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In Vitro Permeation and Skin Retention of α-Mangostin Proniosome

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The current investigation evaluated the potential of proniosome as a carrier to enhance skin permeation and skin retention of a highly lipophilic compound, α-mangostin. α-Mangostin proniosomes were prepared using the coacervation phase seperation method. Upon hydration, α-mangostin loaded niosomes were characterized for size, polydispersity index (PDI), entrapment efficiency (EE) and ζ-potential. The *in vitro* permeation experiments with dermis-split Yucatan Micropig (YMP) skin revealed that proniosomes composed of Spans, soya lecithin and cholesterol were able to enhance the skin permeation of α-mangostin with a factor range from 1.8- to 8.0-fold as compared to the control suspension. Furthermore, incorporation of soya lecithin in the proniosomal formulation significantly enhanced the viable epidermis/dermis (VED) concentration of α-mangostin. All the proniosomal formulations (except for S20L) had significantly ($p<0.05$) enhanced deposition of α-mangostin in the VED layer with a factor range from 2.5- to 2.9-fold as compared to the control suspension. Since addition of Spans and soya lecithin in water improved the solubility of α-mangostin, this would be related to the enhancement of skin permeation and skin concentration of α-mangostin. The choice of non-ionic surfactant in proniosomes is an important factor governing the skin permeation and skin retention of α-mangostin. These results suggested that proniosomes can be utilized as a carrier for highly lipophilic compound like α-mangostin for topical application.

Key words α-mangostin; proniosome; permeation; skin retention; lipophilic

α-Mangostin is a xanthone obtained from mangosteen pericarp and has indicated for strong anti-melanogenic activity on B16F1 melanoma cells. Without compromising the cell viability, α-mangostin (5 μg/mL) was capable of inhibiting tyrosinase enzyme activity and down-regulating genes expression involved in the melanogenesis pathways.[1] Despite its strong biological activities, α-mangostin is a highly lipophilic compound, with an estimate log*P* value of 4.64. Malkia *et al.*[2] suggested that skin permeation is generally increased with lipophilicity, but a further increase in log*P* to more than 4.1 was reported to decrease the skin permeability. Highly lipophilic drugs may be retained in the lipophilic stratum corneum and resist partitioning into the more hydrophilic viable epidermis.[3] Thus, clearance by desquamation of the stratum corneum rather than diffusion across the stratum corneum may then become the rate-limiting step for highly lipophilic drugs.[3] For potent whitening effect, lipophilic α-mangostin should permeate and retain in the hydrophilic basal epidermis where the melanocytes are located. It is a clear fact that delivery of a natural compound to its targeted site is very important to expect the biological action.

Several nanocarrier systems *e.g.* liposome, niosome, ethosome *etc.* have been proposed to overcome the limitation in skin delivery. Niosomes are non-ionic surfactant based vesicles formed by the self-assembly of non-ionic surfactants in aqueous media. Niosomes were first reported in the 1970's by the researcher in cosmetic industry,[4] and since then have widely been used as a drug carrier. This vesicular system is capable of encapsulating both hydrophilic and lipophilic compound and therefore is analogous to liposomes (phospholipid vesicles).[5] Niosomes pose several advantages over liposomes as they require less purity variability, offer higher stability, and have greater availability of surfactants.[6–8] The non-ionic nature of niosomes also offers relatively non-toxic, flexibility in their structural constitution, and controlled delivery at a particular site.[9] Besides, niosomes do not require special preparation and storage conditions, therefore are relatively inexpensive and more attractive for industrial manufacturing than liposomes.[7,10]

Despite the advantages offered by the niosomal systems, they still have drawbacks such as (1) physical and chemical instability, (2) leakage and fusion of encapsulated drug from the vesicles, and (3) hydrolysis of the encapsulated drug which leads to decreasing shelf life.[8] These problems are avoided by the introduction of provesicular approach known as proniosome. Proniosome exists in several forms, which are liquid crystalline compact proniosomal gels, alcoholic solutions of the non-ionic surfactant (liquid form),[11] or dry granular powder.[12] Proniosomes offer greater stability due to very little water content in the system and can transform into niosomal vesicles immediately upon hydration. Proniosomes also provide ease of manufacture and scale up, convenience of transportation, distribution, storage, and dosing. Fang *et al.*[13] reported that proniosome gel and niosome suspension of estradiol showed different permeation profile through the skin. Estradiol proniosome composed of Span 40 showed enhanced skin permeation while estradiol niosome did not show enhancement in skin permeation as compared to control (free estradiol). High concentration of phospholipids and non-ionic surfactant is necessary to provide penetration enhancer effect and vesicle–skin interaction that can enhance the permeation of drugs from vesicles. Therefore, in this study, instead of niosome, proniosome was chosen as the vesicle carrier of α-mangostin to enhance skin permeation.

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Several research attempts had been made to enhance topical delivery of α-mangostin using different strategies, including liposome[14,15] and niosome.[16] However, most of these efforts require sophistication and lengthy techniques, also involved the use of harmful solvent in large volume. No report has been found that investigated the feasibility of proniosomes as a carrier for topical delivery of α-mangostin although there are a few examples of lipophilic compound encapsulated in proniosomes. Documented were the levonorgestrel ($\log P$=3.06),[17] estradiol ($\log P$=3.91),[13] flurbiprofen ($\log P$=3.94),[11] carvedilol ($\log P$=3.12),[18] and mefenamic acid ($\log P$=4.03).[19] However, development of proniosomal systems is challenging as a selection of suitable formulation ingredients may affect the formation, stability, characteristics and performance of the vesicles.[17] The development of proniosome is still in infancy and requires further exploration in the field for topical delivery.

