Evaluation of the Mechanism of Skin Enhancing Surfactants on the Biomembrane of Shed Snake Skin

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The aim of the present work was to investigate the effects of different surfactants at various concentrations as a skin penetration enhancer through the biomembrane of the shed skin of Naja kaouthia. Additionally, the enhancer mechanism(s) of each class of surfactants were evaluated using physical characterization techniques, such as scanning electron microscopy (SEM), attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy, and small and wide angle X-ray scattering (SWAXS). Our results showed that skin permeability increased with increasing concentrations of surfactants and the degree of increase was higher for the model hydrophilic permeant, deuterium dioxide (D₂O), than the lipophilic permeant, ketoprofen (KP). Ionic surfactants, sodium lauryl sulfate (SLS) and cetyl trimethyl ammonium bromide (CTAB), demonstrated higher enhancement ability than the polyoxyethylene (20) sorbitan mono-oleate (Tween 80) non-ionic surfactant, which was consistent with the results from physical characterization studies. Increasing amounts of permeated drug resulted in an increase in membrane interactions. From our observations, it can be assumed that SLS and CTAB can be localized inside the biomembrane and thereby enhance drug permeation mainly through interactions with intercellular lipids in the stratum corneum (SC) and the creation of a perturbed microenvironment among lipid alkyl chains and polar head groups.

Key words surfactant; shed snake skin; enhancer mechanism; permeation; attenuated total reflectance-Fourier transform infrared spectroscopy; small-wide angle X-ray scattering

The barrier function of the skin resides in the stratum corneum (SC), the outermost layer of the skin. Its multilayered wall-like structure contains terminally differentiated keratin-rich epidermal cells that are embedded in an intercellular lipid-rich matrix. The unique constituents and structural organization of the SC play a major role in the percutaneous absorption of any substances, including drugs, applied onto the skin. It is generally accepted that the intercellular lipid domain is composed mainly of free fatty acids (FFAs), cholesterol, and at least nine classes of ceramides (CER), referred to as CER1 to CER9. The molecular structures of these lipids are illustrated in Fig. 1. The CER are composed of a sphingosine to which a non-hydroxy or α-hydroxy fatty acid is linked. R in the structure of CER represents a fatty acid which has a chain length distribution. Phospholipids, which are the most abundant in all cell membranes, are a minor component of the SC lipid domain.

Although human skin is an ideal model membrane for transdermal research, the ethical considerations and limited availability of human skin present major problems. Animal skin, such as hairless rat, pig, rabbit, and shed snake skin, has been generally used as an alternative. Snakes shed their skins periodically, allowing the skin membrane to be obtained without killing the animals. Multiple shed skins can be acquired from the same individual snake. Since shed snake skin is not a living tissue, it can be stored for a long period at room temperature and is easily transported. It has been claimed that stored and fresh snake skins show no differences in permeability. The principal advantage for using this membrane is that it provides a permeability barrier similar to that of human skin. Shed snake skin consists of three distinctive layers and the main barrier to water permeation resides in the mesos layer of the epidermis, which contains the extremely flat and thin cells surrounded by intercellular lipids, similar to the human SC. The potential of shed snake skin as a model biological membrane for transdermal research has been reported. Therefore, we have used shed snake skin for our studies.

Surfactants are often used to increase the physical stability of many topical pharmaceutical dosage forms and cosmetic products. These categories of substances are also the most useful enhancers to incorporate in formulations. Commonly, surfactants may be classified according to the nature of the head group as anionic, cationic, non-ionic, or zwitterionic. In general, cationic surfactants cause a greater increase in the flux of drugs than anionic surfactants, which, in turn, produce greater increases in flux than nonionic surfactants. Understanding the mechanism of action of these substances

Fig. 1. The Chemical Structures of Ketoprofen and the Major Biological Lipids That Exist in Skin

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acting on the skin at the molecular level is important to ensure the proper selection of surfactants that are incorporated into transdermal formulations to achieve therapeutic effects. However, the mechanism(s) of skin penetration enhancement of surfactants remains to be clearly elucidated.

In this study, the skin permeation of ketoprofen (KP, lipophilic permeant, Fig. 1) and deuterium oxide (D₂O, hydrophilic permeant) through shed snake skin was investigated. The effects of nonionic surfactant (polyoxyethylene (20) sorbitan mono-oleate, Tween 80), anionic surfactant (sodium lauryl sulfate, SLS) and cationic surfactant (cetyl trimethyl ammonium bromide, CTAB) were evaluated as skin penetration enhancers at 0.1, 0.5, 1, 2 and 5% (w/v). The chemical structures of surfactants used in this study are illustrated in Fig. 2. The enhancement ratios by these enhancers were assessed and compared. Surfactant-treated shed skins were also examined using physical characterization techniques, such as attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy, scanning electron microscopy (SEM), and small and wide angle X-ray scattering (SWAXS), to identify the molecular mechanism of surfactants as skin penetration enhancers.

