

Destabilization of Whole Skin Lipid Bio-liposomes Induced by Skin Penetration Enhancers and FT-IR/ATR (Fourier Transform Infrared/Attenuated Total Reflection) Analysis of Stratum Corneum Lipids

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Whole skin lipid bio-liposomes (skin bio-liposomes), in size ranging from 2 to 8 μm , were prepared by a reverse phase evaporation technique using rat full thickness skin. Leakage of an encapsulated fluorescence probe, ANTS (δ -amino-1,3,6-naphthalenetrisulfonate), was measured by adding transdermal penetration enhancers (penetrants) into the medium where the skin bio-liposomes were present. Oleylamine induced a fast release of ANTS from the liposomes compared to laurylamine which showed a weak action. With these penetrants, the degree of ANTS release from the prepared bio-liposomes was found to correlate well with the results of frequency changes in the CH-asymmetric stretching band near 2920 cm^{-1} in the rat stratum corneum. The penetrant which caused relatively strong leakage of ANTS induced the significantly large shift of the peak toward the higher wavenumbers due to the perturbation in the structure of lipids of the stratum corneum. The skin bio-liposomes prepared from the rat full thickness skin could be useful in evaluating the penetrants.

Keywords whole skin lipid bio-liposome; stratum corneum; skin penetration enhancer; ANTS; perturbation; FT-IR/ATR measurement; oleylamine; laurylamine; propylene glycol

Introduction

The intercellular spaces of the stratum corneum are filled with multiple-lipid lamellae.¹ The stratum corneum is known to act as the barrier for drug penetration across the skin and into the circulatory system.² Transdermal penetration enhancers (penetrants) have been introduced to enhance the transdermal flux of drugs.³ These have greatly elucidated the mechanism of transdermal drug penetration associated with membrane disordering induced by penetrants. However, a systemic approach of the characterization of penetrants has not yet been accomplished. The liposomes from stratum corneum lipids were prepared as with the hope of understanding of the barrier function of the stratum corneum. The major lipids present in the stratum corneum are ceramides, cholesterol, fatty acids and cholestyl sulfate. By using these mixtures without phospholipids, several workers have investigated the formation of liposomes.^{4,5} The purpose of these studies was to confirm the bilayer-forming capability of stratum corneum lipids without any phospholipids. The usefulness of such stratum corneum-simulated synthetic liposomes as an evaluation system for penetrants has not yet been reported. The problems to be solved regarding stratum corneum liposomes and stratum corneum-simulated synthetic liposomes for the evaluation of penetrants are possibly due to insufficient stability of these formed liposomes.

In this study, we have tried to form fluorescence encapsulated stable whole skin lipid bio-liposomes (skin bio-liposomes) using rat full thickness skin. The leakage of the fluorescence compound was measured by adding penetrants into the medium where the skin bio-liposomes were present. The obtained results were compared with the data of frequency changes in C-H asymmetric vibration associated with lipid hydrocarbon chains in the rat stratum corneum, using fourier transform infrared/attenuated total reflection (FT-IR/ATR) spectroscopy. We also compared two different skin samples prepared by *in vitro* and *in vivo* methods for their usefulness in measuring penetrant-

induced steric disordering by FT-IR/ATR spectroscopy. Oleic acid, and two fatty amines, laurylamine and oleylamine (structural analogs of fatty acids) were used as penetrants.

Materials and Methods

Materials Reagent grade laurylamine, oleylamine and oleic acid were all purchased from Nakarai Tesque. Propylene glycol (PG) and sodium δ -amino-1,3,6-naphthalenetrisulfonate (ANTS) were also obtained from Nakarai Tesque, P-xylene-bis-pyridinium bromide (DPX) was obtained from Micro Probes (Junction City, OR). All other chemicals were reagent grade commercial products.