This study aimed to develop a convenient and low-cost topical delivery system of α-mangostin using proniosome as a novel carrier. Several non-irritant, non-toxic, and relatively cheap non-ionic surfactants were screened for α-mangostin proniosome preparations. The influence of formulation components on the characteristics of α-mangostin proniosome such as vesicle size, polydispersity index (PDI), encapsulation efficiency (EE), and ζ-potential was investigated. Furthermore, *in vitro* permeation and skin retention of α-mangostin proniosome were also studied using dermis-split Yucatan Micropig (YMP) skin. The performances of α-mangostin proniosome were related to the solubility of α-mangostin in the non-ionic surfactants and soya lecithin.

Experimental

Materials α-Mangostin (98% purity) was purchased from Biopurify Phytochemicals Ltd., Chengdu, China. Span 60, cholesterol (from sheep wool, ≥ 92.5% [GC]) and soya lecithin were procured from Sigma-Aldrich Co., LLC, MO, U.S.A. Others non-ionic surfactants (Span 20, 40, 80, 85, Tween 20, 40, 60, Tween 80) were obtained from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. Water (HPLC grade) was produced directly from Millipore filter (Millipore S.A.S., Molsheim, France). All others chemicals and solvents were of reagent grade and were obtained commercially, with majority from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Log *P* Calculation of α-Mangostin Chemical structure of α-mangostin was drawn using software ChemBioDraw Ultra 12.0 to determine the $\log P$ value of α-mangostin. The estimate $\log P$ value of α-mangostin is 4.64, indicated that it is a highly lipophilic compound.

Determine the Distribution to Octanol of α-Mangostin To develop proniosome formulation, non-ionic surfactants (Spans/Tweens) and soya lecithin were selected for solubility screening of α-mangostin. Four groups of solutions were prepared, that are solution containing 1% (w/v) Spans, solution containing both 1% (w/v) Spans and 1% (w/v) soya lecithin, solutions containing 1% (w/v) Tweens and solutions containing 1% (w/v) soya lecithin. Excess α-mangostin powder (98% purity) was added to a vial containing the solution and stirred for 24 h at 32°C. α-Mangostin was allowed to dissolve until its saturation point. The saturated suspension was filtered using 0.20 μm disposable membrane filter (Advantec, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and the concentration of solubilized α-mangostin was assayed using HPLC system. The same procedure was carried out to determine the solubility of α-mangostin in *n*-octanol. Experiments were carried out in triplicate. The distribution to octanol of α-mangostin was calculated as following equation:

$$\text{Distribution to octanol} = \frac{\text{Concentration of } \alpha\text{-mangostin in } n\text{-octanol}}{\text{Concentration of } \alpha\text{-mangostin in the aqueous phase}}$$

Preparation of α-Mangostin Proniosome Coacervation phase separation method modified from Alsarra *et al.*[20] was adopted for the preparation of α-mangostin proniosomes. The compositions of different proniosomal formulations were listed in Table 1. Non-ionic surfactants, cholesterol, soya lecithin and α-mangostin were accurately weighed and mixed with 400 μL of absolute ethanol in a wide-mouth glass tube. The total lipid was fixed at 570 mg. The open end of the wide-mouth glass tube was screwed and warmed in a water bath at 70±5°C until all the ingredients were completely dissolved. Hot distilled water (100 μL) was added and the mixture was warmed at 70±5°C for 2 min. The clear solution formed was allowed to cool down to room temperature for the formation

Table 1. Compositions and Appearance of α-Mangostin Proniosomes (mg)

Formulation code	α-Mangostin	Non-ionic surfactant		Lecithin	Cholesterol	Appearance
S20	5	Span 20	513	—	57	YL
S40	5	Span 40	513	—	57	BG
S60	5	Span 60	513	—	57	SWG
S80	5	Span 80	513	—	57	BL
S85	5	Span 85	513	—	57	BL
S20L	5	Span 20	270	270	30	BL
S40L	5	Span 40	270	270	30	BG
S60L	5	Span 60	270	270	30	BG
S80L	5	Span 80	270	270	30	BL (2 phases)
S85L	5	Span 85	270	270	30	BL (3 phases)
T20	5	Tween 20	513	—	57	YL
T40	5	Tween 40	513	—	57	YL
T60	5	Tween 60	513	—	57	YL
T80	5	Tween 80	513	—	57	YL

Abbreviations: YL: yellowish liquid, BG: brownish gel, SWG: solid white gel, and BL: brownish liquid.

of proniosome.

Characterization of α-Mangostin Loaded Niosome Prior to characterization, α-mangostin proniosomes (100 mg) were hydrated with 10 mL of water, warmed in a water bath (70±5°C, 5 min) with manual shaking to form α-mangostin loaded niosome.

Vesicle Size, Size Distribution and ζ-Potential

The size, polydispersity (PDI) and ζ-potential of the α-mangostin loaded niosome were determined using Zetasizer (Malvern Zetasizer Nano S; Maver Instruments, Worcestershire, U.K.). The α-mangostin loaded niosome was diluted 10-fold with distilled water before measurement. The size and PDI of α-mangostin loaded niosome were determined using dynamic light scattering (DLS) based on photon correlation spectroscopy while the ζ-potential measurement was based on electrophoretic light scattering. The analysis was performed at 25°C, the material refractive index at 1.45 and absorption index 0.001. All samples were measured 3 times. Results were expressed as the mean±standard deviation (S.D.).