MATERIALS AND METHODS

Chemicals and Skin Membrane  Ketoprofen (KP; molecular weight, 254.28 Da) and deuterium oxide (D₂O; 254.32 Da) were purchased from Sigma Aldrich Chemical Company, Inc. (U.S.A.). Sodium lauryl sulfate (SLS, 288.38 Da), cetyl trimethyl ammonium bromide (CTAB, 364.46 Da) and polyoxyethylene (20) sorbitan mono-oleate (Twee 80, 1310 Da) were provided from Ajax Finechem (Australia). All other chemicals were of reagent grade and used without further purification. Shed snake skin of *Naja kaouthia* was donated by the Queen Saovabha Memorial Institute, Thai Red Cross Society (Bangkok, Thailand).

Solubility Studies  The saturated solubility of KP in water and at various concentrations of enhancers was determined. Saturated solutions were prepared by adding excess drug to the solvents and stirring for 48 h at 37°C. After the solutions were centrifuged, the KP concentration was determined by measuring the intensity of the O–D stretching vibrational band at 2512 cm⁻¹ by Fourier transform infrared spectroscopy (FTIR).

In Vitro Skin Permeation  The method used to measure percutaneous absorption followed Test Guideline 428 of the Organization for Economic Cooperation and Development. The biomembrane of shed snake skin used in this study was obtained from the natural shedding of the snake. Therefore, no ethical approval was required in Thailand. Dry shed skin of *Naja kaouthia* was used as a model membrane for our skin permeation study. The shed skin was obtained from 3–4 different snakes and stored at -18°C prior to use. The dorsal portion was cut into an appropriate size. Each shed snake skin can be divided into 10–12 pieces. The skin was mounted between two half cells of a side-by-side diffusion cell with a water jacket connected to a water bath at 37°C, each having 4.0 mL volume and 0.78 cm² effective diffusion area. The receiver and donor compartments were filled with distilled water and stirred with a Teflon magnetic stirrer at 600 rpm. After 1 h of equilibration, the receiver compartment was filled with freshly distilled water. The donor compartment was replaced with compounds in various concentrations (0, 0.1, 0.5, 1, 2, 5%, w/v) of enhancers, such as SLS, CTAB, and Tween 80. Skin permeation was performed at 37°C for 8 h. Samples were taken every 1 h until 8 h. A part (1.0 mL) of the receiver solution was withdrawn and replaced with the same volume of distilled water to maintain a constant volume. The concentration of KP in all samples was assayed by HPLC, whereas D₂O was assayed by Fourier transform infrared spectroscopy (FTIR).

Analytical Methods for KP and D₂O  To determine the concentration of KP, a High Performance Liquid Chromatograph (HPLC; Perkin Elmer, MA, U.S.A.) equipped with a UV detector (Perkin Elmer, MA, U.S.A.) at 254 nm was used in the reversed-phase mode with a Spherisorb ODS-5 (C18) column (4.6 mm i.d. × 250 mm; Scitronic Co., Ltd., Bangkok, Thailand) at 25°C. A mixture of 1.0% (v/v) phosphoric acid and methanol (75:25, v/v) was employed as the mobile phase at a flow rate of 1.0 mL min⁻¹. D₂O was quantified by measuring the intensity of the O–D stretching vibrational band at 2512 cm⁻¹ in infrared spectra.

Permeation Data Analysis  The cumulative amount of drug permeated through a unit area of shed skin was plotted as a function of time. The flux of the compounds from solutions containing various concentrations of surfactants was also compared. The permeability coefficient (Kᵣ) and enhancement ratio (ER) were calculated and compared with the control sample.

The permeability coefficient (Kᵣ) was calculated from the following enhancement equation:

\[
Kᵣ = \frac{J_s}{C_d}
\]

where \( J_s \) is the steady state permeation rate or flux obtained from the slope of the linear portion of the permeation profile and \( C_d \) is the saturated solubility of the drug in the donor compartment.

