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Research Article

Topical Application of Yolk Lecithin Liposomes Reinforces Skin Barrier Function Against Chemical Agents Such as Psoriasis-inducing IMQ and Alleviates Disease Phenotype

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Abstract

Background: Compromised skin barrier is a major driver of skin diseases such as psoriasis. We hypothesized that Yolk Lecithin Liposomes (YLLs) being water soluble bi-lipid membrane vesicles rich in skin barrier lipid and their precursors would be efficient in enhancing skin barrier. We tested this hypothesis by applying YLLs topically to Imiquimod (IMQ)-induced mouse model of psoriasis for evidence of skin barrier protection against IMQ and alleviation of the disease phenotype.

Materials and Methods: 100 nm YLLs were first tested for effects on human MSC proliferation, collagen production in human dermal fibroblasts and penetration into human skin. They were then tested for efficacy in enhancing skin barrier against IMQ infiltration in a psoriasis mouse model. After 6 daily topical applications of IMQ and then a cream with YLLs or cream alone, barrier efficacy was assessed by the severity of psoriatic symptoms and cytokine induction in the skin lesion.

Results: YLLs are non-toxic, and can enhance cell proliferation and collagen production. They readily permeated the stratum corneum. They alleviated psoriatic skin pathology and reduced IL-23 and TNF-α in the skin lesion of an IMQ-induced psoriasis mouse model.

Conclusion: YLLs are effective in enhancing the skin barrier as evidenced by the alleviation of IMQ-induced psoriasis.
Introduction

The skin barrier is critical to the terrestrial survival of mammals as a first line of defense against excessive water loss and microbial entry [1]. Compromises in skin barrier function leading to excessive loss of water and infiltration of microbes or environmental irritant have been implicated in many skin disorders such as Atopic Dermatitis (AD) and psoriasis. Consistent with this, enhancing skin barrier function through the use of moisturizers and emollients is a mainstay of disease management for these diseases.

The structural organization and composition of the skin have been extensively documented and reviewed [2]. Briefly, the skin consists of three structural layers, namely epidermis, dermis, and hypodermis. The outermost epidermis constitutes the skin barrier and has four different layers, the Stratum Basale (SB), Stratum Spinosum (SS), Stratum Granulosum (SG), and Stratum Corneum (SC). The cells in the SC are dead cornified keratinocytes formed by a progressive differentiation program via homeostatic growth of keratinocytes in the SB to spinous and then granular keratinocytes in the SS and SG, respectively [2]. As they reach the outermost SC, the granular keratinocytes undergo its final maturation to become dead, keratin-filled corneocytes. The SC which represents the waterproof physical barrier to permeability and microbial entry is composed of ~15 layers of corneocytes embedded in a specialized matrix of lipid membranous bilayer [2]. The corneocytes which are interconnected via ‘corneodesmosomes’ are enveloped by densely cross-linked cornified proteins and a covalently attached lipid monolayer (the cornified lipid envelope), making the corneocytes impermeable to diffusion [2]. Therefore, the route for water loss and microbial invasion via the skin is through the intercellular lipid matrix. This matrix consists of highly ordered multi-lamellar lipid sheets. Despite constituting only 5-15% of the dry weight of the SC against a 75-80% protein dry weight [2], the SC lipid sheets are critical in determining the barrier function of SC. Perturbations in these lipid sheets such as lipid depletion are known to disrupt barrier function and contribute to skin diseases such as psoriasis and atopic dermatitis [2].

The lipid bilayers in the SCs have also been extensively studied and reviewed [2,3]. The main lipids in the SC are ceramides (50%), cholesterol (25%), and free fatty acids (15%), and are derived from the enzymatic hydrolysis of membrane lipids in lamellar bodies produced by keratinocytes in the SS and SG [4]. The relative abundance of ceramides, cholesterol and free fatty acids are critical to skin barrier function [5] and topical application of such lipids e.g. ceramides generally enhances barrier function [2]. However, SC lipid precursors such as sphingomyelins were reportedly more efficient in increasing SC lipid content and the density of lamellar-related structures if they are delivered close to the SG-SC interface such as through the use of liposomes [6,7,8].

