Triterpenoid-Containing Liposome by Micelle-to-Vesicle Transition and Their Biological Activities

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Keywords: Liposomes, micelle-to-vesicle transition, vesiculation, oleanolic acid, solubilization

Summary

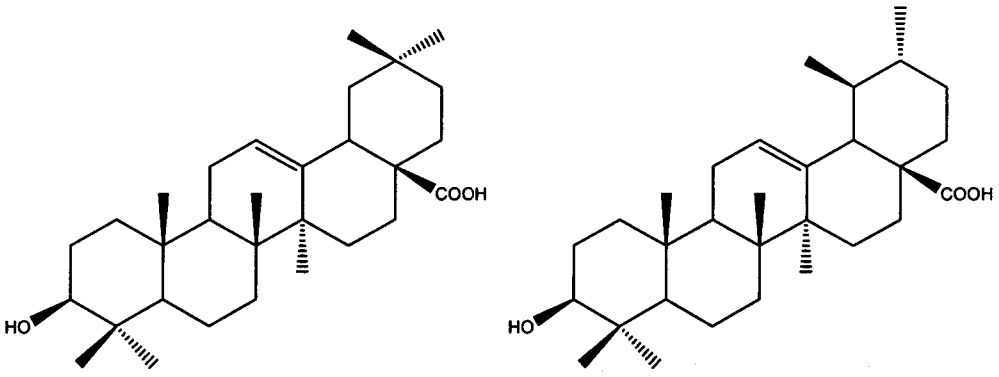
Ursolic acid (UA) and oleanolic acid (OA) are pentacyclic triterpenoids which are widely distributed in plants, and their derivatives are aglycones of many naturally occurring saponins. It is known that pentacyclic acids may possibly enhance the mechanical barrier functions of cell membranes in plants. Recently, it has been reported that OA and UA have interesting biological activities on skin, such as anti-inflammatory and anti-wrinkle activities. Since triterpenoids are extremely insoluble and their solubility problem limits skin-care application, OA and UA were encapsulated in liposomes via micelle-to-vesicle transition to overcome poorly soluble property and enhance biological efficacy. Optimal molar ratio of OA to lecithin was found to exist for producing liposomes of small hydrodynamic size and liposomal suspensions without recrystallized precipitation of OA. From electron micrograph and dynamic light scattering studies, reconstituted OA-containing liposomes without severe mechanical treatment showed small hydrodynamic size about 150 nm. Wide-angle X-ray diffraction coupled with dynamic light scattering revealed that optimal amount of OA in liposome was 25.4 mole %. In biological evaluation, OA-containing liposome significantly increased filaggrin and transglutaminase as markers of keratinocyte differentiation in epidermal layer of hairless mouse, whereas ursolic acid-containing liposome did not show noticeable increase of filaggrin and transglutaminase compared to empty liposome. It is concluded that nano-scaled liposomes containing triterpenoids were spontaneously prepared by vesicular transition from mixed micelle and liposomal triterpenoids can enhance skin absorption of triterpenoid and biological efficacy.

Introduction

Liposomes have been widely studied owing to the inherent structure of lipophilic membrane bilayer and water-containing core. This unique vesicular property has triggered the numerous studies related to delivery vehicles of proteins and highly hydrophobic drugs or medical diagnostics. In the earlier time, liposomes showed physical instability that made it difficult to be used in biological application. As physical stability became enhanced by modifying mechanical and interfacial properties, liposome products of antifungal and anticancer drugs were commercially developed [1-

3].

Liposome can be generally prepared by mechanical treatment such as extrusion, sonication, and high-pressure homogenization. All mechanical treatments are the same in that high energy is used for reducing a hydrodynamic dimension of large liposomes. Otherwise, small unilamellar or multilamellar liposomes can be spontaneously prepared by changing pH and detergent depletion techniques [1,4,5]. While these methods are useful for preparing small liposomes of narrow particle distribution, there are some disadvantages that specific lipids must be used or some chemicals must be removed for liposome constitution. It is desirable to prepare small liposomes of narrow distribution without mechanical treatment and organic additives in the aspects of human safety and colloidal stability [6,7]. However, it has not been reported that small liposomes of narrow distribution using lipids and therapeutic agents simultaneously were prepared without mechanical treatments and organic solvents. Therefore, a simple preparation of small liposomes encapsulating therapeutic agents can be versatily utilized in various applications.



