

Spontaneous Vesicle Formation from Sodium Salt of Acidic Sophorolipid and Its Application as a Skin Penetration Enhancer

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Abstract: In this study, spontaneous vesicle formation from the sodium salt of acidic sophorolipid (SLNa) was observed, and its potential application as a skin penetration enhancer for triterpene glycosides extracted from the fruits of *Siraitia grosvenorii* Swingle was then investigated. Dynamic light scattering (DLS) measurements of the SLNa assemblies prepared by the gentle mixing of SLNa with water (1%) showed their hydrodynamic radius (R_h) to be 96.2 nm, and their structure was assigned to be vesicles by freeze-fracture electron microscopy (FF-TEM). DLS and FF-TEM also revealed that the size of the vesicles increased with an increase in the concentration of the triterpene glycosides, indicating that the triterpene glycosides were incorporated into the SLNa vesicles. The results of an *in vitro* skin permeation assay, after loading the SLNa vesicles on a 3D cultured skin model, showed that the amount of SLNa that penetrated through the skin model increased with time. It was also found that the amount of permeated mogroside, which is the main active component of triterpene glycosides, was significantly enhanced by the SLNa vesicle formulation. These results clearly demonstrated that spontaneously formed vesicles composed of the bolaamphiphile SLNa are useful for application as penetration enhancers for active ingredients such as mogroside V.

Key words: glycolipid biosurfactant, sophorolipid, spontaneous vesicle, penetration enhancer, triterpene glycosides

1 INTRODUCTION

Bio-based surfactants (BSs) are natural amphiphiles that are abundantly produced from a variety of renewable resources by microorganisms. They have received great attention because of their unique properties, including biodegradability, low toxicity, and versatile biological functions, compared to petroleum-based surfactants¹⁻³. The other advantage of BSs is their structural diversity, which is naturally designed and engineered by microorganisms; their unique and complex structures enable them to build up distinct self-assembled structures^{4,5}. Recently, we reported the self-assembly properties of mannosyl erythritol lipids (MELs) produced by the genus *Pseudozyma*; they self-assembled into giant micelles⁶, vesicles⁷, and sponge particles⁸ depending on the concentration or the functional groups on the head group. Among a variety of assemblies, we have particularly focused on vesicle structures in

recent years because they can encapsulate both hydrophobic and hydrophilic compounds, and are known to act as effective skin penetration enhancers^{9,10}. It is also well-known that conventional vesicles are thermodynamically unstable. Kaler *et al.* first reported that thermodynamically stable vesicles were spontaneously formed by two synthetic single-tailed surfactants¹¹. These vesicles are more stable than conventional vesicles and are easily prepared by gently mixing the two surfactants with water. We also obtained spontaneously formed vesicles by mixing MEL with a phospholipid such as dimyristoyl phosphatidylcholine (DMPC)¹². Although the spontaneous vesicles reported so far are generally composed of multicomponent synthetic surfactant systems, some bolaform surfactants having two hydrophilic groups at both ends of a hydrocarbon chain generated unique spontaneous vesicles composed of monolayers and not bilayers^{13,14}.

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On the other hand, sophorolipids (SLs) are promising glycolipids BSs, generally produced by *Starmerella bombicola*¹⁵. They exhibit considerably high productivity with yields over 100 g/L from different carbon sources, and are efficiently recovered from culture broths, mainly by precipitation¹⁶. Unlike other BSs, SLs are produced as a mixture of acid- and lactone-type sophorolipids. Because of this, SLs display a high detergency, ready biodegradability, low foaming, and low cytotoxicity¹⁷. Furthermore, they are also characterized by high tolerance to hard water and have been used as commercial detergents in households and healthcare facilities in Japan^{18,19}. SLs and their derivatives have also been found to display anti-microbial²⁰, anti-cancer²¹, and anti-virus activities²². Thus, they are of growing commercial interests in the cosmetics, food, and detergent industries. Of all the SL homologues, acidic SL is an asymmetric bolaform structure with two different hydrophilic groups (Fig. 1). The disaccharide polar head and carboxyl group provide many sites for intermolecular association through hydrogen bonding.

