Droplet surface makes skin absorption different

: A study of skin penetration in biosurfactant glycolipids-incorporated liposomes

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Abstract

In the contemporary era, there is a growing demand for natural, safe, and efficacious cosmetic products. To better exert the function of active ingredients, transdermal delivery systems such as liposome technology were developed. The objective of this work is to prepare a biosurfactant-introduced liposome with biosurfactants and investigate the possible correlations between surface activity and skin penetration capability. The liposome, composed of glycolipids, hydrogenated lecithin and niacinamide, was demonstrated to exhibit excellent stability through the monitoring of particle size and zeta potential by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) respectively. A morphological study was performed using cryogenic transmission electron microscopy (cryo-TEM) to reveal the microstructure of the liposome. The incorporation of glycolipids into liposomes resulted in a favorable increase in surface activity, as evidenced by the measurement of the contact angle. An *in vitro* skin penetration study was conducted using Franz diffusion cell method and the glycolipids-incorporated liposome showed superior skin penetration compared to the control.

Confocal Raman spectroscopy was employed to investigate the *in vivo* skin penetration efficiency of liposomes, with the result that the liposome with lower surface tension demonstrated superior skin absorption.

Keywords: Glycolipids; Liposome; Skin penetration; Skin delivery; Niacinamide.

Introduction.

After COVID-19 pandemic, people are interested in the skin absorption of cosmetic actives. There are several factors that can enhance skin absorption for active ingredients, including the formulation type, the presence of enhancers, and the use of beauty devices. Transdermal drug delivery systems can be divided into three generations: First generation delivery candidates are low-molecular-weight, lipophilic and efficacious at low doses [1]. Second generation technologies increase skin permeability and provide a driving force to transport active ingredients into the skin. Third generation systems are more effective but disrupt the *stratum corneum* barrier and may cause skin irritation. It can be reasonably assumed that consumers would like to choose safe, efficacious and convenient ways of skin penetration to improve their skin status.

Liposome technology, as the 2nd generation approach to transdermal delivery, is a conventional but safe technology for transdermal delivery. Due to its bilayer vesicle structure, active ingredients can be loaded inside or on the vesicle, making it suitable for both hydrophilic and hydrophobic active ingredients [1,2]. Lecithin and cholesterol are common ingredients for the preparation of liposomes in pharmaceutical industry. The delivery efficiency of liposome is influenced by a number of factors, including particle size, lamellar structure, charge, edge activator and lipid composition [3]. However, there is a paucity of research investigating the impact of droplet surface properties on the process.

Recently, biosurfactant glycolipids have attracted considerable attention due to their favorable sustainability, low toxicity and high biodegradability [4]. Mannosylerythritol lipids, as a category of glycolipids, generated by yeast strains, have been found to exhibit outstanding surface activity, including the capability to reduce surface tension at low concentration and the self-assembling property to form vesicles, sponge (L_3), bicontinuous cubic (V_2) or lamellar (L_α) structures [5,6]. Moreover, they have been demonstrated beneficial properties for the skin, including barrier repairing, moisturizing and the ability to increase penetration of active ingredients [7–9]. Some research has indicated that the combination of glycolipids and other surfactants can be used to prepare transdermal drug delivery systems. However, the mechanism by which glycolipids-incorporated liposomes increase skin permeation remains to be elucidated [9,10].

In this study, we introduced biosurfactant glycolipids into liposome system with the objective of developing a novel transdermal delivery system. Considering the cosmetical application of liposomes, a stability test was conducted to obtain the optimal composition of liposomes. Furthermore, droplet size and zeta potential were also measured in order to confirm the stability of liposomes. A morphological study was conducted in order to gain a deeper understanding of the liposome system. The surface activity of liposomes and its potential correlation with skin permeability were investigated through the use of contact angle measurements and the application of Franz diffusion cell method in conjunction with confocal Raman spectroscopy.

Materials and Methods.

