

As shown in Figure 5, the amount of AsP permeated across the skin from the liposomes redispersed after freeze-drying was less than that measured with the liposomes prepared without freeze-drying process but the cumulative amount of AsP permeated for 1 h was the same, regardless

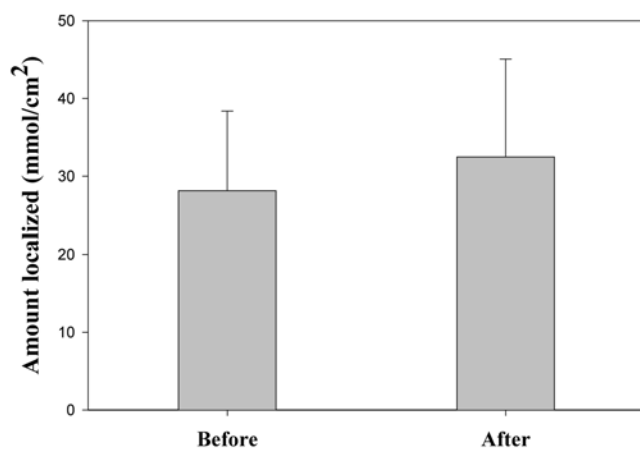

Figure 6. The skin localization test result of ascorbyl palmitate loaded liposomes before and after freeze-drying.

of the liposomes studied. However, the amount of AsP localized in the skin from the liposomes redispersed after freeze-drying was similar to each other (Fig. 6). Thus, although the amount of AsP permeated from the freeze-dried liposomes was lower than that of AsP from fresh liposome, ultimately the total amount accumulated for 1 h was statistically the same as fresh liposome. If there were significant physical changes in liposome such as fusion or leakage of encapsulated AsP during freeze-drying or reconstitution process, the skin permeation and localization results would considerably be different. Moreover, as AsP is a neutral drug having low water solubility, the skin permeation and localization of AsP is mainly dependent on the solubility of AsP into the bilayer of liposome. Therefore, if AsP was leaked out from the freeze-dried liposomes, the skin permeation and localization property of AsP would be different from those obtained with freshly prepared liposomes.

Conclusions

AsP-encapsulated liposomes were prepared, freeze-dried, and the feasibility of freeze-dried liposome as a topical formulation with an instant reconstitution before use was evaluated. To prove that no changes occurred before and after freeze-drying of the liposomes containing AsP, particle size and time required for reconstitution were measured. The stability of AsP in accelerated condition, skin permeation and localization properties before and after freeze-drying were also tested. From the results obtained, the freeze-dried liposomes did not show any physical changes and demonstrated short reconstitution time, enhanced stability and similar skin permeation and localization characteristics with freshly dispersed liposomes. It was therefore expected that the freeze-dried liposome can be a desirable AsP formulation for the skin delivery of AsP for anti-aging and skin whitening therapy.

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