Siraitia grosvenori Swingle (SG) is a traditional Chinese fruit, and a herbaceous perennial vine of the Cucurbitaceae family. SG has been used as a folk medicine in China for cough, sputum, asthma, bronchitis, pharyngitis, acute gastritis, and constipation. The most remarkable characteristic of SG is its unique property of sweetness. The chemical structure of the sweetening component in SG has been identified to belong to the triterpenoid named as mogroside for various glycosylated compounds and as mogrol for aglycones²³. The sweetening components in this plant include mogroside V and 11-oxo-mogroside V; tetraglucose conjugated mogrosides include mogroside IV and siamenoside I, and triglucose conjugated mogrosides include mogroside III. Previous studies on the pharmacological activities of SG extracts revealed anti-atherosclerotic effects²⁴, anti-cancer activity^{25, 26}, anti-allergy activity²⁷, and anti-diabetic effects^{28, 29} in animal models. Thus, they are of growing commercial interests in cosmetic industry.

In this study, we report the spontaneous vesicle formation from the sodium salt of acidic SLs (SLNa) for the first time, and their characterization using dynamic light scattering (DLS) and freeze-fracture electron microscopy (FF-

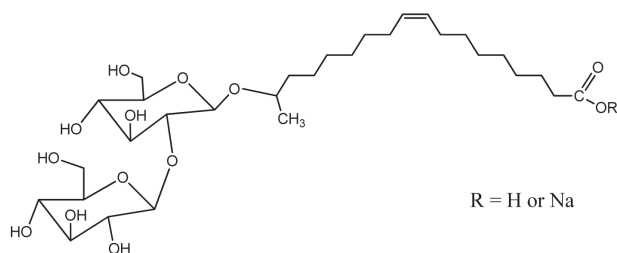


Fig. 1 The structure of acidic form of sophorolipid (SLH) and its sodium salt (SLNa).

TEM). Moreover, SL vesicles including triterpene glycosides extracted from the fruits of SG were newly prepared, and their penetration ability through a 3D cultured human skin model was evaluated by an *in vitro* penetration assay.

2 EXPERIMENTAL

2.1 Microorganism and culture conditions

The SL mixture was microbially produced by the following procedure³⁰: a seed culture (1.5 mL) of the yeast strain of *Starmerella bombicola* NBRC 10243 was transferred to a 300 mL Erlenmeyer flask containing 30 mL of a fermentation medium [1% (w/v) glucose, 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, and distilled water], followed by incubation at 30 °C on a rotary-shaker (300 rpm). The seed cultures were transferred into a mini-jar fermenter (TBR-2-3, Sakura S. J., Tokyo) with 2.5 L fermentation medium [10% (w/v) soybean oil, 10% (w/v) glucose, 0.25% (w/v) yeast extract, 0.1% (w/v) urea, 0.5% (w/v) MgSO₄·7H₂O, 0.1% (w/v) NaCl, and 1% (w/v) KH₂PO₄]. The cultivation was performed at 30 °C, 500 rpm, and 1 vvm of aeration for 7 days.

2.2 Purification and alkaline hydrolysis of the SLs.

The culture broth was then extracted with an equal volume of ethyl acetate. The organic layer was separated and evaporated. The resulting yellow oil (50 g) was dissolved in chloroform and placed on a column (5 × 40 cm). The mixture of SL was then chromatographed with a close gradient elution of chloroform – acetone (10 : 0 to 0 : 10). Alkaline hydrolysis of the SLs mixture led to the deacetylated acidic structures. The SLs mixture (1.0 g) was dissolved in 0.5 mL of 5N NaOH (aq) and placed in a test tube, and then the test tube was tightly sealed with a screw cap and heated at 80 °C in a water bath for 1 h. After cooling, the reaction medium was carefully acidified to pH 1.0 by the dropwise addition of HCl (aq) and then centrifuged (9000 rpm, 5 min). The upper layer was then removed, and the lower layer including the acidic SL was washed with water three times. The acidic SL was neutralized by the addition of 3N NaOH. SLNa was obtained by freeze-drying. The structure was assigned by ¹H-NMR and MALDI-TOF-MS as previously reported³¹.

2.3 Dynamic Light Scattering

The size of the assembled structures was measured with a DLS-7000 (Otsuka Electronics Co., Japan) using a He-Ne laser of 633 nm wavelength as a light source at 25 °C. Triterpene glycosides extracted from the fruits of SG were kindly supplied from Saraya Co., Ltd. (Osaka, Japan).