Materials

Hydrogenated lecithin was employed due to its well-documented property to form liposomes and its superior stability in comparison to unhydrogenated lecithin. Glycolipids, specifically mannosylerythritol lipids, were derived from the fermentation of fatty acids and glucose, both of

which were naturally occurring compounds. Niacinamide, as a common brightening agent, was selected for convenient quantitative analysis. Other raw materials were of cosmetic grade.

Preparation of liposomes

A group of liposomes was prepared with the following ingredients: hydrogenated lecithin, cholesterol, glycolipids, polyol, preservatives and niacinamide, as presented in **Table I**. After preliminary tests, a full factorial experiment was conducted with two factors both at three levels each: the concentration of glycolipids (0%, 0.05%, and 0.1%) and the concentration of cholesterol (0%, 0.1%, and 0.2%). During the process, Phase A and phase B were heated to 85 °C in order to facilitate the complete dissolution of solid materials. Subsequently, phase B was added into phase A, which was then homogenized at 4500 rpm for 5 min. After cooling the mixture to 45 °C, phase C was added with gentle mixing to obtain a crude emulsion. Then a high-pressure homogenizer was employed at a working pressure of 100 MPa to generate small-sized liposomes. To assess the stability of the samples, they were placed in PET bottles and stored at 4 °C, 25 °C and 45 °C for four weeks.

Table I. Composition of liposomes

Phase	Ingredients	mass%
A	Water	Up to 100
	Niacinamide	10
B	Cholesterol	0-0.2
	Hydrogenated lecithin	0.5
	Glycol lipids	0-0.1
	Dipropylene glycol	7
C	Preservatives	q.s.

Hydrodynamic diameter and zeta potential measurements

The hydrodynamic diameter was determined by dynamic light scattering (DLS). The excitation light source was a 40-mW semiconductor laser at 638 nm, and the scattered light was measured at an angle of 90°. All samples were diluted with purified water to the concentration

of 10% (w/w). The zeta potential was measured by electrophoretic light scattering (ELS). Both DLS and ELS measurements were conducted three times at 25 °C and the results were presented as means ± standard deviation (SD).

Morphological characterization by cryogenic transmission electron microscopy (Cryo-TEM)

The morphology of the liposome was characterized by Cryo-TEM. The liposome sample was diluted with buffer to minimize droplet overlays and therefore facilitate size measurements. The diluted sample was then loaded onto a lacey formvar/carbon film on a 200 mesh Cu-grid. Subsequently, the grid was plunged into liquid ethane, which rapidly vitrified the sample. The cryo-TEM imaging was conducted at an acceleration voltage of 200 kV.

Surface activity measurements by contact angle method

The surface activity was measured by contact angle method, specifically, sessile drop method. A drop of sample was placed on a coated lipophilic substrate at 25 °C, and a telescope goniometer was used to view the liquid drop profile positioned over the surface. Then the angle formed between the two interfaces (air/liquid and liquid/solid) can be measured. In order to compare the surface activity of different liposomes, the contact angle of water was introduced into the measurements. Each sample was observed for a period of 10 seconds.

***In vitro* skin penetration test by Franz diffusion cell method**

To investigate the skin penetration ability of liposomes, the Franz diffusion cell method was conducted with artificial membranes (transdermal diffusion test model, diameter 25mm), which has been widely adopted for skin permeation studies [11,12]. Each cell was composed of a donor chamber and a receptor chamber, with a membrane fixed between them. The receptor chamber was filled with 7 mL solution of PBS (pH 7.4) and ethanol in a weight ratio of 1:1 and

a magnetic stirrer was placed inside. The experiment run continuously at a temperature of 37 ± 0.5 °C and a speed of 250 rpm for 24 hours. At 2, 4, 8 and 24-hour intervals, 1 mL of receptor solution was sampled from the receptor chamber and 1 mL of PBS-ethanol solution was refilled inside to maintain a constant volume. All the collected solutions were sent for measurements of niacinamide contents using high-performance liquid chromatography (HPCL) with a UV detector. The cumulative infiltration per unit area, Q_n, can be calculated using the following equation:

$$Q_n = \frac{C_n V + \sum_{i=1}^{n-1} C_i V_i}{A}$$

Here, C_n represents the concentration of niacinamide in the receptor solution at the *n*th sampling point (µg/mL); V is the volume of the receptor chamber (7 mL); C_i is the concentration of niacinamide in the receptor solution from the 1st to the last sampling (µg/mL); V_i is the sampling volume (1 mL); A is the effective skin permeation area (1.767 cm^2).

