Penetration route of functional molecules in stratum corneum studied by time-resolved small- and wide-angle x-ray diffraction

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Abstract. We studied effects of functional molecules on corneocytes in stratum corneum using time-resolved small- and wide-angle x-ray diffraction after applying a functional molecule. From these results it was revealed that in the stratum corneum a typical hydrophilic molecule, ethanol, penetrates via the transcellular route and on the other hand a typical hydrophobic molecule, d-limonene, penetrates via the intercellular route.

1. Introduction
The outermost layer of skin, stratum corneum (SC), is composed of corneocytes and intercellular lipid matrix. The structure of the SC plays an important role in the barrier function [1,2]. The intercellular lipids in the SC form lamellar structures. In the lamellar repeating direction the long and the short lamellar structures which have the repeat distances of 13.5 nm and 6 nm, respectively, have been detected by the small-angle x-ray diffraction (SAXD) and in the orthogonal direction the lattice spacings of the hydrocarbon-chain packing structure, 0.41 nm and 0.37 nm, have been observed by the wide-angle x-ray diffraction (WAXD). Recently it has been proposed that in the intercellular lipid matrix there are two domains: A domain with hydrophobic character consists of the long lamellar structure with hexagonal hydrocarbon-chain packing in which the lattice spacing is 0.41 nm and another domain with hydrophilic character consists of the short lamellar structure having water layers with orthorhombic hydrocarbon-chain packing in which the lattice spacing is 0.41 nm and 0.37 nm [3]. Furthermore, it has been pointed out that very diffuse peaks with the lattice spacing of 1 nm and 0.46 nm observed by the x-ray diffraction are related to the structure of soft keratin in the corneocytes in the SC [2]. The SAXD and WAXD measurements on the SC are a powerful tool since the molecular arrangements resulting from the structural modification in the SC can be measured when functional molecules such as penetration enhancers, cosmetics, etc. are applied to the SC. In general there exist two potential pathways; one is an intercellular route in which the penetration of the functional molecule occurs via the intercellular lipid matrix lying between the corneocytes and the other is a transcellular route in which the penetration takes place across both the corneocytes and the intercellular lipid matrix. However, whether the former is the fundamental route as claimed by the majority opinion or the both play a complementary role as pointed out by some researchers is still
controversial. Therefore, it is highly important to make clear the structural modification at the molecular level caused by application of the functional molecules to the SC.

Here we investigated effects of hydrophilic or hydrophobic molecule on the SC. As a typical hydrophilic or hydrophobic molecule we applied ethanol [4,5] or \(\text{d-limonene} [6-9]\), respectively. For this purpose we have developed an x-ray diffraction method which can perform the SAXD and WAXD measurements successively for a single SC sample as a function of time after applying the functional molecules [10]. From these measurements we are able to elucidate the possible penetration routes of a hydrophilic or hydrophobic molecule.

It is expected that incorporation of a hydrophilic molecule into the corneocytes modifies the structure of the soft keratin but that of a hydrophobic molecule does not. Thus, the structure of the soft keratin in the corneocytes is crucial in the study on the penetration route in the SC. After the application of hydrophilic or hydrophobic molecule, we focused our attention to the detection of the changes in the x-ray diffraction profile with about 1 nm spacing where a characteristic broad peak associated with the soft keratin takes place [2].

2. Experimental

Eight-week-old male hairless mice (HR-1, Hoshino, Japan) were used. The sample preparation has been described in the previous paper [10]. The small- and wide-angle x-ray experiments were performed at BL40B2 of SPring-8 (Japan). The experimental procedure was mentioned also in the previous paper [10]. The diffraction profile for the entire angular range was obtained with an imaging plate every 210 s. The exposure time was 30 s. Through the figures of the paper, the profile was shown every 210 s successively from red to blue curves. A sample cell developed by us has been described in the previous paper [10]. A stratum corneum sample was embedded in a central hollow with cylindrical shape surrounded by a solution-permeable support. The front and the rear surfaces of the hollow were sealed by a pair of thin-polyimide-film windows with a thickness of 7 µm. In the present study the sample cell was filled by a solution at the beginning of the measurement. As a hydrophilic molecule, we used ethanol (reagent grade, Wako Pure Chemical Industries, Ltd., Osaka) and as a hydrophobic molecule, \(\text{d-limonene} \) (reagent grade, Tokyo Chemical Industries, Co. Ltd., Tokyo).

3. Results and Discussion

To describe the overall tendencies, the successive changes of the x-ray diffraction profile for the whole angular range are shown in Figs. 1a and b. Profiles after applying ethanol and \(\text{d-limonene} \) to the SC sample are shown together with the diffraction profiles of pure ethanol and pure \(\text{d-limonene} \), respectively. The abscissa is \(S=(2/\lambda)\sin(\theta/2)\), where \(\lambda(=0.1 \text{ nm})\) is the wavelength of x-ray and \(\theta\) is the scattering angle. The apparent peak at 0.6 nm\(^{-1}\) is due to the scattering from the polyimide-film windows of the sample cell. The profile of pure ethanol exhibits extremely broad peaks near \(S=1.25 \text{ nm}^{-1}\) and \(S=2.4 \text{ nm}^{-1}\). The diffraction profile of pure \(\text{d-limonene} \) exhibits an extremely broad peak near \(S=1.9 \text{ nm}^{-1}\).