Based on the above studies and observation, we therefore hypothesized that topical application of liposomal SC lipid and their precursors will deliver SC lipid precursors close to the SG-SC interface and enhance barrier function to alleviate skin diseases such as atopic dermatitis or psoriasis. The pathophysiology of these diseases is complex and are thought to involve an “inside-out” where systemic immune dysregulation leads to defective epidermal defects and disease manifestation or an “outside-in” mechanism where a defective epidermal barrier precedes immune sensitization [9].

Here, we used a commercially available yolk lecithin liposomes to evaluate their efficacy in protecting skin against water loss in human subjects and penetration of external toxic agents such as psoriasis-inducing agent, Imiquimod (IMQ) in a mouse model. Liposomes first described in 1964 [10] are widely used in pharmaceutical and cosmetic applications. Their synthesis using both synthetic and natural phospholipids is now a fairly generic process [4]. Yolk lecithin liposomes which are made using phospholipid-rich lecithin, is widely used as excipients in oral and parenteral drugs [11] and is generally considered safe even for those with egg allergy [12]. In the cosmetic industry, egg yolk lecithin is recognised as a safe skin conditioning agent [7] and egg yolk lecithin liposomes have also been shown to be superior to soy lecithin liposomes as they are physiologically more compatible with human skin [8].

Materials and Methods

Determination of Particle Size and Concentration in Egg Lecithin-derived Liposomes

Yolk lecithin liposomes (YLLs) were provided by Vesiderm Pte Ltd, Singapore, and were characterized for particle size distribution and concentration by Zetaview® (#PMX120, Particle Metrix GmbH, Meerbusch, Germany) according to the manufacturer’s protocol.

Liposome sample was pre-diluted for 1:10 and 1:100 (in dH2O). The sample was deposited on freshly glow-discharged EM grids (Cu200-Formvar-carbon), stained with 4% uranyl acetate (UAc) for 2min twice, and embedded in a 1:9 (v/v) ratio of 4% UAc to 2%Methylcellulose (MC). Grids were analysed with a JEM-1010 JEOL transmission electron microscope operating at 80 kV. Images were acquired with SIA model 12C high resolution full-frame CCD camera at 10k and 40k magnification. This study was performed by IMB-IMCB Joint Electron Microscopy Suite, A*STAR Singapore.

Biochemical Composition

Phospholipids were quantified by measuring total choline-containing phospholipids using the Phospholipid Assay Kit according to the manufacturer’s protocol (#KA1635, Abnova,
Since phosphatidylcholine (PC) is the major constituent of lecithin, the quantity of YLLs will be represented as “mM PC” equivalents. Total cholesterol, triglyceride, and sphingomyelin levels were assayed using the Total Cholesterol and Cholesteryl Ester Colorimetric/Fluorometric Assay Kit, Triglyceride Quantification Colorimetric/Fluorometric Kit and the Sphingomyelin Quantification Colorimetric Assay Kit, respectively according to the manufacturer’s instructions (#K603, #K622, #K600, BioVision, Milpitas, CA, USA). A more detailed lipid analysis was performed by Avanti Polar Lipids, Inc. Briefly, the liposomes were lyophilized. ∼30 mg was dissolved in 1 mL detergent and measured by quantitative 31P-NMR on a Bruker AVANCE III 400MHz NMR spectrometer with CryoProbe™ Prodigy (according to protocol reference #790233, Avanti Polar Lipids, Inc.). Neutral lipid of triglycerides (TG), diglycerides (DG), monoglycerides (MG) and fatty acids (FA) were measured by normal phase HPLC/ELSD using a 5 mg/mL sample (according to protocol reference #790208, Avanti Polar Lipids, Inc.)

**Mesenchymal Stem Cell Proliferation Assay and Assessment of Liposome Uptake**

Immortalized E1-MYC 16.3 human ESC-derived mesenchymal stem cells [13] were seeded in DMEM with 10% fetal calf serum at a density of 2 x 10⁴ cells per well in a 24-well plate and allowed to attach overnight. They were then treated with a chemically-defined medium [14] in the presence and absence of increasing concentrations of YLLs for 5 days, with medium change every 2 days. Cell proliferation was assayed using the CellTiter 96® AQ solution Cell Proliferation Assay (MTS) according to the manufacturer’s protocol (#G3582, Promega, Fitchburg, WI, USA).