The data were presented as means ± SD and the results were analyzed statistically for significance using Student's *t*-test.

***In vivo* skin penetration test by confocal Raman spectroscopy**

To investigate the *in vivo* skin penetration effect of the liposomes, confocal Raman spectroscopy was employed. The liposome samples were incorporated into serums at a concentration of 50% (w/w) for this test. Prior to the commencement of the measurements, volunteers (*n*=6) were asked to clean the volar forearms and to stay in an environment where constant temperature and humidity were maintained for 30 min. The liposome formulations were applied to the volar forearms on the test area for absorption over a period of 1 hour. Then the area was gently cleaned with wet wipes. Raman spectra were obtained at a wavelength of 800-3,700 cm^{-1}, from the surface of skin (0 µm) to a depth of 30 µm with a step size of 2 µm. The skin surface (0 µm) was defined as the position where the area under the curve (AUC) of the peak of 1,650 cm^{-1} Amide I band reached half of its maxima [13]. The relative content of

niacinamide was calculated according to the intensity of the peak at 1,033-1,053 cm^{-1}. The data were presented as means ± SD and were analyzed with software to generate a heat map according to the intensity of niacinamide in a more readily comprehensible format [14].

Results.

Preparation and characterization of liposomes

The stability of liposomes was monitored for 4 weeks as illustrated in **Table II**. The liposome sample containing 0.05% glycolipids (G-LS) or the sample without glycolipids (LS) exhibited favorable stability. At temperatures of 4 °C and 25 °C, formulations with cholesterol exhibited a tendency to crystallize, regardless of the concentration of glycolipids. Furthermore, the presence of glycolipids appeared to accelerate the process. At 45 °C, the formulation containing 0.1% glycolipids exhibited slight precipitation while all other formulations demonstrated good stability.

Table II. The stability results of liposomes in 4 weeks

		w% ratio of glycolipids and cholesterol								
		0:0	0:0.1	0:0.2	0.05:0	0.05:0.1	0.05:0.2	0.1:0	0.1:0.1	0.1:0.2
1 w	4 °C	o	x	xx	o	x	xx	o	xx	xx
	25 °C	o	o	xx	o	o	xx	o	o	xx
	45 °C	o	o	o	o	o	o	o	o	o
2 w	4 °C	o	x	-	o	x	-	o	-	-
	25 °C	o	x	-	o	x	-	o	x	-
	45 °C	o	o	o	o	o	o	o	o	o
4 w	4 °C	o	-	-	o	xx	-	o	-	-
	25 °C	o	xx	-	o	xx	-	o	xx	-
	45 °C	o	o	o	o	o	o	*	*	*

o: No significant change; x: Crystal formation on the surface; xx: Crystal formation on the surface and in the liquid; *: Slight precipitation. If the samples were not stable, further observation would not be conducted.

The appearances of G-LS and LS at 4 °C, 25 °C, and 45 °C after 4 weeks were shown in **Figure 1**. All initial samples were light blue liquids with a high transparency. The samples at 45 °C exhibited a slight cloudiness after 4 weeks. All the samples demonstrated a high degree of stability.

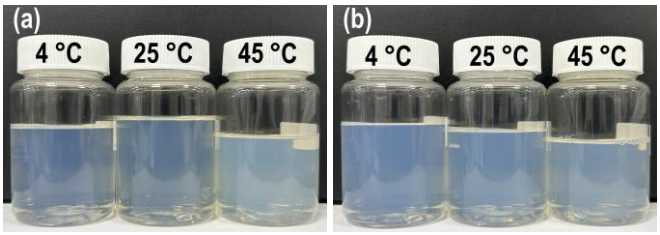

Figure 1. The appearances of G-LS (a) and LS (b) after stability tests at 4 °C, 25 °C and 45 °C for 4 weeks.