The time-dependent correlation function of the scattered light intensity was measured at a scattering angle of 90°. The DLS intensity data were processed using the in-

strumental software to obtain the hydrodynamic diameter, polydispersity index, and mass diffusion coefficient of the samples. The mass diffusion coefficient D was derived from the decay time (τ_c) of the intensity correlation function as $D = (2k_L^2\tau_c)^{-1}$, where k_L is the scattering wave vector. The hydrodynamic mass diffusion coefficient D_0 was obtained as the limit of D as k_L goes to zero. D_0 was found to obey the Stokes-Einstein relation, $D_0 = kT/6\pi\eta R_H$, where k is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the solution, and R_H is the hydrodynamic radius.

2.4 Freeze-Fracture Electron Microscopy

FF-TEM was used to determine the structure of the assemblies. Some samples were frozen with liquid nitrogen at $-189\text{ }^\circ\text{C}$. The fracture process was performed with a JFD-9010 (JOEL, Japan) at $-130\text{ }^\circ\text{C}$, and the fractured surface was then replicated by evaporating the platinum at an angle of 60° , followed by carbon at an angle of 90° to strengthen the replica. The replicate was placed on a 400 mesh copper grid after being washed with water, methanol, and chloroform. It was then examined and photographed using a JEM-1010 (JOEL, Japan) transmission electron microscope.

2.5 Determination of the surface activity

The surface tension of SLH and SLNa was determined by the pendant drop method at $25\text{ }^\circ\text{C}$, which was performed using an apparatus consisting of an automatic interfacial tension meter (DM500, Kyowa Interface Science, Japan) and the Drop Shape Analysis software of FAMAS ver. 2.01. The drop was formed at the tip of the syringe by pressing the solution out by means of a setscrew. The drop shape analysis was performed as follows: a drop profile was extracted from the drop image; then, a curve fitting program compared the experimental drop profile with a theoretical one (Young-Laplace method) and gave the corresponding surface tension value. For each concentration of the purified glycolipid solution, the evolution of the drop surface tension was followed over 30 min.

2.6 *In vitro* skin permeation study

The transdermal absorption of SLNa and mogroside V, which is an effective ingredient of SG, was measured using the Upright Franz cell assembly (PermeGear Inc., Hellertown, Pa.). TESTSKIN LSE-high, purchased from Toyobo Co., Ltd. (Osaka, Japan), was mounted between the donor and receptor compartments. The donor consisted of 1.0 mL of the vehicle containing either SG (0.1%) alone or SG (0.1%) with SLNa (1%). The receptor medium (4.5 mL) was phosphate-buffered saline (PBS, pH 7.2). The available diffusion area between compartments was 0.64 cm^2 . The stirring rate was kept constant and the temperature of the receptor was maintained at $37\text{ }^\circ\text{C}$. At 30, 60, and 120 min,

300 μL aliquots of the receptor medium were withdrawn for the quantitation of the SLs or mogroside V. The accumulated amounts of SLs and mogroside V were determined by RP-HPLC as described previously^{28,29}.

3 RESULTS

3.1 Surface activity of SLH and SLNa

The lowering of the surface tension is the most important physical property of surfactants, and the surface tension in aqueous solution was measured by the pendant drop method at $25\text{ }^\circ\text{C}$. Figure 2 shows the surface tension plotted as a function of SLH or SLNa concentration. The surface tensions of both decreased with an increase in the concentration and became constant at their critical aggregation concentrations (CAC). The CAC and γCAC were determined by the intersection of two fitted lines, and the estimated CAC and γCAC for SLH were 0.034% and 35.3 mN/m, which agree well with the values reported in the literature³⁰. The CAC and γCAC of the sodium salt of SL, SLNa, were 0.083% and 32.4 mN/m, indicating that SLNa can lower the surface tension more effectively than SLH. The higher hydrophilicity of SLNa resulted in its higher CAC value. Because we found that SLNa possesses a high surface activity and a high aqueous solubility, SLNa was used for further experiments.