The enhancement ratio (ER) was calculated from Eq. 2:

\[
ER = \frac{Kᵣ \text{ (with enhancer)}}{Kᵣ \text{ (without enhancer)}}
\]

Statistical Analysis  In vitro drug permeation measurements were collected for four to six experiments. These values are expressed as the mean±standard deviation (S.D.). The statistical significance of the differences between groups, such as untreated shed skin, 5% SLS-treated shed skin, 5% CTAB-treated shed skin, and 5% Tween 80-treated shed skin,
in the amount of model drugs permeated through shed skin was examined using a one-way analysis of variance (ANOVA) followed by a Student’s *t*-test. The significance level was set at *p*<0.05.

**Characterization of Surfactant-Treated Shed Skins**  The effect of different enhancers was determined by soaking the model membrane in each surfactant solution (*i.e.* SLS, CTAB, Tween 80) for 8 h, the same time period in which the skin permeation study was performed. Afterwards, the shed snake skins were wiped gently with Kimwipes®, rinsed in 3 aliquots of distilled water, softly patted with Kimwipes®, and then stored in an electric dry cabinet set at 24°C, 19% RH for 24 h prior to use. The same method was used for water-treated shed snake skin. The skin samples were subsequently characterized by the following methods.

**Scanning Electron Microscopy (SEM)**  The surface morphology of the shed snake skin was investigated using SEM. Shed skin samples were mounted on an aluminum sample holder and sputter-coated with gold in a vacuum evaporator. SEM imaging was performed using a Model Maxim-2000 SEM (CamScan Analytical, Cambridge, England) at an accelerating voltage between 15 and 25 kV. The surface morphology of the skin was observed at 500×, 5000×, and 10000× magnification.

**Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) Spectroscopy**  ATR-FTIR spectra were obtained using a Perkin-Elmer spectrophotometer (Spectrum 100) equipped with a crystal diamond universal ATR sampling accessory (UATR). Before each measurement, the ATR crystal was carefully cleaned with acetone. During each measurement, the outer surface of the skin sample was in contact with the Universal diamond ATR-top-plate. For each sample, the spectrum obtained represented an average of 4 scans recorded within the range of 4000–400 cm\(^{-1}\) with a 4 cm\(^{-1}\) resolution.

**Small and Wide Angle X-Ray Scattering (SWAXS)**  The experiments were performed with small and wide angle X-ray scattering (SAXS and WAXS) instrument, beamline B1, installed at the DORIS III synchrotron source at HASYLAB/DESY in Hamburg, Germany. SAXS scattering patterns (0–1 Å) were acquired using a large area pixel detector (PILATUS 1m, Dectris, Switzerland) with a pixel size of 172µm×172µm. WAXS (1–4 Å) was measured simultaneously using a Mythen strip detector (Dectris, Switzerland). The distance from the sample to detector was 0.885 m, and the X-ray energy was 14 keV. Samples of both parts (hinge and scale areas) of the model membrane were folded 4–5 times (about 4×4 mm) and fixed onto a holder, which was then placed into a vacuum chamber and measured for SWAXS. The raw scattering data were background corrected, integrated and calibrated using a MATLAB-based analysis suite, which is available at the beamline. Peak positions were determined by using Origin software to fit a Gaussian equation to the data. Considering the lamellar phase of the lipid determined by the Bragg equation, the repeat distance or the length of the unit cell “*d*” was calculated as follows\(^20\),

\[
d = \frac{2\pi d}{q_o}
\]

where *n* = the order of the diffraction peak, and *q*\(_o\) = scattering vector at the particular order.

**RESULTS AND DISCUSSION**

**Effects of Surfactants on Drug Solubility and Skin Permeation**  KP and D\(_2\)O were used as model hydrophobic and hydrophilic drugs, respectively, to evaluate the enhancement effect of various surfactants on drug solubility and skin permeation. The addition of surfactants in vehicles enhanced the solubility of KP significantly (Table 1). The highest solubility was measured for the vehicle containing 5% (w/v) SLS (1.19 mg/mL), which was more than 6-fold greater than the solubility of KP in water (0.18 mg/mL). The flux of KP and D\(_2\)O in the vehicle (water) and in the presence of various concentrations (0.1–5%, w/v) of SLS, CTAB, and Tween 80 is shown in Fig. 3. All surfactants used in this study increased the permeation rate of the model drugs. As shown in Figs. 3 and 4, the skin permeation enhancement effect of surfactants was