Entrapment Efficiency (EE)

The EE of α-mangostin loaded niosomes was determined using the ultracentrifugation method. The α-mangostin loaded niosome (1 mL) was separated from the unentrapped drug by ultracentrifuged at 80000 rpm, 4°C for 45 min (CS150 GXII micro ultracentrifuge, Hitachi Koki Co., Ltd., Tokyo, Japan). The supernatant was recovered and assayed by HPLC system for free α-mangostin content. The total content of drug in the niosomal suspension was also determined. The EE of α-mangostin loaded niosomes was calculated as the following equation:

$$\mathrm{EE}(\%) = \frac{C_t - C_f}{C_t} \times 100\%$$

where C_t is the concentration of total α-mangostin and C_f is the concentration of free α-mangostin. Experiments were conducted in triplicate and results were expressed as the mean±S.D.

Observation with Field Emission Scanning Electron Microscopy (FESEM)

FESEM was used to analyze the morphology of α-mangostin loaded niosome. The α-mangostin niosomal dispersion hydrated from proniosome S85L was dropped on a clear glass cover and dried in the oven at 45°C for overnight. The sample was coated with platinum (Pt) with a vacuum evaporator. The sample observation was carried out using ZEISS Crossbeam 340, with an accelerating voltage of 5 kV.

Skin Sample Frozen Yucatan micropig (YMP) skin sets (female pigs: 5 months old, 22 kg) were obtained from Charles River Japan Inc. (Yokohama, Kanagawa, Japan). Each YMP skin set consisted of 16 skin sheets (sheet size: approximately 10×10 cm) and was enclosed in a plastic bag identified by numbers. The frozen YMP skin sets were stored at −80°C until the permeation studies (maximum period of six months). The YMP dorsal and shoulder skin sheets were selected for the permeation studies. All animal experiments were performed according to the ethics committee of Josai University.

***In Vitro* Permeation Experiment** YMP skin was prepared using the method as described by Takeuchi *et al.*[21] YMP skin was thawed for 15 min and excess subcutaneous fat was trimmed off from the skin sheets. Intact YMP skin was around 2.4–2.8 mm in thickness. Dermis-split YMP skin (0.40±0.05 mm) was prepared using an electric dermatome (Acculan® 3Ti Dermatome; Aesculap, Inc., U.S.A.) and the thickness of skin samples were measured using a dial thickness gauge (Teclock Corporation, Advic Co., Ltd., Amagasaki, Hyogo, Japan). Skin piece was mounted on the diffusion cell with the epidermis side facing donor compartment. The control suspension (α-mangostin suspended in water) and proniosomal liquids were studied using side-by-side diffusion cell (effective diffusion area, 0.95 cm^2) while proniosomal gels were studied using vertical-type diffusion cell (effective diffusion area, 1.77 cm^2). The receiver solution was ethanol–water [40 : 60% (v/v)] to maintain a sink condition. The skins were allowed to equilibrate for 1 h using water before the experiment. After equilibration, 3 g of the proniosomal formulation was placed in the donor compartment and all donor compartments were covered with parafilm to minimize solvent evaporation from the formulation. Receiver solution was kept agitated and warmed at 32°C throughout the experiments. Sample aliquot (500 μL) was withdrawn from the receiver compartment at predetermined time intervals (0, 12, 24, 36, 48 h). The permeant concentration in the receiver chamber was determined by LC-MS/MS. Each experiment was carried out four times (n=4).

Sample Preparation, LC-MS/MS Instrumentations and Conditions The concentration of α-mangostin permeated into the receiver compartment was determined using LC-MS/MS with positive ion electrospray ionization (ESI). LC-MS/MS conditions were modified from Li *et al.*[22] Briefly, 100 μL sample aliquot was added to 100 μL of mobile phase (acetonitrile–water [80 : 20, v/v]) containing 0.1% formic acid and vortex-mixed. After centrifugation at 15000 rpm at 4°C for 5 min (Hitachi Inimac CT15RE; Hitachi Koki Co., Ltd.), the resulting supernatant was injected into an LC-MS/MS system. The LC-MS/MS system was equipped with a SIL-20 A prominence autosampler (Shimadzu, Kyoto, Japan), a LC-20 AD pumps (Shimadzu) and an API 3200™ LC-MS/MS system equipped with a Turbo Ion Spray interface (Applied Biosystems, Foster, CA, U.S.A.). The instrument was controlled by Analyst® Software (Version 1.4.1). Chromatographic separation was performed using a Shodex ODP2 HP-2B (2.0 mm i.d. × 50 mm L, SUS 316) (Shoko Co., Ltd., Tokyo, Japan) at 40°C using a flow rate of 0.2 mL/min. The instrument parameters included an ion transfer tube temperature of 700°C and a spray voltage of 5.5 kV. The collision energy was 21 eV for α-mangostin. The selected reaction monitoring scheme followed transitions of the precursor to selected product ions with the following values: m/z 412.1→ 356.1 for α-mangostin.