3.2 SLNa assemblies including triterpene glycosides characterized by DLS and FF-TEM

The structure of the SLNa assemblies above the CAC and the effect of the addition of triterpene glycosides on

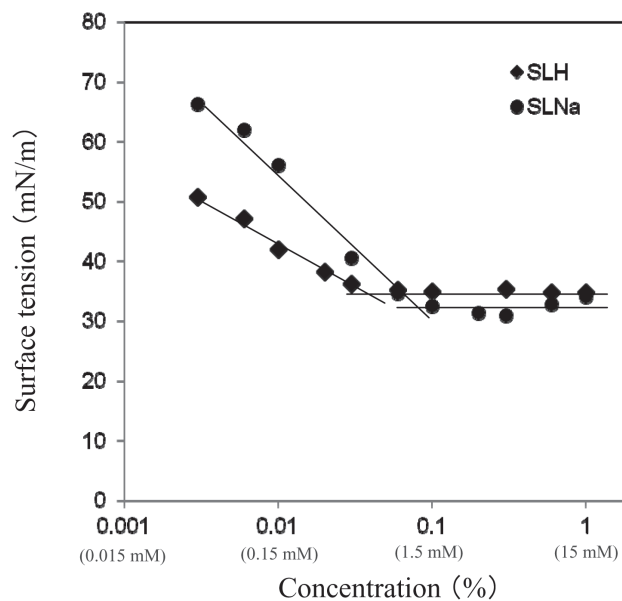


Fig. 2 Relationship between surface tension and surfactant concentration. ◆SLH ●SLNa.

the structure were investigated by DLS and FF-TEM. The triterpene glycosides extracted from the fruits of SG are potentially useful as important sources of pharmaceutical and sweetener compounds. They are known to be about a hundred times sweeter than sucrose but low in calories²³⁾. Mogroside V, which is one of the major components, inhibits the initiation and promotion of cancer, and exhibits antioxidant and antiallergic effects³⁵⁾.

The hydrodynamic radii (R_h) of the SLNa assemblies measured by DLS are shown in Table 1. The R_h of the pure SLNa assemblies at a concentration of 1% was 96.2 nm, and the R_h increased with an increase in the triterpene glycosides concentration. Zhou *et al.* reported on self-assembling structures of SLNa in dilute solutions ($<0.17\%$)⁴⁾, where SLNa self-assembles into giant aggregates whose R_h were below 100 nm; the size was found to dependent on the concentration. However, the detailed structure of the SLNa assemblies is still unclear because they used only DLS to determine the structure.

To address this, we directly observed the SL assemblies by FF-TEM. Figure 3(a) shows the FF-TEM images of the SL assemblies at a concentration of 1%. The image clearly indicates a vesicle structure whose size is similar to that predicted by the DLS measurements. Interestingly, Guilbot *et al.* reported the spontaneous formation of vesicles composed of unsymmetrical bolaamphiphiles bearing a neutral glycosidic polar and an electropositive ammonium group¹³⁾. Chen *et al.* also reported thermodynamically stable vesicles formed with orotic acid-derived bolaamphiphiles¹⁴⁾. The

observed SLNa vesicles were spontaneously formed by gently mixing with water, and the SLNa vesicle solution was stable for over one month at room temperature, demonstrating the formation of thermodynamically stable vesicles probably composed not of an ordinary bilayer but a monolayer structure.

Moreover, based on both the DLS and FF-TEM measurements, the size of the SLNa vesicles increased with an increase in the concentration of triterpene glycosides extracted from the fruits of SG, suggesting that the formation of the SL vesicles included the triterpene glycosides, even though the solubilization locus of the triterpene glycosides in the SLNa vesicles is unclear. These results clearly demonstrated that thermodynamically stable vesicles including triterpene glycosides were prepared for the first time by using a naturally derived bolaamphiphile bearing sophorose and electronegative carboxylate groups at both ends.

3.3 *In vitro* skin permeation study

To enhance the transdermal absorption of active ingredients, vesicle formulations have recently attracted attention^{9,10)}; vesicles, often called liposomes, are known to penetrate well when their size is about 300 nm³⁶⁾. Even though most vesicles reported as penetration enhancers have been conventional vesicles composed of bilayer membranes, in this study, the thermodynamically stable vesicles formed by the bolaform amphiphile SLNa were used as a penetration enhancer for triterpene glycosides extracted from the fruits of SG.