Oleanolic acid Ursolic acid

Figure 1. Chemical structures of oleanolic acid (OA) and ursolic acid (UA)

Oleanolic acid (OA) is a kind of pentacyclic triterpenoid acids which are widely distributed in plants, and their derivatives are aglycones of many naturally occurring saponins. It is known that OA has anti-viral, hepatoprotective, anti-proliferative, and anti-inflammatory activities [8-11]. In spite of the described biological activities of OA, the extremely low solubility of OA in aqueous and organic media has restricted its clinical application. Since it is reported that OA stimulated adipocyte differentiation and liposomal triterpenoid in topical application increased ceramides and collagen in vitro and in vivo, OA is highly needed to be solubilized in liposomal membrane for enhancing its biological efficacy and overcoming solubility problem [9,11-13].

This study demonstrates that OA as a therapeutic agent was solubilized in liposomal bilayer without organic solvents and mechanical treatment. In addition, spontaneous vesiculation of OA-containing liposomes by adjusting solution pH is discussed in the point of colloidal and biological aspects.

Materials and Methods

Liposome preparation

Average molecular weight of soybean lecithin (Phospholipon 90G; 90-95% phosphatidylcholine content) was found to have 780 Da, which was calculated by considering weight fraction of each fatty acid in lecithin. 5 g of soybean lecithin was solubilized in 15 mL of cosolvent (ethanol:propylene glycol=2:1), followed by the addition of oleanolic acid (OA). After the equivalent mole of potassium hydroxide to OA was applied in the solution, the solution of lecithin and OA was poured into 72 g of distilled water. The mixtures were stirred for 30 min at room temperature. Citric acid was dropwisely added in the viscous mixture until the pH of the mixtures reached 7.0. In high-pressure homogenization, the mixtures were passed through high-pressure homogenizer (Microfluidics) two times. The resulting liposome suspensions were stored in 4℃ prior to use. As a structural analog of OA, ursolic acid (UA)-containing liposomes were prepared with the same procedure. The structures of OA and UA are shown in Fig. 1.

Measurements

The critical micelle concentration (CMC) was obtained from fluorescence excitation spectra of pyrene as a fluorescent probe. A known amount of pyrene in acetone was added to each 10 ml vial and dried in 50°C. The sample solutions were introduced to the vials to give pyrene concentration in the final solution of 6×10^{-7} M. Steady-state fluorescence spectra were measured using Hitachi fluorescence spectrophotometer (F-4500) with bandwidth of 2.5 nm for excitation and emission. The excitation spectra were obtained using emission wavelength of 390 nm.

Size distribution and zeta potential of liposomes were measured by a light scattering method equipped with a He-Ne laser (Zetasizer 3000HS, Malvern, UK). The scattering angle was fixed at 90° and temperature was maintained at 25℃. The hydrodynamic diameter (D_p) was calculated by Stokes-Einstein equation (1).

$$D = \frac{k_B T}{3\pi\eta D_p} \quad (1)$$

where k_B is the Boltzmann constant, D is the diffusion coefficient (cm^2s^{-1}), T is the temperature [K], and η is the aqueous solution viscosity ($gcm^{-1}s^{-1}$). The Doppler electrophoretic light scattering measures the electrophoretic mobility of colloid particles in an applied electric field. The velocity of moving liposomes is calculated based on Doppler frequency shifts of scattered laser light. All of the measurements were performed by diluting samples in the KCl (5mM) solution. The electrophoretic mobility ($m^2V^{-1}s^{-1}$) of liposomes was obtained from an average of at least 8 measurements.

The X-ray diffraction (XRD) pattern and lattice spacing were indentified by a powder XRD with a wide-angle diffractometer (Rigaku/USA, D/max-RB) of CuKα radiation, λ=1.54 Å). XRD analysis was carried out after lyophilization of the samples. The scanning rate was 4°/min, and X-ray data were analyzed by the Bragg's equation.

For visualizing liposome morphology, freeze-fracturing procedure was performed on a liquid

nitrogen-cooled support by BAF 060 (BAL-TEC). The images were analyzed on a Philips CM 200 transmission electron microscope.