Skin Retention Study After completion of the *in vitro* permeation experiment, the skin samples were removed from the diffusion cells and washed briefly with distilled water on both stratum corneum (SC) side and the viable epidermis/dermis (VED) side. Excess water was blotted off. Each harvested skin sample was divided into two parts, in which one-half was used to determine the total concentration of α-mangostin in the split skin while another half was tape stripping 30 times to remove the SC and studied for the concentration of α-mangostin in the VED only. The skin was weighed 0.05 g and cut into small pieces using scissors. Methanol (450 μL) was added and the skin was homogenized in ice using ergonomic homogenizer (Polytron®, PT 1200 E, Kinematica AG, Schweiz, Switzerland). Then, 500 μL of methanol was added

to the homogenate, vortexed for 15 min and centrifuged (Hitachi Inimac CT15RE; Hitachi Koki Co., Ltd.) at 15000 rpm, 4°C for 5 min. The supernatant was collected. Each experiment was carried out four times (n=4).

Sample Preparation and HPLC Instrumentations and Conditions The concentration of α-mangostin retained in the dermis-split YMP skin was determined using a Shimadzu LC-20AD system (Shimadzu). Briefly, 100 μL sample aliquot was added to 100 μL of methanol and vortex-mixed. The mixture was centrifuged (Hitachi Inimac CT15RE; Hitachi Koki Co., Ltd.) at 15000 rpm, 4°C for 5 min to remove protein. Supernatant was injected into the Shimadzu LC-20AD system (Shimadzu), which mainly consisted of a SCL-10A VP system controller, a LC-20 AD pump, a SIL-20 A prominence auto sampler, a DGU-20 A_3 prominence degasser, a CTO-20 A prominence column oven, and a SPD-M20A UV detector. The separation was performed using a reverse phase C-18 column (Type UG120, 5 μm, 4.6×250 mm) (Shiseido Co., Ltd., Tokyo, Japan). The mobile phase consisting of acetonitrile and 0.1% orthophosphoric acid with the mixing ratio 80 : 20 for 10 min, delivered at the flow rate of 1 mL/min. The temperature was maintained at 40°C. The injection volume was 20 μL. UV detector was monitored at 320 nm.

Data Analysis The cumulative amount of α-mangostin (ng/cm^2) permeated through the dermis-split YMP skin was calculated and expressed as the mean±S.D. The permeation rate or flux (J, ng/cm^2/h) was determined based on the slope of linear regression of the cumulative amount of α-mangostin (ng/cm^2) plotted against flux time. The *in vitro* permeation enhancing effect of α-mangostin was determined in terms of enhancement ratio (ER) using the following equation:

$$\mathrm{ER} = \frac{\text{Flux of } \alpha\text{-mangostin from proniosomal formulations}}{\text{Flux of } \alpha\text{-mangostin from control}}$$

For skin retention study, the concentrations of α-mangostin deposited in the skin (the 'total concentration' and 'VED concentration') were calculated and expressed in μg/g. The skin retention enhancing effect of α-mangostin in the VED layer was determined using the following equation:

$$\mathrm{ER} = \frac{\text{The VED concentration of } \alpha\text{-mangostin from proniosomal formulations}}{\text{The VED concentration of } \alpha\text{-mangostin from control}}$$

Statistical Analysis All the experimental data were tested for statistical significance (p<0.05) using one-way ANOVA with a *post hoc* multiple comparison tests (Tukey test) (IBM SPSS© Statistics 20).

Results

Determine the Distribution to Octanol of α-Mangostin Screening of the formulation ingredients for the development of α-mangostin proniosomes was performed by determining the distribution to octanol of α-mangostin in solutions containing 1% (w/v) non-ionic surfactants and/or 1% (w/v) soya lecithin. Nine types of non-ionic surfactants (Spans/Tweens) and soya lecithin were selected for screening. Figure 1 shows the distribution to octanol of α-mangostin in solutions containing 1% (w/v) non-ionic surfactants with or without 1% (w/v) soya lecithin solutions. Due to its highly lipophilic nature, α-mangostin was found completely insoluble in water. Solubility of α-mangostin increased with the addition of non-ionic surfactants and/or soya lecithin. The distribution to octanol values of α-mangostin decreased as soya lecithin was added in the Span solutions. Tween solutions exhibited lower distribution to octanol values as compared to Span solutions.

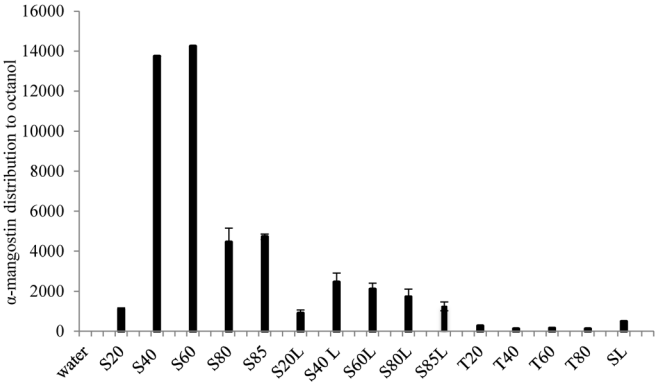

Fig. 1. Distribution to Octanol of α-Mangostin

α-Mangostin was dissolved in water, n-octanol, or solution containing 1% (w/v) surfactants/soya lecithin. α-Mangostin distribution to octanol=[n-octanol]/ [aqueous phase]. Each value represents the mean±S.D. (n=3). Solubility of α-mangostin in n-octanol was 158.85±6.86 mg/mL while solubility of α-mangostin in water was 0 μg/mL (completely insoluble). 'S' refers to solution containing Span [1% w/v]; 'S_L' refers to solution containing both Span [1% w/v] and soya lecithin [1% w/v]; 'T' refers to Tween solution [1% w/v]; and 'SL' refers to soya lecithin solution [1% w/v]).