Lipid Extraction from Rat Skin Seven- to eight-week male rats (Wistar strain) were used. The skin was obtained from the abdomen under anesthesia with pentobarbital. It was shaved lightly with an electric clipper and then with an electric razor. The freshly excised full thickness skin, with subcutaneous fat removed, obtained from two rats was dipped into CHCl_3 -MeOH (2:1, by volume) solution for 48 h. The area of the excised skin for each was 50 cm^2 . After the above treatment, the skin was removed from the extraction solvent. The lipid extract was filtered with filter paper (Advantec filter paper (qualitative) No. 2, 90 mm), and used to prepare the skin bio-liposomes.

Preparation of Skin Bio-liposomes for Leakage Determination The skin bio-liposomes were prepared using a reverse-phase evaporation technique. Ten milliliters of the skin lipid extract obtained from rat skins were evaporated under a stream of nitrogen and then under vacuum in a rotary evaporator which was placed in a bath maintained at 75°C so as to deposit the lipid as a film on the wall of the flask. The obtained preparation was stored in a freezer at -25°C overnight. The prepared lipid film was dissolved in 2.0 ml of diethylether, then 0.5 ml of a 10 mM triethyl silane (TES) buffer solution composed of 12.5 mM ANTS, 45 mM DPX and 68 mM NaCl, (pH 7.4) was added to the flask. The mixture was sonicated in a bath sonicator (W-113, Honda) at room temperature. The resulting emulsion was evaporated in a rotary evaporator at an ambient temperature under vacuum to remove the bulk of the ether until a gel formed. Collapse of the gel was then initiated by a brief vortex mixing, and evaporation was continued under gentle reduced pressure for approximately 3 min until the skin bio-liposomes were formed. The liposomes were separated from unencapsulated materials (ANTS and DPX) by centrifugation at $56350 \times g$ for 60 min. The obtained pellets of the skin bio-liposomes containing ANTS and DPX were then placed in a tube and kept in ice until they were used. To confirm the formation of the skin bio-liposomes and monitor the size, a micrograph assay was carried out. The pellets were suspended in 6 ml of a buffer solution composed of 150 mM NaCl/10 mM TES/0.1 mM ethylenediaminetetra acetic acid (EDTA), (pH 7.4), and the obtained suspension was then

diluted with the same buffer 200 times. One drop of suspension was placed on a glass plate and the formation of the skin bio-liposomes was examined using an Olympus VANOX-T, model AH2-FL/AH-RFL.

Measurement of Leakage of Liposome Contents For the leakage assay, the pellet was suspended with 6 ml of the above TES buffer solution (pH 7.4), then 0.3 ml of this skin bio-liposome suspension was diluted again with 5 ml of the same buffer solution. This diluted skin bio-liposome suspension (3 ml) was used for the measurement of ANTS leakage from the skin bio-liposomes. The leakage of the contents was measured as an increase of fluorescence of ANTS in the surrounding medium. This is because the leakage of ANTS and DPX from liposomes into the surrounding medium, resulting in dilution, increases the fluorescence intensity of ANTS.⁶⁾ A Hitachi F-3000 fluorescence spectrophotometer was used for this assay. Excitation and emission of ANTS were at 360 and 545 nm, respectively. Leakage assay of ANTS was started by the addition of 50 μ l of distilled water to a 3 ml aliquot of the above diluted skin bio-liposomes, followed by adding 100 μ l of ethanol containing either oleic acid (150, 300 and 500 μ M) or fatty amines (750 μ M). The solution was continually mixed with a magnetic stirrer. Fluorescence changes were measured for about 10 min. At the end of this period, 50 μ l of 2% Triton X-100 was added to the cuvette to determine the total fluorescence. The percentage of ANTS leaked out from the liposomes was determined by the following equation.⁷⁾

$$\% \text{ of leakage} = (F - F_0) / (F_t - F_0) \times 100$$

where F_0 = initial fluorescence, F_t = fluorescence with Triton X-100 added, F = fluorescence at every minutes after addition of fatty acid or fatty amine.