The hydrodynamic diameter and zeta potential of liposomes were measured by DLS and ELS respectively. The average particle size of G-LS and LS distributed from 90 nm to 120 nm and the zeta potential of G-LS and LS distributed from -25 mV to -40 mV as presented in **Figure 2**. Both G-LS and LS exhibited a preferred stability at 4, 24, and 45 °C in 4 weeks.

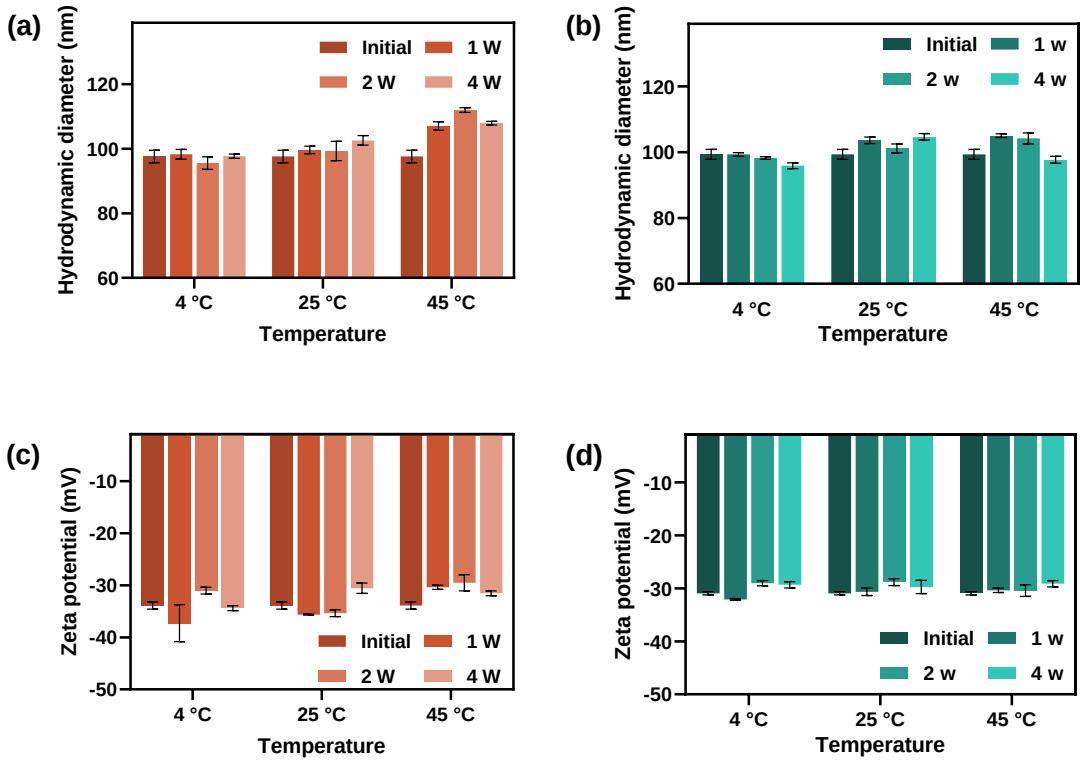

Figure 2. The average hydrodynamic diameters of LS (a) and G-LS (b); the zeta potentials of LS (c) and G-LS (d) at 4 °C, 25 °C and 45 °C in 4 weeks.

Given that LS was prepared with only one surfactant, hydrogenated lecithin, which was well-known for its ability to form liposomes, the cryo-TEM image of LS was not measured. The morphological characterization of G-LS by cryo-TEM was shown in **Figure 3**. The circular shape represented the vesicular structure of liposomes, and the size of G-LS vesicles was about 40-200 nm. The majority of the vesicles were unilamellar and a small proportion of the particles were multilamellar.