Proniosome Preparation Table 1 summarized the appearance of α-mangostin proniosome formulations. Most of the α-mangostin proniosomes appeared as gel or liquid with a yellowish or brownish colour. It was also observed that Span 40 and 60 could formed gel with or without the presence of soya lecithin while the other formulations existed as proniosomal alcoholic solutions (liquid phase). Two phases and three phases proniosomal liquids were observed in S80L and S85L proniosomes, respectively.

Characterization of α-Mangostin Loaded Niosome Table 2 shows the physicochemical characteristics of α-mangostin loaded niosomes. Although proniosomes that composed of Tweens produced small vesicles with no significance difference (p>0.05) among themselves, no entrapment of α-mangostin was observed. On the other hand, all the α-mangostin proniosomes formulated from Spans with or without soya lecithin exhibited high EE (*ca*. 100%). S40 and S60 proniosomes produced significantly larger vesicle (p<0.05) as compared to S20, S80, and S85 proniosomes. The presence of soya lecithin had significantly decreased (p<0.05) the particle size of S40L and S60L proniosomes but increase the size of S20L proniosome (p<0.05). Besides, vesicles composed of Spans with soya lecithin (except S85L) was found to display more negative ζ-potential values than those without. Proniosomes S85, S80L and S85L recorded low PDI values (PDI <0.33), while the other proniosomes composed of Spans indicated higher PDI values (PDI >0.5). Figure 2 shows the observation of S85L under FESEM. The observed particle size of S85L was corresponded with the result of DLS.

***In Vitro* Permeation Study** Figure 3 showed the permeation profile of α-mangostin across the dermis-split YMP skin. α-Mangostin proniosomes composed of Spans and soya lecithin was tested for *in vitro* permeation study. α-Mangostin suspended in water was used as control. The control suspen-

Table 2. Physical Characteristics of α-Mangostin Loaded Niosomes

Formulation code	Size (μm)	PDI	ζ-Potential (mV)	EE (%)
S20	0.52±0.06	0.55±0.07	−57.1±7.9	98.7±1.42
S40	3.93±0.39	0.62±0.10	−53.6±7.8	99.5±0.32
S60	4.15±0.57	0.67±0.10	−60.4±3.3	99.6±0.45
S80	0.40±0.08	0.56±0.13	−59.5±4.3	98.8±1.49
S85	0.23±0.01	0.30±0.03	−59.1±7.2	99.7±0.15
S20L	1.77±0.20	0.78±0.07	−78.6±5.7	98.6±2.27
S40L	1.57±0.04	0.55±0.03	−86.6±7.0	99.7±0.25
S60L	2.14±0.80	0.86±0.15	−75.0±5.0	100±0.00
S80L	0.17±0.01	0.29±0.05	−67.6±4.8	99.8±0.29
S85L	0.18±0.03	0.28±0.03	−39.9±4.5	99.8±0.29
T20	0.18±0.02	0.33±0.07	−24.1±2.6	0.0±0.0
T40	0.22±0.03	1.00±0.00	−36.4±2.1	0.0±0.0
T60	0.36±0.06	1.00±0.00	−39.8±2.4	0.0±0.0
T80	0.29±0.01	0.84±0.06	−19.1±1.6	0.0±0.0

Each value represents the mean±S.D. (n=3).

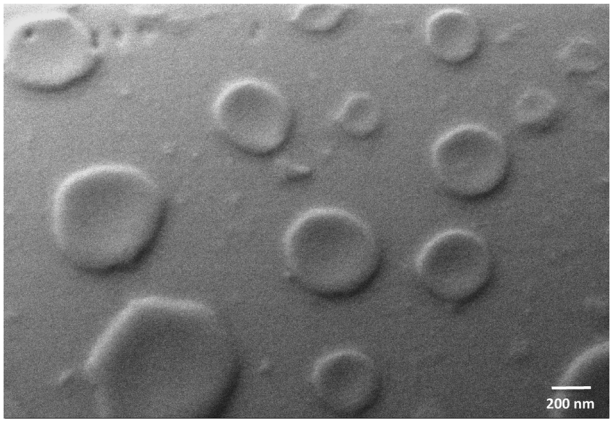

Fig. 2. Observation of Proniosome S85L under Field Emission Scanning Electron Microscopy (FESEM)

Microscopic image was generated under magnification of 21 K X. The scale bar indicates 200 nm.

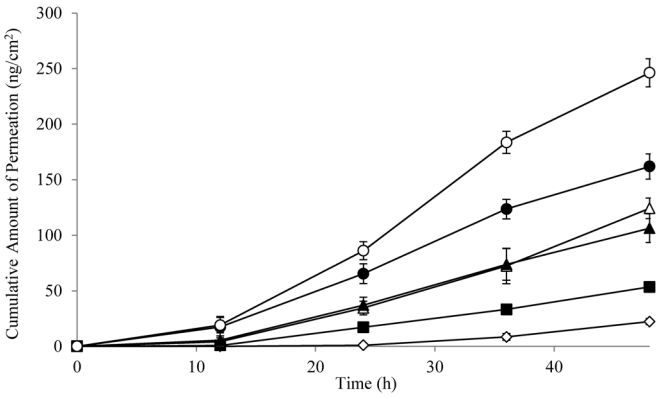

Fig. 3. Permeation Profiles of α-Mangostin from Control Suspension and Proniosomal Preparations across the Dermis-Split YMP Skin (48 h)

Symbols; ◇: control, ■: S20L, △: S40L, ●: S60L, ▲: S80L, ○: S85L. Each value represents the mean±S.D. (n=4).