### Table 1. The Solubility and Skin Permeation Parameters of Ketoprofen in Various Concentrations of Surfactants

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Concentration (%w/v)</th>
<th>Solubility of KP at 37°C (mg/mL) (^{120})</th>
<th>Flux ((\mu g cm^{-2} h^{-1}))</th>
<th>(K_p) ((\times 10^2, cm^2 h^{-1}))</th>
<th>ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>0.18±0.02</td>
<td>4.18±0.42</td>
<td>2.32±0.16</td>
<td>1</td>
</tr>
<tr>
<td>CTAB</td>
<td>0.1</td>
<td>0.19±0.02</td>
<td>4.50±1.37</td>
<td>2.37±0.11</td>
<td>1.02</td>
</tr>
<tr>
<td>CTAB</td>
<td>0.5</td>
<td>0.25±0.03</td>
<td>10.05±2.98</td>
<td>4.06±0.23</td>
<td>1.75</td>
</tr>
<tr>
<td>CTAB</td>
<td>1</td>
<td>0.41±0.04</td>
<td>29.48±3.31(^*)</td>
<td>7.19±0.42</td>
<td>3.1</td>
</tr>
<tr>
<td>CTAB</td>
<td>2</td>
<td>0.65±0.05</td>
<td>47.1±5.35(^*)</td>
<td>7.26±0.45</td>
<td>3.13</td>
</tr>
<tr>
<td>CTAB</td>
<td>5</td>
<td>0.81±0.07</td>
<td>106.6±12.36(^*)</td>
<td>13.51±0.98</td>
<td>5.82</td>
</tr>
<tr>
<td>SLS</td>
<td>0.1</td>
<td>0.21±0.01</td>
<td>5.71±1.61</td>
<td>2.62±0.19</td>
<td>1.13</td>
</tr>
<tr>
<td>SLS</td>
<td>0.5</td>
<td>0.42±0.05</td>
<td>16.0±1.91(^*)</td>
<td>3.83±0.21</td>
<td>1.65</td>
</tr>
<tr>
<td>SLS</td>
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<td>0.58±0.05</td>
<td>43.72±6.12(^*)</td>
<td>7.54±0.68</td>
<td>3.25</td>
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<tr>
<td>SLS</td>
<td>2</td>
<td>0.78±0.08</td>
<td>99.29±5.25(^*)</td>
<td>12.73±1.06</td>
<td>5.51</td>
</tr>
<tr>
<td>SLS</td>
<td>5</td>
<td>1.19±0.11</td>
<td>194.7±32.12(^*)</td>
<td>16.93±1.23</td>
<td>7.3</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.1</td>
<td>0.19±0.02</td>
<td>4.54±0.95</td>
<td>2.39±0.12</td>
<td>1.03</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.5</td>
<td>0.25±0.01</td>
<td>7.82±2.51</td>
<td>3.13±0.11</td>
<td>1.35</td>
</tr>
<tr>
<td>Tween 80</td>
<td>1</td>
<td>0.37±0.03</td>
<td>19.9±2.93(^*)</td>
<td>5.38±0.29</td>
<td>2.32</td>
</tr>
<tr>
<td>Tween 80</td>
<td>2</td>
<td>0.48±0.05</td>
<td>23.7±2.61(^*)</td>
<td>4.94±0.30</td>
<td>2.13</td>
</tr>
<tr>
<td>Tween 80</td>
<td>5</td>
<td>0.61±0.06</td>
<td>33.1±6.52(^*)</td>
<td>5.43±0.41</td>
<td>2.34</td>
</tr>
</tbody>
</table>

\(a\) Each value represents the mean±S.D. of three experiments. \(b\) Each value represents the mean±S.D. of four to six experiments. \(*p*<0.05 compared with control.)
in the order of SLS>CTAB>Tween 80. These results were inconsistent with the previous report, mentioned in the introduction part, indicating that the cationic surfactants are generally more effective as penetration enhancer than the anionic ones. This difference may be explained by the specific interaction of SLS with the shed skin which was further described. In all surfactant-treatments, the flux of KP that permeated through the shed skin increased when the concentration of surfactant was gradually increased. In contrast, for D2O, a small increase in the concentration of surfactant did not enhance the flux of this permeant. This is, in part, due to the very small molecular size of D2O, which can easily penetrate through the skin without enhancers. However, at high concentrations (5% w/v), SLS and CTAB significantly enhanced the penetration of both KP and, especially, D2O. The maximum enhancing effect was achieved at the highest concentration of SLS and CTAB (5% w/v). When Tween 80 was used as a penetration enhancer, drug permeation increased considerably at 1% (w/v). Using higher concentrations of Tween 80 did not increase the permeation of drugs. The ER values of KP and D2O through the shed skin are summarized in Table 1 and Fig. 4. The types and concentrations of surfactants play an important role in the ER. The KP increased with increasing concentrations of surfactants. Furthermore, the degree of increase was higher for D2O than that for KP. The highest ER values were observed in the presence of 1% (w/v) Tween 80 (2.32 for KP and 3.84 for D2O). In higher concentrations of Tween 80, ER values were almost constant. The highest permeation rates of KP and D2O were obtained from the solution containing 5% (w/v) SLS (7.3, 10.30-fold, respectively, over control) and 5% (w/v) CTAB (5.82, 8.22-fold, respectively, over control). The ER of KP and D2O in the presence of SLS were larger than those of Tween 80 by almost 3 times. With the increase in the concentration of CTAB, the ER values of KP and D2O were more than 2.5 times higher than Tween 80. These data are in agreement with a previous report in which cationic surfactants were shown to be more destructive to skin tissues, causing a greater increase in flux than nonionic surfactants. Anionic surfactants, such as SLS, were also reported to penetrate and function by the reversible modification of lipid in the SC barrier and alteration of SC barrier properties.