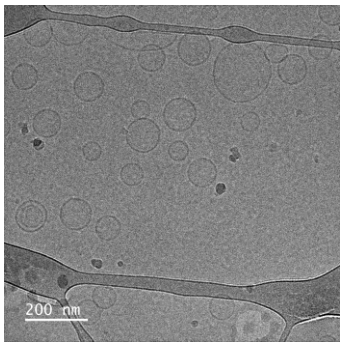

Figure 3. Cryogenic transmission electron microscopy (Cryo-TEM) image of G-LS.

Surface activity study of liposomes

A comparative study on the surface activity of G-LS and LS was conducted using the contact angle method. As illustrated in **Figure 4**, the contact angle of water was approximately 73° and barely changed in 10s. The contact angle of LS decreased from 64° to 58.8° in 10 s while the contact angle of G-LS decreased significantly from 59° to 35.4°. The contact angle images of water, LS, and G-LS at 10 s intuitively demonstrated the differing surface activities. In a previous report, the contact angle of forearm in human skin is about 30° [15]. Because G-LS exhibited lower surface tension, it would be superior compatibility on the skin in comparison to LS.

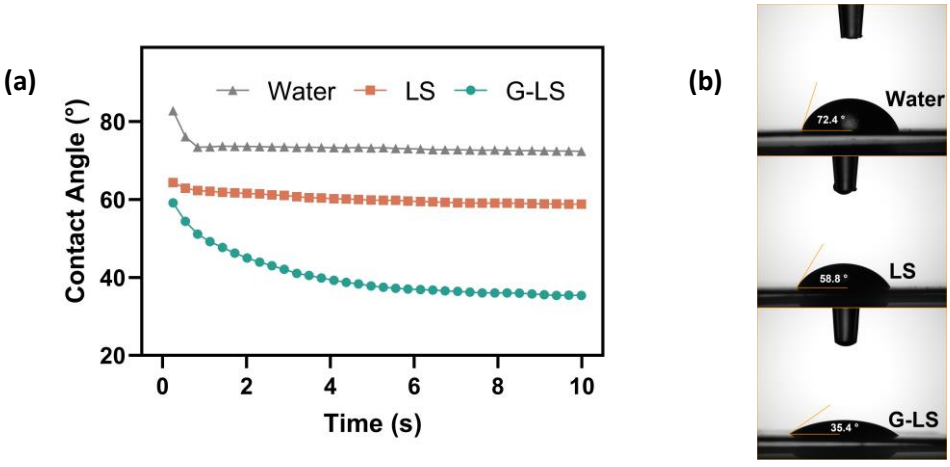

Figure 4. (a) The dynamic contact angles of water, LS and G-LS at 25 °C for 10 s and (b) the drop profile images of water, LS and G-LS at 10 s.

In vitro skin penetration study by Franz diffusion cell method

In vitro skin penetration efficiency of liposomes was evaluated by Franz diffusion cell method using artificial membrane. The concentrations of niacinamide in the receptor chambers were analyzed by HPLC. As presented in **Figure 5**, during the initial 8 hours, the penetration of G-LS and LS exhibited a low penetration rate, with no significant difference between the two samples despite the mean value of G-LS being higher. At 24 hour, the cumulative infiltration of G-LS (12.46±0.97 mg/cm^2) was significantly higher than that of LS (6.62±1.44 mg/cm2), revealing that G-LS exhibited superior penetration ability (p<0.01).

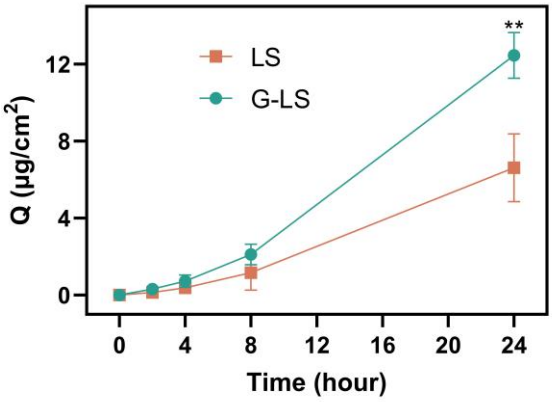

Figure 5. The cumulative penetration amount of G-LS and LS in 24 hours. The data are presented as means ± SD (n = 3), ** p<0.01.