sion recorded a flux of 0.81±0.23 ng/cm^2 over 48 h (Table 3). All the proniosomes exhibited higher (p<0.05) skin permeation as compared to the control containing an equivalent amount of α-mangostin. Among the proniosomal formulations, the skin permeation profile increased in an order as shown in

Table 3. Skin Permeation Profile of α-Mangostin Suspension and α-Mangostin Proniosomes

Formulation	Flux (ng/cm^2/h)	Enhancement ratio (ER)
Control	0.81±0.23	1.0
S20L	1.46±0.12*	1.8
S40L	3.32±0.25*	4.1
S60L	4.04±0.32*	5.0
S80L	2.81±0.33*	3.5
S85L	6.49±0.17*	8.0

ER=Flux of α-mangostin from proniosome/Flux of α-mangostin from control. Each value represents the mean±S.D. (n=4). (*) indicated significant different (p<0.05) to control.

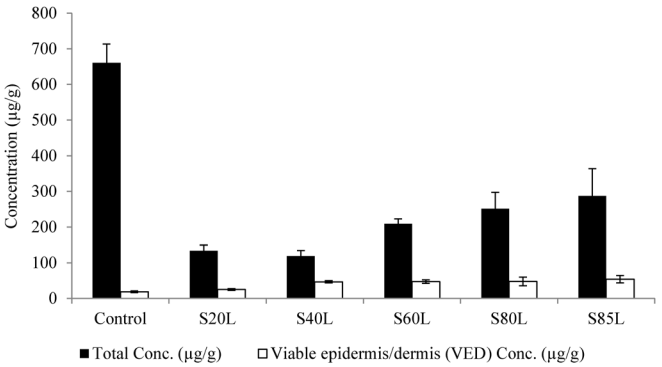

Fig. 4. Skin Concentration of α-Mangostin after 48 h *in Vitro* Permeation Experiment

Each value represents the mean±S.D. (n=4).

Fig. 3: S85L>S60L>S40L>S80L>S20L. S85L proniosome indicated the highest flux (8.0-fold) while S20L showed the lowest flux (1.8-fold), significantly different (p<0.05) to other formulations.

Skin Concentration of α-Mangostin Skin concentration measurement was performed to determine the deposition of α-mangostin in the SC and the VED. Figure 4 shows the concentration of α-mangostin retained in the dermis-split YMP skin 48 h after *in vitro* permeation experiment. "Total skin concentration" is referred to as the concentration of α-mangostin retained in both the SC and the VED, while

"VED concentration" is referred to as the concentration of α-mangostin recovered in the VED layer only. The total skin concentration of α-mangostin after application of control suspension was significantly higher ($p<0.05$) than that after application of proniosomes. Meanwhile, in term of the retention of α-mangostin in the VED layer, control showed significantly lower ($p<0.05$) VED concentration as compared to all the proniosomal formulations, except for S20L proniosome ($p>0.05$). The concentration of α-mangostin in the VED layer increased in an order of S85L>S80L>S60L>S40L>S20L. Non-significance difference ($p>0.05$) was, however, observed for the VED concentration between S40L, S60L, S80L, and S85L proniosomes.

Discussion

Proniosome Preparation The efficacy and toxicity of topical drugs and active cosmetics ingredients are determined by their concentrations at the skin target site. Thus, it is very important to develop topical formulations by the evaluation of skin permeation and skin concentration of drugs and active ingredients. In this study, proniosome was developed to deliver α-mangostin into the VED by enhancement of its skin permeation.

Proniosomes were successfully prepared with Spans with or without soya lecithin. Most of the α-mangostin proniosomes appeared as yellowish/brownish gel or liquid, attributed to the colour of non-ionic surfactants, the yellowish α-mangostin and/or the brownish soya lecithin. It was also observed that Span 40 and 60 could form gel with or without the presence of soya lecithin while the other formulations existed as proniosomal alcoholic solutions (liquid phase). This result was consistent with the report by Ibrahim *et al.*[11] Both Span 40 and 60 have high transition temperatures (T_c=42, 53°C, respectively) and are solids at room temperature. Thereby, they act as gelators by themselves, producing thermo-reversible proniosome gel systems. On the other hand, S20, S80, and S85 proniosomes were found in liquid phases, attributed to the low transition temperatures of surfactants, *i.e.* Span 20 (T_c=16°C), Span 80 (T_c=−12°C), and Span 85 (T_c=−23°C). They are liquids at room temperature and could not form gels at less than 20 mM % of cholesterol.[11] Two phases and three phases proniosomal liquids were observed in S80L and S85L proniosomes, respectively. Similar observation was reported by Ibrahim *et al.*[11] in which Span 80 which is more hydrophobic (HLB=4.3) than Span 20 (HLB=8.6) produced two phases proniosomal liquid at cholesterol concentrations below 30%. Similarly, this might explain the reason for Span 85 (HLB=1.8) which is more hydrophobic than Span 80 (HLB=4.3) to produce three phases proniosomal liquid in this study.