It is clearly seen in Fig. 1a and b that, by applying ethanol and \(\text{d-limonene} \) to the SC, the broad peaks originating from these molecules increased gradually. These results indicate the uptake of these molecules in the SC. We have already analyzed and discussed the behavior in the small angle range of \(0.0<S(\text{nm}^{-1})<0.4\) and in the wide angle range of \(2.0<S(\text{nm}^{-1})<3.0\) in the previous paper [10]. In this paper, since a broad peak of the soft keratin in the corneocytes appears near \(S=1.25 \text{ nm}^{-1}\) and \(S=2.4 \text{ nm}^{-1}\), we focused our attention to the range of \(0.5<S(\text{nm}^{-1})<1.5\) and to comparing the changes caused by ethanol and \(\text{d-limonene} \). To show the minute changes in the diffraction profiles we calculated the intensity difference \(\Delta I(S,t)\) given by

\[
\Delta I(S,t) = I(S,t) - I(S,0),
\]

where \(I(S,t)\) is the intensity at time \(t\) and \(I(S,0)\) is the intensity just after applying the functional molecule to the SC. In Fig. 2a and b, the intensity difference is shown for ethanol and \(\text{d-limonene} \) in the range of \(0.5<S(\text{nm}^{-1})<1.5\), respectively. With \(\text{d-limonene} \), the intensity did not change near \(1 \text{ nm}^{-1}\) except the increase in the range larger than \(1 \text{ nm}^{-1}\) as a result of the uptake of \(\text{d-limonene} \) in the SC.
Figure 1a and b. a exhibits the whole x-ray diffraction profile changes with time on applying ethanol to the stratum corneum (from red to blue curves, see Sec. Exp.) with the profile for pure ethanol (black curve). b indicates the whole x-ray diffraction profile changes with time on applying $d$-limonene to the stratum corneum (from red to blue curves, see Sec. Exp.) with the profile for pure $d$-limonene (black curve).

Figure 2a and b. a exhibits the successive change in the profiles of the intensity difference near 1 nm$^{-1}$ on applying ethanol to the stratum corneum (from red to blue curves, see Sec. Exp.) [10]. b indicates the successive change in the profiles of the intensity difference near 1 nm$^{-1}$ on applying $d$-limonene to the stratum corneum (from red to blue curves, see Sec. Exp.).

On the other hand, with ethanol, the intensity increased over the entire angular range. The increment was bigger in the smaller angular range. However, in superposition on this behavior the decrease of the intensity occurred near 1 nm$^{-1}$ clearly where the broad peak of the soft keratin lay and additionally the increase of the very broad peak near 1.25 nm$^{-1}$ occurred as a result of the uptake of ethanol in the SC. Furthermore the behavior in the smaller angular range was distinctly different between ethanol and $d$-limonene as seen in Fig. 3a and b, respectively, in which a peak at about 0.07 nm$^{-1}$ corresponds to the first order diffraction of the long lamellar structure. The behavior of this reflection after application of ethanol or $d$-limonene has been discussed in the previous paper in detail [10]. In addition, the distinct increase of the intensity in the smaller angle region occurred on applying ethanol, but no significant change took place on applying $d$-limonene.

Based upon the present results, we can point out clearly that ethanol affects the structure of the soft keratin in the corneocytes, while $d$-limonene does not. Therefore, ethanol is incorporated into the corneocytes and then the x-ray diffraction intensity increases in the smaller angular range. Finally by referring to the results obtained in the previous paper [10] we can propose two penetration routes: One is the transcellular route as observed in the application of hydrophilic ethanol and the other is the intercellular route as observed in the application of hydrophobic $d$-limonene. Then, not only the well-
Figure 3a and b. a and b exhibit the time course of the profiles in the small angle region after applying ethanol and \(d\)-limonene to the stratum corneum, respectively (from red to blue curves, see Sec. Exp.).

known intercellular route that is schematically shown as

\[
\text{Penetration of hydrophobic molecule, preferentially via long lamellar structure} \quad \Rightarrow \quad \text{Pool of hydrophobic molecule}
\]

but also the transcellular route that is schematically shown as

\[
\text{Penetration of hydrophilic molecule, preferentially via short lamellar structure} \quad \Rightarrow \quad \text{Corneocyte} \quad \Rightarrow \quad \text{Pool of hydrophilic molecule}
\]

plays an indispensable role in the penetration of the functional molecules through the SC.

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