Characterization of Surfactant-Treated Shed Skin

The section above (i.e. the permeation experiment), revealed the variation in the abilities of surfactants to enhance the flux of permeants. The techniques of SEM, ATR-FTIR spectroscopy, and modern characterization technique, SWAXS, were further used to characterize shed skin samples in this study. Different techniques obtain complementary information used to clearly understand the mechanism of each surfactant as skin penetration enhancers. Dry samples were used in these comparisons to avoid any discrepancies that might arise from variations in the degree of hydration of samples. All samples were stored in an electric dry cabinet for at least 24h before analysis.

SEM Studies

SEM was used to image the shed skin surface and any alterations that might be observed from the surfactant-treated shed skins. SEM micrographs of dorsal shed skin samples (Fig. 5) revealed characteristics of the shed skin surface. The pigmentation of scales arose from the keratin folds, whereas the hinges were quite colorless. The image exhibits finer structural detail with 500× and 5000× magnification of the hinge and 5000× magnification of the scale.
regions. At high magnification, electron microscopic examination of large areas of the dorsal scales and hinges of shed skin revealed that there were no pores. The surface of surfactant-treated shed skins was investigated and compared with control shed skin samples. Structural changes of the shed skin, which may result from the action of surfactants, could not be observed by SEM. However, surfactants caused a change in the hydration of the treated shed skin, which is noticeable in Figs. 5a–e). Micropits were clearly observed for the scales of shed skin that had been treated with surfactant as shown in Figs. 5A–E). Micropits of the hinges of treated shed skins observed at 5000 × were also clearer than those of untreated shed skin (data not shown).

**ATR-FTIR Studies** Figure 6 shows the ATR-FTIR spectra of shed skins in the presence of various surfactants at 5% (w/v). The bands of lipids between 2800 and 3000 cm⁻¹ are due to C–H stretching vibrations primarily associated with the lipid alkyl chains, which are of particular interest for our studies. These bands occurred near 2920 and 2850 cm⁻¹, which correspond to asymmetric υ(CH₂) and symmetric υ(CH₃) vibrations, respectively.²⁴–²⁶ The C–H₂ symmetric and asymmetric stretches are often used to study the alterations of SC lipid hydrocarbon chain packing and possible conformational changes induced by chemical enhancers. Normally, hydrocarbons within the lipid domain below their transition temperature exist in a trans conformation. When the lipid domain becomes fluidized, a trans gauche shift to a higher frequency (blue shift) occurs.²⁴–²⁷ The intensity of a band (height and

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak No.</th>
<th>q (1/Å)</th>
<th>d (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry shed snake skin</td>
<td>1</td>
<td>0.16</td>
<td>39.27</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.665</td>
<td>9.45</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.414</td>
<td>4.44</td>
</tr>
<tr>
<td>5% SLS-treated shed skin</td>
<td>1</td>
<td>0.167</td>
<td>37.62</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.321</td>
<td>19.57</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.485</td>
<td>12.96</td>
</tr>
<tr>
<td>5% SLS solution</td>
<td>1</td>
<td>0.179</td>
<td>35.10</td>
</tr>
<tr>
<td>5% CTAB-treated shed skin</td>
<td>1</td>
<td>0.089</td>
<td>70.60</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.665</td>
<td>9.45</td>
</tr>
<tr>
<td>5% CTAB solution</td>
<td>1</td>
<td>0.056</td>
<td>112.20</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.092</td>
<td>68.30</td>
</tr>
<tr>
<td>5% Tween 80-treated shed skin</td>
<td>1</td>
<td>0.128</td>
<td>49.09</td>
</tr>
<tr>
<td>5% Tween 80 solution</td>
<td>1</td>
<td>0.135</td>
<td>46.54</td>
</tr>
</tbody>
</table>