Characterization of α-Mangostin Loaded Niosome For vesicles composed of Spans without soya lecithin, the vesicle size increased in the order as S85<S80<S20<S40<S60. Small vesicle by surfactant Span 85 (T_c=−23°C) and Span 80 (T_c=−12°C) might be attributed to the low transition temperature of surfactant, which tends to form more disordered bilayer and small size vesicle.[5] The size of vesicles is dependent on the length of the alkyl chain of the surfactants.[4] This might be the reason Span 60 and 40 which has longer alkyl chain than Span 20 produced larger vesicle size. Similar observation was reported by Balakrishnan *et al.*[23]

In addition, it was observed that the presence of soya lecithin decreased the size of S40L and S60L proniosomes ($p<0.05$), as well as size of S80L and S85L proniosomes ($p>0.05$). The negative charge of soya lecithin might increase the curvature of the bilayer through the effect on electrostatic repulsion between the ionized head group, and therefore create smaller vesicles. However, the incorporation of soya lecithin increased the size of S20L proniosome ($p<0.05$). Span 20 (HLB=8.6) has a relatively high HLB compared to other Spans (HLB=1.8 to 6.7). The addition of soya lecithin may increase the overall hydrophilicity, subsequently increase the water intake into the bilayers and lead to enlargement of the vesicle.

All the α-mangostin proniosomes formulated from Spans with or without soya lecithin exhibited high EE (*ca.* 100%). The high EE observed might be attributed to the lipophilic nature of the drug (α-mangostin) and the low HLB of Spans surfactants.[17] Similar observations have been previously reported in levonorgestrel proniosome of Span 40 (HLB=6.7),[17] ketorolac proniosomes of Span 60 (HLB=4.7)[20] and piroxicam proniosome of Spans.[24]

Incorporation of soya lecithin in the proniosomal formlations results in more negative ζ-potential value. This might be attributed to the existence of negatively charged phospholipids such as phosphatidic acid in the soya lecithin.[25] This finding is in accordance with report by Wen *et al.*[19] The high negative charge on the surface of α-mangostin vesicles indicated a high repulsive force between the vesicles which provide stability and devoid of agglomeration.

***In Vitro* Permeation and Skin Retention Study** In our preliminary experiment, proniosome formulation that composed of Span 60 and soya lecithin (S60L) displayed two times higher α-mangostin concentration in VED than that without soya lecithin (S60) (data not shown). Thus, the skin permeation and retention of α-mangostin were evaluated with proniosomes that composed of Spans and soya lecithin. Although skin permeation and VED concentration of α-mangostin were significantly improved by proniosomes composed of Spans and soya lecithin, the total skin concentration after application of control suspension was higher than those of proniosomes. To clarify this discrepancy, the skin surface was observed with an optical microscope after finishing skin permeation experiment to reveal the reason for high concentration of α-mangostin after application of control suspension. Figure 5 shows the microscopic image of harvested dermis-split YMP skin after washed using water. The yellow spots observed (Fig. 5a) were α-mangostin residues that cannot be completely removed by water solely, since α-mangostin is completely insoluble in water. On the other hand, no α-mangostin residue was observed on the skin surface after removal of applied S85L (Fig. 5b). This might be a reason for high concentration of α-mangostin in the total skin after application of control suspension.

The enhancement of α-mangostin concentration in the VED layer might be attributed to the enhancement of permeation by proniosomes (S40L, S60L, S80L, S85L). Although ER values obtained from skin permeation with S20L, S40L, S60L, S80L and S85L proniosomes were 1.8, 4.1, 5.0, 3.5 and 8.0-fold (Table 3), respectively, the value were not in correspondent with the ER value that obtained from VED concentration (1.3-fold for S20L, 2.5-fold for S40L, 2.5-fold for S60L, 2.5-fold for S80L, 2.9-fold for S85L) (Table 4). According to Fick's

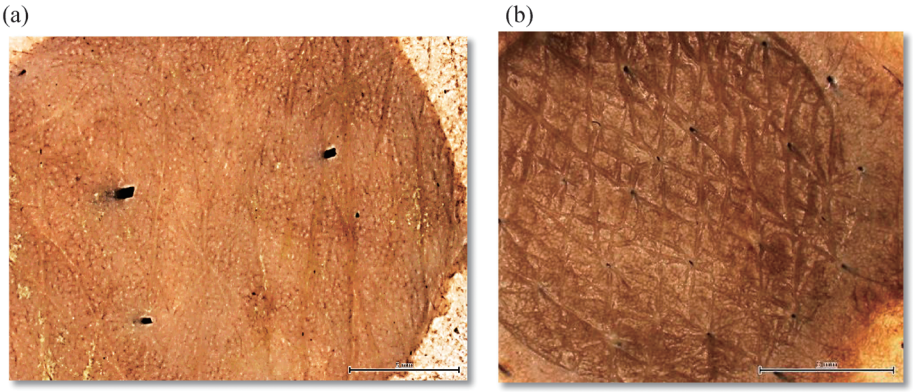

Fig. 5. Microscopic Image of Dermis-Split YMP Skin Treated with (a) α-Mangostin Suspension and (b) α-Mangostin Proniosome (S85L)

The skin samples were harvested after 48 h *in vitro* permeation experiment and washed using water on both stratum corneum (SC) and viable epidermis/dermis (VED) sides. Yellowish α-mangostin residues were observed in the region surrounded by the circle in (a). The scale bar indicated 5 mm.