For the skin permeation study, a 3D cultured human skin model, TESTSKIN LSE-high, was used. It consists of a multilayer of stratum corneum (SC), viable epidermis, and a dermis for which skin fibroblasts are embedded in a collagen gel. The skin properties are close to those of biogenic skin, and the characteristics of the *in vitro* skin penetration correlate well with those of the *in vivo* situation³⁷⁾.

The amount of permeation of SLNa vesicles through the skin was investigated first. SLNa vesicles (1%) without triterpene glycosides were applied to the test skin, where the

Table 1 The effect of addition of triterpene glycosides on particle size of SLNa assemblies.

Triterpene glycosides	Particle size (nm)
0 %	96.2
0.02%	114.7
0.1 %	182.6

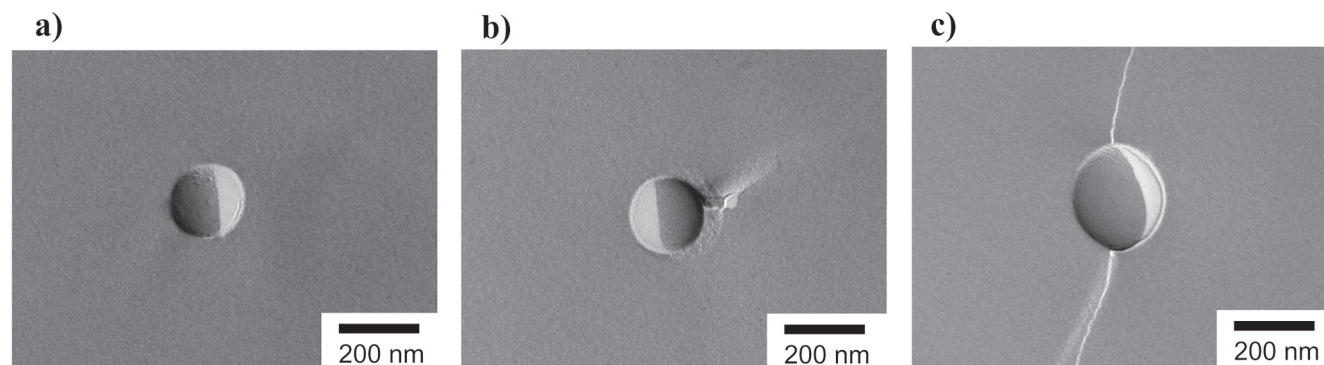


Fig. 3 Freeze-fracture electron micrographs of SLNa assemblies including triterpene glycosides. a) 0 % b) 0.02 % c) 0.1 % (Triterpene glycosides conc).

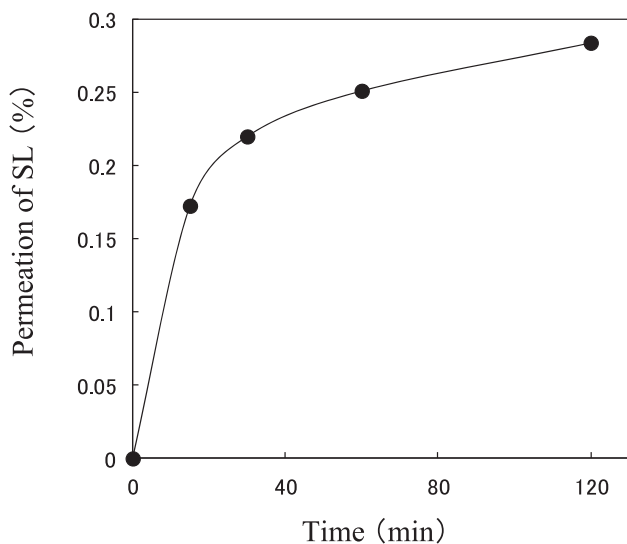


Fig. 4 Time dependence of transdermal absorption of SLNa having vesicle structure without triterpene glycosides.

vesicle size was 96.2 nm, as described in Table 1. Figure 4 shows the time dependence of the amount of SLNa permeated through the skin. The figure clearly indicates that the amount of penetrated SLNa increased with an increase in time, suggesting that SLNa could act as a penetration enhancer. We further investigated the effect of the SLNa vesicle formulation on the permeation of triterpene glycosides extracted from the fruits of SG. The SLNa vesicles containing 0.1% triterpene glycosides were prepared and loaded on the test skin. The size of the vesicles was 182.6 nm, as described in Table 1. Among the triterpene glycosides, the amount of permeated mogroside V, which is one of the major active and representative components and

water-soluble, was determined by HPLC, and the results are shown in Fig. 5(a). The structure of mogroside V is shown in Fig. 5(b). As shown in the figure, the amount of permeated mogroside V increased by the SLNa vesicle formulation after 2 h. This clearly demonstrates that the SLNa vesicles could act as a penetration enhancer for active ingredients.