Biological evaluations

After treating samples in hairless mouse for 48 hours, biopsy of hairless mouse skin was performed and stored in –20℃. Epidermis and dermis were separated after harvesting the skin in trypsin/EDTA 2.5% solution at 4℃ overnight. Subsequently, the separated epidermal layers were homogenized in lysis buffer (Cell Signaling Co.) and proteins of epidermal layers were extracted for Western blotting. The extracted proteins were electrophoresed on SDS-PAGE gels. Following electrophoresis, filaggrin was transferred to nictrocellulose paper, followed by blocking (5% non-fat milk), labeling with filaggrin antibodies (primary) and HRP anti-mouse antibodies (secondary) supplied with an ECL western blotting detection reagent (Amersham Pharmacia Biotech Co.). Transglutaminase expression was quantitated using the similar procedure.

Results and Discussion

Hydrodynamic properties of OA and lecithin mixtures

Potassium salt of OA was prepared by reacting OA with potassium hydroxide in order to have surfactant nature at high pH. Intensity ratios at 338 and 335 nm in fluorescence excitation spectra, I_{338}/I_{335} at each concentration were used to determine the critical micelle concentration (CMC). The CMC was determined as the intersection point of the slopes of a flat region in the dilute concentration and a dramatic rising region in the plot. Even though OA is not soluble in aqueous media and do not micellize, potassium salt of OA showed micellization behavior over narrow range of the concentration in Fig. 2, which means the traditional nature of conventional surfactants [14]. The CMC of potassium salt of OA was found to have 2.23×10^{-6} M.

Since aqueous mixtures of lecithin and potassium salt of OA exhibited highly basic state of pH 10 to 11, citric acid was added to adjust the solution pH to 7.0. Fig. 3 shows electrophoretic mobility (EPM) of the aggregate of lecithin and potassium salt of OA. Highly negative EPM in basic state should be only owing to potassium salt of OA in lecithin aggregate because lecithin as a zwitterionic molecule is neutrally charged. On pH titration, the EPM of the aggregate sharply increased around pH 9, meaning that potassium carboxylate of OA, origin of negative charge, was changed to neutral carboxylic acid.

Various molar ratios of OA to lecithin were examined for colloid properties of OA-containing liposomes. After pH titration, low molar ratios of OA to lecithin induced larger hydrodynamic diameter of liposomes and showed relatively high suspension viscosity, while liposomes in the intermediate molar ratio showed small hydrodynamic diameter with relatively low viscosity in Fig. 4. However, high molar ratios of OA increased hydrodynamic diameter of liposomes again, resulting from recrystallized precipitate of OA during pH titration. The OA precipitate in higher molar ratios was originated from the reason that OA amount exceeded the solubilizing capacity of liposomal

hydrophobic bilayer. Therefore, it is suggested that optimal molar ratio of OA and lecithin exists for preparation of small liposomes without OA recrystallization. In addition, the reason that the size of liposomes decreased as increasing the OA amount of should be due to the surfactant nature of potassium salt of OA.

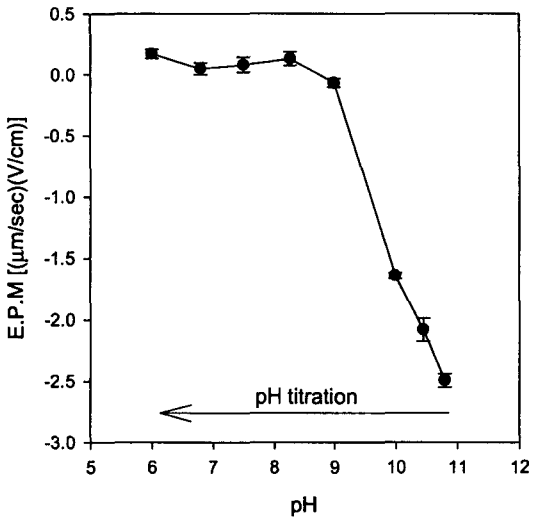


Figure 2. I_{338}/I_{335} ratio of potassium salt of oleanolic acid with varying concentration

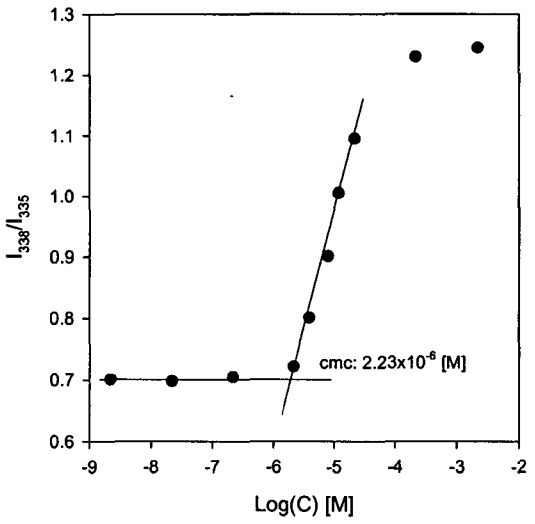


Figure 3. Electrophoretic mobility of the aggregate of lecithin and oleanolic acid with varying pH (OA: 25.4 mole%)