To assess liposome uptake, YLLs were labeled with Alexa Fluor 488 amine-reactive probe (#A30005, ThermoFisher Scientific) according to the manufacturer’s protocol. Labeled YLLs were separated from the unreacted free probe using the BioGel P30 gel filtration medium (#7326231, Bio-Rad Laboratories, Hercules, CA, USA) and filtered through 0.22 µm. They were added to E1-MYC cells in serum-free DMEM and the cells were washed, harvested and fixed in 1% paraformaldehyde at the indicated timepoints. Fluorescence intensity was quantified on the BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

**Collagen Induction in Human Dermal Fibroblast**

Adult human dermal fibroblasts (HDFa) were purchased from Gibco (#C0135C, ThermoFisher Scientific, Waltham, MA, USA) and cultured according to the supplier’s instructions. Cells were seeded at 2 x 10⁴ cells per well in a 24-well plate. After 24 hrs, the medium was replaced, and cells were incubated in chemically-defined medium with and without YLLs (0.25 mM PC) or Matrixyl (peptide amphiphile C₁₆KTTKS) for 48 h. For collagen quantification, cells were washed, fixed in 70% ethanol and stained with picro-sirius red solution (1 mg/ml Direct Red 80 in 1.3% picric acid in water, Sigma, St Louis, MO, USA). Excess dye was washed off using 5% acetic acid, and the collagen-dye complex in cells was solubilized in 0.1M sodium hydroxide and quantified by measuring absorbance at 550 nm using a spectrophotometer. Cell numbers were estimated using the CellTiter 96® AQ solution Cell Proliferation Assay ((#G3582, Promega, Fitchburg, WI, USA).

**Human Skin Permeation**

The Human Skin (Hospital Consent Donor, sourced by DeNOVA Sciences Pte. Ltd.) when received from hospital was first trimmed off all the fats leaving only the intact skin of dermis and epidermis. The skin was soaked in 70% ethanol for 2 seconds to remove contamination and then rinsed in 1x PBS for 3 min. The skin is then placed in decontamination medium (in-house recipe) for 2 hr in 37°C CO₂ incubator. Thereafter, the skin was cut (into strip of 0.5 cm width and 2.5 cm length) and prepared in 6 well plate with 1 ml of Skin explant medium (in-house recipe). These organ-cultured human skin were then topically treated with 50 µl of PBS or labeled YLLs (prepared as described above) for 2 hrs in a 37°C incubator. Thereafter, they were gently washed with PBS and placed in OCT quick-freeze medium compound and snap-frozen in liquid nitrogen. 3 µm sections were mounted on glass slides and subjected to hematoxylin and eosin staining (H&E) analysis and green fluorescence compound screening. Images were taken using EVOS microscope for H&E and Carl Zeiss microscope for fluorescence (DAPI and FL 488 detection). This assay was performed by DeNOVA Sciences Pte Ltd.

**IMQ Mouse Model of Psoriasis**

This animal study was obtained ethical approval for animal use and performed by WASHINGTON BIOTECHNOLOGY, INC.6200 Seaford Street Baltimore, MD 21224, IACUC no: 17-003. On day 0, 70 mice (7-8 weeks, balb/c, male, 10 mice per group) were weighed and back skin (1.5 cm x 2 cm) was shaved. Thickness of back skin and right ear were measured. IMQ (Aldara 5% cream) was applied on shaved back and right ear (50 mg for back skin and 12.5 mg for right ear). Immediately after IMQ application, liposome cream or the base cream (supplementary table 1) was applied to back skin (dosage: 80 ul) and to right ear (dosage: 20 ul) every day (day 0-5: total 6 doses). The positive control is dexamethasone (3 mg/Kg, IP). Erythema, scaling, and edema were scored independently daily on a scale from 0 to 4 [15]. Ear and back skin thickness were measured by electronic calipers as an indicator of edema. The shaved back skin was collected from each animal at termination (day 6), weighed and homogenized in tris buffer with protease inhibitor in cold condition. It was then centrifuged (14,000 rpm, 5 min, 4°C), and the supernatant was used for cytokines (TNF-a, IL-17 and IL-
23) measurement using commercially available ELISA kit (#MTA00B, #1700 and M2300 respectively, R&D Systems, Minneapolis, MN, USA) by WASHINGTON BIOTECHNOLOGY, INC.6200 Seafort Street Baltimore, MD 21224. Scoring (erythema, scaling, thickness, and cumulative score), thickness measurements (back fold and ear), spleen weights and cytokine results were analyzed using Student’s t-test (Microsoft Excel 2013, two-tailed). P values <0.05 were considered as statistically significant.