In Vitro Skin Preparation for FT-IR/ATR Spectroscopy Measurement

Seven- to eight-week old male rats (Wistar strain) were used. The rat was anesthetized with pentobarbital and the skin was shaved lightly with an electric razor, taking care to prevent damage to the surface of the skin. The freshly excised full thickness abdominal skins with subcutaneous fat removed were incubated in PG with or without skin penetration enhancers for 2 h at 37°C. After this treatment, the skin surface was gently wiped with Kimwipes® to remove the solvent existing on the skin surface and then rinsed with ethanol for 10 s. This sample was then vacuum dried for 1 h. The control sample without any treatment of either PG or the penetrant was also prepared in the same manner as described above. For the FT-IR/ATR measurement, each of the skin samples was then placed in a chamber maintained at 95% relative humidity and 25°C for 3 d. By this treatment, the skin sample was equilibrated to a water content of about 50%.

In Vivo Skin Preparation for FT-IR/ATR Spectroscopy Measurement

Seven- to eight-week old rats (Wistar strain) were anesthetized with pentobarbital and the skin was shaved lightly with an electric razor, taking care to prevent damage to the surface of the skin. Each rat was then fixed on its back on a board. A glass cell (effective diffusion area: 1.05 cm²) was attached to the abdominal skin with instant adhesive bonding. The skin penetration enhancer, 0.15 M oleylamine or 0.15 M laurylamine dissolved in PG (1 g), was applied to the skin by adding it into the cell. After a 2-h treatment, the sample applied to the skin was gently removed, then the skin was wiped with ethanol to clean the surface. Immediately after this treatment, the skin sample was excised and the FT-IR spectrum was measured on the epidermis side of the skin.

Measurement of FT-IR/ATR Spectra Infrared spectra of the stratum corneum were obtained with a JEOL JIR-100 Fourier transform spectrometer equipped with a liquid nitrogen cooled, narrow band mercury-cadmium-telluride detector (MCT detector). The internal reflection element was KRS-5 (52 \times 20 \times 2 mm trapezoid cut at 45°). The sample was determined at ambient temperature. The internal reflection element permitted the infrared beam to penetrate to a depth between 0.58 and 5.8 μ m into the stratum corneum.⁸⁾ The thickness of the rat stratum corneum was about 20 μ m,⁹⁾ so FT-IR/ATR measurement can permit one to examine the molecular motion of the lipid domains in the stratum corneum near the surface.

Results and Discussions

A reverse phase evaporation technique and sonication of the lipid mixtures extracted from rat whole skins yielded large unilamellar bio-liposomes ranging in size from approximately 2–10 μ m as shown in Fig. 1. Wertz *et al.*⁵⁾ successfully prepared stable and unilamellar liposomes

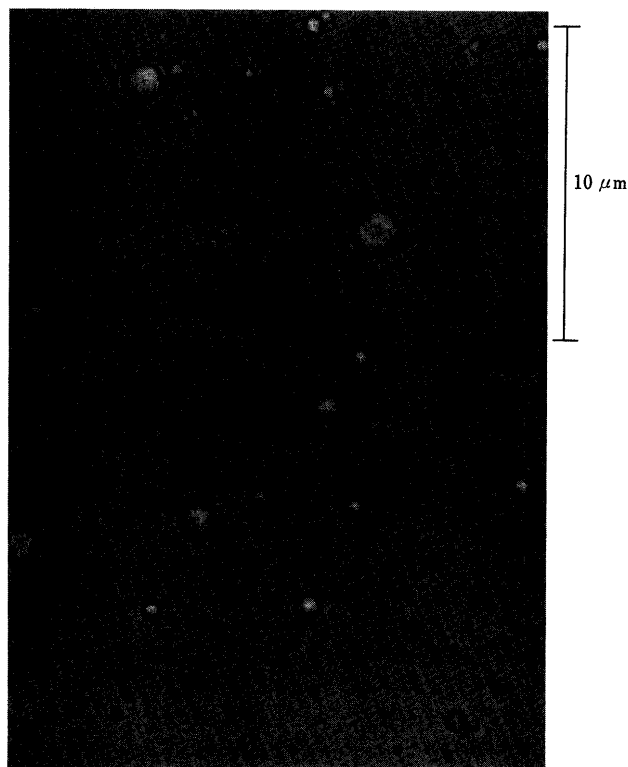


Fig. 1. Photograph of a Large Unilamellar Lipid Liposome Prepared from Rat Whole Skin by the Reverse Phase Evaporation Method

One drop of the suspension of the liposomes-encapsulated fluorescence probe, ANTS and DPX, was coated with a cover glass and examined in an Olympus VANOX-T, model AH 2-FL/Ah-RFL.