Although liposomes have been widely studied in biomedical applications, there is a remaining problem that the integrity becomes weakened by several bile salts and micellization occurs subsequently, where bile salts are acidic derivatives of cholesterol and strongly negative surfactants [15]. Therefore, the mixture of lipids and bile salts tend to form the mixed micelle of smaller hydrodynamic dimension. In the same principle, potassium salt of OA has the same character of bile salt, resulting in the micellization of liposomes. The mixture of potassium salt of OA and lecithin would constitute the mixed micelle of smaller hydrodynamic dimension in aqueous solution than liposomes of lecithin only. As reported earlier, the mixture of lecithin and surfactants do not micellize directly and shows a time-dependent structural change and/or coexisting micellar and bilayered assemblies with changing composition and dilution [5].

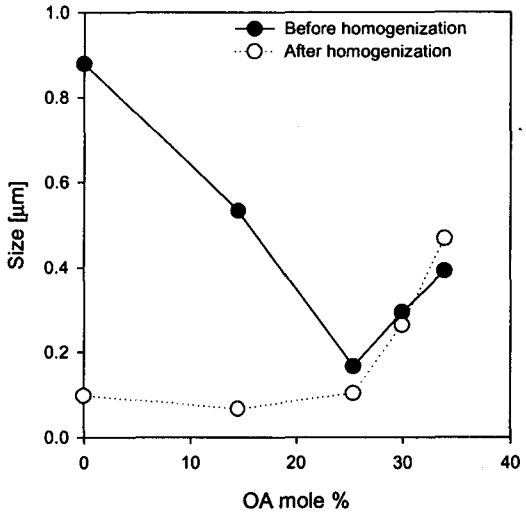


Figure 4. Hydrodynamic size of oleanolic acid-containing liposomes as a function of oleanolic acid content

Therefore, the liposomal assembly of large hydrodynamic dimension evolves to the small micellar assembly as the molar ratio of OA to lecithin increases. However, potassium salt of OA lost the surfactant nature when pH was adjusted to 7.0 by the addition of citric acid, and the structure of mixed micelle was converted to liposomes containing OA in the membrane bilayer. After liposomes were reconstituted with OA by adjusting pH, OA-containing liposomes were treated by high-pressure homogenization. Liposomes in the region of low molar ratio of OA showed significant difference between the sizes before and after high-pressure homogenization, while liposome in higher molar ratio of OA showed little difference. The result indicates that the mixture of lecithin and potassium salt of OA forms a mixed micellar phase of small hydrodynamic dimension and small liposomes from the mixed micelle are spontaneously vesiculated by micelle-to–vesicle transition after pH titration. Freeze-fractured electron micrographs of liposome containing 25.4 mole% of OA are shown in Fig. 5. Even though high-pressure homogenization was not applied, liposomes constituted from the mixed micelle were found to have relatively narrow size distribution. In addition,

recrystallized OA precipitate was not found in the picture. The electron micrograph was consistent with the result of dynamic light scattering.

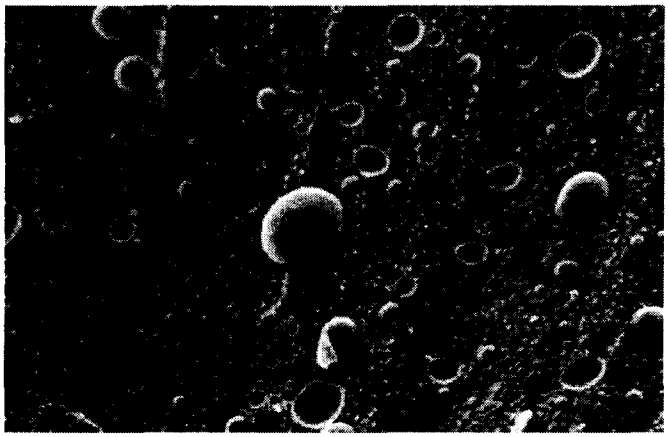


Figure 5. Electron microscope picture of oleanolic acid-containing liposomes (OA: 25.4 mole %)