### Supplementary Table 1: Composition of the liposome and base cream.

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<th>Ingredients</th>
<th>Liposome (%)</th>
<th>Base (%)</th>
</tr>
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<tr>
<td>Liposome solution in 10% (v/v) Ethanol (2.6 x 10E13 particles/mL)</td>
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<td>0</td>
</tr>
<tr>
<td>10% (v/v) Ethanol</td>
<td>0</td>
<td>50</td>
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<td>Distilled Water</td>
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<tr>
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<td>1.00</td>
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<tr>
<td>Glycerin (Making Cosmetics, HUM-GLYC-01)</td>
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<tr>
<td>Vitamin E (dl-alpha tocopherol) (Making Cosmetics, VIT-VITE-01, Activity 750 IU per 1 ml)</td>
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### Results

#### Characterization of Egg Lecithin-derived Liposomes

The hydrodynamic particle size distribution of the YLLs was analyzed over a size range of 5 to 1000 nm on a nanoparticle tracking analyzer. The median size of the YLLs is 105.5 nm (Figure 1A), and this was verified by transmission electron microscopy (Figure 1B).

![Size Distribution Histogram](image1.png)

![80k TEM Magnification: 40lx, scale bar: 50nm](image2.png)

**Figures 1 (A-B):** YLLs are nano-sized lipid vesicles. (A) Size distribution of YLLs as measured by Nanoparticle Tracking Analysis on a Zetaview over a size range of 5 to 1000 nm at 25°C. (B) YLLs as observed under TEM.
Using colorimetric or fluorescent enzyme-based lipid analysis, YLLs were determined to contain 7.62 mM phospholipids, 134.82 µM sphingomyelin, 1.12 mM triglycerides and 5.48 mM cholesterol. A more extensive lipid analysis using chromatographic and mass spectrometry assay revealed that the main lipid classes were phosphatidylcholine (56%), free fatty acids (11.9%), phosphatidylethanolamine (9.6%), cholesterol (9.3%), triglycerides (5.9%), sphingomyelin (2.5%), lysophosphatidylcholine (2.3%). The major FFAs are oleic acid (cis18:1), palmitic acid (16:0), linoleic acid (18:2) and stearic acid (18:0).

Egg Lecithin Liposome is Non-toxic and a Cell Nutrient

To test if YLLs have biological effects on human cells, they were tested on human mesenchymal stem cells. We have previously reported that when the serum-containing culture medium of human mesenchymal stem cells was replaced with a serum-free Chemically-Defined Medium (CDM), the cells lose their proliferative activity but remain viable for about a week [14]. When YLLs were added to the culture medium, we observed increased cellular proliferation with increasing YLL concentration. Specifically, 0.375 mM PC YLLs induced a 60% increase in proliferation demonstrating that YLLs are non-toxic to human cells and provide nutrients that could partially substitute for serum in cell culture (Figure 2A). When the cells were incubated with fluorescence-labelled YLLs and analysed by flow cytometry, we observed cellular internalization of YLLs within 30 minutes and this internalization increased with time (Figure 2B).

![Figures 2(A-B): Cellular growth promotion and uptake of YLLs](image)
Egg Lecithin-derived Liposomes Increase Collagen Content in Dermal Fibroblasts

When the medium of human dermal fibroblast cultures was supplemented with YLLs, collagen content of the cells increased by 50% as determined by picro-sirius red staining. This increase was similar to that elicited by Matrixyl 3000, a cosmetic ingredient consisting of 2 palmitoylated matrikines by Sederma (Figures 3A and B). Matrikines are small biologically active peptides produced from limited enzymatic degradation of the extracellular matrix and have been reported to regulate different aspects of the extracellular matrix including collagen production [16,17]. However, the increased collagen production in Matrixyl 3000-treated fibroblasts was accompanied by a 16.3% reduction in cell viability as measured by MTS assay. In contrast, the viability of YLL-treated fibroblasts was not compromised (Figure 3C).