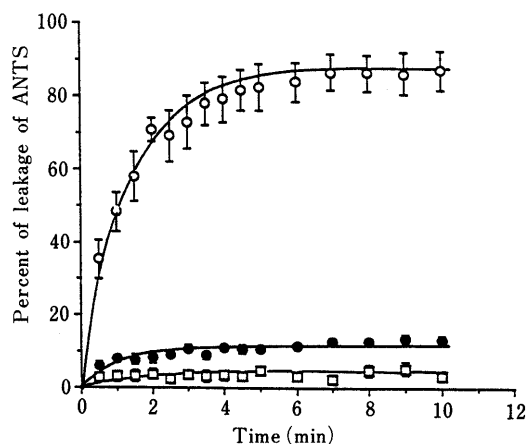


Fig. 2. Leakage of ANTS from Whole Skin Lipid Bio-liposomes Under Treatment with Either Laurylamine or Oleylamine

Each value represents the average of three experiments. The detailed experimental conditions are described in the experimental section.

approximating the composition of stratum corneum lipids by hydration technique. They ranged in size from 20–150 nm, and this size was exclusively smaller than that we obtained. The composition of the lipids of the skin bio-liposomes was different from the simulated stratum corneum lipid liposomes obtained by Wertz, but the size difference between them could be considered due partly to the different processes of forming the liposomes. Generally, the larger unilamellar liposomes should be more thermodynamically stable as compared with the smaller ones.¹⁰⁾ Thus, it is probable that aggregation and

fusion of such large liposomes is somewhat less. The large unilamellar liposomes are able to encapsulate a larger amount of fluorescence substance compared to the small ones. These considerations would suggest that the obtained large unilamellar liposomes might be superior to the smaller ones in measuring quantitatively the time profiles of fluorescence leakage from the inside of the liposomes to the outside solution.

As shown in Fig. 2, the ANTS release from the skin bio-liposomes after the addition of either oleylamine or laurylamine at the concentrations of $23.8 \mu\text{M}$ was accelerated, compared to the release without these penetrants (control). The ANTS release started immediately after the addition of oleylamine, and this was successively followed by the massive release. However, complete leakage of ANTS was not observed. This phenomenon appeared to be the result of either a partial collapse of the skin bio-liposomes after the intensive interaction of oleylamine with the lipids, or denaturation of lipid membranes of the skin bio-liposomes which enhanced the penetration characteristics of the lipid membranes. As indicated in Table I, the leakage of ANTS from the skin bio-liposomes treated with laurylamine was significantly ($p < 0.001$) smaller than that treated with oleylamine. PG did not cause any release of ANTS from the skin bio-liposomes in the medium. Its value was significantly different from that

TABLE I. Leakage of ANTS from Skin Bio-liposomes Caused by Either Oleylamine or Laurylamine and the Asymmetric C-H Stretching Frequency for Rat Stratum Corneum Lipids Treated with the Corresponding Fatty Amines

Penetrants	Leakage of ANTS (%) ^a	Frequency ^b	Frequency ^c
Oleylamine	72.9 ± 7.0^d (3)	2924.2 ± 0.3^f (3)	2923.6 ± 0.3^h (3)
Laurylamine	10.3 ± 1.2^e (3)	2921.5 ± 0.2^g (3)	2921.8 ± 0.3^i (3)
PG	3.6 ± 1.1 (3)	2919.5 ± 0.2 (4)	2920.4 ± 0.2 (4)
No treatment	2.7 ± 1.6 (3)	2919.8 ± 0.2 (7)	2919.6 ± 0.2 (5)

a) The leakage percent of ANTS at 2 min after the initiation of the reaction by adding each of the penetrants was calculated. b, c) Wave numbers for asymmetric C-H stretching vibrations of rat stratum corneum samples prepared by the *in vitro* and *in vivo* methods, respectively. d-f and h) Significantly different from both the PG treated sample and the untreated sample (no treatment), ($p < 0.001$). g and i) Significantly different from both the PG treated sample and the untreated sample ($p < 0.05$). The values given in parentheses represent mean \pm S.D.