**Table 2. Scattering Vectors (q) of Different Samples and Their Repeat Distances (d)**

![Image of SEM micrographs showing dendal scales and hinges](image)

**Fig. 5. Low Magnification (7×) SEM Micrographs of Dorsal Shed Skin Samples of Naja kaouthia Showing the Hinge and Scale Construction**

The images a) to e) and A) to E) of the following shed skin represent the hinge region at 5000 × and the scale region at 5000 ×, respectively: a) and A), dry shed snake skin (no treatment); b) and B), water-treated shed skin; c) and C), 5% (w/v) SLS-treated shed skin; d) and D), 5% (w/v) CTAB-treated shed skin; and e) and E), 5% (w/v) Tween 80-treated shed skin. Black arrows indicate the micropits on hinge and scale construction.
area) is related to the concentration of the energy absorbing component. When an enhancer extracts SC lipids, the lipid bands decrease in band height and/or area or the bands completely disappear.29) Surfactant-treated shed skins, which manifested the higher enhancement in skin penetration compared with water-treated shed skin, were supposed to exhibit changes in lipid bands of the shed skins. However, based on our results, there was no significant difference in the frequency shift of $\nu(CH_2)$ and $\nu(CH_3)$ vibrations. Nevertheless, an alteration of the intensity of these bands was observed. Surfactant-treated shed skins exhibited an increase in band height and area of these lipid bands. CTAB-treated shed skin (5%, w/v) exhibited a greater increase in band height than shed skin treated with 5% (w/v) SLS, which, in turn, had much greater band height than 5% (w/v) Tween 80-treated shed skin and the controls. These results differed from previous studies, which reported a band shift and/or band height reduction in the ATR-FTIR spectra of skin induced by enhancers.27) The implications of these results may be explained by the interactions of surfactants with the skin membrane. SLS and CTAB were inserted into the intercellular lipid domain of the shed skin and interacted with the lipid alkyl chains, resulting in polarity alteration. This may cause a large change in the dipole moment of the molecules during vibration, resulting in the observation of more intense absorption bands. The alkyl side chain of CTAB is composed of 16 carbon atoms, which is larger than that 12 carbon atoms of SLS, and may explain the greater disturbance effect of CTAB on the lipid alkyl chains of SC than SLS.

The impact of surfactants on the SC protein conformation was also examined. The amide bands arising from $-CONH-$ moieties, which are the most intense features in the IR spectra, typically occur at 1500–1700 cm$^{-1}$. Specifically, the amide I and the amide II bands occur at 1657 and 1547 cm$^{-1}$, respectively.23,27,29) These two amide bands are sensitive to the changes in protein conformation and have been the most frequently used for conformation studies.29) From our results shown in Fig. 6, the amide I and amide II bands appeared around 1630 and 1520 cm$^{-1}$. A decrease in the wavenumbers of these peaks may be attributed to the different species of snake used in this study, Naja kaouthia, compared to the previous report on Elaphe obsoleta.25) Surfactants did not alter the protein conformation in the non-lipid domain of SC of shed skin when compared to that of water regardless of considering other amide bands.

The region between 400–1500 cm$^{-1}$ contained some interesting bands. For the spectrum of 5% (w/v) SLS-treated shed skin, a band at 1219 cm$^{-1}$ was observed, showing a band shift to a lower frequency and a considerable increase in the band intensity. This band could be assigned to either $\delta(CH_2)$ wagging mode or $\nu(CN)$ amide III disordered.23,30) The result may suggest the interaction of a high concentration (5%), w/v) of SLS with both SC lipid and protein domains for permeation enhancement across the SC. However, the changes in the amide III C–N stretching frequency and intensity were not completely associated with the protein conformation in the SC. These changes may reflect the change in polarity of the surrounding environment of the ceramides and/or some bonding interactions of ceramides with SLS. On the other hand, the assignment at 1219 cm$^{-1}$ together with 1083 cm$^{-1}$ could be attributed to the asymmetric $\nu(RO-SO_3^{-}\,M^+)$ vibration between 1315–1220 cm$^{-1}$ and the symmetric $\nu(RO-SO_3^{-}\,M^+)$ vibration between 1140–1050 cm$^{-1}$.30) These assignments may correlate with the sulfate group of SLS. It is possible that the changes in these characteristic bands may indicate interactions of the sulfate groups of SLS with the polar head groups of the lipid or protein components in the SC of shed skin via hydrogen bonds. CTAB-treated shed skin (5%, w/v) showed two weak bands at 964 and 912 cm$^{-1}$, which were associated to the $P-$NC$_{dihedral}$ stretching band in the 1110–930 cm$^{-1}$ region.30) The presence of CTAB with a quaternary amine group has been reported to cause the formation of positive charge, promoting strong electrostatic interactions between CTAB and the anionic phosphate of phospholipids in shed snake skin.8,31)