Recently, Ishii *et al.* reported that vesicle-like structures composed of the acid form of sophorolipid, whose size ranges from 40 to 2700 nm, also act as a penetration enhancer for lactoferrin³⁸⁾. Compared with their system, the vesicles we prepared were much smaller and had a narrower size distribution. Thermodynamically stable vesicles are also known to be easily prepared compared with typical vesicles. Although the detailed mechanism of the penetration enhancement is still unclear, and further studies including *in vivo* experiments are necessary to clarify the mechanism, the spontaneously formed vesicles composed of bolaamphiphiles presented herein would be useful biomaterials for exploring transdermal applications. We will continue with further detailed studies to clarify the mechanism of the penetration enhancement.

4 CONCLUSION

Spontaneous vesicle formation from the bolaform amphiphile, SLNa, was observed, and the potential application of the vesicles as a skin penetration enhancer for triterpene glycosides extracted from the fruits of SG was then investigated. DLS measurements of the SLNa assemblies prepared by gently mixing SLNa with water (1%) revealed their hydrodynamic radius to be 96.2 nm, and their structure was determined to be vesicles using FF-TEM.

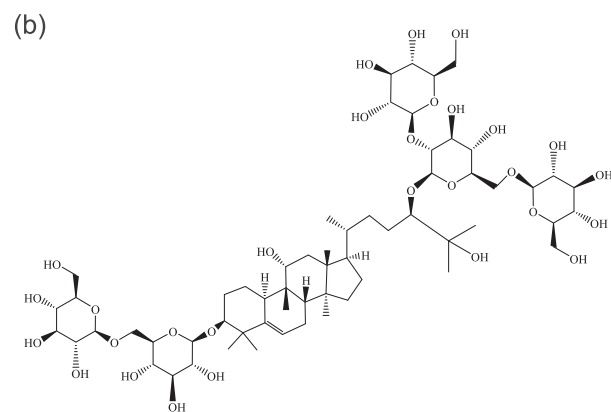
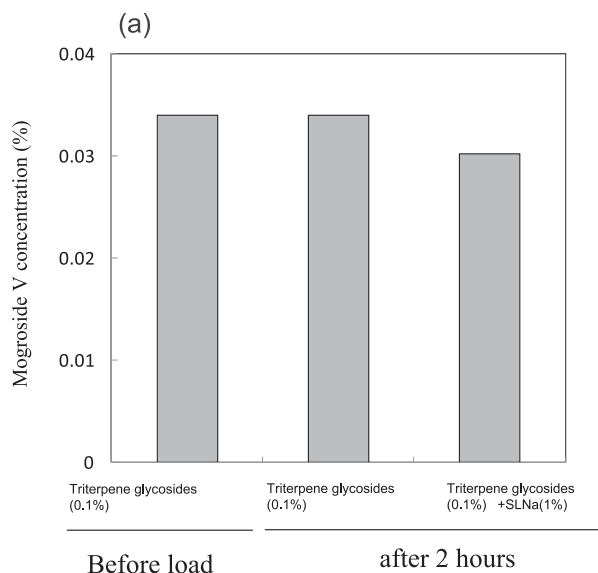


Fig. 5 (a) Transdermal absorption of mogroside V with or without SLNa assemblies. (b) Structure of mogroside V.

DLS and FF-TEM measurements also revealed that the size of vesicles increased with an increase in the concentration of triterpene glycosides, indicating that the triterpene glycosides were incorporated into the SLNa vesicles. An *in vitro* skin permeation assay, after loading the SLNa vesicles on a 3D cultured skin model, showed that the amount of SLNa that penetrated through the skin model increased with time. It was also found that the amount of permeated mogrosin, which is the main active component of triterpene glycosides, was significantly enhanced by the SLNa vesicle formulation.

Our results clearly demonstrated that spontaneously formed SLNa vesicles are useful as a penetration enhancer for active ingredients such as mogrosin V.

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