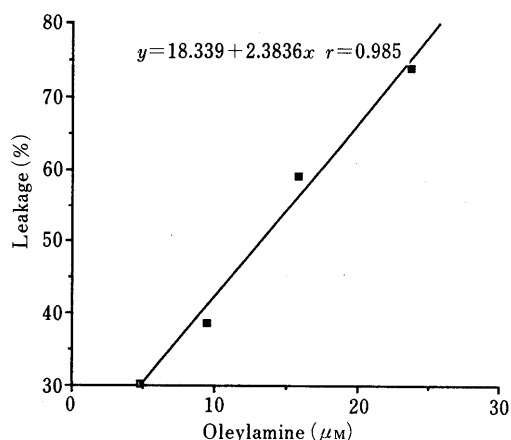


Fig. 3. Relationship between Leakage of ANTS from Whole Skin Lipid Bio-liposomes and Oleylamine Concentrations

The data was the value at 2 min after initiation of the leakage reaction in the medium, pH 7.4.

of the sample treated with either oleylamine ($p < 0.001$) or laurylamine ($p < 0.001$), while the leakage value with PG was not significant from the value of the untreated sample (Table I). From this data, it was verified that no collapse or denaturation of the skin bio-liposomes occurred in the PG-containing medium free from fatty amines. The oleylamine-induced leakage of ANTS from the skin bio-liposomes, expressed as percent of leakage during 2 min from the initiation of the leakage assay by adding fatty amines dissolved in PG, was clearly concentration-dependent in the range from 4.76 to $23.8 \mu\text{M}$, as shown in Fig. 3.

Oleic acid also induced the leakage of ANTS significantly in the manner similar to that observed with oleylamine and laurylamine (Fig. 4). Oleic acid was reported to reduce the transition temperature of the stratum corneum of porcine lipids, and thereafter the amount of oleic acid was taken up by the stratum corneum paralleling with the enhancement of drug flux.¹¹⁾ Consequently, our results with the leakage experiment of ANTS using skin bio-liposomes suggested that oleylamine and other penetrants

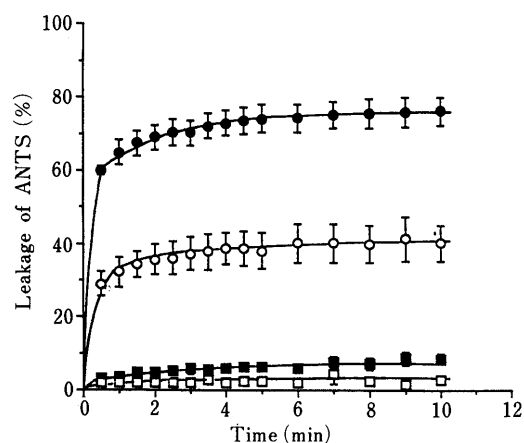


Fig. 4. Effect of Oleic Acid Concentrations on Leakage of ANTS from Whole Skin Lipid Liposomes

Each value represents the average of three experiments. The detailed experimental conditions are described in the experimental section.