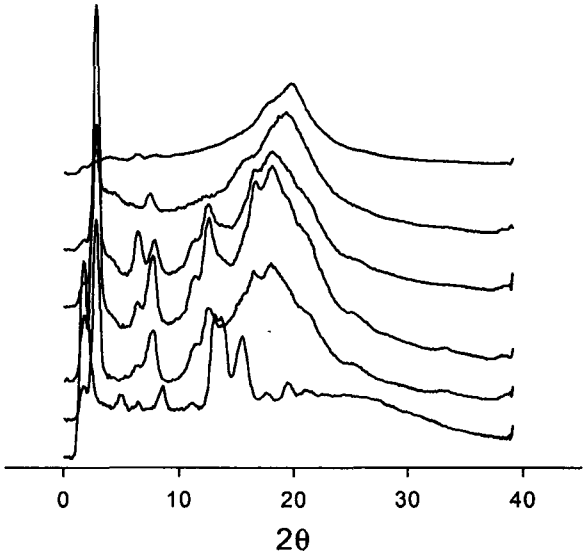


Figure 6. XRD pattern of oleanolic acid and oleanolic acid-containing liposomes

X-ray diffraction of OA-containing liposomes

X-ray diffraction in the wide-angle region (θ=2–50°) provides information about the conformation of hydrocarbon chains and the distances between individual molecules. Fig. 6 shows the diffraction pattern of pure OA and lyophilized OA-containing liposomes, indicating that the characteristic crystal pattern of OA was disappeared in OA-containing liposomes. Lamellar phase was showed in a very broad peak in the range of 15-25° in 2θ (3.5-6Å). Since this is due to intermolecular distances, maximal 2θ in the broad peak range was found to calculate intermolecular distances (d-spacing) of lecithin molecules as shown in Fig. 7. It is interesting that d-spacing of lecithin

molecules increased up to 25.4 mole % of OA and was constant beyond the OA amount. Increase of d-spacing is possibly attributed to enlarged intermolecular spacing of lecithin molecules by insertion of OA into liposomal bilayer membrane. The result also reveals that OA beyond 25.4 mole % cannot be inserted into liposomal bilayer and the 25.4 mole % of OA is an optimal amount which can be loaded in liposomes. This is consistent with the result of liposome size as a function of OA, showing that the OA above 25.4 mole % was recrystallized during pH titration and resulted in larger hydrodynamic size of liposomes.

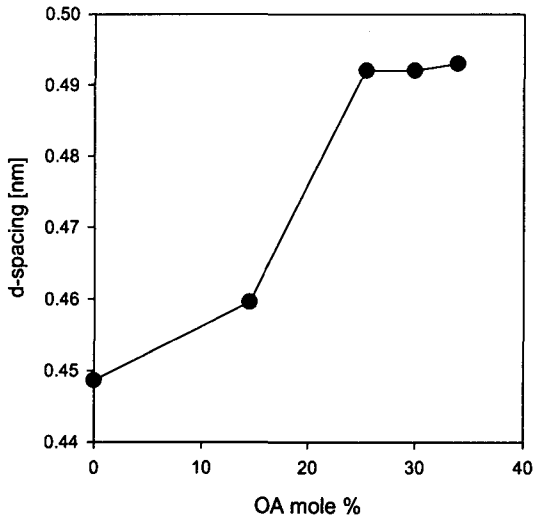


Figure 7. Intermolecular spacing of lecithin molecules as a function of oleanolic acid content