Figures 3(A-C): YLLs increased collagen production in dermal fibroblasts
(A) Phase contrast images of primary human dermal fibroblasts cultured in the presence of vehicle, 0.25 mM PC YLLs, 0.1% or 1% Matrixyl, fixed and stained with picro-sirius red solution to visualize collagen. Scale bar = 100 µm. (B) Collagen content per cell in each culture was normalized to that in untreated control. Collagen content per cell was determined by normalizing the amount of stain in each culture against cell numbers by MTS assay. Data are presented as mean ± SD (C) Cell numbers relative to untreated control, as estimated by MTS assay. Data are presented as mean ± SD.
Dermal Penetrance Potency of YLLs

Liposomes were previously thought to be efficient transdermal delivery vehicles. However, careful investigations over the years have demonstrated that skin penetration by nanoparticles such as liposomes is highly inefficient [18]. In fact, liposomes have been observed to be disintegrated in the SC [19], making the use of liposomes as impractical transdermal vehicles for systemic drug delivery. However, these impractical features make liposomes attractive vehicles for replenishing SC lipids. To assess the effectiveness of YLLs in localizing to the SC, fluorescence-labeled YLLs were topically applied to intact human skin culture. Within 2 hrs, YLLs effectively permeate the entire breadth and depth of the SC but not the underlying nucleated SG (Figure 4). This illustrates the potential efficiency of YLLs in replenishing SC lipids through topical applications.

Figures 4(A-B): Topical application of YLLs on skin organ culture. Human skin organ culture was topically treated with PBS (Control) or Alexa Fluor 488-labeled YLLs, washed, frozen in OCT medium and sectioned. (A) H&E staining with arrowheads indicating position of stratum corneum. Scale bar = 100 µm. (B) Fluorescence imaging of sections counterstained with DAPI (blue). White broken lines denote epidermal-dermal junction. Scale bar = 50 µm.

Protection Against a Topically Applied Psoriasis-inducing Agent, Imiquimod (IMQ)

Based on the efficient permeation of the SC by topically applied YLLs, we hypothesized that topically applied YLLs could potentially enhance barrier function and mitigate the disease-inducing insult of environmental agents such as psoriasis-inducing IMQ. IMQ was topically applied to shaved backs and right ears of mice every day for six days. After every IMQ application, one group of mice were given topical applications of the liposome cream, another group was given the base cream i.e. cream without liposome, and the third group which is the positive control, was given 3 mg/Kg dexamethasone ip.

Generally, all three groups exhibited a small decrease in body weight. The dexamethasone group consistently lost more weight than the other two groups, suggesting that both liposome and base cream treatments are not toxic or at least less toxic than topical
application of dexamethasone (Figure 5A). The spleen, which is
the largest secondary lymphoid organ, is often used as a surrogate
to evaluate the histopathology of the immune system [20]. The
spleen/body weight ratio of liposome- and base cream-treated
animals was significantly higher than that in the dexamethasone-
treated animals (Figure 5B). This observation suggested that
unlike dexamethasone, liposome and the base cream have little
or no direct impact on the immune system. The cumulative score
for erythema, scaling, and thickness was similar for both liposome
and dexamethasone treatment groups but was lower than that in
the base cream, reaching statistical significance on day 3 (p=0.011
and p= 0.000, respectively by Student’s t-test) (Figure 5C). The
IMQ-treated backs of all the animals were assayed for IL-17,
TNF-α, and IL-23 by ELISA(Figure 5D) which represent the
key immune mediators and also therapeutic targets in psoriasis
[21]. Both dexamethasone and liposome treatment groups have
exhibited reduced levels of IL-17, TNF-α and IL-23 relative to the
base cream group. However, only the reduction of IL-17, TNF-α,
and IL-23 in the dexamethasone group, and TNF-α in the liposome
group, were statistically significant. As the level of TNF-α and IL-
23 were statistically highly similar in both dexamethasone and
liposome-treated animals, the reduced IL-23 level in the liposome
treatment group is probably real.