In vivo skin penetration study by confocal Raman spectroscopy

To investigate the human skin penetration efficacy of liposomes, *in vivo* skin penetration study was conducted using the confocal Raman spectroscopy method. The concentration of niacinamide was calculated and is shown in **Figure 6**, from the skin surface (0 μm) to a depth of 30 μm. At skin depth of 0-4 μm, the intensity of niacinamide signal was markedly high and then decreased markedly with the increasing depth. The absorption of G-LS was found to be higher than that of LS from 0 μm to 20 μm, with the intensities being similar at 20-30 μm.

Moreover, the absorption of niacinamide was still observed at a skin depth of 30 μm, as evidenced by the baseline and sample spectra. This result shows G-LS would be a potential skin delivery system.

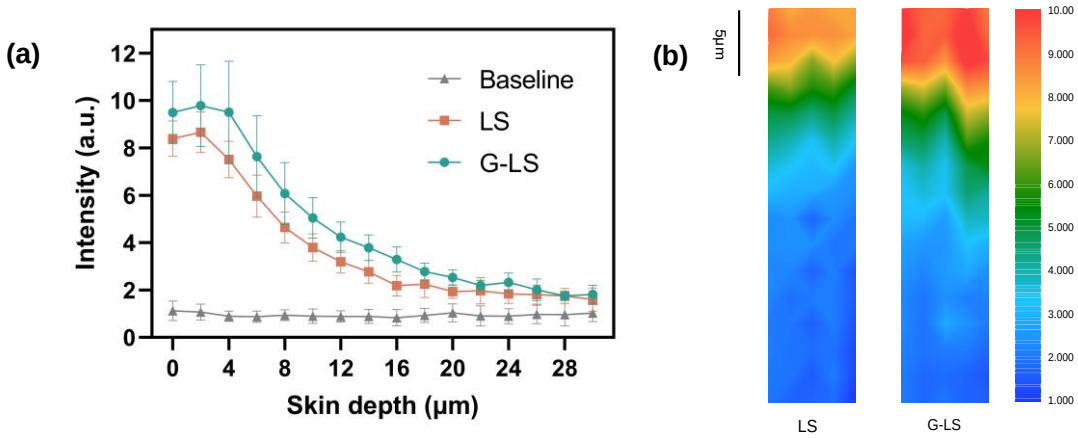

Figure 6. (a) Average penetration intensities of niacinamide plotted against the skin depth at 1 hour, the results are presented as means ± SD. (b) Penetration intensity map of niacinamide after 1 hour. (*n*=6)

Discussion.

Liposomes are nanosized vesicles comprising a hydrophobic tail and a hydrophilic head, which together constitute a phospholipid membrane [16]. Hydrogenated lecithin and cholesterol are two common materials for preparation of liposomes. In the present study, glycolipids were introduced into the system with the objective of modifying the surface of liposomes. However, during the experiments, all liposomes prepared with cholesterol exhibited poor stability at 4 °C and 25 °C. One possible reason is that the melting point of cholesterol is very high, which gives it a strong tendency to crystallize in the phospholipid bilayers [17]. It has been reported that solid lipids are more easy to form larger liposomes compared to liquid lipids and excessive solid lipids can result in instability of liposome systems [18]. Samples prepared with hydrogenated lecithin and 0% or 0.05% glycolipids exhibited superior stability. The glycolipids

used in this research were viscous liquids, which were less likely to cause crystallization and instability.