Table 4. Skin Concentration of α-Mangostin after 48 h *in Vitro* Permeation Experiment

Formulation	Total conc. (μg/g)	VED conc. (μg/g)	Enhancement ratio (ER)
Control	660.44±53.10	18.85±2.41	1.0
S20L	133.56±16.19*	25.11±2.52	1.3
S40L	118.84±15.25*	46.67±3.16*	2.5
S60L	208.99±14.25*	47.35±5.10*	2.5
S80L	251.42±45.74*	47.63±11.96*	2.5
S85L	287.45±76.39*	54.16±10.40*	2.9

ER=VED concentration of α-mangostin from proniosome/VED concentration of α-mangostin from control. Each value represents the mean±S.D. (n=4). (*) indicated significantly different ($p<0.05$) to control.

first law of diffusion, skin permeation enhancement effect could be expressed by either or both the increase of partition coefficient (K) and diffusion coefficient in the SC (D).[26] On the other hand, steady-state of skin concentration (C_{ss}) of topically applied chemicals could be expressed by the function of K value, but not by D.[27] Thus, there is no linear relationship between the ER values of skin permeation with the ER values of skin concentration as the increase of D value was only taken into account in the permeation enhancement mechanism, not in the skin retention.

It is suggested that diameter of proniosome might be one of the factor that could modulate the vesicle–skin interaction, thereby different permeation profile was observed among the α-mangostin proniosomal formulations. In this study, S85L proniosome exhibited the highest flux of α-mangostin among others ($p<0.05$) perhaps due to more disordered bilayer and small size of vesicle. Yoshioka *et al.*[5] reported that Span 85 showed faster release rate of the drug than Span 40 and 60 niosome as Span 85 exhibited more disordered bilayer than the latter at room temperature due to its low transition temperature. In addition, Fang *et al.*[13] suggested that proniosomes should be hydrated *in situ* by the dissolution medium to form niosomal vesicles prior to the release and permeation of drug across the skin. Small vesicle size of S85L could provide maximum surface area exposed to the dissolution medium. Same might apply to S80L proniosome which also had small vesicle size (Table 2). However, it was observed that proniosomal size alone might not determine the drug permeation process, as S40L proniosome which was significantly larger ($p<0.05$) than S80L proniosome also portrayed enhanced permeation profile with no significance different from S80L ($p>0.05$). The enhancement effect of S40L might be due to its lipophilic nature of vesicle which could interact and fuse with lipophilic SC.[24]

In this study, soya lecithin was selected over egg lecithin because the former was reported to contain unsaturated fatty acids, oleic and linoleic acid, which have better skin penetration enhancing properties as compared to egg lecithin which contains saturated fatty acids.[28,29] Therefore, the vesicles may act as a penetration enhancer and reduce the barrier properties of SC through structure modification. The vehicles may interact and bind with keratin filaments resulting in a disruption within the corneocyte.[30] Fang *et al.*[13] highlighted that sufficient amount of non-ionic surfactant and lecithin was required to interact with lipid bilayers of SC. Non-ionic surfactants and/or lecithin may increase the fluidity, solubilize, and extract lipid component in the SC,[24,30] resulting a looser and more permeable skin barrier. Besides, the enhancement of α-mangostin permeation might also be related to its distribution to octanol. The distribution to octanol of α-mangostin decreased in the presence of Spans and soya lecithin as compared to with only presence of Spans. A certain amount of α-mangostin solubility in water would be important for the increase of its distribution into SC from the formulation and into viable epidermis from its distributed SC. Manconi *et al.*[31] suggested that vesicles could form a lipid film on the skin, subsequently improve the SC intra- and intercellular hydration. This would lead to the open of SC compact structure and improve the barrier permeability, therefore, enhance the retention of lipophilic drug (*e.g.* tretinoin, $\log P$=4.65) in the SC.[31] Thus, improvement of aqueous solubility of α-mangostin caused by non-ionic surfactants and/or soya lecithin might be a reason for the changes of barrier function of SC and subsequent increase of its concentration at the VED.

Further experiment should be carried out to evaluate the pharmacological effects after topical application of α-mangostin proniosomes to show the usefulness of formulations. As aforementioned, 5 μg/mL of α-mangostin is optimum and necessary at the VED where melanocytes are located to expect its anti-melanogenic effect.[1)] Thus, topical delivery of α-mangostin could be greatly achieved with proniosome by enhancement of skin permeation and a higher localized α-mangostin concentration at the VED. Improvement of α-mangostin solubility at the VED could be achieved by the addition of Spans and soya lecithin into the proniosomal formulation.

Conclusion

The results of this research have demonstrated that α-mangostin can be formulated in proniosome composed of non-ionic surfactants (Spans), soya lecithin and cholesterol using coacervation separation method. With or without soya lecithin, Spans produced α-mangostin proniosome with good entrapment efficiency and physical characteristics. The preliminary study of skin retention suggested that the α-mangostin proniosomes composed of Spans exhibited better localization of α-mangostin at the hydrophilic VED layers than Tween did (data was not shown). The incorporation of soya lecithin in formulations also significantly improved the deposition of α-mangostin at the VED. Different permeation profile was observed among proniosomal formulations prepared from different Spans suggested that the delivery of α-mangostin across the skin might be modulated by the solubility of α-mangostin in different vesicles, as well as by the partitioning of vesicle through the SC. Besides, the nature of surfactants and the characteristics of vesicles might also affect the skin permeation profile. Among the tested proniosomal formulations, S85L showed the highest permeation profile (8.0-fold) and the highest enhancement of VED concentration (2.9-fold). The data justifies our conclusion that skin permeation and retention of highly lipophilic drug such as α-mangostin could be improved by incorporated in the proniosome system. Further investigations should be fueled to understand the possible skin enhancement mechanism by the α-mangostin proniosome. *In vivo* study using animal model might also provide stronger evidence supporting the application of proniosome as a topical delivery vesicle for highly lipophilic compound.

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Conflict of Interest The authors declare no conflict of interest.

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