Figures 5 (A-D): Topical application of YLLs on an IMQ-induced mouse model of psoriasis. IMQ was applied to the shaved backs and right ears of mice from day 0 to day 5. This was followed by the liposome cream, base cream (vehicle control), or 3 mg/Kg dexamethasone, i.p. (positive control). (A) Weight of mice over six days of study; (B) The spleen from each mouse was removed, weighed and the ratio of spleen to body weight for each mouse was calculated; (C) Erythema, scaling, and thickness in each mouse were determined daily on a scale from 0 to 4, and combined to generate the cumulative score; (D) The back skin of each mouse was harvested on day 6, homogenized and the homogenized solution was assayed for TNF-α, IL-17 and IL-23 by ELISA. Data are presented as mean ± SE, * P< 0.05.
Discussion

In summary, this study demonstrated that 100 nm water-soluble YLLs can be taken up by cells in a time-dependent manner and are non-toxic to human cells. YLLs enhance proliferation of human mesenchymal stem cells and dermal fibroblasts. They also enhanced collagen synthesis in human dermal fibroblasts. YLLs have a lipid composition highly to plasma membrane with the major species being phosphatidylcholine, free fatty acids, phosphatidylethanolamine, cholesterol, triglycerides, sphingomyelin and lysophosphatidylcholine. Notably, the major FFAs present in the YLLs, namely oleic acid (cis18:1), palmitic acid (16:0), linoleic acid (18:2) and stearic acid (18:0) are also the same four major FFAs found in mouse [22] and human skin [23]. Although YLL membrane lipids are different from SC lipids which are mainly ceramides (50%), cholesterol (25%), and free fatty acids (15%), they are similar to that of lamellar bodies from which the SC lipids are derived through enzymatic hydrolysis the SC. Specifically, SC ceramides, cholesterol and free fatty acids are derived from the hydrolysis of lamellar body lipids, sphingomyelins, cholesterol esters and phosphatidylcholine, respectively [4]. In addition, it has been reported that topical applications of 110nm liposomal sphingomyelins have been reported to increase SC ceramides possibly through enzymatic hydrolysis [23,24]. Together, these data demonstrated the potential of YLLs to contribute to the SC lipids in SC and enhance skin barrier function.

This potential was evidenced by the efficacy of YLLs in enhancing barrier function against environmental insults such as psoriasis-inducing IMQ cream (Aldara 5% cream) in a mouse model. This cream was optimised to compromise the skin barrier to enhance penetration of IMQ, a TLR7/8 ligand that stimulates the immune system and induces psoriasis-like pathology [25]. As expected, mice treated with dexamethasone, a potent immune suppressor manifested a less severe disease phenotype with little or no immune activation as evidenced by the normal spleen to body weight ratio. While the YLL-treated animals also manifested a similar alleviation of IMQ-induced psoriasis disease phenotype, their immune system was not suppressed to the same extent as revealed by a less attenuated reduction of TNF-α and IL-23levels, and increased spleen to body weight ratio. These manifestations were consistent with a fortified barrier where less IMQ penetrated the skin barrier to induce cytokines in the lesion rather than a suppression of the immune system as the spleen to body weight ratio in the YLL treated animals. Together, these observations implicate a role for both the skin barrier and the immune system in skin diseases such as psoriasis, and demonstrate that the pathophysiology of such diseases probably involve both an “inside-out” mechanism where systemic immune dysregulation leads to defective epidermal defects and disease manifestation and also an “outside-in” mechanism where a defective epidermal barrier precedes immune sensitization [9].

In conclusion, YLLs are effective in reinforcing skin barriers and would be efficacious in alleviating diseases or conditions such as psoriasis [26], atopic dermatitis [27], ichthyosis [28], sunburn [29], first-degree burn, melasma [30] where skin barriers are compromised.

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Disclosures

Conflict of Interest: SKL owns stock in Vesiderm Pte Ltd.

References