**SWAXS Studies** In order to determine the structure of these biological membranes at the nanoscale, small and wide angle X-ray scattering (SWAXS) at the synchrotron beamline B1 at HASYLAB, DESY, Hamburg was applied. The SWAXS data showed the differences between the untreated shed skin and the shed skin treated with surfactants (Fig. 7). The X-ray pattern of the SLS 5% (w/v) solution in water can form a lamellar ordered system (SAXS-region) with the scattering vector, $q$, at 0.179 Å$^{-1}$, which can be calculated to the lipid repeat distance, $d$, of 35.10 Å. However, the 5% (w/v) SLS solution did not exist in the gel phase, because no peaks were observed under the WAXS-region ($q$, 1 to 4 Å$^{-1}$). For 5% (w/v) SLS-treated shed skin, all peaks representing the shed skin membrane did not significantly shift. Only the peak indicating the SLS solution was observed in the X-ray pattern of the SLS-treated shed skin. However, a small shift of the significant peak to the lower $q$ occurred, resulting in higher $d$-spacing. This shift may be due to the broadness peak of the treated shed skin, which may have caused the difficulty in fitting to the Gaussian equation. The characterization was processed after washing the SLS solution from the shed skin. These data may imply that SLS penetrated into the skin and localized inside the membrane, which can interact with the lipid components in the skin membrane. The 5% (w/v) CTAB solution in water can form a hexagonal phase with the $q$ at 0.056 and 0.092 Å$^{-1}$ and the $d$-spacing of 112.20 and 68.30 Å. In a previous report,32) at 30% (w/w) CTAB in water, the hexagonal phase with the $d$-spacing of 67.4 Å was formed. The $d$-spacing of CTAB in our studies differed from the literature.
which may be due to the difference in concentrations between the two studies. The scattering pattern of membrane treated with CTAB demonstrated less order and more diffusion, especially in the WAXS-region ($q$ higher than 1 Å$^{-1}$), indicating a strong interaction with SC lipids. However, the significant peak representing CTAB can be well observed with the lamellar structure at $q$ of 0.089 Å$^{-1}$ and $d$ of 70.60 Å, demonstrating that the structure of CTAB changed from hexagonal to a lamellar phase after incorporation into the skin. This observation may imply that CTAB could significantly destroy the secondary structure of the skin, by altering the alkyl chains so that they stretch more and cause higher $d$-spacing. The X-ray pattern of a 5% (w/v) solution of Tween 80 in water can form a lamellar ordered system (SAXS-region) with $q$ at 0.135 Å$^{-1}$, which can be calculated to the $d$ of 46.54 Å. For 5% (w/v) Tween 80-treated shed skin, all peaks representing the shed skin membrane did not significantly shift. Only a small peak, which may indicate Tween 80, was observed at $q$ of 0.128 Å$^{-1}$ in the X-ray pattern of the Tween 80-treated shed skin. However, the significant peak had a small shift to a lower $q$, which

Fig. 7. Scattering Patterns of SAXS and WAXS of the Following Shed Skin of *Naja kaouthia* and the Effect of Surfactants over 8 h Treatment

a) Dry shed snake skin (control); b) 5% (w/v) SLS-treated shed skin; c) 5% (w/v) CTAB-treated shed skin; and d) 5% (w/v) Tween 80-treated shed skin. The numbers corresponding to each scattering pattern are as follows: 1) SAXS-WAXS patterns of skin; 2) magnification of the SAXS-region; 3) SAXS patterns of 5% (w/v) surfactant solution.
may be due to the difficulty in the fitting of the broad peak. The scattering vector \( q \) and their repeat distance \( d \) of each sample are summarized in Table 2.

The diffusion of liquid into a model membrane can be also confirmed by the small characteristic signal of water at \( q 0.14 \) Å\(^{-1} \) (data not shown). If the model membrane was soaked in water for 8 h and measured by SWAXS, the membrane should be carefully making contact between liquid and skin, as liquid can penetrate into the skin. However, the amount of contact and effect may be different depending on the liquid type. The data from SWAXS corresponded to our FTIR data, which showed that SLS and CTAB could interact and/or form bonds with the skin membrane, whereas Tween 80 had a less altering effect on the skin. This difference may be due to less Tween 80 being able to penetrate into the membrane or the inability of Tween 80 to form a strong interaction with membrane components.