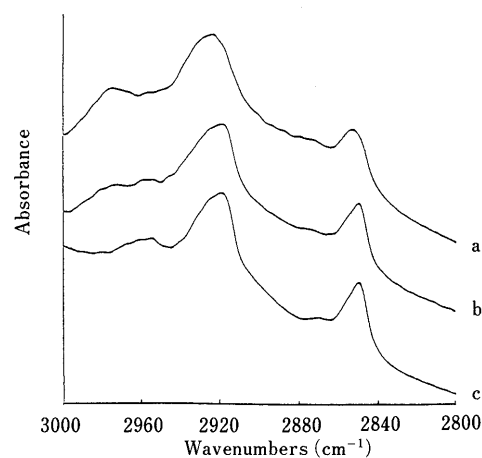


Fig. 5. Representative FT-IR Spectra of Rat Abdominal Stratum Corneum in the C-H Stretching Region Following Treatment with Oleylamine in PG, PG Alone, and Untreated Skin

(a) Spectrum obtained from rat skin stratum corneum treated with oleylamine, (b) with PG and (c) with nontreated skin. The conditions for FT-IR measurement were described in detail in the experimental section.

such as oleic acid primarily caused denaturation of the lipid membranes by their penetration into the lipids-forming skin bio-liposomes. It was also understood that the degree of denaturation caused by the penetrants was attributed to the degree of interaction of a penetrant with the lipids.¹²⁾ Figure 5 shows a representative spectrum of the rat skin stratum corneum, which was treated *in vitro* with 0.15 M oleylamine in PG (a) together with the spectra obtained from the untreated stratum corneum sample (b and c) prepared *in vitro*. The C–H asymmetric stretching frequency near 2920 cm^{-1} originates primarily from methylene groups in the stratum corneum lipid hydrocarbon chains.^{13–15)} As indicated in Table I, *in vitro* treatment of the skin sample with oleylamine dissolved in PG caused a significant shift ($p < 0.001$) in the peak of the spectrum toward higher wavenumbers from 2919.8 to 2924.2 cm^{-1} due to the asymmetric C–H stretching frequency as compared to the peak in the spectrum obtained from the untreated skin sample. Laurylamine-induced shift was not as pronounced as seen with oleylamine, nevertheless a significant small shift toward the higher wavenumbers ($p < 0.05$). As to the skin sample treated with PG and the untreated skin sample, there was no significant difference in the value between them. A similar trend was also observed with the skin samples prepared *in vivo*. This coincidence in the *in vivo* and *in vitro* experimental observations suggested that the procedure described here could be useful for characterizing the penetrants using the FT-IR/ATR method. The advantage of skin samples prepared *in vivo* is that the complicated and time-consuming technique to prepare samples is avoided. Table I also shows the results describing the relation between the leakage of ANTS from the skin bio-liposomes in the medium containing laurylamine or oleylamine, as well as the asymmetric C–H stretching frequency for the rat stratum corneum lipids treated with either laurylamine or oleylamine. It was mentioned that an increase in wavenumbers for the C–H asymmetric stretching peak near 2920 cm^{-1} reflects the lipid structure perturbation in the stratum corneum.¹⁵⁾ Based on this finding, it was understood that oleylamine caused the stronger perturbation in the structure of the stratum corneum, compared to laurylamine. The degree of the above penetrant-induced steric disordering measured by FT-IR/ATR spectroscopy correlated well with the data where the oleylamine-induced leakage of ANTS from the skin lipid bio-liposomes was

definitely larger in percentage than the laurylamine-induced leakage. This implied that the skin bio-liposomes could be sufficiently useful for evaluation of the penetrant-induced enhancement of drug delivery through the skin. This observation may also suggest the existence of a possible relationship between the degree of frequency changes of C–H asymmetric vibrations of hydrocarbon chains of the stratum corneum lipids and transdermal drug delivery in the presence of penetrants.

In conclusion, the reverse phase evaporation technique enabled us to prepare stable and large unilamellar vesicles of rat whole skin lipids ranging in size from approximately 2 to $8\text{ }\mu\text{m}$. According to the FT-IR/ATR analysis for molecular mobility of the stratum corneum, prepared skin bio-liposomes were useful for quantitative evaluation of the skin penetration enhancers which affect the stratum corneum lipid domains causing the steric disordering of the lipids and subsequent enhancement of the skin penetration of drugs.

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