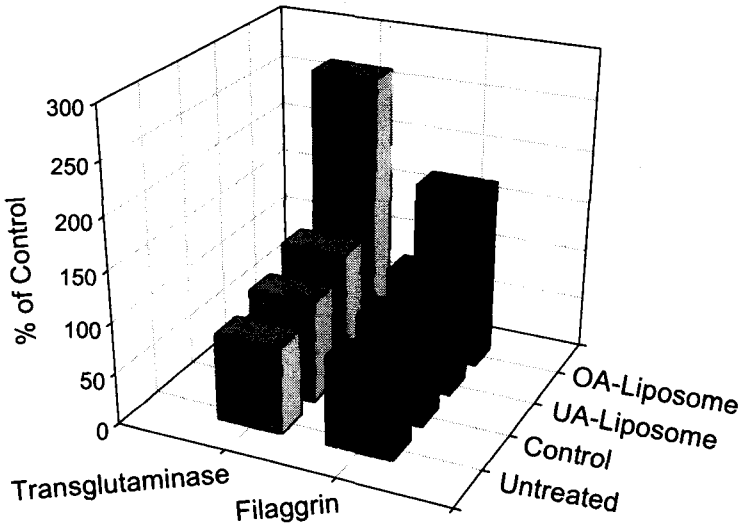


Figure 8. Transglutaminase and filaggrin expression of UA- and OA-containing liposomes

Skin cell differentiation of UA and OA-containing liposomes

Western blotting of proteins extracted from epidermal layers of hairless mouse was carried out to reveal the degree of keratinocyte differentiation of triterpenoid-containing liposomes. Only OA-containing liposome showed distinctive protein expressions, whereas UA-containing liposome and control (empty liposome) showed the similar results to untreated sample in Fig. 8. Among expressed proteins, filaggrin is a precursor of natural moisturizing factors that results from keratinocyte differentiation, and transglutaminase is a kind of differentiation markers. It is noted that UA-containing liposomes as a similar triterpenoid to OA did not make a difference compared to control.

However, both of OA- and UA-containing liposomes significantly increased the amount of total ceramides in hairless mouse skin as shown in Fig. 9, and UA-containing liposomes in other report increased ceramides in human skin [13]. It seems contradictory that ceramides were produced in epidermal layer without expression of marker proteins related to keratinocyte differentiation. It is supposed that lipid synthetic pathway of UA in keratinocyte is different with OA and a different unknown receptor was related to keratinocyte differentiation. Elucidation of differentiation mechanism of UA and OA is under progress and will be reported elsewhere.

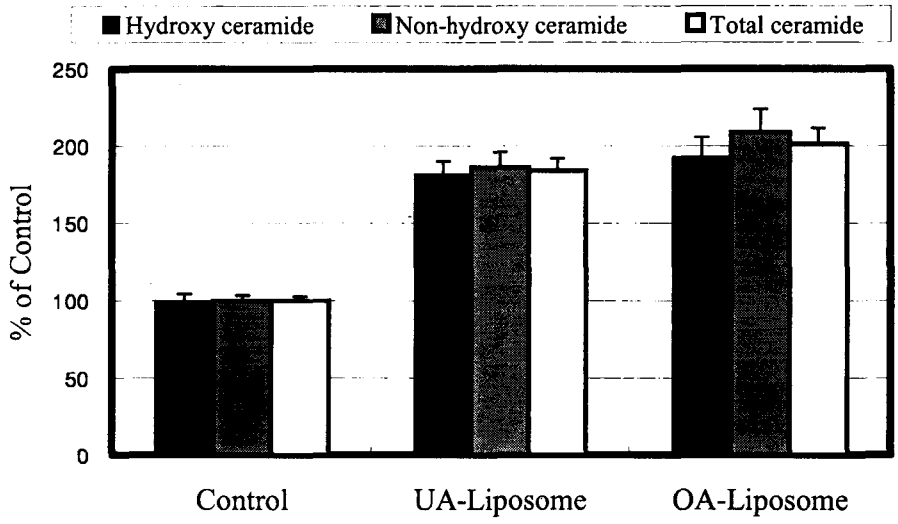


Figure 9. Ceramide synthesis of UA- and OA-containing liposomes and control (empty liposome)

Conclusions

Oleanolic acid (OA) and ursolic acid (UA) which have extremely low solubility were successfully solubilized in liposomal bilayer. Liposomes containing these triterpenoids were reconstituted from mixed micelles and showed nano-scaled hydrodynamic dimension without severe mechanical treatment. These reconstituted liposomes had optimal molar amount of triterpenoid for exhibiting small hydrodynamic size and suspension without recrystallization. Liposomal triterpenoids enhanced skin absorption of triterpenoid and biological efficacy. OA and UA in liposome increased

the amount of total ceramide in hairless mouse skin. OA-containing liposome significantly increased filaggrin and transglutaminase as markers of keratinocyte differentiation in epidermal layer of hairless mouse, whereas UA-containing liposome did not show significant increase of filaggrin and transglutaminase compared to empty liposome.

Acknowledgements

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