**Mechanism(s) of Surfactants as SkinPermeation Enhancers** The present study investigated a number of surfactants to act as penetration enhancers and examined the resulting membrane alteration in order to provide some insight on the enhancer mechanism(s) of various types of surfactants. From our discussion, it should be noted that biomembranes are complex heterogeneous systems containing many different lipids and proteins. Various compounds in the SC of shed skin could affect the present investigation, and it is hard to assess the results in any specific way. Furthermore, one should consider the possibility of reversible effects because the physical characterizations were processed in a dry condition, which is not a practical circumstance. ATR-FTIR and SWAXS results demonstrated the interactions with the model membrane in the presence of different surfactants. Based on the results of the present study, the mechanism(s) by which these surfactants induced permeation enhancement could be attributed to the following actions: (i) surfactants, particularly the ionic surfactants, interacted with the model drugs. For example, CTAB could form a complex with the model drug, such as KP, by an ionic interaction between CTAB and the aromatic and carboxyl groups of KP.\(^{33} \) The complexation between the drug and surfactant was expected to increase the drug solubility, alter the partitioning of the drug between the SC and the drug solution, and affect the model drug transport kinetics\(^{33} \); (ii) surfactants interacted with the lipid components of the SC. The surfactant interactions may occur through superficial deposition over the epidermis and even penetration into the biological membranes. SLS probably penetrated into the SC and interacted with the lipid components for both of the hydrophobic tails, the lipid alkyl chains, and the hydrophilic head groups, preferably with the ceramides, the major lipid class in the SC. The formation of hydrogen bonding between SLS and ceramide head groups may consequently induce the disruption of lateral hydrogen-bond networks of the SC lipid bilayer as previously described for terpene penetration enhancers.\(^{24} \) At the highest concentration of 5% (w/v) SLS, the permeation enhancement effect could also be possibly related to the interaction of SLS with the protein domain in the SC. The cationic surfactant, CTAB, facilitated model drugs across shed skin by interacting with the SC through electrostatic and hydrophobic links. These interactions led to the creation of a disordering effect among the alkyl chains of skin lipids, which can be also confirmed by FTIR and X-ray scattering. In addition, the presence of the quaternary amine group in the CTAB molecule may create anionic interaction with phospholipids in the skin. From the results of ATR-FTIR and SWAXS studies, it seems that CTAB was more effective on structural changes than SLS. However, SLS showed the higher effective on skin penetration enhancement than CTAB. These results suggest the most critical mechanism for penetration enhancement of SLS as the interactions of the sulfate group with the polar head group of the ceramides because these interactions are specific for SLS. Tween 80 showed a slight increase in permeation experiments compared to SLS and CTAB. However, no significant differences on the ATR-FTIR study of Tween 80-treated shed skin were observed. From the SWAXS findings, it is possible that Tween 80 could penetrate the skin membrane slightly due to the bulkiness of the molecule. Moreover, non-ionic surfactants may form only weak bonds with lipid and/or protein components in the SC.

**CONCLUSION**

The effect of surfactants on skin permeation and surfactant mechanism(s) were investigated in the present study. Our results revealed that the surfactant enhancers varied in their abilities to enhance the flux of permeants through the shed skin of *Naja kaouthia*. The permeability coefficient increased with increasing concentration of surfactants, and the degree of increase was higher for D\(_2\)O than that for KP, SLS and CTAB (5% w/v) exhibited the greatest increase in flux of permeants. The mechanism of action(s) of these surfactants was examined by the following physical characterization techniques: SEM, ATR-FTIR, and SWAXS. Considering all the data collected, the results from the physical characterization techniques were consistent with the skin permeation studies. The higher the amount of model drugs permeated, the greater the membrane interaction observed. The ionic surfactants, SLS and CTAB, showed large permeation enhancements and major effects on the SC lipid domain with both lipid alkyl chains and polar head groups. The major critical mechanism for penetration enhancement of SLS may be explained by the specific interaction of sulfate group of SLS with the polar head group of the ceramides presented in the skin. This study also suggests that SC lipid extraction was not the major mechanism of action of the surfactants. In order to choose a skin penetration enhancer, not only the permeation enhancement but also the interaction between enhancer and skin must be considered to avoid toxicity and skin irritation.

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