The cryo-TEM image clearly demonstrated the vesicular structure of G-LS. The particle size distribution ranged from 40 nm to 200 nm. Mannosylerythritol lipids are a mixture of four distinct chemical types, which can be distinguished by the degree of acetylation on the mannosyl groups [19]. As amphiphilic materials, mannosylerythritol lipids exhibit adjustable spontaneous curvature near zero, depending on the structure, which makes them ideal lipids for liposome preparation [20].

The surface of human skin is hydrophobic, uneven and distributed with appendages [21]. The existence of sulcus cutis and arista cutis greatly expands the surface area of skin. There are three principle routes for the transfer of drug through the *stratum corneum*: intercellular, transcellular (paracellular) and transappendageal routes [22]. The intercellular way is less effective than the other two. When the cosmetic product is applied on skin, bigger contact area means higher possibility for the active ingredients penetrating into the skin. Small contact angle promises enhanced spreadability and can facilitate an expanded contact area. Moreover, liposomes with small contact angle also helps actives spread into the sweat ducts, hair follicles and sebaceous glands. The glycolipids exhibited a favorable capability to decrease contact angle. Additionally it has been documented in the literature that glycolipids exhibit superior spreadability when compared to other surfactants, for example, polysorbate 20 and laureth-10 [23].

Therefore, there are two possible approaches by which glycolipids-incorporated liposome facilitates skin penetration. 1) Improved spreadability increases the contact area of the formulation with the skin. 2) Improved spreadability promotes active ingredients to flow into appendages and sulcus cutis, thereby enhancing skin absorption.

The schematic diagram of this study is presented in **Figure 7**.

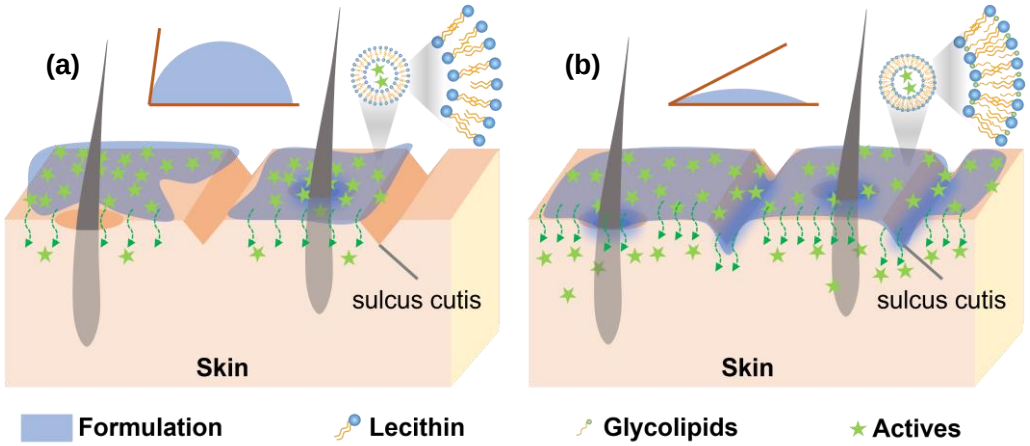

Figure 7. Schematic diagram of differences between liposomes with poor spreadability (a) and superior spreadability (b) while penetrating through the skin.

Conclusion.

In this study, we successfully prepared a biosurfactant glycolipids-incorporated liposome (G-LS). During the course of the experiment, it was observed that cholesterol had a negative effect on the stability of liposomes. In contrast, the glycolipids-incorporated liposome exhibited a preferred stability profile, as evidenced by particle size and zeta potential monitoring. The cryo-TEM image revealed that the structure of G-LS was mainly unilamellar vesicles with a particle size distribution of 40-200 nm. The droplet surface property was investigated using dynamic contact angle method. G-LS exhibited favorable surface activity and spreadability on lipophilic surface, which was mostly attributed to the presence of glycolipids. Liposome with small contact angle showed superior *in vitro* skin penetration ability. Similar result was observed in the *in vivo* skin penetration test conducted using the confocal Raman spectroscopy method.

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Conflict of Interest Statement.

NONE.

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